Team Results Document (TRD)

TU/e Sensing Team

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1. Summary for the SensUs website

T.E.S.T. presents a novel biosensor to measure adalimumab concentrations in blood plasma. This biosensor is based on a ratiometric bioluminescent measurement using the target molecule of adalimumab (TNFα) and a TNFα/adalimumab complex specific antibody as reporter molecules. As a result of the formation and subsequent recognition of formed TNFα/adalimumab complexes, a blue light signal is generated by the recombination of two protein fragments into a bioluminescent protein. To increase the reproducibility of the assay under variable sample conditions, a ratiometric measurement approach was developed. For this, a protein complex was used that emits green light independent of the adalimumab concentration. The ratio of the measured intensities of both colors yields a more robust measure proportional to the adalimumab concentration. A sample of blood plasma and sensor can easily be put into a uniquely designed cartridge which is then inserted into the biosensor. Within 5 minutes, the bioluminescent signal is detected using an optical system by photodiodes and converted to an adalimumab concentration. Therefore, T.E.S.T. developed a biosensor which provides decisive information on the current and future treatment, and realizes precision and personalized medicine to improve therapy of rheumatoid arthritis and other anti-inflammatory diseases.

2. Biosensor System and Assay

2.1 Molecular recognition and assay reagents

T.E.S.T. developed a novel bioluminescent assay to detect adalimumab (ADL) directly in human blood plasma, which is also suitable to be implemented in a small point-of-care device. Compared to commercially available ELISA assays [1], [2], [3], this method requires no washing steps and very short incubation steps, which significantly reduces the total measurement time.

The bioluminescent assay comprises two protein engineered and chemically modified components; tumor necrosis factor alpha (TNFα) and an anti-adalimumab/TNFα (anti-AT) antibody (HCA204, Bio-Rad Laboratories). These components form the molecular recognition of the assay using the affinity of TNFα to adalimumab and subsequently anti-AT antibody to the adalimumab/TNFα complex (AT-complex). In order to generate a bioluminescent signal proportional to the concentration adalimumab, the split reporter enzyme NanoBiT was used. NanoBiT is an engineered form of NanoLuc luciferase, a bright bioluminescent enzyme that has already been used in many applications in the field of bioluminescent imaging due to its high stability, sensitivity and its small size [4]. NanoBiT consists of two protein fragments, SmallBiT (1.3 kDa) and LargeBiT (18 kDa), which have an inherent affinity to each other. Recombination of both fragments will lead to the formation of the complete bioluminescent enzyme, which is able to catalyze the oxidation of its substrate Furimazine to produce Furimadine and a photon of blue light.

The molecular recognition and bioluminescent signal are coupled by attaching the SmallBiT (SB) and LargeBiT (LB) to TNFα and the anti-AT antibody respectively. This way, SB and LB can be brought in each other's vicinity, facilitating the formation of the NanoBiT complex and thereby creating a light signal upon formation of the ADL/TNFα/anti-AT complex (see Figure 1).
TNFα-SmallBiT was obtained through recombinant protein expression while LargeBiT was attached to the anti-AT antibody using LASIC technology [5]. This method relies on the non-covalent binding of Protein G to the C_H2-C_H3 junction of human IgGs, after which a covalent conjugation is possible after activation of photoreactive non-natural amino acid benzoylphenylalanine (BPA) with UV-light. By recombinantly expressing LargeBiT with Protein G containing BPA, it is therefore possible to obtain anti-AT-LargeBiT after photoconjugation of anti-AT with Protein G. This specific technology has been developed in the Merkx group (TU/e) [6]. Both SmallBiT and LargeBiT are linked to their respective capture molecules through a semi-flexible glycine-serine linker, which allows for enough freedom to form the NanoBiT complex. A schematic representation of the assay is given in Figure 1.

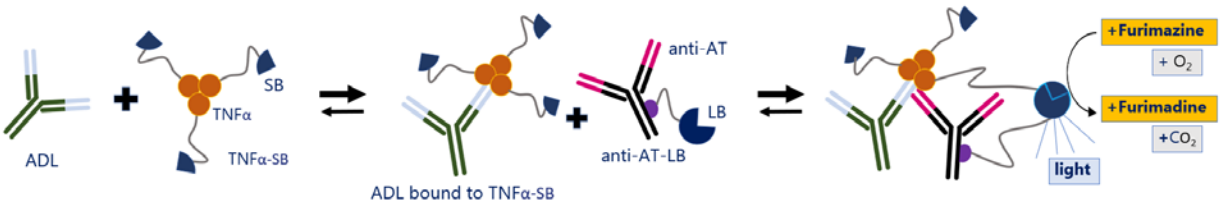

Figure 1: The assay principle for the detection of adalimumab. Adalimumab first binds to the trimeric TNFα-SmallBiT (TNFα-SB) complex, after which an anti-adalimumab/TNFα (anti-AT) antibody with LargeBiT (LB) can bind. This facilitates the formation of the NanoBiT complex, which generates light by catalyzing Furimazine.

The final component of the assay is the addition of a NanoLuc-mNeonGreen protein (NL-mNG) to the system. T.E.S.T. uses a variant of this protein that has a 95% BRET efficiency [3], [4]. As the emission of mNeonGreen relies on the same experimental conditions as the NanoBit enzyme that is used in the assay, it can correct for effects on NanoBiT intensity such as substrate concentration, pH and temperature. By choosing a constant mNeonGreen concentration and varying the ADL concentration, we can obtain a constant green emission peak at 517 nm with a varying blue emission peak at 460 nm. Shifts in the ratio of blue and green light will therefore be only dependent on the ADL concentration. Therefore, this ratiometric calibration corrects for inter-experimental variabilities and serves as a robust measure for the analyte concentration.

