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Print Protocol

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DIG High Prime DNA Labeling and Detection Starter Kit I

Version 12

Content version: July 2012

For color detection with NBT/BCIP.
Random primed DNA labeling with digoxigenin-dUTP, alkali-labile, and detection of hybrids by enzyme immunoassay

Cat. No. 11 745 832 910
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

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1. Preface

1.1 Table of Contents

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 ×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	▪ 100 µl ▪ [750 U/ml] ▪ from sheep, Fab-fragments, conjugated to alkaline phosphatase ▪ clear solution
5	NBT/BCIP	▪ 6 × 1 ml ▪ 50x conc. stock solution [18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 67% (v/v) DMSO] ▪ can vary between light yellow and brown, clear solution ▪ reacts with alkaline phosphatase.
6	Blocking solution	▪ 4 × 100 ml ▪ 10 × conc. ▪ yellow, viscous solution
7	DIG Easy Hyb Granules	▪ to give 4 × 100 ml (preparation page 17) ▪ Hybridization solution

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
3.2 DIG-DNA labeling	Water bath	▪ PCR grade water ▪ EDTA, 0.2 M, pH 8.0, sterile
3.3 Semi-quantitative determination of labeling efficiency	Nylon membranes positively charged*	▪ DIG Wash and Block Buffer Set* ▪ TE-buffer or
		▪ Washing buffer ▪ Maleic acid buffer ▪ Detection buffer ▪ TE-buffer
3.4 DNA transfer and fixation	▪ UV-transilluminator ▪ commercial available UV-cross linker	▪ 2 × SSC or ▪ 10 × SSC
3.5 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	
3.6 Immunological detection	▪ Container of appropriate size in relation to filter size ▪ Hybridization bags*	▪ DIG Wash and Block Buffer Set* ▪ TE-buffer or ▪ Washing buffer ▪ Maleic acid buffer ▪ Detection buffer ▪ TE-buffer
3.7 Stripping and reprobing of DNA blots	▪ Large tray ▪ Water bath	▪ 10 × SSC ▪ 10% SDS ▪ 0.2 M NaOH

2. Introduction

2.1 Product overview

Test principle

The DIG High Prime DNA Labeling and Detection Starter Kit I uses digoxigenin (DIG), a steroid hapten, to label DNA probes for hybridization and subsequent color detection by enzyme immunoassay [1].

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. 2) 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 colorimetric substrates NBT/BCIP.

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 λ 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
Color development	0.5 - 16 h

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 100 cm^2.

Quality Control

Using unlabeled control-DNA (pBR 328), labeled as described in the protocol, 0.1 pg of homologous DNA is detected in a dot blot after 16 h color development (1 pg of homologous DNA can be detected after 1h color development).

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 cap 4	+2 to +8°C, stable
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 kept sterile ▪ working solution should always be prepared fresh
NBT/BCIP cap 5	▪ +2 to +8°C, stable ▪ or at least 4 weeks at +15 to +25°C ***Note:*** During shipment of the kit on dry ice, a precipitate may occur which is easily dissolved by briefly warming to 37°C

Sensitivity and specificity

A single copy human gene is detected in a Southern blot of 1 µg digested placenta DNA.

Note: Sensitivity depends both on the concentration of labeled DNA in the hybridization and on the time of color reaction.

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 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

Overview

Section 3.2	**DIG-DNA labeling**
Section 3.3	**Quantification of labeling efficiency**
Section 3.4	**DNA fixation**
Section 3.5	**Hybridization**
Section 3.6	**Immunological detection**
Section 3.7	**Stripping and reprobing of DNA blots**

3.2 DIG-DNA Labeling

Introduction

DNA is random primed labeled with Digoxigenin-11-dUTP using DIG-High Prime, a 5x 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	Use
Water	PCR grade water	+15 to +25°C, stable	Dilution of DNA
EDTA	0.2 M ethylenediamino- tetraacetic acid, pH 8.0	+15 to +25°CC, stable	Stopping the labeling reaction

Template DNA

The following table lists the recommended features of the template DNA

Feature	Detail
Purity	For plasmid DNA use the High Pure Plasmid Isolation Kit * for purification. 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 – 3 µg 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.

