919 Fraser Drive Unit 11 Burlington ON L7L 4X8
Tel: 866-344-3954 Fax: 289-204-9100
www.cytodiagnostics.com

PRODUCT SHEET

Human IgG Fc ELISA Kit

Catalogue No. EL-003

Background

Cytodiagnostics Human IgG Fc ELISA (Enzyme Linked Immunosorbent Assay) kit is designed for the quantitative measurement of human IgG Fc protein in serum, plasma and cell culture supernatants.

The Human IgG Fc ELISA kit is based on the antibody sandwich principle. A microtiter plate coated with a capture antibody specific to human IgG Fc has been blocked and stabilized to create the solid phase of the assay. To perform the assay, samples, standards, and controls are added directly to the wells of the plate. After washing away unbound IgG, a Horse Radish Peroxidase (HRP) -conjugated Detection Antibody Solution is added and binds to the heavy chains of the captured human IgG molecules that were immobilized by the capture antibody, completing the sandwich. The wells are washed and a tetramethylbenzidine (TMB) Substrate Solution is added. A blue colour develops in proportion to the amount of bound human IgG. This blue end product is soluble and can be read kinetically for up to 60 minutes using a microtiter plate reader at either 370 nm or 655 nm wavelengths. Alternatively, the colour development may be stopped using Stop Solution, which turns the blue end product yellow and the optical density (OD) of the yellow product is measured at 450 nm on a microtiter plate reader.

The discovery and development of hybridoma technology for the generation of monoclonal antibodies (mAbs) in mice by Georges Kohler and Cesar Milstein in 1975 has had a huge impact on basic research and modern medicine (Kohler & Milstein, 1975). As a result of this technology, it became possible to generate antibodies that are highly specific to their target and in large quantities. In 1986, muromonab-CD3 became the first mouse monoclonal antibody to be approved by the US Food and Drug Administration (FDA) for therapeutic use for the prevention of kidney transplant rejection (Lu *et al.*, 2020). When given to human patients as therapeutics, however, mouse mAbs rapidly produce a human anti-mouse antibody (HAMA) response. HAMAs produce allergic reactions and decrease the mAb's effectiveness as a treatment (Lu *et al.*, 2020). To overcome this disadvantage, Gregory P. Winter applied the phage display technique for the rapid evolution of peptides and proteins that was developed by George P. Smith in 1985 (Smith, 1985; Jones *et al.*, 1986) and apply it to the discovery and isolation of antibodies (McCafferty et al., 1990). Together, these discoveries led to the development of the first fully human therapeutic antibody to be approved by the US FDA; adalimumab (Humira), for the treatment of rheumatoid arthritis (Kempini, 1999). Another method for producing fully human mAbs is using transgenic animals. Two transgenic mouse lines, the HuMabMouse (Lonberg *et al.*, 1994) and the XenoMouse (Mendez *et al.*, 1997) were created by replacing the mouse genes immunoglobulin genes (Ig) with human IgG genes making these animals capable of producing fully human antibodies after immunization (Lonberg *et al.*, 1994; Green *et al.*, 1994). These advances led to the generation of the first human antibody therapeutic generated in a transgenic mouse, anti-epidermal growth factor receptor, panitumab (Moroni *et al.*, 2005; Gibson *et al.*, 2006). In 1984, Aman *et al.* developed the single B cell antibody technology, where single B cells are immortalized with Eppstein-Barr virus and used for the production of human monoclonal antibodies (Aman *et al.*, 1984). In 2004, Traggiai *et al.* used this technique on memory B cells isolated from a SARS-CoV1 patient, and produced human monoclonal antibodies capable of potent neutralization of the SARS coronavirus (Traggiai *et al.*, 2004). In the face of novel emergent pathogens, single B cell antibody technology has proven invaluable for the rapid generation of potent human monoclonal neutralizing antibodies against Human Immunodeficiency virus (Bonsignori *et al.*, 2011), *Bacillus anthracis* (Chi *et al.*, 2015), Ebolavirus (Bornholdt *et al.*, 2016) and Zika virus NS1 (Bailey *et al.*, 2018).

