For life sciences research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

DIG Gel Shift Kit

For nonradioactive detection of sequence specific DNA binding proteins

Cat. No. 1 635 352 Store this kit at -15 to -25° C

Kit for 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

Instruction Manual

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Roche

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1.2 Kit contents

Caution

Vial 1 and vial 4 contain toxic material (potassium cacodylate). Use gloves during handling of these substances. Pay attention to the following risk and safety phrases:

Toxic by inhalation and if swallowed. Keep locked up and out of reach of children. When using do not eat, drink or smoke. After contact with skin, wash immediately with plenty of water.

R 21-23/25 S1/2-20/21-28-44

Collect the supernatants from the labeling reactions in a tightly closed, non-breakable container and indicate contents. Discard as regulated for toxic waste.

Bottle/ Cap	Label	Content including function	Cat. No. (if available separately)
1	**Labeling buffer 5x**	• 80 µl • [1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6 (25° C)].	
2	**$CoCl_2$ solution**	• 80 µl • 25 mM $CoCl_2$-solution	
3	**DIG-ddUTP solution**	• 20 µl • 1 mM Digoxigenin-11-ddUTP (DIG-ddUTP), in double dist. water	1 363 905
4	**Terminal transferase**	• 20 µl terminal transferase, in 0.2 M potassium cacodylate, 1 mM EDTA, 200 mM KCl, 4 mM 2-mercaptoethanol, pH 6.5 (25° C), glycerol, 50% (v/v) • 50 units/µl	220 582
5	**Binding buffer 5x**	• 800 µl, [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]	
6	**Control oligonucletide ds 39mer, unlabeled**	• 40 µl • 0.1 µg/µl • 3.85 pmol/µl • containing the binding site for Oct2A (8), as specific competitor for the binding reaction with Oct2A • Sequence: 5'-GTACGGAGTATCCAGCTCCGTAGC**ATGCAAAT**CCTCTGG-3' 3'-CCTCATAGGTCGAGGCATCG**TACGTTTA**GGAGACCAGCT-5'	
7	**DIG-labeled control oligonucleotide ds 39mer**	• 140 µl • 0.4 ng/µl • 15.54 fmol/µl • in TE buffer, 0.1 M NaCl. • The sequence is the same as for the unlabeled control oligonucleotide	
8	**Control factor Oct2A**	• 40 µl • 25-75 ng/µl • in 30 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 2 mM DTT, 0.2 M NaCl,	

continued on next page

Bottle/ Cap	Label	Content including function	Cat. No. (if available separately)
9	**Poly [d(I-C)]**	• 200 µl • 1 µg/µl in double dist. water	108 812
10	**Poly [d(A-T)]**	• 200 µl • 1 µg/µl in double dist. water	108 740
11	**Poly L-lysine**	• 200 µl • 0.1 µg/µl in double dist. water	Sigma P-0879
12	**Loading buffer without bromophenol blue**	• 1 ml • 0.25 x TBE buffer, 60%; glycerol, 40%	
13	**Loading buffer with bromophenol blue**	• 1 ml • 0.25 x TBE buffer, 60%; glycerol, 40%, bromphenol blue, 0.2% (w/v)	
14	**Anti-Digoxigenin-AP**	• 40 µl • 750 U/ml polyclonal sheep, Anti-Digoxigenin, Fab fragments conjugated with alkaline phosphatase	1 093 274
15	**CSPD**[1)]	• 440 µl • 10 mg/ml	1 655 884
16	**Blocking reagent**	• one bottle • 50 g powder	1 096 176

Additional equipment and reagents required

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

Detailed information is given in front of each procedure.

Procedure	Equipment	Reagents
3.3 Annealing and labeling of oligonucleotide	water bath	• sterile, double distilled water • EDTA, 0.2 M, pH 8.0, sterile TEN buffer
3.4 Determination of labeling efficiency	Nylon membranes positively charged* (Cat. No. 1 417 240)	• DIG Wash and Block Buffer Set* (Cat. No. 1 585 762) or
		• Washing buffer • Maleic acid buffer • Detection buffer
3.5 Gel shift reaction	Novex Retardation Gel (6%) ready to use	• TEN buffer
3.6 Polyacrylamide gel electrophoresis		• 0.25 TBE buffer • Acrylamide, bis-acrylamide, • TEMED • Ammonium sulfate
3.7 Blotting and cross-linking	• Nylon membranes, positively charged* (Cat. No. 1 417 240) • Whatmann 3 MM paper • electro-blotting device • transilluminator or • commercially available UV-cross linker	• 0.25 TBE buffer • 2x SSC or • 10x SSC
3.8 Chemiluminescent detection	• temperature resistant plastic bags or roller bottles • Hybridization bags* (Cat. No. 1 666 649)	DIG Wash and Block Buffer Set*(Cat. No. 1 585 762) or
		• Washing buffer • Maleic acid buffer • Detection buffer

2. Introduction

2.1 Product overview

Introduction

The study of DNA-protein interactions has been significantly facilitated in recent years by the "gel retardation" or "gel mobility shift" assay (1,2). 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. Reviews of the gel mobility shift assay have been published (3,4,5).

Test principle

The DIG Gel Shift Kit uses the DIG-endlabeling technique for detecting sequence-specific DNA-binding proteins.

Stage	Description
Annealing and labeling of oligonucleotides	Annealing of ss 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 unspecific binding of the analyzed protein or extract to the DNA fragment or oligonucleotide, non-specific 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 non-specific competitor. ***Note***: 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 by • electro-blotting or • contact blotting to Nylon membranes, positively charged.
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 with the Lumi-Imager.

Application

Detection of sequence specific DNA binding proteins.

Sample material

- 5' -or 3'-overhanging ends as well as blunt-ended oligonucleotides, since labeling is performed with terminal transferase and DIG-11-dUTP (6,7)

or

- single and double-stranded DNA with a fragment size between 30 - 200 bp

Note: The electrophoresis assay works best with shorter DNA fragments to reduce non-specific interactions of proteins with sequences which flank the specific binding site.

Assay time

This table lists the reaction time of the single steps

Step	Reaction time
Oligonucleotide annealing and labeling	10 min.
Formation of oligonucleotide-protein complexes	25 min.
Electrophoresis	Depending on gel system 1-2 h to O/N.
Blotting	Depending on device used, 1 - 2 h
Immunological detection	2 h
Exposure to X-ray film	15-40 min

Number of tests

1 kit is sufficient for

- 20 reactions for labeling DNA at the 3'end with digoxigenin (DIG),
- 200 binding reactions,
- Chemiluminescent detection reaction for 20 blots,
- DNA binding protein and oligonucleotide for 20 control reactions

Quality Control

At least 50% of the control oligonucleotide (vial 7) is shifted by the control factor (vial 8) under the conditions described in the protocol. 20 fmol of control oligonucleotide labeled according to the kit protocol can be detected.

Kit storage/ stability

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

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

Kit component	Storage
Control factor Oct2A vial 8	• Repeated freezing and thawing should be avoided • Aliquot the factor solution after the first thawing and store the aliquots at -15 to -25° C
Anti-Digoxigenin-AP Conjugate vial 14	• 2-8° C, stable • ***Note***: Do not freeze!
CSPD ®, ready-to-use vial 15	2-8° C, stored protected from light.
Blocking reagent powder bottle 16	• unopened, stable at 15 to 25° C • working solution should always be prepared fresh.

Advantages

Benefits	Feature
Stability of the DIG- labeled probe	No labeling of same oligo for each new experiment.
Upscaling of labeling reaction	Make a large quantity of labeled oligo and use it for the project, get your experiment more standardized, by using same oligo-labeling reaction for several experiments.
Short exposure times	Faster results.
Sensitivity	At least equal to isotopic procedures.
Time saving	No need to remove unincorporated DIG-ddUTP.
No waste disposal problems, no health hazard	Non-radioactive labeling.

3. Procedures and required materials

3.1 Before you begin

General handling recommendations

This table describes general hints for DIG-labeling and detection.

Recommendation	Guideline
Work under clean conditions, use good laboratory practice	• Autoclave DIG-System solutions. • Filter-sterilize solutions containing SDS. • Tween® 20[2] should be added to previously sterilized solutions.
Use clean incubation trays	• Rigorously clean and rinse laboratory trays before each use. • Use sterile disposable plastic wear.
Membrane handling requirements	• Wear powder-free gloves. • Handle membrane only on the edges and with clean forceps.

3.2 Flow chart

Section 3.3	**Annealing and labeling of oligonucleotides**
	↓
Section 3.4	**Determination of labeling efficiency**
	↓
Section 3.5	**Gel shift reaction**
	↓
Section 3.6	**Polyacrylamide gel electrophoresis**
	↓
Section 3.7	**Blotting and crosslinking**
	↓
Section 3.9	**Chemiluminescent detection**

3.3 Annealing and labeling of oligonucleotides

Control reaction

For the control reaction with the control oligonucleotide (vial 6) 100 ng (= 3.85 pmol of a ds 39mers) are used for the labeling reaction.

Conversion of ng to pmol

ng ds DNA = pmol x 0.66 x N (N= length of the ds fragment)
Note: Please also compare to formulas in LabFAQS "Find a quick solution" from Roche Molecular Biochemicals / Printed Material No. 2 115 972

Additional reagents required

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

Solution	Composition	Storage	Use
Water	Autoclaved, double distilled water	15 to 25° C stable	Dilution of buffers and preparation of labeling/ binding reactions and of the oligonucleotide
EDTA	0.2 M ethylenediamino- tet-raacetic acid, pH 8.0	15 to 25° C stable	Stopping of the reaction
TEN-buffer	10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0	15 to 25° C, stable	For the annealing reaction and dilution series of the oligonucleotide

Procedure

Please refer to the following table.

***Note*:** 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 increase of incubation time to 1 hour.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Mix ss oligonucleotide in a molar ratio of 1:1 in TEN-buffer.</td></tr>
<tr><td>2</td><td>Incubate 10 min at 95° C.</td></tr>
<tr><td>3</td><td>Cool slowly to 15 -25° C.</td></tr>
<tr><td>4</td><td>Dilute with sterile TEN-buffer to 3 - 4 pmol/µl.</td></tr>
<tr><td>5</td><td>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 1µl control oligonucleotide (vial 6) and 9 µl sterile double distilled water to a reaction vial.</td></tr>
<tr><td>6</td><td>Add the following on ice:
<table>
<tr><th>Reagent</th><th>Volume</th><th>Final conc.</th></tr>
<tr><td>5x Labeling buffer (vial 1)</td><td>4 µl</td><td>1x</td></tr>
<tr><td>$CoCl_2$-solution (vial 2)</td><td>4 µl</td><td>5 mM</td></tr>
<tr><td>DIG-ddUTP solution (vial 3)</td><td>1 µl</td><td>0.05 mM</td></tr>
<tr><td>Terminal transferase (50 units) (vial 4)</td><td>1 µl</td><td>2.5 units/µl</td></tr>
</table>
• Mix and centrifuge briefly.
• Incubate at 37° C for 15 min, then place on ice.</td></tr>
<tr><td>7</td><td>Stop the reaction by adding 2µl 0.2 M EDTA (pH 8.0).</td></tr>
<tr><td>8</td><td>Add 3 µl double dist. 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.</td></tr>
</table>

Labeling efficiency

The efficiency of the labeling reaction must be checked by comparison of spotted dilution series of labeling reaction with one of the labeled control-oligonucleotide (vial 7) in a direct detection assay (see section 3.4).

3.4 Determination of labeling efficiency

Introduction

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

Test principle

Direct detection method

Stage	Description
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 and the working solution of CSPD® . • Both dilution series should show a signal with the 4 pg spot.

Additional reagents required

- Nylon membrane, positively charged (Cat. No. 1 209 272)
- DIG Wash and Block Buffer Set (Cat. No. 1 585 762)

Preparation of additional solutions required

The Washing buffer, Maleic acid buffer, and Detection buffer are also available as 10x stock solutions in the DIG Wash and Block Buffer Set.

Solution	Composition / Preparation	Storage and stability	Use
Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; pH 7.5 (20° C); 0.3% (v/v) Tween® 20	15 to 25° C, stable	Removal of unbound antibody
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20° C)	15 to 25° C, stable	Dilution of Blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20° C)	15 to 25° C, stable	Equilibration of membrane for adjustment of pH to 9.5
TEN-buffer	10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0	15 to 25° C, stable	Dilution of oligonucleotide

Preparation of kit working solutions

Please refer to the following table:

Solution	Composition / Preparation	Storage and stability	Use
Blocking stock solution (10x conc.)	Dissolve Blocking reagent (bottle 12) 10%(w/v) in Maleic acid buffer under constantly stirring on a heating block (65°C) or heat in a microwave oven, autoclave. The solution remains opaque.	4 weeks at 2 to 8° C if kept sterile ***Note:*** Freeze aliquots at -15 to -25° C°	Preparation of Blocking solution
1x Blocking solution	Prepare a 1x working solution by diluting the 10x Blocking solution 1:10 in Maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites on the membrane
Antibody solution	Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:10 000 (75 mU/ml) in Blocking solution.	12 h at 2 to 8° C	Binding to the DIG-labeled probe
CSPD working solution	Dilute 0.1 mg/ml stock solution 1:100 in Detection buffer.	2 to 8° C protected from light	Chemiluminescent detection

Dilution series

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

Note: The concentration of the labeled DNA is 4 ng/µl or 0.155 pmol/µl after labeling as described in section 3.3 step 8

.

Tube	Oligo (µl)	From tube #	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

Procedure

The following procedure describes the direct detection.

Step	Action
1	Apply a 1µl spot of tubes 1-5 from your labeled oligonucleotide and the labeled control to the nylon membrane.
2	Fix the nucleic acid to the membrane by cross linking with UV-light or baking for 30 min at 120° C.
3	• Transfer the membrane into a plastic container with 20 ml **Washing buffer**. • Incubate under shaking for 2 min at 15 to 25° C.
4	Incubate for 30 min in 10 ml **Blocking solution**.
5	Incubate for 30 min in 10 ml **Antibody solution.**
6	Wash with 10 ml **Washing buffer**, 2 x 15 min.
7	Equilibrate 2-5 min in 10 ml **Detection buffer**.
8	• Place membrane with DNA side facing up on a development folder (or hybridization bag) and apply 0.1 ml **CSPD® working solution**. • **Immediately** cover the membrane with the second sheet of the folder to spread the substrate evenly and without airbubbles over the membrane. • Incubate for 5 min at 15 to 25° C.
8	Squeeze out excess liquid and seal the edges of the development folder. ***Note***: Drying of the membrane during exposure will result in dark background.
9	Incubate the damp membrane for 10 min at 37° C to enhance the CSPD chemi-luminescent reaction.
10	Expose to Lumi-Imager™ for 5-20 min or to X-ray film. ***Note***: Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it will reach a plateau where signal intensity remains almost constant during the next 24 - 48 hours. Multiple exposures can be taken to achieve the desired signal strength.

Analyzing the results

You can now determine the quantity of DIG labeled DNA by comparing the dilution series with that of the DIG labeled control oligo (vial 7). The 1 fmol e.g. 4 pg spots should be visible.

3.5 Gel shift reaction

Before you begin

We recommend NOVEX Retardation Gel (6%), ready to use.

Note: Precast gels should be used.

Modification of reaction conditions

Control reaction is optimized for the factor Oct2A (vial 8).

