

Applied Biosystems
47 Wiggins Avenue
Bedford, MA 01730
Phone: (800) 542-2369
or (781) 271-0045
FAX: (781) 275-8581
email: tropix@appliedbiosystems.com

CSPD[®] and CDP-*Star*[®] Substrates for Alkaline Phosphatase

Instructions for Use of Substrate Concentrate and Ready-to-Use Formulations
with or without Chemiluminescence Enhancers

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I. Introduction

CSPD[®] and CDP-*Star*[®] substrates are chemiluminescent substrates for alkaline phosphatase (AP). These reagents are intended for use in solution- or membrane-based detection of AP or AP-conjugates in applications including ELISA, microplate-based nucleic acid detection, western blotting and Southern blotting.

CSPD[®] and CDP-*Star*[®] substrates produce a luminescent signal when acted upon by AP, which dephosphorylates the substrates and yields anions that ultimately decompose, resulting in light emission. Light production resulting from chemical decomposition exhibits an initial delay followed by a persistent glow that lasts as long as free substrate is available. The glow signal may endure for hours or even days if signal intensity is low; signals with very high intensities may only last for a few hours. With CSPD[®] substrate, peak light emission is obtained in 10-20 min in solution assays, or in about four hours on a nylon membrane; CDP-*Star*[®] substrate exhibits solution kinetics similar to CSPD[®] substrate, but reaches peak light emission on a membrane in only 1-2 hours. Despite these long times to peak signal intensity, however, X-ray film exposure usually only requires 15 sec to 15 min with standard X-ray film. Both substrates provide high detection sensitivity, fast X-ray film exposure, superior band resolution, and glow light emission kinetics, enabling acquisition of multiple film exposures and use of luminometers without automatic reagent injectors. CDP-*Star*[®] substrate exhibits a brighter signal (5-10-fold) and a faster time to peak light emission on membranes, making CDP-*Star*[®] substrate the preferred choice when imaging membranes on digital signal acquisition systems.

In order for efficient light emission to occur, CSPD[®] and CDP-*Star*[®] substrates require an alkaline hydrophobic environment. Ready-to-Use substrate formulations are provided in an alkaline buffer solution, and it is recommended that substrate concentrates be diluted in an alkaline buffer. The requirement for a hydrophobic environment for light emission is fulfilled by use of a luminescence enhancer (for solution-based assays or for blotting on nitrocellulose membranes) or by use of nylon membranes. Luminescence enhancers (and nylon membranes) provide an environment that permits chemical decomposition of CSPD[®] and CDP-*Star*[®] substrates with high efficiency, resulting in fast reaction kinetics and bright chemiluminescent signals. Luminescence enhancers have been developed specifically for use in solution assays or for membrane blotting, and may be used to modulate the output wavelength of light emission, allowing optimization of signal intensity based upon the instrumentation being used for chemiluminescence detection.

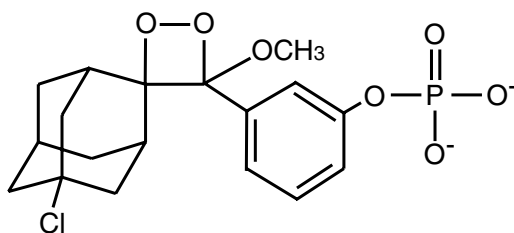
II. Chemical Information

CSPD[®] substrate CAS No. 142849-53-4 MW = 461 g/mol

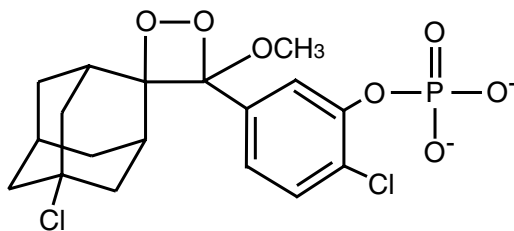
Disodium 3-(4-methoxy Spiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate

CDP-*Star*[®] substrate CAS No. 160081-62-9 MW = 496 g/mol

Disodium 2-chloro-5-(4-methoxy Spiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate



CSPD[®] Substrate



CDP-*Star*[®] Substrate

III. Substrate Formulations

Shelf-life for all substrate formulations is 1 year when stored at 4°C.

