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DIG High Prime DNA Labeling and Detection Starter Kit II

Version 14

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Random primed DNA labeling with digoxigenin-dUTP, alkali-labile, and chemiluminescent detection with CSPD, ready-to-use

Cat. No. 11 585 614 910

Kit for 12 labeling reactions of 10 ng to 3 μg DNA and detection of 24 blots of 100 cm^2

Store the kit at −15 to −25°C

1. Preface

1.1 Table of Contents

1. Preface 2
1.1 Table of Contents 2
1.2 Kit contents 3

2. Introduction 5
2.1 Product overview 5

3. Procedures and required materials 8
3.1 Before you begin 8
3.2 Flow chart 8
3.3 DIG-DNA Labeling 9
3.4 Determination of labeling efficiency 12
3.5 DNA transfer and fixation 15
3.6 Hybridization 16
3.7 Immunological detection 17
3.8 Stripping and reprobing of DNA blots 19

4. Appendix 20
4.1 Troubleshooting 20
4.2 References 21
4.3 Ordering Information 22

1.2 Kit contents

Bottle/ Cap	Label	Content including function
1	DIG-High Prime	• 50 µl DIG-High Prime • 5 × conc. labeling mixture containing optimal concentrations of random primers, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme and buffer components • ready-to-use • clear, viscous solution • for efficient random primed labeling of DNA
2	DIG-labeled Control DNA	• 20 µl • [5 µg/ml] pBR328 DNA (linearized with Bam HI) • clear solution • determination of labeling efficiency
3	DNA Dilution Buffer	• 3 vials of 1 ml • [50 µg/ml fish sperm DNA in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 at 25°C] • clear solution
4	Anti-Digoxigenin-AP Conjugate	• 50 µl • [750 U/ml] • from sheep, Fab-fragments, conjugated to alkaline phosphatase • clear solution
5	CSPD ready-to-use	• 50 ml CSPD • clear solution • Chemiluminescent substrate for alkaline phosphatase
6	Blocking solution	• 4 × 100 ml • 10 × conc. • yellow, viscous solution
7	DIG Easy Hyb granules	4 bottles for 100 ml DIG Easy Hyb buffer each, for the hybridization of DNA

Additional equipment and reagents required

In addition to the reagents listed above, you have to prepare several solutions. In the table you will find an overview about the equipment which is needed for the different procedures.

Detailed information is given in front of each procedure.

Procedure	Equipment	Reagents
3.3 DIG-DNA labeling	water bath	• sterile double distilled water • EDTA, 0.2 M, pH 8.0, sterile
3.4 Semi-quantitative determination of labeling efficiency	Nylon membranes positively charged*	DIG Wash and Block Buffer Set* or
		• Washing buffer • Maleic acid buffer • Detection buffer
3.5 DNA transfer and fixation	• UV- light box or • commercially available UV-cross linker	• 2 × SSC or • 10 × SSC
3.6 Hybridization	• Nylon membranes, positively charged* • Hybridization bags* or temperature resistant, sealable plastic bags or roller bottles ***Note:*** Do not use open trays when working with DIG Easy Hyb buffer	
3.7 Immunological detection	• temperature resistant plastic bags or roller bottles • Hybridization bags*	DIG Wash and Block Buffer Set* or
		• Washing buffer • Maleic acid buffer • Detection buffer
3.8 Stripping and reprobing of DNA blots	• Large tray • Water bath	• 10 × SSC • 10% SDS • 0.2 M NaOH

* available from Roche Diagnostics

2. Introduction

2.1 Product overview

Test principle

The DIG High Prime DNA Labeling and Detection Starter Kit II uses digoxigenin (DIG), a steroid hapten, to label DNA probes for hybridization and subsequent chemiluminescence detection by enzyme immunoassay (1,2,3).

Stage	Description
DNA labeling	DIG-labeled DNA probes are generated with DIG-High Prime according to the random primed labeling technique. DIG-High Prime is a specially developed reaction mixture containing digoxigenin-dUTP, alkali-labile (Fig. 1) and all reagents, including enzyme necessary for random primed labeling, premixed in an optimized 5 × concentrated reaction buffer.
Hybridization	DIG-labeled probes are used for hybridization to membrane blotted nucleic acids according to standard methods. The use of the alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization with a second DIG-labeled probe.
Immunological detection	The hybridized probes are immunodetected with anti-digoxigenin-AP, Fab fragments and are then visualized with the chemiluminescence substrate CSPD, ready-to-use. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a light emission at a maximum wavelength of 477 nm (Fig. 2) which is recorded with a appropriate imager or on X-ray films. Film exposure times are in the range of 5 to 30 min only.

