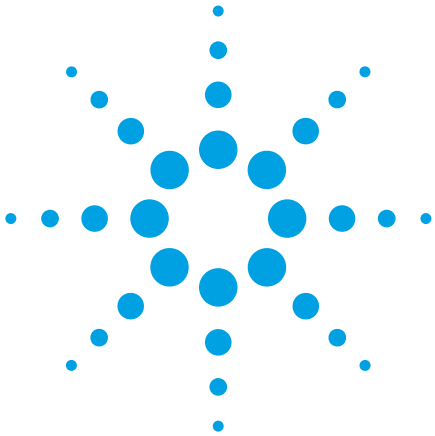
Agilent Fluorescent Linear Amplification Kit



Protocol

Product Number G2554A or P/N G2556-66002
Supports Agilent's Oligonucleotide Microarrays

Version 3.0
June 2002

See list of components or package insert for storage conditions.

RESEARCH USE ONLY

Agilent Technologies

Manual Part Number

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Technical Support

Technical product support may be obtained by contacting your local Agilent Support Services representative.

The following URL will direct you to a website that lists telephone information for you local area.

www.agilent.com/chem/dnasupport

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to something that, if not correctly performed, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until you meet the conditions it describes.

Table of Contents

Introduction

The Fluorescent Linear Amplification Kit generates fluorescent cRNA (complimentary RNA), from poly A+ RNA, for use with all of Agilent's Custom Oligonucleotide Microarrays.

RNA isolated from two samples is amplified and converted to fluorescently labeled cRNA – either cyanine 3-cRNA (which is excited by a 532 nm laser), or cyanine 5-cRNA (which is excited by a 633 nm laser). A primer, which contains poly dT and a T7 polymerase promoter, is annealed to the poly A+ RNA. Reverse transcriptase (MMLV-RT) is added to the reaction to synthesize the first strand of cDNA. Random hexamers are included in the reaction to prime a second strand of cDNA. At this point, double-stranded cDNA has been synthesized. Next, cRNA is synthesized using T7 RNA polymerase, which simultaneously incorporates cyanine 3- or cyanine 5-labeled CTP. The labeled cRNA samples are combined and hybridized to the microarray. Genes whose expression differs between the samples are easily identifiable by scanning the microarray with a laser-based detection system.

The diagram on Page 5 provides an overview of the procedure and shows a sample microarray.

A feature extraction software package links a feature to a design file and determines the relative fluorescence intensity of the two different dyes after normalization. This process allows you to quantitate differential gene expression between the two samples when compared.

An experienced user should require approximately 7 hours to complete this procedure from start to finish, depending on the number of samples being processed. If necessary, the procedure may be broken down into two steps with a break overnight at step 17.

Although the amount of poly A + RNA varies between cell types, 200 ng of poly A + RNA is approximately equivalent to the amount of total poly-A + RNA in 10^6 cells, assuming:

- There are 100,000 – 600,000 mRNA molecules per cell, and
- The average molecular weight per RNA molecule is 5.77 x 10^5 Da (with an average length of 1.75 kb and 330 Da per base).

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.

Reading the entire protocol before you commence work is essential for success.

