GE Healthcare

Amersham Megaprime DNA Labeling Systems

Product Booklet

Codes: RPN1604
RPN1605
RPN1606
RPN1607

Page finder

1. Legal

http://www.gelifesciences.com

GE Healthcare UK Limited.
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Buckinghamshire, HP7 9NA
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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.* Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: For use with radioactive material.
This product is to be used with radioactive material. Please follow the manufacturer's instructions relating to the handling, use, storage and disposal of such material.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes, wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage and stability

Upon receipt of these systems components should be stored at -15°C to -30°C. The components are stable for at least 3 months when stored under recommended conditions.

2.3. Quality control

The Megaprime™ DNA Labeling Systems are tested by our quality control group to ensure an incorporation rate greater than 55% after 10 minutes at 37°C.

The performance of RPN1604/1605 is tested with the standard DNA provided

using 17 pmol/25 ng DNA of [α-^{32}P] labeled nucleotides, specific activity 3000 Ci/mmol and RPN 1606/1607 are tested using 17 pmol/25 ng DNA of [α-^{32}P]dCTP, 3000 Ci/mmol. Incorporations greater than 55% are achieved after 10 minutes incubation at 37°C, as assayed by thin-layer chromatography on PEI cellulose in 1.25M KH_2PO_4. pH3.4.

In addition components of the kits are checked for identity by HPLC and the DNA solutions for concentration by UV spectrophotometry.

3. System components

Megaprime DNA labeling	RPN1604	RPN1605	RPN1606	RPN1607
Primer solution: Random nonamer primers in an aqueous solution	150 µl	300 µl	150 µl	300 µl
Labeling buffer; dATP, dGTP and dTTP in Tris/HCl pH7.5, 2-mercaptoethanol and $MgCl_2$	–	–	300 µl	600 µl
Nucleotide solutions				
(a) dATP	120 µl	240 µl	–	–
(b) cCTP	120 µl	240 µl	–	–
(c) dGTP	120 µl	240 µl	–	–
(d) dTTP in Tris/HCl pH8.0, 0.5 mM EDTA	120 µl	240 µl	–	–
Reaction buffer: A 10× concentrated buffer containing Tris/HCl pH7.5, 2-mercaptoethanol and $MgCl_2$	150 µl	300 µl	–	–

Megaprime DNA labeling	RPN1604	RPN1605	RPN1606	RPN1607
Enzyme solution; 1 unit/µl DNA polymerase 1 Klenow fragment (cloned in 100 mM potassium phosphate pH6.5, 10 mM 2-mercapto-ethanol and 50% glycerol	60 µl	120 µl	60 µl	120 µl
Standard DNA solution; 5 ng/µl *Hin*d III digested lambda DNA in 10 mM Tris/HCl pH 8.0, 1 mM EDTA	25 µl	50 µl	25 µl	50 µl
Carrier DNA solution; 500ng/ml sonicated fish sperm DNA in 10 mM Tris/HCl pH 8.0, 1 mM EDTA	1.25 ml	2.5 ml	1.25 ml	2.5 ml

3.1. Megaprime DNA Labeling Systems

30 standard labeling reactions – for use with any radioactive nucleotide	RPN1604
60 standard labeling reactions – for use with any radioactive nucleotide	RPN1605
30 standard labeling reactions – for use with radioactively labeled dCTP	RPN1606
60 standard labeling reactions – for use with radioactively labeled dCTP	RPN1607

4. Introduction

Feinbereg and Vogelstein (1,2) introduced the use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The primer-template complex is a substrate for the 'Klenow' fragment of DNA polymerase 1. By substituting a radiolabeled nucleotide for a non-radioactive equivalent in the reaction mixture newly synthesized DNA is made radioactive (see Figure 1). The absence of the 5'–3' exonuclease activity associated with DNA polymerase 1 ensures that labeled nucleotides incorporated by the polymerase are not subsequently removed as monophosphates. Very small amounts of input DNA can be labeled, enabling very high specific activity DNA probes to be produced with relatively small quantities of added nucleotides. These radioactive labeled fragments can then be used as sensitive hybridization probes for a wide range of filter based applications (3–6).

Previous protocols for the random primer labeling of DNA have required reaction times of at least 30 minutes. GE Healthcare's Megaprime DNA Labeling System allows the labeling of template DNA to the same high specific activity but at a greatly accelerated rate. Probes of specific activity 1.9×10^9 dpm/µg can be produced with the majority of DNA substrates, using the standard protocol, after 10 minutes incubation at 37°C. This rapid labeling is achieved by the use of nonamer primers rather than the conventional hexamers (Figure 1). Nonamers allow for more efficient priming from the template DNA at 37°C, resulting in fast and efficient labeling of the DNA. A new alternative protocol has further reduced the variability in labeling which can occur with DNA template from a variety of sources. Both the standard Megaprime protocol and the new protocol are given as options in this booklet. The labeling of DNA in low melting point agarose takes only 15–30 minutes in contrast to conventional systems where overnight incubation are necessary.

