Pregnostic®-PE *IIp*

Enzyme-linked Immunosorbent Assay Kit

to determine Endothelial Cell-Specific Molecule 1 (ESM-1)

Package insert

REF IQP-304

96 Tests

For Research Use Only

Not for use in Clinical Diagnostic Procedures

Intended use

This Pregnostic®-PE *IIp* kit is intended for the quantification of ESM-1 in EDTA plasma samples and cell culture supernatants. It is designed for research purposes only. The Pregnostic®-PE *IIp* kit has been validated for EDTA plasma measurements and cell culture supernatants. The Pregnostic®-PE *IIp* kit is completed within 5 hours with less than 1 hour of hands-on-time.

Background information

Endothelial cell-specific molecule 1 (ESM-1), also called Endocan, was originally discovered in endothelial cells[1,2]. ESM-1 is described as an inflammatory factor that is increased in cardiovascular diseases, sepsis[3] and lung diseases[4,5]. Next to this, the role of ESM-1 is already described in several other clinical contexts such as: tumors[6,7], hypertension[8,9] and preeclampsia[10,11,12].

ESM-1 is a 50 kDa proteoglycan constituted of a 165 amino acid mature protein core (20 kDa), and a unique chondroitin/dermatan sulfate chain linked to the serine residue[2,13]. ESM-1 can be found in plasma and there is a spontaneous synthesis and secretion by (highly specialized) endothelial cells. The production is increased in the presence of pro-angiogenic molecules such as VEGF and FGF-2[14], pro-inflammatory cytokines[2] and bacterial lipopolysaccharide[15]. ESM-1 is involved in a wide range of biological processes, such as proliferation, endothelial cells migration, cell adhesion and neovascularization[16,17,18,19].

Principle of Pregnostic®-PE *IIp* kit

This Pregnostic®-PE *IIp* kit is based on the principle of the robust and well-described quantitative sandwich enzyme immunoassay technique. Briefly, a monoclonal antibody specific for human ESM-1 has been coated on the surface of a 96-well microplate. After incubation with the sample or recombinant ESM-1 standard a second biotinylated monoclonal antibody recognizing human ESM-1 is added to the wells. Detecting of the latter is done by primary adding streptavidin-HRP and secondly a substrate solution to each well. A color should develop in proportion to the amount of human ESM-1 present in the sample. The color development is stopped by acid solution and the intensity of the color is measured by spectrophotometry. The intensity of the samples is compared to that of the standard curve resulting in the quantification of ESM-1.

Kit content

Item	Description	Amount
Reagent A	Recombinant ESM-1 standard **Please check the label on the vial for the correct reconstitution volume of reagent E (1x)**	2x vial
Reagent B	Biotinylated detection antibody Contains 0.02% Sodium azide	135 µL
Reagent C	Streptavidin-HRP	150 µL
Reagent D (10x)	10x Washing buffer	40 mL
Reagent E (10x)	10x Dilution buffer	10 mL
Reagent F	TMB substrate. Contains <0.02% w/w TMB	11 mL
	*WARNING*	
Reagent G	Stop Solution (0.2 M H_2SO_4)	11 mL
	*WARNING*	
Reagent H	10x Blocking buffer	2 mL
Pre-coated microtiter plate	12x 8 well pre-coated strips	1x
Adhesive plate seals	Plate covers	4x
Package insert	Instructions for use	1x

Laboratory material required but not included

- Laboratory centrifuge for 1.5 mL vials
- 1.5 mL reaction tubes
- Distilled or demineralized water
- Adjustable micropipettes and tips
- Vortex mixer
- ELISA shaker
- Stopwatch or timer
- Microtiter plate reader with 450 nm (and 620, 650, or 690 nm) filter and software to evaluate ELISA data

Warning and precautions ⚠ ☣ ✳

- Please read carefully and completely this manual before use.
- All reagents should be handled in accordance with good laboratory practices using appropriate precautions.
- All patient samples should be handled with appropriate precautions.
- Always use polypropylene tubes for serial dilution.
- Do not pipette by mouth and wear gloves during the procedure.
- Do not freeze reconstituted standard.
- Always avoid foaming when reconstituting standards and when mixing solutions.
- Reagents containing sodium azide (reagent B [0,002%]) may react with lead or copper plumbing to form explosive metal azides. On disposal, flush with large amounts of water to prevent azide build-up.
- Reagent E contains Thimerosal (0.01%). Thimerosal may enter the body through the skin, is toxic, alters genetic material, May be irritating to the eyes and can cause allergic reactions. Avoid skin and eye contact and handle in accordance with good laboratory practices using appropriate precautions.
- Reagent F contains 3, 3', 5, 5'-tetramethylbenzidine (TMB; concentration <0.02%). The original manufacturer states that the preparation is not to be classified according to EEC directive 88/379/EEC. It is suggested to act in case of emergency as if the material should be. Avoid skin and eye contact and handle in accordance with good laboratory practices using appropriate precautions.
- Always keep TMB solution protected from light.
- Reagent G, the Stop Solution provided with this kit, is an acid solution. Protect face, eyes, hands and clothing by wearing appropriate protection when working with Reagent G (Stop Solution). Avoid skin and eye contact in accordance with good laboratory practices using appropriate precautions.
- The test must be performed by well-trained and authorized laboratory technicians.
- Do not substitute or mix reagents with those from other origins.
- Please contact the manufacturer if the original test kit is damaged upon receipt.

