# ab324510 – Human/Rat Tyrosine Hydroxylase SimpleStep ELISA® Kit

For the quantitative measurement of Tyrosine Hydroxylase in human/rat cell extract and tissue extract.
For research use only - not intended for diagnostic use.

**For overview, typical data and additional information please visit: www.abcam.com/ab324510**

This kit is available in a 384-well plate format. This plate utilises smaller volumes of standards and samples per well. Directions for using this format can be found on pages 6-7.

**Storage and Stability:** Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

## Materials Supplied

| Item | Quantity 1 x 96 tests | Quantity 10 x 96 tests | Storage Condition |
|---|---|---|---|
| Human/Rat Tyrosine Hydroxylase Capture Antibody 10X | 600 µL | 10 x 600 µL | +4°C |
| Human/Rat Tyrosine Hydroxylase Detector Antibody 10X | 600 µL | 10 x 600 µL | +4°C |
| Human/Rat Tyrosine Hydroxylase Lyophilized Recombinant Protein | 2 Vials | 10 x 2 Vials | +4°C |
| Antibody Diluent CPR2 | 6 mL | 10 x 6 mL | +4°C |
| Cell Extraction Buffer SSW | 10 mL | 2 x 50 mL | +4°C |
| Cell Extraction Enhancer Solution 50X | 1 mL | 10 x 1 mL | +4°C |
| Denaturant | 500 µL | 10 x 500 µL | +4°C |
| Sample Diluent NS | 12 mL* | N/A | +4°C |
| Wash Buffer PT 10X | 20 mL | 200 mL | +4°C |
| TMB Development Solution | 12 mL | 120 mL | +4°C |
| Stop Solution | 12 mL | 120 mL | +4°C |
| SimpleStep Pre-Coated 96-Well Microplate | 96 wells | 10 x 96 wells | +4°C |
| Plate Seal | 1 | 10 | +4°C |

*Sample Diluent NS is provided but not necessary for this product.

## Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

## Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

The provided Cell Extraction Enhancer Solution 50X may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The Cell Extraction Enhancer Solution 50X can be stored at room temperature to avoid precipitation.

**1X Wash Buffer PT:** Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

**1X Cell Extraction Buffer SSW:** Prepare 1X Cell Extraction Buffer SSW by diluting Cell Extraction Buffer SSW Stock to 1X with 1X Wash Buffer PT. To make 10 mL 1X Cell Extraction Buffer SSW combine 9.6 mL 1X Wash Buffer PT and 0.4 mL Cell Extraction Buffer SSW Stock. Mix thoroughly and gently. If required protease inhibitors can be added.

**1X Cell Extraction Buffer SSW + Enhancer:** Prepare 1X Cell Extraction Buffer SSW + Enhancer by diluting Cell Extraction Buffer SSW Stock and Cell Extraction Enhancer Solution 50X to 1X with 1X Wash Buffer PT. To make 10 mL 1X Cell Extraction Buffer SSW combine 9.4 mL 1X Wash Buffer PT, 0.4 mL Cell Extraction Buffer SSW Stock, and 0.2 mL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.

**Antibody Cocktail:** Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPR2. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent CPR2. Mix thoroughly and gently.

## Standard Preparation

Always prepare a fresh set of standards for every use. Do not freeze and reuse resuspended lyophilized protein. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute the Tyrosine Hydroxylase standard sample by adding the volume of 1X Cell Extraction Buffer SSW + Enhancer indicated on the protein vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 400 ng/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 352 µL of 1X Cell Extraction Buffer SSW + Enhancer into tube number 1 and 150 µL of 1X Cell Extraction Buffer SSW + Enhancer into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

| Standard # | Dilution Sample | Volume to Dilute (µL) | Volume of Diluent (µL) | Starting Conc. (ng/mL) | Final Conc. (ng/mL) |
|---|---|---|---|---|---|
| 1 | **Stock Standard** | 48 | 352 | 400 | 48 |
| 2 | Standard#1 | 150 | 150 | 48 | 24 |
| 3 | Standard#2 | 150 | 150 | 24 | 12 |
| 4 | Standard#3 | 150 | 150 | 12 | 6 |
| 5 | Standard#4 | 150 | 150 | 6 | 3 |
| 6 | Standard#5 | 150 | 150 | 3 | 1.5 |
| 7 | Standard#6 | 150 | 150 | 1.5 | 0.75 |
| 8 | Blank Control | 0 | 150 | 0 | 0 |

