MAQC Main Study Plan (Draft)

Applied BioSystems Gene Expression Arrays and Assays

Part I: Microarray Study

A. Test sites:

1. Test site I: Applied BioSystems R&D
   Contact Person: Lu Zhang
   Address: 850 Lincoln Centre Drive, MS447, Foster City, CA 94404
   Phone: 650-638-5045

2. Test site II: EPA/NHEERL
   Contact Person: David Dix
   Address: Reproductive Toxicology Division (MD-72), Nat'l Health&Environmental Effects Research Lab/Office of R&D, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711
   Phone: 919-541-2701

3. Test site III: Vanderbilt University
   Contact Person: Shawn Levy
   Address: Vanderbilt Microarray Shared Resource, Vanderbilt University, 465 21st Ave South, MRBIII Room 9274, Nashville, TN 37232
   Phone: 615-936-3003

B. Materials and Equipment Required:

- ***RT-IVT labeling:***
  1. NanoAmp™ RT-IVT labeling kit (P/N 4365715) **(Provided by ABI)**
  2. NanoAmp™ Rt-IVT labeling kit protocol (P/N 4365710)
  3. Quick Reference Card, NanoAmp™ RT-IVT labeling kit (P/N 4365711) **(Provided by ABI)**
  4. 96-Well GeneAmp PCR System 9700 (P/N4314878) or other equivalent PCR machine
     All other reagents and equipments required for RT-IVT labeling are described in NanoAmp™ Rt-IVT labeling kit protocol (P/N 4365710) Materials and Equipment section (Page 3-7)

- ***Microarray hybridization and Chemiluminescent detection***:
  5. Chemiluminescent detection Kit (P/N 4342142) **(Provided by ABI)**

6. Protocol, Chemiluminescent detection Kit (P/N 4339627)
7. Quick Reference Card, Chemiluminescent detection Kit (P/N 4346875) **(Provided by ABI)**
8. Applied BioSystems Microarray Wash Tray (P/N 4352927)
9. Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (P/N 4338036)
10. Expression Array System Analyzer Software Version 1.1.1
11. Human Genome Survey Microarray Version 2.0 (12 pack, P/N 4359031) **(Provided by ABI)**
12. Anti-Digoxigenin-AP, Fab fragments: 150 U (200 ul) (By Roche Applied Science, P/N 1093274) **(Provided by ABI)**
13. Digoxigenin-UTP:200nmol (57 ul) (By Roche Applied Science, P/N 3359247) **(Provided by ABI)**
14. Chemiluminescent Microarray Analyzer User Guide (P/N 4338852)
15. Chemiluminescent Microarray Analyzer Chemistry Guide (PN 4338853)
16. Hybridization Oven at 55^0C (Recommended: Signature Benchtop Shaking Incubator, Model 1575 by VWR Scientific, or Infors HT Minitron Incubator Shaker)
17. VWR Rocking Platform Shaker, Single-Tier Platform (VWR, P/N 40000-300 for North America, P/N 40000-302 for Europe and other regions)

All other reagents and equipments required for microarray hybridization and chemiluminescent detection are described in Chemiluminescent detection Kit protocol (P/N 4339627) Materials and Equipment section (Page 3-7)

- ***Other Materials:***

Four Total RNAs with each greater than 15 ug (Provided by Ambion and Stratagene): Universal Human Reference RNA (Stratagene, SUHRR) (**A**), Brain (Ambion) (**B)**, 25% Brain / 75 % SUHRR (**C**), 75% Brain / 25 % SUHRR (**D**)
Agilent Bioanalyzer 2100 and RNA 6000 Nano kit

C. Experimental Design:

Five technical replicates, which include labeling and corresponding microarray hybridization reactions, will be processed for all four total RNA samples (A, B, C and D respectively). One individual will label all samples on one day. The microarray hybridizations will be split into two groups run on two separate days. The same person should hybridize microarrays on the two separate days. On day 1, three replicates for all four samples (A, B, C and D) will be hybridized and detected. On day 2, the remaining two replicates for all four samples (A, B, C and D) will be hybridized and detected. The hybridization day for each array will be documented and submitted to the MAQC along with the primary data.

D. Experimental Procedures:

1. Total RNA QC:
   Upon receiving, all four total RNA samples (A, B, C and D) will be quantified with a spectrophotometer. The concentration and OD260/OD280 ratio for each sample will be documented in the template provided by MAQC project (MAQC_RNA_Quality_Report_Template.xls).
   RNA integrity will be checked by running 250ng total RNA sample on Bioanalyzer system. 28S/18S ratio and RIN numbers will be documented in the same template as above.