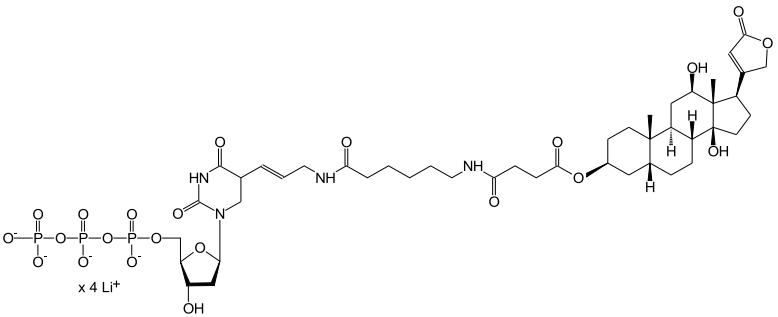


Fig. 1: DIG-dUTP, alkali labile

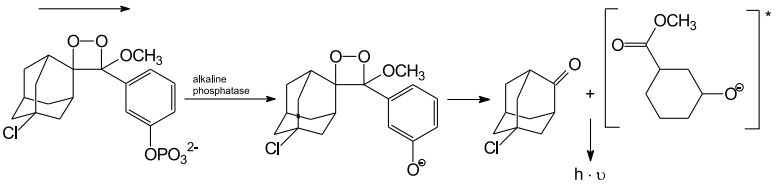


Fig. 2: Reaction of CSPD

Application

DIG-labeled DNA probes can be used:

- for all types of filter hybridization
- for single copy gene detection in total genomic DNA, even from organisms with high complexity, *e.g.* human, barley, and wheat.

Sample material

- DNA fragments of at least 100 bp
- linearized plasmid, cosmid or DNA
- supercoiled DNA

Assay time

This table lists the reaction time of the single steps

Step	Reaction time
DNA labeling	1 h-O/N
Hybridization	6 h or O/N
Immunological detection	1.5 h
Chemiluminescent signal detection	5-30 min

Number of tests

1 kit is sufficient for

- 12 standard labeling reactions of up to 3 μg template DNA
- and detection of
- 24 blots of 10 x 10 cm^2.

Quality Control

Using unlabeled control DNA pBR 328] labeled as described in the protocol, 0.1 pg homologous DNA diluted with 50 ng heterologous DNA are detected in a dot blot with CSPD, ready-to-use after 30 min exposure to X-ray film, following the standard detection protocol.

Kit storage/ stability

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

Once opened, please refer to the following table for proper storage.

Kit component	Storage
Anti-Digoxigenin-AP Conjugate vial 4	+2 to +8°C, stable ***Note***: Do not freeze!
CSPD, ready-to-use vial 5	+2 to +8°C, stored protected from light.
Blocking solution bottle 6	• unopened, stable at + 15 to +25°C • once opened, it should be aliquoted and stored at −15 to −25°C or at +2 to +8°C up to one month when keeping sterile • working solution should always be prepared fresh
DIG Easy Hyb Granules	• stable at +15 to +25°C • once opened, the solution is stable for 1 month, when kept sterile
DIG-High Prime Mixes	12 month; -15 to -25°C. Avoid repeated freezing and thawing!

Sensitivity and specificity

A single copy gene (tissue plasminogen activator, tPA) is detected in a Southern blot of 0.3 µg *Bgl* II or *Eco* RI digested human placenta DNA.

Advantages

This table describes benefits and features of the kit.

Benefit	Feature
Accurate and fast	The use of premixed DIG-High Prime minimizes the hands-on-time required to label DNA probes and increases yields and reproducibility.
Sensitive	Single-copy genes can be detected in total human DNA complex and plant genomes.
Time-saving	DIG-labeled probes can be stored frozen for at least one year. Hybridization solutions can be reused 3 – 5 times, depending on the amount of labeled probe used for signal generation in each hybridization.

