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master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Genetic modification of T-cell receptors for whole cell biosensor development

Promotor :
Prof. dr. Luc MICHIELS

Tijs Louwies

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

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UNIVERSITEIT VAN DE TOEKOMST

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Preface

During the last 30 weeks, I had the opportunity to complete my internship at the research group Biosensors at the Department Immunology/Biochemistry of Hasselt University. The last 8 months were an eye-opener. I was lucky to work with very driven people, whom I joined in the exciting world of research. All these people collaborated in their own way to my thesis, some more than others, but all deserve my gratitude.

First of all, I would like to thank my promoter Prof. Dr. Luc Michiels for granting me the opportunity to be a temporary member of his research group. Thanks for your insight and advice and the well-appreciated criticism.

If there is one person that I will not easily forget, then it will be my daily supervisor Karolien Deprez. Karolien, thank you for putting up with me, thank you for your patience and forgiveness, it must not have been easy to cope with me and my habits. Thank you to keep me motivated. Thanks for listening to my doubts and frustration, the nonsense and relevant things. I would like to thank you even more for all the things you have taught me, your guidance and support in and out the lab.

Furthermore I would like to thank all the people who offered a little of their time to help me. I cannot list you all, but know that I owe you my gratitude. Thank you for the details that made the difference!

There is a special group of people I would like to thank: my fellow students Lise, Sohie, Winde and Petra. I will forget most the small talk, but I will not forget the impression that you left on me. Thanks for the help with my research and thesis, also thanks for the amusing discussions

Last, but not least, my gratitude goes to my parents. Thanks for all the opportunities, support and advice you have given me. Thank you for all the things I am forgetting now. Thank you for being.

Tijs Louwies; June 13th, 2011.

List of abbreviations

cTCR	chimeric T-cell Receptor
PSA	Prostate Specific Antigen
CD28	Cluster of Differentiation 28
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
IL-2	Interleukin-2
GFP	Green Fluorescent Protein
scFv	Single-chain variable fragment
APC	Antigen Presenting Cell
MHC	Major Histocompatibility Complex
IFN	Interferon
PCR	Polymerase Chain Reaction
PBMC	Peripheral Blood Mononuclear Cell

Abstract

Allergens, toxins and disease markers are difficult to detect in a sample when present in low concentrations. Nevertheless, these molecules can exert their effect. It would be beneficial to detect these substances before harm is done. Nowadays, most affinity sensors cannot detect these low concentrations. This research topic focuses on the development of a novel kind of whole cell biosensor in which a chimeric T-cell receptor is used for detection of PSA. It is expected that via the use of a cTCR, lower concentrated substances can be detected. cTCR are fusions of extracellular antigen binding domains (derived from antibodies) and intracellular signal transduction domains (derived from activation or stimulatory T-cell receptors). The cTCR is constructed as a receptor gene cassette. The genes for the single chain antibody against PSA, CD28 and CD3ζ are flanked with restriction sites.

Development of a functional biosensor requires three major steps. First, this cassette structure will be introduced in a lentivirus. The lentivirus will be used to infect Jurkat T-cells that do not express a functional T-cell receptor. Second, infected Jurkat cells will be tested for the expression of the cTCR. Third, a functional cTCR will, upon antigen encounter and binding, activate the Jurkat cell via signal transduction pathways started through CD3ζ and CD28. Activated Jurkat cells will start producing IL-2 which this will be measured.

The principle of our new whole cell biosensor is that the presence of an antigen will not be measured directly, but through the secretion of a reporter protein (in the case of the Jurkat cells IL-2). This is the strength of our whole cell biosensor; measurement of a higher concentrated reporter protein will indicate the presence of the lower concentrated antigen. cTCR are able to detect lower concentrated antigens via its specificity and affinity for the antigen.

Due to encountered problems with the construction of the lentivirus, expression and functionality of the cTCR was not addressed. The cTCR, when cloned in the pLenti6/V5 TOPO vector, proved to be lethal to the Stbl3 E.coli bacterial strain that had to be used to produce the vector needed for the assembly of the lentivirus. The receptor cassette structure had to be modified before the lentivirus could be constructed. A leader sequence was introduced in the cTCR-construct. The leader sequence ensured bacterial survival. After modification of the cTCR-construct, the lentivirus will be constructed.

Samenvatting

Allergenen, toxines of ziektemerkers zijn, indien aanwezig in een zeer lage concentratie, zeer moeilijk te detecteren in stalen. Desondanks zijn deze moleculen in staat hun effect uit te oefenen, daarom zou het voordelig zijn deze te ontdekken voordat er schade wordt aangericht. De meeste conventionele affiniteitsensoren zijn niet in staat deze concentraties te detecteren. Dit onderzoek spitst zich toe op de ontwikkeling van een nieuw soort cellulaire biosensor, waarin een chimere T-cel receptor gebruikt zal worden om PSA te detecteren. Er wordt gesteld dat het via de cTCR mogelijk wordt om laag-geconcentreerde antigenen te detecteren.

cTCR zijn fusies van extracellulaire antigenbindingsdomeinen (afgeleid van antilichamen) en intracellulaire signaaltransductie domeinen (afgeleid van activatie- of stimulatie TCR). Het cTCR-construct is opgebouwd als een receptor gen cassette. De genen voor het single chain antilichaam tegen PSA, CD28 en CD3ζ worden geflankeerd door restrictiesites. Ontwikkeling van een functionele biosensor vereist drie grote stappen. Ten eerste wordt de cassette structuur geïntroduceerd in een lentivirus. Dit lentivirus zal gebruikt worden om Jurkat T-cellen te infecteren. Ten tweede zullen geïnfecteerde Jurkat-cellen getest worden op de expressie van de cTCR. Ten derde zal de functionaliteit getest worden; een functionele cTCR zal indien er een antigen gebonden wordt, de Jurkat-cel activeren via signaaltransductiepaden opgestart door CD3ζ en CD28. Geactiveerde Jurkat-cellen zullen IL-2 produceren.

De aanwezigheid van het antigen zal niet rechtstreeks gemeten worden, maar door de secretie van een reporterproteïne (in dit geval IL-2). Hierin ligt de kracht van deze biosensor, de detectie van een hoger geconcentreerd reporterproteïne wijst op de aanwezigheid van een lager geconcentreerd antigen. Een cTCR kan laaggeconcentreerde antigenen detecteren dankzij zijn specificiteit en affiniteit voor het antigen.

Wegens onvoorziene obstakels tijdens het maken van het lentivirus, is de expressie en functionaliteit van de cTCR niet onderzocht kunnen worden. De cTCR, gekloond in de pLenti6/V5 TOPO vector bleek toxisch te zijn voor de bacteriën die deze vector moesten produceren voor de assemblage van het lentivirus. De receptor cassette structuur moest aangepast worden opdat het lentivirus alsnog gemaakt kon worden. Een leader-sequentie werd aan het cTCR-construct gehecht waardoor de bacteriën wel overleefden. Na aanpassingen aan het cTCR-construct, zal het lentivirus gemaakt kunnen worden.

1 Introduction

1.1 Biosensors: an introduction

1.1.1 Biosensor shortcomings

Molecules present in low concentrations (allergens, toxins, disease markers) are difficult to detect in a sample, but still they can exert effects or indicate (early stages of) diseases. It would be beneficial to trace these substances before they can cause harm. Biosensors can be used as diagnostic tools by making use of biological components to trace targets in a sample (1). These biological components (enzymes, antibodies, subcellular components or microorganisms) are used for their specificity and sensitivity in order to detect small amounts of chemicals or to report physiological or biochemical characteristics of a sample (1). An important problem of most biosensors lies with the practical detection limit. For instance, the detection limit of the commercially available glucose sensors Precision QID Meter from Exactech ranges between 20 and 600mg glucose per dl blood. This is a good practical detection limit for diabetics, but the working principle of these glucose sensors cannot be used for other sensors which require lower detection limits. For instance, some allergens provoke a reaction when present in xx - xx nM-range. The detection limit for these allergen targeted biosensors remains too high, therefore low concentrations cannot be detected. We aim to construct a novel type of whole cell biosensor in which a chimeric T-cell receptor (cTCR) is used for antigen binding and the cell for amplification of the signal generated by the binding of the antigen to the cTCR. This way, due to the high specificity and sensitivity of cTCR, specific low concentrated antigens can be captured in a sample. Antigen binding on the cTCR will result in the production of a reporter protein. This protein production will reach a higher concentration than the antigen, and therefore it will be easier to detect. Instead of direct antigen detection, the protein will be detected and thus antigen presence will be visualized.

Historically Clarck and Lyons demonstrated the modern concept of the biosensor by integrating an enzyme into an electrode. This gave a rapid, accurate and simple biosensor for glucose. The modern concept gave rise to a still expanding field of instruments to determine concentrations of substances or other parameters of biological interests (2-5). Nowadays, biosensors consist of a detection platform, a transducer and an electronic or optical reporter. These biosensors have their advantages and drawbacks. The main advantages are: the low costs and the simplicity to operate. Most types of biosensors perform better than the conventional analytical instruments (for instance, gas chromatography) (6-10). Although the

main advantages seem convincing, biosensors also suffer limitations. In comparison with sensors made entirely out of polymers, biosensors often have a limited time span, due to their decreasing stability in time. Another problem is stability of the immobilization techniques, used to immobilize the antibodies or enzymes on the detection platform. Antibodies and enzymes need to be directed in a specific direction for their active site to interact properly with the antigen to give the optimal result. Incorrect immobilization biases the sensitivity of the sensor and can lead to reliability problems (11-13).

1.1.2 Whole cell biosensors

Whole cell biosensors are a good alternative to the problem of immobilization because cells do not necessarily need to be immobilized. Whole cell biosensors can be (engineered) microorganisms or cultured cells. The working principle of whole cell biosensors does not differ from standard cellular responses (see figure 1). An antigen can bind to a (engineered) cellular (membrane) receptor. This interaction starts a signal transduction cascade, which eventually leads to the synthesis of proteins.

