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Implementation of proximity ligation assay to quantify low protein concentrations in low volume samples

Bachelor assignment

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Commission

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Abstract

It is of great importance to understand the influence of different substances on the human body. Vessels-on-a-chip are used to mimic the blood vessels and find the influence of the substances on the endothelial cells covering the inside. Several studies have shown that inflammatory cytokines in blood plasma can disrupt these endothelial cells. A different student at AST used ELISA to quantify the concentration of these cytokines with ELISA but one cytokine, IL-1β could not be measured due to its low concentration, therefore another method, SP-PLA, is set up. This method is able to quantify low concentration and low volume samples. When implemented successfully this method could later be used to quantify a broader range of proteins.

PLA probes were used to label the IL-1β antigen with oligonucleotides that were amplified with qPCR. A standard series was made to check the overall fitness of the SP-PLA. The qPCR data showed that the basics of the SP-PLA are working but it still has to be improved. The standard deviations of the standard series are low but a significant calibration curve could not be constructed.

IL-1β samples could not yet be measured, some further research is necessary to make the SP-PLA operable. However, when this is the case, the SP-PLA has the potential to become the standard to quantify low concentration and low volume samples.

Samenvatting

Het is van groot belang om de invloed van verschillende stoffen op het menselijk lichaam te begrijpen. Vessels-on-a-chip kunnen worden gebruikt om de bloedvaten na te bootsen en de invloed van de stoffen op de endotheelcellen die de binnenkant bedekken te vinden. Verschillende studies hebben aangetoond dat ontsteking cytokines in het bloedplasma deze endotheelcellen kunnen verstoren. Een andere student van AST heeft ELISA gebruikt om de concentratie van deze cytokines te kwantificeren, maar één cytokine, IL-1β , kon niet worden gemeten vanwege de lage concentratie, daarom is een andere methode, SP-PLA, opgezet. Deze methode is in staat om monsters met een lage concentratie en een laag volume te kwantificeren. Als deze methode met succes is geïmplementeerd, kan deze later worden gebruikt om een breder scala aan eiwitten te kwantificeren. PLA-probes werden gebruikt om het IL-1β -antigeen te labelen met oligonucleotiden die werden geamplificeerd met qPCR. Er is een standaardserie gemaakt om de algemene werking van de SP-PLA te controleren. De qPCR-gegevens toonden aan dat de basis van de SP-PLA werkt, maar er nog wel verbeterpunten zijn. De standaarddeviaties van de standaardreeksen zijn laag, maar een significante kalibratiecurve kon niet worden geconstrueerd. IL-1β monsters konden nog niet gemeten worden en verder onderzoek is nodig om de SP-PLA operationeel te maken. Wanneer dit echter het geval is, heeft de SP-PLA het potentieel om de standaard te worden voor het kwantificeren van monsters met een lage concentratie en een laag volume.

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Contents

1 Introduction

The possibility to research the impact of different substances on the human body without having to actually test it on a real person is, needless to say, something of great importance. Currently there are a few ways to kind of mimic the human body and allow us to test a broad spectrum of things what could otherwise possibly hurt a human being.

1.1 In vivo, in vitro and organ-on-chip models

In vivo (within the living) animal models are across the most reliable methods to mimic the human body[1]. Experimental drugs, new treatments and research protocols are normally tested in vivo before being used on humans because the effect of the drugs on the different tissue levels can be monitored[2]. In vivo models have been on the foreground in genetic regulation and disease modelling[3]. But there are some disadvantages with animal model systems. It is expensive to set up, the results take long to arrive and although the models contain most of the complexity of the human body it still faces translatability issues since there are still differences between the two. And there is the moral part of using live animals for research, which also results in stricter testing regulations[4].

Another method to mimic the human body is the use of in vitro (within the glass) models, where cells are grown in a Petri dish under controlled circumstances. This method has been used for decades and proved to be especially useful to understand signalling mechanisms, cell proliferation and the influence of chemicals on specific cells[5]. It also allows the use of human cells which eliminates the translatability problem and also creates the possibility of personalized medicine when specific donor cells are used. Thereby is this method of testing relatively cheap, results are in quick and the cells used are often derived from cell-lines that are infinite. This all comes at the cost that in vitro models lack overall complexity and are too simple to be used as a standalone method, therefore in vitro findings will almost always be confirmed by in vivo studies[2,4].

Organs-on-chips are able to combine the advantages of both in vivo and in vitro studies. These are microfluidic systems that offers a microenvironment for cells that resembles complex in vivo circumstances better than in vitro does, while also maintaining the ability to use human tissue and to control the microenvironment[6]. The chips are used not to mimic a whole organ or tissue but to create small functional units that summarizes its function. Simple systems mimic only one tissue by using a single microfluidic unit with one cell type. More complex systems can exist of many connected microfluidic units inhibited by different cell types, this can be used to mimic interactions between different tissues, for instance to monitor the oxygen release through blood vessels. A disadvantage from the use of the chips is its small scale, therefore they yield very little sample to analyse.

Figure 1 In vivo, in vitro and organ-on-chip diagnostics. Image created in BioRender.

1.2 Vessel-on-a-chip models

One of the most important tasks of the human body is the supply of nutrients and disposal of waste products. This transport is mostly done via a complex network of blood vessels, the blood vasculature. Studies have shown that abnormalities in the formation of this network can lead to severe disorders or even the inability to live[3]. With organs-on-chips blood vessels can be modelled which in this case is referred to as vessel-on-a-chip models. Currently the use of soft-lithography with poly(dimethyl siloxane), a clear gas-permeable silicone elastomer, is the widely adapted method to create microfabricated vessels. One of the most recurring problems with blood vessels is the improper function and damage of the endothelial cells covering the inside of all blood vessels[7]. The microfabricated vessels can be coated with endothelial cells which allows researching the impact of different substances on the endothelial cells and the accessory response. Studies already found out that the endothelial cells excrete increased amounts of different inflammatory cytokines when being harmed[8].

1.3 Protein quantification methods

It is necessary to be able to quantify inflammatory cytokines in order to create chips that can compare the results of patients with different inflammatory cytokine concentrations. There are methods that have been well known for the past years such as ELISA and mass spectrometry, but the last years new methods have seen the light of day for instance solid-phase proximity ligation assay (SP-PLA) and bioluminescent technology.

1.3.1 ELISA

ELISA stands for Enzyme Linked Immunosorbent Assay and is a labelled immunoassay that is currently regarded to as the gold standard of immunoassays[9]. An ELISA is a very specific test due to the use of antibodies and is used to quantify a wide range of proteins, including cytokines. ELISA is based on four main general steps, coating, blocking, detection and a final read. First a plate is coated with either antibodies or antigens after which unbound sites of the plate are blocked with blocking agents such as BSA or other animal proteins. Followed by the incubation of the antibody with an enzyme connected to it to make the reaction detectable. Thereafter detection finds place with the use of a substrate that can generate a colour. Most commonly used is horseradish peroxidase, which uses hydrogen peroxide as a substrate and generates a blue colour change, and alkaline phosphatase which uses P-Nitrophenyl-phosphate as a substrate and generates a yellow colour change. Finally the colour generation is stopped using a stop solution and the plate is examined with a fluorescence plate reader at the corresponding wavelengths. In between each step the plates are washed to reduce background signal and unspecific binding. There are a few different types of ELISA with different protocols but they are all based on the four steps stated above. ELISA is an overall good technique, its lowest limit of detection for some cytokines is around 0.5 pg/ml with the use of a 100 µl[10] sample[11]. For some techniques it may be difficult to obtain such large values for the purpose of measuring only one type of cytokine.

1.3.2 Mass spectrometry

Mass spectrometry (MS) is technique that is able to distinguish different samples based on a difference in charge and weight[12]. There is a wide variety of MS methods with different aspects and advantages these methods will be divided in two methods that are able to quantify proteins, with and without labelling of the samples. With the first method the sample will be

labelled with a stable isotope while sample with a known concentration, will be labelled with a stable isotope with a slightly different weight. The two samples are then mixed and analysed. The corresponding graph will show two intensity peaks and the ratio between the two can be used to calculate the concentration of the first sample. It is also possible to quantify the samples when they cannot be labelled because it is impractical or too expensive. Normally when non-label methods are used samples will be analysed apart from each other and the peptide ion peaks will be integrated and used as a measure of quantity. Different samples can be relatively compared to each other or a standard curve can be made to calculate the exact values. Mass spectrometry does have a quite high limit of detection with 900 pg/ml[13][14]. This is above cytokine concentrations in blood plasma so it can be hard to use this method to detect cytokines.

1.3.3 Bioluminescent immunoassay

The bioluminescent immunoassay is a new technique that uses the bioluminescence of a luciferase to quantify proteins[15]. The luciferase is split into a big and a small subunit, these subunits are not bioluminescent and only become an active form of luciferase when they are in proximity of each other. The subunits are coupled to two different antibodies that are directed against the target of interest, when these antibodies bind to the target of interest the subunits will be close enough to form the luciferase. A substrate can then be added and the samples can be analysed with a fluorescence plate reader. The loose subunits have a low affinity for each other and will only form their active luciferase when both of the antibodies are coupled to the same antigen, so almost no background noise will occur. Bioluminescent assays have a limit of detection of around 6 pg/ml[16]. Also a 100 µl sample is needed so it has the same problem as ELISA that it can be difficult to obtain such volumes with for instance organs-on-chip.

1.3.4 Proximity ligation assay

Proximity ligation assay (PLA) is a new detection method that combines the specificity of an immunoassay with the sensitivity of DNA replication techniques[11][17]. There are a few PLA variations but they are all based on the use of PLA probes(antigen specific antibodies linked with oligonucleotides), whom will allow a cascade of reactions when in proximity of each other, and the use of a DNA replication technique. The PLA probes are added to samples containing antigen. When the probes are bound to the antigen the oligonucleotides on the probes are in proximity to each other. Different methods use different read-out systems, one of the methods uses a connector piece oligonucleotide to form one nucleotide which is then ligated and detected with qPCR. The other method is based on rolling circle DNA synthesis with the two pieces of nucleotides, two other DNA nucleotides are added together with specific substrates and enzymes. The substrates and enzymes form high concentration of fluorescence the more the DNA is replicated and this signal can be read with a fluorescence plate reader. PLA has a very low limit of detection, due to the use of DNA replication techniques, it is possible to detect cytokines with a concentration of 0.1 pg/ml[18]. Also it only needs 1 µl of sample which is much easier to gather from experiments which yield little sample volume[11].

1.4 Problem analysis

For a vessel-on-a-chip model different concentrations of cytokines need to be measured. Experiments done before showed that for a specific cytokine, IL-1β, concentrations could not be measured since its concentration was below the range of the ELISA kit. A variation of the proximity ligation assay, solid-phase proximity ligation assay (SP-PLA) will be used to detect the values of this cytokine. This SP-PLA needs little sample volume and has a lower limit of detection well below that of ELISA. The implementation of SP-PLA has been tried before but the results were not as expected, meaning the protocol has to be improved. When the SP-PLA has been proved successfully for IL-1β this method can be implemented on a bigger scale to make it possible to measure a lot of different chemicals in organ-on-chip models despite their low sample yield.

1.5 Goal of the research and hypothesis

The main goal of this research is to set up SP-PLA to measure the values of IL-1β in blood plasma. First a standard series will be made to prove the method has been performed correctly. Thereafter the SP-PLA can be used to measure the blood plasma samples. The bigger goal is to implement SP-PLA as the new standard for quantifying proteins in small samples. The overall research question is:

What are the concentrations of the cytokine IL-1β in blood plasma samples quantified with solid-phase proximity ligation assay (SP-PLA)? Can this method be used as the new standard in protein quantification when sample volumes and concentrations are too low to use ELISA?

The expectation is that with SP-PLA the quantification of IL-1β in blood plasma will be successful. The lower limit of detection from PLA is lower than ELISA and include the range of cytokine concentrations in blood plasma. It is possible to modify the antibodies used in the PLA which makes it possible to quantify other proteins. This is the only change in the protocol that should be necessary and therefore the SP-PLA could be used as the new standard of quantifying proteins.

2 Theoretical framework

In this part the theory behind sandwich ELISA and solid-phase PLA will be discussed, these are specific ways to perform the general methods as explained above. The different crucial steps for each method will be further elaborated, the use of the different reagents will be clarified and the analysis of the data will be explained.

2.1 Sandwich ELISA

A sandwich ELISA is one of the four ELISA methods as explained in the introduction. It is called sandwich ELISA because the protein of interest in the final product is captured in between two antibodies. This ELISA uses two different antibodies, capture and detection. These antibodies are first diluted with their corresponding dilution solution before use. The capture antibodies are first coated on a microwell plate and they will stay in that place during the whole process. After this coating the rest of the binding sites of the plate will be blocked after which samples containing the antigen are added to the wells and these are captured on the capture antibody. Finally the detection antibodies, labelled with biotin, are added and bound to the antigen.

The antigen samples are Human IL-1β and the antibodies and reagents are provided by a standard IL-1β ELISA kit. The enzyme used with this ELISA is streptavidin coated Horseradish Peroxidase (HRP), this enzyme is bound to the biotin coated detection antibody after which a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution is added. This solution is oxidized by the HRP which yields a blue colour change that is dependent on the amount of HRP in each well which is a gauge for the amount of antigen in each well. The incubation time of the oxidation reaction between HRP and the substrate has an optimum for good interpretation within it linear range, therefore it has to be stopped.

The reaction is stopped with stop solution, this will change the blue colour of the wells to yellow and it makes the whole plate stable for approximately an hour. It is necessary to add the stop solution to each well at the same time to prevent a difference in reaction time between wells which will result in altered results. During the stable period the plate will be read using a fluorescence plate reader. Two measurements will be made for each plate, one at 450 nm for the primary signal and one at 570 nm for the background signal. The 570 nm values are then subtracted from the 450 nm signal to get the final results. This whole reaction can be seen schematically in figure 2[19] . In between each step all the wells are washed with washing buffer to remove unbound reagents and reduce background noise.

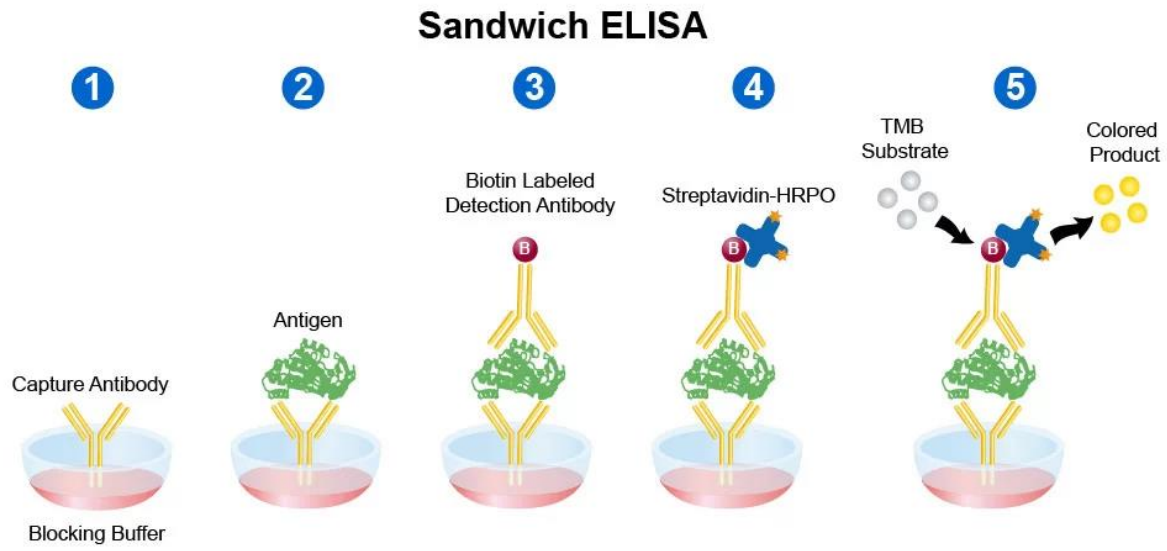

Figure 2 Different steps of the sandwich ELISA shown schematically. 1: Antibody is captured on the well plate. 2: Antigen is bound to the antibody. 3: Biotin labelled antibody is bound to the antigen. 4: Via streptavidin-biotin coupling HRP is bound to the antibody. 5: With TMB substrate a coloured product is formed.

2.2 Solid-phase PLA

Solid-phase PLA (SP-PLA) is a specific way of PLA which uses a solid support were the whole complex with antigen, antibodies and PLA probes is captured on. This allows the addition of washing steps which washes away unbound substances and thus reduce background noise. There are a lot of different components and incubation steps all with their own importance for the SP-PLA to work. The process is shown schematically in figure 3[11].

2.2.1 Solid support

Streptavidin-coated magnetic Dynabeads will be used as solid support. A biotin-coated antibody specific for mouse IL-1β will be immobilized on these magnetic beads via the streptavidin-biotin binding, which is one of the strongest non-covalent bindings in nature. The magnetism of the beads allows them to be pulled away with a magnet so that the supernatant can be removed and the beads can be washed. All of the non-bound sites on the beads will be blocked with BSA .

2.2.2 PLA probes

The PLA probes consist of a mouse IL-1β specific antibody which are chemically crosslinked with pieces of oligonucleotides. There are two oligonucleotides which both will be bound to a different antibody to get two different probes. The two oligonucleotides are both 50 to 60 bp long and they contain both a ligation and a PCR primer site. The ligation side is complementary to another oligonucleotide, this is the connector that binds both oligonucleotides with each other. One oligonucleotide both has a free 5'end that is phosphorylated which, in combination with the free 3' end (non-phosphorylated) of the other oligonucleotide, allows the ligation reaction to happen. The other free end of both oligonucleotides is thiol-modified, this allows for crosslinking to the antibody. For this crosslinking sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) will be used, this is an agent that is able to connect the thiol group of the nucleotides to the free amines in the antibody.

2.2.3 Quantitative PCR

After the ligation of the oligonucleotides, they will be detected using quantitative PCR (qPCR). A qPCR probe will be used to detect the amount of DNA after each PCR cycle. The probe contains a fluorescent dye, FAM, which fluorescence is normally decreased due to the presence of a dark quencher in the same probe. This probe anneals to its complementary sequence in between the forward and reverse primer binding sites on the single stranded DNA, when this piece of DNA is elongated the fluorescent dye will be released. Since the dark quencher is not in proximity of the dye anymore, it will become fluorescent.

