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TGF beta 1 Rat ELISA Kit

For the quantitative measurement of Rat TGF beta 1 concentrations in Cell culture supernatant, serum and plasma (EDTA and heparin).

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

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1. Overview

Abcam's TGF beta 1 Rat *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Rat TGF beta 1 concentrations in Cell culture supernatant, serum and plasma (EDTA and heparin).

Rat TGF beta 1 specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells and then incubated at room temperature. After washing, a Biotin-conjugated anti-Rat TGF beta 1 detection antibody is added then incubated at room temperature. Following washing Streptavidin-HRP conjugate is added to each well, incubated at room temperature then again washed. TMB is added and then catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Rat TGF beta 1 captured on the plate.

Transforming growth factor β (TGF β) is a pleiotropic cytokine that exhibits a broad spectrum of biological and regulatory effects on the cellular and organism level. It plays a critical role in cellular growth, development, differentiation, proliferation, extracellular matrix (ECM) synthesis and degradation, control of mesenchymal-epithelial interactions during embryogenesis, immune modulation, apoptosis, cell cycle progression, angiogenesis, adhesion and migration and leukocyte chemotaxis. It has both tumor suppressive and tumor promoting activities and is highly regulated at all levels (e.g. mRNA turnover, latent protein activation or post-translational modifications).

TGF β is the first recognized protein of at least 40 of the TGF β superfamily of structurally related cytokines.

Three isoforms (TGF beta 1-3) have been described in mammals. (Each isoform is encoded by a unique gene on different chromosomes. All

bind to the same receptors.) They are synthesized by most cell types and tissues. Cells of the immune system mainly express TGF beta 1.

The initially sequestered, inactive LTGF β (latent TGF β) requires activation (cleavage and dissociation of its LAP (latency associated peptide) region) before it can exert biological activity. LTGF- β can also be bound to LTB (latent TGF β binding protein) to form a large latent complex (LLC). TGF β forms homodimers, and its subunits of 12.5 kDa each are bound via disulphide bridges.

TGF β signal transduction is mediated via the TGF β receptors Type II and I, phosphorylation and conformational changes, followed by different pathways:

SMAD (-pathway: TGF β recruitment finally leads to phosphorylation of receptor-regulated SMADs (R-SMADs = SMAD 2, 3) and binding of common SMAD (coSMAD = SMAD 4). The R-SMAD/ coSMAD complex enters the nucleus and interacts with a number of transcription factors, coactivators and corepressors.

TGF β induces MAPK- and MAP/ERK kinase dependent signal transduction (JNK/MAPK-, JNK/SPAK-, p38-, ERK1/2 - pathway) and the NF-κB – pathway. TGF β mediates cell cycle growth arrest via the phosphoinositide 3-kinase/Akt pathway. TGF β signaling is highly regulated e.g. via interaction with inhibitory SMADs (I-SMADs = SMAD 6, 7) or binding of the E3-ubiquitin ligases Smurf1 and Smurf2 or/and coreceptors.

TGF beta 1 is the key mediator in the pathophysiology of tissue repair and human fibrogenesis: balance between production and degradation of type I collagen and fibrosis and scarring in organ and tissue.

TGF beta 1 exhibits important immunoregulatory features of partially adverse character: TGF beta 1 inhibits B and T cell proliferation,

differentiation and antibody production as well as maturation and activation of macrophages. It inhibits activity of NK cells and lymphokine activated killer cells and blocks production of cytokines. TGF beta 1 promotes Treg cell differentiation resulting in IL-10/TGF beta 1 production and Th1 cell and Th2 cell suppression.

TGF beta 1 was recently shown to promote Th17 development in the presence of IL-6 or IL-21 in mice and probably plays a role in human Th17 development together with IL-1 β, IL-21 and IL-23. In this context TGF beta 1 is involved in induction and mediation of proinflammatory and allergic responses.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add standard or sample to appropriate wells.

Incubate the plate.

Wash and add Biotin-Conjugated anti-rat TGF beta 1 antibody to appropriate wells. Incubate the plate.

