DeQuanto® Rituximab (Mabthera®) Immunogenicity ELISA Kit

IM5004

USER MANUAL

Immunoassay for qualitative determination of Antibodies to Rituximab drug in human serum or plasma

(Mabthera® is a registered trademark of Roche inc.)

1 X 96- Well Microtiter plate

Research Use Only (RUO)

Please read this user's manual carefully before using the kit

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Version 1.1

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INTENDED USE

DeQuanto® Rituximab (Mabthera®) immunogenicity ELISA kit is intended for qualitative determination of antibodies to Rituximab in human serum/plasma.

INTRODUCTION

Rituximab (MabThera®) is a genetically engineered chimeric murine/human monoclonal antibody specific to CD20. CD20 is an approximately 35 KDa transmembrane phosphoprotein involved in the activation, proliferation, and differentiation of B-lymphocytes. It is absent in terminally differentiated plasma cells.

The Fab domain of Rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effectors functions to induce apoptosis in B cells and is used in the treatment of leukemia s and lymphomas, some autoimmune disorders, and organ transplant.

However the use of Rituximab is associated with the development of anti Rituximab antibodies. The consequences of an immune response range from transient appearance of antibody without any clinical significance to severe life threatening condition. The **DeQuanto®** Rituximab (Mabthera®) Immunogenicity ELISA kit can be efficiently used for monitoring the Anti Rituximab antibodies.

ASSAY PRINCIPLE

The **DeQuanto®** Rituximab (Mabthera®) immunogenicity ELISA kit is an ELISA based immunoassay. The antibodies to Rituximab present in the sample binds to pre coated Rituximab on polystyrene microtiter plate. Non bound or the excess substances are removed by washing. Horseradish Peroxidase (HRP) labeled Rituximab is added to the wells, which bind to the Anti Rituximab antibody in the micro- wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the micro-wells. A color product is formed in proportion to the amount of Anti Rituximab Antibody present in the sample. The color development is stopped by addition of a stop solution. The absorbance is measured at 450 nm in a microtiter plate reader.

MATERIALS PROVIDED AND STORAGE

The DeQuanto® Rituximab (Mabthera®) immunogenicity ELISA kit is recommended to be stored as mentioned in table below.

Sl. No.	Description	Quantity	Volume	Strength/Conc.	Storage
1	Pre Coated 96-well microtiter plate	1 plate	NA	NA	-20°C
2	Positive Control	1 Vial	1 ml	NA	-20°C
3	Negative Control	1 Vial	1 ml	NA	-20°C
4	Drug Conjugate	1 Vial	20 µl	2000X	-20°C
5	Assay Diluent	1 Bottle	100 ml	1X	2-8°C
6	TMB Substrate	1 Vial	1 ml	20X	2-8°C
7	Wash Buffer-A	1 Bottle	70 ml	20X	2-8°C
8	Plate sealer	1 Nos	NA	NA	RT

MATERIALS REQUIRED BY END-USER

All the following materials are required but not available with the kit.

- Human Serum or Plasma
- Tween-20
- 2N H_2SO_4
- Deionized or ultra pure water
- Microtiter plate reader capable of measuring absorbance at 450 nm (Reference wave length 600 nm is optional)

ASSAY DURATION

The approximate time taken for each step of the assay are shown in the table below

Sl. No.	Step	Duration
1	Reagent thawing and preparation	30 minutes
2	Positive control, Negative Control and Sample addition and incubation	90 minutes
3	Drug Conjugate Addition and Incubation	30 minutes
4	Substrate Addition and Measurement	30 minutes
5	Calculation of Results	15 minutes

PRECAUTIONS

1. User should be trained with ELISA based assays and test procedure
2. All reagents have to be at room temperature before use, except the detection antibody (should be stored at -20°C to ensure the stability)
3. Avoid repeated freeze/thaw cycles for all reagents
4. Handle all reagents wearing gloves and other protective gears
5. Do not pipette any reagents by mouth
6. Use calibrated pipettes and devices only
7. Sodium azide inactivates HRP, do not use sodium azide-containing solutions
8. Any modification in the standard assay procedure may influence the kit performance
9. The described pipetting volume, incubation time, temperature steps should be performed according to the user manual
10. TMB/H_2O_2 Substrate and Drug Conjugate are light sensitive, hence should not be exposed to light
11. Avoid physical contact with Stop solution
12. Centrifuge all vials briefly before use
13. Use disposable pipette tips for each transfer to avoid cross contamination
14. Any samples which are out of assay range should be repeated using different dilutions
15. Reagents from different batch/lot are not interchangeable
16. Do not mix reagents from different kit batches/lots
17. The waste disposal should be performed according to your laboratory regulations

SPECIMEN COLLECTION AND STORAGE

SERUM

Use serum clot tube and allow the blood sample to coagulate at room temperature (RT) for 30 minutes. Centrifuge at 5000 RPM for 10 minutes at RT. Aliquot the clear serum and store at -20°C. Avoid repeated freeze/ thaw cycles.

