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The Localization of the Mitochondrial Proteins MRP1, KREL2 and LSU1 of *Trypanosoma brucei*

Bachelor Thesis

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Biological Chemistry

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ANNOTATION

The aim of this thesis was to investigate the localization of the mitochondrial proteins MRP1, KREL2 and LSU1 of *Trypanosoma brucei*. Furthermore, the proper functioning of the eYFP-tagged protein MRP1 was investigated.

AFFIRMATION

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act. No. 111/1998. I also agree to the comparison of the text of my thesis with the Thesis.cz thesis database operated by the National Registry of University and a plagiarism detection system.

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ACKNOWLEDGEMENT

1. ABSTRACT

Trypanosomiasis (Sleeping sickness) is a disease caused by parasites of the genus *Trypanosoma*. In order to find drug targets for this disease, unique features of trypanosomes are searched for. The investigated mitochondrial proteins of this thesis, namely MRP1, KREL2 and LSU1 all exhibit such features. Hence, they have the potential to get drug targets. In addition, the temporal and spatial regulation of gene expression is not well understood in trypanosomes. The exact coordination of transcription, RNA editing, its processing, stability and translation in the mitochondria have yet to be elucidated.

Here, we successfully incorporated eYFP into the three above-mentioned proteins of *Trypanosoma brucei* for the investigation of their respective locations within the cell. In addition, for one of the tagged proteins, namely MRP1-eYFP, the proper functioning was examined. The findings of the practical work of this thesis confirmed that all three proteins are situated inside the mitochondria. Furthermore, MRP1 and KREL2 are homogeneously distributed throughout the organelle and the eYFP-tagged version of MRP1 was found to be able to form the same complex as its endogeneous version.

2. AIM AND OBJECTIVES OF THE PROJECT

The aim of the project was the tagging of mitochondrial proteins of *Trypanosoma brucei* with eYFP by homologous recombination into the endogenous gene locus.

Those tagged genes were then localized via an immunofluorescent assay and validated by western blotting. Furthermore, the proper functioning of one of the tagged proteins was investigated with a co-immunoprecipitation.

3. INTRODUCTION

3.1.   Trypanosomes in general

The order Trypanosomatida are protozoan, single-cell flagellates. They are parasites, whose hosts are predominantly insects. However, many have life-cycles with a secondary host [1]. Among those are *Trypanosoma brucei*, *Trypanosoma cruzi* and *Trypanosoma Leishmania*, which are pathogens that give rise to severe diseases in humans. For instance, *T. brucei gambiense* and *T. brucei rhodesiense* cause African sleeping sickness and Chagas disease is caused by *T. cruzi*.

Trypanosomes belong to the class kinetoplastida. The name kinetoplastid is derived from a special feature that all members of the class share: the kinetoplast, which is a DNA containing granule situated in their single, large mitochondrion.

3.2.   Trypanosoma brucei

The parasite *T. brucei* is heteroxenous [1], which means that it has more than one host in its life cycle: The tse-tse fly (*Glossinidae Glossina*) as the vector and vertebrates as an intermediate host. When switching from one host to the other, *T. brucei* undergoes big changes. Three different conformations can be distinguished, depending on the environment they are situated in: procyclic (found in the tse-tse midgut), metacyclic (found in the fly's salivary gland) and bloodform (in the bloodstream of mammals) [2].

When changing from one stage to the other, the mitochondrion changes from a full-functioning, cristae-rich organelle in the procyclic stage into a reduced one, which is smaller in size and has just a few cristae in the bloodform stage [2, 3].

A subspecies of *T. brucei,* namely *Trypanosoma brucei brucei*, causes the disease nagana in rurant animals but is not infectious for humans. Due to this fact and also because both life stages are easy to cultivate in the laboratory, *T. brucei brucei* is a good model organism.

3.3.   Mitochondrial DNA

The mitochondrial DNA (kinetoplast DNA or in short kDNA) of *Trypanosoma brucei* is a network of concatenated, circular DNA, situated at the basal body of the flagellum [4]. It consists of two kinds of concatenated DNA circles: minicircles and maxicircles [5]. The maxicircles (size: about 23 kb) contain mostly genes for mitochondrial rRNA and proteins [6], while the minicircles (size: about 1 kb) encode for small guide RNAs (gRNAs), which are essential for the translation of the genes on the maxicircles [7, 8].

3.4.   RNA editing in *Trypanosoma brucei*

RNA editing in trypanosomes is the insertion and deletion of uridines in a precursor mRNA at specific sites [9]. This mechanism corrects frameshifts, creates new start codons or sometimes even generates an entire ORF (**O**pen **R**eading **F**rame). [10, 11, 12]

Blocking of editing is lethal to the parasite as it is needed to produce the right, translatable ORFs of essential proteins [10]. Because it is a unique and essential process, it serves as a target for drugs against the diseases trypanosomes are causing [13].

3.5.   The mechanism of RNA editing in *Trypanosoma brucei*

The process of RNA editing is directed by small guide RNAs, which are about 50-70 nucleotides long [14, 15]. In general, a gRNA molecule consists of three parts:

1. the **anchor-sequence** at the 5'-end, which consists of the complementary sequence of the mRNA just before the site to be edited.

2. the **coding region**, which contains the information about how many uridines will be inserted or deleted into the pre-mRNA and

3. the **3' oligo(U)tail** which is believed to stabilize the interaction between gRNA and pre-mRNA [14].

The mechanism of RNA-editing is depicted in figure 3.1.
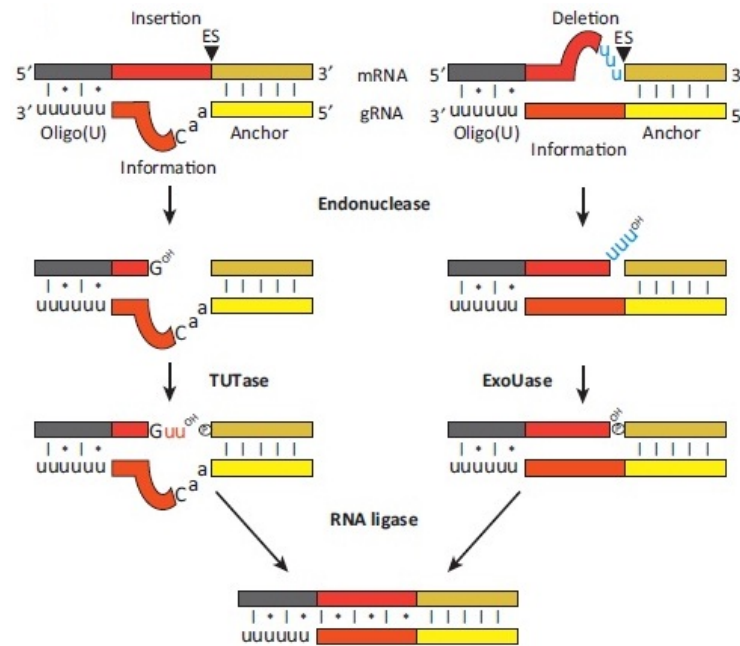
Fig. 3.1.: Mechanism of RNA-editing; After hybridization of the gRNA to the pre-mRNA with its anchor sequence, a few catalytic steps occur as following: An endonuclease cleaves at the editing site. Afterwards, a **T**erminal **U**ridylyl **T**ransferase (TUTase) inserts uridines in the case of uridine addition. In the case of uridine deletion, an **U**-specific **EXO**nuclease (ExoUase) deletes uridines. Finally, a RNA ligase joins the modified fragments together [8].
Picture adopted from [16].

3.6.   Facts about studied proteins and their respective complexes

3.6.1. MRP1 (synonyms: GBP21; Tb427.tmp.55.0009)

Length: Gene: 621 bps   Protein: 23 kDa

Function:

MRP1 (**M**itochondrial **R**NA binding **P**rotein1) builds a heteromeric, tetrameric complex with the protein MRP2 [17]. The interaction of these two proteins is necessary for the stability of the complex [18].

The complex can bind gRNAs with nM affinity [19]. The nature of the binding is mostly electrostatic and nonsequence-specific [17]. The complex promotes the annealing of gRNA to the cognate pre-mRNAs in the initial stages of RNA editing [19].

Suspected localization: Mitochondria

3.6.2. LSU1 (synonyms: ribosomal protein L3; TbMRPL3)

Size: Gene: 1422 bps   Protein: 55 kDa

Function:

LSU1 is a component of the 60S ribosome.

The difference between the prokaryotic and eukaryotic ribosome is that the eukaryotic version has so-called ESs (**E**xpansion **S**egments) [20], which are additional rRNA insertions. Kinetoplastids have extraordinarily large ESs and ribosomal protein extensions [21]. Those unique features of the mitochondrial ribosome are possible drug targets.

Suspected localization: Mitochondria

3.6.3. KREL 2 (Tb427.01.3030)

Size: Gene: 1251 bps   Protein: 47 kDa

Function:

KREL2 (**K**inetoplast **R**NA **E**diting **L**igase 2) belongs of the 20S editosome and is part of the U insertion subcomplex [22]. The protein belongs to the nucleotidyltransferase superfamily, which also contains ATP and NAD-dependent DNA ligases as well as eukaryotic mRNA capping enzymes. All DNA ligases and mRNA capping enzymes have a common catalytic core structure, which contains an **O**ligonucleotide-**B**inding (**OB**)-fold domain, which is needed for the initial auto-adenylation step [8]. KREL2 lacks this domain. However, the missing OB-domain is provided by other proteins, which are also embedded in the editosome [22].

Suspected localization: Mitochondria

4. MATERIALS AND METHODS

> In the following section, all information specific for the conducted experiments are marked with an arrow like the one at the beginning of this sentence.

In order to tag the proteins in question with eYFP, their open reading frames (ORFs) were cloned without their stop codon into the "Locus for ORF of proteins" of the p2937 vector, which contains the gene-sequence for eYFP directly adjacent to it. (See figure 4.1). In this way, the proteins will be expressed with the eYFP-tag.
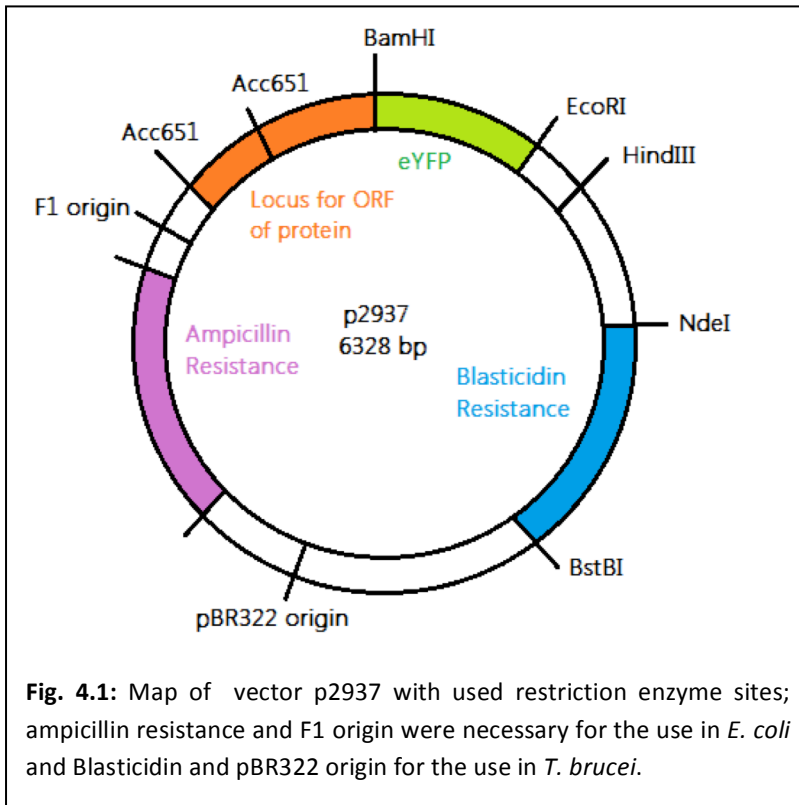
Fig. 4.1: Map of vector p2937 with used restriction enzyme sites; ampicillin resistance and F1 origin were necessary for the use in *E. coli* and Blasticidin and pBR322 origin for the use in *T. brucei*.

4.1. Design of the vector

PCR was performed to generate inserts corresponding to the ORFs of MRP1, KREL2 and LSU1 for cloning into p2937 (Section 4.1.2). As GeneArt® (section 4.1.6) was the chosen method for cloning these inserts into the locus for the ORF's of the respective protein, the PCR required the design of special primers providing the needed homology for the respective gene with the vector (see Section 4.1.1). At the same time, the p2937 plasmid was produced in *Escherichia coli* and extracted by the miniprep method (Section 4.1.9). The plasmid p2937 was then linearized (section 4.1.5) to make the cloning sites for the homologous recombination done by GeneArt® accessible. The plasmid was then mixed together with the DNA of MRP1, KREL2 and LSU1, respectively, and GeneArt® was performed (section 4.1.6). With the reaction mixture, a heat-shock transformation into competent *E. coli* XL-blue 100 was performed (section 4.1.7). Transformed bacteria were then spread on agar plates containing ampicillin and colonies were grown overnight (section 4.1.8). The resulting single-cell colonies were then screened for positive transformants by first doing colony-PCR (Section 4.1.2.5.2). The colonies most likely

containing the right insert were then grown in liquid media (section 4.1.8), DNA was isolated with the miniprep method (section 4.1.9) and a restriction digestion was performed (section 4.1.5.5.2) in order to confirm the presence of the right insert. DNA of positive transformants was then sent for sequencing to ensure that no errors occurred during the PCR-step.

4.1.1. Design of PCR-primers for GeneArt and sequencing of p2937

4.1.1.1. Principle

A primer for PCR is a single-stranded piece of DNA that is complementary to the starting point, called the 5'-end, of the DNA-fragment to be amplified. PCR-primers are necessary because DNA polymerases can only add nucleotides to an already existing strand of DNA. During the process of the PCR-reaction, the primers will hybridize to the DNA, thus providing the needed free –OH group for the polymerase to add further nucleotides.

Primers for GeneArt require a 15 base-pair overhang at their 5' end, which provide the necessary homology for the homologous recombination (see section 4.1.6.1).

4.1.1.2. Protocol

The primers were designed with the help of the online-tool OligoCalc.

(http://www.basic.northwestern.edu/biotools/OligoCalc.html)

The forward primer should generally align to the first nucleotides of the sense strand, which corresponds to the template for a gene's mRNA. The reverse primer should generally align to the last nucleotides of the anti-sense strand (complement to antisense strand), and anneals downstream of the forward primer. To enable the homologous recombination, 15 base-pairs complementary to the vector p2937 from the place the gene should be incorporated were added to the sequence of the primer (See table 4.1, small letters).

The whole primer-sequence was inserted and melting temperatures of the primers were calculated by the online-tool OligoCalc to keep the difference between the forward and reverse primers as small as possible. With the "Check Self-Complementary" function, potential, interfering secondary structures, like hairpins (intramolecular interactions) or primer-dimers (intermolecular interactions) were predicted.

