Team Results Document TU Eindhoven Sensing Team (T.E.S.T.)

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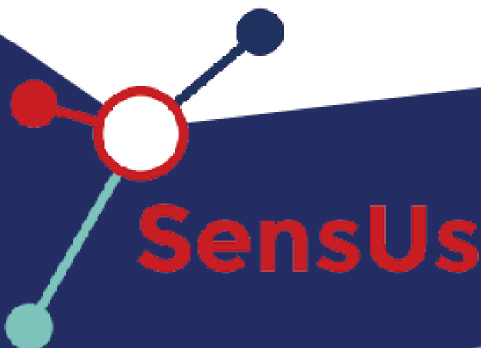
09-08-2024



SensUs 2024
Acute Kidney Injury

1. Abstract

The desired treatment for patients suffering from kidney failure is a kidney transplantation. However, the waiting list for kidney transplantation is increasing due to a shortage of viable donor kidneys. T.E.S.T. has designed an antibody-switch assay as a monitoring technique to assess the quality of ex-vivo kidneys during preservation before transplantation. As a result, the occurrence of delayed graft function and the rejection of kidneys post-transplantation can be lowered, since the quality of kidneys can be improved. By doing so, higher amounts of available kidneys for transplantation can be achieved. To achieve this, T.E.S.T has designed a completely new molecular construct from the ground up which is robust for the application in human serum. The antibody-switch complex is designed in a competition assay format. The molecular competitor is bound to an antibody in the absence of creatinine. In the presence of creatinine, the bait is being displaced by a target with higher affinity to the antibody. The physical transduction method relies on Förster Resonance Energy Transfer (FRET). The antibody is labeled with a donor fluorescent dye and the bait with an acceptor fluorescent dye.

2. Biosensor

2.1 Overview

T.E.S.T. has developed a biosensor to continuously measure creatinine for monitoring the functioning of a transplanted kidney. The technique used for this consists of an antibody-based molecular switch. The design is inspired by the publication of Thompson et al. (2023)[2] from Stanford University, however we have introduced multiple improvements and adjustments to fit our concept.

In the design, the switch opens when the antibody complex is in the presence of creatinine, therefore inducing a competitive assay, and closes in the absence of creatinine. The measurements are quantified via the Förster Resonance Energy Transfer (FRET) technique.

2.2 Molecular recognition

In order to functionalize the antibody to the substrate and to bind the antibody with creatinine acid, i.e. the analog of the creatinine molecule, a DNA scaffold is designed. This enables the opening and closing of the switch. The design of the DNA scaffold takes into account the balance between flexibility and stability, which was ensured by including both single stranded DNA (ssDNA) and double stranded DNA (dsDNA) (Appendix 1).
After addition of the creatinine sample, a competition assay is obtained as the analog is competing against the free creatinine present in the sample for the binding spot at the fragment antigen binding region (FAB fragment). The fluorophores are of significance in this context. The donor fluorophores are located on the antibody and the acceptor fluorophore is coupled to the DNA scaffold. To enable easy attachment of the DNA scaffold, protein M is used, a novel, recently discovered antibody-binding protein showing no dissociation despite non-covalent binding. This enables easy attachment to off-shelf antibodies without tedious chemical conjugation. To our knowledge, this is a first known attempt to attach DNA to antibodies via protein M [27].

As seen in Figure 1, protein M is attached to the FAB-fragment of the antibody and an acceptor fluorophore is part of the DNA scaffold. The assay complex is functionalized to the surface via a biotin molecule attached to the DNA scaffold.

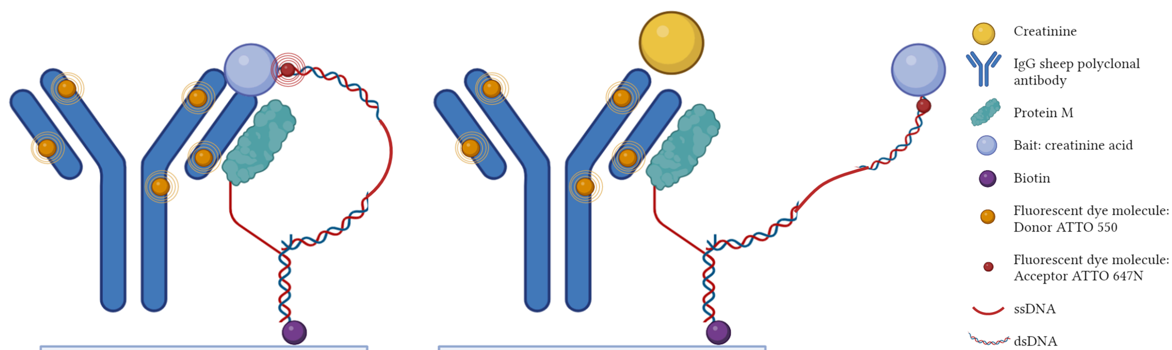


Figure 1: *Schematic drawing of the assay design including an antibody switch on a glass plate (the switch is closed on the left and open on the right).*

The switching events are quantified by using a FRET read-out. The antibody-switch described above has two different states, of which the underlying mechanisms are explained in Section 2.3. In the presence of creatinine, the switch will open, causing displacement of the analog. Figure 1 presents the principle of the biosensor and its two states.

2.3 Physical transduction

The underlying mechanism that is used for the physical transduction of the signal is FRET. This mechanism corresponds to a photophysical phenomenon where non-radiative transfer of energy between two fluorophores takes place, mainly achieved by dipole-dipole interactions [3]. The energy is transferred from a donor fluorophore, in an

excited state, to an acceptor fluorophore, resulting in the acceptor being excited. When the distance between fluorophores decreases, more energy transfer from donor to acceptor takes place.

The dyes that are chosen are ATTO-550 and ATTO-647N for the donor fluorophore and acceptor fluorophore, respectively. The amount of overlap between spectra determines the rate of (observable) energy transfer (see Figure 2). The molecular recognition works by means of a switch. The donor is located on the antibody, whereas the acceptor is located on the antibody. In the presence of the creatinine, the switch will open, leading to an increased distance between the two fluorophores. Due to this increased distance between DNA scaffold and protein M, the FRET ratio will decrease, as less energy transfer takes place. The other state, in the absence of creatinine, the bait is attached to the antibody, resulting in the close proximity of donor and acceptor, leading to a higher FRET ratio. Therefore, the FRET ratio between acceptor and donor is inversely proportional to the opening and closing of the antibody switch, which is determined by the concentration of creatinine.

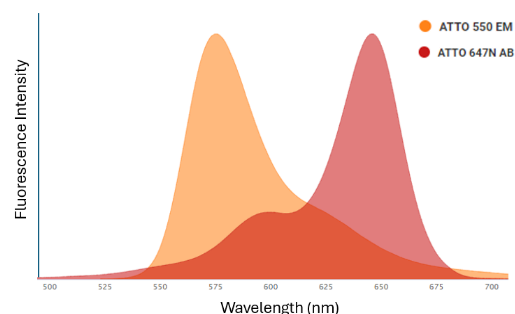


Figure 2: *Emission spectrum (EM) of ATTO 550 (orange) and the absorption spectrum (AB) of ATTO 647N (red); y-axis represents the FRET signal magnitude and the x-axis is the wavelength of light.*

2.4 Cartridge technology

The cartridge consists of a glass plate with a flow cell sticker attached to it. The molecular construct is inserted prior to testing by pipetting, and will remain in the flow cell during the tests. The cartridge can be re-used for multiple tests due to the reversibility of the molecular switch. For testing, 40µl of the creatinine sample is pipetted in the flow cell, after which creatinine can bind to the antibody construct and can be determined based on the FRET signal as described in the previous section. Upon addition of a new creatinine sample, fluid replacement in the flow cell takes place, allowing the new sample to be measured in the flow cell. No intermediate fluid additions are used.

2.5 Reader instrument

The excitation of the donor is achieved by using a laser with a center frequency of 520 nm (FL-525-1200), in combination with a collimator to collimate the laser light. The laser light moves horizontally, and then passes through a lens and an excitation filter (Chroma ET510/80m). The light is reflected by a dichroic mirror (Chroma AT565DC), which bends the light with a 90 degree angle upwards towards the objective, which focuses the light on the sample. The excitation of the donor leads to the excitation of the acceptor, ultimately leading to the emission of the acceptor. Both the donor and acceptor emission are measured separately, which are distinguished by different filters placed in a filter wheel (Chroma ET575/50m & Chroma ET665lp). These emissions are captured using a camera, which transmits the input to the connected laptop.

For the image processing, leading to the output concentration, a MATLAB code is written, using the software and drivers of the camera manufacturer FLIR. An average value of a field of view (FOV) of the FRET signal (i.e. ratio between donor and acceptor signal) is calculated. This value is compared with the calibration curve obtained from known creatinine concentration, giving the creatinine concentration of the measured sample.

2.6 User interaction

The dimensions of the biosensor are 25 cm in depth, 30 cm in width and 55 cm in height. The hardware of the biosensor itself is primarily made of aluminum, while the exterior case consists of MDF. During usage, our sample needs to be pipetted in the flow cell, after which the cartridge needs to be inserted manually via an opening on top of the biosensor, where it needs to be put on the sample stage. To stop the detection temporarily, the laser block needs to be moved manually via a small opening, located at the top of the biosensor, which is kept small to ensure the safety of bystanders. Automatization of the biosensor can be improved: an autonomously working laser block could be installed and a pump can be integrated to replace manual pipetting.

3. Technological feasibility

An important requirement for continuous sensing is that there is no carry-over effect between samples. Carry-over leads to a decrease in accuracy over time, and would require washing of the flow cell to restore this accuracy. To test this, 4 solutions of donor fluorophores were created: 2 low concentration and 2 high concentration. For each sample, 5 different FOVs were measured. Signals were measured from the average light intensity captured by the camera for one FOV for 2 minutes. Figure 3 shows the average light intensities for each measurement. There is no carry-over between samples, since the mean pixel intensity of samples are independent of preceding samples. An independent two-tailed t-test confirms that the second sample of high concentration (measurement 11 to 15) is not significantly different from the first (measurement 1 to 5) (p = .11). The second sample of low concentration (measurement 16 to 20) is significantly lower than the first (measurement 6 to 10) (p = .003), indicating that there is no carry-over. The area of the flow cell that was studied was not consistent between samples, which could have caused this inconsistency if dyes are spread non-homogeneously across the flow cell.

