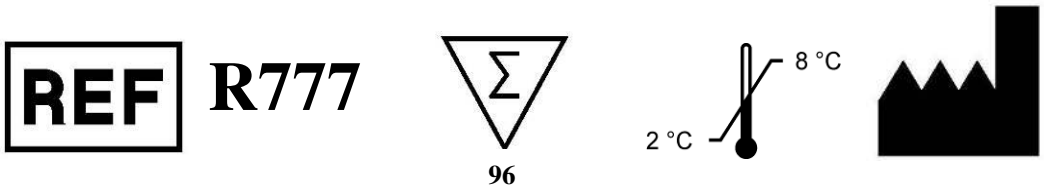
*recovery*ELISA

PCSK9 Neutralization Rate/Evolocumab Kit (RPE)

Instruction

Enzyme immunoassay for the quantitative in-vitro determination of free PCSK9, the PCSK9 neutralization rate and the free therapeutic antibody Evolocumab in human serum samples



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Note: Read the instruction carefully before conducting the test!

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has medical importance because it interferes with the lipoprotein in particular the lipoprotein particles (LDL) homeostasis. PCSK9 binds to the lipoprotein particles receptor (LDLR) which transports fat molecules within the extracellular fluid. After LDL binding to the LDL receptor the complex of LDLR/PCSK9/LDL gets degraded in the hepatocytes upon internalization. Importantly, if PCSK9 does not bind to the complex, the LDLR can return to the surface of the cell and can thereby continue to remove LDL-particles from the bloodstream.
Agents which block PCSK9 can lower LDL particle concentrations in the blood. The therapeutically monoclonal antibody against PCSK9 Evolocumab, was approved by the U.S. Food and Drug Administration in 2015 for lowering LDL-particle concentrations when statins and other drugs were not sufficiently effective or poorly tolerated.
To monitor the therapeutic antibody (Evolocumab) concentration and the degree of neutralization of PCSK9 during Evolocumab therapy is beneficial to identify the ideal drug does for each patient individually. The *recovery*ELISA (RPE) allows the simultaneously determination of the free PCSK9 and the free Evolocumab in one ELISA assay.

Intended use

The *recovery*ELISA RPE kit allows the simultaneous quantitative determination of free PCSK9, the PCSK9 neutralization rate and the available therapeutic antibody Evolocumab in human serum samples. This test should only be used for patients treated with Evolocumab as mono biological therapy. It is a manual, non-automated kit for the determination of 7 samples.

User

The test is intended for doctors and specialist personnel 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 contrast to a classic sandwich ELISA, a two-dimensional calibration is carried out for *recovery*ELISA.

The following calibrations are performed:

1. PCSK9 levels without and with additional Evolocumab against extinction (optical density)
2. Evolocumab levels against PCSK9 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 PCSK9 that binds free PCSK9 from the patient sample. A simultaneous incubation of the PCSK9 calibrators (with and without the addition of Evolocumab), the samples and the detection conjugate (Evolocumab peroxidase conjugate) is carried out. The incubation time is 16 to 22 hours at 2 to 8°C. After washing, the colour substrate TMB (tetramethylbenzidine) is added to the wells. The enzymatic colour reaction is stopped using a sulphuric acid solution. A colour change from blue to yellow 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, an evaluation procedure can be carried out to determine the concentrations of free PCSK9 and Evolocumab in the patient samples using the measured OD values. Determination of the therapeutic antibody is based on the principle that the presence of Evolocumab in patient samples leads to a systematic reduction in the recovery of PCSK9. The evaluation is performed using non-linear regression (Marquardt-Levenberg algorithm) of a logarithmic model from enzyme kinetics and the Langmuir isotherm from surface binding.