PLASMA

Use K_2 EDTA as anticoagulant for blood collection and allow at RT for 30 minutes. Centrifuge the sample at 5000 RPM for 10 minutes at RT. Aliquot the clear plasma and store at -20°C. Avoid repeated freeze/ thaw cycles.

NOTE: Grossly haemolyzed, lipemic or contaminated sample (serum or Plasma) may lead to inaccurate results and is not recommended to use with this procedure.

It is recommended that the end user must validate assay procedure using either serum or plasma.

REAGENT PREPARATION

WASH BUFFER-A

1. Thaw the wash buffer at RT until it is a clear solution
2. Prepare 1000 ml of 1X wash buffer-A, using 50 ml of 20X Wash buffer-A and make up the volume to 1000 ml with ultra pure water

WASH BUFFER-B

1. Aliquot 500 ml of wash buffer-A
2. Add 0.05% (500 µl) of Tween-20 solution and mix it for 10 minutes on magnetic stirrer at RT

ASSAY DILUENT

1. Thaw the wash buffer at RT until it is a clear solution
2. Use this assay diluent buffer for assay matrix and test sample dilution

ASSAY MATRIX

1. Dilute human serum or plasma at MRD (1:200) in assay diluent

CAUTION ⚠
▪ *Human serum or plasma used for assay matrix must be from healthy volunteers*

TMB SUBSTRATE

1. Dilute the 20X TMB substrate to 1X solution in ultra pure water (600 µl of TMB substrate to 11.4 ml of ultra pure water)
2. Mix the 1X solution thoroughly on vortex mixer or manually

DRUG CONJUGATE

1. Dilute the 2000X drug conjugate in assay diluent at 1:2000 (take 5 µl of 2000X drug conjugate and add to 20 ml of assay diluent)
2. Gently mix the detection antibody before use

ASSAY PROCEDURE

PRE-PROCESSING OF 96-WELL MICROTITER PLATE

1. Thaw the precoated 96-well microtiter plate at room temperature for 15 minutes
2. Wash the plate with 1X wash buffer-A, allowing 2 minutes for soaking
3. Decant off the contents of the plate

CAUTION

- ***Use multichannel pipette***
- ***Keep the programs for plate washer ready (if automated washer is used)***

SAMPLE AND CONTROLS ADDITION

1. Prepare positive, negative and test samples
2. Add 100 µl of positive, negative and test or specimen samples to the wells of the microtiter plate as per the plate template
3. Seal plates with adhesive plate sealer
4. Incubate the microtiter plate at RT (~25°C) for 1 hour

CAUTION

- ***Refer the sample plate layout given at the end of this user manual, for standards and control***
- ***Before addition of samples to the microtiter plate, keep the Plate template ready.***

DRUG CONJUGATE ADDITION

1. Discard the contents of each well and wash 3 times with 1X wash buffer-B and followed by 3 washes with 1X wash buffer-A, allowing 2 minutes for soaking between each wash step
2. Blot the microtiter plate on absorbent paper to remove any residual reagent from the wells
3. Add 100 µl of drug conjugate solution to each well of the microtiter plate
4. Seal the microtiter plate with adhesive plate sealer
5. Incubate the microtiter plate at RT (~25 °C) for 30 minutes

SUBSTRATE ADDITION AND MEASUREMENT

1. Discard the contents of each well and wash 3 times with 1X wash buffer-B and followed by 3 washes with 1X wash buffer-A, allowing 2 minutes for soaking between each wash step
2. Blot the microtiter plate on absorbent paper to remove any residual reagent from the wells
3. Add 100 µl/well of 1X TMB substrate solution and incubate the plate in dark at RT (~25°C) for 15 minutes
4. Add 50µl/well of Stop solution to stop the reaction
5. Read the absorbance in micro plate reader set to 450 nm, set the reference wavelength to 600 nm

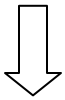
CAUTION

- ***Add stop solution in the same order of addition of TMB substrate to the plate***

ASSAY SUMMARY

1. Prepare all reagents as instructed in the user manual

2. Add 100 µl of Positive, Negative control and test/specimen samples to each well of microtiter plate



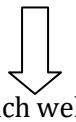
3. Seal the microtiter plate with adhesive plate sealer and incubate at RT (~25°C) for 1 hour

4. Decant the contents of the microtiter plate and wash the microtiter plate 3 times with wash buffer-B, followed by 3 times with wash Buffer-A, allowing 2 minutes of soaking between each wash step