MRP1 Forward	aattgggtaccatttATGATTCGACTCGCATGCCTGCGT
MRP1 Reverse	gccattccgccaccgGAATGGTATCGCGATGTGTCACTTAC
KREL2 Forward	aattgggtaccatttATGTTGCGTCGCCTCGGTGTACGT
KREL2 Reverse	gccattccgccaccgGATTCGCTAAAGTCAGGAGACTGACG
LSU1 Forward	aattgggtaccatttATGCGCTCGCTCATAGCCATGAGG
LSU1 Reverse	ccattccgccaccgGACTTCATACGTTTGTACTTCAGTAT

4.1.1.3. Troubleshooting

To create well working primers, there are several things to take into consideration:

1. The GC-content of the primer should be about 40-60%. As G and C are binding stronger (3 H-bonds) to the template than A and T (only 2 H-bonds), the GC-content directly affects the annealing temperature of the primers. A too high annealing temperature might cause insufficient primer-template hybridization and lead to a low yield. On the other hand, a too low annealing temperature might lead to nonspecific products.

2. The presence of G and C within the last 5 bases of the 3' end helps to promote specific binding.

4.1.2. Polymerase Chain Reaction (PCR)

4.1.2.1. Principle

The **P**olymerase **C**hain **R**eaction (in short PCR) is a technique to amplify a short piece of DNA. To perform it, a DNA polymerase is used to replicate the DNA. It is crucial that this polymerase is not destroyed at high temperatures. Therefore, usually a Taq-polymerase is used (taken from the thermophilic organism *Thermus aquaticus*).

"Chain Reaction" means that the products of former cycles are used as templates for the next cycles. The method is based on thermal cycles - repeated changes in temperature – which consist of the following steps:

1. Initiation step, which is to heat the reaction to a temperature of 94-98 °C for several minutes. This step is only required for hot-start polymerases, which need a certain

temperature to be activated. During the heating, the polymerase undergoes a conformational change. The heat-activation is usually incorporated in polymerases to avoid non-specific amplification during the assembly of the reaction system.

2. Denaturation step, which heats the reaction to 94-98 °C for 15 to 30 seconds usually. It is the first step of the cycle and serves to melt the DNA template by disrupting the hydrogen bonds between Watson and Crick bases. Single-stranded DNA molecules are the result.

3. Annealing step, which lowers the temperature to 50-65 °C. It allows the primers to anneal to the single stranded DNA template. Primers are single-stranded, short pieces of DNA complementary to the 5'-end or beginning of the DNA fragment to be amplified. DNA replication begins.

4. Elongation step, in which the polymerase synthesizes the DNA strand by adding dNTPs complementary to the template in 5' to 3' direction. The temperature of this step depends on the optimum activity temperature of the DNA polymerase. This is the last step of the cycle.

5. Final elongation: This step is necessary to enable all single-stranded DNA fragments to be finished.

4.1.2.2. Used Devices and amounts of Chemicals

- Devices: Biometra T3000 Thermocycler

- Recipe for a general 50 µL-reaction:

1	µL	Forward-primer (20 µM)
1	µL	Reverse-primer (20 µM)
10	µL	Buffer (5x)
1	µL	dNTPs (10 mM each dNTP)
0.5	µL	Polymerase
1	µL	DNA template (final [c] = 20-30 ng/µL)
35.5	µL	MiliQ

➢ The template used was genomic DNA of *T. brucei* strain 427.

4.1.2.3. Protocol

For more than one reaction, a "master-mix" is prepared. Therefore, the MiliQ, buffer, dNTPs and polymerase (if possible, also the primers) are mixed together. Approximately 0.5 - 1 µL of the mixture (excluding the template) are prepared additionally to compensate for pipetting losses. It should be paid attention to that after adding the polymerase, the reaction should be kept on ice. As a negative control, MiliQ is added instead of the template. When the reactions are mixed, they are put into the thermocycler. Usually, the following program is adjusted to the right annealing-temperatures:

Lid temperature: 99 °C (to avoid condensation of liquid on the lid of the PCR tubes)

1. 94 °C for 2 minutes
2. 94 °C for 30 seconds
3. 55 °C for 1 minute
4. 72 °C for 1 minute (cycle back to step 2 for 28 cycles)
5. 72 °C for 5 minutes
6. 14 °C (to cool down after the reaction until the reaction is taken up)

Usually, the 3rd step is altered according to the melting temperature of the primers.

> ➢ The annealing temperatures for the primers for MRP1, KREL2 and LSU1 were all 55 °C.

4.1.2.4. Troubleshooting

1. It should be checked if the concentration of the DNA is optimal. Too much DNA can lead to severe interferences and hence no amplification. 20 to 30 ng of template are recommended.

2. The secondary structure due to so-called hairpins (base-pairing occurs between nucleotides on the same strand of the molecule) is another source of error. This can be solved by adding DMSO (**DiM**ethyl **S**ulph**O**xide), which will interrupt the secondary structure. Usually, 2.5 µL of 100% DMSO are added to a 50 µL reaction.

3. All used chemicals should be checked for possible contamination and appropriate negative controls should be set up.

4. Mg^{++} is required as a cofactor for the polymerase. Mg^{++} stabilizes dsDNA and raises the melting temperature. It is important for controlling the specificity of the reaction. A low concentration requires more stringent base pairing in the annealing step and may result in a low yield of PCR product. However, too much Mg^{++} increases the products of non-specific products and promotes misincorporation. The $MgCl_2$ concentration should normally be between 1mM and 4mM. Special care should be taken when the dNTP-concentration is changed as it sequesters Mg^{++} ions.

4.1.2.5. Variations

4.1.2.5.1.   Phusion High Fidelity PCR

The Phusion High Fidelity DNA Polymerase has an increased fidelity and speed. Its error rate is 50 times lower than that of Taq DNA polymerase. The buffer used for this polymerase is the 5x Phusion HF buffer, commercially available together with the Phusion High Fidelity DNA Polymerase.

> ➢ The lower error rate is the reason why it was used for the amplification of cloning-educts.

4.1.2.5.2.   Colony PCR for identification

This technique is an excellent choice for screening for the right colonies on an agar-plate after performing a Ligation or GeneArt®. As it is time- and cost-intense to do first minipreps of all colonies to check if they contain the right insert, colony-PCR is a cheap and quick method to screen a lot of them. Usually, 20 µL of a Taq-reaction-system are prepared per colony. Instead of the template, one additional µL MiliQ is added. To add the template, the colony is slightly touched with a small pipet-tip and dipped in the corresponding reaction. The program for the thermocycler is also slightly adjusted: The very first step (heating up to 94 °C) is extended to about 3 minutes. This step will lyse the cell and make the contained DNA accessible to the primers.

4.1.3. <u>PCR clean-up</u>

The used kit was the GenElute PCR Clean-up Kit from SIGMA.

www.sigmaaldrich.com/life-science/molecular-biology/dna-and-rna-purification/pcr-clean-up-kit.html

4.1.3.1. Principle

The Clean-up kit works on the basis of an affinity chromatography: The negatively charged DNA in solution (= mobile phase) binds to a silica membrane within the column (= stationary phase) in the presence of chaotropic salts (eg.: guanidine HCl, guanidine thiocyanate, urea and lithium perchlorate). Usually, nucleic acids are covered by a hydrate shell, which consists of water molecules that maintain the solubility of DNA in aqueous solutions. When adding chaotropic ions, this hydrate shell is destroyed by creating a hydrophobic environment. Under this condition, the silica membrane is the most suitable binding partner for the nucleic acids. Proteins, metabolites and other contaminants do not bind to the membrane and therefore are washed away during the following washing steps. In addition, the cations of the salt saturate the silica membrane with positive charges, which improves the binding of nucleic acids under hydrophobic conditions. Chaotropic salts denature proteins because they disrupt noncovalent interactions. Hence, DNAses and RNAses will be also denatured. To enhance the binding, alcohol is added. (Usually ethanol or isopropanol).

After binding to the column, one to two washing steps are performed. The first washing step is optional and the buffer used for it usually contains a low concentration of chaotropic salts. It is used to remove proteins. The second washing step - done with ethanol - is to remove the salts, which is important to get a high yield and purity of the DNA. If salt remains behind, the elution will be poor and the spectroscopic A230-reading will be high.

The next step is to spin the column down one additional time to get rid of all residual ethanol. For the last step, water or elution buffer is added. This step will hydrate the nucleic acids. If some ethanol is left behind, the DNA can't be dehydrated completely and therefore will not become soluble.

To judge the quality of the clean-up, the A230, A260 and A280-values are measured with a spectrometer. The 260/280 ratio tells about contaminations from proteins. For pure DNA, it

should be around 1.8. On the other hand, the 260/230-ratio tells about contaminations from salts or from the washing buffer.

4.1.3.2. Used Devices and amounts of Chemicals

➢ Devices: Eppendorf Minispin Centrifuge

➢ Used Chemicals per sample:

    500   µL Column Preparation Solution

              Binding Solution: 5x the Volume of the PCR-Reaction Volume of

    500   µL Wash Solution

    50   µL MiliQ for Elution

4.1.3.3. Protocol

1. Of the Column Preparation Solution, 500 µL are added to the column. The column is spinned at maximum speed for 1 minute and the flow-through is discarded. Then, 5 volumes of the Binding solution are added to the PCR reaction and mixed. The solution is transferred to the column and centrifuged at maximum speed for 1 minute. The flow-through is discarded.

2. Of the washing solution, 500 µL are added to the column. The column is then centrifuged 2 times at full speed for 1 minute to get rid of all residues.

3. The column is placed into a different eppendorf tube and 50 µL of MiliQ are added to the center of the column. After waiting for approximately one minute, the column is centrifuged at maximum speed for 1 minute and the column is discarded.

4.1.3.4. Troubleshooting

1. It should be taken into consideration that the columns are made for 10 µg of DNA in the range of 100 bps to 10 kb. Products at the edges or outside that range will be difficult or impossible to recover.

2. More elution steps increase the yield. Hence, two elutions with 25 µL might be better than just one with 50 µL.

4.1.3.5. Variations

The PCR clean-up can be used to purify DNA not only after a PCR-reaction but also after other procedures containing chemicals which might interfere with following experiments. In many cases, this interfering chemical is glycerol, which is added to many enzymes for storage. In large concentrations, it will decrease the activity of other enzymes.

4.1.4. <u>Gel Extraction</u>

The used kit was the Agarose Gel DNA Extraction Kit from Roche.

http://www.roche-applied-science.com/proddata/gpip/3_6_8_32_1_1.html

4.1.4.1. Principle

First, an agarose gel electrophoresis is run to separate the DNA fragments. Then, the desired band is cut out and dissolved. This solution is then mixed with a silica-suspension, which contains a chaotropic salt.

The principle is the same as in the PCR-clean-up: DNA binds to the silica, is washed once with Binding Buffer, washed with a washing buffer and eluted with MiliQ.

4.1.4.2. Used Devices and Chemicals

<u>Devices:</u> Eppendorf Minispin Centrifuge

<u>Chemicals:</u>

 10 μL Silica Matrix

   Solubilization Buffer: 3 x the Volume of the Agarose gel

 500 μL Nucleic Acid Binding Buffer

 500 μL Washing Buffer

4.1.4.3. Protocol

1. The agarose gel is run to separate the DNA and the desired band is cut out.

2. The slice of gel is transferred into a pre-weighted eppendorf tube.

3. The eppendorf tube containing the gel is weighed.

4. Per 1 mg of agarose gel, 3 μL of Solubilization Buffer are added.

5. The tube is warmed up to 50-60 °C until the gel is dissolved. This might take about 10 minutes. From time to time, the tube is vortexed to speed up the process.

6. The silica suspension is homogenized and 10 µL of it are added to the sample.

7. The mixture is incubated for 10 minutes at 56 – 60 °C and vortexed every 2 to 3 minutes.

8. The tube is centrifuged for 30 seconds at max. speed and the supernatant is discarded.

9. The silica matrix is resuspended in 500 µL of Nucleic Acid Binding Buffer and centrifuged at the same conditions as before. The supernatant is discarded.

10. The pellet is washed with 500 µL Washing Buffer. The eppendorf tube is centrifuged and the supernatant is discarded. The centrifugation is repeated and the remaining liquid is pipetted out as good as possible.

11. The tube is inverted on a paper towel and left to dry at room temperature for 15 minutes.

12. For the elution of the DNA, 50 µL of MiliQ are used. The pH of the MiliQ should be between 8 and 8.5. The tube is vortexed and incubated for 10 minutes at 56 to 60 °C. From time to time, it is vortexed.

13. The tube is centrifuged at maximum speed and the solution is transferred to a new eppendorf tube.

4.1.4.4. Troubleshooting

1. The recovery efficiency of a gel-extraction is usually low. It is normal to get concentrations below 10 ng/µL. When dealing with concentrations below the detection limit of the spectrophotometer (NanoDrop 1000 in my case), the measurement will most probable give wrong values. Sometimes, a concentration of 0 or even below is measured although the solution contains some DNA. In such cases, an aliquot can be run on an agarose gel. For experiments requiring only a small amount of DNA (such as ligations), a visible faint band is good enough to get a positive result.

2. To increase the yield, more elution steps can be carried out.

3. The MiliQ for the elution step should be prewarmed to at least room temperature if it is kept in a fridge at 4 °C. The elution time can be increased to 20 minutes.

4. It should be kept in mind that the kit is designed to isolate fragments in the range from 400 bps to 5 kb.

4.1.5. <u>Digestion</u>

The used enzymes were purchased from New England Biolabs.

http://www.neb.com/nebecomm/default.asp

4.1.5.1. Principle

Restriction endonuclease enzymes originate from bacteria, which protect themselves against invading DNA with these enzymes. There are three different types of restriction endonucleases: Type I, II, and III. Type I and III are not very useful for most molecular biology techniques as they cut either randomly (Type I) or far away from the recognition site (Type III). Only type II is suitable and its mechanism works as follows: The enzyme recognizes a certain nucleotide sequence in the foreign DNA and binds to it tightly. The binding of the enzyme at the recognition site causes a conformational change, which brings the catalytic part close to the DNA strand. Then, it hydrolyzes the phosphodiester backbone of the DNA. Thus, the bond between the 3' oxygen atom and the phosphorus atom is broken.

Restriction enzymes can cut in several ways: They can either create so-called sticky ends which means 3' or 5' overhangs or they can produce blunt ends, which means cuts without overhangs.

4.1.5.2. Used Chemicals

- Restriction endonuclease enzymes with corresponding buffers (see table 4.1) from New England Biolabs, stored in 50% glycerol;
- 100 x BSA, diluted to 10 x;

4.1.5.3. Protocol

➢ A summary of the used restriction enzymes used in the practical work of this thesis can be found in table 4.1.

According to table 4.1., the right buffer is chosen and looked up if BSA is needed or not. BSA stabilizes the enzyme during the reaction and its effect is most pronounced during overnight-digestions.

Tab. 4.2.: List of used restriction enzymes with respective buffers, needed incubation temperatures and need for BSA (+) or not (-)

Restriction enzyme	Buffer	Temperature	BSA
Acc65I	NEB3	37 °C	+
BamHI	NEB3	37 °C	+
EcoRI	NEB EcoRI	37 °C	-
HindIII	NEB2	37 °C	-
SmaI	NEB4	25 °C	-
NcoI	NEB3	37 °C	-

Overall recipe for a 50 µL reaction:

1-2	µL	Restriction Enzyme (depends on the amount of DNA to digest)
5	µL	10x NEBuffer (1, 2, 3, 4 or EcoRI-buffer, depending on the used enzyme)
5	µL	10x BSA, if necessary
X	µL	DNA (to get 1-2 µg of DNA, depending on the actual needs)
Y	µL	MiliQ (fill up to 50 µL)

The reaction is then incubated at the required temperature (see table 4.1.) for 1 to 3 hours or longer, depending on the actual needs.

4.1.5.4. Troubleshooting

1. All available information about the digestion enzyme should be checked. If the right buffer and incubation temperature were used, it should be also checked whether the enzyme needs BSA or not. Some enzymes even need some ATP to be added to the reaction system.

