TECHNICAL BULLETIN

Prime-a-Gene® Labeling System

Instructions for Use of Product
U1100

Revised 2/15
TB049

Prime-a-Gene® Labeling System

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Prime-a-Gene® Labeling System is based on the method developed by Feinberg and Vogelstein (1,2), in which a mixture of random hexadeoxyribonucleotides is used to prime DNA synthesis in vitro from any linear double-stranded DNA template. With this method, it is possible to generate probes of high specific activity (>1 × 10^9cpm/µg), even using DNA fragments cut from agarose gels (2). Since the input DNA serves as a template and remains intact during the reaction, minimal amounts of DNA (25ng) can be labeled to a high specific activity. Typically, greater than 60% of the labeled deoxyribonucleotide can be incorporated into the Prime-a-Gene® Control DNA using the labeling reaction described here. Incorporation may vary from 40–80% with other samples, depending on the template and reaction conditions used. Using a template greater than 500bp, probes generated with the Prime-a-Gene® Labeling System are generally 250–300bp in length and are suitable for a variety of applications (3–8).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Prime-a-Gene® Labeling System	**30 reactions**	**U1100**

Each system contains sufficient reagents for 30 labeling reactions, including 5 reactions with the control DNA. Includes:

- 150u DNA Polymerase I, Large (Klenow) Fragment
- 40µl Each of 4 Prime-a-Gene® dNTP Solutions, 1.5mM
- 300µl Labeling 5X Buffer including 26.0 A_{260}u/ml Random Hexadeoxyribonucleotides
- 75µl BSA, Nuclease-Free
- 125ng Prime-a-Gene® Control DNA
- 1.25ml Nuclease-Free Water

Note: The Prime-a-Gene® Control DNA contains lambda DNA.

Storage Conditions: Store at –20°C. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes.

3. Labeling Protocol

Materials to Be Supplied by the User

(Solution compositions are provided in Section 8.)

- TE buffer
- 0.2M EDTA (pH 8.0)
- radiolabeled dNTP

The following reaction conditions are optimized for labeling 25ng of DNA template. To label other amounts of DNA, adjust the reaction volume proportionally to these conditions. Larger amounts of DNA used in the standard 50µl reaction volume will result in lower specific activities and shorter average probe lengths, while smaller amounts of template DNA will result in a slower reaction.

For example, the standard reaction with 25ng DNA is essentially complete in 60 minutes, whereas a reaction with 10ng of DNA in 50µl may take as long as 5 hours to go to completion. If necessary, the reaction can be allowed to proceed overnight at room temperature without harming the product.

1. Thaw all system components on ice except the Klenow Fragment. **Keep the Klenow Fragment at –20°C, and return it to the freezer immediately after use.**
2. Dissolve the DNA in deionized water or TE buffer at 1–25µg/ml, and denature the sample by heating in a microcentrifuge tube at 95–100°C for 2 minutes. Rapidly chill the tube in an ice bath. Assemble the reaction in a microcentrifuge tube, on ice, in the order shown:

Component	Add	Final Concentration
Nuclease-Free Water to achieve final volume of 50µl	____µl	
Labeling 5X Buffer	10µl	1X
mixture of the unlabeled dNTPs (see Note 1)	2µl	20µM each
denatured DNA template* (see Note 2)	25ng	500ng/ml
Nuclease-Free BSA	2µl	400µg/ml
[α-^{32}P]dNTP, 50µCi, 3,000Ci/mmol (see Note 3)	5µl	333nM
DNA Polymerase I Large (Klenow) Fragment	5 units	100u/ml
Final volume	**50µl**	

*A control reaction using 25ng (5µl) of the provided Prime-a-Gene® Control DNA may be performed to verify reaction kinetics. Denature the control DNA prior to beginning the reaction as described.

3. Mix gently, and incubate the reaction tube at room temperature for 60 minutes.
4. Terminate the reaction by heating at 95–100°C for 2 minutes and subsequently chilling in an ice bath. Add EDTA to 20mM, and use directly in a hybridization reaction or store at –20°C for later use.

