Ph. Eur. Reference Standard – LEAFLET

Erysipelas ELISA Coating Antigen BRP batch 1

Erysipelas ELISA coating antigen BRP batch 1 consists of 0.5 ml (0.367 mg protein) of a freeze-dried *Erysipelothrix rhusiopathiae* preparation per vial.

It is intended for use in the serology based ELISA potency assay of inactivated erysipelas vaccines for veterinary use as mentioned in the Ph. Eur. monograph *erysipelas vaccine (inactivated) for veterinary use (0064)*. A standard operating procedure (SOP) for the assay is annexed. Aliquots should be diluted in phosphate buffered saline before use. Individual laboratories should validate that antigen dilutions used provide OD values in the linear range for their own reference sera. Recommended dilution for the coating antigen before use is in the range of 1:200 to 1:800.

**STORAGE**

Keep vials unopened at -20 °C.
The reconstituted material should be aliquoted as described below and stored at -20 °C.

**USE**

- Allow the vial and content to reach room temperature.
- Tap vial gently to collect material at the bottom.
- Using an appropriate syringe reconstitute the reference preparation by injecting 0.5 ml of distilled water
- Aliquots should be dispensed by pipette in to suitable containers and stored at -20°C until use (up to 6 months).

**CAUTION**

Erysipelas ELISA coating antigen Ph. Eur. BRP batch 1 is not appropriate for administration to humans and/or to animals. This preparation must be handled according to the appropriate QA system for biological testing laboratories. Apply state-of-the-art safety precautions. Please refer to the corresponding safety data sheet, which...
can be downloaded from the internet web site of the EDQM (http://www.edqm.eu) or is delivered upon request.

LITERATURE

Standard Operating Procedure

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5. STATISTICAL ANALYSIS
1. SUMMARY OF PROCEDURE

Separate groups of mice are immunised with erysipelas vaccine (inactivated) test material. Three weeks after immunisation they are bled. Sera from each group are pooled and the quantity of induced antibodies in each pool is determined by enzyme linked immunosorbent assay (ELISA) using the erysipelas ELISA coating antigen Ph. Eur. BRP Nº1. Relative potency is determined against a reference sera prepared using a standard reference vaccine. A mouse serum free of antibodies against *E. rhusiopathia* is also included on each plate as a negative control.

The following procedure was used in the international collaborative study to establish the erysipelas ELISA coating antigen Ph. Eur. BRP Nº1 and is provided as a guide. It is recommended that each user should perform an in house validation study to demonstrate the suitability of the ELISA for their specific products and to optimise the protocol as required.

2. IMMUNISATION OF LABORATORY ANIMALS

It is recommended to use female NMRI mice with a body weight of 17-20 g at the time of immunisation. Mice should receive one subcutaneous injection of 1/10 the pig dose in an inoculation volume of 0.2 mL. The group size recommended for each vaccine is 10.

Reference serum is prepared by injecting mice subcutaneously with a suitable amount (e.g. one tenth of a pig dose) of a vaccine which has been shown efficacious in pigs according to the procedure outlined in the Ph. Eur. Monograph (0064).

Negative serum is prepared using pooled serum from a group of non-immunised mice. If possible it should be produced in a large volume, aliquoted and freeze-dried for future use.

3. BLEEDING AND SERUM PREPARATION

Three weeks after immunisation the animals are bled by cardiac puncture under anaesthesia.

3.1. Material
- Individual mouse blood samples in micro-vials with serum gel coagulation activator (Sarstedt, Nr. 41.1500.005)
- Cryovials
- Eppendorf centrifuge (6.000 x g)
- Pipette (500 µl)

3.2. Method

Blood samples of individual mice are kept at room temperature for at least 30 minutes and then centrifuged for 2 minutes at 6.000 x g to separate blood clots. The individual sera of one treatment group is pooled.

Note:
- An identical quantity of each individual serum should be used for pooling
- If possible, the minimal quantity of 0.2 ml per animal should be used
- The serum samples are labelled and kept at -20°C.
Reference serum and negative serum may be aliquoted and kept at -20°C for future use.

