



Standard Operating Procedure
Version 3.8

January 1, 2005

Table of Contents

Table of Contents	i
Table of Tables	1
Table of Figures	1
Chapter 1: Compound Library	1
1.1. PURPOSE	1
1.2. REFERENCE	1
1.3. RESPONSIBILITY	1
1.4. REQUIRED MATERIALS AND EQUIPMENT	1
1.5. PROCEDURE.....	1
1.5.1. Compound Acquisition.....	1
1.5.2. Compound Storage.....	2
1.5.3. MDS Sample Selection and Preparation Details.....	2
1.5.4. Shipping Information for MDS Collaborations	4
Chapter 2: Range-Finding Study	11
2.1. PURPOSE	11
2.2. RESPONSIBILITY	11
2.3. REQUIRED MATERIALS AND EQUIPMENT	11
2.4. PROCEDURE.....	12
2.4.1. Tracking of Compound Availability for RF study	12
2.4.2. The “LD50 Info” Spreadsheet	13
2.4.3. Choosing Compounds for the Upcoming RF study	13
2.4.4. Curation for Relevant Route of Administration.....	15
2.4.5. Finding LD50 Information.....	15
2.4.6. Solubility Information	16
2.4.7. Choosing the Vehicle.....	17
2.4.8. Setting the Doses	18
2.4.9. Rationale Report.....	19
2.4.10. Entering Information into the VV Database	20
2.4.11. Entering Dose Values into the VV Database.....	22
2.4.12. Updating the RF_Tracking Database	23
2.4.13. Compound Amounts to Drug Informatics for Weighing	23
2.4.14. Upon Completion of the RF Studies at the CRO.....	24
2.4.15. Update of VV and RF_Tracking Databases.....	25

2.4.16.	Update of VV and RF_Tracking Databases for Repeat RF Studies	26
Chapter 3:	Range Finding Compound Preparation for Delivery to CRO	27
3.1.	PURPOSE	27
3.2.	RESPONSIBILITY	27
3.3.	REQUIRED MATERIALS AND EQUIPMENT	27
3.4.	PROCEDURE.....	27
3.4.1.	Retrieving Information from FileMaker Pro Database.....	27
3.4.2.	Filling the Dosing Table	28
Chapter 4:	Weighing Compounds for <i>In Vivo</i> and <i>In Vitro</i> Studies	30
4.1.	PURPOSE	30
4.2.	REFERENCE	30
4.3.	RESPONSIBILITY	30
4.4.	REQUIRED MATERIALS AND EQUIPMENT	30
4.5.	PROCEDURE.....	30
4.5.1.	Preparation of Samples for In Vivo Studies	30
4.5.2.	Preparation of Samples for In Vitro Studies	31
Chapter 5:	<i>In Vivo</i> Array Study Experimental Design.....	33
5.1.	PURPOSE	33
5.2.	RESPONSIBILITY	33
5.3.	REQUIRED MATERIALS AND EQUIPMENT	33
5.4.	PROCEDURE.....	33
5.4.1.	Preparation of New Record in VV database	33
5.4.2.	Preparation of the Rationale Report.....	34
5.4.3.	Maximum Tolerated Dose	34
5.4.4.	Maximum Feasible Dose.....	36
5.4.5.	Fully Efficacious Dose Recommendation.....	37
5.4.6.	Searching the Literature	39
5.4.7.	Using MedMiner	39
5.4.8.	Working with the PubMed Abstracts	40
5.4.9.	Stanford Library	42
5.4.10.	Fully Efficacious Dose Recommendation.....	43
5.4.11.	Summary	44
5.4.12.	Preparation for the Dose-Setting Meeting.....	45
5.4.13.	At the Dose-Setting Meeting	47
5.4.14.	After the Dose-Setting Meeting.....	47

5.4.15.	Array Tissues	48
5.4.16.	Compound Amounts to Drug Informatics for Weighing	49
5.4.17.	Outcome of the Study	50
Chapter 6: Printing Compound Experimental Detail Tables for CROs		51
6.1.	PURPOSE	51
6.2.	RESPONSIBILITY	51
6.3.	REQUIRED MATERIALS AND EQUIPMENT	51
6.4.	PROCEDURE.....	51
6.4.1.	Enter the VV_Exp_Design in FileMaker Database.....	51
6.4.2.	Fill the Dosing and Tissue Tables	52
Chapter 7: Generate Labels and Upload Sample files		55
7.1.	PURPOSE	55
7.2.	RESPONSIBILITY	55
7.3.	REQUIRED MATERIALS AND EQUIPMENT	55
7.4.	PROCEDURE.....	55
7.4.1.	'Tissue', 'Animal', and 'Codes' File Generation	55
7.4.2.	Printable Barcodes Generation.....	57
Chapter 8: <i>In Vivo</i> Expression Array Experiment Execution		59
8.1.	PURPOSE	59
8.2.	REFERENCES.....	59
8.3.	RESPONSIBILITY	59
8.4.	REQUIRED MATERIALS AND EQUIPMENT	59
8.5.	PROCEDURE.....	60
8.5.1.	Experimental Design	60
8.5.2.	Test and Control Articles	60
8.5.3.	Test System Rat	61
8.5.4.	In-Life Evaluations.....	63
8.5.5.	Necropsy	64
8.5.6.	Histopathologic Examination.....	67
8.5.7.	Statistical Analysis	68
8.5.8.	Control of Bias	68
8.6.	REPORTING.....	68
8.6.1.	CRO Report.....	68
8.6.2.	Iconix Audit of CRO	68
Chapter 9: Reception and Storage of Frozen Tissues.....		70
9.1.	PURPOSE	70

9.2.	RESPONSIBILITY	70
9.3.	REQUIRED MATERIALS AND EQUIPMENT	70
9.4.	PROCEDURE.....	70
9.4.1.	Receiving Tissues	70
9.4.2.	Tissue Storage.....	72
9.4.3.	Entering Information in Database	72
Chapter 10: Database Upload of <i>In Vivo</i> Data.....		74
10.1.	PURPOSE	74
10.2.	RESPONSIBILITY	74
10.3.	REQUIRED MATERIALS AND EQUIPMENT	74
10.4.	PROCEDURE.....	74
10.4.1.	Importing Experimental Details into FileMaker Pro Database	74
10.4.2.	Importing the Tissue ID into FileMaker Pro Database.....	75
10.4.3.	Importing Sample Location into FileMaker Pro Database.....	75
10.4.4.	Entering Dose solution ID	76
10.4.5.	Uploading Information into Tissue Tracking Database (FileMaker Pro) 76	
10.4.6.	RNA Sample Release for Motorola platform	77
10.5.	APPENDIX.....	79
10.5.1.	Assigning Control Animal ID	79
Chapter 11: Mammalian Cell Culture		80
11.1.	PURPOSE	80
11.2.	REFERENCE	80
11.3.	RESPONSIBILITY	80
11.4.	REQUIRED MATERIALS AND EQUIPMENT	80
11.5.	GENERAL PROCEDURES	81
11.5.1.	Aseptic Technique.....	81
11.5.2.	Preparation of Collagen-Coated Tissue Culture Vessels	82
11.5.3.	Thawing DMSO-Tolerant Cells from a Liquid Nitrogen Stock	82
11.5.4.	Thawing DMSO-Sensitive Cells from a Liquid Nitrogen Stock.....	83
11.5.5.	Cell Counting and Viability.....	84
11.5.6.	Handling of Adherent Cells	85
11.5.7.	Trysinizing Cells	86
11.5.8.	Freezing Cells for Liquid Nitrogen Stocks	86
Chapter 12: Primary Rat Hepatocyte Culture and <i>In Vitro</i> Cytotoxicity and Proliferation Assays.....		88

12.1.	PURPOSE	88
12.1.1.	Reference	88
12.2.	RESPONSIBILITY	88
12.3.	REQUIRED MATERIALS AND EQUIPMENT	88
12.4.	PROCEDURE.....	89
12.4.1.	Primary Rat Hepatocyte Treatment	89
12.4.2.	Obtain Cells from SRI and Cell Count.....	90
12.4.3.	Plating Cells for LDH and MTS Assays- DAY 1	91
12.4.4.	Dosing – DAY 2.....	92
12.4.5.	LDH and MTS Assays – DAY 3	94
Chapter 13: <i>In Vitro</i> Array Experiment		97
13.1.	PURPOSE	97
13.2.	REFERENCE.....	97
13.3.	RESPONSIBILITY	97
13.4.	REQUIRED MATERIALS AND EQUIPMENT	97
13.5.	PROCEDURE.....	98
13.5.1.	Plating Cells	98
13.5.2.	Compound Preparation	99
13.5.3.	Dosing.....	102
13.5.4.	Harvesting Cell Samples.....	102
13.6.	CREATE RECORDS AND UPLOADE TO FILEMAKER PRO	103
13.6.1.	Entering Information into Excel.....	103
13.6.2.	Uploading to the UV_Main Database	104
13.6.3.	Filling in the UV Database.....	105
Chapter 14: Retrieving Tissues.....		106
14.1.	PURPOSE	106
14.2.	RESPONSIBILITY	106
14.3.	SAFETY PRECAUTIONS.....	106
14.4.	REQUIRED MATERIALS AND EQUIPMENT	106
14.5.	PROCEDURE.....	106
14.5.1.	Tissue Release List Generation.....	106
14.5.2.	Worksheet Preparation.....	107
14.5.3.	Retrieving Tissues.....	109
14.5.4.	Weighing Tissue Samples	110
14.5.5.	Entering Information in the Database.....	110
Chapter 15: Sample Preparation for RNA Isolation.....		112

15.1.	PURPOSE	112
15.2.	RESPONSIBILITY	112
15.3.	SAFETY PRECAUTIONS.....	112
15.4.	REQUIRED MATERIALS AND EQUIPMENT	112
15.5.	PROCEDURE.....	113
15.5.1.	Tissue Homogenization	113
15.5.2.	Syringing of Tissue and Cell Culture samples	114
15.5.3.	Uploading Raw Tissue Data into Tissue Tracking DB (FileMaker Pro) 114	
Chapter 16: RNA Isolation		116
16.1.	PURPOSE	116
16.2.	REFERENCE	116
16.3.	RESPONSIBILITY	116
16.4.	SAFETY PRECAUTIONS.....	116
16.5.	REQUIRED MATERIALS AND EQUIPMENT	116
16.6.	PROCEDURE.....	117
16.6.1.	Preparation for a Run	117
16.6.2.	Robot Run.....	120
16.6.3.	Used lysate storage	120
16.6.4.	Post-elution	121
16.7.	CONTACT INFORMATION	123
Chapter 17: Ethanol Precipitation of mRNA		124
17.1.	PURPOSE	124
17.2.	RESPONSIBILITY	124
17.3.	SPECIAL NOTES	124
17.4.	REQUIRED MATERIALS	124
17.5.	PROCEDURE.....	124
17.5.1.	Ethanol Precipitation	124
Chapter 18: Running the Agilent 2100 Bioanalyzer		126
18.1.	PURPOSE	126
18.2.	REFERENCE	126
18.3.	RESPONSIBILITY	126
18.4.	SAFETY PRECAUTIONS.....	126
18.5.	REQUIRED MATERIALS	126
18.6.	PROCEDURE.....	127
18.6.1.	96-well plate Preparation	127
18.6.2.	Print the Agilent Run List	128

18.6.3.	Run the Bioanalyzer Program	128
18.6.4.	Preparing the Gel	128
18.6.5.	Prepare the Gel-Dye Mix	128
18.6.6.	RNA Ladder Denaturation.....	129
18.6.7.	Electrode Decontamination	129
18.6.8.	Load the RNA chip	129
18.6.9.	Running the Bioanalyzer.....	130
Chapter 19: Ribogreen Analysis.....		133
19.1.	PURPOSE	133
19.2.	RESPONSIBILITY	133
19.3.	SAFETY PRECAUTIONS.....	133
19.4.	REQUIRED MATERIALS	133
19.5.	PROCEDURE.....	133
19.5.1.	Thawing Reagents.....	133
19.5.2.	96-well plates Preparation	134
19.5.3.	Ribogreen Dye Preparation	134
19.5.4.	Reading the Plate Using Fluorescent Plate Reader	136
19.5.5.	Entering Information into the Batch Worksheet	136
19.5.6.	Data Upload into UNV_Main.FP5 database	138
19.5.7.	Data Upload into Tissue Tracking DB	138
19.6.	APPENDIX.....	139
19.6.1.	1X TE Buffer Preparation	139
19.6.2.	Preparation of Ribogreen Standard Aliquots	139
19.6.3.	Preparation of Fluorescent Dye Aliquots	140
19.6.4.	Ribogreen Solution Preparation.....	140
Chapter 20: Release mRNA Samples		141
20.1.	PURPOSE	141
20.2.	RESPONSIBILITY	141
20.3.	REQUIRED MATERIALS AND EQUIPMENT	141
20.4.	PROCEDURE.....	141
20.4.1.	Sample Release	141
20.4.2.	Releasing Re-Run Samples to mRNA	142
Chapter 21: Target Preparation.....		146
21.1.	PURPOSE	146
21.2.	REFERENCES.....	146

21.3.	RESPONSIBILITY	146
21.4.	REQUIRED MATERIALS AND EQUIPMENT	146
21.4.1.	Reagents for Synthesis of First Strand DNA	146
21.4.2.	Reagents for Synthesis of Second Strand DNA	146
21.4.3.	Reagents for Purification of Double Stranded cDNA.....	147
21.4.4.	Reagents for In Vitro Transcription.....	147
21.4.5.	Reagents for Electrophoresis of ds-cDNA for Quality Control.....	147
21.4.6.	Reagents for Recovery of Biotin-Labeled cRNA	148
21.4.7.	Reagents for Agilent 2100 Bioanalyzer Protocol	148
21.4.8.	Other Equipment and Materials	148
21.5.	PROCEDURE.....	150
21.5.1.	Sign up for mRNA Samples	150
21.5.2.	cRNA Template	150
21.5.3.	Robot Flushing	151
21.5.4.	First Strand cDNA Synthesis.....	151
21.5.5.	Second Strand cDNA Synthesis	154
21.5.6.	QIAquick® cDNA Purification	156
21.5.7.	In vitro Transcription Reaction	158
21.5.8.	RNeasy® cRNA Purification	160
21.6.	APPENDIX A.....	162
21.6.1.	Electrophoresis of ds-cDNA.....	162
21.6.2.	Preparation of ds-cDNA Samples and DNA Ladder	162
21.6.3.	Electrophoresis and Analysis of ds-cDNA Samples.....	162
21.7.	APPENDIX B	163
21.7.1.	Bioanalyzer 2100 Analysis of cRNA.....	163
21.8.	APPENDIX C.....	163
21.8.1.	Stock Solution Preparation	163
21.9.	CONTACT INFORMATION	164
Chapter 22:	cRNA Quality Control and Precipitation	166
22.1.	PURPOSE	166
22.2.	REFERENCES.....	166
22.3.	RESPONSIBILITY	166
22.4.	SAFETY PRECAUTIONS.....	166
22.5.	REQUIRED MATERIALS AND EQUIPMENT	166
22.6.	PROCEDURE.....	167

22.6.1.	Quantification of cRNA.....	167
22.6.2.	Precipitation/Archive	169
22.6.3.	Uploading Completed Worksheet.....	171
22.7.	APPENDIX.....	172
22.7.1.	70% Ethanol	172
Chapter 23:	Release cRNA Samples	173
23.1.	PURPOSE	173
23.2.	RESPONSIBILITY	173
23.3.	REQUIRED MATERIALS AND EQUIPMENT	173
23.4.	PROCEDURE.....	173
23.4.1.	Releasing Samples to Hybridize	173
23.4.2.	Releasing cRNA Failures as cRNA or mRNA reruns.....	174
23.4.3.	Filling out the “mRNA Re-Runs Template2003”	174
Chapter 24:	cRNA Fragmentation	176
24.1.	PURPOSE	176
24.2.	REFERENCES.....	176
24.3.	RESPONSIBILITY	176
24.4.	SPECIAL NOTES	176
24.5.	REQUIRED MATERIALS AND EQUIPMENT	177
24.6.	PROCEDURE.....	177
24.6.1.	Obtaining Samples for Fragmentation.....	177
24.6.2.	Fragmentation of Samples	179
Chapter 25:	Array Hybridization	182
25.1.	PURPOSE	182
25.2.	REFERENCES.....	182
25.3.	RESPONSIBILITY	182
25.4.	SPECIAL NOTES	182
25.5.	REQUIRED MATERIALS AND EQUIPMENT	182
25.6.	PROCEDURE.....	184
25.6.1.	Preparation of Hybridization Reaction Mixture	184
25.6.2.	Loading of Reaction Mixtures into Array Chambers	185
25.6.3.	Entering Slide Information in the Database.....	186
25.6.4.	Post-Hybridization Wash	188
25.6.5.	Detection with Streptavidin-Alexa Fluor® 647	189
25.6.6.	Database Upload of Hybridization Conditions.....	193
25.6.7.	Slide Scanning	194

25.6.8.	Gridding	197
25.6.9.	Gridding Review	198
25.6.10.	Gridding CodeLink-Slides Manually	199
25.6.11.	QC Report Generation and Gridded File Export	202
25.7.	EQUIPMENT MAINTENANCE.....	205
25.7.1.	Daily Maintenance	205
25.7.2.	Weekly Maintenance	205
25.8.	APPENDIX.....	205
25.8.1.	TNT Buffer Preparation (20L)	205
25.8.2.	0.75X TNT Buffer Preparation.....	205
25.8.3.	TNB Buffer (2.0 L)	205
25.8.4.	TNB Buffer Aliquots	206
25.8.5.	Streptavidin-Alexa 647 Stock Solution.....	207
25.8.6.	Streptavidin-Alexa 647 Dye Aliquots	207
25.8.7.	0.05% Tween 20 Solution (20 L)	207
Chapter 26:	Validation	208
26.1.	PURPOSE	208
26.2.	RESPONSIBILITY	208
26.3.	REQUIRED MATERIALS AND EQUIPMENT	208
26.4.	PROCEDURE.....	208
26.4.1.	Running Validator	208
26.4.2.	Running Filemover	209
Chapter 27:	Array Grading and QC.....	211
27.1.	PURPOSE	211
27.2.	RESPONSIBILITY	211
27.3.	REQUIRED MATERIALS AND EQUIPMENT	211
27.4.	PROCEDURE.....	211
27.4.1.	Download Array QC from LIMS System	211
27.4.2.	First Round Grading.....	213
27.4.3.	Second Round Grading.....	213
27.4.4.	Third Round Grading.....	215
27.4.5.	Fourth Round Grading.....	216
27.4.6.	Weekly QC Status Report	220
Chapter 28:	Releasing Rejected Hybs	221
28.1.	PURPOSE	221

- 28.2. RESPONSIBILITY 221
- 28.3. REQUIRED MATERIALS AND EQUIPMENT 221
- 28.4. PROCEDURE..... 221
 - 28.4.1. Releasing Rejected Hybs 221
- Chapter 29: MagNA Pure LC Robot Maintenance..... 223**
 - 29.1. PURPOSE 223
 - 29.2. RESPONSIBILITY 223
 - 29.3. SAFETY PRECAUTIONS..... 223
 - 29.4. REQUIRED MATERIALS AND EQUIPMENT 223
 - 29.5. PROCEDURE..... 224
 - 29.5.1. General Daily 224
 - 29.5.2. Weekly 224
 - 29.5.3. Periodic..... 225
 - 29.6. CONTACT INFORMATION 225
- Chapter 30: QIAgen BioRobot Maintenance 226**
 - 30.1. PURPOSE 226
 - 30.2. REFERENCES..... 226
 - 30.3. RESPONSIBILITY 226
 - 30.4. REQUIRED MATERIALS AND EQUIPMENT 226
 - 30.5. PROCEDURE..... 226
 - 30.5.1. Equipment Maintenance 226
 - 30.5.2. Prior to Each BioRobot Use: 227
 - 30.5.3. After Each BioRobot Use:..... 227
 - 30.5.4. Weekly Maintenance Procedure 227
 - 30.5.5. Periodic and Corrective Maintenance..... 228
 - 30.6. CONTACT INFORMATION 228
- Chapter 31: Hazardous Materials Guidelines 229**
 - 31.1. PURPOSE 229
 - 31.2. RESPONSIBILITY 229
 - 31.3. SAFETY PRECAUTIONS..... 229
 - 31.4. PROCEDURE..... 229
 - 31.4.1. General Issues..... 229
 - 31.4.2. Disposal of Waste..... 230
 - 31.4.3. Biohazard Waste 231
 - 31.4.4. Chemical Waste 231
 - 31.4.5. Labeling Requirements 231

Summary of Changes between V 3.2 and V 3.7..... 232
Summary of Changes between V 3.7 and V 3.8..... 233

Table of Tables

TABLE 2.4-A: RF STUDY DATABASE CONTENT	14
TABLE 8.5-A: ARRAY EXPERIMENT EXPERIMENTAL DESIGN	60
TABLE 8.5-B: TISSUE HARVESTING INSTRUCTIONS.....	66
TABLE 13.5-A: ARRAY EXPERIMENT CELL PLATING DENSITIES	99
TABLE 16.6-A: MAGNA PURE LC BUFFER CHART	119
TABLE 21.8-A: 1 ST STRAND cDNA SYNTHESIS BACTERIAL CONTROL SPIKES EXAMPLE ..	164
TABLE 24.6-A: FRAGMENTATION MASTER MIX	180
TABLE 25.6-A: HYBRIDIZATION REACTION MASTER MIX.....	184
TABLE 25.6-B: DETECTION MASTER MIX	189
TABLE 25.6-C: EXAMPLE OF GRIDDING FILE LIST.....	201
TABLE 27.4-A: FIRST ROUND ARRAY GRADING.....	213
TABLE 27.4-B: SECOND ROUND ARRAY GRADING.....	214
TABLE 27.4-C: THIRD ROUND ARRAY GRADING	216

Table of Figures

FIGURE 1.5-A: SHIPPING WARNING LABEL WARNING	5
FIGURE 1.5-B: EXAMPLE OF SHIPPING COVER LETTER	6
FIGURE 1.5-C: SHIPPING CHECKLIST.....	7
FIGURE 1.5-D: EXAMPLE OF PROFORMA INVOICE FOR SHIPMENT TO TAIWAN.....	9
FIGURE 11.5-A: COUNTING CELLS USING A HEMACYTOMETER	85
FIGURE 12.4-A: SET -UP OF ASSAY PLATE.....	94
FIGURE 13.5-A: FLUVASTATIN PROLIFERATION ASSAY RESULTS	100
FIGURE 13.5-B: LABETALOL (FD) PROLIFERATION ASSAY RESULTS.....	100
FIGURE 13.5-C: IFOSFAMIDE (MF) PROLIFERATION ASSAY RESULTS	101
FIGURE 16.6-A: MAGNA PURE LC SAMPLE TRAY LOADING.....	120
FIGURE 16.6-B: POST ELUTION PROTOCOL SAMPLE TRANSFER SCHEMATIC	123
FIGURE 18.6-A: AGILENT PLATE PREPARATION.....	127
FIGURE 19.5-A: AGILENT PLATE.....	135
FIGURE 19.5-B: PLATE #1.....	135
FIGURE 19.5-C: PLATE #2	136
FIGURE 25.6-A: AVOID PIPETTING THE PRECIPITATE.....	191
FIGURE 27.4-A: FALSE-COLOR IMAGES AND SCATTER PLOTS OF PASSED ARRAYS	217
FIGURE 27.4-B: FALSE-COLOR IMAGES AND SCATTER PLOTS OF FAILED ARRAYS	217

Chapter 1

Compound Library

1.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for acquisition of Drug Matrix compounds, and preparation of samples for collaboration with MDS Pharma Services and other contracts research organizations (CRO).

1.2. REFERENCE

Drug Matrix Compound Library Protocol

1.3. RESPONSIBILITY

The Drug Informatics group is responsible for implementation of this protocol.

1.4. REQUIRED MATERIALS AND EQUIPMENT

- Access to CEXSmpl_Received.FP5 database
- Access to CEX_MDS.FP5 database
- Analytical balance located in Fume hood
- 96-position deep-tube racks (E&K Scientific, part# 684512-S)
- Cap strips for deep-well racks (E&K Scientific, part# 480005-S)
- 1-dram vials (VWR, part# 66010-522)
- Teflon-lined 1-dram vial caps (VWR, part# 66012-350)

1.5. PROCEDURE

1.5.1. *Compound Acquisition*

In general, if a compound is available from commercial sources (as indicated by its presence in the latest version of ACD3Dfinder (MDL Information Systems; a database providing chemical pricing for multiple suppliers, as well as the necessary catalogue numbers, and available quantities)), all efforts are made to acquire 100 mg to 50 g of a sample with the highest purity. The exact amount that will be acquired for each depends on the cost and minimum package size. For most of the compounds, 500 mg to 5 g are purchased. Compounds not available via ACD are sourced via Sequoia Biochemical or custom synthesized

1.5.2. *Compound Storage*

- 1.5.2.1. In general, compounds are stored in a -20°C freezer unless “do not freeze” or “desiccate” is specified on the package. A desiccator is available for samples that must be kept moisture-free below 0°C (check inside -20°C freezer) or for those chemicals requiring a moisture-free environment above 0°C (check the 4°C refrigerator). Class II DEA controlled substances are stored in a safe at room temperature; class III, IV, and V DEA-controlled substances are stored in a designated location in a locked -20°C freezer.
- 1.5.2.2. Samples are logged into CEXSmpl_Received.FP5 database, preferably on the same day they are received. The following information (usually found on the sample vials) is entered into CEXSmpl_Received, using the “Login_New_samples” layout:
- Sample source (vendor, person or group that prepared the compounds)
 - Vendor catalog number (when the catalog number is entered in the correct format, associated information on “ordered compounds” will appear)
 - Batch/lot number (as on the vial; if the sample is prepared for Iconix, the notebook page number is recorded here)
 - Sample name (as given on the vial; this name may be a synonym of the molname, or a salt form of the molname)
 - Molecular formula of sample (as given on the vial)
 - Molecular weight of sample (as given on the vial)
 - ICX Number (copied from “CEXSmpl_Ordered”)
 - Storage Location (an appropriate storage location is assigned, based upon the container size, and specified storage conditions)

1.5.3. *MDS Sample Selection and Preparation Details*

- 1.5.3.1. Samples to be sent in each shipment are selected based on priorities established by the Drug Matrix project team.
- 1.5.3.2. Lists of compounds ready to be weighed (and the batch number of the samples) are prepared and released by the Director of Drug Informatics.
- 1.5.3.3. Three samples of each compound are weighed into 1-dram vials with Teflon-faced caps and labeled with ICX Numbers.
- MDS-Taiwan: 15 mg, record exact weight.

- MDS-Bothell: enough (20 μ mole) to make 2 ml of 10 mM solution, record exact weight.
 - Iconix-Analytical: 5 mg, record exact weight. These samples are kept in storage for in-house purity checks, to be done by the appropriate Iconix staff.
- 1.5.3.4. Enter the weight of each sample into CEX_MDS.FP5; the weights should be in milligram quantities.
- 1.5.3.5. Finalize the list of compounds for the next shipment and log the information in CEX_MDS.FP5.
- 1.5.3.6. Preparation for MDS_Taiwan shipment
- Prepare and label each vial with the ICXNumber.
 - Prepare Excel spread sheet containing:
 - ICX number
 - Sample name
 - Sample weight
 - Compound molecular weight
 - ml_DMSO to be added for 10 mM solution
 - Shipping date of each sample
 - MDS_Taiwan sample ID
 - Ship samples in DOT certified container.
 - Ship via Federal Express on a Thursday.
- 1.5.3.7. Assign each sample for MDS_bothell with a Rack_Well ID (e.g. 4_A8, 4_B12).
- 1.5.3.8. Dissolve each sample for MDS_Bothell by
- Adding the required amount of DMSO directly into the 1-Dram vial.
 - Bouncing samples in ultrasonic bath to break up solids, if necessary.
 - Allowing the samples to sit overnight at room temperature if necessary.
- 1.5.3.9. Array MDS_Bothell samples in 96-position deep-tube racks
- Label each tube of two sets of 12-tube deep-tube cassettes with Rack_Well_ID.

- Rack one set for MDS_Bothell (24 samples in each rack) the other set for retention sample to be kept at Iconix.
- Transfer 1 ml each of the DMSO solution from 1-dram vial to the corresponding deep-tubes.
- Cap tubes with cap strips.

1.5.3.10. Prepare for MDS_Bothell shipment.

- Prepare Excel spreadsheet to contain Tube_ID, ICX Number, sample name, Salt-free molecular weight, molecular weight of salt form, shipping date of each sample, and ICN#.
- Ship samples, via Federal Express, to MDS Bothell on dry ice on a Monday or Tuesday.

1.5.4. Shipping Information for MDS Collaborations

1.5.4.1. Compounds are sent in sets of multiples of 24 to MDS-Bothell for *in vitro* metabolism assays and to MDS-Taiwan for pharmacological assays. In general,

- MDS-Taiwan requires 15 mg of each sample in a 1-dram vial with barcode.
- MDS-Bothell requires 1 ml of a 10 mM solution in DMSO arrayed in Row-A and Row-B of a 96-position deep-well rack.
- DEA controlled samples will not be sent but will be purchased by the two MDS-labs directly from the vendors.
- MDS-Taiwan samples are shipped on dry ice on a Friday for Tuesday delivery.

1.5.4.2. The following outlines the procedure for compound shipments that are made to collaborators and contractors. In summary:

- MSDS sheets for all compounds are included in each shipment.
- All compounds are packed in DOT certified containers.
- A “Package Warning” indicating that the package must be opened in a hood in case of breakage is prominently displayed (Figure 1.5-A).
- MSDS sheets are reviewed for three exceptional classes of compounds:
 - Compounds that are extremely flammable or explosive
 - Compounds that are extremely toxic
 - Toxic compounds being provided as large quantities

- Exceptional compounds will be flagged on the compound list with notes describing the specific danger.
- The compound list includes the names of all compounds.

**Please open internal
container in a fume
hood in case of
damage during
shipping!**

Figure 1.5-A: Shipping Warning Label Warning

- A general warning is included in the cover letter for the shipment (Figure 1.5-B).
- The receiver is notified in advance of intentions to send a large amount of a toxicant and must approve the shipment of this material.
- The Compound Shipment Checklist (Figure 1.5-C) is used to facilitate the shipping procedure and provide compliance documentation.
- Every shipment must be approved by a Director level or above staff member, once prepared but before it is sealed. Several senior staff members will be trained on this procedure to assure compliance and availability. The checklist will be filed with copies of all sent paperwork (except MSDS sheets) to have a precise record of each shipment.

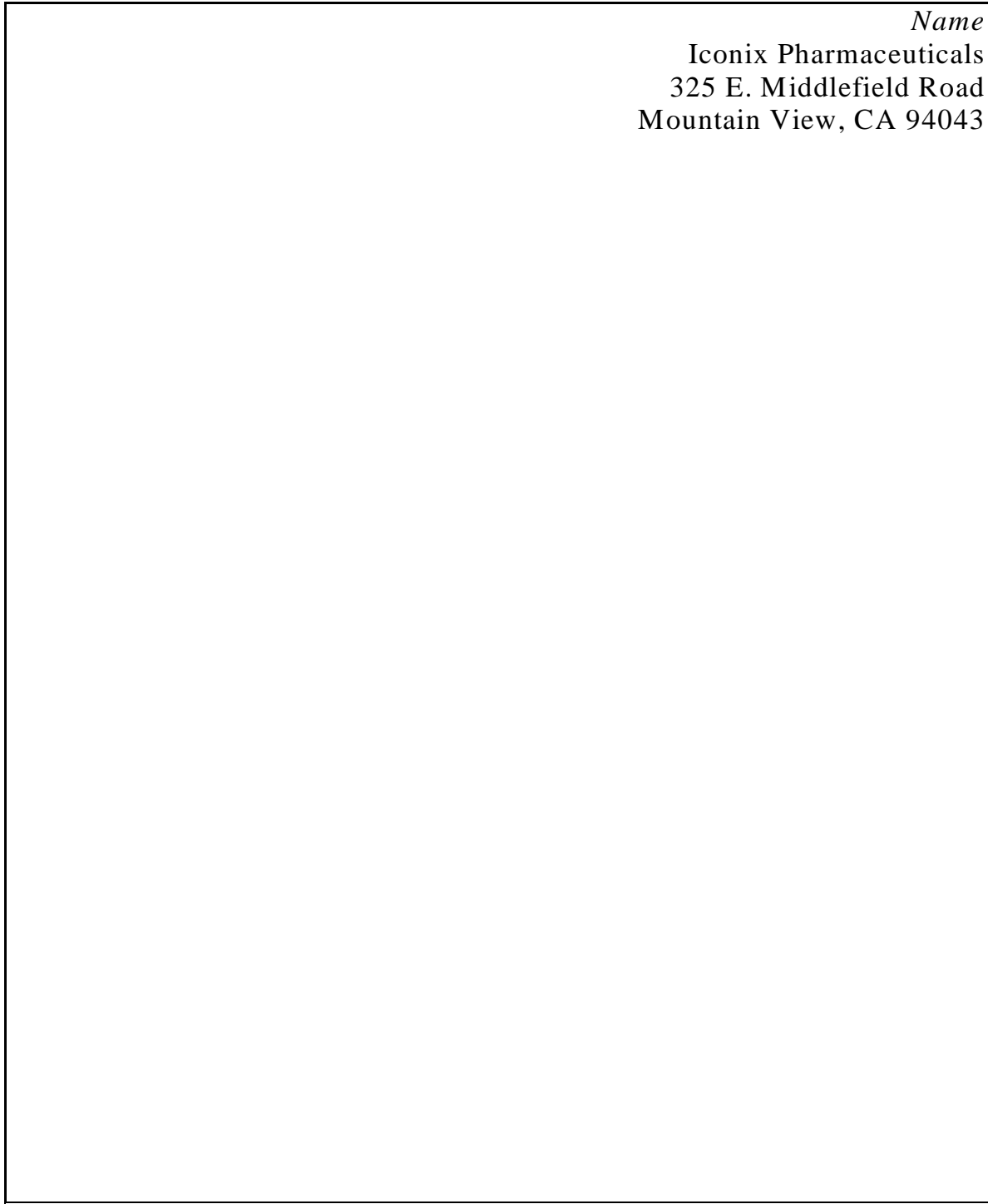



Figure 1.5-B: Example of Shipping Cover Letter



Compound Shipment Checklist

Planned Ship Date _____

Destination _____

- Compound list finalized (Number of Compounds: _____)
- Compound list sent to Receiver and approval received
- MSDS's collected and copied
- MSDS's checked for highly flammable or explosive compounds
- MSDS's checked for highly toxic compounds
- MSDS's checked for toxins in excessive amounts
- Unusual compounds noted on compound list
- Cover letter prepared
- Package warning prepared
- Compound list prepared
- Pro forma invoice prepared (international only)
- Commercial invoice prepared (international only)
- Fedex Airbill prepared
- DOT paperwork prepared
- Samples packed in DOT certified container
- Container packed in dry ice
- MSDS's and other paperwork enclosed
- Shipment and paperwork approved

} Shipping Paperwork

Signed & Dated by Director Level Staff

- Shipment sealed
- Copies of Cover letter, Compound List and Shipping Paperwork attached and filed

Signed & Dated by Shipper

Figure 1.5-C: Shipping Checklist

- 1.5.4.3. Procedure for non-hazardous compounds to Taiwan
- Fill out the Proforma Invoice (Figure 1.5-D).
 - Address sample to:
 - Enter the following in the Commercial Invoice (for customs)
 - No Commercial Value
 - Value for Customs Purpose only
 - For research use only
 - Not for sale, No commercial value
 - Determine the dimensions of the package, the total weight and the weight of the dry ice.
 - Fill out the Commercial Invoice according to previous shipments.
 - Fill out the 023 air waybill according to previous shipments with the exception of the "Chargeable Weight", "Rate/Charge", "Total", "Other Charges" and the payment fields in the bottom left corner of the document.
 - Call FedEx the day before the shipment should go out at 1-800-332-0807 and ask for help in completing the 023 air waybill. They will determine the "Chargeable weight" (the greater of either the total weight of the package or the dimensional weight $\{[l \times w \times d] / 166\}$) as well as the applicable charges.
 - Ask FedEx to schedule a pickup for the shipment. They will give you an estimated pickup time (you may be able to choose) and will give you a confirmation number.
 - The shipment will be picked up by FedEx at receiving.
- 1.5.4.4. Keep the FedEx Tracking number until after the package has been delivered.
- 1.5.4.5. MDS-Bothell samples are shipped on dry ice on a Thursday.

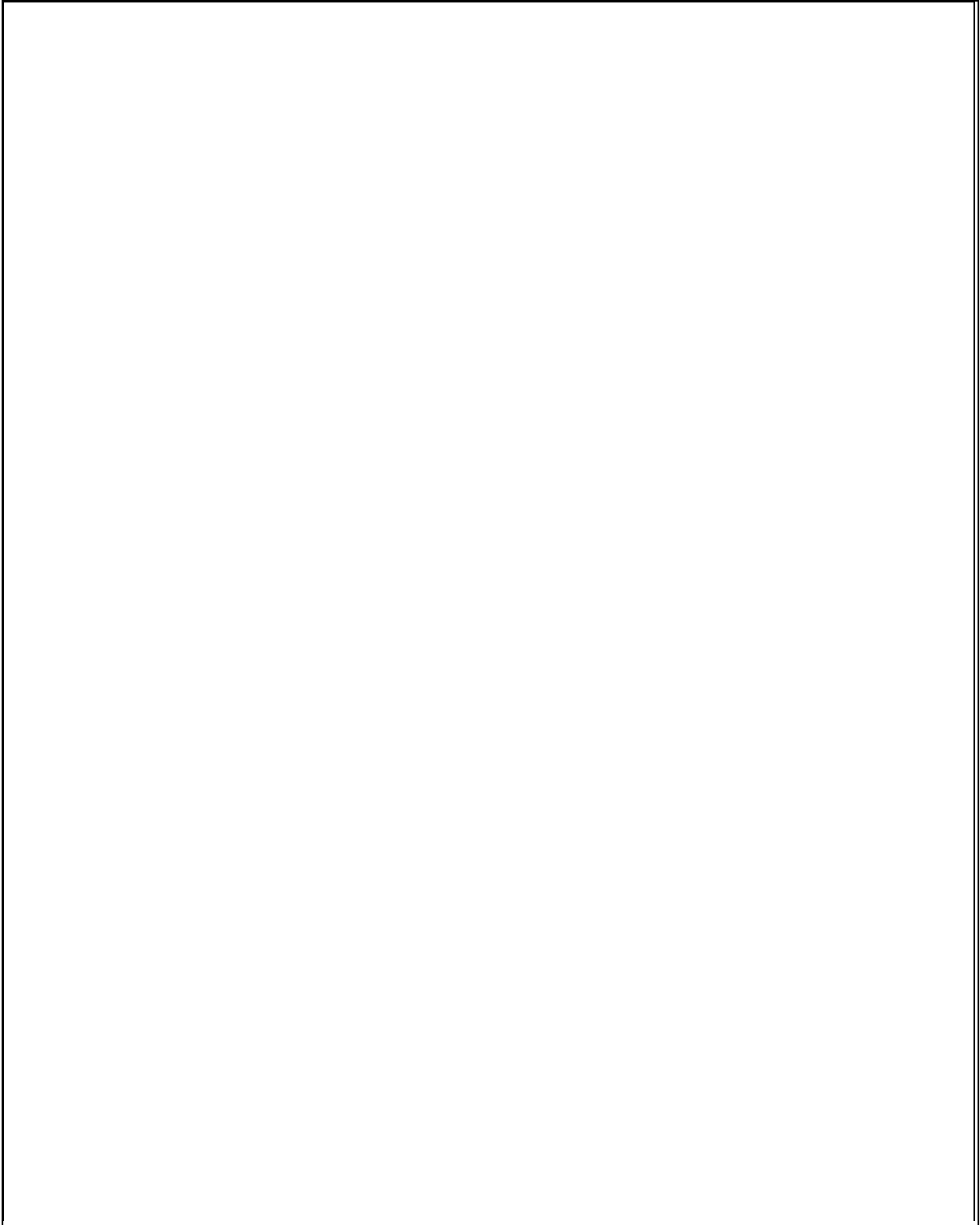


Figure 1.5-D: Example of Proforma Invoice for Shipment to Taiwan

Chapter 2

Range-Finding Study

2.1. PURPOSE

Range-Finding (RF) Studies determine the maximum tolerated dose (MTD) and are a pre-requisite to conducting Array Studies. This dose is operationally defined at Iconix as the maximal amount of drug administered that does not lead to life-threatening clinical signs or severe body weight loss (see details below). An appropriate vehicle and route of administration are selected after careful review of the relevant literature. A report of the selection of the doses, vehicle, and route of administration is provided for each compound.

2.2. RESPONSIBILITY

The Toxicology Division is responsible for implementation of this SOP.

2.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to CEX_Main database
- Access to VV_Exp_Design database
- Access to RF_Tracking Database
- Access to LD50 info Excel spreadsheet located on N:\ChemExpress\
In Vivo Biology\RF Study Preparation
- Access to CEX Smpl_Received located at VV_Exp_Design\FileMaker 1
- Access to relevant dose setting resources
 - PDR CD-ROM
 - Martindale CD-ROM
 - Access to RTECS (www.nisc.com)
 - Merck Index
 - PubMed
- Scaling Formula located on N:\ChemExpress\In Vivo Biology\RF
Study Preparation

2.4. PROCEDURE

2.4.1. *Tracking of Compound Availability for RF study*

- 2.4.1.1. The CEX Smpl_Received database located at VV_Exp_Design\FileMaker 1 contains the list of compounds available for Range Finding Studies.
- 2.4.1.2. The database is queried for specific compounds of interest, which information about the compound [ICXNumber, MolName, Amount Received (grams), and CAS Number] is exported into a Tab delimited file.
- 2.4.1.3. For compounds with more than one CAS Number, the CAS Numbers will appear on multiple lines.
- 2.4.1.4. Within the tab-delimited file, cut and paste the CAS Numbers into the cell on the first line (which contains the rest of the compound information) and separate them with commas.
- 2.4.1.5. Delete the empty lines.
- 2.4.1.6. Make sure that only the NUMBER is present in the "Amount Received" column.
- 2.4.1.7. Create a new column entitled "Compound Rank" beside the "Amount Received" column.
- 2.4.1.8. Change the field format of this new column to text
- 2.4.1.9. Fill in 01 in the cells of this column beside each compound then save the entire file as an Excel workbook entitled "new compounds"
- 2.4.1.10. Open the RF_Tracking Database and import the Excel file containing the new compounds into the RF_Tracking Database as follows:
 - File Import
 - Choose the "update existing records" option.
 - Tick the "add new records" box.
 - Match ICXNumber fields
 - Do NOT import MolName. The RF_Tracking database gets this information automatically.
 - Check for and eliminate duplicates being sure to preserve the most current and relevant information for each compound.

2.4.2. *The "LD50 Info" Spreadsheet*

This spreadsheet is the information source for the RF study compounds. Compounds are designated "high", "medium", or "low" priority in this spreadsheet. To prepare for the RF study, essential information for the compounds is curated into this spreadsheet. Information for the compounds destined for the upcoming range finding study is copied and pasted from the LD50 info spreadsheet into a "working" spreadsheet. Then, the information contained in the "working" spreadsheet is imported in batch into the RF_Tracking database AND the VV_Exp_Design database.

- 2.4.2.1. Open the LD50 info Excel spreadsheet located on Iconix-filesrv/ ChemExpress/In Vivo Biology/RF Study Preparation
- 2.4.2.2. Cut and paste the ICXNumber, MolName, and Amount Received from your "new compounds" Excel spreadsheet into the appropriate columns of the list of compounds present in the "compound information" worksheet of the LD50-info Excel spreadsheet.
- 2.4.2.3. LD50 spreadsheet column C is used when compounds have been designated high priority. Fill in this information for each compound if necessary.

2.4.3. *Choosing Compounds for the Upcoming RF study*

- 2.4.3.1. Refer to the current schedule [located on Iconix-filesrv1; ChemExpress/In Vivo Biology/Animal Studies and Compound Weighing Schedules folder to determine the number of compounds needed for the upcoming study.
- 2.4.3.2. Check the RF_Tracking Database to see if any repeats need to be conducted.
- 2.4.3.3. Determine how many compounds need to be researched by defining the total number of doses that can be done as follows:
 - Maximum doses available = total number of compounds for the RF study X 3
 - Repeat compounds and MFD test compounds do NOT always need 3 doses
 - Plan to use up the maximum doses available accordingly:
 - For a study of 21 compounds, maximum doses = 21 X 3 = 63

- Example of distribution: 18 new compounds X 3 = 54 doses,
3 repeats X 2 = 6 doses; 3 MFD tests X 1 = 3 doses

2.4.3.4. Fill in the appropriate information for the following columns following the detailed instructions below:

Table 2.4-A: RF Study Database Content

Column Letter	Contents
F	Human route of administration (ROA)
G	Human ROA alt (alternative clinical ROA, if available)
H	Value of the relevant ROA, no units (the ROA most frequently used in the clinic and/or which suits the amount of compound we have available, scaled to rat if necessary)
I	Oral ROA (if F and/or G applies)
J	IV ROA (if F and/or G applies)
K	Subcutaneous ROA (if F and/or G applies)
L	Inhaled ROA (if F and/or G applies)
M	IP ROA (if F and/or G applies)
N	LD50 information source
O	(yes/no) if LD50 information on the compound came from the CEX databases
P	Solubility information
Q	Solubility information source
R	Vehicle
S	Rationale
T	High Dose (automatically calculated when column H is filled in)
U	Mid Dose (automatically calculated when column H is filled in)
V	Low Dose (automatically calculated when column H is filled in)

2.4.4. Curation for Relevant Route of Administration

(Columns F and G)

- 2.4.4.1. Using the PDR CD-ROM or an Internet source of information (e.g., Google); enter the name of the compound to find the route of administration and primary indication for the compound in humans.
- 2.4.4.2. If the compound is a toxicant, use the available resources to find how a person is typically exposed to it (*i.e.* inhalation or ingestion).
- 2.4.4.3. Enter the relevant route of administration (ROA) into the spreadsheet using one of the following EXACT terms:
 - Oral gavage
 - Intraperitoneal
 - Intravenous
 - Subcutaneous
 - Intramuscular
 - Inhale
 - Infusion
- 2.4.4.4. Any other versions will have to be changed to these terms before importing this information into the VV_Exp_Design database.

2.4.5. Finding LD50 Information

(Columns N–O)

- 2.4.5.1. Check Databases.
- 2.4.5.2. Go to CEX_Main and click on Compound Information; then In Vivo/Clinical Pharmacology & Toxicity Data (long green button near bottom left).
- 2.4.5.3. The “Animal Toxicology Curation” section contains LD50 information.
- 2.4.5.4. In the “LD50 Info Source” column (N), cite their source for the LD50 (e.g., RTECS, HSDB, PubMed, etc.).
- 2.4.5.5. Since RTECS is a commonly used resource, the specific procedure for identifying LD50 information from RTECS is detailed below:
 - Go to www.nisc.com.

- Under NISC highlights, go to the GRC homepage.
 - Click Login (Subscriber) on the left side of page
 - Enter the user ID and password.
 - Locate the CAS Number in the RF spreadsheet.
 - Paste the CAS Number into the search box beside “limit to Chemical Names or CAS Registry Numbers” in the RTECS database search sheet.
 - Click Search.
 - Scroll to the “Acute Toxicity Data” section.
 - Find relevant the LD50.
- 2.4.5.6. Enter rat LD50 information into the spreadsheet (Columns I – M) with units, converting immediately to mg/kg.
- 2.4.5.7. If rat information is NOT available, take the following species in order of preference: mouse, guinea pig, hamster, rabbit, dog, cat, sheep, swine, and horse.
- 2.4.5.8. Note the species to which the LD50 belongs if not rat.
- 2.4.5.9. Note the species to which the LD50 belongs if not rat. Write yes/no in Column O, to show if the LD50 information was obtained from our databases or not.
- 2.4.5.10. Exit RTECS by clicking on Exit

2.4.6. Solubility Information

(Columns P and Q)

- 2.4.6.1. Paraphrase the solubility description into Column P and put the source for the information in Column Q
- 1. PDR
 - 2. Martindale
 - 3. Merck Index
- 2.4.6.2. Theoretical Water Solubility (used if there is NO solubility information present in sources 1 or 2):
- Open CEX_Main, click on Compound Information tab, and Drug Physico-chemical Properties tab.
 - Under “Solubility in Water” are three rows of boxes; the first row (pink boxes) gives the experimentally obtained water

solubility; the second and third give theoretically calculated water solubility using two independent algorithms.

- The concentrations at which the compound is soluble in water are given in these boxes and can be used to determine if the doses chosen will accommodate a water vehicle. Values in the pink boxes are given priority.
- Copy the lowest of these in Column P.
- Note the following in Column Q: Drug Matrix theoretical solubility calculation.

2.4.6.3. Internet Searches (e.g., Google, HSDB, PubMed) (used if there is no solubility information present in 1, 2, or 3)

- Cite the solubility description in Column P and copy and paste the web address for the information into Column Q.

