Preparation of RNA Spike Controls and Amino-Allyl, Dye-labeled cDNA for use with MI PathArray ™

Description

MI has developed a mixture of the Ambion ArrayControl™ RNA Spikes that can be used to normalize hybridization data from the MI PathArray™ system. A mixture of the ArrayControl spikes covering a range of concentrations is added to the sample RNA to be tested. The resulting labeled target cDNA corresponds to specific control probes in the MI PathArray. Hybridization signals from the known concentration of the different spike controls can be used normalize signals from the sample RNA.

This protocol has been developed by MI and is a starting point for using the spike controls for data normalization. Further optimization of the spike dilutions may be necessary for your experiments depending on the source of the RNA. Many of the protocol steps are taken directly from the Invitrogen SuperScript™ and ArrayControl kits and are reproduced here for convenience.

A. Preparation of RNA Spike Control Master Mix (optional, skip to section B if not using)

***Note**: For proper handling of RNA, follow precautions outlined in the "ArrayControl Spots and Spikes" Instruction Manual from Ambion, Inc.*

Recommended Kits/Reagents

***Note**: The following materials are purchased directly from the manufacturer. Please see our Purchasing Guide, below, for contact information to order items listed.*

MATERIAL	CATALOG #	SOURCE
Ambion ArrayContro Spikes Kit	AM1780	Ambion/Applied Biosystems

1) Prepare the following RNA Spike Master Mixture:

Spike Length	Spike #	Dilution	Master Mix Final Formulation	Moles/µl in Master Mix (moles x 1.0E-14)
750 nt	1	1µl RNA in 249µl TE Buffer	1µl of 250:1 dilution	0.08
752 nt	2	1µl RNA in 9.6µl TE Buffer	1µl of 10.6:1 dilution	1.68
1000 nt	3	1µl RNA in 2.95µl TE Buffer	1µl of 3.95:1 dilution	3.22
1000 nt	4	1µl RNA in 2µl TE Buffer	1µl of 3:1 dilution	4.80

1034 nt	5	None	0.92µl RNA	14.42
2000 nt	8	0.72µl in 0.28µl TE Buffer	0.72µl in 0.28µl TE Buffer	4.80
			Total Volume: 6µl	

- **Spikes 1 through 5** were designed to produce hybridization signals creating an expression curve covering the full dynamic range of standard 16-bit fluorescent scanners.
- **Spike 8** matches the molar concentration of spike 4 and provides a comparable hybridization signal for longer RNA transcripts.
- The spike volumes are adjusted for transcript size and dye incorporation efficiency.
- The amount of master mix to add to the RT reaction will need to be optimized for your RNA source to match the range of expression levels of genes in your source RNA.
- MI recommends that a dilution series of the master mix be tested within your system to establish the optimal dilution for use in routine hybridizations.

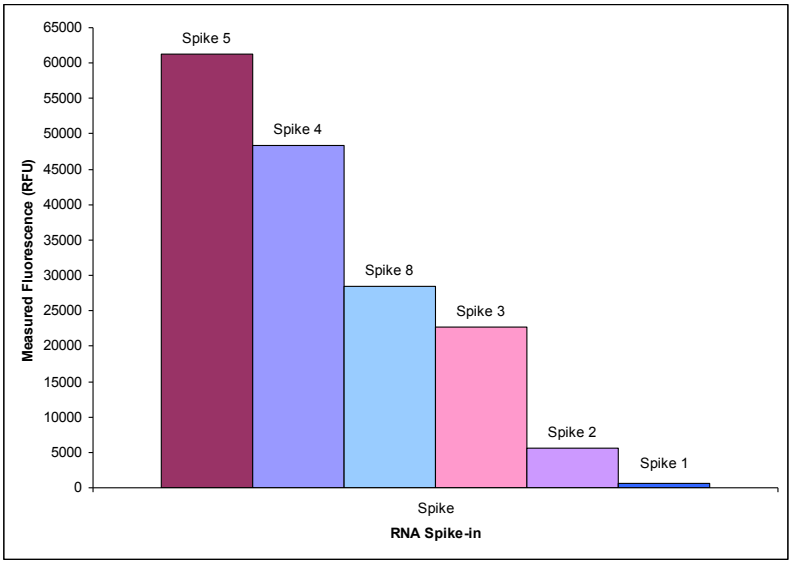

Fig 1. Results from undiluted spike master mix at 1.45µl in 5µg RNA, hybridized at 25ng cDNA

B. Reverse Transcription Reaction

The Invitrogen SuperScript Indirect cDNA Labeling System is designed for use with 1-5µg total RNA or 0.4-2µg of mRNA, isolated using a method of the user's choice. It is advised to proceed directly to "First-Strand cDNA Synthesis Reaction" following RNA isolation. If this is not possible, store RNA at -80˚C.

