Quick Reference Card

Affymetrix®

GeneChip® WT PLUS Reagent Kit

Additional Reagent Required

- Absolute Ethanol, Molecular Biology Grade

Input RNA Quantity

IMPORTANT: The RNA volume must be ≤ 5 µL (≤ 3 µL if poly-A RNA controls are used).

Table A: Total Input RNA Limits

RNA Input	Total RNA
Recommended	100 ng
Minimum	50 ng
Maximum	500 ng

Table B: Thermal Cycler Programs

Program	Heated Lid Temp	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C or 105°C	25°C for 60 min	42°C for 60 min	4°C for 2 min		10 µL
Second-Strand cDNA Synthesis	RT or disable	16°C for 60 min	65°C for 10 min	4°C for 2 min		30 µL
In Vitro Transcription cRNA Synthesis	40°C or 50°C	40°C for 16 hr	4°C hold			60 µL
2nd-Cycle Primers-cRNA Annealing	70°C or 105°C	70°C for 5 min	25°C for 5 min	4°C for 2 min		28 µL
2nd-Cycle ss-cDNA Synthesis	70°C or 105°C	25°C for 10 min	42°C for 90 min	70°C for 10 min	4°C hold	40 µL
RNA Hydrolysis	70°C or 105°C	37°C for 45 min	95°C for 5 min	4°C hold		44 µL
Fragmentation	93°C or 105°C	37°C for 60 min	93°C for 2 min	4°C hold		48 µL
Labeling	70°C or 105°C	37°C for 60 min	70°C for 10 min	4°C hold		60 µL
Hybridization Control	65°C or 105°C	65°C, 5 min				Variable
Hybridization Cocktail	99°C or 105°C	95°C or 99°C, 5 min	45°C, 5 min			Variable

Section 1: Prepare RNA with Poly-A RNA Controls

Table 1: Serial Dilution of Poly-A RNA Control Stock

Total RNA Input Amount	Serial Dilutions				Volume of 4th Dilution to Add to Total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
50 ng	1:20	1:50	1:50	1:20	2 µL
100 ng	1:20	1:50	1:50	1:10	2 µL
250 ng	1:20	1:50	1:50	1:4	2 µL
500 ng	1:20	1:50	1:50	1:2	2 µL

Table 1 provides a guideline when 50, 100, 250 or 500 ng of total RNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

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

Table 2: Total RNA/Poly-A RNA Control Mixture

Component	Volume for One Reaction (µL)
Total RNA Sample (50-500 ng)	variable
Diluted Poly-A RNA Controls (Fourth Dilution)	2
Nuclease-free Water	variable
Total Volume	**5**

TIPS for Reagent Preparation

- Mix Enzymes by gently vortexing and mix Buffers by thoroughly vortexing to dissolve precipitates.
- Include ~5% overage to correct for pipetting losses when preparing Master Mixes.
- Master Mixes and samples should be mixed thoroughly by gently vortexing followed by a quick spin to collect contents of tube or well.
- Immediately after incubation, spin the sample tubes briefly, then place on ice before proceeding to the next step.
- Enzyme should be added last and just before adding the Master Mix to the reaction.

Section 2: Synthesize First-Strand cDNA

1. On ice, prepare First-Strand Master Mix.

Table 3: First-Strand Master Mix

Component	Volume for One Reaction (µL)
First-Strand Buffer	4
First-Strand Enzyme	1
Total Volume	**5**

2. On ice, transfer 5 µL of the First-Strand Master Mix to individual tube or well. Add 5 µL of the Total RNA/Poly-A Control Mixture (Table 2).
3. Incubate for 60 min at 25°C, then for 60 min at 42°C in a thermal cycler using the "First-Strand cDNA Synthesis" program (Table B).
4. Proceed immediately to Second-Strand cDNA Synthesis.

Section 3: Synthesize Second-Strand cDNA

1. On ice, prepare Second-Strand Master Mix.

   NOTE: Pre-cool the thermal cycler block to 16°C while you are preparing the Second-Strand Master Mix.

