

Version 3 Last updated 3 November 2021

# ab229399 Human TNF alpha CatchPoint<sup>®</sup> SimpleStep ELISA<sup>®</sup> Kit

For the quantitative measurement of TNF alpha in human serum, plasma, and cell culture supernatant.

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

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# 1. Overview

TNF alpha *in vitro* CatchPoint SimpleStep ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of TNF alpha protein in human serum, plasma, and cell culture supernatant.

The CatchPoint 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. CatchPoint HRP Development Solution containing the Stoplight Red Substrate is added. During incubation, the substrate is catalyzed by HRP generating a fluorescent product. Signal is generated proportionally to the amount of bound analyte and the intensity is measured in a fluorescence plate reader at 530/570/590 nm Excitation/Cutoff/Emission.

TNF alpha, also known as cachectin or TNFSF1A, is the prototypic ligand of the TNF superfamily which plays a central role in inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis and morphogenesis. It is expressed on macrophages, endothelial, epithelial and tumor cells as a 26kDa transmembrane protein. TNF alpha is cleaved by proteolytic processing into six chains: (1) TNF membrane form, (2) Intracellular domain 1, (3) Intracellular domain 2, (4) C-domain 1, (5) C-domain 2 and (6) TNF soluble form. Signaling from TNF alpha differs depending on the type of ligand initiating the signaling event (intracellular, membrane or soluble). As an example, the membrane form of TNF alpha appears to mediate anti-tumorigenic therapeutic responses whereas the soluble ligand is linked to inflammation and proliferation.

## 2. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50  $\mu$ L standard or sample to appropriate wells



Add 50  $\mu$ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350  $\mu$ L 1X Wash Buffer  
PT



Add 100  $\mu$ L of prepared CatchPoint HRP Development Solution to  
each well and incubate for 10 minutes



Read fluorescence at Ex/Cutoff/Em 530/570/590 nm

### 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.
- All data, except Typical Standard Curve and Sensitivity were collected using the colorimetric version of this kit (ab181421).

## 6. Materials Supplied

Item	Quantity	Storage Condition
Human TNF alpha Capture Antibody 10X	600 µL	+4°C
Human TNF alpha Detector Antibody 10X	600 µL	+4°C
Human TNF alpha Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BR	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
Stoplight Red Substrate Buffer	12 mL	+4°C
100X Stoplight Red Substrate	120 µL	+4°C
500X Hydrogen Peroxide (H <sub>2</sub> O <sub>2</sub> , 3%)	50 µL	+4°C
Sample Diluent NS*	50 mL	+4°C
SimpleStep Pre-Coated Black 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

\*Sample Diluent NS is provided but not necessary for this product.

## 7. Materials Required, Not Supplied

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

- Fluorescence microplate reader Ex/Cutoff/Em 530/570/590 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

## 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.

- The incubation times provided in this protocol were optimized for fastest results with good signal. It is possible to increase the signal with longer incubation times, further optimization might be necessary.
- Keep in mind any RFU values shown are relative, NOT absolute. RFU from one plate reader are not comparable to another, especially if different make or model.
- 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 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.

### 9.2 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. To make 3 mL of the Antibody Cocktail combine 300  $\mu$ L 10X Capture Antibody and 300  $\mu$ L 10X Detector Antibody with 2.4 mL Antibody Diluent 4BR. Mix thoroughly and gently.

### 9.3 CatchPoint HRP Development Solution

Just prior to use prepare CatchPoint HRP Development Solution by diluting the 100X Stoplight Red Substrate and the 500X Hydrogen Peroxide in Stoplight Red Substrate Buffer.

For example, to make 6 mL of the CatchPoint HRP Development Solution combine 60  $\mu$ L 100X Stoplight Red Substrate and 12  $\mu$ L of 500X Hydrogen Peroxide with 5.928 mL Stoplight Red Substrate Buffer. Mix thoroughly and gently.

## 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 TNF alpha by adding that volume of Sample Diluent NS indicated on the label.

Alternatively, if the vial has a mass identified, reconstitute the TNF alpha standard by adding 1000  $\mu\text{L}$  sample diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 10000  $\text{pg/mL}$  **Stock Standard Solution**.

**10.2** Label 14 tubes, Standards 1– 14.

**10.3** Add 80  $\mu\text{L}$  Sample Diluent NS into tube number 1 and 150  $\mu\text{L}$  of Sample Diluent NS into numbers 2-14.

**10.4** Use the Stock Standard to prepare the following dilution series. Standard #14 contains no protein and is the Blank control.

Standards will be added to the plate in step 13.3. If desired all 14 standards can be used for a full standard curve.

Alternatively, to commit fewer wells to the standard curve, select a subset of at least 7 standards plus the blank control. If 7 standards are desired, we recommend standards #3-9.

Standard #	Dilution Sample	Volume to Dilute (μL)	Volume of Diluent (μL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	320	80	10000	8000
2	Standard#1	150	150	8000	4000
3	Standard#2	150	150	4000	2000
4	Standard#3	150	150	2000	1000
5	Standard#4	150	150	1000	500
6	Standard#5	150	150	500	250
7	Standard#6	150	150	250	125
8	Standard#7	150	150	125	62.5
9	Standard#8	150	150	62.5	31.25
10	Standard#9	150	150	31.25	15.63
11	Standard#10	150	150	15.63	7.81
12	Standard#11	150	150	7.81	3.91
13	Standard#12	150	150	3.91	1.95
14	None	0	150	0	0

## 11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Serum*	≤ 1:40
Plasma – Citrate*	≤ 1:40
Plasma – EDTA*	≤ 1:40
Plasma – Heparin*	≤ 1:40
PBMC cell culture supernatant**	1:80 -1:10

\*Based on spiked sample

\*\*Range can vary depending on stimulation factors.

### 11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:40 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C 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 samples at least 1:40 into Sample Diluent NS and assay. Store un-diluted serum at -20°C 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 and assay. Or dilute samples at least 1:10 into Sample Diluent NS and assay. Store un-diluted 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 fluorescence or "edge effects" have not been observed with this assay.
- Ensure plate and all materials are equilibrated to room temperature during use.
- Cover the plate with a plate seal during incubation steps.

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

- 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 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. 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.
- 13.7** Add 100 µL of prepared CatchPoint HRP Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm. Further optimization of incubation time vs signal strength can be performed if needed.
- 13.8** Record the fluorescence at Ex/Cutoff/Em 530/570/590 nm. If using a Molecular Devices' plate reader supported by SoftMax® Pro software, a preconfigured protocol for these CatchPoint SimpleStep ELISA Kits is available with all the protocol and analysis settings at [www.softmaxpro.org](http://www.softmaxpro.org).

Mode:	Fluorescence
Instrument settings:	Endpoint
Excitation:	530 nm
Cutoff:	570 nm
Emission:	590 nm
Sensitivity:	6 flashes/read or 200ms
PMT:	Auto
Auto calibrate:	On
Read:	Top
Read Height:	1*

\*For microplate readers with Pre-Read Optimization option, the Read Height as well as Microplate Optimization is recommended before the first read.