2.2 Physical transduction

The chemical reaction occurs in a microfluidic cartridge. To convert the analogue light signal into a digital signal, the emitted light from a cartridge is led through an optical setup in a cage system. First, the light from the cartridge, which is considered as a point source, is collimated by a plano-convex lens (f=25.4 mm). The emission spectrum of the bioluminescent protein has two maxima as it consists of the signal from both mNeonGreen-Nanoluc and NanoBiT. For this reason, the spectrum is split at the minimum between these maxima into a green and a blue beam. This is done through means of a long-pass dichroic mirror at 490 nm at an angle of 45 degrees. Afterwards, the green and blue beams are focused (plano-convex, f=25.4 mm) on the photodiodes (Thorlabs PDF10A, Si fW Sensitivity Fixed Gain Detector 320-1100 nm). An overview of the optical setup is given in figure 2 on the next page. The photodiodes provide an analog signal that is converted with an ADC and processed in a Raspberry-Pi computer.

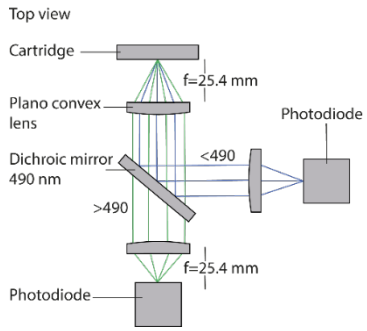

Figure 2: Schematic of optical setup. The light from the cartridge is collimated and split into a blue and green beam, which is separately measured by the photodiodes.

2.3 Cartridge technology

For the sample handling a disposable cartridge with one chamber, an inlet and an outlet is used. The prepared sample, described in section 3.4, is pipetted in the inlet and flows from there into the chamber. The design of the cartridge can be seen in Figures 3A and 3B. All three layers of the cartridge consist of poly(methyl methacrylate) (PMMA) material. The middle and bottom layer are made from white instead of transparent PMMA to obtain more signal due to diffuse reflection. The cartridge can be placed vertically in the biosensor to measure light.

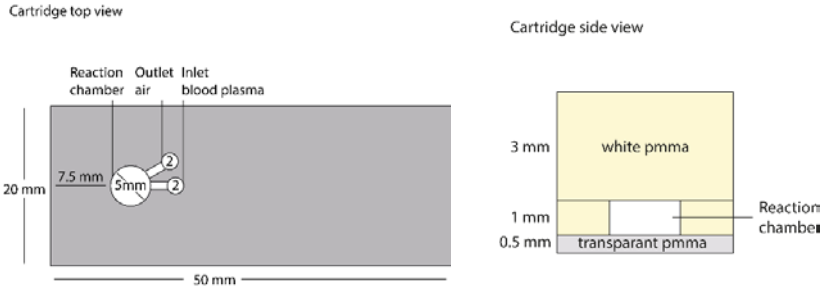

Figure 3a: Top view cartridge design, diameter of the reaction chamber is 5 mm. Figure 3b: Side view cartridge design, the reaction chamber is placed in the middle layer.

2.4 Reader instrument and user interaction

Reader instrument

The used photodiodes provide an analog output signal between 0-10V. A voltage divider is used to transform the actual voltage range to the range of 0-5V. This signal is converted by the ADC (ADS1115 16-bit) to a digital signal, so it can be processed by the Raspberry Pi (3B+). The photodiodes and Raspberry Pi are powered by one power supply (Farnell RPT-60B). A 7-inch LCD touchscreen makes it possible to interact with the device.

When a cartridge is entered in the device and the measurement is started, the signal of the photodiode is measured 15 times per second. The average value per two seconds is saved in a CSV file. This is done to minimize noise. Since a ratiometric approach is used, the converted signal of the blue light is divided by the converted signal of green light. The ADL concentration is determined by a calibration curve of the ratio.

Performing a measurement

Several practical handling steps are needed to perform a measurement: **Step 1**: Add the ADL sample to the tube containing the sensor protein. **Step 2**: Wait for three minutes (incubation time). **Step 3**: Add substrate (Furimazine) to the tube containing sensor protein and ADL. **Step 4**: Pipet contents of step 3 (the prepared sample containing sensor proteins, ADL and Furimazine) into cartridge, **Step 5**: Put the cartridge into the device and start the measurement (takes 1 minute).

A zero-measurement averaging 10 seconds of data is started to compute and eliminate the offset of the photodiodes. The time set to perform steps 3, 4 and 5 is set at 40 seconds, after which the measurement starts. After one minute the measurement is finished and the ADL concentration is displayed, see Figure 4. It is important that the device is turned on for at least five minutes before starting the measurements to obtain valid results. This is due to the temperature sensitivity of the photodiode causing an offset. The overall dimensions of the device can be seen in Figure 5.

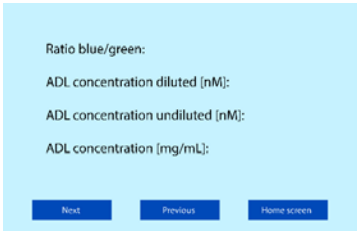

Figure 4: GUI design.

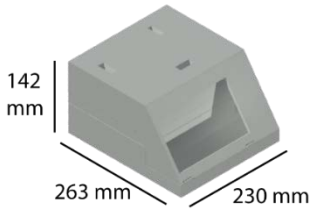

Figure 5: 3D model of the sensor

3. Novelty and Creativity

3.1 Already available

Detection of adalimumab (ADL) in commercially available assays relies on sandwiching ADL in a multi-step assay, by using the binding sites on its paratope and the Fc-region. Adalimumab is commonly bound to the solid phase using its original target, TNFα, or an anti-adalimumab antibody, while a non-specific antibody with a reporter enzyme can be used to bind to the Fc domain. These types of ELISA assays are characterized by long measurement times due to the multiple washing/incubation steps that are needed [1], [2], [3].
Our biosensor makes use of a protein-fragment recombination enzyme (NanoBiT) which enables the assay to take place in only one step. Here, ADL first binds to recombinant TNFα, after which an anti-AT antibody can bind. Both steps take directly place in solution.

