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Not for use in diagnostic procedures.

DIG Gel Shift Kit, 2nd Generation

Version: 13
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For nonradioactive detection of sequence-specific DNA binding proteins, containing recombinant Terminal Transferase.

Cat. No. 03 353 591 910 1 kit
20 Oligonucleotide 3'-end labeling reactions with DIG-11-ddUTP, 200 binding reactions, chemiluminescent detection reaction for 20 blots, DNA binding protein and oligonucleotide for 20 control reactions

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Label	Function / Description	Content
1	DIG Gel Shift Kit, 2nd Generation, Labeling Buffer, 5x conc.	Contains 1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/mL bovine serum albumin, pH 6.6 (25°C). ⚠ ***Please follow the instructions in the section Safety Information.***	1 vial, 80 µL
2	DIG Gel Shift Kit, 2nd Generation, Cobalt dichloride solution	Contains 25 mM $CoCl_2$ solution.	1 vial, 80 µL
3	DIG Gel Shift Kit, 2nd Generation, Digoxigenin-ddUTP solution	Contains 1 mM Digoxigenin-11-ddUTP in double-distilled water.	1 vial, 20 µL
4	DIG Gel Shift Kit, 2nd Generation, Terminal Transferase	▪ Contains terminal transferase in 60 mM K-phosphate (pH 7.2 at 4°C), 150 mM KCl, 1 mM 2-mercaptoethanol, 0.1% Tween 20, 50% glycerol. ▪ 400 U/µL	1 vial, 20 µL
5	DIG Gel Shift Kit, 2nd Generation, Binding Buffer, 5x conc.	Contains 100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2$ $S0_4$, 5 mM DTT, Tween 20, 1% (w/v), 150 mM KCl.	1 vial, 800 µL
6	DIG Gel Shift Kit, 2nd Generation, Control Oligonucleotide, unlabeled	▪ 0.1 µg/µL ▪ 3.85 pmol/µL ▪ Contains the binding site for Oct2A, as specific competitor for the binding reaction with Oct2A. ▪ Sequence of the double stranded 39mer: 5′-GTACGGAGTATCCAGCTCCGTAGC**ATGCAAAT**CCTCTGG-3′ 3′-CCTCATAGGTCGAGGCATCG**TACGTTTA**GGAGACCAGCT-5′	1 vial, 40 µL
7	DIG Gel Shift Kit, 2nd Generation, Control Oligonucleotide, DIG labeled	▪ 0.4 ng/µL ▪ 15.54 fmol/µL ▪ Supplied in TE buffer, 0.1 M NaCl. ▪ The sequence is the same as for the unlabeled control oligonucleotide.	1 vial, 140 µL
8	DIG Gel Shift Kit, 2nd Generation, Control Factor Oct2A	▪ 25 – 75 ng/µL ▪ Supplied in 30 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 2 mM DTT, 0.2 M NaCl.	1 vial, 40 µL
9	DIG Gel Shift Kit, 2nd Generation, Poly [d(I-C)]	1 µg/µL in double-distilled water.	1 vial, 200 µL
10	DIG Gel Shift Kit, 2nd Generation, Poly [d(A-T)]	1 µg/µL in double-distilled water.	1 vial, 200 µL
11	DIG Gel Shift Kit, 2nd Generation, Poly L-lysine	0.1 µg/µL in double-distilled water.	1 vial, 200 µL
12	DIG Gel Shift Kit, 2nd Generation, Loading Buffer, without bromphenol blue	0.25x TBE buffer, 34% glycerol.	1 vial, 1 mL

13	DIG Gel Shift Kit, 2nd Generation, Loading Buffer, with bromphenol blue	0.25x TBE buffer, 34% glycerol, 0.2% (w/v) bromphenol blue.	1 vial, 1 mL
14	DIG Gel Shift Kit, 2nd Generation, Anti-Digoxigenin-AP Fab fragments	750 U/mL polyclonal sheep, Anti-Digoxigenin, Fab fragments conjugated with alkaline phosphatase.	1 vial, 40 µL
15	DIG Gel Shift Kit, 2nd Generation, CSPD	10 mg/mL	1 vial, 440 µL
16	DIG Gel Shift Kit, 2nd Generation, Blocking Reagent	Supplied as a powder.	1 bottle, 50 g

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.
Store all kit components at −15 to −25°C, except for those shown below.

