Research

Standard Operating Procedure for Quantifying Growth and Inflammatory Factors in-Cell Culture Supernatants, and Plasma via ELISA within the Nanostructurome Methods Pipeline

Michelini Sara[1], Drobne Damjana[1,*]

1. University of Ljubljana, Biotechnical faculty, Department of Biology, Ljubljana, Slovenia
* Correspondence: damjana.drobne@bf.uni-lj.si

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Abstract

Here we present the Standard operating procedure (SOP) used used to quantify growth factors and inflammatory markers in extracellular vesicles (EVs) - containing cell culture supernatants, plasma samples and using enzyme linked Immunosorbent assay (ELISA). The targets investigated in plassma and supernatant include interleukin 6 (IL-6), tumor necrosis factor α (TNFα), platelet-derived growth factor-BB (PDGF-BB), transforming growth factor β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF). These soluble mediators are crucial for cell proliferation, differentiation, and immune response regulation. They may also play a pivotal role in determining the efficacy of EV-based regenerative therapies. This SOPs was implemented within the Nanostructurome methods pipeline.

Keywords: Growth factors; ELISA; Plasma; Inflammatory factors; Supernatants.

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1. Definitions:

Ab: antibody; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CV: coefficient of variation; EGF: endothelial growth factor; ELISA: Enzyme Linked Immunosorbent Assay (ELISA); EV: extracellular vesicle; HRP: Horseradish Peroxidase, IGF-1: insulin-like growth factor 1; IL-6: Interleukin 6; O.D.: optical density; PDGF-BB: platelet-derived growth factor-BB; LLC: Large Latent Complex; PBS: phosphate buffered saline; PRP: platelet-rich plasma; RT: room temperature; TMB: 3,3, 5,5'-tetrametilbenzidine; TGF-β: transforming growth factor; TNFα: tumor necrosis factor α; VEGF: vascular endothelial growth factor

2. Background

Extracellular vesicles (EVs) are nanometer-sized vesicles secreted by virtually all cell types across a wide range of organisms, from plants to bacteria (Gill et al., 2019; Kumar et al., 2024; Peregrino et al., 2024; Bayat et al., 2021; Hu et al., 2024). In humans, EVs serve as carriers for various biomolecules, including proteins (such as cytokines, growth factors, receptors, etc.), mRNAs, miRNAs, DNA, and lipids (Kumar et al., 2024). As a result, they play a crucial role in both short- and long-range cell-to-cell communication in health and disease (Kumar et al., 2024; Mohammadipoor et al., 2023; Chang et al., 2021). The composition and quantity of EVs produced can vary depending on the stimuli a cell encounters, which in turn influences their biological effects and the cells they target. Plasma is a particularly rich source of EVs, primarily produced by hematopoietic cells, with platelets being a key contributor (Li et al., 2020; Aberro et al., 2021). Platelet-rich plasma (PRP) of autologous origin is being extensively studied in clinical dermatology because of its positive effect on skin regeneration and more applications are now being considered. The positive effect of PRP can be linked to the presence of different active molecules in platelets; but also, to the high concentration of biologically active EVs. Depending on the protocol used for plasma preparation, the concentration and type of EVs might vary, and thus the efficacy of the preparation. This is applicable also to cell-derived supernatant samples.

3. Purpose, Scope and Applicability

The purpose of this SOP is to describe the procedures for quantifying soluble mediators—such as IL-6, TNFα, PDGF-BB, TGF-β, EGF, IGF-1, and VEGF—in EV-containing plasma and cell-culture supernatants, using commercially available ELISA kits from R&D and Peprotech. This protocol was implemented within the Nanostructurome methods pipeline.

4. Health & Safety Warning

The hazards and safety measures regarding working with blood-derived products are described in Guidance on Working Safely with Human Blood and Plasma (https://www.sgul.ac.uk/about/our-professional-services/safety-health-and-environment/documents/guidance-on-working-safely-with-human-blood-or-plasma.pdf.) and must be carefully followed by the staff which should always wear suitable DPI during the procedure to reduce risk.

5. Cautions

The disposal of consumables should be in accordance with applicable laws and good research and laboratory practices. All non-disposable equipment should be disinfected at the beginning and end of the procedure. Adequate aeration of the room should be provided. An electronic or paper register for user reservations of the spectrophotometer should be provided. Operating procedures of the spectrophotometer, safety cabinet, pipettes and vacuum pump should be available for the users.

6. Personnel Qualifications / Responsibilities

When testing plasma samples, the staff should be informed regarding the hazards connected to testing these samples. The staff should undergo training on how to handle blood-derived samples. Usually, staff members with work experience in a biological/cell laboratory such as re-searchers are qualified enough to test these samples.

7. Materials, Equipment and Supplies

7.1 Samples:

Plasma samples must be transported to the laboratory at room temperature and processed immediately. If immediate processing is not possible, samples should be stored at -80*C and used immediately after tawing. Multiple freezing and thawing steps must be avoided. The samples should be tested as soon as possible after thawing. The same principles apply to cell-culture supernatants.

7.2 Other Equipment, Materials and Consumables:

Consumables usually provided within the ELISA kits or ELISA accessory kits: 96-well plate, standards, blocking buffer, sample diluent concentrate, assay buffer concentrate, capture and biotinylated detection antibodies, streptavidin or avidin – HRP, wash buffer, TMB or ABTS (chromogen), stop solution, adhesive plate covers.

Extra equipment and consumables: reagent reservoirs, PBS, spectrophotometer, multichannel and single-channel pipettes, tips, safety cabinet, waste disposal bins, protective gloves, goggles, lab coats, glass bottles to dilute diluents and buffer concentrates, - 80*C freezer and fridge for sample and material storage, plastic tubes, ddH_2O and sterile milliQ water to dilute reagents, vacuum pump with container to collect liquid waste.

8. Computer Hardware & Software

Hardware: PC, plate reader. Software: Microsoft Excel, GraphPad Prism, online data analysis resources such as assayfit pro. Data saving/sharing: Cloud and/or Drive documents.

9. Step by Step Procedure

The procedure is highly dependent on the type of kit used and the specific analyte/sample being investigated. These steps are always thoroughly detailed in the kit protocol.

For example, for measuring the analytes in EVs-containing plasma (cytokines and growth factors), we use Peprotech and R&D kits. Below, we provide a general step-by-step procedure; for more detailed instructions, please refer to the respective kit protocol.

9.1 Reconsituting and Storage of the Reagents:

- Reconstitute capture Ab and detection Ab reconstituted in the most appropriated solvent and store the aliquots at -20*C.
- Reconstitute standards in the most appropriate solvent and store the aliquots at -20*C.
- Dilute streptavidin-HRP or avidin-HRP in the most appropriate solvent and store the aliquots at 2-8˚C or at -20*C respectively.

9.2 Plate Coating with Capture Ab and Blocking:

- Dilute capture Ab in the most appropriated solvent to the concentration specified in the kit and add it to each well.
- Cover the plate with adhesive covers and incubate overnight at RT.
- Wash the plate 4x with the wash buffer and add the blocking buffer to each well. This step will reduce nonspecific binding.
- Incubate for at least 1 hour at RT and then repeat the washing steps.

