cDNA Synthesis Kit

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Introduction

Genisphere’s cDNA Synthesis Kit is designed to produce labeled cDNA from SenseAMP™ or RampUP™ amplified senseRNA, for gene expression analysis on Affymetrix GeneChips or glass arrays.

Any labeled dUTP can be incorporated into the cDNA. Biotin-deoxynucleotide is incorporated into the cDNA for hybridization to Affymetrix Gene Expression Analysis Arrays. Recent publications (Barker C.S., *et al*. and Eklund, A.C., *et al.*) have shown that labeled cDNA produces expression profiles equal to or better than labeled aRNA on Affymetrix GeneChips. Fluorescent- or aminoallyl-deoxynucleotide may be incorporated into the cDNA for hybridization to glass arrays such as those manufactured by Agilent or printed in-house.

Kit Specifications

Components and Storage

Green cap	Random 12-mer Primer (15μg/μl)
White cap	Superase-In™ RNase Inhibitor
Purple cap	Low dTTP dNTP mix (2mM dTTP, 10mM each dATP, dCTP, dGTP)

Microcon® YM-50 columns (10) and collection tubes (20)

Store all vials at -20°C.
Store Microcon YM-50 columns and collection tubes at room temperature.

Handling:

Green cap vial:
Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.

White and Purple cap vials:
Thaw on ice and briefly centrifuge. Keep on ice at all times. Do not vortex.

Additional Materials/Equipment Required

- 20μg SenseAMP or RampUP senseRNA (minimum concentration of 1.25μg/μl)
  OR
  10μg SenseAMP or RampUP senseRNA (minimum concentration of 1.54μg/μl)
- SuperScript™ II Reverse Transcriptase (Invitrogen cat. no. 18064-014) or equivalent
- Biotin 16-dUTP, 1mM (Roche cat. no. 11093070910) or other labeled dUTP
- 1X TE Buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) (Ambion cat. no. AM9858 or equivalent)
- Nuclease-Free Water (Ambion cat. no. AM9934 or equivalent)
- Heating devices for incubations at 42°C, 65°C, and 80°C
- 0.5M NaOH, 50mM EDTA (cDNA stop solution)
- 1M Tris-HCl, pH 8.0
- 10mM Tris-HCl, pH 8.0
- UV/Vis Spectrophotometer for cDNA quantitation (or other quantitation instrument)

Procedure for Use

cDNA Synthesis from 20µg of senseRNA

Note: This procedure is recommended for Affymetrix GeneChip applications to ensure maximum yield of labeled cDNA for hybridization. If 20µg of senseRNA is not available, please refer *cDNA Synthesis from 10µg of senseRNA* on page 5.

1. Bring the volume of 20µg of senseRNA (minimum concentration of 1.25µg/µl) to 16µl with Nuclease-Free Water. If necessary concentrate the senseRNA sample to a minimum concentration of 1.25µg/µl using one of the following methods:
   - -SpeedVac or similar vacuum concentrator
   - -Lyophilization
   - -Evaporation in a 60°C heat block
   - -Microcon YM-3 concentrator (Millipore™ cat. no. 42404)

2. Add 2µl Random 12-mer Primer (Green cap, 15µg/µl).

3. Incubate the senseRNA/primer mix at 80°C for 5 minutes.

4. During the 5 minute senseRNA/primer mix incubation at 80°C, prepare a 22µl reverse transcription reaction mix on ice for each reaction:
   - 8.0µl 5X First Strand Buffer, included with SuperScript II
   - 4.0µl 0.1M DTT, included with SuperScript II
   - 1.0µl Superase-In RNase Inhibitor (White cap)
   - 2.0µl low dTTP dNTP mix (Purple cap)
   - 5.0µl Biotin 16-dUTP, 1mM (or other labeled-dUTP)
   - 2.0µl SuperScript II Reverse Transcriptase

5. Immediately ice the senseRNA/primer mix for 2 minutes. Briefly centrifuge.

6. Incubate the senseRNA/primer mix at room temperature for 3 minutes.

7. Add the 22µl reverse transcription reaction mix to each reaction for a final volume of 40µl.

8. Incubate the samples at 42°C for 2.5 hours.

9. Add 7µl of cDNA stop solution (0.5M NaOH, 50mM EDTA).

10. Incubate the samples at 65°C for 30 minutes.

11. Add 10µl of 1M Tris-HCl, pH 8.0.

12. Add 43µl of 1XTE Buffer to adjust the volume of each reaction to 100µl.

13. Proceed to *Microcon YM-50 Purification and Concentration* on page 6.

cDNA Synthesis from 10µg of senseRNA

1. Bring the volume of 10µg of senseRNA (minimum concentration of 1.54µg/µl) to 6.5µl with Nuclease-Free Water. If necessary concentrate the senseRNA sample to a minimum concentration of 1.54µg/µl using one of the following methods:
   - -SpeedVac or similar vacuum concentrator
   - -Lyophilization
   - -Evaporation in a 60°C heat block
   - -Microcon YM-3 concentrator (Millipore™ cat. no. 42404)

2. Add 1µl Random 12-mer Primer (Green cap, 15µg/µl).

3. Incubate the senseRNA/primer mix at 80°C for 5 minutes.

4. During the 5 minute senseRNA/primer mix incubation at 80°C, prepare a 12.5µl reverse transcription reaction mix on ice for each reaction:
   - 4.0µl 5X First Strand Buffer, included with SuperScript II
   - 2.0µl 0.1M DTT, included with SuperScript II
   - 1.0µl Superase-In RNase Inhibitor (White cap)
   - 1.0µl low dTTP dNTP mix (Purple cap)
   - 2.5µl Biotin 16-dUTP, 1mM (or other labeled-dUTP)
   - 2.0µl SuperScript II Reverse Transcriptase

5. Immediately ice the senseRNA/primer mix for 2 minutes. Briefly centrifuge.

6. Incubate the senseRNA/primer mix at room temperature for 3 minutes.

7. Add the 12.5µl reverse transcription reaction mix to each reaction for a final volume of 20µl.

8. Incubate the samples at 42°C for 2.5 hours.

9. Add 3.5µl of cDNA stop solution (0.5M NaOH, 50mM EDTA).

10. Incubate the samples at 65°C for 30 minutes.

11. Add 5µl of 1M Tris-HCl, pH 8.0.

12. Add 71.5µl of 1XTE Buffer to adjust the volume of each reaction to 100µl.

13. Proceed to *Microcon YM-50 Purification and Concentration* on page 6.

Microcon YM-50 Purification and Concentration

Purify the 100µl of cDNA using a Microcon YM-50 column (provided) as described below.

Note: Do <u>not</u> use alternative columns for this step.

1. Place a Microcon YM-50 column into a collection tube provided.

2. Add the 100µl cDNA to the sample reservoir. Do not touch the membrane with the pipet tip. Secure the tube cap and centrifuge for 6 minutes at 13,000g.

3. Add 200µl of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 minutes at 13,000g.

4. Carefully separate the sample reservoir from the collection tube. Discard the flow-through. Place the YM-50 column into the same collection tube.

5. Add 200µl of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 minutes at 13,000g.

Note: If using fluorescent labeled deoxynucleotide to generate labeled cDNA, repeat steps 3 and 4 two additional times to remove excess fluorescent label.

6. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.

7. Add 10-20µl 10mM Tris-HCl, pH 8.0 to the sample reservoir without touching the membrane. Gently tap the side of the reservoir to mix.

8. Carefully place the sample reservoir **upside-down** in a **new collection tube** provided. Centrifuge for 3 minutes at 10,000-13,000g.

