*recovery*ELISA

TNFα Neutralization Rate/Infliximab Kit (RTI)

Instructions

Enzyme immunoassay for the quantitative in-vitro determination of free TNFα, the TNFα neutralization rate and the available therapeutic antibody Infliximab in human serum samples

REF R444

Σ 96

2 °C 8 °C

For research use only
Not for diagnostic use

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Note:

Read the instructions carefully before conducting the test!

Instructions
BioTeZ *recovery*ELISA TNFα Neutralization Rate/Infliximab Kit (RTI)

Introduction

Tumour necrosis factor (TNFα) is a signalling substance of the immune system which can cause, among other things, the formation of inflammatory mediators in cells. In inflammatory diseases such as rheumatoid arthritis, there is overproduction of TNFα, which can be inhibited by using therapeutic antibodies.
Infliximab is an chimeric monoclonal antibody which is used as a TNFα inhibitor. Infliximab binds specifically to the TNFα protein and neutralises the biological effect of TNFα.
Infliximab is approved for the treatment of the following diseases: rheumatoid arthritis, psoriatic arthritis, psoriasis, ankylosing spondylitis, Crohn's disease and ulcerative colitis.
In therapy using Infliximab, contraindications such as hypersensitivity to Infliximab and also serious infections, sepsis, tuberculosis and cases of opportunistic infections have been noted. Weakening of the body's defence is produced by the immunosuppressive effect of Infliximab. Excessive neutralization of TNFα during treatment with Infliximab increases the risk of developing opportunistic infections.
In view of this, monitoring the treatment antibody levels and degree of neutralization of TNFα would appear useful during therapy using the TNFα inhibitor Infliximab.
In comparison with the tests available on the market, the *recovery*ELISA TNFα Neutralization Rate/Infliximab (RTI) Kit can be used to simultaneously determine the free TNFα target antigen, the available therapeutic antibody Infliximab and its ability to neutralise TNFα.

Intended use

The *recovery*ELISA RTI is an in-vitro diagnostic agent for the simultaneous quantitative determination of free TNFα, the TNFα neutralization rate (Infliximab activity) and the available therapeutic antibody Infliximab in human serum samples. It consists of a manual, non-automated kit for the determination of 7 samples.

User community

The test is intended for doctors and specialist personnel in clinical chemical laboratories with experience of conducting immunoassays. The test is intended for research purposes only.

Methodology

The *recovery*ELISA (Enzyme-Linked Immunosorbent Assay) is an immunological quantitative detection method based on a sandwich ELISA. In comparison to a classic sandwich ELISA, a two-dimensional calibration is carried out for *recovery*ELISA obtaining two analysis results within the same assay. The following calibrations are performed:

1. TNFα levels without and with additional Infliximab against extinction (optical density)
2. Infliximab levels against TNFα recovery

*Recovery*ELISA is performed in a 96-well microplate. The wells of the microplate are pre-coated with a specific capture antibody against human TNFα that binds free TNFα from the patient sample. There occurs a simultaneous incubation of the TNFα calibrators (with and without the addition of Infliximab) of the samples and of the detection conjugate (peroxidase conjugate of an anti-TNFα antibody). Incubation takes place over 16 to 22 hours at 2 to 8°C. After washing, the colour substrate TMB (tetramethylbenzidine) is added to the wells. After incubation the enzymatic colour reaction is stopped using a sulphuric acid solution. A colour change from blue to yellow occurs that is optically detected. Using a wavelength of 450 nm (reference value 620 nm), the optical density (OD) of the reaction product is measured with a suitable microplate reader.
With the aid of the two calibrations to be performed, an evaluation procedure can be carried out to determine the concentrations of free TNFα and Infliximab in the patient samples using the measured OD values. Determination of the therapeutic antibody is based on the principle that the presence of Infliximab in patient samples leads to a systematic reduction in the recovery of TNFα. The evaluation is performed using non-linear regression (Marquardt-Levenberg algorithm), the Michaelis-Menten model from enzyme kinetics and the Langmuir isotherm from surface binding.