2.4.6.4. Default vehicle is 0.5% CMC.

2.4.7. *Choosing the Vehicle*

(Column R)

- 2.4.7.1. For all administration routes except “IV”, if the compound displays sufficient water solubility, water is selected as the vehicle.
- 2.4.7.2. If the compound is not soluble in water, 0.5% carboxymethylcellulose (0.5% CMC) is the second choice.
- 2.4.7.3. “IV” compounds are always given in saline (unless specifically dictated otherwise by a credible source e.g. Merck/PDR).
- 2.4.7.4. If an “IV” compound cannot be dissolved in saline and must be formulated in a special way, find the compound in the RF_Tracking database and change its ranking to 08 (Formulation Problem) and note what must be done for formulation in the “Notes” box.
- 2.4.7.5. Enter the vehicle choice into the spreadsheet, choosing from the following EXACT terms:
 - 0.5% CMC
 - Saline
- 2.4.7.6. For customer compounds, the vehicle choice is identified by the customer and entered directly into the spreadsheet as noted above. However, if the vehicle has been previously run by Iconix,

all appropriate parties of the project team, from Database support to Array processing are notified of the addition. This ensures the proper changes to the appropriate databases are included as well as inclusion of a sufficient number of control animals in the subsequent array study.

2.4.8. *Setting the Doses*

(Columns H, T-V)

- 2.4.8.1. Enter the value (no units) of the relevant LD50 into Column H of the spreadsheet
- 2.4.8.2. If the LD50 isn't rat, scale the appropriate value to rat using the appropriate conversion formula found in the "Scaling Formulae" spreadsheet located on Iconix-filesrv1; ChemExpress; In Vivo Biology; RF Study Preparation folder then enter the value into Column H.
- 2.4.8.3. When LD50 is available, RF doses are set according to the following algorithm and are automatically calculated and entered into Columns T, U, and V:
 - High RF dose = LD50
 - Mid RF dose = LD50/2
 - Low RF dose = LD50/4
- 2.4.8.4. If LD50 values from various sources differ, there are several options:
 - Average the LD50 values and use the above-mentioned algorithm to set the RF doses.
 - Set the doses to encompass both values (*i.e.* the lower LD50 could be the mid RF dose).
 - Do the RF study basing doses on the LOWER LD50 value.
- 2.4.8.5. If LD50 is unknown, RF doses are chosen based on the following:
 - IP route of administration in rat or mouse (scaled) - used as low RF dose.
 - Long-term toxicity information mined from RTECS (TDLo/ReproTox dosing will give the total amount administered, the length of time of the study, and, in some cases, descriptive terms from their observations of the animals); used as low RF dose.

- Toxicity information mined from PubMed or other internet sources.
 - Helpful search strings for PubMed include:
 - Compound AND toxicity AND route of administration.
 - Compound AND route of administration AND primary indication.
- 2.4.8.6. If LD50 is >2500mg/kg, test the Maximum Feasible Dose (MFD):
- Search our databases, PDR, Martindale, and internet (as described above) for half-life information.
 - The MFD should never be set above 1500mg/kg/day with only rare exception.
 - If possible, consider the half life of the compound and the established LD50 as follows:
 - If LD50 > 5000mg/kg/day AND half life is > 8 hours, set the MFD at 1500mg/kg/day
 - If LD50 > 5000mg/kg/day AND half life is < 8 hours, set the MFD at 2000mg/kg/day
 - If 2500 < LD50 < 5000 AND half life is > 8 hours, set the MFD at 1000mg/kg/day
 - If 2500 < LD50 < 5000 AND half life is < 8 hours, set the MFD at 1500mg/kg/day
- 2.4.8.7. This value is entered into Column H
- 2.4.8.8. Once the spreadsheet calculates High, Mid, and Low doses for these values, delete them and the formulae.
- 2.4.8.9. Copy and paste the MFD value from Column H into the Low dose column V.

2.4.9. Rationale Report

(Column S)

- 2.4.9.1. A dose-selection justification report is written in the “Rationale for choosing each dose” box on the T page for the compound.
- 2.4.9.2. Cut and paste the information from the Excel spreadsheet as follows:
- Look at a previous report to see the format.
 - List the LD50 used to set the doses into the report.

- List the vehicle chosen and the justification
- Beside the LD50 and the vehicle, cite all sources/references used (*i.e.* Merck, RTECS, and PubMed).
- If clarification required, write a phrase justifying the vehicle and LD50 choices.
- Use <alt> <enter> to create line breaks within the Excel cell in which you are entering the rationale.
- The following is the format for citing PubMed references (if used to set RF doses):
 - (PMID: _____ abstract only) - if information directly obtained from the abstract, print out the abstract; write the compound name and ICXNumber at the top and file in Iconix library
 - (PMID: _____ article retrieved) - if online copy of article obtained at library or article retrieved from Stanford , print out this article; write the compound name, ICXNumber, AND PMID Number at the top and file in the Iconix library

2.4.10. *Entering Information into the VV Database*

- 2.4.10.1. Once the spreadsheet has been completed for the required number of compounds, enter YES into Column D beside each compound that will be put into the RF study.
- 2.4.10.2. Cut and Paste the lines containing the compound information for the upcoming RF study from the LD50 spreadsheet into a new Excel spreadsheet.
- 2.4.10.3. Save this spreadsheet as RF<date>.xls
- 2.4.10.4. Immediately Paste this same information into the "RF DONE" worksheet of the LD50 spreadsheet below the compounds that have already been done.
- 2.4.10.5. Return to the RF spreadsheet and insert a column in front of the ICXNumber.
- 2.4.10.6. Go to VV_Main.
- 2.4.10.7. Click on the "Experiment Design" button.
- 2.4.10.8. Click on "Experiment List→ Experimental Design" button.
- 2.4.10.9. You will see a "list" view of all RF and Array experiments.

- 2.4.10.10. If this page is already open, press <ctrl>J to make sure that all records are displayed
- 2.4.10.11. Create as many new records as necessary for the required number of compounds in the upcoming RF study. (If your password doesn't allow this, ask Database Support to change your password privileges OR to create the new records for you.)
- 2.4.10.12. Click the sort button above the ICXNumber.
- 2.4.10.13. Scroll down until you reach the first record which is blank in the Exp/Ctl/Tox box.
- 2.4.10.14. Copy the Exp# of this record into the first cell of the newly created column (A) in the RF Excel spreadsheet
- 2.4.10.15. Fill in the rest of Column A with numbers increasing sequentially by 1 from the Exp# for each compound in the spreadsheet
- 2.4.10.16. To the right of the Exp# column, insert a new column and type T in each cell of the column for each compound in the spreadsheet.
- 2.4.10.17. To the right of the T column, insert a new column and type in the projected RF study date for each compound using the following format: XX/XX/XXXX (month/day/year)
- 2.4.10.18. To the right of the date column, insert a new column and type the name of the RF dose setting scientist in each cell of the compound for each compound in the spreadsheet
- 2.4.10.19. Import the new compounds and their information into the VV_Exp-Design file.
 - Use the “update existing records” option
 - Match Exp# with the actual number in Column A (= sign)
 - Do NOT tick the “add new records” box
 - Use arrows to direct the appropriate information from the RF worksheet into the corresponding fields in VV_Exp_Design.
- 2.4.10.20. Once the import is complete, FileMaker will show you the records containing the information on the RF compounds.
- 2.4.10.21. Click on the gray box beside the “T” in a particular compound record. You must only write into the T page.
- 2.4.10.22. You will pull up a complex page containing many fields.

- 2.4.10.23. Verify that all information from your spreadsheet has been placed in the correct fields.
- 2.4.10.24. Make corrections if necessary.
- 2.4.10.25. Choose Sprague-Dawley Rat from the pull-down menu in the "Animal_Strain_ID" box.
- 2.4.10.26. Choose 2M from the pull-down menu in the Animal_Group_Size box.
- 2.4.10.27. Enter the volume administered in the "Dose_Vol" box located on the left side of the T page as follows:
 - 10 if compound is orally administered
 - 2.5 if compound is administered IV
 - 5 if compound is administered SC
 - 2.5 if compound is administered IP
- 2.4.10.28. Copy and paste the Smpl_LogNo from the "Available Compounds" box in the middle of the T page into the "Drug_Smpl_LogNo" box located on the left near the top of the T page.
- 2.4.10.29. Below the dose boxes of the T page for the compound is a box entitled "Use for T, E, T&E"
- 2.4.10.30. Click on this box and choose T from the pull-down menu
- 2.4.10.31. Enter the date of the dose-setting meeting in the "mm/dd/yyyy Finalized" box using / to separate month, day, and year (refer to the schedule).
- 2.4.10.32. Enter the shipping date in the "mm/dd/yyyy Shipped" box (e.g. 06/22/2002 Shipped).
- 2.4.10.33. Click on the appropriate CRO name in the pull-down menu of the "CRO" box.

2.4.11. *Entering Dose Values into the VV Database*

- 2.4.11.1. Dose values are imported from the Excel spreadsheet.
- 2.4.11.2. If not, enter the doses in the "High_Dose", "Mid_Dose", and "Low_Dose" boxes of the T page for the compound in the VV database manually.

- 2.4.11.3. The total compound amount required for the RF study appearing in the "Total amount needed for range finding" box is automatically calculated.

2.4.12. *Updating the RF_Tracking Database*

- 2.4.12.1. The records in the RF_Tracking Database are updated in batch by importing relevant information from your RF Excel spreadsheet.
- 2.4.12.2. Create a new column in the RF Excel spreadsheet.
- 2.4.12.3. Change the field format of this new column to text.
- 2.4.12.4. Fill in 02 in the cells of this column beside each compound
- 2.4.12.5. Import the new compounds and their information into the RF_Tracking database.
- Use the "update existing records" option
 - Match ICXNumber fields (= sign)
 - Do NOT tick the "add new records" box
- 2.4.12.6. Use arrows to direct the appropriate information from the RF worksheet into the corresponding fields in RF_Tracking

2.4.13. *Compound Amounts to Drug Informatics for Weighing*

The *In Vivo* Compound Dispenser (Drug Informatics Group) is in charge of weighing compounds for RF studies.

- 2.4.13.1. Send an email to the *In Vivo* Compound Dispenser (cc: Director Toxicology, Director of Drug Informatics, *In Vivo* Experiment Designer, CRO Liaison, MDS Assay Compound Dispenser) containing the following information:
- Date Finalized
 - Projected RngFd date
 - Number of compounds he should expect to pull up
 - Date that the compounds must be ready in time for delivery
 - Any other essential details
 - Note in the email that he should look in CEX_invivo, Planning, and search under BOTH "Date_Finalized" AND Projected_RngFd_Date to pull up the compounds.

2.4.14. Upon Completion of the RF Studies at the CRO

- 2.4.14.1. The CRO Study Director will transmit the clinical observations from the RF studies to the CRO Liaison
- 2.4.14.2. The CRO Liaison will collect all data for each compound RF Experiment and format it for analysis. The data from the CRO is transferred electronically to a separate labeled folder on the server.

- Concatenate body weight data files from the CRO if necessary using the Excel macro “concatBW”.
- Open the concatenated data file and the appropriate compound dose-level table for the RF study. Edit the dose level table so that the compounds on the table are in the same order as the compound treatments in the body-weight data file. Save the dose level table to the desktop (or other location from which it can be conveniently deleted later) with the name “RFDosetable.xls”.
- Add rows to the body weight data file so that each compound name occurs in six contiguous rows.

****Note: Column A in each added row must be nonblank. The macro will not work properly if there are empty cells in Column A or if there are not exactly six rows for each compound.***

- Generate the RF data reports by running the Excel macro “CopyWeight”.
- The macro saves the generated data reports in the N:\ChemExpress\In Vivo Biology\InVivo_Clinical_Data\Range Finding Studies\DoneGraphs folder. Move the report files from this folder to a subfolder of the folder containing the RF data from the CRO.
- Concatenate the clinical observations files from the CRO if necessary using the Excel macro “concat Obs”.
- Copy and paste each day’s observation for each animal from the clinical observations data file to the appropriate box on the appropriate RF data report.
- Enter the gross necropsy findings if any in the appropriate blank on the RF data report.
- Save and close each RF data report as it is completed.

2.4.14.3. Print out the graphs and tables for each drug and evaluate for success or failure.

2.4.14.4. Criteria for success:

- A dose-response in body weight change with respect to compound dose administered is observed AND at least one dose shows an average of 5 – 10% body weight gain.
- Body weight gains are greater than 10% for the highest RF dose administered AND this was the LD50 of the compound.

2.4.14.5. Criteria for failure/repeat:

- If animal(s) are lost in all doses, the RF study is repeated.
- If animals gained greater than 10% body weight at the highest RF dose AND LD50 was NOT used, the RF study is repeated.

2.4.14.6. Annotate “success” or “repeat” on the graph.

2.4.15. *Update of VV and RF_Tracking Databases*

2.4.15.1. Go to the “list” page in the VV database and find all compounds associated with the RF study.

2.4.15.2. In the box entitled “Exp_Outcome”, select the appropriate designation from the pull-down menu. (“Successful”, “To be repeated”, “Feasible dose applied”, *etc.*)

2.4.15.3. Go to VV_Main.

2.4.15.4. Click on the “Experiment Design” button.

2.4.15.5. Click on “Experiment List → Experimental Design” buttons.

2.4.15.6. You will see a “list” view of all RF and Array experiments.

2.4.15.7. If this page is already open, press <ctrl>J to make sure that all records are displayed.

2.4.15.8. Click the sort button above the ICXNumber.

2.4.15.9. Scroll down until you reach the first record, which is blank in the Exp/Ctl/Tox box.

2.4.15.10. Choose T from the pull down menu in the Exp/Ctl/Tox box.

- 2.4.15.11. Paste the ICXNumber for the repeat compound in the "ICXNumber" box.
- 2.4.15.12. Doing a "Find" for this ICXNumber will pull up records of all studies done for this compound including the most recently created "T" record.
- 2.4.15.13. In the "Animal Grp Size" field for the new T record, select 2M from the pull-down menu.
- 2.4.15.14. Find the record for the RF study which is to be repeated for this compound
- 2.4.15.15. Click on the "Vehicle", "Exp Duration", "Dosing_Route" boxes and select from the pull-down menu such that these fields exactly match those in the previous RF study.

**Note: The default "CRO" is Stanford Research Institute, International, Menlo Park, CA.*

2.4.16. Update of VV and RF_Tracking Databases for Repeat RF Studies

- 2.4.16.1. When repeating the RF study, increase/decrease the doses in increments of 1.5 from the high/low RF dose from the unsuccessful study.
- 2.4.16.2. Verify the amount of compound remaining in-house.
- 2.4.16.3. BE SURE to consider the amount of compound required for the Array study. If insufficient compound is available, notify the Director of Toxicology to approve the order for more compound.
- 2.4.16.4. Set up to two doses depending on the amount of compound in-house.
- 2.4.16.5. These repeat compounds should be included in the next RF study.

Refer to the schedule and insert the appropriate date in the Projected_RngFd_Date field in BOTH VV_Exp_Design AND RF_Tracking

Chapter 3

Range Finding Compound Preparation for Delivery to CRO

3.1. PURPOSE

Provide our Contract Research Organizations (CROs) with the material and information they need to successfully complete a Range Finding Study.

3.2. RESPONSIBILITY

The Toxicology group is responsible for implementation of this SOP.

3.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to FileMaker Pro
- Access to InVivoRangeFindingStudy folder located at ChemExpress/ChemExpress Worksheets/Biology Worksheets.
- Weighed compounds list

3.4. PROCEDURE

3.4.1. *Retrieving Information from FileMaker Pro Database*

- 3.4.1.1. Open VV_Exp_Design FileMaker database. Log into the VV_Main database, Click the "Experimental Design" button, enter password, and then click the "Experiment List → Experimental Design" button.
- 3.4.1.2. Find the compound information for the current Range Finding (RF) study by activating the Find tool by clicking on the View menu and selecting "Find Mode" or by pressing Ctrl+F.
- 3.4.1.3. Click in the blank field in the "Projected RngFind Date" column, enter the start date of the Range Finding Study in mm/dd/yyyy format, and press ENTER.
- 3.4.1.4. Sort the resulting list of compounds, click the Records menu and select "Sort..." Select these field names in order in the VV_Exp_Design file (should be the default): "Dosing Route", "Vehicle", and "MolName".
- 3.4.1.5. Export the compound list: click the File menu and select "Export Records" Chose a location to save the exported file to, name the

file "RFStudyymmdd" and select "Tab-Separated Text Files (*.tab)" as the Save As file type. Click the "Save" button.

- 3.4.1.6. Export the following fields in order from the VV_Exp_Design file (should still be the default):
- ExpNum
 - Exp_Control_Tox
 - ICXNumber
 - MolName
 - Dosing Route
 - Vehicle
 - Tox_Dose_Vol_ml_per_kg
 - Tox_Low_Dose_mg_per_kg
 - Tox_Mid_Dose_mg_per_kg
 - Tox_High_Dose_mg_per_kg
 - Animal_Group_Size

3.4.2. *Filling the Dosing Table*

- 3.4.2.1. Launch Excel and open the InVivoMacro.xls workbook.
- 3.4.2.2. To run a macro in Excel, under the "Tools" menu, select the "Macro" sub menu and select "Macros..."
- 3.4.2.3. Run the "RFMasterList" macro. When prompted, select the tab delimited file that was generated in step 3.4.1.5. After the macro has finished, the RangeFinding Master List.xls will be open with the new data pasted in and highlighted.
- 3.4.2.4. Double-check this table to ensure that the data fall under the proper columns.
- 3.4.2.5. Enter the appropriate SRI study code and study start-date in the "SRI Code" and the "Date" columns, then save and close the RangeFinding Master List.xls file.
- 3.4.2.6. Run the RFDoseTable Macro. Check the resulting table to ensure that the data fall under the proper columns.
- 3.4.2.7. Enter the appropriate SRI study code and study start date under the "SRI Code" and the "Date" columns.
- 3.4.2.8. Enter the correct Information in the cells that read: "CRO Here", "Date Here", and "Study Code Here". Double check the table to

ensure that the columns are wide enough that all of the data will be legible when printed, then save and close the workbook.

- 3.4.2.9. Save the Dosage Table file in the appropriate Range Finding folder in ChemExpress/InVivo Biology/ Sample Tracking/ Delivered Compounds. Name the file "CROcpdsRFyyyymmdd"
- 3.4.2.10. Run the "RFDosePrep" macro. Check the resulting table to ensure that all of the dose levels are represented with the proper number of significant figures, that the data fall under the proper columns, and that the columns are wide enough that all of the data will be legible when printed.
- 3.4.2.11. Enter the appropriate SRI study code and study start date under the "SRI Code" and the "Date" columns.
- 3.4.2.12. Enter the correct Information in the cells that read: "CRO Here", "Date Here", and "Study Code Here". Save and close the table.
- 3.4.2.13. Print the Dosage Table and the Dose Prep Table to accompany the compounds to the CRO and copy Iconix's Director of Toxicology.
- 3.4.2.14. Send an electronic copy of the Dose Table and the Dose Prep Table to the CRO as an e-mail attachment and copy Iconix's Director of Toxicology.
- 3.4.2.15. Print the weighed out compounds list sent by the In Vivo Compound Dispenser/MDS Assay Compound Dispenser.
- 3.4.2.16. Compare weighed compounds list against the list of compounds in the Dosage Table. There must be 100% concordance between the table and the physical vials of compounds. If there is not, notify the Group responsible for weighing the compound and resolve the discrepancy.
- 3.4.2.17. Email the Dosage table to the 'Study Director' of the CRO and copy Iconix's Director of Toxicology.
- 3.4.2.18. Deliver compounds, Dosage table, and weighed compounds list to the CRO.

Chapter 4

Weighing Compounds for *In Vivo* and *In Vitro* Studies

4.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for weighing compounds for *in vivo* and *in vitro* studies.

4.2. REFERENCE

Drug Matrix Compound Library Protocol

4.3. RESPONSIBILITY

The Drug Informatics group is responsible for implementation of this protocol.

4.4. REQUIRED MATERIALS AND EQUIPMENT

- Access to CEX_invivo.FP5 database
- Access to CEX_CellBio database
- Analytical balance
- Amber 4-dram vials (VWR Part No. 66010-408)
- Amber 125 ml glass jar (VWR Part No. 16154-449)
- Amber 60 ml glass jar (VWR Part No. 16154-448)
- Teflon-lined caps for 4-dram vials (VWR Part No. 66012-394)

4.5. PROCEDURE

4.5.1. *Preparation of Samples for In Vivo Studies*

- 4.5.1.1. The Biology department decides each week's set of samples.
- 4.5.1.2. Once the samples have been decided, the Drug Matrix librarian will be notified, by e-mail, when they are ready.
- 4.5.1.3. The samples to be prepared will be listed in CEX_invivo.FP5; the librarian will be notified of which Date_Finalized the samples are associated with. The Biology department is responsible for deciding upon the preferred manufacturer, lot number, and sample amount to be dispensed; this information will be entered into VV_Exp_Design.FP5, by the member of the Biology

department responsible for setting doses, and can be retrieved from CEX_invivo.

- 4.5.1.4. For each week's samples, part of the set will be weighed out for an array study, and part of the set will be weighed out for a range-finding study – the librarian will be notified of which experiment dates each subset of samples is associated with.
- 4.5.1.5. In CEX_invivo, the particular stock the sample should be taken from will also be noted (*i.e.* manufacturer, lot, *etc.*)
- 4.5.1.6. Each sample should be labeled with the sample name, molecular weight, manufacturer, and lot number (use the "Bottle label" layout in CEXSmpl_Received.FP5).
 - Use a 4-dram vial if the sample weight is 7 grams or less (this may vary depending on the density of individual compounds).
 - Use the large glass amber jars for samples of greater than 7 grams (this may vary, depending on the density of individual compounds).
 - For each sample, keep a temporary record of the sample weight, which will later be logged into the Drug Matrix database.
- 4.5.1.7. Log all sample weights into CEX_invivo as gram quantities.
- 4.5.1.8. After all samples have been weighed, leave them in a designated area of one of the Drug Matrix freezers for delivery to the CRO by the CRO Liaison.

4.5.2. *Preparation of Samples for In Vitro Studies*

The Iconix Biology department conducts these studies in-house.

- 4.5.2.1. The Biology department will determine each experiment's set of samples.
- 4.5.2.2. Once the samples have been decided upon, they will be entered into CEX_CellBio by the Biology department.
- 4.5.2.3. The Biology department is responsible for selecting from which supplier and lot the sample will be drawn from, and is responsible for specifying this preference in CEX_CellBio.
- 4.5.2.4. If a sample request is e-mailed to the Drug Matrix librarian, the librarian is responsible for entering these requests into CEX_CellBio. In either case, the Biology department is responsible for letting the librarian know of any manufacturer

and lot preferences. All requests made for Cell Biology experiments will be in milligram quantities.

- 4.5.2.5. Weigh the samples into amber 1-dram vials, and cap with a Teflon-lined cap; each sample should be labeled with its ICX number. Enter the sample weights, in milligrams, into CEX_CellBio.
- 4.5.2.6. Leave the samples in a designated area of the Drug Matrix freezer, or a designated area of the shared lab refrigerator for the person working on the in vitro experiments.

Chapter 5

In Vivo Array Study Experimental Design

5.1. PURPOSE

Provide the appropriate documentation for the dose selection and other study design details for *In Vivo* Array Study conduct.

5.2. RESPONSIBILITY

The Toxicology group is responsible for implementation of this SOP.

5.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to VV_Exp_Design database
- Access to Internet resources (e.g., PubMed, MedMiner at <http://discover.nci.nih.gov/textmining/filters.html>)
- Access to Stanford library
- Access to “mortality Report” located at ChemExpress/In Vivo Biology/Sample Tracking/Array Study Mortality
- Scaling Formulae located on Iconix-filesrv1/ ChemExpress/In Vivo Biology/RF Study Preparation

5.4. PROCEDURE

5.4.1. *Preparation of New Record in VV database*

- 5.4.1.1. Open the VV_Exp_Design Database.
- 5.4.1.2. Search for the compounds in the latest Range-Finding (RF) study by using the “find” feature and typing the date of this study in the “Projected RngFd Date” field.
- 5.4.1.3. For each compound that was designated “successful” in the “Exp_Outcome” field in RF studies, create a new record in the VV_Exp_Design database as follows:
 - Copy the ICXNumber of the compound
 - Click “Ctrl-J” to make sure that all records are being displayed.
 - Click the “sort’ button above the “ICXNumber” field.
 - Scroll down until you see a record that is blank in the “Exp/Ctl/Tox” column (2nd from the left).
 - Click on this box and select “E” from the pull-down menu.

- Paste the ICXNumber into the ICXNumber field of that record.
- Search for the ICX Number using the “Find” feature to identify the records of all completed studies for this compound including the most recently created “E” record.
- In the “Animal Grp Size” field for the “E” record, select 3M from the pull-down menu.
- Identify the record for the RF study, which was designated “successful” in the “Exp_Outcome” field for this compound.
- In the new E record, click on the “Vehicle”, “Exp Duration”, and “Dosing_Route” boxes and select from the pull-down menu such that these fields exactly match those in the successful RF study.

5.4.2. *Preparation of the Rationale Report*

- 5.4.2.1. The entire report for ALL compounds in the Array Study is prepared in a Word document.
- 5.4.2.2. The Rationale Report contains the Maximum Tolerated Dose (MTD) Recommendation and the Fully Efficacious Dose (FED) Recommendation.
- 5.4.2.3. Upon completion, the report for each drug is copied and pasted into the “Rationale for choosing each dose” box on the E page in the VV_Exp_Design database.

5.4.3. *Maximum Tolerated Dose*

Choosing the Maximum Tolerated Dose (MTD) Based on RF Study Results

- 5.4.3.1. The acceptable level of toxicity for Array Studies is a 5 – 10 % body weight GAIN in the absence of clinical signs throughout the 6 days of observation.
- 5.4.3.2. If any of the RF doses meets the defined level of toxicity, this dose becomes the recommended high array dose. If multiple doses meet this criteria, the HIGHEST of the acceptable doses is selected.
- 5.4.3.3. Otherwise, use the following as guidelines for choosing the high array dose:
 - Normal male rat body-weight gain is 3-4% per day, thus over a 5d study, the range is 14-20%.

- If the high RF dose = LD50 for rat and animals gained weight normally, use this high RF dose as the MTD. In situations where additional information is available, the MTD may be exceeded, see Dose Setting Meeting below.
 - Each RF dose is evaluated individually, at least initially. When 2 or more RF doses lead to the same decision point (see below), the HIGHER dose is selected.
 - Use the RF dose as the MTD dose under the following circumstances:
 - 1st animal body weight gain 5 – 10%, 2nd animal 0 – 5%, +/- mild clinical signs
 - Both animals have a body weight gain 5 – 10% + mild clinical signs
 - Divide the RF dose by 2 under the following circumstances:
 - 0 – 5% body weight gain, moderate/severe clinical signs
 - 0 – 10% body weight loss, mild/moderate clinical signs
 - Divide the RF dose by 4 under the following circumstances:
 - >10% body weight LOSS +/- clinical signs
 - If a Higher RF dose gives 0 – 5% body weight loss/gain +/- mild/moderate clinical signs AND the next lower RF dose gives 10% - normal body weight gain +/- mild clinical signs THEN the recommended MTD array dose is ½ way between the 2 doses.
- 5.4.3.4. Prepare a concise paragraph of the RF study results describing the following (refer to previous reports to see how this has been done):
- Doses administered
 - Average body weight change for all dose groups
 - Body weight loss for each animal if there are substantial inter-individual variation
 - If and when unscheduled deaths occurred
 - A description of the clinical observations which are defined as follows plus when they occurred during the study:
 - Level 1 = mild
 - Level 2 = moderate
 - Level 3 = severe
 - Level 1 eye discharge is always considered moderate

- At any level, blood in the stool or urine is always considered severe
- If no clinical signs were observed
- Definitions for abbreviations used can be found on Iconix-filesrv1/ChemExpress/In Vivo Biology/InVivo Clinical Observations/Observations Dictionary/ InLifeObservationsSRI (Excel spreadsheet) and Location.tsv (use Excel to open)
- Recommended MTD or MFD (see 5.4.3 OR 5.4.4).

5.4.4. *Maximum Feasible Dose*

5.4.4.1. The Maximum Feasible Dose (MFD) is chosen under the following conditions:

- When the RF study does not identify a dose which causes body weight change 50% less than control or shows clinical signs of toxicity.
- When limited supply of compound is available and an RF study can't be done. In this case, doses are selected based on available literature information.
- When not enough compounds have been tested in RF to complete the required amount of compounds for the Array study AND the compound's LD50 > 2500mg/kg.

5.4.4.2. The MFD is set as follows:

- The MFD is NEVER above 2000mg/kg/day.
- A 10X spread between the MFD and the low array dose is the goal. In the case of highly toxic compounds, this might not be feasible.
- In rare instances, MFDs must be derived in the absence of a RF. In such cases, consider the half life of the compound and the established LD50 as follows:
 - If LD50 > 5000mg/kg/day AND half life is > 8 hours, set the MFD at 1500mg/kg/day
 - If LD50 > 5000mg/kg/day AND half life is < 8 hours, set the MFD at 2000mg/kg/day
 - If 2500 < LD50 < 5000 AND half life is > 8 hours, set the MFD at 1000mg/kg/day
 - If 2500 < LD50 < 5000 AND half life is < 8 hours, set the MFD at 1500mg/kg/day

5.4.5. *Fully Efficacious Dose Recommendation*

This part of the rationale report is divided into several sections:

- Summary
- Background Information
- Target/Toxicity Tissue Candidates
- Animal Studies

5.4.5.1. Background Information

This section provides a concise paragraph describing the compound. The purpose is to allow the reader of the rationale report to have an idea of what the compound does and to be able to put the MTD and the Fully Efficacious Dose (FED) information into context.

The Background Information Section includes the following information:

- Molecular Target
- Indication
- Mode of Action (MOA)
- Clinical Dose

The above-listed information is found by searching the following sources:

- DrugMatrix™
- PDR (CD Rom)
- Martindale (CD Rom)
- Internet searches
- Once the information is obtained, summarize it in a paragraph which presents the molecular target, lists the indications for the compound, describes the MOA, and finally gives the dose used in the clinic (drugs).
- Cite the source of information of each fact used.
- Scale the clinical dose to the rat equivalent using standard scaling formulae.
- If the compound is a toxicant, summarize, in a descriptive paragraph, its uses, the possible routes of exposure, as well as any other relevant information found in the various sources examined.

- For compounds where FED is irrelevant (e.g., toxicants), the FED should be 10x lower than the high dose in the array study.

5.4.5.2. Target/Toxicity Tissue Candidates

This section lists potential tissues for the array studies by priority of toxicity or target.

- Review “adverse effect” information provided on the compound in the sources listed above.
- From this information and from other sources of toxicity and MOA information (DrugMatrix™, PDR, Martindale, Internet searches), select tissues and rank them as follows, based on severity of side effects, type of warnings, percentages of patients affected:
 - 1'tox: effect is noted in a “black box” warning; compound specifically acts in this tissue; compound was withdrawn by FDA for adverse effects on this tissue; literature information specifically states that the drug has serious adverse effects on this tissue
 - 1'target: compound specifically acts in this tissue;
 - 2'tox: adverse effects on this tissue (system to which the tissue belongs) occur in a high percentage of patients taking the drug; literature information states that this tissue is indirectly affected by the compound in either an adverse or beneficial way
 - 2'target: literature information states that this tissue is indirectly affected by the compound in either an adverse or beneficial way
 - 3'tox: adverse effects on this tissue (system to which the tissue belongs) occur in a low percentage of patients taking the drug
- Note that any combination of tox/target ranking can be used (e.g. 1'tox/2'target).
- Besides each ranking for the tissue, state the facts that support this ranking.
- Cite the source for the information for each fact used.

5.4.5.3. Animal Studies

This section lists animal models in which the drug/compound was used to ameliorate a disease state, in a toxicity study, in a study

involving investigation into mode of action, and/or in a pharmacokinetic study. The purpose of this section is to gather enough information from the literature on how the compound has been used in research to define the FED.

5.4.6. *Searching the Literature*

- 5.4.6.1. Open the Lane Medical Library homepage:
<http://lane.stanford.edu/index.html>
- 5.4.6.2. Click on "PubMed" (located at the top of the page).
- 5.4.6.3. This opens up an embellished version of PubMed, which states whether the journal is present at the library and if the article can be obtained on-line.
- 5.4.6.4. Search the literature using MedMiner (5.4.7) OR PubMed and various combinations of keywords (examples listed below):
 - Drug AND species AND route of administration
 - Drug AND animal AND route of administration NOT human NOT patient
 - Feel free to create your own keyword combinations or use PubMed in any possible way to obtain relevant results

5.4.7. *Using MedMiner*

(Optional search tool)

- 5.4.7.1. On the Internet, go to the MedMiner homepage:
<http://discover.nci.nih.gov/textmining/filters.html>.
- 5.4.7.2. Click on the "General Query" button.
- 5.4.7.3. Scroll down to the "Abstract must contain one of these comma-separated (OR) terms" box and type in the compound name.
- 5.4.7.4. Scroll down to the "Abstract must also contain these comma-separated (AND) terms" and type in "rat" then comma then route of administration (*e.g.* oral, intravenous, subcutaneous).
- 5.4.7.5. Click on the "Submit Query" button.
- 5.4.7.6. In the center of the page, make sure that the "Select results format" choice is set to "Keyword Categories".
- 5.4.7.7. Click on the "Filter X abstract(s)" box
 - If this box says 0 abstracts, click the back button and replace "rat" with "mouse"

- If the box still says 0, click the back button and try the following species in order of preference: guinea pig, hamster, dog, cat, sheep, pig/swine, and cow.
- 5.4.7.8. Scroll down the “Filtered Results” page.
- 5.4.7.9. Abstracts are divided according to the MedMiner categories and each abstract may show up in many different categories.
- 5.4.7.10. The filter provides some context, which can be used to decide if the abstract is useful. If the abstract is useful, it **MUST** be reviewed. If usefulness is not evident from the context, the abstract **MUST** be reviewed.
- 5.4.7.11. Tick the box beside the PMID number. This automatically puts a tick mark beside this abstract for all the times that it is listed. This way, you will know that you have already reviewed this particular abstract.
- 5.4.7.12. Click on “PMID” to open the abstract in PubMed together with the MeSH terms.
- 5.4.7.13. A box will appear marked “Stanford Print Holdings” if the journal is available at Lane AND a second box will appear marked “Stanford Online” if the article can be downloaded from the journal website at the Stanford library.
- 5.4.7.14. Review the abstract for relevance. The rat strain can sometimes be obtained from the MeSH term list.
- 5.4.8. *Working with the PubMed Abstracts***
- 5.4.8.1. If the abstract contains dose and time information, it is included in the Animal Studies section for the compound:
- Describe the purpose of the study
 - Describe the animals used including species if possible, how much compound was used for dosing, and the length of the dosing period
 - Describe the outcome of the study
 - Note (PMID: _____, abstract only)
 - Print out a copy of the abstract
 - Write the ICXNumber and MolName at the top
 - Deliver the abstract for filing and addition to the Iconix hard copy library

- 5.4.8.2. If the abstract does not mention route of administration or dose amount or time of dosing, the article must be obtained
- 5.4.8.3. Construct a list in an Excel spreadsheet named "array XX-XX-XX (date)" name the worksheet containing the articles to be obtained "references"
- For articles to retrieve from Stanford, list the ICXNumber, MolName, Reference (cut and pasted from the PubMed abstract), PMID (cut and pasted form the PubMed abstract)
 - Provide a column entitled "Obtained?" so that the article can be checked off as it's retrieved and photocopied when at the library.
- 5.4.8.4. When the article is obtained, follow the steps listed above in "Working with the PubMed abstracts", section 1 to write a descriptive paragraph of the study. Note (PMID: _____, article retrieved)
- 5.4.8.5. Write the ICXNumber, MolName, and PMID at the top of the front page of the article.
- 5.4.8.6. Deliver the abstract for filing and addition to the Iconix hard copy library.
- 5.4.8.7. Requirements for recommending the FED:
- 2 – 5 studies describing use of the compound in a relevant (clinically related) animal disease model
 - If this many studies cannot be obtained from abstracts only, 4 – 5 articles must be obtained to ensure that at least 1 – 2 have the relevant information
 - If nothing relevant is available in PubMed, write a statement to this effect in the rationale report
- 5.4.8.8. Note that supplemental information can be obtained for the "Target/Toxicity Tissue Candidates" section as well as the mode of action part of the "Background Information" section.
- State the fact(s) for the relevant tissue or as part of the Background Information paragraph.
 - Note (PMID: _____, abstract only)
 - Print out and write the ICXNumber and MolName at the top of the abstract or article as described above.

- Deliver the abstract or article for filing and addition to the Iconix hard copy library

5.4.9. *Stanford Library*

- 5.4.9.1. Obtain the library pass from the receptionist.
- 5.4.9.2. If the library pass is not available, you will have to pay the \$10.00 entrance fee. Keep the receipt for reimbursement.
- 5.4.9.3. If driving, keep track of the mileage between Iconix and the Stanford library. Use the parking lot associated with the hospital.
- 5.4.9.4. Be sure to have at least \$20.00 available for copies.
- 5.4.9.5. When at the library, go first to the computer room on the right hand side as you pass through the entry turnstile to obtain the articles from online journals.
- 5.4.9.6. Find a computer, which is ALREADY logged into the Lane Library homepage. You will NOT be allowed to login to the system since you are NOT a Stanford affiliate.
- 5.4.9.7. Click on the "PubMed" button.
- 5.4.9.8. Type in the PMID and click "Go"
- 5.4.9.9. Click the "Stanford Online" box and obtain the reference in the PDF format.
- 5.4.9.10. If this doesn't work, return to the Lane Library home page and click on "E-journals" located on the left side of the page.
- 5.4.9.11. Find the relevant journal and click on it. Obtain the reference in the PDF format.
- 5.4.9.12. Print this reference.
- 5.4.9.13. Keep track of how many pages you print.
- 5.4.9.14. Deposit 5 cents per page into the moneybox beside the printer.
- 5.4.9.15. Keep track of how much money you spend.
- 5.4.9.16. For "shelf" articles, continue to the back of the library to the stacks. Journals including 1980 and onwards are on the main floor. Journals before 1980 are located downstairs in the compact shelving.

- 5.4.9.17. Obtain the relevant volume and photocopy the entire article taking care to be certain that ALL of each page is clear and legible.
- 5.4.9.18. Keep track of how much money you spend to charge the photocopy card or feed directly into the machine.
- 5.4.9.19. When leaving the parking lot, obtain a receipt for reimbursement.

5.4.10. *Fully Efficacious Dose Recommendation*

- 5.4.10.1. Review all studies written up in the "Animal Studies" section
- 5.4.10.2. Select an exact dose used in one of the studies using the following criteria as priority
 - Double an ED50 value
 - The ROA in the animal study should be the same as that used in our studies.
 - Chronic rat dose in a model reflecting the clinical indication
 - Chronic rat dose in an alternative disease/MOA model
 - Single rat dose in a model reflecting the clinical indication
 - Single rat dose in an alternative disease model
 - Chronic mouse dose in a model reflecting the clinical indication
 - Single mouse dose in a model reflecting the clinical indication
 - Chronic/single mouse dose in an alternative disease/MOA model
 - Chronic dose in other RODENT species (guinea pig, hamster, rabbit) in a model reflecting the clinical indication
 - Single dose in other RODENT species (guinea pig, hamster, rabbit) in a model reflecting the clinical indication
 - Chronic/single dose in other RODENT species (guinea pig, hamster, rabbit) in an alternative disease/MOA model
 - Single rat dose in a pharmacokinetic study keeping the spread between MTD and FED ≥ 10
 - Single other RODENT species (mouse, guinea pig, hamster, rabbit) dose in a pharmacokinetic study keeping the spread between MTD and FED ≥ 10
 - Chronic dose in other species (dog, cat, sheep, swine, horse, cow) in a model reflecting the clinical indication

- Single dose in other species (dog, cat, sheep, swine, horse, cow) in a model reflecting the clinical indication
 - Chronic/single dose in other species (dog, cat, sheep, swine, horse, cow) in an alternative disease/MOA model
 - If NONE of the above is available, FED = 10X less the MTD
- 5.4.10.3. In the Dose Selection rationale, underline AND italicize the study used to define the FED.

5.4.11. *Summary*

- 5.4.11.1. State the recommended FED and briefly describe the study that was used in the choice.
- 5.4.11.2. State the compound's DrugMatrix™ chemical structure classification group, the primary toxicity tissue associated with this group, and, any other noted toxicities as follows:
- Open CEX_Main
 - Click on "Compound Information"
 - Click on "Drug Matrix Case Study"
 - Do a "find" and cut and paste the ICXNumber into the relevant box
 - The compound's study group, primary toxicity tissue, other noted toxicities will appear if they have been decided by the committee appointed for this task.
 - If there is no information on your particular compound, do a more general search in this database using the structure class (CEX_Cmpd, MOA/Target/Pathway Curation layout)
 - Base primary toxicity tissue choice on that which was chosen for a compound of the same structure class and/or whose chemical structure is closely related to that of your compound
- 5.4.11.3. Liver, Kidney, Heart, Bone Marrow are always collected at each time point.
- 5.4.11.4. Choose the target/toxicity tissues in order of preference as follows:
- Primary toxicity tissue according to Drug Matrix chemical structure classification (5.4.11.2).
 - Tissue in which the compound acts if not the Primary toxicity tissue

- Refer to the list of Target/Toxicity Tissue Candidates to add more tissues from the standard harvested tissues according to their rank.
- The floater tissue is included in this list and is prioritized according to its rank in the list of Target/Toxicity Tissue Candidates.

5.4.11.5. Choose the floater tissue as follows:

- If the primary toxicity tissue is NOT always collected, this is the floater tissue.
- If the pharmacologic target tissue is NOT always collected, this is the floater tissue.
- Refer to the list of Target/Toxicity Tissue Candidates to choose a floater tissue according to its rank.
- Intestine is the default tissue for the compounds unless a strong case from the literature or other reference sources is made.

5.4.11.6. List these tissues in order of preference in a descriptive phrase noting which tissue is the floater.

5.4.12. *Preparation for the Dose-Setting Meeting*

5.4.12.1. Enter all recommended dose information into the dose boxes on the compound's E page in VV_Exp_Design.

5.4.12.2. Enter the volume administered in the "Dose_Vol" box located on the left side of the E page right below the dose boxes as follows:

- 10 if compound is orally administered
- 5 if compound is administered SC
- 2.5 if compound is administered IV
- 2.5 if compound is administered IP

5.4.12.3. Enter the array start date in the "Projected_Exp_Date" box at the bottom left of the E page.

5.4.12.4. Enter your name in the "Array_Exp_Designer" field.

5.4.12.5. Select the floater tissue from the pull down menu in the "Floater_Tissue" field.

5.4.12.6. Enter the tissue choices in order of preference, ONE tissue per line, in the "Tox_Target Animal Tissue" box.

- 5.4.12.7. Copy and Paste the Rationale Report from the Word document in which you are working, into the "Rationale for choosing each dose" field.
- 5.4.12.8. Copy and paste the Smpl_LogNo from the "Available Compounds" box in the middle of the E page into the "Drug_Smpl_LogNo" box located on the left near the top of the E page.
- 5.4.12.9. Below the dose boxes of the E page for the compound is a box entitled "Use for T, E, T&E".
- 5.4.12.10. Click on this box and choose E from the pull-down menu.
- 5.4.12.11. Enter the date of the Dose Setting meeting in the "mm/dd/yyyy Finalized" box (e.g. 06/22/2002) using slashes as separators.
- 5.4.12.12. Enter the shipping date in the "mm/dd/yyyy Shipped" box (e.g. 06/22/2002) using slashes as separators.
- 5.4.12.13. Click on the appropriate CRO name in the pull-down menu of the "CRO" box.
- 5.4.12.14. Return to List Mode.
- 5.4.12.15. Sort the compounds of the array study by Dosing_Route AND Vehicle AND ICXNumber.
- 5.4.12.16. Export the following information into a tab-separated text file:
 - ICXNumber
 - MolName
 - Nml_High_Dose_mg_kg
 - Nml_Low_Dose_mg_kg
 - Dosing_Route
 - Vehicle
 - Nml_Dose_Vol_ml_kg
 - Floater_Tissue_Code
 - Floater_Tissue_Name
 - Tox_Target_Tissue
 - CRO
- 5.4.12.17. Copy and Paste this table into a new worksheet entitled "doses" in the array mm-dd-yy Excel spreadsheet that already contains

the list of references obtained from the library for this set of compounds.

- 5.4.12.18. Format the table so that it is easily read and so that it fits on one page when printed.
- 5.4.12.19. Print enough copies of this table for all members of the group attending the Dose-Setting meeting.
- 5.4.12.20. Re-sequence the rationale reports for each of these compounds in the Word Document in the order in which they appear in the table.
- 5.4.12.21. Print enough copies of this Word Document for all members of the group attending the Dose-Setting meeting.
- 5.4.12.22. Distribute the Word Document and the Table to all members of the group attending the Dose-Setting meeting as early as possible prior to the meeting.
- 5.4.12.23. Assemble the RF graphs for the compounds and arrange them in the order in which they appear in the table.
- 5.4.12.24. Bring these to the meeting.

5.4.13. *At the Dose-Setting Meeting*

- 5.4.13.1. The Toxicology group will go over each compound, the dose selection rationale, the floater tissue selection, and any additional study design issues. At the discretion of the Toxicology group and approval of the Director of Toxicology, slight deviations from the standard protocol can be determined at this meeting.

Examples include but are not limited to:

- Inclusion of additional animals at later time points to ensure at least 3 animals survive to the end of the study.
- Collecting more tissues at the earlier timepoint.
- Increasing the dose level greater than the MTD.

5.4.14. *After the Dose-Setting Meeting*

- 5.4.14.1. When compounds have been chosen and doses verified, select a CRO for each compound from the pull-down menus located:
 - VV_Exp_Design “CRO” field
 - E page for the compound – “CRO” field (left center of page)
 - If more than one CRO is to be used:

- Change the "Date Finalized" for the second CRO to one day later than that of the dose-setting meeting.
- 5.4.14.2. Correct all typographical errors in both the Word Document and in each rationale report in VV_Exp_Design.
- 5.4.14.3. At the foot of the rationale report for the compound in VV_Exp_Design states the following in bold: All recommendations accepted at the dose-setting meeting XX-XX-XX (date) (YOUR NAME).
- 5.4.14.4. If changes were made to the doses or tissue choices, at the foot of the rationale report, in bold, describe the changes and reasons behind them. Then state the following: All other recommendations accepted at the dose-setting meeting mm-dd-yy (YOUR NAME).
- 5.4.14.5. If the CRO reports any problems with the animals after the study has started, discuss with the Director of Toxicology the possible solutions (lowering dose, restarting study, etc.).
- 5.4.14.6. Report this change immediately to the CRO.
- 5.4.14.7. Save ALL emails as Word-documents to a subfolder titled with the CRO Study ID.
- 5.4.14.8. This folder should be added to the "Study Email Folder" located on Iconix-filesrv1/ChemExpress/In Vivo Biology.
- 5.4.14.9. At the foot of the rationale report for the compound, enter, in bold, the date, the new dose, and the reason for the change along with your name. Save an electronic copy of the rationale report (Word document in which you have been working) and the Excel spreadsheet containing the references, dose, and array tissue information on Iconix-filesrv1/ChemExpress/In Vivo Biology/DosesRationale
- 5.4.14.10. Save a hard copy of the rationale report (Word document in which you have been working), the Excel spreadsheet containing the references, dose, and array tissue information, and the RF study graphs in the Doses/Rationale Binder.

5.4.15. *Array Tissues*

After the dose-setting meeting, the array tissue is finalized by the Director of Toxicology using the recommendations made in the Rationale Report.

- 5.4.15.1. The Director of Toxicology will inform you of the precise tissue that is to be processed in arrays.
- 5.4.15.2. In the array mm-dd-yyyy spreadsheet, copy and paste the ICXNumber and the compound name into a new worksheet called array tissues.
- 5.4.15.3. Insert the specific tissue into the column beside its corresponding compound.
- 5.4.15.4. Add the array date in a new column beside each compound.
- 5.4.15.5. Return to FileMaker and select Array_Tissues.
- 5.4.15.6. Import the data from the array tissues worksheet into the Array_Tissues:
 - Select the “Add New Records” option
 - Use arrows to match the corresponding fields

5.4.16. *Compound Amounts to Drug Informatics for Weighing*

- 5.4.16.1. Transmit the compound information to the *In Vivo* Compound Dispenser (Curation Group) in an email containing the following information (cc: Director of Toxicology, Director of Drug Informatics, *In Vivo* Experiment Designer, CRO Liaison, MDS Assay Compound Dispenser):
 - Date Finalized
 - Projected Array Study Date
 - Number of compounds he should expect to pull up
 - Date that the compounds must be ready in time for delivery
 - Any other essential details
 - Note in the email that he should look in CEX_invivo, Planning, and search under BOTH “Date_Finalized” AND Projected_Exp_Date to pull up the compounds
 - The *In Vivo* Compound Dispenser provides a hard copy of the actual compound amounts weighed out and shipped to the CRO.
 - Save this hard copy in the Doses/Rationale Binder with the other material for the study.

