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ab171577–HIF1a Human SimpleStep ELISA® Kit

For the quantitative measurement of HIF1a in human cell extracts.

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

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1. Overview

HIF1a *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of HIF1a protein in human cell extracts.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Hypoxia-inducible factor 1-alpha (HIF1 alpha) is a constitutively expressed transcription factor that is degraded under normal oxygen tensions but stabilized when oxygen is limiting (hypoxia). Under hypoxic conditions, stabilized HIF1 alpha translocates to the nucleus and promotes the transcription of a host of genes that enable the cell to adapt to the lack of oxygen. Aspects of the HIF1 alpha mediated hypoxic response include promotion of angiogenesis and the switch from aerobic respiration to anaerobic glycolysis. Many of the HIF1 alpha responsive genes encode proteins that promote glycolysis and/or inhibit oxidative phosphorylation (known as the Warburg effect). An exciting and developing area of current cancer research is examining how HIF-mediated metabolic reprogramming promotes tumor growth and survival.

In most cases, HIF1 alpha will need to be stabilized to be measured (steady state levels of HIF1 alpha in non-hypoxic environments is exceeding low in most cell lines). This can be achieved by (a) creating a hypoxic environment (e.g. using a hypoxia chamber) or (b) by using chemical treatments that mimic hypoxia (e.g. cobalt chloride or deferoxamine). The sample data in this assay protocol was generated using deferoxamine (DFO). DFO is an iron chelator and disrupts the function the prolyl hydroxylases that degrade HIF1 alpha in normoxia. By disrupting the enzymes that degrade HIF1 alpha, DFO increases the abundance of HIF1 alpha protein.

2. Protocol Summary

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

Add standard or sample to appropriate wells.

Incubate at room temperature.

Add Antibody Cocktail to all 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.

Alternatively, record color development kinetically after TMB substrate addition.

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 handled 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.

4. Storage and Stability

Store kit at +4°C 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.

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)
10X HIF1a Capture Antibody	600 µL	+2-8°C
10X HIF1a Detector Antibody	600 µL	+2-8°C
HIF1a Human Lyophilized Recombinant Protein	2 Vials	+2-8°C
Antibody Diluent 5B	6 mL	+2-8°C
10X Wash Buffer PT	20 mL	+2-8°C
5X Cell Extraction Buffer	10 mL	+2-8°C
50X Cell Extraction Enhancer Solution	1 mL	+2-8°C
TMB Substrate	12 mL	+2-8°C
Stop Solution	12 mL	+2-8°C
Sample Diluent NS	12 mL	+2-8°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8°C
Plate Seal	1	+2-8°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 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

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.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- 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.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- When generating positive control samples, it is advisable to change pipette tips after each step.

  As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.

9. 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.

9.1 1X Wash Buffer PT

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

9.2 Antibody cocktail

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5B. 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 5B. Mix thoroughly and gently.

9.3 1X Cell Extraction Buffer PTR (For cell and tissue extracts only)

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue. Refer to note in the Troubleshooting section.

10. 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).

10.1 IMPORTANT: If the protein standard vial has a volume identified on the label, reconstitute the HIF1a standard by adding that volume of 1x Cell Extraction Buffer PTR indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the HIF1a standard by adding 1 mL 1x Cell Extraction Buffer PTR. Mix thoroughly and gently at room temperature for 10 minutes. This is the 200 ng/mL **Stock Standard** Solution.

