ab201275 – EGFR Mouse SimpleStep ELISA® Kit

Instructions for Use

For the quantitative measurement of EGFR in mouse cell culture supernatant, serum and plasma samples.

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

Version 5a Last Updated 16 April 2024

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1. BACKGROUND

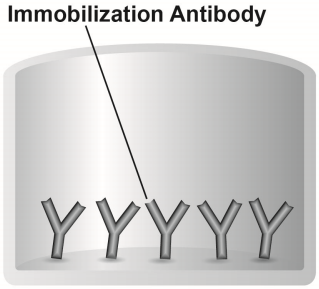
EGFR *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of EGFR protein in mouse cell culture supernatant, serum and plasma samples.

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 Development Solution 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.

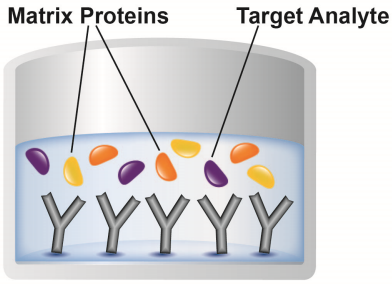
EGFR is a receptor tyrosine kinase that binds ligands of the EGF family and activate several signaling cascades to convert extracellular cues into appropriate cellular responses. Known ligands include EGF, TGFA/TGF-alpha, amphiregulin, epigen/EPGN, BTC/betacellulin, epiregulin/EREG and HBEGF/heparin-binding EGF. The ligand binding triggers receptor homo- and/or hetero-dimerization and autophosphorylation on key cytoplasmic residues. The phosphorylated receptor recruits adapter proteins like GRB2 which in turn activates complex downstream signaling cascades. EGFR activates at least 4 major downstream signaling cascades including the RAS-RAF-MEK-ERK, PI3 kinase-AKT, PLC gamma-PKC and STATs modules. EGFR may also activate the NF-kappa-B signaling cascade. EGFR also directly phosphorylates other proteins like RGS16, activating its GTPase activity and probably coupling the EGF receptor signaling to

the G protein-coupled receptor signaling. EGFR also phosphorylates MUC1 and increases its interaction with SRC and CTNNB1/beta-catenin. Endocytosis and inhibition of the activated EGFR by phosphatases like PTPRJ and PTPRK constitute immediate regulatory mechanisms. Upon EGF-binding EGFR phosphorylates EPS15 that regulates EGFR endocytosis and activity. Moreover, inducible feedback inhibitors including LRIG1, SOCS4, SOCS5 and ERRFI1 constitute alternative regulatory mechanisms for the EGFR signaling.

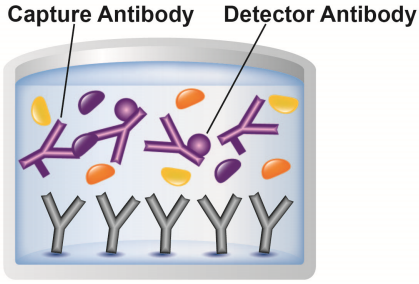
2. ASSAY SUMMARY



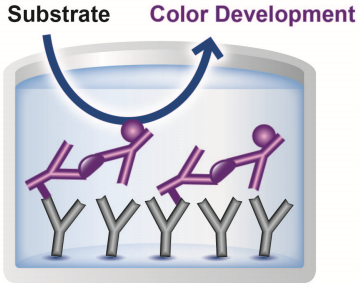
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.



Add standard or sample to appropriate wells.



Add Antibody Cocktail to all wells. Incubate at room temperature.