2. Some enzymes are prone to exhibit star activity, which leads to more pieces of DNA than there should be. Under certain conditions, some enzymes are capable of cleaving sequences, which are similar but not identical to their defined recognition sequence. Conditions that contribute to star activity are for example:
   - glycerol concentrations above 5%,
   - a high concentration of enzyme compared to DNA,

- a prolonged reaction time

- the presence of organic solvents (DMSO, EtOH, etc.)

- Substitution of Mg^{2+} with other divalent cations as other divalent cations may not fit correctly into the recognition site of the restriction enzyme, interfering with a proper recognition.

4.1.5.5. Variations

4.1.5.5.1. Double digestion

It is possible to use two restriction enyzmes at once. However, it can only be done if there is a suitable buffer for both enzymes. This can be easily checked with the following New England Biolabs online-tool:

http://www.neb.com/nebecomm/DoubleDigestCalculator.asp#.UCT5VloinU8

For some couples of enzymes, a double digestion is not recommended. In this case, digests have to be done sequentially, which may lead to a high loss of DNA during the process.

➢ A summary of the double digests performed during the practical work of this thesis can be found in table 4.2.

Tab. 4.3.:   List of used restriction enzymes for double digestion with respective buffers, needed incubation temperatures and need for BSA (+) or not (-)

Restriction enzyme 1	Restriction enzyme 2	Buffer	T [°C]	BSA
Acc651	BamHI	NEB3	37	+
BamHI	EcoRI	NEB EcoRI	37	+

4.1.5.5.2. Digestion for identification

After cloning an insert to a plasmid and transforming it into competent *E. coli*, a digestion can help to identify positive transformants. To do so, one or more restriction enzymes are chosen that will cut only if the desired insert is present. NEBcutter is a handy tool to choose restriction enzymes for identification: http://tools.neb.com/NEBcutter2/

After inserting the sequence and submitting it, it shows all possible restriction enzyme sites. Also, it lists all enzymes, which will cut once, twice, three times or not at all. For identification,

a complete digestion is not necessary. Therefore, an incubation time of one hour or even less is good enough to get the result.

4.1.6. GeneArt®

GeneArt® was used for cloning of the amplified inserts produced by the PCR into the vector p2937. Used was the GeneArt® Seamless Cloning and Assembly Kit from Invitrogen[TM]:

http://products.invitrogen.com/ivgn/product/A13288?ICID=cvc-seamless-assembly-c2t1

4.1.6.1. Principle

The GeneArt® -kit contains a mix of enzymes that fuses together DNA fragments that share terminal end-homology. The principle behind it is homologous recombination. Normally, homologous recombination is used by cells to repair harmful double-strand breaks or to produce new combinations of DNA sequences during meiosis. The general mechanism is the following:

1. After a break of the double-stranded DNA happened, the region around the break is cut away. This process is called resection. In the case of GeneArt® , the break is done artificially by a digestion.

2. The next step is the invasion of a 3' end of the broken DNA molecule into the DNA-strand, which is not broken.

3. A holliday junction is formed between the two DNA-molecules.

4. The DNA is extended and thereby repaired.
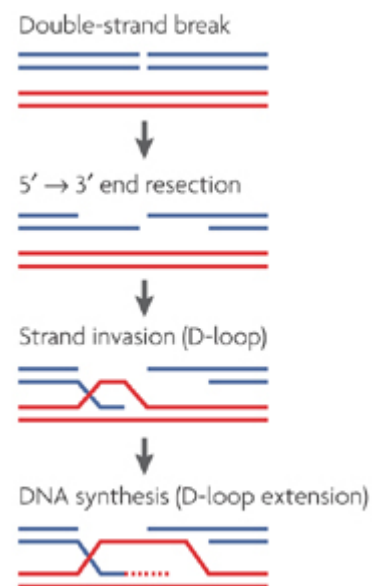
Fig. 4.2: Procedure of homologous recombination. Picture acquired from:
http://www.nature.com/nrg/journal/v8/n10/images/nrg2193-f1.jpg

4.1.6.2. Used Chemicals

- Enzyme Mix, 10x
- Enzyme Buffer, 5x

4.1.6.3. Protocol

1. First, the vector is linearized.

2. A three times higher molar concentration of the insert is taken und put together with the vector. As a negative control, MiliQ is added to the vector instead of the insert.

3. The reaction is filled up with MiliQ to 14 µL.

4. Then, 4 µL of the enzyme buffer are added.

5. The very last step is to add 2 µL of the enzyme-mix.

After GeneArt® was performed, the next step is the transformation of the formed plasmids into competent cells.

4.1.6.4. Troubleshooting

1. If a lot of false positives were obtained, it should be checked if the linearization of the vector has worked out properly.

2. It is crucial that the enzyme-mix is added at the very end.

4.1.7. Transformation of plasmids into competent cells with heat-shock

4.1.7.1. Principle

To transfer plasmids into competent *E. coli*, a technique called heat-shock is performed. Therefore, the cell wall of *E. coli* is chemically weakened, mixed together with the desired plasmid and put on ice. The plasmids will stick to the cell wall of the bacteria. The next step is to incubate the bacteria with the plasmids in a water-bath at 42 °C for one minute. This will cause the cell walls to expand, swallowing thereby the plasmid.

4.1.7.2. Used Cell-line:

➢ Competent *E. coli*, strain XL-blue 100

4.1.7.3. Protocol

1. The competent cells are slowly thawed on ice.

2. The desired plasmid (resulting from a ligation or performed GeneArt®) is mixed together with about 60 μL of the competent cells in an eppendorf tube and put on ice for 20 minutes.

3. The eppendorf tube is incubated for 1 minute at 42 °C.

4. The bacteria are put on ice for 5 minutes.

4.1.8. Growing of transformed cells on agar plates and transfer of colonies to liquid media

4.1.8.1. Principle

Competent *E. coli* do not contain a resistance against any antibiotic. Therefore, transformed cells are grown in media, which contains one. Only bacteria with a plasmid containing the resistance against the antibiotic will be able to divide. However, freshly transformed cells need some time to express the protein for the resistance. Therefore, cells are usually firstly grown in media containing no drugs and only after that, they are transferred to either agar plates or liquid media containing an antibiotic.

4.1.8.2. Used Chemicals and recipes

Agar plates, per 100 mL:		Liquid media, per 100 mL:		Other reagents used:
1.0 g	Bactotryptone	1.0 g	Bactotryptone	Ampicillin:
0.5 g	Bacto yeast extract	0.5 g	Bacto yeast extract	100 μg/mL for agar plates
1.0 g	NaCl	1.0 g	NaCl	6 μg/mL for liquid media
1.0 g	Bacto Agar	100 mL	distilled water	
100 mL	distilled water			

➢ As the plasmid p2937 contains the ampicillin-resistance, only this antibiotic was used for the practical work of this thesis.

4.1.8.3. Protocol

1. After the transformation via heat-shock, the bacteria are transferred to liquid media without drugs and incubated on a shaker at 37 °C for about 30 minutes.

2. About 100 to 150 µL of the mixture are spread on an agar-plate containing the antibiotic.

3. The plates are incubated for 12 hours at 37 °C.

4. Single-cell colonies are picked with a pipet-tip and put into test tubes containing liquid media with the selective drug.

5. The test tubes are incubated for 12 hours at 37 °C.

4.1.8.4. Troubleshooting

When the cells are incubated longer than 12 hours, the ampicillin is degraded in a circle around the colony. The longer the incubation, the bigger the circle of degraded ampicillin gets. Within the area without the drug, so-called satellite-colonies are able to grow. Those are cells without the resistance against the drug.

4.1.9. Extraction of DNA by means of minipreps

4.1.9.1. Principle

Minipreps were used to extract plasmid DNA from *E. coli*. The principle behind this method is to lyse the cells and apply the lysate to a column containing a silica membrane. The principle of DNA binding to and its elution from the column is the same as for the PCR clean-up (Section 4.1.3). The used kit was the QIAprep Spin Miniprep Kit from Qiagen®.

4.1.9.2. Used Devices and Chemicals

Devices: Eppendorf Minispin Centrifuge

Used Chemicals:

    250 µL of Buffer P1 (resuspension buffer)

    250 µL of Buffer P2 (lysis buffer)

    350 µL of Buffer N3 (neutralization buffer)

    750 µL of Buffer PE (washing buffer)

    MiliQ for elution

4.1.9.3. Protocol

1. Bacteria are pelleted by centrifuging them at maximum speed for 1 minute.

2. The cells are resuspended in 250 µL of Buffer P1 and transferred to a microcentrifuge tube. No cell clumps should be visible after the resuspension of the pellet.

3. To the cells, 250 µL of buffer P2 are added and it is mixed thoroughly by inverting the tube 4–6 times. The tube is mixed gently by inverting until the solution becomes viscous and slightly clear. The lysis reaction should not proceed for more than 5 minutes.

4. To the cells, 350 µL of buffer N3 are added and the tube is mixed immediately and thoroughly by inverting it 4 to 6 times. The solution should become cloudy.

5. The tube is centrifuged for 10 min at maximum speed. A white pellet forms, which contains the precipitated proteins of the sample. The supernatant containing the DNA is applied to the column.

6. The tube is centrifuged for 1 minute at maximum speed and the flow-through is discarded.

7. The column is washed by adding 750 µL of Buffer PE and centrifuging for 1 minute at maximum speed.

8. The flow-through is discarded and the column is centrifuged at full speed for an additional 1 min to remove all residual washing buffer.

9. The column is placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 µL MiliQ are added. It is left for 1 minute and then centrifuged for 1 minute at maximum speed.

4.2.   Transformation of vectors into *Trypanosoma brucei*

After the result of the sequencing confirmed that the genes of MRP1, KREL2 and LSU1 were error-free incorporated into the plasmid p2937, the respective colonies of *E. coli* containing the correct plasmid were grown in liquid media to amplify the DNA. (Chapter 4.1.8) The plasmids were then extracted by the miniprep method (section 4.1.9) and linearized (section 4.1.5). The digested DNA was then precipitated with ethanol and washed with the CytoMix Buffer. Afterwards, electroporation was performed to transfect the DNA into procyclic *T. brucei*. (section 4.2.1). The electroporated cells were incubated overnight in a media containing no selective drugs as they need some time to express the protein for the resistance. After 18 hours, the selective drug was added and the cells were transferred to a 24-wells plate

in 4 different dilutions. The plates were screened for 2 weeks for surviving cells, which contain the resistance against the selective drug and hence incorporated successfully the electroporated vector. To confirm the presence of the eYFP-tagged proteins, cells from positively transfected colonies were lysed, the lysate was run on a SDS-PAGE gel and a western blot was performed (Chapter 4.2.4).

4.2.1. Electroporation of procyclic *Trypanosoma brucei*

4.2.1.1. Principle

Electroporation is a mechanical method, which is used to introduce polar DNA into a host cell through the cell membrane. As the phospholipid bilayer of the plasma membrane is hydrophobic at the outside and the inside, any polar molecules (like DNA) are not able to freely pass through it.

During the procedure of electroporation, a large electric pulse disturbs temporarily the phospholipid bilayer's hydrophobic and hydrophilic interactions, which allows the DNA to pass into the cell. Afterwards, the membrane can reseal quickly and thus, the cell stays intact.

4.2.1.2. Chemicals:

Digestion:	**CytoMix Buffer:**		**Selective drugs and media:**
BamHI	25 mM	HEPES, pH 7.6	2.5 mg/mL Blasticidin in MiliQ
NEBuffer 3	120 mM	KCl	SDM-79 [23]+ 10% FBS
	0.15 mM	$CaCl_2$	
Precipitation of DNA:	10 mM	K_2HPO_4/KH_2PO_4, pH 7.6	
2 M NaCOOH	2 mM	EDTA	
96% EtOH	6 mM	Glucose	
76% EtOH	5 mM	$MgCl_2$	

4.2.1.3. Devices:

Eppendorf Centrifuge 5810 R for 15 mL falcons or 50 mL falcons

Eppendorf Centrifuge 5424 R for 1.5 mL eppendorf tubes

ECM650 Electroporator (BTX)

27 °C Incubator

4.2.1.4. Protocol

4.2.1.4.1. Preparation of DNA

1.1. In a 1.5 mL eppendorf tube, about 10 μg of DNA are linearized by an appropriate digestion enzyme.

> The used digestion enzyme was BamHI with NEBuffer 3 (see protocol in chapter 4.1.5). The digestion was done in a 250 μL reaction system with 6 μL of enzyme (units per μL). The digestion was carried out for 6 hours and complete digestion was confirmed by running an agarose gel together with the uncut plasmid as a negative control.

2.1. To the digested DNA, 25 μL of the 2 M NaCOOH and 625 μL of 96% EtOH are added, gently mixed and incubated at -20 °C for 20 minutes.

3.1. The DNA is centrifuged for 15 minutes at 4 °C and maximal speed.

4.1. The ethanol is aspirated and 1 mL of 76% EtOH are added. At this point, the DNA can be stored at -20 °C for later usage.

5.1. The eppendorf tube is centrifuged at maximal speed at 4 °C for 10 minutes.

6.1. The ethanol is aspirated and the eppendorf tube is left open for drying. After all of the ethanol has evaporated, the DNA is resuspended in 400 μL of the CytoMix Buffer.

4.2.1.4.2. Preparation of Cells and electroporation

2.1. Per electroporation, 10 mL of procyclic *Trypanosoma brucei* (density: $1\text{-}2 * 10^7$) are collected.

2.2. The cells are spinned down at 1300 g, at 4 °C, washed once by resuspending them in 5 mL of CytoMix Buffer and spinned down at the same conditions again.

2.3. The supernatant is aspirated and the cells are resuspended in the DNA-CytoMix Buffer from step 1.6. The negative control-cells are resuspended in an eppendorf tube containing only 400 μL CytoMix Buffer, without DNA.

2.4. The pre-cooled cuvettes are loaded with the cell-DNA and the cell-CytoMix Buffer only, respectively.

2.5. One by one, the cuvettes are put into the electroporation machine and 1 pulse (at 1600 V, 25 Ω and 50 μF) is applied.

2.6. The cells are then immediately resuspended in 10 mL of prewarmed SDM-79 medium and incubated for 18 hours at 27 °C under 5% CO_2.

4.2.1.4.3. Plating of electroporation

3.1. After 18 hours, the appropriate amount of the selective drug is added to both the sample and the negative control. In addition, to 30 mL of new media, the appropriate amount of selective drug is added.

➢ The used selective drug was Blasticidin, diluted 1:1000 (1 µL stock-solution per mL media)

3.2. The 4 rows (A-D) of two 24-well plates (One for the sample, one for the negative control) are prepared in the following way:

Row A: The first row is loaded with 1.5 mL of the cells.

Row B and C: the second and third rows are loaded with 1 mL of SDM-79, containing the selective drug.

Row D: The last row is loaded with 0.5 mL of SDM-79, containing the selective drug.

3.3. Starting from the first column, 0.5 mL of the first well (row A) are transferred to the second in the same column (row B). The well is mixed and 0.5 mL are then transferred to row C. The well in row C is mixed and 0.5 mL of this well are transferred to the well in the row D.

3.4. The same as in 3.3. is done for all 6 columns.

3.5. The points 3.3 and 3.4 are repeated for the negative control.

4.2.1.4.4. Screening for clones and confirmation of the right clone

4.1. The screening for positive clones for procyclic trypanosoma brucei takes about two weeks. During this time, two things should happen:

- all cells in the negative control should die due to no resistance against the selective drug

- one or more clones – (Row D preferred over C or B)– should survive in the sample.

