Random Primer Extension Labeling System

Catalog Number NEP103L

Contains reagents for the incorporation of radiolabeled deoxynculeoside triphosphates into a single stranded or denatured double stranded DNA in the preparation of radiolabeled DNA probes.

The following deoxynucleotides are used with this system:

[α-^{32}P] dCTP, BLU5/NEG-013A, 800 Ci/mmol

[α-^{32}] dCTP, BLU5/NEG-013H, 3000 Ci/mmol

[α-^{32}P] dCTP, BLU5/NEG-013Z, 6000 Ci/mmol

[^{35}S] dCTPαS, BLU/NEG7-036H, 1250 Ci/mmol

[5,5'-^{3}H] dCTP, NET-601A, 40-70 Ci/mmol

5'-[^{125}I] iodo-dCTP, NEX074, 2200 Ci/mmol

Note: Tracer must be ordered separately.

I. INTRODUCTION

The NEN® Life Science Product's Random Primer Extension Kit allows the researcher to conveniently label DNA to very high specific activity. This technique, first introduced by Feinberg and Vogelstein (1,2), uses a mixture of hexanucleotides to prime DNA synthesis randomly on single stranded DNA. DNA synthesis, which requires a primer and a template, can be accomplished by either the holoenzyme DNA polymerase I, or the Klenow fragment (large fragment) of DNA polymerase I. Because Klenow fragment lacks the 5' → 3' exonuclease activity, the use of the Klenow fragment in primer extension avoids loss of incorporated label. With the availability of cloned forms of the Klenow enzyme, with greatly diminished the 3' → 5' exonuclease activity, the reaction pH is now adjusted to 7.6 which is the pH optimum for the polymerase activity. This allows routine incorporation of radiolabeled nucleotide at greater than 60% in less than 30-60 minutes, providing DNA probe specific activities of greater than 109 dpm/µg DNA.

II. PRINCIPLE OF RANDOM PRIMER EXTENSION

Random oligonucleotide primers were originally derived from DNase I digestion of calf thymus DNA. Fractionation of this digest allowed the recovery of short oligonucleotides consisting primarily of hexamers of random sequences. With the development of DNA synthesizers these random hexanucleotides can now be more efficiently synthesized and sized to a specific length. The Random Primer Extension Buffer contained in this kit includes enough of these random hexamer primers to enable hybridization of a hexamer to every region within the template DNA thereby eliminating the requirement for primers that are strictly complementary to a unique target DNA sequence. The hybridized hexamer is recognized by Klenow fragment and elongation of the hexamer synthesizes a complementary strand of DNA. When one or more deoxynucleoside triphosphates is radioactively labeled, the newly synthesized strand will also be radioactive. Labeled DNA prepared in this manner can be used a DNA probe.

III. EXPLANATION OF THE SYSTEM

The NEN's Random Primer Extension Labeling System (NEP103) contains the large fragment of DNA polymerase I (Klenow fragment), water, control DNA, buffer containing random hexamer oligonucleotide primers and nonradioactive deoxynucleoside triphosphates (minus dCTP). For maximum flexibility, the choice of isotope is left to the researcher (see cover page). The kit is designed to be used with either [α-^{32}P] dCTP, [^{35}S]dCTPαS, [^{3}H]dCTP or [^{125}I]dCTP.

IV. KIT COMPONENTS

<u>This system contains research and development reagents for Laboratory use only, and is not for use in humans or for clinical diagnosis.</u> The components provided in the Random Primer Extension Labeling System allow for at least 20 reactions.

1. <u>5X Random Primer Extension Buffer:</u>

   Two vial containing 150µl random hexanucleotides in a buffer containing Tris-HC1, (pH 7.6), magnesium chloride, bovine serum albumin and 2-mercaptoethanol is provided ready for use. The preparation is stable for at least 3 months at -20°C. Repeated freeze thaw cycles have limited effect on the performance of this preparation.

2. <u>5X DEOXYNUCLEOSIDE TRIPHOSPHATE MIXTURE (minus dCTP):</u>

   Two vial containing 150µl of a mixture of dNTPs at a concentration of 100 µM each in water is provided ready for use. The solution provided in this kit lacks dCTP and contains dATP, dGTP, and TTP. The preparation is stable for at least 3 months at-20°C. Repeated freeze thaw cycles have limited effect on the performance of this preparation.

3. <u>LARGE FRAGMENT DNA POLYMERASE I, (KLENOW FRAGMENT):</u>

   One vial containing 150 µl of Klenow fragment at 1.5-2.5 units/µl in a buffer containing Tris-HC1, pH 7.5, 2-mercaptoethanol and 50% glycerol is provided ready for use. The preparation is stable for at least 3 months at-20°C. Do not allow this solution to warm above 4°C at any time or significant loss of enzyme activity will result. It is strongly recommended that this enzyme solution be removed from the freezer and put directly on ice immediately prior to its addition to the reaction mixture and returned to -20°C immediately after use.

4. <u>CONTROL DNA:</u>

   One vial containing 50 µl of linearized pBR322 at 10 µg/ml is supplied.

5 <u>WATER:</u>

   One vial containing 2 mL of deionized water is supplied. This component is provided to minimize interfering substances (e.g. metals, ions, and enzymes) present in some water supplies that might inhibit enzymatic reactions.