Recommended Kit

***Note**: The following materials are purchased directly from the manufacturer. Please see our Purchasing Guide, below, for contact information to order items listed.*

MATERIAL	CATALOG #	SOURCE
SuperScript Indirect cDNA Labeling System	L1014-02	Invitrogen Corp

Other materials supplied by the user (in addition to the SuperScript™ Indirect Labeling System)

Vortex mixer
Microcentrifuge
Incubator or heat blocks
1N NaOH
1N HCl
Sterile, 1.5ml microcentrifuge tubes
100 % Isopropanol
100% Ethanol
75% Ethanol
Aerosol-resistant pipette tips

First-Strand cDNA Synthesis Reaction

1) Mix and briefly centrifuge each reagent before use.

2) MI recommends using 1-5µg total RNA in each reverse transcription reaction. Combine the following in a sterile, 1.5ml RNAse-free microcentrifuge tube.

Reactant	Amount
1-5µg total RNA	X µl (no greater than 14.5µl)
RNA spike mixture dilution (optimized)	1.45 µl (optimized, if using spike controls)
*Anchored Oligo$(dT)_{20}$ Primer (2.5µg/ µl)	2 µl
DEPC-treated water	to 18 µl

***NOTE**: Kit contains both anchored oligo$(dT)_{20}$ primers and random hexamers. *You may add both primer sets for use with mRNA (random hexamers at 1µl + anchored oligo $(dT)_{20}$ primers at 2µl). For total RNA reactions, use 2µl of the anchored oligo$(dT)_{20}$ primer only.*

3) Incubate tube at 70˚C for 5 minutes in a heat block or incubator.

4) Incubate tube on ice for ≥1 minute.

5) Add the following to each reaction tube on ice:

Reactant	Amount
5X First-Strand Buffer	6 µl
0.1 M DTT	1.5 µl
dNTP mix (contains amino-modified nucleotides)	1.5 µl
RNaseOUT™ (40 U/µl)	1 µl
SuperScript III RT (400 U/µl)	2 µl
Final Reaction Volume	30 µl

6) Mix gently by tapping the tube and briefly centrifuge.

7) Incubate tube at 46˚C for 2-3 hours.
Note: *It is recommended that you allow the reverse transcription reaction to proceed for 3 hours for best cDNA yield. After incubation proceed to Alkaline Hydrolysis and Neutralization.*

C. Alkaline Hydrolysis and Neutralization

8) Add 15µl of 1N NaOH to each tube and mix thoroughly.

9) Incubate the tube at 70˚C for 10 minutes.

10) Add 15µl 1N HCl to each tube and mix gently by tapping the tube.

11) Add 20µl 3M sodium acetate, pH 5.2. Mix gently.

D. SNAP™ Column Purification

12) To each tube, add 500µl Loading Buffer (prepared with addition of 30ml of 100% isopropanol to the 13ml Loading Buffer bottle, as per manufacturer's recommendation) to the neutralized cDNA.

13) Vortex the tube.

14) Place a SNAP Column on a collection tube and load the sample onto the column.

15) Centrifuge at 14,000 x *g* at room temperature (RT) for 1 minute. Remove the collection tube and discard the flow-through.

16) Place the SNAP Column onto the same collection tube and add 700µl of Wash Buffer to the column (prepared with the addition of 75ml of 100% ethanol to the 25ml bottle of Wash Buffer, as per the manufacturer's recommendation).

17) Centrifuge at 14,000 x g at RT for 1 minute.

18) Remove the collection tube and discard the flow-through.

19) Repeat steps 16-18 above.

20) Centrifuge once more at 14,000 x *g* at RT for 1 minute.

21) Remove the collection tube and discard the flow-through.

22) Place the SNAP Column onto a new sterile 1.5ml microcentrifuge tube.

23) Add 50µl of DEPC-treated water to the SNAP Column and incubate for 1 minute at RT.

24) Centrifuge at 14,000 x *g* at RT for 1 minute.

25) Repeat step 23-24, using the same microcentrifuge tube.

26) Proceed to Ethanol Precipitation.

E. Ethanol Precipitation

27) Add 10µl 3M sodium acetate, pH 5.2, to the tube.

28) Add 2µl glycogen (20mg/ml) to the tube and mix gently.

29) Add 300µl ice-cold 100% ethanol to the tube.

30) Incubate at -20˚C for ≥ 30 minutes.
Note*: Overnight ethanol precipitation can be performed*.

31) Centrifuge the tube at 14,000 x *g* at 4˚C for 20 minutes.

