Universität für Bodenkultur Wien

MASTERARBEIT

“Protein synthesis and detection in a wheat germ derived *in vitro* protein synthesis system”

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Wien, 27.12.2016

Acknowledgements

I would like to thank Darren Tan for the enthusiasm he showed in supporting me with the work related to this thesis. Also, to Eva-Kathrin Sinner for enabling me to pursue this work in a field of sciences that matches my personal interests and fascination. My work would have been much more difficult without the supportive and encouraging discussions with all group members. Among these, a special thank you goes to Fabio Bisaccia for his never ending fascination for science.

Abstract

Membrane proteins perform important tasks like communication and transport in and between single cells. Cells that lack functional membrane proteins will cease to work properly, resulting in a variety of human diseases. Membrane protein research is thus a necessity for the progress of developing medical treatment. Synthetic production of membrane proteins could replace the demanding task of protein extraction from cells. *In vitro* protein synthesis describes the process of protein expression without living cells. Supplementing the reaction with membranes leads to integration of the proteins into them, so providing a minimal cell membrane model consisting of a membrane with embedded protein. Yet, it is not clear how this integration process occurs. Establishment of reproducible methods for *in vitro* protein expression and detection is critical for research in this field. The choice of expression system, among many available, and the design of a suitable expression vector are the first steps towards successful *in vitro* protein synthesis. From available eukaryotic systems, one derived from wheat germ was chosen due to its comparatively low levels of contaminating messenger RNA. Protein detection with Western blot has proven to be inadequate in this case, as the specificity of most antibodies used is not high enough and multiple reaction components interfered with the desired binding events. The incorporation of fluorophore-labelled lysine into the proteins produced in the *in vitro* synthesis reaction seems to be a more suitable method. Reproducible expression and detection of the AIDS-related protein CD4 was achieved this way.

1. Introduction

1.1. What are membrane proteins?

The number of cells in the human body is approximately 3.72 x 10^{13} (1). A cell can be regarded as a functional unit of life in eukaryotes. This functional unit can be further divided into functional subunits, the cell organelles (Figure 1). Both single cells and cell compartments are surrounded by a bilayer of lipid molecules - the cell membrane. A cell membrane thus represents a border between systems that can vary in multiple characteristics.

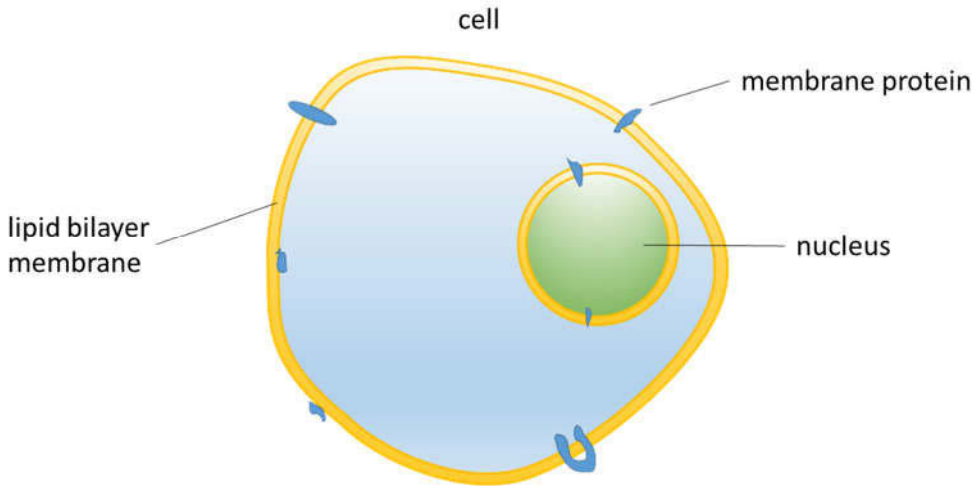

Figure 1 Schematic of a cell surrounded by a lipid bilayer membrane with proteins embedded in or attached to the membrane. As a representative for a cell organelle, the nucleus is shown. It is also surrounded by a membrane with membrane proteins.

At these borders, molecules with functions like energy storage or messengers need to be transported into and out of the cell. This task is accomplished by proteins located on or in the cell membranes. These membrane proteins are encoded by as much as 30% of the human genome (2). Proteins that span the bilayer are called integral membrane proteins or transmembrane proteins, whereas proteins located on the surface of a membrane, or embedded in only one of the layers of the bilayer are called peripheral membrane proteins.

Their variability and abundance leads them to be involved in many diseases. As such, they also have an important role in disease and medication. Half of therapeutics present on the market target membrane proteins (2).Still, much more research needs to be done in order to continuously improve on existing therapeutics.

1.2.Membrane protein research

To carry out such studies, living cells that express proteins of interest can be used. To obtain sufficient amounts of the desired protein, such cells have to express it at high levels.

Since membrane proteins occur at a low concentration in their natural environment, overexpression is indispensable. This is achieved by transfecting cells with plasmid or viral DNA that carries the gene of interest under the control of a powerful promoter. Unfortunately, membrane protein overexpression is connected to particular difficulties: (i) non-specific interactions with other membrane proteins; (ii) pore-forming activities and (iii) overloading of the transport system can lead to significant stress for the host cells. High-expression clones will thus have a low chance of surviving in cell culture (3). Further issues connected to cell-line maintenance and the complexity of a cell culture system gave rise to the idea of artificial vesicles with proteins incorporated into the vesicle membrane as an alternative. With the membrane protein of interest being the only protein present, non-specific interactions can be eliminated. Furthermore, interference from a host cell's native mechanisms are avoided by using a non-living system. A model for studying membrane protein behaviour could basically be made up of two parts: a vesicle and the membrane protein incorporated in its membrane.

1.3.Protein translocation

But how does a protein even get into the membrane in cells? In living cells, synthesis of membrane proteins takes place at the ribosomes, which are located at the endoplasmic reticulum (ER). The ER is a complex structure consisting of bilayered lipid membranes. From the ribosomes, the nascent protein is inserted into the ER membrane in a process called translocation. Translocation usually already starts during protein synthesis; the process is thus referred to as co-translational translocation. The exact mechanism of the translocation depends on the number of times the protein spans the membrane. Single-pass membrane proteins carry an N-terminal signal sequence that directs them to the ER membrane. They also have an internal stop-transfer membrane-anchor sequence. This sequence remains in the membrane due to its hydrophobic character (Figure 2). It is called the transmembrane domain.

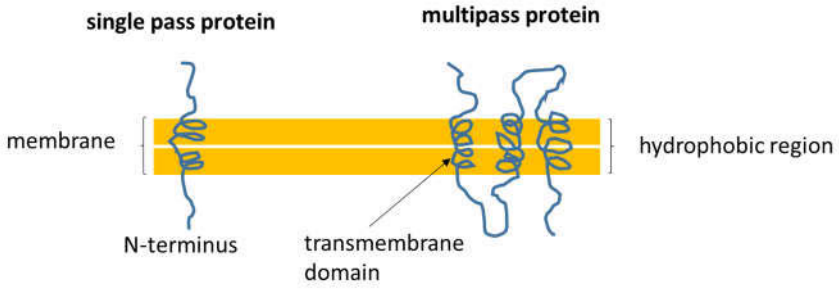

Figure 2 Transmembrane proteins with varying numbers of membrane spanning domains. The part of the protein that is embedded in the hydrophobic membrane has a hydrophobic character.

The N-terminal signal sequence is recognized by the signal recognition particle (SRP). This ribonucleoprotein consists of a single 300-nucleotide RNA and six proteins of different molecular masses (4). Binding of the SRP to the signal sequence, that emerges from the ribosome, results in formation of the ribosome-nascent-chain complex (RNC) which associates with the ER. The RNC is recognized by the SRP receptor, a protein in the ER membrane. Elongation of the polypeptide chain is then arrested. Both SRP and SRP receptor dissociate from the RNC and the polypeptide is then transferred to the multi-protein complex called the translocon. The mammalian translocon consists of the protein complex comprising Sec61 and TRAM (translocating chain-associated membrane) protein which are ER membrane spanning proteins (5).

The translation of the protein continues until its cytosolic domain is complete. When a protein has more than one membrane-spanning domain, it is called a multi-pass protein. Its first transmembrane domain will be recognized as a signal-anchor sequence. It initiates translocation into the membrane. Translation and insertion of the protein continues, until the second transmembrane domain is translated. It is integrated into the membrane and serves as a stop-transfer membrane-anchor sequence. The transfer is arrested and translation continues. When a third transmembrane domain is recognized, translocation starts again. The fourth transmembrane domain serves as the next stop-transfer signal, and so on (5).

The required energy for this whole process is provided by GTP hydrolysis accomplished by SRP and SRP receptor. Once the translocation process is completed, the proteins are subject to targeted transport to their final destination, which can be within the ER membrane itself or any other part of the cell's membrane system (5). The final product is a fully functional membrane protein integrated with a membrane.

This process of protein insertion is crucial for constructing an artificial membrane-protein system. However, the first thing to start with is the construction of an artificial membrane.

1.4. Artificial membranes

Synthetic vesicles mimic their natural counterpart in both the amphiphilic structure of their monomers as well as the behaviour of self-assembly by which the monomers spontaneously form well-organised structures (6).

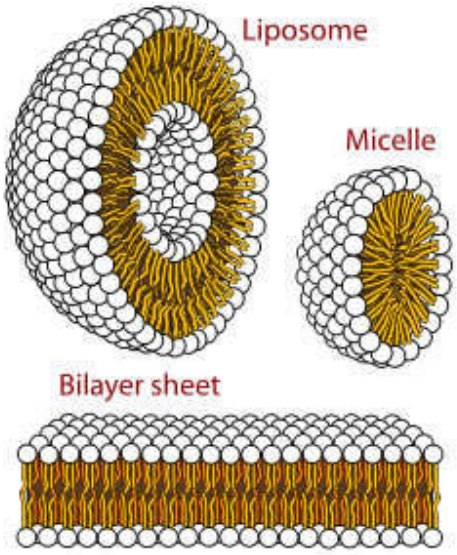

Figure 3 Structures formed from self-assembly of amphiphilic molecules. A bilayer sheet has the lipid bilayer structure of a cell membrane. Liposomes and micelles can spontaneously form upon exposure of amphiphilic monomers to an aqueous environment. The monomers' hydrophilic headgroups forms a polar shell. The polarity of the core of the vesicle (micelle or liposome form) depends both on the lipid structure and the environment (7).

Instead of lipid molecules, different amphiphilic polymers can be used to form the structures described in Figure 3. Amphiphilic polymers undergo self-assembly to form a structure called "polymersomes". Their behaviour in this respect thus equals liposomes. However, the polymers that are used for the synthesis of described polymersomes result in higher membrane thickness and increased stability (8). Biocompatible and biodegradable examples of such polymersomes have been reported. They could therefore provide an alternative to liposomes in various aspects of medical treatment (9) (10). A polymersome that carries membrane proteins of interest on its surface presents a minimal cell membrane model. It is reduced to the two parts that define the system: a spherical membrane system and the protein embedded in it. To synthetize an artificial membrane protein is a task that is to be accomplished by the means of synthetic biology. A protein can be synthesized without a cell in a so-called *in vitro* protein synthesis approach.

1.5. *In vitro* protein synthesis

Proteins in cells are synthesized at the ribosomes of the rough endoplasmatic reticulum. The ribosomes as well as other components that are required for successful protein synthesis can be purified and used to make proteins from a DNA template (Figure 4). *In vitro* protein synthesis (IVS) systems usually comprise a polymerase, amino acids, as well as an extract from either *E. coli*, rabbit reticulocytes or wheat germ (11). Upon addition of adequate template DNA, a protein can be synthesized in such mixtures.

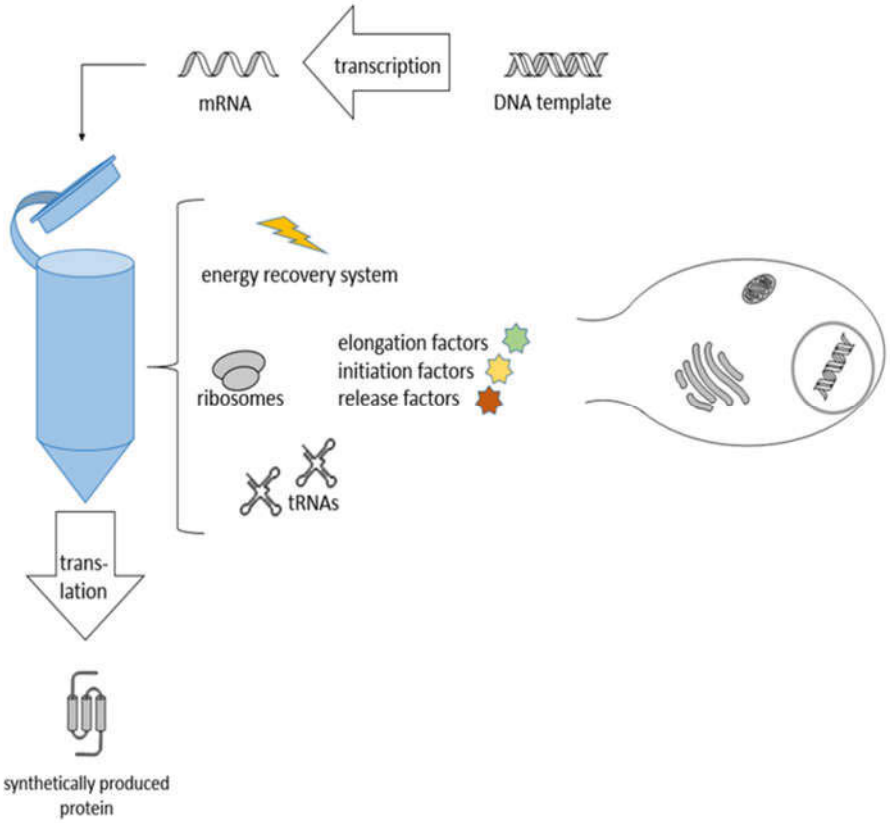

Figure 4 *In vitro* protein synthesis. The whole system consists of a cell extract and supplemented components.

Avoiding the need for host cells and the expression of proteins that would be toxic to a cellular environment, are the main advantages of using IVS. However, the attempt to isolate certain biological functions from such a reaction mix comes with certain difficulties. The presence of cellular proteins, nucleic acids and probably membrane compounds makes the environment for IVS an undefined one. The way in which these compounds interfere with the protein synthesis is not predictable. A step towards control has been made with the development of the PURE system by Shimizu et al. Instead of using crude cell extract, only the ribosomes and t-RNAs present in the system are derived from an *E. coli* cell extract. The other proteins contained in this system are produced recombinantly

and carry a His-tag. Due to this tag, the synthesized protein can be purified by removing these reaction components by affinity chromatography.

If a membrane protein is to be produced with such an *in vitro* system, it ought to be attached to or integrated into a membrane in order to fold and orientate correctly. For most of the *in vitro* protein synthesis systems described above, the expression of membrane proteins in the presence of membrane forming substances has been reported (12).

1.6. *In vitro* membrane-assisted protein synthesis (iMAPS)

When a membrane protein is produced in the presence of amphiphilic bilayer structures, their spontaneous integration into the structure has repeatedly been reported. For example, the light-harvesting complex II protein (LHCII) has been shown to integrate into polymeric membrane structures (13). This phenomenon is not understood yet.

Understanding of why and how membrane proteins are integrated into artificial membranes would lead to an advance in membrane protein research. On the one hand, understanding of the insertion process would facilitate the synthesis of artificial vesicles with embedded membrane proteins. On the other hand, understanding of the insertion process would provide important basic information about how it occurs, presumably without translocation machinery. The term "*in vitro* membrane-assisted protein synthesis (iMAPS)" is suggested for this process. iMAPS, therefore, is IVS carried out in the presence of artificial membranes (13).

For these reasons, our general aim is to investigate and understand the process of co-translational insertion during *in vitro* protein synthesis. Probing for components of the translocation machinery, that might influence the insertion process, in our reaction mixes will be carried out in the course of the project. This will be performed using electron microscopy to detect membranous structures as well as antibodies targeted against components of the translocation machinery. Comparisons of *in vitro* protein synthesis reactions with and without supplemented membranes will also be performed.

In order for this work to be feasible, reproducible membrane protein synthesis and detection needs to be achieved. In order to determine how reliable our processes are, the detection methods we hope to use need to be validated first. This was the specific aim of my work.

1.7. Proteins included in this study

This study is concerned with four membrane proteins that show a varying number of membrane spanning domains. Claudin 2 is a protein of the claudin family that constitute the main components of tight junctions (14). The term “tight junction” refers to special contact areas between two cells that are formed by proteins such as claudin and provide controlled mass transport and polarity between neighbouring cells. The three other proteins are CD4, CXCR4 expressed on T-helper cells and CCR5 expressed on macrophages of the immune system. These proteins play a major role in the infection of these cells with the human immunodeficiency virus (HIV). CD4 (cluster of differentiation 4) is a single-pass membrane protein whose natural function in the cell is to recognize and bind the antigen-presenting major histocompatibility complex II (MHCII). Upon infection of the body with HIV, CD4 serves the virus as a receptor for attachment onto host cells (15). If either one of its co-receptors, the CXC-motif chemokine receptor 4 (CXCR4) or the CC-motif chemokine receptor 5 (CCR5), are present, the virus might be able to penetrate the cell and use it for its reproductive cycle (16). CXCR4 and CCR5 belong to the class of G-protein coupled receptors (GPCRs). They both have seven transmembrane domains (16).

