ANTI-BORRELIA (LYME) MICROPLATE EIA
Cat. No. 32507

For the qualitative and/or semiquantitative detection of Borrelia burgdorferi total antibodies (IgG, IgM, and IgA) in serum by enzyme immunoassay (EIA) to be used as an aid in the diagnosis of Lyme disease.

SUMMARY AND EXPLANATION
Lyme borreliosis is reported to occur in North America, Europe, and Asia (1,2,3,4,5). Lyme disease has been present in Europe for years, but was first recognized in the United States in 1975 at Lyme, Connecticut. The disease, transmitted through the bite of a tick infected with the spirochete, Borrelia burgdorferi, exhibits a variety of symptoms which may be confused with immune and inflammatory disorders. Inflammation around the tick bite eventually causing skin lesions, erythema chronic migrans (ECM), is the first stage of disease. B. burgdorferi disease is also associated with neurologic or cardiac symptoms (stage 2) or arthritic symptoms (stage 3). A definitive distinction between stages is not always seen. In some cases, these secondary symptoms may occur even though the patient does not remember a tick bite or rash.

The criteria for the diagnosis of Lyme borreliosis are not clearly defined. Unless the typical ECM lesions are present, serological diagnosis is necessary to identify patients exposed to the agent. However, cross-reactions within the Borrelia genus and other cross-reactions (e.g., flagellin reactions with spirichetes and membrane reactions with bacterial membrane proteins) have limited the reliability of B. burgdorferi serology. The Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA combines purified Borrelia burgdorferi cell lysate and the recombinant 39 kilodalton (P39) protein as antigens (6,7). Therefore, human antibodies against any Borrelia burgdorferi antigen (i.e., flagellin [41Kd], outer surface proteins [Osa and Osb], etc.) are detected using this test kit.

Antibodies against P39, unlike antibodies to flagellin which cross-react with other spirochetal flagellins, are specific to Borrelia burgdorferi and are conserved among North American and European isolates (7). Because P39 protein is highly antigenic, but constitutes only a small fraction of the protein in the organism, it may not be detected in other assay systems (e.g., western blots, IFA, IFA, etc.) which are not enriched with P39 protein.

PRINCIPLES OF THE PROCEDURE
The Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA utilizes an EIA microtiter plate technique for the detection of antibodies. Because the B. burgdorferi-specific P39 protein represents a small fraction of borrelia protein, assay sensitivity is improved by addition of recombinant P39 antigen to the entire borrelial cell extract. To improve assay specificity serum is absorbed in a blocking solution containing E. coli proteins. The blocked serum is added to antigen coated microtiter wells and allowed to react. After removal of unbound antibodies, horseradish peroxidase-conjugated antihuman antibodies are allowed to react with bound antibodies. The bound peroxidase reacts with 2-2’-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS®), the chromogenic substrate, developing a color. Finally, the substrate reaction is stopped and the optical density is read with a spectrophotometric microewell reader.

PRODUCT INFORMATION
96 determinations

Kit is stored at +2-8°C until stated expiration date. Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA reagents have been optimized for use as a system. Do not substitute other manufacturers’ reagents or other Microrit Tes reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use kit if evidence of microbial contamination (cloudiness) is present. The Color Developer should be used within one hour after preparation. Do not ingest reagents. Do not use any kits beyond the stated expiration date. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since this may result in poor assay performance.

R1: Microplate: 12 x 8 microwell strips
Coated with Borrelia burgdorferi sensu strictu (strain B31) extract and P39 recombinant protein. Reaction Wells are removed from the foil pouch and unused wells are resealed in the foil pouch using the integral zip-lock.

R2: Specimen Diluent: 1 x 50 ml
0.01 M phosphate buffered saline (PBS, pH 6.2-7.6) and carrier protein containing <0.1% NaN₃.

S1: Calibrator: 1 x 0.3 ml
Human anti-B. burgdorferi serum (prediluted to 1:20) in Specimen Diluent.

C0: Negative Control: 1 x 0.1 ml
Nonreactive human serum containing <0.1% NaN₃.

C1: Positive Control: 1 x 0.1 ml
Human anti-B. burgdorferi serum containing <0.1% NaN₃.

R3: Borrelia Blocker: 1 x 25 ml
E. coli protein in 0.01 M PBS (pH 6.2–7.6) with carrier protein and <0.1% NaN₃.

R4: Wash Buffer Concentrate: [20X], 1 x 50 ml
0.2 M PBS and 1.0% Tween. Final solution (pH 6.2–7.6).

R5: Conjugate: 1 x 12 ml
Peroxidase-conjugated goat anti-human antibodies (IgG, IgM and IgA) in 0.01 M PBS (pH 6.2–7.6) and carrier protein containing preservatives.

R6: Substrate Buffer: 1 x 25 ml
0.1 M sodium citrate (pH 4.4–4.6) and 0.01% hydrogen peroxide.

R7: Substrate Concentrate: 1 x 1.5 ml
2.19% 2-2’-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS®) in 0.1 M sodium citrate (pH 4.4–4.6).

R8: Stop Solution: 1 x 25 ml
0.25 M Oxalic Acid.

WARNINGS AND PRECAUTIONS
1. For in vitro diagnostic use.
2. This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Handle appropriately with the requisite Good Laboratory Practices (GLP). Do not eat, drink or smoke when using this product. Wear appropriate protective clothing, including lab coat, eye/face protection and disposable gloves (synthetic, non-latex gloves are recommended) while handling kit reagents and patient samples. Wash hands thoroughly after performing the test.

3. Human source material. Material used in the preparation of this product has been tested and found non-reactive for hepatitis B surface antigen (HBsAg), antibodies to hepatitis C virus (HCV), and antibodies to human immunodeficiency virus (HIV-1 and HIV-2). Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting infectious disease (8). Follow recommended Universal Precautions for bloodborne pathogens as defined by OSHA (14), Biosafety Level 2 guidelines from the current CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (15), WHO Laboratory Biosafety Manual (16), and/or local, regional and national regulations.

4. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. It may be harmful if enough is ingested (more than supplied in kit). On disposal of liquids, flush with a large volume of water to prevent azide build-up (9). This dilution is not subject to GHS, US HCS and EU Regulation 2008/1272/EC labeling requirements.

5. 0.25M Oxalic acid [-3% C₂H₂O₄, CAS# 144-62-7, EC No 205-634-3] may be harmful if swallowed so handle according to GLP. This dilution is not subject to GHS, US HCS and EU Regulation 2008/1272/EC labeling requirements.

The safety data sheet (SDS) is available at www.bio-rad.com or upon request.

SPECIMEN COLLECTION AND PREPARATION
The Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA is performed on serum. Hemolyzed or lipemic serum has not been shown to be an acceptable specimen. The test requires 10 µL of serum. Serum is collected according to standard practices and may be stored at +2-8°C for up to five days. Serum may be frozen below -20°C for extended periods.

Regulation 2008/1272/EC labeling requirements.

1. 4

2. 3

3. 2
PROCEDURE

Materials Provided
R1: Microplate
R2: Specimen Diluent
C0: Negative Control
C1: Positive Control
S1: Calibrator
R3: Borrelia Blocker
R4: Wash Buffer Concentrate (20X)
R5: Conjugate
R6: Substrate Buffer
R7: Substrate Concentrate
R8: Stop Solution

Materials Required But Not Provided
1. Distilled or deionized water
2. Pipets
3. Microwell washer
4. Test tubes
5. Microwell spectrophotometer (405 nm)

Procedural Comments
1. Reproducibility of the assay is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the assay procedure.
2. Positive and Negative Control Sera (Undiluted) – These control sera are used to assure test performance.
3. Calibrator (Prediluted to 1:20) – Used to standardize between run values.
4. Substrate Blank – All reagents, except serum, are added to the substrate blank well. This blank well is intended to baseline (zero) the microwell spectrophotometer.

Wash Buffer Preparation
Wash Buffer (pH 6.2–7.6) is prepared by adding the contents of the Wash Buffer Concentrate (20X) bottle into 1 liter of distilled/deionized water. After reconstitution, the 1X solution is stored at +2-8°C. Discard if visibly turbid.

NOTE: In some instances the Wash Buffer Concentrate (20X) may develop crystals upon storage at +2-8°C. It is important that these crystals be completely redissolved before dilution of the Concentrate. This can be accomplished by warming the Concentrate to +37°C in a water bath with occasional mixing.

Color Developer Preparation
Color Developer is prepared by adding one (1) drop of Substrate Concentrate to 1ml of Substrate Buffer. One ml of Color Developer is sufficient for one eight-well strip. Use within one hour.

Procedure
1. Allow all components including diluted Wash Buffer to warm to room temperature (+22-27°C).
2. Determine the total number of specimens to be run. In addition to the specimens, one blank, duplicates of calibrator, and duplicates of control are included in each run.
3. For each specimen and control, pipet 10 µL serum into a clean tube containing 200 µL Specimen Diluent and mix (1:20 dilution).
   CAUTION: Do not dilute Calibrator in Specimen Diluent. It is provided ready to use at a 1:20 dilution in Specimen Diluent.
4. Pipet 10 µL of each 1:20 diluted specimen or control (step 3) into a clean tube containing 200 µL Borrelia Blocker and mix gently. Pipet 20 µL predilute Calibrator into a clean tube containing 400 µL Borrelia Blocker and mix gently.
5. Determine the total number of wells to be run including blank, specimens, calibrators, and controls. Well strips can be broken to the exact number needed to conserve reagent wells. Strips need to be completed with empty wells to facilitate washing procedures.
6. Add 100 µL of Borrelia Blocker into the first well as a Substrate Blank. Using a pipet, transfer 100 µL of blocked samples to each assigned well.
7. Incubate at room temperature (+22-27°C) for 30±5 minutes.
8. Aspirate the samples out of the wells. Do not allow the wells to dry.
9. Wash the wells three (3) times by completely filling the wells with Wash Buffer (See Wash Buffer Preparation) and aspirate the wells completely after washes.
10. Pipet 100 µL Conjugate into all wells.
11. Incubate the wells at room temperature (+22-27°C) for 30±5 minutes.
12. Aspirate the conjugate out of the wells. Do not allow the wells to dry.
13. Wash the wells three (3) times as described in step 9.
15. Pipet 100 µL of Color Developer into each well.
16. Incubate at room temperature (+22-27°C) for 30±5 minutes.
17. Add 100 µL of Stop Solution to each well.
18. Inspect the outside bottom surface of the microwells for the presence of condensation, dried buffer salts or wash solution which might interfere with the spectrophotometric reading. Carefully clean the well bottoms with a soft tissue.
19. Using the Substrate Blank to zero the spectrophotometer, read the optical density of each well at 405 nm within 30 minutes of completion of step 17.

NOTE: If blank can not be automatically subtracted by the instrument, the value for the blank well must be manually subtracted from each control, calibrator, and sample value before calculating results.

QUALITY CONTROL
1. Calibrator O.D. ≥ 0.600 absorbance.
2. Determine the activity of the positive and negative controls using the method described in the next section. Each control must be within the expected range given in the Package Insert Supplement included in this reagent kit.

INTERPRETATION
The assigned value (U/ml) of the calibrator used in the calculation below will vary by lot number. Please verify that the lot number on the vial matches the lot number on the Package Insert Supplement to assure the proper value is used in the calculation.

In order to eliminate the effects of washing variation, instrument variability, etc. specimen values are normalized according to the following calculation:

\[ A_N = \frac{A_s}{A_c} \times AV_c \]

Where:
\[ A_N = \text{Normalized activity of the specimen (U/ml)} \]
\[ A_s = \text{Absorbance of the specimen} \]
\[ A_c = \text{Mean absorbance of the Calibrator obtained in the assay} \]
\[ AV_c = \text{Assigned Value (U/ml) of the Calibrator given in the Supplement} \]

The interpretive ranges are:

<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
<th>U/ml</th>
<th>CLINICAL INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONREACTIVE</td>
<td>&lt;120</td>
<td>Antibody to B. burgdorferi not detected. If symptomology is suggestive of Lyme disease, redraw in 2–4 weeks and retest.</td>
</tr>
<tr>
<td>BORDERLINE</td>
<td>120 – 159</td>
<td>Repeat the test using the same sample. If result repeats as either reactive or non reactive, report the definitive result. If test repeats as borderline, report test as “borrelia antibody detected at borderline (equivocal) level.” Suggest that another sample be tested 2–4 weeks later.</td>
</tr>
<tr>
<td>REACTIVE</td>
<td>≥160</td>
<td>Antibody to B. burgdorferi detected.</td>
</tr>
</tbody>
</table>

This assay, like all tests which use borrelia proteins (flagellin, outer membrane antigens, etc.) has cross-reactions with antibodies against some other agents. Further testing with other methods (western blot analysis (10), specific recombinant proteins, RPR to rule out syphilis) may be indicated.
LIMITATIONS OF THE PROCEDURE

1. Positive results should be interpreted with caution. Sera from patients with other spirochetal diseases (syphilis, yaws, pinta, leptospirosis, relapsing fever, peridontal disease, etc.) may also give positive results. The diagnosis of Lyme Disease should be based on interpretation of test results in combination with patient clinical signs and symptoms, other clinical and laboratory test results, and epidemiological data.

2. Patients with connective tissue autoimmune diseases (rheumatoid arthritis, ANA, SLE, etc.) may have antibodies which cross-react with B. burgdorferi antigens.

3. Patients with other bacterial and viral infections such as Rocky Mountain Spotted Fever, EBV, CMV, and HIV may also have antibodies which cross-react with B. burgdorferi antigens.

4. Negative results do not rule out the diagnosis of Lyme Disease. Early antibiotic therapy may suppress antibody response, and some individuals may not develop antibodies above detectable levels. Negative results in suspected early Lyme Disease should be repeated in 4-6 weeks.

5. The continued presence or absence of antibodies cannot be used to determine the success or failure of therapy.

6. Testing should not be performed as a screening procedure for the general population. The predictive accuracy of a positive or negative serologic result depends on the pretest likelihood of Lyme disease being present. Testing should only be done when clinical evidence suggests the diagnosis of Lyme disease.

7. A single positive result only indicates previous immunologic exposure; level of antibody response or class of antibody response may not be used to determine active infection or disease stage.

EXPECTED RESULTS

In general, three types (stages) of Lyme disease are recognized: erythema chronicum migrans (ECM), neurologic, and arthritic. Antibody levels are generally low or absent during early (ECM) infection. Most symptomatic patients will have either no antibody or highly cross-reactive antibody during the first 1–2 weeks after tick bite and the antibody titer will rise and become more specific with time. Highest antibody levels are seen in chronic arthritis subjects.

The number of antibody positive subjects in a population depends on several factors: 1) prevalence of the causative agent, 2) assay used to detect antibody, and 3) clinical screening criteria to select tested subjects. Because early assays lacked accuracy (11), the number of antibody positive subjects in a population at present (1991) is highly dependent on the assay used. Whenever a suitably accurate test is used, few positives should be detected in a randomly screened population in a non-endemic area. On the other hand, if patients with typical ECM signs in an endemic region are tested, many positive results are expected.

Disagreement between assays which do not use an absorbant and those assays like the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA which do use an absorbant are expected. Fawcett (12,13) has shown that assays using an absorbant were equally sensitive to those without an absorbant and that the absorbed assays were significantly more specific. Addition of recombinant P39 protein does not affect assay specificity, but does increase the absorbance reading for some samples. Cross reactions with other borrelia or closely related species (treponema and leptospira) are expected because of the common flagellin protein.

SPECIFIC PERFORMANCE CHARACTERISTICS

Reproducibility

The within-run and between-run variability were determined using five different runs of reference samples tested in triplicate. The results are shown in Table 1.

Table 1 ANTI-BORRELIA (LYME) MICROPLATE EIA REPRODUCIBILITY

<table>
<thead>
<tr>
<th>MEAN</th>
<th>WITHIN ASSAY VARIATION (%CV)</th>
<th>BETWEEN ASSAY VARIATION (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1140</td>
<td>4%</td>
<td>9%</td>
</tr>
<tr>
<td>800</td>
<td>3%</td>
<td>5%</td>
</tr>
<tr>
<td>580</td>
<td>5%</td>
<td>11%</td>
</tr>
<tr>
<td>440</td>
<td>4%</td>
<td>10%</td>
</tr>
<tr>
<td>300</td>
<td>5%</td>
<td>13%</td>
</tr>
<tr>
<td>150</td>
<td>7%</td>
<td>20%</td>
</tr>
<tr>
<td>20</td>
<td>21%</td>
<td>57%</td>
</tr>
</tbody>
</table>

Correlation

The specificity of the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA using 250 sera from asymptomatic blood donors collected from areas other than the upper Midwestern and Northeastern United States (hyperendemic regions) is 100% specific. Six samples (2.4%) tested borderline.

The specificity was also determined using sera from Lyme disease negative, symptomatic subjects (Table 2). Because of the high frequency of false positive reactions with samples from syphilis patients, subjects suspected to have syphilis or related disorders must also be tested with a non-treponemal test (e.g. RPR) to rule out this cause.

Table 2 SPECIFICITY – LYME DISEASE NEGATIVE PATIENTS

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Nonreactive</th>
<th>Borderline</th>
<th>Reactive</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA +</td>
<td>37</td>
<td>1</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>CMV IgM +</td>
<td>24</td>
<td>0</td>
<td>1</td>
<td>96%</td>
</tr>
<tr>
<td>Heterophile +</td>
<td>20</td>
<td>4</td>
<td>1</td>
<td>96%</td>
</tr>
<tr>
<td>RF +</td>
<td>23</td>
<td>1</td>
<td>1</td>
<td>96%</td>
</tr>
<tr>
<td>Syphilis +</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>8%</td>
</tr>
<tr>
<td>HIV+</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>100%</td>
</tr>
</tbody>
</table>

Sera from seventy-seven patients diagnosed with Lyme borreliosis were used to assess assay sensitivity. Diagnoses were based on epidemiological, clinical, and serological criteria. These studies were conducted by two outside laboratories. The results are summarized in Table 3, and site specific results follow.

Table 3 SENSITIVITY – DIAGNOSED LYME DISEASE PATIENTS

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Nonreactive</th>
<th>Borderline</th>
<th>Reactive</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 (Early)</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>88%</td>
</tr>
<tr>
<td>Stage 2 (Neurological)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>Stage 3 (Arthritic)</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>100%</td>
</tr>
<tr>
<td>Unknown Stage</td>
<td>0</td>
<td>0</td>
<td>47</td>
<td>100%</td>
</tr>
</tbody>
</table>

Each trial site used clinical, epidemiologic, and laboratory criteria to classify the patients as Lyme disease cases. Site 1 subdivided the patients into the three stages of disease which are indicated in Table 3. Four Site 1 patients are classified as unknown stage, and the balance of the other unknown stage patients are from Site 2. At Site 1, the laboratory performed an absorbed and unabsorbed EIA test as well as a western blot analysis. The mean absorbance values for the three EIA assays performed at Site 1 are shown in Table 4. These data further support that the use of E. coli absorption does not interfere with assay sensitivity, and show that higher antibody reactivity is associated with later stage disease.

Table 4 SITE 1 RESULTS

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Stage 1</th>
<th>Stage 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard EIA</td>
<td>560</td>
<td>1160</td>
</tr>
<tr>
<td>Absorbed EIA</td>
<td>530</td>
<td>1220</td>
</tr>
<tr>
<td>Bio-Rad Lyme</td>
<td>550</td>
<td>1020</td>
</tr>
</tbody>
</table>

Site 1 also tested fifty sera from Lyme disease negative subjects. This control group was comprised of sera from normal volunteers and patients with diagnosed viral infections (Epstein-Barr virus) and rheumatic disorder (juvenile arthritis and systemic lupus erythematosus) and sera from patients seen at the rheumatology clinic with other unclassified disorders among whom past or current Lyme disease was ruled out. None of the sera reacted in the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA (100% specificity). Two sera (4%) reacted as borderline.

Site 2 tested sera from 43 Lyme disease patients and all reacted positive in Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA. This site also tested a select group of 55 sera from patients who had been originally classified as possibly having Lyme disease, but were subsequently determined to have western
The Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA identified two sera originally classified as negative by the alternate EIA as reactive and agreed with the negative classification of the remaining 51 samples. Agreement between samples classified as reactive by the alternate assay and the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA was poor. This discrepancy is expected because the alternate EIA does not use an absorbent to remove cross-reactive antibody. Loss of test sensitivity is not anticipated by use of the absorbent.

Thirty-nine proficiency samples from two state public health laboratories and a national proficiency program were also used to evaluate the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA performance. The Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA identified 17 out of 17 nonreactive samples correctly and 17 of 18 reactive samples correctly. One reactive sample was determined to be borderline by the Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA. In addition, three of four borderline proficiency samples were identified as nonreactive.

### REFERENCES


### Table 5  SUSPECTED LYME DISEASE SUBJECTS

<table>
<thead>
<tr>
<th>Bio-Rad Laboratories Anti-Borrelia (Lyme) Microplate EIA</th>
<th>EIA</th>
<th>Nonreactive</th>
<th>Borderline</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonreactive</td>
<td>51</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Reactive</td>
<td>49</td>
<td>11</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

### PROCEDURE SUMMARY

1. Prepare Wash Buffer from Wash Concentrate

2. Pipet 10 µL each patient specimen or control into 200 µL Specimen Diluent (1:20 dilution).

3. Add 10 µL of each 1:20 diluted specimen or control to 200 µL Borrelia Blocker and mix. Add 20 µL of each prediluted calibrator to 400 µL Borrelia Blocker and mix.

4. Add 100 µL of Borrelia Blocker into the first well as a substrate blank.

5. Pipet 100 µL of the prepared calibrator, controls, and patient sera to coated microwells and incubate 30 min at room temperature.

6. Aspirate microwells and wash microwells three times with Wash Buffer.

7. Pipet 100 µL of Conjugate to microwells and incubate 30 min at room temperature.

8. Aspirate microwells and wash microwells three times with Wash Buffer.

9. Prepare fresh Color Developer

10. Pipet 100 µL of Color Developer to microwells and incubate 30 min at room temperature.

11. Pipet 100 µL of Stop Solution to microwells and read results at 405 nm.