Vial / Bottle	Label	Storage
8	Control Factor Oct2	⚠ ***Avoid repeated freezing and thawing.*** After first thawing, aliquot and store at −15 to −25°C. If possible, store the aliquoted Oct2A factor at −70°C.
14	Anti-Digoxigenin-AP Fab fragments	Store at +2 to +8°C. ⚠ ***Do not freeze.***
15	CSPD	Store at +2 to +8°C. ⚠ ***Store protected from light.***
16	Blocking Reagent	Store at +15 to +25°C. ⚠ ***Always prepare fresh working solution.***

1.3. Additional Equipment and Reagent required

In addition to the reagents listed below, you will need to prepare additional solutions specific to each protocol. Detailed information can be found at the beginning of each protocol.

Annealing and Labeling of Oligonucleotide

- Water bath
- Double-distilled water
- Sterile 0.2 M EDTA, pH 8.0
- TEN buffer

Determination of Labeling Efficiency

- Nylon Membranes, positively charged*
- DIG Wash and Block Buffer Set*, or washing buffer, maleic acid buffer, and detection buffer

Gel Shift Reaction

- Novex Retardation Gel (6%) ready to use
  ℹ *Precast gels should always be used.*
- TEN buffer

Polyacrylamide Gel Electrophoresis

- 0.5x TBE buffer
- Acrylamide, bis-acrylamide
- TEMED
- Ammonium sulfate

Blotting and Crosslinking

- Nylon Membranes, positively charged*
- Whatman 3MM paper
- Transfer buffer: 0.5x TBE buffer
- Electroblotting device, such as NOVEX System (XCell II Blot Module), LKB 2117-250, Novablot Electrophoretic Electroblotting Transfer Kit
- Transilluminator or commercially available UV crosslinker
- 0.5x TBE buffer
- 2x SSC or 10x SSC

Chemiluminescent Detection

- Temperture-resistant plastic bags or roller bottles
- Hybridization Bags
- DIG Wash and Block Buffer Set*, or washing buffer, maleic acid buffer, and detection buffer

1.4. Application

The DIG Gel Shift Kit can be used to label the 3′ ends of oligonucleotides, whether they have 5′- or 3′-overhanging ends or blunt ends. This is because labeling is performed with recombinant Terminal Transferase and DIG-11-ddUTP. Both single- and double-stranded DNA can be labeled. Ideally, fragments should be between 30 and 100 bp. The electrophoresis assay works best with shorter DNA fragments in order to reduce nonspecific interactions of proteins with sequences that flank the specific binding site.

1.5. Preparation Time

Assay Time

Step	Reaction Time
Oligonucleotide annealing and labeling	10 minutes
Formation of oligonucleotide-protein complexes	25 minutes
Electrophoresis	1 to 2 hours to overnight, depending on gel system
Blotting	1 to 2 hours, depending on device used
Immunological detection	2 hours
Exposure to X-ray film or imaging device	15 to 40 minutes

2. How to Use this Product

2.1. Before you Begin

Sample Materials

- 5′- or 3′-overhanging ends as well as blunt-ended oligonucleotides.
- Single- and double-stranded DNA with a fragment size between 30 to 200 bp.

i *The electrophoresis assay works best with shorter DNA fragments, to reduce nonspecific interactions of proteins with sequences which flank the specific binding site.*

General Considerations

Precautions

- Work under clean conditions and use good laboratory practice.
- Autoclave DIG-System solutions.
- Filter sterilize solutions containing SDS.
- Add Tween 20 to previously sterilized solutions.
- Rigorously clean and rinse laboratory trays before each use.
- Use sterile disposable plasticware.
- Wear powder-free gloves when handling membranes.
- Handle membranes only on the edges and with clean forceps.