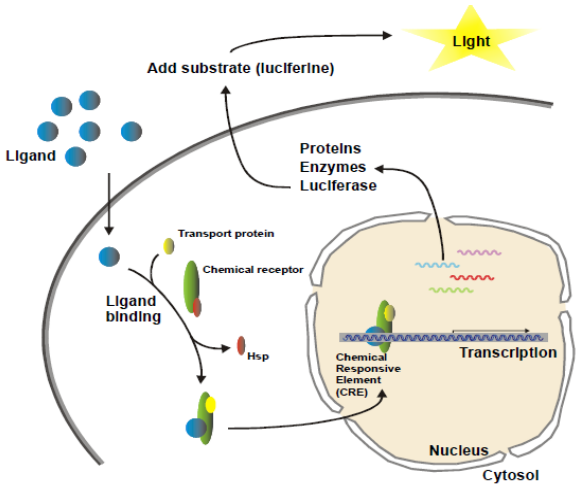

Figure 1 Working principle of a whole cell biosensor, antigen encounter leads to protein production that can be measured instead of the antigen concentration itself

The protein production can be usefully employed as a parameter to obtain the desired information (14). In order to do so, cells can be genetically engineered to respond to the presence of certain chemicals or physiological stress and synthesize reporter genes (for instance luciferase, beta-galactosidase, GFP) to visualize antigen presence (1, 14). In this way, a cellular biosensing system is created: specific stimuli regulate gene expression which is monitored with specific protein production.

Nowadays, most whole cell biosensors are (genetically altered) bacterial cells. We propose a novel whole cell biosensor, in which a higher eukaryotic cell will be used to amplify the signal generated by target detection. Although this is the same principle as a bacterial cell, working with eukaryotic cells has several advantages. Bacterial sensors are mainly used as toxicity monitors because several bacterial strains can cope with toxins. Minor genetic alterations, like the introduction of a reporter system, turn the cell into a biosensor. The

drawback is that if a bacterial strain does not possess the toxin resistance gene, it cannot be used to monitor that toxin (1).

Eukaryotic cells on the other hand can be genetically changed to react to a target of choice (14). Eukaryotic cells possess numerous receptor molecules, a result from evolution. Certain membrane receptors in particular are valuable candidates for modification. Genetic altering of membrane receptors will change the recognition machinery of eukaryotic cells. A possible candidate is the T-cell receptor. In this project, we will use a cTCR, which is target specific, but target specificity can be altered easily if needed. A cTCR for target detection will function better in eukaryotic cells. Bacterial cells lack the signal transduction machinery needed for the cTCR to give rise to protein expression, whereas higher eukaryotic cells possess all these molecules needed for successful signal cascades. The use of cTCR enables us to reach lower detection limits than bacterial sensors can achieve. Most bacterial sensors can only detect bioavailable concentrations up to 0,1µM (15). A eukaryotic cell with a cTCR will be able to register even lower concentrations, up to 2,5µg/ml (16). This is due to the fact that the eukaryotic cell functions as an amplifier of the signal generated by the cTCR: few ligand-cTCR interactions can be able to evoke a (measurable) cellular response. The cTCR uses the signal transduction machinery of the cell which will amplify the generated signal.

In summary, we suggest a whole cell biosensor in which a chimeric T-cell receptor (cTCR) will serve for antigen recognition. Activation of the cellular component of the biosensor will become dependent on the interaction between the cTCR and the antigen. This new whole cell biosensor combines the best of several worlds. The cTCR, which can be regarded as an affinity sensor which responds only to specific target molecules, in combination with a cell for signal amplification will result in a highly specific and very sensitive biosensor, adaptable to a wide range of targets.

1.2 Chimeric T-cell receptors: a brief history

The idea of a cTCR originates from structural studies that compared differences in specificity between T-cell receptors and antibodies. These differences are not evident from the differences in their molecular structures. Both molecules consist of two disulfide-linked polypeptide chains, with constant and variable domains. The variable domains of the TCR are called the α- and β-chains, while an antibody has a light and heavy chain. The variable

domains form the antigen binding parts and thus determine the antigen specificity. From a structural point of view the resemblance of the TCR and immunoglobulins lead to the idea to confer antibody specificity on T-cells by replacing variable TCR-domains with variable antibody domains (17).

cTCRs have mainly been used in cancer research. Scientists were looking for a way to direct T-cells against tumor antigens and tumor masses. Activation of these T-cells would lead to a T-cell mediated destruction of the tumor (18). T-cell activation requires signals from the TCR and costimulatory receptors. Because normal TCR do not recognize most tumor antigens, the TCR does not provide the signals needed for T-cell activation. Therefore it was a challenge to make the T-cell able to recognize tumor antigens via its TCR. The encountered challenge was that T-cells, with the normal TCR, are dependent on antigen presenting cells (APC) for their activation for at least two reasons. First, the antigen needs to be presented on a Major Histocompatibility Complex-molecule (MHC), expressed on APC. Most tumor antigens will not be displayed by APC's because MHC-molecules do not recognize tumor antigens (19, 20). Second, T-cells need costimulation from APC's and tumor cells to become fully activated. Tumor cells lack costimulatory molecules and thus evade T-cell mediated killing (21, 22). Despite these limits, the normal TCR held promise of a novel range of applications. Chimeric TCR's were developed to overcome the restrictions of the normal TCR (23).

cTCR's are fusions between antigen recognizing ectodomains and signaling endodomains (see figure 2). Eshhar et al. was one of the first to propose an alternative TCR. Based on the idea of structural similarities Esshar et al. tried to overcome the limitations by introducing new components in the TCR (17). The first problem Eshhar et al. needed to tackle was the antigen specificity of the receptor. Eshhar et al. constructed a TCR in which the antigen recognition unit of the TCR was replaced by a single chain variable fragment (scFv) of an antibody. A scFv-domain is a combination of the specific binding residues of the light and heavy chain of a monoclonal antibody. The chains

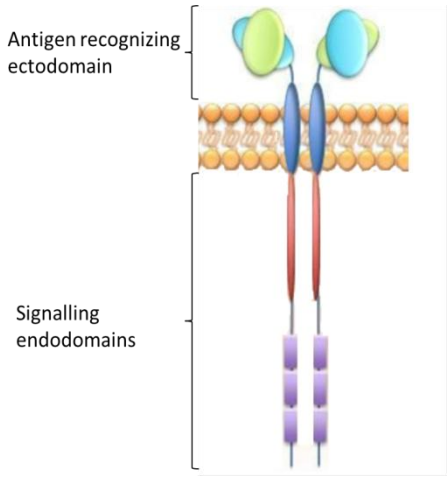

Figure 2 Schematic overview of a cTCR. The antigen recognizing ectodomain of the cTCR is derived from an antibody, whereas the signalling endodomains are derived from activation or stimulatory T-cel receptors.

are linked with a flexible linker region, which preserves the antigen binding domain. scFv-domains have proven to exhibit the same specificity and affinity for antigens as the natural antibody (24). Replacing the alpha- and beta-chains of the TCR by the heavy and light chain/the scFv-domain yielded a new type of TCR, the scFv-receptor, which enabled activation of the T-cell without antigen presentation by other cells. scFv-fragments were genetically attached to the cytoplasmic domain of the TCR ζ –chain. Z-chains are regarded as the common signal transducing subunits of TCRs. Upon ligand engagement, the T-cell was stimulated by this ζ-chain (18, 24). The generated signal can initiate tumor target cell killing and promotes IFNγ release (25). Given the fact that numerous antibodies, and their corresponding scFv-domains exist, a whole range of new scFv-receptors was designed to target T-cells against specific antigens.

Numerous studies relied on this principle, but the other limitation of the TCR was not overcome: cTCR made use of only a restricted subset of the TCR signal transduction machinery. Because there were no costimulatory molecules involved, the costimulatory signals needed for proper T-cell activation remained absent. The fact that no MHC-molecules were involved in cTCR-signaling meant that no CD4 or CD8 coreceptors or other stimulatory receptors were recruited. These receptors enhance the T-cell sensitivity for their antigens (26, 27). The cTCRs were only able to produce weak activation signals when antigens were encountered. These signals were normally amplified by costimulatory molecules that were absent on cTCRs. In order to provide a sufficient amount of costimulation to reach the activation threshold, Esshar et al. linked (co)stimulatory domains to the scFv-receptor (24, 28). The resulting cTCR could recognize a chosen antigen and produce an activation signal through the antigen-binding-induced-activation of the incorporated (co)stimulatory domains. In this way, costimulation provided by other cells was not necessary anymore. The signal would be established when a specific tumor antigen binds on the antigen recognition domain. The resulting activation of the T-cell leads to the destruction of tumor cells (29).

1.3 Chimeric T-cell receptors and biosensors

The development of the cTCR created new opportunities for new cancer treatment therapies, but its usefulness can be extended to other research areas. During this project we aim to construct a novel whole cell biosensor. The biosensor will be used to detect the presence of low concentration antigens in samples. For this detection, a cTCR will be used. Finney et al.

developed a functional cTCR. They proposed and proved that the combination of P67scFv (the extracellular antigen recognizing domain), an h.28 spacer, CD28 and CD3ζ (both intracellular (co)stimulatory domains (30, 31)) yielded a functional cTCR in terms of signal transduction and activation of T-cells (16).

CD3ζ is regarded as an activation domain from T-cells. Nevertheless, this domain alone fails to fully activate T-cells. This is due to impaired signal transduction because important TCR proximal kinases are not recruited to the cTCR. CD28 is a molecule with costimulatory signaling capacity in T-cells. CD28 is responsible for the activation of naive T-cells and rescues T-cells from apoptosis (32, 33). The short intracellular domains can initiate signal transduction cascades which are distinct from primary signals delivered by TCRs (33). Finney et al. proved that constructs with CD28 and the ζ-chain distal to the cell membrane produces more IL-2 than constructs with only the ζ-chain. Our biosensor will not be directed against P67, instead a scFv-fragment derived from the antibody against PSA will be used. The cTCR will be expressed in J.RT3-T3.5 Jurkat cells. These cells exhibit the function to efficiently express cTCR constructs (16).

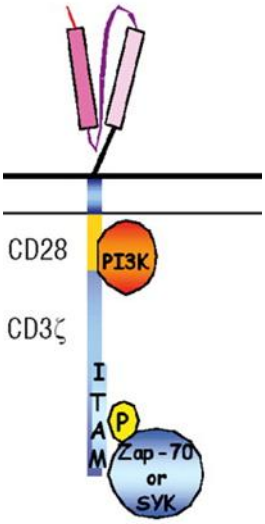

Figure 3 A cTCR combines different cellular pathways in one receptor molecule. Both activation and stimulatory signal can be generated if the antigen is encountered. These signals will establish a signal transduction cascade in the T-cell that will eventually lead to the production of IL-2.

The biosensor cell serves to amplify the signal that comes from antigen binding on the cTCR. An antigen encounter will activate the signal properties of CD3ζ and CD28. The generated signal transduction cascade will lead to the activation of the biosensor cell. This process of antigen mediated stimulation of the cell will be visualized with a reporter system and the consequent synthesis of the specific reporter protein will be measured. In this lies yet another strength of the biosensor: the concentration of the produced protein will be higher than the concentration of the antigen that was detected. This amplification allows to measure antigens that are present in samples at very low concentrations. A whole cell biosensor with a cTCR, as described above, will be able to detect these molecules and thus will function as a valuable diagnostic tool.

