Prime-It RmT Random Primer Labeling Kit

Single-Use dCTP-Labeling Reactions

INSTRUCTION MANUAL

Catalog #300392

Revision A

For In Vitro Use Only

300392-12

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Prime-It RmT Random Primer Labeling Kit

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MATERIALS PROVIDED

Materials provided[a]	Quantity
Dehydrated control DNA (linearized pBluescript II DNA, 3 kb in length) **supplied in a red, 0.2 ml microcentrifuge tube**	250 ng
Magenta DNA polymerase	320 U (4 U/µl)
Stop mix [0.5 M EDTA (pH 8.0)]	100 µl
Single-use reaction tubes containing a dehydrated mixture of random primers, dNTPs, buffer and cofactors for use with [α-^{32}P]dCTP[b] **supplied in clear, 0.2 ml microcentrifuge tubes**	24 (3 strips; 8 reaction tubes in each strip)

[a] The Prime-It RmT random primer labeling kit contains reagents for 24 [α-^{32}P]dCTP-labeling reactions and enough control DNA for 10 control reactions.

[b] The [α-^{32}P]dCTP is not provided in this kit.

STORAGE CONDITIONS

Control DNA: Room temperature until rehydrated. After rehydration store at –20°C.

All Other Components: Room temperature

ADDITIONAL MATERIALS REQUIRED

[α-^{32}P]dCTP (3000 Ci/mmol)

Revision A

INTRODUCTION

The Prime It RmT random primer labeling kit uses random oligonucleotides as primers for labeling DNA to produce high-specific-activity probes.[1,2] The procedure relies on the ability of random 9-mers to anneal to multiple sites along the length of a DNA template. The primer–template complexes formed represent a substrate for the magenta DNA polymerase, a thermostable polymerase that allows the Stratagene Prime-It RmT random primer labeling kit to be stored at room temperature.[3] 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 radiolabeled [α-^{32}P]dCTP for unlabeled dCTP in the reaction mixture. The resulting labeled DNA serves as a sensitive hybridization probe for screening gene libraries,[4] probing Southern and Northern blots,[5,6] and *in situ* hybridization techniques.

DNA labeling reaction components (random primers, dNTPs, buffers and cofactors) are supplied as a dehydrated reaction mixture, pre-aliquoted in 24 single-use reaction tubes. The Prime-It RmT random primer labeling kit also includes magenta thermostable polymerase, and dehydrated control DNA, allowing storage of the kit at room temperature.

Conventional protocols for random oligonucleotide labeling of DNA require a minimum of 30 minutes to perform, and many suggest an overnight incubation.[7] The Prime-It RmT random primer labeling kit produces high-specific-activity probes (>1 × 10^9 dpm/μg) in just 5 minutes.

PROTOCOL

Note *See the last page of this instruction manual for a condensed quick-reference protocol that can be detached and used when detailed instructions are no longer needed.*

The standard protocol is designed to label a DNA probe with [α-^{32}P]dCTP (3000 Ci/mmol) to a specific activity of >1 × 10^9 dpm/μg. An alternate protocol is provided for generating probes with specific activities of >2 × 10^9 dpm/μg using 6000 Ci/mmol [α-^{32}P]dCTP and providing the label at a higher concentration in the reaction mixture.

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

Rehydrating the Control DNA

When using the Prime-It RmT random primer labeling kit for the first time, follow steps 1 and 2 below:

1. Rehydrate the control DNA in 10 μl of sterile distilled water (dH_2O).

2. After rehydration, store the control DNA at –20°C.

Labeling the DNA Probe

1. Prepare a 37°C water bath and a boiling water bath. Defrost the [α-^{32}P]dCTP label, if necessary.

2. Remove the required number of single-use reaction tubes from the tube strips provided. Add the components listed in the table below to each of the reaction tubes, according to the desired probe specific activity. Prepare reactions for all samples and controls in separate tubes.

Sample Reactions

Component	Standard protocol (for >1 × 10^9 dpm/μg)	Alternate protocol (for >2 × 10^9 dpm/μg)
Template DNA	25–50 ng	25–50 ng
dH_20	X μl to a final volume of 42 μl	X μl to a final volume of 37 μl

Control Reactions

Component	Standard protocol (for >1 × 10^9 dpm/μg)	Alternate protocol (for >2 × 10^9 dpm/μg)
Rehydrated control DNA	1 μl (25 ng)	1 μl (25 ng)
dH_20	41 μl	36 μl

For guidelines on the preparation of the DNA template, see *Appendix I: Preparation of Template DNA by Low-Melting-Temperature Agarose Gel Electrophoresis* and *Appendix II: Preparation of DNA Templates from PCR Products*. For a DNA restriction fragment isolated by low-melting-temperature (LMT) agarose gel electrophoresis, up to 42 µl of sample can be added to achieve the required 25 ng of template DNA.

