4x44K: Modified Agilent New Kit Fluorescent Linear Amplification Protocol

Revised by Olga on February 11, 2009

This protocol is 1/2 of Regular Reaction Volume. It was tested on MCF7 and SUM102 cells. RNA yield, Dye Incorporation Rates and Arrays were compared to the Perou Old Amplification Protocol and Agilent New Kit Amplification Protocol.

<table>
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<tr>
<th>Experiment</th>
<th>IntraClass Correlation Summary</th>
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<td>New Kit vs Modified New Kit</td>
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Regent preparation:
Cyanine Dye Mix (-20oC, kept in the dark, aliquot to minimize freeze/thaw cycles)

- Cy3-CTP 10.0mM (enough to do ~10-24 reactions) 1.0ul/reaction.
- Cy5-CTP 10.0mM (enough to do ~10-24 reactions) 1.0ul/reaction

RNA Input Range: Sample: 200ng-2.0ug total RNA. Standard amount for the Sample is 1ug of total RNA.
Reference: Standard amount for the Reference is 2ug of total RNA.
Each tube of Reference contains 2ul (2.0ug) of total RNA.

Preparation of Spike A Mix (Cy-3) or Spike B Mix (Cy-5) by Serial Dilutions.

The Agilent RNA Spike-In Kit was developed to provide positive controls for monitoring the microarray workflow from sample amplification and labeling to microarray processing. These controls are labeled and amplified together with the RNA samples of interest being analyzed. After hybridization to the complementary probes on the microarray, the log ratios of red to green signal intensities for each Spike-In transcript can be used to monitor the sample amplification and labeling and microarray processing procedures used in the experiment, which are independent from the quality of the starting RNA sample.

1. 1st Dilution 1:20 dilution: Add 2ul Spike A or B to 38ul Dilution Buffer in 1.5 ml tube Mix and centrifuge (briefly)
2. 2nd Dilution 1: 20 dilution: Add 2ul Spike A or B from 1st Dilution to 38ul of Dilution Buffer in new 1.5 ml tube. Mix and centrifuge briefly.
3. 3rd Dilution 1:4: Make enough for all samples: for each sample you need to use 1ul of 3rd Dilution Spike A or B, so If you are running 4 samples to make 3rd Dilution you would add 2ul from 2nd Dilution and 6ul of Dilution Buffer.

STORAGE:
- Original Spike-Ins can be stored @ -80ºC up to 1 year
- 1st Dilution can be stored @ -80ºC up to 3 month and freeze/thawed up to 8 times
- 2nd Dilution can be stored @ -80ºC up to 1 month and used at least once after defrosting
- 3rd Dilution do not store

Step 1. cDNA synthesis and 2nd strand DNA synthesis from total RNA (~3hrs)

Use Thermal Cycler (Make sure to heat the lid to prevent condensation except when noticed otherwise)
1 reference is usually enough for 8-10 samples when doing hybridization step.

1. To a 250ul PCR tube, add total RNA. Add Rnase-free water to 5.8ul total volume.
2. Add 1.2ul of T7 promoter primer (from kit).
3. Add 1ul of appropriate 3rd Dilution of Spike-Ins (A=reference or B=your sample)
4. Heat at 65ºC for 10 min with cap and block lead open to reduce volume by evaporation.
5. Run Program Ag.Heat or Ag.step4B). Note: usually 6.0ul will be left, 2.0ul will be evaporated. Cool to 4ºC for at least 5 min.
6. Pre-warm 5x First Strand buffer at 80°C for 1-4 minutes.
Make Master Mix for cDNA and 2nd strand synthesis according to the table below.
Mix all reagents well.

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7. Add 6.0ul of Master Mix to each reaction and mix well gently. Total Rxn Vol=12ul
Incubate at 40°C for 2hrs, then 65°C for 15 min and 4°C for at least 5 min
8. Run program: Ag-FSS or Ag.Step7. If necessary, spin sample briefly

Step 2. Fluorescent cRNA Synthesis: *in vitro* transcription and Cy-dye incorporation (~2.5 hrs)

9. Immediately prior to use, gently mix the following components by pipetting, in the order indicated, at room temperature. Pre-warm PEG @ 40°C, 1 min; vortex; spin briefly

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10. Add 1.0ul cyanine 3-CTP (10mM) to each reference sample. Add 1.0ul cyanine 5-CTP (10mM) to each of the other samples.
11. To each sample, add 27ul of the above transcription mix. Mix gently by pipetting.
12. Incubate sample at 40°C for 2hrs at dark. Then 4°C for 1 min, no longer than 30 minutes
Run program: Ag-IVT or Ag.STEP12.
Step 3. Purification of labeled cRNA using Qiagen PCR Purification Kit

13. Add 500ul of Buffer PB to the spin column.
14. Add 60ul of Nuclease-free water to your labeled cRNA (total volume 100ul)
15. Transfer cRNA (100ul) to the column with PB buffer and mix well by pipetting.
16. Centrifuge 30 seconds at 9000xg.
17. Wash column with 500ul PE buffer (containing Ethanol) and centrifuge 30 seconds at 9,000xg.
18. Add another 500ul PE Buffer to the column, centrifuge sample for 2 min at 9,000xg.
19. Transfer column to a clean 1.5mL tube and add 50ul of EB buffer, let sit 1 min, then centrifuge 9,000xg for 1 min.
20. Repeat elution with another 50ul EB. Final volume will be ~100ul; put samples on ice.
21. Use 1.5 ul to measure cRNA concentration with Nanodrop, “Microarray” setting.