V. ADDITIONAL SUPPLIES AND EQUIPMENT NEEDED

Vortex mixer
Pipetting equipment
Waterbath for 90-100°C denaturation of template DNA
Plastic gloves
Polypropylene microcentrifuge tubes
Sorvall@ Microspin or equivalent microcentrifuge
Methanol
Water
Isotope

VI. PRIMER EXTENSION REACTION PROTOCOL

1. Linearized DNA is required for efficient random primer extension. If the DNA is a circular or supercoiled plasmid, the DNA must first be cut with a restriction endonuclease to produce linear DNA. The amount of DNA usually used in a primer extension reaction is 25-50 ng in 7-12 µl.

2. Denature a 2-3 fold excess of DNA by heating the sample in a boiling water bath for 2-5 minutes and chill on ice. The microcentrifuge tube is centrifuged briefly to collect sample on the bottom of the tube.

3. Add 25-50 ng of the denatured DNA (in a volume no greater than 12µl) to 6 µl 5X Random Primer Extension Buffer, in a 1.5 ml polypropylene microcentrifuge tube.

4. Add 6 µl of 5X Deoxynucleotide Triphosphate Mixture to the reaction tube.

(NOTE: This mixture is a minus dCTP solution, e.g., 100 µM dATP, dGTP, TTP (each). If another labeled nucleoside triphosphate or a mixture of several labeled nucleoside triphosphates are to be used in the reaction, replace this solution with a solution minus the corresponding labeled nucleoside triphosphates.)

5. Add the required amount of water to the reaction tube, such that the final reaction volume will be 30 µl.

6. Add 5-10 µl of radiolabeled dCTP to the reaction tube.

(NOTE: 50 ng of DNA contains only 40 pMol of dC residues, the amount of 'radiolabeled dCTP should not exceed this molar level by more than 10-20% to obtain maximal per cent incorporation.)

The reaction is started by adding 1 µg of Klenow fragment to the reaction solution.

8. Mix and centrifuge briefly. The reaction is allowed to proceed for minimally 30 to 60 minutes at room temperature.

(NOTE: The reaction pH has now been increased to 7.6, which allows for a much faster DNA labeling reaction. The reaction mixture can be left for several hours with no loss of incorporated radioactive dCTP or overnight with only minimal (<10%) breakdown of radiolabeled DNA.)

9. The reaction is terminated by the addition of 5 µl of a 250 mM EDTA solution. Purify immediately on cartridge or freeze sample.

VII. REAGENT SUMMARY

REAGENT		VOLUME (µl)
5X Random Primer Extension Buffer		6.0
DNA	X =	7.0 - 12.0
5X Deoxynucleoside Triphosphate Mixture		6.0
Labeled dCTP, (^{32}P, ^{35}S, ^{125}I, or ^{3}H)*	Y =	5.0 - 10.0
Water		30 - (13 + X + 4)
Klenow Enzyme (DNA Polymerase I)		1.0
Final reaction volume		30.0µl

Mix and centrifuge briefly to collect reagents in the bottom of tube. The labeling reaction should be 30 - 60 minutes at room temperature, minimally.

Stop the reaction by adding 5 µl of a 0.1M EDTA solution.

*SPECIAL NOTE: If isotope is received in ethanol: water the material must be taken to dryness under a stream of nitrogen or under vacuum and subsequently reconstituted with water.

ADDITIONAL NOTE: The addition of reagents does not need to be done in any particular order as long as Klenow fragment is added last to initiate the reaction. The volume of the DNA solution to be added in the reaction mixture must be in the range of 7.0-12.0 µl. The amount of tracer to be added to the reaction mixture may also be adjusted to be within the range of 5.0-10.0 µl. These are the only two parameters that may be adjusted as long as the final reaction volume does not exceed 30µl.

VIII. PRIMER EXTENSION REACTION PROTOCOL FOR LABELING RESTRICTION FRAGMENT IN LOW MELTING POINT AGAROSE

1. Cut circular DNA with restriction enzyme(s) to obtain a linear DNA molecule.

2. Separate the restriction fragments by agarose gel electrophoresis using low melting point agarose. (we recommend using concentrations of agarose 2% or less. Above this concentration, inhibition of the reaction has been observed.)

3. Visualize the restriction digest by a standard method (e.g. ethidium bromide staining) and excise the band of interest with the minimum amount of extraneous agarose.

4. Place the band(s) in a pre-weighed microcentrifuge tube and add distilled water at a ratio of 1.5:1 (w/w) agarose. Determine the final concentration of DNA. You will need between 2.5-5.0 ng DNA/gl final concentration

5. Heat the tube in a boiling water bath for 7-10 minutes to melt the agarose and denature the DNA.

6. Transfer the tube to a 30°C water bath for a minimum of 10 minutes before adding to the reaction mixture. Alternatively, the restriction fragment can be stored at -20°C; repeat steps 5 and 6 before each subsequent primer extension reaction.

7. Refer back to Section VI and proceed with steps 3-9.

8. Purify by method of choice.

X. REFERENCES

1. Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., 132; 6-13.

2. Feinberg, A. P. and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum Anal. Biochem., 137; 266-267.

3. Johnson, M.T., Read, B.A., Monko, A.M., Pappas, G., and Johnson, B.A. (1986). A convenient, new method for desalting, deproteinizing, and concentrating DNA or RNA. Biotechniques 4; (1), 64-70.

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