5.4.17. Outcome of the Study

- 5.4.17.1. When the study is complete, its outcome must be entered into its record in VV_Exp_Design.
- 5.4.17.2. Use the "find" feature to identify the records of the compounds in the relevant study.
- 5.4.17.3. Check the CRO Liaison's mortality report sheet, found at N:\ChemExpress\In Vivo Biology\Sample Tracking\Array Study Mortality (show all files), to find if any animals were lost.
- 5.4.17.4. Go to the bottom of "sheet 1" and check for any array compounds.
- 5.4.17.5. If no deaths have been reported for a particular compound, select "Successful" from the pull-down menu in the "Exp_Outcome" box.
- 5.4.17.6. If deaths were reported, select the appropriate description from the pull-down menu in the "Exp_Outcome" box: 1 death in array study; multiple deaths in array study, no samples received from CRO.

Chapter 6

Printing Compound Experimental Detail Tables for CROs

6.1. PURPOSE

Provide our Contract Research Organizations (CROs) with the compound, materials and information that they need to successfully complete an *in vivo* Array study, and to make a record of each experiment as it is initiated.

6.2. RESPONSIBILITY

The Toxicology group is responsible for implementation of this SOP.

6.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to FileMaker Pro
- Access to Study Tracking folder located at N:\ChemExpress\In Vivo Biology.
- Weighed compounds list
- “InVivoMacros.xls” located at N:\ChemExpress\In Vivo Biology\Protocols

6.4. PROCEDURE

6.4.1. *Enter the VV_Exp_Design in FileMaker Database*

- 6.4.1.1. Log into the VV_Main database, click the “Experimental Design” button and click the “Experiment List → Experimental Design” button.
- 6.4.1.2. Find the Records for the current Array study. Use the Find feature under the “View” menu by selecting “Find Mode” or by pressing CTRL+F.
- 6.4.1.3. Enter the start date of the Array study in mm/dd/yyyy format under the “Projected Exp_Date” column then press ENTER.
- 6.4.1.4. Sort the resulting list of compounds and controls by selecting the Records menu and selecting “Sort...” or by clicking the sort icon in the tool bar.
- 6.4.1.5. In the “Sort Records” dialog that appears, select these field names in order in the VV_Exp_Design file (should be the default):

- Dosing_Route
 - Vehicle
 - MolName
- 6.4.1.6. Export the Experimental Details from the File Maker Data Base, click the File menu and select “Export Records”.
- 6.4.1.7. Save the file to N:\ChemExpress\In Vivo Biology\Sample Tracking, name the file “Arrayyyymdd” and select “Tab-Separated Text Files (*.tab)” as the Save As file type.
- 6.4.1.8. Click the “Save” button. Select the following fields to export in order from the VV_Exp_Design file (should still be the default):
- ExpNum
 - Exp_Control_Tox
 - ICXNumber
 - MolName
 - Dosing_Route
 - Vehicle
 - Nml_Dose_Vol_mL_per_kg
 - Nml_Low_Dose_mg_per_kg
 - Nml_High_Dose_mg_per_kg
 - Floater_Tissue_Code
 - Floater_Tissue_Name
 - Array_Tissue:Array_Tissue
 - Animal_Group_Size

6.4.2. *Fill the Dosing and Tissue Tables*

- 6.4.2.1. Open the InVivoMacros.xls workbook and run a macro by selecting “Macros...” from the “Macro” submenu under the “Tools” menu in Excel and clicking the relevant macro name.
- 6.4.2.2. Run the “MasterList” macro. When prompted, select the tab delimited file that was generated in step 6.4.1.6. After the macro has finished, the Array Study Master-list worksheet will be open with the new data pasted in and highlighted. Double-check this table to ensure that the data fall under the proper columns. Enter the appropriate SRI study code and study start-date under the “Iconix Study Code”, the “CRO Study Code” and the “Exp. Start Date” columns, then save and close the product file.

- 6.4.2.3. Run the "DoseTable" macro. Check the resulting table to ensure that the data fall under the proper columns. Enter the correct information in the cells that read: "CRO Here", "Date Here", "Iconix Study Code", and "CRO Study Code". Double check the table to ensure that the columns are wide enough so that all of the data will be legible when printed, then save the product file into N:\ChemExpress\InVivo Biology\Sample Tracking\Delivered Compounds as "CROcpdsArrayyyyymmdd" and close.
- 6.4.2.4. Run the "DosePrep" macro. Check the resulting table to ensure that all of the dose levels are represented with the proper number of significant figures, that the data fall under the proper columns, and that the columns are wide enough so that all of the data will be legible when printed. Enter the correct information in the cells that read: "Enter CRO Here", "Enter Date Here", "Enter Iconix Study Code Here", and "Enter CRO Study Code Here". Save and close the product file.
- 6.4.2.5. Run the "TissueScheme" macro. Select the tissue scheme that is specific for the CRO that will receive the compounds in the current list.
- 6.4.2.6. Check the resulting table to ensure that all of the compounds have the correct floater tissues specified.
- 6.4.2.7. Enter the control vehicle and route in the top most yellow cell under the "Drug" column.
- 6.4.2.8. Enter the group number in the "group number" column and enter the appropriate animal sex under the "sex (M/F)" column.
- 6.4.2.9. Enter the correct information in the cells that read: "Enter CRO Here", "Enter Date Here", "Enter Iconix Study Code Here", and "Enter CRO Study Code Here".
- 6.4.2.10. Assign the correct floater tissues to the control animal groups. Enter the correct number of drugs, controls, and animals in the appropriate cells at the top of the table. Check the table to ensure that the columns are wide enough so that all of the data will be legible.
- 6.4.2.11. Save the worksheet, minimize the columns with grey cells, and print the table on a color printer.

- 6.4.2.12. Print the Dosage Table, the Dose Prep Table, and the Tissue Scheme Table to accompany the compounds to the CRO.
- 6.4.2.13. Send an electronic copy of the Dose the Dose Prep Table and the Tissue Scheme Table to the CRO as an e-mail attachment.
- 6.4.2.14. Print the weighed out compounds list sent by the In Vivo Compound Dispenser/MDS Assay Compound Dispenser.
- 6.4.2.15. Double check the compound amounts on the weighed compound list against the amounts calculated in the Dosage Table. There must be 100% concordance between the table and the physical vials of compounds.
- 6.4.2.16. Deliver of ship via overnight express service the compounds, weighed compounds list and Dosage table to the CROs.

Chapter 7

Generate Labels and Upload Sample files

7.1. PURPOSE

Automatically generate three data files for tracking samples in the database. A Codes file lists the code for each sample that will be produced in the study which is used for printing the bar-coded sample tube labels, an Animal file lists each animal code and experimental details, and a tissue file lists each tissue code with the corresponding experiment number. An additional purpose is to produce printed sample tube labels.

7.2. RESPONSIBILITY

The Toxicology Group is responsible for implementation of this SOP.

7.3. REQUIRED MATERIALS AND EQUIPMENT

- "InVivoMacros.xls located in N:\ChemExpress\In Vivo Biology\Protocols
- Word file 'MMAnimalTissues.doc located in N:\ChemExpress\In Vivo Biology\StudyTracking\Labels\Barcode label tools
- Labelmaker batch execution file located in N:\ChemExpress\In Vivo Biology\LabelMaker\labelmaker.bat
- Personal Computer
- Laser Cryotag 52 1.69"x0.75" labels per sheet (Diversified Biotech catalog # LCRY-1100)

7.4. PROCEDURE

7.4.1. *'Tissue', 'Animal', and 'Codes' File Generation*

- 7.4.1.1. Working from a PC, save the "TissueScheme" file to be processed as tab delimited text file (.txt).
- 7.4.1.2. Open the text file by double clicking the icon (it should open in Notepad) and delete all empty lines/spaces after the last animal number.
- 7.4.1.3. Save and close the "TissueScheme" text file.

- 7.4.1.4. Open the labelmaker execution folder. Right mouse click on the file (labelmaker.bat) and select edit. The file will open in a Notepad window.
- 7.4.1.5. The command line looks as follows:
"cd/D "N:\ChemExpress\In Vivo Biology\LabelMaker\"java-classpath %CLASSPATH%;.;iconix.jar;gnu.jar;classes12.zip com.iconix.lims.AnimalTissueParser 020422 S "N:\ChemExpress\In Vivo Biology\Sample Tracking\Tissue schemes Label sheets\SRI_TissueScheme20020422W.txt" pause.
- 7.4.1.6. Edit the three underlined text strings; the first string reflects the study start-date (yyymmdd format) for which the files are being created, the second is the single letter code for the CRO executing the experiment, and the third string identifies the tissue scheme to be processed (must be located in the specified folder).
- 7.4.1.7. Save and close the labelmaker.bat file after having made the necessary changes. The command lines should be thoroughly inspected to ensure that the Date code, CRO code, and file path are correct.
- 7.4.1.8. Run labelmaker by double-clicking on the icon.
- 7.4.1.9. Check the information that is being displayed in the run window for errors as follows:
- Check for 'exception'; any exception indicates an error
 - Check that all tissue codes that were used are displayed in the list
- 7.4.1.10. Three files are generated from the labelmaker parser, namely XyymmddANIMAL, XyymmddTISSUE, and XyymmddCODES, where Xyymmdd is the Study Code indicating the CRO and the study start date.
- 7.4.1.11. Check the three output files for completeness and accuracy as follows:
- Animal numbers need to match with the numbers from the tissue scheme file.
 - Tissue numbers need to match with the numbers of tissue samples from the tissue scheme file.
 - Codes numbers need to match with the numbers of tissue samples collected.

- Correct floater tissues are indicated in the tissue codes in the 'codes' file.
- Correct study code is indicated in all of the file names and in the bodies of the files.

7.4.1.12. Move the output files to the db upload folder located at N:\ChemExpress\In Vivo Biology.

7.4.2. *Printable Barcodes Generation*

- 7.4.2.1. Go to the file MManimalTissues.doc located at N:\ChemExpress\In Vivo Biology\Sample Tracking\Labels\Barcode label tools\MManimalTissues.doc.
- 7.4.2.2. Open the file and click "cancel" in the dialog window that appears, and then click the "find data source" button.
- 7.4.2.3. Choose the XyymmddCODES file generated previously (located in the db upload folder) by selecting "text files" in the Files to open window and click open.
- 7.4.2.4. Choose plain text.
- 7.4.2.5. Click the Merge button on the tool bar, and then click "Merge" in the dialog box that appears.
- 7.4.2.6. Check that the expected number of labels has been created using the following calculation: $\text{Number of expected labels} / 52$ (codes/page) = number of pages expected.
- 7.4.2.7. Create a header to appear on the top of every page of labels: Under the View Menu, select "Header and Footer". In the header edit-field, press return in order to type text in the second line from the very top. Text in the very top line will not be printed.
- Type the Iconix Study Code (Xyymmdd).
 - Press tab to move the cursor to the center position and type study code designated by the CRO.
 - Press tab to move the cursor to the Right justified position to enter the section numbering of the label sheets. MS Word treats the label document as one page and each label sheet as a section. To take advantage of this and add pagination information to the header, go to the "Insert" menu in the main menu bar and select "Field...", select "Numbering" in the Categories list and select "Section" under the Field names list.

Type “_of_” where “_” is a space and click the “Insert number of pages” button on the Header/Footer tool bar. The pagination should look similar to “1 of 231”. Finally, exit Header edit mode by clicking the “Close” button.

- 7.4.2.8. Select “Save As” and change the name of the label file to XyymmddBARCODE.doc and save it in the Labels folder in Sample Tracking folder.
- 7.4.2.9. Print the labels: Select “Print...” from the File menu or click the Print icon on the tool bar. Click the Print button on the dialog box. By default, the labels print from the manual paper feed tray. Open the manual feed door and load about 20 blank label sheets onto the tray. Reload the tray as necessary.

Chapter 8

In Vivo Expression Array Experiment Execution

8.1. PURPOSE

Array studies are conducted to obtain tissue samples from test compound-treated and vehicle-treated rats for expression array analysis. As part of the Array study, clinical, hematological, and pathological effects of test compounds are also determined.

8.2. REFERENCES

SRI M-193-01 Protocol
Ricerca BioSciences Study Protocol

8.3. RESPONSIBILITY

The CRO is directly responsible for implementation of this SOP, but the Toxicology Group is responsible for the oversight of the CROs. The CRO Liaison is responsible for delivering materials to the CRO, and for receiving results and data from the CRO.

8.4. REQUIRED MATERIALS AND EQUIPMENT

The following materials are required by the CRO to execute this SOP:

- Compound dosage sheet
- Floater Tissue Table printed in color
- Compound Vials - crosschecked against Compound dosage sheet
- Compound weigh out table
- Sample tube labels ****Check the Date in the Tissue Code****
- A vehicle large enough to transport 6 large foam-shipping cartons
- Safety Labels for the boxes
- Sterile, disposable 6 mm biopsy punch (VWR cat# 21909-144)

8.5. PROCEDURE

8.5.1. *Experimental Design*

- **Route:** Oral by gavage, intravenous injection, or subcutaneous injection. Route of administration will be defined by protocol amendment for each experiment.
- **Frequency:** Daily for 5 consecutive days
- **Study Length:** 5 days of treatment.
- **Necropsy:** Study Days 1, 2, 4, and 6

Table 8.5-A: Array Experiment Experimental Design

Group	Test Article	Dose Level (mg/kg)	No. Animals	No of Animals/Sacrifice Interval			
				6 hr	Day 1	Day 3	Day 5
1	TBD	0	12-24	3-6	3-6	3-6	3-6
2	TBD	0	12-24	3-6	3-6	3-6	3-6
3	TBD	Low	12	3	3	3	3
4	TBD	High	12	3	3	3	3
↓	TBD	Low	12	3	3	3	3
▼ <i>etc.</i>	TBD	High	12	3	3	3	3
11	TBD	Low	12	3	3	3	3
12	TBD	High	12	3	3	3	3

8.5.2. *Test and Control Articles*

(For one study)

Once a set of compounds is received, the CRO documents all of the following pertinent information regarding each test article and vehicle in the study records.

- **Test Article:** Document in the study records
- **Lot No.:** Document in the study records
- **Physical Description:** Document in the study records
- **Storage Conditions:** Document in the study records
- **Characterization of Test Articles:** Iconix is responsible for characterization of the identity, purity, and stability of the test articles.
- **Control Articles:** Document in the study records

- **Lot No.:** Document in the study records
- **Physical Description:** Document in the study records
- **Storage Conditions:** Document in the study records
- **Characterization of Control Article:** Characterization of the control article/vehicle may be attained by recording all pertinent information provided on the container labels or by retaining the container labels themselves as raw data.
- **Preparation of Dose Formulations:** Dosages will be prepared by accurately weighing out the appropriate amount of the test article, placing it in a glass vial, and mixing with the specified amount of vehicle.
- **Dose Formulations:** Characterization of the identity, purity, homogeneity, and stability of the test articles will not be performed.
- **Disposition:** Unused samples will be returned to Iconix.
- **Test Article Handling:** The test article will be handled according to the safety procedures specified by Iconix. If available, Iconix will provide an MSDS at the time of test article shipment.

8.5.3. *Test System Rat*

(For one study)

The CRO documents all of the following for test system Rat.

- **Species:** Document in the Study Records
- **Strain:** Sprague-Dawley (Ctrl: CD (SD)|GS BR) rat
- **Supplier:** Charles River Labs
- **Sex:** Document in the Study Records
- **Age at Initiation:** Males are 6-7 weeks old and weigh between 120 and 175 g on arrival; females are slightly older to ensure that their weight is similar to the males.
- **Weight at Initiation:** Document in Study Records
- **Number of Animals:** Document in the Study Records
- **Animal Care:** General procedures for animal care and housing will be in accordance with DHHS Publication No. (NIH) 86-23 (Revised, 1985) and the U.S. Department of Agriculture through the Animal Welfare Act (7 USC 2131), 1985 and Animal Welfare Standards incorporated in 9 CFR Part 3, 1991. No contaminants

are expected to be present in the food, water, or bedding, which could interfere and affect the results of the study. Include copies of appropriate feed and water analysis in the study record.

- **Quarantine:** 5 days
- **Housing:** 1-3/cage
- **Cages:** Suspended 22 x 12-1/2 x 8-in. polycarbonate or stainless steel cages
- **Bedding:** Sani-Chips hardwood bedding (P. J. Murphy Forest Products, Montville, NJ)
- **Light Cycle:** 12 hr light/12 hr dark (6:30 am to 6:30 pm)
- **Temperature:** 72 ± 5°F
- **Humidity:** 30-70%, brief excursions outside this range may occur (*e.g.*, when animal facility hallways are washed); excursions of less than 4 hr/day will be noted in the study records, but will not be considered deviations from the study protocol.
- **Ventilation:** At least 10 room volumes per hour, with no recirculation of air.
- **Food:** Purina Certified Rodent Chow, #5002, (Richmond, IN), *etc.* Copies of log-specific feed analysis reports will be archived for reference.
- **Water:** Purified (filtered, deionized, and UV-treated) tap water, *etc.*
- **Assignment of Animals to Study:**
 - **Day:** No more than 3 days before start of treatment.
 - **Randomization:** Randomly assigned to treatment groups using a computerized body-weight stratification procedure.
 - **Identification:** Individually identified by a unique, consecutively numbered ear punch.
- **Test System Justification:** Document in the study records.
- **Dosing:** Dosing of all animals occurs at the same time relative to the day night cycle, thus animals are dosed starting at 6:30 am to be completed in 2-4 hours depending on number of animals in the study with the dose time recorded ± 15 min. Dosing of animals is staggered based on the intended harvest

order so that the animals can be sacrificed and necropsied at the designated time point:

- **Blood:** Collected at time point + ≤ 5 minutes and never early.
- **Animals:** Euthanized at time point ± 30 minutes.

8.5.4. *In-Life Evaluations*

(For one study)

The CRO documents all of the following:

- **Mortality/Morbidity:** Twice daily on weekdays, once daily on weekends and holidays.
- **Clinical Signs:** 3-6 hr after dose administration on Days 1-7 or more often as clinical signs warrant. Time of observation is recorded ± 15 mins.
- **Body Weights:** Pretest, days 1, 3, 5, and prior to necropsy. Body weight recorded for found dead or unscheduled sacrifice animals.
- **Clinical Pathology:**
 - **Route of Collection:** Retro-orbital sinus or abdominal aorta under CO₂/O₂ anesthesia.
 - **Frequency:** Blood for clinical laboratory evaluation will be collected at necropsy.
- **Hematology:** Measure and record the following:
 - RBC count
 - Hematocrit
 - Hemoglobin
 - MCV
 - MCC
 - MCH
 - WBC count
 - Platelet count
- **Clinical Chemistry:** Measure and record the following:
 - Alanine aminotransferase (ALT/SGPT)
 - Albumin
 - Alkaline phosphatase
 - Aspartate aminotransferase (AST/SGOT)

- Bilirubin, total
- Blood urea nitrogen (BUN)
- Chloride
- Cholesterol
- Creatinine
- Creatine phosphokinase (CPK)
- Glucose
- Lactate dehydrogenase (LDH)
- Sodium
- Phosphorus
- Potassium
- Uric Acid
- **Plasma Collection for Plasma Drug Levels:**
 - **Frequency:** Blood for plasma drug levels will be collected at necropsy.
 - **Method of Collection:** Orbital Sinus/Abdominal Aorta. Samples will be collected using EDTA as the anti-coagulant.
 - **Sample Size:** 0.5 ml whole blood; blood will be processed to plasma and frozen at -70°C for possible future analysis.

8.5.5. *Necropsy*

The CRO documents all of the following:

- **Interval:** 3 individuals of each dose group will be sacrificed unfasted on for each of the following dose groups; 6 hr, 24 hr, Day 3, Day 5. Sacrifice and necropsy of every animal must occur at the same time relative to the day night cycle to maintain consistency between experiments:
 - The 6-hour and 24-hour animals are sacrificed and necropsied starting at 1 pm and completed in 2-4 hours depending on number of animals in the study. The 3-day and 5-day animals are sacrificed and necropsied starting at 7 am on the relevant day and completed in 2-4 hours depending on number of animals in the study. The time is recorded \pm 15 min. Necropsy will also be performed on animals found dead or sacrificed in moribund condition. However, frozen samples will not be retained on these animals.

- **Euthanasia:** Exsanguination under CO₂/O₂ or 60% CO₂/ 40% O₂ to effect.
- **Observations:** External examination of all body orifices and surfaces and an examination of all cranial, thoracic, and abdominal organs. Gross pathology findings will be recorded.
- **Blood Retained:** Blood is retained for three purposes:
 - Plasma separated for Array studies (frozen on liquid nitrogen)
 - Serum separated for clinical chemistry (kept at 4°C)
 - Whole blood for hematology (kept at 4°C)
- **Tissues Retained:** Sections of tissues will be retained (Table 8.5-B):
 - Section will be fixed in phosphate-buffered 10% formalin.
 - Additionally, sections will also be placed into 4 ml cryovials, snap frozen, and stored at -70 °C for shipment to Iconix. Time between sacrifice and the last tube being frozen in liquid nitrogen should be < 30 min. Frozen tissue sample tubes will be loaded into 3" high standard freezer boxes fitted with 100 cell dividers. The label on each tube will be read with a barcode scanner in order to record its position in the freezer boxes. The table of tissue sample tubes will be transmitted electronically on disk to Iconix at the same time as the frozen tissue samples.
- **Bone Marrow Collection:** As specified in Table 8.5-B, bone marrow will be collected from each animal by:
 - Both femurs will be removed and placed in a collection tube on water ice.
 - The marrow will be flushed with a hypodermic needle and syringe with 1 ml cold Phosphate Buffered Saline (PBS) by forcing the liquid through each bone at least 3-5 times to ensure maximum recovery.
 - The bones will be removed from the tube and the tube centrifuged for at least 5 minutes at 3000g.
 - The supernatant will be removed by either decanting or aspiration with care taken to remove as much liquid as possible.

- **Organ Weights:** Organ weights (paired organs will be weighed together) of each tissue retained will be recorded for all surviving animals at terminal sacrifice. The snap-frozen samples will not be weighed.

Table 8.5-B: Tissue Harvesting Instructions

Order of tissue Harvest	Label Code	Tissue	10% Formalin	Snap-frozen (note: when multiple tissue punches are taken, each punch will be placed in a separate tube)	Target sample weight
0	BL	Blood Plasma	N/A	1000uL of plasma in a single tube	1000mg aliquot
	BC	Whole Blood	N/A	0.2mL of whole blood will be added to each of 4 separate tubes (200uL per tube)	200mg aliquot
1	LI	Liver	Left lobe	3 x 6-mm punches from medial lobe will be retained in 3 separate tubes (one punch per tube). A 4th tube will be used for retention of the residual tissue.	100mg each punch
2	KI	Kidney	Right	Left - 3 x 6-mm in 3 separate tubes. A 4th tube will be used for retention of the residual tissue.	100mg each punch
3	SP	Spleen	1/3 spleen	2 x 6mm punches will be retained in 3 separate tubes. A 3rd tube will be used for retention of the residual tissue.	100mg each punch
4	BM	Bone marrow	N/A	Both femurs will be flushed with sterile saline to remove the marrow into a single tube. The sample will centrifuged, and all supernatant removed before freezing.	As much material as possible
5	BR	Brain	Right side	Left side - 3 longitudinal sections will be retained in 3 separate tubes. A 4th tube will be used for retention of the residual tissue.	100mg each section
6	HE	Heart	Right side	Left side - 3 x 6-mm punches in 3 separate tubes. A 4th tube will be used for retention of the residual tissue.	100mg each punch

Order of tissue Harvest	Label Code	Tissue	10% Formalin	Snap-frozen (note: when multiple tissue punches are taken, each punch will be placed in a separate tube)	Target sample weight
7	GS	Glandular Stomach	Remaining tissue	Cut lengthwise, rinsed with cold PBS. section tissue into up to 4 samples.	100mg each section
8	FS	Forestomach	Remaining tissue	Section tissue into up to 4 samples	100mg each section
9	LU	Lung	Right side	Left side section into up to 4 samples	100mg each section
10	MU	Thigh muscle	Right side	Left side - 3 x 6-mm punches in 3 separate tubes. A 4th tube will be used for retention of the residual tissue	100mg each punch
11	RO	Gonads	Right	Left side - will be retained in single tube	
12	IN	Intestine	>5 Cm distal to stomach	5 cm distal to stomach, section will be opened lengthwise and rinsed with cold PBS. 3 sections will be retained in 3 separate tubes. A 4th tube will be used for retention of the residual tissue.	100mg each section
13	CM	Cecum		Section tissue into up to 4 samples	100mg each section
14	CO	Colon		Section tissue into up to 4 samples	100mg each section
15	DU	Duodenum		Section tissue into up to 4 samples	100mg each section
16	IL	Ileum		Section tissue into up to 4 samples	100mg each section
17	JE	Jejunum		Section tissue into up to 4 samples	100mg each section

8.5.6. *Histopathologic Examination*

- **Groups Evaluated:** Animals in Day 6 necropsy groups only.
- **Tissues:** Iconix will determine tissues. Three sections will be prepared and evaluated.
- **Performed by:** Board-certified veterinary pathologist.

- **Method:** Each lesion will be listed and coded by the most specific topographic and morphologic diagnoses, severity, and distribution by using Systematized Nomenclature of Medicine (SNOMED, Rothwell and Cote, Eds, College of American Pathologists, 1993) codes as a guide. A four-step grading system (minimal, mild, moderate, and marked) will be used to define gradable lesions for comparison between treated and control groups. Records of gross findings for a specimen from postmortem observations will be available to the pathologist when examining that specimen microscopically.

8.5.7. *Statistical Analysis*

- **Parameters:** Body weight, clinical pathology data, absolute organ weight and organ-weight ratios.
- **Statistical Test:** One-way ANOVA followed by Dunnett's test to compare the mean of each dose group with the control.
- Hypothesis Rejection: $p < 0.05$.

8.5.8. *Control of Bias*

While evaluating the responses of the animals, the technical staff should be aware of the treatment history of each animal. However, based on the relatively objective endpoints to be examined, bias is not expected to influence the results of the study.

8.6. REPORTING

8.6.1. *CRO Report*

The CRO will report all data to Iconix electronically and in print. Electronic data tables will be formatted in a way specified by Iconix to facilitate automatic loading to a database.

8.6.2. *Iconix Audit of CRO*

Iconix will perform an annual audit of the CRO to ensure compliance with the Iconix Standard Operating Procedure. This audit includes the following:

- Dosing time +/- 15 min recorded
- Clinical observations 3-6h after dosing, time recorded +/-15min
- Necropsy time recorded
- CO₂ used to euthanize animals

- Necropsy tissues harvested in following order:
 - blood plasma
 - spleen
 - intestine (20-30cm starting 5cm downstream of the duodenum, cut lengthwise and rinsed with PBS)
 - glandular stomach (cut lengthwise and rinsed with PBS)
 - fore stomach (cut lengthwise and rinsed with PBS)
 - liver (median lobe for array, small lobe for histopathology)
 - kidney (left for array, right for histopathology)
 - heart
 - lung (left for array, right for histopathology)
 - thigh muscle (left for array, right for histopathology)
 - bone marrow (marrow from both femurs for array)
 - testes (left for array, right for histopathology)
 - brain (left for array, right for histopathology)
- For paired organs, correct side used for array
- Each punch only used for one tissue from one animal
- Longitudinal sections taken from brain
- Organs kept sterile (*e.g.* on parafilm)
- Organ weights are recorded
- Time between sacrifice and last tube per animal in liquid nitrogen <30min
- No possibility of mix-up between organs or between animals
- Tube labels double-checked before tissue is added to tube.

Chapter 9

Reception and Storage of Frozen Tissues

9.1. PURPOSE

Safely transport, store, and track frozen tissue samples used in expression array experiments.

9.2. RESPONSIBILITY

The Toxicology group is responsible for implementation of this SOP.

9.3. REQUIRED MATERIALS AND EQUIPMENT

- Inventory disk from the CRO
- Portable PC
- Access to Chemexpress/In Vivo Biology/Sample Tubes Received

9.4. PROCEDURE

9.4.1. *Receiving Tissues*

- 9.4.1.1. Receive tissue samples packed on dry ice and inventory disks with tissue location tables from the CRO.
- 9.4.1.2. Create a new folder in Chemexpress/In Vivo Biology/Sample Tubes Received named CROyyyymmddcode, where CRO is a three-letter abbreviation for the name of the CRO where the tissue samples are from, yyyymmdd is the start date of the experiment, and code is any CRO generated identifier for the experiment. Create a sub folder named Disksyymmdd and copy all of the files from the disks received into that folder. All of the tissue location data should be supplied by the CRO on a single disk in a single file.
- 9.4.1.3. Combine the tissue location data into a new workbook from the Inventory template located in the Sample Tubes Received folder. Open each file in the disks folder and copy and paste the data in the "Box Code", "Position", and "Tissue" columns to the same columns on the "tubes" page and save the workbook as Inventoryyyyymmdd in the CROyyyymmddcode folder.
- 9.4.1.4. Generate the list of Box Codes expected. Fill in the Box Code column of the table. If the Box Code column has not been

completely filled in by the CRO, it will have to be done at this point. Copy the Box Code (TB000000) from the cell to the left of the "A2" under the Position column and paste it into all of the cells that correspond to tissue samples in the same box (there should be 98 positions in each box). The keyboard shortcuts to do the copy-paste operation are the following:

- Select the first BoxCode entered in the table.
- Press CTRL+SHIFT+DownArrow. This selects the active cell, the next non-empty cell (the next BoxCode), and every empty cell in between.
- Press SHIFT+UpArrow. This deselects only the bottom cell of the selection; the cell containing the next Box Code
- Press CTRL+d. This copies the value of the active cell to all of the selected cells below it (this action is called "copy down".)
- Press CTRL+DownArrow. This selects the last non-empty cell in a column of contiguous cells (the next BoxCode).
- Repeat steps (except the first) until there is a BoxCode for each tissue sample code.

9.4.1.5. Select the "Count of Box Code" pivot table and activate the pivot table tool under the "Data" menu. Click the "Back" button and edit the formula in range field so that it completely includes the data. The formula will look like this: Tubes!\$E\$1:\$F\$9213, change the last number so that it is equal to the row number of the sample at the bottom of the table. Click the "Finish" button

9.4.1.6. Sort the resulting table in numerical order using "sort..." under the Data menu.

9.4.1.7. The CRO may not have scanned in every Box Code. Find missing Box Code numbers in the pivot table by finding numbers under the "total" column that are larger than 98. Find the Box Code numbers that occur more than 98 times in the sample table, and replace Box codes that are wrong with a letter of the alphabet working backwards from "z". Refresh the data in the pivot table and sort the Box Codes again. This will result in a list of Box Code numbers that are known and a list of letters representing boxes with unknown box codes.

9.4.1.8. Convert the list of Box Codes into a format that is more convenient to print. Break the list of Box codes into columns of

no more than 20 boxes by pasting the box codes into the cells to the left of the pivot table.

- 9.4.1.9. Find the missing samples. Copy all of the tissue codes and paste under the Tissue Code column on the "Samples" sheet. Make sure that all of the tissue codes have an animal number in the cell to the right by using "fill down" to copy the formula "=MID(A2,9,3)" to the right of every tissue code. Select the "Count of Animal" pivot table and activate the pivot table tool under the "Data" menu. Click the "Back" button and edit the range field so that it completely includes the data. The formula will look like this: Samples!\$A\$1:\$B\$9175, change the last number so that it is equal to the row number of the sample at the bottom of the table. Click the "Finish" button.
- 9.4.1.10. Check the pivot table for missing animal numbers. If numbers are out of sequence, sort the list to make finding missing numbers easier. Note any missing numbers and call the CRO to determine positively what happened to that animal.

9.4.2. *Tissue Storage*

- 9.4.2.1. Go to any freezer with room with the dry ice shipping cartons, box code list and partial box list. Open the shipping cartons and put the tissue boxes in order by box code. Separate out all of the partially empty boxes so that they can be consolidated. Re-pack the now ordered boxes on dry ice in the shipping cartons. Identify any Box numbers that are on the Box code list and are not in the shipping cartons. Call the CRO immediately if boxes are missing.
- 9.4.2.2. Load the Tissue sample boxes from the shipping cartons to the freezer racks. Note down the box codes that are loaded into each freezer rack. Enter this information in the database.

9.4.3. *Entering Information in Database*

- 9.4.3.1. Enter the proper freezer and Rack Codes next to each Tissue Code entry referring to the notes from step 9.4.2.2.
- 9.4.3.2. Copy the data on the "tubes" sheet including Freezer, Rack Code, Box Code, Position and Tissue Code to the proper file in the "Freezers" folder.

- 9.4.3.3. Open a new file from the "Received" template located in ChemExpress/ChemExpress/In Vivo Biology/Sample Tubes Received and paste in the Freezer, Rack Code, Box Code, Position and Tissue Code data from the "Tubes" sheet. It is critical that the data fall under the proper column headings.
- 9.4.3.4. Enter Y for "Received (Y/N) for all of the samples. Save the file as Xyymmddreceived where X is the one-letter CRO code and yymmdd is the experiment start date. Proceed with "Load VV data" protocol to upload files into the database.

Chapter 10

Database Upload of *In Vivo* Data

10.1. PURPOSE

The purpose of the SOP is to populate the In Vivo database with treatment, animal ID and frozen tissue storage location data, and to release tissue samples to the mRNA group for processing.

10.2. RESPONSIBILITY

The Toxicology group is responsible for implementation of this SOP.

10.3. REQUIRED MATERIALS AND EQUIPMENT

- Make sure that the following files are available in the folder db upload located at ChemExpress/ChemExpress/In Vivo biology:
- **Animal file** (identified by CRO initial, study date + animal, contains all animal assignments to drug/dose/time).
- **Tissue file** (identified by CRO initial, study date + tissue, contains all tissue assignments).
- **Received file** (identified by CRO initial, study date + received, contains sample storage location).

**Note: Files 1 and 2 are created from the tissue scheme source-file using a parser written by the VP of Informatics. File 3 is generated at the CRO by scanning in barcodes and assigning box locations to tissues and by Iconix where freezer location is added.*

- Access to FileMaker Pro
- Access to VV_Main database

10.4. PROCEDURE

10.4.1. *Importing Experimental Details into FileMaker Pro Database*

- 10.4.1.1. Open VV_Main, select “animal log”, enter password, and select “animal list”.
- 10.4.1.2. Locate “Scripts” on the menu and select “Import animal data file” from the pull down menu.

- 10.4.1.3. Find the animal file to be imported by CRO initial and date (example: AYYMMDDanimal) in the folder db upload on ChemExpress/ChemExpress/In vivo biology.
- 10.4.1.4. In the Import Field Mapping window, align corresponding data columns together then click the Import button or hit the enter key.
- 10.4.1.5. Make sure that the number of animals imported corresponds to the expected number.

10.4.2. *Importing the Tissue ID into FileMaker Pro Database*

- 10.4.2.1. If the Animal Log file is still open, select "animal tissue log" on the display bar on the top of the page, otherwise open VV_Main, select "animal tissue log", enter password.
- 10.4.2.2. Locate "Scripts" on the menu bar, and from the scripts pull down menu select "Import Tissue Data File".
- 10.4.2.3. Find the file to be imported by CRO initial and date (example: AYYMMDDtissue) in the db upload folder located at ChemExpress/ChemExpress/In vivo biology.
- 10.4.2.4. In the Import Field Mapping window, align corresponding data columns together then click the Import button or hit the enter key.

10.4.3. *Importing Sample Location into FileMaker Pro Database*

- 10.4.3.1. If the Animal Tissue Log file is still open find "Scripts" on the menu bar otherwise open VV_Main, select "animal tissue log", enter password.
- 10.4.3.2. Locate "Scripts" on the menu bar, and from the scripts pull down menu select "Import tissue location".
- 10.4.3.3. Find the file to be imported by CRO initial and date (example: AYYMMDDreceived) in the db upload folder on ChemExpress/ChemExpress/ ChemExpress/In vivo biology.
- 10.4.3.4. In the First Row Option dialog box, select "Field Names" then click "OK".
- 10.4.3.5. In the Import Field Mapping window, align corresponding data columns together, selecting the 'Import Data' single arrow for Freezer ID, Rack ID, Box ID, Position, and Received, select 'Match Fields' double arrow for Tissue ID, then click the Import button or hit the enter key.

10.4.4. Entering Dose solution ID

- 10.4.4.1. Go to Window VV-Experiment Design.FP5 and click "Experiment List".
- 10.4.4.2. Find all experiments performed on the date of interest. On the menu bar locate "view" and select "Find mode" from the pull down menu; or use ctrl F.
- 10.4.4.3. Enter the desired date (format MM/DD/YYYY) into the column "Exp/RF Start Date" and click find (or hit return). This will pull up the records of all studies performed on that day.
- 10.4.4.4. Locate and select (click) "Dose solution" on the top of the page to move to the dose formulation sheets for each of the compounds. To navigate through the individual compounds use the Records tool (left upper corner on the page). Click on the small page icon to move from one record to the next.
- 10.4.4.5. With one record, open locate the field "Exp_week".
- 10.4.4.6. Click in the field and select "Wk1" from the pop up menu.
- 10.4.4.7. Move to the next field "Dose H/M/L" and select "H" (for high dose).
- 10.4.4.8. Repeat selection of "Wk1" in the next line and select "L" (low dose) for the second dose solution entry.

**Note: Entering this information is required and will write to tissue RNA file*

10.4.5. Uploading Information into Tissue Tracking Database (FileMaker Pro)

- 10.4.5.1. Double click the Tissue Tracking DB icon shortcut on desktop.
- 10.4.5.2. Click gray "Open Database" button. Enter "mRNA" as password.
- 10.4.5.3. From the Main Menu, click "Freezer Locations" button. There is no password, click "ok" when prompted for one.
- 10.4.5.4. Click the dark grey button "Import new tissue locations."
- 10.4.5.5. Using the 'Open File' box, find the Excel worksheet that has the new freezer location data at N:\ChemExpress\In Vivo Biology\SamplesReceived\FrozenTissue. Click open.

- 10.4.5.6. From the dialog box that opens, entitled 'Specify Excel Data,' choose the worksheet "tubes" and click "ok."
- 10.4.5.7. A second dialog box will then open entitled 'First Row Option.' Choose "Field Names" and click "ok."
- 10.4.5.8. Verify the data on the left hand side matches with the fields on the right hand side of the dialog box 'Import Field Mapping,' and that they are aligned with bold one-way arrows. Click "Import."
- 10.4.5.9. Verify the import was successful and all the tissues represented in the Excel sheet have been uploaded into the Tissue Tracking DB. Close Database.

10.4.6. RNA Sample Release for Motorola platform

- 10.4.6.1. Locating experiment numbers for drug treatment samples to be released:
 - In FileMaker Pro VV_Main, go to VV_Experiment.
 - Select "View" and "find mode" from the menu bar.
 - Enter the desired date (format MM/DD/YY) into the column "Exp/RF start" and the CRO identifier in the "CRO" column.
 - Click Find (or hit return). This will pull up the records of all studies performed on that day at that CRO.
 - Select "export records" from the file menu.
 - In the pop up window select the following fields for export:
 - Experiment number (required)
 - ICX number
 - Mol name
 - Route
 - Vehicle
 - Array tissue (required)
 - Day
 - Select file "tab delimited text" as the export format and type in a file name.
 - Save the file and open it in Ms-excel to verify that the information is present.
 - Print the records (or work directly with the electronic copy).

- 10.4.6.2. Assigning RNA numbers to the treatment tissue to be released for RNA extraction:
- In FileMaker Pro VV_Main, select “Animal Tissue Log”.
 - Select “View” and “find mode” on the menu bar or press Ctrl+F.
 - Type the experiment number of the first compound into the column “Exp_number”; type following into the column “tissue id”: *tissue code 1 (example: *Li1).
 - This will pull up all liver samples generated with this particular compound; in general, you will pull up 24 records.
 - Use the “omit” button to hide all of the tissues that will not be released.
 - Locate the last RNA number used (located in the Last RNA file in ChemExpress/ChemExpress/In Vivo biology/db upload).
 - Type the next RNA number in to the RNA number field using R for rat and H for human samples.
 - Select the number and copy it.
 - Hit Ctrl= to pull up an auto fill window.
 - Select the radio button in front of “Replace with serial number” and paste in the RNA number.
 - Select “replace” to automatically fill in the selected records with serial RNA numbers.
 - Note the last RNA number used.
 - Move to the right of the displayed entries and find the column “Ready for mRNA”.
 - Before releasing a sample make sure that a freezer location is noted which indicates that the sample is available.
 - To release a sample, click in the sample record row in the field under the column header “Ready for mRNA”.
 - Enter the release date in the following format:
Y_MM/DD/YY_Firstlast initial
 - Repeat process for all Experiment samples (treatment/tissue combinations) to be released to the RNA processing group.
- 10.4.6.3. Identify the number and type of drug/tissue combinations released, and notify the person responsible for coordinating the RNA extraction. Indicate the date and number of samples that were released.

10.5. APPENDIX

FOR INCYTE GEM samples with matched controls only

10.5.1. *Assigning Control Animal ID*

- 10.5.1.1. Use find by "Animal date" and "Vehicle"
- 10.5.1.2. Sort for Ctl_animal_assignment
- 10.5.1.3. Manually assign control animal ID to each treated animal sorting by time point and vehicle (repeat 4 times for experiments with 4 time points)
- 10.5.1.4. Go back to VV_Tissue_RNA, and select the desired Day_0_Date.
- 10.5.1.5. Run the following three scripts using the buttons provided:
 - "Enter Exp_Num"
 - "Enter Animal Group"
 - "Enter Ctl_Tissue_ID"
- 10.5.1.6. Assign RNA# to tissues to be processed (make sure to check for the last RNA number used!)
- 10.5.1.7. Import Ctl_Tissue_ID into VV_RNA_Ctl.FP5.
- 10.5.1.8. Go to VV_Tissue_RNA.FP5.
- 10.5.1.9. Find based on Date, RNA# and "C" (for control experiment) – this should pull up all the samples that are ready for RNA extraction (check whether the number of samples that come up correspond to the expected).
- 10.5.1.10. Go to VV_RNA_Ctl.FP5, run the "Import Ctl_Tissue_ID" script.
- 10.5.1.11. You will be asked to identify the correct data to load.
- 10.5.1.12. Go to host, select VV_Tissue_RNA and accept the auto-entry option, which loads the dataset previously loaded into that file (above).
- 10.5.1.13. Release RNA numbers from VV_Tissue_RNA into mRNA_Exp.FP3 (this happens automatically).

Chapter 11

Mammalian Cell Culture

11.1. PURPOSE

Provide a reference set of general mammalian cell culture techniques.

11.2. REFERENCE

Protocolversion_v2.1_ (ADME-R2) mammalian cell culture chapter

11.3. RESPONSIBILITY

The *In Vitro* Biology Team Leader is responsible for implementation of this protocol.

11.4. REQUIRED MATERIALS AND EQUIPMENT

- Fetal Bovine Serum (Hyclone Cat# SH30071.03, lot# ALA12963)
- Trypsin (0.25%): EDTA (0.1%) (Cellgro Cat# 25-053-C1)
- Phosphate-Buffered Saline (Gibco Cat # 14190-144)
- Sterile Deionized Water (Gibco Cat# 15230-162)
- Trypan Blue (Sigma Cat# T8154)
- T-175 Tissue Culture Flasks (VWR Cat# 29560-970)
- T25 Tissue Culture Flasks (VWR Cat# 29186-002)
- 100 mm Corning plate (VWR Cat# 25382-428)
- Cell scrapers with long handles (VWR Cat# 29442-204)
- Cell lifter (Costar-VWR Cat# 29442-200)
- 0.22 μ M filter flasks (VWR Cat# 28199-764)
- Rat Tail collagen Type I (Sigma Cat# C7661)
- Aspiration pipettes, 2.0 ml (VWR Cat# 29442-462)
- 1 ml Sterile pipette (VWR Cat# 53283-708)
- 2 ml Sterile pipette (VWR Cat# 53283-704)
- 5 ml Sterile pipette (VWR Cat# 53283-706)
- 10 ml Sterile pipette (VWR Cat# 29442-430)
- 25 ml Sterile pipette (VWR Cat# 53283-710)
- 50 ml Sterile pipette (VWR Cat# 53283-712)
- 15 ml Centrifuge Tube (VWR Cat# 21008-103)

- 50 ml Centrifuge Tube (VWR Cat# 21008-147)

11.5. GENERAL PROCEDURES

The procedures outlined below are general cell culture methods.

11.5.1. *Aseptic Technique*

- 11.5.1.1. Carry out all culture operations in a laminar flow hood.
- 11.5.1.2. Wipe down work surface liberally with 70% ethanol.
- 11.5.1.3. Periodically spray 70% ethanol over gloves to minimize contamination. Replace if torn.
- 11.5.1.4. In case of spill, spray with 70% ethanol and wipe immediately with paper towels.
- 11.5.1.5. Discard gloves after use.
- 11.5.1.6. Bring only those items needed for a particular procedure into the hood.
- 11.5.1.7. Leave a wide, clear space in the center of the hood (not just front edge) to work in. Do not clutter area to prevent blockage of proper airflow.
- 11.5.1.8. Spray all glassware, plastic ware, pipettors, *etc.* with 70% ethanol before placing inside hood.
- 11.5.1.9. Arrange work area to have easy access to all supplies and materials within the hood without having to reach over items to get to others.
**Note: It is especially important not to reach over open bottles or flasks.*
- 11.5.1.10. Use sterile wrapped pipettes and discard after use into biohazard waste container.
**Note: Check that the wrapping of the sterile pipette is not broken or damaged before using.*
**Note: Pipettes contaminated with compound should be disposed in hazardous chemical waste containers.*
- 11.5.1.11. Inspect vessels to be used:
 - T-flasks must be free from visible contamination or breakage.
 - Medium bottles must be free from cracks and visible contamination and must be within the expiration date.

- 11.5.1.12. Properly discard all biohazard or chemically contaminated material immediately and properly.
- 11.5.1.13. When handling sterile containers with caps or lids, place cap on side if it must be laid on work surface.
- 11.5.1.14. Make sure not to touch pipette tip to rim of any flask or sterile bottle. If the pipette tip touches any surface, it is considered contaminated and must be replaced with a fresh one.
- 11.5.1.15. Clean work area when finished by spraying down UV-hood with 70% alcohol.

11.5.2. *Preparation of Collagen-Coated Tissue Culture Vessels*

All operations should be carried out under strict aseptic conditions.

- 11.5.2.1. Solubilize 100 mg rat-tail collagen type I in 66 ml of 1% (v/v) acetic acid. Stock solution can be stored at 4°C.
- 11.5.2.2. Prepare a 1:100 dilution of collagen stock with sterile distilled water.
- 11.5.2.3. Filter sterilize with a 0.22 micron filter (additional filter insert should be used). 250 ml are needed to coat 90-100 150 mm plates.
- 11.5.2.4. Coat the 150 mm plates in sets of five.
 - Using a 50 ml pipette, add 10 ml of the diluted collagen solution to each plate, swirl to coat evenly.
 - Let sit while pipetting 10 ml of the collagen dilution into 5 more plates, swirl to coat evenly.
 - Remove as much collagen solution from the first set as possible.
 - Pipette from collagen dilution to regain a volume of 50 ml for the next round. Repeat.
 - After all dishes are coated, discard collagen dilution.
- 11.5.2.5. Prop each plate lid slightly open and leave in the tissue-culture hood (no UV) to dry.

11.5.3. *Thawing DMSO-Tolerant Cells from a Liquid Nitrogen Stock*

This protocol is for cells that can easily withstand DMSO

**Note: This is the default protocol unless noted otherwise.*

- 11.5.3.1. Preferentially, cells should be thawed in the morning so that medium can be replaced in the afternoon.

11.5.3.2. Remove vial from liquid nitrogen storage and immediately place it on dry ice. Keep the vial on dry ice until ready to thaw.

11.5.3.3. Thaw cells by gentle agitation in 37°C water bath. When cells have thawed, spray vial with 70% ethanol.

**Note: To reduce the possibility of contamination, keep the tube O-ring and cap above the water.*

11.5.3.4. In a laminar flow hood, transfer the vial contents to a T25 tissue culture flask and dilute with the recommended culture medium.

**Note: To avoid excessive alkalinity of culture medium during cell recovery, it is suggested that the culture flask containing growth medium be placed in an incubator for 15 minutes to allow the medium to reach its normal pH prior to adding to contents from vial.*

11.5.3.5. Incubate cells at 37°C and %CO₂.

11.5.3.6. Replace medium with fresh warm growth medium after cells have attached. Preferentially this is done the same day.

11.5.4. Thawing DMSO-Sensitive Cells from a Liquid Nitrogen Stock

This protocol is for cells that are sensitive to DMSO.

11.5.4.1. Remove the vial from liquid nitrogen storage and immediately place it on dry ice. Keep the vial on dry ice until ready to thaw.

11.5.4.2. Thaw cells by gentle agitation in 37°C water bath. When cells have thawed, spray vial with 70% ethanol.

**Note: To reduce the possibility of contamination, keep the tube O-ring and cap above the water.*

11.5.4.3. Add 9 ml warm medium to a sterile 15 ml centrifuge tube, immediately add the vial contents, and swirl gently to mix.

11.5.4.4. Centrifuge cells at 1000 RPM for 5 minutes at room temperature, remove supernatant and dilute with the recommended culture medium.

11.5.4.5. Count number of viable cells (section 11.5.5)

11.5.4.6. In a laminar flow hood, transfer the contents of the 15 ml centrifuge tube to a T25 tissue culture flask.

**Note: To avoid excessive alkalinity of culture medium during cell recovery, it is suggested that the culture flask containing growth*

medium be placed in an incubator for 15 minutes to allow the medium to reach its normal pH prior to adding to contents from vial.

