The Ambion® WT Expression Kit

For safety and biohazard guidelines, refer to the "Safety" appendix in the *The Ambion® WT Expression Kit Protocol* (PN 4425209). For all chemicals in **bold red** type, read the MSDS and follow the instructions. Wear appropriate protective eyewear, clothing, and gloves.

Before you begin

- Prepare the **Control RNA**.
- Prepare your total RNA according to your laboratory's procedure.
- Determine your input RNA quantity.
- Prepare the Poly-A RNA Controls.
- Evaluate RNA quality by determining its A_{260}/A_{280} ratio. RNA of acceptable quality is in the range 1.7 to 2.1.
- Evaluate RNA integrity by microfluidic analysis or denaturing agarose gel electrophoresis.
- Program your thermal cycler.

Method	Heated lid temp.‡	Step 1	Step 2	Step 3	Step 4
First-Strand cDNA Synthesis	50 °C	25 °C, 60 min	42 °C, 60 min	4 °C, ≥2 min	
Second-Strand cDNA Synthesis	RT or disable	16 °C, 60 min	65 °C, 10 min	4 °C, ≥2 min	
In Vitro Transcription cRNA Synthesis	50 °C	40 °C, 16 hrs	4 °C, ∞		
2nd-Cycle cRNA Denaturation	75 °C	70 °C, 5 min	25 °C, 5 min	4 °C, 2 min	
2nd-Cycle cDNA Synthesis	75 °C	25 °C, 10 min	42 °C, 90 min	70 °C, 10 min	4 °C, ≥2 min
RNase H Hydrolysis	75 °C	37 °C, 45 min	95 °C, 5 min	4 °C, ≥2 min	

‡ For MJ Research/BioRad thermal cyclers, engage the heated lid.

Day 1 workflow

1 Synthesize first-strand cDNA

a. Prepare a First-Strand Master Mix, then dispense 5 µL into a reaction tube/plate.

1. Thaw the First-Strand synthesis reagents at room temperature.
2. Prepare the First-Strand Master Mix in a nuclease-free tube.

First-Strand Master Mix component	Volume for one reaction (µL)
First-Strand Buffer Mix	4
First-Strand Enzyme Mix	1
Total Volume	5

3. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec).
4. Transfer 5 µL of the First-Strand Master Mix to the supplied PCR tubes.

b. Add 5 µL of total RNA, mix thoroughly by gently vortexing, centrifuge briefly, then proceed immediately to the next step.

c. Incubate for 1 hour at 25 °C, then for 1 hour at 42 °C, then for at least 2 min at 4 °C in a thermal cycler. After the incubation, centrifuge briefly (~5 sec), then place the sample on ice for 2 min.

2 Synthesize second-strand cDNA

a. Prepare a Second-Strand Master Mix, then add 50 µL to each sample.

1. On ice, prepare the Second-Strand Master Mix in a nuclease-free tube.

Second-Strand Master Mix component	Volume for one reaction (µL)
Nuclease-free Water	32.5
Second-Strand Buffer Mix	12.5
Second-Strand Enzyme Mix	5
Total Volume	50

2. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec) and place on ice.
3. Transfer 50 µL of the Second-Strand Master Mix to each (10 µL) first-strand synthesis cDNA sample. Mix thoroughly by gently vortexing or flicking the tube 3 or 4 times. Centrifuge briefly and proceed immediately to the next step.

b. Incubate for 1 hr at 16 °C, then for 10 min at 65 °C, then for at least 2 min at 4 °C.

1. Incubate for 1 hr at 16 °C, then for 10 min at 65 °C, then for at least 2 min at 4 °C in a thermal cycler.

IMPORTANT! Disable the heated lid of the thermal cycler or keep the lid off during the second-strand cDNA synthesis.

2. After the incubation, centrifuge briefly (~5 sec). Place the sample on ice.

3 Synthesize cRNA by In Vitro Transcription

a. Prepare an IVT Master Mix, then dispense 30 µL to each sample.

