

Analyzing RNA with Microarrays

Overview

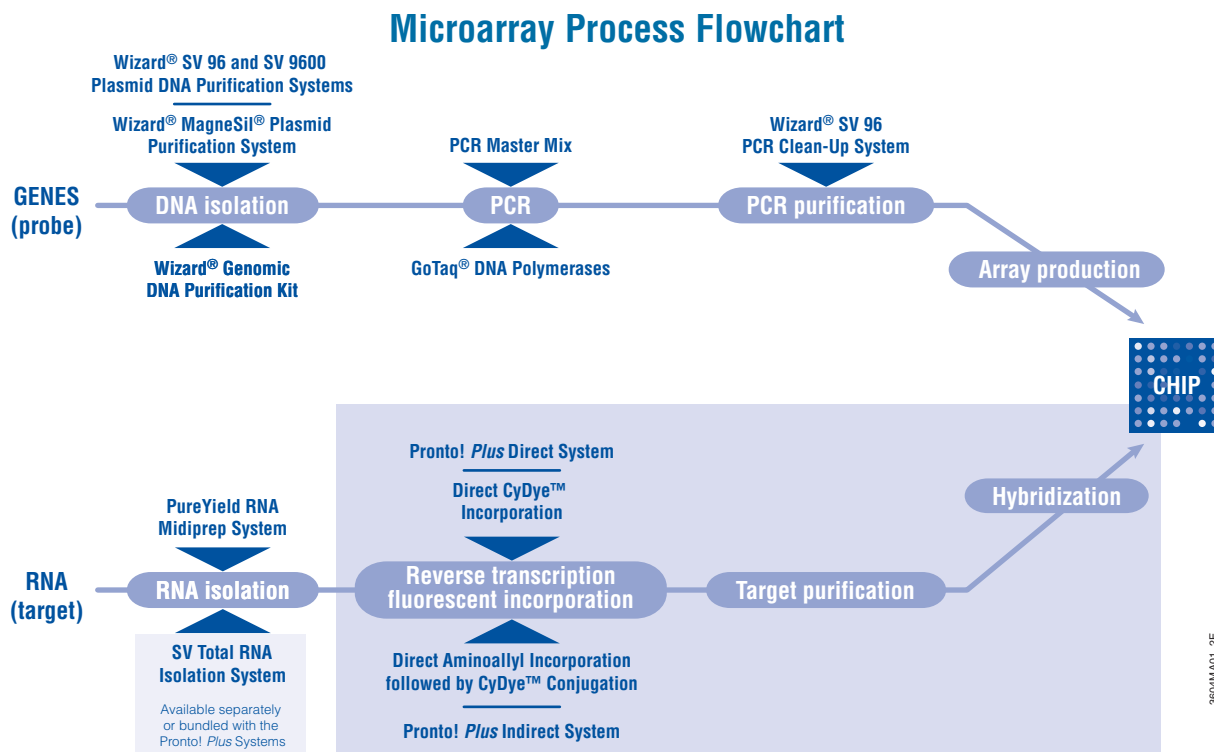
The use of DNA microarrays for transcriptional profiling remains the main application of this powerful technology. Genome-wide monitoring of gene transcription has become a standard tool in research laboratories.

Biologists increasingly use DNA microarrays to understand processes as diverse as the mechanisms of toxicity, embryogenesis and disease progression. The quality and consistency of the reagents and protocols used to process these arrays have a substantial impact on the reliability of the data generated.

Microarray analysis can be divided into two main steps: probe (gene) production and target (cDNA) production. Probe production is generally a high-throughput process involving plasmid isolation from a library of 10,000 or more clones, followed by PCR amplification of the library, purification of the PCR product and printing the purified products onto an array substrate. Oligonucleotides can also be printed on slides and used as probes.

Target production, also critical to microarray success, is commonly lower throughput. Generally, two to four targets are produced from the control and experimental RNAs. The cDNA is labeled through direct or indirect labeling methods. For direct labeling, the RNA is reverse transcribed in the presence of fluorescent dNTPs. For indirect labeling, the RNA is transcribed in the presence of aminoallyl dNTPs, which are subsequently labeled with reactive fluorescent NHS-ester. The resulting cDNA is purified and hybridized to the microarray.