- 11.5.4.7. Incubate cells at 37°C and %CO₂.
- 11.5.4.8. Replace medium with fresh warm growth medium after cells have attached. Preferentially this is done the same day.

11.5.5. Cell Counting and Viability

- 11.5.5.1. Make a 10-fold dilution of cell suspension by adding 100 µl cell suspension to 200 µl Trypan Blue and 700 µl PBS.
- 11.5.5.2. Load 12.5 µl of the 10-fold dilution to each half of the hemacytometer.
- 11.5.5.3. Count the cells and determine the number of cells per ml (Figure 11.5-A).
 - Load both sides of hemac
 - Hemacytometer with cell suspension, covering both counting grids.
 - Count the number of cells in 5 of the 9 squares in each counting grid (total of 10 squares).
 - Divide the total number of cells counted by 10 (to get the average number of cells per square).
 - Multiply by dilution factor (usually a 1:10 dilution).
 - Multiply by 1e⁴ (to get the number of cells per ml of original cell suspension).
 - To determine the total number of cells, multiply the concentration (cells/ml) by the total volume (ml) of cells suspension.
- 11.5.5.4. Tally dead cells and calculate percent viability using Equation 11.5-A.

$$\% \text{ cell viability} = \frac{\# \text{ viable cells}}{\# \text{ viable cells} + \# \text{ dead cells}} \times 100$$

Equation 11.5-A: Cell Viability Count

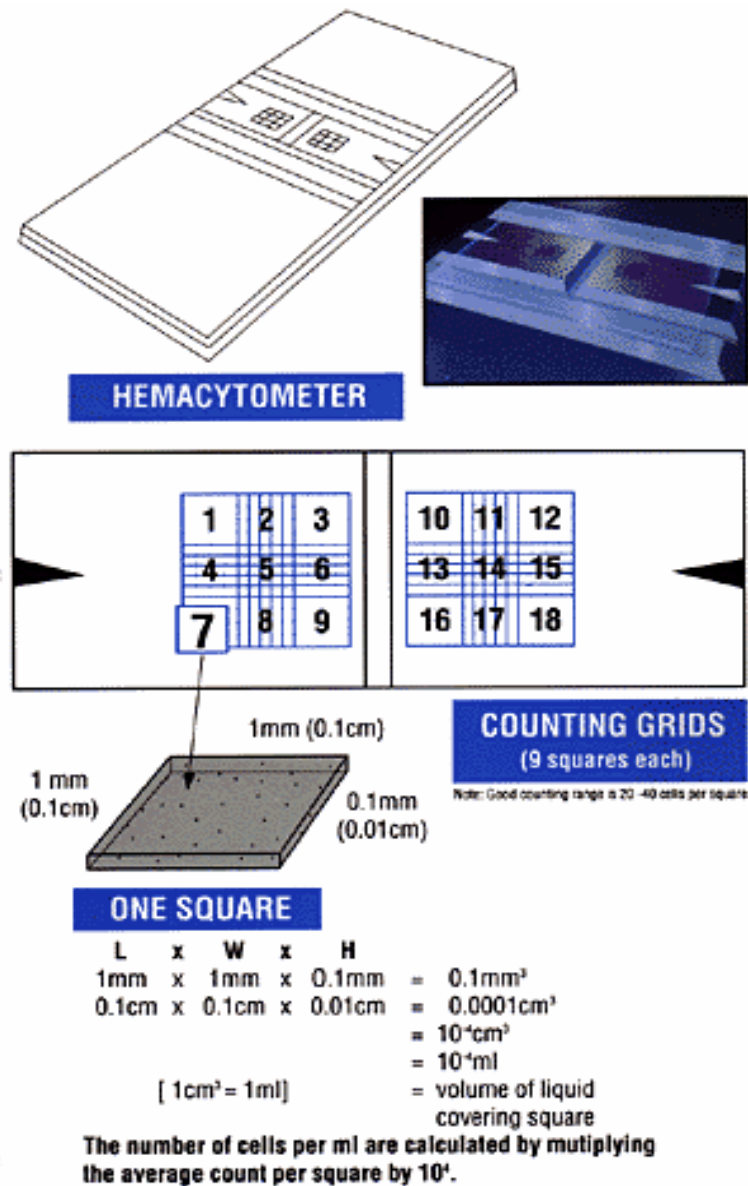


Figure 11.5-A: Counting Cells using a Hemacytometer

11.5.6. Handling of Adherent Cells

When handling adherent cell lines it is important to avoid disturbing the monolayer using the following techniques:

- Be careful not to touch the cell monolayer with pipettes or aspirators.
- To add solutions to the flask, hold the flask upright and squirt the solution to the side of the flask opposite the monolayer.
- To aspirate solutions from the flask, hold the flask upright and tip it so that the liquid flows into a corner opposite the cell

monolayer, then put the aspirator into the corner until all the liquid is gone.

11.5.7. *Trypsinizing Cells*

The amounts in this protocol are designed for trypsinizing cells from T175 flasks. Reagent amounts should be reduced for smaller flasks.

- 11.5.7.1. Aspirate medium from flask being careful not to touch the cell monolayer.
- 11.5.7.2. Rinse cells by adding 5 ml PBS carefully to each flask and gently rocking flasks so that PBS covers the cell monolayer.
- 11.5.7.3. Aspirate the PBS.
- 11.5.7.4. Add trypsin (2 ml per T-175).
- 11.5.7.5. Gently rock the flasks to evenly distribute trypsin. Place flasks in 37°C incubator and %CO₂ for 3 minutes.
- 11.5.7.6. Following the 3-minute incubation, add relevant medium to each flask.
- 11.5.7.7. Rock the flasks so growth medium covers surface and neutralizes all the trypsin.
- 11.5.7.8. Dislodge the cells by using a pipette to squirt the medium onto the cell monolayer until all the cells have come off of the wall.

**Note: For particularly adherent cells such as HepG2 cells, it is sometimes necessary to scrape the cells from flask with a long handled cell scraper.*

11.5.8. *Freezing Cells for Liquid Nitrogen Stocks*

- 11.5.8.1. Trypsinize cells (section 11.5.7).
- 11.5.8.2. Count cells and determine total number of cells.
- 11.5.8.3. Prepare freezing medium by adding 9 ml growth medium to 1 ml tissue culture grade DMSO.
- 11.5.8.4. Transfer cells to a sterile centrifuge tube and pellet at 1000 RPM for 5 min at room temperature.
- 11.5.8.5. Resuspend cells to a concentration of 1e⁶ cells/ml in freezing medium

**Note: Avoid creating air bubbles when resuspending cells.*

- 11.5.8.6. Aliquot 1ml cells per freezing vial and note the following information on vial (use CryoTags) and in notebook:
- Cell line
 - Passage number (passage number from flask +1)
 - Number of cells per vial
 - Freeze Date
 - Your initials
- 11.5.8.7. Transfer the vials to a freezing vessel containing isopropanol and label with initials and the date, then freeze at -80°C.
- *Note: The isopropanol should only be used 4 times. It is extremely important that the freezing take place in the freezing vessels to ensure that the cells are slowly frozen.*
- 11.5.8.8. Leave at -80°C for a minimum of 16 hours before transferring cells to liquid nitrogen
- *Note: NEVER leave cells at -80°C for more than 2 weeks, as viability will be significantly reduced.*
- 11.5.8.9. Remove vials from freezing vessel and place in the liquid nitrogen tank.
- 11.5.8.10. Note the location of vials and record this information, together with the information from the vial, to ChemExpress/ChemExpress/In Vitro Biology/Frozen Cell Stock.

Chapter 12

Primary Rat Hepatocyte Culture and *In Vitro* Cytotoxicity and Proliferation Assays

12.1. PURPOSE

- To provide rat hepatocyte culture procedures for *in vitro* cytotoxicity assay (LDH) and proliferation assay (MTS).
- *In vitro* LDH and MTS assays are used to determine the relevant test compound doses at TC20 (cell inhibition level at 20%). A measured dose will be used for *in vitro* array experiment.

12.1.1. Reference

Protocolversion_v2.1_(ADME-R2) mammalian cell culture chapter

12.2. RESPONSIBILITY

The *In Vitro* Biology Team Leader is responsible for implementation of this protocol.

12.3. REQUIRED MATERIALS AND EQUIPMENT

- Primary Rat Hepatocytes (supplied by SRI)
- InVitroGRO™ HI Medium (IN VITRO Technologies Product# Z990002), store at 4°C, shelf life 60 days.
- Williams Medium E (Gibco Cat# 12551-032, lot# 11292275), store at 4°C
- Fetal Bovine Serum (Hyclone Cat# SH30071.03, lot# ALA12963), store at -20°C
- Penicillin/Streptomycin, 5,000IU/ml and 5,000 µg/ml (Cellgro Cat# 30-001-CI), store at -20°C
- Phosphate-Buffered Saline (Gibco Cat # 14190-144)
- Sterile Deionized Water (Gibco Cat# 15230-162)
- Trypan Blue solution 0.4% (IN VITRO Technologies Cat# 15250-061)
- 96-well plate, BD BIOCOAT collagen coated (VWR# 62405-419)
- 100 mm dish, BD BIOCOAT collagen coated (VWR# 62405-425)
- CoStar 96-well plate flat bottom with lid (VWR Cat# 29443-954)
- Costar cell lifter (VWR Cat# 29442-200)
- 0.22 µm filter flasks (VWR Cat# 28199-764)

- 5 ml polypropylene tubes (VWR Cat# 60819-728)
- Reagent Reservoir (VWR Cat# 29442-478)
- CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega Catalog #TB163 or VWR Catalog #PAG1780) (Storage conditions: two parts /kit. Part one including Substrate Mix and Assay Buffer, store at -20°C; Part two including Positive Control, Lysis Solution (10X) and Stop Solution, store at 4°C;)
- CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega Catalog #G3581 or VWR Catalog #PAG3581), store at -20°C
- Aspiration pipettes, 2.0 ml (VWR Cat# 29442-462)
- 1 ml Sterile pipette (VWR Cat# 53283-708)
- 2 ml Sterile pipette (VWR Cat# 53283-704)
- 5 ml Sterile pipette (VWR Cat# 53283-706)
- 10 ml Sterile pipette (VWR Cat# 29442-430)
- 25 ml Sterile pipette (VWR Cat# 53283-710)
- 50 ml Sterile pipette (VWR Cat# 53283-712)
- 15 ml Centrifuge Tube (VWR Cat# 21008-103)
- 50 mL Centrifuge Tube (VWR Cat# 21008-103)
- 250 ml Corning sterile bottle (VWR Cat# 28199-758)
- 500 ml Corning sterile bottle (VWR Cat# 28199-760)
- 2 L Corning sterile bottle (VWR Cat# 62404-446)
- Forma Scientific CO₂ water jacketed incubator, Model 3110
- Forma Scientific Class II A/B3 biological safety cabinet, Model 1186
- SORVALL RT7 Centrifuge
- Molecular Devices Spectra MAX 340 plate reader, S/N# 01562

12.4. PROCEDURE

12.4.1. *Primary Rat Hepatocyte Treatment*

12.4.1.1. Making media

- Make IVT Growth Medium (InVitroGRO™ HI Medium (IVT medium) with 2% Penicillin/Streptomycin (100 IU/ml and 100,000 µg/ml)) by doing the following:
 - Add 20 ml Penicillin/Streptomycin into 1000 ml IVT medium, mix well.
 - Label with initial and expiration of date, store at 4°C.

- Medium expires 7 days after preparation
- Make FBS Aliquots
 - Thaw 1L bottle of FBS at 37°C bath
 - Filter and make 6 ml aliquots
 - Store aliquots at -20°C
 - Do not thaw aliquots more than one time.
- Make IVT Plating Medium (IVT Growth medium with 3% Fetal Bovine Serum (FBS)) by doing the following:
 - Add 6 ml FBS and 194 ml IVT Growth medium to obtain IVT plating medium
 - Discard any unused medium.

12.4.2. Obtain Cells from SRI and Cell Count

- 12.4.2.1. Determine how many cells will be needed for an experiment and ask SRI two weeks in advance to make the primary cells from the necessary number of rats.

$$1 \text{ liver} \approx 3 \times 10^8 \text{ cells}$$

12.4.2.2. Obtain cells from SRI

- For each rat liver to be harvested, take 200 ml IVT growth medium in a bottle at room temperature to SRI.
- Upon arrival of cells at Iconix, immediately proceed to count cells.

12.4.2.3. Cell Count

****Note: Since cells settle in the bottle while traveling from SRI to Iconix, it is necessary to thoroughly swirl the bottle to resuspend the cells evenly.***

- Centrifuge a 5 ml aliquot of cells at 600 rpm for 3 minutes at room temperature.
- Remove the medium and resuspend cells in 5 ml Williams Medium E

****Note: It is important to use the WME (no additives) as BSA interferes with cell counts.***

- Make a 10-fold dilution by adding 100 μ l cell suspension to 200 μ l Trypan Blue and 700 μ l PBS. Load 12.5 μ l of the 10-fold dilution in to each half of the hemocytometer (Section 11.5.5, page 84).

- Record all the cell count, density and viability into “Trypan Blue Viability” worksheet and along with other paperwork obtained from SRI to “SRI Forms” binder.

12.4.3. *Plating Cells for LDH and MTS Assays- DAY 1*

12.4.3.1. Prepare cell suspension for plating

- For each 96-well plate, a volume of 10 ml is required
- Adjust the cell concentration to 150,000 cells/ml in plating medium.
- Calculate the dilution and amount of medium necessary to get the correct cell density using Equation 12.4-A.

$$V1 = \frac{1.5e^5 \text{ (cells/ml)} \times V2 \text{ (ml)}}{M1 \text{ (cells/ml)}}$$

where V2 = ml needed for X plates at 10 ml/plate
and M1 = cell count (cells/ml)

$$\text{Medium to add} = V2 - V1$$

Equation 12.4-A: Plating Medium Calculation

12.4.3.2. Seeding the plate

- Add 100 μ l of the cell suspension into a 96-well collagen coated microtiter plate to get a 15,000 cells/well density.

$$\frac{15,000 \text{ cells}}{\text{well}} \times \frac{\text{well}}{100 \mu\text{l}} \times \frac{1000 \mu\text{l}}{\text{ml}} = 1.5e^5 \frac{\text{cells}}{\text{ml}}$$

Equation 12.4-B: Cell suspension density calculation for plating

- For each drug to be tested, plate 2 plates with 100 μ L/well cell suspension in rows A-H and columns 2-12, leaving the first column no cells, but medium only for Blank. Labeled Plate I (for LDH assay) and Plate II (for MTS assay).
- Do medium replacement following 3-hour (+/- 10 minutes) attachment with IVT growth medium by quickly inverting the plates to remove the media from the plates.

****Note: The above procedures should be carried out in the hood and all waste media should be poured into the Biohazard Waster container.***

****Note: Do NOT use the multi-channel aspirator to minimize cell loss.***

12.4.3.3. Incubate plates for twenty-four hours at 37°C under a 5% CO₂ humidified atmosphere.

12.4.4. Dosing – DAY 2

12.4.4.1. Test compound preparation

- One week before the experiment, send a compound request to the Compound Dispenser. Request 10 mg/compound for the assays.
- Utilize Cytotox_1.5fold.set-up spreadsheet (ChemExpress/ChemExpress/In Vitro biology/Biology Worksheets).
- Assume 1000 mM initial concentration. Fill in yellow highlighted fields for Date, Drug Name, ICX Number, Drug Lot Number, Formula Weight, Total Weight (≥5 mg), Initial Volume (to get 1000 mM initial concentration), and Vehicle (want DMSO primarily, water used as well).
- Based on Initial Volume determined by Cytotox1.52fold.set-up spreadsheet, solubilize compound in DMSO.

Note: The compound may go into 100% DMSO, but precipitate in media with 0.1% DMSO. If 1000-fold dilution does result in precipitation, dilute the stock further (in two fold dilution steps) with 100% DMSO and repeat solubility test. Continue diluting the initial stock solution until compound dissolves completely in media. If a compound, for example Valproic acid is not soluble in DMSO, solubilize the compound in water first and make a 1000-fold dilution with incubation medium containing 0.1% DMSO. The highest concentration of a compound solubilized in 0.1% DMSO in incubation media is defined as the maximum feasible dose (MFD).

12.4.4.2. Control compound preparation

Chlorpromazine is included in each assay as a reference compound to monitor reproducibility of the system. The starting concentration of Chlorpromazine is 500 µM.

12.4.4.3. Highest dose preparation

- Add 5 ml IVT growth medium into a 50 ml centrifuge tube, label each tube. If a test compound is dissolved in water, add 5 ml IVT growth medium with 0.1% DMSO.
- For each drug to be tested, add 5 µl of compound into 5 ml media for a 1000-fold dilution. Vortex the tube while adding the compound.

12.4.4.4. Compound serial dilution preparation

- Set up nine 5 ml polypropylene tubes in a microcentrifuge tube rack for each compound, labeled with the drug name and a number 1-9.
- Fill tube 1 with 3.6 ml of the highest dose either 1 mM or the MFD.
- Fill each of tubes 2-9 with 1,200 µl IVT growth medium with 0.1% DMSO.
- Using an adjustable Matrix multi-channel pipettor, perform 1.5-fold dilution from tube 1 to tube 2 by pipetting 2,400 µl from tube 1 to tube 2. Continue 1.5-fold dilutions through the remaining tubes ending with 3,600 µl in tube 9.

****Note: A filter tip is not necessary for doing serial dilutions, but it is necessary to change tips before each step of mixing.***

12.4.4.5. Dosing the cells

- Remove the microtiter plates plated in Section 12.4.3.3 from incubator when ready to dose.
- Remove the medium.
- Add compound dilution series to cells in microtiter plates (100 µl per well) using the adjustable Matrix multi-channel pipettor. Each drug should be dosed in quadruplicate, with two drugs per plate (Figure 12.4-A). The compounds should be added to the columns as follows:
 - 1) No cells + Vehicle
 - 2) Cells + Vehicle (NOT USED in analysis - Cytotoxicity Assay ONLY)
 - 3) Cells + (Growth Medium, no Drugs or) Vehicle
 - 4) Cells + Compound dose 1 (lowest dose)
 - 5) Cells + Compound dose 2
 - 6) Cells + Compound dose 3

- 7) Cells + Compound dose 4
- 8) Cells + Compound dose 5
- 9) Cells + Compound dose 6
- 10) Cells + Compound dose 7
- 11) Cells + Compound dose 8
- 12) Cells + Compound dose 9 (highest dose)

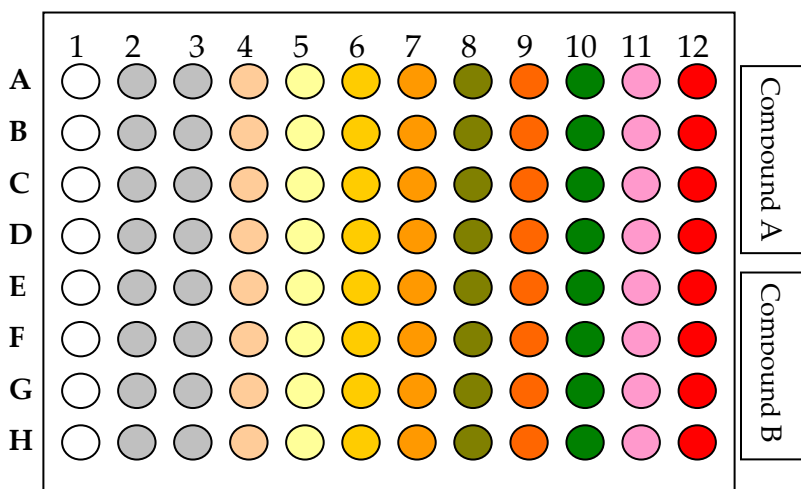


Figure 12.4-A: Set-up of Assay Plate

12.4.4.6. Return plates to the incubator for additional twenty-four hours at 37°C under a 5% CO₂ humidified atmosphere.

12.4.5. LDH and MTS Assays – DAY 3

12.4.5.1. Check wells for compound precipitation

- Remove plates from incubator and check all wells for compound precipitation under microscope.
- Note precipitation in lab book and on results page and graph.

12.4.5.2. DH assay (24 hour post dosing)

Use CytoTox96® Non-Radioactive Cytotoxicity Assay kit

- Add 10 µl of Lysis Solution (10x) per 100 µl of medium to control wells (Column 2) to lyse cells and return plates to the incubator for additional 45 minutes incubation.
- Transfer 50 µl aliquots from all wells to a fresh 96-well flat bottom plate – Assay Plate.

- Add 12 ml of room temperature Assay Buffer to a bottle of Substrate Mix. Invert and shake gently until the substrate is dissolved.
- Add 50 µl of the reconstituted Substrate Mix to each well of the assay plate.
- Cover the plates and incubate plates at room temperature for 30 minutes without exposure to light.
- Add 50 µl of Stop Solution to each well.
- Pop any large bubbles using a syringe needle and record the absorbance at 490 nm using Molecular Devices plate reader (Softmax Pro 2.2.1) within one hour after the addition of Stop Solution.

12.4.5.3. MTS Assay (24 hour post dosing)

Use CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit

- Thaw MTS and PMS solutions.
- For each plate, combine 100 µl PMS, 2.0 ml MTS, and 10 ml IVT Growth Medium.
- Remove the microtiter plates from incubator and remove medium from the plates.
- Add 100 µl per well PMS/MTS/growth medium solution to all wells in the microtiter plate using Matrix multi-channel pipettor.
- Incubate plates at 37°C for 2 hours.
- Retrieve plates from the incubator, remove lid, and read absorbance at 490 nm with Molecular Devices plate reader (Softmax Pro 2.2.1).

12.4.5.4. Data analysis

- Save the file and export as text.
- Open “toxdatagraph” Excel sheet found in ChemExpress/ChemExpress/In Vitro biology/Biology Worksheet and paste the new plate data from the text file into cell C3 on the relevant time point tab. The data should line up with the column heading numbers at the top of the sheet. Create a new workbook for each compound.

- Save graph in ChemExpress/ChemExpress/In Vitro biology/
New Toxicity (in the relevant cell line folder) as "ICX
Number_Date_Cell line."

Chapter 13

In Vitro Array Experiment

13.1. PURPOSE

The Array Experiment is performed to obtain cell sample from test compound treated and vehicle control cell for expression array analysis.

13.2. REFERENCE

Protocolversion_v2.1_(ADME-R2) mammalian cell culture chapter

13.3. RESPONSIBILITY

The *In Vitro* Biology Team Leader is responsible for implementation of this protocol.

13.4. REQUIRED MATERIALS AND EQUIPMENT

- Primary Rat Hepatocytes (supplied by SRI)
- HepG2 (ATCC Cat# HB-8065, lot#1659255)
- HEK-293 (ATCC Cat# CRL-1573, lot#2310360)
- HK-2 (ATCC Cat# CRL-2190, lot#2061647)
- Dulbecco's Modified Eagle's Medium (Cellgro Cat# 10-013-CV)
- Fetal Bovine Serum (Hyclone Cat# SH30071.03, lot# ALA12963)
- Penicillin/Streptomycin (Gibco Cat# 15140-122)
- Trypsin (0.25%):EDTA (0.1%) (Cellgro Cat# 25-053-C1)
- Williams Medium E (Gibco Cat# 12551-032, lot# 11292275)
- 0.2% Fatty Acid Poor BSA (Sigma Cat# A-6003, lot# 91K7435)
- 1 mM L-glutamine (Gibco Cat# 25030-081, lot# 1131728)
- 0.1 μ M Dexamethasone (Sigma Cat# D-4902, lot# 81K1005)
- Insulin, Transferrin, Selenium supplement (Sigma Cat# I-1884)
- Phosphate-Buffered Saline (Gibco Cat # 14190-144)
- Sterile Deionized Water (Gibco Cat# 15230-162)
- Trypan Blue (Sigma Cat# T8154)
- Keratinocyte Serum Free Medium (Gibco Cat#17005-042)
- T-175 Tissue Culture Flasks (VWR Cat# 29560-970)
- T75 Tissue Culture Flasks (VWR Cat# 29185-306)

- T25 Tissue Culture Flasks (VWR Cat# 29186-002)
- BIOCOAT collagen T175 Flasks (VWR# 62405-431)
- BIOCOAT collagen T75 Flasks (VWR Cat# 62405-428)
- BIOCOAT collagen T25 Flasks (VWR Cat# 62405-539)
- 100 mm Corning plate (VWR Cat# 25382-428)
- 100 mm BD-Biocoat, collagen plates (VWR Cat#62405-424)
- Cell scrapers with long handles (VWR Cat# 29442-204)
- Cell lifter (Costar-VWR Cat# 29442-200)
- 0.22 μ M filter flasks (VWR Cat# 28199-764)
- Corning 120 ml Sterile Bottles (VWR Cat# 28199-758)
- Micro-Centrifuge Tubes 1.5 ml purple (VWR Cat# 53550-930)
- Reagent Reservoir (VWR Cat# 53504-035)
- TC Roller Bottles 1750 cm² (VWR Cat# 62404-440)
- Rat Tail collagen Type I (Sigma Cat# C7661)
- Aspiration pipettes, 2.0 ml (VWR Cat# 29442-462)
- 1 ml Sterile pipette (VWR Cat# 53283-708)
- 2 ml Sterile pipette (VWR Cat# 53283-704)
- 5 ml Sterile pipette (VWR Cat# 53283-706)
- 10 ml Sterile pipette (VWR Cat# 29442-430)
- 25 ml Sterile pipette (VWR Cat# 53283-710)
- 50 ml Sterile pipette (VWR Cat# 53283-712)
- 15 ml Centrifuge Tube (VWR Cat# 21008-103)
- 50 ml Centrifuge Tube (VWR Cat# 21008-147)

13.5. PROCEDURE

The following protocol applies to all cell lines used by the *In Vitro* Biology Department.

13.5.1. *Plating Cells*

- 13.5.1.1. For each drug to be tested, plate 9 plates (3 time points in triplicate).
- 13.5.1.2. Seed the 100 mm plates according to Table 13.5-A.

****Note: Use collagen coated plates for HepG2 and Primary Rat Hepatocytes.***

Table 13.5-A: Array Experiment Cell Plating Densities

Cell Line	Seeding/Plating Density
Primary Rat Hepatocytes	6.0e ⁶
HepG2	1.0e ⁷
HEK-293	1.0e ⁷
HK-2	1.0e ⁷ *

*It may be necessary to decrease cell density and use 2-3 plates/triplicate/time point.

- 13.5.1.3. To calculate the volume of medium to add to the cell suspension use Equation 13.5-A.

$$M_i V_i = M_f V_f$$

$$V_i = \frac{M_f (\text{cells/ml}) \times V_f (\text{ml})}{M_i (\text{cells/ml})}$$

where V_i = volume (ml) of cell suspension

V_i = ml needed for X plates at 15 ml/plate

and M_f = concentration of cell suspension (cells/ml)

M_f = desired seeding density from Table 1.6 - A

Medium to add = $V_f - V_i$

Equation 13.5-A: Array Experiment Plating Medium Calculation

- 13.5.1.4. Incubate plates overnight at 37°C under CO₂.

13.5.2. Compound Preparation

- 13.5.2.1. Determine array experiment dose based on the drug's proliferation assay:
- The ideal array experiment dose is the TC20 dose, the dose that produced 20% cell inhibition as shown in Figure 13.5-A.
 - A formula derived (FD) dose is warranted when one concentration exhibits no effect and the next concentration (2-fold difference), results in TC50 or more inhibition as shown in Figure 13.5-B. The FD dose is the average of the two concentrations before the drastic increase in inhibition.

- If the compound exhibited no effect, use the maximum feasible (MF) dose as shown in Figure 13.5-C.

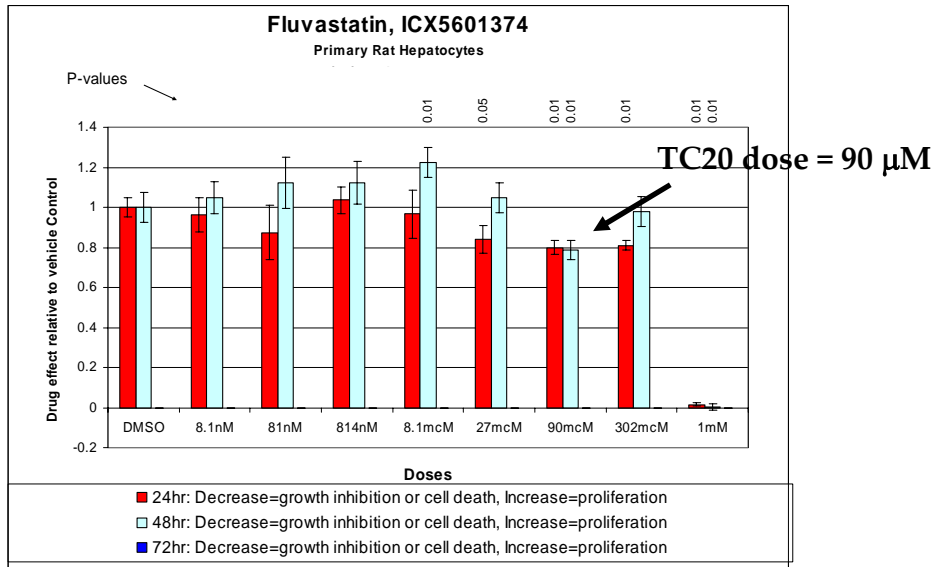


Figure 13.5-A: Fluvastatin Proliferation Assay Results

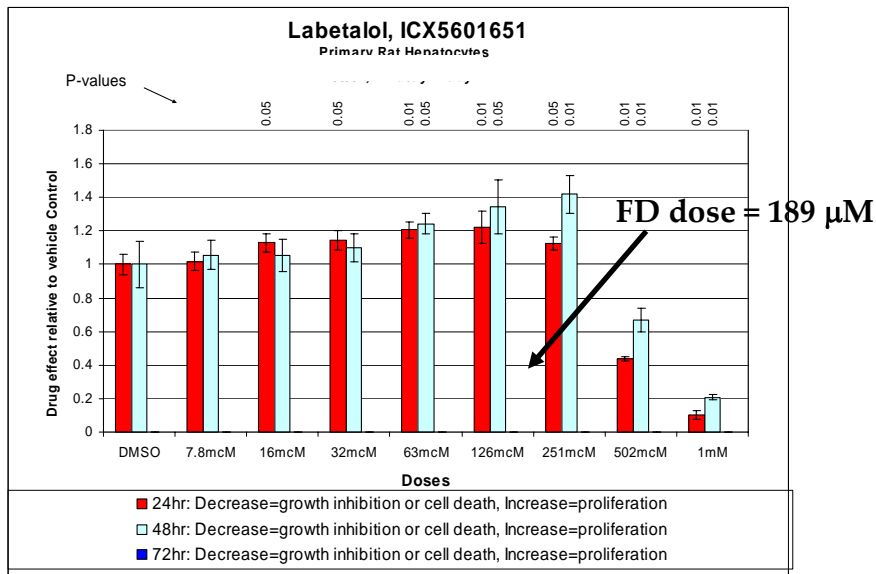


Figure 13.5-B: Labetalol (FD) Proliferation Assay Results

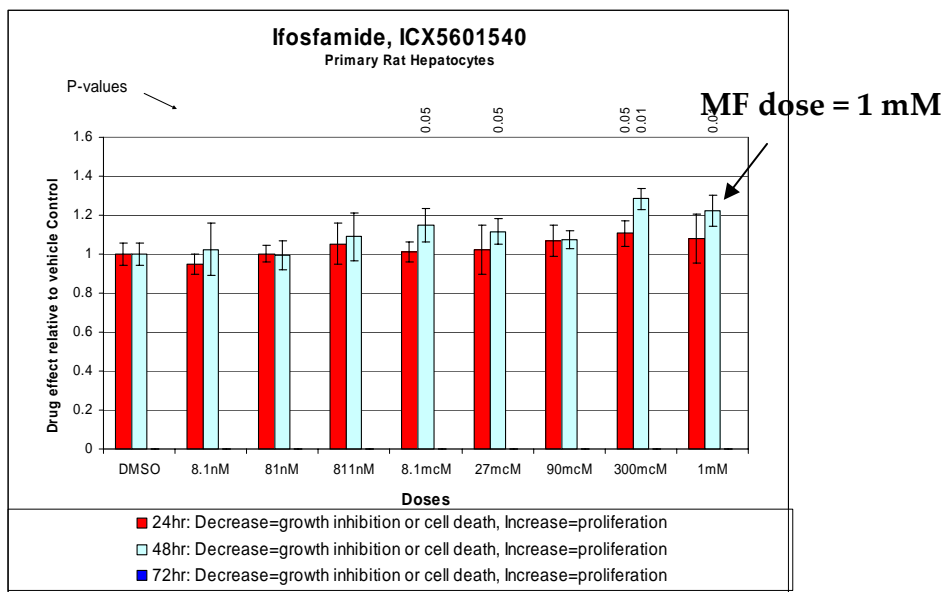


Figure 13.5-C: Ifosfamide (MF) Proliferation Assay Results

- 13.5.2.2. Calculate the amount of compound needed for the experiment based on the doses chosen in step 13.5.2.1 using Equation 13.5-B.

$$W(g) = D(\text{mol} / \text{ml}) \times V_t(\text{ml}) \times MW(\text{g} / \text{mol})$$

where W = Compound Weight (g)

D = Dose (mol/ml)

V_t = Total Volume needed = 120 ml (9 plates \times 12 ml/plate + 10%)

MW = Molecular Weight of compound (g/mol)

Equation 13.5-B: Compound Weight Calculation

- 13.5.2.3. One week before implementing the experiment, send a compound request to the Compound Dispenser. Request double the amount of compound needed for the Array Experiment.
- 13.5.2.4. Approximately 24 hours post-plating, prepare compound doses.
- Solubilize the compound in 120 μ l DMSO (compound amount determined in step 13.5.2.2). If <5 mg compound is necessary for a particular dose, weigh out at least 5 mg. If more compound is weighed out than necessary, use Equation 13.5-C to determine the DMSO solubilization volume necessary.

$$V_{DMSO}(\mu l) = \frac{W_{actual}(mg) \times 120\mu l}{W_{calculated}(mg)}$$

where V_{DMSO} = DMSO volume (μl) used to solubilize compound

W_{actual} = Exact compound weight (mg)

$W_{calculated}$ = Weight compound necessary calculated in Equation 13.6 - B

Equation 13.5-C: Calculation of DMSO Solubilization Volume

Fluvastatin Example :

$$W_{calculated} = 4.4mg, W_{actual} = 6.2mg$$

$$\text{so } V_{DMSO} = \frac{6.2mg \times 120\mu l}{4.4mg} = 169\mu l$$

- For each compound, add 120 ml warm growth medium to a 120 ml sterile bottle.
- Add 120 μl DMSO-solubilized compound, resulting in a DMSO concentration 0.1%.

13.5.3. *Dosing*

- 13.5.3.1. Retrieve the 100 mm plates in sets of nine from incubator.
- 13.5.3.2. Aspirate medium from the plate using a 2 ml aspiration pipette.
- 13.5.3.3. Add 12 ml medium/dose to each 100 mm plate and label with labels printed from FileMakerPro.
- 13.5.3.4. Return plates to the incubator and harvest 3, 12, and 24 hours later respectively.

13.5.4. *Harvesting Cell Samples*

Cells are harvested at 3, 12 and 24 hrs post-treatment.

- 13.5.4.1. At the appropriate time point, retrieve plates from incubator in sets of three.
- 13.5.4.2. Pour the medium/dose into a glass container to later dispose in Hazardous Waste. Tip the plate on its side and aspirate the remaining medium/dose with an aspiration pipette (

**Note: Place a 40-200 μl non-plugged pipette tip on the aspiration pipette and change it for each plate.*
- 13.5.4.3. Add 1 ml MagNA Pure Lysis Buffer to each plate. Swirl the plate to cover with buffer.

- 13.5.4.4. Use a Cell Lifter to scrape cells from the plate.
- 13.5.4.5. Pipette the lysed cell/buffer mixture into corresponding pre-labeled 1.5 ml microcentrifuge tube labeled with RNA#, ICX Number, and time point.
- 13.5.4.6. Place tubes in a storage box (9X9 grid) numerically by RNA number and store at -20°C.
- 13.5.4.7. Release the samples in FileMakerPro (section 13.6).

13.6. CREATE RECORDS AND UPLOADE TO FILEMAKER PRO

13.6.1. *Entering Information into Excel*

- 13.6.1.1. Using Microsoft Excel, open the "CellGrowthInputSheet.xls" found in ChemExpress/ChemExpress/InVitroBiology/UniversalDatabaseMisc/CellGrowthInputSheet.xls and select the tab pertaining to the species of the cell line (rat or human).
- 13.6.1.2. Starting with the next available RNA Prep Number and the controls, enter the following information by drug as time points are listed sequentially for each drug.
 - C/E
 - Drug Iconix Number
 - Drug Name
 - Lot Number
 - Drug Concentration
 - Drug Concentration Unit
 - Drug Solvent
 - Solvent Concentration
 - Solvent Concentration Unit
 - Cell Growth Notebook Page Number
 - Drug Treatment Time
 - Scientist Name
- 13.6.1.3. Click the "list exp#s" tab and assign experiment numbers to the control and each subsequent drug. The control receives one experiment number and each drug receives its own experiment number. In the same field, enter the date of the experiment number and the name of the scientist who assigned it

- 13.6.1.4. Return to the Rat or Human page, and paste the experiment numbers in the “experiment number” column.
- 13.6.1.5. Assign Replicate ID Numbers to the control and the drugs.
 - For controls: each time point (*i.e.* 3, 12, and 24 hour) receives a separate Replicate ID Number.
 - For drugs:
 - One dose/drug: each time point receives a separate RepID Number
 - Two doses/drug: the low and high concentrations in each time point receive a separate Replicate ID Number
- 13.6.1.6. Copy all information pertaining to that RNA experiment and paste it into the “Current Rat” (or “Current Human”) tab. This is the page that will be used to upload into the UV_Main Database.

13.6.2. *Uploading to the UV_Main Database*

- 13.6.2.1. Open the UV_Main Database.
- 13.6.2.2. Under Cell Biology, click the “Cell Growth Setup Conditions and Results” button and enter the password.
- 13.6.2.3. Under Human or Rat (depending on the species of the cell line), click the “Compound Treated and Control Cell Growth” button.
- 13.6.2.4. Click the “Cell Growth Conditions” button and enter the following information:
 - Cell Growth Notebook Page Number
 - Cell Growth Start Date
 - Cell Stock Number
 - Cell Passage Number
 - *Note: Primary Rat Hepatocytes will not have a passage number***
 - Medium
 - FBS Lot Number
 - Medium Temperature
 - Cell Growth Plate Size
 - % CO₂
 - Initial Cell Density
- 13.6.2.5. Return to the Human or Rat page and click the “Import CGR Data” button. In the pop-up box browse to N:\ChemExpress\

InVitroBiology\UniversalDatabaseMisc\CellGrowthInputSheet.xls and select the "Current Human" or "Current Rat" tab (depending on cell line) then click "Import" and accept the auto data option during the upload process.

13.6.3. Filling in the UV Database

- 13.6.3.1. Click the "Cell Stock ID" button then the "Replace" button. This fills in the cell line name (PrimRatHep, HepG2, HK-2, or HEK-293).
- 13.6.3.2. Click the "Species" button then the "Replace" button. This fills in the species of the cells (Human or Rat).
- 13.6.3.3. Click the "Add ExpNum Prefix" button to add C (Control) or E (Experiment) to the beginning of the Experiment Number. On the new screen, click the "Continue" and the "Replace" buttons, respectively.
- 13.6.3.4. Click the "Update Exp_Rep_ID" button to add Replicate ID Numbers to the end of the Experiment Number. On the new screen, click the "Continue" and the "Replace" buttons, respectively.
- 13.6.3.5. Upon completion of harvesting cells for the array experiment, release the samples by enter "Y-yyyymmdd-initials" for each relevant sample in the "Ready for RNA_Ext" column.
- 13.6.3.6. Enter any necessary comments (*e.g.* MagNA Pure Buffer volume, contamination, SOP, *etc.*) in the "CGR Comments" column.
- 13.6.3.7. For controls, enter Y-yyyymmdd-initials in the "For Each "C" RNA Prep # Enter "Y" to Create Ctrl RNA# in CGR_Ctrl" column.
- 13.6.3.8. After the samples have been released to the RNA group they can be processed starting with Section 15.5.2, page 114.

Chapter 14

Retrieving Tissues

14.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for retrieving tissues from the freezers.

14.2. RESPONSIBILITY

The RNA Team Lead is responsible for enforcing the execution of this protocol.

14.3. SAFETY PRECAUTIONS

Always wear your lab coat and goggles when working in the lab.

14.4. REQUIRED MATERIALS AND EQUIPMENT

- Small and big styrofoam boxes
- Large ice bucket
- Hemostats
- Box (9x9 grid)
- Analytical balance (Homogenization Lab)
- Dry Ice
- Access to "In Vitro Run List Master.xlt" located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run list\In Vitro_Cells\New List.
- Access to "run list master_111004.xlt" located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run list\In Vivo_Tissue\New List.
- Access to "Tissue Homog Master_Any tube type.xlt" located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Homogenized Box Weights\Homogenization Worksheets.

14.5. PROCEDURE

14.5.1. *Tissue Release List Generation*

- 14.5.1.1. Obtain a summary list containing the following information from the Study Coordinator:

- Exp#
 - ICX#
 - Drug name
 - Route
 - Vehicle
 - Array tissue
 - Sample release date
 - # of samples released
 - Time points released
- 14.5.1.2. Go to UNV_Main, choose mRNA extraction and type in the default password to gain access. Choose "Review Animal mRNA Extraction" and then press "Esc" to cancel upload (a find will be performed so it is not necessary to see all uploaded data).
- 14.5.1.3. Go to find mode (Ctrl F) and type in the sample release date (Y_mm/dd/yy_initials) in the "ready_for_mRNA_ext" field. The number of records found should match the number released by the Study Coordinator.
- 14.5.1.4. Export the list by selecting the "Export Rat in vivo RNA tissue list" button on the top right of the page. Name the file "Tissue REL DB_mmddyy_initials", save to appropriate folder, and select the export function.
- 14.5.1.5. Open the saved text file from the previous step and check the list against the list sent by the Study Coordinator, making sure that all drug names are present and that the correct time points were released.
- 14.5.1.6. Paste file into Tissue REL_Template.xlt found at:
P:\ChemExpress\ChemExpress Worksheets\Sample Release Worksheets. Send the list to the RNA Group Team Leader and to the Array Group Manager. Print the list and the email sent by the Study Coordinator, and file in the Sample tracking binder (by phase).
- 14.5.2. Worksheet Preparation**
- 14.5.2.1. Open "In Vitro Run List Master.xlt" for InVitro samples or "run list master_111004.xlt" for InVivo samples located at
P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run

list\ (In Vitro_Cells or In Vivo_Tissue)\New List, respectively. Save it as "Run list_ 'date' (mmddyyyy)" (e.g. "Run list_01012005") in the monthly folder located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run list\ (In Vitro_Cells or In Vivo_Tissue)\New List\Run Lists 2005.

- 14.5.2.2. Copy Tissue IDs and mRNA Numbers from the Tissue REL file into the "Release List" and "Run List" tabs of the worksheet. Sort both by RNA number.
- 14.5.2.3. In the "Release List" worksheet, assign a unique release date to the samples (mm/dd/yyyy). This worksheet will be used to upload into the Tissue Tracking DB.
- 14.5.2.4. Save file as "Run List_mmddy_initials" to P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run list\ In Vivo_Tissue\New List (and put in the appropriate monthly file).
- 14.5.2.5. Open Tissue Tracking DB from shortcut on desktop.
- 14.5.2.6. Click gray "Open Database" button and when prompted for password, enter "mRNA."
- 14.5.2.7. Click on the "mRNA Group" button.
- 14.5.2.8. To find import a new release list and find freezer locations, click "Import New mRNA List" button. You will then be asked whether your file contains three important pieces of information: a unique release date, mRNA numbers, and tissue ID numbers. At this time, click either cancel or continue.
- 14.5.2.9. Click button number one, "Import List from Excel Worksheet."
 - Find the Excel file you want to upload (Run List_mmddy_initials), using the 'open file' dialog box. Click "Open."
 - Select the "Release List" tab of the worksheet, and click "ok."
 - Select "Field Names" in the dialog box titled *First Row Option* if it is not already selected for you, click "ok."
 - Verify the information on the right and left sides of the dialog box 'Import Field Mapping' match. Verify under "import action," "add new records" is selected. Click "import."
- 14.5.2.10. From the layout, 'Temporary Hold Import List Database 2,' click the button number two, "Goto Freezer Location DB."

- 14.5.2.11. Next, click button three, "Find Tissue Sets forNew mRNA List."
- 14.5.2.12. On the next screen, click the fourth button, "Open Tissue Tracking Main DB."
- 14.5.2.13. Click button five, "Import Tissue Sets."
- 14.5.2.14. From the layout 'Import New list 1st Step', click button six, "Find Tissue Sets for Import."
- 14.5.2.15. Click button seven, "Import New mRNA Run List."
- 14.5.2.16. Lastly, click the red button (button eight), "Delete All Temporary Records."
- 14.5.2.17. From the layout 'List – Tissue & mRNA Data,' verify the release list you started with is completely represented in the found records. As a new release list, all tissues should be punch piece one.
- 14.5.2.18. From FileMaker toolbar, click on "Scripts" and select "Export Freezer Location List." Save this file to your desktop. This export contains all the mRNA numbers, Tissue ID's, and Freezer location information for your list of samples on your release list.
- 14.5.2.19. Open the freezer file from your desktop and paste all the information into the "Pull List" tab of the Run List worksheet. Sort by freezer, rack and box for ease in pulling tissues from freezers.

14.5.3. *Retrieving Tissues*

- 14.5.3.1. Obtain hemostats and prepare a (9x9) tissue box.
- 14.5.3.2. Obtain 2 Styrofoam boxes (1 large and 1 small), and one large green ice bucket. Fill up the large box and green bucket with dry ice and place a layer of dry ice in the small box. Place the tissue box in the small box and fill dry ice around it. Fill tissue box lid with dry ice.
- 14.5.3.3. Follow the "Pull List" for tissue sample number 1, go to the Freezer number on the list, open the freezer, go to the appropriate rack label and pull out the appropriate box (you may pull up to 7 or 8 boxes at one time. Place said boxes in large Styrofoam box). Close the freezer and place the box on dry ice. Go to the grid location in the box, pull out the tissue sample, place it in the new

(9x9) box (position A1), and place the dry ice filled lid on top of the box. Check it off on the list.

****Note: Remember to orient the box with the arrow positioned in the (upper left corner) before pulling samples. Position A1 and J10 are always empty. Work efficiently to make sure that no sample thaws.***

- 14.5.3.4. Repeat for every tissue sample.
- 14.5.3.5. Verify tissue number and tissue type against the list and note down any discrepancy. If a tissue sample cannot be located, skip the corresponding space in the box and continue on to the next tissue. Remember to follow the order of the list.

14.5.4. *Weighing Tissue Samples*

- 14.5.4.1. Take the box (always on dry ice) to the analytical balance located in the homogenization room.
- 14.5.4.2. Follow proper analytical balance technique and zero the balance before weighing the samples.
- 14.5.4.3. Start at A1 corner, pull out sample with a hemostat, knock the tube on the counter a couple times (to remove any ice), weigh it and put it back into the box as quickly as possible.
- 14.5.4.4. Record the weight of the tissue on the list next to the appropriate sample number.
- 14.5.4.5. Note down the tube type (Round or Regular).
- 14.5.4.6. Repeat the above steps for all the tissue samples.
- 14.5.4.7. Keep all the samples on dry ice.

14.5.5. *Entering Information in the Database*

- 14.5.5.1. Take the list to your computer. Open Tissue Homog Master_Any tube type.xlt located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Homogenized Box Weights/ Homogenization Worksheets.
- 14.5.5.2. Enter the tube weight (from step 14.5.4.4), and tube type (from step 14.5.4.5) in the appropriate fields.
- 14.5.5.3. Go to the "Print Form" worksheet in the spreadsheet. Sort the list by "Homogenize in".

- 14.5.5.4. If any volume in the "Homogenize in" column exceeds 3200 μ l, go to the "Tissue Worksheet" worksheet and under "Run/well", enter 75 if the "Homogenize in" volume is between 3200 – 6400, 50 if between 6500 – 9600, or 25 if between 9700 – 19200.
- 14.5.5.5. Return to "Print Form" worksheet and sort by "Homogenize in".
- 14.5.5.6. Note on the run list in the mRNA notebook any modified volumes in the "Vol Run/Well" column.
- 14.5.5.7. Print this sheet to be used for Homogenization of tissues. Take this sheet and your samples to the "Homogenization" station and proceed with homogenization as detailed in "Sample Preparation for RNA Isolation" protocol (Chapter 15, page 112).

Chapter 15

Sample Preparation for RNA Isolation

15.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for preparation of *In Vivo* samples by homogenization and syringing and of *In Vitro* samples by syringing.

15.2. RESPONSIBILITY

The RNA Team Leader is responsible for enforcing the execution of this protocol.

15.3. SAFETY PRECAUTIONS

Wear your lab coat, safety glasses, ear shields, and face shields at all times.