3. Heat the reaction tubes to 95–100°C in a boiling water bath for 5 minutes and then centrifuge the tubes briefly at room temperature to collect the liquid, which may have condensed on the caps of the tubes. If the DNA was added to the reaction in LMT agarose, place the reaction tube at 37°C after centrifugation, then proceed to step 4. If the DNA was added in aqueous solution, leave the reaction tube at room temperature after centrifugation then proceed to step 4.

4. Add [α-^{32}P]dCTP label to each of the reaction tubes according to the table below. Mix the contents of the tube *thoroughly* with a pipet tip.

Sample and Control Reactions

Component	Standard protocol (for >1 × 10^9 dpm/µg)	Alternate protocol (for >2 × 10^9 dpm/µg)
[α-^{32}P]dCTP	5 µl **(3000 Ci/mmol)**	10 µl **(6000 Ci/mmol)**

5. Add 3 µl of magenta DNA polymerase (4 U/µl) to each of the reaction tubes. Mix the reaction components *thoroughly* with a pipet tip.

6. Incubate the reactions at 37–40°C for 5–10 minutes. For probes made from LMT-agarose-purified DNA, allow the reaction to continue for 10 minutes for maximum specific activity (see *Appendix I: Preparation of Template DNA by Low-Melting-Temperature Agarose Gel Electrophoresis*).

   Note *For the 3-kb control template DNA, a specific activity of 1 × 10^9 dpm/µg is expected after 5 minutes at 37° and a specific activity of ~1.6 × 10^9 dpm/µg is expected after 10 minutes at 37°.*

7. Following the incubation period, stop the reaction by adding **2 µl of stop mix** to each reaction tube.

See *Appendix III: Measurement of Probe Specific Activity* for measurement of specific activity and to determine optimal labeling time for a specific DNA template.

Removal of Unincorporated Nucleotides

Probes that have been purified from unincorporated nucleotides generate higher signal-to-background ratios on Southern and Northern blots compared to probes that have not been purified before use. Stratagene NucTrap probe purification columns are ideal for this purpose, removing unincorporated nucleotides in <2 minutes with 99% efficiency. Additional probe purification protocols are available in reference 7.

APPENDIX I: PREPARATION OF TEMPLATE DNA BY LOW-MELTING-TEMPERATURE AGAROSE GEL ELECTROPHORESIS

Linearized and denatured plasmid or phage DNA, containing the desired insert, can be labeled and used as probes for hybridization experiments. Sometimes it is desirable to isolate part or all of the insert from the vector, since the vector sequence in the probe may bind to similar vector sequences on the target membrane. Isolating insert DNA also allows all 25 ng of the DNA to be converted into useful probe by eliminating the production of unused vector probe. Low-melting-temperature agarose gel electrophoresis allows the separation of insert or insert fragments from the vector. The fragment(s) of choice can be excised from the gel and prepared as described below:

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 500 ng of the insert.

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

3. Visualize the isolated insert DNA on a long-wavelength ultraviolet (UV) light box.

   Note *Do not expose the DNA to short-wavelength UV rays, because this may damage the template DNA and reduce the specific activity of the probe.*

4. Cut out the desired DNA fragment from the gel and remove as much extraneous agarose as possible. Place this gel slice, containing the DNA fragment of interest, into a preweighed microcentrifuge tube. Weigh the microcentrifuge tube again to obtain the weight of the gel slice.

5. Add 3 ml of dH_2O/g of gel (e.g., for a 100-mg gel slice, add 300 µl of dH_2O) and heat at 65°C for 5 minutes to melt the agarose. Mix the sample thoroughly and then store the tubes at –20°C until required for the labeling reaction. It may be necessary to heat the sample to 65°C before aliquoting into the reaction tube for labeling.

APPENDIX II: PREPARATION OF DNA TEMPLATES FROM PCR PRODUCTS

PCR products make excellent substrates for the labeling reaction if residual free nucleotides are removed from the sample. The remaining PCR primers do not hinder the labeling reaction. If the amplification reaction contains spurious DNA products, the intended PCR product should be gel isolated. DNA fragments as small as 222 bp have been used successfully to generate high specific activity probes. Free nucleotides can be removed by using a desalting resin such as Sephadex® G-50 or by using Stratagene NucTrap probe purification columns.