4.2. If necessary, the plates are diluted with new SDM-79 media, containing the selective drug.

4.3. After about two weeks, cells of each surviving clone are taken, lysed and with a western blot and the appropriate primary antibody, the presence of the desired protein is confirmed.

4.2.2. Handling of procyclic *Trypanosoma brucei*

4.2.2.1. Chemicals and Devices

Used Media: SDM-79, according to [23] + 10% FBS

Devices: Z2 cell counter (Beckman Coulter Inc.)

27 °C Incubator

4.2.2.2. Protocol

Every 48 hours, the following procedure is conducted:

1. Cells are checked under the microscope for vitality.

2. The well with the cells is mixed with a pipet, 50 μL from that are taken, diluted to 10 mL of and counted with the cell counter.

3. For normally growing procyclic *T. brucei*, the density of the cells after 48 hours should be around $1\text{-}2*10^7$. The cells are then diluted to a concentration of approximately $2 * 10^6$.

4.2.3. Principles of Antigens and Antibodies

Antibodies are proteins that are produced by B-lymphocyte cells in response to the presence of foreign molecules or organisms in the body. The basic structure of an antibody is Y-shaped (see figure 4.3). It consists of two copies of a heavy polypeptide chain and two copies of a light polypeptide chain, which are held together by disulfide
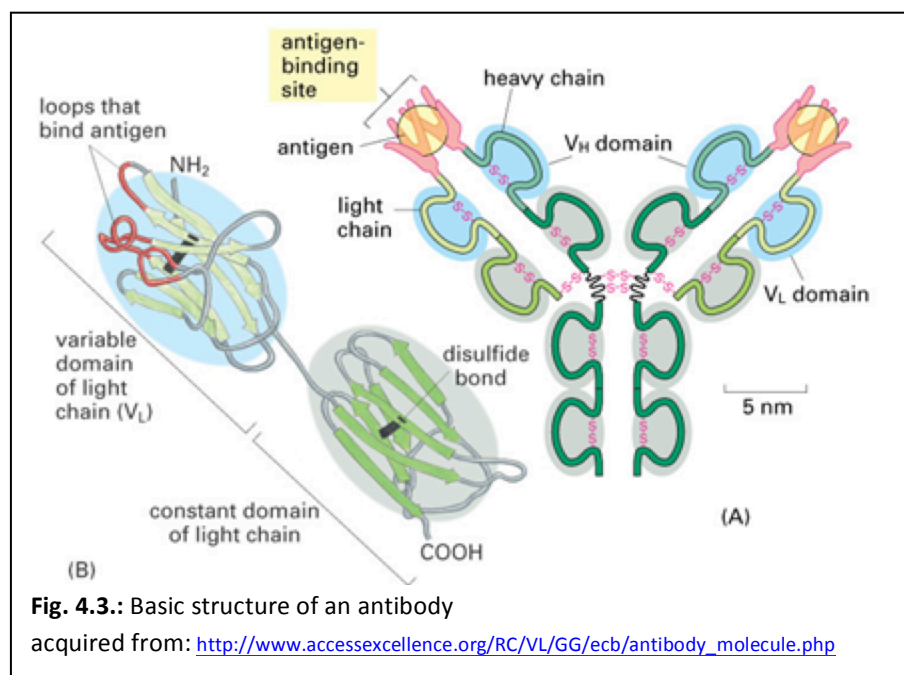

Fig. 4.3.: Basic structure of an antibody
acquired from: http://www.accessexcellence.org/RC/VL/GG/ecb/antibody_molecule.php

27

bridges. The heavy and light chains are composed of a variable and constant region. The variable region bears the antigen-binding site, which will bind to the particular part of the antigen it can recognize, the so-called epitope.

To produce a specific antibody, the antigen is injected into an animal and serum samples are collected. The animal produces a large group of antibodies that recognize independent epitopes on the antigen. Each antibody that recognizes a particular epitope is produced by a different clone of B-cells. By taking the whole serum, so-called polyclonal antibodies, which are against a lot of different epitopes are collected. The other type of antibodies is called monoclonal and recognizes only a single epitope.

Tagging of antibodies and types of immunodetection

Antibodies can be used for the detection of a specific protein. For that, it must be possible to visualize the interaction of protein and antibody. Antibodies can be labeled, most often by a fluorescent tag that emits light upon illumination with a specific wavelength. An enzyme can also be used to catalyze a reaction that results in a colored precipitate or in emission of light when supplied with an appropriate substrate.

For detecting a particular antigen, it is incubated with a monoclonal or polyclonal antibody specific for it. This antibody is called "primary antibody". When the primary antibody is tagged with one of the above mentioned labels, it allows a direct immunodetection. However, in most cases, the primary antibody is not labeled and the antigen is visualized by the use of a secondary antibody, which bears a tag. The secondary antibody is raised against the antibodies from a particular animal and can be used to detect different primary antibodies that were produced by this animal.

The most important advantage of the usage of a secondary antibody is the enhancing of the signal: usually, more than one secondary antibody can bind to a single primary antibody. Hence, the sensitivity of the antigen detection is increased.

4.2.4. <u>Western Blotting</u>
4.2.4.1. Principle

The Western Blot is used for the detection of a specific protein in a hetrogenous sample, such as cell lysates. The general procedure is to first separate the proteins in the sample by running

it on a SDS-PAGE (**S**odium-**D**odecyl-**S**ulfate-**P**oly**A**crylamide **G**el **E**lectrophoresis) gel. Afterwards, the separated proteins are transferred to either a nitrocellulose or PVDF (**P**oli**V**inyli**D**ene **F**luoride) membrane, which is then incubated in a blocking solution. This is done because the membrane has presumably a high binding affinity to proteins and the antibodies, which are later on applied, are nothing else than proteins. Without blocking, antibodies would bind randomly all over the membrane, which would result in a high background noise. Hence, the membrane is incubated in a non-fat milk solution containing a lot of proteins, which will bind to all unoccupied space. Antibodies will then be able to only bind to their respective antigens and not unspecifically all over the membrane. Afterwards, the membrane is probed with a primary antibody against either a single (monoclonal antibody) or multiple (polyclonal antibody) antigens of the protein of interest. After that, the membrane is incubated with a secondary antibody, which is linked to a reporter enzyme. This enzyme in most cases is horseradish peroxidase. To visualize the protein, a mixture of luminol and peroxide is applied on the membrane. The horseradish peroxidase on the secondary antibody then catalyzes the oxidation of luminol by the peroxide, which is a chemiluminescent reaction: The oxidized luminol emits light as it decays to its ground state. This emitted light can then be detected.

If more than one protein has to be visualized from the same sample, PVDF membranes make it possible to strip-off all antibodies from the membrane and repeat the procedure of visualization for another protein.

4.2.4.2. Used Chemicals:

Acrylamide resolving and stacking gel:
30% Acrylamide
1 M Tris, pH 8.8
1M Tris, pH 6.6
10% SDS
10%APS
TEMED

Blotting Buffer :
25 mM Tris

SDS-PAGE loading buffer:
4% SDS
100 mM Tris pH 6
200 mM DTT
0.014% Bromophenol blue
20% Glycerol
ddH$_2$O

Running buffer
25 mM Tris

Chemicals for antibodies:
5% Non-fat milk in PBS
1° Antibodies (see protocol)
PBS-T (PBS with 0.05% Tween-20)
2° Antibodies (see protocol)

Clarity^{TM} Western ECL Substrate kit:
Luminol (unknown conc.)
Peroxide (unknown conc.)

Stripping buffer:
62.5 mM Tris, pH 6.7

192 mM Glycine	192 mM Glycine	100 mM 2-mercaptoethanol
20 % Methanol (v/v)	1% SDS (w/v)	2% SDS (w/v)
1 % SDS (w/v)		MiliQ

4.2.4.3. Used Devices:

Multiple Gel Caster (AA Hoefer)

Gel Electrophoresis Unit SE260 (AA Hoefer)

Mini-PROTEAN® tetra system (Biorad)

ChemiDoc^{TM} MP Imaging System (Biorad)

4.2.4.4. Protocol

4.2.4.4.1.Preparation of sample

1.1. For the preparation of the protein-sample, cells were taken and lysed by incubating them together with SDS-sample buffer at 100 °C for 10 minutes.

4.2.4.4.2.Preparation of a 12% SDS-PAGE resolving gel and a 5% stacking gel:

The chosen concentration of the resolving gel (not the stacking gel) depends on the size of the protein(s) to be visualized. If the size of the proteins is small, the percentage of the resolving gel should be higher and vice versa.

2.1. All parts of the gel casting form (glass slides, comb, etc.) are cleaned well with ethanol and rinsed with dH₂O. The casting form for pouring the gel is assembled and checked for leakage with dH₂O.

2.2. For each 12% acrylamide-gel (with 0.75 mm spacers) 10 mL of a solution containing 12% Acrylamide, 375 mM Tris of pH 8.8 and 0.1% of SDS is made with MiliQ.

2.3. The solution is mixed well. Immediately before pouring 100 μL APS (Ammonium Persulfate) and 8 μL of TEMED are added. The gel is then poured bubble-free into the casting form and immediately overlayered with dH₂O. After about 30 minutes, the gel is polymerized.

2.4. For the stacking-gel, 4 mL of a solution containing 5% Acrylamide, 125 mM Tris of pH 6.8, 0.1% of SDS are prepared with MiliQ.

2.5. The solution is mixed well. Just before pouring, 40 µL of APS and 4 µL TEMED are added. The gel is then poured onto the resolving gel and the comb is immediately inserted. The gel has polymerized after about 20 minutes.

4.2.4.4.3.   Running of the SDS-PAGE gel:

3.1. The buffer for running the gel is prepared (see "running buffer" in "used chemicals") and poured into the electrophoretic apparatus.

3.2. The protein-sample is mixed with the SDS-PAGE loading dye and heated to 95 - 100 °C for 10 minutes. This step disrupts the secondary structure of the protein and coats the polypeptides with an even, negative charge, provided by the SDS.

3.3. The wells of the gel are washed once with running buffer, the samples and a pre-stained standard are loaded and the gel is placed in the electrophoretic apparatus.

3.4. The machine is set to 80 V for the time the proteins are running through the stacking gel (about 5 minutes) and put to 120 V for the rest of the run, which takes about 1 hour.

4.2.4.4.4.   Transfer of proteins to the membrane (Blotting):

3.1. The blotting buffer is prepared and poured into the wet blot apparatus. Two extra thick filter papers and the membrane are cut to the right size.

3.2. The PVDF membrane is incubated in methanol for about 1 minute to activate it

3.3. Special care should be taken that neither the gel nor the membrane are getting dry at any point. Therefore, the used sponges and filter papers are soaked in blotting buffer.

3.4. The gel is placed bubble-free on a wet filter paper. The activated membrane is placed on top. A wet filter-paper is placed on top and by rolling over it with a glass rod all bubbles are removed.

3.5. The filter-paper sandwich is placed in between two wet sponges and this assembly is clamped tightly together in a western-cassette.

3.6. The cassette is placed in the wet-blot-machine in a way that the gel is facing the cathode and the membrane is facing the anode.

3.7. The machine is run at 85 V for 2 hours. It is possible that the voltage drops during the run, so it is necessary to check the voltage from time to time.

4.2.4.4.5.   Visualization of the desired protein:

5.1.   After two hours of blotting, the membrane is taken out. The pre-stained protein marker should be completely transferred to the membrane.

5.2.   The membrane is then blocked in 5% non-fat milk solution for at least one hour or overnight at 4 °C.

5.3.   The appropriate amount of primary antibody (this varies from antibody to antibody) is pipetted to 5% non-fat milk in PBS. The membrane is incubated in this mix for 1 hour.

5.4.   The membrane is washed 3 times with PBST.

5.5.   The appropriate amount of secondary antibody is pipetted to 5% non-fat milk in PBS. The membrane is incubated in this mix for 1 hour.

5.6.   The membrane is washed 3 times with PBST.

5.7.   A double-layered piece of a sheet protector foil, bigger than the membrane, is cut and the membrane is placed in between the sheets, which is open at three sides.

5.8.   The reagents from the Clarity^{TM} Western ECL Substrate kit are mixed in a ratio 1:1. The used volumes depend on the size of the membrane. For small membranes, 250 µL of each component are sufficient. For bigger membranes, up to 500 µL should be used.

5.9.   The mix is spread over the membrane and incubated for one minute.

5.10. Afterwards, the sheet protector foil is closed and the liquid is striped off the membrane.

5.11. Pictures are taken at several, different exposure times. The picture containing the most information (no over- or underexposure) is taken.

4.2.4.4.6.   Stripping the membrane

6.1. The membrane is incubated in stripping buffer at 50 °C for 30 minutes.

6.2. Then, it is washed at least 3 times with PBST.

6.3. The steps from d, Visualization of the desired protein are repeated with the primary antibody of the other protein of interest.

4.2.4.5. Troubleshooting

4.2.4.5.1. Preparation of the SDS-PAGE gel:

If the gel is not polymerizing well, it might be due to a too old APS. The chemical provides sulfate radicals, which start the reaction. Try to prepare a new solution of APS.

4.2.4.5.2. Transfer of proteins to the membrane:

Touching the membrane should be avoided as much as possible. Completely avoided should be bare hands as this would lead to dirty blobs on the membrane from the fat and proteins present on bare skin. The usage of tweezers is highly recommended.

4.3. Localization of MRP1-eYFP, KREL2-eYFP and LSU1-eYFP

Although it theoretically would be possible to detect eYFP directly with a fluorescence microscope, an Indirect Fluorescent Antibody-assay (IFA-assay) was performed to localize the eYFP-tagged proteins. This indirect assay was done to increase the fluorescent signal. For the assay, *T. brucei* containing MRP1-eYFP, KREL2-eYFP and LSU1-eYFP respectively, were incubated with Mitotracker Red to stain the mitochondria. Then, cells were fixed, put on a glass slide, permealized, incubated with a primary antibody against GFP and then with a secondary antibody bearing GFP to visualize the eYFP-tagged proteins. Afterwards, DAPI-stain (4',6-**DiA**midino-2-**P**henyl**I**ndole) applied for the visualization of the nucleus and the kinetoplast (section 4.3.1.3.1).

So-prepared glass slides were then observed with a confocal microscope and pictures from the Mitotracker-, GFP- and DAPI-channel were taken and overlain (See figure 5.7). From this overlay, the locations of the tagged proteins were approximated. To confirm the observations, intensities along a line at the same position in all 3 channels were measured and put together in a distance versus intensity graph. (see sections 4.3.2 and 5.4. for details).

4.3.1. Indirect Fluorescent Antibody-assay (IFA-assay)

4.3.1.1. Principle

In the Indirect Fluorescent Antibody assay, the cells are incubated with a primary antibody against the protein of interest and afterwards with a secondary antibody conjugated to a fluorescent dye. To visualize the protein of interest, so-prepared cells are then observed with a confocal microscope.

4.3.1.2. Used Chemicals (per sample):

1 Superfrost® plus microscope slide from Thermo Scientific

1 x PBS

4% paraformaldehyde in PBS

Mitotracker Red (Invitrogen)

5 % non-fat milk in PBS-T (0.05% Tween 20)

Primary antibody in 5% non-fat milk (concentration 10 times higher than for western blot)

> ➢ The primary antibody used was A6455, anti-GFP, rabbit serum (from life technologies^{TM})

Secondary antibody in 5% non-fat milk

> ➢ The secondary antibody used was Anti-rabbit Alexa Fluor® 488 from Invitrogen.

