INTENDED USE

The Rituximab Anti-CD20 ELISA Kit # 200-210-RAG is an immunoassay for quantifying Rituximab (Rituxan) anti-CD20 activity in mouse serum or plasma, or in other appropriately qualified samples.

GENERAL INFORMATION

CD20 is a 33-36-kDa (297-aa) transmembrane phosphoprotein involved in the activation, proliferation, and differentiation of B-lymphocytes. It is absent in terminally differentiated plasma cells. Rituximab is a genetically engineered chimeric murine/human monoclonal IgG1 kappa antibody (~145 kda), produced by mammalian cell (Chinese Hamster Ovary) suspension culture. The mouse/human chimeric CD20 mAb rituximab was the first cancer therapeutic mAb to be given Food and Drug Administration (FDA) approval and since then has become an important new treatment for B lymphocyte malignancies. Administration of Rituximab leads to destruction of B lymphocytes, and is therefore used to treat diseases which are characterized by having excess, overactive or dysfunctional B cells. This includes many lymphomas and leukemias, transplant rejection and some autoimmune disorders. Rituximab has been shown to be an effective treatment for rheumatoid arthritis and other autoimmune diseases.

The ADI Rituximab Anti-CD20 ELISA is designed to measure the active CD20-binding antibody activity of the drug in mouse serum or plasma.

PRINCIPLE OF THE TEST

The Rituximab Anti-CD20 ELISA kit is based upon capture of active Rituximab (CD20-binding) to the CD20 antigen coated on the plate. Bound Rituximab is then detected by anti-Rituximab HRP that binds to Rituximab captured on the plate. 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 Rituximab 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 Rituximab in samples and control is calculated from a curve of standards containing known concentrations of Rituximab.

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.

KIT CONTENTS

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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.
Antibody-HRP Conjugate Concentrate (100x) Part No. 200-214, 0.15ml	Peroxidase conjugated anti-rituximab 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
Antigen Coated Strip Plate	200-211	8-well strips (12)	Coated with CD-20 antigen and post-coated with stabilizers.
Rituximab Anti-CD20 Standards			
50 ng/ml 100 ng/ml 200 ng/ml 400 ng/ml 750 ng/ml	200-213B 200-213C 200-213D 200-213E 200-213F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing purified recombinant Rituximab with designated concentrations; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
Positive Control [Rituximab] range on label	200-212	0.65 ml	Rituximab 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.

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

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 Rituximab 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 Rituximab in the sample relative to the Rituximab Standards.

Prepare and run a series of dilutions of the Rituximab sample (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 greater dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Rituximab was spiked into dilutions of mouse serum, 3 pools and 6 individual samples, or Sample Diluent (Control), at a final concentration of 475 ng/ml.

Results: recovered values ranged from **73** to **107%** of Control with sera diluted 1/100. Recovery was **less** when serum was diluted less than 1/100. Low recovery suggests serum factors that interfere with Rituximab binding to the CD20 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 200-210ul 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 **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.

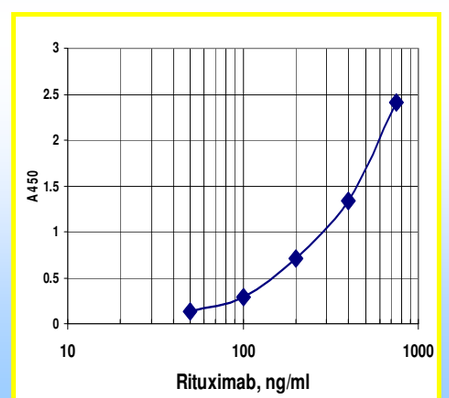
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Rituximab 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 Rituximab (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 Rituximab 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**		0.04
B1,2	50 ng/ml	**Standard**	0.14
C1,2	100 ng/ml	**Standard**	0.29
D1,2	200 ng/ml	**Standard**	0.71
E1,2	400 ng/ml	**Standard**	1.34
F1,2	750 ng/ml	**Standard**	2.40
G1,2	**Positive**	**Control**	1.70
H1,2	Sample	**1:100**	1.21

Sample Result: **35** ng/ml x 100 dilution = **3.5** ug/ml



PERFORMANCE CHARACTERISTICS

Specificity
The plate is coated with CD20 antigen to which Rituximab binds with high affinity. Other antibodies or binding proteins in mouse serum may also bind to the CD20 coated plate; however, the Antibody-HRP conjugate will not bind to mouse antibodies or proteins. Therefore, the assay would be specific for measuring Rituximab activity only.

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

Rituximab concentrations were measured with good between-assay (2.1 to 5.1 %CV) reproducibility.

Sample	Rituximab ng/ml	Inter-assay %CV
High Concn	494	**5.1**
Medium Concn	254	**2.1**
Low Concn	148	**3.5**

Recovery
Rituximab was diluted into Sample Diluent containing 1% mouse serum (3 pooled and 6 individual samples, each diluted 1/100), and assayed in duplicate. **Recovery** was calculated comparing the observed (O) values to the expected (E) values for each diluted sample. O/E values ranged from **73%** to **107%**.

Mouse Serum Sample	Rituximab Concn (E) = 475 ng/ml	
	Observed (O)	O/E %
BM Pool	385	**81**
UK Pool	355	**75**
SM Pool	460	**97**
A	460	**97**
B	390	**82**
C	505	**106**
D	510	**107**
E	475	**100**
F	348	**73**

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 Rituximab 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 50 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 Rituximab activity, i.e., antibody that actually binds to the C20 antigen coated plate, relative to Rituximab standards that are presumed to be 100% active antibody. Factors in the sample that diminish Rituximab binding, e.g., CD20 antigen, other Rituximab-binding molecules, or CD20-binding molecules, may reduce apparent Rituximab concentration in the assay (**Recovery**).

2. Assays that measure Rituximab mass concentration (Cat# 200-215-RHG) may not have a tight correlation with the Rituximab activity assay, e.g., full Rituximab recovery may be determined by different factors.

3. The **recovery** (accuracy of Rituximab measurement in stored mouse serum) may be diminished if not diluted at least 1/100 (1%) in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual mouse serum or plasma samples may differ, and has not been determined.

4. Single dose, intravenous administration of Rituxan in humans peaks around 157 ug Rituximab/ml of serum (mean); the ELISA assay detection range is 50 - 750 ng IgG/ml, a 200 to 3000-fold **sensitivity** window.

Instruction Manual No. M-200-210-RAG

Rituximab (Rituxan) Anti-CD20

ELISA Kit # 200-210-RAG

For Quantitation of Rituximab Anti-CD20 in Mouse Serum

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ELISA Kit Components	Amount	Part
CD20 Antigen Coated Microwell Plate	8-well strips (12)	200-211
Rituximab Anti-CD20 Positive Control	0.65 ml	200-212
Rituximab Anti-CD20 Standard 50 ng/ml	0.65 ml	200-213B
Rituximab Anti-CD20 Standard 100 ng/ml	0.65 ml	200-213C
Rituximab Anti-CD20 Standard 200 ng/ml	0.65 ml	200-213D
Rituximab Anti-CD20 Standard 400 ng/ml	0.65 ml	200-213E
Rituximab Anti-CD20 Standard 750 ng/ml	0.65 ml	200-213F
Antibody-HRP Conjugate (100X)	0.15 ml	200-214
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
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