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

# DIG-High Prime DNA Labeling and Detection Starter Kit II

**Version: 15**

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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** 1 kit
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. General Information

## 1.1. Contents

| Vial / Bottle | Label | Function / Description | Content |
|---|---|---|---|
| 1 | DIG-High Prime DNA Labeling and Detection Starter Kit II, DIG-High Prime, 5x conc. | ▪ Random prime labeling mixture: Optimal concentrations of random primers, nucleotides, DIG-dUTP, alkali-labile, Klenow enzyme, and buffer components.<br>▪ Clear, viscous ready-to-use solution.<br>▪ For efficient random primed labeling of DNA. | 1 vial, 50 µl |
| 2 | DIG-High Prime DNA Labeling and Detection Starter Kit II, DIG-labeled control DNA | ▪ 5 µg/ml pBR328 DNA, linearized with Bam HI.<br>▪ Clear solution.<br>▪ For the determination of labeling efficiency. | 1 vial, 20 µl |
| 3 | DIG-High Prime DNA Labeling and Detection Starter Kit II, DNA dilution buffer | ▪ 50 µg/ml fish sperm DNA in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0 at +25°C.<br>▪ Clear solution. | 3 vials, 1 ml each |
| 4 | DIG-High Prime DNA Labeling and Detection Starter Kit II, Anti-DIG AP conjugate | ▪ 750 U/ml, from sheep, Fab fragments, conjugated to alkaline phosphatase.<br>▪ Clear solution. | 1 vial, 50 µl |
| 5 | DIG-High Prime DNA Labeling and Detection Starter Kit II, CSPD, ready-to-use | ▪ Clear solution.<br>▪ Chemiluminescent substrate for alkaline phosphatase. | 1 bottle, 50 ml |
| 6 | DIG-High Prime DNA Labeling and Detection Starter Kit II, Blocking solution, 10x conc. | Viscous solution. | 4 bottles, 100 ml each |
| 7 | DIG-High Prime DNA Labeling and Detection Starter Kit II, DIG Easy Hyb Granules | ▪ Each bottle contains granules for the preparation of 100 ml DIG Easy Hyb buffer.<br>▪ For the hybridization of DNA. | 4 bottles |

## 1.2. Storage and Stability

### Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiry date printed on the label.

| Vial / Bottle | Label | Storage |
|---|---|---|
| 1 | DIG-High Prime, 5x conc. | Store at −15 to −25°C.<br>⚠ ***Avoid repeated freezing and thawing. To avoid contamination, aliquot the DIG-High Prime solution into 2 to 3 vials.*** |
| 2 | DIG-labeled control DNA | Store at −15 to −25°C. |
| 3 | DNA dilution buffer | |
| 4 | Anti-DIG AP conjugate | Once opened, store at +2 to +8°C.<br>⚠ ***Do not freeze.*** |
| 5 | CSPD, ready-to-use | Store at +2 to +8°C.<br>⚠ ***Keep protected from light.*** |
| 6 | Blocking solution, 10x conc. | Store at −15 to −25°C or +15 to +25°C.<br>⚠ ***Once opened, aliquot and store at −15 to −25°C or +2 to +8°C up to one month when kept sterile.*** |
| 7 | DIG Easy Hyb Granules | Store at +15 to +25°C.<br>⚠ ***Once opened, the solution is stable for 1 month when kept sterile.*** |

# 1.3. Additional Equipment and Reagent required

### For random primed DNA labeling

*See section,* **Working Solution** *for additional information on how to prepare solutions.*

- Water bath
- Ice water
- Water, PCR Grade*
- 0.2 M EDTA, pH 8.0, sterile-filtered

### For labeling DNA isolated from agarose

- High Pure PCR Product Purification Kit*

### For direct detection

*See section,* **Working Solution** *for additional information on how to prepare solutions.*

- Nylon Membranes, positively charged*
- UV light or oven (+120°C)
- Plastic container
- Hybridization Bags
- Imager
- X-ray film or Lumi-Film*
- DIG Wash and Block Buffer Set*, or
- Washing buffer
- Maleic acid buffer
- Detection buffer
- Tween 20*
- Tris-HCl*

### For DNA transfer and fixation

- Nylon Membranes, positively charged*
- 10x and 2x SSC*
- UV-transilluminator
- Commercially available UV-crosslinker
- Whatman 3MM paper, or oven