4. ANALYSIS OF SERUM ANTIBODY CONTENT BY ELISA

Summary

Mutiwell plates are coated with the erysipelas ELISA coating antigen Ph. Eur. BRP No.1 preparation. Test sera are then incubated on the plate (a dilution series of each sera is used). Each plate should also include reference sera and negative sera. The reaction is visualised by goat anti-mouse IgG conjugate with tetramethyl-benzadine as substrate. Serum potency is estimated by comparing curves of test and reference serum.

4.1 Instrumentation

- Multiwell Platewasher (Platewasher 96 PW, SLT Labinstruments GmbH)
- Multiwell plate thermostaker (Thermostat, SLT Labinstruments Deutschland GmbH) or incubator Brutschrank
- Multiwell plate reader (filter 450-620 nm) (Rainbow-Reader, SLT Labinstruments Deutschland GmbH Software:EasyFit)
- Magnetic stirrer with hotplate
- Precision balance

4.2 Material

- Eppendorf pipettes
- Multichannel pipette (12-channels, 50 μL-300 μL)
- Reagent reservoir for multichannel pipette
- Graduated cylinders
- Serological pipettes
- Assorted reaction tubes
- Shake flasks
- Multiwell plates (Immuno-Platte, No. 655001, F-Form, medium binding capacity, Greiner GmbH)
- Sealing film for multiwell plates (SLT Labinstruments Deutschland GmbH)

4.3 Reagents

4.3.1 Erysipelas ELISA coating antigen Lyophilate stored at -20 °C (Lyophilate resuspended in 0,5 ml distilled water and aliquoted –refer to note for use), protein content 0,367 mg/0,5 ml
Only a part of the resuspended lyophilate is required per plate. The remaining resuspended antigen should be aliquoted and stored at -20 °C for further use.

4.3.1.2 **Reference serum**
Per plate 40 µl of the sera is needed (for determination in triplicate per dilution). The remaining resuspended reference serum should be aliquoted and stored at -20 °C for further use.

4.3.1.3 **Negative control serum**
Per plate 20 µl of the sera is needed (for determinations in triplicate) The remaining resuspended negative control serum should be aliquoted and stored at -20°C for further use.

4.3.1.4 **Test serum** (-20°C). The maximal storage time of test sera is 2 years following bleeding.
Per assay and serum: 40 µl (for 3 replicates per plate).
Note: Serum should be aliquoted to avoid repeat freeze-thaw cycles of the material.

4.3.1.5 **Conjugate** (Peroxidase-labelled goat-anti-mouse IgG (H and L) (Fa. Dianova)), 1:100 diluted in Gelatine and Glycerin, 1 ml Aliquot

4.3.2 **Chemicals and other substances**

- *Aqua bidest., sterile, store at room temperature*
- Ethanol highest purity, non-sterile, store at room temperature
- Skim milk powder (Skim Milk, Difco Nr.:0032-17-3), store at room temperature
- Tween 20 (Sigma, Nr.:P-1379), store at room temperature
- Sodium acetate anhydrous Suprapur (Merck 106264), store at room temperature
- Hydrochloric acid 1N (Merck 1.09057.1000), store at room temperature
- Hydrochloric acid 32% (Merck 1.00319.1000), store at room temperature
- 3,3’,5,5’-Tetramethyl-Benzidine [TMB] (Sigma, Nr.:T-2885), store at 4°C
- H₂O₂ 30% (Merck 822287), store at 4°C
- H₂SO₄ 2,5 mol/l (Merck 1.09912.0001), store at room temperature
- Glycerol 87%, M.W.92.09 (Pharmacia Biotech, Nr.:17-1325-01), store at room temperature
- Gelatine highest purity (SERVA, Nr.:22151), store at room temperature

4.3.3 **Buffers and solutions**
4.3.3.1 Phosphate buffered saline (PBS) without Ca and Mg

\[ \text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O} \text{ (di-sodium hydrogen phosphate)} \quad 1,15 \text{ g} \]
\[ \text{KH}_2\text{PO}_4 \times 2\text{H}_2\text{O} \text{ (potassium hydrogen phosphate)} \quad 0,20 \text{ g} \]
\[ \text{KCl} \text{ (Potassium chloride)} \quad 0,20 \text{ g} \]
\[ \text{NaCl} \text{ (Sodium chloride)} \quad 8,00 \text{ g} \]
Aqua double distilled \quad \text{ad 1 L}

Adjust pH to within 7,0 - 7,2.

