

ab323516 – Human Cathepsin D (mature, single chain) SimpleStep ELISA® Kit

For the quantitative measurement of Cathepsin D in human serum, plasma (citrate), plasma (heparin), cell culture supernatant, urine, saliva, and milk.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab323516

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 Cathepsin D Capture Antibody 10X	600 µL	10 x 600 µL	+4°C
Human Cathepsin D Detector Antibody 10X	600 µL	10 x 600 µL	+4°C
Human Cathepsin D Lyophilized Recombinant Protein	2 Vials	10 x 2 Vials	+4°C
Antibody Diluent CPI2	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
Sample Diluent NS	50 mL	2 x 250 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.
Deionized water.
PBS.
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.

Sample Diluent SSW: Prepare Sample Diluent SSW by diluting Cell Extraction Buffer SSW Stock to 1X with 1X Wash Buffer PT. To make 10 mL Sample Diluent SSW, combine 9.6 mL 1X Wash Buffer PT and 0.4 mL Cell Extraction Buffer SSW Stock. Mix thoroughly and gently.

Sample Diluent NS + 1X Enhancer: Prepare Sample Diluent NS + 1X Enhancer by combining Sample Diluent NS and 50X Cell Extraction Enhancer Solution. To make 5 mL Sample Diluent NS + 1X Enhancer, combine 4.9 mL Sample Diluent NS and 100 µL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently.

Antibody Cocktail: Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPI2. 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 CPI2. 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 Cathepsin D standard sample by adding the volume indicated on the protein vial label. For **serum, plasma, supernatant, urine, and saliva samples measurements**, use Sample Diluent NS + 1X Enhancer. For **milk samples measurements**, use Sample Diluent SSW. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 30 ng/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Use the same Sample Diluent as used to resuspend the Stock Standard to prepare the standard curve. Add 371 μ L of Sample Diluent into tube number 1 and 150 μ L of Sample Diluent 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 (μ L)	Volume of Diluent (μ L)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock Standard	49	371	30,000	3,500
2	Standard#1	150	150	3,500	1,750
3	Standard#2	150	150	1,750	875
4	Standard#3	150	150	875	437.5
5	Standard#4	150	150	437.5	218.75
6	Standard#5	150	150	218.75	109.38
7	Standard#6	150	150	109.38	54.69
8	Blank Control	0	150	0	0

Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Serum	$\leq 50\%$
Plasma – Citrate	$\leq 50\%$
Plasma – Heparin	$\leq 50\%$
A431 Cell Culture Supernatant	0.39 - 6.25%
HepG2 Cell Culture Supernatant	3.13 - 25%
Urine	$\leq 12.5\%$
Saliva	3.13 - 25%
Milk	$\leq 25\%$

Serum Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:2 into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma Collect plasma using citrate or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:2 into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. Note: This kit is incompatible with plasma (EDTA) samples.

Cell Culture Supernatants Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Assay, or dilute samples into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Urine Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Dilute samples at least 1:8 into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted urine samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Saliva Centrifuge saliva at 800 x g for 10 minutes to remove debris. Collect supernatants. Dilute samples at least 1:4 into Sample Diluent NS + 1X Enhancer and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Milk De-fat milk samples as follows. Centrifuge milk samples at 500 x g for 15 minutes at 4°C and collect the aqueous fraction using syringe attached to needle. Centrifuge the aqueous fraction at 3,000 x g for 15 minutes at 4°C and collect the final aqueous fraction (de-fatted milk) using syringe attached to needle. Dilute samples at least 1:4 into Sample Diluent SSW and assay. Store un-diluted de-fatted milk at -20°C or below. Avoid repeated freeze-thaw cycles.

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 mature human Cathepsin D. The antibodies in this kit are specific to the mature, single chain form of human Cathepsin D and do not recognize full pre-pro Cathepsin D or pro Cathepsin D.

The standard protein in this kit is recombinant mature human Cathepsin D.

