CHALMERS

Initial study of truncated human β-tropomyosin with mutations related to neromuscular disorders

Master thesis in Biotechnology

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Abstract

This report regards an initial part of a larger study aiming towards structure determination of six β-tropomyosin mutations, mapped during clinical investigations of congenital myopathies performed at Sahlgrenska university hospital. This initial project includes methods and suggestions as to how the tropomyosin protein molecule can be truncated to produce a suitable platform for high resolution structure determination, using x-ray crystallography.

During this study Ligation Independent Cloning, LIC, was evaluated as cloning method, and this method is discussed with practical conclusions presented in the end of the report. For the tropomyosin truncation design a thorough literature study was conducted, over viewing all presently published material of x-ray crystallography regarding tropomyosin. Cloning and vector amplification were performed in *Escherichia coli*.

This study points out the necessity of making a thorough investigation of present information to be able to deduce protein truncations, which not only will have good prerequisites for high resolution structure determination, but also include as many intact interaction sites with other sarcomeric proteins as possible. Also, this study shows that LIC can be an efficient and high throughput method for cloning gene fragments.

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Abbreviations

ACTA1 – α-actin
CM – Congenital myopathies
LIC – Ligase Independent Cloning
NEB – Nebulin
TPM2 – β-tropomyosin
TPM3 – γ-tropomyosin
TnT – Troponin T
TnT1 – Troponin T1

1. Introduction

1.1. Background

Neuromuscular disorders comprise a group of disorders with muscle weakness as a common predominant symptom and have a prevalence of approximately 1/1000. Many muscle diseases lead to severe physical handicap of affected individuals. The sarcomere is the functional unit of striated muscle contraction. The sarcomere is a highly ordered structure composed of the thin and thick filaments. The thin filament is composed of three components in striated muscle, actin, tropomyosin and troponin with the three subunits (troponin I, troponin C and troponin T). Mutations in sarcomeric proteins are important causes of cardiac and skeletal muscle diseases. The diseases vary in severity from life threatening at birth to mild conditions compatible with normal life span. The front line in research on muscle diseases is at present to define the genetic background and pathogenesis of these disorders.

Recently, different mutations in the gene encoding ß-tropomyosin (*TPM2*), have been identified in association with a range of clinical and morphological phenotypes, including unspecific congenital myopathy, nemaline myopathy, cap disease, and distal arthrogryposis (DA)

Congenital myopathies are muscle diseases present at birth. Congenital myopathies are in turn divided into four main subclasses, classified in regard to morphology: myopathy with protein accumulations, myopathy with cores, myopathy with central nuclei and myopathy with fiber size variations. The clinically features for congenital myopathies include: muscle weakness, hypotonia, and will usually have a non progressive clinical course. For congenital myopathies many disease genes have been identified with aid of genetic and prenatal diagnosis. (Goebel 2005; North 2008)

One of the recently discovered congenital myopathies is the nemaline myopathy. Nemaline myopathy is characterized by abnormal, thread-like rods – called nemaline bodies – present in biopsy samples of muscle fibers. People diagnosed with nemaline myopathy often experience delayed motor development and weakness in the arm, leg, trunk, and face muscles. Clinical investigations have determined mutations in genes coding for proteins involved with the sarcomeric unit during cases of nemaline myopathy. These mutations have in turn been associated with the following genes: skeletal α-actin (ACTA1), nebulin (NEB), β-tropomyosin (TPM2), γ-tropomyosin (TPM3) and troponin T1 (TnT1). (Tajsharghi et al 2007, Ohlsson 2008).

Cap disease is a rare congenital myopathy with well demarcated and peripherally located cap-like structure consisting of disarranged myofibrils with enlarged Z-disks. The clinical features are similar to those of typical nemaline myopathy, with infantile onset of hypotonia and muscle weakness, predominantly involving the proximal muscles, neck flexors and facial muscles. Scoliosis and respiratory insufficiency are common. Cap disease has so far been associated with mutations in three different genes: *TPM2, TPM3* and *ACTA1*, all associated with nemaline myopathy as well.

Distal Arthrogryposis (DA) syndrome is an unusual autosomal dominant disorder, with a prevalence of 1 in 3000 birth. DA is a clinically and genetically heterogeneous group of disorders. The clinical features characterize by flexion of the joints, primarily hands and feet, with congenital contractions where the hands and feet are tightly clenched and the fingers overlapping each other. DAs have been associated with mutations in different sarcomeric proteins including beta-tropomyosin (*TPM2*),

The key role of TM in the regulation of striated muscle contraction has been highlighted by the identification of mutations TM genes associated with inherited human diseases of cardiac and

skeletal muscle. Mutations in the β-TM are particularly interesting because of their clinical and morphological heterogeneity.

To further understand how mutations in these genes affect the pathology of congenital myopathies – and how they give rise to clinically and morphologically different phenotypes such as Cap disease, nemaline myopathy, congenital myopathy, and distal arthrogryposis syndromes it is incentive to study how the proteins are structurally affected by these mutations. In this project a platform for efficiently transforming and expressing six presently known mutations of TPM2 is evaluated, as a step towards crystallization and structure determination of mutant constructs, provided by Homa Tajsharghi, associate professor at Sahlgrenska, department of pathology.

1.2. Aim

This project aims to build a platform for efficient and high resolution structure determination of TPM2 mutations. The project can be divided into the following segments:

- Based on current literature, design TPM2 truncations that will be able to give useful structural information for the six mutations, as well for the interaction sites present on the molecule.
- Evaluate and determine protocol for high efficiency Ligase Independent Cloning, to be able to readily transform TPM2 truncations into expression vectors for expression in *E. coli*.
- Based in current literature design an expression platform for expression of protein construct interacting with tropomyosin in the sarcomeric unit, namely ACTA1 and TnT1.

1.3. Outline of project execution

Based on current literature tropomyosin will be truncated into three separate and overlapping regions that are representative for the protein as a whole, and also include undisturbed binding sites for affiliation with ACTA1 and TnT. These fragments will be produced with PCR, with the addition of LIC compatible ends, using mutated TPM2 templates provided by Homa Tajsharghi, associate professor at Sahlgrenska department of pathology. Each construct will then be inserted into a LIC compatible expression vector and expressed with chemically competent *E. coli*. When all expression plasmids are amplified and confirmed, an expression platform for wild type ACT1 and TnT1 will be built based on a similar literature study.

2. Theoretical background

2.1. Vector

The plasmid used for this project was the pNIC28-BSA41 vector. It is a low copy number pET expression vector, with a T7 promoter and a lac operator – inducible by IPTG. Target protein sequence is transcribed including a N-terminal 6x His-tag and a TEV protease cleavage site. The vector also includes negative selection using Kanamycin at 50 µg/mL. The SacB gene allows you to perform negative selection with 5% sucrose to assure of successful ligation with your target gene. The vector is constructed with a bacterial origin of replication (ColE1 pBR322), for amplification in a bacterial host. This plasmid is compatible with LIC cloning – see latter segment 2.8 Ligase Independent Cloning (LIC) – and includes several restriction enzyme sites for traditional cloning techniques, see figure 1.

