NEBlot® Kit

Instruction Manual

NEB #N1500S/L
Store at –20°C

NEBlot® Kit

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Kit Includes:

	#N1500S	#N1500L
Octadeoxyribonucleotides in 10X Labeling Buffer	150 µl	300 µl
dATP (0.5 mM in Tris-HCl pH 7.0)	50 µl	125 µl
dCTP (0.5 mM in Tris-HCl pH 7.0)	50 µl	125 µl
dGTP (0.5 mM in Tris-HCl pH 7.0)	50 µl	125 µl
dTTP (0.5 mM in Tris-HCl pH 7.0)	50 µl	125 µl
Klenow Fragment (3´→ 5´ exo⁻) (5 units/µl)	125 units	250 units
Control DNA (Hind III digested lambda DNA; 25 ng/µl)	20 µl	50 µl
Nuclease-free dH_2O	1.8 ml	1.8 ml

Introduction:

The New England Biolabs NEBlot® Kit is designed to produce labeled DNA probes with a specific activity of >1 x 10^9 dpm/µg. Using the method of Feinberg and Vogelstein (1,2), random sequence octadeoxynucleotides serve as primers for DNA synthesis *in vitro* from denatured double-stranded template DNA. One labeled deoxyribonucleotide is used in the dNTP reaction mixture and is incorporated during primer directed DNA synthesis by DNA polymerase. The resulting labeled DNA can be used as hybridization probes for screening gene libraries (3), probing of Southern and Northern blots (4,5), and *in situ* hybridization (6).

The method permits the labeling of small quantities of DNA (< 25 ng). It also enables the labeling of DNA in the presence of low-melting-point (LMP) agarose.

The NEBlot® Kit is available in two sizes. Order catalog #N1500S for reagents sufficient for 25 reactions and catalog #N1500L for reagents sufficient for 50 reactions.

Discussion:

The NEBlot® Kit is designed to generate high specific activity probes (>10^9 dpm/µg) from minimal amounts of DNA (< 25 ng). Linearized double stranded template DNA is first denatured, then random octadeoxyribonucleotide sequences are added. The random sequences anneal to the denatured template and serve as primers for DNA synthesis by the DNA Polymerase I - Klenow Fragment (3´→ 5´ exo⁻).

Optimization

Quantities of DNA from 5 ng to 2 µg have been labeled without varying the standard NEBlot® Kit protocol. However, the reaction conditions are optimized for labeling 25 ng to 100 ng of DNA to specific activity of (> 1 x 10^9 dpm/µg) using the recommended 50 µCi of ^{32}P radiolabel. To label quantities of DNA less than 25 ng, an incubation time greater than one hour is recommended. To label quantities of DNA greater than 100 ng, add more radiolabel if a specific activity of > 1 x 10^9 dpm/µg is required (see Figure 1, page 4). High specific activity ^{32}P probes degrade quickly due to radiolysis and should be used immediately.

Preparation of Template

The template DNA should be linear. Labeling only the insert DNA, rather than insert and vector will enhance the specificity of the resultant probe. It is essential that template DNA be DENATURED initially as outlined in the NEBlot® Kit protocol. If the template is not denatured, incorporation is greatly reduced (see Figure 1, page 4). The volume of the DNA solution will depend on the volume of labeled nucleotide solution required; the combined volume of the two should not exceed 38 µl.

Table 1: Recommended Incubation Times for Standard 50 µl Reactions

LABELED NUCLEOTIDE	SPECIFIC ACTIVITY	REACTION TIME
[α^{32}P]dCTP	3000 Ci/mmol	60 minutes
[^{3}H]dCTP	50 Ci/mmol	20 hours
[α^{32}P]dATP	3000 Ci/mmol	60 minutes
[α^{35}S]dCTPαS	1000 Ci/mmol	6 hours
Agarose/DNA		6 hours

Specific activities of probes labeled with 50 µCi of [^{3}H]dCTP or [α^{35}S]dCTPαS will be less than 1 x 10^{9}.
Label dNTPs supplied in 50% ethanol should be evaporated to dryness and resuspended in dH_2O before use in the reaction.

Choice of Label

NEBlot® Kit can be used with the labeled nucleotide of your choice. Listed below are guidelines for the use of some common nucleotide triphosphates used in labeling reactions.

Random Priming Reaction:

Allow system components to thaw on ice. Klenow enzyme, however should be kept at –20°C except when being used.