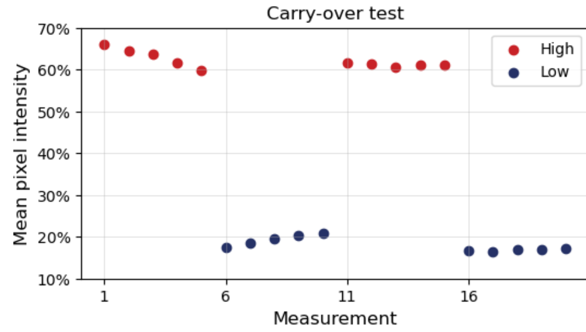


Figure 3: *Carry-over test results. Samples of high (red) and low (blue) concentrations of dyes were measured using the same flow cell. Signal is measured from mean pixel intensity, captured by the camera.*

Fluorescent dyes have a photobleaching property, which describes how their light emission intensity decreases as a result of excitation. It is important to map this property, since it determines how long continuous measurements can be done with the same dyes. Figure 4 shows the photobleaching process of our donor dyes, measured over 30 minutes with constant laser excitation of 60 mW . There is no laser excitation in the first seconds of the measurement, which provides a reference point for the amount of signals if the dyes are entirely bleached. Donor dyes are measured because they are excited by the laser directly, making them more prone to bleaching than the acceptor. What can be concluded from the graph is that the dyes are stable over a timeframe of at least 30 minutes. Furthermore, taking measurements does not require constant laser excitation, because the laser can be blocked in between measurements. The amount of photobleaching over time is therefore reduced even further during usage. If too much bleaching does occur, the FOV of can be changed by moving the sample stage, which brings unbleached dyes into view.

In order to design an optimally functioning assay, it is crucial to know in which concentration fluorescent dyes can be measured best. This was approached by measuring 4 different donor concentrations. For each concentration, the average pixel intensity was measured over 3 minutes and divided this by the average pixel intensity without laser excitation. The camera exposure time is increased with a 10-fold for each 10-fold dilution.

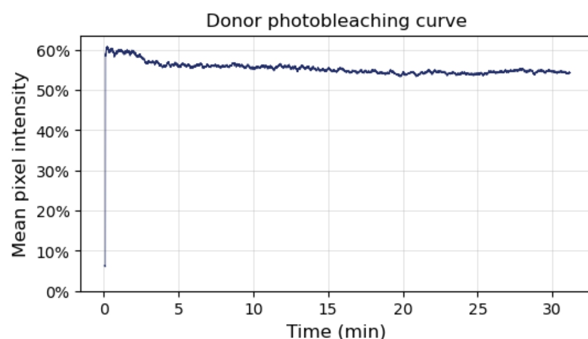


Figure 4: *Donor photobleach test results. Light intensity from donor dyes with constant laser excitation is measured over 30 minutes. The dye is stable over this time period.*

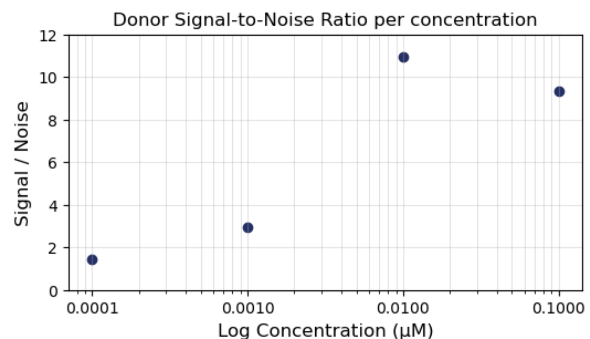


Figure 5: *Signal-to-Noise Ratios for 4 concentrations of donor dyes were measured. It shows that 0.1 μM has an optimal SNR.*

This is corrected for in the signal calculation. The results in Figure 5 show that a concentration of 0.01 μM has an optimal signal to noise ratio.

It is important during the development of the switch construct to confirm that every step of construction showed desirable results.

First, protein M was mutated to be able to attach the DNA scaffold at the right position. There is limited information regarding the protein because of its novelty, which made the process of mutating it more challenging. However, after numerous trials the mutations were eventually successful. These mutations were verified by GENEWIZ of Azenta Life Sciences. A more detailed explanation of the taken steps and the resulting sequence can be found in appendix 2.

This protein needed to be attached to an ssDNA piece. The conjugation was verified by an SDS page gel. The gel and analysis can be seen in appendix 3. When comparing the sample with just protein M and de sample of the product after the reactions a difference could be seen which confirmed the product had formed. The yield was not as desired and optimizations and retrials have not shown an improvement in yield, which means there is still protein M in the product that is not bound to ssDNA.

An analogue, creatinine acid, was synthesized to be able to create a competition assay (reaction mechanism in appendix 4). This synthesis was confirmed with GC-MS for the intermediate product, the creatinine ester, and LC-MS to confirm the correct synthesis of the creatinine acid; these can be seen in appendix 5. Both spectra showed that the right products had formed.

The creatinine acid was also attached to a ssDNA piece. This conjugation was checked with a bioanalyzer, which performs capillary electrophoresis. The conjugation also took multiple iterations with differing concentrations before the final product was visible. The results, which can be seen in appendix 6, show that the product is formed but unbound ssDNA was still present. In a similar fashion to the protein M conjugation the yield was not as desired, and optimization has not rendered better results yet.

Another important part of the assay is the fluorophore dyes. The acceptor dye was ordered readily attached to the scaffold, while the donor dye was manually attached to both the antibody and protein M via a labeling protocol. Both were tried to see which labeling gave better results. The degree of labeling (DOL), which is the amount of fluorophore dyes per antibody or protein, was determined using a NanoDrop spectrophotometer and a DOL formula. The results showed a DOL of 4.1 for the antibody and 0.6 for protein M. The desired DOL is 3-4 so the experiment with the antibody gave the desired results while the protein did not. This protocol was optimized, giving a DOL of 1.8 and after further trial 2.17. The decision was made to continue with the donor fluorophores on the antibody, not on the protein because of these results. See appendix 7 for the DOL formula and the values used in the calculation.

All the 4 different ssDNA pieces were assembled with a thermocycler that went from very high temperatures to room temperature. By using high temperatures, the ssDNA pieces are not in any secondary structures, and it is definitely above melting temperature in which nothing is hybridized. By then slowly lowering the temperature the pieces start to hybridize. The DNA attached to protein M was added later at a temperature of 37 degrees Celcius to prevent protein denaturation. An SDS page gel (Appendix 9) showed that this process rendered a product with a larger structure than just the protein M with the single ssDNA strand. This indicates that some form of hybridization took place, and the process might have worked.

The affinity of the antibody for the target, creatinine, was tested with a surface plasmon resonance machine. The results from this experiment (Appendix 8) were not as desired, there could be two reasons for this. It could be because the creatinine molecule is too small for the machine to accurately give an affinity or because there is actually very low affinity between the antibody and creatinine. Further testing will be done with this antibody but also a backup antibody that might show better results.

4. Originality

4.1 Team captain

At the start of the project, elaborate literature research was conducted. We found that the continuous measurement of creatinine would be possible by various methods. Possible techniques to continuously monitor molecules are biosensing by free particle motion (f-BPM) [4][5][6] and an antibody-based molecular switch [2]. We decided to develop an antibody-based molecular switch, as we believed that this choice would give us more design freedom. Moreover, it has better generalizability, because the antibody can easily be adjusted to other targets by exchanging the bait and antibody pair. The antibody-based switch is a novel technique to continuously measure biomolecules. In Thompson et al. (2023), they specifically show that this design is applicable for cortisol. The team modified the molecular switch in such a way that it is viable for the detection of creatinine, while maintaining high accuracy, sensitivity and reversibility, with the support of the Molecular Biosensing and Protein Engineering research groups within the Eindhoven University of Technology. We decided to use a recently discovered protein M, as it can be used with polyclonal antibodies. To use the protein within the switch construct, it had to be modified. The modification of this protein was incredibly challenging and took numerous trials and methods due to the novelty of the protein. The DNA scaffold has been designed from scratch by the team to ensure flexibility of the scaffold. A scaffold on which the acceptor dye is located, and a scaffold including a biotin molecule for functionalization to the substrate are ordered. The whole scaffold assembly, attachment of the analog, creatinine acid, and of protein M is done by our team. The fluorophores used in our design are chosen by the team in such a way that maximum energy transfer takes place when these are in proximity to each other. The fluorophores are attached to protein M by the team. The functionalization of the construct to the surface coated with Neutravidin is done via biotin which is connected to the DNA scaffold. The optical read-out mechanism is FRET, using inverted microscopy. The microscope is based on an existing design within the Molecular Biosensing group, although the equipment differs significantly. The detection team has decided which equipment to use. The custom sample stage has been designed by the team and 3D printed using the facilities of TU/e Innovation Space. The choice of the dyes and filter was made by the team. The software consists of image processing code, sensor control code and a GUI, which were all written from scratch in MATLAB and Python. The bottom up assembly of the biosensor and the testing of the samples has been done by the team.

4.2 Team's supervisor

After an extensive literature review, the team decided to follow a challenging route by selecting a FRET-based competitive assay principle instead of the BPM technique that has been developed at TU/e. This choice required the design of a molecular construct specific for the creatinine assay. After consulting biochemists (group Merkx), they decided to use a DNA scaffold, designed from the ground up, that can be assembled after synthesis out of the individual components. Main non-trivial challenges, taken up by the assay sub-team, have been coupling (including characterization) of DNA and fluorophores to a low affinity antibody (finally achieved with protein-M) and the synthesis of a suitable analog with both a fluorophore and DNA attached. A second sub-team designed an optical breadboard based sensor including software inspired by the knowledge in the biosensor group at applied physics. The team has clearly chosen an admirable challenging and innovative approach taking a risk regarding the available time for testing sensor performance.

4.3 Signatures

dr. L.J. (Leo) van IJzendoorn (Supervisor T.ES.T.)	D.M. (Danny) Struijk (Team Captain T.E.S.T.)	A.F. (Aukje) Wakkerman (Team Vice-Captain T.E.S.T)

5. Translation potential

5.1 Introduction

Over 10% of the Dutch population suffers from kidney disease [1]. It is an incurable disease in which the kidney functioning slowly degrades. There are various stages of the disease, of which the latest stage is kidney failure. Kidney failure can be detrimental for the patient if left untreated [7]. The patient therefore needs a kidney-replacing treatment: dialysis or a kidney transplantation. A transplantation is the desired treatment of the two, due to the high burden dialysis puts on the patient's health and day to day life. For the majority of patients, a kidney transplant is not possible as the queue is tremendously long: in 2023, in the Netherlands, the average waiting time for a transplant was 2 years and 3 months [8].

