DECAprime™ II Kit

(Part Number AM1455, AM1456)

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I. Introduction

A. Overview of the Procedure

The Ambion® DECAprime™ II Random Primed DNA Labeling Kit is designed for rapid production of radiolabeled probes for a variety of uses such as Northern, Southern, and dot blotting. The kit is optimized to produce DNA probes with high specific activity in just 10 min. The reaction is performed using random decamer (10-mer) oligonucleotide primers and exonuclease-free Klenow enzyme (Exo–Klenow). The DECAprime II Kit is available in either 30 or 100 reaction sizes, and offers the convenience of using either radiolabeled dCTP or dATP (not supplied).

The DECAprime II Kit is based on methodology developed by Feinberg & Vogelstein (1983). A mix of all possible decamers is hybridized to the DNA template by heating at 100°C for 10 min, then flash-freezing. The complementary strand is then synthesized from the 3'-hydroxyl termini of random decamer primers by adding buffer, the nucleotides (three nonlabeled, one radiolabeled) and Exo– Klenow. The 3' to 5' editing function of Ambion Exo– Klenow is genetically engineered to remove all exonuclease activity (Derbyshire et al., 1988). The absence of exonuclease activity dramatically increases the stability of probes during the synthesis reaction (no decrease in specific activity over time), so that very small amounts of template DNA (25 ng) can be used. The Exo– Klenow incorporates labeled nucleotide ([α-^{32}P] dNTP) very efficiently, resulting in probes with specific activities routinely $\geq 1 \times 10^9$ cpm/µg, and labels even small fragments of ≤500 bp effectively. A control DNA is included for testing the performance of the system.

B. Reagents Provided with the Kit and Storage

For storage at –20°C, use non frost-free freezer.

30 Rxns P/N AM1455	100 Rxns P/N AM1456	Component	Storage
30 µL	100 µL	Exo– Klenow (5 Units/µL)	–20°C
75 µL	250 µL	10X Decamer Solution	–20°C
150 µL	500 µL	5X Reaction Buffer minus dATP (–dATP)	–20°C
150 µL	500 µL	5X Reaction Buffer minus dCTP (–dCTP)	–20°C
10 µL	10 µL	DECAtemplate™ GAPDH-M (10 ng/µL)	–20°C
400 µL	400 µL	0.5 M EDTA	–20°C
1 mL	1 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C, or room temp.

C. Materials Not Provided with the Kit

- Linear template DNA
- [α-^{32}P]dCTP or [α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/mL)
- TE (10 mM Tris-HCl, pH 8, 1 mM EDTA)
- (optional) 10% trichloroacetic acid (TCA)
- (optional) Carrier nucleic acid for TCA precipitation (e.g. salmon sperm DNA or yeast RNA)
- (optional) Materials for G-25 Sephadex™ spin column
- (optional) 5 M ammonium acetate and 100% ethanol

D. Related Products Available from Applied Biosystems

Product	Description
DECAtemplates™ See web or print catalog for P/Ns	Gel-purified plasmid inserts ready for use in random-primed labeling reactions. The insert sequences are derived from mouse, but they will also hybridize with rat and human sequences.
NorthernMax® Kits P/N AM1940, AM1946	Ambion NorthernMax Kits: NorthernMax, and NorthernMax-Gly, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time.
ULTRAhyb® Ultrasensitive Hybridization Buffer P/N AM8670	Ultra sensitive hybridization solution that provides ten- to one hundred-fold greater sensitivity than standard hybridization buffers.
NucAway™ Spin Columns P/N AM10070	Guaranteed RNase- and DNase-free, Ambion's NucAway Spin Columns provide a fast, efficient way to remove unicorporated nucleotides, and to effect buffer exchange after probe synthesis and other reactions.

II. DECAprime II Procedure

A. Amount of Template DNA

The amount of probe synthesized, the specific activity of the probe, and the reaction rate are all affected by the amount of template DNA used in the reaction. Adding more template DNA increases the reaction rate and the amount of probe synthesized, but decreases the specific activity of the probe. For example, after a 10 min incubation about 15% of the ^{32}P labeled dATP will be incorporated into probe when 6.25 ng of template DNA is used, about 45% is incorporated using 12.5 ng of template and about 70% incorporation is achieved using 25, 50 or 100 ng of template (Figure 1). However, the specific activity of the probe synthesized with 6.25 ng of template DNA is about 2.5 times higher than that of the probe synthesized with 100 ng template. This ratio increases to 5:1 after a 6 hr incubation period (Figure 2).