1.4 Experimental outline

In order to develop a new, functional whole cell biosensor, we expect that a cTCR proves to be a valuable membrane receptor to bind specific antigens. This project focuses on three major objectives. First, the constructed cTCR gene cassette will be introduced in the J.RT3-T3.5 Jurkat cell line. Second, after successful transfection, the expression of the introduced cTCR gene will be addressed. Third, the expressed gene will be tested for its functionality.

The cTCR gene cassette has already been created and is composed of the PSA-antibody derived scFv-domain, an h.28 spacer region and two signal domains CD28 and CD3ζ. All these domains are linked through specific restriction sites in one long gene (see figure 4 or supplemented figure 1). The gene cassette is incorporated in the pCR2.1 TOPO vector, a plasmid that is stored in TOP10 E.coli cells.

In first instance the whole cell biosensor will be based on J.RT3-T3.5 Jurkat cells. Because this cell line does not express a functional cTCR, these cells are well suited to study the effect of the introduced cTCR. In order to transfect Jurkat cells, a lentivirus will be developed. This guarantees a stable, genomic expression of the cTCR-gene in the Jurkat cells.

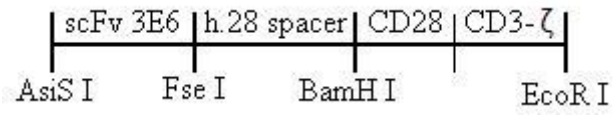

Figure 4 Schematic representation of the gene cassette of the cTCR that will be used in order to develop a functional whole cell biosensor. Note that every domain is flanked by restriction sites which have no effect on the final conformation of the cTCR.

The lentiviral transfection will introduce the cTCR-gene in the genome. Specific promoter sequences, delivered from the lentivirus, can initiate cTCR gene expression without the need for additional factors. The expression of the gene will be tested at several levels. Firstly, reverse transcription will be performed on the mRNA of J.RT3-T3.5 Jurkat cells to ensure that the cTCR-gene is expressed. Cells that express the cTCR-gene are further studied. Secondly, the cTCR's location in the cell will be investigated. For the biosensor to work, the cTCR must be located in the cell membrane. Therefore, transfected J.RT3-T3.5 Jurkat cells will be analyzed with flow cytometry. Fluorescent labeled PSA, which will bind the cTCR, will be used to identify the presence of the cTCR in the cell membrane. In both cases non-transfected J.RT3-T3.5 Jurkat cells will be used as control cells.

T-cells start secreting IL-2 when activated. If the membrane cTCR is functional, the J.RT3-T3.5 Jurkat cells will start producing IL-2 upon stimulation with the antigen. To assess IL-2 production a cytokine assay (ELISA) will be performed. J.RT3-T3.5 Jurkat cells will be evaluated in three different ways. First, J.RT3-T3.5 Jurkat cells are derived from T-cell leukaemia, these cells are in a naturally activated state. To assess the effect of this naturally occurring activation, non-stimulated cells will be assessed for their IL-2 production. Second, another Jurkat cell line, the Jurkat clone E6-1 will be stimulated with anti-CD3 and anti-CD28. The Jurkat E6-1 harbors the same features as the J.RT3-T3.5 Jurkat cells, except for the fact that this cell line does express a TCR. Therefore the cells can be stimulated with anti-CD3. J.RT3-T3.5 Jurkat cells would not respond to anti-CD3 because the lack of the TCR-complex. These antibodies will activate CD3 and CD28 signaling pathways in Jurkat clone E6-1 cells, the activated signal pathway is the same as our cTCR will activate. In this way, important data will be collected on what might be expected when the cTCR will be used. The data collected from the different Jurkat cell lines will be compared. If the cTCR is functional, antigen stimulation of the J.RT3-T3.5 Jurkat cells harboring the cTCR will produce the same results as the anti-CD3/antiCD-28 stimulation of the Jurkat E6-1 cells. Third, T-cells from peripheral blood mononuclear cells will be stimulated with anti-CD3 and anti-CD28. IL-2 production will be determined and compared to stimulated Jurkat E6-1 cells. This population acts as a positive control to evaluate the effect of the CD3/CD28 stimulation. Fourth, when the cTCR-gene is introduced in the J.RT3-T3.5 Jurkat cells, PSA will be presented in solution. If the cTCR is functional, IL-2 will be produced. Stimulation will occur through antigen and non-specific antigen stimulation. Non-specific antigen stimulation will act as a control to determine the specificity of the cTCR.

Once the cTCR proves to react to the specific antigens, the lowest concentration that evokes a response from the cell will be determined. The antigen concentration will be lowered in progressing steps to determine the minimal concentration at which the biosensor is functional. To address functionality, IL-2 production will be determined.

The possibility remains that (lower concentrations of) dissolved antigens cannot provoke a (higher) production of IL-2. This can be due to the low concentration which limits antigen binding or the signal generated by the cTCR is insufficient. If so, the antigens will be precipitated and thus concentrated. Bound antigens will bind more easily on cTCR and should evoke a larger IL-2 response (16).

If the signal derived from ligand-receptor interaction, is too low, the sensitivity of the cTCR must be altered. A feeble or undetectable signal can be generated due to: too low antigen concentrations or too weak signal properties of the intracellular components of the cTCR. If this happens, the receptor cassette will be optimized by use of additional or alternative signaling components.

2 Materials and methods

2.1 Cell Lines

The human cell lines HTB-22 (MCF-7, an adenocarcinoma of the mammary gland), TIB-152 (Clone E6-1, derived from an acute T-cell leukaemia) and TIB-153 (J.RT3-T3.5, derived from an acute T-cell leukaemia), CCL-121 (HT1080, derived from a sarcoma cell line) were purchased from American Type Culture Collection and cultured in RPMI (GIBCO, Merelbeke, Belgium) supplemented with: 10% FCS (Invitrogen, Merelbeke, Belgium), 0,1mM Non-Essential Amino Acids (GIBCO), 1mM sodium pyruvate (GIBCO) and 1% penicillin/streptomycin (GIBCO). Cells were incubated at 37°C and 5% CO2.

The human cell line 293FT was obtained from Invitrogen-Life Sciences (Invitrogen, Merelbeke, Belgium) and cultured in DMEM supplemented with: 2% L-glutamine (GIBCO) and, 10% FCS, 0,1mM Non-Essential Amino Acids (GIBCO), 1mM sodium pyruvate (GIBCO) and 1% penicillin/streptomycin (GIBCO). Cells were incubated at 37°C and 5% CO_2. Subculturing of the cells was done by using trypsin to loosen the cells from the flasks.

2.2 Construction of the lentivirus

The cTCR was generated using splice-overlap PCR. The construct was cloned into the pCR2.1 TOPO vector (Invitrogen) and stored in TOP10 E.coli cells (Invitrogen). The sequence of the construct was controlled by primer extension sequencing reactions. The lentivirus was constructed with the Vira Power Lentiviral kit (Invitrogen). The lentivirus was constructed according to the protocols provided with the kit. Miniprep was performed on the colonies which contained a mutation free cTCR-construct. PCR with Hotstar Hifidelity Polymerase (Qiagen) was performed on the isolated plasmids, using 001cTCR and 014cTCR primers, generating the desired cTCR-PCR product. PCR products were TOPO cloned into the pLenti6/V5-D-TOPO vector. Stbl3 Chemically Competent E.coli (Invitrogen) were transformed with the vector using heat shock. Transformants were analysed by restriction digest, primer extension sequencing and PCR analysis.

2.3 Sequencing of the cTCR construct

The chimeric construct was stored in the pCR2.1 TOPO vector in TOP10 E.coli cells or in the pLenti6/V5 TOPO vector in Stbl3 E.coli cells. Colonies were controlled by primer extension sequencing, in order to choose the colonies that carried a mutation free construct. Both external (M13 forward and reverse primers, CMV forward and V5 reverse primers) and internal primers (001cTCR, 002cTCR, 006cTCR and 014cTCR) were used. Primers were purchased from Eurogentec (Seraing, Belgium). For each sample 2µl Big Bye buffer (Applied Biosystems, Halle, Begium), 1µl Big Dye sequence mix (Applied Biosystems) and 1 µl of each primer was used. Every sample contained 50 to 100ng of plasmid DNA. Water was added to a total volume of 10µl. The sequencing reaction was performed in a thermal cycler MyCycler (BioRad, Nazareth Eke, Belgium). A hotstart of 96°C was performed, the initial denaturation lasted 1', 25 cycles were run in which each cycle had a 10" 96°C denaturation step, a 5'' annealing step (see table 1 for annealing temperatures) and a 4' 72°C elongation step. Afterwards the PCR-products were held on 4°C. Sequences were analysed by a Abi Prism 310 Genetic Analyser (Applied Biosystems).

Primer	Sequence	$T_{annealing}$ (°C)
001cTCR	5'-CACCATGCGCGATCGCACCCAGTCTCCAGCCTCCCTA-3'	62
005cTCR	5'-ACGGCCGGCCACAAAACTCACAC-3'	62
006cTCR	5'-TTGGGATCCAGGGGCTTAGAAGGTCCCGGAAATAG-3'	62
014cTCR	5'-TATGAATTCTTAGCGAGGGGGCAGGGCCTGCATG-3'	62
M13-forward	5´-GTAAAACGACGGCCAG-3´	55
M13-reverse	5´-CAGGAAACAGCTATGAC-3´	55
CMV-forward	5'-CGCAAATGGGCGGTAGGCGTG-3'	56
V5-reverse	5'-ACCGAGGAGAGGGTTAGGGAT-3'	56
Leader	5'-CACCATGGGATGGAGCTGTA-3'	51
002cTCRcorrect	5'-TGAGGAGACGGCCGGCCGTGGTCCCTTGGCCCC-3'	51

Table 1 Primers used during sequencing and PCR reactions. All cTCR primers are primers that anneal on their complementary sequences of the cTCR-construct. M13-primers anneal on the pCR2.1 TOPO vector, whereas CMV/forward and V5-reverse anneal on the pLenti6-V5 TOPO expression vector. Only the leader primers anneals on the leader sequence.

2.4 Restriction digest

The rearrangements of the LTR-regions of the pLenti6 TOPO expression vector were controlled by restriction digest using the restriction enzymes Xho I and Afl II, both provided

by New England BioLabs (Leiden, The Netherlands). For each sample 2µl 10x NEBuffer (New England BioLabs), 0,5µl of each enzyme and 3µl of Midiprep isolated plasmid was used. Water was added to a final volume of 20µl. Samples were held at 37°C for 2 hours before being analysed by gel electrophoresis.

