LISA TRACKER Duo Rituximab

English

REF **LTR 005**

2 x 48

42 determinations

DEFINITION

LISA-TRACKER Duo Rituximab (Theradiag) is an enzyme linked immunoassay (ELISA) for the quantitative determination of Rituximab (anti-CD20) and anti-Rituximab antibodies in human serum samples. These tests can be separatly or simultaneously done by following the standardized assay protocole.

DESCRIPTION AND DIAGNOSTIC VALUE

Rituximab is indicated for the treatment of Non-Hodgkin's Syndrome, Chronic Lymphocytic Leukemia and Rheumatoid Arthritis in association with Methotrexate in patients with an insufficient response or an intolerance to conventional treatments (DMARDS) or anti-TNF treatments.

Rituximab is a monoclonal antibody directed against transmembrane CD20 molecule expressed on the surface of B-cell (pre-B state to mature B-lymphocyte). Rituximab is a chimeric antibody constituted with human constant domains (γ1 heavy chain and kappa light chain) and murine variable domains. During the course of the treatment, some patients can develop antibodies directed against Rituximab

LISA-TRACKER Duo Rituximab (Theradiag) allows the detection of 2 parameters: Rituximab and anti-Rituximab antibodies. This kit allows the physician to monitor the level of these 2 parameters in patient sera.

SAMPLES COLLECTION AND HANDLING

- The test should be performed on serum or on plasma.
- Lipemic sera should be avoided, as well as samples which have been frozen and defrosted more than once.
- To avoid any non-specific binding, samples which have been frozen for more than 6 months or which are cloudy, should be centrifuged and filtered.

ASSAY PRINCIPLE

A. Dosage of Rituximab

Anti-rituximab antibody (anti-idiotype) is coated onto a polystyrene microtiter plate (6 strips of 8 wells).

- First, the diluted sample is added to the antibody coated well, which allows to bind. After incubation, unbound proteins are removed by washing.
- Anti-human IgG biotinylated antibodies is added. After incubation, unbound antibodies are removed by washing.
- Then horseradish peroxydase labelled streptavidin is added. The streptavidin binds to the complex formed with biotinylated anti-IgG antibodies. After incubation, the wells are washed again to eliminate any excess of conjugate.
- The bound enzyme is revealed by addition of substrate TMB (3,3',5,5' tetramethylbenzidin). The colour intensity is proportional to the amount of Rituximab.
- Adding H_2SO_4 allows to stop the enzymatic reaction.
- After stopping the reaction by H_2SO_4, the optical density is read by a spectrophotometer at 450nm.

A range of calibration allows to define the quantity of Rituximab of each patient samples expressed in µg/mL.

B. Dosage of anti-Rituximab

Rituximab is coated onto a polystyrene microtiter plate (6 strips of 8 wells).

- First, the diluted sample is added to the antibody coated well, which allows to bind. After incubation, unbound proteins are removed by washing.
- Biotinylated Rituximab is added. After incubation, unbound antibodies are removed by washing
- Then horseradish peroxydase labelled streptavidin is added. The streptavidin binds to the complex formed with biotinylated Rituximab. After incubation, the wells are washed again to eliminate any excess of conjugate.
- The bound enzyme is revealed by addition of substrate TMB (3,3',5,5' tetramethylbenzidin). The colour intensity is proportional to the amount of anti-Rituximab antibodies.
- Adding H_2SO_4 allows to stop the enzymatic reaction.
- After stopping the reaction by H_2SO_4, the optical density is read by a spectrophotometer at 450nm.

A range of calibration allows to define the quantity of anti-Rituximab antibodies of each patient samples expressed in ng/mL.

REAGENTS

3 reagent families :

Color	Rituximab reagents	anti-Rituximab antibodies reagents	Common reagents
cap of vials	**Blue**	**Yellow**	**Green, White, Black or Purple**
microwells	**Blue**	**Yellow**	**-**

A) Specific reagents for Rituximab determination

Strips of individual breakaway blue wells coated with anti-Rituximab antibody (anti-idiotype). MP	6 strips
5 vials of « Rituximab » Standards, (µg/mL). Ready to use. The vials can be reused several times. ***The quantity of Rituximab is indicated on the vial label.*** ***Blue caps*** RIT CAL n	5 x 1.5mL
«Positive control - Rituximab», (µg/mL). To dilute. The vials can be reused several times. ***The quantity of Rituximab is indicated on the vial label.*** ***Blue cap*** RIT CONTROL +	1 x 250µL
Biotinylated antibody vial. Ready to use. ***Blue cap*** RIT Ab BIOT	1 x 7.5mL

