Purification of Fluorescent Dye Labeled cDNA Using a Superparamagnetic Particle Based Kit

Lisa R. Booth*, Dwayne T. Campogan, Karin A. Hughes,
Douglas A. Spicer, and Amy L. Springer

Prolinx Inc., 22322-20th Ave SE, Bothell, WA 98021 USA

http://prolinx.com

Presented at Transcriptome 2002:

From Functional to Systems Biology

Seattle, WA Mar. 10-13, 2002

Abstract

Nucleic acid microarrays allow for the profiling of thousands of genes in a single experiment and are an increasingly valuable tool for analyzing gene expression. A typical microarray is composed of a 3 X 1" slide containing an array of nucleic acid fragments to which fluorescently-labeled cDNAs are hybridized. cDNA is the product of reverse transcription reactions where mRNA is converted to cDNA. The removal of excess dyes from these reverse transcription reactions is vital to the accuracy and efficiency of experimental results. Prolinx® Inc., has developed a new method using superparamagnetic beads for the removal of excess Cy3- and Cy5-dye labeled dNTP precursors from these reactions. The protocol affords minimal loss of product, making it ideal when sample size is limited. High cDNA recoveries reduce the potential for introduction of variability, and concomitant loss of data reliability, by enabling replicates using the same cDNA sample and increase the possibility of improved detection of rare message. Studies presented will compare and contrast methods of cDNA purification in expression analysis microarrays. Effect of cDNA recovery on signal intensity and assay sensitivity will be included.

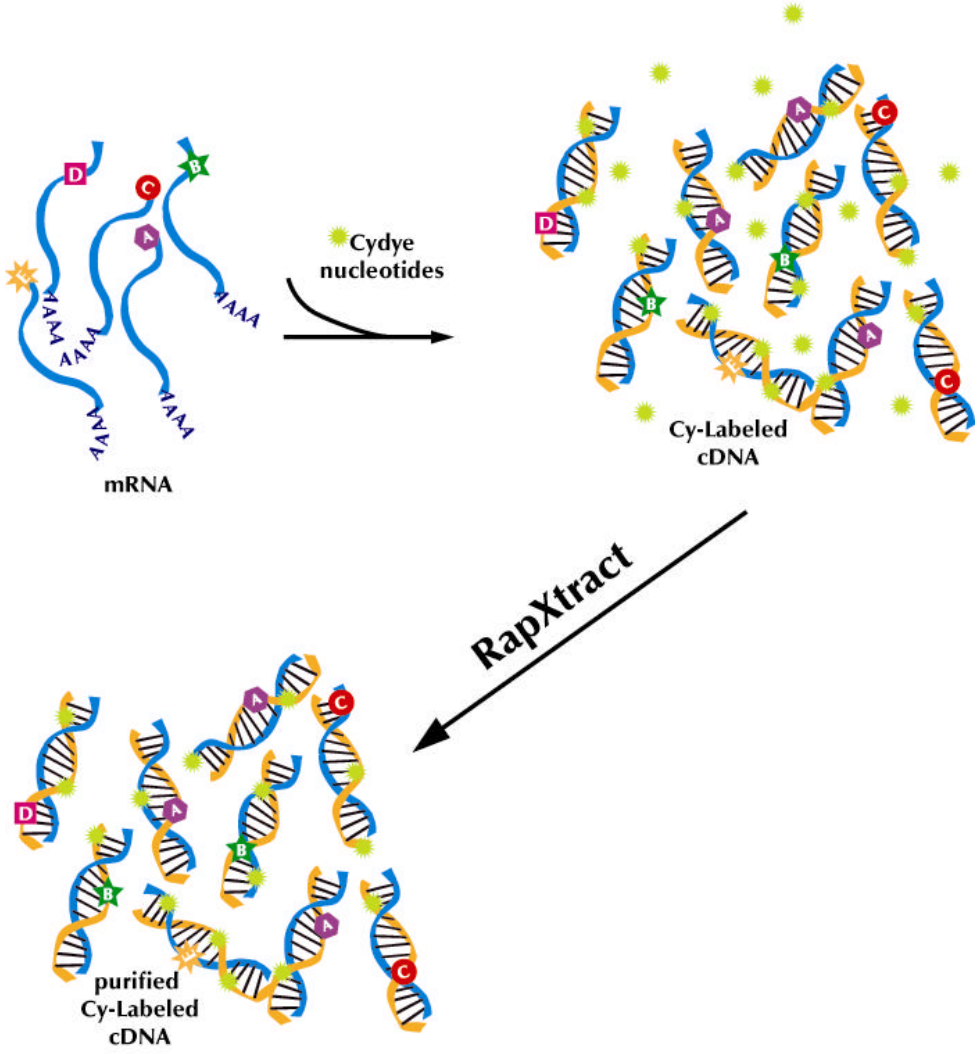
Introduction: Microarrays

- DNA, RNA, or oligonucleotide *probes* are immobilized on a microscope slide in an array of spots, representing a series of genes
- Fluorescently labeled cDNA or cRNA (synthesized RNA from DNA using an RNA polymerase such as T7) *targets* are then hybridized to the spots on the slide
- Expressed sequences present in the target bind to the complimentary spots on the slide
- Binding is detected in a fluorescent scanner and intensity of spot color allows quantitation of expressed sequences
- Arrays can analyze the expression of up to 60,000 genes at one time

