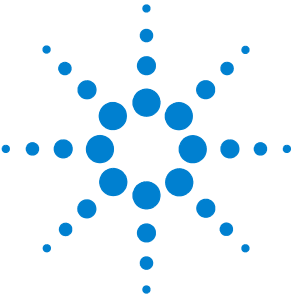
Agilent Low RNA Input Linear Amplification Kit PLUS

Agilent Low RNA Input Linear Amplification Kit (20 reactions)



Protocol

Optimized protocol for use with Agilent oligonucleotide microarrays

Version 4.0	Added Low RNA Input Linear Amplification Kit PLUS
	Added RNA Spike Mix Controls
	Added Optional Thermocycler Program

January 2006

See list of components or package insert for storage conditions.

Research Use Only

Agilent Technologies

Notices

Manual Part Number

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Acknowledgement

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Contents

1 Introduction

Agilent's Low RNA Input Linear Amplification kits generate fluorescent complimentary RNA (cRNA) for use with Agilent's oligonucleotide microarrays. The kit may be used for both one-color and two-color labeling experiments. Total RNA input of 200 to 1000 ng for one-color experiments and 50 to 5000 ng for two-color experiments, or a minimum of 10 ng of PolyA$^+$ RNA may be used with the kit. For more details, consult Agilent's One-Color (p/n G4140-90040) or Two-Color (p/n G4140-90050) Microarray-Based Gene Expression Analysis processing guide.

The Agilent Low RNA Input Linear Amplification Kit PLUS is offered in two formats. These kits contain the cyanine CTP required for the generation of labeled cRNA for one-color or two-color experiments.

An experienced user should require approximately 6–10 hours to complete this procedure from start to finish, depending on the number of samples being processed and the procedure being performed. If necessary, the procedure may be broken down into two steps with a break overnight at step 11 on page 15. Store samples overnight at –20°C.

There is routinely at least a 100-fold RNA amplification with use of this kit. Your results may vary with the purity of the RNA preparation that you use.

NOTE This kit contains enough reagent for 20 reactions, assuming a minimum batch size of 6.

READING THE ENTIRE PROTOCOL BEFORE YOU BEGIN IS ESSENTIAL TO SUCCESS.

Kit Contents

Agilent Low RNA Input Linear Amplification Kit (5184-3523)

Component	Volume (µL)
T7 Promoter primer	110
5X First strand buffer	195
0.1 M DTT	230
10 mM dNTP mix	25
MMLV RT	45
RNaseOUT	25
4X Transcription buffer	430
NTP mix	175
Inorganic pyrophosphatase	15
T7 RNA Polymerase	20
50% PEG (polyethylene glycol)	140
CTP	**125**
Random hexamers	**25**
dNTP	**25**
RNAse I "A"	**25**

Agilent Low RNA Input Linear Amplification Kit PLUS, One-Color (5188-5339)

Component	Volume (µL)
T7 Promoter primer	110
5X First strand buffer	195
0.1 M DTT	230
10 mM dNTP mix	25
MMLV RT	45
RNaseOUT	25
4X Transcription buffer	430
NTP mix	175
Inorganic pyrophosphatase	15
T7 RNA Polymerase	20
50% PEG (polyethylene glycol)	140
Cyanine 3-CTP	48
CTP	**125**
Random hexamers	**25**
dNTP	**25**
RNAse I "A"	**25**

Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color (5188-5340)

Component	Volume (µL)
T7 Promoter primer	110
5X First strand buffer	195
0.1 M DTT	230
10 mM dNTP mix	25
MMLV RT	45
RNaseOUT	25
4X Transcription buffer	430
NTP mix	175
Inorganic pyrophosphatase	15
T7 RNA Polymerase	20
50% PEG (polyethylene glycol)	140
Cyanine 3-CTP	24
Cyanine 5-CTP	24
CTP	**125**
Random hexamers	**25**
dNTP	**25**
RNAse I "A"	**25**

NOTE Recommended storage of all components is –20 °C in a non-defrosting freezer.

CAUTION The components marked in bold type are used only when generating fluorescently-labeled cDNA.

Make sure you use the correct dNTP (blue-capped tube) for the first RT reaction.