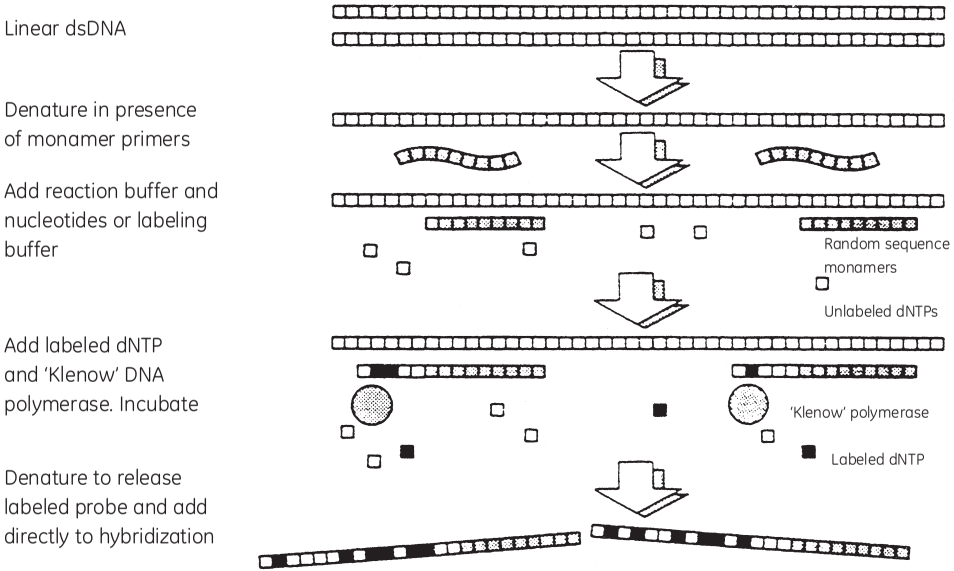


Figure 1. Preparation of labeled probes using GE Healthcare's Megaprime DNA Labeling Systems.

5. Megaprime DNA labeling protocols

The Megaprime systems allow DNA from a variety of sources to be labeled *in vitro* to high specific activity with ^{32}P and other radionuclides. The specific activity of the probes generated by these systems will vary according to the specific activity of the labeled dNTP used.

The standard Megaprime protocol is presented, together with a new protocol which reduces the variation in labeling efficiency that can occur with DNA template from a variety of sources.

The protocols given here are for use with 17 pmol[α-^{32}P]dNTP, specific activity 3000 Ci/mmol. For alternative reaction conditions refer to page 20.

DNA prepared by standard minilysate methods may be used in either protocol. DNA solutions which are too dilute to be used directly should be concentrated by ethanol precipitation followed by redissolution in an appropriate volume of water or 10 mM Tris/HCl, pH 8.0, 1 mM EDTA. DNA in restriction enzyme buffers may be added directly to the reaction. The reaction can also be performed with DNA in agarose gel slices (see note 3 and Appendix 1).

5.1. Standard Megaprime protocol

Protocol	Notes
1. Dissolve the DNA to be labeled to a concentration of 2.5–25 ng/µl in either distilled water of 10 mM Tris/HCl, pH8.0, 1 mM EDTA (TE buffer).	**1.** If desired, the labeling efficiency of a DNA sample can be compared with that of the standard DNA supplied with the kit. In this case 5 µl of standard DNA should be used.

Protocol	Notes
2. Place the required tubes from the Megaprime system, with the exception of the enzyme, at room temperature to thaw. Leave the enzyme at -15°C to -30°C until required, and return immediately after use.	
3. Place 25 ng of template DNA into a microcentrifuge tube and to it add 5 µl of primers and the appropriate volume of water to give a total volume of 50 µl in the final Megaprime reaction. Denature by heating to 95–100°C for 5 minutes in a boiling water bath.	**3.** When labeling DNA in low melting point agarose, first place the tube containing the stock DNA in a boiling water bath for 30 seconds to melt the agarose before removing the required volume. The volume of low melting point agarose DNA should not exceed 25 µl in a 50 µl reaction.
4. Spin briefly in a microcentrifuge to bring the contents to the bottom of the tube.	
5. Keeping the tube at room temperature, add the nucleotides and reaction buffer (RPN1604/5) or the labeling buffer (RPN1606/7) followed by the radiolabeled dNTP(s) and enzyme as follows:	**5.** The reaction volume may be scaled up or down if more or less than 25 ng of DNA is to be labeled.

