# ab210603 Nitrotyrosine ELISA Kit

Instructions for use:

For quantitative measurement of Nitrotyrosine in a variety of biological samples.

This product is for research use only and is not intended for diagnostic use.

Version 2 Last Updated 28 January 2019

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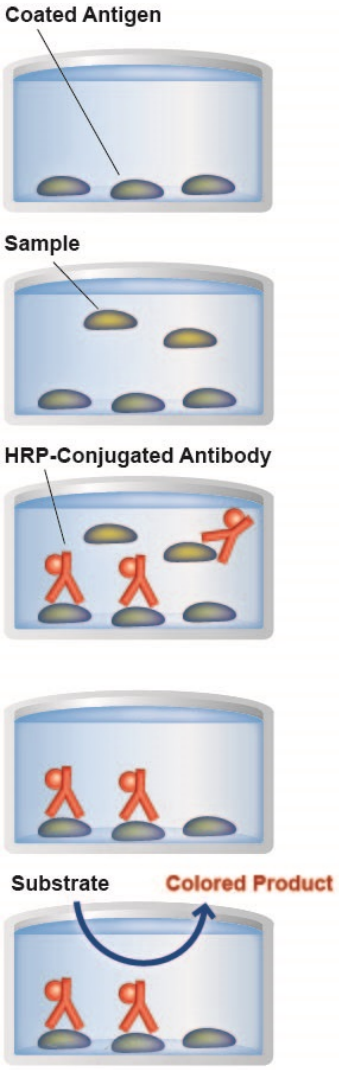
## 1. BACKGROUND

Abcam's Nitrotyrosine *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit (ab210603) is designed for the competitive measurement of Free Nitrotyrosine protein in plasma, serum, cell lysates, urine, and other sample matrices. The ELISA utilizes an Nitrotyrosine-coated plate and an HRP-conjugated antibody for detection which allows for an assay range of 62.5 to 8000 nM Free Nitrotyrosine, with a sensitivity of 50 nM. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

Nitrotyrosine has been identified as a marker of inflammation and NO production. Nitrotyrosine is formed in presence of the active metabolite NO. Various pathways including the formation of peroxinitrite lead to nitrotyrosine production. Since nitrotyrosine is a stable end product of peroxynitrite oxidation, assessment of its plasma concentration may be useful as a marker of NO-dependent damage in vivo. Since $NO_X$ is only an indicator for enhanced NO production, protein associated nitrotyrosine might be a more suitable marker for damage induced by reactive nitrogen intermediates derived from NO. Furthermore, most proteins have a longer half-life in the circulation than $NO_X$ levels. The presence of nitrotyrosine has been detected in various inflammatory processes including atherosclerotic plaques, celiac disease, rheumatoid arthritis, chronic renal failure and septic shock. In normal plasma low, undetectable, levels of nitrotyrosine are present.

Nitrosylation of the amino acid tyrosine occurs both for free tyrosine and for protein bound tyrosine.

## 2. ASSAY SUMMARY




Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.

Add sample and conjugate to appropriate wells. Incubate at room temperature

Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint. Measure absorbance on a plate reader at 450 nm.

## 3. PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- All ELISA reagents must be at room temperature before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

- Take care not to contaminate the TMB Substrate. Do not expose the TMB solution to glass, foil or metal. If the solution is blue before use, do NOT use it.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in triplicate.
- Buffers may crystallize over time. Warm crystallized buffer until the salt crystals return to solution. Ensure that your components return to RT before use in the assay.

## 4. STORAGE AND STABILITY

**Store kit at 4ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

## 5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. MATERIALS SUPPLIED

| Item | Amount | Storage Condition (Before Preparation) | Storage Condition (After Preparation) |
|---|---|---|---|
| Nitrosylated BSA Coated Plate | 96 tests | 4°C | 4°C |
| Nitrotyrosine Standard (Stock) | 110 µL | 4°C | 4°C |
| Anti-Nitrotyrosine:HRP Conjugated Detection Antibody | 75 µL | 4°C | 4°C |
| Sample and Standard Diluent | 50 mL | 4°C | 4°C |
| Nitrotyrosine Antibody Diluent | 13 mL | 4°C | 4°C |
| Wash Buffer Concentrate (10X) | 50 mL | 4°C | 4°C |
| TMB Substrate | 13 mL | 4°C | 4°C |
| Stop Solution | 13 mL | 4°C | 4°C |
| Plate Cover | 2 | 4°C | 4°C |