Native signal was detected in serum, plasma (citrate), plasma (heparin), cell culture supernatant, urine, saliva, and milk sample types.

Spiked protein experiments were used to validate serum, plasma (citrate), plasma (heparin), urine and milk sample types.

CSF, cell extract, and tissue extract samples have not been tested with this kit.

This kit is incompatible with plasma (EDTA) samples.

For the measurement of Cathepsin D in cell extract sample types, use Human Cathepsin D ELISA kit ab323517.

CROSS REACTIVITY

100 ng/mL full-length human pre-pro Cathepsin D (aa1-412) and 3 ng/mL human pro Cathepsin D (aa21-412) were tested for cross-reactivity. No cross reactivity was observed.

SPECIES REACTIVITY

Other species reactivity was determined by measuring 50% serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution.

Reactivity < 3% was determined for the following species: Rat, Cow, Monkey, Goat, Minipig

This kit is incompatible with the following species: Mouse, Rabbit

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.

△ **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	0.049	0.048	0.049
54.69	0.109	0.107	0.108
109.38	0.170	0.173	0.171
218.75	0.287	0.281	0.284
437.5	0.486	0.513	0.499
875	0.912	0.963	0.938
1,750	1.724	1.727	1.725
3,500	3.204	3.145	3.174

Table 1. Example of human Cathepsin D standard curve in Sample Diluent NS + 1X Enhancer. The Cathepsin D standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

Standard Curve Measurements			
Concentration (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.067	0.062	0.065
54.69	0.144	0.149	0.146
109.38	0.211	0.214	0.213
218.75	0.348	0.343	0.345
437.5	0.603	0.595	0.599
875	1.062	1.044	1.053
1,750	1.873	1.941	1.907
3,500	3.458	3.414	3.436

Table 2. Example of human Cathepsin D standard curve in Sample Diluent SSW. The Cathepsin D standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

TYPICAL SAMPLE VALUES

Sensitivity:

The minimal detectable dose (MDD) was determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentration.

Sample Diluent Buffer	N=	Minimal Detectable Dose
Sample Diluent NS + 1X Enhancer	24	12.58 pg/mL
Sample Diluent SSW	24	8.61 pg/mL

Recovery

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

Sample Type	Average % Recovery	Range (%)
50% Serum	87	80 - 91
50% Plasma – Citrate	85	81 - 87
50% Plasma – Heparin	85	83 - 88
2.5% A431 Supernatant	113	104 - 117
5% HepG2 Supernatant	104	100 - 107
12.5% Pooled Gender Urine	102	101 - 102
12.5% Male Saliva	92	81 - 108
25% Milk	96	95 - 96

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 Cathepsin D was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 1X Enhancer.

Dilution Factor	Interpolated value	50% Human Serum	50% Human Plasma (Citrate)	50% Human Plasma (Heparin)	12.5% Human Urine (Pooled Gender)
Undiluted	pg/mL	197.7	208.9	267.2	133
	% Expected value	100	100	100	100
2	pg/mL	86.8	94.9	114.4	55.4
	% Expected value	88	91	86	83
4	pg/mL	ND	ND	58.1	ND
	% Expected value	ND	ND	87	ND

ND – Not Detected – below product dynamic range

Native Cathepsin D was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 1X Enhancer.

Dilution Factor	Interpolated value	6.25% A431 Supernatant	25% HepG2 Supernatant	25% Human Male Saliva
Undiluted	pg/mL	2,306.0	952.4	1,164.5
	% Expected value	100	100	100
2	pg/mL	1,127.7	419.8	601.5
	% Expected value	98	88	103
4	pg/mL	467.9	221.3	307.2
	% Expected value	81	93	106
8	pg/mL	246.0	96.5	160.9
	% Expected value	85	81	111
16	pg/mL	114.8	ND	ND
	% Expected value	80	ND	ND

ND – Not Detected – below product dynamic range

Recombinant Cathepsin D was spiked into the following biological samples and then diluted in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS + 1X Enhancer.

