NanoAmp™ RT-IVT Labeling Kit

RT-IVT Labeling Process for **Two Rounds** of Amplification

Safety

For safety and biohazard guidelines, refer to the "Safety" section in the *NanoAmp™ RT-IVT Labeling Kit Protocol* (PN 4365710). For all chemicals that appear in **bold** type throughout this document, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

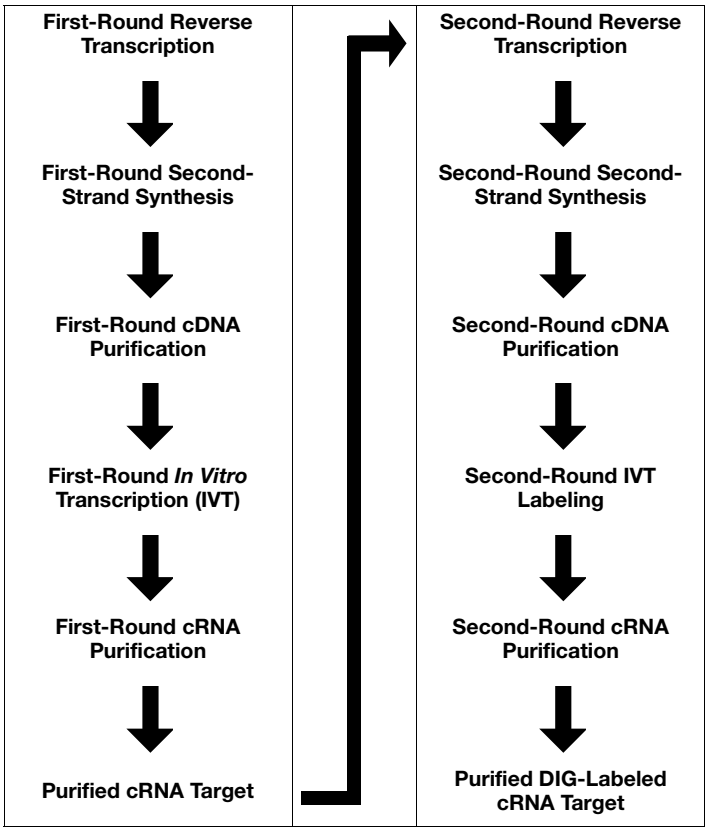
Overview

The purpose of the NanoAmp™ RT-IVT Labeling Kit (PN 4365715) is to convert RNA into digoxigenin (DIG)-labeled cRNA for hybridization to Applied Biosystems microarrays. You can use the kit to perform:

- One round of amplification (standard), *or*
- Two rounds of amplification

This Quick Reference Card briefly describes how to use the kit for two rounds of amplification. For one round of amplification, refer to Quick Reference Card PN 4365711. For a detailed protocol of both processes, refer to the *NanoAmp™ RT-IVT Labeling Kit Protocol* (PN 4365710).

Workflow for Two Rounds of Amplification



Estimated completion times:

- First round: 5–6 hours plus one overnight incubation
- Second round: 5–6 hours plus one overnight incubation

Handling the Enzymes

IMPORTANT! Keep all enzymes on ice at all times throughout these procedures. Do not vortex the enzymes.

Performing First-Round Reverse Transcription

Required Materials

- RNA sample: up to 0.1 µg of total RNA (for the first round of amplification)
- Reverse transcription reagents (blue-capped tubes):
  - Control RNA
  - T7-Oligo (dT) Primer
  - **10× 1st Strand Buffer**
  - dNTP Mix
  - **RT Enzyme**
  - **RNase Inhibitor**
- Nuclease-free water (gray-capped tube)
- 0.2-mL MicroAmp® reaction tube(s)
- Ice bucket
- Pipettor: 1- to 20-µL range
- Pipette tips
- Vortexer
- Microcentrifuge
- Thermal cycler

Procedure

1. Dilute the control RNA 1:50,000:
   a. Add 2 µL of control RNA to 98 µL of nuclease-free water, then mix by vortexing (1:50).
   b. Add 2 µL of the first dilution to 198 µL of nuclease-free water, then mix by vortexing (1:100).
   c. Add 5 µL of the second dilution to 45 µL of nuclease-free water, then mix by vortexing (1:10, final is 1:50,000).
2. Thaw the RNA sample and all reagents on ice.
3. Vortex separately the RNA sample, T7-Oligo (dT) Primer, **10× 1st Strand Buffer**, and dNTP Mix.
4. Centrifuge all reagent tubes briefly.
5. In the order given below, add the components to a 0.2-mL MicroAmp reaction tube on ice, then mix by pipetting up and down:

Component	Volume (µL)
T7-Oligo (dT) Primer	1.0
Diluted control RNA (1:50,000)	2.0
RNA sample (up to 0.1 µg of total RNA); bring volume to 9.0 µL with nuclease-free water	9.0
Total	12.0

6. Heat and then cool the mixture in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	70	5 min	12 µL
2	4	Indefinite hold	

7. After the run, place the reaction tube on ice.
8. If you see precipitate in the **10× 1st Strand Buffer**, warm it at 37 °C for 5 min, then vortex briefly.
9. In the order given below, add the components to the reaction tube on ice, then mix thoroughly by pipetting up and down:

Component	Volume (µL)
T7-Oligo (dT) Primer, diluted control RNA, and RNA sample	12.0
10× 1st Strand Buffer	2.0
dNTP Mix	4.0
RT Enzyme	1.0
RNase Inhibitor	1.0
Total	20.0

Note: Alternatively, you can prepare a master mix of the **10× 1st Strand Buffer**, dNTP Mix, **RT Enzyme**, and **RNase Inhibitor** up to 15 min before use. Add the primer/RNA mixture to an 8-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 10.

10. Perform reverse transcription in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	25	10 min	20 µL
2	42	2 hours	
3	70	5 min	
4	4	Indefinite hold	

11. After the run, place the reaction tube on ice.

Performing First-Round Second-Strand Synthesis

Required Materials

- Second-strand synthesis reagents (yellow-capped tubes):
  - **10× 2nd Strand Buffer**
  - **DNA Polymerase**
  - **RNase H**
- dNTP Mix (blue-capped tube)
- Nuclease-free water (gray-capped tube)
- Ice bucket
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw all reagents on ice.
2. Vortex separately the **10× 2nd Strand Buffer** and dNTP Mix.
3. Centrifuge all reagent tubes briefly.
4. If you see precipitate in the **10× 2nd Strand Buffer**, warm it at 37 °C for 5 min, then vortex briefly.
5. In the order given below, add the components to the cDNA mixture on ice, then mix gently:

Component	Volume (µL)
cDNA mixture	20.0
Nuclease-free water	63.0
10× 2nd Strand Buffer	10.0
dNTP Mix	4.0
DNA Polymerase	2.0
RNase H	1.0
Total	100.0

Note: Alternatively, you can prepare a master mix of the water, **10× 2nd Strand Buffer**, dNTP Mix, **DNA Polymerase**, and **RNase H** up to 15 min before use. Add the cDNA mixture to an 80-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 6.

6. Perform second-strand synthesis in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	16	2 hours	100 µL
2	70	5 min	
3	4	Indefinite hold	

7. After the run, place the reaction tube on ice.

Purifying First-Round cDNA

Required Materials

- cDNA purification components:
  - **DNA Binding Buffer**
  - **Wash Buffer**
  - Nuclease-free water
  - DNA purification column(s) and tube(s)
  - DNA elution tube(s)
- **100% ethanol**, ACS-grade or better
- Pipettors: 20- to 200-µL range, 100- to 1000-µL range
- Pipette tips
- Microcentrifuge

IMPORTANT! Before you use the kit for the first time, reconstitute the **Wash Buffer** by adding 16 mL of **100% ethanol** to the **Wash Buffer** bottle. Mix well, then mark the label to indicate that **100% ethanol** was added.

Procedure

1. If you see precipitate in the **DNA Binding Buffer**, warm it at 37 °C for 5 min, then vortex briefly.
2. In a new 1.5-mL nuclease-free microcentrifuge tube:
   a. Combine the **DNA Binding Buffer** (250 µL) and the entire second-strand synthesis reaction (100 µL).

b. Mix thoroughly by pipetting up and down.

IMPORTANT! Do not use the DNA elution tubes provided in the kit until step 6. Use other nuclease-free microcentrifuge tubes for mixing the **DNA Binding Buffer** and second-strand synthesis reaction.

3. Begin the cDNA purification:

   IMPORTANT! Before using the columns, inspect each one to be sure the filter is flush against the bottom of the column. If necessary, push it into place with a pipette tip.

   a. Seat the DNA purification column in its wash tube.

   b. Pipette the **DNA Binding Buffer**/reaction mixture (350 µL) onto the DNA purification column.

   c. Centrifuge the column and tube at 10,000 × *g* for 1 min.

   d. Make sure that the entire volume passes through the column. If it does not, centrifuge the column and tube at 10,000 × *g* for 1 min.

   e. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.

