# Human CD62P ELISA Kit

Instructions for use

| | | |
|---|---|---|
| Catalogue numbers: | 1x96 tests: | 850.930.096 |
| | 2x96 tests: | 850.930.192 |

**For research use only**

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# Human CD62P ELISA KIT

## 1. Intended use

The Diaclone Human CD62P ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of CD62P in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human CD62P.

**This kit has been configured for research use only. Not suitable for use in therapeutic procedures.**

## 2. Introduction

### 2.1. Summary

P-selectin (CD62P, LECAM-3) was originally purified from platelets and later found to be expressed also in endothelial cells. In both cell types, P-selectin is constitutively expressed and stored in secretory granules – α-granules in platelets and Weibel-Palade bodies in endothelial cells (1).

A soluble form of P-selectin, which might represent a proteolytic fragment or a soluble splice variant lacking the transmembrane domain, is found in serum and plasma (2). High expression of P-selectin or sP-selectin has been implicated in several inflammatory disorders, including adult respiratory distress syndrome, acute lung injury, ischemia-reperfusion injury, Gram-negative septic shock, thrombotic diseases, malaria, systemic sclerosis, connective tissue disease and rheumatoid arthritis. Furthermore, lung injury scores correlate significantly with sP-selectin in plasma of patients with acute lung injury (3) and (4).

### 2.2. Principle of the method

A capture Antibody highly specific for CD62P has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of CD62P in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed.
During the next incubation period the binding of the Biotinylated anti-CD62P secondary antibody to the analyte occurs. Any excess unbound secondary antibody is then removed.
The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing.
A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of CD62P present in the samples and standards.
The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of CD62P in any sample tested.

## 3. Reagents provided and reconstitution

| **Reagents** (Store@2-8°C) | **Quantity 1x96-well kit** Cat no. 850.930.096 | **Quantity 2x96-well kit** Cat no. 850.930.192 | **Reconstitution** |
|---|---|---|---|
| Anti-CD62P Coated Plate | 1 | 2 | Ready to use (96-well strip pre-coated plate) |
| Plastic plate covers | 2 | 4 | n/a |
| CD62P Standard: 140 ng/ml | 2 | 4 | Reconstitute as directed on the vial (see Assay preparation, section 8) |
| Standard Diluent | 1 (15ml) | 1 (25ml) | 10x concentrate, dilute in distilled water (see Assay preparation, section 8) |
| Biotinylated Anti-CD62P | 1 (0.4ml) | 2 (0.4ml) | Dilute in Biotinylated Antibody Diluent (see Assay preparation, section 8) |
| Biotinylated Antibody Diluent | 1 (7ml) | 1 (13ml) | Ready to use |
| Streptavidin-HRP | 2 (5µl) | 4 (5µl) | Add 0.5ml of Streptavidin-HRP Diluent prior to use (see Assay preparation, section 8) |
| Streptavidin-HRP Diluent | 1 (12ml) | 1 (23ml) | Ready to use |
| Wash Buffer | 1 (10ml) | 2 (10ml) | 200x concentrate dilute in distilled water (see Assay preparation, section 8) |
| TMB Substrate | 1 (11ml) | 1 (24ml) | Ready to use |
| $H_2SO_4$ Stop Reagent | 1 (11ml) | 2 (11ml) | Ready to use |

## 4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450 nm required with optional 620 nm reference filter)
- Microtiter plate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

## 5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

**Wash Buffer 1X:** Once prepared, store at 2-8°C for up to 1 week.

**Standard Diluent Buffer 1X:** Once prepared, store at 2-8°C for up to 1 week.

**Reconstituted Standard:** Once prepared use immediately and do not store.

**Diluted Biotinylated Anti-CD62P:** Once prepared use immediately and do not store.

**Diluted Streptavidin-HRP:** Once prepared use immediately and do not store.

## 6. Specimen collection, processing & storage

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

**Cell culture supernatants:** Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

**Serum:** Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

**Plasma:** EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

**Storage:** If not analysed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

**Recommendation:** Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

## 7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual: "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with $H_2SO_4$ and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of $H_2SO_4$ and TMB Substrate solutions, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB Substrate solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB Substrate solution with metal to prevent colour development. Warning TMB Substrate is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB Substrate solution has been contaminated and must be discarded. Read absorbances within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB Substrate within 15 min of the washing of the microtiter plate.