For analysis of your binding protein, reaction conditions might be modified as follows:

- With purified DNA binding proteins no or low concentration of unspecific competitor DNA are required.
- If factors are analyzed from crude extracts, it is absolutely essential to add non-specific competitor DNA to the binding reaction.
- Some binding reactions may require a different reaction temperature (range 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 non-specific is dependent on the buffer conditions. Therefore it could also be important to use additions like 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 (9). Albumin can also be used to improve formation of specific protein/DNA complexes (10).
- The order of probe and nuclear protein extract addition can determine the specificity of protein-DNA complexes formed (11).

Additional reagents required

TEN buffer: 10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0.

Dilution of labeled oligonucleotide and control

1. Dilute the own labeled oligonucleotide to a concentration of 15-30 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 already DIG-labeled control oligonucleotide (vial 7).

Sample preparation

Prepare three samples for the control reaction and for the analysis of your factor accordingly:

Sample	Description
1	Labeled oligonucleotide without factor.
2	Labeled oligonucleotide with factor.
3	Labeled oligonucleotide with factor and with an 125-fold excess of unlabeled oligonucleotide for specific competition. ***Note***: The addition of poly-L-lysin is optional, it improves binding of the factor to the oligonucleotide.

Protocol

Standard protocol for the Gel shift reaction.

Step	Action
1	Mix on ice:

	sample 1	sample 2	sample 3
Binding buffer (vial 5)	4 µl	4 µl	4 µl
poly [d(I-C)] (vial 9), [1µg/µl]	1 µl	1 µl	1 µl
poly L-lysine (vial 11), [0.1 µg/µl]	1 µl	1 µl	1 µl
DIG-labeled oligo-nucleotide (vial 7) [0.4 ng/µl]	2 µl	2 µl	2 µl
double dist. water	12 µl	11 µl	10 µl
unlabeled oligo-nucleotide (vial 6) [0.1 µg/µl]	-	-	1 µl
Oct2A factor (vial 8) [25-75 ng/µl]	-	1 µl	1 µl

2	Mix carefully and incubate for 15 min at 15-25° C.
3	Place tube on ice.
4	Add to each sample 5 µl of Loading buffer with bromophenol blue (vial 13). ***Note:*** When using the NOVEX System instead add the recommended high density TBE sample buffer.
5	Apply sample immediately to a pre-electrophoresed polyacrylamide gel.

3.6 Polyacrylamide gel electrophoresis

Adjustment of acrylamide concentration

The appropriate acrylamide concentration depends on the size of oligonucleotide or factor complexes (12). For most cases a 6%-8% native polyacrylamide gel in 0.5 x TBE buffer is optimal.

Additional reagents required

Running buffer: 0.5 x TBE buffer (Tris-borate-EDTA buffer, 10x conc.: 890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0).

Protocol

Please refer to the following table.

Step	Action
1	**one day before** Prepare a native polyacrylamide gel of 6% or 8% acrylamide in 0.5 x TBE buffer. ***Note:*** 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 min).
3	Load the samples to the gel. ***Note:*** Before loading samples, clean sample wells to remove APS, urea and residual polyacrylamide to ensure sample application without diffusion.
4	Run a 10 cm x 10 cm x 0.1 cm PAGE at 80 V. ***Note***: For other gel sizes use 8 V/cm.
5	Run dye 2/3 of the way to the bottom of the plates. (NOVEX System 50-60 min, 70 V). ***Note:*** Make sure that the gel does not overheat up during the run!

3.7 Blotting and crosslinking

Blotting techniques and results

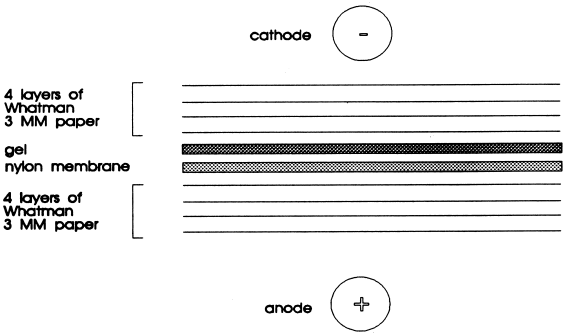
Two transfer techniques are possible:

- Electro-blotting,
- Contact-blotting.

The best results are obtained by electro-blotting, Contact-blotting produces slightly lower signal intensities.

3.7.1 Electro-blotting

Fig. 1 Electro-blotting



Additional equipment and reagents required

- Nylon membranes, positively charged* (Cat. No. 1 417 240)
- Whatman 3MM paper
- Transfer buffer: 0.5x TBE buffer
- for electro-blotting: blotting device like e.g.
  - NOVEX System (XCell II Blot Module No. EI 9051)
  - LKB 2117-250
  - Novablot Electrophoretic Electroblotting Transfer Kit.

Protocol

Please refer to the following table for the standard protocol of the electroblotting.

Step	Action
1	After electrophoresis: Remove one glass plate carefully from the gel.
2	Equilibrate a sheet of nylon membrane trimmed to the size of the gel for 5 min in transfer buffer (0.5x TBE buffer).
3	Place equilibrated nylon membrane carefully onto the gel. ***Note:*** Avoid air bubbles between gel and filter.
4	Place 4 layers of gel-sized Whatman 3MM papers, presoaked in transfer buffer on the filter.
5	Roll with a glass rod or a pipette over the 4 layers Whatman 3 MM paper to remove air bubbles.
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 on the other side of the gel.
8	Place resulting sandwich between the electrodes of the electroblotting device (see fig. 1).
9	Transfer is performed for a 10 cm x 10 cm x 0.1 cm PAGE for 30 min at 400 mA (NOVEX System: 60 min; 30 V; 300 mA).

3.7.2 Contact-blotting

Protocol

After electrophoresis use the following protocol for the contact blotting.

Step	Action
1	Remove one glass plate carefully from the gel.
2	Place a dry nylon membrane trimmed to the size of the gel onto the gel.
3	Put 3 layers of dry Whatman 3 MM paper on the membrane.
4	Put a glass plate on the 3 layers of Whatman 3MM paper.
5	Place a load of 100 g on the glass plate.
6	Transfer is completed after 20-30 min.

3.7.3 Crosslinking of oligonucleotides

Fixation methods

Please refer to the following table.

Bake at 120° C	bake the membrane for 15-30 min
Cross-link	• place the membrane on a Whatman 3 MM paper presoaked with 2x SSC • cross-link at 120 mJ in e.g. a Stratalinker or with a transilluminator for e.g. 3 min which has to be tested empirically.

Storage of the membrane

Please refer to the following table.

IF...	THEN...
You want to go ahead to the chemiluminescent detection	Use the membrane immediately.
You want to work later on	Store the membrane air dried at 4° C between two sheets of Whatman 3 MM paper.

3.8 Chemiluminescent 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 (Cat. No. 1 585 762) DNase and RNase free.

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

Preparation of kit working solutions

Please refer to the following table:

Solution	Composition / Preparation	Storage and stability	Use
Blocking stock solution (10x conc.)	Dissolve Blocking reagent (bottle 12) 10%(w/v) in Maleic acid buffer under constantly stirring on a heating block (65°C) or heat in a microwave oven, autoclave. The solution remains opaque.	4 weeks at 2 to 8° C if kept sterile ***Note:*** Freeze aliquots at -15 to -25° C°	Preparation of Blocking solution
1x Blocking solution	Prepare a 1x working solution by diluting the 10x Blocking solution 1:10 in Maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites on the membrane
Antibody solution	Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:10 000 (75 mU/ml) in Blocking solution.	12 h at 2 to 8° C	Binding to the DIG-labeled probe
CSPD working solution	Dilute 0.1 mg/ml stock solution 1:100 in Detection buffer.	2 to 8° C protected from light.	Chemiluminescent detection

Procedure

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

Note: The volumes suggested below are for a 100 cm^2 blot processed in a small plastic container, such as a petri dish. Use sufficient buffer volumes to cover the membrane completely during all steps. All incubations should be performed at 15 to 25°C with agitation.

Step	Action
1	Rinse membrane briefly (1-5) min in **Washing buffer**.
2	Incubate for 30 min in 100 ml **Blocking solution**.
3	Incubate for 30 min in 20 ml **Antibody solution.**
4	Wash 2 x 15 min in 100 ml **Washing buffer**.
5	Equilibrate 2-5 min in 20 ml **Detection buffer**.
6	• 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 airbubbles over the membrane. • Incubate for 5 min at 15 to 25° C.
7	Squeeze out excess liquid and seal the edges of the development folder. ***Note***: Drying of the membrane during exposure will result in dark background.
8	Incubate the damp membrane for 10 min at 37° C to enhance the luminescent reaction.
9	Expose to Lumi-Imager for 5-20 min or to X-ray film for 15-25 min at 15 to 25° C. ***Note***: Luminescence continues for at least 48 hours. The signal increases in the first few hours after initiation of the detection reaction until it will reach a plateau where signal intensity remains almost constant during the next 24 – 48 hours. Multiple exposures can be taken to achieve the desired signal strength.

4. Appendix

4.1 Trouble shooting

Trouble shooting table

This table describes various troubleshooting parameters concerning band shift experiments using DIG-labeling and detection

Problem	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 3.4 • Purify DNA fragments by phenol/chloroform extraction and ethanol precipitation before labeling. • Oligonucleotides have to be HPLC purified.
	Inefficient transfer	We recommend electro-blotting for best results.
	To short exposure time	Increase time of exposure to X-ray film or Lumi Imager.
No shift observed	Insufficient amount of protein	• The ratio 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 – 20 µg of extract should give a good band shift result. • Most purified proteins will not be 100% active. Titration should range from equimolar protein-DNA ratio up to five-fold 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 non-specific competitor, such as poly[(d(I-C)] or poly[d(A-T)] for every 2-3 µg of extract. • With purified DNA binding proteins no or low concentrations of non-specific 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 obtain binding sites for the protein of interest.
	Binding buffer is not optimal or missing components	• Typically the binding buffer contains 25 mM - 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. ***Note:*** The standard 1x binding buffer delivered with this kit contains 1 mM EDTA. • The addition of basic peptides, as poly-L- lysin can increase the binding affinity of some proteins.

continued on next page

Problem	Possible cause	Recommendation
	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 (14).
DNA/protein complex does not run into the gel but remains in slot	Too much protein used, or insufficient amounts of non-specific competitor added	Titrate the amount of protein and nonspecific competitor.
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 stick 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/ wrong buffer	• Spotty background may be caused by precipitates in the anti-DIG-AP conjugate: Centrifuge anti-digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. • Use detection buffer without Mg^{++}.
	Contamination of trays	When using laboratory trays for the detection procedure, they should be rigorously cleaned before use.

4.2 References

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4.3 Related products

Single reagents

Product	Pack Size	Cat. No.
Anti-Digoxigenin-AP	150 U (200 µl)	1 093 274
Blocking reagent	50 g	1 096 176
DIG Wash and Block Buffer Set	30 blots (10 x 10 cm^2)	1 585 762
DIG-11-ddUTP	25 nmol (25 µl)	1 363 905
EDTA	250 g 500 g 1 kg	808 261 808 270 808 288
Hybridization bags	50 bags	1 666 649
Lumi-Film Chemiluminescent Detection Film	100 sheets (20.3 x 25.4 cm) 100 sheets (18 x 24 cm) 100 sheets (35 x 43 cm)	1 666 657 1 666 916 1 666 711
Lumi-Imager F1	240 Volt 120 Volt	2 015 170 2 012 847
Nylon Membrane, positively charged (20 x 30 cm) (10 x 15 cm) (0.3 x 3 m roll)	 10 sheets 20 sheets 1 roll	 1 209 272 1 209 299 1 417 240
Terminale Transferase	500 U	220 582

* available from Roche Applied Science

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