Protocol

Component	RPN1604/5	RPN1606/7
Labeling buffer		10 µl
Unlabeled dNTPs	4 µl of each omitting those to be used as label	–
Reaction buffer	5 µl	–
Radiolabeled (dNTP)	5 µl	5 µl (dCTP)
Enzyme	2 µl	2 µl

6. Mix gently by pipetting up and down and cap the tube. Spin for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube.

7. Incubate at 37°C for 10 minutes

Notes

6. Avoid vigorous mixing of the reaction mixture as this can cause severe loss of enzyme activity.

7. Purified DNA can be labeled to high specific activity in 10 minutes at 37°C but, if desired, can be labeled for up to 1 hour at this temperature. When labeling DNA in low melting point agarose, longer incubation of 15–30 minutes at 37°C are required for optimum labeling. Longer incubation

Protocol	Notes
7. Incubate at 37°C for 10 minutes *continued*.	**7.** ***Continued.*** times (up to 60 minutes) are required when nucleotide analogues (e.g. [^{35}S]dNTPαS) are used.
8. Stop the reaction by the addition of 5 µl of 0.2 M EDTA. For use in a hybridization, denature the labeled DNA by heating to 95–100°C for 5 minutes, then chill on ice.	**8.** Labeled probe can be stored at -15°C to -30°C in a non frost-free freezer. Prolonged storage of ^{32}P-labeled probes can lead to substantial probe degradation (7). High specific activity probes should be stored for no longer than 3 days. Although probe purification is not usually necessary for most membrane applications, the removal of unicorporated nucleotide is sometimes useful to reduce background in filter hybridizations for probes >10^9 dpm/µg or when the reaction yields an incorporation of less than 50%. This procedure is described in Appendix III. Calculation of probe specific activity is described in Appendix II. Extensive experimentation with **Rapid-hyb buffer (RPN1635/6)** has shown that probe purification, even

Protocol	Notes
8. Stop the reaction by the addition of 5 µl of 0.2 M EDTA. For use in a hybridization, denature the labeled DNA by heating to 95–100°C for 5 minutes, then chill on ice *continued*.	**8.** ***Continued*** under the conditions given above is not required with the isotopes ^{32}P and ^{33}P. Purification of ^{35}S labeled probes is however required to reduce filter background.

5.2. New Megaprime protocol

Protocol	Notes
1. Dilute the DNA to a concentration of 5 ng/µl in either distilled water or 10 mM TE buffer.	**1.** DNA solutions at concentrations in the range 5–25 ng/µl can be used if desired. However the denaturing volume (step 3) should not be less than 10 µl to maximize the efficiency of primer annealing. The labeling efficiency of a DNA sample can be compared with that of the standard DNA supplied with the kit. In this case 5 µl of standard DNA should be used.
2. Place the required tubes from the Megaprime system with the exception of the enzyme at room temperature to thaw. Leave the enzyme at -15°C to -30°C until required, and return immediately after use.	

Protocol	Notes
3. Place 25 ng (5 µl) of template DNA into a clean microcentrifuge tube and to it add 5 µl of primers. Denature by heating to 95–100°C for 5 minutes in a boiling water bath.	**3.** If the volume of DNA and primers is less than 10 µl make up to this volume with water. When labeling DNA in low melting point agarose first place the tube containing the stock DNA in a boiling water bath for 30 seconds to melt the agarose before removing the required volume. The volume of low melting point agarose DNA should not exceed 25 µl in a 50 µl reaction.
4. Spin briefly in a microcentrifuge to bring the contents to the bottom of the tube.	
5. Keeping the tube at room temperature add the nucleotides and 10× reaction buffer (RPN1604/5) or the labeling buffer (RPN1606/7), water and enzyme:-	**5.** The enzyme can be added directly to the reaction mix or pipetted on to the side of the microcentrifuge tube and "washed" down with the water.

Component	RPN1604/5	RPN1606/7
Labeling buffer		10 µl
Unlabeled dNTPs	4 µl of each – omitting those to be used as label	
Reaction buffer	5 µl	–
Enzyme	2 µl	2 µl
Water*	as appropriate for a final reactionvolume of 50 µl*	

*When calculating this volume remember to allow for the volume of radioactive nucleotide to be added.

Protocol	Notes
6. Cap the tube and spin for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube.	
7. Add the radiolabeled dNTP, for example 5µl [α–^{32}P]dNTP, specific activity 3000 Ci/mmol. Mix by gently pipetting up and down. Spin for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube.	**7.** Avoid vigorous mixing of the reaction mixture as this can cause severe loss of enzyme activity.
8. Incubate at 37°C for 10 minutes.	**8.** Purified DNA can be labeled to high specific activity in 10 minutes at 37°C but, if desired can be labeled for up to 1 hour at this temperature. When labeling DNA in low melting point agarose, longer incubation of 15–30 minutes at 37°C are required for optimum labeling. Longer incubation times (up to 60 minutes) are required when nucleotide analogues (e.g. [^{35}S]dNTP(S) are used.