Probes are the known entity in microarray research. Specific sequences are immobilized to a surface and reacted with labeled cDNA targets. A signal resulting from hybridization of the labeled target with the specific, immobilized probe identifies which RNAs are present in the unknown target sample.



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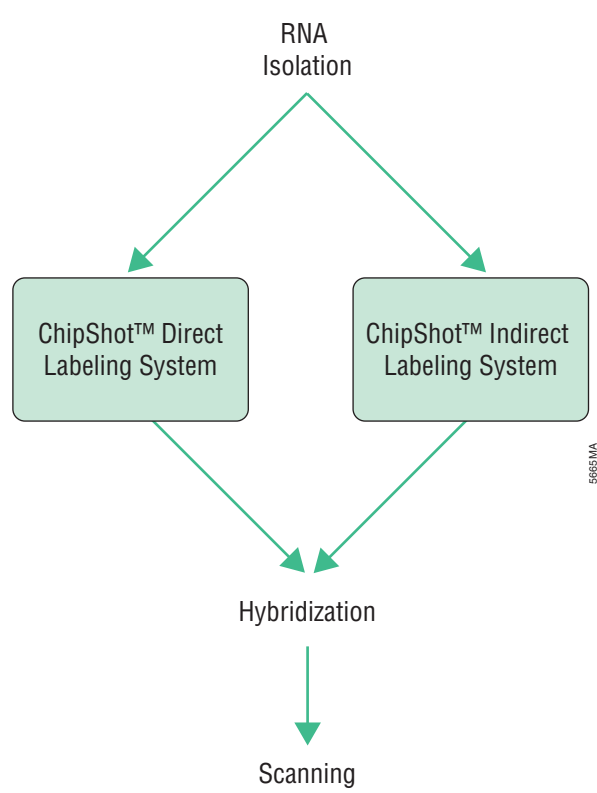
Target Production and Hybridization

Promega Corporation and Corning Incorporated have developed two new systems that provide fully integrated sets of reagents for use in microarray analysis. The Pronto![™] Plus Direct System provides reagents for generating fluorescent cDNA by direct incorporation of CyDye[™] labeled dCTP (ChipShot[™] Direct Labeling System). The Pronto![™] Plus Indirect System allows users to generate fluorescent cDNA via synthesis of aminoallyl-modified cDNA, followed by post-synthesis conjugation of CyDye[™] NHS ester (ChipShot[™] Indirect Labeling System). Both systems provide reagents and protocols specifically optimized for microarray application and include components for cDNA labeling and cDNA cleanup (ChipShot[™] Membrane Clean-Up System).

Optimized reagents for arrays printed on Corning UltraGAPS[™] or Epoxide Coated Slides

Pronto![™] Plus Indirect System Protocol available at: www.promega.com/tbs/tm261.tm261.html
System with SV Total RNA Isolation System: Cat. # 40075
System alone: Cat. # 40076
Sufficient reagents for 25 reaction

Pronto![™] Plus Direct System Protocol available at: www.promega.com/tbs/tm243.tm243.html
System with SV Total RNA Isolation System: Cat. # 40055
System alone: Cat. # 40056
Sufficient reagents for 25 reaction



Explore the Pronto![™] Plus Systems more fully at: www.prontosystems.com

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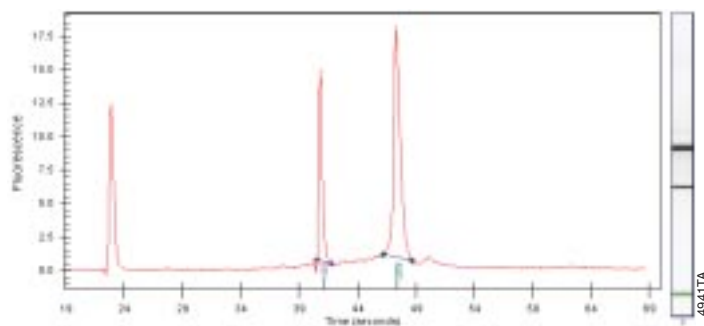
RNA Purification