We recommend 25 ng as the ideal amount of template DNA for optimizing probe specific-activity, yield and reaction time. For synthesis of larger amounts of probe, it is better to scale up the standard reaction, rather than to simply increase the template DNA quantity, so that probe specific-activity remains high.

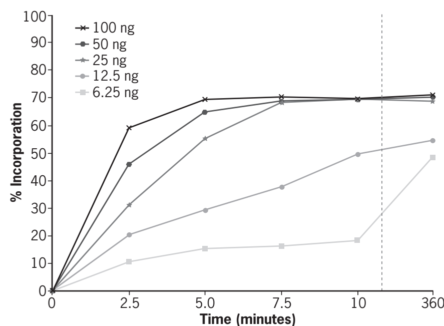

Figure 1. Kinetics of Polymerization using DECAprime II

Time course of incorporation of [α-^{32}P]dATP in a DECAprime II reaction using the indicated amounts of a 3 kb linearized plasmid template. Aliquots were removed at the indicated times to assess total and acid precipitable counts.

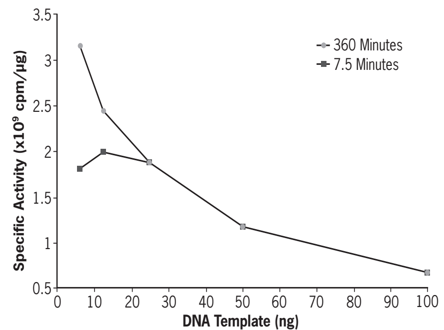

Figure 2. DNA Template Concentration and Probe Specific Activity

The specific activity of probes produced using decreasing amounts of a 3 kb template DNA in DECAprime II reactions was determined. Probe specific-activity decreases with increased DNA template input.

B. Preparation of Template DNA

IMPORTANT

It is important to linearize plasmid template DNA before labeling; supercoiled DNA is labeled inefficiently.

1. Dilute template DNA to approximately 1–2.5 ng/µL

Dilute linear template DNA in water or TE to a concentration of about 1–2.5 ng/µL. The standard DECAprime II reaction can accommodate approximately 6–100 ng of DNA, in a volume of up to 11.5 µL.

2. Add 2.5 µL of 10X Decamer Solution, and incubate at 95–100°C for 3–5 min

Denature template DNA by mixing approximately 25 ng of linearized DNA template (in TE or water, in a volume not to exceed 11.5 µL) with 2.5 µL of 10X Decamer Solution and heating in a heat block or water bath at 95–100°C for 3–5 min.

3. Snap-freeze the mixture, then thaw and place on ice

Freeze the denatured DNA/decamer mixture in liquid nitrogen, powdered dry ice, or dry ice/ethanol. This will prevent self-annealing of the template DNA to allow more efficient decamer binding, thus increasing product yield.

Thaw the mixture, briefly centrifuge to collect sample, then place on ice.

C. Probe Synthesis Reaction

1. Assemble the Klenow reaction on ice

On ice, add the following components to the denatured DNA/decamer mixture. Use the appropriate 5X Reaction Buffer for the labeled nucleotide being used; –dCTP 5X Reaction Buffer with [α–^{32}P]dCTP, and –dATP 5X Reaction Buffer with [α–^{32}P]dATP.

NOTE

We recommend using –20°C storage-type labeled dNTPs. The stabilizer used in 4°C storage type dNTPs can inhibit DECAprime II reactions when >5 µL dNTP is added.

Amount	Component
– µL	Denatured DNA/Decamer Mix (from previous step)
5 µL	5X Reaction Buffer (–dCTP or –dATP)
5 µL	[α-^{32}P]dCTP or [α-^{32}P]dATP
to 24 µL	Water*
1 µL	Exo– Klenow

* Additional labeled nucleotide may be substituted for all or part of the water.

2. Mix reaction gently

Mix contents of tube by gentle flicking or pipetting.

3. Incubate at 37°C for 5–10 min

Incubate the labeling reaction for 5–10 min at 37°C. If less than 12.5 ng of template DNA is used, the incubation can be extended up to 6 hr to maximize radiolabel incorporation (see Figure 1 on page 3).

4. Add 1 µL of 0.5 M EDTA to stop the reaction

Stop the reaction by adding 1 µL of 0.5 M EDTA.

5. (optional) Remove an aliquot of the crude reaction mix

At this point an aliquot of the reaction can be removed to determine the specific activity of the probe (see section III.B on page 7). This should be done before removing unincorporated nucleotides.

NOTE

The probe can generally be used for hybridization without removal of unincorporated nucleotides, since the efficiency of incorporation of radiolabeled precursor into probe is high, approximately 40% after 5 min and 70% after 10 min when using 25 ng of template DNA. However, to obtain the lowest background, we recommend separating the probe from the unincorporated nucleotides (see section III.A).

6. Use the probe or store it at –20°C

The probe is now ready for use (see section *III.D. Preparing Probes for Hybridization* on page 9) or storage. Due to the high specific activity of the probe, radiolytic degradation will occur rapidly. It is therefore advisable to use the probe within 1 week after synthesis. The probe may be stored at –20°C until use.

III. Additional Procedures

A. Removal of Unincorporated Nucleotides

Below are two procedures for removing unincorporated nucleotides. In addition, Ambion® offers the NucAway™ Spin Column for removal of unincorporated nucleotides and salts after probe sythesis.

Ethanol precipitation

1. Add ammonium acetate to a final concentration of 0.5 M (for example add 2.5 µL of 5 M ammonium acetate to a 25 µL labeling reaction) and mix well.
2. Add 2 volumes of 100% ethanol (for example, add 55 µL ethanol in example above) and mix well.
3. Chill tube on ice for 15 min.
4. Centrifuge the tube for 15 min at maximum speed (~12,000 rpm).
5. Carefully remove the supernatant by gentle aspiration.
6. Wash the pellet in 500 mL of 70% ethanol and microfuge the tube for 5 min at maximum speed.
7. Carefully remove the supernatant by gentle aspiration then centrifuge the tube again for a few seconds and remove the residual supernatant.
8. Resuspend the pellet in about 50 µL of TE and store at –20°C until use.

Spin-column preparation and use

Unincorporated labeled nucleotides can be removed by size exclusion chromatography on Sephadex G-25 or G-50 spin columns. The following is a procedure for the preparation and use of spin columns:

1. Resuspend and equilibrate Sephadex G-25 or G-50 resin with 2 volumes of TE, then wash with several volumes of TE.
2. Resuspend the washed resin in 1.5 volumes of TE in a glass bottle and autoclave. Store at 4°C until use.
3. Rinse a 1–3 mL spin column thoroughly with distilled water; frits may be pre-installed, or made by plugging the bottom of a 1 mL syringe with a support such as siliconized glass beads.
4. Mix the prepared resin to resuspend before use.
5. Pipet 1–3 mL of the prepared resin into the washed spin column. Place the column in a 15 mL plastic centrifuge tube and spin at 2,000 rpm for 10 min in a centrifuge with a swinging-bucket rotor.
6. Place the end of the spin column containing the spun resin into an appropriate microfuge tube (typically 0.5 mL) and insert the assembly into a new 15 mL centrifuge tube.

7. Load 20–100 µL of the sample onto the center of the resin bed (dilute sample with water or TE if necessary), and spin at 2,000 rpm for 10 min. The eluate collected in the microfuge tube should be approximately the same volume as the sample loaded onto the column, and it will contain about 75% of the nucleic acid applied to the column.

NOTE

The centrifugation conditions for column preparation and sample purification should be identical; varying them could lead to either incomplete recovery or dilution of the sample. The spin column can be tested by loading it with 100 µL of TE, and centrifuging; 100 µL of eluate should be recovered. If recovery is much greater or less than 100 µL, the column is not equilibrated.