3. Procedures and required materials

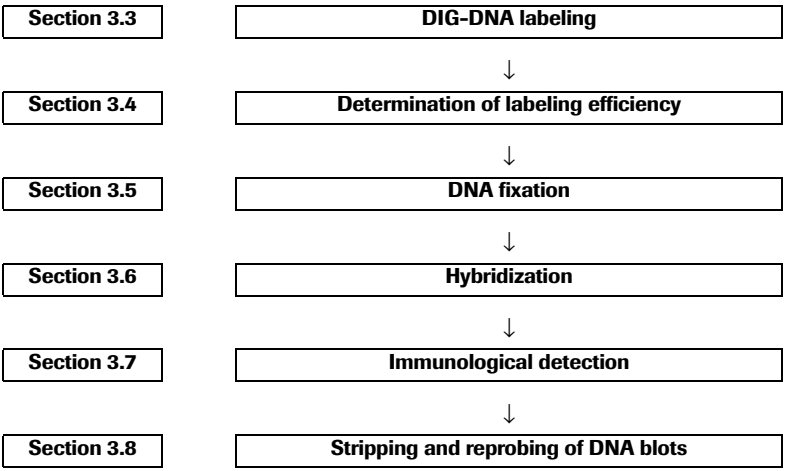
3.1 Before you begin

General handling recommendations

This table describes general hints for DIG labeling and detection.

Recommendation	Guideline
Work under clean conditions	Autoclave DIG System solutions Filter-sterilize solutions containing SDS Tween 20 should be added to previously sterilized solutions
Use clean incubation trays	Rigorously clean and rinse laboratory trays before each use.
Membrane handling requirements	Wear powder-free gloves Handle membrane only on the edges and with clean forceps

3.2 Flow chart



3.3 DIG-DNA Labeling

Introduction

DNA is random primed labeled with Digoxigenin-11-dUTP using DIG-High Prime, a 5 × concentrated labeling mixture of random hexamers, dNTP mix containing alkali-labile Digoxigenin-11-dUTP, labeling grade Klenow enzyme and an optimized reaction buffer.

Additional equipment and reagents required

- water bath
- ice/water

This table lists composition, storage and use of the required reagents in addition to kit components.

Solution	Composition	Storage/ Stability	Use
Water	Autoclaved, double distilled water	+15 to +25°C, stable	Dilution of DNA
EDTA	0.2 M ethylenediamino- tetracetic acid, pH 8.0	+15 to +25°C, stable	Stopping the labeling reaction

Template DNA

The following table lists the recommended features of the template DNA

Feature	Detail
Purity	Template DNA should be prepared with the High Pure Plasmid Isolation Kit*. When other commercially available purification kits are used, we recommend to do an additional phenol/chloroform extraction to remove residual protein. This step is also necessary when templates have been treated with restriction or other modifying enzymes before labeling.
Size	To obtain optimal results, template DNA should be linearized and should have a size of -100 or larger. Template DNA >5 kb should be restriction-digested using a 4 bp cutter (*e.g., Hae* III), prior to labeling
Amount	With the procedure described below principally 10 ng – 3μg of template can be labeled, however, please check in the given table the necessary amount of probe needed for your size of blot. By scaling up of all volumes and components accordingly this procedure can be used for labeling of larger amounts. If single-copy gene detection in complex genomes is performed at least 300 ng of template DNA (probe concentration: 25 ng/ml hybridization solution) should be labeled.

Labeling of DNA isolated from agarose

If you intend to perform genomic Southern blotting, you should separate the template insert DNA from the vector by agarose gel electrophoresis.

To isolate DNA from the gel, you can use the Agarose Gel DNA Extraction Kit* for DNA fragments in the range of 400 bp to 5 kbp. It is applicable for standard agarose gels as well as low melting point agarose gels. Afterwards, the DNA fragments are efficiently labeled with digoxigenin without further purification. However, labeled probes should be purified with the High Pure PCR Product Purification Kit* to remove residual agarose particles.

Procedure

This procedure is designed for 10 ng-3 µg of DNA. Larger amounts (up to 10 µg) can be labeled by scaling up of all components and volumes.

Step	Action
1	Add 1 µg **template DNA** (linear or supercoiled) and autoclaved, double distilled water to a final volume of 16 µl to a reaction vial.
2	Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath. ***Note***: Complete denaturation is essential for efficient labeling.
3	Mix **DIG-High Prime** (vial 1) thoroughly and add 4 µl to the denatured DNA, mix and centrifuge briefly. Incubate for 1 h or O/N at +37°C. ***Note:*** Longer incubations (up to 20 h) will increase the yield of DIG-labeled DNA (see table below).
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. ***Note:*** The lengths of the DIG labeled fragments obtained with DIG-High Prime range from 200 bp to 1,000 bp or larger, depending on the lengths of the original template.