The reporter enzyme used for our assay is NanoBiT, which emits a spectrum with a peak at 460 nm. This protein has already been frequently used to monitor protein-protein interactions and proximity-based assays. This is due to its high stability, sensitivity and the small size of the protein fragments compared to other protein-fragment complementation assays [5, 6]. NanoBiT has also been used in ratiometric BRET assays for the detection of therapeutic antibodies. Ni et al. combined this enzyme with their LUMABS-technology, where they labelled SmallBiT with a Cy3 fluorophore, that served as a BRET acceptor [10]. Binding of a target antibody to their protein construct disrupted the binding of LargeBiT with the labelled SmallBiT and enabled it to bind with an unlabeled SmallBiT fragment, resulting in a shift of the emitted light from red to blue.

The conjugation of a protein to an antibody is realized using LASIC-technology, which was used to attach a protein fragment of NanoBiT to our detection antibodies. This method makes use of the affinity of Protein G, an immunoglobulin-binding protein, to the C_H2-C_H3 junction of human IgGs [5]. Protein G can be expressed with photoreactive non-natural amino acid benzoylphenylalanine (BPA), which is used to form a covalent bond with the Fc domain of a humane antibody. This is achieved by treating the antibodies and Protein G-constructs with UV light. As Protein G can be bacterially expressed, it is possible to design DNA sequences in which Protein G can be coupled with a protein of interest and as such attachment of this protein to the regarded antibody.

3.2 New developments

In contrast to conventional bioluminescent assay methods, our assay takes place in solution without the need for a solid phase. This could be achieved by choosing two binding proteins that selectively bind to ADL. TNFα and an anti-AT antibody fulfill these criteria, and thus were chosen to be used in our assay. The use of these high affinity proteins ($K_{D,\ WT\text{-}TNF\alpha}$ = 0.11 nM [11], $K_{D,\ anti\text{-}AT}$ = 67 nM) also result in a very fast two-step binding where incubation times in the order of minutes are possible.

We have also developed unique sensor proteins by conjugating NanoBiT protein fragments to TNFα and the anti-AT antibody, meaning that these capture molecules are also serving as reporters in our assay. This in contrast to all available bioluminescent ADL assays, as TNFα is used exclusively to isolate ADL from other proteins in the solution [1, 2, 3], while none of these assays have incorporated the use of similar anti-AT antibody strategies.
We designed a DNA sequence for our TNFα-SmallBiT construct, which was inserted into a pET28a vector and used to express the protein in *E. coli*. The DNA sequence of TNFα was taken from Hoffmann et al., where they also used a N-terminal His-SUMO-tag to obtain a high yield of the properly folded protein [12]. As such, we have attached SmallBiT to the C-terminus of the protein, such that TNFα is still able to form its native trimeric complex without any steric hindrance of the fragment. Moreover, it was also important to provide sufficient steric freedom to both fragments such that the NanoBiT complex was able to form. This was achieved by adding a semi-flexible glycine-serine linker between the SmallBiT and TNFα.
Conjugation of LargeBiT to the anti-AT antibody was done using LASIC-technology. For this, a plasmid had already been designed containing LargeBiT attached to Protein G. LargeBiT was connected to Protein G through a glycine-serine linker, which again served to provide freedom for the NanoBiT recombination. The conjugation itself was performed by mixing the antibody with two molar equivalents of Protein G-LargeBiT constructs and treating this with UV light, which resulted in stable and active antibody conjugates.

Ratiometric approaches for bioluminescent assays are very attractive as it is possible to correct the reporter signal for variable experimental factors such as pH, temperature and substrate concentration. While the ratiometric approach of Ni et al. is not possible for our assay system since we use two binding proteins, the same effects could still be achieved by adding an independent bioluminescent enzyme that is able to oxidize the same substrate but emits light at a different wavelength. Ongoing research by the group of Maarten Merkx (TU/e) investigated the use of mNeonGreen-NanoLuc construct in ratiometric assays, of which the construct itself was developed by Suzuki et al. [7]. mNeonGreen serves as a BRET acceptor for the Furimazine substrate, that is oxidated by NanoLuc and emits a spectrum with a peak at 517 nm. The intensity of this light is completely independent of the analyte concentration, meaning that there is always a stable green peak present in our luminescence spectrum. This in contrast to the intensity of the light that comes from NanoBiT, which scales as a function of the ADL concentration. The ratio of blue and green light is therefore also solely dependent on the ADL concentration and is constant regardless of the experimental conditions in which the assay is taking place, most notably incubation time.

4. Analytical Performance

4.1 Off-chip performance

The biochemical assay was first tested in an experimental setup using a well plate reader (Tecan M10 Spark). Two ratiometric assays were performed in PBS with 1 mg/ml BSA and 1:5 diluted blood plasma. For a measurement, 10 µL TNFα-SB, anti-AT-LB NL-mNG solution was added to 5 µL of an ADL sample. After an incubation step of 30 minutes, 5 µL of substrate was added to start the measurement. Intensities were measured 5 minutes after adding the substrate. The final concentrations in the solution were 100 nM TNFα-SB, 10 nM anti-AT-LB, 100 pM NL-mNG and 1/6th of an ADL-spiked sample. These off-chip measurements can be seen in Figure 6a and 6b.

