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DIG Luminescent Detection Kit

Version 08

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Chemiluminescent detection of digoxigenin labeled nucleic acids by enzyme-immunoassay

Cat. No. 11 363 514 910

Kit for the detection of 50 blots of
100 cm^2

Store the kit at −15 to −25°C

Please note:

The antibody (vial 3) and the substrate CSPD (vial 5), once opened, should be stored at +2 to +8°C.
Please also refer to Kit storage/stability information on page 6.

1. Preface

1.1 Table of contents

1.2 Kit contents

Bottle/ Cap	Label	Content including function
1	Labeled control DNA	• 50 µl linearized pBR328 DNA, labeled with digoxigenin according to the standard protocol containing 1 µg template DNA and approx. 260 ng synthesized labeled DNA. • clear solution • for estimation of labeling efficiency
2	DNA dilution buffer	• 1 ml fish sperm* DNA (50 µg/ml) in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (20°C). • clear solution
3	Anti-digoxigenin-AP, Fab fragments	• 100 µl polyclonal sheep anti -digoxigenin, Fab fragments, conjugated to alkaline phosphatase • clear solution
4	Blocking Reagent	Two bottles with 50 g powder each
5	CSPD	• 1 ml CSPD Disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2´-(5´-chloro) tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate, molecular weight: 461. • chemiluminescent substrate for alkaline phosphatase

2. Product overview

Test principle

The nonradioactive DIG system uses digoxigenin, a steroid hapten, coupled to dUTP, UTP or ddUTP to label DNA, RNA or oligonucleotides for hybridization and subsequent luminescent detection (1–4).

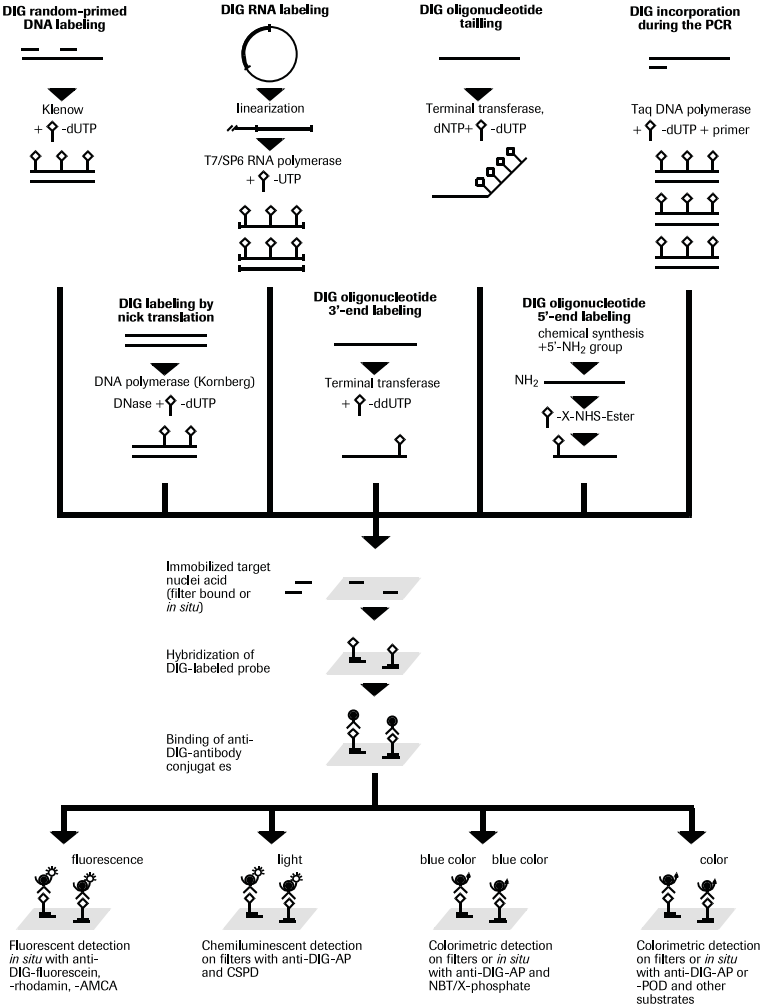

Fig. 1:

DIG-labeling reaction

Please find in the following table the labeling techniques for DNA, RNA, and oligonucleotides.

Nucleic acid	Labeling reaction
DNA probes	Labeling with DIG-dUTP via random-primed labeling, nick translation or PCR.
Oligonucleotides	3'-end labeling with DIG-ddUTP or tailed with DIG-dUTP using terminal transferase. 5'-end labeling of oligonucleotides is done with DIG-NHS-ester (digoxigenin-3-O-methylcarbonyl-ε-aminocaproic-acid-N-hydroxysuccinimide ester).
RNA probes	Synthesis in an *in vitro* transcription reaction with SP6, T7 or T3 RNA polymerases.

Application

Highly sensitive detection of DIG-labeled nucleic acids on all types of membrane blots, using anti-digoxigenin, alkaline phosphatase conjugates and the chemiluminescent substrate CSPD.

Immunological detection

The hybridized probes are immunodetected with anti-digoxigenin, Fab fragments conjugated to alkaline phosphatase and are then visualized with the chemiluminescence substrate CSPD. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a light emission at a maximum wavelength of 477 nm which is recorded on X-ray films.
The chemiluminescent signal from CSPD persist for days on nylon membranes.
Since film exposures of a few minutes are usually sufficient for signal detection, multiple images may be acquired. (Fig. 2)

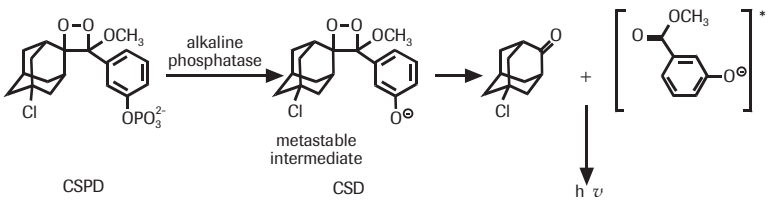

Fig. 2

Sample material

DIG labeled nucleic acids.

Assay time

Step	Reaction time
Immunological detection	1.5 h
Signal detection	5 - 30 min

Number of detections

50 blots with a size of 10 × 10 cm^2.

Quality control

Each lot of CSPD is tested for purity: CSPD (NMR) > 98%

Using DIG-labeled control DNA (pBR328/*Bam* HI) as hybridization probe, 0.03 pg homologous DNA diluted with 50 ng heterologous DNA are detected in a dot blot with CSPD after < 30 min exposure to X-ray film, following the standard detection protocol.

Kit storage/ stability

The unopened kit is stable at −15 to −25°C until the expiration date printed on the label.
All components of the kit are stable at −15 to −25°C.
In the following table storage and stability instructions for several kit components are listed:

Reagent	Storage/Stability
Antibody conjugate (vial 3)	once opened, should be stored at +2 to +8°C
Blocking Reagent (bottle 4)	dry at +2 to +8°C or at +15 to +25°C
CSPD (vial 5)	• at +2 to +8°C when frequently used. • Repeated freeze/thaw cycles should be avoided. ***Note***: Store protected from light

Sensitivity and specificity

A single copy gene (tissue plasminogen activator, tPA) is detected in a Southern blot of 0.3 µg *Bgl* II or *Eco* RI digested human placenta DNA. The same sensitivity is obtained, when using DIG-labeled RNA probes.

3. Procedures and required materials

3.1 Immunological detection with CSPD

Time requirements

Time requirements for each of the listed steps are indicated below:

Step	Time
Washing and Blocking of membrane	32 min
Antibody binding	30 min
Washing and equilibration of membrane	32 min
Luminescent reaction	5 min
Preincubation at +37°C	10 min
Film exposure	20 min
Total time	**130 min**

Additional equipment and reagents required

- Hybridization bags*
  or
- Development folders
- plastic or glass boxes or petri dishes
- DIG Wash and Block Buffer Set*
  or
- Washing buffer
- Maleic acid buffer
- Detection buffer

Preparation of additional solutions required

Washing buffer, Maleic acid buffer, and Detection buffer are also provided in the DIG Wash and Block Buffer Set*.

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

Preparation of kit working solutions

Note: Please refer to the following table:

Solution	Composition / Preparation	Storage/ stability	Use
CSPD (vial 5)	Dilute CSPD 1:100 in Detection buffer. [0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)]. ***Note***: The solution can be reused one to two times.	+2 to +8°C in the dark	Chemiluminescence detection
Antibody solution (vial 3)	Centrifuge Anti-Digoxigenin-AP (vial 3) 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
Blocking stock solution (10 × conc.) (bottle 4)	Dissolve Blocking Reagent 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.	+2 to +8°C or −15 to −25°C	Preparation of Blocking solution
Blocking solution	Prepare a 1 × working solution by diluting the 10 × Blocking solution 1:10 in Maleic acid buffer.	Always prepare fresh	Blocking of unspecific binding sites on the membrane

Procedure

All incubations should be performed at +20 to +25°C with agitation. The volumes are calculated for a membrane size of 100 cm^2.

Step	Action
1	After hybridization and stringency washes, rinse membrane briefly (1-5) min in **Washing buffer**.
2	Incubate for 30 min in 100 ml **Blocking solution**.
3	Incubate for 30 min in 20 ml **Antibody solution.**
4	Wash 2 × 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 2 ml diluted **CSPD** 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.
7	Squeeze out excess liquid and seal the edges of the development folder.
8	Incubate the damp membrane for 5-15 min at 37°C to enhance the luminescent reaction.
9	Expose to imaging instrument or to X-ray film for *e.g.*, 5–25 min at +15 to +25°C. ***Note***: Luminescence continues for at least 24 hours and signal intensity remains almost constant during the first hours. Multiple exposures can be taken to achieve the desired signal strength.

