Defined Epitope Blocking (DEB)-ELISA for the detection of Ross River Virus Specific Neutralising Antibody in Serum or Plasma

Ross River Virus Total Antibody
DEB-ELISA

CATALOGUE CODE: 7013

Manufactured for Biocene Pty Ltd by Meddens Diagnostics BV
1. SYMBOLS
The symbols used in the instructions for use and labels are illustrated below (not all will appear):

- “Attention, See Instructions for Use”
- “Use by”
- “Do Not Reuse”
- “Batch Code”
- “Catalogue number”
- “Manufacturer”
- “In Vitro Medical Device”
- “The CE mark”
- “Temperature limitation for storage conditions”
- “Contains sufficient for <n> tests”
- “Irritant”
- “20 x concentrated”
- “Ready to Use”
- “Positive Control”
- “Negative Control”
- “Negative Calibrator”
- “Control Antigen”
- “Antibody conjugated to peroxidase”
- “Dilution Buffer”
- “Conjugate Diluent”
- “Microtiterplate coated with antigen”
- “Wash buffer”
- “TMB Solution”
- “Sulphuric Acid, 0.5 M”
- “20 x concentrated”
- “100 x concentrated”
2. INTENDED USE

The Ross River Virus Total Antibody DEB-ELISA is a Defined Epitope Blocking enzyme immunoassay for the detection of Ross River virus specific neutralising antibody in serum or plasma. It is an aid in the diagnosis of Ross River virus infections and for the detection of immune status. The assay must be performed strictly in accordance with the instructions set out in this protocol. No responsibility is taken for any loss or damage (except as required by statute) caused or arising from non-compliance with the instructions provided.

3. INTRODUCTION

Ross River virus belongs to the alphavirus group of arboviruses and causes a syndrome known as "epidemic polyarthritis". Ross River virus is transmitted via mosquito bite, and is the most common and widespread arboviral disease in Australia. Disease occurs most commonly in adults 20 to 50 years of age. Symptoms of Ross River virus disease include arthritis, fatigue, rash, myalgia and mild fever. A significant proportion of patients will have symptoms for more than 12 months, emphasising the significant morbidity of the disease. Outbreaks of disease have been reported throughout Australia, Papua New Guinea and the Solomon Islands. A single major epidemic also occurred in Fiji and various Pacific Islands. Epidemic activity is associated with periods of heavy rainfall, flooding and tidal inundation of salt marshes and coastal wetlands. In northern, tropical Australia, transmission occurs year round. In southern Australia, transmission typically occurs in spring summer. Diagnosis is often made serologically.

4. PRINCIPLE OF THE ASSAY

Ross River Virus Total Antibody DEB-ELISA detects a single type specific neutralising epitope (2). The test measures total antibody and is species independent. Purified, inactivated Ross River whole virions are coated onto the surface of microtiter wells. Test samples and controls diluted 1/11 are added to the plate and incubated for 1 hour. This is followed by the addition of Ross River specific HRP labelled, purified monoclonal antibody and further incubated for 1 hour. All unbound material is washed away. Colour is developed by addition of TMB substrate. The reaction is stopped and optical densities measured at 450 nm. If specific antibody is present in the sample it will bind to the antigen on the plate and effectively "block" the attachment of HRP labelled monoclonal antibody, thus reducing the amount of colour produced. Negative samples have high colour. Positive samples have low or no colour.

5. REAGENTS AND ACCESSORIES

5.1 Reagents provided in the kit

The kit contains the following reagents. A distinction can be made between reagents that are specific for the assay and universal reagents.

<table>
<thead>
<tr>
<th>SYMBOL</th>
<th>CAT. CODE</th>
<th>DESCRIPTION</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL</td>
<td>7013-26</td>
<td>Negative calibrator (100 x concentrated, contains bovine serum)</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>CAL</td>
<td>7013-27</td>
<td>Positive Calibrator (100 x concentrated, contains equine serum)</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>CONJ Ab</td>
<td>7013-05</td>
<td>HRP Labelled Ross River Neutralising Epitope Specific Monoclonal Antibody (ready to use, obtained through tissue culture)</td>
<td>7.0 mL</td>
</tr>
<tr>
<td>MTP Ag</td>
<td>7013-08</td>
<td>Microtiter plate (12 x 8 break apart wells) coated with inactivated, purified Ross River virus antigen</td>
<td>1 plate</td>
</tr>
<tr>
<td>SOLN TMB</td>
<td>9000-19</td>
<td>TMB Substrate Solution (ready-to-use)</td>
<td>15 mL</td>
</tr>
<tr>
<td>DILAS</td>
<td>7013-28</td>
<td>Dilution buffer (BLUE, ready-to-use)</td>
<td>60 mL</td>
</tr>
<tr>
<td>WASHBUF</td>
<td>9000-07</td>
<td>Wash buffer (20 x concentrated)</td>
<td>60 mL</td>
</tr>
<tr>
<td>H2SO4 0,5M</td>
<td>9000-08</td>
<td>Stop solution (ready-to-use)</td>
<td>20 mL</td>
</tr>
</tbody>
</table>
5.2 Materials provided with the kit
- Resealable bag, 2x
- Instructions for use, 1x
- Certificate of Analysis, 1x