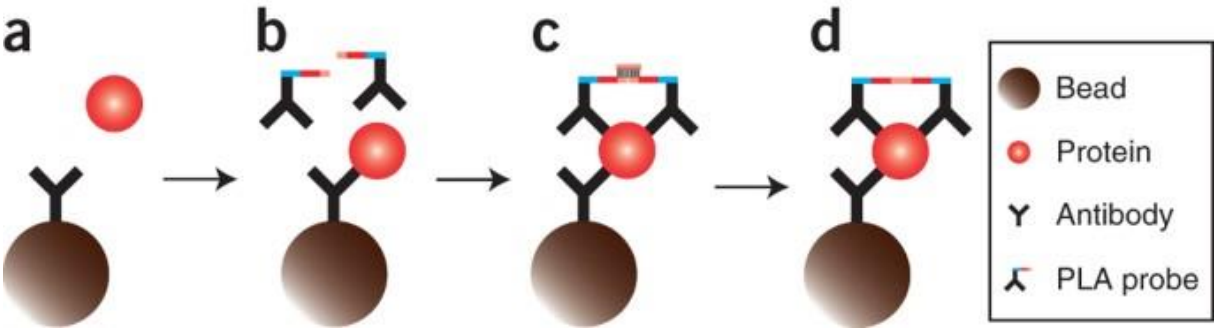

Figure 3 SP-PLA shown schematically. a: antigen is captured on antibody coated beads. b: PLA probes are introduced and bound to the antigen. c: Oligonucleotides are in proximity and will be ligated. d: Oligonucleotides are ligated and qPCR can be performed

2.3 Data analysis

From both the sandwich ELISA and SP-PLA a data set is obtained with a set of points corresponding with the concentration of IL-1β. These points are fluorescence units for the ELISA and the threshold cycle in which the signal came above the base line for the PLA. These points are then plotted against the known IL-1β concentrations, after which a regression line is drawn through these points. Since the samples tested were only standard series this line is regarded to as the standard curve. Then the coefficient of determination (R^2) is calculated of this regression line, this is a statistical method that shows the correlation between the data points and the regression line. Ideally this factor has a value of 1, which means that 100% of the variance of the dependent variable being studied is explained by the variance of the independent variable.

3 Material and methods

In this section all the reagents and equipment used during the experiments will be listed. Also the used procedures will be further elaborated.

3.1 Materials

3.1.1Elisa reagents

Table 1 ELISA reagents

Component	**Supplier**	**Catalog Number**
Human IL-1β ELISA MAX™ Deluxe Set	BioLegend	437004
Human IL-1β ELISA MAX™ Capture Antibody (200X)	ELISA kit	
Human IL-1β ELISA MAX™ Detection Antibody (200X) Avidin-HRP (1000X) Substrate Solution F Coating Buffer A (5X) Assay Diluent A (5X) Assay Buffer D	ELISA kit	
Human IL-1β antigen (0.2 µg/ml)	PeproTech	200-01β
PBS tablets	Thermo Scientific	P4417
Tween-20	Sigma-Aldrich	P9416
ELISA stop solution	Thermo Scientific	SS04
High purity water	mQ device	

3.1.2 Elisa equipment

Table 2 ELISA equipment

Component	**Supplier**	**Catalog Number**
Microwell plate	Thermo Scientific	44-2404-21
Self-adhesive PCR film	Bio-Rad	MSB-1001
Multiskan™ GO Microplate Spectrophotometer	Thermo Scientific	p-4530546
Various adjustable pipettes (0.2 µl – 1000 µl)	Gilson	
Multichannel pipettor	Eppendorf	3125000060
Maximum recovery filter tips	Axygen	TF-200-L-R-S
Pipette tips PCR grade	Tip one	10 µl: S1121-3810 200 µl: S1120-1810 1000 µl: S1122-1830
1.5 ml tubes (sterilized)	Eppendorf	0030120086
Stericup 0.45 µM filter	Sigma-Aldrich	S2HVU11RE
Orbital shaker	IKA	KS 260

3.1.3 SP-PLA reagents

Table 3 SP-PLA reagents

Component	Supplier	Catalog number
BSA (20 mg/ml)	New England Biolabs	B9000S
EDTA disodium salt dehydrate	Sigma-Aldrich	E5134
Goat IgG	Sigma-Aldrich	I5256-50MG
Dynabeads MyOne streptavidin T1 (10 mg/ml)	Thermo Scientific	65601
Salmon sperm DNA (10 mg/ml)	Thermo Scientific	15632-011
T4 DNA ligase (1U/µl)	Thermo Scientific	EL0016
ATP solution (100 mM)	Thermo Scientific	R0441
GeneAmp dNTP blend with dUTP (12,5 mM)	Thermo Scientific	N8080270
Uracil-DNA glycosylase (1 U/µl)	Thermo Scientific	EN0361
Oligonucleotides, specified in table 4	IDT	Custom order
Mouse IL-1 beta /IL-1F2 Biotinylated Antibody (capture)	RND	BAF401
Mouse IL-1 beta /IL-1F2 Antibody CONTAINS TREHALOSE <5%	RND	AF-401-NA
IL-1 beta antigen (0,2 µg/ml)	Pepratech	200-01β
Sulfo-SMCC (Mw 436,4 g/mol)	Thermo Scientific	22322
Dithiothreitol (DTT)	Thermo Scientific	D0632-1G
PBS (made from tablets, 0.45 µM filtered)	Thermo Scientific	18912014
D-Biotin	Life Technologies	B-1595 (1G)
Tween-20	Sigma-Aldrich	P9416
High purity water	mQ device	
UltraPure™ DNase/RNase-Free Distilled Water, molecular biology grade	Thermo scientific	10977015
Platinum taq polymerase (300 rxn)	Thermo scientific	10966026
Oligonucleotides	IDT	Custom order, details see table
Tris base	Sigma-Aldrich	1.08382.0500
Glycine	Sigma-Aldrich	G8898-500G
HCl		
NaOH		
Glycerol	Sigma-Aldrich	356350-1000mL
Bromophenol blue	Bio-Rad	161-0404

Novex 4-12% precast gel	Invitrogen	XPO4125BOX
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771
Pageruler TM Plus, prestained protein ladder 250 kDA	Thermo Scientific	26619
Methanol	VWR	20847.295
Acetic acid (HAc)	Sigma-Aldrich	100056.25003
Coomassie Brilliant Blue	Sigma-Aldrich	1154440025

3.1.4 SP-PLA oligonucleotides

Table 4 SP-PLA oligonucleotides

Oligonucleotide	**Function**	**Sequence 5' to 3'**
SLC1 thiol	PLA-probe arm left	/5Thiol /CG CAT CGC CCT TGG ACT ACG ACT GAC GAA CCG CTT TGC CTG ACT GAT CGC TAA ATC GTG
SLC2 thiol	PLA-probe arm right	/5Phos/TC GTG TCT AAA GTC CGT TAC CTT GAT TCC CCT AAC CCT CTT GAA AAA TTC GGC ATC GGT GA/3thioMC3D/
Bio forward	Primer	CATCGCCCTTGGACTACGA
Bio reverse	Primer	GGGAATCAAGGTAACGGACTTTAG
Connector Biosplint	Connector oligo	TACTTAGACACGACACGATTTAGTTT
qPCR probe	Detection with FAM dye and double quencher	5'-6-FAM-TGACGAACC/ZEN/GCTTTGCCTGA/IBFQ/-3'

3.1.5 SP-PLA equipment

Table 5 SP-PLA equipment

Component	**Supplier**	**Catalog number**
Zeba Spin desalting columns	Thermo scientific	89882
1,5 ml tubes (sterilized)	Eppendorf	0030120086
Slide-A-Lyzer Mini dialysis devices	Thermo scientific	69560
Float buoys for slide-a-lyzer	Thermo scientific	69588
Rotator end-over-end	Grant Instruments	PTR-60
PCR strip tubes with attached caps	VWR	211-0339
Self-adhesive PCR film	Biorad	MSB-1001
Multichannel pipettor (30-300 µl)	Eppendorf	3125000060
Maximum recovery filter tips	Axygen	10 µl: TXLF-10-L-R-S 200 µl:TF-200-L-R-S
Non-filter pipette tips	Tip one	200 µl: S1111-0700
Pipette tips PCR grade	Tip one	10 µl: S1121-3810 200 µl: S1120-1810 1000 µl: S1122-1830

Real time thermo cycler	Bio-Rad	C1000 CFX96
Heat block	Grant Instruments	QBT2
Hard-Shell 96 well PCR plate	Biorad	HSP9645
DynaMag-96 Side Magnet	Thermo scientific	12331D
Fluor Chem imager	Cell Biosciences	95051
Mini gel tank	Thermo Scientific	A25977

3.2 ELISA

For the ELISA wash buffer was prepared by mixing PBS and 0.05% (v/v) Tween-20. The buffer could be stored for 6 months at room temperature. Prior to the ELISA the provided reagents were diluted to the working concentrations according to the protocol provided by the manufacturer.

3.2.2 ELISA procedure

The 96-microwell plate was coated by adding 100 µl 1x capture antibody to the wells that would contain standard dilutions or controls, these were the only wells used in all subsequent steps. Plate was sealed and incubated overnight at 4°C.

The plate was washed four times, this was done by adding 300 µl wash buffer to each well and shaking the plate on the bench. Thereafter the plate was emptied by firmly tapping it on a piece of absorbent paper. To reduce the background signal and block non-specific binding 200 µl 1x assay diluent was added to each well, plate was sealed and incubated at room temperature for one hour while on a plate shaker at 200 rpm. All subsequent wash and incubation steps were performed similarly.

A standard series was prepared by an initial solution of 200 ng/ml IL-1β, this was further diluted in assay diluent to get the final concentration of 125 pg/ml. Thereafter a nine two-fold serial dilutions of this 125 pg/ml standard was performed. The 1x assay diluent was used as the zero standard.

After incubation the plate was washed four times. 50 µl of assay buffer D and 50 µl of each standard dilution was added to the appropriate wells and incubated for two hours. Plate was washed four times and 100 µl of the 1x detection antibody solution was added to each well afterwards, before an incubation step of one hour. Plate was washed 4 times and 100 µl of the 1x Avidin-HRP was added to each well after which the plate was incubated for 30 minutes. For the next five wash steps the wells were soaked with the wash buffer for at least 30 seconds before removing. After the fifth wash 100 µl of substrate solution F was added to each well, the plate was packed in aluminium foil and incubated for 20 minutes. With a multichannel pipet 100 µl of stop solution was added to each well containing the substrate solution, after which the absorbance of the plate was read within 15 minutes using the Multiskan™ GO Microplate spectrophotometer. The absorbance was read at 450 nm and 570 nm, where the 570 nm was subtracted from the 450 nm to eliminate background noise.

3.3 SP-PLA

3.3.1 Preparation of reagents

A selection of buffers and reagents had to be made for this research. For the PAGEs this selection consisted of SDS running buffer, Laemlli sample buffer, native running buffer, native sample buffer, Coomassie Brilliant Blue(CBB) staining and destaining solutions. For the PLA conjugation buffer, PLA buffer, storage buffer and wash buffer had to be made.

The native running buffer was made by mixing high purity water with 25 mM TRIS-base and 19.2 mM Glycine. The 2x native sample buffer consisted of high purity water with 200 mM tris HCL, 20% glycerol and 0.0005% bromophenol blue. The running buffer was composed of high purity water with 25 mM TRIS-base, 19.2 mM Glycine and 10% SDS. The Laemlli buffer was homemade and consisted of 0.3 M tris solution, 10% SDS, 50% glycerol and 25% β-mercapto ethanol. CBB staining solution contained 0.25% CBB and 10% acetic acid in a 1:1 methanol/high purity water solution.

Wash buffer was made as described in the ELISA protocol. The conjugation buffer consisted 5 mM EDTA in 1x PBS. Storage buffer was made by adding BSA to 1x PBS to reach a final concentration of 1 mg/ml BSA in PBS. PLA buffer was composed of 1x PBS with 0.046% (v/v) Tween-20, 1 mM D-Biotin, 100 nM Goat IgG, 5mM EDTA, 1 mg/ml BSA and 0.1 mg/ml Salmon sperm DNA.

3.3.2 Crosslinking antibodies with oligonucleotides

Antibodies had to be crosslinked with sulfo-SMCC and thiol-oligonucleotides in order to create the bifunctional PLA probes. Per probe the mouse IL-1β antibodies (AF-401-NA) were activated by mixing 10 µl 13.3 µM antibody with 1uL 4 mM sulfo-SMCC in a 1.5 ml tube. Tubes were mixed gently and incubated for 2 hours at room temperature. The reduction of both oligonucleotides was initiated 40 minutes after the incubation of the antibodies started. DTT was prepared fresh by adding 2.58 mg DTT to 167.3 µl conjugation buffer for a final concentration of 100 mM DTT. 4 µl 100 µM thiol-modified oligonucleotides was mixed with 11 µl conjugation buffer and 15 µl DTT solution in a 1.5 ml tube. This was done separately for both SLC1 and SLC2. Both tubes were incubated for 1 hour at 37° C to make 13.3 µM activated oligonucleotide. The surplus of DTT, sulfo-SMCC and storage buffer needs to be removed to obtain pristine probes. This was done by changing the storage buffer for both the oligonucleotides and the antibodies to conjugation buffer with the use of four Zeba desalting columns. The buffer change was done according to the accessory protocol. After the buffer was changed antibodies were mixed with the oligonucleotides and dialyzed in 7-kDa MWCO cups against PBS. After the dialysis the remnant was topped up to 100 µl with storage buffer creating two 1.33 µM probe mixes.

3.3.3 Polyacrylamide gel electrophoresis (PAGE)

After the conjugation of the mouse IL-1β antibodies to the SLC1 and SLC2 oligonucleotides two PAGEs were run to check if the conjugation was successful. The SDS running and Laemmli sample buffer were used for the SDS-PAGE and the native running and native sample buffer were used for the native-PAGE. The gels used were Novex 4-12% tris/glyc gels. The gels were taken out of the plastic and prepared according to the manufacturer guidelines. The samples that were analysed with the gel were diluted with high purity water and sample buffer. Sample containing the Laemmli sample buffer were cooked for 5 minutes at 95°C. Sample were loaded in the wells on top of the gel together with a prestained protein ladder used for reference. The lid was placed on top and connected to an Amersham power supply which was run at 30 mA for approximately 70 minutes with a voltage cap of 150V. After the run was done the gel was removed from the cassette and stained for 30 minutes with the CBB staining solution whereafter it was destained three times with the CBB destaining solution. A kim-wipe was put in the box during the destaining to enhance the reaction. After the destaining gel was imaged on the Fluor Chem imager.

3.3.4 Immobilize capture antibody on magnetic beads

Streptavidin-coated Dynabeads were resuspended carefully to achieve a homogeneous suspension. 100 µl was transferred to an 1.5 ml tube and washed three times, during the washing steps a magnet was kept to the side of the tube to keep the beads inside. IL-1β antibody (BAF401) was diluted to 50 nM in storage buffer. Antibodies were added to the beads and incubated for one hour after which the now antibody covered beads were washed again and stored in storage buffer.

3.3.5 Preparation of the standard series

Two different IL-1β antigen standard series were made as a control for the PLA. All standard series were made from a 0.2 ug/ml IL-1β standard. PLA buffer served as the zero standard and was used to dilute the IL-1β antigen. The first IL-1β standard series used was a two-fold dilution series from 125 pg/ml to 2.0 pg/ml. The second IL-1β standard series used was a five-fold dilution series from 1562 pg/ml to 0.1 pg/ml.

3.3.6 Capture IL-1β antigen on antibody-coated beads

The antibody-coated beads were carefully resuspended to reach a homogeneous suspension. Per reaction 1 µl of beads was transferred to a new 1.5 ml tube and a magnet was placed to the side after which the storage buffer was removed. Per reaction 5 µl of PLA buffer was added and the beads were resuspended. 5 µl of the beads suspension was added to each well of a PCR strip together with 45 µl standard series sample. After the strips were closed they were vortexed gently and incubated at room temperature for 90 minutes on an end-over-end rotator at 18 rpm.

3.3.7 Detection of the captured proteins

After the incubation of the IL-1β antigen on the beads the PCR strips were centrifuged for 5 seconds at 1000x g. Strips were placed on a 96-well plate magnet for 30 seconds to pull all beads to the side. Supernatant was removed with a multichannel pipettor. Strips were washed by adding 100 µl of wash buffer with the same pipettor, strips were vortexed gently, centrifuged for 5 sec at 1000x g and again placed on the magnet. Supernatant was removed and strips were washed two more times. All subsequent wash steps were performed similarly. After the last wash 50 µl of PLA probe mix was added to each well. This mix was prepared by diluting the 1.33 µM PLA probes in two step to 500 pM. After a 5 minute incubation at room temperature to pre-block the probes both probes were mixed in one 1.5 ml tube to achieve a final

concentration of 250 pM per probe. After the strips were closed they were vortexed gently and incubated at room temperature for 90 minutes on an end-over-end rotator at 18 rpm.

3.3.8 Preparing ligation and real-time PCR mix

For each reaction 50 µl of PCR mix was needed, 1250 µl PCR mix was made for 3 PCR strips (24 reactions). The PCR mix for 1 reaction consisted of: 5 µl 10x PCR buffer, 2.5 µl 50 mM $MgCL_2$, 0.5 µl 10 µM qPCR probe, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.5 µl 10 µM connector bio splint, 0.04 µl 100 mM ATP, 0.8 µl 12.5 mM dNTPs, 0.3 µl 8 U/µl Taq polymerase, 0.5 µl 1 U/µl T4 DNA ligase, 0.1 µl 1 U/µl Uracil-DNA glycosylase and 38.16 µl ultra-pure H_2O. All the components were thawed on ice except for the enzymes, they were constantly kept inside a -20°C carrier. After all the components were added PCR mix was gently vortexed and put on ice.

3.3.9 Performing ligation and real-time PCR

After incubation of the PLA probes beads were washed 3 times after which 50 µl of PCR mix was added to each well with beads. Strips were mixed and incubated for 5 minutes to perform the ligation of the oligonucleotides from the PLA probes that were bound in close proximity on the beads. After the incubation strips were centrifuged for 5 seconds at 1000x g and put in the real-time thermo cycler. The sides of a hard shell 96 well PCR plate were also put in the cycler to prevent the strips from being crushed, this can be seen in appendix 7.1 figure 12. The real-time PCR was performed in three steps. For the first step everything was heated to 95°C to activate the Taq polymerase. This was followed by another 15 seconds at 95°C and then 1 minute at 60°C, this step was repeated 40 times. During the 95° step DNA is denaturated after which, at 60°C primers are bound and the single stranded DNA is elongated. The real-time PCR finished with a melting curve analysis.

4 Results

In this section alle the relevant results obtained during the research will be showed. First the ELISA standard series is shown. Thereafter the results of both the native- and SDS-PAGE are shown and finally the standard series from the solid-phase proximity ligation assay is shown.

4.1 ELISA standard series

The standard curve obtained from the human IL-1β ELISA is shown in figure 4. The data set with all the values and controls can be found in appendix 7.2.1.

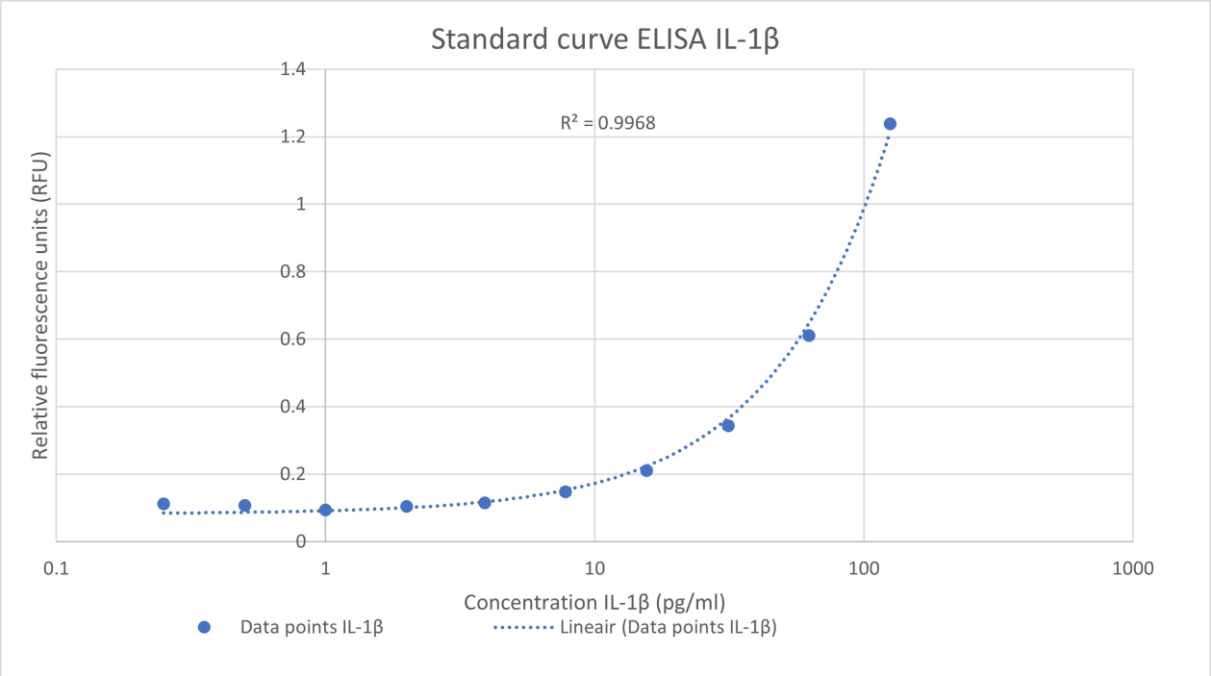

Figure 4: Calibration curve of the standard series. The concentrations are in pg/ml and on a logarithmix x-axes. On the y-axes the relative fluorescence units are shown. Standard deviations of the RFU values are included. A regression line is drawn through the data points and the corresponding coefficient of determination is shown in the graph.

The regression line has a coefficient of determination of 0.9968, which means that 99.68% of the variance of the RFU values are explained by the variance of the IL-1β standard series. The negative control was sample without IL-1β , this value is in the same range as the four lowest dilutions. The signal from the negative control is the assay background signal and since this is in the same range as the four lowest concentrations these concentrations are all outside the range of this assay. This assay shows that the used human IL-1β antigen is pristine, but the lower range of the assay is not low enough to measure the IL-1β values found in blood plasma. Therefore a SP-PLA will be performed.

4.2 Native- and SDS-PAGE

For the first SP-PLAs previously conjugated probes were used, to check if the oligonucleotides were still conjugated correctly to the antibodies two gels were run. The results of the native PAGE are shown in figure 5 and the results of the SDS-PAGE are shown in figure 6.

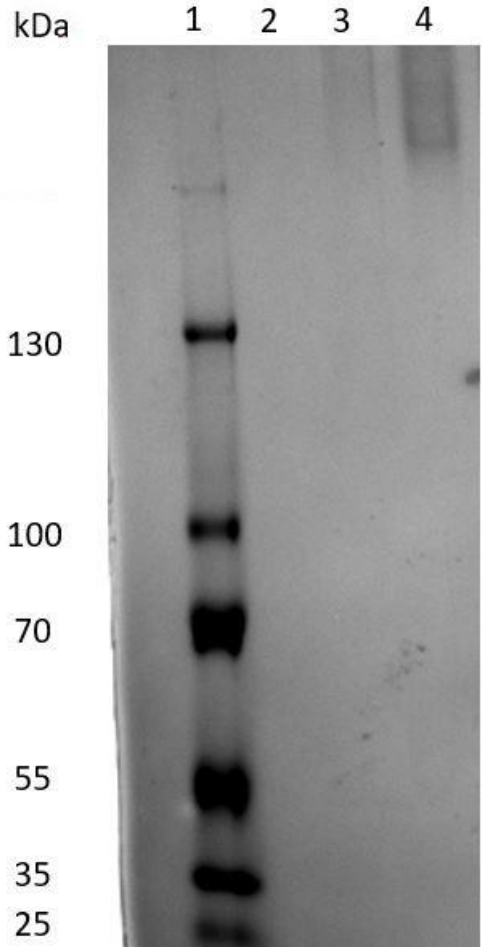

Figure 5 Native PAGE of the SLC1 probe (lane 2), SLC2 probe (lane 3) and IL-1β antibody (AF-401-NA) (lane 4). A prestained protein ladder is run in lane 1. Gel was stained with Coomassie Brilliant Blue

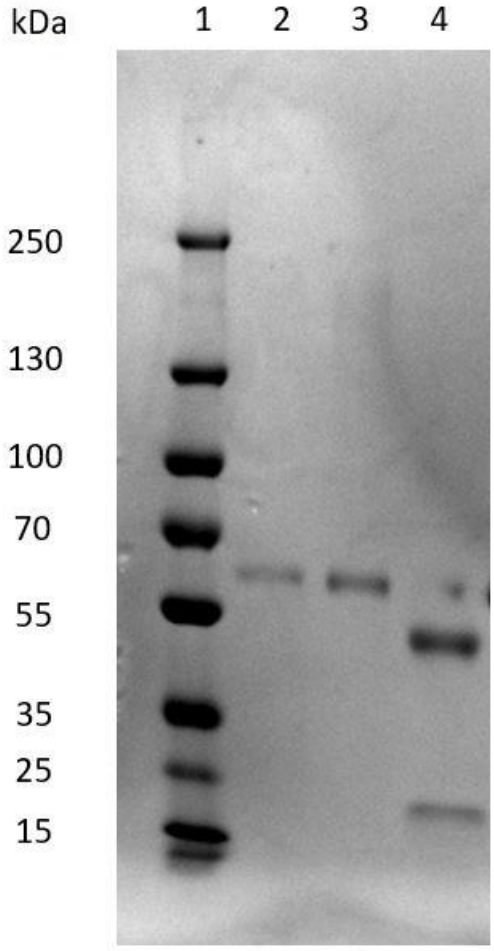

Figure 6 SDS-PAGE of the SLC1 probe (lane 2), SLC2 probe (lane 3) and IL-1β antibody (AF-401-NA) (lane 4). A prestained protein ladder is run in lane 1. Gel was stained with Coomassie Brilliant Blue

In both PAGEs a prestained protein ladder is shown with its corresponding molar mass values. In figure 5 three smeared bands can be seen. The band in lane 2 is barely visible while the bands in lane 3 and 4 are clearly visible, the band in lane 4 is thicker than the band in lane 3. In figure 6 two bands around 65 kDa can be seen in lane 2 and 3. In lane 4 there are two bands approximately at 50 and 20 kDa.

New probes were made during the research, these were also checked with a native- and a SDS-PAGE to see if the oligonucleotides were correctly conjugated to the antibodies. The results of the native PAGE can be seen in figure 7 and the SDS-PAGE can be seen in figure 8.

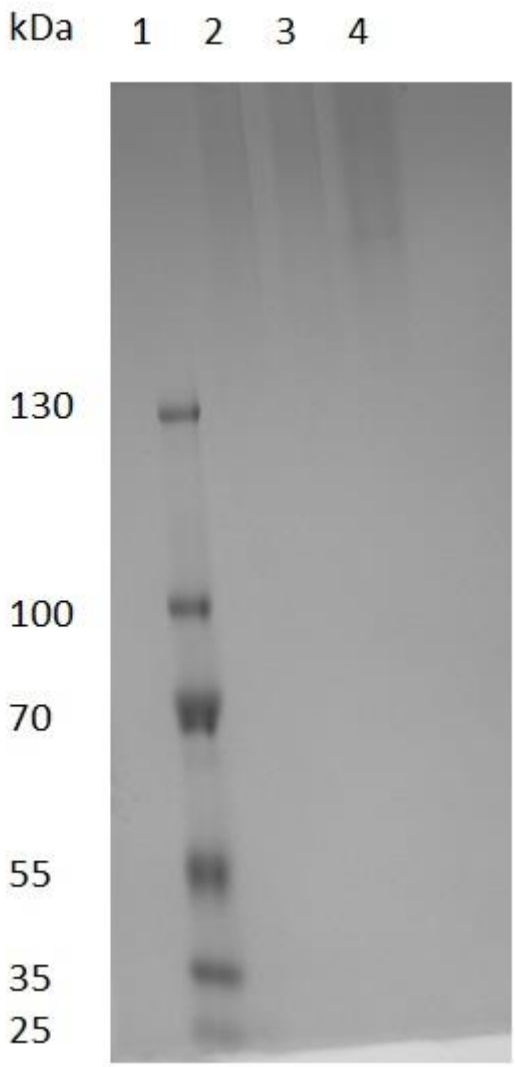

Figure 7 Native PAGE of the SLC1 probe (lane 2), SLC2 probe (lane 3) and IL-1β antibody (AF-401-NA) (lane 4). A prestained protein ladder is run in lane 1. Gel was stained with Coomassie Brilliant Blue.

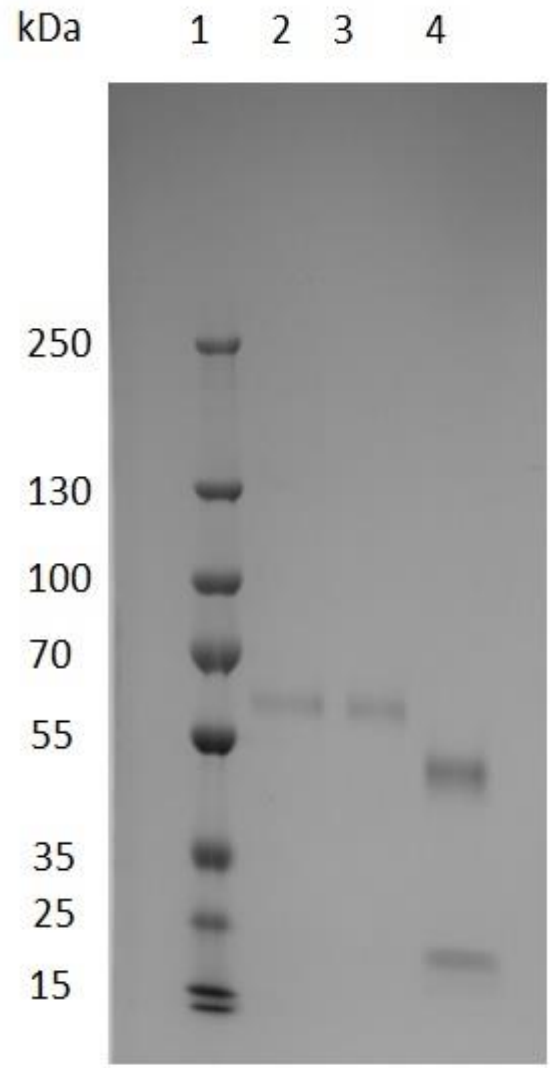

Figure 8 SDS-PAGE of the SLC1 probe (lane 2), SLC2 probe (lane 3) and IL-1β antibody (AF-401-NA) (lane 4). A prestained protein ladder is run in lane 1. Gel was stained with Coomassie Brilliant Blue.

There is a slight difference between the PAGEs of the old and the new probes. In figure 7 there are still three smeared bands in lanes 2, 3 and 4 but the intensity of lane 2 and 3 is the same and the difference of intensity is way less with band 4 than that was the case with the old probes in fig 5 . Figure 8 has exact the same result as figure 6, in lane 2 and 3 a band around 65 kDa is visible and in lane 4 two bands are visible around 50 and 20 kDa.

4.3 SP-PLA

After the PAGEs the probes were used to create a standard curve with the SP-PLA.

4.3.1 SP-PLA with a PCR-plate

The first SP-PLA was done according to the existing protocol, this method varies from the method described above. Instead of using PCR-strips in the thermal cycler the samples from the PCR-strips were transferred to a PCR-plate. The results of this first SP-PLA can be seen in figure 9. The whole dataset including the controls can be found in table appendix 7.2.2.

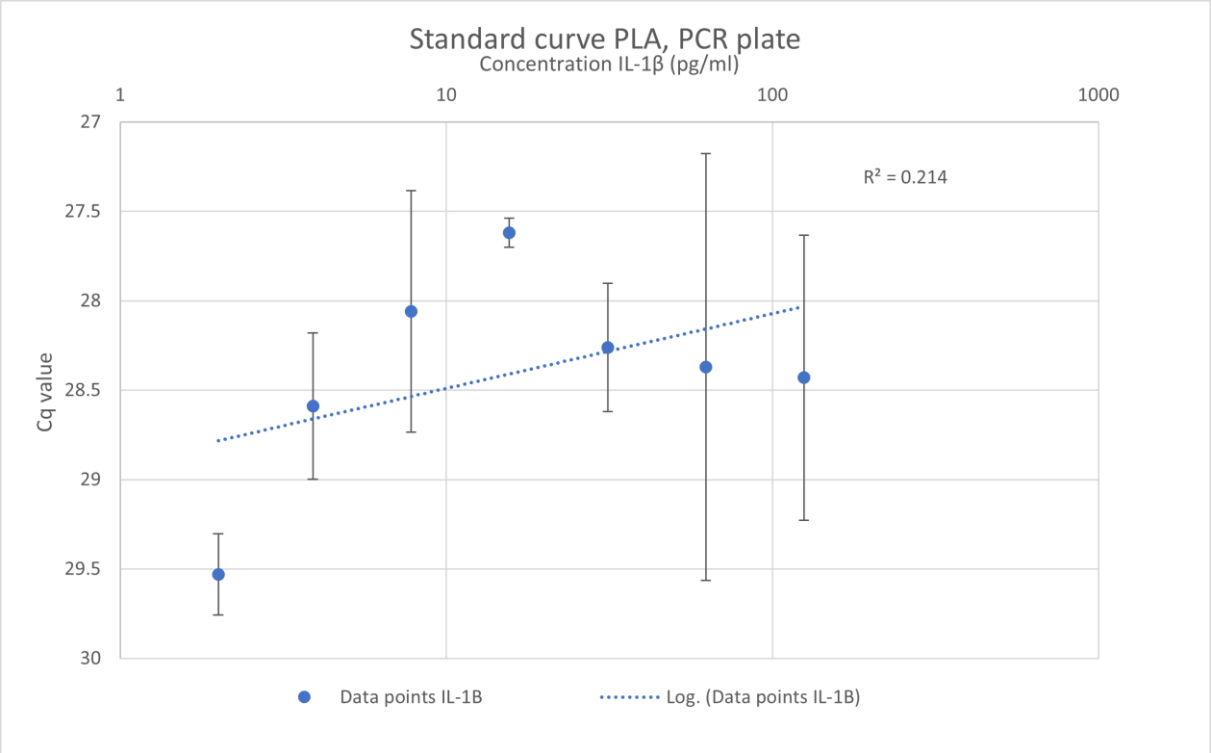

Figure 5 Calibration curve of the PCR-plate standard series. The concentrations are in pg/ml and on a logarithmic x-axes. On the y-axes the C_q are shown. Standard deviations of the C_q values are included. A regression line is drawn through the data points and the corresponding coefficient of determination is shown in the graph.

A regression line is drawn through the obtained data points, the coefficient of determination of this line is 0.214. The standard deviations are mostly between 0.4 and 1.2 this is too high to say something concrete about this data.

4.3.2 SP-PLA with PCR-strips

A second SP-PLA was done were the samples were directly analysed in the PCR-strips. This eliminated the transferring step in which sample could be lost. The range of the standard series was adjusted to focus on the lower concentrations, it ranged from 15.6 to 0.25 pg/ml The results of this second SP-PLA can be seen in figure 10. The complete dataset including the controls can be found in appendix 7.2.3.

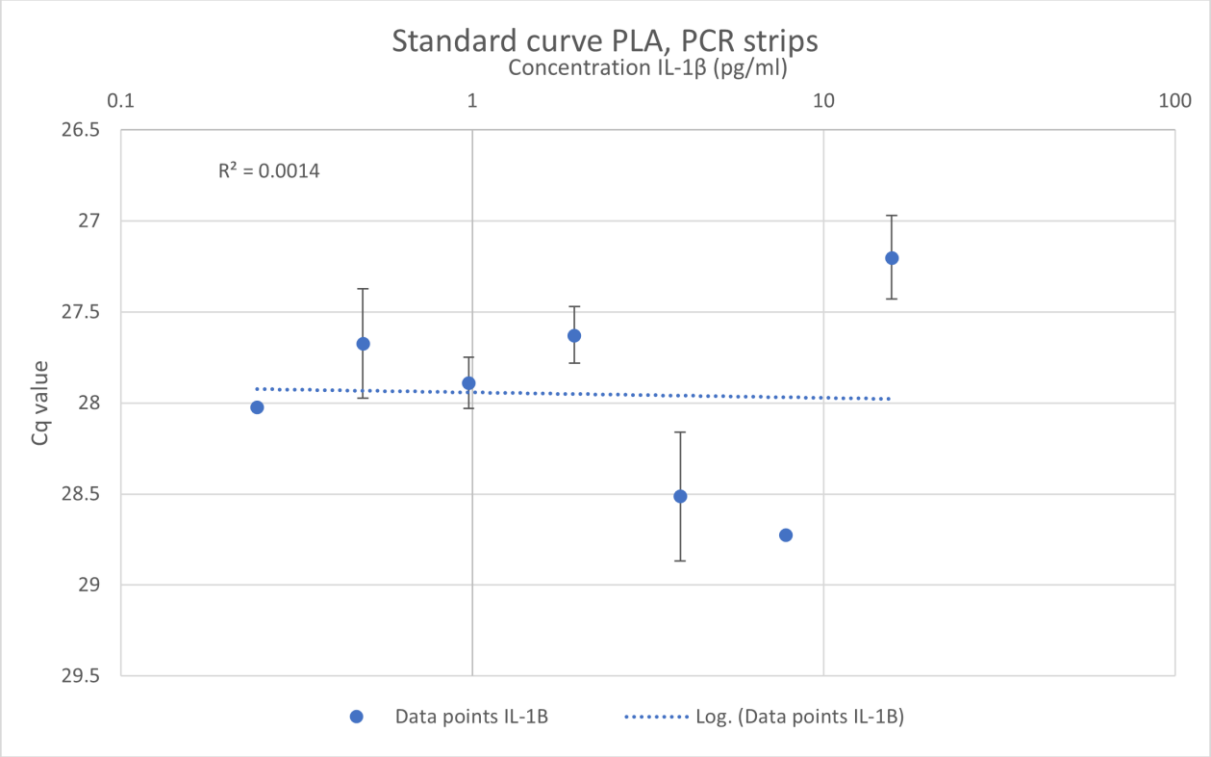

Figure 6 Calibration curve of the PCR-strip standard series. The concentrations are in pg/ml and on a logarithmic x-axes. On the y-axes the C_q are shown. Standard deviations of the C_q values are included. A regression line is drawn through the data points and the corresponding coefficient of determination is shown in the graph.

A regression line is drawn through the data points with a R^2 of 0.0014. All the standard deviations lie between values of 0 and 0.35. The regression line is almost a horizontal line and the Cq values lie very close together, within a two point range. To broaden this range to get a non-horizontal regression line and more spread Cq values a broader standard series was made and more qPCR probe was added. Also new PLA probes were made for this SP-PLA.

