MATRIKS BIOTEK
innovation for health & wellness

"trace & catch"

Instructions for Use

Rituximab (Rituxan®, Mabthera®) ELISA

SHIKARI®
Q-RITUX

Enzyme immunoassay for the quantitative determination of Rituximab (Rituxan®, Mabthera®) in serum and plasma

REF **TR-RTXv2** Σ 12 x 8 2-8°C

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CE
IVD

Matriks Biotek® Laboratories
www.matriksbiotek.com

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	SHIKARI Q-RITUX
	Free rituximab (Rituxan®, Mabthera®) quantitative analyses
Required Volume (µl)	10
Total Time (min)	135
Sample	Serum, plasma
Sample Number	96
Detection Limit (ng/mL)	3
Spike Recovery (%)	Between 85-115
Shelf Life (year)	1

Intended Use

Enzyme immunoassay for the quantitative determination of free rituximab (Rituxan®, Mabthera®) in serum and plasma. Matriks Biotek® Rituximab ELISA has been especially developed for the quantitative analysis of free rituximab in serum and plasma samples. It is for professional use only.

* Rituxan® is a registered trademark of Biogen Idec, Inc., Mabthera® is a registered trademark of Roche, Inc.,

Summary and Explanation

Rituximab is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. The antibody is a glycosylated IgG1 kappa immunoglobulin containing murine light- and heavy-chain variable region sequences (Fab domain) and human constant region sequences (Fc domain). Rituximab is composed of 1,328 amino acids and has an approximate molecular weight of 144 kD. Rituximab has a high binding affinity for the CD20 antigen of 5.2 to 11.0 nM.

Rituximab binds specifically to the antigen CD20, a transmembrane molecule located on pre-B and mature B lymphocytes. The antigen is expressed on > 95% of all B-cell non-Hodgkin's lymphomas (NHL). CD20 (human B lymphocyte-restricted di erenti tion anti en, Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD. This non-glycosylated phosphoprotein is found on both normal and malignant B cells, but not on haematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues. CD20 regulates (an) early step(s) in the activation process for cell cycle initiation and diferentiation, and possibly functions as a calcium ion channel. CD20 does not internalise upon antibody binding and is not shed from the cell surface. This antigen does not circulate in the plasma. Thus, free antigen does not compete for rituximab binding.

The Fab domain of rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effector functions to mediate B-cell lysis. Possible mechanisms of cell lysis include complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The antibody also induces apoptosis in the DHL-4 human B-cell lymphoma line and *in vitro* studies have demonstrated that rituximab sensitises drug-resistant human B-cell lymphoma lines to the cytotoxic effects of some chemotherapeutic agents. In human tissue, the expression of the CD20 antigen is highly restricted; rituximab binding to CD20 was found only on lymphoid cells in the thymus,

the white pulp of the spleen and a majority of B lymphocytes in peripheral blood and lymph nodes. Little or no non-specific bindingwas observed.

Rituximab is a CD20-directed cytolytic antibody indicated for the treatment of patients with: Non-Hodgkin's Lymphoma (NHL), Chronic Lymphocytic Leukemia, Rheumatoid Arthritis (RA) in combination with methotrexate in adult patients with moderately-to severely-active RA who have inadequate response to one or more TNF antagonist therapies, Wegener's Granulomatosis (WG) and Microscopic Polyangiitis (MPA) in adult patie ts in combination with glu ocorti oids.

Pharmacokinetics

Non-Hodgkin's Lymphoma(NHL): Rituximab at a dose of 375 mg/m2 was administered as an IV infusion at weekly intervals for 4 doses to 203 patie ts with NHL naive to rituximab. The mean Cmax following the fourth infusion was 486 µg/mL (range 77.5 - 996.6 µg/mL). The peak and trough serum levels of rituximab were inversely correlated with baseline values for the number of circulating CD19-positive B-cells and measures of disease burden. Median steady-state serum levels were higher for responders compared with non-responders. Serum levels were higher in patients with International Working Formulation (IWF) subtypes B, C, and D as compared with those with subtype A. Rituximab was detectable in the serum of patients 3 – 6 months after completion of last treatment. Rituximab at a dose of 375 mg/m2 was administered as an IV infusion at weekly intervals for 8 doses to 37 patients with NHL. The mean Cmax increased with each successive infusion, spanning from a mean of 243 µg/mL (range, 16 – 582 µg/mL) after the first infusion to 550µg/mL (range 171 – 1177 µg/mL) after the eighth infusion. The pharmacokinetic profile of rituximab when administered as 6 infusions of 375 mg/ m^2 in combination with 6 cycles of CHOP chemotherapy was similar to that seen with rituximab alone.

