Promega

Technical Manual

ChipShot™ Indirect Labeling and Clean-Up System

INSTRUCTIONS FOR USE OF PRODUCT Z4000.

www.promega.com

PRINTED IN USA.
Revised 3/09

Part# TM287

ChipShot™ Indirect Labeling and Clean-Up System

All technical literature is available on the Internet at: www.promega.com/tbs/
Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

1. Description

The ChipShot™ Indirect Labeling[a] and Clean-Up System generates fluorescent cDNAs by synthesizing an aminoallyl cDNA and then conjugating the CyDye™ NHS ester to the cDNA. Either total RNA or poly(A)+ mRNA can be used with these systems. Resulting cDNAs are labeled to provide efficient microarray hybridization results. The ChipShot™ Indirect Labeling System includes a human Total RNA Positive Control to evaluate the performance of the synthesis, labeling and clean-up procedures. The ChipShot™ Membrane Clean-Up System produces highly purified, labeled cDNAs. Amplification of RNA is not required. All reagents are specifically optimized for microarray applications.

2. Product Components and Storage Conditions

Product	Size	Cat.#
ChipShot™ Indirect Labeling and Clean-Up System	25 reactions	Z4000

This system contains sufficient reagents to label and purify 25 cDNAs (24 experimental and 1 control). Includes:

- 1 ChipShot™ Indirect Labeling System (25 reactions; Z3604)
- 1 ChipShot™ Membrane Clean-Up System (50 columns; Z3613)
- 1 Protocol

Storage Conditions: Store the ChipShot™ Indirect Labeling System (Z3604) at –20°C, except for the RNase Solution, which should be stored at room temperature, and the Total RNA Positive Control, which should be stored at –70°C. Store the ChipShot™ Membrane Clean-Up System (Z3613) at room temperature.

3. ChipShot™ Indirect cDNA Labeling and Clean-Up Protocols

The quality and cleanliness of the starting RNA and the resulting cDNA are critical factors for successful use of arrays. We recommend that RNA quality be thoroughly checked before attempting to synthesize cDNA and that the labeled cDNA be purified and quantified using a spectrophotometer. Minimize the exposure of solutions containing fluorescent nucleotides to light to prevent photobleaching. CyDye™-labeled cDNA is stable at 4°C for several weeks.

For more information on RNA isolation, please see the *SV Total RNA Isolation System* (Cat.# Z3100) *Technical Manual* #TM048 or the *PureYield™ RNA Midiprep System Technical Manual* #TM279 (Cat.# Z3740, Z3741).

For information on mRNA isolation, please see the *PolyATtract® mRNA Isolation System* (Cat. Z5200, Z5420) *Technical Manual* #TM021.

Note: RNA prepared using these systems has been used with microarrays; see references 1–6.

Materials to Be Supplied by the User

- water bath or heating block, preheated to 70°C
- water bath or heating block, preheated to 42°C
- water bath or heating block, preheated to 37°C
- 80% ethanol, molecular biology grade
- 100mM sodium bicarbonate (pH 9.0)
- 4M hydroxylamine

3. ChipShot™ Indirect cDNA Labeling and Clean-Up Protocols (continued)

Materials to be Supplied by the User (continued)

- microcentrifuge
- spectrophotometer
- microcuvette
- CyDye™ Post-Labeling Reactive Dye Pack (GE Healthcare Bio-sciences Cat.# RPN5661)

Notes:

Prepare fresh sodium bicarbonate before proceeding to the purification step. Efficiency of the dye coupling may be affected if the sodium bicarbonate is not at pH 9.0.

Protect reactive dye packs from light at all times to prevent photobleaching.

Materials Supplied in the System

ChipShot™ Indirect Labeling System (Z3604)

- $MgCl_2$ (25mM)
- Oligo(dT) Primer
- Random Primers
- Aminoallyl-dNTP mix
- ChipShot™ Reverse Transcriptase 5X Reaction Buffer
- ChipShot™ Reverse Transcriptase
- Nuclease-Free Water
- Total RNA Positive Control
- RNase H
- RNase Solution (RNase A)

ChipShot™ Membrane Clean-Up System (Z3613)

- Binding Solution
- ChipShot™ Membrane Columns
- Elution Buffer
- Collection Tubes
- Sodium Acetate, 3M (pH 5.2)

Notes:

Never store the ChipShot™ Reverse Transcriptase at room temperature.

The Total RNA Positive Control is provided as a control for the labeling and clean-up procedures. It is not intended as a control for subsequent array hybridization.