Safety Information

Vial 1 contains toxic material (potassium cacodylate). Use gloves during handling of these substances (see main label on the outer packaging).
Collect the supernatants from the labeling reactions in a tightly closed, non-breakable container and label the contents. Discard as regulated for toxic waste.

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

For Determination of Labeling Efficiency Protocol and for the Chemiluminescent Detection Protocol

Prepare the following kit working solutions prior to starting the protocol.

Solution	Composition/Preparation	Use	Storage and Stability
Blocking stock solution, 10x conc.	Dissolve Blocking reagent (Bottle 16) 10% (w/v) in Maleic acid buffer with constant stirring on a heating block (65°C), or heat in a microwave oven, autoclave. The solution remains opaque.	Preparation of blocking solution.	Store 4 weeks at +2 to +8°C if kept sterile. ⚠ ***Freeze aliquots at −15 to−25°C.***
1x Blocking solution	Prepare a 1x working solution by diluting the 10x Blocking solution 1:10 in Maleic acid buffer.	Blocking of nonspecific binding sites on the membrane.	Always prepare fresh.
Antibody solution	Centrifuge Anti-Digoxigenin-AP for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/mL) in Blocking solution.	Binding to the DIG-labeled probe.	Store 12 hours at +2 to +8°C.
CSPD working solution	Dilute CSPD (Vial 15) 1:100 in Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C).	For chemiluminescent detection.	Store at +2 to +8°C. ⚠ ***Keep protected from light.***

2.2. Protocols

Overview

A brief overview of the gel mobility shift assay is shown below.

① Annealing and Labeling of Oligonucleotides

② Determination of Labeling Efficiency

③ Gel Shift Reaction

④ Polyacrylamide Gel Electrophoresis

⑤ Blotting and Crosslinking

⑥ Chemiluminescent Detection

Annealing and Labeling of Oligonucleotides

For the control reaction with the Control Oligonucleotide (Vial 6), 100 ng (= 3.85 pmol of a ds 39-mer) is used for the labeling reaction.

For the conversion of ng to pmol: ng dsDNA = pmol× 0.66× N (N = length of the ds fragment).

Additional Solutions Required

Prepare the following solutions prior to starting the annealing and labeling protocol.

Solution	Composition/ Preparation	Use	Storage and Stability
Water	Autoclaved, double-distilled	Dilution of buffers and preparation of labeling/ binding reactions and of the oligonucleotide.	Stable at +15 to +25°C.
EDTA	0.2 M EDTA, pH 8.0	Stops the reaction.	
TEN buffer	10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0	Annealing reaction and dilution series of the oligonucleotide.	

Follow the steps below when setting up the labeling reaction.

It is not recommended to increase the amount of oligonucleotide in the labeling reaction. Larger amounts of oligonucleotide can be labeled by increasing the reaction volume and all components proportionally, and by increasing the incubation time to 1 hour.

1. Mix solutions of complementary oligonucleotides in TEN buffer in a molar ratio of 1:1.

2. Incubate 10 minutes at +95°C.

3. Cool slowly to +15 to +25°C.

4. Dilute with sterile TEN buffer to 3 –4 pmol/µL.

5. Add 3.85 pmol or 100 ng ds oligonucleotide and sterile, double-distilled water to a final volume of 10 µL to a reaction vial.
   – For the control reaction, add 1 µL Control Oligonucleotide (Vial 6) and 9 µL sterile, double-distilled water to a reaction vial.

6. Add the following on ice:

Reagent	Volume [µL]	Final conc.
5x Labeling Buffer (Vial 1)	4	1x
$CoCl_2$ dichloride solution (Vial 2)	4	5 mM
DIG-ddUTP solution (Vial 3)	1	0.05 mM
Terminal Transferase, 400 U (Vial 4)	1	20 U/µL
Total Volume	**10**	

   –Mix and centrifuge briefly.
   – Incubate at +37°C for 5 minutes, then place on ice.

7. Stop the reaction by adding 2 µL 0.2 M EDTA (pH 8.0).

8. Add 3 µL double-distilled water to a final volume of 25 µL to obtain a final concentration of 4 ng/µL or 0.155 pmol/µL of the labeled oligonucleotide.

*The efficiency of the labeling reaction must be checked by comparison of a spotted dilution series of the labeling reaction with one of the labeled Control Oligonucleotides (Vial 7) in a direct detection assay, see section **Determination of Labeling Efficiency**.*

Determination of Labeling Efficiency

The determination of the efficiency of the DIG-labeling reaction is most important for optimal and reproducible results.

Direct Detection Method

① A series of dilutions of DIG-labeled oligonucleotides are applied to a small strip of Nylon Membrane, positively charged* in direct comparison with the DIG-labeled Control Oligonucleotide (Vial 7).

② The Nylon Membrane is subjected to immunological detection with Anti-Digoxigenin-AP conjugate (Vial 14) and the working solution of CSPD (Vial 15).
– Both dilution series should show a signal with the 4 pg spot.

Additional Solutions Required

Prepare the following solutions prior to determining the labeling efficiency.

i *The Washing buffer, Maleic acid buffer, and Detection buffer are also available as 10x stock solutions in the DIG Wash and Block Buffer Set*, guaranteed DNase- and RNase-free according to the current quality control procedures.*

Solution	Composition/Preparation	Use	Storage and Stability
Washing buffer	0.1 M maleic acid, 0.15 M NaCl; pH 7.5 (20°C), 0.3% (v/v) Tween 20	Removal of unbound antibody.	Stable at +15 to +25°C.
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl; adjust with NaOH (solid) 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)	Equilibration of membrane for adjustment of pH to 9.5.	
TEN buffer	10 mM Tris, 1 mM EDTA, 0.1 NaCl, pH 8.0	Dilution of oligonucleotide.	

i *See section,* **Working Solution** *for preparation of kit working solutions.*

Preparation of Dilution Series

Prepare a dilution series of the labeled control oligonucleotide and your labeling reaction as described in the table below.

i *The concentration of the labeled DNA is 4 ng/µL or 0.155 pmol/µL after labeling as described in section,* **Annealing and Labeling of Oligonucleotides***, Step 8.*

Tube	Oligo [µL]	From Tube No.	TEN Buffer	Dilution	Final Concentration [fmol/µL]	[ng/µL]
1	–	Original	0	–	155	4
2	2	1	18	1:10	15.5	0.4
3	2	2	18	1:100	1.55	0.04
4	2	3	18	1:1000	0.15	0.004
5	–	–	20	–	0	0

Direct Detection Protocol

1. Apply a 1 µL spot from tubes 1 – 5, from your labeled oligonucleotide and the labeled control, 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 Washing 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 with 10 mL Washing buffer, 2 × 15 minutes.

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 working solution.
   – 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. Incubate the damp membrane for 10 minutes at +37°C to enhance the CSPD chemiluminescent reaction.

10. Expose to X-ray film or imaging device.

    Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it reaches 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

You can determine the quantity of DIG-labeled DNA by comparing the dilution series with that of the DIG-labeled Control Oligo (Vial 7). The 4 pg spot (0.155 fmol) should be visible.

Gel Shift Reaction

Modification of Reaction Conditions

Control reaction is optimized for the Factor Oct2A (Vial 8). For analysis of your binding protein, reaction conditions can be modified as follows:

- With purified DNA binding proteins, no or low concentration of nonspecific competitor DNA is required.
- If factors are analyzed from crude extracts, it is absolutely essential to add nonspecific competitor DNA to the binding reaction.
- Some binding reactions may require a different reaction temperature (+4°C to +37°C).