## Sample Preparation

| Typical Sample Dynamic Range | |
|---|---|
| **Sample Type** | **Range** |
| U-87 MG Cell Extract* | ≤ 183 µg/mL |
| PC-12 Cell Extract | 62.5 - 500 ng/mL |
| Rat Brain Stem Tissue Extract | 24 - 194 µg/mL |

*Based on spiked sample

**Preparation of extracts from cell pellets** Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C. Rinse cells twice with PBS. Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer SSW + Enhancer. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Once quantified, add 1 volume of Denaturant to 35 volumes of extract (example: 5 µL Denaturant to 175 µL extract). Mix thoroughly and gently. Incubate at room temperature for 10 minutes. Dilute samples 10-fold in 1X Cell Extraction Buffer SSW. Mix thoroughly and gently. Dilute samples to desired concentration in 1X Cell Extraction SSW + Enhancer. See table below as an example.

**Preparation of extracts from adherent cells by direct lysis (alternative protocol)** Remove growth media and rinse adherent cells 2 times in PBS. Solubilize the cells by addition of chilled 1X Cell Extraction Buffer SSW + Enhancer directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer SSW + Enhancer per confluent 15 cm diameter plate). Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Once quantified, add 1 volume of Denaturant to 35 volumes of extract (example: 5 µL Denaturant to 175 µL extract). Mix thoroughly and gently. Incubate at room temperature for 10 minutes. Dilute samples 10-fold in 1X Cell Extraction Buffer SSW. Mix thoroughly and gently. Dilute samples to desired concentration in 1X Cell Extraction Buffer SSW + Enhancer. See table below as an example.

**Preparation of extracts from tissue homogenates** Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended). Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer SSW + Enhancer. For lower amounts of tissue adjust volumes accordingly. Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay. Once quantified, add 1 volume of Denaturant to 35 volumes of extract (example: 5 µL Denaturant to 175 µL extract). Mix thoroughly and gently. Incubate at room temperature for 10 minutes. Dilute samples 10-fold in 1X Cell Extraction Buffer SSW. Dilute samples to desired concentration in 1X Cell Extraction Buffer SSW + Enhancer. See table below as an example.

**Example dilution scheme for sample treatment with Denaturant:**

| Tube # | Sample to Dilute | Volume to Dilute (µL) | Volume of Denaturant (µL) | Volume of 1X Cell Extraction Buffer SSW (µL) |
|---|---|---|---|---|
| 1 | Prepared Cell or Tissue Extract | 175 | 5 | 0 |
| 2 | Tube #1 | 20 | 0 | 180 |

## Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

## Assay Procedure

Equilibrate all materials and prepared reagents to room temperature prior to use.
We recommend that you assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
3. Add 50 µL of all sample or standard to appropriate wells.
4. Add 50 µL of the Antibody Cocktail to each well.
5. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
6. Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
7. Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
   *Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.*
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
8. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
9. Alternative to 7 – 8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

| Mode | Kinetic |
|---|---|
| Wavelength: | 600 nm |
| Time: | up to 20 min |
| Interval: | 20 sec - 1 min |
| Shaking: | Shake between readings |

   **Note** that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

**Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:**
www.abcam.com/protocols/the-complete-elisa-guide

**For technical support contact information, visit:** www.abcam.com/contactus


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## Additional information

### ASSAY SPECIFICITY

This kit is designed for the quantification of human/rat Tyrosine Hydroxylase.

The standard protein in this kit is full length human Tyrosine Hydroxylase (Uniprot P07101). Homology of this sequence to rat is 88.7%.

Native signal was detected in cell extract and tissue extract sample types.

Spiked protein experiments were used to validate cell extract sample types.

Serum, plasma (citrate), plasma (EDTA), plasma (heparin), cell culture supernatant, urine, saliva, milk, and CSF samples have not been tested with this kit.

### SPECIES REACTIVITY

Native signal was measured in rat cell and tissue extracts.

Other species reactivity not determined.

### CALCULATION

- Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

  Δ Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g., linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
- Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at absorbance values less than that of the lowest standard should be retested in a less dilute form.

