Prime-It® II Random Primer Labeling Kit

INSTRUCTION MANUAL

Catalog #300385

Revision #112001b

For In Vitro Use Only

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Prime-It® II Random Primer Labeling Kit

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MATERIALS PROVIDED

Materials provided[a]	Quantity
Control DNA—buffered aqueous solution of a 3-kb linearized plasmid (25 ng/µl)	10 µl
Random 9-mer primers—buffered aqueous solution of random oligodeoxy-ribonucleotides (27 OD units/ml)	350 µl
5× *dCTP buffer (for use with [α–^{32}P]dCTP)— buffered aqueous solution containing dATP, dGTP and dTTP (0.1 mM of each)	350 µl
5× *dATP buffer (for use with [α–^{32}P]dATP)— buffered aqueous solution containing dCTP, dGTP and dTTP (0.1 mM of each)	350 µl
Exo(–) Klenow—buffered glycerol solution (5 U/µl)	175 units
Stop mix—0.5M EDTA, pH 8.0.	100 µl

[a] Enough reagents are provided for 30 reactions.

* Indicates missing nucleotide in buffer.

STORAGE CONDITIONS

All components: –20°C

ADDITIONAL MATERIALS REQUIRED

[α–^{32}P]dCTP
[α–^{32}P]dATP

Revision #112001b

INTRODUCTION

Random oligonucleotides have been used as primers in a method for labeling DNA to produce high specific-activity probes.[1,2] The procedure relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The primer–template complexes formed represent a substrate for the Klenow fragment of DNA polymerase I. The enzyme synthesizes new DNA by incorporating nucleotide monophosphates at the free 3´–OH group provided by the primer. The newly synthesized DNA is made radioactive by substituting a radiolabeled nucleotide for a nonradioactive one in the reaction mixture. The resulting labeled DNA can serve as a sensitive hybridization probe for screening gene libraries,[3] probing Southern and northern blots,[4,5] and in situ hybridization techniques.

Conventional protocols for random oligo-labeling of DNA require a minimum of 30 minutes to perform, and many suggest an overnight incubation. Stratagene's Prime-It® II random primer labeling kit makes it possible to produce high-specific-activity DNA probes within two minutes. To achieve this rate of incorporation, we used a 3´ exonuclease-deficient mutant of the Klenow fragment of DNA polymerase I [Exo(–) Klenow] and random nonamer primers (9-mers). High-specific-activity probes can be produced from fragments purified by a variety of techniques, including low-melting-temperature (LMT) agarose gels.

Stratagene's Prime-It Fluor fluorescence labeling kit* generates directly fluoresceinated DNA probes using random 9-mer primers and Exo$^-$ Klenow to incorporate fluor-12-dUTP into the probe fragments.

* Available from Stratagene, catalog #300380.