3.A. Aminoallyl cDNA Synthesis Protocol for Total RNA

We recommend using both random primers and oligo(dT) for labeled cDNA synthesis. Using both random primers and oligo(dT) to synthesize cDNA from total RNA maximizes the coverage of transcribed sequences and their detection by hybridization to array probes representing upstream sequences. Using random primers also results in the synthesis of large amounts of ribosomal cDNA, which may cross-hybridize to related array probes and contribute nonspecific signal intensities. Cross-hybridization events can be detected and measured by including negative control probes (e.g., sequences that have no known homology with the target) in the arrays. Alternatively, the synthesis of cDNA from total RNA may be initiated using oligo(dT) alone, in which case the expected cDNA yield and frequency of incorporation (FOI) will correspond to those of reactions using mRNA.

See *Promega Notes* **91**, 10–12, for additional information on primers and template RNA (www.promega.com/pnotes/)

1. For each indirect synthesis reaction, assemble the following reagents in a microcentrifuge tube. Keep the reagents on ice, and mix the total RNA and primers as follows:

Total RNA or Total RNA Positive Control	5µg
Random Primers (3µg/µl)	1µl
Oligo(dT) Primer (2µg/µl)	1µl
Nuclease-Free Water to a total volume of	**20µl**

2. Incubate RNA/primer solution at 70°C for 10 minutes, then place on ice.
3. While the RNA/primer solution is incubating at 70°C, prepare extension mix as follows:

Component	
ChipShot™ Reverse Transcriptase 5X Reaction Buffer	8µl
$MgCl_2$, 25mM	4.8µl
Aminoallyl-dNTP mix	4µl
ChipShot™ Reverse Transcriptase	3.2µl
Nuclease-Free Water to a final volume of	**20µl**

4. Add the entire 20µl extension mix to each tube of RNA/primer solution, (40µl total volume) vortex, spin briefly and incubate at room temperature (22–25°C) for 10 minutes.
5. Incubate at 42°C for 2 hours.
6. Add 1.0µl RNase H and 0.35µl RNase Solution to each cDNA-synthesis reaction. Mix gently and incubate at 37°C for 15 minutes.

   Note: To obtain maximum day-to-day reproducibility, incubate the tubes in a thermal cycler.

3.B. Aminoallyl cDNA Synthesis Protocol for mRNA

1. For each indirect synthesis reaction, assemble the following reagents in a microcentrifuge tube. Keep the reagents on ice, and mix the mRNA and primers as follows:

mRNA	1.5µg
Random Primers (3µg/µl)	1µl
Nuclease-Free Water to a total volume of	**20µl**

2. Incubate RNA/primer solution at 70°C for 10 minutes, then place on ice.

3. While the RNA/primer solution is incubating at 70°C, prepare extension mix:

Component	
ChipShot™ Reverse Transcriptase 5X Reaction Buffer	8µl
$MgCl_2$, 25mM	4.8µl
Aminoallyl dNTP mix	4µl
ChipShot™ Reverse Transcriptase	3.2µl
Nuclease-Free Water to a final volume of	**20µl**

4. Add the entire 20µl extension mix to each RNA/primer solution (40µl total volume), vortex, spin briefly and incubate at room temperature (22–25°C) for 10 minutes.

5 Incubate at 42°C for 2 hours.

6. Add 1.0µl RNase H and 0.35µl RNase Solution to each cDNA-synthesis reaction. Mix gently and incubate at 37°C for 15 minutes.

   Note: To obtain maximum day-to-day reproducibility, incubate tubes in a thermal cycler.

3.C. Purifying Aminoallyl cDNA

1. To 40µl of the synthesized cDNA, add the following components in the order listed:

Sodium Acetate, 3M (pH 5.2)	4µl
Binding Solution	225µl

   Note: Prepare fresh sodium bicarbonate before proceeding to the purification step. Efficiency of the dye coupling may be affected if the sodium bicarbonate is not at pH 9.0.

2. Vortex gently for 5–10 seconds to mix.
3. Place a ChipShot™ Membrane Column into a Collection Tube. Apply solution to the column and cap the tube.
4. Let the column stand at room temperature for 5 minutes, and then spin at 10,000 × *g* for 1 minute.
5. Discard the column flowthrough.
6. Wash column with 500µl of 80% ethanol, cap the tube, and centrifuge at 10,000 × *g* for 1 minute.
7. Discard the column flowthrough.
8. Repeat Steps 6 and 7 twice for a total of 3 washes.
9. Centrifuge column at 10,000 × *g* for 1 minute to remove traces of ethanol.
10. Place column in a clean Collection Tube (provided).
11. To elute purified cDNA, add 65µl of 100mM sodium bicarbonate (pH 9.0), and let the column stand at room temperature for 1 minute. Centrifuge at 10,000 x *g* for 1 minute. Approximately 60µl will be recovered after centrifugation. Proceed immediately to the dye conjugation.

