# MicroArray Protocol

Modified by Ryoji Kojima, Takumi Yoshida and Jeffrey Randall, 8.20.2000

## 1. Total RNA Isolation from cultured cells.

1. Wash cultured cells 2X with 8 ml PBS (without calcium, without magnesium) @ RT.
   (For 100 mm dish use 5 ml PBS)
2. Add 8 ml TRIzol reagent (Gibco/BRL) to cells in T-75 flask.
   (For 100 mm dish use 5 ml TRIzol reagent.)
3. Transfer lysed cell solution to a 15 ml flacon tube.
4. Incubate @ RT for 5 minutes.
5. Add 1.6 ml Chloroform:Iso-Amyl alcohol (24:1) to each tube.
6. Shake vigorously for 30 seconds.
7. Incubate at RT for 3 minutes.
8. Spin in centrifuge @ 4$^{o}$C for 15 minutes @ 4000 RPM.
9. Transfer upper phase (clear solution only; no white goop) to a new 15 ml tube.
10. Add 4 ml of Isopropanol (2-propanol) to each tube.
11. Mix by inverting several times.
12. Incubate @ RT for 10 minutes.
13. Spin for 30 minutes @ 4$^{o}$C @ 4000 RPM.
14. Remove supernatant with a pipette very carefully.
15. Resuspend pelleted RNA using pipette in 1 ml of 70 % Ethanol.
16. Transfer to a microfuge tube.
17. Spin for 10 minutes @ 4$^{o}$C @ 14,000 RPM.
18. Remove supernatant with a pipette.
19. Aspirate remaining drops.
20. Air-dry pellet for 5-10 minutes @ RT.
21. Resuspend RNA pellet in 80 ul DEPC treated water.
22. Spec 2 ul in 198 ul of water.
23. Store RNA @ -80$^{o}$C.

## 2. DNase Treatment of Total RNA

1. Reaction solution:
    60 ug Total RNA
    3 ul RNase Inhibitor
    10 ul 10X PCR Buffer
    6 ul $MgCl_2$ (25 mM)
    20 ul DNase (Promega, 10U/ul)
    X ul DEPC Treated water
    100 ul total reaction volume

2. Incubate @ 37$^{o}$C for 30 minutes.
3. Add 100 ul Phenol:Chloroform:Iso-Amyl Alcohol (25:24:1) pH 4.5-5.2
4. Vortex for 30 seconds
5. Spin for 5 minutes @ RT @ 14,000 RPM.
6. Transfer upper phase (clear solution; no white goop) to a new microfuge tube.
7. Add:
    10 ul of 3 M NaOAc (pH 4.5-5.2)
    200 ul 100% ethanol

8. Mix by inverting several times.
9. Put in DRY ICE/Ethanol bath for 30 minutes or @ -80$^{o}$C for 1 hour.
10. Spin for 15 minutes @ 4$^{o}$C @ 14,000 RPM.
11. Remove supernatant with a pipette very carefully.
12. Resuspend pelleted RNA using pipette in 500 ul of 70 % Ethanol.
13. Spin for 10 minutes @ 4$^{o}$C @ 14,000 RPM.
14. Remove supernatant with a pipette.
15. Aspirate remaining drops.
16. Air-dry pellet for 5-10 minutes @ RT.
17. Resuspend RNA pellet in 40 ul DEPC treated water.
18. Spec at $OD_{260}$ and $OD_{260/280}$ using 2 ul in 198 ul of water.

## 3. Making the single strand cDNA probe.

In an autoclaved PCR tube Add:

| Cy3 (Red) | |
|---|---|
| Total RNA | 10 ug |
| RT primer (Cy3,1pmole/ul) | 1.0 ul |
| DEPC treated water | x ul |
| Total reaction volume | 10 ul |

In an autoclaved PCR tube Add:

| Cy5 (Green) | |
|---|---|
| Total RNA | 10 ug |
| RT primer (Cy5, 1pmole/ul) | 1.0 ul |
| DEPC Treated water | x ul |
| Total reaction volume | 10 ul |

Heat in PCR machine @ 80$^{o}$C for 10 minutes.
Chill to 42$^{o}$C and hold.
Use **new PCR caps** as heat denatures plastic and allows evaporation!