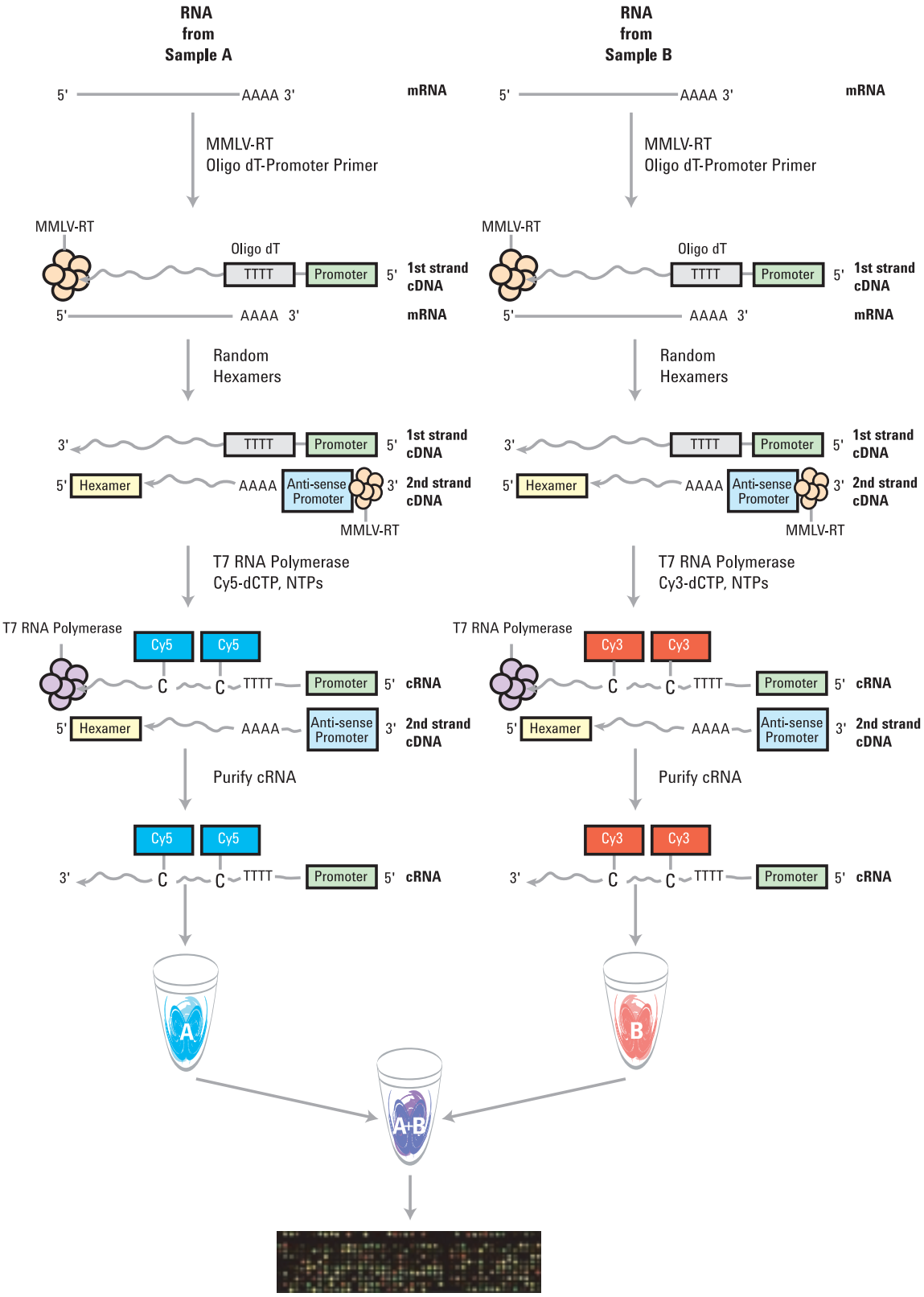


Figure 1. Procedural overview.

Workflow Timeline Table

Step	Temperature	Time
cDNA Synthesis		
Denature Primer and Template	65°C	10 min.
Snap Cool	ice	5 min.
Double-stranded cDNA Synthesis	40°C	120 min.
MMLV-RT Inactivation	65°C	15 min.
Snap Cool	ice	5 min.
cRNA Synthesis		
cRNA Synthesis	40°C	60 min.
cRNA Precipitation		
cRNA Precipitation	-20°C	60 min.-Overnight
Centrifuge	4°C	20 min.
Air Dry cRNA Pellet	Room Temp	10 min.

Kit Contents

Component	Part number
T7 Promoter Primer (100 ul)	54172
5x First Strand Reaction Buffer (80 ul)	54173
0.1 M DTT (160 ul)	54174
10 mM dNTP mix(20 ul)	54175
Random Hexamers (200 ng/µL; 20 ul)	54176
MMLV-RT (200 U/µL; 20 ul)	54177
RNaseOUT (40 U/µL; 20 ul)	54178
4x Transcription Buffer (400 ul)	54180
NTP Mix (160 ul)	54181
Inorganic Pyrophosphatase (12 ul)	54182
T7 RNA Polymerase (2500 U/µL; 16 ul)	54183
4 M Lithium Chloride (1.6 ml)	54243

Table 1. Kit contents and part numbers.

Store all kit components at – 20°C.

