BDL-ECP ELISA Kit

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1. Intended Use

The anti-human ECP ELISA Kit from BDL is an immunoassay designed for the determination of the concentration of Eosinophil Cationic Protein (ECP) in human serum. This test kit should only be used according to the terms of the *in-vitro* diagnostic (IVD) regulations.

2. Introduction

Eosinophil Cationic Protein (ECP) is a 20 kDa, highly cationic protein (pI = 10.8) with cytotoxic activity. Besides Eosinophil Derived Neurotoxin (EDN) and Major Basic Protein (MBP), ECP is an important protein mediator released from activated eosinophils. Here, ECP and EDN are located in the matrix of granules whereas MBP is restricted to the core of the granules. Activated eosinophils play a fundamental role in inflammatory conditions like acute asthma. Especially in the late asthmatic response the release of ECP from activated eosinophils can serve as a diagnostic marker for activation and degranulation of these cells. Additionally, if human patients are treated with anti-inflammatory drugs, the decline of inflammation reflected by the level of ECP in serum or plasma is of value in monitoring the effect of therapy.

3. Assay Principles

The anti-human ECP ELISA kit from BDL is a sandwich ELISA which is performed in a microplate format. The ELISA is able to quantify ECP concentrations in human serum

between 6 ng/mL and 200 ng/mL. The detection limit for ECP in human serum is approximately 2.27 ng/mL. Cross-reactivity to EDN could not be detected.

The surface of the microplate wells is coated with a monoclonal antibody against human ECP. First, diluted human samples, standards and controls are added to the microplate and incubated at room temperature for one hour. After a following washing step to remove unbound sample a peroxidase conjugated anti-human ECP antibody solution is transferred to the microplate wells, and incubated as mentioned above. After a last washing step to get rid of unbound peroxidase conjugated antibodies, peroxidase substrate reagent is added to the wells, and incubated for 10 minutes at room temperature. The presence of human ECP, captured by the coated monoclonal anti-ECP antibody and the peroxidase labeled anti-ECP antibody, can be recognized by an increase in blue colour. The addition of stop solution changes the colour to yellow. The intensity of the yellow colour is proportional to the amount of ECP in the samples and standards. The colour intensity can be read with a microplate reader equipped with a 450 nm filter (optional reference wavelength is 620 nm). From the measured standards a calibration curve can be created, which allows the calculation of the ECP concentration in human serum samples.

4. Test Procedures

Preparations:

1. All assay components should be equilibrated to room temperature.
2. Dilute 10X-Wash Buffer Concentrate WASHBUF | 10x with deionized or ultrapure water (1 part of concentrate + 9 parts of water).

Assay Procedure:

Step 1: Sample incubation

1. Add 100 µL of standards: STD | 1 ; STD | 2 ; STD | 3 ; STD | 4 and controls: CONTROL | + ; CONTROL | – to the appropriate wells of the microplate strips. Do not dilute the controls.
2. Add 20 µL of human serum samples to the intended sample wells.
3. Add 80 µL of Assay Diluent DILBUF to each sample well.
4. Cover the microplate and mix the solutions by lateral shaking of the plate on the bench.

5. Incubate at room temperature for one hour.

Step 2: Washing

Manual wash: In case of a manual wash procedure wash each well for a total of four times with 300 µL of wash buffer. Invert and tap dry the plate after each washing step. Automated Wash: If an automated plate washer is used, wash each well four times with 400 µL of wash buffer in overflow mode. It is important for the automated washer to conduct a final aspirate cycle to eliminate residual amounts of wash buffer because this will affect assay performance.

Step 3: Conjugate incubation

1. Add 100 µL of Conjugate Solution | CONJ | HRP | to each well.
2. Cover plate and mix the solution by lateral shaking of the plate on the bench.
3. Incubate the microplate for 1 hour at room temperature.

Step 4: Washing

Wash the microplate wells as described in step 2.

Step 5: Colour development

1. Add 100 µL of Substrate Reagent | SUB | TMB | to each well.
2. Cover the plate and incubate for 10 min. at room temperature in the dark.
3. Add 100 µL of Stop Solution | STOP | H_2SO_4 | to each well.

Step 6: Measurement and calculation

1. Measure the absorbance at a wavelength of 450 nm (reference wavelength 620 nm). Wells should be read within 1 hour after stopping the reaction.
2. Plot the absorbance value (vertical-axis) of each standard versus its ECP concentration in ng/mL (horizontal-axis) on a semi-log graph paper and construct a standard curve.
3. Calculate the ECP concentration of samples and controls by reading the value from the standard curve. The negative control should result in an ECP concentration lesser or equal to 1.2 ng/mL whereas the positive control results in an ECP concentration between greater than 30 ng/mL. The concentration of ECP in the serum samples must be multiplied by 5, resulting from the sample dilution in *Step 1*.

The concentration of ECP in serum from healthy blood donors is < 16 ng/mL.

Flow chart:

Sample Incubation

1. 100 µL standards **STD**
2. 100 µL controls **CONTROL**
3. 20 µL serum samples + 80 µL Dilution Buffer **DILBUF**

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Washing (4 times)

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Conjugate Incubation

1. 100 µL conjugate **CONJ** | **HRP**
2. 1 hour at room temperature

↓

Washing (4 times)

↓

Colour Development

1. 100 µL substrate **SUB** | **TMB**
2. 10 minutes at room temperature
3. 100 µL stopping solution **STOP** | **H_2SO_4**

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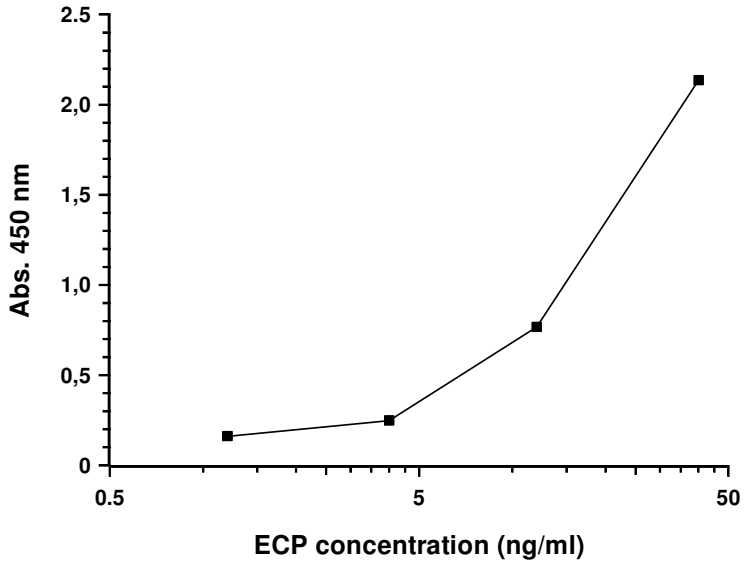
Measurement and Calculation

1. 450 nm (reference wavelength 620 nm)
2. Calibration Curve
3. multiply ECP concentration of serum samples by 5

Calibration curve:



5. Materials provided

- Assay Diluent DILBUF: 20 mL (ready to use) tris buffered saline solution with bovine serum albumin and a preservative.
- Wash buffer concentrate (10X) WASHBUF | 10x: 75 mL phosphate buffered saline solution with a surfactant. Dilute 10 fold with deionized or ultrapure water before use.
- Conjugate reagent CONJ | HRP: 12 mL (ready to use) horseradish peroxidase conjugated anti-human ECP antibodies in tris buffered saline solution with bovine serum albumin and a preservative.
- Substrate reagent SUB | TMB : 20 mL (ready to use) stabilized TMB/H_2O_2 solution.
- Stop solution STOP | H_2SO_4: 20 mL (ready to use). 0.25 mol/L H_2SO_4.
- ECP standards STD | 1 ; STD | 2 ; STD | 3 ; STD | 4: 4 x 1 mL vials with different ECP concentrations (1.2 / 4 / 12 / 40 ng/mL); ready to use. ECP stabilized in tris buffered saline solution with bovine serum albumin and a preservative.
- Negativ control CONTROL | –: 1 mL (ready to use) 20% (v/v) horse serum in tris buffered saline solution with bovine serum albumin and a preservative.
- Positiv control CONTROL | +: 1 mL (ready to use) ECP in tris buffered saline solution with bovine serum albumin, 20% horse serum and a preservative.

- Microwell strips: 12 strips with 8 wells coated with anti-human ECP antibodies.

6. Materials needed but not provided

- Precision pipettes that range from 10 µl to 100 µl and disposable tips.
- Deionized or ultrapure water.
- Graduated cylinder to dilute and mix wash buffer.
- Plate cover
- Microplate reader with a 450 nm filter.
- Microplate washer (for automated washing).
- Multichannel micropipette (can be used for manual washing).
- Vacutainer SST blood collection vials.
- Laboratory centrifuge

7. Precautions and Notes

- For reliable determinations of the ECP concentration in human serum one calibrator series for each individual microplate is necessary.
- Do not expose kit components to direct sunlight.
- Do not use kit or components beyond the expiration date.
- Microwell strips which are not immediately required should be returned to the original bag to prevent moisture absorption. Used microwell strips must be discarded.
- Microplate wells should not run completely dry during the entire test procedure.
- Microplate wells should be free of impurities. Before measurement of the absorption, it must be ensured that the wells are free from air bubbles and that the bottom of the plate is clean and dry.
- All specimens should be considered potentially infectious. Exercise proper handling precautions.
- Keep plate covered except when adding reagents, washing or reading.
- Deviations from described incubation temperatures or incubation times may result in incorrect ECP determinations.
- This kit is only for in vitro diagnostic use.

- Excessive or reduced ECP values may occur if incorrect blood collection tubes are used, the clotting time or temperature was not strictly considered, serum transfer to a new tube has not occurred, or did not occur timely.
- The detection of increased ECP levels only exhibits enhanced activation of eosinophil cells. Clinical statements must include additional diagnostic findings.
- Positive results in ECP *in vitro* tests may not accompany necessarily clinical manifestations.
- The release of ECP may be influenced by corticosteroid therapy, cytotoxic and immunosuppressive drugs (see references 5).

8. Specific Performance

The anti-human ECP ELISA kit from BDL is able to quantify ECP concentrations in human serum between 6 ng/mL and 200 ng/mL. The limit of detection of ECP is LoD = 0,68 ng/mL and the limit of quantitation is LoQ = 2,27 ng/mL. Cross-reactivity to EDN could not be detected. Reproducibility data are CV = 2.6 % for intra-assay reproducibility (determined by testing individual samples 6 times); inter-assay reproducibility CV = 12.1 % (determined by 5 independent assays of individual samples) and lot-to-lot reproducibility CV = 5.1 % (determined by testing the same sample with three different lots).

9. Restrictions and Interferences

Icterus:	0 – 18.3 mg/dl Bilirubin F; without impairment
	0 – 19.0 mg/dl Bilirubin C; without impairment
Chyle:	up to 1390 Units (Formazine); without impairment
Rheumatoid Factor:	0 – 5000 IU/ml; without impairment

Do not use hemolytic or highly lipemic sera.

10. Specimen collection and storage

ECP concentrations may be influenced by sampling and storage. A release of ECP from eosinophil granulocytes can also occur during blood clotting. Following the instructions of sample extraction and storage is therefore essential. For blood collection, the Vacutainer SST blood collection vials (glass separator, e.g. order number 367 785) from Becton

Dickinson, Heidelberg are recommended. After the uptake of blood in the Vacutainer SST-blood collection tube the content must be mixed by repeated inversion of the tubes (6 times). Each variation in temperature, time, or serum tubes may influence the ECP release. One hour after blood collection the sample must be positioned in a vibration-free place at room temperature for clotting. Direct sunlight should be avoided.

Immediately after clotting the blood has to be centrifuged for 10 minutes at 1200 g (g = 9.81 m/s ²). After the centrifugation serum must be transferred to a new tube (glass or polystyrene without gel barriers or other additives). For shipping purposes, serum is stable for at least 24 hours at room temperature. If the ECP concentration is not determined within 24 h after blood collection serum must be frozen at -20 °C. Repeated freeze-thaw cycles should be avoided.

11. Stability and Storage

All kit components are stable for at least one year and should be stored at 2 °C to 8 °C.

The expiration date is printed on the individual test components.

12. Literature

1. Gleich, G. et al., *Proc. Natl. Acard. Sci. USA* **83**, 3146-3150, (1986)
2. Krisyjansson, S., et al., *Annals of medicine* **28**, 395-399, (1996)
3. Peterson, C., et al., *Eur J Haematol*. **40**, 415-423, (1988)
4. Zimmerman, B., et al., *Clin. Exp Allergy* **23**, 564-570, (1993)
5. Ren-Bin Tang, et al., *Pediatric Pulmology* **31**, 121-125 (2001)

13. Validity

This instruction protocol is valid from April 19th, 2012.

14. Manufacturer

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