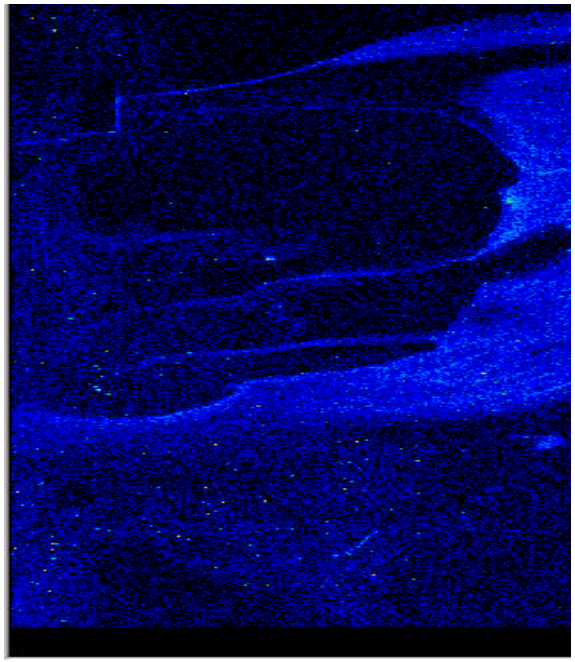
Procedure : cDNA Synthesis

- Generate cDNA from mRNA or total RNA with reverse transcriptase
- Incorporate fluorescently-labeled nucleotides during cDNA synthesis using 1-step “direct” protocol
- Purify cDNA transcripts (utilizing one of following)
  - Size exclusion chromatography
  - Ethanol precipitation
  - RapXtract Fluorescent cDNA Purification Kit
- Quantify purified dye-labeled products
- Hybridize purified targets to pre-spotted slide
- Wash unbound targets from slide
- Scan and analyze results

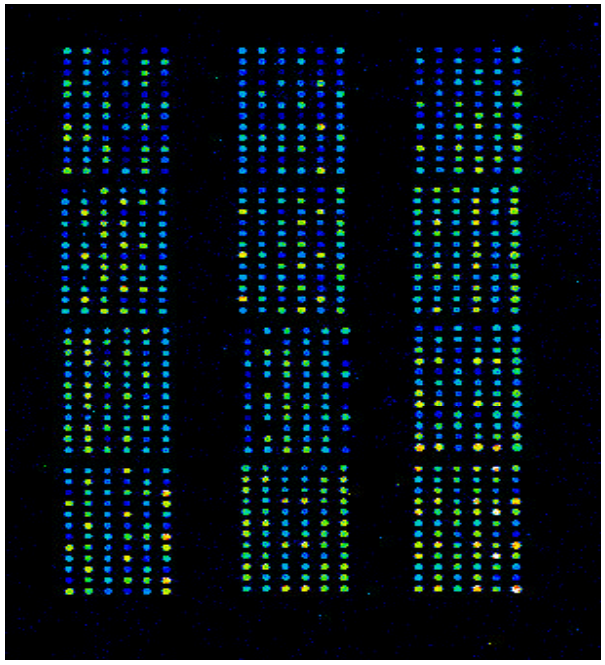
cDNA Synthesis: 1-Step “Direct” Protocol



The Need for Purification



Unpurified targets



Purified targets

- Purification removes the excess dye-labeled nucleotides, which create background and loss of resolution
- Scanners have difficulty focusing on spots when background signal interferes with spot resolution
- Improved signal-to-noise improves quantification by maximizing dynamic range

Purification of Dye-Labeled Targets

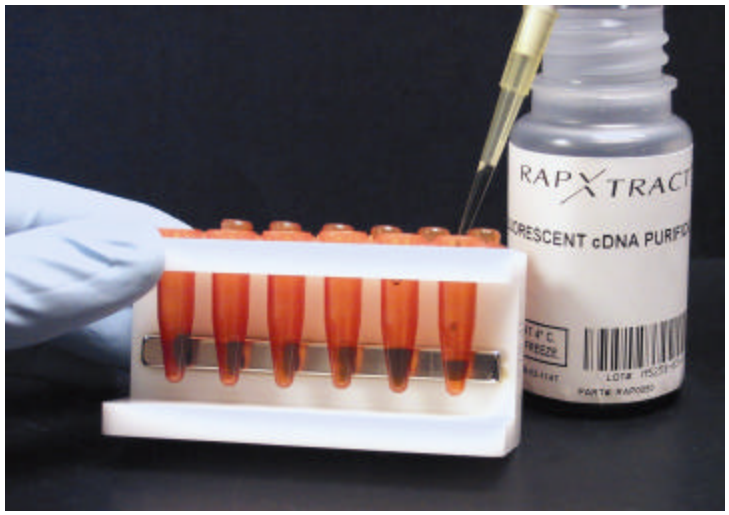
- Purification method should provide for maximum product recovery and be rapid and easy to implement.
- Current methods are labor intensive and can result in substantial loss of product, potentially making experimental conclusions erroneous.
- Prolinx, Inc. has developed a unique superparamagnetic particle-based clean-up method for direct labeling protocols that is fast, easy to perform and allows for high recovery of product.
- The method can be optimized for variations in protocols, including differing dye-labeled nucleotide concentrations.
- The method can be automated, making it ideal for high-throughput laboratories.