### For hybridization

- Ice/water
- Nylon Membranes, positively charged*
- Shaking water bath, or
- Hybridization oven
- Hybridization bags, or
- Temperature-resistant, plastic or glass boxes, sealable plastic bags, or petri dishes or roller bottles

***Do not use open containers with DIG Easy Hyb buffer.***

### For stringency washes after hybridization

- Shaking water bath
- 2x SSC*
- 0.1% SDS*
- 0.5x SSC*

### For immunological detection

*See section,* **Working Solution** *for additional information on how to prepare solutions.*

- Container of appropriate size in relation to filter size
- Hybridization bags, or
- Temperature-resistant, plastic or glass boxes, sealable plastic bags, or petri dishes or roller bottles
- Shaking water bath
- Imager
- X-ray film or Lumi-Film*
- DIG Wash and Block Buffer Set*, or
- Washing buffer
- Maleic acid buffer
- Detection buffer

### For stripping and reprobing of membranes

- Large tray
- Water bath
- Autoclaved,double-distilled water
- 0.2 M NaOH
- 0.1% SDS* (w/v)
- 2x SSC*

### For storage of stripped membranes

- Maleic acid buffer, or
- 2x SSC*

## 1.4. Application

DIG-labeled DNA probes can be used in a variety of applications:

- All types of filter hybridization.
- Single-copy gene detection in total genomic DNA, even from organisms with high complexity, such as human, barley, and wheat.

## 1.5. Preparation Time

## Assay Time

| Step | Reaction Time [Hours] |
|---|---|
| DNA labeling | 1 to overnight |
| Hybridization | 6 or overnight |
| Immunological detection | 1.5 |
| Chemiluminescent signal detection | 5 to 30 minutes |

# 2. How to Use this Product

## 2.1. Before you Begin

## Sample Materials

### Templates for labeling reaction

- DNA fragments of at least 100 bp.
- Linearized plasmid, cosmid, or λDNA.
- Supercoiled DNA

## General Considerations

### Precautions

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

### Template DNA requirements

| Feature | Detail |
|---|---|
| Purity | ▪ For plasmid DNA, use the High Pure Plasmid Isolation Kit* for purification.<br>▪ When other commercially available purification kits are used, perform an additional phenol/ chloroform extraction to remove residual protein.<br>*i 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.<br>▪ Template DNA >5 kb should be restriction digested using a 4 bp cutter, such as Hae III prior to labeling. |
| Amount | For the Random Primed DNA Labeling protocol, 10 ng to 3 µg of template can be labeled.<br>*i Larger amounts can be labeled by scaling up of all components and volumes. 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.* |

### Hybridization temperature

The appropriate hybridization temperature is calculated according to GC content and percent homology of probe to target according to the following equation (Fig. 1).

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

$T_{opt.} = T_m - 20$ to $25°C$

**Fig. 1:** Calculation of the hybridization temperature.
The given numbers of the equation were calculated according to a standard equation for hybridization solutions containing 50% formamide.

*i The actual hybridization temperature $T_{opt}$ for hybridization with DIG Easy Hyb is +20 to +25°C below the calculated Tm 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%, lower the $T_{opt}$ accordingly (approximately 1.4°C below Tm per 1% mismatch) and also adjust the stringent washing steps accordingly, that is, increase SSC concentration and lower washing temperature.***

# Safety Information

## For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

## Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis/ Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink, or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

## Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

# Working Solution

The solutions used for the **Direct detection** are also used in the **Immunological detection** procedure and can be prepared in larger quantities.

*The buffers for both protocols are also available in the DIG Wash and Block Buffer Set*, tested on the absence of DNases and RNases.*

## Random Primed DNA Labeling

| Solution | Composition/Preparation | Storage and stability | For use in... |
|---|---|---|---|
| Water | Water, PCR Grade* | Store at +15 to +25°C. | Dilution of DNA. |
| EDTA | 0.2 M ethylenediaminetetraacetic acid, pH 8.0 | | Stops the labeling reaction. |