2.5 Hotstar Hifidelity PCR: "construction PCR"

The Hotstar Hifidelity proofreading DNA-polymerase (QIAGEN, Venlo, The Netherlands) was used to generate a mutation free PCR-product of the cTCR-gene, this product would be used in TOPO-cloning reactions. The PCR was conducted on the isolated pCR2.1 TOPO vector with 001cTCR and 014cTCR primers (see table 1 for primer sequences). For each sample of 100-150ng pCR2.1 vector: 10µl 5x PCR buffer (Qiagen), 5µl of each primer (Eurogentec) and 1µl Hotstar Hifidelity Polymerase was used. Water was added to a total volume of 50µl. The PCR was performed in a thermal cycler MyCycler (BioRad, Nazareth Eke, Belgium) A hot start of 95°C was performed, the initial denaturation lasted 5', 40 cycles were run in which each cycle had a 15" 94°C denaturation step, a 1' annealing step (see table 1 for annealing temperatures) and a 90" 72°C elongation step, the final elongation lasted 10' on 72°C. Afterwards the PCR-products were held on 4°C.

2.6 Colony PCR

Bacteria were grown in LB-medium containing ampicillin at 37°C. On bacteria containing the cTCR construct colony PCR was performed. 001cTCR and 014cTCR or CMV-forward and V5-reverse primers (Eurogentec) were used to obtain the cTCR-construct. For each sample of 1µl bacterial cells: 5µl PCR-buffer (Roche Diagnostics, Brussels, Belgium) 0,5µl dNTP-mix (Roche Diagnostics), 1,4µl of each primer (see table 1 for primer sequences) and 0,4µl Tag-polymerase (Roche Diagnostics) was used. Water was added to a total volume of 50µl. The PCR was performed in a thermal cycler MyCycler (BioRad). A hot start of 95°C was performed, the initial denaturation lasted 10', 35 cycles were run in which each cycle had a 30" 94°C denaturation step, a 30'' annealing step (see table 1 for annealing temperatures) and a 90" 72°C elongation step, the final elongation lasted 10' on 72°C. Afterwards the PCR-products were held on 4°C.

2.7 Plasmid isolation

2.7.1 Miniprep

Transformed Stbl3 E.coli were cultured overnight in 2ml LB-medium with 100µg/ml ampicillin. Dry cells were treated with Resuspension buffer P1 (which contained RNase), Lysis buffer P2 and buffer P3. Cell debris was removed and supernatant was purified with sephadex. After purification, 100% ethanol was added to the mixture. The plasmid precipitated. The pellet was resuspended in 70% ethanol. After centrifugation, the pellet containing the plasmid was dried and solved in 50µl H2O.

2.7.2 Midiprep

Before plasmid isolation, transformed Stbl3 E.coli were cultured overnight in 100ml LB-medium with 100µg/ml ampicillin. The S.N.A.P. Midiprep Kit (Invitrogen) was used for plasmid isolation, the procedure was conducted as described in the supplemented manual.

2.8 Ligation

PCR-products were treated with the restriction enzyme Fse I (New England BioLabs). For each sample 2µl 10x NEBuffer (New England BioLabs), 0,5µl enzyme and 1µl of PCR-product was used. Water was added to a final volume of 20µl. Samples were held for 2 hours on 37°C and afterwards 20 minutes on 65°C. The PCR-products were ligated with T4 DNA-ligase (Promega, Leiden, The Netherlands). For each sample of 100-200ng DNA: 3µl ligation-buffer and 1µl T4 DNA-ligase (Promega) was used. Water was added to a final volume of 20µl. Samples were held for 3 hours on room temperature.

2.9 Blasticidin resistance

Resistance to the antibiotic blasticidin was determined on J.RT3-T3.5 Jurkat and HT1080 cells. Cells were grown at 500000cells/ml in T_{25}-flasks and subjected to cell culture medium supplemented with various concentrations of blasticidin (Invitrogen). The tested concentrations ranged from 2µg/ml to 10µg/ml. The medium was changed every 3 days and after 12 days the concentration at which no cells were alive was determined with tryptan bleu staining of dead cells.

2.10 ELISA

IL-2 production of stimulated and non-stimulated Jurkat, Clone E6-1 cells was measured with the human IL-2 ELISA kit from Invitrogen. Jurkat, Clone E6-1 cells were grown at 4000cells/ml in 200µl culture medium. Cells were stimulated with 2µl Dynabeads Human T-activator CD3/CD28 (Invitrogen). Supernatants were harvested after 12h, 24h, 48h and 72h. PBMC acted as controls, supernatants were harvested 24h past stimulation with Dynabeads Human T-activator CD3/CD28. The ELISA-procedure was conducted as described in the supplemented manual. ELISA-plates were analysed with BioRad Microplate Manager (BioRad).

3 Results and discussion

3.1 Construction of the lentivirus

3.1.1 TOPO-cloning reactions

In order to develop a functional whole cell biosensor, there was need of a proof of principle. This proof would be obtained when the three major research objectives were reached. Firstly, the cTCR-construct had to be expressed by the Jurkat cells. Secondly and thirdly, the expression and functionality of the cTCR had to be tested. Due to the lack of a TCR in J.RT3-T3.5 Jurkat cells, these cells were chosen to examine expression and functionality of the cTCR.

The cTCR-gene had to be stably expressed in the Jurkat cell line. Stable expression would give rise to a functional cTCR. Therefore, to meet the first research objective, a lentiviral expression vector was constructed to successfully deliver the cTCR-gene. The Vira Power Lentiviral directional TOPO expression kit from Invitrogen was used to create the lentivirus. According to the protocol, the construction of the lentivirus consists of four major steps. First, a "construction PCR" is performed to yield a mutation free construct. Second, a directional TOPO cloning will introduce the cTCR-construct in the pLenti6/V5 TOPO vector. Third, after successful transfection of Stbl 3 E.coli, the pLenti6/V5 TOPO expression vector is isolated. Fourth, the pLenti6/V5 TOPO expression vector is used to transfect 293FT cells which will produce the lentivirus. Every step is accommodated with several controls (PCR or sequencing reaction) to guarantee the correctness and integrity of the cTCR-construct.

3.1.1.1 The wrong vector?

The cTCR-receptor cassette structure was constructed by K. Deprez (see figure 5 for a schematic overview or see supplemented figure 1 for the sequence). The gene was stored in the pCR2.1 TOPO vector inside TOP10 E.coli cells. After successful colony PCR with 001cTCR and 014cTCR primers and gel electrophoresis, a band of +/- 1300bp was observed. The sequence of the cTCR-gene was controlled by primer extension sequencing, no deletions or substitutions were observed.

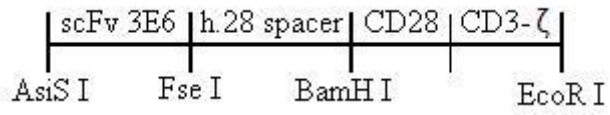

Figure 5 Schematic overview of the cTCR-receptor cassette structure. For directional TOPO-cloning a CACC TOPO-overhand is required. This sequence is present in front of the AsiS I restriction site. The detailed sequence of the cTCR-receptor cassette structure can be found in the supplemented figures.

The cTCR-gene was already constructed to be cloned in TOPO-vectors. The required CACC TOPO-overhang was present (see supplemented figures for a detailed sequence of the cTCR-gene), therefore additional modification with special primers was not needed. The first step was to generate a mutation free cTCR-construct. The gene was amplified during a PCR in which the proof reading polymerase Hotstar Hifidelity was used. Gel electrophoresis showed a discrete band at the right height which indicated that the gene was amplified successfully (see figure 6A).

During the second step, the gene is introduced in the pLenti6/V5 TOPO expression vector. Different molar ratios of cTCR-gene/pLenti6 V5 TOPO expression vector (10:1, 5:1, 2:1, 1:1) were used to clone the cTCR-gene directly in the pLenti6/V5 TOPO expression vector. The cloning reaction inserted the gene downstream of the CMV-promoter and upstream of the V5-sequence. One Shot Stbl3 Chemically Competent E.coli cells were transfected, using heat shock, with the pLenti 6/V5 TOPO expression vector and grown on ampicillin selection plates. Stbl3 E.coli that had successfully taken up the pLenti 6/V5 TOPO expression vector became ampicillin resistant (the vector harboured ampicillin resistance genes) and formed distinct colonies on the selection plate.

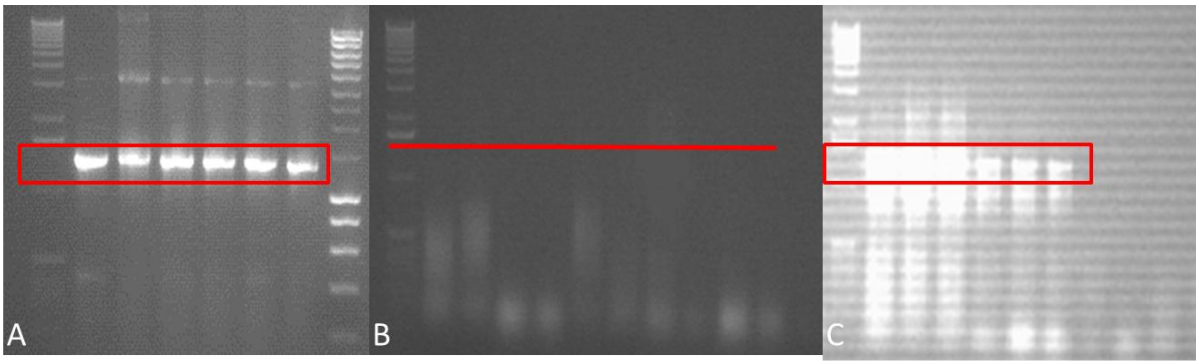

Figure 6. Construction of the lentivirus: construction and colony PCR. In figure 6A the results of the construction PCR with the proof reading polymerase Hotstar Hifidelity are visualized using gel electrophoresis. The red box indicates where the expected band should be. As can be seen, the PCR generated the cTCR-construct of 1,3kb. In figure 6B, the results of the colony-PCR with the CMV-forward and V5-reverse primers can be seen on gel. The red bar, at a height of 1,5kb, indicates where the expected band should be. Gel electrophoresis revealed that no bands were present. When the same colony PCR was repeated with different primers (001cTCR forward and 014cTCR reverse primers), gel electrophoresis indicated that the cTCR-construct was present in the Stbl3 E.coli cells, as can be seen on figure 6C. The red box indicates the position of the expected bands, 1,3kb.

