KRISHGEN BioSystems

KRIBIOLISA
Human Immunoglobulin G ELISA

Cat. No: KBBP08

Ver2.2

Immunoassay for the quantification of human IgG

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Introduction:
Human IgG is a monomeric immunoglobulin, and the most abundant one constituting approximately 75% of the serum. Presence of such trace contaminants in the products may lead to potential health hazards, especially when the product is intended for therapeutic use.

Intended Use:
This generic kit is intended in determining the presence of Total human Immunoglobulin G contamination in variety of sample types. A highly sensitive and specific Enzyme linked immunosorbent assay can be used for the quantitation of low levels of human IgG.

Principle:
This assay is based on the Sandwich ELISA procedure. Samples containing hIgG are reacted with anti hIgG-HRP antibody simultaneously in the microtiter wells already coated with affinity purified capture IgG antibody. This immunological reaction results in formation of a sandwich complex of solid phase antibody-hIgG-enzyme labeled antibody. The wells are washed to remove any unbound reactants as per the wash procedure (see Assay procedure section mentioned below). The substrate 3, 3', 5, 5' Tetramethyl Benzidine is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and it is directly proportional to the concentration of hIgG present.

Materials Provided:

1. Anti-IgG Coated Microtiter Plate (96 wells) – 1 no
2. hIgG Standards, (1ml/vial) – 0, 0.5,1.5, 5,15 and 50ng/ml
3. Anti-hIgG:HRP Conjugate, 12ml
4. Wash Buffer (20X), 50ml
5. Sample Diluent, 12ml
6. TMB Substrate, 12ml
7. Stop Solution, 12ml
8. Instruction Manual

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25µl to 1000µl
3. Deionized (DI) water
4. Wash bottle or automated microplate washer
5. Semi-log paper or software for data analysis
6. Timer
7. Absorbent Paper

Handling/Storage:

1. All reagents should be stored at 2^0C to 8^0C for stability.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. Prior to use, bring all components to room temperature (18-25 ºC). Store all the components of the kit at its appropriate storage condition.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. All the reagents provided may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research or Manufacturing use only.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label Bring all reagents to Room Temperature before use.
2. To make Wash Buffer (1X), dilute 20ml of Wash Buffer (20X) in 380 ml of DI water and store at 4ºC.

Procedural Notes:

1. For good assay reproducibility and sensitivity, proper washing of the ELISA plate to remove excess/unbound reagents is essential.
2. If the IgG concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect. High Dose Hook Effect may be observed in samples with very high concentrations of IgG, usually in samples from the initials stages of purifications. To overcome Hook Effect samples to be assayed should be sufficiently diluted with our recommended diluent.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of hIgG.
4. All Standards and Samples should be assayed at least in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. Bring all reagents to Room Temperature prior to use. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.

2. Pipette out 25µl of **Standards** and **Samples** into the respective wells as mentioned in the work list.

3. Pipette out 100µl of **Anti-hIgG:HRP Conjugate** into each well.

4. Cover the plate and incubate it on a plate shaker at ~180rpm for 1 hour at room temperature, 22°C±4°C.

5. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.

6. Add 100µl of **TMB Substrate** in each well.

7. Incubate the plate at Room Temperature for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

8. Pipette out 100µl of **Stop Solution**. Wells should turn from blue to yellow in color.

9. Read the absorbance at 450 nm blanking on the zero standard

Example of a Work list

Well #	Contents	Abs at 450nm	Mean Absorbance	ng/ml hIgG equiv.
1A 2A	Zero Std Zero Std			
1B 2B	0.5ng/ml 0.5ng/ml			
1C 2C	1.5ng/ml 1.5ng/ml			
1D 2D	5ng/ml 5ng/ml			
1E 2E	15ng/ml 15ng/ml			
1F 2F	50ng/ml 50ng/ml			
1G 2G	Sample A Sample A			
1H 2H	Sample B Sample B			

Calculation of Results:

It is recommended to use the data reduction program (logit-log) in the reader to determine the IgG concentration in the unknown samples. Plot the % bound on the vertical axis (logit) against the IgG concentrations on the horizontal axis (log) for each standard (except the zero standard).

