For life science research only.
Not for use in diagnostic procedures.

BM Chemiluminescence ELISA Substrate (POD)

Version: 07
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Chemiluminescent substrate system for highly sensitive detection of peroxidase in ELISA

Cat. No. 11 582 950 001	250 ml 2,500 wells or 1,000 tubes

Store the product at +2 to +8°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	brown	BM Chemiluminescence ELISA Substrate (POD), Substrate reagent A	▪ Buffered solution containing luminol and 4-iodophenol. ▪ Filtered through 0.2 µm pore-size membrane, without addition of preservatives.	1 bottle, 250 ml
2	white	BM Chemiluminescence ELISA Substrate (POD), Starting reagent B	Buffered solution containing a stabilized form of H_2O_2.	1 bottle, 5 ml

1.2. Storage and Stability

Storage Conditions (Product)

When stored at +2 to +8°C, the product is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	brown	Substrate reagent A	Store at +2 to +8°C. ⚠ ***Keep protected from light.*** ⚠ ***Avoid contamination when handling. Commonly used biocides, such as NaN_3 or Kathon CG at active concentrations negatively influence the sensitivity of the reagent.*** When stored at −15 to −25°C, equilibrate the reagent to +15 to +25°C before use.
2	white	Starting reagent B	Store at +2 to +8°C. ⚠ ***Keep protected from light.***

Storage Conditions (Working Solution)

The premixed solution is stable for 1 week when stored at +2 to +8°C.

1.3. Additional Equipment and Reagent required

For quantification of luminescence

- Luminometer with photomultiplier technology
  Commercially available camera luminometers can also be used.
- Instruments for tubes (12 mm vials) and 96-well microplates
  The BM Chemiluminescence ELISA Substrate (POD) has been evaluated with different immunoassays on the LB 96 P microplate reader and the LB 953 tube chemiluminescence analyzer from EG&G Berthold.
- White or black microplates for chemiluminescent detection

1.4. Application

The BM Chemiluminescence ELISA Substrate provides a substrate solution for peroxidase-based (POD, HRP) secondary detection systems by highly sensitive enhanced chemiluminescence. This reagent has been optimized especially for ELISA applications to be run on a microplate chemiluminescence reader (96-well format) or tube-format luminometer.

2. How to Use this Product

2.1. Before you Begin

General Considerations

Sensitivity

Sensitivity-limiting factors for enzyme immunoassays include the:
- Affinity of the specific interacting components, such as antigen/antibody, biotin/streptavidin, receptor/ligand, etc.
- Sensitivity of the secondary detection system (marker enzyme/substrate/detection principle).
- Fractional contribution of nonspecific binding.

Comparing the sensitivity in an artificial system using purified peroxidase (serial dilutions), luminol/iodophenol is 10 to 100 times more sensitive (Fig. 1) compared with commonly used chromogenic substrates, for example, ABTS and TMB. This maximal sensitivity can only be reached when the affinities of the interacting components are not limiting for the assay and the total nonspecific binding (background) is below 0.1% of total binding.

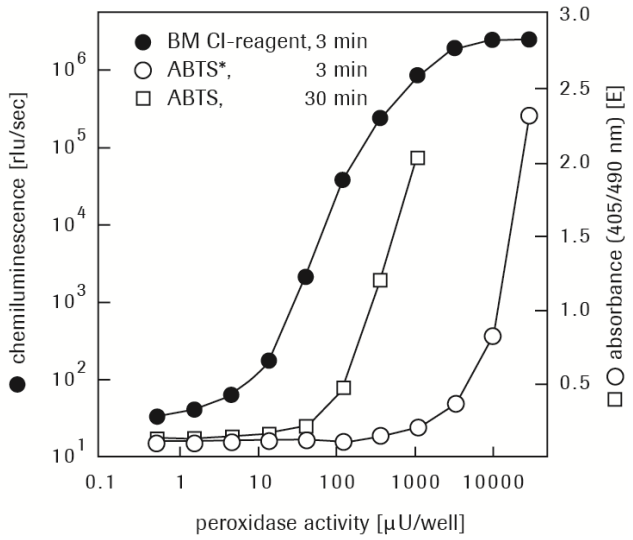

Fig. 1: Sensitivity of BM Chemiluminescence ELISA Substrate. Comparison of photometric detection using ABTS with chemiluminescence detection.

Signal kinetics

The rate of signal generation in an immunoassay has to be directly proportional to the amount of marker enzyme bound to the solid phase. Using chemiluminescence substrates, the velocity of the light reaction (half-life of the light emitting intermediate) largely influences the kinetics of signal generation. Using a slowly decaying substrate results in a complex kinetic with a non-linear relationship between signal and enzyme activity. Due to the very rapid decay of oxidized luminol, the peroxidase reaction reaches its steady state (= constant and proportional signal) within 2 to 3 minutes (Fig. 2).