Required Equipment

- Ice and ice bucket
- Powderless gloves
- UV-Vis scanning spectrophotometer and 0.1-mL volume quartz microcuvettes (1-cm path length) or the NanoDrop ND-1000 Spectrophotometer. We recommend using the NanoDrop Spectrophotometer because the amount of RNA used in the measurement is very low. Traditional UV-Vis spectrophotometer cuvettes consume too much sample unless a microcuvette can be used.
- Timer or clock
- Black waterproof marking pen
- Micropipettors to pipette a range of 0.1-µL to 1-mL volumes
- Sterile, nuclease-free 1.5-mL microcentrifuge tubes
- Sterile, nuclease-free aerosol barrier pipet tips
- Microcentrifuge
- Circulating water baths (if unavailable, use heating blocks)
- Vortex mixer
- Benchtop cooling rack, for example Stratagene catalog number 410094 or equivalent (optional)
- Thermocycler with 96-well block and heated lid (optional)
- Sterile, nuclease-free PCR tubes (0.2 mL) or 96-well plates (optional)

Other Required Reagents - Not Included in Kit

- Cyanine 3-CTP (10.0 mM), PerkinElmer/NEN Life Sciences catalog number NEL580[*, †]
- Cyanine 5-CTP (10.0 mM), PerkinElmer/NEN Life Sciences catalog number NEL581[*, †]
- Agilent RNA Spike-in Kit, One-Color, part number 5188-5282
- Agilent RNA Spike-in Kit, Two-Color, part number 5188-5279[‡]
- Total RNA. We recommend a concentration between 6 ng/µL and 5000 ng/µL.
- Poly A^+ RNA. We recommend a concentration between 2 ng/µL and 200 ng/µL.
- DNase/RNase-free distilled water, Invitrogen catalog number 10977015
- Qiagen RNeasy Kit, catalog number 74104
- 100% Ethanol, Amresco, catalog number E193

* Quote promotional number PerkinElmer/NEN AG2001 to receive a 30% discount on these products. Discount not applicable in Japan or other Pacific Rim countries served through Perkin-Elmer's distribution network (for example, Taiwan, Korea, India, and New Zealand).

† Required if purchasing 5184-3523. PerkinElmer/NEN provides sufficient cyanine 3-CTP and cyanine 5-CTP for FOUR reactions; if you wish to do more than four cyanine 3-CTP and cyanine 5-CTP reactions, be sure to purchase more than one tube.

‡ Agilent RNA Spike-in Kit should only be used with the entire Agilent Two-Color Microarray-Based Gene Expression Analysis, version 4.0 workflow. This version 4.0 workflow is based on Agilent's oligonucleotide microarrays to quickly generate the most accurate and reliable results with highest data confidence.

General Procedural Notes

- Follow Biosafety Level 1 (BL1) safety rules.
- Our cDNA master mix procedure specifies reagent volumes for 1 reaction and 6.5 reactions. We have added an extra half-reaction to the recipe to ensure that you do not run out of reagents in your final master mix.
- We recommend preparing amplified RNA in batches of no less than six. This minimizes errors associated with pipetting small volumes of enzyme solutions.
- To specify reagent volumes for more than 6 reactions, multiply the volumes for 1 reaction by (n + 10%).
- To prevent contamination of reagents by ribonucleases, always wear powderless laboratory gloves. Use dedicated, nuclease-free solutions, microcentrifuge tubes and pipettors with nuclease-free aerosol tips.
- Cyanine 3 and cyanine 5 are photolabile. Minimize exposure to light.
- Stock solutions that are stored in 1.5-mL microcentrifuge tubes should be prepared for use as follows:
  - Thaw the aliquot as rapidly as possible without heating it above room temperature.
  - Vortex briefly. Microcentrifuge for 5–10 seconds to drive tube contents off the tube wall and lid.
  - Store on ice until use.

NOTE Agilent cannot guarantee microarray performance using non-Agilent protocols in microarray labeling and hybridization and it does not provide support to any non-Agilent protocols.

Safety Notes

- Wear appropriate protective equipment when working in a laboratory.

