Rheumatoid Factor (RF)

Cat # 2550Z

INTENDED USE
The Diagnostic Automation Rheumatoid Factor (RF) Enzyme-Linked Immunosorbent Assay (ELISA) is intended for the detection of IgM antibodies in human serum to RF antigen and as an aid in the diagnosis of rheumatoid arthritis. For in vitro diagnostic use. High complexity test.

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology (1). Rheumatoid arthritis is a systemic disease characterized by chronic proliferation and inflammation of joint cartilage and supporting structures. RA is mainly defined by clinical criteria, in which systematic pathogenetic studies have been hampered by doubts about the presence of common pathogenetic mechanisms and the relative lack of unique laboratory findings (2). IgG rheumatoid factor has been reported to be present in sera of patients with rheumatoid arthritis both with and without IgM rheumatoid factor activity (3,4).

RFs are immunoglobulins of any isotype with antibody activity directed against antigenic sites on the Fc portion of IgG molecules. Because of its pentavalent structure and ability to cross-link immunoglobulin G antigen, IgM-RF is the main isotype identified by clinically available diagnostic assays for RF detection (5). Rheumatoid factors may exist as the mu, gamma, alpha, and epsilon isotypes (6).

Rheumatoid factors are found in 1 to 4% of the general population. RFs are present in 75% of adult patients with the highest incidence of rheumatoid factors occurring in persons over 65 years of age and nearly all patients with Felty and Sjogren syndrome. The clinical correlation of an elevated rheumatoid factor should be interpreted cautiously. Increased titers may accompany a variety of acute immune responses, particularly viral infections and a number of other diseases (e.g., infectious mononucleosis, tuberculosis, leprosy, various parasitic diseases, liver disease, sarcoidosis, and lymphoproliferative syndromes) (6).
The earliest tests and those still most widely used rely on the agglutinating properties of the IgM class of rheumatoid factors. Sensitized sheep red blood cell and latex agglutination tests have been developed and routinely employed. These assays are most sensitive for the detection of RF that is of the IgM isotype because of its multivalent structure. These tests provide a dilution which is difficult to standardize and have laborious processing and poor reproducibility. Enzyme immunoassays are more sensitive than agglutination and very specific due to use of purified antigen (5).

Principle of the Assay

The DAI Rheumatoid Factor test uses the ELISA technique for the detection of IgM antibodies to IgG antigen. The purified antigen is bound to a solid phase microassay well. Patient serum samples to be assayed for antibody are first diluted and added to each well. If antibody is present in the patient's serum, antigen-antibody complexes are formed. After washing the unbound serum from the well, horseradish peroxidase conjugated anti-human IgM is added to the wells and allowed to incubate. The conjugate will bind to human antibody which is present. After washing the unbound conjugate from the wells, TMB substrate solution is added and incubated. The enzyme conjugate present will react with the H2O2 substrate and tetramethylbenzidine (TMB) chromogen, resulting in blue color development. The addition of 1N H2SO4 stops the enzymatic reaction and turns the blue color to yellow. The absorbance of the solution, measured at 450 nm, is directly related to the concentration of IgM antibody bound to the well. (9, 10, 11, 12)

Kit Presentation

Materials Supplied

Each kit contains the following components in sufficient quantities to perform the number of tests indicated on the package label.

1. Purified Rheumatoid Factor IgG antigen coated microassay plate: 96 wells, configured in twelve 1x8 strips, stored in a foil pouch with desiccant. (96T: one plate)
2. Serum Diluent Type II: Ready to use, contains proclin (0.1%) as preservative. (96T: one bottle, 30 mL)
3. Calibrator: Human serum or defibrinated plasma. Sodium azide (< 0.1%) and pen/strep (0.01%) added as preservatives, with kit specific factor printed on vial label. The Calibrator is used to calibrate the assay to account for day-to-day fluctuations in temperature and other testing conditions. (96T: one vial, 0.4 mL) *
4. Positive Control: Human serum or defibrinated plasma. Sodium azide (< 0.1%) and pen/strep (0.01%) added as preservatives, with established range printed on vial label. The Positive Control is utilized to control the upper dynamic range of the assay. (96T: one vial, 0.4 mL) *
5. Negative Control: Human serum or defibrinated plasma. Sodium azide (< 0.1%) and pen/strep (0.01%) added as preservatives, with established range printed on vial label. The Negative Control is utilized to control the negative range of the assay. (96T: one vial, 0.4 mL) *
6. Horseradish-peroxidase (HRP) Conjugate: Ready to use. Goat anti-human IgM, containing proclin (0.1%) and gentamicin as preservatives. (96T: one bottle, 16 mL)
7. Chromogen/Substrate Solution Type I: Tetramethylbenzidine (TMB), ready to use. The reagent should remain closed when not in use. If allowed to evaporate, a precipitate may form in the reagent wells. (96T: one bottle, 15 mL)
8. Wash Buffer Type I (20X concentrate): Dilute 1 part concentrate + 19 parts deionized or distilled
water. Contains TBS, Tween-20 and proclin (0.1%) as a preservative. (96T: one bottle, 50 mL)

9. Stop Solution: Ready to use, contains a 1N H₂SO₄ solution. (96T: one bottle, 15 mL)

*Note: serum vials may contain excess volume.

The following components are not kit lot # dependent and may be used interchangeably within the DAI ELISA assays: Chromogen/Substrate Solution Type I, Wash Buffer Type I, and Stop Solution. Please check that the appropriate DAI Reagent Type (Type I, Type II, etc.) is used for the assay.

Additional Requirements

1. Wash bottle, automated or semi-automated microwell plate washing system.
2. Micropipettes, including multichannel, capable of accurately delivering 10-200 µL volumes (less than 3% CV).
3. One liter graduated cylinder.
5. Test tube for serum dilution.
6. Reagent reservoirs for multichannel pipettes.
7. Pipette tips.
8. Distilled or deionized water (dH₂O), CAP (College of American Pathology) Type 1 or equivalent. (15,16).
9. Timer capable of measuring to an accuracy of +/- 1 second (0 – 60 minutes).
10. Disposal basins and 0.5% sodium hypochlorite (50 mL bleach in 950 mL dH₂O).
11. Single or dual wavelength microplate reader with 450 nm filter. If dual wavelength is used, set the reference filter to 600-650 nm. Read the Operator's Manual or contact the instrument manufacturer to establish linearity performance specifications of the reader.

Note: Use only clean, dry glassware.

Storage and Stability

1. Store unopened kit between 2° and 8° C. The test kit may be used throughout the expiration date of the kit. Refer to the package label for the expiration date.
2. Unopened microassay plates must be stored between 2° and 8° C. Unused strips must be immediately resealed in a sealable bag with desiccant and returned to storage between 2° and 8° C.
3. Store HRP Conjugate between 2° and 8° C.
4. Store the Calibrator, Positive Control, and Negative Control between 2° and 8° C.
5. Store Serum Diluent Type II and 20X Wash Buffer Type I between 2° and 8° C.
6. Store the Chromogen/Substrate Solution Type I between 2° and 8° C. The reagent should remain closed when not in use. If allowed to evaporate, a precipitate may form in the reagent wells.
7. Store 1X (diluted) Wash Buffer Type I at room temperature (21° to 25° C) for up to 5 days, or up to one week between 2° and 8° C.

Note: If constant storage temperature is maintained, reagents and substrate will be stable for the dating period of the kit. Refer to package label for expiration date. Precautions were taken in the manufacture of this product to protect the reagents from contamination and bacteriostatic agents have been added to the liquid reagents. Care should be exercised to protect the reagents in this kit from contamination.
Precautions

1. For *in vitro* diagnostic use.

2. The human serum components used in the preparation of the Controls and Calibrator in this kit have been tested by an FDA approved method for the presence of antibodies to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen and found negative. Because no test method can offer complete assurance that HIV, HCV, hepatitis B virus, or other infectious agents are absent, specimens and human-based reagents should be handled as if capable of transmitting infectious agents.

3. The Centers for Disease Control & Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2 (7).

4. The components in this kit have been quality control tested as a Master Lot unit. Do not mix components from different lot numbers except Chromogen/Substrate Solution Type I, Stop Solution, Wash Buffer Type I, and Serum Diluent Type II. Do not mix with components from other manufacturers.

5. Do not use reagents beyond the stated expiration date marked on the package label.

6. All reagents must be at room temperature (21° to 25° C) before running assay. Remove only the volume of reagents that is needed. **Do not pour reagents back into vials as reagent contamination may occur.**

7. Before opening Control and Calibrator vials, tap firmly on the benchtop to ensure that all liquid is at the bottom of the vial.

8. Use only distilled or deionized water and clean glassware.

9. Do not let wells dry during assay; add reagents immediately after completing wash steps.

10. Avoid cross-contamination of reagents. Wash hands before and after handling reagents. **Cross-contamination of reagents and/or samples could cause false results.**

11. If washing steps are performed manually, wells are to be washed three times. Up to five wash cycles may be necessary if a washing manifold or automated equipment is used.

12. **Sodium azide inhibits Conjugate activity.** Clean pipette tips must be used for the Conjugate addition so that sodium azide is not carried over from other reagents.

13. It has been reported that sodium azide may react with lead and copper in plumbing to form explosive compounds. When disposing, flush drains with water to minimize build-up of metal azide compounds.

14. Never pipette by mouth or allow reagents or patient sample to come into contact with skin. Reagents containing proclin, sodium azide, and TMB may be irritating. Avoid contact with skin and eyes. In case of contact, flush with plenty of water.

15. If a sodium hypochlorite (bleach) solution is being used as a disinfectant, do not expose to work area during actual test procedure because of potential interference with enzyme activity.

16. Avoid contact of Stop Solution (1N sulfuric acid) with skin or eyes. If contact occurs, immediately flush area with water.

17. **Caution:** Liquid waste at acid pH must be neutralized prior to adding sodium hypochlorite (bleach) solution to avoid formation of poisonous gas. Recommend disposing of reacted, stopped plates in biohazard bags. See Precaution 3.

18. The concentrations of anti-Rheumatoid Factor IgM in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.
Specimen Collection and Storage

1. Handle all blood and serum as if capable of transmitting infectious agents (7).
2. Optimal performance of the DAI ELISA kit depends upon the use of fresh serum samples (clear, non-hemolyzed, nonlipemic, non-icteric). A minimum volume of 50 µL is recommended, in case repeat testing is required. Specimens should be collected aseptically by venipuncture (8). Early separation from the clot prevents hemolysis of serum.
3. Store serum between 2° and 8° C if testing will take place within two days. If specimens are to be kept for longer periods, store at -20° C or colder. Do not use a frost-free freezer because it may allow the specimens to go through freeze-thaw cycles and degrade antibody. Samples that are improperly stored or are subjected to multiple freeze-thaw cycles may yield erroneous results.

Methods for Use

Preparation for the Assay

1. All reagents must be removed from refrigeration and allowed to come to room temperature before use (21° to 25° C). Return all reagents to refrigerator promptly after use.
2. All samples and controls should be vortexed before use.
3. Dilute 50 mL of the 20X Wash Buffer Type I to 1 L with distilled and/or deionized H2O. Mix well.

Assay Procedure

1. Place the desired number of strips into a microwell frame. Allow five (5) Control/Calibrator determinations (one Negative Control, three Calibrators, and one Positive Control) per run. A reagent blank (RB) should be run on each assay. Check software and reader requirements for the correct Control/Calibrator configuration. Return unused strips to the sealable bag with desiccant, seal and immediately refrigerate.

Example Configuration:

<table>
<thead>
<tr>
<th>Plate Location</th>
<th>Sample Description</th>
<th>Plate Location</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>RB</td>
<td>2A</td>
<td>Patient #3</td>
</tr>
<tr>
<td>1B</td>
<td>NC</td>
<td>2B</td>
<td>Patient #4</td>
</tr>
<tr>
<td>1C</td>
<td>Cal</td>
<td>2C</td>
<td>Patient #5</td>
</tr>
<tr>
<td>1D</td>
<td>Cal</td>
<td>2D</td>
<td>Patient #6</td>
</tr>
<tr>
<td>1E</td>
<td>Cal</td>
<td>2E</td>
<td>Patient #7</td>
</tr>
<tr>
<td>1F</td>
<td>PC</td>
<td>2F</td>
<td>Patient #8</td>
</tr>
<tr>
<td>1G</td>
<td>Patient #1</td>
<td>2G</td>
<td>Patient #9</td>
</tr>
<tr>
<td>1H</td>
<td>Patient #2</td>
<td>2H</td>
<td>Patient #10</td>
</tr>
</tbody>
</table>

RB = Reagent Blank - Well without serum addition run with all reagents. Utilized to blank reader.
NC = Negative Control
Cal = Calibrator
PC = Positive Control
2. Dilute test sera, Calibrator and Control sera 1:21 (e.g., 10 µL + to 200 µL) in Serum Diluent Type II. Mix well. (For manual dilutions it is suggested to dispense the Serum Diluent into the test tube first and then add the patient serum.)

3. To individual wells, add 100 µL of the appropriate diluted Calibrator, Controls and patient sera. Add 100 µL of Serum Diluent Type II to reagent blank well. Check software and reader requirements for the correct reagent blank well configuration.

4. Incubate each well at room temperature (21° to 25° C) for 30 minutes +/- 1 minute.

5. Aspirate or shake out liquid from all wells. If using semi-automated or automated washing equipment add 250-300 µL of diluted Wash Buffer to each well. Aspirate or shake out and turn plate upside down and blot on paper toweling to remove all liquid. Repeat the wash procedure two times (for a total of three (3) washes) for manual or semi-automated equipment or four times (for a total of five (5) washes) for automated equipment. After the final wash, blot the plate on paper toweling to remove all liquid from the wells.

**IMPORTANT NOTE:** Regarding steps 5 and 8 - Insufficient or excessive washing will result in assay variation and will affect validity of results. Therefore, for best results the use of semi-automated or automated equipment set to deliver a volume to completely fill each well (250-300 µL) is recommended. A total of up to five (5) washes may be necessary with automated equipment. **Complete removal of the Wash Buffer after the last wash is critical for the accurate performance of the test. Also, visually ensure that no bubbles are remaining in the wells.**

6. Add 100 µL Conjugate to each well, including reagent blank well. Avoid bubbles upon addition as they may yield erroneous results.

7. Incubate each well at room temperature (21° to 25° C) for 30 minutes +/- 1 minute.

8. Repeat wash as described in Step 5.

9. Add 100 µL Chromogen/Substrate Solution (TMB) to each well, including the reagent blank well, maintaining a constant rate of addition across the plate.

10. Incubate each well at room temperature (21° to 25° C) for 5 – 15 minutes.

11. Stop reaction by addition of 100 µL of Stop Solution (1N H₂SO₄) following the same order of Chromogen/Substrate addition, including the reagent blank well. Tap the plate gently along the outsides, to mix contents of the wells. The plate may be held up to 30 minutes after addition of the Stop Solution before reading.

12. The developed color should be read on an ELISA plate reader equipped with a 450 nm filter. If dual wavelength is used, set the reference filter to 600-650 nm. The instrument should be blanked on air. The reagent blank must be less than 0.150 Absorbance at 450 nm. If the reagent blank is ≥ 0.150 the run must be repeated. Blank the reader on the reagent blank well and then continue to read the entire plate. Dispose of used plates after readings have been obtained.

**Quality Control**

For the assay to be considered valid the following conditions must be met:

1. Calibrator and Controls must be run with each test run.
2. Reagent blank (when read against air blank) must be < 0.150 Absorbance (A) at 450 nm.
3. The mean O.D. for the Calibrator should be ≥ 0.250 at 450 nm (when read against reagent blank).
4. The Index Values for the Positive and Negative Controls should be in their respective ranges printed on the vial labels. If the control values are not within their respective ranges, the test should be considered invalid and should be repeated.

5. Additional Controls may be tested according to guidelines, or requirements of local, state, and/or federal regulations or accrediting organizations.

6. Refer to NCCLS C24-A for guidance on appropriate QC practices (14).

7. If above criteria are not met upon repeat testing, contact DAI Technical Services.

**Interpretation**

1. **Mean Calibrator O.D.** - Calculate the mean value for the Calibrator from three Calibrator determinations. If any of the three Calibrator Values differ by more than 15% from the mean, discard that value and calculate the average of the two remaining values.

2. **Correction Factor** - To account for day-to-day fluctuations in assay activity due to room temperature and timing, a Correction Factor is determined by DAI for each lot of kits. The Correction Factor is printed on the Calibrator vial.

3. **Cutoff Calibrator Value** - The Cutoff Calibrator Value for each assay is determined by multiplying the Correction Factor by the mean Calibrator O.D. determined in Step 1.

4. **Index Value** - Calculate an Index Value for each specimen by dividing the specimen O.D. value by the Cutoff Calibrator Value determined in Step 3.

Example:

O.D.s obtained for Calibrator = 0.38, 0.42, 0.40
Mean O.D. for Calibrator = 0.40
Correction Factor = 0.50
Cutoff Calibrator Value = 0.50 x 0.40 = 0.20
O.D. obtained for patient sera = 0.60
Index Value = 0.60/0.20 = 3.00

**Analysis**

1. The patients' Index Values are interpreted as follows:

<table>
<thead>
<tr>
<th>Index</th>
<th>Results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.90</td>
<td>Negative</td>
<td>No detectable antibody to Rheumatoid Factor by the ELISA test.</td>
</tr>
<tr>
<td>0.91-1.09</td>
<td>Equivocal</td>
<td>Equivocal Samples should be retested in this assay or analyzed in a different assay system.</td>
</tr>
<tr>
<td>≥ 1.10</td>
<td>Positive</td>
<td>Indicates presence of detectable antibody to Rheumatoid Factor by the ELISA test.</td>
</tr>
</tbody>
</table>
**International Unit Conversion**

International unit (IU) reactivity is determined relative to the IU standard. Conversion of Index values to international units is accomplished by using an exponential regression analysis. Each lot is standardized versus international units and provided with a lot specific conversion table (Conversion of International Units (IU) per mL for RF IgM) For example:

<table>
<thead>
<tr>
<th>Index Value</th>
<th>IU</th>
<th>Index Value</th>
<th>IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>7.7</td>
<td>3.0</td>
<td>189.8</td>
</tr>
<tr>
<td>1.5</td>
<td>16.6</td>
<td>3.5</td>
<td>368.7</td>
</tr>
<tr>
<td>2.0</td>
<td>36.0</td>
<td>4.0</td>
<td>800.5</td>
</tr>
<tr>
<td>2.5</td>
<td>78.2</td>
<td>4.5</td>
<td>1738.0</td>
</tr>
</tbody>
</table>

See attached addendum for the lot specific conversion table.

**Expected Values**

Rheumatoid arthritis is a chronic inflammatory disease afflicting about 1-4% of the population. The highest incidence of rheumatoid factors occurs in persons over 65 years of age, reaching approximately 20% when latex fixation is used. The clinical correlation of an elevated rheumatoid factor should be interpreted cautiously. Increased titers may accompany a variety of acute immune responses, particularly viral infections and a number of other diseases (e.g., infectious mono-nucleosis, tuberculosis, leprosy, various parasitic diseases, liver disease, sarcoidosis, and lymphoproliferative syndromes) (6).

**Limitations of Use**

1. Only if test instructions are rigidly followed will optimum results be achieved.
2. Reproducible results depend on careful pipetting, observation of incubation periods and temperature, as well as washing the test strips and thorough mixing of all prepared solutions.
3. If comparisons with other methods are required, always perform both tests simultaneously.
4. Do not scratch coated wells during washing and aspiration. Wash and fill all reagents without interruption. While washing, check that all wells are filled evenly with washing solution, and that there are no residues in the wells.
5. Instructions for using appropriate photometers are to be observed; check adjustment of proper wavelength (450 nm) and reference wavelength (600-650 nm, optional) respectively.
6. The values obtained from this assay are intended to be an aid for diagnosis only. Each physician must interpret the results in conjunction with the patient's history, physical findings and other diagnostic procedures.

**Performance Characteristics**

**Sensitivity and Specificity**

A study was performed using 204 patient sera obtained from outside clinical laboratories. These samples were tested using both the DAI RF ELISA test and a commercially available RF ELISA...
test following the manufacturers’ package inserts. Forty-five samples were found positive by the ELISA test, the remaining 157 samples were negative by the reference ELISA test. Two samples were found to be false positive and none found false negative on the DAI test as compared to the ELISA reference method. Using the above data criteria, the DAI RF ELISA test has a 100% sensitivity and 98.7% specificity as compared to the results obtained on the reference ELISA method. The following data were obtained:

<table>
<thead>
<tr>
<th>DAI Rheumatoid</th>
<th>Reference +</th>
<th>ELISA -</th>
<th>Relative Sensitivity</th>
<th>Relative Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45</td>
<td>2</td>
<td>0</td>
<td>100%</td>
</tr>
</tbody>
</table>

98.7% Factor

(45/45) (157/159)

Agreement = 99.0%

**Reproducibility**

Studies were performed to assess the precision of the test using five patient sera run in 10 wells each in one assay. The Intra-assay results are as follows:

<table>
<thead>
<tr>
<th>Serum 1</th>
<th>Serum 2</th>
<th>Serum 3</th>
<th>Serum 4</th>
<th>Serum 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.23</td>
<td>.57</td>
<td>.53</td>
<td>2.46</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.02</td>
<td>0.03</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>C.V.</td>
<td>10.4%</td>
<td>5.4%</td>
<td>4.2%</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

Another run was performed to evaluate the Inter-assay precision using five patient sera run over five days each. The following results were obtained:

<table>
<thead>
<tr>
<th>Serum 1</th>
<th>Serum 2</th>
<th>Serum 3</th>
<th>Serum 4</th>
<th>Serum 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.11</td>
<td>0.64</td>
<td>1.47</td>
<td>2.25</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.01</td>
<td>0.03</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>C.V.</td>
<td>14.2%</td>
<td>4.6%</td>
<td>6.1%</td>
<td>3.6%</td>
</tr>
</tbody>
</table>

**International Unit Conversion**

The data in Table 1 illustrate RF Index Values for the serially diluted international unit standard, obtained from the World Health Organization. The RF Index values are compared to serial dilutions of the international unit standard serum by linear regression (exponential regression analysis). The data indicate that international units can be determined from the Index value.
Table 1
International Unit Conversion

<table>
<thead>
<tr>
<th>International Unit Standard Units / mL</th>
<th>Index Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.8</td>
</tr>
<tr>
<td>12.5</td>
<td>1.2</td>
</tr>
<tr>
<td>6.30</td>
<td>0.8</td>
</tr>
<tr>
<td>3.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\[ r^2 = 0.979 \quad a = 0.633 \quad b = 0.311 \quad Y = \text{Index} \quad X = \text{IU} / \text{mL} \]

Exponential Regression Equation Calculation

\[ X = \frac{(y+b)}{e^x} = \text{derived IU} / \text{mL} \]

References


DAI Code # 1

<table>
<thead>
<tr>
<th>Date Adopted</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008-05-01</td>
<td>DA-Rheumatoid Factor IgM-2008</td>
</tr>
</tbody>
</table>

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Revision Date: 8/18/08

DAI Rheumatoid Factor IgM Summary of Assay Procedure

1. Dilute serum
2. Add to micro wells 100µL
3. WASH
4. Add conjugate 100µL
5. WASH
6. Add TMB solution 100µL
7. Add stop 100µL 1N H₂SO₄
8. Read at 450nm