GeneChip™ Pico Reagent Kit

Pub. No. MAN0027197 Rev. C00

Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *GeneChip™ 3′ IVT Pico Kit Manual Workflow User Guide* (Pub. No. 703308). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Input RNA quantity and thermal cycler programs

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

Table 1 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 to 10 ng	2–50 ng
Maximum	10 ng	50 ng

Table 2 Thermal cycler programs.

Program	Heated lid temperature	Step 1	Step 2	Step 3	Step 4	Volume
First-Strand cDNA Synthesis	42°C or 105°C	25°C for 5 minutes	42°C for 60 minutes	4°C for 2 minutes		10 µL
Cleanup	80°C or 105°C	37°C for 30 minutes	80°C for 10 minutes	4°C for 2 minutes		12 µL
3' Adaptor cDNA Synthesis	RT, disable, or left open	15°C for 15 minutes	35°C for 15 minutes	70°C for 10 minutes	4°C for 2 minutes	20 µL
Pre-IVT Amplification	105°C	95°C for 2 minutes	6, 9, or 12 cycles of 94°C for 30 seconds, 70°C for 5 minutes	4°C for 2 minutes		50 µL
In Vitro Transcription cRNA Synthesis	40°C or 105°C[1]	40°C for 14 hours	4°C hold			80 µL
2nd-Cycle ds-cDNA Synthesis2nd-Cycle	70°C or 105°C	25°C for 10 minutes	42°C for 50 minutes	70°C for 10 minutes	4°C hold	40 µL
RNA Alkaline Hydrolysis	70°C or 105°C	65°C for 20 minutes	4°C hold			47 µL
Fragmentation and Labeling	93°C or 105°C	37°C for 60 minutes	93°C for 2 minutes	4°C hold		30 or 60 µL
Hybridization Control	65°C or 105°C	65°C for 5 minutes				Variable
Hybridization Cocktail	99°C or 105°C	95°C or 99°C for 5 minutes	45°C for 5 minutes			Variable

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

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Table 3 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
2–10 ng	6	9
10–50 ng	N/A	6

Note: One or more PCR cycles can be added to the cycling guidelines for the 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 can cause non-target amplification.

Table 4 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 4 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 perform the appropriate dilutions to arrive at the same proportionate final concentration of the spike-in controls in the samples.

Note: Avoid pipetting solutions <2 µL in volume to maintain precision and consistency when preparing the dilutions.

Table 5 Total RNA/Poly-A RNA Control mixture.

Component	Volume for 1 reaction
Total RNA Sample (100 pg–50 ng)	variable
Diluted Poly-A RNA Controls (fourth dilution)	2 µL
Nuclease-free water	variable
Total volume	**5 µL**

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, briefly centrifuge the sample tubes, then place on ice before proceeding to the next step.
- Enzyme should be added last and immediately before adding the master mix to the reaction.

IMPORTANT! Master mixes and samples should be mixed thoroughly by gently vortexing followed by briefly centrifuging to remove air bubbles and collect contents of tube or well.

Section 2: Synthesize First-Strand cDNA

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

   Table 6 First-Strand Master Mix.

Component	Volume for 1 reaction
Pico First-Strand Buffer	4 µL
Pico First-Strand Enzyme	1 µL
Total volume	**5 µL**

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 5.)

3. Incubate for 5 minutes at 25°C, and then for 60 minutes at 42°C in a thermal cycler using the "First-Strand cDNA Synthesis" program. (Table 2.)

4. On ice, add 2 µL of Pico Cleanup Reagent to each (10 µL) cDNA sample, pipette up and down twice, then mix by gently vortexing.

   Note: Air bubbles that form during mixing should be removed by briefly centrifuging.

5. Incubate for 30 minutes at 37°C, and then for 10 minutes at 80°C in a thermal cycler using the "Cleanup" program. (Table 2.)

6. Proceed immediately to "Section 3: Synthesize 3' Adaptor cDNA" on page 3.

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 7 Adaptor Master Mix.

Component	Volume for 1 reaction
Pico 3' Adaptor Buffer	7 µL
Pico 3' Adaptor Enzyme	1 µL
Total volume	**8 µL**

2. On ice, transfer 8 µL of the 3' Adaptor Master Mix to each (12 µL) cDNA sample.

3. Incubate for 15 minutes at 15°C, for 15 minutes at 35°C, and then for 10 minutes at 70°C in a thermal cycler using the "3' Adaptor cDNA Synthesis" program. (Table 2.)

   Note: Disable the heated lid of the thermal cycler or keep the lid off during the "3' Adaptor cDNA Synthesis" program.

4. Proceed immediately to "Section 4: Synthesize ds-cDNA" on page 4.

Section 4: Synthesize ds-cDNA

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

   Table 8 Pre-IVT Master Mix.

Component	Volume for 1 reaction
Pico PCR Buffer	29 µL
Pico PCR Enzyme	1 µL
Total volume	**30 µL**

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 minutes at 95°C, and then for 6, 9, or 12 cycles of 30 seconds at 94°C and 5 minutes at 70°C in a thermal cycler using the "Pre-IVT Amplification" program (Table 2). See Table 3 for cycling guidelines based on the sample type and amount of starting total RNA input.

4. Proceed immediately to "Section 5: Synthesize cRNA by In Vitro Transcription" on page 4.

Note: One or more PCR cycles can be added to the cycling guidelines for the "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.

   Table 9 IVT Master Mix.

Component	Volume for 1 reaction
Pico IVT Buffer	24 µL
Pico IVT Enzyme	6 µL
Total volume	**30 µL**

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 hours at 40°C in a thermal cycler using the "*In Vitro* Transcription cRNA Synthesis" program. (Table 2.)