Alternatively, calculate percent conjugate bound (%B) for each standard, and sample relative to the maximum binding (B_0, Zero standard) wells as follows:

$$\%B/B_0 = \frac{\text{Mean Absorbance (highest standard) - Mean Absorbance (standard/sample)}}{\text{Mean Absorbance (highest standard) - Mean Absorbance (Zero standard)}} \text{ X } 100$$

Using semi-log graph, plot % bound on the vertical axis against IgG concentration on the horizontal axis for each of the calibrators and draw a smooth line curve through the points. IgG concentrations for the unknown may then be estimated from the line by interpolation.

Of the methods surveyed the logistic-log and fully specified logit-log functions are the most accurate models for forming standard curves and for interpolating IgG concentrations from the standard curve. The accuracy of the fully specified logit-log function is highly dependent on the precise specification of two unknown quantities, the optical densities at zero and infinite concentrations, prior to fitting the model to a typical set of calibration data. The function does not require pre-specification of any parameters before estimating the standard curve, and the four parameters are readily interpretable in terms of identifiable physical quantities. This model also has the advantage that it is easiest to visualize since it does not incorporate complex transformations of the optical density scale.

Typical Data:
This data is for illustration only and must not be used for the calculation of any sample result.

Std. (ng/ml)	Abs1	Abs2	Mean Abs.	B/Bo %	Result (X with corrected dilution Factor (ng/ml)
0	0.086	0.081	0.084	100.0	
0.5	0.136	0.111	0.123	98.4	
1.5	0.160	0.169	0.165	96.6	
5	0.322	0.324	0.323	90.1	
15	0.755	0.887	0.821	69.5	
50	2.239	2.190	2.215	11.7	
Sample 1 (1:200,000)	1.705	1.726	1.716	32.5	7635714

For Example
Sample 1 = (2.215 – 1.716) / (2.215 –0.084) x 100 = 32.5 %

Precautions:

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

1. **Sensitivity:**

a) **Limit Of Detection:** It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD.
10 replicates of '0' standards were evaluated and the LOD was found to be 0.17ng/ml.

b) **Limit of Quantitation:** It is defined as the lowest concentration for which Coefficient of Variation is <20%. The LOQ is found to be <0.5ng/ml.

2. **Specificity / Cross reactivity:** Specificity of an analytical method is defined as its ability to measure an analyte accurately in the presence of interference**.**
By immunoelectrophoresis and ELISA the antibodies in this set react specifically with Human Immunoglobulin G, not with other human immunoglobulins or other human serum proteins. Cross-reactivity with other species has not been tested.

3. **Precision:**
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100.
Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (10 ng/ml), medium (20 ng/ml) and high (30 ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	8.5%	8.8%
Medium	3.0%	1.5%
High	0.7%	5.4%

4. Recovery by Spiking:

In spike and recovery, a known amount of analyte is added (spiked) into the natural test sample matrix and its response is measured (recovered) in the assay by comparison to an identical spike in the standard diluent.

Sample Buffer Matrix	Pure Antigen Added (ng/ml)	Expected	Observed	Recovery (%)
0.1 M PBS	10	10	10	100
	20	20	19	95
	30	30	30	100
Assay Diluent	10	10	9.8	98
	20	20	19	95
	30	30	28	93.3
Stabilzyme Noble	10	10.1	9	89.1
	20	20.1	21	104.5
	30	30.1	30	99.7
ELISA Diluent	10	10.2	11	107.8
	20	20.2	22	108.9
	30	30.2	32	106.0
Sample Diluent	10	10	9.8	98
	20	20	16	80
	30	30	30	100

5. Standard Curve Characteristics:

Correlation Coefficient (r)	Intercept (A)	Slope (B)
0.990	**0.175**	**-0.038**

Troubleshooting:

