

## ab322366 – Human Tau (phospho T231) SimpleStep ELISA® Kit - Intracellular

For the quantitative measurement of Tau (phospho T231) in human 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/ab322366](http://www.abcam.com/ab322366)

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 5-6.

**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<br>1 x 96 tests | Quantity<br>10 x 96 tests | Storage<br>Condition |
|---|--------------------------|---------------------------|----------------------|
| Human Tau (phospho T231) Lyophilized Capture Antibody                       | 1 Vial                   | 10 x 1 Vial               | +4°C                 |
| Human Tau (phospho T231) Detector Antibody 10X                              | 600 µL                   | 10 x 600 µL               | +4°C                 |
| Human Tau (phospho T231) Lyophilized Chemically Ligated Recombinant Protein | 2 Vials                  | 10 x 2 Vials              | +4°C                 |
| Antibody Diluent 4BR  | 6 mL                     | 10 x 6 mL                 | +4°C                 |
| Cell Extraction Buffer PTR 5X   | 10 mL                    | 2 x 50 mL                 | +4°C                 |
| Sample Diluent NS   | 12 mL                    | 12 mL                     | +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                 |

### 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.

**1X Cell Extraction Buffer PTR (For cell and tissue extracts only):** Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 8 mL deionized water and 2 mL Cell Extraction Buffer PTR 5X. Mix thoroughly and gently. If required protease inhibitors can be added.

**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.

**10X Capture Antibody:** To reconstitute the lyophilized capture antibody, centrifuge at 10,000 g for 2 minutes. Add 660 µL of Sample Diluent NS, let sit at room temperature for 10 minutes and resuspend well by inverting the tube by hand and gently pipetting. Unused reconstituted antibody can be stored frozen at -20°C. Avoid repeated freeze-thaw cycles.

**Antibody Cocktail:** Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. 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 4BR. Mix thoroughly and gently.

### Standard Preparation

Always prepare a fresh set of standards for every use. 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 Tau (phospho T231) standard sample by adding the volume of 1X Cell Extraction Buffer PTR indicated on the protein vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 160,000 pg/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1–8.
3. Add 385 µL of 1X Cell Extraction Buffer PTR into tube number 1 and 150 µL of 1X Cell Extraction Buffer PTR 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. (pg/mL) | Final Conc. (pg/mL) |
|------------|-----------------|-----------------------|------------------------|------------------------|---------------------|
| 1          | Stock Standard  | 15                    | 385                    | 160,000                | 6,000               |
| 2          | Standard#1      | 150                   | 150                    | 6,000                  | 3,000               |
| 3          | Standard#2      | 150                   | 150                    | 3,000                  | 1,500               |
| 4          | Standard#3      | 150                   | 150                    | 1,500                  | 750                 |
| 5          | Standard#4      | 150                   | 150                    | 750                    | 375                 |
| 6          | Standard#5      | 150                   | 150                    | 375                    | 187.5               |
| 7          | Standard#6      | 150                   | 150                    | 187.5                  | 93.75               |
| 8          | Blank Control   | 0                     | 150                    | 0                      | 0                   |

### Sample Preparation

| Typical Sample Dynamic Range           |                 |
|--|-----------------|
| Sample Type                            | Range           |
| SH-SY5Y Cell Extract                   | 0.78 - 25 µg/mL |
| Human Brain Cortex Tissue Extract      | 0.38 - 12 µg/mL |
| Human Brain Hippocampus Tissue Extract | 0.47 - 15 µg/mL |

**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<sup>7</sup> cell/mL in chilled 1X Cell Extraction Buffer PTR. 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. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

**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 PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR 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. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

**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 PTR. 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. Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

### 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:

| <b>Mode</b> | <b>Kinetic</b>         |
|-------------|------------------------|
| 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  $\mu$ L 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](http://www.abcam.com/protocols/the-complete-elisa-guide)

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### Additional information

#### ASSAY SPECIFICITY

This kit is designed for the quantification of human Tau (phospho T231). This pair does not recognize Tau that is non-phosphorylated at T231. The amino acids numbering is based on Tau-F (2N4R, Uniprot P10636-8).