1. At room temperature, prepare an IVT Master Mix in a nuclease-free tube.

IVT Master Mix component	Volume for one reaction (µL)
IVT Buffer Mix	24
IVT Enzyme Mix	6
Total Volume	30

2. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec), then proceed immediately to the next step.
3. Transfer 30 µL of the IVT Master Mix to each 60-µL Second-Strand cDNA sample. Mix thoroughly by gently vortexing, then centrifuge briefly.

b. Incubate for 16 hr at 40 °C, then overnight at 4 °C.

1. Incubate the IVT reaction for 16 hr at 40 °C, then overnight at 4 °C in a thermal cycler.
2. After the incubation, centrifuge briefly (~5 sec).

c. Place the cRNA on ice briefly, or freeze immediately.

Day 2 workflow

1 Purify cRNA

Before beginning the cRNA purification:

- Preheat the bottle of Elution Solution to 50 to 58 °C for at least 10 min.
- Make sure to add 100% ethanol (ACS reagent grade or equivalent) to the bottle of **Nucleic Acid Wash Solution Concentrate** before use.
- Make sure that the **Nucleic Acid Binding Buffer Concentrate** is completely dissolved. If not, warm the solution to <50 °C until the concentrate is solubilized.
- Vortex the Nucleic Acid Binding Beads vigorously before use to ensure that they are fully dispersed.

To purify the cRNA:

a. Prepare the cRNA Binding Mix.

cRNA Binding Mix component	Volume for one reaction (µL)
Nucleic Acid Binding Beads	10
Nucleic Acid Binding Buffer Concentrate	50

b. Add 60 µL of cRNA Binding Mix to each sample. Transfer each sample to a well of a U-Bottom Plate.

c. Add 60 µL of isopropanol to each sample, then shake gently for 2 min.

d. Capture the Nucleic Acid Binding Beads and discard the supernatant.

1. Move the plate to a magnetic stand to capture the magnetic beads.
2. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.

e. Wash twice with 100 µL of Nucleic Acid Wash Solution.

1. Add 100 µL of Nucleic Acid Wash Solution to each sample, then shake at moderate speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).
2. Move the plate to a magnetic stand and capture the Nucleic Acid Binding Beads.
3. Carefully aspirate and discard the supernatant without disturbing the Nucleic Acid Binding Beads, then remove the plate from the magnetic stand.
4. Repeat step 1 to step 3 to wash each sample again with 100 µL of Nucleic Acid Wash Solution.
5. Move the plate to a shaker and shake the plate vigorously for 1 min (setting 10 on the Lab-Line Titer Plate Shaker).

f. Elute cRNA with 40 µL of preheated Elution Solution.

1. Add to each sample 40 µL of preheated (55 to 58 °C) Elution Solution. Incubate without shaking for 2 min.
2. Vigorously shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker), then check to make sure that the Nucleic Acid Binding Beads are fully dispersed. If they are not, continue shaking until the beads are dispersed and/or pipette up/down 3 times.
3. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads.
4. Transfer the supernatant, which contains the eluted cRNA, to a nuclease-free multiwell plate.

g. Place the cRNA on ice briefly, or freeze immediately.

2 Assess cRNA yield and size distribution

Determine cRNA yield by UV absorbance or by using Quant-iT™ RiboGreen® RNA Reagent. Optionally, use a bioanalyzer to determine cRNA size distribution.

3 Synthesize 2nd-cycle cDNA

a. On ice, prepare 455 ng/µL cRNA. This is equal to 10 µg cRNA in a volume of 22 µL. If necessary, use nuclease-free water to bring the cRNA sample to 22 µL.

b. Combine 10 µg of cRNA and the **Random Primers**.

1. Thaw the 2nd-cycle synthesis reagents and place them on ice.
2. On ice, using supplied PCR tubes or plate, combine:
   - 22 µL of cRNA (10 µg)
   - 2 µL of **Random Primers**
3. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec). Place on ice.

c. Incubate for 5 min at 70 °C, then 5 min at 25 °C, then 2 min at 4 °C. After the incubation, centrifuge briefly (~5 sec).

d. Prepare the 2nd-Cycle Master Mix on ice, then add 16 µL to each sample.

