University of Crete

School of Sciences and Engineering

Department of Biology

“Development of enzymatic molecular tools for RNA analysis.”

Master of Science in Protein Biotechnology

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Heraklion 2019

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ACKNOWLEDGMENTS

First, and most of all I would like to express the deepest appreciation to my principal supervisor and Scientific Director of Minotech Biotechnology, Dr. D. Koutsioulis for his help, support and great patience at all times on both academic and personal level.

The present thesis would not have been possible without the good advice, support and friendship of my second supervisor Dr. Ioannis Vlatakis. I thank him for his patience, motivation, enthusiasm and knowledge.

Besides my advisors, I would like to express my special gratitude to the professors of my tripartite committee, Professor V. Bouriotis, M. Kokkinidis and G. Chalepakis. Without their participation I would be able to conduct my master thesis.

My sincere thanks also goes to my fellow labmates and friends. I thank the technician of our laboratory Mrs.Pepi for her useful advice, Ioanna M., Ioanna K., Konstantina and Lilian. We shared a pleasant and constructive year.

ABSTRACT

Synthesis of RNA by *in vitro* transcription is essential in many analytical techniques. Transcript generation is a simple and efficient process, requiring only an RNA polymerase to transcribe the gene of interest, DNA template downstream of a T7 promoter, ribonucleotides and a ribonuclease inhibitor to protect the RNA product. The current Master Thesis focuses on the development of two enzymatic molecular tools essential for RNA analysis, T7 RNA Polymerase and Human Placental Ribonuclease Inhibitor.T7RNAP is a single subunit RNA polymerase, extremely promoter-specific and able to complete RNA synthesis without additional protein factors. These properties make T7RNAP an ideal enzyme for *in vitro* transcription. Both the isolation and quality controls have been successfully performed and the enzyme is commercially available by Minotech Biotechnology. Ribonuclease inhibitor binds to pancreatic-type ribonucleases with high affinity and renders them inactive. For this work, an hRI variant resistant to oxidation was selected. Due to the enzymes hydrophobicity and instability that are strongly connected with the existence of 31 cysteine residues, the development of the product was incomplete. Extensive troubleshooting was applied including expression and purification conditions in order to isolate an active enzyme.

ΠΕΡΙΛΗΨΗ

Η *in vitro* σύνθεση του RNA είναι μια διαδικασία απαραίτητη σε πολλές αναλυτικές τεχνικές της μοριακής βιολογίας. Πρόκειται για μία απλή μέθοδο που μπορεί να αποδώσει αρκετά mgs RNA απαιτώντας μόνο μία RNA πολυμεράση, ένα DNA εκμαγείο κάτωθεν ενός T7 υποκινητή, ριβονουκλεοτίδια και αναστολέα ριβονουκλεασών για την προστασία του συντιθέμενου μετάγραφου.
Η παρούσα Μεταπτυχιακή Εργασία έχει στόχο την ανάπτυξη δύο μοριακών ενζυμικών εργαλείων για την ανάλυση RNA μορίων, την T7 RNA πολυμεράση και τον ανθρώπινο αναστολέα ριβονουκλεασών. Η υψηλή ειδικότητα της T7 RNA πολυμεράσης για τον T7 υποκινητή και η ικανότητά της να καταλύει τη μεταγραφή χωρίς την παρουσία συμπαραγόντων, καθιστούν το ένζυμο ιδανικό για την *in vitro* μεταγραφή. Τόσο ο καθαρισμός όσο και οι ποιοτικοί έλεγχοι για τη διασφάλιση της ποιότητας και λειτουργίας το ενζύμου διεξήχθησαν με επιτυχία και το προϊόν είναι εμπορικά διαθέσιμο από τη Minotech Biotechnology. Σε ότι αφορά τον αναστολέα ριβονουκλεασών, το ένζυμο που επιλέχθηκε είναι μια μεταλλαγή του ανθρώπινου αναστολέα ριβονουκλεασών ανθεκτική στην οξείδωση. Παρά την εκτεταμένη έρευνα για την εύρεση των κατάλληλων συνθηκών έκφρασης και καθαρισμού της πρωτεΐνης, η απομόνωση ενός λειτουργικού ενζύμου δεν επετεύχθη. Σημαντικός συντελεστής δυσκολίας είναι η παρουσία 31 καταλοίπων κυστεϊνης που καθιστούν το ένζυμο υδρόφοβο και ασταθές.

1. INTRODUCTION

1.1 *IN VITRO* TRANSCRIPTION

1.1.1 CHARACTERISTICS OF *IN VITRO* TRANSCRIPTION

In vitro transcription is a simple procedure that allows template-directed synthesis of RNA molecules of any sequence, from short oligonucleotides to those of several kilobases, in μg to mg quantities. It is based on the engineering of a template that includes a bacteriophage promoter sequence upstream of the sequence of interest, followed by transcription using the corresponding RNA polymerase. The most frequently used systems are the T3, T7 and SP6. Current thesis focus on the T7 system derived from the T7 phage of *E.coli*.

The basic strategy is to place the sequence of interest downstream from the T7 promoter. The promoter sequence covers a range from -17 to +6, with +1 being the first nucleotide transcribed. Thus, there is a limitation on the choice of the sequence at the very 5' –end of the i*n vitro* transcript. Class III promoters have G's at +1, +2 and +3, and the first two G's are crucial for the efficiency of the transcription. In class II promoters, an A is placed in position +1, while the preference for G's in position +2 is the same with class III (Figure 1).

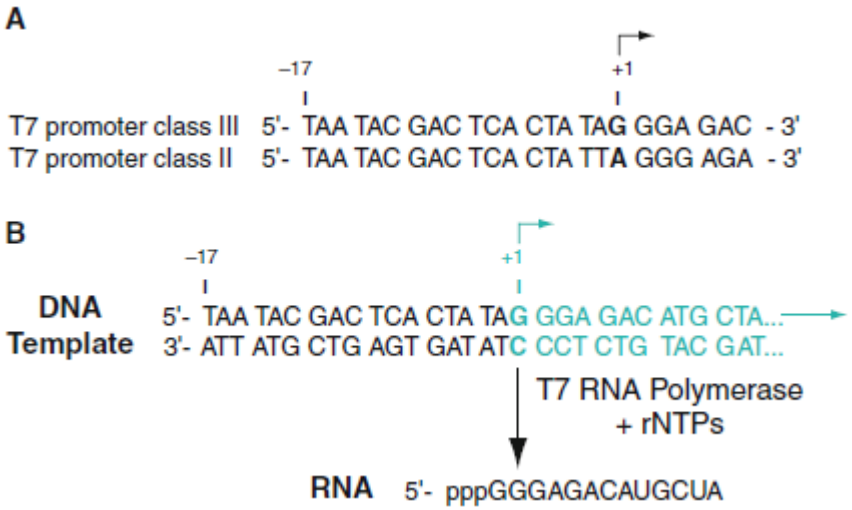


Fig.1 : A. The sequence of class III and class II T7 promoterB. DNA template incubated with T7 RNA polymerase and rNTPs resulting in a transcript with a triphosphate at the 5'-end. (Beckert and Masquida, 2011)

Regarding to the DNA template needed for transcription it can be a) a plasmid, containing the promoter sequence upstream from MCS for cloning the sequence to be transcribed, b) a PCR product that has the T7 promoter as part of the 5'- primer used in the PCR reaction and c) two annealed oligonucleotides that carry the T7 promoter and the sequence of interest (Figure 2).

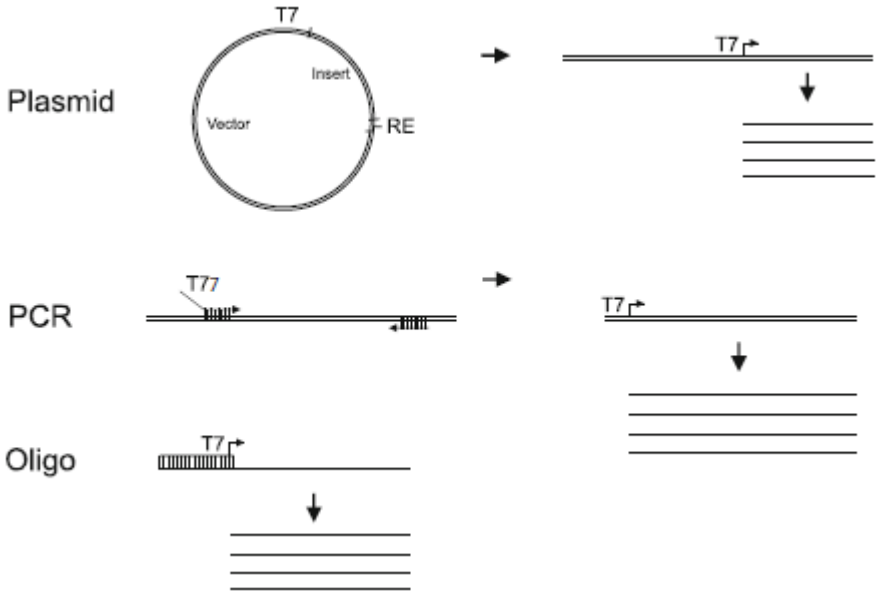


Fig.2: Three different types of DNA template used for *in vitro* transcription. (Beckert and Masquida, 2011)

In vitro transcription is a cell free transcription system. All the essential components for the reaction are contained in a reaction tube. The enzymes needed for the efficient conduction of the reaction include a) the T7 RNA polymerase (T7 RNAP), to transcribe the DNA of interest, b) an RNase Inhibitor to protect the transcript from nucleases and c) a DNase to hydrolyze the template, so that the only nucleic acid included is the desired transcript (Figure 3).

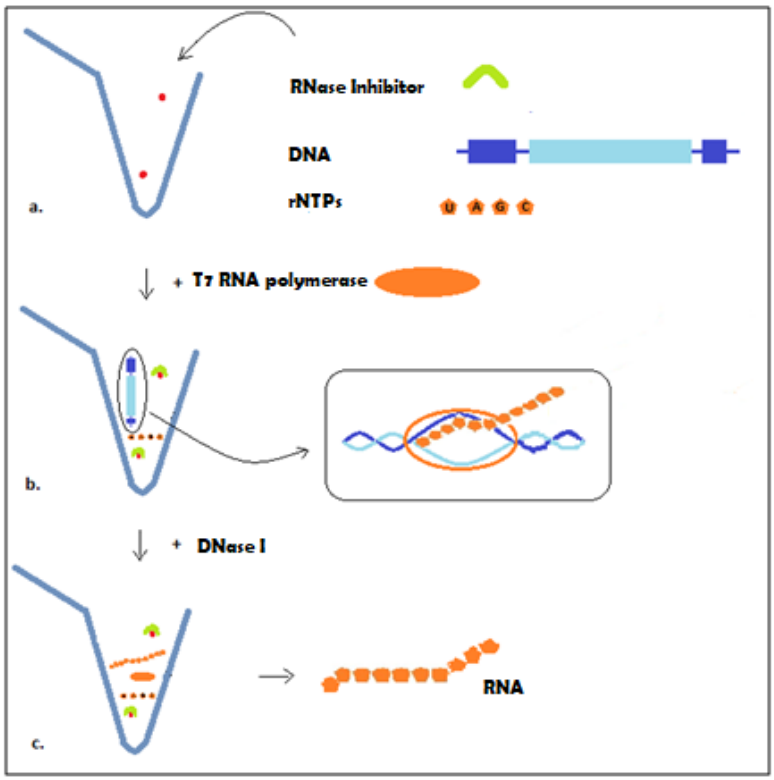


Fig. 3: The components needed for *in vitro* transcription. (Image adapted from Maria's Tsontaki Thesis)

1.1.2 APPLICATIONS OF *IN VITRO* TRANSCRIPTION IN MOLECULAR BIOLOGY.

The ability to synthesize RNA in the laboratory is critical to many techniques. Radiolabeled and nonisotopically labeled RNA probes, generated in small scale transcription reactions, can be used in blot hybridizations and nuclease protection assays. Such probes are much more sensitive than random-primed DNA probes. Small scale reactions may also be used to synthesize RNA transcripts containing modified nucleotides for various biochemical and molecular biology studies. Large scale transcription reactions, generating up to 200 µg of RNA per reaction can be used for RNA amplification, expression studies (microinjection, infection with viral transcripts, *in vitro* translation), structural analysis (NMR, X-ray crystallography), and mechanistic studies (ribozyme analyses).(Beckert and Masquida, 2011)

Summarizing, some of the application features are:

- Radiolabeled RNA probes
- Non-isotopic RNA labeling
- Preparation of RNA vaccines
- Guide RNA for gene targeting
- mRNA for *in vitro* translation and micro injection
- RNA structure, processing and catalysis studies
- RNA amplification
- Anti-sense RNA for gene expression experiments

1.1.3 FACTORS THAT AFFECT IN VITRO TRANSCRIPTION

As mentioned, *in vitro* transcription requires a purified DNA template containing a promoter (T7 promoter), ribonucleotide triphosphates, a buffer system that includes DTT and magnesium ions and an appropriate phage RNA polymerase, in our case T7 RNAP. The exact conditions used in the transcription reaction depend on the amount of RNA needed for the respective application. Initiation of RNA synthesis by T7 RNAP is abortive if the concentration of pyrimidine triphosphates is limiting (Ling *et al.*, 1989). Under insufficient initiation conditions the transcription takes place but produces oligonucleotides that are terminated just before the first occurrence of the limiting nucleotide. Abortive initiation is most pronounced if the limiting substrate occurs within the first 10-12 nucleotides of the RNA chain. Regarding the magnesium, T7 RNAP requires divalent cation for RNA synthesis. The steps monitored by Kcat and optimal binding as reflected by Km, require magnesium. Sulfhydryl reducing agents, such as DTT is required for enzyme activity and also stabilizes very long term storage of the enzyme. Studies of T7 RNA polymerase report a requirement for BSA or spermidine for optimal activity in transcription of full length templates. In the absence of spermidine the removal of carrier protein from the protein buffer impairs *in vitro* transcription. (Maslaki and Martin, 1994) The reason for this requirement may be related to the extreme sensitivity of the enzyme in the ternary RNA polymerase-DNA-RNA complex to inhibition by polyanions (Lin, 1973). Other factors that affect RNA synthesis are temperature, pH and salt concentration. Specifically, the rate of synthesis is decreased when occurring at low temperatures ($T<20^{o}C$), since RNAP is not able to anneal the DNA strands. The maximum of enzymatic activity is exhibited between pH 7.7 and 8.3. High salt concentration decreases enzyme activity. T7 RNAP is extremely sensitive to salt inhibition. The overall salt concentration should not exceed 50 mM. (Ikeda and Richardson, 1986) (Table 1).

Factors	
Increasing Efficiency	**Decreasing Efficiency**
BSA	>50mM [NaCl]
Spermidine	Temperature <20˚C
Reducing reagents	pH<7, pH>9
(b-mercaptethanol, DTT)	↓ [rGTP]
↑ [Mg^{++}]	

Table 1: Factors that affect the efficiency of *in vitro* transcription.

1.2 T7 RNA POLYMERASE

1.2.1 BIOLOGICAL ROLE OF T7 RNA POLYMERASE

In 1970, three different laboratories reported the identification of a novel RNA polymerase activity encoded by bacteriophage T7 (Chamberlin, 1970:Summers, 1970:Hayashi, 1970) . Bacteriophage T7 has been well characterized and has been widely used as a model system to study transcription. Two different RNA polymerases have been identified to play the main role in transcription of the virus genome during infection. The host RNA polymerase catalyzes the transcription of the early genes while T7 RNAP is responsible for the late genes transcription (Bailey, Klement and Mgallistert, 1983). T7 RNA polymerase is known to be one of the simplest enzymes catalyzing RNA synthesis as it is a single-subunit protein able to perform the complete transcriptional cycle in the absence of additional protein factors (Figure 4). T7 RNAP is a DNA-dependent RNAP sharing a common two-metal-ion mechanism for phosphodiester bond formation with DNA polymerases. RNAPs initiate transcription by recognizing a specific DNA sequence promoter followed by annealing of the DNA strands close to the active site and precise positioning of the single stranded template at positions +1 and +2 for base pairing with the incoming rNTPs. The intermediate RNA-DNA heteroduplex cannot extend beyond a total of four base pairs. Extension of the template-RNA complex by even one additional base pair would result in clashes with the polymerase NH2- terminal domain, so the products starts to peel away from the template. After RNA extension to a length of 9nts the polymerase releases the promoter, while RNA extension to a length of 14nts confers full stability on the elongation complex, escaping from initiation phase to progressive elongation. The transition between initiation and elongation involves extensive conformational changes within the N-terminal domain. Other modifications occur in the structure of the T7 RNAP, in particular, at the level of the specificity loop: it releases the promoter, moves to the open exit tunnel and becomes a part of it. Finally, the specificity loop contacts the 5- end of the RNA. The T7 transcription reaction ends when the polymerase meets a terminator and releases the RNA. Two distinct types of terminators have been described for T7 RNA polymerase (Macdonald et al., 1994). Class I terminators contain a sequence that forms a G-C rich hairpin followed by a U-rich element. The hairpin formation disrupts RNAP interactions with the single stranded RNA 8-14 nt away from the RNA 3'-end. Once the interactions are disrupted and the U-rich domain is weak to hold the RNA in the transcription complex, termination occurs (Hartvig and Christiansen, 1996). Class II

terminators do not exhibit any secondary structure. A class II terminator studied, revealed a sequence ATCTGTTTTTCTTGC with termination occurring at the underlined nucleotide (Lyakhov *et al.*, 1998).

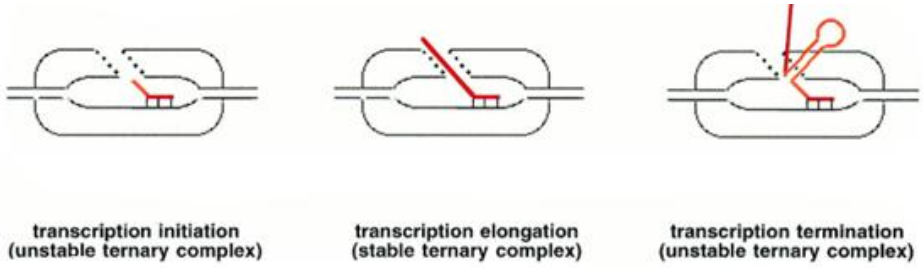


Fig.4 : Transcription catalyzed by RNAPs. A schematic view of the transcription initiation, elongation and termination (Class I terminator).

1.2.2 STRUCTURE

In comparison to the multisubunit DNA-dependent RNAPs that synthesize messenger RNA in prokaryotes and eykaryotes, T7 RNA polymerase is a simple enzyme of 100kD molecular weight, consisting of 883 amino acid residues, that is completely capable of transcribing T7 phage DNA. T7 RNAP is homologous to eykaryotic mitochondrial, chloroplast and other phage-like RNAPs and to the well-studied DNA polymerase I (Cheetham, 2002). Crystal structures of T7 RNAP alone (Sousa et al, 1993) reveal an enzyme organized around a cleft to accommodate a duplex DNA molecule. As mentioned, a large part of the enzyme displays extensive structural similarities to the polymerase domain of DNAP I (Figure 5).

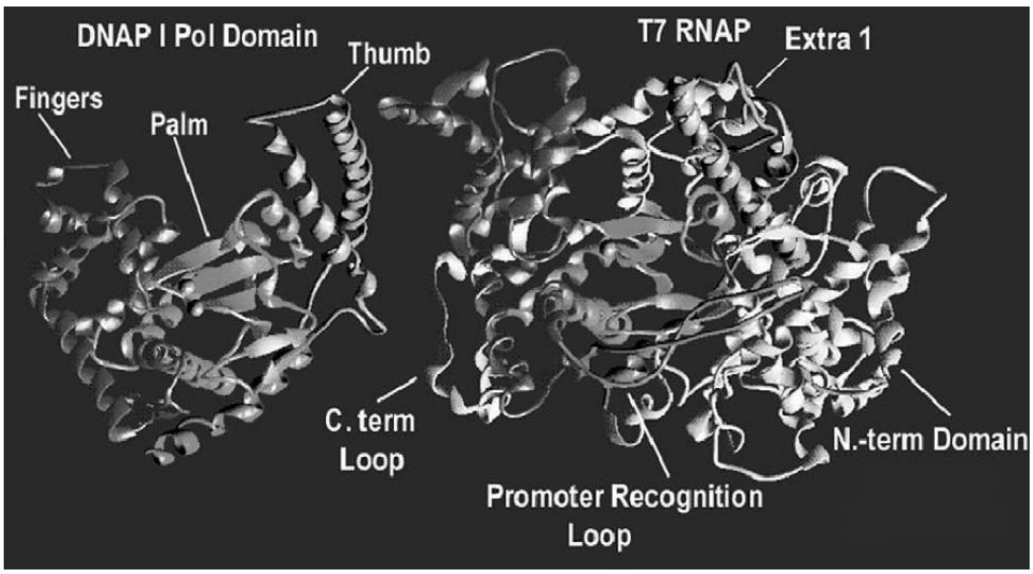


Fig. 5: Ribbon diagrams depicting the polymerase domain of DNA polymerase I (left) and the entire T7 RNA polymerase molecule (right). (Sousa, 2013)

The shape of T7 RNAP is similar to a cupped right hand composed of three subdomains that have been named “thumb”, “palm” and “fingers”. T7 RNAP contains four additional structural elements that are indicated as the N-terminal domain, the extra 4-helix bundle, the promoter recognition loop and the C-terminal loop. The active site of the enzyme is located in a deep slop, which is delimited by the fingers, the thumb, the subunits of the palm and a NH_2- terminal subunit (Figure 6).