Purification Methods

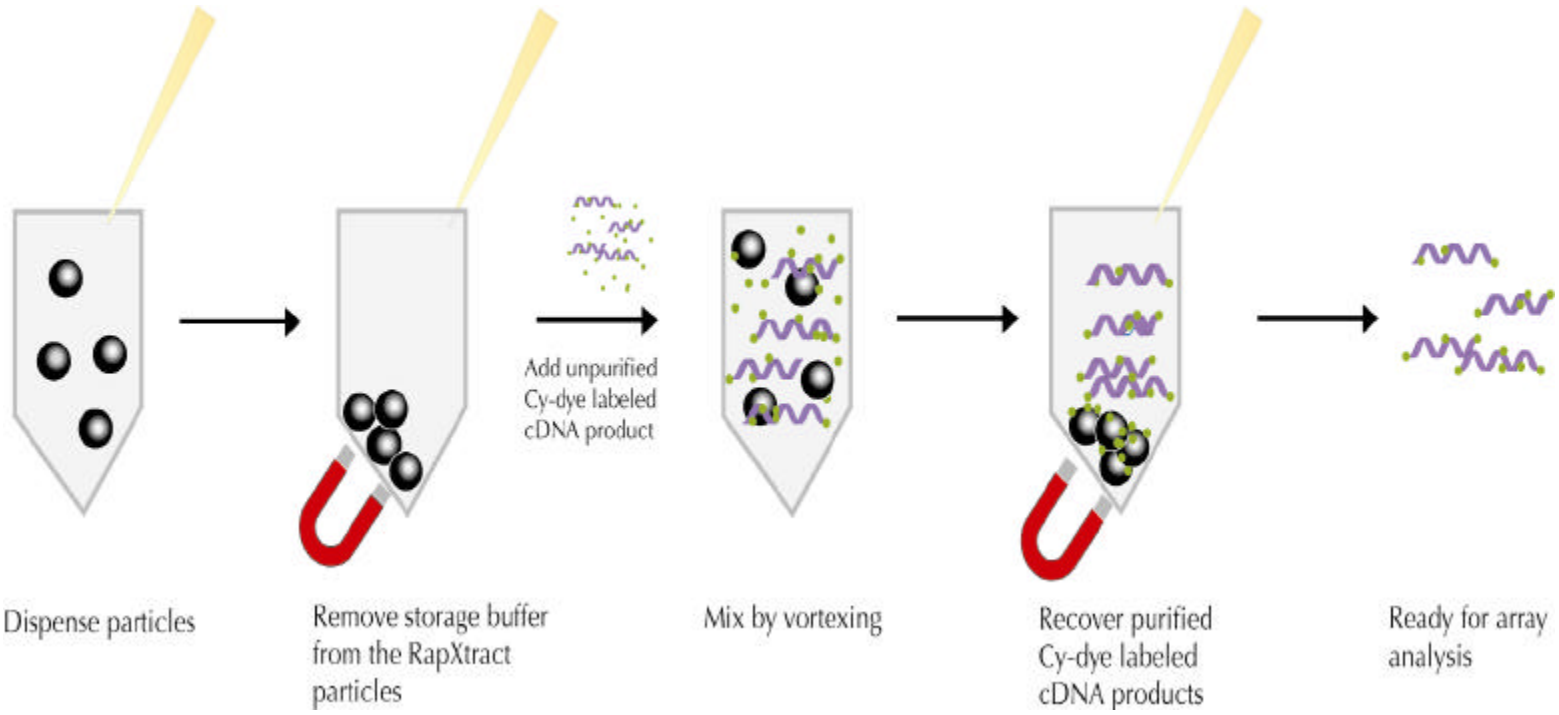
- Size Exclusion Method:
  - Individual tubes or 96-well format with either a membrane filter or a gel matrix
  - Requires vacuum filtration or centrifugation
  - May require 2-3 hours to hydrate matrix
  - Recovery of product is low; $\leq$25%
- Alcohol Precipitation Method:
  - Format selected by user
  - Requires refrigerated centrifugation
  - Takes 1.5 - 3 hours
  - Potential for low recovery
- RapXtract:
  - Reverse purification
  - Requires vortex
  - Takes 30 seconds
  - High recoveries

RapXtract™ Fluorescent cDNA Purification Kit

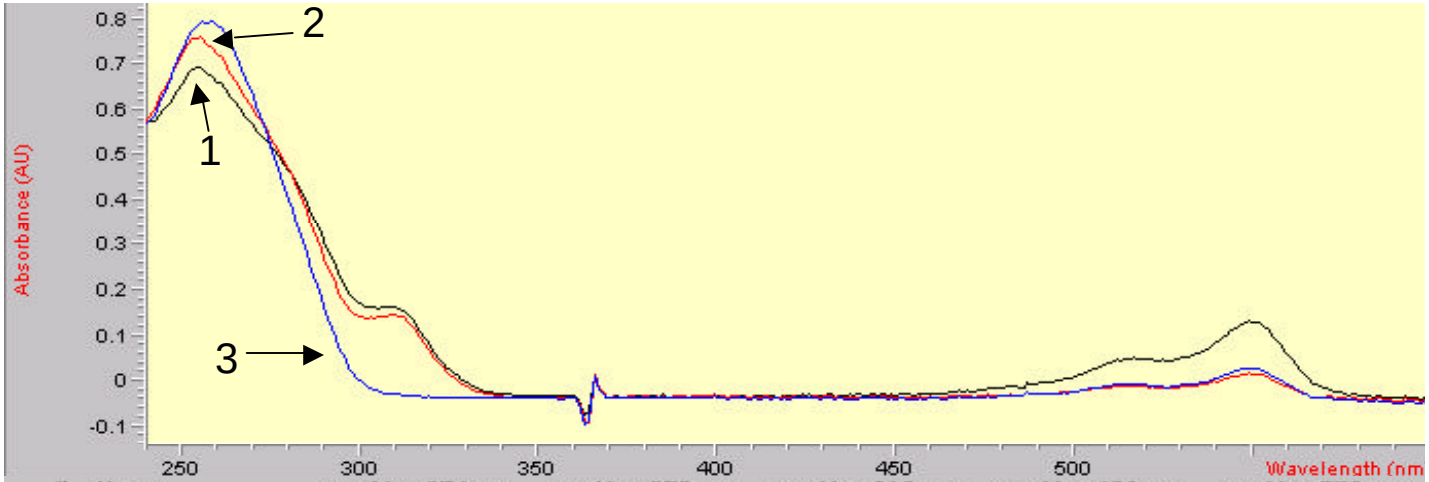
- Superparamagnetic particle technology (patent pending)
- "Reverse purification" process extracts unincorporated dyes from reaction
- Can be optimized to remove excess dyes while allowing for high target recovery rates
- Effective in removing CyDye and Fluorescein.
- Method is easily automated
- Rapid protocol; only 30 seconds