This solution is stable for one year maximally at room temperature. Approximately \(300 \text{ ml required per plate.}\)

4.3.3.2 Wash buffer (PBS-Tween 20, 0,1%)

Pre-dilute 1:5 (to improve pipetting)

Tween 20 \quad 30 \text{ ml}
PBS, non-sterile (bulk) \quad 120 \text{ ml}

This solution is \textit{stable for 1 year at 4°C.}\n
Approximately 1 ml required per plate.

\textit{Working solution 0,1%}

Pre-dilution 1:5 \quad 5 \text{ ml}
PBS, non-sterile (bulk) \quad 995 \text{ ml}

This solution is \textit{stable for 4 weeks at 4°C.}\n
160 ml required per plate.

4.3.3.3 Dilution buffer- Blocking solution (PBSM)

(Skim milk powder 5% dissolved in PBS)

Skim milk powder \quad 12,5 \text{ g}
PBS, sterile \quad \text{ad 250 ml}

This solution is \textit{stable for max. 3 days at 4°C.}\n
40 ml is required per plate.
4.3.3.4 Conjugate solution

a) Peroxidase labelled goat-anti-mouse IgG (H+L) (Dianova:115-035-146) dissolve in double-distilled water according to instructions from manufacturer.

b) Gelatine solution (0,25%)
   Gelatine 0,25 g
   PBS, sterile ad 100 ml
   Dissolve at 60°C whilst stirring

c) Pre-dilution (1:50)
   Dissolved conjugate 1 ml
   Gelatine solution, 0,25% 49 ml

d) Working solution (1:2)
   Pre-dilution (1:50) 50 ml
   Glycerol, 87% 50 ml
   (based on the steps c) and d) a the final dilution is 1:100

Aliquot in Cryovials (1 ml)
Store at -20°C.

4.3.3.5. Preparation of final conjugate solution (1:100 dilution of working solution results in 1:10,000 final dilution)

Aliquot conjugate working solution 100 µl
PBSM 9,900 µl
To be prepared immediately before use. Remaining solution not to be re-used.
10 ml required per plate

4.3.3.6. Sodium acetate buffer

Sodium acetate anhydrous 90,2 g
double distilled water. ad 1,0 L
Adjust pH to 5.5 with 32% and 1 N hydrochloric acid.

**Stable at room temperature for max. 1 year**

4.3.3.7 TMB-Stock solution

12 mg TMB/2 ml Ethanol highest purity.

For flushing the TMB from the weigh boat, at least 2 ml ethanol is required. This minimal quantity is sufficient for ca 10 plates. Shake the solution for ca 30 min (protect vials against light by wrapping with aluminium foil), because TMB dissolves slowly.

The solution can be kept at room temperature in the dark for a maximum of 2 weeks. If the solution shows yellow colour it has to be discarded.

4.3.3.8 TMB-Substrate Working solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>double-distilled water</td>
<td>9 ml</td>
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<tr>
<td>Sodium acetate buffer</td>
<td>1 ml</td>
</tr>
<tr>
<td>TMB-Stock solution</td>
<td>167 µl</td>
</tr>
<tr>
<td>H₂O₂ 30%</td>
<td>2 µl</td>
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</tbody>
</table>

H₂O₂ (Starting reagent) add immediately before use and mix carefully.

10 ml required per plate.

4.3.3.9. Stop solution (1 M H₂SO₄)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>double-distilled water</td>
<td>300 ml</td>
</tr>
<tr>
<td>2,5 M H₂SO₄</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

The solution can be stored up to 1 year at room temperature.

5 ml required per plate.

4.4 Procedure

Note: With the exception of step 9 (substrate incubation) for each incubation step the multiwell plate should be covered with sealing film during the incubation periods (in order to avoid evaporation during incubation).

**Step 1: Antigen coating procedure**

Resuspend lyophilised antigen in 0.5 ml distilled water.