The correct clones were determined with PCR through a combination of the CMV-forward primer and the V5-reverse primer. The obtained PCR-product should have a length of 1,5kb. Gel electrophoresis indicated that no bands were present (figure 6B). The colony PCR with the CMV-forward and the V5-reverse primers was repeated numerous times (under different conditions), but did not show any bands.

When a colony PCR was performed with the internal 001cTCR and 014cTCR primers, discrete bands appeared at the right height of 1,3kb (figure 6C). Although the CMV and V5 primers did not work, the colony PCR with the 001cTCR and 014cTCR primers confirmed the presence of our insert.

The third step was to isolate the pLenti6/V5 TOPO expression vector .The colonies which contained the cTCR-insert were cultured and midiprep plasmid isolation was performed. The isolated plasmids were controlled with gel electrophoresis. The pLenti 6/V5 TOPO expression vector with the cTCR-gene should be 8,3kb long, on the gel can be seen that this is not the case (figure 7A). Due to plasmid supercoiling, the migration properties of plasmids in agarose

gels are altered, therefore the plasmid can travel further in the gel and appears to be smaller than it should be. The problem of supercoiling can be made undone. The pLenti6/V5 TOPO expression vector harbours different restriction sites. Several of these restriction sites only appear once in the plasmid. One of these restriction sites is Xho I. A restriction digest with the Xho I restriction enzyme would cut the plasmid once and yield a linearized vector. The results of the restriction digest with Xho I can be found in figure 7C. When these results are compared with the supercoiled plasmid (figure 7A), a shift towards a higher position on the gel is observed. This indicates that the pLenti6/V5 TOPO expression vector is cut by Xho I. Despite the linearization of the vector, the plasmid still not reached the required 8,3kb on the gel.

When a PCR was performed on the isolated plasmid with the CMV-forward and V5-reverse primers, multiple discrete bands emerged on the gel. None of these bands could be found on the exact height of 1,5kb (Figure 7B). When this PCR-product was used as a template for a PCR with the 001cTCR and 014cTCR primers, a single discrete band of 1,3kb appeared at the gel, indicating that the cTCR-insert was present in one of the PCR-products generated with the CMV and V5 primers (figure 7C). It could be due to nonspecific binding that these four bands appeared. Despite optimisation of the PCR-protocol with different Mg^{2+}-concentration and temperature gradients, the four bands kept appearing on the gel.

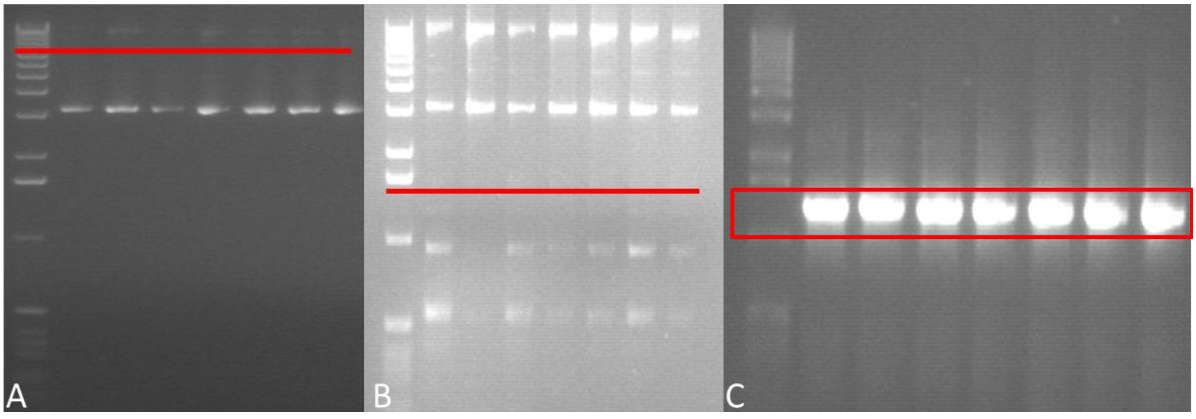

Figure 7 Construction of the lentivirus: plasmid isolation and PCR. Figure 3A shows the results of the SNAP Midiprep plasmid isolation. The plasmid should be 8,2kb long (the red bar) but gel electrophoresis indicated that the isolated plasmid was only 3kb in length. When a PCR was performed on the isolated plasmid with CMV-forward and V5-reverse primers, a band of 1,5bp was expected (red bar). As can be seen in figure 7B, four unexpected bands were revealed with gel electrophoresis. When these PCR-products were used as samples in a new PCR with 001cTCR and 014cTCR primers to test the presence of the cTCR-construct in one of the unexpected bands, gel electrophoresis indicated the presence of cTCR at a height of 1,3kb (red box), as can be seen in figure 7C.

When sequencing reactions were performed on the isolated plasmid, both cTCR-construct (001cTCR, 005cTCR, 006cTCR, 014cTCR) and pLenti6/V5 TOPO expression vector primers (CMV-forward and V5-reverse) were used. Sequencing with internal cTCR-primers indicated that the construct was present in the plasmid. No substitutions or deletions were found. On the other hand, sequencing with the CMW-forward and V5-reverse primers did not work. If these primers were malfunctioning, this could be the explanation why the colony PCR did not work and why nonspecific binding was observed when these primers were used on the isolated plasmid.

In order to confirm the integrity of the isolated plasmid, which was needed to ensure that no recombination had taken place in the long terminal repeats of the pLenti 6/V5 TOPO expression vector, a restriction digest had to be performed. The restriction enzyme Xho I cuts once in the plasmid, whereas Afl II cuts the plasmid twice (Figure 8A). When the restriction digest was visualised on gel, discrete bands were observed. The digestion with Xho I should undo the supercoiling and therefore have yielded a linear plasmid of 8,3kb. As seen on the gel, this was not the case, the plasmid was only around 6kb long (Figure 8C). The digestion with Afl II was supposed to cut the plasmid in two separate pieces, but only one discrete band was observed (Figure 8B).

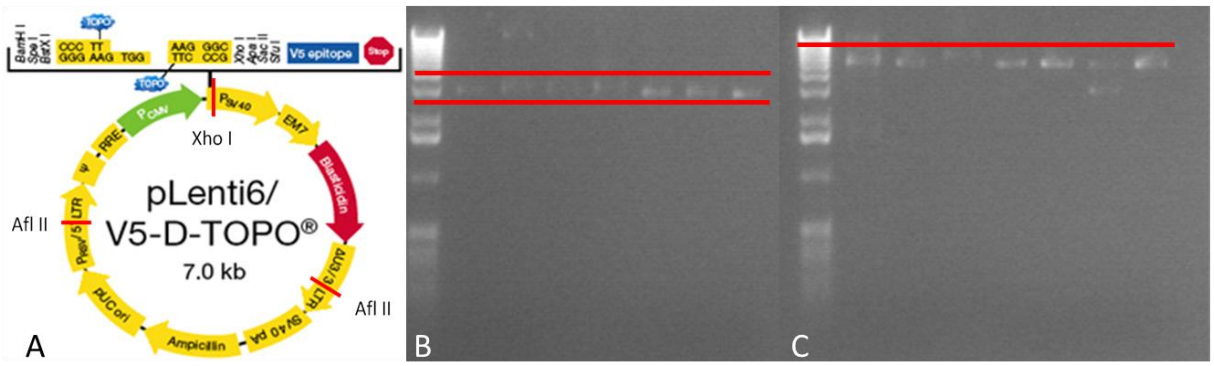

Figure 8. Construction of the lentivirus: restriction digest. In figure 8A the pLenti6/V5 TOPO expression vector is depicted. The red bars indicate where the three restriction sites for the restriction enzymes Xho I (one cut) and Afl II (two cuts) can be found. When TOPO cloning reactions were performed, the resulting plasmid had a length of 8,3kb. Digestion with Afl II should yield two bands, one band should be 3,6kb and the other bad should be 4,7kb long. Figure 8B shows the result of the restriction digest with Afl II. The red bars indicate where the observed bands should have been. As can be seen on the photo, only one ban appeared on the gel, whereas there should have been two. Figure 8C shows the restriction digest with Xho I, this yielded a different restriction pattern than Afl II. The red bar shows where the expected band should have been, as can be seen on the photo, all bands are shorter than expected.

All the results (the colony PCR with the CMV-forward and V5-reverse primers that did not function, the non-specific binding of the CMV-forward and V5-reverse primers, the sequencing reaction with the CMV-forward and V5-reverse primers and the restriction digest that gave the wrong results) were put together and taken into account. These results pointed out that we were not working with the pLenti6/V5 TOPO expression or that the vector sequence was not as it should be. After discussing the encountered problems with the technical service of Invitrogen a new Vira Power Lentiviral directional TOPO expression kit was provided.

3.1.1.2 A toxic cTCR-construct?

In our second attempt, the same procedure, as described above, was followed. The cTCR-gene was amplified during a "construction PCR", in which the proof reading polymerase Hotstar Hifidelity was used. Gel electrophoresis showed a discrete band at the right height which indicated that the gene was amplified successfully. Different molar ratios of cTCR-gene/pLenti6 (10:1, 5:1, 2:1, 1:1) were used to clone the cTCR-gene directly in the pLenti6/V5 TOPO expression vector. The cloning reaction inserted the gene downstream of the CMV-promoter and upstream of the V5-sequence. One Shot Stbl3 Chemically Competent E. coli cells were transfected with the pLenti 6/V5 TOPO expression vector and grown on ampicillin selection plates. Stbl3 E.coli cells that had successfully taken up the pLenti 6/V5 TOPO expression vector became ampicillin resistant and formed distinct colonies on the selection plate.

The correct clones were determined with PCR through a combination of the CMV-forward primer and the V5-reverse primer. The obtained PCR-product should have a length of 1,5kb. Gel electrophoresis indicated that the PCR-products of the selected clones had the right height (Figure 9A).

