UPPSALA UNIVERSITET

Optimization and Establishment of a Micro Solid Phase Proximity Ligation Assay (micro SP-PLA)

Βελτιστοποίηση και καθιέρωση μιας βασιζόμενης σε μεθόδους μικρορευστομηχανικής έκδοσης της ανάλυσης PLA στερεάς φάσης (micro SP-PLA)

Georgios Flamourakis

Department of Genetics and Pathology
University of Uppsala

‘Science is about figuring out your mistakes’

Saul Pelmatter, 2011 Nobel Prize in Physics

Table of Contents

Abbreviations

PLA	Proximity Ligation Assay
SP-PLA	Solid Phase proximity ligation assay
qRT-PCR	Quantitive real time polymerase chain reaction
RCA	Rolling circle amplification
μTAS	Micro total analysis system
IHC	Immunohistochemistry
AppDNA	Adenylylated DNA
RCP	Rolling circle product
sRCP	Super rolling circle amplification

Abstract

In the 21st century the diagnostic needs are getting bigger and bigger. Methods that are established exceed in great sensitivity and efficiency, parameters that are crucial for a successful assay. One of the most innovative assays is the proximity ligation assay, a method which takes advantage of the proximity distance that two oligonucleotides can be in and upon ligation eventually will give a strong signal. Such method reduces background greatly while improves signal to noise ratios. For that reason solid phase proximity ligation assay was chosen to be optimized by further establishment of the microfluidic version of it. During SP- PLA, capture antibodies are immobilized on a solid support and the antigen binds on them making it possible for a PLA to take place. By the end of all reactions the signal is detected on a fluoresce microscope, were each product is distinguished as an individual spot. The ease and speed of this method implied that it could be applied into microfluidics, an application that could push the assay to its limits. Consequently, after optimizing the method on slides we were able to detect concentration of antigen as low as 1 pM. Then the optimized protocol was applied on microfluidic making the method much faster but not more sensitive due to restricted knowledge over this subject. The biggest advantage was the signal to noise ratio which was raised in very satisfactory levels. Further studies and experiments could make micro SP-PLA one of the most sensitive methods ever been made.

Περίληψη

Τον 21 αιώνα οι διαγνωστικές ανάγκες αυξάνονται όλο και περισσότερο. Οι τρέχοντες μέθοδοι υπερέχουν σε ευαισθησία και αποδοτικότητα – παράμετροι καθοριστικοί για μια επιτυχημένη μέθοδο. Η ανάλυση PLA στερεάς φάσης (SP-PLA) είναι μία από τις πιο καινοτόμες μεθόδους, η οποία εκμεταλλεύεται την κοντινή απόσταση στην οποία μπορεί να βρεθούν δύο ολιγονουκλεοτίδια, τα οποία υπό μία αντίδραση σύνδεσης τελικά θα δώσουν ένα ισχυρό σήμα. Κατά συνέπεια, ο θόρυβος μειώνεται σημαντικά ενώ παράλληλα βελτιώνεται η σχέση θορύβου - σήματος. Για αυτό το λόγο η ανάλυση PLA στερεάς φάσης (SP-PLA) επιλέχθηκε για να βελτιστοποιηθεί με περεταίρω καθιέρωση της μικρορευστομηχανικής έκδοσής της. Συγκεκριμένα, κατά την SP-PLA, τα αιχμαλωτίζωντα αντισώματα ακινητοποιούνται πάνω σε ένα στερεό υπόστρωμα και το αντιγόνο συνδέεται σε αυτά καθιστώντας δυνατή μία μέθοδο PLA. Μετά το πέρας όλων των αντιδράσεων, το σήμα ανιχνεύεται μέσω ενός μικροσκοπίου φθορισμού, όπου κάθε προϊόν διακρίνεται ως μία ξεχωριστή κουκίδα. Η άνεση και η ταχύτητα αυτής της μεθόδου συνιστούν ότι μπορεί να εφαρμοστεί σε μικρορευστομηχανική, μία εφαρμογή που θα μπορούσε να πιέσει τη μέθοδο στα όριά της. Στη πράξη, αφού βελτιστοποιήσαμε τη μέθοδο σε γυάλινα πλακίδια είχαμε τη δυνατότητα να ανιχνεύσουμε μέχρι και 1 pM συγκέντρωσης αντιγόνου. Έπειτα, το βελτιστοποιημένο πρωτόκολλο εφαρμόστηκε σε μικρορευστομηχανική καθιστώντας τη μέθοδο πιο γρήγορη, ωστόσο όχι πιο ευαίσθητη λόγο περιορισμένης γνώσης επί του συγκεκριμένου θέματος. Το μεγαλύτερο πλεονέκτημα ήταν το γεγονός ότι η σχέση θορύβου - σήματος βελτιώθηκε σε ικανοποιητικό βαθμό. Περαιτέρω μελέτες και πειράματα θα μπορούσαν να κάνουν την micro SP-PLA μία από τις πιο ευαίσθητες μεθόδους που επινοήθηκε.

1 Introduction

1.1 PLA

The *Proximity Ligation Assay* (PLA) is an immunoassay where pairs of oligonucleotide-labeled antidodies- PLA probes- are employed to detect an antigen of interest. When two PLA probes bind the same antigen, the attached oligonucleotides are brought in proximity and can be ligated upon addition of a short complementary oligonucleotide. The ligated DNA strands serve as reporter molecules that can be readily detected using a variety of methods such us *quantitative Real- Time PCR* (qPCR) or *Rolling Circle Amplification* (RCA) [1].

The technique used in this thesis is the *solid phase proximity ligation assay* (SP-PLA) in which an antibody is immobilized on a solid support (ex. the surface of a glass slide) and acts as a capture reagent for the antigen [1]. Then the antigen is been added and the PLA assay is performed over the antibody- antigen complex (***figure 1***).

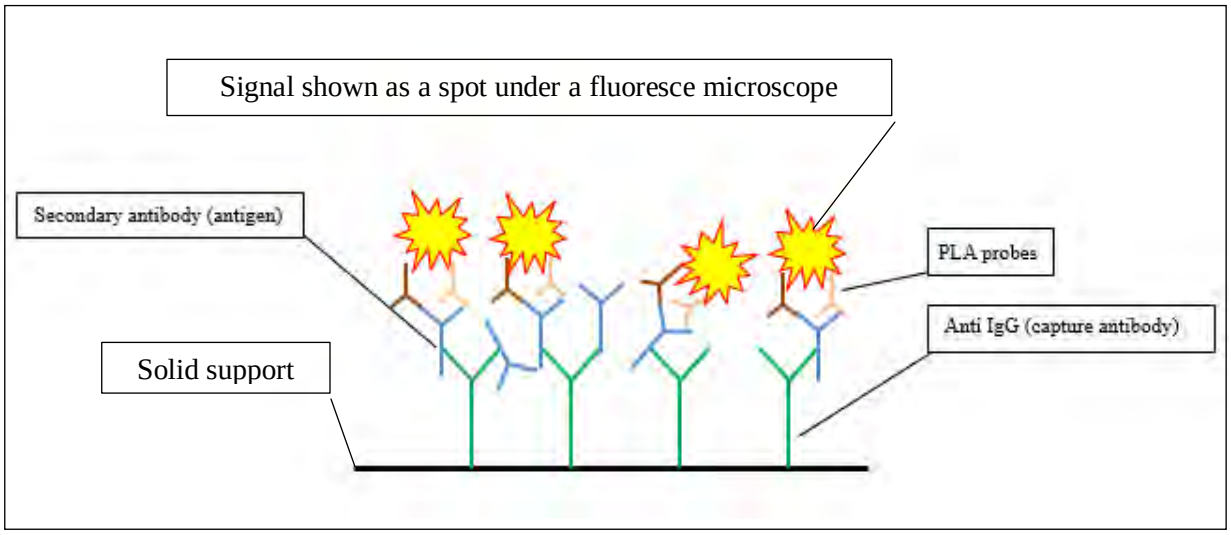


Figure 1.*The PLA assay. Hybridization of the primary antibody and recognition of the antigen proceed at the first place. The PLA probes which are conjugated antibodies with two different oligonucleotides are then added and the strands of DNA are coming in a proximity distance and with connector oligos it is possible for a ligation reaction to take place. For the final step, a polymerase is been used to amplify the circle that has been created by the ligation process creating a template of thousands of repetitive sequences, which are complementary with fluorescent oligos that eventually create a spot. The signal can be detected with a fluoresce microscope.*

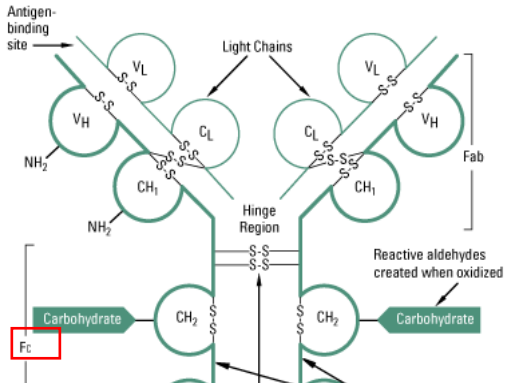


Figure 2. *The antibody with its primary amines which are indicated in red boxes. These are the residues where the amide bond will be created between NHS and the antibody*

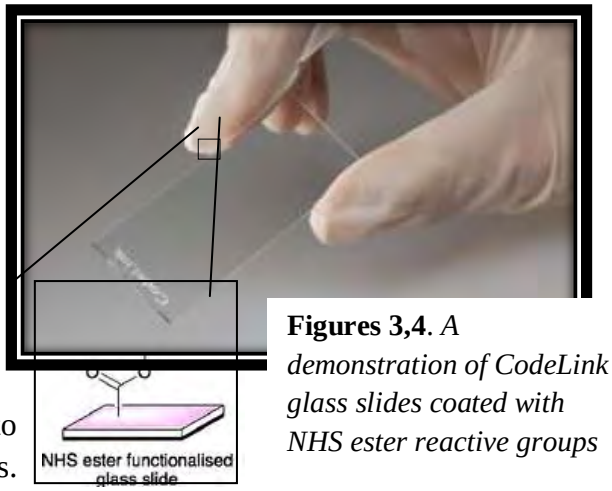
1.2 Primary amines

SP- PLA is a variation of the classical method of PLA, where a solid support is been used and a capture antibody is immobilized on it. The immobilization takes place by through the reaction of the primary amines (***figure 2***) on the antibody

(NH_2) with *N-hydroxysuccinimide esters* (NHS) on the solid support. NHS is commonly found in organic chemistry or biochemistry where it is used as an activating reagent for carboxylic acids. Activated acids can react with amines to form amides, a very stable bond.

1.3 Solid Phase

The solid phase of the method is a glass slide coated with a hydrophilic polymer containing N-hydroxysuccinimide (NHS) ester reactive groups. Planar glass slides constitute one of many solid supports used. One of the most commonly used support is paramagnetic microparticles (beads). Microparticles are commonly used as solid supports in immunoreactions to capture and separate target molecules. Generally, SP-PLA is very well suited for analyses of proteins present at low concentrations in complex biological materials such as undiluted plasma or serum or whole blood [2].



Figures 3,4. *A demonstration of CodeLink glass slides coated with NHS ester reactive groups*

1.4 Fighting background: the golden mean

Immunoassays in general rely heavily on the specificity of affinity reagents and detection strategies to report targets. This fact by itself reports that the likelihood of background creation can be excessively high. Background is influenced by a variety of factors:

- Antibodies may recognize and bind to non intended proteins.
- Antibodies may stick nonspecifically to the surface of the solid phase and generate signal in absence of antigen.
- Probes may come in proximity in solution and generate signal in absence of an antigen

A sandwich immunoassay (such as SP-PLA) which uses capture antibodies to recognize and bind the antigen as well as secondary detection antibodies, can improve detection limits especially in blood where high sensitivity is required and where background is a problem due to the presence of high amounts of proteins over a broad dynamic range. Minimizing

background then can be easier. We struggle to have zero false positive signal by altering time of incubations, concentration of reagents and efficient blocking methods. Nonetheless, background reduction usually lead to decrease of the true signal. The 'golden' ratio between signal and background should be found for any immunoassay to be efficient and extremely sensitive, a discovery that takes huge amounts of time and effort because of the vast number of combination of conditions that can be used for that purpose.

1.5 Rolling Circle Amplification (RCA)

The RCA method is the most important tool for a succesfull PLA. It is the last step of the method during which Φ29 polymarase replicates a circural DNA thousands of times, creating a very long linear single- stranded DNA which contains many repetative sequences of the circural DNA. Then, using small single- standred fluorecenced complementary DNA oligonucleotides, it is possible to detect the whole rolling circle product (RCPs), which can be seen under a fluorence microscope as an individual dot.

1.6 Φ29 DNA polymerase: a peculiar enzyme

In order to perform RCA, this very special polymerase is being used. *Φ29 DNA polymerase* is an enzyme from the bacteriophage Φ29

Φ29 is a bacteriophage of *Bacillus subtilis* with a sequenced, linear, 19,285 base pair DNA genome [3]. Each 5' end is linked to a terminal protein, which is essential in the replication process. A symmetrical mode of replication has been suggested, whereby protein-primed initiation occurs non-simultaneously from either end of the chromosome; this involves two replication origins and two distinct polymerase monomers. Synthesis is continual and involves a strand displacement mechanism. This was demonstrated by the ability of the enzyme to continue to copy the singly primed circular genome of the M13 phage more than tenfold in a single strand (over 70kb in a single strand) [4]. In vitro experiments have shown that Φ29 replication can proceed to completion with the sole phage protein requirements of the polymerase and the terminal protein. The polymerase catalyses the formation of the initiation complex between the terminal protein and the chromosome ends at an adenine residue. From here, continual synthesis can occur.

The polymerase (***figure 5***), is a monomeric protein with two distinct functional domains. Site-directed mutagenesis experiments support the proposition that this protein displays a structural and functional similarity to the Klenow fragment of the *Escherichia coli* Polymerase I enzyme [5]. It comprises a C-terminal polymerase domain and a spatially separated N-terminal domain with a 3'-5'

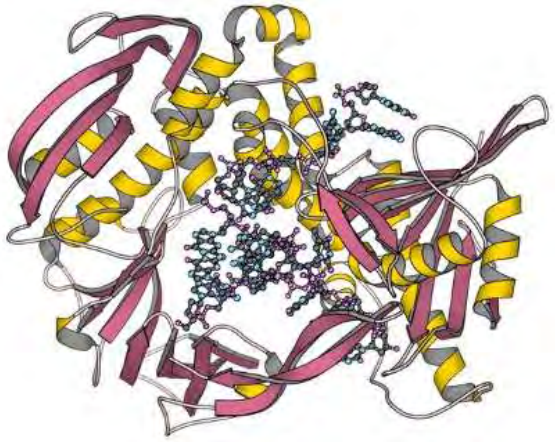


Figure 5.*Phi29 DNA Polymerase with Substrate*

exonuclease activity. The isolated enzyme has no intrinsic helicase activity, but may carry out an equivalent function by way of its strong binding to single stranded DNA, particularly in preference to double stranded nucleic acid. The exonuclease activity of the enzyme is, like its polymerisation activity, highly processive and can degrade single stranded oligonucleotides without dissociation [6].

It is being increasingly used in molecular biology for multiple displacement DNA amplification procedures, and has a very unique characteristic that makes it the ideal enzyme for this method: constant polymerization of a circular, single strand DNA into a long linear repetitive one. This long linear DNA is actually the amplified product that can be observed microscopically.

1.7 T4 DNA ligase: a crucial enzyme for a successful PLA

DNA ligases are divalent metal cation-dependent enzymes that utilize ATP or NAD+, depending on the ligase, to catalyze phosphodiester bond formation between adjacent double-stranded polynucleotide termini possessing a 3′-hydroxyl and a 5′-phosphate [7]. A typical DNA ligase catalyzes the formation of the phosphodiester bond in 3 steps: during step 1, an adenylyl group is transferred from ATP to the lysine in the active site of the enzyme. Then, in step 2, the adenylylated enzyme transfers the adenylylate group to the 5'- phosphate end of DNA creating a media form of adenylylated DNA (AppDNA). In step 3, a nucleophilic attack of 3'-hydroxyl on the App group causes the formation of the phosphodiester bond and the release of AMP (***figure 6***). All steps are metal cation- dependent, by the ion Mg^{+2} [7], [8].

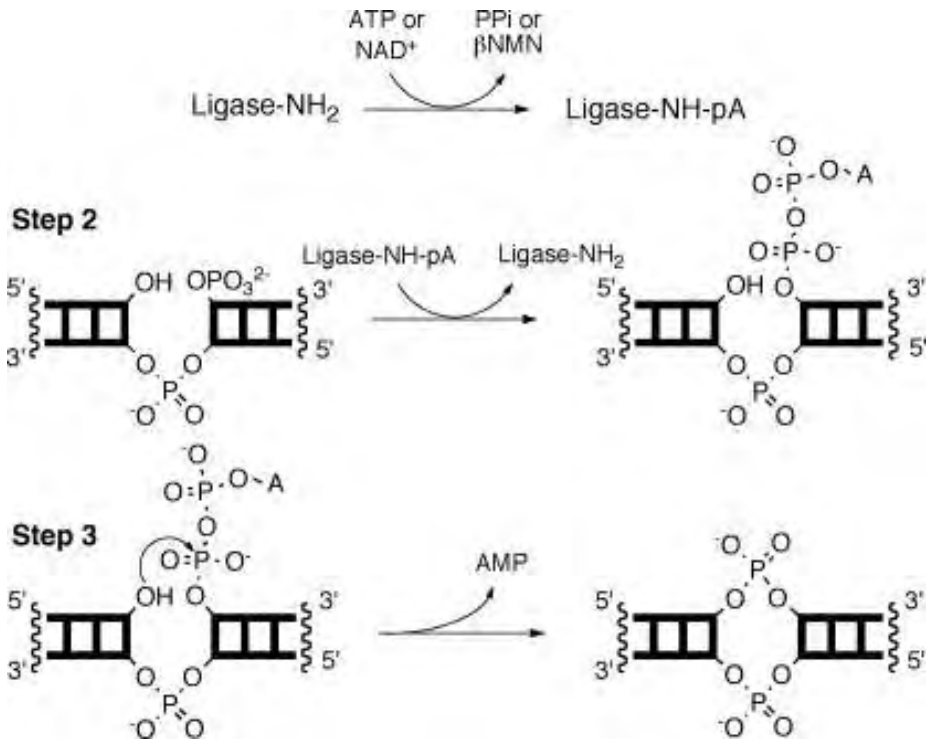
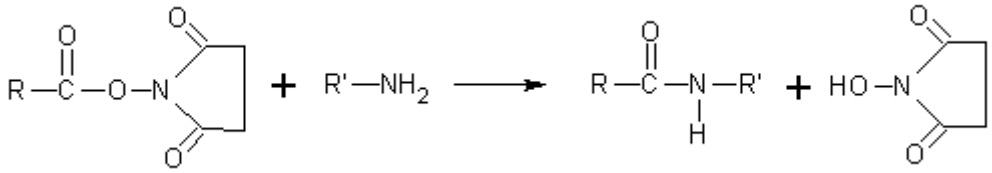


Figure 6. *The 3 steps of the reaction of formation of phosphodiester bond on DNA by DNA ligase*

1.8 A closer look to the method

Everything beggins with the immobilization of the capture antibody on the glass surface. Using NHS esters and the NH_2 groups of the antibody the following reaction takes place:



NHS-Esters react with α-amine groups of the antibody to form amide bonds. A covalent amide bond is formed during the reaction, releasing N-hydroxysuccinimide. This bond is a covalent bond, so it is very unlikely that the antibody will be washed away.

The second step of the method is the capture of the antigen by the capture antibody on the surface. The reaction time can be minimized inside the microfluidic channel because of the very small dimensions of it. The diffusion time is given by the equation:

$$t = \frac{x^2}{2D}$$

D is the diffusion coefficient which is $4{,}6.10^{-7}$ cm^2/sec for an IgG antibody of 150kDa weight. This equation implies that the time that a capture antibody takes to bind to an antigen is only 2,4 sec in a distance of 15μm.

PLA probes are then added. These probes consists of 2 antibodies, one PLUS anti-mouse IgG and one MINUS anti-mouse IgG. The terms PLUS and MINUS refer to two poluclonal antibodies spefied for a very spesific antigen and they cary two different strands of DNA (olus and minus). For the formation of the circle 2 additional oligos are required: S3 backbone and S3 splint. Backbone has a small part of its 3' and 5' ends complementary with the DNA on the 2 probes. Splint is complimentary to the probe's DNA but is located between the two ends of the backbone, as it shown in ***figure 7***. These two DNA pieces have their 5' ends phosphorilated for a successful ligation to be performed. By a ligation process, the whole circle closes and it is ready for the amplification process through an RCA.

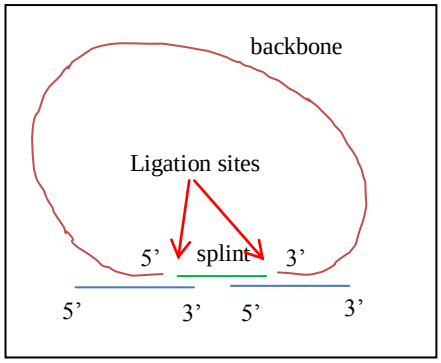


Figure 7. *structure of the circle*

1.9 Microfluidics: Lab on a chip

The earliest microfluidic devices demonstrated that fluidic components could be miniaturized and integrated together, leading to the idea that one could fit an entire "lab on a chip", in much the same way that a microelectronic circuit is an entire computer on a chip. Since then, there has been tremendous interest in harnessing the full potential of this approach and,

consequently, the development of countless microfluidic devices and fabrication methods. Microfluidics have many advantages, which makes them ideal for using in many applications.

One obvious advantage is that miniaturized components and processes use smaller volumes of reagents, thus leading to reduced reagent consumption. This decreases costs and permits small quantities of precious samples to be used more effectively (ex. antibodies, probes, samples etc.).

At the small scales in microfluidic devices, diffusive mixing is fast, often increasing the speed of reactions. Dramatic performance improvements are often seen in microfluidic assays such as: reduced measurement times, improved sensitivity, higher selectivity, and greater repeatability.

Many microfluidic technologies permit the construction of devices containing multiple components with different functionalities. A single integrated chip could perform significant biological or chemical processing from beginning to end, for example the sampling, pre-processing, and measurement involved in an assay. This is the kind of vision that led to the terms "lab-on-a-chip" and "micro total analysis system (μTAS)".Such system offers the potential for high efficiency, simultaneous analysis of a large number of biologically important molecules in genomic, proteomic and metabolic studies [9]. Performing all fluid handling operations within a single chip saves time, reduces risk of sample loss or contamination, and can eliminate the need for expensive laboratory robots. Furthermore, operation of microfluidic devices can be fully automated, thus increasing throughput, improving ease of use, improving repeatabilty, and reducing the element of human error.