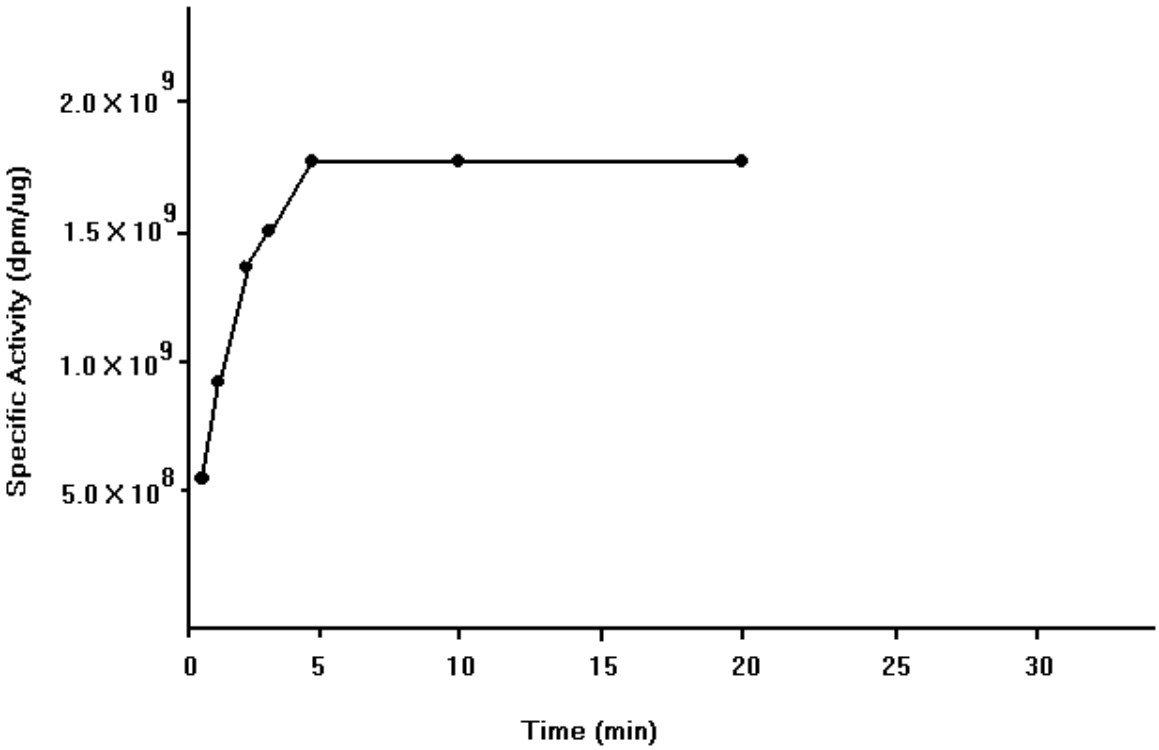

FIGURE 1 The rate of radionucleotide incorporation.

Time Course of the Reaction

The rate of radionucleotide incorporation into a 3 kb template was measured using the Exo(–) Klenow enzyme–9-mer primer combination. Figure 1 above demonstrates that, within 2 minutes after initiating the polymerization reaction, 59% of the total labeled nucleotide was incorporated, and a specific activity of 1.7 x 10^9 dpm/µg was achieved.

Sensitivity

The sensitivity of a probe generated in 2 minutes was tested in a hybridization experiment. We used a Southern transfer blot containing various quantities of human genomic target DNA immobilized on Duralon-UV™ nylon membranes.* The Southern blot data revealed that as little as 1.0 pg of target DNA could be detected from 0.3 µg of human genomic DNA, using a 16-hour exposure of the blot to X-ray film with a single intensifying screen at –80°C.

Probe Fragment Lengths

The most useful length of DNA probes for hybridization studies is approximately 500–1500 nucleotides.[6] Alkaline agarose gel electrophoresis was used to verify that Exo(–) Klenow generated probes from 200–1000 nucleotides in length.

* Available from Stratagene.

Specific Activities

Specific activities of $\geq$1 x 10^9 dpm/µg have been achieved with templates ranging in size from 400 bp to 10 kb. The method also labels DNA templates which have been isolated by a variety of techniques, including low-melting-temperature (LMT) agarose gel electrophoresis. Probes with specific activities $\geq$1 x 10^9 dpm/µg have been produced when as much as 22 µl of 1% LMT agarose (final agarose concentration 0.44%) is present in the labeling reaction mixture.

In addition, the method performs equally well with [α–^{32}P]dCTP (3000 Ci/mmol) or [α–^{32}P]dATP (3000 Ci/mmol). Probes with specific activity of $\geq$2 x 10^9 dpm/µg may be obtained using [^{32}P]dCTP (~6000 Ci/mmol).

Note *To reduce hybridization time from 12 to 24 hours to only 1 hour, we recommend using Stratagene's QuikHyb® hybridization solution.**

* Available from Stratagene, catalog #201220 and #201221.

PROTOCOL

This protocol is designed to label a DNA probe, at a fixed timepoint, with [α–^{32}P]dATP (3000 Ci/mmol) or [α–^{32}P]dCTP (3000 Ci/mmol), resulting in maximum specific activity.

Note *If using [a-32P]dCTP (6000 Ci/mmol) to generate probes with specific activities ≥2 × 10^9 dpm/μg, the amount of label per reaction should be 10 μl (or 100 μCi), and the volume of water should be reduced by 5 μl.*

To determine the optimal labeling time for a specific DNA template, see *Appendix II: Measurement of Probe Specific Activity*.