10.2 Label eight tubes, Standards 1– 8.

10.3 Add 370 µL 1X Extraction Buffer PTR into tube number 1 and 150 µL of 1X Extraction Buffer PTR into numbers 2-8.

10.4 To prepare tube **#1** add 30 µL from the stock standard into tube **#1.** To prepare tube **#2** add 150 µL from tube **#1** into tube **#2.** Repeat for tubes **#3-7**

10.5 Use the Stock Standard to prepare the following dilution series. Standard **#8** contains no protein and is the Blank control:

Standard #	Volume to dilute (µL)	Volume Diluent N (µL)	HIF1a (ng/mL)
1	Step 10.4		15
2	150 µL Standard #1	150	7.5
3	150 µL Standard #2	150	3.75
4	150 µL Standard #3	150	1.88
5	150 µL Standard #4	150	0.94
6	150 µL Standard #5	150	0.47

7	150 µL Standard #6	150	0.23
8 (Blank)	N/A	150	0

11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
HeLa (Deferoxamine treated)	50 – 500 µg/mL

11.1 Preparation of extracts from cell pellets

11.1.1 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.

11.1.2 Rinse cells twice with PBS.

11.1.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.1.4 Incubate on ice for 20 minutes.

11.1.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.1.6 Transfer the supernatants into clean tubes and discard the pellets.

11.1.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

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

11.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol)

11.2.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.2.2 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).

11.2.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

11.2.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.2.5 Transfer the supernatants into clean tubes and discard the pellets.
11.2.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
11.2.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.3 Preparation of extracts from tissue homogenates

11.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
11.3.2 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.
11.3.3 Incubate on ice for 20 minutes.
11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
11.3.5 Transfer the supernatants into clean tubes and discard the pellets.
11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

Guidelines for Dilutions of 100-fold or Greater *(for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
4 µl sample + 396 µl buffer (100X) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl*	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl*
1000x	**100000x**
A) 4 µl sample + 396 µl buffer (100X) B) 24 µl of A + 216 µl buffer (10X) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl*	A) 4 µl sample + 396 µl buffer (100X) B) 4 µl of A + 396 µl buffer (100X) C) 24 µl of A + 216 µl buffer (10X) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl*

Refer to Dilution Guidelines for further instruction.

12. 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.

13. Assay Procedure

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 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.

13.3 Add 50 µL of all samples and standards to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal or cover plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 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. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Substrate 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.

13.8 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.

Alternative to 13.7 – 13.8: 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

Δ 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.*

13.9 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.

14.2 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.

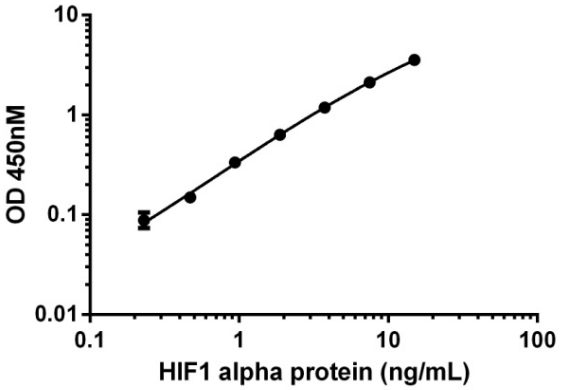
> **Δ Note**: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.

14.3 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.

14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

15. 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			
Conc. (ng/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.08	0.09	0.09
0.23	0.18	0.16	0.17
0.47	0.24	0.23	0.23
0.94	0.42	0.42	0.42
1.88	0.71	0.73	0.72
3.75	1.32	1.23	1.27
7.50	2.20	2.21	2.20
15	3.62	3.68	3.65

Figure 1. Example of IL-1 beta standard curve prepared in Sample Diluent NS. The IL-1 beta standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable (MDD) dose is typically 42 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=24) and adding 2 standard deviations then extrapolating the corresponding concentrations.

RECOVERY –

(Sample spiking in representative sample matrices)

Sample Type	Average % Recovery	Range (%)
50% Cell Culture Media	112	94 – 129
10% FBS	109	102 – 120
5% BSA	97	93 – 106

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.

Dilution Factor	HeLa lysate (µg/mL)	Interpolated value (ng/mL)	% Expected Value
Undiluted	50	1.95	100
2	25	1.13	116
4	12.50	0.63	129
8	6.25	0.34	138

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of HeLa lysates (treated with DFO) within the working range of the assay.

