

MICROARRAY PROTOCOLS

cDNA Synthesis Protocol with Genisphere 900MPX Kit

Revision September 2 2008

****NOTE-do cDNA synthesis protocol in RNase-free zone and remember to use Nuclease-free water only!**

1. Check to see if you have enough of the reagents needed for cDNA synthesis protocol:

- Reagents needed in Genisphere Kit (in -20°C freezer)-
- Reagents needed with SuperScript Reverse Transcriptase enzyme (in -20°C freezer)-
 - RT enzyme
 - 0.1 M DTT
 - 5X Reaction Buffer
- Reagents that need to be prepared-
 - RNA sample(s)
 - 0.5 M NaOH / 50 mM EDTA
 - 1 M Tris-HCl, pH 7.5
 - 10 mM Tris, pH 8 / 1 mM EDTA

2. **Calculate the volume (μl) needed to give 2 μg of total RNA per sample/condition. You need concentrations that are > 286 ng/ μl to ensure you can do this in the required volume. Remember! You need 2 μg of each RNA sample if you are doing dual assays so that in the end it adds up to 4 μg of starting RNA per array (slide).**

3. **Prepare RNA-Primer mix in a PCR tube (one for each RNA sample).**

1-7 μl	RNA (2 μg total)
4 μl	Random RT primer (Vial 2) \rightarrow [2 μl per μg of RNA]
	X μl Nuclease-free water (Vial 10) \rightarrow to get up to V _{final}
11 μl	V_{final}

- Mix and spin each PCR tube.
- Heat to 80°C for 10 minute in thermalcycler
- Ice immediately for 2 minutes.

4. **Make a Reaction Master Mix - do this while RNA primer mix is incubating!**

Master Mix Per sample

4 μl	5X SuperScript II First Strand Buffer
2 μl	0.1M DTT (supplied with Superscript enzyme)
1 μl	dNTP mix (Vial 3)
1 μl	Superase-In RNase inhibitor (Vial 4)
1 μl	Superscript II enzyme, 200 units

9 μl	V_{final}
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- KEEP EVERYTHING ON ICE!
 - DO NOT VORTEX ENZYME!
 - Gently mix, microfuge, and KEEP ON ICE until ready to use.
5. **Combine RNA-Primer mix with corresponding Reaction Mix (if using a Master Mix instead, then add appropriate volume to RNA-Primer mix).** If you have followed Genisphere's recommendations for RNA-Primer and Reaction Master Mix, your final volume after combining should be 20 μ l. Gently mix via pipetting, then microfuge BRIEFLY.
 6. **Incubate at 42°C for 1.5-2 hours.**
 7. **Stop Reaction with 0.5 M NaOH/ 50 mM EDTA-**
 - If you have a 20 μ l rxn, add 3.5 μ l per reaction
 - If you have a 25 μ l rxn, add 4.38 μ l per reaction
 8. **Incubate at 65°C for 15 minutes.**
 9. **Neutralize Reaction with 1M Tris-HCl (pH 7.5) /10 mM Tris (pH 8), 1mM EDTA**
 - For 20 μ l rxns add
 - 5 μ l 1 M Tris-HCl, pH 7.5
 - 21.5 μ l 10 mM Tris, pH 8, 1 mM EDTA

So total volume for each reaction ends as 50 μ l
 - For 25 μ l rxns add (use this only if you increased volume of RNA-primer mix)
 - 6.25 μ l 1 M Tris-HCl, pH 7.5
 - 26.88 μ l 10 mM Tris, pH 8, 1 mM EDTA

So total volume for each reaction ends as ~58 μ l

Purification of cDNA with Qiagen Minelute Kit

Purify each sample of RNA separately
 This protocol is for one 50 μ l reaction

1. **Add 250 μ l Buffer PB to sample**
2. **Mix and microfuge**
 - a. Apply DNA/PB to MinElute column
 - b. Centrifuge for 1 minute at 13,000 rpm
 - c. Discard flow-through.
3. **Add 750 μ l Buffer PE to column**
 - a. Centrifuge for 1 minute at 13,000 rpm
 - b. Discard flow-through and centrifuge for an additional 2 minutes, 13,000 rpm
 - c. Discard flow-through and replace column into appropriately labeled 1.5mL tube

