For general laboratory use.
FOR *IN VITRO* USE ONLY.

CSPD, ready to use

Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate

Cat. No. 1 755 633 2 × 50 ml (0.25 mM; 0.116 mg/ml) with dropper

Version 3, July 1999

Store at 2-8° C

1. Introduction

1.1 Product overview

Caution

The toxicological properties of CSPD[1)] have not been investigated. Avoid contact and handle with care. Wear gloves and a laboratory coat

Contents

Cat. No	Label	Contents
1 755 633	CSPD, ready-to-use	• 50 ml • 0.25 mM, 0.116 mg/ml

Product description

CSPD is a chemiluminescent substrate for alkaline phosphatase that enables extremely sensitive and fast detection of biomolecules by producing visible light which is recorded with film or instrumentation (1).

Application

CSPD can be used for the detection of alkaline phosphatase and alkaline phosphatase conjugates either in solution or on solid supports.

It is especially suited for highly sensitive and fast detection of nonradioactively labeled nucleic acids in

- Southern blots
- Northern blots
- Colony or plaque hybridizations
- Gel shift assays
- Sequencing

Note: For chemiluminescent detection of blots with CSPD a nylon membrane should be used for blotting of nucleic acids. When using nitrocellulose membranes a drastic reduction in sensitivity must be accepted.

Assay time

In the following table the time requirements for the single steps are listed:

Step	Time
Washing and Blocking of membrane	30 min
Antibody binding	30 min
Washing and equilibration of membrane	30 min
Luminescent reaction	5 min
Preincubation at 37°C	10 min
Film exposure	20 min
Total time	**125 min**

Quality control

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.

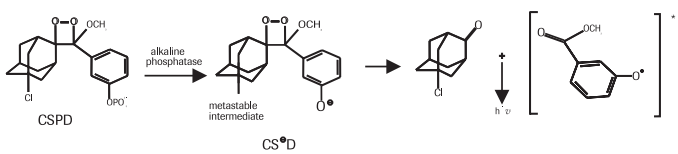
Storage/ Stability

The unopened bottle is stable at 2-8° C through the expiration date printed on the label.

Note: Store protected from light.

1.2 Product characteristics

Reaction scheme



Reaction principle

Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to the metastable phenolate anion which decomposes and emits light at a maximum wavelength of 477 nm. The luminescent light emission is recorded on X-ray films or by suitable cameras or instruments.

Formula

$C_{18}H_{20}ClO_7PNa_2$

Molecular weight

461

Purity

CSPD (NMR) > 98%

Sensitivity

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

Signal stability

The chemiluminescent signal from CSPD persists for days on nylon membranes. Since film exposures of a few minutes are usually sufficient, multiple images may be acquired.

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2. Procedures and required materials

2.1 Detection procedure for blot application

Detection of DIG-labeled nucleic acids with CSPD

Nucleic acid probes can be labeled very efficiently with digoxigenin (DIG) and be used as hybridization probes in various membrane blot applications. After stringency washes, the blots are subjected to immunological detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and CSPD. Detailed protocols for DIG labeling and hybridization are available in the product descriptions of various DIG labeling and detection kits (see below) and the DIG User's Guide for Filter Applications.

Additional equipment required

- Hybridization bags* (Cat. No. 1 666 649) or
- Temperature resistant plastic or glass boxes, petri dishes or roller bottles

Additional reagents required

- Anti-digoxigenin-AP, Fab fragments (Cat. No. 1 093 274)
- DIG Wash and Block Buffer Set* (Cat. No. 1 585 762) or
- Washing buffer
- Maleic acid buffer
- Detection buffer

Preparation of additional solutions required

The Washing buffer, Maleic acid buffer, and Detection buffer are available DNase- and RNase free in the DIG Wash and Block Buffer Set (Cat. No. 1 585 762).

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[2)] 20	15-25° 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)	15-25° C, stable	Dilution of Blocking solution
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20° C)	15-25° C, stable	Adjustment of pH to 9.5
Blocking stock solution (10 × conc.)	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.	2-8° C	Preparation of Blocking solution
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 the antibody for 5 min at 10 000 rpm in the original vial prior to each use, and pipet necessary amount carefully from the surface. Dilute anti-digoxigenin-AP 1:10 000 (75 mU/ml) in Blocking solution.	2-8° C	Binding to the DIG-labeled probe

Procedure

The volumes are calculated for a membrane size of 100 cm^2. All incubations should be performed at 15-25° C with agitation

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 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 , ready-to-use** (20-30 drops) out of the dropper bottle. • **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 min at 15-25° C.
7	Squeeze out excess liquid and seal the development folder completely. 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 15-25° 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.