WARNING **Cyanine 3-CTP and cyanine 5-CTP are possible carcinogens. Avoid inhalation, swallowing, or contact with skin.**

WARNING **Dithiothreitol (DTT) causes irritation to the skin, eyes, and respiratory tract and is harmful if swallowed or inhaled. It may also be harmful if absorbed through the skin, and may affect the central nervous system.**

2 Fluorescent cRNA Synthesis Procedure

For use with Agilent oligo microarrays

The fluorescent cRNA synthesis procedure was optimized to work with Agilent's oligo microarrays. It features an efficient, single tube, single amplification, one-or two-color labeling method to create ample cyanine 3- and/or cyanine 5-labeled cRNA. In this procedure, one sample is labeled with cyanine 3 (which is excited by a 532-nm laser) and a second sample may be labeled with cyanine 5 (which is excited by a 633-nm laser). A primer containing poly dT and a T7 polymerase promoter is annealed to the poly A^+ RNA. Reverse transcriptase is added to the reaction to synthesize the first and second strands of cDNA. Next, cRNA is synthesized from the double-stranded cDNA using T7 RNA polymerase, which simultaneously incorporates cyanine 3- or cyanine 5- labeled CTP. Once labeling is complete, samples are hybridized to the microarray. Genes whose expresssion differs between samples are easily identifiable by scanning the microarray with a laser-based detection system. See Table 1 for the Workflow Time Table for preparation of fluorescent cRNA.

Please see Figure 1 on page 13 for a schematic drawing depicting the entire Two-Color amplified cRNA Procedure.

NOTE For applications requiring one color gene expression analysis please see Agilent's One-Color Microarray-Based Gene Expression Analysis processing guide (part number G4410-90040). For applications using two-color gene expression analysis, please see Agilent's Two-Color Microarray-Based Gene Expression Analysis processing guide (part number G4410-90050).

READING THE ENTIRE PROTOCOL BEFORE YOU BEGIN IS ESSENTIAL TO SUCCESS

Workflow Time Table for Preparation of Fluorescent cRNA

Table 1 Preparation of fluorescent cRNA

cDNA Synthesis (Total time: 155 minutes)		
Step	**Temperature**	**Time (minutes)**
Denature primer and template	65°C	10
Snap cool	Ice	5
Double-stranded cDNA Synthesis	40°C	120
MMLV-RT Inactivation	65°C	15
Snap cool	Ice	5
cRNA Synthesis (Total time: 120 minutes)		
cRNA Synthesis	40°C	120
cRNA Purification (Total time: 30 minutes)		
cRNA Purification	RT	30

Schematic of amplified cRNA procedure

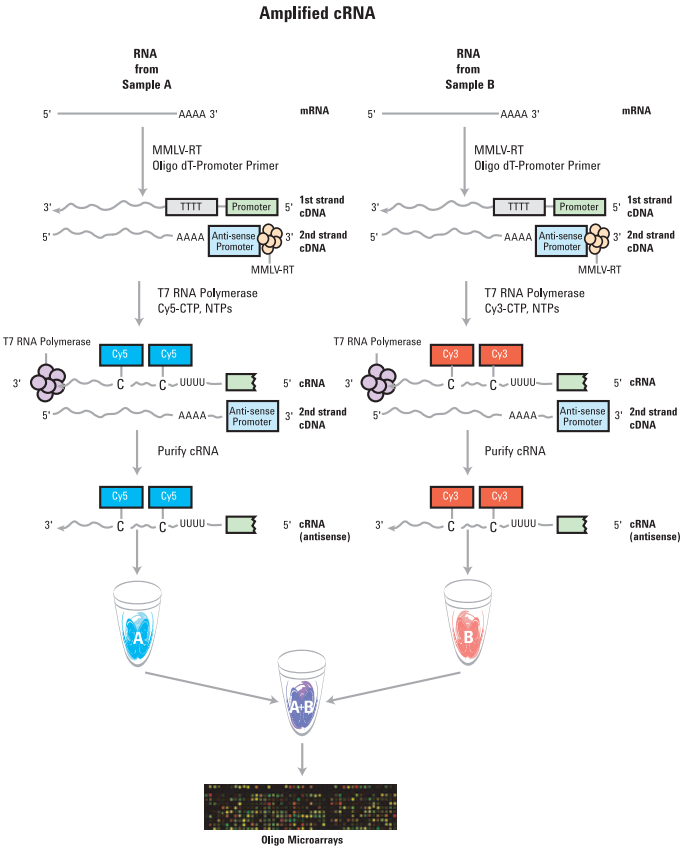


Figure 1 Schematic of amplified cRNA procedure.