Excise the DNA fragment from an agarose gel. For best results use either our High Pure PCR Product Purification Kit * or our Agarose Gel DNA Extraction Kit* to separate the DNA from the agarose.

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 1µg **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 length of the DIG-labeled fragments obtained with DIG-High Prime range from 200 bp to 1000 bp or larger, depending on the length 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	1050 ng
100 ng	270 ng	1500 ng
300 ng	450 ng	2000 ng
1000 ng	850 ng	2300 ng
3000 ng	1350 ng	2650 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)

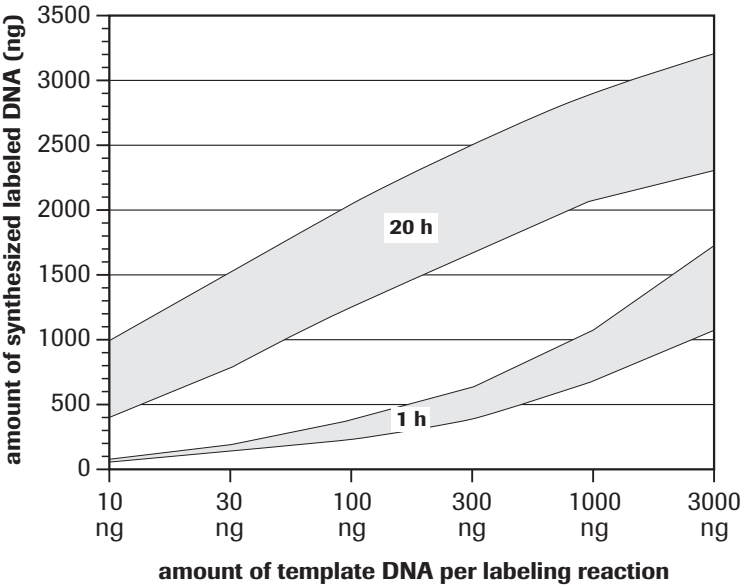


Fig. 2: Yield of DIG-labeled DNA from different amounts of template DNA after 1 and 20 h incubation of the DIG-High Prime reaction at 37°C.

3.3 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 the premixed stock solution of NBT/BCIP (vial 5). ▪ The intensities of the dilution series of DIG-labeled DNA and control DNA are compared.

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.*
Please note: These solutions are also used in the detection procedure of chapter 3.6. and can be prepared in larger quantities.

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	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)	+15 to +25°C, stable	Adjustment of pH to 9.5
TE-buffer	10 mM Tris-HCl, 1 mM EDTA, pH 8.0	+15 to +25°C, stable	Stopping color reaction

Preparation of kit working solutions

The following table shows the preparation of kit working solutions

Solution	Composition/ preparation	Storage and stability	Use
Blocking solution	Prepare a 1 × working solution by diluting the 10x 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: 5 000 (150 mU/ml) in Blocking solution.	12 h at +2 to +8°C	Binding to the DIG-labeled probe
Color-substrate solution	Add 40 µl of NBT/BCIP stock solution (vial 5) to 2 ml of Detection buffer. ***Note:*** Store protected from light!	always prepare fresh	Visualization of antibody-binding

Probe quantification

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 table 1 in chapter 3.2. 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.

Dilution series

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	Incubate membrane in 2 ml freshly prepared **color substrate solution** in a appropriate container in the dark. **Do not shake** during color development. ***Note:*** The color precipitate starts to form within a few minutes. The membrane can be exposed to light for short time periods to monitor color development.
9	Stop the reaction, when desired spot or band intensities are achieved, by washing the membrane for 5 min with 50 ml of PCR grade water or with TE-buffer. Results can be documented by photocopying the wet filter or by photography.