Table 4: Second-Strand Master Mix

Component	Volume for One Reaction (µL)
Second-Strand Buffer	18
Second-Strand Enzyme	2
Total Volume	**20**

2. On ice, transfer 20 µL of the Second-Strand Master Mix to each (10 µL) first-strand cDNA sample.
3. Incubate for 60 min at 16°C, then for 10 min at 65°C in a thermal cycler using the "Second-Strand cDNA Synthesis" program (Table B).

   NOTE: Disable the heated lid of the thermal cycler or keep the lid off during the Second-Strand cDNA Synthesis.
4. Proceed immediately to In Vitro Transcription.

Section 4: Synthesize cRNA by In Vitro Transcription

1. Transfer the second-strand cDNA sample to room temperature while preparing IVT Master Mix.
2. At room temperature, prepare the IVT Master Mix.

   NOTE: Transfer the IVT Buffer to room temperature for ≥ 10 min before preparing the IVT Master Mix.

Table 5: IVT Master Mix

Component	Volume for One Reaction (µL)
IVT Buffer	24
IVT Enzyme	6
Total Volume	**30**

3. At room temperature, transfer 30 µL of the IVT Master Mix to each (30 µL) second-strand cDNA sample.
4. Incubate the IVT reaction for 16 hr at 40°C in a thermal cycler using the "In Vitro Transcription cRNA Synthesis" program (Table B).
5. Proceed to cRNA Purification, or freeze immediately.

   TIP: STOPPING POINT. The cRNA sample can be stored at –20°C.

Section 5: Purify cRNA

Before beginning the cRNA purification:

- Preheat the Nuclease-free Water to 65°C for at least 10 min.
- Mix the Purification Beads thoroughly before use. Aliquot the appropriate amount, and keep at room temperature. For each reaction, 100 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage will be needed.

NOTE: This entire procedure is performed at room temperature.

1. Bind cRNA to Purification Beads.
   - **A.** Mix the Purification Beads container to resuspend the magnetic particles. Add 100 µL of the magnetic beads to each (60 µL) cRNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.
   - **B.** Mix well by pipetting up and down 10 times. Incubate for 10 min.
   - **C.** Move the plate to a magnetic stand for ~5 min to capture the magnetic beads.
   - **D.** Carefully aspirate and discard the supernatant without disturbing the magnetic beads. Keep the plate on the magnetic stand.
2. Wash the Purification Beads.
   - **A.** While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 sec.
   - **B.** Slowly aspirate and discard the 80% ethanol wash solution without disturbing the magnetic beads.
   - **C.** Repeat Step A and Step B twice for a total of 3 washes. Completely remove the final wash solution.
   - **D.** Air-dry on the magnetic stand for 5 min until no liquid is visible. Additional time may be required. Do not over-dry the beads.
3. Elute cRNA.
   - **A.** Remove the plate from the magnetic stand. Add 27 µL of the preheated (65°C) Nuclease-free Water to each sample and incubate for 1 min.
   - **B.** Mix well by pipetting up and down 10 times.
   - **C.** Move the plate to the magnetic stand for ~5 min to capture the magnetic beads.
   - **D.** Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free tube.
   - **E.** Place the purified cRNA on ice, and proceed to quantitation.

   TIP: STOPPING POINT. The purified cRNA sample can be stored at –20°C.

Section 6: Assess cRNA Yield

Determine the concentration of a cRNA solution by measuring its absorbance at 260 nm using a NanoDrop® Spectrophotometer or equivalent quantitation instrument.

Section 7: Synthesize 2nd-Cycle ss-cDNA

1. On ice, prepare 15 µg of cRNA in a volume of 24 µL with Nuclease-free Water.
2. Add 4 µL of 2nd-Cycle Primers to each (24 µL) cRNA sample.
3. Incubate for 5 min at 70°C, 5 min at 25°C, then for 2 min at 4°C in a thermal cycler using the "2nd-Cycle Primers-cRNA Annealing" program (Table B).
4. Place the cRNA/2nd-Cycle Primers sample on ice.
5. On ice, prepare 2nd-Cycle ss-cDNA Master Mix.