Additional Required Equipment and Reagents

Equipment

- Ice and ice bucket
- Powderless gloves
- UV-Vis Scanning Spectrophotometer with cuvettes
- Timer or clock
- Black waterproof marking pen
- Micropipettors
- Sterile, nuclease-free 50 mL conical centrifuge tube
- Sterile, nuclease-free 1.5 mL microcentrifuge tubes
- Sterile, nuclease-free aerosol barrier pipet tips
- Heating block, **temperature set to 65°C**
- Refrigerated microcentrifuge, **temperature set to 4°C**
- Circulating water bath, **temperature set to 40°C**
- Vortex mixer

Reagents

- **TE Buffer** (10 mM Tris; 1mM EDTA; pH 8.0, **nuclease-free**), Amresco cat. no. E112
- **70% ethanol**, diluted from Amresco cat. no. E193
- **10% Triton X-100,** proteomics grade, diluted from Amresco cat no. M143[****]
- **1mM EDTA,** RNAse free sterile solution, diluted from Amresco cat. no. E522-100ML[****]
- **Cyanine 3-CTP** (10.0 mM), PerkinElmer/NEN Life Sciences cat. no. NEL580[*], [**]
- **Cyanine 5-CTP** (10.0 mM), PerkinElmer/NEN Life Sciences cat. no. NEL581[*], [***]
- **Poly A+ RNA**. We recommend a concentration between 36.4 µg/mL and 200 µg/mL.
- **Total RNA.** We recommend a concentration between 1.11 mg/mL and 5 mg/mL.
- **DNase/RNase-free distilled water**, Invitrogen cat. no. 10977015

[*] Quote promotional number **PerkinElmer/NEN AG2001** to receive a 30% discount on these products.

[**] PerkinElmer/NEN provides sufficient cyanine 3-CTP for **FOUR** reactions; if you wish to do more than four cyanine 3-CTP reactions, be sure to purchase more than one tube.

[***] PerkinElmer/NEN provides sufficient cyanine 5-CTP for **SIX** reactions; if you wish to do more than six cyanine 5-CTP reactions, be sure to purchase more than one tube.

[****] Triton X-100 and 1mM EDTA are necessary only if you plan to amplify from total RNA

Safety Notes

1. Wear appropriate protective equipment when working in a laboratory.
2. Cyanine 3-CTP and cyanine 5-CTP are possible carcinogens. Avoid inhalation, swallowing or contact with skin.
3. This kit contains lithium chloride (LiCl):
   - LiCl is toxic. Its target organ is the central nervous system.
   - It is a potential teratogen, and may cause harm to breastfed babies.
   - It also may impose a risk of impaired fertility.
   - LiCl is harmful by inhalation, by contact with skin, and if swallowed. Wear suitable protective equipment.
   - For the Material Safety Data Sheet (MSDS) for this compound, please visit www.chem.agilent.com.

General Procedural Notes

- Follow Biosafety Level 1 (BL1) safety rules.
- We recommend preparing amplified RNA in batches of **no less than 6**.
  - This policy minimizes errors associated with pipetting small volumes of enzyme solutions.
  - Our 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.
  - To specify reagent volumes for ***n*** reactions, multiply the volumes for 1 reaction by ***n*** **+ 0.5.**
- To prevent contamination of reagents by ribonucleases, always wear powderless laboratory gloves. Use dedicated, nuclease-free solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Cyanine 3 and cyanine 5 are photolabile: **minimize exposure to light**.
- Stock solutions that are stored frozen 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 in a cold block until use.

PLEASE READ THE ENTIRE PROTOCOL BEFORE YOU BEGIN

I. Reagent preparation

Notes:

- These reagents may be prepared during the 2 hour incubation period in step 8 (cDNA synthesis).
- Diluted cyanine 3-CTP and cyanine 5-CTP solutions may be prepared ahead of time and stored at –80°C.
- You will need to add 4.0 µL of diluted cyanine 3- or cyanine 5-CTP to each sample. Be sure to dilute enough dye-labeled CTP to accommodate your reactions.

Cyanine Dye Mix (Time required: 5 – 10 minutes)

Cyanine 3-CTP, 6.0 mM
(Enough for **4** labeling reactions.)

1. Thaw 10 mM cyanine 3-CTP stock and place on ice.
2. Vortex briefly.
3. Spin briefly (1 – 2 seconds) at maximum speed in a microcentrifuge to drive contents off the tube wall and lid.
4. Pipet 10 µL cyanine 3-CTP stock solution into a microcentrifuge tube.
5. Add 6.7 µL nuclease-free water and mix gently.
6. Store frozen in the dark at –80° C. Consult your cyanine dye supplier for long term storage and stability conditions.

Cyanine 5-CTP, 4.0 mM
(Enough for **6** labeling reactions.)