## 7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 nm
- MilliQ water or other type of double distilled water ($ddH_2O$)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Timer
- Centrifuge capable of spinning at 2000 x g
- 0.2 µm filter
- Log-log graph paper or computer and software for ELISA data analysis

## 8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Completely aspirate all solutions and buffers during wash steps.
- When preparing your standards, it is critical to briefly spin down the vial first.
- The Standard should be aliquoted into smaller portions; it should be used right away or else frozen for later use.
- Be sure to discard the working standard dilutions after use – they do not store well.

## 9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

### 9.1. 1X Wash Buffer

9.1.1. Prepare 1X wash buffer by diluting 10X wash buffer in distilled or deionized water.

For example, if preparing 500 mL of 1X wash buffer, dilute 50 mL of 10X wash buffer into 450 mL of distilled water. Mix well.

*NOTE: Store reconstituted 1X wash buffer at 4ºC for up to one (1) month. Do not use 1X wash buffer if it becomes visibly contaminated during storage.*

### 9.2. Anti-Nitrotyrosine:HRP Conjugated Detection Antibody

9.2.1. Determine the amount of Antibody Preparation required. For every strip-well used (8-wells), prepare 0.5 mL of Antibody Preparation.

9.2.2. Prepare Antibody Preparation by diluting the Anti-Nitrotyrosine:HRP Conjugated Detection Antibody Concentrate 1:100 with Nitrotyrosine Antibody Diluent. For example, if 6 mL of Antibody Preparation is required (one whole plate), dilute 60 µL of Antibody in 6 mL of Nitrotyrosine Antibody Diluent. Mix well prior to use.

## 10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be prepared immediately prior use. Do not store for future use.

10.1. Centrifuge the Nitrotyrosine Standard (stock) vial before removing the cap. This process with assure that all of the standard is collected and available for use.

10.2. Label eight (8) polypropylene tubes, each with one of the following standard values: 8000 nM, 4000 nM, 2000 nM, 1000 nM, 500 nM, 250 nM 125 nM and 62.5 nM.

10.3. Add 500 µL of Sample and Standard Diluent to Tube #1.

10.4. Add 250 µL of Sample and Standard Diluent to Tube #2, 3, 4, 5, 6, 7 and 8.

10.5. Add 10 µL of the 408 µM Nitrotyrosine Standard to Tube #1. Mix well.

10.6. Transfer 250 µL from Tube #1 to Tube #2. Mix well.

10.7. Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix well, etc.) up to and including Tube #8.

10.8. Finally, add 250 µL Sample and Standard Diluent to another 1.5 mL polypropylene tube (Tube #9), which is the zero standard (0 ng/mL).

| Standard # | Standard volume | Assay Buffer volume | Final volume | End Conc. (nmol) |
|---|---|---|---|---|
| Stock | N/A | N/A | N/A | 408 µM |
| S1 | 10 µL of stock | 500 µL | 250 µL | 8000 |
| S2 | 250 µL of S1 | 250 µL | 250 µL | 4000 |
| S3 | 250 µL of S2 | 250 µL | 250 µL | 2000 |
| S4 | 250 µL of S3 | 250 µL | 250 µL | 1000 |
| S5 | 250 µL of S4 | 250 µL | 250 µL | 500 |
| S6 | 250 µL of S5 | 250 µL | 250 µL | 250 |
| S7 | 250 µL of S6 | 250 µL | 250 µL | 125 |
| S8 | 250 µL of S7 | 250 µL | 500 µL | 62.5 |
| S9 (Blank) | N/A | 250 µL | 250 µL | 0 |


Each dilution has enough amount of standard to set up triplicate readings

## 11. SAMPLE PREPARATION

**General Sample Information**

- We recommend that you use fresh samples. We suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- Please be advised that all suggested dilutions below are simply recommended as a starting point, and it may be necessary to adjust the dilution based on experimental results.