The rapid development these various techniques for the large-scale production of human monoclonal antibodies has created a need for a rapid and simple method for accurately quantifying human antibody production both *in vitro* (i.e., cell culture supernatant) and *in vivo* (i.e., serum, plasma, ascites). The Cytodiagnostics Inc. anti-human IgG Fc ELISA kit is a sensitive and accurate assay for the determination of human IgG levels produced *in vivo* and for assessing the level of immunoglobulin secretion by a hybridoma *in vitro*.

Assay Summary

The assay is summarized in Figure 1 and involves a detection system that uses an HRP-conjugated Detection Antibody Solution and TMB Substrate Solution. The Substrate Solution turns blue when modified by HRP. The final step is to stop the reaction with Stop Solution, which turns the solution yellow. The optical density (OD) of the yellow solution is read at 450 nm using a microtiter plate reader.

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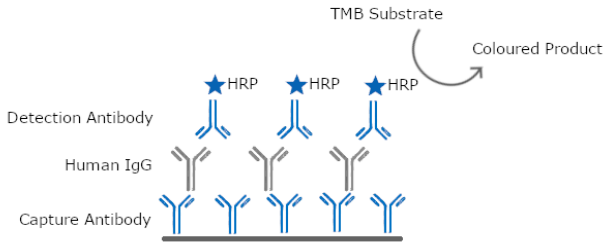

Figure 1. *Schematic overview of the Human IgG ELISA Kit.*

Precautions

Please read all instructions described in this document prior to performing the assay.

All components of this kit have been formulated and quality control tested to perform as described in this document. Substituting buffers or reagents provided or modifications to the procedures may result in loss of performance of the assay.

Storage and Stability

Kit should be stored at 2-8°C upon receipt.

Follow the storage conditions for all kit components and prepared components as described in sections below.

Materials Supplied

Item	Quantity	Storage Condition
[1X] Diluent Buffer	20 mL	+2-8°C
[10X] Wash Buffer 1	12 mL	+2-8°C
[10X] Wash Buffer 2	2 mL	+2-8°C
Human IgG, lyophilized	1 vial	+2-8°C
[500X] Detection Antibody Solution	30 µl	+2-8°C
[10X] TMB Substrate Solution	1.2 mL	+2-8°C
TMB Substrate Diluent	12 mL	+2-8°C
Enhancer Solution	12 µl	+2-8°C
Stop Solution	12 mL	+2-8°C
Pre-Coated and Blocked 96-Well Microtiter Plate (12 x 8-well strips)	1 plate	+2-8°C
Plate Seal	1	

Materials Required, Not Supplied

These materials are required to perform the assay and are to be provided by the end-user.

- Assorted adjustable volume pipettes.
- Multi-channel and single channel pipettes.
- Pipette tips.
- Polypropylene tubes.
- Deionized water.
- Reagent reservoirs.
- Microtiter plate reader capable of measuring absorbance at 450, 540 and 655 nm.

Limitations

- Assay kit is intended for research use only. Do not use for diagnostic procedures.
- Do not substitute reagents or components of the kit with other lots, other vendors or end-user made reagents. This kit has been QC tested with the components provided and performance cannot be guaranteed if any single component has been substituted.

Technical Tips

- Avoid creating bubbles or foaming when mixing or reconstituting components and during preparation of standards.
- Avoid contamination of reagents or samples by changing tips between addition of standards, samples, reagents and wash buffers.
- Samples generating A450 or A655 values that are higher than the highest IgG standard should be further diluted to fall within the range of the assay. Conversely, samples generating A450 or A655 values that are below the lowest IgG standard should be concentrated prior to dilution in Diluent Buffer.
- Because of variations in laboratory temperatures and environments, stop solution may need to be added sooner than 20 or 30 minutes of incubation with substrate. To ensure the standards and samples do not overshoot the endpoint of the assay, add stop solution before the highest standard reached an OD = 1.0 at 655 nm.
- Ensure ELISA plate is properly sealed during incubation steps and protected from light during incubation with Detection Antibody Solution.