The standard protein in this kit is full length human Tau-A (Uniprot P10636-3) ligated with a peptide identical to the region surrounding T231, containing phosphorylated T231.

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

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

For the measurement of Tau (phospho T231) in serum, plasma (citrate), plasma (EDTA), plasma (heparin), cell culture supernatant, and CSF sample types, use Human Tau (phospho T231) ELISA kit ab322367.

#### CROSS REACTIVITY

30 ng/mL of the following recombinant full length human Tau-A proteins each ligated with a peptide identical to the region surrounding the respective modified amino acid and containing the phosphorylated amino acid were tested for cross reactivity:

Human Tau-A non-phosphorylated T231  
Human Tau-A phospho T181  
Human Tau-A phospho S199  
Human Tau-A phospho T217  
Human Tau-A phospho S396

No cross reactivity was observed.

#### INTERFERENCE

30 ng/mL of recombinant full length human Tau-A proteins each ligated with a peptide containing a specific phosphorylated amino acid was tested for interference with 3 ng/mL of full length human Tau-A ligated with a peptide containing phosphorylated T231 (human Tau phospho T231). See results in the table below:

| Tested Interferent                  | Decreased Interpolated Value of Human Tau-A phospho-T231 |
|-------------------------------------|--|
| Human Tau-A non-phosphorylated T231 | 1.07-fold (7%)   |
| Human Tau-A phospho T181            | 1.08-fold (8%)   |
| Human Tau-A phospho S199            | 1.11-fold (10%)  |
| Human Tau-A phospho T217            | 1.04-fold (4%)   |
| Human Tau-A phospho S396            | 1.01-fold (1%)   |

#### SPECIES REACTIVITY

Other species reactivity was determined by measuring brain tissue extract samples of various species. Reactivity was observed for the following species: Human, Rat, Mouse  
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.  
 $\Delta$  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 (pg/mL)       | O.D 450 nm |       | Mean O.D |
|                             | 1          | 2     |          |
| 0                           | 0.046      | 0.045 | 0.045    |
| 93.75                       | 0.092      | 0.091 | 0.091    |
| 187.5                       | 0.145      | 0.142 | 0.143    |
| 375                         | 0.242      | 0.239 | 0.240    |
| 750                         | 0.450      | 0.487 | 0.468    |
| 1,500                       | 0.849      | 0.850 | 0.849    |
| 3,000                       | 1.665      | 1.646 | 1.655    |
| 6,000                       | 3.005      | 3.077 | 3.041    |

Table 1. Example of human Tau (phospho T231) standard curve in 1X Cell Extraction Buffer PTR. The Tau (phospho T231) 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 21 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=16) and adding 2 standard deviations then extrapolating the corresponding concentration.

### Recovery

Three concentrations of Tau (phospho T231) were spiked into the indicated biological matrix to evaluate signal recovery in the working range of the assay.

| Sample Type                                    | Average % Recovery | Range (%) |
|--|--------------------|-----------|
| 10 µg/mL SH-SY5Y Cell Extract                  | 112                | 102 - 119 |
| 4 µg/mL Human Brain Cortex Tissue Extract      | 116                | 107 - 121 |
| 5 µg/mL Human Brain Hippocampus Tissue Extract | 116                | 115 - 118 |

### 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 Tau (phospho T231) was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR.