2 Materials and methods

The whole experimental procedure was divided into several phases, each of which was aimed to optimize different experimental conditions. We started by immobilizing antibodies on plastic slides and continued with glass slides. In an attempt to compare the two types of solid supports. In addition, we investigated different blocking approaches for each type of support. The next phase intended to optimize probing dilutions and eventually combine everything into a single optimized protocol, which would be used for the last phase, which was integration to microfluidics.

2.1 Immobilization protocol (Sigolis plastic slides)

The antibodies were initially immobilized on a plastic slide provided by Sigolis (Uppsala, Sweden). The slides were coated with *PhenylDextran* in the following way: a drop of water was dropped on the surface of the slide for the hydrophobicity to be evaluated, followed by a wash with distilled water. The slides were then dipped into 60% and then 96% ethanol and after the ethanol evaporated, they were dipped into 0.1% of Dextran for 30 minutes under soft agitation on a Stuart® mini orbital shaker, SSM1 in room temperature. Finally, a last wash with distilled water took place and the hydrophobicity of the slide was evaluated again with a drop

of water and by comparing it with the previous result, before the coating procedure. The coated slides were stored at 4°C.

Different hybridization chambers were used according to the needs of each individual experiment:

- ✓ Secure seal™ hybridization chamber gasket, eight chambers, 9mm diameter, 0,8mm deep (Invitrogen™, Eugene, Oregon, USA)
- ✓ Secure seal™ hybridization chamber gasket, one chamber, 22mm x 22mm, 0,8 deep (Invitrogen™, Eugene, Oregon, USA)
- ✓ Secure-Seal™ Hybridization chamber gasket, one chamber, 20 mm diameter, 0.8 mm deep (Invitrogen™, Eugene, Oregon, USA)

The appropriate mask was placed on the slides and then *Sodium Periodate* 5mM ($NaIO_4$) was added until the mask was full and incubated for 30 min in the dark. After a wash with 1x PBS pH=7.4 for 1- 3 mins, 50% of glycerol was added for 10min followed by another wash with 1x PBS. Then the antibody was added. The antibody that was used, was a mouse IgG (16.6 μM diluted in 0.1nM sodium bicarbonate, pH= 9.6). The incubation time was 2 hours at room temperature. After the time elapsed, the antibodies were removed from the mask. 10μL of cyanoborohydride ($NaCNBH_3$) 5 M were added and the mixture was placed back in the masks for 30 min at room temperature. All procedures took place in a hood because of the high toxicity of $NaCNBH_3$. After a wash with 1x PBS, Tris-HCl was added and the incubation was held for 10 min at room temperature.

2.2 PLA probes and detection protocol

All the reagents and enzymes that were used, were provided by Olink Bioscience (Uppsala, Sweden), as a part of *Duolink In situ PLA kit*. At the beginning, PLA probes that were diluted in 1:5 ratio were prepared in Antibody Diluent. Before addition of the PLA probes, the masks were washed with 1x TBS. Incubation took place in a pre- heated humidity chamber for 1 hour at 37°C.

The first step of the detection protocol was the ligation, in which the enzyme T4 ligase was used. The enzyme was added in the Olink's Ligation stock and incubated for 30 min in a preheated humidity chamber at 37ºC. For the amplification step, Φ29 polymerase was used in olink's amplification stock with high purity water. The incubation time was 100 min in a preheated humidity chamber at 37ºC. In each individual step washings were performed with 1x PBST (1x PBS 0.1%, Tween 20%).

At the final step, detection of the product was performed. Before the masks were removed, the slides were engraved with a diamond pen on the opposite surface at the places there the masks were, so that it would be easier to macroscopically detect the sites that the products were without the masks. Then the slide was dipped consecutively into 70%, 85% and 99% of ethanol for 2 min each, to dehydrate. The slide was let to dry and then a drop of vectashield mounting medium for fluorescence H-1000 (Vector Laboratories INC. CA, USA) was added on top of the slide. The slide was then covered with a glass cover slip (Menzel- Gläser, 24x55

mm) and observed in a fluorescence microscope at Cy 3.5 and magnification 20x. To count the rolling circle products (RCPs), Image J was used.

2.3 Immobilization protocol (CodeLink™ glass slides)

Because of the fact that CodeLink™ Activated glass slides (SurModics INC. USA) were coated with NHS, the immobilization protocol needed to be adapted. The activation of chemical groups was not necessary anymore and the glass was hydrophilic by itself. Therefore there was no need of making it hydrophilic as with the hydrophlic Sigolis plastic slides.

After the appropriate mask was applied, the antibody was added inside the chambers and immobilization took place at room temperature for 2 hours. In order to test which concentration of capture antibody (anti mouse IgG) is the most efficient, 3 different concentrations were prepared: 5, 50 and 500 nM. All were diluted in carbonate buffer pH=9.6.

2.4 Blocking on Sigolis slides

An 8- chamber mask was placed onto the sigolis plastic slide so that different blocking conditions could be tested. For the experiment 0.1%, 1% and 10% of BSA were used. After activating the Dextran on the slide as described above, the mouse IgG antibody was mixed with the different concentrations of BSA to a final concentration of 1nM. A layout of the experiment can be seen below:

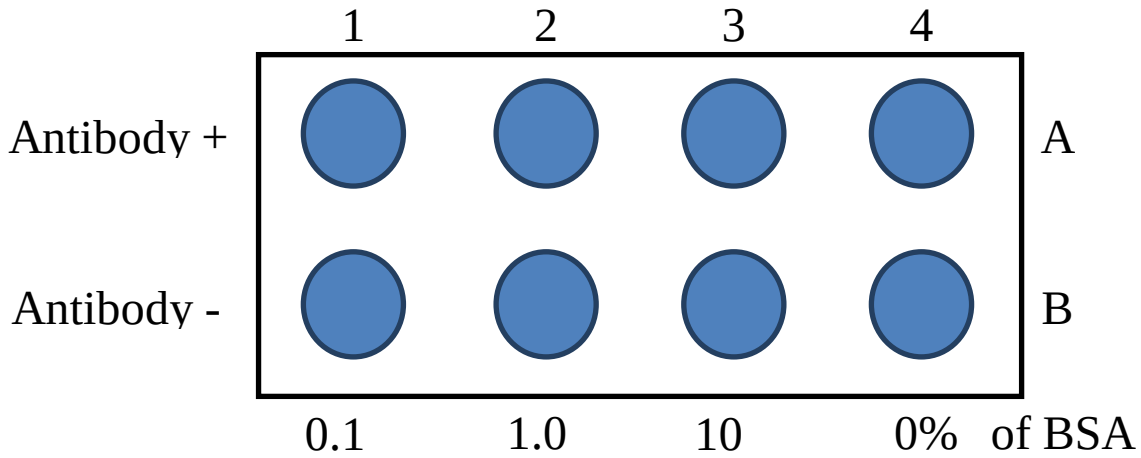


Figure 8. *layout of the blocking conditions in the mask*

All reactions in row A contained mouse IgG, while in row B were the antigen independent background was tested, no antibody was added. Incubation was performed at room

temperature for 1 hour. After a wash with 1x PBS, PLA probes and detection protocol was performed, as described in materials and methods.

2.5 Blocking on CodeLink™ slides

To test blocking on Codelink slides, first the capture antibody was added. This was an anti-mouse IgG in concentration 2μg/μL, diluted in Carbonate Buffer pH=9.6. For the experiment, the antibody was diluted in carbonate buffer to 500nM (for 400μL of reaction 15,2 μL of antibody were diluted in 384,8μL Carbonate Buffer). Immobilization took place for 2 hours at room temperature. After immobilization, various blocking reagents were used, to evaluate which gave the best results taking into consideration true signal and noise. Blockings that used were:

1. *Starting Block (*Thermo Scientific)
2. *Blocking buffer (*Thermo Scientific)
3. *SEA block (*Thermo Scientific)
4. *Super Block (*Thermo Scientific)
5. *DuoLink blocking buffer* (Olink)

In all blocking solutions, 0.1% mg/ml salmon sperm DNA was added and all were incubated for 30 min at room temperature. After removing the blocking solutions, a wash with 1x PBS was performed and the mouse IgG antibody, 1nM, was inserted. Then the regular protocol of detection was performed.

2.6 Different concentrations of antigen[1]

The sensitivity of the method was evaluated. Different concentrations of antigen were used: 0, 1, 10, 50 pM and the layout of the experiment is shown in ***figure 9***:

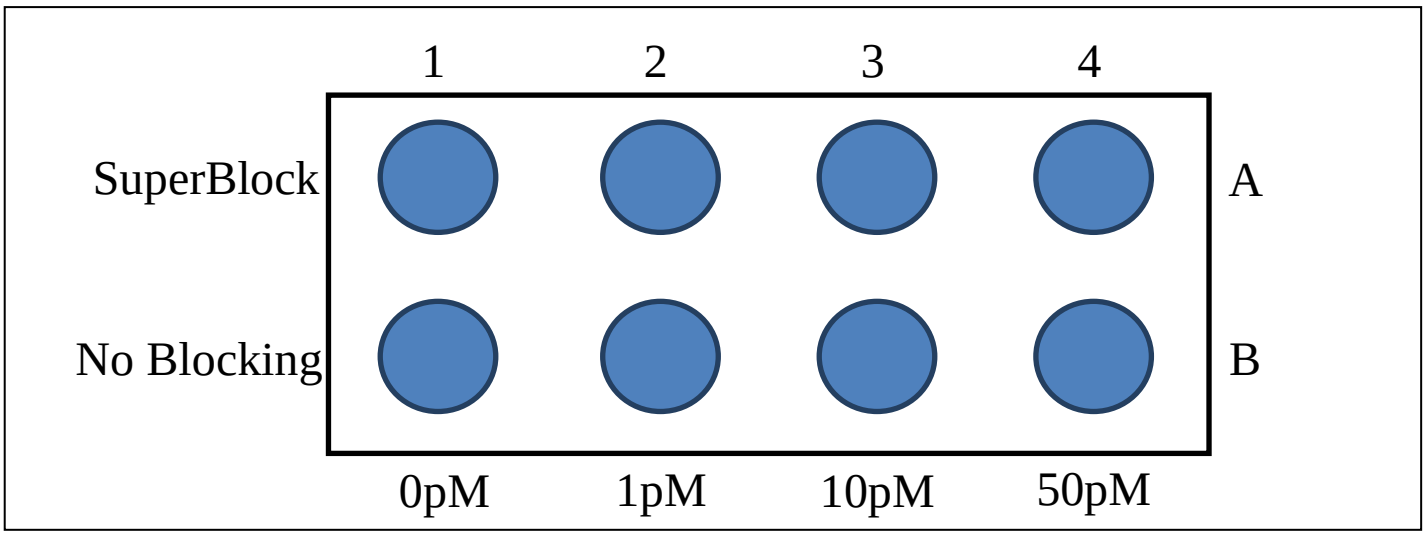


Figure 9. *layout of the experiment with different concentrations of antigen*

[1] Mouse IgG is referd to as antigen not to be confused with the capture antibody (anti- mouse IgG).