Yield of labeling reaction

Table 1:

This table shows you the yield of DIG-High Prime labeling under optimal conditions.

In the standard reaction with 1 µg DNA per assay approx. 15% of the nucleotides are incorporated into about 0.8 µg of newly synthesized DIG-labeled DNA within 1 h and approx. 38% of the nucleotides into about 2 µg after 20 h.

Template DNA	1 h	20 h
10 ng	45 ng	600 ng
30 ng	130 ng	1,050 ng
100 ng	270 ng	1,500 ng
300 ng	450 ng	2,000 ng
1000 ng	850 ng	2,300 ng
3000 ng	1,350 ng	2,650 ng

Using DIG-High Prime solution, reactions were performed with increasing amounts of different template DNAs for 1 h and 20 h. The yield of DIG-labeled DNA was determined by incorporation of a radioactive tracer and confirmed by a dot blot (Average of 10 independent labeling assays).

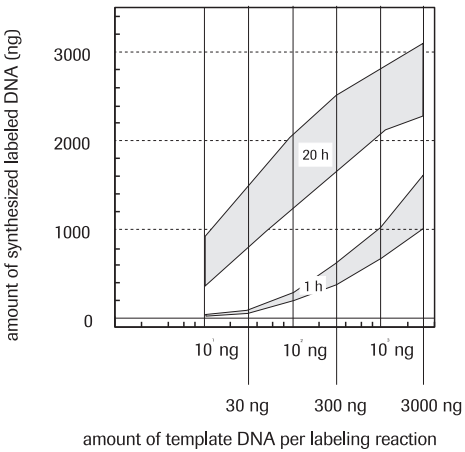


Fig. 3: Yield of DIG-labeled DNA from different amounts of template DNA after 1 and 20 h incubation of the DIG-High Prime reaction at 37°C

3.4 Determination of labeling efficiency

Introduction

Determination of the yield of DIG-labeled DNA is most important for optimal and reproducible hybridization results. Too high of a probe concentration in the hybridization mix causes background, while too low of a concentration leads to weak signals.

Test principle

The preferred method for quantification of labeled probes is the direct detection method.

Stage	Description
1	A series of dilutions of DIG-labeled DNA is applied to a small strip of nylon membrane positively charged*. Part of the nylon membrane is preloaded with defined dilutions of DIG-labeled control DNA (vial 2) which are used as standards.
2	The nylon membrane is subjected to immunological detection with anti-digoxigenin-AP conjugate (vial 4) and CSPD ready-to-use. The intensities of the dilution series of DIG-labeled DNA and control DNA are compared by exposure to a appropriate imager or X-ray film.

Preparation of additional solutions required

Please find in the following table composition and preparation of additional reagents required. The following buffers are also available in the DIG Wash and Block Buffer Set* DNase and RNase free, according to the current quality control procedures.
Please note: Solutions and working solutions required for the detection part of the determination of labeling efficiency are identical to those for the chemiluminescent detection of your blot (please see chapter 3.7) and can be prepared in amount, that will also cover the detection procedure in chapter 3.7.

Solution	Composition / Preparation	Storage/ stability	Use
Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20	+15 to +25°C, stable	Removal of unbound antibody
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20°C)	+15 to +25°C, stable	Dilution of Blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	1+15 to +25°C, stable	Adjustment of pH to 9.5

Preparation of kit working solutions

The following table shows the preparation of kit working solutions.

Solution	Composition/preparation	Storage/ stability	Use
Blocking solution	Prepare a 1 × working solution by diluting the 10 × Blocking solution (vial 6) 1:10 in maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites on the membrane
Antibody solution	Centrifuge anti-digoxigenin-AP (vial 4) for 5 min at 10,000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute anti-digoxigenin-AP 1:10,000 (75 mU/ml) in blocking solution.	12 h at +2 to +8°C	Binding to the DIG-labeled probe

Dilution series

Labeled probes and the DIG-labeled control DNA (vial 2) must be diluted to 1 ng/µl, according to the expected yield of synthesized nucleic acid to start the dilution series below. The expected yield of DIG-labeled DNA in your probe can best be estimated by using the chart in chapter 3.3. The yield depends on the starting amount of template and incubation time.