| Dilution Factor | Interpolated value | 25 µg/mL SH-SY5Y Cell Extract | 12 µg/mL Brain Cortex Tissue Extract | 15 µg/mL Brain Hippocampus Tissue Extract |
|-----------------|--------------------|-------------------------------|--------------------------------------|---|
| Undiluted       | pg/mL              | 5,862.11                      | 4,807.78                             | 5,534.12                                  |
|                 | % Expected value   | 100%                          | 100%                                 | 100%                                      |
| 2               | pg/mL              | 2,891.93                      | 2,413.14                             | 2,759.92                                  |
|                 | % Expected value   | 99%                           | 100%                                 | 100%                                      |
| 4               | pg/mL              | 1,498.55                      | 1,136.54                             | 1,318.76                                  |
|                 | % Expected value   | 102%                          | 95%                                  | 95%                                       |
| 8               | pg/mL              | 689.33                        | 545.79                               | 637.24                                    |
|                 | % Expected value   | 94%                           | 91%                                  | 92%                                       |
| 16              | pg/mL              | 313.98                        | 268.61                               | 280.03                                    |
|                 | % Expected value   | 86%                           | 89%                                  | 81%                                       |

### Precision

Mean coefficient of variations of interpolated values of Tau (phospho T231) from two concentrations of human brain cortex tissue extract within the working range of the assay.

|        | Intra-assay | Inter-assay |
|--------|-------------|-------------|
| N=     | 8           | 3           |
| CV (%) | 4.1         | 6.4         |

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:

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## DIRECTIONS FOR 384-WELL PLATE FORMAT:

### Materials Supplied for 384-well Format

| Item  | Quantity  | Storage Condition |
|---|-----------|-------------------|
| Human Tau (phospho T231) Lyophilized Capture Antibody                       | 1 Vial    | +4°C              |
| Human Tau (phospho T231) Detector Antibody 10X                              | 600 µL    | +4°C              |
| Human Tau (phospho T231) Lyophilized Chemically Ligated Recombinant Protein | 2 Vials   | +4°C              |
| Antibody Diluent 4BR  | 6 mL      | +4°C              |
| Cell Extraction Buffer PTR 5X   | 50 mL     | +4°C              |
| Sample Diluent NS   | 12 mL     | +4°C              |
| Wash Buffer PT 10X  | 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.

**1X Cell Extraction Buffer PTR (For cell and tissue extracts only):** Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X to 1X with deionized water. To make 250 mL 1X Cell Extraction Buffer PTR combine 200 mL deionized water and 50 mL Cell Extraction Buffer PTR 5X. Mix thoroughly and gently. If required protease inhibitors can be added.

**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.

**10X Capture Antibody:** To reconstitute the lyophilized capture antibody, centrifuge at 10,000 g for 2 minutes. Add 660 µL of Sample Diluent NS, let sit at room temperature for 10 minutes and resuspend well by inverting the tube by hand and gently pipetting. Unused reconstituted antibody can be stored frozen at -20°C. Avoid repeated freeze-thaw cycles.

**Antibody Cocktail:** Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. 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 4BR. Mix thoroughly and gently.

### Standard Preparation

Always prepare a fresh set of standards for every use. 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 Tau (phospho T231) standard sample by adding the volume of 1X Cell Extraction Buffer PTR indicated on the protein vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 160,000 pg/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1–8.
3. Add 385 µL of 1X Cell Extraction Buffer PTR into tube number 1 and 75 µL of 1X Cell Extraction Buffer PTR 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. (pg/mL) | Final Conc. (pg/mL) |
|------------|-----------------------|-----------------------|------------------------|------------------------|---------------------|
| 1          | <b>Stock Standard</b> | 15                    | 385                    | 160,000                | 6,000               |
| 2          | Standard#1            | 75                    | 75                     | 6,000                  | 3,000               |
| 3          | Standard#2            | 75                    | 75                     | 3,000                  | 1,500               |
| 4          | Standard#3            | 75                    | 75                     | 1,500                  | 750                 |
| 5          | Standard#4            | 75                    | 75                     | 750                    | 375                 |
| 6          | Standard#5            | 75                    | 75                     | 375                    | 187.5               |
| 7          | Standard#6            | 75                    | 75                     | 187.5                  | 93.75               |
| 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.

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:

[www.abcam.com/protocols/the-complete-elisa-guide](http://www.abcam.com/protocols/the-complete-elisa-guide)

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