Chronic Lymphocytic Leukaemia (CLL): Rituximab was administered as an IV infusion at a first-cycle dose of 375 mg/m2 increased to 500 mg/m2 each cycle for a further 5 doses in combination with fludarabine and cyclophosphamide (FC) in CLL patients. The mean Cmax (N=15) was 408 µg/mL (range, 97 – 764 µg/mL) a er the fift 500 mg/m2 infusion.

Rheumatoid Arthritis (RA): The pharmacokinetics of rituximab were assessed following two IV doses of 500 mg and 1000 mg on days 1 and 15 in four studies. In all these studies, rituximab pharmacokinetics were dose proportional over the limited dose range studied. Mean Cmax for serum rituximab following first infusion ranged from 157 to 171 µg/mL for 2 x 500 mg dose and ranged from 298 to 341 µg/mL for 2 x 1000 mg dose. Following second infusion, mean Cmax ranged from 183 to 198 µg/mL for the 2 x 500

mg dose and ranged from 355 to 404 µg/mL for the 2 x 1000 mg dose. Mean terminal elimination half-life ranged from 15 to 16.5 days for the 2 x 500 mg dose group and 17 to 21 days for the 2 x 1000 mg dose group. Mean Cmax was 16 to 19% higher following second infusion compared to the first infusion for both doses. Upon re-treatment with a second course the pharmacokinetics of rituximab were again assessed following two IV doses of 500 mg and 1000 mg. Mean Cmax for serum rituximab following first infusion was 170 to 175 µg/mL for 2 x 500 mg dose and 317 to 370 µg/mL for 2 x 1000 mg dose. Cmax following second infusion, was 207 µg/mL for the 2 x 500 mg dose and ranged from 377 to 386 µg/mL for the 2 x 1000 mg dose. Mean terminal elimination halflife after the second infusion, following the second course, was 19 days for 2 x 500 mg dose and ranged from 21 to 22 days for the 2 x 1000 mg dose. PK parameters for rituximab were comparable over the two treatment courses.

Wegener's Granulomatosis(WG) and Microscopic Polyangiitis(MPA): Based on the population pharmacokinetic analysis of data in 97 WG and MPA patients who received 375 mg/m^2 rituximab once weekly by intravenous infusion for four weeks, the estimated median terminal elimination half-life was 23 days (range, 9 to 49 days). Rituximab mean clearance and volume of distribution were 0. 312 L/day (range, 0.115 to 0.728 L/day) and 4.50 L (range, 2.21 to 7.52 L) respectively. Male patients and patients with higher BSA or positive human anti-chimeric antibody (HACA) levels have higher clearance. However, further dose adjustment based on gender or HACA status is not necessary.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. The observed incidence of antibody (including neutralizing antibody) positivity in an assay is highly dependent on several factors including assay sensitivity and specificity, assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. It was reported that, using an ELISA assay, anti-human anti chimeric antibody (HACA) was detected in 4 of 356 (1.1%) patients with low-grade or follicular NHL receiving single-agent rituximab. Three of the four patients had an objective clinical response. A total of 273/2578 (11%) patients with RA tested positive for HACA at any time after receiving rituximab. HACA positivity was not associated with increased infusion reactions or other adverse reactions. Upon further treatment, the proportions of patients with infusion reactions were similar between HACA positive and negative patients, and most reactions were reported to be mild to moderate. Four HACA positive patients had serious infusion reactions, and the temporal relationship between HACA positivity and infusion reaction was variable. A total of 23/99 (23%) rituximab-treated patients with WG and MPA tested positive for HACA by 18 months. The clinical relevance of HACA formation in rituximab-treated patients is unclear.