Binding Buffer Conditions

- Conditions such as salt concentration and pH have been shown to affect the protein/DNA interaction. Whether the complexes formed are specific or nonspecific is dependent on the buffer conditions. Therefore, it could also be important to use additions, such as Mg^{2+}, Zn^{2+}, Ca^{2+}, detergent, or spermidine.
- Poly-L-lysine is provided separately in the kit to allow individual optimization of the formation of the specific protein/DNA complexes, because basic peptides can also increase the apparent DNA binding affinity. Albumin can also be used to improve formation of specific protein/DNA complexes.
- The order of probe and nuclear protein extract addition can determine the specificity of protein-DNA complexes formed.

Additional Solutions Required

Solution	Composition/Preparation	Use	Storage and Stability
TEN buffer	10 mM Tris, 1 mM EDTA, 0.1 MNaCl, pH 8.0	For the dilution of the labeled oligonucleotide and control.	Stable at +15 to +25°C.

Dilution of Labeled Oligonucleotide and Control

1. Dilute your labeled oligonucleotide to a concentration of 15 to 30 fmol/µL in TEN buffer.

2. Dilute the Control Oligonucleotide (Vial 6) labeled with DIG with TEN buffer to 0.4 ng/µL (15.5 fmol/µL) or use the supplied DIG-labeled Control Oligonucleotide (Vial 7).

Sample Preparation

Prepare three samples for the control reaction and for the analysis of your factor according to the following table.

Sample	Description
1	Labeled oligonucleotide without factor.
2	Labeled oligonucleotide with factor.
3	Labeled oligonucleotide with factor and with a 125-fold excess of unlabeled oligonucleotide for specific competition. *The addition of Poly-L-lysine (Vial 11) is optional; it improves binding of the factor to the oligonucleotide.*

Gel Shift Reaction Standard Protocol

1. Mix the following on ice:

Reagent	Volume [µL] Sample 1	Volume [µL] Sample 2	Volume [µL] Sample 3
Binding Buffer (Vial 5)	4	4	4
Poly [d(I-C)] (Vial 9), 1 µg/µL	1	1	1
Poly L-lysine (Vial 11), 0.1 µg/µL	1	1	1
Dig-labeled Control Oligonucleotide (Vial 7), 0.4 ng/µL	2	2	2
Double-distilled water	12	11	10
Unlabeled Control Oligonucleotide (Vial 6), 0.1 µg/µL	–	–	1
Oct2A Control Factor (Vial 8), 25 to 75 ng/µL	–	1	1

2. Mix carefully and incubate for 15 minutes at +15 to +25°C.

3. Place tubes on ice.

4. Add 5 µL of Loading buffer with bromphenol blue (Vial 13) to each sample.

   When using the NOVEX System, replace the Loading buffer with the recommended high density TBE sample buffer.

5. Apply sample immediately to a pre-electrophoresed polyacrylamide gel.

Polyacrylamide Gel Electrophoresis

Adjustment of Acrylamide Concentration

The appropriate acrylamide concentration depends on the size of oligonucleotide or factor complexes. For most case, a 6% to 8% native polyacrylamide gel in 0.5x TBE buffer is optimal.

Additional Solutions Required

Solution	Composition/Preparation	Use
0.5x Tris-borate-EDTA (TBE) buffer, 10x conc.	890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0.	Running buffer

Electrophoresis Protocol

1. One day prior to the start of the gel electrophoresis, prepare a native polyacrylamide gel of 6% or 8% acrylamide in 0.5x TBE buffer.

   Prepare the gel the day before use to make sure that the gel is completely polymerized.