3.D. Conjugation of CyDye™ NHS Ester to Aminoallyl cDNA

Note: Protect the reactive dye packs from light at all times to prevent photobleaching.

1. Add 60µl of the eluted aminoallyl cDNA to one dried aliquot of CyDye™ NHS ester.
2. Vortex gently for 5–10 seconds to ensure that the CyDye™ NHS ester is thoroughly resuspended.
3. Incubate at ambient temperature (22–25°C) for 1 hour, protected from light.

   Note: To obtain maximum day-to-day reproducibility, use a thermal cycler set to 23°C for this step.
4. Add 20µl of 4M hydroxylamine (80µl total volume).
5. Vortex gently to mix.
6. Incubate at ambient temperature (22–25°C) for 15 minutes, protected from light. Proceed immediately to final purification.

3.E. Purifying CyDye™-Labeled cDNA

1. To the 80µl of the synthesized, labeled cDNA add the following components in the order listed:

Sodium Acetate, 3.0M (pH 5.2)	8µl
Binding Solution	440µl

Note: Protect the labeled sample from light as much as possible.

2. Vortex gently for 5–10 seconds to mix.
3. Place a ChipShot™ Membrane Column into a Collection Tube. Apply solution to the column and cap the tube.
4. Let column stand at room temperature for 5 minutes and then spin at 10,000 × *g* for 1 minute.
5. Discard the column flowthrough.
6. Wash column with 500µl of 80% ethanol, cap the tube and centrifuge at 10,000 × *g* for 1 minute.
7. Discard the column flowthrough.
8. Repeat Steps 6 and 7 twice, for a total of 3 washes.
9. Centrifuge column at 10,000 × *g* for 1 minute to remove traces of ethanol.
10. Place column in a clean Collection Tube.
11. To elute labeled cDNA: Add 60µl Elution Buffer, let column stand for 1 minute, then centrifuge at 10,000 × *g* for 1 minute. Eluted cDNA can be stored in a light-proof container at 4°C for several weeks.

3.E. Purifying CyDye™-Labeled cDNA (continued)

12. Quantitate absorbance at 260, 550 and 650nm, and calculate frequency of incorporation.

    Note: Absorbance readings should be taken using undiluted cDNA directly in a microcuvette. Diluting the cDNA prior to reading the absorbance may give inaccurate readings due to low concentration. The cDNA used for spectrophotometry should be recovered for use in the hybridization reaction. Clean the cuvette thoroughly between samples to prevent cross-contamination.

    Frequency of incorporation (FOI) is defined as the number of CyDye™-labeled nucleotides per 1,000 nucleotides of cDNA. Best results are obtained with cDNAs having an FOI as recommended in Table 1. The FOI can be calculated as follows:

$$\text{FOI} = \frac{\text{pmol of dye incorporated} \times 324.5}{\text{ng of cDNA}}$$

$$\text{Amount of labeled cDNA (ng)} = A_{260} \times 37 \times \text{total volume } (\mu\text{l})$$

$$\text{For Cy}^{®}3\text{: pmol of dye incorporated} = \frac{A_{550} \text{ x total volume } (\mu\text{l})}{0.15}$$

$$\text{For Cy}^{®}5\text{: pmol of dye incorporated} = \frac{A_{650} \times \text{total volume } (\mu\text{l})}{0.25}$$

Note: These equations were generated using the following constants: Average Molar Mass of dNTP = 324.5; one A_{260} unit of single-stranded DNA = 37µg/ml; extinction coefficient of Cy®3 = $150{,}000M^{-1}cm^{-1}$ at 550nm; extinction coefficient of Cy®5 = $250{,}000M^{-1}cm^{-1}$ at 650 nm.

Best results are obtained with cDNAs that fall into the following ranges:

Table 1. Expected cDNA Yields and FOI.

5µg total RNA	**ng yield**	**pmol**	**FOI**
Cy®3	1,750–5,000	100–300	12–35
Cy®5	1,750–5,000	100–320	12–40
1.5µg mRNA	**ng yield**	**pmol**	**FOI**
Cy®3	500–1,500	50–100	12–25
Cy®5	500–1,500	75–130	15–30

4. Preparing cDNA for Hybridization

The labeled cDNA is suitable for use with many different hybridization solutions and protocols. Here are some suggestions for preparing the cDNA for hybridization.

