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Human IFNγ Autoantibody Assay ELISA Kit

Catalog No: CKH022 **Lot No:** TBD **Size:** 96 tests **Expiration Date:** TBD

NOTE: this is a sample protocol which is subject to variation by Lot Number. Refer to the protocol inserted in your package for the current lot number specifications and expiration date or contact our technical support at tech@cellsciences.com

Specificity:	Native human auto-antibodies directed against human IFNγ
Assay Sensitivity:	0.5 U/mL
Assay Range:	0.5-16 U/mL
Sample Type:	Plasma and serum (heparin-treated). Not compatible with EDTA.
Expected Values:	Normal value in serum < 1000 U/mL; Elevated levels in serum ≥ 1000 U/mL

Introduction

Naturally occurring autoantibodies are antibodies that bind "self" molecules originating from the body's own cells, tissues and organs. An increasing number of reports describe the presence of naturally occurring autoantibodies to a variety of cytokines in the circulation of human individuals without any obvious adverse health effects. Dysregulation of this immune network, leading to enhanced autoantibody production have also been described, resulting in a pathogenic effect. For example, high levels of neutralizing anti-IFN-γ autoantibodies (IFAAs) have been described to underlie disseminated non-tuberculous mycobacterial infections. The increased autoantibody production may be linked with a genetic predisposition combined with an environmental trigger, such as a viral infection or prolonged exposure to certain toxic compounds.

In order to measure IFN-γ autoantibodies, a highly sensitive immuno-enzymatic capture assay was developed for the quantitative determination of IFAAs in human serum and plasma. The assay provides an attractive tool to monitor disease progression and response to therapy. The performance of the assay is straightforward and therefore simple to implement. The design of the supplied pre-coated plate is optimized to preserve the native conformation of the IFN-γ molecule and avoids steric hindrance of antibody binding. In addition, the assay includes an internal negative control for each sample analysis, thereby minimizing the chance of false positivity. Overall, the Human IFN-γ Autoantibody Assay is a highly sensitive, reproducible assay providing maximal flexibility to study IFAAs in health and disease.

Principle of the Test

The kit is an antigen capture assay for autoantibody determinations in human serum and plasma samples. Streptavidin-coated 96-well strip plates are used to immobilize biotin-labeled IFN-γ molecules. Control wells contain an immobilized control agent for measuring non-specific binding. Standards, controls and samples are added to the wells, and (auto)antibodies present in the samples bind to the captured antigen. Next, wells are washed and incubated with an alkaline phosphatase (AP)-labeled anti-human IgG conjugate. After washing away unbound conjugate, the enzymatic activity is detected by addition of a ready-to-use p-NitroPhenyl Phosphate substrate. Finally, the enzymatic reaction is stopped and the optical density (OD) is read at 405 nm (reference 650 nm).

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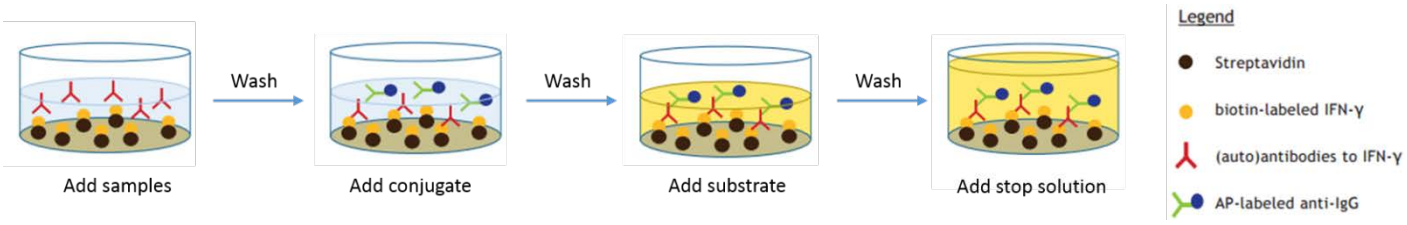

Warnings and Precautions

This kit is designed for research use only, and not for diagnostic or therapeutic procedures.

When blood components or other biological materials are used, these materials should be considered as potentially infectious and handled with the usual precautions under Bio-Hazard conditions. Follow universal precautions as established by the US government agencies, Centers for Disease Control and Prevention and Occupational Safety and Health Administration, when handling and disposing of (potentially) infectious waste.

Do not use reagents after the kit has exceeded the expiration date.

Reagents and Materials Supplied with the Kit:

Reagents (Store @ 2-8 °C)	**Quantity** (1 x 96 test kit)	**Reconstitution**
CKH002-P: 96 well microtiter strip plate	1	Ready to use (Pre-coated)
CKH002-Z: Plastic plate covers	5	n/a
CKH002-A: Standard: 640 U/mL, lyophilized	1 vial	Resuspend in 1 mL Sample Dilution Buffer (1X)
CKH002-B: Control High, lyophilized	1 vial	Resuspend in 1 mL Sample Dilution Buffer (1X)
CKH002-C: Control Low, lyophilized	1 vial	Resuspend in 1 mL Sample Dilution Buffer (1X)
CKH002-D: Sample Dilution Buffer (5X)	1 bottle (10 mL)	Dilute 1:5 with distilled water
CKH002-E: AP Conjugate (100X)	1 vial (150 µL)	Dilute 1:100 with diluted Conjugate Buffer
CKH002-F: Conjugate Buffer (5X)	1 bottle (2.5 mL)	Dilute 1:5 with distilled water
CKH002-G: pNPP Substrate Solution	1 bottle (14 mL)	Ready to use
CKH002-H: Stop Solution	1 bottle (14 mL)	Ready to use
CKH002-I: Wash Buffer (20X)	2 bottles (30 mL)	Dilute 1:20 with distilled water

Materials/Reagents Required but not Provided:

- Sterile distilled water
- Pipetting devices for the accurate delivery of volume required for the assay performance
- Tubes and containers/plates to make solutions
- 37 °C incubator
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Reading device for microtiter-plate set to 405/650 nm
- Vortex mixer

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Storage and Stability:

Precoated Plate:

The precoated 96-well strip plate in the vacuum-closed bag can be safely stored at 4 ºC until the kit expiration date. *Do not use the plate when the bag has lost vacuum.* After opening, return any unused strips to the provided self-seal plastic bag including desiccant and seal with adhesive tape. Store at 4 °C and use within 10 weeks.

Standard:

The vial with lyophilized standard can be safely stored at 4 ºC until the kit expiration date. After reconstitution, the standard is stable for 10 weeks at 4 ºC when kept sterile, or in aliquots at -20 ºC for up to 1 year.

Control - High and Control - Low:

The vials with lyophilized controls can be safely stored at 4 ºC until the kit expiration date. After reconstitution, the vials with controls are stable for 10 weeks at 4 ºC when kept sterile, or at -20 ºC for up to 1 year.

Sample Dilution Buffer (5x), Conjugate Buffer (5x), and Wash Buffer (20x):

The bottles with Sample Dilution Buffer, Conjugate Buffer and Wash Buffer can be safely stored at 4 ºC until the kit expiration date. After opening, these solutions are stable for 6 months at 4 ºC, when kept sterile.

AP conjugate (100x):

The vial with AP conjugate is stable until the kit expiration date, when stored at 4 ºC in the dark. After opening, the reagent is stable for 6 months at 4 ºC in the dark when kept sterile.

pNPP Substrate Solution (ready-to-use):

The ready-to-use pNPP Substrate Solution should be stored at 4 ºC in the dark and is stable until the kit expiration date. Avoid exposure to direct light (sunlight and UV sources) and heat.

Stop Solution (0.1 N NaOH):

The ready-to-use Stop Solution should be stored at 4 ºC and is stable until the kit expiration date.

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Preparation of Solutions and Reagents

Precoated Plate

Bring plate (vacuum-sealed, pre-coated) to room temperature (RT) prior to use. The standards/controls/samples can be added directly into the wells, without prior washing. Ensure that strip-wells are properly labeled and firmly fixed into the frame provided. After opening, return any unused strips to the provided plastic bag, including desiccant, seal completely with adhesive tape and store at 4 °C.

Wash Buffer (20x)

Dilute Wash Buffer 1:20 in distilled water and mix thoroughly. At least 300 mL diluted Wash Buffer is required for one plate. Always use freshly prepared Wash Buffer.