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- It is necessary to completely remove all solutions and buffers after each wash step in order to minimize background.
- As a general guide, typical ranges of IgG concentration in commonly used sample types and starting dilution factors are given in Section 11 of this document entitled Sample Preparation.
- The provided Diluent Buffer for diluting standards and samples does not contain protease inhibitors. Protease inhibitors may be added to samples by the end-user, if necessary.
- Ensure that the [10X] TMB Substrate Solution has been brought to room temperature and is completely in solution before use. DO NOT heat the solution to facilitate this process.
- To avoid high background, allow Wash Buffer 2 to remain in wells for a minimum of 10 seconds to a maximum of 5 minutes.
- Review this document completely to confirm that this kit meets your research needs. Please contact our Technical Support Department with any questions.

  Tech Support:
  tech_support@cytodiagnostics.com

Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) before using the assay. This kit contains sufficient reagents for 96 wells.

The sample and buffer volumes given below are enough for 96 wells (12 x 8-well strips). Adjust the volumes given below as needed for the number of strips to be used in your experiment.

[1X] Wash Buffer 1:

Add 10 mL of [10X] Wash Buffer 1 to 90 mL of dH_2O.

[1X] Wash Buffer 2:

Add 1.5 mL of [10X] Wash Buffer 2 to 13.5 mL of dH_2O.

[1X] Detection Antibody Solution:

Add 20 µL of [500X] Detection Antibody Solution to 9,980 µL of Diluent Buffer.

[1X] TMB Substrate Solution:

Add 1 mL of [10X] TMB Substrate Solution to 9 mL of TMB Substrate Diluent.

Add 2 µL of Enhancer Solution per 10 mL of [1X] TMB Substrate Solution, immediately prior to use.

Standard Preparation

Always prepare a fresh set of standards and positive controls for each assay. Prepare serially diluted standards immediately prior to performing the assay. This section describes the preparation of a standard curve for triplicate measurements (recommended) from a [1 µg/mL] Stock Standard Solution.

1. [1 µg/mL] Stock Standard Solution:

   Reconstitute the lyophilized human IgG standard by adding 1 mL of ultrapure water to the vial. Mix by pipetting up and down or by vortexing until the lyophilized protein pellet has been completely dissolved. This is the [1 µg/mL] Stock Standard Solution.

2. Label 7 tubes, Standards 1 – 7.
3. Add 590.4 µL of Diluent Buffer to tube number 1 and 300 µL of Diluent Buffer into tubes numbered 2-7.
4. Add 9.6 µL of Stock Standard Solution to tube number 1. Mix well by vortexing or gently pipetting up and down.
5. Transfer 300 µL from tube number 1 to tube number 2 and mix well.
6. Continue to serially transfer 300 µL from tube number 2 to 6.
7. Standard tube number 7 contains no protein and is the blank control for the assay.

Standard Vial #	1	2	3	4	5	6	7
Diluent Buffer (µl)	590.4	300	300	300	300	300	300
Standard Solution (µl)	9.6 Stock Standard Solution	300 Vial #1	300 Vial #2	300 Vial #3	300 Vial #4	300 Vial #5	0
Resulting Human IgG conc. (ng/ml)	16	8	4	2	1	0.5	0

Sample Preparation

Dilute samples in Diluent Buffer to fall within the 0.5 ng/mL - 16 ng/mL range of the assay. If the concentration of IgG is unknown, try multiple dilutions

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of the sample in Diluent Buffer. Human IgG can be accurately quantified in the most common sample types at the dilutions indicated below.

Typical IgG Concentrations and Recommended Dilution for Common Sample Types

Sample Type	Concentration	Dilution Range Required
Normal Human Serum	5 – 22 mg/ml	1:600,000 – 1:10,000,000
Cell Culture Supernatant	0.005 – 0.05 mg/ml	1:5,000 – 1:10,000

Serum

Samples should be collected in a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes at 4°C and collect serum. Dilute samples in the Diluent Buffer provided with the kit. Store undiluted serum samples at -20°C. Avoid repeated freeze-thaw cycles. Avoid hemolysis during whole blood collection and serum separation as excessive hemolysis in serum samples has been shown to interfere with the detection of human IgG in this assay.

