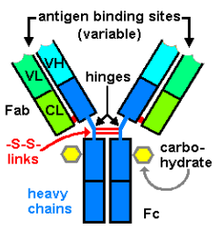
INTENDED USE

The **Horse IgG-(Fab')$_2$** ELISA Kit is an immunoassay for quantifying IgG in serum or plasma, or the (Fab')$_2$ fragment in pepsin digests of horse IgG.

GENERAL INFORMATION

Passive immunity is the transfer or infusion of pre-made antibodies. Passive immunity also occur naturally, when maternal antibodies are transferred to the fetus through the placenta. Typically, high levels of antibodies specific to a pathogen (rabies), (diphtheria) or venom are made in animals that can provide large volumes (Horse or sheep or goat). Antibodies can be used infused an unpurified antiserum, purified IgG or IgG-(Fab')$_2$ fragment to animals) are transferred to non-immune persons through infusion of immunoglobulin called antiserum therapy or immunoglobulin for intravenous (IVIG). Passive immunization is used when there is a high risk of infection and insufficient time for the body to develop its own immune response, or to reduce the symptoms of ongoing or immunosuppressive diseases. Passive immunization can be provided when people cannot synthesize antibodies, and when they have been exposed to a disease that they do not have immunity against.

IVIG may be obtained from human (CMV, Hepatitis A, B, rabies, tetanus, vaccinia or varicella) or made in large donor species such as horse (Botulism, diphtheria, rabies, tetanus, and anti-venom). Infusion of large volumes of IVIG from animals to humans may invoke prophylactic shock. Therefore, many IVIG use partially purified whole IgG or fragments of IgG-(Fab')$_2$ (e.g. equine rabies anti-tetanus, equine anti-diphtheria, and FAV Africa, equine (Fab')$_2$ against the snake venom). ADI has developed antibody ELISA kits to measure antibody titers in various IVIG and also develop an ELISA kit to determine the concentration of equine (Fab')$_2$. Equine IgG-(Fab')$_2$ ELISA will work with purified antibodies or when they contain other protein additives.



Immunoglobulin G (IgG)

Immunoassays using heavy-chain specific antibodies provide for selective, sensitive quantification of Horse immunoglobulins IgG, IgA and IgM, as found circulating in blood or as present in other body fluids, including saliva, milk/colostrum, ascites, tears and mucosa of linings of the gut, respiratory or urogenital tracts. The quantitative immunoassays measure Horse (Fab')$_2$ and Horse IgG with high sensitivity; this allows dilution beyond interference from the sample matrix for samples derived from any of the above specimen types. Expected performance of each kit relative to precision and linearity of dilution is presented as guidance for use and experimental design.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8° C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8° C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-Horse IgG-(Fab')$_2$ - HRP Conjugate Concentrate (100x) Part No. 7714, 0.15ml	Peroxidase conjugated anti-Horse IgG-(Fab')$_2$ in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8° C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Anti-Horse IgG-(Fab')$_2$ Coated Microwell Strip Plate	7711	8-well strips (12)	Coated with anti-horse IgG-(Fab')$_2$ antibodies, and post-coated with stabilizers.
Horse IgG-(Fab')$_2$ Standards			
5 ng/ml 12.5 ng/ml 25 ng/ml 50 ng/ml 100 ng/ml	7713B 7713C 7713D 7713E 7713F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing horse serum with calibrated IgG-(Fab')$_2$ concentrations; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
Horse IgG-(Fab')$_2$ Positive Control [IgG-(Fab')$_2$] range on label	7712	0.65 ml	Horse IgG-(Fab')$_2$ of stated concentration range; in buffer with detergents and non-azide antimicrobials as stabilizers.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Horse IgG-(Fab')$_2$ HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

PRINCIPLE OF THE TEST

The **Horse IgG-(Fab')$_2$** ELISA kit is based on the binding of horse IgG-(Fab')$_2$ in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to horseradish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of IgG-(Fab')$_2$ present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of IgG-(Fab')$_2$ in samples and control is calculated from a curve of standards containing known concentrations of IgG-(Fab')$_2$.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum, culture medium and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature.

For all samples, clarify by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Assay Design

Dilute **Serum Samples** in **Working Sample Diluent.** Dilutions above 100k-fold are appropriate for most normal horse sera. For accuracy, three dilution steps are recommended, as follows;

1) 10ul serum + 990ul diluent = [1:100]
2) 10ul [1:100] + 990ul diluent = [1:10k]
3) 20ul [1:10k] + 380ul diluent = [1:200k]

Run as duplicates for best precision.

Note: Serum diluted in the **Working Sample Diluent** is stable for several years stored at 2-8° C, or frozen.

- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy. Blank OD should be <0.3.
- Run the **Standards** in duplicate to provide sufficient precision for accurate sample quantitation.
- Run the Horse IgG-(Fab')$_2$ **Positive Control**. A value within the range stated on the vial demonstrates proper assay performance. Also, run internal controls that represent the lab's expected sample population and are maintain stabilized.

Plate Set-up

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- o Add 100ul of standards, samples and controls each to pre-determined wells.
- o Tap the plate gently to mix reagents and incubate for **60** minutes.
- o Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- o Add 100ul of diluted Anti-Horse IgG-(Fab')$_2$-HRP Conjugate to each well.
- o Incubate for **30** minutes.
- o Wash wells 5 times as in step 1.