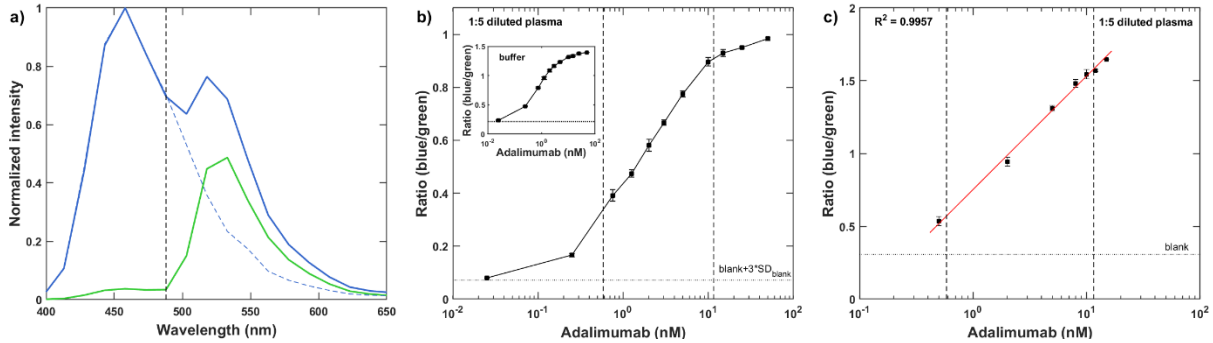

Figure 6: Detection of ADL in PBS buffer and blood plasma. (a) Luminescence spectrum of the assay in the presence of 0 nM (green curve) and 50 nM (blue curve) ADL in 1:5 diluted blood plasma and 100 pM NanoLuc-mNeongreen. The blue dashed line represents the signal of NanoBiT in the same circumstances without the presence of NL-mNG. The black dashed line indicates the wavelength at which the dichroic mirror is placed in the biosensor. Intensities summed before and after this line are used to obtain the ratio of blue and green light. (b) Dose-response curve of the intensity ratio of blue and green light as function of the ADL concentration in 1:5 diluted blood plasma. Ratios were measured 5 minutes after adding the substrate. The inset shows a dose-response curve of the bioluminescent assay in PBS buffer. The dashed lines represent the relevant clinical concentration range (0.5 – 10 µg/ml ≡ 3.5 – 69.4 nM) when diluting the sample 6 times. (c) Detection of ADL in blood plasma on-chip; Dose-response of the intensity ratio of blue and green light as function of the ADL concentration in 1:5 diluted blood plasma. Ratios were measured after 10 minutes of incubation and adding substrate. An exponential equation was used to fit the data. The dashed lines represent the relevant clinical concentration range (0.5 – 10 µg/ml ≡ 3.5 – 69.4 nM) when diluting the sample 6 times.

Figure 6a shows the luminescence spectrum with (blue curve) and without (green curve) 50 nM ADL. It can be seen that without ADL, the spectrum is dominated by the signal of NanoLuc-mNeongreen with a peak around 530 nm. However, it can also be seen that there is a slight signal at 460 nm, due to the residual enzymatic activity of LargeBiT and the NanoLuc that is attached to mNeongreen. After addition of the analyte, the ADL/TNFα/anti-AT complex can form which leads to an increase in NanoBiT formation, significantly increasing the signal at 460 nm. The corresponding luminescence spectrum is the result of summing the intensities of the NanoBiT and NanoLuc-mNeonGreen spectra.

Taking the area under the curve before and after 490 nm will result in the intensities of blue and green light, which can be used to calculate the ratio of blue and green intensities. This can be done for each ADL concentration, resulting in the calibration curve in Figure 6b. It can be seen that there is a distinct shift from green light (low ratio) to blue light (high ratio) with increasing ADL concentrations. The assay also performs very well in both PBS buffer and diluted blood plasma, with a calculated limit of detection of 26 pM (≡ 3.7 ng/ml) in blood plasma.

4.2 On-chip performance

The ratiometric assay on-chip was performed using 100 nM TNFα-SB, 10 nM anti-AT-LB and 30 pM NL-mNG. 21 µL of The solution was added to 7 uL ADL, and 14 uL of substrate. After 10 minutes of incubation the solution was put into the cartridge and immediately after insertion the measurement was performed for 1 minute. The corresponding calibration curve can be seen in Figure 6c.
The output of the photodiode measuring light under 490 nm integrated over 1 minute is divided by the output of the photodiode measuring light above 490 nm integrated over 1 minute, representing the ratio of blue and green intensities. It can be observed yet again that there is a distinct shift from green light (low ratio) to blue light (high ratio) with increasing ADL concentrations, while the device itself is able to perform measurements with high precision and reproducibility. Fitting the data with an exponential equation results in a very reliable fit (R^2 = 0.9957).
We have also observed that a good distinction between ADL concentrations can already be reliably made after only 3 minutes of incubation, leading to a significantly shorter time to result of 4 minutes and 40 seconds.

5. Translation Potential

5.2 Stakeholder Desirability

Adalimumab is an anti-inflammatory drug used in the treatment of rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, Crohn's disease, psoriasis and ulcerative colitis.
In the Netherlands, 250.000 patients are (approximately 77 million patients worldwide) suffering from rheumatoid arthritis and approximately 90.000 people are diagnosed with Crohn's disease (type of Inflammatory Bowel Disease or IBD). Adalimumab is highly effective in the treatment of these diseases. Despite its overall effectiveness adalimumab does not lead to an adequate clinical response in a significant part of the patients [13]. Lack of response may be due to formation of anti-drug-antibodies (ADA), high disease burden, or pharmacodynamic factors such as the (re)activation of inflammation by alternative pathways [14]. Different causes of non-response to anti-TNFα drugs require distinct approaches to treatment optimization.
In current clinical practice, increasing dosage is often the first step in treatment optimization when loss of response occurs, while other options such as adding immunosuppressive co-medication, changing to a different anti-TNFα drug, and changing to a different class of immunosuppressive drug should all be considered. To consider these options, measurement of drug levels, ADA and investigation of pharmacodynamic factors are required [15]. This process takes precious time, resulting in high costs for hospitals and insurance companies, and lowers the quality of life of the patients, as their treatment remains ineffective and diagnostic methods are demanding.
It appears in interviews that all stakeholders involved in the treatment of adalimumab would benefit from ways to make dosage optimization of adalimumab more time and cost-efficient.