1. Add the following components to the bottom of a clean microcentrifuge tube:

   Sample:

   - 25 ng (1–23 μl) of DNA template to be labeled. See *Appendix I: Preparation of Template DNA by LMT Agarose Gel Electrophoresis* for guidelines on the preparation of template DNA. If the DNA template concentration is 25 μg/ml, then 1 μl of template will give the required 25 ng.
   - 0–23 μl of high quality H_2O. For a DNA restriction fragment isolated by LMT agarose gel electrophoresis, as much as 23 μl of sample can be added to the microcentrifuge tube to achieve the required 25 ng of template DNA. The H_2O is not required under these circumstances.
   - 10 μl random oligonucleotide primers.

   The total reaction volume should be 34 μl at this point. Proceed to step 2.

   Control:

   - 25 ng (1 μl) of control DNA template.
   - 23 μl of high quality H_2O. (If preparing probes of specific activity ≥2 × 10^9 dpm/μg, add 18 μl of high-quality water, as described in the note above).
   - 10 μl random oligonucleotide primers.

   Total reaction volume is 34 μl at this point. Proceed to step 2.

2. Heat the reaction tubes in a boiling water bath for 5 minutes and then centrifuge briefly at room temperature to collect the liquid, which may have condensed on the cap of the tubes. If the DNA sample is in LMT agarose, after removal from the boiling water bath and centrifugation, place the reaction tube at 37°C and then proceed to step 3. If the DNA sample is in aqueous solution, leave the reaction tube at room temperature and then proceed to step 3.

3. Add the following reagents to the reaction tubes:

   - 10 µl of 5× primer buffer: Two vials of 5× primer buffer (5× PB) have been supplied.
   - The 5× *dCTP primer buffer contains dATP, dGTP and dTTP, and it should be used when [α–^{32}P]dCTP or a dCTP analog is to be incorporated. The 5× *dATP primer buffer contains dCTP, dGTP and dTTP and it should be used when [α–^{32}P]dATP or a dATP analog is to be incorporated.
   - 5 µl of labeled nucleotide: Use either [α–^{32}P]dCTP at 3000 Ci/mmol or [α–^{32}P]dATP at 3000 Ci/mmol. For probes with specific activity $\geq 2 \times 10^9$ dpm/µg, add 10 µl of [α–^{32}P]dCTP at 6000 Ci/mmol. Mix the contents of the tube thoroughly with your pipet tip.
   - 1 µl Exo(–) Klenow enzyme (5 U/µl).

   Mix the reaction components *thoroughly* with your pipet tip.

4. Incubate the reactions at 37–40°C for 2–10 minutes. See Figure 1.

   LMT agarose reactions: Incubate for at least 10 minutes to ensure high specific activity.

   Note *When the 3-kb control template DNA is used, a specific activity of 1 x 10^9 dpm/µg is achieved within 2 minutes of incubation at 37°C. At the 10 minute timepoint, the specific activity is approximately 1.6 x 10^9 dpm/µg.*

5. Following the incubation period, stop the reaction by adding 2 µl of stop mix.

   Note *Purified probes generate higher signal-to-background ratios on Southern and Northern blots, compared to probes which have not been purified before use. Stratagene's NucTrap® probe purification columns* are ideal for this purpose, removing unincorporated nucleotides in less than 2 minutes with 99% efficiency.*

* Available from Stratagene, catalog #400701 and #400702.

APPENDIX I: PREPARATION OF TEMPLATE DNA BY LMT AGAROSE GEL ELECTROPHORESIS

Linearized and denatured plasmid or phage DNA, containing the desired insert, can be labeled and used as a probe for hybridization experiments. However, it may be necessary to isolate the insert DNA away from vector sequences. LMT agarose gel electrophoresis is a convenient method for accomplishing this isolation.

1. Digest the plasmid or phage DNA, containing the insert of interest, with the appropriate restriction enzyme(s). Enough DNA should be digested to provide at least 250 ng of the insert.

2. Electrophorese the sample on a LMT agarose gel containing 0.05 µg/ml of ethidium bromide (EtBr).

3. Destain the gel in distilled H_2O.

4. Visualize the isolated insert DNA on a long-wavelength ultraviolet light box.

   Note *Do not expose the DNA to short-wavelength ultraviolet rays, because low-specific-activity probes will result.*

5. Cut out the desired DNA fragment from the gel and remove as much extraneous agarose as possible. Cut this gel slice, containing the DNA fragment of interest, into equal portions and place each portion in a preweighed tube. Weigh the tube again to obtain the weight of the gel.