3. Substrate Incubation [100ul – 15 min]

- o Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- o Incubate for **15** minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- o Add 100ul of Stop Solution to each well.
- o Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- o Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- o Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

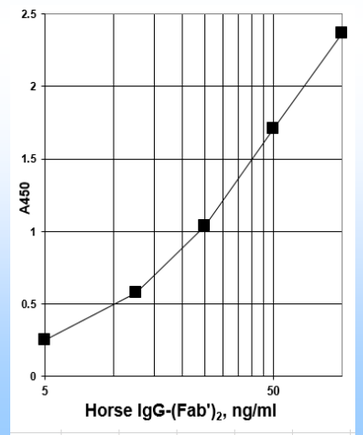
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, horse IgG-$(Fab')_2$ concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of horse IgG-$(Fab')_2$ (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The horse IgG-$(Fab')_2$ concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 100 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators & Samples	A450 nm
A1, A2	Diluent Blank	0.08
B1, B2	**5** ng/ml **Standard**	0.25
C1, C2	12.5 ng/ml **Standard**	0.58
D1, D2	25 ng/ml **Standard**	1.04
E1, E2	50 ng/ml **Standard**	1.71
F1, F2	100 ng/ml **Standard**	2.37
G1, G2	**Positive Control** [28 – 52 ng/ml]	1.37

Positive Control = **35.6** ng/ml



PERFORMANCE CHARACTERISTICS

Specificity
The antibodies used in this kit have been shown by immunoelectrophoresis and ELISA to react specifically with horse IgG, and to have minor reactivity with IgM, IgA, and IgE via common light chain, but with no other horse serum proteins. Antibodies also react with Fab and $(Fab')_2$ fragments, but no reactivity with Fc fragments.

Linearity of Dilution: IgG types
Seven (7) horse IgG sample types were diluted to 2 levels for testing, and concordance of the assay values with the IgG-(Fab')2 standard curve was compared:

- Commercial Antitoxins: $(Fab')_2$ preparations of a) anti-snake venom (Vins Bio) and b) anti-tetanus toxin (Vins Bio). Results: **95-97%** parallel.
- Horse IgG: a) Fab fragments – **80%** parallel.
  b) Purified intact IgG – **92%** parallel.
- Horse Serum: **90 – 93%** parallel.

Sample	Dilution	Assay Value ng/ml	Serum Value mg/ml	Concord
Anti-Snake Venom $(Fab')_2$	1:400k 1:1000k	64.8 24.3	25.9 24.3	**97 %**
Anti-Tetanus Toxin (Fab')2	1:10k 1:40k	48.9 13.5	0.49 0.54	**95 %**
Fab Fragment	1:10k 1:40k	53.2 20.3	0.53 0.81	**80 %**
Intact IgG	1:40k 1:200k	73.6 12.4	2.94 2.48	**92 %**
Horse Serum 1	1:125k 1:500k	59.4 17.4	7.4 8.7	**93 %**
Horse Serum 2	1:125k 1:500k	54.3 16.7	6.8 8.35	**90 %**
Horse Serum 3	1:125 1:500k	56.8 17.5	7.1 8.75	**90 %**

Precision
Samples containing low, medium and high concentrations of IgG-$(Fab')_2$, were assayed for precision, as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficient of variations were calculated for the concentrations using a 4PL curve-fitting program.

IgG-$(Fab')_2$ concentrations were measured with good between-assay (**4.7** to **5.8** %CV) reproducibility.

Sample	IgG-$(Fab')_2$ ng/ml	Inter-assay %CV
Low	15	**4.7**
Medium	34	**4.8**
High	68	**5.4**

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls A Positive Serum Control is provided with the kit, assigned with a Horse IgG-$(Fab')_2$ concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Sample Diluent blank should also be run; OD should be <0.2 and lower than the 5 ng/ml Standard OD.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{O} C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

Instruction Manual No. M-7710-Fab

Horse IgG-$(Fab')_2$

ELISA Kit Cat. No. 7710-Fab

For Quantitation of Horse IgG-$(Fab')_2$ in Serum or Plasma

For research use only, not for diagnostic or therapeutic use.

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ELISA Kit Components	Amount	Part
Anti-Horse IgG-$(Fab')_2$ Coated Microwell Plate	8-well strips (12)	7711
Horse IgG-$(Fab')_2$ Positive Control	0.65 ml	7712PC
Horse IgG-$(Fab')_2$ Standard 5 ng/ml	0.65 ml	7713B
Horse IgG-$(Fab')_2$ Standard 12.5 ng/ml	0.65 ml	7713C
Horse IgG-$(Fab')_2$ Standard 25 ng/ml	0.65 ml	7713D
Horse IgG-$(Fab')_2$ Standard 50 ng/ml	0.65 ml	7713E
Horse IgG-$(Fab')_2$ Standard 100 ng/ml	0.65 ml	7713F
Anti-Horse $(Fab')_2$ HRP Conj (100X)	0.15 ml	7714
Sample Diluent Concentrate (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual Fab	1 ea	M-7710-