B. TCA Precipitation to Determine Radiolabel Incorporation

1. Dispense 150 µL of carrier DNA or RNA (1 mg/mL) into a nuclease-free 1.5 mL microfuge tube. (Ambion Sheared Salmon Sperm DNA, P/N AM9680, can be used.)
2. Add 2 µL of the DECAprime II reaction (***before*** removal of unincorporated nucleotides) and mix thoroughly.
3. Transfer 50 µL of the diluted DECAprime II reaction from step 2 to aqueous scintillation cocktail and count in a scintillation counter. This will measure the total amount of radiolabel present in the reaction mixture (unincorporated and incorporated counts).
4. Transfer another 50 µL of the diluted DECAprime II reaction from step 2 to a 12 x 75 mm glass tube, and add 2 mL of cold 10% trichloroacetic acid (TCA). Mix thoroughly and place on ice for 10 min. This will precipitate nucleic acids, but not free nucleotides.
5. Collect the precipitate via vacuum filtration through a Whatman® GF/C glass fiber filter (or its equivalent).
6. Rinse the tube twice with 1 mL of 10% TCA and then rinse once with 3–5 mL of 95% ethanol. Pass each of the rinses through the GF/C filter.
7. Place the filter in a scintillation vial, add aqueous scintillation cocktail, and count in a scintillation counter. The number will reflect the amount of radiolabel that was incorporated.
8. Multiply the cpm measured in step 7 by 1.5 to calculate the cpm/µL of probe.
9. Divide the cpm in step 7 by the cpm in step 3 to determine the fraction of label incorporated (multiply by 100 for percent incorporation).
10. Multiply the TCA precipitable cpm/µL (from step 8) by 26 (final volume of reaction + EDTA) to calculate the total number of cpm of probe synthesized.

C. Calculating Probe Specific-Activity

Probe specific-activity is the ratio of the counts of TCA precipitable product (cpm) to the total mass (µg) of DNA in the sample.

total DNA mass = mass of template + mass of product

$$\text{probe specific-activity} = \frac{\text{cpm of product (as measured in step 10)}}{\text{total DNA mass}}$$

The TCA precipitable product is calculated in step 10 above. Since it is problematic to directly quantitate DNA amounts in the range produced by this reaction, the total DNA mass is calculated from the percent incorporation of labeled nucleotide and the initial template input. An online calculator to determine the specific activity of a radiolabeled probe can be found at:

www.ambion.com/techlib/tips/DNA_specific_activity_calculator.html

Example calculations

Calculate moles of labeled nucleotide incorporated

For this sample calculation we assume:

- Radioisotope specific activity = 3000 Ci/mmol = 3 µCi/pmol
- Radioisotope concentration = 10 mCi/mL = 10 µCi/µL
- Percent incorporation = 50%

$$\frac{\text{\% incorporation from step B.9 (0.5)} \times \text{Vol isotope in reaction (5 µL)} \times \text{isotope conc (10 µCi/µL)}}{\text{Radioisotope specific-activity (3 µCi/pmol)}} =$$

$$\frac{0.5 \times 5\ \mu L \times 10\ \mu Ci/\mu L}{3\ \mu Ci/pmol} = 8.33\ \text{pmol labeled nucleotide incorporated}$$

Calculate DNA mass

Calculate the mass of newly synthesized product from the moles of product calculated above, then add this to the mass of input DNA to get the total mass of DNA (for this example assume input DNA was 25 ng).

Assuming that all four nucleotides are incorporated in roughly equal amounts, we can use the average molecular mass of all four nucleotides (330 g/mole) to calculate mass of DNA.

8.33 pmol X 330 g/mol X 4 = 11 ng of synthesized DNA

11 ng synthesized DNA + 25 ng template DNA = 36 ng total DNA

Calculate probe specific-activity

Calculate the specific activity of the probe from the values calculated above, and assuming that a cpm of 5.5×10^7 was determined in step 10.

$$\text{probe specific-activity} = \frac{5.5 \times 10^7 \text{ cpm}}{36 \text{ ng}} = 1.53 \times 10^6 \text{ cpm/ng}$$

$$1.53 \times 10^6 \text{ cpm/ng} = 1.53 \times 10^9 \text{ cpm/µg}$$

D. Preparing Probes for Hybridization

DECAprime II probes can be used in a variety of hybridization applications. Below is a standard procedure for denaturing probes in preparation for blot hybridization.

1. Dilute the probe 10 fold in 10 mM EDTA

Transfer an appropriate amount of probe (generally 1–5 $\times$ 10^6 cpm is used per mL of hybridization solution) to a separate tube. Dilute the probe 10-fold in 10 mM EDTA (i.e. if your probe is in 2 µL, add 18 µL of 10 mM EDTA).

2. Incubate diluted probe at 90°C for 10 min

This heat denaturation maximizes the amount of probe that will be available for hybridization with the nucleic acids on the blot.

3. Add the probe to the prehybridized blot

Transfer the probe solution directly to a container with a prehybridized blot. Mix well.

Follow standard procedures for hybridization, washing, and detection.

