invitrogen QUICK REFERENCE

# WT Expression Kits

**Catalog Numbers** 4411973, 4411974, 4440536, and 4440537

**Pub. No.** 4425210 **Rev.** C

**Note:** For safety and biohazard guidelines, see the "Safety" appendix in the *WT Expression Kit User Guide* (Pub. No. 4425209). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

## Before you begin

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

| Method | Heated lid temp. | Alternate Protocol[1] | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|---|---|
| First-Strand cDNA Synthesis | 50°C | 105°C | 25°C, 60 minutes | 42°C, 60 minutes | 4°C, 2 minutes | — |
| Second-Strand cDNA Synthesis | RT or disable | Lid open | 16°C, 60 minutes | 65°C, 10 minutes | 4°C, 2 minutes | — |
| In Vitro Transcription cRNA Synthesis | 50°C | 40°C oven | 40°C, 16 hours | 4°C, Hold | — | — |
| 2nd-Cycle cRNA Denaturation | 75°C | 105°C | 70°C, 5 minutes | 25°C, 5 minutes | 4°C, 2 minutes | — |
| 2nd-Cycle cDNA Synthesis | 75°C | 105°C | 25°C, 10 minutes | 42°C, 90 minutes | 70°C, 10 minutes | 4°C, 2 minutes |
| RNase H Hydrolysis | 75°C | 105°C | 37°C, 45 minutes | 95°C, 5 minutes | 4°C, 2 minutes | — |

[1] For thermal cyclers that lack a programmable heated lid.

**For Research Use Only. Not for use in diagnostic procedures.**

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## Day 1 workflow

**1 Synthesize first-strand cDNA**

a. At room temperature, prepare the First-Strand Master Mix in a nuclease-free tube.

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

b. Mix thoroughly by gently vortexing. Centrifuge briefly. Proceed immediately to the next step.
c. Transfer 5 µL of the First-Strand Master Mix to the supplied PCR tubes.
d. Add 5 µL of total RNA, mix thoroughly by gently vortexing, centrifuge briefly, then proceed immediately to the next step.
e. Incubate for 1 hour at 25°C, then for 1 hour at 42°C, then for at least 2 minutes at 4°C in a thermal cycler. Immediately after the incubation, centrifuge briefly (~5 seconds), then place the sample on ice for 2 minutes.

**2 Synthesize second-strand cDNA**

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

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

b. Mix thoroughly by gently vortexing. Centrifuge briefly and proceed immediately to the next step.
c. 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.
d. Incubate for 1 hour at 16°C, for 10 minutes at 65°C, then for at least 2 minutes 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.

e. Immediately after the incubation, centrifuge briefly (~5 seconds). Place the sample on ice.

**3 Synthesize cRNA by in vitro transcription**

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

| IVT Master Mix component | Volume for one reaction |
|---|---|
| IVT Buffer Mix | 24 µL |
| IVT Enzyme Mix | 6 µL |
| Total volume | 30 µL |

b. Mix thoroughly by gently vortexing. Centrifuge briefly, proceed immediately to the next step.
c. Transfer 30 µL of the IVT Master Mix to each 60-µL Second-Strand cDNA sample. Mix thoroughly by gently vortexing, then centrifuge briefly.
d. Incubate the IVT reaction for 16 hours at 40°C, then overnight at 4°C in a thermal cycler.
e. After the incubation, centrifuge briefly (~5 seconds).
f. 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 50–58°C for at least 10 minutes.
- Add 100% 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 that they are fully dispersed.

To purify the cRNA:

a. Prepare the cRNA Binding Mix.

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

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 minutes.

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 minute (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 substep e1 to substep e3 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 minute (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 (55–58°C) Elution Solution. Incubate without shaking for 2 minutes.
2. Vigorously shake the plate for 3 minutes (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. On ice, using supplied PCR tubes or plate, combine:

- 22 µL of cRNA (10 µg)
- 2 µL of Random Primers

c. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 seconds). Place on ice.

d. Incubate for 5 minutes at 70°C, then 5 minutes at 25°C, then 2 minutes at 4°C. After the incubation, centrifuge briefly (~5 seconds).

e. At room temperature, prepare the 2nd-Cycle Master Mix in a nuclease-free tube.

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

f. Mix thoroughly by gently vortexing. Centrifuge briefly (~5 seconds). Proceed immediately to the next step.

g. Transfer 16 µL of 2nd-Cycle Master Mix to each (24-µL) cRNA/Random Primer sample. Mix thoroughly by gently vortexing. Centrifuge briefly. Proceed immediately to the next step.

h. Incubate for 10 minutes at 25°C, then 90 minutes at 42°C, then 10 minutes at 70°C, then for at least 2 minutes at 4°C. Immediately after the incubation, centrifuge briefly (~5 seconds), 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. Proceed immediately to the next step.<br>**b.** Incubate for 45 minutes at 37°C, then 5 minutes at 95°C, then for at least 2 minutes at 4°C. After the incubation, centrifuge briefly (~5 seconds), then place the sample on ice. |

**5** **Purify 2nd-cycle cDNA**

Before beginning the cDNA purification:

- Preheat the bottle of Elution Solution to 50–58°C for at least 10 minutes.
- 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 |
|---|---|
| Nucleic Acid Binding Beads | 10 µL |
| Nucleic Acid Binding Buffer Concentrate | 50 µL |

b. 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.

c. Add 120 µL of ethanol to each sample, then shake gently for 2 minutes.

d. 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.

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 the samples at moderate speed for 1 minute (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 substep e1 to substep e3 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 minute to evaporate residual ethanol from the beads (setting 10 on the Lab-Line Titer Plate Shaker).

f. 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 (55−58°C) Elution Solution to each sample. Incubate for 2 minutes at room temperature without shaking.
   2. Vigorously shake the plate for 3 minutes (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.

g. 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. We recommend evaluating the absorbance of 1.5 µL of cDNA sample using a NanoDrop™ Spectrophotometer.<br>**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, see the *Affymetrix™ GeneChip™ WT Terminal Labeling and Hybridization User Manual* (Pub. No. 702880). |

## Limited product warranty

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