Aspirate and wash each well. Add TMB Development Solution 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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC 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 Reagent and Standard Preparation sections.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Mouse EGFR Capture Antibody	600 µL	+2-8ºC
10X Mouse EGFR Detector Antibody	600 µL	+2-8ºC
Mouse EGFR Lyophilized Recombinant Protein	2 Vials	+2-8ºC
Antibody Diluent CP2*	6 mL	+2-8ºC
10X Wash Buffer PT	20 mL	+2-8ºC
TMB Development Solution	12 mL	+2-8ºC
Stop Solution	12 mL	+2-8ºC
Sample Diluent NS	50 mL	+2-8ºC
Sample Diluent 25BS	20 mL	+2-8ºC
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8ºC
Plate Seal	1	+2-8ºC

***Note: Antibody Diluent CP2- This buffer has been reformulated to enhance stability after freeze-thaw cycles while producing data equivalent to the original formulation of antibody diluent CP previously used in this kit.**
While we run stock down, you may receive kits containing antibody diluent CP. This does not affect the way you should use the kit.
If you have any questions please contact Abcam Scientific Support.

6. 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.
- 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).

7. 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.

8. TECHNICAL HINTS

- 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.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- 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.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **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.**

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 CP2. 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 CP2. Mix thoroughly and gently.

10. STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following table 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 EGFR standard by adding that volume of Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the EGFR standard by adding 500 µL Diluent,

10.2 For **cell culture supernatant** samples reconstitute the EGFR standard by adding Sample Diluent NS by pipette.

For **serum and plasma** samples reconstitute the EGFR standard by adding Sample Diluent 25BS by pipette.

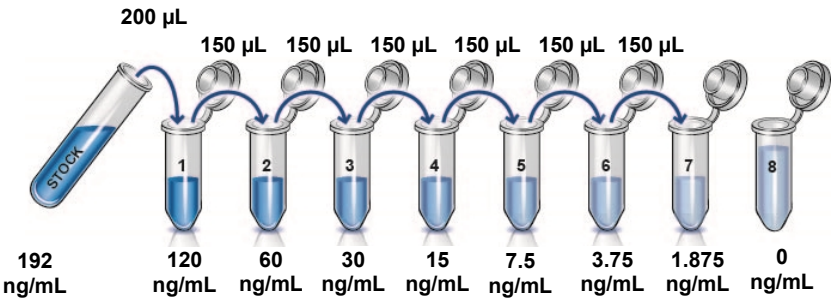
Hold at room temperature for 10 minutes and mix gently. This is the 192 ng/mL **Stock Standard** Solution.

10.3 Label eight tubes, Standards 1– 8.

10.4 For **cell culture supernatant** samples add 120 µL of Sample Diluent NS into tube number 1 and 150 µL into tube numbers 2-8.

For **serum and plasma** samples add 120 µL of Sample Diluent 25BS into tube number 1 and 150 µL into tube numbers 2-8.

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



11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	**Range**
Liver Cell Culture Supernatant	1.56 – 100%
Mouse Serum	200X - 6,400 diluted
Mouse Plasma - Citrate	200X - 3,200 diluted
Mouse Plasma - EDTA	200X - 128,000 diluted
Mouse Plasma - Heparin	200X - 128,000 diluted

11.1 Plasma

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute plasma samples at least 200 X into Sample Diluent 25BS and assay. Store un-diluted plasma samples at -20ºC or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 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 serum samples at least 200X into Sample Diluent 25BS and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants. Cell culture supernatant samples can be assayed without dilution. If needed, dilute cell culture supernatant samples in Sample Diluent NS and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

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

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

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 4ºC storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

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

13.5 Seal the 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 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.

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

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.

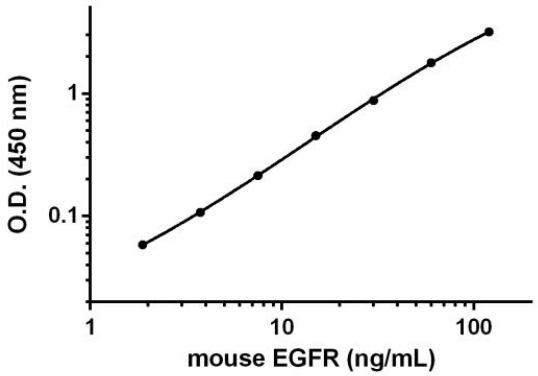
13.9 Analyze the data as described below.