Dilute antigen in sterile PBS according to the dilution factor determined during method validation. (prepare approximately 10 ml of diluted antigen per plate)

Add 100 µl of the diluted coating antigen to each well with a micropipette

**Incubation:** Over night (ca. 18 hours), at 4° C
Step 2: Washing procedure
Buffer: PBS without Tween 20
Discard the content of the plate
Add 200 µl buffer to each well
Repeat wash 3 times
After washing dry by tapping the plates vigorously on absorbent paper

Step 3: Blocking procedure
Buffer: PBSM
Add 150 µl per well
Incubation: 1 hour at 37°C on a thermoshaker (150 rpm)

Step 4: Washing procedure
Buffer: PBS-Tween 20, 0,1%
Discard the content of the plate
Add 200 µl buffer to each well
Repeat wash 3 times
After washing dry by tapping the plates vigorously on absorbent paper

Step 5: Sera dilutions
Test sera, reference sera and negative control sera are diluted 1:20 before use.
For 3 replicates 800µL of each test sera type and reference are required
Dilute the appropriate serum 1:20 with PBSM
Quantity per plate:

<table>
<thead>
<tr>
<th>Serum</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>PBSM</td>
<td>760 µl</td>
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<tr>
<td>Test serum</td>
<td>40 µl</td>
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<tr>
<td>Negative</td>
<td>20 µl</td>
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</tbody>
</table>

For Negative serum (3 wells) prepare 400 µl per plate

<table>
<thead>
<tr>
<th>Serum</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Negative</td>
<td>20 µl</td>
</tr>
<tr>
<td>PBSM</td>
<td>380 µl</td>
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</table>

Plate layout (refer to example Figure 1)
6 dilution steps of 1:2 for each of the test sera and the reference sera are performed as follows.
Add 100 µl of PBS skimmed milk buffer to wells B to G (1 – n as required) (ie: 1-12 in figure 1 example).
Add 200 µl of the sera (test or reference as appropriate)(prediluted to 1:20) to well A(x).
(ie: in figure 1 example; Test serum 1; A1-A3, Test serum 2; A4-A6, Test serum 3; A7-A9, Reference serum; A10-A12)
Remove 100 µl from well A1-An and add into well B1-Bn. Repeat this procedure until well G1-Gn has been treated. Discard the last 100 µl of solution.
Add 100 µl of negative serum to wells H1-H3.
Add 100 µl of PBS skimmed milk 5% to wells H4-H6 (conjugate control)
Incubation: 1 hour at 37°C on a thermoshaker (150 rpm)
Figure 1: Example of assay plate for 3 test sera

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<tbody>
<tr>
<td>Dil’n</td>
<td>Test serum 1</td>
<td>Test serum 2</td>
<td>Test serum 3</td>
<td>Reference serum</td>
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Negative Serum | Conjugate Control
Step 6: Washing procedure
   Buffer: PBS-Tween 20, 0.1%
   Discard the content of the plate
   Add 200 µl buffer to each well
   Repeat wash 3 times
   After washing dry by tapping the plates vigorously on absorbent paper

Step 7: Conjugate procedure
   Prepare final conjugate solution immediately before use according to section 4.3.3.5.
   Add 100 µl of conjugate solution to each well
   Incubation: 1 hour at 37°C (150 rpm)

Step 8: Washing procedure
   Buffer: PBS-Tween 20, 0.1%
   Discard the content of the plate
   Add 200 µl buffer to each well
   Repeat wash 3 times
   After washing dry by tapping the plates vigorously on absorbent paper

Step 9: Substrate incubation
   Prepare immediately before use TMB-Substrate solution according to section 4.3.3.8.
   Approximately 10mL required per plate:
   Mix solution carefully and use immediately
   Add 100 µl to each well
   Incubation: 5 minutes at room temperature in the dark

Step 10: Stopping the reaction
   Prepare Stop solution (1 M H₂SO₄) according to section 4.3.3.9.
   Add 50 µl buffer to each well

Step 11: Photometric reading procedure
   Reading OD values at 450 nm (reference 620nm if available)

Criteria for validity of experiments
Limit for the negative serum: Extinction ≤ 0.150
Limit for the conjugate control: Extinction ≤ 0.0480
Limit for variation coefficient of extinction values for reference- and test sera ≤ 15 % (2 or 3 replicates)
The relative value of the reference serum is set to 1. The test sera comply with the requirements if their relative value is ≥ 1.
5. STATISTICAL ANALYSIS

The potency of test preparations should be calculated by analysing individual assays applying a four parameter logistic model, taking the response against the log of the dilution. Curves of test and reference serum are compared to give a value expressed as relative potency. The reference has an arbitrary potency of 1, which represents the pass mark for the vaccines being tested.

If the results from a single assay does not fall within acceptable confidence limits the assay should be repeated.