

Random Primer Extension Labeling

1. Introduction

The basic technique for primer extension labelling was first introduced by Feinberg and Vogelstein, and used 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 of DNA polymerase I. Because Klenow fragment lacks the 5' → 3' exonuclease activity, the use of Klenow fragment in primer extension avoids the loss of incorporated label. 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.

2. Principle

Random oligonucleotide primers were originally derived from DNase I digestion of calf thymus DNA. With the development of DNA synthesizers, random oligonucleotides can be more efficiently synthesized and sized to a specific length. The ability to synthesize random oligos of various lengths has allowed optimization of the primer length in random oligonucleotide priming. Hybridized primers are recognized by Klenow fragment, which elongates the primer to synthesize 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 as a DNA probe.

3. Radiochemical

The choice of radioisotope is left to the researcher. The protocol is designed to be used with either [α -³²P] dCTP, [³⁵S] dCTP α S, [³H] dCTP, or [¹²⁵I] dCTP.

| Chemical | Isotope | Specific activity | Catalog number |
|---|------------------|-------------------|----------------------------|
| α -dCTP (labeled on the alpha phosphate) | ³² P | 800 Ci/mmol | NEG/BLU013A NEG/BLU513A |
| | ³² P | 3000 Ci/mmol | NEG/BLU013H NEG/BLU513H |
| | ³² P | 6000 Ci/mmol | NEG/BLU013Z NEG/BLU513Z |
| dCTP α S | ³⁵ S | 1250 Ci/mmol | NEG036H |
| dCTP | ³ H | 40-70 Ci/mmol | NET601A |
| | ¹²⁵ I | 2200 Ci/mmol | NEX074 |

4. Materials needed

- Radiolabeled nucleotide appropriate for assay (refer to above table)
- Klenow fragment enzyme (1.5 – 2.5 U/ μ L)
- 5X reaction buffer containing random primers
- Deoxynucleoside Triphosphate mixture (containing 100 μ M each of dATP, dGTP, and dTTP but lacking dCTP)
- Purified water
- Template DNA

5. Primer extension reaction

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 μ L.
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 12 μ L) to 6 μ L of 5x reaction buffer in a microfuge tube.
4. Add 6 μ L of 5x triphosphate mixture to the tube.

Note: This mixture is a minus dCTP solution, e.g. 100 μ M dATP, dGTP, and dTTP 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 nucleoside labeled triphosphates.

5. Add the required amount of deionized water to the reaction tube such that the final reaction volume will be 30 μ L.
6. Add 5-10 μ L 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 percent incorporation.

7. The reaction is started by adding 1 μ L of Klenow fragment to the reaction.
8. Mix and centrifuge briefly. Incubate at the appropriate temperature for the appropriate length of time as recommended by the enzyme supplier.
9. The reaction is terminated by freezing or by the addition of 5 μ L of a 250 mM EDTA solution.

Reaction summary:

| <u>Reagent</u> | <u>Volume (μL)</u> |
|--|-----------------------------------|
| 5x reaction buffer | 6 μ L |
| DNA plus distilled water | 7 - 12 μ L |
| 5X deoxynucleoside triphosphate mixture | 6 μ L |
| Labeled dCTP (32 P, 35 S, 125 I, or 3 H)* | 5 – 10 μ L |
| Klenow fragment enzyme | <u>1 μL</u> |
| Final reaction volume | 30 μ L |

Mix and centrifuge briefly to collect reagents to bottom of tube. Stop reaction by freezing or adding 5 μ L 250 mM EDTA.

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

Additional note: The addition of reagents does not need to be done in any particular order as long as the Klenow fragment is added last to initiate the reaction. The volume of DNA to be added must be in the range of 7 – 12 μ L. The amount of tracer to be added to the reaction mixture may also be adjusted to be within the range of 5 – 10 μ L.

6. Primer extension protocol for labelling 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 microfuge 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 and 5 ng of DNA/ μ L final concentration.
5. Heat the tube in a boiling water bath for 7 -10 minutes to melt the agarose and denature the DNA.
6. Immediately after boiling, add an aliquot of denatured DNA (25-50 μ g) into a vial containing the rest of the reaction mixture. Alternatively, add the reaction mixture containing the random primers then the rest of the reagents to the vial containing the denatured DNA. Let the mixture cool to room temperature, then add the enzyme. If the reaction mixture has a tendency to re-gel, incubate the mixture at higher temperature. Any residual DNA in agarose may be stored at -20°C for future labelling. Steps 5 and 6 must be performed before each subsequent primer extension reaction.
7. Refer back to Section 5 and proceed with steps 3-9.

7. References

1. Feinberg, A.P. & Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem* **132**, 6-13 (1983).
2. Feinberg, A.P. & Vogelstein, B. "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity". Addendum. *Anal. Biochem* **137**, 266-267 (1984).
3. Johnson, M., Read, B., Monko, A., Pappas, G. & Johnson, B. A convenient, new method for desalting, deproteinizing, and concentrating DNA or RNA. *BioTechniques* **4**, 64-70