14. CALCULATIONS

Subtract average zero standard from all readings. Average the duplicate readings of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

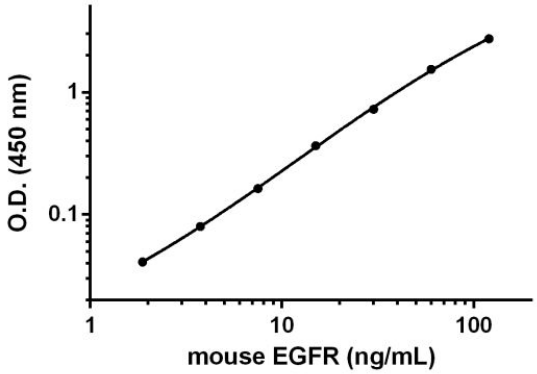
15. TYPICAL DATA

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	0.051	0.050	0.051
1.87	0.107	0.111	0.110
3.75	0.157	0.159	0.158
7.5	0.268	0.263	0.266
15	0.506	0.502	0.504
30	0.931	0.928	0.930
60	1.844	1.829	1.837
120	3.248	3.210	3.229

Figure 1. Example of EGFR standard curve prepared in Sample Diluent NS. The EGFR 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.



Standard Curve Measurements			
Conc. (ng/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.102	0.098	0.101
1.87	0.142	0.140	0.142
3.75	0.182	0.179	0.181
7.5	0.269	0.259	0.264
15	0.472	0.459	0.466
30	0.834	0.822	0.828
60	1.607	1.665	1.637
120	2.825	2.847	2.836

Figure 2. Example of EGFR standard curve prepared in Sample Diluent 25BS. The EGFR 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 dose (MDD) is determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentrations. The MDD is dependent on the Sample Diluent buffer used:

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	27	0.06 ng/mL
Sample Diluent 25BS	31	0.98 ng/mL

RECOVERY –

For **cell culture supernatant, serum and plasma** samples measurements, three concentrations of mouse recombinant EGFR were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
100% Cell Culture Media	116.1	114.3 – 119.3
800X diluted Mouse Serum	102	97.1 – 106.7
800X diluted Mouse Plasma - Citrate	100.5	96.2 – 105
800X diluted Mouse Plasma - EDTA	101.1	97.5 – 106
800X diluted Mouse Plasma - Heparin	96.9	95.2 – 99.7

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 EGFR was measured in the following biological samples in a 2-fold dilution series. Serum and plasma sample dilutions were made in Sample Diluent 25BS. Liver supernatant sample dilutions were made in Sample Diluent NS.

Dilution Factor	Interpolated value	0.5% Mouse Serum	0.5% Mouse Plasma (Citrate)	0.5% Mouse Plasma (EDTA)	0.5% Mouse Plasma (Heparin)	100% Mouse Liver Supern.
Undiluted	ng/mL	100.97	80.95	103.75	139.94	50.29
	% Expected value	**100**	**100**	**100**	**100**	**100**
2	ng/mL	50.14	39.84	46.83	58.87	24.59
	% Expected value	**99.3**	**98.4**	**90.3**	**84.1**	**97.8**
4	ng/mL	24.13	19.54	22.67	29.36	12.76
	% Expected value	**95.6**	**96.6**	**87.4**	**83.9**	**101.5**
8	ng/mL	11.98	9.44	11.33	14.15	6.29
	% Expected value	**94.9**	**93.3**	**87.3**	**80.9**	**100.1**
16	ng/mL	5.75	4.31	5.69	6.98	2.89
	% Expected value	**91.1**	**85.2**	**87.8**	**79.8**	**91.8**
32	ng/mL	2.98	NL	3.02	3.89	1.49
	% Expected value	**94.4**	**NL**	**93.1**	**89.0**	**94.6**
64	ng/mL	NA	NA	1.51	2.03	0.65
	% Expected value	**NA**	**NA**	**93.4**	**93.0**	**82.9**

