DIG High Prime DNA Labeling and Detection Starter Kit I

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 Store at −15 to −25°C
Kit for 12 labeling reactions of 10 ng-3 μg DNA
and detection of 24 blots of 100 cm²

Instruction Manual
Version November 2009

www.roche-applied-science.com
# Preface

## Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preface</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>2</td>
</tr>
<tr>
<td>Kit contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Product overview</td>
<td>5</td>
</tr>
<tr>
<td>Procedures and required materials</td>
<td>8</td>
</tr>
<tr>
<td>Before you begin</td>
<td>8</td>
</tr>
<tr>
<td>DIG-DNA Labeling</td>
<td>9</td>
</tr>
<tr>
<td>Determination of labeling efficiency</td>
<td>12</td>
</tr>
<tr>
<td>DNA transfer and fixation</td>
<td>15</td>
</tr>
<tr>
<td>Hybridization</td>
<td>17</td>
</tr>
<tr>
<td>Immunological detection</td>
<td>19</td>
</tr>
<tr>
<td>Stripping and re-probing of DNA blots</td>
<td>21</td>
</tr>
<tr>
<td>Results</td>
<td>22</td>
</tr>
<tr>
<td>Typical results</td>
<td>22</td>
</tr>
<tr>
<td>Appendix</td>
<td>23</td>
</tr>
<tr>
<td>Trouble shooting</td>
<td>23</td>
</tr>
<tr>
<td>References</td>
<td>24</td>
</tr>
<tr>
<td>Ordering Information</td>
<td>25</td>
</tr>
</tbody>
</table>
### 1.2 Kit contents

<table>
<thead>
<tr>
<th>Bottle/Cap</th>
<th>Label</th>
<th>Content including function</th>
</tr>
</thead>
</table>
| 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 |
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.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equipment</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 DIG-DNA labeling</td>
<td>Water bath</td>
<td>• H$_2$O, sterile, double distilled water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• EDTA, 0.2 M, pH 8.0, sterile</td>
</tr>
<tr>
<td>3.3 Semi-quantitative determination of labeling efficiency</td>
<td>Nylon membranes positively charged*</td>
<td>• DIG Wash and Block Buffer Set*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• TE-buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Washing buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Maleic acid buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Detection buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• TE-buffer</td>
</tr>
<tr>
<td>3.4 DNA transfer and fixation</td>
<td>UV-transilluminator</td>
<td>• 2 × SSC</td>
</tr>
<tr>
<td></td>
<td>commercial available UV-cross linker</td>
<td>• 10 × SSC</td>
</tr>
<tr>
<td>3.5 Hybridization</td>
<td>Nylon membranes, positively charged*</td>
<td>• Hybridization bags*</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td>• Temperature resistant, sealable plastic bags or roller bottles</td>
</tr>
<tr>
<td></td>
<td>Note: Do not use open trays when working with DIG Easy Hyb buffer</td>
<td></td>
</tr>
<tr>
<td>3.6 Immunological detection</td>
<td>Container of appropriate size in relation to filter size</td>
<td>• DIG Wash and Block Buffer Set*</td>
</tr>
<tr>
<td></td>
<td>Hybridization bags*</td>
<td>• TE-buffer</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td>• Washing buffer</td>
</tr>
<tr>
<td></td>
<td>Temperature resistant, sealable plastic bags or roller bottles</td>
<td>• Maleic acid buffer</td>
</tr>
<tr>
<td></td>
<td>Note: Do not use open trays when working with DIG Easy Hyb buffer</td>
<td>• Detection buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• TE-buffer</td>
</tr>
<tr>
<td>3.7 Stripping and reprobing of DNA blots</td>
<td>Large tray</td>
<td>• 10 × SSC</td>
</tr>
<tr>
<td></td>
<td>Water bath</td>
<td>• 10% SDS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 0.2 M NaOH</td>
</tr>
</tbody>
</table>
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].