Notes:

1. To prepare the unlabeled dNTPs, mix 1µl of each nonisotopically labeled dNTP to yield 3µl of a premix containing the three dNTPs, each at 500µM. Also see Note 3.
2. The DNA template should be linear. It is usually best to label only the insert DNA, rather than the entire vector. This considerably increases the signal-to-noise ratio of the resultant probe (1,2). While DNA can be labeled in molten agarose (2), labeling efficiencies may be lower than with purified template DNA. Reference 2 includes a protocol for radiolabeling DNA in molten agarose.
3. Although [α-^{32}P]dCTP is used in the standard reaction, optimal labeling can be achieved using a variety of isotopes. To achieve optimal probe length, the amount of [^{3}H, ^{35}S, ^{32}P, ^{33}P or ^{125}I] radiolabeled dNTP should be 10–125pmol. However, the highest incorporation efficiency occurs when ≤30pmol of a labeled dNTP is present. This is an important consideration when the background contributed by unincorporated label is an issue. The final specific activity of the DNA is influenced by two factors: 1) the specific activity of the labeled dNTP (Ci/mmol), and 2) how many of the 4 dNTPs (at 10–125pmol each) in a reaction are radiolabeled. The latter observation refers most specifically to reactions that contain more than one [α-^{35}S]- or [^{3}H]dNTP.
4. The volume of aqueous labeled dNTP should not exceed 50% of the total reaction volume. Labeled dNTPs supplied in 50% ethanol must be evaporated to dryness and redissolved in deionized water before use in the reaction.

4. Removal of Unincorporated Label (Optional)

Unincorporated, labeled nucleotides can be removed by size exclusion chromatography using Sephadex® G-50 spin columns (9) or by selective precipitation of the labeled DNA. This step usually is not required unless incorporation levels are low. In most applications, if incorporation is greater than 60%, removal of unincorporated, labeled nucleotides is not necessary.

4.A. Size Exclusion Chromatography

The advantages of Sephadex® G-50 columns include probe yields virtually free of unincorporated dNTPs and substantially reduced amounts of very short DNA oligomers. This is useful when generating hybridization probes, since optimal signal-to-noise ratios are achieved with probes 500–1,500 bases in length (10). Sephacryl® S-400 spin columns can be used to separate labeled DNA greater than 270 bases from smaller fragments.

4.B. Selective Precipitation of Labeled DNA with Ammonium Acetate and Ethanol

This method results in the precipitation of DNA >20 nucleotides in length, while free dNTPs remain in the supernatant. Recovery levels of labeled DNA precipitated by this method depend on length and concentration but can be as low as 50%.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- 4M ammonium acetate (pH 4.5)
- ethanol
- 0.67M ammonium acetate (pH 4.5), 67% ethanol
- TE buffer

1. Add 1 volume of 4M ammonium acetate (pH 4.5) to the probe and vortex.
2. Add 2 volumes (1 volume = total volume in Step 1) of ethanol. Mix and chill in an ice bath for 15 minutes.
3. Heat at 37°C for 2 minutes with occasional gentle mixing. This step redissolves free deoxyribonucleotide precipitated in Step 2.
4. Centrifuge at 12,000 × *g* for 15 minutes, and carefully aspirate the supernatant.
5. Wash the pellet once in 0.5ml of 0.67M ammonium acetate (pH 4.5), 67% ethanol at room temperature with gentle shaking. Then centrifuge as in Step 4, and again carefully aspirate.
6. Wash the pellet once in 90% ethanol and dry under vacuum.
7. Redissolve the labeled DNA in TE buffer and use for probe hybridization.

5. Determination of Percent Incorporation

The percent of label incorporated may be determined either by a filter-binding assay (see Section 5.A) or by trichloroacetic acid (TCA) precipitation (see Section 5.B).