1. Thaw 10 mM cyanine 5-CTP stock and place on ice.
2. Vortex briefly.
3. Spin briefly (1 – 2 seconds) at maximum speed in a microcentrifuge to spin down contents.
4. Pipet 10 µL cyanine 5-CTP stock solution into a microcentrifuge tube.
5. Add 15 µL nuclease-free water and mix gently.
6. Store frozen in the dark at –80° C. Consult your cyanine dye supplier for long term storage and stability conditions.

70% ethanol (Time required: 5 minutes)

1. In a 50 mL conical tube, mix 35 mL 100% ethanol and 15 mL nuclease-free water.
2. Store at room temperature.

0.3% Triton X-100 (Time required: 5 minutes)
This is only needed when amplifying from Total RNA.

1. Pipette 970 uL of 1mM EDTA in a nuclease free eppendorf tube
2. Add 30 uL of 10% Triton X-100

RNA Preparation and Qualification

It is essential that your total or poly-A^+ RNA be of high quality that meets these specifications:

1. **Size distribution (see Appendix for illustrations):**

   Analyze using a denaturing gel or Agilent's 2100 bioanalyzer.

   For RNA analysis, we recommend Agilent's 2100 bioanalyzer with an RNA6000 Nano LabChip® Kit (pr. no. 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.

   - For total RNA, ribosomal RNA should be visible at approximately 1.9 and 5 kb. The bands should be sharp and clear. If they are smeared or there are multiple peaks, 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 a nucleic acid concentration of approximately **40 µg/mL.**

II. Amplification from mRNA or Total RNA

cDNA synthesis from mRNA (Time required: ~3 hours)

1. To a 1.5 mL microcentrifuge tube, add 200 ng poly A^+ RNA in a volume of **5.5 µL or less**. The mRNA sample concentration should be at least **36.4 µg/mL**. See the ***Introduction*** for details.
2. Add 5.0 µL of T7 Promoter Primer (from kit).
3. Use nuclease-free water to bring the total reaction volume to 10.5 µL.
4. Denature the primer and the template by incubating the reaction at 65°C in a heating block for 10 minutes.
5. Place the reactions on ice and incubate for 5 minutes.
6. In a separate 1.5 mL microcentrifuge tube, mix the following components in the order indicated and maintain on ice:

Kit 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
Random Hexamers	1.0	6.5
MMLV-RT	1.0	6.5
RNaseOUT	0.5	3.3
Total volume	**9.5**	**61.8**

Table 2. Preparation of cDNA mix. for labeling from mRNA

7. To each sample tube, add 9.5 µL cDNA Mix.
8. Incubate samples at 40°C in a circulating water bath for 2 hours.
9. Move samples to 65°C. Incubate at 65°C for 15 minutes.

NOTE Incubation at 65°C inactivates MMLV-RT.

10. Move samples to ice. Incubate on ice for 5 minutes.
11. Spin samples briefly in a microcentrifuge to drive tube contents off the tube wall and lid.

cDNA synthesis from total RNA (Time required: ~5 hours)

1. To a 1.5 mL microcentrifuge tube, add 5.0 µg Total RNA in a volume of 4.5 µL or less. The total concentration should be at least **1.11 mg/mL.** See the ***Introduction*** for details.
2. Add 5.0 µL of T7 Promoter Primer (from kit).
3. Use nuclease-free water to bring the total reaction volume to 9.5 µL.
4. Denature the primer and the template by incubating the reaction at 65°C in a heating block for 10 minutes.
5. Place the reactions on ice and incubate for 5 minutes.
6. In a separate 1.5 mL microcentrifuge tube, mix the following components in the order indicated and maintain on ice:

Kit 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
Random Hexamers	1.0	6.5
MMLV-RT	1.0	6.5
RNaseOUT	0.5	3.3
0.3% Triton X-100	1.0	6.5
Total volume	**10.5**	**68.3**

Table 3. Preparation of cDNA mix. for labeling from total RNA

7. To each sample tube, add 10.5 µL cDNA Mix.
8. Incubate samples at 40°C in a circulating water bath for 4 hours.
9. Move samples to 65°C. Incubate at 65°C for 15 minutes.

NOTE Incubation at 65°C inactivates MMLV-RT.

10. Move samples to ice. Incubate on ice for 5 minutes.
11. 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: 1.5 hours)

12. To each sample tube, add **EITHER** 4.0 µL cyanine 3-CTP (6.0 mM) **OR** 4.0 µL cyanine 5-CTP (4.0 mM).
13. Immediately prior to use, gently mix the followng components by pipetting, in the order indicated, at room temperature:

NOTE **Do not** add enzymes until just before you do the reaction.