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA labeling</td>
<td>DIG-labeled DNA probes are generated with DIG-High Prime according to the</td>
</tr>
<tr>
<td></td>
<td>random primed labeling technique. DIG-High Prime is a specially developed</td>
</tr>
<tr>
<td></td>
<td>reaction mixture containing digoxigenin-dUTP, alkali-labile (Fig. 2) and all</td>
</tr>
<tr>
<td></td>
<td>reagents, including enzyme necessary for random primed labeling, premixed</td>
</tr>
<tr>
<td></td>
<td>in an optimized 5 × concentrated reaction buffer.</td>
</tr>
<tr>
<td>Hybridization</td>
<td>DIG-labeled probes are used for hybridization to membrane blotted nucleic</td>
</tr>
<tr>
<td></td>
<td>acids according to standard methods. The use of the alkali-labile form of</td>
</tr>
<tr>
<td></td>
<td>DIG-11-dUTP enables easier and more efficient stripping of blots for</td>
</tr>
<tr>
<td></td>
<td>rehybridization with a second DIG-labeled probe.</td>
</tr>
<tr>
<td>Immunological</td>
<td>The hybridized probes are immunodetected with anti-digoxigenin-AP, Fab</td>
</tr>
<tr>
<td>detection</td>
<td>fragments and are then visualized with the colorimetric substrates NBT/BCIP.</td>
</tr>
</tbody>
</table>

www.roche-applied-science.com
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

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA labeling</td>
<td>1 h-O/N</td>
</tr>
<tr>
<td>Hybridization</td>
<td>6 h or O/N</td>
</tr>
<tr>
<td>Immunological detection</td>
<td>1.5 h</td>
</tr>
<tr>
<td>Color development</td>
<td>0.5 - 16 h</td>
</tr>
</tbody>
</table>

Number of tests
1 kit is sufficient for
• 12 standard labeling reactions of up to 3 μg template DNA
• detection of
• 24 blots of 100 cm².

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 1 h 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.

<table>
<thead>
<tr>
<th>Kit component</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Digoxigenin-AP Conjugate</td>
<td>2-8° C, stable</td>
</tr>
<tr>
<td>cap 4</td>
<td></td>
</tr>
<tr>
<td>Blocking solution</td>
<td>• unopened, stable at 15-25° C</td>
</tr>
<tr>
<td>bottle 6</td>
<td>• once opened, it should be aliquoted and</td>
</tr>
<tr>
<td></td>
<td>• stored at -15 to -25° C or at 2-8° C up to</td>
</tr>
<tr>
<td></td>
<td>• working solution should always be prepared</td>
</tr>
<tr>
<td></td>
<td>• one month when keeping sterile</td>
</tr>
<tr>
<td></td>
<td>• fresh</td>
</tr>
<tr>
<td>NBT/BCIP cap 6</td>
<td>• 2-8° C, stable</td>
</tr>
<tr>
<td></td>
<td>• or at least 4 weeks at 15-25° C</td>
</tr>
<tr>
<td></td>
<td><strong>Note:</strong> During shipment of the kit on dry</td>
</tr>
<tr>
<td></td>
<td>ice, a precipitate may occur which is easily</td>
</tr>
<tr>
<td></td>
<td>dissolved by briefly warming to 37°C</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Benefit</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accurate and fast</td>
<td>The use of premixed DIG-High Prime minimizes the hands-on-time required to label DNA probes and increases yields and reproducibility.</td>
</tr>
<tr>
<td>Sensitive</td>
<td>Single-copy genes can be detected in total human DNA complex and plant genomes.</td>
</tr>
<tr>
<td>Time-saving</td>
<td>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.</td>
</tr>
</tbody>
</table>
3. Procedures and required materials

3.1 Before you begin

General handling recommendations

This table describes general hints for DIG labeling and detection.