Purification with the RapXtract Fluorescent cDNA Purification Kit



RapXtract Fluorescent cDNA Purification Kit Removes Dyes Efficiently



#	Name	net Abs<254nm>	net Abs<259nm>	net Abs<310nm>	net Abs<400nm>	net Abs<550nm>
1	Unpurified 1:20	0.72579	0.70552	0.19498	0.00000	0.16347
2	Column 1:10	0.79552	0.77117	0.18001	0.00000	5.0266E-2
3	RapXtract 1:10	0.82104	0.83501	9.1004E-3	0.00000	6.5597E-2

1. Unpurified sample (1:20 dilution) (black) shows excess dye that absorbs (A_{550}) and a contaminant peak (A_{310}).
2. Size exclusion-purified sample (1:10 dilution) (red) eliminates excess dye that absorbs (A_{550}) , but contaminant peak remains (A_{310}).
3. RapXtract-purified sample (1:10 dilution) (blue) eliminates excess dye at 550 nm. Contaminant peak (A_{310}) also removed.

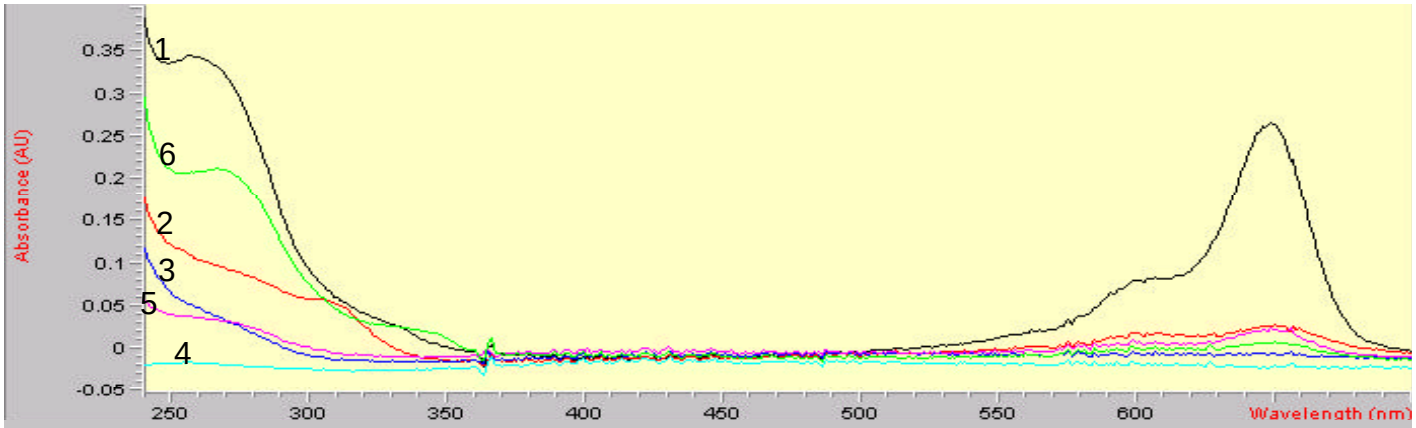
RapXtract Fluorescent cDNA Purification Kit Removes Dyes Efficiently

Three duplicate cDNA target reactions were prepared using a *Label*-IT kit for Cy3 (Mirus) and mouse brain mRNA (Ambion). Each reaction was prepared using sterile 0.2 mL PCR tubes into which was added 35 µL sterile ddH_2O, 5 µL of 10X Mirus Labeling Buffer A, 5 µL of 1 mg/mL mRNA and 5 µL *Label*-IT Cy3 Reagent reconstituted in 25 µL Mirus Reconstitution Solution. The reactions were incubated at 37°C for 1 hour in a thermalcycler.

The reactions were combined, vortexed, then re-aliquotted into 3 clean PCR tubes in equal amounts. One reaction was left unpurified, and one was purified using a size-exclusion column included with the *Label*-IT kit. The third reaction was purified with the RapXtract Fluorescent cDNA Purification Kit using 600 µg particles with agitation for 30 seconds.

Dilutions for spectrophotometer scanning were made using 10 mM Tris-hydrochloric acid, 140 mM sodium chloride, pH 8.

