cellsciences.com

Human IFNγ Autoantibody Assay ELISA Kit

Catalog No: CKH022 **Size:** 1 x 96 tests

Range:	0.5-16 U/ml
Sensitivity:	0.3 U/ml
Specificity:	Human IFNγ Autoantibody
Calibration:	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.
Type of sample:	Plasma and serum
Expected Values:	Normal value in serum < 1000 U/ml Elevated levels in serum ≥ 1000 U/ml

Introduction

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 (1). For example, high levels of neutralizing anti-IFN-γ autoantibodies (IFAAs) have been described to underlie disseminated non-tuberculous mycobacterial infections (2, 3). Other anti-cytokine autoantibodies associated with disease pathogenesis have been described for GM-CSF, Interleukin 17A/F (IL-17A/F) and IL-22, which are involved in pulmonary alveolar proteinosis (PAP), chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I, respectively (4,5).

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 easy and 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 on 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.

1. Browne S.K., et al., Immunodeficiency secondary to anti-cytokine autoantibodies. Curr Opin Allergy Clin Immunol, 2010. 10(6): 534-41.

2. Chi C.Y., et al., Anti-IFN-gamma autoantibodies in adults with disseminated nontuberculous mycobacterial infections are associated with HLA-DRB1*16:02 and HLA-DQB1*05:02 and the reactivation of latent varicella-zoster virus infection. Blood, 2013. 121(8): 1357-66.

3. Patel S.Y., et al., Anti-IFN-gamma autoantibodies in disseminated nontuberculous mycobacterial infections. J Immunol, 2005. 175(7): 4769-76.

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

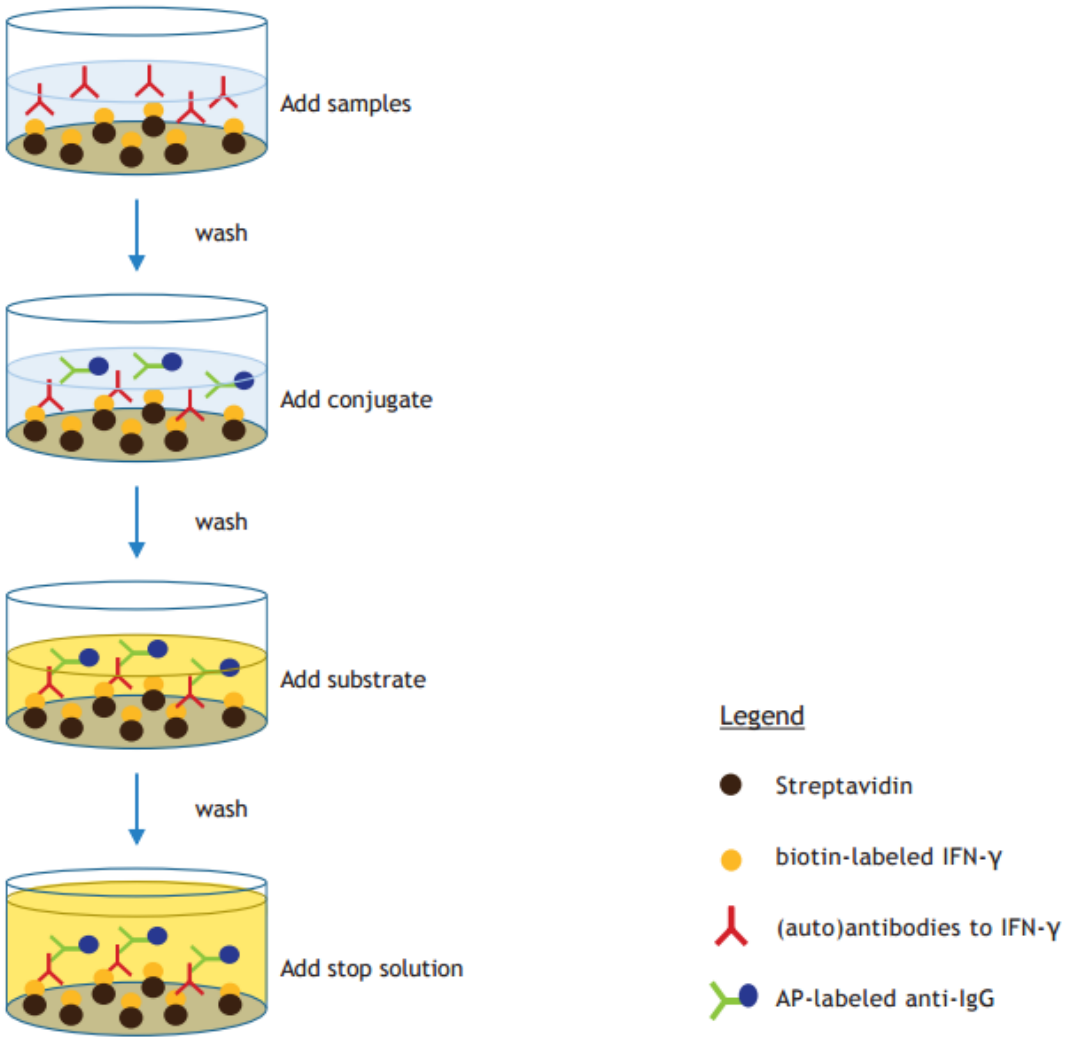
E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

4. Puel A., et al., Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. J Exp Med, 2010. 207(2): 291-7.

5. Uchida K., et al., High-affinity autoantibodies specifically eliminate granulocyte-macrophage colonystimulating factor activity in the lungs of patients with idiopathic pulmonary alveolar proteinosis. Blood, 2004. 103(3): 1089-98.

Principle of the test

The kit is a sensitive antigen capture assay for autoantibody determinations in human serum and plasma samples. The assay consists of streptavidin-coated 96-well strip plates with immobilized 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 diluted 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 (pNPP) substrate. Finally, the enzymatic reaction is stopped and the optical density (OD) is read at 405 nm (reference 650 nm).



Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

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, then please note that all 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:

Items	Quantity (5 Plates)	Storage conditions
Pre-coated 96-well strip plate* (sensitized by human IFN-γ and a control agent)	1 Plate	4 ℃
Standard (lyophilized)	1 vial	4 ℃
Control High (lyophilized)	1 vial	4 ℃
Control Low (lyophilized)	1 vial	4 ℃
Sample Dilution Buffer (5x)	1 vial (10 ml)	4 ℃
AP Anti-human IgG Conjugate (100x)	1 vial (150 µl)	4 ℃
Conjugate Buffer (5x)	1 vial (2.5 ml)	4 ℃
pNPP Substrate Solution (ready-to-use)	1 vial (14 ml)	4 ºC in the dark
Stop Solution (0.1 N NaOH)	1 vial (14 ml)	4 ℃
Wash buffer (20x)	2 vials (30 ml)	4 ℃
Adhesive cover slips	5 pieces	RT

* Consists of 12 separate strips of 8 wells fixed in a frame.

Hazard Information:

All kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

Storage and Stability:

Precoated Plate

The precoated 96-well strip plate in the vacuum-closed sachet can be safely stored at 4ºC until the expiration date (indicated on the label of the sachet). Do not use the plate when the sachet 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℃ and use within 4 weeks.

Standard

The vial with lyophilized standard can be safely stored at 4ºC until the expiration date (indicated on the vial). After reconstitution, the standard is stable for at least 4 weeks at 4ºC when kept sterile. The standard can also be divided into small aliquots for single use. These aliquots should be stored at ≤-20ºC (stable for at least two years).

Control High and Control Low

The vials with lyophilized controls can be safely stored at 4ºC until the expiration date (indicated on the vials). After reconstitution, the vials with controls are stable for at least 4 weeks at 4ºC when kept sterile. The controls can also be stored at ≤-20 ºC (stable for at least two years).

Sample dilution buffer (5x), Conjugate buffer (5x), and Wash buffer (20x)

The vials with Sample dilution buffer, Conjugate buffer and Wash buffer can be safely stored at 4ºC until the expiration date (indicated on the vials). After opening, these solutions are stable for at least 6 months at 4ºC when kept sterile.

AP Anti-human IgG conjugate (100x)

The vial with AP anti-human IgG conjugate is stable until the expiration date (indicated on the vial) when stored at 4ºC in the dark. After opening, the reagent is stable for at least 6 months at 4ºC in the dark when kept sterile.

pNPP substrate solution (ready-to-use)

The ready-to-use pNPP substrate solution should be stored at 4ºC in the dark and is stable until the expiration date (indicated on the vial). 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 4ºC and is stable until the expiration date (indicated on the vial).

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

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℃ incubator.
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc).
- Reading device for microtiter-plate set to 405/650 nm.
- Vortex mixer

Preparation of solutions and reagents

Precoated plate

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

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 min 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 indicated on the vial and specified on the Certificate of Analysis. 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 min at RT.