2.2 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 experiments.

Southern blots hybridized with DIG-labeled RNA probes can be stripped following the same procedure.

Additional equipment and reagents required

- Large tray
- Water bath
- 10 × SSC
- 10 % SDS
- 0.2 N NaOH

Procedure

This procedure describes the stripping of a membrane.

Note: Alternative stripping protocols, as mentioned in the "DIG System User´s Guide 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 x 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 2x SSC.
4	Prehybridize and hybridize with a second probe.

3 Appendix

3.1 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 of your DIG DNA or RNA labeling by comparison to the labeled control DNA or RNA.
	Wrong type of membrane	The quality of the membrane used as support for dot, Southern or northern blotting influences sensitivity and speed of detection. We recommend the nylon membrane , positively charged, from Roche Diagnostics, specially tested for chemiluminescent detection. Other types of nylon membranes like e.g. Biodyne A (Pall) are also suitable but need longer exposure times to X-ray film. Some membranes may cause strong background formation. Nitrocellulose membranes can not be used with the protocol described.
	Inefficient hybridization	Increase the concentration of DIG-labeled probe, but do not exceed 25 ng/ml for DNA probes and 100 ng/ml for RNA probes in the hybridization solution. Check hybridization and washing conditions
	Low antibody concentration	Make sure, that the recommended dilution of 1:10 000 was used.
	To short exposure time	• Increase time of exposure to X-ray. • The type of film may also influence the observed signal strength.
High background	Inefficient labeling	• Purify DNA/RNA 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	• Although 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. When using the recommended nylon membrane*, from Roche Diagnostics which is function tested with the DIG system these problems are avoided.
	Concentration of labeled probe to high	• Important: It can be necessary to decrease concentration of DIG- labeled DNA or RNA probe. Standard probe concentration for a DNA probe is 25 ng/ml for a RNA probe 100 ng/ml. The critical probe concentration limit (concerning background formation) can be determined by a mock hybridization with increasing probe concentrations using unloaded membrane • Care should be taken not to permit the membranes to dry throughout the whole procedure.
	Exposure too long	• Shorten exposure time. The signal intensity increases with time.

3.2 How to contact Roche Molecular Biochemicals

Three ways to contact us To contact Roche Molecular Biochemicals for technical assistance, please choose one of the following

IF you are using...	THEN...
the Internet	type our web-site address: http://biochem.roche.com
E-mail	please refer to the address which corresponds to your particular location (see below).
telephone	please refer to the telephone number which corresponds to your particular location(see below)

3.3 Reference

1 Bronstein, I. et al.(1991) Novel chemiluminescent adamantyl 1,2-dioxetane enzyme substrates in: Stanley P. and Kricka L. J. (eds.), Bioluminescence and Chemiluminescence, Current Status, pp 73-82, John Wiley, Chichester England

*available from Roche Molecular Biochemicals

1) CSPD is a trademark of Tropix, Inc. Bedford, MA, USA and covered by European patent application 0 497 972 and US patent 5 112 960, both assigned to Tropix Inc. USA.

2) Tween is a trademark of ICI Americas Inc., Wilmington, USA

3.4 Related products

Kits

Product	Pack Size	Cat. No
DIG Luminescent Detection Kit for Nucleic acids (with CSPD)	1 kit (50 blots)	1 363 514
DIG High Prime Labeling and Detection Starter Kit II	12 labeling reactions and 24 blots	1 585 614
DIG DNA Labeling and Detection Kit	25 labeling reactions and 50 blots	1 093 657
DIG DNA Labeling Kit	40 labeling reactions	1 175 033

Single reagents

Product	Pack Size	Cat. No.
Blocking reagent	50 g	1 096 176
Anti-DIG-AP conjugate, Fab fragments	150 U (200 µl)	1 093 274
CSPD	1 ml 2 × 1 ml 4 × 1 ml	1 655 884 1 759 035 1 759 043
DIG Easy Hyb (ready-to-use hybridization solution without formamide)	500 ml	1 603 558
DIG Easy Hyb Granules	1 set (6 × 100 ml)	1 796 895
DIG Wash and Block Buffer Set	30 blots (10 × 10 cm^2)	1 585 762
Hybridization bags	50 bags	1 666 649
Lumi-Imager F1	240 volt 120 volt	2 015 170 2 012 847
Lumi-Film, for chemiluminescent detection	100 sheets (20.3 × 24.4cm) 100 sheets (18 × 24 cm) 100 sheets (35 × 43 cm)	1 666 657 1 666 916 1 666 711
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
Tween 20	5 × 10 ml	1 332 465

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