## Direct Detection

| Solution | Composition/Preparation | Storage and stability | For use in... |
|---|---|---|---|
| Washing buffer | 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (+20°C), 0.3% (v/v) Tween 20* | Store at +15 to +25°C. | Removal of unbound antibody. |
| Maleic acid buffer | 0.1 M maleic acid, 0.15 M NaCl, adjust with solid NaOH to pH 7.5 (+20°C) | | Dilution of Blocking solution. |
| Detection buffer | 0.1 M Tris-HCl*, 0.1 M NaCl, pH 9.5 (+20°C) | | Adjustment of pH to 9.5. |
| **Preparation of kit working solutions** | | | |
| Blocking solution, 1x | Dilute the 10x Blocking solution (Bottle 6) 1:10 with Maleic acid buffer. | ⚠ ***Always prepare fresh.*** | Blocking of nonspecific binding sites on the membrane. |
| Antibody solution | ▪ Centrifuge Anti-Digoxigenin-AP (Vial 4) for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface.<br>▪ Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/ml) in 1x Blocking solution. | Store for 12 hours at +2 to +8°C. | Binding to the DIG-labeled probe. |

## Hybridization

| Solution | Composition/Preparation | For use in... |
|---|---|---|
| DIG Easy Hyb working solution | ▪ Add carefully 64 ml autoclaved, double-distilled water in two portions to the DIG Easy Hyb Granules (Bottle 7).<br>▪ Dissolve by stirring immediately for 5 minutes at +37°C. | Hybridization |

## Immunological Detection

| Solution | Composition/Preparation | Storage and stability | For use in... |
|---|---|---|---|
| Washing buffer | 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (+20°C), 0.3% (v/v) Tween 20* | Store at +15 to +25°C. | Washing of membrane. |
| Maleic acid buffer | 0.1 M maleic acid, 0.15 M NaCl, adjust with solid NaOH to pH 7.5 (+20°C) | | Dilution of Blocking solution. |
| Detection buffer | 0.1 M Tris-HCl*, 0.1 M NaCl, pH 9.5 (+20°C) | | Alkaline phosphatase buffer. |
| **Preparation of kit working solutions** | | | |
| Blocking solution, 1x | Dilute the 10x Blocking solution (Bottle 6) 1:10 with Maleic acid buffer. | ⚠ ***Always prepare fresh.*** | Blocking of nonspecific binding sites on the membrane |
| Antibody solution | ▪ Centrifuge Anti-Digoxigenin-AP (Vial 4) for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface.<br>▪ Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/ml) in 1x Blocking solution. | Store for 12 hours at +2 to +8°C. | Binding to the DIG-labeled probe. |

# 2.2. Protocols

## Random primed DNA labeling

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.

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

1. To a reaction vial, add 1 µg template DNA (linear or supercoiled) and PCR-grade water* to a final volume of 16 µl.

2. Denature the DNA by heating in a boiling water bath for 10 minutes.
   – Chill quickly in an ice water bath.
   *i Full 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 hour or overnight at +37°C.
   *i Longer incubations up to 20 hours increase the yield of labeled DNA, see* **Table, Labeling reaction yield**.

4. Stop the reaction by adding 2 µl 0.2 M EDTA, pH 8.0, and/or by heating to +65°C for 10 minutes.

*i The length of the DIG-labeled fragments obtained with DIG-High Prime range from 200 bp to 1,000 bp or larger, depending on the length of the original template.*

### Labeling DNA isolated from agarose

1. For hybridization of genomic Southern blots, separate the template insert DNA from the vector by agarose gel electrophoresis.

2. To isolate DNA from the gel, use the High Pure PCR Product Purification Kit* or an agarose gel DNA extraction kit for fragments in the range of 400 bp to 5 kbp.
   *i The kits can be used with standard agarose gels as well as low melting point agarose gels.*

3. 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.

