Quick Reference Card

Affymetrix® Gene Profiling Reagents

Intended Use

For In Vitro Diagnostic Use

Affymetrix® Gene Profiling Reagents are intended for the preparation of labeled complementary RNA target from purified total RNA from fresh or frozen clinical tissue specimens for hybridization to Affymetrix GeneChip® microarrays and the measurement of fluorescence signals of labeled RNA target using the Affymetrix GeneChip® Microarray Instrumentation System.

Intended for use with separately FDA-cleared Affymetrix GeneChip microarray assays specifying the use of Affymetrix Gene Profiling Reagents.

This quick reference guide is for experienced users only and is supplemental to the *Affymetrix® Gene Profiling Reagents User Guide* (P/N 702749).

Procedure 1: Thermal Cycler Setup

Thermal Cycler programs utilized are listed at each Procedure in this QRC.

See Chapter 2 of the *Affymetrix® Gene Profiling Reagents User Guide* (P/N 702749).

Procedure 2: Preparation of Poly-A Controls

The Affymetrix® RNA Control Kit (P/N 901285) is used for this procedure.

Table 1: Serial Dilution of Poly-A Control Stock

Starting Amount of Total RNA	Serial Dilution				Volume to Add to Total RNA
	First	Second	Third	Fourth	
100 ng	1:20	1:50	1:50	1:5	1 µL
200 ng	1:20	1:50	1:50	1:2.5	1 µL
500 ng	1:20	1:50	1:50		1 µL
1,000 ng	1:20	1:50	1:25		1 µL

Table 1 provides a guideline when 100, 200, 500 or 1,000 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, the appropriate dilutions giving the same final concentration of spike-in controls in the samples must be calculated.

TIP: Avoid pipetting solutions in volumes smaller than 2 µL to maintain precision and consistency when preparing the dilutions.

1. Use the fourth dilution (for 100 and 200 ng of total RNA) to prepare the solution described next in *Procedure 3: Preparation of 1st-Strand cDNA Synthesis Reaction*.

Procedure 3: Preparation of 1st-Strand cDNA Synthesis Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

Table 2: 1st-Strand cDNA Synthesis Thermal Cycler Settings

Step / Method	Incubation Program	Incubation Program	Hold Program	Reaction Volume
1st-Strand cDNA Synthesis	42°C for 2 hours	4°C for 10 minutes	4°C hold	10 µL

Table 3: Preparation of 1st -Strand Master Mix

Component	Working Master Mix Volume Sufficient for 1 Reaction (V)	Working Master Mix Volume Sufficient for 1 Reaction x 1.15 (V x 1.15)	Desired Number of Reactions (R)	Total Volume Required (V x 1.15) x R
1st-Strand Synthesis Buffer	4 µL	4.6 µL		
1st-Strand Synthesis Enzyme Mix	1 µL	1.15 µL		
Diluted Poly-A Control (from Procedure 2)	1 µL	1.15 µL		
Total Volume	**6 µL**	**6.9 µL**		

TIP: Take one reagent out at a time and return to the appropriate storage place when done. Use a benchtop cooler to transport enzymes. Gently vortex and briefly spin down each reagent, including the enzyme, before adding to the master mix.

1. Turn on thermal cycler at least 15 minutes before use.
2. Prepare 1st-Strand Master Mix at room temperature according to Table 3.
3. Transfer the appropriate volume of diluted Poly-A Control from Procedure 2 to the 1st-Strand Master Mix according to Table 3.
4. Mix 1st-Strand Master Mix by gently vortexing. Briefly spin down the tube.

5. Add 6 µL of 1st-Strand Master Mix to bottom of wells of 96-well plate.
6. Add 4 µL of total RNA samples into the appropriate wells. Use a total of 100 to 1000 ng per reaction; if the total RNA is in less than 4 µL, add nuclease-free water up to 4 µL. Gently mix by pipetting up and down several times.
7. Cover plate with aluminum adhesive foil, spin at 370 x g for 10 seconds at room temperature, then incubate using 1st-Strand cDNA Synthesis Program. Use compression pad and heated lid.
8. Remove within 10 minutes of 4°C hold at the end of the program and proceed immediately to *Procedure 4: Preparation of 2nd-Strand cDNA Synthesis Reaction*.