In a separate microfuge tube make the reaction buffer (# of samples + 1).

| | Single channel | Double channel (Cy3 and Cy5) |
|---|---|---|
| 5x RT Buffer (Gibco/BRL) | 4 ul | 8 ul |
| 0.1 M DTT (Gibco/BRL) | 2 ul | 4 ul |
| 10 mM dNTP's | 1 ul | 2 ul |
| RNase Inhibitor (40 U/ul, Promega) | 1 ul | 1 ul |
| DEPC treated water | 1 ul | 2 ul |
| Superscript II (200 U/ul, Gibco/BRL) | 1 ul | 2 ul |
| Total reaction volume | 10 ul | 20 ul |

Add 10 ul of Reaction Buffer to each PCR tube.
Mix well with pipette and incubate at 42$^{o}$C in PCR machine for 2 hours.
**Use new PCR caps to prevent evaporation!**
Add 3.5 ul of 0.5 M NaOH/50 mM EDTA to each tube.
Incubate @ 65$^{o}$C for 10 minutes to denature DNA/RNA hybrids.
**Add**:
5 ul of 1 M Tris/HCl, pH 7.5 to neutralize the reaction mixture.
70 ul of DEPC treated water (to dilute reaction and bring volume to 100 ul)
Combine all the Cy3 and Cy5 RT reaction mixtures in a microfuge tube.
**Add**:
0.1 volumes (20 ul) of 3 M NaOAc
2.5 volumes (550 ul) of 100% ethanol (-20$^{o}$C)
Mix by inversion several times.
Place in DRY ICE/Ethanol bath for 30 minutes (label with tape as marker will run)
or place in –80$^{o}$C for 1 hour.
Spin @ 14,000 RPM for 20 minutes at Room Temp.
Remove supernatant with pipette carefully.
Add 500 ul of 70% Ethanol (-20$^{o}$C)
Spin down @ 14,000 for 15 minutes @ Room temp.
Remove the supernatant using a pipette, carefully.
Air-Dry the pellet for 2 - 5 minutes at RT.

Re-suspend the pellet in 15 µl Dendrimer hybridization buffer (pre-warmed to 65$^oC$).
(Tube #6 in Genisphere kit, 0.25 M $NaPO_4$, 4.5% SDS, 1 mM EDTA, 1X SSC).

## 4. Automated Slide Processor (ASP) Version for hybridization.

Incubate "Express Hyb"-hybridization solution (Clonetech) at 65$^oC$.

**Preparation of Dendrimer Cy3 and Cy5**.
Prepare a mixture of Cy3 dendrimer and Cy5 dendrimer in a 1:1 ratio.
(***a stock solution of 36 ul (18ul each) can be made and kept in a light block tube at +4$^o$C)***

Incubate the dendrimer mixture @ 65$^oC$ for 10 minutes.
Vortex dendrimer mixture for 30-60 seconds to break up aggregates.
Incubate @ 65$^oC$ until use (Should be about 10 more minutes).
Denature the probe @ 95$^oC$ for 3-5 minutes in PCR machine.
Transfer immediately to 65$^oC$ waterbath.
**Add**:
  3 ul Dendrimer mix to each probe and mix well.
Incubate @ 65$^oC$ for 30 minutes to 1 hour to facilitate probe-dendrimer hybridization.
**Add**:
  200 ul ExpressHyb to each probe and mix well.
  Quick spin down and return to 65$^oC$.
Using the Hamilton syringe take up entire probe and inject into chamber.
Wash the syringe 3X with water.
Continue loading all probes into respective chambers.
Hybridize O/N.

## 5. Washing microarrays in ASP.

ASP will automatically wash slides as follows:
  Flush with 2 ml of 2X SSC + 0.2% SDS @ 55$^oC$.
  Flush with 2 ml of 1X SSC @ Room Temp.
  Flush with 2 ml of 0.1X SSC @ Room Temp.
Air Dry slides for 120 seconds each. END PROGRAM.

Remove slides from chambers and place in stainless steel slide rack.
Spin for 1 minute at 500-1000 rpm in Jouan centrifuge.

SCAN in ScanArray 5000.

## 6. Processing of Array slide

Place the spotted chip on plastic tray (**DNA SIDE UP**).
Cross-link the DNA to the chip using Stratagene Stratalinker.
Turn Power on.
Press RESET
Press ENERGY
Press 5500
Press START
Put slide in 80$^{o}$C oven for two hours (DNA SIDE UP).
Using slide holder, place arrays in 0.2% SDS for 10 minutes with gentle mixing/stirring.
Wash the arrays in R/O water 2x.
Place arrays in plastic container for 2 minutes in fresh R/O water.
Transfer the arrays to 100% ethanol (–35$^{o}$C) for 2 minutes.
Spin slides for 1 minute at 500-1000 RPM in Jouan centrifuge.
Dry slide at RT and place in light block slide box.