### Labeling reaction yield

In the standard reaction with 1 µg DNA per assay, approximately 15% of the nucleotides are incorporated into approximately 0.8 µg of newly synthesized DIG-labeled DNA within 1 hour, and approximately 38% of the nucleotides into approximately 2 µg after 20 hours.
The labeling efficiency of DIG-High Prime labeling under optimal conditions is shown in the following table and in Figure 2.

| Template DNA [ng] | Template DNA [ng] and Labeling Time | |
|---|---|---|
| | 1 Hour | 20 Hours |
| 10 | 45 | 600 |
| 30 | 130 | 1,050 |
| 100 | 270 | 1,500 |
| 300 | 450 | 2,000 |
| 1,000 | 850 | 2,300 |
| 3,000 | 1,350 | 2,650 |

*Using DIG-High Prime solution, reactions were performed with increasing amounts of different template DNA for 1 hour and 20 hours. The yield of DIG-labeled DNA was determined by incorporation of a radioactive tracer and confirmed by a dot blot. Numbers shown are the average of 10 independent labeling assays.*

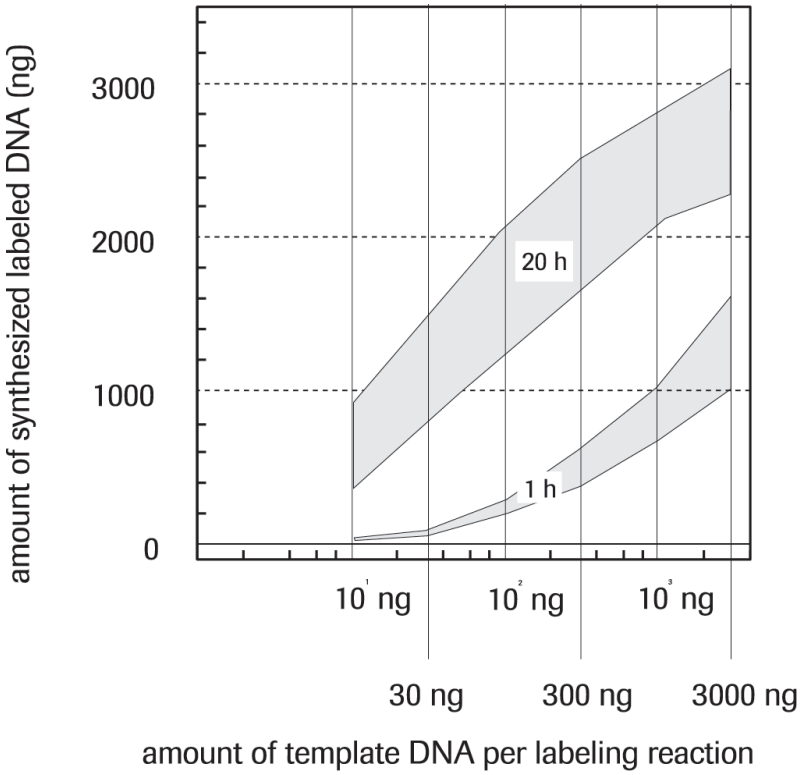


**Fig. 2:** Yield of DIG-labeled DNA from different amounts of template DNA after 1 and 20 hours incubation time of the DIG-High Prime reaction at 37°C.

## Semi-quantitative determination of labeling efficiency

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 step causes background, while too low of a concentration leads to weak signals.
The preferred method for quantification of labeled probes is the direct detection method.

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-DIG AP conjugate (Vial 4) and CSPD, ready-to-use (Vial 5).
– The intensities of the dilution series of DIG-labeled DNA and control DNA are compared by exposure to an appropriate imager or X-ray film or Lumi-Film*.

### 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 referring to the table in, **Labeling reaction yield**. The yield depends on the starting amount of template and incubation time.

*The yields given in the* **Table, labeling reaction yield** *were achieved under optimal conditions using highly purified template DNA.*

### Dilution series

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

| Tube | DNA [µl] | From tube No. | 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 |

### Direct detection

*See section,* **Working Solution** *for additional information on preparation of solutions.*
*Use sufficient buffer volumes to cover the membrane completely during all steps.*

1. Apply 1 µl spots of Tubes 2 to 9 from your labeled probe and control DNA to the Nylon Membrane*.

2. Fix the nucleic acid to the membrane by crosslinking with UV light or baking for 30 minutes at +120°C.

3. Transfer the membrane into a plastic container with 20 ml Maleic acid buffer.
   – Incubate with shaking for 2 minutes at +15 to +25°C.

4. Incubate for 30 minutes in 10 ml Blocking solution.

5. Incubate for 30 minutes in 10 ml Antibody solution.

6. Wash 2 × 15 minutes with 10 ml Washing buffer.

7. Equilibrate 2 to 5 minutes 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, that is, about 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 air bubbles over the membrane.
   – Incubate for 5 minutes at +15 to +25°C.

