Identification and subsequent optimization of RKD1, RKD2 and RKD4 fusion protein expression regarding post-induction incubation temperature

Submitted by

Marcel Piepers

Department of Biochemistry

In partial fulfilment of the requirements

For the degree of Bachelor of science

Wageningen University

Wageningen, The Netherlands

30-09-2016

Supervisor: Prof. Dr. S.C. de Vries

Inhoud

Abstract

RKD1, RKD2 and RKD4 are plant specific transcriptional regulators. All three genes are involved in embryo formation. Recently, RKD1 has been exposed as a novel inducer of suspensor-derived embryogenesis when expressed ectopically in suspensor cells. However, it's family members RKD2 and RKD4 do not possess this capability. To elucidate why RKD1 is able to induce suspensor-derived embryogenesis and it's family members are not, here an ongoing process of isolating RKD1, RKD2 and RKD4 is continued with regards to analyzing and optimizing the expression of soluble RKD1, 2 and 4 in Rosetta BL21 E.coli cells. Rosetta BL21 E.coli cells are transformed with a pTWIN1d plasmid containing either a RKD1, RKD2 or RKD4 gene insert. Subsequently expression of the gene insert in tandem with a CBD tag was induced and cells were incubated at 20°C, 25°C, 32°C or 37°C. To analyse the results, western blots and coomassie stains were performed on protein samples obtained from expression tests.

From analysis it was concluded that recombinant protein is expressed in both soluble and non-soluble form. Soluble and non-soluble RKD fusion protein(RKD+CBD-tag) was identified at a MW of 68.2 kDa(RKD1), 54,9 kDa(RKD2), and 68,5 kDa(RKD4). Separation of the RKD protein from the tag was observed and caused by exposure of the intein linker to the redox potential inside the E. coli cells. Maximum soluble RKD1, RKD2 and RKD4 fusion protein was observed when cells were incubated at 20°C, 20°C and 25°C respectively. While more protein cleavage was observed, the highest amount of RKD fusion protein was produced at 25°C.

Introduction

Basic seed structure

Seeds are an essential part of nature. They form the means of reproduction for plants and are an important part of human and animal diet. Seeds consist of a miniature plant, the embryo, surrounded by a nutrient rich tissue, endosperm, both enclosed in a protective coat, see Figure 1. The purpose of the seed is to disperse the embryo to land with favourable environmental conditions for germination. Upon germination the protective coat ruptures, and the embryo is allowed to develop into a mature plant.

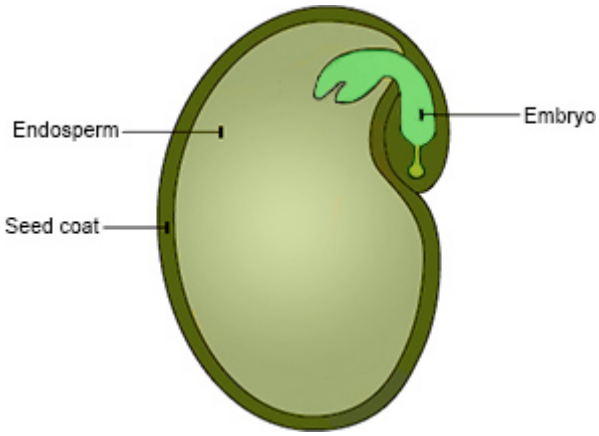


Figure 1: Image of a seed, showing its basic structure, consisting of the embryo, endosperm and its protective coat.

Origin of the embryo

Traditionally in seed plants, the plant embryo originates from a plant's egg cell once it is fertilized. Fertilization is initiated when the male gametophyte, pollen grains, land on the *stigma* of a flower, see Figure 2. The pollen grains consist of a tube cell and a generative cell. Upon landing the tube cell elongates down the plant's *style,* through the *micropyle* into the *embryo sac.* Meanwhile, the generative cell divides into two sperm cells. One of the sperm cells fuses with the egg cell to form a diploid zygote (Copeland & McDonald, 1999). After fusion of the two gametes the zygote will elongate and divide asymmetrically into a larger basal and a smaller apical cell. Most of the apical cell will develop into the mature embryo, whereas the basal cell will form the extra-embryonic suspensor, see Figure 3. The extra-embryonic suspensor is believed to aid in positioning the developing embryo within the seed (Kawashima & Goldberg, 2010). The second sperm cell fuses with the polar nucleus of the embryo sac forming a triploid nucleus cell. This cell will grow separately from the embryo into endosperm which offers mechanistic support and provides nutrients (through a second function of the suspensor) to the developing embryo (Yan, Duermeyer, Leoveanu, & Nambara, 2014).

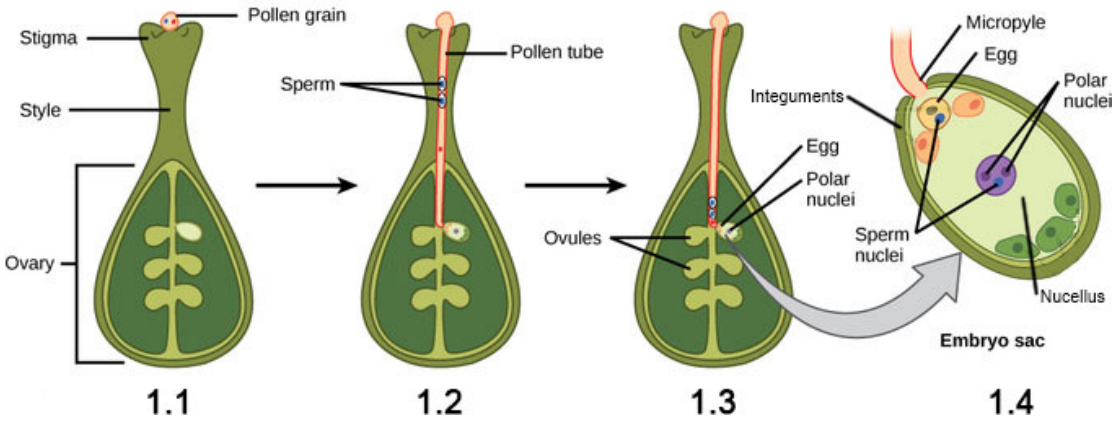


Figure 2: Double fertilization occurring in a plant's pistil. This Figure shows the path the pollen grain takes through the plant's pistil to fertilize the egg cell. All the plant organs, cells and tissues are labeled.

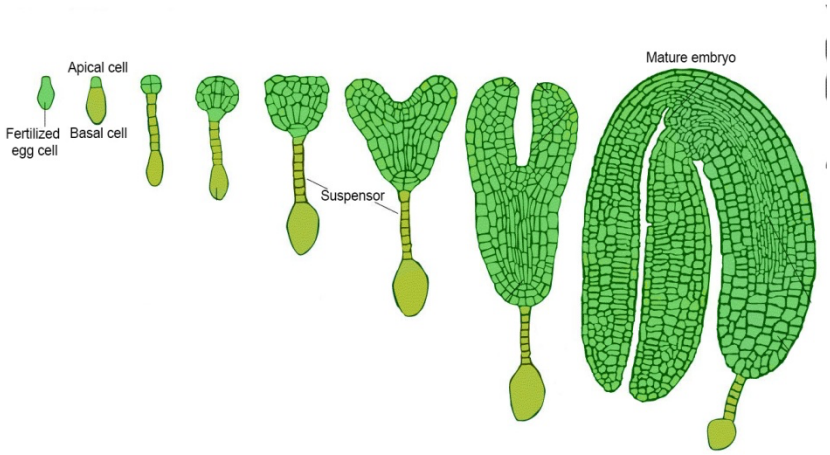


Figure 3: Starting on the left side of the Figure, it can be seen how the fertilized egg cell(zygote) divides asymmetrically into a larger basal and smaller apical cell. Following the embryo development, multiple rounds of coordinated cell division take place. The various stages of embryo development are shown including the formation of the extra-embryonic suspensor from the basal cell and the apical cell's growth into most of the mature embryo.

Origin of embryo initiation

In flowering plants, embryo development, also known as embryogenesis, generally follows the pattern of sexual reproduction induced by (double) fertilization. However, the initiation of embryogenesis is not restricted to the fertilized egg cell. A variety of different origins of embryogenesis have been reported. Somatic cells, primordial seed cells, and microspores have the capability to differentiate and adopt an embryo identity. Thereafter developing into a mature embryo (Asker & Jerling, 1992; Reinert, 1959; Steward, Mapes, & Mears, 1958; Touraev et al., 1997). Somatic *Daucus carota* cells, which for years only formed roots were the first to develop into complete embryos on a synthetic medium (Steward et al., 1958). The primordial seed cells; the egg cell, nucellus, or integuments(see Figure 1.4 for cell/tissue location) also have been shown to be capable of initiating embryogenesis. These cells can grow into a mature embryo through various pathways resulting in genetically identical offspring (Maheshwari & Maheshwari, 1950). This capability of primordial seed cells to initiate embryogenesis extends to the precursor cells of pollen grains, microspores. Microspores have been shown to be able to generate haploid embryos when exposed to stress treatment in culture (e.g. temperature, nutrient or osmotic stress)(Soriano, Li, & Boutilier, 2013; Touraev et al., 1997). However, the underlying genetic processes that trigger these cells to differentiate and adopt an embryo identity are elusive, and are thought to vary based on cell type. Therefore a fundamental question is addressed; whether a set of genes exists that can initiate a transition to embryo identity, or if misexpression of specific genes disrupts cellular homeostasis in such a way that cells revert back to an undifferentiated state and subsequently adopt an embryo identity (T. Radoeva & Weijers, 2014).

Trying to find an answer

To elucidate the answer to the question asked, recently, 15 genes that are known to induce embryogenesis, see Table 1, were ectopically misexpressed in *Arabidopsis Thaliana* suspensor cells (Ikeuchi, Sugimoto, & Iwase, 2013; T. M. Radoeva, 2016). Previously, these embryogenesis inducers were tested in various experimental models which complicated the issue whether they are part of a coherent genetic network (Boutilier et al., 2002; Hecht et al., 2001; Waki, Hiki, Watanabe, Hashimoto, & Nakajima, 2011; Zuo, Niu, Frugis, & Chua, 2002). To combat this, a uniform and proven model (*Arabidopsis Thaliana* suspensor specific GAL4/GFP driver line M0171) was used (Rademacher et al., 2012; Weijers, Van Hamburg, Van Rijn, Hooykaas, & Offringa, 2003). Misexpression of the

transcription factors was accomplished using the two component GAL4/UAS system. Expected was that misexpression of the transcription factors leads to the development of a second embryo within the seed. This expectation is based on previous misexpression of the auxin-inhibitor *bdl* in the same M0171 suspensor driver line. Misexpression of *bdl* led to the formation of con-joined twin embryos (Rademacher et al., 2012). Surprisingly, misexpression of the 15 known embryogenesis inducers only led to the discovery of one novel suspensor-derived embryogenesis inducer. This novel gene belonged to the family of RWP-RK domain(RKD) containing transcription factors.

Gene	Function	Gene	Function
LEC1		RKD1	Regulators of egg cell fate
LEC2		RKD2	
BBM	Regulators for re-acquisition of embryonic cell fate		
AGL15			
PLT5		WUS	Regulators of meristem fate
RKD4		WOX5	
SERK1			
SERK2	Somatic embryogenesis receptor kinases		
SERK3			
SERK4			
SERK5			

Table 1: 15 ectopically misexpressed genes in *A. thaliana* suspensor specific GAl4/GFP driver live M1701 (T. M. Radoeva, 2016) **and their regular function.**

Among the 15 misexpressed genes were RKD1, RKD2, and RKD4. Both RKD1 and its closest homolog RKD2 (50% homology) are involved in regulation of an egg cell-related gene expression program (Koszegi et al., 2011), whereas RKD4 is involved in zygote elongation and suspensor formation (Jeong, Palmer, & Lukowitz, 2011). However, only RKD1, was able to induce suspensor-derived embryogenesis (T. M. Radoeva, 2016). This finding that among the 15 misexpressed genes only RKD1 was able to induce embryogenesis, and even its closest homolog, RKD2, is not, suggests that suspensor-derived embryogenesis requires a specific set of genetic regulators to initiate the transition from suspensor to embryo identity.

Importance of isolating RKDs

To investigate why RKD1 is able to initiate suspensor-derived embryogenesis and its family members, RKD2 and RKD4, are not, the structural and domain specific properties of the three proteins have to be determined. Comparing these properties could elucidate differences in their role during suspensor-derived embryogenesis. Bioinformatics software has been used to get an overview of the structural and functional properties of the three proteins (Wenzel, 2016). It has been predicted that all three RKD proteins have a DNA-interacting RWP-RK domain with a conserved binding profile. RKD4 might have a different DNA binding stability or recognition site because the predicted profile of the RWP-RK domain has a slight difference in amino acid sequence. In addition, prediction of the secondary structure were made with some overlapping structural elements. All these predictions were based on homology to existing solved protein structures. Therefore the predictions cannot be assigned as correct. The prediction profile of the RWP-RK domain only had a probability of 50% of being correctly assigned (Weertman, 2016; Wenzel, 2016). To gather detailed knowledge of the proteins structural properties a crystallographic study has to be carried out. A crystallographic study

requires proteins to be isolated in soluble form. To obtain soluble protein, heterogeneous gene expression of the RKD genes can be carried out.

Heterogenous gene expression

In principle heterogeneous gene expression is simple. Insert the RKD gene in an expression vector, transform the vector into a host, allow the host to grow in nutrient rich medium, induce expression of the gene and purify the RKD protein.

Bacterial growth

The life cycle of a bacterial culture can be divided into four phases; lag phase, log phase, stationary phase, and death phase, see Figure 4. During the lag phase, bacteria adapt to growth conditions(e.g.temperature, pH, salt concentration, O_2 availability, humidity) and synthesis of RNA, enzymes and other molecules required for reproduction occurs. Once adapted, the bacteria divide, and the culture enters the exponetial phase. The exponential phase can be measured with an optical density of the culture at 600 nm(OD600), usually the culture is present in the log phase when the OD600 is between 0.6 - 0.8. However bacteria require nutrients and nutrients in the growth medium are not endless, therefore after a while there won't be enough nutrients to sustain growth of the bacterial culture. The growth rate and death of the bacteria will be equal and the culture will be present in a stationary phase. When nutrients in the growth medium are completely depleted, or if environmental conditions (e.g. temperature) are not suiTable for growth anymore, the death rate will overtake the growth rate and the bacterial culture will enter the death phase.

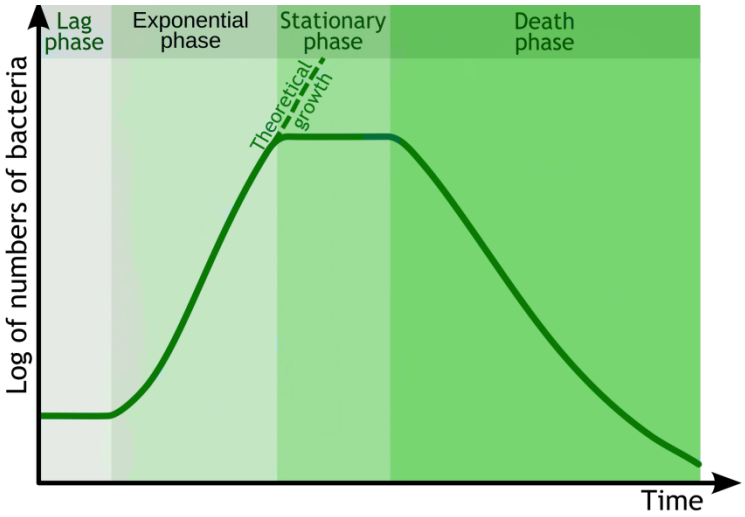


Figure 4: A visual representation of the bacterial life cycle. The logarithm of the number of bacterial cells is plotted against time. This graph gives an idea of the amount of bacterial cells in each phase.

Host organism

In terms of heterogeneous gene expression E.coli has always been the preferred host. It has fast growth kinetics and can reach a high cell density within a short period of time (Sezonov, Joseleau-Petit, & D'Ari, 2007). In addition, E.coli cells have been generated that harbour a plasmid_(pRARE) that encodes for several rare codon tRNA's. This increases the likelihood that an eukaryotic (plant) gene is expressed correctly. The Rosetta E.coli strain is an example of harbouring this pRARE plasmid.

Growth medium

To sustain growth of E.coli bacteria a nutrient rich medium is required. LB (Luria Bertani) medium is the most commonly used medium for bacterial growth. It contains the enzymatic reaction product of the milk protein casein (peptone), yeast extract and sodium chloride. Peptone is rich in amino acids and peptides whereas yeast extract contains nucleic acids, lipids and other nutrients needed for bacterial growth. Because LB allows growth of an E.coli culture up to an OD_{600} of 2 to 3, specific

growth medium to reach higher cell densities have been designed. Terrific Broth (TB) medium is a phosphate buffered rich medium. In addition to 20% more peptone and 380% more yeast extract than LB, TB also has added glycerol as a carbon source. These added nutrients allow growth up to an OD_{600} of 5 to 8(Expression Technologies Inc., 2003).

Plasmids

Plasmids are the means of heterogeneous expression. Various components of a plasmid allow for easier and controlled expression of the inserted gene. Taking a pTWIN1Δ1 vector as an example, see Figure 5, the components of a plasmid are discussed. One of the things to consider is the plasmid's copy number because too many copies of the plasmid may impair bacterial growth. pTWIN1Δ1 contains an additional *rop*(repressor of primer) gene that is responsible for keeping the copy number of the vector low (~15-20)(Shetty, Endy, & Knight, 2008).