B) Specific reagents for anti-Rituximab antibodies determination

Strips of individual breakaway yellow wells coated with Rituximab. MP	6 strips
5 vials of « anti-Rituximab » Standards, (ng/mL). Ready to use. The vials can be reused several times. ***The quantity of anti-Rituximab is indicated on the vial label.*** ***Yellow caps*** A-RIT CAL n	5 x 1.5mL
« Positive control – anti-Rituximab», (ng/mL). To dilute. The vial can be reused several times. ***The quantity of anti-Rituximab is indicated on the vial label.*** ***Yellow cap*** A-RIT CONTROL +	1 x 1mL
Biotinylated antibody vial. Ready to use. ***Yellow cap*** A-RIT Ab BIOT	1 x 7.5mL

C) Common reagents

HRP labelled Streptavidin. Ready to use. ***Green cap*** CONJ HRP	1 x 12mL
Phosphate-Tween Buffer pH 7,2 (10x) – To reconstitute with distilled water. ***White cap*** BUF WASH 10x	1 x 100mL
Substrate (TMB). Ready to use. ***Black cap*** SUBS TMB	1 x 12mL
Stop solution - H_2SO_4 (0.25 N). Ready to use. ***Purple cap*** SOLN STOP	1 x 15mL

MATERIAL REQUIRED BUT NOT PROVIDED

- distilled water
- precision pipettes
- microplate spectrophotometer with 450 nm filter
- 8 channel pipettes

STABILITY AND STORAGE

- Store reagents and micro-wells at +2°C/+8°C in their own package.
- Do not use kits beyond the expiration date.
- Store unused strips into their plastic bag with the desiccant.
- Store all components immediately after use again at +2°C/+8°C.

SETUP

Except the TDL, which can be prepare in advance, all reagents must be prepared extemporaneously.

1. Dilution and Wash buffer (TDL)

- Dilute concentrated Phosphate-Tween Buffer 1/10 in distilled water.

BUF WASH 10x

- Shelf life : 3 months at +2°C/+8°C (avoid to use if signs of contamination or other visible changes occur).

NB. If there are crystals in the concentrated solution, warm the bottle up to +37°C for 15 minutes before use.

2. Preparation of samples and positive controls

a. Samples

- Rituximab determination

- Dilute to 1/1001 in TDL

Ex : 3µL sample + 3mL TDL

If a micropipette is not available to take 3µl, a serial dilution of the sample should be done. In a first time make a 1/101 dilution of the sample (10µl + 1ml of TDL). In a second time, make a 1/10 dilution of the 1/101 dilution (100µl of the 1/101 dilution + 900µl TDL).

- Vortex vigorously.

- Anti-Rituximab determination

- Dilute to 1/2 in TDL

Ex : 100µL sample + 100µL TDL

- Vortex vigorously.

b. Positive controls

- Rituximab determination

- Dilute to 1/1001 in TDL

Ex : 3µL positive control + 3mL TDL

If a micropipette is not available to take 3µL, a serial dilution of the sample should be done. In a first time make a 1/101 dilution of the sample (10µL + 1mL of TDL). In a second time, make a 1/10 dilution of the 1/101 dilution (100µL of the 1/101 dilution + 900µL TDL).

- Vortex vigorously.

- anti-Rituximab determination

- Dilute to 1/2 in TDL

Ex : 100µL positive control + 100µL TDL

- Vortex vigorously.

3. Use of ready-to-use biotinylated antibody.

- Estimate the amount required for handling and transfer to a tube.

4. Use of ready-to-use HRP Streptavidin conjugate.

- Estimate the amount required for handling and transfer to a tube.

5. Use of ready-to-use substrate.

- Estimate the amount required for handling and transfer to a dark tube.

PRECAUTIONS

- Unpack all reagents in order to let them warm at room temperature (+18°C/+25°C) at least half an hour before starting the test.

☝ The temperature of the reagents can impact the final result.

- Check that all plates are well drained after each wash.

- Avoid to use reagents if signs of contamination or other visible changes occur.

- Human sources for the preparation of standards and controls have been tested and found negative for antibody to HIV 1 and 2, antibody to hepatitis C virus and hepatitis B virus antigen. Nevertheless, no test can offer complete assurance that HIV, hepatitis B virus or other infectious agents are absent. Therefore, the reagents should be handled as potentially infective materials.