4. Wash the cDNA:

   a. Add 500 µL of **Wash Buffer** (containing **100% ethanol**) to the column.

   b. Centrifuge the column and tube at 10,000 × *g* for 1 min.

   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.

5. Centrifuge the column and tube again at 10,000 × *g* for 1 min.

6. Elute the cDNA (elution #1):

   a. Transfer the column to a new DNA elution tube (use the tubes provided in the kit).

   b. Pipette 10 µL of nuclease-free water onto the center of the fiber matrix at the bottom of the column.

   c. Incubate the column at room temperature for 2 min.

   d. Centrifuge the column and tube at 10,000 × *g* for 1 min for an elution volume of approximately 9 µL.

7. Elute the cDNA (elution #2):

   a. Pipette 10 µL of nuclease-free water onto the center of the fiber matrix at the bottom of the column.

   b. Incubate the column at room temperature for 2 min.

   c. Centrifuge the column and tube at 10,000 × *g* for 1 min for a total elution volume of approximately 18 µL.

8. Discard the column, then close the tube.

Note: If desired, you can stop at this point and store the purified cDNA at –20 °C overnight.

Performing First-Round IVT

Required Materials

- IVT labeling reagents (green-capped tubes):
  - **10× IVT Buffer**
  - **NTP Mix**
  - **IVT Enzyme Mix**
- Nuclease-free water (gray-capped tube)
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw the **10× IVT Buffer** at room temperature; thaw the remaining reagents on ice.
2. Vortex separately the **10× IVT Buffer** and **NTP Mix**.
3. Centrifuge all reagent tubes briefly.
4. If you see precipitate in the **10× IVT Buffer**, warm the buffer at 37 °C for 5 min, then vortex briefly.
5. In the order given below, add the components to the cDNA output at room temperature. Mix gently, then briefly centrifuge the tube.

Component	Volume (µL)
ds cDNA output; bring volume to 28 µL with nuclease-free water	28.0
10× IVT Buffer	4.0
NTP Mix	4.0
IVT Enzyme Mix	4.0
Total	40.0

Note: Alternatively, you can prepare a master mix of the **10× IVT Buffer**, **NTP Mix**, and **IVT Enzyme Mix** up to 15 min before use. Add the ds cDNA output to a 12-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 6.

6. Perform IVT in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	37	9 hours	40 µL
2	4	Indefinite hold	

7. After the run, remove the tube from the thermal cycler.

Purifying First-Round cRNA

Required Materials

- cRNA purification components
  - **RNA Binding Buffer**
  - **Wash Buffer**
  - Nuclease-free water
  - RNA purification column(s)
  - RNA collection tube(s)
- **100% ethanol**, ACS-grade or better
- Ice bucket
- 1.5-mL nuclease-free microcentrifuge tube(s)
- Pipettors: 20- to 200-µL range, 100- to 1000-µL range
- Pipette tips
- Microcentrifuge

Procedure

1. In a new 1.5-mL nuclease-free microcentrifuge tube:

   a. Combine the nuclease-free water (60 µL) and the entire IVT reaction (40 µL).

   b. Vortex briefly to mix.

   IMPORTANT! Do not use the RNA collection tubes provided in the kit until step 6. Use other nuclease-free microcentrifuge tubes for mixing the water and IVT reaction.

2. To the water/IVT reaction mixture:
   a. Add the **RNA Binding Buffer** (350 µL) and the **100% ethanol** (250 µL).
   b. Mix by pipetting up and down (do not vortex).
3. Begin the cRNA purification:

   IMPORTANT! Before using the columns, inspect each one to be sure the filter is flush against the bottom of the column. If necessary, push it into place with a pipette tip.

   a. Insert an RNA purification column into an RNA collection tube.
   b. Pipette the IVT reaction/**RNA Binding Buffer**/**ethanol** mixture (700 µL) onto the column, then close the tube.
   c. Centrifuge the column and tube at 10,000 × *g* for 1 min.
   d. Make sure that the entire volume passes through the column. If it does not, centrifuge the column and tube at 10,000 × *g* for 1 min.
   e. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
4. Wash the cRNA:
   a. Add 650 µL of **Wash Buffer** to the column, then close the tube.
   b. Centrifuge the column and tube at 10,000 × *g* for 1 min.
   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
5. Close the tube, then centrifuge the column and tube at 10,000 × *g* for 1 min.
6. Elute the cRNA:
   a. Transfer the column to a new RNA collection tube (use the tubes provided in the kit).
   b. Pipette 100 µL of nuclease-free water onto the fiber matrix at the bottom of the column, then close the tube.
   c. Incubate the column at room temperature for 2 min.
   d. Centrifuge the column and tube at 10,000 × *g* for 1 min.
7. Discard the column, then close the tube containing the eluted RNA.
8. Store the cRNA product on ice while you assess its quantity and quality.