Kidney preservation is an important aspect of kidney transplantations. It ensures the viability and functionality of the organ until it can be transplanted into the recipient. Normothermic Machine Perfusion (NMP) is an advanced method of preservation in which kidneys are continuously perfused with a solution to remove waste products and provide nutrients, while maintaining metabolic activity and minimizing tissue damage. This method can prevent kidney damage due to a lack of oxygen, increase preservation time and even regenerate kidney function [9]. NMP has been shown to improve functioning of the transplanted kidney, especially from Extended Criteria Donors [10]. However, NMP does not provide the clinician information on the well-being of the kidney, which limits the amount of interpretable data for the clinician to use for the transplantation.

There has been a shift towards the usage of post-mortem kidneys for transplantation: in the Netherlands, it is calculated that in 5 years the number of post-mortem kidney donors will increase by 50%, resulting in 250 extra kidney transplants per year. Of all post-mortem kidneys, 18% is not used due to a lower quality of the kidneys [11]. Research is being conducted to realize the regeneration of post-mortem kidneys, enabling the kidneys to be eligible for kidney transplantation, which would increase the amount of possible kidney transplantation. However, this research is still in a very early stage. It is predicted that the regeneration of kidneys will take place in 5 to 10 years [11].

T.E.S.T. proposes a product that enhances the process of kidney transplantation, by providing clinicians and researchers with more interpretable data about the functioning of post-mortem kidneys. This is achieved by designing a product which is capable of continuously sensing the level of creatinine in the perfusion fluid that the kidney is filtering during MP. In Figure 4, the MP circuitry can be observed. In the stage where sample collection takes place, our device will be implemented. Our product is plug-and-play: the perfusion fluid only needs to flow through the sensor and it will not have influence on the MP circuitry. The measured sample is discarded after measurement. This leads to two main benefits: The first is that a more accurate quality assessment, by analyzing biomarkers in the perfusion fluid of kidneys can take place.

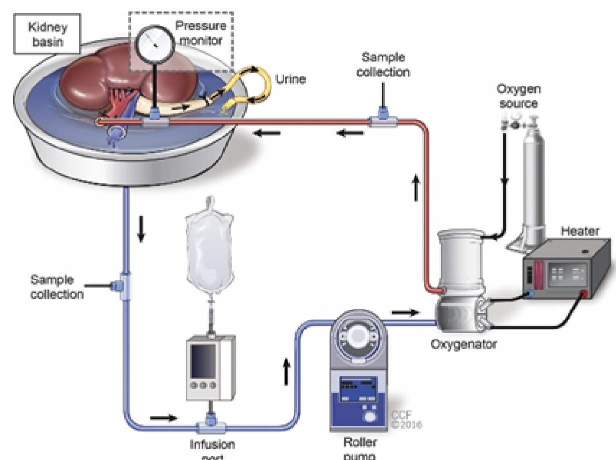


Figure 6: *Machine perfusion circuitry, Image retrieved from Kidney News Online [25]*

This results in a lower rate of kidney refusal. Secondly, interpretable data is provided to the researchers, which has a significant possible impact on the research being conducted on the regeneration of post-mortem kidneys.

Ultimately, this leads to an increase of usable kidneys, as the quality of post-mortem kidneys is increased By doing this, T.E.S.T. wants to decrease the strain on the healthcare system and improve the quality of life of patients with kidney failure.

5.2 Business model canvas

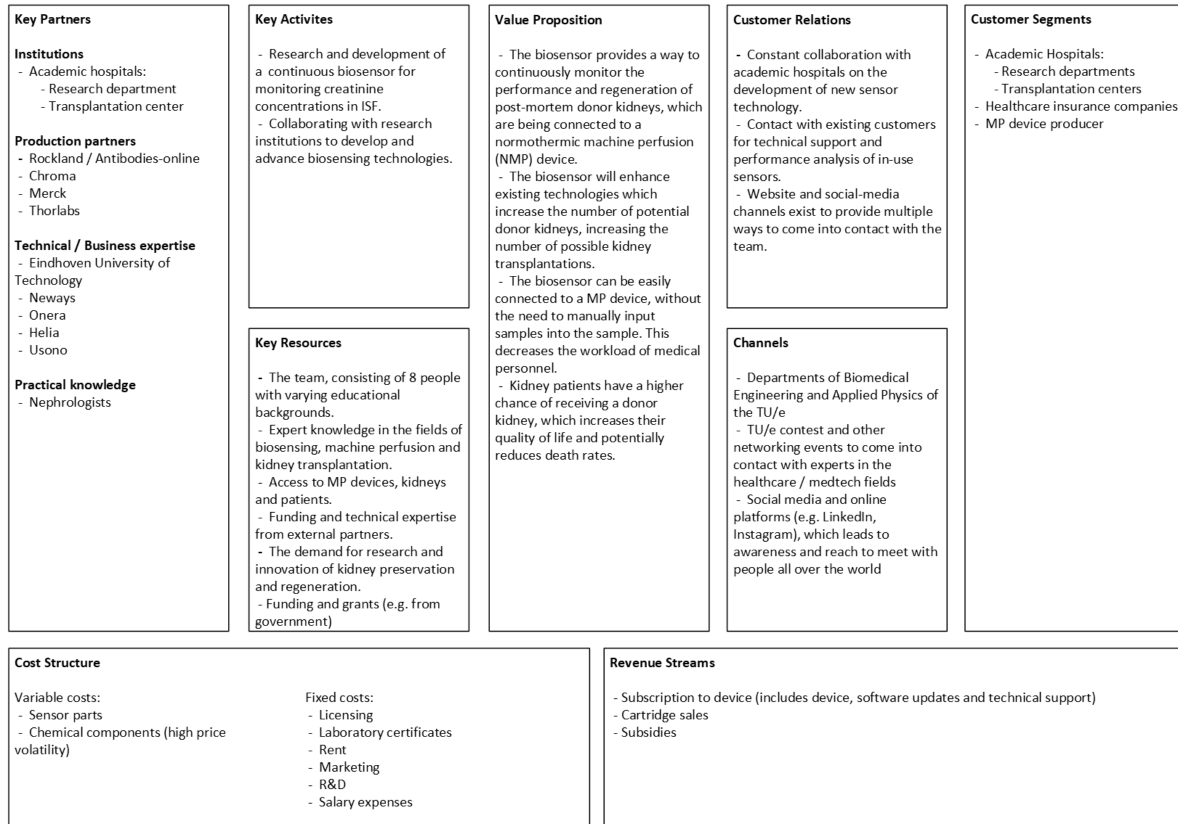


Figure 5: *Business model canvas*

5.3 Stakeholder desirability

The group which profits from kidney transplantation are the kidney failure patients. The group consists mostly of older people with underlying health issues, such as cardiovascular diseases or diabetes. The patient requires kidney-replacing treatment when his/her kidneys work for less than 15%. In 2023, 1019 kidney transplants took place in the Netherlands. On top of that, in the same year, 1084 people were waiting for a kidney transplant, of which 184 people have died due to kidney failure [8]. Patients who are on the waiting list usually have to undergo dialysis to bridge the gap between failure and transplantation. A shortening of the waiting list greatly increases the comfort of the patient and it saves people from death due to longevity of the waiting list. In addition, the increased data about the transplanted kidney results in a better prevention of possible post-transplantation complications, such as delayed graft function, which is a complication possibly resulting in death [12].

The Dutch healthcare system operates on a regulated free-market model. These companies are motivated to make a profit, primarily through the premiums paid by citizens. The insurance companies aim to minimize costs and maximize efficiency. The high costs of kidney replacement treatments, including dialysis and transplantation, are covered by healthcare insurance companies, with dialysis alone costing €100.000 per year per patient [8]. As mentioned priorly, the waiting list for kidney transplantations can be significantly reduced with our device, as more kidneys become viable for kidney transplantation. Assuming that 80% of these patients are on dialysis, reducing the waiting time for a kidney transplant by just three months would lead to significant cost savings. Specifically, it could save healthcare insurance companies approximately €22 million annually (calculation in Appendix 8). These savings could be used to lower insurance premiums for payers or redirect to other healthcare needs, ultimately reducing pressure on the overall healthcare system in the Netherlands.

Academic hospitals are affiliated with universities, through which they facilitate research and innovation alongside regular hospital activities. Seven academic hospitals

exist in the Netherlands, which each have a transplantation center [13]. Moreover, they perform research on novel transplantation methods and technologies [14]. The preservation technique for kidney transplantation specifically is crucial for donor organ quality, which is directly related to morbidity and survival after transplantation [15]. Because of this, academic hospitals strive for high-end technologies to optimize transplantation for success and patient quality of life. Academic hospitals are described as our end-user, because they are the users of Machine Perfusion (MP) devices. We aim to partner with an academic hospital such that we can provide the technology in return for scientific research and validation of the value of our product. Once the safety, reliability and value are proven, the product can be used for transplantation procedures, which would also start with trials at the partnering hospital. During this partnership, we aim to adopt an agile strategy to align our product with the needs of the user. The Netherlands has a highly developed knowledge-based economy, which fosters innovation. Government funding accounts for 73% of the total revenue that the academic hospitals generate for research purposes [16]. This income is steadily increasing, which enables academic hospitals to drive innovation in the medical sector. Especially as these hospitals are at the global top of medical innovation, meaning their research can drive global change. High-end research is required in order to remain a global innovator, in which our product can play a crucial role. During the research phase of our product at an academic hospital, the product is not yet CE certified. At this point, it is classified as an Investigational Medical Device, which requires an Investigational Medical Device Dossier and approval from an accredited METC for clinical research with human subjects [17]. Clinical tests can also be conducted with donated kidneys that are unsuitable for transplantation. This method cannot induce harm in any way. However, it should be considered that this is also only possible with consent from the kidney donor [18].

Value Proposition

As the innovation we are developing is a pioneering, non-existent technique, we are competing with the current state of kidney preservation. In current practice, the most used techniques are Static Cold Storage (SCS) and Hypothermic Machine Perfusion (HMP) [19]. In SCS, the kidneys are flushed with preservation fluid and then placed on ice until kidney transplantation takes place. In HMP, the kidneys are constantly flushed and a low temperature is maintained. These techniques are based on the principle that the reduction in temperature enables the cells to survive for a longer period, therefore maintaining cellular stability. NMP, which we will complement with our device, is an emerging technique, due to the ability of NMP to recreate the in-vivo environment of the kidney more closely than SCS and HMP. This ensures optimal oxygen delivery and supports metabolic function. Currently, NMP does not contain components to simulate kidney function, meaning that the kidney is maintained in a healthy state, but is not functioning. Novel research shows that NMP can be further enhanced by analyzing other biomarkers and reduces the rate of kidney refusal and delayed graft function (DGF) [19][20]. In combination with the discussed advantages of NMP, the occurrence of DGF and the rejection of kidneys can be significantly improved relative to the current techniques, being SCS and HMP.

The development of our product is a combination of existing, public techniques and newly developed techniques. Currently, a US provisional patent is being requested for a specific technique used in the product by the protein engineering group of TU/e. Regular consults with legal experts will take place to verify that we are compliant with intellectual property laws during the process. As we offer a pioneering solution in a heavily researched, but novel topic, it is relatively difficult to evaluate our product. Extensive market research in similar fields will be conducted to gain knowledge about the monetary valuation of T.E.S.T. In addition, expertise will be attained by talking with entrepreneurs in the biomedical field.

The predicted costs and profits are based on the cost of the prototype. This calculation includes the benefits of the acquired partnership, as equipment becomes cheaper. This does however lead to a percentage of the sales going to the corresponding partners. Based on measures which are to be explained further on, the price can be

brought down to €1.484,89. As discussed earlier, the product that we offer is integrated into the machine perfusion circuitry in a plug-and-play fashion: no other modifications need to be made to the circuitry for it to work adequately. The prototype showcases that the technique of continuously measuring creatinine is possible. In the next phases of the product, the focus will be on the user-friendliness of the product. Developments will take place to ensure that the product works autonomously. Input setting and output creatinine values are both communicated via laptop, which will be replaced in the future by an interactive LCD screen.

Business feasibility

An extra 6-year research phase is needed for the development of our product. This estimate is based on an average of 6.5 years from concept to certification for biosensors that are not based on existing devices. During the research phase, it is most necessary to have access to MP machines and kidneys. Kidneys can be sourced from laboratory animals or donor kidneys that are not used for transplantation. In the last stage of research, clinical trials will be conducted which require access to transplantation centers and consenting patients. Medical personnel such as organ perfusionists are the end-users of our product and are therefore needed early in the design process to align the sensors with their needs. They are also needed to execute the clinical trials. Lastly, expertise from medical experts and researchers should be acquired complementary to our own expertise. We aim to reach these resources through a partnering academic hospital. Since each academic hospital in the Netherlands has a considerable share in kidney transplantation, we can access the required resources through any of them as partners and each would allow for initial upscaling [21]. A market penetration phase in succession to the research phase requires a reliable supply of sensor parts, which will be established through existing partners. The core resource we possess throughout the entire process is our team's time and expertise. Since this is a complex resource in terms of value, a SWOT analysis was carried out to identify its strengths and weaknesses (Appendix 9). Lastly, funding is required to perform research and to enter the market. This will be acquired from investment rounds and subsidies from the government and health insurance companies.

In the early phase of development, product optimization and partner acquisition are key activities. The product needs to be optimized to be fully suited for integration in the MP circuitry, and tested extensively for performance, safety and reliability. This requires constant research, with the guidance of TU/e. Another key activity is the acquisition of partnerships. It is important to consolidate the partnerships for a long-term commitment. These partnerships result in the supply of materials. The needed money for development, as calculated in the financial viability section, will be acquired via several investment rounds throughout the year: to this end it is important for T.E.S.T. to network in the field of biotechnology, talk to entrepreneurs in Biotech and to inquire whether investors are interested.

The first step in marketing and sales is to contact each academic hospital in the Netherlands to inform them about our product. An agreement will be offered to the hospitals where they can receive the sensor and cartridges for free in return for access to their resources described previously. Coming to such an agreement can be complex, as the decision-making unit usually consists of personnel from varying departments. Thirdly, we aim to convert academic hospitals to revenue sources after clinical trials have proven the potential of our product, which leads to a willingness of academic hospitals to pay for the sensor and cartridges. To achieve continued engagement from customers, it is important that kidney transplantations benefit from the use of our sensor. We aim to evaluate continuously how our sensor performs in practice, and what can be done to improve it. Customers will also receive software updates and technical support in case of unsatisfactory performance or technical failure.

Before our sensor can be brought to the market, existing patents should be considered. Our sensor design currently conflicts with the requested patent for protein M from the protein engineering research group at TU/e and the antibody-switch design from the Soh Lab research group at Stanford university [2][22]. Our first option is to make patent

licensing agreements with both research groups such that we can proceed with our sensor design. Licensing costs have been accounted for in the financial viability section. A secondary option is to alter our design.

The development of the cartridges relies on creatinine antibodies from antibodies-online; chemicals from Merck; essential filters from Chroma and materials from Thorlabs. These materials have been sponsored in the current prototype and a partnership is mutually beneficial for T.E.S.T. and the sponsoring companies, as T.E.S.T. becomes a reliable sales channel for these manufacturers. Other key partners include Neways Electronics and Onera for expertise. For development, the academic hospitals are a key partner, since they supply us with necessary knowledge and expertise, as all academic hospitals are involved in the process of kidney transplantations. They can provide insights regarding their needs as customers, as well as the resources and research capabilities in order to develop the product.

In order to have a well-functioning product, it is important that the device has high durability. The materials being used for our biosensor are of high quality: the skeleton of the biosensor consists of solid aluminum and the remaining hardware is obtained via reliable partners. All different hardware components and the cartridge can be easily detached and replaced if needed. To compensate for the heating of the laser, it is constantly cooled during its runtime. Both the interior and the exterior of the biosensor consists of MDF, preventing damage caused by the laser. Finally, the software is developed with minimal errors, reducing the chances of bugs and resulting in efficiently running code.

Financial Viability

As of now, the manufacturing cost of one cartridge is €3,16. For the prototype, the cost of manufacturing one sensor is €6.456,13. These costs are expected to decline to 20% of the current price when production of cartridges increases, due to company growth. The expected production cost of one cartridge becomes €0,72, while for the device this becomes €1.484,89. Included in these prices is that 15% of the material price is needed for marketing of the sensor and payrolls of the employees. Calculations on these prices can be found in Appendices 3 through 6.

Before entering the market with a user-ready sensor, developments will need to be made. Expected is that over the next 6 years, annually €30.000 will have to be invested in R&D of the sensor technology. Secondly, patents and intellectual property rights will cost €1.000 annually. This brings the total investment needed over the next 6 years to €186.000. Thereafter, when a market-ready sensor has been constructed, €10.000 will be spent yearly on further R&D. Since the current market is research-oriented, it is difficult to come up with a competitive price point. For income generation, a yearly subscription model has been chosen. This subscription includes installation and services for sensor maintenance and software support. Also, should one sensor become inoperable, T.E.S.T. will provide a new sensor as a part of the subscription model. The price of one yearly subscription will be €4.000. This is fit to current NMP device costs [23]. Next to this, cartridges for the sensor will be sold separately for the price of €200 each. One cartridge will be sufficient to use for one transplantation session, which can take a few hours.

In 2023, 515 kidney transplantations were carried out where NMP was being applied in the Netherlands [24]. Assuming we will start with one partnering academic hospital, this will come down to 72 yearly transplants in which our sensor and cartridge is being used. One academic hospital will therefore create a revenue of roughly €18.000 yearly. As stated in the introduction, the number of post-mortem donor kidneys will increase, which will positively impact our revenue stream. Our short-term goal is 1 partnering academic hospital in the Netherlands. After twelve years we expect to have 3 partnering hospitals. With these goals, the expected break-even point (BEP) is expected to be reached in 2039. Calculation of the BEP can be found in Appendix 7.

6. Team and support

6.1 Contribution of the team members

The team was divided into two sub-teams: the assay team was responsible for the development of the assay and the detection team was responsible for the development of the physical transduction and the reader instrument of the biosensor. Furthermore, a Translational Potential team was formed to develop the business model. Next to being part of a sub-team, each member had an organization function: team captain, secretary, treasurer, external relations and public relations.

Team member	Function within the team
Mélissa Bröring-Kerkhof	Member of assay, member of Translational Potential, public relations
Luisa Koppers	Head of assay, external relations
Sjoerd Offringa	Head of Translational Potential, member of detection
Jelle van der Pas	Head of detection team, member of Translational Potential, treasurer
Frederieke Serrarens	Member of assay, member of Translational Potential, public relations
Danny Struijk	Member of detection, member of Translation Potential, team-captain
Mara Teodorescu	Member of assay, secretary
Aukje Wakkerman	Member of assay, vice team-captain, external relations

6.2 People who have given support

Throughout this project, our team has received great support from a number of people, who we would like to thank given this opportunity. First and foremost, we would like to thank our general supervisor **dr. Leo van IJzendoorn** who guided us along this product development process, as well as in other matters, in (bi-)weekly meetings. Next to him, PhD. candidates **ir. Selina Janssen, Anna Świetlikowska, MSc** and **ir. Koen Valk** supervised the weekly meetings and provided the team with advice and support. **Anna Świetlikowska**, **MSc** was the primary point-of-contact for the assay sub-team and helped with lab protocols and any other questions surrounding this matter. **ir. Selina Janssen** was of help for both the assay and detection sub-teams by giving advice and suggestions on how we should further proceed as a team numerous times. **ir. Koen Valk** was the contact person for the detection team, giving advice and solutions to any technical problem that might have arisen. We would like to thank the members of the **MBx group** and **dr. ir. Arthur de Jong** for guiding us in this process and providing any advice or material that was needed. Next we would like to thank **dr. Peter Zijlsta** for providing additional advice and solutions to the detection sub-team.

Further, we would like to thank the members of the **Protein Engineering Research Group** from TU/e and the **Merkx lab** for the access to the laboratories, machines and materials. From this group, we would like to thank **prof. dr. Maarten Merkx, ir. Harm van der Veer, dr. Alexander Gräwe** and **Alexander Stephany, MSc** for the great support they have given to the assay sub-team. Moreover, we would like to thank **ir. Sander Huisman** for the help related to the creation of creatinine acid. Last, we would also like to thank **drs. C.R. Susanto** and **Maurits Overmans, BSc**, for providing information, advice and new insights for the translational potential sub-team.