- Reagents in solution (except for substrate buffer and stop solution) contain <0.1% of sodium azide and <0.6% of ProClin® 300. Do not eat and avoid contact with skin and eyes. Azide can form explosive mixtures in copper or lead piping. Rinse thoroughly after flushing.

- ⚠ At this concentration, ProClin® 300 is irritating to eyes and skin, and may be detrimental if enough quantity is ingested. It is a skin sensitizer; prolonged or repeated exposure may cause allergic reaction in certain sensitive individuals.

- LISA-TRACKER Duo Rituximab (Theradiag) has been developed according CE directives 67/548/EEC and 1999/45/EC relating to the classification, packaging and labeling of dangerous preparations.

- LISA-TRACKER Duo Rituximab (Theradiag) has been optimized for the use as describe in this procedure. Do not substitute other manufacter's reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Close adherence to the test procedure will assure optimal performance.

METHOD

1. Preparing the test

Use the work sheet to note the sample locations.

Set out:

- 5 "standard" wells
- 1 well for positive control
- 1 well for each sample

For a simultaneous testing of the 2 parameters, detach the exact number of wells needed. Return unused wells to plastic pouch provided in the kit, with the desiccant bag.

Remark :

If a dispensing/diluting device is used, place the specific wells in the following order : dosage of Rituximab then dosage of anti-Rituximab.

2. Samples, positive controls and standards incubation

Add 100 µL of standards, diluted controls or samples.

Incubate for 60 minutes at room temperature (+18°C/+25°C).

Wash step:

Remove the content of the wells by rapid inversion.

Wash 3 times with 300µL of TDL buffer.

Dry the microplate by tapping it gently on an absorbent paper to eliminate the excess of liquid.

3. Incubation of biotinylated antibodies

Add 100µL of **specific** biotinylated antibodies in identified wells.

Incubate for 60 minutes at room temperature (+18°C/+25°C).

Wash step:

Remove the content of the wells by rapid inversion.

Wash 3 times with 300µL of dilution and washing buffer.

Dry the microplate by tapping it gently on an absorbent paper to eliminate the excess of liquid.

4. Incubation of Conjugate

Add 100µL of conjugate.

Incubate for 30 minutes at room temperature (+18°C/+25°C).

Wash step:

Remove the content of the wells by rapid inversion.

Wash 3 times with 300µL of dilution and washing buffer.

Dry the microplate by tapping it gently on an absorbent paper to eliminate the excess of liquid.

5. Incubation of Substrate

Add 100µL substrate into each well.

Incubate for 15 minutes at room temperature (+18°C/+25°C), in the dark.

6. Stop of the reaction

Add 100µL of H_2SO_4 to each well.

7. Reading

Read the optical density of each well at 450nm within 30 minutes after stopping reaction.

RESULTS AND INTERPRETATION

A. Dosage of Rituximab

- The OD of the standard 1 should be at least 0.8.
- The positive control value should be comprised into the range indicated on the vial label.
- Trace a degree 4 polynomial standard curve or a 4PL standard curve, plotting the units of the 5 standard points (µg/mL) along the abscissa (X axis) and the corresponding OD values along the ordinate (Y axis).
- The Rituximab value can be directly read on the curve.
- Samples with values greater than that of standard 1 may be diluted to obtain a more precise result. The number of units should be multiplied by the selected dilution.

B. Dosage of anti-Rituximab

- The OD of the standard 1 should be at least 0.8.
- The positive control value should be comprised into the range indicated on the vial label.
- Trace the standard curve (polynomial curve), plotting the units of the 5 standard points (ng/mL) along the abscissa (X axis) and the corresponding OD values along the ordinate (Y axis).
- The anti-Rituximab value can be directly read on the curve.

- Samples with values greater than that of standard 1 may be diluted to obtain a more precise result. The number of units should be multiplied by the selected dilution.

CHARACTERISTICS AND PERFORMANCE OF THE TEST

Limits of detection / threshold values

Estimated on 154 healthy patient samples.

Limit of detection Rituximab	Limit of detection Anti-Rituximab
2 µg/mL > 99th percentile	5 ng/mL > 99th percentile

Assay range

Rituximab	Anti-Rituximab
2 µg/mL - 50 µg/mL	5 ng/mL - 100 ng/mL

Interfering Substances Study

LISA-TRACKER Duo Rituximab (Theradiag) was evaluated to assess potential cross reactivity to other antibodies and interference from serum components (cryoglobulins, rheumatoid factors, heterophilic antibodies, high levels of triglycerides, bilirubin, IgG and/or IgM, and C1q proteins, autoantibodies). No interference was detected.

Precision

	Intra-run (4 tests in a same assay)		Inter-runs (4 tests 4 different assays)	
Parameters	**Mean**	**CV (%)**	**Mean**	**CV (%)**
Rituximab (µg/mL)	48.1	7.6	48.8	4.6
	28.0	4.9	28.1	7.4
	9.6	4.0	10.7	9.8
	3.8	1.4	4.0	5.1
Anti-Rituximab (ng/mL)	98	1.4	96	6.0
	53	1.4	47	10.6
	22	6.8	23	4.5
	12	9.1	12	12.8

LIMITS

Sera of patients treated with Ibritumomab tiuxetan or Tositumomab, 2 others anti-CD20, may cross-react with Rituximab test.

REFERENCES

Magda Nakou, Georgios Katsikas, Prodromos Sidiropoulos, George Bertsias, Eva Papadimitraki, Amalia Raptopoulou, Helen Koutala, Helen A Papadaki, Herakles Kritikos and Dimitrios T Boumpas. Rituximab therapy reduces activated B cells in both the peripheral blood and bone marrow of patients with rheumatoid arthritis: depletion of memory B cells correlates with clinical response. Arthritis Research & Therapy 2009, 11:R131.

Dieter Huhn, Christoph von Schilling, Martin Wilhelm, Anthony D. Ho, Michael Hallek, Rolf Kuse, Wolfgang, Knauf, Ute Riedel, Axel Hinke, Stefanie Srock, Stefan Serke, Christian Peschel and Bertold Emmerich. Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. Blood, 2001; 98: 1326-1331.

Stanley B. Cohen, Paul Emery, Maria W. Greenwald, Maxime Dougados, Richard A. Furie, Mark C. Genovese, Edward C. Keystone, James E. Loveless, Gerd-Rudiger Burmester, Matthew W. Cravets, Eva W. Hessey, Timothy Shaw and Mark C. Totoritis, for the REFLEX Trial Group. Rituximab for Rheumatoid Arthritis Refractory to Anti–Tumor Necrosis Factor. Arthritis Rheum. 2006 Sep;54(9):2793-806.

R. John Looney, Jennifer H. Anolik, Debbie Campbell, Raymond E. Felgar, Faith Young, Lois J. Arend, James A. Sloand, Joseph Rosenblatt and Inaki Sanz. B Cell depletion as a novel treatment for Systemic Lupus Erythematosus : a Phase I/II dose-escalation trial of Rituximab. Arthritis & Rheumatism.Vol. 50, No. 8, August 2004, 2580–2589.

Cartron G, Blasco H, Paintaud G, Watier H, Le Guellec C. Pharmacokinetics of rituximab and its clinical use : Thought for the best use ? Critical Reviews in Oncology/Hematology 62 (2007) 43-52.

Mark S. Cragg, Mike B. Bayne, Alison L. Tutt, Ruth R. French, Stephen Beers, Martin J. Glennie and Timothy M. Illidge. A new anti-idiotype antibody capable of binding rituximab on the surface of lymphoma cells. Blood. 2004;104:2540-2542.

Tobinai K, Y. Kobayashi, M. Narabayashi, M. Ogura, Y. Kagami, Y. Morishima, T. Ohtsu, T. Igarashi, Y. Sasaki, T. Kinoshita, T. Murate. Feasibility and pharmacokinetic study of a chimeric anti-CD20 monoclonal antibody (IDEC-C2B8, rituximab) in relapsed B-cell lymphoma. Annals of Oncology 1998; 9: 527-534.

Ferdinand Breedveld, Sunil Agarwal, Ming Yin, Song Ren, Nicole F. Li, Tim M. Shaw, and Brian E. Davies. Rituximab pharmacokinetics in patients with Rheumatoid Arthritis: B-Cell levels do not correlate with clinical response. Journal of Clinical Pharmacology, 2007; 47:
1119-1128.

Thurlings RM, Teng O, Vos K, Gerlag DM, Aarden L, Stapel SO, JM van Laar, Tak PP, Wolbink GJ. Clinical response, pharmacokinetics, development of human anti-chimaeric antibodies, and synovial tissue response to rituximab treatment in patients with rheumatoid arthritis. Ann Rheum Dis 2010; 69: 409–412.

Schmidt E, Hennig K, Mengede C, Zillikens D, Kromminga A. Immunogenicity of rituximab in patients with severe pemphigus. Clinical Immunology, 2009; 132: 334–341.

Saito K, Nawata M, Iwata S, Tokunaga M, Tanaka Y. Extremely high titer of anti-human chimeric antibody following re-treatment with rituximab in a patient with active systemic lupus erythematosus. Rheumatology (Oxford). 2005 Nov;44(11):1462-4. Epub 2005 Aug 16.

Koren et al. Recommendation on risk-based strategies for detection and characterization of antibodies against biotechnology products. Journal of Immunological Methods, 333 (2008) 1-9.

Mire-Sluis et al. Recommandation for the design and optimization of immunoassays used in the dectection of host antibodies against biotechnology products. Journal of Immunological Methods, 289 (2004) 1-16.

SUMMARY OF METHOD

A) Sample Dilution

Rituximab	Anti-Rituximab
1/1001	1/2

B) Positive Control Dilution

Rituximab	Anti-Rituximab
1/1001	1/2

C) Procedure

Reagents	Procedure
Standards	100µL / well
Diluted positive controls	
Diluted samples	
Incubation	1 h at room temperature
Washing*	Wash 3 times with TDL buffer : 3 x 300µL / well
Biotinylated antibodies	100µL / wells **(specific reagents)**
Incubation	1 h at room temperature
Washing*	Wash 3 times with TDL buffer : 3 x 300µL / well
HRP-Streptavidin	100µL / well
Incubation	30 minutes at room temperature
Washing*	Wash 3 times with TDL buffer : 3 x 300µL / well
Substrate (TMB)	100µL / wells
Incubation	15 minutes at room temperature. Protect from light.
Stop solution	100µL / well

**** Dry the microplate by tapping it gently on a towel to eliminate the excess of liquid.***

D) Configuration of the assays

a. 42 sera for Rituximab and anti-Rituximab

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Standard 5**	Sera 3	Sera 11	Sera 19	Sera 27	Sera 35	**Standard 5**	Sera 3	Sera 11	Sera 19	Sera 27	Sera 35
B	**Standard 4**	Sera 4	Sera 12	Sera 20	Sera 28	Sera 36	**Standard 4**	Sera 4	Sera 12	Sera 20	Sera 28	Sera 36
C	**Standard 3**	Sera 5	Sera 13	Sera 21	Sera 29	Sera 37	**Standard 3**	Sera 5	Sera 13	Sera 21	Sera 29	Sera 37
D	**Standard 2**	Sera 6	Sera 14	Sera 22	Sera 30	Sera 30	**Standard 2**	Sera 6	Sera 14	Sera 22	Sera 30	Sera 30
E	**Standard 1**	Sera 7	Sera 15	Sera 23	Sera 31	Sera 39	**Standard 1**	Sera 7	Sera 15	Sera 23	Sera 31	Sera 39
F	**C+**	Sera 8	Sera 16	Sera 24	Sera 32	Sera 40	**C+**	Sera 8	Sera 16	Sera 24	Sera 32	Sera 40
G	Sera 1	Sera 9	Sera 17	Sera 25	Sera 33	Sera 41	Sera 1	Sera 9	Sera 17	Sera 25	Sera 33	Sera 41
H	Sera 2	Sera 10	Sera 18	Sera 26	Sera 34	Sera 42	Sera 2	Sera 10	Sera 18	Sera 26	Sera 34	Sera 42

Rituximab assay

anti-Rituximab assay

b. 2 sera for Rituximab and anti-Rituximab

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 5	Standard 5										
B	Standard 4	Standard 4										
C	Standard 3	Standard 3										
D	Standard 2	Standard 2										
E	Standard 1	Standard 1										
F	C+	C+										
G	Sera 1	Sera 1										
H	Sera 2	Sera 2										

Rituximab assay	anti-Rituximab assay

SYMBOLS USED

CE	EC Declaration of conformity	▽	Number of test
	ELISA Test		Consult Instructions
REF	Catalogue number		Temperature limitation
LOT	Lot Number		Biological hazard
	Expiry Date	CONT NaN_3	Contains sodium azide
IVD	*In Vitro* Diagnostic Device	RCNS	Reconsitute with
	Manufacturer		Warning

Theradiag

IVD

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