9. Measure the volume of cDNA collected in the bottom of the tube (10-20µl).

Quantitation and Visualization of cDNA

Quantitate the cDNA by using a spectrophotometer or other instrument. From the OD, determine the nucleic acid concentration of each labeled sample:

A260nm x 33 (single-stranded DNA extinction coefficient) x dilution factor = concentration of cDNA in ng/µl

The cDNA should range from 50 to 600 bases. The average fragment size should be 50-250 bases, which is ideal for microarray hybridization. If cDNA fragments are too long, contact Genisphere Technical Support for recommendations for cDNA fragmentation.

Array Hybridization Recommendations

Affymetrix GeneChip

Hybridize approximately 3-6μg of biotinylated cDNA to the Affymetrix GeneChip.
Note: Do not apply less than 2.5μg of biotinylated cDNA to an Affymetrix GeneChip.

For example:

Prepare labeled cDNA to 40μl: Combine 3-6μg of biotinylated cDNA sample with Nuclease-Free Water to give a volume of 32μl. Add 8μl Affymetrix 5X Fragmentation Buffer. Vortex to mix and briefly centrifuge. Incubate at 94°C for 35 minutes. (Note: the cDNA is not being fragmented during this incubation because the 5X Fragmentation Buffer is designed to fragment RNA. However, this step is important to reduce array background.)
Immediately place tubes on ice for 2 minutes and briefly centrifuge.

Allow GeneChips to equilibrate to room temperature (20-25 °C).

Prepare cDNA Hybridization Buffer for Midi Array:

100.0μl 2X Hybridization Buffer
22.7μl Nuclease-Free Water
20.0μl DMSO
3.3μl Control Oligonucleotide B2
10.0μl 20X Eukaryotic Hybridization Controls
2.0μl 10mg/ml Herring Sperm DNA
2.0μl 50mg/ml Acetylated BSA
160μl (sufficient hybridization buffer for single reaction)

Add 160μl of cDNA hybridization buffer to each 40μl labeled cDNA sample for a final hybridization volume of 200μl. Incubate samples at 99°C for 5 minutes. Transfer tubes to 45°C for 5 minutes. Briefly centrifuge samples at 13,000g for 5 minutes.

Pre-Hybridization of GeneChip: Load 140μl of pre-hybridization buffer into each Midi GeneChip and rotate in an Affymetrix oven at 60rpm for 10 minutes at 45°C.

cDNA Hybridization: Remove chips from oven, remove pre-hybridization buffer, and load 140μl of each 200μl fragmented cDNA sample into the GeneChip. In an Affymetrix oven, hybridize with rotation at 60rpm for 16 hours at 45-47°C.

Washing and Staining: Prepare staining vials as recommended in the standard Affymetrix protocol. For U133Av2.0 midi chip, use fluidics protocol Midi_euk2v3.

Scan GeneChips using Affymetrix scanner.

Glass Array

Hybridize approximately 5-20µg of direct fluorescent or aminoallyl labeled cDNA to the glass array. **Note:** Due to the presence of ribosomal RNA in the amplified senseRNA labeled, it may be necessary to increase the stringency of the post cDNA hybridization wash procedure to preserve differential data.

For example:

cDNA Hybridization

Perform cDNA hybridization according to standard protocol for the array. Depending on array type, the effective hybridization temperature should be 55-65°C.

Post cDNA Hybridization Wash and Scan

Prewarm 2X SSC, 0.2% SDS wash buffer to:

55°C for oligo arrays
65°C for cDNA arrays

Remove coverslip and wash the array in the prewarmed buffer for 15 minutes. Transfer the array to 2X SSC wash buffer at room temperature and wash for 10 minutes. Transfer the array to 0.2X SSC wash buffer at room temperature and wash for 10 minutes.

Dry and scan the array following standard protocol.

References

Barker, C.S., *et al*. Increase DNA microarray hybridization specificity using sscDNA targets, BMC Genomics, 6:57 (2005).

Eklund, A.C, *et al.* Replacing cRNA targets with cDNA reduces microarray cross-hybridization, Nature Biotechnology, 24:1071 (2006).

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