Note *The desalting process should be carried out in the presence of water instead of a buffer such as 1× STE, which may decrease the activity of the polymerase during the labeling reaction.*

APPENDIX III: MEASUREMENT OF PROBE SPECIFIC ACTIVITY

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

1. Complete steps 1–7 of the main protocol.

2. Remove 1 µl of the reaction mixture at the 5- and 10-minute time points and dilute the reaction mixture with 99 µl of 0.2 M EDTA.

3. For each sample or time point, label two Whatman® DE81 filter paper disks with a "U" to designate unwashed filters and label two filters with a "W" to designate washed filters. If more than one sample or time point requires quantification, add an identifying prefix to the label (e.g., 2-W or 2-U).

4. Spot 3 µl of the diluted probe from step 2 above onto each of the four filters.

5. Dry the filters completely for 15 minutes under a heat lamp.

6. Wash the filters labeled "W" two times for 5 minutes with 50 ml of 2× SSC buffer (0.3 M NaCl and 0.03 M trisodium citrate) and then rinse the filters once briefly with ice-cold 100% (v/v) ethanol.

7. Dry the "W" filters completely under a heat lamp.

8. Place each filter (washed and unwashed) into a separate scintillation vial and cover the filters with scintillation fluid. The average counts per minute (cpm) of the washed duplicate filters represents the amount of radionucleotide incorporated into the probe DNA. The average counts per minute of the unwashed duplicate filters represents the total amount of radioactivity in the reaction mixture.

Calculating Specific Activity

9. Use the following formula to calculate the specific activity (S_A):

$$S_A = \frac{(\mu\text{Ci})(2.2 \times 10^9)(P)}{M_i + [(1.3 \times 10^3)(P)(\mu\text{Ci}/S_a)]}$$

S_A = the specific activity in disintegrations per minute per microgram (dpm/µg)

µCi = the amount of radiolabeled nucleotide in microcuries in the reaction mixture

P = the proportion of radiolabeled nucleotide incorporated into the probe DNA, calculated by dividing the average counts per minute counted on the washed Whatman DE81 filter paper disks divided by the average counts per minute counted on the unwashed Whatman DE81 filter paper disks

M_i = the mass of input of the DNA template in nanograms (ng)

S_a = the specific activity of radiolabeled nucleotide in curies per millimole (Ci/mmol) [curies per millimole (Ci/mmol) equals microcuries per nanomole (µCi/nmol)]

Multiply the microcuries (µCi) by 2.2×10^9 to calculate the total number of disintegrations per minute (dpm) in the reaction. This calculation also converts the final value for S_A from disintegrations per minute per nanogram (dpm/ng) to disintegrations per minute per microgram (dpm/µg). Multiply the resulting value by P to calculate the proportion of disintegrations per minute (dpm) incorporated into the probe DNA.

To compute the S_A, 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 (M_i) DNA template plus the mass of the newly synthesized DNA. The latter value is obtained by multiplying the number of nanomoles of dCMP incorporated [$(P)(\mu\text{Ci}/S_a)$] 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 counts per minute measured on the washed Whatman DE81 filter paper disks and 35,516 represents the average counts per minute on the unwashed Whatman DE81 filter paper disks. The reactions were performed for 2 minutes with ^{32}P[dCTP] (50 µCi, 3000 µCi/nmol), using 25 ng of the control template DNA.

$$S_A = \frac{(50)(2.2\times10^9)(20{,}577/35{,}516)}{25+[(1.3\times10^3)(20{,}577/35{,}516)(50/3000)]}$$

$$S_A = \frac{6.4\times10^{10}}{25+12.6} \quad (12.6 = \text{the amount of newly synthesized DNA in nanograms})$$

$$S_A = \frac{6.4\times10^{10}}{37.6}$$

$$S_A = 1.7\times10^9 \text{ dpm}/\mu\text{g}$$

REFERENCES

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ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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Catalog #300392

QUICK REFERENCE PROTOCOL

Prepare a 37°C water bath and a boiling water bath

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Remove single-use reaction tubes from the reaction tube strips

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Add the following to each single-use reaction tube:

- dH_2O to a final volume of 42 µl
- 25–50 ng of DNA

↓

Boil the reaction for 5 minutes

↓

Centrifuge briefly to collect the condensate

↓

Add the following:

- 5 µl of labeled nucleotide
- 3 µl of magenta DNA polymerase (4 U/µl)

↓

Mix well and incubate at 37°C for 5–10 minutes
(Probes prepared from LMT-agarose-purified DNA require incubation for 10 minutes)

↓

Add 2 µl of stop mix