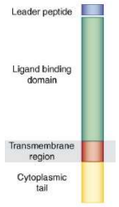
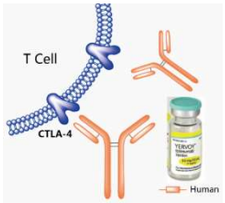
INTENDED USE

The **Yervoy** (Ipilimumab) Kit is an indirect ELISA for quantifying active (CTLA-4-binding) Yervoy in biological solutions including serum or plasma or other biological fluids. This test is designed for human samples but can be used for other species (mouse, rat etc) after proper validations. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

GENERAL INFORMATION

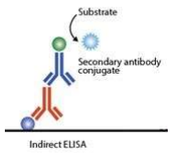


CTLA4 or CTLA-4 (cytotoxic T-lymphocyte-associated protein 4/CD152) is a receptor that, functioning as an immune checkpoint, downregulates immune responses. CTLA4 is a member of the immunoglobulin superfamily that is expressed by activated T cells and transmits an inhibitory signal to T cells. It acts as an "off" switch when bound to CD80 or CD86 on the surface of antigen-presenting cells. CTLA4 (human 223-aa, ~25 kda) contains an extracellular V domain, a transmembrane domain, and a cytoplasmic tail. It is widely expressed with highest levels in lymphoid tissues.



The comparatively higher binding affinity of CTLA4 has made it a potential therapy for autoimmune diseases. The fusion protein CTLA4-Ig is commercially available as Orencia (**Abatacept**). A second generation form of CTLA4-Ig known as **belatacept** was recently approved. Antagonistic intervention of CTLA4 using antibodies such as **ipilmumab** is being used as a means of inhibiting immune system tolerance to tumors and thereby providing a potentially useful immunotherapy strategy for patients with cancer. This is the first approved immune checkpoint blockade therapy. Another drug is **tremelimumab**. Ipilimumab (trade name Yervoy/MDX-010, by BMS, approved in 2011), is a **human antibody** that works to activate the immune system by targeting CTLA-4 and blocking the inhibitory signal, which allows the CTLs to destroy the cancer cells. It is indicated for unresectable or metastatic melanoma. Yervoy, like other humanized antibodies, has the potential to be immunogenic and induce anti-drug antibodies (6.9% patients).

PRINCIPLE OF THE TEST



The Yervoy ELISA kit is based upon capture of active Yervoy to CTLA-4 antigen coated on the plate. Bound Yervoy is then detected by anti-Yervoy IgG HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color (blue), which is directly proportional to the amount of antibody present in the sample. Stop Solution is added (converts blue to yellow color), and A450nm is then measured using an ELISA reader. The activity of antibody in samples is calculated relative to supplied calibrators..

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.#. 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. # 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. 210-204, 0.15ml	in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10 ul of concentrate to 1 ml of **Working Sample Diluent** (**WSD**) 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
Antigen Coated Strip Plate	210-201	8-well strips (12)	Coated with CTLA-4 antigen and post-coated with stabilizers.
Yervoy Standards			
1 ng/ml 2.5 ng/ml 5 ng/ml 12 ng/ml 30 ng/ml	210-203B 210-203C 210-203D 210-203E 210-203F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing purified recombinant Yervoy with designated concentrations; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
Positive Control [Yervoy] range on label	210-202	0.65 ml	Yervoy of stated 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.
- 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.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Culture medium, 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. If samples will not be assayed immediately, store frozen for long-term storage.

DILUTE serum samples in Working Sample Diluent. Dilutions of 1:1000–1:10,000 may be appropriate for standard drug treatment regimens or for drug production processing. For accuracy, multiple dilution steps are recommended, as follows:

1) 10ul serum + 990ul diluent = [1:100],
2) 50 ul [1:100] + 950ul diluent = **[1:1000].**

Prepare additional dilutions as required. Diluted samples are stable for several months refrigerated. All samples must be diluted to bring them within the range of the standard curve. Samples reading >2.00 should be diluted further and re-tested.

Assay Validation

Validate the performance of the Yervoy sample and matrix in the assay system for recovery (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 Yervoy in the sample relative to the Yervoy Standards.

Prepare and run a series of dilutions of the Yervoy sample (within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. Serum and plasma may require greater than 1/500 dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Yervoy was spiked into dilutions of human serum & plasma, 1 pool and 5 individual samples, or Sample Diluent (Control), at a final concentration of 15 ng/ml.

Results: recovered values ranged from **48** to **107%** of Control with sera diluted 1/500. Recovery was **less** when serum was less dilute (<1:500). Low recovery suggests serum factors that interfere with Yervoy binding to the antigen on the plate.