The RapXtract Fluorescent cDNA Purification Kit Affords High Recovery of Labeled Targets



#	Name	net Abs<260nm>	net Abs<280nm>	Bkgd<400nm>	Abs<260nm>	Abs<280nm>
1	Unpurified sample	0.35313	0.26475	-1.1112E-2	0.34202	0.25364
2	Size excl. type 1	0.11715	9.6140E-2	-1.3469E-2	0.10368	8.2672E-2
3	Size excl. type 2	5.6996E-2	2.7665E-2	-1.0165E-2	4.6831E-2	1.7499E-2
4	Size excl. type 3	-4.9543E-4	-5.0721E-3	-1.6418E-2	-1.6913E-2	-2.1490E-2
5	Size excl. type 4	3.9135E-2	2.7151E-2	-3.4647E-3	3.5671E-2	2.3686E-2
6	RapXtract	0.21488	0.18769	-6.9566E-3	0.20793	0.18073

1. Unpurified sample
2. Size exclusion type 1
3. Size exclusion type 2
4. Size exclusion type 3
5. Size exclusion type 4
6. RapXtract Kit; 600 µg particles, 30 seconds

The RapXtract Fluorescent cDNA Purification Kit Affords High Recovery of Labeled Targets

Six replicate cDNA reactions were prepared using a CyScribe First Strand Labelling Module (Amersham Pharmacia) and mouse brain mRNA (Ambion). Each reaction was prepared in sterile 0.2 mL PCR tubes into which was added 1 µg of mRNA, 1 µL random nonamers, 1 µL anchored oligo (dT), and 7 µL sterile water. The reactions were incubated for 5 minutes at 70 °C then cooled to room temperature for 10 minutes to allow primer annealing. To each tube the following was added in sequence: 4 µL CyScript buffer, 2 µL 0.1 M dithiothreitol, 1 µL dUTP nucleotide mix, 1 µL Cy5-labeled dUTP, and 1 µL CyScript reverse transcriptase. The reactions were then incubated for 90 minutes at 42 °C. 2 µL 2.5 M sodium hydroxide was added, and reactions were incubated for 15 minutes at 37 °C to hydrolyze the RNA template. Reactions were neutralized by adding 10 µL 2 M HEPES.

All six reactions were combined, and 1 µL removed to determine A_{260}. Reactions were then re-aliquotted into six equal parts and purified with each of the methods listed above.

Dilutions for spectrophotometer scanning were made using 10 mM Tris, 140 mM sodium chloride, pH 8.

Visualizing Excess Dye in cDNA Labeling Reactions on Slide Gels by Agarose Gel Electrophoresis

- Dye blob from excess CyDye apparent in lane of unpurified material
- Lanes of purified materials indicate no residual dye
- Better product recovery is apparent for RapXtract purified product

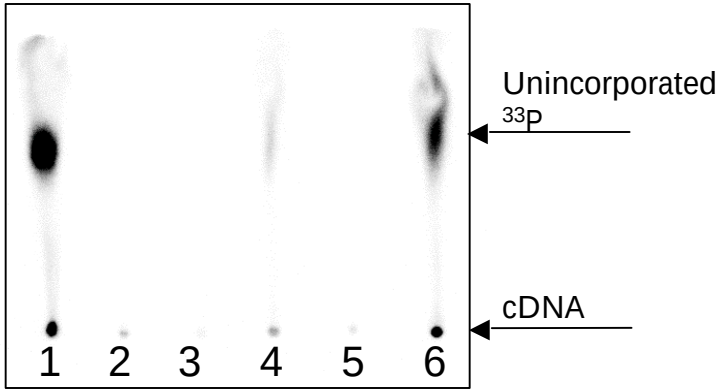
Visualizing Excess Dye in cDNA Labeling Reactions by Agarose Gel Electrophoresis

Reactions were prepared using a Cyscribe First-Strand cDNA Labelling Module (Amersham Pharmacia) and mouse embryo mRNA (Ambion). Three reactions were prepared in sterile 0.2 mL PCR tubes into which was added 1 µg mouse embryo mRNA, 1 µL random nonamers, 1 µL anchored oligo (dT), and 7 µL sterile water. The reactions were incubated for 5 minutes at 70 °C then cooled to room temperature for 10 minutes to allow the primers to anneal. To each tube was added in order: 4 µL CyScript buffer, 2 µL 0.1 M dithiothreitol, 1 µL dUTP nucleotide mix, 1 µL Cy3-labeled dUTP, and 1 µL CyScript reverse transcriptase. The reactions were then incubated for 90 minutes at 42 °C. 2 µL 2.5 M sodium hydroxide was added, and the reactions were incubated for 15 minutes at 37 °C to hydrolyze the RNA template. Reactions were then neutralized by adding 10 µL 2M HEPES and subjected to (1) column purification, (2) RapXtract Fluorescent cDNA Purification Kit, 600 µg particles, 30 seconds, and (3) no purification.