Several methods to measure the concentration of adalimumab are currently available. However, these devices are focused on end-users that have expertise in laboratory procedures. None of these devices accommodate patient-use, as they require several steps (mixing, dilution, adding reagents) before a measurement is achieved.

T.E.S.T. strives to deliver a new biosensing technology that will enable point-of-care (at home) measurement of the concentration of adalimumab and its ADA's in serum, allowing for efficient therapeutic drug monitoring (TDM) of adalimumab. This should provide a way to optimize the dosage, in order to achieve and maintain an adequate clinical response in more patients. Also, it should provide a way for medical doctors to lower the dose of adalimumab in a controlled fashion.

The sensor is unique in several ways. Measurement will require only one step (putting a finger prick blood sample on the device), it will take no more than 10 minutes to complete, and the ADL concentration will be accurately measured. The measurement should be performed on a whole blood sample. Clinical studies and cost-efficiency analysis will determine whether TDM will be deployed at the home of the patient and/or at the clinical healthcare facilities.

T.E.S.T. has decided to initially focus on delivering their sensor into two fields: Rheumatology and Gastro-enterology. This strategic choice is elaborated upon in the passage below.

Currently, in the treatment of rheumatoid arthritis, the concentration of adalimumab in the blood is typically not measured throughout the treatment period. In other words, therapeutic drug monitoring is not applied in the treatment of Rheumatoid Arthritis. Patients visit the treating rheumatologist every 3 to 6 months depending on the severity of inflammatory symptoms of the joints. Dosage adjustments occur mostly in early stages of treatment. T.E.S.T. provides a tool which makes therapeutic drug monitoring of rheumatoid arthritis possible. Clinical studies will be required to prove the proposed benefits that are validated in our interviews at MUMC+ and Zuyderland hospital. The staff in these hospitals are willing to cooperate and form partnerships in order to realize this. However, the implementation of TDM in rheumatoid arthritis will take time and until this is successful, no profit is generated.

The strategy of T.E.S.T. is to initially focus on validating the use of this sensor to monitor treatment of inflammatory bowel diseases. TDM in IBD is currently already applied, as the clinical relevance of TDM in these diseases is already established: In IBD the symptoms are difficult to track with standard clinical evaluation [16]. Unlike inflamed joints (as in RA), inflamed bowels do not show on the outside of the patient. Treatment efficacy is therefore often uncertain and adalimumab treatment modality is challenging to optimize without application of invasive methods such as colonoscopies. Gastroenterologists are readily looking for ways to improve current methods of TDM, since the methods that are currently available are lacking (time to result is too long, costs are high). The above-mentioned device, which allows low-invasive rapid TDM of adalimumab, which can be delivered cheaply, can be used without much expertise, and in a point-of-care setting directly at the patient's bedside, will dramatically improve IBD monitoring and care.

5.3 Financial viability

To ensure the financial viability for the solution provided by T.E.S.T. a cost projection was drawn. The manufacturing price of one microfluidic chip is €0,72. The major costs per chip are €0,16 for the anti-TNFα-ADL-antibody (for the detection of ADL), and €0,32 for the ADL (for the detection of ADA). The total costs of the components of the prototype device are €2500. The photodiodes, dichroic mirror, and lenses have been obtained from ThorLabs a research-oriented company and are representing the largest part of these costs. T.E.S.T. should strive for a partnership with an original equipment manufacturer (OEM) of optical and electrical components. For a profitable business model, it is necessary to cut the costs for the biosensor device components to a price of €250, 10% of total costs of the prototype. This can be achieved

by using cheaper hardware components, large scale orders and mass producing the device. Moreover, it is estimated that the labor costs of the device are 10% of the price of the raw production materials. For marketing and distribution another 10% costs per device is estimated. This results in a total production price per device of €300. For of an overview of all the costs, see appendix, table 1.
The device will be leased for free to our customers: the hospitals and private healthcare facilities. These healthcare facilities will pay for each measurement (per microfluidic chip) a fee of €5. This will be our main revenue stream, see appendix table 1. The chosen price per measurement is competitive, a commercial ELISA kit, for example the one from Biovision, costs €10,10 per measurement [22], only providing a measurement for ADL and not ADL and its ADAs.

T.E.S.T. will provide a solution that should lead to a cost reduction of the treatment with ADL. The biosensor device will be used 1) to assist in dose-down policy, 2) which should lead to a volume reduction usage of ADL. The treating physician at a healthcare facility will be responsible for distributing the biosensor device to the patients, who will perform the measurements at their home. The data of the measurements will be sent to the treating physician.
Furthermore, a market analysis is needed to make a sales estimation and determine a business strategy. In 2017, before the expiration of the patent of Abbvie, the main producer of ADL (trade name Humira), the total revenue of ADL in the Netherlands was €252.000.000 to €315.000.000 (based on the number of patients prescribed to ADL and average treatment prices) [17], [18]. The annual revenue of Humira only was €230.000.000 in the Netherlands [17], making Abbvie by far the biggest player on the market. In Europe the revenue of Humira was about $4 billion in 2017.
After the expiration of the patent of ADL (Humira), the price for ADL has been dropped 80-85%, according to insurance companies VGZ, Menzis and CZ. A price reduction of 80%, still leaves an adalimumab market revenue of €46.000.000. According to our interview with the insurance company Zilveren Kruis (Achmea), this still can be considered as quite a large market, which could still be interesting to pursuit.
Only clinical trials can determine how often the adalimumab and ADAs concentrations should be measured in the patient. This therefore determines the number of chips sold and therefore our revenue stream.