Kit components RTI

No.	Components	Marking	Volume	Colour of top cap	Con-dition **
1	Microplate coated with anti-TNFα antibody	**RT-PLATE**	12 Strips á 8 well	-	G
2	Sample dilution buffer	**RTI-SAMPLE-BUF**	1 x 6.0 mL	colourless	G
3	TNFα concentrate (2000 ng TNF/ml)*	**RT-TNF-CONC**	1 x 0.15 mL	black	R
4	TNFα dilution buffer	**RT-TNF-DILUTION-BUF**	1 x 7.0 mL	blue	G
5	Therapy antibody calibrator 0 (0 µg Infliximab/mL)	**RTI-TAK 0**	1 x 0.8 mL	yellow	G
6	Therapy antibody calibrator 1 (0,2 µg Infliximab/mL)	**RTI-TAK 1**	1 x 0.8 mL	yellow	G
7	Therapy antibody calibrator 2 (0,6 µg Infliximab/mL)	**RTI-TAK 2**	1 x 0.8 mL	yellow	G
8	Therapy antibody calibrator 3 (2,4µg Infliximab/mL)	**RTI-TAK 3**	1 x 0.8 mL	yellow	G
9	Control	**RTI-CONTROL**	1 x 0.8 mL	red	G
10	Anti-TNF antibody HRP conjugate	**RTI-CONJ**	1 x 6.0 mL	green	G
11	TMB reagent	**RT-TMB**	1 x 11.0 mL	brown	G
12	Stopping solution (1M H_2SO_4)	**RT-STOP-H_2SO_4**	1 x 3.0 mL	colourless	G
13	Washing buffer concentrate (10x)	**RT-WASHBUF 10x**	1 x 30.0 mL	colourless	R
14	Cover sheeting for microplate	**-**	1 piece	-	G

*** Concentration of TNFα is calibrated against WHO Reference Reagent**
**** Condition: G = ready for use; R = reconstitution required**

Test procedure

The test sequence comprises 2 reaction steps. A washing stage must be performed between these reaction steps:

Reaction step 1: Incubation of the following assay components:
anti-TNF antibody HRP conjugate **RTI-CONJ**, calibrators **RTI-TNF-CAL 0 bis 4** and **RTI-TAK 0 bis 3**, samples 1-7, control **RTI-CONTROL**

Washing stage: 1:10 diluted **RT-WASHBUF 10x**

Reaction step 2: Colour reaction with the components **RT-TMB** and **RT-STOP-H_2SO_4**

Once the kit reagents have been prepared and brought to temperature for reaction step 1 and following completion of the dilution of the samples, the assay is pipetted according to the microplate schematic for TNFα calibrators, samples, control and therapeutic antibody calibrators.
The wells A1-H4 of the microplate are designated for the calibration curve with TNFα calibrators **RTI-TNF-CAL 0 to 4** and the therapy antibody calibrators **RTI-TAK 0 to 3**. The samples are pipetted in rows into wells A5-H12 (8 wells per sample) and topped up with the TNFα calibrators **RTI-TNF-CAL 0, 1, 2 and 4**. Repeat determinations are performed for each calibrator.
1x 50 µL and 2x 25 µL of the specified solutions are pipetted into each well with a Multipette and a Combitip with pipette tip.

Layout of the microplate

	Strip	1	2	3	4	5	6	7	8	9	10	11	12	
Row	RTI-TAK calibrators	Field of calibrators Strip 1-4 TNFα calibrators				Field of samples Strip 5-12 Topping up the samples with TNFα calibrators								Samples 1:10 diluted
		RTI-TNF-CAL 0	RTI-TNF-CAL 1	RTI-TNF-CAL 2	RTI-TNF-CAL 3	RTI-TNF-CAL 4		RTI-TNF-CAL 2		RTI-TNF-CAL 1		RTI-TNF-CAL 0		
A	RTI-TAK 0													Sample 1
B														Sample 2
C	RTI-TAK 1													Sample 3
D														Sample 4
E	RTI-TAK 2													RTI-CONTROL
F														Sample 5
G	RTI-TAK 3													Sample 6
H														Sample 7

The kit is delivered cooled and must be stored until ready for use without any interruption of the cold chain at a temperature of 2 to 8 ° C.
All kit components are designed for single use only.