5.A. Filter-Binding Assay

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- 0.2M EDTA (pH 8.0)
- 0.5M sodium phosphate (pH 6.8)
- Whatman® GF/B or GF/C 24mm circular filters

1. Dilute 1µl of the labeling reaction 1:100 in 0.2M EDTA (pH 8.0). In duplicate, spot 3µl of the diluted sample on Whatman® GF/B or GF/C circular filters.
2. Dry the filters briefly under a heat lamp. Set one filter aside to use directly for the determination of total cpm in the sample.
3. Wash the remaining filter in 50ml of 0.5M sodium phosphate (pH 6.8) twice for 5 minutes to remove the unincorporated dNTPs.
4. Dry the washed filter under a heat lamp.
5. Add the appropriate amount of scintillation fluid to each filter and count in a scintillation counter.

   Note: It is not necessary to use scintillation fluid for counting ^{32}P-labeled samples. The Cerenkov radiation emitted from samples without scintillation fluid can be detected by a scintillation counter set to monitor the tritium window. Although the absolute number of counts is not the same with and without scintillation fluid (because Cerenkov counting is less than half as efficient), the counts will be proportional from sample to sample.

5.B. TCA Precipitation

Materials to Be Supplied by the User

- 0.2M EDTA (pH 8.0)
- 20mM EDTA containing 0.1mg/ml carrier DNA or BSA
- ice-cold 10% trichloroacetic acid, 1% sodium pyrophosphate
- ice-cold 10% trichloroacetic acid
- acetone or 95% ethanol (see Step 4)
- scintillation fluid

1. Dilute 1µl of the labeling reaction 1:100 in 0.2M EDTA (pH 8.0). Spot 3µl of this diluted sample on a glass fiber or nitrocellulose filter for determination of total cpm in the sample. Let the filter air-dry.
2. Transfer 3µl of the same dilution to a tube containing 100µl of 0.1mg/ml carrier DNA or BSA and 20mM EDTA. Mix well.

5.B. TCA Precipitation (continued)

3. Add 1.3ml of ice-cold 10% TCA, 1% sodium pyrophosphate to the mixture and precipitate on ice for 20 minutes.

   Note: The sodium pyrophosphate reduces the nonspecific binding of unincorporated nucleotides to the filter.

4. Collect the precipitated DNA by vacuum filtration onto a glass fiber or nitrocellulose filter. Wash the filter a minimum of 3 times with 5ml of cold 10% TCA and briefly rinse with acetone (glass fiber only) or 95% ethanol. Let air-dry completely.

5. Add an appropriate amount of scintillation fluid (optional) to each filter and count both total and incorporated (filter-bound or TCA-precipitated) cpm using a scintillation counter.

   Note: It is not necessary to use scintillation fluid for counting ^{32}P-labeled samples. The Cerenkov radiation emitted from samples without scintillation fluid can be detected by a scintillation counter set to monitor the tritium window. Although the absolute number of counts is not the same with and without scintillation fluid (because Cerenkov counting is less than half as efficient), the counts will be proportional from sample to sample.

6. Calculation of Specific Activity

1. Random-primed labeling results in net DNA synthesis. To calculate the specific activity of labeled probe DNA, first calculate the amount of DNA generated in the reaction assuming 100% incorporation:

$$\frac{\mu\text{Ci dNTP added} \times 4 \times 330\text{ng/nmol}}{\text{specific activity of dNTP (Ci/mmol} = \mu\text{Ci/nmol)}} = \text{ng theoretical yield}$$

2. Next, calculate the percent incorporation from the filter-binding or TCA precipitation results:

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 100 = \%\text{ incorporation}$$

3. Determine the amount of DNA synthesized:

   % incorporation × 0.01 × theoretical yield (from Step 1, above) = ng DNA synthesized

4. Next, calculate the total cpm incorporated:

   cpm incorporated × 33.3 × 50 = total cpm incorporated

   Note: The factors 33.3 and 50 correct for using 3µl of a 1:100 dilution for the filter-binding or TCA precipitation and converting this back to a 50µl total reaction volume.