Kit Component	Volume (µL)/ reaction	Volume (µL)/ 6.5 reactions
Nuclease-free water	20.1	130.7
4x Transcription Buffer	20	130
0.1 M DTT	6.0	39
NTP Mix	8.0	52
RNaseOUT	0.5	3.3
Inorganic Pyrophosphatase	0.6	3.9
T7 RNA polymerase	0.8	5.2
Totals	**56.0**	**364.1**

Table 4. Preparation of Transcription Mix

14. To each sample tube, add 56 µL of Transcription Mix. Mix gently by pipetting.
15. Incubate samples in a circulating water bath at 40°C for 1 hour.

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

Precipitation of fluorescent-labeled cRNA (antisense RNA; time required: 2.5 hours – overnight)

16. Add 80 µL 4.0 M LiCl (from kit) to each sample. Mix gently by pipetting.

CAUTION LiCl is toxic. Please observe the precautions listed in the ***Safety Notes*** section of this manual.

17. Store at **–20°C** overnight (or for at least 1 hour) to precipitate the labeled RNA products.

NOTE Do not freeze at –80°C or on dry ice — the LiCl may crystallize.

18. Spin the LiCl precipitates in a microcentrifuge set to 4°C, at full speed, for 20 minutes.
19. Remove supernatants by pipetting or decanting carefully.

CAUTION Dispose of LiCl according to your institution's guidelines.

20. Rinse each pellet once with 1.0 mL of 70% room temperature ethanol. Re-pellet in a microcentrifuge. Remove ethanol by aspirating the supernatant or by decanting it.
    - If desired, re-spin for 30 seconds at maximum speed to consolidate the residual ethanol. Remove it with a small bore pipet tip.
    - The pellet should be easily visible. It will be **bright pink** if it contains **cyanine 3-CTP** or *bright blue* if it contains *cyanine 5-CTP*.
21. Dry the pellet briefly at room temperature for approximately 10 minutes.
22. Resuspend each sample pellet in 100 µL of nuclease-free water.

NOTE You may perform this step by adding 100 µL nuclease-free water to the pellet, allowing it to absorb the water for 5 minutes, then gently pipetting several times to re-suspend the cRNA pellet.

III. Quantitating fluorescent-labeled cRNA products

23. Calculate the amplification yield:
    - Make a 1:60 dilution of your sample. Add 5.0 µL of the sample to a 1.5-mL microcentrifuge tube, followed by 295 µL of TE (pH 8.0).
    - Measure the OD at 260nm, using TE buffer for the blank measurement. Calculate the concentration of cRNA by using the formula

      1 OD260 = 40 µg/mL RNA.

    - Calculate amplification yield by multiplying cRNA concentration (µg/mL) by the sample volume (0.1 mL) and dividing by the amount of poly A^+ RNA initially added to the reaction (0.2 µg):

      cRNA conc (µg/mL) x 0.1 mL / 0.2 µg

24. Calculate the amount of cyanine 3 and cyanine 5 incorporated into your labeled products:

    - Make a 1:20 dilution of your sample by adding a 5.0 µL aliquot of the product to 95 µL of TE (pH 8.0).
    - Scan the 1:20 dilution in a spectrophotometer in the range of 350 – 700 nm.
    - Calculate the concentration of cyanine 3-CTP:

$$\textbf{concentration Cy3-CTP (mM)} \frac{\mathbf{A_{552nm}}}{\mathbf{(150\ mM^{-1}\ cm^{-1})\ (1cm\ pathlength)}} \text{ X 20 (dilution factor)}$$

    - Calculate the concentration of cyanine 5-CTP:

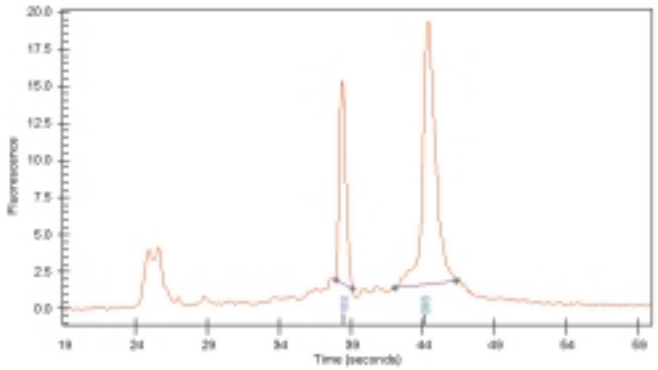
$$\textbf{concentration Cy5-CTP (mM) =} \frac{\mathbf{A_{650nm}}}{\mathbf{(250\ mM^{-1}\ cm^{-1})\ (1cm\ pathlength)}} \text{ X 20 (dilution factor)}$$

25. Calculate the amount of cRNA product required for each microarray to be hybridized. If not using the cRNA immediately, aliquot it into smaller volumes, freeze on dry ice and store in the dark at -80°C."