Mounting media with DAPI-stain (from Invitrogen^{TM})

4.3.1.3. Protocol

4.3.1.3.1. Staining-procedure with Mitotracker Red and preparation of the slides

1. Per sample, 1 mL of cells at the concentration of approximately 10^7 cells are taken. To that, Mitotracker Red is added to get a final concentration of 200 nM. The mixture is incubated at 27 °C for 20 – 30 minutes.

2. The glass slides are prepared by appropriately labeling them and scratching a square in the middle to mark the location of where the cells are placed afterwards.

3. The cells are centrifuged for 60 s at 3000 rpm at room temperature and the supernatant is removed.

4. Then, they are resuspended in 1 mL of 1x PBS for washing.

5. The supernatant is aspirated and the cells are resuspended in 1 mL of 4% paraformaldehyde in an eppendorf tube, for fixing them. The tube is set aside for approximately 20 to 30 minutes.

6. A humid chamber is prepared (This is basically a light-proof box with a sponge inside, which will humidify the air inside the chamber)

7. The paraformaldehyde is removed and the cells are washed once by applying point 4 and 5.

8. The cells are centrifuged again as in step 4 and resuspended in 400 µL of 1 x PBS. One hundred µL of this mixture are applied on the prepared glass slide and incubated in the humid chamber for 10 minutes. This steps allows the cells to attach to the surface of the glass.

9. The cells are permeabilized by either covering them with ice-cold methanol for 20 minutes or with a 0.15% TritonX-100 in PBS solution for about 10 to 20 minutes.

10. The methanol/TritonX-100-solution is removed. For the rest of the protocol, all incubations are done in the humid chamber.

11. The cells are washed by covering the cells with 1 x PBS, letting it stand for about 5 minutes to let loosened cells settle down again and removing the PBS carefully.

12. Step 12 is repeated once more and after that, the slides are incubated in 5% non-fat milk in 1x PBST (0.05% Tween-20) for 45-60 minutes for blocking.

13. The milk is removed and the cells are washed by applying step 12.

14. On each slide, 250 µL of 5% non-fat milk in PBST containing the primary antibody at the desired concentration are added.

15. The slides are incubated overnight at 4 ^{o}C in the humid box.

16. The next day, the milk with the primary antibody is removed and the slides are washed three times by applying step 12.

17. The secondary antibody is prepared at the desired concentration in 5.0% non-fat milk in 1xPBS-T (0.05% Tween-20). On each slide, 250 µL of this mixture are applied and incubated for 1 hour at room temperature. From now on, it is recommended to expose the slides as few as possible to sunlight (as it can excite the chromophore at the secondary antibody, which will shorten its lifetime.)

18. The milk-mixture is removed and the slides are washed 3 times by applying point 12.

19. Finally, 1 drop of mounting media with DAPI-stain is applied to each slide and a coverslide is carefully placed on the drop.

20. The slide can be kept in the dark up to a week at 4 °C.

As there is a special training needed before working with the confocal microscope, pictures were taken by my skolitel specialista Zhenqiu Huang.

4.3.2. <u>Distance-Intensity graphs for IFA pictures</u>

4.3.2.1. Principle

The co-localization of proteins by only looking at merged IFA-pictures can be quite subjective as it is difficult for the human eye to see very small differences in variations of color shades. To overcome this problem, pixel-intensities of Mitotracker- and GFP-channels are measured along a line at the same position in the respective channels. Afterwards, the measured intensity-values are plotted versus the distance of the line. The resulting graph shows the quantified co-localization of Mitotracker and GFP.

4.3.2.2. Used Programmes

Fiji (Fiji Is Just ImageJ) (See Reference [28])

Excel (Microsoft)

4.3.2.3. Protocol

For a better understanding, a theoretical example was prepared: In this example, the mitochondria of a self-drawn cell was colored red and called "Mitotracker-channel" (see figure 4.4, left panel). The distribution of a protein was drawn in green and called "GFP-channel". (see figure 4.4, middle panel).

1. For measuring co-localization by means of a distance-intensity graph, one picture of each channel is needed and an additional one, in which all channels are merged:
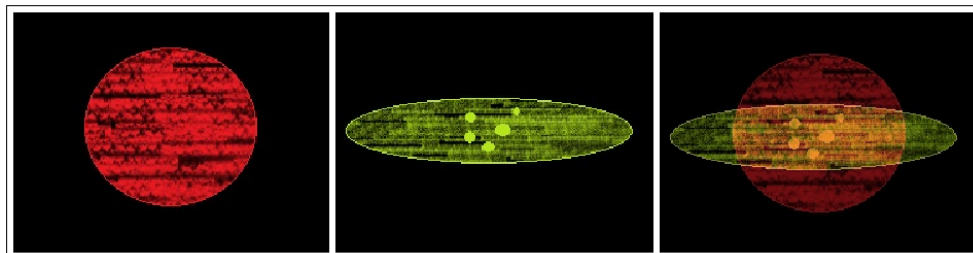
Fig. 4.4.: Theoretical cell for demonstration of the distance-intensity graph; *left:* mitochondria in Mitotracker-channel; *middle:* visualized protein in GFP-channel; *right:* Overlay of Mitotracker and GFP-channel

2. The Fiji program is started and all necessary pictures are opened.

3. A stack is made out of all pictures (Image → Stacks → Images to Stack)

4. The straight-line selection tool is chosen and a line is drawn in the merged picture over which pixel intensity will be measured in all channels.

5. At the bottom of the stacks is a scrollbar, which enables switching between all the single pictures of the stack. The line, which was drawn in the merged picture, is situated at the same coordinates in every picture of the stack. By scrolling though the pictures, the line can be adjusted if needed. Adjusting the line alters it in all the stacks.
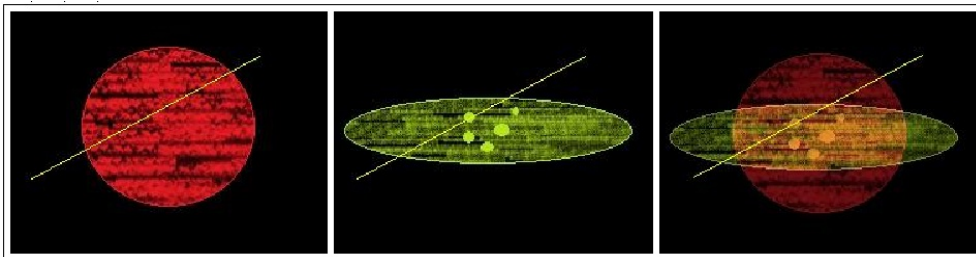

Fig. 4.5.: Theoretical cell including line for distance-intensity graph; *left:* mitochondria in Mitotracker-channel; *middle:* visualized protein in GFP-channel; *right:* Overlay of Mitotracker and GFP-channel

6. For each picture of the stack (except the merged one), the following is done:

7. Analyze → Plot profile (or shortcut: Ctrl + K). This gives a graph with grey value versus distance.

8. To get the raw data, the "List"-button is pressed. The X-values represent the distance on the line and the Y-values the corresponding intensity.

    8.1. Excel is opened and the values are copy-pasted.

9. With the data, a graph is made in Excel (See figure 4.6).

10. To mark the line from which the intensities were measured, the following is done:

    10.1.1. Edit → Draw (or Ctrl + D) (See figure 4.5 for resulting line)

11. The Images are now removed from the stack (Image → Stacks → Stacks to images) and the pictures containing the lines are saved.

4.3.2.4. Interpretation of the result

The measured intensities can only be interpreted relative to each other.
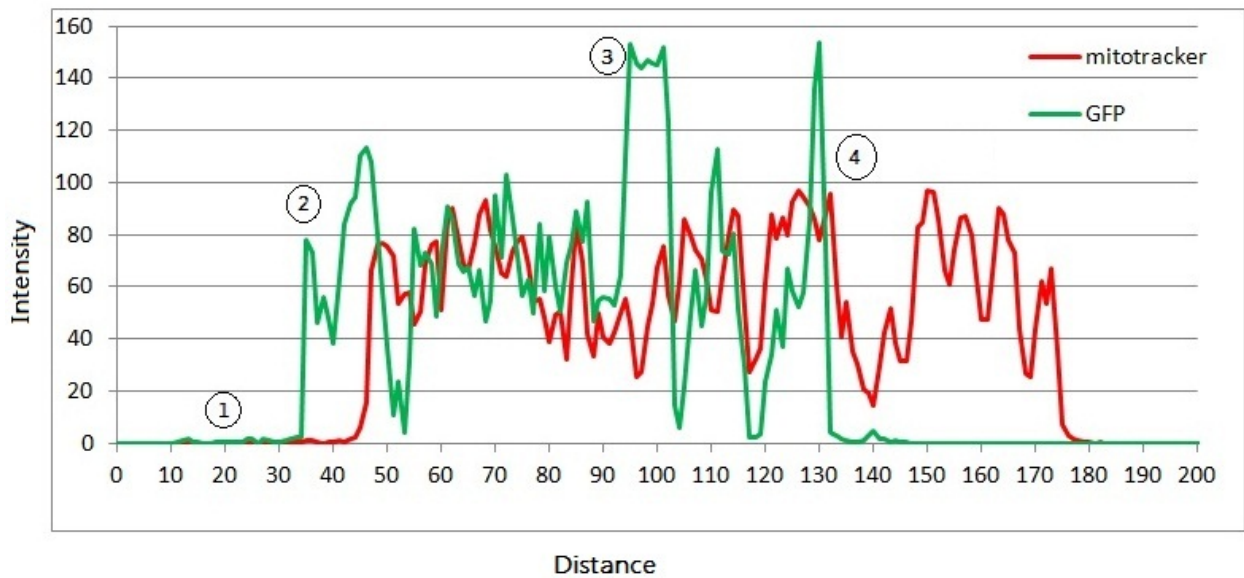
Fig. 4.6.: Distance-Intensity graph for the theoretical cell over the line shown in figure 4.5. Numbers are explained in the text directly below.

From the graph of the theoretical cell, the following can be interpreted: (Numbers below correspond to the numbers in figure 4.6.)

1. Neither GFP nor the Mitotracker show any signal. Small changes in the line are due to background-noise.

2. A signal of GFP, but no signal of the Mitotracker can be observed. The GFP-linked protein seems to be present outside the mitochondria.

   In between point 2 and 4: Both of the two channels show a signal: The protein is also located inside the mitochondria.

3. A high spike in the GFP-channel in comparison to the Mitotracker-channel. It seems that the protein is concentrated there in a foci.

4. The GFP-channel abruptly goes to zero but the Mitotracker channel still shows a signal.

   In conclusion, the protein is distributed inside as well as outside of the mitochondria. However, as the GFP- and Mitotracker-line are not perfectly in proportion to each other, one conclusion is that the distribution of the protein is not homogeneous throughout the mitochondria.

4.4. Proof of functionality of MRP1-eYFP

To confirm that the eYFP-tagged protein MRP1 is still functional and hence able to form the same complex as the endogeneous protein, a co-immunoprecipitation was performed. (see following chapter).

4.4.1. <u>Co-Immunoprecipitation (CIP) with Protein G Dynabeads</u>
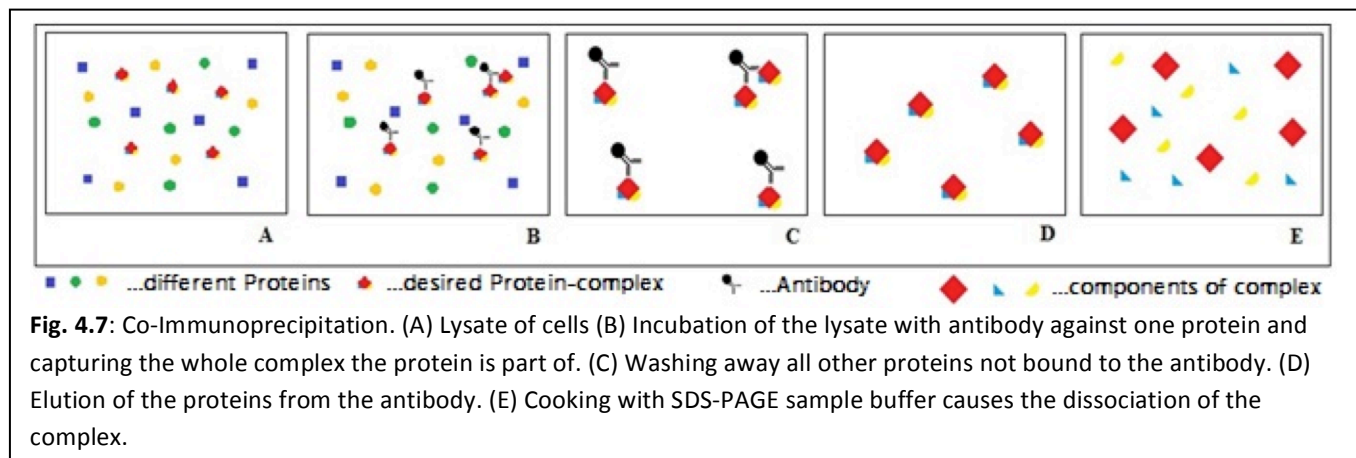
4.4.1.1. Principle

Immunoprecipitation is a technique that allows the purification of a protein of interest. Dynabeads (Invitrogen) are polymer-coated magnetic particles that allow a quick separation from a liquid or suspension by applying a magnetic field from a magnet. The ones used in this experiment are coupled to protein G, which enables an antibody against the protein of interest to attach to it. The antibody will then couple to the desired protein and captures it. All the proteins which are not attached to the beads are then washed away. In the end, only the protein of interest will be left over.

As the antibody is not covalently bound to the protein, a denaturing buffer can be used to elute the protein from the Dynabeads. The eluate can then be analyzed by western blotting.

Co-Immunoprecipitation (see figure 4.7) is in general the same as immunoprecipitation but is used to indirectly capture proteins that are bound to a specific target protein. Hence, not only one protein will be captured by the beads but a whole protein-complex. One requirement for catching a whole complex is not to disrupt the protein-protein interactions during the process. Therefore, the used lysis and washing buffers are key factors for a successful co-immunoprecipitation. To maintain the native state of the proteins as good as possible, empirical testing might be necessary to determine the best composition of the buffers for a specific complex of interest.

In the final step, when preparing the sample for SDS-PAGE electrophoresis, heating with SDS in the sample buffer causes the dissociation of interactions in the complex. The individual proteins of the complex can then be identified by western blotting.

Fig. 4.7: Co-Immunoprecipitation. (A) Lysate of cells (B) Incubation of the lysate with antibody against one protein and capturing the whole complex the protein is part of. (C) Washing away all other proteins not bound to the antibody. (D) Elution of the proteins from the antibody. (E) Cooking with SDS-PAGE sample buffer causes the dissociation of the complex.

4.4.1.2. Used Chemicals

Lysis-Buffer for 50 mL

20 mM Tris-HCl

137 mM NaCl

0.1% NP-40 (IGEPAL)

2 mM EDTA

Glycine-Elution-Buffer

0.1 M Glycine

pH adjusted to 2.6 with HCl

Washing-Buffer

10 mM Tris, pH 7.4

1   mM EDTA

1 mM EGTA

150 mM NaCl

0.1%   Triton X-100

SDS-PAGE loading buffer (2x):

see recipe in the chapter western blotting.

