Quick Reference Card

affymetrix
Biology for a better world

GeneChip® 3' IVT 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	Volume
First-Strand cDNA Synthesis	42°C or 105°C	42°C for 2 hr	4°C for 2 min		10 µL
Second-Strand cDNA Synthesis	RT or disable	16°C for 1 hr	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 4 - 16 hr[1]	4°C hold		60 µL
Fragmentation	94°C or 105°C	94°C for 35 min	4°C hold		Variable
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

1. 4 hr for 250 - 500 ng RNA input, and 16 hr for 50 - 250 ng RNA input.

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)
3′ First-Strand Buffer	4
3′ 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 2 hr 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)
Nuclease-free Water	13
3′ Second-Strand Buffer	5
3′ 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 1 hr 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 Labeled 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)
3′ IVT Biotin Label	4
3′ IVT Buffer	20
3′ 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 4 - 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 Labeled 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 labeled 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) labeled 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 Labeled 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: Fragment Labeled cRNA

1. On ice, prepare the amount of labeled cRNA needed for hybridization with your GeneChip® array format.

Table 6: One Labeled cRNA Fragmentation Reaction by Array Format

Component	49/64-Format		100-Format or 81/4-Format	169, 400-Format, or Array Plate	Array Strip
Labeled cRNA	15 µg (in 32 µL)	12 µg[1] (in 25.6 µL)	12 µg (in 25.6 µL)	7.5 µg (in 16 µL)	9.4 µg (in 20 µL)
3′ Fragmentation Buffer	8 µL	6.4 µL	6.4 µL	4 µL	5 µL
Total Volume	**40 µL**	**32 µL**	**32 µL**	**20 µL**	**25 µL**

1. Alternative protocol for samples with cRNA yields below 15 µg.

2. Incubate for 35 min at 94°C in a thermal cycler using the "Fragmentation" program (Table B).
3. Place the reaction on ice immediately after the incubation.
4. (Optional) Evaluate a sample of the reaction on a Bioanalyzer.
5. Proceed to 3′ IVT Cartridge Array Hybridization.

   TIP: STOPPING POINT. The fragmented cRNA sample can be stored at –20°C (or –70°C for long-term storage).

Section 8: 3′ IVT Cartridge Array Hybridization

Please refer to *Affymetrix® 3′ IVT 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).
- Equilibrate the array and the pre-hybridization buffer to room temperature.
- Set the Hybridization Oven temperature to 45°C.

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 7: Hybridization Master Mix for a Single Reaction

Component	49/64-Format	100-Format	169/400-Format	Final Concentration
Fragmented and Labeled cRNA	**11 µg**	**10 µg**	**5 µg**	**50 ng/µL**
Control Oligonucleotide B2 (3 nM)	3.7 µL	3.3 µL	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	11 µL	10 µL	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	110 µL	100 µL	50 µL	1X
DMSO	22 µL	20 µL	10 µL	10%
Nuclease-free Water	43.9 µL	40 µL	20 µL	
Total Volume	**190.6 µL**	**173.3 µL**	**86.7 µL**	

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

3. Inject the appropriate amount (Table 8) of the Pre-Hybridization Mix into the array.

Table 8: Array Cartridge Volumes for Pre-Hybridization Mix

	49/64-Format	100-Format	169/400-Format
Volume to Load on Array	200 µL	130 µL	80 µL

4. Pre-hybridize with rotation at 60 rpm for 10 - 30 min at 45°C.
5. Add the Hybridization Master Mix to individual tube containing the labeled cRNA sample to prepare Hybridization Cocktail.

Table 9: Hybridization Cocktail for a Single Array

Component	49/64-Format	100-Format	169/400-Format
Hybridization Master Mix	190.6 µL	173.3 µL	86.7 µL
Fragmented and Labeled cRNA	29.4 µL (11 µg)	26.7 µL (10 µg)	13.3 µL (5 µg)
Total Volume	**220 µL**	**200 µL**	**100 µL**

6. 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).
7. Inject the appropriate amount (Table 10) of the specific sample into the array.

Table 10: Array Cartridge Volumes for Hybridization Cocktail

	49/64-Format	100-Format	169/400-Format
Volume to Load on Array	200 µL	130 µL	80 µL

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

Table 11: Fluidics Protocol

Component	49/64-Format	100-Format	169/400-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0003

Limited License

Subject to the Affymetrix terms and conditions that govern your use of Affymetrix products, Affymetrix grants you a non-exclusive, non-transferable, non-sublicensable license to use this Affymetrix product only in accordance with the manual and written instructions provided by Affymetrix. You understand and agree that except as expressly set forth in the Affymetrix terms and conditions, that no right or license to any patent or other intellectual property owned or licensable by Affymetrix is conveyed or implied by this Affymetrix product. In particular, no right or license is conveyed or implied to use this Affymetrix product in combination with a product not provided, licensed or specifically recommended by Affymetrix for such use.

Patents

This product may be covered by one or more of the following patents: U.S. Patent Nos. 6,864,059; 6,965,020; 7,282,327; 7,291,463 and 7,468,243 and other U.S. and foreign patents.

Trademarks

Affymetrix®, Axiom®, Command Console®, CytoScan®, DMET™, GeneAtlas®, GeneChip®, GeneChip-compatible™, GeneTitan®, Genotyping Console™, myDesign™, NetAffx®, OncoScan™, Powered by Affymetrix™, PrimeView®, Procarta®, and QuantiGene® are trademarks or registered trademarks of Affymetrix, Inc. 3DNA®, FlashTag™ and Genisphere® are trademarks or registered trademarks of Genisphere, LCC. All other trademarks are the property of their respective owners.

Contact Information

Affymetrix, Inc.
3420 Central Expressway
Santa Clara, CA 95051 USA
E-mail: support@affymetrix.com
Tel: 1-888-362-2447 (1-888-DNA-CHIP)
Fax: 1-408-731-5441

Affymetrix UK Ltd
Voyager, Mercury Park,
Wycombe Lane, Wooburn Green,
High Wycombe HP10 0HH
United Kingdom
E-mail: supporteurope@affymetrix.com
UK and Others Tel: +44 (0) 1628 552550
France Tel: 0800919505
Germany Tel: 01803001334
Fax: +44 (0) 1628 552585

Affymetrix Japan, K. K.
ORIX Hamamatsucho Bldg, 7F
1-24-8 Hamamatsucho, Minato-ku
Tokyo 105-0013, Japan
E-mail: supportjapan@affymetrix.com
Tel: +81-3-6430-4020
Fax: +81-3-6430-4021

For Research Use Only
Not for use in Diagnostic Procedures.