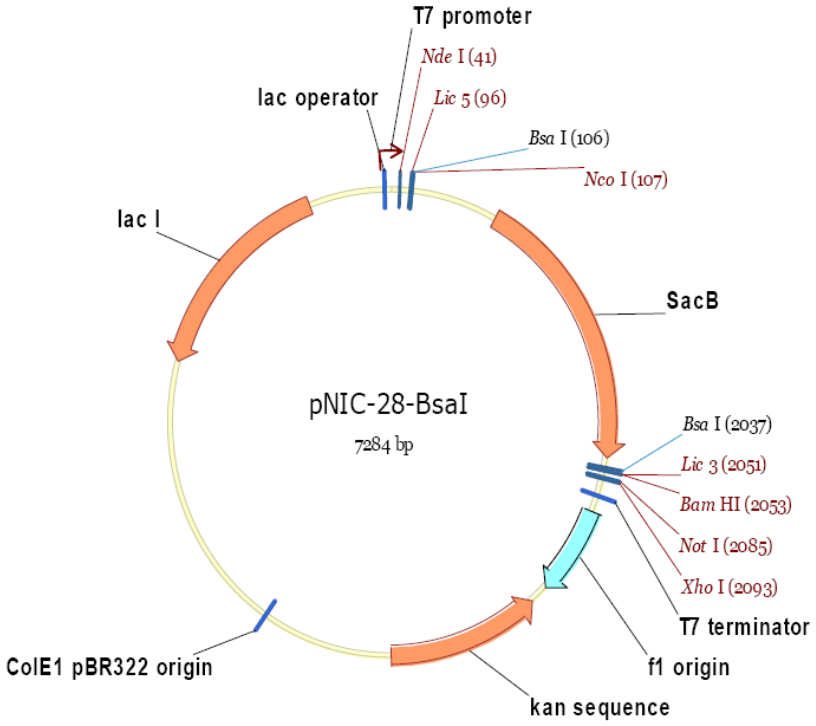

Figure 1: the pNIC-28-BsaI plasmid

2.2. Sarcomere

The sarcomere is the basic contractile unit of a muscle cell. Muscle cells are composed of tubular myofibrils, which in turn are composed of repeating sections of sarcomeres, held together by Z discs. The sarcomere is a highly ordered structure composed of the thin and thick filaments (Fig 2). The thin filament is composed of three components in striated muscle, actin, tropomyosin and troponin with the three subunits (troponin I, troponin C and troponin T). When muscles contract the myosin heads travel along the actin filament – composed of actin, encompassed with tropomyosin and troponin, along a line of nebulin. This interaction is activated when the presence of calcium ions promote a

conformational change in troponin, adjusting the tropomyosin fibers along the actin filament, so that the myosin heads can interact with the actin filament. (The Cell, 4 Ed.) See figure 2 for an illustration.

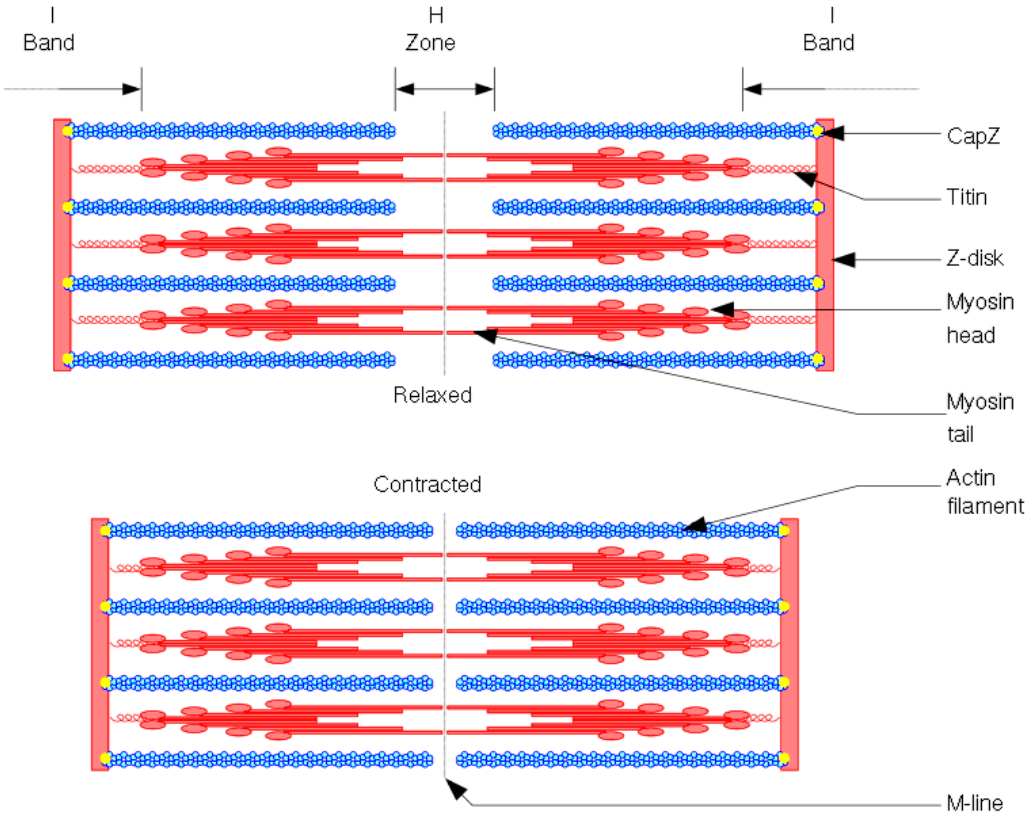

Figure 2: Schematic representation of the sarcomeric unit, in relaxed and contracted state

2.3. Tropomyosin

Tropomyosins comprise a family of actin-binding proteins encoded by four different genes (TPM1, TPM2, TPM3 and TPM4). Each gene uses alternative splicing, alternative promoters, and differential processing to encode multiple striated muscle, smooth muscle and cytoskeletal transcripts {Gunning, 2008 ;Gunning, 2005 }. In vivo TM exists as a rod-shape a-helical coiled-coil dimer that forms a head-to-tail polymer along the length of an actin filamet, providing stability and is essential for myosin-actin interaction. In mammals there are more than 40 tropomyosin isoforms, derived from alternative splicing of four genes (Gunning et al. 2005). TM isoforms in human, are highly homologous but are thought to exhibit unique physiologic properties. The native protein is dimerized into an approximately 420 Å long coil-coil structure, consisting of around 280 residues, depending on isoform. (Whitby & Phillips 2000)

The tropomyosin coil-coil dimer includes seven actin binding sites and one troponin binding site. The dimer interacts with the consecutive tropomyosin dimer by a scissor like overlap at the C-terminal end. (Frye et al. 2010) Below, in figure 3, is an illustration of how the actin filaments are built up inside the sarcomere:

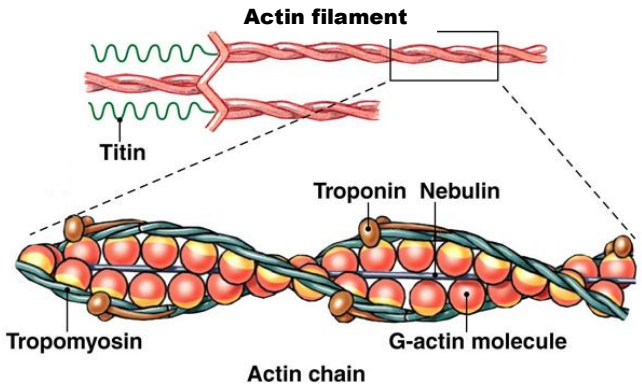

Figure 3: Schematic representation of thin filament composition, illustrating how the tropomyosin dimer encompasses polymerized actin along the nebulin line. Courtesy of colorado.edu

The first x-ray crystallography study of tropomyosin were published 1986 and yielded low resolution representations (15Å) of the whole tropomyosin dimer. This manifestation helped verify previous predictions of the coil-coil structure of tropomyosin. (Phillips 1986) Having greater resolution proved difficult because the long and flexible protein molecule will naturally have much variability within the crystal cell, and therefore disrupt the imaging. This problem was overcome with the help of small stabilizing protein fragments, which locked the tropomyosin dimers in a more unison state. With this method Whitby and Phillips managed to get 7Å resolution of the whole β-tropomyosin molecule. Still this was not enough to make any crucial conclusions of atomic interactions within the molecule, or with adjacent proteins. (Whitby & Phillips 2000)

Figure 4: The tropomyosin dimer, clearly showing its coil, coil properties

To drastically increase resolution there were projects where the tropomyosin molecule was truncated into smaller segments: Brown et al produced an 81 amino acid long N-terminal fragment that diffracted to a resolution of 2.0 Å in 2001. And Li et al produced a 31 amino acid long C-terminal fragment that diffracted to a resolution of 2.7 Å in 2002, and thereby managed to reveal the key recognition site for troponin. In 2005 Brown et al published another article in which they present a 120 amino acid long middle fragment, with three actin binding zones, that diffracted to a resolution of 2.3 Å. In 2010 Frye et al. managed to have the overlapping regions between tropomyosin fragments to diffract down to 2.1 Å, thereby eluding the interaction by which the tropomyosin dimers are polymerized.