5. Blot the plate on absorbent paper to remove any residual reagent from the wells

6. Add 100 µl of drug conjugate to each well of the microtiter plate and incubate for 30 minutes at RT (~25°C)

7. Repeat the steps 4 and 5



8. Add 100 µl of 1X TMB solution to each well of the microtiter plate and incubate for 15 minutes in dark at RT (~25°C)

9. Add 50 µl of stop solution (2N H_2SO_4) to each well

10. Read the absorbance at 450 nm and 600 nm as reference wave length

INTERPRETATION OF RESULTS

The results are calculated and reported as positive or negative relative to a cut-off point. The cut-off point may be determined in each plate by running 4-6 replicates of negative control. The cut-off point is calculated by calculating the mean of the negative controls and by adding 2*standard deviations to the calculated mean.
i.e.
If a sample OD at 450 nm is ≥ cut-off value then the sample is POSITIVE
If a sample OD at 450 nm is ≤ cut-off value then the sample is NEGATIVE

ABBREVIATIONS

RT	Room Temperature
Abs	Absorbance
Conc.	Concentration
HRP	Horse Radish Peroxidase
TMB	3, 3', 5, 5'-Tetramethylbiphenyl-4, 4'-Diamine
TNF	Tumor Necrosis Factor
ELISA	Enzyme Linked Immunosorbent Assay
%CV	Coefficient of variations
%RE	Relative Error
MRD	Minimum Required Dilution
CTRL	Control
°C	Degree Celsius
µg	Microgram
mg	Milligram
µl	Microliter
Eg.	Example
ml	Milliliter
W/V	Weight by Volume
IgG	Immunoglobulin
ng	Nanogram
nm	Nanometer
2N H_2SO_4	2 Normal Sulphuric Acid
K_2 EDTA	Ethylene Di-amine Tetra Acetic acid (Di-Potassium salt)
RPM	Revolutions Per Minute

TROUBLESHOOTING

Problem	Probable Causes	Solution
High or Low OD value of test samples	Test samples contain anti drug antibody concentrations greater than assay range	Repeat the assay with multiple dilutions of the test samples.
	Test Samples contain no or below detectable levels of anti drug antibody	Test sample contains the anti drug antibody level below detection limits
High OD value of blank/ controls (-ve)	Matrix interference	Perform higher dilution than MRD (minimum required dilution). Ensure the diluent buffer blank/ controls (-ve) OD are acceptable.
High % CV (High variability in duplicate OD values)	Unequal volumes	Ensure the calibrated pipettes
		Ensure pipette tips are tightly secured while adding the solution to each well
	Inadequate washing	Ensure the wash system is working properly if the automated wash station is used. Also ensure the manual washes
	Non-homogenous solution	Ensure that all solution prepared are adequately mixed.
	Edge effect	Use plate sealer and maintain the temperature as recommended
	Cross-well contamination	Ensure the good pipetting practice
	Inadequate mixing of TMB substrate solution	Ensure the TMB substrate solution is mixed thoroughly before use
Low signal	Positive control was incompletely reconstituted or the storage was inappropriate	Reconstitute positive control according to the user's manual Ensure the kit reagents are not expired
	Reagents added to wells with incorrect concentrations	Cross check for calculation and pipetting errors
	Incorrect incubation time or temperature	Adhere to the recommended assay procedure
Standard curve with R^2 value <0.95	Pipetting error	Ensure the pipetting and the analyst training

PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	P CTRL	P CTRL										
B	N CTRL	N CTRL										
C	N CTRL	N CTRL										
D	BLANK	BLANK										
E												
F												
G												
H												

P CTRL: Positive Control

N CTRL: Negative Control

NOTES

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CUSTOMIZED SERVICES

PRECLINICAL/CLINICAL SAMPLE ANALYSIS
ELISA based validation and clinical or pre-clinical sample analysis.

POLYCLONAL ANTIBODY DEVELOPMENT
PAb development against peptide or protein, peptide designing, high titer antibody purification, characterization.

MONOCLONAL ANTIBODY DEVELOPMENT
MAb development against peptide or protein, peptide designing, best reacting/stable hybridoma development, sub cloning, small scale/large scale hybridoma culture and antibody purification, characterization.

CLONING, PROTEIN EXPRESSION PURIFICATION
Gene synthesis, site directed mutagenesis, cloning and expression in bacterial systems and other customized molecular biology services.

ANTIBODY CONJUGATION
With HRP, FITC, Biotin, ALP and many more molecules.

CELL BASED ASSAYS
Cell based assays, neutralization assays and potency ratio estimation of drugs with reference drugs, drug efficacy assessment and customized in-vitro, in-vivo assay development.

CUSTOMIZED ELISA BASED ASSAY/METHOD DEVELOPMENT
Different formats of ELISA based assay development.

PHARMACOVIGILENCE
Clinical/post marketing drug safety reporting medical writing

For any queries/enquiries related to our products or services, please contact us by mailing us at info@denovobiolabs.com or call us at +91-80-29575711