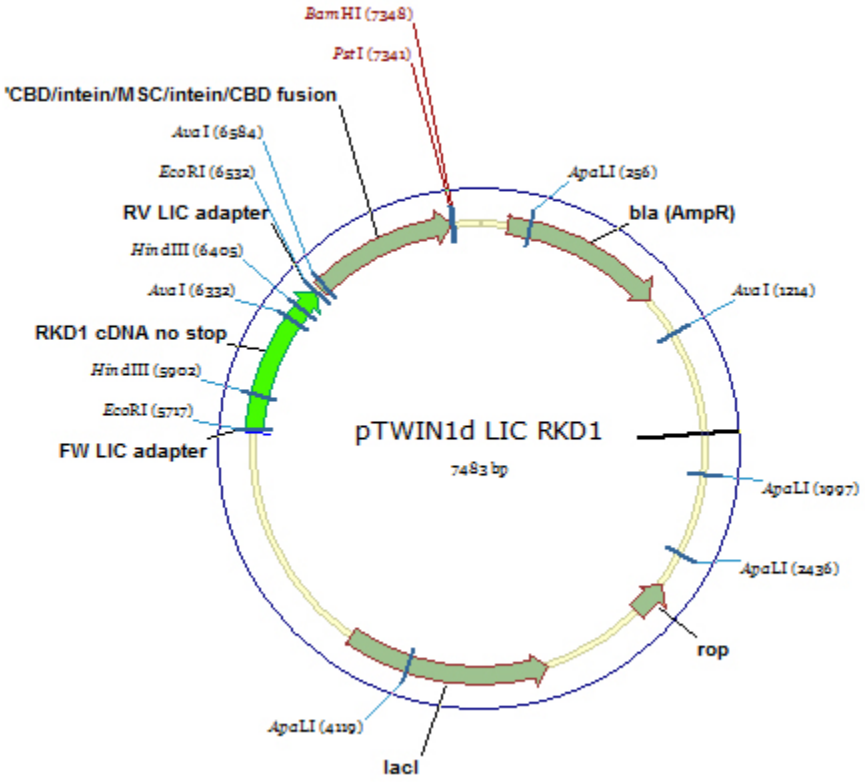


Figure 5: A pTWIN1d plasmid. The plasmid contains a RKD1 gene insert linked to a CBD sequence, a lacI gene and a bla gene. In addition, all recognition sites for restriction enzymes are indicated.

Promoter

Gene expression of the inserted RKD gene in the pTWIN1Δ1 plasmid is regulated under control of a T7 promoter. The T7 promoter is recognized by the highly active T7 RNA polymerase. T7 RNAP synthesizes RNA at a rate several times higher than E.coli's RNA polymerase and terminates transcription less frequently (Tabor, 2001). T7 RNAP is therefore preferred for high volume protein production. The T7 RNAP gene is present in the Rosetta E.coli genome and its expression is under control of a *lac*UV5 promoter. The lacUV5 promoter is a variant of the *lac* promoter that is less sensitive to catabolite regulation (induced in the presence of glucose in growth medium)(Silverstone, Arditti, & Magasanik, 1970). The lacUV5 promoter is inhibited by LacI present in the genome of E.coli.

However the one chromosomal copy of LacI is not able to prevent expression at all times which sometimes lead to basal expression of T7 RNAP in the absence of inducer(IPTG). Therefore the Rosetta E.coli cells carry a mutated version of the LacI gene; $LacI^q$. $LacI^q$ has a 10-fold higher expression than LacI and thus controls basal expression (Calos, 1978). In addition, pTWIN1Δ1 plasmids contain an additional LacI gene further increasing basal expression control. T7 RNAP expression is thus inhibited by the LacI genes until expression is induced with lactose or Isopropyl β-D-1-thiogalactopyranoside(IPTG). IPTG binds to the lac inhibitor allowing expression of T7 RNAP which in turn binds to the T7 promoter on the plasmid inducing expression of the inserted RKD gene.

Selection marker

To prevent growth of plasmid-free cells or other microorganisms in the growth medium, antibiotics are added. The bacteria require a resistance gene for the added antibiotic. In the case for pTWIN1Δ1 the *bla* gene is inserted. The *bla* gene confers resistance to ampicillin by expression of an pleriplasmic enzyme that degrades ampicillin.

Affinity tag

To attain the purified (soluble) recombinant protein it is of importance to have means to detect it along the expression and purification scheme. The expression of a polypeptide (fusion partner) in tandem allows for this (Nilsson et al. 1997). In the pTWIN1Δ1 vector, an intein-Chitin Binding Domain(CBD) fusion tag is cloned in frame at the C-terminus of the RKD insert for this purpose, expression results in a RKD-intein-CBD fusion protein. The weights of the expected expression products are seen in Table 2. An intein is a segment of a protein that is able to excise itself under specific conditions and join the remaining parts (exteins) with a peptide bond. Intein-mediated splicing occurs after intein-containing mRNA has been translated into a precursor protein. The precursor protein contains three segments; an N-extein, followed by the intein followed by a C-extein. After splicing, the resulting protein contains the N-extein linked to the C-extein, and the intein is removed. Figure 6.1 shows a visual representation of this mechanism.

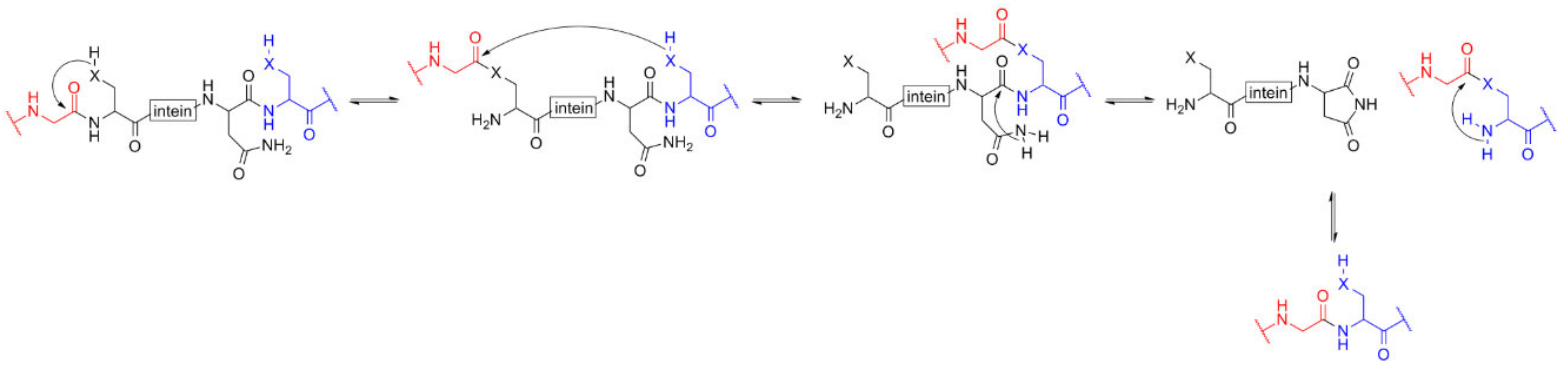


Figure 6: Mechanism of protein splicing. N-exteins are shown in red, and C-exteins in blue. X represents either an oxygen or sulfur atom.

Protein	MW (kDa)
CBD intein tag	27.8
RKD1	30.6
RKD2	33.9
RKD4	29.9
RKD1 + CBD tag	58.4
RKD2 + CBD tag	61.7
RKD4 + CBD tag	57.7

Table 2: Expected molecular weights of RKD fusion proteins, RKD proteins, and CBD-tag. This data was obtained from Weertman's report.

This mechanism of protein splicing is modified in the intein-CBD affinity tag so that cleavage only occurs at the N-terminal induced by 1,4-dithiothretol, β-mercaptoethanol or cysteine at low temperatures and over a broad pH range (Chong et al., 1997). This causes RKD fusion protein to splice into RKD, and the intein-CBD fusion. However, for crystallographic studies this process is unnecessary and the tag can be left in place.

Previous isolation work

In May 2016 work has been started to isolate RKD1, RKD2 and RKD4 (Weertman, 2016). Three types of pTWIN1Δ1 plasmids have been generated containing either a RKD1, RKD2 or RKD4 gene insert. Rosetta BL21 E.coli cells were transformed with these plasmids and an expression test(ET) was carried out. The resulting protein samples were loaded on an acrylamide gel, run and subsequently coomassie stained. However, none of the RKD fusion proteins could be identified with certainty and it remained uncertain if the fusion proteins were expressed. To conclude if any recombinant protein was expressed it was recommended to perform a western blot on the extracted protein samples with antibodies specific for CBD.

Road to isolation of RKD

The next step in isolating RKD fusion protein would be to examine Weertman's samples by carrying out the recommended western blot. The examination is carried out here. Knowing that Weertman's generated plasmids are suiTable for heterogeneous gene expression, new Rosetta BL21 E.coli cells are transformed with Weertman's plasmids. The newly transformed cells were used to do part of the optimization for maximum expression of soluble recombinant RKD protein. Part of the optimization included finding the optimum temperature for expression of RKD post induction. Therefore, multiple ETs are carried out in which RKD expression is induced in the cells and subsequent incubation of the cell is done at various temperatures. The results are analyzed using a western blot procedure and a conclusion regarding the optimum temperature post induction is draw.

Materials and Methods

Throughout the course of this project Rosetta BL21 E.coli cells were transformed with pTWIN1Δ1 plasmids, transformed cultures were grown from which glycerol stocks were stored and plasmids were isolated. The plasmid's gene insert was amplified using PCR amplification. Expression tests were carried out on transformed cells and analysis was done using SDS-PAGE gel electrophoresis, western blots and coomassie staining. These methods are described below. In addition, a step-by-step protocol for each method described in this chapter can be found in Appendix A.

Transformation of Rosetta BL21 E.coli cells (with pTWIN1Δ1 plasmids)

Competent Rosetta BL21 E.coli cell stocks and pTWIN1Δ1 plasmids were available and stored at -80°C and 4°C respectively. The Rosetta BL21 E.coli cell stocks were generated by WAM van den Berg at the department of Agrotechnology and Food Sciences of Wageningen University, The Netherlands. The pTWIN1Δ1 plasmids were generated by Naomi Weertman, a Master student Molecular Life Sciences at Wageningen University. Figures 7 shows the plasmid used and it's assigned a codename which will be referred to. Likewise for a RKD2, RKD4 or no gene insert, the corresponding plasmid has the codename pTWIN1d-RKD2, pTWIN1d-RKD4 and pTWIN1d respectively.

Competent Rosetta BL21 E.coli cells were diluted 1:1 in 50% glycerol. The diluted cells were transformed with pTWIN1d plasmid(pTWIN1d-RKD1, pTWIN1d-RKD2, pTWIN1d-RKD4, pTWIN1d) using electroporation. Once transformed, the cells were incubated in LB medium for 45min at 37°C. The grown cells were plated on a LB agar plate containing ampicillin(1:1000) and incubated O/N at 37°C.

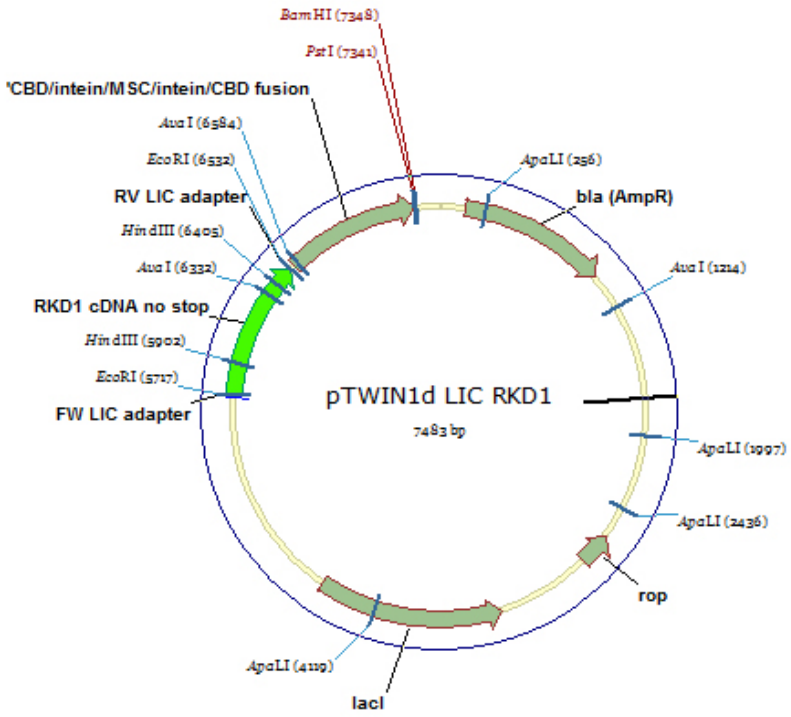


Figure 7: pTWIN1d-RKD1, containing a RKD1 gene insert linked to a CBD affinity tag.

Glycerol stock storage

Colonies were picked from generated agar plates containing transformed Rosetta BL21 E.coli cells. Picked cells were used for the growth of a culture O/N in LB medium containing ampicillin(1:1000) at 37°C. A small volume of the culture is diluted 2:3 in 100% glycerol to prevent the formation of ice crystals at freezing temperatures. The stock is labelled and frozen by dipping in liquid nitrogen. Subsequently, the stock is stored at -80°C. In appendix B a Table is shown of the glycerol stocks generated and corresponding labels.

Plasmid isolation

In parallel with the glycerol stock storage, plasmids are isolated from grown cultures generated during the glycerol stock storage. In Appendix B a Table is included showing which cultures are used for plasmid isolation. The isolation is carried out using *GeneJET Plasmid Miniprep Kit*. The plasmid isolation is carried out analogous to the protocol included in this miniprep kit. *GeneJET's plasmid miniprep kit* utilizes a silica based membrane in a spin column. Grown cells are pelleted, lysed and neutralized to create appropriate conditions for bindings of plasmid DNA to the silica membrane. The neutralized cell fragments are run through the spin column, and the plasmid DNA remains loaded on the silica membrane. Subsequent elution with 10mM Tris-Hcl(pH 8.5) results in isolated plasmid DNA. The plasmid concentration was measured using *Nanodrop*^{TM}'s 2000/2000c spectrophotometer, with 10mM Tris-HCL (pH 8.5) as a blanco. Subsequently, the plasmids were stored at 4°C. In appendix B a Table is included showing the measured plasmid concentrations.

PCR amplification

PCR amplification is done to identify if transformed cells are correctly transformed with a (RKD1, RKD2 or RKD4) gene insert. Isolated plasmid samples(pTWIN1d-RKD1, pTWIN1d-RKD2 and pTWIN1d-RKD4) were used for PCR amplification of the gene insert. Forward and reverse primers with sequences specific for the gene inserts were used. The PCR amplification is carried out like described in Appendix A under *PCR amplification* protocol, this protocol also included the primer sequences. The resulting PCR amplification products were loaded on a 1% agarose gel containing 20µl ethidium bromide. The gel was run in 0.5x Tris-acetate-EDTA(TAE) at 100V for 30min.

Expression tests (at various post induction incubation temperatures)

ETs(ET) were done to induce the expression of recombinant protein in the transformed (Rosetta BL21 E.coli) cells. A small amount of cells was taken out of the glycerol stocks containing for the growth of a culture in LB medium containing ampicillin(1:1000) O/N at 37°C. A Table showing which glycerol stocks are used is included in Appendix B. The O/N grown culture was diluted 100x in TB medium containing ampicillin(1:1000) and allowed to grow to an OD_{600} of 0.8 at 37°C. All incubations(20, 25,32, 37°C) of cell cultures are done in a variable shaker at 150 rpm to aerate the cells in the growing culture. Expression of fusion protein was induced with 0.4mM IPTG. Appendix B contains a Table indicating the cell densities in terms of OD_{600} of the cultures before induction. The induced cultures were incubated O/N at 20°C, 25°C, 32°C, or 37°C. The following day the cells were spun down(4500 rpm) for 10min at 4°C. The pellet was resuspended in 50mM NaPi and sonicated 3 x 10sec while kept on ice. The sonicated samples were spun down(13000 rpm) at 4°C. The supernatant(SN) was separated from the pellet(P) and the P was resuspended in 50mM NaPi equal to the volume of SN. The extracted samples were stored at -20°C or used directly for SDS-PAGE gel electrophoresis. In appendix B a Table is included indicating all generated samples separated based on their gene insert and post-induction incubation temperature(PIIT).

SDS-PAGE gel electrophoresis

SDS-PAGE gel electrophoresis was done to separate proteins based on their molecular weight. In every case protein samples were obtained from ETs. These samples were prepared for loading by addition of a 4x sample buffer(SB) containing: 12% sodium dodecyl sulfate(SDS, unfolds and adds a negative charge to proteins), 5% β-mercaptoethanol(β-ME, breaks protein's di-sulfide bonds), 25% glycerol(adds weight to the samples to allow for easier loading), and 0.02% bromophenol blue(stains proteins to allow for tracking through acrylamide gel) dissolved in 150mM Tris/HCl(pH 7.0). In some cases the protein samples were diluted in MilliQ water before addition of SB(5x, 10x, 20x, 100x, 400x, 500x, 700x, 1000x). After addition of SB, the samples were heated at 95°C for 10min to denature the proteins and subsequently spun down for 30sec at 14000 rpm to pellet all contaminations(e.g. genomic DNA). Prepared samples were loaded on 12%, 1mm gels. For 15 wells gels 10µl sample was loaded, and for 10 wells gels 15µl sample was loaded. The loading order of every gel, and dilutions(dil.) of every protein sample can be found in Appendix B. Loaded gels were run at constant ampere and variable voltage. The gels were run at 90V throughout the stacking gel, and the voltage is increased to 120V when the stacked protein samples cross the stacking to running gel interface to increase migration speed.

Western blot

Western blot was used to visualize recombinant protein that may have been expressed by transformed cells. To transfer proteins from acrylamide gel to nitrocellulose membrane, *Bio-rad's Transblot-Turbo Transfer System* is used. The nitrocellulose membrane is used for its increased retention abilities compared to the optional PVDF membrane. The gel with the membrane on top is sandwiched between two stacks of ion reservoir paper. The stack is soaked in *Trans-blot Turbo buffer* and loaded into *Bio-rad's Transblot Turbo Transfer System's* cassette. The *turbo mode*(7min) for 1 mini gel is used. After transfer, the membrane is washed shortly in Tris-Buffered Saline Tween 20(TBST) to remove excess transfer buffer. Subsequently, the membrane is blocked in blocking solution consisting of 3% non-fat milk dissolved in TBST for 60min at room temperature(RT) or at 4°C O/N (/over the weekend). This is done to prevent non-specific binding of primary antibody to the nitrocellulose membrane. After blocking, the membrane is washed shortly in TBST and subsequently incubated with the primary antibody(monoclonal mouse-derived anti-CBD(E8034) diluted (1:1000) in blocking solution) O/N at 4°C (/over the weekend).