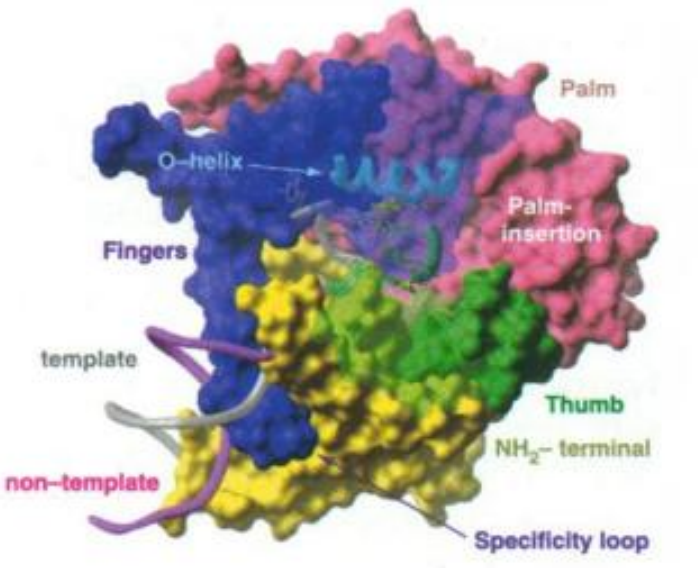


Fig.6 : The structure of a transcribing T7 RNAP complex (Cheetham, 2002)**.**

1.2.3 GENE EXPRESSION APPLICATION

Although T7 RNAP functions *in vivo* to transcribe the bacteriophage T7 genome, it can also transcribe a variety of DNA templates that contain T7 promoters *in vitro.* T7RNAP is widely used for heterologous protein expression in bacteria (William Studier *et al.*, 1990). Due to its high selectivity for specific promoters that are rarely encountered in DNA unrelated to T7 DNA and its unique, efficient termination signals, T7 RNAP is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. The high activity of the enzyme increases its efficiency to elongate chains during transcription about five times faster than *E.coli* RNA polymerase. These properties have highlighted T7 RNAP as the basis for expression systems in *E.coli* and in a variety of cell types. In the basic protocol, two engineered DNA domains are maintained within the same *E.coli* cell. The first one, which is anexpression vector that contains the T7 promoter upstream the gene to be expressed. The second one, that is inserted in the *E.coli* genome contains the gene of T7 RNA polymerase under the control of an *E.coli* promoter.

The most popular strategy for regulating gene expression is by using the pET-expression system (Figure 7). The gene coding for T7 RNAP itself has been engineered into many commercially available *E. coli* strains under a modified lac operon system. Therefore, it is expressed only if the inducer IPTG is added to the growth medium. Isopropyl β-D-1-thiogalactopyranoside(IPTG) is a non hydrolysable molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator. With the addition of IPTG T7 RNA polymerase is expressed and able to bind on the T7 promoter lying upstream the cloned gene into the pET vector, leading to the transcription of the target gene.

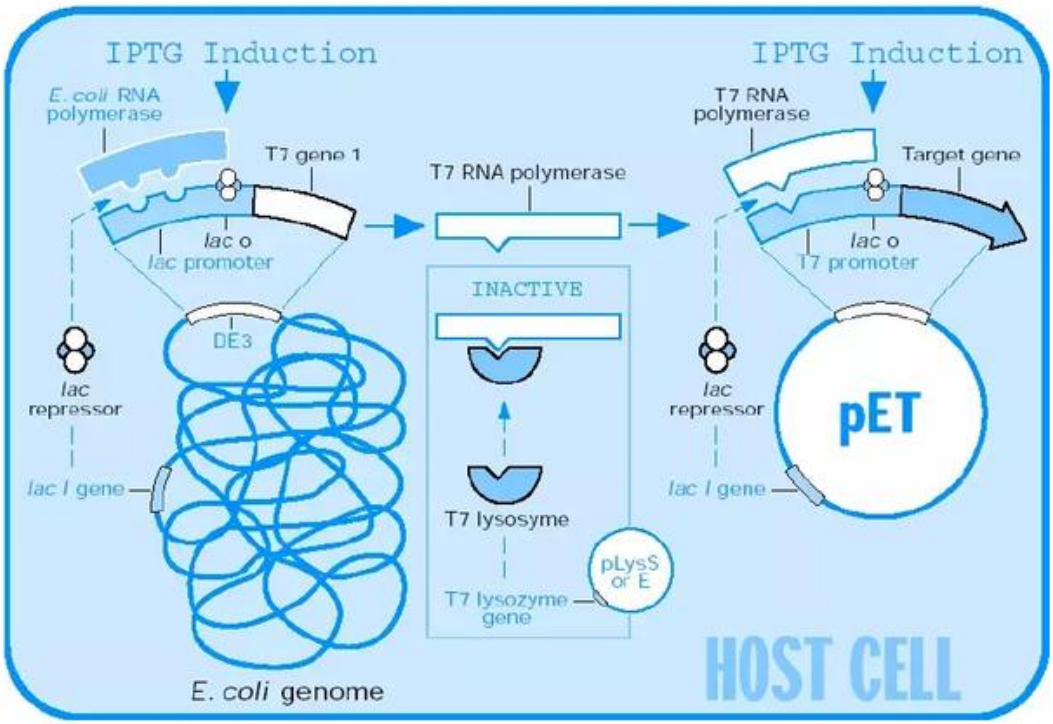


Fig. 7: The mechanism of induction by IPTG. Some strains of *E. coli* include an additional gene coding for T7 lysozyme which inactivates the T7 promoter i.e. strains like BL21(DE3)pLysS. Furthermore, the presence of a lac operator between T7 promoter and the cloned gene reduces transcription of the cloned gene in the absence of the inducer IPTG. This keeps the basal expression levels of T7 RNAP extremely low. (Image adapted from Novagen pET expression manual)

1.3 RIBONUCLEASES AND RIBONUCLEASE INHIBITOR

1.3.1 RIBONUCLEASES

Ribonucleases are enzymes that catalyze RNA degradation by cleaving on single stranded, double stranded or DNA-RNA hybrid molecules. All organisms studied so far, protozoans, plants, bacteria, animals and viruses contain RNases in their genomes, indicating that RNA degradation is an ancient and important process. Ribonucleases can be separated in endo-and exonucleases. They function in a variety of cellular processes including DNA synthesis, RNA processing, cytoplasmic or nuclear RNA degradation, RNAi, and antiviral defense (Luhtala and Parker, 2010).
Ribonucleases comprise several subclasses. In the endonucleases RNase A, RNase P, L, H, RNase III, RNase T1,T2 and U2 are included. RNase A (Fig.7) is specific for single-stranded RNAs. It cleaves the 3'-end of unpaired C and U residues, ultimately forming a 3'-phosphorylated product via a 2',3'-cyclic monophosphate intermediate (Cuchillo and Raines, 2011). In the human genome eight RNase A genes have been identified, encoding eight different RNase proteins (RNase 1,2,3,4,5,6,7,8)(Cho et al., 2005). RNase A is one of the most stable enzymes at molecular biology laboratory level, as it remains active even in extreme temperatures, does not require cofactors for its activity and can be secreted from human skin (Figure 8).

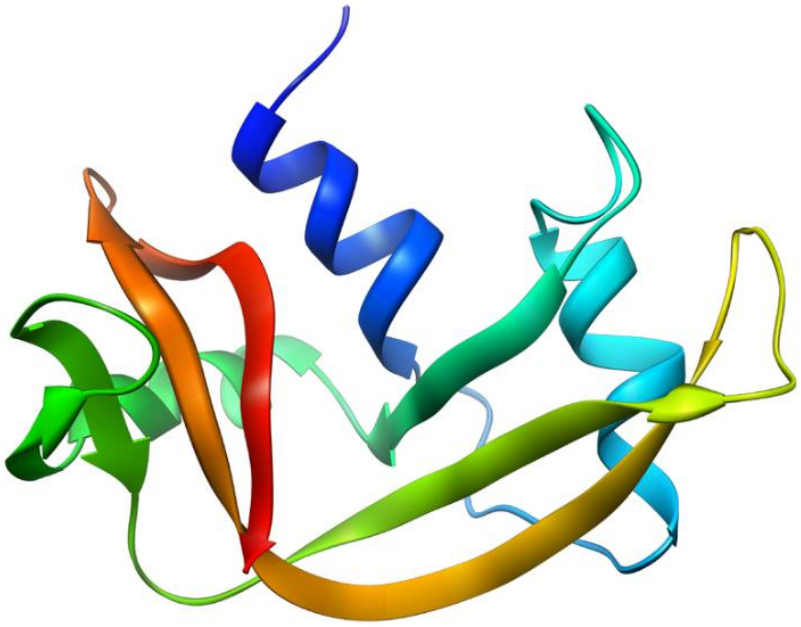


Fig. 8: Tree-dimensional structure of bovine pancreatic RNase A.

T1, T2 and U2 RNases are sequence specific for single stranded RNA. While T1 RNase exists only in bacteria, T2 RNase has been found in protozoa, bacteria, plants, viruses and animals (Luhtala and Parker, 2010). RNase III exists in prokaryotes and cleaves double-stranded RNA (Nicholson, 2014). In the case of exoribonucleases RNase R, D as well as Polynucleotide Phoshorylase(PNPase) are included (Table 2).

Family	Organismal Distribution	Optimum pH of Activity	Substrate Specificity
RNaseT2	Broadly distributed across kingdoms	Acidic (pH4–5)	Little substrate specificity
RNaseA	Primarily found in animals	Weakly acidic (pH6.5–7) or alkaline activity (pH7–8)	Pyrimidine base specificity
RNaseT1	Found in certain species of fungi and bacteria	Alkaline (pH7–8)	Guanylic-acid specificity

Table 2: Characteristics of RNase A, T1 and T2. (Luhtala and Parker, 2010)

1.3.2 RIBONUCLEASE INHIBITOR

The mammalian ribonuclease inhibitor(RI) is a 50-kDa major cellular protein, comprising ~0,1% of all cellular protein by weight (Blackburn, 1970). It binds to pancreatic-type ribonucleases with femtomolar affinity and renders them inactive. Complexes formed by RI and its target ribonucleases are among the tightest of known biomolecular interactions. RI serves to protect the RNA in the cytosol of mammalian cells from the invasion of ribonucleases. The enzyme is also widely used for protecting RNA during laboratory experiments.

The inhibitory activity of RI was discovered in 1952, in guinea pig liver extracts (Pirotte et al., 1952). In the 1970's, the techniques developed to purify RI enabled its biochemical characterization. Since then, RI has been isolated from numerous sources including brain, liver, and placenta. The tight complex formed by RI and bovine pancreatic ribonuclease (RNase A) – 1:1 stoichiometry (Rockefeller, 1979), has been exploited to achieve high purity of RI in one chromatographic step by using immobilized RNase A. Today most purification methods rely upon this affinity chromatography followed by ion-exchange chromatography.

RI is an acidic (pI 4.7) cytosolic protein. Members of the RNase A superfamily, are unable to bind or degrade RNA when complexed with RI, include RNase A, human pancreatic ribonuclease (RNase I), ANG, RNase 2, 4 and monomers of bovine seminal ribonuclease (BS-RNase).

Regarding the amino acid sequence of RIs, it seems to be conserved, as sequences of human, porcine, mouse and rat RI share 66% identity. (Figure 9) One third of the residues that differ are conservative substitutions.

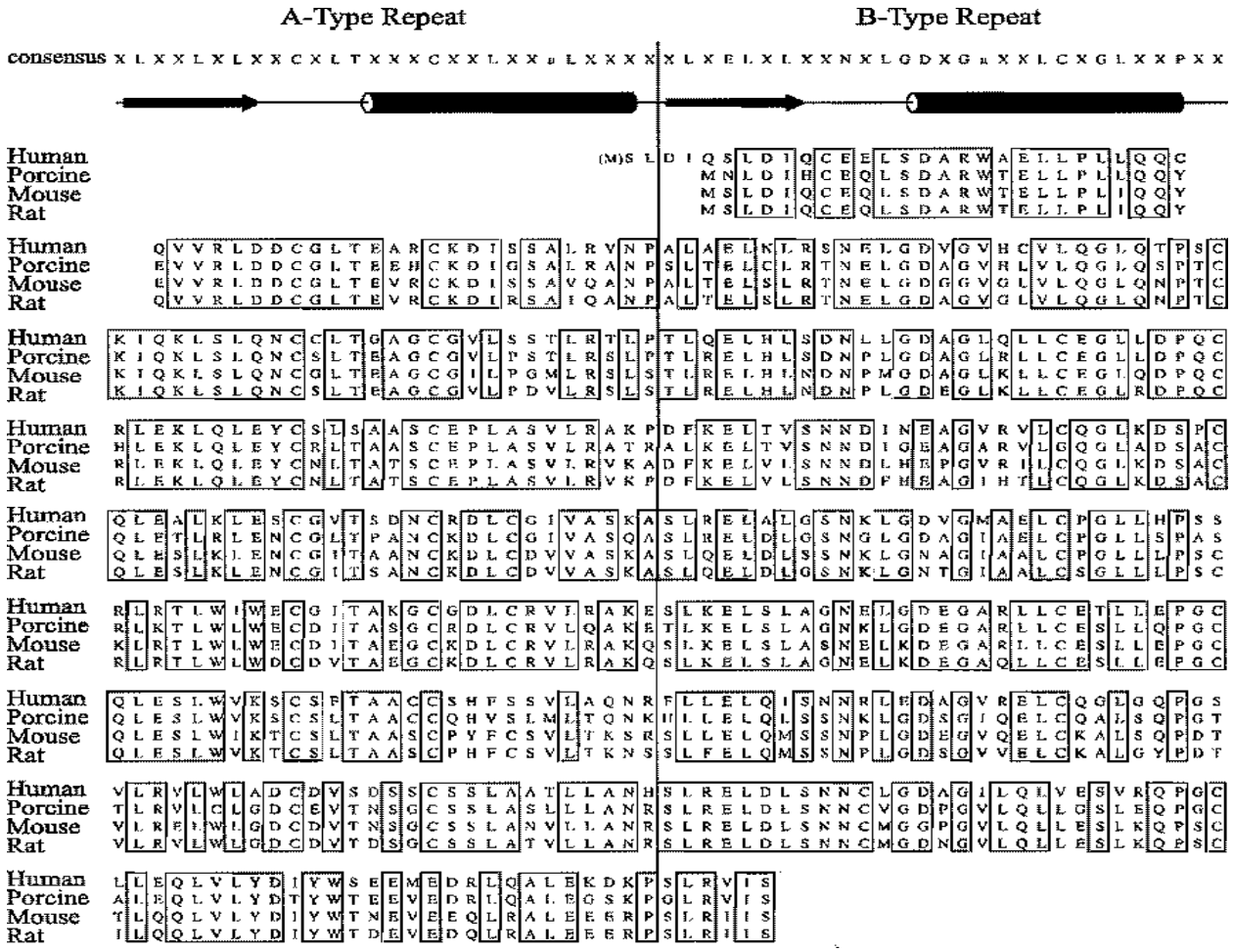


Fig. 9 : Alignment of the amino acid sequences of human, porcine, mouse and rat RI. The consensus sequence for the A-type and B-type repeats are indicated. Conserved residues are in boxes. (Dickson, Haigis and Raines, 2005)

1.3.3 STRUCTURE OF RI

The three-dimentional structure of RI reveals alternating units of a-helix and β-strand that form a striking horseshoe shape (Figure 10).

Fig. 10: Top view of porcine ribonuclease inhibitor showing the horseshoe shape. The outer layer is composed of a-helices and the inner layer of parallel β-strands. (Image adapted by https://en.wikipedia.org/wiki/Ribonuclease_inhibitor)

The repeating structural unit posses a repetitive amino acid sequence rich in leucine residues. RI features 15 leucine rich repeats (LRR) alternating 28 and 29 residues. These LRR motifs seem to participate in protein-protein interactions. The β-strand and the α-helix are roughly parallel in individual β-α units and the strands and helices are aligned parallel to a common axis. A similar conformation as the repeats is formed by bordering sequences, resulting in parallel β-sheet on the inner circumference of the horseshoe, providing a vast surface for interactions with other proteins, and α-helices on the outer side (Kobe, 1993). RI is also rich in cysteine residues (30 in porcine RI, 32 in human placental RI). In the active inhibitor all cysteine residues are in the free thiol form, while in absence of reducing agent the enzyme undergoes a conformational change and becomes inactive. The three dimensional structure of porcine RI-RNase A complex was determined in 1995 by Kobe et al. (Figure 11). The RI-RNase A interaction is relied on Coulombic forces more than do most protein-protein interactions. Only two contact residues belong in the α-helix of RI, while the remaining 17 contacts are found in loops connecting the C-termini of the β-strands with the N-termini of the α-helices.

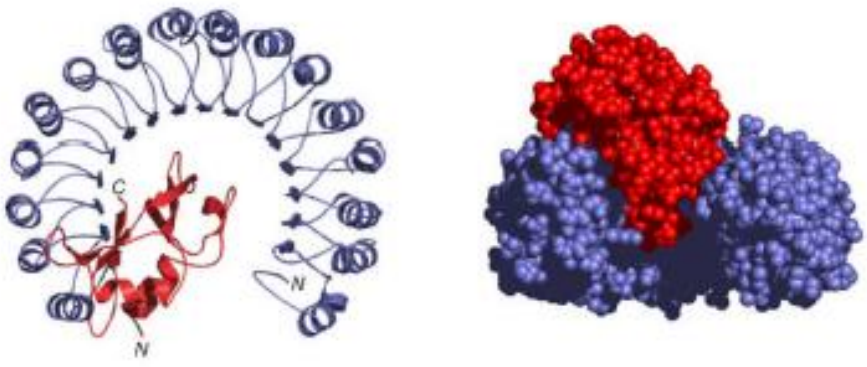


Fig.11 : Three dimensional structure of porcine RI in complex with RNase A. (Dickson, Haigis and Raines, 2005)

1.3.4 VARIANTS OF RIBONUCLEASE INHIBITORS RESISTING OXIDATION

RI is widely used to prevent the enzymatic degradation of RNA. The sensitivity of RI to oxidation limits its application as a reagent, as many reducing reagents are not compatible with all laboratory techniques.

In absence of reducing environment, after the initial oxidation of a small number of cysteine residues the reactivity of the remaining thiols increases resulting in the formation of 15 disulfide bonds and inactive RI (Fominaya and Hofsteenge, 1992). Replacing proximal cysteine residues of human RI, that do not intervene in the RI-RNase complex, with alanine, results in RI variants with oxidation resistance (Kim, Schultz and Raines, 1999).

Considering the tree-dimentional structure of human RI (Fig. 8) the adjacent amino acids are Cys 94 and Cys 95 (which are in a loop) and Cys 328 and Cys 329 (which are in an α-helix). The substitution of Cys 328 and Cys 329 with alanine residues results in 10-15 fold more oxidation resistant hRI with negligible effect on the RI-RNase A affinity. In contrast, C94A/C95A hRI variants impair the ability of the inhibitor to bind RNase A (Figure 12).

The human placental ribonuclease inhibitor that was studied in the current thesis has been codon optimized for *E.coli* expression and bears this oxidation resistance by having Cys 328 substituted with alanine (C328A). (Anna Stathi Thesis, 2017)

Fig. 12: Tree-dimensional structure of human RI. All 32 cysteine residues with their side chains are depicted. The residues that could be substituted with alanine are in yellow. (Kim, Schultz and Raines, 1999)

2. THE AIM OF THE STUDY

The aim of the study is to develop an *in vitro* transcription kit, that will be commercially exploited by MINOTECH biotechnology, IMBB-FORTH. Considering the numerous applications of *in vitro* RNA synthesis, an advanced and efficient transcription kit containing the enzymatic tools essential for this process, would be a competitive product.
The enzymes developed in this work include:

- T7 RNA polymerase
- Human Placenta Ribonuclease Inhibitor

while DNAase I development is in progress by other members of the laboratory.

The respective enzymes will be heterologously expressed using *E.coli* expression system. Protein purification through liquid chromatography methodology and quality controls will be thoroughly investigated.

3. MATERIALS AND METHODS

3.1 PREPARATION OF *E.COLI* COMPETENT CELL LINES FOR PLASMID TRANSFORMATION

- Perform a fresh streaking on LB agar /respective antibiotic (if necessary) from a glycerol stock (20% glycerol) and incubate O/N at 37^{o}C.
- Transfer a single colony in 5ml liquid LB medium (5 gr NaCl, 5gr Yeast Extract and 10 gr Tryptone, pH=7/ lt) O/N at 37^{o}C, under stirring.
- Use the pre-culture to inoculate 50ml LB and incubate at 37^{o}C, under stirring, until OD_{600nm} reaches 0.5.
- Centrifuge at 3000g for 15min at 4^{o}C and discard supernatant.
- Gently resuspend the cell pellet in 40ml 100mM $CaCl_2$ on ice, until the cell solution is homogenized and incubate on ice for 20 min.
- Centrifuge at 2000g for 15 min at 4^{o}C and discard supernatant.
- Gently resuspend cell pellet in 10ml buffer A (100mM $CaCl_2$ and 15% glycerol).
- Centrifuge again at 2000g for 15min at 4^{o}C and discard supernatant.
- Resuspend the cell pellet in 400ml buffer A.
- Aliquot the solution rapidly (50ml per aliquot), freeze in a dry ice-ethanol bath and store competent cells at -80^{o}C.
- All steps are performed under sterile conditions.