Dilution Factor	Interpolated value	50% Human Serum	50% Human Plasma (Citrate)	50% Human Plasma (Heparin)	12.5% Human Urine (Pooled Gender)
Undiluted	pg/mL	1,734.8	1,677.9	1,645.7	1,959.8
	% Expected value	100	100	100	100
2	pg/mL	915.6	900.4	825.7	897.9
	% Expected value	106	107	100	92
4	pg/mL	462.1	456.3	464.5	476.0
	% Expected value	107	109	113	97
8	pg/mL	245.1	252.2	224.3	219.0
	% Expected value	113	120	109	89
16	pg/mL	125.1	118.8	113.9	109.4
	% Expected value	115	113	111	89

Native Cathepsin D was measured in the following biological sample in a 2-fold dilution series. Sample dilutions are made in Sample Diluent SSW.

Dilution Factor	Interpolated value	50% Human Milk
Undiluted	pg/mL	274
	% Expected value	100
2	pg/mL	158.2
	% Expected value	115
4	pg/mL	ND
	% Expected value	ND

ND – Not Detected – below product dynamic range

Recombinant Cathepsin D was spiked into the following biological samples and then diluted in a 2-fold dilution series. Sample dilutions are made in Sample Diluent SSW.

Dilution Factor	Interpolated value	25% Human Milk
Undiluted	pg/mL	1,663.8
	% Expected value	100
2	pg/mL	924.7
	% Expected value	111
4	pg/mL	456.6
	% Expected value	110
8	pg/mL	233.9
	% Expected value	112
16	pg/mL	113.9
	% Expected value	110

Precision

Mean coefficient of variations of interpolated values of Cathepsin D from a single concentration of human serum within the working range of the assay.

	Intra-assay	Inter-assay
N=	8	3
CV (%)	8.3	6.9

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

DIRECTIONS FOR 384-WELL PLATE FORMAT:

Materials Supplied for 384-well Format

Item	Quantity	Storage Condition
Human Cathepsin D Capture Antibody 10X	600 µL	+4°C
Human Cathepsin D Detector Antibody 10X	600 µL	+4°C
Human Cathepsin D Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent CPI2	6 mL	+4°C
Cell Extraction Buffer SSW	50 mL	+4°C
Cell Extraction Enhancer Solution 50X	5 mL	+4°C
Sample Diluent NS	250 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.

Deionized water.

PBS.

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 50 mL 1X Wash Buffer PT, combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

Sample Diluent SSW: Prepare Sample Diluent SSW by diluting Cell Extraction Buffer SSW Stock to 1X with 1X Wash Buffer PT. To make 50 mL Sample Diluent SSW, combine 48 mL 1X Wash Buffer PT and 2 mL Cell Extraction Buffer SSW Stock. Mix thoroughly and gently.

Sample Diluent NS + 1X Enhancer: Prepare Sample Diluent NS + 1X Enhancer by combining Sample Diluent NS and 50X Cell Extraction Enhancer Solution. To make 250 mL Sample Diluent NS + 1X Enhancer, combine 245 mL Sample Diluent NS and 5 mL Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently.

Antibody Cocktail: Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPI2. 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 CPI2. 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 Cathepsin D standard sample by adding the volume indicated on the protein vial label. For **serum, plasma, supernatant, urine, and saliva samples measurements**, use Sample Diluent NS + 1X Enhancer. For **milk samples measurements**, use Sample Diluent SSW. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 30 ng/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Use the same Sample Diluent as used to resuspend the Stock Standard to prepare the standard curve. Add 159 µL of Sample Diluent into tube number 1 and 75 µL of Sample Diluent 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	21	159	30,000	3,500
2	Standard#1	75	75	3,500	1,750
3	Standard#2	75	75	1,750	875
4	Standard#3	75	75	875	437.5
5	Standard#4	75	75	437.5	218.75
6	Standard#5	75	75	218.75	109.38
7	Standard#6	75	75	109.38	54.69
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

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