Standard 50 µl Reaction using ^{32}P dCTP

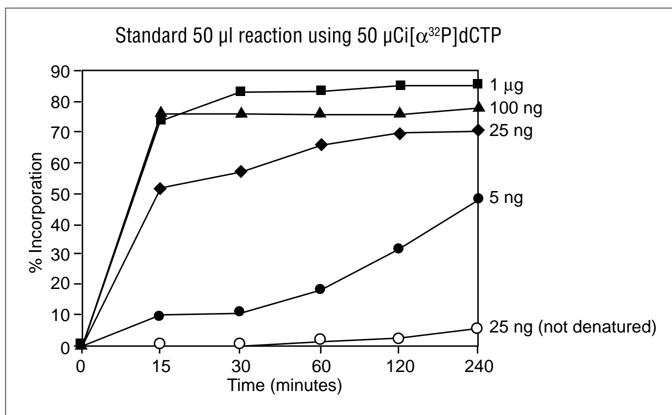
For a standard 50 µl reaction, total volume of DNA and label should be 38 µl.

In a microcentrifuge tube:

1. Dissolve 25 ng of template DNA in nuclease free H_2O (1–33 µl).
   For the control reaction, add 1 µl of control DNA to 32 µl of H_2O
2. Denature in **boiling H_2O bath** for 5 minutes (see Figure 1, page 7).
3. Quickly place in ice for 5 minutes.
4. Centrifuge briefly in the cold.
5. Add the following reagents to your DNA in the order listed.
   5 µl Octadeoxyribonucleotides in 10X Labeling Buffer
   6 µl dNTP mixture (2 µl of dATP, dTTP, and dGTP)
   5 µl α ^{32}P dCTP (3,000 ci/mmol, 50 µCi)
   1 µl DNA Polymerase I - Klenow Fragment (3´→ 5´ exo^{-}) (5 units)
6. Incubate at 37°C for 1 hour (see Figure 1, page 4 for other labels)
   Incubation time for DNA/Agarose 6 hours for ^{32}P [ ]
7. Terminate reaction by adding 5 µl of 0.2 M EDTA (pH 8.0).

We recommend purification of probes to reduce non-specific background and limit exposure of lab personnel to high levels of radioactivity during hybridization experiments.

Figure 1. Incorporation of Label at Varying Template Concentrations



Purification and Use of Labeled Probes

Probes synthesized can be separated from unincorporated nucleotides by gel filtration on Sephadex® G-50, Spin Column Elutips® or similar size exclusion media. Following purification, labeled DNA should be prepared for hybridization as follows:

1. Denature by heating in boiling H_2O bath 95–100°C for 5 minutes.
2. Quickly place in ice bath for 5 minutes. DNA may be used directly in hybridization experiment or store at –20°C.

Preparation of DNA in LMP Agarose:

1. Digest DNA with appropriate restriction endonuclease(s).
2. Purify restriction digest by electrophoresis using a low-melting-point agarose (recombinant DNA grade) gel containing 0.5 µg/ml of ethidium bromide.
3. Excise desired band and place in a pre-weighed conical tube.
4. Add 3 ml H_2O per gram of gel slice.
5. Incubate at 70°C for 2 minutes or until gel is completely melted. Place aliquot to be used in microcentrifuge tube.

   Up to 25 µl of the DNA/Agarose solution may be used in each oligolabeling reaction.
6. Denature DNA in boiling H_2O bath at 95–100°C for 5 minutes.
7. Transfer to 37°C bath and incubate for 10 minutes. Centrifuge briefly (at room temperature).
8. Proceed with addition of reagents for oligolabeling reaction, as outlined on page 3.

Determination of % Incorporation:

TCA Precipitation Method

1. Remove a 1 µl sample from the reaction mixture and dilute to 100 µl with 0.2 M EDTA.
2. Spot 5 µl of the diluted sample onto each of two glass fiber filters, nitrocellulose discs or 3 mm paper discs.

   One of the filters is used to measure total activity in the reaction. The other filter is used to measure only acid-precipitable radioactivity. Under these conditions DNA more than 50 nucleotides long will be precipitated on the surface of the filter.
3. Add one filter to a beaker containing 100 ml of ice-cold 5% Trichloroacetic acid (TCA) and 1% Sodium Pyrophosphate. Let sit for 10 minutes.

   TCA is extremely corrosive and should be handled carefully.
4. Wash two times with fresh, cold TCA/Pyrophosphate solution.
5. Remove the filter from TCA/Pyrophosphate solution. Place the filter in a beaker containing 200 ml of ethanol or isopropanol and agitate briefly.
6. Remove the filter from the alcohol and dry both filters under a heat lamp for 15 minutes.