Wash and add prepared Streptavidin-HRP Conjugate to appropriate wells. Incubate at room temperature.

Wash and add TMB Substrate to each well.

Add Stop Solution to each well. Read immediately.

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	Quantity	Storage Condition
Microplate coated with monoclonal antibody to rat TGF beta 1 (12 x 8 wells)	96 wells	4°C
Biotin-Conjugate anti-rat TGF beta 1 monoclonal antibody	120 µL	4°C
Steptavidin-HRP	150 µL	4°C
TGF beta 1 Standard Lyophilized	2 vials	4°C
20X Assay Buffer	2 x 5 mL	4°C
20X Wash Buffer	50 mL	4°C
TMB Substrate Solution	15 mL	4°C
Stop Solution (1M Phosphoric acid)	15 mL	4°C
Plate seal	6 x 1 unit	4°C

7. Materials Required, Not Supplied

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

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis
- 1N HCl
- 1N NaOH

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.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- 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.
- Prepare only as much reagent as is needed on the day of the experiment.
- If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

9.1 1X Wash Buffer

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Δ Note: The 1X Wash Buffer should be stored at 4 °C and is stable for 30 days.

9.2 1X Assay Buffer

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer, combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Δ Note: The 1X Assay Buffer should be stored at 4 °C and is stable for 30 days.

9.3 1X Biotin Conjugate

To prepare the 1X Biotin Conjugated Antibody, dilute the monoclonal anti-Rat TGF beta 1 Antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Biotin Conjugated Antibody as needed by adding the required volume (µL) of the monoclonal anti-rat TGF beta 1 Antibody to the required volume (mL) of distilled water. Mix gently and thoroughly.

Number of strips	Volume of Biotin-Conjugate Concentrate (µL)	Volume of 1X Assay Buffer (mL)
1-6	60	5.94
7-12	120	11.88

Δ Note: The 1X Biotin Conjugate should be used within 30 minutes after dilution.

9.4 1X Streptavidin-HRP Conjugate

To prepare the Streptavidin-HRP Conjugate, dilute the anti-Streptavidin-HRP Conjugate 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Streptavidin-HRP Conjugate as needed by adding the required volume (µL) of the Streptavidin-HRP Conjugate to the required volume (mL) of distilled water. Mix gently and thoroughly.

Number of strips	Volume of Streptavidi-HRP solution Concentrate (µL)	Volume of 1X Assay Buffer (mL)
1-6	60	5.94
7-12	120	11.88

Δ Note: The 1X Streptavidin HRP should be used within 30 minutes after dilution.

All other solutions are supplied ready to use.

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 Prepare a 4,000 pg/mL **Stock Standard** by reconstituting one vial of the Rat TGF beta 1 standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 4,000 pg/mL **Stock Standard** cannot be stored for later use.

10.2 Label eight tubes with numbers 1 - 8.

10.1 Add 225 µL 1X Assay Buffer to all tubes.

10.4 Prepare a 2,000 pg/mL **Standard #1** by transferring 225 µL of the 4,000 pg/mL Stock Standard to 225 µL sample diluent to tube 1. Mix thoroughly and gently.

10.5 Prepare **Standard #2** by transferring 225 µL from **Standard #1** to tube 2. Mix thoroughly and gently.

10.6 Prepare **Standard #3** by transferring 225 µL from **Standard #2** to tube 3. Mix thoroughly and gently.

10.7 Using the table below as a guide, repeat for tubes number 4 through to 7.

10.8 **Standard #8** contains no protein and is the Blank control

Standard	Sample to	Volume	Volume of	Starting	Final

#	Dilute	Diluent N (µL)	Diluent (µL)	Conc. (pg/mL)	Conc. (pg/mL)
1	Stock standard	225	225	4000	2000
2	Standard #1	225	225	2000	1000
3	Standard #2	225	225	1000	500
4	Standard #3	225	225	500	250
5	Standard #4	225	225	250	125
6	Standard #5	225	225	125	62.5
7	Standard #6	225	225	62.5	31.3
8 (Blank)	None	-	225	-	0

11. Sample Preparation

- Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot or cells as soon as possible after clotting and separation.

- Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Rat TGF beta 1. If samples are to be run within 24 hours, they may be stored at 4°C.

- Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent.

- Aliquots of serum samples (spiked or unspiked) were stored at
- -20°C and thawed several times, and the Rat TGF beta 1 levels determined. There was no significant loss of Rat TGF beta 1 immunoreactivity detected by freezing and thawing.

- Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 4°C and room temperature (RT), and the rat TGF beta 1 level determined after 24 h. There was no significant loss of rat TGF beta 1 immunoreactivity detected during storage under above conditions.

Refer to Dilution Guidelines for further instruction.

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*

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use.
- Unused well strips should be returned to the plate packet and stored at 4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

13. 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.

13.1 Prepare serum and plasma samples before starting the test procedure. Dilute serum and plasma samples with 1X Assay Buffer according to the following scheme: 20 µL sample + 920 µL 1X Assay Buffer Add 30 µL 1N HCl to 940 µL prediluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 30 µL 1N NaOH.
Prepare cell culture supernatant samples before starting the test procedure. Dilute cell culture supernatant samples with 1X Assay Buffer according to the following scheme: 20 µL sample + 180 µL 1X Assay Buffer Add 20 µL 1N HCl to 200 µL prediluted sample, mix and incubate for 1 hour at room temperature. Neutralize by addition of 20 µL 1N NaOH.

Sample Matrix	Sample Volume (µL)	1X Assay Buffer (µL)	HCl 1 N (µL)	NaOH 1 N (µL)	Predilution
Serum and Plasma	20	920	30	30	1:50
Cell culture supernatant	20	180	20	20	1:12

Δ Note: Pay attention to a possibly elevated blank signal in cell culture supernatant samples containing serum components (e.g. FCS), due to latent TGF-β levels in animal serum.

13.2 Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.

13.3 Wash the microplate twice with approximately 400 µL 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells

for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.

13.4 After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively, the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

13.5 Add 100 µL of the prepared Standard Dilutions to appropriate wells

13.6 For serum and plasma samples add 80 µL of 1X Assay Buffer to the sample wells.
For cell culture supernatant samples add 60 µL of 1X Assay Buffer to the sample wells.

13.7 For serum and plasma samples add 20 µL of each pretreated sample in duplicate to the sample wells.
For cell culture supernatant samples add 40 µL of each pretreated sample in duplicate to the sample wells.

13.8 Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).

13.9 Remove adhesive film and empty wells. Wash microplate strips 5 times according to step 13.4. Proceed immediately to step 13.10.

13.10 Add 100 µL of Biotin-Conjugated Antibody to all wells.

13.11 Cover with adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).

13.12 Remove adhesive film and empty wells. Wash microplate strips 5 times according to step 13.2. Proceed immediately to step 13.13.

13.13 Add 100 µL of Streptavidin-HRP to all wells.

13.14 Cover with adhesive film and incubate at room temperature (18° to 25°C) for 30 minutes (microplate can be incubated on a shaker set at 400 rpm).

13.15 Remove adhesive film and empty wells. Wash microplate strips 5 times according to step 13.2. Proceed immediately to step 13.16.

13.16 Add 100 µL of TMB Substrate Solution to all wells.

13.17 Incubate the microplate strips at room temperature (18 to 25°C) for 30 minutes. Avoid direct exposure to intense light.

Δ Note: The color development on the plate should be monitored and the substrate reaction stopped (see step 13.18) before the signal in the positive wells becomes 9saturated.

Determination of the ideal time period for color development should to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively, the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be
stopped as soon as Standard 1 has reached an OD of 0.9 - 0.95.

13.18 Stop the enzyme reaction by adding 100 µL of Stop Solution into each well.

ΔNote: It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

13.19 Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

ΔNote: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless, the results are still valid.