3.2 BACTERIAL CELL TRANSFORMATION

- Transfer the chemically competent cells from -80^{o}C to 4^{o}C for 5 min.
- Add the appropriate amount of DNA (less than 200ng, less than 10% of competent cell volume) to the cells and let in ice for 30 min.
- Perform heat-shock by incubating the cells at 42^{o}C for 1.5 min. and transfer immediately in ice for 2 min.
- Incubate cells with 900µl LB (or SOC) at 37^{o}C for 1h shaking.
- Centrifuge the cells for 5 min. at 1100g at RT.
- Discard 800 µl of the supernatant
- Gently dissolve and plate cells on LB/ respective antibiotics-agar plates
- Incubate cells at 37^{o}C O/N.
- All steps are performed under sterile conditions.

3.3 CLONING PROCEDURE-GENOME ASSEMBLY NEB BUILDER KIT FOR CLONING

The kit is a modified version of Gibson Assembly cloning method. The fragments, vector and gene, are amplified by PCR using the appropriate primer sequence. Subsequently, a ligation reaction is performed according to the instructions of the protocol for two DNA fragments. The reaction is consisted of three different enzymes:

- An exonuclease which creates 3' prominent ends so as to facilitate the hybridization of the complementary fragments,
- A polymerase which leads to a double-stranded DNA
- A ligase which binds the produced fragments.

The method was used to insert hRI gene from pET24b-hRI in the plasmid vector PJSC011. The reaction prepared is the following. Tables 3 and 4 present the reaction composition and primer characteristics respectively.

NEBuilder Reaction	
Recommended DNA Ration	vector:insert=1:2
Total Amount of Fragments	0.03-0.2 pmols
Assembly Master Mix	10μl
ddH_2O	10-xμl
Total Volume	20μl

Table 3: NEBuilder general reaction composition

Overlaps	Oligo (Uppercase=gene-specific primer)	Anneals	F/R	Tm (°C)	Ta (oC)
hRI	CATTGCATTGGATTGGAAG (Primer B)	Pjsc011	Rev	58.8	58
Pjsc011	acttccaatccaatgcaatgAGCCCTGGATATTCAGAGC (Primer C)	hRI	Fwd	60.9	59.8
Pjsc011	atccacttccaatgttattaTTAGCTAATAACACGCAGG (Primer D)	hRI	Rev	58.8	59.8
hRI	TAATAACATTGGAAGTGGATAAC (Primer A)	Pjsc011	Fwd	57	58

Table 4. : Primers required for NEBuilder Assembly. Ta temperatures are the recommended annealing temperatures for PCR.

3.3.1 POLYMERASE CHAIN REACTION (PCR)

PCR method was used to amplify the plasmid vector PJSC011 and the gene encoding Human Placental RI as part of the genome assembly Neb builder kit (Tables 5 and 6).

Reaction Components
5x Minopol reaction buffer
0.2μM Primer A (for hRI) or C (for Pjsc011)
0.2μM Primer B (for hRI) or D (for Pjsc011)
0.2μM dNTPs
1U Minopol
10ng PJSC011/1ng Hri
ddH_20 until final volume is 50μl

Table 5: Reaction composition

Reaction conditions	
hRI gene	PJSC011 vector
98 °C for 30''	98°C for 30''
98 °C for 10''	98°C for 10''
59,8°C for 30''	58°C for 30''
72°C for 35''	72°C for 1:35
8°C O/N	8°C O/N
Repeat steps 2-4 x31 cycles	

Table 6: Reaction conditions

F' I^q *E.coli* competent cells were transformed with 2μl of the reaction and plated on LB-agar plates containing 100μg/ml Ampicillin. Respective colonies were tested by colony PCR (Section 3.1.2) and the positive ones were grown in 5ml liquid LB with 100μg/ml Ampicillin for 16h at 37°C. The desirable plasmid constructs were purified using Plasmid DNA Purification kit from Macherey-Nagel (NucleoSpin Plasmid). The positive constructs were verified by sequencing and restriction digest screening (Section 3.1.3).

3.3.2 COLONY PCR

After each cloning step selected colonies are tested through a PCR reaction for the existence of the target DNA insert. Each colony was diluted in 20μl ddH_2O and 5μl of each resuspension were used as a template in colony PCR screening reaction. Two PCR were performed, using Taq and Minopol DNA polymerase in each reaction respectively. The results were analyzed in 1% agarose electrophoresis gel (Tables 7 and 8).

Reaction Components	
5x Minopol reaction buffer	10x Taq Polymerase reactionbuffer
0.2μM Primer A	0.2μM Primer A
0.2μM Primer B	0.2μM Primer B
0.2μM dNTPs	0.2μM dNTPs
1U Minopol	1U Taq Polymerase
5μl of the colony	5μl of the colony
ddH_20 until final volume is 50μl	ddH_20 until final volume is 50μl

Table 7. Reaction composition

PCR Steps for Minopol DNAP	PCR Steps for Taq DNAP
98 °C for 30''	98 °C for 30''
98 °C for 10''	98 °C for 10''
59,8°C for 30''	52°C for 30''
72°C for 35''	72°C for 1:20
repeat steps 2-4 x 31 cycles	repeat steps 2-4 x 31 cycles
8°C O/N	8°C O/N

Table 8: Reaction conditions

3.3.3 PLASMID DIGESTS TO IDENTIFYSUCCESFUL CLONING

Using NEBcutter tool (http://nc2.neb.com/NEBcutter2/) the restriction sites are analyzed. The restriction enzyme used is BamHI. In order to distinguish the PJSC011 vector containing the hRI gene, PJSC011_Cas9 vector was digested with the same restriction enzyme. In case there is no insert the BamHI digest profile is as in Table 9. In case the correct plasmid construction is tested the BamHI digest profile is as in Table 10.

#	Ends	Coordinates	Length (bp)
1	BamHI-BamHI	6768-6017	9323
2	BamHI-BamHI	6018-6767	750

Table 9 : Pjsc011_hRI-cas9 digest profile. Digest results in two DNA fragments, 9323 and 750 bps.

#	Ends	Coordinates	Length (bp)
1	BamHI-BamHI	25-6383	6359
2	BamHI-BamHI	6384-24	993

Table 10 : Pjsc011_hRI-BamHI digest profile. Digest results in two DNA fragments, 6,359 and 999 bps.

- The reaction was incubated at 37°C for 1h (Table 11).
- The sample was analyzed in 1% agarose gel to verify digest results.

Reaction
1µg template
12 U BamHI
10x K Buffer
ddH_2O until Vt 30µl

Table 11 : Digest reaction composition

3.4 HETEROLOGOUS EXPRESSION SCHEME OF T7 RNA POLYMERASE

- From a fresh plate of transformed cell culture (BL21 Star (DE 3) pLys cells transformed with T7plasmid) a single colony was picked and grown O/N, at 37^{o}C, , in 1L liquid ampicillin-chloramphenicol LB under vigorous shaking (250rmp)
- After 16-18 hrs (OD_{600nm} 1.8-2.0) IPTG was added at final concentration 0.5mM and the culture was further incubated for 3hrs at 30^{o}C, 250 rpm.
- Cells were collected by centrifugation at 5000 g for 15 min at 4^{o}C and stored at -80^{o}C. On average 1lt cell culture resulted in 2.6gr cell paste.

3.5 HETEROLOGOUS EXPRESSION SCHEME OF HRI

- From a fresh plate of transformed cell culture (BL21 Star (DE 3) pLys cells transformed with PJSC011-hRI) a single colony was picked and grown O/N, at 37^{o}C, 250rmp, in 100ml liquid ampicillin-chloramphenicol LB under vigorous shaking (250rpm).
- After 16-18h the OD_{600nm} was counted and part of the preculture was added in 1L cell culture (ampicillin-chloramphenicol liquid LB) so that the final OD_{600nm} was 0.05.
- The culture was incubated at 30^{o}C, under vigorous shaking until the OD_{600nm} reached 0.4-0.6.
- IPTG was added at final concentration 0.5mM and induction was conducted for 3h at 30^{o}C, under vigorous shaking (250rpm).
- Cells were harvested by centrifugation at 5000 g for 15 min. at 4^{o}C and cell pellet was stored at -80^{o}C. On average 1lt cell culture resulted to 2gr cell paste.

3.6 HETEROLOGOUS EXPRESSION SCHEME OF TEV PROTEASE

- Cells of a fresh plate (B-971 *E.coli* strain transformed with TEV plasmid) were grown on an O/N 100 ml kanamycin-chloramphenicol liquid LB preculture at 37^{o}C, under vigorous shaking (250rmp).
- Media from preculture was inoculated by transferring 1/20 of preculture volume to 1L liquid kanamycin-chloramphenicol LB and cells were grown at 30^{o}C, under shaking (250rmp) until OD reached 0.6-0.8.
- Then, temperature was reduced to 20^{o}C and cells were let to adjust before 0,1-0,2mM IPTG was added. Incubation followed, O/N at 20^{o}C, under shaking (250rmp).
- Cells were harvested by centrifugation at 5.000g for 15 min. at 4^{o}C and cell pellet was stored at -80^{o}C. On average 1lt cell culture resulted to 3.3gr cell paste.

3.7 PROTEIN SOLUBILITY TEST

Before the identification of the optimum purification scheme the solubility of the heterologously expressed proteins were tested. The steps followed were as below:

- From 50ml cell culture cells were aliquoted in 1.5ml eppendorf tube according to the rule $1ml1OD_{600nm}=xmlOD_{600nm}$.
- Cells were harvested by centrifugation at full speed for 1min at 4^{o}C.
- 500µl of the lysis buffer tested were added and cells were disrupted by sonication (10x30 seconds sonication steps, with 30sec resting intervals on a dry ice- ethanol bath).
- Crude lysate was centrifuged at 15.000g for 30min at 4^{o}C.
- Soluble supernatant and insoluble fraction were separated.
- The soluble supernatant was precipitated with acetone (Section 3.5.1) and resuspended in 15µl 5x protein dye.
- Cell pellet was resuspended in 20µl 5x SDS PAGE loading dye.
- Finally, SDS-PAGE electrophoresis was performed to identify whether the protein lies in pellet or in soluble supernatant.

3.7.1 ACETONE PRECIPITATION

- 100µl of soluble supernatant and 400µl of 100% acetone were added in an eppendorf tube.
- The sample was then incubated at -20^{o}C for 10 min.
- A centrifugation at 16.000g for 5min. at 4^{o}C followed and supernatant was discarded in order to get the precipitated proteins.
- Sample was let to air dry in hood and resuspended in 15µl 5x protein loading dye.

3.8 PURIFICATION SCHEME OF T7 RNA POLYMERASE

3.8.1 T7RNAP NI-NTA AFFINITY CHROMATOGRAPHY

- Frozen cell paste (2.6gr) expressing T7 RNA polymerase was thawed on ice and resuspended in 9ml equilibration buffer at 4° C (Table 12).
- Cells were disrupted by sonication (10x30 seconds sonication steps, with 30sec resting intervals on a dry ice- ethanol bath).
- The lysate was centrifuged at 15000 g, 4 °C for 30 minutes.
- The cytoplasmic supernatant was collected and diluted in a ration 1:3 with equilibration buffer before loading to the subsequent chromatography column.

Preparation of Ni-NTA beads

- In 1ml Ni-NTA agarose beads (1 Column Volume, 1CV). 2CV 0,1M NaOH were added and incubated for 30min.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV ddH_2O were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV equilibration buffer were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV of Equilibration Buffer was added and beads were let stirring for 10min.
- Beads were centrifuged at 1200 rpm for 1 min. and supernatant was discarded.

Ni-NTA Chromatography

- The diluted cytoplasmic supernatant was loaded onto the equilibrated Ni-NTA beads in a 50ml falcon tube.
- Incubation at 4^{o}C for 1hr, under stirring followed.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (flow through sample).
- 5CV Wash buffer were added on the beads and the solution was stirred for 10 minutes.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (wash sample).
- 4 step elutions were performed. a)25mM imidazole (3CV, 1 fraction), b)50mM imidazole (3CV, 1 fraction), c)75mM imidazole (3CV, 1 fraction), d)200mM imidazole (3CV, 1 fraction). Between step elutions stirring (10 min) and centrifugation steps were performed (1200 rpm, 1 min) while the respective supernatants were collected.

Ni-NTA Chromatography (T7 RNAP)							
Lysis	**Equilibration**	**Wash 1**	**Wash 2**	**Elution 1**	**Elution 2**	**Elution 3**	**Elution 4**
20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol
20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6	20 mM Tris-HCl pH 7.6
500mM NaCl	500mM NaCl	500mM NaCl	100mM NaCl	100mM NaCl	100mM NaCl	100mM NaCl	100mM NaCl
1mM PMSF	0,1% Triton-X	0,1% Triton-X	0,1% Triton-X	0,1% Triton-X	0,1% Triton-X	0,1% Triton-X	0,1% Triton-X
10% glycerol	10% glycerol	10% glycerol	10% glycerol	10% glycerol	10% glycerol	10% glycerol	10% glycerol
				25mM imidazole	50mM imidazole	75mM imidazole	200mM imidazole

Table 12. Buffer composition used in Ni NTA chromatography for T7 RNAP purification

3.8.2 T7 RNAP ION EXCHANGE CHROMATOGRAPHY

Sample preparation

- Step elutions a, b and c from Ni-NTA chromatography were merged in a single falcon (total volume 9ml) and diluted in a ratio 1:1 with Dilution Buffer (Table 13) to decrease NaCl concentration to 50mM before loading to the subsequent chromatography column.

Preparation of Q-Sepharose beads

- In 3ml Q-Sepharose beads (1 Column Volume, 1CV) 2CV 0,1M NaOH were added and incubated for 30min.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV ddH_2O were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 20CV equilibration buffer was added and incubated for 10 min
- Equilibrated Q-Sepharose beads were loaded onto a gravity flow column.

Q-Sepharose Chromatography

- The diluted sample from Ni-NTA chromatography was loaded onto the gravity flow column and flow through sample was collected.
- 5CV Wash buffer were added on the beads and wash sample was collected.
- One step elution (5CV) was performed at 500mM NaCl and collected in two fractions (7.5ml each).

Q- Sepharose fast flow chromatography (T7 RNAP)		
Dilution	**Equilibration and Wash**	**Elution**
20mM b-mercaptethanol	20mM b-mercaptethanol	20mM b-mercaptethanol
20mM Tris-HCl pH 7.6	20mM Tris-HCl pH 7.6	20mM Tris-HCl pH 7.6
0mM NaCl	50mM NaCl	500mM NaCl
0,1% Triton-X	0,1% Triton-X	0,1% Triton-X
10% glycerol	10% glycerol	10% glycerol

Table 13. Buffer composition used in ion exchange chromatography for T7 RNAP purification

3.9 PURIFICATION SCHEME OF HUMAN PLACENTAL RI

3.9.1 HRI NI-NTA AFFINITY CHROMATOGRAPHY

Sample preparation

- Frozen cell paste (6gr) expressing hRI was thawed on ice and resuspended in 18ml equilibration buffer (Table 14) at 4° C.
- Cells were disrupted initially by French press (sample was passed through the press 3 times). Sonication followed (5x30 seconds sonication steps, with 30sec resting intervals on a dry ice-ethanol bath).
- The lysate was centrifuged at 15000 g, 4 °C for 30 minutes.
- The cytoplasmic supernatant was collected and diluted in a ration 1:1 with equilibration buffer before loading to the subsequent chromatography column.

Ni-NTA agarose beads preparation

- In 3ml Ni-NTA agarose beads (3 Column Volume, 3CV) 2CV 0,1M NaOH were added and incubated for 30min.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV ddH_2O were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV equilibration buffer were added and incubated for 10 min before loaded onto the chromatography column.
- Ni-NTA beads were loaded carefully with the use of a Pasteur pipette onto the column.

Column preparation

- 0,1M NaOH were passed through the column's connectivity which was then washed with ddH20.
- Columns connectivity was equilibrated by passing Equilibration Buffer.

FPLC Ni-NTA Chromatography

- A chromatography column containing 3ml Ni-NTA agarose beads was packed and equilibrated with 10CV Equilibration Buffer.
- Next, the diluted cytoplasmic supernatant was loaded onto the column using a peristaltic pump with 0,5ml/min flow rate. Flow through sample was reloaded onto the column.
- After the sample was loaded the column was washed with 10CV Equilibration Buffer (flow rate: 2ml/min) and washed with 10CV Wash Buffer (flow rate: 2ml/min). Wash samples were collected.
- The bonded proteins were eluted by a linear gradient of imidazole concentration in Elution Buffer, starting from 50 and reaching 300 mM imidazole (10 CV) with a flow low rate of 2ml/min and collected in 15 fractions of 2ml each.
- One step elution at 600mM imidazole (flow rate 2ml/min) was also performed. 4 fractions were collected, 2ml each.
- DTT was added in all the elutions in final concentration 8mM.
- The fractions were analyzed by SDS-PAGE in order to assess their relative concentration in hRI and choose which ones will be further purified.

Ni-NTA Chromatography (hRI)				
Lysis	**Equilibration**	**Wash**	**Elution**	**Step Elution**
50 mM Tris-HCl pH 7.6	50 mM Tris-HCl pH 7.6	50 mM Tris-HCL pH 7.6	50 mM Tris-HCl pH 7.6	50 mM Tris-HCl pH 7.6
50mM NaCl	50mM NaCl	50mM NaCl	50mM NaCl	50mM Nacl
15mM KCl	15mM KCl	15mM KCl	15mM KCl	15mM KCl
5 mM Imidazole	5 mM Imidazole	20mM Imidazole	50-300 mM Imidazole	600 mMImidazole
10% glycerol	10% glycerol	10 % glycerol	10% glycerol	10% glycerol
0.1% Triton-X	0.1% Triton-X	0.1% Triton-X	0.1% Triton-X	0.1% Triton-X
1mM PMSF				

Table 14. Buffer composition used in Ni NTA chromatography for hRI purification

3.9.2 HRI BUFFER – EXCHANGE CHROMATOGRAPHY

Sample preparation

- Elutions 1-5 from Ni-NTA chromatography were merged in a single falcon (10ml total) and diluted 1:1 with dilution buffer (Table 15) before loaded onto the buffer-exchange Q-sepharose column.

Preparation of Q-Sepharose beads

- In 3ml Q-Sepharose beads (1 Column Volume, 1CV) 2CV 0,1M NaOH were added and incubated for 30min.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV ddH_2O were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 20CV equilibration buffer was added and incubated for 10 min
- Equilibrated Q-Sepharose beads were loaded onto a chromatography column.

Buffer-Exchange Chromatography

- The diluted sample from Ni-NTA chromatography was loaded onto the column using a peristaltic pump and flow through sample was collected.
- 5CV Wash1 buffer were added on the beads and wash1 sample was collected.
- 5CV Wash2 buffer were added onto the beads and wash2 sample was collected.
- 5CV Wash3 buffer were added onto the beads and wash3 sample was collected.
- 3 step elutions were performed at 300mM NaCl (5CV), 500mM NaCl (5CV) and 1M NaCl (2CV) and elution fractions were collected (3ml each).
- In all steps the flow rate was stable at 2ml/min.

Ion Exchange Chromatography (hRI)							
Dilution	**Equilibration**	**Wash 1**	**Wash 2**	**Wash 3**	**Elution 1**	**Elution 2**	**Elution 3**
15mM KCl	25mM Tris-HCl pH 7.6	25mM Tris-HCl pH 7.6	25mM Hepes pH 7.6	50mM Hepes pH 7.6	50mM Hepes pH 7.6	50mM Hepes pH 7.6	50mM Hepes pH 7.6
10% glycerol	25mM NaCl	25mM NaCl	25mM Tris-HCl pH 7.6	25mM NaCl	300mM NaCl	500mM NaCl	1M NaCl
0.1& Triton-X	15mM KCl	15mM KCl	25mM NaCl	15mM KCl	15mM KCl	15mM KCl	15mM KCl
	10% glycerol	10% glycerol	15mM KCl	10% glycerol	10% glycerol	10% glycerol	10% glycerol
	0.1% Triton-X	0.1% Triton-X	10% glycerol	0.1% Triton-X	0.1% Triton-X	0.1% Triton-X	0.1% Triton-X
			0.1% Triton-X				

Table 15. Buffer composition used in ion exchange chromatography for hRI purification

3.10 PURIFICATION SCHEME OF TEV PROTEASE

3.10.1 NI-NTA CHROMATOGRAPHY

Sample preparation

- Frozen cell paste (4gr) expressing TEV protease was thawed on ice and resuspended in 12ml Buffer A at 4^{o}C (Table 16).
- Cells were disrupted by sonication (10x30 seconds sonication steps, with 30sec resting intervals on a dry ice- ethanol bath).
- The lysate was centrifuged at 15000 g, 4 °C for 30 minutes.
- The cytoplasmic supernatant was collected and diluted in a ratio 1:1 with Buffer A before loaded to the subsequent chromatography column.