After the first incubation, the membrane is washed 3 x 10min in TBST to remove unbound primary antibody and incubated with the secondary antibody(HRP-conjugated polyclonal rabbit anti-mouse (RαM) diluted (1:1000) in blocking solution) for 45 - 60 min at RT. After the second incubation, the membrane is washed 3 x 10 min in TBST to remove unbound secondary antibody.

The membrane is now analysed using chemiluminescence detection. For chemiluminescence detection *Bio-Rad's ChemiDoc™ imaging system* is used in combination with the software program *image lab*. The membrane is exposed to peroxidase and luminol in a 1:1 ratio as a substrate for horseradish peroxidase(HRP), conjugated to the secondary antibody. HRP catalyses the oxidation reaction of luminol by peroxidase with light emission as a by-

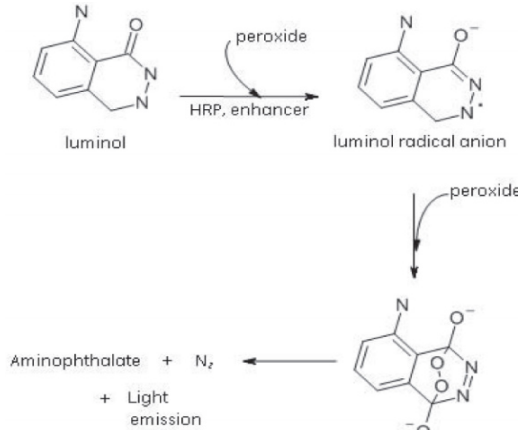


Figure 8: 3-step oxidation reaction mechanism of luminol by peroxidase, cataylsed by HRP, resulting in light emission as a by-product.

product(see Figure 8). HRP greatly enhances the intensity and duration of light emission. The enhanced chemiluminescence(ECL) activity is captured after 20 sec of exposure using no filter on the imager. In addition, an image is made of the developed membrane after ~10 min of exposure using filter 1.

The obtained images are enhanced in *Adobe Photoshop CS6*, the colors of the images are inverted, and the black/white levels enhanced.

Coomassie staining

Coomassie stainings were done to stain all proteins expressed by the transformed cells. Acrylamide gels are stained in coomassie brilliant blue for 10min. Subsequently, the gels are destained O/N in 10% glaual acetic acid, 60% ddH_2O and 30% ethanol. Both incubations are done on an electric shaker bed(40 swings/min) to homogenize the solutions across the surface of the gel. Destained gels are imaged in *Bio-Rad's ChemiDoc^{TM} imaging system* using filter 1, and the colors of the image are inverted using *Adobe Photoshop CS6*.

Results

Examination of Weertman's samples

To examine if Weertman's transformed Rosetta BL21 E.coli cells expressed any recombinant protein, six protein extracts were available. The extracts were obtained from ETs carried out on 21/04/16 (preparation is carried out on 13/06/16, 52 days later). The induction of recombinant gene expression of two differently transformed Rosetta BL21 E.coli cells was carried out by Weertman. The cell were either transformed with a pTWIN1d-RKD1 or pTWIN1d-RKD2 plasmid, the expression of non-transformed competent Rosetta BL21 E.coli cells was also tested. The non-transformed cells served as a negative control(NC), to exclude that a genomic Rosetta BL21 E.coli gene interferes with the results. Weertman induced the cells to express the inserted gene with 0.3M IPTG and subsequent incubation was done O/N at 20 °C. Soluble protein(SN) fractions and insoluble(P) fractions were obtained per cell culture. This resulted in the six samples; SN and P fraction of pTWIN1d-RKD1 transformed cells, SN and P fraction from pTWIN1d-RKD2 transformed cells, and SN and P fraction from non-transformed cells.

The protein extracts were undergone SDS-PAGE gel electrophoresis followed by western blot analysis. The membrane on which the ECL activity is captured is seen in Figure 9.

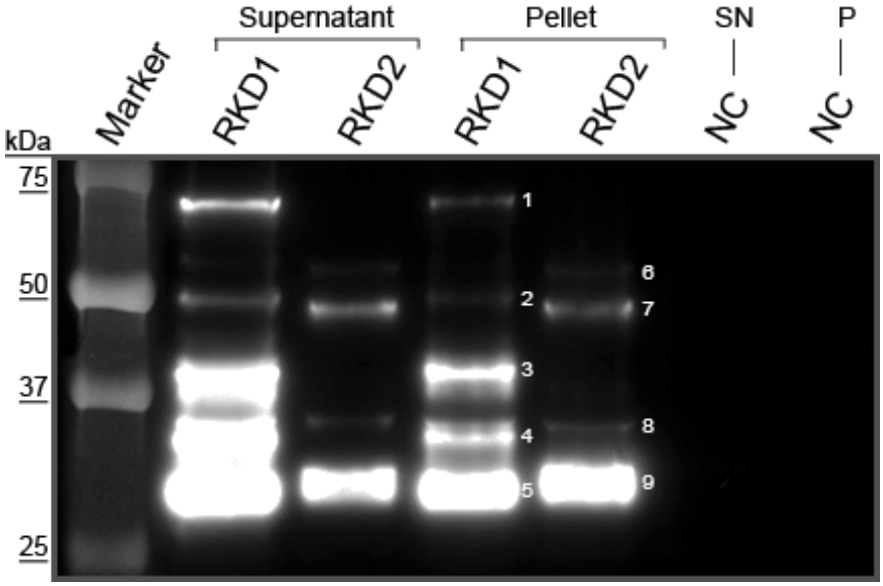


Figure 9: Western blot with samples obtained from Naomi Weertman. 12%, 1mm gel loaded with samples from Weertman's ET(5x dil.), transferred to a nitrocellulose membrane, incubated with anti-CBD followed by RαM-HRP and ECL activity is captured after 20sec of exposure to luminol and peroxide. The loading order is indicated with the gene insert present in the pTWIN1d plasmid the Rosetta BL21 E.coli cells were transformed with. The ECL activity is captured after 20 seconds of exposure.

First, it is important to mention that the negative control(NC) lanes contain no bands. In the remaining RKD1 and RKD2 lanes, bands are seen at various locations, and the band pattern is identical in SN and P fractions. In the RKD1 lane bands are seen at 67.8 kDa(1), 50,8 kDa(2), 40,0 kDa(3), 34,5 kDa(4) and 29,5 kDA(5). In the RKD2 lane bands are seen at 54,4(6) kDa, 48,6 kDa(7), 35,0 kDa(8) and 31,2 kDa(9)(Table 3).

	Band	MW(kDA)
RKD1	1	67.8
	2	50,8
	3	40,0
	4	34,5
	5	29,5
RKD2	6	54,5
	7	48,6
	8	35,0
	9	31,2

Table 3: Molecular weights of the bands visible in Figure 9. For the determination of the weights seen in this Table the mass of the proteins present in the marker lane bands are plotted against their relative run distance(retardation factor, Rf). This graph is included in Appendix B (molecular weight graphs – Weertman western blot).

Insert check of newly transformed Rosetta BL21 E.coli cells

Once it was confirmed that Weertman's samples contained recombinant protein, new Rosetta BL21 E.coli cells were transformed with plasmids previously generated by Weertman (on 18/04/16, transformation done on 24/06/16, 66 days later). Cells were transformed with pTWIN1d-RKD1, pTWIN1d-RKD2, pTWIN1d-RKD3 and pTWIN1d. Five agar plates with transformed cells were generated; one plate with bacteria with an insertionless plasmid(pTWIN1d). One plate with bacteria transformed with a pTWIN1Δ1-RKD2 plasmid. And two plates with bacteria transformed with a pTWIN1d plasmid. Ten colonies were picked per plate(fifty in total), and were used for the growth of a culture, resulting in ten RKD1, ten RKD2 and ten NC cultures and twenty RKD4 cultures. From each of these fifty cultures glycerol stocks were stored, and from twenty-five of these cultures plasmids were isolated. Five pTWIN1d-RKD1 transformed cells, five pTWIN1d-RKD2 transformed cells; ten pTWIN1d-RKD4 transformed cells, and five pTWIN1d transformed cells were picked for the isolation of plasmid. The glycerol stocks and isolated plasmids were stored, and from six of the isolated plasmid samples the gene insert was checked using PCR amplification of the supposed gene insert(RKD1, RKD2 or RKD4). Two plasmid samples containing pTWIN1d-RKD1, two samples containing pTWIN1d-RKD2, and two samples containing pTWIN1d-RKD4. The resulting PCR amplification products are seen in Figure 11. In addition, Figure 10 shows a visual representation of the procedure described above.

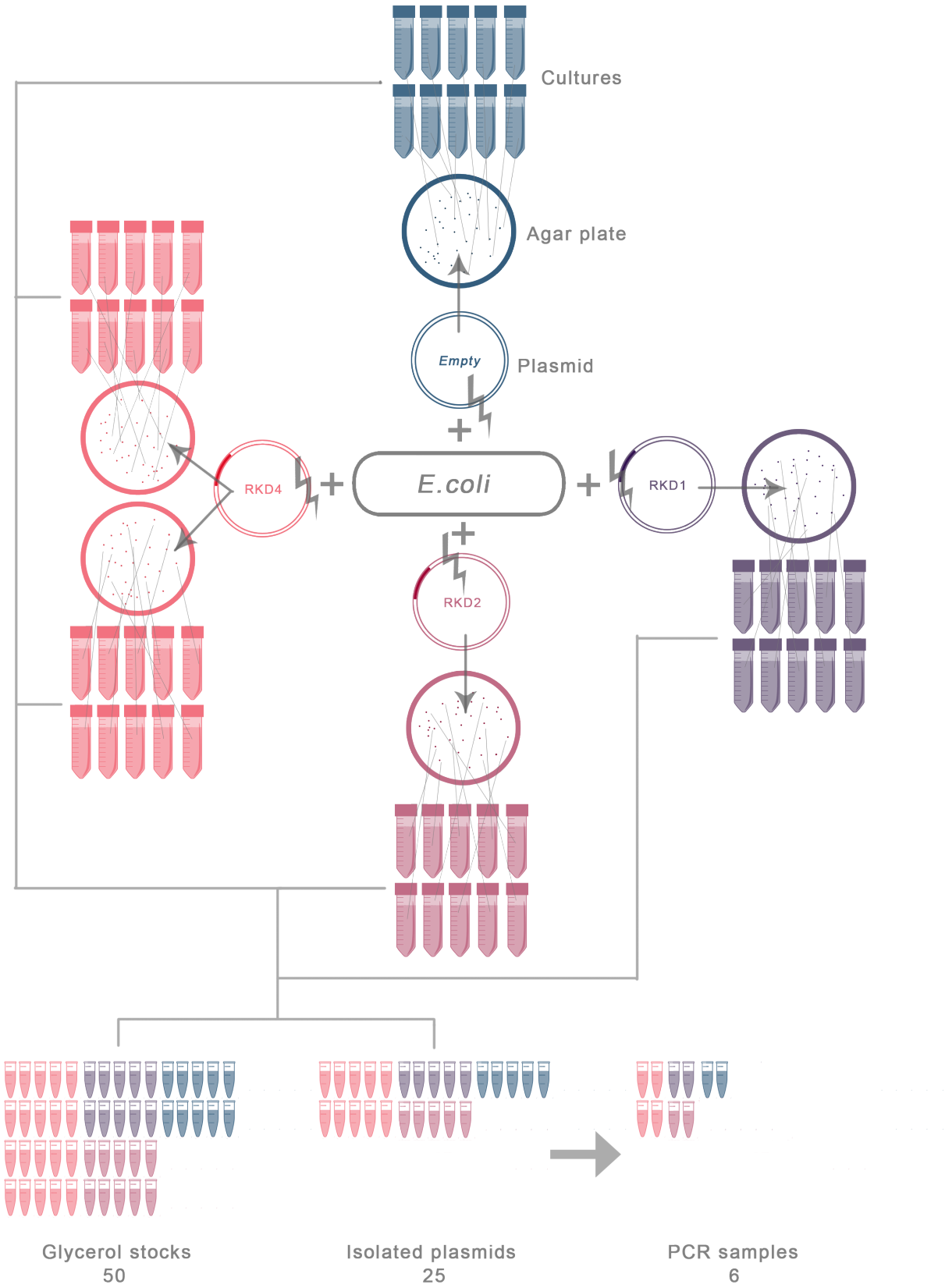


Figure 10: Visual representation of the procedure to generate PCR samples, starting from the transformation of Rosetta BL21 E.coli cells(in the middle). The competent Rosetta BL21 E.coli cells were transformed using electroporation with one of the pTWIN1d(-RKD) plasmids. The cells were plated on an agar plate and subsequently ten colonies were picked for the growth of a culture. Glycerol stocks were stored from each culture, and from half of the cultures plasmids were isolated. Six isolated plasmid samples were in turn used for PCR amplification.

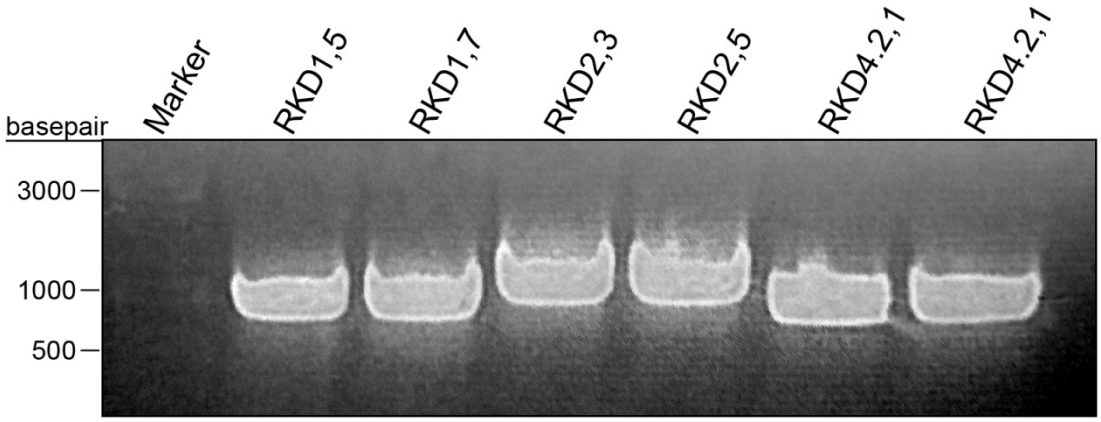


Figure 11: PCR products loaded on a 1% agarose gel. This image was taken with a mobile phone of a printed image of the agarose gel taken under a CCD image developer. The colors of the image were inverted and the black/white levels enhanced in Adobe Photoshop CS6 to obtain the image seen. The loading order is included on top. Although the marker lane is barely visible the length of the nucleotides present in the marker lane band are indicated on the left.

Figure 11 shows that both the expected RKD1 containing plasmid samples resulted in PCR amplification products with a length of ~900bp, both the expected RKD2 products a length of ~1050bp and both the expected RKD4 products a length of ~850bp.

Expression tests at various post-induction incubation temperatures

After having transformed Rosetta BL21 E.coli cells and stored transformed cells in glycerol stocks, the frozen cells were used for a variety of ETs with variable temperature post induction. pTWIN1d-RKD1, pTWIN1d-RKD2, pTWIN1d-RKD4 and pTWIN1d transformed cells(RKD1, RKD2, RKD4 and NC respectively) were induced to express recombinant protein. This was done four times, each time at a different PIIT; 20°C, 25°C, 32°C and 37°C. To determine if the bacteria expressed any recombinant (RKD fusion) protein, SN and P samples obtained from the ETs were undergone SDS-PAGE gel electrophoresis followed by western blot analysis and coomassie staining separately. Western blots were carried out to visualize the recombinant protein expressed by the transformed Rosetta BL21 E.coli cells. The coomassie stained gel visualizes all proteins expressed by the transformed cells and served as a loading control to compare the quantity of loaded samples. All results are summarized in Figure 13. In addition, Figure 12 shows a visual representation of the procedure described above.

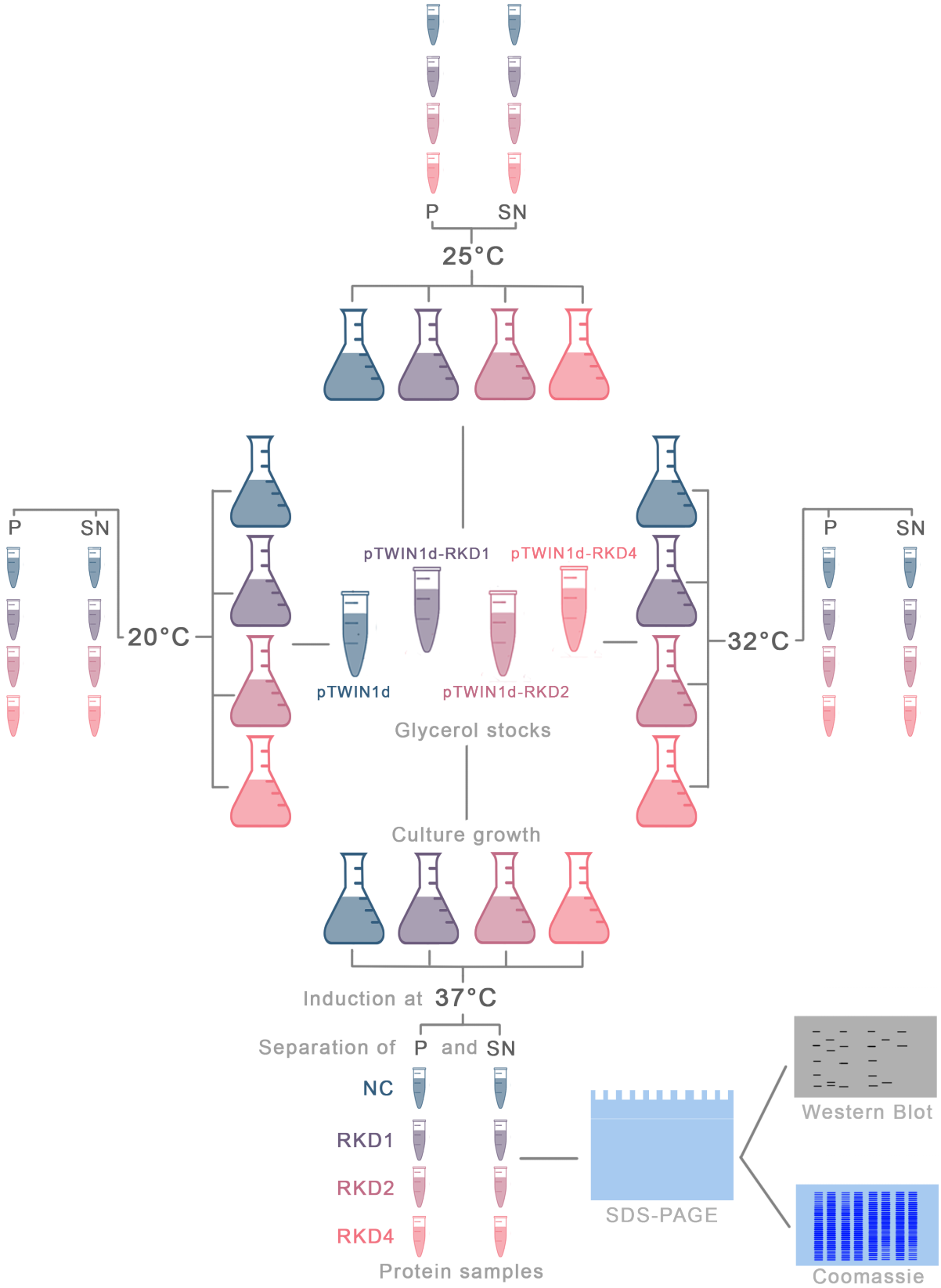


Figure 12: Visual representation of the ET procedure carried out starting from the generated glycerol stocks(middle) that are used to grow a (pre)culture. Transformed cells were grown and expression was induced at variable temperature (with 0.4mM IPTG). The resulting P and SN samples were then undergone SDS-PAGE gel electrophoresis followed by western blot or coomassie stain analysis. The samples are labeled by the gene insert present in the pTWIN1d plasmid. It should be noted that this Figure only shows the analytic western blot and coomassie procedures for the obtained 37°C samples, but the procedures are done on all generated samples.

