QuickReferenceCard

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GeneChip® Pico Reagent Kit

Additional Reagent Required

- 100% Ethanol, Molecular Biology Grade
- GeneChip® Hybridization, Wash, and Stain Kit

Input RNA Quantity

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

Table A: Input RNA Limits

RNA Input	Total RNA from Fresh-Frozen Cells or Tissues	Total RNA from Formalin-Fixed, Paraffin-Embedded Tissues
Minimum	100 pg	500 pg
Recommended	500 pg - 10 ng	2 ng - 50 ng
Maximum	10 ng	50 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 5 min	42°C for 60 min	4°C for 2 min		10 µL
Cleanup	80°C or 105°C	37°C for 30 min	80°C for 10 min	4°C for 2 min		12 µL
3′ Adaptor Synthesis	RT, disable, or left open	15°C for 15 min	35°C for 15 min	70°C for 10 min	4°C for 2 min	20 µL
Pre-IVT Amplification	105°C	95°C for 2 min	6, 9 or 12 cycles of 94°C for 30 sec, 70°C for 5 min	4°C for 2 min		50 µL
In Vitro Transcription cRNA Synthesis	40°C or 105°C*	40°C for 14 hr	4°C hold			80 µL
2nd-Cycle ds-cDNA Synthesis	70°C or 105°C	25°C for 10 min	42°C for 50 min	70°C for 10 min	4°C hold	40 µL
RNA Alkaline Hydrolysis	70°C or 105°C	65°C for 20 min	4°C hold			47 µL
Fragmentation and Labeling	93°C or 105°C	37°C for 60 min	93°C for 2 min	4°C hold		30 or 60 µL
Hybridization Control	65°C or 105°C	65°C for 5 min				Variable
Hybridization Cocktail	99°C or 105°C	95°C or 99°C for 5 min	45°C for 5 min			Variable

** Use 0.2 mL or larger volume tubes or plates when using heated-lid setting at 105°C.*

Table C: Pre-IVT Amplification Cycling Guidelines Based on Sample Type and the Amount of Starting Total RNA Input

RNA Input	Typical Number of PCR Cycles for Fresh- Frozen Cell or Tissue Samples	Typical Number of PCR Cycles for Formalin-Fixed, Paraffin-Embedded Tissue Samples
100 pg to <500 pg	12	N/A
500 pg to <2 ng	9	12
2 ng to 10 ng	6	9
>10 ng to 50 ng	N/A	6

TIP: One or more PCR cycles may be added to the cycling guidelines for Pre-IVT Amplification program to improve cRNA yield of poor quality RNA sample.

Section 1: Prepare RNA with Poly-A RNA Controls

NOTE: Do not use Poly-A Control Dil Buffer to prepare serial dilution of Poly-A RNA Controls because it may cause non-target amplification.

Table 1: Serial Dilution of Poly-A RNA Control Stock with **Nuclease-free Water**

Total RNA Input Amount	Serial Dilutions				Volume of Fourth Dilution to Add to Total RNA
	First Dilution	Second Dilution	Third Dilution	Fourth Dilution	
≤1 ng	1:50	1:100	1:100	1:100	2 µL
2 ng	1:50	1:100	1:100	1:50	2 µL
5 ng	1:50	1:100	1:100	1:20	2 µL
10 ng	1:50	1:100	1:100	1:10	2 µL
20 ng	1:50	1:100	1:100	1:5	2 µL
50 ng	1:50	1:100	1:100	1:2	2 µL

Table 1 provides a guideline when the indicated amount of total RNA is used as starting material. For starting sample amounts other than those listed here and ≥1 ng, 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 (100 pg - 50 ng)	variable
Diluted Poly-A RNA Controls (Fourth Dilution)	2
Nuclease-free Water	variable
Total Volume	**5**

TIPS for Reagent Preparation

- Mix Enzymes and Reagents by gently vortexing and mix Buffers by thoroughly vortexing to dissolve precipitates.
- Include ~10% overage to correct for pipetting losses when preparing Master Mixes.
- Immediately after incubation, spin the sample tubes briefly, and 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.