9. Squeeze out excess liquid and seal the edges of the development folder.
   ⚠ ***Drying of the membrane during exposure will result in dark background.***

10. Expose to a appropriate imager for 5 to 20 minutes or to X-ray film or Lumi-Film* for 15 to 25 minutes at +15 to +25°C.

*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 to 48 hours. Multiple exposures can be taken to achieve the desired signal strength.*

### Analyzing the results

Compare the intensity of the spots from 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, see Table, **Labeling reaction yield**, and can be used in the recommended concentration in the hybridization.

## DNA transfer and fixation

Standard protocols for gel electrophoresis, denaturation, and neutralization of the gel are described in the literature. Do not use gels containing ethidium bromide, as ethidium can cause uneven background problems. All common types of DNA transfer methods are suitable for subsequent DIG hybridization. Ideal results are obtained when gels are blotted by capillary transfer with 20x SSC* on Nylon Membranes*, positively charged.

⚠ ***Alkali transfer, for example, using 0.4 M NaOH, is not suitable for the transfer of DIG-labeled Molecular Weight Markers*.***

### Fixation procedure

Fix the DNA to the membrane using any of the following methods:

| Method | Steps |
|---|---|
| UV crosslinking (nylon membrane) | ▪ Place the membrane on Whatman 3MM paper soaked with 10x SSC.<br>▪ UV crosslink the wet membrane without prior washing.<br>▪ After the UV crosslinking, rinse the membrane briefly in double-distilled water and allow to air-dry. |
| Baking at +120°C (nylon membrane) | ▪ Wash the membrane briefly in 2x SSC.<br>▪ Bake the nylon membrane at +120°C for 30 minutes or according to the manufacturer's instructions. |
| Baking at +80°C (nylon membrane) | ▪ Wash the membrane briefly in 2x SSC.<br>▪ Bake at +80°C for 2 hours under vacuum. |

### Membrane storage

| IF... | THEN... |
|---|---|
| you want to continue, | use the membrane immediately for prehybridization. |
| you want to use it later, | store the membrane dry at +2 to +8°C. |

## Hybridization

1. Pre-heat an appropriate volume of DIG Easy Hyb buffer (10 ml/100 cm² membrane) to hybridization temperature (+37 to +42°C).
   – Prehybridize membrane for 30 minutes with gentle agitation in an appropriate container.
   ℹ *Membranes should move freely, especially if you use several membranes in the same prehybridization solution.*

2. Denature approximately 25 ng/ml DIG-labeled DNA probe by boiling for 5 minutes and rapidly cooling in ice/water.
   ⚠ ***Since 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 cm² membrane) and mix well.
   ⚠ ***Avoid foaming as bubbles may lead to background.***

4. Pour off prehybridization solution and add probe/hybridization mixture to membrane.
   – Incubate 4 hours to overnight with gentle agitation.

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

⚠ ***Do not boil DIG Easy Hyb buffer.***

### Stringency washes after hybridization

For most DNA:DNA applications, a stringency wash with 0.5x SSC is sufficient. The correct post washing conditions must be determined empirically for each probe. Suggestions are shown below:

| Probe Type | Washing Conditions |
|---|---|
| Human genomic DNA | Use 0.5x SSC and +65°C. |
| Probes >150 bp and with a high G/C content | Wash at +68°C. |
| Shorter probes ≤100 bp | Wash temperature must be lowered. |

1. Wash 2 × 5 minutes in ample 2x SSC, 0.1% SDS at +15 to +25°C under constant agitation.

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

## Immunological detection

*See section,* **Working Solution** *for additional information on preparation of solutions.*

Perform the immunological detection on a 100 $cm^2$ membrane according to the following steps.

⚠ ***Perform all incubations at +25 to +50°C with agitation. If the membrane is to be reprobed, do not allow the membrane to dry at any time.***

1. After hybridization and stringency washes, rinse membrane briefly 1 to 5 minutes in Washing buffer.

2. Incubate for 30 minutes in 100 ml Blocking solution.

3. Incubate for 30 minutes in 20 ml Antibody solution.

4. Wash 2 × 15 minutes in 100 ml Washing buffer.

5. Equilibrate 2 to 5 minutes 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 air bubbles over the membrane.
   – Incubate for 5 minutes at +15 to +25°C.