6.3 Partners of T.E.S.T. 2024

T.E.S.T. 2024 is extremely grateful to the partners of this year whose support has made this project possible. We would like to thank TU/e Innovation space and Thorlabs for providing financial support; Merck, Chroma, Antibodies-online and Rockland for in-kind contribution via products; Onera for in-kind contribution via translational potential advice and Neways and YER for providing both financial support and in-kind contribution via technical advice and team training respectively.

7. Final Remarks

It is hard to believe that the time spent on developing and testing our biosensor is close to an end as the SensUs competition approaches us. The success of this project is a testament to the collaborative efforts and diverse skill sets of our team members, ranging from biochemistry to physics and data analysis. Being part of T.E.S.T. had an immense impact on all of our lives for the better, as it allowed us to get a sense of what it actually entails working in research and the engineering field. Nonetheless, we also got an insight on how it is to run a start-up by dividing concrete roles and tasks among us so that everything would run as smoothly as possible along the way. During our journey we have challenged ourselves by opting for a creative but rather difficult design. We have developed ourselves greatly, but most importantly, we have grown as a team and had lots of fun in the process. We are happy with the choice of participating in the challenge and look back on great memories.

We would, once again, like to express our gratitude towards our supervisors who facilitated this process. It could not have been possible without your effort.

8. References

[1] Nierstichting.(n.d.).Nierziekten. https://nierstichting.nl/over-nieren/oorzaken-nierschade/nierziekten/#:~:text=de%20nieren%20kwetsbaar.-,Nierschade%20door%20nierziekten,nierschade%20is%20de%20oorzaak%20onbekend.

[2] Thompson, I. A., Saunders, J., Zheng, L., Hariri, A. A., Maganzini, N., Cartwright, A. P., Pan, J., Yee, S., Dory, C., Eisenstein, M., Vuckovic, J., & Soh, H. T. (n.d.). *An antibody-based molecular switch for continuous small-molecule biosensing*. Science Advances. https://doi.org/10.1126/sciadv.adh4978

[3] Beating kidney disease Jares-Erijman, E. A., & Jovin, T. M. (2003). FRET imaging. *Nature Biotechnology*, *21*(11), 1387–1395. https://doi.org/10.1038/nbt896

[4] Buskermolen, A. D., Lin, Y., Van Smeden, L., Van Haaften, R. B., Yan, J., Sergelen, K., De Jong, A. M., & Prins, M. W. J. (2022). Continuous biomarker monitoring with single molecule resolution by measuring free particle motion. *Nature Communications*, *13*(1). https://doi.org/10.1038/s41467-022-33487-3

[5] Buskermolen, A. D., Michielsen, C. M., De Jong, A. M., & Prins, M. W. (2024). Towards continuous monitoring of TNF-α at picomolar concentrations using biosensing by particle motion. *Biosensors and Bioelectronics*, *249*, 115934. https://doi.org/10.1016/j.bios.2023.115934

[6] Michielsen, C. M. S., Buskermolen, A. D., De Jong, A. M., & Prins, M. W. J. (2023). Sandwich Immunosensor based on particle motion: How do reactant concentrations and reaction pathways determine the Time-Dependent response of the sensor? *ACS Sensors*, *8*(11), 4216–4225. https://doi.org/10.1021/acssensors.3c01549

[7] Norton, W. G., Pearson, R., Devlin, J., Nicholson, M. L., & Hosgood, S. A. (2022). Normothermic machine perfusion in renal transplantation. *Current Transplantation Reports*, *9*(4), 308–317. https://doi.org/10.1007/s40472-022-00378-x

[8] Nierstichting. (n.d.). *Feiten en cijfers*. Nierstichting. https://nierstichting.nl/nierdonatie/feiten-en-cijfers/

[9] *Exploring the benefits of machine perfusion in organ transplantation*. (n.d.). UC Health. https://www.uchealth.com/en/media-room/articles/exploring-the-benefits-of-machine-perfusion-in-organ-transplantation

[10] Bellini, M. I., Tortorici, F., Amabile, M. I., & D'Andrea, V. (2021). Assessing Kidney Graft Viability and Its Cells Metabolism during Machine Perfusion. *International Journal of Molecular Sciences*, *22*(3), 1121. https://doi.org/10.3390/ijms22031121

[11] *Niertransplantatie | LUMC*. (n.d.). https://www.lumc.nl/afdelingen/transplantatie-centrum/diagnoses-en-behandelingen/niertransplantatie/#:~:text=De%20gemiddelde%20wachttijd%20voor%20een%20niertransplantatie%20is%20ruim%20drie%20jaar.

[12] Perico, N., Cattaneo, D., Sayegh, M. H., & Remuzzi, G. (2004b). Delayed graft function in kidney transplantation. *The Lancet*, *364*(9447), 1814–1827. https://doi.org/10.1016/s0140-6736(04)17406-0

[13] Fabrique, Kings, T., & Ontwerpwerk. (n.d.). *Alle transplantatiecentra | NTS*. NTS. https://www.transplantatiestichting.nl/page/alle-transplantatiecentra

[14] Organ Donation and Transplantation Alliance. (2022, October 24). *Keeping Up with Technical Innovations in Organ Transplantation: Perfusion and Preservation Technology (Part 1) | The Organ Donation and Transplantation Alliance*. The Organ Donation and Transplantation Alliance. https://www.organdonationalliance.org/insight/keeping-up-with-technical-innovations-in-organ-transplantation-perfusion-and-preservation-technology-part-1/

[15] Jing, L., Yao, L., Zhao, M., Peng, L., & Liu, M. (2018). Organ preservation: from the past to the future. *Acta Pharmacologica Sinica*, *39*(5), 845–857. https://doi.org/10.1038/aps.2017.182

[16] *Inkomsten, onderzoek en zorg van de universitair medische centra | Rathenau Instituut*. (n.d.). https://www.rathenau.nl/nl/wetenschap-cijfers/geld/inkomsten-uitgaven-van-universiteiten-umcs-en-hogescholen/inkomsten

[17] Ministerie van Volksgezondheid, Welzijn en Sport. (2021, June 15). *D2. Investigational Medical Device Dossier (IMDD)*. Investigators | the Central Committee on Research Involving Human Subjects. https://english.ccmo.nl/investigators/clinical-investigations-with-medical-devices/standard-research-file-medical-devices/d-product-information/d2-investigational-medical-device-dossier-imdd

[18] Ministerie van Volksgezondheid, Welzijn en Sport. (2020, July 1). *Transplantatie-onderzoek bij donatie*. Uw Keuze Maken | Donorregister. https://www.donorregister.nl/uw-keuze-maken/welke-keuzes-heeft-u/transplantatie-onderzoek-bij-orgaandonatie

[19] Norton, W. G., Pearson, R., Devlin, J., Nicholson, M. L., & Hosgood, S. A. (2022b). Normothermic machine perfusion in renal transplantation. *Current Transplantation Reports*, *9*(4), 308–317. https://doi.org/10.1007/s40472-022-00378-x

[20] De Almeida Leite, R. R., Leite, M., Jr, Einicker-Lamas, M., Valverde, R. H. F., Miranda, L. C. D., & Schanaider, A. (2024). Clinical outcomes prediction in kidney transplantation by use of biomarkers from hypothermic machine perfusion. *International Braz J Urol*, *50*(4), 470–479. https://doi.org/10.1590/s1677-5538.ibju.2024.0166

[21] Fabrique, Kings, T., & Ontwerpwerk. (n.d.-b). *Alles over orgaan- en weefseldonatie | NTS*. NTS. https://www.transplantatiestichting.nl/

[22] *WIPO - Search international and national patent collections*. (n.d.). https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2022081541&_cid=P11-LZ8F92-18426-1

[23] *Machinale perfusie van donor-nieren – een Rapid Assessment*. (n.d.). https://kce.fgov.be/nl/publicaties/alle-rapporten/machinale-perfusie-van-donor-nieren-een-rapid-assessment

[24] Fabrique, Kings, T., & Ontwerpwerk. (n.d.-c). *Cijfers over organen en weefsels | NTS*. NTS. https://www.transplantatiestichting.nl/medisch-professionals/cijfers-nts

[25] Nori, U. (2018, February 1). Novel normothermic perfusion technique for preserving donor organs. KidneyNews. https://www.kidneynews.org/view/journals/kidney-news/10/2/article-p17_13.xml

[26] van Smeden, L. (2018). Continuous creatinine monitoring with particle mobility via a competition immunoassay.

[27] Grover, R. K., Zhu, X., Nieusma, T., Jones, T., Boero, I., MacLeod, A. S., Mark, A., Niessen, S., Kim, H. J., Kong, L., Assad-Garcia, N., Kwon, K., Chesi, M., Smider, V. V., Salomon, D. R., Jelinek, D. F., Kyle, R. A., Pyles, R. B., Glass, J. I., . . . Lerner, R. A. (2014). A Structurally Distinct Human Mycoplasma Protein that Generically Blocks Antigen-Antibody Union. Science, 343(6171), 656–661. https://doi.org/10.1126/science.1246135

9. Appendix

Appendix 1 - DNA scaffold design

To design the DNA scaffold the analyzer tool from Integrated DNA Technologies and the Nupack program were used. Repeated iterations of designs gave the following final scaffold pieces.

1. ssDNA with 3'-ATTO 647N fluorophore:

5'-CAC AGA CAT CAA GGG AGA CAC ACT TCG CAC ATT TAC TAC TAC TGA CTG ACA CTG AAT CTT /3ATTO647NN/

2. ssDNA with 3'-Biotin:

5'-GTG TGT CTC CCT TGA TGT CTG TGT TAT CTG ATG AAC AAC TTA AAT TT/3Bio/

3. ssDNA with 3'-amine group for attachment to protein M:

5'- GT GTG TCT CCC TTG ATG TCT GTGTT-3' – prot M (25 bases)

4. ssDNA with 5'-amine group for attachment to creatinine acid:

/5AmMC6/TT GAT TCA GTG TCA GTC AGT AGT AGT-3'

Attachment of these pieces gives the following scaffold:

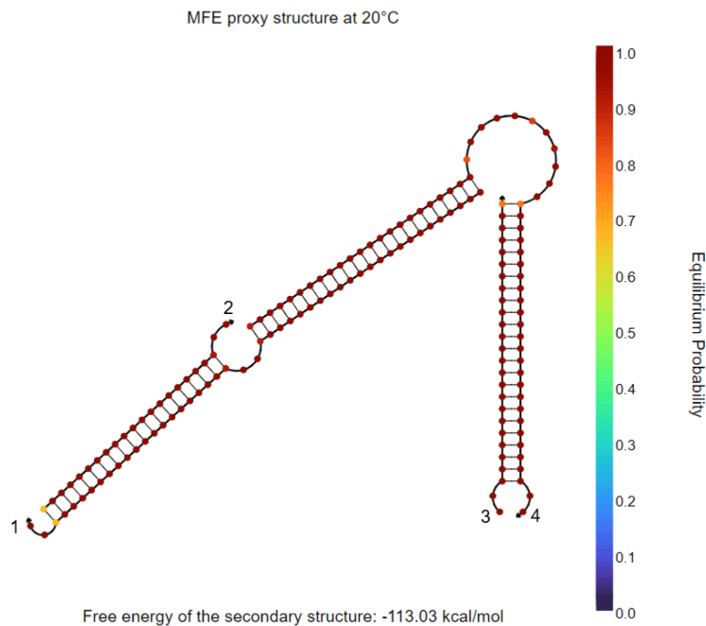


Figure 7: *DNA scaffold - short version. Equilibrium probability at 20 degrees according to the Nupack program. 1: attachment to Biotin; 2: attachment to protein M; 3: attachment to creatinine acid; 4: attachment to ATTO 647N..*

A longer version of the scaffold was designed by designing 2 extra ssDNA pieces to insert between the attachment of the ssDNA with acceptor to the ssDNA with biotin. These pieces are complementary to each other.

1. ssDNA for attachment to ssDNA with acceptor

5'-GTG TCT CCC TTG ATG TCT GTG TTT TCT TTT TTT TCT TTT TTG TTT TTT GTT TTT TCT TTT TTT TAT-3'

2. ssDNA for attachment to ssDNA with Biotin

5'-AGA CAT CAA GGG AGA CAC ACA TAA AAA AAA GAA AAA ACA AAA AAC AAA AAA GAA AAA AAA GAA AA-3'

This gave the following two complexes that can be attached together to form a longer scaffold:

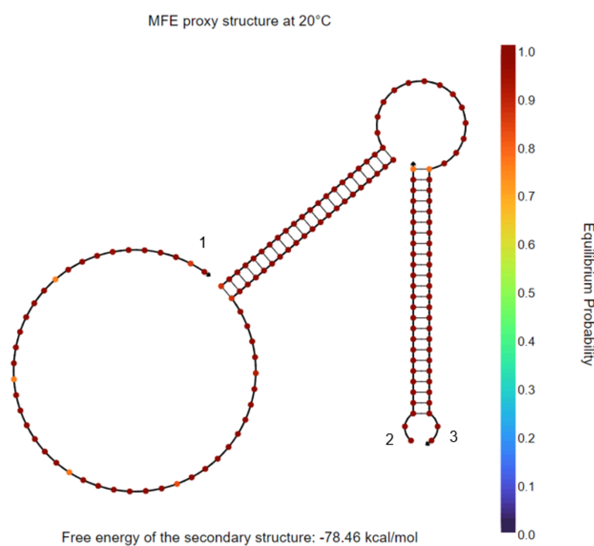


Figure 8: *DNA scaffold - long version part 1. Equilibrium probability at 20 degrees according to the Nupack program. 1: attachment to complex 2; 2: attachment to creatinine acid; 3: attachment to ATTO 647N.*

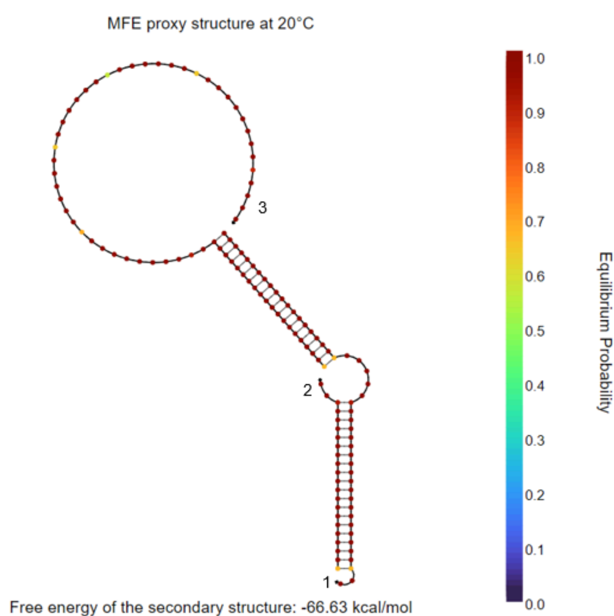


Figure 9: *DNA scaffold - long version part 2. Equilibrium probability at 20 degrees according to the Nupack program. 1: attachment to Biotin; 2: attachment to protein M; 3: attachment to complex 1.*

Appendix 2 - Mutagenesis protein M

Protein M is a protein that can attach to an antibody at the FAB fragment, which is why it is used in the switch construct. To this protein DNA can be attached via a cysteine on the protein. However, since the cysteine is located on the C-terminus of the protein it does not work in the construct. The reason being that when the protein binds to the antibody, the C-terminus is at the top of the antibody. If the scaffold is bound at this location it would need to bend almost 180 degrees for the analogue to interact with the antibody.

To lower the degree that the scaffold bends, the scaffold should be attached at the N-terminus. This also prevents hindering of the blocking site of the protein at the C-terminus. For this, two mutations are needed, one to change the cysteine at the C-terminus to a serine and another to change the serine at the N-terminus into a cysteine.

The C-terminal cysteine was removed by KLD methods. Two primers were designed that contained the desired mutation, after which PCR amplification was performed on a plasmid containing the protein M gene as a template. Then the KLD treatment was performed to phosphorylate the product with kinase; ligate the plasmid with ligase and degrade the template DNA by Dpnl.

When trying this method for the N-terminus it did not render suitable results. After trying multiple methods, the N-terminal cysteine was introduced by oligo cloning which gave the desired results. Two custom DNA sequences, oligos, were designed to insert the desired sequence into a plasmid vector. These oligos were annealed (paired) to form a double stranded sequence and then ligated into the plasmid vector after it was cut at the right places. The plasmid was then introduced into genetically modified E. coli bacteria, allowing these bacteria to replicate the plasmid.

The mutated sequence of the protein is presented in Figure 9.

MGSSHHHHHHKLGSCGASGTLVPRGSHMSLSLNDGSYQSEIDLSGGANFREKFRNFANEL
SEAITNSPKGLDRPVPKTEISGLIKTGDNFITPSFKAGYYDHVASDGSLLSYYQSTEYFNNRV
LMPILQTTNGTLMANNRGYDDVFRQVPSFSGWSNTKATTVSTSNNLTYDKWTYFAAKGSPL
YDSYPNHFFEDVKTLAIDAKDISALKTTIDSEKPTYLIIRGLSGNGSQLNELQLPESVKKVSLY
GDYTGVNVAKQIFANVVELEFYSTSKANSFGFNPLVLGSKTNVIYDLFASKPFTHIDLTQVTL
QNSDNSAIDANKLKQAVGDIYNYRRFERQFQGYFAGGYIDKYLVKNVNTNKDSDDDLVYRS
LKELNLHLEEAYREGDNTYYRVNEFGGSGGSWSHPQFEKS

Figure 10: *Mutated protein M sequence. Marked are the mutated positions into the right amino acid.*

Appendix 3 - SDS page results and analysis of conjugation ssDNA to protein M

The conjugation of ssDNA to protein M is needed because it is the bridge between the scaffold with the acceptor dye and bait and the antibody. The ssDNA has an amine group on one end so it can be coupled to maleimide. After this conjugation is successful the DNA can be attached to protein M. To verify the conjugation of ssDNA to protein M an SDS-page gel was used. This gave the following result.

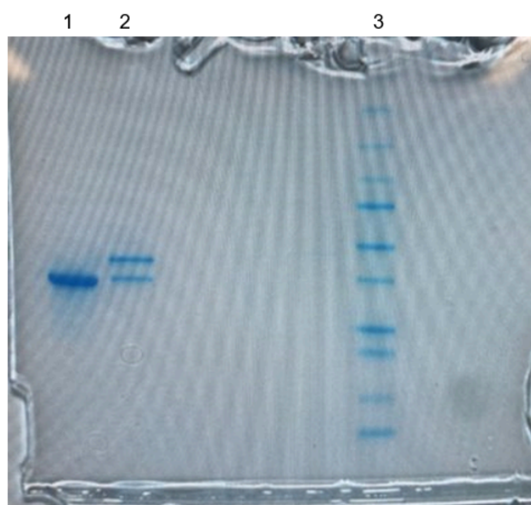


Figure 11: *SDS page gel of conjugation of ssDNA to protein M. Coomasie staining was used to make the protein bands visible. 1: protein M; 2: product of conjugation to DNA; 3 protein ladder. The visibility of two bands in sample 3 show that the attachment took place.*

The gel shows that the conjugation worked since there are 2 bands in the product. One at the same height as just the protein and one higher which means the DNA is attached. However, the yield is not great as can be seen by the 2 bands still in the product. There is still protein M not attached to DNA in the product, which is less desirable. With optimization this has not been improved yet.

Appendix 4 - Synthesis mechanism creatinine acid

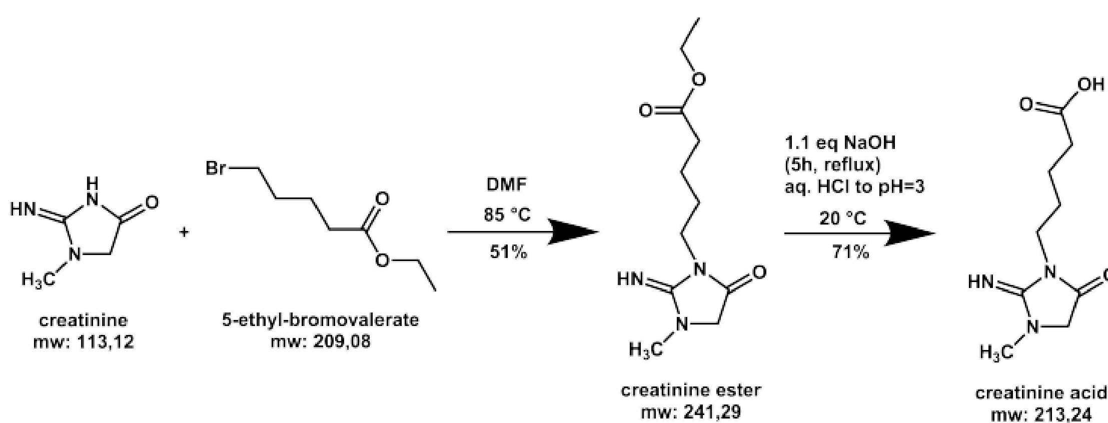
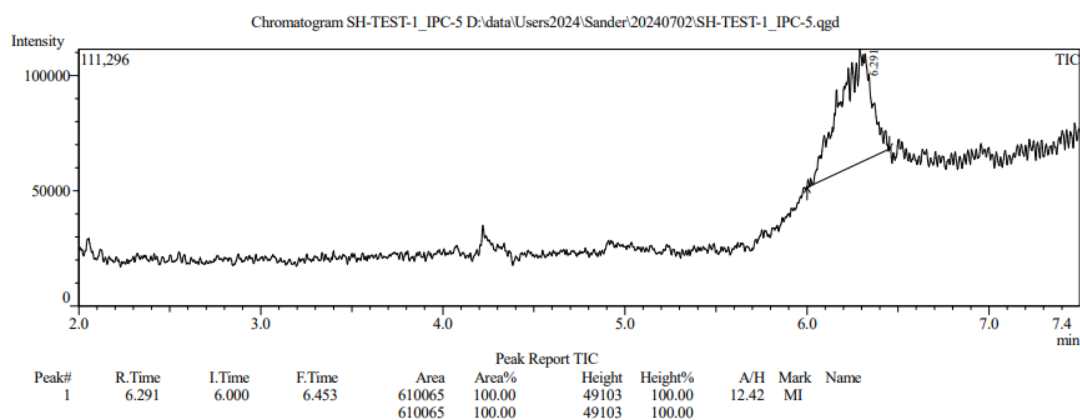


Figure 12: *Synthesis mechanism of creatinine acid starting with creatinine and 5-ethyl-bromovalerate. [26]*

Appendix 5 - LC-MS and GC-MS graphs of creatinine acid synthesis



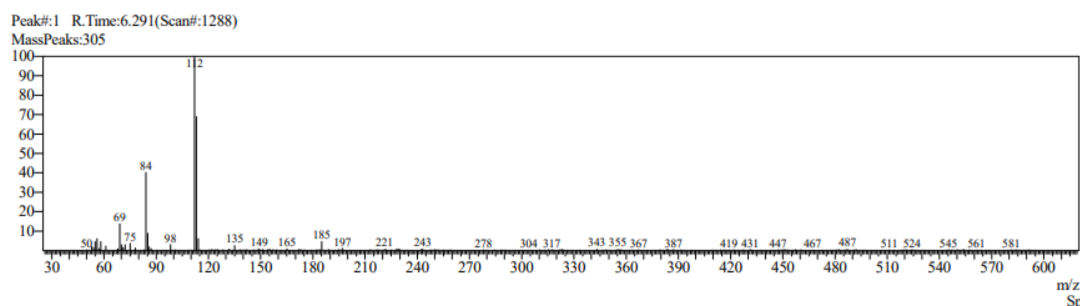


Figure 13: GC-MS graphs of product after creatinine ester synthesis.

The gas chromatography showed only one significant peak, as can be seen in Figure 11, which means there was only one product in the solution. This already gave an indication that the synthesis between creatinine and 5-bromovalerate had worked to form the creatinine ester. The MS spectrum of this peak confirmed this. Creatinine has a molar mass of about 113 g/mol and a peak is seen at 112 m/z so this corresponds. Ethyl 5-bromovalerate has a molar mass of about 209 g/mol, when removing the bromide ion the molar mass is about 130 which corresponds to the peak seen at 135 m/z. The 69 and 84 m/z peaks can be explained by the separate bromide ions.

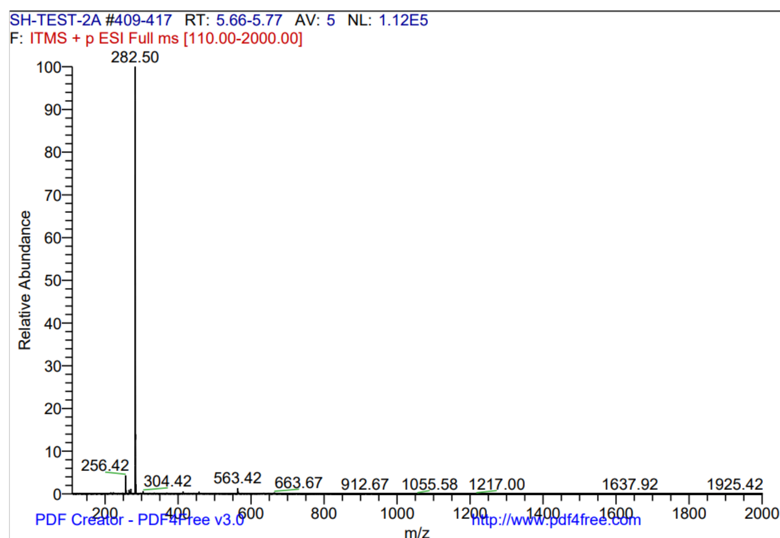


Figure 14: Mass spectrum of peak in LC-MS of product after creatinine acid synthesis from creatinine ester.

Based on the starting products and the final peak that showed in the MS in Figure 13, it can be assumed that the correct product was formed.

Appendix 6 - Bioanalyzer result of ssDNA to creatinine conjugation

To test if the conjugation of ssDNA to creatinine worked a Bioanalyzer was used. This gave the following result:

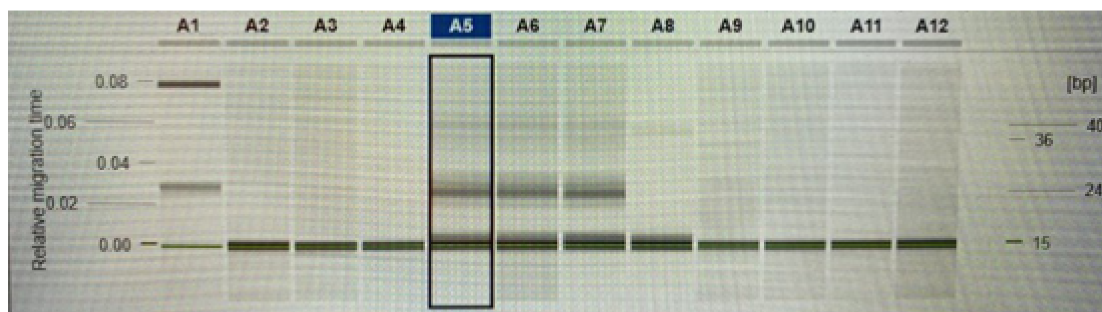


Figure 15: *Relative migration time of the components of the product after ssDNA to creatinine acid conjugation. A1: size marker; A2-A4: dilutions of first attempt at conjugation; A5-A7: dilutions of second attempt at conjugation; A8: ssDNA.*

In A5-A8 a band can be seen right above the 15 bp edge, this is the ssDNA unbound to creatinine. There is also a band higher at about 40 bp, this is assumed to be a secondary structure since it is also in the sample with only ssDNA. As can be seen when comparing the second attempt dilutions to the sample with only ssDNA, a new product is formed of a higher size than the unbound ssDNA which means there is a high probability that the conjugation worked. The first attempt dilutions show nothing, the concentrations in this attempt were 1000x smaller than first thought.

Appendix 7 - Degree of labeling formula

The spectrophotometer gives the absorbance values which can be used to calculate the degree of labeling (DOL) in the following formula.

$$DOL = \frac{concentration\ dye}{concentration\ protein} = \frac{A_{max}/\varepsilon_{max}}{A_{prot}/\varepsilon_{prot}} = \frac{A_{max}/\varepsilon_{prot}}{(A_{280} - A_{max} \cdot CF_{280}) \cdot \varepsilon_{max}}$$

The extinction coefficients that were used are $\varepsilon_{max} = 1.2 \cdot 10^5\ M^{-1}cm^{-1}$ and $\varepsilon_{prot} = 53750\ M^{-1}cm^{-1}$. The $CF_{280} = 0.10$ The absorbance values were derived from the NanoDrop spectrophotometer (A_{max} and A_{280}).

The degrees of labeling were achieved are:

	Antibody	Protein M trial 1	Protein M trial 2	Protein M trial 3
DOL	4.1	0.6	1.8	2.17

Appendix 8 - SPR results

The affinity of the antibody is a crucial part of the switch construct. If the affinity is too high the creatinine will not dissociate which results in an assay that would only be usable once. So no continuous monitoring would be possible. On the other hand there does need to be affinity for both creatinine as well as the creatinine acid.
The affinity for creatinine was tested with surface plasmon resonance. There were many antibodies immobilized on a protein G dimer chip which was then flushed with creatinine.

The experiment gave the following graph:

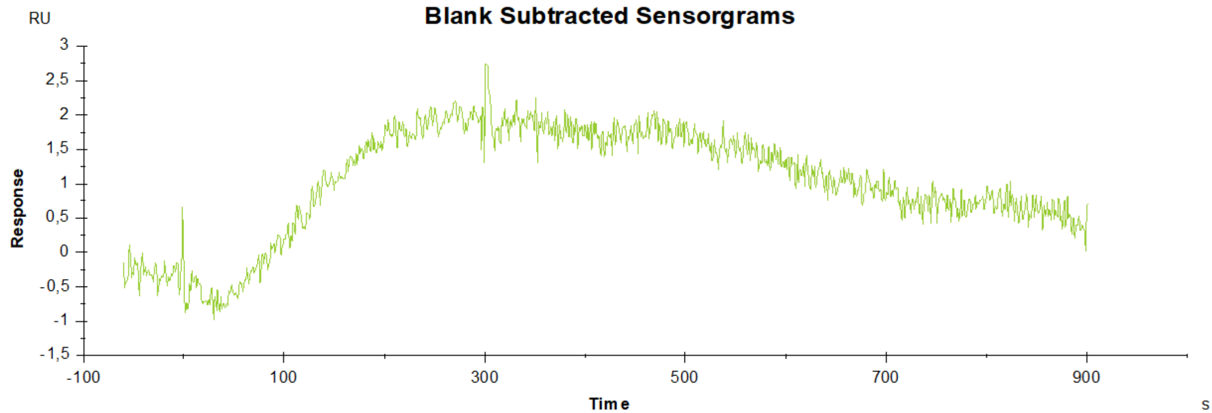


Figure 16: *Response of the creatinine and antibody over time.*

These values gave an Rmax, the maximal feasible SPR signal generated by an interaction between a ligand – analyte pair. The desired Rmax was six if you assume a 1:1 binding. However, an antibody has two FAB fragments so it was also possible the Rmax would go up to twelve. The response from the experiment was much lower than this at around 2. This means that either the affinity of the antibody to creatinine is very low, or that the experiment failed because creatinine is too small of a molecule.