14. Calculations

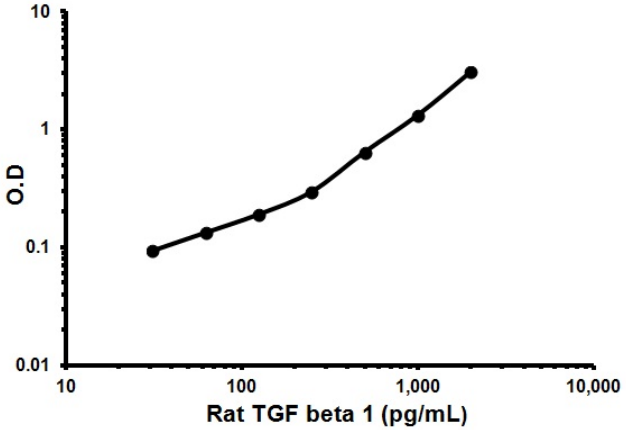
14.1 Average the duplicate reading for each standard, sample and control blank. Subtract the control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A five-parameter algorithm (5PL) 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, 5-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

14.2 If instructions in this protocol have been followed serum and plasma samples have been diluted 1:250 (1:50 predilution as per Step 13.1 followed by 1:5 dilution as per Step 13.8) and cell culture supernatant samples have been diluted 1:30 (1:12 predilution as per Step 13.1 followed by 1:2.5 as per step 13.8), the concentration read from the standard curve must be multiplied by the dilution factor (x 250 or x 30, respectively). This is in addition to any dilution factor imposed by the user, but not called for by this protocol.

14.3 Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low Rat TGF beta 1 levels. Such samples require further external predilution according to expected Rat TGF beta 1 values with 1X Assay Buffer in order to precisely quantitate the actual Rat TGF beta 1 level.

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.	O.D. 450 nm		Mean
(pg/mL)	1	2	O.D.
0	0.056	0.058	0.057
31.25	0.090	0.098	0.094
62.5	0.133	0.135	0.134
125	0.191	0.191	0.191
250	0.306	0.287	0.297
500	0.636	0.651	0.644
1,000	1.363	1.257	1.319
2,000	2.979	3.178	3.069

Figure 1. Example of Rat and the Rat TGF beta 1 standard protein standard curve.

16. Typical Sample Values

SENSITIVITY –

The limit of detection for TGF beta 1 defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 7.8 pg/mL (mean of 6 independent assays).

PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Rat TGF beta 1.

	Intra-assay Precision	Inter-Assay Precision
n =	4	4
% CV	6.8	6.8

SPIKE RECOVERY –

The spike recovery was evaluated by spiking 3 levels of Rat TGF beta 1 into serum, plasma and cell culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous Rat TGF beta 1 in unspiked samples was subtracted from the spike values. For recovery data see the table below.

Sample Matrix	Spike High (%)	Spike Medium (%)	Spike Low (%)
Serum	98	97	112
Plasma (EDTA)	77	81	71
Plasma (Citrate)	72	78	71
Cell culture supernatant	87	85	95

Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of Rat TGF beta 1 were analyzed at serial 2-fold dilutions with 4 replicates each. For recovery data see the table below. Plasma and serum samples were serially-diluted to test for linearity.

Sample Matrix	Recovery of Exp. Value	
	Range (%)	Mean (%)
Serum	95 – 106	100
Plasma (EDTA)	76 – 92	85
Plasma (Citrate)	69 – 92	82
Cell culture supernatant	95 -118	105

17. Assay Specificity

The assay detects both natural and recombinant Rat TGF beta 1. The cross reactivity of TGF- β 2 and TGF- β 3, and of TNF- β, IL-8, IL-6, IL-2, TNF-α, IL-1 β, IL-4, IFN-γ, IL12p70, IL-5 and IL-10 was evaluated by spiking these proteins at physiologically relevant concentrations into serum. There was no cross reactivity detected.

Please contact our Technical Support team for more information.

18. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards 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; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
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 kit	Store the all components as directed.

19. Notes

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