32) Carefully remove and discard the supernatant.
Note*: if a refrigerated centrifuge is not available, tubes may be spun at RT however, there may be a loss in yield.*

33) Add 250µl of ice-cold 75% ethanol and centrifuge the tube at 14,000 x g for 2 minutes.

34) Carefully remove and discard the supernatant.

35) Air dry the sample for 10 minutes.
Note: *be sure all traces of ethanol are removed prior to beginning the labeling reaction.*

F. Labeling Reaction

***Note**: MI developed the MI PathArray product using Cy3 Dye. If a different NHS-ester dye is used, consult the Invitrogen user manual for* the *"SuperScript Indirect cDNA Labeling System" regarding use of an alternate dye.*

Recommended Kits/Reagents

***Note**: The following materials are purchased directly from the manufacturer. Please see our Purchasing Guide, below, for contact information to order items listed.*

MATERIAL	CATALOG #	SOURCE
Amersham Cy™3 Mono-Reactive Dye Pack [1]	PA23001	GE Healthcare

[1] *Other dyes compatible with amino-allyl labeling methods may be used with the Invitrogen kit.*

36) Resuspend the sample in 5µl of 2X Coupling Buffer.

37) Prepare the Cy3 Dye Solution (PA23001) by adding 45µl DMSO directly to the dye vial. Mix well.

38) Add 5µl Dye Solution to the reaction tube containing the resuspended cDNA in the 2X Coupling Buffer. Mix thoroughly.

39) Incubate the tube at RT, protected from light, for ≥1 hour.
***Note**: the reaction can be stored overnight if desired.*

G. SNAP™ Column Purification

40) Add 20µl of 3M sodium acetate, pH 5.2, to the dye reaction.

41) Add 500µl of prepared Loading Buffer to the solution. Vortex mixture.

42) Place a SNAP Column on a clear collection tube and pipette the solution onto the column.

43) Centrifuge the tube at 14,000 x *g* at RT for 1 minute. Remove the collection tube and discard the flow-through.

44) Place the SNAP Column on the same collection tube and add 700µl prepared Wash Buffer to the column.

45) Centrifuge at 14,000 x *g* at RT for 1 minute. Remove collection tube and discard the flow-through.

46) Repeat steps 44-45, using the same collection tube.

47) Centrifuge once more at 14,000 x *g* at RT for 1 minute. Remove the collection tube and discard the flow-through.

48) Place the SNAP Column onto a new amber 1.5ml centrifuge tube.

49) Add 50µl of DEPC-treated water to the SNAP Column and incubate at RT for 1 minute.

50) Centrifuge at 14,000 x *g* at RT for 1 minute.

51) Collect the flow-through which contains your purified, dye-coupled cDNA.

52) Proceed directly to assessment of labeling efficiency/quantitation of cDNA for hybridization or freeze sample at -20˚C for up to one week. Avoid multiple freeze-thaw cycles of the sample as this may degrade the cDNA.

H. Assessment of cDNA Yield and Labeling Efficiency

To calculate the amount of labeled cDNA:

53) Transfer an appropriate amount (based on the cuvette size used) of purified, undiluted, labeled cDNA sample to the spectrophotometer and measure the absorbance at 260nm and 320nm.

54) Use the following calculation to determine the amount of labeled cDNA in the sample:

$$\text{cDNA (ng)} = (A_{260} - A_{320}) \times 37 \text{ ng/µl} \times \text{Volume in µl}$$

55) Using the sample from above, measure absorbance at 550nm and 650nm if using Cy3™. If using another dye, measure at the manufacturer's recommended wavelengths. See "SuperScript Indirect cDNA Labeling System" User Manual from Invitrogen for alternative calculations using other dyes.

56) Use the following calculation to determine the amount of dye incorporation:

$$\text{Cy3 (pmole)} = (A_{550} - A_{650})/0.15 \times 50 \text{ (elution volume)}$$

I. Hybridization of the Purified, Dye-coupled cDNA to the Microarray

Following purification and quantitation of the dye-labeled cDNA, you may proceed directly to hybridization onto a prepared microarray. Follow the specific hybridization protocol for your application.

Protocols for hybridization to MI PathArray using static, dynamic mixing, or Tecan HS Pro Hybridization Station™ methods can be found at www.microarrays.com/mipatharrayinfo

References

"Amersham Cy™ 3 Mono-Reactive Dye Pack" Product Specification Sheet, GE Healthcare, PA23001PS Rev G 2007.

"ArrayControl™ Spots and Spikes" Instruction Manual, Ambion, Inc. (Applied Biosystems), Manual1780M Revision C, July 16, 2007.

"SuperScript™ Indirect cDNA Labeling System" User Manual from Invitrogen Corporation (Life Technologies), Version F, 7 May 2007, 25-0655.

Purchasing Guide

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