Procedure 4: Preparation of 2nd-Strand cDNA Synthesis Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

Table 4: 2nd-Strand cDNA Synthesis Thermal Cycler Settings

Step / Method	Incubation Program	Incubation Program	Hold Program	Reaction Volume
2nd-Strand cDNA Synthesis	16°C for 1 hour	4°C for 10 minutes	4°C hold	30 µL

Table 5: Preparation of 2nd-Strand Master Mix

Component	Working Master Mix Volume Sufficient for 1 Reaction (V)	Working Master Mix Volume Sufficient for 1 Reaction X 1.10 (V x 1.10)	Desired Number of Reactions (R)	Total Volume Required (V x 1.10) x R
2nd-Strand Synthesis Buffer	18 µL	19.8 µL		
2nd-Strand Synthesis Enzyme Mix	2 µL	2.2 µL		
Total Volume	**20 µL**	**22.0 µL**		

TIP: Take one reagent out at a time and return to the appropriate storage place when done. Use a benchtop cooler to transport enzymes. Gently vortex and briefly spin down each reagent, including the enzyme, before adding to the master mix.

1. Prepare 2nd-Strand Master Mix at room temperature according to Table 5. Mix by gently vortexing. Briefly spin down the tube.
2. Transfer 20 µL of 2nd-Strand Master Mix to side wall of appropriate wells.
3. Cover plate with new aluminum adhesive foil, spin at 370 x g for 10 seconds at room temperature, then incubate using 2nd-Strand cDNA Synthesis Program.

   IMPORTANT: Do not use heated lid for this incubation.
4. Remove within 10 minutes of 4°C hold at end of program.
5. Proceed immediately to *Procedure 5: Preparation of the In Vitro Transcription (IVT) Reaction*.

Procedure 5: Preparation of the In Vitro Transcription (IVT) Reaction

Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) and Kit B (901298) are used for this procedure.

Table 6: IVT Reaction Thermal Cycler Settings

Step / Method	Incubation Program	Incubation Program	Hold Program	Reaction Volume
IVT Reaction	40°C for 16 hours*		4°C hold	60 µL
** Incubation time is specific for 100 ng of total RNA. The time should be adjusted based on the amount of starting total RNA used in these procedures*				

Table 7: Recommended Incubation Times

Amount of Total RNA	Recommended Incubation Time
100 ng	16 hours
500 ng	4 to 8 hours
1,000 ng	2 to 4 hours

Table 8: IVT Master Mix Preparation

	Working Maser Mix Volumes Sufficient for:		Desired # of Rxns	Total Volume Required
Component	1 Rxn (V)	1 Rxn x 1.10 (V x 1.10)	(R)	(V x 1.10) x R
In Vitro Transcription Buffer	22 µL	24.2 µL		
RNA Label	2 µL	2.2 µL		
In Vitro Transcription Enzyme Mix	6 µL	6.6 µL		
Total Volume	**30 µL**	**33 µL**		

TIP: Remove RNA label tube and allow contents to thaw at room temperature.

1. Prepare IVT Master Mix at room temperature according to Table 8. Mix by gently vortexing. Briefly spin down the tube
2. Transfer 30 µL of IVT Master Mix to the side wall of each well containing 30 µL of 2nd-strand reaction.
3. Incubate the IVT reaction according to Tables 6 and 7. Use compression pad and heated lid.
4. After incubation, proceed to *Procedure 6: Purification of cRNA from the IVT Reaction*.

Procedure 6: Purification of cRNA from the IVT Reaction

The Affymetrix® Transcript Synthesis and Labeling Kit A (P/N 901293) is used for this procedure.

TIP:

- **Aliquot 400 µL of Nuclease-free Water in a Nuclease-free 1.5 mL tube. This volume is sufficient to process 8 reactions. Place the Nuclease-free Water at 60°C for at least 10 minutes on a heat block.**
- **Add 12.6 mL of 100% ethanol to the bottle of Beads Wash Buffer before the first use, and mix by inversion**.

1. cRNA Binding
   - **A.** Gently shake the bottle of Magnetic Beads to resuspend any magnetic particles that may have settled. Transfer 975 µL of magnetic beads into the small compartment of the trough.
   - **B.** Add 108 µL Magnetic Beads to each sample, mix, and transfer to a separate well of a U-bottom plate.
   - **C.** Shake for 2 minutes at medium speed on plate shaker.
   - **D.** Move the plate to a magnetic stand and capture the magnetic beads for 5 to 10 minutes, until a pellet forms and the solution is clear.
   - **E.** Once solution is transparent, remove and discard the supernatant without disturbing the beads.
2. Washing the Beads
   - **A.** Transfer 4 mL of the Beads Wash Buffer into the large compartment of the trough.
   - **B.** With the plate on a magnetic stand, add 200 µL Bead Wash Solution to each sample, without disturbing the beads. Incubate 25 to 35 seconds at room temperature.
   - **C.** Aspirate off the supernatant without disturbing the beads and discard it.
   - **D.** Repeat steps 2A and 2B above once more.
   - **E.** Allow the plate to air-dry for 5 to 7 minutes sitting on the magnetic stand. Do not cover plate.
   - **F.** Remove plate from the magnetic stand.
3. cRNA Elution

   IMPORTANT: When using the repeater pipette, allow for two extra 30 µL volumes: discard the first 30 µL before adding to the samples and do not use the last 30 µL of the elution solution.