1. Dry the appropriate amount of each dye-labeled cDNA using a speed-vacuum concentrator.

   Calculating the Volume of Hybridization Solution to Use

   The volume of hybridization solution needed depends on the size of the printed area and cover glass. We recommend using a glass coverslip, such as Corning 2870 and 2940 cover glass product lines. Use 2.5–3.5µl of hybridization solution per cm^2 of surface area. This range of volume will accommodate differences in humidity conditions and hybridization times. The fluorescence strength required to achieve high levels of sensitivity and broad dynamic range depends on the template used to synthesize the CyDye™-cDNA.

Table 2. Examples of Recommended Hybridization Solution Volumes and Amount of cDNA Based on Varying Coverslip Sizes.

Coverslip Size	Surface Area (cm^2)	Volume of Hybridization Solution	Amount of Labeled cDNA from Total RNA (per slide)	Amount of Labeled cDNA from mRNA (per slide)
22 × 22mm	4.84	12–17µl	12–17pmol	3–4pmol
24 × 40mm	9.60	24–33µl	24–33pmol	6–8pmol
24 × 60mm	14.4	36–50µl	36–50pmol	9–12pmol

*If doing a two-color hybridization, combine the recommended amount of both dye-labeled cDNAs. For example, for a 22 × 22mm coverslip with a two-color hybridization using total RNA-derived cDNA, combine 12–17pmol of Cy®3-labeled and 12–17pmol of Cy®5-labeled cDNAs.

   Calculating the Amount of cDNA to Use

   Total RNA. For CyDye™-labeled cDNA made from total RNA, dry down an amount of cDNA containing 1.0pmol labeled nucleotides per microliter of hybridization solution that will be used per dye.

   mRNA. For CyDye™-labeled cDNA made from mRNA, dry down an amount of cDNA containing 0.25pmol of labeled cDNA per microliter of hybridization solution that will be used per dye.

2. Resuspend the labeled cDNA in the required volume of hybridization solution.

3. Incubate the labeled cDNA solution at 95°C for 5 minutes, protecting samples from light.

4. Centrifuge the cDNA at 13,500 × *g* for 2 minutes to collect condensation. Do not place the solution on ice because this will cause some components to precipitate.

5. Apply the labeled cDNA to the surface of the printed slide and place the coverglass on the array.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

5.A. Troubleshooting, cDNA Labeling and Clean-Up

Symptoms	Possible Causes and Comments
Low cDNA yield	RNA degradation/RNase introduced during handling: • Use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol-barrier tips and gloves. • Ensure that reagents, tips and tubes are kept RNase-free by using sterile technique. • RNA storage conditions are important. Store at –70°C. Keep RNA target in single-use aliquots to minimize freeze-thaw cycles. Once thawed, keep RNA on ice. • Use RNasin® Ribonuclease Inhibitor (Cat.# N2511, N2515) to inhibit degradation of target during cDNA synthesis (40 units/40µl reaction). • Use DEPC-treated glassware and solutions when manipulating and storing RNA. Wear gloves at all times. RNases introduced after elution will degrade the RNA. • Work quickly during sample preparation.
	cDNA degradation. DNase contamination from the RNA preparation may be digesting the cDNA.
	Inhibitors may be present in the RNA preparation. Inhibitors such as SDS, EDTA, polysaccharides, heparine, guanidine isothiocyanate or other salts may carry over from some RNA preparations and interfere with cDNA labeling. To determine if the experimental RNA preparation contains an inhibitor, set up a spiking experiment by adding control RNA to experimental RNA and assess inhibition of cDNA synthesis.
	Incorrect primer:RNA ratio. Confirm RNA conc centration and use the recommended primer concentration and the recommended amount of template RNA. Mix well.
	Insufficient reverse transcriptase activity. Use the recommended amount of ChipShot™ Reverse Transcriptase in the labeling reaction.
	Insufficient mixing of reagents. Vortex all reagents (except enzymes) before use.

5.A. Troubleshooting, cDNA Labeling and Clean-Up (continued)

Symptoms	Possible Causes and Comments
Low yield of cDNA (continued)	Poor elution of the cDNA from the ChipShot™ membrane. Perform recommended ambient temperature incubation of the elution buffer with the cDNA/ChipShot™ Membrane before the elution spin.
cDNA yield acceptable, but Frequency of Incorporation (FOI) is low	Insufficient RNase activity. Residual RNA present in the cDNA sample can result in an A_{260} absorbance reading that does not accurately represent the amount of labeled cDNA present. Perform the recommended treatment step before ChipShot™ Membrane clean-up. Refer to Table 1 for expected yield ratios.
cDNA yield acceptable, but FOI is high	Incomplete removal of CyDye™ esters. Follow the cDNA purification protocol (Section 3.C).

5.B. General Suggestions for Troubleshooting Hybridization

Based on our experience, here are some suggestions for troubleshooting hybridization.

Symptoms	Possible Causes and Comments
Spots appear smeared as comets	DNA concentration is too high. Print using a lower DNA concentration. Make note of UV energy required for cDNA and long oligonucleotides from the slide manufacturer.
	Coverslip slid into place over labeled cDNA solution. Drop coverslip squarely on the array. Do not slide coverslip across the array. Practice coverslip placement with water and plain slide before performing on an array.
Low fluorescent signal	Too little or too much incorporation of fluorescent dye in sample probe. Check the FOI of cDNA before using for hybridization. If the appropriate dye incorporation is obtained, less labeled cDNA is used, and the highest signals and lowest background are attained (Table 1).
	Degradation of the fluorescent dye in the cDNA. Keep the labeled dyes and labeled cDNA protected from light.

5.B. General Suggestions for Troubleshooting Hybridization (continued)

Symptoms	Possible Causes and Comments
Fluorescent spots in the background	Powder from gloves can contaminate slides during printing or hybridization. Use powder-free gloves during the microarray process.
	Dust has settled on the slide. Work in a dust-free hood or environment.
Uneven or high background	Air bubbles trapped under coverslip during hybridization may prevent labeled cDNA from contacting the arrayed nucleic acid. Small bubbles dissipate during hybridization; no action necessary. Hold slide and coverslip so that larger bubbles rise to the top of the slide and escape from under the coverslip. Practice coverslip placement with water and a plain slide before performing on an array.
	Incomplete washing. Use clean washing vessels for each run. Washing steps are critical for low backgrounds. Slides should not be allowed to dry until the final wash step.
	Incomplete or improper drying. Immediately blow-dry or spin-dry slides after last wash step.
	Excess amounts of labeled cDNA with poorly incorporated dye. Check FOI of labled cDNA before using it for hybridization (Table 1).
Black holes	Low expressors surrounded by background fluorescence. Depending upon the appearance of the background around the black hole, refer to the appropriate background comments (above). Black holes appear as dark spots within the background field. These spots have the expected size, shape and placement of printed spots.
Unexpected hybridization patterns	Arrays that were hybridized with different cDNAs were washed in the same bath and wash solutions.
Intense uniform fluorescence around outer edge of coverslip	Hybridization solution dried out. Maintain proper humidity during hybridization.
	Be sure to drop coverslip into place. Sliding the coverslip can leave some cDNA uncovered on the arrayed slide. Be sure to match cDNA volume with coverslip size so that it does not wick to the underside of the slide.
	Slides were hybridized too long.

6. References

1. Ishida, S. *et al*. (2001) Role for e2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol. Cell Biol*. **21**, 4684–99.

2. Maleck, K. *et al*. (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet*. **26**, 403–10.

3. Morgan, R.W. *et al.* (2001) Induction of host gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus. *J. Virol*. **75,** 533–9.

4. Travers, K.J. *et al*. (2000) Functional and genomic analyses reveal essential coordination between unfolded protein response and ER associated degradation. *Cell* **101**, 249–58.

5. Ferbeyre, G. *et al*. (2000) PML is induced by oncogenic ras and promotes premature senescence. *Gen. Dev*. **14**, 2015–17.

6. Brisco, P. *et al.* (2006) PureYield™ RNA Midiprep System: Isolating Pure Total RNA without DNase. *Promega Notes* **92**, 14–19

7. Related Products

Product	Size	Cat.#
PureYield™ RNA Midiprep System*	10 preps	Z3740
	50 preps	Z3741
SV Total RNA Isolation System		Z3100
PolyATtract® mRNA Isolation System II with Magnetic Stand*	3 isolations	Z5200
PolyATtract® System 1000*	scalable	Z5420
RNAgents® Total RNA Isolation System*	scalable	Z5100
ChipShot™ Direct Labeling and Clean-Up System	25 reactions	Z4100

*For Laboratory Use.