15.4. REQUIRED MATERIALS AND EQUIPMENT

- Micropipettes and sterile, aerosol resistant tips
- Bottle of Lysis Buffer (Roche Part No. 3 172 627 – MagNa Pure LC mRNA Isolation Kit II (Tissue) or Roche Part No. 3 004 015 – MagNa Pure LC mRNA Isolation Kit I (Blood, Blood Cells))
- Syringes (VWR Part No. BD309585)
- Needles (VWR Part No. BD 305167)
- Homogenizer (Omni Type No. TH115)
- Homogenizer Probe (Omni Part No. 34725)
- Ear and face shields
- Lab Coat with Special Arm Protection (VWR Part No. CV9403N)
- SORVALL Centrifuge at 0°C
- Dry Ice
- Safety glasses
- Access to “Homogenize In” list located at P:\ChemExpress\
Module.Lab.RNA\mRNA FOLDER\Homogenized Box
Weights\Homogenization Worksheets.

15.5. PROCEDURE

15.5.1. *Tissue Homogenization*

This section applies only to *In Vivo* tissue samples.

- 15.5.1.1. Obtain the "Homogenize In" list that was prepared in the "Pulling Tissues" protocol section 14.5.5.7 (page 111).
- 15.5.1.2. Wear the required and proper protection.
- 15.5.1.3. Take the box with weighed tissue samples maintained on dry ice to the "Homogenization" station.

****Note: It is very important the tissue samples do not thaw prior to homogenization to avoid degradation by RNases! It is important to process only one sample at a time.***

- 15.5.1.4. Insert the probe into the homogenizer.
- 15.5.1.5. Use the list to measure the appropriate volume of lysis buffer.
- 15.5.1.6. Pull out the appropriate tissue sample and verify the tissue number against the list.
- 15.5.1.7. Add the lysis buffer to the tube, throw away the tip in the hazardous waste container, and homogenize immediately, quickly, and thoroughly.
- 15.5.1.8. Push tissue down from the sides of the tube using the probe on homogenizer.
- 15.5.1.9. Turn the homogenizer ON from minimum speed to maximum speed.
- 15.5.1.10. Homogenize until the tissue is completely macerated.
- 15.5.1.11. Discard the probe into a hazardous waste container.
- 15.5.1.12. Place homogenized sample in a tissue box with a 9X9 grid (sample order doesn't need to be maintained), and let sit at room temperature.
- 15.5.1.13. Repeat steps 15.5.1.3 – 15.5.1.12 for all the samples.
- 15.5.1.14. After homogenizing all the samples, take them to the Sorvall RT7 centrifuge located in the Main Lab and spin the tubes for 3 minutes at 4000rpm.

****Note: Always balance the centrifuge.***

15.5.2. Syringing of Tissue and Cell Culture samples

This protocol is used for preparing both tissue and cell culture samples.

- 15.5.2.1. Syringe each sample 5-6 times to break up small cell debris.
**Note: Syringe cell culture samples immediately after thawed, and store on ice immediately after syringing*
- 15.5.2.2. If there are tissue chunks left in the tube, dispose of the tissue chunk and note it in the lab notebook notes section next to the corresponding tissue number as "chunk".
**Note: This applies only to In Vivo tissue samples.*
- 15.5.2.3. Dispose of syringes by placing in the hazardous chemical sharps container.
- 15.5.2.4. Order the tubes in 9x9 boxes following the order on the "Run List". At this time you may proceed to either section 16.6.1.2 or 15.5.2.5 if time doesn't allow.
- 15.5.2.5. Label the top of the box with the next day's date and place it in the appropriate -80°C mRNA freezer until the next morning run.
- 15.5.2.6. Next morning, take the samples out of -80°C freezers and proceed with "RNA Isolation" protocol (Chapter 16, page 116).

15.5.3. Uploading Raw Tissue Data into Tissue Tracking DB (FileMaker Pro)

- 15.5.3.1. Upon completion of homogenization, upload homogenization data into the Tissue Tracking Database using both the "Homogenize In" worksheet and the file it is apart of "Tissue Homog Master."
**Note: Any changes in homogenization volume that were made at the homogenization station are to be written on the "Homogenize In" worksheet and subsequently changed in the "Tissue Homog Master" before uploading.*
- 15.5.3.2. Open Tissue Tracking DB from shortcut on desktop.
- 15.5.3.3. Click gray "Open Database" button and when prompted for password, enter "mRNA."
- 15.5.3.4. Click on the "mRNA Group" button.
- 15.5.3.5. Click button "Import Raw Tissue Data."

- Find the Excel file you want to upload (Tissue Homog Master_mmddy_initials), using the 'open file' dialog box. Click "Open."
 - Select the "For Import" tab of the worksheet, and click "ok."
 - Select "Field Names" in the dialog box titled 'First Row Option' if it is not already selected for you, click "ok."
 - Verify the information on the right and left sides of the dialog box 'Import Field Mapping' match. Verify that under "import action," "update matching records" is selected. Click "import."
- 15.5.3.6. From layout "List – Tissue & mRNA data" verify the import of tissue data was successful. Close DB.

Chapter 16

RNA Isolation

16.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for the operation of the MagNA Pure LC robot for RNA isolation.

16.2. REFERENCE

MagNA Pure LC Manuals

16.3. RESPONSIBILITY

The RNA Isolation Team leader is responsible for the implementation and the correct execution of this protocol. In-house MagNA Pure LC Consultant can be asked regarding any problems with the MagNA Pure LC.

16.4. SAFETY PRECAUTIONS

- Always wear your lab coat and protective eyewear, as well as gloves when working in the lab.
- In an emergency, immediately turn the power switch off and unplug the instrument.

16.5. REQUIRED MATERIALS AND EQUIPMENT

(For 1 run)

- There are two MagNA Pure LC robots at Iconix. Both are currently running the MagNA Pure software program version 3.03 (floppy and CD). These disks are located with the instruction manuals.
- Roche MagNA Pure LC mRNA Isolation Kit II (Tissue) part No. 3 172 627
- Roche MagNA Pure LC mRNA Isolation Kit I (Blood, Blood Cells) part No. 3 004 015
 - Bottle of Streptavidin-coated beads
 - Wash buffer I and II
 - Elution Buffer
 - DNase solution
 - DNase Incubation Buffer

- Lysis Buffer
- Oligo-dT
- 4 Processing Cartridges (Roche Part No. 3004147)
- 3 Sample Cartridges (Roche Part No. 3004112)
- 2.5 Cartons of large tips (Roche Part No. 3004171)
- 1 Carton of Small tips (Roche Part No. 3004236)
- 2 Large Tub lids (Roche Part No. 3004074)
- 3 Medium tub lids (Roche Part No. 3004082)
- 1 Small tub lid (Roche Part No. 3004082)
- Micropipettes and sterile, aerosol resistant tips
- Centrifuge with 96-well rotor
- 1.5 ml microcentrifuge tubes (Ambion Part No. 12400)
- 5 mg/ml Glycogen (Roche Part No. 901393)
- 5 M NH₄OAc (Ambion Part No. 9070G)
- Absolute ethanol (Aldrich No. 459828)
- 75% ethanol (Prepared from absolute ethanol)
- Label Sheet (TTSW-2240 Size .94x.50 inches)
- Dry Ice
- Micropipettes and sterile, aerosol resistant tips
- 25 ml reservoirs
- Tube Rotator
- Parafilm
- Lab Coat
- Safety glasses
- Samples from -80°C mRNA freezer (These samples were stored in -80°C freezer when "Homogenization" protocol was finished).
- *In Vitro* standard

16.6. PROCEDURE

16.6.1. *Preparation for a Run*

- 16.6.1.1. Obtain sample box labeled with the correct date (see section 15.5.2.5, page 114) from the appropriate -80°C mRNA freezer and thaw in a rack at room temperature during machine set-up.

****Note: Standing the tubes in room temperature water will expedite the thawing process.***

- 16.6.1.2. Obtain bottle of streptavidin-coated magnetic beads from the 4°C deli box and place on the rotator (at 4°C) during machine set-up.
- 16.6.1.3. Using the MagNA Pure computer, click on “Sample Ordering” from the Main Menu Screen. Select “Load Sample Order” from the “File” menu and select the appropriate pre-established run template from either the cell protocols or tissue II protocols folders.
 - In each folder there are protocols written for the following four run scenarios: 1-8, 1-16, 1-24, and 1-32 samples.

****Note: The protocols in the cell protocol folder run the mRNA I cells program, whereas the protocols in the tissue II folder run mRNA II tissue program***

- 16.6.1.4. A warning that says “Existing screen data will be lost.” will appear. Click “OK”.
- 16.6.1.5. Click the “Stage Setup” button on the Sample Ordering Screen to move on to the Start Information Screen. The stage layout graphic will indicate the disposable plastics and reagent volumes needed for the run.
- 16.6.1.6. Place all the necessary plastics on the stage according to the computer graphic and click on each icon as completed.

****Note: Do not fill in all the items and then click on the icons as this will result in errors. Always double-check all items have been loaded before walking away.***
- 16.6.1.7. Place the reagent-tub rack on the lab bench and add the appropriate reagent tubs, reagents, and tub lids according to the computer graphic. The order of reagents in the holder is important.

****Note: To solubilize the lyophilized DNase, add 4 ml DNase incubation buffer to the glass vial of lyophilate. Before beginning the run add an additional 3.8 ml DNase incubation buffer, mix well, and pour into appropriate tub.***

****Note: DO NOT add the beads or DNase to their respective reagent tubs until ready to start the run***

Table 16.6-A: MagNA Pure LC Buffer Chart

# Wells:	1-8	9-16	17-24	25-32
L - Hyb Buffer:	8.8 ml	13.6 ml	18.4 ml	23.2 ml
+ oligo-dT	7.3 µl	11.3 µl	15.3 µl	19.3 µl
L - Wash II:	10.4 ml	16.8 ml	23.2 ml	29.6 ml
M – DNase:	7.8 ml	7.8 ml	7.8 ml	7.8 ml
M - Wash I:	4.4 ml	7.6 ml	10.8 ml	14.0 ml
S - Streptavidin. Beads:	0.9 ml	1.3 ml	1.7 ml	2.3 ml
M – Elution Buffer:	2.6 ml	3.7 ml	4.9 ml	6.0 ml

} Not for tissue samples

L= large, M= medium, S= small reagent tub

16.6.1.8. Add samples to the 32-well sample tray:

- Run in double-well operation mode for all tissue samples using Tissue II Kit:
 - Add 150 µl of clear tissue lysis buffer to each well
 - Add 150 µl of each sample to two wells of the sample tray, filling the tray from H1 to A4 (Figure 16.6-A).

**Note: Actual lysis buffer and sample run volumes can differ, so always refer to the lab notebook.*

- Add 600 µl of Capture Buffer (Oligo-dT and Hybridization Buffer combined in a 25 ml reservoir) to each well.
- Incubate at room temperature for 5 minutes.
- Run in double-well operation mode for all primary rat hepatocytes and blood samples using mRNA Isolation Kit I

**Note: Remember to keep cell culture samples on ice at all times.*

- Add 150 µl of blue cell/blood lysis buffer to each well
- Add 150 µl of standard to wells H1 and G1 if running primary rat hepatocytes.

**Note: Standard is periodically obtained in bulk from the In Vitro Biology group, syringed, aliquoted 350 µl per Eppendorf tube, and stored at -80.*

- Add 150 µl of each sample to two wells of the sample tray, filling the tray from H1 to A4, or F1 to A4 if running primary rat hepatocytes (Figure 16.6-A).

**Note: Actual lysis buffer and sample run volumes can differ, so always refer to the lab notebook.*

- For other cell lines as specified by *In Vitro* Biology group use single-well operation and mRNA Isolation Kit I:
 - Add 300 μ l of sample to one well of the sample tray, filling the tray from H1 to A4 (Figure 16.6-A).

16.6.1.9. Place the tray in the appropriate spot on the machine stage

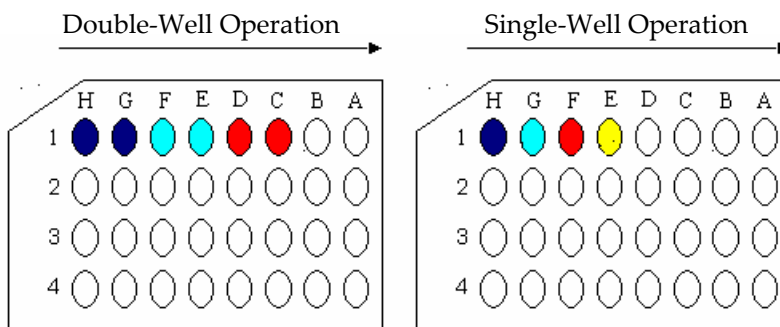


Figure 16.6-A: MagNA Pure LC Sample Tray Loading

16.6.1.10. Add the correct amount of streptavidin-coated magnetic beads (SMPs) and the solubilized DNase to their respective reagent tubs, put the reagent tub rack on the machine stage, and close lock bar. Close MagNA Pure robot door and click on the remaining icons.

16.6.2. Robot Run

- 16.6.2.1. Click the OK button in the lower right corner of the Start Information Screen to start the run.
- 16.6.2.2. A 32-well run will last approximately 1.5 hours.

16.6.3. Used lysate storage

***Note: 16.6.3.1 – 16.6.3.5 applies to tissue samples. Cell culture samples are placed (no scanning) into boxes and stored at -80 in the appropriate rack.**

- 16.6.3.1. Examine the used lysate tubes and dispose of tubes containing less than 150 μ l lysate.
- 16.6.3.2. Open P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\ used lysate boxes and find the next available used tissue box file.
- 16.6.3.3. Tubes containing more than 150 μ l lysate are stored in the next available used lysate box. (Check the -80° for the box number corresponding to the open file in the previous step).

- 16.6.3.4. Make sure the cursor is in the next empty slot on the used box file and then scan the barcode of the used lysate tubes as you place them in the corresponding slot of the used lysate box.

****Note: For Tissue Tracking Database purposes, do NOT put used lysates back in their original location if running reruns.***

- 16.6.3.5. Replace the used lysate box in the -80°C freezer.

16.6.4. Post-elution

This step is the transfer of samples from plate to tubes.

- 16.6.4.1. Once the run is complete, the Result Screen will appear. Remove the sample tray from the back righthand corner, and cover with parafilm. Centrifuge for 3 min @ 4000 rpm in the Sorvall (RT7) tabletop centrifuge in the Main Lab.

****Note: Make sure to balance the centrifuge.***

- 16.6.4.2. Print mRNA labels by opening PC Labels PLUS Excel file located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER.

- 16.6.4.3. Type mRNA sample numbers and the Notebook Page Number onto this sheet and print this list on Labels worksheet.

- 16.6.4.4. Label 1.5 ml microcentrifuge tubes with mRNA labels printed in the previous step. Write the sample number on the top of the tube. Include R for rat or H for Human.

- 16.6.4.5. Make NH₄OAc -glycogen Master Mix (per sample) as follows:

– For double-well operation samples:

- 1 µl glycogen
- 20 µl 5M NH₄OAc

– For single-well operation samples:

- 1 µl glycogen
- 10 µl 5M NH₄OAc

- 16.6.4.6. Add Ammonium Acetate-glycogen mix to each labeled tube:

- 21 µl/tube for double-well operation
- 11 µl/tube for single-well operation

- 16.6.4.7. Sample transfer

– Manual transfer of samples for single-well operation:

- Transfer each sample to the appropriately labeled tube by carefully pipetting the sample without disturbing the bead pellet at the bottom of the well.

****Note: DOUBLE CHECK to make sure the samples in the sample tray corresponds to the correctly labeled tube.***

- Automated transfer of samples for double-well operation:
 - Load the appropriately labeled tubes into the left cooling block in row 1 and 17 as shown in Figure 16.6-B (for 16 samples, load row 1 only).

****Note: DOUBLE CHECK to make sure the samples in the sample tray corresponds to the correctly labeled tube.***

- Following centrifugation put the elution tray back in the robot in the same position.
- Put two rows of small tips into the robot.
- Select "Actions" from the toolbar of the Results Screen, and click on "Post Elution".
- Under "File," click the "Load Post Elution" button and select the appropriate protocol: 1-8, 1-16, 1-24, 1-32.

****Note: These protocols are designed to combine two sample wells from the sample tray into one eppendorf tube.***

- Click on the "Start Post Elution" button. It will ask you if you want to start, then hit "Start". The protocol will take 20 minutes.

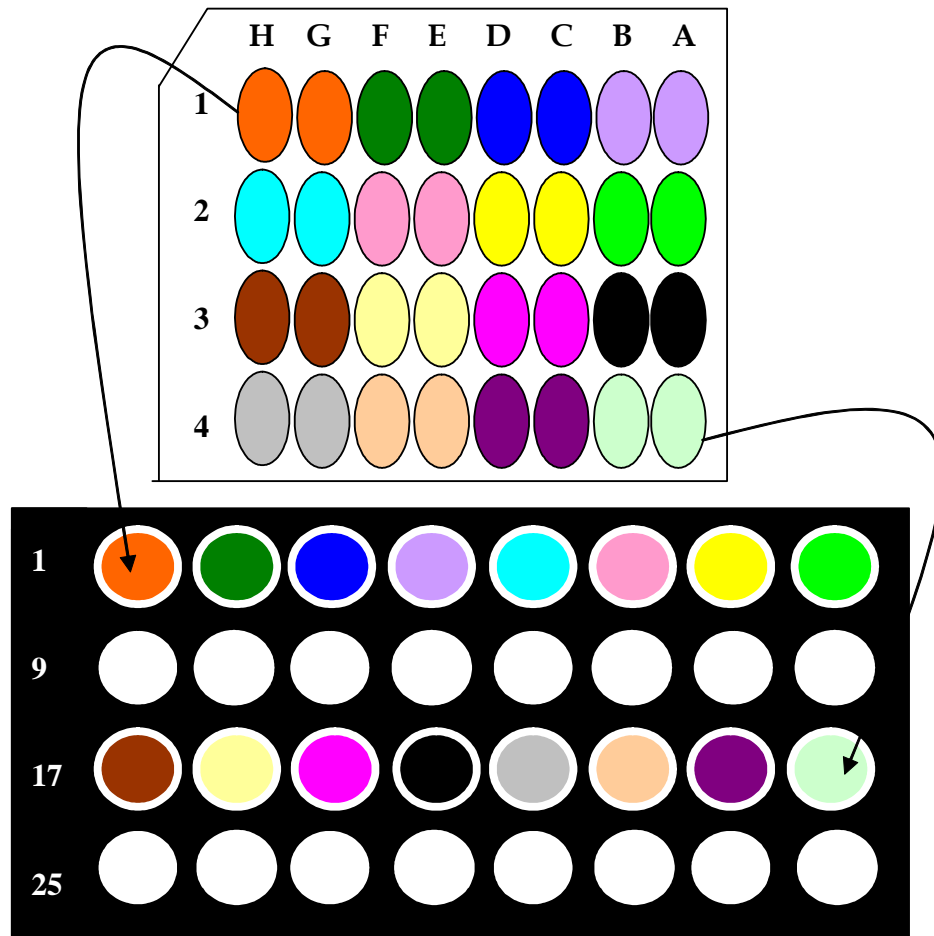


Figure 16.6-B: Post Elution Protocol Sample Transfer Schematic

16.6.4.8. When the transfer of sample into the tubes is complete, proceed with ethanol precipitation (Chapter 17, page 124).

16.7. CONTACT INFORMATION

Roche Field Service Representative
 Eriberto A. Aranjuez, Jr.
 1-800-845-7355 ext 5653

Chapter 17

Ethanol Precipitation of mRNA

17.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for concentration of mRNA samples by ethanol precipitation.

17.2. RESPONSIBILITY

The RNA Team Leader is responsible for enforcing the execution of this protocol.

17.3. SPECIAL NOTES

- Always wear your lab coat and goggles when working in the lab.
- Precipitation is a critical step and if not done properly might result in loss of sample. Once the centrifugation has stopped, work quickly because the pellet could loosen if not processed immediately especially if 12 or more samples are processed at a time. This is true for both the ethanol precipitation and the 75% ethanol wash.
- Drying RNA pellets should be done by air dry or with vacuum, but not by centrifugation under vacuum. If the RNA pellet is too dry, it will be difficult to resuspend.

17.4. REQUIRED MATERIALS

- Absolute ethanol (Aldrich No. 459828) (stored at -20°C)
- 75% ethanol (Prepared from absolute ethanol) (stored at -20°C)
- Dry Ice
- Micropipettes and sterile, aerosol resistant tips
- Refrigerated Microcentrifuge capable of 15000rpm
- Labtop mini-centrifuge
- Lab Coat
- Safety glasses

17.5. PROCEDURE

17.5.1. *Ethanol Precipitation*

- 17.5.1.1. Add cold 100% ethanol to the tubes obtained from the "RNA Isolation" protocol section 16.6.4.8 (page 123) then shake/invert tubes and vortex:
 - 250 µl for single-well operation
 - 500 µl for double-well operation
- 17.5.1.2. Place the tubes on dry ice for 15 minutes or in -80°C freezer for 30-40 minutes or at -20°C for 1 hour to overnight.
- 17.5.1.3. Spin in a microcentrifuge at maximum speed for 20 min at 4°C. Carefully decant the supernatant into Alcohol Waste Container (glass jar with green lid) without decanting the pellet.
**Note: If sample is frozen, spin for 25 minutes at 4°C.*
- 17.5.1.4. Add 400 µl of 75% ethanol to wash the pellet and spin at maximum speed at 4°C for another 5 minutes.
- 17.5.1.5. Carefully decant the supernatant into Alcohol Waste Container (glass jar with green lid) without decanting the pellet. Quick spin tubes for 5 seconds. Go back to the first tube and carefully remove the remaining ethanol with a 200 µl-tip without disturbing the pellet. Using 10 µl tip to remove any left over ethanol. Air-dry on bench top for 10 min.
- 17.5.1.6. Completely dissolve the pellet in 7 µl of DEPC-H₂O (depending on the RNA, the re-suspension volume may vary, refer to the lab notebook.) Make sure that you have not missed the pellet somewhere on the wall of the tube, if this is the case make sure to re-suspend. Vortex and quick spin each sample in the tube.
- 17.5.1.7. Store the samples in an appropriate -80°C mRNA freezer in a temporary RNA box labeled with day's Date.
- 17.5.1.8. Follow the "Running the Agilent 2100 Bioanalyzer" protocol (Chapter 18, page126) to check for mRNA integrity and ribosomal contamination.

Chapter 18

Running the Agilent 2100 Bioanalyzer

18.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for assessment of mRNA quality using the 2100 Bioanalyzer.

18.2. REFERENCE

Agilent 2100 Bioanalyzer Manual and mRNA Nano kit instructions.

18.3. RESPONSIBILITY

The RNA Team Lead is responsible for enforcing the execution of this protocol.

18.4. SAFETY PRECAUTIONS

Always wear your lab coat and goggles when working in the lab!!

18.5. REQUIRED MATERIALS

- 96-well V-bottom plate (VWR Part No. 29443-954)
- DEPC-H₂O
- 25 ml Reservoir
- Syringe and chip priming station (Part of Agilent Part No. 5065-4476)
- RNA 6000 Nano Assay Kit (Agilent Part No. 5065-4476)
- RNA ladder (Ambion Part No. 7152)
- Agilent 2100 Bioanalyzer
- Agilent Vortexer
- Seal film (MJ-MSA 5001)
- Micropipettes and sterile, aerosol resistant tips
- Microcentrifuge
- Wet Ice
- Timer
- Heat block at 70°C
- Access to Agilent Plate Master Worksheet.xls located at
P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\mRNA Agilent

- Samples stored in Temp RNA box located in appropriate mRNA -80°C freezer

18.6. PROCEDURE

18.6.1. 96-well plate Preparation

- 18.6.1.1. Obtain a bucket of ice.
- 18.6.1.2. Obtain the samples from temporary RNA box (section 17.5.1.7, page 125) and arrange them on ice according to the RNA number on Run List.
- 18.6.1.3. Add 4 μ l of DEPC-H₂O to per well (up to 80 wells) to a 96-well V-bottom plate. For more than 80 samples, start a new plate.
- 18.6.1.4. Make sure samples are completely thawed and thoroughly mixed (if not briefly vortex or “flick” the tube and spin down). Add 1 μ l of sample to each well of the 96-well plate in row order (A1-H1, A2-H2...A12-H12) (Figure 18.6-A).
- 18.6.1.5. Cover the plate with seal film and lid. Place the plate on ice.
**Note: Work quickly but accurately.*
- 18.6.1.6. Label the plate as “Agilent plate”.
- 18.6.1.7. Put the samples back according to the mRNA number into numbered mRNA boxes located in appropriate -80°C mRNA freezer.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 18.6-A: Agilent plate preparation

18.6.2. *Print the Agilent Run List.*

- 18.6.2.1. Open Agilent Plate Master Worksheet.xls located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\mRNA Agilent.
- 18.6.2.2. In the Data worksheet, type in mRNA numbers from the "Run List" located in lab notebook.
- 18.6.2.3. Go to the Plate worksheet and print this sheet. Refer to the print out and check off the samples as you run Agilent.
- 18.6.2.4. Name the file as "Agilent Plate_'mmddy'_initials", e.g. Agilent Plate_06.26.03_PQ).

18.6.3. *Run the Bioanalyzer Program*

- 18.6.3.1. Turn on the computer and the Bioanalyzer.
- 18.6.3.2. Double click on the "2100 Expert" icon. Upon opening the program, the Instrument screen will appear.

18.6.4. *Preparing the Gel*

(Sufficient for 10 chips)

- 18.6.4.1. Remove the necessary reagents from 4°C and allow equilibration room temperature for 30 minutes before use.
- 18.6.4.2. Put 550 µl of RNA gel matrix (red cap) into the top receptacle of a spin filter.
- 18.6.4.3. Centrifuge at 1500g ± 20% for 10 minutes (~4000 rpm).
- 18.6.4.4. Aliquot 65 µl filtered gel into 0.5 ml microfuge tubes provided in the kit.
- 18.6.4.5. Store the aliquots at 4°C and use them within one month of preparation.

18.6.5. *Prepare the Gel-Dye Mix*

- 18.6.5.1. Remove the necessary reagents from 4°C and allow equilibration room temperature for 30 minutes before use.
**Note: Protect the dye concentrate from light while bringing it to temperature.*
- 18.6.5.2. Vortex the RNA 6000 Nano dye concentrate (blue cap) for 10 seconds then spin down.

- 18.6.5.3. Add 1 μ l of dye to a 65 μ l aliquot of filtered gel (prepared in section 18.6.4). This aliquot is sufficient to run two chips.
- 18.6.5.4. Vortex the mixture thoroughly then visually inspect for proper mixing.

**Note: Protect the gel-dye mixture from light.*

- 18.6.5.5. Centrifuge the mixture for 10 minutes at room temperature at 14,000 rpm.

- 18.6.5.6. Use the gel-dye mixture within one day.

**Note: A larger volume of gel-dye mix can be prepared in multiples of 65+1 if more than two chips will be used within one day. Always re-spin the gel-dye mix at 14,000 rpm before each use.*

18.6.6. RNA Ladder Denaturation

- 18.6.6.1. RNA 6000 Ladder from Ambion comes in a vial of 20 μ l that must be divided into 10 μ l aliquots and stored at -80°C .
- 18.6.6.2. Remove a 10- μ l aliquot from the freezer and heat the tube at 70°C for 2 minutes.
- 18.6.6.3. Place the tube directly on ice for 5 minutes to snap cool the ladder. Briefly centrifuge to clear any condensate from the tube's walls and cap. Keep the RNA 6000 ladder on ice throughout the day and return to -80°C at the end of the day.

18.6.7. Electrode Decontamination

- 18.6.7.1. Add 350 μ l RNase Zap to the RNase Zap chip and 350 μ l DEPC- H_2O to the DEPC- H_2O chip.
- 18.6.7.2. Decontaminate the machine's electrodes by first placing the RNase Zap chip in the machine for 1 minute followed by the DEPC- H_2O chip for 10 seconds. Allow the electrodes to dry with the lid open for 10 seconds after cleaning with water.

**Note: Save these chips as they can be reused for the entire box (25 runs).*

18.6.8. Load the RNA chip

- 18.6.8.1. Place a new chip on the Chip Priming Station.
- 18.6.8.2. Pipette 9 μ l of gel-dye mix (prepared above in step 18.6.4) into the third well marked with a highlighted G. Be sure to place the tip

all the way to the bottom of the well to avoid introducing air bubbles.

- 18.6.8.3. Make sure that the Chip Priming Station Plunger is at 1 ml then close the Chip Priming Station securely.
- 18.6.8.4. Press the plunger down until caught by the syringe clip and wait exactly 30 seconds. Replace the plunger biweekly and record the date replaced in the "Plunger Replacement Sheet" posted next to the Agilent station.
- 18.6.8.5. Release the plunger with the clip release mechanism and allow the plunger to come up then pull it back to the 1-ml position.
- 18.6.8.6. Pipette 9 μ l of the gel-dye mix into the other two wells marked G.
- 18.6.8.7. Add 5 μ l of RNA 6000 Nano Marker (green cap) to each of the 12 sample wells and to the ladder well.
**Note: All 12 wells must be filled with RNA 6000 Nano Marker, even if running fewer than 12 samples.*
- 18.6.8.8. Add 1 μ l of denatured RNA6000 Ladder to the ladder well.
- 18.6.8.9. Transfer 1 μ l of the sample from Agilent Plate prepared at step 18.6.1.4 to the chip wells. Use new pipette tip every time. Make sure the plate is on ice at all times.
- 18.6.8.10. Vortex the chip in the special adapter for 1 minute at a setting of 5. The chip must be used within 5 minutes of vortexing.

18.6.9. Running the Bioanalyzer

- 18.6.9.1. Place the chip in the Bioanalyzer and close the lid.
- 18.6.9.2. Go to "Context" and then "Instrument." Then click the Instrument tab.
- 18.6.9.3. Under All Instrument, click Instrument 1 or 2, depending on which machine you placed the chip in. Instrument 1 (DE23101934) is on the left, and Instrument 2 (DE04700541) is on the right.
**Note: It is possible to run up to four machines at one time with this one program.*
- 18.6.9.4. Select "mRNA Nano" assay under Assays -> Electrophoresis -> RNA -> mRNA Nano.

- 18.6.9.5. Under Destination, click Custom. Click the browse key and select P:\Agilent\2100 bioanalyzer\2100 expert\data. This should already be set as the default save location.
- 18.6.9.6. Also under Destination, enter the File Prefix in the following format: mRNA.dd.MMM.yy.LETTER, e.g. mRNA.01.JAN.05.A. It must be 2 digit day, 3 CAPS month, 2 digit year, and CAPS letter.
- 18.6.9.7. Under Run sample, select the number of samples present on that chip (between 1 and 12).
- 18.6.9.8. Click "Start".
- 18.6.9.9. Go to "Context" and then "Data and Assay".
- 18.6.9.10. Click the file prefix.
- 18.6.9.11. Under the Chip Summary tab, enter the RNA Prep numbers from the "Agilent Run list" into the Sample Names column. Click "OK".
- 18.6.9.12. When the run is over, a folder with the day's date, e.g. "2005-01-01", is automatically created within P:\Agilent\2100 bioanalyzer\2100 expert\data. The results are automatically saved as Agilent .xad files in the newly created folder.
**Note: At the end of every month, create a new folder in "data" folder and name it as "month_year" e.g. "January_2005". Place all the files for that month into the folder you just created. At the end of the year, create a new folder with the year, and drag into it all the month folders.*
- 18.6.9.13. Print out "Run Summary". Click on the Data and Assay button on the left-hand side of the screen. On the menu go to "File", "Print", and select "Run Summary" (should already be selected). Under Page Range, select "Pages" and type 1. Click "Print". Paste all printouts into mRNA notebook.
- 18.6.9.14. Remove the chip from the Bioanalyzer. Rinse the electrodes with the DEPC-H2O chip for 5 seconds. Allow the electrodes to dry with the lid open for 10 seconds. Repeat this for every run.
**Note: DO NOT leave the Lab chip in the Bioanalyzer for more than 30 min. after it has finished running as it can contaminate the pins in the cartridge.*

- 18.6.9.15. At the end of the day, decontaminate the machine electrodes by first placing the RNase Zap chip in the machine for 1 minute followed by the DEPC-H₂O chip for 10 seconds. Allow the electrodes to dry with the lid open for 10 seconds after cleaning with water.
- 18.6.9.16. Make sure to empty out the cleaning chips at the end of each day to prevent build up of contaminants. Wash the RNase Zap chip in the sink. Save these chips as they can be reused for the entire box (25 runs).
- 18.6.9.17. Keep the "Agilent Plate" on ice and proceed to the "Ribogreen Analysis" protocol (Chapter 19, page 133).

Chapter 19

Ribogreen Analysis

19.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for assessment of mRNA quality using the Ribogreen Analysis.

19.2. RESPONSIBILITY

The RNA Team Leader is responsible for enforcing the execution of this protocol.

19.3. SAFETY PRECAUTIONS

Always wear your lab coat and goggles when working in the lab!!

19.4. REQUIRED MATERIALS

- 1x TE (Made from 20x TE (Molecular probe Part No. R-11490))
- Agilent Plate (Prepared section 18.6.1, page 127)
- Flat bottom 96-well Plates (VWR Part No. 29443-954)
- Ribogreen Kit (Molecular probe Part No. R-11490)
- Multi-channel pipette
- Fluorescent Plate Reader with 483/538 filter sets
- Micropipettes and sterile, aerosol resistant tips
- 3 x 25 ml Reservoir
- Access to database
- Access to Ribogreen Raw Data Worksheet located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\mRNA Ribogreen Raw Data.
- Access to AAA.Batchload Master.xls located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\mRNA Batchload\Batch Lists\2005.

19.5. PROCEDURE

19.5.1. *Thawing Reagents*

- 19.5.1.1. Pull out RNA standard and fluorescent dye aliquots from Ribogreen kit and let them thaw. Be sure to protect fluorescent dye from light and to keep the RNA standard on ice.

19.5.2. 96-well plates Preparation

- 19.5.2.1. Add 199 μ l of 1xTE to wells A1-H10 of a flat bottom 96-well plate. Label the plate as plate #1 (Figure 19.5-B: Plate #1).
- 19.5.2.2. Completely thaw and mix the mRNA sample. Transfer 1 μ l of sample by row from "Agilent Plate" (saved from step 18.6.1.4) to sample plate #1 using multi-channel pipette
- 19.5.2.3. Transfer 100 μ l of sample by row from Plate #1 to a new flat bottom 96-well plate. Label the plate as plate #2 (Figure 19.5-C: Plate #2).
- 19.5.2.4. Add 100 μ l of 1xTE to wells B11-H11 and B12-H12 of Plate #2.
- 19.5.2.5. Add 196 μ l of 1xTE and 4 μ l of RNA standard to wells A11 and A12 of Plate #2.
- 19.5.2.6. Transfer 100 μ l from each well of A11 and A12 to wells B11 and B12, respectively. Mix by pipetting up and down.
- 19.5.2.7. Transfer 100 μ l from B11 and B12 to next wells. Repeat this until wells G11 and G12.
- 19.5.2.8. Take 100 μ l out of wells G11, and G12 and dispose.

The concentrations of standards are:

A11-12	1 μ g/ml
B11-12	0.5 μ g/ml
C11-12	0.25 μ g/ml
D11-12	0.125 μ g/ml
E11-12	0.0625 μ g/ml
F11-12	0.0313 μ g/ml
G11-12	0.01565 μ g/ml
H11-12	0 μ g/ml

19.5.3. Ribogreen Dye Preparation

- 19.5.3.1. Refer to "Dye Preparation Sheet" on Ribogreen workstation to figure out how much dye and TE buffer is needed for the assay.
- 19.5.3.2. Refer to the worksheet located at Ribogreen station to make enough diluted solution (TE + Dye) in a sterile reservoir to run the assay. Be sure to use the solution immediately.
- 19.5.3.3. Use a multi-channel pipette to transfer 100 μ l of diluted solution from the reservoir into each well of Plate #2.

19.5.3.4. Cover the plate with foil and incubate at RT in the dark for 4 minutes. Take the plate to the Fluorescent Plate Reader.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D				5 μ l sample per well								
E												
F												
G												
H												

Figure 19.5-A: Agilent Plate

Transfer 1 μ l and
Add 199 μ l of 1X TE ↓

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D				200 μ l sample per well								
E												
F												
G												
H												

Figure 19.5-B: Plate #1

Transfer 100 μ l ↓

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

100 μ l sample per well

Figure 19.5-C: Plate #2

19.5.4. Reading the Plate Using Fluorescent Plate Reader

- 19.5.4.1. Remove the lid and place the plate in the proper position on the machine stage.
- 19.5.4.2. Start the fluorescent plate reader by clicking on "Wallace 1420" icon. Choose "Ribo-Top" protocol from the drop down menu and click the start button.
- 19.5.4.3. Once the machine has finished reading the assay plate, it will automatically save the file to the My Documents \ mRNA group folder located on the desktop.
- 19.5.4.4. Drag the file into the mRNA Ribogreen Raw Data folder located on the desktop. This folder is a shortcut to P:\ChemExpress\ Module.Lab.RNA \ mRNA FOLDER \ mRNA Ribogreen Raw Data. Rename file as "Ribogreen_ddmmyy_initials."
- 19.5.4.5. Save plate#1 until data has been entered into Batchload worksheet and it is determined the reading was "good" (is determined by RNA standard).

19.5.5. Entering Information into the Batch Worksheet

- 19.5.5.1. Open the Ribogreen Raw Data Worksheet saved in step 19.5.4.4, located at P:\ChemExpress\ Module.Lab.RNA \ mRNA FOLDER \ mRNA Ribogreen Raw Data.
- 19.5.5.2. Select: "List; Plates 1 – 1" worksheet.

- 19.5.5.3. There are 6 columns of information on this worksheet. Select all the contents of column "F" (excluding the header) and copy.
- 19.5.5.4. Once copied onto the clipboard, close the Ribogreen file.
- 19.5.5.5. Go to AAA.Batchload Master.xls located at P:\ChemExpress\ Module.Lab.RNA \mRNA FOLDER \mRNA Batchload \Batch Lists\2005.
- 19.5.5.6. Save the file as "Batchload_mmddy_initials", e.g. Batchload_06.03.02_PQ.
- 19.5.5.7. Select the "1.Ribogreen Plate Data" tab. Paste the data copied from the Ribogreen data file into column "B" it is labeled as "Ribogreen – Top (RFU)".
- 19.5.5.8. Under the "D" column, type the Agilent file date. (This is the date that you saved the Agilent files under, e.g. 10.APR.02.A).
- 19.5.5.9. Under column "F", enter the lab Notebook Page Number.
**Note: Make sure that a notebook page number is only used once and that it is not used in the database already).*
- 19.5.5.10. Under the "I" column, type mRNA sample numbers in the order that they were in on the Ribogreen assay plate.
- 19.5.5.11. Go to the next worksheet labeled as "2. Data Analysis" and enter the RNA Standard and Ribogreen Dye lot numbers in the yellow cells.
- 19.5.5.12. Verify Ribogreen data by checking that the calculations were made. If the RFU/concentration values are acceptable, the concentrations of the samples will automatically be calculated. If the standard curve is unacceptable, the word "REDO" will appear in the RFU column and the concentrations will not be calculated.
- 19.5.5.13. If Ribogreen data is not acceptable, delete the RFU information from the worksheet and run another Ribogreen plate using plate #1.
- 19.5.5.14. Go to the next tab labeled "3. mRNA Data"
- 19.5.5.15. Go to column "E" labeled "Volume of recovered mRNA" Make sure the values reflect the resuspension volume used.
- 19.5.5.16. Go to "RNA QC" column and fill in "P" for "Pass" or "F" for "Fail" for all degraded Agilent data.

19.5.5.17. Go to column "I" labeled as "mRNA comments". Fill in the comments for each sample(all reruns must have "rerun" in mRNA comments section). Save your worksheet in P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\mRNA Batchload\Batch Lists\2005 in the appropriate folder for the month.

19.5.6. Data Upload into UNV_Main.FP5 database

- 19.5.6.1. Open the UNV_Main.FP5 database.
- 19.5.6.2. Choose mRNA extraction tab, enter password "mRNA", then click on Batch Load mRNA Extraction Data tab.
- 19.5.6.3. Select "Scripts" from the menu then choose "Merge_RNA Info_1" script.
- 19.5.6.4. Under RNA prep number, enter the range of the mRNA numbers for which you wish to import data, starting with the lowest number and ending with the largest, separated by three periods. For example, if your lowest numbered sample is R94128, and the highest numbered is R94226, enter R94128...R94226. Select "Continue", and then "Find."
- 19.5.6.5. Again, from the menu select "Scripts". Choose "Merge_RNA Info_2" script. Follow the import instructions as the computer prompts you. Import the saved Batchload worksheet.
**Note: Make sure the import fields are matching correctly before you press enter.*
- 19.5.6.6. Verify the number of samples in database match the number of samples in the worksheet.
- 19.5.6.7. Send an email to the Team leader of the Target Prep group and to your supervisor that includes the batch worksheet in it.
- 19.5.6.8. You can now throw away all Ribogreen plate.

19.5.7. Data Upload into Tissue Tracking DB

- 19.5.7.1. Open Tissue Tracking DB from shortcut on desktop.
- 19.5.7.2. Click gray "Open Database" button and when prompted for password, enter "mRNA."
- 19.5.7.3. Click on the "mRNA Group" button.

- 19.5.7.4. From the mRNA Group main page, click the button "Import Batch mRNA Data."
- 19.5.7.5. Next, click button number one, "Import List from Excel Sheet." Using the 'Open File' dialog box, locate Batchload worksheet, and click "open."
- 19.5.7.6. In the 'Import mRNA Data – Temporary Hold DB' are two questions. Who ran the MagNA Pure and Who ran the Agilent and Ribogreen. Using the pull down menu, enter the correct scientist. Then click "Continue."
- 19.5.7.7. Click button two, "Goto Tissue Tracking DB."
- 19.5.7.8. Click button three, "Import mRNA Data."
- 19.5.7.9. Lastly, click the red button "Delete All Temporary Records."

19.6. APPENDIX

19.6.1. *1X TE Buffer Preparation*

Add the following reagents in the order:

- 25 ml of 20X TE
- 475 ml of DEPC-H₂O

19.6.2. *Preparation of Ribogreen Standard Aliquots*

- 19.6.2.1. Retrieve all standard tubes of the same lot # from the kits and combine them into one RNase-free tube. There should be five 200 µl aliquots in each kit.
- 19.6.2.2. Make three 1:20 dilutions by carefully pipetting 5 µl in each of three 1.5 ml tubes then carefully adding 95 µl 1X TE buffer.
- 19.6.2.3. For each dilution, measure the absorbance at 260 nm using a UV spectrophotometer.
- 19.6.2.4. Calculate the average of the concentration of the standard.
- 19.6.2.5. Aliquot the standard into 100 µl aliquots and label with:
 - Lot #
 - Aliquot #
 - Concentration (µg/ml)
- 19.6.2.6. Store the standard aliquots at -20°C.

19.6.3. Preparation of Fluorescent Dye Aliquots

19.6.3.1. Retrieve all the dye from all kits of the same lot#. Each kit should contain one 1 ml tube of fluorescent dye.

**Note: The dye is photosensitive. Always protect the tubes from light.*

19.6.3.2. Prepare enough RNase-free microcentrifuge tubes to aliquot 200 μ l per tube.

- Cover the tube with aluminum foil secured to the tube by glue and a label.
- Cover the cap with a label circle.
- Label each tube with:
 - Lot #
 - Aliquot #

19.6.3.3. Pipette 200 μ l of dye into each tube.

19.6.3.4. Store the dye aliquots in a dark box at -20°C.

19.6.4. Ribogreen Solution Preparation

Example for 20 samples:

- 20 samples in duplicate = 40 wells
- Plus 8 standards in duplicate = 16 wells
- Totaling 56 wells x 1.1 (10% extra) = 61.6 rounded down = 61 wells
- 100 μ l per well = 6100 μ l 1X TE buffer
- Dilute the concentrate 1:200 = $6100/200 = 30.5$ μ l reagent

Chapter 20

Release mRNA Samples

20.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for releasing mRNA samples.

20.2. RESPONSIBILITY

The Array Target Prep group leader is responsible for the implementation and for the correct execution of this protocol.

20.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to FileMaker Pro
- Access to “Phases 1-3 RNA#sDONOTneedtorepeat.xls” file which is located at P:\ChemExpress\ChemExpress Worksheet\Sample Release Worksheets
- Access to “In Vivo_ReRun List_Master.xlt” located at P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Run list\In Vivo_Tissue\Rerun List
- Access to “In Vitro_ReRun List_Master.xlt” located at P:\ChemExpress\ Module.Lab.RNA\mRNA FOLDER\Run list\In Vitro_Tissue\Rerun List

20.4. PROCEDURE

20.4.1. *Sample Release*

- 20.4.1.1. Open “Batch Load Sheet” emailed by mRNA group. Note down notebook page number, and total number of samples processed.
- 20.4.1.2. Go to “Data”, “Filter”, and “Auto filter”. Filter “mRNA QC” field for “Failed”. Write down the number of failed samples.
- 20.4.1.3. Now to filter for “Passed”, go to “Custom filter” and select “is less than” and type in 0.095 µg/µl. Note down the number of samples that are less than 0.095 µg/µl.
- 20.4.1.4. Go to “Custom filter” again and select “is greater or equal to” and type in 0.095 µg/µl. Note down the number of samples that have a concentration greater than or equal to 0.095 µg/µl.

****Note: The total number of samples from steps 20.4.1.2 to 20.4.1.4 should match the total number samples from step 20.4.1.1.***

20.4.1.5. Open FileMaker Pro. Go to mRNA extraction tab and type in the password given to you by your supervisor. Then go to Condensed_Target_Prep_Summary.

20.4.1.6. Go to View, Find and enter the following:

- >0.095 under “mRNA conc”
- Pass under “mRNA QC”
- Enter Notebook page number from step 20.4.1.1 under “mRNA NB_Page”

****Note: Make sure number of samples found on left hand corner of this page matches the number you wrote down in step 20.4.1.1***

20.4.1.7. To release samples to Target Prep group, enter day's date as “Y_yyyymmdd_initials” in “Process into cRNA” field. Do this for all the samples.

20.4.2. Releasing Re-Run Samples to mRNA

20.4.2.1. Open ReRun List_Master.xlt for the appropriate sample type, i.e. either In Vivo or In Vitro.

20.4.2.2. In File Maker Pro, go to View, Find and enter the following:

- < 0.095 under mRNA conc field
- Pass under mRNA QC field
- Enter Notebook page number from step 20.4.1.1 under NB_Page

****Note: This is to obtain the mRNA failures due to low concentrations.***

20.4.2.3. Export the following fields: Combi_RNA_Prep_Num, Tissue_ID, mRNA_Stock_Conc_mcg_mcl, mRNAQC

****Note: Tissue_ID will be substituted with Suffix_Cont_Exp for in vitro data.***

20.4.2.4. Open the exported file from desktop, copy the data and paste it in Cell A2 of the Run List worksheet of ReRun List_Master.xlt.

****Note: Headers will not match up with the four exported fields. Data such as concentration and QC will be used to determine method of resuspension and then deleted.***

- 20.4.2.5. In ReRun List_Master.xlt, check for "Method of Resuspension". If failed sample is Bone Marrow (BM) tissue, and it failed at mRNA for low concentration, ALWAYS recombine with old mRNA for method of resuspension.
- 20.4.2.6. In FileMaker Pro, go to View, Find, and enter the following:
- Failed under mRNA QC field
 - Enter Notebook page number from step 20.4.1.1 under NB_Page
- *Note: This is to obtain the mRNA failures due to degradation.***
- 20.4.2.7. Export the appropriate data for the template from FileMaker Pro onto your Desktop (see step 20.4.2.3) .
- 20.4.2.8. Open the exported file from desktop, copy the data and paste it in ReRun List_Master.xlt.
- 20.4.2.9. Sort compiled reruns by concentration. Samples that are ≥ 0.065 $\mu\text{g}/\mu\text{l}$ are resuspended in a "whole new" 7ul (put comment under Notes header), as are samples that failed QC. Samples that are ≥ 0.065 $\mu\text{g}/\mu\text{l}$ and pass QC are recombined with the original mRNA sample (highlight these samples gray).
- *Note: Upon precipitation of the rerun, the remaining 6ul of the original mRNA sample is used to resuspend the RNA pellet, the volume of the rerun is then brought up to 7 μl with DEPC-H₂O.***
- 20.4.2.10. Sort by mRNA Prep Number to obtain Run List order.
- 20.4.2.11. For In Vivo reruns, copy sorted mRNA Prep Number and Tissue ID into the Release List worksheet of ReRun List_Master file.
- 20.4.2.12. Assign a Release Date to the samples (ex. 01/27/2000). This worksheet will be used to upload into the Tissue Tracking Database.
- 20.4.2.13. Save file to P:\ChemExpress\Module.Lab.RNA\mRNA folder\Run List\ (In Vivo_Tissue or In Vitro_Cells)\Rerun List\Rerun2005\ folder for particular month) as "rerun list_mmddyy".
- 20.4.2.14. The following information is for In Vivo reruns only. Open Tissue Tracking Database from link on desktop.
- 20.4.2.15. Click gray "Open Database" button and when prompted for password enter "mRNA".
- 20.4.2.16. Click "mRNA Group" button.