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 3.2.) and can be used in the recommended concentration in the hybridization.

The following spots should be visible

Incubation time	Appearance
5-10 min	30 pg spot
30 min	3 pg spot

3.4 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. (2). 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 (4,5).

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.

3.5 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 solution

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 is 20-25° 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 - 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) 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 hours - O/N with gentle agitation.

Storage of hybridization solution

DIG Easy Hyb 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 2x SSC, 0.1% SDS at +15 to +25°C under constant agitation.
2	Wash 2 × 15 min in 0.5x SSC, 0.1% SDS (prewarmed to wash temperature) at 65 - 68° C under constant agitation.

3.6 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*.

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
TE-buffer	10 mM Tris-HCl, 1 mM EDTA, pH 8.0	+15 to +25°C, stable	Stopping color reaction

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 1x 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 1:5,000 (150 mU/ml) in Blocking solution.	12 h at +2 to +8°C	Binding to the DIG-labeled probe
Color-substrate solution	Add 200 µl of NBT/BCIP stock solution (vial 5) to 10 ml of Detection buffer. ***Note:*** Store protected from light!	Always prepare fresh	Visualization of antibody-binding

Procedure

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

Note: All incubations should be performed at 15-25° 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 x 15 min in 100 ml **Washing buffer**.
5	Equilibrate 2-5 min in 20 ml **Detection buffer**.
6	Incubate membrane in 10 ml freshly prepared **color substrate solution** in a appropriate container in the dark. **Do not shake** during color development. ***Note:*** The color precipitate starts to form within a few minutes and the reaction is usually complete after 16 h. The membrane can be exposed to light for short time periods to monitor color development.
7	Stop the reaction, when desired spot or band intensities are achieved, by washing the membrane for 5 min with 50 ml of PCR grade water or with TE-buffer. Results can be documented by photocopying the wet filter or by photography.

Storage of membrane

Please refer to the following table.

IF...	THEN...
you want to reprobe the membrane	the membrane should not dry off at any time, store in sealed plastic bag. ***Note***: If you want to maintain the color, store membranes in TE buffer do not allow the membrane to dry.
you don´t want to reprobe	dry the membrane at +15 to +25°C for storage. ***Note:*** Color fades upon drying, to revitalize the color, wet the membrane in TE buffer.

3.7 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 reagents required

- Dimethylformamid (DMF)
- 0.2 N NaOH, 0.1% SDS (w/v)
- 2 × SSC

Protocol

Please refer to the following table.

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

CAUTION: Work under a fume hood

Step	Action
1	▪ Heat **DMF** in a large glass beaker in a water bath under a fume hood to 50-60° C. ▪ Incubate the membranes in the heated DMF until the blue color precipitate is removed from the filter. **CAUTION:** DMF is volatile and can be ignited above 67° C.
2	Rinse membrane briefly in **double distilled water**.
3	Wash for 2 × 15 min in **0.2 N NaOH, SDS, 0.1%** (w/v) at 37° C under constant agitation.
4	Equilibrate briefly in **2 × SSC**.
5	Prehybridize and hybridize with a second probe.

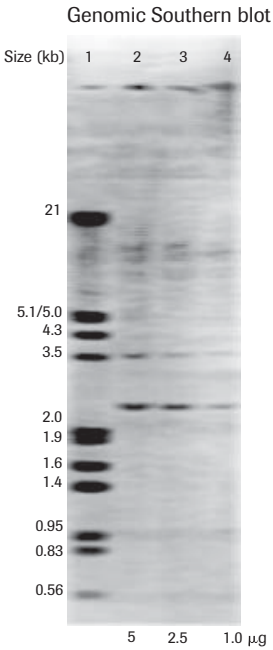
Storage of stripped membrane

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

4. Results

4.1 Typical results

Genomic Southern blot



Probe: DIG label β-actin DNA fragment
Lane 1: 100 ng DNA molecular weight marker III, digoxigenin-labeled.
Lane 2: 5 µg
Lane 3: 2.5 µg
Lane 4: 1 µg
} human placenta DNA, *Eco* R1

Figure 3: This figure shows you the detection of a single-copy gene (β-actin) in total human DNA using the standard protocol.