**13.9** Analyze the data as described below.

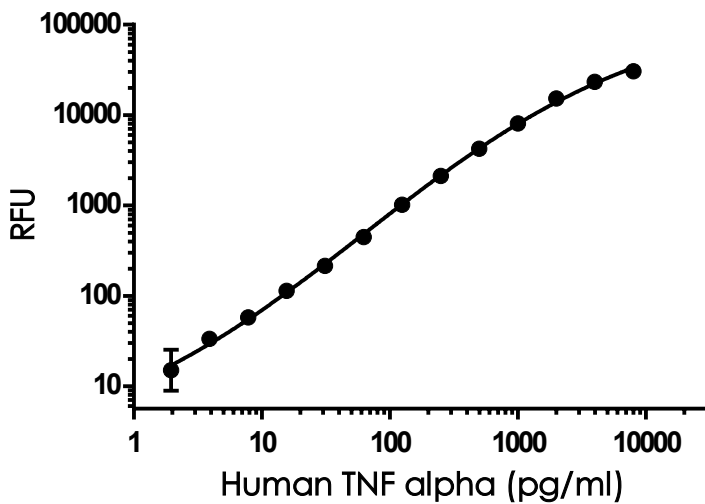
## 14. Calculations

- 14.1 Preconfigured protocols are available when using SoftMax Pro software from Molecular Devices
  - 14.2 Calculate the average fluorescence value for the blank control (zero) standards. Subtract the average blank control standard fluorescence value from all other fluorescence values.
  - 14.3 **Create a standard curve** by plotting the average blank control subtracted fluorescence 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 **fluorescence** 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.
- 14.4 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted fluorescence **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.
  - 14.5 Samples generating fluorescence values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at fluorescence 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			
Concentration (pg/mL)	RFU		Mean RFU
	1	2	
0	48	55	52
1.95	74	63	69
3.91	86	86	86
7.81	109	112	110
15.63	167	165	166
31.25	258	277	268
62.5	492	510	501
125	1040	1100	1070
250	2158	2174	2166
500	4250	4351	4300
1000	8185	8121	8153
2000	15500	15125	15312
4000	23515	23281	23398
8000	30687	30233	30460

**Figure 1.** Example of human TNF alpha standard curve in Sample Diluent NS. The TNF alpha standard curve was prepared as described in Section 10. Raw data generated on SpectraMax M4 Multi-Mode Microplate Reader is shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

## 16. Calibration

This immunoassay is calibrated against a highly purified human TNF alpha. The NIBSC/WHO unclassified purified human TNF alpha preparation 12/154 was evaluated in this kit.

The dose response curve of the unclassified standard TNF alpha parallels the CatchPoint SimpleStep ELISA kit standard curve. To convert sample values obtained with the CatchPoint SimpleStep Human TNF alpha ELISA kit to approximate NIBSC 12/154 units, use the equation below.

NIBSC (12/154) approximate value (IU/mL) = 0.094 x CatchPoint SimpleStep Human TNF alpha value (pg/mL).

## 17. Typical Sample Values

### SENSITIVITY –

The calculated minimal detectable dose (MDD) is 1.23 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=18) and adding 2 standard deviations then extrapolating the corresponding concentration.

### RECOVERY –

Three concentrations of TNF alpha recombinant protein 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 (%)
2.5% Serum	88	83-93
2.5% Plasma-Citrate	91	86-97
2.5% Plasma-EDTA	100	95-104
2.5% Plasma-Heparin	98	89-110
5% PHA-M treated PBMC Cell culture supernatant*	110	105-113

\*Media is RPMI 1640 containing 10% fetal calf serum.

## 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 TNF alpha was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	10% PHA-M Treated PBMC Cell Culture Supernatant
Undiluted	pg/mL	726.56
	<b>% Expected value</b>	100
2	pg/mL	355.55
	<b>% Expected value</b>	98
4	pg/mL	183.66
	<b>% Expected value</b>	101
8	pg/mL	94.78
	<b>% Expected value</b>	104

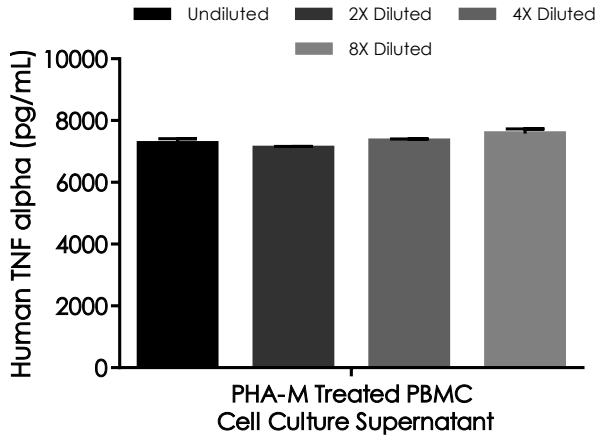
Recombinant TNF alpha was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	2.5% Human Serum	2.5% Human Plasma (Citrate)	2.5% Human Plasma (EDTA)	2.5% Human Plasma (Heparin)
Undiluted	pg/mL	447.20	451.09	483.71	499.11
	% Expected value	100	100	100	100
2	pg/mL	223.35	234.34	244.59	237.17
	% Expected value	100	104	101	95
4	pg/mL	119.50	127.27	123.94	120.73
	% Expected value	107	113	102	97
8	pg/mL	60.23	59.54	62.05	60.11
	% Expected value	108	106	103	96
16	pg/mL	28.78	NL	NL	NL
	% Expected value	103	NL	NL	NL