Before Getting Started

NOTE The following reagents are used for generating fluorescently labeled cDNA from cRNA (red caps): dNTPs, CTP, Random hexamers, RNase 1 "A". If you are generating labeled cRNA, please place these reagents in a separate box to avoid inadvertently using the wrong reagent. Store at –20°C.

NOTE Many of the kit components are viscous in nature. Care must be taken to ensure accurate pipetting of the 5X first strand buffer, PEG, RT, RNaseOUT, and T7 reagents.

RNA Preparation and Qualification

High-quality RNA starting material is essential for successful microarray results. It is essential that your total or poly A^+ RNA be of high quality. We recommend the Agilent Total RNA Isolation Mini kit (product number 5185-6000) for purification of total RNA from mammalian tissue samples. For purification of total RNA from plant tissue, we recommend the Agilent Plant RNA Isolation Kit (product number 5188-2780).

1 **Size distribution (Appendix A and Appendix B for illustrations):** Analyze using a denaturing gel or Agilent's 2100 Bioanalyzer.

- For routine RNA QC analysis, we recommend Agilent's 2100 Bioanalyzer with an RNA 6000 Nano LabChip® Kit (product number 5065-4776). The bioanalyzer provides a complete RNA profile with as little as 5 ng/µL total or poly A^+ RNA and can quickly reveal sample degradation. Where sample is limited, we recommend the RNA 6000 Pico LabChip Kit (product number 5065-4473) for qualitative measurement of RNA (200-5000 pg/µL total RNA) (500-5000 pg/µL mRNA).
- The latest version of Agilent's 2100 Bioanalyzer software (Expert version 2.0, product number G2946CA) includes an algorithm to calculate a robust quality score called the RNA Integrity Number (RIN). RIN allows researchers to compare reproducibility of RNA isolations and downstream applications. More detail can be found at http://www.chem.agilent.com/scripts/pcol.asp?lpage=50.
- For total RNA, ribosomal RNA should be visible on a gel at approximately 1.9 and 5 kb. The bands should be sharp and clear. If the gel bands are smeared or there are multiple peaks on the 2100 Bioanalyzer electropherogram, then the RNA has been degraded*. In addition, high molecular weight bands (>9000 kb) indicate DNA contamination in the sample.
- For poly A^+ RNA, a faint smear in the range of 0.5 to 2 kb should be detectable.

2 **Determine the RNA concentration:**

Measure UV absorbance at 260 nm using a spectrophotometer. An A_{260} of 1 equals an RNA concentration of approximately **40 µg/mL**.

3 **Plant RNA**: Consult Appendix B for guidance.

* Intact plant total RNA may contain additional peaks or bands depending on tissue type.

cDNA Synthesis from Total RNA (Time required: ~3 hours)

1. Add total or poly A^+ RNA to a 1.5 mL microcentrifuge tube in a volume of 8.3 µL or less. Samples should be diluted such that at least 2 µL is pipetted.
2. Add 1.2 µL of T7 Promoter Primer (from kit).
3. Add the appropriate volume of One- or Two-Color Spike-In mix. Please refer to our protocols for One-Color and Two-Color Spike-In mixes (publications 5188-5977 and 5188-5928, respectively) for detailed instructions on the preparation and use of spike-ins.
4. Use nuclease-free water, if necessary, to bring the total reaction volume to 11.5 µL.
5. Denature the primer and the template by incubating the reaction in a 65°C water bath for 10 minutes.
6. Place the reactions on ice and incubate for 5 minutes.
7. Immediately prior to use, gently mix the following components by pipetting, in the order indicated in Table 2, and put on ice.

NOTE Pre-warm the 5X first strand buffer by incubating the vial in an 80°C waterbath for 3–4 minutes to ensure adequate resuspension of the buffer components. For optimal resuspension, vortex briefly and spin the tube briefly in a microcentrifuge to drive the contents off the tube walls. Keep at room temperature until use.

NOTE Keep MMLV-RT and RNaseOUT in the freezer until ready to use. Do not add enzymes to the master mix until just before starting reactions.