- 20.4.2.17. Click "Import Used Lysate List" to import freezer, rack, box, and tube position information.
- 20.4.2.18. Click "Import List from Excel Sheet" button and import any files found in P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\used lysate boxes (one file at a time). Full files (those with 81 entries) are then put into P:\ChemExpress\Module.Lab.RNA\mRNA FOLDER\Full Used Boxes.
- Find the Excel file you want to upload, using the 'open file' box. Click "Open."
 - Select the "Used Lysates" worksheet of the file and click "ok."
 - Select "Field Names" in the pop-up box titled 'First Row Option' if it is not already selected for you, click "ok."
 - Verify the fields on the right and left sides of the pop-up box 'Import Field Mapping' match. Verify under "import action," "add new records" is selected. Click "import."
- 20.4.2.19. Click "Goto_Tissue_Tracking_DB" button.
- 20.4.2.20. Click "Import_Used_Lysates" button
- 20.4.2.21. Click the red button "Delete_All_Temporary_Records." New records will have now been created in the Tissue Tracking DB for each individual used lysate.
- 20.4.2.22. Go back to the Tissue Tracking DB Main Menu by clicking the dark gray "Main Menu" button at the top of the page. Click button "mRNA Group."
- 20.4.2.23. To import reruns and have the TTDB assign either a new tissue piece or a used lysate, click "import new mRNA list." You will then be asked whether your file contains three important pieces of information: a unique release date, mRNA numbers, and tissue ID numbers. Click either cancel or continue.
- 20.4.2.24. Click button "Import List from Excel Worksheet."
- Find the Excel file you want to upload (rerun list_mmddy) using the 'open file' box. Click "Open."
 - Select the "Release List" worksheet of the file and click "ok."
 - Select "Field Names" in the pop-up box titled 'First Row Option' if it is not already selected for you, click "ok."

- Verify the fields on the right and left sides of the pop-up box 'Import Field Mapping' match. Verify under "import action," "add new records" is selected. Click "import."
- 20.4.2.25. Click button two, "Goto Freezer Location DB."
 - 20.4.2.26. Skip button three by clicking the button in the top left hand corner titled "Main." Select "Open Tissue Tracking Main DB."
 - 20.4.2.27. Click button four "Open Tissue Tracking Main DB."
 - 20.4.2.28. Skip button five by clicking the "Import" button at the top left hand corner. Select "Import New List 2nd Step."
 - 20.4.2.29. Click button six "Find Tissue Sets for Import" and then button seven "Import New mRNA Run List."
 - 20.4.2.30. Click the red button, "Delete All Temporary Records."
 - 20.4.2.31. From the layout "List-Tissue & mRNA Data" verify the release list excel file is completely represented in the found records and that either a new tissue piece or a used lysate was selected for each rerun.
 - 20.4.2.32. From FileMaker toolbar, click on "Scripts" and select "Export Freezer Location List." Save this file to your desktop. This export contains all the mRNA numbers, Tissue ID's, and Freezer location information for your list of reruns on your release list.
 - 20.4.2.33. Open the exported file and paste into "rerun list_mmddyy" in the Pull List worksheet of the file. First sort by RNA Prep number. Copy Tissue ID and RNA number into "Run List" worksheet to accurately reflect which tissues/lysates will be run. Go back to the "Pull List" worksheet and sort by box, rack, and freezer, respectively, for ease in pulling tissues from freezer.

Chapter 21

Target Preparation

21.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for the running of automated target preparation.

21.2. REFERENCES

Motorola CodeLink Expression User Menu

21.3. RESPONSIBILITY

The Target Prep Array group leader is responsible for the implementation and for the correct execution of this protocol. The Target Prep Array group leader should also be consulted with any problems regarding the Bio Robots as well as responsible for any manuals and literature.

21.4. REQUIRED MATERIALS AND EQUIPMENT

There are two machines located in Iconix that are used for automated target preparation (QIAgen BioRobot 9604 - #2 and QIAgen BioRobot 3000 -#1). Reagents and Materials can be used for both machines

21.4.1. *Reagents for Synthesis of First Strand DNA*

- Nuclease-free H₂O (Ambion No. 9915G)
- 100 pmol/μl T7-(dT)₂₄ Oligonucleotide primer (GenSet Oligos Part No. Affymetrix Primers T7-(dT)₂₄Primer 50 O3)
- 5X First-strand buffer (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- 0.1M DTT (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- 10mM dNTP mix (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- SuperscriptTMII RNaseH- reverse transcriptase (200 U/μl) (Amersham Biosciences cDNA synthesis kit Part No. 320000)

21.4.2. *Reagents for Synthesis of Second Strand DNA*

- Nuclease-free H₂O (Ambion No. 9915G)

- 5X Second-strand buffer (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- 10mM dNTP mix (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- *E.coli* DNA polymerase I (10 U/μl) (Amersham Biosciences cDNA synthesis kit Part No. 320000)
- RNaseH (2 U/μl) (Amersham Biosciences cDNA synthesis kit Part No. 320000)

21.4.3. Reagents for Purification of Double Stranded cDNA

- Absolute Ethanol (Gold Shield ETH-200P) (Stored at -20°C)
- PM Buffer (QIAGEN No.19083)
- QIAquick® 96 BioRobot kits (4x96; QIAGEN No. 963141)- “96” represents plate format for 96 reactions per plate. Four plates come in the kit.

21.4.4. Reagents for In Vitro Transcription

- 10X Reaction buffer (Amersham Biosciences IVT kit Part No. 320001)
- 75 mM ATP (Amersham Biosciences IVT kit Part No. 320001)
- 75 mM GTP (Amersham Biosciences IVT kit Part No. 320001)
- 75 mM CTP (Amersham Biosciences IVT kit Part No. 320001)
- 75 mM UTP (Amersham Biosciences IVT kit Part No. 320001)
- 10 mM Biotin-11-CTP (Perkin Elmer; NEL No. 999)
- 10 mM Biotin-11-UTP (Perkin Elmer; NEL No. 999)
- T7 Enzyme Mix (Amersham Biosciences IVT kit Part No. 320001)
- DEPC-H₂O

21.4.5. Reagents for Electrophoresis of ds-cDNA for Quality Control

- Agarose (Invitrogen No. 15510-027)
- Ethidium bromide - 10 mg/ml (Invitrogen No. 15585011)
- 1 kb DNA ladder (BioNexus No. BN2050)
- 1X TAE running buffer (Invitrogen Gibco No. 24710-030)
- 3.3X gel loading buffer (diluted from 10X loading buffer)
- Becton Dickinson 96-well U-bottom Flexible plate (VWR No. 62406-220)

21.4.6. Reagents for Recovery of Biotin-Labeled cRNA

- RNeasy® 96 BioRobot kits (24x96; QIAgen No. 967143)- “96” represents plate format for 96 reactions per plate. 24 plates come in the kit.
- RLT Buffer (QIAgen No. 79216)
- 14.3 M β -Mercaptoethanol (β -ME) (Sigma No. M7522)
- Absolute Ethanol (Gold Shield ETH-200P)

21.4.7. Reagents for Agilent 2100 Bioanalyzer Protocol

- Becton Dickinson 96-well U-bottom Flexible plate (VWR No. 62406-220)
- DEPC-H₂O
- Syringe and chip priming station (Part of Agilent Part No. 5065-4476)
- RNA 6000 Nano Assay Kit (Agilent Part No. 5065-4476)
- RNA ladder (Ambion Part No. 7152)
- Agilent 2100 Bioanalyzer
- Agilent Vortexer
- Micropipettes and sterile, aerosol resistant tips
- Microcentrifuge
- Wet Ice
- Timer
- Heat block at 70°C

21.4.8. Other Equipment and Materials

- Thermo Savant SpeedVac (No. SPD111V-115)
- Thermo Savant Universal Vacuum Source (No. UVS400-115)
- Thermo Savant Rotor, Deep-well Microplates (No. RHDW2MP)
- Thermo Savant Tubing and Fittings Kit (No. TFK100)
- Thermo Savant Cryocool Heat Transfer Fluid (No. SCC1)
- Micropipettes and sterile, aerosol resistant tips
- Microcentrifuge tubes-2ml VWR brand, nuclease free (VWR No.20170-170)
- 15 ml conical tubes Nuclease-Free VWR brand (VWR No.21008-103)
- DNA Engine® Thermal Cycler (MJ Research No. PTC-200)

- Single Alpha® module heat block, flat surface (MJ Research No. ALS-1200)
- PCR sealing tape (MJ Research No. MSA-5001)
- Clear Adhesive Film (VWR No. BD-352142)
- Greiner round-bottom microtiter plates w/ lids (QIAgen No.19581)
- Table Top Centrifuge (Sigma 4-15C) or (QIAgen No. 81010)
- Centrifuge Plate Rotor-2x96 (QIAgen No.81031)
- QIAgen BioRobot 3000 (Configuration-S0017) including:
 - Varitherm unit, Shaker-4 microplate, dilutors (X4), 1000µl syringes (X4), tip adapter set (X4), high-speed peristaltic pump system, vacuum manifold system, tip disposal unit, 120 cm length.
- Tall MP-Slot (QIAgen No. 9010636)
- Short MP-Slot (QIAgen No. 9011686)
- QIAvac 96 Vacuum Manifold Top & Base (QIAgenNo.19504)
- 96-well channeling block (QIAgen No. 9232720)
- Microplate adapter (acrylic spacer block) (QIAgenNo.9232627)
- 24-well aluminum block (QIAgen No. 9232721)
- Reagent Bottle Holder, B1-B10 block (QIAgen No. 9011930)
- Tip rack slots (6) (QIAgen No. 2000473)
- Buffer bottle with straws, 500ml size (5) (QIAgen No. 9232631)
- QIAgen 300µl conductive filtered tips (QIAgen No. 9012599)
- QIAgen 1100µl conductive filtered tips (QIAgen No. 9012598)
- QIASoft operating system, version 4.0 or better
- UV spectrophotometer
- Vortexer
- Microcentrifuge, room temperature and 4°C
- Access to "cRNA 96Plate Templ 05.01.03.xlt" located at P:\ChemExpress\ChemExpress Worksheets\Codelink BioRobot Worksheets\96 well plate worksheets
- Access to "cRNA Reagent Volume Robot(11.02).xlt" located at P:\ChemExpress\ChemExpress Worksheets\Codelink BioRobot Worksheets\Reagent Worksheets

- Access to "cRNA PC Labels Plus" located at P:\ChemExpress\
ChemExpress Worksheets\Codelink BioRobot Worksheets
- Access to FileMaker Pro

21.5. PROCEDURE

21.5.1. *Sign up for mRNA Samples*

- 21.5.1.1. Open FileMaker Pro.
- 21.5.1.2. Click on "cRNA Preparation" tab and enter the password "cRNA".
- 21.5.1.3. For Motorola rat samples, click on "cRNA Preparation Sign-up" tab under Motorola Rat.
- 21.5.1.4. Enter your next available lab notebook page number under "cRNA Prep NB-Page" for the appropriate number of samples.
- 21.5.1.5. Click the "cRNA Prep Conditions" button at the top of the sign-up page. Create a new record by typing your NB page number and info in the appropriate fields. Close the page when finished.
- 21.5.1.6. To view samples you signed up for, go to View, Find Mode and enter your NB page number. Then sort by RNA number by clicking on the pink button (Sort RNA#) on top of the page.
- 21.5.1.7. To export data, go to "File" and "Export Records" and save onto your desktop.
- 21.5.1.8. In next window, from the current file select the following:
 - Combi_RNA_prep_Num
 - Exp_mRNA_Conc
- 21.5.1.9. Click on the pull-down menu on the top-left corner of the window. Choose VV_Tissue_RNA and select the following:
 - Tissue_ID
 - Ctl_Exp_Tox
- 21.5.1.10. For In Vitro samples, choose UNV_CGr_Exp on the pull-down menu and select the following:
 - Suffix_Cont_Exp

**Note: This field is the equivalence to In Vivo's Ctl_Exp_Tox.*
- 21.5.1.11. Then hit "Export" and a text file will appear on your desktop.

21.5.2. *cRNA Template*

- 21.5.2.1. Copy the text file from the desktop and paste onto "cRNA 96Plate Templ 05.01.03.xlt" file under "mRNA sheet" worksheet.
- 21.5.2.2. Go to "Robot Sheet" worksheet where it is automatically updated but check it regardless.
- 21.5.2.3. Enter notebook page in cell B1 of Robot Sheet.
- 21.5.2.4. Save the file as "cRNA_mm_dd_yy" to a location on P:\cRNA Module for open accessibility by other members of the group when required.
- 21.5.2.5. Print "Robot Sheet", paste it in your notebook, and take it with you into the Target Prep Lab.
- 21.5.2.6. Find the appropriate cRNA Robot Reagent Volume worksheet. This sheet details the master mixes required for target preparation. There is a printed set of master worksheets in the "cRNA reagent volume worksheets" folder located in the Biorobot lab. This set includes worksheets for most plate sizes.
- 21.5.2.7. If you cannot find a printed sheet for your number of reactions, open cRNA Reagent Volume Robot (11.02).xlt located at P:\ChemExpress\ChemExpress Worksheets\CodeLink Biorobot Worksheets\Reagent Worksheets and enter the appropriate number of reactions to be run in cell E3. Print the worksheets for use in the lab and put in the folder with the others when done.

21.5.3. *Robot Flushing*

General Note: The robot must be flushed before each use.

- 21.5.3.1. Flush the robot by selecting "Flush Robot" on the "Tools" menu.
- 21.5.3.2. If air bubbles remain in the line, repeat the flushing step, 100 ml at a time until the bubbles are gone.

**Note: It may take many flushes to remove bubbles from the line especially if the robot has not been run recently.*

**Note: It is important to flush the robot weekly even if it is not in use.*

21.5.4. *First Strand cDNA Synthesis*

General Note: This target preparation protocol has been optimized to process 24-96 samples in a single run on the QIAgen BioRobot 3000 (Robot #1) or QIAgen BioRobot 9604 (Robot #2).

- 21.5.4.1. Open "QIAsoft 4.exe" program (shortcut found on desktop).
- 21.5.4.2. Locate Protocol Window from drop down menu and open "First Strand cDNA Synthesis" found in Motorola Applications folder.
- 21.5.4.3. Press "Run".
- 21.5.4.4. If prompted to "Continue stopped...protocol at...(Press 'No' to start protocol from beginning). Click "No".
- 21.5.4.5. Select the number of wells that will be run by highlighting the corresponding wells.
**Note: Always highlight from left to right.*
- 21.5.4.6. Enter "plate ID" as "Iconix_mmddyy_initials,", e.g. Iconix_010105_AA.
- 21.5.4.7. Place 300 µl tips in specified locations (A3 and B3) & allow for the minimum number of tips needed.
- 21.5.4.8. Fill system liquid bottle and liquid reservoir with deionized water, fit cassettes to the Peri Pump, empty the waste container and vacuum trap.
**Note: If the waste container is full, pour contents down the sink.*
**Note: If the vacuum trap is full, exchange with the empty trap and write the date and safety info on the new trap. Place old, full trap in red hood for disposal pick up.*
**Note: If stock DI water is empty, fill it up at DI station.*
- 21.5.4.9. Place a 24-well aluminum block on a tall MP-slot in Location C2 of the robot deck.
- 21.5.4.10. Get out two tubes of standard (1 µl each) from the -80°C freezer. Add 5 µl DEPC-H₂O to each tube then add the entire 6 µl to the first and last well on your plate.
**Note: Do not add perform step 21.5.4.12 for these wells.*
- 21.5.4.11. Get out round-bottom 96-well plate and use the light box to verify sample order is maintained.
- 21.5.4.12. Add each mRNA sample for target preparation to a well of a 96-well microtiter plate according to your cRNA template worksheet. (Add standard to A1 then add samples into wells in sequential columnar order, *i.e.* B1...H1, A2...H2, etc.)
 - 3 µl mRNA

- 3 µl DEPC-H₂O.

**Note: Use the multi-channel pipette to add water to the plate.*

- 21.5.4.13. Dilute stock working solution of bacterial control mRNAs (already prepared in section 21.8.1.1) by adding 145 µl of DEPC-H₂O to 5 µl of stock solution. This is now the Bacterial Control Spike Working Solution.
- 21.5.4.14. According to the cRNA Reagent Volume Robot (11.02).xlt, on ice assemble the Primer-Bacterial Control Spike Working Solution-Water Mix in a 2 ml conical bottom VWR tube.
- 21.5.4.15. Bring round-bottom 96-well robot microtiter plate to BioRobot when prompted and place it in shaker front/right position.
- 21.5.4.16. Pen mark the plate on the top-left corner.
- 21.5.4.17. Place the First-Strand Synthesis Tube#1 (Primer-Bacterial Control Spike Working Solution-Water Mix) in A1 position of the 24-well block on Tall MP Slot and press Continue.
- 21.5.4.18. When prompted, seal the plate with PCR Sealing Tape (MJ Research No. MSA-5001), and bring plate to the MJ DNA Engine® thermal cycler.
- 21.5.4.19. Run the thermocycler as follows:
 - Select “Run” by pushing “Proceed”. Adjust setting using “Select” keys.
 - Select “<MOTORA>” or “<MOT*EX>” depending on thermocycler
 - Select “70-10 MIN”
 - Select “Yes” to use heated lid.
 - Prepare the first three reagents in step 21.5.4.20 while waiting for the thermocycler to finish, but do not add the Superscript™ II RNaseH- reverse transcriptase.
 - When program has reached “0.0° forever”, “Proceed” to end program. Press “Proceed” once more to return to main menu.
- 21.5.4.20. According to the cRNA Reagent Volume Robot (11.02).xlt, on ice assemble the First-Strand Synthesis Tube#2 (dNTP/SSII-RT Enzyme Mix) in a 2 ml conical bottom VWR tube.
 - 5X 1st strand buffer
 - 0.1M DTT

- 10 mM dNTP
 - Superscript™ II RNaseH- reverse transcriptase (200 U/μl) (**Add right before the run**)
 - Record all lot #s in "Robot Sheet".
- 21.5.4.21. Remove the microtiter plate from the thermocycler once it has reached 0°C and bring it to the BioRobot.
- 21.5.4.22. Remove the sealing tape and place the microtiter plate with denatured T7/RNA in the shaker's front/right position.
- 21.5.4.23. Place the First-Strand Synthesis Tube#2 (dNTP/SSII-RT Enzyme Mix) in B1 position of the 24-well block on the Tall MP Slot. Press Continue.
- 21.5.4.24. When the protocol has finished, seal the plate with PCR Sealing Tape and place into the MJ DNA Engine® thermal cycler. Open the peristaltic pump and finish the program.
- 21.5.4.25. Run the thermocycler as follows:
- Select "Run"
 - Select "<MOTORA>"
 - Select "37-1 HR"
- *Note: The robot message calls for 47°C-1 HR. Disregard this message.*
- Select "Yes" to use heated lid.
 - Prepare the reagents in step 21.5.5.1 while waiting for the thermocycler to finish, but do not add the RNaseH or the DNA polymerase.
 - When program has reached "0.0° forever", "Proceed" to end program. Press "Proceed" once more to return to main menu.
- 21.5.4.26. Remove the microtiter plate from the thermocycler once it has reached 0°C and place in -80°C freezer until ready to process or proceed to section 21.5.5 (Second Strand cDNA Synthesis).

21.5.5. Second Strand cDNA Synthesis

General Note: This target preparation protocol has been optimized to process 24-96 samples in a single run on the QIAgen BioRobot 3000.

- 21.5.5.1. In a VWR 15 ml conical tube, on ice assemble the Second Strand Master Mix using volumes outlined on the cRNA Reagent Volume Robot (11.02).xlt (also specified by prompts in protocol).

- 5X second strand buffer
- 10 mM dNTP mix
- *E.coli* DNA polymerase (10U/μl)
- RNase H (2 U/μl) (Add to mixture just prior to starting next step)
- Nuclease-free water

****Note: Invert tube gently to mix and visually inspect to verify the enzyme has been thoroughly mixed.***

- 21.5.5.2. Open "QIAsoft 4.exe" program and locate the Protocol Window. Open "2 Second Strand cDNA Synthesis" found in Motorola Applications folder.
- 21.5.5.3. Press "Run"
- 21.5.5.4. If prompted to "Continue stopped...protocol at...(Press 'No' to start protocol from beginning)". Click "No".
- 21.5.5.5. Select the number of wells that will be run by highlighting the corresponding wells with your mouse.
****Note: Always highlight from left to right.***
- 21.5.5.6. Enter a "plate ID" as "Iconix_mmddy_initials,", e.g. Iconix_010105_AA.
- 21.5.5.7. Place 1100 μl tips in specified locations (A3 and B3) & allow for the maximum number of tips needed.
- 21.5.5.8. Fill system liquid bottle and the liquid reservoir with deionized water, fit cassettes to the Peri Pump, empty the waste container and vacuum trap.
- 21.5.5.9. Place Reagent B1-B10 Block on Short MP-Slot in Location C1 and place the 15 ml tube of Second Strand Mix in B1 of that block (ensure cap is removed).
- 21.5.5.10. Remove the sealing tape and place the First Strand cDNA microtiter plate in the Shaker front/right position.
- 21.5.5.11. Press the "Continue" button.
- 21.5.5.12. After protocol has finished place a HARD lid on the microtiter plate (do NOT use sealing tape) and bring to MJ DNA Engine® thermal cycler.

21.5.5.13. Run the thermocycler as follows (Ensure the heated lid option is not used):

- Select “Run”
- Select “<MOTORA>”
- Select “16-2 HRs”
- Select “No” to not use heated lid.
- When program has reached “0.0° forever”, “Proceed” to end program. Press “Proceed” once more to return to main menu.

21.5.5.14. While waiting for the program to finish, prepare:

- Agarose gel according to Appendix A section 21.6.1
- Tube stickers using cRNA PC Labels PLUS.xls located at P:\ChemExpress\ChemExpress Worksheets\CodeLink Biorobot Worksheets
- Turn on thermal vacuum; press “preheat” to preheat SpeedVac to 45°C. Adjust Heat Time for heating to “C.CC” by going one point below “0.01.”

21.5.5.15. Remove the microtiter plate after reaching 0°C and place in –80°C until ready to process or proceed to cDNA purification (section 21.5.6).

21.5.6. QIAquick® cDNA Purification

General Note: This target preparation protocol has been optimized to process 24-96 samples in a single run on the QIAgen BioRobot 3000.

21.5.6.1. Turn on vacuum source then turn on SpeedVac at least 1 hour prior to use.

21.5.6.2. Prepare the PE buffer as suggested on the bottle by QIAgen with the addition of the appropriate volume of 100% ethanol and mark the tube to indicate you have added the ethanol.

21.5.6.3. Open “QIAsoft 4.exe” program (shortcut found on desktop).

21.5.6.4. Locate Protocol Window and open “Motorola QIAquick DNA Purification_old_121101” found in Custom Applications folder.

21.5.6.5. Press “Run”.

21.5.6.6. If prompted to “Continue stopped...protocol at...(Press ‘No’ to start protocol from beginning). Click “No”.

- 21.5.6.7. Select the number of wells that will be run by highlighting the corresponding wells.
**Note: Always highlight from left to right.*
- 21.5.6.8. Enter a "plate ID" as "Iconix_mmddyy_initials,", e.g. Iconix_010105_AA.
- 21.5.6.9. Place 300 µl tips in specified locations (A3, B3 & C3) & allow for the maximum number of tips needed.
- 21.5.6.10. Fill system liquid bottle with deionized water, fit cassettes to the Peristaltic Pump, and empty the waste container and vacuum trap.
- 21.5.6.11. Connect external lines to the appropriate buffer bottles (Line 1 = PM; Line 2 = PE).
- 21.5.6.12. Place Reagent B1-B10 Block on the Tall MP Slot and in position B9, place a 55 ml bottle of EB Buffer (ensure cap is removed and bottle is full).
- 21.5.6.13. Set up vacuum manifold by placing the Vacuum Manifold Base over the porthole, placing the 96-well aluminum channeling-block in the base, placing the Acrylic Vacuum Manifold Top over the base, and placing a cDNA QIAquick filter plate (purple) inside the top. Mark the top-left corner of the plate with a marker.
**Note: If running a partial microtiter plate, use a partially used filter, if available. Prepare the microtiter plate to reflect the columns available on the filter. Partially used filters should be labeled with number of wells left and which wells were used.*
**Note: Ensure all unused wells are taped with clear adhesive film. Otherwise, proper vacuum pressure will not be reached.*
- 21.5.6.14. Place the microtiter plate containing ds-cDNA in the Shaker front/right position. (Ensure that the lid is removed.)
- 21.5.6.15. Allow robot to run until user interaction is prompted.
- 21.5.6.16. Tap the QIAquick filter plate firmly onto a stack of paper towels until all residual ethanol is removed.
**Note: Only use each area of the paper towels ONCE to minimize cross contamination.*

- 21.5.6.17. Replace QIAquick filter plate into manifold with 96-well channeling block below it.
- 21.5.6.18. Allow robot to run until user interaction is prompted.
- 21.5.6.19. Tap the QIAquick filter plate firmly onto a stack of paper towels until all residual ethanol is removed.
***Note: Only use each area of the paper towels ONCE to minimize cross contamination.**
- 21.5.6.20. Remove the 96-well channeling block; rinse with tap water and dry vacuum manifold base and place clear acrylic spacer block inside the base.
- 21.5.6.21. Mark the top-left corner with a marker then place a round-bottom microtiter plate on top of the clear acrylic spacer block and replace top manifold with the dried QIAquick filter plate. Press "Continue".
- 21.5.6.22. When the process has finished remove the QIAquick filter plate from the top position and remove the round-bottom microtiter plate from the manifold base.
- 21.5.6.23. When prompted, press 'Yes' to run wash protocol.
- 21.5.6.24. Ensure flushing bottle is filled with deionized water and connect lines 1 and 2 back to flushing bottle and press Continue.
- 21.5.6.25. Run an Agarose gel (See Appendix A, page 162) for quality purposes. DO NOT proceed to IVT if a smear is not apparent. Rerun gel and stop protocol if no sample is apparent.
- 21.5.6.26. After Protocol is finished, bring the plate to the SpeedVac (pre-heated for 1 hour) and completely dry using heat ~45°C (approximately 2.5 hours depending on how many samples).
- 21.5.6.27. Press Manual Run and check back in 2.5 hrs.

21.5.7. *In vitro* Transcription Reaction

General Note: This target preparation protocol has been optimized to process 24-96 samples in a single run on the QIAgen BioRobot 3000.

- 21.5.7.1. As shown in the "cRNA Reagent Volume Robot(11.02)", assemble the *In Vitro* Transcription (IVT) Mix in a VWR 15 ml conical tube (volumes are also specified by prompts in protocol) and gently mix well by pipetting. Remove the following components from -

20°C freezer 20 minutes prior to use to allow thawing (exceptions being Biotins and T7 Polymerase).

- Nuclease-free water
- 10X Reaction Buffer
- 75 mM ATP, GTP, CTP, and UTP
- 10 mM Biotin-11 UTP (Store at 4°C)
- 10mM Biotin-11 CTP (Store at 4°C)
- T7 Polymerase

****Note: Assembling the reaction components in the suggested order ensures stabilization of the nucleotides before the addition of the enzyme mix and double-stranded cDNA.***

DO NOT ASSEMBLE REACTION ON ICE. The Spermidine in the reaction buffer can precipitate causing co-precipitation of template DNA.

- 21.5.7.2. Open "QIAsoft 4.exe" program (shortcut found on desktop)
- 21.5.7.3. Locate Protocol Window from drop down menu and open "4 in vitro Transcription_OLD_121101" found in Custom Applications folder.
- 21.5.7.4. Press "Run"
- 21.5.7.5. If prompted to "Continue stopped...protocol at...(Press 'No' to start protocol from beginning)". Click "No".
- 21.5.7.6. Select the number of wells that will be run by highlighting the corresponding wells with you mouse.
****Note: Always highlight from left to right.***
- 21.5.7.7. Enter "plate ID" as ""Iconix_mmdyyy_ initials," e.g. Iconix_01012005_AA.
- 21.5.7.8. Place 1100 µl tips in specified locations (A3 and B3) & allow for the minimum number of tips needed
- 21.5.7.9. Fill system liquid bottle with deionized water, fit cassettes to the Peristaltic Pump, empty the waste container and vacuum trap
- 21.5.7.10. Place Reagent B1-B10 Block on Short MP-Slot in Location C1 and place the 15 ml tube of the IVT Mix in B1 of that block (ensure cap is removed).
- 21.5.7.11. Place the dried ds-cDNA in Shaker front/right Position.

- 21.5.7.12. After the protocol has finished place PCR Sealing Tape on microtiter plate and bring the plate to the MJ DNA Engine® thermal cycler.
- 21.5.7.13. Run the thermocycler as follows:
- Select “Run”
 - Select “<MOTORA>”
 - Select “37-IVT 14 hours”
 - Select “Yes” to use heated lid.
 - When program has reached “0.0° forever”, “Proceed” to end program. Press “Proceed” once more to return to main menu.

****Note: The IVT reaction MUST be taken out the next day. It CANNOT stay in the thermocycler for extended periods of time (i.e. over the weekend).***

- 21.5.7.14. Remove the microtiter plate from the thermocycler after it has reached 0°C, and either:
- Proceed to cRNA Purification (step 21.5.8) or
 - Tape a new plate lid on the plate and secure it on the 4 sides with tape. Write your name, date and IVT on the plate and place in -80°C freezer until ready to process.

21.5.8. RNeasy® cRNA Purification

General Note: This target preparation protocol has been optimized to process 24-96 samples in a single run on the QIAgen BioRobot 3000.

- 21.5.8.1. Prepare the following buffers: RLT+ β -ME, RPE+100%Ethanol, 70% Ethanol (See Appendix C, page 163).
- *Note: β -ME must be opened in a fume hood only.***
- 21.5.8.2. Open “QIASoft 4.exe” program (shortcut found on desktop).
- 21.5.8.3. Locate Protocol Window from drop down menu and open “Motorola RNeasy RNA Purification_OLD_121101” found in Custom Applications folder.
- 21.5.8.4. Press “Run”.
- 21.5.8.5. If prompted to “Continue stopped...protocol at...(Press ‘No’ to start protocol from beginning)”. Click “No”.
- 21.5.8.6. Select the number of wells that will be run by highlighting the corresponding wells with your mouse.

****Note: Always highlight from left to right.***

- 21.5.8.7. Enter "plate ID" as ""Iconix_mmddyy_initials,"", e.g. Iconix_010105_AA.
- 21.5.8.8. Place the aluminum 30 ml bottle holder in the VariTherm Position and in it place a 30 ml bottle of RNase-free water (ensure cap is removed and bottle is full).
- 21.5.8.9. Place 300 and 1100 µl tips in specified locations within Robotic protocol (A3, B3, C3, A4) & allow for the minimum number of tips needed.
- 21.5.8.10. Fill system liquid bottle with deionized water, fit cassettes to the Peri Pump, empty the waste container and vacuum trap.
- 21.5.8.11. Connect external lines to the appropriate buffer bottles (Line 1 = 70% ethanol; Line 2 = RPE; Line 3 = RLT).
- 21.5.8.12. Place the microtiter plate containing IVT reactions in the Shaker front/right position.

Note: Remove sealing tape prior to positioning the plate in the shaker).

- 21.5.8.13. Set up vacuum manifold by placing the Vacuum Manifold Base over the porthole, placing the 96-well aluminum channeling-block in the base, placing the Acrylic Vacuum Manifold Top over the base, and placing an RNeasy 96 filter plate (pink) inside the top. Mark the top-left corner of the plate.

****Note: If running a partial microtiter plate, use a partially used filter, if available. Prepare the microtiter plate to reflect the columns available on the filter. Partially used filters should be labeled with number of wells left and which wells were used.***

****Note: Ensure all unused wells are taped with clear adhesive film. Otherwise, proper vacuum pressure will not be reached.***

- 21.5.8.14. Allow robot to run until user interaction is prompted.
- 21.5.8.15. Tap the RNeasy 96 filter plate firmly into a stack of paper towels until all residual ethanol is removed. Replace RNeasy 96 filter plate into manifold with 96-well channeling block below it.
****Note: Only use each area of the paper towels ONCE to minimize cross contamination.***

- 21.5.8.16. Allow robot to run until user interaction is prompted.

- 21.5.8.17. Repeat steps 21.5.8.15 and 21.5.8.16.
- 21.5.8.18. Tap the RNeasy 96 filter plate firmly into a stack of paper towels.
**Note: Only use each area of the paper towels ONCE to minimize cross contamination.*
- 21.5.8.19. Remove the 96-well channeling block; rinse with tap water and dry vacuum manifold base and place clear acrylic spacer block inside base.
- 21.5.8.20. Place a round-bottom microtiter plate on top of the spacer block into vacuum manifold base position. Replace RNeasy 96 filter plate into manifold so that the round-bottom microtiter plate is aligned below it.
- 21.5.8.21. When the process is done, remove the RNeasy 96 filter plate from the top position and remove the round-bottom microtiter plate from the manifold base.
- 21.5.8.22. When prompted, press 'Yes' to run wash protocol.
- 21.5.8.23. Ensure flushing bottle is filled with deionized water and connect lines 1, 2 and 3 back to the flushing bottle and press Continue.
- 21.5.8.24. Pick 3 columns to run on Agilent Bioanalyzer for cRNA quality control (See Appendix B, page 163).

21.6. APPENDIX A

21.6.1. *Electrophoresis of ds-cDNA*

- 21.6.1.1. Preparation of Agarose Gel
- 21.6.1.2. Prepare a 1% agarose 1X TAE gel (*e.g.* add 1.0 g agarose to 100 ml 1X TAE). Bring to boil in a microwave oven until all agarose is melted (~1.50 minutes).
- 21.6.1.3. Allow agarose solution to cool to approximately 55°C and add 3.5- μ l ethidium bromide (10mg/ml).
- 21.6.1.4. Pour agarose into the gel tray and insert 30-well comb into gel. Allow the gel to solidify for 30 minutes.

21.6.2. *Preparation of ds-cDNA Samples and DNA Ladder*

Place 7 μ l ds-cDNA from columns 1, 7, 12 (if possible) and two Clontech standards in a 96 well plate and add 3 μ l 3.3X loading buffer.

21.6.3. *Electrophoresis and Analysis of ds-cDNA Samples*

- 21.6.3.1. Submerge agarose gel in 1X TAE. Remove comb and load samples.
- 21.6.3.2. Add 10 μ l/sample to respective gel wells starting with the fourth well from the left. Add 5 μ l of DNA standard to the left of the first sample well and to the right of the last sample well.
- 21.6.3.3. Run gel at 135 volts and 400 mA for 35 minutes.
- 21.6.3.4. Remove and photograph the gel under UV light. Save the gel image in your folder (create one if it does not already exist) located at P:\ChemExpress\Gel Images. There should be a smear of cDNA corresponding to a length of 500-2000 base pairs. If there is no smear, do not continue with the sample.
- 21.6.3.5. Discard gel in Chemical Hazard Waste.

21.7. APPENDIX B

21.7.1. *Bioanalyzer 2100 Analysis of cRNA*

- 21.7.1.1. Add 4 μ l DEPC-H₂O to 3 columns of a 96-well plate using a multi-channel pipettor.
- 21.7.1.2. Add 1 μ l of the chosen samples (22 samples from columns 1, 7, 12 and 2 controls) by transferring from the robot plate to the Agilent plate using a multi-channel pipettor. These should be the same columns chosen at step 21.6.2.
- 21.7.1.3. Run the samples according to sections 18.6.3-18.6.9 (page 128).

21.8. APPENDIX C

21.8.1. *Stock Solution Preparation*

- 21.8.1.1. Clontech mRNA Standard
 - Pool Clontech tubes (5 μ g/ μ l in 5 μ l)
 - Add equal volume of DEPC-H₂O
 - Aliquot 1 μ l into empty sample tube and label as “Clontech Standard” and date.
 - Record lot # and prep date in Notebook
 - Store at -80°C
- 21.8.1.2. Working Solution of Bacterial Control for first strand cDNA synthesis

**Note: The In-house QIAgen BioRobot Consultant is responsible for preparation of this solution.*

Table 21.8-A: 1st Strand cDNA Synthesis Bacterial Control Spikes Example

Type	Final Amount	Lot#
FixA	1.5 pg	070600
Yjek	5 pg	070P05A
EntF	15 pg	070P011
FixB	50 pg	070P03A
HisB	150 pg	111B06A
LeuB	500 pg	111B06A
Gnd	1500 pg	070600

- Thaw each control mRNA on ice (Table 21.8-A).
- Prepare solution to working solution to above recipe and aliquot into individual microcentrifuge tubes @ 150 µl/tube.

Note: The lot# changes according to the lot used.

21.8.1.3. Buffer RLT+ β-ME

- Add 10 µl β-Mercaptoethanol per 1 ml of buffer RLT.

**Note: This step must be performed in a fume hood. The solution is stable for one month at room temperature.*

21.8.1.4. Buffer RPE

- Add 4 volumes of absolute ethanol (100%) as indicated on the bottle to obtain a working solution. With an alcohol proof marker, note on the bottle "EtOH added", the date it was added, and your initial.

**Note: Buffer RPE is supplied as a 5X concentrate.*

21.8.1.5. TAE Buffer Working Solution

- Add 163 µl of 50X TAE buffer to 8L MQ water.

21.9. CONTACT INFORMATION

QIAgen BioRobot

Ed Almodovar, Field Engineer

800-362-7737 X-22225

*Only the Target Prep team lead may contact

QIAgen

Michelle Reid, Technical Service

800-362-7737 (1-800-DNA PREP) X-23516

Press 1 for "Instruments"

*Only the Target Prep team lead may contact

Emergency Contact

In-house QIAgen BioRobot Consultant

Chapter 22

cRNA Quality Control and Precipitation

22.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for concentration of cRNA sample by precipitation.

22.2. REFERENCES

CodeLink Expression User Menu

22.3. RESPONSIBILITY

The Target Prep Array group leader is responsible for the implementation and for the correct execution of this protocol.

22.4. SAFETY PRECAUTIONS

Always wear your lab coat and protective eyewear, as well as gloves when working in the lab.

22.5. REQUIRED MATERIALS AND EQUIPMENT

- 96-well UV Plate (Costar 3635)
- Absolute Ethanol (Gold Shield ETH-200P) (stored at -20°C)
- 70 % cold Ethanol (stored at -20°C)
- 5M Ammonium Acetate (Ambion 9070G)
- 96-well UV spectrophotometer (Bio-Tek μ Quant MQX200)
- Microcentrifuge (refrigerated)
- DEPC or RNase Free water
- Micropipettes and sterile, aerosol resistant tips
- Multi-channel pipette
- Lab Coat
- Safety glasses
- Access to "cRNA 96PlateTempl 05.01.03.xlt" located at
P:\ChemExpress\ChemExpress Worksheets\Codelink BioRobot
Worksheets\96 well plate worksheets.

22.6. PROCEDURE

22.6.1. Quantification of cRNA

22.6.1.1. Obtain the 96-well plate from the "Target Preparation" protocol (section 21.5.8.21, page162).

22.6.1.2. Make a 1:30 dilution of your cRNA samples to a final volume of 150 μ l into a Costar 96-Well Flat Bottom Plate. To do this, use a multi-channel pipette to transfer 5 μ l of sample keeping the same sample location on both plates, and then add 145 μ l DEPC-H₂O. Mix well by pipetting up and down five times. This will be the plate for reading the A₂₆₀ and A₂₈₀. Label the plate ABS.

****Note: It is important to use the UV transparent 96-well plate to obtain accurate readings. Regular plates will absorb UV light increasing readings.***

*****Note: Do not place plate on ice.***

22.6.1.3. Transfer the remaining volume of the cRNA samples from the QIAGEN 96-Well Microplate RB onto a Costar 96-Well Flat Bottom UV Plate using a multi-channel pipette keeping the same sample location on both plates. This plate will be used to determine the sample volumes. Label the plate VOLUME.

****Note: It is important to use the UV transparent 96-well plate to obtain accurate readings. Regular plates will absorb UV light increasing readings.***

*****Note: Do not place plate on ice***

22.6.1.4. Open "cRNA Plate Spec Abs" located on the desktop of the μ Quant computer. Use the 96-Well UV-Spectrophotometer to obtain A₂₆₀ and A₂₈₀ of the samples on the ABS plate.

22.6.1.5. Press the "Read" button (third from the left).

22.6.1.6. Enter your name and the date in the "Name" and "Date" fields, respectively.

22.6.1.7. Press the "Start Reading" button.

22.6.1.8. A Bio Windows Application window will open with the message "Insert blank plate and press OK." Place a blank plate containing 200 μ l of DEPC-H₂O in each well (with no cover) into the UV-Spectrophotometer and press "OK".

- 22.6.1.9. Once the blank plate has been read, a Bio Windows Application window will open prompting you to insert the sample plate. Remove the blank plate (replacing the MJ Research PCR Sealing Tape cover on top of it) and insert the sample plate ABS into the UV-Spectrophotometer. Press "OK".
- 22.6.1.10. Once the ABS plate has been read, a "save" window will open. Save the file to the default save location, P:\ChemExpress\ Module.Lab.Motorola\cRNA Plate Spec Folder, in the KC4 Data format: mm.dd.yy initials assay# using the following assay abbreviation:
e.g. 06.30.03.JB.Vol
.Spec
.Ppt
.Respec
- 22.6.1.11. An Excel file containing the data will open once the KC4 Data file has been saved. Go to "File" and then choose "Save Copy As..." in order to save this Excel file. Save the file in the same format to the same default location as step 22.6.1.10.
- 22.6.1.12. Remove the ABS plate from the UV-Spectrophotometer, cover, and save in case a second A_{260} and A_{280} reading is necessary.
- 22.6.1.13. Close the cRNA Plate Spec Abs KC4 program.
- 22.6.1.14. Open "cRNA Plate Spec Volume" located on the desktop of the μ Quant computer and use the 96-Well UV Spectrophotometer to obtain the volume (μ l) of the samples on the VOLUME plate.
- 22.6.1.15. Repeat steps 22.6.1.5-22.6.1.12.
- 22.6.1.16. Enter the Spectrophotometer data from both the cRNA Plate Spec ABS and cRNA Plate Spec VOLUME Excel files into the cRNA 96PlateTempl 05.01.03.xlt Excel file saved in step 21.5.2.4.
- 22.6.1.17. The sample A_{260} and A_{280} data is found in the "A₂₆₀ Corr" and "A₂₈₀ Corr" columns, respectively, in the Table Form worksheet of the cRNA Plate Spec ABS Excel file. Copy and paste these data into the appropriate columns (A_{260} and A_{280}) in the Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file.
- 22.6.1.18. The sample volume data is found in the "Volume (μ l)" column in the Table Form worksheet of the cRNA Plate Spec VOLUME

Excel file. Copy and paste this data into the "Volume of recovered cRNA" column in the Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file.

22.6.1.19. The A_{260}/A_{280} ratio, cRNA Concentration ($\mu\text{g}/\mu\text{l}$), and cRNA Yield (μg) will be calculated automatically by the worksheet. In addition, samples which require precipitation and those which Fail will be flagged accordingly:

- If the A_{260} nm is > 1.0 , add an additional 150 μl DEPC-H₂O to the relevant sample well on the ABS plate prepared at step 22.6.1.2 and re-measure the UV absorbance. Be sure to adjust the result and dilution ("Dil. of cRNA (UV Spec)" to 60) in the Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file.
- If cRNA concentration $\geq 0.521 \mu\text{g}/\mu\text{l}$ then a sample passes and is released to the Hyb group.
 - Transfer cRNA sample from Costar 96-Well Flat Bottom UV Plate VOLUME to their respective, labeled 1.5 ml microcentrifuge tubes.
 - Put away in cRNA archive boxes stored at -80°C .
 - Go to step 22.6.3.
- If cRNA yield is $\geq 15 \mu\text{g}$ and cRNA concentration is $< 0.521 \mu\text{g}/\mu\text{l}$, then the sample must be precipitated to concentrate it.
 - On the cRNA worksheet, filter for samples that need precipitating and recolor these rows orange.
 - Change the dilution on the sheet from 30 to 200 and change the volumes to 20 μl for these samples.
 - Go to step 22.6.2.
- If a cRNA yield is $< 15 \mu\text{g}$ the sample will automatically be flagged as failure. Do not continue processing the failed sample.

22.6.1.20. Save the file.

22.6.2. *Precipitation/Archive*

22.6.2.1. Label the top of each 1.5 ml eppendorf tube with its respective cRNA number and attach tube label.

**Note: Each tube label has three pieces of information. Sample type (mRNA or cRNA), RNA number, and notebook page number.*

- 22.6.2.2. Transfer cRNA sample from Costar 96-Well Flat Bottom UV Plate VOLUME to their respective, labeled 1.5 ml microcentrifuge tubes.
- 22.6.2.3. Add 60 μ l of 5M Ammonium Acetate to each tube.
- 22.6.2.4. Mix well by pipetting up and down eight times.
- 22.6.2.5. Add 500 μ l of ice-cold absolute ethanol to the tube containing the sample and vortex the mixture for 5 seconds at maximum speed.
- 22.6.2.6. Incubate the sample at least 30 minutes at -20°C .
- 22.6.2.7. Centrifuge at maximum speed for 20 minutes at 4°C . Carefully remove and discard the supernatant into alcohol waste container.
- 22.6.2.8. To wash the pellet, add 500 μ l of ice-cold 70% ethanol to the tube and invert the tube back and forth five times.
- 22.6.2.9. Centrifuge at maximum speed for 5 minutes at 4°C . Carefully remove and discard the supernatant into the alcohol waste container then air-dry the pellet for 20 minutes, or until completely dry.
- 22.6.2.10. Resuspend cRNA in 20 μ l nuclease-free water at room temperature for at least 10 minutes. Make sure pellet is completely resuspended before quantification.
- 22.6.2.11. Transfer 1 μ l of each sample using a Pipet-Lite 2 μ l pipettor to make a 1:200 dilution of your cRNA sample to a final volume of 200 μ l into a Costar 96-Well Flat Bottom UV Plate.
- 22.6.2.12. Quantify the cRNA samples using the μ Quant 96-well UV Spectrophotometer. Open the program template "cRNA Plate Spec Abs" and obtain A_{260} and A_{280} of the samples on the ABS plate.
- 22.6.2.13. Repeat steps 22.6.1.5-22.6.1.12.
- 22.6.2.14. Enter the A_{260} and A_{280} data of the precipitated sample(s) to their respective fields in the Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file saved in step 21.5.2.4.
- 22.6.2.15. The A_{260}/A_{280} ratio, cRNA Concentration ($\mu\text{g}/\mu\text{l}$), and cRNA Yield (μg) will be calculated automatically by the worksheet and samples which Fail will be flagged:
 - If the A_{260} nm is > 1.0 , add an additional 100 μ l DEPC-H₂O to the relevant sample well on the ABS plate prepared at step

22.6.2.11 and re-measure the UV absorbance. Be sure to adjust the result and dilution ("Dil of cRNA (UV Spec) value to 300) in the Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file.

- If cRNA concentration $\geq 0.521 \mu\text{g}/\mu\text{l}$ then a sample passes and is released to the Hyb group. Go to step 22.6.3.

22.6.2.16. Save the file and send it to the Team Leader.

22.6.2.17. Upload completed Final worksheet of the cRNA 96PlateTempl 05.01.03.xlt Excel file into FileMaker Pro.

22.6.3. *Uploading Completed Worksheet*

- 22.6.3.1. Open the "cRNA 96PlateTempl 05.01.03.xlt" file you used to prepare your 96-well plate and saved on your desktop at step 21.5.2.4. Click on the "Final" worksheet of the file. This file was initially found in P:\ChemExpress\ChemExpress Worksheets\CodeLink BioRobot Worksheets\96 well plate worksheets.
- 22.6.3.2. Open the Excel file and confirm all the data in the Final worksheet have been entered correctly. Once this is done, close the Excel file.
- 22.6.3.3. Open FileMaker Pro file UNV_Main.FP5 from the link on your desktop. If you do not have the link on your desktop copy it to your desktop from S:\Share\DB Access Files\FileMaker.
- 22.6.3.4. Click on "cRNA Preparation" tab and enter the password "cRNA".
- 22.6.3.5. For each sample, click on the appropriate species "Review/ Batchload cRNA Data" and then "Batchload Rat cRNA Data".
- 22.6.3.6. Enter your lab notebook page number under "cRNA prep NB - Page" and click "enter" or "continue". Click "enter" or "continue" once more to confirm sample numbers.
- 22.6.3.7. This will open UNV_cRNA_Exp_Import. Click on "Import cRNA data".
- 22.6.3.8. Find and open your Excel file.
- 22.6.3.9. The "Specify Excel Data" window will come up. Select Final worksheet and press "OK".

- 22.6.3.10. The "First Row Option" window will come up. Press "OK".
- 22.6.3.11. Hit "Final" and then "OK".
- 22.6.3.12. Match information in the "Data In" field with information in "Fields In". Click on the "Import" button.
- 22.6.3.13. Click cRNA_Exp.FP3 tab (middle, gray button at top of page).
- 22.6.3.14. Go to Scripts and select "Fill cRNA data". Click "OK" to confirm importing the data according to RNA number.
- 22.6.3.15. Click "Continue" once the data has been uploaded. This will bring you back to the Import sheet. In the import sheet, click "Delete All". Click "Delete" to confirm the deletion. The data has now been uploaded into FMP.

22.7. APPENDIX

22.7.1. 70% Ethanol

- 22.7.1.1. Add 35 ml of absolute (100%) ethanol to 15 ml of nuclease-free H₂O and mix well by inversion. Store at -20°C.