Protocol	Notes
9. Stop the reaction by the addition of 5 µl of 0.2 M EDTA. For use in a hybridization, denature the labeled DNA by heating to 95–100°C for 5 minutes, then chill on ice.	**9.** Labeled probe can be stored at -15°C to -30°C in a non frost-free freezer. Prolonged storage of ^{32}P-labeled probes can lead to substantial probe degradation(7). High specific activity probes should be stored for no longer than 3 days. Although probe purification is not usually necessary for most membrane applications the removal of unincorporated nucleotide is sometimes useful to reduce background in filter hybridizations for probes >10^9 dpm/µg or when the reaction yields an incorporation of less than 50%. This procedure is described in Appendix III. Calculation of probe specific activity is described in Appendix II. Extensive experimentation with **Rapid-hyb buffer (RPN1635/6)** has shown that probe purification, even under the conditions given

Protocol	Notes
9. Stop the reaction by the addition of 5 µl of 0.2 M EDTA. For use in a hybridization, denature the labeled DNA by heating to 95–100°C for 5 minutes, then chill on ice *continued*.	9. ***Continued*** above is not required with the isotopes ^{32}P and ^{33}P. Purification of ^{35}S labeled probes is however required to reduce filter background.

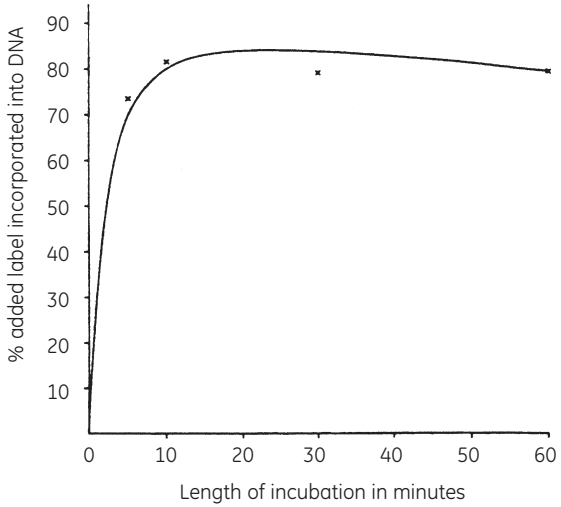


Figure 2. Time course of incorporation of [α–^{32}P]dCTP (17 pmoles) in a Megaprime reaction at 37°C. The DNA used was the standard DNA supplied with the system.

5.3. Use of alternative reaction conditions

a. Use of more than one labeled [α–^{32}P]dNTP.

Table 1 lists the results of a selection of standard reactions, using a variety of input labels under optimum conditions. Figure 3 gives more complete information on their use in Megaprime reactions. Reactions were carried out at 37°C for 5 minutes.

b. Use of alternative specific activity [α–^{32}P]dNTPs.

When using [α–^{32}P]dNTPs of specific activity <3000 Ci/mmol the incubation time should be extended to 1 hour at 37°C.

c. Use of [^{35}S]dNTPαS.

When using ^{35}S-labeled radionucleotides the incubation time should be extended to 1 hour at 37°C.

d. Labeling at room temperature.

If desired, labeling reactions can be carried out at room temperature. Maximum incorporation occurs after an incubation time of 45–60 minutes. A decline in incorporation can be observed if reactions are left overnight.

e. Factors affecting the labeled DNA.

1. Specific activity

Figure 3a should be used to ascertain the number and quantity of labeled dNTP's required in order to prepare a probe of the desired specific activity.

2. Efficiency

Figure 3b indicates the efficiency of the chosen reaction conditions, and thus permits a balance of specific activity and economy.

3. Probe length

Figure 3c gives a measure of mean probe lengths obtained under standard conditions. Probe lengths were measured by denaturing agarose gel electrophoresis followed by autoradiography with reference to molecular weight standards.

Probe length can be affected by the concentration of DNA, primer and nucleotide, the size of the template DNA and also radiolysis of the labeled probe. The data in the figure was obtained using linearized plasmid DNA, 4.5 Kb in length under the standard labeling conditions.

It is recommended that not less than 10 pmol and not more than 125 pmol of any labeled dNTP is used in the reaction and combinations shown offer optimum balance of stability, specific activity and economy.

Table 1

Compounds	Specific activity (see note a)		Formulation (see note b)	Quantity of each dNTP required				Specific activity of probe (see notes c,d and e)
	TBq/mmol	Ci/mmol		MBq	µCi	µl	pmol	dpm/ug
[α-^{32}P]dCTP	~111	~3000	1	1.85	50	5	17	1.9×10^9
	~222	~6000	1	7.4	200	20	32	5.3×10^9
[α-^{32}P]dATP	~111	~3000	1	1.85	50	5	17	3.4×10^9
[α-^{32}P]dCTP	~111	~3000	1	1.85	50	5	17	
[α-^{32}P]dATP	~111	~3000	1	1.85	50	5	17	3.7×10^9
[α-^{32}P]dCTP	~111	~3000	1	1.85	50	5	17	
[α-^{32}P]dGTP	~111	~3000	1	1.85	50	5	17	

a. At the specific activity reference date of the labeled nucleotide.

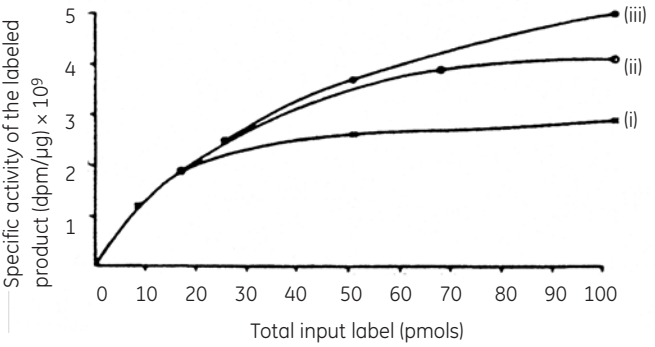
b. Formulation code 1 = 370 MBq/ml, 10 mCi/ml in stabilized aqueous solution.

c. The probe specific activities were calculated using observed incorporation levels which are similar to those found in figure 3b.

d. It is important to note that the specific activity of probes made from different amounts of labeled dNTP cannot be calculated on a proportional basis, because net DNA synthesis occurs.

e. Brackets enclose nucleotides used in combination.

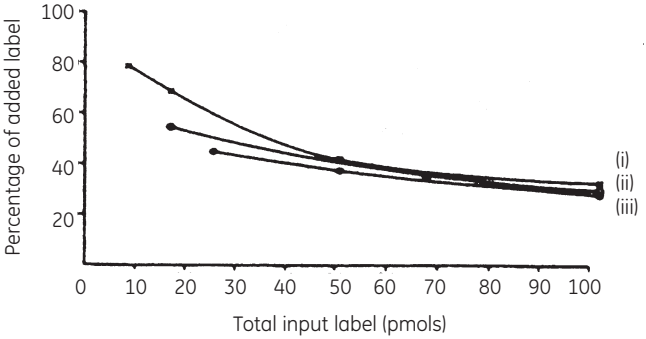
a) Specific activity



i) One labeled dNTP
ii) Two labeled dNTP
iii) Three labeled dNTP

Figure 3. The use of [α–^{32}P]dNTPs in the Megaprime DNA Labeling System (see notes on page 26).

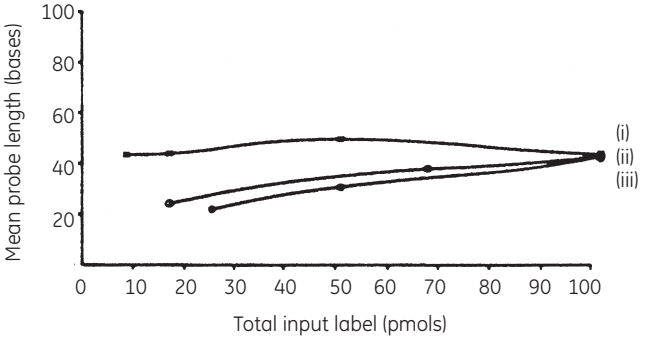
b) Incorporation efficiency



i) One labeled dNTP
ii) Two labeled dNTP
iii) Three labeled dNTP

Figure 3. The use of [α-^{32}P]dNTPs in the Megaprime DNA Labeling System (see notes on page 26).

c) Probe length



i) One labeled dNTP
ii) Two labeled dNTP
iii) Three labeled dNTP

Figure 3. The use of [α–^{32}P]dNTPs in the Megaprime DNA Labeling System (see below).

Notes to figure 3

a. The results shown are the means of a number of experiments in which different nucleotides and combinations of nucleotides were used. Observed results may deviate ± 10% from those shown.

b. As the number of different labeled nucleotides is increased, at a given level of total input label, the net synthesis of DNA is reduced.
Although the overall incorporation efficiency is reduced the labeled product is of a higher specific activity.

c. The data was generated using the standard labeling protocols. If dNTPs <3000 Ci/mmol are to be used, then the desired probe specific activity must be multiplied by a conversion factor, before determining the amount of input label.

For a single labeled dNTP:-

$$\text{Total input label (pmols)} = \frac{3000\ \text{Ci/mmol}}{\text{specific activity of dNTP to be used}} \times \text{required probe specific activity}$$

For more than one labeled dNTP the mean specific activity of the labeled dNTP to be used should be inserted in the above calculation.

Having determined the required number of pmols of input label with reference to figure 3a, the required volume of each labeled dNTP can be calculated. Note that the figures give the **total** amount of input label required. If more than one labeled dNTP is to be used, this figure should be divided by the number of labeled dNTPs to be used to give the required number of pmols of each labeled dNTP.

Volume of each labeled dNTP required in µl =

$$\frac{\text{pmol of dNTP required} \times \text{specific activity of dNTP (Ci/mmol)} \times 10^{-3}}{\text{radioactive concentration of dNTP (mCi/ml)}}$$

6. Appendices

6.1. Appendix I. Labeling of DNA fragments in low melting point agarose

The DNA samples produced by the following protocol have been found to be labeled to approximately the same extent as purified DNA. 15–20 minutes at 37°C is optimum for labeling. The standard labeling protocol may be found to be more appropriate for labeling DNA in agarose as the volume of DNA to be added using the new protocol is limited to 5 µl, requiring a relatively high initial DNA concentration.

Protocol	Notes
1. Fractionate restriction endonuclease digested DNA in a suitable low melting point agarose gel containing 0.5 µg/ml ethidium bromide. Estimate the DNA content of the band by reference to a set of standards of known concentration on another track. 250 ng should allow 25 ng to be used in the standard labeling protocol without further concentration	**1.** A low melting point agarose of high purity for example SepRate-LMP is recommended for maximum labeling efficiency.
2. Excise the desired band cleanly, with the minimum of excess agarose and transfer to a pre-weighed 1.5 ml microcentrifuge tube.	**2**. It is recommended that the exposure to UV light is minimized, as prolonged exposure can damage the DNA.

Protocol	Notes
3. Add water to a ratio of 3 ml per gram of gel and place in a boiling water bath for 5 minutes to melt the gel and denature the DNA.	**3.** If the DNA is not to be used immediately divide the boiled samples into suitably sized aliquots and store at -15°C to -30°C in a non frost-free freezer.
4. If the DNA is to be used immediately remove the appropriate volume containing 25 ng, add to the primers as indicated in the labeling protocol (page 11, step 3). The volume of DNA should not exceed 25 µl for the standard labeling protocol.	**4.** When using DNA which has been previously boiled and then stored at -15°C to -30°C, first place the tube in a boiling water bath for 30 seconds to melt the agarose, before removing the required volume containing 25 ng. Do not reboil DNA aliquots more than twice.
5. Incubate the labeling reaction for 15–20 minutes at 37°C.	

6.2. Appendix II. Monitoring the reaction and calculating the specific activity of the labeled DNA

A. Adsorption to DE81 paper

Monitoring of the progress of the labeling reaction and measurement of probe specific activity can be achieved by determining the proportion of the radionucleotide incorporated during the Megaprime reaction.

Protocol	Notes
1. Remove a 1 or 2 µl aliquot of the reaction mixture to a clean microcentrifuge tube containing 20 µl of water or 10 mM Tris/HCl pH.8.0. 1 mM EDTA buffer. Mix well by pipetting up and down.	
2. Spot, in quadruplicate, 5 µl aliquots of this dilution on to Whatman DE81 chromatography paper squares (minimum size 1 × 1 cm), placed on a non-absorbent backing. These squares may be marked with a pencil for identification if required.	
3. Take two of the filters and dry under a heat lamp. 10–15 minutes should be adequate.	
4. Wash the remaining two filters twice for 5 minutes each, at room temperature in excess 2 × SSC (30 mM Na_3 citrate, 300 mM NaCl pH7.0) using gentle agitation. Rinse briefly in distilled water and then once with ethanol for 5 minutes. Then dry the filters under a heat lamp.	**4.** In aqueous solution DE81 paper becomes fragile and care should be taken when handling. In order to stabilize the paper the squares are rinsed in ethanol.