2. The gel must be pre-run (NOVEX System: 5 minutes).

3. Load the samples into the gel.

   Before loading samples, clean sample wells to remove ammonium persulfate (APS), urea, and residual polyacrylamide to ensure sample application without diffusion.

4. Run a 10 cm ×10 cm × 0.1 cm PAGE at 80 V.

   For other gel sizes, use 8 V/cm.

5. Run dye 2/3 of the way to the bottom of the plates. (NOVEX System: 50 to 60 minutes, 70 V).

   Make sure that the gel does not overheat during the run.

Blotting and Crosslinking

Two transfer techniques are possible: electroblotting and contact blotting. The best results are obtained by electroblotting; contact blotting produces slightly lower signal intensities.

Electroblotting

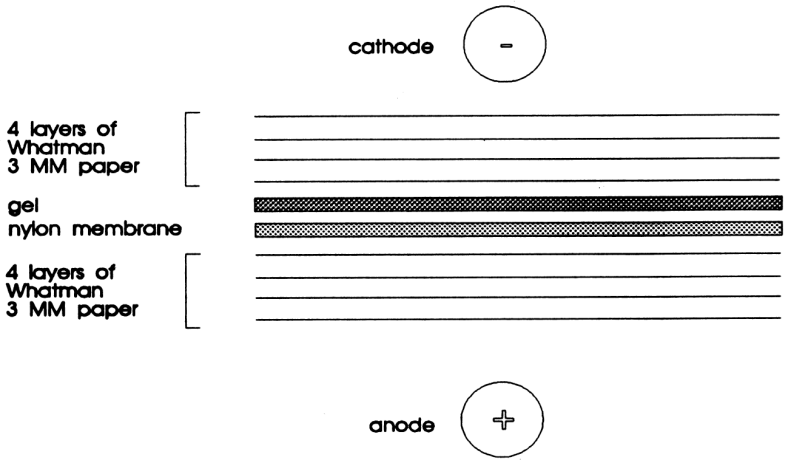

Fig. 1: Diagram of a sandwich between electrodes during electroblotting.

Additional Solutions Required

Solution	Composition/Preparation	Use
0.5x Tris-borate-EDTA (TBE) buffer, 10x conc.	890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0.	Transfer buffer

The standard electroblotting protocol is shown below.

1. After electrophoresis, carefully remove one glass plate from the gel.
2. Equilibrate a sheet of Nylon Membrane, trimmed to the size of the gel, for 5 minutes in transfer buffer (0.5x TBE buffer).
3. Place equilibrated Nylon Membrane carefully onto the gel.

   Avoid air bubbles between gel and filter.
4. Place 4 layers of gel-sized Whatman 3MM papers, presoaked in transfer buffer onto the filter.
5. Remove air bubbles by rolling a glass rod or a pipette over the 4 layers of Whatman 3 MM paper.
6. Remove pad of Whatman 3MM paper/nylon membrane/gel from the other glass plate.
7. Add 4 layers of pre-soaked Whatman 3MM papers onto the other side of the gel.
8. Place resulting sandwich between the electrodes of the electroblotting device (see Figure 1).
9. Transfer is performed for a 10 cm x 10 cm x 0.1 cm PAGE for 30 minutes at 400 mA (NOVEX System: 60 minutes, 30 V, 300 mA).

Contact Blotting

After electrophoresis, use the following protocol for the contact blotting.

1. Carefully remove one glass plate from the gel.
2. Place a dry Nylon Membrane trimmed to the size of the gel onto the gel.
3. Place 3 layers of dry Whatman 3MM paper onto the membrane.
4. Place a glass plate onto the 3 layers of Whatman 3MM paper.
5. Place a weight of 100 g onto the glass plate.
6. Transfer is completed after 20 to 30 minutes.