In rituximab-treated cynomolgus monkeys B lymphocyte numbers were reduced by 99% or more in comparison with pre-test values in the peripheral blood of all monkeys, approximately 24 hours after the first dose. Two weeks after the last dose, B lymphocyte numbers were still reduced by more than 99% in 3/6 monkeys dosed for four weeks and in 4/6 monkeys dosed for eight weeks, and B lymphocyte numbers were also depleted in the mandibular lymph nodes and femoral bone marrow. However, a partial recovery of B lymphocyte numbers in the peripheral blood of some monkeys in both dose groups was correlated with the development of antibodies against rituximab.
The use of rituximab (Rituxan®, Mabthera®) was associated to the development of anti-rituximab antibodies, even some might be neutralizing, in various percentages of patients during therapy with the drug. The *Matriks Biotek®* Rituximab-ELISA and Antibody to rituximab ELISA Kits can be efficiently used, for monitoring serum through levels and the presence of anti-rituximab antibodies respectively, during therapy and offers the scientist a tool for decision on possible preventive measures.

Test Principle

The *Matriks Biotek®* rituximab (Rituxan®, Mabthera®) solid phase enzyme-linked immunosorbent assay (ELISA) is based on rituximab-specific monoclonal antibody (mAb). Standards and diluted samples are incubated in the microtitre plate coated with mAb. After incubation, the wells are washed. Anti-human IgG Fc-specific mAb conjugated to horse radish peroxidase (HRP) is added and binds to rituximab specifically captured by the human monoclonal antibody on the surface of the wells. Following incubation, wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of rituximab in the sample or standard. Results of samples can be determined directly using the standard curve.

Warnings and Precaution

1. For professional use only.

2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.

3. In case of severe damage of the kit package please contact Matriks Biotek® or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.

4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.

6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.

7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.

8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. Some reagents contain sodium azide (NaN_3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN_3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up.

10. All reagents of this test kit containing human serum or plasma have been tested and were found negative for HIV I/II, HBsAg and HCV by FDA approved procedures. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin)*

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light
Stability:	7 d	6 mon	Avoid repeated freeze-thaw cycles

*. Rituximab (Rituxan®,Mabthera®) infusion camouflages/masks the presence of antibody to rituximab in serum/plasma samples. Therefore, blood sampling time is critical for detection of rituximab. Matriks Biotek® Laboratories propose to obtain blood sample just before the infusion of Rituximab (Rituxan®,Mabthera®) or at least 2 weeks after the infusion of rituximab (Rituxan®,Mabthera®).

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with human monoclonal antibody.
8 x 1 mL	STND A-F HIGH CNTRL LOW CNTRL	**Rituximab Standards A-F, High Level Control, Low Level Control** 300;100; 30; 10; 3; 0 nanogram/mL Ready to use. Used for construction of the standard curve. Contains rituximab, human serum, stabilizer and <0.1% NaN_3.
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains proteins and <0.1% NaN_3.
1 x 12 mL	HRP CONJ	**Horse radish peroxidase-Conjugated Probe** Red colored. Ready to use. Contains HRP-probe, stabilizer and preservatives.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB

1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer, Concentrate (20x)** Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation

Materials Required But Not Supplied

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Calibrated measures.
3. Tubes (1 mL) for sample dilution.
4. Wash bottle, automated or semi-automated microtiter plate washing system.
5. Microtiter plate reader capable of reading absorbance at 450/650 nm.
6. Bidistilled or deionised water, paper towels, pipette tips and timer.

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.

2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.

3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.

4. Use a pipetting scheme to verify an appropriate plate layout.

5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipes or for pipetting of solutions in all wells.

6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.

7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Preparation of Component

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	bidist. Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	2 w

*****Prepare Wash Buffer before starting assay procedure.

Dilution of Sample

Sample	To be diluted	With	Diluent	Relation	Remarks
Serum/ Plasma	1:1000	Assay Buffer	bidist. Water	1:1000	First; for dilution at 1:10; 10 µl Serum/Plasma + 90 µl Assay Buffer Second; for dilution at 1:100; 5 µl 1:10 diluted Serum/ Plasma + 495 µl Assay Buffer

Patient samples with a concentration of rituximab above the measuring range are to be rated as > "Highest Standard (Standard A)". The result must not be extrapolated. The patient sample in question should be further diluted with Assay Buffer and retested.