IMPORTANT: Master Mixes and samples should be mixed thoroughly by gently vortexing followed by a quick spin to remove air bubbles and collect contents of tube or well.

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)
Pico First-Strand Buffer	4
Pico 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 5 min at 25°C, and then for 60 min at 42°C in a thermal cycler using the "First-Strand cDNA Synthesis" program (Table B).
4. On ice, add 2 µL of Pico Cleanup Reagent to each (10 µL) cDNA sample, pipette up and down twice, and mix by gently vortexing.

   NOTE: Air bubbles that may form during mixing should be removed by a quick spin.
5. Incubate for 30 min at 37°C, and then for 10 min at 80°C in a thermal cycler using the "Cleanup" program (Table B).
6. Proceed immediately to 3' Adaptor Synthesis.

Section 3: Synthesize 3′ Adaptor cDNA

1. On ice, prepare 3′ Adaptor Master Mix.

   NOTE: Pre-cool the thermal cycler block to 15°C while you are preparing the 3′ Adaptor Master Mix.

   Table 4: 3′ Adaptor Master Mix

Component	Volume for One Reaction (µL)
Pico 3′ Adaptor Buffer	7
Pico 3′ Adaptor Enzyme	1
Total Volume	**8**

2. On ice, transfer 8 µL of the 3′ Adaptor Master Mix to each (12 µL) cDNA sample.
3. Incubate for 15 min at 15°C, for 15 min at 35°C, and then for 10 min at 70°C in a thermal cycler using the "3′ Adaptor cDNA Synthesis" program (Table B).

   NOTE: Disable the heated lid of the thermal cycler or keep the lid off during the 3′ Adaptor cDNA Synthesis.
4. Proceed immediately to Pre-IVT Amplification.

Section 4: Synthesize ds-cDNA

1. On ice, prepare Pre-IVT Amplification Master Mix.

   Table 5: Pre-IVT Master Mix

Component	Volume for One Reaction (µL)
Pico PCR Buffer	29
Pico PCR Enzyme	1
Total Volume	**30**

2. On ice, transfer 30 µL of the Pre-IVT Master Mix to each (20 µL) cDNA sample.

   NOTE: The following steps should be performed in Post-Amplification Area using dedicated supplies and equipment.
3. Incubate the Pre-IVT Amplification reaction for 2 min at 95°C, and then for 6, 9 or 12 cycles of 30 sec at 94°C and 5 min at 70°C in a thermal cycler using the "Pre-IVT Amplification" program (Table B). See Table C for cycling guidelines based on the sample type and amount of starting total RNA input.
4. Proceed immediately to *In Vitro* Transcription.

NOTE: One or more PCR cycles may be added to the cycling guidelines for Pre-IVT Amplification program to improve cRNA yield of poor quality RNA sample.

Section 5: Synthesize cRNA by *In Vitro* Transcription

1. Transfer the ds-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 6: IVT Master Mix

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

3. At room temperature, transfer 30 µL of the IVT Master Mix to each (50 µL) ds-cDNA sample.
4. Incubate the IVT reaction for 14 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 6: 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, 140 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 and transfer 140 µL of the Purification Beads to a clean well of a round bottom plate. Add 80 µL of cRNA sample to each (140 µL) Purification Beads, and mix by pipetting up and down 10 times.
   - **B.** 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:
   - Add an additional 10 to 70 µL of the preheated Nuclease-free Water to the well to elute the sample with high concentration cRNA.
   - STOPPING POINT. The purified cRNA sample can be stored at –20°C.

Section 7: 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.

NOTE: Samples with cRNA concentrations greater than 3,000 ng/µL should be diluted with Nuclease-free Water before measurement and reaction setup.

Section 8: Synthesize 2nd-Cycle ds-cDNA

1. On ice, prepare 20 µg of cRNA in a volume of 24 µL with Nuclease-free Water.
2. On ice, prepare 2nd-Cycle ds-cDNA Master Mix.

