

MAQC Study – Data Generation Plan

Agilent Technologies Gene Expression Platform

Test Sites

1. Test site: Agilent Technologies R&D
Site Code: AGL_1
Contact person: Jim Collins
Address: 3500 Deer Creek Road, MS 24M, Palo Alto, CA 94304
Phone: 650/485-5048
2. Test site: FDA/NCTR
Site Code: AGL_2
Contact person: Tucker Patterson
Address: Division of Neurotoxicology, HFT-132, National Center for Toxicological Research, U.S. Food & Drug Administration, 3900 NCTR Rd., Jefferson, AR 72079
Phone: 870/543-7427
3. Test site: Icoria, Inc.
Site Code: AGL_3
Contact person: Ed Lobenhofer
Address: 108 T. W. Alexander Drive, P.O. Box 14528, Research Triangle Park, NC 27709-4528
Phone: 919/425-2904

Materials Required

Agilent Products

- Agilent 2100 Bioanalyzer and consumables (Not provided by Agilent)
- RNA 6000 Nano LabChip Kit (P/N 5065-4476) (Not provided by Agilent)
- 2 x Agilent Low RNA Input Fluorescent Linear Amplification Kit (P/N 5184-3523) = 40 reactions (**3 kits will be provided**)
- 1 x Agilent RNA Spike-in Kit (5188-5279)(**1 kit will be provided**)
- 4 x Whole Human Genome Oligo Microarray Kit (P/N G4112A) = 20 microarrays (**5 kits will be provided**)
- 4 x Hybridization Backing Kit - SureHyb enabled, 1 x 22K or 44K format; 5/pack (P/N G2534-60003) = 20 gasket slides (**5 kits will be provided**)
- 2 x Gene Expression Hybridization Kit (P/N 5188-5242)(**3 kits will be provided**)
- 1 x Gene Expression Wash Buffer 1 (P/N 5188-5325) = 4L (**1 cubitainer will be provided**)
- 1 x Gene Expression Wash Buffer 2 (P/N 5188-5326) = 4L (**1 cubitainer will be provided**)
- Stabilization and Drying Solution (5185-5979) = 500 mL (**2 bottles will be provided**)
- Hybridization Oven (P/N G2505-80086)–set at 65°C (Not provided by Agilent)

- Oven Hybridization Rotator for use with Agilent Microarray Hybridization Chambers (P/N G2530-60029) (Not provided by Agilent)
- 20 x Hybridization Chamber – SureHyb (P/N G2534A)(Not provided by Agilent)
- Agilent DNA Microarray Scanner BA (P/N G2565BA)
- Agilent Feature Extraction Software, version 8.1 (P/N G2567AA)

Non-Agilent Products

- Total RNAs
 - RNA A: Ambion Human Brain Reference RNA (**AHBRR**)
 - RNA B: Stratagene Universal Human Reference RNA (**SUHRR**)
 At least 12 µg of each RNA will be provided.
- All other non-Agilent reagents and equipment required are described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit manual (P/N 5185-5818) and the Two-Color Microarray-Based Gene Expression Analysis v 4.0 manual (P/N G4140-90050).

User Manuals

- Stratagene Universal Human Reference RNA – Catalog # 740000
- Agilent RNA Spike-In Kit – P/N 5188-5279
- Agilent Low RNA Input Fluorescent Linear Amplification Kit, Version 4.0 Early Access – P/N 5185-5818
- Agilent Two-Color Microarray-Based Gene Expression Analysis, Version 4.0 Early Access – P/N G4140-90050

Experimental Design

Five replicate hybridizations will be processed for each of the following:

RNA Pair Code	RNA Pair Description
A	SUHRR-Cy3 vs. SUHRR-Cy5
B	AHBRR-Cy3 vs. AHBRR-Cy5
C	SUHRR-Cy3 vs. AHBRR-Cy5
D	AHBRR-Cy3 vs. SUHRR-Cy5

RNA Codes are modified from those outlined in Table 2 of the spreadsheet labeled “Naming convention” in the “RNA quality report template.xls” file distributed by Leming Shi. Codes are modified to represent the fact that Agilent test sites will only use two of the RNA samples provided and these will be processed using Agilent’s recommended design.

Replicates will be labeled as 1, 2, 3, 4 and 5.

Total RNA Preparation and Analysis

SUHRR (RNA A) will be provided by Stratagene in a solution of 70% ethanol and 0.1 M sodium acetate. This should be prepared as per the procedure recommended in Stratagene’s Catalog #740000.

AHBRR (RNA B) will be provided in aqueous solution ready for use.

RNA quantitation and purity assessment: RNA samples should be quantitated using a NanoDrop ND-100 UV-VIS or equivalent. Three replicate measurements should be taken for each sample and reported as average \pm standard deviation. For the NanoDrop ND-100, sample volumes of 1.5 μ L should be used.

RNA intactness assessment: 200 ng quantities of SUHRR and AHBRR should be run on the Bioanalyzer 2100, three replicates each (all samples on one chip). Bioanalyzer traces must be saved. rRNA Ratio [28S/18S] and RIN values should be reported as reported as average \pm standard deviation.

Quality control cutoffs:

Acceptable A_{260}/A_{280} ratios are in the range 1.8 – 2.2. Samples with A_{260}/A_{280} ratios outside this range must be requantitated to confirm, and then be replaced with higher quality samples from RNA vendor.

