For life science research only. Not for use in diagnostic procedures.

Roche

DIG RNA Labeling Kit (SP6/T7)

RNA labeling with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase

Cat. No. 11 175 025 910

Kit for 2× 10 labeling reactions

Version 12
Content version: January 2013
Store at −15 to −25°C

1. What this Product Does

Number of Tests

1 kit is sufficient for 2× 10 labeling reactions.

Kit Contents

Bottle	Label	Contents Including Function
1	pSPT18 DNA	• 40 μl (0.25 mg/ml) • Cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase
2	pSPT19 DNA	• 40 μl • [0.25 mg/ml] • Cloning/transcription vector; subclones are transcribed into RNA probes by T7 or SP6 RNA polymerase
3	Control DNA 1 pSPT18-Neo	• 20 μl • [0.25 mg/ml] cleaved with *Pvu* II • Transcription of control DNA 1 by T7 RNA polymerase according to the standard protocol produces DIG-labeled "antisense" Neo transcripts of 760 bases in length. • Use as control in labeling and hybridization reactions • contains DNA fragments of 798 and 3,281 bp due to cleavage at the 2 *Pvu* II sites on the plasmid
4	Control DNA 2 pSPT19-Neo	• 20 μl • [0.25 mg/ml] cleaved with *Pvu* II • Transcription of control DNA 2 by SP6 RNA polymerase according to the standard protocol produces DIG-labeled "antisense" Neo transcripts of 760 bases in length. • Use as control in labeling and hybridization reactions • contains DNA fragments of 316 and 3,761 bp due to cleavage at the 2 *Pvu* II sites on the plasmid (one at pos. 761 within the neo gene)
5	Labeled control RNA*	• 100 μl • (100 ng/μl) • DIG-labeled "antisense" Neo RNA (7) (760 bases), transcribed with T7 RNA polymerase from 4 μl (equivalent to 1 μg) *Pvu* II-linearized pSPT18-Neo DNA (vial 3) according to the standard protocol. The transcription assay was not treated with DNase I. After ethanol precipitation, it was dissolved in dimethylpyrocarbonate-treated water. • The solution contains approx. 10 μg of DIG-labeled Neo RNA and 1 μg pSPT18-Neo template DNA • For semi-quantitative estimation of DIG-labeled RNA and use as a hybridization control • contains DNA fragments of 798 and 3,281 bp.
6	Unlabeled control RNA	• 20 μl, unlabeled control RNA, • [200 μg/ml] • Unlabeled Neo poly (A) "sense" RNA in dimethylpyrocarbonate-treated water. • The Neo poly (A) RNA is synthesized by *in vitro* transcription and is 1 kb long • Target RNA to practice RNA/RNA hybridizations; when applied to a membrane, this RNA will hybridize with the labeled control RNA (vial 5)
7	NTP labeling mixture*	• 40 μl nucleotide mixture, • 10 ×, [10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP, pH 7.5 (20°C)]
8	Transcription buffer	• 40 μl • 10 × conc.
9	DNase I, RNase-free*	• 40 μl • [10 U/μl] • Degrades DNA template after the labeling reaction
10	Protector RNase Inhibitor*	• 20 μl • [20 U/μl] • Prevents the degradation of RNA during the labeling reaction
11	SP6 RNA Polymerase*	• 20 μl • [20 U/μl] • Synthesizes RNA from a DNA template
12	T7 RNA Polymerase*	• 20 μl • [20 U/μl] • Synthesizes RNA from a DNA template

* *available from Roche Applied Science*

Storage/Stability

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

Additional Equipment/Reagents Required

In addition to the reagents listed above, you must prepare several solutions. In the table you will find an overview of the equipment and reagents needed for the different procedures.

Detailed information is given at the beginning of each procedure.

Procedure	Equipment	Reagents
2.2 DIG RNA labeling	water bath	• Water, PCR Grade treated with DMPC • EDTA, 0.2 M, pH 8.0
2.3 Determination of labeling efficiency	• nylon membranes positively charged* • UV-transilluminator or • UV cross-linker	• RNA dilution buffer • DIG Wash and Block Buffer Set* • Anti-digoxigenin-AP* • CSPD* or CDP-*Star** • DIG Luminescent Detection Kit*

Applications

DIG-labeled RNA is used for hybridization to

- Northern blots
- Southern blots
- *in situ* hybridization
- plaque or colony lifts
- RNase protection experiments

As the linkage between DIG and UTP is resistant to alkali, DIG-labeled RNA can be fragmented by alkaline treatment. Slightly reducing the size of the DIG-labeled RNA probe may make it more suitable for certain applications in *in situ* hybridization (5, 6).

2. How to Use this Product

2.1 Before You Begin

General Handling Recommendations

This table gives some general tips for successful DIG labeling and detection.

Recommendation	Guideline
Work under clean conditions	• Autoclave DIG System solutions • Filter-sterilize solutions containing SDS • Do not add Tween 20 to solutions until after they have been sterilized
Use clean incubation trays	• Rigorously clean and rinse laboratory trays before each use • When performing northern blots use sterilized glass trays and solutions for all washing and detection steps.
Membrane handling requirements	• Wear powder-free gloves • Handle membrane only on the edges and with clean forceps

Sample Material

- linearized plasmid DNA
- PCR product

Features of the Template DNA

The following table lists the recommended features of the template.

Feature	Detail
Purity	We recommend using the High Pure Plasmid Isolation Kit* to isolate plasmid DNA and the High Pure PCR Product Purification Kit* for purification of the linearized template.
Size	For optimal results, template DNA should be linearized and should be approx. 100 bp or larger. Template DNA >10 kb should be restriction-digested with a 4 bp cutter prior to labeling.
Amount	The procedure described below can produce up to 10 μg of labeled RNA from 1 μg of plasmid DNA template. If you scale up all volumes and components, you can use this procedure to label even larger amounts of DNA.

Sample Preparation

- Linearize the DNA template by cutting at a restriction enzyme site downstream from the cloned insert.
- To avoid transcription of undesirable sequences, **use a restriction enzyme that creates 5´ overhangs.**
- After restriction digest, purify the DNA with the High Pure PCR Product Purification Kit* or via phenol/chloroform extraction, and subsequent ethanol precipitation.

Preparation of Working Solutions

This table lists composition, storage and use of the other reagents you will need in addition to kit components.

Solution	Preparation	Storage/ Stability	Use
Water, PCR Grade* or DMPC-treated water	Double distilled water treated with 0.1% methylpyrocarbonate	+15 to +25°C, stable	Adjust reaction volume and/or resuspend RNA
EDTA	0.2 M ethylene-diamino-tetraacetic acid, pH 8.0	+15 to +25°C, stable	Stopping the reaction

Control Reactions

For checking if the DIG labeled RNA probe has been labeled efficiently please refer to chapter 2.3 "Determination of Labeling Efficiency*.
Optional: *In vitro* transcription of linearized control plasmid (vial 3 or 4) is possible using T7 or SP6 RNA Polymerase according to the standard protocol.

** available from Roche Applied Science*

2.2 Procedure

Standard RNA labeling reaction

Step	Action
1	Add 1 μg **purified template DNA** or 4 μl **control DNA** (vial 3 or 4) to a sterile, RNase-free reaction vial. Then, add enough water (Water, PCR Grade* or DMPC-treated water) to the vial to make the total sample volume up to 13 μl.
2	• Place the reaction vial on ice, then add the following reagents: Reagent / Vial / Volume: 10× NTP labeling mixture / 7 / 2 μl 10× Transcription buffer / 8 / 2 μl Protector RNase inhibitor / 10 / 1 μl RNA Polymerase SP6 **or** RNA Polymerase T7 / 11, 12 / 2 μl • Mix gently and centrifuge briefly. • Incubate for 2 h at +37°C. Longer incubations do not increase the yield of labeled RNA
3 (optional†)	• Add 2 μl **DNase I**, **RNase-free** to remove template DNA. • Incubate for 15 min at +37°C. † Recommended for RNase-protection experiments!
4	Stop the reaction by adding 2 μl 0.2 M **EDTA** (pH 8.0). The RNA transcripts can be analyzed by agarose gel electrophoresis (*e.g.*, formaldehyde gels or native gels) and ethidium bromide staining.