Test Procedure

1	Pipette **100 µL** of each **ready-to use Standards, High level control, Low level control and Diluted Samples** into the respective wells of microtiter plate. **Wells** A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1: Standard F G1: High Level Control H1: Low Level Control A2 and on: Sample (Serum/Plasma)
2	Cover the plate with adhesive film. **Incubate 60 min** at room temperature (18-25°C).
3	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
4	Pipette **100 µL** of ready-to use **Peroxidase Conjugate** into each well.
5	Cover the plate with adhesive film. **Incubate 60 min** at room temperature (18-25°C).
6	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
7	Pipette **100 µL** of **TMB Substrate Solution** into each well.
8	**Incubate 15 min** (without adhesive film) at room temperature (18-25°C) in the dark.
9	Stop the substrate reaction by adding **100 µL** of **Stop Solution** into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
10	**Measure** optical density (OD) with a photometer at **450/650 nm** within **30 min** after pipetting of the Stop Solution.

Interpretation & Calculation of Results

1. Using the standards (300; 100; 30; 10; 3; 0 ng/mL) disregarding zero standard, construct a standard curve by plotting the OD450/650 nm for each of 5 standards on the vertical (Y-axis) axis versus the corresponding rituximab concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of rituximab from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the rituximab concentration for the unknown sample.

3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

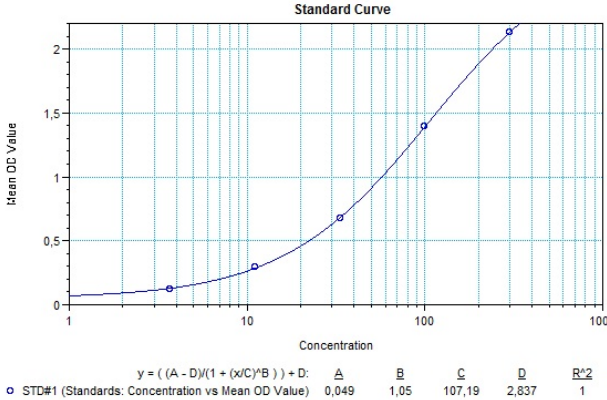
4. To obtain the exact values of the samples, the concentration determined from the standard-curve must be multiplied by the dilution factor (1000x). Any sample reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. Therefore, if the pre-diluted samples have been further diluted, the concentration determind from the standard curve must be multiplied by the further dilution factor.

   E.g.; *If the pre-diluted sample further diluted in a ratio of 1:5 then results should be multiplied by 5000.*

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve

(Example. Do not use for calculation!)



Standard	Concentration (ng/mL)	Mean OD 450/650nm
A	300	2,130
B	100	1,395
C	30	0,676
D	10	0,293
E	3	0,124
F	0	0,050

Assay Characteristic

Specificity
There is no cross reaction with any other proteins present in native human serum. In addition, there is **no cross reaction** with the other therapeutic immunoglobulins tested (infliximab (Remicade®), etanercept (Enbrel®), adalimumab (Humira®), bevacizumab (Avastin®) and trastuzumab (Herceptin®)

Sensitivity
The lowest detectable level that can be specifically distinguished from the zero standard is 3 ng/mL.

Precision of Kit
Intra-assay CV: <15% at the range of 3-300 ng/mL.

Inter-assay CV: <15% at the range of 3-300 ng/mL.

Recovery
Recovery rate was found to be between 85-115% with native human serum spiked with rituximab at concentrations of 100, 10 ng/mL.

Automation

Experiments have shown that the *Matriks Biotek*® SHIKARI® Rituximab ELISA is also suitable to run on an automated ELISA processor.