Sample collection and storage

Plasma – Collect blood in tube with EDTA as anticoagulant. Centrifuge at 1500 x g for 10 minutes at 4°C. Remove plasma and aliquot before storage at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatant – Remove cellular debris by centrifugation and aliquot the supernatant before storage at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Reagents preparation

- Before use, all buffers should be allowed to reach room-temperature (20-25 °C).
- Dilute the Washing buffer (reagent D) 10 times with demineralized water before use.
- Dilute the Dilution buffer (reagent E) 10 times with demineralized water before use.
- Dilute the Blocking buffer (reagent H) 10 times with demineralized water before use.

Prior to the Pregnostic®-PE *IIp* assay performance it is recommended to dilute the samples. It is recommended to dilute at least 1:2 in 1x reagent E. As the samples may derive from various sources, the correct dilution has to be determined for each type of experiment.

Pregnostic®-PE *IIp* Assay procedure

The procedure roughly consists of the following steps

· Washing pre-coated microtiter plate	step 1
· Blocking pre-coated microtiter plate	step 2
· ESM-1 standard preparation	step 4
· Sample preparation	step 5
· Sample and Standard incubation	step 6
· Detection-antibody incubation	step 8
· Streptavidin-HRP incubation	step 10
· TMB substrate reaction	step 12
· Adding Stop Solution	step 13
· Data analysis	

Preparations prior to Pregnostic®-PE *IIp* Assay Procedure

- The incubation steps as described in this section may be performed on a microplate shaker under gentle shaking. This is not necessary for a successful assay performance.
- Prior to their use *reagents A, B and C should be centrifuged* for 30 seconds at full speed before opening.

Technical hints

- If samples generate higher OD values than the highest standard value, dilute the samples with 1x Dilution buffer (1x Reagent E) and repeat the assay.
- Avoid cross-contamination by changing pipette tips between standard, samples and reagents addition.
- Variations in pipetting, washing techniques, incubation time and room temperature can cause variations in results.
- Avoid repeated freeze-thaw cycles of your samples.
- Always add the Stop Solution in step 11 (Reagent G) in the same order as the TMB solution (Reagent F).

Assay procedure

1. Washing pre-coated microtiter plate

- Dispense 200 µL 1x reagent D into each well and aspirate the washing solution.
- Invert the plate on a lint-free tissue and tap dry.

2. Blocking pre-coated microtiter plate

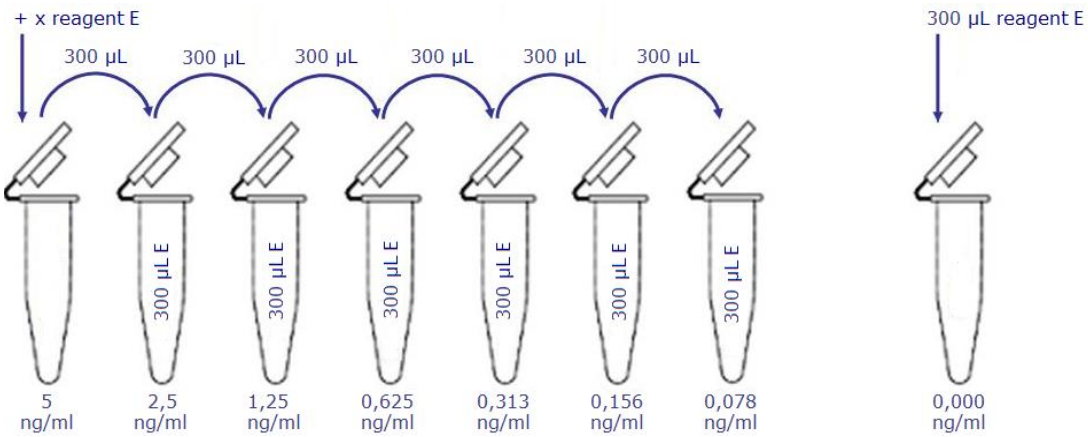
- Dispense 200 µL 1x reagent H into each well and seal the plate.
- Incubate on an ELISA shaker at room temperature (20-25 °C) for 1 hour.
- Invert the plate on a lint-free tissue and tap dry.

