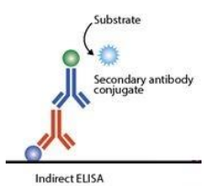
INTENDED USE

The Humira/adalimumab (Anti-TNFα) ELISA Kit is an immunoassay for quantifying Humira activity in serum or plasma, or in other appropriately qualified samples from cell culture, bioprocessing solutions, or tissue fluids (e.g., saliva, mucosa). The assay has been specifically validated for quantifying Humira in human and dog serum. It may be used in other species such as mouse and rat. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

GENERAL INFORMATION

Humira (adalimumab) is a recombinant human IgG1/k monoclonal antibody produced in a mammalian cell expression system, with antibody activity specific for binding to human tumor necrosis factor (TNF-alpha), a naturally occurring cytokine involved in a wide range of normal inflammatory and immune responses. The binding of adalimumab to TNF-alpha blocks its interaction with the p55 and p75 cell surface TNF receptors; also, lyses surface TNF-expressing cells *in vitro* in the presence of complement; and modulates biological responses, such as adhesion molecule levels, that are induced or regulated by TNF. Adalimumab/Humira was constructed from a fully human monoclonal antibody, while infliximab is a mouse-human chimeric antibody and etanercept is a TNF receptor-IgG fusion protein. Humira has been approved for the treatment of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, moderate to severe chronic psoriasis and juvenile idiopathic arthritis. Because adalimumab/humira suppresses TNF, which is part of the immune system, latent infections, such as tuberculosis, can be reactivated, and the immune system may be unable to fight new infections. Therefore, it is necessary to carefully monitor the concentration of TNF-alpha (total and free), Humira, and if patients are making antibodies to the drug (Human anti-Humira antibodies). Humira is a trademark of Abbott Labs.

PRINCIPLE OF THE TEST



The Humira ELISA kit is based on the binding of Humira in samples to hTNAα coated on the plates. Bound Humira is detected with anti-Humira IgG conjugated to horseradish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate is added and color is developed using HRP –substrate, TMB substrate that produced blue color. Stopping Solution is added to terminate the reaction, and color (yellow) A450nm is then measured using an ELISA reader. The concentration of Humira in samples and control is calculated from a curve of standards containing known concentrations of Humira.

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-Human IgG - HRP Conjugate Concentrate (100x) Part No. 1754, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with protein, 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
TNFα coated Microwell Strip Plate	200-311	8-well strips (12)	Coated with human TNFα, and post-coated with stabilizers.
Humira Standards			
5 ng/ml 10 ng/ml 25 ng/ml 50 ng/ml 100 ng/ml	200-303B 200-303C 200-303D 200-303E 200-303F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing purified recombinant Humira with designated concentrations; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
Positive Control [Humira] range on label	200-302	0.65 ml	Humira of stated IgG concentration range; diluted in buffer with protein, 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 Antibody HRP Concentrate.
- 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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% 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 or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Culture medium, bioprocessing preparations, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). 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 Validation
Validate the performance of the sample antigen and matrix in the assay system for recovery and parallelism (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of the Humira sample relative to the Standard curve.

Prepare and run a series of dilutions of the sample antigen (concentrations that will fall within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. For most buffer solutions a minimum 5-fold sample dilution is usually sufficient. Serum and plasma require at least a 1/100 dilution to obtain consistent quantitation or complete antigen recovery (see graph on page 6).

Parallelism – dilutions of the sample should read equivalent values from the top and bottom of the Standard curve to provide good assay precision.

Prepare a dilution series of the sample antigen that gives complete recovery and falls within the full range of the Standard curve. Sample readings from the upper and lower regions of the curve should differ by less than 25%.

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 200-310ul 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]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- 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]

- Add 100ul of diluted Anti-Human IgG HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- 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]

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

5. Absorbance Reading

- 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.
- 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.