   - **A.** Add 30 µL preheated (60°C) Nuclease-free Water, using a repeater pipette to the side wall of each well without disturbing the pellet.
   - **B.** Cover the plate with the lid and transfer to the shaker. Shake at high speed for one minute on plate shaker.
   - **C.** Check that the pellet is fully dispersed. Move the plate to a magnetic stand, and allow beads to settle for 3 to 4 minutes. The solution should be clear, with all the beads pelleted against the magnet.
   - **D.** Transfer 30 µL of the supernatant, which contains the eluted cRNA, to a nuclease-free PCR tube.
   - **E.** Proceed to *Procedure 7: Quantitation of the cRNA*, or store at –80°C.

Procedure 7: Quantitation of the cRNA

Refer to Chapter 2 of the *Affymetrix® Gene Profiling Reagents User Guide* (P/N 702749).

Use adjusted yield in Procedure 8: Preparation of the cRNA Fragmentation Reaction.

Procedure 8: Preparation of the cRNA Fragmentation Reaction

Nuclease-free Water and the 5X Fragmentation Buffer from the Transcript Detection Kit A (P/N 901307) are used for this procedure.

Table 9: Fragmentation Thermal Cycler Setting

Step / Method	Incubation Program	Incubation Program	Hold Program	Reaction Volume
Fragmentation	94°C for 35 minutes	4°C for 10 minutes	4°C hold	30 µL

Table 10: Preparation of Fragmentation Reaction

Component	Amount or Volume
cRNA	15 µg
5X Fragmentation Buffer	6 µL
Nuclease-free water	Variable; to 30 µL final volume
Total Volume	**30 µL**

1. Set up reaction in 0.2 mL strip tube according to Table 10, using the adjusted cRNA yield from Procedure 7 to calculate the volume of cRNA required to add 15 µg to the fragmentation reaction.
2. Mix by gentle vortexing and briefly spin down to collect the contents at the bottom of the tube.
3. Incubate in thermal cycler using the Fragmentation method. Cover with the heated lid.

Procedure 9: Preparation of the Target Hybridization Cocktail

Affymetrix® Transcript Detection Kit A (P/N 901307) and Kit C (P/N 901312) are used for this procedure.

Table 11: Preparation of Hybridization Master Mix

Component	Working Master Mix Volumes Sufficient for 1 Probe Array (V)	Working Master Mix Volumes Sufficient for 1 Probe Array x 1.10 (V x 1.10)	Desired Number of Probe Arrays (R)	Total Volume Required (V x 1.10) x R	Final Dilution/ Concentration
Oligo B2 (3 nM)	4.2 µL	4.62 µL			50 pM
20X Hybridization Control (bioB, bioC, bioD, cre)	12.5 µL	13.75 µL			1.5, 5, 25 and 100 pM respectively
2X Hybridization Mix	125 µL	137.5 µL			1X
DMSO	25 µL	27.5 µL			10%
Nuclease-free Water	58.3 µL	64.13 µL			
Total Volume	**225.0 µL**	**247.5 µL**			

TIP:

- **Remove Oligo B2 and 20X Hybridization Control from the freezer and thaw at room temperature.**
- **DMSO will solidify at 4°C; ensure that the reagent is completely thawed prior to use.**
- **Set the temperatures of the heat blocks to 45°C, 65°C and 99°C.**

IMPORTANT: It is imperative that stocks of the 20X Hybridization Control are heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquoting.

1. Prepare Hybridization Master Mix at room temperature for 1 or more arrays according to Table 11.
2. Aliquot 225 µL of the Hybridization Master Mix into a Nuclease-free 1.5 mL tube.
3. Add 25 µL of fragmented cRNA from Procedure 8 to prepare the Hybridization Cocktail for one probe array. The final concentration of cRNA in the Hybridization Cocktail is 0.05 µg/µL.
4. Equilibrate probe array to room temperature immediately before use.
5. Heat the Hybridization Cocktail to 99°C for 5 minutes in a heat block.
6. Meanwhile, wet the probe array with 200 µL of Pre-Hybridization Mix by filling it through one of the septa.
7. Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes at 60 rpm.
8. Transfer the Hybridization Cocktail that has been heated in step 5 to a 45°C heat block for 5 minutes.
9. Spin the Hybridization Cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.
10. Remove the array from the hybridization oven and remove the Pre-Hybridization Mix. Refill the array with the appropriate volume of the clarified Hybridization Cocktail, avoiding any insoluble matter at the bottom of the tube.
11. Place probe array into a hybridization oven set to 45°C. Rotate at 60 rpm.
12. Hybridize for 17 ± 1 hours.

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