Step 4. Hybridization in of 4x44k Arrays (17 hours) Final Step.

→ Prepare 10x blocking reagent: dissolve a tube of 10xBlocking reagent (5188-5281) in 1250ul of H2O, vortex well; heat at 37^oC, 4-5 min, spin briefly. Store -20^oC, up to 2 months.

4x44K 0.825ug of cRNA is required for each sample. Hybridization Volume ~ 110 ul. Load 100ul.

22. Combine 0.825ug Cy-3 cRNA, and 0.825ugCy-5 cRNA, 11ul 10X Blocking Agent, then bring to 55ul with nuclease-free water.
23. Add 2.2ul of 25 x Fragmentation Buffer to each tube. (total volume=55ul)
24. Mix well by gentle pipetting.
25. Incubate at 60C in dark for exactly 30 minutes using a PCR machine (Program AG60C).
26. Immediately add 55ul of 2 x Hybridization Buffer (GEx HI-RPM). (This step will stop fragmentation.) Repeat for each sample.
27. Mix well by careful pipetting. Use ASAP.
28. Disassemble the Chamber. Put the Gasket in. Pipette samples onto gasket avoiding bubbles. NOTE that if you are scanning with Agilent scanner the Array #1 is the array closest to the BARCODE. Overlay the actual array slide Agilent side DOWN. Close the Chamber by moderately tightening the screw.
29. If bubbles did form during loading, verify that they rotate freely in the hybridization chamber by viewing the solution from the back while slowly rotating the chamber.
30. Place the hybridization chamber on the hybridization rotator rack in the hybridization oven, at 65C.
31. Set the hybridization rotator to rotate at 15 rpm.
32. Hybridization at 65C for overnight or 17hrs.
Step 5. Wash Slides: MINIMIZE ATMOSPHERIC OZONE EXPOSURE

! If Wash Buffer 1 and Wash Buffer 2 are new packs- add 2ml of 10% Triton X-102 into each Buffer. Mix.  
! Wash Buffer 2 needs to be warmed to 37oC overnight prior to the wash.

33. Remove up to 4 hybridization chambers from the oven. Leave them on a leveled platform.  
34. Separate array slide from gasket slide while holding UNDER Wash Buffer 1.  
35. Quickly transfer the slide to a new container with fresh Wash Buffer 1 rocking gently for 1 min.  
36. Quickly transfer the slide to a new container with WARM Wash Buffer 2 and rock gently for 1 min.  
37. Take out slides one at a time using forceps.  
38. BRIEFLY wipe off excess buffer on the 4 edges and barcode areas.  
39. Immerse the slide into Agilent’s stabilization and drying solution for 10 to 20 seconds.  
   Note: Do not stay in the solution more than 30 seconds and do this step in a ventilated hood.  
   Acetonitrile is toxic.  
40. SCAN ASAP on Agilent Scanner in room 11-112.

Step 6. Scanning on Agilent Scanner

41. Prepare the scanner to operate:  
   1. First, turn on the scanner; allow 2-3 min for the scanner to initialize. Once the yellow and green  
      lights are solid, double Click “Agilent Scan Control”. At this point the lasers will be turn on and start  
      to warm up. From this point, the lasers need 20-30 min to warm up.  

   2. Select the appropriate profile. The drop down menu contains four profiles (CGH, 1-color gene expression, 2-color gene expression, and miRNA).  

   3. Default settings are The Sure Scan High Resolution Technology can achieve the extended dynamic range in a single scan. To do this, the 20 bit TIFF must be selected. This is a default for those platforms that previously used the XDR. This creates a single TIFF file that is 25% larger in file size. Run times are 8min for 5um scan. The Scan Control displays the total run time.  

   4. We have direct writing ability to Samba. Just navigate to your folder on the S:\ drive to save the data.  
      NOTE: Currently we are scanning at a 5uM resolution. When we move up to higher density arrays,  
      we will need to scan at a 2 or 3uM resolution. We will make Profiles specific to the higher  
      dimension platforms so there is no confusion about which resolution to scan at.  

42. Place slide in scanning slide holder Agilent-side up  
43. Place holder/slide into slots 1-n, barcode faces user (only fits in one direction)  
44. Select number of Slots to scan.  
45. Close lid and start scanning.  
   Important! When you are finished scanning, close the Agilent Scan Control.  

Step 7. Feature Extraction

46. Open feature extraction software  
47. Drag and drop your .tif files from scanned arrays into program  
48. Protocol File: GE2 v5_95_Feb07  
49. Under “Project” choose “start extracting~20m/slide.  
   Important! When you are finished extracting, close the Agilent Scan Control.