Reagents for step 2:

Reagent	Vial	Volume
10× NTP labeling mixture	7	2 μl
10× Transcription buffer	8	2 μl
Protector RNase inhibitor	10	1 μl
RNA Polymerase SP6 **or** RNA Polymerase T7	11 12	2 μl

DNase Treatment

If you are using the DIG-labeled RNA for hybridization to northern or Southern blots, plaque or colony lifts, you do not need to treat the sample with DNase (optional step 3 above), since the amount of DIG-labeled RNA transcript is far in excess of the template DNA.

Handling and Storage of Labeled Probe

Do not phenol/chloroform extract your DIG-labeled probe because it will partition into the organic phase.

Labeled probes are stable for a minimum of 1 year when stored at −15 to −25°C.

Note: Avoid repeated freezing and thawing of the labeled probe.

Scaled Up Reaction

Reaction may be scaled up to increase the yield of labeled RNA.

By keeping the amount of template DNA constant while increasing the amount of the other components in the labeling reaction, you can generate larger amounts of labeled probe.

In a 5 × scaled-up reaction with 1 μg linear control DNA (vial 3) as template, you can synthesize more than 40 μg of DIG-labeled RNA in a 2 hour incubation at +37° C.

Hybridization

If you are performing northern analysis we recommend loading the following amounts of target RNA onto the gel: 1 μg of total RNA, 100 ng mRNA.

DIG-labeled RNA can be used in standard hybridization protocols. Use of DIG Easy Hyb buffer* will decrease the hybridization time (less than 6 h) and the hybridization background. Use 20-100 ng of the DIG-labeled RNA probe per ml hybridization mix. For best results we recommend using Roche positively charged nylon membranes*, which are function-tested for optimal results with the DIG System. Hybridization solutions containing labeled RNA can be stored frozen and reused, provided that all solutions are RNase-free. If you used DIG Easy Hyb buffer denature the probe for 10 min at +65°C before using it again (do not boil!).

Immunological Detection

Either analyze the hybridized membrane immediately (using the immunological detection procedure) or store the membrane sealed in a plastic bag for later analysis.

The immunological detection procedure involves blocking the membrane and then using the anti-DIG-AP conjugate* to detect DIG-labeled RNA on the membrane. Use a chemiluminescent substrate (CSPD* or CDP-*Star**) to visualize the antibody-probe complexes on the membrane.

Expose the membrane to X-film or imaging device for 10-30 min. The membranes can be stripped and reused for hybridization, provided the membrane did not dry at any time and it was handled under RNase-free conditions throughout the procedure.

Detection reagents must be purchased separately. A detailed detection procedure is included in the DIG Luminescent Detection Kit*, which we recommend for easy and sensitive detection of DIG-labeled RNA probes.

2.3 Determination of Labeling Efficiency

Introduction

It is important to determine the yield of DIG-labeled RNA for optimal and reproducible hybridization results. Using too much probe in the hybridization mix causes background, while using too little concentration leads to weak signals.

Test Principle

The preferred method for quantification of labeled probes is direct detection via spot test. An overview of the test is given below .

Stage	Description
1	• Apply a series of dilutions of DIG-labeled RNA to a positively charged nylon membrane*. • Also spot several known dilutions of the control RNA (vial 5) on the membrane. These serve as standards.
2	• Perform an immunological detection reaction on the membrane with anti-digoxigenin-AP conjugate* and the premixed stock solution of CSPD ready-to-use* or CDP-*Star**. • Expose the membrane to X-ray film or imaging device to visualize the spots.

Preparation of Probe Stock Solution for the Spot Test

The efficiency of the labeling reaction depends on the starting amount of template and the incubation time.

To perform the spot test, initially assume that the yield of the labeling reaction was 10 μg of labeled RNA/1 μg of template. Then, dilute your labeled probe accordingly to a starting concentration of 10 ng/μl.