Table 2 cDNA Master Mix

Component	Volume (µL)/reaction	Volume (µL)/ 6.5 reactions
5X First strand buffer	4.0	26
0.1 M DTT	2.0	13
10 mM dNTP mix	1.0	6.5
MMLV-RT	1.0	6.5
RNaseOUT	0.5	3.3
Total volume	8.5	55.3

NOTE **There are two vials of dNTPs. Be sure to use the 10 mM dNTP mix (blue cap).**

8 Briefly spin each sample tube in a microcentrifuge to drive the contents off the tube walls and lid. Return to ice.

9 To each sample tube, add 8.5 µL of cDNA Master Mix and pipette up and down to mix.

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

11 Move samples to a 65°C water bath and incubate for 15 minutes.

NOTE **Incubation at 65°C inactivates MMLV-RT.**

12 Move samples to ice. Incubate on ice for 5 minutes.

13 Spin samples briefly in a microcentrifuge to drive tube contents off the tube wall and lid.

Fluorescent cRNA Synthesis: *in vitro* Transcription and Incorporation of Cyanine 3- or Cyanine 5-CTP (Time required: ~2.5 hours)

NOTE Cyanine 3 is bright pink and cyanine 5 is bright blue. Both are light sensitive. Minimize light exposure.

NOTE Prewarm the 50% PEG solution by incubating the vial in a 40°C waterbath for 1 minute. To ensure optimal resuspension, vortex briefly and spin the tube briefly in a microcentrifuge to drive the contents off the tube walls. PEG is viscous. Careful pipetting is required to ensure accurate volume. Keep at room temperature until use.

14 Immediately prior to use, gently mix the following components by pipetting, in the order indicated in Table 3, at room temperature.

NOTE **Do not add enzymes to Transcription Master Mix until just before starting the reaction.**

Table 3 Transcription Master Mix

Component	Volume (µL)/reaction	Volume (µL)/ 6.5 reactions
Nuclease-free water	15.3	99.4
4X Transcription buffer	20	130
0.1 M DTT	6.0	39
NTP mix	8.0	52
50% PEG	6.4	41.6
RNaseOUT	0.5	3.3
Inorganic pyrophosphatase	0.6	3.9
T7 RNA Polymerase	0.8	5.2
Cyanine 3-CTP or cyanine 5-CTP	2.4	15.6
Total volume	60.0	390

15 To each sample tube, add 60 µL of Transcription Master Mix. Gently mix by pipetting.

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

NOTE **Protect the samples from light by covering the water bath.**

Purification of Amplified RNA

We support the use of Qiagen's RNeasy mini spin columns for purifying amplified cRNA samples. Unincorporated dye-labeled nucleotides in the hybridization solution significantly increases background fluorescence on the microarray. Please follow the RNeasy mini kit protocol (reproduced here) with our modifications.

1. Add 20 µL of nuclease free-water to your cRNA sample, to obtain a total volume of 100 µL.
2. Add 350 µL of Buffer RLT and mix well.
3. Add 250 µL of ethanol (96-100% purity) and mix thoroughly by pipetting. DO NOT centrifuge.
4. Transfer 700 µL of cRNA sample to an RNeasy mini column in a 2 mL collection tube. Centrifuge the sample for 30 seconds at 13,000 rpm. Discard the flow-through and collection tube.
5. Transfer the RNeasy column to a new collection tube and add 500 µL of buffer RPE to the column. Centrifuge the sample for 30 seconds at 13,000 rpm. Discard the flow-through. Re-use the collection tube.
6. Again, add 500 µL of buffer RPE to the column. Centrifuge the sample for 60 seconds at 13,000 rpm. Discard the flow-through and the collection tube.
7. Elute the cleaned cRNA sample by transferring the RNeasy column to a new 1.5 mL collection tube. Add 30 µL RNase-free water directly onto the RNeasy filter membrane. Wait 60 seconds before centrifuging for 30 seconds at 13,000 rpm. Discard the RNeasy column.

We have observed higher yields when using a refigerated centrifugation procedure, Therefore, we highly recommend using a refrigerated microcentrifuge set at 4°C for the centrifugation steps listed above. Alternatively, you could locate your microcentrifuge in a 4°C cold room to accomodate this recommended procedure.

Quantitating cRNA Products

A typical cRNA amplification reaction, starting with 50 ng of total RNA, with Agilent's kit will yield 0.2– 2.0 µg of cRNA (2.0– 4.0 µg when starting with 500 ng of total RNA) depending on the purity and quality of the input sample RNA. This amount of cRNA is typically too low to be quantitated using a spectrophotometer with a standard cuvette. We recommend quantitating cRNA using a ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE) to minimize the amount of sample consumed by the measurement.