Other reagents used:

1 x PBS

PBST: 1x PBS + 0.02% Tween 20

PBSG: 1x PBS with 1% glucose

Protease-inhibitor cocktail:

Roche, cOmplete ULTRA Tablets, Mini, EDTA-free, EASY pack; prepared according to instructions.

Phosphatase-inhibitor:
0.2 mM Sodium-ortho-Vanadate

4.4.1.3. Used Devices:

Eppendorf Centrifuge 5810 R for 15 mL falcons or 50 mL falcons

Eppendorf Centrifuge 5424 R for 1.5 mL eppendorf tubes

4.4.1.4. Protocol

4.4.1.4.1.   Dynabead preparation

1.1. The beads are resuspended in the vial by rotation for 5 minutes (alternatively, it can be vortexed shortly)

1.2. Of this suspension, 50 μL are pipetted into a microfuge tube.

1.3. The beads are sedimented by placing them into a magnetic stand for 1 minute.

1.4. The supernatant is aspirated without touching the beads.

1.5. The beads are resuspended in 500 µL PBS.

1.6. Then, the beads are washed three times by repeating steps 1.5, 1.3 and 1.4.The last step is to resuspend them in 200 µL PBST.

1.7. To the 200 µL of PBST, 10 µg of the antibody are added. (This amount can range from 1-10 µg or more).

> In the experiment, 5 µg of anti-GFP mouse, isotype IgG 2a, monoclonal 3EG (Invitrogen) were used per vial.

1.8. The tubes are rotated for 30 minutes at 4 °C.

1.9. The beads are washed once with PBS by applying steps 1.3, 1.4 and 1.5.

4.4.1.4.2. Harvesting cells

2.7. The amount of cells depends on the expression level of the desired protein. For a start, 10^8 to $2*10^8$ procyclic trypanosome cells can be tried, which corresponds to approximately 10 mL of cells in the log phase. For bloodform *T. brucei*, the volume is usually about 10 times higher as they are growing at a lower density.

2.8. For the experiment, 10 mL of procyclic *T. brucei* at a density of about $1.5 * 10^7$ cells were used per sample.

2.9. The desired protein was the GFP-tagged MRP1. As a negative control served the procyclic parental cell-line 427 from which the MRP1-eYFP cell line is derived.

2.10.   The cells are centrifuged for 10 minutes at 1300 g. The supernatant is discarded.

2.11.   Resuspend in 1 mL of ice-cold PBSG and transferred to a 1.5 mL eppendorf tube.

2.12.   The cells are centrifuged for 10 minutes at 1300 g at 4 °C and the supernatant is discarded.

4.4.1.4.3.   Cell lysis

3.1. Always performed on ice.

3.2. One mL of the lysis buffer is pipetted per sample into a separate flask and to that, protease/phosphatase inhibitors are added. This step should be done only shortly before the experiment. The lysis buffer is put on ice.

3.3. The cell pellet is resuspended in 1 mL of ice-cold lysis buffer.

3.4. The eppendorf tubes are kept on constant agitation for 30 minutes at 4 °C.

3.5. The tubes are centrifuged at 10 000 g at 4 °C for 15 minutes.

3.6. Twenty µL of the lysate (the supernatant above the cell pellet) are taken. This is the "IP INPUT" for the Western blot afterwards.

3.7. The rest of the supernatant is transferred to the tube with the prepared beads.

3.8. The pellet is discarded.

4.4.1.4.4.   *Immunoprecipitation*

4.1. The beads are incubated overnight with the cell lysate at 4 °C and rotating.

4.2. The next day, the beads are sedimented and the supernatant is collected as "**IP supernatant**" for the Western.

4.3. Per sample, 900 µL are pipetted into a separate flask and to that, the appropriate amount of protease/phosphatase inhibitors are added. This step should be done only shortly before usage. The buffer is then put on ice.

4.4. The beads are washed three times by sedimenting the beads, aspirating the supernatant and resuspending them in 300 µL of washing buffer.

4.5. From the washing buffer, as much as possible is aspirated from the beads.

4.4.1.4.5.   *Elution with the Glycine-Buffer:*

5.1. The beads are eluted three times with 150 µL of 0.2 M glycine pH 2.6 (1:1) by incubating the sample for 10 minutes with frequent agitation before sedimenting the beads and transferring the supernatant to a new eppendorf tube.

5.2. The eluate is pooled and neutralized by adding an equal volume of Tris pH 8.0.

5.3. The beads are washed two times with PBS and can be reused after removal of the glycine buffer.

5.4. The eluate is run in the Western as "**IP"**.

4.4.1.5. Troubleshooting

1. It should be kept in mind that the used antibodies for capturing the protein are present in the final eluate. Hence, for western blotting, the light and heavy chain of the primary antibody will be present at 25 and 50 kDa at the membrane. To avoid the unwanted detection of the light and heavy chain, the primary antibodies can be directly cross-linked to the Dynabeads. Another approach to solve this problem is to use antibodies from one animal for pulling down the protein of interest and afterwards primary antibodies from another animal to detect the protein on the western membrane. The latter approach was used in this experiment.

2. If a lot of proteins are lost during the process (comparison of input and eluted protein), all liquids, which were in contact with the Dynabeads during the protocol should be kept and run on a SDS-PAGE gel followed by a western blot to investigate, at which exact step the loss happens. The protocol can then be adjusted accordingly.

3. Optimization for the used lysis and washing buffers can be achieved by varying the following concentrations:

   - Salts: 0 – 1 M
   - Non-ionic detergents: 0.1 – 2.0%
   - EDTA: 0 – 5 mM
   - Divalent cations: 0 – 10 mM
   - pH: 6 – 9

4. If the protein is present in the supernatant after the incubation of the lysate with the Dynabeads (after step 4.1), either the amount of antibody on the beads or the amount of beads itself should be increased. If all the protein is found in the supernatant and none binds to the Dynabeads, the antibody is most likely problematic. If a monoclonal antibody was used, the corresponding epitope was unavailable due to a conformational change of the protein. So either the composition of the lysis buffer should be changed or another monoclonal or polyclonal antibody could be used instead.
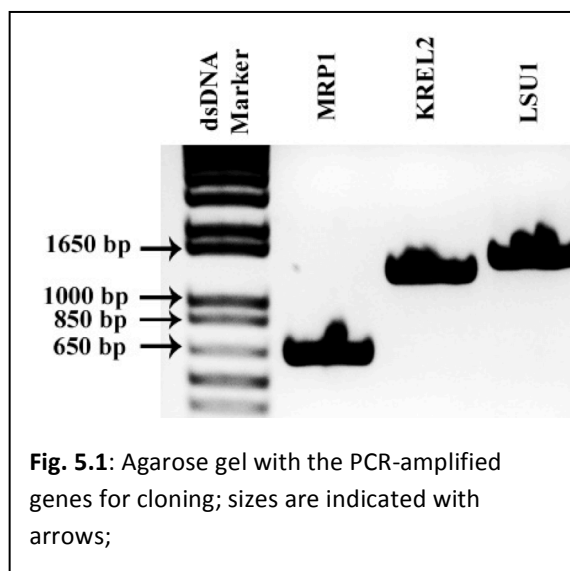
5. RESULTS AND DISCUSSION

5.1. Cloning of MRP1, KREL2 and LSU1 into p2937, transfection in *E. coli*

5.1.1. <u>Amplification of the genes by PCR</u>

In the agarose gel below (see figure 5.1), the successfully amplified PCR-products of the open reading frames of MRP1, KREL2 and LSU1 can be observed. As the GeneArt-technique works by homologous recombination, the primers contain 5' overhangs that provide the amplicons with the required homology to the sequence of the vector (see chapter Materials and Methods, section 4.1.1). The base-pair lengths of the respective genes are given in table 5.1. The numbers in brackets give the expected lengths of the bands, which include the added homology-basepairs.

Tab. 5.1: Expected length of MRP1, KREL2 and LSU1 Numbers in brackets include the length of the gene including the added base-pairs needed for GeneArt-mediated cloning.
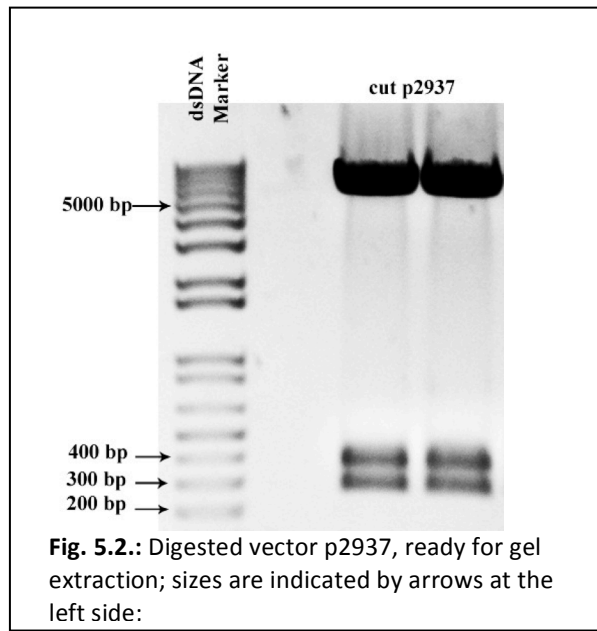
Gene	Length in bps
MRP1	621 (651)
KREL2	1251 (1281)
LSU1	1422 (1451)

Fig. 5.1: Agarose gel with the PCR-amplified genes for cloning; sizes are indicated with arrows;

As the DNA-bands in the agarose gel are at their respective correct sizes, it is most likely that the PCR-amplification resulted in the expected products.
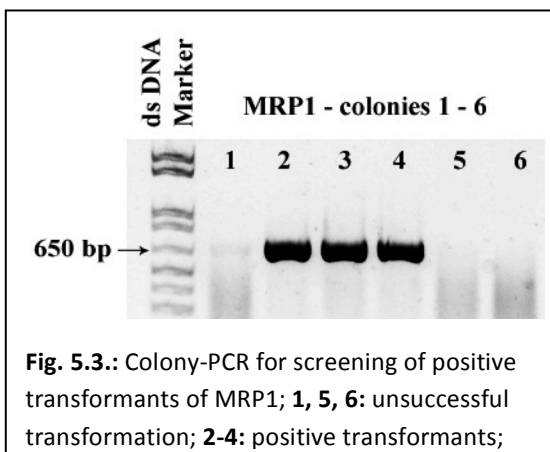
5.1.2. <u>Linearization and purification of the plasmid p2937</u>

After production of the plasmid p2937 in *E. coli*, it was extracted by the miniprep method as described in "Materials and Methods". Following this procedure, the plasmid was digested with the enzymes Acc651 and BamHI (see section 4.1.5). As the enzyme Acc651 cuts 2 times and BamHI cuts once in the sequence of the plasmid, the plasmid should theoretically be cut into 3 pieces. The theoretical sizes of these pieces are 270, 365 and around 6000 bps. As can be seen in the agarose gel in figure 5.2, the DNA-bands exhibit the correct sizes. The DNA from the band with about 6000 bps was then recovered by gel extraction as described in chapter 4.1.4.

Fig. 5.2.: Digested vector p2937, ready for gel extraction; sizes are indicated by arrows at the left side:

5.1.3. <u>Identification of positively transformed bacteria</u>

After the PCR-amplification of MRP1, KREL2 and LSU1 with the 5' overhang-primers and the restriction digest of the vector p2937, GeneArt-mediated cloning was performed as described in the chapter "Materials and Methods" to clone the PCR-products into the vector p2937. Following that, the obtained vectors were transfected into competent *E. coli* XL-blue 100 cells, which were then grown on agar plates containing ampicillin as the selective drug. The resulting colonies were then screened for positive transformants by colony-PCR. An example for the result of this colony-PCR, resolved on an agarose gel, can be seen below in figure 5.3.

Fig. 5.3.: Colony-PCR for screening of positive transformants of MRP1; **1, 5, 6:** unsuccessful transformation; **2-4:** positive transformants;

After this first screening, positive colonies were transferred to liquid LB-media containing ampicillin, grown for 12 hours and the plasmids were extracted by the miniprep method. Next, the isolated plasmids were digested to identify clones harboring the right plasmid with insert. The expected sizes of the respective cut plasmids can be observed in table 5.2. The agarose gels seen in figures 5.4 and 5.4 show that the constructs do bear the right inserts. To finally check whether the inserts did not get altered by any errors introduced by the DNA polymerase during PCR, the plasmids were then sent for sequencing. The result of the sequencing (data not shown) confirmed that the error-free MRP1, KREL2 and LSU1 ORFs were incorporated into the plasmid p2937.
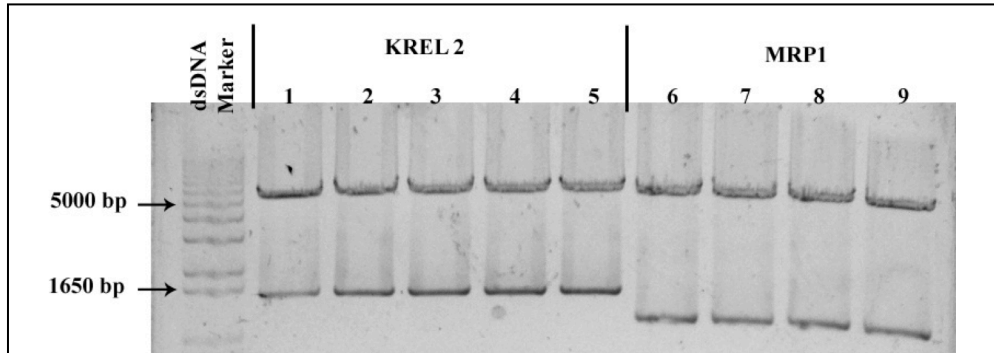
Fig. 5.4.: Restriction digestion to check for positive transformants; **1-5:** digested KREL2; **6-8:** digested MRP1;
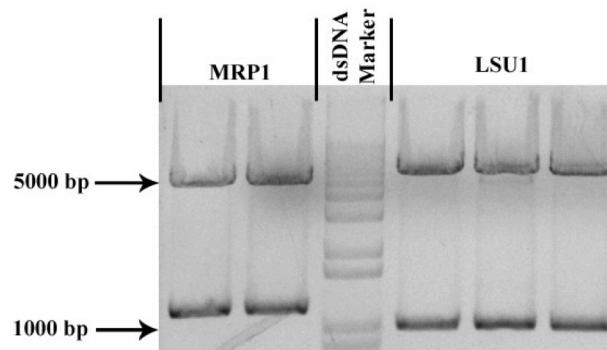
Fig. 5.5.: Restriction digestion to check for positive transformants; left of dsDNA Marker: digested MRP1; right to dsDNA Marker: digested LSU1

Tab. 5.2.: Used Restriction enzymes and expected sizes of fragments for the restriction digestion of MRP1, KREL2 and LSU1 bearing constructs for positive transformants.

Gene	Restriction Enzyme(s)	Band 1	Band 2
MRP1	Hind III	5400	1537
KREL2	BamHI + EcoRI	5200	1155
LSU1	EcoRI	6200	998

5.2. Electroporation and confirmation of successful incorporation of MRP1, KREL2 and LSU1 by a western blot

Procyclic *T. brucei* were then transfected with the respective plasmids via electroporation as described in section 4.2.1. Three different drug-resistant clones of each of the MRP1, KREL2 and LSU1 electroporations were taken. To confirm the presence of the tagged MRP1, KREL2 and LSU1 proteins in the cells, a western blot was performed. To visualize the proteins on the membrane, a primary antibody against the GFP-tag was used. As GFP differs from eYFP only at the chromophore [27], which is situated in the center of the molecule, the antibody is able to recognize eYFP. The western blot was then
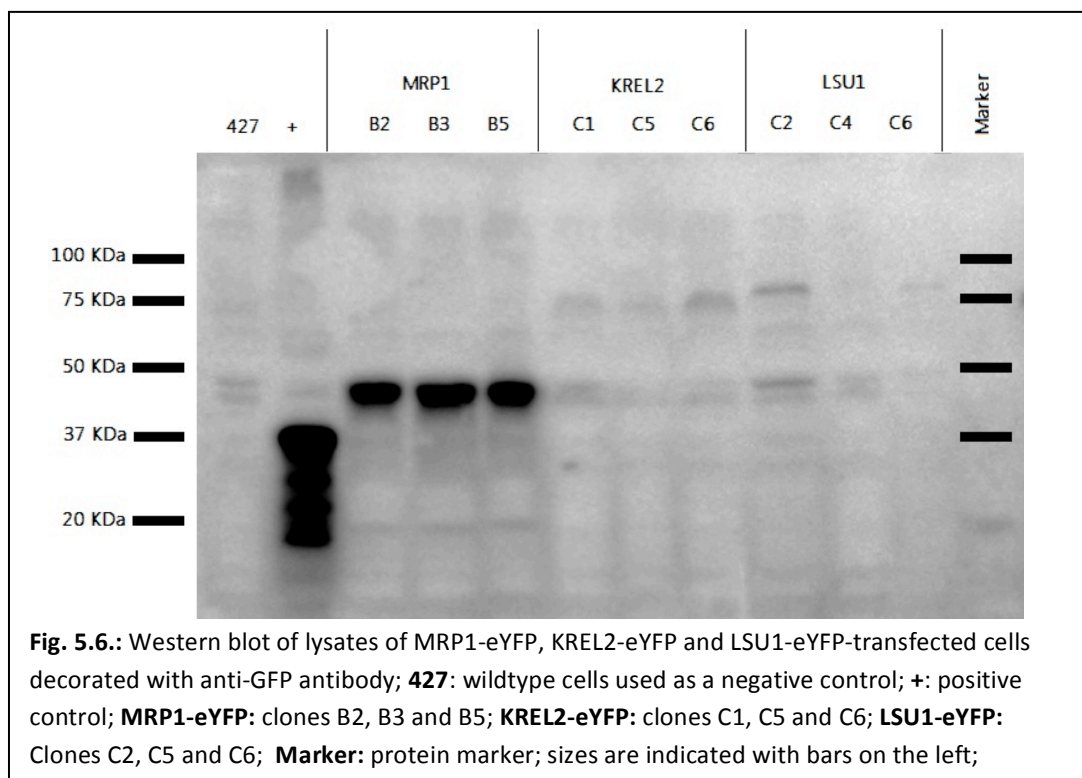
performed as described in section 4.2.4. The expected sizes of the bands can be observed in the table 5.3 and a picture of the membrane can be seen in figure 5.6.

Table 5.3.: Expected sizes of eYFP and the worked-on proteins coupled to eYFP

Protein	Expected size
eYFP alone	27 kDa
MRP1-eYFP	50 kDa
KREL2-eYFP	74 kDa
LSU1-eYFP	82 kDa

The intensity of the observed bands are directly linked to the expression levels of the respective protein. The expression levels of KREL2-eYFP and LSU1-eYFP are considerably weaker than the expression level of MRP1-eYFP.


Fig. 5.6.: Western blot of lysates of MRP1-eYFP, KREL2-eYFP and LSU1-eYFP-transfected cells decorated with anti-GFP antibody; **427**: wildtype cells used as a negative control; **+**: positive control; **MRP1-eYFP:** clones B2, B3 and B5; **KREL2-eYFP:** clones C1, C5 and C6; **LSU1-eYFP:** Clones C2, C5 and C6; **Marker:** protein marker; sizes are indicated with bars on the left;

5.3. Localization of MRP1-eYFP, KREL2-eYFP and LSU1-eYFP with IFA

To localize the eYFP-linked proteins MRP1, KREL2 and LSU1, cells expressing the respective proteins and parental line cells as a negative control were taken and IFA (see section 4.3.1) was performed.

During the process, the DNA of the cells was stained with DAPI (see figure. 5.7, column A). For each cell, two blue spots can be observed: The bigger one is the nuclear DNA and the smaller one is the kDNA in

the mitochondria. Some cells may contain a third small blue circle. Those cells were in the process of dividing. As the kinetoplast divides first in the cell cycle of T. brucei [26], the third blue circle corresponds to a second kDNA in the cell. To see whether the proteins localize to the mitochondria, the cells were stained with Mitotracker Red (see figure 5.7, Column B). For the localization itself, the proteins were tagged with primary antibodies against GFP and a secondary antibody bearing Alexa Fluor® 488, which has an emission wavelength of 488 nm that results in emission of green light (see figure 5.7, Column C). As expected, the parental cell lines, from which the ones expressing the tagged proteins are derived, do not show any fluorescence. Co-localization of the respective protein, the mitochondria and DNA was observed by merging the pictures of the respective channels together (see figure 5.7, Column D). As an overlay of the red Mitotracker and the green GFP results in a yellow color, all three proteins seem to localize to the mitochondria. To confirm this finding and also address the distribution of these tagged proteins in the cell, distance-intensity line scans were performed (see next subchapter).
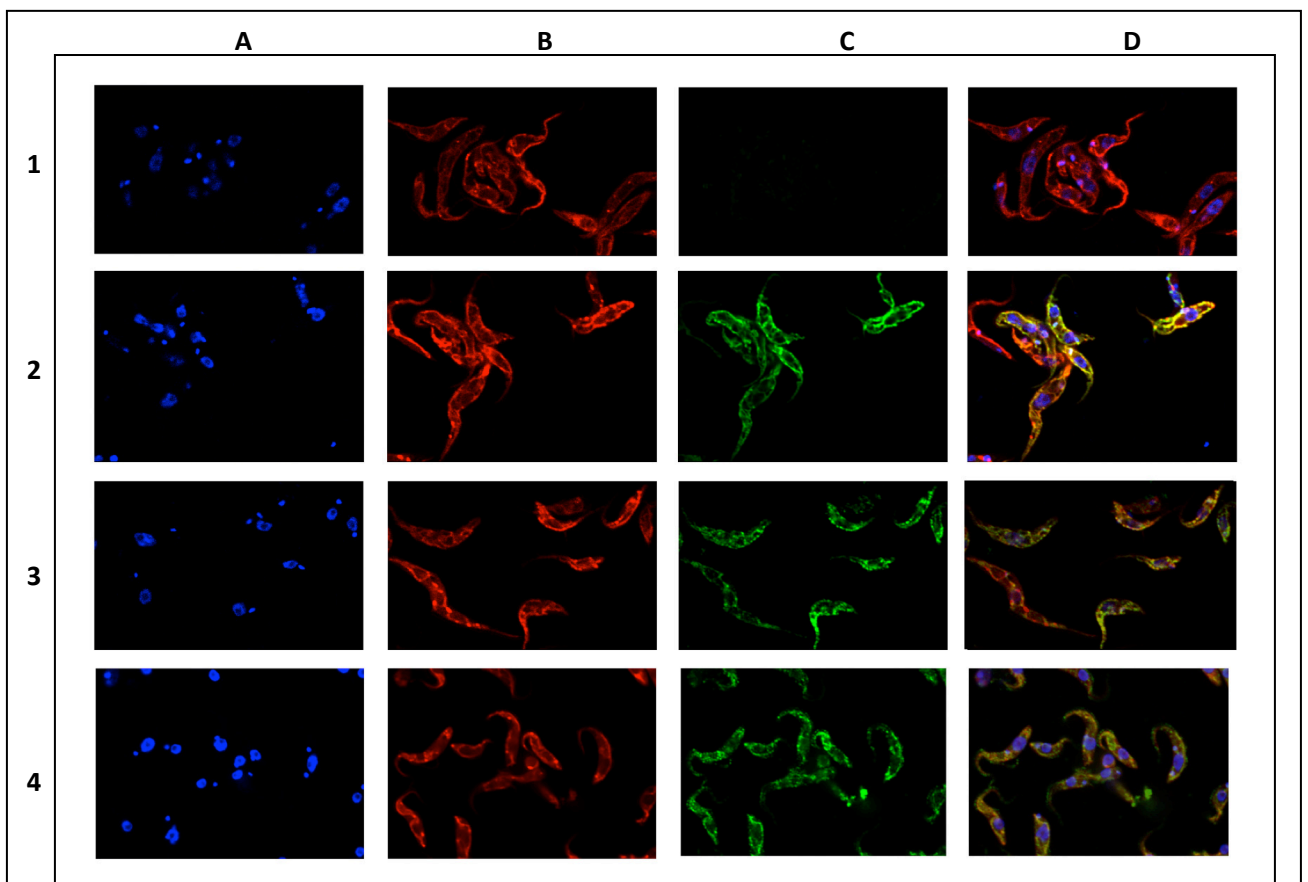
Fig. 5.7.: IFA-pictures**; Row 1:** parental cell line as a negative control; **Row 2:** MRP1-eYFP transfected cells; **Row 3:** KREL2-eYFP transfected cells; **Row 4:** LSU1-eYFP transfected cells; **Column A:** DAPI-stained DNA; **Column B:** Mitotracker-stained mitochondria; **Column C:** respective, eYFP-tagged protein visualized with GFP secondary antibody; **Column D:** merged pictures from Column A, B and C;

The intensity of the YFP-channels confirm the data from the western blots: The expression levels of KREL2 and LSU1 are weaker in comparison to MRP1-eYFP. As future experiments require a relatively high expression level of the protein to be studied, the proteins KREL2 and LSU1 were not investigated further.
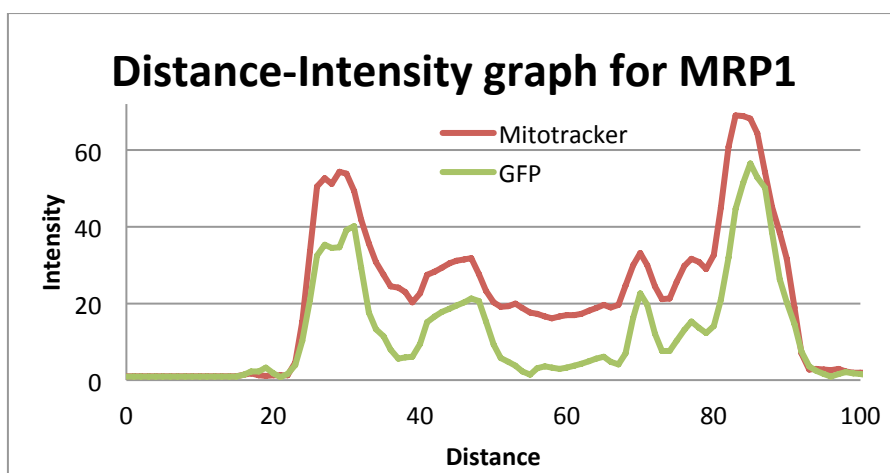
5.4. Distance - Intensity -graphs

As described in chapter 4.3.2, pictures from the IFA-assay were overlain in a stack with the Fiji program [28]. The intensities of Mitotracker and GFP were measured along a line scan. The resulting data was copied to Excel, in which a diagram with distance versus intensity was drawn.
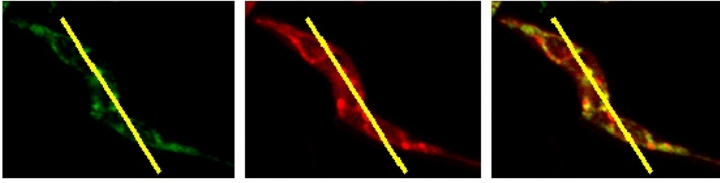
5.4.1. <u>MRP1-eYFP</u>

Fig. 5.8.: Fluorescence image IFA from MRP1-eYFP; yellow bars indicate the line scan over which intensity was measured; *left*: localization of MRP1; *middle:* Mitotracker; *right:*overlay of   GFP, Mitotracker and DAPI;

Fig. 5.9.: Distance-Intensity graph for MRP1; *Green line*: measured intensity of GFP-channel;
Red line: measured intensity of Mitotracker;

As can be seen in figure 5.9, there is no signal of GFP outside the Mitotracker-signal. Hence, the protein is located within the mitochondria. An additional observation is that when the intensity of the Mitotracker rises or falls, the intensity of the GFP rises and falls in the same way. From this, it can be concluded that the protein is evenly distributed throughout the mitochondria.

5.4.2. __KREL2-eYFP__

Fig. 5.10: Fluorescence image IFA from KREL2-eYFP; yellow bars indicate line scan over which intensity was measured; *left*: localization of MRP1; *middle:* Mitotracker; *right:*overlay of   GFP, Mitotracker and DAPI;
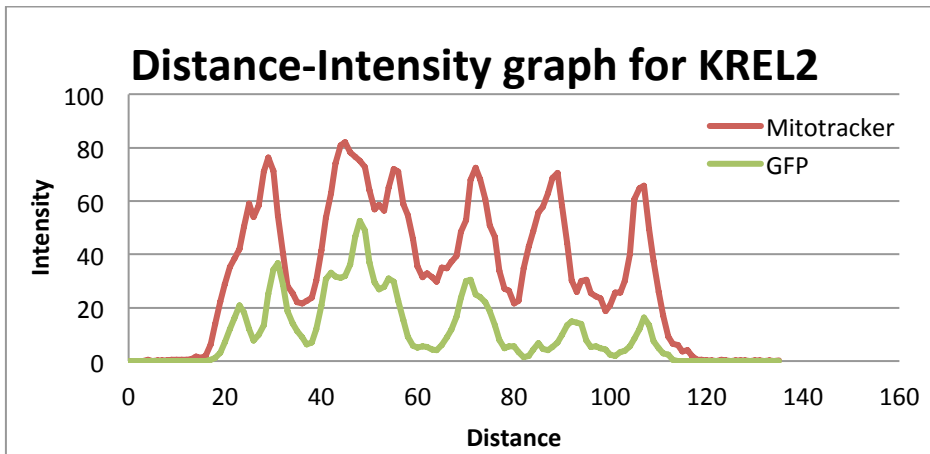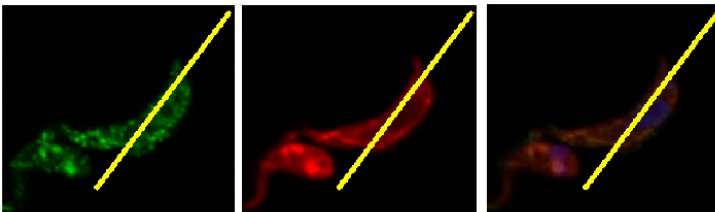

Fig. 5.11: Distance-Intensity graph for KREL2; *Green line*: measured intensity of GFP-channel; *Red line*: measured intensity of Mitotracker;

The distribution of KREL2 is similar to MRP1 (See figure 5.11). The protein is also located within the mitochondria, as no signal of GFP can be observed outside of the signal of the Mitotracker. By comparing the intensities of GFP and Mitotracker relative to each other, it can be concluded that this protein is as well distributed evenly throughout the mitochondria.

5.4.3. __LSU1-eYFP__

Fig. 5.12: Fluorescence image IFA from MRP1-eYFP; yellow bars indicate line, over which the intensity was measured; *left*: localization of MRP1; *middle:* Mitotracker; *right:*overlay of   GFP, Mitotracker and DAPI;
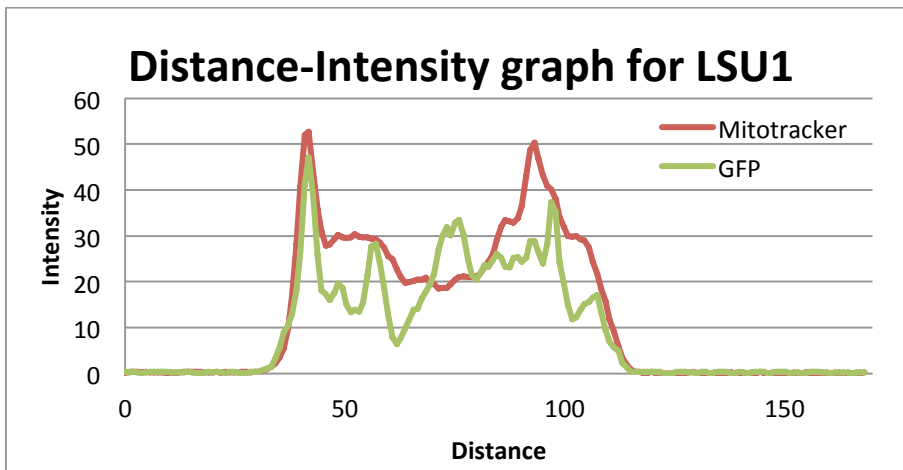
Fig. 5.13.: Distance-Intensity graph for LSU1; *Green line*: measured intensity of GFP-channel; *Red line*: measured intensity of Mitotracker;

The protein LSU1 is also situated within the mitochondria, as no GFP-signal can be observed outside of the signal of the Mitotracker (See figure 5.13). However, when comparing the intensities of the GFP and the Mitotracker signals, there are significant differences between the rise and fall of their respective intensities. Hence, this protein is not evenly distributed within the mitochondria.

5.5. Confirmation of the functionality of MRP1-eYFP by co-immunoprecipitation

The function of the MRP1 protein may be affected by the C-terminal eYFP tag. For example, the tagged form could be misfolded, hence affecting the protein's function. Secondly, as eYFP has approximately the same size as MRP1, the additional bulk could affect the accessibility of the protein to interacting partners.

The endogeneous version of MRP1 forms a complex together with the protein MRP2. In the complex, two subunits of each MRP1 and 2 build a tetramer [19]. We assume that the function of MRP1 is still maintained if the tagged version incorporates into the complex as well. To test this hypothesis, immunoprecipitation of the MRP1-eYFP was performed as described in "Materials and Methods". The antibody for pulling down the presumptive complex was against GFP, thus targeting only eYFP-MRP1. As a negative control, the parental cell line from which MRP1-eYFP were used. As these cells do not contain an eYFP-tagged protein, the eluate from this sample should not contain any co-immunoprecipitated proteins.

The eluate from the Co-IP was then run on a 12% SDS-PAGE gel and western blotting was performed. To see whether the MRP1-eYFP protein was able to incorporate into the MRP1/2 complex, the membrane was first incubated with a primary antibody against MRP1 and further developed as described in section 4.2.4. In figure 5.14, two bands can be seen in the lane "IP, MRP1-eYFP": A 50 kDa band corresponding to MRP1-eYFP and a 25 kDa band corresponding to the endogeneous MRP1. Hence, the eYFP linked MRP1 can still bind endogenous MRP1-proteins. As there is no band in the negative control (figure 5.14, "IP parental line"), the observed bands are not due to some unspecific binding to the Dynabeads or the antibody.

The western blot also makes clear that the immunoprecipitation was efficient: In figure 5.14, the supernatant after the incubation of the lysate with the Dynabeads can be seen. It shows all proteins that are not bound by the antibodies. As there is no band at the size of eYFP-MRP1, all of this protein was pulled down by the immunoprecipitation. In the lanes of the three washing steps of the Dynabeads is also no band. Hence, no protein was lost during the washing-steps.

In the lane of the Input of MRP1-eYFP, the lysate itself can be observed. Bands at 25 and 50 kDa show the endogeneous and eYFP bound MRP1, respectively. A band slightly above the endogeneous MRP1
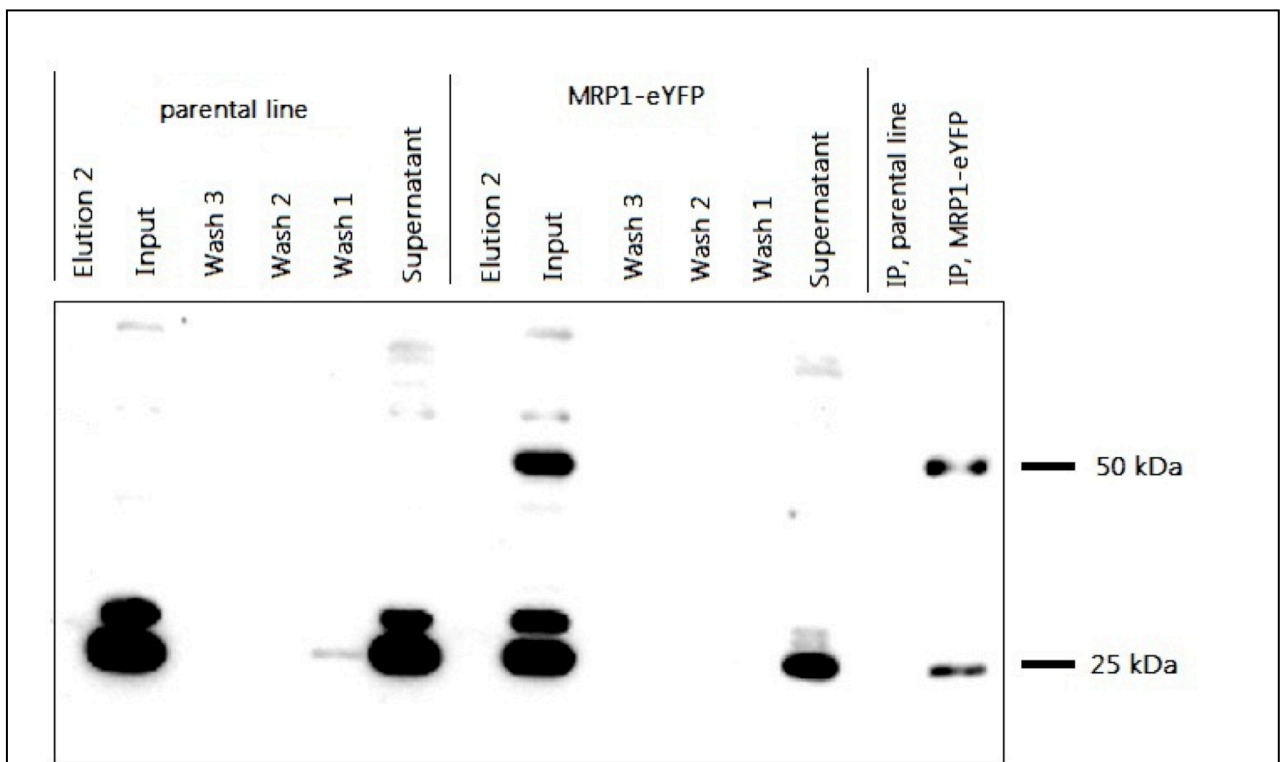
Fig. 5.14.: Western blot of membrane incubated with 1° AB against MRP1; sizes indicated with bars on the right;
IP, MRP1-eYFP: Eluate from the IP; **IP, parental line:** Eluate from the negative control containing only endogeneous MRP1; **Supernatant:** Supernatant above Dynabeads after incubation with the lysate; **Wash 1-3:** Wash solution removed from Dynabeads after these steps; **Input:** Pure lysate before immunoprecipitation; **Elution 2:** Second elution of the beads with PBS and long elution time;

might be due to an unspecific binding of the MRP1 antibody.

To ensure that the whole MRP1/MRP2 complex was able to form, the membrane was stripped as described in the "Materials and Methods" section. Afterwards, the membrane was incubated with the
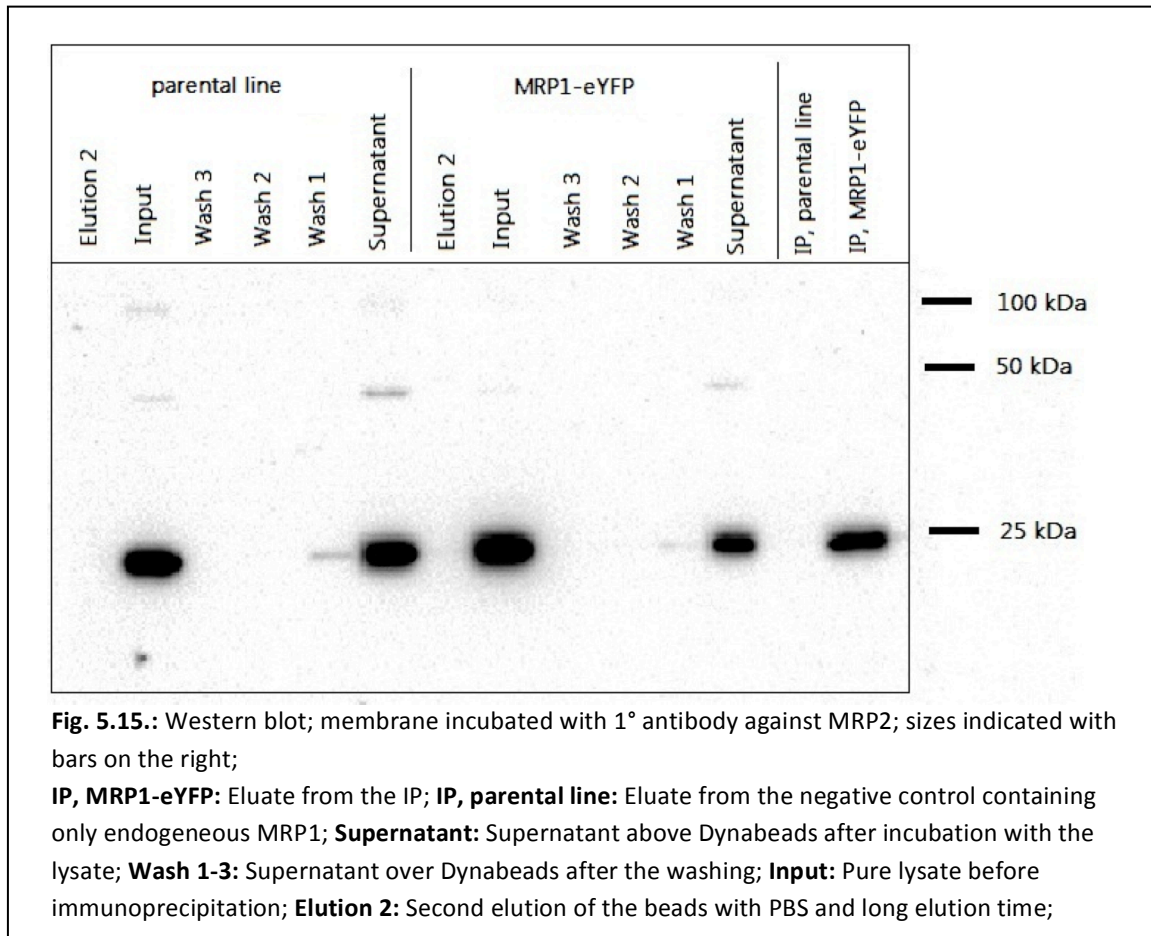

Fig. 5.15.: Western blot; membrane incubated with 1° antibody against MRP2; sizes indicated with bars on the right;
IP, MRP1-eYFP: Eluate from the IP; **IP, parental line:** Eluate from the negative control containing only endogeneous MRP1; **Supernatant:** Supernatant above Dynabeads after incubation with the lysate; **Wash 1-3:** Supernatant over Dynabeads after the washing; **Input:** Pure lysate before immunoprecipitation; **Elution 2:** Second elution of the beads with PBS and long elution time;

primary antibody against the protein MRP2 and further developed. The size of the protein MRP2 is approximately the same as MRP1, at around 25 kDa. As this is indeed the case, as can be observed in figure 5.15, the complex of MRP1/MRP2 was formed. It can be concluded that the protein MRP1-eYFP is functional.

6. CONCLUSION AND FUTURE EXPERIMENTS

One of the main goals of the project, namely the tagging with eYFP of the mitochondrial proteins MRP1, KREL2 and LSU1 of *Trypanosoma brucei*, was achieved. The successful tagging was validated by western blotting, as can be viewed in figure 5.6, section 5.2. The tag on the proteins can now be used for several further applications. For instance, it can serve as bait for co-immunoprecipitations to purify the complexes the proteins are part of. At the same time the proper functioning of the proteins can also be validated by this method, as it has been done for MRP1-eYFP (see sections 4.4 and 5.5).

With the help of the eYFP-tags on the proteins, their respective localization was investigated with a confocal microscope (see section 5.3.). The eYFP-tag also allows the use of several further microscopy techniques. For instance, the lateral mobility of the proteins can be investigated by **FRAP** (**F**luorescence **R**ecovery **A**fter **P**hotobleaching). To conduct a FRAP-experiment, a small area with the fluorescent molecule of interest is treated with an intense light pulse, which leads to bleaching of the molecules within the area. The result is a black area without fluorescence. Due to the lateral movement of fluorescent molecules outside the bleached are, the fluorescence will slowly recover. (See [23] for further reading).

Another possible microscopy technique, which can be exploited in a future experiment, is **FRET** (**F**luorescent **R**esonance **E**nergy **T**ransfer). Therefore, another protein of interest in the same cell has to be tagged with a different chromophore. Crucial is here that the emission spectrum of one chromophore has to overlap with the excitation spectrum of the other. When the two proteins come into close proximity, the energy of the light absorbed of one chromophore can be directly transferred to the other one. Hence, the excitation of the sample at the excitation wavelength of the first chromophore leads to an emission of light at the wavelength of the second one. (See [24] for further reading).

7. APPENDIX

List of abbreviations

IFA-assay	Indirect Fluorescent Antibody-assay
CIP	Co-Immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
SDS-PAGE	Sodium Dodecyl Sulphate- PolyAcrylAmide Gel Electrophoresis
APS	Ammonium PerSulphate
MRP1	Mitochondrial RNA binding Protein1
MRP2	Mitochondrial RNA binding Protein2
KREL2	Kinetoplast RNA Editing Ligase 2
LSU1	Ribosomal Large Subunit 1
eYFP	enhanced Yellow Fluorescent Protein
kDNA	kinetoplast DNA
rRNA	ribosomal RNA
kb	Kilobases
gRNA	guide RNA
mRNA	messenger RNA
ORF	Open Reading Frame
TuTase	Terminal Uridylyl Transferase
ExUase	U-specific EXOnuclease
ES	Expansion Segment
OB-fold-domain	Oligonucleotide-Binding-fold domain
PCR	Polymerase Chain Reaction
DMSO	Di Methyl Sulf Oxide
bps	base-pairs
BSA	Bovine Serum Albumin
HEPES	N-2-HydroxyEthylPiperazine N`-2-EthaneSulfonic Acid
EDTA	EthyleneDiamineTetraAcetic Acid
EGTA	Ethylene Glycol TetraAcetic Acid
PVDF	PoliVinyliDene Fluoride
TEMED	TEtraMethylEthyleneDiamine
PBST	Phosphate Buffered Saline containing Tween-20
PBSG	Phosphate Buffered Saline containing glucose
FRAP	Fluorescence Recovery After Photobleaching
FRET	Förster Resonance Energy Transfer

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