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

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.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Storage</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Autoclaved, double distilled water</td>
<td>15-25°C, stable</td>
<td>Dilution of DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2 M ethylenediamine- tetraacetic acid, pH 8.0</td>
<td>15-25°C, stable</td>
<td>Stopping the labeling reaction</td>
</tr>
</tbody>
</table>

**Template DNA**
The following table lists the recommended features of the template DNA.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>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.</td>
</tr>
<tr>
<td>Size</td>
<td>To obtain optimal results, template DNA should be linearized and should have a size of 100 or larger. Template DNA &gt;5 kb should be restriction-digested using a 4 bp cutter (e.g. Hae III) prior to labeling.</td>
</tr>
<tr>
<td>Amount</td>
<td>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.</td>
</tr>
</tbody>
</table>

**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.
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.

### Procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add 1μg template DNA (linear or supercoiled) and autoclaved, double distilled water to a final volume of 16 μl to a reaction vial.</td>
</tr>
<tr>
<td>2</td>
<td>Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath. <strong>Note:</strong> Complete denaturation is essential for efficient labeling.</td>
</tr>
</tbody>
</table>
| 3    | Mix DIG-High Prime (vial 1) thoroughly and add 4 μl to the denatured DNA, mix and centrifuge briefly.  
**Note:** 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.

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>1 h</th>
<th>20 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ng</td>
<td>45 ng</td>
<td>600 ng</td>
</tr>
<tr>
<td>30 ng</td>
<td>130 ng</td>
<td>1050 ng</td>
</tr>
<tr>
<td>100 ng</td>
<td>270 ng</td>
<td>1500 ng</td>
</tr>
<tr>
<td>300 ng</td>
<td>450 ng</td>
<td>2000 ng</td>
</tr>
<tr>
<td>1000 ng</td>
<td>850 ng</td>
<td>2300 ng</td>
</tr>
<tr>
<td>3000 ng</td>
<td>1350 ng</td>
<td>2650 ng</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>• A series of dilutions of DIG-labeled DNA is applied to a small strip of nylon membrane positively charged.* &lt;br&gt; • Part of the nylon membrane is preloaded with defined dilutions of DIG-labeled control DNA (vial 2) which are used as standards.</td>
</tr>
<tr>
<td>2</td>
<td>• 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). &lt;br&gt; • The intensities of the dilution series of DIG-labeled DNA and control DNA are compared.</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition / Preparation</th>
<th>Storage and stability</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing buffer</td>
<td>0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 (20°C); 0.3% (v/v) Tween 20</td>
<td>15-25°C, stable</td>
<td>Removal of unbound antibody</td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td>0.1 M Maleic acid, 0.15 M NaCl, adjust with NaOH (solid) to pH 75 (20°C)</td>
<td>15-25°C, stable</td>
<td>Dilution of Blocking solution</td>
</tr>
<tr>
<td>Detection buffer</td>
<td>0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)</td>
<td>15-25°C, stable</td>
<td>Adjustment of pH to 9.5</td>
</tr>
<tr>
<td>TE-buffer</td>
<td>10 mM Tris-HCl, 1 mM EDTA, pH 8.0</td>
<td>15-25°C, stable</td>
<td>Stopping color reaction</td>
</tr>
</tbody>
</table>
Preparation of kit working solutions

The following table shows the preparation of kit working solutions:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition/preparation</th>
<th>Storage and stability</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution</td>
<td>Prepare a 1x working solution by diluting the 10x Blocking solution (vial 6) 1:10 in Maleic acid buffer.</td>
<td>Always prepare fresh</td>
<td>Blocking of unspecific binding sites on the membrane</td>
</tr>
<tr>
<td>Antibody solution</td>
<td>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 ml/ml) in Blocking solution.</td>
<td>12 h at 2-8°C</td>
<td>Binding to the DIG-labeled probe</td>
</tr>
<tr>
<td>Color-substrate solution</td>
<td>Add 40 µl of NBT/BCIP stock solution (vial 5) to 2 ml of Detection buffer.</td>
<td>always prepare fresh</td>
<td>Visualization of antibody-binding</td>
</tr>
</tbody>
</table>

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

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:

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

The following procedure describes the direct detection.