1 Use 1.5 µL of nuclease-free water to blank the NanoDrop instrument. Then use 1.5 µL of amplified cRNA for analysis. Calculate the concentration of cRNA by using the following formula:

$$\text{conc. cRNA (µg/µL)} = \frac{OD_{260} \times 10 \times 40\ \text{µg/mL}}{1000}$$

- The reaction is multiplied by 10 because the pathlength of analysis is 1 mm.

2 Calculate the specific activity of cRNA by using the following formula:

Specific Activity = pmol dye/µg cRNA

$$\text{pmol Cyanine 3/µL} = \frac{OD_{550} \times 10 \times 10^6}{150000}$$

$$\text{pmol Cyanine 5/µL} = \frac{OD_{650} \times 10 \times 10^6}{250000}$$

$$\text{Specific Activity Cyanine 3-cRNA} = \frac{\text{pmol Cyanine 3/µL}}{\text{conc. cRNA (µg/µL)}}$$

$$\text{Specific Activity Cyanine 5-cRNA} = \frac{\text{pmol Cyanine 5/µL}}{\text{conc. cRNA (µg/µL)}}$$

NOTE When using the ND-1000, choose the Microarray tab to scan the appropriate wavelengths.

Excessive freeze-thaw cycles may reduce cRNA integrity. If you are unsure about your product, we suggest evaluation on an Agilent 2100 Bioanalyzer. See Appendix A on page 21 for a representative profile of intact, labeled cRNA.

Specific activity from reactions starting with 500 ng of input RNA should be between 10–15 pmole/µg. cRNAs with specific activities less than 8 pmole/µg should not be used as microarray targets.

B
Plant RNA Quality

Consistent with all RNA handling procedures, be careful when working with plant RNA to avoid introducing nucleases that will degrade your sample. Before labeling, be sure to check the quality and integrity of your sample via gel electrophoresis or using the Agilent 2100 Bioanalyzer and RNA 6000 Nano or RNA 6000 Pico kit (www.agilent.com/chem/chip). Pictured here are examples of Arabidopsis RNA which may give you guidance when analyzing other plant RNA samples. High-quality Arabidopsis RNA will have two prominent ribosomal bands accompanied by a series of smaller plastid ribosomal peaks that are isolated from the Arabidopsis chloroplasts. Notice the difference in the baseline fluorescence of the degraded Arabidopsis RNA in comparison to the high quality RNA.

RNA purified from plant and fungal samples by methods other than the Agilent Plant RNA Isolation Mini Kit may contain cellulose, polysaccharides, and other interfering substances that are co-isolated with the RNA. Polysaccharides and secondary metabolites can interfere with the OD readings leading to errors in concentration determination. Cellulose, polysaccharides and some other plant RNA compounds will make the labeling reaction less efficient and should be removed prior to labeling.

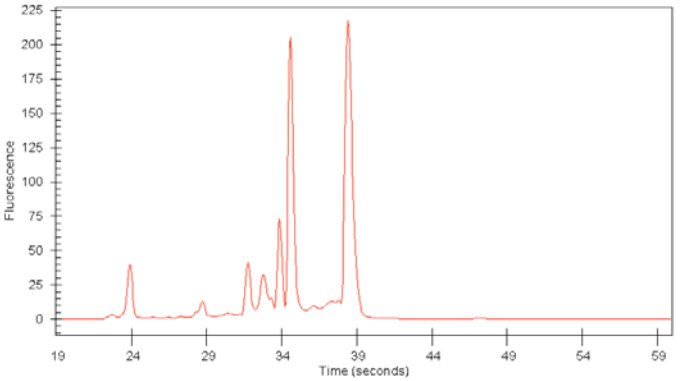
1 It is essential that you properly prepare your plant RNA for use in labeling reactions. We recommend the Agilent Plant RNA Isolation Mini Kit (part number 5180-2770).