Super block was tested compared to no blocking. Column 1 served as a background test. 1μL of antibody was added in 1660 μL of carbonate buffer to a final concentration of 10nMfollowed by a second dilution 1:10 dropped the concentration in 1nM. Consequently, this dilutant was used to make all the different concentrations, as shown on the table:

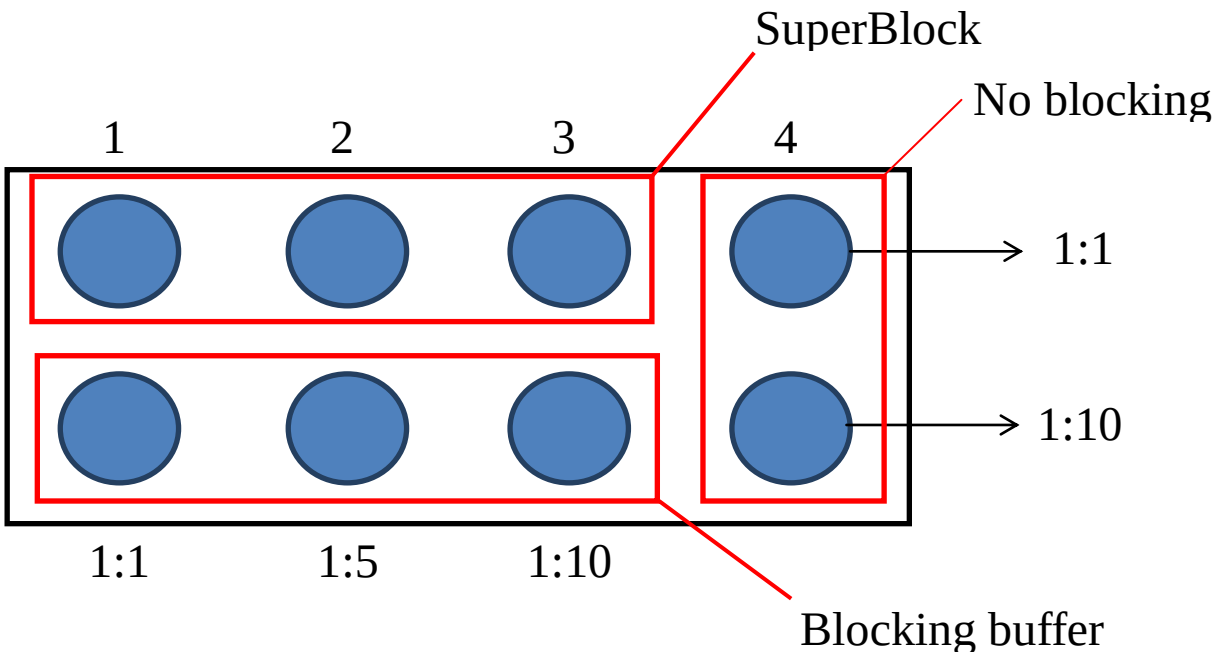
Table 1 dillutants of antigen

Concnentration (pM)	$V_{antigen\ 1nM}$ (μL)	$V_{carbonate}$ (μL)	V_{final} (μL)
0	0	100	100
1	1	999	1000
10	1	99	100
50	5	95	100

Furthermore, the efficiency of storing the already immobilized slide in 4ºC was tested. So, 2 slides where prepared, but slide no.1 was kept in the freezer for the whole weekend, whereas slide no.2 was used the same day (no storage). Slide no.1 was kept in the freezer with the mask on it and the capture antibodies inside the chambers.

2.7 Probe titrations

Figure 10. *layout of the probe experiment's mask*



The efficiency of using different dilutions of the Olink's Duolink in situ PLA probes PLUS and MINUS (see introduction 1.8, closer look to the method) was tested. Dilutions used were: 1:10, 1:5 and 1:1. The layout of the mask was as it is illustrated. Furthermore, the dilutions were tested alongside with two Blockings that gave the best results in previous experiments so that it is easier to reach an optimized protocol. Again, two slides were used, the one with antigen and the other without.

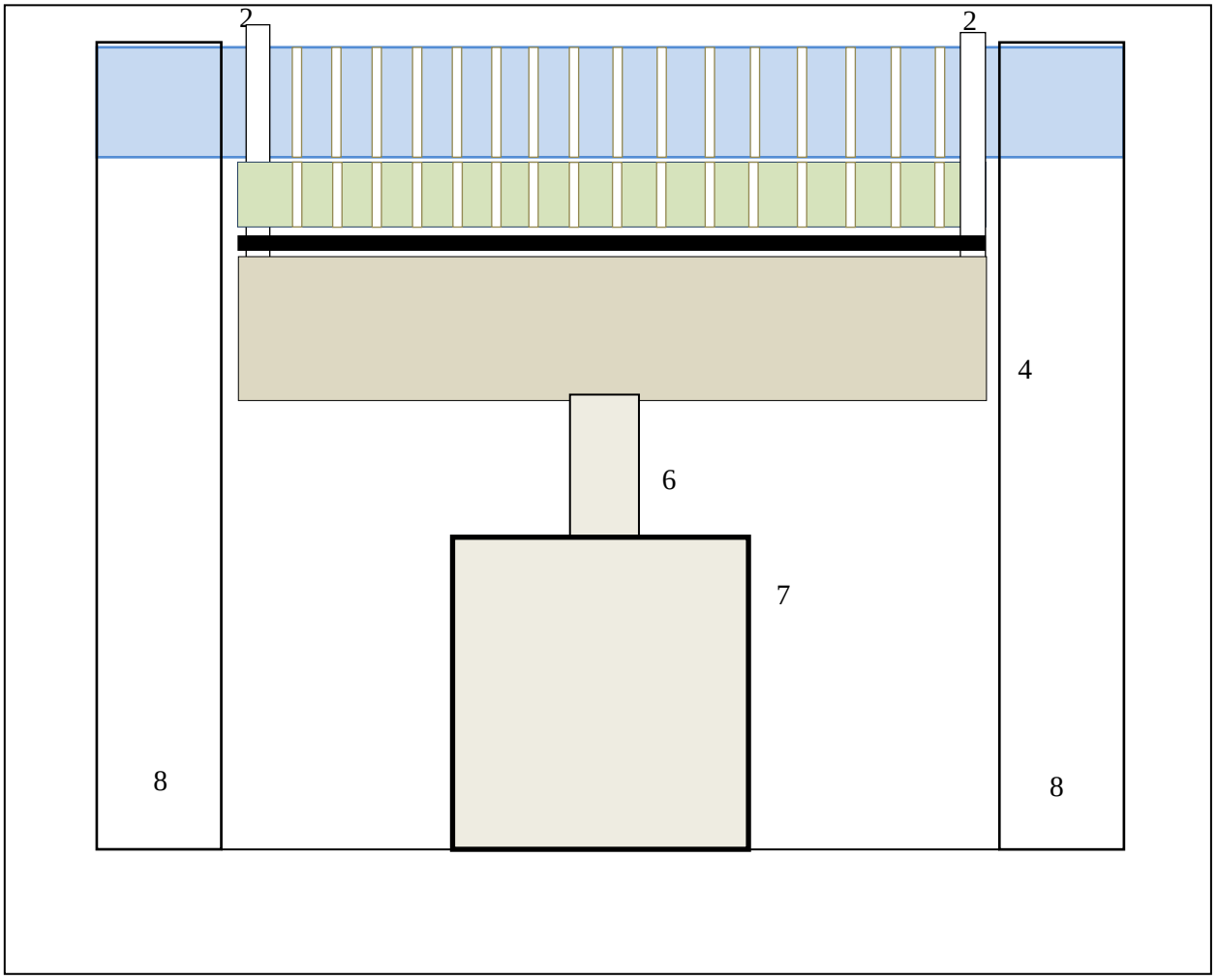
2.8 Moving into microfluidics

Having optimized conditions for blocking, capture and detection we proceeded to perform SP-PLA in microfluidics.

The following components were used, and assembled in order to run the microfluidic experiments

- ✓ Automated compressed air slide holding device

This device was used in order to hold together and with great pressure the slides, the PDMS mask where the microfluidic channels were in and the guides for the tubes. All the components were aligned together by the assistance of two metallic cylinder holders, which guide all the components through two holes on each side of the device. The slide and all the other components were held by an aluminum platform which was raised to the level of the plastic selling by a hydraulic cylinder, which moved upwards and downwards with the assistance of compressed air. Compressed air was provided by a Biltema compressor OL 15-6 and 0.4MPa of pressure was applied.



At the design above, it is demonstrated how the layout of the device is:

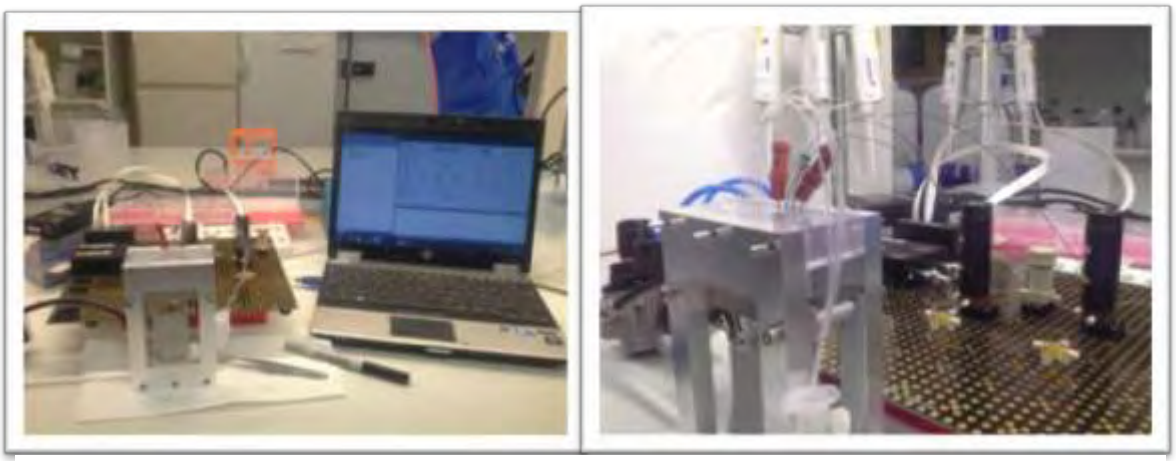
1. Plastic selling with 15 guide holes
2. Metallic guide cylinders
3. Silicon mask with 15 microfluidic channels
4. Slide
5. Aluminum holding platform
6. Piston
7. Hydraulic cylinder device
8. Aluminum holdings

✓ Syringe pump

The stable flow through the channels was succeeded by the help of a programmable PHD 2000 syringe pump (Harvard Apparatus 84, October Hill Roa, Holliston MA 01746, United States) and a 1mL plastic syringe (diameter 5mm). With the command 'withdraw' the syringe sucked the reagents with a tube (1/32'') and by the command 'diffuse' the liquid was lead into the channels.

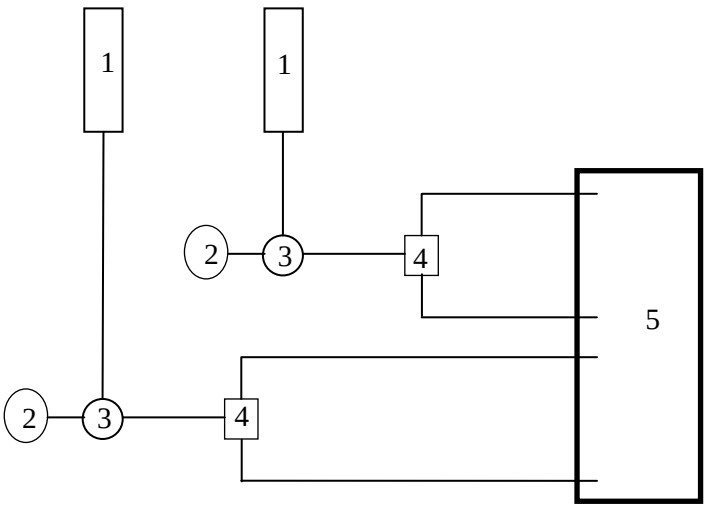
✓ Standard uProcess™ Kit

This kit was provided by Labsmith (LabSmith, Inc. | 6111 Southfront Road, Suite E | Livermore, CA 94551) and it is a breadboard on which the whole microfluidic circuit could be applied. It was modified to be able to test 4 different channels simultaneously. The kit contained 2 reservoirs, 2 pumps with two 80μL syringes, two automated valves and some adaptors to connect it to the pc.