Protocol	Notes
5. Place the squares in separate vials with at least 5 ml of scintillation fluid and count.	**5.** Determination of the proportion of the ^{32}P labeled nucleotide incorporated may be achieved using Cerenkov counting if desired in this case drying the filter is not necessary.
6. Efficiency of counting will vary, but the percentage incorporation can be used to calculate probe specific activity. Unlike the nick translation labeling reaction, Megaprime labeling leads to net DNA synthesis, and so the total amount of DNA at the end of the reaction must be calculated. Total amount of DNA (A) ng = $\frac{\text{Total number of µCi added} \times 13.2^* \times \% \text{ incorporation}}{\text{Number of radioactive dNTPs added} \times \text{average specific activity of dNTPs added}} + 25$ This assumes a 25% content of any one dNTP in the newly synthesized DNA, and 25 ng of template DNA. *13.2 equals four times the average molecular weight of the four dNTPs divided by 100.	**6.** The mean value of the counts on the washed filter represents the proportion of the radionucleotide incorporated into the DNA probe, while the mean of the unwashed filters represents the total amount of radioactivity in the reaction mix, such that; $\% \text{ incorporation} = \frac{\text{mean counts on washed filters}}{\text{mean counts on unwashed filters}} \times 100$

6. *Continued.*

The amount of radioactivity incorporated during the reaction (B) in dpm.

B = total number of µCi added × 2.2×10^4 × % incorporation

Thus the specific activity of the labeled DNA is

$$\text{specific activity} = \frac{B}{A} \times 10^3 \text{ dpm per µg}$$

B. Precipitation with trichloroacetic acid

Plastic or siliconized glass tubes must be used to avoid adsorption of DNA.

1. Dilute an appropriate aliquot of the reaction mixture as described in section A1.

2. Transfer 1–10 µl of diluted reaction mixture to two duplicate tubes containing 200 µl water or 0.2M EDTA and 50 µl carrier DNA solution. Mix well. Use this mixture (less any set aside in step 3) for the TCA precipitation described in step 4 below.

3. Set aside an appropriate aliquot from each tube in step 2 for the determination of total input radioactivity.

4. To the diluted samples from step 2, add 2 ml ice-cold 10% trichloroacetic acid (TCA) solution, vortex, and allow to stand in an ice-bath for 10–15 minutes. The labeled and carrier DNA will co-precipitate. **Note that TCA is corrosive, and care should be taken in its handling.**

5. Collect the precipitated DNA by vacuum filtration on a glass fibre or nitrocellulose filter disc.

6. Wash the filter discs six times with 2 ml 10% TCA solution and dry the filter discs thoroughly, for example using an infra-red lamp. Avoid overheating and possible charring of the discs.
7. Count the dried filter discs by liquid scintillation or Cerenkov (^{32}P) and count with the samples set aside in step 3.
8. Determine % incorporation and probe specific activity as in section A6.

6.3. Appendix III. Removal of unincorporated nucleotides

Removal of unincorporated nucleotides is sometimes desirable to reduce background produced by the probe during hybridization. It is considered important to remove these free nucleotides particularly if the radioactive probe is to be kept for several days before use or the incorporation is less than 50%. If ^{32}P or ^{33}P-labeled probes are to be used in combination with GE Healthcare's new Rapid-hyb buffer (RPN1635/6), purification is not required unless the probe is to be used more than 24 hours after preparation. Probes can be purified by Sephadex chromatography or selective precipitation (8,9).

A. Sephadex™G-50 spin columns

Probe reaction are passed through columns packed with Sephadex G-50, which retains the free nucleotides within the column matrix. A number of pre-packed columns are commercially available. However columns may also be prepared as indicated below:

1. Equilibrate Sephadex G-50 in TE buffer either overnight or at 65°C for 1–2 hours.
2. Plug a 1.0 ml syringe with a piece of siliconized glass wool.
3. Fill the syringe with the equilibrated Sephadex. Place in a 15 ml conical tube, in which a decapped 1.5 ml microcentrifuge tube has been inserted. Centrifuge at 1600 g for 5 minutes. Remove

any liquid from the microcentrifuge tube. Refill with Sephadex and centrifuge as before. Continue until the column is packed to a volume of 1 ml.

4. Add a volume of TE buffer equal to the reaction volume, to the top of the column and centrifuge, as in step 3. A minimum of 50 µl should be applied to the column.
5. Repeat once more to ensure fractions of the correct size are collected from the column.
6. Place the column in a clean 15 ml conical tube containing a decapped 1.5 ml microcentrifuge tube.
7. Apply the DNA sample to the column. Centrifuge as before. The purified probe is collected in the microcentrifuge tube.

B. Selective precipitation of labeled DNA

The following protocol leads to precipitation of DNA greater than about 20 nucleotides in length with unincorporated nucleotides remaining in solution. Recovery of the labeled DNA by this method varies according to the DNA concentration and size, and may be as low as 50%.

1. Add one volume of 4 M ammonium acetate, pH4.5 to the nick translation reaction, and mix gently by pipetting up and down.
2. Add four volumes of ethanol, mix by inversion. Chill the mixture for 15 minutes in a dry-ice ethanol bath or place at -70°C for at least 30 minutes.
3. Thaw the mixture if necessary by placing at 37°C for 2 minutes.
4. Spin in a microcentrifuge for 15 minutes. Carefully aspirate and dispose of supernatant in a suitable manner.
5. Wash the pellet once in 0.5 ml of 0.67 Mammonium acetate, pH 4.5, 67% ethanol at room temperature by gentle inversion, centrifugation and aspiration.

