4. Add 100µL of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 45 minutes at 37°C.
5. Repeat the aspiration/wash process for five times as conducted in step 3.
6. Add 50µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30 minutes at 37°C. Protect from light.
7. Add 50µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well at once, using a microplate reader set to 450 nm.

Calculation of results

Average the duplicate readings for each standard, control, and sample and sample, then subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the target antigen concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. It is recommended to use professional software to do this calculation, such as CurveExpert. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Important note

1. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. The reconstituted Standards, Detection Reagent A and B can be used only once.
2. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay.

Precaution

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES
This immunoassay kit allows for the in vitro quantitative determination of target antigen concentrations in serum, plasma, tissue homogenates, cell culture supernates or other biological fluids.

### Components and Storage

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay plate</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sample Diluent A</td>
<td>1 x 10mL</td>
<td>Stored at –20°C</td>
</tr>
<tr>
<td>Sample Diluent B</td>
<td>1 x 10mL</td>
<td></td>
</tr>
<tr>
<td>Detection Reagent A</td>
<td>1 x 60μL</td>
<td></td>
</tr>
<tr>
<td>Detection Reagent B</td>
<td>1 x 120μL</td>
<td></td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>1 x 30mL</td>
<td>Stored at 4°C</td>
</tr>
<tr>
<td>Substrate</td>
<td>1 x 10mL</td>
<td></td>
</tr>
<tr>
<td>Stop Solution</td>
<td>1 x 10mL</td>
<td></td>
</tr>
<tr>
<td>Plate sealer</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

### Intended use

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### Test principle

The ELISA is based on the competitive binding enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with an antibody specific to target antigen. During the reaction, target antigen in the sample competes with a fixed amount of biotin-labeled target antigen for sites on a pre-coated antibody specific to target antigen. Excess conjugate and unbound sample or standard are washed from the plate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microwell and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of target antigen in the samples is then determined by comparing the O.D. of the samples to the standard curve.

### Sample collection and storage

#### Serum
- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C.

#### Plasma
- Collect plasma using EDTA or heparin as an anticoagulant. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C.

#### Cell culture supernates and Other biological fluids
- Cell culture supernates or other biological fluids.

#### Tissue homogenates
- The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissue was rinsed with ice-cold 1×PBS, homogenized in ice-cold 1×PBS and stored overnight at -20°C. In most cases, 10% homogenate (eg.1g of tissue in 10mL of ice-cold 1×PBS) is recommended. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Remove the supernate and assay immediately or aliquot and store at -20°C or -80°C.

### Reagent preparation

**Standard**
- Please refer to the Data Sheet inserting in the kit.

**Detection Reagent A and B**
- Dilute to the working concentration using Assay Diluent A and B (1:100), respectively.

**Wash Buffer**
- If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.
- Dilute 30 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 750 mL of Wash Buffer.

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**Assay procedure**

- Allow all reagents to reach room temperature (Please do not dissolve the reagents at 37°C directly). All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Remove strips should be resealed and stored at -20°C until the kits expiry date. Prepare all reagents, working standards and samples as directed in the previous sections. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

1. Add 50 μL of Standard, Blank, or Sample per well.
2. Immediately add 50 μL of Detection A working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 μL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

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