

Standard Operating Procedure for One-Color Agilent 4x44K Microarray

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Purpose

This protocol describes the required procedure for microarray hybridization of total RNA samples isolated from human kidney tissue

Scope

This procedural format is utilized for processing human kidney RNA samples from the 'SysKID' and 'Genome Wide Gene Expression Profiles Associated with Renal Aging' projects. Adherence to the protocol will minimise technical variation in microarray data obtained for these projects.

Materials

Reagents and hardware from Agilent Technologies:

- Whole Human Genome (4x44K) Oligo Microarray kit (G4112F)
- Gasket slides, 4/Microarrays per slide (G2543)
- Hybridization chamber (G2534A)
- Quick-Amp Labeling Kit (5190-0424)
- Cyanine CTP Dye Pack (5188-1169-P)
- Gene Expression Hybridization Kit (5188-5242)
- Gene Expression Wash Buffer 1 & 2

QIAGEN RNeasy Mini Kit

96-100% ethanol

Procedure

The following procedure is a modification of the Agilent One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) Protocol, Version 5.7 March 2008

Before starting the protocol

- Set 2 heatblocks (65°C and 80°C) and a waterbath (40°C).
- The first dilution of spike-in mix can be prepared in advance and stored at -80°C for up to 2 months or 8 freeze/thaws.

Prepare One-Colour Spike-Mix

Mix stock solution on vortex.

Heat at 37°C for 5 min, vortex solution again and zip-spin.

Dilute Spike-Mix in 3-step serial dilution:

1. 2 ul + 38 ul dilution buffer
2. 2 ul + 48 ul dilution buffer
3. 2ul + 18 ul dilution buffer

Prepare labeling reaction

Add: 200 ng of total RNA to a 1.5 ml tube in a total volume of 8.3 ul

1.2 ul of T7 Promoter Primer

2.0 ul diluted Spike-Mix

Denature primer and template by incubation at 65°C for 10 min and cool on ice for 5 min

Prewarm 5x First strand buffer at 80°C for 3-4 min, briefly vortex and zip-spin

cDNA Master Mix:

Component	x1	x4.5	x8.5
5x First strand buffer	4	18	34
0.1M DTT	2	9	17
10mM dNTP	1	4.5	8.5
MMLV-RT	1	4.5	8.5
RNase Inhibitor	0.5	2.3	4.3
TOTAL	8.5	38.3	72.3

Add 8.5 ul of cDNA Master Mix to each sample and mix by pipetting. Incubate samples in waterbath at 40°C for 2 hours.

Transfer samples to 65°C for 15 min, then cool samples on ice for 5 min and zip-spin.

Prewarm the 50% PEG solution at 40°C for 1 min, briefly vortex and zip-spin.

Transcription Master Mix:

Component	x1	x4.2	x8.5
H ₂ O	15.3	64.3	130
4x Transcription buffer	20	84.0	170
0.1M DTT	6	25.2	51
NTP Mix	8	33.6	68
50% PEG	6.4	26.9	54.4
RNase Inhibitor	0.5	2.1	4.3
Inorg. pyrophosphate	0.6	2.5	5.1
T7 RNA polymerase	0.8	3.4	6.8
Cyanine 3-CTP	2.4	10.1	20.4
TOTAL	60	252	510

Add 60 ul of Transcription Master Mix to each sample, mix gently by pipetting. Incubate in waterbath at 40°C for 2 hours.

Purify the labeled / amplified RNA

- Add 20 ul H₂O to the cRNA samples (brings sample volume to 100 ul)
- Add 350 ul RLT buffer (QIAGEN) and mix well by pipetting
- Add 250 ul of 100% ethanol and mix thoroughly by pipetting (Do NOT centrifuge)
- Transfer the 700 ul sample to an RNeasy mini column, centrifuge (4°C, 30 sec, 13,000 rpm) and discard flow-through and collection tube
- Transfer column to new tube, add 500 ul buffer RPE (QIAGEN) and centrifuge (4°C, 30s, 13,000 rpm). Discard flow-through , re-use collection tube
- Add another 500 ul of buffer RPE to the column, centrifuge (4°C, 30s, 13,000 rpm). Discard flow-through and collection tube.
- Elute the cleaned cRNA sample by transferring the RNeasy column to a new collection tube. Add 30 ul H₂O directly to the filter membrane, wait for 1 min then centrifuge (4°C, 30s, 13,000 rpm).
- Store the cleaned cRNA on ice, discard the column.