### 11.1. Plasma/Serum

11.1.1. Storage: Collect plasma using established methods and store at - 80°C.

11.1.2. Dilution: Serum samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

### 11.2. Cell Lysates

11.2.1. Storage: Collect lysates using established methods and store at -80°C until use.

### 11.3. Urine

11.3.1. Storage: Fresh urine samples should be centrifuged at 2,000 x g for 10 minutes or filtered with a 0.2 µm filter before this assay, and stored at -20°C immediately after collection.

11.3.2. Dilution: Urine samples may be diluted 1:4 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

## 12. PLATE SET UP

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents.

*NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

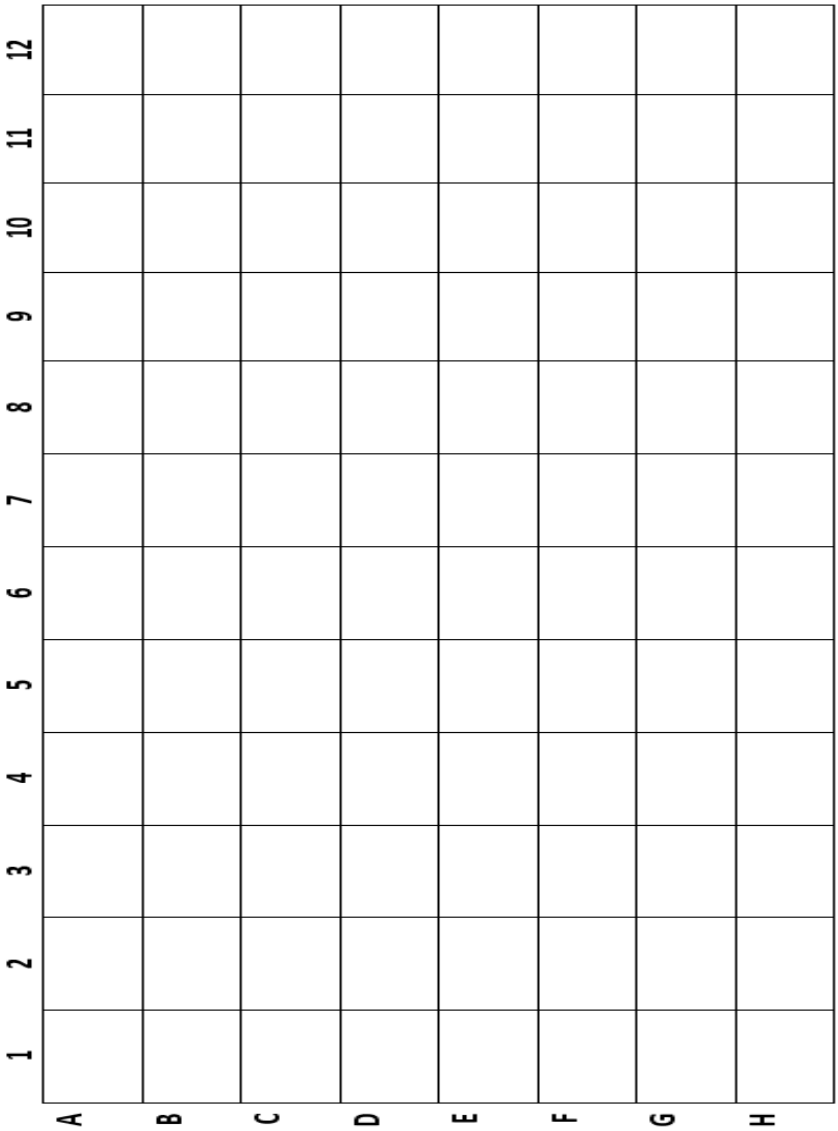
For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis. We suggest you record the contents of each well on the template sheet provided.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | S1 | S1 | S1 | S9 | S9 | S9 | 7 | 7 | 7 | 15 | 15 | 15 |
| B | S2 | S2 | S2 | Blk | Blk | Blk | 8 | 8 | 8 | 16 | 16 | 16 |
| C | S3 | S3 | S3 | 1 | 1 | 1 | 9 | 9 | 9 | 17 | 17 | 17 |
| D | S4 | S4 | S4 | 2 | 2 | 2 | 10 | 10 | 10 | 18 | 18 | 18 |
| E | S5 | S5 | S5 | 3 | 3 | 3 | 11 | 11 | 11 | 19 | 19 | 19 |
| F | S6 | S6 | S6 | 4 | 4 | 4 | 12 | 12 | 12 | 20 | 20 | 20 |
| G | S7 | S7 | S7 | 5 | 5 | 5 | 13 | 13 | 136 | 21 | 21 | 21 |
| H | S8 | S8 | S8 | 6 | 6 | 6 | 14 | 14 | 14 | 22 | 22 | 22 |