4. **Add 10 μ l Buffer EB to center of column**
 - a. Incubate at room temperature for 2 minutes
 - b. Centrifuge for 2 minutes at 13,000 rpm

Terminal Deoxynucleotidyl Transferase Tailing reaction

1. **To purified DNA**
 - a. Transfer all your purified cDNA (10 μ l) to a PCR tube. Do each sample separately.
 - b. Add nuclease Free water to bring final volume to 16.5 μ l
2. **Heat cDNA in thermocycler**
 - a. Heat for 10 minutes at 95°C
 - b. Immediately transfer to ice for 2 minutes
3. **Add the following components for a final volume of 25 μ l**

16.5 μ l	purified cDNA
2.5 μ l	10X Tailing buffer (vial 13)
4 μ l	10 mM dTTP (Vial 14)
2 μ l	Terminal Deoxynucleotidyl Transferase (Vial 15)

4. **Incubate in thermocycler at 37°C for 30 minutes**

→ Proceed immediately to ligation!

Ligation of Capture Sequences

1. **Denature tailed DNA for 10 minutes at 95°C and then ice samples immediately for 2 minutes.**
2. **Determine which dye (Cy3 or Cy5) you will use for each sample and write it down!**
3. **Per sample of cDNA (for a volume of 32 μ l): Mix gently and microfuge.**

25 μ l	Tailed cDNA
5 μ l	6X ligation mix (Vial 11) either Cy 5 or Cy 3
2 μ l	T4 DNA Ligase (Vial 12)

4. **Incubate at 23°C for 2 hours.**

Genisphere recommends 30 minutes, but we have modified protocol to do 2 hours. Not sure why we did 2 hours, but have never tested to see if a shorter time works and have kept with the longer time period.

5. To STOP reaction:

- Add 3.5 μ l of 0.5M EDTA to each tube and vortex thoroughly (5-10 sec)
- Add 14.5- μ l 1X TE buffer for a total volume of 50 μ l.

2nd Purification of cDNA

Now, you will purify your tailed cDNA (i.e. cDNA with TTTT tail AND either Cy3 or Cy5 capture SEQUENCE) with Qiagen MinElute PCR Purification Kit.

Purify each reaction separately. This protocol is for one 50 μ l reaction

1. Add 250 μ l Buffer PB to sample

2. Mix and microfuge

- a. Apply DNA/PB to MinElute column
- b. Centrifuge for 1 minute at 17900 rcf
- c. Discard flow-through

3. Add 750 μ l Buffer PE to column

- a. Centrifuge for 1 minute @ 17900 rcf
- b. Discard flow-through and centrifuge for an additional 2 minutes, max speed
- c. Discard flow-through and replace column into appropriately labeled 1.5mL tube

4. Add 10 μ l Buffer EB to center of column

- a. Incubate at room temperature for 2 minutes
- b. Centrifuge for 2 minutes @ max speed

→ Proceed to cDNA Hybridization. Samples can be frozen at -80°C until ready to do hybridization.

****When you are ready to proceed to Hybridization #1 (i.e. hybridization of tailed cDNA to array), COMBINE both types of cDNA (labeled Cy 5 and Cy 3) into a PCR tube for a final volume of 20 μ l.**

cDNA Hybridization to Array - Protocol for Microarrays

Revision September 2 2008

FIRST HYBRIDIZATION

1. Thaw and resuspend the 2X hybridization mix (Vial 6) by heating to 65-70°C in heat block for at least 10 minutes. Vortex to ensure all components are resuspended evenly.
2. Thaw at room temp (Vial 9) LNA™ dT Blocker.
3. Prepare Cot-1 DNA (Rat Hybloc)
 - a. Dilute Cot-1 DNA 1:5 from 1 mg/ml stock (final concentration: 200 ng/μl)
 - b. Denature at 95°C for 10 minutes
4. Prepare cDNA hybridization mix, gently vortex and briefly microfuge cDNA hybridization mix.