5. Proceed to "Section 6: Purify cRNA" on page 5, or freeze immediately.

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 minutes.
- Mix the Purification Beads thoroughly before use. Aliquot the appropriate amount, then keep at room temperature. For each reaction, 140 µL plus ~10% overage is needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage is 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, then 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, then mix by pipetting up and down 10 times.
   b. Incubate for 10 minutes.
   c. Move the plate to a magnetic stand for ~5 minutes to capture the magnetic beads.
   d. Carefully aspirate, then 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, then incubate for 30 seconds.
   b. Slowly aspirate, then discard the 80% ethanol wash solution without disturbing the magnetic beads.
   c. Repeat substep 2a and substep 2b twice for a total of 3 washes. Completely remove the final wash solution.
   d. Air-dry on the magnetic stand for 5 minutes until no liquid is visible. Extra 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, then incubate for 1 minute.
   b. Mix well by pipetting up and down 10 times.
   c. Move the plate to the magnetic stand for ~5 minutes 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, then proceed to quantitation.

   Note: (Tip) Add an additional 10–70 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: Evaluate the 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 >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 10 2nd-Cycle ds-cDNA Master Mix.

Component	Volume for 1 reaction
Pico 2nd-Cycle Primers	4 µL
Pico 2nd-Cycle ds-cDNA Buffer	8 µL
Pico 2nd-Cycle ds-cDNA Enzyme	4 µL
Total volume	**16 µL**

3. Transfer 16 µL of the 2nd-Cycle ds-cDNA Master Mix to each (24 µL) 20 µg cRNA sample.
4. Incubate for 10 minutes at 25°C, for 50 minutes at 42°C, and then for 10 minutes at 70°C in a thermal cycler using the "2nd-Cycle ds-cDNA Synthesis" program. (Table 2.)
5. Proceed immediately to "Section 9: Hydrolyze RNA using Hydrolysis Buffer" on page 6.

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, then briefly centrifuge.
3. Incubate for 20 minutes at 65°C in a thermal cycler using the "RNA Alkaline Hydrolysis" program. (Table 2.)
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, then proceed to "Section 10: Purify 2nd-Cycle ds-cDNA" on page 7, or freeze immediately.

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 minutes.
- Mix the Purification Beads thoroughly before use. Aliquot the appropriate amount, then keep at room temperature. For each reaction, 100 µL plus ~10% overage is needed.
- Prepare fresh dilutions of 80% ethanol wash solution each time. For each reaction, 600 µL plus ~10% overage is 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, then 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, then mix by pipetting up and down 10 times.

      Note: Do not add ethanol to ds-cDNA samples.

   b. Incubate for 10 minutes.
   c. Move the plate to a magnetic stand for ~5 minutes to capture the magnetic beads.
   d. Carefully aspirate, then 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, then incubate for 30 seconds.
   b. Slowly aspirate, then discard the 80% ethanol wash solution without disturbing the magnetic beads.
   c. Repeat substep 2a and substep 2b twice for a total of 3 washes. Completely remove the final wash solution.
   d. Air-dry on the magnetic stand for 5 minutes until no liquid is visible. Extra 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, then incubate for 1 minute.

      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 minutes 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, then proceed to quantitation.

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

Section 11: Evaluate the 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 11 ds-cDNA normalization.

	For 1 reaction			
Array	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 12 Fragmentation and Labeling Master Mix.

	For 1 reaction			
Array	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 minutes at 37°C, and then for 2 minutes at 93°C in a thermal cycler using the “Fragmentation and Labeling” program. (Table 2.)

5. Proceed to “Section 13: Cartridge Array Hybridization” on page 9.

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

Section 13: Cartridge Array Hybridization

See the *GeneChip™ WT Pico Reagent Kit User Guide* (Pub. No. MAN0017893) for array plate hybridization information.

Note:

- Ensure that the reagents are completely thawed before use. Store DMSO at room temperature after the first use. DMSO solidifies when stored at 2–8°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 minutes at 65°C in a thermal cycler using the "Hybridization Control" program. (Table 2.)

2. At room temperature, prepare the Hybridization Master Mix.

Table 13 Hybridization Master Mix for a single reaction (3' cartridge arrays).

Array	3' 49 or 64-format	3' 100 or 81/4-format	3' 169-format	
Component				**Final concentration**
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	22 µL	15 µL	10 µL	10%
Nuclease-free water	13.3 µL	9 µL	6.3 µL	
Total volume	**160 µL**	**109 µL**	**73 µL**	

Table 14 Hybridization Master Mix for a single reaction (WT cartridge arrays).

Array	WT 49 or 64-format	WT 100 or 81/4-format	WT 169-Format	
Component				**Final concentration**
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**	

Table 15 Hybridization Master Mix for a single reaction (Clariom™ S cartridge arrays).

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 the Hybridization cocktail.

Table 16 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 ng)	41 µL (4.5 ng)	27 µL (3 ng)	60 µL (11 ng)	41 µL (7.5 ng)	27 µL (5 ng)	27 µL (5 ng)
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 minutes at 99°C (tubes) or 95°C (plates), and then for 5 minutes at 45°C in a thermal cycler using the "Hybridization Cocktail" program. (Table 2.)

5. Inject the appropriate amount of the specific sample into the array. (Table 17.)

Table 17 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 speed at 60 rpm for 16 hours at 45°C.

Table 18 Fluidics protocol.

	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

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Affymetrix Pte Ltd | 7 Gul Circle #2M-01 | Keppel Logistics Building | Singapore 629563

Thermo Fisher Scientific Baltics UAB | V.A. Graiciuno 8, LT-02241 | Vilnius, Lithuania

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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