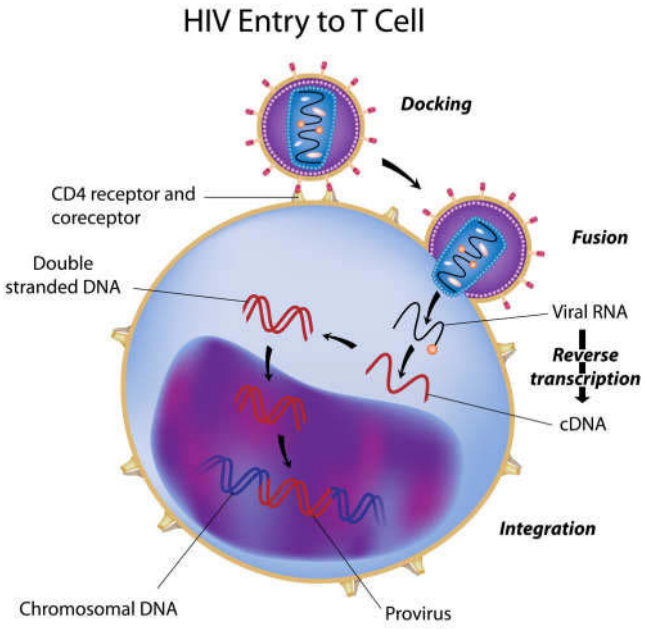

Figure 5 HIV entry mediated by CD4 and a GPCR chemokine receptor, which can be either CXCR4 or CCR5 depending on the type of virus (18)

1.8. General experimental approach

For producing these proteins, cloning work for integrating the DNA coding for the target protein sequences into appropriate expression vectors was performed. These expression vectors were used for *in vitro* protein synthesis. Supplementing these synthesis reactions with membranes ought to lead to protein integration. These will be added in the form of polymersomes. Comparing reactions with or without membranes can provide information on the membranes' influence on protein location. It is assumed that in the presence of membranes, the protein will integrate into them (Figure 6).

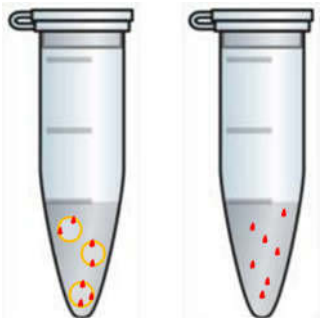

Figure 6 *In vitro* protein synthesis with and without polymersomes. It is assumed that in the presence of polymersomes, proteins integrate into the membrane.

A straight forward way of monitoring the proteins' location would be to compare the quantity of free protein in the samples as shown in Figure 6. If the membrane proteins preferably integrate into the membranes provided, the level of free protein in solution will be low. Proteins integrated into the membranes can be separated by ultracentrifugation and pelleting of the membranes. Consequently, a reliable method of protein detection is needed. Only then will it be possible to compare relative protein quantities from *in vitro* protein synthesis, under different conditions that might assist or inhibit co-translational insertion.

1.9. Specific experimental approach

Quality control of the protein detection process is therefore an important part of the iMAPS protocol. Only if protein detection for the *in vitro* synthesis reaction mix is reliable can statements about potential insertion into membranes be made.

For insertion-related experiments, only un-modified proteins should be used, in case the modifications influence the insertion process. However, for the early stages of the project and to evaluate the success of the protein syntheses *per se*, proteins with a His-tag were

included. Tags are commonly used for detecting proteins, due to the availability of high-affinity antibodies that target tags.

In order to facilitate detection of the protein produced by IVS, proteins in the IVS reaction mix were first partially separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, Western botting was used to detect the protein of interest specifically. When this proved inconsistent, the less specific method of Coomassie Blue staining was performed. Eventually, detection of synthesized proteins via incorporated fluorophore-labelled amino acids was used.

Whether an experiment yields a signal indicating the presence of synthesized protein depends on the processes of 1) protein synthesis 2) protein preparation and 3) protein detection (Figure 7).

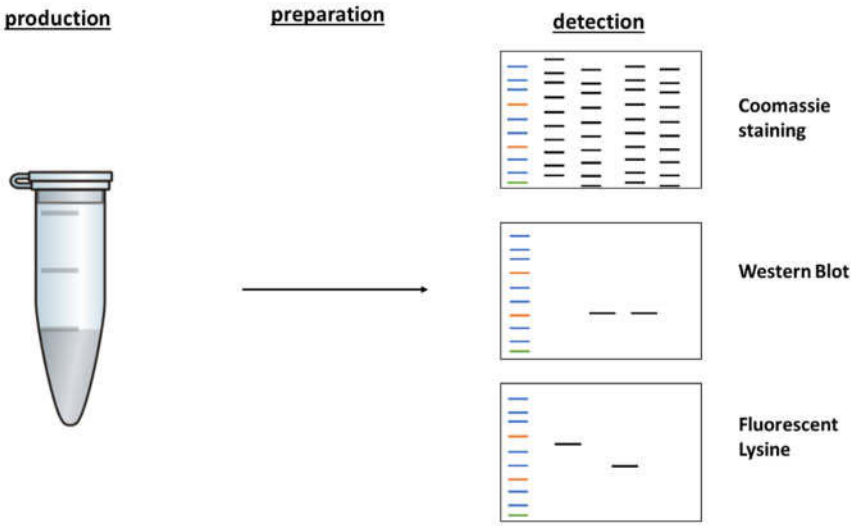

Figure 7 The three critical steps in the course of the study are protein production, preparation and detection. Inconsistency in one or more of these steps leads to the inability of drawing conclusions from the experiments.

Only the last step, protein detection, can directly be observed. If protein detection is prone to errors, no statements about the success of protein synthesis or protein preparation can be made. Comparative studies that involve IVS reactions under different conditions require detection methods that provide clear conclusions about the experiment's outcome. Validation of protein detection methods was thus the specific aim of this work.

2. Materials and Methods

2.1.Cloning of the inserts into the pTNT vector

The *in vitro* synthesis of a protein requires the gene that encodes the protein, including a promoter that can be recognized by the RNA polymerase used in the system. The genes of the proteins of interest were cloned into pTNT® plasmids (Promega). These plasmids carry a T7 promoter, for expression of circular DNA, and a multi-cloning site (MCS). Xho I and Not I in the MCS were chosen for inserting the genes of interest into the pTNT® vectors.

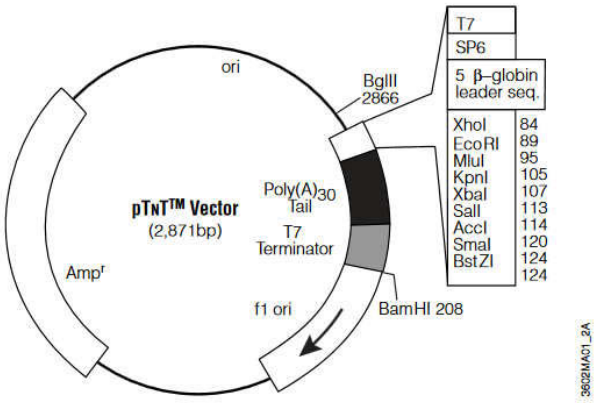

Figure 8 pTNT® vector with restriction sites, ampicillin resistance and origin of replication.

2.1.1. PCR reaction

Each gene of interest must be flanked by Xho I and Not I restriction sites in order for it to be inserted into the MCS. Polymerase chain reactions (PCR) were carried out in order to add the sequences for these two restriction sites to each gene of interest, as well as to produce sufficient amounts of each modified gene for insertion into the pTNT® vectors. This was done by designing primers that include the sequences for Xho I (forward primer) and Not I (reverse primer).

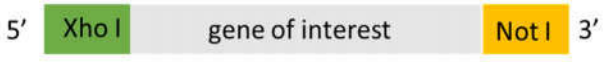

Figure 9 Gene of interest with Xho I at the 5' end and Not I at the 3' end.

The primers for CD4, CXCR4 and CCR5 were ordered from Integrated DNA Technologies. Apart from the His-tagged CXCR4, one more tag-carrying protein was used. The protein CLDN2 with a VSV-tag was used as a control for synthesis of CLDN2. The primers for

CLDN2-VSV and His-tagged CXCR4 (His-CXCR4) were ordered from Sigma-Aldrich. Because six codons for the six histidine amino acids as well as the Xho I restriction site needed to be added to the 5' end of CXCR4, two forward primers for His-CXCR4 were designed with overlaps, for two subsequent rounds of PCR. This was done to avoid using primers more than 30 bases long. One forward primer that adds part of the His tag and a second forward primer that adds the rest of the His tag as well as the restriction site were designed. The reverse primer was the same for both PCR reactions.

Figure 10 Addition of a 5' His-tag comprising six histidine residues, a 5' Xho I restriction site and a 3' Not I restriction sites in two rounds of PCR. In round 1, part of the His-tag is added to the 5' end of the CXCR4 gene. In round 2, the rest of the tag as well as the Xho I restriction site was added.

Table 1 Primers for the amplification of genes of interest. Highlights in yellow indicate the Xho I restriction site sequence, in blue indicates the Not I restriction site and in green the sequences encoding histidine.

Construct	Primer	Sequence
pTNT-CD4	Forward	ATACTCGAGCCCTGCCATTTCTGTGG
	Reverse	ATAGCGGCCGCCTCTAGAGG
pTNT-CXCR4	Forward	ATACTCGAGTAACGGCCGCC
	Reverse	ATAGCGGCCGCGTGATGG
pTNT-CCR5	Forward	ATACTCGAGGATGGATTATCAAGTG
	Reverse	ATAGCGGCCGCACAACTC
pTNT-CLDN2	Forward	ATACTCGAGGGCCTCTCTTGGCC
	Reverse	ATAGCGGCCGCTCACACATACCC
pTNT-His-CXCR4	Forward I	CATCATCATCATATGGAGGGGATCAGTAT
	Forward II	ATACTCGAGATGCATCATCATCATCATCATATGGA
	Reverse	ATAGCGGCCGCTGCCTAGACACACATC

The PCR reaction mix contained 10x PCR buffer (Sigma Aldrich P2192), 200 µM deoxynucleotide mix (Sigma Aldrich, D4788, D4913, D5038, T9656), 0.5 µM of each primer, 3% v/v DMSO (omitted for His-CXCR4), 0.025 U/µL Taq polymerase (Sigma Aldrich D6677, D1806) and 200 pg/µL template DNA in plasmid form. Donor plasmids encoding each gene were used as template DNA for PCR. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pT4B (Cat# 157) from Dr. Richard Axel (19) (donor plasmid for CD4). The following reagent was

obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pc-Fusin (Cat# 3326) from Dr. Nathaniel Landau (20), (21) (donor plasmid for CXCR4). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pcCCR5 (Cat #3325) from Dr. Nathaniel Landau (20), (21) (donor plasmid for CCR5). For cloning CCR5 into the pTNT vector, Phusion buffer (Thermo Fisher Scientific F518) and Phusion Polymerase (Thermo Fisher Scientific F530S) were used. The annealing and extension temperatures were optimized for high levels of specific amplification and low unspecific amplification resulting from mispriming. The reactions were carried out in a thermocycler (Peqlab, Primus 96 advanced).

Table 2 PCR reaction conditions

1	Lid on 99°C								
2	95°C						30 sec		initial denaturation
	CD4	CXCR4	CCR5	CLDN2	His-CXCR4 I	His-CXCR4 II			
3	95°C						30 sec	**30x***	**denaturation**
4	55°C	62,4 °C	56°C	57,8 °C	64°C	62,5 °C	30 sec		**primer annealing**
5	60,4°C	60°C	60°C	57,8°C	61,9°C	65°C	3min		**extension**
6	60,4°C	60°C	60°C	57,8°C	61,9°C	65°C	5min		final extension
7	Cool down to 4°C						1°C per sec		
8	storage at 4°C								

*for His-CXCR4, 32 cycles were run.

2.1.2. Agarose gel electrophoresis of PCR products

The PCR amplicons were electrophoresced on a 1% agarose gel (Sigma Aldrich, A9539-5006) in TAE buffer (Gibco, 2410-030) at 85 V for 45 mins. The electrophoresis was carried out with the Powerpack300 from Biorad. For staining the DNA, the gel was supplemented with SYBR® Safe (Thermo Fisher Scientific, S33102) at a ratio of 1:10000 v/v. Prior to loading onto the gel, the samples were diluted with DNA Gel loading dye (Thermo Fisher Scientific, R0611) at a ratio of 1:6 v/v for visual tracking of DNA migration. For size determination, 5 µL of GeneRuler 1 kB Plus (Thermo Fisher Scientific, SM1331) was used as a molecular weight ladder. For visualization of the stained DNA, a transilluminator (Vilber, CN 3000) was used. Successful amplification was determined by the presence of DNA at the expected amplicon size.

2.1.3. Gel extraction of PCR products

The modified genes must first be purified from the agarose gel before they can be inserted into the pTNT® vector. For extracting the DNA from the agarose gel, the MiniElute Gel Extraction Kit from QIAGEN (28604) was used. The DNA fragment was excised from the agarose gel and weighed. Three volumes of Buffer QG were added to the gel (100 mg gel correspond to 100 µL). The sample was incubated at 50°C with thorough shaking. Additional vortexing ensured complete dissolving of the gel. One gel volume of isopropanol was added and the sample mixed by inverting several times. The sample was loaded onto a MiniElute column (provided) and centrifuged for 1 min. If the sample volume was higher than 800 µL, the column was loaded and centrifuged several times. The flow-through was discarded after each step. 500 µL of Buffer QG were loaded onto the column and centrifuged for 1 min. The flow-through was discarded and 750 µL of Buffer PE were added. After 1 min centrifugation, the flow-through was discarded and the empty column centrifuged for 1 min to remove residual ethanol from the column. For elution, 10 µL H_2O was added onto the column and after 1 min, the column was centrifuged for 1 min. The concentration of the DNA solution was determined with a spectrophotometer (Thermo Fisher Scientific, Nanodrop 2000 Spectrophotometer).

2.1.4. Restriction digestion of PCR product

The purified PCR products with the attached restriction sites had to be digested with Xho I and Not I in order to create complementary overhangs needed for the ligation of the PCR product and the pTNT® vector backbone. The amplicons were digested by Xho I (Thermo Fisher Scientific FD0694) and Not I (ThermoFisher FD0593) in FastDigest buffer (Thermo Fisher B72) according to the manufacturer's protocol (22) at 37°C for 15 min.

2.1.5. Ethanol precipitation of digested PCR product

To remove the restriction enzymes as well as buffer salts from the restriction digestion reaction, the restricted amplicons were purified by ethanol precipitation. After addition of 1/3 vol of 3 M sodium acetate, pH = 5.2, 2 vol absolute ethanol were added and the solution was centrifuged at 184, 000 x *g* for 20 min (Hermle Z 233 MK). The supernatant was carefully discarded and the pellet was washed with 1 mL 70% ethanol. The sample was centrifuged again for 15 min at 184, 000 x *g*. Part of the supernatant was carefully discarded, the rest was dried at 65°C under vacuum (Thermo Fisher Scientific, Savant ISS110 SpeedVac Concentrator) to prevent accidental discarding of the pellet. The pellet was dissolved in 10 µL nuclease-free H_2O and used as inserts for ligation with the pTNT vector backbone.

2.1.6. Restriction digestion and purification of the pTNT vector backbone

The pTNT backbone was digested with Xho I and Not I as described in section 2.1.4 Restriction digestion of PCR product. The resultant reaction mix was electrophoresced (using 1% agarose in TAE as described above. The digested plasmid was then purified by gel extraction using the QIAGEN kit as described.

2.1.7. Ligation of PCR product and pTNT backbone

The mass ratio of pTNT backbone to insert was 1:3. For ligation, T4 ligase buffer (Thermo Fisher Scientific B69) and T4 ligase (Thermo Fisher Scientific EL0011) were used according to the manufacturer's protocol (23). The ligation was performed at 16°C over night.

2.1.8. Transformation of chemically-competent bacteria

One Shot® TOP 10 chemically-competent *E. coli* (Thermo Fisher Scientific C404010) were transformed with the ligation product according to the manufacturer's protocol (24). For the transformation, 5 µL of the ligation product was used. The transformation process allows plasmid DNA to enter the bacterial cells after their membranes have been made permeable by heat shock. As a control to see if the transformation had worked, either 200 ng of pTNT-CLDN2-VSV (for transformation of ligation products carrying CD4, CXCR4 and CCR5) or 200 ng of the empty pTNT vector (for transformation of ligation products carrying CLDN2 and CXCR4-His) were used. To exclude contamination with ampicillin-resistant bacteria that do not carry the desired plasmid, a control sample with 5 µL of H_2O added to the bacteria was used for transformation. 200 µL of the reactions were plated onto LB agar (LB broth base from Thermo Fisher Scientific, 12780-052, agar from Sigma Aldrich, A5306) supplemented with an ampicillin (Sigma Aldrich, A0166) concentration of 100 µg/mL. The plates were incubated at 37°C overnight. Colonies from the transformed bacteria were used to inoculate liquid cultures.