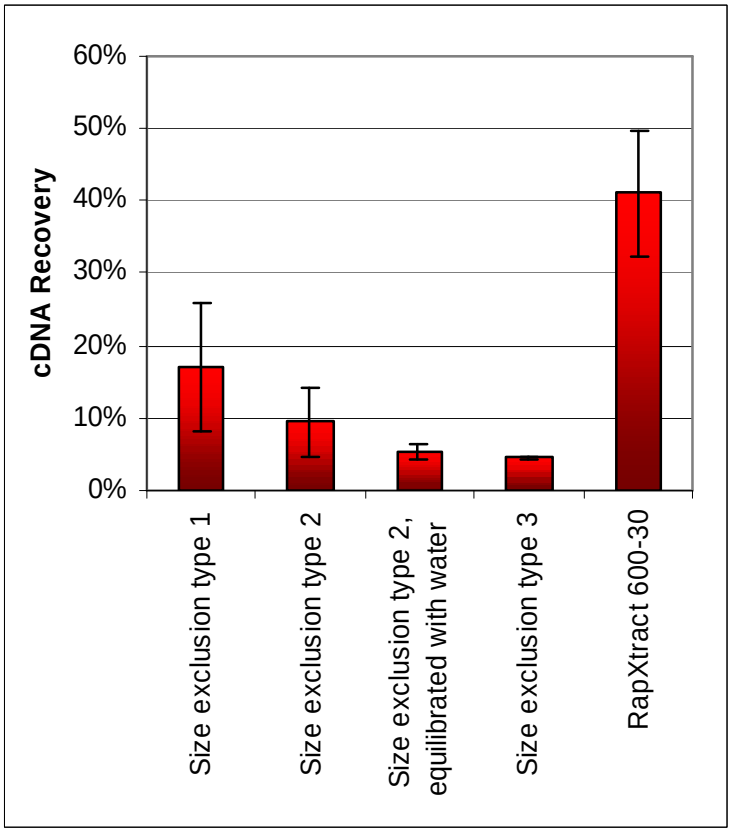
Agarose gel (2%, 5 mL) was poured on 3 X 1microscope slide and polymerized for 15 minutes. 1 µL of a 1:4 dilution in 30% glycerol in ddH_2O was loaded and run at 150 kV for 25 minutes. The gel was dried on blotting paper in gel dryer at 65 °C for 25-30 minutes, then transferred from the paper to the slide using 40% glycerol in ddH_2O. Some gels required additional soaking in the glycerol solution to remove paper fibers before pasting onto the slide.

The slide was then loaded onto a ScanArray L*ite* laser scanner, and scanned at 550 nm, 80 % laser output and PMT gain, 50 µm resolution.

Determination of Product Recovery by ^{33}P Incorporation



1. Unpurified
2. Size exclusion type 1
3. Size exclusion type 2
4. Size exclusion type 2, equilibrated with water
5. Size exclusion type 3
6. RapXtract, 600 µg particles, 30 seconds



Determination of Product Recovery by ^{33}P Incorporation

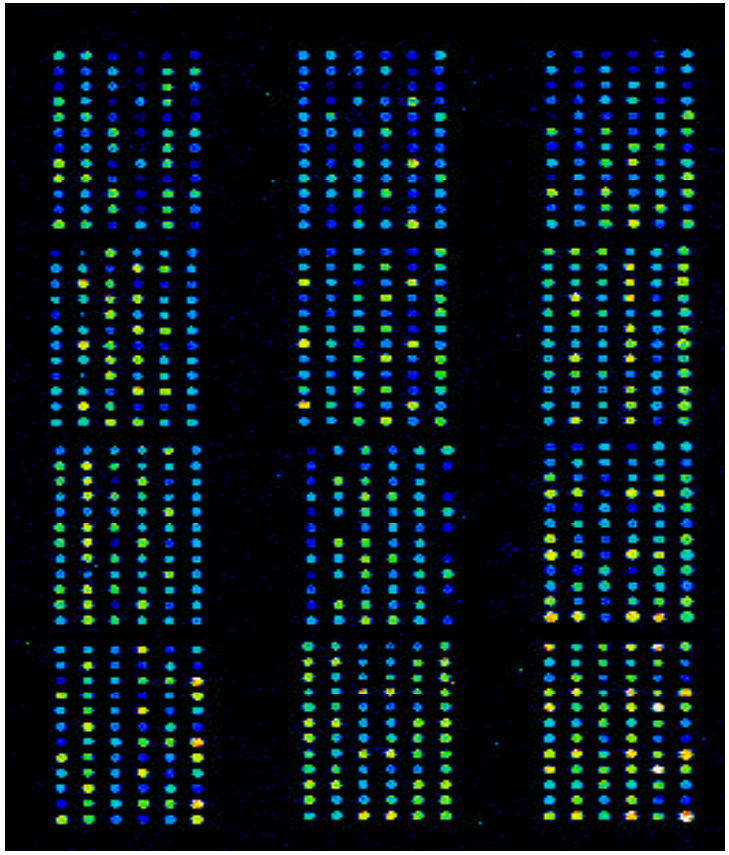
Reactions were prepared using a CyScribe First Strand Labelling Module (Amersham Pharmacia) and mouse brain mRNA (Ambion). Each of the six reactions was set up in sterile 0.2 mL PCR tubes into which was added 1 µg mRNA, 1 µL random nonamers, 1 µL anchored oligo (dT), and 7 µL sterile water. The reactions were incubated for 5 minutes at 70 °C then cooled to room temperature for 10 minutes to allow the primers to anneal. To each tube, the following was added in sequence: 4 µL CyScript buffer, 2 µL 0.1 M Dithiothreitol, 1 µL dUTP nucleotide mix, 1 µL Cy3-labeled dUTP, and 1 µL CyScript reverse transcriptase. The reactions were then incubated for 90 minutes at 42 °C. 2 µL 2.5 M sodium hydroxide was added, and reactions were incubated for 15 minutes at 37 °C to hydrolyze the RNA template. Reactions were neutralized by adding 10 µL 2 M HEPES free acid.

All reactions were pooled and re-aliquotted before purification to ensure equal starting amounts of product, then purified according to the manufacturer's instructions for the methods listed.