Other materials and equipment required

Multipette and Combitips, 10-100 µL and 100-1000 µL pipettes and tips, timer, measuring cylinder, distilled water, vortexer, microplate shaker, washing device for microplates (where available), microplate reader (450 nm / 620 nm), computer with Microsoft Excel, version from 2003 and **evaluation software** ©***recovery*ELISA TNFα Neutralization Rate/Infliximab**, download from www.biotez.de

Preparation of Samples

This test must only be used for human blood serum. The blood may be collected with any ordinary serum collecting tubes (e.g. Vacutainer, Sarstedt serum monovette). The blood sample should be left to stand for at least 20 minutes, but for no longer than one hour, to ensure the full coagulation of the sample. The blood samples should always stand dark. After centrifugation (e.g. 10 mins at 1500 g, or see the table for the rpm for the centrifuge used), transfer the supernate obtained (serum) into a neutral test tube. Please do not forget to give all samples a unique identification, even where used in your own laboratory, i.e. each should be labelled in such a way as to prevent confusion. It is recommended that you note the date and time of collection, so that should discrepancies later occur, you are able to ensure traceability.
Haemolytic or lipaemic sera may not be used.

When the sera can not be tested within 2 hours, so they must be kept in the dark at a storage temperature of -20 ° C in portions (servings recommended 100-200µL).
You should, however, avoid repeatedly freezing and thawing the samples. Before conducting the tests, allow the serum samples to reach room temperature (20-25 °C) and mix them well. This kit allows 7 samples (in repeat determination) to be measured.
The samples should generally be diluted 1:10 with the sample diluent **RTI-SAMPLE-BUF** before performing the test as follows: 60 µL human serum sample and 530 µL **RTI-SAMPLE-BUF**.

Test sequence: *Steps A to I*

A. Preparation of the kit

The following reagents should be brought to room temperature (20-25 °C) before use in reaction step 1.

- 1 microplate **RT-PLATE**
- 1 sample dilution buffer **RTI-SAMPLE-BUF**
- 1 Anti-TNF antibody HRP conjugate **RTI-CONJ**
- 1 TNFα concentrate **RT-TNF-CONC**
- 1 TNFα dilution buffer **RT-TNF-DILUTION-BUF**
- 4 calibrators **RTI-TAK 0,1,2,3**
- 1 control **RTI-CONTROL**

The reagents **RTI-WASHBUF 10x, RT-TMB und RT-STOP-H_2SO_4** are required on the second day and must be stored at 2-8 ° C.

B. Preparation of Combitips with pipette tips

1 Combitip with tip for 50 µL pipetting
17 Combitips with tip for 25 µL pipetting

C. Diluting the samples

Dilute the serum samples 1:10 with the diluent **RTI-SAMPLE-BUF**. Each 60 µL serum sample should be diluted with 530 µL **RTI-SAMPLE-BUF**.

D. Reconstitution of RT-TNF-CONC with RT-TNF-DILUTION-BUF and preparation of RTI-TNF-CAL 0-4

The TNFα concentrate **RT-TNF-CONC** must be reconstituted with reagent TNFα dilution buffer **RT-TNF-DILUTION-BUF** immediately before use. Prepare the **RTI-TNF-CAL 0-4** as follows:

At first, provide 4 vessels for the preparation of **RTI-TNF-CAL 1-4**. Pipette the following volume of **RT-TNF-DILUTION-BUF** in the corresponding bottles:

Reagent	Volume of **RT-TNF-DILUTION-BUF** [µl]
RTI-TNF-CAL 3 (32 ng/ml)	**2460**
RTI-TNF-CAL 4 (24 ng/ml)	**500**
RTI-TNF-CAL 2 (16 ng/ml)	**600**
RTI-TNF-CAL 1 (8ng /ml)	**600**

Start the dilution as follows:

1. Pipette 40 µL **RT-TNF-CONC** into the reagent **RTI-TNF-CAL 3 (32ng/ml)** and mix.

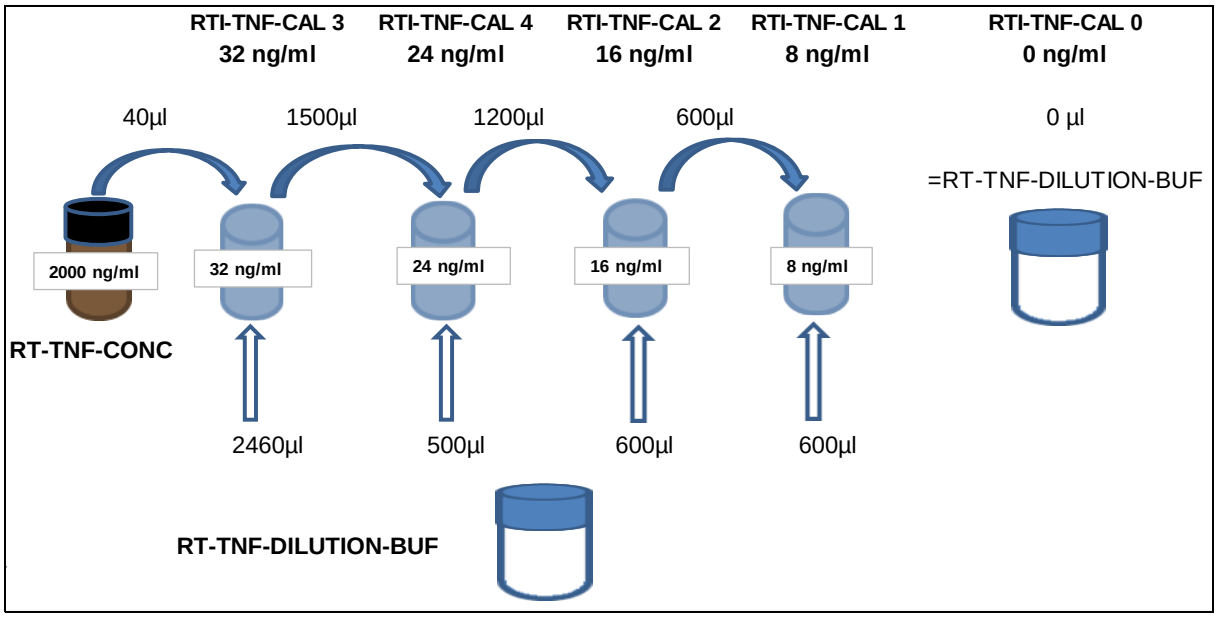
2. Pipette 1500 µL **RTI-TNF-CAL 3 (32ng/ml)** into the reagent **RTI-TNF-CAL 4 (24ng/ml)** and mix.

3. Pipette 1200 µL **RT-TNF-CAL 4 (24ng/ml)** into the reagent **RTI-TNF-CAL 2 (16ng/ml)** and mix.

4. Pipette 600 **µL RTI-TNF-CAL 2 (16ng/ml)** into the reagent **RTI-TNF-CAL 1 (8ng/ml)** and mix.

The calibrators **RTI-TNF-CAL 1, 2, 3** and **4** are now ready to use immediately. They can not be stored. The **RT-TNF-DILUTION-BUF** serves as the calibrator **RTI-TNF-CAL 0** (0ng/ml) and is ready to use.

Scheme of Dilution Procedure:



E. Microplate

The microplate **RT-PLATE** should be removed from its packaging immediately before start of pipetting.

F. Pipetting

The entire pipetting process should be carried out within a time of not more than 20 minutes.

Note: The individual pipetting steps 1 to 12 must be followed exactly as described below. Any non-observance of these instructions will compromise the accuracy of the assay and the software is likely to deliver false results in the evaluation.

Pipette carefully into the middle of the wells. Always use a Combitip with tip!

1. Pipette 50 µL Anti-TNFα antibody HRP conjugate **RTI-CONJ** into each of the 96 wells (A1-H12) of the **RT-PLATE.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

2. Pipette 25 µL therapy antibody calibrator 0 **RTI-TAK 0** into the 8 wells A1 - A4 and B1 - B4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

3. Pipette 25 µL Therapy antibody Calibrator 1 **RTI-TAK 1** into the 8 wells C1 - C4 und D1 - D4 pipettieren.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

4. Pipette 25 µL Therapy antibody Calibrator 2 **RTI-TAK 2** into the 8 wells E1 - E4 und F1 - F4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

5. Pipette 25 µL Therapy antibody Calibrator 3 **RTI-TAK 3** into the 8 wells G1 - G4 und H1 - H4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

6. Pipette 25 µL of a 1:10 dilution of each sample into 8 wells by row.
   Sample 1: A5-A12;
   Sample 2: B5-B12;
   Sample 3: C5-C12;
   Sample 4: D5-D12;
   Sample 5: F5-F12;
   Sample 6: G5-G12;
   Sample 7: H5-H12
   Note: The wells E5-E12 are reserved for the control value and must not be used for the determination of samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

7. Pipette 8x 25 µL of the control **RTI-CONTROL** into the 8 wells E5-E12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

8. Pipette 25 µL **RT-DILUTION-BUF**= **RTI-TNF-CAL 0** into the 8 wells A1 - H1 and into the 16 wells A11 – H11 and A12 – H12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

9. Pipette 25 µL **reconstituted** TNFα calibrator 1 **RTI-TNF-CAL 1** into the 8 wells A2 - H2 and into the 16 wells A9 – H9 and A10 – H10.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

10. Pipette 25 µL **reconstituted** TNFα calibrator 2 **RTI-TNF-CAL 2** into the 8 wells A3 - H3 and into the 16 wells A7 – H7 and A8 – H8.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

11. Pipette 25 µL **reconstituted** TNFα calibrator 3 **RTI-TNF-CAL 3** into the 8 wells A4 - H4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

12. Pipette 25 µL **reconstituted** TNFα Calibrator 4 **RTI-TNF-CAL 4** into the 16 wells A5 – H5 and A6 – H6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

G. Incubation

Cover the microplate with adhesive film and briefly agitate on a microplate shaker. Cover with a light-tight covering and incubate in the dark in a refrigerator at 2-8 °C for 16 hours (overnight; minimum 16 h, maximum 22 h).

H. Washing step after at least 16 h incubation at +2-8 °

The washing buffer concentrate (10x) **RT-WASHBUF10x** must be brought to room temperature before use and then diluted with distilled water 1:10 as follows: to 25 mL **RT-WASHBUF 10x** add distilled water to 250 mL volume = **reconstituted washing solution.**

After incubation remove the microplate from the refrigerator and wash 3x with 300 µL **reconstituted washing solution** per well by hand (with Multipette and Combitip) or wash with washing device for microplates. Lightly tap off excess liquid.

I. Enzymatic colour reaction

1. Pipette 100 µL TMB substrate **RT-TMB** into each well with Multipette and Combitip + tip.
2. Cover microplate again with adhesive film and briefly agitate on the microplate shaker.
3. Incubate for 30 min at room temperature, keeping the microplate in the dark.

4. To halt incubation, pipette 25 µL stop solution **RT-STOP-H_2SO_4** into each well using Multipette and Combitip + tip. Briefly agitate on the microplate shaker.
5. Measure the OD values of the 96 wells using a microplate reader at 450 nm (reference wavelength 620 nm).

Test evaluation

The measured values are evaluated using the evaluation software ©***recovery*ELISA TNFα Neutralization Rate/Infliximab** (Download from the homepage www.biotez.de).

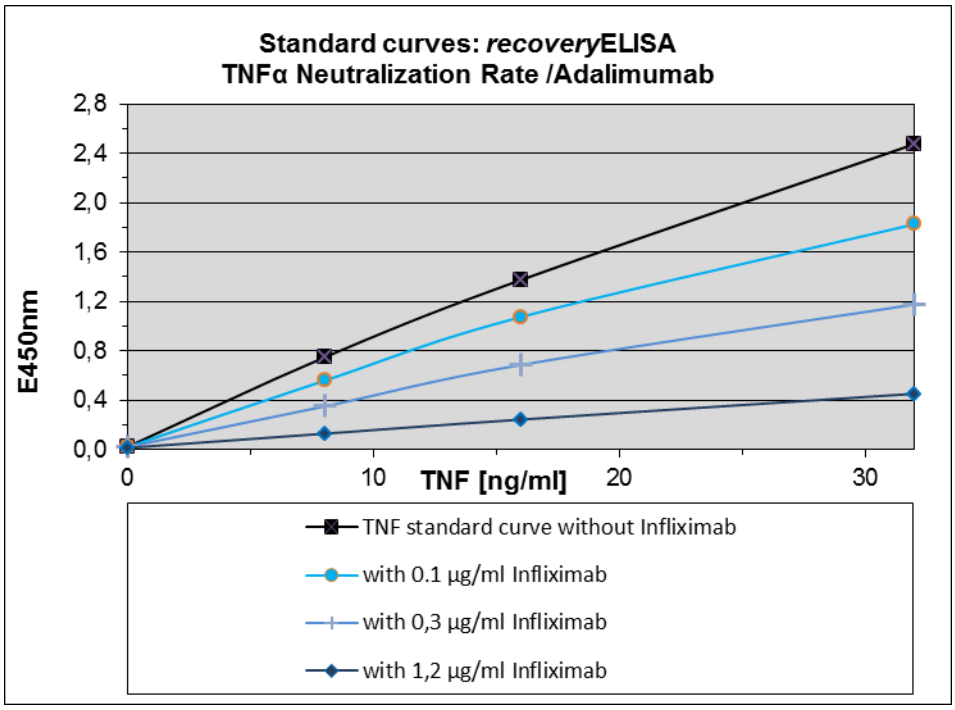
Calculation steps from the extinction values to the determining of the free TNFα, the available therapeutic antibody Infliximab content in the serum, TNFα recovery and TNFα neutralization rate

- Calculation of average values of all repeat determinations
- Correction of average values by unspecific binding
- Determination of the TNFα therapeutic antibody reference curve for the relationship of RTI-TNF-CAL 0-3 and RTI-TAK 0 using non-linear regression
- Calculation of antigen concentration and the average recovery of the antigen from the corrected extinction via reference curve
- Determination of the assay-specific recovery curve for Infliximab with reference to the average recovery of the antigen in standard curves
- Calculation of Infliximab content in the samples with reference to the recovery curve
- Calculation of the **TNFα recovery (%)**
- Calculation of the **TNFα neutralization rate (%)**
- Conversion of the TNFα and Infliximab levels to undiluted samples

The extinctions obtained from the reader file of the microplate reader should be transferred to the input template of the evaluation software ©***recovery*ELISA TNFα Neutralization Rate/Infliximab** by means of Copy and Paste. The output of measurement values for TNFα and Infliximab can then be seen on the program page “Evaluation”.
The TNFα level is given in ng/mL. The Infliximab level is given in µg/mL
Other impoRTInt results of recoveryELISA include determining the "TNFα recovery (%)" and "TNFα neutralization rate”.
"**TNFα recovery**” refers to the TNFα recovery in *recovery*ELISA. This value is determined using the standard addition of TNFα (TNFα increase) for the serum sample. The value is shown in %.
The "**TNFα neutralization rate**" is calculated based on the difference between the "**TNFα recovery** " and 100%. This value refers to the serum sample and indicates the percentage of TNFα bound by the therapeutic antibody at the time of blood collection.

Typical standard curves with TNF and Infliximab calibrators:

	TNF concentration [ng/ml]			
Infliximab [µg/ml]	**0**	**8**	**16**	**32**
0	0.023	0.748	1.376	2.477
0.1	0.022	0.559	1.076	1.828
0.3	0.023	0.351	0.687	1.177
1.2	0.015	0.130	0.244	0.453



Important information for supervising doctors:

The *recovery*ELISA TNFα Neutralization Rate/Infliximab Kit (RTI) delivers the parameters mentioned above. The test result does not represent a recommendation for adjusting doses or treatment. Evaluation of the test result is the responsibility of the supervising doctor.

Capability characteristics of the method

Measurement range

The *recovery*ELISA TNFα Neutralization Rate/Infliximab Kit (RTI) has the following measurement range:

Analyt		**Measurement Range** (for undiluted sample)
TNFα	Samples without Infliximab	2 – 400 ng/mL
	Samples with Infliximab	2 – 160 ng/mL
Infliximab		0.1 – 15 µg/mL

Please note, that samples are routinely measured with recoveryELISA RTI in a 1:10 dilution. Samples whose TNFα or Infliximab concentrations lie above the measurement ranges should be further diluted and tested again. However, a maximum dilution of 1:40 should not be exceeded to prevent errors occurring in the assay. The dilutions of the sample should be carried out as follows:

Dilution 1:20: Dilute 30 µL of human serum sample in 570 µL **RTI-SAMPLE-BUF**.
Dilution 1:30: Dilute 20 µL of human serum sample in 580 µL **RTI-SAMPLE-BUF**.
Dilution 1:40: Dilute 20 µL of human serum sample in 780 µL **RTI-SAMPLE-BUF**.

Precision

Intra-Assay Variance (3 tests in 7fold determination)

Sample	Mean Value TNFα (ng/mL)	CV (%)	Mean Value Infliximab (µg/mL)	CV (%)
serum, undiluted (after recalculation)				
1	< 2	-	5.7	4.9
2	153.0	2.2	< 0.1	-

Inter-Assay Variance (3 tests in 7fold determination)

Sample	Mean Value TNFα (ng/mL)	CV (%)	Mean Value Infliximab (µg/mL)	CV (%)
serum, undiluted (after recalculation)				
1	< 2	-	5.6	5.1
2	153.0	3.9	< 0.1	-

Linearity

The assay should be conducted with a serum dilution of 1:10. For dilutions of 1:20 to 1:40, the linearity of the assay results to their dilution is assured.

Specificity

The *recovery*ELISA RTI detects specifically human TNFα. Cross-reactions with other serum proteins cannot be verified in physiological concentrations.

References

1. Strohner P: Patent: EP 1957980B1
Immunoassay for the simultaneous immunochemical determination of an analyte (antigen) and a treatment antibody targeting the analyte in samples (Recovery immunoassay)

2. Strohner P, Sarrach D, Reich JG, Becher G, Staatz A, Schäfer A, Steiß J-O, Häupl T:
The Recovery-ELISA – a novel Assay Technique for the Therapy Control of Therapeutic Antibodies
Am J Respir Crit Care Med 2010;181:A5674

3. Strohner P, Staatz A, Schäfer A, Becher G, Sarrach D, Reich JG, Steiß J-O, Häupl T:
Monitoring of Monoclonal Antibody Therapy with a new Recovery ELISA Assay Technique (R-ELISA®)
ERS Barcelona 2010, Abstract-No: 3399

4. Strohner P, Staatz A, Sarrach D, Steiß J-O, Becher G:
The *recovery*ELISA – a newly devloped immunoassay for measurement of therapeutic antibodies and the target antigen during antibody therapy
Clin Chem Lab Med 2012;50(7):1263-1269

Important points and precautions

1. This kit has been manufactured in compliance with the EU in-vitro diagnostic guidelines (98/79/EG). The data provided by the manufacturer, particularly concerning its use, user community and intended purpose, must be strictly observed. The test must be conducted solely in accordance with these instructions, which contain the necessary instructions for use and warnings. Any modification to the test not authorised by the manufacturer, including with regard to its procedure or the reagents and materials used, is prohibited.
2. The manufacturer assumes no liability and indicates that the user is solely responsible for the consequences of any alterations made, for non-observance of instructions or for performing the tests without paying due attention.
3. The equipment used must be maintained in accordance with the manufacturers' instructions and any applicable guidelines. Before equipment is used it should be checked for fault-free operation.
4. The materials and reagents included in the kit are intended for single use only. Excess material and materials and reagents that have exceeded their expiry date/lifetime should be disposed of correctly. You should observe the regulations that apply to you.
5. Do not perform the test if the packaging or contents are damaged.
6. The test may only be carried out by trained specialists. Pregnant women should not perform the test.
7. The test is validated for use at room temperature (20 to 25°C), where the incubation following the first reaction step is carried out at a temperature of 2 to 8°C. Deviations in the climatic conditions can negatively influence the results.
8. You should exclusively use the materials and reagents included in the kit. Do not mix these with materials and reagents from other kits, even where such kits are from the same manufacturer and for the same purpose. Similarly, the use of materials and reagents from other manufacturers instead of those contained in the kit is forbidden.
9. Ensure that the materials, equipment and reagents are clean, paying particular attention e.g. to sample vessels and pipette tips.
10. Before use, check the materials and reagents for any visible contamination.
11. Observe general health and safety regulations.
12. Follow the instructions for this test very closely. The washing procedure, in particular, represents a source of error if the washing is not performed adequately.
13. Pipette carefully into the wells (always use a Combitip with tip), as otherwise excessive deviations in the results may occur.
14. The kit contains material of animal origin that should be regarded as potentially infectious. You should therefore observe the appropriate protection regulations concerning the handling and disposal of these materials. In the event of injury a medical specialist should always be consulted.
15. Note that waste that contains serum should be collected and disposed of separately. Pay attention to the regulations that apply to you. Disinfect thoroughly.
16. The kit contains substances such as 1M sulphuric acid, ProClin 300 (maximum 0.05%) as a preservative in reagents and TMB that are corrosive and/or toxic. Avoid contact with eyes and skin! Wear protective gloves!
17. Observe the following safety information as per the EU regulation no. 1907/2006 when handling 1M sulphuric acid "RT-STOP-H_2SO_4": Wear protective gloves.
    In the event of contact with the eyes: rinse carefully with water for several minutes. Remove contact lenses if possible. Rinse again. If irritation persists, seek medical advice/attention. In case of contact with skin: wash with plenty of soap and water.
    Classification of risk according to EU regulation no. 1272/2008: can be corrosive to metals. Causes skin irritation. Causes eye irritation.
18. When disposing of the materials and reagents, observe any potential harm they may cause to the environment. Observe the regulations that apply to you.
19. Observe the fundamentals of good diagnostic laboratory practice.
20. Observe safety regulations, e.g. do not eat, drink or smoke in the workplace; keep materials and reagents away from foods and feeding stuffs; wear protective clothing (lab coat, safety glasses and gloves).
21. Never carry out pipetting operations using the mouth; always use suitable equipment or devices.
22. In the event of a warranty claim the entire kit should be returned to the manufacturer, BioTeZ Berlin-Buch GmbH, within 14 days with a written explanation.

Symbols:

European conformity	For in-vitro-diagnostic use	Manufactured by	Catalog Number	Lot Number	Do not reuse	Use before	Consult instructions for use	Refer accompanying documents	Sufficient for <n> tests	Tempe-rature limit
										

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