   Note: If a RIN number < 9.0 is observed, report the finding to MAQC team and do not proceed further.

2. Total RNA labeling:

   1 ug of each total RNA sample will be used as the starting material and the labeling reaction will be performed according to the procedure described in the Applied BioSystems NanoAmp™ Rt-IVT labeling kit protocol (P/N 4365710) and Quick Reference Card, NanoAmp™ RT-IVT labeling kit (P/N 4365711). All 24 samples will be labeled at the same process by same person.

3. Labeled cRNA QC:

   All the labeled cRNA will be quantified using the spectrophotometer and cRNA quality will be checked using Bioanalyzer system with 250ng samples. The cRNA concentration, cRNA size distribution and a median size value will be reported in the template provided by MAQC project (MAQC_RNA_Quality_Report_Template.xls).

4. Microarray Hybridization:

   The hybridization reaction will be performed according to the procedure described in the Protocol, Chemiluminescent detection Kit protocol (P/N 4339627) and Chemiluminescent detection Kit Quick Reference Card (P/N 4346875)

5. Chemiluminescent detection:

   The Chemiluminescent detection will be performed according to the procedure described in the Protocol, Chemiluminescent detection Kit protocol (P/N 4339627) and Chemiluminescent detection Kit Quick Reference Card (P/N 4346875) with the following modifications (Quick Reference Card has been referred here).
   At the Performing Hybridization Washes step, these changes should be followed:
   3. Perform hybridization wash 1:

a. Cover the wash tray with the wash tray cover, then agitate on the rocking platform shaker for 10 minutes, instead of 5 minutes

4. Perform hybridization wash 2:

a. Cover the wash tray with the wash tray cover, then agitate on the rocking platform shaker for 10 minutes, instead of 5 minutes

6. Data Acquisition:

After the microarray has been processed, the images will be generated according to the guidance described in the Protocol, Chemiluminescent detection Kit protocol (P/N 4339627) and Chemiluminescent detection Kit Quick Reference Card (P/N 4346875). The samples should following the naming convention guide provided by MAQC documents (MAQC_RNA_Quality_Report_Template.xls). The output data will be generated using the Expression Array System Analyzer Software Version 1.1.1 and both microarray images (.tif) and output data will be delivered to the MAQC.

7. Data Analysis Guide:

The initial data analysis could follow the guidance in the following documents: Chemiluminescent Microarray Analyzer User Guide (P/N 4338852) and Chemiluminescent Microarray Analyzer Chemistry Guide (PN 4338853). Advanced data analysis guide will be provided separately.

Part II: TaqMan RT-PCR validation

A. Test sites

8. Test site I: Applied BioSystems R&D
   Contact Person: Lu Zhang
   Address: 850 Lincoln Centre Drive, MS447, Foster City, CA 94404
   Phone: 650-638-5045

B. Materials Required

***Applied Biosystems Products*:**

- TaqMan Gene Expression Assays (P/N 43311882) -1000 Inventoried Assays
- Applied Biosystems 7900HT Fast Real-Time PCR System
- SDS Software v2.2.1 or higher for the 7900HT System (P/N4352620)
- *RNAse Inhibitor (P/N 8080119)
- *High-Capacity cDNA Archive Kit (P/N 4322171)
- *TaqMan® Universal PCR Master Mix (2X), No AmpErase® UNG (P/N 4352042)
- *ABI Prism® 96-Well Optical Reaction Plate with Barcode (code 128) (P/N 4306737)
- *ABI Prism® 384-Well Optical Reaction Plate with Barcode (code 128) (P/N 4309849)
- *ABI Prism® Optical Adhesive Covers (P/N 4311971)
- TaqMan Low Density Array Endogenous Control Panel (P/N 4367563)
- 7900HT Low Density Array Upgrade (P/N 4329012)
- TaqMan® Gene Expression Assays Protocol (P/N 4333458)
- High-Capacity cDNA Archive Kit Protocol (P/N 4322169)
- Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide (P/N 4347828)
- TaqMan Low Density Array User Guide (P/N 4319399)

- *Reagents and consumables provided for > 4000 TaqMan Assays

Non-Applied Biosystems Products:

- Total RNA, Brain (Ambion) - 150 µg
- Total RNA Universal Human Reference RNA (UHR) (Stratagene) - 150µg
- Total RNA, Mixture 25% Brain, 75% UHR – 150µg
- Total RNA, Mixture 75% Brain, 25% UHR – 150µg
- All other non-Applied Biosystems reagents and equipment required are described in Applied Biosystems Documents TaqMan® Gene Expression Assays Protocol (P/N 4333458), High-Capacity cDNA Archive Kit Protocol (P/N 4322169), TaqMan Low Density Array User Guide (P/N 4319399)

C. Experimental Design

1000 genes will be selected by MAQC for QRT-PCR analysis with TaqMan® Gene Expression Assays from a list of >5000 RefSeqs that showed perfect match to a Taqman® Assay and to the probe sequences on the seven microarray platforms (Affymetrix, Agilent, AB Microarray, Combimatrix, GE Healthcare, Illumina, and NCI_Operon). The final selection will be based on expression data generated in Pilot Study 1 and will include 100 genes which showed the most variability and 100 genes with the best sample specificity (50 brain, 50 UHR) across platforms from Pilot Study 1. ~175 genes with RefSeqs that match Eppendorf and Genospectra probe sets are also included. The remainder will include a random selection of genes that cover whole intensity and log ratio ranges.

TaqMan® Gene Expression Assays will be done in four replicates for each RNA sample. 10ng total cDNA (converted from total RNA) in 10µl final volume will be used for each replicate assay. 4 selected endogenous controls to use for normalization will be run on each 384 reaction plate: 18S(Hs99999901_s1), UBC(Hs00824723_m1), HPRT(Hs99999909_m1), POLR2A(Hs00172187_m1).

D. Total RNA Preparation and QC process

- The RNA samples used for microarray analysis will also be used for TaqMan® Assay. Please refer to previous section on Microarray Study for RNA preparation and QC process.

E. cDNA preparation and QC process

Synthesis of cDNA from total RNA samples is the first step in TaqMan Gene Expression Assay.

- Synthesis will be carried out according to High-Capacity cDNA Archive Kit Protocol (P/N 4322169) .
- 10µg total RNA per 100µl reactions for each sample (10 reactions per sample).
- All reactions from the same RNA sample will be pooled.
- cDNA will be tested by running each pooled sample on a TaqMan® Low Density Endogenous Control Panel. Refer to TaqMan® Low Density Array User Guide for detailed protocol..
  100ng cDNA is made up to 50µl in TE final volume. The sample is then combined with 50µl TaqMan® Universal PCR Master Mix (2X), No AmpErase® UNG (P/N 4352042). Final concentration is 100ng cDNA /100ul 1X Master Mix.
  100µl of each sample is then added into a sample port on the low density array.
  The array is centrifuged, sealed, and run on 7900HT as described in User Guide.
  Data is analyzed with SDS 2.1 or higher software.

C_T values will be compared to results generated from the Pilot Study II_Tissue Titration Study.

F. TaqMan Gene Expression Assay

- The procedure described in TaqMan® Gene Expression Assays Protocol (P/N 4333458) will be followed with the following modifications:
  - Use 10 ng cDNA per reaction.
  - The final volume is 10µl for each reaction.
  - Reaction mixture for 4 reactions (plus additional volume for overage) will be prepared in a 96-well plate and replicate assays transferred to 384-well Optical Reaction Plate using robotic methods (Biomek FX).
  - Reaction Plate will be run on 7900HT System using Standard Thermal Cycler mode, Sample Volume set at 10µl.
- Universal Cycling conditions: 10' at 95ºC; 15" at 95ºC, 1' 60ºC, 40 cycles.

G. Data Acquisition and Analysis

Data will be generated and output using SDS 2.2 or higher software in text format. Data will be analyzed using ΔCt (absolute Ct value – endogenous control Ct value) and ΔΔ C_T (Δ C_T for each gene – Δ C_T for a common reference gene) methods. The endogenous control with the least variation across all the samples and plates within one sample will be used for normalization to calculate Δ C_T t for each individual gene.

Our brief guidelines for validation of microarray results using TaqMan® Assay data as standard are in three categories:

1. Validate detection sensitivity and accuracy from each microarray platform:

2. Fold change correlation with TaqMan® data (ΔΔ C_T method) for all microarray platform
3. Differential expression sensitivities and accuracy by each microarray platform

The genes could be evaluated as a whole set (1041 total) or divided into subcategories according to the original design:

Subcategory 1: Total randomized genes (Number to be finalized)
Subcategory 2: Sample specific genes (110)
Subcategory 3: Most variable genes (100)

More details will be available in the upcoming Data Analysis guide.