5. Calculate the specific activity of the product:

$$\frac{\text{total cpm incorporated}}{(\text{ng DNA synthesized} + \text{ng input DNA}) \times 0.001\mu\text{g/ng}} = \text{cpm/}\mu\text{g}$$

Sample Calculation:

Using 50µCi of [α-^{32}P]dCTP (3,000Ci/mmol) in a standard reaction, the calculation is as follows:

$$\frac{50\mu Ci \times 4 \times 330ng/nmol}{3{,}000\mu Ci/nmol} = 22ng \text{ theoretical yield}$$

Assume that 3.17×10^4 cpm were TCA-precipitated and that the unprecipitated sample had 5.28×10^4 cpm:

$$\frac{3.17 \times 10^4}{5.28 \times 10^4} \times 100\% = 60\% \text{ incorporation}$$

$$60 \times 0.01 \times 22ng = 13.2ng \text{ DNA synthesized}$$

Therefore, the specific activity is:

$$\frac{3.17 \times 10^4 \text{ cpm} \times 33.3 \times 50}{(13.2ng + 25ng) \times 0.001\mu g/ng} = 1.4 \times 10^9 \text{ cpm}/\mu g$$

7. 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**

Symptoms	Causes and Comments
Low amounts of or no DNA labeled	DNA not linear or not denatured. • Labeling of circular plasmid DNA is approximately 50% less efficient than labeling linear templates. • Check that protocol was followed as specified and, particularly, that DNA was linear and denatured prior to use.
	Inactive enzyme mix, incorrect amount of DNA, or insufficient reaction time. Use the Prime-a-Gene® Control DNA to monitor the progress of the reaction. Make sure the control DNA is denatured before random-primed labeling.
	Labeled dNTP lost during manipulation of reagents supplied in aqueous ethanol. Repeat the experiment.
	Counts quenched by moisture retained in sample filters. Dry the filters completely before counting.
High background during hybridization	Excessive unincorporated [α-^{32}P]dNTP. Purify the probe. See Section 4, Removal of Unincorporated Label.
	Too much template DNA in the reaction. Excess template DNA in the reaction yields shorter probes, which have reduced sequence specificity. Lower amount of DNA included in the reaction.

8. Composition of Buffers and Solutions

Labeling 5X Buffer

250mM Tris-HCl (pH 8.0)
25mM $MgCl_2$
10mM DTT
1M HEPES (pH 6.6)
26 A_{260}u/ml random hexadeoxyribonucleotides

0.5M sodium phosphate (pH 6.8) (per liter)

47.25g NaH_2PO_4
22.35g Na_2HPO_4

TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)

9. Related Products

Product	Size	Cat.#
dATP, 100mM	40µmol	U1201
dCTP, 100mM	40µmol	U1221
dGTP, 100mM	40µmol	U1211
dTTP, 100mM	40µmol	U1231
dUTP, 100mM	40µmol	U1191
dATP, dCTP, dGTP, dTTP, 100mM each	40µmol each	U1240
	10µmol each	U1330
dUTP, dCTP, dGTP, dATP, 100mM each	40µmol each	U1245
	10µmol each	U1335
Random Primers (hexadeoxyribonucleotides, 0.67 A_{260}u)	20µg	C1181
Labeling 5X Buffer (includes 26 A_{260}u/ml random hexadeoxyribonucleotides)	300µl	U1151
DNA Polymerase I Large (Klenow) Fragment	150u	M2201
	500u	M2206
Spin Columns	10 each	C1281
Sephacryl® S-400 Matrix	10ml	V3181

10. References

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11. Summary of Changes

The following changes were made to the 2/15 revision of this document:

1. The suggested circular filters in Section 5.A were updated to replace a discontinued item on the materials list.
2. The document design was updated.