Procedural Notes

- Determine the integrity and purity of the input RNA for labeling and hybridization prior to use to increase the likelihood of a successful experiment.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature, unless otherwise indicated.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use, unless otherwise indicated.
- In general, follow Biosafety Level 1 (BL1) safety rules.

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2 Procedures

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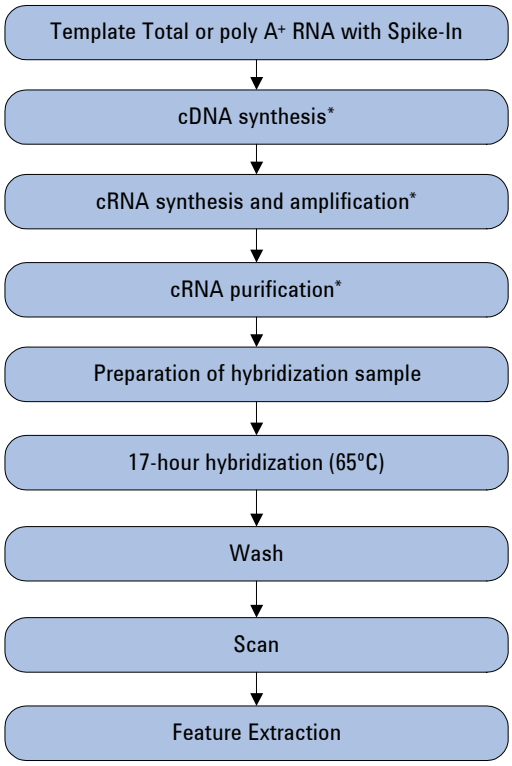
The Agilent One-Color Microarray-based Gene Expression Analysis uses cyanine 3-labeled targets to measure gene expression in experimental and control samples. Figure 1 is a standard workflow for sample preparation and array hybridization design.



* Samples can be stored frozen at -80°C after these steps, if needed.

Figure 1 Workflow for sample preparation and array processing.

Sample Preparation

The Low Input Quick Amp Labeling Kit, One-Color generates fluorescent cRNA (complimentary RNA) with a sample input RNA range between 10 ng and 200 ng of total RNA or a minimum of 5 ng of poly A^+ RNA for one-color processing. The method uses T7 RNA Polymerase Blend (red cap), which simultaneously amplifies target material and incorporates Cyanine 3-CTP. Amplification is typically at least a 100-fold from total RNA to cRNA with the use of this kit.

NOTE

For optimal performance, use pure high quality, intact template total or poly A+ RNA. RNA that is not pure, as measured by A260/A230 ratio, can lead to poor results and must be purified. Please refer to "Quality Assessment of Template RNA and Labeled cRNA" on page 73 for general guidance and procedural recommendations on quality assessment of template RNA.