NOTE

If the probe is not going to be used directly in hybridization it should be frozen in dry ice or liquid nitrogen after heat denaturation in step 2 to prevent reannealing of complementary strands. After this, the probe can be stored at –20°C.

IV. Troubleshooting

A. Positive Control Reactions

Use the control DNA template

A control DNA (DECAtemplate GAPDH-M) is included with the kit to aid in troubleshooting possible problems. Should unexpected results occur using your own sample DNA template, repeat the labeling reaction using 2.5 µL of the DECAtemplate GAPDH-M control template to distinguish between problems with the template, and problems with the reagents.

Expected results

In a typical 25 µL probe synthesis reaction using 2.5 µL DECAtemplate GAPDH-M control DNA incubated for 10 min, you should see approximately 50% radiolabel incorporation when the products are analyzed by TCA precipitation, or 40–50% incorporation if using a size exclusion column followed by scintillation counting. Most of the probe synthesized should be between 250–600 bases when analyzed by denaturing acrylamide gel electrophoresis.

Control DNA is labeled well, but sample DNA is not

If the reaction works using the control DNA, but not with the sample DNA template, there may be a problem with the sample DNA template you are using (see section IV.B).

Control DNA is not labeled well

If the reaction does not work using the control DNA, this could indicate that the procedure was not followed correctly or that there is a problem with one of the reagents. In this case, try using a different lot of radiolabled NTP.

Make unlabeled product to visualize on gel

The DECAprime II reaction can also be performed with all four unlabeled dNTPs by adding 2.5 µL of each of the –dATP and –dCTP 5X Reaction Buffer, and no radiolabeled nucleotides in a 25 µL reaction. Under these conditions, 300–600 ng of DNA will be synthesized and can be visualized on a 3% (nondenaturing) agarose gel stained with ethidium bromide. The product will be a range of sizes (roughly 200–600 bp) extending even longer than the size of your input template.

B. Problems with Template Preparation

- The DNA to be labeled should be linearized by restriction endonuclease digestion prior to labeling. Supercoiled DNA is labeled inefficiently, if at all.
- If the template DNA was gel purified, the fragment may be labeled directly in melted agarose (Feinberg & Vogelstein 1984, Nolan 1989) or recovered by electroelution into a small dialysis bag in a small volume of 0.5X electrophoresis buffer.
- Be sure the input DNA was completely denatured with added decamers and snap-frozen as described in section II.B.
- There are a variety of methods for rapid purification of DNA, and some of them yield DNA that is purer than others. If sample purity is suspected to be a problem, then a phenol:chloroform extraction followed by precipitation in the presence of ammonium acetate should yield DNA suitable for efficient labeling.

C. Other Considerations

Amount of template input

Accurate quantitation of the DNA template is important. The DECAprime II reaction has been optimized to label 6–100 ng of DNA. A good estimate of the amount of input DNA used in the DECAprime II reaction is necessary to ensure optimal labeling. The DNA should be quantitated in a spectrophotometer, if possible.

Low specific activity

The detection of a very small amount of nucleic acids in blot hybridization is frequently limited by both the total amount, and the specific activity of the probe used for the hybridization. Avoid the temptation to add additional template to the labeling reaction to boost the mass yield of probe. While this will increase the total amount of labeled probe, it will decrease the specific activity (Figure 2 on page 3). To increase the limit of detection we recommend scaling up the reaction volume 3 to 5-fold (i.e. label 75–125 ng of template DNA in a reaction volume of 75–125 µL) and use the entire labeling reaction in the hybridization.

Probe denaturation

DNA probes must be denatured before use in hybridization applications (see section III.D on page 9).

V. Appendix

A. References

Derbyshire V, Freemont PS, Sanderson MR, Beese L, Friedman JM, Joyce CM, and Steitz TA (1988) Genetic and Crystallographic Studies of the 3', 5'-Exonucleolytic Site of DNA Polymerase I. *Science* **240**:199–201.

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Feinberg AP, and Vogelstein B (1984) A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity:Addendum. *Analyt. Biochem.* **137**:266–7.

Nolan C (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed, Cold Springs Harbor (NY):Cold Springs Harbor Laboratory Press.

B. Quality Control

A 25 µL probe synthesis reaction with 2.5 µL DECAtemplate GAPDH-M control DNA resulted in >50% incorporation in 10 min. Most of the probe synthesized was between 250–600 nucleotides as analyzed by denaturing acrylamide gel electrophoresis.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

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