6. Wash the pellet once in 90% ethanol, in the same manner. Dry the pellet.
7. Finally redissolve the DNA pellet in TE buffer for use as a probe and for storage.

6.4. Appendix IV. Additional equipment and reagents

TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA)
0.2 M EDTA solution
Adjustable pipettes for example Pipetman™
Sterile pipette tips
Waterbaths at 37°C and 100°C
Polypropylene microcentrifuge tubes
Microcentrifuge
Gloves
Radiation safety equipment
DE81 ion-exchange chromatography paper (Whatman)
Trichloroacetic acid (TCA) solution: 10% (w/v) TCA in water
Filter discs; glass fibre or nitrocellulose
Plastic or siliconized glass tubes, capacity ~5 ml
Filtration apparatus
2× SSC (30 mM Na_3 citrate, 300 mM NaCl, pH 7.0)

7. Troubleshooting guide

If poor results are obtained, the following guide may help to determine the cause of the problem.

Problem	Possible cause	Remedy
1. Low signal	**1.** Incomplete denaturation of template DNA	**1.** Ensure denaturation protocol is followed.
	2. Low probe concentration	**2.** Accurately measure the concentration of template DNA used in the labeling reactions. Check recovery of probe if purification is performed to remove unincorporated nucleotide.
	3. Low probe specific activity	**3.** If the specific activity of the labeled DNA is lower than expected, a labeling reaction should be carried out using a sample of the control DNA supplied with the system. If this proceeds satisfactorily, check the concentration and purity of your DNA.

Problem	Possible cause	Remedy
	4. Loss of dNTP during evaporation	**4.** If the dNTP solution has been evaporated to dryness prior to use, handling losses may have occurred. Check this loss has not occurred during lyophilization of the solvent, during transfer of the reconstituted dNTP solution or by adsorption of the dNTP onto the walls of the tube. If necessary the reconstituted dNTP solution may be counted and an adjustment made before setting up the labeling reaction.
2. Non-specific background over whole of filter	**1.** Presence of unincorporated label	**1.** Unincorporated nucleotides can give high backgrounds. Remove by Sephadex G-50 spin columns or ethanol precipitation (see page 32 for protocol)

Problem	Possible cause	Remedy
	2. Concentrated probe has contacted membrane directly during probe addition	**2.** It is suggested that up to 1.0 ml of the buffer used for prehybridization is withdrawn for mixing with the probe. The mixture should then be added back to the hybridization container in an area away from the filter.
	3. Probe concentration is too high	**3.** Ensure measurement of template DNA concentration is accurate
	4. Probe not denatured	**4.** Non-denatured double-stranded probes often give high backgrounds.

8. References

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9. Related Products

Labeling systems

Nick Translation Kits	N5000/5500
Rediprime II DNA Labeling System	RPN1633/4
Ready-To-Go DNA Labeling Beads (-dCTP)	27-9240-01

Hybridization buffers

Rapid-hyb buffer	RPN1635/6

Hybridization products

Hybond™ - Range of nylon and nitrocellulose blotting membranes

Hybridisation oven/shaker (220/240V 50Hz)	RPN2510
Hybridisation oven/shaker (110/115V 60Hz)	RPN2511E

Autoradiography products

Hyperfilm™ - high performance autoradiography films

Hypercassettes™ and Hyperscreens™ - available from stock

Sensitize™ preflash gun.	RPN2051
TrackerTape™	RPN2050

An adhesive waterproof tape that phosphoresces to give a permanent written image on autoradiography film

Safety Products

Radiation safety products for safe handling and storage of $^{32}P/^{33}P/^{35}S$ and ^{125}I, liquid scintillation products

Labeled dNTPs

See Table 2

Table 2. Labeled dNTPs and analogues available from GE Healthcare

Compound	Specific Activity		Product code
	TBq/mmol	Ci/mmol	
[8–^{3}H]dATP	0.37–1.1	10–30	TRK 347
[1',2',5–^{3}H]dCTP	1.85–3.14	50–85	TRK 625
[5–^{3}H]dCTP	0.55–1.1	15–30	TRT 352
[8–^{3}H]dGTP	0.185–0.740	5–20	TRK 350
[1'2,[–^{3}H]dGTP	0.9–1.85	25–50	TRT 627
[*methyl*, 1',2'–^{3}H]TTP	3.3–4.8	90–130	TRK 576
[*methyl*–^{3}H]TTP		40–60	TRK 424
		30	TRK 354

See GE Healthcare Products catalogue for further details.

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