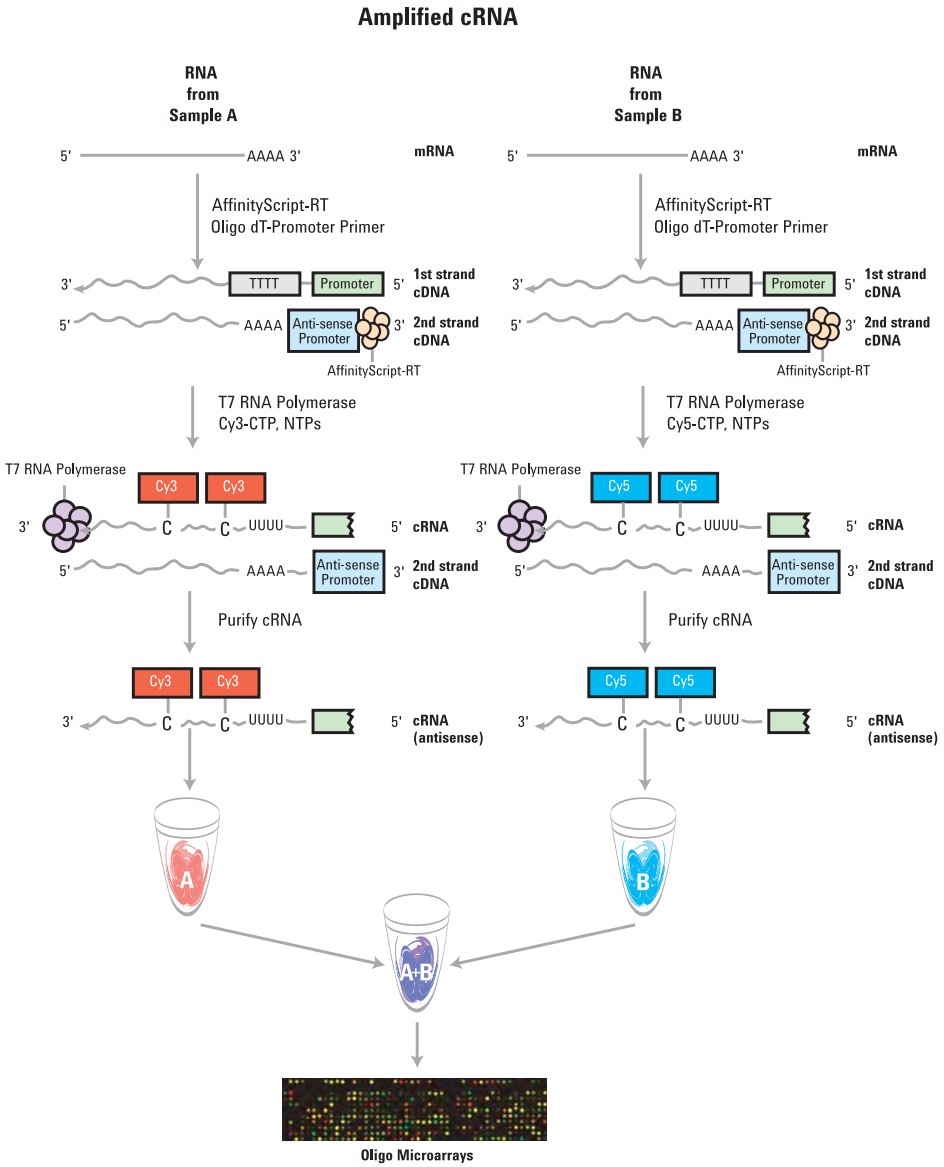


Figure 2 Schematic of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown.

Step 1. Prepare Spike Mix

(Time required: ~0.5 hours)

Refer to the protocol for RNA Spike-In Kit, One-Color for in-depth instructions and troubleshooting advice on how to use one-color spike mixes. This protocol is available with the RNA Spike-In Kit, One-Color and can also be downloaded from the Agilent web site at www.agilent.com/chem/dnamanuals-protocols.

1 Equilibrate water baths to 37°C, 40°C, 65°C, 70°C, and 80°C.

2 Vigorously mix the Spike Mix solution on a vortex mixer.

3 Heat at 37°C for 5 minutes, and mix on a vortex mixer once more.

4 Briefly spin in a centrifuge to drive contents to the bottom of the tube prior to opening. Settlement of the solution on the sides or lid of the tubes may occur during shipment and storage.

Table 13 provides the dilutions of Spike Mix for a range of total RNA input amounts. For inputs not shown Table 13, make sure that the amount of spike mix is proportional to the amount of RNA input. If you start with 5 ng mRNA as the input mass, follow the dilution scheme as described in Table 13.

Table 13 Dilutions of Spike Mix for Cyanine 3-labeling

Starting Amount of RNA		Serial Dilution				Spike Mix Volume to be used in each labeling reaction (µL)
Total RNA (ng)	PolyA RNA (ng)	First	Second	Third	Fourth	
10		1:20	1:25	1:20	1:10	2
25		1:20	1:25	1:20	1:4	2
50		1:20	1:25	1:20	1:2	2
100		1:20	1:25	1:20		2
200		1:20	1:25	1:10		2
	5	1:20	1:25	1:20		2

NOTE Use RNase-free microfuge tubes and tips. Make sure you dispense at least 2 µL with a pipette to ensure accuracy.

For example, to prepare the Spike Mix dilution appropriate for 25 ng of total RNA starting sample:

1 Create the First Dilution:
   a Label a new sterile 1.5-mL microcentrifuge tube “Spike Mix First Dilution.”
   b Mix the thawed Spike Mix vigorously on a vortex mixer.
   c Heat at 37°C in a circulating water bath for 5 minutes.
   d Mix the Spike Mix tube vigorously again on a vortex mixer.
   e Spin briefly in a centrifuge to drive contents to the bottom of the tube.
   f Into the First Dilution tube, put 2 µL of Spike Mix stock.
   g Add 38 µL of Dilution Buffer provided in the Spike-In kit (1:20).
   h Mix thoroughly on a vortex mixer and spin down quickly in a microcentrifuge to collect all of the liquid at the bottom of the tube. This tube contains the First Dilution.
2 Create the Second Dilution:
   a Label a new sterile 1.5-mL microcentrifuge tube “Spike Mix Second Dilution.”
   b Into the Second Dilution tube, put 2 µL of First Dilution.
   c Add 48 µL of Dilution Buffer (1:25).
   d Mix thoroughly on a vortex mixer and spin down quickly in a microcentrifuge to collect all of the liquid at the bottom of the tube. This tube contains the Second Dilution.
3 Create the Third Dilution:
   a Label a new sterile 1.5-mL microcentrifuge tube “Spike Mix Third Dilution.”
   b Into the Third Dilution tube, put 2 µL of Second Dilution.
   c Add 38 µL of Dilution Buffer (1:20).
   d Mix thoroughly on a vortex mixer and spin down quickly in a microcentrifuge to collect all the liquid at the bottom of the tube. This tube contains the Third Dilution.

4 Create the Fourth Dilution:

   a Label a new sterile 1.5-mL microcentrifuge tube “Spike Mix Fourth Dilution.”

   b Into the Fourth Dilution tube, add 10 µL of Third Dilution to 30 µL of Dilution Buffer for the Fourth Dilution (1:4).

   c Mix thoroughly on a vortex mixer and spin down quickly in a microcentrifuge to collect all of the liquid at the bottom of the tube. This tube contains the Fourth Dilution (now at a 40,000-fold final dilution).

5 Add 2 µL of Fourth Dilution to 25 ng of sample total RNA as listed in Table 13 and continue with cyanine 3 labeling using the Agilent Low Input Quick Amp Kit protocol as described in “Step 2. Prepare labeling reaction” on page 26.

Storage of Spike Mix dilutions

Store the RNA Spike-In Kit, One-Color at –70°C to –80°C in a non-defrosting freezer for up to 1 year from the date of receipt.

Store the first dilution of the Spike Mix positive controls for up to 2 months in a non-defrosting freezer at -70°C to -80°C. Do not freeze/thaw more than eight times. After use, discard the second, third and fourth dilution tubes.

Step 2. Prepare labeling reaction

(Time required: ~5.5 hours)

For each assay, make sure that the volume of the total RNA sample plus diluted RNA spike-in controls does not exceed 3.5 µL. Because the 1× reaction involves volumes of less than 1 µL, prepare components in a master mix and divide into the individual assay tubes in volumes >1 µL. When preparing 4 samples, use the 5× master mix. When preparing 8 samples, use the 10× master mix.

NOTE

The starting input for the Low Input Quick Amp Labeling Kit, One-Color ranges from 10 ng to 200 ng of total RNA. For best results, start with at least 25 ng of total RNA for the 4-pack and 8-pack formats, and 50 ng of total RNA for the 1-pack and 2-pack formats. For the 8-pack microarray format, as little as 10 ng of total RNA can be used to generate high quality data.

1. Add 10 to 200 ng of total RNA to a 1.5-mL microcentrifuge tube in a final volume of 1.5 µL. If samples are concentrated, dilute with water until 10 to 200 ng of total RNA is added in a 1.5 µL volume. Dilute the total RNA just prior to use and store the total RNA at concentrations over 100 ng/µL.
2. Add 2 µL of diluted Spike Mix to each tube. Each tube now contains a total volume of 3.5 µL.

3 Prepare T7 Primer Mix and add to sample:

a Mix T7 Primer (green cap) and water as listed in Table 14.

Table 14 T7 Primer Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
T7 Primer (green cap)	0.8	4	8
Nuclease-free Water	1	5	10
Total Volume	**1.8**	**9**	**18**

b Add 1.8 µL of T7 Primer Mix into each tube that contain 3.5 µL of total RNA and diluted RNA spike-in controls. Each tube now contains a total volume of 5.3 µL.

c Denature the primer and the template by incubating the reaction at 65°C in a circulating water bath for 10 minutes.

d Put the reactions on ice and incubate for 5 minutes.

4 Prewarm the 5× First Strand Buffer (green cap) at 80°C for 3 to 4 minutes to ensure adequate resuspensions of the buffer components. For optimal resuspension, briefly mix on a vortex mixer and spin the tube in a microcentrifuge to drive down the contents from the tube walls. Keep at room temperature until needed.

5 Prepare and add cDNA Master Mix:

a Immediately prior to use, add the components in Table 15 to a 1.5-mL microcentrifuge tube. Use a pipette to gently mix. Keep at room temperature.

The Affinity Script RNase Block Mix (violet cap) is a blend of enzymes. Keep the Affinity Script RNase Block Mix (violet cap) on ice and add to the cDNA Master Mix immediately prior to use.

Table 15 cDNA Master Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
5× First Strand Buffer (green cap)	2	10	20
0.1 M DTT (white cap)	1	5	10
10 mM dNTP Mix (green cap)	0.5	2.5	5
Affinity Script RNase Block Mix (violet cap)	1.2	6	12
Total Volume	**4.7**	**23.5**	**47**

b Briefly spin each sample tube in a microcentrifuge to drive down the contents from the tube walls and the lid.

c Add 4.7 µL of cDNA Master Mix to each sample tube and mix by pipetting up and down. Each tube now contains a total volume of 10 µL.

d Incubate samples at 40°C in a circulating water bath for 2 hours.

e Move samples to a 70°C circulating water bath and incubate for 15 minutes.

NOTE Incubation at 70°C inactivates the AffinityScript enzyme.

f Move samples to ice. Incubate for 5 minutes.

g Spin samples briefly in a microcentrifuge to drive down tube contents from the tube walls and lid.

Stopping Point If you do not immediately continue to the next step, store the samples at -80°C.

6 Prepare and add Transcription Master Mix:

a Immediately prior to use, add the components in Table 16 in the order listed into a 1.5 mL microcentrifuge tube. Use a pipette to gently mix. Keep at room temperature.

The T7 RNA Polymerase Blend (red cap) is a blend of enzymes. Keep the T7 RNA Polymerase Blend (red cap) on ice and add to the Transcription Master Mix just before use.

Table 16 Transcription Master Mix

Component	Volume (µL) per reaction	Volume (µL) per 5 reaction	Volume (µL) per 10 reactions
Nuclease-free water (white cap)	0.75	3.75	7.5
5× Transcription Buffer (blue cap)	3.2	16	32
0.1 M DTT (white cap)	0.6	3	6
NTP Mix (blue cap)	1	5	10
T7 RNA Polymerase Blend (red cap)	0.21	1.05	2.1
Cyanine 3-CTP	0.24	1.2	2.4
Total Volume	**6**	**30**	**60**

b Add 6 µL of Transcription Master Mix to each sample tube. Gently mix by pipetting. Each tube now contains a total volume of 16 µL.

c Incubate samples in a circulating water bath at 40°C for 2 hours.

Stopping Point If you do not immediately continue to the next step, store the samples at -80°C.

Step 3. Purify the labeled/amplified RNA

(Time required: ~0.5 hours)

Use the RNeasy Mini Kit to purify the amplified cRNA samples.

If sample concentration causes difficulty, you can use the Absolutely RNA Nanoprep Kit as an alternative. See "Absolutely RNA Nanoprep Purification" on page 58.

NOTE Make sure that ethanol was added to the RPE buffer as specified in the Qiagen manual before you continue.

1. Add 84 µL of nuclease-free water to your cRNA sample, for a total volume of 100 µL.
2. Add 350 µL of Buffer RLT and mix well by pipetting.
3. Add 250 µL of ethanol (96% to 100% purity) and mix thoroughly by pipetting. Do *not* spin in a centrifuge.
4. Transfer the 700 µL of the cRNA sample to an RNeasy Mini Spin Column (pink) in a Collection Tube (2 ml). Spin the sample in a centrifuge at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through and collection tube.
5. Transfer the RNeasy column to a new Collection Tube (2 ml) and add 500 µL of Buffer RPE (containing ethanol) to the column. Spin the sample in a centrifuge at 4°C for 30 seconds at 13,000 rpm. Discard the flow-through. Re-use the collection tube.
6. Add another 500 µL of Buffer RPE to the column. Centrifuge the sample at 4°C for 60 seconds at 13,000 rpm. Discard the flow-through and the collection tube.
7. If any Buffer RPE remains on or near the frit of the column or on the outside of the column, transfer the RNeasy column to a new Collection Tube (1.5 ml) and spin the sample in a centrifuge at 4°C for 30 seconds at 13,000 rpm to remove any remaining traces of Buffer RPE. Discard this collection tube and use a fresh Collection Tube (1.5 ml) to elute the cleaned cRNA sample.

CAUTION *Do not discard the final flow-through in the next step*. It contains the cRNA sample.

8 Elute the purified cRNA sample by transferring the RNeasy column to a new Collection Tube (1.5 ml). Add 30 µL RNase-Free Water directly onto the RNeasy filter membrane. Wait 60 seconds, then centrifuge at 4°C for 30 seconds at 13,000 rpm.

9 Maintain the cRNA sample-containing flow-through on ice. Discard the RNeasy column.

Step 4. Quantify the cRNA

Use the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (or higher) to quantify the cRNA.

1. Start the NanoDrop software.
2. Click the **Microarray Measurement** tab.
3. Before initializing the instrument as requested by the software, clean the sample loading area with nuclease-free water.
4. Load 1.0 to 2.0 µL of nuclease-free water to initialize. Then click **OK**.
5. Once the instrument has initialized, select **RNA-40** as the **Sample type** (use the drop down menu).
6. Make sure the **Recording** button is selected. If not, click **Recording** so that the readings can be recorded, saved, and printed.

CAUTION Failure to engage recording causes measurements to be overwritten, with no possibility of retrieval.

7. Blank the instrument by pipetting 1.0 to 2.0 µL of nuclease-free water (this can be the same water used to initialize the instrument) and click **Blank**.
8. Clean the sample loading area with a laboratory wipe. Pipette 1.0 to 2.0 µL of the sample onto the instrument sample loading area. Type the sample name in the space provided and click **Measure**.

   Be sure to clean the sample loading area between measurements and ensure that the baseline is always flat at 0, which is indicated by a thick black horizontal line. If the baseline deviates from 0 and is no longer a flat horizontal line, reblank the instrument with nuclease-free water, then remeasure the sample.
9. Print the results. If printing the results is not possible, record the following values:
   - Cyanine 3 dye concentration (pmol/µL)
   - RNA absorbance ratio (260 nm/280 nm)
   - cRNA concentration (ng/µL)

10 Determine the yield and specific activity of each reaction as follows:

a Use the concentration of cRNA (ng/µL) to determine the µg cRNA yield as follows:

$$\frac{(\text{Concentration of cRNA}) \times 30\ \mu\text{L (elution volume)}}{1000} = \mu\text{g of cRNA}$$

b Use the concentrations of cRNA (ng/µL) and cyanine 3 (pmol/µL) to determine the specific activity as follows:

$$\frac{\text{Concentration of Cy3}}{\text{Concentration of cRNA}} \times 1000 = \text{pmol Cy3 per µg cRNA}$$

11 Examine the yield and specific activity results. See Table 17 for the recommended cRNA yields and specific activities for hybridization.

CAUTION If the specific activity does not meet the requirements listed in Table 17, do not continue to hybridization. Repeat preparation of cRNA.

Table 17 Recommended Yields and Specific Activity

Microarray format	Yield (µg)	Specific Activity (pmol Cy3 per µg cRNA)
1-pack	5	≥6
2-pack	3.75	≥6
4-pack	1.65	≥6
8-pack	0.825	≥6

NOTE Please refer to "Quality Assessment of Template RNA and Labeled cRNA" on page 73 for general guidance and procedural recommendations on quality assessment of labeled cRNA.