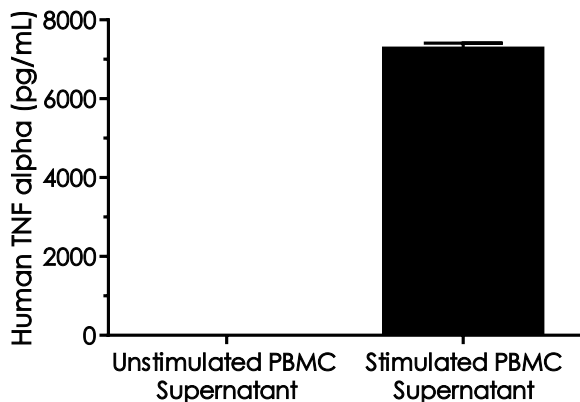
#### PRECISION –

Mean coefficient of variations of interpolated values of TNF alpha from a single concentration of PHA-M treated PBMC cell culture supernatant within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	8	3
CV(%)	2.5	3.1



**Figure 2.** Interpolated concentrations of native TNF alpha in human PBMC cell culture supernatant samples. PBMC cells were treated with 1.5% PHA-M for 46 hours in 10F RPMI. The concentrations of TNF alpha were measured in duplicates, interpolated from the TNF alpha standard curve and corrected for sample dilution. Undiluted samples are PHA-M treated PBMC cell culture supernatant 10%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was determined to be 7326.31 pg/mL in PHA-M treated PBMC cell culture supernatant.



**Figure 3.** Interpolated concentrations of native TNF alpha PBMC cell culture supernatant samples. PBMC cells were stimulated with 1.5% PHA-M or vehicle control in 10F RPMI and incubated for 46 hours. The concentrations of TNF alpha were measured in duplicate and interpolated from the TNF alpha standard curves. Undiluted samples are PHA-M stimulated PBMC cell culture supernatant 10% and unstimulated PBMC cell culture supernatant 10%. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean TNF alpha concentration was back calculated determined to be 7265.71 pg/mL in PHA-M stimulated PBMC cell culture supernatant and undetectable in the unstimulated PBMC control.

## 18. Assay Specificity

This kit recognizes both native and recombinant human TNF alpha protein in serum, plasma, and cell culture supernatant samples only.

### CROSS REACTIVITY

Recombinant proteins were prepared at 1000 pg/mL and assayed for cross reactivity. No cross reactivity was found for the following targets:

- Human IL-2
- Human IL-4
- Human IL-1a
- Human IFN gamma
- Human TNF beta
- Human TNF R1

### INTERFERENCE

Recombinant Human TNF R1 was prepared at 1000 pg/mL and tested for interference. No interference with was observed.



## 19. Species Reactivity

This kit recognizes human TNF alpha protein.

Other species reactivity was determined by measuring 1000 pg/mL recombinant proteins of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration assayed at the same dilution.

Reactive species:

- Primate

Reactivity < 3% was determined for the following species:

- Mouse
- Rat

Please contact our Technical Support team for more information.

## 20. Troubleshooting

Problem	Reason	Solution
<b>Poor standard curve</b>	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
<b>Low Signal</b>	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 CatchPoint HRP Development Solution too brief	Read plate again after longer incubation time
<b>Large CV</b>	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
<b>Low sensitivity</b>	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep Stoplight Red Substrate protected from light.
<b>Precipitate in Diluent</b>	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

# 21. Notes

## Technical Support

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