Sample Dilution Buffer (5x)

Before use, mix the solution gently. Dilute the buffer 1:5 in distilled water. Mix gently but thoroughly. Always use freshly prepared Sample Dilution Buffer.

Standard

Reconstitute the lyophilized Standard by adding 1 mL of diluted Sample Dilution Buffer (1x) into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 30 minutes at RT. Avoid vigorous shaking. Thereafter, the reconstituted Standard is diluted as described in “Preparing the Standards.”

Control High

The Control High contains high levels of anti-human IFN-γ antibodies. **The concentration is 9 U/mL**. The vial with lyophilized control should be reconstituted in 1 mL diluted Sample Dilution Buffer (1x). Mix gently for approximately 15 seconds and allow to stand for 30 minutes at RT. Add to plate without further dilution.

Control Low

The Control Low contains low levels of anti-human IFN-γ antibodies. **The concentration is 0.5 U/mL** The vials with lyophilized control should be reconstituted in 1 mL diluted Sample Dilution Buffer (1x). Mix gently for approximately 15 seconds and allow to stand for 30 minutes at RT. Add to plate without further dilution.

Conjugate Buffer (5x)

Before use, mix the solution gently. Dilute the Conjugate Buffer 1:5 in distilled water. Mix thoroughly. This buffer is required for the preparation of the AP conjugate working solution. (e.g., For one plate, mix 2 ml Conjugate Buffer (5x) with 8 ml distilled water.)

AP Antibody Conjugate

Gently mix 1 volume of AP antibody conjugate and 99 volumes of diluted Conjugate Buffer (1x). (e.g., For one plate, mix 100 µL AP conjugate (100x) with 9.9 mL diluted Conjugate Buffer [1x]). DO NOT use a Vortex mixer

pNPP Substrate Solution (ready-to-use)

Bring pNPP substrate solution to RT prior to use (keep in the dark).

Stop Solution (ready-to-use)

Bring Stop Solution to RT prior to use.

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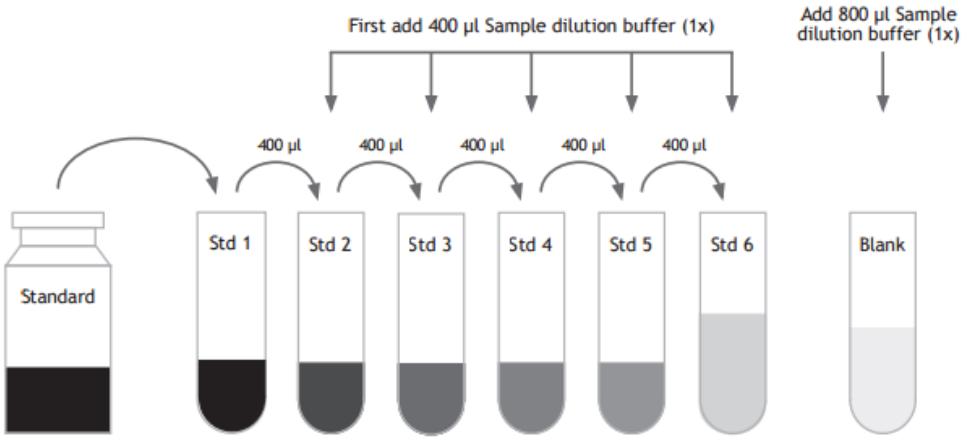
Preparing the Standards

By making use of a standard curve, the autoantibody concentration can be determined in serum and plasma samples. The standard curve is generated from the data of 6 two-fold serial dilutions of the Standard (Std 1-6). Always include a blank control (diluted Sample dilution buffer [1x] only). (See also figure in the "Plate Layout" section of this protocol).

For one precoated Autoantibody plate:

- Label seven tubes: Std 1 through 6, and Blank. Add 400 µL Sample Dilution Buffer (1x) to the tubes for Std 2 through 6. Add 800 µL Sample Dilution Buffer (1x) to the tube for the Blank.
- In the tube for Std 1, prepare the highest concentration to be used in the standard curve (see "Assay Range") by mixing an appropriate volume of Standard with Dilution buffer. The final volume of Std 1 should be 800 µL. Allow the mixture to stand for at least 15 seconds before adding it to the next tube.
- Perform serial two-fold dilutions by transferring 400 µL of Std 1 to the next tube (Std 2). Mix well and transfer 400 µL from Std 2 to the next tube (Std 3), and so on until Std 6.

***Note**: A standard curve, including a blank, should be run on each plate.*



Specimen Collection and Handling:

Specimens should be clear, non-hemolyzed and non-lipemic. Excessive hemolysis and the presence of large clots or microbial growth in the sample may interfere with the performance of the test.

- **Serum:** use a clot tube and allow sample to clot for 30-45 minutes at RT, then centrifuge for 10-15 minutes at 1,000 to 2,000 x *g* (RT) and collect serum immediately.
- **Plasma:** collect plasma by using anticoagulant, such as heparin (DO NOT use EDTA). Mix well immediately after collection. Centrifuge for 10-15 minutes at 1,000-2,000 x *g* at RT and collect plasma.

Samples should be aliquoted and stored frozen at -20 ºC to -80 ºC. If samples are run within 5 days, they may be stored at 2-8 ºC. Avoid repeated freeze-thaw cycles. DO NOT heat serum or plasma samples. Prior to assay, frozen samples should be completely thawed and mixed well.

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Sample Preparation

Both serum and plasma can be used, but **DO NOT use EDTA as anticoagulant**. Make sure the samples are clear and homogenous (if not, filter or centrifuge the sample briefly for 5 minutes at 10,000 x *g*).

Use polystyrene tubes to prepare dilutions.

When the levels of autoantibodies present in the samples are unknown, it is recommended to prepare and analyze a series of dilutions to ensure that sample measurements fall within the assay range (see also "Performance Characteristics" section of this protocol).

Dilute samples 100 to 500-fold in 1x Sample Dilution Buffer. Mix well and incubate for 30 minutes at RT. Apply 100 µL to each well.

The diluted samples are tested in triplicate as illustrated in the Plate lay-out as shown below (two wells are autoantibody-specific; one well is for the internal negative control).

Plate Layout

Columns 1, 2, 4, 5, 7, 8, 10, and 11 of the pre-coated plate are sensitized with human IFN-γ (Antigen). Columns 3, 6, 9 and 12 are sensitized with a Control agent, which serves as a negative internal control (to visualize false positive signals).

	1	2	3	4	5	6	7	8	9	10	11	12
	Antigen	Antigen	Control agent	Antigen	Antigen	Control agent	Antigen	Antigen	Control agent	Antigen	Antigen	Control agent
A		Std 1			S1			S9			S17	
B		Std 2			S2			S10			S18	
C		Std 3			S3			S11			S19	
D		Std 4			S4			S12			S20	
E		Std 5			S5			S13			S21	
F		Std 6			S6			S14			S22	
G		Blank			S7			S15		Control Low		
H					S8			S16		Control High		

Use columns 1 – 3 (pink wells) for Std 1 through 6 and the Blank. Samples S1 through S22 (green wells) are pipetted in three neighboring columns (triplicates). For example: sample S1 is in positions A4, A5 and A6. This results in two specific determinations for antibody binding to human IFN-γ and one non-specific determination as an internal negative control. Include on each plate one Control Low (triplicate blue wells) and one Control High (triplicate yellow wells).

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Directions for Washing

- Incomplete washing of the wells will adversely affect the assay. All washing steps should be performed with diluted Wash Buffer (1x).
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering the tip of an aspiration device into each well (without touching the bottom). After aspiration, fill the wells with at least 250 µL Wash Buffer and then aspirate the liquid. Repeat these steps at least 6 times. After washing, the plate is inverted and tapped dry on absorbent tissue paper.
- Alternatively, the Wash Buffer may be put into a squirt bottle. If a squirt bottle is used, empty the wells and flood the plate with Wash Buffer, completely filling all wells. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper. *Note: If you have excessive air bubbles in wells due to squirt bottle, replace Wash Buffer with sterile PBS during the last wash step.*
- When using an automated washing device, follow operating instructions carefully.

Assay Procedure

Vacuum-packed plate and all solutions should be at RT prior to use.