Crosslinking of Oligonucleotides

Fixation Methods

1. Bake the membrane at +120°C for 15 to 30 minutes.
2. Place the membrane on a Whatman 3MM paper presoaked with 2x SSC.
3. Crosslink at 120 mJ in, for example, a Stratalinker or with a transilluminator for, for example, 3 minutes.

   Times must be determined empirically.

Membrane Storage

Store the membrane as shown in the following table.

If...	Then...
You want to proceed to the chemiluminescent detection,	use the membrane immediately.
You want to work at a later time,	store the membrane air dried at +2 to +8°C between two sheets of Whatman 3MM paper.

Chemiluminescent Detection

Additional Solutions Required

Prepare the following solutions prior to performing the chemiluminescent detection.

i The Washing buffer, Maleic acid buffer, and Detection buffer are also available as 10x stock solutions in the DIG Wash and Block Buffer Set, DNase- and RNase-free according to the current quality control procedures.*

Solution	Composition/Preparation	Use	Storage and Stability
Washing buffer	0.1 M maleic acid, 0.15 M NaCl; pH 7.5 (20°C), 0.3% (v/v) Tween 20	Washing of membrane.	Stable at +15 to +25°C.
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl; adjust with NaOH (solid) 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	

i See section, **Working Solution** *for preparation of kit working solutions.*

The following protocol describes the immunological detection on a 100 cm^2 membrane.

i The suggested volumes are for the 100 cm^2 membrane processed in a small plastic container such as a petri dish. Use sufficient buffer volumes to cover the membrane completely during all steps. Perform all incubations at +15 to +25°C with agitation.

1. 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 working solution.
   – 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.

   i 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 X-ray film for 15 to 25 minutes or an imaging device at +15 to +25°C.

   i Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it reaches 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.

3. Troubleshooting

Observation	Possible cause	Recommendation
Weak or no signals.	Inefficient probe labeling.	Check labeling efficiency of your DIG-labeled DNA fragments or oligonucleotides by comparison to the labeled Control Oligonucleotide as recommended in section,***Determination of Labeling Efficiency.***
		Purify DNA fragments before labeling with the High Pure PCR Product Purification Kit* or by phenol/chloroform extraction and ethanol precipitation.
		Oligonucleotides must be HPLC purified.
	Inefficient transfer	We recommend electroblotting for best results.
	To short of exposure time.	Increase time of exposure to X-ray film or imaging device.
No shift observed.	Insufficient amount of protein.	The ratio of DNA to protein has to be estimated empirically for best results.
		Using nuclear protein extracts, the amount of extract will vary greatly depending on the extract preparation, DNA binding affinity of the protein, and quality of the extract. Usually 1 µg to 20 µg of extract should give a good band-shift result.
		Most purified proteins will not be 100% active. Titration should range from an equimolar protein-DNA ratio up to fivefold molar excess of protein.
	Too much nonspecific competitor DNA, or inappropriate type of competitor DNA.	Using nuclear protein extracts, a general rule is to use 1 µg of nonspecific competitor, such as poly[(d(I-C)] or poly[d(A-T)] for every 2 to 3 µg of extract.
		With purified DNA binding proteins, no or low concentrations of nonspecific competitor DNA are required. If used, the amount should be carefully titrated.
		Other types of competitor DNA such as calf thymus DNA or *E. coli* DNA should be avoided because they may contain binding sites for the protein of interest.
	Binding buffer is not optimal or missing components.	Typically, the binding buffer contains 25 mM to 50 mM K^+ or Na^+. In rare cases, it may be necessary to adapt the binding buffer to the ion (Na^+, K^+) present in the protein extract.
		Do not use more than 150 mM salt for optimal binding.
		For some proteins, the formation of specific DNA/protein complexes is dependent on additional factors, such as magnesium, zinc, calcium ions, detergent, or spermidine. *The standard 1x Binding Buffer delivered with this kit contains 1 mM EDTA.*
		The addition of basic peptides, such as Poly-L- lysine (Vial 11) can increase the binding affinity of some proteins.
	Unstable DNA protein complex.	Perform the binding reaction and electrophoresis at +4°C.
		Decrease the ionic strength of the electrophoresis gel system (gel and buffer) from 0.5x to 0.25x TBE.
		Decrease the electrophoresis time by increasing the voltage.