Description	Size	Cat. No.
CSPD [®] Concentrate (25 mM)	0.5 mL	CD005
	1 mL	CD010
	2.5 mL	CD025
	5 mL	CD050
	10 mL	CD100
	25 mL	CD250
	100 mL	CDX
CSPD [®] Ready-to-Use (0.25 mM)	50 mL	CD050R
	100 mL	CD100R
	250 mL	CD250R
	1 L	CD1000R
CSPD [®] Ready-to-Use (0.25 mM) with Nitro-Block [™] Enhancer	100 mL	CD100RN
CSPD [®] Ready-to-Use (0.4 mM) with Sapphire-II [™] Enhancer	25 mL	CD025RX
	100 mL	CD100RX
	1 L	CD1000RX
CSPD [®] Ready-to-Use (0.4 mM) with Emerald-II [™] Enhancer	100 mL	CD100RY
	1 L	CD1000RY
CDP-Star [®] Concentrate (12.5 mM)	1 mL	MSC010
	2 mL	MSC020
	5 mL	MSC050
	10 mL	MSC100
	20 mL	MSC200
	50 mL	MSC500
	200 mL	MSC2000
CDP-Star [®] Ready-to-Use (0.25 mM)	50 mL	MS050R
	100 mL	MS100R
	250 mL	MS250R
	1 L	MS1000R
CDP-Star [®] Ready-to-Use (0.25 mM) with Nitro-Block-II [™] Enhancer	100 mL	MS100RN2
CDP-Star [®] Ready-to-Use (0.4 mM) with Sapphire-II [™] Enhancer	100 mL	MS100RX
	1 L	MS1000RX
CDP-Star [®] Ready-to-Use (0.4 mM) with Emerald-II [™] Enhancer	100 mL	MS100RY
	1 L	MS1000RY
Related Products		
Nitro-Block [™] Enhancer	LN200	20 mL
Nitro-Block-II [™] Enhancer	LN200	20 mL
Emerald-II [™] Enhancer	LAY250	25 mL
Sapphire-II [™] Enhancer	LAX250	25 mL
Diethanolamine Buffer Concentrate	AD120	120 mL

IV. Instructions for Use

A. Selection of a Substrate / Enhancer Formulation

Applied Biosystems offers two substrates and four enhancers for use in solution- or membrane-based detection of AP. Which combination is best for your application? The following suggestions should facilitate the selection of an appropriate substrate / enhancer combination:

1. **CSPD[®] or CDP-Star[®] Substrate?** CSPD[®] and CDP-Star[®] substrate are both very sensitive for detection of AP activity, however, CDP-Star[®] substrate produces a more intense (brighter) signal than does CSPD[®] substrate in either solution or blotting assays. In addition, compared with CSPD[®] substrate in a membrane-based assay, CDP-Star[®] substrate exhibits a faster time to peak light emission. The brighter signal and faster kinetics of CDP-Star[®] substrate enable shorter X-ray film exposure times, which may be beneficial in some applications, and make CDP-Star[®] substrate ideal for digital image acquisition. For most purposes, CSPD[®] substrate is a satisfactory detection reagent. However, if maximum signal intensity is required, if shorter film exposure times are desired, or if digital imaging of membrane blots is to be performed, CDP-Star[®] substrate may be a better choice.
2. **Chemiluminescence Enhancers for Blotting Assays.** If blotting on nylon membranes (usually Southern or northern blotting), a chemiluminescence enhancer is unnecessary. For blotting on nitrocellulose membranes (usually western blotting), an enhancer is necessary. There are two enhancers for use in blotting applications, Nitro-Block[™] or Nitro-Block-II[™] enhancer. CSPD[®] substrate may be used with either, but CDP-Star[®] substrate should only be used with Nitro-Block-II[™] enhancer. No enhancer should be used with CDP-Star[®] substrate for blotting on PVDF membranes, but either Nitro-Block[™] or Nitro-Block-II[™] enhancer may be used with CSPD[®] substrate on PVDF membrane.
3. **Enhancers for Solution Assays.** A chemiluminescence enhancer is required for use with CSPD[®] and CDP-Star[®] substrate in solution-based assays (i.e., ELISA). There are two enhancers to choose from: Sapphire-II[™] and Emerald-II[™] enhancer. Sapphire-II[™] enhancer is the same basic compound as Emerald-II[™] enhancer, but Emerald-II[™] enhancer also incorporates fluorescein, which provides a more intense signal (and a slightly higher background) with a green-shifted wavelength; the benefit of the brighter “greener” signal may compensate for the increased background in some applications. Sapphire-II[™] substrate-enhancer formulations typically emit light with a λ_{\max} (wavelength of maximum light intensity) at 461 nm, while Emerald-II[™] formulations emit light with a λ_{\max} near 542 nm. The λ_{\max} of a formulation may be important for some applications, as there are some preferences for λ_{\max} with some detection instrumentation. For example, photo-multiplier tube-based detection systems often exhibit a higher sensitivity (due to the quantum efficiency of detection) for blue versus green light, thus substrate formulations containing Sapphire-II[™] enhancer may exhibit improved performance on photo-multiplier tube-based detection systems. Substrate formulations containing Emerald-II[™] enhancer may exhibit improved performance on CCD-camera-based imaging systems.