Control Low

The Control Low contains low levels of anti-human IFN-γ antibodies. The concentration is indicated on the vial and specified on the Certificate of Analysis. The vials with lyophilized controls should be reconstituted in 1 ml diluted Sample dilution buffer (1x). Mix gently for approximately 15 seconds and allow to stand for 30 min at RT. **Note:** Use vials with Standard and Controls within 4 weeks.

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. For one plate: 2 ml Conjugate buffer (5x) is mixed with 8 ml distilled water.

AP antibody conjugate

Mix gently 1 volume of AP antibody conjugate and 99 volumes of diluted Conjugate buffer (1x). Do not use a Vortex mixer. For one plate: 100 µl AP conjugate (100x) is gently but thoroughly mixed with 9.9 ml diluted Conjugate buffer (1x).

pNPP substrate solution (ready-to-use)

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

Stop solution (ready-to-use)

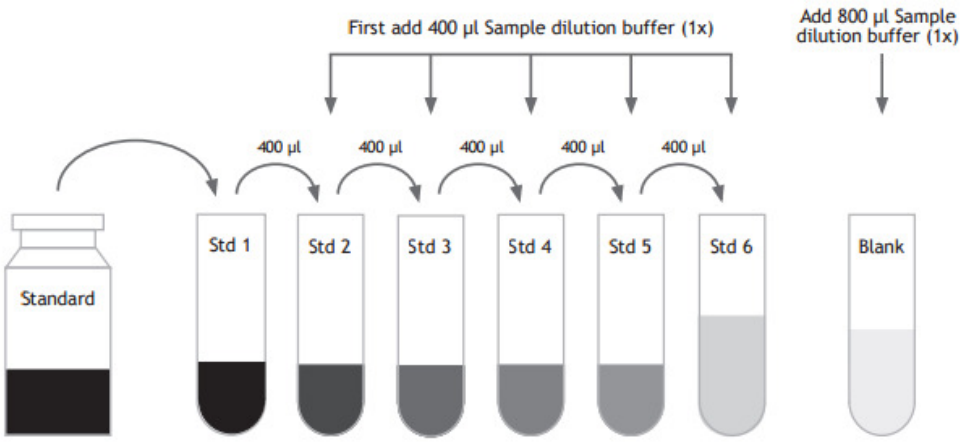
Bring Stop solution to RT prior to use.

Preparing the standards

By making use of a standard curve, the IFAA concentration can be determined in serum and plasma samples. The standard curve is generated from the data of 6 two-fold serial dilutions (Std 1-6) of the Standard. The recommended standard range of the Human IFN-γ Autoantibody Assay is 0.5-16 U/ml. Always include a blank control (diluted Sample dilution buffer [1x] only) (See also figure for “Plate Layout” shown below).

For one precoated Autoantibody plate:

- Take 7 tubes: Mark them Std 1 till Std 6 and blank. Add 400 µl Sample dilution buffer (1x) to 5 of these tubes (Std 2 till Std 6) and 800 µl to the last tube (Blank).
- Prepare in the remaining tube (Std 1) the highest concentration to be used in the standard curve (see Certificate of Analysis) 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 min at RT, then centrifuge for 10-15 min at 1,000 to 2,000 x g (RT) and collect serum immediately.
- **Plasma:** collect plasma by using anticoagulant, such as EDTA or heparin (Do Not Use EDTA). Mix well immediately after collection. Centrifuge for 10-15 min at 1,000-2,000 x g at RT and collect plasma.

Samples should be aliquoted and stored frozen at -20ºC to -80ºC. 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.

Note: Specimen collection from humans should be carried out in accordance with the Clinical and Laboratory Standards Institute document M29-T2, "Protection of laboratory workers from infectious diseases transmitted by blood and tissue".

Sample preparation

Both serum and plasma (do not use EDTA as anticoagulant) can be used. Make sure the samples are clear and homegenous (if not, filtrate or centrifuge the sample briefly for 5 min at 10,000 x g).

Use polystyrene tubes to prepare dilutions.

When the levels of IFAA 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” below).

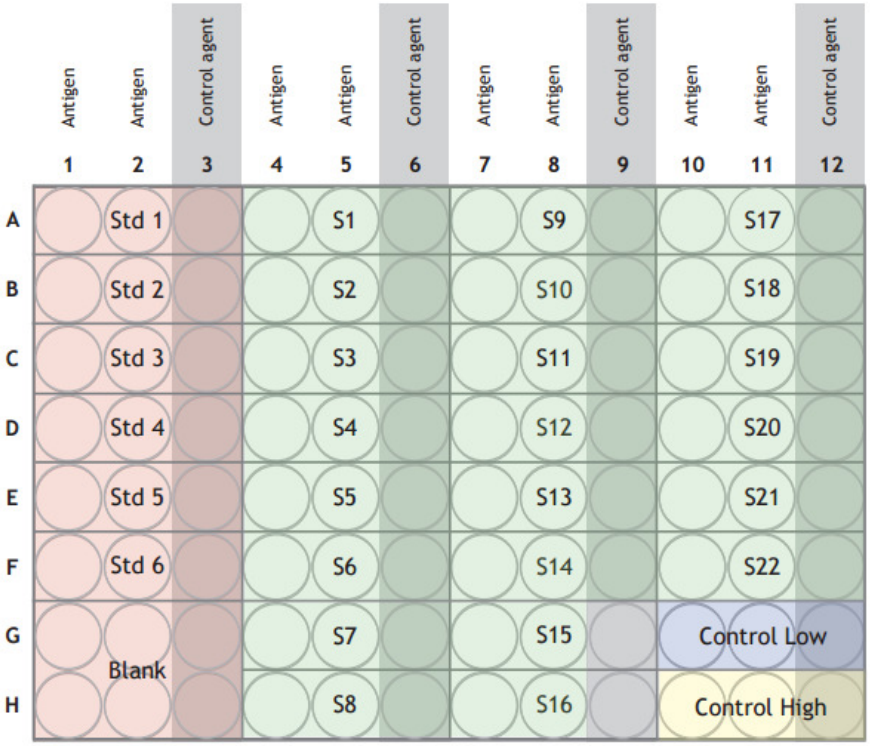
Dilute samples 100- to 500-fold in diluted Sample dilution buffer (1x). Mix well and incubate for 30 min at RT. Apply 100 µl to each well.

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

The columns 1, 2, 4, 5, 7, 8, 10, 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 (blue wells).

The control agent serves as a negative internal control (visualizing false positive signals).



Use the first three columns (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 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 pale yellow wells).

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

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 six 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.

• 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 sachet.
2. Add 100 µl of diluted standards/controls/samples to each well, without prior washing. Check “Plate layout” above.
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 six times with Wash buffer (1x).
5. Add 100 µl of diluted AP antibody 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 six times with Wash buffer (1x).
8. Add 100 µl of pNPP substrate to each well.
9. Leave the plate for 20 min 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.
11. Read the plate at 405 nm and for reference at 650 nm within 15 min after stopping the reaction.

Assay time: Maximal 4 h

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 ℃. Use the unused strips within 4 weeks after opening of the vacuum closed sachet.*

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

Data analysis

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

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 (for example, see Certificate of Analysis).

For samples, calculate the mean OD for each sample and subtract the $OD_{control\ agent}$. The IFAA 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 OD for the highest standard, blank and calculated values of the Control High and Control Low comply with the reference values indicated under Specification on the Certificate of Analysis enclosed in each Autoantibody kit. 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.*

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 0.3 U/ml.

Expected values

In a normal range study with serum from healthy donors and patients with proven increased IFAA 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.

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

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

Linearity

Serum samples from patients with proven elevated levels of IFAAs were serially diluted in Sample dilution buffer (1x) to demonstrate the dynamic range of the assay and the upper / lower end of linearity. The relative arbitrary units were calculated for each dilution from the calibration curve using a 4-parameter logistic regression curve.

Sample	Dilution	Observed U/ml	Expected U/ml	Observed/Expected (%)
1	1:30,000	11.2	11.2	100
	1:60,000	5.6	5.3	95
	1:120,000	2.8	2.4	86
	1:240,000	1.4	1.2	88
	1:480,000	0.7	0.6	88
2	1:100	12.4	12.4	100
	1:200	6.4	6.2	104
	1:400	3.0	3.1	97
	1:800	1.6	1.5	102
	1:600	0.9	0.8	113

Note: *not all serum samples may dilute in a linear way according to the standards of the kit.*

Reproducibility

Intra-assay precision: Coefficient of variation (CV) was calculated for three positive IFAA 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

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.

Abbreviations

AP	Alkaline Phosphatase
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic acid
h	hour
IFAA	IFN-γ autoantibody
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
min	minutes
N	Negative control
OD	Optical Density
pNPP	p-NitroPhenyl Phosphate
RT	Room temperature
S	Sample
Std	Standard
U	Unit(s)

NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.

Cell Sciences®
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com