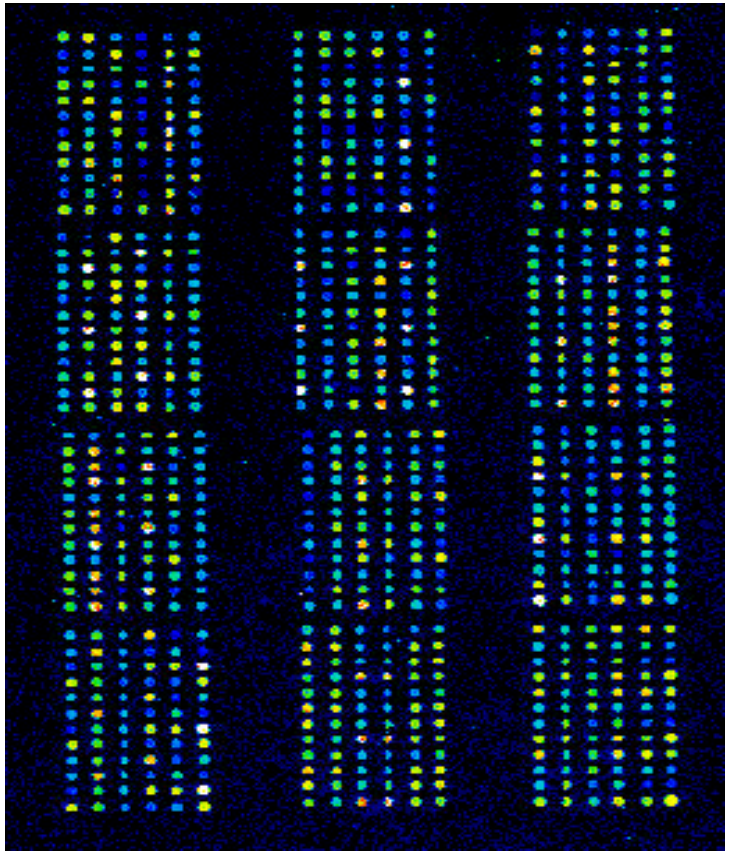
The thin layer chromatography was performed on cellulose polyethylene imine chromatography plates (Selecto Scientific). The reactions were spotted with 0.5 µL sample and run for 90 minutes using 1 M potassium phosphate, pH 9.3 as the mobile phase. The plate was exposed for 5-7 days in a phosphoimager (Bio-Rad), then analyzed using Bio-Rad MultiAnalyst software version 1.1.

Quantitation was done by comparing the recovered amount of cDNA in each individual sample to the amount of cDNA in the unpurified sample.

RapXtract Purified cDNA Targets Successfully Hybridize to Oligonucleotide Probes



RapXtract Purified



Column Purified

Arrays hybridized utilizing 100% of the recovered cDNA from the column-purified reaction, and only 33% of the recovered data from the RapXtract-purified reaction.

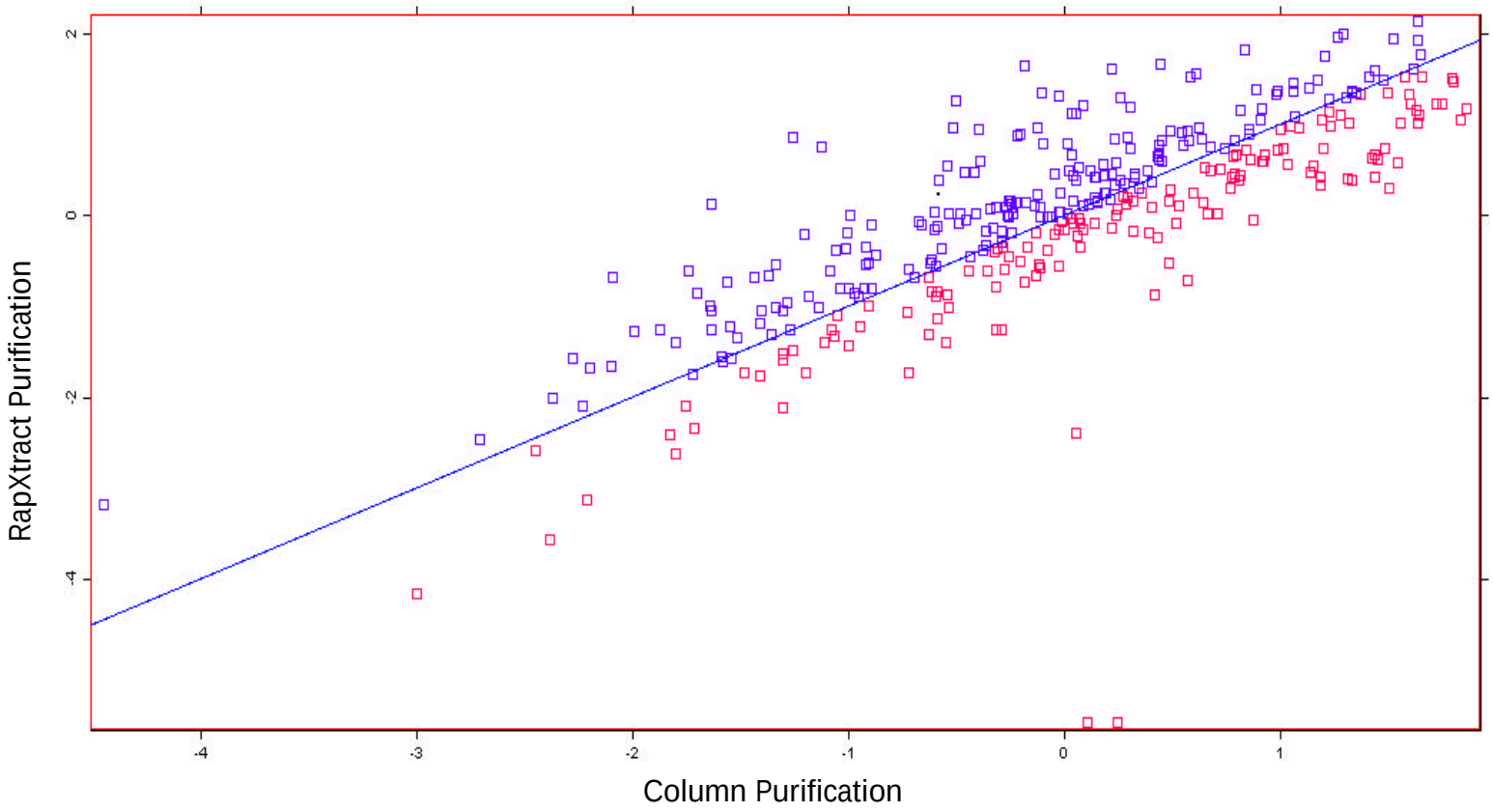
RapXtract Purified cDNA Targets Successfully Hybridize to Oligonucleotide Probes

