Investigating the potential role of Cadherin-23 as a Calcium sensor

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Certificate of Examination

This is to certify that the dissertation titled **"Investigating the potential role of Cadherin-23 as a Calcium sensor"** submitted by **Ms. Shweta Mishra** (Reg. No. MS15084) for the fulfillment of **BS-MS dual degree programme** of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Sudip Mandal Dr. Lolitika Mandal Dr. Sabyasachi Rakshit
(Supervisor)

Dated: 24/04/2020

Declaration

The work presented in the dissertation has been carried out by me under the guidance of Dr. Sabyasachi Rakshit at the Indian Institute of Science Education and Research, Mohali.

This work has not been submitted in part or in full for a degree, a diploma or a fellowship to any university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed withing have been detailed in the bibliography.

Shweta Mishra
(Candidate)
April 24, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sabyasachi Rakshit
(Supervisor)

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Abstract

Cadherin-23, a non-classical Cadherin protein with twenty seven extracellular domains, is well studied for its role in hearing loss. It is involved in both homophilic and heterophilic interactions depending on the concentration of extracellular Calcium ions. In this thesis we standardise and try to utilise a physical chemistry based technique, to visualise the changes in the transmembrane domain of this membrane spanning protein upon ligand binding. In the scope of this thesis, we also attempt at understanding how this signal is further transmitted to the cell cytoskeleton.

Chapter 1

Introduction

Cadherins are calcium-dependent adhesion receptor proteins that mediate cell-cell adhesion and are known to be involved in cell proliferation, cell polarity and motility. They play a crucial role during embryogenesis and morphogenesis of organisms, and are also involved in intracellular as well as intercellular signaling.[1, 2, 3] Cadherins are broadly divided into classical and non-classical cadherins based on the length of their extracellular domains. Classical cadherins, otherwise known as typical cadherins, for instance, E-Cadherin and N-Cadherin, have a higher expression across cell types, and their role linked to actin cytoskeleton is well-studied.[4, 2, 5] Non-classical cadherins or atypical cadherins, on the other hand, have a lower endogenous expression. The extracellular domains of these atypical cadherins vary in domain length, with as long as 34 extracellular domains in the case of Fat Cadherins. These cadherins carry out a diverse set of functions, ranging from cell polarity to force receptor.[6]

Cadherin-23 is a long chain non-classical cadherin protein with twenty-seven extracellular domains. It mediates both homophilic and heterophilic interactions. At the tip links of neuroepithelial hair cells, Cadherin-23 interacts with another non-classical Cadherin protein Protocadherin-15, where it acts as a gating spring.[7, 8] In contrast, in the epithelial tissues of the lungs, liver, and kidney, Cadherin-23 mediates homophilic interaction.[7, 9, 10] The extracellular Calcium ion concentrations in these tissue niches are drastically different, with a low Calcium concentration in the endolymph (20-50uM) and a high Calcium concentration in epithelial tissue (10mM).[11, 12] Thus, it is prudent to study if Cadherin-23 can act as a sensor of Calcium in the extracellular environment, in addition to being Calcium-dependent for its functioning.

Chapter 2

Deciphering allostery in Cadherin-23 using Liquid Crystals

2.1 Introduction

The complex organization in higher-order organisms demands coordinated communication among cells. Cells perceive and respond to signals from their immediate neighbors as well as from distant sources. Communication between cells primarily takes place through signaling molecules like proteins, small peptides, amino acids, nucleotides, fatty acids, steroids, ions, small molecules, and non-coding RNAs, and many others, that are secreted into the extracellular environment.[13] These extracellular signaling molecules are recognized by receptor proteins of the receiving cells. Receptors for water-soluble ligands are present on the plasma membrane, whereas receptors for lipid-soluble ligands that are transported inside the cell are found on the membrane of intracellular compartments such as the nucleus. Receptor proteins usually consist of an extracellular domain that binds to the signaling molecule, one or more membrane-spanning transmembrane domains and an intracellular cytoplasmic domain. Upon ligand binding to the extracellular receptor domain, a cascade of downstream intracellular signaling takes place, often triggered by conformational changes in the cytoplasmic domain of the receptor protein. This phenomenon of signal transduction where the binding of a ligand to the receptor at one site causes changes at a distal site, thereby rendering function to the receptor, is termed as allostery.

Numerous instances of allostery are seen in biological systems. G-protein coupled receptors (GPCRs) are a family of seven- transmembrane cell surface receptor

proteins that play a crucial role in cell-cell communication and signaling. GPCRs exist as heterotrimers of three protein subunits (alpha, beta and gamma). The span of seven transmembrane domains extend to both intracellular and extracellular regions. The extracellular loops bind to specific ligand, whereas the intracellular loops bind to the G-proteins upon external trigger. In basal conditions, G-proteins are bound to guanosine diphosphate (GDP) and all the subunits of the G-protein are together. Upon ligand binding in the extracellular domain, there is a quick transmission of signal to the cytoplasmic domain through the transmembrane region. Such external chemical cue. Consequently,the affinity to GDP is dropped and towards GTP is enhanced. Henceforth GTP binds to the intracellular transmembrane and triggers disengagement of the subunits.Disengagement of the heterotrimer complex initiates further downstream signaling.[14, 15]

In Epidermal growth factor receptor (EGFR), the rotation of the linkage between the transmembrane domain with the kinase domain leads to transphosphorylation and activation of the intracellular tyrosine kinase domain.[16] EGFR is known to promote cell growth, differentiation, and proliferation.

Integrins are a family of cell surface receptors involved in cell adhesion to the extracellular matrix and migration. Heterodimeric integrins transmit signals bidirectionally into and out of the cell depending on whether the signal is extracellular or intracellular. These signaling events correlate with the association state of the transmembrane segments of their alpha and beta subunits.[17, 18]

Fas, a member of Tumor Necrosis Factor (TNF) receptor family, binds to Fas ligand and promotes apoptosis. Upon ligand binding, the Fas receptor trimerizes further transmitting the pro-apoptotic signal.[19, 20, 21, 22] In cancer, a soluble antagonist of the Fas receptor protein binds to the Fas ligand and prevents its binding to the transmembrane Fas, thereby preventing apoptosis and promoting uncontrolled cell growth.[23] In addition to this, somatic mutations in the transmembrane domain of the Fas receptor have been shown in cancer cells. [24]

Allostery thus is a widespread phenomenon involved in signaling. Since membrane proteins are exposed to the surrounding, manipulation of the extracellular conditions is easy, making membrane proteins the primary targets for many drugs

and therapy. For instance, specific targeting of receptor tyrosine kinases using monoclonal antibodies has emerged as a new therapy for malignancies. [25, 26]

2.2 Hypothesis

We propose a simple method to monitor the conformational alterations or allostery in the trans-membrane domain upon ligand binding using liquid crystals based sensors. The extent of altered optical polarization of liquid crystals can act as a quantitative readout of the conformational change in transmembrane domain. (Figure 2.1)

Aqueous-liquid crystal interface mimic the dynamic plasma membrane. Alterations in the transmembrane domain leads to orientational ordering transition of the liquid crystal, the optical output of which can be observed with the naked eye. [25, 26]

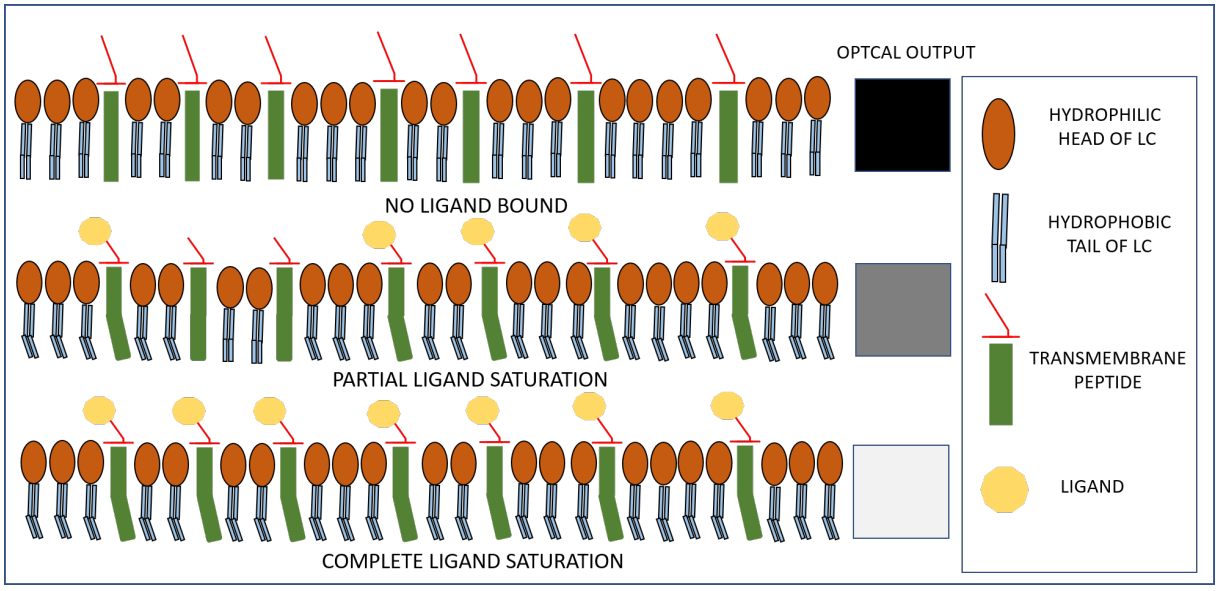


Figure 2.1: Schematic Representation of the Liquid Crystal Experiment

2.3 Experimental Procedure

2.3.1 Cloning, Expression and Purification of Cdh-23 Tm domain

Transmembrane domain of a protein is highly hydrophobic as it is surrounded by the lipid moieties in the membrane. As such transmembrane peptide has to be expressed along with a soluble protein lest it will go into the inclusion bodies and be proteolytically cleaved. For this purpose, a soluble tag protein, Maltose-binding protein (MBP) was used.

Mouse Cadherin-23 (Cdh-23) transmembrane domain (L3065 to L3085) was PCR amplified using primer pair Cdh23TM FP1 and Cdh23TM RP. Cdh23 full length sequence was used as template for this purpose. Cdh23TM FP1 has an overhanging sort tagging site along with the overlapping sequence of the transmembrane domain. Cdh23TM RP has an overhanging XhoI recognition sequence (Figure 2.3A). The amplicon from this round of PCR amplification was further used as template for the second round of PCR amplification using primer pair Cdh23TM FP2 and Cdh23TM RP. Cdh23TM FP2 has an overhanging TEV Protease cleavage site and BamHI recognition sequence (Figure 2.3A). The first two rounds of PCR were carried out with Q5 High-fidelity DNA polymerase. Amplicon from the second round of PCR amplification was then PCR amplified using the primer pair Cdh23TM FP2 and Cdh23TM RP with Taq DNA polymerase. Using Taq DNA polymerase enzyme ensures the addition of an overhanging A-tail to the 3′ end, required for cloning the PCR product into a pGEM-T Easy Vector (Promega) (Figure 2.3C). This ensures proper digestion of the insert using restriction endonucleases. This T-vector provides a double screening of positive clones with antibiotic resistance (Ampicillin) and blue-white screening. Positive colonies (Figure 2.3D) were then confirmed using colony PCR with vector specific universal primer pair M13 Forward and Reverse Primer.

Cdh23 TM FP1	CAGGGCGGCGGCGGGCCGAAACGCATGTCTGCCCTGCAGA
Cdh23 TM FP2	GAATTCGGATCCGAAAATCTGTACTTCCAGGGCGGAGGCGGG
Cdh23 TM RP	TATATACTCGAGTTAGAGGACGAAGAGCATGGCAGCCAA
Cdh23 TM FP3	CCGAAACGCATGTCTG

Table 2.1: Primers for Cdh23TM Cloning

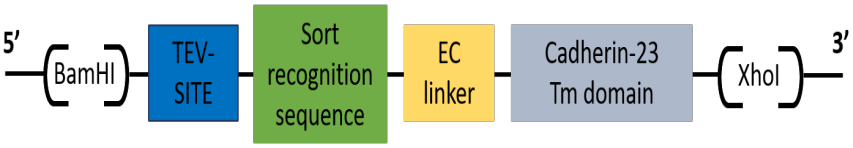


Figure 2.2: Schematic representation of Cdh23 TM construct

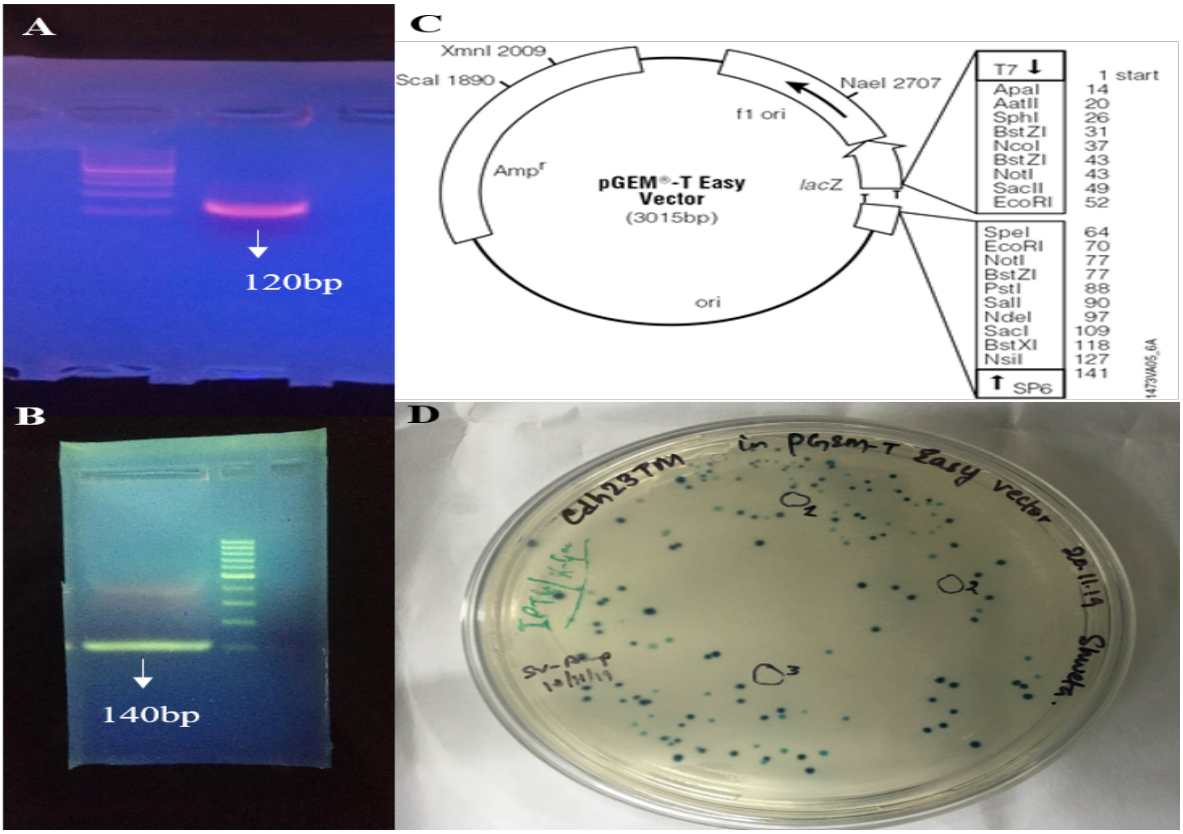


Figure 2.3: PCR Amplification of Cdh23 TM and cloning into T-vector: (A) PCR amplification to include sort-tagging sequence and XhoI sequence to Cdh-23 Tm sequence, Amplicon length 120 bp (B) PCR amplification to include TEV protease cleavage sequence and BamHI sequence to Amplicon 1. Amplicon length 140 bp (C). pGEM-T Easy Vector map (D). Transformed colonies on a X-Gal coated Ampicillin plate for blue-white screening,

Plasmid from positive clones was then isolated and digested with BamHI and XhoI to give an insert of 140 base pairs (Figure 2.4A), which was then cloned into pMAL-c2X (Figure 2.4C) vector digested with the same restriction enzymes (Figure 2.4B). The pMAL-c2X vector used for this purpose is modified to have an XhoI

recognition site downstream of BamHI and upstream of HindIII and a His- affinity tag at the N-terminal of MBP (for flexibility in affinity purification). For protein expression, the plasmid was then expressed in *E.coli* Lemo21(DE3) cells.

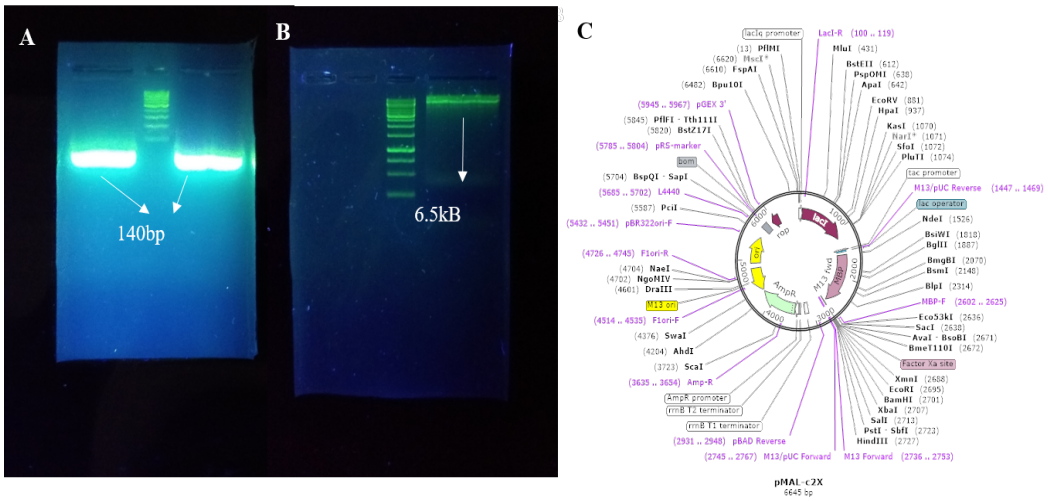


Figure 2.4: Sub-cloning of Cdh23TM insert from pGEM-T vector into pMAL vector: (A) Double digestion of insert from pGEM-T Clone using BamHI and XhoI, insert length 140 bp (B) Double digestion of pMAL-c2X EC3 with BamHI and XhoI, digested vector length 6.5 kB,(C) Original pMAL-c2X Vector map, XhoI restriction site has been inserted between BamHI and HindIII

For optimal expression, the cells were grown in LB Media (Hi-Media) supplemented with 1% Glucose, 750 μM Rhamnose at 37 ^{0}C till an OD_{600} of 0.5 was reached and then induced with 0.4 mM IPTG (Hi-Media) at 37 ^{0}C for 2.5 hours. Then cells were pelleted down and resuspended in HEPES buffer (50 mM NaCl, 50 mM KCl, 25mM HEPES, pH 7.5, all from HiMedia). Chemical lysis was done using Lysozyme (2mg per gram of pellet). Protease inhibitor PMSF (1mM) was added to prevent proteolytic degradation. This was followed by sonication till the solution is translucent and centrifugation at 4 ^{0}C for 30 minutes at 10000g. The supernatant was then loaded onto amylose column (Polypropylene column: Qiagen, Amylose resin: NEB) that had previously been equilibrated with HEPES buffer for binding

followed by washing with HEPES buffer. The protein was eluted with 10 mL HEPES buffer supplemented with 10mM Maltose (Hi-Media) (Figure 2.5). The eluents were then pooled together for buffer exchange and concentration of the desired protein (Amicon® Ultra-4 Centrifugal Filter Units 10 NMWL).

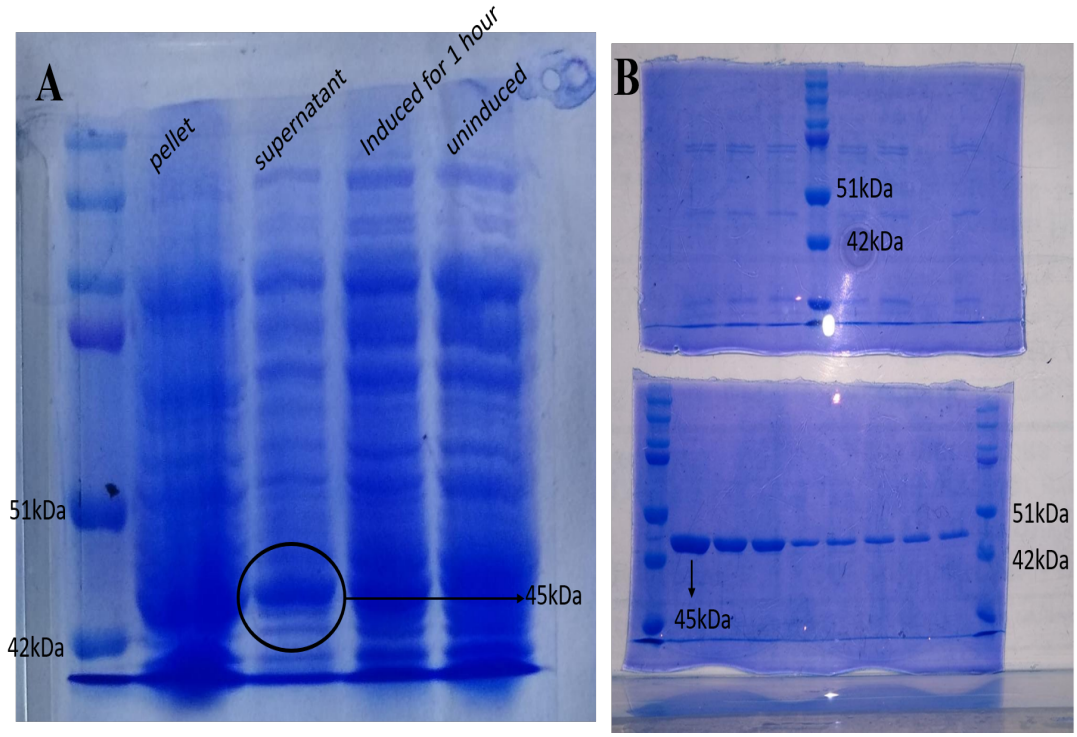


Figure 2.5: Coommassie stained SDS PAGE after purification: (A) Encircled in supernatant well is Cdh23TM-pMAL (45 kDa) (B) Purification using Nickel-NTA (top) and Amylose (bottom) resin

2.3.2 Reaction with Liquid Crystal

The concentrated protein was then put on liquid crystal embedded grids (Figure 2.6A). Insertion of the hydrophobic transmembrane domain into the hydrophobic layers of liquid crystal should stabilize it. However,the liquid crystal did not stabilize even after 24 hours (Figure 2.6B). Since the hydrophobic transmembrane peptide is tiny (3kDa) as compared to the soluble MBP (42kDa), there is a possibility the fused protein is soluble in aqueous environment, unable to insert itself into the liquid crystal. The peptide was then cleaved from the soluble MBP using TEV Protease (purification protocol AppendixA) at an enzyme: protein molar ratio of 1:20 at 4 ^{0}C overnight in the presence of 0.1% Triton-X. The resulting solution when put

on liquid crystal grids bleached the liquid crystal probably due to the presence of Triton-X (Figure 2.6C).

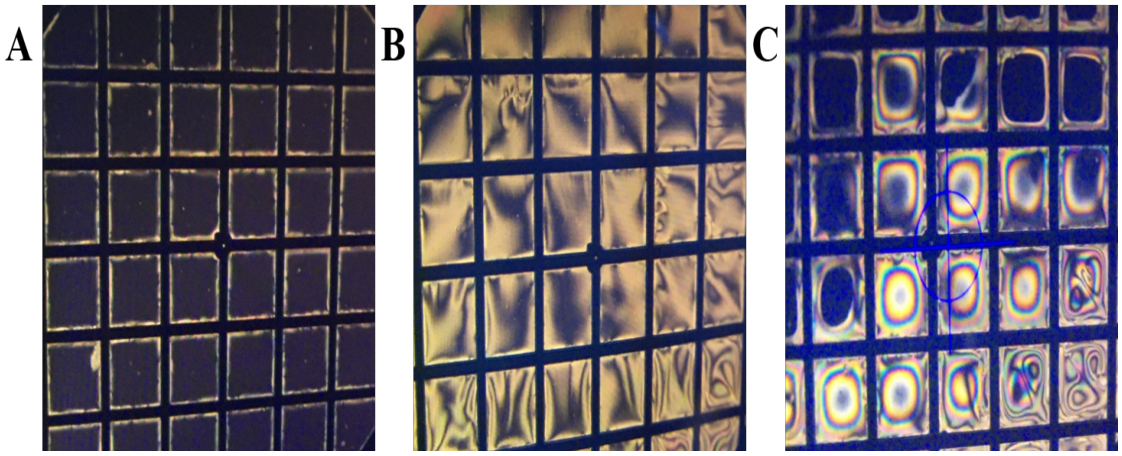


Figure 2.6: Cdh23TM-pMAL on Liquid Crystal: (A) Liquid crystal in the absence of any aqueous solution, ordered liquid crystal, dark optical output (B) Disordered liquid crystal on addition of protein dissolved in water (24 hours after addition) (C) Bleaching of liquid crystal from the edges of the grid upon contact with detergent

To purify the peptide, concentrated fusion protein after TEV proteolytic cleavage was passed through Amicon® Ultra-4 Centrifugal Filter Units with cutoff of 10 kDa. Desired peptide is 31 amino acid long (approximately 3kDa) and thus was found in the flowthrough. To visualize the peptide Tris-Tricine gel was run (Appendix B) and bands were visualized using silver staining (Figure 2.7) (Appendix C). The bands could not be reproduced in further experiments.

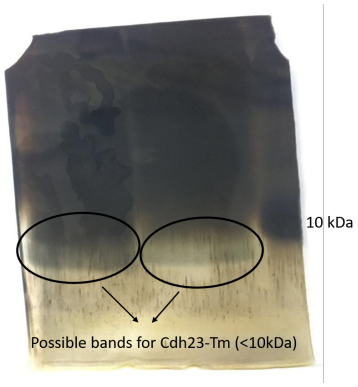


Figure 2.7: Silver staining of TEV-cleaved Cdh23TM-pMAL: Encircled: bands below 10 kDa as seen in SDS- polyacrylamide gel stained using silver staining after the fused protein is cleaved using TEV Protease

2.4 Future Directions

The transmembrane domain of Cadherin-23 needs to be cleaved from the fused protein and embedded into the liquid crystal. One way to do this would be to isolate the peptide, lyophilize it and mix it with liquid crystal before putting it on the grid. After that, using sort-tagging an extracellular calcium-sensing domain has to be attached to this transmembrane domain which will then be subjected to varying Calcium concentrations.

Chapter 3

Understanding mechanochemical signalling via Cadherin-23

3.1 Introduction

Interaction of classical cadherins with the actin cytoskeleton is well studied. Proteins such as α-catenin and β-catenin link these cadherins to the actin filaments.[27, 28, 29] Non-classical cadherins on the other hand have different cytosolic domains and their downstream interaction is not well known. Cadherin-23 is known to interact with the cytoskeleton proteins via different intermediary proteins such as MAGI-1 (Cdh23 binds to PDZ4 domain of MAGI-1 which can interact with β-catenin via its PDZ5 domain),[30, 31] Harmonin A (to F-Actin)[32] and Harmonin B (to Myosin 7a).[33] The regulation of these interactions is not well studied.

3.2 Hypothesis

We hypothesise that Cadherin-23 senses the extracellular force via its EC domains and transmits this signal to its cytosolic domain. This in turn influences the interatcion of the cytosolic domain with the actin cytoskeleton via different binding partners.

Binding of Calcium ions to the EC domains of Cadherin-23, provides rigidity to the protein (Figure 3.1). Hence, changing the calcium concentration should change the force experienced by the protein and affect its downstream interactions.

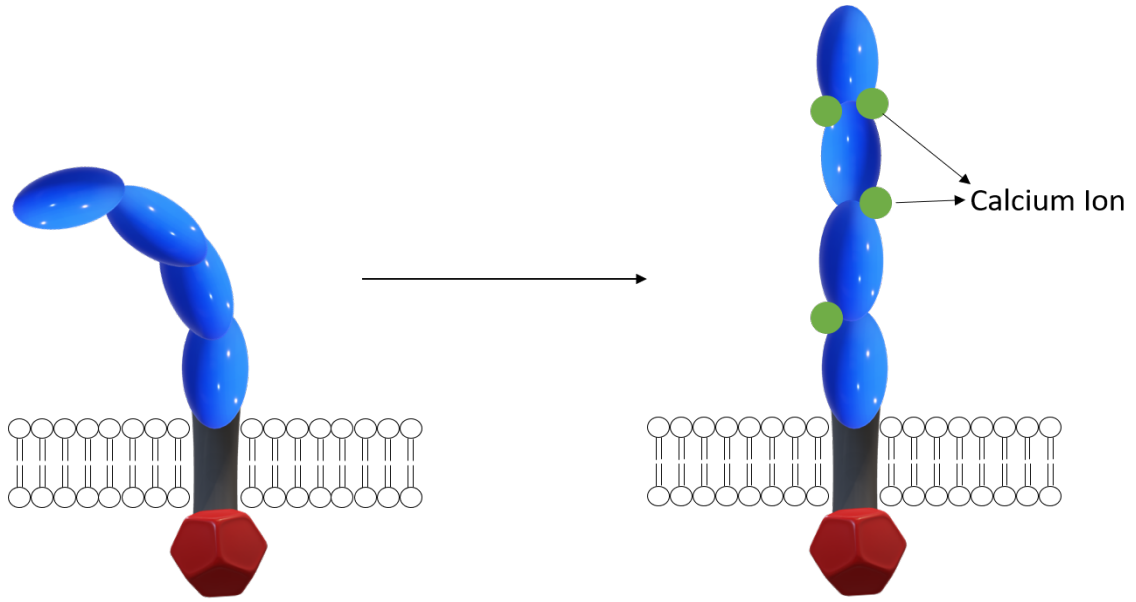


Figure 3.1: Schematic Representation of the Effect of Calcium binding to EC domain of Cadherin-23

For the proof of principle, first we wanted to see the effect of varying force on the downstream targets of E-Cadherin. Interaction of E-Cadherin with actin cytoskeleton is very well documented. The goal was to vary the force experienced by the extracellular domains of the protein and see the effect it has on the arrangement of the actin filaments.

3.3 Experimental Procedure

3.3.1 Cloning

E-Cadherin had to be modified to include a fluorescent marker and an Avitag sequence at the N-terminus of the protein, between the signal peptide and EC1 domain. The clone was designed as three different fragments and assembled using Gibson reaction.

FRAGMENT 1: It has the signal sequence of Cadherin-23 to ensure localization of the protein to the cell membrane and an Avitag sequence for biotinylation and attachment of magnetic bead. This was amplified using GBSN ECdh FP1 and GBSN

ECdh RP1 from Avitag SGH Vector in the lab. The forward primer contains overlapping sequence with the vector and reverse primer has sequence overlapping with Fragment 2. The resulting amplicon was 120 basepairs long (Figure 3.3A)
FRAGMENT 2: This fragment has ECadherin full length sequence with mCherry, amplified from murine ECadherin mCherry plasmid using primer pair GBSN ECdh FP2 and GBSN ECdh RP2. Forward primer bind to N-terminal sequence of E-Cadherin after its signal sequence whereas the reverse primer contains sequence overlapping with the Vector backbone (Figure 3.3B).
FRAGMENT 3: This is the vector backbone that contains antibiotic resistance cassette and promoter sequences. The murine ECdh mCherry plasmid was digested with HindIII and NotI to take out the ECadherin mCherry insert (Figure 3.3 C,D).

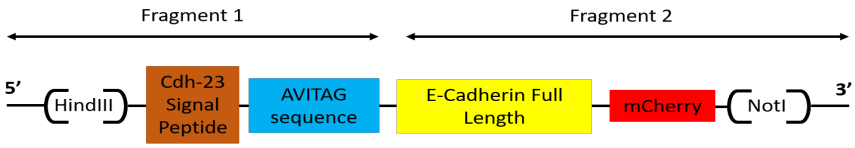


Figure 3.2: Schematic Representation of Avitag E-Cadherin mCherry construct

Fragments 1, 2 and 3 were then joined using Gibson reaction at 50^0C for 1hour. Gibson reaction mixture (In-Fusion ® Cloning, Takara) contains low-affinity DNA Polymerase and Ligase to join overlapping fragments without the need for restriction digestion. The DNA-Gibson reaction mixture was then transformed into E. coli DH5α cells and screened for positive colony using GBSN ECdh FP2 and ECdh TA RP.

GBSN ECdh FP1	TCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGATGAGGTACTCCCTGGTCAC
GBSN ECdh RP1	GGGACTGCAGGACTCAGGCTCCAGCTCCTGATCACGCAGTTCCATTTTCA
GBSN ECdh FP 2	CAGGAGCTGGAGCCTGAGTC
GBSN ECdh RP2	TATGGCTGATTATGATCTAGAGTCGCGGCCGCTTTACTTGTACAGCTCGTCC
ECdh TA RP	GGGTCGTCCTCACCACCGC

Table 3.1: Primers for Avitag ECdh mCherry cloning

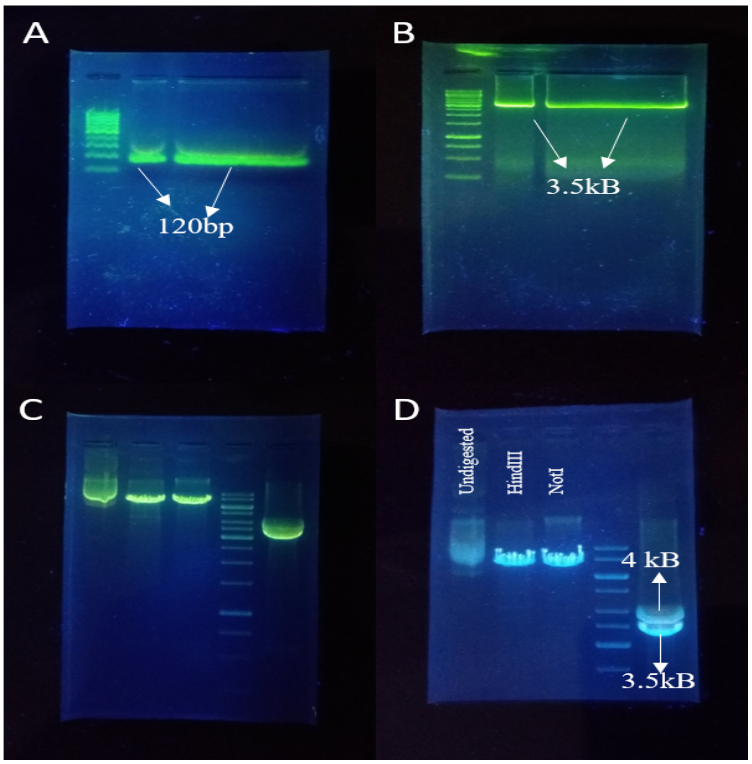


Figure 3.3: PCR amplification and cloning of Avitag ECadherin mCherry: (A) PCR Amplification of Fragment 1, AVITAG and Cadherin-23 Signal Peptide, 120 bp amplicon length (B) PCR Amplification of Fragment 2, ECadherin mCherry, 3.5kB amplicon length (C) Double digestion of murine ECadherin mCherry by HindIII and NotI to yield two fragments of length 4kB and 3.5 kB, 4 kB fragment equivalent to pmCherry-N1 vector used for cloning our Gibson clone (D) C further resolved to show two separate bands

3.3.2 Expression in mammalian cells

For further experiments, the clones needed to be expressed in mammalian cells. For this, MDCK cells were at first transiently transfected with Avitag ECdh mCherry and GFP Actin (for live cell imaging of actin filaments) using liposome mediated delivery system (Lipofectamine^{TM} 3000 Transfection Reagent, K2 Transfection System – Biontex). For stable cell line, MDCK cells were first transfected with GFP Actin and selected on G418 (600μg/mL in DMEM media). The cells at first showed signs of stress. Selection was continued till cells recovered from this stress and assumed their normal cell shape. Thereafter, cells were transfected with Avitag ECdh mCherry and selected on G418 (600μg/mL) (Figure 3.4). Stable cells with GFP Actin and Avitag ECdh mCherry were then maintained at a G418 concentration of 200μg/mL in DMEM media.

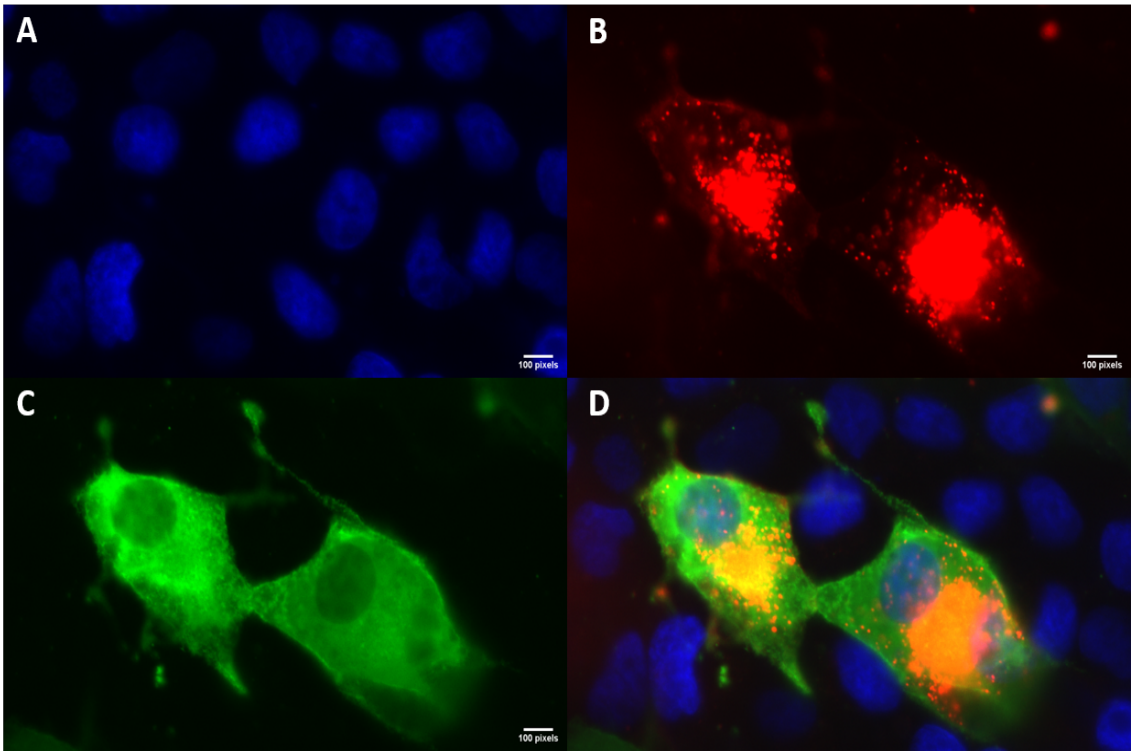


Figure 3.4: MDCK cells transfected with E-Cadherin mCherry and GFP Actin, image taken at 100X magnification: (A) Hoechst stained nucleus (B) E-Cadherin mCherry (C) Actin GFP (D) Merged channels

3.4 Future Directions

Following biotinylation, the force exerted on the EC domain of E-Cadherin has to be varied to see the effect on the interacting actin filaments. This requires a combination of force spectroscopy and fluorescence microscopy. In future, different constructs of Cadherin-23 are to be used to study the effect of force on its interaction to the cytoskeleton. At constant external force, the effect of varying extracellular Calcium concentrations on the interaction of Cadherin-23 to its downstream binding partners also need to be seen to determine if Cadherin-23 indeed acts as a calcium sensor.

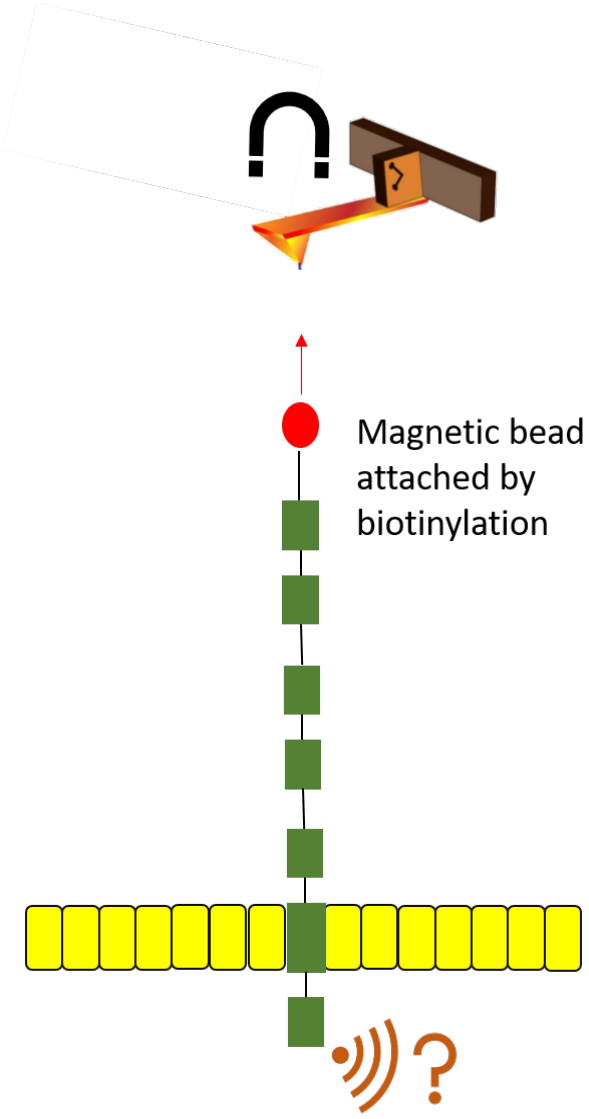


Figure 3.5: Schematic Representation of pulling experiment with magnetic tweezer

Appendix A

TEV Protease Purification

- Primary inoculation of TEV Protease Rosetta cells at 37^0C overnight in LB media with Ampicillin and Chloramphenicol.
- Secondary culture in LB media with Ampicillin and Chloramphenicol, at 37^0C till the OD_{600} reaches 0.4.
- Induce protein expression with 0.3mM IPTG. Incubate at 16^0C for 20 hours.
- Pellet down the cells at 4^0C at 10000g for 30 minutes.
- Resuspend the pellet in lysis buffer (25 mM HEPES, 20 mM KCl, 150 mM NaCl, 20 mM $MgCl_2$, pH 7.4)
- Add lysozyme (2mg/gram of pellet) and PMSF (1mM) and shake at 4^0C for 1 hour.
- Sonicate the cells, followed by centrifugation at 4^0C for 1 hour at 10000g.
- Pass the supernatant through equilirated Nickel-NTA column.
- Wash with washing buffer (25 mM HEPES, 20 mM KCl, 650 mM NaCl, 20 mM $MgCl_2$, 20 mM Imidazole pH 7.4), 50 times the column volume.
- Elute slowly with elution buffer (20 mM HEPES, 20 mM KCl, 20 mM $MgCl_2$, 300 mM Imidazole pH 7.4)
- Dialyse in lysis buffer. Change the dialysis buffer every four hours.
- Store the enzyme at -80^0C with 10% glycerol and 5mM DTT.

Appendix B

Tris-Tricine SDS PAGE

Reagents required

- 3X Gel Buffer (3M Tris HCl, 0.3% SDS, pH 8.45)
- Cathode Buffer (100 mM Tris, 100 mM Tricine. 0.1% SDS, pH 8.25)
- Anode buffer (100 mM Tris HCl, pH 8.9)
- AB-3 (48 gm Acrylamide and 3 gm bis-acrylamide in 100 mL water)
- AB-6 (46.5 gm Acrylamide and 6 gm bis-acrylamide in 100 mL water)
- 3X Sample Lysis Buffer(6% β-mercaptoethanol, 150 mM Tris-HCl, 30% Glycerol)

Stacking Gel (4%, 5 mL)

Component	Volume (in ml)
3X Gel Buffer	1.2
AB-3	0.4
Water	3.36
APS	0.04
TEMED	0.004

Resolving gel (16%, 10 mL)

Component	Volume (in ml)
3X Gel Buffer	3.3
AB-6	3.3
Water	3.36
APS	0.03
TEMED	0.004

Appendix C

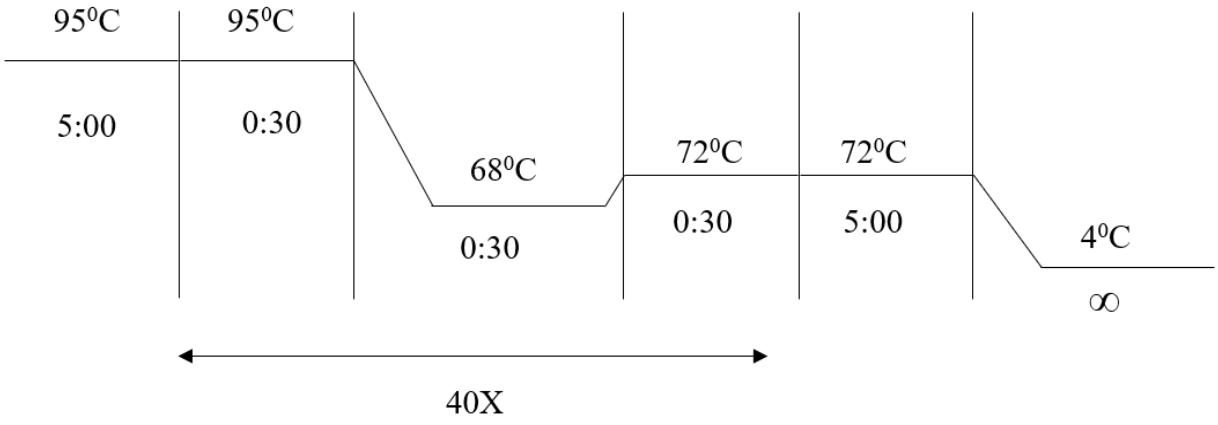
Silver Staining

- Fix the gel in fixing solution (10% Acetic acid, 30% ethanol) overnight.
- Sensitize for 1 minute in sensitizing solution (0.02% w/v Sodium thiosulphate pentahydrate).
- Rinse twice with 20% ethanol for 10 minutes each.
- Rinse four times in water for 10 minutes each.
- Stain the gel with 12 mM Silver nitrate (add 0.02% formaldehyde just before use) for 2 hours.
- Rinse in water for 10 seconds.
- Transfer to basic developer solution (3% Sodium carbonate, 0.05% formaldehyde to be added just before use) and shake gently. Bands development may take upto 5 minutes.
- Transfer to Tris stop solution (4% Tris w/v, 2% Acetic acid).
- Wash gels with water twice for 30 minutes each.

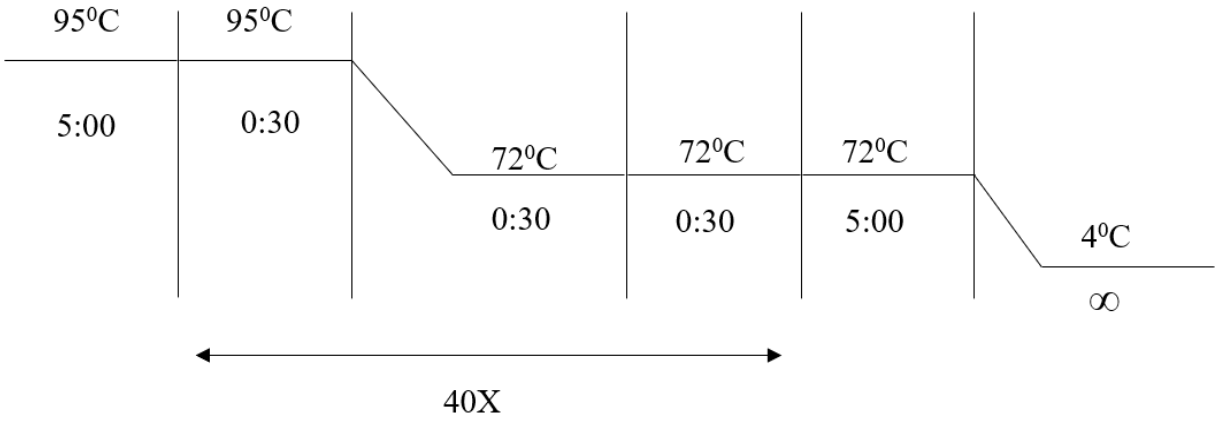
Appendix D

PCR Conditions

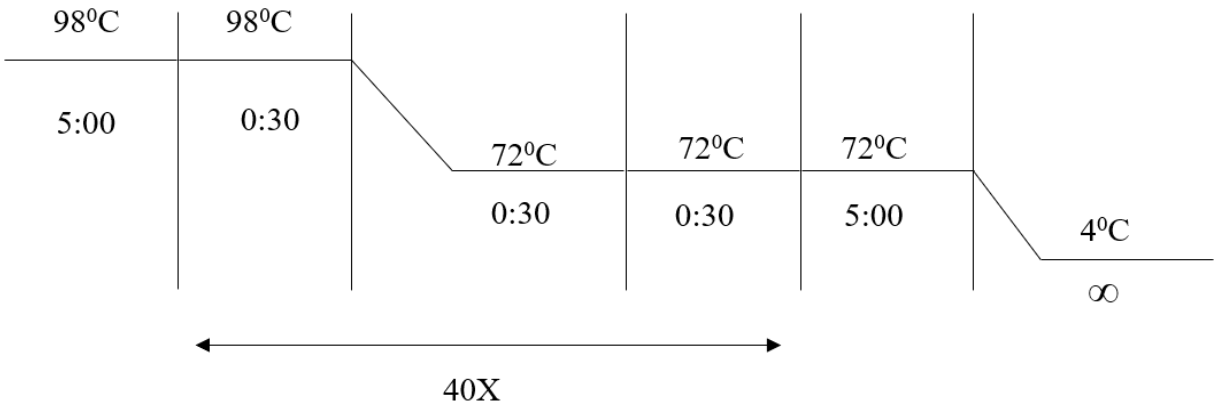
PCR Amplification 1 for Cdh23-TM



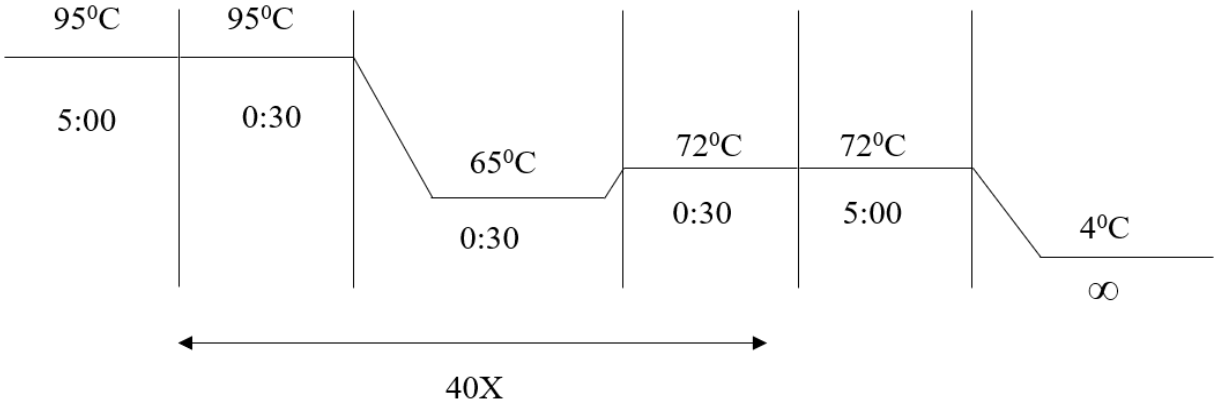
PCR Amplification 2 for Cdh23-TM



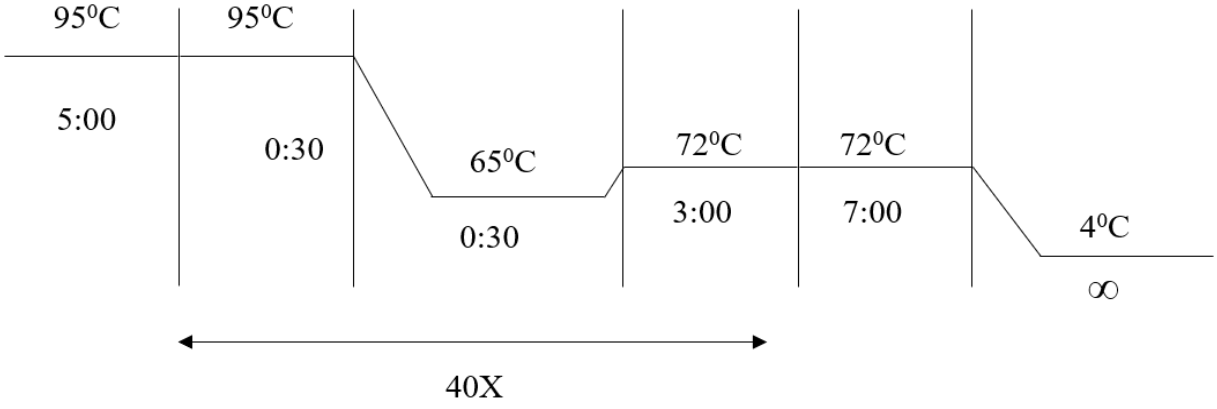
PCR Amplification 3 for Cdh23-TM, Poly-A tail generation



PCR Amplification for Gibson Fragment 1



PCR Amplification for Gibson Fragment 2



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