All tropomyosin mutations that have been eluded by clinical investigations are presented in table 1, below:

Tabell 1: tropomyosin mutations represented in this project, their position, change and biochemical effect

Mutation position	Change	Biochemical effect	Reference
41	Glutamic acid changed to Lysine	Negatively charged residue changed for positively charged	(Tajshargi et al. 2007)
49	Lysine deletion	Hydrophobic residue removed	(Ohlsson et al. 2008)
53	Glycine insertion	Insertion of a Small residue	(Ohlsson et al. 2008)
122	Glutamic acid changed to Lysine	Negatively charged residue changed for positively charged	Still to be published
202	Aspargine changed to Lysine	Polar uncharged residue changed for positively charged	(Ohlsson et al. 2008)
261	Tyrosine changed to Cysteine	Hydrophobic residue changed for sulphur containing residue	Still to be published

2.4. Ligase Independent Cloning (LIC)

LIC is a cloning technique that takes advantage of the simultaneous 3' to 5' exonuclease and 5' to 3' endonuclease activity of T4 DNA polymerase. By treating your DNA with T4 DNA polymerase together with a single nucleotide, the T4 DNA polymerase will remove nucleotides from the 3' end until coming upon a nucleotide corresponding to the one put in the mix. Then the T4 DNA polymerase will remove and add this nucleotide at the same phase, thereby resulting in a stop in the exonuclease activity. By adding LIC overhang on the primers, the protein fragment will be produced with LIC homology by PCR. When treating the insert with T4 DNA polymerase, in the presence of for example dATP, the polymerase will remove nucleotides until the first dATP – strategically positioned close to the end of the LIC fragment. See figure 5 for an example.

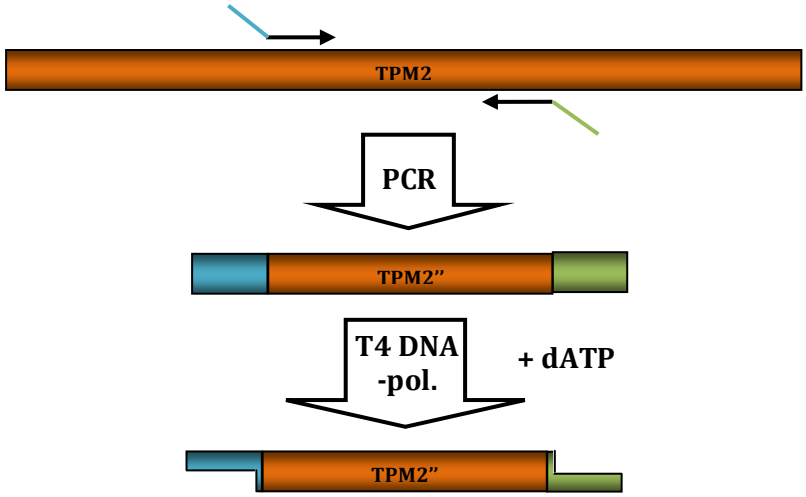

Figure 5: Preparing gene fragment for vector transformation using LIC

When preparing the vector a similar procedure is used: First the vector is linearized, with Eco31I if you take the case with the pNIC28-BSA41 plasmid, removing the SacB gene. The cleaved vector is

then treated with T4 DNA polymerase in the presence of the nucleotide corresponding to the one used to treat the insert. This procedure is illustrated in figure 6, below.

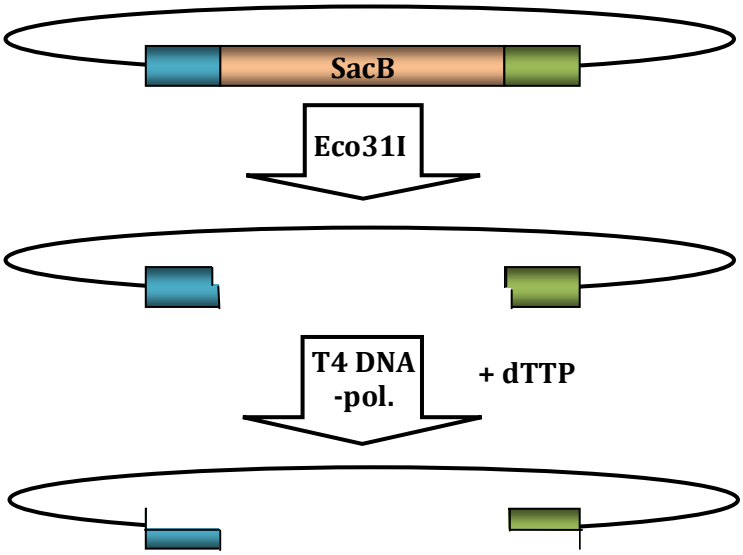

Figure 6: Cuting and treating the pNIC-BSA plasmid with Eco31I and T4 polymerase will result in a frame with ends complementary to the LIC ends at the insert

Ligation is then simply performed by mixing the two parts, and alignment will follow due to sequence homology in the LIC regions, illustrated below in figure 7. After transforming the constructs into cells, the cellular machinery will take care of completing the ligation by adding phosphate groups, making the constructs ready for expression.

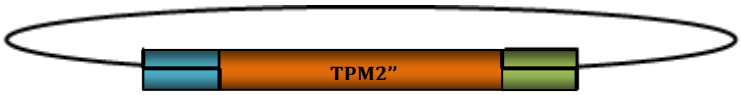

Figure 7: pNIC plasmid fused with TPM2” segment

3. Experimental methods

3.1. PCR

For PCR a standard protocol together with reagents provided by Finnzymes was used. The following was mixed for each PCR reaction, and scaled up or down in regard to what the products should be used to; PCR for confirmation was preformed with 20µL working volume, PCR for further product purification was performed with 2*40µL working volume.

Tabell 2: PCR mixture composition

Component	Volume (for a 20 µL reaction)	Final Concentration
5X Phusion HF Buffer	4 µL	1X
Deoxynucleotide Solution Mix	0.4 µL	200 µM
Upstream Primer	1 µL	0.5 µM
Downstream Primer	1 µL	0.5 µM
DNA Template	2.5 µL	
DMSO	0.6 µL	3%
Phusion DNA Polymerase	0.2 µL	0.02 units/µL
Nuclease free water	10.3 µL	

Phusion HF Buffer, Deoxynucleotide Solution, DMSO and nuclease free water were mixed together to form a Master mixed. To each tube master mix, codon optimized DNA template and respective primers were mixed, and finally the Phusion DNA polymerase was added.

The PCR was run on an Eppendorf Mastercycler gradient, with the following settings:

C for 3 min

C for 30 s

C for 30 s

C for 30 s (+30 s/kb)

The last three steps of the program were cycled 25 times until completion.

3.2. Dpn1 treatment

To eliminate template plasmid present in the PCR product, the solution was treated with the Dpn1 restriction enzyme. This was done so that the kanamysin resistance present in the template plasmid would not account for any background during later transformation.

3.3. Protein design

As mentioned in the theoretic background – 2.3. Tropomyosin – previous studies have shown that there are difficulties to obtain high structural resolution when performing crystallization with the whole tropomyosin protein molecule. To overcome this, the tropomyosin molecule was truncated into overlapping segments, each containing complete actin interaction sites. Segment one and two

contain two actin interaction sites, and segment three contain three actin interaction sites and one troponin interaction site.

The β-tropomyosin mutations that have been inclined to be involved in clinically and morphologically different phenotypes are illustrated below; figure 8, with corresponding amino acid position.

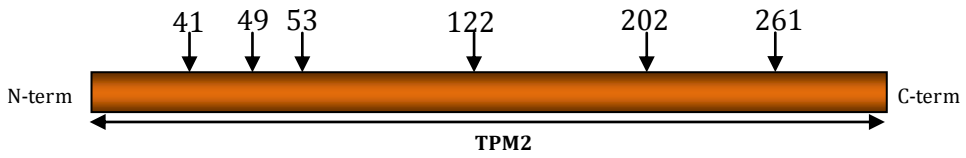

Figure 8: Schematic representation of β-tropomyosin mutations

The truncations consists of three fragments, with 33 amino acids overlapping between fragments one and two, and 10 amino acids overlapping between fragments two and three. The difference in overlap depends on how the mutation density differs between each fragment, and is illustrated in figure 9 below. For further reference the fragments are denominated TPM2', TPM2'' and TPM2'''.

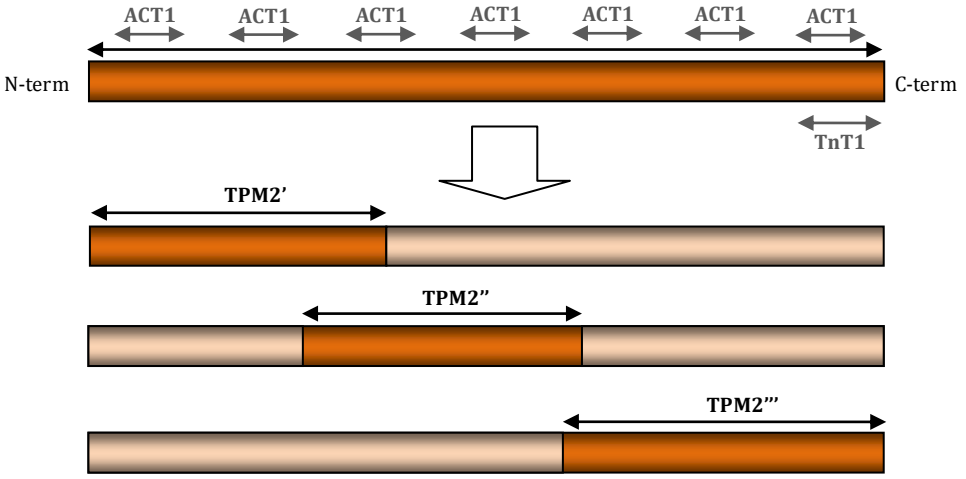

Figure 9: Wild type TPM2 gene with resulting fragments. Actin and troponin interaction sites are marked out with gray

TPM2' consists of β-tropomyosin residues 1-109. Four TPM2' constructs were created: Wild Type and mutations: E41K, K49del and G53ins.

TPM2'' consists of β-tropomyosin residues 77-178. Two TPM2'' constructs were created: Wild Type and mutation: E122K.

TPM2''' consists of β-tropomyosin residues 171-285. Three TPM2''' constructs were created: Wild Type and mutations: N202K and Y261C.

3.3. Cloning

For treating the insert with T4 DNA polymerase the following were mixed in a 1.5 mL Eppendorf tube:

5 µL 5X Fermentas Reaction Buffer

1 µg purified PCR product

1 µL dATP

0.2 µL Fermentas T4 DNA polymerase

ddH_2O was added to a total volume of 20 µL and the reaction mix was incubated at room temperature for 30 min. The T4 DNA polymerase was then inactivated by heating the sample to 75°C on a heating block for 10 minutes.

For linearizing the plasmid, the following were mixed in a 1.5 mL Eppendorf tube:

5 µL 10X Fermentas Fast Digest Buffer

5 µg pNIC-BSA plasmid

5 µL Fermentas Fast Digest Eco31I

ddH_2O was added to a total volume of 50 µL and the reaction mix was incubated in water bath at 37°C for 30 min. The Eco31I was inactivated by heating the sample to 65°C on a heating block for 10 minutes.

For treating the opened plasmid with T4 DNA polymerase the following were mixed in a 1.5 mL Eppendorf tube:

5 µL 5X Fermentas Reaction Buffer

1 µg linear vector DNA

1 µL dTTP

0.2 µL Fermentas T4 DNA polymerase

ddH_2O was added to a total volume of 20 µL and the reaction mix was incubated at room temperature for 30 min. The T4 DNA polymerase was inactivated by heating the sample to 75°C on a heating block for 10 minutes.

After T4 DNA polymerase treatment both PCR product and cut plasmid was purified with PCR clean up kit to prevent nucleotide contamination and remove enzymes that could be affecting the ligation step. Finally 0.02 pmol digested PCR fragment are mixed with ~50 ng digested plasmid. The sample was heated to 90°C to assure that eventual nucleotide contaminants remaining from the PCR cleanup will not interfere with the ligation step.

3.4. Primer design

With the protein literature study as background, primers were designed using the codon optimized gene sequence for each protein, available at the Qiagen homepage. Each primer was then adjusted for melting temperature, dimer- and hairpin formation using the Vector NTI software, version 4.0. To the 5' end of each primer a LIC sequence was added for downstream ligation of gene sequence into a LIC vector – see 2.4. Ligase Independent Cloning (LIC) – and then rechecked with Vector NTI so that the addition would not add any new dimer- or hairpin formations.

Primers with dimer- or hairpin formation calculated to be present at a ΔG falling below -2.0 kcal/mol were redesigned. Taking into account that addition of the LIC sequence would to some extent distort binding to the gene during PCR a melting temperature of 65± C w
C as is common for this type of PCR application.

Forward

	LIC	Gene fragment	Calculated T_m
Pro41	TACTTCCAATCCATG	GATGCCATTAAAAAAAAAATGCAGATGCTGAAAC	66.1
Pro49	TACTTCCAATCCATG	GATGCCATTAAAAAAAAAATGCAGATGCTGAAAC	66.1
Pro53	TACTTCCAATCCATG	GATGCCATTAAAAAAAAAATGCAGATGCTGAAAC	66.1
Pro122	TACTTCCAATCCATG	GCAACCGATGCCGAAGCAGATGT	63.9
Pro202	TACTTCCAATCCATG	GAAGGTGAACTGGAACGTAGCGAAGAACG	65.5
Pro261	TACTTCCAATCCATG	GAAGGTGAACTGGAACGTAGCGAAGAACG	65.5

Reverse

	LIC		
Pro41	TATCCACCTTTACTG	TTACTACAGAGCGGTTGCCAGACGTTCCTG	65.6
Pro49	TATCCACCTTTACTG	TTACTACAGAGCGGTTGCCAGACGTTCCTG	65.6
Pro53	TATCCACCTTTACTG	TTACTACAGAGCGGTTGCCAGACGTTCCTG	65.6
Pro122	TATCCACCTTTACTG	TTACTAGCTACGTTCCAGTTCACCTTCCAGAATCAC	64.7
Pro202	TATCCACCTTTACTG	TTACTACAGGCTGGTAATATCATTCAGGGCATTATC	64.5
Pro261	TATCCACCTTTACTG	TTACTACAGGCTGGTAATATCATTCAGGGCATTATC	64.5

Table 1: Primers for each mutation, based on codon optimized sequence provided by Qiagen. Green represent start codon and red represent stop codons that was added to each fragment

3.5. Host organism

For plasmid amplification and initial protein expression *E. coli* was used. The reasons for using *E. coli* in this kind of initial study is mainly that there are many different commercial variants available, they are relatively cheap and cultures are able to be grown to high concentrations. (Friehs & Reardon 1993)

3.6. Determination of DNA concentration

Concentration measurements of plasmids, PCR fragments and eluted DNA from any purification step were performed with a NanoDrop™ ND-1000 spectrophotometer.

3.7. Transformation of cells

Transformation of chemically competent cells was performed with the heat chock method, described below:

To 50µL of thawed chemically competent BH5-α *E. coli* -
C for 45s in water bath.
Cell tubes were put on ice for 2 min, to prevent further damage to the transforme
C.

3.8. Plasmid amplification

Plasmids were amplified using chemically competent BH5-α *E. coli* cells, prepared according to Inue et al. 1990. The QIAGEN QIAprep Spin Miniprep Kit™ was used to purify plasmid constructs.

3.9. Making expression cells chemically competent

BL21 *E. coli* cells from agar plates were grown over night in 5mL LB with 20mM $MgSO_4$, cells were centrifuged and the pellets resuspended in 1mL LB. From this broth 250µL were added to 150 mL LB medium with 20mM $MgSO_4$ and grown until OD_{600} reached around 0.6. Then the growth vessel was immediately put on ice, centrifuged in sterile tubes at 4°C and resuspended with 100 mL pre chilled salt solution, consisting of 30mM CH_3CO_2K, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% glycerol.

The cell broth was centrifuged once again and resuspended in 10 mL pre chilled salt solution, consisting of 10 mM MOPS, 75 mM $CaCl_2*2H_2O$, 10 mM KCl and 15% glycerol. After incubating on ice for 15 minutes the cell broth was divided to 20 pre chilled cryogenic tubes with 0.5 mL cell broth into each, frozen with liquid nitr - freezer.

3.10. Small scale protein expression

Proteins were expressed with chemically competent BL21 *E. coli* cells. Seed cultures were grown
C. The following morning OD_{600} was measured and cell broth was inoculated so that OD_{600} corresponded to 0.1 in 100 mL LB, cultivated in 500 mL shake flasks. When OD_{600} reached 0.6 protein expression was induced by adding IPTG to a total concentration of 1 mM. Cell samples were taken before induction and once every hour until three hours after protein induction.

4. Results of experimental part

4.1. Primers

From annealing temperature experiments to optimize tropomyosin fragments these three gels, figure 10-12, were produced by loading 5 µL sample volumes onto a 0.7% agarose gel, together with Fermentas Generuler™ 1kb ladder. Each gel shows distinct bands between the 250-500bp markings, which would indicate that the tropomyosin fragments were successfully produced with LIC extensions. The expected band size is around 350bp for each fragment. The whole gradient interval

C.

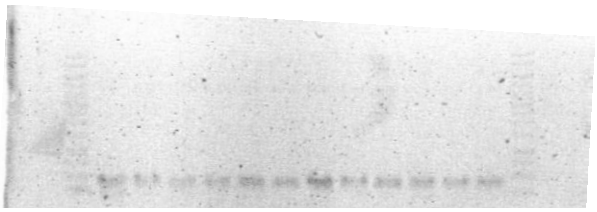

Figure 10 - C with Tropomyosin primer 1 and 1'

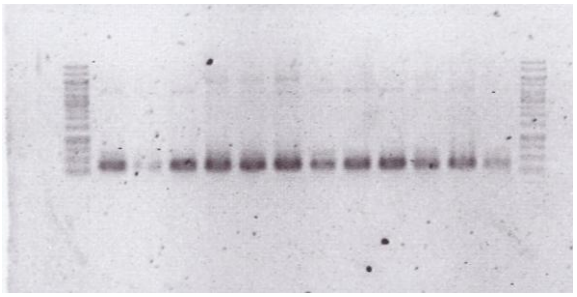

Figure 11: Temperature gradient r - C with Tropomyosin primer 2 and 2'

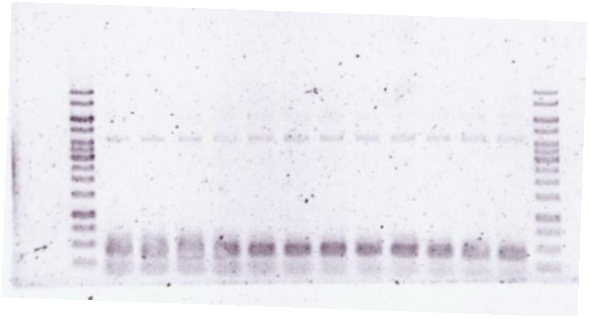

Figure 12 - C with Tropomyosin primer 3 and 3'

4.2. Constructs

Insert fragments treated with DpnI is shown in figure 13. By comparing to for example figure 12, it is apparent that the template plasmid is successfully digested. This was performed to avoid downstream contamination, since both the template plasmid and the pNIC28-BSA41 share kanamycin resistance as means for negative selection.

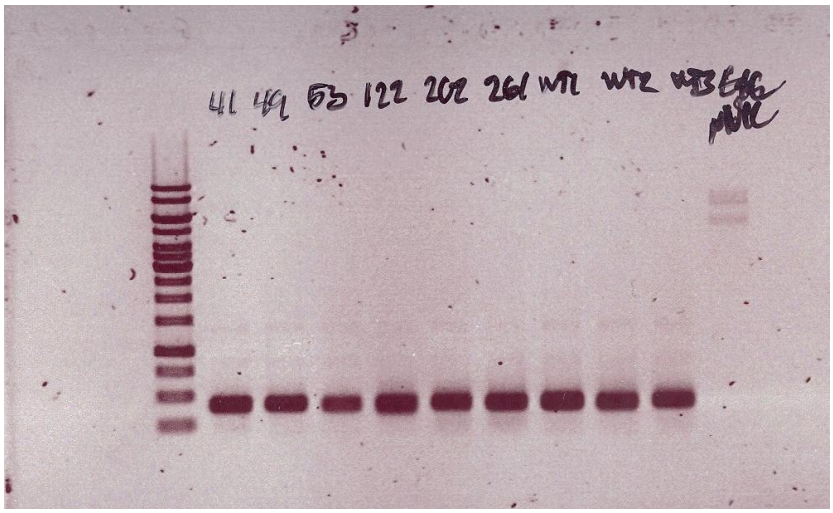

Figure 13: Gene fragments treated with DpnI to remove remnants of the originating plasmid, in the gel bands corresponding to metylated plasmids are removed

4.3. Plasmid digestion

pNIC28-BSA41 plasmid was digested with Eco31I and run on 0.7% agarose gel, alongside Fermentas Generuler™ 1 kb ladder. Single cut plasmid is present just below the 8000 bp mark, which corresponds well to the full size plasmid (7284 bp). With double cut, the SacB gene is removed, shortening the fragment of 2031 bp, and yielding a fragment just above the 5000 bp indication. On the picture to the right the cut fragment without SacB gene is extracted.

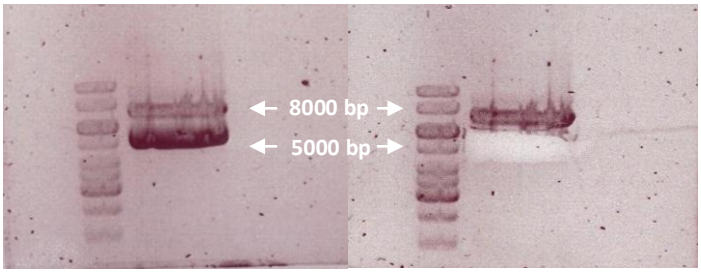

Figure 14: pNIC plasmids digested with Eco31I.

4.4. Sequencing

After Ligase Independent Cloning was successfully performed with each construct, the plasmids were transformed into chemically competent BH5-α *E. coli*. Plasmids were extracted using the Sigma-Aldrich Miniprep Kit™, resulting in 70 µL samples with between 40-70 ng plasmid DNA / µL. From these samples 30 µL was sent to Eurofins MWG Operon™ for sequencing, yielding results depicted below in figure 15:

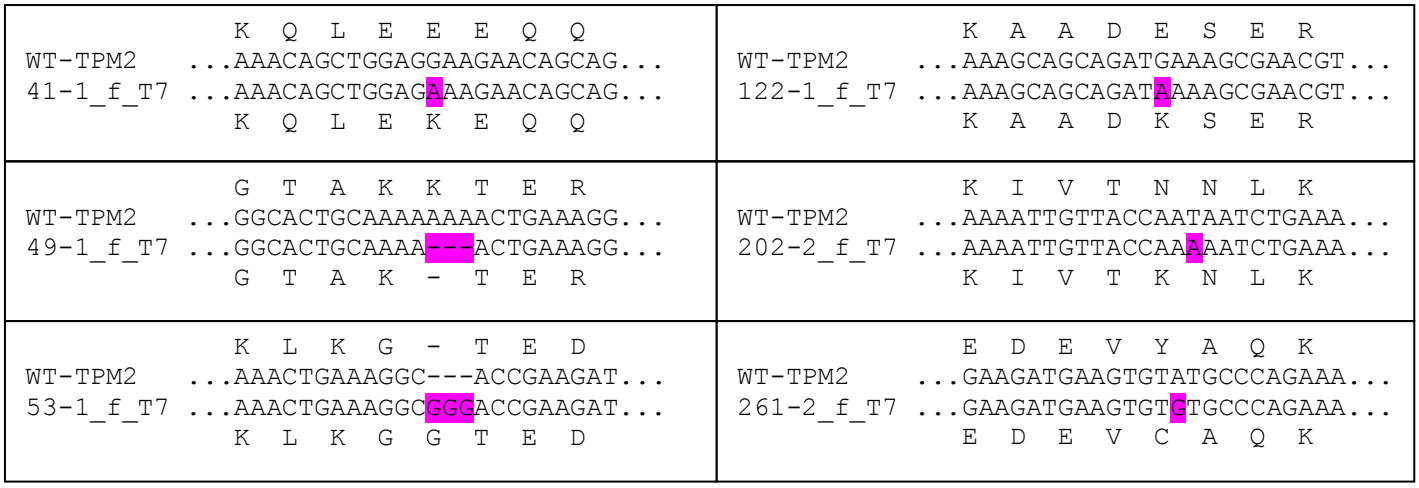

Figure 15: Resulting DNA sequencing of the mutants compared to the codon optimized wild type genome. Sequencing of other parts than the site of mutation was perfectly aligned with the codon optimized wild type genome, and therefore omitted

4.5. Small scale protein production

Small scale protein production with the TPM2'-WT construct resulted in the gel depicted in figure 16. A band just above the 15 kDa marking of the prestained PageRuler™ ladder, corresponding to the calculated molecular weight of TPM2'-WT (16.22 kDa) was found to be induced by the IPTG.

Figure 16: Boiled cell lysate from TPM2'-WT

5. Discussion

There are several aspects to take into account for the x-ray crystallography work line to be as efficient as possible, and the initial steps are in their way critical for success of the later. Initially the protein construct must be determined taking in account what knowledge is presently available of the protein; solubility, active sites, truncations etc. During this project this was a major part as there is presently much literature available regarding structure determination of tropomyosin. Even as this initial study have not resulted in any actual protein crystallization, there is still no redundancy in pressing the importance of doing initial work thoroughly, and basing further attempts on a broad ground of previously published notions regarding the protein of interest.

A significant notion from during the practical work with this project is that DNA purification steps need to be rationalized: Each DNA prep would reduce the sample concentrations, and after consecutive enzyme treatments, followed by DNA purification, the final DNA concentration would be so low that it had a critical effect on LIC ligation efficiency. From PCR to PCR clean up; from DpnI treatment to PCR clean; from T4 DNA polymerase treatment to PCR clean up; in this process a large extent of DNA originating from PCR would be lost. I found that the buffer supporting Fermentas DpnI was also compatible with Fermentas T4 DNA polymerase, and with that in mind I would run the T4 DNA polymerase treatment directly after thermally inactivating the DpnI, and could thereby skip one out of three clean up preps. Keeping in mind that as much as half of the originating DNA concentrations would be lost in each clean up step this would yield twice as much DNA in the final product. Also, by consequently reducing the elution volume – inside the tolerated interval – for each step; this procedure would still yield a product with high enough concentration for efficient LIC cloning procedures from 80 µL PCR production.

Another important note to working with LIC is that it is hard to tell whether the T4 DNA polymerase is active or not. We had difficulties with batches of T4 DNA polymerase that had no detectable activity, and the drawback is that it is not until you transform the construct into cells you can tell if it worked – and at that stage it could still be a matter of DNA or dNTP concentration. With the LIC method you have, to my knowledge, no reliable way of determining the ligation success until after transformation into a host organism. With classical cloning procedures, using restriction enzymes and ligase, it is possible to test if the ligation was successful before transforming the construct into an organism. Still, as long as you can trust the T4 DNA polymerase activity, LIC is a very useful method for making inserts that are compatible to a wide range of different expression vectors within the pET system.

5.1. Future perspective

From the platform of vectors that were constructed in this study, the obvious next step is to go ahead with protein production. I would suggest that a new batch of chemically competent BL21 cells is used when moving forward with expression of truncated β-tropomyosin. The literature suggests that tropomyosin should be expressed in low temperature to obtain high stability, but this has been emphasized mostly when expressing the whole tropomyosin molecule. It will be interesting to see how stability and solubility is affected with the three truncations constructed in this project.

For future optimization of protein expression it is a useful effort to have all six mutations placed on three fragments, as there is a larger possibility that conclusions made from the three wild type fragments will to some extent be applicable for the mutations as well. Also, one should keep in mind that the structural affect inflicted by the mutations will likely prove a stable protein expression more difficult than with the wild type fragments.

If all β-tropomyosin constructs would eventually be crystallized and high resolution structures are derived from these crystals, then it is still not certain that we can make any conclusions as to the mutations involvement in myoline myopathy. Such information is more likely to be eluded from how the interaction between β-tropomyosin and adjacent sarcomeric proteins is affected, rather than from the β-tropomyosin structure in itself. So, after the β-tropomyosin constructs have successfully been crystallized it would be very interesting to try and co crystallized the protein together with actin and troponin. For this I have designed a primer set up with codon optimized ACTA1 and TnT1 for production of actin, and interacting sites of troponin T, for cloning based on the LIC system. These primers are disclosed in Appendix A1.

6. Conclusions

As proven by literature and present drug development, structural knowledge is an essential foundation for understanding disease. Centuries of clinical observations have led to understanding much of the pathology of disease – now structural determination of protein can help us understand the very essence of how genomic mutations affect us at the atomic level.

In conclusion this initial study show, with the help of previous findings, how protein molecules that have structural or chemical properties that prevent them from giving high resolution x-ray diffraction can, with much success be truncated into smaller, still representative domains. Even if these truncations presented in this report are still to be crystallized, literature gives us much hope that these protein not only will have good prerequisite to crystallize, but also to give valuable information about how mutation affect structure, and also the interaction with other sarcomeric proteins.

Finally, it is incentive that structural genomics and clinical pathology use the possibility to interact clinical findings and structural knowledge, for I am certain that it will synergetically help bringing both fields forward toward future findings.

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Appendix

A1. Primer design

ACT1 primers

Forward

	Lic	Gene fragment	Tm	Dimers	ΔG	Hair
Act_f	TACTTCCAATCCATG	TGTGATGAAGATGAAACCACCGCACTG	64.6	0	n.a.	0

Reverse

	Lic					
Act_r	TATCCACCTTTACTG	TTACTAAAAGCATTTACGATGAACAATACTCGGACC	64.6	0	n.a.	0

Troponin T primers

Forward

	Lic	Gene fragment	Tm	Dimers	ΔG	Hairp
TropoT_N_f	TACTTCCAATCCATG	AGCGATACCGAAGAACAAGAATATGAAGAAGAACA	65.3	3	-1,8	0
TropoT_C_f	TACTTCCAATCCATG	GTGCTGTATAATCGCATTAGCCATGCCC	64.9	1	-1,3	0

Reverse

	Lic					
TropoT_N_r	TATCCACCTTTACTG	TTACTAATAATCAATATCCAGCGGTTTTTTACGTTCGC	64.6	0	n.a.	0
TropoT_C_r	TATCCACCTTTACTG	TTACTATTTCCAACGACCACCAACACGACC	65.2	0	n.a.	0

A2. Sequencing results

TPM2'-WT

```
TPM2'_f_T7          --------------------------------ATTCCCCTCTAGAA-TAATTTTGTTTAA 27
TPM2'_r_T7term      ACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAA 360
Tropomyosin         ------------------------------------------------------------


TPM2'_f_T7          CTTTAAGAAGGAGATATACATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTG 87
TPM2'_r_T7term      CTTTAAGAAGGAGATATACATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTG 420
Tropomyosin         --------ATGAAA----------CACCATCACCATCACCAT-------------AT--- 26
                            * ** *          ******** ***** ***             **


TPM2'_f_T7          GGTACCGAGAACCTGTACTTCCAATCCATGGATGCCATTAAAAAAAAAAATGCAGATGCTG 147
TPM2'_r_T7term      GGTACCGAGAACCTGTACTTCCAATCCATGGATGCCATTAAAAAAAAAAATGCAGATGCTG 480
Tropomyosin         ------GAAA---------------CA-GGATGCCATTAAAAAAAAAATGCAGATGCTG 63
                          ** *                ** ******************************


TPM2'_f_T7          AAACTGGATAAAGAAAATGCCATTGATCGTGCAGAACAGGCAGAAGCAGATAAAAAACAG 207
TPM2'_r_T7term      AAACTGGATAAAGAAAATGCCATTGATCGTGCAGAACAGGCAGAAGCAGATAAAAAACAG 540
Tropomyosin         AAACTGGATAAAGAAAATGCCATTGATCGTGCAGAACAGGCAGAAGCAGATAAAAAACAG 123
                    ************************************************************


TPM2'_f_T7          GCCGAAGATCGTTGTAAACAGCTGGAGGAAGAACAGCAGGCACTGCAAAAAAAACTGAAA 267
TPM2'_r_T7term      GCCGAAGATCGTTGTAAACAGCTGGAGGAAGAACAGCAGGCACTGCAAAAAAAACTGAAA 600
Tropomyosin         GCCGAAGATCGTTGTAAACAGCTGGAGGAAGAACAGCAGGCACTGCAAAAAAAACTGAAA 183
                    ************************************************************


TPM2'_f_T7          GGCACCGAAGATGAAGTGGAAAAATATAGCGAAAGCGTGAAAGAAGCACAGGAAAAACTG 327
TPM2'_r_T7term      GGCACCGAAGATGAAGTGGAAAAATATAGCGAAAGCGTGAAAGAAGCACAGGAAAAACTG 660
Tropomyosin         GGCACCGAAGATGAAGTGGAAAAATATAGCGAAAGCGTGAAAGAAGCACAGGAAAAACTG 243
                    ************************************************************


TPM2'_f_T7          GAACAGGCCGAAAAAAAAGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGT 387
TPM2'_r_T7term      GAACAGGCCGAAAAAAAAGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGT 720
Tropomyosin         GAACAGGCCGAAAAAAAAGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGT 303
                    ************************************************************


TPM2'_f_T7          ATTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGTAG 447
TPM2'_r_T7term      ATTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGTAG 780
Tropomyosin         ATTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGCAA 363
                    ************************************************************ *


TPM2'_f_T7          TAAC----------AGTAAAGGTGG-ATACGGATCCGAATTCGAGCT-----CCGTCGAC 491
TPM2'_r_T7term      TAAC----------AGTAAAGGTGG-ATACGGATCCGAATTCGAGCT-----CCGTCGAC 824
Tropomyosin         AAACTGGAAGAAGCAGAAAAAGCAGCAGATGAAAGCGAACGTGGCATGAAAGTGATTGAA 423
                     ***          ** *** *  * * * * *  ****   *   *        * **


TPM2'_f_T7          AAGCTTGCGGCCGCACTCGA-GCACCACCACCACCACCACTGAGATCCGGCTGCTAACAA 550
TPM2'_r_T7term      AAGCTTGCGGCCGCACTCGA-GCACCACCACCACCACCACTGAGATCCGGCTGCTAACAA 883
Tropomyosin         AATCGTGCCATGAAAGATGAAGAAAAAATGGAACTGCAA--GAAATGCAGCTG--AAAGA 479
                    ** * ***      *   ** * *  *     **  * *  ** ** * ****  **  *


TPM2'_f_T7          AGCCCGAAAGGAAGCTGA-GTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCC 609
TPM2'_r_T7term      AGCCCGAAA---------------------------------------------------- 892
Tropomyosin         AGCTAAACATATTGCCGAAGATAGCGATCGCAAATATGAAGAAGTTGCCCGTAAA----- 534
                    ***   * *
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TPM2’’-WT

```
TPM2’’_f_T7          ---------------------------CTAGAA-TAATTTTGTTTAACTTTAAGAAGGAG 32
TPM2’’_r_T7term      GAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAG 480
Tropomyosin          AGCTGGAGGAAGAACAGCAGG----CACTGCAAAAAAAACTGAAAGGCACCGAAGATGAA 198
                                              **  **  **   **     *    *  * **

TPM2’’_f_T7          ATATACATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACC 92
TPM2’’_r_T7term      ATATACATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACC 540
Tropomyosin          GTGGAAAAATATAGCGAAAGCGTGAAAGA------AGCACAG----GAAAAACTGGAACA 248
                      *  * * **  * *   * * * *           *   **    *   * *  ****

TPM2’’_f_T7          TGTACTTCCAATCCATGGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGTA 152
TPM2’’_r_T7term      TGTACTTCCAATCCATGGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGTA 600
Tropomyosin          GGC----CGAAAAAAAAGCAACCGATGCCGAAGCAGATGTTGCAAGCCTGAATCGTCGTA 304
                      *     * **   *  ****************************************

TPM2’’_f_T7          TTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGCAAA 212
TPM2’’_r_T7term      TTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGCAAA 660
Tropomyosin          TTCAGCTGGTTGAAGAAGAACTGGATCGCGCACAGGAACGTCTGGCAACCGCTCTGCAAA 364
                     ************************************************************

TPM2’’_f_T7          AACTGGAAGAAGCAGAAAAAGCAGCAGATGAAAGCGAACGTGGCATGAAAGTGATTGAAA 272
TPM2’’_r_T7term      AACTGGAAGAAGCAGAAAAAGCAGCAGATGAAAGCGAACGTGGCATGAAAGTGATTGAAA 720
Tropomyosin          AACTGGAAGAAGCAGAAAAAGCAGCAGATGAAAGCGAACGTGGCATGAAAGTGATTGAAA 424
                     ************************************************************

TPM2’’_f_T7          ATCGTGCCATGAAAGATGAAGAAAAAATGGAACTGCAAGAAATGCAGCTGAAAGAAGCTA 332
TPM2’’_r_T7term      ATCGTGCCATGAAAGATGAAGAAAAAATGGAACTGCAAGAAATGCAGCTGAAAGAAGCTA 780
Tropomyosin          ATCGTGCCATGAAAGATGAAGAAAAAATGGAACTGCAAGAAATGCAGCTGAAAGAAGCTA 484
                     ************************************************************

TPM2’’_f_T7          AACATATTGCCGAAGATAGCGATCGCAAATATGAAGAAGTTGCCCGTAAACTGGTGATTC 392
TPM2’’_r_T7term      AACATATTGCCGAAGATAGCGATCGCAAATATGAAGAAGTTGCCCGTAAACTGGTGATTC 840
Tropomyosin          AACATATTGCCGAAGATAGCGATCGCAAATATGAAGAAGTTGCCCGTAAACTGGTGATTC 544
                     ************************************************************

TPM2’’_f_T7          TGGAAGGTGAACTGGAACGTAGCTAGTAAC-------AGT----AAAGGTGGATACGG-- 439
TPM2’’_r_T7term      TGGAAGGTGAACTGGAACGTAGCTAGTAAC-------AGT----AAAGGTGGATACGG-- 887
Tropomyosin          TGGAAGGTGAACTGGAACGTAGCGAAGAACGTGCCGAAGTTGCAGAAAGCAAATGTGGCG 604
                     ********************** *  ***       ***     ** *   **  **

TPM2’’_f_T7          ATCCG-AATTCGAGCTCCGTCGACAAGCTTGCGGCCGC--ACTCGAGCA--CCACCACCA 494
TPM2’’_r_T7term      ATCCG-AATTCGAGCTCCGTCGACAAGCTTGCGGCCGC--ACTCGAGCA--CCACCACCA 942
Tropomyosin          ATCTGGAAGAGGAACT-----GAAAA--TTGTTACCAATAATCTGAAAAGCCTGGAAGCA 657
                     *** * **   ** **     ** **  ***   **    *   **  *  *    * **

TPM2’’_f_T7          CCACCACTGAGATCCGGCTGCTAACAA----AGCCCGAAAGGAAGCTGAGTTGGCTGCTG 550
TPM2’’_r_T7term      CCACCACTGAGATCCGGCTGCTAACAA----AGCACGAA-------------------- 977
Tropomyosin          CAGGCAGATAAATATAGCACCAAAGAAGATAAATACGAAGAAGAAATTAAACTGCTG--- 714
                     *   **   * **   **  * ** **    *   ****
```

TPM2’’’-WT

```
TPM2’’’_f_T7        ------------------------------------------------TAGAA-TAAT 9
TPM2’’’_r_T7term    AGGGGAATTGTGAGCGG-----------------ATAACAATTCCCCTCTAGAAATAAT 349
Tropomyosin         GCAAAAACTGGAAGAAGCAGAAAAAGCAGCAGATGAAAGCGAACGTGGCATGAAAGTGAT 419
                                                                *  ** * **

TPM2’’’_f_T7        T-----TTGT----TTAACTTTAAGAAGGAGATA----TACAT---ATGCACCATCATCA 53
TPM2’’’_r_T7term    T-----TTGT----TTAACTTTAAGAAGGAGATA----TACAT---ATGCACCATCATCA 393
Tropomyosin         TGAAAATCGTGCCATGAAAGATGAAGAAAAAATGGAACTGCAAGAAATGCAGCTGAAAGA 479
                    *     * **    * **   * *  *  * **     * **    ***** *   *  *

TPM2’’’_f_T7        ---TCATCATTCTTCTGGTG-TAG--ATC-TGGGTACCGAGAA----CCTGTA--CTTCC 100
TPM2’’’_r_T7term    ---TCATCATTCTTCTGGTG-TAG--ATC-TGGGTACCGAGAA----CCTGTA--CTTCC 440
Tropomyosin         AGCTAAACATATTGCCGAAGATAGCGATCGCAAATATGAAGAAGTTGCCCGTAAACTGGT 539
                       * * ***  * * *  * ***  ***     **   ****    ** ***  **

TPM2’’’_f_T7        AATCCATGGAAGGTGAACTGGAACGTAGCGAAGAACGTGCCGAAGTTGCAGAAAGCAAAT 160
TPM2’’’_r_T7term    AATCCATGGAAGGTGAACTGGAACGTAGCGAAGAACGTGCCGAAGTTGCAGAAAGCAAAT 500
Tropomyosin         GATTC-TGGAAGGTGAACTGGAACGTAGCGAAGAACGTGCCGAAGTTGCAGAAAGCAAAT 598
                     ** * ************************************************

TPM2’’’_f_T7        GTGGCGATCTGGAAGAGGAACTGAAAATTGTTACCAATAATCTGAAAAGCCTGGAAGCAC 220
TPM2’’’_r_T7term    GTGGCGATCTGGAAGAGGAACTGAAAATTGTTACCAATAATCTGAAAAGCCTGGAAGCAC 560
Tropomyosin         GTGGCGATCTGGAAGAGGAACTGAAAATTGTTACCAATAATCTGAAAAGCCTGGAAGCAC 658
                    ************************************************************

TPM2’’’_f_T7        AGGCAGATAAATATAGCACCAAAGAAGATAAATACGAAGAAGAAATTAAACTGCTGGAAG 280
TPM2’’’_r_T7term    AGGCAGATAAATATAGCACCAAAGAAGATAAATACGAAGAAGAAATTAAACTGCTGGAAG 620
Tropomyosin         AGGCAGATAAATATAGCACCAAAGAAGATAAATACGAAGAAGAAATTAAACTGCTGGAAG 718
                    ************************************************************

TPM2’’’_f_T7        AAAAACTGAAAGAAGCCGAAACACGCGCAGAATTTGCAGAACGTAGCGTTGCAAAACTGG 340
TPM2’’’_r_T7term    AAAAACTGAAAGAAGCCGAAACACGCGCAGAATTTGCAGAACGTAGCGTTGCAAAACTGG 680
Tropomyosin         AAAAACTGAAAGAAGCCGAAACACGCGCAGAATTTGCAGAACGTAGCGTTGCAAAACTGG 778
                    ************************************************************

TPM2’’’_f_T7        AAAAAACCATTGATGATCTGGAAGATGAAGTGTATGCCCAGAAAATGAAATATAAAGCCA 400
TPM2’’’_r_T7term    AAAAAACCATTGATGATCTGGAAGATGAAGTGTATGCCCAGAAAATGAAATATAAAGCCA 740
Tropomyosin         AAAAAACCATTGATGATCTGGAAGATGAAGTGTATGCCCAGAAAATGAAATATAAAGCCA 838
                    ************************************************************

TPM2’’’_f_T7        TTAGCGAAGAACTGGATAATGCCCTGAATGATATTACCAGCCTGTAGTAACAGTAAAGGT 460
TPM2’’’_r_T7term    TTAGCGAAGAACTGGATAATGCCCTGAATGATATTACCAGCCTGTAGTAACAGTAAAGGT 800
Tropomyosin         TTAGCGAAGAACTGGATAATGCCCTGAATGATATTACCAGCCTGTAGTAA---------- 888
                    ********************************************

TPM2’’’_f_T7        GGATACGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCA 520
TPM2’’’_r_T7term    GGATACGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCA 860
Tropomyosin         ------------------------------------------------------------

TPM2’’’_f_T7        CCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGC 580
TPM2’’’_r_T7term    CCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAA---------------------- 899
Tropomyosin         ------------------------------------------------------------
```