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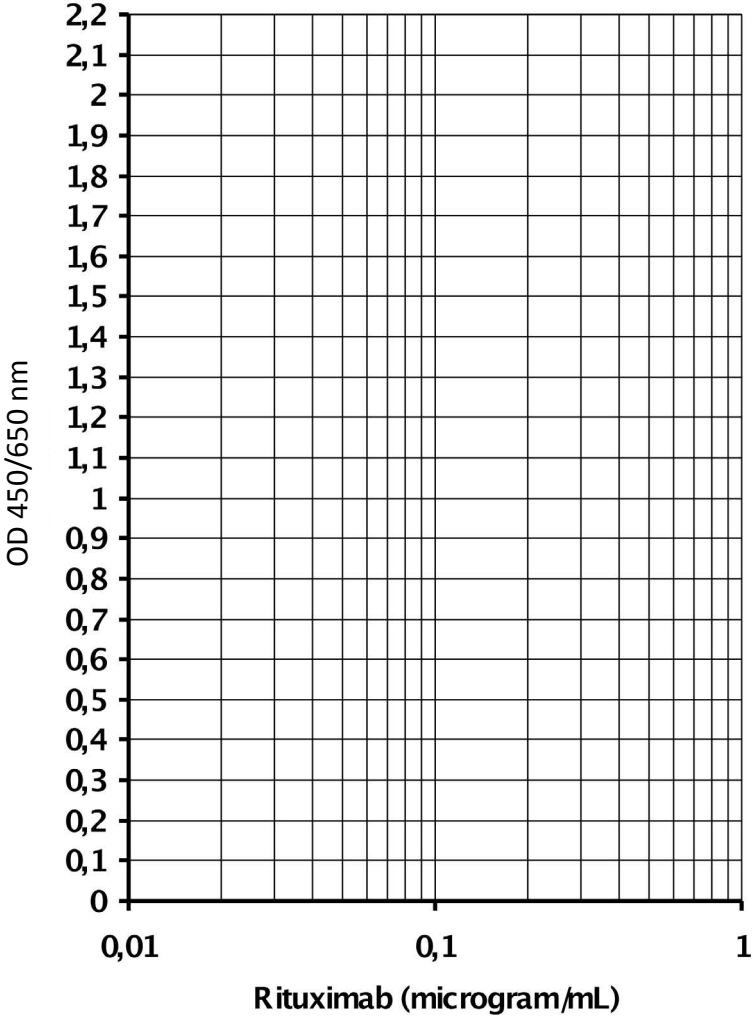
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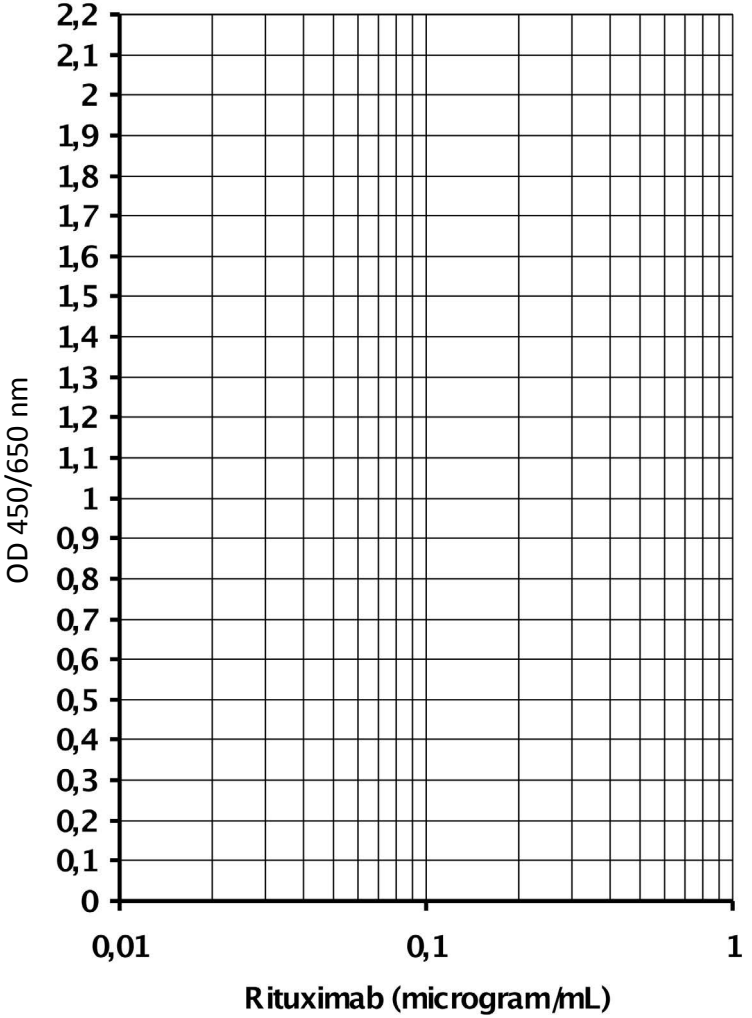
Rituximab (microgram/mL)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