**S1 – S8:** 8000- 62.5 nM Standards
**S9:** Zero Standard
**Blk:** Blank
**1 – 22:** Samples

**Plate template sheet**



## 13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to correct temperature prior to use.**
- **We recommended to assay all standards, controls and samples in triplicate.**
- **Prepare all reagents, working standards, and samples as directed in the previous sections.**
- **Always add the Antibody Preparation after the rest of the reagents, as this is a competitive assay.**
- **Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.**

13.1. **Addition of the Reagents**

13.1.1. Add 50 µL (in triplicate) of each of the following to appropriate wells:

13.1.2. Prepared Nitrotyrosine Standard (Tube #1 through Tube #8) into wells labelled S1-S8

13.1.3. Zero Standard (Tube #9- Sample and Standard Diluent, which represents 0 nM into wells labelled S9.

13.1.4. Samples (previously prepared) into wells labelled 1-22.

13.1.5. Add 50 µL of the previously diluted Nitrotyrosine Antibody Preparation to each well, except the blank.

13.1.6. Add 50 µL of Standard and Sample Diluent and 50 µL of Antibody Diluent into wells labelled as the blank.

13.2. **Incubate the Plate**

13.2.1. Cover each plate with the plate cover and incubate 1 hour at room temperature (20- 25˚C).

13.3. **Plate Washing**

13.3.1. Carefully remove adhesive plate cover. Gently squeeze the long sides of the plate frame before washing to ensure all strips remain securely in the frame.

13.3.2. Empty plate contents. Use a multi-channel pipette to fill each well completely (300 µL) with 1X Wash buffer, then empty plate contents. Repeat procedure three additional times, for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

*NOTE: Follow the same procedure when using an automated plate washer as well. Take care to avoid microbial contamination of equipment. Automated plated washers can easily become contaminated thereby causing assay variability.*

13.4. **TMB Substrate Incubation and Reaction Stop**

13.4.1. Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used.

13.4.2. Do NOT use a glass pipette to measure the TMB Substrate solution. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT.

13.4.3. Add 100 µL of TMB Substrate into each well.

13.4.4. Cover carefully with the second provided plate cover.

13.4.5. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. The substrate reaction yields a blue solution.

13.4.6. After 30 minutes, carefully remove the plate cover, and stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

13.5. **Absorbance Measurement**

*NOTE: Evaluate the plate within 30 minutes of stopping the reaction.*

13.5.1. Wipe underside of wells with a lint-free tissue.

13.5.2. Measure the absorbance on an ELISA plate reader set at 450 nm.

## 14. CALCULATIONS

14.1. Many plate readers come with data reduction software that plot data automatically

14.2. The following procedure is recommended for preparation of the data prior to graphical analysis.

a. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD bound.

b. Plot Net OD versus Concentration of Nitrotyrosine for the standards. Sample concentrations may be calculated off of Net OD values using the desired curve fitting.

c. Samples that read at concentrations outside of the standard curve range will need to be re-analyzed using a different dilution. Make sure to multiply sample concentrations calculated off the curve by the dilution factor used during sample preparation to get starting sample concentration.