Note: The yields given in table 1 were achieved under optimal conditions with highly purified template DNA.

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

Tube	DNA (µl)	From tube #	DNA Dilution Buffer (vial 3) (µl)	Dilution	Final concentration
1		diluted original			1 ng/µl
2	2	1	198	1:100	10 pg/µl
3	15	2	35	1:3.3	3 pg/µl
4	5	2	45	1:10	1 pg/µl
5	5	3	45	1:10	0.3 pg/µl
6	5	4	45	1:10	0.1 pg/µl
7	5	5	45	1:10	0.03 pg/µl
8	5	6	45	1:10	0.01 pg/µl
9	0	-	50	-	0

Procedure

The following procedure describes the direct detection.

Note: Use sufficient buffer volumes to cover the membrane completely during all steps.

Step	Action
1	Apply a 1 µl spot of tubes 2-9 from your labeled probes and the labeled control to the 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	• Transfer the membrane into a plastic container with 20 ml **Maleic acid buffer**. • Incubate under shaking for 2 min at +15 to +25°C.
4	Incubate for 30 min in 10 ml **Blocking solution**.
5	Incubate for 30 min in 10 ml **Antibody solution.**
6	Wash with 10 ml **Washing buffer**, 2 × 15 min.
7	Equilibrate 2-5 min in 10 ml **Detection buffer**.
8	• Place membrane with DNA side facing up on a development folder (or hybridization bag) and apply 0.1 ml **CSPD ready-to-use** (i.e. 4 drops from the dropper bottle 5) to the membrane. • **Immediately** cover the membrane with the second sheet of the folder to spread the substrate evenly and without airbubbles over the membrane. • Incubate for 5 min at +15 to +25°C.
9	Squeeze out excess liquid and seal the edges of the development folder. ***Note*:** Drying of the membrane during exposure will result in dark background.
10	Expose to a appropriate imager for 5-20 min or to X-ray film for 15-25 min at +15 to +25°C. ***Note***: Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it will reach a plateau where signal intensity remains almost constant during the next 24 – 48 hours. Multiple exposures can be taken to achieve the desired signal strength.

Analyzing the results

Compare the intensity of the spots out of your labeling reaction to the control and calculate the amount of DIG-labeled DNA. If the 0.1 pg dilution spots of your probe and of the control are visible, then the labeled probe has reached the expected labeling efficiency (pls. see table 1 in 3.3.) and can be used in the recommended concentration in the hybridization.

3.5 DNA transfer and fixation

Transfer methods and membranes

Standard protocols for gel electrophoresis, denaturation and neutralization of the gel are described in Sambrook et al. (6). Gels lacking ethidium bromide are preferred, because ethidium can cause uneven background problems. All common types of DNA transfer methods are suitable for subsequent DIG hybridization (7,8).

In our experience, best results are obtained when gels are blotted by capillary transfer with 20 × SSC on nylon membranes*, positively charged.

Note: Alkali transfer (*e.g.*, in 0.4 M NaOH) is not suitable for the transfer of DIG-labeled molecular weight markers*.

Fixation procedure

Fix the DNA to the membrane by any of the following procedures:

IF you want to...	THEN...
UV-crosslinking (nylon membrane)	• place the membrane on Whatman 3MM-paper soaked with 10 × SSC. • UV-crosslink the wet membrane without prior washing. • after the UV-crosslinking, rinse the membrane briefly in double distilled water and allow to air-dry.
bake at +120°C (nylon membrane)	• wash the membrane briefly in 2 × SSC. • bake the nylon membrane at +120°C for 30 min or according to the manufacturer`s instructions.
bake at +80°C (nylon membrane)	• wash the membrane briefly in 2 × SSC. • bake at 80°C for 2 h under vacuum.

Storage of the membrane

Please refer to the following table.

IF...	THEN...
you want to go ahead.	Use the membrane immediately for prehybridization.
you want to work later on	store the membrane dry at +2 to +8°C.

3.6 Hybridization

Additional equipment required

- ice/water
- shaking water-bath
- or hybridization oven
- Temperature resistant plastic or glass boxes, petri dishes, roller bottles or sealable plastic bags.