Ni-NTA agarose beads preparation

- In 4ml Ni-NTA agarose beads (1 Column Volume, 1CV). 2CV 0,1M NaOH were added and incubated for 30min.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV ddH_2O were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.
- 10CV equilibration buffer were added and incubated for 5 min
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was discarded.

Ni-NTA Chromatography

- The diluted cytoplasmic supernatant was loaded onto the equilibrated Ni-NTA beads in a 50ml falcon tube.
- Incubation followed at 4^{o}C for 1hr, under stirring followed.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (flow through sample).
- 10 CV of buffer A were added on the beads and the solution was stirred for 10 minutes.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (wash A sample).
- 10 CV of buffer B were added on the beads and the solution was stirred for 10 minutes.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (wash B sample).
- 10 CV of buffer C were added on the beads and the solution was stirred for 10 minutes.
- Beads were centrifuged at 1200 rpm, 1 min, RT and the supernatant was collected (wash C sample).
- One step elution was performed by adding 7CV of Buffer D. Elutions were collected in 4 fractions (1CV, 2CV, 2CV, 2CV respectively).
- All samples were analyzed by SDS-PAGE.
- All elution fractions were merged and dialyzed O/N in 1lt dialysis buffer

Ni NTA chromatograpgy (TEV)				
Buffer A	**Buffer B**	**Buffer C**	**Buffer D**	**Dialysis Buffer**
50mM Tris-HCl pH 8	50mM Tris-HCl pH8	50mM Tris-HCl pH8	50mM Tris-HCl pH8	50mM Tris-HCl pH8
1M NaCl	50mM NaCl	50mM NaCl	50mM NaCl	100mM NaCl
20% Glycerol	20% Glycerol	20% Glycerol	20% Glycerol	10% /50% Glycerol
5mM imidazole	5mM imidazole	30mM imidazole	300mM imidazole	5mM b-mercaptethanol

Table 16. Buffer composition used in Ni NTA chromatography for TEV purification

3.11 WESTERN BLOT

This method (also called protein immunoblot) is an analytical technique widely used to detect specific proteins in a sample. A synthetic or animal-derived antibody (known as the primary antibody) is created that recognizes and binds to a specific target protein. A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods such as staining, immunofluorescence, and radioactivity. The steps performed are the following:

- Transfer samples from the SDS-PAGE to a nitrocellulose membrane using Transfer Buffer (Table 17).
- Perform transfer (Biorad Transfer kit) for 1h and 15 min. at 4^{o}C, at 138 Volt.
- Transfer the nitrocellulose membrane into a shaking tank and add 50ml Blocking Buffer
- Incubate at RT for 1h.
- Wash three times with TTBS at RT while shaking. (1 x 15 min, 4 x 5 min)
- Add the first antibody and shake O/N at 4^{o}C. (In our case Serotech's mouse anti-His antibody in dilution 1:200)
- Wash three times with TTBS at RT while shaking. (1 x 15 min, 4 x 5 min)
- Add the second antibody and incubate 1h at RT while shaking. (In our case Amersham Scientific sheep anti-mouse IgG HRP antibody in dilution 1:200)
- Wash three times with TTBS at RT while shaking. (1 x 15 min, 4 x 5 min)
- Wash with TBS for 5 min. at RT while shaking
- Load ECL mix (Thermo Scientific, SuperSignal West Pico Chemiluminescence Substate) on the membrane and incubate 1 min. without shaking.
- In our case images were acquired by Chemi-Doc (Biorad) after 30 seconds exposure time.

Electrophoresis Buffer (1L)	Transfer Buffer (1L)	TBS 1x (1L)	Blocking Buffer	TTBS
14.4gr glycine	100ml electrophoresis buffer	200ml Tris-HCl pH 7.5-8	1x TTBS	970ml TBS
1gr SDS	200ml methanol	87.4gr NaCl	5% dry milk	30ml Tween-20
3.03gr Tris Acetate	700ml ddH_2O	ddH_2O until final volume is 1L		
ddH_2O until 1L				

Table 17. Buffer composition used in western blot experiment.

3.12 RNA MANIPULATION

As mentioned, ribonucleases are stable enzymes with high enzymatic activity and usually they do not need cofactors for their activity. Even in minimum quantities they can cause RNA degradation. For these reasons the sample as well as all the components evolved in RNA analysis (enzymes, buffers etc.) must be RNase free. In order to achieve an RNAse free environment the following steps must be followed:

- Wash the laboratory bench extensively with 0,1M NaOH, 70% EtOH, 1mM EDTA and double sterile ddH_2O (40min/121^oC), before and after the experiment.
- Clean pipettes with 0,1M NaOH and double sterile ddH_2O before and after usage.
- Sterilize all buffers in order to be RNase free.
- Always wear gloves.
- Always wear laboratory outfit.
- Use filter tips and one use stripettes.
- Wash RNA electrophoresis tank with soap and water.
- Prepare agarose gel with double sterile, RNase free ddH_2O.

3.13 TRIZOL EXTRACTION

This method offers the ability to isolate total RNA of high purity (also DNA and proteins) from cells or tissues. Components:
a)Trizol
b)Chloroform
c)Isopropanol
d)80% ethanol
e)RNase free ddH_2O

Procedure:

- Remove cell culture medium
- Wash with 1.5ml/cm petri cold PBS (4^oC) and remove it.
- Add 1ml Trizol/10cm petri and transfer cells in a 2ml tube.

- Add 0,2ml chloroform and vortex vigorously for 15min.
- Incubate at RT for 3-4 min.
- Centrifuge at 13.000 rmp for 15 min. at 4^{o}C.
- After centrifugation the mixture is separated in two phases, a soluble (RNA) and an organic (proteins). Transfer the upper phase in a new tube.
- Precipitate RNA by adding 0,5ml isopropanol per ml Trizol.
- Incubate for 10 min at RT and centrifuge at 13.000 rpm, for 10 min. at RT.
- Discard supernatant and add at least 0,2ml 75% ice cold ethanol per ml Trizol.
- Centrifuge at 13.000 rpm, for 10 min, at 4^{o}C.
- Discard ethanol, spin down and let the rest ethanol to air dry.
- Resuspend RNA pellet in 50-100μl ddH2O. (volume depends on the pellet's size)
- Quantify RNA concentration using NanoDrop.
- Perform sample electrophoresis in 1% agarose gel to verify RNA quality and quantity.
- Store sample at -20^{o}C.

3.14 PHENOL-CHLOROFORM EXTRACTION

This method is a liquid – liquid extraction technique in molecular biology, used to separate nucleic acids from proteins and lipids.

- Add water until final volume is 500μl.
- Add 0,1V CH3COONa (stock 3M, pH 5,2) for later precipitation.
- Add 550μl of phenol (pH 7)
- Vortex vigorously for 1 min.
- Centrifuge at 16,000g for 5 min, at 4^{o}C.
- Transfer aquatic phase in a new sterile tube.
- Add 550μl chloroform and vortex vigorously for 1 min.
- Centrifuge at 16,000g for 5min, at 4^{o}C.
- Transfer carefully only the aquatic phase in a new sterile tube.
- Add 1ml 100% absolute ethanol and incubate at -20^{o}C for 15 min.
- Centrifuge at 16,000g for 30min, at 4^{o}C.
- Remove supernatant and add 1ml of 70% absolute ethanol to the pellet.
- Vortex briefly and centrifuge at 16,000g for 2min, at 4^{o}C.
- Discard supernatant and perform a short spin to discard the remaining ethanol by pipetting.
- Allow to air dry under hood for 5 min.
- Resuspend pellet in ddH_2O.
- Measure concentration in nanodrop.
- Run sample in agarose electrophoresis gel.

3.15 QUANTIFICATION OF DNA/RNA/PROTEIN CONCENTRATION USING NANODROP.

The method lies on the property of nucleic acids to absorb ultraviolet radiation at 260nm. The more light is absorbed, the higher the DNA/RNA concentration. The same applies to proteins, which have the ability to absorb light at280nm, due to the aromatic side chains of their amino acids. In order to calculate the protein concentration, the molecular weight and the extinction coefficient of the protein must be known.
(https://tools.thermofisher.com/content/sfs/manuals/nd-1000-v3.8-users-manual-8%205x11.pdf)

3.16 QUALITY CONTROLS

3.16.1 QUALITY CONTROLS FOR T7 RNA POLYMERASE

3.16.1.1 T7 RNAP QUALITY CONTROLS FOR NUCLEASE DETECTION (ENDONUCLEASES/EXONUCLEASES)

In case of endonucleases the enzyme sample was incubated with the plasmid pUC19. For exonuclease detection λDNA was used (Table 18).

- The assay was prepared in an eppendorf tube.
- Reaction was incubated for 1.5h at 37°C.
- Sample was analyzed in 1% agarose gel.

Assay	T7 1x Reaction Buffer
10x Reaction Buffer	40mM Tris-HCl pH 8
0.5 of each rNTPs	6mM $MgCl_2$
1μg DNA	10mM DTT
100U T7 RNA polymerase	
ddH_2O until final volume 20μl	

Table 18. Reaction conditions for nuclease detection assay.

3.16.1.2 T7 RNAP FUNCTIONAL ASSAY

In order to test the activity of T7 RNAP a functional assay was prepared (Table 19).

- Reaction was incubated at 37°C for 2h.
- Sample was analyzed in 1% agarose gel.

Assay
10x Reaction Buffer
0.5 of each rNTPs
0.2-1µg DNA template
20U RI
ddH_2O until final volume 20µl

Table 19. Reaction conditions for T7 RNAP functional assay.

3.16.2 QUALITY CONTROLS FOR HRI

3.16.2.1 HRI FUNCTIONAL ASSAY

- In order to test the activity of hRI a functional assay was prepared .10U of hRI were incubated at RT for 10min in an eppendorf tube containing 10x hRI reaction buffer(50mM Tris-HCl pH 7.4, 50mM NaCl, 10mM DTT), 5ng RNase A and ddH_2O until the final volume reaches 10µl.
- 200ng Hela RNA were added and incubated for 10 more minutes at RT.
- 10µl of 5x DNA loading dye were added and sample was analyzed in 1% agarose gel to assess RNA protection.

4. RESULTS

4.1 T7 RNA POLYMERASE

4.1.1 T7 RNA POLYMERASE PURIFICATION

After the overexpression of T7 RNAP the cell pellet (2,6gr) was resuspended in 9ml Lysis Buffer and lysed by sonication. Centrifugation followed and the soluble supernatant was diluted 3 times (Total volume 27ml) with Equilibration Buffer and loaded onto 1ml Ni-NTA agarose beads. Protein was eluted from Ni-NTA resin by addition of Elution Buffer containing imidazole, which mimics the side chain of His residues and displaces the protein. The four (4) step elutions performed, containing imidazole in ascending concentrations (25mM, 50mM, 75mM, 200mM) resulted in 4 protein fractions (3ml, 3ml, 3ml, 5ml respectively). SDS-PAGE analysis (12,5% gel) followed for all samples from His-tag affinity chromatography in order to verify their purity (Figure13).

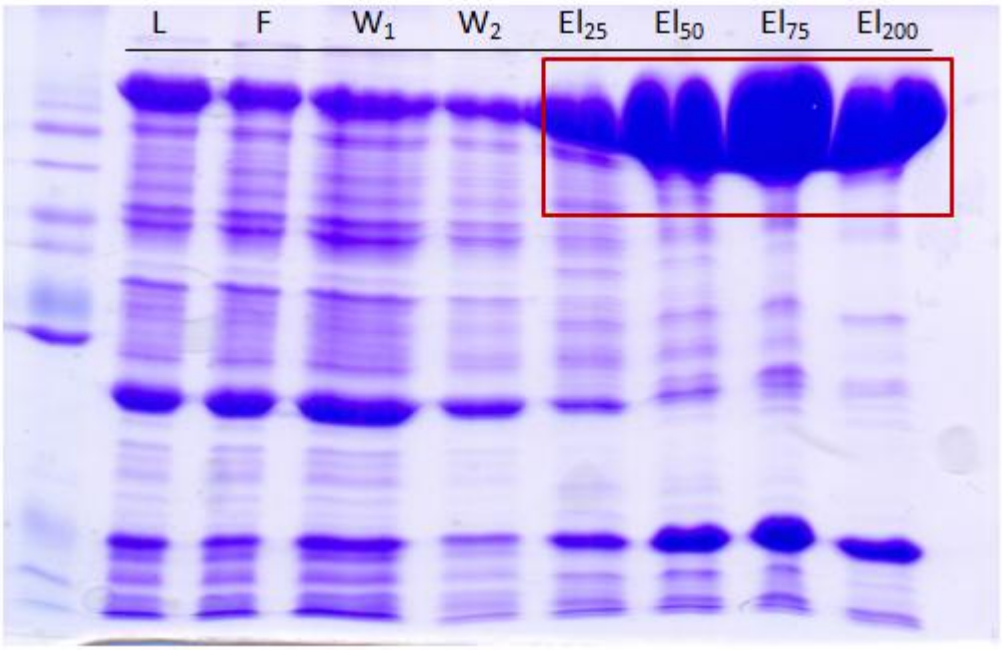


Fig. 13 : SDS-PAGE electrophoresis for all samples collected from Ni-NTA affinity chromatography. L:, the soluble supernatant loaded onto the beads, F:, the sample collected after Load addition, $W_{1/2}$: the samples collected after the addition of Wash Buffer, $El_{25/50/75/200}$: The step elutions performed.

As presented in Figure 13, fractions El $_{50/75/200}$ contain the highest amount of the protein. All four elutions were merged (total volume 14ml) for further analysis.

The merged sample (referred as Q_{load}) went under a second purification step, an Ion-Exchange chromatography. Before loading onto Q-Sepharose beads, Q_{load} was diluted with one volume of dilution buffer (V_{total} 28ml) in order to decrease NaCl concentration in half (50mM). We performed one step elution at 500mM NaCl, separated in two fractions, 7.5ml each. As shown in Q-Sepharose SDS-PAGE electrophoresis (Figure 14), El_1 contains the most of the eluted protein in high purity. El_1 sample went under further analysis and is referred as Minotech's T7 RNAP ($T7_M$ RNAP).

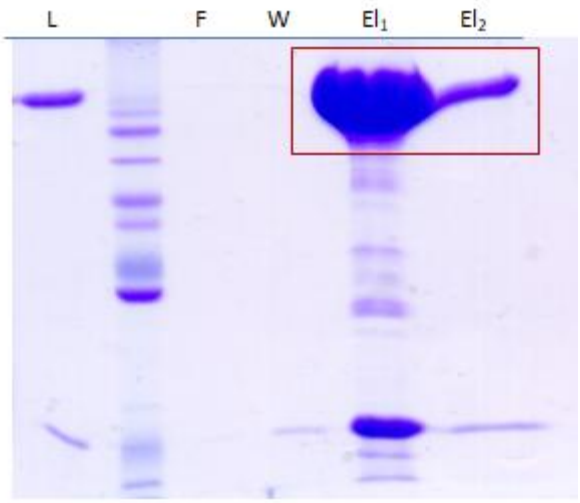


Fig.14 : Q-Sepharose samples SDS-PAGE electrophoresis. L: the merged Ni-NTA elutions, F: the sample collected after Load addition, W: the sample collected after wash buffer addition, $El_{1/2}$: the samples collected after protein elution.

4.1.2 PRELIMINARY UNIT DEFINITION

After $T7_M$ RNAP purification, preliminary unit definition was conducted by SDS-PAGE. $T7_M$ serial dilutions were analyzed (1/10, 1/20, 1/30) and compared against NEB's T7 RNAP. 1µl of each dilution and 2µl of NEB's T7 RNAP (50U/µl) were analyzed in 12.5% SDS-PAGE (Figure 15).

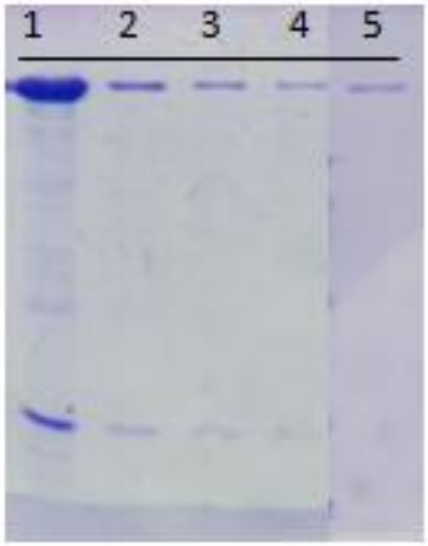


Fig.15 : Approximate unit definition by SDS-PAGE analysis
1: $T7_M$, 2: $T7_M$ 1/10, 3: $T7_M$ 1/20, 4: $T7_M$ 1/30, 5: NEB's T7 RNAP.

As determined by SDS-PAGE electrophoresis, 1μl $T7_M$ 1/30 contains the same protein amount with 2μl NEB's T7 Since 1μl NEB's T7RNAP contains 50 enzyme units we assume that $T7_M$ RNAP 1/30 dilution contains 100U/μl.

4.1.3 QUALITY CONTROLS

4.1.3.1 NUCLEASE DETECTION

In order to detect the presence of undesired nuclease activities in $T7_M$, 2μl of the enzyme was incubated with 1μg nucleic acid. The reaction was set as mentioned in 3.14.1.1 section. In the case of endonuclease detection assay the enzyme sample was incubated with plasmid PBR322 while for exonuclease detection λDNA and λDNA/StyI templates were used. For ribonuclease detection Hela RNA was selected. As depicted in Figure 16. the enzyme is nuclease free.

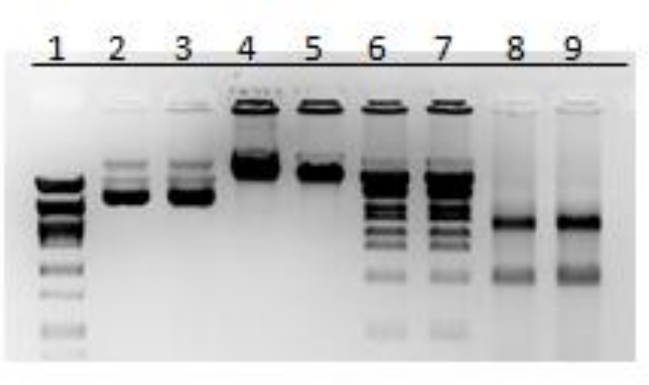


Fig.16 : 1% agarose gel electrophoresis for nuclease detection.
1: λDNA/PstI DNA ladder, 2: PBR322, 3:PBR322-T7 RNAP, 4:λDNA, 5:λDNA-T7 RNAP, 6:λDNA/StyI, 7:λDNA/StyI-T7, 8:Hela RNA, 9: Hela RNA-T7.

4.1.3.2 FUNCTIONAL ASSAY

Since the enzyme is free of unwanted enzymatic activities, the next step was to examine the enzyme activity of RNA polymerase. The reactions were set as in 3.14.1.2 section. 100ng Pjsc011 plasmid (results to 5.601 bs transcript) was used as a template in order to check the T7RNAP ability to transcribe supercoiled DNA. Regarding linearized templates, 100ng Pjcs011/BamHI digest (results to 4.727 bs transcript) and 100ng Pjsc011/EcoRI digest (results to 2.689 bs transcript) were tested. The efficiency of $T7_M$ RNAP was compared to NEB's T7 RNA pol.

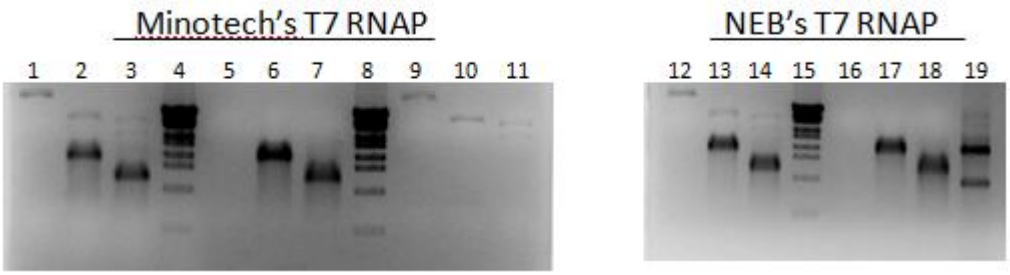


Fig.17 : Functional Assay of $T7_M$ RNAP compared to NEB's.
1: Pjsc011 +$T7_M$, 2:Pjsc011/BamHI + $T7_M$, 3: Pjsc011/EcoRI + $T7_M$, 4:λDNA/StyI DNA ladder, 5: Pjsc011 + $T7_M$ + DNaseI, 6:Pjsc011/BamHI + $T7_M$ + DNase I, 7: Pjsc011/EcoRI + $T7_M$ + DNaseI, 8:λDNA/StyI DNA ladder, 9: Pjsc011, 10: Pjsc011/BamHI, 11: Pjsc011/EcoRI, 12: Pjsc011 + T7, 13: Pjsc011/BamHI + T7, 14:Pjsc011/EcoRI + T7, 15: λDNA/StyI DNA ladder, 16: Pjsc011 + T7 + DNase I, 17: Pjsc011/BamHI + T7 + DNase I, 18: Pjsc011/EcoRI + T7 + DNase I, 19: 250ng Hela RNA.