Acceptable rRNA Ratio [28S/18S] values are > 0.9 . Samples with ratios < 0.9 must be rerun on the Bioanalyzer to confirm and then be replaced with higher quality samples from RNA vendor.

Acceptable RIN values are > 8.0 . Samples with RIN values < 8.0 must be rerun on the Bioanalyzer to confirm and then be replaced with higher quality samples from RNA vendor.

Data reporting: The following information should be input to the spreadsheet labeled “total RNA QC” in the “RNA quality report template.xls” file distributed by Leming Shi:

- Investigator
- A_{260}/A_{280}
- Concentration (μ g/ μ l)
- RIN
- 28S/18S Ratio
- BioAnalyzer Filename

Sample Labeling

Ten replicate labeling reactions should be set up per dye for each sample as follows:

10 x SUHRR-Cy3
10 x SUHRR-Cy5
10 x AHBRR-Cy3
10 x AHBRR-Cy5

- Procedure described in Agilent Low RNA Input Fluorescent Linear Amplification Kit (Version 4.0 Early Access) manual must be followed. When using a thermocycler, reactions should be incubated in single tubes rather than in 96-well reaction plates. [Exception – If a thermocycler is not available, waterbaths/ice may be used to substitute for incubations.]
- 500 ng of total RNA must be input per reaction.
- Spike-in mix preparation should follow the procedure described in Agilent RNA Spike-In Kit manual. For a 500 ng input amount, this specifies doing a three step serial dilution (1:20, 1: 40, 1:4) for each spike mix and inputting 2 μ L of dilution 3 per reaction.
- Qiagen RNeasy columns should be used for purifying cRNA samples (rather than Agilent Cleanup Module) following the procedure outlined in the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Version 4.0 Early Access) manual,

Appendix C. NOTE: cRNA should be eluted from Qiagen RNeasy columns in one volume of 30 μ L.

- If possible, all samples should be labeled in one batch, i.e., set up at same time, same master mixes, etc.

cRNA Analysis

Assessment of cRNA yield and specific activity: cRNA yields and dye incorporation rates should be determined spectrophotometrically using a NanoDrop ND-100 UV-VIS or equivalent. For the NanoDrop ND-100, a sample volume of 1.5 μ L should be used. Specific activity values should be calculated as pmol of dye/ μ g cRNA.

cRNA size distribution assessment: 200 ng quantities of SUHRR and AHBRR cRNAs must be run on the Bioanalyzer 2100, one replicate per labeled cRNA. Bioanalyzer traces must be saved.

Quality control cutoffs:

Acceptable cRNA yield and specific activity values are:

cRNA yield: > 2 μ g

cRNA specific activity: 8-15 pmol dye/ μ g cRNA

cRNA samples that do not meet both of these criteria should be regenerated. Values for both passing and failing samples must be recorded and reported to the MAQC study.

Data reporting: The following information should be input to the spreadsheet labeled “target prep QC” in the “RNA quality report template.xls” file distributed by Leming Shi:

- Investigator
- cRNA yield (μ g), replicates 1-5
- BioAnalyzer Filename
- cRNA specific activity (pmol dye/ μ g), replicates 1-5 (under site-specific QC data)

Sample Hybridization

Five replicate 1X Hybridization Solutions should be prepared for each sample pairing as follows:

5 x SUHRR-Cy3 vs. AHBRR-Cy5

5 x AHBRR-Cy3 vs. SUHRR-Cy5

5 x SUHRR-Cy3 vs. SUHRR-Cy5

5 x AHBRR-Cy3 vs. AHBRR-Cy5

- Procedure described in Agilent Two-Color Microarray-Based Gene Expression Analysis (Version 4.0 Early Access) manual must be followed.
- 1.5 μ g of cRNA must be hybridized per dye channel per microarray.
- All hybridizations must be processed in one batch, i.e., set up at same time and incubated in same oven.
- During all processing steps, the order of the 20 microarrays being used should be randomized, i.e., Hybridization Solutions should be hybridized to randomly selected microarrays, microarrays for washing batches should be selected randomly, scan order should be randomized.
- Microarrays must be hybridized at 65°C for 17 hours.
- Microarrays must be washed in two batches of 10 slides each.

- Microarrays must be washed with Agilent Gene Expression Wash Buffers 1 (room temperature) and 2 (elevated temperature) as described in user manual.
- Final microarray wash must use Stabilization and Drying Solution as described in user manual.
- Slides must be scanned as soon as possible after washing.

Data Acquisition

Microarray scanning: All 20 microarrays must be scanned in one batch in random order using default settings:

- PMT gain setting = 100% PMT for both channels
- Scan area = 61 x 21.6 mm
- Scan Resolution = 10 μ m

Data extraction: Data must be extracted using Feature Extraction Software, version 8.1 using default settings. Select all output formats (Text, MAGE, JPEG, GEML, Visual Results, QC Report, Grid).

Data reporting: All output files must be renamed as per the convention specified in the spreadsheet labeled "Naming convention" in the "RNA quality report template.xls" file distributed by Leming Shi. Examples of file names for RNA Pair Code A for replicate 1 processed by the FDA/NCTR will be:

- AGL_2_A1.txt
- AGL_2_A1_MAGEML.xml
- AGL_2_A1.jpg
- AGL_2_A1.xml
- AGL_2_A1.shp
- AGL_2_A1.QCReport.HTML
- AGL_2_A1_grid.csv

All files (including completed "RNA quality report template.xls") should be transferred to the FDA/NCTR as per instructions to be distributed by Leming Shi.