All target production begins with RNA isolation. High-quality RNA is important for successful target production. Versions of both the Pronto![™] Plus Direct and Indirect Systems are available that include the SV Total RNA Isolation System. The affinity purification method eliminates the need for phenol:chloroform extractions and is compatible with a wide variety of sample types. A single SV Total RNA Isolation procedure routinely provides sufficient total RNA for multiple cDNA labeling reactions.

If you need to isolate total RNA from a larger sample than the miniprep scale offered by the SV Total RNA Isolation System, the PureYield[™] RNA Midiprep System also produces high quality RNA for use in microarray analysis.

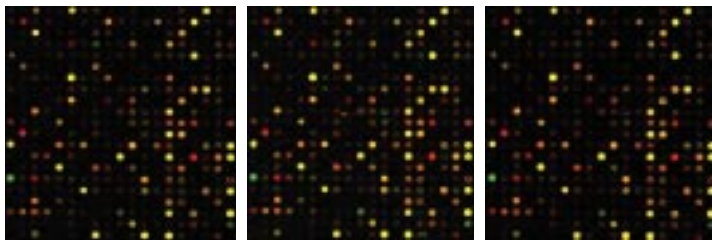
SV Total RNA Isolation System for:
up to 5×10^6 cultured cells
up to 60mg tissue

PureYield RNA Midiprep System for:
up to 5×10^7 cultured cells
up to 300mg tissue



Purity and integrity of total RNA isolated using the SV Total RNA Isolation System. Total RNA was isolated from 2×10^6 293T cultured cells as described in the Pronto![™] Plus Direct and Pronto![™] Plus Indirect Systems Technical Manuals (#TM243 and #TM261, respectively). A small aliquot was run on the Agilent Bioanalyzer.

See Chapter 2 for more information on both the SV Total RNA Isolation and PureYield[™] RNA Midiprep Systems

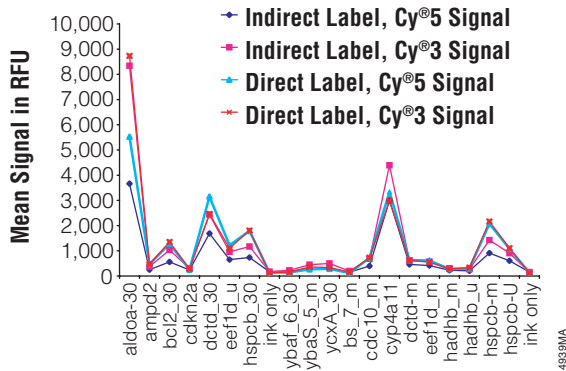


Reproducible microarray analysis with total RNA purified with the PureYield[™] RNA Midiprep System. Three separate Total RNA isolations were performed from 5×10^7 HEK 293T cells and 5×10^7 HeLa cells. HEK 293T RNA was labeled with CyDye[™]3 and HeLa RNA was labeled with CyDye[™]5 using the Pronto![™] Plus Direct System. Labeled cDNA was hybridized to 4K human cancer arrays (provided by Corning[®] Incorporated). Each panel above shows the results for subgrids from these 3 arrays. Reproducibility was assessed by determining the coefficient of variance for 4,000 features from the three arrays. Interslide %CV was 6.6%, indicating a high degree of reproducibility. Arrays were scanned using an Axon GenePix[®] 4000B scanner.