NOTE Excessive freeze-thaw cycles may reduce cRNA integrity. If you are unsure about your product, we suggest re-evaluation on an Agilent 2100 Bioanalyzer.

Appendix 1: RNA Quality

Mouse Spleen Total RNA

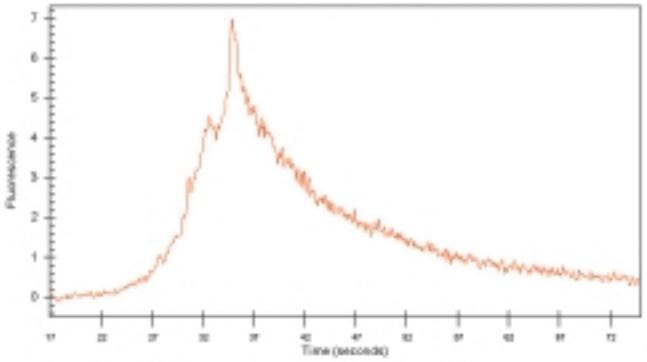


Data from a high quality total RNA preparation

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 are the 18S and 28S ribosomal peaks (16S & 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.

Bovine Brain mRNA



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 percent of the total mRNA concentration or greater.

Notes

Notes

Agilent's Printed Microarray Solutions

Design it!	**Microarray Design Services**
G2560A	Microarray Design and Basic QC
G2561A	Probe Selection
G2562A	Probe Curation
G2563A	Professional Consulting Service
Print it!	**cDNA and Custom Microarrays**
G2506A	25-mer Custom Oligonucleotide Microarray (8.4K)
G2507A	25-mer Custom Oligonucleotide Microarray (22K)
G2508A	60-mer Custom Oligonucleotide Microarray (8.4K)
G2509A	60-mer Custom Oligonucleotide Microarray (22K)
G4100A	Human 1 cDNA Microarray Kit
G4101A	Human 2 cDNA Microarray Kit
G4104A	Mouse cDNA Microarray Kit
G4105A	Rat cDNA Microarray Kit
G4135A	Arabidopsis 1 Microarray Kit
Run it!	**Microarray Processing Tools**
G2554A	Fluorescent Linear Amplification Kit
G2556A	Fluorescent Linear Amplification Kit with Hyb'n Reagent
G2559A	*in situ* Hybridization Reagent Kit
G2557A	Fluorescent Direct Label Kit
G2555A	Fluorescent Direct Label Kit with Hybridization Reagent
G2558A	Deposition Hybridization Reagent Kit
G4145A	Large Volume Deposition Hybridization Kit
G2530A	Microarray Hybridization Chamber (8.4K format)
G2530-60002	Hybridization (8.4K format) Septa, Backings & Gasket
G2533A	Microarray Hybridization Chamber (16.2K format)
G2533-65002	Hybridization (16.2K format) Septa, Backings & Gasket
G2531A	Microarray Hybridization Chamber (22K format)
G2531-60002	Hybridization (22K format) Septa, Backings & Gasket
G2940BA	2100 Bioanalyzer Instrument System Bundle
5065-4476	RNA 6000 Nano LabChip Kit (messenger & total RNA)
5064-8230	DNA 7500 LabChip Kit (100 - 7500 bp)
5064-8231	DNA 12000 LabChip Kit (100 - 12000 bp)
5064-8284	DNA 500 LabChip Kit (25 - 500 bp)
5065-4449	DNA 1000 LabChip Kit (25 - 1000 bp)
G2565AA	48-slide, Dual Laser DNA Microarray Scanner
(# varies)	Microarray Technology Transfer, Services and Support Packages
Discover it!	**Microarray Analysis**
G2567AA	Feature Extraction Software License
(# varies)	Rosetta Resolver® Gene Expression Data Analysis System

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http://www.agilent.com/chem/dna
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