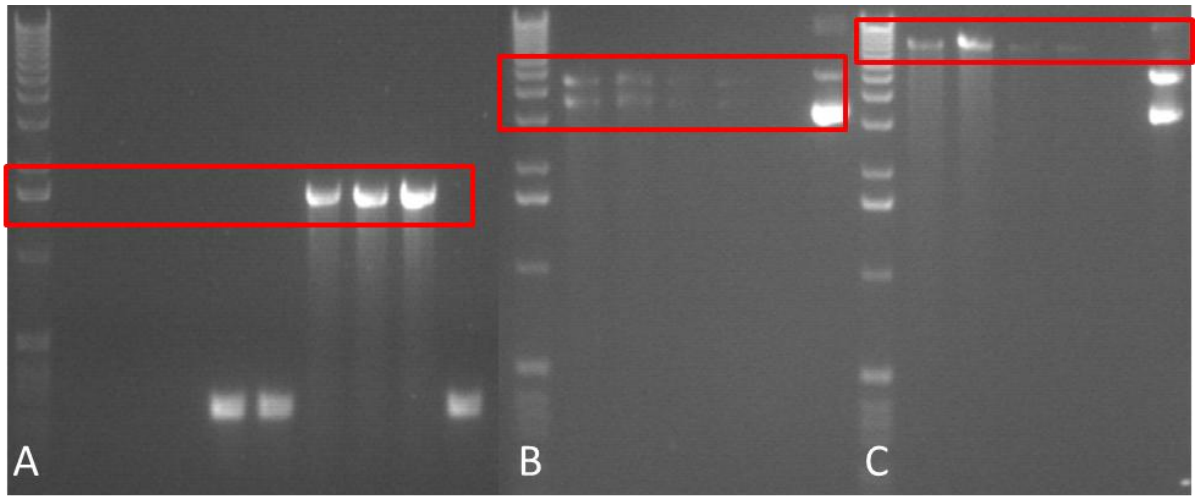

Figure 9. Construction of the lentivirus, second attempt: colony PCR and restriction digests. Figure 9A shows the results of the colony PCR with the CMV-forward and V5-reverse primers on Stbl3 E.coli cells that were transfected with the pLenti6/V5 TOPO expression vector which. The generated band should be 1,5kb long and this was confirmed with gel electrophoresis. The result indicated the presence of the cTCR-construct in the pLenti6/V5 TOPO expression vector. Colonies that were positive for the cTCR-construct were cultured and the plasmid was isolated. Figure 9B and 9C show the results of the restriction digest of the isolated plasmid. The restriction digest of Afl II is shown in Figure 9B. The bands were expected to have a length of 3,6kb and 4,7kbbp and this was confirmed by restriction digest. The red box indicates the right height at which the band lies. Figure 9C shows the results of the restriction digest with Xho I. As expected, the digest yielded a band with a length of 8,3kb (the red box).

Correct clones were cultured and a midiprep plasmid extraction was performed. Isolated pLenti 6/V5 TOPO expression vectors were controlled with gel electrophoresis and bands were observed at a height of 8,3kb, which was the length of the vector with the inserted cTCR-gene.

To verify the correctness of the pLenti6/V5 TOPO expression vector, a restriction digest was performed. The full length of the vector with the cTCR-insert was 8,3kb. After digestion with Xho I, a linearised fragment of 8,3kb was observed after gel electrophoresis (Figure 9C). After digestion with Afl II, two fragments of 6,1kb and 2,2kb were observed after gel electrophoresis (Figure 9B). These lengths of the bands were the same as the lengths that should be observed.

After successful plasmid isolation and restriction digests of three positive colonies, the vector was sequenced. Sequencing with CMV-forward and V5-reverse primers confirmed the correct orientation and reading frame of the cTCR-insert. Sequencing with cTCR-construct primers however showed that there was a base deleted in the insert. This deletion would cause a

reading frame shift and this would render a useless cTCR. Strikingly, this deletion was present in all three colonies, while it was not present it the original insert of the pCR2.1 TOPO vector (Figure 10). The sequencing results indicated that either the starting material, which was used for the initial PCR with Hotstar Hifidelity, was damaged or a selection for a mutated construct was taking place.

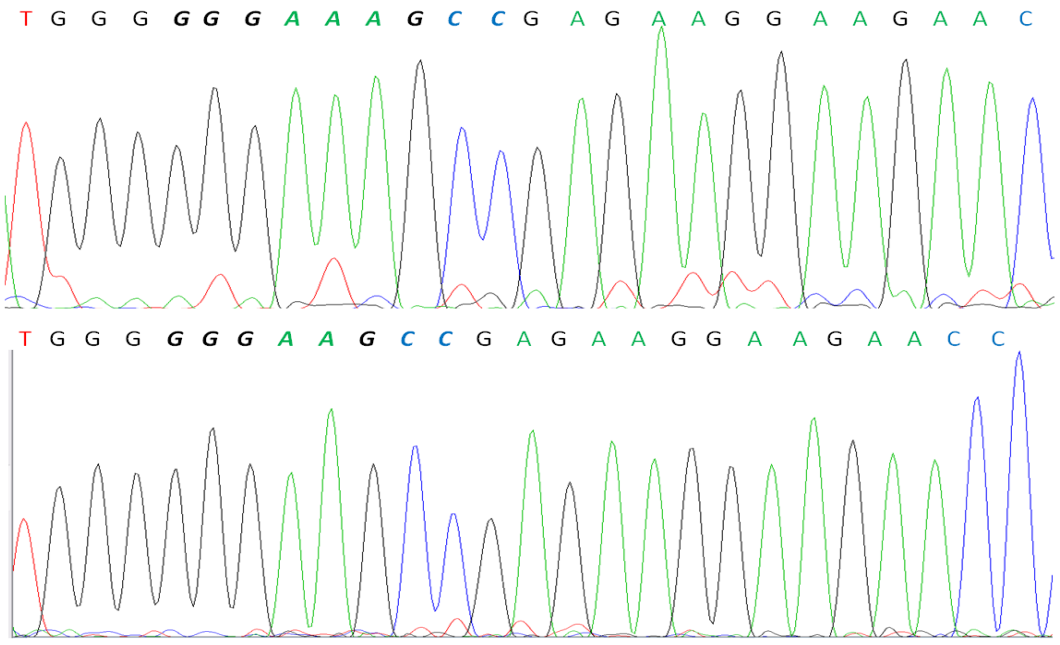

Figure 10. Construction of the lentivirus, sequencing results. The cTCR construct was sequenced with external plasmid and internal construct primers. Sequencing revealed that a mutation had occurred in the cTCR-construct. The first sequence is the unmutated sequence GGGAAAGCC of the cTCR-construct in pCR2.1 TOPO vector. During the process a deletion of one A-base had occurred which resulted in the wrong sequence GGGAAGCC in the pLenti6/V5 TOPO expression vector. This deletion resulted in a frame shift and rendered the cTCR-construct non-functional.

Before a third attempt to produce a lentivirus was started, several sequencing control reaction were performed. The correctness of the cTCR-construct, in the pCR2.1 TOPO vector that was stored inside TOP10 E.coli cells, was confirmed. The basic cTCR-construct did not carry the mutation. Starting with this mutation free construct, the same procedures as described above were followed. Surprisingly, the third attempt to create a pLenti6/V5 TOPO expression vector failed. After the TOPO-cloning reaction, the Stbl3 E.coli cells were heat shocked in the presence of the vector. These cells were cultured on ampicillin selection plates. Afterwards, no colonies were observed.

The observation that a mutated cTCR-construct could yield plenty colonies, whereas the correct cTCR-construct could not indicated that the correct cTCR-construct in combination with the pLenti6/V5 TOPO expression vector could be toxic to the Stbl3 E.coli cells. This assumption was controlled in two ways. First, if the cTCR combined with the vector would result in a toxic event for the Stbl3 E.coli cells, another combination of the cTCR with a different vector might not. The pCR2.1 TOPO vector which contained the correct cTCR-construct was introduced in the Stbl3 E.coli cells via heat shock. These cells received the same treatment as the cells that were transformed with the pLenti6/V5 TOPO expression vector. When these E.coli cells were cultured on ampicillin selection plates, colonies were observed. Second, when a small part of the cTCR-construct was introduced in the pLenti6/V5 TOPO expression vector and this vector was used to transform Stbl3 E.coli, an abundant number of colonies were generated. These observations lead to the conclusion that the combination of the mutation-free cTCR and the intact pLenti6/V5 TOPO expression vector were toxic for the Stbl3 E.coli cells. This could explain why the mutated cTCR yielded plenty of colonies, while the correct insert did not produce any colonies. An inactive cTCR (like the mutated construct) would not lead to a toxic reaction and the Stbl3 E.coli cells would grow normally.

3.1.2 Modification of the cTCR cassette structure

The cTCR-gene proved to be toxic to Stbl3 E.coli cells when it was used in combination with the pLenti6/V5 TOPO expression vector. Despite the encountered problems, the lentiviral approach was chosen above other possibilities. Lentiviral introduction of the cTCR-gene in the cellular genome of J.RT3-T3.5 Jurkat cells would ensure a stable expression of the receptor cassette. In order to introduce the receptor cassette in J.RT3-T3.5 Jurkat cells, modification of the cTCR-gene was required. A modified cTCR might be tolerated by the Stbl3 E.coli cells and from that point a lentivirus can be constructed.

The receptor cassette could be modified in two different ways. First, a bacterial transcriptional stop sequence could be incorporated in front of the cTCR-gene. Transcription would be terminated before the cTCR-gene. Thus, the lethal cTCR would not be generated. Second, a leader sequence could be introduced in front of the cTCR-gene. The prime function of this leader sequence is to target the protein to the cell membrane. A second feature of the leader sequence is that it is not transcribed in bacteria. Thus, with the leader sequence the cTCR would not be expressed in bacteria.

Previous researchers in our research group (K. Motmans) had worked with the leader sequence, therefore the decision was made to incorporate the leader sequence in the cTCR-construct. K. Motmans had designed a PSA-single chain antibody with leader sequence (34). The single chain was flanked with the Fse I restriction site and stored in the pFvMycH6 vector. The single chain of the original cTCR would be changed for the PSA-single chain antibody with the leader sequence that Motmans had designed.

After the sequence of the PSA-single chain antibody was controlled, a "construction PCR" with Hotstar Hifidelity was conducted on the plasmid with Leader and 002cTCRcorrect primers (Figure 11A). Another "construction PCR" with Hotstar Hifidelity was conducted on pCR2.1 TOPO vector with 001cTCR and 014cTCR primers to yield the cTCR-construct. Both PCR-products were brought together with an abundance of leader-PSA compared to cTCR. Molar ratios ranged from 1:1 to 10:1. A restriction digest with the restriction enzyme Fse I was performed on the mixture. The fragments were ligated by T4 DNA ligase. The higher concentration of leader-PSA compared to original PSA, gave leader-PSA a competing advantage over the standard PSA for the ligation place with the remaining cTCR-construct. In this way, the original PSA was swapped for the leader-PSA. A "construction PCR" with Hotstar Hifidelity polymerase was performed on the ligation mixture. The used primers, Leader and 014cTCR, led to an amplification of the leader-PSA cTCR-construct (Figure 11B).