7. Place each of the filters (washed and unwashed) into scintillation vials.
8. Measure radioactive decay as shown on page 6.

Absorption to DE-81 Filter Method

1. Remove a 1 µl sample from the reaction mixture and dilute to 100 µl with 0.2 M EDTA.
2. Spot 5 µl of the diluted sample onto each of two DE-81 (2.4 cm) filters.

   One of the filters is used to measure total radioactivity in the reaction and is not subsequently washed. The other filter is used to measure radioactivity incorporated.

3. Dry the filters under a heat lamp for 10 minutes.
4. Add one filter to a beaker containing 100 ml of 0.5 M Na_2HPO_4. Wash for 5 minutes using mild agitation. Wash two additional times with fresh 0.5 M Na_2HPO_4.
5. Next wash the filter twice with dH_2O then repeat wash two additional times with ethanol.
6. Dry filter under a heat lamp for 15 minutes then place in scintillation vial.
7. Measure radioactive decay as shown on page 6.

Measurement of Radioactive Decay:

It is important that the user be aware that the efficiency with which radioactive decay is measured varies according to the method used, the isotope incorporated, and the individual instrument in use.

Use One of the Following Methods

a. Dissolve the nitrocellulose filters in 10 ml of toluene based liquid scintillation cocktail.

b. Add 10 ml of a toluene based liquid scintillation cocktail directly to the glass fiber filter or paper filter and count using the appropriate channel of the liquid scintillation counter.

c. To measure the radioactivity directly from the dry filters (Cerenkov counting) use the tritium channel of the liquid scintillation counter to measure ^{32}P decay.

Calculation of Probe Specific Activity:

$$\text{Fraction of Radioactivity Incorporated (F)} = \frac{\text{Acid-precipitable Counts}}{\text{Total Counts}}$$

$$\text{\% Incorporation} = \text{Fraction of Radioactivity Incorporated (F)} \times 100\%$$

$$\text{Specific Activity (dpm/}\mu\text{g)} = \frac{\text{Acid-precipitable Counts} \times 10^3}{\text{Input DNA + Newly Synthesized DNA}}$$

$$= \frac{(\mu Ci)(2.2 \times 10^9)(F)}{Mi + [(1.3 \times 10^3)(F)(\mu Ci/Sn)]}$$

μCi = μCi radiolabeled nucleotide in reaction mixture.

F = Fraction of input label incorporated into DNA.

Mi = Mass of input DNA (ng)

Sn = Specific Activity of d[ ]TP expressed in (μCi/nmol)

The factor 2.2×10^9 in the numerator represents the conversion of μCi to dpm. [(μCi) 2.2×10^6 = dpm] multiplied by 10^3 to convert dpm/ng to dpm/μg.

The factor 1.3×10^3 in the denominator represents 4 times the average molecular weight of the four dNMPs [(4)(325) = 1.3×10^3]

The denominator of the specific activity calculation represents the total amount of DNA present after the reaction.

Mi (Input DNA) + [(4)(325)(F)(μCi/Sn)] (newly synthesized DNA)

For an approximation of specific activity, omit Newly Synthesized DNA from the denominator of the specific activity calculation.

References:

1. Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6–13.
2. Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.* 137, 266–267.
3. Grunstein, M. and Hogness, D.S. (1975) *Proc. Natl. Sci.* USA 72, 3961–3965.
4. Southern, E.M. (1975) *J. Mol. Biol.* 98, 503–517.
5. Smith, G.E. and Summers, M.D. (1980) *Anal. Biochem.* 109, 123–129.
6. Hasse, A. et al. (1984) *Methods of Virology 7*, 189–226.

Quality Control Specifications:

Each lot of the NEBlot® Kit is assayed to ensure its ability to label 25 ng of control DNA to a specific activity > 1×10^9 dpm/µg.

Storage recommendations: Avoid multiple freeze-thaw cycles.
Store at –20°C.

Ordering Information

PRODUCT	NEB #	SIZE
NEBlot® Kit	N1500S/L	25/50 Reactions
KIT COMPONENTS SOLD SEPARATELY		
Klenow Fragment (3´→5´ exo⁻)	M0212S	200 units, 5,000 units/ml
Klenow Fragment (3´→5´ exo⁻)	M0212M	1,000 units, 50,000 units/ml
Klenow Fragment (3´→5´ exo⁻)	M0212L	1,000 units, 5,000 units/ml
Octadeoxyribonucleotides in 10X Labeling Buffer	N1501S	25 reactions/ 400 mg/ml
Octadeoxyribonucleotides in 10X Labeling Buffer	N1501L	50 reactions/ 400 mg/ml

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