2 **Determining the RNA Concentration:** Measure the UV absorbance of the purified RNA sample at 230 nm, 260 nm, 280 nm, and 320 nm, either as single wavelength determinations or as part of a wavelength scan (220 nm to 320 nm) using a UV/Visible spectrophotometer. An $\mathbf{A_{260}}$ of **1** equals an RNA concentration of approximately **40 µg/mL.**

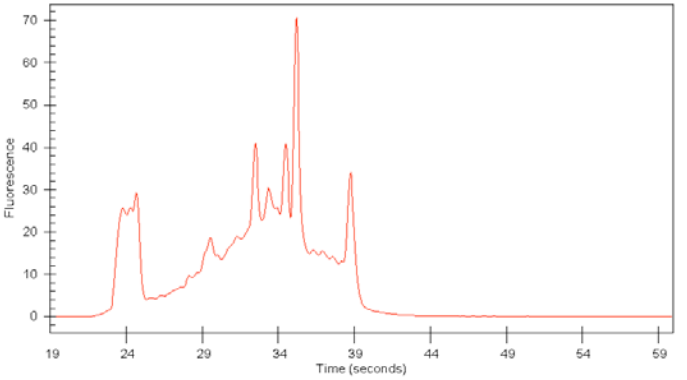
- The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid. Pure preparations of RNA (and DNA) have OD ratio values ($\mathbf{A_{260}/A_{280}}$) of between 1.8 and 2.0. Values below this indicate contamination of the sample with either phenol and/or protein. Values above this may indicate significant contamination of the sample with soluble carbohydrates.
- OD ratio values ($\mathbf{A_{260}/A_{230}}$) less than 2.0 may indicate contamination by polysaccharides.
- Significant absorption caused by light scattering at 320 nm indicates the presence of particulate matter in the sample.

For reliable estimation, concentrations of at least 1 µg/mL are required.

High quality Arabidopsis RNA



Degraded Arabidopsis RNA

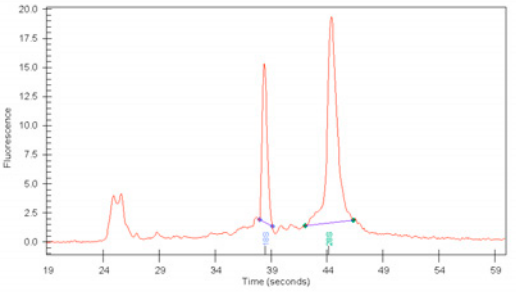


A
RNA Quality

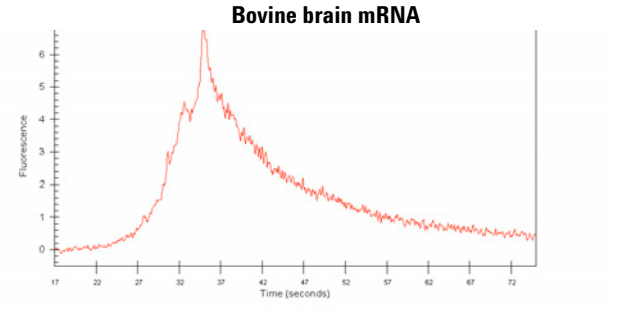
Data from a high quality total RNA preparation

Although a wide variety of ribosomal peak heights and ratios exist across a broad range of RNA sample types, most high quality RNA samples share the same common features. The most distinct and intense features on a 2100 Bioanalyzer electropherogram are the 18S and 28S ribosomal peaks (16S and 23S, prokaryotic). The baseline between 29 seconds and the 18S ribosome is relatively flat and free of small rounded peaks corresponding to smaller RNA molecules and there is an absence of smaller well-defined peaks between the two ribosomes. Any RNA migrating between the ribosomal peaks will be smooth and lack distinct peaks. Finally, depending on the RNA extraction method, the small 5S, 5.8S and tRNA may be present in the electropherogram from 24–27 seconds.

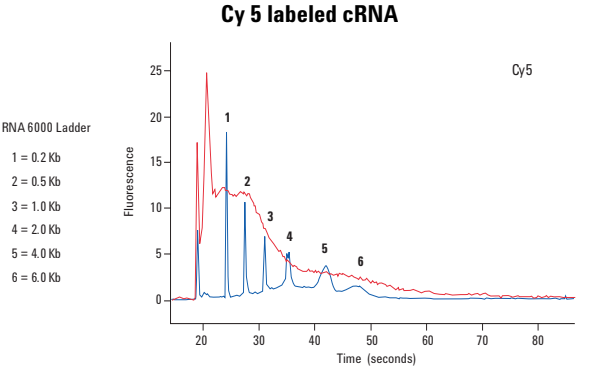
Mouse spleen total RNA



Data from a high quality mRNA preparation
High quality mRNA run on the Agilent 2100 Bioanalyzer typically has the shape of a broad peak, with transcripts falling in the range of 500–9000 bases long. The majority of transcript density falls in the size range of 1000–4000 bases. The electorpherograms of high quality samples are generally smooth and free of multiple large peaks. It is common for high quality mRNA samples to contain low levels of ribosomal RNA contamination, which are characterized by the presence of one to two large, well-defined ribosomal RNA peaks. The bioanalyzer software identifies and quantitates ribosomal peaks that are 5% of the total mRNA concentration or greater.



Data from a high quality cRNA preparation
High quality, fluorescently labeled cRNA run on Agilent's 2100 Bionalyzer typically has a profile that includes transcripts with a broad range of sizes. Shown here is Cy labeled HeLa cRNA overlaid with Ambion's RNA 6000 ladder (sizes as indicated).



C
Optional Thermocycler Protocol

Thermocycler Programs

Store the following programs into your thermocycler:

- Program 1: 65°C for 10 minutes, 4°C forever
- Program 2: 40°C for 2 hours, 65°C for 15 minutes, 4°C forever
- Program 3: 40°C for 2 hours, 4°C forever

Five minutes at 4°C is enough; forever is used in case the reagents for the next step are not ready.

cDNA Synthesis from Total RNA (Time required: ~3 hours)

1. Add 50 to 1000 ng of total RNA to a 0.2 mL PCR tube or the well of a 96-well PCR plate in a volume of 8.3 µL or less. The total concentration should be at least 6 ng/µL. For optimal performance, at least 500 ng of input total RNA should be used.
2. Add 1.2 µL of T7 Promoter Primer (from kit).
3. Add the appropriate volume of One- or Two-Color Spike-In mix. Please refer to our protocols for One-Color and Two-Color Spike-In mixes (publications 5188-5977 and 5188-5928, respectively) for detailed instructions on the preparation and use of spike-ins.
4. Put the tubes in the thermocycler and run Program 1 to denature the template and anneal the primer.
5. Keep the reaction tubes from step 4 in the thermocycler at 4°C or move to benchtop rack on ice.
6. Immediately prior to use, gently mix the components in Table 2 on page 17 in the order listed by pipetting, and put on ice.

NOTE Pre-warm the 5X first strand buffer by incubating the vial in an 80°C waterbath for 3–4 minutes to ensure adequate resuspension of the buffer components. For optimal resuspension, vortex briefly and spin the tube briefly in a microcentrifuge to drive the contents off the tube walls. Keep at room temperature until use.

NOTE Keep MMLV-RT and RNaseOUT on ice. Do not add enzymes until just before starting reactions.

NOTE **There are two vials of dNTPs. Be sure to use the 10 mM dNTP mix (blue cap).**

7. To each sample tube, add 8.5 µL of cDNA Master Mix and pipette up and down to mix. Total reaction volume is 20 µL.
8. Place reaction tubes in thermocycler and run Program 2 to synthesize double-stranded cDNA.

NOTE Incubation at 65°C inactivates MMLV-RT.

Fluorescent cRNA Synthesis: *in vitro* Transcription and Incorporation of Cyanine 3- or Cyanine 5-CTP (Time required: ~2.5 hours)

1 Immediately before use, make one Master Mix for each cyanine dye by adding the components listed in Table 3 on page 18 in the order shown to 1.5 mL nuclease-free microfuge tubes on ice. Vortex thoroughly after adding the first five components. Add enzymes and cyanine dyes and mix gently, but completely, by pipetting up and down without introducing bubbles.

NOTE

Prewarm the 50% PEG solution by incubating the vial in a 40°C waterbath for 1 minute. To ensure optimal resuspension, vortex briefly and spin the tube briefly in a microcentrifuge to drive the contents off the tube walls. PEG is viscous. Careful pipetting is required to ensure accurate volume. Keep at room temperature until use.

NOTE

Do not add enzymes to Transcription Master Mix until just before you do the reaction.

2 Keep the reaction tubes (step 8, above) in the thermocycler at 4°C or move to benchtop rack on ice. To each sample tube, add 60 µL of Transcription Master Mix. Gently mix by pipetting up and down. The final volume of the reaction is now 80 µL.

3 Return the reaction tubes to the thermocycler and run Program 3 (see page 16) to synthesize labeled cRNA.

4 Purify the labeled cRNA as described on page 17.

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