Table 6: 2nd-Cycle ss-cDNA Master Mix

Component	Volume for One Reaction (µL)
2nd-Cycle ss-cDNA Buffer	8
2nd-Cycle ss-cDNA Enzyme	4
Total Volume	**12**

6. Transfer 12 µL of the 2nd-Cycle ss-cDNA Master Mix to each (28 µL) cRNA/2nd-Cycle Primers sample.
7. Incubate for 10 min at 25°C, 90 min at 42°C, then for 10 min at 70°C in a thermal cycler using the "2nd-Cycle ss-cDNA Synthesis" program (Table B).
8. Proceed immediately to RNA Hydrolysis.

Section 8: Hydrolyze RNA using RNase H

1. On ice, add 4 µL of RNase H to each (40 µL) 2nd-Cycle ss-cDNA sample.
2. Mix thoroughly, followed by a quick spin.
3. Incubate for 45 min at 37°C in a thermal cycler using the "RNA Hydrolysis" program (Table B).
4. Place the hydrolyzed 2nd-Cycle ss-cDNA sample on ice.
5. On ice, add 11 µL of Nuclease-free Water to each (44 µL) hydrolyzed 2nd-Cycle ss-cDNA sample.
6. Mix thoroughly and proceed to ss-cDNA Purification, or freeze immediately.

TIP: STOPPING POINT. The hydrolyzed ss-cDNA samples can be stored at –20°C.

Section 9: Purify 2nd-Cycle ss-cDNA

Before beginning the ss-cDNA purification:

- Preheat the Nuclease-free Water to 65°C for at least 10 min.
- Mix the Purification Beads thoroughly before use. Aliquot the appropriate amount, and keep at room temperature. For each reaction, 100 µL plus ~10% overage will be needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage will be needed.

NOTE: This entire procedure is performed at room temperature.

1. Bind ss-cDNA to Purification Beads.
   - **A.** Mix the Purification Beads container to resuspend the magnetic particles. Add 100 µL of the magnetic beads to each (55 µL) hydrolyzed ss-cDNA sample, mix by pipetting up and down, and transfer to a well of a U-bottom plate.
   - **B.** Add 150 µL of 100% ethanol to each sample. Mix well by pipetting up and down 10 times. Incubate for 20 min.
   - **C.** Move the plate to a magnetic stand for ~5 min to capture the magnetic beads.
   - **D.** Carefully aspirate and discard the supernatant without disturbing the magnetic beads. Keep the plate on the magnetic stand.
2. Wash the Purification Beads.
   - **A.** While on the magnetic stand, add 200 µL of 80% ethanol wash solution to each well and incubate for 30 sec.
   - **B.** Slowly aspirate and discard the 80% ethanol wash solution without disturbing the magnetic beads.
   - **C.** Repeat Step A and Step B twice for a total of 3 washes. Completely remove the final wash solution.
   - **D.** Air-dry on the magnetic stand for 5 min until no liquid is visible. Additional time may be required. Do not over-dry the beads.
3. Elute ss-cDNA.
   - **A.** Remove the plate from the magnetic stand. Add 30 µL of the preheated (65°C) Nuclease-free Water to each sample and incubate for 1 min.
   - **B.** Mix well by pipetting up and down 10 times.
   - **C.** Move the plate to the magnetic stand for ~5 min to capture the magnetic beads.
   - **D.** Transfer the supernatant, which contains the eluted ss-cDNA, to a nuclease-free tube.
   - **E.** Place the purified ss-cDNA on ice, and proceed to quantitation.

   TIP: STOPPING POINT. The purified cDNA sample can be stored at –20°C.

Section 10: Assess ss-cDNA Yield

Determine the concentration of a ss-cDNA solution by measuring its absorbance at 260 nm using a NanoDrop® Spectrophotometer or equivalent quantitation instrument.