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apply a 1 μl spot of tubes 2-9 from your labeled probes and the labeled control to the nylon membrane.</td>
</tr>
<tr>
<td>2</td>
<td>Fix the nucleic acid to the membrane by cross linking with UV-light or baking for 30 min at 120 °C.</td>
</tr>
</tbody>
</table>
| 3    | • Transfer the membrane into a plastic container with 20 ml Maleic acid buffer.  
      • Incubate under shaking for 2 min at 15-25 °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 sterile double dist. 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

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10 min</td>
<td>30 pg spot</td>
</tr>
<tr>
<td>30 min</td>
<td>3 pg spot</td>
</tr>
</tbody>
</table>
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:

<table>
<thead>
<tr>
<th>IF you want to...</th>
<th>THEN...</th>
</tr>
</thead>
</table>
| 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.

<table>
<thead>
<tr>
<th>IF...</th>
<th>THEN...</th>
</tr>
</thead>
</table>
| you want to go ahead. | Use the membrane immediately for prehybridization.  
| you want to work later on | store the membrane dry at 2-8° 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:

\[ T_m = 49.82 + 0.41 \times (\% \text{ G} + \text{ C}) - (600/l) \quad [l = \text{length of hybrid in base pairs}] \]

\[ T_{opt} = T_m - 20 \text{ to } 25^\circ \text{C} \]

(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 allowing 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
</table>
| 1    | • Pre-heat an appropriate volume of **DIG Easy Hyb** (10 ml/100 cm² 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 (3.5 ml/100 cm² 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**.

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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wash 2 × 5 min in ample 2x SSC, 0.1% SDS at 15-25°C under constant agitation.</td>
</tr>
<tr>
<td>2</td>
<td>Wash 2 × 15 min in 0.5x SSC, 0.1% SDS (prewarmed to wash temperature) at 65-68°C under constant agitation.</td>
</tr>
</tbody>
</table>
### 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.*

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition / Preparation</th>
<th>Storage and stability</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing buffer</td>
<td>0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20</td>
<td>15-25°C, stable</td>
<td>Washing of membrane</td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td>0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20°C)</td>
<td>15-25°C, stable</td>
<td>Dilution of Blocking solution</td>
</tr>
<tr>
<td>Detection buffer</td>
<td>0.1 M Tris·HCl, 0.1 M NaCl; pH 9.5 (20°C)</td>
<td>15-25°C, stable</td>
<td>Alkaline phosphatase buffer</td>
</tr>
<tr>
<td>TE-buffer</td>
<td>10 mM Tris·HCl, 1 mM EDTA, pH 8.0</td>
<td>15-25°C, stable</td>
<td>Stopping color reaction</td>
</tr>
</tbody>
</table>

#### Preparation of kit working solutions

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

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition / Preparation</th>
<th>Storage and stability</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution</td>
<td>Prepare a 1x working solution by diluting 10 × Blocking solution (vial 6) 1:10 with Maleic acid buffer.</td>
<td>Always prepare fresh</td>
<td>Blocking of unspecific binding sites</td>
</tr>
<tr>
<td>Antibody solution</td>
<td>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 1:5000 (150 mU/ml) in Blocking solution.</td>
<td>12 h at 2-8°C</td>
<td>Binding to the DIG-labeled probe</td>
</tr>
<tr>
<td>Color-substrate solution</td>
<td>Add 200 μl of NBT/BCIP stock solution (vial 5) to 10 ml of Detection buffer.</td>
<td>Always prepare fresh</td>
<td>Visualization of antibody-binding</td>
</tr>
</tbody>
</table>

*Note: Store protected from light!
Procedure

This table describes how to perform the immunological detection on a 100 cm² 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>After hybridization and stringency washes, rinse membrane briefly (1-5) min in <strong>Washing buffer</strong>.</td>
</tr>
<tr>
<td>2</td>
<td>Incubate for 30 min in 100 ml <strong>Blocking solution</strong>.</td>
</tr>
<tr>
<td>3</td>
<td>Incubate for 30 min in 20 ml <strong>Antibody solution</strong>.</td>
</tr>
<tr>
<td>4</td>
<td>Wash 2 x 15 min in 100 ml <strong>Washing buffer</strong>.</td>
</tr>
<tr>
<td>5</td>
<td>Equilibrate 2-5 min in 20 ml <strong>Detection buffer</strong>.</td>
</tr>
</tbody>
</table>
| 6 | Incubate membrane in 10 ml freshly prepared **color substrate solution** in an 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 sterile double dist. 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.