Multiple shifted bands produced with addition of protein.	Protein degradation	Avoid multiple freeze-thawing of the protein extracts or purified proteins.
		Add protease inhibitors to protein extract preparations or purified proteins and binding buffer.
	Several proteins in extract recognize DNA sequence.	Use antibody supershift reactions to identify the protein of interest.
DNA/protein complex does not run into the gel but remains in the slot.	Too much protein used, or insufficient amounts of nonspecific competitor added.	Titrate the amount of protein and nonspecific competitor.
	DNA fragments are too large and have multiple binding sites.	Reduce size of DNA fragment.
General high background.	Drying out of membrane during detection procedure.	Membrane should not be allowed to dry through all detection steps. If it dries or if membrane sticks together in the dishes, high background will result.
	Inefficient blocking and washing.	Increase volumes of the washing and blocking solution and duration of the washing and blocking steps.
	Exposure too long.	Shorten exposure time. The signal intensity increases with time.
Spotty background	Precipitates in buffer or wrong buffer.	Spotty background may be caused by precipitates in the Anti-DIG-AP conjugate. Centrifuge Anti-Digoxigenin-AP (Vial 14) for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface.
		Use detection buffer without Mg^{2+}.
	Contamination of trays.	When using laboratory trays for the detection procedure, they should be rigorously cleaned before use.

4. Additional Information on this Product

4.1. Test Principle

The study of DNA-protein interactions has been significantly facilitated by the gel retardation or gel mobility shift assay. This rapid and simple technique is based on the separation of free DNA from DNA-protein complexes due to the differences in their electrophoretic mobility in native (nondenaturing) polyacrylamide or agarose gels.

The DIG Gel Shift Kit uses the DIG end-labeling technique to detect sequence-specific DNA-binding proteins.

Stage	Description
Annealing and labeling of oligonucleotides	Annealing of single-stranded oligonucleotides and 3′-end labeling with Digoxigenin-11-ddUTP.
Formation of oligonucleotide-protein complexes	The labeled DNA fragment containing the sequence of interest is incubated with a cell extract or a purified DNA binding protein. To prevent nonspecific binding of the analyzed protein or extract to the DNA fragment or oligonucleotide, nonspecific competitor nucleic acid is added to the binding reaction. If a GC-rich binding sequence is to be expected, poly [d(I-C)] should be applied; if an AT-rich binding sequence is expected, poly [d(A-T)] is applied as a nonspecific competitor. *The optimum amount of competitor DNA must be determined empirically.*
Electrophoresis	The mixture is transferred to a native polyacrylamide gel and submitted to gel electrophoresis.
Blotting	Following the electrophoretic separation, the oligonucleotide-protein complexes are blotted to Nylon Membranes, positively charged* by ▪ Electroblotting, or ▪ Contact blotting
Immunological detection	The DIG-labeled DNA fragments or oligonucleotides are visualized by an enzyme immunoassay using Anti-Digoxigenin-AP, Fab fragments* and the chemiluminescent substrate CSPD*.
Chemiluminescent signal detection	The generated chemiluminescent signals are recorded either on X-ray film or an imaging device.

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

Editorial changes.
Updated the section 1.1 “contents”: 0.5% Triton X-100 is replaced with 0.1% Tween 20.
Removed information related to the REACH Annex XIV.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Consumables		
Hybridization Bags	50 bags, 25 cm x 23 cm	11 666 649 001
Reagents, kits		
DIG Wash and Block Buffer Set	1 set, 30 blots (100 cm^2)	11 585 762 001
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
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

5.4. Trademarks

All 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:
Product Disclaimers.

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.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany