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Bioluminescent determination of protein using a capture / signal complex

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Bioluminescent determination of protein using a capture / signal complex

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Abstract

In the present project a method for detection of proteins has been developed. The aim of this project has been to use the developed method together with a device originally created for bioluminescent analysis of DNA sequences to measure the amount of proteins. The protein chosen in this study was C-reactive protein. This protein is prevalent for diagnosis in the healthcare. If the amount of CRP is known it will help to make a diagnosis on the patient. The detection has been carried out by using plastic beads, antibodies and enzymes. The antibodies have make protein capture, to plastic beads possible and have made it able to mark the protein with an enzyme. A system with enzymes which can produce light have then been used (pyruvate kinase and luciferase). The produced light has finally been detected at the instrument developed for DNA detection. The results prove that the principle for protein detection works.

Keywords

Antibody, beads, C-reactive protein, CRP, IgY, luciferase, pyruvate kinase, pyrosequencing

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Bioluminescent determination of protein using a capture / signal complex

Kaleb Eriksson

Sammanfattning

Om ett träd faller i skogen och ingen hör det falla, har det då fallit? Det finns en mängd händelser som sker i mikrokosmos utan att vi känner till dem. Ett område där vi skulle ha nytta av att kunna registrera de här händelserna är inom sjukvårdsdiagnostiken. Tanken med den här metoden är att den i framtiden ska kunna användas inom sjukvården för att ställa proteindiagnoser. Det som gör det här arbetet särskilt intressant är att utrustningen som använts egentligen är utvecklad för ett helt annat ändamål och det faktum att vi har kunnat visa att den kan användas i en helt ny tillämpning. Principen som gjort det här möjligt är utrustningens förmåga, att med hjälp av enzymerna luciferas och pyruvatkinas detektera proteiner genom att mäta ljus. En annan intressant aspekt är att alla reaktioner skett på små plastkulor. Genom att plastkulornas yta har modifierats så har olika verktyg kunnat fästas in, till exempel antikroppar, som bland annat gjort det möjligt att fånga upp proteinerna. Marknaden i världen för proteindiagnostik, som den här tekniken tillhör, är enorm och som ett exempel kan nämnas att enbart i Sverige görs 10 miljoner tester av proteinet CRP per år.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala Universitet, Maj 2007

1. INTRODUCTION

Is detection of proteins, with a device developed for bioluminescent analysis of DNA sequences possible? In this project the goal has been to investigate if technology from the Centre for Surface Biotechnology is possible to use in cooperation with devices from Biotage AB to detect proteins. Since immunoassays with C-reactive protein (CRP) are robust, well standardized, reproducible and prevalent for diagnostics in the healthcare, CRP was chosen in this study as the protein to be detected. The protein is a sensitive marker for inflammation, infection and tissue damage. The production of CRP is a part of the non-specific acute phase response. Together with other clinical and pathological results it can provide useful information about a patient's condition [1]. In detection of CRP a blood sample is drawn by needle from a vein in the arm. The sample is then analyzed by either a rapid test, which has medium sensitivity or by an advanced highly sensitive test, which requires analytical instruments. The sensitive test will predict the risk of cardiovascular diseases, heart attacks, and strokes [2]. There are several kits on the market for measuring CRP. Two companies which provide highly sensitive tests are Randox laboratories and Thermofisher. Their assays have a dynamic detection range for CRP of 0.15 mg/L to 320 mg/L.

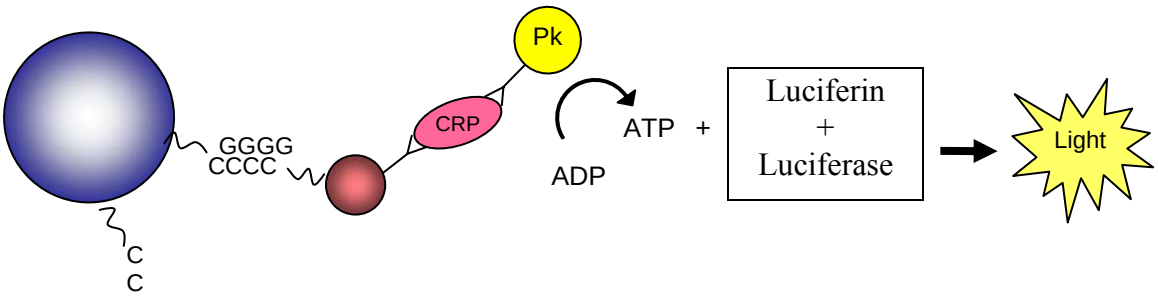

Fig. 1: Shows the protein capture/signal complex. The complex is composed of many small beads linked to a large one. Antibodies attach CRP to the small bead as well as pyruvate kinase (Pk) to CRP. Pyruvate kinase converts ADP to ATP. Luciferase mediates a light emitting reaction when ATP is produced.

There were two challenges in the present project. The first was to develop a method to separate the CRP from other substances in a suspension and the second was to detect and quantify the purified CRP sample. The procedure of isolating the target molecule is normally to fasten it to a solid phase and to rinse away everything which is not bound. A common way is attaching the target molecule to the walls in a well. The problem with this method is that the wall surface is limited. In order to increase the available surface, the Centre for Surface Biotechnology attaches the sample to small plastic beads. The equipment provided by Biotage for handling beads requires beads with a larger diameter. The advantage of increased surface area would be lost if the size of the beads is increased. One way to solve this problem is to attach the small beads to larger beads (Fig. 1). This procedure has been studied in this project.

The second challenge was to detect the target molecule. This could possibly be achieved by attaching to the target molecule an enzyme which produces light. Luciferase is such an enzyme, used both in Pyrosequencing® and in the technology from the Centre for Surface Biotechnology. However, luciferase is sensitive to immobilisation. Luciferase requires ATP for its reaction, therefore pyruvate kinase, an enzyme which produces ATP could be attached instead. Thus the combination of attached pyruvate kinase and free luciferase is one

possibility (Fig. 1). An advantage of the Pyrosequencing® instrument used for detection is the real time monitoring of the reaction. This can provide several ways of determining the amount of the target molecule.

Finally the method is required to be specific, which is achieved by using antibodies. The same sort of antibodies is used both for attaching the sample to the beads and for attaching the enzymes to the target molecule.

1.1 Challenges

- Find an appropriate solid phase for attaching the small beads
- Get activity of pyruvate kinase in the system used for Pyrosequencing®
- Which amount of pyruvate kinase is needed? Dynamic range of pyruvate kinase detection.
- Is there a clear correlation between the amount of pyruvate kinase and the signal?
- Prepare an appropriate strong linkage between the large and small beads
- Prevent the filter from harming the enzymes

1.2 Aim of the project

The aim of this project was to purify and move proteins by using a Vacuum Prep Tool and detect proteins by using a Pyrosequencing® instrument, originally developed for DNA analysis.

2. THEORY

Why are beads needed?

A solid phase such as a bead is needed for isolating the target molecule. The advantage of using small beads compared to other commonly used flat surfaces e.g. microtiter plates, is that the available surface area is greater [3]. If the beads are attached to a wall the area will increase many times and if they are suspended, the available surface will increase even more, due to the fact that the whole surface is used. Besides the increase in surface area the mass transport and kinetics will increase due to the curvature of the beads. The mass transport is favoured by the fact that the reagents are captured from a cone-shaped volume. By contrast on a flat surface the accessible liquid volume is cylindrical and covers a smaller part of the solution. Furthermore on a flat surface steric hindrance will block optimal kinetics [4]. Previous studies have shown a 10-fold increase in binding constant of the streptavidin-biotin complex with a corresponding increase in bead surface curvature [3]. The small beads chosen in the current study had a diameter of 0.24 µm.

Why are two sizes of beads utilized?

The filter in the Vacuum Prep Tool provided by Biotage has an average pore size of 10 µm. The small beads used in the study would pass through the filter and making the pores narrower would decrease the liquid flow through the filter. Such a loss could be prevented by attaching the small beads to larger beads. The pore size of the filter varies between 6 µm to 14 µm [5]. To prevent loss due to this variation the larger beads chosen had a mean diameter of 19 µm. Both beads were made of polystyrene, which gives them hydrophobic properties, that had to be treated using Pluronic (see below).

The use of Pluronic

Pluronic F108 is a polymer, consisting of three blocks $(PEO)_{129}$-$(PPO)_{56}$-$(PEO)_{129}$. Poly(ethylene oxide) (PEO) is a hydrophilic block and poly(propylene oxide) (PPO) is a hydrophobic block [7]. The hydrophobic block provides the means to attach the polymer to a hydrophobic plastic surface, for example that of polystyrene beads. In an aqueous solution the hydrophilic tails will extend and prevent unspecific protein adsorption to the beads, and at the same time protect the attached proteins from denaturation. A pyridyldisulfide (PDS) group was attached to the hydrophilic end. The thiol group allowed it to bind other thiolated molecules, for example thiolated oligonucleotides, by forming disulfide bridges [3].

The use of oligonucleotides

Oligonucleotides were used to link the small and large beads through hybridisation [3]. The oligonucleotide consisted of 15-mers of either guanine (dG) or cytosine (dC) bases. The oligonucleotides were thiolated at the 5´-end, which allowed covalent attachment to the Pluronic F108-PDS coated beads. In previous studies the strength of the oligolinkage has been

shown by attaching beads of 0.24 μm to a plane surface by using the oligonucleotide linkage. The arrangement was stable in a flow of 6 mL/min, equivalent to shear forces on the beads between 0.44 and 0.64 pN [4].

The use of Cy5 as a tracer

The beads were labelled with Cy5 to help the study of bead-bead coupling and their transfer using the Vacuum Prep Tool. Cy5 is a water soluble fluorescent dye that is widely used for labelling proteins. The Cy5 used in this project was a monofunctional NHS ester. Cy5-NHS ester is excited maximally at 649 nm and emits maximally at 670 nm. The scanner used for Cy5-NHS ester detection will preferably have a wavelength emission of around 635 nm and filter with wavelengths of 655-695 nm to reduce the background. The optimum pH for protein labeling using Cy5-NHS ester is 9.3 but labeling down to pH 7.3 is possible. The molecular weight of the dye is 791.99 g/mol [8]. The fluorescence measurements were performed in a Perkin Elmer1420 Victor2^{TM} instrument which consists of three essential parts: a lamp, a photocathode, and a photomultiplier. The lamp emits light which a fluorophore (Cy5) can absorb and then emit a photon at a longer wavelength. The photocathode is a negatively charged electrode covered with a photosensitive compound. When the photon reaches the photocathode the cathode emits an electron. The signal is increased by a photomultiplier tube where the electrons are accelerated and their kinetic energy increased [9]. Due to the higher energy more electrons will be released when they strike the cathode again. By repeating the last step more electrons are released. Finally the current is measured, which will result in a value with the unit of counts per second.

The enzymes

The detection of the protein was performed by pyruvate kinase and luciferase. The overall reaction where luciferase produces light in cooperation with pyruvate kinase is seen in the scheme below.

$$\text{Phospho(enol)pyruvate} + \text{ADP} + Mg^{2+} + K^{+} \xrightarrow{pyruvatekinase} \text{pyruvate} + \text{ATP}$$

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow{luciferase} \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

Pyruvate kinase is an enzyme that mediates the reaction where ADP is converted to ATP. It is a tetramer with a molecular weight of 237 kDa. The enzyme is built up of four equal subunits, each with a molecular weight of 57 kDa [10]. The kinase is an essential enzyme in glycolysis. It catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, yielding a pyruvate molecule. This produces one molecule of ATP from one ADP molecule. In this reaction the enzyme requires two cofactors, a bivalent cation such as Mg^{2+} and a monovalent cation, preferably K^{+}. The optimum operating pH for pyruvate kinase is 7.5 [11] but the initial velocity of the reaction is also high in the range between pH 7.2 and pH 8.2 [12]. The catalysis by the kinase can be enhanced by including proline, glycine or sorbitol in the reaction [13]; Ca^{2+} inhibits the enzyme [10].

Luciferase mediates the reaction whereby light is produced by the oxidation of a luciferin molecule. The enzyme is commonly associated with fireflies. Male flies emit a flash at regular intervals. If a female fly recognises the flash she will respond to the male by flashing. The reaction of the enzyme is seen below, where luciferin and ATP reacts to give luciferyl

adenylate, in the first stage. In the second stage the luciferyl adenylate reacts with oxygen and light is emitted [14].

$$\text{luciferin} + \text{ATP} \xrightarrow{luciferase} \text{luciferyl adenylate} + \text{PPi}$$
$$\text{luciferyl adenylate} + O_2 \xrightarrow{luciferase} \text{oxyluciferin} + \text{AMP} + \text{light}$$

Antibody

IgY antibodies were used to capture the proteins. They had a mass of 180 kDa and were produced in eggs. The antibodies were attached to the beads through disulphide linkage to the Pluronic and to the enzymes through a disulphide linkage to N-sucinimidyl 3-(2-pyridyldithio)propionate (SPDP). Approximately 500-1000 antibodies were loaded on each small bead [15].

C-reactive protein

The production of CRP is a part of the non-specific acute phase response. Together with other clinical and pathological results it can provide useful information about a patient's condition. If there is an inflammation the CRP level can reach high levels in a few hours. Different amounts of CRP in a blood sample will point to [16]:

- No infection: 3 mg/L or less
- Indicates an infection: Above 5 mg/L
- Indicates a bacterial infection which must be treated: more than 50 mg/L
- Points towards pneumonia and other severe infections: Above 100 mg/L

The CRP levels can also predict the risk of cardiovascular disease, heart attacks, and strokes [2]. In the list below are the guiding values for the risk of cardiovascular disease [6].

- Low risk: less than 1.0 mg/L
- Average risk: 1.0 to 3.0 mg/L
- High risk: above 3.0 mg/L

CRP is built up of five subunits and has a molecular mass of 115 kDa and a high affinity for alkaline earth metals [1].

Detection by Pyrosequencing® equipment

Two pieces of equipment were essential to the project: the Vacuum Prep Tool, which provides sample purification and a means of moving the sample between different buffers and reagents, and the PSQ 96MA instrument, which performs the bioluminescent analysis of the sample (Fig. 2). The Vacuum Prep Tool consists of a hand-grip with 96 filter probes and is connected to a vacuum source. The probes are placed in wells containing the sample that is immobilised on a solid-phase. By applying the vacuum, the tool will aspirate the liquid in the wells and the sample/solid-phase will get caught at the surface of the filter at the probes. The sample can be released by placing the probes in new wells, turning off the vacuum and shaking the tool.

To trace possible loss of the sample, liquid which passed the filters had to be collected. In order to perform the collection of liquid the probes were moved from the Vacuum Prep Tool and individually connected to 2 mL syringes.

The PSQ 96MA instrument consists of a CCD-camera which registers light. Consequently, the emission of light can be used to indicate the presence of a target molecule. The light is produced by the oxidation of luciferin, catalyzed by the enzyme luciferase.

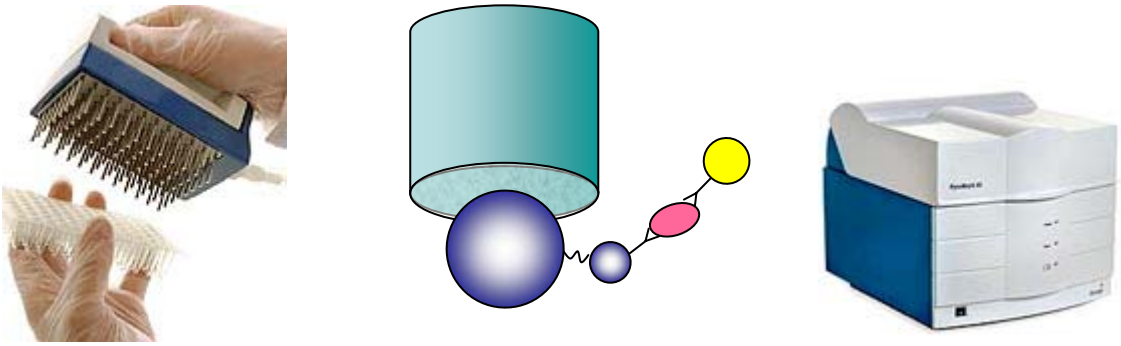

Fig. 2: The protein capture/signal complex is trapped by a Vacuum Prep Tool (left) and analyzed in a PSQ 96MA instrument (right). The central figure shows the protein capture/signal complex and one of the probes of the Vacuum Prep Tool. (The illustration was used with permission from Biotage AB).

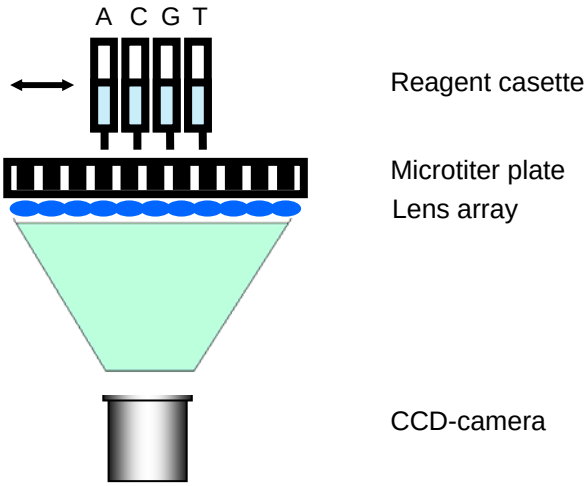

Fig. 3: The principle of detection in the PSQ 96MA instrument.

In the PSQ 96MA instrument it is possible to dispense the reagents from six positions in a reagent cassette (Fig. 3). The volume added can be determined through the opening times for each position. The cassette moves over a microtiter plate and adds the reagents in a predetermined order. To ensure a good mixing the microtiter plate is agitated continuously during the run. The light which is generated in the wells is directed to a CCD camera by a lens array located below the microtiter plate [9]. All the reactions are measured in real time.

The Vacuum Prep Tool is a handheld device with 96 probes. Each probe has a filter of an ultra high molecular weight polyethylene (UHMWPE) [5]. The filter pores vary from 6 µm to 14 µm in size and the median size is 10 µm. UHMWPE is synthesized from monomers of ethylene and the polymer chains are extremely long with high molecular weights. The long chains make the material very strong. Due to the polyethylene the filter is hydrophobic. In this project, the filter was covered with hydrophilic Pluronic to prevent denaturation of the protein samples by the hydrophobic surfaces.

3. CY5 AS A TRACER

The intention of the Cy5-tracer study was to find out how sensitive the instrument Perkin Elmer 1420 Victor2 ™ was and to determine the relationship between the signal and the amount of Cy5. This would indicate whether Perkin Elmer 1420 Victor2™ and Cy5-NHS ester could be used to trace beads transferred by Vacuum Prep Tool.

3.1 Experimental

Different amounts of Cy5-NHS esters were dissolved in 50 µL 10 mM HEPES adjusted to pH 7.65±0.05 with HCl and HAc (referred to below as HEPES buffer) and analyzed using the Perkin Elmer 1420 Victor2™ instrument (for further description of the material, see section 11).

3.2 Results

The results from the fluorescence measurement of Cy5-NHS ester are shown in Fig. 4-5. Each point in the graphs is a mean value of two samples prepared at the same concentration. A signal from the background of 700 was subtracted from each value. A linear function could be fitted to the points in the range 0.05 pmol to 0.2 pmol (Fig. 4). The function intersects the fluorescence axis in 82.5. The R^2 of the linear function was 0.997, compared to a R^2 of 1.0 for a second order polynomial function. In the range 0.5 pmol to 2 pmol (Fig. 5) the intersection value was -2563. The R^2 of the fitted linear function was 0.998, compared to an R^2 of 1.0 for a second order polynomial function.

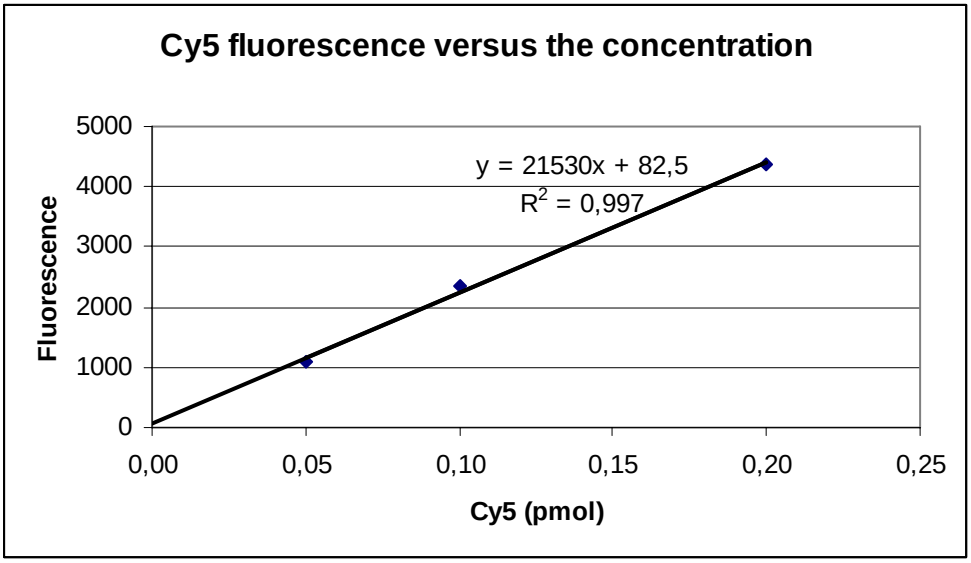

Fig. 4: Fluorescence as a function of amount of Cy5-NHS ester. Values are in the range 0.05 pmol to 0.2 pmol. Two samples of each concentration were prepared. The values were corrected by subtracting the background signal of 700.

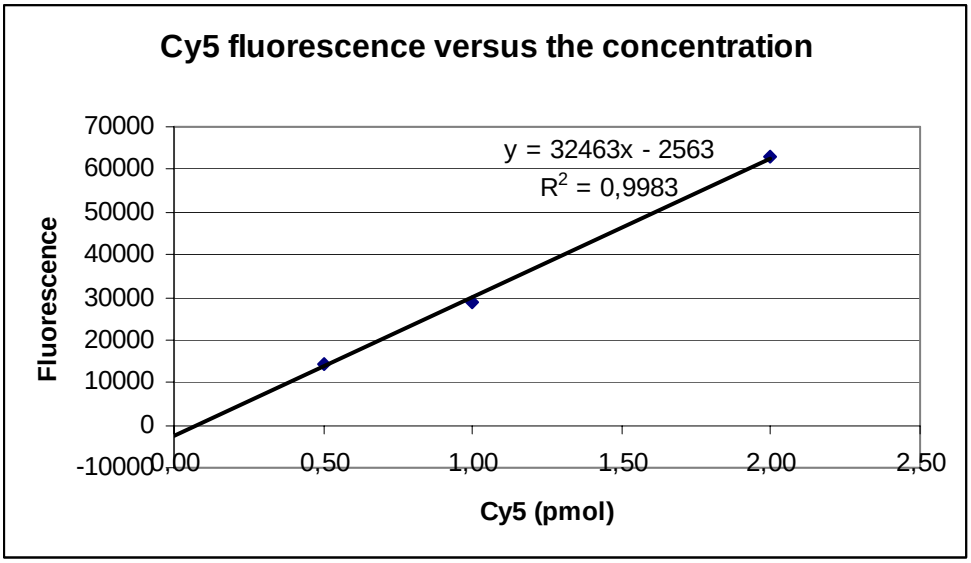

Fig. 5: Fluorescence as a function of amount of Cy5-NHS ester. Values are in the range 0.5 pmol to 2 pmol. Two samples of each concentration were prepared. The values were corrected by subtracting the background signal of 700.

3.3 Discussion

There was a linear correlation between amount of Cy5 and fluorescence signal in the range 0.05 pmol to 0.2 pmol of Cy5-NHS ester. The regression line intersected the fluorescence axis in a point closer to the origin, in comparison with the line fitted to the values in the interval 0.5 pmol to 2.5 pmol. If it is assumed that the signal versus the Cy5-NHS ester concentration is linear, the line should preferably intersect the fluorescence axis at the origin. Due to the intersection value, the range between 0.05 pmol and 0.2 pmol was preferable to the range 0.5 and 2.5 pmol. The highest fluorescence signal in the range 0.05 pmol to 0.2 pmol was about 5000 units and therefore measurements in further experiments should be lower than 5000 units. The linear function in the range 0.05 pmol to 0.2 pmol of Cy5-NHS ester, had an R^2 value of 0.997; if instead a polynomial function of second order was fitted to the points in the graph, the R^2 value was 1.The accuracy was higher with the polynomial function, but every $R^2 \geq 0.99$ was accepted as being sufficiently accurate. The linear regression line is easier to handle and was therefore chosen in the present project.

4. CY5 BSA BOUND TO 19 µm BEADS

The purpose of binding Cy5-stained BSA (Bovine Serum Albumine) to beads was to find out if 19 µm beads could be captured and released using Vacuum Prep Tool in a quantitative manner. Before the investigation, it was necessary to ensure that there was no unbound material in the suspension which could affect the signal. A Nanosep® centrifugal device 0.45 µm was used to rinse away the unbound material from the beads. Everything which passed through the filter was discarded and the material which did not pass through was retained.

4.1 Experimental

4.1.1 Pluronic adsorption to polystyrene beads

100 µL 1% (w/v) Pluronic F108-PDS in MilliQ water was mixed with 25 µL 19 µm beads and left to attach to the beads by hydrophobic interactions overnight (for further description of the material, see section 11).. The unbound F108-PDS was rinsed away with MilliQ water by centrifugation using an Eppendorf centrifuge 5415C; this device was used for all centrifugation steps in the present project and with the same rotation speed of 14000 rpm. After each centrifugation the liquid was discarded and the pellet was resuspended in MilliQ water; the centrifugation was performed three times, 8 minutes for each time.

4.1.2 Binding of Cy5 to BSA

BSA 1 mL (2.0 mg/mL) was dissolved in 10 mM HEPES buffer. A volume of 75 µL 30 mM SPDP in 99.7% EtOH (the same concentration of SPDP was used in all steps) was added to the BSA solution and then left to bind to an amine group on the BSA. After 10 minutes, the unbound SPDP was removed by gel filtration using a NAP-10 column and 10 mM HEPES buffer. After removal of unbound SPDP, the solution was mixed with Cy5-NHS ester for 30 minutes, allowing the Cy5-NHS ester dye to bind to an amine group on the BSA. The excess Cy5-NHS ester dye was removed from the BSA by gel filtration using a NAP-10 column. In order to form a thiol group on the SPDP molecule, the BSA solution was treated with 100 µL of DTT (100 mM in MilliQ water) for 20 minutes. The DTT was then rinsed away by gel filtration using a NAP-5 column followed by a NAP-10 column.

4.1.3 Binding of BSA to 19 µm beads

The thiol group on the BSA was now able to bind to the thiol on the PDS-modified Pluronic F108 on the larger beads. The BSA and the beads were allowed to react for 1h. Afterwards the excess of BSA was rinsed away with 10 mM HEPES buffer by means of centrifugation (after each centrifugation the liquid was discarded and the pellet was resuspended in 10 mM HEPES buffer; the centrifugation was performed three times, 8 for minutes each time).

4.1.4 Transferring beads with Vacuum Prep Tool

To ensure that no free BSA was left in the suspension the large beads covered with BSA were filtered with 10 mM HEPES buffer by using a Nanosep® centrifugal device 0.45 µm. After removal of the unbound BSA the suspension was added to wells of a PCR plate (the amount

of beads in each well corresponded to a suspension of 25500 19 µm beads). The beads were captured on the filters by a Vacuum Prep Tool and released into Microtiter wells by turning the vacuum off and shaking the tool. As a control if the beads were released from the filter, the tool was shaken two more times into two other Microtiter wells. The remaining beads in the PCR wells, not captured the first time by the Vacuum Prep Tool, were also transferred to the Microtiter plate with a pipette. The samples were then analyzed with the Perkin Elmer 1420 Victor2^{TM} instrument.

4.2 Results

A suspension containing 25500 19 µm beads was taken for each sample. A background of 640 was subtracted. All samples were measured in a Microtiter plate where each well in the plate contained 100 µL liquid. The bars shown in Fig.6 are the mean values of two samples filtered using a Nanosep® centrifugal device 0.45 µm. The "1st", "2nd" and "3rd" filtrate consisted of unbound material which had passed through the filter. "1st", "2nd" and "3rd" filtrate indicated how many washing steps which were needed. The outcome, the purified material, the "Filtrand", gave a signal of 1297 which could be compared with the signal of 11761 before the filtration. The bar of "The sum" is the summation of the "filtrand" and the"1st", "2nd" and "3rd" filtrate.

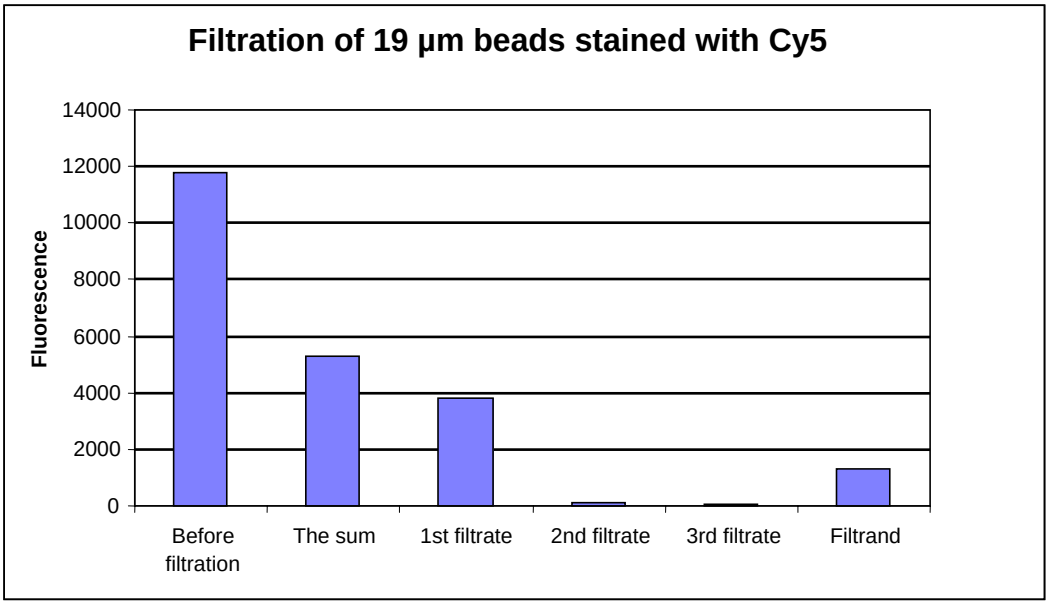

Fig. 6: The bar chart shows the signal from Cy5-NHS ester stained beads, filtered by Nanosep® centrifugal device 0.45 µm. The amount of beads in the filtered material corresponded to a suspension of 25500 19 µm beads.

The purified sample the "filtrand" was then investigated with the Vacuum Prep Tool. After removal of the sample from the well by the Vacuum Prep Tool, any remaining material in the well was suspended in 10 mM HEPES buffer and measured using the Perkin Elmer 1420 Victor2^{TM}. The signal corresponded to a loss of 9.5%. Each bar in Fig. 7 is the mean value of three samples treated with the Vacuum Prep Tool. "1st release" is the sample captured by using the Vacuum Prep Tool and then releasing into a Microtiter well. "Left in PCR tube" is the sample left in the PCR tube not captured by the Vacuum Prep Tool. The "2nd release" is the sample released by shaking the Vacuum Prep Tool a second time. The second release is

performed using the same filter directly after the "1st release". "The sum" is the sum of all the bars shown in Fig. 7 except the "filtrand" bar.

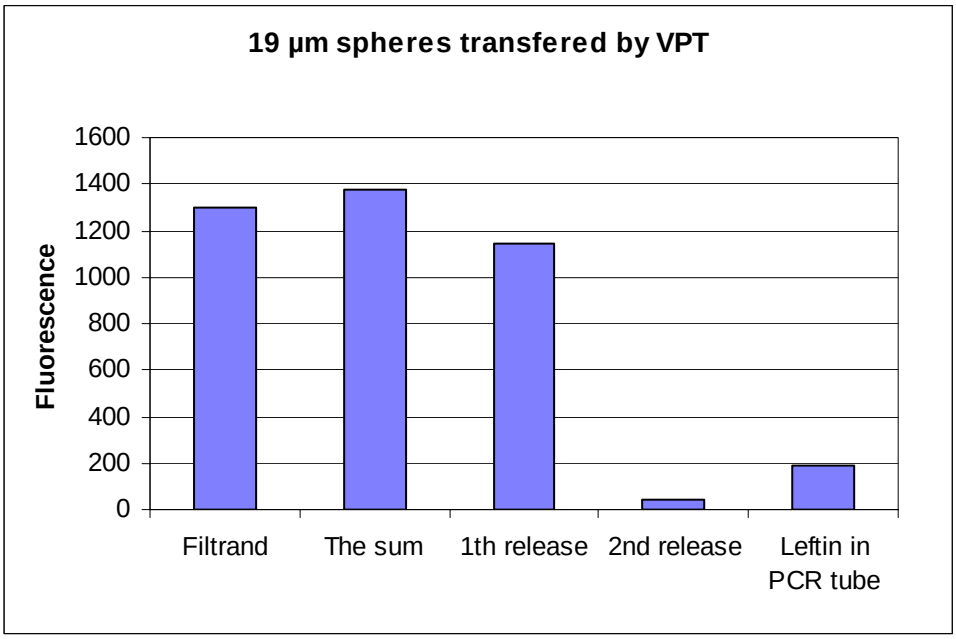

Fig. 7: The bar chart shows the signal from Cy5-NHS ester stained beads released by Vacuum Prep Tool. The material contained 25500 19 µm beads.

4.3 Discussion

The purpose of this investigation was to find out if the greater part of the beads were able to be captured and released by the Vacuum Prep Tool. The results show that almost 89% of the beads were successfully transferred by the Vacuum Prep Tool. If the signals from the samples moved and then released by the first, second and third shaking were added to the signal from the loss, it corresponded to 1379. This could be compared with the signal from the start material of 1297. This implied that no sample was attached to the filter of the Vacuum Prep Tool. The loss of 9.5% of the material when using the Vacuum Prep Tool might be addressed to at least three sources. One possibility was that the filter of the probe was overloaded. Another explanation could be that the beads were too heavy and that the probe was not able to reach the bottom of the tube. The final reason could be an effect of the probe touching the brim of the PCR tube. About 0.1 µL densely packed beads were loaded. This volume was not an excess of capacity in comparison to the volume of beads loaded during Pyrosequencing® and therefore the hypothesis of an overloaded filter could be excluded. The possibility that the filter did not reach the bottom of the tube can be investigated further by increasing the vacuum.

5. 0.24 µM BEADS LABELED WITH CY5 AND LINKED TO 19 µM BEADS

The purpose of connecting 0.24 beads to 19 µm beads was to find out if the oligonucleotide linkage between the small and large beads was sufficiently strong to permit processing of the complex by the Vacuum Prep Tool. Due to the need of traceability the Vacuum Prep Tool was replaced by a filter probe from the Vacuum Prep Tool and a syringe. Everything which passed the filter was collected by the syringe and could therefore be measured using the Perkin Elmer 1420 Victor2 ™ instrument.

5.1 Experimental

5.1.1 Pluronic adsorption to Polystyrene beads

100 µL 1% (w/v) Pluronic F108-PDS in MilliQ water was mixed with 25 µL of 10% (w/v) 19 µm beads in MilliQ water and left to bind by hydrophobic interactions overnight (for further description of the material, see section 11). The same procedure was performed with the 0.24 µm beads, by mixing 6.5 µL of 10% (w/v) 0.24 µm beads in MilliQ water and 25 µL of 1% (w/v) F108-PDS in MilliQ water. The unbound F108-PDS was washed away with MilliQ water by centrifuging the mixture three times for 20 minutes for the 0.24 µm beads and 8 minutes for the 19 µm beads (after each centrifugation the liquid was discarded and the pellet was resuspended in MilliQ water).

5.1.2 Attachment of oligonucleotides to the Pluronic F108-linker

A thiolated oligomer with either dG or dC (90 pmol/µL in MilliQ water) was added to the beads prepared in 5.1.1 and allowed to bind to the thiol in Pluronic F108-PDS. The volume of dC added to the 0.24 µm beads was 12.5 µL and the volume of dG added to the 19 µm beads was 100 µL. The reaction was allowed to proceed at room temperature. For 20 minutes between the nucleotides and small beads and for 1.5 hours between the large beads and nucleotides. The excess of unbound oligomers was washed away with MilliQ water by centrifugation three times for 20 minutes for the 0.24 µm beads and 8 minutes for the 19 µm beads (after each centrifugation the liquid was discarded and the pellet was resuspended in MilliQ water).

5.1.3 Binding of BSA to 0.24 µm polystyrene beads

The BSA was bound to the Cy5-NHS ester and the SPDP using the same procedure and amounts as before (see section 4.1.2 Binding of Cy5 to BSA) and then mixed with the 0.24 µm beads for one hour. The BSA which was not bound to the beads was washed away with 10 mM HEPES buffer by centrifugation of the suspension three times (after each centrifugation the liquid was discarded and the pellet was resuspended in 10 mM HEPES buffer; the centrifugation was performed for 20 minutes each time).

5.1.4 Attachment of 0.24 µm beads to 19 µm beads

After centrifugation the 0.24 µm beads were allowed to bind to 19 µm beads via oligonucleotide hybridization by mixing the beads for about two hours in 10 mM HEPES buffer. To investigate how to wash away the unbound 0.24 µm beads without losing the 19 µm beads, naked 19 µm beads were shaken in a 1.5 mL microcentrifuge tube at 350 cycles per minute. After one hour, a pellet had formed at the bottom of the tube. The supernatant was removed from the pellet and centrifuged for 3x 8 minutes. The supernatant contained almost no beads. Therefore, the suspensions of 19 µm and 0.24 µm beads were treated in the same way. The mixing kept the free 0.24 µm beads in suspension but the 0.24 µm beads bound to the 19 µm beads formed a pellet at the bottom of the tube. The suspension was removed and the pellet was stored in a microcentrifuge tube.

5.1.5 Treatment of filter probes

The Vacuum Prep Tool filter probes were removed from the tool and flushed with unmodified Pluronic (10 mg/mL in MilliQ water) and then left overnight in the Pluronic solution. The excess of Pluronic was then washed away from the filter by flushing 10 mM HEPES buffer through the probe. The filter probe was then connected to a 2 mL syringe.

5.1.6 Moving of beads by using Vacuum Prep Tool filter probes

The suspension of the 0.24 µm beads bound to the 19 µm was purified by using the filter probe and a syringe. A dilution of the original suspension in 10 mM HEPES buffer was poured into three wells in a PCR plate (approximately 20400 19 µm beads in each well). The beads were moved to a Microtitre plate by applying vacuum over the filter using a syringe. The beads were released into the wells by releasing the vacuum and shaking the probe. Each well in the Microtiter plate contained 100 µL 10 mM HEPES buffer. As a control, the filter was shaken in two more wells. Samples, which passed the filter, were collected in the syringe and poured into the Microtiter wells. All the samples were then analyzed by the Perkin Elmer 1420 Victor2^{TM} instrument.

5.2 Results

Three samples were prepared, the amount of beads in each sample corresponded to a suspension of 20400 19 µm beads. The mean value of these samples is shown in Fig. 8. Every bar in the figure is in percent of the start material, the bar “before VPT”. The amounts of sample transferable by the filter probes were, 90.5% shown in Fig. 8 as “1st release”. Remains in the PCR tube after capture and release by the filter probes corresponded to 7%: see the bar “remaining in tube”. The mean value of the amount of fluorescence in the liquid which passed through the filter probes, the bar “Filtrate”, corresponded to about 1%. The “2nd release” was the value obtained after the probe was shaken a second time, this value corresponds to 4% “The sum” is the summation of “1st release”, “2nd release”, “Remaining in tube” and “Filtrate”. The summation corresponded to 102%.

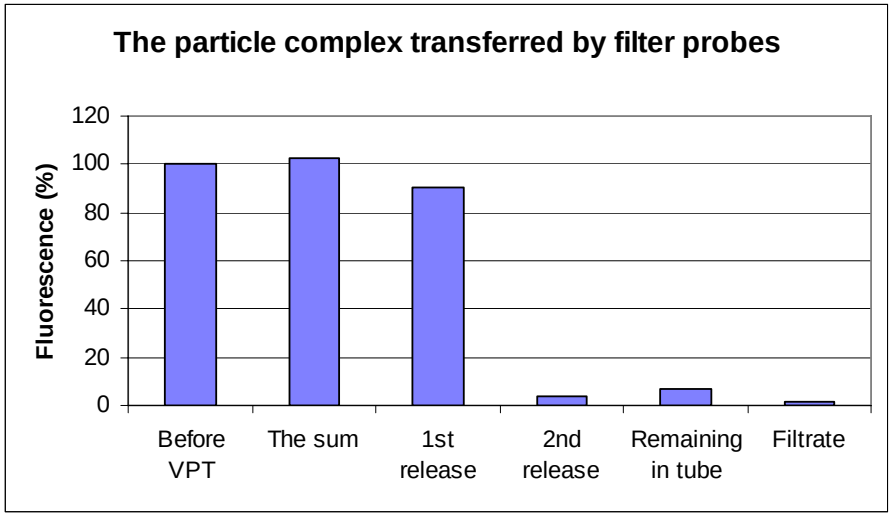

Fig. 8: The bar chart shows the signal from Cy5-NHS ester stained beads released by the filter probes. The material contained a suspension of 20400 19 µm beads.

5.3 Discussion

The purpose of this study was to investigate if the linkage between the small and large beads was sufficiently strong to permit treatment with the filter probes. In this study the amount of 90.5% transferred beads, was in the same range compared to the value of 89% transferred beads in section 4. The comparison of the results from section 4 and these results proof that the linkage between the beads is sufficiently strong. This is due to the observation that it was no more loss of beads in this investigation compared to the investigation in section 4.

Was the amount of transferred beads sufficient in the investigation, for use of the particle complex in the protein capture/signal complex, as described below in section 7? If 7 µL of the 19 µm beads could be captured by the filter probe and 50% of the surface of the large beads was covered width small beads, this would correspond to 0.5 µg pyruvate kinase. Furhermore if 90.5% of this was then transferred, this would correspond to 0.45 µg pyruvate kinase. If the surface instead was covered to 30% and 41% was transferred this would correspond to 0.14 µg pyruvate kinase. Activities of as little as 0.01 µg free pyruvate kinase could be detected, as shown in section 6. Consequently, in this project the mean value of 90.5% transferred beads was sufficient for use of the particle complex in the protein capture/signal complex.

6. PYRUVATE KINASE DETECTION BY LUCIFERASE AND PSQ 96MA

The intention of this study was to find detection limits of pyruvate kinase and to determine if there was a simple correlation between concentration and signal. This information would be of interest in the further investigation of protein detection by the protein capture signal complex.

6.1 Experimental

In order to detect pyruvate kinase activity Annealing buffer and potassium acetate were added directly to a PSQ plate according to Table 1 (for further description of the material, see section 11). Different volumes of 0.5 mg/mL pyruvate kinase in one part E-buffer (double concentration) and one part glycerol (87%) were also included. The other reagents were dispensed in the order: luciferin, luciferase, ADP, PEP and ATP by the instrument PSQ 96MA (for concentration and volumes see Table 1).

30 µl	Annealing buffer
5 µl	KAc 500 mM in annealing buffer
5 µl	Luciferin 1.5 mg/mL in Ebuffer
5 µl	Luciferase 0.1 mg/mL in Ebuffer
200 nl	ADP 7.5 mM in annealing buffer
200 nl	PEP 25 mM in annealing buffer
200 nl	ATP various concentrations

Table 1. Reagents added when measuring the pyruvate kinase activity.

6.2 Results

The amount of pyruvate kinase was in the range 0.01 µg to 2.5 µg. Two methods have been used to calculate the amount of pyruvate kinase, as shown in the figures. In the first case, the rate of change of signal was calculated by subtracting the signal at a certain time from the signal measured the next time. The maximum value from each concentration (denoted as maximum gradient) was then plotted against the concentration. In the second case the background from ADP was subtracted from the peak height (or the highest value) and the result was plotted against the concentration. Each point in the Figures 9 and 10 is the mean value of three samples. In the case where the weakest signal for a specific concentration has been lower than the median signal for some of the lower concentration, the weak signal has been erased. Figure 9 shows the maximum gradient as a function of the concentration and Figure 10 shows the peak height as a function of the concentration.

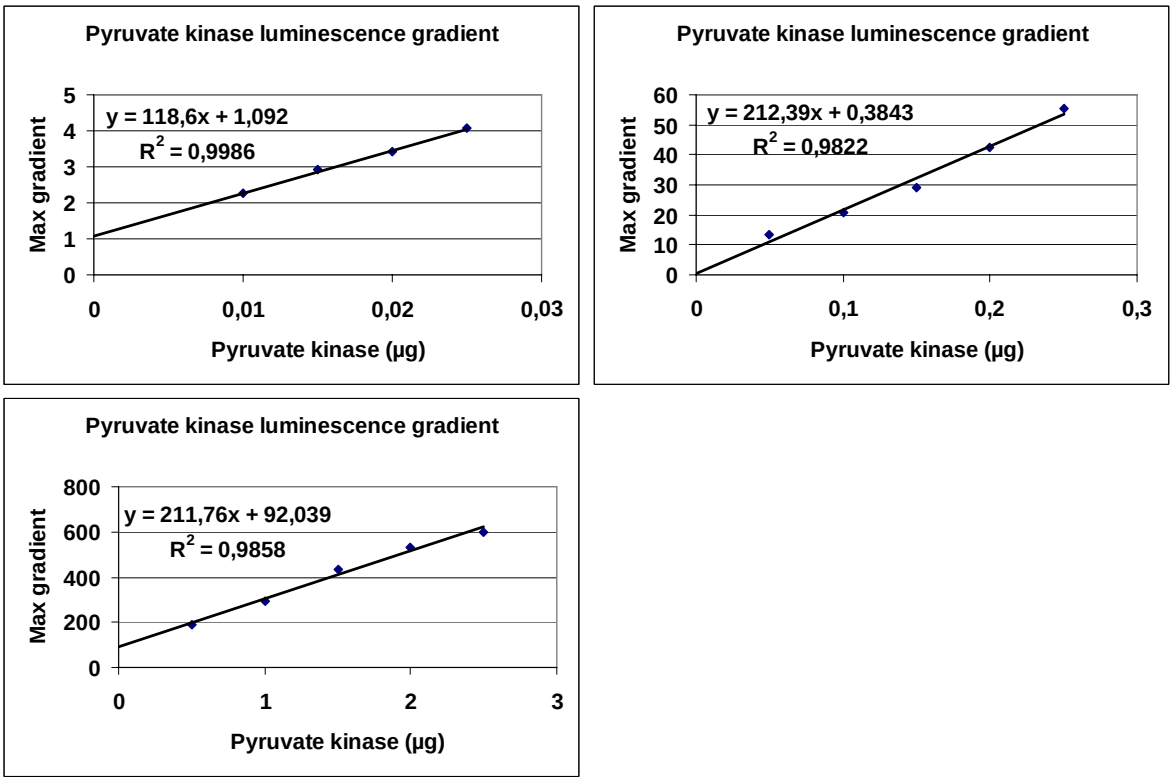

Fig. 9: The graphs show the maximum gradients of the signals as a function of the amount of pyruvate kinase.

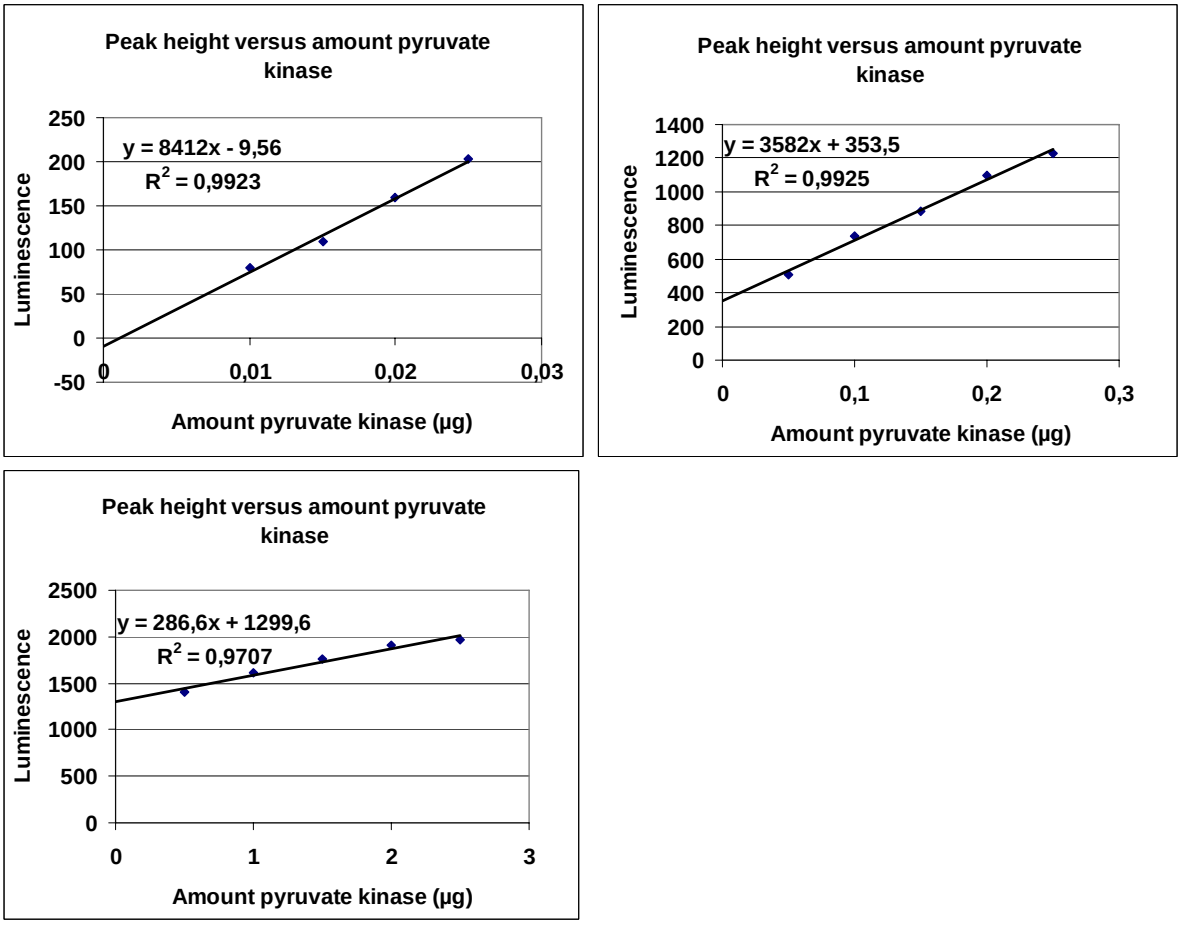

Fig. 10: The graphs show the peak height as a function of the amount of pyruvate kinase.

6.3 Discussion

The aim of this investigation was to find a method to predict the relationship between amount of pyruvate kinase and signal. Preferably, a linear correlation between the amount of pyruvate kinase and signal was wanted. The observed results showed a linear correlation. In limited intervals this observation was true both for the method with “peak height” and for the method with “gradient”. However within wide intervals the linearity was dependent on the method. For the “peak height” the slope was decreasing and thus it was no linearity for this method in a wide range of pyruvate kinase. For the “gradient” the slope had the same value, both for low and high amounts of pyruvate kinase (not for the lowest interval of pyruvate kinase) and thus this method was linear in a wide interval. An explanation to the difference between these methods was observed when the signal was plotted against the time. When the concentration of pyruvate kinase was increased the signal reached a maximum and then decreased. This was not observed for lower concentrations of the enzyme. Consequently the activity was decreased after a certain time. The time until the decrease in signal was dependent on the amount of pyruvate kinase. Thus one solution is to note the highest signal at an earlier time. For the “gradient” in contrast to the “peak height” the highest value was achieved in the beginning and therefore the decrease in the activity did not affect these values. Since it is not obvious at which time the highest value should be noted to improve the linearity of “peak height” the gradient method is preferable. For the method with gradient the dynamic range of pyruvate kinase detection were 0.01 µg to at least 2.5 µg. The value of 0.01 µg corresponds to a concentration of CRP as low as 0.01 mg/L in blood (if 50 µl blood is taken). In comparison to other companies on the market, this value is in the same range as the high sensitive CRP tests.

7. CRP DETECTION BY USING THE PROTEIN CAPTURE/SIGNAL COMPLEX

The purpose of the project was to detect CRP by using the protein capture signal/complex. The 0.24 µm beads were used to bind CRP via antibodies. Other antibodies conjugated with pyruvate kinase were then bound to the CRP. Detection of the pyruvate kinase thus indicated the presence of CRP. The smaller beads were bound to larger beads that in turn facilitate handling the beads using the Vacuum Prep Tool.

7.1 Experimental

7.1.1 Modifying of antibodies

1 mL of IgY antibodies (0.44 mg/mL) in PBS were mixed with 16 µL SPDP. After one hour the excess of unbound SPDP was removed by washing the antibodies with PBS in a NAP-10 column (for further description of the material, see section 11). Pyruvate kinase 0.5 mL (1.8 mg/mL) in PBS was mixed with SPDP. After 20 minutes, the unbound SPDP was rinsed by using a NAP-5 column and A buffer. The antibodies were split in two equal volumes and each volume was then mixed with 100 µL DTT (100 mM in MilliQ water) for 20 minutes. The DTT was then rinsed by using a NAP-10 column and A buffer for half of the antibodies and PBS buffer for the other half.

7.1.2 Binding of antibodies to enzymes and polystyrene beads

Pyruvate kinase (0.5 mL 1.8 mg/mL) in PBS was mixed with SPDP. After 20 minutes, the unbound SPDP was rinsed by using a NAP-5 column and A buffer. The SPDP modified antibodies (those stored in A buffer) were then bound to the pyruvate kinase by disulphide bridge formation. The other half of the SPDP modified antibodies were bound by disulphide bridges to the 0.24 µm beads (prepared with F108-PDS and dC as before). After one hour, the unbound antibodies were rinsed away from the beads by centrifugation with 10 mM HEPES buffer (after each centrifugation the liquid was discarded and the pellet was resuspended in 10 mM HEPES buffer; the centrifugation was performed three times, 20 minutes for each time).

7.1.3 Attachment of 0.24 µm beads to 19 µm beads

The 19 µm beads were prepared as before with F108-PDS and dG and then mixed with the 0.24 µm beads with bound antibodies and dC. Excess of unbound 0.24 µm beads were removed using the same method as before (described in section 5.1.4). The suspensions of beads and antibodies were then purified by the filter probe.

7.1.4 Transferring beads by using filter probes

After purification, the suspension (about 102000 19 µm beads covered with small beads and IgY and stored in 32.5 µL A buffer) was mixed with CRP (9 µL CRP 2.3 mg/mL in PBS dissolved in 22.75 µL A buffer). The reaction between CRP and antibodies took place in A buffer. After 15 minutes free CRP was removed from the mixture using the syringe and the filter. In order to attach the pyruvate kinase antibody conjugate to CRP, 310 µL of 0.25

mg/mL antibody-pyruvate kinase conjugate in A buffer was mixed with the beads to which the anti-CRP antibodies plus CRP were attached. A control was also prepared with the same amount of beads with antibodies and conjugate, but without CRP. After about 20 minutes, the beads were captured with the filter probe, by applying vacuum over the probe with a syringe, and moved to PSQ wells. As a control, 5 µL of the conjugate solution was added to other wells (about 0.8 µg pyruvate kinase in E-buffer) and 5 µL free pyruvate kinase (0.8 µg in E-buffer) were added to other wells. The reagents listed in Table 1 were included in all wells and also 5 µL E-buffer to the wells containing the beads.

7.1.5 Analyzing the bead protein complex on the PSQ 96MA instrument

The samples were analyzed using a PSQ 96MA instrument. Pyruvate kinase and the reagents potassium acetate and Annealing Buffer (see Table 1) were added directly into the wells and the other reagents were dispensed by the instrument in the following order luciferin, luciferase, ADP, PEP, ATP (see Table 1).

7.2 Results

Two samples of the protein capture/signal complex containing CRP and two of the one without CRP were prepared. Before the background had been subtracted, the signal from the CRP sample was 119 and for the sample without CRP, the signal was 114.5 (Fig. 11-12). After the background had been subtracted, the signal from the CRP sample was 26% in comparison with its ATP peak and in the sample without CRP 11% in comparison with its ATP peak.

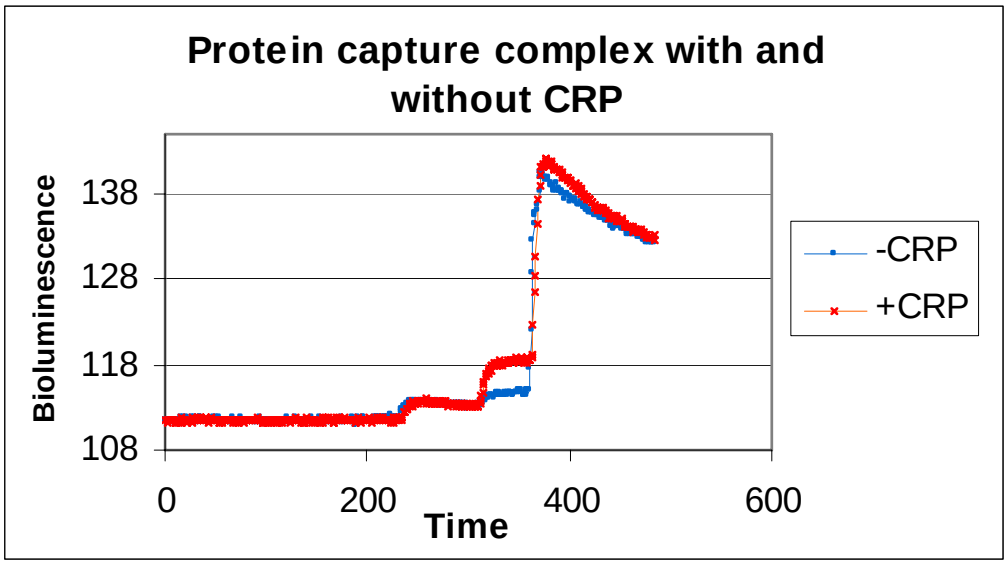

Fig. 11: The graph shows pyruvate kinase activity. The upper red line is bead complex bound to conjugate by CRP. The lower blue line is bead complex and conjugate without CRP. The first elevation is due to ADP addition, the second is due to PEP addition and the last is due to ATP addition.

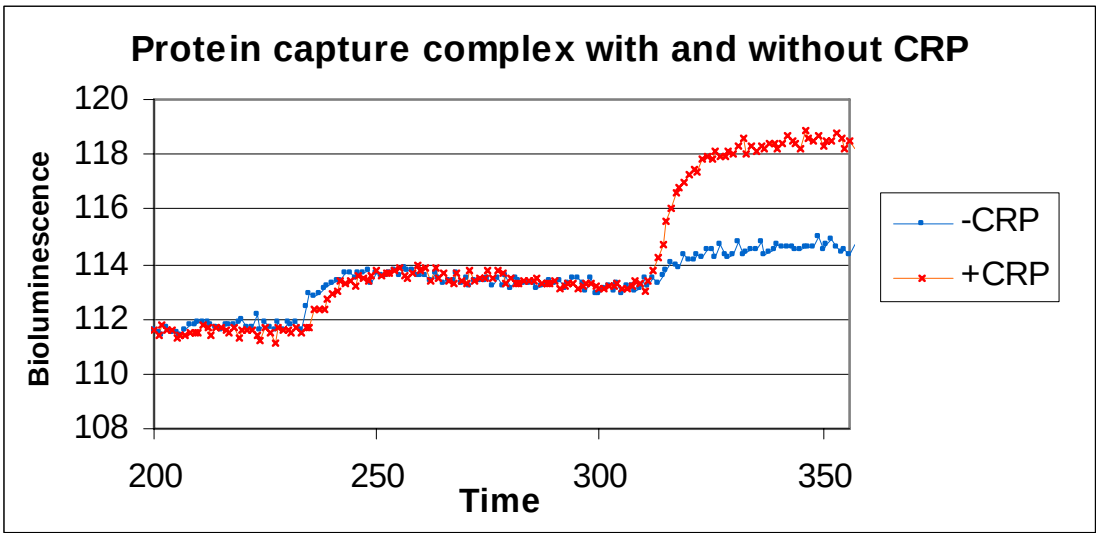

Fig. 12: The graph shows pyruvate kinase activity. The red upper line is bead complex bound to conjugate by CRP. The lower blue line is bead complex and conjugate without CRP. The first elevation is due to ADP addition and the second is due to PEP addition.

In the wells with free pyruvate kinase, the ATP peak was 2844 (the background subtracted) (Fig. 13). The corresponding ATP value for the samples with and without the CRP was 29.

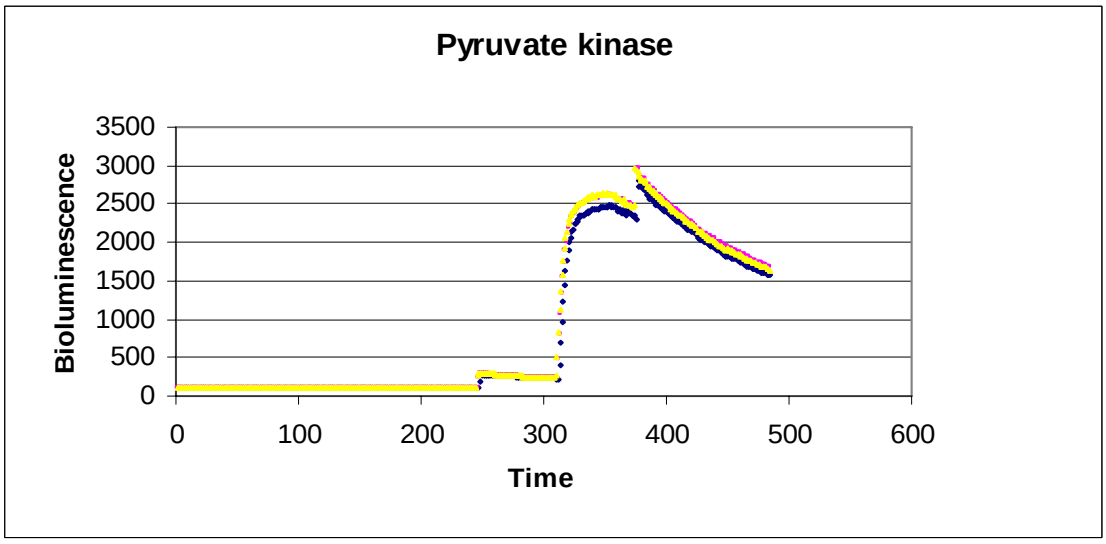

Fig. 13: The graph shows pyruvate kinase luminescense. The first elevation is due to ADP addition the second is due to PEP addition and the last is due to ATP addition.

The signal from the sample with the mixture of free pyruvate kinase and pyruvate kinase bound to IgY was 262 over the background (Fig. 14) that is 12% compared to the ATP peak with the signal of 2100. The total amount of pyruvate kinase in this sample was 0.8 µg.

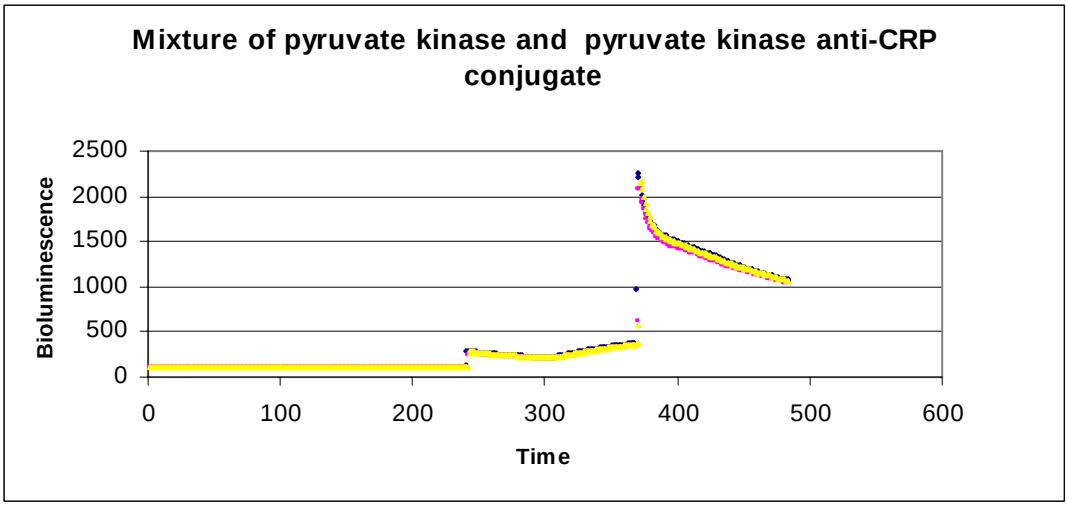

Fig. 14: The first elevation is due to ADP addition the second is due to PEP addition and the last is due to ATP addition. The amount of kinase in the mixture of conjugate and free pyruvate kinase should correspond to 0.8 µg.

7.3 Discussion

The signals were not as high as expected in either the capture/signal complex with CRP or without CRP. The signal was low when PEP was added as well as when ATP was added. If the ATP signal was corrected to the expected level of about 2840, the value of the signal after PEP was added would be 739 for the CRP sample. The signal from the sample without CRP would then correspond to 312. The same relation was also achieved if the peak when ADP was added was used as the standard level. A number of 102000 19 µm beads with attached 0.24 µm beads and antibodies were loaded in every well. If 50% of the surface of the large beads were covered with small beads and if every small bead bound 500 molecules of pyruvate kinase, this would correspond to 0.25 µg pyruvate kinase. The signal from the conjugate was 262 after subtraction of background. The assumed amount of kinase in the conjugate well was about 0.8 µg. The signal from the 0.8 µg free pyruvate kinase was 2411. The weak signal in the conjugate mixture could be a result of either low enzymatic activity or loss of enzyme due to the chromatography used (NAP-5 and NAP-10 columns). The corrected value of 739 was high when considering the low activity in the conjugate well.

8. CONCLUSIONS

It is possible to measure pyruvate kinase in a Pyrosequencing® system. The enzyme kept a high activity and even at low amount of pyruvate kinase, the kinase could provide luciferase with enough ATP to generate a detectable signal. Further, it was possible to draw a conclusion regarding the amount of pyruvate kinase with the PSQ 96MA instrument, the amount versus the signal was linear both with respect to the peak height and with respect to the maximum gradient. This meant that pyruvate kinase could be used to mark and detect an analyte with a PSQ 96MA instrument.

It was possible to transfer 19 µm beads with proteins attached on their surfaces with the Vacuum Prep Tool. Furthermore 0.24 µm beads could be linked to the 19 µm beads through oligonucleotides. This linkage was strong enough for keeping the beads attached to each other even during rough treatment. The Vacuum Prep Tool could be used to transfer the bead complex with proteins attached to the small beads. Consequently the complex could be transferred with Vacuum Prep Tool and used for detection.

Separating unbound 0.24 µm beads from those adhered to 19 µm beads was best performed through sedimentation by simple gravitation (1g). The small beads could be kept in suspension through continuous shaking while the large beads formed a pellet in the bottom.

It was possible to measure amounts of free pyruvate kinase as low as 0.01 µg. This corresponded to 5 ng CRP. If 50 µL blood [18] is taken from a patient this would correspond to 0.01 mg/L of CRP.

Finally, although the present study was of preliminary nature, we are encouraged to note that the protein capture/signal complex appears to work.

9. FURTHER INVESTIGATIONS

First of all the goal will be to determine how to increase the signal from the protein capture/signal complex. In these studies two types of investigations may be necessary, first a study of the effects on the protein capture/signal complex by the use of different equipments at Biotage and at the Center for Surface Biotechnology, second the effect each part in the protein capture/signal complex has on the signal. The difference with respect to the equipment could include studies of the plastic material in the PSQ plate and the agitation in the PSQ 96MA machine and its influences at the enzymatic activity. In the study of the parts in the protein capture/signal complex, the activity of the enzyme in the conjugate shall be studied and then an investigation if the conjugate attaches to its antigen. Before an extensive investigation of the different parts in the protein capture signal complex a comparison with conjugate attached to CRP at small beads shall be done between the equipments at Biotage and at the Center for Surface Biotechnology. If the signal became high at the Centre for Surface Biotechnology but low at Biotage, differences in equipment shall be further investigated, if the signal is low at the Centre for Surface Biotechnology the different parts in the complex can be further investigated.

In order to confirm the investigation with the Cy5-NHS ester, further research could also include determination by optical microscopy of the amount of large beads transferred by the Vacuum Prep Tool. Additionally the amount of small beads attached to every large bead could be determined for example by analysis by a field-flow fraction (FFF) technique. The outcome of that investigation would enable the determination of the amount of CRP bound to the protein capture complex. Another important point is to determine if the large beads can reduce the signal. This investigation could be performed by comparing the signal from the enzymes attached to the small beads in the protein capture/signal complex with the signal from the same amount of small beads in a suspension. Further the environment for the enzymes could be optimised. This should take into consideration buffers, pH and reagents.

10. ACKNOWLEDGEMENTS

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11. MATERIAL

A buffer; (50 mM glycine, 5 mM Tris, 5 mM $MgSO_4$, 5 mM KCl) in MilliQ water pH 7.7

Adenosine 5-diphosphate sodium salt (ADP) from Sigma

Adenosine 5′-triphosphate (ATP) from Sigma

Annealing buffer (20 mM Tris hydroxymethylaminoethane, 2mM Magnesium acetatetetrahydrate) pH 7.6 from Biotage AB

Beads; polystyrene latex beads with an average diameter of 0.24 µm and 19 µm, both beads were purchased from Bangs Laboratories.

Bovine serum albumin (BSA) provided by Maria Lönnberg at Pharmacia

Columns; NAP-10 column and NAP-5 purchased from GE Healthcare

C-reactive protein (CRP) from human pleural fluid was purchased from Chemicon

Cy5 Mono-Reactive Dye Pack from GE Healthcare

Dithiothreitol (DTT) from Sigma

E-buffer from Biotage AB

HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) from Sigma

IgY provided by Anders Larsson, department of medical sciences, Uppsala University Hospital

KAc potassium acetate

Luciferase from Promega

Luciferin Beetle potassiumsalt from Promega

Nanosep® centrifugal device 0.45 µm from PALL Corporation

Oligonucleotides of guanine (dG) and cytosine (dC) thiolated at the 5-end delivered from Thermo BioSciences GmbH, Ulm, Germany.

Perkin Elmer1420 Victor2^{TM} from Perkin Elmer

Phosphate buffered saline (PBS)

Phospho(enol)pyruvic acid monosodium salt hydrate (PEP) from Sigma

Pluronic F108-PDS, modified with a thiol-specific pyridyl disulfide group from Allvivo Inc. Lake Forest, CA

Pyruvate kinase from rabbit muscle purchased from Sigma

PSQ 96MA instrument from Biotage AB

N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) from Sigma

PyroMark Vacuum Prep Tool (Vacuum Prep Tool) from Biotage AB

12. ABBREVIATIONS

ADP adenosine diphosphate

AMP adenosine monophosphate

ATP adenosine triphosphate

BSA Bovine Serum Albumine

CCD charge-coupled device

CRP C-reactive protein

dC deoxycytidine

dG deoxyguanosine

DTT dithiothreitol

EtOH ethanol

F108 a coblock polymer

IgY immunoglobuline yolk

KAc Potassium acetate

PBS phosphate buffered saline

PCR polymerase chain reaction

PDS pyridyl disulfide

PEP phosphoenolpyruvate

PPi pyrophosphate

PSQ pyrosequencing

rpm revolutions per minute

SPDP N-succinimidyl 3-(2-pyridyldithio)propionate

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