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 ul 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 sample diluent (blank), 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 **Antibody-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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, 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 or obtained from the ADI website: http://4adi.com/objects/catalog/product/extras/ELISA-Kit-SDS-MSDS-Set-1.pdf

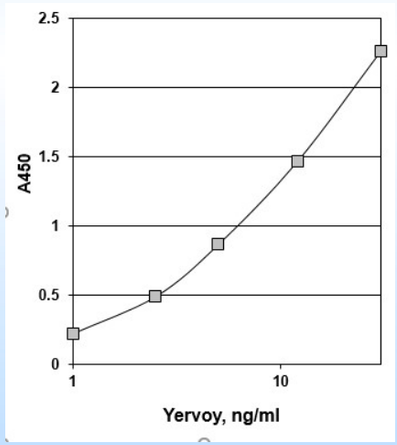
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Yervoy 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 Yervoy (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 Yervoy 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		A450 nm
A1,2	**Negative Diluent Blank**		0.02
B1,2	1 ng/ml	**Standard**	0.20
C1,2	2.5 ng/ml	**Standard**	0.40
D1,2	5 ng/ml	**Standard**	0.86
E1,2	12 ng/ml	**Standard**	1.61
F1,2	30 ng/ml	**Standard**	2.61
G1,2	**Positive Control** [7 – 13 ng/ml]		1.33

Positive Control Result = **10.1** ng/ml



PERFORMANCE CHARACTERISTICS

Specificity
The plate is coated with recombinant CTLA-4 antigen to which Yervoy binds with high affinity. Other antibodies or binding proteins may also bind to the CTLA-4-antigen coated plate; however the Anti-Human IgG-HRP conjugate will not bind to non-human antibodies or non-antibody human serum proteins. Therefore, the assay is highly specific for measuring Yervoy activity only.

Precision
Samples containing low, medium and high concentrations of Yervoy 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.

Yervoy concentrations were measured with good between-assay (**6.1** to **9.1** %CV) reproducibility.

Sample	Yervoy ng/ml	Inter-assay %CV
High Concn	17.3	**9.1**
Medium Concn	10.3	**6.1**
Low Concn	2.83	**7.2**

Recovery
Yervoy was spiked into human serum or plasma diluted 1:20 to 1:500 in Sample Diluent (1 pooled and 5 individual samples), and assayed for anti-CTLA-4 activity. **Recovery** was calculated comparing the observed (O) values to the expected (E) values for each diluted sample. All serum and plasma samples were 0 Yervoy (E).

O/E values at 1:500 dilution ranged from **74%** to **102%**. See Limits of the Assay.

Sample	Dilution	Yervoy Concn (E) = 15.5 ng/ml	
		Observed ng/ml	O/E %
BC pool	**1:20**	n.d.	-
A	**1:20**	2.5	**16**
B	**1:20**	1.3	**8**
C	**1:20**	0.7	**5**
D	**1:20**	2.0	**13**
E	**1:20**	1.5	**10**
BC pool	**1:100**	5.9	**38**
A	**1:100**	3.3	**21**
B	**1:100**	2.5	**16**
C	**1:100**	3.1	**20**
D	**1:100**	2.8	**18**
E	**1:100**	1.7	**11**
BC pool	**1:500**	7.2	**48**
A	**1:500**	10.5	**70**
B	**1:500**	16.2	**107**
C	**1:500**	8.9	**59**
D	**1:500**	83	**55**
E	**1:500**	10.8	**72**

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 Yervoy 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 0.5 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.

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.

LIMITS OF THE ASSAY

1. The assay measures Yervoy activity, i.e., antibody that actually binds to the CTLA-4-antigen coated plate, relative to Yervoy standards that are presumed to be 100% active IgG. Factors in the sample that diminish Yervoy binding, e.g., CTLA-4 antigen or other Yervoy -binding molecules, may reduce apparent Yervoy concentration in the assay (see **Recovery,** page 6).

2. Assays that measure Yervoy mass concentration may not have a tight correlation with the Yervoy activity assay, e.g., full Yervoy recovery may be determined by different assay factors.

3. The **recovery** (accuracy of Yervoy measurement in stored serum) may be diminished if not diluted at least 1/500 in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual human or animal serum or plasma samples may differ, and has not been determined.

4. Multiple-dose intravenous administration of Yervoy in humans has resulted in mean minimal steady-state concentrations of 19.3 and 58.1 ug Yervoy /ml of serum (low & high dose level: product data). The ELISA assay detection range is 1 - 30 ng IgG/ml, so Yervoy samples with the above levels will require dilutions of 700 to 60,000-fold to be within the testing range.

Related Item

210-210-AIG	Human Anti-Ipilimumab (Yervoy) IgG (Anti-drug antibodies/ADA) ELISA Kit

Instruction Manual No. M-210-200-IHG

Yervoy (ipilimumab/anti-CTLA-4) humanized IgG) ELISA Kit

Cat # 210-200-IHG, 96 Tests

For Quantitation of Active Yervoy in Processing and/or Biological Solutions

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive
San Antonio• Texas 78244 • USA.
Phone (210) 561-9515 • Fax (210) 561-9544
Toll Free (800) 786-5777

Email: service@4adi.com
www.4adi.com

ELISA Kit Components	Amount	Part
CTLA-4 Antigen Coated Microwell Plate	8-well strips (12)	210-201
Yervoy Control	0.65 ml	210-202
Yervoy Standard 1 ng/ml	0.65 ml	210-203B
Yervoy Standard 2.5 ng/ml	0.65 ml	210-203C
Yervoy Standard 5 ng/ml	0.65 ml	210-203D
Yervoy Standard 12 ng/ml	0.65 ml	210-203E
Yervoy Standard 30 ng/ml	0.65 ml	210-203F
Anti-Human IgG-HRP Conjugate (100X)	0.15 ml	210-204
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-210-200-IHG