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cDNA Synthesis & Clean-Up

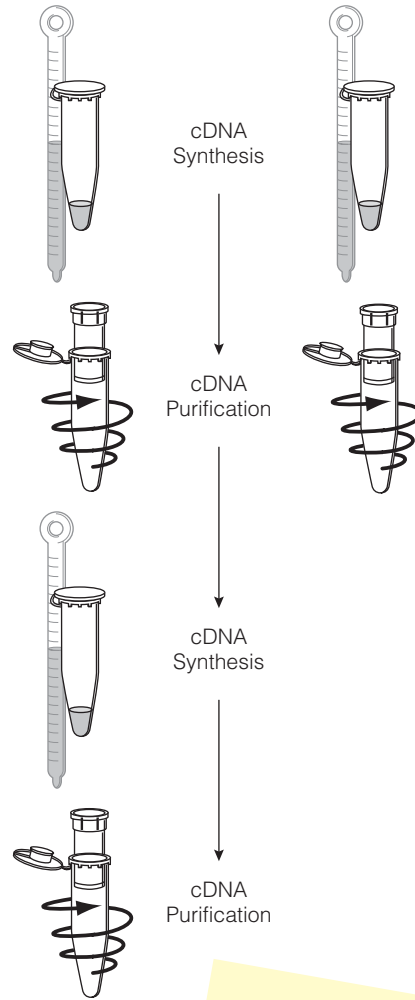
The Pronto!™ Plus Systems offer reagents and tested protocols to ensure premium results. These systems come with a certificate of analysis highlighting functional performance and a guarantee of quality and results. The Pronto! Plus Systems offer two methods for incorporation of dyes into your cDNA. As the name implies, the Direct System includes protocols and reagents to directly incorporate CyDye™3-dCTP or CyDye™5-dCTP into the cDNA using the ChipShot™ Direct Labeling System. The Indirect System first incorporates aminoallyl dUTP into the cDNA then the CyDye™3 or CyDye™5 is conjugated to the purified cDNA using the ChipShot™ Indirect Labeling System. Both systems use the ChipShot™ Membrane Clean-Up System to remove unincorporated reagents.



Correlation between hybridization patterns from Cy®-labeled cDNA synthesized using the ChipShot™ Direct and Indirect Labeling Systems on covalently bound short oligonucleotides. Twenty-one 30mer unmodified oligonucleotides, fifteen of which represented human genes, were purchased from commercial vendors and spotted onto Corning Epoxide Coated Slides. The arrays were hybridized with target cDNA dissolved in Pronto!™ Short-Oligo Hybridization Buffer. Arrays were scanned with an Axon 4000B scanner set at 100% power for both channels and PMT of 700 for Cy®3 and 800 for Cy®5.


ChipShot™ Indirect Labeling System

ChipShot™ Direct Labeling System



5666MA

Using aminoallyl dCTP followed by post-synthesis coupling of CyDye™ NHS esters can remove any bias from using direct CyDye™3- & CyDye™5-dCTP incorporation



For more information on the target cDNA labeling with the Pronto!™ Plus Systems, request:

Canel, C. and Nassif, N. (2005) Synthesizing fluorescent target cDNA for microarray-based transcriptional profiling: Considerations regarding primers and template RNA. *Promega Notes* 91, 10–12.

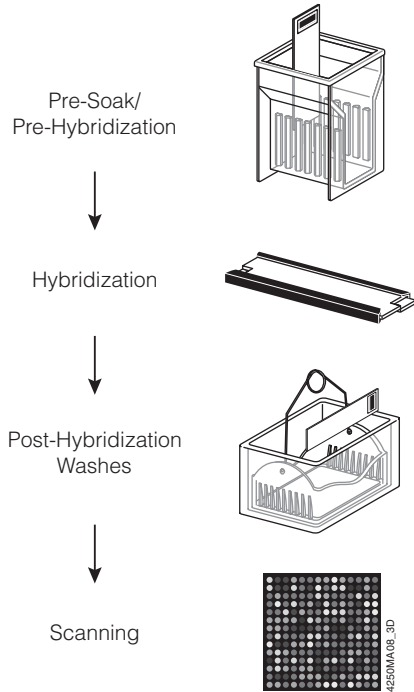
Available online at:
www.promega.com/pnotes/