B. Dilution of Substrate and Enhancer Concentrates

Applied Biosystems provides CSPD[®] and CDP-*Star*[®] substrates at several concentrations. The recommended working concentration of Ready-to-Use formulations is based upon the biochemical and kinetic properties of AP and the substrates, and is designed to provide a linear relationship between signal intensity and enzyme concentration in the nanogram (femtomole) to femtogram (zeptomole) range. Use of substrate concentrations lower than that recommended may result in non-linear relationships, making interpretation of results much more difficult.

CSPD[®] or CDP-*Star*[®] substrate should be used at a concentration of 0.4 mM for solution-based assays or at 0.25 mM for blotting assays. Sapphire-II[™] and Emerald-II[™] enhancers should be used at a final concentration of 10% (vol/vol) in solution assays, while Nitro-Block[™] and Nitro-Block-II[™] enhancers should be used at a final concentration of 5% (vol/vol) in blotting.

CSPD[®] substrate concentrate is provided as a 25 mM solution, requiring either a 62.5-fold or 100-fold dilution, to 0.4 mM or 0.25 mM, for a solution or for a blotting assay, respectively. CDP-*Star*[®] concentrate is provided as a 12.5 mM solution, requiring a 31-fold or 50-fold dilution, for a 0.4 mM or 0.25 mM solution, for solution assay or for blotting, respectively. The following table provides an example of recommended dilutions:

	CSPD[®] Substrate		CDP-<i>Star</i>[®] Substrate	
Stock Concentration	25 mM		12.5 mM	
Assay Type	Solution	Blotting	Solution	Blotting
Working Conc.	0.4 mM	0.25 mM	0.4 mM	0.25 mM
Dilution Buffer	0.1 M Diethanolamine containing 1 mM MgCl ₂ (see Appendix)			
pH	9.5	10	9.5	8.5
Substrate Dil. Factor	62.5	100	31	50
Lumin. Enhancer	Sapphire-II [™] Emerald-II [™]	Nitro-Block [™] Nitro-Block-II [™]	Sapphire-II [™] Emerald-II [™]	Nitro-Block-II [™]
Enhancer Conc.	10% (vol/vol)	5% (vol/vol)	10% (vol/vol)	5% (vol/vol)
Example	To make 10 mL of Working Solution			
Vol. Dil. Buffer	8.84 mL	9.4 mL	8.68 mL	9.3 mL
Vol. Substrate Conc.	0.16 mL	0.1 mL	0.32 mL	0.2 mL
Vol. Enhancer	1 mL	0.5 mL	1 mL	0.5 mL

C. Procedures for Solution Assays (Immunoassay or ELISA)

There are a wide variety of possible formats for immunoassay applications, including both competitive and sandwich immunoassay formats. It is beyond the scope of this document to describe and contrast all the variations on immunoassay procedures, so only a direct sandwich immunoassay, which is the most sensitive format for detecting soluble antigen, will be described. This format is recommended for antigens for which two distinct antibodies are available. The protocol for the ELISA-Light™ Immunoassay System from Applied Biosystems provides more complete immunoassay instructions.

1. Coat plate with capture antibody, then wash coated plate 3X with Wash Buffer.
2. Incubate wells with Blocking Buffer, then wash blocked plate 3X with Wash Buffer.
3. Dilute antigen samples in Blocking Buffer and add 100 µL/well. Incubate 1 hr with shaking. Wash wells 3X with Wash Buffer.
4. Dilute detector antibody-AP conjugate in Blocking Buffer and add 100 µL/well. Incubate for at least 1 hr with shaking. The optimal conjugate dilution must be empirically determined.
5. Wash wells 4X with Wash Buffer then twice with 1X Assay Buffer.
6. Add 100 µL/well Substrate/Enhancer Solution. Incubate for 5-10 min and then measure at 5 min intervals until light emission has peaked (usually 20-30 min after substrate addition at room temperature).

D. Procedures for Blotting Assays

The example provided below is for western blotting on a nitrocellulose membrane. It is beyond the scope of this document to explore the possible variations on this procedure for blotting on different membrane types, and for western, northern or Southern blotting. The protocols for the Southern-Light™ & Southern-Star™ Nucleic Acid Detection Systems or Western-Light™ & Western-Star™ Immunodetection Systems from Applied Biosystems provides more complete instructions for these applications.