2.1.9. Inoculation of bacteria and expression plasmid extraction

The colonies were each inoculated in 5 mL LB medium with 100 µg/mL ampicillin and incubated for 16h at 37°C with shaking at 170 rpm. The expression plasmids were then isolated with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, K0502) according to the manufacturer's protocol (25) ("Protocol A. Plasmid DNA purification using centrifuges"). The DNA was eluted with 50 µL H_2O.

2.1.10. Analysis of the expression plasmids

The expression plasmids were sequenced by the company Microsynth Austria. Their restriction sites were analyzed by restriction digestion with Xho I and Not I (see "Restriction digestion of PCR produts") to see if the correct restriction sites were present and if the restriction products have the expected sizes in agarose gel electrophoresis.

2.2. *In vitro* protein synthesis

2.2.1. *In vitro* protein synthesis with a wheat germ extract kit

The expression plasmids encoding different proteins of interest were used for *in vitro* protein synthesis, with the TNT® T7 coupled Wheat Germ Extract System (Promega, L4140, Lot 0000126248, Lot 0000155365, Lot 000019763). The *in vitro* protein synthesis was carried out according to the manufacturer's protocol (without the use of [^{35}S]) (26). In some samples, the *in vitro* protein synthesis was carried in the presence of fluorescent lysine (Promega, L5001). This supplement consists of a t-RNA loaded with lysine that is coupled to a fluorophore. The incubation time for the reaction mix was typically 90 min at 30°C. In some experiments, the incubation was carried out overnight at room temperature. This was done to test if changed incubation conditions could lead to a difference in protein expression. In other experiments, the incubation was carried out at 37°C for one hour to test if increased incubation temperatures can change the protein expression.

2.2.2. *In vitro* protein synthesis with a rabbit reticulocyte kit

To compare the plant-derived wheat germ extract system with a protein synthesis system derived from mammalian cells, an extract from rabbit reticulocytes (Promega, L4611) was also tested. The *in vitro* protein synthesis reaction was carried out according to the manufacturer's protocol (27) using the expession plasmid for CCR5. The incubation was performed for 90 min at 30°C.

2.2.3. SDS-PAGE

To determine if the desired proteins had been successfully synthesized, it was necessary to partially separate them from contaminating proteins in the *in vitro* synthesis reaction mix. In this process, a uniform negative charge is introduced to all proteins by adding sodium dodecyl sulfate (SDS) to the IVS mixture once the synthesis reaction is completed. In our experiments, lithium dodecyl sulfate was used instead of sodium dodecyl sulfate, as its precipitation occurs at higher concentrations compared to SDS. However, the process will still be referred to as “SDS-PAGE”. The association of LDS molecules with protein molecules imparts a negative charge to the molecular aggregate formed. This negative

charge is significantly greater than the protein's original charge. On average, all proteins now have an equal, negative charge density. Hence, when an electric field is applied during polyacrylamide gel electrophoresis (PAGE), the proteins' movement through this field is only determined by their size - smaller proteins show higher migration velocities. In a subsequent staining process with Coomassie Blue the protein bands that form can be visualized and their size determined by the position of the protein on the gel. Proteins will appear as visible bands on the gel when stained, allowing for qualitative detection. Samples were first treated with a reducing reagent (NuPAGE Sample Reducing Agent 10x, Thermo Fisher Scientific, NP0004) and an SDS-containing reagent (NuPAGE LDS sample buffer 4x, Thermo Fisher Scientific, 525186). Subsequently, the samples were incubated at 70°C for 10 min. Up to 25 µL of sample were loaded onto a 10% Bis-Tris gel (NuPAGE 10% Bis-Tris gel, Thermo Fisher Scientific, NP0301BOX). PAGERuler™ Plus (Thermo Fisher Scientific, 26619) was used as a molecular weight reference. A voltage of 180 V was applied to the gel ("Powerpack 300", Biorad) for electrophoresis.

2.2.4. Detection of fluorescent lysine

For the detection of proteins with incorporated fluorescent lysine, the gel was exposed to light with a wavelength of 470 nm (Invitrogen, Safe Imager™) following electrophoresis. Any resultant fluorescence in the gel was imaged using a CCD camera.

2.2.5. SimplyBlue™ staining

SimplyBlue™ SafeStain solution (Thermo Fisher Scientific, LC6060) is a dye that binds to alkaline side chains of amino acids and leads to unspecific staining of proteins. Following electrophoresis, the Bis-Tris gel with the proteins were stained with described reagent according to the manufacturer's protocol (28). Images were captured with the LI-COR Odyssey infrared scanner.

2.2.6. Western blot

For specific detection of proteins, Western blots were performed. Following electrophoresis, the proteins in the gel were first blotted onto a polyvinylidene fluoride (PVDF) membrane. Blotting of the proteins was carried out with the iblot™ dry blotting system (Thermo Fisher Scientific, IB401002) with a seven-minute blotting program. During this time, an electric field is used to transfer the proteins from the gel onto a polyvinylidene fluoride membrane. All areas of the membrane not blotted with a protein were then blocked from additional blotting with a blocking solution (LI-COR, 927-40000) at room temperature. The blocking buffer was then removed and a primary antibody binding to one or more epitopes of the protein was applied. The membrane was incubated with the primary

antibody overnight at 4°C. It was then washed three times for 7 min each in phosphate-buffered saline (PBS) supplemented with 0.01% v/v Tween20. A secondary antibody that is labelled with a fluorophore, and which is specific to the species of the primary antibody host, was then applied. The membrane was incubated with the secondary antibody for 2 h at room temperature. The membrane was washed twice in PBS with 0.01% v/v Tween20 for 7 min each and, finally, once in PBS for 7 min to remove excess Tween20. An image was captured after excitation of the fluorophore. The fluorophore coupled to the anti-rabbit secondary antibody has its maximum excitation wavelength at 700 nm and the anti-mouse secondary antibody at 800 nm.

The diagram in Figure 11 shows the protein bovine serum albumin (BSA) being detected via Western blot.

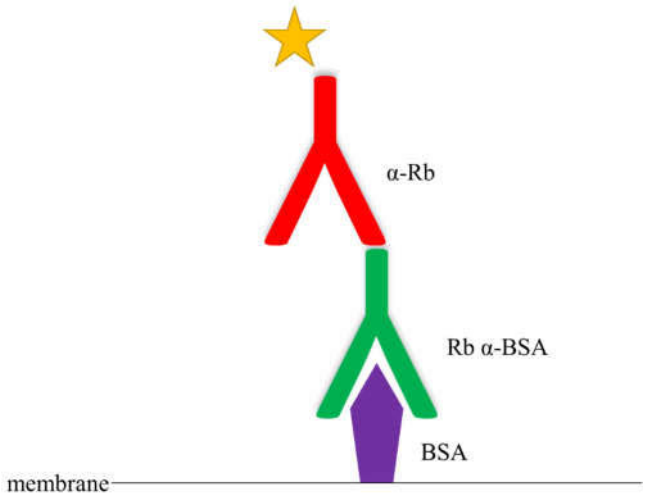

Figure 11 Detection of BSA via Western blot. Commercially obtained BSA was blotted onto a membrane and detected via binding of a set of two antibodies. The secondary antibody (red) creates a signal via a coupled fluorophore (star).

For detecting CD4, the antibody raised in mouse against CD4 (Thermo Fisher Scientific MA5-15775) was diluted 10,000 times in blocking buffer (LI-COR, 927-40000). An antibody raised in rabbit against CXCR4 was obtained from the NIH AIDS Reagent Program and used to probe for CXCR4. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS from ProSci Inc.: Anti-Human CXCR4 Polyclonal (EL) (29), (30), (31), (32), (33), (17), (34) (35), (36), (37), (38). This antibody was diluted 10,000 times in LI-COR blocking buffer. An antibody raised in rabbit against CCR5 was used to probe for CCR5. The following reagent was obtained through the NIH AIDS Reagent

Program, Division of AIDS: Anti-Human CCR5 Polyclonal (NT) (29), (39), (40), (22), (41), (42), (43), (44), (45), (46), (47). This was diluted 10,000 times in LI-COR blocking buffer. Other antibodies raised against CCR5 were purchased from Thermo Fisher Scientific (rabbit PA1-41303) and similarly diluted 10,000 times in LI-COR blocking buffer. Those from ABNOVA (rabbit PAB0177 and mouse H00001234-B02P) were diluted 200 or 1,000 times in LI-COR blocking buffer. Antibodies raised in rabbit against CLDN2 (Invitrogen, 51-6100), as well as antibodies raised in mouse against the VSV tag (Sigma Aldrich, V5507) were diluted 20,000 times in LI-COR blocking buffer. These antibodies were used for detection of the control protein, VSV-tagged light-harvesting complex II (LHCII-VSV). An antibody raised against BSA raised in rabbit (Sigma Aldrich B7276) was diluted 5,000 times in LI-COR blocking buffer.The secondary antibodies were obtained from LI-COR. Antibodies raised against rabbit antibodies (anti-rabbit: 926-68021) were diluted 30,000 times in blocking buffer (LI-COR, 927-40000. Antibodies raised against mouse antibodies (anti-mouse: 926-32210) were diluted 10,000 times in LI-COR blocking buffer.

2.3. Controls

From protein synthesis to detection by Western blot there are many working steps. Each working step constitutes a potential source of error and procedural errors might lead to inconsistent results. Inconsistent results in protein detection prevents one from drawing conclusions about the success of protein synthesis. Since Western blotting is an indirect method of detection that requires several actions before detection can take place, validation is of high importance. As a consequence, our methods of protein detection by Western blot were validated. This means that our Western blot protocols must be able to reliably detect a protein when it is present, and not when it is absent.

To do this, we have to ensure that the secondary antibodies used are able to bind to their respective primary antibodies, and that the primary antibodies used are able to bind to their respective targets. Validating the primary antibodies is critical and this is done using samples spiked with the target protein. A sample that has been supplemented with target protein is a positive control: only if the target protein is present, is the detection method supposed to yield a signal. If the target protein is not present, the detection method must not yield a signal. A sample without target protein is a negative control. To confirm a detection method's suitability, both negative and positive controls need to be used in all experiments.

2.3.1. Ascertaining ability of secondary antibodies to bind targets.

The last step in Western blotting is the binding of a fluorophore-tagged antibody. For all experiments, only two types of secondary antibodies will be used: an antibody targeting rabbit-antibodies and an antibody targeting mouse-antibodies. It will be determined if they are able to bind to their respective targets. A signal from the secondary antibody is detected only if the secondary antibody is able to bind a target, the appropriate primary antibody, on the blotted membrane and if its fluorophore is excited by the detector's radiation.

Ability of anti-rabbit antibody to bind primary antibody

We tested our anti-rabbit secondary antibody (α-Rb) by using it to detect an antibody raised against bovine serum albumin (BSA). The primary antibody used (Rb α-BSA) is rabbit-derived and can thus be recognized by the secondary antibody, α-Rb, that is targeted against rabbit-specific protein domains on the Rb α-BSA. All images were captured with the LI-COR Odyssey infrared scanner. Briefly, 150 ng BSA were dissolved in H_2O and loaded onto the gel after 10 min of incubation with NuPAGE LDS Sample buffer and NuPAGE sample reducing agent. For one sample, this incubation was carried out at room temperature, for another sample, the incubation was carried out at 70°C. These different conditions were tested in case that BSA aggregates at 70°C. Protein aggregation prevents solubilisation and therefore detection. The detection was carried out with an antibody raised against BSA (Sigma Aldrich B7276) produced in rabbit.

Ability of anti-mouse antibody to bind primary antibody

The anti-mouse secondary antibody (α-Ms) was tested in a similar kind of experiment. In this case, the primary antibody was raised in mice against the vesicular stomatitis virus (VSV) glycoprotein. The VSV-tagged protein light-harvesting complex II (LHCII-VSV), was used as the target. LHCII-VSV has been shown to be reliably produced by *in vitro* protein synthesis and reliably detected using the mouse anti-VSV antibody. A second protein that had also previously been expressed and detected in our laboratory is Claudin 2 with a VSV tag (CLDN2-VSV). The primary antibody used to probe for CLDN2-VSV is the same as that used for the detection of LHCII-VSV.

Ability of primary antibodies to bind their targets

In the case of CD4, CXCR4 and CCR5, the antibodies raised against them have not been tested in our laboratory. We needed to know if the antibodies are able to detect their respective targets when they are present and not when the targets are absent.

To test our mouse/rabbit antibody raised against CCR5, it was used to probe for commercially-obtained CCR5, as well as CCR5 that had been extracted from living cells. Validated anti-mouse/rabbit secondary antibody was then used to probe for the primary antibody being tested. These CCR5 proteins were intended to serve as positive controls for the detection of CCR5 expressed in IVS.

CCR5 protein obtained from commercial source

CCR5 protein was purchased from ABNOVA (H00001234-G01). The CCR5 protein provided by this company was produced by *in vitro* protein synthesis with wheat germ extract.

CCR5 protein extracted from A3R5.7 cells

Alternatively, CCR5 protein was stripped from A3R5.7 cells to produce the pure protein. The transformed cell line A3R5.7 expresses CCR5 under geneticin selection. A3R5.7 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID (NIH: Cat#12386, A3R5.7 from Dr. Robert McLinden) (48), (49). Lysis of the cells and protein solubilization was performed using RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma Aldrich, R0278). 1 x 10^7 cells were resuspended in 100 µL RIPA buffer supplemented with 10 µL phospholipase C (PLC) (Calbiochem, 525186, 250 U/mL),10 mM Tris-HCl, 144 mM NaCl, 0.05% BSA and 1 µL benzonase (Sigma Aldrich, E1014). The samples were incubated for 60 min at 37°C and an aliquot was taken for Western blotting. The rest of the sample was centrifuged at 9, 000 x *g* at 4°C for 5 min and an aliquot of the supernatant was taken for Western blotting. Both aliquots (before and after centrifugation) were prepared for SDS-PAGE with 10x LDS sample buffer (Thermo Fisher Scientific, 525186) and 4x reducing agent (Thermo Fisher Scientific, NP0004). Both aliquots were exposed to ultrasonic treatment with an ultrasonication probe (Branson, Sonifier 250). In total, the samples were exposed to 50 seconds of ultrasonication while being cooled with ice to avoid excessive heat. To test whether sonication and enzymatic treatment could have an effect on detectability of the ABNOVA CCR5, 300 ng ABNOVA CCR5 (6 µL) were incubated with 2 µL PLC, 0.2 µL benzonase and 11.8 µL H_2O for 60 min at 37°C. After denaturing ABNOVA CCR5 by 10 min incubation with 8 µL LDS sample buffer (Thermo Fisher Scientific, 525186) and 3.2 µL reducing agent (Thermo Fisher Scientific, NP0004), sonication was carried out. The proteins were loaded onto an SDS-PAGE gel and electrophoresced. Detection of the CCR5 with Western blotting was then performed using the antibody from ABNOVA (H00001234-B02P).

Due to time limitation and the high costs of proteins from commercial sources, these experiments were carried out for CCR5 only.

3. Results

3.1. Controls for protein detection and expression

3.1.1. Anti-rabbit secondary antibody (α-Rb) is able to bind antibodies raised in rabbit.

The quality of the secondary antibody was validated by probing for rabbit-derived primary antibody raised against BSA. One sample of BSA was incubated at 70°C with reducing agent and LDS-containing buffer for 10 min. A similar sample was denatured at room temperature. A major band corresponding to proteins of 70 kDa was observed in the Western blot (Figure 12). The molecular weight of BSA is 66.5 kDa. The additional bands of higher molecular weight might have resulted from overloading.

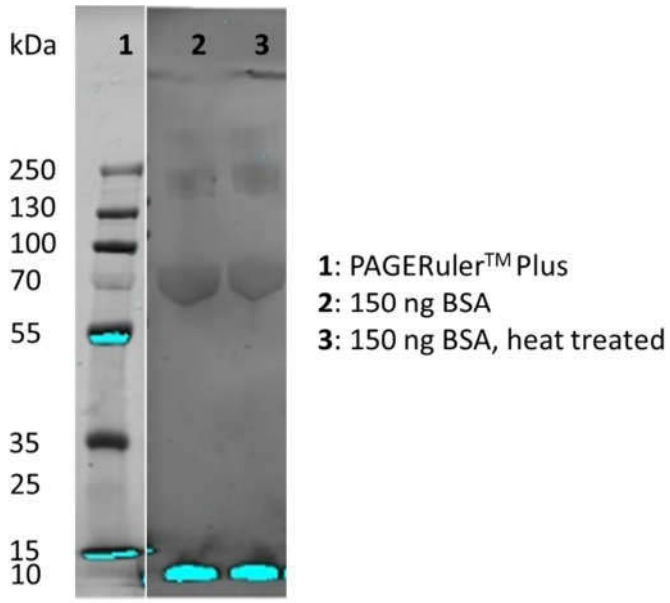

Figure 12 Western blot detection of commercially-obtained BSA. The primary antibody raised against BSA is rabbit-derived. The secondary antibody is fluorophore-labelled and creates a signal.