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Target Hybridization

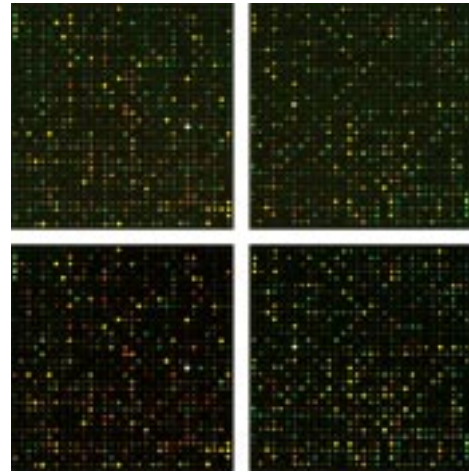
The Pronto![™] Hybridization Solutions are optimized to create the most favorable environment for hybridization between labeled cDNA and spotted DNA, while minimizing cross-hybridization. The Pronto! Hybridization and Wash Solutions combine to deliver a high level of reproducibility. Using these solutions, interslide CVs of 10% or less have been obtained. Two hybridization solutions are provided. The Pronto! Long Oligo/cDNA Hybridization Solution is provided for cDNA or long oligonucleotide arrays (>50mer). The Pronto! Short Oligo Hybridization Solution is provided for short oligonucleotide arrays (~30–50mers). The Wash Solutions are formulated to reduce background signal.

Includes specialized hybridization solutions for short oligos (30–50mers) & long oligos (>50mer) or cDNA

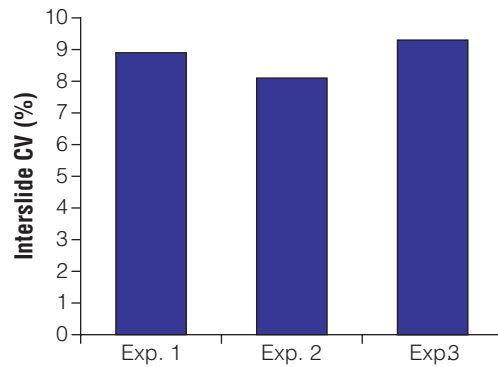


Improved reproducibility with the reagents optimized together by Promega and Corning!

A.



B.



Experimental reproducibility when using the Pronto![™] Plus Systems.

Panel A. Subgrids from four representative 4K human cancer arrays provided by Corning[®] Incorporated) are shown. Labeled cDNA was generated with the ChipShot[™] components of the Pronto![™] Plus System using 5µg of total RNA template. RNAs were from cultured HeLa cells (Cy[®]3) or 293T cells (Cy[®]5) isolated with the SV Total RNA Isolation System components. **Panel B.** Reproducibility was assessed by determining the coefficient of variation (%CV = Std. Dev/Mean) for 4,000 features from n = 3–4 arrays, in three independent experiments (including independent cDNA labelings). Interslide %CV for each experiment was below 10%, indicating a high degree of reproducibility both within and between experiments. Hybridization was performed using the Pronto![™] Universal Reagent System, as described in the *Pronto![™] Plus Systems Technical Manual #TM243*. Arrays were scanned using an Axon GenePix[®] 4000B microarray scanner and analyzed using the Axon GenePix[®] software.

Analyzing RNA with Microarrays

Probe Production

Custom arrays can be produced to meet a researcher's specific requirements, or researchers may choose to make the arrays in-house rather than purchase pre-made arrays that may not specifically meet their probe requirements. Typically, probes are first generated by RT-PCR and cloned into a plasmid vector. A bank of probes is then created and maintained in *E. coli* cells. Whenever the researcher needs to make a new array, the *E. coli* are grown, plasmids are isolated and the inserts are amplified with primers common to the plasmid backbone. The amplification products are purified and spotted onto the array.