## 7. Pre-hybridization of the processed slides (NON-Automated version).

Incubate "Express Hyb"-hybridization solution (Clonetech) at 65$^{o}$C.
Place chamber cover (Secure Seal, SA 500, GRACE bio-labs) on the array.
(Make sure diamond scratches are within the cover chamber).
Add 500 ul of pre-warmed hybridization buffer to the a hole in chamber.
Wipe any spilled solution away from hole gently.
Seal holes with provided seals.
Incubate the array in the hybridization oven for at least 30 minutes.

## 8. Hybridization of Cy3 + Cy5 probe to glass array (NON-Automated version).

Denature the probe @ 95$^{o}$C for 3-5 minutes in PCR machine.
Transfer immediately to 65$^{o}$C waterbath.
Remove the pre-hybridization solution from array slide cover chamber using P-1000 pipette.
Add:
15 ul of probe to 500 ul (**pre-warmed @ 65$^{o}$C**) hybridization solution.(Clonetech, ExpressHyb)
Mix well and **Add directly** to array cover chamber.
Wipe any spill gently with a Kimwipe.
Re-Seal the chamber.
Incubate at 65$^{o}$C, overnight in hybridization oven.

## 9. Preparation of Dendrimer Cy3 and Cy5.

Place 3DNA dendrimer hybridization buffer @ 65$^{o}$C to incubate until use (about 10 minutes).
Prepare a mixture of Cy3 dendrimer and Cy5 dendrimer in a 1:1 ratio.
(a stock solution of 36 ul (18ul each) can be made and kept in a light block tube at +4$^{o}$C)
Incubate the dendrimer mixture @ 65$^{o}$C for 10 minutes.
Vortex the mixture again for 30-60 seconds to break up aggregates.

Incubate @ 65$^{o}$C until use (Should be about 10 more minutes).

## 10. Washing unbound probe from glass array (NON-Automated version).

Remove chamber cover from probed array over garbage can as hybridization solution will drip.
Carefully remove any remaining adhesive material from the array with a razor blade.
Places arrays in Wheaton glass slide holder.
Wash in pre-warmed 2X SSC, 0.2% SDS washing solution @ 65$^{o}$C for 5 minutes.
(Every minute lift the tray up and down 2-3x to aid in washing)
Wash the array in 2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Wash the array in 0.2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Wash the array in 0.2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Air Dry the array.

## 11. Hybridization of Dendrimers (Cy3 and Cy5) to Array (NON-Automated version).

Place circular filter papers (about 3-4) into both reservoirs of pre-warmed incubation chamber.
Add 40-50 ul of 2X SSC onto each filter paper stack.
Place the air-dried array into the chamber. (DNA side up)
Add 2 ul of 65$^{o}$C dendrimer mixture into 13 ul of 65$^{o}$C 3DNA hybridization buffer and mix well.
(These values are for a 25 mm x 25 mm cover slip)
(Double the volumes for a 40 mm x 25 mm cover)
Place the 15 ul hybridization solution and dendrimers on the center of the array.
(Between the scratches made with the diamond pencil)
Carefully place the ethanol wiped and dried glass cover slip (Corning) on the drop of solution.
If necessary, remove bubbles by gentle tapping on glass with a pipette tip.
Assemble the incubation chamber cover on the base. (MAKE SURE GASKET IS SEATED)
Apply a binder clip at each end, and fold the metal hinges toward the middle.
Place the chamber and array @ 65$^{o}$C for 6 hours.

## 12. Washing unbound dendrimer from glass array(NON-Automated version).

Remove incubation chamber from 65$^{o}$C incubator.
Carefully remove binder clips and cover from incubation chamber.
Places arrays in Wheaton glass slide holder.
Wash in pre-warmed 2X SSC, 0.2% SDS washing solution @ 65$^{o}$C for 10 minutes **AFTER COVER SLIP FALLS OFF**!
(Every minute lift the tray up and down 2-3x to aid in washing)
(Make sure that the cover slip falls off, do not help in any other way)
Wash the array in 2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Wash the array in 0.2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Wash the array in 0.2X SSC, 0.2% SDS washing solution @ RT for 2 minutes.
Air Dry the array.
Wipe the back of the array with a Kimwipe (70 % ETOH treated).
Scan the array.