Note: Do not use open containers with DIG Easy Hyb buffer.

Preparation of DIG Easy Hyb working buffer

Add carefully 64 ml sterile double distilled water in two portions to the DIG Easy Hyb Granules (bottle 7), dissolve by stirring immediately for 5 min at +37°C.

Hybridization temperature

The appropriate hybridization temperature is calculated according to GC content and percent homology of probe to target according to the following equation:

T_m = 49.82 + 0.41 (% G + C) - (600/l) [l = length of hybrid in base pairs]

$T_{opt.}$ = T_m – 20 to 25°C

(The given numbers of the equation were calculated according to a standard equation for hybridization solutions containing formamide, 50%.)

The actual hybridization temperature $T_{opt.}$ for hybridization with DIG Easy Hyb buffer is +20 to +25°C below the calculated T_m value. $T_{opt.}$ can be regarded as a stringent hybridization temperature allows up to 18 % mismatches between probe and target. When the degree of homology of your probe to template is less than 80%, you should lower $T_{opt.}$ accordingly (approx. 1.4 °C below T_m per 1 % mismatch) and also adjust the stringent washing steps accordingly (i.e. increase SSC concentration and lower washing temperature).

Procedure

Please refer to the following table.

Step	Action
1	• Pre-heat an appropriate volume of **DIG Easy Hyb** buffer (10 ml/100 cm^2 filter) to hybridization temperature (+37 to +42°C). • Prehybridize filter for 30 min with gentle agitation in an appropriate container. ***Note:*** Membranes should move freely, especially if you use several membranes in the same prehybridization solution.
2	Denature **DIG-labeled DNA probe** (about 25 ng/ml DIG Easy Hyb buffer) by boiling for 5 min and rapidly cooling in ice/water. ***Note:*** As DIG-11-dUTP is alkali-labile, DNA probes cannot be denatured by alkali treatment (NaOH).
3	Add denatured DIG-labeled DNA probe to pre-heated DIG Easy Hyb buffer (3.5 ml/100 cm^2 membrane) and mix well but avoid foaming (bubbles may lead to background).
4	• Pour off prehybridization solution and add probe/hybridization mixture to membrane. • Incubate 4 h- O/N with gentle agitation.

Storage of hybridization solution

DIG Easy Hyb buffer containing DIG-labeled probe can be stored at −15 to −25°C and be reused several times when freshly denatured at +68°C for 10 min before use.

Note: Do not boil DIG Easy Hyb buffer.

Stringency washes

For most DNA:DNA applications, a stringency wash with 0.5 × SSC is sufficient. The correct post washing conditions have to be determined empirically for each probe.

- For human genomic DNA use 0.5 × SSC and +65°C.
- Probes > 150 bp and with a high G/C content should be washed at 68°C.
- For shorter probes around 100 bp or shorter, the wash temperature must be lowered.

This table describes how to perform post-hybridization washes.

Step	Action
1	Wash 2 × 5 min in ample 2 × SSC, 0.1% SDS at +15 to +25°C under constant agitation.
2	Wash 2 × 15 min in 0.5 × SSC, 0.1% SDS (prewarmed to wash temperature) at +65 to +68°C under constant agitation.

3.7 Immunological detection

Additional reagents required

Please find in the following table composition and preparation of additional reagents required. The following buffers are also available in the DIG Wash and Block Buffer Set* DNase and RNase free, according to the current quality control procedures.

Solution	Composition / Preparation	Storage and stability	Use
Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20	+15 to +25°C, stable	Washing of membrane
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20°C)	+15 to +25°C, stable	Dilution of Blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)	+15 to +25°C, stable	Alkaline phosphatase buffer

Preparation of kit working solutions

In the following table the preparation of kit working solutions is described.

Solution	Composition / Preparation	Storage and stability	Use
Blocking solution	Prepare a 1 × working solution by diluting 10 × Blocking solution (vial 6) 1:10 with Maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites
Antibody solution	Centrifuge Anti-Digoxigenin-AP (vial 4) for 5 min at 10,000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/ml) in Blocking solution.	12 h at +2 to +8°C	Binding to the DIG-labeled probe

Procedure

This table describes how to perform the immunological detection on a 100 cm^2 membrane.

Note: All incubations should be performed at +25 to +50°C with agitation. If the membrane is to be reprobed, do not allow the membrane to dry at any time.

