**BM Chemiluminescence ELISA Substrate (POD)**

For peroxidase-based secondary detection systems

**Cat. No. 11 582 950 001**

250 ml, for 2500 wells or 1000 tubes

1. **Introduction**

The sensitivity of enzyme-linked immuno sorbent 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 (Law of Lambert & Beer). With chemiluminescence technology, a non-radioactive method has become available which combines the convenience of tube or microtiter plate-based immunoassays with the advantages of isotopic assays:

- improved sensitivity
- large dynamic range
- rapid and constant signal

The BM Chemiluminescence ELISA Substrate provides a substrate solution for peroxidase-based (POD, HRP) secondary detection systems by highly sensitive enhanced chemiluminescence (1). This reagent has been optimized especially for ELISA applications to be run on a micro plate chemiluminescence reader (96-well format) or tube-format luminometer.

2. **Description of the method**

**Principle**

The signal (photons) generated in enzyme-catalyzed light emitting reactions (luminescence) is identical with the signal generated during radioactive decay in scintillation counting. Thus, chemiluminescence detection has features comparable to radioactive methods (fig.1).

Horseradish peroxidase (POD, HRP) in the presence of hydrogen peroxide (H$_2$O$_2$) catalyzes the oxidation of diacylhydrazides like luminol (see fig.2). A reaction product in an excited state is thus formed, which then decays to the ground state by emitting light (2). 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 (3). Hydrogen peroxide H$_2$O$_2$ is provided in stable form in starting reagent B.

**Fig. 1**: Enzyme-induced generation of chemiluminescence and detection by photomultiplier/counter unit.

**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 oxidised luminol, the peroxidase reaction reaches its steady state (= constant and proportional signal) within 2 - 3 minutes (fig. 3).

**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 x10$^6$ relative light units/second (rlu/s). Therefore, depending on the background of a given immunoassay, the linear range can cover more than four orders of magnitude.

**Fig. 2**: Reaction scheme

**Fig. 3**: Kinetics of signal development.
Sensitivity

The sensitivity-limiting factors for enzyme immunoassays are:

a) the affinity of the specific interacting components (e.g., antigen/antibody, biotin/streptavidin, receptor/ligand, etc.),
b) the sensitivity of the secondary detection system (marker enzyme/substrate/detection principle)
c) the fractional contribution of non-specific binding.

Comparing the sensitivity in an artificial system using purified peroxidase (serial dilutions), luminal/iodophenol is 10 - 100 times more sensitive (fig. 4) compared with commonly used chromogenic substrates (e.g. ABTS, TMB). However, this maximal sensitivity of the method can only be reached if the affinities of the interacting components are not limiting for the assay and the total non-specific binding (background) is well below 0.1% of total binding.

Stability and storage

The set contains 250 ml buffered solution containing luminol and 4-iodophenol.

Equipment

For quantification of luminescence, a luminometer with photo-multiplier technology is required (also commercially available camera luminometer may be used). Instruments for tubes (12 mm vials) and 96-well microtiter plates are available from various suppliers (2).

The BM Chemiluminescence ELISA Substrate has been evaluated with different immunoassays on the LB 96 P microplate reader and the LB 953 tube chemiluminescence analyzer from EG&G Berthold.

For chemiluminescent detection the use of white or black microplates is recommended.

Note: Roche Applied Science offers a wide selection of Streptavidin-coated Microplates (StrepatWell). Please refer to our Biochemicals Catalog.

3. Product description

The set contains

Substrate reagent A
250 ml buffered solution containing luminol and 4-iodophenol.

Starting reagent B
5 ml buffered solution containing a stabilized form of H2O2.

Preparation of working solution

The BM Chemiluminescence ELISA Substrate is supplied as a set of two stable solutions. Depending on the scale of the assay, the appropriate amount of substrate solution has to be prepared 15 min before use:

- Add to 100 parts of solution A one part of solution B.
- Stir the mixture for at least 15 min at +15 to +25°C to equilibrate the components.

Premixed working solution stored at +2 to +8°C should be warmed up to +15 to +25°C to avoid temperature effects during measurement.

4. Application

According to our experience, the colorimetric substrate in most ELISA protocols can easily be replaced by BM Chemiluminescence ELISA Substrate without changing any further parameters. Standard ELISA conditions are given in the table below.