3. Washing (manual)

- Aspirate the blocking buffer after the 1 hour incubation.
- Invert the plate on a lint-free tissue and tap dry.
- Dispense 200 µL 1x reagent D into each well and aspirate the washing solution.
- Invert the plate on a lint-free tissue and tap dry.
- Repeat this step for 2 more times.

4. Preparation of the recombinant ESM-1 standard (reagent A)

- Shortly spin the reagent A vial (recombinant ESM-1 standard) to make sure all material is at the bottom of the vial before opening. One vial is enough to prepare one standard curve in duplicate.
- After warming of the lyophilized ESM-1 Standard at room temperature (RT), carefully open the vial to avoid any loss of material. Then reconstitute each vial of lyophilized ESM-1 Standard with the volume of 1x reagent E indicated on the vial and directly in the vial, to obtain a solution of 5 ng/mL (highest concentration of the standard curve).
- After complete reconstitution, the standard solution should never be frozen a second time.
- Add 300 µL of 1x reagent E to 6 tubes (always use polypropylene tubes).
- Perform serial dilutions by adding 300 µL of each Standard (2-fold dilution) to the next tube and mix each tube thoroughly between each dilution. 1x reagent E serves as the zero standard (0 ng/mL).



5. Sample preparation

- Use polypropylene tubes for sample dilution.
- Dilute samples in 1x reagent E.
- Cell culture supernatants and EDTA plasma samples may require dilution optimization according to experimental set up.
- Duplicate measurements are recommended.

6. Adding standards and samples to the pre-coated wells

- Add 100 µL of each standard dilution (Reagent A, prepared in step 4), or sample (prepared in step 5) in duplicate to each well.
- Add 100 µL of 1x reagent E to two blank wells.
- Seal the plate.
- Incubate on an ELISA shaker at room temperature (20-25 °C) for 1 hour.
- During this incubation, the detection antibody (reagent B) is prepared (next step).

Preparation of the Detection-antibody (Reagent B)

- Dilute the 100x reagent B (100 x detection antibody) in 1x reagent E. For example: Add 100 µL of 100x reagent B to 10 ml of 1x reagent E. This is sufficient for a full microtiter plate.

7. Washing (manual)

- Aspirate the samples/standards after the 1 hour incubation.
- Invert the plate on a lint-free tissue and tap dry.
- Dispense 200 µL 1x reagent D into each well and aspirate the washing solution.
- Invert the plate on a lint-free tissue and tap dry.
- Repeat this step 2 more times.

8. Detection-antibody incubation

- Add 100 µL of the 1x reagent B (detection antibody) to each well using a multi-channel pipette.
- Seal the plate with the plate sealer.
- Incubate on an ELISA shaker at room temperature (20-25 °C) for 1 hour.
- During this incubation the streptavidin-HRP solution (reagent C) is prepared (next step).

Preparation of the streptavidin-HRP solution (Reagent C)

- Dilute the 100x reagent C (100 x streptavidin-HRP solution) in 1x reagent E. For example: Add 100 µL of 100x reagent C to 10 ml of 1x reagent E. This is sufficient for a full microtiter plate.

9. Washing (manual)

- Aspirate the samples/standards after the 1 hour incubation.
- Invert the plate on a lint-free tissue and tap dry.

- Dispense 200 µL 1x reagent D into each well and aspirate the washing solution.
- Invert the plate on a lint-free tissue and tap dry.
- Repeat this step 2 more times.

10. Streptavidin-HRP incubation

- Add 100 µL 1x reagent C (streptavidin-HRP solution) to each well using a multi-channel pipette.
- Seal the plate with the plate sealer.
- Incubate on an ELISA shaker at room temperature (20-25 °C) for 1 hour.

11. Washing (manual)

- Aspirate the samples/standards after the 1 hour incubation.
- Invert the plate on a lint-free tissue and tap dry.
- Dispense 200 µL 1x reagent D into each well and aspirate the washing solution.
- Invert the plate on a lint-free tissue and tap dry.
- Repeat this step 5 times.

12. Adding the TMB (reagent F)

- Make sure the TMB (reagent F) is at room temperature (20-25 °C) before use.
- Add 100 µL of TMB (reagent F) to each well using a multi-channel pipette.
- Seal the plate with a *new* plate sealer.
- Incubate at room temperature (20-25 °C) for 30 min in the dark.