Appendix 9 - SDS-page gel of DNA scaffold assembly

To test if the assembly of the scaffold was successful a SDS-page gel was performed. The following image shows the result.

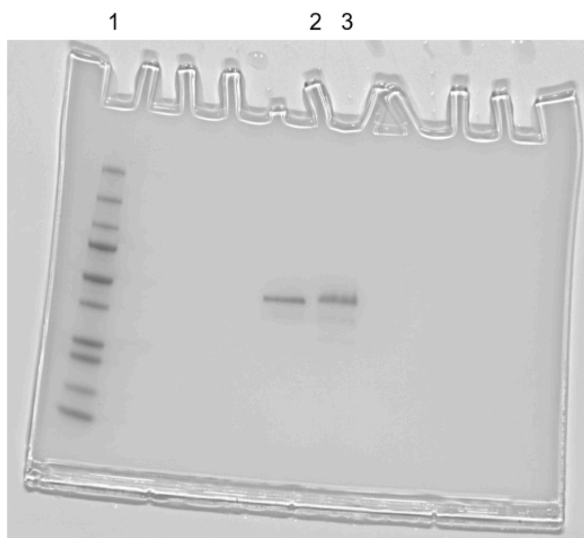


Figure 17: *SDS-page gel of assembled scaffold with Coomassie protein staining. 1: protein ladder; 2: protein M with one piece of ssDNA attached; 3: product of scaffold assembly of protein M with all 4 DNA pieces.*

As can be seen in Figure 13, the product of the assembly shows a band slightly higher on the gel than just the protein with 1 piece of DNA. This means that something did hybridize

to that single piece of ssDNA. From this the conclusion can be drawn that it is possible that the scaffold hybridization rendered the desired results, an entire assembled scaffold.

Appendix 10 - Cartridge costs

Product	**Unit price**	**Amount / cartridge**	**Price / cartridge**
ATTO550 with NHS-ester	€ 160 (1 mg)	16 ng	< € 0,01
Ethyl 5-Bromovalerate	€ 49 (5 g)	8 ng	< € 0,01
Neutravidin	€ 274 (10 mg)	1,2 µg	€ 0,03
Creatinine	€ 31,60 (10 g)	4 ng	< € 0,01
Sulfo MMC	€ 232 (20 mg)	17 ng	< € 0,01
Custom Oligo	€ 1002,32 (1 µmol)	0,02 nmol	€ 0,02
Primers	€ 15,09 (5 mg)	4 ng	< € 0,01
Anti-Creatinine antibodies	€ 994,09 (1 mg)	3 µg	€ 2,98
Other*	-	-	~ € 0,10
TOTAL			~ € 3,16

*Other chemicals are used to develop the assay, but are not present on the cartridge.

Appendix 11 - Sensor costs

Product	**Price**
Camera	€ 523,50
Laser	€ 1.272,01
Optical filters	€ 1.334,70
Lenses	€ 725,06
Electronics	€ 358
Mounting	€ 2.242,86
TOTAL	**€ 6.456,13**

Appendix 12 - Cartridge production costs

Subject	Cost
Cartridge costs	€ 0,63
Labor costs*	€ 0,06
Marketing and distribution**	€ 0,03
TOTAL	**€ 0,72**

*labor costs are estimated to be 10% of the device costs

**marketing and distribution costs are estimated to be 5% of the device costs

Appendix 13 - Sensor production costs

Subject	Cost
Device costs	€ 1291,23
Labor costs*	€ 129,12
Marketing and distribution**	€ 64,56
TOTAL	**€ 1484,91**

*labor costs are estimated to be 10% of the device costs

**marketing and distribution costs are estimated to be 5% of the device costs

Appendix 14 - Break even point

Balance x€1000		**2031**	**2032**	**2033**	**2034**	**2035**	**2036**	**2037**	**2038**	**2039**
Costs	Production costs	-€1,54	-€0,05	-€0,05	-€0,05	-€0,05	-€3,13	-€0,15	-€0,15	-€0,15
	R&D	-€10	-€10	-€10	-€10	-€10	-€10	-€10	-€10	-€10
Revenue	Subscriptions	€4	€4	€4	€4	€4	€12	€12	€12	€12
	Cartridge sales	€14	€14	€14	€14	€14	€42	€42	€42	€42
Balance		**-€179,54**	**-€171,59**	**-€163,64**	**-€155,69**	**-€147,74**	**-€106,87**	**-€63,02**	**-€19,17**	**€24,68**

Appendix 15 - Calculation saving insurance companies

Patients on Waiting List: The total number of patients awaiting kidney transplants, 1084
Percentage of Patients Undergoing Dialysis: The proportion of patients on the waiting list who are receiving dialysis treatment, 80%
Cost of Dialysis per Month: The average monthly cost of dialysis treatment per patient, 8333 euros.
Amount of Months Reduced: The reduction in time (in months) on dialysis due to improved kidney preservation techniques, potentially leading to quicker transplantation, 3 months.

Total Amount Saved=Patients on Waiting List × Percentage of Patients Undergoing Dialysis × Cost of Dialysis per Month × Amount of Months Reduced

Filling in the values, gives:

$1084 \ * \ 0.8 \ * \ 8333 \ * \ 3 \ = \ 21.7 \, million$

Appendix 16 - SWOT analysis

In order to prepare a market strategy for the upcoming years, the current position of team T.E.S.T. needs to be determined. To do this a SWOT analysis has been used. Determining strengths, weaknesses, opportunities and threats can be used in this team's advantage. Knowing where the team is still lacking gives the opportunity to improve in these areas and knowing where the team is strong makes sure that T.E.S.T. can reinforce those parts. This analysis will help in the market strategy and therefore give a better position to enter the market.

Strengths

- T.E.S.T. consist of members from diverse backgrounds with diverse interests. There is knowledge in multiple fields of biosensing within the team.
- All members proudly represent their university with great ambition and motivation to develop the sensor and keep improving the technology and user experience.
- The team is supported by research groups at the University of Technology. This provides opportunity for further research and feedback from different viewpoints.
- The team has multiple partners, also in different fields of the biosensing market, who give materials and expertise in both the technological and business sector. This gives a good opportunity to keep improving technology and to prepare well for market entry.
- The team has established contact with multiple consumers, health professionals regarding kidney transplants in the hospital. This provides a good opportunity for market research and research on making the sensor user-friendly.

Weaknesses

- While there is diverse interest and background within the team, most are biomedical students. Missing disciplines like electrical engineering and chemical engineering makes us vulnerable in development of the biosensor.
- Partnering is needed to integrate the sensing mechanism in machine perfusion technology. This makes T.E.S.T. dependent and choosing the wrong partner can lead to problems and a copy of our product.
- The market in kidney transplants is relatively small which causes a higher need for expansion to a different market

Opportunities

- There is a great need for innovative technology in the healthcare industry since it is overworked and the desire for good care is increasing.
- Continuous detection is possible and it offers great chances to reduce the need for point of care testing.
- The cartridge can be made for other biomarkers than creatinine while the detection method can stay the same which gives opportunity for expansion to other transplants for a bigger market.

Threats

- There are other biomarkers and ways to measure kidney function. Currently in machine perfusion there is no competitor in creatinine however since there are other biomarkers the competition can get high.

Appendix 17 - Sensor model

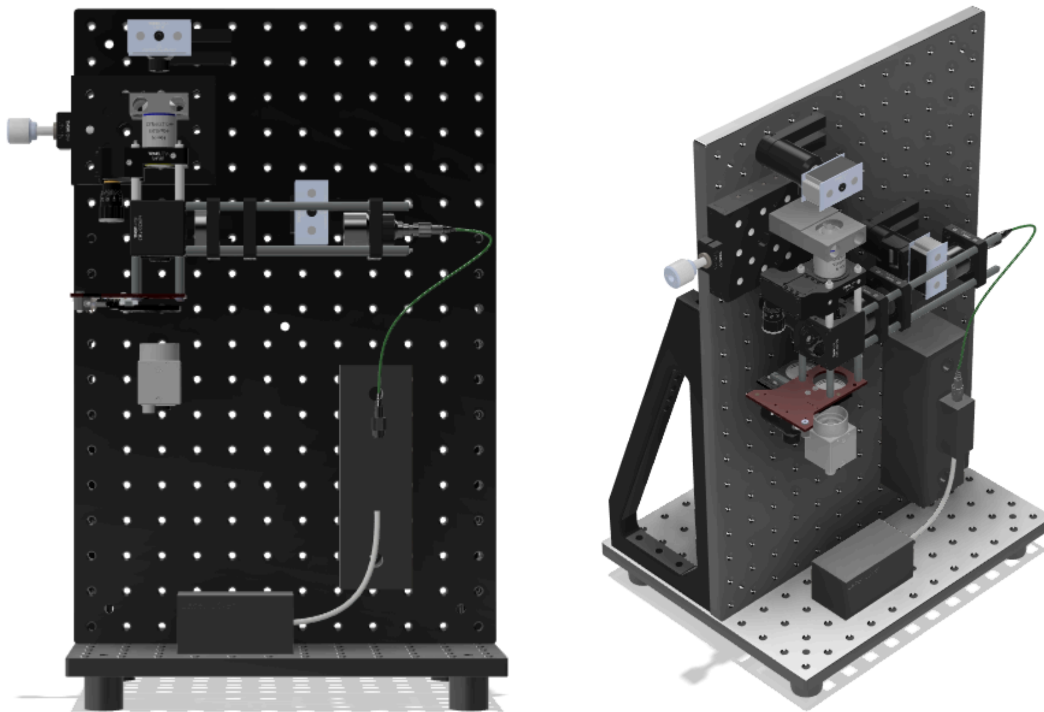


Figure 18: *Model of the sensor without case. Front view and isometric view of the left, top and front.*

Figure 19: *Model of the sensor case closed, with the top lid opened, and with the front and top lid opened*