Cell Culture Supernatant

Collect cell culture media, or supernatant, and centrifuge at 2,000 x g for 10 minutes at 4°C to remove debris. Dilute samples in the Diluent Buffer provided with the kit. Store undiluted media or supernatant samples at -20°C. Avoid repeated freeze-thaw cycles.

Plate Preparation

- The 96-well plate strips included in this kit are ready-to-use. It is not necessary to rinse the plate or perform a blocking step prior to adding standards and samples.
- For each assay performed, a standard curve should be performed in triplicate wells as well as triplicate blank. Unknown samples being quantified should be performed in at least duplicate wells.
- Remove the number of strips required for the experiment. Immediately return unused strips back to the bag containing the desiccant pouches, reseal and store at 4°C.
- Edge effects have not been observed for this assay.

ELISA Protocol

Equilibrate all materials, buffers and prepared reagents to room temperature prior to performing the assay.

1. Add 100 µL of sample or standards per well. Mix by gently tapping the plate frame for 1 minute. Cover with an adhesive seal and incubate for 2 hours at room temperature.
2. Wash each well with 3 x 150 µL of Wash buffer 1. Wash by removing liquid from wells and then dispensing 150 µL of Wash buffer 1. Wash buffer 1 should remain in the wells for at least 10 seconds. Complete removal of liquid from wells after each step is essential to ensure proper washing and good performance of the assay.
3. Add 100 µL of the [1X] HRP-conjugated Detection Antibody Solution (see Reagent Preparation) to each well. Cover with adhesive seal and incubate for 1 hour at room temperature in the dark.
4. Repeat the aspiration/wash as in Step 2.
5. Add 150 µL of Wash buffer 2 into each well. Incubate for 1 minute at room temperature before removal from wells. Ensure complete removal of liquid from wells before adding substrate.
6. Add 100 µL of [1X] TMB Substrate Solution to each well.
7. Incubate for 20 - 30 minutes at room temperature. Avoid placing the plate in direct light.

   *NOTE: Stop solution (step 9) may need to be added sooner, if blue colour develops rapidly. Stop solution should be added before the highest IgG standard concentration reads an OD = 1.0 at 655 nm.
8. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Measure the OD at 450 nm and 540 nm. This is an endpoint reading.
10. Subtract readings at 540 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plates. Readings made directly at 450 nm without correction may be higher and less accurate.

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11. Analyze the data as described in the next section.

Alternative to Steps 8 – 10

Instead of taking a single endpoint reading at 450 nm, the development of the TMB substrate may be recorded kinetically. After the addition of the TMB Substrate Solution, the blue colour development may be measured every 5 or 10 minutes at 370 nm or 655 nm for up to 60 minutes.

An endpoint measurement may be taken by adding 100 µL of Stop Solution to each well and measuring the OD at 450 and 540 nm. Subtract readings at 540 nm from the readings at 450 nm and 655 nm.

Calculation of Results

Use a curve-fitting software to generate a standard curve from the analyzed standard samples. It is recommended to use a 4 parameter logistic curve fit, figure 2.

The values of unknown samples are assigned and interpolated from the generated standard curve.

Note: The quality of the standard curve fit can be checked by backfitting of standard curve OD values. To do this, first plot the standard curve. Next, treat standards as unknowns and interpolate the OD values from your standard curve. They should read close to the expected values (+/-10%).

A typical standard curve is shown below. Data is provided for demonstration purposes only. A standard curve must be generated for each ELISA performed with the kit.