Figures 4,5*: The main layout of the microfluidic experiment. Pc with uprocess program controlled pumps and valves.*

All fittings of the kit were for 360μm tubings. The microfluidic circuit was:



1. 80μL syringe
2. Reservoir
3. Automated 3way valve
4. Spliter
5. slide

2.9 Microfluidic experiment

2.9.1 With 15 minutes of antigen wash through

For the first experiment of the micro SP-PLA a channel of 15μm in depth and 30μm in width was used alongside with 1/32'' tubings and a 1mL syringe attached to the Harvard apparatus pump device. The table shows duration, flow rates and volumes of each reagent:

	antigen	*wash*	*probes*	*wash*	*ligation*	*wash*	*amplification*
total volume (μL)	80	30	50	25	50	25	90
Flow rate (μL/min)	5	5	5	5	5	5	1
duration (min)	15	6	10	5	10	5	90

Before the microfluidic procedure, the capture antibody was immobilized the previous day in a squared mask 22mm x 22mm and let for an overnight immobilization in 4ºC. Then, blocking buffer with salmon sperm DNA (0.1 mg/ml) was added and the incubation took place for 30min in room temperature. The mask was then removed and the slide got into the automated compressed air slide holding device with all the other components.

2.9.2 With 1 minute of antigen wash through

By keeping all the durations the same, only the one of antigen was changed just to push the method even further. So the duration that was chosen was only 1 minute, in a flow rate of 1 μL/min and the concentration of antigen 100pM.

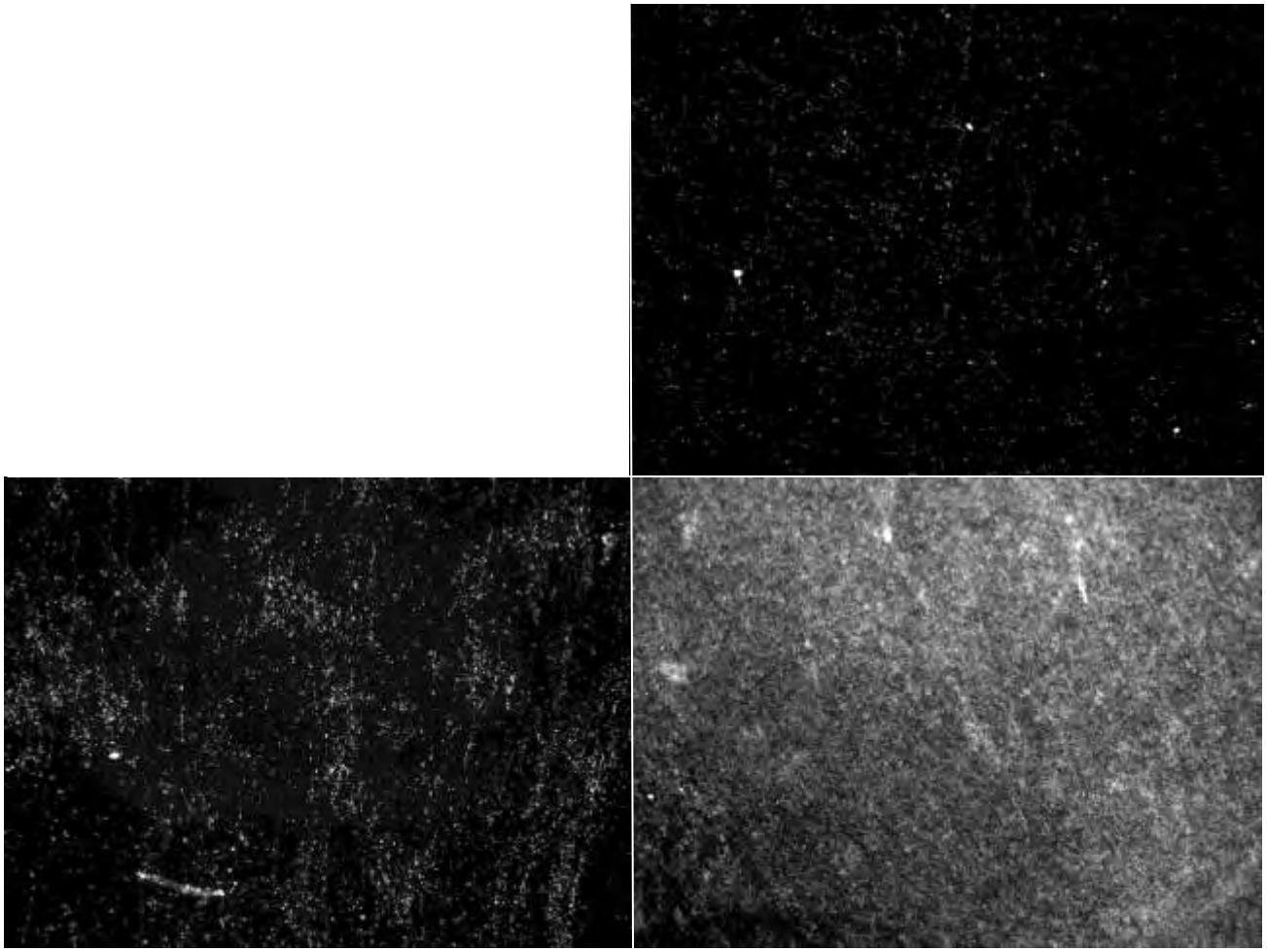
2.10 Same duration in masks

What makes a microfluidic experiment successful? Better signal, zero background, lower volumes and even lower duration. We wanted to assess the performance of the assay in a microfluidic environment and compare it to the results we already had. The background was tested alongside with 1 and 15 minutes of antigen incubation. All the other incubation times were the same as the ones in the microfluidic experiment.

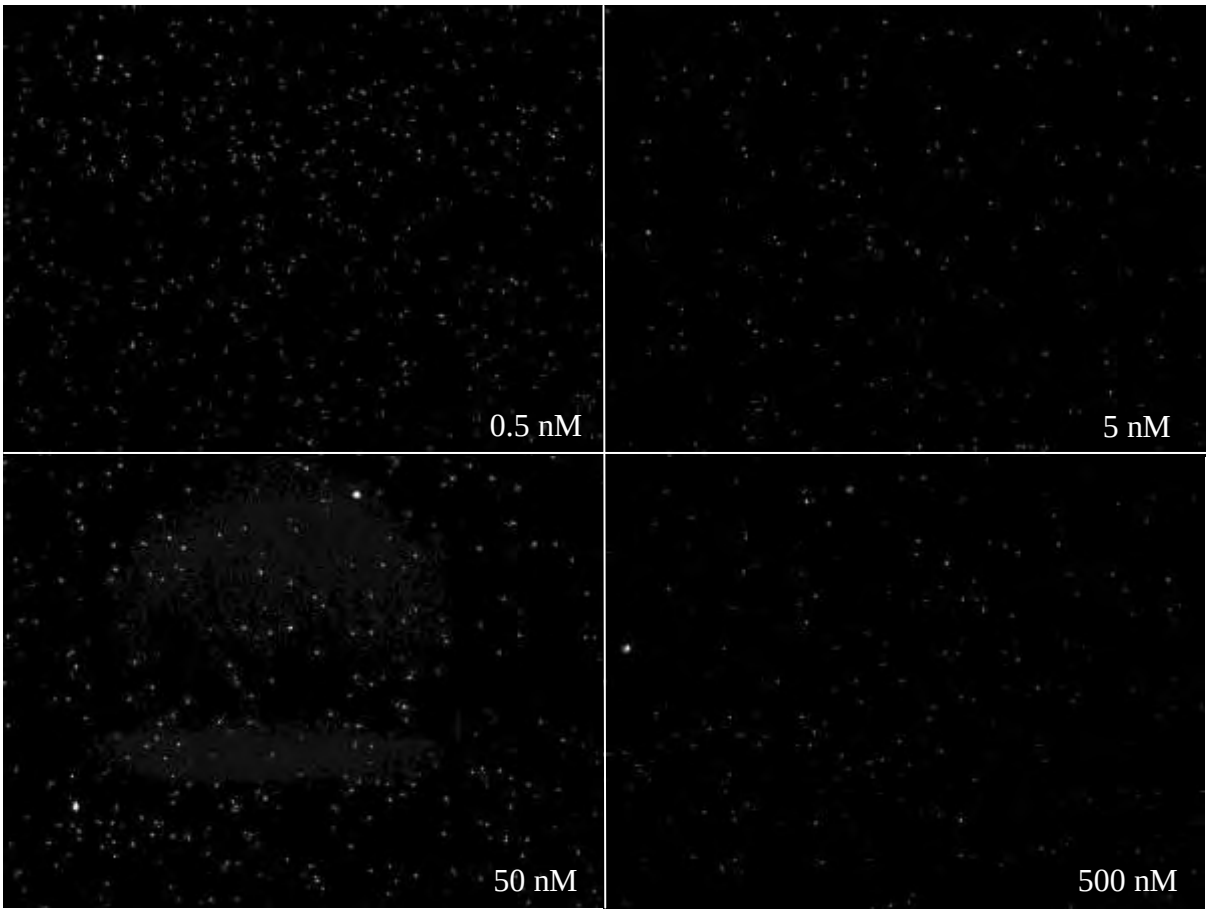
3 Results

3.1 The right concentration of capture antibody

The right concentration of capture antibody was one of the most important parameters in the whole experiment because that would define how much of the antigen could be captured and detected. From the 4 concentrations used (0.5, 5, 50, 500 nM), 500 nM gave the best results according to the following images:



The density of RCPs was so high that it was impossible to be counted. Though, background kept really low in all 4 concentrations.



Background images (antigen minus)

3.2 CodeLink vs. Sigolis

The optimization of this method was performed only on the CodeLink slides because it was more efficient to use them and much easier. The signal was higher compared to Sigolis slides and the protocol time was significantly reduced by 90 mins. The diagram (***figure 11***) shows how the number of the counted RCPs changes with the different concentration of antibody in both slides. Sigolis slides had a very high signal in 10nM on antibody but less signal in 1nM, which is the concentration we used. The big drawback of the CodeLinks slides is the high background, which needed to be fixed by applying a very efficient blocking method.

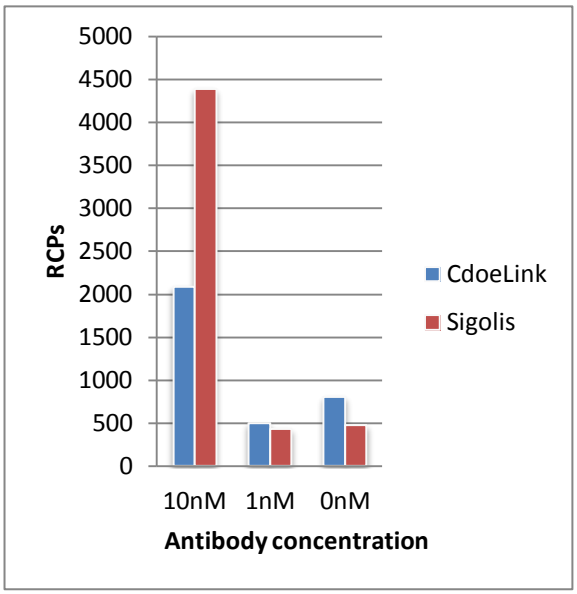


Figure 11. *diagram of the antibody concentration and signal in Codelink and sigolis slides*

3.3 Blocking with BSA

Blocking with BSA was the first option in the blocking techniques. It is used widely in many immunoassays with good results. The diagram shows how number of RCPs changes with each different concentration of BSA. As it is demontrated, the more BSA added the less the signal. That suggests that perhaps BSA interacts with the antigen binding sites of the antibody causing block and making the antibody unable to detect and bind the antigen. Also the background seemed to deminish which means that BSA can actually block the surface and reduce non specific binding, but the fact that true signal diminish made us explore alternative blocking strategies.