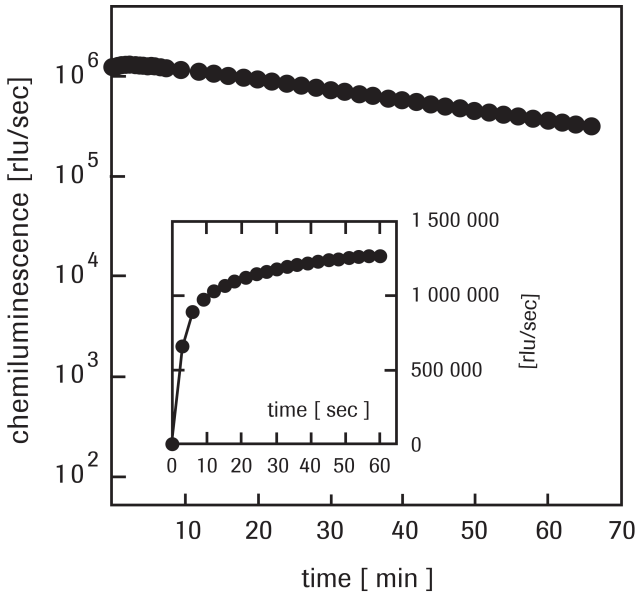

Fig. 2: Kinetics of signal development.

Dynamic range

The quantification of chemiluminescence is limited by the resolution of the primary detection unit, the photomultiplier. Commercially available luminometers have a dynamic range between 10 and 2 × 10^6 relative light units/second (rlu/s). Depending on the background of a given immunoassay, the linear range can cover more than four orders of magnitude.

Optimization of ELISA for chemiluminescence

Initially, use the recommended conjugate concentration or as optimized for the individual assay. If the enzyme conjugate contributes to nonspecific binding, lower the concentration to 1:10.

Observation	Possible Cause	Recommendation
High background signal.	Nonspecific binding	Use the Blocking Reagent for ELISA*.
		Add additional components to washing, incubation, and conjugate buffer. The following additives may be used: ▪ Salt: 0.5 to 1.0 M NaCl ▪ Complexing agent: 1 to 5 mM EDTA ▪ Detergent: 0.05 to 0.1%Tween 20* ▪ Protein: 0.1 to 1% BSA, serum, casein, milk powder, IgG from non-cross-reacting species, hydrophobized proteins.
		Lower the concentrations of the specific interacting components.
	Washing conditions	Prolonged intervals between individual washes (at least 3 repeated washes) favor dissociation from nonspecific binding sites.
Weak signal present.	Low affinity antibodies.	Replace low affinity antibodies by high affinity systems if possible. For example, by conjugating a component with biotin or digoxigenin. The haptenized molecules can then be bound with high affinity using streptavidin or anti-digoxigenin antibodies.

In a colorimetric assay, 1% nonspecific binding accounts for approximately 0.02 OD at a maximal signal of 2.0 OD. This effect is within the range of experimental error. In a chemiluminescent assay, 1% nonspecific binding accounts for 10,000 rlu at a total signal of 10^6 rlu. In this case, potentially two orders of magnitude in the high sensitivity range are lost. Using Anti-Digoxigenin-POD, Fab fragments results routinely in nonspecific binding in the range of 100 to 1,000 rlu, varying between different ELISAs.*

Working Solution

The BM Chemiluminescence ELISA Substrate (POD) is supplied as a set of two stable solutions. Depending on the scale of the assay, prepare the appropriate amount of substrate solution as shown in the following table:

Solution	Preparation	Storage and Stability	For use in...
Premixed	▪ Mix Starting reagent B (Bottle 2) 1:100 with Substrate reagent A (Bottle 1). ▪ Stir the mixture for at least 15 minutes at +15 to +25°C to equilibrate the components. *Premixed working solution stored at +2 to +8°C must be prewarmed to +15 to +25°C to avoid temperature effects during measurement.* ▪ For one 96-well microplate, dilute 100 µl Starting reagent B (Bottle 2) in 10 ml Substrate reagent A (Bottle 1).	Store the premixed solution at +2 to +8°C for one week. ⚠ ***Prepare 15 minutes before use.***	Labeling

2.2. Protocols

The colorimetric substrate in most ELISA protocols can easily be replaced by the BM Chemiluminescence ELISA Substrate (POD) without changing any parameters. Standard ELISA conditions are given in the table below.

Using an automatic luminometer equipped with injectors, addition of reagent and measurement will be performed by the instrument in a synchronized manner.

Procedure	Buffer	Additives (optional)	Volume [µl]	Incubation Time	Incubation Temperature [+°C]
Coating of microplates	$NaHCO_3/Na_2CO_3$, pH 9.6, or PBS or TBS, pH 7.0 to 8.0	–	50 – 250	0.5 – 18 hours	4 – 35
Washing steps	PBS, pH 7.2 to 8.0 or TBS, pH 7.2 to 8.0	0.1 – 1.0% BSA 0.05 – 0.1% Tween 20	300 (3 times)	Leave buffer 0.5 – 5 minutes in the wells between each washing step.	20 – 35
Blocking of nonspecific binding sites	Blocking Reagent for ELISA*; PBS containing 0.5 to 2% BSA, pH 7.2 to 8.0, or TBS/BSA pH 7.2 to 8.0	Casein, gelatine serum, dry milk, hydrophobized proteins, 0.05% Tween 20	250	10 – 30 minutes	20 – 35
▪ Primary/ secondary antibody ▪ Standard/ sample ▪ Peroxidase conjugate	PBS, pH 7.2 to 8.0 or TBS, pH 7.2 to 8.0	Casein, gelatine serum, casein, dry milk, hydrophobized proteins, 0.05% Tween 20, EGTA, salt	50 – 250	1 – 4 hours or overnight at +2 to +8°C.	20 – 35