1. Take the pre-coated strip plate out of the vacuum-packed bag. Label the Control Agent strips carefully.
2. Add 100 µL of diluted standards/samples and undiluted controls (High and Low) to appropriate wells, without prior washing of wells. See “Plate Layout.”
3. Seal the plate and incubate for 2 hours at 37 ºC.
4. Discard the content of the wells and wash the wells at least 6 times with Wash Buffer (1x).
5. Add 100 µl of diluted AP conjugate to each well.
6. Seal the plate and incubate for 1 hour at 37 ºC.
7. Discard the content of the wells and wash the wells at least 6 times with Wash Buffer (1x).
8. Add 100 µl of pNPP substrate to each well.
9. Leave the plate for 20 minutes at 37 ºC in the dark.

   Note: The substrate produces a soluble yellow end-product.
10. After substrate incubation, DO NOT empty the wells. Stop the reaction by adding 100 µL of Stop Solution. The color remains yellow, but does not fluctuate.
11. Read the plate at 405 nm, and for reference at 650 nm, within 15 min after stopping the reaction.

Assay time: Maximum 4 hours.

Note: If you only use a part of the plate, put the remaining unused strips in the accompanying plastic bag with desiccant and store at 4 °C. *Use the unused strips within 10 weeks after opening of the vacuum closed bag.*

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Data analysis

First, subtract the reference OD_{650nm} from each OD_{405nm} (=OD).

Then, calculate the mean OD for each standard concentration (n=2): Std 1-6 (see sections "Preparing the Standards" and "Plate Layout").

Next, calculate the mean OD_{blank} (n=4) and subtract this value from the mean OD of each standard concentration (see formula below).

Formula: OD = mean $OD_{std\ 1\text{-}6}$ - mean OD_{blank}

To create the standard curve, plot the standard concentration (x-axis) versus the corresponding OD (y-axis). Draw the standard curve, using a 4-parameter logistic regression curve (see below).

For samples, calculate the mean OD for each sample and subtract the $OD_{control\ agent}$. The autoantibody concentration of samples can be determined from the standard curve by interpolation. The calculated concentration must be multiplied by the dilution factor.

Test results are valid if the data are as follows:

- $OD_{405/650\ nm}$ for the highest standard is > 0.9
- $OD_{405/650\ nm}$ for the blank is < 0.2
- Calculated values of the Control High and Control Low comply with the reference values indicated:
  - Control High: 5 – 13 U/mL
  - Control Low: < 2 U/mL

If these quality control criteria are not met, the assay run is invalid and should be repeated.

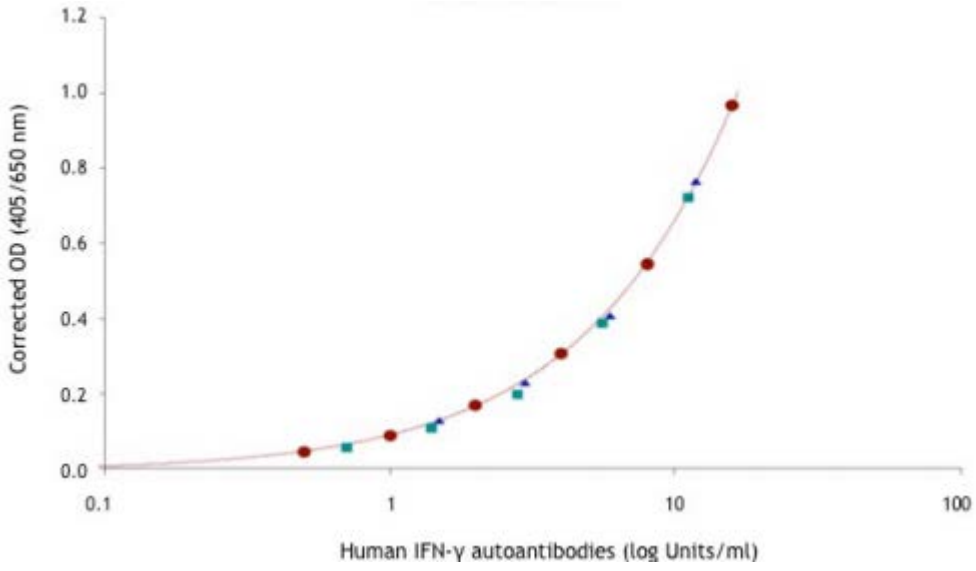
Note: *The OD values of the standards in the column with control agent (plate layout, column 3) are not used for calculations. The OD values should be < 0.200.*

Sample Standard Curve:

(Example only. Each lab must produce their own standard curve for each assay.)

● - Human IFN-γ standard

□ & Δ - Two positive serum samples, serially diluted through the quantitative range of the assay.



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Performance characteristics

Calibration

The human IFN-γ Autoantibody assay is calibrated in relative arbitrary units, since no international reference preparation is available for this assay. One Unit (U) is arbitrarily defined as the amount of anti-human IFN-γ binding activity that equals the binding activity of 1 ng of mouse anti-human IFN-γ monoclonal antibody in the same assay.

Quantification Range and Sensitivity

The quantification range of the Human IFN-γ Autoantibody Assay is: 0.5-16 U/mL.

The sensitivity is 0.5 U/mL.

Expected Values

In a normal range study with serum from healthy donors and patients with proven increased natural autoantibodies directed to human IFN-γ values, the following cutoff has been established with this Human IFN-γ Autoantibody Assay using a serum dilution of 1:300:

Normal value in serum < 1000 U/mL

Elevated levels in serum ≥ 1000 U/mL

Please note that each laboratory should establish its own normal and pathological reference ranges for IFAA levels. Also, it is recommended to use your own panel of control serum or plasma samples in this assay.

Interfering substances

Interfering effects have been found with the use of EDTA as an anticoagulant and should therefore not be used. In addition, it is recommended to avoid hemolyzed or lipemic samples.

Reproducibility

Intra-Assay Precision: Coefficient of variation (CV) was calculated for three positive autoantibody samples from the results of 8 determinations in a single run. Results for the precision within one assay are shown in the Table below.

Inter- Assay Precision: CV was calculated for three samples from the results of 24 determinations in 3 different runs. Results for run-to-run are shown in Table below.

Sample	Intra-Assay (n=8)		Inter-Assay (n=24)	
	Mean U/ml	CV (%)	Mean U/ml	CV (%)
1	2.3	6.9	2.4	7.5
2	13.3	3.1	13.5	8.8
3	7.0	4.2	7.8	11.9

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Troubleshooting

Problem	Possible cause	Solution
Poor consistency of replicates	Inaccurate pipetting	- Ensure accurate pipetting of volume and avoid air bubbles. - Check pipettes.
	Inadequate mixing of reagents	- Mix reagents adequately.
	Inadequate washing	- Increase the stringency of washes (particularly after the AP conjugate incubation step).
	Too much airbubbles after using a squirt bottle for washing	- You can add a PBS soak step after 5 washing steps with Wash buffer. Use commercially available liquid PBS (pH 7.4).
	Evaporation of solutions	- Ensure precise sealing of the plate.
	Non-homogenous samples or with high particulate matter	- Mix samples thoroughly and remove particulates by centrifugation.
OD_{blank} values higher than 0.2	Incubation time of pNPP substrate solution is too long	- Incubation time of substrate should be 20 min.
	Improper storage of pNPP	- Store pNPP at 4 °C and protected from light (unreacted pNPP substrate appears colorless to pale yellow).
	Working solutions were contaminated	- Solutions should be clear and colorless. Use a clean container before addition into wells.
	AP conjugate dilution was too concentrated or left too long on the plate	- Ensure proper dilution of AP conjugate and incubation time.
No signal or low OD values for standards	Improper storage of AP conjugate	- Avoid prolonged exposure to light and heat. - Store conjugate always at 4 °C.
	Incorrect incubation times or temperature	- Ensure proper incubation times. - Reagent solutions should be at RT before use.
	Improper quality or pH of distilled water	- Use distilled water, and not tap water. - Check quality and pH of distilled water.
	Improper standard dilution	- Ensure proper dilution of standard.
	Degradation of antibodies	- Follow recommended storage conditions.
	Overly high washing / aspiration pressure from automated plate washer.	- Check function of washing system or apply manual washing.
Poor standard curve (linearity and dynamic range)	Improper standard dilutions	- Ensure proper dilution of standards (follow 'two-fold dilutions' guidelines).
	Inaccurate pipetting	- Ensure accurate pipetting of volume and avoid air bubbles. - Check pipettes.

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