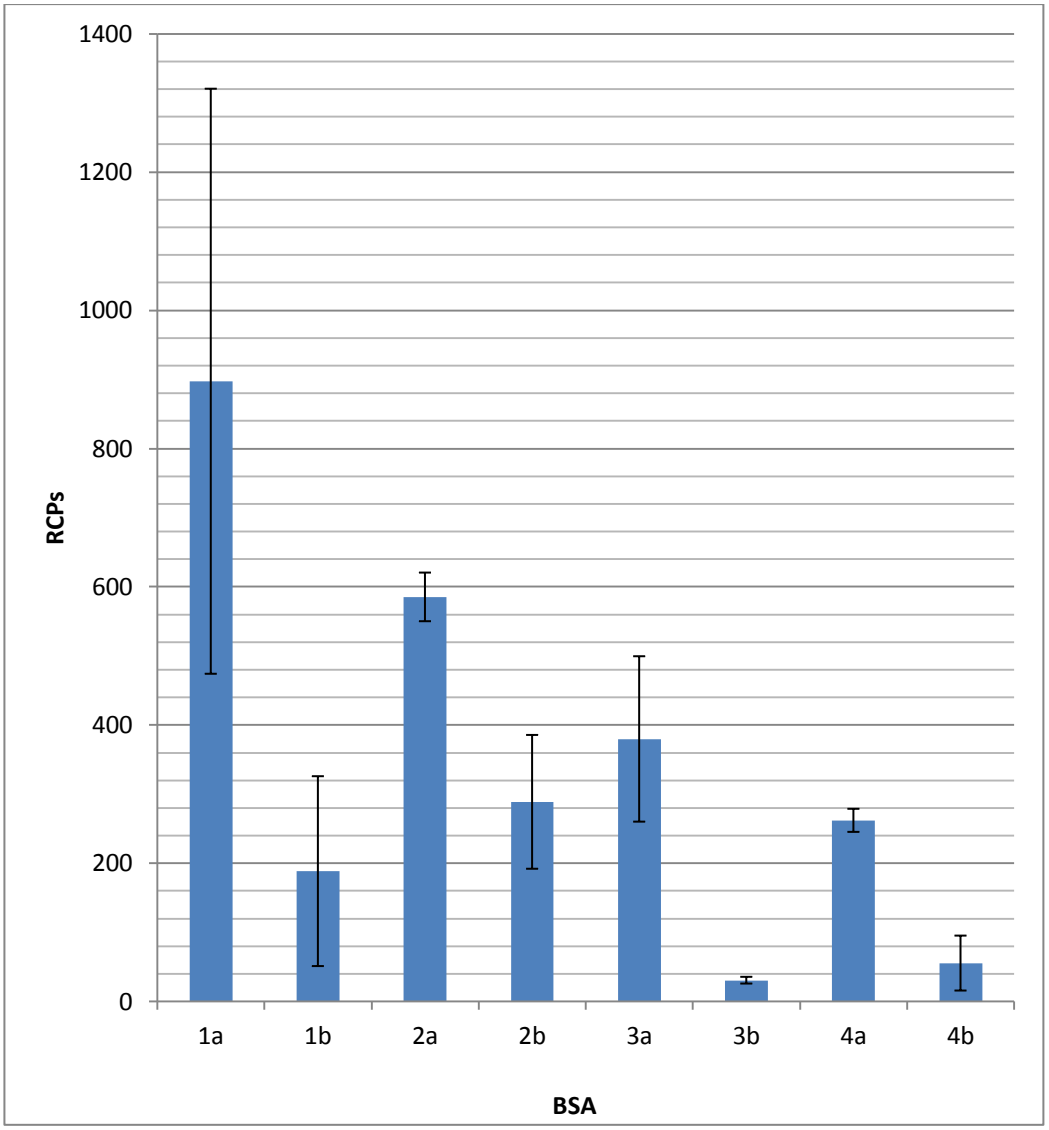


Figure 12 *diagram of signal and noise between different concentrations of BSA*

1: 0% BSA a: antigen +
2: 0.1% BSA b: antigen -
3: 1% BSA
4 10% BSA

3.4 Testing different blocking

Different blocking solutions were used in order to find one that keeps the background in low levels while maintaning true signal. 3 different blockings were used: Blocking buffer, Super Block, Duolink's blocking as well as no blocking.

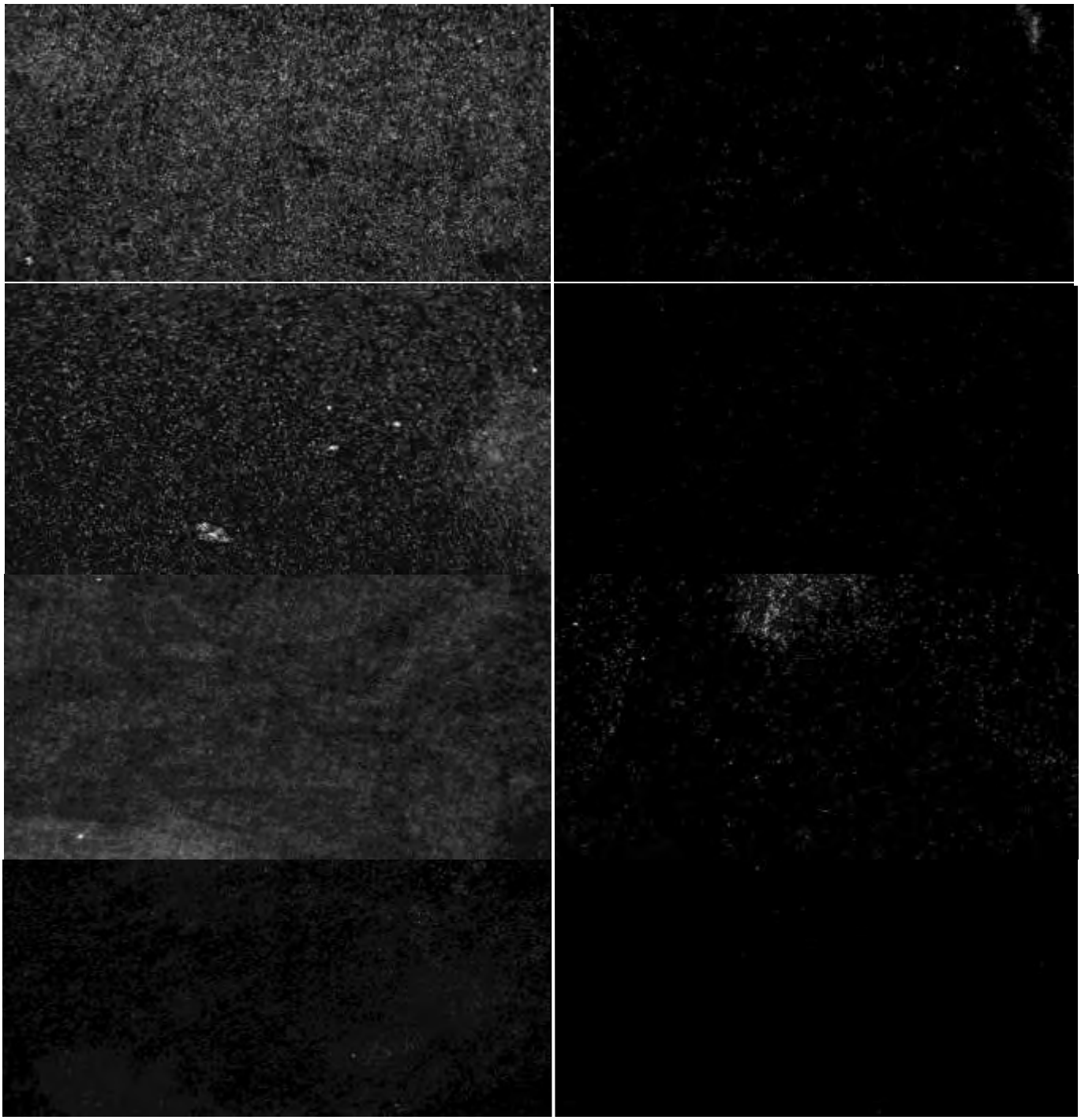


Figure 13. *Pictures of results of different blockings. The signal is shown in the left images whereas the background on the right ones.*

Antigen +

Antigen –

On ***figure 13,*** the images of the results in different blockings are demonstrated. Notice that the signal is still too high to count RCPs, so in such concentrations of antigen the actual number of them cannot be estimated. Here we observed a tremendous difference between the signal that Super Block gives and the others. Super block was able to eliminate the background (***figure 14***) and make the all RCPs clear.

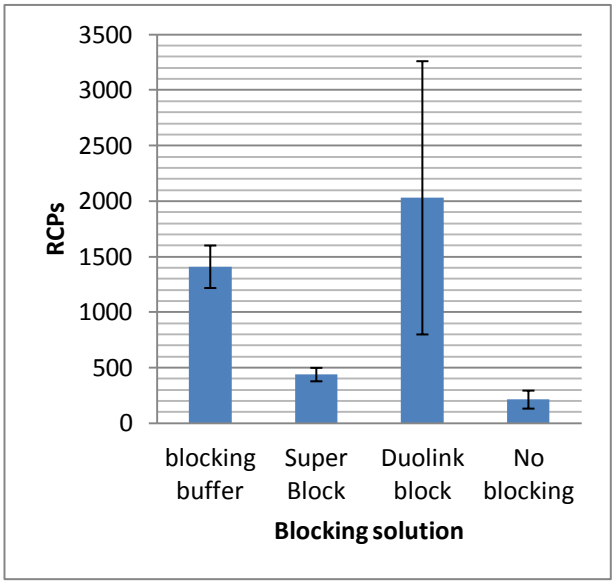
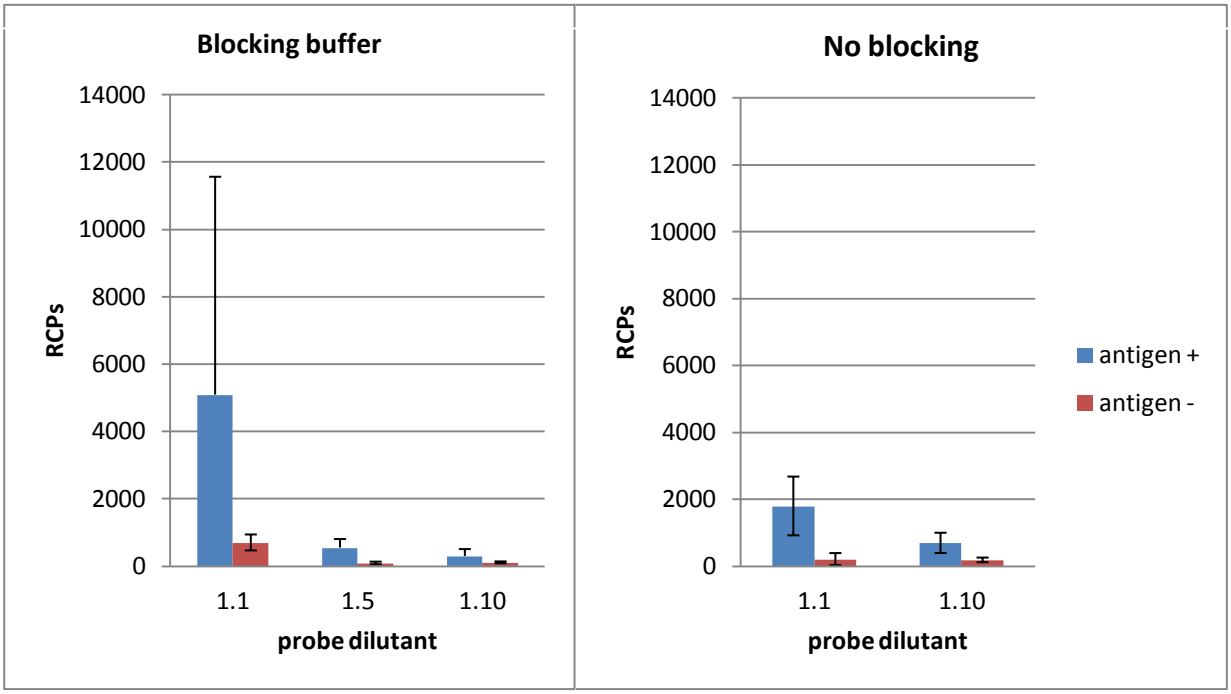
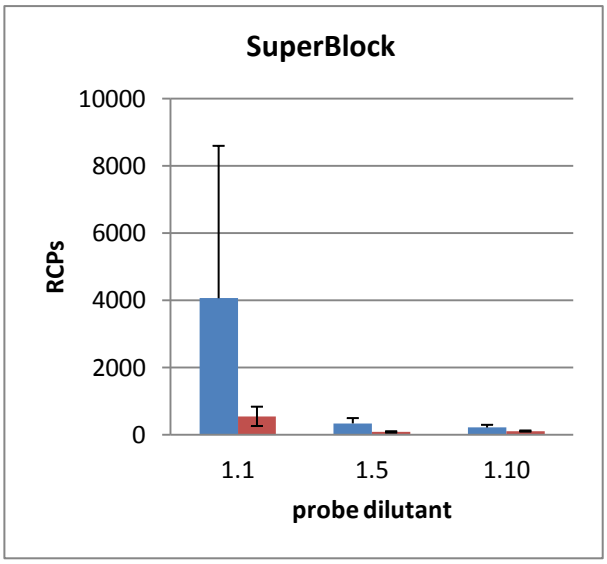


Figure 14. *diagram of the background using different blockings*

3.5 Probe titrations

The right concentration of probes will affect assay performance. Too high concentration could cause high background, and too low could cause loss of true signal.





Figures 15, 16, 17: *Different blockings with different probe dilutions*

Figures 15, 16, 17 show how the number of RCPs is affected by the different concentrations of probes alongside with different blocking buffers. With the bocking buffer, the 1:1 dilutant gave a maximum signal of approximately 12,000 RCPs, while the others failed to raise the signal in such high levels. The background in the 1:1 dilutant is also kept on low levels, making it possible for the signal to noise ratio to be in the highest levels.

Assessing the effect of blocking, we observed that the difference is quite big. The average difference is approximately 3,000 RCPs making blocking buffer more efficient than no blocking in the 1:1 dilutant of probes.

Last, we checked SuperBlock but the results showed that it is not as efficient as the Blocking Buffer. Consequently, the best dilution of probes to use was 1:1, corresponding to a concentration of 6,25μg/ml.

3.6 Different concentrations of antigen with optimized conditions

Having established different experimental conditions, we proceeded by assessing the sensitivity of the method. We were able to

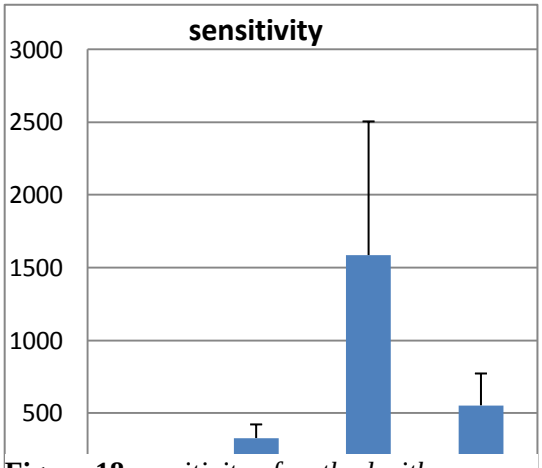


Figure 18 *sensitivity of method with optimized conditions*

accurately detect concentrations of the antigen as low as 1 pM (***figure 18***).

3.7 Optimized protocol for SP-PLA on CodeLinks' glass slides

Optimized experimental conditions are as described:

1. Dilute the capture antibody in carbonate buffer (pH=9,6) using the preferable concentration (500 nM)
2. Place the antibody dilutant in the mask and let the immobilization proceed for 2 hours at Room Temperature (RT).
3. Wash once with 1X PBS for 1-3 min
4. Insert Blocking Buffer with salmon sperm DNA 0.1mg/ml and incubate for 30min at RT
5. Wash once with 1X PBS for 1-3min
6. Add antigen. Then incubate for 2h in 37ºC
7. Wash with Tween PBS for 1-3min once
8. Dilute DuoLinks' probes at a 1:1 ratio and incubate for 60 min in 37ºC
9. Wash once with Tween PBS for 1-3min
10. Procced with the ligation reaction, using Ligation stock from DuoLink in 1:5 dilution according to manufacturer's instructions.
11. Wash once with Tween PBS for 1-3min
12. Add the amplification stock in dilution 1:5 according to manufacturer's instructions.
13. Wash with Tween PBS for 1-3min once (last wash)
14. Put the slides in ethanol series (70%, 85%, 99%) for 2 min respectively
15. Use vectashield mounting medium and a cover glass to cover the slide
16. Detect the signal in a fluorescence microscope in Cy 3.5
17. Store slides in the dark at 4ºC for further analysis

3.8 Micro SP- PLA

With the optimized protocol for SP PLA it was possible to move and apply the method into microfluidics. In figure 19, point 1 represents the infusion point and point 15 the exit point of the microfluidic channel.

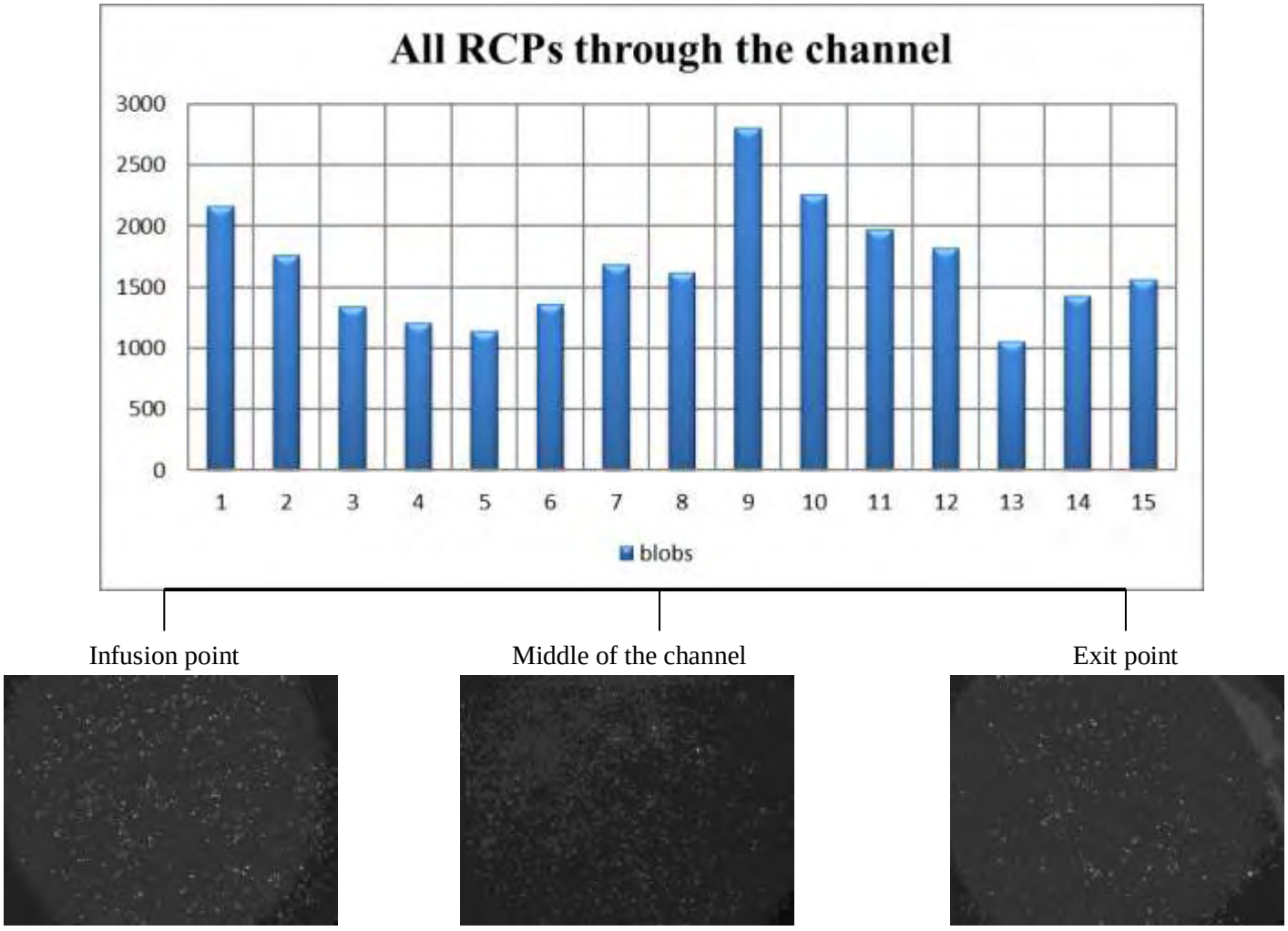


Figure 19. *RCPs through the channel with images of 3 points*

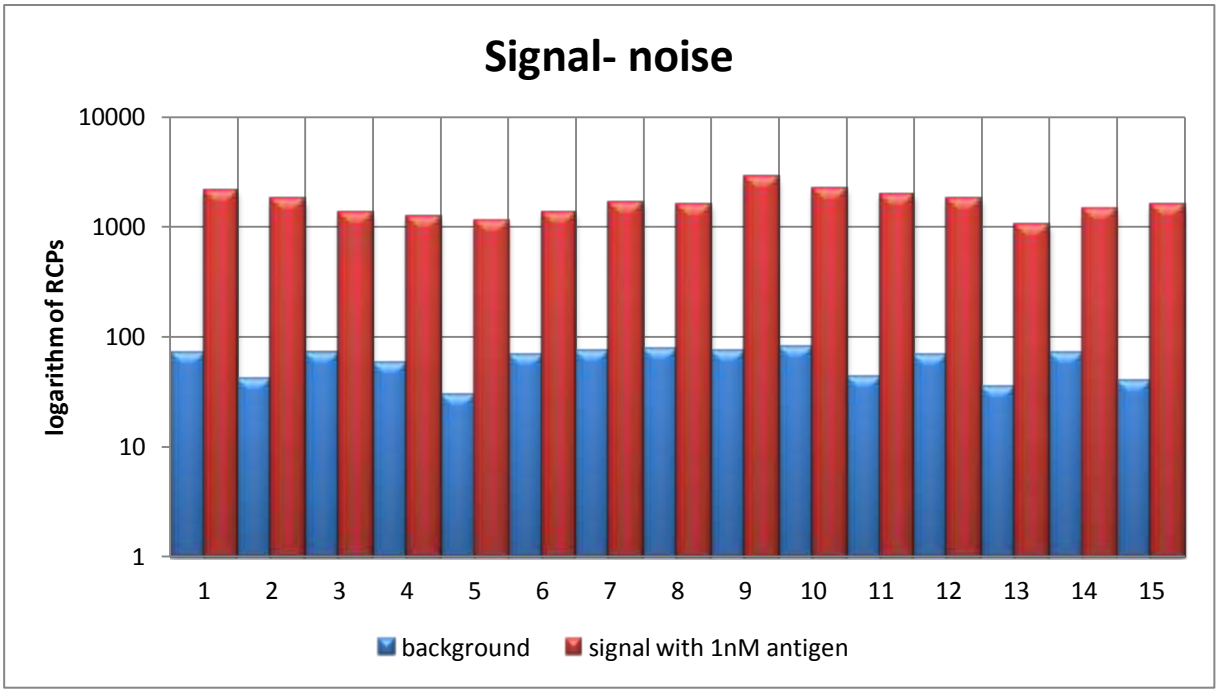


Figure 20. *Signal to noise ratio in logarithmic scale*

Another advantage of the microfluidic method is the fact that the background is almost eliminated (figure 20). The ratio between true signal and noise is very high and that gives promising results for the future. It will be really encouraging to use even lower concentrations of antigen and even lower volumes of all reagents.

Next step was to reduce the antigen duration to only 1 minute, and concentration in 100pM. The diagram in that case was completely different (***figure 21***). All the RCPs in the whole channel were as low as the background, a clue that implies the inefficiency of the method to detect the antigen in such short duration.

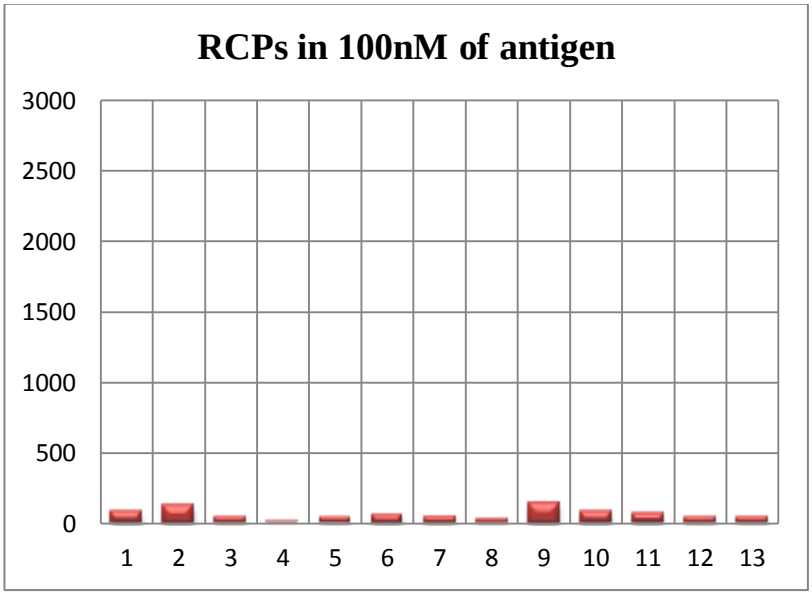


Figure 21 *diagram of reduced time*

We run the same experiment without the use of microfluidics in a 50μL mask.We were able to detect signals over background in the samples that were incubated for both 15 and 1 minute, as shown in ***figure 22***:

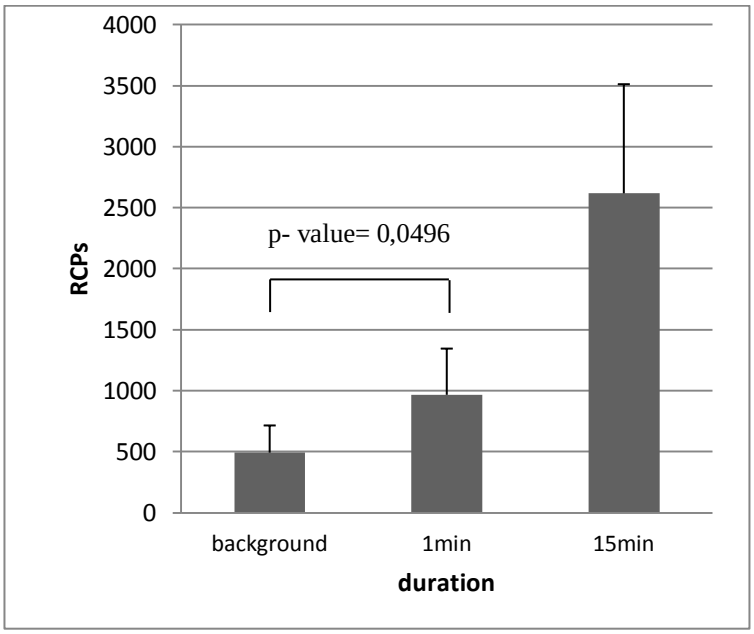


Figure 22[2] *results with same durations in masks*

3.9 Efficiency of the method

The fact that the signal is lower than the one in the mask, is concerning. Because of the nature of microfluidics, reactions times should be much lower, and efficiency of binding the antigen better. This suggests that perhaps because of the flow rate some of the RCA products that are bind with the capture antibody are washed away and so we lose most of the signal. It is sure though, that the background is almost zero, so we have gains in signal to noise ratio.

4 Discusion

The design of the PLA technology demonstrates excellent sensitivity of detection which can reach even 1000-fold greater sensitivity than the commercially available ELISA. So, trying to optimize even further such techniques holds the key for creating one of the world's most sensitive method. Diagnostics could benefit from an improvement in assay performance. Taking for example troponin I and T, molecules that are released in blood during myocardial infraction, are excellent biomarkers for it. The only problem is that they are found in the blood in extremely low concentrations (50-100 ng/dl) and a big amount of them is not so long after cardiac damage. Consequently, micro SP-PLA should be used as an excellent paradigm of such method. With its low duration and high sensitivity, this method could be commercially available to public and hospitals, making it possible for many human lives to be saved.

[2] The p- value was calculated by t- test

Unfortunately we are still to very beginning and we currently try to understand how microfluidics exactly work and behave under various conditions. The prototype device we made had some faults like inefficient way to hold tubes inside the microfluidic channels causing leaking of reagents. This, could cause non efficient flow through the channel and consequently stationary reagents. Furthermore it would be better for more flow rates and volumes to be tested. So far we tested 2 conditions, but the limited time didn't gave us the chance to test more. Flow rates are really important because that could affect directly how efficient the reactions could be. For instance, high flow rates with high volumes of reagents could cause molecules to pass the channel so quickly that it would be impossible for any reaction to be completed. On the other hand, low flow rates with lower volumes could cause lack of the quantity of the reagents.

We were able to overpass this problem by binding a new mask made of silicon on the glass slide by oxidation. The silicon mask was thicker than the one we used from sigolis, and also the entrance holes were tighter so that the tube could firmly fit in them. The only drawback was the fact that the immobilization of the capture antibody had to take place inside the microfluidic channel, which mend that the antibody should flow through it. So, practically that was a 'flow immobilization' with non-predictable results. Also, because of the inability to remove the silicon mask, the signal should be detected with a confocal microscope. The first results we got from this new approach were satisfying and promising (***figure 23***). Nevertheless more experiments and analysis need to be done to evaluate the efficiency and convenience of the new method.

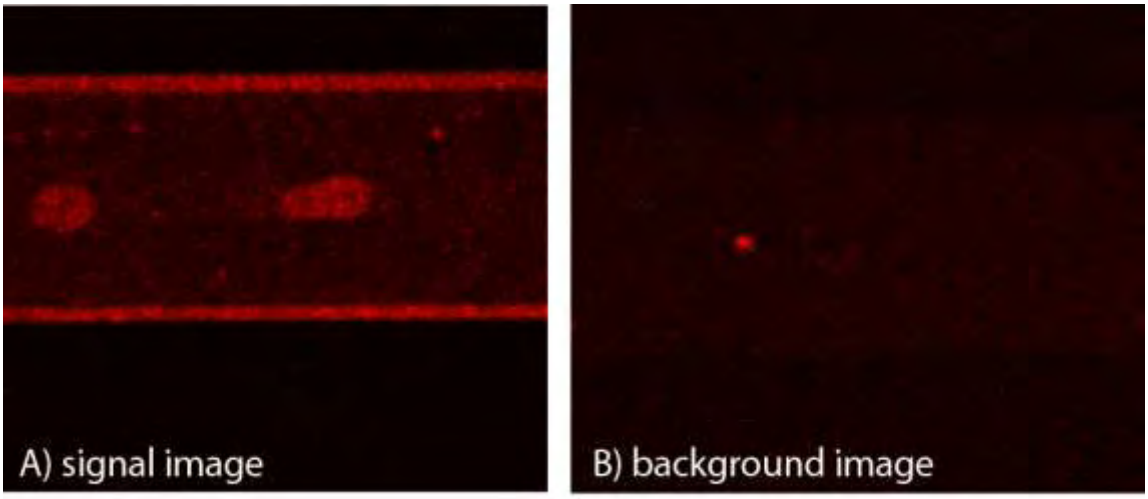


Figure 23 *images demonstrating the results from the latest microfluidic experiment*

Another parameter that could be changed is the amplification process. We currently use RCA in order to amplify the signal into an RCP. This method takes about 1.5 hours to be accomplished, time that should be reduced. Future plans includes reducing the amplification duration by taking advantage of a new method called super rolling circle amplification (sRCA). During this assay, the time of reaction could reduce to only 15 minutes and also RCPs could be the same size and quality as the ones in the traditional RCA.

That consists the first step for this super-efficient method that can actually replace robotics or make them obsolate. Microfluidics gives us the advantage of tiny quantities of reagents and space. So, multiple samples could be analyzed simultaneously and automatically without any great effort from the scientist.

5 References

[1] Darmanis S, Nong RY, Vänelid J, Siegbahn A, Ericsson , *et al*. (2011). PreoteiSeq: High-Performance Proteomic Analyses by Proximity Ligation and Next Generation Sequencing. PLoS ONE 6(9): e25583

[2] Kamali-Moghaddam *et al*. (2010). Sensitive detection of Aβ protofibrils by proximity ligation- relevance for Alzheimer's disease. BMC Neuroscience 2010, 11:124

[3] Vlcek C, Paces V (1986). Nucleotide sequence of the late region of Bacillus phage Φ29 completes the 19,285-bp sequence of Φ29 genome. Comparison with the homologous sequence of phage PZA. Gene 46 (2-3): 215–25

[4] Blanco L, Bernad A, Lázaro JM, Martín G, Garmendia C, Salas M (1989). Highly efficient DNA synthesis by the phage Φ29 DNA polymerase. Symmetrical mode of DNA replication. J. Biol. Chem. 264 (15): 8935–40

[5] Bernad A, Blanco L, Salas M (1990). Site-directed mutagenesis of the YCDTDS amino acid motif of the phi 29 DNA polymerase. Gene 94 (1): 45–51

[6] Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M (1992). The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. J. Biol. Chem. 267 (4): 2594–9

[7] Gregory J. S. Lohman, Lixin Chen, and Thomas C. Evans, Jr. (2011). Kinetic Characterization of Single Strand Break Ligation in Duplex DNA by T4 DNA ligase. JBiol Chem. 288 (51): 44187- 44196

[8] Cherepanov AV, de Vries S. (2002) Kinetic mechanism of the Mg2+-dependent nucleotidyl transfer catalyzed by T4 DNA and RNA ligases. J Biol Chem. 18;277(3):1695-704

[9] S. J. Lee, S. Y.Lee (2004). Micro total analysis system (μ-TAS) in biotechnology. Appl Microbiol Biotechnol 64: 289- 299