Turn on Hybridization oven to pre-heat (65°C).

Quantify the cRNA

Use the NanoDrop Spectrophotometer Microarray measurement feature. Load 1.5 ul H₂O onto pedestal and initialise instrument.

Select RNA-40 as the sample type and blank the instrument with 1.5 ul H₂O, measure each sample using 1.5 ul volume and print results.

Calculate yield: cRNA (ng/ul) x 30 (elution volume), / 1000 = ug of cRNA.

Calculate specific activity: Cy3 (pmol / ul) / cRNA (ng / ul), x 1000 = pmol Cy3 / ug cRNA

If the yield is <1.65 ug and SA is <9.0 pmol Cy3 / ug cRNA do not proceed.

Hybridization

Prepare 10x Blocking Agent:

Add 500 ul of H₂O to the vial containing lyophilized 10x Blocking Agent, mix by gentle vortexing and zip-spin. If pellet does not dissolve heat at 37°C for 4 to 5 min, vortex and zip-spin. Note: 10x Blocking Agent can be made up in advance and stored at -20°C.

Prepare the hybridization samples:

Equilibrate heatblock to 60°C

Fragmentation Mix per sample:

Component	
Cy3-labelled cRNA	1.65 ug
10x Blocking agent	11 ul
Nuclease-free H ₂ O	bring volume to 52.8 ul
25x Fragmentation buffer	2.2 ul
TOTAL Volume	55 ul

Vortex each sample gently and thoroughly. Incubate at 60°C for exactly 30 min.

Add 55 ul 2x GEx hybridization buffer HI-RPM to stop the fragmentation reaction, mix well by pipetting (DO NOT use vortex – no bubbles allowed). Centrifuge at room temperature (1 min, 13,000 rpm). Place samples on ice and load onto the array as soon as possible.

Prepare hybridization assembly

Load clean gasket slide into chamber base with label facing up. Slowly dispense 100 ul of sample onto the gasket well in a “drag and dispense” manner, take care not to wet the gasket as this may cause leakage.

Carefully place an array active side down onto the gasket slide so that the numeric barcode is facing up, ensure the slides are properly aligned. Place the chamber cover over the slides and secure with the clamp assembly. Vertically rotate the assembled chamber to wet the gasket and check the mobility of bubbles (If required tap the assembly on a hard surface to move stationary bubbles).

Load slide chamber into rotisserie of hyb oven set to 65°C. Set rotational speed to 10 rpm and hybridize at 65°C for 17 hours.

Place Wash Buffer 2 into 37°C waterbath to pre-heat overnight.

Washing the Microarray Slide

Three washing dishes, a slide rack and a magnetic stirring bar should be cleaned in three changes of RNase-free water.

Use Wash Buffer 1 to ½ fill dish 1. Into dish 2 place the stirring bar and slide rack and fill to $\frac{2}{3}$ with Wash Buffer 1.

Remove the hybridization chamber from the oven and inspect the slide to confirm that the bubble in each array slot was able to rotate freely and that no leakage occurred. Release the slide sandwich from the hybridization chamber, submerge both slides into dish 1 and use plastic forceps to separate the array from the gasket slide.

Transfer the array slide to the rack in dish 2 ensuring it is covered fully by Wash Buffer 1 and stir at speed 4 for 1 minute.

Pour pre-warmed Wash buffer 2 (37°C) into dish 3 until it is $\frac{2}{3}$ full.

Transfer the slide rack (with array slide) and the stirring bar to dish 3 and stir at speed 4 for 1 minute.

Slowly remove the array slide from Wash buffer 2 (should take 5-10 seconds) to minimise droplets on the slide. Gently touch one edge of the slide to absorbent paper to remove any excess liquid.

The slide can be scanned immediately or stored in a plastic slide box in the dark.