Step	Action
1	After hybridization and stringency washes, rinse membrane briefly (1-5) min in **Washing buffer**.
2	Incubate for 30 min in 100 ml **Blocking solution**.
3	Incubate for 30 min in 20 ml **Antibody solution.**
4	Wash 2 × 15 min in 100 ml **Washing buffer**.
5	Equilibrate 2-5 min in 20 ml **Detection buffer**.
6	Place membrane with DNA side facing up on a development folder (or hybridization bag) and apply 1 ml **CSPD ready-to-use** (bottle 5). **Immediately** cover the membrane with the second sheet of the folder to spread the substrate evenly and without airbubbles over the membrane. Incubate for 5 min at +15 to +25°C.
7	Squeeze out excess liquid and seal the edges of the development folder. ***Note***: Drying of the membrane during exposure will result in dark background.
8	Incubate the damp membrane for 10 min at +37°C to enhance the luminescent reaction.
9	Expose to a appropriate imager for 5-20 min or to X-ray film for 15-25 min at +15 to +25°C. ***Note***: Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it will reach a plateau where signal intensity remains almost constant during the next 24-48 hours. Multiple exposures can be taken to achieve the desired signal strength.

3.8 Stripping and reprobing of DNA blots

General

The alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization experiment.

Additional equipment and reagents required

- large beaker
- water bath
- 10 × SSC
- 10 % SDS
- 0.2 N NaOH

Procedure

This procedure describes the stripping of a membrane.

Note: When stripping and rehybridization of blots is planned, the membrane should not dry off at any time.

Alternative stripping protocols, as mentioned in the "DIG Application Manual for Filter Hybridization" (available via internet) can also be used with high efficiency.

Step	Action
1	Rinse membrane thoroughly in double distilled water.
2	Wash for 2 × 15 min at +37°C in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled probe.
3	Rinse thoroughly 5 min in 2× SSC.
4	Prehybridize and hybridize with a second probe.

Storage of stripped membrane

Once the membrane is stripped, it can be stored in Maleic acid buffer or 2 × SSC until used again.

4. Appendix

4.1 Troubleshooting

Troubleshooting table

This table describes various troubleshooting parameters for DIG-labeling and detection

Problem	Possible cause	Recommendation
Low sensitivity	Inefficient probe labeling	Check labeling efficiency of your DIG DNA by comparison to the labeled control DNA.
	Wrong type of membrane	The quality of the membrane used as support for dot, Southern blotting influences sensitivity and speed of detection. Other types of nylon membranes like e.g. Biodyne A (Pall) are also suitable but might need longer exposure times to X-ray film. Some membranes may cause strong background formation. Nitrocellulose membranes can not be used with the protocol described.
	Inefficient hybridization	Increase the concentration of DIG-labeled DNA probe in the hybridization solution.
	Low antibody concentration	Increase the concentration of the anti-DIG-AP conjugate.
	Preincubation before exposure	Increase the duration of preincubation before exposure to X-ray film to > 30 min up to 12 h.
	To short exposure time	Increase time of exposure to X-ray film. The type of film may also influence the sensitivity.
High background	Inefficient labeling	Purify DNA/RNA by phenol/chloroform extraction and/or ethanol precipitation before labeling. Make sure that the probe does not contain crosshybridizing vector sequences.
	Wrong type of membrane	Although the protocol is optimized for the use of positively charged nylon membranes, some types which are very highly charged can cause background. Lot-to-lot variations in some membranes may also cause problems. When using the recommended function tested nylon membrane*, these problems are avoided.
	Concentration of labeled probe to high	Important: It can be necessary to decrease concentration of DIG- labeled DNA. The critical probe concentration limit (concerning background formation) can be determined by hybridization with increasing probe concentrations to unloaded membrane. Care should be taken not to permit the membranes to dry throughout the whole procedure.
	Antibody concentration too high	Decrease concentration of anti-DIG-AP conjugate. Increase volumes of the washing and blocking solution and duration of the washing and blocking steps. Spotty background may be caused by precipitates in the anti-DIG-AP conjugate: remove by a short centrifugation step. ***Note***: Several centrifugation steps can cause a certain loss of material, which must be compensated by use of larger amounts.
	Preincubation before exposure	Shorten the time of preincubation.