## 8. Assay Preparation

**Bring all reagents to room temperature before use**

### 8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

**Example plate layout** (example shown for a 6 point standard curve)

| | Standards | | Sample Wells | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Std1 | Std1 | | | | | | | | | | |
| B | Std2 | Std2 | | | | | | | | | | |
| C | Std3 | Std3 | | | | | | | | | | |
| D | Std4 | Std4 | | | | | | | | | | |
| E | Std5 | Std5 | | | | | | | | | | |
| F | Std6 | Std6 | | | | | | | | | | |
| G | zero | zero | | | | | | | | | | |
| H | | | | | | | | | | | | |

*All remaining empty wells can be used to test samples in duplicate*

### 8.2. Preparation of Wash Buffer

If crystals have formed in the concentrate Wash Buffer, warm it gently until complete dissolution.

Dilute the (200X) concentrate Wash Buffer 200 fold with distilled water to give a 1X working solution. Pour entire contents (10 ml) of the concentrate Wash Buffer into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle.

### 8.3. Preparation of Standard Diluent Buffer 1X

If crystals have formed in the concentrate Standard Diluent, warm it gently until complete dissolution.

Dilute the (10X) concentrate Standard Diluent 10 fold with distilled water to give a 1X working solution. Pour entire contents of the concentrate Standard Diluent into a clean appropriate graduated cylinder. Bring to final volume with glass-distilled or deionized water. Transfer to a clean wash bottle.
Please see example volumes below:

| Standard Diluent concentrate (ml) | Distilled water (ml) |
|---|---|
| 15 | 135 |
| 25 | 225 |

## 8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of Standard Diluent Buffer 1X shown on the vial immediately prior to use. This reconstitution gives a stock solution of 140 ng/ml of CD62P. Mix the reconstituted standard gently by inversion only. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 140 to 4.4 ng/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 140 ng/ml.
- Add 100µl of Standard Diluent Buffer 1X to the remaining standard wells B1 and B2 to F1 and F2.
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 140 ng/ml to 4.4 ng/ml.
- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred into the relevant wells.

## 8.5. Preparation of Samples

Before testing, human serum or plasma samples have to be diluted 1:5 in Standard Diluent Buffer 1X.

## 8.6. Preparation of Biotinylated Anti-CD62P

It is recommended this reagent is prepared immediately before use. Dilute the Biotinylated Anti-CD62P with the Biotinylated Antibody Diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

| Number of wells required | Biotinylated Antibody (µl) | Biotinylated Antibody Diluent (µl) |
|---|---|---|
| 16 | 40 | 1060 |
| 24 | 60 | 1590 |
| 32 | 80 | 2120 |
| 48 | 120 | 3180 |
| 96 | 240 | 6360 |

## 8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5µl vial with 0.5ml of Streptavidin-HRP Diluent **immediately before use.** Do not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

| Number of wells required | Streptavidin-HRP (µl) | Streptavidin-HRP Diluent (ml) |
|---|---|---|
| 16 | 30 | 2 |
| 24 | 45 | 3 |
| 32 | 60 | 4 |
| 48 | 75 | 5 |
| 96 | 150 | 10 |

## 9. Method

**We strongly recommend that every vial is mixed thoroughly without foaming prior to use.**

**Prepare all reagents as shown in section 8.**
**Note: final preparation of Biotinylated Antibody (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.**

| Assay Step | | Details |
|---|---|---|
| 1. | Addition | **Prepare standard curve** as shown in section 8.4 above and add in duplicate to appropriate wells |
| 2. | Addition | Add 100µl of each **Sample and zero (Standard Diluent Buffer 1X)** in duplicate to appropriate number of wells |
| 3. | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** |
| 4. | Wash | Remove the cover and wash the plate as follows:<br>a) Aspirate the liquid from each well<br>b) Dispense 0.3 ml of **1x Wash Buffer** into each well<br>c) Aspirate the contents of each well<br>d) Repeat step b and c another two times |
| 5. | Addition | Add 50µl of diluted **Biotinylated Anti-CD62P** to all wells |
| 6. | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** |
| 7. | Wash | Repeat wash step 4. |
| 8. | Addition | Add 100µl of diluted **Streptavidin-HRP** solution into all wells |
| 9. | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min** |
| 10. | Wash | Repeat wash step 4. |
| 11. | Addition | Add 100µl of ready-to-use **TMB Substrate** solution into all wells |
| 12. | Incubation | Incubate in the dark for **10-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil. |
| 13. | Addition | Add 100µl of **$H_2SO_4$ Stop Reagent** into all wells |
| **Read the absorbance** value of each well (immediately after step 13.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable). | | |

**Incubation time of the TMB Substrate is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.*

## 10. Data Analysis

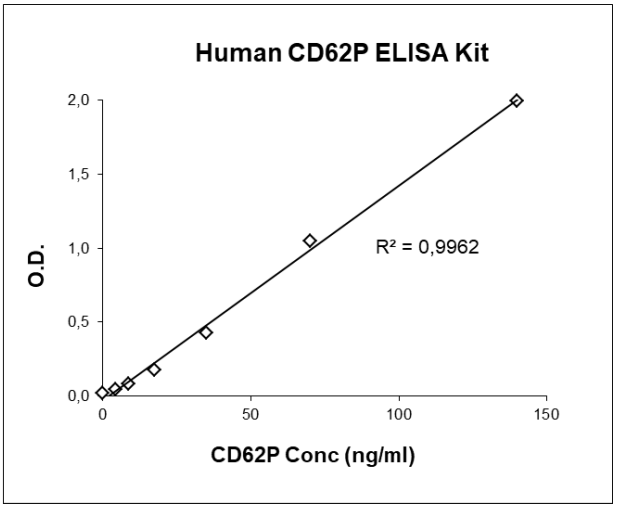
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding CD62P standard concentration on the horizontal axis.

The amount of CD62P in each sample is determined by extrapolating OD values against CD62P standard concentrations using the standard curve**.**

**Example CD62P Standard curve**

| Standard | CD62P Conc (ng/ml) | OD (450nm) mean | CV (%) |
|---|---|---|---|
| 1 | 140 | 1.992 | 2.0 |
| 2 | 70 | 1.050 | 0.7 |
| 3 | 35 | 0.429 | 0.0 |
| 4 | 17.5 | 0.172 | 2.9 |
| 5 | 8.8 | 0.079 | 2.1 |
| 6 | 4.4 | 0.046 | 7.8 |
| zero | 0 | 0.020 | - |




**Note:** curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For samples human serum or plasmas which have been diluted 1:5 according to the protocol, the calculated concentration should be multiplied by the dilution factor (x5).

## 11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard Diluent Buffer or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

# 12. Performance Characteristics

## 12.1. Sensitivity

The sensitivity or minimum detectable dose of CD62P using this Diaclone Human CD62P ELISA kit was found to be **3.6 ng/ml**. This was determined by adding 2 standard deviations to the mean OD obtained when the zero standard was assayed in 6 independent experiments.

## 12.2. Specificity

The assay recognizes both natural and recombinant human CD62P. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested: MICA, gp130, GM-CSF, IL-2, IL-6, TRAIL, IL-13, IL-8, Perforin and CD31.

## 12.3. Precision

| | Intra-assay | | | | | Inter-assay | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **n** | **Mean (ng/mL)** | **SD** | **CV%** | **Sample** | **n** | **Mean (ng/mL)** | **SD** | **CV%** |
| A | 6 | 90.4 | 5 | 5.6% | A | 18 | 92 | 6.9 | 7.5% |
| B | 6 | 39.6 | 3.1 | 7.8% | B | 18 | 42.3 | 1.8 | 4.4% |

## 12.4. Dilution Parallelism

In two independent experiments two spiked human serum samples with different levels of CD62P were analysed at different serial two fold dilutions (1:2 to 1:8) with two replicates each.
Recoveries ranged from 95 to 113% with an overall **mean recovery of 101%.**

## 12.5. Spike Recovery

The spike recovery was evaluated by spiking 3 concentrations of CD62P in human serum and culture medium in 3 separate experiments.
The calculated **mean recovery is 101%.**

## 12.6. Stability

**Storage Stability**
Aliquots of spiked serum and spiked medium were stored at –20°C, +2-8°C, room temperature (RT) and at 37°C and the CD62P level determined after 24h. There was no significant loss of CD62P reactivity during storage at +2-8°C, RT and 37°C.

**Freeze-thaw Stability**
Aliquots of spiked serum and spiked medium were stored frozen at –20°C and thawed up to 5 times and the CD62P level was determined. There was no significant loss of CD62P reactivity after 5 cycles of freezing and thawing.

## 12.7. Expected serum values

A panel of 22 sera from apparently healthy blood donors (male and female) was tested for CD62P. The detected CD62P levels ranged between 124 and 539.8 ng/ml with a mean level of 348.5 ng/ml and a standard deviation of ± 116.6 ng/ml.

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## 15. Assay Summary

**Total procedure length : 2h45min**

**Add 100µl of diluted Samples and diluted Standards**

↓

**Incubate 1 hour at room temperature**

↓

**Wash three times**

↓

**Add 50µl of diluted Biotinylated Antibody**

↓

**Incubate 1 hour at room temperature**

↓

**Wash three times**

↓

**Add 100µl of diluted Streptavidin-HRP**

↓

**Incubate 30 min at room temperature**

↓

**Wash three times**

↓

**Add 100µl of TMB Substrate**
**Protect from light. Let the color develop for 10-15 min.**

↓

**Add 100µl of Stop Reagent**

↓

**Read Absorbance at 450 nm**

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