Reactions were prepared using a CyScribe First Strand Labelling Module (Amersham Pharmacia) and *Saccharomyces cerevisiae* mRNA (Clontech). The reactions were prepared in sterile 0.2 mL PCR tubes into which was added 1 µg mRNA, 1 µL random nonamers, 1 µL anchored oligo (dT), and 7 µL sterile water. The reactions were incubated for 5 minutes at 70 °C then cooled to room temperature for 10 minutes to allow the primers to anneal. To each tube the following was added in sequence: 4 µL CyScript buffer, 2 µL 0.1 M dithiothreitol, 1 µL dUTP nucleotide mix, 1 µL Cy3-labeled dUTP, and 1 µL CyScript reverse transcriptase. The reactions were then incubated for 90 minutes at 42 °C. 2 µL 2.5 M sodium hydroxide was added, and reactions were incubated for 15 minutes at 37 °C to hydrolyze the RNA template. Reactions were neutralized by adding 10 µL 2M HEPES. The reactions were then combined and re-aliquotted into two equal parts. One reaction was purified using the RapXtract Fluorescent cDNA Purification Kit, 600 µg particles, with agitation for 30 seconds, and the other using the size exclusion column recommended in the CyScribe Module.

The purified Cy-labeled cDNA targets were quantified by A_{260}, and total recovery calculated. The RapXtract purification resulted in a 40% recovery, and the column recovered 15% of the original product. The arrays would be hybridized with 500 ng of cDNA; all of the column purified reaction, and 33% of the RapXtract purified reaction. The 500 ng of cDNA was added to 390 mM sodium chloride, 39 mM sodium citrate, 10 µg poly dA, and 0.2% (w/v) sodium dodecyl sulfate. The cDNA was denatured at 95°C for 2 minutes then spun briefly to cool. The cDNA was then hybridized to a Yeast Collage array (Operon) as follows: a cover slip was placed on a pre-spotted array, and each reaction added under the cover slip by capillary action. The slides were placed into hybridization chambers with 15 µL 45 mM sodium chloride, 4.5 mM sodium citrate in each humidity well. The chamber was sealed, wrapped in plastic wrap and placed in a 67 °C water bath for 12 hours.

After hybridization, the slide was washed for 5 minutes in each of (1) 150 mM sodium chloride, 15 mM sodium citrate , 0.03 % (w/v) sodium dodecyl sulfate, pH 8, (2) 30 mM sodium chloride, 3 mM sodium citrate, pH 8 and (3) 75 µM sodium chloride, 7.5 µM sodium citrate, pH 8 . Each slide was then spun dry in a vacuum extractor and visualized on a Packard ScanArray *Lite* scanner at a laser output of 80 %, a PMT gain of 80 %, and a resolution of 50 µm.

The RapXtract Kit Affords Strong Signal Intensities While Recovering More Sample



A scatterplot generated by BioDiscovery ImaGene v4.1 of the signal intensities from the arrays pictured above shows good correlation between the two purification methods. These data taken from 100% of the recovered cDNA from the column-purified reaction (red), and only 33% of the recovered data from the RapXtract-purified reaction (blue).

Data courtesy of BioDiscovery, Inc.

www.BioDiscovery.com

Conclusions

The RapXtract Fluorescent cDNA Purification kit is:

- Effective
  - Reverse purification technique efficiently binds and removes excess dye-labeled nucleotides, NOT labeled product
  - Allows for recovery of substantially more cDNA than other methods evaluated
  - Increased purified cDNA recovery allows replicates to be performed with the same sample, increasing data reliability
  - Increased recovery allows for the possibility of improved detection of rare message
- Rapid & Easy
  - 5-step protocol with brief 30-second agitation
  - Rapidly optimized to remove dye while retaining maximum cDNA recovery
  - Compatible with a variety of robotic platforms for high-throughput laboratories

Acknowledgements

The authors would like to gratefully acknowledge Alexander Kuklin, Shelley Cao, and Jeff Smith from BioDiscovery for their assistance in the microarray image analysis and quantification.