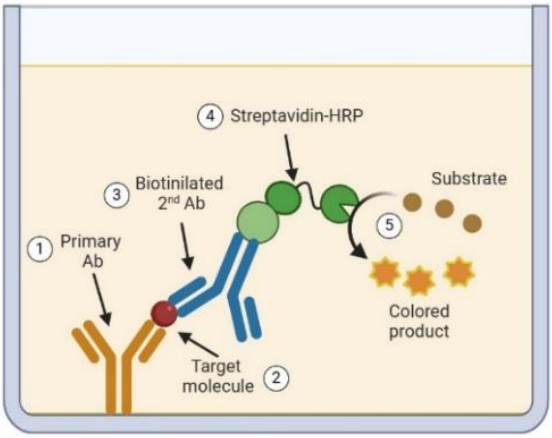

Figure 1: steps of a sandwich ELISA. 1) Plate coating with capture, 2) Incubation with the sample containing the target molecule, 3) addition of biotinylated detection Ab, 4) Incubation with the streptavidin-horseradish peroxidase, 5) colorimetric reaction induction by addition of the HRP substrate (e.g. TMB). Data are then acquired with a spectrophotometer after stopping the reaction (if necessary) using HCl. Image created with Biorender.com

9.3 Standard and Sample Preparation:

- Unfreeze and vortex the samples. Depending on the analyte under investigation, the sample should be diluted differently, For, example, the analytes mentioned in section 3 are diluted as shown in **Table 1**.

Table 1: table showing the most appropriated sample dilution factors for a given analyte.

Target	Dilution Factor	KIT
IL-6	Pure	ELISA TMB Peprotech
TNF-α	Pure	ELISA TMB Peprotech
VEGF	Pure	ELISA TMB Peprotech
EGF	1:2	ELISA ABTS Peprotech
PDGF-BB	1:50	ELISA ABTS Peprotech
IGF-1	1:50	ELISA TMB R&D
TGF-β1	Free, pure/ Total - 1:80	ELISA TMB R&D

- The dilution factors were determined by performing pilot experiments. *NOTE*: TGF-β1 can be found in the plasma both in an "active" (or free) form or in an "inactive" form which is bound to proteins of the Large Latent Complex (LLC) (11). The inactive form cannot be measured by the ELISA unless the sample is exposed to 1N HCI to allow LLC denaturation and protein release. After neutralization with 1N NaOH the sample can be added to the plate and measured. This process will allow to detect the total amount of TGF-β1 present in the sample (active + inactive).
- Add undiluted or diluted samples in the plate at least in duplicates.
- Unfreeze the standard aliquots and prepare 8 serial dilutions from the highest (concentration is kit dependent) to zero in the most appropriated solvent. Add the standard to the plate at least in duplicates.
- Incubate the plate at RT for 2 h.

9.4 Analyte Detection and Colorimetric Reaction:

- Wash the plate 4 times.
- Prepare the detection Ab by diluting the stock solution in diluent to the concentration specified within the kit. Add 100µl per well.
- Keep the plate at RT for 2 h then wash again 4 times.
- Prepare the Streptavidin-HRP or Avidin-HRP Conjugate by diluting the stock in The concentration specified in the kit and aliquot it in each well and incubate 30 min at RT.
- Wash the plate 4 times. Then add TMB (for plates incubated with streptavidin-HRP) or ABTS (to plates incubated with Avidin-HRP) to each well and allow the colorimetric reaction to develop at RT.
- For TMB kits wait at least 20 min, then add the stop solution and read absorbance at 450nm with wavelength correction set at 620nm.
- For ABTS kits instead measure the plate over time at 405nm with wavelength correction at 650nm. Save the data when the O.D of the highest standard in the curve reaches 1.4 units.

9.5 Data Acquisition

Save the excel file containing the results provided by the spectrophotometer. Copy the obtained O.D. values in the favorite analysis software. For example, use the online ELISA assay results calculator: assayfit pro. In assayfit pro insert the plate layout, the samples dilution factors and the standard curve fit input values. Analyze the data using a four-parameter logistic (4-PL) curve-fit. Ensue that the R2 value of the curve fir is > 0.99. Analyze and plot the obtained concentration data with GraphPad Prism.

9.6 Troubleshooting

High background:

- No/not enough washing steps.
- No/incorrect/insufficient plate blocking.
- Longer incubation times.
- Cross-reactivity / contaminated buffer / sample cross-contamination.

The signal is weak or absent:

- Sample not enough concentrated. Consider using a kit with a lower detection range.
- Incorrect storage, preparation or dilution of the components.
- Expired or omitted reagents.
- Enzyme inhibitor present or the assay is performed at too low temperatures.
- The plate reader is not set to read the correct wavelength.

The signal is too high:

- Incorrect dilution/preparation of reagents.
- Not enough washing steps.
- Too long incubation of the reagents (e.g. HRP)

Edge effects:

- Different temperature across plate.
- Evaporation – use adhesive plates covers.

Poor standard curves:

- Pipetting errors or incorrect calculations/pipetting technique.
- Reduced capture Ab binding: use a proper plate.
- Wells not properly aspirated or poorly mixed reagents.
- High coefficient of variation (CV) among sample replicates:
- Pipetting errors
- Contamination of plates or reagents.
- Different temperature across plate or evaporation – use adhesive plates covers.

10. Data and Records Management

All experimental details must be recorded in detail in the lab book. Both raw and processed data must be stored electronically both in the staff computers, in the cloud and in a physical backup for 10 years after generation.

11. Waste Management

Gloves, liquid waste, plates, tubes, tips and all plasticware which was in contact with the samples must be disposed of in biological sanitary waste, in compliance with applicable laws and proper research laboratory practices.

12. Related Protocols or SOPs

Not applicable.

13. Quality Control and Quality Assurance

Usually linearity range, linearity, lower limit of detection, precision, recovery and specificity tests are routinely performed by the kit producer and results are described within the data sheet of the specific kit. Some of these parameters change depending on the kit lot number and thus must be always carefully consulted before assay performance and purchase of the kit. To ensure the generation of quality data during the assay is important to: 1) run standard curves on every plate, 2) run samples/standards in duplicates/triplicates. The coefficient of variation (CV) of the samples/standard should be ≤ 20%, 3) include background controls and (if possible) positive controls, 4) Analyze the data using a 4-parameter curve fit, and 5) ensure that the O.D. values of the samples fall in the linear range of the standard curve. This will ensure the correct quantification of the analyte of interest. To allow this, consider sample dilution.

It is important to note however that human – derived samples composition might strongly differ because of donor-to-donor variance. To obtain reliable results with O.D. values that are in the linear range, is important to either: 1) perform a detailed literature research to determine the expected range of analyte concentration in healthy/non-healthy plasma in order to define (if necessary) the most suitable dilution factor of the samples, 2) if point one is not possible, perform a pre-test of the sample at different concentrations in order to define the dilution factor.

13.1. Instrument Calibration

Periodically calibrate the spectrophotometer using the appropriate calibration microplate. Calibration must be performed by a laboratory technician or by trained personnel

13.2. Critical Processes Parameters and Checkpoints

The temperature and incubation time of samples and capture/detection antibodies are critical during the assay performance. Additionally, sample / reagent storage and preparation can strongly influence the results.

14. Data on procedures and samples

Data on procedures and samples are given in **Table 2**.

Table 2. Data on procedures and samples

Description of the sample	Liquid. If not tested immediately, must be stored frozen at -80°C. Repeated freeze-thaw cycles mush be avoided.
Aliquots needed	Depends on the number of analytes to be investigated. If IL-6, TNFα, PDGF-BB, TGF-β, EGF, IGF-1, and VEGF are under investigation: 3 aliquots with at least 1ml, 200 µl and 400 µl are needed.
Total volume of the sample	300 µl per type of analyte (if tested in triplicates and undiluted).
Time required to obtain results	2 days
Manpower	Skilled researcher
Estimated cost per kit without manpower	Kit price: 300-600 euro each.
Contact person	Damjana Drobne, damjana.drobne@bf.uni-lj.si

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Conflicts of Interest:

The authors declare no conflict of interest.

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