1x244K: Modified Low RNA Input Fluorescent Linear Amplification Protocol
(1/2 of Regular Reaction Volume) (Revised by Aaron on February 11, 2008)

Regent preparation:
Cyanine Dye Mix (-80C, kept in dark, aliquot to minimize freeze/thaw cycles)

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

Cy5-CTP, 10.0mM (PerkinElmer NEL581, enough to do ~10 reactions)
1.0 ul/ reaction

RNA Input Range: 50ng – 2.5 ug total RNA. 10-200 ng mRNA.

Preparation of Spike A Mix (Cy-3) or Spike B Mix (Cy-5)

1. 1:20 dilution: Add 2ul Spike A or B to 38ul dilution buffer in 1.5 ml microcentrifuge tube
2. Mix and centrifuge (briefly)
3. 1: 40 dilution: Add 2ul from 1st dilution to 78ul of dilution buffer in new 1.5 ml microcentrifuge tube
4. Mix and centrifuge (briefly)
5. 3rd dilution (if necessary):
   1:16 of 2nd dilution if total RNA is 50-200ng
   1:4 of 2nd dilution if total RNA is 201-2000ng
   No 3rd dilution for >2000ng total RNA or 200ng mRNA
6. First dilution can be stored @ -80° C up to 1 year or stored @ -20 in a non-defrosting freezer and freeze/thawed up to 8x; discard 2nd and 3rd dilutions after one use

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)

1. To a 250ul PCR tube, add 50ng to 2.5 ug total RNA (standard amount=2.5 ug), or 10-200ng mRNA (standard amount=200ng), in a volume of 4.5 ul or less. Add Rnase-free water to 4.5ul total volume.
2. Heat at 65C for 10 min with cap open to evaporate possible residual EtOH (Ag.Heat or Ag.step4). Note: usually 2.5 ul will be left, 2.0ul will be evaporated.
3. Add 2.5ul of T7 promoter primer (from kit).
4. Add 2ul of appropriate (see above) Spike-Mix dilution (A=reference or B=your sample)
5. Heat at 65C for 10 min (Run Ag.Heat or Ag.step4).
6. Cool to 4C for at least 5 min

7. Make Master Mix for cDNA and 2nd strand synthesis. Pre-warm 5x First Strand buffer at 80C for 1-4 minutes. Keep master mix at room temperature.

Component	1X	2X	3X	4X	5X	6X	7X	8X
5x FS Buffer	2.4	4.8	7.2	9.6	12	14.4	16.8	19.2
0.1M DTT	1	2	3	4	5	6	7	8
10mM dNTP	0.5	1	1.5	2	2.5	3	3.5	4
MMLV-RT	0.8	1.6	2.4	3.2	4	4.8	5.6	6.4
RnaseOut	0.25	0.5	0.75	1	1.25	1.5	1.75	2

Note: MMLV-RT is 0.3ul more than the kit suggests.

8. Add 5.0ul of master mix to each reaction and mix well gently. Total Rxn Vol=12ul
9. Incubate at 40C for 2hrs, then 65C for 15 min and 4C for at least 5 min (Run program: Ag-FSS or Ag.Step7).
10. If necessary, spin sample briefly

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

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

Component	1X	2X	3X	4X	5X	6X	7X	8X
RNase-Free H2O	5.85	11.7	17.55	23.4	29.25	35.1	40.95	46.8
4x Trans. Buffer	10	20	30	40	50	60	70	80
0.1M DTT	3	6	9	12	15	18	21	24
NTP mix	4	8	12	16	20	24	28	32
50% PEG	3.2	6.4	9.6	12.8	16	19.2	22.4	25.6
RNaseOut	0.25	0.5	0.75	1	1.25	1.5	1.75	2
Inorganic Pyrophosphatase	0.3	0.6	0.9	1.2	1.5	1.8	2.1	2.4
T7 RNA Polymerase	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2

12. Add 1.0ul cyanine 3-CTP (10mM) to each reference sample. Add 1.0ul cyanine 5-CTP (10mM) to each of the other samples.
13. To each sample, add 27ul of the above transcription mix. Mix gently by pipetting.
14. Incubate sample at 40C for 2hrs at dark. Then 4C 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