5.3 Reagents and materials needed but not provided
- Pipettes to deliver volumes between 50 and 1000 µL
- Volumetric laboratory glassware
- Distilled (or deionised) water
- 37°C Incubator
- Clean disposable plastic tubes for sample dilution (approx. 3 mL capacity)
- Clean disposable plastic tubes for TMB (12 mL capacity)
- Disposable absorbent towels
- Automatic plate washer (optional)
- Microtiter Plate Reader, equipped for measurement at 450 nm (optional with 620 nm reference filter)
- Vortex tube mixer
- Timer

6. COLLECTION, HANDLING, STORAGE OF SAMPLES
Serum or plasma from any species can be used in this method. Samples may be stored at 4°C for up to 7 days. If longer storage is required, store at -20°C to -70°C. Avoid repeat freezing and thawing. Do not use grossly haemolysed or lipemic sera/plasma.

**NOTE: Treat all human samples as potentially infectious.**

7. PROCEDURE

7.1 Wash Procedure
Efficient washing is a fundamental requirement of EIAs. It is essential that the wash procedure be carried out with care to obtain reproducible inter and intra assay results. Both manual and automatic washing can be performed.

7.1.1 Manual Wash
1. Empty the contents of each well by turning the strips in the holder upside down followed by a firm short vertical movement. Keep the strips tightened by pressing the sides of the strip holder.
2. Fill all wells to the rim (300-350 µL) with wash buffer. Take care to avoid carry-over.
3. Turn the strips upside down and empty the wells by a firm vertical movement.
4. Repeat this wash cycle (steps 2 and 3) 4 times.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Do not allow any of the wells to dry out before the next reagent is dispensed. Therefore, proceed with the next step immediately.

7.1.2 Automatic Plate Wash
When using automatic plate wash equipment, check that all wells can be aspirated completely, that the wash buffer is accurately dispensed reaching the rim of each well during each wash cycle. The plate washer should be programmed to execute 5 (five) cycles. After the last cycle, remove the wash buffer from the wells by tapping firmly the plate on absorbent towels.
7.2 Assay and reagent preparation procedure

**Note: Allow all reagents to reach room temperature before use.**

1. Dilute the controls and samples 1:11 with Dilution Buffer (50 µL test sample or control mixed with 500 µL Dilution Buffer). The Dilution Buffer contains a sample delivery indicator: a colour change from purple to clear blue can be seen upon delivery of the sample and control.

2. Dispense 50 µL negative calibrator and positive calibrator in duplicate, see scheme below. Dispense 50 µL of each diluted patient serum (BLUE) into a well. Insert the plates into the resealable bag or cover to avoid evaporation.

```
1 2 3 4 5 6 7 8 9 10 11 12
A - S5
B - S6
C + S7
D + S8
E S1 S9
F S2 S10
G S3 S11
H S4 S12
```

+ = Positive Calibrator  - = Neg. Calibrator  S1 = Diluted Sample  S3 = Diluted Sample  S2 = Diluted Sample  Etc.

3. Incubate for 1 hour at 37°C in a 100% moist environment.

4. Remove plate from the Incubator. **DO NOT WASH.** Add 50 µL of HRP conjugate to each well. Tap plate gently to mix. Insert the plate into the resealable bag or cover to avoid evaporation.

**Note: be aware of preventing carry-over when dispensing conjugate. Transferring trace amounts of RRV antibodies will induce significant inhibition.**

5. Incubate for 1 hour at 37°C in a 100% moist environment.

**Note: use only clean disposable containers.**

6. Prepare the wash buffer: for each 8 well strip mix 1.5 mL of the wash buffer (20x) with 28.5 mL distilled water. The stability of the working solution is one month at room temperature or one year at +4°C.

7. Wash the plate 5 times with 300 -350 µL wash buffer per well. Blot dry.

8. Add 100 µL of the ready-to-use TMB substrate solution to each well and incubate in the dark for 30 minutes at room temperature.

9. Stop the reaction by adding 100 µL of Stop Solution to each well.

10. Measure the absorbance of specimens with a photometer at 450 nm (optionally with a 620 nm
reference filter) within 10 minutes of adding the stop solution.
8. CALCULATION, VALIDATION AND INTERPRETATION

The colour produced is inversely proportional to the amount of Ross River specific neutralizing antibody present in the sample. Negative samples develop intense colour. Positive samples develop no colour or a low colour level. The results are expressed as % Inhibition.