Chapter 23

Release cRNA Samples

23.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for releasing cRNA samples.

23.2. RESPONSIBILITY

The Array Target Prep group leader is responsible for the implementation and for the correct execution of this protocol.

23.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to FileMaker Pro
- Access to “mRNA Re-Runs Template2003.xlt” located at P:\ChemExpress\ChemExpress Worksheets\Sample Release Worksheets.

23.4. PROCEDURE

23.4.1. *Releasing Samples to Hybridize*

- 23.4.1.1. Open “cRNA template” completed in section 22.6.3 (page 171). Go to “Final” sheet. Record the notebook page, total number of samples processed, total number of passing, and total number of failing.
- 23.4.1.2. To filter for “Failed” samples, go to “Custom filter” and select “is less than” and type in 0.53 µg/µl. Note down the number of samples that are less than 0.53 µg/µl.
- 23.4.1.3. To filter for “Passed” samples, go to “Custom filter” again and select “is greater or equal to” and type in 0.53 µg/µl. Note down the number of samples that are greater than or equal to 0.53 µg/µl.
- 23.4.1.4. Open FileMaker Pro. Go to mRNA Extraction tab and type in the password given to you by your supervisor. Go to Condensed_Target _ Prep_ Summary.
- 23.4.1.5. Go to View, Find and enter the following:
 - > 0.53 under “cRNA conc.”
 - Enter Notebook page number from step 23.4.1.1 under “cRNA NB_Page”

- 23.4.1.6. To release samples to Hybridization group, enter day's date and initials as "Y-yyyymmdd_XX" under "Hybridize", e.g. Y-20050131_MJ.
- 23.4.1.7. If a sample was released by mistake or changes in sample release occur after the fact, NB Page 000-000 can be used to "remove the record" from view (please check with Target Prep Team Leader for the corrected version). Otherwise, the NB page number can be deleted using a special password.
- *Note: If the cRNA number was on "DONOTneedtorepeat" list, do not enter the date under "Hybridize".*

23.4.2. Releasing cRNA Failures as cRNA or mRNA reruns

- 23.4.2.1. Open FileMaker Pro. Go to mRNA extraction tab and type in the password given to you by your manager. Go to Condensed_Target_Prep_Summary.
- 23.4.2.2. Go to View, Find and enter the following to view all the cRNA failures:
- < 0.53 under "cRNA conc."
 - Enter Notebook page number from step 23.4.1.1 under "cRNA NB_Page"
- 23.4.2.3. If a sample has 3 µl mRNA remaining (as indicated under "cRNA comments"), the sample can be released back to the target prep group. To release cRNA reruns, enter day's date and initials as "Y-yyyymmdd_XX" under "Process into cRNA".
- 23.4.2.4. If a sample has no more mRNA sample remaining (as indicated under "cRNA comments"), the sample has to be released back to the mRNA isolation group. To release mRNA reruns, fill out the "mRNA Re-Runs Template2003.xlt", send the data to the mRNA group leader and save a copy for yourself in a folder.

23.4.3. Filling out the "mRNA Re-Runs Template2003"

- 23.4.3.1. Open "mRNA Re-Runs Template2003.xlt" located at P:\ChemExpress\ChemExpress Worksheets\Sample Release Worksheets.
- 23.4.3.2. Open FileMaker Pro. Go to mRNA extraction tab and type in the password given to you by your manager. Go to Condensed_Target_Prep_Summary.

- 23.4.3.3. Go to View, Find and enter the following to view all the cRNA failures:
 - < 0.53 under “cRNA conc.”
 - Enter Notebook page number from step 23.4.1.1 under “cRNA NB_Page”. Omit any records that have been released back to the cRNA or hyb groups already.
- 23.4.3.4. Go to File, Export Records. Save file onto desktop. Export the appropriate fields as indicated in the “mRNA Re-Runs Template”, *i.e.* mRNA#, Tissue ID#, mRNA NB Page, mRNA Conc, and mRNA QC.
- 23.4.3.5. Open the Exported file from your desktop. Copy the data and paste into the “mRNA Re-Runs Template2003.xlt” in the “From DB” worksheet. The information provided on the “From DB” worksheet is automatically linked to the “RNA isolation” worksheet.
- 23.4.3.6. Under “Comments”, indicate the reason for failure, *i.e.* failed in mRNA, failed in cRNA, or failed in hyb.
- 23.4.3.7. Complete the worksheet, save the file into a personal folder and send a copy to the mRNA group leader to release samples to the mRNA Group.

Chapter 24

cRNA Fragmentation

24.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for fragmenting cRNA samples.

24.2. REFERENCES

CodeLink Expression User Menu

24.3. RESPONSIBILITY

The Array Hyb group leader is responsible for the implementation and for the correct execution of this protocol.

24.4. SPECIAL NOTES

- Always wear your lab coat and protective eyewear, as well as gloves when working in the lab.
- Avoid physical contact with the probe-containing area of the bioarray to prevent damage to the microarray.
- Minimize exposure of the slides to ambient light.
- Place the Streptavidin-Alexa conjugate in a dark place to avoid photo bleaching.
- Handle slides by the label throughout the procedure.
- No cell phones are allowed in the lab area. Exceptions will apply to emergency cases.
- Any immediate work should NOT be delayed or jeopardized due to impromptu conversations.
- A single black box containing slides should be handled at any given time.
- Each person is to use the checklist worksheet during processing in the lab at any time. If one person finishes up an experiment started by someone else, the worksheet will need to be handed over to the next person. Both scientists share the responsibility that this happens.
- Have all required worksheets printed and ready to be taken into the lab and use accordingly when required.

24.5. REQUIRED MATERIALS AND EQUIPMENT

- 5x Fragmentation Buffer (Amersham Biosciences IVT kit Part No. 320001)
- Centrifuge Plate Rotor-2x96 (QIAgen No.81031)
- Nuclease-free H₂O (Ambion No. 9915G)
- Pipette tips, sterile, RNase-free, and aerosol-resistant
- Microcentrifuge tubes, sterile, RNase-free, 1.7 ml
- Micropipettes
- Powder-free gloves
- Microcentrifuge
- Heat block at 94°C
- Wet and dry Ice
- Lab Coat
- Safety glasses
- Access to “HybModWorksheet.doc” located at P:\ChemExpress\ChemExpress worksheets\CodeLink Hyb Worksheets
- Access to FileMaker Pro
- Access to “RU1 Hyb Template 0303.xlt” located at P:\ChemExpress\ChemExpress worksheets\CodeLink Hyb Worksheets
 - “FRAG” worksheet to fragment samples
 - “REAGENTS” worksheet

24.6. PROCEDURE

24.6.1. *Obtaining Samples for Fragmentation*

- 24.6.1.1. Open FileMaker Pro/UNV_Main.FP5
- 24.6.1.2. Click on “Hybridization” tab and enter the password.
- 24.6.1.3. For rat samples, click on “Rat Hybridization Sign-up” tab. When dealing with human samples, click on the “Human Hybridization Sign-up” tab.
- 24.6.1.4. Click on the “Records” button at the top of the toolbar and then select “Sort”.
- 24.6.1.5. Click on the “Clear All” button then select “Combi_cRNA_Prep_Num” from the left panel and click on the “Move” button.

- 24.6.1.6. Sort the samples by clicking on the "Sort" button, then check the samples are in ascending cRNA preparation number.
- 24.6.1.7. Select the "Hyb Conditions" tab on the top of the page and enter your lab notebook page number in "Hyb-NB-Page" field for the appropriate number of samples (*i.e.* when working with 20 samples the notebook page would appear 20 times).
- 24.6.1.8. Fill in the following fields (some are filled via pull-down menu options and others via manual input):
 - Scientist
 - Hybridization Date (date arrays started hybridization)
 - Amount of cRNA Labeled (*e.g.* "10 µg" for RU1 arrays)
 - Hyb Time (hr) (*e.g.* "20 " for RU1 arrays)
 - Post Hyb Wash Protocol ID (*e.g.* "0" for RU1 arrays)
 - Hyb Temp (C) (*e.g.* "37" for RU1 arrays)
 - Hyb Protocol (*e.g.* "S-Alexa" for RU1 arrays)
 - RNA Type (*e.g.* "sub" for RU1 arrays)
 - Slide_Lot (*e.g.* "18" for current RU1 arrays)
 - Array-Chip Type (*e.g.* "BioChip" for RU1 arrays)
- 24.6.1.9. Select the "Window" pull-down menu and choose "UNV_Hyb_Exp.FP5."
- 24.6.1.10. Go to View, Find Mode, and enter the lab notebook number you signed up for in the "Hyb_NB_Page" field. Hit Enter.
- 24.6.1.11. Sort the records again as done in steps 24.6.1.4-24.6.1.6.
- 24.6.1.12. Choose "Export Records..." from the File menu. When the "Export Records to File" window appears, name the file and save it onto the desktop
- 24.6.1.13. When the "Specify Field Order for Export" window appears, choose the following fields from the left panel:
 - Hyb_Num
 - Combi_cRNA_Prep_Num
 - cRNAConc
- 24.6.1.14. Choose "UNV_cRNA_Exp_cRNAConc" from the pull-down menu on the top-left hand side of the window. Then choose

“cRNA_Yield_mcg” from the left panel. All 4 fields should be visible on the Field Order panel.

- 24.6.1.15. Export the information by clicking on the “Export” button.
- 24.6.1.16. Open “RU1 Hyb Template 0303.xlt” file found in P:\ChemExpress\ChemExpress Worksheets\CodeLink Hyb Worksheets, and go to “HYB” worksheet.
- 24.6.1.17. Open the exported file from step 24.6.1.15 with Excel and copy the Hyb number and cRNA numbers along with the concentration of cRNA by highlighting the relevant cells and choosing copy from the “Edit” pull-down menu..
- 24.6.1.18. Paste the information in the “HYB” worksheet of the RU1 Hyb Template 0303.xlt file in the B2 field by choosing “Paste” from the “Edit” pull-down menu.
- 24.6.1.19. Save the file into the “Hyb batchload ‘current month and year’” (e.g. Hyb batchload 01.2003) folder located at P: ChemExpress\ Module.Lab.Hyb\Hyb Batchload worksheet. Name the file using the standard naming format, i.e. "Hyb batchload mm.dd.yy (e.g. Hyb batchload 01.01.03). From this point on in the SOP, this saved file will be referred as Hyb batchload file.
- 24.6.1.20. Check the calculated values for the cRNA yield, amount of cRNA needed for 12.5 µg and water needed to 25 µl are correct.
- 24.6.1.21. Save and go to the “FRAG” worksheet of the Hyb batchload file. Print this page and take it to the lab.
- 24.6.1.22. Print out the “HybModWorksheet.doc” checklist located at P:\ChemExpress\ChemExpress Worksheets\CodeLink Hyb Worksheets, and take this checklist with you to the lab.
- 24.6.1.23. Go to the “REAGENTS” worksheet of the Hyb batchload file and enter the number of hybs you are processing on the B2 field. This worksheet will calculate the amount fragmentation buffer, bacterial control spike, Hyb Buffer A, Hyb Buffer B, and DEPC-H₂O you will need. Print this sheet out as well and take it with you to the lab.

24.6.2. *Fragmentation of Samples*

- 24.6.2.1. Verify the heat block temperature is 94°C and make adjustments if necessary.
- 24.6.2.2. Refer to the "FRAG" worksheet printed in step 24.6.1.21 to obtain cRNA samples from appropriate -80°C freezer. Place the box, from which you are pulling samples, on dry ice until you are done with that box. Place sample tubes that you pull out on wet ice and take it to the Hyb workstation.
- 24.6.2.3. Put these samples on ice while you prepare tubes.
- 24.6.2.4. Label empty 1.5 ml microcentrifuge tubes with cRNA number.
- 24.6.2.5. Make a master mix with the appropriate amounts of Bacterial control spike and fragmentation buffer according to the "REAGENTS" worksheet printed out in section 24.6.1.23. The sheet automatically calculates enough for ten percent extra as shown in Table 24.6-A.

Table 24.6-A: Fragmentation Master Mix

Component/Sample Number	1X	40X	80X	100X
Fragmentation Buffer	6.33 µl	278.5 µl	557.04 µl	696.3 µl
Bacterial Control Spike	1.02 µl	44.88 µl	89.76 µl	112.2 µl
Total Volume	7.35 µl	323.4 µl	646.8 µl	808.5 µl

**Note: For each component, designate and label a pipetman which will always be used to prepare the master mix. Label a 1000 µL Pipet-Lite "Frag Buffer" and a 200 µL Pipet-Lite "BC Spike". Only these pipetmans should be used for the pipetting steps in this protocol.*

- 24.6.2.6. Add 7.35 µL of this mix to each empty, labeled microcentrifuge tube.

**Note: With a marker cross out each column after each solution has been added to the microcentrifuge Tube.*

- 24.6.2.7. Add the appropriate amount of water to each tube according to the "FRAG" worksheet printed in section 24.6.1.21.

**Note: Pipet the EXACT amount of volume calculated by the worksheet.*

24.6.2.8. Make sure each cRNA sample is completely thawed. Vortex and quick spin the cRNA sample and then add the appropriate amount of each to the appropriately labeled tube using the "FRAG" worksheet as reference.

24.6.2.9. Vortex, quick spin, and incubate samples for EXACTLY 20 minutes at 94°C.

**Note: Always check temperature of the heat block to make sure it is correct before adding the samples.*

24.6.2.10. Quick spin the tubes and place them on ice.

24.6.2.11. Proceed with "Array Hybridization" protocol (Chapter 25, page182).

Chapter 25

Array Hybridization

25.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for hybridization of arrays.

25.2. REFERENCES

CodeLink Expression User Menu

25.3. RESPONSIBILITY

The Array Hyb group leader is responsible for the implementation and for the correct execution of this protocol.

25.4. SPECIAL NOTES

- Always wear your lab coat and protective eyewear, as well as gloves when working in the lab.
- Ensure temperature of the laboratory is 23°C +/- 2°C. Higher temperatures can lead to increased background and/or excessive signal intensity. If the temperature is out of range, immediately inform the Facilities Manager.
- Prepare adequate TNT buffer for the entire procedure. Calculations are provided for slides in sets of 12: 12 slides or less = 1.5 liters, 24 slides = 3 liters, etc.
- At the beginning of each wash step, the slide rack should be agitated gently.
- Avoid contact with the probe-containing surface of the microarray throughout the detection assay.
- Clean the shaking incubator and the slide holder with deionized H₂O after each use.
- Streptavidin-Alexa 647 and TNB Buffer aliquots must be prepared on the afternoon of the last working day of the week for the following week. See Appendix sections

25.5. REQUIRED MATERIALS AND EQUIPMENT

- Hybridization Buffer Component A (Amersham Biosciences IVT kit Part No. 320001)

- Hybridization Buffer Component B (Amersham Biosciences IVT kit Part No. 320001)
- Codelink Expression Bioarrays - Uniset Rat 1(Amersham Biosciences No. 300012 – 6pk; No. 300012-24pk)
- Codelink Expression Bioarrays - Uniset Human 1(Amersham Biosciences No. 300007 – 6pk; No. 300007-24pk)
- Sealing strips (Amersham Biosciences Codelink Expression Bioarrays No. 300007; No. 300012)
- Shaker Kit (Amersham Biosciences No. 310016):
 - 12-slide shaker tray (Amersham Biosciences No. 600017)
 - Universal Platform Assembly (Amersham Biosciences No. 600007)
 - Threaded posts (Amersham Biosciences No. 600018)
- Parallel Processing Kit (Motorola No. 310010)
 - Hybridization Chamber Removal Tool (Amersham Biosciences No. 600021)
 - Bioarray Rack (Amersham Biosciences No. 600010)
 - Small Reagent Reservoir (Amersham Biosciences No. 600011)
 - Medium Reagent Reservoir (Amersham Biosciences No. 600012)
 - Large Reagent Reservoir (Amersham Biosciences No. 600013)
 - Bioarray Removal Tool (Amersham Biosciences No. 600015)
 - Bioarray Position Tool (Amersham Biosciences No. 600016)
- 1 X PBS; pH 7.4 (Invitrogen/LTI No. 10010-023)
- Blocking Reagent (NEL704A)
- S -Alexa 647 (Molecular Probes No. S-21374)
- 1M Tris-HCl, pH 7.6 (Sigma No. T-2788)
- 5M NaCl (Sigma No. S-5150)
- Tween®-20 (Sigma No. P-7949)
- Axon GenePix 4000B scanner
- Sigma/QIAGEN Centrifuge (4°-15°C) (QIAGEN No. 81010)
- Computer configured for Axon 4000B Scanner
- New Brunswick Innova™ 4080 shaking incubator
- Sigma/QIAGEN Centrifuge (4°-15°C) (QIAGEN No. 81010)
- Centrifuge Plate Rotor-2x96 (QIAGEN No.81031)

- Pipette tips, sterile, RNase-free, and aerosol-resistant
- Microcentrifuge tubes, sterile, RNase-free, 1.7 ml
- Micropipettes
- Powder-free gloves
- Tabletop centrifuge
- Microcentrifuge
- Mettler Toledo Top Loading Balance (model PB 3002)
- Microtiter plate lid, Black (Corning, # 3935)
- Lab Coat
- Safety glasses
- Access to “RU1 Hyb Template0303.xlt” and “HybModWorksheet.doc” files located at P:\ChemExpress\ChemExpress worksheets\CodeLink Hyb Worksheets
- Access to FileMaker Pro

25.6. PROCEDURE

25.6.1. *Preparation of Hybridization Reaction Mixture*

- 25.6.1.1. Ensure the temperature of the Innova™ 4080 shaker is 37°C for the hybridization.
- 25.6.1.2. Prepare Hybridization Master Mix in a conical tube according to the “REAGENTS” worksheet of the Hyb batchload file you saved on section 24.6.1.19. For each sample, prepare 300.00 µl of hybridization solution as shown in Table 25.6-A.

Table 25.6-A: Hybridization Reaction Master Mix

Component/Sample Number	1X	40X	80X	100X
Hyb Buffer Component A	99.57 µl	4,082 µl	8,762 µl	10,953 µl
Hyb Buffer Component B	165.96 µl	6,805 µl	14,605 µl	18,255 µl
Nuclease-free water	34.47 µl	1,413 µl	3,033 µl	3,792 µl
Total Volume	300.00 µl	12,300 µl	26,400 µl	33,000 µl

- 25.6.1.3. Vortex the master mix for 5 seconds.
- 25.6.1.4. Dispense 300.00 µl of the Hybridization master mix into each labeled tube obtained from the “cRNA Fragmentation” protocol section 24.6.2.10 (page 181).

****Note: Process in batches of 20 tubes at one time.***

- 25.6.1.5. Vortex the hybridization mixture tubes for 5 seconds and incubate at 90°C for 5 minutes to denature the cRNA.
- 25.6.1.6. Spin the hybridization reaction-mixture tubes for 5 minutes at maximum speed.

****Note: DO NOT place the hybridization mixture on ice as components may precipitate.***

25.6.2. Loading of Reaction Mixtures into Array Chambers

- 25.6.2.1. Pull out the number of slides you will process that day. Select slides by receiving date (*i.e.* Use slides received earliest first) and in sequential numerical order (use slides by box).

- 25.6.2.2. Number and label the slides.

****Note: Only process a single batch of 20 tubes at a time.***

- 25.6.2.3. Place 10 slides into the 12-slide shaker tray, which is placed in turn, on a level surface, with the input/output ports facing up. Slides should be loaded in sets of 10 or less. Save the slide cases because the slide barcodes and lot numbers will be entered in the database later.
- 25.6.2.4. Aspirate 250 µl of hybridization reaction mixture into a 1 ml pipette tip for each array chamber. Place the 1 ml pipette tip containing the hybridization solution over the appropriate array input port and press until the tip forms a seal with the chamber.
- 25.6.2.5. Slowly inject the entire sample into the array chamber without using the blowout feature of the pipette. When the chamber is full, maintain thumb pressure on the pipette and remove the pipette tip from the input port. Discard any excess target mix in the pipette tip.
- 25.6.2.6. Cover the hybridization chamber ports with the 1 cm sealing strips, using the provided tabs.
- 25.6.2.7. To prevent evaporation during hybridization, ensure the entire port is sealed by gently pressing the port-sealing device over the port, taking care not to touch the center of the slide.
- 25.6.2.8. Load the 12-slide shaker tray loaded with slides (10 or less) into the Innova™ incubator aligned on front and back posts, with the hybridization chamber facing up.

- 25.6.2.9. Record the rack position of each slide.
- 25.6.2.10. Set the shaker to 300 rpm and incubate for 20 hours at 37°C.
**Note: Before walking away ensure the shaker settings are correct.*
- 25.6.2.11. In preparation for the next step, fill a Large Reagent Reservoir with 240 ml of filtered 0.75X TNT buffer, cover and place in a 46°C water bath, overnight.
**Note: One large reagent reservoir is needed for each set of 10 slides.*

25.6.3. *Entering Slide Information in the Database*

This step must be done before slides can be scanned the following day.

- 25.6.3.1. Open the Hyb batchload file you saved on section 24.6.1.19.
- 25.6.3.2. Enter the slide numbers and the slide lot number from the slide case in the corresponding field in the HYB worksheet of the Hyb batchload file.
- 25.6.3.3. Enter Position on Slide, which is always 1 (For Rat or Human Uniset; for ADME-R2 there are 2 possible positions).
- 25.6.3.4. Enter the shaker temperature (in °C) in the corresponding field.
- 25.6.3.5. Save this file.
- 25.6.3.6. Open UNV_Main.FP5.
- 25.6.3.7. Click on "Hybridization" button and enter the password.
- 25.6.3.8. For CodeLink rat samples, click on "Review/Batch Load Hybridization Data" button.
- 25.6.3.9. Click on the "Batch Load Rat Hyb Data" button at the top of the page and enter information in Hyb_NB_Page. Hit the "Continue" button.
- 25.6.3.10. Verify the correct Hyb numbers are shown and then click on the "Continue" button a second time. You should now be in UNV_Hyb_Exp_Import.FP5.
- 25.6.3.11. Click on the blue "Import Hyb Data" button and when the "Open File" window appears, select the Hyb batchload file you saved in step 25.6.3.5.
- 25.6.3.12. When prompted in the "Specify Excel Data" window, select the "HYB" worksheet to import and click "OK."

- 25.6.3.13. In the “First Row Option” window, make sure “Field names” is selected then click “OK.”
- 25.6.3.14. Verify the following fields are checked for import and then click on the “Import” button:
- Hyb_Number
 - Slide_Barcode
 - Slide_Lot_Number
 - Shaker_T
- 25.6.3.15. Click on the gray “Hyb_Exp.FP3” button then go to the “Scripts” pull-down menu and select “Fill Hyb Data” to upload the information.
- 25.6.3.16. Click on the “Continue” button.
- 25.6.3.17. Once done, go to the “Windows” pull-down menu, select “UNV_Hyb_Exp_Import”, and click on the pink “Delete All” button.
- *Note: This step is extremely important to ensure records do not get confused.*
- 25.6.3.18. Close this file by selecting “File” pull-down menu and choosing “Close”. You should now be back at UNV_Hyb_Exp.FP5.
- 25.6.3.19. Go to the right side of the page and enter information in the “Ready for Array Reading” field. Enter the date hybs will be scanned as Y-yyyymmdd (e.g. Y-20030201).
- *Note: You must enter this information the DAY hybridization is set up or the hybridization will fail Validation the following day.*
- 25.6.3.20. Go back to UNV_Main.FP5. Choose “Array Detection” and enter the password.
- 25.6.3.21. Choose Array Sign-up (Under Rat for rat samples and under Human for human samples).
- 25.6.3.22. Enter the **new lab notebook page** for the arrays you will scan the next day (corresponding cRNA#s).
- 25.6.3.23. Go to “Array Conditions” and enter the Array NB_Page, Scientist (your name from the pull-down menu), and Detection Date (i.e. the scan date).
- *Note: You must enter this information the DAY hybridization is set up or the hybridization will fail Validation the following day.*

- 25.6.3.24. Close the FileMaker Pro database.
- 25.6.3.25. Go back to the Hyb batchload file and print out the "For Notebook" worksheet and the "SCAN" worksheet. Paste these two documents into the lab notebook. Use the "SCAN" worksheet as a scan checklist for the next day.

25.6.4. Post-Hybridization Wash

(Next day after the 20-hour incubation)

- 25.6.4.1. Next day, ten minutes before the 20-hour incubation is done, fill each slot (except slot 6 and 7) in the Medium Reagent Reservoir with 13 ml of filtered TNT. Place the Bioarray Rack into the Reservoir. Leave at room temperature.
- 25.6.4.2. Record the shaker temperature in the "HybModWorksheet" checklist. Remove the 12-slide holder from the Innova™ 4080 shaker.
- 25.6.4.3. Place the first slide to be processed into the Hybridization Chamber Removal Tool.
**Note: Process in batches of 20 slides at one time. Use a staggered method between each batch, i.e. wait 10 minutes after processing last slide in one batch before starting with the first slide of the next batch.*
- 25.6.4.4. Remove the hybridization chamber by lifting the tab and pulling up and back the hybridization chamber at a 60° angle, slowly and gently, without creasing the hybridization chamber.
- 25.6.4.5. Place the slide into a slot of the Bioarray Rack, which was placed in the Medium Reagent Reservoir containing TNT in step 25.6.4.1. Move the slide into place using the Bioarray Position Tool, tooth-side down.
**Note: Do NOT place any slides in slot 6 or 7 of the Bioarray Rack.*
- 25.6.4.6. Rinse the surface of the Hybridization Chamber Removal Tool with 5.5 ml of TNT buffer at room temperature to avoid potential cross-contamination, using a squirt bottle. Let the reservoir stand at room temperature until all slides have been processed.
- 25.6.4.7. Repeat steps 25.6.4.3-25.6.4.6 for each hybridized slide of the current batch.

25.6.4.8. In "HybModWorksheet" checklist, record the temperature of water bath and TNT Buffer in the Large Reagent Reservoir in the "Water Bath" and "Init TNT Buffer" fields, respectively. Remove the Bioarray Rack containing slides from the Medium Reagent Reservoir and place into the Large Reagent Reservoir containing TNT at 46°C. Replace the lid on the Large Reagent Reservoir and then on the water bath. Incubate for exactly 1 hour.

**Note: Wait 10 minutes after placing a batch of slides in the 46 °C TNT before repeating steps 25.6.4.3-25.6.4.6 for the next batch of slides.*

25.6.5. *Detection with Streptavidin-Alexa Fluor® 647*

The Streptavidin-Alexa Fluor 647 Working Solution should be dispensed using the Dispensette pump attached to an amber bottle. Do NOT use clear bottles that cannot protect the solution from light or serological pipettes/Pipetman to dispense reagent.

25.6.5.1. Prepare Streptavidin-Alexa 647 Working Solution according to the "REAGENT" worksheet in the Hyb batchload file. This solution should be prepared 10 minutes before the 1-hour incubation is done (Table 25.6-B).

Table 25.6-B: Detection Master Mix

Component/Sample Number	1X	10X
Streptavidin-Alexa 647 dye solution (100 µl aliquot/tube)	6.8 µl	2 tubes
Filtered TNB Buffer (50 ml aliquot/tube)	3,400 µl	2 tubes
Total Volume	3,406.8 µl	100.2 ml

- Obtain the appropriate number of Streptavidin-Alexa 647 dye aliquots from the 4°C fridge, *i.e.* 2 tubes of the 100 µl aliquot are required to process up to 10 slides. To process more than 10 slides, obtain an additional aliquot of Streptavidin–Alexa 647 for every additional set of 10 slides or fraction thereof (*e.g.* 3 tubes of Streptavidin-Alexa 647 are required to process 11-20 slides; 4 tubes for 21-30 slides, *etc.*)

- Centrifuge the Streptavidin-Alexa 647 dye aliquot(s) for 1 minute at 10,000 rpm to remove precipitates and collect all of the condensation.
- Obtain the appropriate number of Filtered TNB Buffer aliquots for the number of slides to be processed from 4°C fridge, *i.e.* 2 tubes of Filtered TNB are required to process up to 10 slides. To process more than 10 slides, obtain an additional aliquot of Filtered TNB for every additional set of 10 slides or fraction thereof (*e.g.* 3 tubes of Filtered TNB are required to process 11-20 slides; 4 tubes for 21-30 slides, *etc.*).
- Centrifuge the Filtered TNB Buffer tubes for 1 minute at 3,000 rpm to collect condensation.
- The entire content of a single S-Alexa aliquot tube is combined with a single Filtered TNB Buffer aliquot in the following manner:
 - Add the 100 µl Streptavidin-Alexa 647 dye aliquot to the 50.0 ml Filtered TNB Buffer aliquot for each set of 10 slides to be processed.

Note: If up to 10 slides are being processed, you must use two tubes of Streptavidin-Alexa 647 and two tubes of filtered TNB. This is to ensure the Dispensette pump delivers the correct volume of reagent. For every additional set of 10 slides or fraction thereof, an additional aliquot of Streptavidin-Alexa 647 and Filtered TNB is required (see above).

- To pipet out the S-Alexa 647 dye aliquot, tilt the microcentrifuge tube to a 45-degree angle with the side that was facing out while inside the centrifuge facing away from you. Place the pipette tip on the opposite side at the bottom of the tube, *i.e.* pipette tip is positioned at bottom of tube at side nearest you as shown in Figure 25.6-A. Avoid pipetting any precipitate.
- Tightly cap the 50 ml Falcon tube containing the mixture, invert 8 times, then vortex at maximum speed for 5 seconds to ensure proper homogenization of the two solutions.

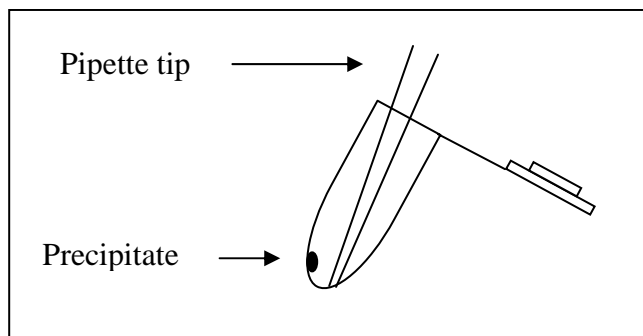


Figure 25.6-A: Avoid Pipetting the Precipitate

- Transfer the mixture from the 50 ml Falcon tube into a 250 ml light-protected bottle using a 50 ml disposable serological pipet (total volume should be 100.2 ml).
- Once the aliquots have been placed into the 250 ml bottle, cap the bottle, invert 8 times, then vortex at maximum speed for 5 seconds to ensure proper homogenization of the two solutions.

****Note: If less than 10 slides will be processed, make the master mix for 10 slides and discard the unused portion of the Streptavidin-Alexa 647 Working Solution. This working solution should be used within 15 minutes of preparation. Keep each master mix inside drawer, hidden from light, until ready to use.***

- 25.6.5.2. Using the Dispensette pump, fill each slot (except 6 and 7) in the Small Reagent Reservoir with 3.4 ml of Streptavidin-Alexa Fluor® 647 working solution (See Appendix). Since arrays are processed in batches of 20, 30 reactions-worth of working solution is required to allow sufficient solution to prime the pump. No slots should contain bubbles after dispensing. Leave at room temperature. Cover with lid, and protect from ambient light.
- 25.6.5.3. After the incubation, remove the Bioarray Rack containing slides from the Large Reagent Reservoir and place into the Small Reagent Reservoir containing Streptavidin-Alexa 647 working solution.
- 25.6.5.4. Agitate the bioarray rack gently (by lifting the rack up and down within the reservoir), cover with lid, and protect from ambient light. Incubate at room temperature (25°C +/- 2° C) for 30 minutes.

- 25.6.5.5. Record the room temperature and temperature of the Large Reagent Reservoir filled with 0.75X TNT in the "Room Temp" and "Final TNT Buffer" fields of the "HybModWorksheet" checklist.
- 25.6.5.6. During incubation, fill a Large Reagent Reservoir with 240 ml of filtered TNT buffer at room temperature.
- *Note: Each set of slides requires 4 washes.*
- 25.6.5.7. After the incubation, remove the Bioarray Rack containing slides from the Small Reagent Reservoir containing Streptavidin-Alexa Fluor® 647 working solution and place immediately into the Large Reagent Reservoir containing the filtered TNT buffer without draining the solution from the slide. Agitate gently and incubate at room temperature for 5 minutes.
- 25.6.5.8. Remove the Bioarray Rack containing slides from the Large Reagent Reservoir with filtered TNT buffer and place into a second Large Reagent Reservoir containing filtered TNT buffer without attempting to drain the solution from the slide. Agitate gently and incubate at room temperature for 5 minutes. Repeat this step with fresh filtered TNT buffer for two more washes.
- 25.6.5.9. Remove Bioarray Rack containing slides from Large Reagent Reservoir and place into a new Large Reagent Reservoir containing 0.05 % Tween 20 in deionized water. Move rack up and down 5 times in 5 seconds. Repeat this step once.
- 25.6.5.10. Remove Bioarray Rack containing slides from Large Reagent Reservoir, and remove excess liquid by gently tapping edges on paper towel.
- 25.6.5.11. Dry slides by centrifugation in the QIAgen Sigma 4-15C (No. 81010) centrifuge, using a swinging bucket rotor 2 x 96 (No. 81031) using the following settings:
- Speed: 2000 rpm
 - Acceleration: 9
 - Deceleration: 9
 - Time: 3 minutes
- 25.6.5.12. Place the dry slide into a light protected slide box until scanned. Dry remaining slides.

25.6.5.13. Rinse out all reservoirs with deionized H₂O repeatedly to clean, and then invert them to dry.

25.6.6. Database Upload of Hybridization Conditions

25.6.6.1. Open the Hyb batchload file that contains the relevant hyb data.

25.6.6.2. Go to the "HYB" worksheet and fill in the following columns and save the file:

- Water Bath temperature
- Initial TNT temperature
- Final TNT temperature
- Room temperature
- Slide Set#
- S-Alexa 647 Prep Date
- S-Alexa 647 Lot#
- TNB Prep. Date
- TNT Prep. date
- Hyb Comments

25.6.6.3. Open UNV_Main.FP5.

25.6.6.4. Click on "Hybridization" button and enter the password.

25.6.6.5. For CodeLink rat samples, click on "Review/Batch Load Hybridization Data" button.

25.6.6.6. Click on "Batch Load Rat Hyb Data" button at the top of the page and enter the notebook page number for which you originally signed up for the samples into the "Hyb_NB_Page" field

25.6.6.7. Click on the "Continue" button to the side. The page should show all values of cRNA prep#, cRNA concentration, Hyb#, etc.

25.6.6.8. Verify the correct Hyb#s are shown, and then click on the "Continue" button a second time to get to UNV_Hyb_Exp_Import.FP5.

25.6.6.9. Click on the blue "Import Hyb Data" button and when the "Open" window appears, select the Hyb batchload file you saved in step 25.6.6.2.

25.6.6.10. When prompted in the "Specify Excel Data" window, select the "HYB" worksheet to import and click "OK".

- 25.6.6.11. In the "First Row Option" window, make sure "Field names" is selected then click "OK."
- 25.6.6.12. Verify the following fields are checked for import then click on the "Import" button:
- Position_on_Slide
 - Water_Bath_T
 - Initial_TNT_T
 - Final_TNT_T
 - Room_T
 - Slide_Set_Number
 - S-Alexa Prep Date
 - S-Alexa Lot #
 - TNB prep date
 - TNT prep date
 - Hyb_Comments
- 25.6.6.13. Click on the gray "Hyb_Exp.FP3" button then go to the "Scripts" pull-down menu and select "Fill Hyb Data" to upload the information.
- 25.6.6.14. Click on the "Continue" button.
- 25.6.6.15. Once done, go to the "Windows" pull-down menu, select "UNV_Hyb_Exp_Import", and click on the pink "Delete All" button.
- *Note: This step is extremely important to ensure records do not get confused.*
- 25.6.6.16. Close the FileMaker Pro database.

25.6.7. Slide Scanning

The Hyb Team Leader can be consulted with questions.

- 25.6.7.1. Take the processed slides to the Axon GenePix Scanner. Until they are scanned, slides should be kept covered in a black, slide box to protect them from dust and light.
- 25.6.7.2. Start the GenePix Pro 4.0 Scanning Software on the computer that controls the Axon GenePix Scanner.
- 25.6.7.3. Calibrate the scanners using the calibration slide accompanying GenePix Pro 4.0 on a DAILY basis as follows:

- Click the "Hardware Diagnostics" button on the right-hand side of the GenePix Pro main window.
- Click the "Calibrate System" button.
- Make sure the 635-laser box is checked, click the start button and follow the "wizard" through the rest of the steps.
- Log in the calibration values of each scanner in the logbook located next to the scanners. Close the calibration window.

****Note: The GenePix Pro software must be running for at least 15 minutes before beginning the calibration procedure. Initiate the calibration step first thing in the morning. When the start button is pressed a countdown window showing the number of minutes left until 15 minutes have elapsed is displayed. Please wait 15 minutes before performing the calibration.***

25.6.7.4. Check the scanning parameters before each slide by clicking the "Hardware Settings" button on the right-hand side:

- Laser setting: 635 nm
- PMT Gain: 600
- Pixel/size (μm): 10 microns.

25.6.7.5. Use the "SCAN" worksheet printed out in step 25.6.3.25, which has information of hyb # and slide barcodes for all slides to be scanned that day. You will visualize each slide after it is scanned and record array comments on this worksheet.

25.6.7.6. Enter slide information and save path /files before scanning:

- Click on the "Report" tab on the top of screen
- Under the "CodeLink Scanning" menu, choose "Scan CodeLink Slide". This will take you to Screen 1.
- Enter Slide ID (Slide barcode, e.g. T00001234), then Click "Next".
- A window pops up with the message "ActiveX control... Do you want to allow this interaction?" Click "Yes".

****Note: This step is extremely important. Double-check the numbers entered are correct before proceeding.***

On Screen 2, enter the experiment and scan information:

- Enter the array type, e.g. Uniset Rat 1, in the "Project Name" field.

- Enter the array type, *e.g.* Uniset Rat 1, in the "Experiment Name" field.
 - Enter the Hyb #, *e.g.* 80000, in the first "Sample Name" field and leave the remaining "Sample Name" fields blank.
 - Select "New Setting File" by clicking on the "Browse..." button and going to C:\Axon\Genepixpro4. Double-click on "motorola.gps" then click "Next".
 - When screen 3 appears, load and scan slide:
 - Choose the Image File Path by clicking on the "Browse..." button and going to P:\IMAGES\GRIDDING_IN PROCESS (the image will be saved in this folder).
 - Click "Scan slide" box.
 - After scanning is done, "Scan done" will appear. Click "OK".
 - This should take you go back to Screen 3. Click on "Save Image".
 - Once the file has been saved, a window containing the path and name of the file directory will appear confirming the file has been saved successfully. Click "OK."
 - You should be back on Screen 3. Click on "New Slide". This will take you back to Screen 1.
- 25.6.7.7. Scan and save the remaining slides using the same scanner settings and file naming convention (*i.e.* change the slide number in the file name).
- 25.6.7.8. Indicate which scanner slide was scanned on "SCAN" sheet printed in step 25.6.3.25.
- 25.6.7.9. Visually inspect all slides and enter comments on:
- Intensity of the signal (*e.g.* signal ok, low/minimal signal, *etc.*). Examples of images for each type are located at folders in P:\Reference for Array Comments.
 - Image imperfections (*e.g.* low signal in mid array, comet tails, streaks, *etc.*)
- *Note: This is a crucial QC step. A comment must be entered for EVERY slide.**
- 25.6.7.10. Enter the array comments in FileMaker Pro in the corresponding "Array Comments Field".

25.6.8. *Gridding*

The Hyb Team Leader can be consulted with questions about the protocol and Computer Support can be consulted for software or computer problems.

- 25.6.8.1. Open Batch Submission 2 by clicking on the icon on the desktop.
- 25.6.8.2. On the 1st window's "Main Menu", select "Create Batch" and click "Next."
- 25.6.8.3. In the "Image List" window, click "Add File."
- 25.6.8.4. Go to the P:\images\gridding_in_process directory and highlight all slides to be gridded. To choose multiple files, hold down the Ctrl-key while selecting files. Click "Open."
- 25.6.8.5. Verify the number of slides you chose as they appear in the window.
- 25.6.8.6. Click "Next" and the window will submit the batch.
**Note: If Microsoft Excel is not open on your desktop, it will open automatically.*
- 25.6.8.7. Once the "Finish" window opens and asks for a Results Destination Path:
 - If the last path used was P:\gridding_output, click "Choose last used path" and then click "Next."
 - Otherwise, browse to P:\gridding_output (by clicking on "...") button) and click on "Next."
 - The images will be gridded and saved in this folder.
- 25.6.8.8. Once the "Batch Details" window opens, click "Next."
- 25.6.8.9. In the "Finish" window, enter the name of the batch gridding ("Daily batch mmddyy, e.g. Daily batch 010203) in the "Batch Name" field
- 25.6.8.10. Verify the "Submit Batch after create" box is checked and the destination path is P:\gridding_output, then click "Create Batch."
- 25.6.8.11. The CodeLink™ - Batch Submission window will open, notifying you the batch has been created and saved. Click "OK."
- 25.6.8.12. Close the Batch Submission 2 program.

25.6.8.13. Submit the batch (the Hyb Team Leader usually submits the batch before he leaves for the day) by clicking on the "Create Batch" button and naming the batch with the current date.

25.6.8.14. The batch loader will email you with a report when it has finished.

25.6.9. *Gridding Review*

The Hyb Team Leader can be consulted with questions.

25.6.9.1. Create an excel worksheet by copying the list of gridded slides from the batch completion notification message that the batch loader sent. Make sure there are columns for "comments" and "gridding status" and print out the worksheet.

25.6.9.2. Open Results Reviewer 5 Software by double-clicking the icon on the desktop.

25.6.9.3. Select an image to review by clicking on "File" and then choosing "review results". Alternatively, click on the folder with star icon on the toolbar at the top of the screen.

25.6.9.4. A dialog box "Select a Review File" will open. Find the file you want to review in P:\gridding_output (you may have to browse to locate this destination).

25.6.9.5. Select the .SST file corresponding to the slide name (barcode_scan date.SST) by double clicking on the file name. A multicolored image of the slide will appear on the right hand portion of the screen. The "composite" tab near the top of the screen should be highlighted light gray.

25.6.9.6. Record "color" observations on the worksheet (e.g. uniformly red/blue; longitudinal dark lines apparent on left side; metagrid 1= red/blue and metagrid 2=green/blue, etc.)

25.6.9.7. Verify the "Brightness/Contrast" control setting is at maximum:

- Click on the "main" tab on the upper left corner of the screen (tab will go from dark gray to light gray).
- Look at the second window called "Brightness/Contrast Control". All 3 triangles should be lined up vertically on the left hand side.
- If they are not, select the triangle on the top and drag it all the way to the left.

- 25.6.9.8. Magnify a part of the image by clicking on the magnifying glass on the tool bar at the top of the screen and selecting an area on the image (click and drag; a box outlined in yellow will identify the area magnified) or by sliding the notch at the bottom of the screen to the right. Adjust the magnification so that gridding can be checked accurately.
- 25.6.9.9. Check the gridding efficiency by selecting the segmented view. This can be done by clicking on the third tab at the top of the screen. This tab has the slide name_date.TIFseg.
- 25.6.9.10. Scroll through the image using the arrow bars on the left hand side and bottom of the screen making sure to view the entire slide. In particular, the top, bottom and sides of the image should be scrutinized carefully. The white spots on the image should be centered within the red circles (grid).
- If the spots are centered within a circle, the slide is gridded correctly.
 - If the spots are NOT contained within a circle, or the grid appears to be irregular, the slide is misgridded.
- 25.6.9.11. Enter the gridding review state on the worksheet. Highlight misgridded images using a blue highlighter on the worksheet. These images will need to be gridded manually.
- 25.6.9.12. Proceed to the next image by clicking on the "results" tab located in the top left corner and clicking on the "cancel" button on the bottom left of the screen.
- 25.6.9.13. A dialog box will appear. Click "Yes" and repeat steps 25.6.9.3-25.6.9.12 until 25% or 20 slides, whichever is greater, have been reviewed.
- 25.6.9.14. Close Result Reviewer 5.0.

25.6.10. *Gridding CodeLink-Slides Manually*

Prior to following the procedure outlined below please consider the following questions.

- Why do you think the software misgridded the image?
- Does your image look typical?
- Did you follow the assay protocol or have you deviated from it?

- Was your slide of good quality? (Did it have any issues to start with?)

****Note: If the image was not able to be gridded automatically, resubmitting the batch will not help.***

- 25.6.10.1. Launch ImaGene 5.5
- 25.6.10.2. Load the image, e.g. T12345678.TIF by using File -> Load Images.
- 25.6.10.3. Load the configuration file by using File -> Setting. Select the "Load" button at the bottom of the screen and locate the appropriate configuration file. (See table below). The configuration files can be found under C:\Program Files\Amersham Biosciences\CodeLink 2_3\ProductFiles\Configuration. You must load the configuration files for the product; otherwise, ImaGene will use default settings or settings from a previous session that are not correct for the product you are about to analyze.
- 25.6.10.4. Load the gridding file by clicking on Grid -> Load Grid and then selecting the appropriate gridding file (Table 25.6-C). The file can be found in C:\Program Files\Amersham Biosciences\CodeLink 2_3\ProductFiles\Grids.
- 25.6.10.5. Load the Gene Ids by clicking on Grid -> Load Gene Ids and then selecting the appropriate Gene ID file (Table 25.6-C). This file can be found under C:\Program Files\Amersham Biosciences\CodeLink 2_3\ProductFiles\Products.
- 25.6.10.6. Manually place the grid using the button along the tool bar at the top of the screen and then use the Automatically Place Grid button.
- 25.6.10.7. Inspect to see if your spots are gridded correctly, if not, adjust the grid manually.
- 25.6.10.8. Press the Auto Adjust Spots button located along the top of the screen.
- 25.6.10.9. On the Main tab at the left, press the Quantify button in the lower part of the screen.
- 25.6.10.10. Once the results are displayed in a table, click on Save and point to a temporary location to save the files. A TXT file, a *final.grd file, and a *.sst file will be created. Copy these files to the location

where the previous files from the image were stored. Overwrite any existing misgridded output files already present.

25.6.10.11.Exit ImaGene 5.0.

25.6.10.12.Reload your batch using Expression 2.3 software (i.e. rebatch the misgridded file).

25.6.10.13.Go to the gridding_output folder and replace the .sst and .txt files generated from the batch-process (misgridded) with the manually gridded files you saved in a temporary location.

25.6.10.14.Check the gridding using Result Reviewer.

25.6.10.15.Export, validate and file move as usual.

**Note: Do NOT save any Configuration, Settings, Gridding, files using the same name as we use for CodeLink. Your changes only apply to the image you are working with. These settings were optimal settings selected based on a large data set.*

**Note: If it is necessary to make changes for a batch of slides, please contact Amersham for suggestions on how to approach that problem. Based on the changes you would like to make, Amersham may be able to suggest a better way to handle your problem.*

Table 25.6-C: Example of Gridding File List

Catalog Number	Product Name	Configuration File
300007-02	UniSet Human I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300007-03	UniSet Human I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300012-02	UniSet Rat I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300012-03	UniSet Rat I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300012-04	UniSet Rat I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300013-00	UniSet Mouse I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300007-04	UniSet Human I	10K_Configuration_AutoSegmentation_NegCtrls.xml
300014-00	UniSet Human II	10K_Configuration_AutoSegmentation_NegCtrls.xml
300019-00	UniSet Human 20K	20K_Configuration_2_Bkg_Buff_4_Bkg_Width.xml
300007-02	UniSet Human I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300007-03	UniSet Human I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300012-02	UniSet Rat I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd

Catalog Number	Product Name	Configuration File
300012-03	UniSet Rat I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300012-04	UniSet Rat I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300013-00	UniSet Mouse I	GRID-2UP-112R-48C-Staggered_639_V5.grd
300007-04	UniSet Human I	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300014-00	UniSet Human II	GRID-2UP-112R-48C-Staggered V5-new_16MinDiameter.grd
300019-00	UniSet Human 20K	grid20K_fields2.grd
300007-02	UniSet Human I	EXP5216x2-506.gid
300007-03	UniSet Human I	exp5280x2-609.gid
300012-02	UniSet Rat I	exp5280x2-584.gid
300012-03	UniSet Rat I	exp5280x2-613.gid
300012-04	UniSet Rat I	EXP5280x2-648.gid
300013-00	UniSet Mouse I	EXP5280x2-639.gid
300007-04	UniSet Human I	exp5280x2-649.gid
300014-00	UniSet Human II	EXP5280x2-690_REV2.gid
300019-00	UniSet Human 20K	EXP10336x2-770.gid

25.6.11. QC Report Generation and Gridded File Export

The Hyb Team Leader can be consulted with questions about the protocol and Computer Support can be consulted for software or computer problems.

25.6.11.1. Open Codelink Expression 2 Software by double clicking on the icon on desktop.

25.6.11.2. Click on the "Batch" button then select "Select Batch File" in the pull down menu and add the specific files to be loaded:

Click on "Add Batch"

A dialog box will appear.

Click on "Show only (name) batches" under Batch Owner Selection.

Click on "Sorted by Batch Name" under Batch Sorting Preference.