For the following sales estimation the assumption is made that the number of measurements (i.e. the number of chips sold) is at least as large as the number of the ADL injections per patient (one average once every two weeks). This would result in 26 measurements per patient per year. This is a potential revenue of €2.673.710 in the Netherlands only, if these measurements are done in all the 20.000 Dutch patients using ADL. 20.000 patients is a high aim, but the market is growing, so in the future this number can be achieved.
As earlier stated, the purchase of our biosensor device should lead to a volume reduction usage of ADL and therefore should lead to a cost reduction for healthcare facilities which use our solution. Therefore, it is important that the expenses for the biosensor device are significantly smaller than the resulting cost reduction for the hospital on treatment with ADL. For this reason, the biosensor device (initially) should be deployed in both the clinic and at home of the patients. It is assumed that all these measurements will be done on 5000 devices (on average 4 patients per device). This would result in an expense of €1.500.000 for the production of the devices.
At least a 5.7% volume reduction usage of ADL is needed to cover for the costs for the chip measurements (at a fee of €5). A 20% volume reduction would save hospitals €6.529.000 on treatment with ADL in the Netherlands only, making the usage of our biosensor device interesting for all parties involved. On top of this financial picture, healthcare costs can be saved by increasing efficiency in consultations, making it even more attractive to implement this solution.
It should be noted that these numbers can change significantly based on clinical trials, only clinical trials can determine 1) how often ADL and ADA's should be measured (i.e. how much microfluidic chips are sold) 2) the potential cost reduction as a result of more efficient dosing. Therefore it is almost impossible to make a good estimation of the expected revenues before clinical trials.

5.4 Business Feasibility

In 2019 and 2020 development of our biosensor device at the Eindhoven University of Technology should be continued for incorporation of a whole blood separation mechanism and the measurement of ADAs, see the final remarks for how we plan to do this. Moreover, other potential markets should be identified. The NanoBiT technology is a very generic technique, which can be used for the detection of other therapeutic antibodies. It is therefore also very attractive to consider TDM for other anti-TNFα antibodies using our biosensor, thereby creating the option to pursue a greater market after successful trials with ADL. For this reason, our assay principle has been put into a provisional patent.
As for partnerships, OEM manufacturer have been selected as the provider of our optical and electrical components. Bio-Rad and Gentaur can provide the necessary capture molecules (anti-AT and ADL respectively) for our cartridge.

From an academic point of view, it is interesting to research how TDM can be deployed to improve therapy with ADL in RA, IBD and other auto-inflammatory diseases. The solution of T.E.S.T. provides a tool which makes it possible to investigate this. MUMC+ and Zuyderland hospital are (potentially) willing to set-up these trials and can potentially (partly) cover the initial research costs.

At the end 2021, clinical trials for ADL for IBD and RA should be finished successfully. After this the markets of the Netherlands, Belgium and Germany are considered as the first to penetrate. Eventually, the American market is financially the most interesting, as the bulk of AbbVie's revenue of $18.4 billion (in 2017) is made in the U.S. Until 2023, Abbvie still holds a patent in the U.S. After the patent expiries for ADL in the U.S., it could be mutually beneficial for both a POC producent like us and a producent of (a biosimilar of) ADL to collaborate to preserve or even improve their market position. Furthermore, on 16 august 2019 AbbVie's successor of Humira, Rinvoq a JAK inhibitor was approved by the FDA [19]. This new drug possibly tops its anti-TNFα predecessor in terms of efficacy, and therefore changes the overall market forecast.

5.1 Business model canvas

<table>
<tr><th>Problem</th><th>Solution</th><th>Unique value proposition</th><th>Unfair Advantage</th><th>Customer segments</th></tr>
<tr><td rowspan="3">1.Adalimumab therapy is expensive and ways to cut down on the treatment modality are lacking.
2. Adequate clinical response with adalimumab is not achieved in a significant part of patients</td><td rowspan="2">1. Point-of-care (POC) biosensor for measuring the concentration of adalimumab.</td><td rowspan="3">Small, easy to use, fast testing time and accurate

Point-of care

At patient's home, making therapeutic drug monitoring (TDM) possible</td><td>Student Dream Team

Professional knowledge

Access to knowledge from multiple departments at TU/e</td><td rowspan="3">1.Hospitals
2.Health Insurance companies
3.Manufacturers of adalimumab/biosimilars
4.Medical doctors
5.Patients
6.Nurse practitioner</td></tr>
<tr><td>Key Metrics</td><td>Channels</td></tr>
<tr><td>Existing alternatives</td><td>At least one hospital wants to preform trials with the usage of our biosensor

Contact at least 5 other hospitals who are willing to use and help in the further development of the biosensor</td><td>Conventions (network events)

1ste degree contest with doctors, companies

LinkedIn, website other social media,</td></tr>
<tr><td>All require expertise in lab work. None accommodate patient-use, they require several steps (mixing, dilution, adding reagents)</td><td></td><td></td><td></td></tr>
<tr><td colspan="2">Cost Structure</td><td colspan="3">Revenue streams</td></tr>
<tr><td colspan="2">Fixed: Employee, Housing, Chemical licensing, Lab maintenance
Variable: device, chemicals needed for testing</td><td colspan="3">Small size device for homecare
Investigate/ talk to target customer
Investigate revenue models of similar companies
Data collection for sales</td></tr>
</table>

6. Team and Support

6.1 Contributions of the team members

For developing the sensor, all team members have been divided over various subteams (assay design subteam 'AD', detection principle subteam 'DP' and sample handling subteam 'SH') that have worked together intensively.