1. Following transfer, rinse blot briefly with PBS or TBS, and then incubate in Blocking Buffer (at least 10 mL) for 30-60 min (see Note 1).
2. Dilute primary antibody in Blocking Buffer (5-10 mL). Incubate with blot 30-60 min.
3. For nitrocellulose and nylon, wash at least 2 x 5 min in Wash Buffer (for PVDF, use Blocking Buffer). Use at least 20 mL for all washes.
4. Dilute secondary antibody-AP conjugate 1:5,000 in Blocking Buffer (5 mL). Incubate with blot for 30-60 min.
5. Wash 3 x 5 min as in Step 3, then rinse 2 x 2 min with 1X Assay Buffer.
6. Drain blots by touching a corner on a paper towel, then place on plastic wrap on a flat surface (do not let blots dry).
7. Pipette a thin layer of substrate solution (3 mL) onto the blot and incubate 5 min (Note 2).
8. Drain excess substrate solution and place blot in Development Folder (after removing anti-static sheet) or wrap in plastic. Smooth out bubbles or wrinkles.
9. Blots may be imaged by placing them in contact with standard X-ray film. Initial exposures of 5 to 30 min are recommended. Exposure times with PVDF or nylon membranes are usually shorter than with nitrocellulose.

Notes

1. The indicated volumes are for a single blot (100 cm²) and should be adjusted for larger or smaller blots. All steps are performed at room temperature with agitation. See the Appendix for solution recipes. PBS or TBS buffers can be used with either nitrocellulose or PVDF membranes. With nylon, background is slightly higher with TBS than with PBS.
2. Use CSPD® substrate with Nitro-Block™ enhancer for nitrocellulose and PVDF, or CDP-Star® substrate with Nitro-Block-II™ enhancer for nitrocellulose membranes (150 µL enhancer with 3 mL of substrate solution for 100 cm² of membrane). Do not use enhancer on PVDF with CDP-Star® substrate or on nylon with either substrate.

APPENDIX

Solution Preparation

All solutions should be made with deionized H₂O. 10X PBS / TBS should be kept sterile at room temperature. Buffers should be prepared fresh daily to prevent bacterial contamination. Blocking buffer may be stored at 4°C if 0.02% NaN₃ is added.

Substrate Dilution Buffer

Deionized H ₂ O	80 mL	
0.1 M Diethanolamine pH as desired	1.1 mL (1.06 g)	Adding MgCl ₂ before adjusting pH may cause the MgCl ₂ to precipitate
1 mM MgCl ₂	0.02 g	

Add deionized H₂O to 100 mL

Diethanolamine is available from Applied Biosystems, Cat. No. is AD120 for 120 mL. Diethanolamine has a formula weight of 105.14 g/mol and its density = 1.097 g/mL.

10X PBS

0.58 M Na ₂ HPO ₄	82.3 g
0.17 M NaH ₂ PO ₄ -H ₂ O	23.5 g
0.68 M NaCl	40.0 g

Add deionized H₂O to 1000 mL

10X Tris Buffered Saline (TBS)

0.2M Tris base	24.2 g
1.37 M NaCl	80 g

Adjust pH to 7.6 with conc. HCl
Add deionized H₂O to 1000 mL

Alternative PBS and TBS recipes may be used.

Wash Buffer (PBS-T or TBS-T)

1X PBS or 1X TBS	10 mL, 10X
0.1% Tween [®] -20 Detergent	0.1 mL

Add deionized H₂O to 100 mL

Blocking Buffer *

1X PBS or TBS	3 mL, 10X
0.2% I-Block [™] Reagent	0.06 g
0.1% Tween [®] -20 Detergent	30 µL

* Add 3 mL of 10X PBS or TBS to 27 mL of H₂O. Microwave for 30 sec, then slowly add I-Block[™] reagent while stirring on a hot plate. DO NOT BOIL. Add Tween[®]-20 detergent after solution has cooled. The solution will remain slightly opaque, but particles should be dissolved. Cool to room temperature before use. I-Block[™] solutions of 0.2% are for use with nitrocellulose and PVDF membranes. For positively charged nylon, use 3% I-Block[™] reagent (3 g/100 mL) in 1X PBS with 0.1% Tween[®]-20 detergent.

I-Block[™] Blocking Reagent is available from Applied Biosystems, Cat. No. is AI300 for 30 g.

10X Assay Buffer

200 mM Tris (pH 9.8)
10 mM MgCl₂
Dilute 1:10 with deionized H₂O for use

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