Also dilute the DIG-labeled control RNA (vial 5) to 10 ng/μl. Use these starting solutions to prepare the dilution series (given in the next column).

Additional Solution Required

RNA Dilution Buffer: Mix RNase free, DMPC treated double dist. water: 20× SSC: formaldehyd in the ratio 5:3:2. Always prepare fresh!

** available from Roche Applied Science*

Dilution Series

Prepare a dilution series of your labeled probe and your control RNA as described in the following table:

⚠ Highly diluted solutions of RNA in water are not stable. Spot the dilutions on the test membrane immediately after preparing them.

Tube	RNA (µl)	From tube no.	RNA Dilution Buffer (µl)	Dilution	Final conc.
1	-	dilution of probe and vial 5	-	-	10 ng/µl
2	2	1	18	1:10	1 ng/µl
3	2	2	198	1:100	10 pg/µl
4	15	3	35	1:3.3	3 pg/µl
5	5	3	45	1:10	1 pg/µl
6	5	4	45	1:10	0.3 pg/µl
7	5	5	45	1:10	0.1 pg/µl
8	5	6	45	1:10	0.03 pg/µl
9	5	7	45	1:10	0.01 pg/µl
10	0	-	50	-	0

Procedure

To perform the spot test, do the following:

Step	Action
1	Apply 1 µl spots of tubes 3–10 from your labeled probe and the labeled control RNA to a strip of nylon membrane*.
2	Fix the nucleic acid to the membrane by cross-linking with UV light or baking for 30 min at 120°C.
3	Follow the chemiluminescent detection procedure described in the instruction for use of the DIG Chemiluminescent Detection Kit* or of the substrates CSPD* or CDP-*Star**. Use volumes appropriate to the size of your membrane strip Colorimetric detection procedure using NBT/BCIP* is described in the pack insert of the DIG Nucleic Acid Detection Kit*.

Analyzing the Result

Compare the intensity of your DIG-labeled probe spots to that of the control spots and calculate the amount of DIG-labeled RNA.

3. Troubleshooting

Problem	Possible Cause	Recommendation
Low sensitivity	Inefficient probe labeling	• Check labeling efficiency. The labeling reaction can be scaled up. • Purify template DNA by High Pure PCR Product Purification Kit * or phenolization. • Make sure that the template was linearized before labeling. • Do not store template in buffers containing more than 0.1 mM EDTA. • Check the amount and quality of target RNA or DNA.
	Low probe concentration in the hybridization	• Increase probe concentration to 100 ng/ml. • Let hybridization continue overnight.
	Target degradation/ weak signals in Northerns	• Work under RNAse free conditions. • Use DIG Wash and Block Buffer Set*.
Background	Concentration of labeled probe is too high	Quantify your labeled probe via a spot test (page 4). Do not use more than 100 ng/ml hybridization mix. a) Decrease probe concentration b) Make sure that the membrane was soaked in sufficient prehybridization solution.
	Wrong type of nylon membrane	Some types of nylon membrane may cause high background. Always use nylon membranes from Roche Applied Science, which are tested for use in the DIG System. ⚠ Do not allow the membrane to dry at any time.
	Ineffective stringency washes	• Check temperature of stringency washes, prewarm wash solution to correct temperature. • Eventually decrease stringency wash from 0.5× SSC to 0.1× SSC
Smear in lanes	Target concentration too high	For a northern blot, do not load more than 1 µg of total or 100 ng of mRNA per lane. The DIG System is very sensitive compared to radioactivity and higher RNA concentrations result in detection of degradation products.

4. Additional Information on this Product

4.1 How this Product Works

Labeling Principle

The DIG RNA Labeling Kit produces DIG-labeled, single-stranded RNA probes of known length. Either SP6 or T7 RNA polymerase transcribes these probes *in vitro* from template DNA (in the presence of digoxigenin-UTP) (1,2).

RNA Labeling by *in vitro* Transcription	The DNA to be transcribed is cloned into the polylinker site of appropriate transcription vectors (*e.g.,* pSPT 18 or 19), which contain promoters for SP6 and T7 RNA polymerases adjacent template DNA is linearized at a suitable site, the RNA polymerases are used to produce "run off" transcripts. DIG-UTP is incorporated into the transcript. Every 20-25th nucleotide of the newly synthesized RNA is a DIG-UTP. Since the nucleotide concentration does not become limiting in the standard transcription reaction, this reaction can generate large amounts of labeled RNA.
Hybridization	When used in standard procedures, DIG-labeled probes will hybridize to nucleic acids on a blot or *in situ*.
Immunological Detection	DIG-labeled RNA probes can be detected with anti-digoxigenin (Fab fragments conjugated to alkaline phosphatase). The bound antibody conjugate is then visualized with the highly sensitive chemiluminescent substrates CSPD or CDP-*Star* (filter hybridization) or NBT/BCIP (*in situ*).

** available from Roche Applied Science*

Background Information

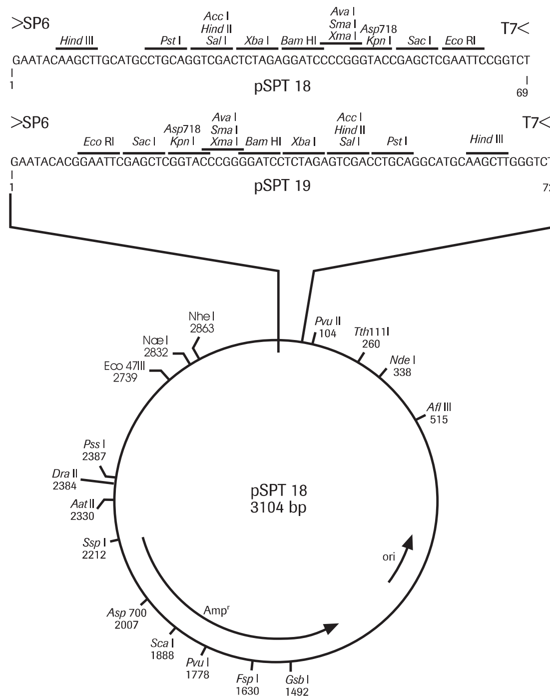

Fig. 1: Polylinker sites of the transcription Vectors pSPT18 and pSPT19.

Cloning into pSPT18/19

The pSPT18/19 plasmids are pUC derivatives, containing the pBR322 origin and the amp gene, but no lac operon. (Therefore, the presence of these plasmids cannot be detected by blue/white selection methods.)

4.2 Product Characteristics

Labeling Efficiency

The amount of synthesized labeled RNA depends on the amount, size (site of linearization) and purity of the template DNA.

Under standard conditions approx. 10 µg of full length DIG-labeled RNA will be transcribed from 1 µg template DNA.

When 1 µg of linearized template DNA (vials 3 or 4) is labeled in the standard reaction, approx. 37% of the nucleotides are incorporated into about 10 µg of DIG-labeled RNA (transcript length, 760 bases).

Sensitivity and Specificity

DIG-labeled RNA probes can detect single-copy genes in as little as 1 µg of mammalian DNA under the following assay conditions: The hybridization mix contains 20-100 ng labeled probe/ml, and the bound probe is detected with anti-DIG-AP and visualized with the chemiluminescent substrate CDP-*Star*.

Advantages

This table describes benefits and features of DIG-labeled RNA probes

Accurate and fast	The optimized kit components reduce the hands-on-time required to label RNA probes and increase efficiency and reproducibility.
Sensitive	DIG-labeled RNA probes can detect rare transcripts in total human or plant RNA.
Time-saving	DIG-labeled RNA probes can be stored at −15 to −25°C in ethanol for at least one year. We recommend freezing the probe in aliquots, to avoid repeated freeze/thaw cycles.
Labeled RNA of known length	• defined length • single-stranded
No probe renaturing	All labeled RNA is available for hybridization and does not renature like labeled DNA.

References

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Please refer to our website for the following informations:

17 http://www.roche-applied-science.com/DIG/
18 DIG Product Selection Guide
19 DIG Application Manual for Filter Hybridization
20 Non-radioactive *In situ* Hybridization Manual
21 Lab FAQS

Quality Control

Control DNAs 1 and 2 (vial 3 and 4) are used as templates for the transcription of labeled RNA as described in the procedure.

This labeled RNA can detect at least 0.1 pg homologous DNA (vial 3 and 4) or 0.1 pg homologous RNA (vial 6) in a dot blot using anti-DIG-alkaline phosphatase and CSPD as detection and visualizing reagents.

5. Supplementary Information

Ordering Information

For a complete overview of related products, please visit and bookmark our Special Interest Sites including:

- DIG Reagents and Kits for Non-Radioactive Nucleic Acid Labeling and Detection:
  http://www.roche-applied-science.com/DIG

Product	Pack Size	Cat. No.
DIG Kits and Reagents		
DIG Luminescent Detection Kit (CSPD)	50 blots	11 363 514 910
DIG Nucleic Acid Detection Kit (NBT/BCIP)	40 blots	11 175 041 910
DIG Northern Starter Kit	10 labeling/detection reactions	12 039 672 910
DIG Easy Hyb (ready-to-use hybridization solution without formamide)	500 ml	11 603 558 001
DIG Wash and Block Buffer Set	30 blots (10× 10 cm^2)	11 585 762 001
DIG RNA Labeling Mix	40 µl (20 reactions)	11 277 073 910
Biotin RNA Labeling Mix	40 µl (20 reactions)	11 685 597 910
Fluorescein RNA Labeling Mix	40 µl (20 reactions)	11 685 619 910
Nucleic Acid Purification		
High Pure Plasmid Isolation Kit	50 purifications 250 purifications	11 754 777 001 11 754 785 001
High Pure PCR Product Purification Kit	1 kit (50 purifications) 1 kit (250 purifications)	11 732 668 001 11 732 676 001
Substrates and Membranes		
CSPD, ready-to-use	2× 50 ml	11 755 633 001
CDP-*Star*, ready-to-use	2× 50 ml	12 041 677 001
NBT/BCIP stock solution	8 ml	11 681 451 001
NBT/BCIP Ready-to-use tablets	20 tablets	11 697 471 001
Nylon Membrane, positively charged (20 × 30 cm) (10 × 15 cm) (0.3 × 3 m roll)	 10 sheets 20 sheets 1 roll	 11 209 272 001 11 209 299 001 11 417 240 001
Nylon Membranes for Colony and Plaque Hybridization	50 discs (each 82 mm) 50 discs (each132 mm)	11 699 075 001 11 699 083 001
Associated Reagents		
SP6 RNA Polymerase	20 µl	10 810 274 001
T7 RNA Polymerase	20 µl	10 881 767 001
T3 RNA Polymerase	1000 U	11 031 163 001
Protector RNase Inhibitor	2 000 U 10 000 U (5 vials of 2000 U)	03 335 399 001 03 335 420 001
DNase I, recombinant, RNase-free	10 000 U	04 716 728 001
Water, PCR Grade	100 ml (4 vials of 25 ml) 25 ml (25 vials of 1 ml) 25 ml (1 vial of 25 ml)	03 315 843 001 03 315 932 001 03 315 959 001
Blocking Reagent	50 g	11 096 176 001
DNA MB grade, fish sperm	500 mg/50 ml	11 467 140 001
COT Human DNA	500 µg (500 µl)	11 581 074 001
COT Human DNA, Fluorometric Grade	1 mg	05 480 647 001
Labeled Control RNA		
Actin RNA Probe, DIG-labeled	2 µg	11 498 045 910
Lumi-Film, for chemiluminescent detection	100 sheets (20.3× 24.4cm) 100 sheets (18× 24 cm)	11 666 657 001 11 666 916 001
Buffers in a box, premixed SSC buffer, 20×	4 l	11 666 681 001

Changes to previous version

- see Kit content: Bottle 1

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