15. Add 500ul of Buffer PB to spin column.
16. Add 60ul nuclease-free water to labeled cRNA tube for 100ul total volume.
17. Immediately transfer sample to spin column, tighten lid, and mix thoroughly by inverting several times.
18. Repeat steps 15-17 for all samples.
19. Centrifuge 15 seconds at 8500xg. Discard flow-through.
20. Wash the column with 500ul Buffer PE (containing EtOH) and centrifuge 15 sec. at 8500xg.
21. Discard flow through and add 300ul Buffer PE, centrifuge 2 min at 8500xg.
22. Transfer column to a clean 1.5mL tube and add 50ul Buffer EB, let sit 1 min, then centrifuge 8500xg for 1 min.
23. Repeat step 22. Final eluate volume will be ~100ul; put samples on ice.

→ Use 1.5 ul to measure cRNA concentration on a Nanodrop, “Microarray” setting.

Step 4. Hybridization in of 4x44k Custom Arrays (17 hours)
Final Hybridization Volume = 100 ul

→Prepare 10x blocking reagent; heat at 37C, 4-5 min, spin briefly. Store -20C, up to 2 months.

24. Combine 10ug Cy-3 cRNA, and 10ug Cy-5 cRNA, 50ul 10X Blocking Agent, then bring up to 240ul with nuclease-free water.

    Note: cRNA concentration needs to be adjusted by deducting the input total RNA amount from the total amount of amplified cRNA.
25. Add 10ul of 25 x Fragmentation Buffer to each tube. (total volume=250ul)
26. Mix well by gentle pipetting.
27. Incubate at 60C in dark for exactly 30 minutes using a PCR machine (Program AG60C).
28. Transfer immediately to a 1.5ml tube which contains 250 ul of 2 x Hybridization Buffer (GEx HI-RPM). (This step will stop fragmentation.)
29. Mix well by careful pipetting, spin briefly. Use ASAP.
30. Pipette samples onto gasket well using drag and dispense method avoiding bubbles.
31. 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.
32. Place the hybridization chamber on the hybridization rotator rack in the hybridization oven, at 65C.
33. Set the hybridization rotator to rotate at 15 rpm.
34. Hybridization at 65C for overnight or 17hrs.

Step 5. Wash Slides: MINIMIZE ATMOSPHERIC (OZONE) EXPOSURE

35. Remove up to 4 hybridization chambers from the oven. Leave them on a leveled platform.
36. Separate array slide from gasket slide while holding UNDER 2x buffer (2XSSC/0.005% Triton X-102 (20xSSC 100ml, TX102 0.5ml, add ddH2O to 1000ml).
37. Quickly transfer the slide to a second wash chamber containing 2x buffer and rock gently for 3-5 MINUTES <u>covered with aluminum foil</u>—be sure that buffer is filled nearly to the top of the chamber and slide is completely submerged during rocking.
38. Wash slides in 0.1XSSC (20xSSC 5ml, add ddH2O to 1000mL) for 2-3 minutes, covered with foil, with rocking.
39. Place into new 0.1xSSC wash buffer and bring to hood keeping foil over chamber.
40. Take out slides one at a time using forceps.
41. BRIEFLY wipe off excess water on the 4 edges and barcode areas.
42. 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.
43. SCAN ASAP on Agilent Scanner in room 11-112.

Step 6. Scanning on Agilent Scanner

44. Turn on scanner and allow to warm up (15-30 minutes)
45. Place slide in scanning slide holder Agilent-side up
46. Place holder/slide into slots 1-n, barcode faces user (only fits in one direction)
47. Close lid
48. Open Agilent Scan Controls and select slot 1-"end slot"
49. Check (select) "eXtend Dynamic Range"
50. Set scan resolution of 5 um and set output path to appropriate user folder; click "Set Value"
51. Check that arrays will be scanned with proper values (5 um, correct output path, Channel: Red/Green, Scan Area: 61 x 21.6 mm, PMT 100/10 for both red and green)
52. Click "Scan Slot 1-n"—scanning takes 15-20 min/slide

Step 7. Feature Extraction

53. Open feature extraction software
54. Drag and drop your .tif files from scanned arrays into program
55. Protocol File: GE2 v5_95_Feb07
56. Under "Project" choose "start extracting", and save to your folder; ~20m/slide