4.2 References

1 Kessler, C. et.al. (1990) *Biol. Chem. Hoppe-Seyler* **371:** 917.

2 Höltke, H.J. et al. (1990) *Biol. Chem. Hoppe-Seyler* **371:** 929.

3 Höltke, H.J. et al. (1995) The Digoxigenin (DIG) System for non-radioactive labelling and detection of nucleic acids-an overview. *Cell. Mol. Biol.* **41:** 883.

4 Bronstein, I. et al. (1991) in: Bioluminescence and Chemiluminescence, Current Status (Stanley, P. & Kricka, L.J., eds), pp 73-82.

5 Schaap, A.P. et al. (1989) *Clin. Chem.* **35:** 1863.

6 Sambrook, J., Fritsch, E.M. and Maniatis,T. (1989) Molecular cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Labor, New York.

7 Southern E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503.

8 Khandijan, E.W. (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Bio/Technology* **5:** 165.

9 Kruchen, B., Rueger, B. (2003) The DIG System — Nonradioactive and Highly Sensitive Detection of Nucleic Acids Biochemica **3,** 13-15.

10 Takada, Y. et. al. (2004) Molecular cloning and characterization of a novel glutathione S-transferase gene induced by light stimulation in the protozoan Blepharisma japonicum. *FEMS Microbiology Letters* **231(2):** 185-189.

11 Kojima, T. et.al. (2004) Expression profiles of polyhydroxyalkanoate synthesis-related genes in Paracoccus denitrificans. J*ournal of Bioscience and Bioengineering,* **97(1)**: 45-53.

4.3 Ordering Information

Kits

Product	Pack Size	Cat. No
DIG-High Prime DNA Labeling and Detection Starter Kit I	1 kit (12 labeling reactions and 24 detection reactions)	11 745 832 910
DNA Isolation Kit for Cells and Tissue for the extraction of genomic DNA from cells and tissue ranging in size from 50 to 150 kb	10 isolations for 400 mg tissue or 5×10^7 cells	11 814 770 001
DNA Isolation Kit for mammalian Blood for the isolation of intact genomic DNA from mammalian whole blood or lymphocyte preparations	25 purifications	11 667 327 001
High Pure PCR Product Purification Kit for the purification of PCR reaction products	50 purifications 250 purifications	11 732 668 001 11 732 676 001
High Pure PCR Template Purification Kit for isolating genomic DNA for PCR	100 purifications	11 796 828 001
High Pure Plasmid Isolation Kit small scale mini-preps for sequencing, PCR, and cloning	50 purifications 250 purifications	11 754 777 001 11 754 785 001
High Pure Viral Nucleic acid Kit for isolating viral DNA and RNA for PCR or RT-PCR	100 purifications	11 858 874 001
PCR Clean Up Kit for post-PCR DNA fragment purification	up to 100 purifications	11 696 513 001

Printed Materials

You can view the following manuals on our website:

DIG Application Manual for Filter Hybridization
Lab FAQS “Find a Quick Solution”
Nonradioactive *In Situ* Hybridization Manual
DIG Product Selection Guide

Single reagents

Product	Pack Size	Cat. No.
Agarose	100 U 500 U	11 417 215 001 11 417 223 001
DIG-High Prime	160 µl (40 labeling reactions)	11 58 5606 910
Blocking reagent	50 g	11 096 176 001
Glycogen, MB grade	20 mg (1 ml)	10 901 393 001
DIG Easy Hyb (ready-to-use hybridization solution without formamide)	500 ml	11 603 558 001
DIG Easy Hyb Granules	1 set (6 × 100 ml)	11 796 895 001
DNA Molecular Weight Marker, Digoxigenin-labeled: DNA Molecular Weight Marker II DNA Molecular Weight Marker III DNA Molecular Weight Marker V DNA Molecular Weight Marker VI DNA Molecular Weight Marker VII DNA Molecular Weight Marker VIII	 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl)	 11 218 590 910 11 218 603 910 11 669 931 910 11 218 611 910 11 669 940 910 11 449 451 910
DIG Wash and Block Buffer Set	30 blots (10 × 10 cm^2)	11 585 762 001
Hybridization bags	50 bags	11 666 649 001
Nylon Membrane, positively charged (20 × 30 cm^2) (10 × 15 cm^2) (0.3 × 3 m roll)	 10 sheets 20 sheets 1 roll	 11 209 272 001 11 209 299 001 11 417 240 001

Changes to previous version

- Editorial changes

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