Section 11: Fragment and Label ss-cDNA

1. On ice, prepare 5.5 µg of ss-cDNA in a volume of 31.2 µL with Nuclease-free Water.
2. On ice, prepare Fragmentation and Labeling Master Mix.

Table 7: Fragmentation Master Mix

Component	Volume for one reaction (µL)
Nuclease-free Water	10
10X cDNA Fragmentation Buffer	4.8
UDG, 10 U/µL	1
APE 1, 1,000 U/µL	1
Total Volume	**16.8**

3. Transfer 16.8 µL of the Fragmentation Master Mix to each (31.2 µL) 5.5 µg ss-cDNA sample.
4. Incubate for 60 min at 37°C, then for 2 min at 93°C in a thermal cycler using the "Fragmentation" program (Table B).
5. On ice, transfer 45 µL of the fragmented ss-cDNA sample to individual tube or well.
6. On ice, prepare Labeling Master Mix.

Table 8: Labeling Master Mix

Component	Volume for one reaction (µL)
5X TdT Buffer	12
DNA Labeling Reagent, 5 mM	1
TdT, 30 U/µL	2
Total Volume	**15**

7. Transfer 15 µL of the Labeling Master Mix to each (45 µL) fragmented ss-cDNA sample.

8. Incubate for 60 min at 37°C, then for 10 min at 70°C in a thermal cycler using the "Labeling" program (Table B).
9. Proceed immediately to WT Cartridge Array Hybridization.

Section 12: WT Cartridge Array Hybridization

Please refer to *Affymetrix® WT PLUS Reagent Kit User Manual* for Array Strip and Array Plate Hybridization.

NOTE:

- Ensure that the reagent is completely thawed before use. Store DMSO at room temperature after the first use. DMSO will solidify when stored at 2-8°C.
- This procedure requires the use of the GeneChip® Hybridization, Wash and Stain Kit (not supplied).

1. Heat the 20X Hybridization Controls for 5 min at 65°C in a thermal cycler using the "Hybridization Control" program (Table B).
2. At room temperature, prepare Hybridization Master Mix.

Table 9: Hybridization Master Mix for a Single Reaction

Component	49 or 64-Format	100 or 81/4-Format	169-Format	Final Concentration
Fragmented and Labeled ss-cDNA	**5.2 µg**	**3.5 µg**	**2.3 µg**	**23 ng/µL**
Control Oligonucleotide B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	11 µL	7.5 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	75 µL	50 µL	1X
DMSO	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	19.9 µL	13.5 µL	9.3 µL	
Total Volume	**160 µL**	**109 µL**	**73 µL**	

Please refer to specific probe array package insert for information on array format.

3. Add the Hybridization Master Mix to individual tube containing the biotin-labeled ss-cDNA sample to prepare Hybridization Cocktail.

Table 10: Hybridization Cocktail for a Single Array

Component	49 or 64-Format	100 or 81/4-Format	169-Format
Hybridization Master Mix	160 µL	109 µL	73 µL
Fragmented and Labeled ss-cDNA	~60 µL (5.2 µg)	41 µL (3.5 µg)	27 µL (2.3 µg)
Total Volume	**220 µL**	**150 µL**	**100 µL**

4. Incubate the Hybridization Cocktail for 5 min at 99°C (tubes) or 95°C (plates), then for 5 min at 45°C in a thermal cycler using the "Hybridization Cocktail" program (Table B).
5. Inject the appropriate amount (Table 11) of the specific sample into the array.

Table 11: Probe Array Cartridge Volumes for Hybridization Cocktail

	49 or 64-Format	100 or 81/4-Format	169-Format
Volume to Load on Array	200 µL	130 µL	80 µL

6. Hybridize with rotation at 60 rpm for 16 hr at 45°C.

Table 12: Fluidics Protocol

Component	49 or 64-Format	100 or 81/4-Format	169-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0007

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Not for use in Diagnostic Procedures.

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