As shown in Figure 17 none of the enzymes transcribed supercoiled DNA. However the linear templates were efficiently transcribed in both enzyme reactions.
Another attempt was made to test the enzyme's ability to transcribe supercoiled DNA. As shown in Figure 18 no transcript was produced. $T7_M$ RNAP capacity to perform long length DNA template transcription was successfully tested by adding 100ng Pjsc011/BseCI (results to 5.582 bs transcript) in the reaction (Fig. 6-9)

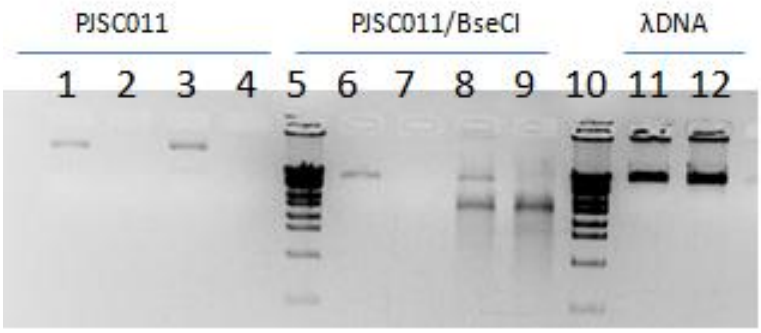


Fig.18 : Functional Assay of $T7_M$ RNAP.
1: Pjsc011, 2: Pjsc011 + DNase I, 3: Pjsc011 + $T7_M$, 4: Pjsc011 +$T7_M$ +DNase I, 5: λDNA/StyI DNA ladder, 6: Pjsc011/BseCI, 7: Pjsc011/BseCI + DNase I, 8: Pjsc011/BseCI + $T7_M$, 9: Pjsc011/BseCI + $T7_M$ + DNaseI, 10: λDNA/StyI DNA ladder, 11: λDNA, 12: λDNA + $T7_M$

The enzymes specificity to T7 promoter was screened by adding 500ng λDNA as a DNA template, which lacks T7 promoter sequence. The enzyme's activity depends on the presence of T7 promoter, as no RNA transcript was produced (Figure 18).

An attempt to increase the transcription efficiency was carried out by adding a) 2mM $MgCl_2$, b) 5mM DTT, c) 2mM $MgCl_2$, 5mM DTT in the reaction mix. As presented by the agarose electrophoresis gel, Figure 19, among the additives, DTT seems to increase the enzyme activity. The higher production rate arise some risks, including prematurely terminated products. Strand displacement may occur faster than usual leading to the unloading of T7 RNAP from the template. The efficiency of the transcription may be increased but the enzyme might be prone to errors.

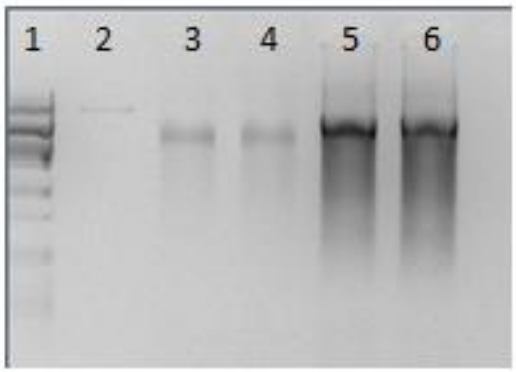


Fig.19 :Attempts to increase T7 $_M$ RNAP activity.
1:λDNA/PstI DNA ladder, 2: 100ng Pjsc011/BseCI DNA template, 3:Standard transcription reaction, 4: addition of 2mM $MgCl_2$, 5: addition of 5mM DTT, 6: addition of 2mM $MgCl_2$ and 5mM DTT.

As long as DTT seems to increase transcription levels, different DTT (1mM, 2mM) concentrations were added to the reaction mix for further optimization. In addition, reaction buffer was prepared and checked against NEB's (Table 20).

Minotech Reaction Buffer 10x	NEB Reaction Buffer 10x
400 mM Tris HCl pH 7.9	400mM TrisHCl pH 7.9
60mM $MgCl_2$	60mM $MgCl_2$
10mM DTT	10mM DTT
	2mM Spermidine

Table 20: Composition of T7 RNAP reaction buffers.

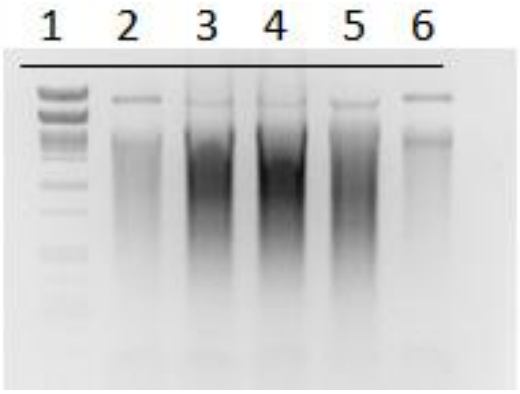


Fig.20 : Transcription reaction in order to check DTT concentrations and Minotech's 10x reaction buffer.
1:λDNA/PstI DNA ladder, 2: Standard transcription reaction, 3: Addition of 1mM DTT, 4: Addition of 2mM DTT, 5: Assay with Minotech's 10x reaction buffer, 6: Assay with NEB's 10x reaction buffer. DNA template: 100ng Pjsc011/BseCI.

According to Figure 20, all samples are RNase contaminated. An exhaustive check was performed on all the components of the reaction to detect the contaminated sample. All additives were incubated with 500ng RNA for 2h at 37^{o} C as shown in 3.14.1.1 section. As concluded by the electrophoresis gel the source of contamination was from the DNA templates (Figure 21).

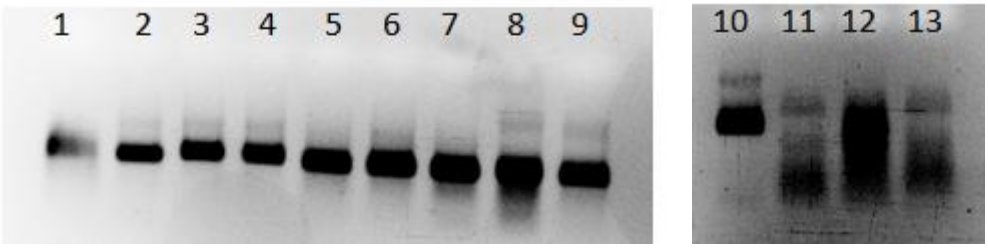


Fig.21 : Ribonuclease control in all transcription reaction components.
1: ddH2O, 2:Minotech's 10x reaction buffer, 3: NEB's 10x reaction buffer, 5: DTT, 6:MgCl2, 7:rNTPs, 8: T7M RNAP, 9: NEB's T7 RNAP, 10: RNA control, 11: Pjsc011/BamHI, 12: Pjsc011/EcoRI, 13: Pjsc011/BseCI.

Once RNase contamination was overcome, we performed a new functional assay to compare Minotech's and NEB's 10x reaction buffer. As shown in Figure 22, more RNA product is observed in the reaction containing Minotech's 10x reaction buffer.
In order to further improve the transcriptional efficiency of T7M reaction, two new reaction buffers were prepared (Table 21) while Takara's reaction buffer was also tested. As shown in Figure 23, $T7_{M}$RNAP efficiency is greater than NEBs 'RNAP in all buffers tested.

Takara Reaction Buffer	Minotech Reaction Buffer 1	Minotech Reaction Buffer 2
400 mM Tris-HCl pH 8.0	400 mM Tris-HCl pH 8.0	400 mM Tris-HCl pH 8.0
80 mM MgCl2	80 mM MgCl2	60 mM MgCl2
50mM DTT	50mM DTT	20mM DTT
20mM spermidine		

Table 21: Composition of T7 RNAP reaction buffers.

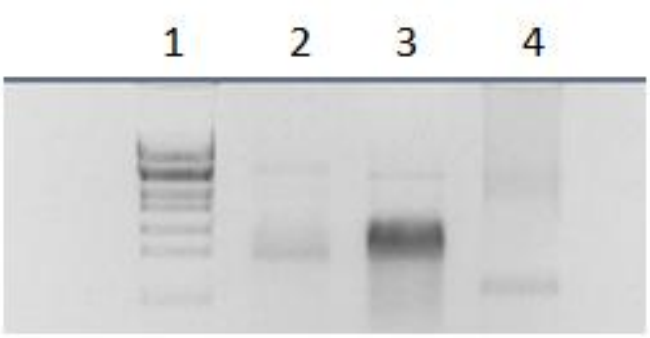


Fig.22 : Functional Assay to compare Minotech's 10x reaction buffer to NEB's. 1: λDNA/StyI DNA ladder, 2: NEB's buffer, 3:Minotech's buffer, 4: Hela RNA. DNA template :100ng Pjsc011/EcoRI

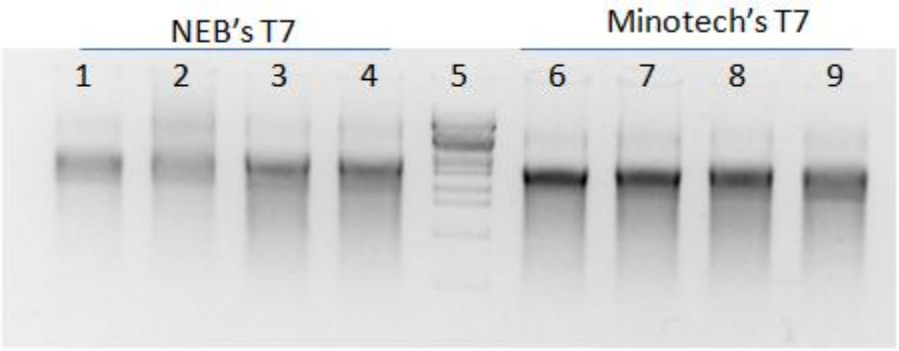


Fig.23 : Functional Assay to compare NEB's and Minotech's T7 RNAP efficiency in different reaction buffers.
1/6: NEB's buffer, 2/7:Takara's Buffer, 3/8:Buffer 1, 4/9: Buffer 2, 5: λDNA/StyI DNA ladder. DNA template: 100ng Pjsc011/BamHI

In order to determine the amount of RNA that was produced during an *in vitro* transcription reaction with $T7_M$ RNAP we performed functional assays using 1/5, 1/10, 1/20 $T7_M$ dilutions from the initial stock (3000U/µl). After the completion of the respective reactions the RNA products were extracted by phenol-chloroform and rediluted in 22µl ddH_2O. All RNA concentrations were determined by NanoDrop. In assay 4 (T7 1/10) which seems to be the most efficient, from 100ng DNA template the total amount of RNA produced is 2.31 µg (Figure 24). The amplification factor of the transcription is over 20X.

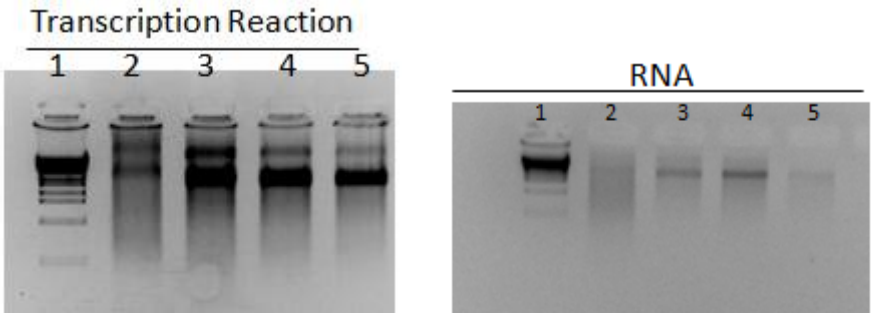


Fig.24 : Transcription reaction and respective purified RNA .
1: λDNA/StyI DNA ladder, 2: T7, 3: T7 1/5, 4: T7 1/10, 5: T7 1/20. DNA template: 100ng Pjsc011/BseCI. 2µl RNA run in 1% agarose gel.

4.2 HUMAN PLACENTAL RIBONUCLEASE INHIBITOR

4.2.1 CONSTRUCTION OF HIS-MBP-HRI

The cloning procedure to insert His-MBP-hRI in the expression vector Pjcs011 was achieved using the NEBuilder DNA Assembly Cloning Kit (section 3.1) (Figure 25).

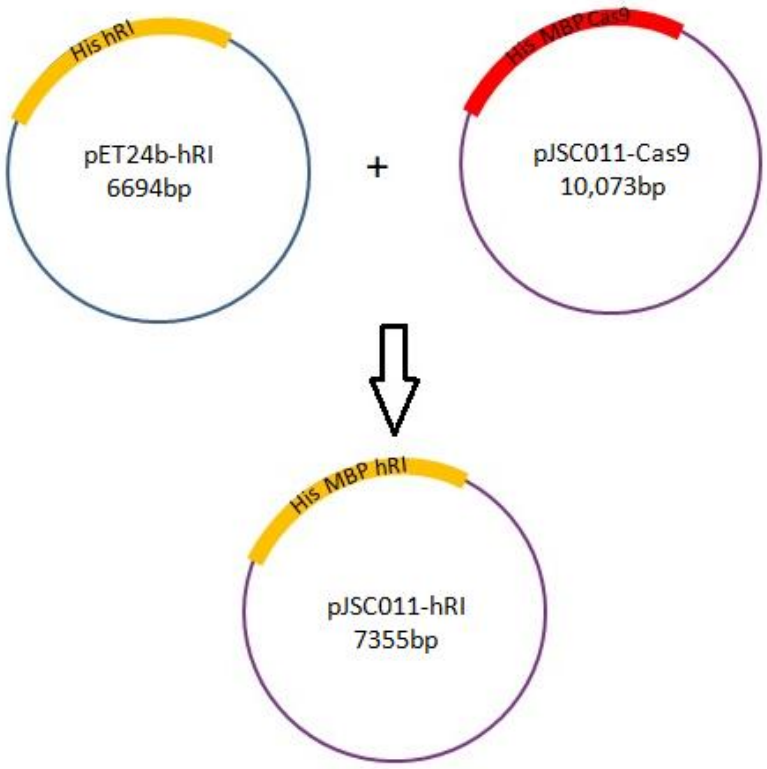


Fig.25 : Final vector arrangement as occurring after NEBuilder reaction.

Both hRI gene and Pjsc011 plasmid vector (initial stock 500ng/µl) were amplified by PCR using the primers according to NEBuilder instructions. A PCR purification step (Figure 26) followed which resulted to 25ng/µl HRI and 30ng/µl Pjsc011 plasmid vector.

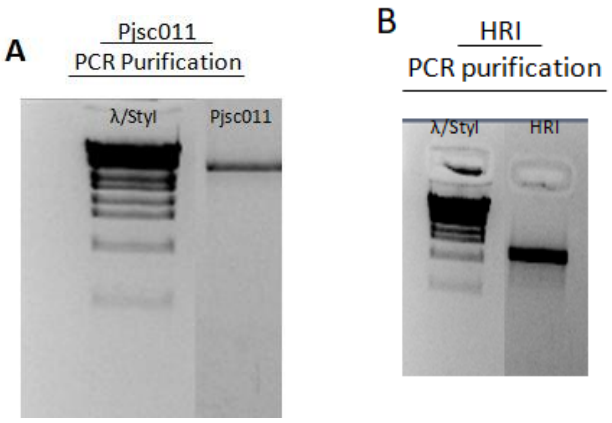


Fig.26 : Pjsc011 (A) and HRI (B) PCR purification. 10ng Pjsc011 and 1ng HRI were added in each PCR reaction. After PCR purification we ended up with 20μl Pjsc011 and 20μl HRI, containing 30ng/μl and 25ng/μl respectively.

The final reaction was set by adding 70ng Pjsc011 and 30ng HRI. After NEBuilder reaction, 2μl were used to transform F' I^q *E.coli* competent cells and the following day three single colonies, HRI-1, HRI-2, HRI-3 were selected. Cloning efficiency was verified by colony PCR, restriction digest screening and sequencing (T7 promoter, T7 terminator sequencing primers, data not shown) (Figure 27).

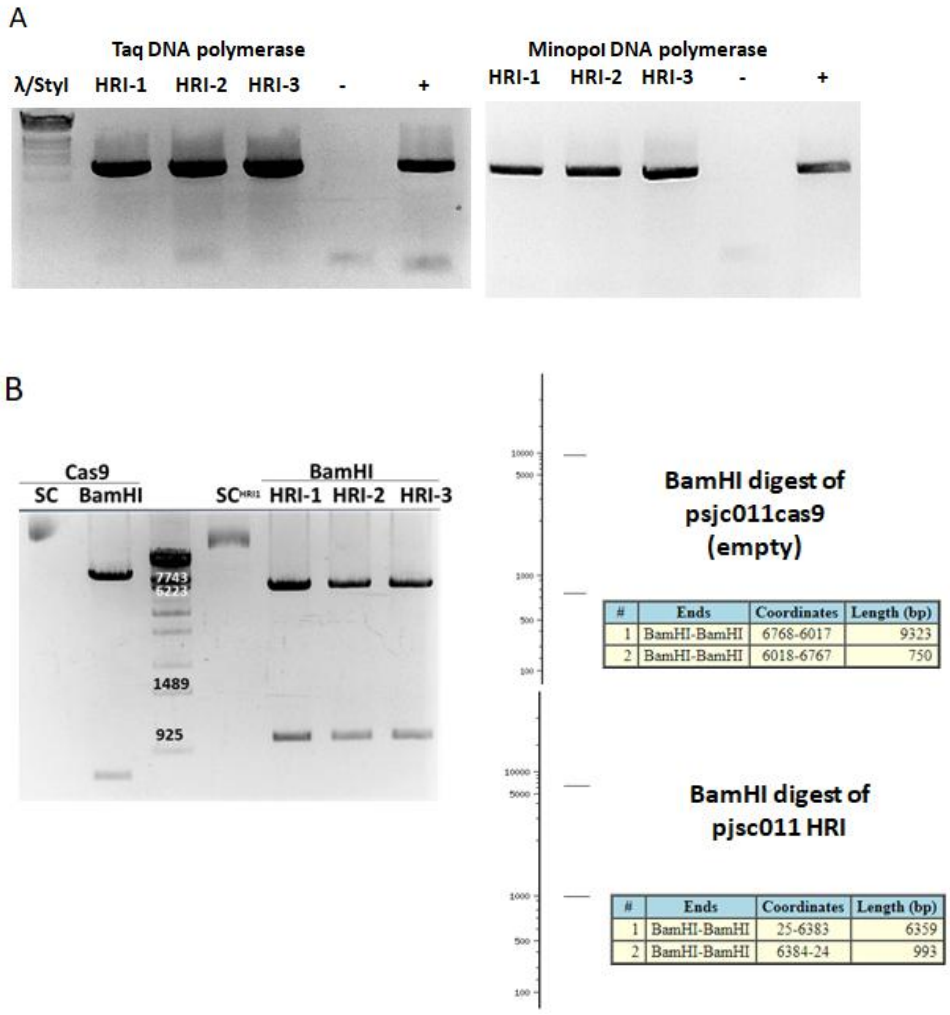


Fig.27 : A. Colony PCR performed for the selected HRI colonies. As a negative control ddH_2O was added while in the positive control we used the initial vector containing HRI gene, pET24b. B. Screening digests for the three HRI vectors. As a control we used pjsc011 vector containing the HYPA Cas9 gene. SC stands for supercoiled plasmid.

As shown in Figure 27 A, hRI gene was detected in all transformants. Furthermore restriction digests, Figure 27 B, confirmed the successful cloning. BamHI digests were expected to create two fragments, 6359bp and 993bp.

4.2.2 OVER EXPRESSION AND PURIFICATION OF HRI

After veryfing that the construct contains the gene of interest, the next step was the heterologous expression of the target protein. The hRI-1, 2, 3 plasmids were transformed into BL 21 Star PLys *E.coli* cells and small scale overexpression was tested (5ml liquid broth). As seen by SDS-PAGE analysis, Figure 28, high level expressions were obtained for all three hRI constructions in BL21 cells. As there was no difference in the expression level between the three constructs, hRI-1 was randomly selected for the following procedures.

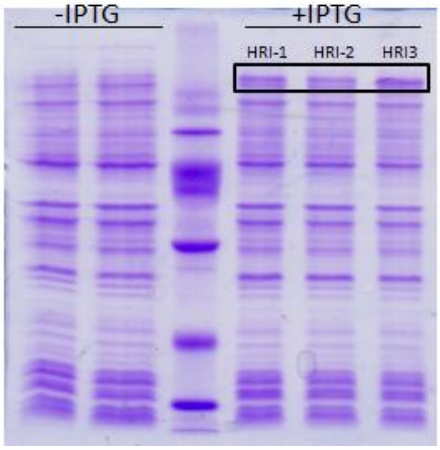


Fig.28 :hRI small scale induction.

A larger scale expression procedure followed using hRI-1 glycerol stock as the starting material No expression level differences were observed at 1.5 and 3h after IPTG induction. Protein solubility was also tested at the same time points. Cell pellets from 1.5ml cell culture were diluted at different lysis buffers in order to identify the most suitable one. (For details see 3.5 section) (Table 22).

Buffer composition					
1	2	3	4	5	6
50mM Tris-HCl	50mM Tris-HCl	50mM Tris-HCl	50mM Tris-HCl	50mM NaH_2PO_4	50mM Tris-HCl
500mM NaCl	50mM NaCl	500mM NaCl	500mM NaCl	500mM NaCl	50mM NaCl
10% glycerol	10% glycerol	0,2% Tween 20	10% glycerol	10% glycerol	10% glycerol
0,1% Tween 20	0.1% Triton-X		0,2% Tween 20	0,1% Triton-X	0,1% Triton-X

Table 22.: Buffers tested for hRI solubility.

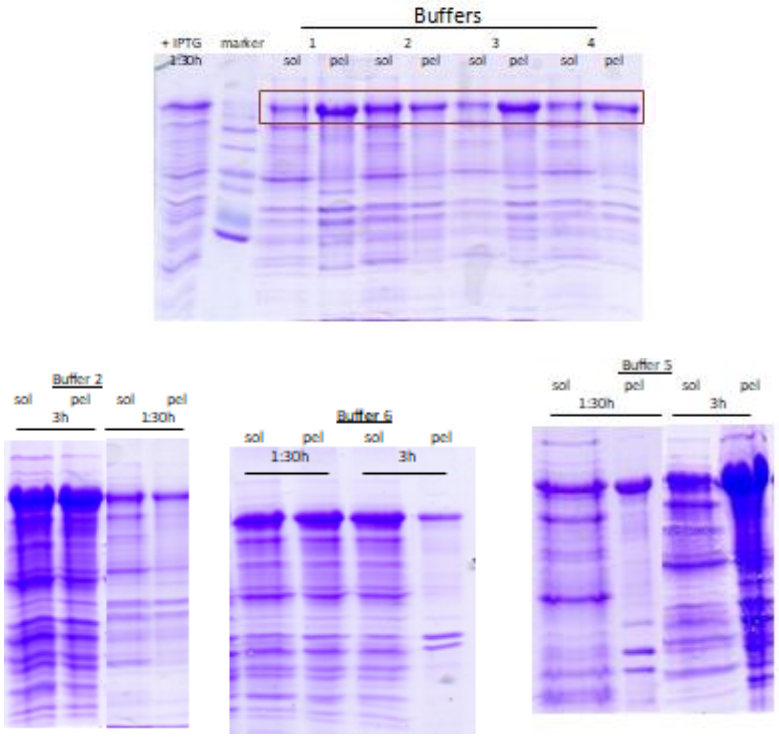


Fig.29 : SDS-PAGE analysis of HRI solubility test. Both soluble and insoluble portion from each buffer, in 1.5 and 3h of induction , was analyzed . Sol. stands for soluble supernatant and pel. for insoluble pellet.

As shown in Figure 29, HRI is less soluble in high salt concentration. In buffer 2 and 6 HRI more than 50% is in the soluble fraction rather than in the insoluble cell debris faction.

4.2.2.1 HRI LARGE SCALE OVER EXPRESSION AND PURIFICATION

LOT1

After standardizing the buffer conditions in which the protein is soluble, a large scale hRI expression was prepared. 100ml culture was grown overnight and used for the inoculation of 4L LB medium (1/.. dilution). Samples before and after induction (time points at 1.5 and 3h) were analyzed by 10% SDS-PAGE, to estimate the induction level of protein expression (Figure 30).

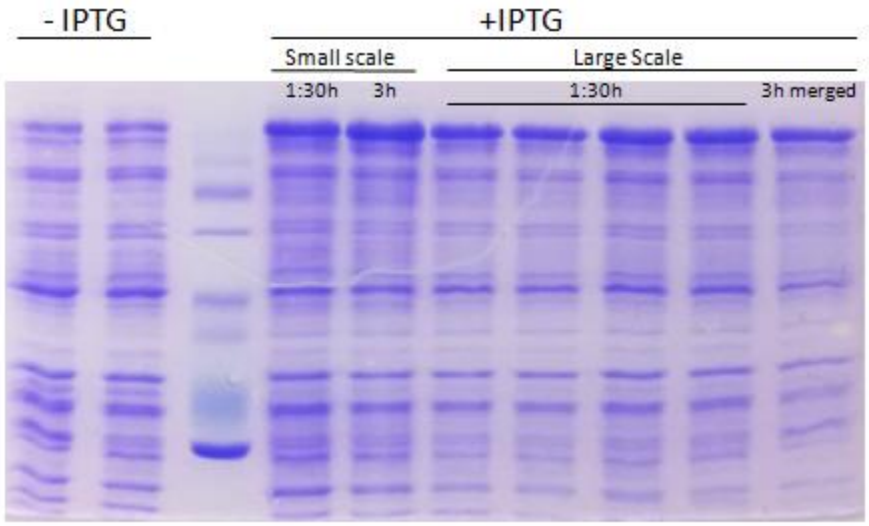


Fig.30 : Large scale induction of HRI expression. On SDS-PAGE minus (-) and plus(+) IPTG samples are analyzed. Induction levels between small scale and large scale expression is compared. After 3h of induction all 4L cell cultures were merged in one.

From 4L cell culture 8.9gr cell pellet were collected. 2gr were lysed by sonication, the soluble supernatant was loaded onto 1ml Ni-NTA agarose beads. and elution was performed at 250mM imidazole. In parallel TEV protease (from 1gr cell paste) was partially purified (TEV purification scheme – section 3.8.3). Both hRI and TEV purification procedures were analyzed in SDS-PAGE (Figure 31).

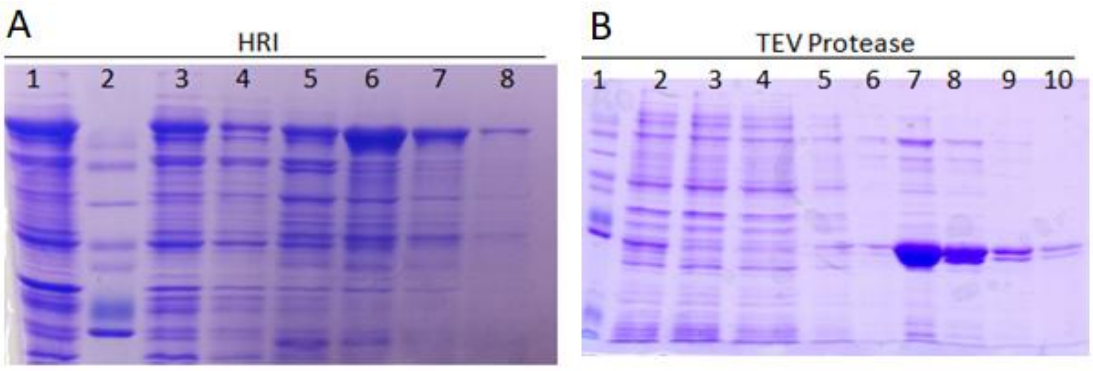


Fig.31 : hRI and TEV protease purification by Ni-NTA chromatography. A) hRI purification. 1, soluble supernatant loaded onto the beads; 2, protein marker; 3, Flow through sample; 4,wash sample; 5-8, step elutions at 250mM imidazole (1CV, 2CV, 2CV, 2CV respectively). B) TEV purification. 1,protein marker; 2, soluble supernatant loaded onto the beads; 3-6, wash samples; 7-10, step elutions at 300mM imidazole (1CV, 2CV, 2CV, 2CV respectively).

Regarding hRI purification, a large amount of protein did not bind on Ni beads (flow through fraction) while elutions appeared to have a lot of protein impurities. Nevertheless, hRI elutions 6-8 were merged in a single falcon. On the other hand TEV protease purification scheme was successful and all elution fractions were joined together. Both TEV and hRI samples were dialyzed overnight (section 3.8.4). After dialysis protein quantitation was determined by Nanodrop. hRI concentration was 0,61mg/ml while TEV protease was 0,2mg/ml.

The first attempt to cleave the His-MBP fusion tag from the hRI protein was by preparing a cleavage reaction following the mass ratio TEV/HRI : 1/4 followed by Ni-NTA chromatography step. The untagged hRI would normally remain in the flow through sample, while His-MBP will bind to the Ni beads and subsequently be present in the elution fractions.

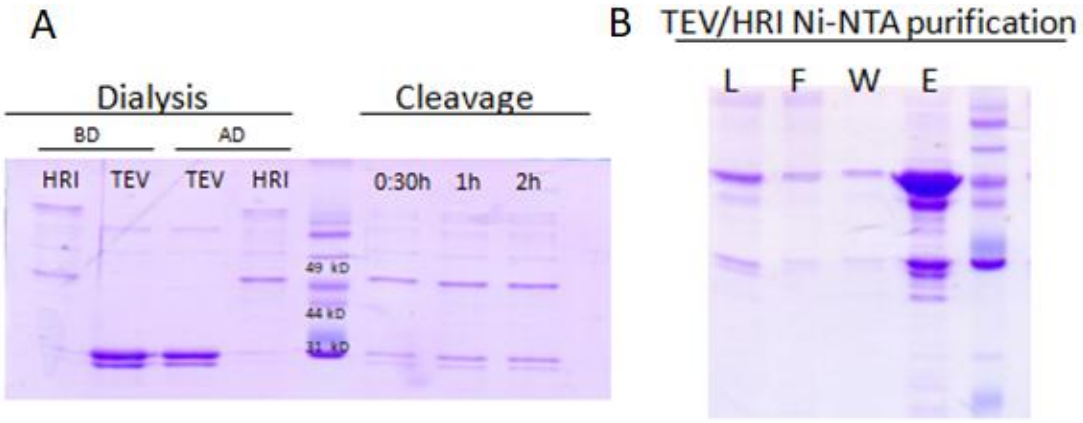


Fig. 32: A) hRI and TEV samples before (BD) and after (AD) dialysis and cleavage reaction in 0.5, 1 and 2h. B) TEV/HRI cleavage reaction purification. L, 2h hRI digested sample was loaded; F, Flow through, where digested hRI is expected to be; W, wash; E, elution, where His-MBP tag is eluted.

hRI even before dialysis seems to be partially degraded and cleavage reaction gives the same results in all three time points. As for cleavage mix Ni purification efficiency, we can not be sure whether the band visible in Flow through, Wash and Elution stands for HRI or His-MBP, as their molecular weights are close enough (hRI 49.9kD, His-MBP 42.3 kD) (Figure 32).

LOT2

Another attempt for over expression of hRI and TEV was performed (Figure 33). 6L HRI cell culture and 3L TEV were harvested by centrifugation and resulted in 14,7gr and 10,7gr cell pellet respectively. Both hRI and TEV were purified as mentioned in section 3.8.2 (hRI purification scheme) and 3.8.3 (TEV protease purification scheme) (Figure 34).

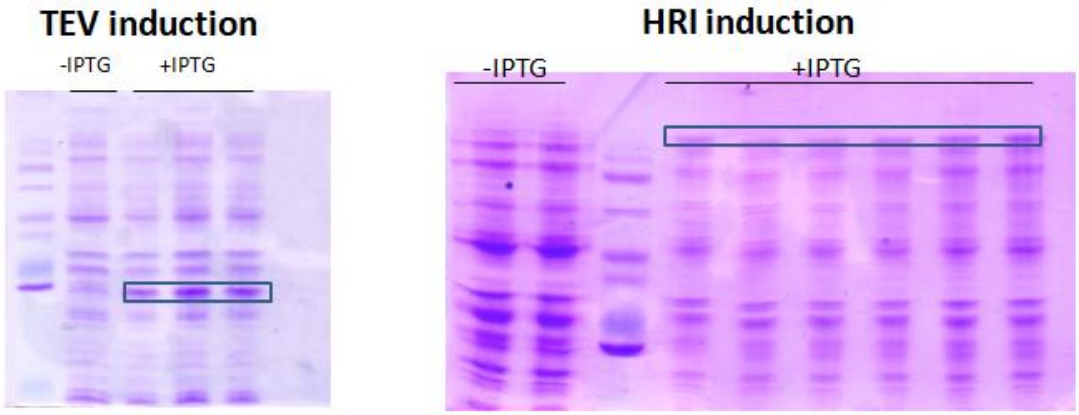


Fig.33 : TEV and hRI induction analyzed on SDS-PAGE.

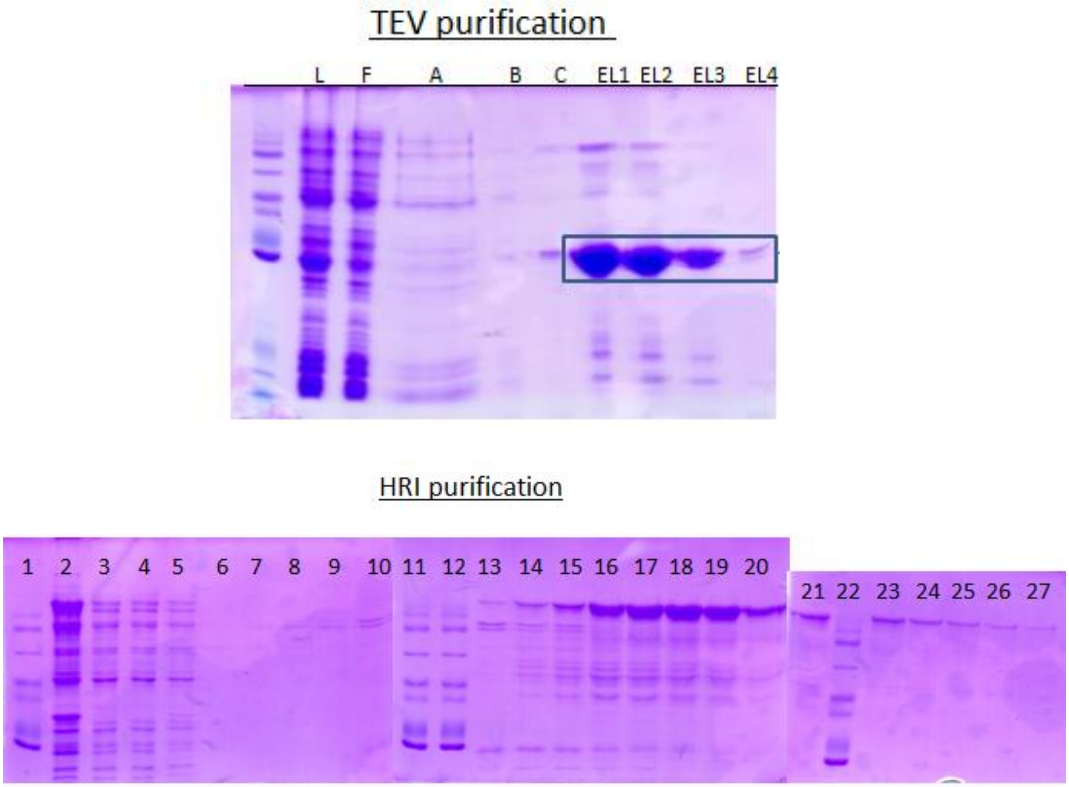


Fig.34 : TEV protease and hRI Ni-NTA chromatography. TEV: L,load; F,Flow through; A/B/C,washes; El1-4, step elutions at 300mM imidazole. hRI: 2,load; 3,Flow through 1; 4, Flow through 2; 5, wash; 6-10/13-21/23, elutions by increasing imidazole gradient; 24-27, step elutions at 600mM imidazole; 1/11/12/22, protein marker.

By using FPLC equipment rather than gravity flow chromatography, hRI purity is much higher than the previous attempt.. HRI starts to elute a 120 mM imidazole and continuous until 600mM. Fractions with the highest protein quantity and purity, 15-19 and 20-25 (hRI_{Nickel}), were selected for further analysis. In particular fractions 15-19 went under a second purification step, ion-exchange chromatography. Due to the protein's inability to remain soluble under high salt concentration, each elution was diluted until the final NaCl concentration is 100mM. That resulted in over-diluted samples, which were not visible in SDS-PAGE analysis (Figure 35). Nevertheless, elutions at 500mM NaCl (50ml) were concentrated until the final volume reached 3ml and were named $hRI_{Q.}$

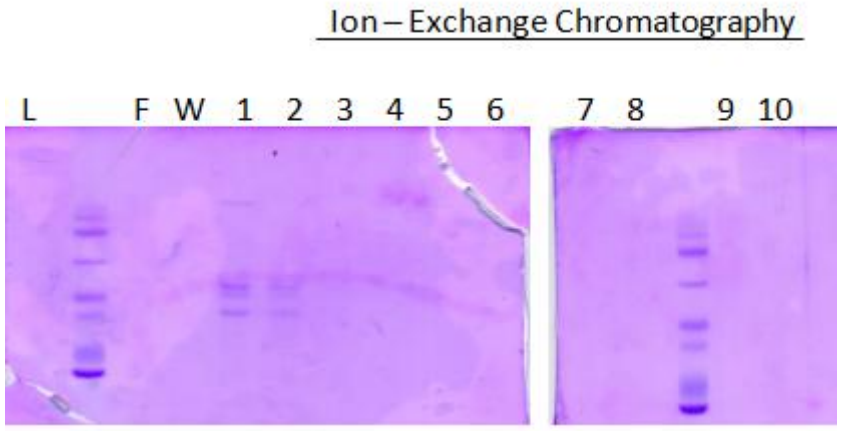


Fig. 35: Ion – exchange chromatography for fractions 15-19 occurred after affinity chromatography. L, load; F,Flow through; W, wash; 1/2, elution at 100mM NaCl; 3/4, elution at 200mM NaCl; 5/6, elution at 300mM NaCl; 7/8, elution at 500mM NaCl; 9/10, elution at 1M NaCl.

hRI_{Nickel}, hRI_Q and TEV protease (elutions 1,2) concentrations were measured by Nanodrop:

- hRI_{Nickel}: 0,6 mg/ml
- hRI_Q: 0,2mg/ml
- TEV: 0,48mg/ml

All three samples were dialyzed overnight. A TEV cleavage overnight dialysis reaction was set (hRI_{Nickel}/TEV) according to Nanodrop measurements in mass ratio 1/4 as in the previous experiment (Table 23). After dialysis, a small amount of TEV protease was stored in 50% glycerol and the following reactions were prepared:

36h TEV/HRI reaction
500µl hRI_Q /153µl TEV
500µl hRI_Q / 306µl $TEV_{50\% \text{ glycerol}}$
500µl hRI_{Nickel} /153µl TEV
500µl hRI_{Nickel} /306µl $TEV_{50\% \text{ glycerol}}$

Table 23.: TEV cleavage reactions.

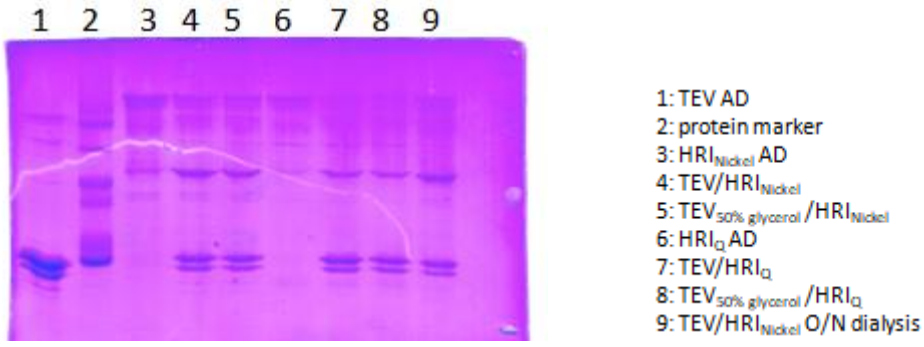


Fig.36 : SDS-PAGE electrophoresis of after dialysis protein samples and TEV reaction samples.

All TEV digests have partially cleaved His-MBP from the hRI and no great difference is observed in the cleavage level between TEV and $TEV_{50\% \text{ glycerol}}$ (Figure 36). TEV reactions were further purified by Ni-NTA chromatography (Figure 37).

1µl of fractions mentioned in Figure 37 were used in functional assays to check RNase inhibition (Figure 38). NEB RNase out was used to tests the inhibitory activity against 10ng, 5ng and 1ng RNase A (Figure 38 A). At the presence of 5ng RNase A, NEB's RI exhibits 50% inhibitory effect. Our sample were tested against5ng RNase A, but no inhibitory effect was observed. RNA was totally degraded in all samples (Figure 38 B).

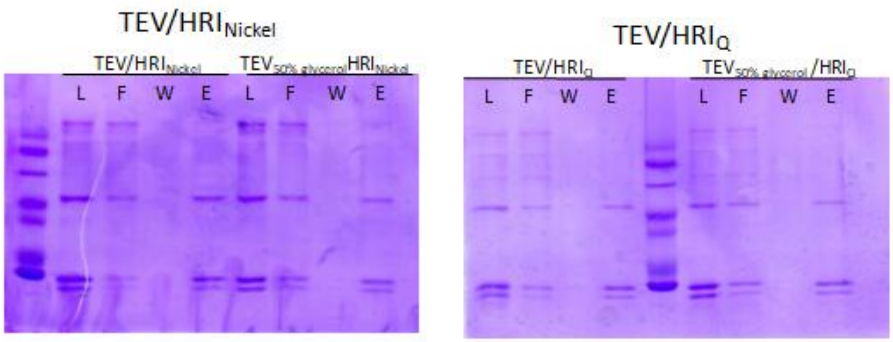
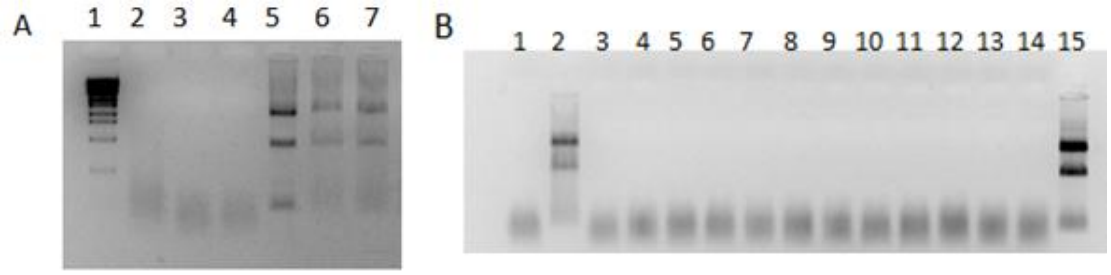


Fig.37 : TEV/hRI reaction Ni-NTA chromatography. L,load; F, flow through; W, wash; E, elution.



Fig,38 : Functional assay of RI A. NEB's RI. 1, λ/StyI DNA ladder; 2, 1ng RNase A; 2, 5ng RNase A; 4, 10ng RNase A; 5, 1ng RNase A/10U RI; 6, 5ng RNase A/10U RI; 7, 10ng RNase A/10U RI. B. Fractions occurred by TEV/HRI affinity chromatography in presence of 5ng RNase A. 1, 5ng RNase A; 2, NEB RNase out; 3, 1µl Load TEV/hRI$_{Nickel}$; 4, 1µl Ft TEV/hRI$_{Nickel}$; 5, 1µl E TEV/hRI$_{Nickel}$; 6, 1µl Load TEV$_{50\% glycerol}$ /hRI$_{Nickel}$; 7, 1µl Ft TEV$_{50\% glycerol}$ /hRI$_{Nickel}$; 8, 1µl E TEV$_{50\% glycerol}$ /hRI$_{Nickel}$;9, 1µl Load TEV /hRI$_Q$; 10, 1µl Ft TEV /hRI$_Q$; 11, 1µl E TEV /hRI$_Q$; 12, 1µl Load TEV$_{50\% glycerol}$ /hRI$_Q$; 13, 1µl Ft TEV$_{50\% glycerol}$ /hRI$_Q$; 14, 1µl E TEV$_{50\% glycerol}$ /hRI$_Q$; 15. . In all samples 200ng Hela RNA was added.

LOT 3

5gr of hRI cell pellet and 2gr TEV protease were lysed by sonication and purified by Ni-NTA chromatography (Figure 39). hRI is detected at 120mM imidazole (fraction 11). Fractions 14-17 and 18-22, which are the most abundant in hRI were merged and quantified by Nanodrop. Regarding TEV protease one step elution was performed at 300mM imidazole, which was quantified by Nanodrop as well.

- hRI$_{14-17}$:0,125mg/ml
- hRI$_{18-22}$: 0,09mg/ml
- TEV: 0,4mg/ml

Overnight dialysis digests were prepared according to the molecular ration 1/2:hRI/TEV (Fig.). hRI$_{14-17}$ and hRI$_{18-22}$ went under dialysis to remove imidazole while TEV protease was dialyzed in 50% glycerol dialysis buffer. After dialysis, digest reactions were purified by Ni-NTA chromatography (Figure 40).

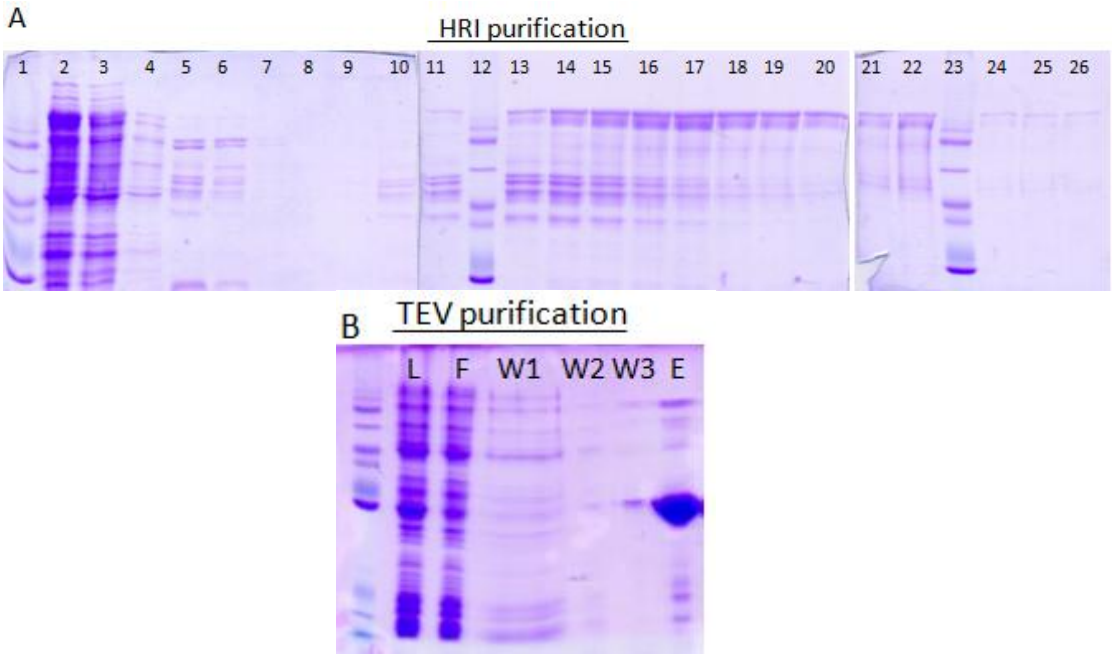


Fig.39 : HRI and TEV Ni-NTA affinity chromatography. A. HRI purification. 2, load; 3, flow through; 4, wash 1; 5, wash 2; 6-11,13-21, elution by imidazole gradient from 50-300mM imidazole; 22,24-26, step elutions at 600mM imidazole. B. TEV purification. L,load; F, flow through; W1,wash 1; W2,wash2; W3, wash 3; E, elution at 300mM imidazole.

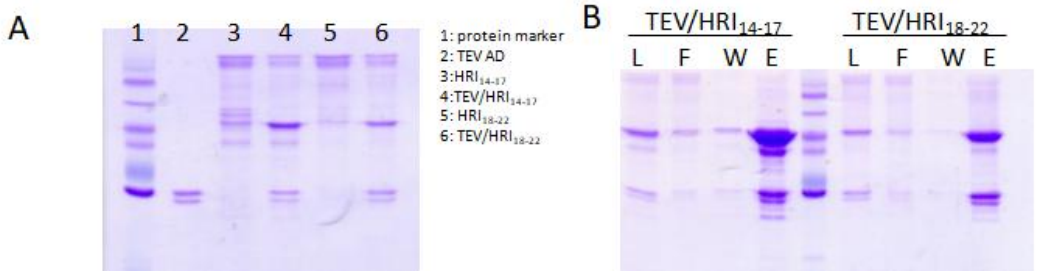


Fig. 40. :A. After dialysis HRI and TEV samples and O/N dialysis TEV/HRI digests. B.TEV/HRI$_{14-17}$ and TEV/HRI$_{18-22}$ purification. L, load; F, flow through; W, wash; E, elution at 200mM imidazole.

As seen in Figure 40, hRI-MBP was not entirely cleaved (fractions 4, 6) as a protein band is still visible at 94kD. In Figure 40B, His-tagged proteins are supposed to be removed through Ni-NTA agarose resin, yielding pure HRI in flow through, but due to the proximal molecular weights it can not be confirmed. For this reason, a Western Blot was performed to detect His-tagged proteins (Figure 41). However, the analytical technique did not indicate pure hRI existence in flow through as a His-tagged protein is detected. The signal in undigested hRI_{14-17} sample designates protein degradation. We would expect a visible protein band higher, at 94kD.

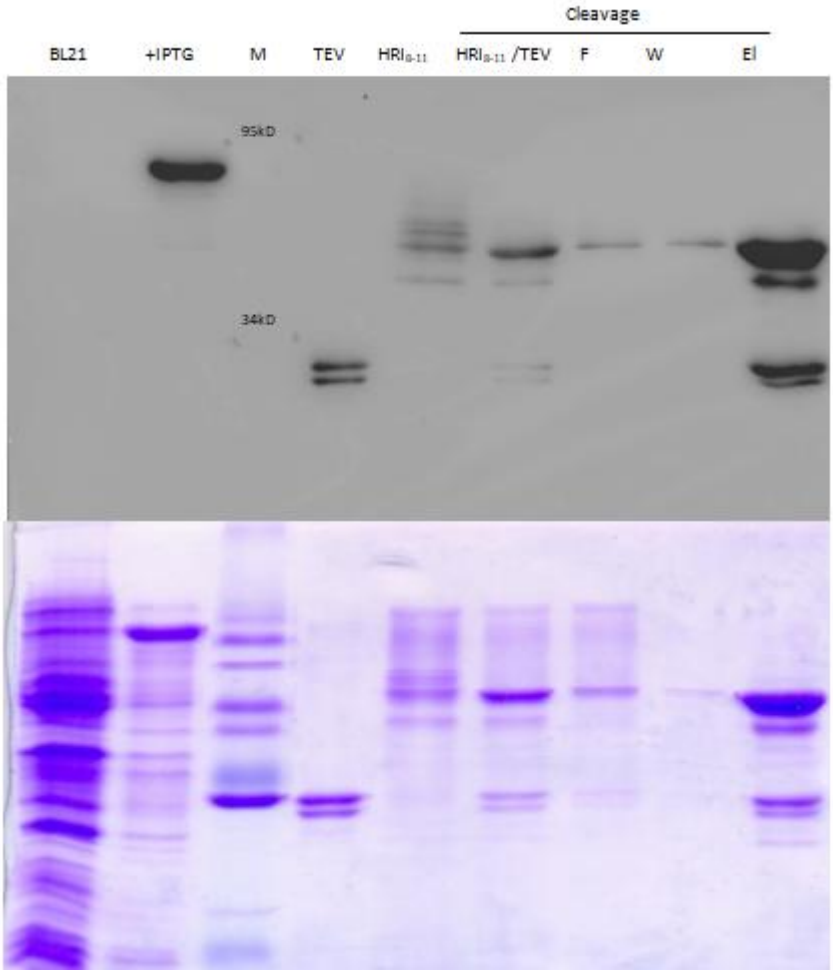


Fig.41 : Western blot to detect His-MBP and His-TEV. The corresponding SDS-PAGE is bellow. BL21 cell extract was added as a negative control while hRI induction sample was the positive control. The prestained protein marker indicated molecular weights at 85kD and 34kD. TEV protease, hRI_{14-17}, hRI_{14-17}/TEV and Flow through, wash, elution samples from cleavage Ni-NTA purification are shown.

All samples from cleavage Ni-NTA purification step were checked for ribonuclease inhibition activity by adding 5ng RNase A, 200ng Hela RNA and 1µl, 2µl and 5µl from each Ni-NTA chromatography sample (Figure 42). None of the samples inhibited the activity of RNase A.

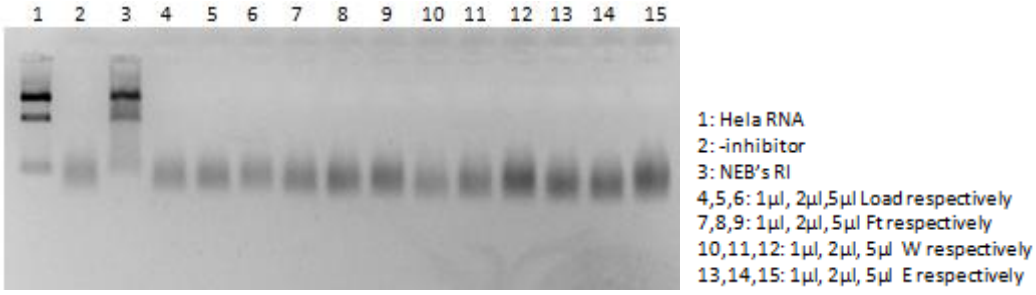


Fig.42 : Functional assay to detect RNase A inhibition. None of the samples was active. .

In an attempt to detect even a low ribonuclease activity, the functional assay was repeated by adding 1ng, 0,1ng and 0,01 ng of RNase A. The only sample tested was the Flow through, were pure hRI is expected to exist. Surprisingly, some activity is detected in 0,01ng RNase A sample, were 5µl of flow through were added (Figure 43). This indicates that our enzyme is too diluted or less active.

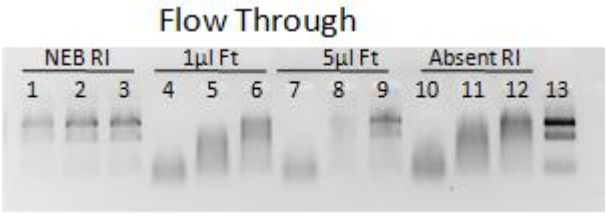


Fig.43 : Functional assay to check flow through for inhibition activity. NEB RI and Hela RNA (13) were used as a control. 1,4,7,10: 1ng RNase A; 2,5,8,11: 0.1ng RNase A; 3,6,9,12: 0.01ng RNase A. . In all samples 200ng Hela RNA was added.

LOT 4

hRI purification was repeated in order to end up with an active enzyme. 6gr of cell pellet were lysed by French press and purified by Ni-NTA affinity chromatography. We performed 2 step elutions at 200mM and 600mM imidazole (5CV each). The results are shown in Figure 44. A large amount of protein is lost in flow through, even though sample was passed twice through the column and the enzyme purity in the elutions is not satisfying. Elutions 1-5 were selected and merged in a single fraction to examine enzyme activity (Fig.).

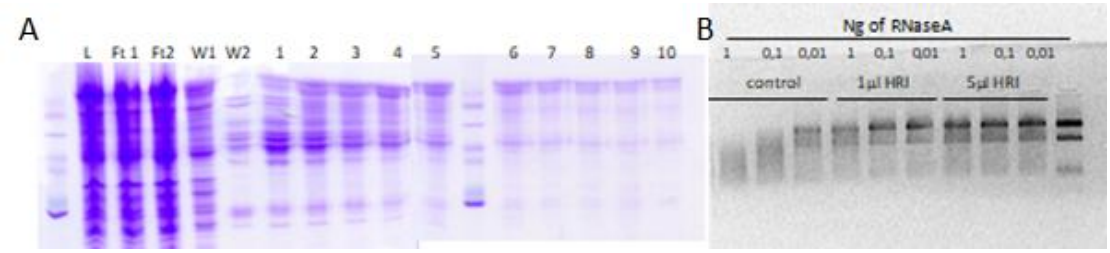


Fig.44 : A) hRI Ni-NTA purification. L,load; Ft1, flow through 1; Ft2, flow through2 (sample was passed twice into the column); W1, wash1-5mM imidazole; W2, wash 2-50mM imidazole; 1-5 step elutions at 200mM imidazole; 6-10, step elutions at 600mM imidazole. B) Functional assay of $hRI_{1-5.}$ 1µl and 5µl of the enzyme was tested to inhibit 1ng, 0.1ng and 0.01ng RNase A. In all samples 200ng Hela RNA was added.

For the first time ribonuclease inhibition is observed (Figure 44 B). 1µl hRI_{1-5} partially inhibited RNase A activity even in presence of 1ng RNase A, while 5µl of HRI_{1-5} completely protected the RNA added. In order to achieve high purity and activity levels, the next day hRI_{1-5} underwent Buffer-exchange chromatography (section 3.9.2). All fractions were analyzed by SDS-PAGE (Figure 45). The whole protein load seems to be lost in flow through. Flow through sample was reloaded onto a new Q-sepharose column. This time not only the sample is lost in flow through, but also the protein amount seems to be less. Nevertheless inhibition assays were performed by adding 1µl of hRI_{1-5}, Q-Sepharose flow through and Ni-NTA elutions 6, 7, 9, 10 (Figure 45). In our surprise none of the samples were active, not even $HRI_{1-5,}$ which inhibited RNase A activity the previous day.

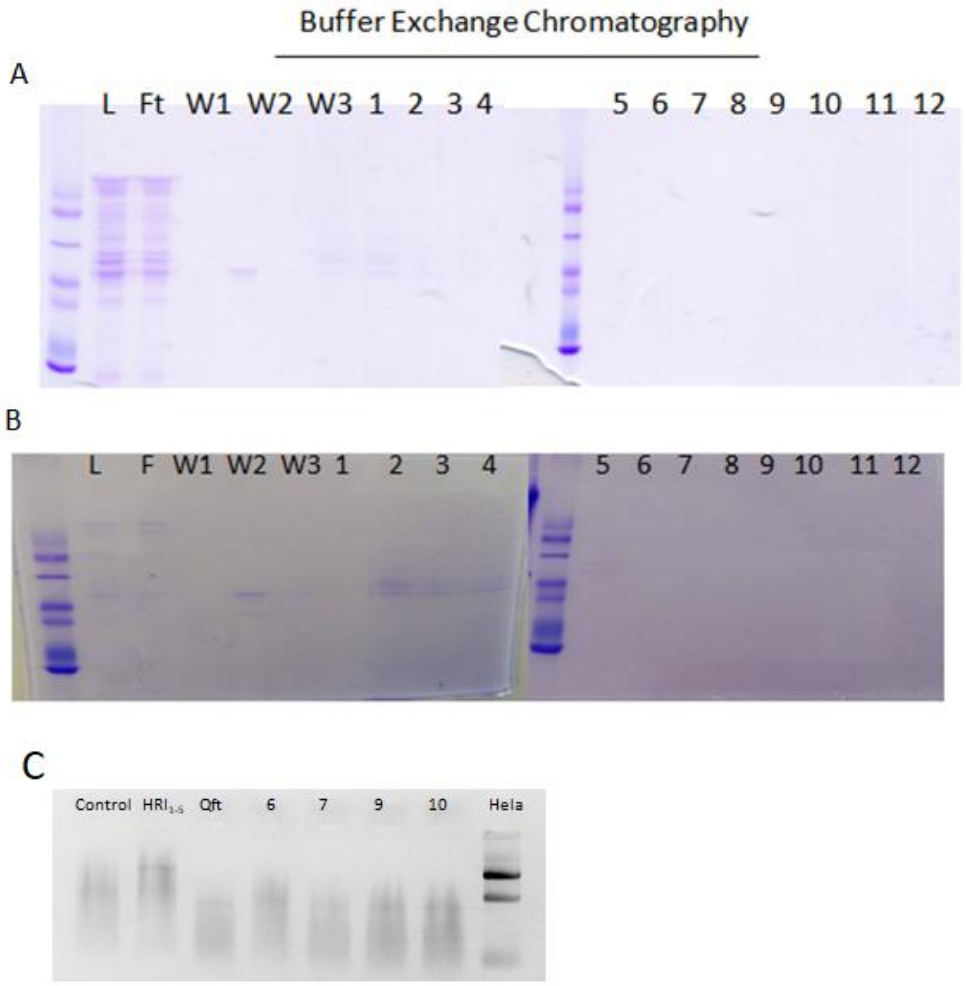


Fig.45 : A)Buffer exchange chromatography. L, load-hRI_{1-5} ; Ft, Flow through; W1, wash 1; W2, wash2; W3, wash3; 1-5, elutions at 300mM NaCl; 6-10, elutions at 500mM NaCl; 11-12, elutions at 1M NaCl. B) Repeat of buffer exchange chromatography. L, Flow through from the previous Q-Sepharose. C)Inhibition assay. Cntrl, 1ng RNase A in absence of inhibitor; 1µl of hRI_{1-5}, flow through sample from Q-sepharose and elutions 6, 7, 9, 10 of Ni-NTA purification in presence of 1ng RNase A. . In all samples 200ng Hela RNA was added.

LOT 5

The same procedure as in LOT5 was repeated again in an attempt to figure out why the enzyme loses its activity after some hours. 2gr of hRI cell pellet were lysed by sonication and purified by Ni-NTA chromatography. This time 8mM DTT were added in the elutions. Ni-NTA elution 1 was tested for RNase inhibition. 1µl and 5µl of the elution sample were added in the reaction, in presence of 1ng ,0.1ng and 0.01ng RNase A. In all reactions hRI showed inhibitory activity (Figur 46 A,B). After

Buffer-Exchange chromatography, flow through sample in which a single band of hRI is observed, elution 1 and 2 as well as elution 1 from Ni-NTA purification were examined in functional assays for inhibition activity. None of the samples protected the added RNA. Not even elution 1 from Ni-NTA purification, which was active some hours ago. (Figure 46 D)

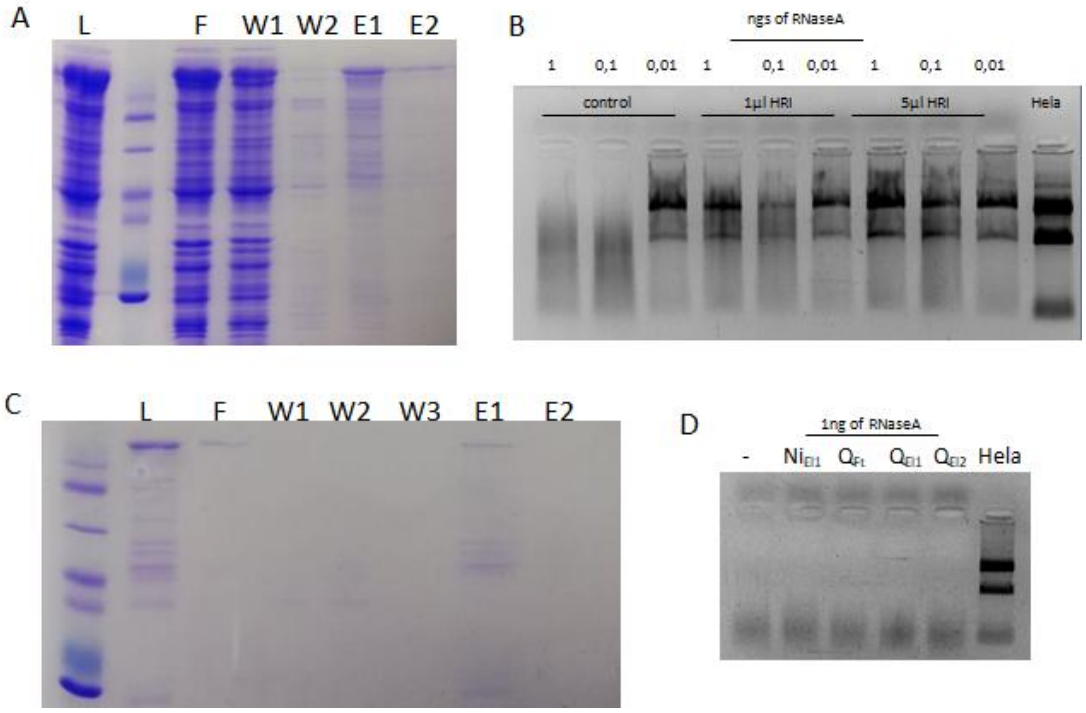


Fig. 46 : A)HRI Ni-NTA purification. B) Functional assay for Ni-NTA El1 sample in presence of 1ng, 0.1ng, 0.01ng RNase A. C) Buffer-exchange chromatography. L, Elution 1 from Ni-NTA purification. D) Functional hRI assay. -, 1ng RNase A in absence of RI; Ni_{El1}, 1µl of Ni-NTA elution 1; Q_{Fl}, 1µl of buffer exchange chromatography flow through; Q_{El1} /Q_{El2}, 1µl of Q-sepharose elutions 1 and 2 respectivelly. In all samples 200ng Hela RNA was added.

4.2.3 PRELIMINARY STRAIN SCREENING

Considering the instability of hRI in all the previous attempts, different expression and purification conditions were screened in SHuffle and Chaperone *E.coli* cells. Both strains were transformed with Pjsc011_hRI vector and protein expression was tested in 30°C and 18°C. As shown in Figure 47, protein induction in SHuffle is observed at 30°C expression temperature, while induction in Chaperone was successful in all temperatures tested.

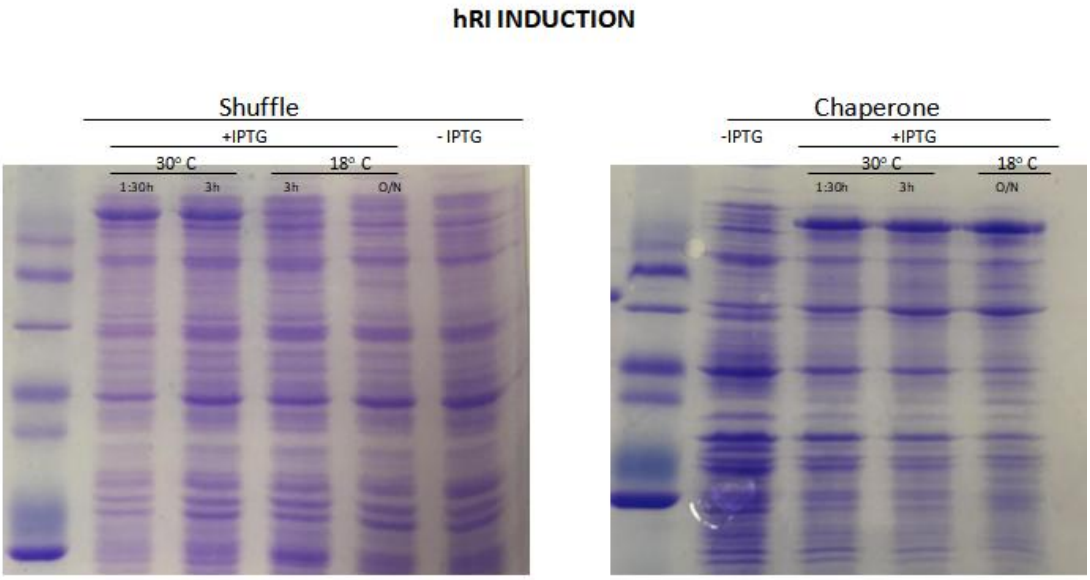


Figure 47: hRI induction in SHuffle and Chaperone *E.coli* cells. A) Protein induction in SHuffle. +IPTG samples in 30°C and 18°C checked in different time points (1.5h, 3h and O/N). B) Protein induction in Chaperone. +IPTG samples in 30°C and 18°C checked in different time points (1.5h, 3h and O/N) are depicted as well.

Four different lysis buffers (Table 24) were screened in order to identify the optimum conditions at which our target protein is stable and active. Four hRI cell pellets from each strain were resuspended in each lysis buffer and Ni-NTA purification was performed (Figure 48). Chaperone expressed hRI crude and elution samples were tested for inhibitory function but no activity was observed (Figure 49).

Buffers tested in Chaperone and Shuffle Cells			
A	B	C	D
50mM Tris-HCl pH 7.6	50mM Tris-HCl pH 7.6	50mM Tris-HCl pH 7.6	50mM Tris-HCl pH 7.6
50mM NaCl	50mM NaCl	125mM NaCl	125mM NaCl
10% glycerol	10% glycerol	10% glycerol	10% glycerol
0.1% Triton-X	0.1% Triton-X	0.1% Triton-X	0.1% Triton-X
5mM b-ME	10mM b-ME	5mM b-ME	10mM b-ME

Table 24: Buffers tested for Ni-NTA chromatography of hRI expressed in SHuffle and Chaperone cells.

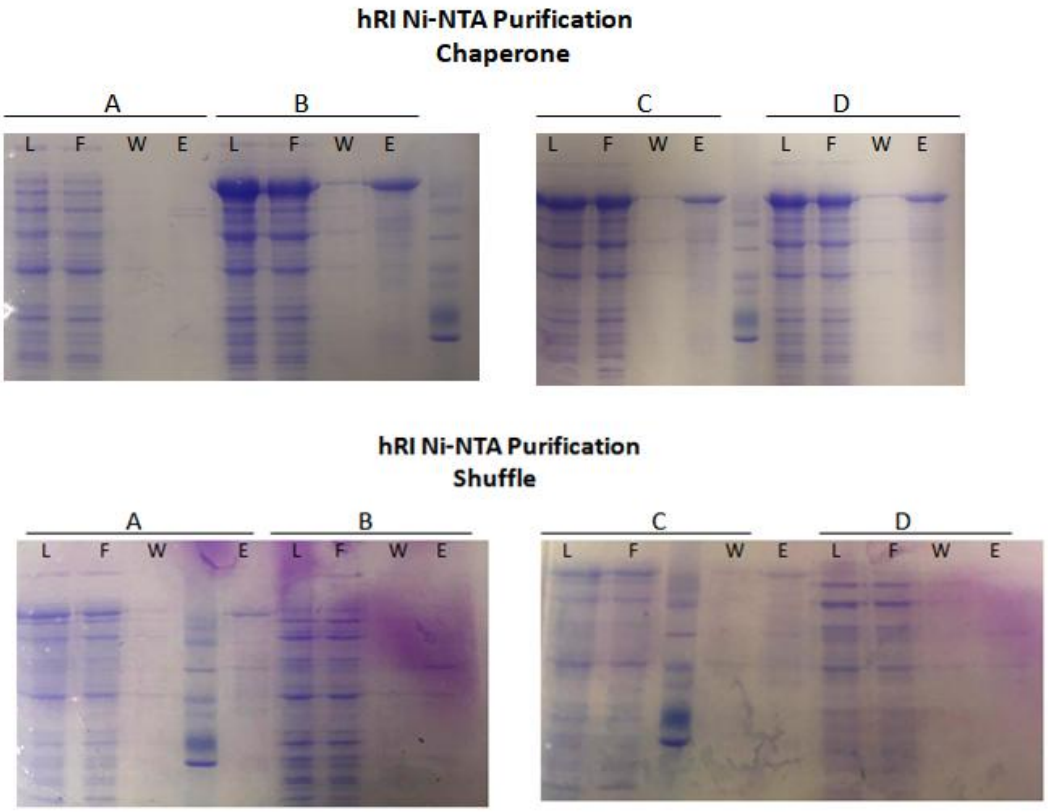


Figure 48: Ni-NTA purification of hRI expressed in Chaperone (I) and SHuffle (II) cells. A,B,C and D stand for the four buffers tested. L, F, W, E stand for Load, Flow Through, Wash and Elution fractions.

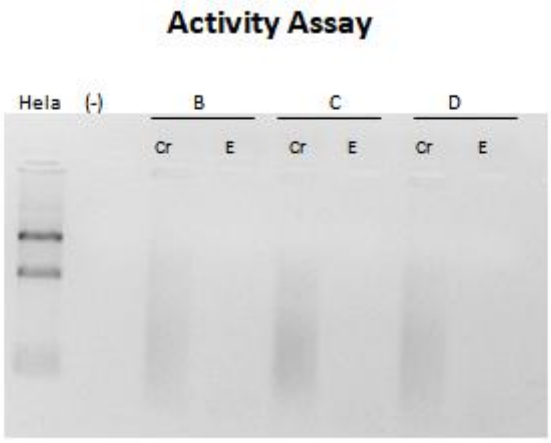


Figure 49: Activity assay of hRI expressed in Chaperone. B,C and D stand for the buffers tested. Cr and E mean crude and elution sample respectively. 1ng RNase A was incubated with 1µl of each hRI sample. Hela RNA was used as a control while (-) sample is RI free.

5. DISCUSSION

The synthesis of RNA by T7 *in vitro* transcription is a standard method of producing up to several mgs of RNA with high quality as compared to solid-phase synthesis, using only a T7 promoter and a purified enzyme. The numerous applications of *in vitro* transcription in molecular biology set the respective kits of the most popular in the molecular biology global market. For that purpose, the present work has focused on the expression, purification and quality control of two enzymes necessary for this process, T7 RNA polymerase and Human Placental Ribonuclease Inhibitor.

5.1 T7 RNA POLYMERASE

T7 RNA Polymerase is a very potent RNA analysis tool. The monomeric enzyme requires no auxiliary transcription factors for activity and is capable of transcribing a variety of DNA templates i*n vitro*. Since the enzyme is well characterized, a standard procedure was followed for the heterologous expression and purification. .

By placing the T7 gene under the control of the T7 promoter and inducing the protein expression by adding IPTG, high expression levels were achieved. In order to isolate the enzyme two consecutive chromatographic steps were performed, Ni-NTA affinity chromatography followed by ion-exchange chromatography. These manipulations led to a final sample with enzyme purity over 95%. After T7RNAP purification, quality controls were thoroughly investigated in order to verify the enzymes quality and efficiency. Neither nuclease activity (endonuclease-exonuclease) nor RNase activity was detected. Regarding promoter specificity, no RNA product was observed when the enzyme was incubated with Lambda DNA, indicating the enzymes ability to transcribe only DNA downstream of a T7 promoter. In order to perform transcriptional assays, of great importance was to determine the enzyme units. One unit of T7RNAP is defined as the amount of enzyme that will incorporate 1nmol ATP into acid-insoluble material in a total reaction volume of 50μl in 1hour at 37^{o}C in 1X RNAP reaction buffer. Minotech's T7RNAP preliminary unit definition was conducted by comparing it with NEB's T7RNAP. It was estimated that Minotech's RNAP contains 50U/μl. Functional assays have been performed in order to verify the enzyme's transcription efficiency. The enzyme can efficiently transcribe large linear DNA templates and the amplification factor of the reaction reaches 20X. T7RNAP activity has also been successfully tested with DNA templates that produce guide RNAs for genome editing applications.

Minotech's T7RNAP has passed through the beta test stage and has been added in Minotech's product catalogue (cat.number 802-1). Future steps include manipulations to increase the enzyme activity, such as increasing dNTPs concentration or using additives such as guanidine hydrochloride. The latter at low concentrations is known to increase the processive transcription .(Mili Das, 1998)

5.2 HUMAN PLACENTAL RNASE INHIBITOR

Considering the limitations occurring due to the sensitivity of wild typehRI to oxidation, the variant of hRI selected for development bears a substitution of Cys328 to Alanine. This particular replacement, conducted by Byung-Moon Kim et al, increases the enzymes resistance to oxidation by 10- to 15 fold and the effect of the enzyme's activity to RNase A is negligent.

The wide usage of RI for protecting RNA during laboratory manipulations result to higher needs for time-saving, high enzyme production yield. On the one hand isolating the inhibitor from natural sources is a labor-intensive process, on the other hand heterologous expression of the protein in *E.coli* results in poor solubility and low yields of active enzyme per liter culture (Kumar et al, 2003). Wanhua et al managed to improve the expression level and solubility of recombinant murine RI in *E.coli* by adding the fusion partner MBP at the N-terminus of the protein and achieved high yield of pure RI in a single IMAC step.

Regarding hRI, all evidence in literature about hRI purification rely upon ribonuclease affinity chromatography, exploiting the tight binding between RI and RNase A. This process is time and money consuming in laboratory routine. In the current Master Thesis Pjsc011_His-MBP-hRI vector has been constructed and transformed in BL21 DE3 Star PLys *E.coli* cells in order to express and purify the hRI by Ni-NTA affinity chromatography.

Although lysis buffer screening revealed high protein solubility, concomitant purification by Nickel affinity chromatography did not give the expected results. Half of the protein load does not bind on Ni-NTA agarose beads as it is detected in the flow through sample. Mutations in the His tag are excluded as an explanation, as the sequencing results verified the existence of the correct sequence. Protein structure may be oriented in a way that His-tag is partially masked and does not come in contact with the chelating agent. Regarding hRI purity, impurities were detected in the elution ftactions. Protein's purity is less than 70%. In order to isolate only the protein of interest, the protein mixture from Ni-NTA chromatography elutions were loaded onto an ion- exchange chromatography. Despite the fact that hRI is negatively charged in basic environment, the protein did not bind efficiently on the anion exchange resin. The protein band visualized by SDS-PAGE indicates about 50% of total hRI content lost in flow through. The formation of aggregates occurring in the soluble fraction, happening during or after purification, could explain the inefficient binding. Efforts to overcome the enzymes instability have been made. Neither different salt concentration, nor adding reducing agents such as DTT or replacing Tris buffer with Phosphate or Hepes optimized protein purification yield. Troubleshooting by changing the expression conditions such as host strains and expression temperatures were not effective as well. The protein's instability has probably compromised the biological function of the enzyme, as no permanent RNA protection has been observed. Freshly purified hRI exhibited its inhibitory function on RNase A., however that activity was lost after less than 24h.. Western blot analysis revealed protein degradation at the C-terminus, as a smeared anti-His tag signal was detected). C-terminus cleavage could explain the loss of function as C-terminal module of RI is forming extensive contacts with RNase A, accounting for the major part of contacts between the two proteins (Kobe, Deisenhofer and Medical, 1996). For this reason, the fusion partner is placed at the N-terminus of hRI,. The immunoassay also showed that degradation occurs after cell lysis, as samples after protein induction give an intact signal at 94kD.

All evidence confirm the fact that recombinant systems for hRI result in low protein solubility. The high sensitivity to oxidation and high hydrophobicity have made the production of the enzyme unsuccessful. Selecting another expression vector, which offers a lower rate of expression, eg utilizing the weak trp promoter might improve protein folding. Future plans include the development of murine or porcine RI, since wild type and engineered variants have been well studied.(Juozas and Neubauer, 2011)(Guo et al., 2011)

5. SUPPLEMENTARY

Amino acid sequence of T7 RNA Polymerase (**UniProt, https://www.uniprot.org/**)

MNTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRKMFERQLKAGEVADNAA
AKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFLQEIKPEAVAYITIKTTLACLTSADNTTVQAVA
SAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNKRVGHVYKKAFMQVVEADMLSKGLLGGEAWSSWHKE
DSIHVGVRCIEMLIESTGMVSLHRQNAGVVGQDSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPK
PWTGITGGGYWANGRRPLALVRTHSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWK
HCPVEDIPAIEREELPMKPEDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKAI
WFPYNMDWRGRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKVPFPERIKFIE
ENHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLPLAFDGSSGIQHFSAMLRDE
VGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNEVVTVTDENTGEISEKVKLGTKALAGQWLA
YGVTRSVTKRSVMTLAYGSKEFGFRQQVLEDTIQPAIDSGKGLMFTQPNQAAGYMAKLIWESVSVTVVA
AVEAMNWLKSAAKLLAAEVKDKKTGEILRKRCAVHWVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPT
INTNKDSEIDAHKQESGIAPNFVHSQDGSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVR
ETMVDTYESCDVLADFYDQFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA

Nucleotide sequence of hRI

https://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS7697

ATGAGCCTGGACATCCAGAGCCTGGACATCCAGTGTGAGGAGCTGAGCGACGCTAGATGGGCCGAGCTC
CTCCCTCTGCTCCAGCAGTGCCAAGTGGTCAGGCTGGACGACTGTGGCCTCACGGAAGCACGGTGCAAG
GACATCAGCTCTGCACTTCGAGTCAACCCTGCACTGGCAGAGCTCAACCTGCGCAGCAACGAGCTGGGC
GATGTCGGCGTGCATTGCGTGCTCCAGGGCCTGCAGACCCCCTCCTGCAAGATCCAGAAGCTGAGCCTC
CAGAACTGCTGCCTGACGGGGGCCGGCTGCGGGGTCCTGTCCAGCACACTACGCACCCTGCCCACCCTG
CAGGAGCTGCACCTCAGCGACAACCTCTTGGGGGATGCGGGCCTGCAGCTGCTCTGCGAAGGACTCCTG
GACCCCCAGTGCCGCCTGGAAAAGCTGCAGCTGGAGTATTGCAGCCTCTCGGCTGCCAGCTGCGAGCCC
CTGGCCTCCGTGCTCAGGGCCAAGCCGGACTTCAAGGAGCTCACGGTTAGCAACAACGACATCAATGAG
GCTGGCGTCCGTGTGCTGTGCCAGGGCCTGAAGGACTCCCCCTGCCAGCTGGAGGCGCTCAAGCTGGAG
AGCTGCGGTGTGACATCAGACAACTGCCGGGACCTGTGCGGCATTGTGGCCTCCAAGGCCTCGCTGCGG
GAGCTGGCCCTGGGCAGCAACAAGCTGGGTGATGTGGGCATGGCGGAGCTGTGCCCAGGGCTGCTCCAC
CCCAGCTCCAGGCTCAGGACCCTGTGGATCTGGGAGTGTGGCATCACTGCCAAGGGCTGCGGGGATCTG
TGCCGTGTCCTCAGGGCCAAGGAGAGCCTGAAGGAGCTCAGCCTGGCCGGCAACGAGCTGGGGGATGAG
GGTGCCCGACTGCTGTGTGAGACCCTGCTGGAACCTGGCTGCCAGCTGGAGTCGCTGTGGGTGAAGTCC
TGCAGCTTCACAGCCGCCTGCTGCTCCCACTTCAGCTCAGTGCTGGCCCAGAACAGGTTTCTCCTGGAG
CTACAGATAAGCAACAACAGGCTGGAGGATGCGGGCGTGCGGGAGCTGTGCCAGGGCCTGGGCCAGCCT
GGCTCTGTGCTGCGGGTGCTCTGGTTGGCCGACTGCGATGTGAGTGACAGCAGCTGCAGCAGCCTCGCC
GCAACCCTGTTGGCCAACCACAGCCTGCGTGAGCTGGACCTCAGCAACAACTGCCTGGGGGACGCCGGC
ATCCTGCAGCTGGTGGAGAGCGTCCGGCAGCCGGGCTGCCTCCTGGAGCAGCTGGTCCTGTACGACATT
TACTGGTCTGAGGAGATGGAGGACCGGCTGCAGGCCCTGGAGAAGGACAAGCCATCCCTGAGGGTCATC
TCCTGA

Amino acid sequence of hRI

https://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS7697

MSLDIQSLDIQCEELSDARWAELLPLLQQCQVVRLDDCGLTEARCKDISSALRVNPALAELNLRSNELG
DVGVHCVLQGLQTPSCKIQKLSLQNCCLTGAGCGVLSSTLRTLPTLQELHLSDNLLGDAGLQLLCEGLL
DPQCRLEKLQLEYCSLSAASCEPLASVLRAKPDFKELTVSNNDINEAGVRVLCQGLKDSPCQLEALKLE
SCGVTSDNCRDLCGIVASKASLRELALGSNKLGDVGMAELCPGLLHPSSRLRTLWIWECGITAKGCGDL
CRVLRAKESLKELSLAGNELGDEGARLLCETLLEPGCQLESLWVKSCSFTAACCSHFSSVLAQNRFLLE
LQISNNRLEDAGVRELCQGLGQPGSVLRVLWLADCDVSDSSCSSLAATLLANHSLRELDLSNNCLGDAG
ILQLVESVRQPGCLLEQLVLYDIYWSEEMEDRLQALEKDKPSLRVIS

Genotypes of *E.coli* strains

- BL21 (DE3) Star PLys

F⁻ompT hsdSB (rB-, mB-) galdcmrne131 (DE3) pLysS (CamR)

- *E.coli* K21 (Shuffle)

F´ lac, pro, lacIq / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC(SpecR, lacIq) ΔtrxB rpsL150(StrR) Δgor Δ(malF)3

- Chaperone pG-KJE8/BL21

E.coli BL21: F- , ompT, hsdSB(rB - mB -), gal, dcm

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