3.1.2. Secondary antibody (α-Ms) creates a signal

Western blotting of LHCII-VSV results in a band corresponding to proteins of 28 kDa in size (Figure 13). This was close to the expected size for LHCII-VSV, which is 30 kDa. As the LHCII-VSV was produced using *in vitro* protein synthesis, it was necessary to exclude the possibility of non-specific binding to wheat germ extract proteins. The case of a false-positive was excluded by the inclusion of an IVS reaction mix without RNA-polymerase. This reaction did not yield a band corresponding to LHCII-VSV.

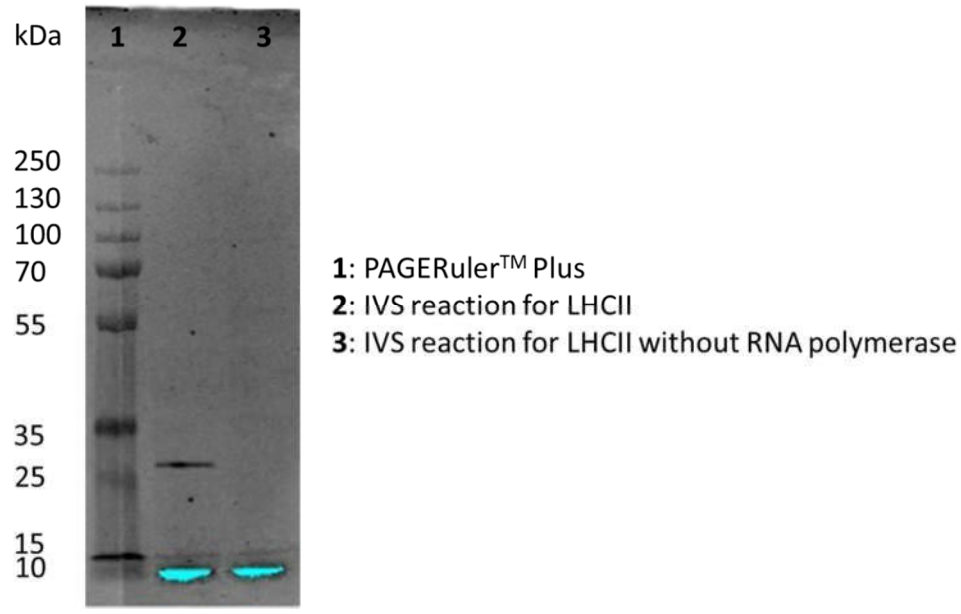

Figure 13 Western blot detection of LHCII-VSV expressed by IVS and detected with a pair of antibodies. The mouse-derived primary antibody targets the VSV-tag of LHCII-VSV, the secondary antibody is targeted against antibodies raised in mouse.

3.1.3. CLDN2-VSV detection is inconsistent

As for LHCII-VSV, CLDN2-VSV that carries a VSV tag for detection was chosen as a reference for detection of CLDN2 produced without a tag. The VSV-tag shows high specificity for binding of the anti-VSV primary antibody. The CLDN2-VSV signal would allow us to determine if untagged CLDN2, probed for using antibodies raised against CLDN2 and not VSV, is produced by the wheat germ kit, by the presence of a band of similar size to CLDN2-VSV in the Western blot. The plasmid with the CLDN2-VSV insert was used for production of CLDN2-VSV with *in vitro* synthesis (IVS). However, detection of the CLDN2-VSV with Western blot was inconsistent. In the first attempts, no signal could be observed. (Figure 14).

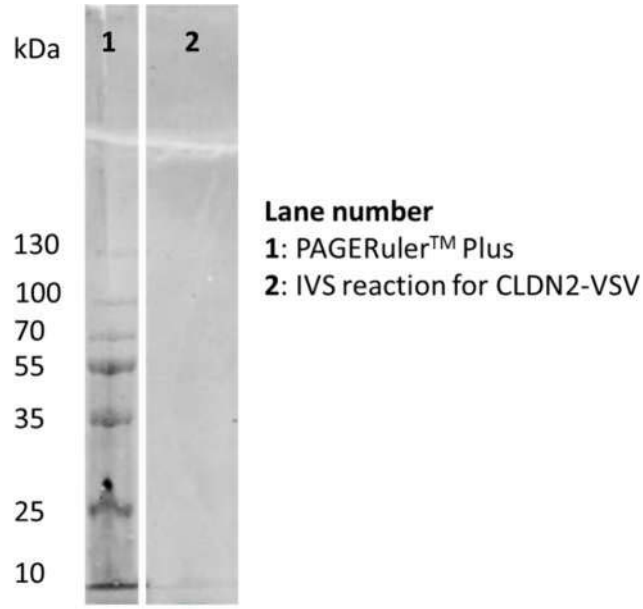

Figure 14 Western blot with CLDN2-VSV from IVS at 800 nm excitation wavelength. The antibody that binds to the primary antibody raised against the VSV tag has its maximum excitation at 800 nm.

Failure to detect CLDN2 is unlikely to be due to handling error.

Either the expression or the subsequent detection of CLDN2-VSV was not successful. In a shadowing experiment carried out by two different people at the same time with the same reagents possible mistakes during handling and preparation of the samples were ruled out. Six different clones of CLDN2-VSV, clones TZ1, 1, 2, 3, 4, 5, and Cl1, were used. To rule out the possibility that the kit used was not able to produce protein at all, LHCII-VSV was included in the experiment as a control. Only LHCII-VSV could be detected in this experiment at a size of 28 kDa (Figure 15). Therefore, the lack of signal for CLDN2-VSV likely does not result from mistakes made during sample handling.

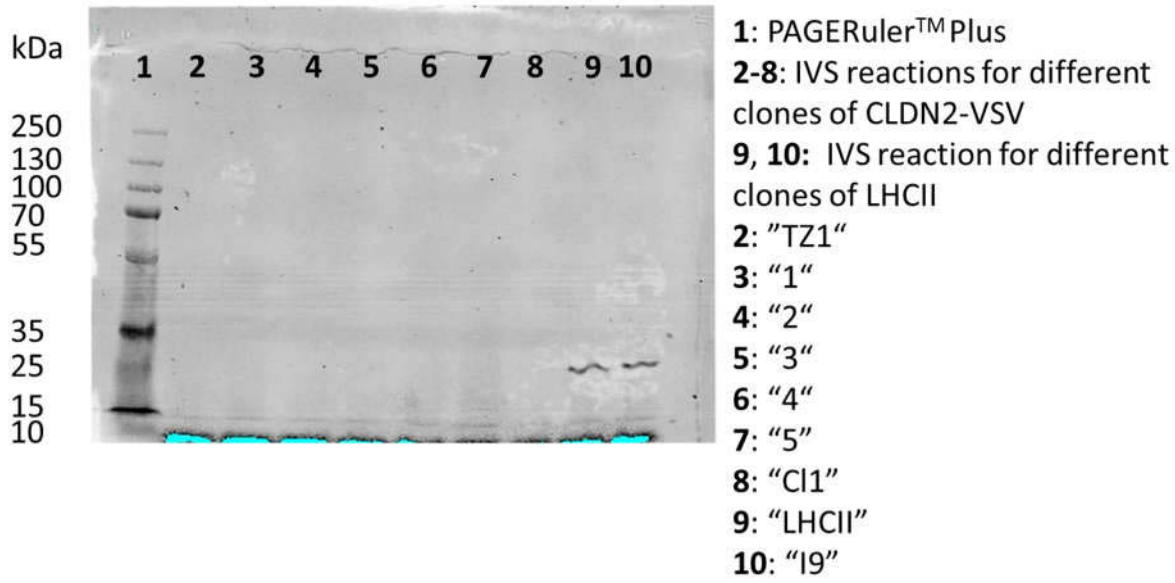

Figure 15 Western blot with CLDN2-VSV and LHCII-VSV from IVS, performer 1.

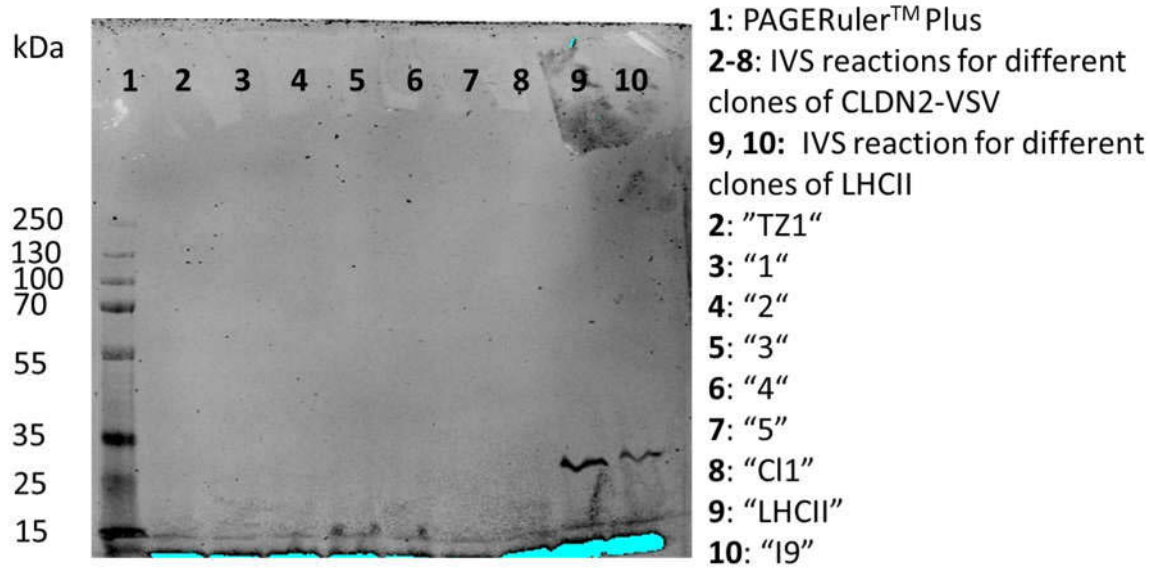

Figure 16 Western blot with CLDN2-VSV and LHCII-VSV from IVS, performer 2.

Failure of CLDN2-VSV to be detected was not wheat germ extract batch-specific

To exclude the possibility of the wheat germ extract used being partially defective, two new *in vitro* synthesis kits were tested. The first, Lot no. 0000126248, was used for *in vitro* synthesis of CLDN2-VSV and LHCII-VSV. Again, only LHCII-VSV could be detected with Western blot at a size of 28 kDa (Figure 17).

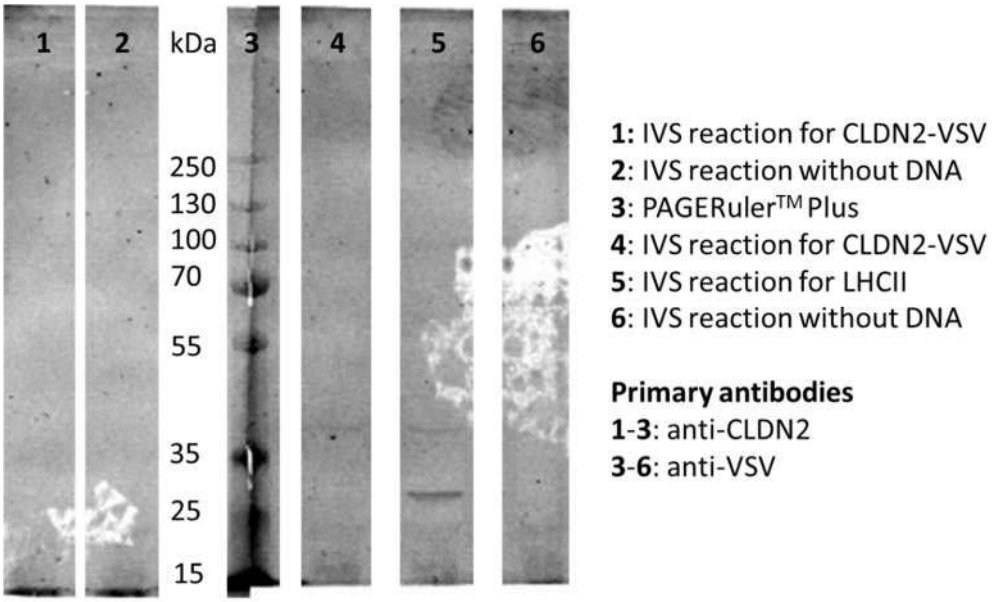

Figure 17 Western blot with CLDN2-VSV and LHCII-VSV from IVS kit Lot no. 0000126248.

The second of the two new kits (Lot no. 0000155365) was also tested for protein production. Different clones of CLDN2-VSV as well as LHCII-VSV were included. Neither CLDN2-VSV nor LHCII-VSV could be detected, so the Western blot was repeated. Both proteins could not be detected (Figure 18). The residual sample from this synthesis reaction was used for one more Western blot, with similar results (Figure 19). Since LHCII-VSV, the control for protein synthesis, did not give a signal, it was concluded that the synthesis reaction had failed.

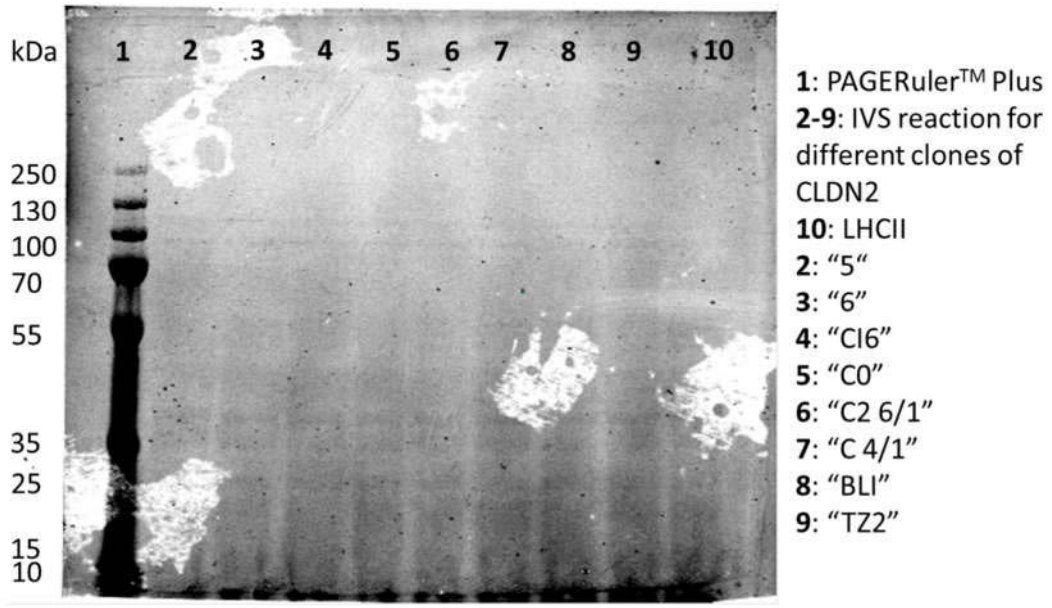

Figure 18 Western blot with CLDN2-VSV and LHCII-VSV from IVS kit Lot no. 0000155365.

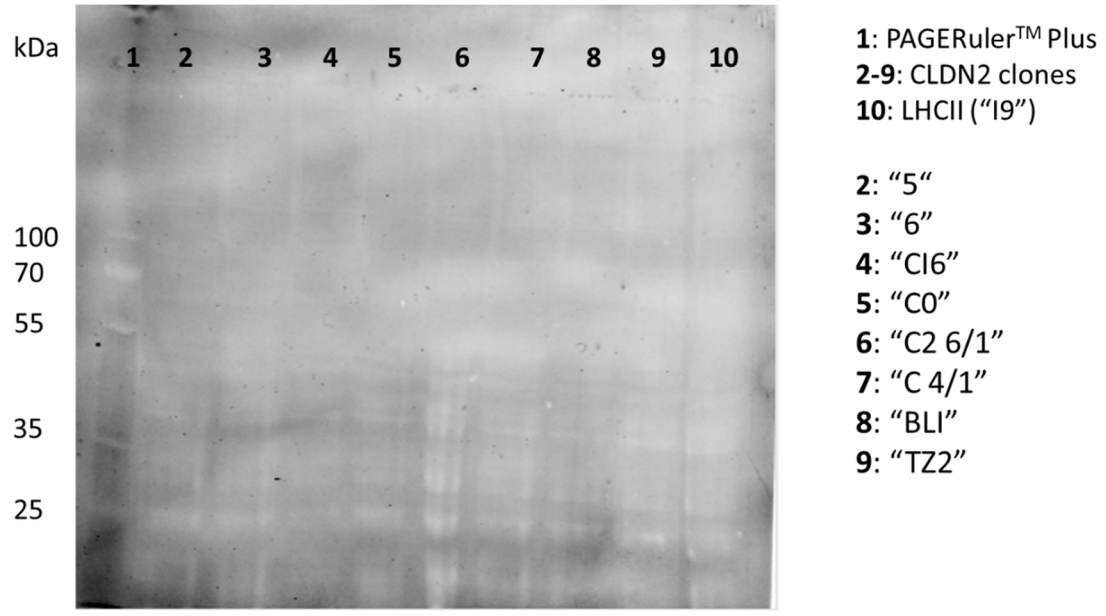

Figure 19 Western blot with CLDN2-VSV and LHCII-VSV from IVS kit Lot no. 0000155365 (repetition).

