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Not for use in diagnostic procedures.

Roche

Random Primed DNA Labeling Kit

For labeling DNA using random oligonucelotides as primers

Cat. No. 11 004 760 001

Kit for 50 labeling assays

Version 23
Content version: September 2016

Store at −15 to −25°C

1. What this Product Does

Number of Tests

1 kit is sufficient for 50 labeling assays.

Kit Contents

Vial	Label	Contents
1	Control DNA	• 20 μl λDNA • 12.5 μg/ml
2	dATP	• 50 μl 2´-deoxyadenosine-5´-triphosphate • 0.5 mM, in 10 mM Tris buffer
3	dCTP	• 50 μl 2´-deoxycytidine-5´-triphosphate, • 0.5 mM, in 10 mM Tris buffer
4	dGTP	• 50 μl 2´-deoxyguanosine-5´-triphosphate • 0.5 mM, in 10 mM Tris buffer
5	dTTP	• 50 μl 2´deoxythymidine-5´-triphosphate • 0.5 mM, in 10 mM Tris buffer
6	Hexanucleotide mixture	• 100 μl hexanucleotide mixture • in 10× concentrated reaction buffer
7	Klenow enzyme	• 50 μl Klenow enzyme labeling grade • 2 U/μl, in glycerol, 50% (v/v)

Storage/Stability

The unopened kit is stable at −15 to −25°C until the expiration date printed on the label. The kit is shipped on dry ice.

Avoid repeated freezing and thawing. We recommend to aliquot the Klenow enzyme (vial 7) and to store in 2 – 3 portions

Additional Equipment/Reagents Required

- Heating block or water bath
- Ice/water bath
- For labeling either radioactively or chemically modified deoxynucleotide triphosphates can be used. We recommend [α-^{32}P] dCTP, 3000 Ci/mmol or Dioxigenin-11-dUTP. However, if any other modified dNTP is used, all dNTPs are supplied individually for optimal convenience.
- 0.2 M EDTA, pH 8

* *available from Roche Diagnostics*

2. How to Use this Product

2.1 Before You Begin

Labeling principle

The method of "random primed" DNA labeling developed by Feinberg and Vogelstein (1,2) is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. All sequence combinations are represented in the hexanucleotide primer mixture, which leads to binding of primer to the template DNA in a statistic manner. Thus an equal degree of labeling along the entire length of the template DNA is guaranteed.

The complementary strand is synthesized from the 3´OH termini of the random hexanucleotide primer using Klenow enzyme, labeling grade. Thus, modified deoxyribonucleoside triphosphates ([^{32}P]-, [^{35}S]-, [^{3}H]-, [^{125}I],digoxigenin- or biotin-labeled) are incorporated into the newly synthesized complementary DNA strand.

Basic steps

Please refer to the following table.

❶	Denaturation of linearized template DNA. For the hybridization of the hexanucleotide primer the template DNA needs to be single stranded.
❷	Hybridization of the hexanucleotide primer to the template DNA. Practically all sequence combinations are represented in the hexanucleoside primer mixture, which leads to binding of primer to template in a statistic manner.
❸	Synthesis of complementary strand from the 3´OH termini of the random hexanucleotide primer using Klenow enzyme. Modified deoxynucleosides are incorporated into the newly synthesized complementary DNA strand.
❹	Stop of reaction by adding EDTA or by heating.
❺	Removal of non-incorporated deoxynucleosides, if necessary.

Application

Labeled DNA probes with high specific activity are used in a variety of hybridization techniques including:

- screening of gene libraries (3)
- Southern and Northern blots (4,5)
- *in situ* hybridizations

Sample material

10 ng - 3 μg of linearized DNA (at least 100-200 bp in length recommended) per reaction.

Assay time

50 min

Specific activity

The described standard assay will routinely give a specific activity of 1.8×10^{9} dpm/μg, corresponding to 65% incorporation with different substrate DNAs in 30 min. When varying the ratio of template DNA to labeled dNTP, similar incorporation rates, but different levels of specific activity of the labeled probe are obtained.

3. Procedures and required materials

Sample material

The features of the sample material are listed in the table.

DNA	Feature
Structure and length	• linear, • at least 200 bp linearized plasmid or linearized lambda DNA
Amount	25 ng for standard labeling reaction Less DNA can also be labeled with the kit, even though maximal incorporation may be achieved only after longer incubation (up to 60 min).
Restriction fragments from agarose gel	DNA fragment excised from gel can be used for labeling, we recommend to use the High Pure PCR Product Purification Kit*. When using DNA/agarose mixture for labeling after low melting point agarose gel extraction at 100°C, be sure to cool down the template to 37°C before adding to the labeling mixture.