Table 7: 2nd-Cycle ds-cDNA Master Mix

Component	Volume for One Reaction (µL)
Pico 2nd-Cycle Primers	4
Pico 2nd-Cycle ds-cDNA Buffer	8
Pico 2nd-Cycle ds-cDNA Enzyme	4
Total Volume	**16**

3. Transfer 16 µL of the 2nd-Cycle ds-cDNA Master Mix to each (24 µL) 20 µg cRNA sample.
4. Incubate for 10 min at 25°C, for 50 min at 42°C, and then for 10 min at 70°C in a thermal cycler using the "2nd-Cycle ds-cDNA Synthesis" program (Table B).
5. Proceed immediately to RNA Alkaline Hydrolysis.

Section 9: Hydrolyze RNA using Hydrolysis Buffer

1. On ice, add 7 µL of Pico Hydrolysis Buffer to each (40 µL) 2nd-Cycle ds-cDNA sample.
2. Mix thoroughly, followed by a quick spin.
3. Incubate for 20 min at 65°C in a thermal cycler using the "RNA Alkaline Hydrolysis" program (Table B).
4. Place the hydrolyzed 2nd-Cycle ds-cDNA sample on ice.
5. On ice, add 10 µL of Pico Neutralization Buffer to each (47 µL) hydrolyzed 2nd-Cycle ds-cDNA sample.
6. Mix thoroughly and proceed to ds-cDNA Purification, or freeze immediately.

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

Section 10: Purify 2nd-Cycle ds-cDNA

Before beginning the ds-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 ds-cDNA to Purification Beads.
   - **A.** Mix the Purification Beads container to resuspend the magnetic particles and transfer 100 µL of the Purification Beads to a clean well of a round bottom plate. Add 57 µL of hydrolyzed ds-cDNA sample to each (100 µL) Purification Beads, and mix by pipetting up and down 10 times.

     NOTE: Do not add ethanol to ds-cDNA samples.
   - **B.** 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 ds-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.

     NOTE: Elute with 25 µL of the preheated (65°C) Nuclease-free Water for samples for array plate hybridization.
   - **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 ds-cDNA, to a nuclease-free tube.
   - **E.** Place the purified ds-cDNA on ice and proceed to quantitation.

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

Section 11: Assess ds-cDNA Yield

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

Section 12: Fragment and Label ds-cDNA

1. On ice, prepare normalized ds-cDNA in appropriate volume of Nuclease-free Water depending on array type.

Table 8: ds-cDNA Normalization

Array	For One Reaction			
	3' Cartridge All formats	WT Cartridge All formats	Clariom™ S Cartridge 400-format	All Plates
Component				
ds-cDNA	6.6 µg	11 µg	5.5 µg	5.5 µg
Nuclease-free Water to	46 µL	44 µL	22 µL	22 µL
ds-cDNA concentration	143.5 ng/µL	250 ng/µL	250 ng/µL	250 ng/µL

2. On ice, prepare Fragmentation and Labeling Master Mix.

Table 9: Fragmentation and Labeling Master Mix

Array	For One Reaction			
	3' Cartridge All formats	WT Cartridge All formats	Clariom™ S Cartridge 400-format	All Plates
Component				
Pico Frag. & Label Buffer	12 µL	12 µL	6 µL	6 µL
Pico Frag. & Label Enzyme	2 µL	4 µL	2 µL	2 µL
Master Mix Total Volume	**14 µL**	**16 µL**	**8 µL**	**8 µL**
Reaction Total Volume	**60 µL**	**60 µL**	**30 µL**	**30 µL**

3. Transfer the Fragmentation Master Mix to each normalized ds-cDNA sample.
4. Incubate for 60 min at 37°C, and then for 2 min at 93°C in a thermal cycler using the "Fragmentation and Labeling" program (Table B).
5. Proceed to Array Hybridization.

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

Section 13: Cartridge Array Hybridization

Please refer to *Affymetrix® Pico Reagent Kit User Guide* (P/N 703262) for Array Plate Hybridization.