<table>
<thead>
<tr>
<th>IF...</th>
<th>THEN...</th>
</tr>
</thead>
</table>
| 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

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
</table>
| 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, 0.1% SDS (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

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.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Recommendation</th>
</tr>
</thead>
</table>
| 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 hybridization | | - Increase probe concentration, but do not use more than 25 ng/ml DNA probe. Check hybridization and washing conditions.  
- Prolong hybridization time.  
- Prolong color development to 16 h. |
| High background | Inefficient hybridization | - Recalculate hybridization temperature.  
- Do not 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 membrane | 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


5.3 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.com.

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel DNA Extraction Kit</td>
<td>100 reactions</td>
<td>11 696 505 001</td>
</tr>
<tr>
<td>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</td>
<td>10 isolations for 400 mg tissue or $5 \times 10^7$ cells</td>
<td>11 814 770 001</td>
</tr>
<tr>
<td>DNA Isolation Kit for mammalian Blood for the isolation of intact genomic DNA from mammalian whole blood or lymphocyte preparations</td>
<td>25 purifications</td>
<td>11 667 327 001</td>
</tr>
<tr>
<td>High Pure PCR Product Purification Kit for the purification of PCR reaction pro-ducts</td>
<td>50 purifications</td>
<td>11 732 668 001</td>
</tr>
<tr>
<td>High Pure Plasmid Isolation Kit small scale mini-preps for sequencing, PCR, and cloning</td>
<td>50 purifications</td>
<td>11 754 777 001</td>
</tr>
<tr>
<td>PCR Clean Up Kit for post-PCR DNA fragment purification</td>
<td>up to 100 purifications</td>
<td>11 696 513 001</td>
</tr>
</tbody>
</table>

www.roche-applied-science.com
### Single reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack Size</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking reagent</td>
<td>50 g</td>
<td>11 096 176 001</td>
</tr>
<tr>
<td>DIG Easy Hyb (ready-to-use hybridization solution without formamide)</td>
<td>500 ml</td>
<td>11 603 558 001</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker; Digoxigenin-labeled:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Molecular Weight Marker II</td>
<td>5 μg (500 μl)</td>
<td>11 218 590 910</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker III</td>
<td>5 μg (500 μl)</td>
<td>11 218 603 910</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker V</td>
<td>5 μg (500 μl)</td>
<td>11 669 931 910</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker VI</td>
<td>5 μg (500 μl)</td>
<td>11 218 611 910</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker VII</td>
<td>5 μg (500 μl)</td>
<td>11 669 940 910</td>
</tr>
<tr>
<td>DNA Molecular Weight Marker VIII</td>
<td>5 μg (500 μl)</td>
<td>11 448 451 910</td>
</tr>
<tr>
<td>DIG Wash and Block Buffer Set</td>
<td>30 blots (10 × 10 cm²)</td>
<td>11 585 762 001</td>
</tr>
<tr>
<td>Nylon Membrane, positively charged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 x 30 cm)</td>
<td>10 sheets</td>
<td>11 209 272 001</td>
</tr>
<tr>
<td>(10 x 15 cm)</td>
<td>20 sheets</td>
<td>11 209 299 001</td>
</tr>
<tr>
<td>(0.3 x 3 m roll)</td>
<td>1 roll</td>
<td>11 417 240 001</td>
</tr>
</tbody>
</table>

* available from Roche Applied Science
1 Tween is a trademark of ICI Americas Inc., USA
2 High Pure is a trademark of a Member of the Roche Group

**DISCLAIMER OF LICENSE**

The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5,344,757, 5,354,657 and 5,702,888 owned by Roche Diagnostics GmbH.
Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help. We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the worldwide research community.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our Online Technical Support Site at:

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed.

On the Roche Applied Science home page select Printed Materials to find:

- in-depth Technical Manuals
- Lab FAQs: Protocols and references for life science research
- our quarterly Biochemica Newsletter
- Material Safety Data Sheets
- Pack Inserts and Product Instructions

or to request hard copies of printed materials.