## 15. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed

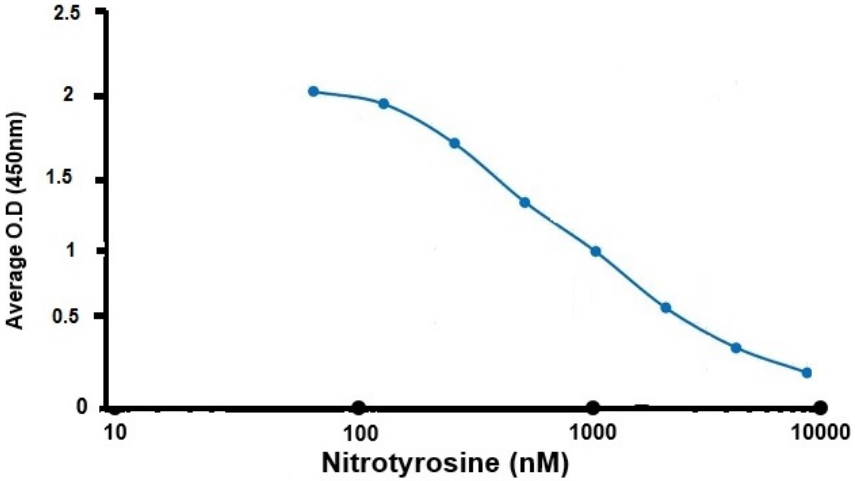



**Figure 1** Standard calibration curve

**Assay Range:** 62.5-8000 nM

**Sensitivity:** The sensitivity of the Nitrotyrosine ELISA kit has been determined to be 50 nM pure nitrotyrosine.

**Intra-Assay Precision:** To determine Intra-Assay Precision, three samples of known concentration were assayed thirty times on one plate. The intra-assay coefficient of variation of the Nitrotyrosine ELISA has been determined to be <10%.

**Inter-Assay Precision:** To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The inter-assay coefficient of variation of the Nitrotyrosine ELISA has been determined to be <15%.

## 16. QUICK ASSAY PROCEDURE

***NOTE:*** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Get equipment ready, prepare reagents, prepare samples in triplicate, prepare standards

| Standard # | Standard volume | Assay Buffer volume | Final volume | End Conc. (nmol) |
|---|---|---|---|---|
| Stock | N/A | N/A | N/A | 408 µM |
| S1 | 10 µL of stock | 500 µL | 250 µL | 8000 |
| S2 | 250 µL of S1 | 250 µL | 250 µL | 4000 |
| S3 | 250 µL of S2 | 250 µL | 250 µL | 2000 |
| S4 | 250 µL of S3 | 250 µL | 250 µL | 1000 |
| S5 | 250 µL of S4 | 250 µL | 250 µL | 500 |
| S6 | 250 µL of S5 | 250 µL | 250 µL | 250 |
| S7 | 250 µL of S6 | 250 µL | 250 µL | 125 |
| S8 | 250 µL of S7 | 250 µL | 500 µL | 62.5 |

16.1. Add 50 µL of prepared standards and samples in triplicate to appropriate wells.

16.2. Add 50 µL of the diluted antibody preparation to the appropriate wells.

16.3. Cover plate with Plate Cover and incubate at room temperature (20-25°C) for 1 hour.

16.4. Wash plate 4 times with 1X Wash Buffer.

16.5. Add 100 µL of TMB Substrate to each well.

16.6. Cover plate and develop the plate in the dark at room temperature for 30 minutes.

16.7. Add 100 µL of Stop Solution to each well.

16.8. Measure absorbance on a plate reader at 450 nm.

16.9. Plot the standard curve and calculate sample concentrations.

## 17. TROUBLESHOOTING

| Problem | Cause | Solution |
|---|---|---|
| Poor Standard Curve | Improper standard solution | Confirm dilutions are made correctly. |
| | Standard degraded | Store and handle standard as recommended. |
| | Curve doesn't fit scale | Try plotting using different scales |
| | Pipetting Error | Use calibrated pipettes and proper pipetting technique. |
| No Signal | Plate washings too vigorous | Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually. |
| | Wells dried out | Do not allow wells to dry out. Cover the plate for incubations. |
| High Background | Wells are insufficiently washed | Wash wells as per protocol |
| | Contaminated wash buffer | Prepare fresh wash buffer |
| | Waiting too long to read the plate after adding stop solution | Read plate immediately |
| Low sensitivity | Standard is degraded | Replace standard |
| | Mixing or substituting reagents from other kits | Avoid mixing components |

## 18. NOTES

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