6. Add 3 ml of distilled H_2O per gram of gel and heat at 65°C for 5 minutes to melt the gel. Mix the sample thoroughly and then store the tubes at –20°C until ready to perform a labeling reaction.

APPENDIX II: MEASUREMENT OF PROBE SPECIFIC ACTIVITY

Accurate monitoring of the progress of the labeling reaction and measurement of probe specific activity can be obtained by determining the proportion of radionucleotide incorporated into newly synthesized DNA. This can be done by performing the following procedure:

1. Complete steps 1–4 of the protocol.

2. Remove a 1-µl aliquot of the reaction mixture at 2-, 5- and/or 10-minute timepoints and dilute it in 99 µl of 0.2 M EDTA.

3a. Spot 3 µl of each dilution in duplicate onto Whatman® DE 81 filter paper disks. Dry the filters for 15 minutes under a heat lamp. Wash the filters twice for 5 minutes each at room temperature in 50 ml of 2× SSC (0.3 M NaCl–0.03 M trisodium citrate) and then once with ice-cold ethanol. Dry the filters again under a heat lamp and place them in separate scintillation vials with 5 ml of scintillation fluid. The average cpm of the washed filters represents the proportion of radionucleotide incorporated into probe DNA.

3b. Spot 3 µl of each dilution in duplicate onto Whatman DE 81 filter paper disks. Dry the filters under a heat lamp. Place the filters in separate vials with 5 ml of scintillation fluid and count. The average cpm of the unwashed filters represents the total amount of radioactivity in the reaction mixture.

4. Use the following formula to calculate the specific activity:

$$SA = [(\mu Ci)(2.2 \times 10^9)(P)] \div \{M_i + [(1.3 \times 10^3)(P)(\mu Ci/S)]\}$$

where SA is the specific activity in dpm/µg; µCi is the µCi radiolabeled nucleotide in the reaction mixture; P is the proportion of radiolabeled nucleotide incorporated into probe DNA, calculated by dividing the average cpm counted on the washed DE 81 filter paper disks divided by the average cpm counted on the unwashed DE 81 filter paper disks; M_i is the mass of input DNA template in ng; and S is the specific activity of radiolabeled nucleotide in Ci/mmol (or µCi/nmol).

Multiply (µCi) by 2.2×10^9 to calculate the reaction's total number of dpm. This calculation also converts the final value for SA from dpm/ng to dpm/µg. Multiply the resulting value by P to calculate the proportion of dpm incorporated into the probe DNA.

To compute SA, divide the value obtained above by the total amount of DNA present at the end of the reaction. The total amount of DNA present at the end of the reaction is the sum of the mass of input DNA template plus the mass of the newly synthesized DNA. The latter value is obtained by multiplying the number of nanomoles of dCMP or dAMP incorporated [$(P)(\mu Ci/S)$] by four times the average molecular weight of the four dNMPs [$(4)(325) = 1.3 \times 10^3$].

In the sample calculation below, 20,577 represents the average cpm measured on the washed DE 81 filters, and 35,516 represents the average cpm on the unwashed filters. The reaction was performed for 2 minutes with [^{32}P]dCTP (50 µCi; 3000 µCi/nmol), using 25 ng of control template DNA.

$$SA = [(50)(2.2 \times 10^9)(20{,}577/35{,}516)] \div \{25 + [(1.3 \times 10^3)(20{,}577/35{,}516)(50/3{,}000)]\}$$
$$SA = (6.4 \times 10^{10}) \div (25 + 12.6)$$
$$SA = 6.4 \times 10^{10} \div 37.6$$
$$SA = 1.7 \times 10^9 \text{ dpm/µg}$$

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ENDNOTES

Prime-It® II Random Primer Labeling Kit

Catalog #300385

QUICK-REFERENCE PROTOCOL

- Add the following to a sterile microcentrifuge tube:

  25 ng of DNA
  0–23 µl of water
  10 µl of random oligonucleotide primers

- Heat the reaction in a boiling water bath for 5 minutes
- Centrifuge briefly at room temperature
- (If DNA is in LMT agarose, incubate the reaction tube at 37°C)
- Add the following:

  10 µl of 5× buffer
  5 µl of labeled nucleotide
  1 µl of Exo(–) Klenow (5 U/µl)

- Incubate at 37–40°C for 2–10 minutes
- Add 2 µl of stop mix
- Purify the probe for best results in hybridization experiments