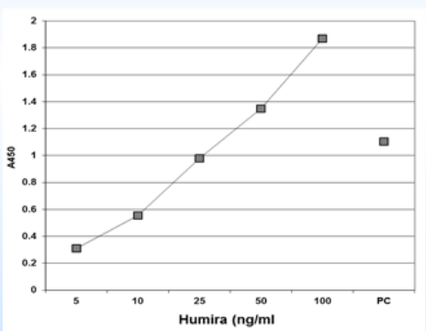
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Humira 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 Humira (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 Humira 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	ng/ml
A1, A2	Diluent Blank		0.03	0
B1, B2	5 ng/ml	**Standard**	0.31	5
C1, C2	10 ng/ml	**Standard**	0.55	10
D1, D2	25 ng/ml	**Standard**	0.98	25
E1, E2	50 ng/ml	**Standard**	1.35	50
F1, F2	100 ng/ml	**Standard**	1.87	100
G1, G2	**Positive Control** [21-37 ng/ml]		1.10	31
H1, H2	Sample [Diluted 1:100]1.41		56	

Calculated: 100-fold dilution x 56 ng/ml = **5.6** ug/ml in serum



PERFORMANCE CHARACTERISTICS

Specificity

The antibodies used in this kit are specific for Humira and normal human IgG, and do not react with IgM, IgA or IgE, or with dog IgG (see Serum: Recovery and Parallelism). Since the humira ELISA is based upon the binding of humira (hIgG1) to the plates, the host specific IgG (human, dog, mouse or rat) will not bind to the coated plates. Therefore, this test is independent of the species. The test has been validated for human and non-human samples (e.g., Dog). This ELISA may be used for other appropriately qualified species such as rat, mouse, and monkey etc.

Precision

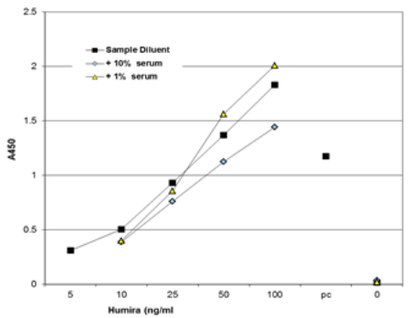
Samples containing low, medium and high concentrations of Humira were assayed as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficients of variation were calculated for the concentrations using a point-to-point curve-fitting program.

Humira concentrations were measured with good between-assay (3.3 to 6.8 %CV) reproducibility.

Sample	Humira ng/ml	Inter-assay %CV
High Concn	59.6	**3.3**
Medium Concn	22.5	**6.8**
Low Concn	9.8	**4.5**

Recovery and Parallelism

Humira was diluted at 4 concentrations into Sample Diluent containing 1% and 10% dog/human serum , and assayed in duplicate. Dilution curves are shown in the following graph:



Humira in 1% dog serum (i.e., dog serum diluted 1/100) was quantified essentially equivalently to Humira in Sample Diluent (Standard Curve). Quantitation was somewhat depressed in 10% dog serum (1/10 dilution). Therefore, dilute samples 1/100 or more for accurate quantitation.

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 an Humira 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 <0.3 and lower than 1 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.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

STORAGE AND STABILITY

The ELISA 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.

LIMITS OF THE ASSAY

1. The **recovery,** or accuracy of Humira measurement in dog/human serum (pooled), appears unaffected when diluted at least 1/100 (1%) in Sample Diluent. Recovery in fresh, individual dog serum or plasma samples has not been determined.

2. Single dose, subcutaneous administration of Humira in humans peaks around 5 ug Humira/ml; the ELISA assay detection range is 5 - 100 ng IgG/ml, a 50 to 1000-fold **sensitivity** window. Humira in circulation may be in complex with TNF and/or cell receptors or other binding molecules; detection & quantitation of complexes with the ELISA assay is unknown.

3. Lower limits of Humira detection is ~1.5 ng/ml.

Instruction Manual No. M-200-310-AHG

Humira/Adalimumab ELISA Kit

Cat. No. 200-310-AHG, 96 tests

For Quantitation of Humira (Anti-TNFα Activity) in Human Serum or plasma or other biological fluids

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

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ELISA Kit Components	Amount	Part
TNFα Antigen Coated Microwell Plate	8-well strips (12)	200-311
Humira IgG Positive Control	0.65 ml	200-302
Humira IgG Standard 5 ng/ml	0.65 ml	200-303B
Humira IgG Standard 10 ng/ml	0.65 ml	200-303C
Humira IgG Standard 25 ng/ml	0.65 ml	200-303D
Humira IgG Standard 50 ng/ml	0.65 ml	200-303E
Humira IgG Standard 100 ng/ml	0.65 ml	200-303F
Anti-Human IgG HRP Conjugate (100X)	0.15 ml	1754
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	1 ea	M-200-310-AHG