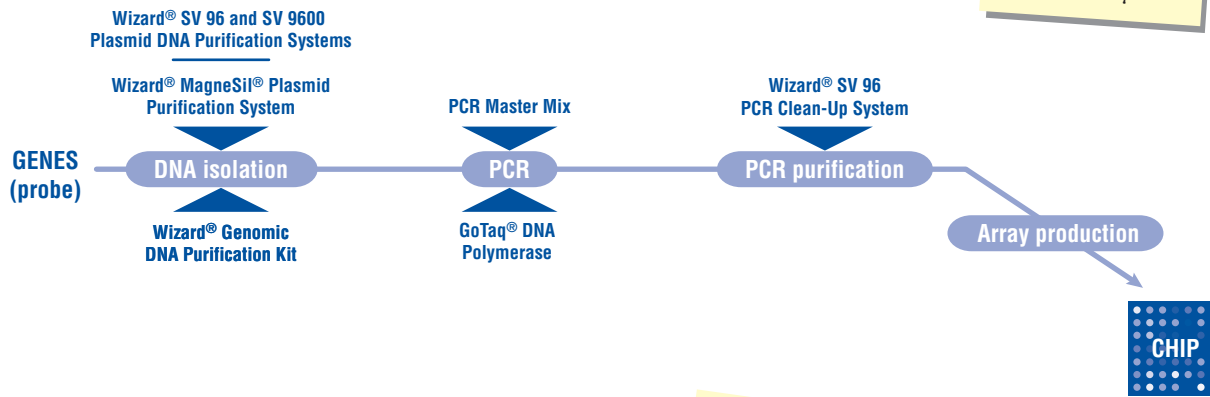
Automation is an obvious choice for processing all of the plasmids needed for amplification. Wizard® MagneSil® Plasmid Purification System and the Wizard® SV 96 Plasmid Purification Systems are automated solutions for this application. Promega PCR Master Mix is a high-throughput solution for mass amplification of thousands of samples, and the Wizard® SV 96 PCR Clean-Up System is an automated partner for purifying the amplification product.



3443CA06_1A

Automated purification of custom probe cDNAs stored in plasmids.

Automated purification of amplified products from custom probe library.



3864MA01_2C

Purified plasmids are a more reliable template for amplification than crude lysates.

Amplification of custom probe library.

Custom probe libraries could be stored in the pGEM®-T Easy Vector.

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Microarray Analysis Systems Target Production & Hybridization Systems	Size	Cat.#
Pronto! Plus Direct System ^(l,m) (with RNA Isolation)	25 reactions	40055
Pronto! Plus Direct System ^(l) (without RNA Isolation)	25 reactions	40056
Pronto! Plus Indirect System ^(l,m) (with RNA Isolation)	25 reactions	40075
Pronto! Plus Indirect System ^(l) (without RNA Isolation)	25 reactions	40076

Probe Production Products	Size	Cat.#
Wizard® SV 96 PCR Clean-Up System ^(m)	1 × 96	A9340
	4 × 96	A9341
	8 × 96	A9342
Wizard® MagneSil® Plasmid Purification System ^(n,o)	4 × 96 preps	A1630
	8 × 96 preps	A1631
Wizard® SV 96 Plasmid DNA Purification System ^{(p)*} (for manual or automated 96-well purifications)	1 × 96 preps	A2250
	5 × 96 preps	A2255
Wizard® Genomic DNA Purification Kit*	100 isolations × 300µl	A1120
GoTaq® Flexi DNA Polymerase ^{(q)*} (Catalog numbers vary for Europe.)	100 units	M8291
	500 units	M8295
	2,500 units	M8296
	5,000 units	M8297
	10,000 units	M8298
GoTaq® DNA Polymerase ^{(q)*} (Catalog numbers vary for Europe.)	100 units	M3001
	500 units	M3005
	2,500 units	M3008
PCR Master Mix ^{(q)*}	100 reactions	M7502
	1,000 reactions	M7505
pGEM®-T Easy Vector System I ^{(i,j)*} (you choose your competent cells)	20 reactions	A1360
pGEM®-T Easy Vector System II ^{(i,j)*} (JM109 Competent Cells provided)	20 reactions	A1380

*For Laboratory Use.

Target Production Products	Size	Cat.#
PureYield™ RNA Midiprep System ^(c,e)	10 preps	Z3740
	50 preps	Z3741
SV Total RNA Isolation System*	10 preps	Z3101
	50 preps	Z3100

*For Laboratory Use.