### TYPICAL DATA

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

| Standard Curve Measurements | | | |
|---|---|---|---|
| Concentration (ng/mL) | O.D. 450 nm | | Mean O.D. |
| | 1 | 2 | |
| 0 | 0.062 | 0.061 | 0.061 |
| 0.75 | 0.116 | 0.117 | 0.117 |
| 1.5 | 0.172 | 0.171 | 0.171 |
| 3 | 0.259 | 0.262 | 0.260 |
| 6 | 0.447 | 0.459 | 0.453 |
| 12 | 0.846 | 0.852 | 0.849 |
| 24 | 1.512 | 1.541 | 1.526 |
| 48 | 2.841 | 2.799 | 2.820 |

Table 1. Example of human/rat Tyrosine Hydroxylase standard curve in 1X Cell Extraction Buffer SSW + Enhancer. The Tyrosine Hydroxylase standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

### TYPICAL SAMPLE VALUES

#### Sensitivity:

The calculated minimal detectable dose (MDD) is 0.15 ng/mL. The MDD was determined by calculating the mean of zero standard replicates (n=24) and adding 2 standard deviations then extrapolating the corresponding concentration.

#### Recovery

Three concentrations of Tyrosine Hydroxylase were spiked into the indicated biological matrix to evaluate signal recovery in the working range of the assay.

| Sample Type | Average % Recovery | Range (%) |
|---|---|---|
| 183 µg/mL U-87 MG Cell Extract | 99 | 93 - 106 |
| 250 ng/mL PC-12 Cell Extract | 94 | 83 - 104 |
| 97 µg/mL Rat Brain Stem Tissue Extract | 97 | 95 - 100 |

## Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Tyrosine Hydroxylase was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer SSW + Enhancer.

| Dilution Factor | Interpolated value | 500 ng/mL PC-12 Cell Extract | 194 µg/mL Rat Brain Stem Tissue Extract |
|---|---|---|---|
| Undiluted | ng/mL | 26.8 | 12.2 |
| | **% Expected value** | 100 | 100 |
| 2 | ng/mL | 13.2 | 6.3 |
| | **% Expected value** | 98 | 103 |
| 4 | ng/mL | 6.2 | 3.0 |
| | **% Expected value** | 93 | 97 |
| 8 | ng/mL | 2.8 | 1.4 |
| | **% Expected value** | 84 | 89 |

Recombinant Tyrosine Hydroxylase was spiked in in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer SSW + Enhancer.

| Dilution Factor | Interpolated value | 183 µg/mL U-87 MG Cell Extract |
|---|---|---|
| Undiluted | ng/mL | 24.3 |
| | **% Expected value** | 100 |
| 2 | ng/mL | 11.7 |
| | **% Expected value** | 96 |
| 4 | ng/mL | 6.0 |
| | **% Expected value** | 98 |
| 8 | ng/mL | 2.6 |
| | **% Expected value** | 84 |

## Precision

Mean coefficient of variations of interpolated values of Tyrosine Hydroxylase from a single concentration of PC-12 cell extract within the working range of the assay.

| | Intra-assay | Inter-assay |
|---|---|---|
| **N=** | 8 | 3 |
| **CV (%)** | 4.7 | 8.8 |

**Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:**
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**For technical support contact information, visit:** www.abcam.com/contactus


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**DIRECTIONS FOR 384-WELL PLATE FORMAT:**

### Materials Supplied for 384-well Format

| Item | Quantity | Storage Condition |
|---|---|---|
| Human/Rat Tyrosine Hydroxylase Capture Antibody 10X | 600 µL | +4°C |
| Human/Rat Tyrosine Hydroxylase Detector Antibody 10X | 600 µL | +4°C |
| Human/Rat Tyrosine Hydroxylase Lyophilized Recombinant Protein | 2 Vials | +4°C |
| Antibody Diluent CPR2 | 6 mL | +4°C |
| Cell Extraction Buffer SSW | 50 mL | +4°C |
| Cell Extraction Enhancer Solution 50X | 5 mL | +4°C |
| Denaturant | 4 x 500 µL | +4°C |
| Wash Buffer PT 10X | 2 x 20 mL | +4°C |
| TMB Development Solution | 2 x 12 mL | +4°C |
| Stop Solution | 2 x 12 mL | +4°C |
| SimpleStep Pre-Coated 384-Well Microplate | 384 wells | +4°C |
| Plate Seal | 1 | +4°C |

### Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm in a 384-well plate.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).
- Optional: Automated liquid handler.

### Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for one full plate. The sample volumes below are sufficient for running all 384 wells; adjust volumes as needed for the number of samples and dilution scheme for your experiment.

Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

The provided Cell Extraction Enhancer Solution 50X may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The Cell Extraction Enhancer Solution 50X can be stored at room temperature to avoid precipitation.

**1X Wash Buffer PT:** Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 200 mL 1X Wash Buffer PT combine 20 mL Wash Buffer PT 10X with 180 mL deionized water. Mix thoroughly and gently.

**1X Cell Extraction Buffer SSW:** Prepare 1X Cell Extraction Buffer SSW by diluting Cell Extraction Buffer SSW Stock to 1X with 1X Wash Buffer PT. To make 10 mL 1X Cell Extraction Buffer SSW combine 9.6 mL 1X Wash Buffer PT and 0.4 mL Cell Extraction Buffer SSW Stock. Mix thoroughly and gently. If required protease inhibitors can be added.

**1X Cell Extraction Buffer SSW + Enhancer:** Prepare 1X Cell Extraction Buffer SSW + Enhancer by diluting Cell Extraction Buffer SSW Stock and Cell Extraction Enhancer Solution 50X to 1X with 1X Wash Buffer PT. To make 50 mL 1X Cell Extraction Buffer SSW combine 47 mL 1X Wash Buffer PT, 2 mL Cell Extraction Buffer SSW Stock and 1 mL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.

**Antibody Cocktail:** Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPR2. To make 6 mL of the Antibody Cocktail combine 600 µL 10X Capture Antibody and 600 µL 10X Detector Antibody with 4.8 mL Antibody Diluent CPR2. Mix thoroughly and gently.

### Standard Preparation

Always prepare a fresh set of standards for every use. Do not freeze and reuse resuspended lyophilized protein. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute the Tyrosine Hydroxylase standard sample by adding the volume of 1X Cell Extraction Buffer SSW + Enhancer indicated on the protein vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 400 ng/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 176 µL of 1X Cell Extraction Buffer SSW + Enhancer into tube number 1 and 75 µL of 1X Cell Extraction Buffer SSW + Enhancer into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

| Standard # | Dilution Sample | Volume to Dilute (µL) | Volume of Diluent (µL) | Starting Conc. (ng/mL) | Final Conc. (ng/mL) |
|---|---|---|---|---|---|
| 1 | **Stock Standard** | 24 | 176 | 400 | 48 |
| 2 | Standard#1 | 75 | 75 | 48 | 24 |
| 3 | Standard#2 | 75 | 75 | 24 | 12 |
| 4 | Standard#3 | 75 | 75 | 12 | 6 |
| 5 | Standard#4 | 75 | 75 | 6 | 3 |
| 6 | Standard#5 | 75 | 75 | 3 | 1.5 |
| 7 | Standard#6 | 75 | 75 | 1.5 | 0.75 |
| 8 | Blank Control | 0 | 75 | 0 | 0 |

### Plate Preparation

The 384-well plate included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
For each assay performed, a minimum of two wells must be used as the zero control.
For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
Differences in well absorbance or "edge effects" have not been observed with this assay.

## Assay Procedure for 384-well Plate Format

Equilibrate all materials and prepared reagents to room temperature prior to use.
We recommend that you assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Add 12.5 µL of all sample or standard to appropriate wells.
3. Add 12.5 µL of the Antibody Cocktail to each well.
4. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 700 rpm.
5. Wash each well with 3 x 100 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 100 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
6. Add 25 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 700 rpm.
   *Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.*
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
7. Add 25 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading. Proper mixing of the Stop Solution is required for proper measurement.
8. Alternative to 6 – 7: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

| Mode | Kinetic |
|---|---|
| Wavelength: | 600 nm |
| Time: | up to 20 min |
| Interval: | 20 sec – 1 min |
| Shaking: | Shake between readings |

   **Note** that an endpoint reading can also be recorded at the completion of the kinetic read by adding 25 µL Stop Solution to each well and recording the OD at 450 nm.

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