Kit components RPE

No.	Components for Day 1	Marking	Volume	Coloured cap	Condition *
1	Microplate coated with anti-PCSK9 antibody	**RPE-PLATE**	12 x 8 wells	-	G
2	Sample dilution buffer	**RPE-SAMPLE-BUF**	1 x 6.0 mL	colourless	G
3	PCSK9 concentrate, lyophilized (300 ng rec. human PCSK9)	**RPE-PCSK9-CONC-L**	1 x	purple	**R**
4	PCSK9 dilution buffer	**RPE-PCSK9-DILUTION-BUF**	1 x 6.0 mL	blue	G
5	Therapy antibody calibrator 0 (0 µg Evolocumab/mL)	**RPE-TAK 0**	1 x 0.8 mL	yellow	G
6	Therapy antibody calibrator 1 (0.2 µg Evolocumab/mL)	**RPE-TAK 1**	1 x 0.8 mL	yellow	G
7	Therapy antibody calibrator 2 (0.5 µg Evolocumab/mL)	**RPE-TAK 2**	1 x 0.8 mL	yellow	G
8	Therapy antibody calibrator 3 (1.5µg Evolocumab/mL)	**RPE-TAK 3**	1 x 0.8 mL	yellow	G
9	Control	**RPE-CONTROL**	1 x 0.8 mL	red	G
10	Anti-PCSK9 antibody HRP conjugate concentrate	**RPE-CONJ-CONC**	1 x 0.08 mL	black	**R**
10	Anti-PCSK9 antibody HRP conjugate dilution buffer	**RPE-CONJ-BUF**	1 x 6.0 mL	green	G
11	Cover for microplate	**-**	1 piece	-	G
	Components for Day 2				
12	TMB reagent	**TMB**	1 x 12.0 mL	brown	G
13	Stopping solution (1M H_2SO_4)	**STOP-H_2SO_4**	1 x 4.0 mL	colourless	G
14	Washing buffer concentrate (10x)	**WASHBUF 10x**	1 x 30.0 mL	colourless	**R**

*** Condition: G = ready to use; R = reconstitution (dilution) required**

Test procedure

The test comprises 2 reactions and a washing step must be performed between these reactions:

Reaction 1: Incubation of the following assay components:
pre-prepared diluted RPE conjugate solution (in bottle **RPE-CONJ-BUF**),
pre-prepared calibrators **RPE-PCSK9-CAL 0 to 4** and **RPE-TAK 0 to 3**,
samples 1-7, control **RPE-CONTROL**

Washing step: 1:10 diluted **WASHBUF 10x**

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

Once the kit reagents have been prepared (at room temperature) reaction 1 can be pipetted according to the microplate scheme for PCSK9 calibrators, samples (diluted), control and therapeutic antibody calibrators.
The wells A1-H4 of the microplate are designated for the calibration curve with PCSK9 calibrators **RPE-PCSK9-CAL 0 to 4** and the therapy antibody calibrators **RPE-TAK 0 to 3**. The samples are pipetted in rows into wells A5-H12 (8 wells per sample) and topped up with the PCSK9 calibrators **RPE-PCSK9-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.

Attention: Pipetting of solutions with Multipette, Combitip and pipette tip

A short movie with more pipetting and assay execution details we can send to you on request. Please contact us via info@biotez.de

Layout of the microplate

	Strip	1	2	3	4	5	6	7	8	9	10	11	12	
		Field of calibrators Strip 1-4 *PCSK9 calibrators*				**Field of samples Strip 5-12 Topping up the samples with** *PCSK9 calibrators*								
	calibrators	**RPE-PCSK9-CAL 0**	**RPE-PCSK9-CAL 1**	**RPE-PCSK9-CAL 2**	**RPE-PCSK9-CAL 3**	**RPE-PCSK9-CAL 4**		**RPE-PCSK9-CAL 2**		**RPE-PCSK9-CAL 1**		**RPE-PCSK9-CAL 0**		**Samples**
Row	***RPE-TAK***	**0 ng/ml**	**15 ng/ml**	**30 ng/ml**	**60 ng/ml**	**45 ng/ml**		**30 ng/ml**		**15 ng/ml**		**0 ng/ml**		**1:10 diluted**
A	**RPE-TAK 0**													Sample 1
B	**0.0 µg/ml**													Sample 2
C	**RPE-TAK 1**													Sample 3
D	**0.2 µg/ml**													Sample 4
E	**RPE-TAK 2**													**RPE-CONTROL**
F	**0.5 µg/ml**													Sample 5
G	**RPE-TAK 3**													Sample 6
H	**1.5 µg/ml**													Sample 7

The kit is delivered cooled and must be stored until use without 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 required equipment

Multipette and combitips, 3 ml container, 10-100 µL and 100-1000 µL pipettes and tips, timer, measuring cylinder, distilled water, vortexer, microplate shaker, washing device for microplates (if available),

microplate reader (450 nm / 620 nm), computer with Microsoft Excel and **evaluation software ©*recovery*ELISA PCSK9 Neutralization Rate/Evolocumab**, download from www.biotez.de.

Sample Preparation

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 stand for at least 20 minutes, but not longer than one hour at room temperature to ensure the full coagulation of the sample. If the sera can not be tested within 2 hours, store the sera in aliquotes (recommended volume 100 to 200µL) at -80 ° C. However avoid repeatedly freezing and thawing the samples.
Haemolytic or lipaemic sera may not be used. After centrifugation (10 min at 1500 g), transfer the supernatant obtained (serum) into a neutral test tube and store at 2 - 8 °C. Please give all samples a unique identification.
Before conducting the tests, allow the serum samples to reach room temperature (20-25 °C) and mix well. The samples should generally be diluted 1:10 with the sample diluent **RPE-SAMPLE-BUF** before performing the test as follows: Use 60 µL human serum sample and add 540 µL **RPE-SAMPLE-BUF.**

Work Flow: *Steps A to I*

A. Preparation of the kit

The following reagents should be at room temperature (20-25 °C) before use.

- 1 microplate **RPE-PLATE**
- 1 sample dilution buffer **RPE-SAMPLE-BUF**
- 1 Anti-PCSK9 antibody HRP conjugate concentrate **RPE-CONJ-CONC**
- 1 Anti-PCSK9 antibody HRP conjugate dilution buffer **RPE-CONJ-BUF**
- 1 PCSK9 concentrate, lyophilized **RPE-PCSK9-CONC-L**
- 1 PCSK9 dilution buffer **RPE-PCSK9-DILUTION-BUF**
- 4 calibrators **RPE-TAK 0,1,2,3**
- 1 control **RPE-CONTROL**

The reagents **WASHBUF 10x, TMB und 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 **RPE-SAMPLE-BUF**. Each 60 µL serum sample should be diluted with 540 µL **RPE-SAMPLE-BUF**.

D. Preparation of RPE-PCSK9-CAL 0-4

Please prepare the **RPE-PCSK9-CAL 0-4** directly before use.

1. Dilution of the lyopilisate **RPE-PCSK9-CONC-L**:
The vial **RPE-PCSK9-CONC-L** contains 300 ng of lyophilized recombinant human PCSK9. Add 1 ml destilled water to the standard tube **RPE-PCSK9-CONC-L** and allow the contents to dissolve for 5-10 minutes. Gently mix, but avoid foaming of the reagent! After the dilution the PCSK9 concentration is 300 ng/ml in this vial.

2. Mix the reagents diluted **RPE-PCSK9-CONC-L** and **RPE-DILUTION-BUF** in the following way:

At first, provide 4 containers for the preparation of RPE-PCSK9-CAL 1-4. Pipette the following volume of **RPE-PCSK9-DILUTION-BUF** in the corresponding bottles:

Reagent	Volume of **RPE-PCSK9-DILUTION-BUF** [µl]
RPE-PCSK9-CAL 3 (60 ng/ml)	**2000**
RPE-PCSK9-CAL 4 (45ng/ml)	**500**
RPE-PCSK9-CAL 2 (30 ng/ml)	**500**
RPE-PCSK9-CAL 1 (15ng/ml)	**500**

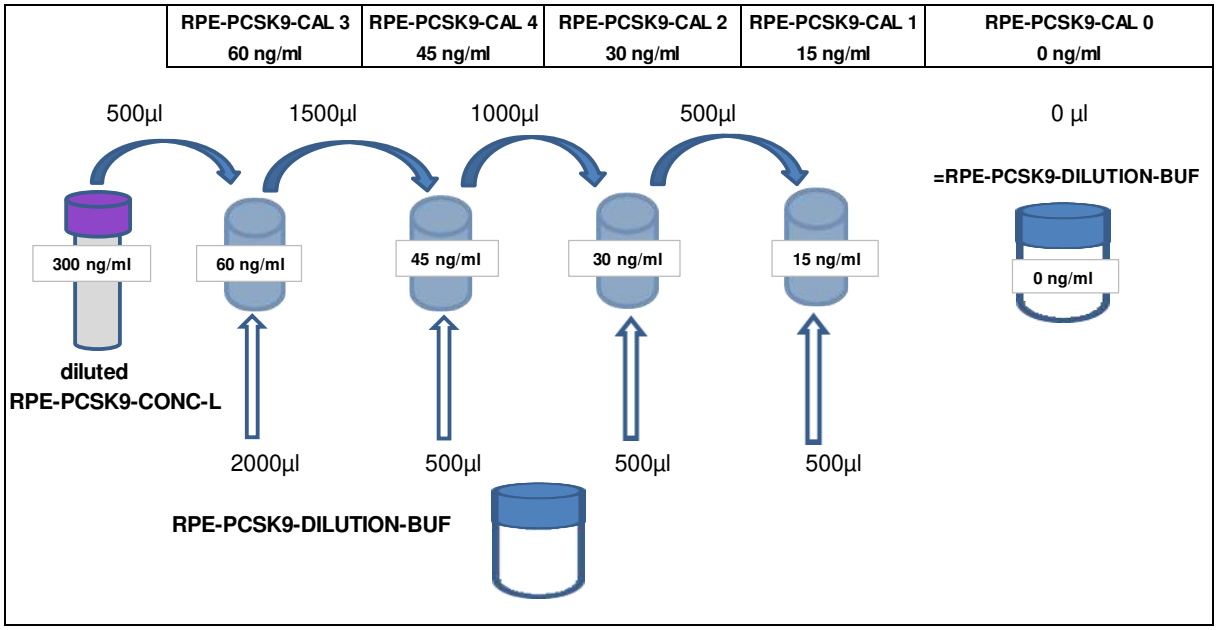
Note:
For the preparation of the RPE-PCSK9-CAL1-4 it is important to pipette with a pre-rinsed tip.

Start the dilution as follows:

1. Pipette **500 µL** diluted **RPE-PCSK9-CONC-L** (300ng/ml) into the reagent **RPE-PCSK9-CAL 3** (60ng/ml) and mix.
2. Pipette **1500 µL** **RPE-PCSK9-CAL 3** (60ng/ml) into the reagent **RPE-PCSK9-CAL 4** (45ng/ml) and mix.
3. Pipette **1000 µL** **RPE-PCSK9-CAL 4** (45ng/ml) into the reagent **RPE-PCSK9-CAL 2** (30ng/ml) and mix.
4. Pipette **500 µL** **RPE-PCSK9-CAL 2** (30ng/ml) into the reagent **RPE-PCSK9-CAL 1** (15ng/ml) and mix.

The calibrators **RPE-PCSK9-CAL 1, 2, 3** and **4** are now ready to use and should not be stored. The **RPE-PCSK9-DILUTION-BUF** serves as the calibrator **RPE-PCSK9-CAL 0** (0ng/ml).

Scheme of Dilution Procedure:



E. Preparation of RPE-CONJ

The **anti-PCSK9 antibody HRP conjugate concentrate (RPE-CONJ-CONC)** must be reconstituted with the reagent **conjugate diluent (RPE-CONJ-BUF)** immediately before use as follows:
Pipette **15 µL** **RPE-CONJ-CONC** into the reagent **RPE-CONJ-BUF** and mix.

The **pre-prepared diluted RPE conjugate solution** is now in the bottle labelled **RPE-CONJ-BUF** and must be used within 30 minutes and cannot be stored.

F. Microplate

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

G. Pipetting

The entire pipetting process should be carried out within 20 minutes.

Note: The individual pipetting steps 1 to 12 must be followed exactly as described below. Any disregard to this 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 pre-prepared diluted conjugate solution from bottle **RPE-CONJ-BUF** after reconstitution Anti-PCSK9 antibody HRP conjugate **RPE-CONJ-CONC** into each of the 96 wells (A1-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

2. Pipette 25 µL therapy antibody calibrator 0 **RPE-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 **RPE-TAK 1** into the 8 wells C1 - C4 und D1 - D4

	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 **RPE-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 **RPE-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 should be used only for the control value.

	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 **RPE-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 **RPE-PCSK9-DILUTION-BUF = RPE-PCSK9-CAL 0 (0ng/ml)** 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 calibrator **RPE-PCSK9-CAL 1 (15ng/ml)** 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 calibrator **RPE-PCSK9-CAL 2 (30ng/ml)** 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 calibrator **RPE-PCSK9-CAL 3 (60ng/ml)** 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 calibrator **RPE-PCSK9-CAL 4 (45ng/ml)** 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

H. Incubation

Cover the microplate with a light-tight cover and briefly (2 minutes) agitate on a microplate shaker. Incubate in the dark at 2-8 °C for 16 hours (min.16 h, max.22 h).

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

The washing buffer concentrate (10x) **WASHBUF10x** must be brought to room temperature before use and then diluted with distilled water 1:10 as follows: to 25 mL **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. Carefully tap off excess liquid.

J. Enzymatic colour reaction

1. Pipette 100 µL **TMB** substrate into each well with Multipette and Combitip + tip.
2. Cover the microplate with a light-tight cover and agitate for 30 minutes on the microplate shaker at room temperature. During agitation keep the microplate in the dark.
3. To stop the reaction, pipette 25 µL stopping solution **STOP-H_2SO_4** into each well using Multipette and Combitip + tip. Briefly agitate on the microplate shaker.
4. 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 PCSK9 Neutralization Rate/Evolocumab** (Download from the homepage www.biotez.de). http://www.biotez.de/index.php/en/company/download-area click on “Company” than “Download Area”; login with Username and Password provided on the single sheet included in the kit.

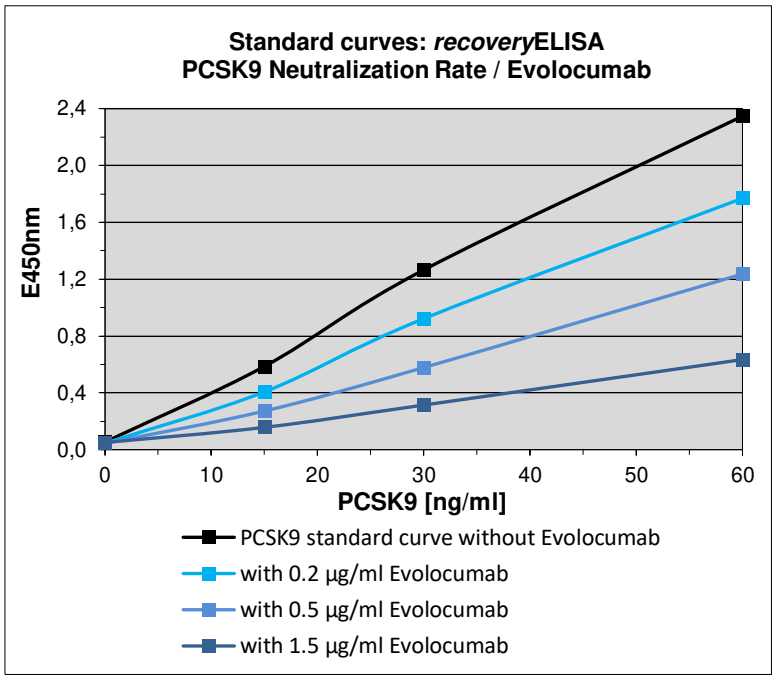
Calculation steps from the extinction values to the determining of the free PCSK9, the available therapeutic antibody Evolocumab content in the serum, PCSK9 recovery and PCSK9 neutralization rate

- Calculation of average values of all repeat determinations
- Determination of the PCSK9 therapeutic antibody reference curve for the relationship of RPE-PCSK9-CAL 0-3 and RPE-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 Evolocumab with reference to the average recovery of the antigen in standard curves
- Calculation of Evolocumab content in the samples with reference to the recovery curve
- Calculation of the **PCSK9 recovery (%)**
- Calculation of the **PCSK9 neutralization rate (%)**
- Conversion of the PCSK9 and Evolocumab levels to undiluted samples

The extinction obtained from the reader file of the microplate reader should be transferred to the input template of the evaluation software **©*recovery*ELISA PCSK9 Neutralization Rate/Evolocumab** by Copy and Paste. The output of measurement values for PCSK9 and Evolocumab can then be seen on the program page “Evaluation”.
The PCSK9 level is given in ng/mL. The Evolocumab level is given in µg/mL
Other importent results of the *recovery*ELISA include determining the "PCSK9 recovery (%)" and "PCSK9 neutralization rate”.
"**PCSK9 recovery**” refers to the PCSK9 recovery in *recovery*ELISA. This value is determined using the standard addition of PCSK9 (PCSK9 increase) for the serum sample. The value is shown in %.
The "**PCSK9 neutralization rate**" is calculated based on the difference between the "**PCSK9 recovery** " and 100%. This value refers to the serum sample and indicates the percentage of PCSK9 bound by the therapeutic antibody at the time of blood collection.

Typical standard curves with PCSK9 and Evolocumab calibrators:

Evolocumab [µg/ml]	PCSK9 concentration [ng/ml] 0	15	30	60
0	0.054	0.586	1.265	2.347
0.2	0.046	0.407	0.922	1.769
0.5	0.044	0.272	0.578	1.236
1.5	0.050	0.157	0.314	0.634



Important information for supervising physician:

The *recovery*ELISA PCSK9 Neutralization Rate/Evolocumab Kit (RPE) 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 physician.

Capability characteristics of the method

Measurement range

The *recovery*ELISA PCSK9 Neutralization Rate/Evolocumab Kit (RPE) has the following measurement range:

Analyt		Measurement Range (for undiluted sample)
PCSK9	Samples without Evolocumab	40 – 800 ng/mL
	Samples with Evolocumab	40 – 350 ng/mL
Evolocumab		0.2 – 24 µg/mL

Please note, that samples are routinely measured with recoveryELISA RPE in a 1:10 dilution. Samples with PCSK9 or Evolocumab concentrations above the measurement ranges should be further diluted and tested again. However, a maximum dilution of 1:20 should not be exceeded to prevent errors occurring in the assay. The 1:20 dilution of the sample should be carried out as follows:
Dilution 1:20: Dilute 30 µL of human serum sample in 570 µL **RPE-SAMPLE-BUF**.

Precision

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

Sample	Mean Value PCSK9 (ng/mL)	CV (%)	Mean Value Evolocumab (µg/mL)	CV (%)
serum. undiluted (after recalculation)				
1	165.8	5.8	2.8	10.6

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

Sample	Mean Value PCSK9 (ng/mL)	CV (%)	Mean Value Evolocumab (µg/mL)	CV (%)
serum. undiluted (after recalculation)				
1	165.8	12.1	2.8	17.7

Linearity

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

Specificity

The *recovery*ELISA RPE was tested for the detection of human PCSK9. The reactivity with PCSK9 of other species was not carried out. Cross-reactions with other serum proteins cannot be verified in physiological concentrations.

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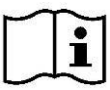
Important points and precautions

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 and equipment 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 (corrosive). ProClin 300 (maximum 0.05%) as a preservative in reagents and TMB (reproductive toxity Repr. 1B.H360D: May damage the unborn child.)
    Precautionary Statement(s): Avoid contact with eyes and skin! Wear protective gloves/protective clothing/eye protection/face protection
17. Observe the following safety information when handling 1M sulphuric acid "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:

Manufactured by	Catalog Number	Lot Number	Do not reuse	Use before	Consult instructions for use	Refer accompanying documents	Sufficient for <n> tests	Temperature limit
								

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