Labeling efficiency

The amount of labeled DNA depends on the:

- amount of template DNA
- purity of template DNA
- fragment size
- conformation of template DNA

Labeling variations

Modified deoxyribonucleoside triphosphates [^{32}P]-, [^{35}S]-, [^{3}H]-, [^{125}I], digoxigenin- or biotin-labeled dUTP can be used in the same way.

3.1 Standard assay

Additional required equipment and reagents

- Heating block or water bath
- Ice/water bath
- 0.2 M EDTA, pH 8.0
- Either radioactively or chemically modified nucleotides. For radioactive labeling, we recommend [α–^{32}P] dCTP, 3000 Ci/mmol. For non-radioactive labeling, please see following table.

Product	Pack size
Digoxigenin-11-dUTP alkali stable	25 nmol (25 µl) 125 nmol (125 µl)
Digoxigenin-11-dUTP alkali labile	25 nmol (25 µl) 125 nmol (125 µl)
Biotin-16-dUTP	50 nmol (50 µl)
Fluorescein-12-dUTP	25 nmol (25 µl)

Preparation of dNTP Stock Mix

To avoid pipetting mistakes, because of low volumes, prepare a stock mix of unlabeled dNTPs. Aliquots should be stored at −15 to −25°C. Avoid repeated freezing and thawing. If a labeled dNTP other than dCTP is used, 1 µl of dCTP (vial 3) has to be added to the mix instead of the corresponding unlabeled dNTP.

dNTP Stock Mix	
Label	**Volume**
dATP (vial 2)	1 µl
dGTP (vial 4)	1 µl
dTTP (vial 5)	1 µl

Procedure for labeling with [α-^{32}P] dCTP

Please refer to the following table:

Larger amounts can be labeled by scaling up of all components and volumes.

1. • Add to 25 ng template DNA (linear) double distilled water to a final volume of 12 µl in a microfuge tube.
   • For the control reaction use 2 µl control DNA (vial 1) and 7 µl double distilled water.
2. Denature the DNA by heating in a boiling water bath for 10 min at 95°C and chilling quickly in an ice/water bath.
   Complete denaturation is essential for efficient labeling. Depending on the DNA used a much shorter denaturing time down to 1 min has proved to be efficient, *e.g.*, for λDNA we recommend 1 to 2 min at 95°C.
3. • Centrifuge briefly and add the components in the following order to the freshly denatured probe on ice:

Reagent	Volume
dNTP Stock Mix	3 µl
Reaction mixture (vial 6)	2 µl
20 µCi [α-^{32}P]dCTP, 3000 Ci/mmol, aqueous solution	2 µl
Klenow enzyme (vial 7)	1 µl

   • Mix and centrifuge briefly.
   • Incubate at 37°C for 30 min.
   Longer incubation can increase the yield of labeled DNA.
4. Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0) and/or by heating to 65 °C for 10 min.

Removal of unincorporated nucleotides

For the removal of unincorporated deoxyribonucleoside triphosphates we recommend to use:

- Quick Spin Column, Sephadex G-50 (Fine)* or
- repeated ethanol precipitation.

3.2 Labeling assay with Digoxigenin-11-dUTP

Before you begin

For random priming of DNA with Digoxigenin, we offer more convenient products like for example DIG DNA Labeling Kit*, DIG-High Prime* or DIG DNA Labeling Mix, 10×* (6).

Additional required equipment and reagents

- Heating block or water bath
- Ice/water bath
- 0.2 M EDTA, pH 8.0
- Digoxigenin-11-dUTP, alkali stable*
- Digoxigenin-11-dUTP, alkali labile*
- Biotin-16-dUTP* can be used in the same way.

Preparation of DIG Stock Mix

To avoid pipetting mistakes, because of low volumes, prepare a stock mix. Therefore mix Digoxigenin-11-dUTP and dTTP (vial 5) 1:1. For each labeling reaction 1.6 µl are needed.

Procedure for labeling with DIG-11-dUTP

Please refer to the following table.

Larger amounts can be labeled by scaling up of all components and volumes.

Step	Procedure
1	• Add to 25 ng template DNA (linear) double distilled water to a final volume of 12.4 µl in a microfuge tube. • For the control reaction use 2 µl control DNA (vial 1) and 10.4 µl double distilled water.
2	Denature the DNA by heating in a boiling water bath for 10 min at 95°C and chilling quickly in an ice/water bath. Complete denaturation is essential for efficient labeling. Depending on the DNA used a much shorter denaturing time down to 1 min has proved to be efficient, *e.g.*, for λDNA we recommend 1 to 2 min at 95°C.
3	• Add the following to the freshly denatured probe on ice: (see table below) • Mix and centrifuge briefly. • Incubate for 1 h to 20 h (overnight) at 37°C. Longer incubation can increase the yield of labeled DNA.
4	Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0) and/or by heating to 65 °C for 10 min.

Reagent	Volume
dNTP Stock Mix	3 µl
DIG Stock Mix	1.6 µl
Reaction Mixture (vial 6)	2 µl
Klenow enzyme (vial 7)	1 µl

Removal of unincorporated nucleotides

When the labeled DNA is used as hybridization probe, removal of unincorporated nucleotides is not necessary. However, if you like to remove non-incorporated Digoxigenin-11-dUTP use:

- Quick Spin Column, Sephadex G-50 (Fine)*
- or repeated ethanol precipitation

4. Results

4.1 Data analysis

Degree of labeling

The degree of labeling is determined by comparison of incorporated to total input radioactivity in an aliquot of the reaction. The kinetics of the reaction may be followed by precipitation of the DNA with trichloroacetic acid of aliquots removed at various time points during the reaction.

Calculation of newly synthesized DNA (ng)

The amount of newly synthesized DNA in the labeling reaction is determined as follows:

$$\frac{\mu\text{Ci dNTP} \times 13.2 \times \%\text{ incorporation}}{\text{specific activity dNTP (Ci/mmol)}}$$

Calculation of incorporated radioactivity (dpm)

The amount of incorporated radioactivity in dpm is calculated as follows:

$$\mu\text{Ci dNTP} \times 2.2 \times 10^6 \times \%\text{ incorporation}$$

Specific activity (dpm/µg)

The specific activity in dpm/µg is calculated according to the following formula:

$$\frac{\text{incorporated radioactivity} \times 10^3}{\text{[input DNA (ng)+ newly synthesized DNA (ng)]}}$$

4.2 Typical results

Specific activity and labeling kinetics

Using the Random Primed DNA Labeling Kit labeling reactions were performed as follows:

25 and 100 ng λDNA with 20, 50, and 100 µCi [α^{32}P] dCTP, 3000 Ci/mmol;

2000 ng λDNA with 50 µCi [α^{32}P] dCTP, 3000 Ci/mmol;

25 ng λDNA with 50 and 100 µCi [α^{32}P] dCTP, 6000 Ci/mmol

λDNA	20 µCi	50 µCi	100 µCi	[α^{32}P] dCTP, 3000Ci/mmol
25 ng	66% 0.9×10^9	65% 1.8×10^9	62% 2.6×10^9	incorporation dpm/µg
100 ng	73 % 0.3×10^9	71% 0.7×10^9	68% 1.1×10^9	incorporation dpm/µg
2000 ng	-	53% 0.3×10^8	-	incorporation dpm/µg
λDNA	-	50 µCi	100 µCi	[α^{32}P] dCTP, 6000Ci/mmol
25 ng	-	68% 2.3×10^9	62% 3.5×10^9	incorporation dpm/µg

The reaction kinetics remain similar with 25 ng or 100 ng DNA (see figure 1 and 2).

The efficiency of the labeling reaction depends on the product used for labeling which may differ in:

- sensitivity
- specificity
- half life
- concentration.

Size of labeled fragments

Using the standard assay, size analysis of the radioactive fragments obtained at various time points of incubation with λDNA was performed by denaturing gel electrophoresis; after 10 – 180 min incubation the length of the fragments was 80 – 120 bp on average.

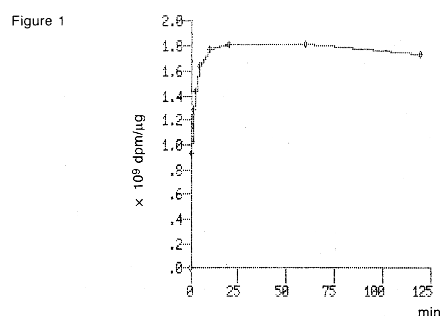

Figure 1: Labeling kinetics of 25 ng λDNA with 20 µCi [α–^{32}P] dCTP, 3000 Ci/mmol

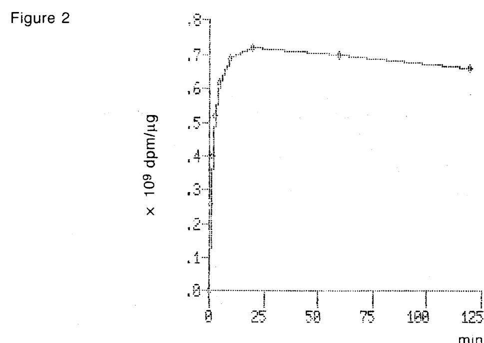

Figure 2: Labeling kinetics of 100 ng λDNA with 20 µCi [α–^{32}P] dCTP, 3000 Ci/mmol

5. Troubleshooting

Problem	Possible cause	Recommendation
Low amount of synthesized labeled DNA.	Purity of DNA	Add an additional purification step for template DNA.
	Repeated freezing and thawing damage the nucleotides	Aliquot the nucleotides and store at −15 to −25°C until use.
	Repeated freezing and thawing damage the Klenow enzyme	Aliquot Klenow polymerase and store at −15 to −25°C until use.
	Order of pipetting steps was not followed	It is important to add Klenow polymerase in the last step after adding all other components.
	DNA not fully denatured	Denaturing time of DNA is crucial. 10 minutes at 95 °C are recommended, but depending on the type of DNA a shortening of denaturing time down to one minute can effectively improve labeling efficiency. After that quickly chill denatured probe in a ice/water bath to avoid renaturation of template DNA. Briefly spin down the probe before adding the labeling components. Continue uninterrupted pipetting to start the labeling reaction.
	Incubation temperature was wrong	Incubate DNA at 37°C.
Low specific activity.	Template concentration was too high	If you use high concentrations of template DNA the degree of incorporation will increase but the specific activity (dpm/µg) decrease
	Modified ratio DNA: labeled NTPs	When varying ratios of DNA to labeled deoxyribonucleotide-triphosphates are used, similar incorporation rates but different levels of specific activity are obtained.
Inefficient labeling of DNA/agarose mixture.	DNA/agarose mixture for labeling was too hot	After extraction of DNA from agarose low melting point at 100°C, be sure to cool the template to 37°C before adding to the labeling mixture.

5.1 References

1 Feinberg, AP. & Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132 (1)**, 6-13.
2 Feinberg, AP. & Vogelstein, B. (1984) „A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity“ Addendum. *Anal. Biochem.*, **137 (1)**, 266-7.
3 Grunstein,M. & Hogness, D. (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **72**, 3961.
4 Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.
5 Smith, G. E. & Summers, M.D. (1980) The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-papier. *Anal. Biochem.* **109**, 123-129.
6 Kruchen, B. & Rueger, B. (2003) The DiG System - Nonradioactive and Highly Sensitive Detection of Nucleic Acids. *Biochemica* **3**, 13-15.

Quality control

Using the standard assay with 25 ng λDNA and 20 µCi [α-^{32}P] dCTP, 3000 Ci/mmol, an incorporation rate of >55% = 1.6 × 10^9 dpm/µg is obtained after 30 min incubation at 37°C.

Changes to previous versions

- see Kit contents Vial 6

5.2 Ordering Information

Kits

Product	Pack size	Cat. No.
High Pure PCR Product Purification Kit	1 kit (50 purifications) 1 kit (250 purifica.)	11 732 668 001 11 732 676 001
Agarose Gel DNA Ext. Kit	1 kit (100 reactions)	11 696 505 001
High Prime DNA Lab. Kit	1 kit for 50 reactions	11 585 584 001
DIG DNA Labeling Kit	1 kit for 40 reactions	11 175 033 910
DIG-High Prime	160 µl (40 reactions)	11 585 606 910
DIG Luminescent Det. Kit	1 kit for 50 blots	11 363 514 910
DIG-High Prime DNA Labeling and Detection Starter Kit II	1 kit for 12 labeling reactions	11 585 614 910

Single reagents

Product	Pack size	Cat. No.
Quick Spin Columns for radio-labeled DNA purification, Sephadex G-50	20 columns	11 273 965 001
Quick Spin Columns for radio-labeled DNA purification, Sephadex G-50	50 columns	11 273 973 001
Digoxigenin-11-dUTP alkali stable	25 nmol (25 µl) 125 nmol (125 µl)	11 093 088 910 11 558 706 910
Digoxigenin-11-dUTP alkali labile	25 nmol (25 µl) 125 nmol (125 µl)	11 573 152 910 11 573 179 910
Biotin-16-dUTP	50 nmol	11 093 070 910
Fluorescein-12-dUTP	25 nmol	11 373 242 910
Tetramethyl-rhodamine-6-dUTP	25 nmol	11 534 378 910
DIG DNA Labeling Mix, 10×	50 µl (25 reactions)	11 277 065 910
dATP, lithium salt	25 µmol (250 µl)	11 051 440 001
dCTP, lithium salt	25 µmol (250 µl)	11 051 458 001
dGTP, lithium salt	25 µmol (250 µl)	11 051 466 001
dTTP, lithium salt	25 µmol (250 µl)	11 051 482 001
Desoxynucleoside-Triphosphate Set	4× 10 µmol (100 µl) 40× 10 µmol	11 277 049 001 11 922 505 001

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