

cDNA preparation for microarray hybridization

We will be analyzing the effects of a newly isolated virus on human cells. We have whole cell RNA samples that have been purified from human cells infected with the virus and from uninfected cells. We will be analyzing the expressed genes by detecting them on microarrays. We expect that infected cells will express a different set of genes than uninfected ones, but we are not sure which genes will be affected. Some genes will be expressed under both conditions; others will not be expressed in both cases, while others will change in expression. We are interested in the ones that are expressed under one condition and not under the other condition, or expressed to significantly different degrees when comparing the two conditions.

To perform the microarray analysis, we convert the cellular RNA into cDNA. As we make the cDNA, we also label it. The cDNA is then hybridized to the microarray. The color is then developed and the microarray sent off to be analyzed. We will obtain a “listing” of which spots on the microarray were seen. This data is then analyzed to determine which genes were affected by the viruses.

This part of the experiment converts mRNA into cDNA by using Reverse transcriptase. We then hybridize the cDNA to a microarray. However, this experiment differs from standard RT-PCR in that the cDNA that we make has an arm attached for labeling the cDNA.

Materials

Genisphere 3DNA Array 900 Submicro Expression Array Detection Kit
cDNA synthesis stop solution: 0.5 M NaOH, 50 mM EDTA
TE (10 mM TrisHCl, pH 8.0, 1 mM EDTA)
1M Tris-HCl, pH 7.5
3M Ammonium acetate
100% ethanol, 95% ethanol, and 70% ethanol

Procedures

Note: Use autoclaved or sterile tubes, tips, and solutions. Wear gloves to protect your samples!

1) If it has not already been performed for you, determine your RNA concentration before continuing. We need 0.5-2 μ g of whole cell RNA (less if it is mRNA) for the experiment.

Note: You will do steps 2 through 11 *once for each dye* (Cy3 and Cy5), for each microarray. One dye will be used for one cell condition and the other cell condition uses the other dye (e.g., infected vs. uninfected cells).

2) In a PCR tube, prepare the **RNA-RT primer mix** by combining:

Reagent	Volume
RNA (0.5-2 µg)	1-7 µl
RT primer (0.2 pmol)*	3 µl
DEPC-treated H ₂ O	to 10 µl total volume

* Cy3 or Cy5 primers (vial 2)

- 3) Make sure the sample is on the bottom of the tube.
- 4) Heat the samples to 80°C for 10 minutes, then 4°C in the thermocycler.
- 5) Add 1 µl of Superase-In RNase inhibitor (Vial 4).

- 6) Place a PCR tube on ice and prepare the **Reaction mix** by combining:

Reagent	Volume
5X RT buffer	4 µl
dNTP mix [vial 3]	1 µl
DEPC-treated H ₂ O	4 µl
Reverse transcriptase (200 units) [Superscript II]	1 µl

- 7) Add the RNA-RT primer mix from step 5 to the Reaction mix from step 6. Your final volume is 20 µl.
- 8) Mix (but don't vortex) and incubate for two hours at 42°C.
- 9) Add 3.5 µl of cDNA synthesis stop solution to stop the reaction (0.5M NaOH/50 mM EDTA).
- 10) Incubate at 65°C for ten minutes to denature the DNA/RNA hybrids.
- 11) Neutralize the reaction by adding 5 µl of 1M Tris-HCl, pH 7.5.

Note: You will now mix the Cy 3 and Cy5 samples, so now you will have one reaction per chip.

- 12) Put the Cy3 cDNA and the Cy5 cDNA mixtures from step 11 into a single microfuge tube.
Rinse the tubes that contained the cDNA with 21 µl each using TE. Combine the wash with the cDNA mixtures.
- 13) Add 5 µl of 5.0 mg/ml linear acrylamide (as a coprecipitate for the ethanol precipitation).
- 14) Add 250 µl 3M Ammonium acetate and mix.
- 15) Add 875 µl of *absolute* ethanol (100%).
- 16) Incubate at -20°C for 30 minutes or more (e.g., in the freezer).
[Stop here if it is getting late]
- 17) Centrifuge the sample at 13K for 15 minutes to pellet the cDNA.
- 18) Remove all the ethanol by inverting and placing, while inverted, on a kimwipe. Let it sit for a few seconds while still touching the kimwipe.
- 19) Add 300 µl of 70% ethanol to the cDNA pellet.
- 20) Centrifuge for 5 minutes and remove the supernatant as in step 18.
- 21) Keep the tube inverted to dry the cDNA pellet until you put it at 65°C for 10 to 30 minutes.
The sample must be completely dry before proceeding.

To be continued...