1. Prepare the 2nd-Cycle Master Mix in a nuclease-free tube.

2nd-Cycle Master Mix component	Volume for one reaction (µL)
2nd-Cycle Buffer Mix	8
2nd-Cycle Enzyme Mix	8
Total Volume	16

2. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 sec).
3. Transfer 16 µL of 2nd-Cycle Master Mix to each (24-µL) cRNA/Random Primer sample. Mix thoroughly by gently vortexing. Centrifuge briefly.

e. Incubate for 10 min at 25 °C, then 90 min at 42 °C, then 10 min at 70 °C, then for at least 2 min at 4 °C. After the incubation, centrifuge briefly (~5 sec), then place the sample on ice.

4 Hydrolyze using RNase H

a. Add 2 µL of **RNase H** to the 2nd-Cycle cDNA. Mix by gently vortexing, then centrifuge briefly.

b. Incubate for 45 min at 37 °C, then 5 min at 95 °C, then for at least 2 min at 4 °C. After the incubation, centrifuge briefly (~5 sec), then place the sample on ice.

5 Purify 2nd-cycle cDNA

Before beginning the cDNA purification:

- Preheat the bottle of Elution Solution to 50 to 58 °C for at least 10 min.
- Make sure to add ethanol to the bottle of **Nucleic Acid Wash Solution Concentrate** before use.
- Make sure that the **Nucleic Acid Binding Buffer Concentrate** is completely dissolved. If not, warm the solution to <50 °C until the concentrate is solubilized.
- Vortex the Nucleic Acid Binding Beads vigorously before use to ensure they are fully dispersed.

To purify the 2nd-cycle cDNA:

a. Prepare the cDNA Binding Mix for the experiment.

cDNA Binding Mix component	Volume for one reaction (µL)
Nucleic Acid Binding Beads	10
Nucleic Acid Binding Buffer Concentrate	50

a. Add 18 µL of nuclease-free water and 60 µL of cDNA Binding Mix to each sample. Transfer each sample to a well of a U-Bottom Plate.

b. Add 120 µL of ethanol to each sample, then shake gently for 2 min.

c. Capture the Nucleic Acid Binding Beads, then discard the supernatant.

1. Move the plate to a magnetic stand to capture the magnetic beads.
2. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.

d. Wash twice with 100 µL of Nucleic Acid Wash Solution.

1. Add 100 µL of Nucleic Acid Wash Solution to each sample, then shake the samples at moderate speed for 1 min (setting 7 on the Lab-Line Titer Plate Shaker).
2. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads.
3. Carefully aspirate and discard the supernatant without disturbing the Nucleic Acid Binding Beads, then remove the plate from the magnetic stand.
4. Repeat step 1 to step 3 to wash a second time with 100 µL of Nucleic Acid Wash Solution.
5. Move the plate to a shaker, then shake the plate vigorously for 1 min to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker).

e. Elute cDNA with 30 µL of preheated Elution Solution.

1. Elute the purified cDNA from the Nucleic Acid Binding Beads by adding 30 µL of preheated (55 to 58°C) Elution Solution to each sample. Incubate for 2 min at room temperature without shaking.
2. Vigorously shake the plate for 3 min (setting 10 on the Lab-Line Titer Plate Shaker).
3. Move the plate to a magnetic stand to capture the Nucleic Acid Binding Beads.
4. Transfer the supernatant, which contains the eluted cDNA, to a nuclease-free multiwell plate.

f. Place the cDNA on ice briefly, or freeze immediately.

6 Assess cDNA yield and size distribution

a. Determine the concentration of a cDNA solution by measuring its absorbance at 260 nm. Applied Biosystems recommends evaluating the absorbance of 1.5 µL of cDNA sample using a NanoDrop® Spectrophotometer.

b. (Optional) Use Quant-iT™ PicoGreen® RNA Reagent to Assess cRNA yield.

7 Fragment and label the single-stranded cDNA

For instructions on how to fragment and label the single-stranded cDNA, refer to the *GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual Version 4* (PN 701880), Affymetrix Procedures G and H on page 29. For hybridization instructions, refer to page 35 of the same manual.

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