A. Calculation.
- Calculate the mean OD of the Negative Calibrator.
- Calculate the % Inhibition of unknown samples and positive calibrator as follows:

\[
\frac{\text{mean OD Negative Calibrator - OD Patient Sample}}{\text{Mean OD Negative Calibrator}} \times 100
\]

B. Validation
- **Negative calibrator:** Mean OD > 1.0 to < 2.0
- **Positive Calibrator:** % Inhibition > 80

C. Interpretation

Results obtained with patient samples are interpreted as follows:

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>Result Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 40</td>
<td>Negative</td>
</tr>
<tr>
<td>&gt; 40 - &lt; 60</td>
<td>Low Positive</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>High Positive</td>
</tr>
</tbody>
</table>

A Negative inhibition is to be considered "zero Inhibition".

The Ross River Virus TAB assay detects only the presence of Ross River virus specific neutralizing antibody. The test does not distinguish between IgG and IgM antibodies- it detects both. To determine recent from past infection an IgM assay may be performed or a second sample obtained (5 to 7 days post onset) to demonstrate an antibody rise in the RRV Total Antibody assay.

9. ASSAY PERFORMANCE DATA

**In house Evaluation**
A total of 200 RRV antibody negative and 90 RRV antibody positive sera as defined by Neutralization were assayed in the RRV Total Antibody DEB ELISA.
- **Specificity.** All 200 Neutralizing antibody negative sera tested negative in the RRV TAB assay.
- **Sensitivity.** 89 of the 90 RRV Neutralizing Antibody positive sera tested positive in the RRV TAB assay.

**External Evaluation** (Arbovirus Unit, C.I.D.M. ICPMR, Westmead Hospital).
The performance of RRV TAB was compared to the Neutralization Test (NT) in 518 samples.
- **Specificity.** 363 samples Negative by NT (titres <10) were also negative in the RRV TAB, indicating a specificity of 100%. Included in the 363 NT negatives were: 10 BFV-IgM+, 10 CMV-IgM+, 10 EBV-IgM+, 8 Q Fever IgM+ and 12 RF+.
- **Sensitivity.** 153 out of 155 NT positive samples (NT titres > 10) were positive in the RRV TAB ELISA, indicating a sensitivity of 98.7%. The 2 negative results were obtained from the acute sample in 2 serum pairs, both with a NT titre of 10. The convalescent sample was positive in both pairs.
- **Seroconversion.** The RRV TAB was positive in the acute sample of 6 NT+ serum pairs, in which IgG was not detectable, thus indicating detection of IgM. The convalescent sample in these pairs had detectable IgG and significantly higher % Inhibition, thus demonstrating the assay’s ability to detect sero-converters and rising titres.

Full evaluation report is available on request.
10. CHANGES TO PROCEDURE
IFU 7013 V03: Instructions for Use are changed as follows: %inhibition calculated by using the negative calibrator. The negative control and the high pos control are removed from the kit.

11. PRECAUTIONS
- All reagents supplied are for in vitro use only.
- Avoid contact of substrate and stop solution with skin and mucous membranes. If these reagents come into contact with skin, wash with abundant tap water.
- Each well is ultimately used as an optical cuvette. Do not touch the under surface of the strips; do not use damaged or soiled wells.
- Use only components that are provided in this kit. Do not mix reagents of different batch numbers.
- The reagents should be used only as indicated in this instruction manual.
- The Negative calibrator is used as reagent blank from which to calculate the % inhibition. The negative serum samples can yield OD levels higher than the negative Calibrator, thus resulting in negative % inhibition.
- Avoid the use of sodium azide as a preservative, as it can inhibit HRP.
- The conjugate and substrate should be protected from prolonged exposure to light.

12. REFERENCES

13. DISCLAIMER
This assay has been standardised for use in human and equine samples. Whilst it is species independent, testing for other species (e.g. bovine), may require a species-specific negative control as some species produce lower OD levels than humans and/or equine. Failure to do this may result in false positive results.
# QUICK REFERENCE PROTOCOL FOR ROSS RIVER TOTAL ANTIBODY ELISA *

## PREPARATION OF REAGENTS

<table>
<thead>
<tr>
<th>A. Dilute Controls and patient test serum : mix 500 µL dilution buffer + 50 µL (Pos and Neg) calibrator and patient test serum, colour shifts from purple to blue</th>
<th></th>
</tr>
</thead>
</table>

## TEST PROCEDURE

<table>
<thead>
<tr>
<th>1. Dispense 50 µL negative calibrator and positive calibrator in duplicate and patient test sera (BLUE) in singlicate. Incubate 60 min at 37°C in the resealable bag or in a 100% moist chamber.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Dispense 50 µL conjugate to each well, colour shifts in the well from blue to green. Incubate 60 minutes at 37°C in the resealable bag or in a 100% moist chamber.</td>
<td></td>
</tr>
<tr>
<td>B. Prepare wash buffer: mix per 8-well strip: 1.5 mL Wash Buffer (20x) + 28.5 mL distilled water</td>
<td></td>
</tr>
<tr>
<td>3. Wash 5 times, Dispense 100 µL ready-to-use TMB to each well. Incubate in the dark, 30 minutes at room temperature.</td>
<td></td>
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<tr>
<td>4. Add 100 µL stop solution to each well. Read absorbance at 450 nm (optionally with 620 nm reference).</td>
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*Read the entire protocol before starting the assay*