NL – Non-Linear; NA – Not Analyzed, measured O.D. values were lower than the O.D. value of the lowest standard

PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of Mouse serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	5	3
CV (%)	1.9	8.5

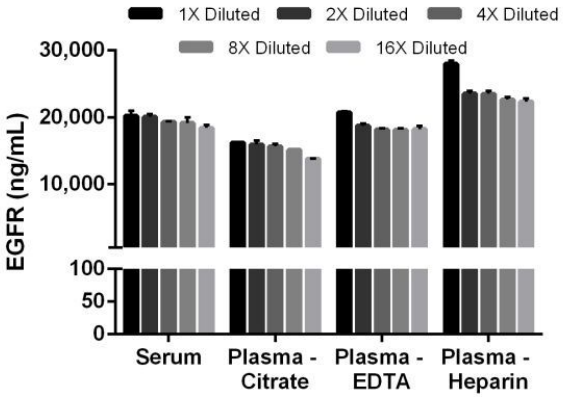


Figure 3. Interpolated concentrations of EGFR in mouse serum and plasma. The concentrations of EGFR were measured in duplicates, interpolated from the EGFR standard curve and corrected for sample dilution. Note that 1X Diluted serum and plasma sample were 200X pre-diluted samples, The interpolated, dilution factor-corrected values are plotted (mean +/- SD, n=2).

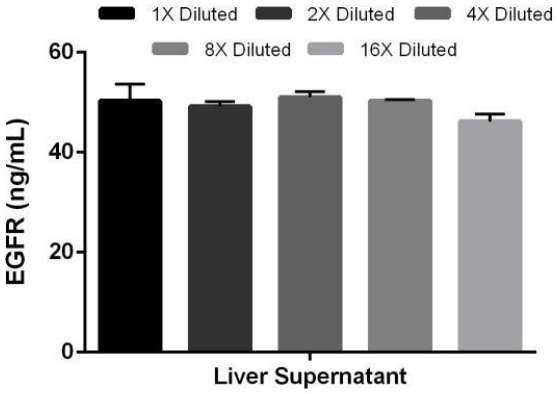


Figure 4. Interpolated concentrations of EGFR in liver cell culture supernatant samples. The concentrations of EGFR were measured in duplicates, interpolated from the EGFR standard curve and corrected for sample dilution. The interpolated, dilution factor-corrected values are plotted (mean +/- SD, n=2).

Serum/Plasma. Pooled female mouse serum and plasma samples were measured in three different dilutions in duplicates for EGFR concentrations using this kit. Means of interpolated sample values adjusted to sample dilution are shown in ng of EGFR per mL of serum/plasma in the table below.

Sample Type	Mean (ng/mL)	Range (ng/mL)
Mouse Serum	19,850	19,300 – 20,194
Mouse Plasma - Citrate	15,920	15,631 – 16,190
Mouse Plasma - EDTA	19,206	18,138 – 20,750
Mouse Plasma - Heparin	25,009	23,491 – 27,988

17. ASSAY SPECIFICITY

This kit recognizes both native and recombinant mouse EGFR protein in cell culture supernatant, serum and plasma samples only.

Saliva and milk samples have not been tested with this kit. Urine, cell and tissue extract samples are not compatible with this kit.

18. SPECIES REACTIVITY

This kit recognizes mouse EGFR protein.

Other species reactivity was determined by measuring 200X diluted serum samples of various species, interpolating the protein concentrations from the mouse standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

Rat
Hamster
Guinea Pig
Rabbit
Dog
Goat
Pig
Cow
Human

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.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37ºC.

Technical Support

For all technical or commercial enquiries please go to:
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www.abcam.co.jp/contactus (Japan)