Note: For a glass cover slip size 22 x 60 coverslip slide, load 51μl total volume onto array. See Genisphere's table of recommended volumes for different coverslip sizes

20 μl	cDNA (includes 10 μl from each condition)
3 μl	Nuclease free water
1 μl	Cot-1 DNA (200)
2 μl	LNA dT Blocker (Vial 9)
25 μl	2X Hybridization Buffer (Vial 6)
51 μl	Vfinal l

5. Incubate the mix in thermocycler for 80°C for 10 minutes. Immediately remove and put in 60°C thermolyne bath to keep at 60°C. Alternatively, have PCR program keep at 60°C.
6. Prewarm metal base of hybridization chamber to 60°C by placing on top of heat block.
7. Prewarm slide by placing directly on top of 60°C heat block for at least 10 minutes.
8. Place a thin strip of filter paper in the groove in the bottom of the hybridization chamber and add 60 μl of nuclease free water to filter paper. This will ensure that the inside of the chamber remains properly humidified during hybridization.
9. Place prewarmed slide into corresponding slot in prewarmed hybridization chamber.
10. If you are using a hybridization chamber that looks old (i.e. the black seal has cracks), carefully apply Dow Corning High Vacuum Silicone-based Grease onto seal using a sterile swab—you don't need to apply too much. Careful to not touch the slide's printed array surface when doing this. It is important that you **DO NOT** use any other type of grease. **YOU MUST USE silicone-based** because it can withstand high temperatures (others melt around 50°C).

11. Gently vortex, briefly microfuge and load cDNA hybridization mix (51µl) to pre-warmed microarray slide, leaving any precipitate in the tube behind.

When loading the array, it is IMPORTANT to keep in mind the following:

- Try to not have any air in your pipet tip because these will become bubbles underneath the coverslip and that is no good.
- Using tweezers to hold the coverslip CAREFULLY and SLOWLY lower it until it comes in contact with the entire hybridization solution (You should practice this technique with a plain microscope slide and water before doing your first array).
- Once you've lowered the coverslip fully, if there are bubbles it is best to NOT MOVE coverslip too much (preferably not at all) to get rid of air bubbles because losing liquid is worse than keeping a few bubbles!

12. Carefully, place plastic hyb chamber top onto array. Engage each screw first, then tighten them all to close the chamber tightly!

When transporting array from heat block to waterbath, keep in mind the following:

- Remember to keep the chamber as horizontal as possible at all times.
- Place chamber in center of bath so it is not touching sides of the waterbath.

13. Incubate the array for 2-3 days in large water bath at 60° C. ** Genisphere recommends overnight at 55-62°C for oligonucleotide spotted DNA, but we have always gone 3 days.

FIRST WASHES

Recipes for washes

Prepare 100ml total volume of each wash buffer. Recipes are given for preparation in 50 ml conical tubes as listed.

Note : DTT is only added to the wash buffers that will be used after second hybridization

20X SSC

17.53 g	NaCl
8.82 g	Sodium citrate
80 ml	RNase-Free Water

- Adjust pH with NaOH to get a pH of 7.0
use 2-point standardization of pH machine (using posted directions, 25°C), make sure electrode port is open and electrode is filled with KCl
- Bring up final volume to 100 mL with RNase-Free Water
- Filter sterilize

2X SSC, 0.2% SDS

5 mL	20X SSC
0.5 mL	20% SDS
45.5 mL	RNase- Free Water
50 µl	0.1M stock DTT (only for 2 nd set of washes)

2X SSC

5 mL	20X SSC
45 mL	RNase-Free Water
50 µl	0.1M stock DTT (only for 2 nd set of washes)

0.2X SSC

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0.5 mL	20X SSC
49.5 mL	RNase-Free Water

Water Baths Needed

- Heat Block to 60°C
- Make sure thermocycler is free
- Large water bath at 60°C (ISOTEMP 210)

FIRST WASHES

Keep the time that array is exposed to air during washes to the absolute minimum (few seconds)!