	Intra-assay Precision	Inter-Assay Precision
N=	5	3
CV (%)	4.3	7

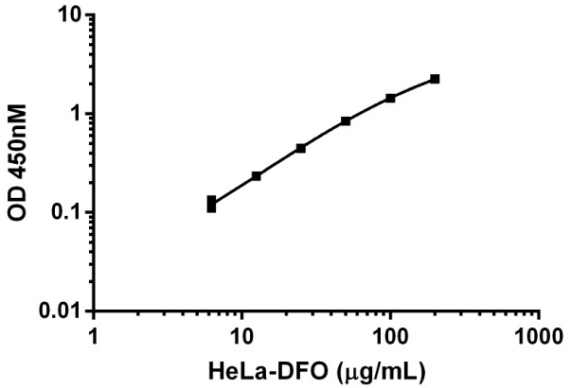


Figure 2. Titration of HeLa-DFO extract within the working range of the assay. Background subtracted data from duplicate measurements are plotted. To induce HIF1 alpha protein levels, HeLa cells were treated with 500 µM Deferoxamine (DFO) for 24 hours.

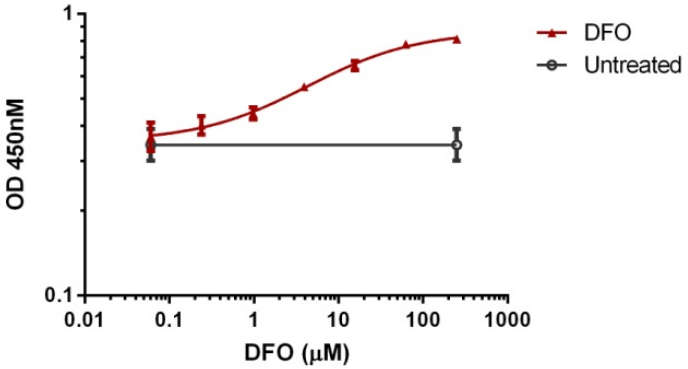


Figure 3. Dose-dependent induction of HIF1 alpha in HeLa cells by deferoxamine (DFO). HeLa cells were cultured in 96-well tissue culture plates and were either untreated or exposed to varying dose of DFO for 24 hours. Cells were extracted directly in the culture plate by overlaying culture media with Extraction Buffer PTR (with Extraction Enhancer) such that the final concentration was 1X Extraction Buffer. Extracts were applied to the HIF1 alpha ELISA. Raw data with standard deviation is plotted from triplicate measurements.

17. Assay Specificity

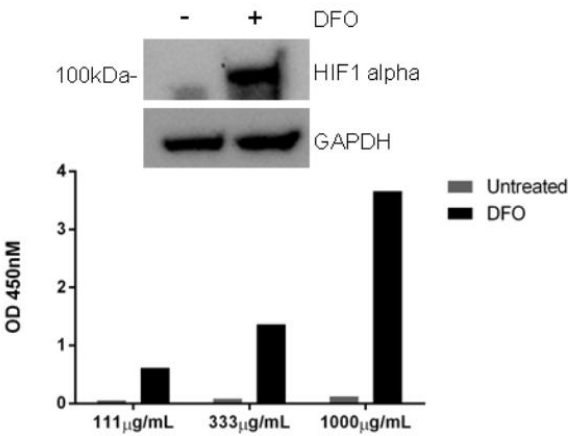


Figure 4. Comparison of HIF1 alpha expression in HeLa cell extracts (with and without DFO treatment) by ELISA (barchart) and western blot (top). Background subtracted OD450 nm data from three loading concentrations are shown. The HIF1 alpha detector antibody was used to blot the same lysates as analyzed by ELISA (40 µg loaded/lane). The GAPDH blot is included to show the relative loads of each lysate. In the HeLa cell line, DFO treatment is required to detect HIF1 alpha protein by both ELISA and western blot.

18. Species Reactivity

This kit detects HIF1a in human samples only. It is not compatible with mouse or rat samples.

Serum and plasma samples have not been tested with this kit.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)