4.3.3 SP-PLA with a broader standard series and more qPCR probe.

Two things were changed with this SP-PLA in comparison with the previous versions. The amount of qPCR per reaction was increased from 0.5 µl 10 µM qPCR probe to 1.1 µl 10 µM qPCR probe. Therefore more dye should be present which should broaden the Cq range from this assay. The two-fold dilution series from 2 to 125 pg/ml was changed to a five-fold dilution series from 0.1 to 1562 pg/ml. The PLA probes used in the previous SP-PLA were over their shelf life so new ones were made and used for this standard curve. The results can be seen in figure 11 the whole dataset including controls can be seen in appendix 7.2.4.

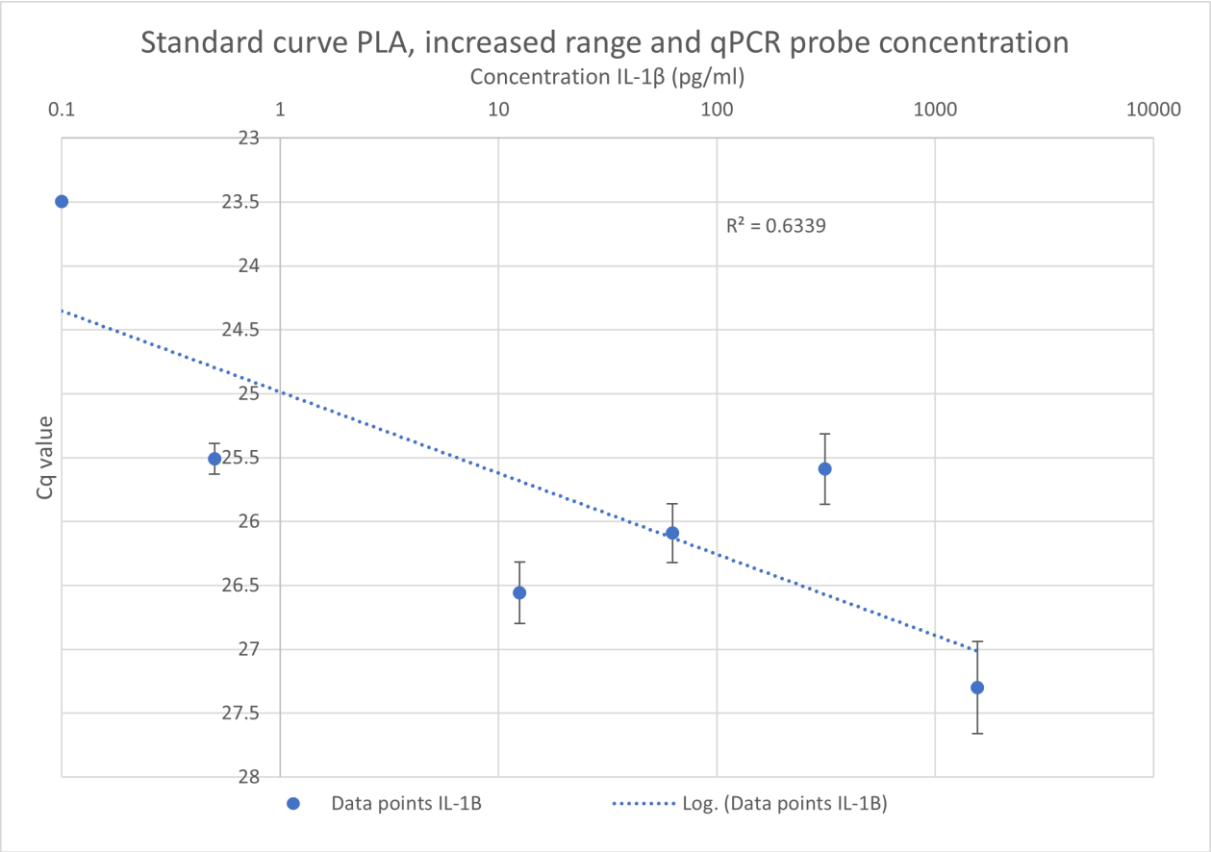

Figure 7 Calibration curve of the standard series with increased range and qPCR probe concentration. The concentrations are in pg/ml and on a logarithmic x-axes. On the y-axes the C_q are shown. Standard deviations of the C_q values are included. A regression line is drawn through the data points and the corresponding coefficient of determination is shown in the graph

A regression line is drawn through the data points and a coefficient of regression of 0.6339 was calculated. All but one standard deviations lie between 0 and 0.36, the standard deviations of the 2.5 pg/ml is significantly higher with a value of 1.7. This data point cannot be seen as an accurate result and therefore was not used in the final graph.

5 Discussion

The goal of this research was to set up the SP-PLA for the quantification of proteins in blood plasma. IL-1β was used as the first protein to test this method.

An ELISA was done to check if the IL-1β antigen used during all the experiments was good quality. The results from the ELISA were as expected, the value of the coefficient of determination was with 0.9968 very close to the perfect value of 1. This means that the concentration difference in the IL-1β antigen was the only thing that caused the difference in the measured fluorescence values. Also the standard deviations were so low, that the error bars could barely be seen. Unfortunately it also lived up to the expectations that it could not accurately measure the four lowest values of IL-1β. This is essential since these are values that normally are found in human blood plasma.

The PLA probes were the first step into performing the SP-PLA. For the first two times the SP-PLA was performed old probes were used. These were made approximately four months by another AST member before being used in this research. The shelf life of these probes is set at six months while kept at 4°C. Just before the final SP-PLA was done the old probes went past their expiration date and since there were enough supplies new ones were made.

The results from the native-PAGE of the old probes, which can be seen in figure 5, were not as expected. In a native PAGE the PLA probes and antibodies (AF-401-NA) are not denatured and therefore a band should have been visible around 168 kDa (150 kDa from the antibody conjugated with an 18 kDa oligonucleotide) in lanes 2 and 3 and a band around 150 kDa in lane 4 from the pure antibody. Instead, only three smears at the top of the gel were visible. This could be the result from aggregation of the antibodies which are then all stuck together which results in a higher molecular weight, causing them to stay higher in the gel. This would also suggest that the SLC1 probe (lane 2) is more aggregated than the SLC2 probe (lane 3) because the smear is less visible, the probe stayed higher in the gel or could not migrate through it at all as a result of the higher molar mass. When the new probes were made, which can be seen in figure 7, this has improved. There were still no bands visible at 168 kDa but the smeared bands from SLC1 and SLC2 had no longer a difference between intensity. This could mean their aggregation levels and thus their functionality are at the same level. This aggregation could be the result from inappropriate storage. The antibodies were ordered around four months before the research started, according to the manufacturer these could be stored at -20°C for up to six months and up to one month at 2 till 8 °C. The PLA probes used in first few experiments were longer in the fridge than one month so it is likely these have been aggregated. When the new probes were made, five months had passed since the antibodies arrived, so this should have been okay. However these antibodies were stored in low concentration and low volume aliquots. The low concentration promotes aggregate formation and the due to the low volume the aliquots were very sensitive for unintentional freeze thaw cycles by for instance the opening of the freezer door or when other aliquots were taken from the same box, freeze thaw cycles are also known for promoting aggregate formation[20].

The results from both SDS-PAGEs, figures 7 and 8, are showing the same results, the bands have the same ratio of intensity and are around the same molecular weights. The Il-1β antibody (AF-401-NA) in lane 4 shows bands around 20 and 50 kDa, which is the result from the heavy and light chains migrating through the chain. The Bèta mercapto-ethanol in the loading buffer is the cause of this, it broke the sulphide bonds holding the heavy and light chains together. The

SDS in the running and loading buffer denaturated the structure of the proteins which made it easier to migrate through the gels. The function of both SDS and Bèta-Mercapto-ethanol in combination with the heating step resulted in the breakup of the protein aggregation and therefore bands are visible in the SDS-PAGE. The SLC1 and SLC2 (respectively lane 2 and 3) both show bands around 65 kDa, this is the result from the conjugation of the 18 kDa oligonucleotides with the 50 kDa heavy chains. This was as expected and this is essential for the function of the antibodies, if the oligonucleotides would have been on the light chain its ability to bind antigen could be compromised. What was not expected is the absence of these light chains in the gel a band was also expected around 20 kDa since nothing should have happened to the light chain. The main goal of this SDS-PAGE was to see the heavy chain shift from 50 to 68 kDa, this was successful.

The qPCR data of the SP-PLA standard series is very incoherent. Only standard series in triplicate have been run and the outcome differed every experiment. The first SP-PLA had very high standard deviations which was probably caused by the need to transfer every sample from the PCR-strip to a PCR-plate, despite the use of maximum recovery tips sample volume was lost with every step and this also differed between the sample and triplicates. Differences in sample volume that need to be analysed resulted in different outcomes. This problem was mostly solved with the discovery that the PCR-strip also fitted directly in the thermal cycler. Although the first time this was tested the strips were crushed under the pressure from the lid, therefore the sides from a PCR-plate were added to the thermal cycler to prevent the lid from crushing the strip, this can be seen in appendix 7.1. With the implementation of this technique the standard deviations were brought back to values lower than 0.5 which is acceptable for technical triplicates.
The data still isn't quite what was expected, despite the low standard deviations, when looking at figure 10 the data points all lie in the same plane around a C_q value of 28. It was expected to see a line with a clear slope where the C_q values were increasing when the concentration of IL-1β antigen was decreasing. To pull the values further from each other the amount of qPCR probe was increased, this was also used in the research from Nong *et al* [11] were the SP-PLA protocol was based on. For the same reason the standard series was broadened, other researches all did standard series ranging from 0.01 pM to 100 pM while this IL-1β standard series only ranged from 0.1 to 7 pM when converted from the 2.0 to 125 pg/ml standard series. And to eliminate another possible cause for the small difference in results new probes were made for the last SP-PLA, if the old probes had aggregated and lost a significant part of their function these probes could have become the limiting factor thus resulting in a low signal variance. The last SP-PLA did have a broader C_q range with values from 23.5 to 27.5. There was also somewhat of a trend visible and the value of the regression coefficient was higher than before with a value of 0.63. However, the trend was the opposite of what was expected. The C_q values where linear with the IL-1β concentration while an inverted linear trend is the logical thing to happen. When the IL-1β concentration rises more signal should be generated earlier in the qPCR and thus the C_q values should decrease.
Beside this there were two other peculiarities with all the SP-PLAs done. When the loose PLA probe mix, containing an even concentration of the work solutions of the SLC1 and SLC2 probe, was mixed with the PCR mix a signal was detected. This was not as expected, when looking at the research and theory this protocol was based on the oligonucleotides on the probes should only be able to ligate if they are in such proximity that could only arise when both probes are bound to the same antigen. Second were the results from the zero standards, this was

pure PLA buffer that was added to the wells instead of sample containing IL-1β antigen. The zero standards should say something about the background signal, but they gave results that were in the same order of magnitude as the samples. This would suggest that all the values found are caused by some sort background signal and not from actual variance in the IL-1β antigen samples.