1. Prewarm the 2X SSC, 0.2 % SDS wash buffer to 60°C (place in large water bath with microarray). Put the wash buffer in 2, 50 mL plastic tubes
2. Remove coverslip by washing array in the prewarmed 2X SSC, 0.2% SDS. Place the microarray in the 50 mL tube with the wash buffer and wait until the coverslip floats off- should take about 2-5 minutes. You REALLY want to make sure that the coverslip floats AWAY from the slide, so pay close attention and BE VERY CAREFUL because the glass coverslip can scratch and damage your array!
3. Pour 50 ml of the warmed 2X SSC, 0.2% SDS into the white, opaque, plastic copland jars.
4. Transfer the microarray to copland jar (white plastic with cover) containing the 2X SSC, 0.2% SDS, wash the microarray for ~10 minutes with GENTLE shaking (put in shaking at room temperature).
5. Wash for 10 minutes in 2X SSC at room temperature- put in shaker (done in copland jar).
6. Wash for 10 minutes in 0.2X SSC at room temperature- put in shaker (done in copland jar).

7. Immediately transfer the array to a dry plastic 50 ml. Centrifuge for 2 minutes at room temperature at 1000 RPM. **IMPORTANT**-Make sure label is at bottom of tube. Careful not to touch the array surface!

Place array in dark box until ready for second hybridization in a non-transparent slide holder, in a closed drawer

SECOND HYBRIDIZATION

Whenever handling dyes avoid exposure to light as much as possible, for example, transport in a foil wrapped box. Light -that includes fluorescent lights- will breakdown dyes, so turn off most or all lights if possible. Second set of washes should also occur in the dark.

Prepare: Heat block and waterbath at 65°C
Make sure thermocycler is free

1. Thaw both 3DNA Array 900 MPX Capture reagents (2 different Vial 1s) in the dark at room temperature for 20 minutes (I just placed them in a drawer).
 - a. Vortex capture reagent for 3 seconds and incubate at 65-70°C in heat block for 10 minutes (place foil over heat block to ensure darkness)
 - b. Vortex capture reagent for 3-5 seconds and microfuge
2. Thaw and resuspend 2X hybridization buffer (Vial 6) by placing it in heat block for at least 10 minutes. Vortex to make sure components are resuspended.
3. Prepare 3DNA Hybridization Mix (for each array)

2.5 µl	3DNA capture reagent 1 (Vial 1)
2.5 µl	3DNA capture reagent 2 (Vial 1)
16 µl	Nuclease free water (vial 10)
30 µl	2X Hybridization buffer (Vial 6)
60 µl	V _{final}

4. Gently vortex hybridization mix and incubate in thermocycler at 80°C for 10 minutes. Move to 60°C waterbath until ready to load.
5. Follow steps 5 thru 11 from “First Hybridization protocol” for prewarming slide, chamber and loading of mix onto array.
6. Incubate for 4 hours at 60°C. (Genisphere Tech support has suggested 65°C, but we have never tried this.)

SECOND WASHES – in the dark

1. Add DTT to first 2 washes to minimize oxidation of Cy dyes.
2. Use 50 µl of 0.1M stock (kept in -20C freezer with Genisphere kit) in each of first 2 washes (2X SC, 0.2% SDS and 2X SSC).
3. Prewarm 2x SSC, 0.2% SDS wash buffer to 65 C.
4. Remove coverslip by washing array in the prewarmed 2X SSC, 0.2% SDS. Place the microarray in the 50 mL tube with the wash buffer and wait until the coverslip floats off- should take about 2-5 minutes. You **REALLY** want to make sure that the coverslip floats

AWAY from the slide, so pay close attention and BE VERY CAREFUL because the glass coverslip can scratch and damage your array!

5. Pour 50 ml of the warmed 2X SSC, 0.2% SDS into the white, opaque, plastic copland jars.

6. Transfer the microarray to copland jar (white plastic with cover) containing the 2X SSC, 0.2% SDS, wash the microarray for ~10 minutes with GENTLE shaking (put in shaking at room temperature).

7. Wash for 10 minutes in 2X SSC at room temperature- put in shaker (done in copland jar).

8. Wash for 10 minutes in 0.2X SSC at room temperature- put in shaker (done in copland jar).

9. Immediately transfer the array to a dry plastic 50 ml. Centrifuge for 2 minutes at room temperature at 1000 RPM. IMPORTANT-Make sure label is at bottom of tube. Careful not to touch the array surface!

10. Store array in dark box until ready to scan * **SHOULD SCAN IMMEDIATELY**