Assessing First-Round cRNA Quantity

IMPORTANT! For optimal array performance and the best second-round results, quantify the first-round yield. Do not amplify more than 100 ng of the first-round cRNA product.

Required Materials

- TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), user-supplied
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Cuvette
- UV spectrophotometer

Procedure

1. Measure the ultraviolet absorbance:
   a. Gently mix the cRNA sample.
   b. Dilute a small amount of cRNA product 1:10 with TE buffer.
   c. Measure the absorbance at 260 nm.
2. Calculate the concentration and yield:
   - cRNA concentration (µg/µL) = A_{260} × 0.04 µg/µL × 10 (dilution factor)
   - cRNA yield (µg) = cRNA conc (µg/µL) × 100 µL total volume of cRNA purified

Note: (Optional) You can use the RiboGreen® Quantitation Assay and Kit to assess low concentrations of cRNA. Refer to the manufacturer's instructions for further information.

(Optional) Concentrating the Purified cRNA

If necessary, concentrate the cRNA by vacuum centrifugation. Use only the medium- or low-temperature settings during the drying process. Remove the sample when the desired volume is achieved.

Performing Second-Round Reverse Transcription

Required Materials

- RNA sample: up to 0.1 µg of first-round cRNA
- Reverse transcription reagents (blue-capped tubes):
  - **10× 1st Strand Buffer**
  - dNTP Mix
  - **RT Enzyme**
  - **RNase Inhibitor**
- Second-round primers (red-capped tubes)
- Nuclease-free water (gray-capped tube)
- 0.2-mL MicroAmp® reaction tube(s)
- Ice bucket
- Pipettor: 1- to 20-µL range
- Pipette tips
- Vortexer
- Microcentrifuge
- Thermal cycler

Procedure

1. Thaw the RNA sample and all reagents on ice.
2. Vortex separately the RNA sample, second-round primers, **10× 1st Strand Buffer**, and dNTP Mix.
3. Centrifuge all reagent tubes briefly.
4. In the order given below, add the components to a 0.2-mL MicroAmp reaction tube on ice, then mix by pipetting up and down:

Component	Volume (µL)
Second-round primers	2.0
RNA sample (up to 0.1 µg of first-round cRNA); bring volume to 10.0 µL with nuclease-free water	10.0
Total	12.0

5. Heat and then cool the mixture in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	70	5 min	12 µL
2	4	Indefinite hold	

6. After the run, place the reaction tube on ice.

7. If you see precipitate in the **10× 1st Strand Buffer**, warm it at 37 °C for 5 min, then vortex briefly.
8. In the order given below, add the components to the reaction tube on ice, then mix thoroughly by pipetting up and down:

Component	Volume (µL)
Second-round primers and RNA sample	12.0
10× 1st Strand Buffer	2.0
dNTP Mix	4.0
RT Enzyme	1.0
RNase Inhibitor	1.0
Total	20.0

Note: Alternatively, you can prepare a master mix of the **10× 1st Strand Buffer**, dNTP Mix, **RT Enzyme**, and **RNase Inhibitor** up to 1 hour before use. Add the primer/RNA mixture to an 8-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 10.

9. Perform reverse transcription in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	25	10 min	20 µL
2	42	2 hours	
3	70	5 min	
4	4	Indefinite hold	

10. After the run, place the reaction tube on ice.

Performing Second-Round Second-Strand Synthesis

Required Materials

- Second-strand synthesis reagents (yellow-capped tubes):
  - **10× 2nd Strand Buffer**
  - **DNA Polymerase**
  - **RNase H**
- T7-Oligo (dT) Primer (blue-capped tube)
- dNTP Mix (blue-capped tube)
- Nuclease-free water (gray-capped tube)
- Ice bucket
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw all reagents on ice.
2. Vortex separately the T7-Oligo (dT) Primer, **10× 2nd Strand Buffer**, and dNTP Mix.
3. Centrifuge all reagent tubes briefly.
4. Add the **RNase H** to the cDNA mixture on ice, then mix gently:

Component	Volume (µL)
cDNA mixture	20.0
RNase H	1.0
Total	21.0

5. Heat and then cool the mixture in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	37	30 min	21 µL
2	4	Hold	

6. After the run, place the reaction tube on ice.
7. Add the T7-Oligo (dT) Primer to the cDNA mixture on ice, then mix gently:

Component	Volume (µL)
cDNA mixture	21.0
T7-Oligo (dT) Primer	5.0
Total	26.0

8. Heat and then cool the mixture in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	70	5 min	26 µL
2	4	Indefinite hold	

9. After the run, place the reaction tube on ice.
10. If you see precipitate in the **10× 2nd Strand Buffer**, warm it at 37 °C for 5 min, then vortex briefly.
11. In the order given below, add the components to the cDNA mixture on ice, then mix gently:

Component	Volume (µL)
cDNA mixture and T7-Oligo (dT) Primer	26.0
Nuclease-free water	58.0
10× 2nd Strand Buffer	10.0
dNTP Mix	4.0
DNA Polymerase	2.0
Total	100.0

Note: Alternatively, you can prepare a master mix of the water, **10× 2nd Strand Buffer,** dNTP Mix, and **DNA Polymerase** up to 15 min before use. Add the cDNA mixture to an 74-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 12.

12. Perform second-strand synthesis in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	16	2 hours	100 µL
2	70	5 min	
3	4	Indefinite hold	

13. After the run, place the reaction tube on ice.

Purifying Second-Round cDNA

Go to "Purifying First-Round cDNA" on page 2 and follow the procedure without adjustment.

Performing Second-Round IVT Labeling

Required Materials

- IVT labeling reagents (green-capped tubes):
  - **10× IVT Buffer**
  - **NTP Mix**
  - IVT Control DNA
  - **IVT Enzyme Mix**
- DIG-UTP (You need two tubes of DIG-UTP for one labeling kit [Roche Molecular Biochemicals, Cat. No. 03359247910].)
- Nuclease-free water (gray-capped tube)
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Thaw the **10× IVT Buffer** at room temperature; thaw the remaining reagents on ice.
2. Vortex separately the **10× IVT Buffer**, **NTP Mix**, IVT Control DNA, and DIG-UTP.
3. Centrifuge all reagent tubes briefly.
4. If you see precipitate in the **10× IVT Buffer**, warm the buffer at 37 °C for 5 min, then vortex briefly.
5. In the order given below, add the components to the cDNA output at room temperature. Mix gently, then briefly centrifuge the tube.

Component	Volume (µL)
ds cDNA output; bring volume to 18 µL with nuclease-free water	18.0
10× IVT Buffer	4.0
DIG-UTP	8.0
NTP Mix	4.0
IVT Control DNA	2.0
IVT Enzyme Mix	4.0
Total	40.0

Note: Alternatively, you can prepare a master mix of the **10× IVT Buffer**, DIG-UTP, **NTP Mix**, IVT Control DNA, and **IVT Enzyme Mix** up to 15 min before use. Add the ds cDNA output to a 22-µL aliquot of this master mix. Add sufficient overage for all reactions. Mix gently, then go to step 6.

6. Perform IVT in a thermal cycler:

Stage	Temp. (°C)	Time	Reaction Vol.
1	37	9 hours	40 µL
2	4	Indefinite hold	

7. After the run, remove the tube from the thermal cycler.

Purifying Second-Round cRNA

Go to "Purifying First-Round cRNA" on page 3 and follow the procedure without adjustment.

Assessing Second-Round cRNA Quantity and Quality

Assess the quantity and quality of the cRNA product before proceeding with the Applied Biosystems Chemiluminescence Detection Kit for Applied Biosystems gene expression microarrays.

Required Materials

- TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), user-supplied
- Pipettors: 1- to 20-µL range, 20- to 200-µL range
- Pipette tips
- Cuvette
- UV spectrophotometer
- 1 to 2% agarose gel

Procedure

1. Measure the ultraviolet absorbance:
   a. Gently mix the cRNA sample.
   b. Dilute a small amount of cRNA product 1:30 with TE buffer.
   c. Measure the absorbance at 260 nm.
2. Calculate the concentration and yield:
   - cRNA concentration (µg/µL) = $A_{260} \times 0.04$ µg/µL × 30 (dilution factor)
   - cRNA yield (µg) = cRNA conc (µg/µL) × 100 µL total volume of cRNA purified
3. Analyze the cRNA: Prepare a 1 to 2% agarose gel, then run 0.5 to 1 µg of cRNA through the gel to visualize the cRNA product.

   Note: You can also analyze an aliquot of cRNA with the Agilent 2100 Bioanalyzer. Refer to the manufacturer's instructions for further information.

(Optional) Concentrating the Purified cRNA

If necessary, concentrate the cRNA by vacuum centrifugation. Use only the medium- or low-temperature settings during the drying process. Remove the sample when the desired volume is achieved.

Storing cRNA Product

- –15 to –25 °C for up to 2 months
- –80 °C for long-term storage

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