Max Bossink	Max is a member of AD and responsible for the contact with sponsors.
Glenn Bouwman	Glenn is a member of DP, focusing on experiments in the optical setup.
Charlotte Janssen	Charlotte is the head of DP and focusses on the software and digital setup.
Selina Janssen	Selina is a member of AD and involved with acquiring sponsors.
Tom Konings	Tom is a member of SH and responsible for our translational potential, and he has worked on hardware (3D printed cage)
Esmee Meijerhof	Esmee is a member of DP and focusses on the optical setup. She is also the secretary of entire team.
Rody Mens	Rody is a member of AD as well as vice-chairman of the entire team.
Roy Nijhuis	Roy is a member of DP, focusing on the software and digital setup.
Thomas Romagnoli	Thomas is a member of AD as well as chairman of the entire team.
Jeannot Stevens	Jeannot is the head of SH and focusses on the cartridges.
Chris Vu	Chris is the head of AD, focusing on the biochemical assay.
Blijke Wessels	Blijke is a member of SH and treasurer of the entire team.
Charlotte Wilbers	Charlotte is a member of AD, focusing on the biochemical assay.

7.2 People who have given support

Throughout the year, T.E.S.T. has received support from many people. The people that have contributed the most are listed below.

Leo van IJzendoorn	Main supervisor of the entire team.
Rafiq Lubken	Supervisor of the entire team.
Claudia Schot	Supervisor of the entire team.

Maarten Merkx	Providing crucial concepts and advice on the biochemical assay.
Yan Ni	Supervisor of the assay design team in the biochemical lab on the TU/e campus.
Bart Timmermans	Providing assistance to the assay design team during lab work.
Sophia Shanko	Supervisor of the sample handling team in the microfluidics lab on the TU/e campus.
Willem Rovers	Providing advice and materials to the detection principle team.
Ivar de Vries	Providing advice about hardware to the detection principle team.

7.3 Sponsors

T.E.S.T. 2019 is very grateful to the sponsors of this year whose support has made this project possible.

Studentenfonds TU/e	Financial aid
Promega	In-kind contribution (Furimazine).
Bio-Rad	In-kind contribution (anti-ADL complex).
ICT	Financial aid.

7. Final Remarks

In 2019 and 2020 development of our biosensor device at the Eindhoven University of Technology could be continued. Now several practical handling steps are required, this should be minimized to one step: a single finger-prick to obtain a single drop of whole blood. For this, several technical developments steps have to be achieved first. A way to meet this one-step test requirement is by drying the materials of the assay (proteins) without requiring washing and/or dilutions steps. For drying the assay materials two factors need to be taken into account; first, it is important that the proteins are not absorbed by the cartridge material. PMMA is hydrophobic material while the proteins are hydrophilic, coating the surface of cartridge can prevent protein absorption. For this DSM's VitroStealthTM coating can be used. Second, denaturation of the proteins must be prevented. Preliminary experiments with VitroStealthTM-coated PMMA well plates have shown that both factors can be reliably achieved with our assay components and that drying in our proteins for the one-step test is indeed very realistic.
Moreover, our current microfluidic chips are only compatible with blood plasma. For a point-of-care biosensor it is necessary to incorporate a blood plasma separation system within the chip. This can be achieved by incorporating a filter in a capillary driven microfluidic device.
An alternative is using paper-based detection devices (µPADs). The µPADs have a plasma separation membrane, and have proved to work for the similar LUMABS technology [20]. The research of µPADs are an active area of investigation of the TU/Eindhoven and its partners.
The next step is incorporating the measurement of anti-drug-antibodies (ADAs) within the same device, where both tests are incorporated in the same chip. ADAs have been identified as one of the causes for primary or secondary non-response. (Early) detection of ADAs will therefore enable specialists to differentiate between different causes of suboptimal response and to adjust the treatment properly [13]. Interviews with hospitals and insurance company Zilveren Kruis (Achmea) both confirmed the added value of measuring both ADL and ADAs in one device.
Research at the TU/e has proven that the same NanoBit technology can be used to measure ADAs of ADL [21]. Utilizing the same LASIC-technology that was used to conjugate NanoBiT fragments to the anti-AT antibody, we can apply the same concept using ADL as a capture molecule to detect ADAs. We have already shown that this very generic concept works for the detection of ADL, which can be seen in Appendix figure A3. Finally, it should be proven that the accuracy of our measurements is similar to commercial ELISA assays.
We would like to end this document by thanking everyone who has supported us during this journey. Leo, Rafiq, Claudia, Yan, Bart and the rest, we could not have done it without you guys. We have learned so much during this project and this has been an unforgettable experience for us.

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9. Appendix

Device costs		Costs per chip	
Photodiodes	€150 *	Furimazine	€ 0,0012
Dichroic mirror + cage system	€40 *	aComplex (detection (ADL)	€ 0,16
Hardware + 3D printed cage	€30 *	ADL (detection ADA)	€ 0,31
		Other	€0,25
Labor costs	€25 **		
Marketing & distribution per device	€25 ***		
Total costs per device	€300	***Total costs per chip***	€0,72
		Sale price per chip	€5,00

Table 1: Overview of production costs and sale price
** Estimated costs are 10% of the total costs of the prototype. **Estimated labor costs of the device are (Europe) 10% of the price of the raw production materials. *** For marketing and distribution another 10% costs per device is estimated (Europe).*

Estimation	Number
Number of patients using ADL in Netherlands	20.567
Number of measurements done per patient per year	26
Production costs per device	€ 300
Patients per device	4
Number of devices (leased for free)	5000
Number of measurements (sold chips)	534742
Revenue sold chips €5 per chip	€ 2.673.710
Total Production costs biosensor device	€ 1.500.000
Profit	€ 1.423.710