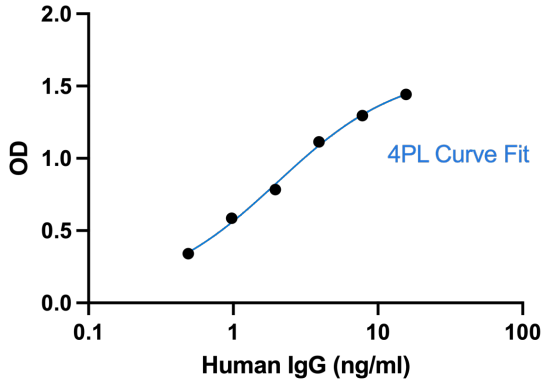

Figure 2. *A 4PL standard curve fit generated from measurement of human IgG standard samples.*

Sensitivity

The calculated minimal detectable dose (MDD) is 0.2 ng/mL of Human IgG in Diluent Buffer. The MDD was determined by calculating the mean of n=12 zero standard replicates and adding 2 standard deviations and extrapolating the corresponding concentrations.

Recovery in Serum

Five concentrations of human IgG were spiked in triplicate into IgG-depleted serum and diluted with Diluent Buffer into the working range of the assay. This assay demonstrated recovery of IgG signal in the range of 77 – 109% with an average recovery of 92%.

Sample	Observed IgG (ng/ml)	Expected IgG (ng/ml) (after dilution)	Recovery
1	11.7	12.5	94%
2	4.49	6.25	77%
3	2.61	3.13	89%
4	1.21	1.56	83%
5	0.79	0.78	109%

Recovery in Cell Culture Medium

Five concentrations of human IgG were spiked in triplicate into conditioned cell culture medium containing 10% FBS to evaluate signal recovery in this biological matrix.

Sample	Observed IgG (ng/ml)	Expected IgG (ng/ml)	Recovery
1	8	7.8	102%
2	3.9	3.9	101%
3	2.3	2.0	118%
4	1.0	1.0	107%
5	0.4	0.5	83%

This assay demonstrated excellent recovery of IgG signal in the range of 83 – 118% with an average recovery of 102%.

Precision

The mean coefficient of variation of interpolated values was calculated from a series of dilutions of IgG in human serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	3	3
CV (%)	3.67	16.6

The assay demonstrates a very good intra-assay precision having a coefficient of variation of 3.76% and a good inter-assay coefficient of variation of 16.6% when assays are performed on different days using freshly prepared dilution series of human serum.

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Species Reactivity

This ELISA assay kit recognizes human IgG protein. Reactivity with other species was determined by measuring immunoglobulin from goat, mouse, rabbit and chicken diluted in Dilution Buffer at 5 different concentrations and interpolating the immunoglobulin concentrations from the standard curve. The interpolated concentrations were then expressed as a percentage of the human IgG concentration assayed at the same dilution.

At the highest concentrations tested, mouse and goat antibodies displayed only 2 and 1%, respectively, of the human IgG signal at A450. No cross-reactivity was detected for rabbit or chicken antibodies.

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Troubleshooting

Problem	Cause	Solution
Low Signal	Incubation time too short	Ensure all standards and samples have been incubated with plates for a sufficient amount of time. A 2-hour incubation time is optimal for this assay. Decreasing the incubation time may result in low signal.
	Improper dilution of standards and samples or inadequate reagent volumes used	Always use properly calibrated pipettes and ensure correct preparation as described in the assay procedure.
	Incubation with the TMB substrate was too short	Ensure sufficient incubation time with the substrate and that blue colour development has occurred in the wells containing the standards proportional to the concentration of IgG added to the wells.
Poor standard curve	Improper dilution of standards	Briefly centrifuge the IgG Stock Standard vial before opening and dissolve the powder completely by vortexing and/or pipetting up and down.
	Inaccurate pipetting	Check pipettors and ensure proper calibration.
Low sensitivity	Improper storage of ELISA kit and reagents	Store reconstituted standards at -20°C and avoid repeat freeze-thaw cycles. Store all other components at 4°C. Ensure TMB solution is properly protected from light during storage. If TMB solution is stored at 4°C or -20°C, ensure that it has been brought to room temperature and is fully dissolved prior to use.
Large Coefficient of Variation (CV)	Plate is improperly or insufficiently washed	Review the ELISA procedure detailed in this manual for proper washing technique. Washing volumes may be increased but not decreased.
	Wash buffers are contaminated or improperly prepared.	Prepare fresh wash buffers.

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