This could be the result, as already discussed with the PAGEs, from compromised PLA probes. When the antibodies are all clustered together their function is decreased and they can therefore become the limiting factor. It is also possible that the crosslinking from the IL-1β antibodies (AF-401-NA) impaired the function of the antibodies. According to the producer of the sulfo-SMCC crosslinking agent the function of the antibody should not be compromised but the amino acid sequence from this specific antibody was not examined. Another possibility is the compatibility of our antibody with our antigen, due to time pressure the AF-401-NA antibodies were purchased these are 100% specific for mouse IL-1β but also have an 67-78% affinity with human IL-1β, the antigen used in this research.

The inaccurate results could also be the result from a few things regarding the qPCR reaction. The qPCR probe used during the qPCR was a probe with a double quencher, the operation of this double quencher is outside the scope of this research but according to the manufacturer this should improve the function of larger qPCR probes (>20 base pairs). But the probe used in this research was a normal qPCR probe with around 20 base pairs so a double quencher was unnecessary and it is unknown if this had any consequences for the final results. This double quencher probe was also different from the probe used in the research this protocol was based on.

For future research into SP-PLA a lot can be investigated, tested and improved to create the optimal assay for sensitive and specific protein detection. The antibodies used are being very sensitive to freeze thaw cycles and long term storage. When a SP-PLA is performed these should be purchased as close to the start of the research as possible and immediately be aliquoted and put inside a freezer, this minimizes the aggregation and will therefore enhance their function and quality of the assay. Further research could also be done in the concentration of all the different reagents used, this could possibly identify limiting factors that are not known yet.

When this is all optimized and an accurate standard curve can be created, this method could be an useful addition to the toolbox of lab researchers. Small samples with low concentration proteins can easily be detected with SP-PLA and it is also possible to detect other proteins beside IL-1β without large changes in the protocol. Organs-on-chip used to yield to little sample to analyse a wide spectrum of proteins but with SP-PLA this is possible. This can even be extended to another form of PLA, multiplex PLA, where multiple proteins can be examined simultaneously.

6 Conclusion

Solid-phase proximity ligation assay was used during this assignment with as main question: What are the concentrations of the cytokine IL-1β in blood plasma samples quantified with solid-phase proximity ligation assay (SP-PLA)? Can this method be used as the new standard in protein quantification when sample volumes and concentrations are too low to use ELISA? A standard series was made with the SP-PLA to test the overall fitness of the method before testing the plasma samples. The standard series was not deemed accurate enough to continue with the samples. The standard deviations of the triplicates were all low enough to draw conclusions from the standard curve, but the measured values were too incoherent to actually construct a standard curve. This could be the result of using old antibodies that have formed aggregated which impaired their function.

The blood plasma samples were not analysed since a proper standard curve could not be constructed, the assay first need to be optimized. SP-PLA could not be performed with relevant results so for now this method cannot be used as a new standard for the protein quantification when concentrations and sample volumes are too low for ELISA.

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7 Appendices

7.1 Thermal cycler set-up

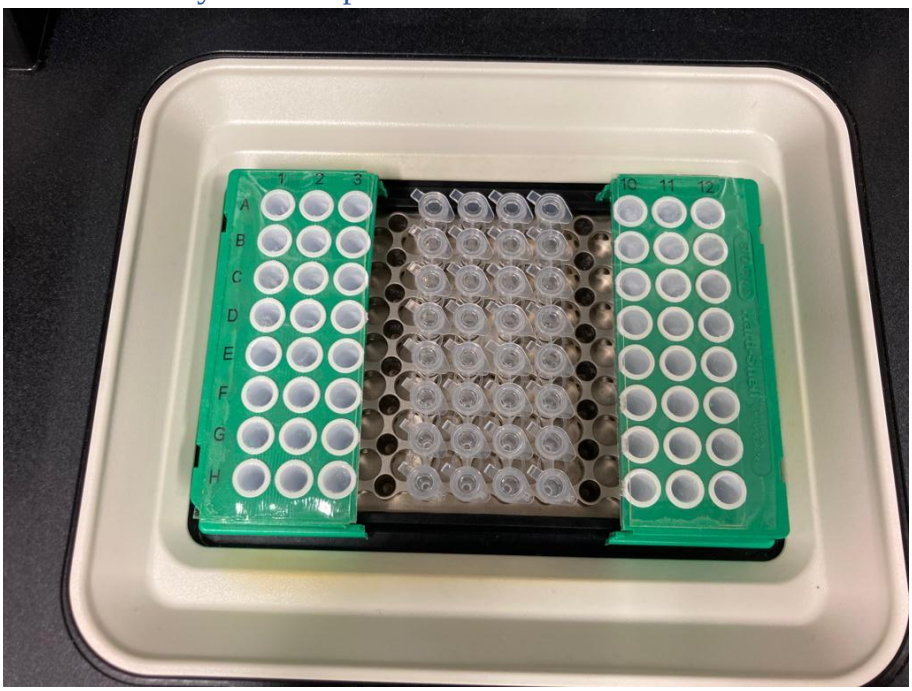

Figure 8 Set-up of the thermal cycler. The sides of a PCR-plate are placed in the machine to prevent the PCR-strip from being crushed.

7.2 Datasets sandwich ELISA and SP-PLA

7.2.1 Data ELISA

Table 6 Quantification data of the ELISA IL-1B standard series in triplicate. The target, sample, RFU mean and standard deviations are presented. The standard series is used as a positive control and MilliQ is the negative control.

Target	Sample	RFU mean	Standard deviation
IL-1β	125 pg/ml	1.239	0.001
IL-1β	62.5 pg/ml	0.610	0.004
IL-1β	31.3 pg/ml	0.344	0.012
IL-1β	15.6 pg/ml	0.211	0.010
IL-1β	7.8 pg/ml	0.148	0.011
IL-1β	3.9 pg/ml	0.115	0.004
IL-1β	2.0 pg/ml	0.105	0.005
IL-1β	1.0 pg/ml	0.094	0.001
IL-1β	0.5 pg/ml	0.107	0.005
IL-1β	0.25 pg/ml	0.112	0.006
IL-1β	0 pg/ml	0.100	0.001
	MilliQ	0.098	0.007

7.2.2 Data PLA, PCR plate

Table 7 Quantification data of the SP-PLA, with PCR plate, IL-1B standard series in triplicate. The target, sample, Cq mean and standard deviations are presented. The standard series is used as a positive control and MilliQ is the negative control.

Target	Sample	C_q mean	Standard deviation
IL-1β	125 pg/ml	28.43	0.80
IL-1β	62.5 pg/ml	28.37	1.19
IL-1β	31.3 pg/ml	28.26	0.36
IL-1β	15.6 pg/ml	27.62	0.08
IL-1β	7.8 pg/ml	28.06	0.69
IL-1β	3.9 pg/ml	28.59	0.41
IL-1β	2.0 pg/ml	29.53	0.23
IL-1β	0 pg/ml	28.91	0.44
	MilliQ	-	-

7.2.3 Data PLA, PCR strips

Table 8 Quantification data of the SP-PLA, with PCR strips, IL-1B standard series in triplicate. The target, sample, Cq mean and standard deviations are presented. The standard series is used as a positive control, MilliQ and the no polymerase control are the negative controls. The PLA probe mix was also analysed.

Target	Sample	C_q mean	Standard deviation
IL-1β	15.6 pg/ml	27.20	0.23
IL-1β	7.8 pg/ml	28.73	0.01
IL-1β	3.9 pg/ml	28.51	0.35
IL-1β	2.0 pg/ml	27.63	0.16
IL-1β	1.0 pg/ml	27.89	0.14
IL-1β	0.5 pg/ml	27.67	0.30
IL-1β	0.25 pg/ml	28.03	0.01
IL-1β	0 pg/ml	28.66	0.1
	MilliQ	-	-
	PLA probe mix	23.82	0.12
	NPC	-	-

7.2.4 Data PLA, increased range and qPCR probe concentration

Table 9 Quantification data of the SP-PLA, with increased range and qPCR probe concentration, IL-1B standard series in triplicate. The target, sample, Cq mean and standard deviations are presented. The standard series is used as a positive control and MilliQ is the negative control.

Target	Sample	C_q mean	Standard deviation
IL-1β	1562.5 pg/ml	27.30	0.36
IL-1β	312.5 pg/ml	25.59	0.28
IL-1β	62.5 pg/ml	26.09	0.23
IL-1β	12.5 pg/ml	26.56	0.24
IL-1β	2.5 pg/ml	25.30	1.71
IL-1β	0.5 pg/ml	25.51	0.12
IL-1β	0.1 pg/ml	23.50	0.01
IL-1β	0 pg/ml	25.30	1.06
	MilliQ	-	-