Since it was not certain whether protein detection or synthesis had failed, *in vitro* synthesis with the kit Lot no. 0000155365 was repeated. This time, LHCII-VSV as well as all the CLDN2-VSV clones could be detected (Figure 20). One of the CLDN2-VSV clones, clone BLI, shows a band at a higher molecular mass than the other clones. This is consistent with previous tests of this specific clone performed in the research group.

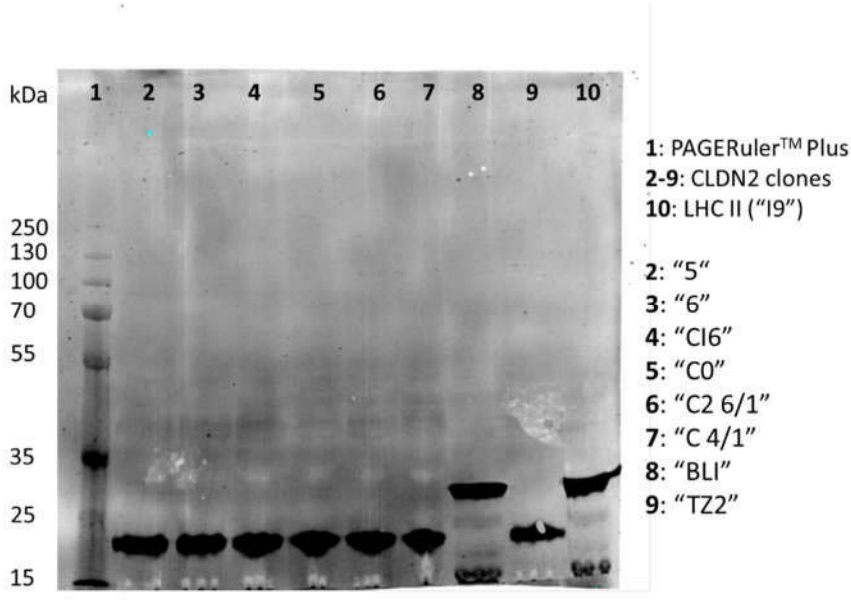

Figure 20 Western blot with CLDN2-VSV and LHCII-VSV from IVS kit Lot no. 0000155365 (repetition).

The lack of signal for LHCII-VSV in Figure 18 and Figure 19 probably resulted from a random mistake during preparation of the IVS reaction. LHCII-VSV yielded a signal in all other experiments, but CLDN2-VSV only yielded a signal in one experiment.

This gave rise to the possibility that the wheat germ extract quality, and the other experimental conditions, had been varying and that although these were sufficient for the expression of LHCII-VSV, they were not for CLDN2-VSV.

3.1.4. Detection of commercial and cell-derived CCR5

Although it had been confirmed that the secondary antibodies are able to bind to their targets, it was still necessary to determine if the primary antibodies could bind their targets. Primary antibodies against one of the protein of interests, CCR5, were tested for their ability to bind the protein. CCR5 protein, purchased from ABNOVA (H00001234-G01) or extracted from a cell line, was used to test different antibodies under different conditions. These experiments were carried out for CCR5 and, due to lack of time and inconsistent results, were not performed for the other proteins.

The protein was treated with LDS sample buffer and reducing agent and then incubated for 10 min at room temperature. Additionally, samples that were incubated at 70°C were tested, since it was not clear whether incubation at room temperature was sufficient for CCR5 denaturation. On the other hand, high temperatures can lead to membrane protein aggregation and therefore prevent protein detection. Testing of these two conditions ought to define the optimal temperature for CCR5 denaturation.

Antibodies 11232 and PAB0177 could not detect ABNOVA CCR5 denatured at room temperature

Detection of the ABNOVA CCR5 was tested with two different antibodies. Antibody NIH 11232 was raised in rabbit and detects a peptide corresponding to amino acids 6 to 20 in human CCR5. Antibody ABNOVA PAB0177 was raised in rabbit and detects a synthetic peptide corresponding to human CCR5. The ABNOVA CCR5 could not be detected with either antibody (Figure 21).

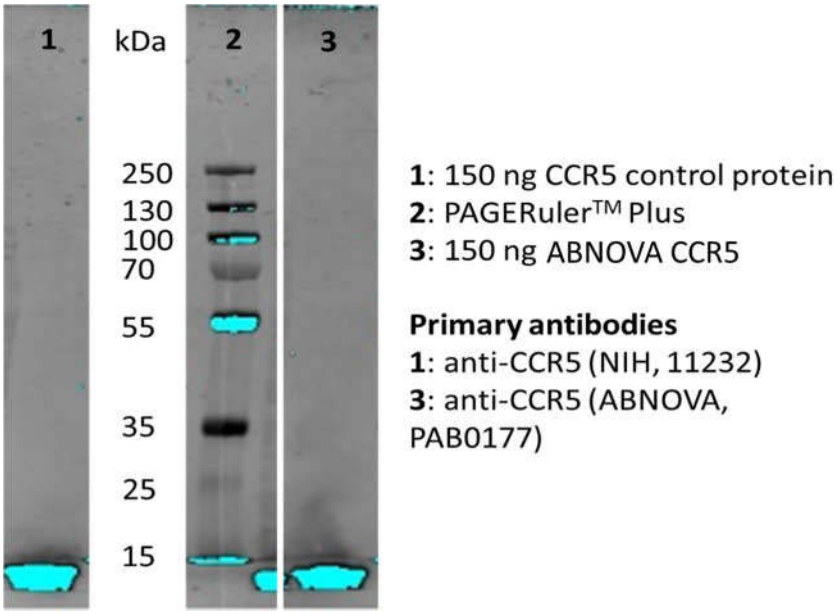

Figure 21 Western blot for ABNOVA CCR5 at 700 nm excitation wavelength. The secondary antibody (anti-rabbit) that binds to the antibodies against CCR5 has its maximum of excitation at 700 nm.

Antibodies 11232 and PAB0177 could not detect heat-denatured ABNOVA CCR5

As denaturation at room temperature might not have been sufficient to prepare the ABNOVA CCR5 for Western blot, it was tested whether incubation of the sample with LDS sample buffer and reducing agent at 70°C instead of room temperature would lead to detectability. Again, CCR5 could not be detected using the same secondary antibodies.

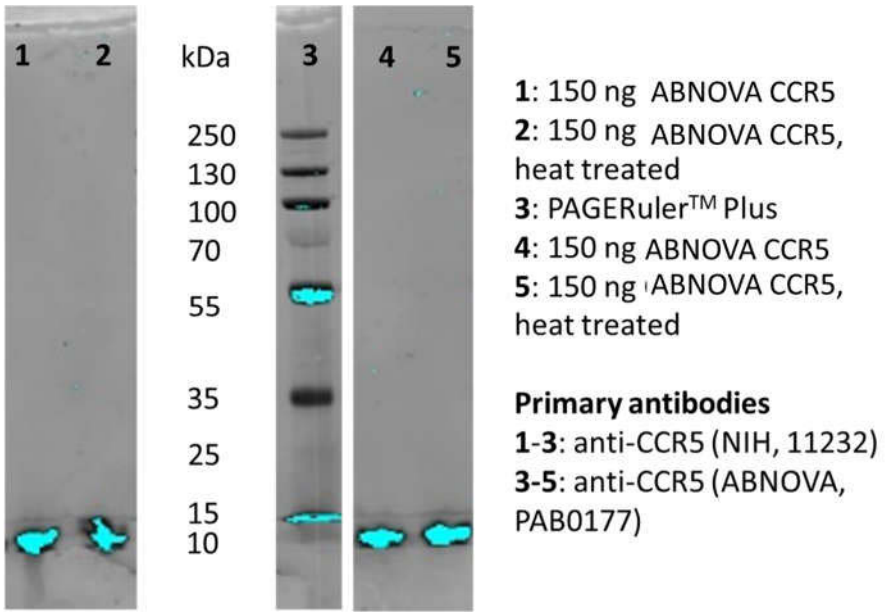

Figure 22 Western blot for ABNOVA CCR5 at 700 nm excitation wavelength. The secondary antibody (anti-rabbit) that binds to the antibody raised against CCR5 has its maximum of excitation at 700 nm.

Antibody PA1-41303 could not detect either room-temperature or heat-denatured ABNOVA CCR5.

An additional primary antibody (Thermo Fisher Scientific, PA1-41303) against the ABNOVA CCR5 was tested. The incubation of the ABNOVA CCR5 with LDS sample buffer and reducing agent was carried out at room temperature or at 70°C. No signal was observed in the Western blot (Figure 23).

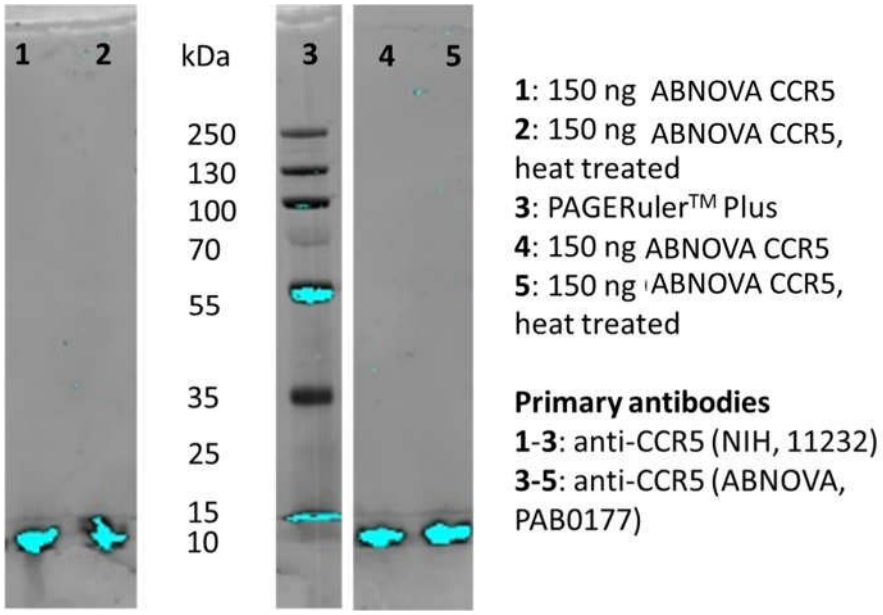

Figure 23 Western blot for ABNOVA CCR5 control protein with antibody from Thermo Fisher Scientific (PA1-41303).

Antibody ABNOVA, H00001234-B02P bound non-specifically to both room-temperature and heat-denatured ABNOVA CCR5, as well as the negative control

After repeated failure in detecting the ABNOVA CCR5 protein, antibody H00001234-B02P, recommended and provided by the manufacturer of the ABNOVA CCR5, was tested. This is different from antibody PAB0177 which was also purchased from ABNOVA. At this time, it was explained by the vendor that the ABNOVA CCR5 was produced by *in vitro* synthesis with wheat germ-derived extract. That way, ABNOVA CCR5 represents exactly, what we were trying to detect: CCR5 protein made from IVS with wheat germ extract. However, the ABNOVA CCR5 sample could contain components from the IVS reaction. These could interfere with the detection. To exclude false-positive signals that may result from the binding of the primary antibody to components of the wheat germ extract, a negative control with all the IVS components including wheat germ extract, but not the expression plasmid, was included in the experiment. This control allows for a comparison between the IVS reaction with and without the presence of expression plasmid. It was not clear whether the wheat germ extract used to produce the ABNOVA CCR5 was the same as that used

in our experiments. As such, the IVS sample without expression plasmid does not exactly represent the microenvironment of the ABNOVA CCR5. However, it was assumed that if the ABNOVA CCR5 sample contains wheat germ derived components, they will be similar to those in our IVS samples. For the ABNOVA CCR5, one band at 65 kDa and one band between 35 kDa and 40 kDa was observed (Figure 24). The expected size for CCR5 is 41 kDa. For the negative control, a band at 40 kDa was also observed. From this signal, it can be concluded that the 40 kDa band results from a component of the reaction mixture and not CCR5. However, the signals were very faint and more than one band could be observed for the ABNOVA CCR5, so the experiment was repeated.

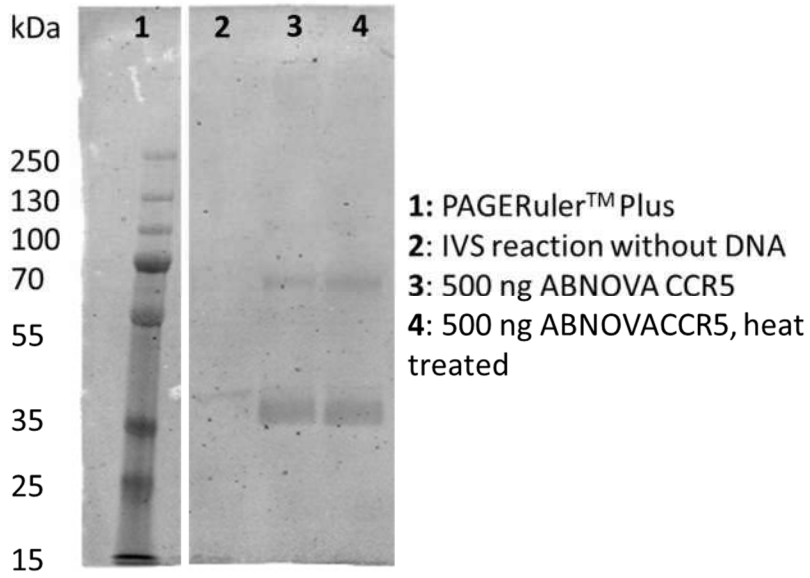

Figure 24 Western blot with ABNOVA CCR5 control protein, detected with antibody H00001234-B02P.

The experiment was repeated with freshly prepared secondary antibody. Since heat treatment did not seem to have an effect on detectability, ABNOVA CCR5 was only incubated at room temperature during denaturation with LDS and reducing buffers. At least three bands could be observed for the ABNOVA CCR5 sample – one corresponding to proteins of size 110 kDa, one of 65 kDa and one between 35 kDa and 40 kDa (Figure 25). The negative control yielded two bands – one at 40 kDa and one at 65 kDa.

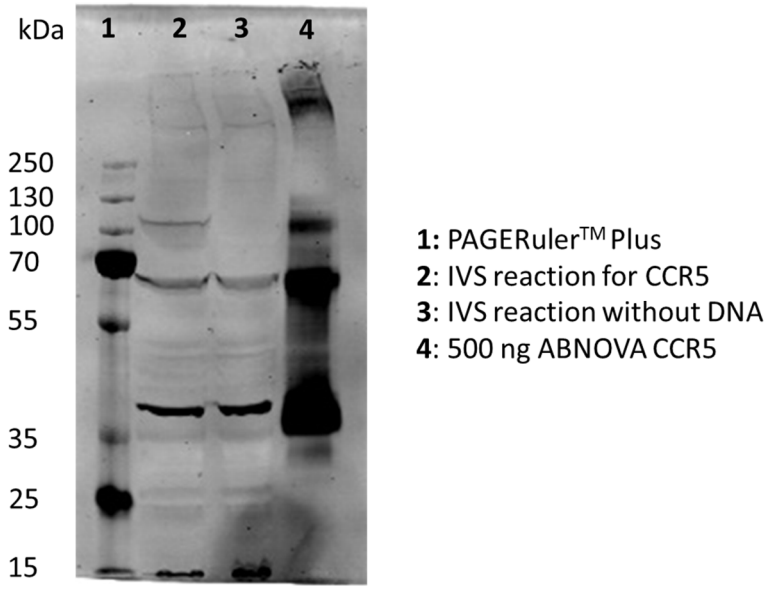

Figure 25 Western blot with CCR5 from IVS and ABNOVA CCR5.

All experiments showed that the negative control (IVS reaction without DNA) yielded a band very similar to the expected size of CCR5 (41 kDa). This band results from a component of the reaction mix. According to the manufacturer's information, ABNOVA CCR5 was produced using IVS with wheat germ extract. As it cannot be excluded that the major band of 40 kDa observed in ABNOVA CCR5 is, at least partly, due to reaction components as well, ABNOVA CCR5 is not suitable as a control for CCR5 detection.

Antibody ANOVA H00001234-B02P could not detect CCR5 extracted from A3R5.7 cells

Since ABNOVA CCR5 was not suitable as a positive control, we tried to use another source of CCR5. CCR5 protein was extracted from cells. After extraction, the sample was centrifuged and aliquots from both supernatant and cell pellets were analysed. ABNOVA CCR5 was included in the experiment. One sample was supplemented with the same enzymes used in the protein extraction process to mimic the conditions. In total, three samples from the cell extract, three supernatant samples, one sample for ABNOVA CCR5 and one ABNOVA CCR5 sample with supplemented enzymes were included. No signal at all or unspecific signals at molecular weights different from the expected weight for CCR5 could be observed (Figure 26).

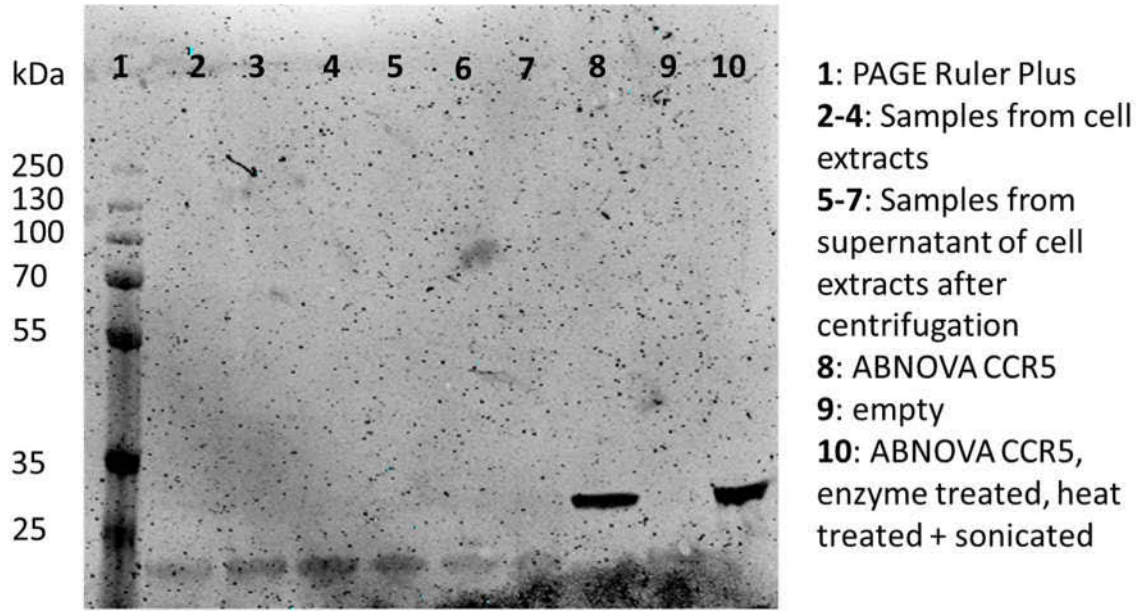

Figure 26 Western blot for CCR5 extracted from A3R5.7 cells. Bands at 30 kDa can be observed for one cell derived sample and for one ABNOVA CCR5 sample.

The expected size for CCR5 is 41 kDa. Two 30 kDa bands can be observed for the ABNOVA CCR5 samples. This suggests an unspecific binding event between the antibody used and components of the same size in both ABNOVA CCR5 samples. A band of this size could not be observed in the previous experiments with ABNOVA CCR5.

The lack of signal for CCR5 from these cell extract samples excludes them as controls for Western blot detection. Neither the commercially obtained CCR5 nor the cell derived CCR5 turned out to be reliable controls for Western blot detection. For this reason and the high costs of commercially obtained protein samples, no controls for CD4, CXCR4 and CLDN2 were validated prior to checking whether their expression in an IVS system yielded any signal.

3.2.Proteins expressed from expression plasmids

Analysis of the plasmids

To analyse whether the inserts coding for the protein of interest had been correctly inserted in the plasmid backbone, the plasmids were digested with restriction enzymes. The enzymes were able to cut out the insert that was flanked by restriction sites. The cut-out inserts and the backbones were separated by electrophoresis. The size of both inserts and backbones showed the expected sizes (Figure 27). To check the integrity of the insert

sequence, the inserts were analysed by Sanger sequencing. The results yielded from the sequencing corresponded to the expected sequences for the inserts.

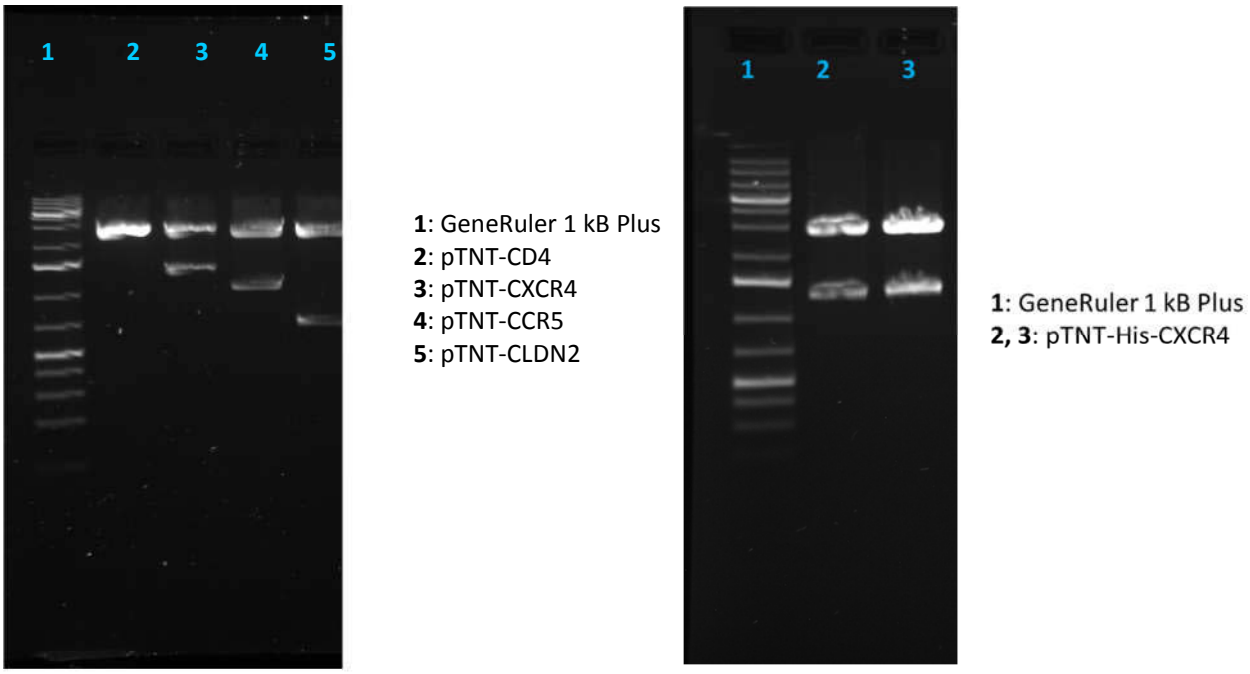

Figure 27 Restriction digestion of pTNT plasmids after insertion of the genes of interest. Since the CD4 insert hast the same size as the pTNT vector backbone (3 kDa), only one band can be observed. However, the signal intensity for this band is greater than that for the vector backbone in the other samples. For all the other plasmids, the 3 kDa backbone can also be observed at 3 kDa. The insert sizes show the expected sizes of 1,1 kDa for CXCR4 and His-CXCR4, 1 kDa for CCR5 and 700 kDa for CLDN2.

3.2.1. Production of CLDN2 from expression plasmids

Production of CLDN2 from the expression plasmid could not be confirmed using Western blot

Plasmids encoding CLDN2 were used for producing the protein in the wheat germ kit. The production of CLDN2-VSV was used as an indicator for successful protein synthesis by the IVS reaction. The Western blot detection of CLDN2 was carried out with an antibody raised against CLDN2 (Invitrogen, 51-6100). For detection of CLDN2-VSV, both the CLDN2-targeting antibody and an antibody raised against the VSV-tag (Sigma Aldrich, V5507) were used. Neither CLDN2, nor CLDN2-VSV could be detected in this experiment (Figure 28). However, it was discovered when validating the secondary antibodies that the detection of CLDN2-VSV by Western blotting was unreliable (See Section 3.1.3). As such, CLDN2-VSV was not suitable as a control protein for CLDN2 production. The use of LHCII-VSV would have been better as an indicator for successful IVS.

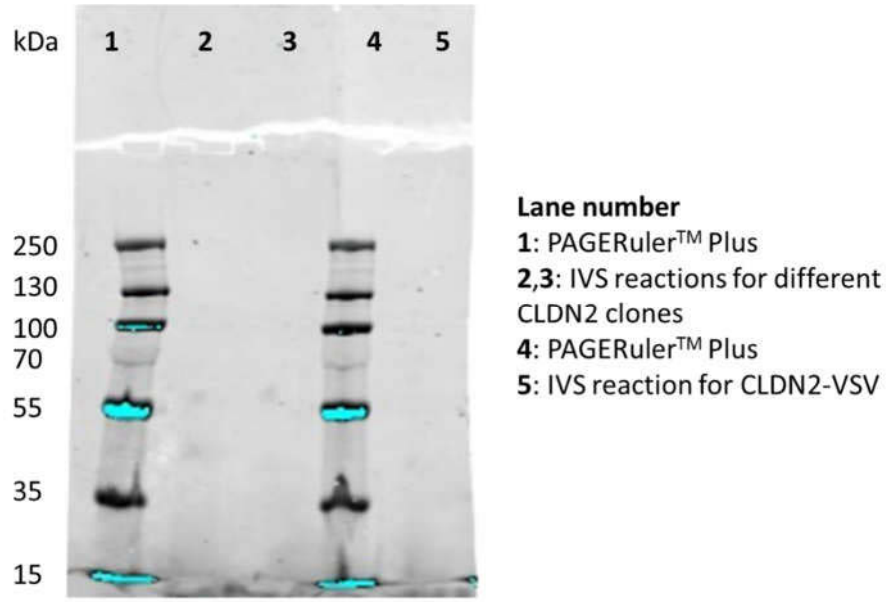

Figure 28 Western blot for CLDN2 and CLDN2-VSV produced by IVS as a control at 700 nm excitation wavelength. The secondary antibody (anti-rabbit) that binds to the antibody raised against CLDN2 has its maximum of excitation at 700 nm. The secondary antibody that binds to the antibody raised against the VSV-tag has its excitation maximum at 800 nm. For a picture under this excitation wavelength, see Figure 14.

Changing in vitro protein synthesis conditions did not improve CLDN2 detectability

As the differences in detection of LHCII-VSV and CLDN2-VSV had shown, the success of protein expression seems to vary for different proteins. Since this could result from different optimal reaction conditions for different proteins, the reaction conditions were varied in subsequent experiments. The experiment was repeated under two different conditions: the incubation for the IVS reaction was carried out at 37°C for 1 h and at 24°C overnight, instead of the usual 90 min incubation at 30°C, to see if different conditions could enhance protein expression. The samples were tested in duplicates from two different plasmids. IVS samples without expression plasmid were included in the experiment as a negative control. CLDN2 could not be detected in Western blots using antibodies raised against CLDN2 (Invitrogen, 51-6100) nor with antibodies raised against VSV (Sigma Aldrich, V5507) and CLDN-VSV as control (See Figure 29).

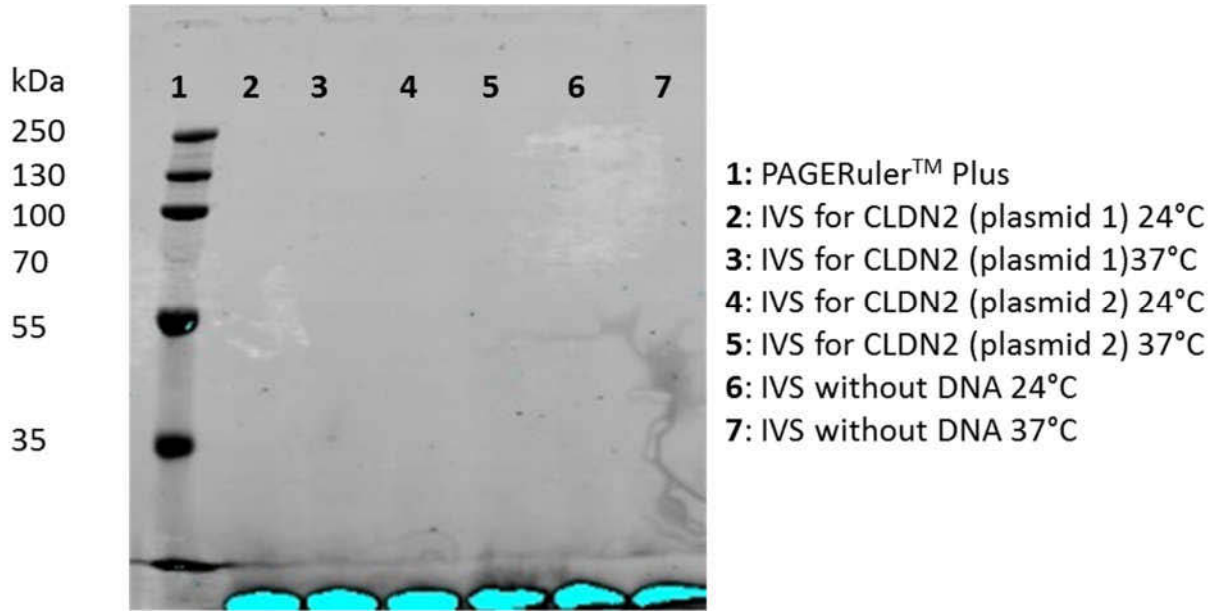

Figure 29 Western blot for IVS with different clones of CLDN2 under different incubation temperatures and durations.

To exclude the possibility that CLDN2 expression had failed because a defective batch of wheat germ extract was used, the production of CLDN2 with IVS was repeated with another batch of wheat germ extract (Lot no. 0000126248). As an indicator of successful protein synthesis by the wheat germ extract, LHCII-VSV was used as a control and probed for with an antibody raised against VSV (Sigma Aldrich, V5507). Only LHCII-VSV could be detected on the Western blot (Figure 30). It was thus concluded that the synthesis reaction had been successful for LHCII-VSV. CLDN2 was either not produced or could not be detected.

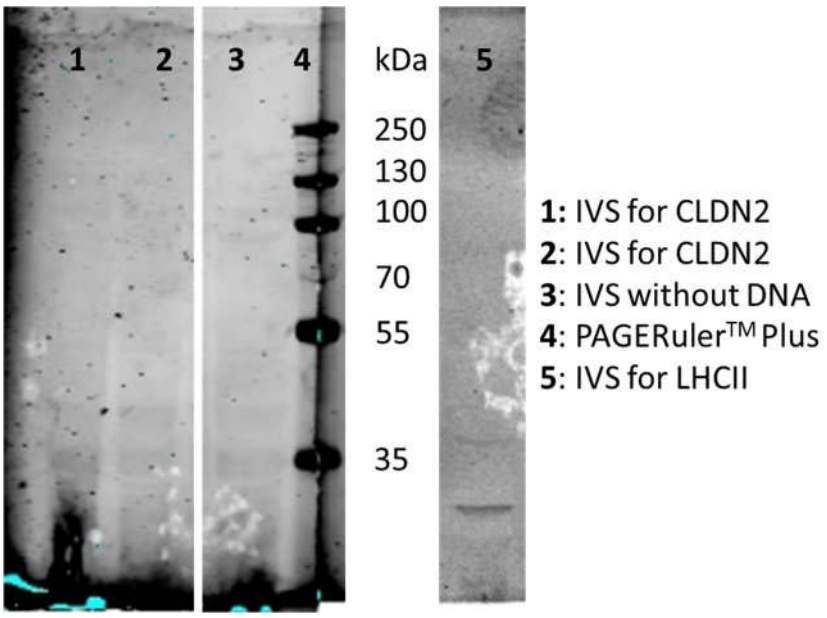

Figure 30 Western blot for CLDN2 at 700 nm excitation wavelength and LHCII-VSV at 800 nm excitation wavelength. The secondary antibody (anti-rabbit) that binds to the antibody raised against CLDN2 has its

maximum of excitation at 700 nm. The secondary antibody that binds to the antibody raised against the VSV-tag of LHCII-VSV has its excitation maximum at 800 nm.

3.2.2. Production of CCR5 from expression plasmids

Production of CCR5 from the expression plasmid could not be confirmed

While the antibodies raised against CCR5 were being tested against the ABNOVA CCR5 (see Section 3.1.4), CCR5 produced through IVS using our own expression plasmid was also tested. However, detection of CCR5 produced from our plasmid was not successful using antibodies NIH 11232, ABNOVA PAB0177 and Thermo Fisher Scientific PA1-41303 (See Figure 31 and Figure 32). The LHCII-VSV sample yielded a band of size 28 kDa detected by an antibody raised against VSV (Sigma Aldrich, V5507). This confirmed that the protein synthesis for LHCII-VSV was successful. CCR5, however, was either not produced or could not be detected.

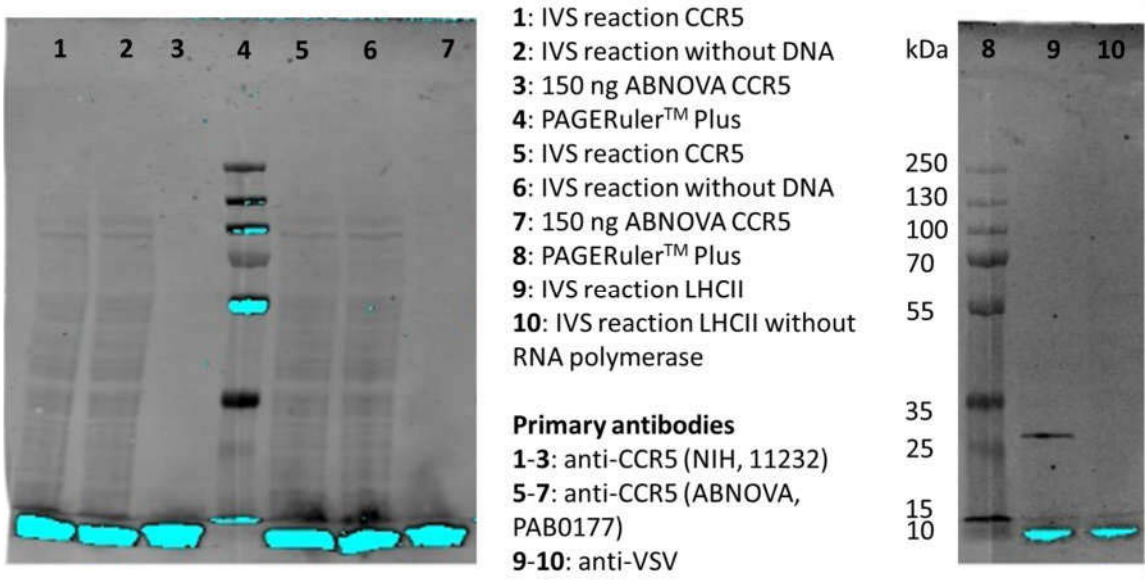

Figure 31 Western blot for CCR5 from IVS and ABNOVA CCR5 at 700 nm excitation wavelength and LHCII-VSV from IVS at 800 nm excitation wavelength.

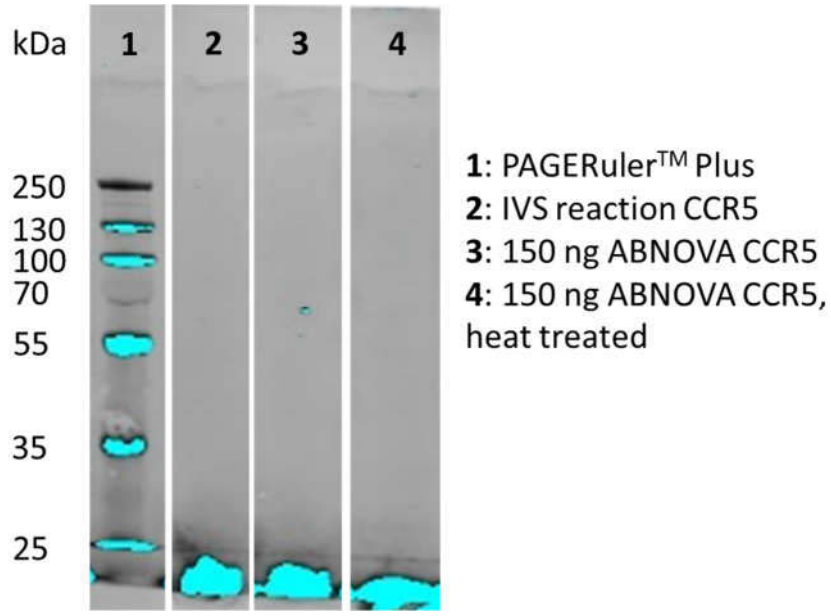

Figure 32 Western blot for CCR5 from IVS and CCR5 control protein at 700 nm excitation wavelength. Antibody PA1-41303 from Thermo Fisher Scientific was used as the primary antibody.

Probing with antibody H00001234-B02P, resulted in a band of size 40 kDa in the CCR5 sample produced using our expression plasmid (Figure 33). However, a band of the same size can also be observed for the negative control. Repetition of the Western blot with the same samples, but with freshly prepared antibody solutions, resulted in a more intense signal, both for the CCR5 produced using our expression plasmid as well as for the ABNOVA CCR5 (Figure 34). For CCR5 produced using our expression plasmid, one band at 110 kDa can be observed that is not present in the negative control. This band was also present in the ABNOVA CCR5 sample. However, since this is not the expected size for CCR5, nor was the data reproducible, CCR5 production could not be confirmed.

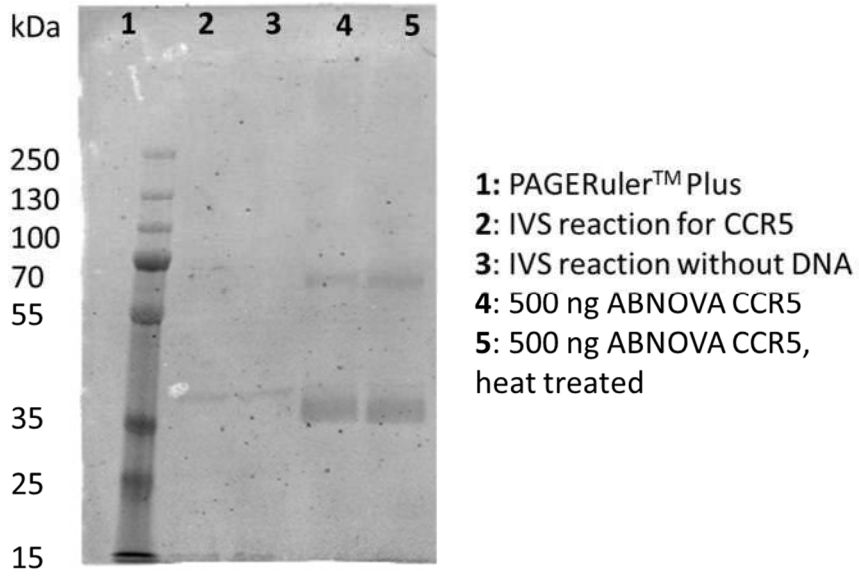

Figure 33 Western blot with CCR5 from IVS and CCR5 control protein.

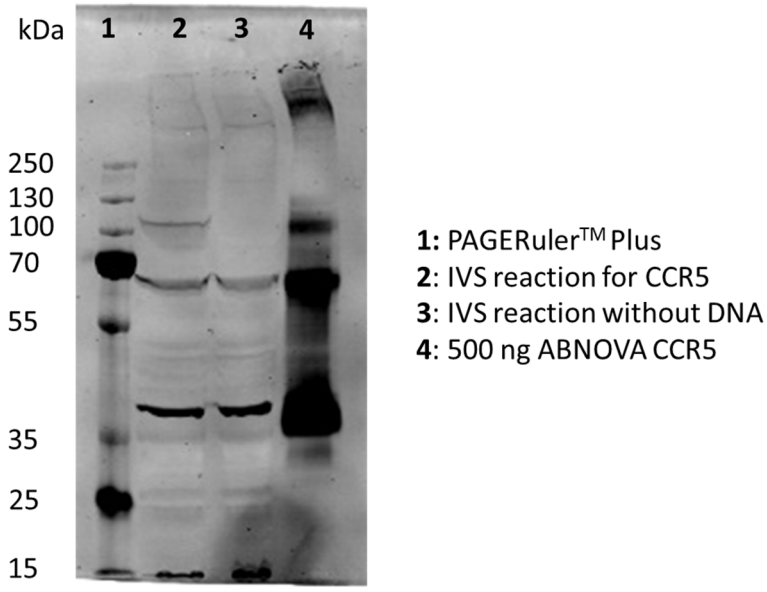

Figure 34 Western blot for CCR5 from IVS and CCR5 control protein (repetition).

ABNOVA CCR5 and CCR5 samples produced using wheat germ extract Lot no. 0000155365 yielded non-specific staining with antibody, H00001234-B02P

Tests with CLDN2-VSV suggested that one batch of wheat germ extract might have been of poor quality. When expression of this protein was successful with a new kit, the same kit, Lot no. 0000155365, was used for the expression of CCR5 using our expression plasmid. ABNOVA CCR5 was included in the experiment as a reference. All of the CCR5 expression plasmids yielded a band at 40 kDa that is also present for ABNOVA CCR5. However, the negative control also shows this band. Thus, the band may not necessarily

indicate the presence of CCR5. The additional bands at 65 kDa and 110 kDa that could be observed in the previous blots were not visible this time.

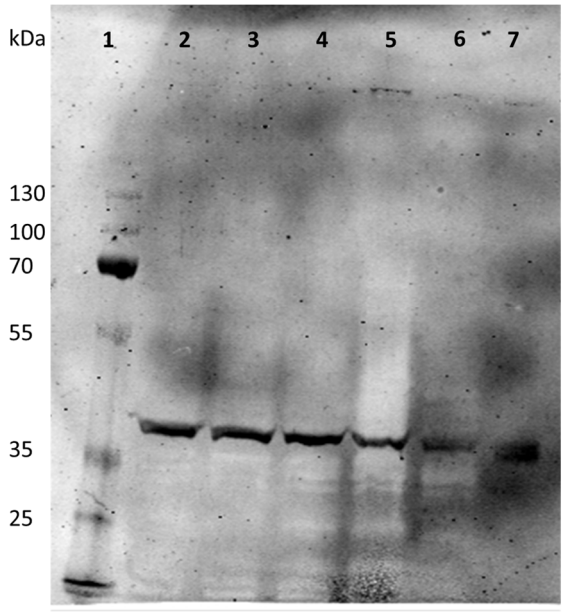

Figure 35 Western blot with CCR5 from IVS and CCR5 control protein

CCR5 was not detected using Coomassie blue staining of ABNOVA CCR5 and CCR5 samples produced using our expression plasmid

The bands that resulted from probing the ABNOVA CCR5 differed greatly in intensity between the Western blots. As such, protein staining with Coomassie Blue was carried out in order to compare the bands from this non-specific stain with those obtained using specific antibodies. The resultant bands for the ABNOVA CCR5 look very similar to those bands seen for the CCR5 produced using our expression plasmid (Figure 36). There were no distinct bands of intensity that would indicate the presence or absence of CCR5. The more numerous bands obtained from Coomassie blue staining indicates impurities in the CCR5 protein samples. These are probably other proteins from the wheat germ extract. The data are consistent with the Western blots, that suggested unspecific antibody binding, perhaps due to high protein load in the samples.

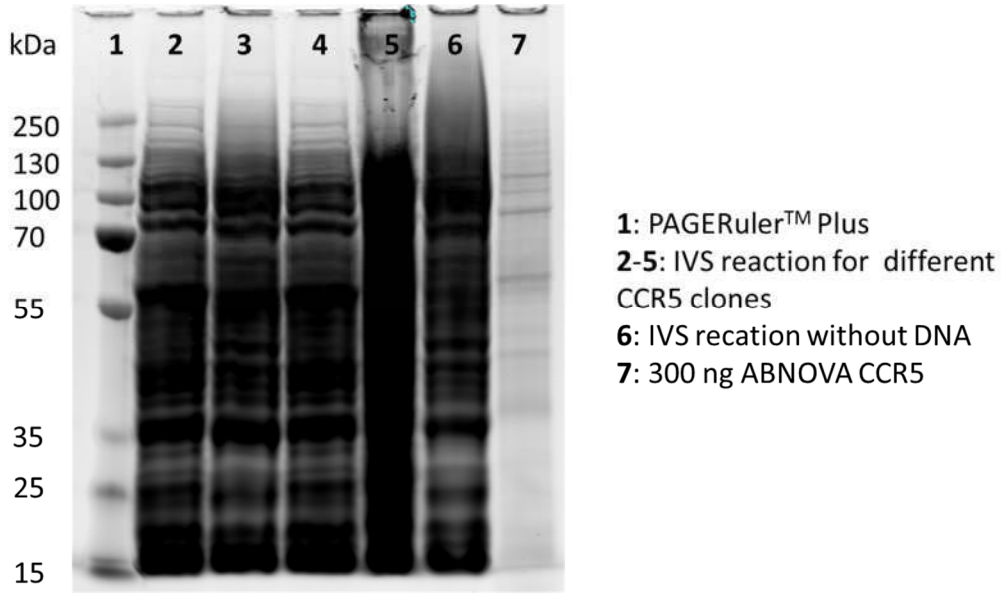

Figure 36 Coomassie Blue staining of SDS PAGE gel with CCR5 from IVS and ABNOVA CCR5

CCR5 samples produced using the rabbit reticulocyte kit could not be detected using antibody, H00001234-B02P

Since the expression of CCR5 was not consistent, expression with a different *in vitro* protein synthesis kit was considered. As CCR5 is a human protein, a system derived from mammalian cells – rabbit reticulocytes – was chosen. ABNOVA CCR5 was included in the Western blots as a reference. The sample produced using our expression plasmid did not yield any signal corresponding to CCR5 (Figure 37). Three bands corresponding to proteins of size 40 kDa, 65 kDa and 110 kDa were again observed for ABNOVA CCR5.

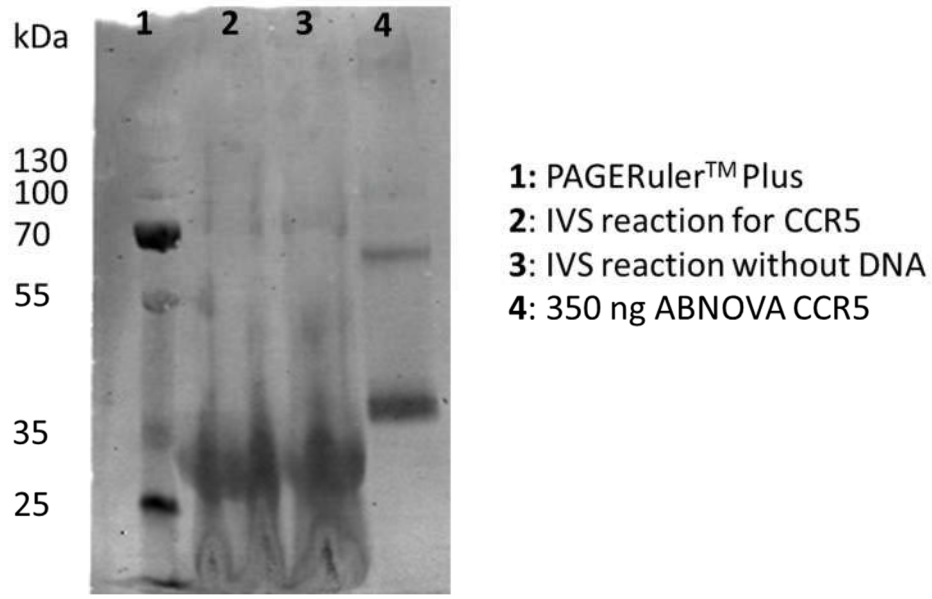

Figure 37 Western blot with CCR from IVS (rabbit reticulocyte kit) and ABNOVA CCR5

3.2.3. Production of CXCR4 from expression plasmids

Production of CXCR4 from the expression plasmid could be confirmed using Western blot

CXCR4 produced using our expression plasmid was not detected by Western blotting using NIH Anti-Human CXCR4 Polyclonal (EL) antibody (Figure 38). The IVS sample without expression plasmid that served as negative control showed multiple bands. This results from unspecific binding of the antibody to the afore-mentioned reaction components. LHCII-VSV was included in the experiment as an indicator for successful protein synthesis. The LHCII-VSV sample yielded a band of 28 kDa.

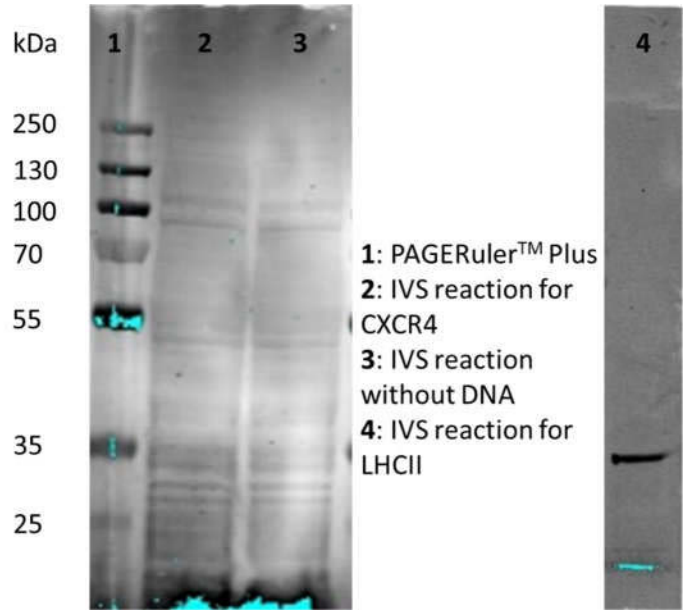

Figure 38 Western blot for CXCR4 from IVS at 700 nm excitation wavelength and LHCII-VSV from IVS at 800 nm excitation wavelength

Production of CXCR4 from the expression plasmid could not be confirmed using Western blot

His-CXCR4 produced using our expression plasmid was not detected by Western blot using NIH Anti-Human CXCR4 Polyclonal (EL) antibody (Figure 39). The antibody raised against the His-tag bound non-specifically to two components of the wheat germ extract. These bands correspond to proteins of size 34 kDa and 24 kDa. The expected size for CXCR4 is 45 kDa.

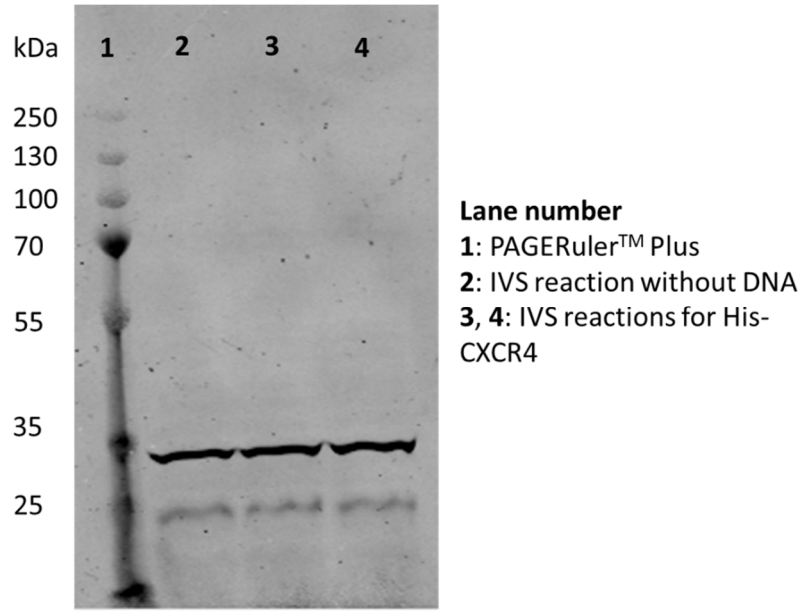

Figure 39 Western blot for His-CXCR4 at 800 nm excitation wavelength

3.2.4. Production of CD4 from expression plasmids

Production of CD4 from the expression plasmid could not be confirmed using Western blot

CD4 produced using our expression plasmid was not detected by Western blot using Thermo Fisher Scientific MA5-15775 antibody (Figure 40) Only a band that was also present for the negative control was observed. LHCII-VSV was included in the experiment as an indicator for successful protein synthesis. The LHCII-VSV sample yielded a band of 28 kDa.

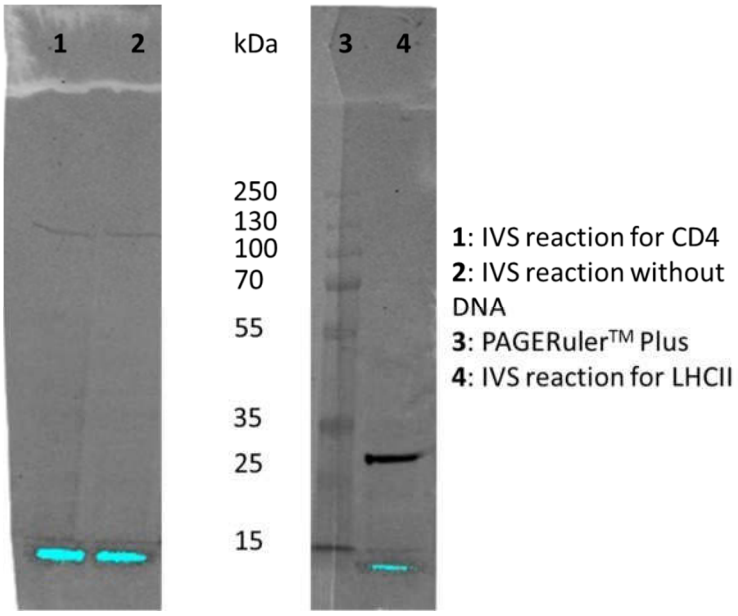

Figure 40 Western blot for CD4 and LHCII-VSV from IVS

Production of CD4 and LHCII-VSV from the expression plasmids was confirmed using incorporation of BIODIPY®-FL-labelled lysine

The wheat germ *in vitro* synthesis reaction mixture was supplemented with tRNAs charged with lysine labelled with the fluorescent dye BIODIPY®-FL. Expression plasmids for CD4, CLDN2, CLDN2-VSV, CCR5, CXCR4, His-CXCR4 and LHCII-VSV were used for protein production in these reactions. Only CD4 and LHCII-VSV could be detected reproducibly in the gels (Figure 41). The bands observed for CD4 are at the expected position corresponding to proteins of size 52 kDa. The bands for LHCII-VSV corresponded to

proteins of size 28 kDa No signals were obtained when no expression plasmids were added to the *in vitro* synthesis reaction mix.

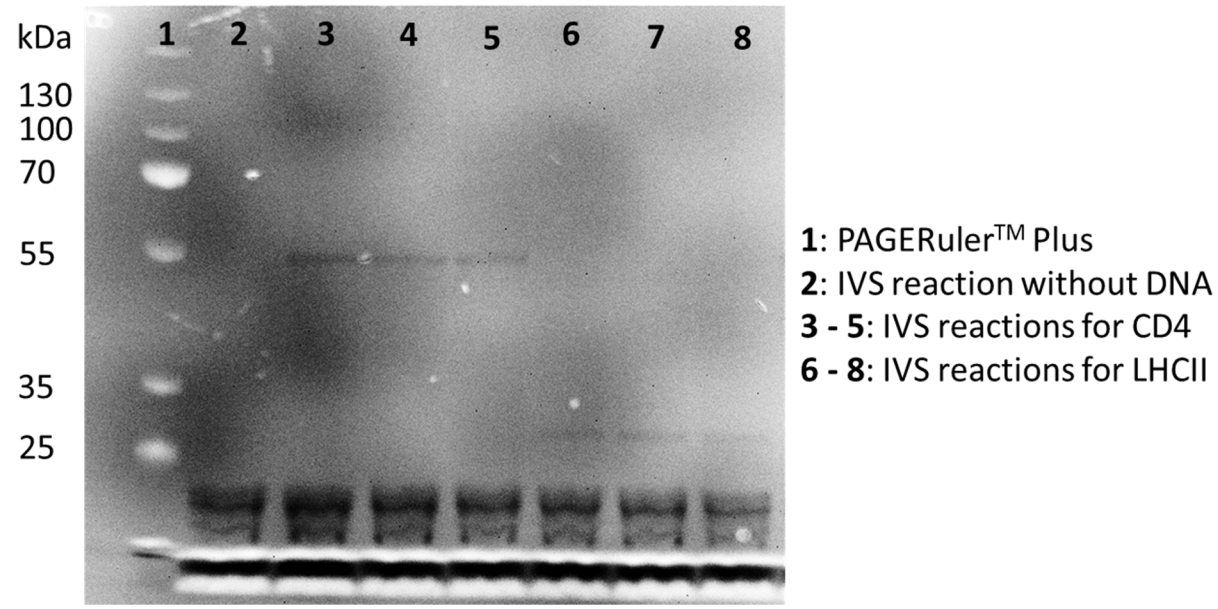

Figure 41 SDS-PAGE of CD4 and LHCII-VSV with BIODIPY®-FL-labelled lysine.

CD4 and LHCII-VSV were the only proteins that could be detected using this method. For CXCR4, CCR5 and CLDN2, no signal could be observed.

Discussion

The general aim of this research project's is the analysis of co-translational insertion of membrane proteins from an *in vitro* protein synthesis system into the membranes of artificial polymersomes. In this study, techniques for both successful protein synthesis and subsequent detection were evaluated. As later experiments will rely on the detection of the proteins of interest in various samples, the methods of their detection must be reliable. As such, it was necessary to validate and optimize the chosen methods. Western blot detection was initially the chosen method of specific protein detection as it is a well-established technique. A variety of antibodies available on the market promised suitable results for protein detection. Consequently, the reliability of Western blot detection was investigated prior to further experiments with IVS.

The two secondary antibodies that were used proved to be suitable for signal creation. Both anti-rabbit and anti-mouse secondary antibodies were shown to be able to bind to antibodies raised in their respective host targets. One of the proteins detected by the validated secondary antibodies, LHCII-VSV, was produced by IVS. Detection of the presence of LHCII-VSV was thus used as an indicator of successful protein synthesis. The primary antibody used was raised against the VSV-tag of the LHCII-VSV used. As CLDN2-VSV was similarly tagged, the same antibody was used to probe for this protein. However, the results were very inconsistent. Based on the fact that the detection method and material were the same, the difference seems to lie in the target protein expression level. It was concluded that the difference in their expression levels lies in the nucleotide sequence. However, to date the nature of this difference is not clear. LHCII-VSV and CLDN2 are similar in size (30 kDa and 25 kDa). It was thus concluded that the difference in size likely did not have an impact on the expression level. It was noted that, unlike LHCII-VSV, CLDN2 was of mammalian origin. The species of the protein might have influenced its expression using an extract of plant origin. Investigation of the nucleotide sequence's influence on protein expression level *in vitro* could provide information of value to future studies and lead to improvement on the expression of proteins of interest.

In order to determine the ability of the primary antibodies to bind to their targets, reliable sources of the antigens were needed. To validate the antibodies raised against CCR5, this protein was obtained from two sources: ABNOVA, a commercial supplier; and protein isolated from CCR5-expressing cells. These CCR5 proteins were intended to serve as controls for the successful detection of CCR5 produced using our expression plasmids.

The CCR5 from ABNOVA was produced from IVS with wheat germ extract. It was considered a suitable positive control, as it is similar to our samples. Detection of CCR5 with Western blot was inconsistent. Not only did the band intensities vary despite constant protein loads, but also the total number of visible protein bands differed from experiment to experiment. CCR5 has a molecular mass of 41 kDa. A band of that size could indeed be observed for the ABNOVA CCR5, in addition to two other bands of 65 kDa and 110 kDa. However, a 41 kDa band could also be observed for an IVS reaction without expression plasmid, and thus presumably without CCR5, treated with the same antibodies. According to these results, the 41 kDa band seems to result from a component of the wheat germ reaction mix to which the primary antibody binds non-specifically. Since the CCR5 control protein obtained from ABNOVA was similarly produced from IVS with wheat germ extract, it cannot be granted that the 41 kDa band arising from it corresponds to the CCR5 protein and not from a component of the extract. One band that could be observed for ABNOVA CCR5 and for CCR5 produced using our expression plasmid, but did not appear for the negative control (IVS reaction without expression plasmid), corresponded to a protein molecular weight of 110 kDa. This weight approximately corresponds to a trimer of CCR5 units. The detection of assumed protein oligomers from *in vitro* protein synthesis has been reported before (3), however this study did not seek to determine whether the higher-molecular weight bands did, indeed, result from CCR5 oligomerization, or from the primary antibody binding to other proteins in the samples. Furthermore, the band was not visible in all performed experiments. In conclusion it can be said that the antibodies against CCR5 seem to bind non-specifically to components of the wheat germ reaction mixture. One, or a group, of the contaminating proteins seems to have the same molecular weight as CCR5 itself, which could lead to false-positive results. Non-specific protein staining of the ABNOVA CCR5 with Coomassie Blue showed various protein bands of different molecular weights. The assumption that ABNOVA CCR5 contained proteins that might interfere with CCR5 detection was confirmed that way. ABNOVA CCR5 is therefore not suitable as a control for CCR5 detection. The detection of CCR5 protein extracted from cells did not lead to consistent results, either. As with the previously described assay, non-specific antibody binding seemed to occur, as bands corresponding to proteins of size 30 kDa were observed for one ABNOVA CCR5 sample and one cell derived sample. CCR5 has an expected molecular weight of 41 kDa. The size of the observed bands of 30 kDa differed from the bands that had previously been observed in Western blots for the ABNOVA CCR5 samples (41 kDa, 65 kDa, 110 kDa). It is not clear why the unspecific signals were different in this case. Explanations for the absence of CCR5 in the cell derived samples could be protein loss during sample preparation or failed

expression of CCR5 in the cells. The used cell line is engineered to express CCR5 under geneticin selection. If this process had failed, CCR5 would not have been produced. If neither of the described cases were valid, the results must be considered as false-negatives.

Since reliable protein detection by Western blot of CLDN2-VSV and CCR5 had failed, reliable statements about their expression could not be made. The problem of non-specific antibody binding came up again with the attempted detection of His-CXCR4. The antibody raised against the His-tag bound to two different components of the wheat germ extract reaction mixture. In contrast, for LHCII-VSV, non-specific or insufficient specific binding of the antibody raised against the VSV-tag did not seem to be the case. It is unclear still why the expression and detection of LHCII-VSV appears significantly better than the detection of other proteins. It could be argued that, compared to the antibody raised against ABNOVA CCR5, the antibody raised against the VSV-tag has a higher affinity for its target. This facilitates binding to the target protein and decreases non-specific binding. However, since the detection of CLDN2-VSV with the same pair of antibodies was not consistent, it can be argued that the problem lies, at least in part, on the consistency of protein expression.

One possible explanation is that LHCII-VSV is a plant protein whose expression might be easier for the transcription and translation machinery derived from wheat germ. To test this hypothesis, expression of the human CCR5 with the mammalian rabbit reticulocyte-derived kit was tested. Despite the non-specific antibody binding observed previously with the wheat germ system, no signal was obtained. One possible source of error that does not lie in the protein expression level, is the protein’s behaviour during the process of preparation for detection and the process of detection itself: Membrane proteins have been shown to behave anomalously during denaturation and binding of SDS (50). This behaviour, called "gel shifting" is caused by membrane proteins' tendency to form so called hairpin structures. These structures comprise two alpha helices connected by a loop (50). The presence of such structures results in an altered ratio of SDS to protein. While studies indicated that the amount of SDS binding to globular proteins is constantly between 1.5 and 2 g SDS/g protein, the amount binding to membrane proteins ranges from 3.4-10 SDS/g protein. If the amount of dodecyl sulphide present in the protein’s environment is too low, its hydrophobic domains might interact with each other instead of the dodecyl sulphide molecules, causing protein precipitation. A different issue connected to dodecyl sulphide-membrane protein interaction is the way in which dodecyl sulphide molecules organize themselves around a polypeptide chain. A study on this topic suggests that

detergent molecules form micelles around hydrophobic domains of the protein (51). The presence of many large hydrophobic domains, as it is the case for membrane proteins, could lead to extended micelle formation that could shield the proteins and interfere with antibody binding. It could also be possible that excessive dodecyl sulphide loading of the proteins results in their migration through the PVDF membrane during blotting. These interactions are one possible source of error in the process of protein detection. They could be the reason why protein detection with Western blot failed in many of our experiments.

Since protein detection with Western blots appeared to be unreliable, a different detection method was needed. Incorporation of fluorophore-labelled amino acids into the protein during synthesis was applied as an additional method for detection of synthesized protein. With this method, LHCII-VSV repeatedly gave a signal, a further indicator for its stable, efficient expression using the wheat germ kit. In addition, reproducible detection of CD4 could be achieved, even though there had never been signals in attempts to detect it using Western blots. Stable expression of this human protein is thus possible with the plant-derived extract. Why the detection of the other human proteins with fluorophore-labelled amino acids failed is not clear at the moment. Barring errors during production of the expression plasmids, an alternative explanation for the lack of signals from CXCR4, CCR5 and CLDN2 is the low total amount of lysine present in their amino acid sequences. The percentage of lysine in their sequences is under 5%, whereas it is 10% for CD4. Furthermore, CD4 is 10 kDa higher in molecular weight than the two G-protein coupled receptors, CXCR4 and CCR5, and 25 kDa higher in molecular weight than CLDN2, further increasing the difference in absolute amount of lysine. However, LHCII-VSV, a protein of 28 kDa has also only 5% lysine in its sequence and still gave a moderate signal. Concentration of the protein synthesis reaction mixture, for example by dialysis and subsequent evaporation, could be a means to increase the sample's protein load and the signal resulting from it. However, difficulties with membrane protein precipitation due to interaction of their hydrophobic domains is increased at higher concentrations.

The results do not make clear whether the source of error lies in protein expression, or detection, or both. Looking for potential sources of error on the expression level started with verifying the quality of the expression plasmids, especially their sequences. As the reliability of Sanger sequencing drops after 800 sequenced nucleotides, it is difficult to make statements about possible sites of premature translation termination. Sequencing with different primers that bind not at the very beginning but in the middle of the expression sequence will provide detailed information on the DNA's quality.

If the detection by fluorophore-labelled lysine turns out to be unreliable as well, the use of radioisotopes represents an alternative labelling method. Instead of a fluorescent tracer, radioactivity is the measurable signal that indicates protein synthesis. This method's sensitivity is higher than a measurement of fluorescence. Isotopes would not alter the protein structure, which would be a critical concern in later experiments. A fluorescent molecule of high molecular weight could interfere with protein insertion into a membrane. However, the use of isotopes would require higher levels of safety precautions and training of the people involved with the project.

Further steps in this project will commence with re-sequencing of the expression plasmids with more than one set of primers to guarantee reliable information. A comparative bioinformatics analysis between the individual sequences, especially the elements related to transcription and translation, could reveal differences that might have an impact on the success of *in vitro* protein expression with a wheat germ system. Of particular interests would be similarities between the sequences of CD4 and LHCII-VSV, which might explain why both proteins could be produced consistently using the wheat germ extract system. If, however, detection of CXCR4, CCR5 and CLDN2 failed merely due to a signal being under the limit of detection, concentrating the samples could improve protein detection.

The reliability of protein detection is crucial for this project, as the results will be used to make qualitative statements about whether or not proteins integrate into artificial membranes. Only a very specific detection method is suited for this purpose. The high background resulting from the environment of the *in vitro* protein synthesis renders unspecific detection methods like Coomassie blue staining unsuitable. Even detection by Western Blot is not sufficient, as unspecific interactions of antibodies occur. The incorporation of fluorophore-labelled amino acids links signal creation directly to protein synthesis and represents a promising tool for further work on this project. Understanding the factors that influence the outcome of *in vitro* protein synthesis could provide useful information, not only for co-translational insertion-related studies, but also for synthetic biology in general.

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