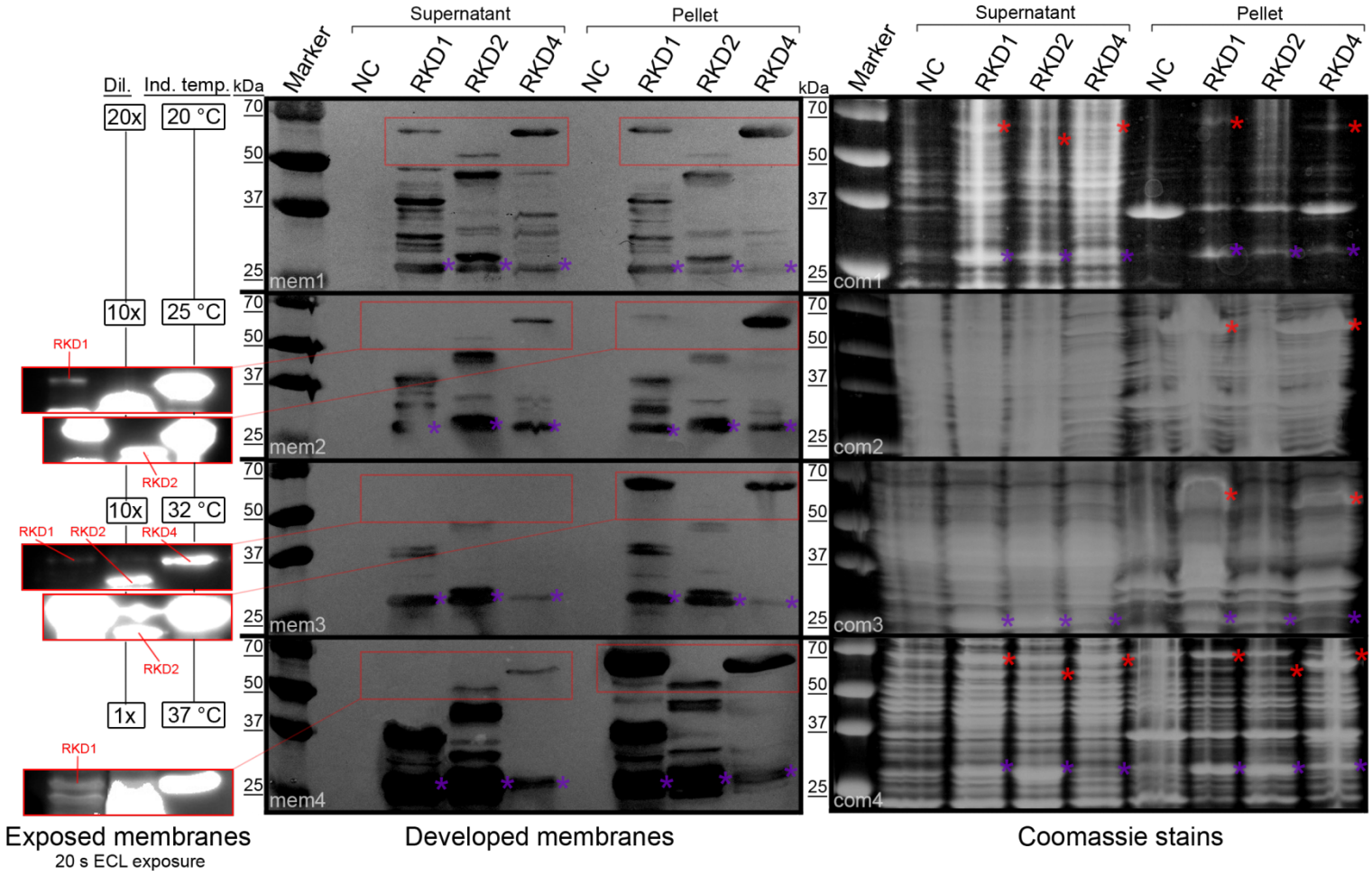


Figure 13: The *developed membranes*(*mem1 - mem5*) shown in the middle are 12%, 1mm gels loaded with samples from ET 20°C(20x dil.), ET 25°C(10x dil.), ET 32°C(10x dil.) and ET 37°C(not diluted), transferred to a nitrocellulose membrane, incubated with anti-CBD followed by RαM-HRP and the membrane is imaged after ~10min of exposure. As some protein bands are not visible on the *developed membrane*, the *exposed membranes* shown on the left are images derived from the same gel as the developed membranes, however the ECL activity is captured after 20sec of exposure to luminol and peroxidase. The *red rectangle region*(RRR) shows the area the *exposed membranes* represent on the *developed membranes*. On the right, *coomassie stains*, are 12%, 1mm gels loaded with samples from ET 20°C, ET 25°C, ET 32°C and ET 37°C(not diluted). Samples are loaded in the same order as the developed membranes. Red stars represent bands on the *coomassie stains*(*com1 - com4*) that are also visible within the RRR on the developed membrane. Purple stars represent bands that are seen both on the *developed membrane* and *coomassie stains*.

In Figure 13, no bands are exposed in any of the NC samples.

com1 Shows a reasonably equal band intensity among SN and among P samples, however the band intensity is lower in P samples compared to the SN samples. *Com2 - com4 show* reasonably equal band intensities among all samples.

The developed membranes shows various bands within the red rectangle region(RRR). The MWs of these bands are shown in Table 4.

MW (kDa)	Supernatant			Pellet		
	RKD1	RKD2	RKD4	RKD1	RKD2	RKD4
Membrane 2, 20°C	67,7	52,3	67,7	67,7	52,3	67,7
Membrane 3, 25°C	71,0	58,2	67,0	71,0	58,2	67,0
Membrane 4, 32°C	66,8	55,0	66,8	66,8	55,0	66,8
Membrane 5, 37°C	62,2	57,0	62,2	62,2	57,0	62,2
Average among membrane 2 - 5	66,9	55,6	65,9	66,9	55,6	65,9

Table 4: Molecular weights of the bands seen in the red rectangle region of Figure 13. These molecular weights are determined by plotting the molecular weights of the proteins present in the marker lane against their relative run distance, the graph are included in Appendix B. The order in which the MWs of the bands are presented in this table are equal to the loading order on the membranes in Figure 13 for easier interpretation.

The bands within the RRR are highlighted in exposed membranes because they are expected to be RKD fusion protein(RKD1: 66.9 kDa vs 58.4 kDa, expected mw)(RKD2: 55.26 kDa vs 61.65 kDa, expected mw) (RKD4: 65,9kDa vs 57,7 kDa, expected mw) and are present at about the same height as bands 1 and 6(Figure 13) exposed during the analysis of Weertman's samples (RKD1: 66,9 kDa vs. 67,8 kDa, RKD2: 55,6 kDa vs 54,5 kDa). During Weertman's sample analysis, RKD4 recombinant protein was not identified, here on the membranes recombinant protein from pTWIN1d-RKD4 transformed cells is identified.

The bands expected to be RKD fusion protein are present in both SN and P fractions. Comparing SN and P bands in the RRR horizontally; In the instance of a PIIT of 20°C the band intensities of RKD fusion protein are equal between P and SN fraction. In the instances of a PIIT of 25 °C, 32 °C and 37 °C, the band intensities are higher in the P samples compared to SN samples. Comparing SN and P bands in the RRR vertically; The bands in the RRR of SN fractions vanish as the PIIT increases(*mem1-mem3*) and return at a PIIT of 37°C(*mem4*). The bands in the RRR of P samples are present regardless of PIIT, apart from RKD2. In addition, bands present in the RRR are also present on the coomassie stained gels(gel stars).

Multiple additional smaller sized bands are seen. The band pattern of these smaller sized bands is not consistent between all RKD samples, each transformant has its distinct set of bands. *Mem1* shows the most bands among all membranes(*mem1-mem4*). As the PIIT increases(*mem2-mem3*) some bands disappear until reaching an induction temperature of 37 °C(*mem4*); here some bands return and the band intensity increases.

Smaller sized bands in RKD1 samples(SN and P) are present at 48 kDA, 40 kDa and 31 kDa. RKD1 20°C sample contains an additional five vague bands located at 44 kDA, 37 kDA, 36 kDa, 29 kDa and 28 kDa. In RKD2 samples(SN and P) bands are seen at 48 kDa, 35 kDa and 33 kDa and 28 kDa. Lastly, in

RKD4 samples bands(SN and P) are seen at 48 kDa, 36 kDa and 33 kDa. Table 5 visualizes the molecular weight of the bands based on their height.

RKD1	RKD2	RKD4
MW (kDa)	MW (kDa)	MW (kDa)
48	46	48
44		
40		
37		
36	34	36
31	33	33
30		
29	28	
27	27	27

Table 5: Molecular weights of the smaller sized bands, ordered based on their size to support the text. In grey are the bands only visible on samples derived from 20°C ETs. It should be noted that these MWs are not determined from the graphs included in appendix B, but are estimated by observation of height compared to the marker lane.

One smaller sized band is present on every membrane and coomassie stain(excluding *com2*) and is marked with a purple star. This band is located at a height corresponding to ~27 kDa.

Expression test comparison

Recombinant (RKD fusion) protein was identified in the samples extracted from all four ETs(20°C, 25°C, 32°C and 37°C). In addition, it was suspected that the amount of insoluble recombinant protein increases with increasing PIIT. To conclude which PIIT resulted in maximum soluble recombinant (RKD fusion) protein and to elucidate whether the amount of insoluble recombinant protein increases with increasing PIIT, samples from each ET (with equal gene insert) were aligned with increasing PIITs. In addition, samples are separated based on their solubility(SN or P fraction) on different gels. The results are shown in Figure 14.

A general observation can be made of the membranes in Figure 14. Both pellet and supernatant membranes are slightly skewed, P samples a bit more and some bands(1(P), 3(P+SN), 4(P+SN), 5(P+SN), 7(P), 9(P+SN), 10(P+SN), 12(SN)) have a very high intensity.

In Figure 14, for RKD1(SN) sample, band 1 decreases in intensity with increasing PIIT(20°C-37°C). Band 2, 3, 4 and 5 first increase(20-25°C), then decrease in intensity(25-37°C).

For RKD2(SN) sample band 6 decreases in intensity with increasing PIIT(20-37°C). Bands 7, 8 and 9 first increase(20-25°C), then decrease in intensity(25-37°C).

RKD4(SN) sample bands 10 and 12, first increase in intensity with decreasing PIIT(20-25°C), then decrease in intensity(25--37°C). Band 11 is vaguely present at a PIIT of 20°C.

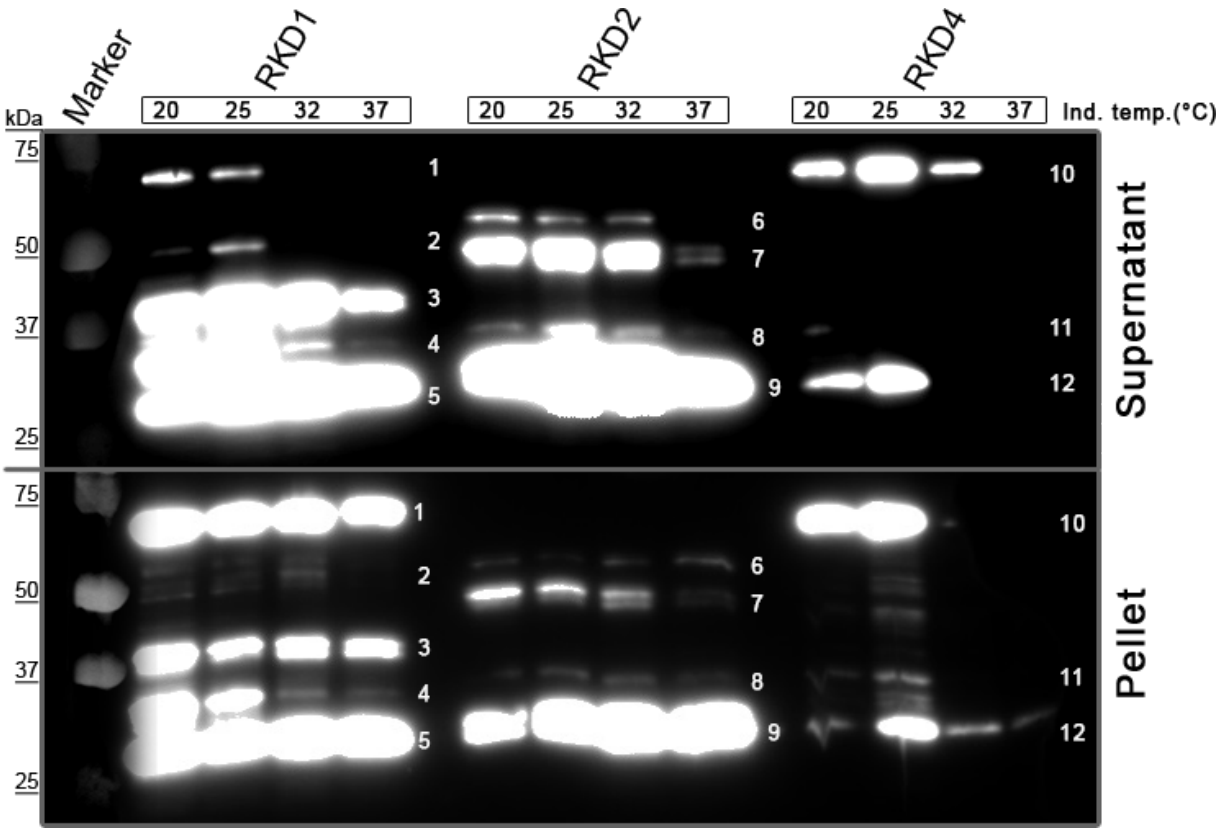


Figure 14: Induction temperature comparison western blot. 12%, 1mm gel loaded with samples from ET 20°C, ET 25°C, ET 32°C and ET 37°C(100x dil.), transferred to a nitrocellulose membrane, incubated with anti-CBD followed by RαM-HRP and ECL activity is captured after 20sec of exposure to luminol and peroxide. The image shows two membranes, supernatant and pellet membrane. Protein samples are aligned based on their gene insert and with increasing PIIT. Each band is assigned a number, and the number of the bands correspond between pellet and supernatant samples. The MWs of the numbered bands are shown in Table 6.

RKD1(P) sample bands 1, 2, and 5 are equal among all PIIT(20-37°C). Band 2 is equal among PIITs of 20-32°C and not present at 37°C. Band 3 is more intense at a PIIT of 20°C and equally intense at PIITs 25-37°C. Band 4 decreases in intensity with increasing PIIT(20-37°C).
RKD2(P) sample band 6 and 8 are equally intense among all PIITs(20-37°C). Band 7 decreases in intensity with increase PIIT(20-37°C). Band 9 increases with increasing PIIT(20-25°C) then remains equally intense(25-37°C).

RKD4(P) sample band 10 and 11 increase with increasing PIIT(20-25°C), then vanish at PIITs of 32°C and 37°C. Band 12 increases with increasing PIIT(20-25°C), then decreases with increasing PIIT(25-37°C). Many additional bands are seen in RKD4(P) samples at PIITs of 20 and 25°C, these first increase in intensity with increasing PIIT(20-25°C), then vanish at PIITs of 32°C and 37°C.

The molecular weights of all numbered bands seen in Figure 14 are shown in Table 6. These bands roughly correspond with the previously observed bands on the developed membranes seen in Figure 14. Bands 1, 6 and 10 correspond with RKD fusion protein.

The captured band intensities of the expected RKD1 and RKD4 fusion proteins, band 1 and 10 respectively, are higher on pellet samples compared to SN samples. Whereas, the intensity of bands 2, 3, 4, 5 and 12 is higher on SN samples compared to P samples. The captured band intensities of the

expected RKD2 fusion protein, band 6, is higher on SN samples, and the intensities of remaining RKD2 sample bands, bands 7, 8 and 9, are also higher on SN samples.

RKD1		RKD2		RKD4	
Bands	MW (kDa)	Bands	MW (kDa)	Bands	MW (kDa)
1	70,0	6	58,0	10	71,0
2	50,0	7	50,0		
3	41,8				
4	33,9	8	37,6	11	36,6
5	29,0	9	30,6	12	31,0

Table 6: Molecular weights of bands seen in Figure 14. For the determination of these MWs the MW of the proteins in the marker lane bands are plotted against their relative run distance, the graphs are included in Appendix B.