Click on the down arrow on the right side of the "Batch Name Selection Field" and select the name of the batch containing the slides of interest. Click "OK". The name of the selected batch

should appear under "Image Selection/Added Batches" and the slides within the batch will be displayed in the right panel.

All images are pre-selected and have a checkmark in the box under "selected". To remove misgridded slides, uncheck the appropriate box in the Gridding Review window then click "OK".

A dialog box named "Ready to move to the next step ..." will appear and ask to load or export the batch. Click "OK".

25.6.11.3. The slides must be loaded in order to obtain the Batch QC report:

- Go to "Batch" at the top of the screen and select "Load Batch Data" from the drop down menu.
- A dialog box called "Progress Report" will appear detailing the progress of data loading. When this process has finished, another dialog box pops up. Click "OK".

25.6.11.4. Before closing the "Codelink Expression Analysis" box, check the status of each step to verify no failures occurred. If failures occurred, an MSR file, or other file is missing or has intrinsic errors. Please contact the Array Group manager, computer support or Amersham.

25.6.11.5. Once the status has been checked, click "close" (do NOT save this document).

25.6.11.6. To look at the Batch QC report, click on the "Batch QC Report" button at the top of the screen. A table will appear and will fill with the name of each slide in the batch and its respective flag count values. Since these values are CALCULATED on the spot, the table may take time to fill in. Be patient and allow the process to finish.

25.6.11.7. To export the table, click the "Export to Excel" button located near the bottom of the screen. An Excel sheet will pop up (may be behind or in front of the initial screen). Click on the worksheet and save this file, then go to "File" and "save as" Batch QC mmddyy (e.g. Batch QC 030403) at P:\Batch QC Reports. DO NOT CLOSE THE "Codelink-System Software-Expression-Analysis" WINDOW.

25.6.11.8. If a large number of slides are selected, you may have to save the reports in parts. If so combine the list and compare it with the

gridding review worksheet to make sure the numbers make sense.

****Note: This step is extremely time-consuming; do NOT try to load more than 30 files at a time.***

25.6.11.9. After the Batch QC information has been saved, the Iconix Format Files and the GEML Files must be exported to P:\iconix_format and to P:\gridding_output, respectively. To export Iconix Format Files to Iconix Format, return to the "Codelink_System Software-Expression Analysis" window and click "Export" at the top of the screen. Select the "Export Batch to Iconix Format" button. When the dialog box appears, click "yes".

25.6.11.10. Save the exported Iconix Format files at P:\iconix_format and click "OK". A progress-report dialog box will appear and will show the progress of the export

****Note: This will take some time.***

25.6.11.11. Once the files have been exported, a dialog box will appear stating that the export has finished. Click "OK" then check the progress report log for errors and click "close".

25.6.11.12. Make sure that no "misgridded" files get exported.

25.6.11.13. Once the export is complete, confirm that the exported files are located at P:\iconix_format. You should find a set of .txt files corresponding to the slides for which you performed the Gridding Review at this location.

25.6.11.14. To export GEML Files to Iconix Format, return to the "Codelink_System Software-Expression Analysis" window and click "Export" at the top of the screen. Select the "Export Batch to XML/GEML" button. When the dialog box appears, click "yes".

25.6.11.15. Save the exported Iconix Format files at P:\gridding_output and click "OK". A progress-report dialog box will appear and will show the progress of the export

****Note: This will take some time.***

25.6.11.16. Once the files have been exported, a dialog box will appear stating that the export has finished. Click "OK" then check the progress report log for errors and click "close".

25.6.11.17. Close Codelink Expression 2.

25.7. EQUIPMENT MAINTENANCE

25.7.1. *Daily Maintenance*

The following equipment should be rinsed thoroughly with DI water on a daily basis to avoid salt build-up and cross-contamination:

- Parallel processing reservoirs
- Chamber removal tools
- Any equipment that comes in direct contact with salty buffers.

25.7.2. *Weekly Maintenance*

The following equipment should be rinsed thoroughly with DI water on a weekly basis to avoid salt corrosion:

- Water baths
- Centrifuge slide holders

25.8. APPENDIX

25.8.1. *TNT Buffer Preparation (20L)*

- 0.1 M Tris-HCl, pH 7.6
- 0.15 M NaCl
- 0.05% Tween-20

25.8.1.1. Rinse a 20 L Carboy out with 300 ml of isopropanol.

25.8.1.2. Rinse the Carboy twice with 4 L of deionized H₂O and completely drain the carboy.

25.8.1.3. Add 2 L 1 M Tris-HCl.

25.8.1.4. Add 600 ml 5 M NaCl.

25.8.1.5. Add 10 ml Tween-20.

25.8.1.6. Add 17.39 L deionized H₂O. Mix well by swirling.

25.8.1.7. Filter TNT through a 0.20 micron filter

This solution can be stored up to 2 weeks at room temperature.

25.8.2. *0.75X TNT Buffer Preparation*

Add 25 ml of deionized water to 75 ml of TNT buffer (from above) per 100 ml of Buffer required.

25.8.3. *TNB Buffer (2.0 L)*

- 0.1 M Tris-HCl, pH 7.6

- 0.15 M NaCl
 - 0.5% NEN Blocking Reagent (Perkin Elmer cat# FP1020)
- 25.8.3.1. Obtain a Corning Stirrer/Hot Plate model # PC-420.
 - 25.8.3.2. Add 1,740 ml of DEPC-H₂O to a 2 L flask containing a stir bar and set the Stir knob of the Stirrer/Hot Plate to 4.
 - 25.8.3.3. Add 200 ml of 1 M Tris-HCl, pH 7.5.
 - 25.8.3.4. Add 60 ml of 5 M NaCl.
 - 25.8.3.5. Slowly add 10g of NEN Blocking Reagent in 0.5g increments until all 10g of the blocking reagent is dissolved. Start by setting the Heat knob of the Stirrer/Hot Plate to 6. Once the temperature is at 65°C, turn down the Heat knob to 4.
 - 25.8.3.6. Filter TNB Buffer through a 0.88-micron filter.
 - 25.8.3.7. This solution can be stored up to 1 week at 4°C and up to 12 weeks at -20°C.
 - 25.8.3.8. Aliquot the TNB buffer into 50 ml tubes as shown in section 25.8.4.

25.8.4. TNB Buffer Aliquots

- Prepare 50 ml aliquots of Filtered TNB Buffer in 50 ml Falcon tubes on the afternoon on the last day of the workweek. Each 50 ml aliquot is sufficient to process 10 slides. Store these aliquots at -20°C.
- 25.8.4.1. Do not throw out any aliquots that are less than 4 weeks old. Use any leftover aliquots from the week first subtracting these from the amount calculated for the following week.
 - 25.8.4.2. A top loading balance (Mettler Toledo model PB 3002) must be used for proper volume measurement of the aliquots. Such a balance may be found in the Media room. Measure out the aliquots in the following way:
 - Place a 50 ml Falcon tube held by a Cube rack on the top loading balance.
 - Unscrew the 50 ml Falcon tube cap and leave it on the balance.
 - Auto zero the balance.
 - Using a disposable serological pipet, fill the 50 ml Falcon tube with Filtered TNB Buffer until the top loading balance reads “50.00 g” (± 0.05 g);

- Repeat the previous four steps for the designated number of aliquots to be prepared, *i.e.* (400 slides/1 aliquot of 50.0 ml TNB per 10 slides) = 40 aliquots.

25.8.4.3. Screw the caps tight back onto the 50 ml Falcon tubes.

25.8.4.4. Label and date each tube.

25.8.4.5. Store the proper number of aliquots sufficient for 5X the daily throughput for the following week at 4°C, *i.e.* if 80 slides are to be processed per day, prepare for 400 slides (40 X 50 ml Filtered TNB Buffer). Store the rest at -20°C.

25.8.5. *Streptavidin-Alexa 647 Stock Solution*

25.8.5.1. Add 1000 µl of 0.1 micron filtered 1X PBS (pH7.4), to the tube of dried Streptavidin-Alexa 647 reagent. This solution can be stored up to 1 week at 4°C and 24 weeks at -20°C. Protect from light.

25.8.6. *Streptavidin-Alexa 647 Dye Aliquots*

Prepare 100 µl aliquots of Streptavidin-Alexa 647 Dye in RNase-free 1.7 ml microfuge tubes on afternoon on the last day of the workweek. Each 100 µl aliquot is sufficient to process 10 slides.

25.8.6.1. Prepare aliquots sufficient for 5X the daily throughput for the following week, *i.e.* if 80 slides are to be processed per day, prepare for 400 slides (40 X 100 µl Streptavidin-Alexa 647 dye solution).

25.8.6.2. Use a designated 200 µl Pipet-Lite labeled "S-Alexa Prep".

25.8.6.3. Store the aliquots to be used for the following week in a light-protected box at 4°C. Store the rest in a light-protected box at -20°C.

25.8.7. *0.05% Tween 20 Solution (20 L)*

25.8.7.1. Rinse a 20 L carboy out with 300 ml of isopropanol.

25.8.7.2. Rinse the carboy twice with 4 L of DI-H₂O and completely drain the carboy.

25.8.7.3. Add 19.99 L of MQ-H₂O to carboy.

25.8.7.4. Add 10.0 ml of Tween 20.

25.8.7.5. Mix well by swirling. This solution can be stored up to 2 weeks at ambient temperature.

Chapter 26

Validation

26.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for running Validator.

26.2. RESPONSIBILITY

The Array Hyb group leader is responsible for the implementation and for the correct execution of this protocol.

26.3. REQUIRED MATERIALS AND EQUIPMENT

Access to P:\FileMover and P:\Validator

26.4. PROCEDURE

26.4.1. *Running Validator*

- 26.4.1.1. Double-click on the Validator Icon located on P:\validator
- 26.4.1.2. Validator will check files in P:\iconix_format.
- 26.4.1.3. Verify that there is one pair of .tif and .txt files for every image scanned.
- 26.4.1.4. Double click on "Validator" and a black box will appear. The validator program is running now.

**Note: The more files within the "ToBeValidated" folder the longer the validator will run.*

- 26.4.1.5. When the validator is finished, a text file will pop up which can look like this:

```
Fri May 03 08:43:39 PDT 2002 : Starting PreLoader with Log File
Fri May 03 08:43:39 PDT 2002 : Reading available image files...
Fri May 03 08:43:45 PDT 2002 : Done reading image files!
Fri May 03 08:43:45 PDT 2002 : Data Files: 6 Hybs are available.
Fri May 03 08:43:45 PDT 2002 : SELECT * FROM
LIMS.PRELOAD_REPORT
Fri May 03 08:43:46 PDT 2002 : LIMS Rows: 6 Hybs are available.
Fri May 03 08:43:46 PDT 2002 : Validating LIMS Data
Fri May 03 08:43:47 PDT 2002 : List Validate error. Value(0.5%
CMC) for Hyb
#80206 RNA #R81975 Array #T00143887 in column
VV ANIMAL.VEHICLE
```

Fri May 03 08:43:47 PDT 2002 : List Validate error. Value(0.5% CMC) for Hyb

#80205 RNA #R81974 Array #T00143885 in column

VV ANIMAL.VEHICLE

Fri May 03 08:43:47 PDT 2002 : **Done with validation phase!**

Fri May 03 08:43:47 PDT 2002 : Replicate Hash contains 0 elements

Fri May 03 08:43:47 PDT 2002 : Validated Hash contains 0 elements

- 26.4.1.6. Look between the "Validating LIMS Data" line and the "Done with Validation phase!" Everything in between tells you what type of error was found. In the above example, there are two validation errors (underlined). Sometimes errors occur because the software does not recognize a value (e.g. 0.5% CMC is not recognized, but is legitimate). If a legitimate value is not recognized, contact Database Support. Blank fields will cause validation errors. If no errors are found, everything passed.
- 26.4.1.7. If an error is found, inform the proper person to correct the error (e.g. mRNA=mRNA team lead, cRNA=cRNA team lead, etc.).
- 26.4.1.8. Pull out files (both .txt and .tiff) that contain the errors from folder and place them in a temporary folder on the desktop until the errors can be fixed.
- 26.4.1.9. If the validation error is due to a mismatch between the slide barcode and hyb number, you must bring up the scanned image, re-enter the data and re-save the image containing the correct information. In addition, you must re-batchload and re-export the iconix format file. Make sure to delete all "old" files and replace with the corrected files. Validate again.

**Note: You must wait until the following day to re-validate so that the change is reflected in Oracle. Double check the "validbarcodes" list in P:\validator. The list contains all of the validated barcodes that will be moved by Filemover.*

26.4.2. Running Filemover

- 26.4.2.1. Double-click on the Filemover icon located on P:\filemover to open the software. Filemover currently does the following:
 - Move Tif. Image Files to P:\done_processing.
 - Move 2 files associated GEML file _for each slide barcode_ into P:\done_processing.
 - Renames the iconix_format files and moves them to P:\done_processing.

- Moves the 3 files associated with gridding to P:\done_processing.
 - Copies the renamed iconix_format files to X:\arraydata\ToBeLoadedin database.
- 26.4.2.2. Confirm all parts of the above list were executed properly by confirming the number of specific files that are moved (.sst, .tif, .txt, .grd, .XML. There should be 2 .txt files, 2.XML files for every barcode) or the total number of files moved (7x number of barcodes).
- 26.4.2.3. Once the moved files have been confirmed, place them in the “daily batch” subfolder in “done_processing” for archiving.

Chapter 27

Array Grading and QC

27.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for array grading and QC report generation.

27.2. RESPONSIBILITY

The Array group is responsible for implementation of this protocol.

27.3. REQUIRED MATERIALS AND EQUIPMENT

- PC or Mac
- Access to the UNV Main.FP5 database
- Access to Iconix LIMS system
- Access to P:\done_processing\Daily batches
- Access to P:\ChemExpress\Module.Lab.Motorola\Motorola QC\DrugMatrix Phase 4

27.4. PROCEDURE

27.4.1. *Download Array QC from LIMS System*

- 27.4.1.1. Open the web browser Internet Explorer (IE) and go to <http://lims> to pull up the Iconix LIMS system.
- 27.4.1.2. Download Array QC Data:
 - Go to the Array menu on the far right-hand side of the screen and choose “Review Array QC”.
 - Enter the appropriate beginning and ending hyb dates under HYB DATE. If searching for a single Hyb Date, put this date for both. Choose “Incomplete (I)” under HYB QC STATUS and press Export, the right gray button on the bottom of the Query Criteria menu. Press Export on the next screen as well and save the file as a text file to the desktop.
 - Open the text file in Excel and delete the first two rows.
 - Type “31% Rule” into cell S1.
 - Sort Column “HYB_NUM” in ascending order and note down the lowest Hyb number. Keep this file open.

- 27.4.1.3. Go to <http://lims> to pull up the Iconix LIMS system.
- 27.4.1.4. Download Array Correlation Data:
 - Go to the Array menu on the far right-hand side of the screen and choose “Review Array Corr.”
 - Enter the lowest Hyb# into the 1st field of HYB ID (second column from top on Review Array Corr). Leave the 2nd field of the HYB ID blank.
 - Choose “Incomplete (I)” under HYB QC STATUS and press Export, the right gray button on the bottom of the Query Criteria menu. Press Export on the following screen as well.
- 27.4.1.5. Once the exporting is done, go to File and choose Save As...
- 27.4.1.6. Save the file onto your desktop as a Text File.
- 27.4.1.7. Open the text file from Step 27.4.1.6 in Excel and delete the first two rows and the first five columns. Name the worksheet containing the data “MotorolaQC data mm.dd.yy”
- 27.4.1.8. The first column now should be HYB_NUM. Sort this column in ascending order.
- 27.4.1.9. Copy ALL columns and ALL rows containing data (including the header row).
- 27.4.1.10. Paste the copied cells into Column U of the file from Step 27.4.1.2. There should be a blank column separating columns QC_COMMENTS and HYB_NUM.
- 27.4.1.11. There should be two Hyb#s within a single row. Double check the Hyb#s match with each other. If there is a mismatch, the sorting of Hyb#s was not done properly. An exception is if there are *in vitro* samples or *in vivo* tissue samples which do not have its tissue control set in the “Review Array Corr” file on LIMS.
- 27.4.1.12. Once the Hyb#s within a single column have been confirmed to match one another, delete the blank column separating the two data sets. In addition, delete columns from 2nd data set up to the “Corr QC” column. Ultimately, there should be 29 data columns. Save the file as an Excel file on your desktop.
- 27.4.1.13. Sort the AVG_NORMAL_BG column in descending order and delete the rows of any arrays which do not have a value in the AVG_NORMAL_BG column.

27.4.1.14. Delete the "I"s in the QC_STATUS column.

27.4.2. First Round Grading

27.4.2.1. Arrays are graded "R" for review, "F" for fail or "P" for pass. All "R" graded arrays must ultimately become "P" or "F". Using the Array QC Data file, assign an Array Fate to each Hyb; all arrays are subjected to further QC Evaluation. If an array fails to meet any one of the conditions, it is designated "F":

- Log Dynamic Range > 1.0
- Fraction Signal < Threshold ≤ 0.6
- Ave. Normalized BG < 2.0
- Median Signal To Threshold > 0.8

27.4.2.2. Color the cell containing any failed value light yellow. Type "F" (F = Fail) in the QC_STATUS column and highlight the cell red (Table 27.4-A).

**Note: If a Hyb has multiple failed values, color each of the cells containing the failed value with Light Yellow.*

Table 27.4-A: First Round Array Grading

Hyb #	QC_STATUS	Ave NormBG	MedSig ToThresh	LogDyn Range	FractSig<Thresh
84216	F	0.299	0.460	3.629	0.541
84223	F	0.448	1.132	3.290	0.650
84217	F	2.513	1.215	3.751	0.700
84227		0.234	4.624	3.665	0.241
84108		0.284	7.798	3.093	0.190
84105		0.235	8.927	3.101	0.178

27.4.3. Second Round Grading

27.4.3.1. In the Array Correlation table, sort the T/C column in ascending order.

27.4.3.2. For a given tissue type, yellow highlights the corresponding Array Correlation value (e.g. If Hyb# 90329 shows HE in its T/C column, highlight its Array Correlation value under the HE column).

Table 27.4-B: Second Round Array Grading

T/C	HYB_NUM	QC_STATUS	BM	BR	HE	IN	KI	LI	SP	RHCYT
HE	90329	P	0.7100	0.6360	0.9720	0.7110	0.7310	0.6330	0.7470	0.6500
HE	90363	P	0.7100	0.6390	0.9680	0.7320	0.7470	0.6420	0.7460	0.6610
KI	89758	P	0.6970	0.6020	0.7430	0.7820	0.9680	0.7460	0.7390	0.7300
LI	90325	F	0.9020	0.5830	0.7360	0.7610	0.7310	0.6890	0.9480	0.6810
LI	90326	P	0.6510	0.4760	0.6410	0.7120	0.7560	0.9600	0.6760	0.8610
RHCYT	90146	P	0.6760	0.5200	0.6400	0.7030	0.7040	0.8060	0.6680	0.9070
RHCYT	90143	P	0.6890	0.5400	0.6920	0.7430	0.7680	0.8600	0.6930	0.9650

27.4.3.3. The Array Correlation Analysis compares the correlation of any test array (experimental or control) to any tissue control array. Each Hyb number is considered a test array. The T/C column indicates each Hyb#s Annotated Tissue type. The two-letter abbreviation Column Headers of each tissue type indicates what type of Tissue Control Array it is.

27.4.3.4. If an array fails to meet either of the following conditions, it is considered a failed array:

- An array is considered a mislabeled array and thus failed, if the correlation between the annotated tissue test array (experimental or control array) and the same tissue control array is < 0.800 **AND** the correlation of the test array to any other tissue control array is > 0.800 .
- An array is considered a poorly processed array and thus failed, if the correlation between the test array and its respective tissue control array is < 0.650 .

**Note: Exceptions to these two rules are Bone Marrow and Spleen samples. Bone marrow and spleen tissues are highly correlated to each other and cannot be used for filtering at this stage of the process. Do NOT fail these two types of samples if they do not meet the set conditions.*

27.4.3.5. In Table 27.4-B, an “F” would be added to the QC_STATUS column for Hyb # 90325 and the cell highlighted in red. In addition, change the annotated tissue correlation cell from yellow to red.

27.4.4. *Third Round Grading*

In the third round of QC Evaluation, Flag Count 2 and Array Comments are examined. Flag Count 2 refers to the number of "Empty" spots: spots where the signal mean is less than the local background mean plus one standard deviation of the local background.

- 27.4.4.1. Sort the CNT 2 column in descending order and for arrays that do not already have an "F" in the QC_STATUS column:
 - *In Vivo* arrays: Enter "R" in the QC_STATUS column if Count 2 $\geq 4,000$.
 - *In Vitro* arrays: If Count 2 $\geq 31.00\%$ of the sum of Counts 0, 2, and 3, enter "F" in the QC_STATUS column. Count 0 spots are "Good Spots" and Count 3 spots are "Poor Spots." To calculate the percent of Count 2 value: Format the "31% Rule" column by highlighting the whole column, pressing Ctrl+1 and choosing Percentage in the Number tab and press OK. For any *In Vitro* array, use the formula "**= 'Count 2 cell' / Sum ('Count 0, 2, and 3 cells')**" in the "31% Rule" column.
- 27.4.4.2. Use the AutoFilter function in Excel to filter the ARRAY_COMMENTS column for the comments shown below and enter "R" in the QC_STATUS column for arrays that do not already have an "F" if any of the following descriptions appear:
 - Low Signal
 - Minimal Signal
 - High Background
 - No/Low Signal in middle of array
 - No/Low Signal top/bottom of array
- 27.4.4.3. Type "P" in the QC_STATUS column for the rest of the arrays which have not been graded either "F" or "R".

Table 27.4-C: Third Round Array Grading

Hyb #	Array Fate	Ave Norm BG	MedSig To Thresh	LogDyn Range	FractSig <Thresh	Count2 (Empty Spots)	Array Comments
83948	F	1.808	0.668	3.188	0.650	7456	Minimal signal
83779	F	0.322	0.773	3.668	0.615	8233	Low Signal
83614	F	2.598	1.133	2.006	0.454	7965	Minimal Signal
83923	R	1.688	0.925	2.883	0.534	7582	Low Signal
84296	R	0.175	8.404	3.497	0.197	2603	Lower signal towards bottom
83735	R	0.176	10.324	3.461	0.192	2486	Areas of Low signal
83740	R	0.146	11.920	3.388	0.168	2594	Low Signal
84165	R	0.115	13.846	3.539	0.152	2377	Area of low signal in mid array
84277	R	0.106	26.812	3.526	0.149	2273	High background half array
84096	R	0.255	11.214	3.196	0.136	1787	Light streaks, Signal ok
83595	R	0.120	18.373	3.436	0.132	2014	High Background upper left corner
84367	P	0.135	13.967	3.440	0.152	2646	Signal ok
84382	P	0.148	14.150	3.457	0.153	2621	Signal ok

27.4.5. Fourth Round Grading

In the fourth round of grading, scatter plots and false-color images are simultaneously examined to determine array fate.

- 27.4.5.1. Go back to <http://lims> to pull up the Iconix LIMS system.
- 27.4.5.2. Go to the Array menu on the far right-hand side of the screen and choose "Array" then "Review Array QC".
- 27.4.5.3. Search for the Hyb ID(s) that were graded "R". Multiple entries may be submitted at once, with a comma (,) separating each entry in the "Hyb ID" field. Press "Query", the middle gray button on the bottom of the Query Criteria menu.
- 27.4.5.4. Click on the "HYB ID" column to sort the column on the Query Result table. Click on a Hyb# in the "HYB ID" column to bring up the False-color image and Scatter plot. These two images are examined simultaneously (Figure 27.4-A and Figure 27.4-B).

- 27.4.5.5. Inspect the false-color image for unevenness. Horizontal bands of green and red due to non-random printing of low and high expressers on the glass slide are “normal” for rat RU1 arrays (this pattern does not apply to human HU1 arrays). Big blotches of green and red colors or vertical bands of green and red colors are indicative of non-random/uneven hybridization.
- 27.4.5.6. Inspect the Scatter plot for a “tight” 45° slope of data points. The tightness is an indication of the overall assay quality.

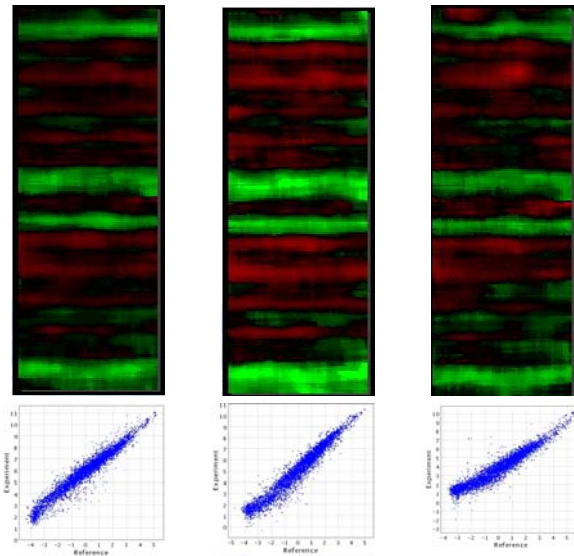


Figure 27.4-A: False-color images and Scatter plots of passed arrays

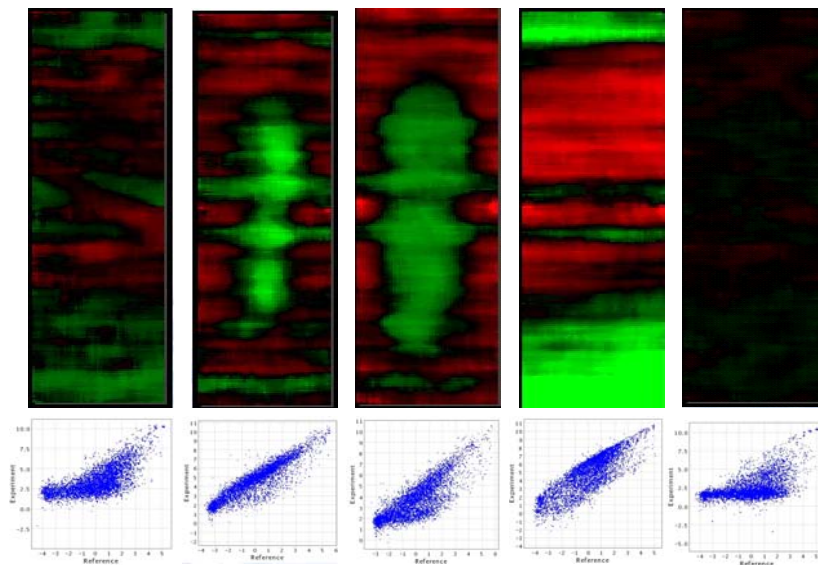


Figure 27.4-B: False-color images and Scatter plots of failed arrays

- 27.4.5.7. If necessary, view the scanned slide images, using GenePix Pro on the Scanner1 and Scanner2 PCs.
- 27.4.5.8. Open the GenePix Pro program and select Open/Save icon located on right-hand side of the Image tab. Choose Open Images. Go to P:\done_processing\Daily batches, and open the relevant array image.
- *Note: Array Images two weeks or older are archived by IS department and must be requested.***
- 27.4.5.9. If uncertainty about Array Fate persists after careful evaluation of the False-color image in combination with the Scatter plot, the array should be passed.
- 27.4.5.10. At the end of the Array QC set, type "P" in the QC_STATUS column for any arrays that were not failed. All arrays must be assigned a "P" or "F" grade.
- 27.4.5.11. For each array with an "F" grade, the reason(s) it failed must be entered at its corresponding QC_COMMENTS field.
- 27.4.5.12. In the Excel file, go to "Insert" menu and choose Worksheet. This new worksheet must be the 1st worksheet (left most) in the Excel file. Name this worksheet "LIMS UPLOAD".
- 27.4.5.13. Copy and paste the "HYB_NUM", "QC_STATUS", and "QC_COMMENTS" columns from the original worksheet to the new worksheet into columns A, B, and C, respectively. There must be a minimum of a single record (e.g. "80006" under HYB_NUM and "F" under QC_STATUS) in each field as blank cells will cause errors in LIMS. In addition, format the cells with the All Borders, even if the QC_COMMENTS cells are blank.
- 27.4.5.14. Save this Excel file in P:\ChemExpress\Module.Lab.Motorola\Motorola QC\DrugMatrix Phase 4 and name it as "MotorolaQC data mm.dd.yy" where the date is the day QC was done, not the date of the file creation.
- 27.4.5.15. If there are any Failed arrays, email the array group with the subject line Failed Hyb(s). State the reason why each hyb failed, e.g. Array Processing (Low signal or Low signal in the middle) or Failed CC QC or Failed 31% Rule. In addition, copy and paste the pertinent data columns from the Excel file from step 27.4.5.14:

RNA#	C/E	Animal	Tissue	Ctl RNA#	Hyb #	Array Fate	QC Comments
R94420	C	RM	TP		90614	F	Low signal in the middle
R94421	C	RM	TP		90615	F	Low signal

- 27.4.5.16. Go back to <http://lims> to pull up the Iconix LIMS system, go to the Array menu on the far right-hand side of the screen, and choose "Load Array QC".
- 27.4.5.17. Click on "Browse", select the Excel file from step 27.4.5.14, and "Load" the file.
- 27.4.5.18. A new page will come up displaying the Hyb #s and their respective Array Fates. Double-check these are the correct Hyb #s to be imported and click on OK. Once LIMS has finished loading the file as indicated by "No Matches" appearing in the blue bar, the IE may be closed.
- 27.4.5.19. Once or twice per week, create Missing Slides file by doing the following:
- 27.4.5.20. Open UNV_Main.FP5. Click the "Array Detection" tab and type in the password when prompted.
- 27.4.5.21. Click the "Review Array Data" tab of the species you are working with.
- 27.4.5.22. Go to "File", "Export Records". Name and save file onto desktop. Export the following fields:
- Array_NB_Page
 - Hyb_Num
 - Combi_RNA_Prep Num
 - Slide_Barcode
 - Array_Data_Fate
 - Array_Comments
 - Ready_for_Array_Reading
 - Array_Detection_Date
- 27.4.5.23. Open the exported text file from the desktop. Create the appropriate header for each column exported (order of data per column is exactly the order of how the fields were exported).

- 27.4.5.24. Delete any row containing "F" or "P" in the Array_Data_Fate column.
- 27.4.5.25. Delete any row containing the date of current export, one day to prior and one day after date of export within Array_Detection_Date column. For instance, date of this Export is 6/6/2002. Thus, delete any row containing dates 6/5/2002-6/7/2002 that is within Array_Detection_Date column.
- 27.4.5.26. Save as an Excel Workbook file into the P:\ChemExpress\Module.Lab.Motorola\Motorola QC\DrugMatrix Phase 4 folder. Name the file "Missing slide mm.dd.yy, e.g. "Missing slides 06.06.02".
- 27.4.5.27. Send this Excel file to Computational Sciences contact(s) to inform them of these slides with missing Array_Data_Fate.
- 27.4.5.28. At the end of each week, create a weekly QC Status.

27.4.6. Weekly QC Status Report

- 27.4.6.1. At the end of each week, create a weekly QC Status according to the following steps:
- 27.4.6.2. Go to the files from step 27.4.5.14 and combine all the Motorola QC data of the week into one file.
**Note: Do not delete any of the individual files.*
- 27.4.6.3. Save the QC data file as a Microsoft Excel Workbook in P:\ChemExpress\Module.Lab.Motorola\Motorola QC\DrugMatrix Phase 4. Name the file with the following method: "QC status of week 'date'", where date was Monday's date of the week (e.g. "QC status of week 06.03.02" for the period covering 06.03.02-06.07.02).

Chapter 28

Releasing Rejected Hybs

28.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for releasing rejected Hybs.

28.2. RESPONSIBILITY

The Array Target Prep group leader is responsible for the implementation and for the correct execution of this protocol.

28.3. REQUIRED MATERIALS AND EQUIPMENT

- Access to UNV_Main.FP5 Database

28.4. PROCEDURE

28.4.1. *Releasing Rejected Hybs*

- 28.4.1.1. Receive a list of samples that have previously failed hyb and need to be rehybed from the Array Group Manager.
- 28.4.1.2. Open FileMaker Pro, click on the “mRNA Extraction” button and type in the password given to you by your supervisor. Click on the pull-down menu in the upper left hand corner of the page titled “mRNA_...” and choose “Condensed_Target_Prep_Hyb_Summary”.
- 28.4.1.3. Perform a find for all the samples that need to be rehybed.
- 28.4.1.4. In UNV_MAIN FileMaker Pro, if the cRNA yield is less than 25µg, the sample needs to be released to either the mRNA group or the cRNA group as a rerun. If the cRNA comments indicate no mRNA sample is left, release the sample to the mRNA group as a rerun. If the cRNA comments indicate 3µl mRNA sample is left, release the sample to the Target prep group as a cRNA rerun. (Refer to Release cRNA samples protocol to release samples).
- 28.4.1.5. In UNV_MAIN FileMaker Pro, if the cRNA yield is greater than 25 µg, the sample can be released to the Hyb group provided that the same sample has not been rejected twice. Confirm cRNA yield by repeating “Quantitation of cRNA” protocol. If the sample has already been rejected twice a two hyb numbers will be associated with the same cRNA sample. These samples must be

sent back to the mRNA group or cRNA group as a rerun. If the cRNA comments indicate no mRNA sample is left, release the sample to the mRNA group as a rerun. If the cRNA comments indicate 3 µl mRNA sample is left, release the sample to the Target prep group as a cRNA rerun. (Refer to Release cRNA samples protocol to release samples).

- 28.4.1.6. If the sample is being released to the Hyb group, compare the old cRNA concentration to the new cRNA concentration. If the concentrations are within 20%, average the concentrations and yields. Enter the averages in FMP. Under cRNA comments, add the following comment "respec'd and changed concentration, mmddy, ave 2/2".
- 28.4.1.7. If the concentrations are not within 20%, repeat "Quantitation of cRNA" protocol. Select the two concentrations that are within 20% of each other. Average the concentrations and yields. Enter the averages in FMP. Under cRNA comments, add comment "respec'd and changed concentration, mmddy, ave 2/2". If all three concentrations are within 20% of each other, average all 3. Under cRNA comments, add comment, "respec'd and changed concentration, mmddy, ave 3/3".
- 28.4.1.8. Do an Agilent Bioanalyzer 2100 analysis of the cRNA sample if it has not been done in the past, i.e. during cRNA Plate processing. Refer to section 21.7 on how to proceed if the cRNA sample has not been precipitated.
- 28.4.1.9. If the cRNA sample has been precipitated, add 6 µl of DEPC-H₂O to 1 µl of the cRNA sample. Run the sample according to sections 18.6.3-18.6.9 (page 128).
- 28.4.1.10. To release samples to the Hyb group, refer to Release cRNA samples protocol.

Chapter 29

MagNA Pure LC Robot Maintenance

29.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for the maintenance of the MagNA Pure LC robot.

29.2. RESPONSIBILITY

The RNA Isolation group leader is responsible for the implementation and the correct execution of this protocol. The In-house MagNA Pure LC Consultant can be contacted regarding any problems with the robot.

29.3. SAFETY PRECAUTIONS

- Always wear your lab coat and protective eyewear, as well as gloves when working in the lab.
- In an emergency, immediately turn the power switch off and unplug the instrument.

29.4. REQUIRED MATERIALS AND EQUIPMENT

(for 1 run)

- There are two MagNA Pure LC robots at Iconix (Oddbot and 2-X-L). Both are currently running the MagaNA Pure software program version 2.0 (floppy and CD). These disks are located with the instruction manuals.
- High vacuum grease (Beckman Coulter Part No. 335148)
- Gloves
- Mask
- Waste bags (Roche Part No. 30004201)
- Lab Coat
- Safety glasses
- RNase Zap (Ambion Part No. 9780)
- Drop Catchers (Tub Lid Seals (Roche Part No. 3004104)

29.5. PROCEDURE

29.5.1. *General Daily*

Daily users are responsible for implementation

- 29.5.1.1. Always wear gloves and a mask when handling specimens and or reagents.
- 29.5.1.2. Keep waste tip slide clean by wiping down with damp paper towel.
- 29.5.1.3. Decontaminate the stage plate by wiping the surface with RNase Zap. ALWAYS spray RNase Zap on a tissue. DO NOT spray the RNase Zap directly on the instrument to avoid damaging electronic components.
- 29.5.1.4. Never operate the instrument without a waste bag secured at the end of the slide
- 29.5.1.5. To avoid collisions between the robotic arm and the cooling blocks, be sure the blocks are seated correctly on the Reagent Stage. Press the cooling blocks firmly to seat the pins on the blocks in the holes of the cooling block 2 Area. After each run, make sure the holes are clean. If necessary, clean these holes with a cotton swab.
- 29.5.1.6. At the end of each day, the robot must be decontaminated with UV light. Remove all plastics from the stage, close the robot door and click on "Decontamination". Set the time to 12 hours and click on the "Start Decontamination" button.
- 29.5.1.7. Dispose of chemically contaminated plastics in solid hazardous waste barrels (large gray metal barrel).

29.5.2. *Weekly*

RNA Team leader is responsible for implementation

- 29.5.2.1. Replace drop catcher every 2 days or when necessary (i.e. when change between sample species or types). To change the drop catcher:
 - Close the robot door and click on "Start Program" then on "Change Drop Catcher". This will bring the robot arm to the front of the stage so that the drop catcher is accessible.
 - Change the drop catcher then close the door and click on "Home" to send the robot arm home.

29.5.2.2. Grease the O-rings with vacuum grease so they don't dry out and crack. This must be done once a week. If the machine is not pipetting well the O-rings must be replaced.

29.5.2.3. Shutting down the robot and computer everyday is not necessary; however, the machines are turned off for the weekend. If the robot was shut down the previous night, allow the machine heating and cooling blocks to equilibrate for 30 minutes after turning on the machine.

29.5.3. *Periodic*

All RNA group members are responsible for implementation.

29.5.3.1. The waste collection paper on the floor should be changed on a monthly basis or as needed.

29.6. CONTACT INFORMATION

Roche Field Service Representative

Eriberto A. Aranjuez, Jr.

1-800-845-7355 ext 5653

MagNA Pure LC manuals and instruction

Manager of Array Group

Chapter 30

QIAgen BioRobot Maintenance

30.1. PURPOSE

Provide a Standard Operating Procedure (SOP) for the maintenance of the QIAgen BioRobot for automated target preparation.

30.2. REFERENCES

Motorola CodeLink Expression User Menu

30.3. RESPONSIBILITY

The Target Prep Array group leader is responsible for the implementation and for the correct execution of this protocol. The Target Prep Array group leader is to be consulted with any problems regarding the Bio Robots as well as responsible for any manuals and literature.

30.4. REQUIRED MATERIALS AND EQUIPMENT

There are two machines located in Iconix that are used for automated target preparation (QIAgen BioRobot 9604 - #2 and QIAgen BioRobot 3000 -#1).

30.5. PROCEDURE

30.5.1. *Equipment Maintenance*

- 30.5.1.1. Flush the peristaltic pump tubing using the wash procedures, which follow all protocols that use external peristaltic pump lines.
- 30.5.1.2. Release the peristaltic pump cassettes from the rollers after each run.
- 30.5.1.3. Check the level of the system liquid and if necessary refill the bottle with distilled water.
- 30.5.1.4. Empty the vacuum trap and empty the waste container.
- 30.5.1.5. Empty the used tip waste bag.
- 30.5.1.6. Disinfect and clean the tip disposal station.
- 30.5.1.7. Make sure the tip adapters are securely fitted and if necessary carefully tighten the retaining screw.
- 30.5.1.8. Disinfect and clean the tip adapters.

30.5.1.9. Disinfect and clean the worktable.

30.5.1.10. Rinse the vacuum manifold.

WARNING: Do not use bleach to clean robot deck as it can react with chaotropic salts to release toxic fumes!

30.5.2. *Prior to Each BioRobot Use:*

30.5.2.1. Follow instructions for BioRobot deck setup at the beginning of each target preparation step.

30.5.2.2. Ensure cassettes are fitted onto the Peristaltic Pump.

30.5.2.3. Empty the waste container and vacuum trap.

30.5.2.4. Fill system liquid and flush bottles with MQ water.

30.5.3. *After Each BioRobot Use:*

30.5.3.1. Turn off SpeedVac when not in use.

30.5.3.2. Cancel MJ programs when complete.

30.5.3.3. Empty the waste container and vacuum trap.

30.5.3.4. Fill system liquid and flush bottles with MQ water.

30.5.3.5. Ensure accessories are thoroughly cleaned of chaotropic salts.

30.5.4. *Weekly Maintenance Procedure*

Users of the robot are responsible for all weekly maintenance procedures.

30.5.4.1. Perform all daily maintenance

30.5.4.2. Visually inspect the dilutor syringe valve, and the syringe for leaks, contaminants, or air bubbles. If there are leaks, make sure all connections and screws are fully tightened. If you detect contaminants or air bubbles (and you have checked all connections, etc.) make sure the system liquid reservoir is full, and try flushing the system repeatedly (use the Flush button in the Execute toolbar). If these problems persist, contact QIAGEN Technical Services.

30.5.4.3. Empty and clean the waste container

30.5.4.4. Check the waste container for contaminants or precipitate build-up. If there is any build-up of contaminants or precipitates, clean the waste container and lid with a mild laboratory detergent, and rinse with water. Repeat as necessary.

- 30.5.4.5. Check the waste tubing for contaminants or precipitates; pour a small amount of cleaning solution down the waste slot in the wash station. Repeat as necessary.
- 30.5.4.6. Empty and clean the vacuum trap
- 30.5.4.7. Check the vacuum trap for contaminants or precipitate build-up. If there is any build-up of contaminants or precipitates, clean the waste container and lid with a mild laboratory detergent, and rinse with water. Repeat as necessary.
- 30.5.4.8. Replace the system liquid with deionized water. Make sure the lid is thoroughly rinsed with distilled or deionized water.

30.5.5. *Periodic and Corrective Maintenance*

- 30.5.5.1. Each BioRobot has a one-year service warranty. Within the warranty period, a QIAGEN service engineer will check the instrument performance and replace parts with normal wear and tear.
- 30.5.5.2. Replace the syringe dilutors as necessary (Qiagen order # 9232771).
- 30.5.5.3. Removing and/or replacing minor parts should only be attempted by experienced users or specially trained personnel.

**Note: When switching off the QIAGEN BioRobot, carefully hold the Z-Bars to stop them from falling into the wash station when the power is off.*

30.6. CONTACT INFORMATION

QIAGEN BioRobot

Ed Almodovar, Field Engineer

800-362-7737 X-22225

*Only the Target Prep team lead may contact

QIAGEN

Michelle Reid, Technical Service

800-426-8157 X-23516

Emergency Contact

In-house Qiagen BioRobot Consultant

Chapter 31

Hazardous Materials Guidelines

31.1. PURPOSE

Provide a Standard Operating Protocol (SOP) for proper handling of Medical and Hazardous waste.

31.2. RESPONSIBILITY

The individual group leaders are responsible for enforcing the execution of this protocol.

31.3. SAFETY PRECAUTIONS

- In case of any incident, ALWAYS notify your supervisor immediately.
- If a waste is BOTH Biohazardous AND Chemically Hazardous, then the Chemical Hazardous Waste regulations take precedence over the Biohazardous Waste regulations, and the waste is to be labeled, packaged and treated as chemical waste and not as biohazard waste.
- The Arrays Group (mRNA Isolation, cRNA Target Prep, Arrays) only produces chemical and hazardous waste.

31.4. PROCEDURE

31.4.1. *General Issues*

- All solutions (only exception is water bottles) need to be in secondary containers.
- ABSOLUTELY NO FOOD IN THE LAB AREA. No food related trash in the lab garbage cans. Dispose food in garbage cans outside the lab.
- EVERY waste container (liquid or solid) has to be labeled. Labeling supplies can be found on top of flammable cabinet.
- Latex gloves ONLY FOR MOLECULAR BIOLOGY WORK, not for work with solutions and chemicals!
- Nitrile gloves for work with SOLUTIONS AND CHEMICALS (change if necessary).
- PPE (Personal Protective Equipment) ALWAYS. Lab coats at all times. Closed-toe shoes at all times. Safety glasses at all times.
- Special PPE for homogenization (always). For homogenization, wear special gear, such as lab coat, protecting shield, nitrile gloves.

31.4.2. *Disposal of Waste*

31.4.2.1. Mixed Solvent Waste

- Covered container on bench top. Empty daily. Tagged with label stating "emptied daily"
- Disposal of empty ethanol bottles: Triple-rinse the bottle with WATER and label as "TRIPLE RINSE" and dispose in the glass disposal container.
- Disposal of empty solution bottles: If water soluble triple rinse and dispose in glass disposal container, if not water soluble, rinse first with ETHANOL OR METHANOL three times and then with WATER three times.
- Ethanol (left over from precipitates) NEVER goes down the sink. It goes into ONE GALLON WIDE MOUTH PLASTIC COATED GLASS "PICKLE JAR" BOTTLES (with Teflon lined cap) (tagged with label).

31.4.2.2. LAB TRASH is different from trash that gets taken care of by the janitor (includes probes, pipette tips, other debris from robot, etc.): Almost everything produced in the ARRAYS-GROUP is contaminated chemically, therefore belongs in the LAB TRASH containers (yellow bins, big blue or gray barrels).

- MagNA Pure and QIAgen solid waste goes into barrel in front of the robot.
- ALL pipette tips waste (no exceptions) is collected in covered container on bench. MUST BE COVERED AT ALL TIMES. (Tagged with label and it must say, "Emptied daily").
- Triple Rinse: every bottle that was triple rinsed needs to be labeled with "triple rinse" before disposing it in the glass disposable container

31.4.2.3. QIAgen robot liquid wash waste (mainly aqueous, resulting from the flushing of the BioRobot lines): Follow the "QIAgen Robot Maintenance" protocol. This waste has been tested and can be dumped down the sink.

31.4.2.4. QIAgen robot liquid waste (waste collected in the smaller vacuum trap container): the waste is collected in the 1-gallon Vacuum rated Nalgene bottles. Once full, the bottle gets swapped with an empty bottle and moved into the Hazardous Waste Cabinet. Always label bottles in use. The Hazardous

Waste contractor will do the decanting from the 1-gallon container into the barrel, and then the empty bottles can be reused.

31.4.3. *Biohazard Waste*

The biohazardous waste is picked up weekly by a medical waste hauler.

- 31.4.3.1. Tissue Culture Waste: flask containing cell debris, serological tips.
- 31.4.3.2. Red Biohazard containers: NOT USED IN ARRAYS GROUP. ONLY non-chemically contaminated waste may be disposed in Red Biohazard containers.
- 31.4.3.3. Yellow waste containers: For chemical waste, but also biohazard waste contaminated with chemically hazardous material LAB TRASH.

31.4.4. *Chemical Waste*

- 31.4.4.1. Homogenization probes: Dispose in CHEMICAL SHARP CONTAINERS FOR MATERIAL CONTAMINATED WITH HAZARDOUS MATERIAL.
- 31.4.4.2. Full yellow hazardous waste containers: Full bags need to be placed in hazardous waste barrels in the closet next to the receiving area (please log in what you deposit).
- 31.4.4.3. Razor blades: collect in 30-gallon barrel after use (CHEMICAL SHARPS CONTAINER).
- 31.4.4.4. Syringes: collect in 30-gallon barrel after use (CHEMICAL SHARPS CONTAINER).
- 31.4.4.5. Tissue Culture Waste: anything treated with DMSO or other compounds. This waste is picked up several times a year..

31.4.5. *Labeling Requirements*

- 31.4.5.1. All internally collected hazardous waste containers are labeled with:
 - The contents, physical state, and hazardous properties (*e.g.* flammable, poison, corrosive, reactive, or biohazard)
 - The accumulation start-date (except for biohazard waste)
 - Iconix Pharmaceuticals, address, phone number and EPA number (except for biohazard waste).

Summary of Changes between V 3.2 and V 3.7

Chapter 12: Cell-Specific Details

- This chapter was excluded from the SOP because we no longer use different cell lines for *in vitro* experiments. The relevant sections pertaining to use of Primary Rat Hepatocytes were combined with chapter 13.
- All the following chapters have changed by one number.

Chapter 13: *In Vitro* Proliferation Assay

- This chapter combined with parts from chapter 12 was renamed “Primary Rat Hepatocyte Culture and *In Vitro* Cytotoxicity and Proliferation Assays”.
- Media and handling details were changed to reflect an updated protocol. The primary rat hepatocytes are no longer plated in WME medium, but rather in IVT medium.

Chapter 15: Retrieving Tissues

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 16: Sample Preparation for RNA Isolation (Now Chapter 15)

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 17: RNA Isolation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 19: Running the Agilent 2100 Bioanalyzer

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 20: Ribogreen Analysis

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Summary of Changes between V 3.7 and V 3.8

Chapter 14: Retrieving Tissues

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 15: Sample Preparation for RNA Isolation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 16: RNA Isolation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 18: Running the Agilent 2100 Bioanalyzer

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 19: Ribogreen Analysis

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 20: Release mRNA Samples

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 21: Target Preparation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 22: cRNA Quality Control and Precipitation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 23: Release cRNA Samples

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 24: cRNA Fragmentation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 25: Array Hybridization

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 26: Validation

- Folder names and directory and pathways were changed to reflect the new location of relevant files.

Chapter 27: Array Grading and QC

- Folder names and directory and pathways were changed to reflect the new location of relevant files.