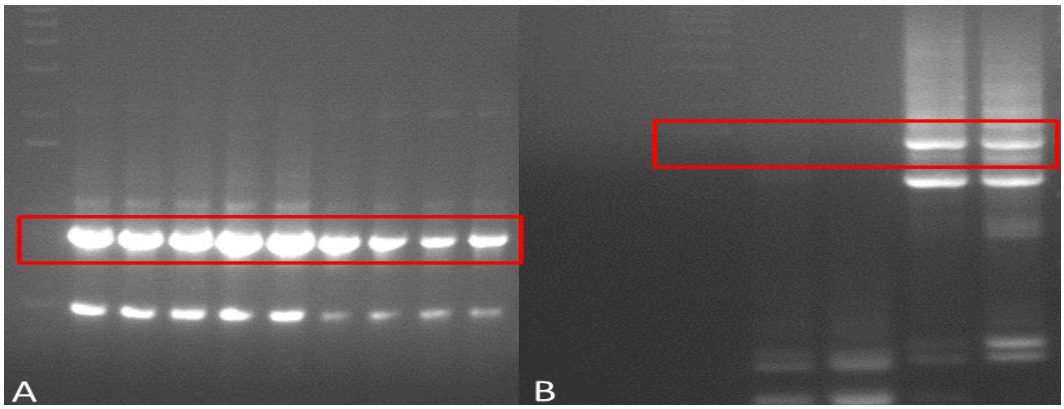

Figure 11 Modification of the cTCR-construct. The cTCR was modified with a leader sequence. Figure 11A shows the PSA-single chain antibody with the leader sequence. It was obtained via a construction PCR with leader and 002cTCRcorrect primers on the pFvMycH6 plasmid. The PSA-single chain antibody and cTCR-construct were brought together and digested by the restriction enzyme Fse I. Both sequences contained the Fse I restriction site. T4 DNA ligase was used to ligate the PSA-single chain antibody with the leader sequence to the cTCR-construct. Figure 11B shows the result of the construction PCR with leader and 014cTCR primers. This PCR was conducted on the ligation reaction. The PCR amplified the cTCR with leader, a product of 1,4kb was generated, as was observed on gel.

3.2 Blasticidin resistance

Blasticidin is an antibiotic and therefore lethal to cells that lack specific resistance genes. Blasticidin prevents cell growth by inhibiting peptide bond formation on the ribosome. Not all cells are equally sensitive to blasticidin, most cells can grow when blasticidin is present in a low concentration. The concentration at which blasticidin completely inhibits cell growth was determined. Lower concentrations only slowed cell proliferation, higher concentrations killed cells. As can be seen on figure 3A, the pLenti6/V5 TOPO expression vector carries blasticidin resistance genes. When the vector is packed into the lentivirus, these genes will also be transferred to host cells and render these cells resistant to blasticidin. Blasticidin selection was performed to determine the minimal concentration at which non-transduced cells were killed by the antibiotic. When the lentivirus transforms host cells and renders them resistant to this blasticidin concentration, all untransformed cells will die. Eventually, only cTCR-expressing cells will remain.

HT1080 cells were needed to determine the virus titer. During 12 days, these cells were subjected to different concentrations of blasticidin. Cells were cultured in T_{25}-culture flasks. The tested concentrations ranged from 2 to 10µg blasticidin per ml of culture medium.

HT1080 cells come from an adherent cell line. Dead cells would detach from the culture flask. After 12 days, no living cells were observed in the culture that received 4µg/ml or more blasticidin, whereas the 2µg/ml blasticidin culture flask still harboured living cells (figure 12, dotted line). This indicated that 4µg/ml was the concentration needed to kill all non-blasticidin resistant cells. When the viral titer will be determined, a concentration of 4µg/ml blasticidin will be used to distinguish transfected living cells from non-transfected dead cells.

J.RT3-T3.5 Jurkat cells will be the cells in which we will test the functionality of the cTCR. The cells were treated under the same conditions as the HT1080 cells. Unlike HT1080, J.RT3-T3.5 Jurkat cells are not adherent. Therefore the cells were counted every two days. After 12 days, no living cells were observed in the culture that received 6µg/ml or more blasticidin (figure 12, dotted line). This indicated that 6µg/ml was the concentration needed to kill all non-blasticidin resistant cells. When the lentivirus introduces the cTCR-gene and the blasticidin resistance gene, a blasticidin concentration of 6µg/ml can be used to select successfully from unsuccessfully transformed cells.

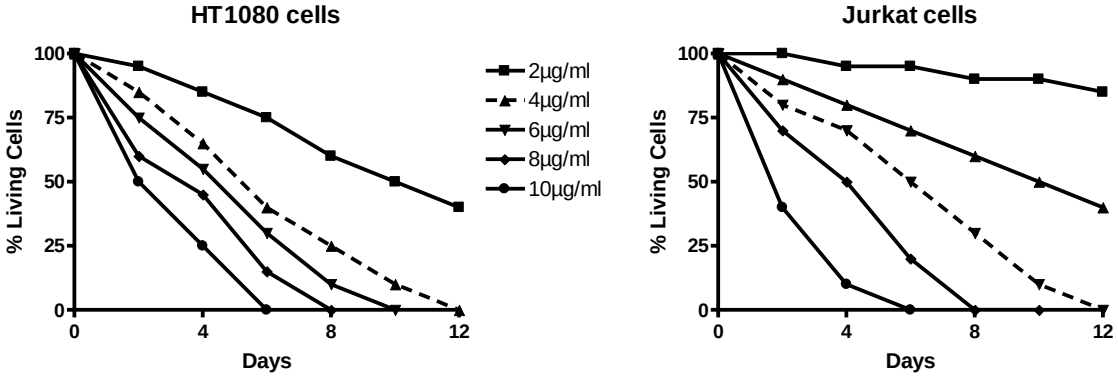

Figure 12 Blasticidin resistance was determined on HT1080 and Jurkat T-cell. From a certain concentration the antibiotic blasticidin is lethal to HT1080 and Jurkat cells. Blasticidin sensitivity was determined in order to determine viral titer (HT1080 cells) or to distinguish transfected from untransfected Jurkat cells. The concentration at which no cells were alive after 12 days indicated the concentration at which transfection with the lentivirus would keep the cells alive. HT1080 cells could survive a blasticidin concentration up to 4µg/ml, whereas Jurkat cells survived 6µg/ml blasticidin.

3.3 IL-2 production of Jurkat cells

T-cells start producing and secreting IL-2 when activated. In order to test the functionality of the cTCR, the production of IL-2 will be measured. Although the cTCR-gene was not introduced in the Jurkat cells, the IL-2 production of untransduced J.RT3-T3.5 Jurkat cells was measured. Jurkat clone E6-1 cells were stimulated with Dynabeads Human T-Activator CD3/CD28 that contained anti-CD3 and anti-CD28. The signals generated by CD3 and CD28 would stimulate the Jurkat clone E6-1 cells to start secreting IL-2. Furthermore, the signal pathways used by CD3 and CD28 are the same as the signal pathways generated by the cTCR, the cTCR is composed of CD3ζ and CD28 domains. The pathways that would be activated in untransduced clone E6-1 Jurkat cells would be the same as cTCR-transduced J.RT3-T3.5 Jurkat cells. Therefore, these results would give an opportunity to compare the effectiveness of the antigen stimulated cTCR-transduced Jurkat cell against the effect of the anti-CD3/anti-CD28 stimulation. A comparison was made between stimulated and unstimulated Jurkat cells. IL-2 production was measured 12hours after stimulation.

Peripheral Blood Mononuclear Cells (PBMC) served as a control population. These cells were treated the same as the Jurkat cells, the stimulation occurred with Dynabeads Human T-Activator CD3/CD28. IL-2 production was determined with ELISA.

Only the IL-2 production of the PBMC and the untransduced J.RT3-T3.5 Jurkat cells could be determined. Basal IL-2 production of J.RT3-T3.5 Jurkat cells was not observed. Stimulation with Dynabeads Human T-Activator CD3/CD28 also did not evoke an IL-2 response. This can be explained by the fact that J.RT3-T3.5 Jurkat cells do not have an active TCR complex and this is required for stimulation with anti-CD3. IL-2 production of PBMC was more than a hundred times the IL-2 production of that J.RT3-T3.5 Jurkat cells (data not shown).

We expect that the IL-2 production of stimulated clone E6-1 Jurkat cells and cTCR-transduced J.RT3-T3.5 Jurkat cells will be comparable to the IL-2 production of the PBMC.

4 Conclusion and future perspectives

This research project focused on the development of a novel whole cell biosensor based on a chimeric T-cell receptor based receptor construct. The construct incorporates an easily interchangeable anti-PSA single chain antibody in a receptor cassette for efficient receptor signalling upon ligand binding. Previous authors have published encouraging results with the combination of cTCR with h.28spacer, CD28 and CD3ζ –domains (16). They proved the expression and functionality of this cTCR (with another single chain antibody), therefore it was a valuable research subject in the development of novel biosensors. The aim of this project was the expression of the cTCR-construct in the human TCR deficient T-cell line JRT3-3.5.

In order to introduce the cTCR-construct in the Jurkat cells, a lentivirus was constructed. Several steps are involved in the stable expression of the construct in J.RT3-T3.5 Jurkat cells. Before actual directional TOPO-cloning took place, the sequence of the cTCR-construct was controlled. Correct constructs were used for TOPO-cloning. The first attempt did not yield plenty Stbl3 E.coli colonies. The colonies that were present on the ampicillin selection plates and which were used in further experiments produced contradictory results. The isolated pLenti6/V5 TOPO expression vector had the wrong length and did not apply to the expected restriction pattern. This led to the conclusion that the vector was not the pLenti6/V5 TOPO expression vector which was required for lentiviral construction.

The second attempt to clone the cTCR-construct in the pLenti6/V5 TOPO expression vector succeeded. Plenty bacterial colonies were generated on the selection plates. Plasmid isolation and subsequent restriction digestion revealed that the vector had the right length and the right restriction pattern. When the vector was sequenced, a frame shift mutation was detected. When this mutated construct would be expressed, the generated cTCR would not function. Before a third attempt was made, the cTCR-construct was sequenced thoroughly to ensure that the frame shift mutation, observed during the second TOPO cloning attempt, was absent. When a third attempt was made, to clone a mutation free cTCR-construct in the pLenti6/V5 TOPO expression vector, no colonies were generated. This led to the hypothesis that the cTCR-construct in combination with the pLenti6/V5 TOPO expression vector was lethal to Stbl3 E.coli cells. The assumption was supported by two facts. First, when a pCR2.1 TOPO vector with the cTCR-construct was used to transform Stbl3 E.coli, plenty colonies were

generated. Second, when the pLenti6/V5 TOPO expression vector was used with a part of the cTCR-construct, it did not kill the Stbl3 E.coli. These results indicated that the cTCR-construct in combination with the pLenti6/V5 TOPO expression vector was toxic to Stbl3 E.coli cells. This would explain the favourable selection for a mutated cTCR-construct.