- Follow your optimized ELISA protocol as optimized for colorimetric detection.
- 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 - 5 min between each individual wash. For this step, an automated washer has been shown to be superior to manual washing.
- Discard the wash solution and tap the MTP on a lint-free, dry, absorbant cloth. Add 100 μl BM Chemiluminescence Substrate, working solution, to each well with a multi-channel pipette (note: all wells should be started within a minimum period of time). Start measurement after a delay of 3 min. To avoid "burning out" of substrate, samples should be quantitated within 30 min after adding the reagent.

Note:

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

- coating of microtiter plates
  - NaHCO3 / Na2CO3
  - pH 9.6
  - (alternatively PBS or TBS pH 7 - 8.0)

- washing steps
  - PBS, pH 7 - 8.0 (alternatively TBS pH 7 - 8.0)
  - BSA (0.1 - 1.0%)
  - Tween 20* (0.05 - 0.1%)
  - leave buffer in the wells for 0.5 - 5 min in between each wash step

- blocking of non-specific binding sites
  - Blocking Reagent for ELISA*: PBS containing 0.5 to 2% BSA, pH 7 - 8.0, (alternatively TBS/BSA pH 7 - 8.0)
  - casein, gelatin serum, casein, dry milk, hydrophobized proteins, Tween 20 (0.05%)
  - 250 μl

- primary/secondary antibody
  - PBS, pH 7 - 8.0 (alternatively: TBS, pH 7 - 8.0)
  - casein, gelatin serum, casein, dry milk, hydrophobized proteins, Tween 20 (0.05%), EGTA, salt

- peroxidase conjugate
  - 50 - 250 μl

Tab.: Standard ELISA conditions.
5. Optimizing ELISA protocols for chemiluminescence detection

POD-conjugate concentration

At the first attempt, the conjugate concentration should be used as recommended or as optimized for the individual assay. If the enzyme conjugate contributes to non-specific binding, its concentration may be lowered to 1:10.

Non-specific binding

Optimizing non-specific binding is a prerequisite for highly sensitive detection. There are different non-specific interactions that may contribute to elevated background levels (ionic interactions, hydrophobic interactions, cross-reactivity). To reduce background:

- use our Blocking Reagent for ELISA
- add additional components to washing, incubation, and conjugate buffer
- lower the concentrations of the specific interacting components.

The following additives may be used:

| Salt: 0.5 - 1.0 M NaCl |
| Complexing agent: 1 - 5 mM EDTA |
| Detergent: Tween 20, 0.05 - 0.1% |
| Protein: BSA, 0.1 - 1%, serum, casein, milk powder, IgG from non cross-reacting species, hydrophobised proteins |

Note

In a colorimetric assay, 1% non-specific binding accounts for approx. 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% non-specific 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 non-specific binding in the range of 100 to 1 000 rlu, varying between different ELISAs.

Low affinity antibodies

Replace low affinity antibodies by high affinity systems if possible. This, for example, can be done by conjugating a component with biotin or digoxigenin. The haptenized molecules can then be bound with high affinity using streptavidin or anti-digoxigenin antibodies.

Washing conditions

Most interactions which contribute to non-specific binding are of low/intermediate affinity and therefore reversible in character. Prolonged intervals between individual washes (we recommend at least 3 repeated washes) favor dissociation from non-specific binding sites.

6. Troubleshooting

1. Weak or no signal

- Check instrument settings
- Check POD-activity of the secondary antibody.
- Check conjugate buffer for incompatible components (e.g., NaN3, SH-reagents)
- Check protocol (incubation times/temperatures, buffer conditions, etc.) and concentrations of primary antibody or antigen.
- Check chemiluminescence reagent for storage conditions and biological contamination. Use freshly prepared reagent.
- Check integrity of positive control.

2. High background signal

- Prolong washing steps (number or time between steps).
- Modulate concentrations for primary/secondary antibody.
- Try different additives with the washing/incubation buffers to block non-specific interactions.

7. References


* available from Roche Applied Science

8. Related Products

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<thead>
<tr>
<th>Product</th>
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<tr>
<td>Anti-Digoxigenin-POD Fab fragments from sheep</td>
<td>150 U</td>
<td>11 207 733 910</td>
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<tr>
<td>BM Chemiluminescent ELISA Substrate (AP)</td>
<td>150 ml</td>
<td>11 759 779 001</td>
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<tr>
<td>Blocking Reagent for ELISA</td>
<td>27 g (for 1 l)</td>
<td>11 112 589 001</td>
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<tr>
<td>Conjugate Buffer, universal</td>
<td>100 ml</td>
<td>11 684 825 001</td>
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Changes to Previous Versions

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