2. How to Use this Product

1. Follow your optimized ELISA protocol as optimized for colorimetric detection.

2. After incubation of the POD-labeled antibody (or streptavidin-POD), discard the solution and wash three times, leaving the buffer in the wells for 0.5 to 5 minutes between each individual wash.
   For this step, an automated washer has been shown to be superior to manual washing.

3. Discard the washing solution and tap the microplate on a lint-free, dry, absorbent cloth.

4. Add 100 µl BM Chemiluminescence Substrate working solution to each well with a multichannel pipette.
   Start all wells within a minimum period of time.

5. Start measurement after a delay of 3 minutes.
   – To avoid “burning out” of substrate, quantify samples within 30 minutes after adding the reagent.

3. Troubleshooting

Observation	Possible cause	Recommendation
Weak or no signal.	Low performance of the luminometer.	Check instrument settings.
	Secondary antibody specificity/ performance too low.	Check POD activity of the secondary antibody.
	Wrong conjugate buffer used.	Check conjugate buffer for incompatible components, for example, NaN_3, SH-reagents.
	ELISA protocol is not optimized.	Check protocol (incubation times/ temperatures, buffer conditions, etc.) and concentrations of primary antibody or antigen.
	Substrate reagent A and Starting reagent B activity is too low.	Check chemiluminescence reagent for storage conditions and biological contamination. *Use freshly prepared reagent.*
	Positive control is degraded.	Check integrity of positive control.
High background signal.	Washing step too short.	Prolong washing steps (number or time between steps).
	Concentration and ratio primary/ secondary antibody not adapted.	Modulate concentrations for primary/ secondary antibody.
	Nonspecific binding.	Try different additives with the washing/incubation buffers to block nonspecific interactions.

4. Additional Information on this Product

4.1. Test Principle

The signal (photons) generated in enzyme-catalyzed light emitting reactions (luminescence) is identical with the signal generated during radioactive decay in scintillation counting. Therefore, chemiluminescence detection has features comparable to radioactive methods (Fig. 3).

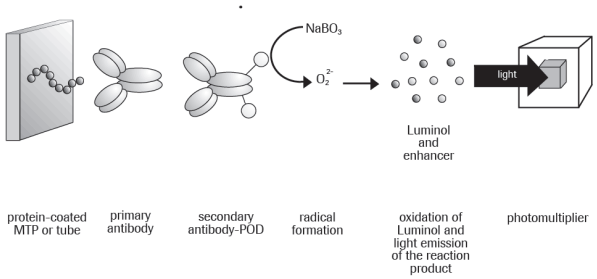

Fig. 3: Enzyme-induced generation of chemiluminescence and detection by photomultiplier/counter unit. Horseradish peroxidase (POD) in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of diacylhydrazides such as luminol (Fig. 4). A reaction product in an excited state is thus formed, which then decays to the ground state by emitting light. Strong enhancement of the light emission is achieved by the agent 4-iodophenol (contained in Substrate reagent A together with luminol), which acts as a radical transmitter between the formed oxygen radical and luminol. Hydrogen peroxide (H_2O_2) is provided in stable form in Starting reagent B.

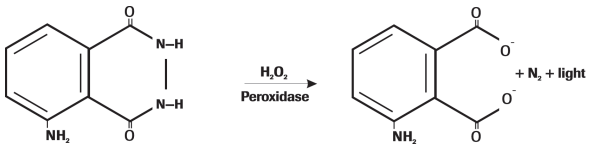

Fig. 4: Reaction scheme

The sensitivity of enzyme-linked immunosorbent assays depends often on the detection limit for the colorimetric substrates used. In addition, the dynamic range of a colorimetric assay is restricted to a maximum of two orders of magnitude due to the physico-chemical limitations of absorbance measurement. The chemiluminescence technology combines the convenience of tube or microplate-based immunoassays with the advantages of isotopic assays:

- Rapid and constant signal
- Large dynamic range
- Improved sensitivity

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

Layout changes.
Editorial changes.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Blocking Reagent	27 g, for one liter blocking solution, *Not available in US*	11 112 589 001
Tween 20	50 ml, 5 x 10 ml	11 332 465 001
Anti-Digoxigenin-POD, Fab fragments	150 U	11 207 733 910
Bovine Serum Albumin Fraction V	50 g	10 735 078 001
	100 g, *Not available in US*	10 735 086 001
	500 g, *Not available in US*	10 735 094 001
	1 kg, *Not available in US*	10 735 108 001

5.4. Trademarks

ABTS is a trademark of Roche.
All other 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:
List of biochemical reagent products.

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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