Table 2: Sale estimation in the Netherlands

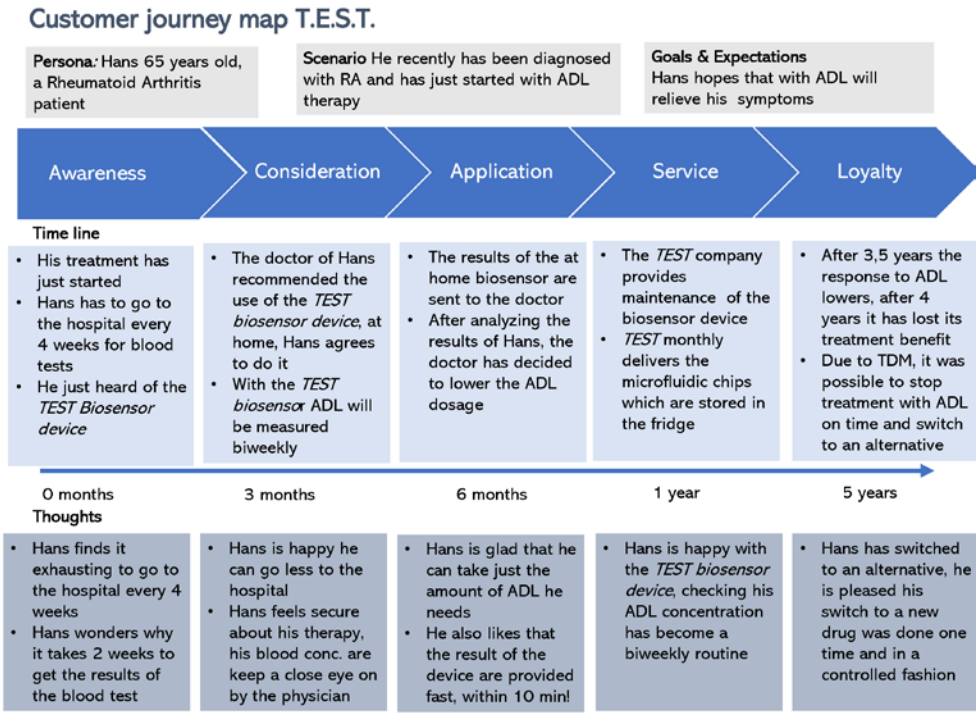

Figure A1; Customer Journey of a patient with Rheumatoid Arthritis (RA). For the IBD patient a comparable value map was created, due to page limitations this figure was not included in this report.

Value Map		Customer profile Map
Product & Services		**Customer Jobs**
• POC biosensor device at home / in clinic, that measures concentrations after addition of whole blood to biosensor • Measurement of ADL concentrations within 5 minutes • Hospital rents POC biosensor device from company, maintenance done by company, every measurement requires a new microfluidic chip (payment per measurement)		• Provide best possible treatment for patients Rheumatoid Arthritis with low risk of side-effects • Maintenance of costs within hospital budget (insurance company gives fixed budget for biologicals) • Avoid long-term effects of rheumatic disease (joint dysfunction and malformation)
Gain Creators		**Gains**
• Measurement of ADL in very short time [minutes], instead of weeks (due to logistics) • Therapeutic drug monitoring (TDM) possible, measurement every 1/ 2 weeks →Possible to lower dosages in a controlled fashion → personalized medicine possible, improvement of treatments patients • The machine is cheap		• Methods to achieve 'dose-down policy' for biologicals as ADL • A measurement of ADL drug level can be done during a policlinic visit. 2 weeks -> 20 minutes. Measurement is more valuable because of this.
Pain relivers		**Pains**
• Measurements of ADL concentration will be a lot quicker, almost direct results [minutes], instead of weeks due to logistics • If POC biosensors are used at home, patient does not have to go to the hospital for blood tests. This makes health care costs lower. • Justifying ADL use and perhaps dosage increase to the insurance companies is easier with a low invasive drug level measurement tool.		• Non-optimal dosage leads to unnecessary side-effects for patients • Non-cost efficient dosages of ADL (only two standard dosages possible) • Patient has to go to hospital for the blood tests • It takes ~ [2 weeks] to get results on ADL and ADA concentrations, *hospital logistics*; blood test at polyclinic, then blood sample to clinical chemistry lab for analysis, results back to physician and patient • Alternatively blood samples are send to companies who preform HTS assays as Sanquin, still results are back within a couple of [days] • Hospitals give fixed budget to doctors for the treatment with biologicals. If doctors exceed this budget, they have to justify this.

Table A3. Value Map & customer profile for the rheumatologist (customer). Blue = 1. most important gain/ pain reliever. Green = 2. Second most important gain/ pain reliever. Red = 1. most important pain. For the Gastroenterologist a comparable value map was created, due to page limitations this table was not included in this report.

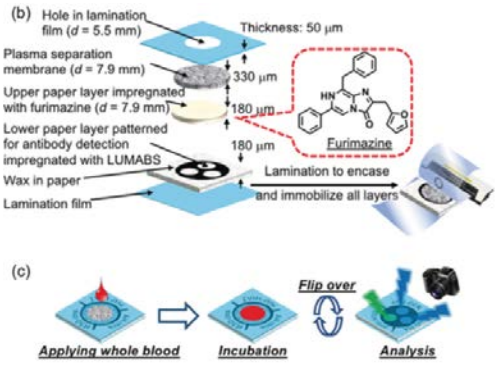

Figure A2. Tenda 2018, paper-based detection devices (µPADs)

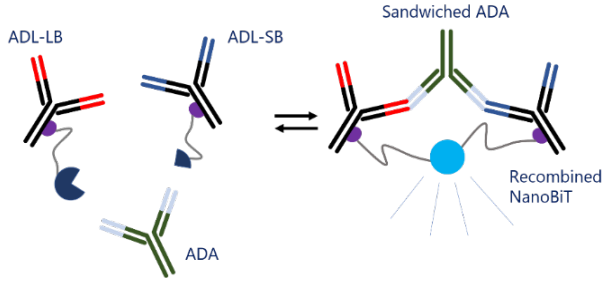

Figure A3, Assay for detection of ADA, ADL as capture molecule coupled to NanoBits.