5. Appendix

5.1 Trouble shooting

Trouble shooting table

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

Problem	Possible cause	Recommendation
Low sensitivity	Inefficient probe labeling	▪ Check labeling efficiency. The labeling reaction can be upscaled. Prolong incubation time to overnight. ▪ Clean up template DNA by phenolization. ▪ Use only fragments < 5 kb or predigest with a restriction enzyme (e.g., four bp cutter) ▪ Make sure that template is efficiently denatured before labeling.
	Low probe concentration in the hybrid-ization	▪ Increase probe concentration, but do not use more than 25 ng/ml DNA probe. Check hybrid-ization and washing conditions. ▪ Prolong hybridization time. ▪ Prolong color development to 16 h.
High back-ground	Inefficient hybridization	▪ Recalculate hybridization temperature. ▪ Do not the allow the membrane to dry between prehybridization and hybridization. ▪ If you use plastic bags, remove all air bubbles prior to sealing.
	Wrong type of nylon mem-brane	Some types of nylon membrane may cause high background: use nylon membrane*, especially tested for the DIG-System from Roche Applied Science.
	Inefficient blocking before immuno-assay	Prolong blocking and washing steps.
	Ineffective stringency washes	Check temperature of stringency washes, prewarm wash solution to correct temperature
	Special hints for immuno-assay	When using laboratory trays for the detection procedure, they should be rigorously cleaned before use. Anti-DIG-AP binding and color development should be done in separate trays.

5.2 References

1 Höltke, H.J., Ankenbauer, W., Mühlegger, K., Rein, R., Sagner, G., Seibl, R., & Walter, T. (1995) The Digoxigenin (DIG) System for non-radioactive labeling and detection of nucleic acids-an overview. *Cell. Mol. Biol.* **41** (7): 883-905.

2 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.

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

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

5.3 Changes to previous version

- Editorial changes

5.4 Ordering Information

Kits

For a complete overview, please visit and bookmark our "DIG Reagents and Kits for Non-Radioactive Nucleic Acid Labeling and Detection" Special Interest Site at http://www.roche-applied-science.

Product	Pack Size	Cat. No.
Agarose Gel DNA Extraction Kit	100 reactions	11 696 505 001
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 Kitfor the purification of PCR reaction pro-ducts	50 purifications 250 purifications	11 732 668 001 11 732 676 001
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
PCR Clean Up Kit for post-PCR DNA fragment purification	up to 100 purifications	11 696 513 001

Single reagents

Product	Pack Size	Cat. No.
Blocking reagent	50 g	11 096 176 001
DIG Easy Hyb (ready-to-use hybridization solution without formamide)	500 ml	11 603 558 001
DNA Molecular Weight Marker, Digoxigenin-labeled:		
DNA Molecular Weight Marker II	5 µg (500 µl)	11 218 590 910
DNA Molecular Weight Marker III	5 µg (500 µl)	11 218 603 910
DNA Molecular Weight Marker V	5 µg (500 µl)	11 669 931 910
DNA Molecular Weight Marker VI	5 µg (500 µl)	11 218 611 910
DNA Molecular Weight Marker VII	5 µg (500 µl)	11 669 940 910
DNA Molecular Weight Marker VIII	5 µg (500 µl)	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 x 30 cm)	10 sheets	11 209 272 001
(10 x 15 cm)	20 sheets	11 209 299 001
(0.3 x 3 m roll)	1 roll	11 417 240 001

* available from Roche Applied Science

5.5 Trademarks

Regulatory Disclaimer

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

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