The toxicity of the vector-construct combination to Stbl3 E.coli cells can be resolved in two ways: the cTCR-construct could be modified or the lentiviral expression system could be changed. The first option was chosen. The cTCR-construct was modified with a leader sequence, a sequence to target the cTCR to the cell membrane. K. Motmans had created an anti-PSA single chain antibody with a leader sequence. The original anti-PSA single chain antibody was swapped with the anti-PSA single chain antibody with leader sequence. In this way, the cTCR was extended with a leader sequence. The leader sequence was constructed with the TOPO-overhang, so it could be cloned in the pLenti6/V5 TOPO expression vector. This particular modification of the cTCR-construct required less structural adjustments than changing the expression system. Another expression system would require its own specific modifications to the cTCR-construct. This would render much of the preliminary work useless.

When this modified construct will be cloned in the pLenti6/V5 TOPO expression vector, the leader ensures that the cTCR-construct will not be transcribed in Stbl3 E.coli cells. If, despite the leader sequence, the cTCR-construct is transcribed, the leader sequence can act as an insertion. This “mutation” might alter the conformation of the protein in a way that it is not toxic to the cells. The leader sequence alters the combination pLenti6/V5 TOPO expression vector/cTCR-construct in a way which is not lethal to Stbl3 E.coli cells.

The interdependency of the research objectives made it hard to obtain results on the following objectives. Therefore the hypothesized development of the whole cell biosensor could not be realised. Despite the encountered problems with the construction of the lentivirus, the first, preparative steps have been taken to realize the following objectives. The sensitivity to the antibiotic blasticidin was determined on HT1080 cells and J.RT3-T3.5 Jurkat cells. The lentivirus would not only integrate the cTCR-gene, but also blasticidin resistance genes in the cells it infects. HT1080 cells will be used to determine viral titer, whereas J.RT3-T3.5 Jurkat

cells will be the cells in which the functionality of the cTCR will be tested. Blasticidin will be used to distinguish transfected from non-transfected cell.

In order to evaluate the functionality of the cTCR, the IL-2 production of the Jurkat cells is being examined. The activation signals of the cTCR leads to the secretion of IL-2, what will be measured through ELISA. The activation signal will be generated by the CD3ζ and CD28 domains of the cTCR. To evaluate and compare the results of Jurkat cells which express the cTCR, non-transfected Jurkat cells were stimulated with anti-CD3 and anti-CD28. IL-2 production of these conditions was measured. If the cTCR works, the transformed Jurkat cells would preferably produce as much IL-2 as the Jurkat cells stimulated with anti-CD3 and anti-CD28.

Unexpected challenges made it impossible to finish the research objectives. In the near future, the formulated research objectives must be reached: the construction of the lentivirus with the modified cTCR, the expression and functionality of the cTCR that must be tested and a reporter system that must be designed. These milestones must be reached in order to develop a functional whole cell biosensor.

Following successful expression of a functional receptor cassette in J.RT3-T3.5 Jurkat cells, the cTCR can be fine-tuned by exchanging signal domains, to optimize the sensor function. In order to decrease the time between detection of the antigen and synthesis/detection of the reporter gene, the Jurkat cell can be traded for another cell type if signal transduction in Jurkat cells is too slow.

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Supplemented Figures

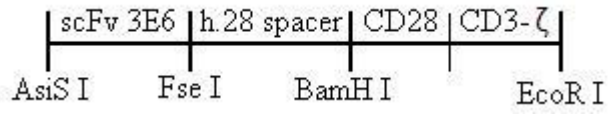

Figure 13 Schematic overview of the cTCR-gene, sequences can be found under this figure. The first sequence is the original cTCR-gene. The second sequence is the cTCR modified with the leader sequence.

Sequence of the cTCR-gene

C ACC ATG CGC GAT CGC ACC CAG TCT CCA GCC TCC CTA CTA TCT GCA TCT GTG GGA GAA
TOPO overhang AsiS I
ACT GTC ACC ATC ACA TGT CGA GCA AGT GGA AAT ATT CAC GAT TAT TTA GCA TGG TAT
CAG CAG AAA CAG GGA AAA TCT CCT CAG CTC CTG GTC TAT AAT GCA AAA ACC TTA GCA
GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA TCA GGA ACA CAA TAT TCT CTC AAG
ATC AAC AGC CTG CAG CCT GAA GAT TTT GGG AGT TAT TAC TGT CAA CAT TTT TGG AGT ACT
ACA TTC ACG TTC GGC TCG GGG ACC AAG CTG GTG ATC AAA GGT GGC GGT GGC TCG GGC
GGT GGT GGG TCG GGT GGC GGC GGA TCT CAG GTC CAA CTG CAG SAG TCW GGA GCT GAG
CTG ATG AAG CCT GGG GCC TCA GTG AAG ATA TCC TGC AAG TCT ACT GGS TAC ACA TTC
AGT GAC TAC TGG ATA GAG TGG GTA AAG CAG AGG CCT GGA CAT GGC CTT GAG TGG ATT
GGA CAG ATT TTA CCT GGA AGT GGT AGT ACT AAC TTC AAT GAG AAG TTC AAG GGC AAG
GCC ACA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG CAG CTC AAC AGC CTG
ACA TCT GAG GAC TCT GCC GTC TAT TTC TGT GCA AGA AGG AAA GTT GGT ACG GTG GAC
TAC TGG GGC CAA GGG ACC AGG CC G GCC AC AAA ACT CAC ACA TGC CCA CCG TGC CCA
Fse I
AAA GGG AAA CAC CTT TGT CCA AGT CCC CTA TTT CCC GGA CCT TCT AAG CCC CTG GAT CCC
BamH I
AAA TTT TGG GTG CTG GTG GTG GTT GGT GGA GTC CTG GCT TGC TAT AGC TTG CTA GTA ACA
GTG GCC TTT ATT ATT TTC TGG GTG AGG AGT AAG AGG AGC AGG CTC CTG CAC AGT GAC
TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC CGC AAG CAT TAC CAG CCC TAT
GCC CCA CCA CGC GAC TTC GCA GCC TAT CGC TCC *AGA GTG AAG TTC AGC AGG AGC GCA GAC*
GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC GAG CTC AAT CTA GGA CGA AGA
GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC CCT GAG ATG GGG GGA AAG CCG
CAG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA CTG CAG AAA GAT AAG ATG GCG
GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG AGG GGC AAG GGG CAC GAT GGC
CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC GAC GCC CTT CAC ATG CAG GCC
CTG CCC CCT CGC TAA GAA TTC ATA
EcoR I

Sequence of the cTCR-gene with leader

CACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT AAG GGG
TOPO overhang
CTC ACA GTA GCA GGC TTG AGG TCT GGA CAT ATA TAT GGG TGA CAA TGA CAT CCA CTT TGC
CTT TCT CTC CAC AGG TGT CCA CTC CGA CAT CCA GCT GAC CCA GTC TCC AGC CTC CCT ACT
ATC TGC ATC TGT GGG AGA AAC TGT CAC CAT CAC ATG TCG AGC AAG TGG AAA TAT TCA
CGA TTA TTT AGC ATG GTA TCA GCA GAA ACA GGG AAA ATC TCC TCA GCT CCT GGT CTA
TAA TGC AAA AAC CTT AGC AGA TGG TGT GCC ATC AAG GTT CAG TGG CAG TGG ATC AGG
AAC ACA ATA TTC TCT CAA GAT CAA CAG CCT GCA GCC TGA AGA TTT TGG GAG TTA TTA
CTG TCA ACA TTT TTG GAG TAC TAC ATT CAC GTT CGG CTC GGG GAC CAA GCT GGT GAT
CAA AGG TGG CGG TGG CTC CGG CGG TGG TGG GTC CGG TGG CGG CGG ATC TCA GGT CCA
ACT GCA GSA GTC WGG AGC TGA GCT GAT GAA GCC TGG GGC CTC AGT GAA GAT ATC CTG
CAA GTC TAC TGG STA CAC ATT CAG TGA CTA CTG GAT AGA GTG GGT AAA GCA GAG GCC
TGG ACA TGG CCT TGA GTG GAT TGG ACA GAT TTT ACC TGG AAG TGG TAG TAC TAA CTT
CAA TGA GAA GTT CAA GGG CAA GGC CAC ATT CAC TGC AGA TAC ATC CTC CAA CAC AGC
CTA CAT GC AGC TCA ACA GCC TGA CAT CTG AGG ACT CTG CCG TCT ATT TCT GTG CAA GAA
GGA AAG TTG GTA CGG TGG ACT ACT GGG GCC AAG GGA CCA GG CC G GCC AC AAA ACT CAC
Fse I
ACA TGC CCA CCG TGC CCA AAA GGG AAA CAC CTT TGT CCA AGT CCC CTA TTT CCC GGA
CCT TCT AAG CCC CTG GAT CCC AAA TTT TGG GTG CTG GTG GTG GTT GGT GGA GTC CTG GCT
BamH I
TGC TAT AGC TTG CTA GTA ACA GTG GCC TTT ATT ATT TTC TGG GTG AGG AGT AAG AGG
AGC AGG CTC CTG CAC AGT GAC TAC ATG AAC ATG ACT CCC CGC CGC CCC GGG CCC ACC
CGC AAG CAT TAC CAG CCC TAT GCC CCA CCA CGC GAC TTC GCA GCC TAT CGC TCC *AGA GTG*
AAG TTC AGC AGG AGC GCA GAC GCC CCC GCG TAC CAG CAG GGC CAG AAC CAG CTC TAT AAC
GAG CTC AAT CTA GGA CGA AGA GAG GAG TAC GAT GTT TTG GAC AAG AGA CGT GGC CGG GAC
CCT GAG ATG GGG GGA AAG CCG CAG AGA AGG AAG AAC CCT CAG GAA GGC CTG TAC AAT GAA
CTG CAG AAA GAT AAG ATG GCG GAG GCC TAC AGT GAG ATT GGG ATG AAA GGC GAG CGC CGG
AGG GGC AAG GGG CAC GAT GGC CTT TAC CAG GGT CTC AGT ACA GCC ACC AAG GAC ACC TAC
GAC GCC CTT CAC ATG CAG GCC CTG CCC CCT CGC TAA GAA TTC ATA
EcoR I

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