Problem	Possible cause	Investigation/Actions
High Absorbances	1. Cross-contamination from other specimens	> Repeat assay taking care when washing and pipetting.
	2. Insufficient or inefficient washing or reading	> Check washer efficiency
	3. Wavelength of filter not correct.	> Check that the wavelength is 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm.
	4. High assay background.	> Repeat assay and include a well that contains only sample diluent or sample absorbent (i.e. a blank well).
	5. Contaminated TMB	> Check that TMB is colorless or faint blue.
	6. Incubation time too long or incubation temperature too high.	> Check incubation time and temperature. > Check incubator is at the correct temperature.
	7. Incorrect dilution of serum	> Repeat assay, ensuring correct serum dilution is used.
Low Absorbances	1. Incubation time too shot or incubation temperature too low.	> Ensure time and temperature of assay incubation are correct. > Check incubator is set at the correct temperature.
	2. Incorrect dilution or pipetting of sera	> Repeat assay ensuring correct dilutions and volumes are used. > Ensure controls are sufficiently mixed.
	3. Incorrect filter wavelength.	> Check the wavelength is set at 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650nm.
	4. Contaminated Conjugate solution.	> Dispense conjugate directly from the bottle using clean pipette tip; avoid transferring Conjugate to another container if possible. > Do not return unused Conjugate to bottle. > Ensure all pipettes and probes used to dispense the Conjugates are clean and free from serum, detergent and bleach.
	5. Kit has expired.	> Check expiration date of kit and do not use if expired.
	6. Air blank reading high.	Investigate causes of high background absorbance.
	7. Incorrect storage of kit.	> Ensure kit is stored at 2-8ºC,plate is sealed in foil pouch and desiccant sachet is blue/purple.
	8. Kit reagents not equilibrated at room temperature	> Allow sufficient time for reagents to equilibrate to room temperature prior to assay.
	9. Incorrect reagents used.	> Check the reagents used match those listed on the specification sheet.
	10. Over washing of plate (e.g. inclusion of a long soak step).	> Repeat assay using recommended wash procedure.
Poor Duplicates	1. Poor mixing of samples.	> Mix reagents gently and equilibrate to room temperature.
	2. Poor pipette precision	> Calibration may need to be checked. > Check pupating technique-change pipette tip for each sample and ensure excess liquid is wiped from the outside of the tip.
	3. Addition of reagents at inconstant timing intervals; reagent addition takes too long, air bubbles when adding reagents.	> Use consistent timing when adding reagents. > Ensure all dilutions are made before commencing addition to plate. > Improve pipetting technique and skill.
	4. Inefficient washing - Wash buffer left in wells, inconsistent washing, inadequate washing.	> Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing.
	5. Reader not calibrated or warmed up prior to plate reading.	> Check reader precision > Check reader manual to ascertain warm up time of instrument.
	6. Optical pathway not clean	> Gently wipe bottom of plate. > Check reader light source and detector are clean.
	7. Spillage of liquid from wells	> Repeat assay, taking care not to knock the plate or splash liquid
	8. Serum samples exhibit microbial growth,	> It is not recommended to use serum samples exhibiting microbial haemolysis or lipaemia. growth, haemolysis or lipaemia.
	9. Uneven well volumes due to evaporation.	> Cover plate with a lid or plate sealer (not provided).

All wells yellow	1. Contaminated TMB.	> Check TMB is colorless or faint blue.
	2. Contaminated reagents (e.g. Conjugate, Wash buffer).	> Check reagents for turbidity.
	3. Incorrect dilution of serum.	> Repeat assay, ensuring correct serum dilution is used.
	4. Incorrect storage of kit.	> Ensure kit is stored at 2-8ºC, plate is sealed in foil pouch and desiccant sachet is blue / purple.
	5. Inefficient washing- Wash buffer left in wells, inconsistent washing, inadequate washing.	> Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled an aspirated when washing.
	6. If Conjugate reconstitute is required – Conjugate reconstituted incorrectly.	> Repeat assay ensuring Conjugate is reconstituted according to assay method.
All wells negative	1. Test not performed correctly – correct reagents not added or not added in the correct sequence.	> Check procedure and check for unused reagents. > Ensure that Stop Solution was not added before Conjugate or TMB. > Ensure that serum was diluted in correct Sample diluent; e.g. do not use Sample Absorbent for an IgG ELISA.
	2. Contaminated Conjugate solution.	> Dispense Conjugate directly from the bottle using a clean pipette tip; avoid transferring Conjugate to another container if possible. > Do not return unused Conjugate to bottle. > Ensure all pipettes and probes used to dispense the Conjugate are clean and free from serum, detergent and bleach.
	3. Over- washing of plate (e.g. inclusion of a long soak step).	> Repeat assay using recommended wash procedure.
	4. Incorrect storage of kit.	> Ensure kit is stored at 2-8ºC, plate is sealed in foil pouch and desiccant sachet is blue / purple.
	5. Wash Buffer made up with Stop Solution instead of Wash Buffer Concentrate	> Ensure Wash Buffer is made up correctly.

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