NOTE:

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

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 10A: Hybridization Master Mix for a Single Reaction

Array	**3' 49 or 64-Format**	**3' 100 or 81/4-Format**	**3' 169-Format**		**WT 49 or 64-Format**	**WT 100 or 81/4-Format**	**WT 169-Format**	
Component				**Final Concentration**				**Final Concentration**
Control Oligonucleotide B2 (3 nM)	3.7 µL	2.5 µL	1.7 µL	50 pM	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"	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	110 µL	75 µL	50 µL	1X
DMSO	22 µL	15 µL	10 µL	10%	15.4 µL	10.5 µL	7 µL	7%
Nuclease-free Water	13.3 µL	9 µL	6.3 µL		19.9 µL	13.5 µL	9.3 µL	
Total Volume	**160 µL**	**109 µL**	**73 µL**		**160 µL**	**109 µL**	**73 µL**	

Table 10B: Hybridization Master Mix for a Single Reaction

Array	**Clariom™ S 400-Format**	
Component		**Final Concentration**
Control Oligonucleotide B2 (3 nM)	1.7 µL	50 pM
20X Hybridization Controls (*bioB, bioC, bioD, cre*)	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	50 µL	1X
DMSO	7 µL	7%
Nuclease-free Water	9.3 µL	
Total Volume	**73 µL**	

3. Add the Hybridization Master Mix to individual tube containing the biotin-labeled ds-cDNA sample from Section 12 to prepare Hybridization Cocktail.

Table 11: Hybridization Cocktail for a Single Array

Array	**3' 49 or 64-Format**	**3' 100 or 81/4-Format**	**3' 169-Format**	**WT 49 or 64-Format**	**WT 100 or 81/4-Format**	**WT 169-Format**	**Clariom™ S 400-Format**
Component							
Hybridization Master Mix	160 µL	109 µL	73 µL	160 µL	109 µL	73 µL	73 µL
Fragmented and Labeled ds-cDNA	60 µL (6.6 µg)	41 µL (4.5 µg)	27 µL (3 µg)	60 µL (11 µg)	41 µL (7.5 µg)	27 µL (5 µg)	27 µL (5 µg)
Total Volume	**220 µL**	**150 µL**	**100 µL**	**220 µL**	**150 µL**	**100 µL**	**100 µL**
ds-cDNA Final Concentration	**30 ng/µL**	**30 ng/µL**	**30 ng/µL**	**50 ng/µL**	**50 ng/µL**	**50 ng/µL**	**50 ng/µL**

4. Incubate the Hybridization Cocktail for 5 min at 99°C (tubes) or 95°C (plates), and then for 5 min at 45°C in a thermal cycler using the "Hybridization Cocktail" program (Table B).
5. Inject the appropriate amount (Table 12) of the specific sample into the array.

Table 12: Probe Array Cartridge Volumes for Hybridization Cocktail

Array	3' 49 or 64-Format	3' 100 or 81/4-Format	3' 169-Format	WT 49 or 64-Format	WT 100 or 81/4-Format	WT 169-Format	Clariom™ S 400-Format
Volume to Load on Array	200 µL	130 µL	80 µL	200 µL	130 µL	80 µL	80 µL

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

Table 13: Fluidics Protocol

Array	3' 49 or 64-Format	3' 100 or 81/4-Format	3' 169-Format	WT 49 or 64-Format	WT 100 or 81/4-Format	WT 169-Format	Clariom™ S 400-Format
Fluidics Protocol	FS450_0001	FS450_0002	FS450_0003	FS450_0001	FS450_0002	FS450_0007	FS450_0007

Affymetrix, Inc. Tel: +1-888-362-2447 ■ **Affymetrix UK Ltd.** Tel: +44-(0)-1628-552550 ■ **Affymetrix Japan K.K.** Tel: +81-(0)3-6430-4020

Panomics Solutions Tel: +1-877-726-6642 panomics.affymetrix.com ■ **USB Products** Tel: +1-800-321-9322 usb.affymetrix.com

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