Samples (RKD1, SN; RKD2, SN, RKD4, SN and P) at a PIIT of 32°C and 37°C show an aberrant band pattern from 20°C and 25°C samples. In addition, the pattern is also not consistent with the previously observed band pattern on the developed membrane seen in Figure 13. On the developed membranes of Figure 13, bands at a height of RKD fusion protein were observed both in P and SN samples at a PIIT of 32°C and 37°C. However, these bands are not seen on the comparison western blot.

Saturation check

Because it was expected that the P samples on the comparison western blot(Figure 14) contain more recombinant protein than their corresponding SN samples, but the observed band intensities in the P samples are too intense, it was checked whether the captured band intensity of band 10(25°C, P, Figure 14) is within the linear range for the detector to detect a difference in emitted photons. To check this, a western blot is carried out with diluted samples of RKD2, and RKD4(P) fractions from ETs with a PIIT of 25°C. For the saturation check the RKD2 samples were diluted, 50x, 100x, 500x and 1000x. The RKD4 samples were diluted 50x, 100x, 400x, 700x and 1000x. Figure 15 shows the resulting western blot.

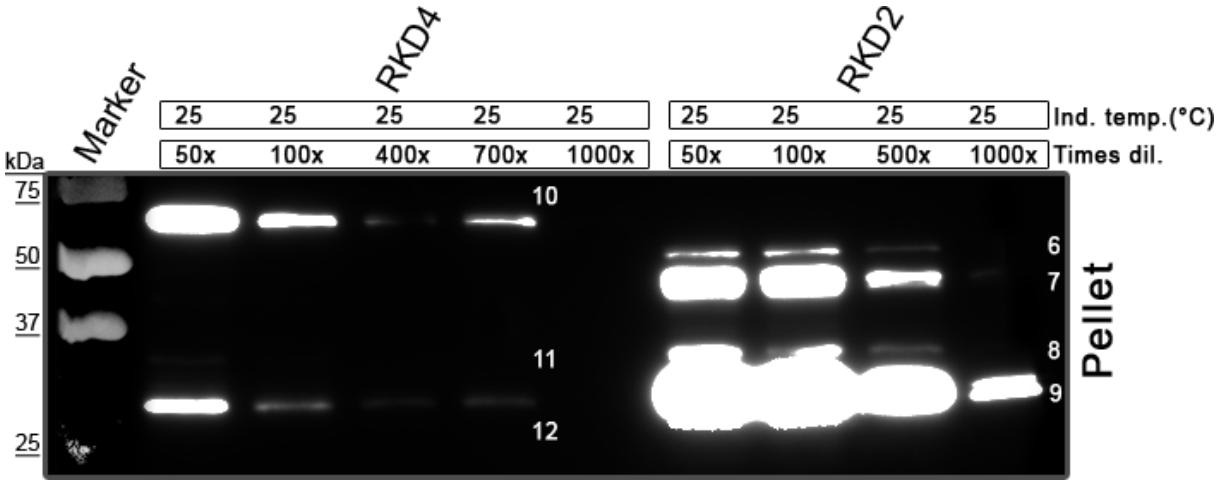


Figure 15: Saturation check western blot. 12%, 1mm gel loaded with samples from ET 25°C(dil. shown in Figure), transferred to a nitrocellulose membrane, incubated with anti-CBD followed by RαM-HRP and ECL activity is captured after 20sec of exposure to luminol and peroxide. Membrane loaded with RKD4(P) and RKD2(P) samples from ETs carried out at a PIIT of 25°C. The ECL activity was captured after 20 seconds of exposure to substrate.

In Figure 15, the RKD4 sample bands shows a decrease in intensity with increasing dilution(50x - 1000x). The RKD4 sample that is 400x diluted has a lower intensity than the RKD4 sample that is 700x diluted. The 1000x diluted sample shows no bands at all.

RKD2 sample bands first remain equally intense(50x - 100x), then decrease in intensity with increasing dilution(100x - 1000x).

Discussion

Gene insert

In Table 7 the lengths of RKD1, RKD2 and RKD4 gene inserts are shown. The lengths of the PCR amplification products of pTWIN1d-RKD1(~900bp), pTWIN1d-RKD2(~1050bp) and pTWIN1d-RKD4(~850bp) roughly correspond with the expected lengths of the RKD1(847bp), RKD2(934bp) and RKD4(808bp) gene insert.

Gene insert	Length (bp)
RKD1	847
RKD2	934
RKD4	808

Table 7: Length of the RKD gene in basepairs(bp). This data is obtained from Weertman's report.

Because it is expected that the primers used during PCR amplification are specific for the RKD gene inserts, it is expected that the plasmids corresponding to the PCR products contain an RKD1, RKD2 or RKD4 gene insert.

The codename of the samples loaded in Figure 11 corresponds with the codename of the glycerol stocks stored (in Appendix B Tables are included that shown which cultures were used for the isolation of plasmid and glycerol stock storage). Although it proven that the six culture samples of which the plasmids shown in Figure 11 were isolated contain a RKD gene insert and therefore the corresponding glycerol stock contains successfully transformed bacteria, it is expected that all glycerol stocks contain successfully transformed bacteria because the colonies isolated for culture growth, and subsequent plasmid isolated were picked from the same agar plate.

Negative controls

During the examination of Weertman's samples a negative control was used that contained non-transformed bacteria. This was done to exclude that anti-CBD binds non-specifcally to a genomic Rosetta BL21 E.coli gene. The NC lane was empty. Therefore, it can be concluded that there is no genomic gene to which anti-CBD binds.

During the analysis of the ETs at variable PIIT(Figure 13) a NC was used that contained bacteria transformed with an insertionless pTWIN1d plasmid. This was done to exclude that in addition to a genomic gene interfering with western blot analysis, also pTWIN1d genes can be excluded from binding non-specifically to anti-CBD. The NC lanes Figure 13 were also empty; concluding that no genomic Rosetta BL21 E.coli gene interferes with the results. However, the pTWIN1d plasmid contains a CBD gene that is present downstream the T7 promoter. As the pTWIN1d(NC) transformed cells were undergone the same procedure to induce expression with IPTG, it was expected that the CBD-tag would be expressed and therefore a band was expected at 27,7 kDa(Table 2). However this band is not identified. The reason for this is uncertain, it is expected that either the CBD gene is inactivated in absence of a gene insert, the NC bacteria did not contain a pTWIN1d plasmid, or isolated pTWIN1d plasmid did not contain a CBD gene.

RKD fusion protein

Bands 1, 6 and 10, and bands present in the RRR(Figure 13 - 14) contain proteins that have the highest MW in their corresponding sample. Because it is not expected that the pTWIN1d-(RKD)

plasmids express a larger gene than RKD(1/2/4) linked to CBD sequence, these bands are expected to be RKD fusion protein. Band 1(RKD1), band 6(RKD2) and band 10(RKD4) have a MW of 68,2 kDa, 54,9 kDa and 68,5 kDa respectively(average MWs between all observations). Their expected MWs are 58,4 kDa(9.8 kDa diff.), 61,7 kDa(6.8 kDa diff.) and 57,7 kDa(10.8 kDa diff.) respectively(Table 2). Although the sizes do not correlate with the expected sizes of the RKD fusion proteins they are still expected to RKD fusion protein. The variation in MW could be attributed to a slower migration profile through the acrylamide gel caused by protein folding.

Smaller (than RKD fusion protein) sized bands

Because anti-CBD specifically binds to CBD and the NCs concluded that there are no genomic and as for now, no pTWIN1d genes(CBD excluded) are present that anti-CBD binds to, the remaining smaller sized protein bands are expected to contain CBD, or fragments of CBD. As described in the introduction, literature mentions that cleavage of the fusion protein may occur induced by β-ME at low temperature and over a wide pH range. Because the protein extracts were present in 50 mM pH 7.4 NaPi, and were unfrozen from -20 °C to ~4 °C, the addition of sample buffer containing β-ME, could have caused the fusion protein to self-cleave during sample preparation. Therefore, the proteins in bands 5, 9 and 12(Figure 9, 14 and 15) and the bands marked with a purple star(Figure 13) may correspond to CBD (+ a few C-terminal amino acids) because they are closest to the expected molecular weight of CBD(27,7 kDa, Table 2).

The self-cleavage could also have occured during incubation of the E.coli cells. Internal hydryolisis of the RKD-intein fusion may have occurred due to a low redox potential in growing E.coli cells.

Cleavage of the RKD-intein fusion however does not explain the bands in between the expected fusion protein and CBD tag, bands 2, 3, 4, 7 and 8. No correlation can be found between the weight of these bands and weights of expected proteins. As the membranes do not show a smear, but rather they show specific bands, and the bands consistently show up at about the same MW among membranes, it is suggested that specific cleavage of the RKD fusion protein occurs in addition to self-cleavage of the RKD-intein fusion. This cleavage may also be caused by specific proteolyse by proteases at set locations in the amino acid sequence of the RKD fusion protein.

Solubility of expressed recombinant protein

All analysed membranes(Figure 9, 13 and 14) show bands present in both SN and P fractions. Therefore, both soluble and insoluble recombinant protein is expressed.

Analyzing *mem1*-mem4(Figure 13), the intensities of cleaved/degradation product bands do not vary much between SN and P fractions. In contrast, the band intensities within the RRR, expected to be RKD fusion protein do vary. The most intense RKD1, RKD2 and RKD4 bands are seen on *mem1* and band intensities are equal among SN and P. Interestingly, the RRR(P) bands remain present and more intense than RRR(SN) bands when increasing the PIIT(*mem2-mem4*). Taking dilution of the protein samples into account, samples on *mem1* are most diluted(20x), whereas samples on *mem2* and *mem3* are less diluted(10x), and *mem4* samples are not diluted at all. This dilution profile would suggest that all RKD fusion protein bands should at least be visible on *mem2-mem4.* However, not all RKD(SN) fusion protein bands are visible on *mem2-mem4* and when visible, they are less intense than their corresponding RKD(P) sample bands. This suggests that RKD fusion protein becomes

increasingly less soluble as the PIIT increases. However, looking at the comparison blots (Figure 14), band 1, expected to be RKD1 fusion protein is equal in intensity in pellet fractions among all PIITs, suggesting that the amount of protein expressed is lower and most of the expressed fusion protein is aggregated into insoluble form. No correlation between the PIITS and increasing aggregation of RKD2 or RKD4 fusion proteins can be seen in the comparison blot(Figure 14). It is expected that RKD2 is expressed in slightly more soluble form than insoluble form because band 6, expected to be RKD2 fusion protein, is slightly more intense on SN samples than P samples. Band 10, expected to be RKD4 fusion protein, is equally intense in SN samples and P samples. Therefore, it is expected that RKD4 fusion protein is expressed in equal amounts of soluble and insoluble protein.

It should be noted that the coomassie stains show reasonably equal amount of proteins between SN and P fractions for *com2-com4*. Whereas, *com1* shows less protein present in the P fractions compared to SN fractions. Therefore, it is expected protein aggregation is less frequent in Rosetta BL21 E.coli cells incubated at a PIIT of 20°C.

Solubility dependant cleavage/degradation

Inspecting the two comparison blots(Figure 14), smaller sized bands, bands 2, 3, 4, and 5 have a higher intensity in SN samples compared to P samples. Therefore, it is expected that (intein) cleavage of RKD fusion protein is more frequent in RKD1(SN) fractions than in RKD1(P) fractions.

Smaller sized bands 7, 8 and 9, have a higher intensity on SN samples compared to P samples. Therefore, a similar phenomenon as in RKD1(SN) fractions is expected in RKD2(SN) fractions; degradation/intein cleavage of RKD2 fusion protein is more frequent than in soluble RKD2(SN) than in RKD2(P).

Smaller sized band 11 is more intense on P samples than SN samples, and smaller sized band 12 is more intense on SN samples than P samples. In addition, P samples show many additional bands between band 10 and 12. No expectation is made on the RKD4 degradation of protein, and the many additional bands are unexplained.

Complication in analyzing membranes

It should be noted that comparisons made between two samples are not necessarily accurate. A general observation about the membranes discussed can be made; most bands produce a very intense signal. The amount of photons generated in these intense bands (during the oxidation of luminol) might exceed the detection limit of *ChemiDoc™'s imaging system*. Therefore, the band intensity is no longer a measure for protein quantity. Thus, when comparing two bands from different samples, the amount of protein in one band may far exceed the quantity of protein in the other band, although band intensities seem alike.

In addition, when comparing different membranes it can complicate to make accurate observations because signal intensity depends on a variety of factors, which include; the quality of protein transfer, washing of the membrane and concentration of primary and secondary antibodies. Because these factors vary (slightly) between each western blot analysis, it further complicates to make just observations when comparing two separate membranes.

Linear detection range

The saturation check membrane(Figure 15) gives insight as to whether the intensity of the RKD2(P) and RKD4(P) bands at a PIIT of 25°C are within the (linear) range in which the detector can detect a difference in the amount of photons emitted. As the RKD4 sample bands decrease in intensity with increasing dilution(50x - 1000x), these samples are within the linear range. (the unexpected pattern of band intensity between the RKD4 samples that are 400x, 700x and 1000x diluted can be attributed to a fault in preparation as it is difficult to transfer little amount of protein(0.2µl) for preparation). This conclusion can be extended to the comparison blot because RKD4(P) 25°C sample is 100x diluted and therefore within the linear range. Thus, the band intensity generated corresponds with the quantity of protein present in this band.

However the bands in the RKD2(P) sample start to decrease in intensity only after 100x dilution. Therefore the linear range is between 100x and 1000x. To extend this observation to the comparison blot, it would conclude that it is uncertain what the quantity of protein is within the RKD2(P) 25°C bands as the signal generated produces too many photons for the detector to detect.

Optimal PIIT for soluble RKD fusion protein expression

From the developed membranes(Figure 13) it was expected that most soluble RKD1, RKD2 and RKD4 fusion protein was present on *mem1*, corresponding to a PIIT of 20°C. This expectation is further supported by the comparison blot(Figure 14) for RKD1 and RKD2 fusion protein. On this blot it is seen that the bands expected to be RKD1(SN) and RKD2(SN) fusion protein, band 1 and band 6, are most intense at a PIIT of 20°C. However, the band expected to be RKD4(SN), band 10, is most intense at a PIIT of 25°C.

Maximum attainable amount of RKD fusion protein

The quantity of degradation product is for one dependent on the amount of RKD fusion protein(RKD+CBD) expressed, as this is the starting product for degradation. Therefore, the smaller sized bands give insight into the amount of RKD fusion protein that has been expressed but was unfortunately degraded. The comparison blot(Figure 14) shows that smaller sized bands, bands 2, 3, 4 and 5, in RKD1(SN) sample are largest at a PIIT of 25°C (it is suspected that these bands are saturated, and therefore this may not be an accurate observation). This observation implies that more soluble RKD1 fusion protein is produced at a PIIT of 25°C compared to other PIITs(20°C, 32°C and 37°C) (but was unfortunately degraded). For RKD2(SN) and RKD4(SN) sample a similar observation can be made at a PIIT of 25°C. Smaller sized bands 7, 8, 9 and 12 are larger at a PIIT of 25°C compared to other PIIT(20°C, 32°C and 37°C). Therefore the maximum attainable amount of soluble RKD2 and RKD4 would be at a PIIT of 25°C.

However, RKD1(P) samples differ from these observations. For RKD1(P)samples, smaller sized bands, bands 2, 3, 4 and 5, are most intense at a PIIT of 20°C. It should also be noted that the intensity of the band expected to be RKD1 fusion protein, band 1, is equal among all PIITs. Therefore, the maximum quantity of attainable insoluble RKD1 fusion protein is at a PIIT of 20°C.

RKD2(P) and RKD4(P) samples, just like their corresponding SN samples, have most protein present in smaller sized bands 7, 8 , 9 and 12 at a PIIT of 25°C. Therefore, the maximum amount of attainable insoluble RKD2 and RKD4 fusion protein is at a PIIT of 25°C.

Weertman's samples

Weertman's samples were stored for a longer time before preparation(52 days) before analysis. Yet, the RKD1 and RKD2 samples show the same band pattern as observed during analysis of newly transformed Rosetta cells. Therefore, RKD fusion protein (intein) cleavage is expected to be minimal during storage at -20°C. This suggests that (intein) cleavage of the samples must occur either before extraction(during cell growth) or after storage(during sample preparation).

Weertman's samples were obtained from ETs carried out at 20°C with 0.3mM IPTG and incubation O/N. On Figure 9(Weertman's samples), the RKD1(SN) band is slightly higher in intensity than the RKD1(P) band. On Figure 16, which represents samples obtained from an ET carried out at 20°C with 0.4mM IPTG and incubation O/N, the RKD1(SN) band is equally intense compared to the RKD1(P) band, and even less intense during chemiluminescnce detection(Figure 14). Because the IPTG concentration is the only variable between these two ETs, a lower IPTG concentration may result in more soluble RKD1 fusion protein.

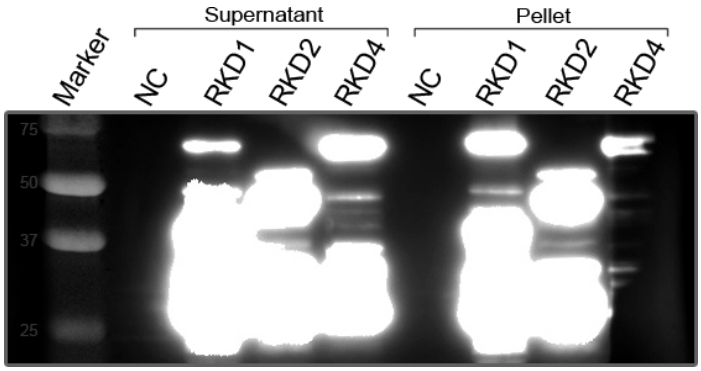


Figure 16: 12%, 1mm gel loaded with samples from ET 20°C(20x dil.), transferred to a nitrocellulose membrane, incubated with anti-CBD followed by RαM-HRP and ECL activity is captured after 20sec of exposure to luminol and peroxide.

Conclusion

Rosetta BL21 E.coli cells that were transformed with a pTWIN1d-RKD1, pTWIN1d-RKD2 or pTWIN1d-RKD4 plasmid were successfully induced to express CBD fused recombinant protein at PIITs 20°C, 25°C, 32°C and 37°C. However, pTWIN1d transformed cells did not express the expected recombinant CBD. Fusion protein with the largest molecular weight in sample is identified at 68,2 kDa, 54,9 kDa and 68,5 kDa for pTWIN1d-RKD1, pTWIN1d-RKD2 and pTWIN1d-RKD4 transformed cells respectively. These proteins are expected to be RKD1, RKD2 and RKD4 fusion protein. Degradation of RKD fusion protein is observed, resulting in additional smaller sized bands at various molecular weights. These bands contain (fragments of) CBD. One prominent band is observed at a MW of 27 kDa, expected to be intein-CBD cleaved off RKD fusion protein. Cleavage at the N-terminal of the CBD-intein fusion may be caused by internal hydrolysis during cell incubation and/or β-ME during sample preparation.

Recombinant protein is expressed in both soluble and insoluble form. Expected is that more insoluble RKD1 fusion protein is expressed than soluble protein at PIITS of 20°C, 25°C, 32°C and 37°C. Whereas, it is expected that more soluble RKD2 protein is expressed at PIITs of 20°C, 25°C and 32°C and equal amounts of soluble and insoluble RKD4 fusion protein at PIIT of 20°C and 25°C. The amount of soluble RKD fusion protein expressed could suffice for isolation and subsequent structure identification. A PIIT of 20°C resulted in the most soluble RKD1 and RKD2 fusion protein. A PIIT of 25°C resulted in the most soluble RKD4 protein. However, the maximum attainable amount of RKD1, RKD2 and RKD4 is expected to be contained at a PIIT of 25°C. However, to attain the maximum amount of RKD1, RKD2 and RKD4 fusion protein degradation has to be prevented.

Recommendations

Justification of results

A general observation about the membranes discussed is made that most bands produce a very intense signal. To prevent that *ChemiDoc™ imaging system*'s detector cannot distinguish differences in protein quantity anymore it is recommended to dilute protein samples obtained from transformed Rosetta BL21 E.coli cells with pTWIN1d-RKD plasmids at least 200x before addition of SB.

Because pTWIN1d transformed cells are expected to express CBD, but no band at the height of CBD was identified(27,7 kDa) in cells expected to contain a pTWIN1d plasmid, it is recommended to PCR amplify the CBD sequence in isolated pTWIN1d plasmids with primers specific for the CBD sequence.

One assumption was made that β-ME could be the cause of RKD fusion protein cleavage. To conclude if β-ME is the cause, it is recommended to prepare stored protein samples(-20°C) in sample buffer without β-ME. However, β-ME breaks up protein's disulphide bonds, and it is expected that in the absence of β-ME the migration profile is altered(or no migration occurs at all) as the pores of the acrylamide gel may be too small for the folded protein to migrate through. However, SDS, also present in the SB, unfolds the proteins which might suffice to separate the proteins based on their MW. A clear separation is not necessary, as long as just one band is exposed it may conclude that B-ME is the cause of RKD fusion protein cleavage. In addition, a lower percentage(7.5%) gel could be used to separate the proteins.

Continue optimization of RKD fusion protein expression

During this thesis the optimum temperature for soluble RKD1, RKD2 and RKD4 is determined to be 20°C, 20°C and 25°C respectively. However, other conditions can be varied during the an ET which might result in more expression of soluble recombinant protein. For example, the cell density(OD_{600} 0.6) before induction with IPTG and the IPTG concentration(0.2 - 0.5 mM) can be varied. Next, compare solubility, quantity and/or cleavage of the RKD fusion protein using SDS-PAGE and western blot analysis.

In addition, if RKD fusion protein is degraded in vivo, the pTWIN plasmid manual recommends to induce expression at lower temperatures, one could test the expression at 12 - 15°C, and conclude if more or less protein degradation occurs. However, this contradicts with the observations that more degradation products are found at 20°C compared to higher PIIT's.

Continue to isolate and identify RKD1, RKD2 and RKD4 structure

To continue on the road to isolate RKD fusion protein, and identify its structure, it is recommended to perform a first isolation of RKD1, RKD2 and RKD4 using stored samples obtained from ETs carried out at a PIIT of 20°C, 20°C, and 25°C respectively.

References

Asker, S. E., & Jerling, L. (1992). *Apomixis in plants*. CRC Press.

Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L., … van Lookeren Campagne, M. M. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *The Plant Cell*, *14*(8), 1737–49. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12172019

Calos, M. P. (1978). DNA sequence for a low-level promoter of the lac repressor gene and an "up" promoter mutation. *Nature*, *274*(5673), 762–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/355890

Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., … Xu, M. Q. (1997). Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene*, *192*(2), 271–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9224900

Copeland, L. O., & McDonald, M. B. (1999). Seed Formation and Development. In *Principles of Seed Science and Technology* (pp. 17–39). Boston, MA: Springer US. http://doi.org/10.1007/978-1-4615-1783-2_2

Expression Technologies Inc. (2003). Bacterial E.coli Growth Media. Retrieved from http://www.exptec.com/Bacterial E.coli Growth Media.htm

Hecht, V., Vielle-Calzada, J. P., Hartog, M. V, Schmidt, E. D., Boutilier, K., Grossniklaus, U., & de Vries, S. C. (2001). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology*, *127*(3), 803–16. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11706164

Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant Callus: Mechanisms of Induction and Repression. *The Plant Cell*, *25*(9), 3159–3173. http://doi.org/10.1105/tpc.113.116053

Jeong, S., Palmer, T. M., & Lukowitz, W. (2011). The RWP-RK factor GROUNDED promotes embryonic polarity by facilitating YODA MAP kinase signaling. *Current Biology : CB*, *21*(15), 1268–76. http://doi.org/10.1016/j.cub.2011.06.049

Kawashima, T., & Goldberg, R. B. (2010). The suspensor: not just suspending the embryo. *Trends in Plant Science*, *15*(1), 23–30. http://doi.org/10.1016/j.tplants.2009.11.002

Koszegi, D., Johnston, A. J., Rutten, T., Czihal, A., Altschmied, L., Kumlehn, J., … Bäumlein, H. (2011). Members of the RKD transcription factor family induce an egg cell-like gene expression program. *The Plant Journal : For Cell and Molecular Biology*, *67*(2), 280–91. http://doi.org/10.1111/j.1365-313X.2011.04592.x

Maheshwari, P., & Maheshwari, P. (Panchanan), 1904-1966. (1950). *An introduction to the embryology of angiosperms.* (1st ed.). New York,: McGraw-Hill,. http://doi.org/10.5962/bhl.title.5681

Rademacher, E. H., Lokerse, A. S., Schlereth, A., Llavata-Peris, C. I., Bayer, M., Kientz, M., … Weijers, D. (2012). Different Auxin Response Machineries Control Distinct Cell Fates in the Early Plant Embryo. *Developmental Cell*, *22*(1), 211–222. http://doi.org/10.1016/j.devcel.2011.10.026

Radoeva, T. M. (2016). Mechanistic dissection of plant embryo initiation. Wageningen University.

Radoeva, T., & Weijers, D. (2014). A roadmap to embryo identity in plants. *Trends in Plant Science*, *19*(11), 709–716. http://doi.org/10.1016/j.tplants.2014.06.009

Reinert, J. (1959). Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. *Planta*, *53*(4), 318–333. http://doi.org/10.1007/BF01881795

Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007). Escherichia coli physiology in Luria-Bertani broth. *Journal of Bacteriology*, *189*(23), 8746–9. http://doi.org/10.1128/JB.01368-07

Shetty, R. P., Endy, D., & Knight, T. F. (2008). Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering*, *2*, 5. http://doi.org/10.1186/1754-1611-2-5

Silverstone, A. E., Arditti, R. R., & Magasanik, B. (1970). Catabolite-insensitive revertants of lac promoter mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *66*(3), 773–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4913210

Soriano, M., Li, H., & Boutilier, K. (2013). Microspore embryogenesis: establishment of embryo identity and pattern in culture. *Plant Reproduction*, *26*(3), 181–96. http://doi.org/10.1007/s00497-013-0226-7

Steward, F. C., Mapes, M. O., & Mears, K. (1958). Growth and Organized Development of Cultured Cells. II. Organization in Cultures Grown from Freely Suspended Cells. *American Journal of Botany*, *45*(10), 705. http://doi.org/10.2307/2439728

Tabor, S. (2001). Expression using the T7 RNA polymerase/promoter system. *Current Protocols in Molecular Biology / Edited by Frederick M. Ausubel ... [et Al.]*, *Chapter 16*, Unit16.2. http://doi.org/10.1002/0471142727.mb1602s11

Touraev, A., Vicente, O., Heberle-Bors, E., Bedinger, P., Heberle-Bors, E., Guha, S., … al., et. (1997). Initiation of microspore embryogenesis by stress. *Trends in Plant Science*, *2*(8), 297–302. http://doi.org/10.1016/S1360-1385(97)89951-7

Waki, T., Hiki, T., Watanabe, R., Hashimoto, T., & Nakajima, K. (2011). *The Arabidopsis RWP-RK Protein RKD4 Triggers Gene Expression and Pattern Formation in Early Embryogenesis*. *Current Biology* (Vol. 21). http://doi.org/10.1016/j.cub.2011.07.001

Weertman, N. (2016). The Twin project.

Weijers, D., Van Hamburg, J.-P., Van Rijn, E., Hooykaas, P. J. J., & Offringa, R. (2003). Diphtheria toxin-mediated cell ablation reveals interregional communication during Arabidopsis seed development. *Plant Physiology*, *133*(4), 1882–92. http://doi.org/10.1104/pp.103.030692

Wenzel, L. N. (2016). Structural prediction of three Arabidopsis thaliana putative transcription factors Written by.

Yan, D., Duermeyer, L., Leoveanu, C., & Nambara, E. (2014). The functions of the endosperm during seed germination. *Plant & Cell Physiology*, *55*(9), 1521–33. http://doi.org/10.1093/pcp/pcu089

Zuo, J., Niu, Q.-W., Frugis, G., & Chua, N.-H. (2002). The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. *The Plant Journal*, *30*(3), 349–359. http://doi.org/10.1046/j.1365-313X.2002.01289.x

Appendix A

Appendix A includes 6 protocols and corresponding recipes that were used to carry out the methods described in the *Materials and Methods* chapter.

Protocol: SDS-PAGE, 12% acrylamide gel preparation

1. Prepare running and stacking gel solutions. Use the scheme below to prepare the solutions in 50 mL Falcon tubes, use the order in the scheme below (top to bottom). Do not add TEMED to the solutions yet. Add TEMED just before you are about to pour in the solutions into the glass plates as TEMED solidifies the gel solution.

RUNNING GEL (10 mL; 12% acrylamide)		STACKING GEL (4 mL)	
component	*volume (ml)*	*component*	*volume (ml)*
demiwater	3.3	demiwater	2.9
30% Acrylamide	4.0	30% Acrylamide	0.5
4x Running separating buffer	2.5	Stacking separating buffer 4x	0.5
10% Aluminium persulfate(APS)	0.1 (100 µl)	10% APS	0.04 (40 µl)
TEMED*	0.01 (10 µl)	TEMED	0.004 (4 µl)

*Use more TEMED (20 µl instead of 10 µl for the running; 10µl instead of 4 µl for the stacking gel) if you want the gel to solidify faster and save time.

2. Assemble the gel preparation setup, put the two glass plates(1mm) on top of each other in the green clamb then put the green clam in the plastic transparent clamb holder.
3. Pipette running gel in between the two glass plates.
4. Add alcohol(isopropanol) on top of the running gel to make the top of the running gel on level and allow for equal transfer of proteins on the border of the running and stacking gel. (15-30 minutes)
5. Wait for the running gel to solidify, use the leftovers of the gel in the tube as an indication for when it has solidified.
6. Once solidified remove the alcohol by gently tipping over the setup to one side and holding filter paper or paper towel next to it so the alcohol will flow out and the paper will absorb it.
7. Pipette the stacking gel in between the two glass plates and put the comb on top(10 /15 wells, 1mm).
8. Wait until the stacking gel has solidified, again use the leftovers of the stacking gel in the tube as an indication as to when the gel has solidified. (15-30minutes) Remove the comb as soon as possible to prevent proteins from clotting when loading (protein) samples.
9. Disassemble the gel from the standard and clean the well with demiwater by gently letting water flow into the wells and prevent the wells from breaking.

10. The gel is now ready for further use or storage. For storage wrap the gel in paper towel and moisten it so it will not dry out. Wrap the moist gel in aluminium foil, as acrylamide is light sensitive, and store it in the fridge at 4 °C.

Recipes: SDS-PAGE, acrylamide gel preparation

- *4x Separating buffer (1.5M Tris HCl (pH 8.8), 0.4% SDS*

Dissolve 46.75 g of Tris in 100 ml of ddH_2O, pH slowly to 8.8 with concentrated HCl, add 1 g SDS and dissolve thoroughly. Adjust final volume to 250 ml with ddH_2O.

- *4x Stacking buffer (1.0M Tris HCl (pH 6.8), 0.1% SDS*

Dissolve 15.13g of Tris in 100 ml ddH_2O, pH slowly to 6.8 with concentrated HCl, add 0.25 g SDS and dissolve thoroughly. Adjust final volume to 250 ml with ddH_2O.

Protocol: Sample preparation and sample loading

1. Thaw stored proteins or use protein solutions directly after extraction.
2. Vortex the proteins to make the solution homogeneous.
3. Transfer the correct amount of protein into an Eppendorf and store the protein samples at -20 °C. For 15 µl prepared samples take 11.25 µl protein samples.
4. Add sample buffer in a 1:4 ratio (1 SB: 3 protein). For 15 µl prepared sample, add 3.75 µl SB to 11.25 µl protein sample.
6. Heat the samples for 5 minutes at 95 °C to denature the proteins.
7. Spin the heated sample for ~30 seconds 14000 RPM (Quick spin mode) to collect junk material at the bottom of the Eppendorf.
8. Load the samples onto an acrylamide gel. In addition to the samples add 10 µl marker.

While waiting for the proteins to unthaw or when they are being denatured at 95 °C for 5 min, there is time to prepare the SDS-PAGE setup. Assemble the prepared acrylamide gel in Bio-Rad vertical gel electrophoresis setup and add 1x Tris glycine running buffer.

9. Run the gel at 90V throughout the stacking gel.
10. Increase the voltage to 120V once the samples pass the stacking/running gel interface.
11. Stop electrophoresis once the samples leave the gel. The gel is now ready for further analysis, western blotting or coomassie staining

Recipes: Sample preparation and sample loading

- 4x sample buffer (10 ml)

Add the following to make 10 ml of 4x SB:

- 2.5 ml 1 M Tris-HCl pH 6.8
- 0.5 ml of ddH20
- 1.0 g SDS
- 0.8 ml 0.1% Bromophenol Blue
- 4 ml 100% glycerol
- 2 ml 14.3 M β-mercaptoethanol(βME) (100% stock)

Adjust the final volume to 10 ml with ddH20

- 1x Tris-glycine running buffer (pH 8.3, 1000 ml)

Add the following to make 1000 ml of 1x Tris glycine running buffer

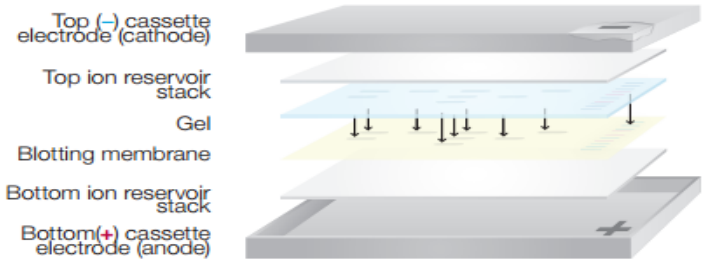
- 3.03 g 25 mM Tris-base
- 14.4 g 192 mM Glycine
- 1.0 g 0.1% SDS

Add ddH_2O to 1000 ml

Protocol: Western blot

Membrane transfer

1. Wear gloves and prepare the Trans-blot Turbo package in Bio-Rad's Trans-blot Turbo setup. Ion reservoir stack and membranes are located in the cupboard below Mark's desk.
2. Prepare the package in the following stacking order, as seen in Figure X. First the ion reservoir stack, secondly the membrane, third the gel and lastly another stack of ion reservoir.
3. Before placing each component on the Trans-blot Turbo plate moisten them in Trans-blot Turbo transfer buffer and when placed on the plate use a roller to eliminate air bubbles to allow for a proper transfer of proteins to the membrane.
4. To start transfer of the proteins use the Turbo mode for 1mini gel. (~7min)



Blocking of the membrane

While the trans-blot turbo is working prepare the blocking buffer. Blocking is done so that the antibody will bind specifically to the protein of interest and reduce background signal generated if antibodies bind non-specifically to the membrane.

5. To prepare 50 ml of 3% non-fat milk blocking solution:
Weight 1.5g of milk powder in the storage room in a 50ml Falcon tube.
Add 20 ml TBST and vortex to dissolve the milk.
Fill the tube up to 50 mL with TBST when all the milk has dissolved.

6. Once the protein transfer is complete gently remove the membrane and put it in a 50 ml Falcon tube and wash it in ~20 mL TBST.
7. Remove the TBST and add the ~50 mL prepared milk blocking solution.
8. Put the prepared tube on a rotation device at a slight angle (to generate small waves) for 1 hour so that the blocking solution is distributed equally over the surface of the membrane.
9. Clean all the used equipment with demiwater and let it dry. Rinse the Trans-blot Turbo plates thoroughly to remove salt (dissolved in the transfer buffer) and prevent it from preciPIITating.

Antibody incubation

The first antibody is monoclonal and only binds to the chitin domain, it is very specific and so will detect the protein, however even if all the protein is bound to the first antibody the signal it produces will not large enough to be detecTable, therefore a second antibody is used that is able to bind to the first antibody. It not only detects the first antibody but also itself. The secondary antibody will therefore form a large root attached to the first antibody that amplifies the signal greatly when imaging later.

First antibody

10. After blocking wash the membrane with TBST shortly.
11. Prepare a 5 mL solution of 3% milk and antibody. If there are leftovers from the blocking solution add antibody to it in 1:1000 ratio. 5 ml 3% non-fat milk + 5 µ anti-CBD.
12. Add the prepared 5 mL solution and incubate the membrane overnight on a rotation device at 4 °C. The antibody can also be prepared beforehand or used multiple times, ~5 times in a 2 week period gives a good result still.

Second antibody

13. After O/N incubation with the first antibody wash the membrane 3 x 10 minutes with TBST at RT.
14. Incubate the membrane with the second antibody for 45 minutes at RT. 6 mL 3% non-fat milk + 6 µL RAM-HRD(1:1000 ratio).
15. After incubation wash 3 x 10 minutes with 5 µL TBST.

Blot imaging

16. Take A(luminol) and B(peroxide) solution bottles out of the fridge in room 3100.
17. Start CCD camera machine by turning on the switch on the left of the machine(black box) and the power button for the lamp at the back of the machine.
18. Run the *image lab* program and start the *western blot white and western blot* protocol. The white protocol is to be run first.
19. Click *position gel* on the *western blot white* protocol. To position the membrane correctly make sure the proteins are facing up and that the top and/or bottom of the membrane are correctly aligned with the roster visible on screen. Alternatively if the blot is rolling up, use sticky notes so the blot stays flat on the surface. After positioning the membrane correctly click on the *run protocol* button to make a screenshot of the blot and export it as a TIFF image.
20. Mix 500 µL of solution A with 500 µL of solution B(1:1, 0.1 mL/cm^2). Use the mixture immediately on blot by applying it drop wise.
21. Run the *western blot* protocol as soon as possible as the reaction has already started. The protocol will make screenshots of the blot after 20 seconds, and every 40 seconds thereafter. Once your result is obtained feel free to end the protocol but make sure to save the images first.
22. After use of the CCD camera make sure to turn off the machine but especially the lamp to save its lifetime.

Recipes: Western blot

- Tris buffered saline Tween 20(TBST) Recipe

To prepare 1000 ml TBST mix the following:

- 100 ml 10x TBS
- 10 ml 10% Tween 20 (polysorbaat 20; soap solution)
- 890 ml ultrapure water

- To prepare 100 ml 10x TBS mix the following:

- 20 ml ultrapure water
- 50 ml 1M Tris.Ck pH 7.4
- 30 ml 5M NaCl (or 9g NaCl crystals)

Protocol: Rosetta BL21 E.coli transformation with pTWIN1Δ1 plasmids

1. Take 50µl competent Rosetta BL21 E.coli cells from -80 °C. Usually the aliquots contain 250 µl.
2. Add 50 µl 10% glycerol to the bacteria (to obtain 100 µl diluted cells) and resuspend, work on ice. Subsequently add 0.5 µl (or less plasmid) and mix gently.
3. Put the bacteria mixture in a 2mm cuvette and elctroporate. Use settings: EC2, bacteria, time in ms.
4. Quickly add 400 µl LB liquid medium to the bacteria and incubate for 45 min at 37 °C.
5. Plate 10 µL bacteria (using plasmid for transformation is very efficient) on LB agar + ampicillin.
6. Leave the plates overnight at 37 °C. The next morning you should have single colonies
7. Use single colonies to prepare liquid cultures in 50 mL falcon tubes: 5 ml LB + 8 µl ampicillin. You can also make 10 ml cultures if you are going to start an ET.

Recipe: Rosetta BL21 E.coli transformation with pTWIN1Δ1 plasmids

- Liquid LB medium

To prepare 1000ml liquid LB medium add the following in a 1l bottle

- 25g LB broth powder
- 1000ml MilliQ

Swirl to mix. Slightly loosen the bottle cap and put autoclave tape on the bottle. Autoclave 45min at 121°C. Cool to RT before tightening the bottle cap.

Protocol: PCR check on RKD1,2 and 4 inserts of pTWIN1Δ1 plasmids

1. Prepare sample(s) for PCR amplification by adding the following in 0.2 ml PCR tube(s):
    - 22 µl MiliQ H_2O
    - 25 µl (2x) Phusion Flash PCR Master Mix
    - 1 µl 20 µM FW primer(sequence in Table 1)
    - 1 µl 20 µM RV primer(sequence in Table 1)
    - 1 µl plasmid DNA
2. Put the samples in Bio-Rad's MyCycler Personal Thermal Cycler and use the following programs:

For RKD1 and RKD4 gene inserts:

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	10 s	1
Denaturation	98 °C	10 s	35
Annealing	55 °C	30 s	
Elongation	68 °C	2 min	
Last elongation	68 °C	3 min	1
Hold	4 °C	∞	1

For RKD2 gene inserts:

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98 °C	10 s	1
Denaturation	98 °C	10 s	35
Annealing	58 °C	30 s	
Elongation	68 °C	2 min	
Last elongation	68 °C	3 min	1
Hold	4 °C	∞	1

3. Once retrieving the PCR products, add 5 µl of 10x DNA loading dye to the products. Load the samples on a 1% agarose gel (containing 20 µl ethidium bromide) with GeneRuler^{TM} DNA ladder for molecular mass determination.
4. Run the get at 100V for 30 min. And put the gel under the imager to make an image.

Primer (Eurogentec):	Sequence (5'-3'):	Product Size (bp):	Code name:
RKD1; FW	TTTAAGAAGGAGAATTCATGAAATCGTTTTGCAAGTTGGA	847	TR476
RKD1; RV	ATTGATTGGGAGTAAGAATTCGCTCTCTCAAAACCCGAAACAGA		TR477
RKD2; FW	TTTAAGAAGGAGAATTCATGGCTGATCACACAACCAA	934	TR478
RKD2; RV	ATTGATTGGGAGTAAGAATTCGCCAAACCACTAGTAAATTCAC		TR479
RKD4; FW	TTTAAGAAGGAGAATTCATGAGTTCGTCAAAACATTCCTC	808	TR480
RKD4; RV	ATTGATTG GGAGTAAGAATTCGCATAATAATCATCACCAAGTG		TR481

Table 1: Forward and reverse primers for PCR amplification of RKD1, 2 and 4.

Protocol: Expression test(s) (at variable temperature)

This protocol is split up in 3 days. Each day a variety of tasks are performed, including continuation of the protocol and preparations for the next day(s). In my experience this day to day protocol was found to work most time efficient as day 1 only required a simple inoculation step, leaving time for preparations for day 2. Day 2 consists of another inoculation step, waiting during bacterial growth (leaving time for preparation for day 3), and induction of expression. Day 3 is the most labour intensive having to isolate the expressed proteins and creating samples for western blot and coomassie (loading control) analysis.

Day 1: Preculture growth

During all these steps work near a flame to keep the medium sterile, and close of the Falcon tubes as soon as possible once finished transferring medium, bacterial cells, antibiotics etc.

1. Prepare 10 ml LB medium + 10 µl ampicillin(1:1000 dil.) in a 50 ml Falcon tube.
2. Inoculate the LB medium with a small amount of bacterial cells (from a glycerol stock) with any pipette tip.
3. Grow the inoculated culture O/N at 37 °C in an incubator shaker (~150 RPM).

Preparation for day 2:

- Terrific Broth(TB) (250 ml)(47.6 g/l)

Weigh 11.9 g TB in a 250 ml bottle and add 250 ml demiwater. Add 1 ml 100% glycerol. Autoclave the bottle, with the lid slightly open, at 120 °C for 45 min.

- (5) sterile 250/300 ml Erlenmeyer flasks

Take (5) Erlenmeyer flasks and fill it with a tiny layer of demiwater on the bottom of the flask. Close of the flask with aluminium foil and autoclave at 120 °C for 45 min (together with the TB medium).

- Sterile water for sodium phosphate buffer(NaPi) preparation

Fill a 250 ml bottle with water and autoclave at 120 °C for 45 min.

Day 2: Dilution, growth and induction

Like day 1, carry out all steps near a flame to keep the medium clear of unwanted microorganisms. Close lids on bottles, wrap aluminium foil on flasks, and close Falcon tubes as soon as possible.

4. Add 50 ml of TB medium in an Erlenmeyer together with 50 µl ampicillin(1:1000 dil.).
5. Inoculate the TB medium with 0.5 ml of the O/N grown culture on day 1.
6. Allow the cells to grow at 37 °C in a shaker incubator(~150 RPM) until an OD_{600} of 0.8 is reached (~3 – 4 hours in case of Rosetta BL21 E.coli cells transformed with a pTWIN1Δ1 plasmid). Measure the OD_{600} using a spectrophotometer and TB medium as a blanco.
7. Induce expression of proteins with 20 µl 1M IPTG or a different amount(corresponds with 0.4 mM in 50 ml medium) O/N at a temperature of choice(e.g. 20, 25, 32 or 37 °C).

Preparation for day 3:

- Sodium phosphate(NaPi) 50mM, pH 7.4

Take 5 mL NaPi 0.5 M and add 40 ml MilliQ in a 50 ml Falcon tube. Adjust the pH to 7.4 with NaOH. End volume should be ~50 ml.

- Acrylamide gel(s) for western blot and/or coomassie stain gel analysis

See protocol *SDS-PAGE 1mm, 12% acrylamide gel preparation* in Appendix A.

Day 3: Extraction of proteins and preparation for analysis

During the course of day 3 it is important to work on ice when extracting the proteins. Keep NaPi, Falcon tubes and Eppendorf tubes on ice at all times. The cold temperature keeps the proteins from denaturing, especially important during sonication of the cells, as sonication generates heat within the sample.

8. Spin down 50 ml of the induced cells at 4500 RPM for 10 min at 4 °C (in 50 ml Falcon tubes).
9. Take of the supernatant(SN) and resuspend the spun down cells in 2 ml 50 mM NaPi. Once resuspended transfer the solution to a 15 ml Falcon tube (pre-cooled on ice).
10. Sonicate the cells in the Falcon tube 3x 10 sec with 1 minute in between each sonication repetition. Keep the Falcon tube surrounded by ice-water throughout the whole sonication procedure.
11. Transfer and divide the sonicated cells to two 2 ml Eppendorf tubes. Try to keep the volumes equal in both Eppendorf tubes to keep the amount of protein equal among the two samples.
12. Spin down the sonicated samples for 10 min, at 13000 RPM and 4 °C to separate the soluble proteins(SN) from the non-soluble proteins(pellet).
13. Mark the height of the solution on the side of the Eppendorf and transfer the supernatant to a clean 2ml Eppendorf tube.
14. Resuspend the pellet up to the marked volume (~2 ml) with 50mM NaPi. The marking is done to keep the concentrations of proteins comparable between supernatant and pellet when analyzing a western blot or coomassie stained gel.

The protein extraction is now complete and the extracted samples can be further prepared for western blot or coomassie staining analysis or stored at -20 °C. The protocols for western blot analysis or coomassie staining can (also) be found in Appendix A.

Appendix B

Appendix B includes, the labels of all glycerol stocks and corresponding grown cultures. Which cultures were picked for plasmid isolation and corresponding plasmid concentrations are included so it can be derived which glycerol stocks contain correctly transformed plasmids. Which glycerol stocks are used for the ETs is also included. The optical densities of each culture before induction is shown, as well as how long it took for the culture to reach this density. An observation about the induction time is made. All generated samples that are stored are listed as well. At last, all loading orders of the gels used are included.

Gene insert	(None)NC	RKD1	RKD2	RKD4	RKD4
Codename of culture/ glycerol stock	NC1	RKD1,1	RKD2,1	RKD4.1,1	RKD4.2,1
	NC2	RKD1,2	RKD2,2	RKD4.1,2	RKD4.2,2
	NC3	RKD1,3	RKD2,3	RKD4.1,3	RKD4.2,3
	NC4	RKD1,4	RKD2,4	RKD4.1,4	RKD4.2,4
	NC5	RKD1,5	RKD2,5	RKD4.1,5	RKD4.2,5
	NC6	RKD1,6	RKD2,6	RKD4.1,6	RKD4.2,6
	NC7	RKD1,7	RKD2,7	RKD4.1,7	RKD4.2,7
	NC8	RKD1,8	RKD2,8	RKD4.1,8	RKD4.2,8
	NC9	RKD1,9	RKD2,9	RKD4.1,9	RKD4.2,9
	NC10	RKD1,10	RKD2,10	RKD4.1,10	RKD4.2,10

Table 1: Codenames of all glycerol stocks created, later used for ETs, and the codename corresponds with the cultures from which plasmids will be isolated.

Gene insert	(None)NC	RKD1	RKD2	RKD4	RKD4
Codename of culture	NC1	RKD1,1	RKD2,1	RKD4.1,1	RKD4.2,1
	NC3	RKD1,3	RKD2,3	RKD4.1,2	RKD4.2,2
	NC5	RKD1,5	RKD2,5	RKD4.1,3	RKD4.2,3
	NC7	RKD1,7	RKD2,7	RKD4.1,5	RKD4.2,5
	NC9	RKD1,9	RKD2,9	RKD4.1,7	RKD4.2,7

Table 2: Cultures from which plasmids are isolated, the codename corresponds with glycerol stock stored.

Codename	NC1	NC3	NC5	NC7	NC9	RKD1,1	RKD1,3	RKD1,5	RKD1,7	RKD1,9
Conc(ng/µl)	5.5	10.4	4.3	9.3	7.2	15.8	17.3	20.4	19.8	19.1
Codename	RKD2,1	RKD2,3	RKD2,5	RKD2,7	RKD2,9	RKD4.1,1	RKD4.1,2	RKD4.1,3	RKD4.1,5	RKD4.1,7
Conc(ng/µl)	16.8	21.8	20.2	20.6	19.7	5.4	5.5	7.3	5.3	33.2
Codename	RKD4.2,1	RKD4.2,2	RKD4.2,3	RKD4.2,5	RKD4.2,7					
Conc(ng/µl)	12.4	12.6	8.1	7.3	7.6					

Table 3: This Table shows the plasmid concentrations in 25 grown transformed cultures. The codename corresponds with the glycerol stocks stored.

Gene insert	RKD1	RKD2	RKD4
Codename culture/glycerol stock	RKD1-5	RKD2-3	RKD4.2-1
	RKD1-7	RKD2-5	RKD4.2-2

Table 4: Plasmids where isolated from these cultures and used for PCR amplification. The codenames correspond with the glycerol stock stored.

	OD_{600}(20°C)	**Time(min)**	**OD_{600}(25°C)**	**Time(min)**
NC	0.830	190	0.846	180
RKD1	0.870	241	0.806	180
RKD2	0.798	190	0.878	180
RKD4	0.788	306	0.877	240
	OD_{600}(37°C)	**Time(min)**	**OD_{600}(37°C)**	**Time(min)**
NC	0.760	180	1.034	207
RKD1	0.790	180	0.740	207
RKD2	0.710	180	0.871	231
RKD4	0.764	270	0.814	277

Table 5: Optical densities of the cell cultures before induction of the samples, and the time it took for the cultures to grow to their corresponding optical density

Observation and conclusion about induction times

In all cases the NC took the least amount of time(~3 hours) to reach an OD_{600} of 0.8 (and in some cases it grew faster than expected reaching an OD_{600} of 1.0 before induction). The growth rate of bacteria transformed with a plasmid containing an RKD1, or RKD2 insert was slightly slower than the NC's. See Figure 5 for a Table of the observed cell densities. The RKD1 and RKD2 cultures also took about 3 hours to grow to an OD_{600} of 0.8. RKD4 in all ETs took the longest, about 4 hours to reach an OD_{600} of 0.8. This observation suggests that RKD4 expression may cause metabolic strain on Rosetta BL21 E.coli cells. This finding could be supported by the fact that RKD1 and RKD2 have a similar function(specification of egg cell) opposed to RKD4, which operates in a different mechanism(zygote elongation).

Soluble protein extracts (supernatant fraction

Gene insert	**Temperature post induction (°C)**			
None (NC)	20	25	32	37
RKD1	20	25	32	37
RKD2	20	25	32	37
RKD4	20	25	32	37

Non-soluble protein extracts (pellet fraction)

Gene insert	**Temperature post induction (°C)**			
None (NC)	20	25	32	37
RKD1	20	25	32	37
RKD2	20	25	32	37
RKD4	20	25	32	37

Table 6: Shown here are the 32 samples obtained from various ETs. They are labelled by their solubility (soluble/non-soluble fraction), the gene insert in the pTWIN1 plasmid that the Rosetta BL21 E.coli cells were transformed with, and the temperature the cells were incubated at after induction with 0.3M IPTG.

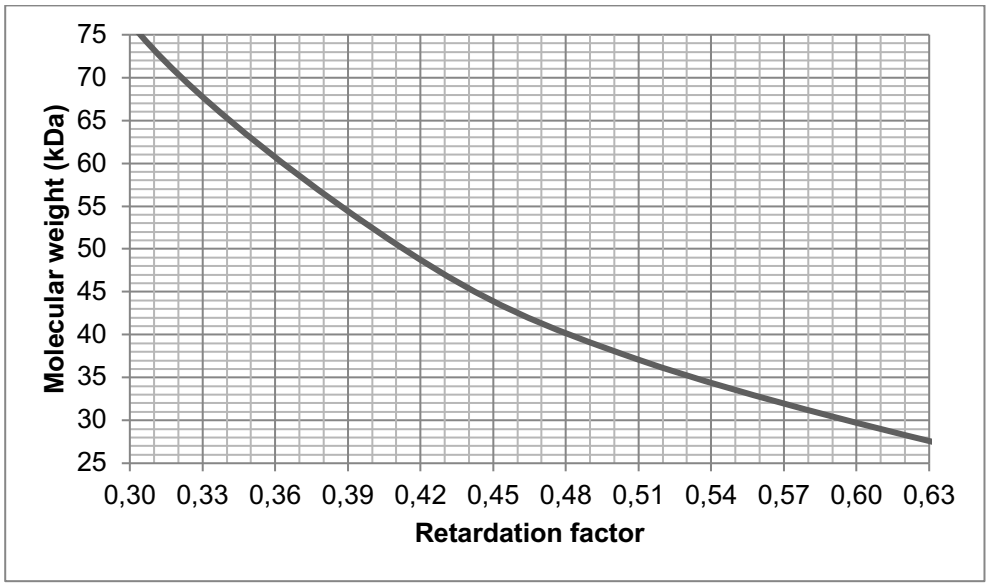
	Gel lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Gene insert	*Marker*	RKD1	RKD2	RKD1	RKD2	NC	NC	*Empty*	*Empty*	*Empty*					
Gel 1, Weertman 20°C (10 wells, 1mm)	**Post-ind. Temp. (°C)**		20	20	20	20	20	20								
	Solubilty/fraction		SN	SN	P	P	SN	P								
	Dilution		5x	5x	5x	5x	5x	5x								
	Gene insert	*Marker*	None(NC)	RKD1	RKD2	RKD4	None(NC)	RKD1	RKD2	RKD4	*Empty*					
Gel 2, ET 20°C (10 wells, 1mm)	**Post - ind. Temp. (°C)**		20	20	20	20	20	20	20	20						
	Solubility/fraction		SN	SN	SN	SN	P	P	P	P						
	Dilution		20x	20x	20x	20x	20x	20x	20x	20x						
Gel 3, ET 25°C (10 wells, 1mm)	**Post - ind. Temp. (°C)**		25	25	25	25	25	25	25	25						
	Solubility/fraction		SN	SN	SN	SN	P	P	P	P						
	Dilution		10x	10x	10x	10x	10x	10x	10x	10x						
Gel 4, ET 32°C (10 wells, 1mm)	**Post - ind. Temp. (°C)**		32	32	32	32	32	32	32	32						
	Solubility/fraction		SN	SN	SN	SN	P	P	P	P						
	Dilution		10x	10x	10x	10x	10x	10x	10x	10x						
Gel 5, ET 37°C (10 wells, 1mm)	**Post - ind. Temp. (°C)**		37	37	37	37	37	37	37	37						
	Solubility/fraction		SN	SN	SN	SN	P	P	P	P						
	Dilution		1x	1x	1x	1x	1x	1x	1x	1x						
	Gene insert	*Marker*	RKD1	RKD1	RKD1	RKD1	*Empty*	RKD2	RKD2	RKD2	RKD2	*Empty*	RKD4	RKD4	RKD4	RKD4
Gel 6, comparison SN (15 wells, 1mm)	**Post-ind. Temp. (°C)**		20	25	32	37		20	25	32	37		20	25	32	37
	Solubilty/fraction		SN	SN	SN	SN		SN	SN	SN	SN		SN	SN	SN	SN
	Dilution		100x	100x	100x	100x		100x	100x	100x	100x		100x	100x	100x	100x
Gel 7, comparison P (15 wells, 1mm)	**Post-ind. Temp. (°C)**		20	25	32	37		20	25	32	37		20	25	32	37
	Solubilty/fraction		P	P	P	P		P	P	P	P		P	P	P	P
	Dilution		100x	100x	100x	100x		100x	100x	100x	100x		100x	100x	100x	100x
	Gene insert	*Marker*	RKD4	RKD4	RKD4	RKD4	RKD	RKD2	RKD2	RKD2	RKD2					
Gel 8, saturation check (10 wells, 1mm)	**Post-ind. Temp. (°C)**		25	25	25	25	25	25	25	25	25					
	Solubilty/fraction		P	P	P	P	P	P	P	p	P					
	Dilution		50x	100x	400x	700x	1000x	50x	100x	500x	1000x					

Table 7: Loading order of every gel during the course of this thesis.

Appendix C

Appendix C includes graphs that are used to determine the molecular weight of the proteins in the bands expected to be RKD fusion protein, proteins in bands in the RRR, and proteins in smaller sized bands/degraded proteins. The molecular weight graphs are determined from the weight of the proteins in the marker lane bands per western blot. These weights are plotted against the relative run distance/retardation factor of the band corresponding with the weight of the proteins in that band.

Weertman's western blot

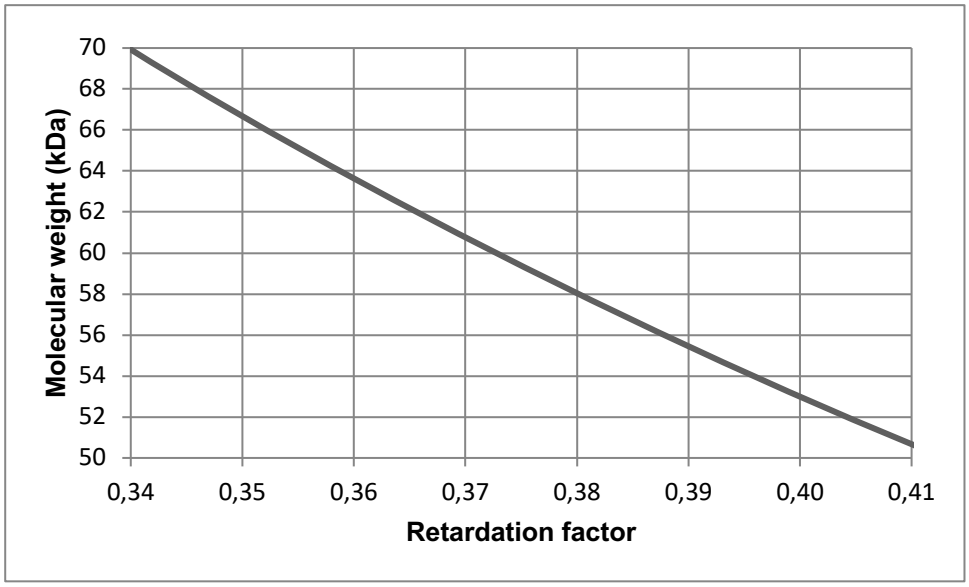


Graph 1: In this graph the molecular weight of the proteins in marker lane band of the membrane on which Weertman's samples were examined, is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
250	0,17
150	0,22
100	0,26
75	0,30
50	0,41
37	0,51
25	0,67
20	0,77
15	0,89

Tabel 1: Values obtained from membrane 1. These values are used to generate Graph 1.

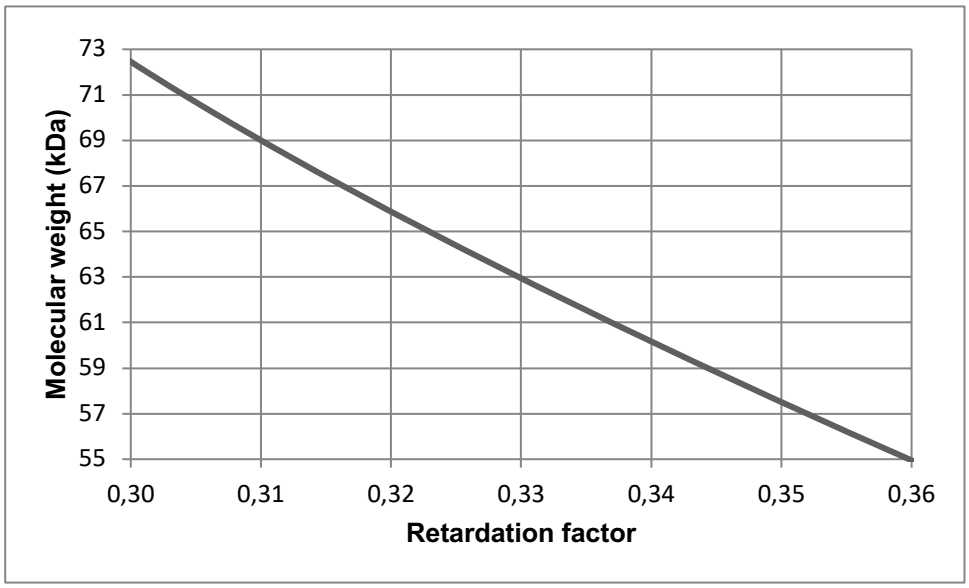
Expression tests at variable temperature post induction



Graph 2: In this graph the molecular weight of the proteins in marker lane band of membrane 1 (20 °C) is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
250	0,23
150	0,25
100	0,29
75	0,33
50	0,41
37	0,50
25	0,65
20	0,74
15	0,85

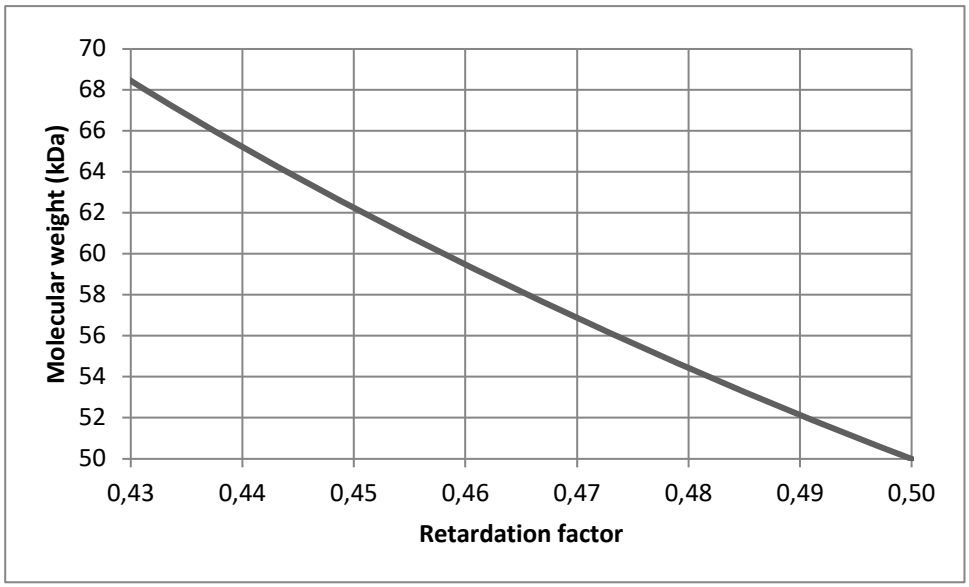
Tabel 2: Values obtained from membrane 1. These values are used to generate Graph 2.



Graph 3: In this graph the molecular weight of the proteins in marker lane band of membrane 2 (25 °C) is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
150	0,22
100	0,26
75	0,29
50	0,38
37	0,46
25	0,57
20	0,67
15	0,78

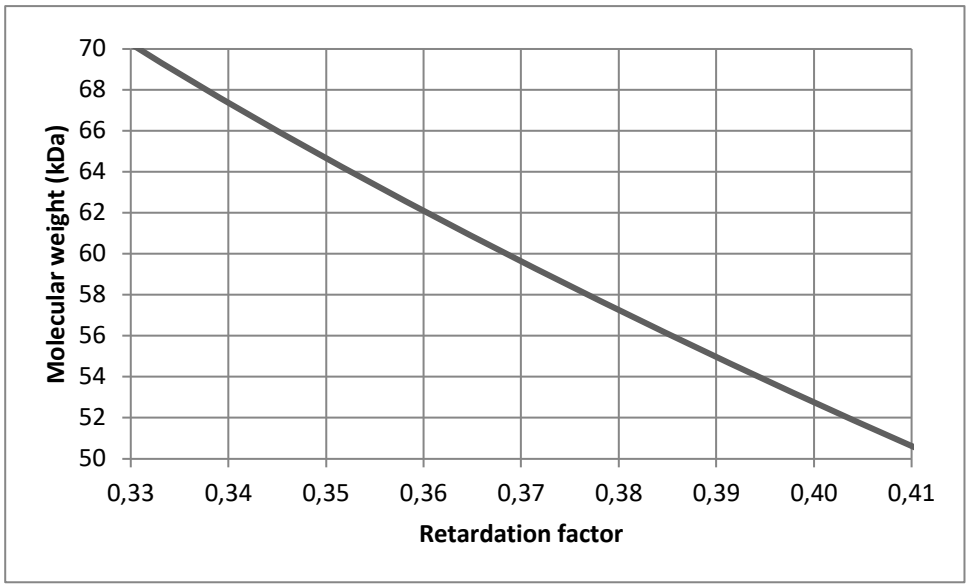
Tabel 3: Values obtained from membrane21. These values are used to generate Graph 3.



Graph 4: In this graph the molecular weight of the proteins in marker lane band of membrane 3 (32 °C) is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
100	0,39
75	0,41
50	0,50
37	0,60
25	0,72
20	0,78
15	0,91

Tabel 4: Values obtained from membrane 3. These values are used to generate Graph 4.

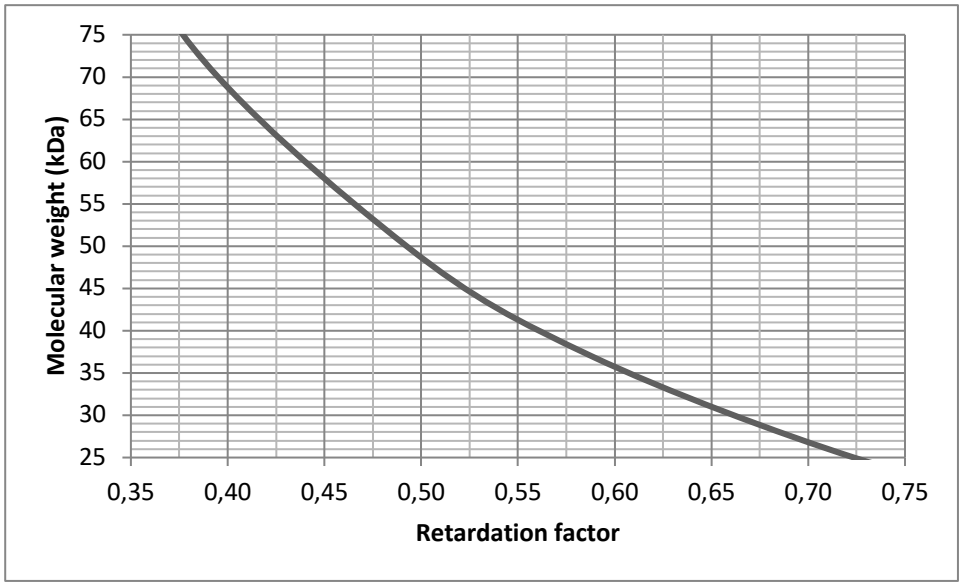


Graph 5: In this graph the molecular weight of the proteins in marker lane band of membrane 4 (37 °C) is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
250	0,20
150	0,23
100	0,27
75	0,32
50	0,41
37	0,50
25	0,65
20	0,74
15	0,78

Tabel 5: Values obtained from membrane 4. These values are used to generate Graph 5.

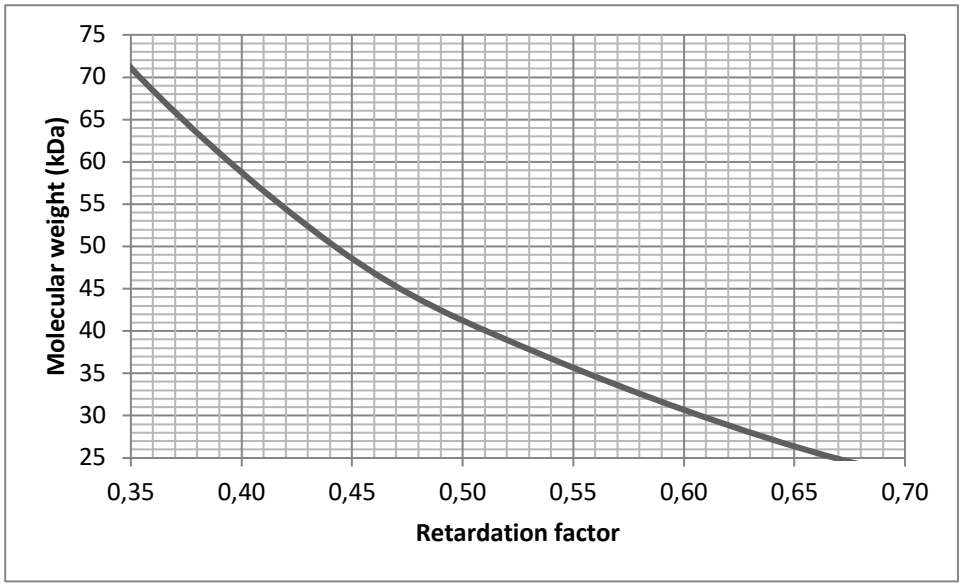
Comparison blots



Graph 6: In this graph the molecular weight of the proteins in marker lane band of comparison blot, supernatant fractions, is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
150	0,28
100	0,33
75	0,38
50	0,49
37	0,59
25	0,72
20	0,82

Table 6: Values obtained from comparison blot, supernatant fractions. These values are used to generate Graph 6.

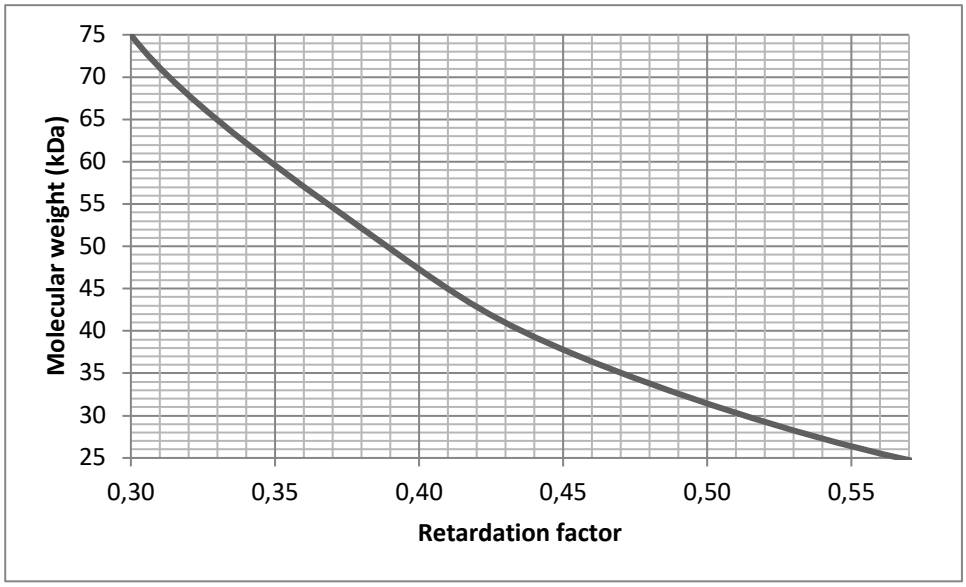


Graph 7: In this graph the molecular weight of the proteins in marker lane band of comparison blot, pellet fractions, is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
150	0,23
100	0,28
75	0,34
50	0,44
37	0,54
25	0,67
20	0,78

Table 7: Values obtained from comparison blot, pellet fractions. These values are used to generate Graph 7.

Saturation check



Graph 8: In this graph the molecular weight of the proteins in marker lane band of the saturation check membrane, is plotted against the relative run distance of the marker lane bands.

mw (kDa)	rf (arb.)
150	0,23
100	0,28
75	0,30
50	0,39
37	0,46
25	0,57
20	0,68

Table 8: Values obtained from saturation check membrane. These values are used to generate Graph 8.