7. Squeeze out excess liquid and seal the edges of the development folder.
   ⚠ ***Drying of the membrane during exposure will result in dark background.***

8. Incubate the damp membrane for 10 minutes at +37°C to enhance the luminescent reaction.

9. Expose to a appropriate imager for 5 to 20 minutes or to X-ray film or Lumi-Film* for 15 to 25 minutes at +15 to +25°C.

*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 to 48 hours. Multiple exposures can be taken to achieve the desired signal strength.*

## Stripping and reprobing

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

*When stripping and rehybridization is planned, do not allow the membrane to dry at any time.*

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

### Storage of stripped membrane

Once the membrane is stripped, store in Maleic acid buffer or 2x SSC until next use.

# 2.3. Parameters

## Sensitivity

The gene for tissue plasminogen activator (tPA) is detected in a Southern blot in 0.3 µg restriction-enzyme digested human placenta DNA.

# 3. Troubleshooting

| Observation | Possible cause | Recommendation |
|---|---|---|
| Low sensitivity observed. | Inefficient probe labeling. | Check labeling efficiency of your DIG DNA labeling by comparison to the labeled control DNA. |
| | Wrong type of membrane. | Use Nylon Membranes, positively charged*, specially tested for chemiluminescent detection.<br>*i The quality of the membrane used as support for dot and Southern blotting influences sensitivity and speed of detection.* |
| | | Other types of nylon membranes are also suitable but need longer exposure times to X-ray or Lumi-Film*.<br>*i Some membranes may cause strong background formation. Nitrocellulose membranes cannot be used with the protocol described.* |
| | Inefficient hybridization conditions. | Check hybridization conditions. |
| | | 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 time before exposure too short. | Increase duration of preincubation before exposure to X-ray film or Lumi-Film* to >30 minutes and up to 12 hours. |
| | Exposure time to short. | Increase exposure time to X-ray film or Lumi-Film*. |
| | | The type of film may also influence the sensitivity. |
| High background present. | Inefficient labeling. | Purify DNA by phenol/chloroform extraction and/or ethanol precipitation before labeling. |
| | | Make sure that the probe does not contain cross-hybridizing vector sequences. |
| | Wrong type of membrane. | Always use Nylon Membranes, positively charged*, specially tested for chemiluminescent detection.<br>*i 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. Avoid this by using Nylon Membranes* which are function tested with the DIG system. |
| | Concentration of labeled probe too high. | Decrease concentration of DIG-labeled DNA probe.<br>*i Determine the critical probe concentration limit by performing a mock hybridization with increasing probe concentrations using an unloaded membrane.* |
| | | Do not allow the membranes to dry at any time in the procedure. |
| | Antibody concentration too high. | Decrease the concentration of the Anti-DIG AP conjugate. |
| | | Increase volumes of the washing and blocking solution and duration of the washing and blocking steps.<br>*i Spotty background may be caused by precipitates in the Anti-DIG AP conjugate; remove by a short centrifugation step.*<br>*i Several centrifugation steps can cause a certain loss of material which must be compensated for by using larger amounts.* |
| | Preincubation before exposure time too long. | Shorten preincubation time. |

# 4. Additional Information on this Product

## 4.1. 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 chemiluminescent detection by enzyme immunoassay.

① 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-11-dUTP, alkali-labile and all reagents, including enzyme necessary for random primed labeling, premixed in an optimized 5x-concentrated reaction buffer.

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② 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 membranes for rehybridization with a second DIG-labeled probe.

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③ The hybridized probes are immunodetected with Anti-Digoxigenin-AP, Fab fragments and are then visualized with the chemiluminescent 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. 3) which is recorded with an appropriate imager or on X-ray film or Lumi-Film*. Film exposure times are in the range of only 5 to 30 minutes.

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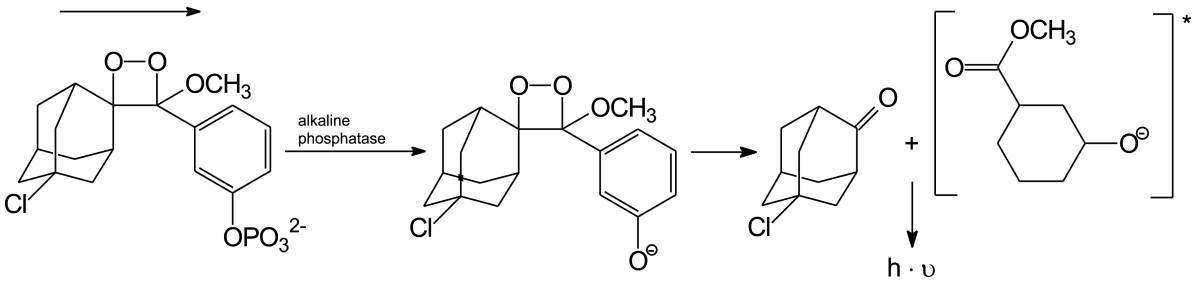


**Fig. 3:** Reaction of CSPD.

## 4.2. Quality Control

For lot-specific certificates of analysis, see section **Contact and Support.**

# 5. Supplementary Information

## 5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

| Text convention and symbols | |
|---|---|
| *Information Note: Additional information about the current topic or procedure.* | |
| **Important Note: Information critical to the success of the current procedure or use of the product.** | |
| ① ② ③ etc. | Stages in a process that usually occur in the order listed. |
| ❶ ❷ ❸ etc. | Steps in a procedure that must be performed in the order listed. |
| * (Asterisk) | The Asterisk denotes a product available from Roche Diagnostics. |

## 5.2. Changes to previous version

Layout changes.
Editorial changes.
New information added related to the REACH Annex XIV.
Update to include new safety Information to ensure handling according controlled conditions.

## 5.3. Ordering Information

| Product | Pack Size | Cat. No. |
|---|---|---|
| Reagents, kits | | |
| Water, PCR Grade | 25 ml, 25 x 1 ml | 03 315 932 001 |
| | 25 ml, 1 x 25 ml | 03 315 959 001 |
| | 100 ml, 4 x 25 ml | 03 315 843 001 |
| High Pure PCR Product Purification Kit | 1 kit, up to 50 purifications | 11 732 668 001 |
| | 1 kit, up to 250 purifications | 11 732 676 001 |
| Nylon Membranes, positively charged | 10 sheets, 20 x 30 cm | 11 209 272 001 |
| | 20 sheets, 10 x 15 cm | 11 209 299 001 |
| | 1 roll, 0.3 x 3 m | 11 417 240 001 |
| DIG Wash and Block Buffer Set | 1 set, 30 blots (100 $cm^2$) | 11 585 762 001 |
| Tween 20 | 50 ml, 5 x 10 ml | 11 332 465 001 |
| Tris hydrochloride | 500 g | 10 812 846 001 |
| Buffers in a Box, Premixed SSC Buffer, 20x | 4 l | 11 666 681 001 |
| Sodium Dodecyl Sulfate (SDS) | 1 kg | 11 667 289 001 |
| DNA Molecular Weight Marker II, DIG-labeled | 5 µg, 500 µl | 11 218 590 910 |
| DNA Molecular Weight Marker III, DIG-labeled | 5 µg, 500 µl | 11 218 603 910 |
| DNA Molecular Weight Marker VI, DIG-labeled | 5 µg, 500 µl | 11 218 611 910 |
| DNA Molecular Weight Marker VII, DIG-labeled | 5 µg, 500 µl | 11 669 940 910 |
| DNA Molecular Weight Marker VIII, DIG-labeled | 5 µg, 500 µl | 11 449 451 910 |
| High Pure Plasmid Isolation Kit | 1 kit, 50 purifications | 11 754 777 001 |
| | 1 kit, 250 purifications | 11 754 785 001 |
| Lumi-Film Chemiluminescent Detection Film | 100 films, 7.1 x 9.4 inches, 18 x 24 cm, *Not available in US* | 11 666 916 001 |

## 5.4. Trademarks

DIG EASY HYB is a trademark of Roche.
All other product names and trademarks are the property of their respective owners.

## 5.5. License Disclaimer

For patent license limitations for individual products please refer to:
**List of biochemical reagent products**.

## 5.6. Regulatory Disclaimer

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

## 5.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

## 5.8. Contact and Support

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

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

1120.11766597001 ®

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany