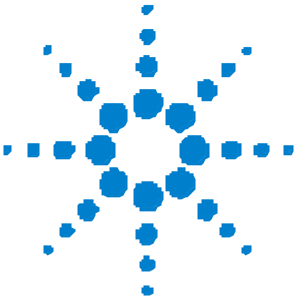

Agilent Direct-Label cDNA Synthesis Kit Protocol

User's Guide

For use with cat. nos. G2555A and G2557A

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Notices

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

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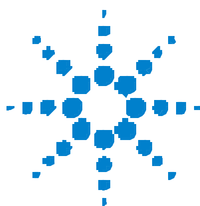


1 Introduction

Agilent's Direct Labeling cDNA Synthesis Kit provides a set of qualified protocols and reagents that enable researchers to generate fluorescent cDNA targets for use with Agilent's cDNA microarrays (cat. no. G4100A). Using this procedure, RNA isolated from two different biological samples is fluorescently-labeled using a reverse transcriptase and cyanine labeled nucleotide analogues. Each sample is labeled with a different fluorescent dye—either cyanine 3 (which is excited by 532 laser line) or cyanine 5 (which is excited by a 633 laser line). After labeling, samples are mixed (reference in one color/test in the second color) and allowed to hybridize to the microarray. Differentially expressed genes are identified by washing the array, scanning it with a laser-based detection system (such as Agilent's Microarray Scanner), using a feature extraction software package that links a feature to a design file and determining the relative fluorescence intensity of the two different dyes after normalization.

Kit Contents

(provided with cat. nos. G2555A and G2557A)

MMLV-RT (200 U/µl)	40	µl
5X First Strand Reaction Buffer	400	µl
0.1 M DTT	200	µl
DNA Primer (100 µM)	40	µl
dNTP mix (-dCTP)	20	µl
5 mM dCTP	10	µl
RNase I "A" (0.05 mg/ml)	40	µl
2x Deposition Hybridization Buffer *(only provided with cat. no. G2555A)*	125	µl

Required Reagents Not Included in Kit

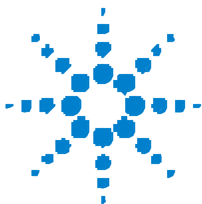
- Cyanine 3-dCTP (1.0 mM) (Perkin-Elmer/NEN, cat. no. NEL 576)
- Cyanine 5-dCTP (1.0 mM) (Perkin-Elmer/NEN, cat. no. NEL 577)
- DNase/RNase-free distilled water (Invitrogen, cat. no. 10813-012)
- 100% ethanol (Amresco, cat. no. E193)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- 35% (w/v) Guanidine Hydrochloride (prepare from Sigma, cat no. G7153)
- To prepare the 35% (w/v) solution, dissolve 35g of guanidine hydrochloride in 100 ml nuclease-free water. Pass through a 0.2 um filter to remove particulates. Guanidine hydrochloride solution should be stored at 4° C. The dry powder is hydroscopic; store in dessicator.

Required Equipment

- Micropipettors to pipette a range of 1 µl to 1 ml volumes
- Sterile, nuclease-free 1.5 ml microcentrifuge tubes
- Sterile, nuclease-free aerosol barrier pipet tips
- Heating block or waterbath temperature set to 70° C
- UV spectrophotometer and 0.1 ml volume quartz cuvettes (1 cm path length)
- Microcentrifuge
- Circulating waterbath, temperature set to 42° C
- Vortex mixer
- SpeedVac rotory dessicator

Safety and General Procedural Notes

- Cyanine 3-dCTP and cyanine 5-dCTP are possible carcinogens. Avoid inhalation, swallowing or contact with skin.
- To prevent contamination of reagents by ribonucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Cyanine 3 and cyanine 5 are photolabile. Minimize exposure to light.



2
Labeling Procedure

RNA Preparation and Labeling

It is essential to start the procedure with high quality RNA. Before starting, ensure that the total or poly-A+ RNA meets the following specifications:

1. Size distribution:

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

   For RNA analysis, we recommend using Agilent's 2100 Bioanalyzer with the RNA6000 LabChip® Kit (product no. 5064-8229). 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. Sample analysis takes approximately 10 minutes, conferring a significant time and sample saving advantage over standard agarose gels.

   - For total RNA, specific bands of highly abundant ribosomal RNA should be visible at approximately 1.9 and 5 kb. These integrity of these bands will reflect the integrity of the mRNA. The bands should be sharp and clear; smearing of the rRNA bands on the gel indicates that the RNA has degraded. In addition, high molecular weight bands (>9000 kb) indicate DNA contamination of the RNA 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 by measuring UV absorbance at 260 nm using a spectrophotometer. An A260 of 1 equals a concentration of approximately 40 µg/ml.

NOTE Labeling of total RNA requires 20 µg per labeling reaction. Labeling poly A+ RNA requires 400 ng units. The RNA should be stored at –80° C until use.

Synthesis of Cyanine 3- and Cyanine 5-labeled cDNA

One tube of cyanine 3-labeled cDNA and one tube of cyanine 5-labeled cDNA must be prepared for each microarray to be hybridized.

1 Add the 20 µg of total RNA or 400 ng of poly-A RNA to each reaction tube

2 Add 2.0 µl DNA Primer and bring the total sample volume to 50 µl in nuclease-free water.

3 Incubate tube at 70° C, 10 minutes, to denature primer and template.

4 Place reaction tubes on ice for 5 minutes.

5 Add 2.5 µl of either cyanine 3-dCTP or cyanine 5-dCTP. Label each tube appropriately.

NOTE Cyanine 3 is bright pink and cyanine 5 is bright blue. Minimize exposure of the cyanine dyes to light.

6 Add the following to each reaction:

Volume	Component
14 µl	Nuclease-free Water
20 µl	5x First Strand Buffer
10 µl	0.1 M DTT
1 µl	dNTP Mix (no dCTP)
0.5 µl	5 mM dCTP
2 µl	MMLV-RT

For efficiency and to minimize pipetting errors when setting up the cDNA synthesis reactions, prepare a single Master Mix of the components above for all reactions. Keep Master Mix on ice until ready to aliquot into each reaction tube.

7 Aliquot 47.5 µl of cDNA Master Mix (prepared in step 6) into each sample tube.

8 Incubate cDNA synthesis reaction at 42 °C in waterbath for 60 minutes.

9 Move reaction tubes to waterbath or heating block at 70 °C for 10 minutes.

10 Place reaction tubes on ice for 5 minutes.

11 Add 2.0 µl RNase I A, 0.05 mg/ml, to each reaction tube. Mix by pipetting then incubate at room temperature for 30 min to degrade the RNA.

NOTE Use caution when handling RNase to avoid contamination of the lab environment.

Purification of Labeled cDNA

Agilent recommends using the Qiagen's QIAquick spin columns for purification of labeled cDNA samples. Unincorporated dye-labeled nucleotides in the hybridization solution will significantly increase background fluorescence on the array. The addition of a wash with 35% (w/v) guanidine hydrochloride (i.e., step 3 below and directions for preparation are in Required Reagents section at the beginning of the protocol) to Qiagen's standard procedure is necessary to ensure efficient removal of the unincorporated nucleotides. For this reason, please follow the QIAquick PCR Purification Kit Protocol with the modifications outlined below:

1 Bind the labeled cDNA to the column as described in the Qiagen protocol.

2 Discard flow-throµgh and place the QIAquick column back into the same collection tube.

3 Add 0.75 ml of 35% guanidine hydrochloride to the QIAquick column and centrifuge in a table-top microcentrifuge at 13,000 rpm for 60 sec.

4 Discard flow-through and place the QIAquick column back into the same collection tube.

5 Wash the column with 0.75 ml of Qiagen's Buffer PE and centrifuge for 60 sec as described in the protocol.

6 Discard flow-through and place the QIAquick column back into a **clean** collection tube.

7 To elute the sample, add 30 µl of the Qiagen Buffer EB (10mM Tris-Cl, pH 8.5) to the center of the column, let sit for 1 minute, then centrifuge for 60 sec.

8 Repeat step 7 with an addition 30 µl of Qiagen Buffer EB and elute into the same tube. Final eluted volume should be approximately 60 µl.

Concentration of Cyanine 3- or Cyanine 5-labeled cDNA

Following purification with the QIAquick columns, the purified, labeled cDNA samples may be concentrated as follows.

1 Combine the appropriate cyanine 3-cDNA and cyanine 5-cDNA targets intended for hybridization to a single array in a 1.5 mL microcentrifuge tube.

2 Dry the solution under vacuum in rotary dessicator until dry (approximately 60 minutes).

CAUTION Do not use heat during drying to prevent degradation of the cyanine dyes.

3 Either proceed directly to hybridization or freeze on dry ice and store at -80 °C. Purified samples can be stored at –80° C.

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