

Procedure 2.4 – Agilent Two-Color Quality Assurance

Date: _____

Experiment ID: 2.4-~~2525~~mmdd

Technician: _____

Project: _____

Client Signature: _____

1. Prepare Spike A Mix and Spike B Mix

1.1 Assign stock solutions LMT numbers (Appendix 7.3). _____

1.2 Dilute the stock solutions according to the following table.

Starting amount of RNA		Serial dilution			Spike A Mix or Spike B Mix volume to be used (µL)
Total RNA (ng)	PolyA RNA (ng)	First	Second	Third	
50-200		1:20	1:40	1:16	2
201-2000		1:20	1:40	1:4	2
>2000	200	1:20	1:40	0	2

1.3 Assign dilutions LMT numbers (Appendix 7.3) and store aliquots as described in Procedure 1.2 _____

2. Prepare Labeling Reactions (Agilent LRILAK Kit) _____

2.1 Combine the following in a PCR tube or plate.
 The following RNA must be used.

Stratagene Universal Human Reference RNA – Cyanine3
 Ambion Human Brain Reference RNA – Cyanine5

Total RNA	500 ng	_____
Appropriate dilution of Spike Mix (A or B)	2ul	_____
T7 Promoter Primer	1.2 ul	_____
Add nuclease free water to a total volume of	11.5 ul.	_____

2.2 Place in thermocycler and run <LRILAK1>

2.3 Prewarm the 5X first strand buffer at 80C for 5 minutes.

2.4 Prepare a First Strand Master Mix of the following components by multiplying the volumes by n.5, where n is the number of reactions.

5X First Strand Buffer	4.0 ul
0.1M DTT	2.0 ul
10mM dNTP mix	1.0 ul
MMLV-RT	1.0 ul
RnaseOut	0.5 ul

2.5 Add 8.5ul of the Master Mix to each reaction.

2.6 Place in thermocycler and run program <LRILAK2>

40C	2 hours
65C	15 minutes
4C	forever

2.7 Prewarm the 50% PEG solution at 40C for 1 minute.

2.8 Prepare a Transcription Master Mix of the following components by multiplying the volumes by n.5, where n is the number of reactions.

Nuclease-free water	15.3 ul	_____
4X Transcription Buffer	20.0 ul	
0.1M DTT	6.0 ul	
NTP mix	8.0 ul	
50% PEG	6.4 ul	
RnaseOut	0.5 ul	
Inorganic Pyrophosphatase	0.6 ul	
T7 RNA Polymerase	0.8 ul	
Cyanine 3-CTP or Cyanine 5-CTP	2.4 ul	

2.9 Add 60ul of Transcription Master Mix to each reaction. Mix well.

2.10 Place in thermocycler and run program <LRILAK3>

40C	2 hours
4C	forever

3. Purify the labeled cRNA using Qiagen RNeasy Mini. _____

3.1 Add 20ul nuclease-free water to the cRNA sample. _____

3.2 Add 350ul Buffer RLT and 250ul 100% EtOH and mix.

3.3 Transfer to RNeasy mini column and centrifuge at 13,000 RPM for 30 seconds at 4C. Discard the flowthrough.

3.4 Add 500ul Buffer RPE to the column and centrifuge at 13,000 RPM for 30 seconds at 4C. Discard the flowthrough.

3.5 Repeat 3.4

3.6 Centrifuge for one minute to dry the column.

3.7 Elute the cRNA into a clean collection tube by pipetting 30ul nuclease-free water onto the column, incubating for 60 seconds at room temperature, and centrifuging at 13,000 RPM for 30 seconds at 4C.

4. Quantify the cRNA

4.1 Determine cRNA, Cy3, and Cy5 values for the sample(s) using the NanoDrop spectrophotometer (Appendix 7.1).

4.2 Save NanoDrop Report as <2.4_yyyymmdd_NanoDrop> in appropriate SOP Reports folder.

4.3 Calculate specific activities (pmol CyDye per ug cRNA) and add to NanoDrop data.

$$\text{Specific activity} = [\text{CyDye}] / [\text{cRNA}] * 1000$$

5. Prepare the 10X Blocking Agent (Stock)_____

5.1 Add 500 ul nuclease-free water to the vial containing lyophilized 10X Blocking Agent.

5.2 Heat the solution at 37C for five minutes.

5.3 10X Blocking Agent can be stored at -20C for 2 months. Assign the blocking agent an LMT number (Appendix 7.3).

6. Prepare Hybridization Samples

(Hybridization Kit) _____

6.1 Combine the following in a PCR tube or plate according to the following chart.

Components	Volume/Mass 4x44K
cyanine 3-labeled, linearly amplified cRNA	825 ng
cyanine 5-labeled, linearly amplified cRNA	825 ng
10X Blocking Agent	11 μ L
Nuclease-free water	bring volume to 52.8 μ L
25X Fragmentation Buffer	2.2 μ L
Total Volume	55 μL

6.2 Incubate at 60C for exactly 30 minutes.

6.3 Scan microarray barcodes into an Excel sheet and record corresponding sample identifications. Save file as <2.4_yymmdd_microarrays> in appropriate SOP Reports Folder.

6.4 Add 55ul 2X GEx Hybridization Buffer. Mix. Use Immediately.

7. Prepare the Hybridization Assembly.

7.1 Load a clean gasket slide into the SureHyb chamber.

7.2 Dispense 100ul of the hybridization sample(s) into the gasket well(s).

7.3 Slowly place the microarray onto the gasket slide so that the barcode is facing up.

7.4 Place the SureHyb cover onto the sandwiched slides and slide the clamp onto both pieces. Hand-tighten the clamp.

7.5 Place assembled slide chamber in rotisserie in a hybridization oven set to 65C. Set rotation speed at 10 RPM.

7.6 Hybridize at 65C for 17 hours.

7.7 Warm Wash Buffer 2 at 37C overnight.

8. Microarray Wash

8.1 Disassemble SureHyb chambers and carefully remove gasket slides under wash buffer 1.

8.2 Place microarrays in steel rack in staining chamber containing wash buffer 1. _____

8.3 Place on rotator in ozone cabinet. Set speed at 3 and rotate in the dark for one minute.

8.4 Transfer slides to a new staining dish containing prewarmed wash buffer 2. Set speed at 3 and rotate in the dark for one minute. _____

8.5 Centrifuge slides at 500 RPM for 5 minutes to dry and scan as described in Appendix 7.4.

9. Data Analysis

9.1 FDA-MAQC data files VARI data files must be adjusted to remove all duplicates and controls and so that all column headers are the same between all arrays.

9.2 Use limmaGUI to normalize and export m-values. Perform global lowess normalization within microarrays and quantile normalization between microarrays.

9.3 Generate a correlation matrix for all of the microarrays and evaluate the data.

9.4 Correlations are expected to fall near these values.

Fold-change greater than 2 – 0.95 correlation_____

Fold-change greater than 1 – 0.94 correlation_____

Fold-change greater than 0.5 – 0.93 correlation_____