13. Adding the Stop solution (Reagent G)

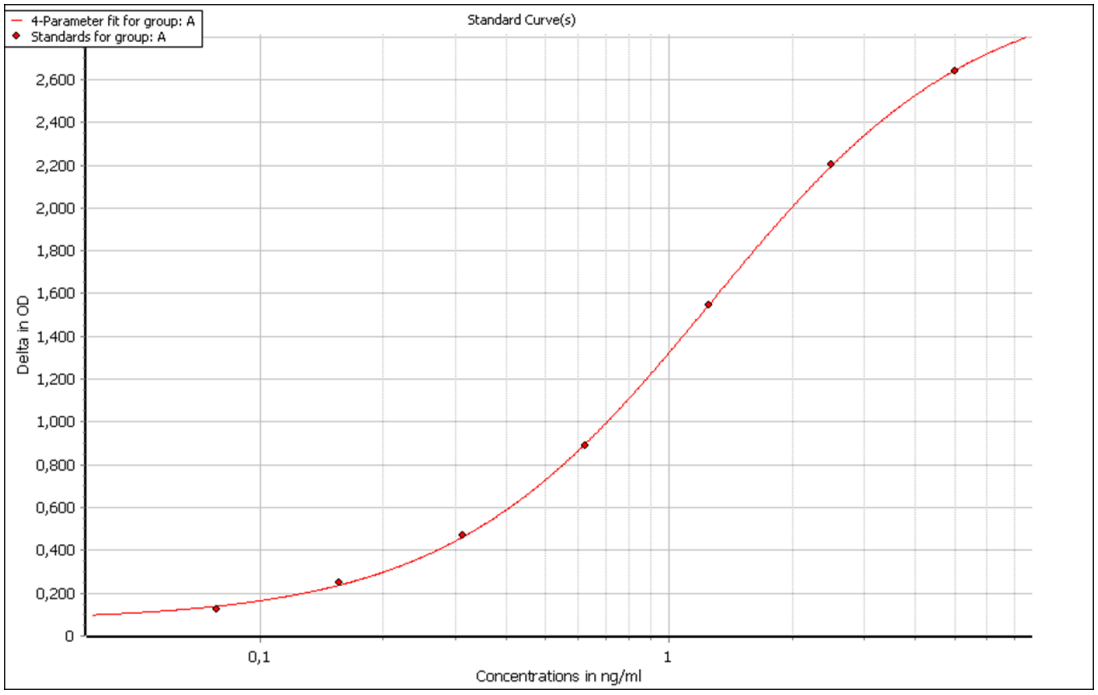
- Add 100 µL Reagent G (Stop Solution) to each well using a multi-channel pipette.
- Mix the fluids gently by tapping the side of the plate or by using a microplate shaker under gentle shaking.
- Read the plate with a 450 nm filter within 15 minutes of adding 0.2 M H_2SO_4 (a 620, 650, or 690 nm filter can be used as reference wavelength).

Data analysis

Subtract the zero standard optical density to the optical density of each Standard and each Sample. Diluted samples should be multiplied by the dilution factor to calculate the concentration read.

i. Standard curve

The standard curve can be generated by linear interpolation or by automated curve fitting methods as supplied by the standard ELISA software programs. The following figure represents a typical example of an ESM-1 ELISA standard curve.



ii. Expected values in pathological conditions

ESM-1 is increased in cardiovascular diseases, sepsis[3], preeclampsia[12] and lung diseases such as pneumonia and acute respiratory distress syndrome.[4,5] Expected values have to be determined for every specific situation by the user.

iii. Traceability/correctness

The assay correlates well (r^2 = 0.97).

iv. Precision of the Pregnostic®-PE *IIp* assay

Intra-assay variability is 5.8%; Inter-assay variability is 7.7 %.

v. Linearity of the Pregnostic®-PE *IIp* assay

It was shown to be linear from 0.3125 – 5 ng/ml (see figure of standard curve).

vi. Detection level of the Pregnostic®-PE *IIp* assay

0.096 ng/mL (background OD + 2x standard deviation).

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Warranty

Products sold hereunder are warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, which extend beyond the description on the label of the product. IQ Products BV is not liable for property damage, personal injury, or economic loss caused by the product.

Explanation of used symbols

	Consult instructions for use
REF	Catalogue number
	Sufficient for
	Caution, consult accompanying document
	Keep away from (sun)light
	Biological risks
	Temperature limitation (°C)
RUO	For Research Use Only
LOT	Batch code
	Use by yyyy-mm-dd
	Manufacturer

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Revision History

Current version + release date	Version 2 12-07-2023 (DD-MM-YYYY)
Previous version	Version 1
Changes	Assay procedure Point 11: wash 5x after strep-HRP step instead of 3x Point 12: 30 min TMB incubation instead of 20 min. Also mentioned that TMB should be at room temp. Sampling scheme deleted on page 13.
Justification	Adjustment of the assay procedure by using another strep-HRP. Most laboratories use their own sampling scheme.
Current version + release date	Version 1 01-05-2023 (DD-MM-YYYY)
Previous version	N/A
Changes	N/A
Justification	N/A