3.2 Stripping and reprobing of DNA blots

General

The alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization experiment.
Note: If filters are to be stripped and reprobed, they should not be allowed to dry out, but should be stored in 2 × SSC or maleic acid.

Additional equipment and reagents required

- Large beaker
- Water bath
- 10 × SSC
- 10% SDS
- 0.2 M NaOH

Procedure

Please refer to the following table.
Note: Alternative stripping protocols, as mentioned in the “DIG Application Manual for Filter Hybridization” (available on request) can also be used with high efficiency.

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

Detection of DIG labeled Nucleic Acids with CSPD

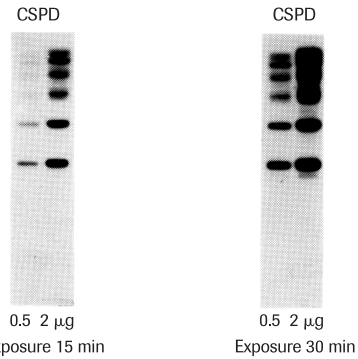

Fig. 3

Human genomic DNA was digested with *Eco* RI, separated on a 1% agarose gel, and blotted onto positively charged nylon membranes. The blots were hybridized with 50 ng/ml DIG-labeled β-actin RNA. Chemiluminescent detection was according to the standard DIG chemiluminescent detection procedure using CSPD at 0.25 mM final concentration.

5. Appendix

5.1 Trouble shooting

Trouble shooting table

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

Problem	Possible cause	Recommendation
Low sensitivity	Inefficient probe labeling	• Check labeling efficiency. The labeling reaction can be upscaled. Prolong incubation time to overnight. • Clean up template DNA by phenolization. • Use only fragments < 10 kb or predigest with a restriction enzyme (*e.g.*, a four bp cutter). • Check the amount and quality of target DNA. • Make sure that template is efficiently denatured before labeling.
	Low probe concentration in the hybridization	• Increase probe concentration (use 25 ng/ml). • Prolong hybridization time to overnight. • Increase concentration of anti-DIG-AP (dilute 1:5,000)
High background	Inefficient hybridization	• Recalculate hybridization temperature. • Do not allow the membrane to dry between prehybridization and hybridization. • If you use plastic bags, remove all air bubbles prior to sealing. • Use DIG Easy Hyb* buffer, especially when other membrane brands are used.
	Concentration of labeled probe is too high	Determine optimal probe concentration as described in section 3.1, do not use more than 25 ng/ml. a) Decrease probe concentration b) Increase volume of prehybridization solution.
	Wrong type of nylon membrane	Some types of nylon membrane may cause high background, use Roche Applied Science nylon membrane, especially tested for the DIG-System.
	Inefficient blocking before immunoassay	Prolong blocking and washing steps.
		When using laboratory trays for the detection procedure, they should be rigorously cleaned before use. Anti-DIG-AP binding and chemiluminescent development should be done; in separate trays.
		Heat treatment of all glass ware is recommended to solve background problems

5.2 References

1 Höltke, H. J. et al. (1995) *Cellular and Molecular Biology* **41**(7), 883-905.

2 Bronstein, I. et al. (1991) in Bioluminescence and Chemiluminescence, Current Statur (Stanley, P. & Kricka, L.J., eds) pp 73-82.

3 Schaap, A. P. et al. (1989) *Clin. Chem.* **35,** 1863

5.3 Ordering Information

Kits

Product	Pack Size	Cat. No
DIG DNA Labeling Kit	40 labeling reactions	11 175 033 910
DIG- RNA Labeling Kit (SP6/T7)	2 × 10 reactions	11 175 025 910

Single reagents

Product	Pack Size	Cat. No.
Anti-digoxigenin-AP, Fab fragments	50 U	11 093 274 910
Blocking Reagent	50 g	11 096 176 001
CDP-Star, ready-to-use	2 × 50 ml	12 041 677 001
CSPD, ready-to-use	2 × 50 ml	11 755 633 001
DIG Easy Hyb	500 ml	11 603 558 001
DIG Wash and Block Buffer Set	30 blots (10 × 10 cm)	11 585 762 001
DIG-11-ddUTP	25 nmol (25 µl)	11 363 905 910
DIG-11-dUTP, alkali-labile	25 nmol (25 µl) 125 nmol (125 µl)	11 573 152 910 11 573 179 910
DIG-11-UTP	250 nmol (25 µl)	11 209 256 910
DIG-DNA Labeling Mix	50 µl (25 reactions)	11 277 065 910
DIG-High Prime	160 µl (40 reactions)	11 585 606 910
DNA, MB grade	500 mg (50 ml)	11 467 140 001
Hybridization Bags	50 bags	11 666 649 001
Nylon membranes, positively charged	10 sheets (20 × 30 cm) 20 sheets (10 × 15 cm) 1 roll (0.3 × 3m)	11 209 299 001 11 209 272 001 11 471 240 001

[1]* *available from Roche Diagnostics*

Changes of previous version

- Editorial changes.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany