Project Report on

# Engineering a mammalian expression construct of SSBP1 and SSBP1

Submitted by

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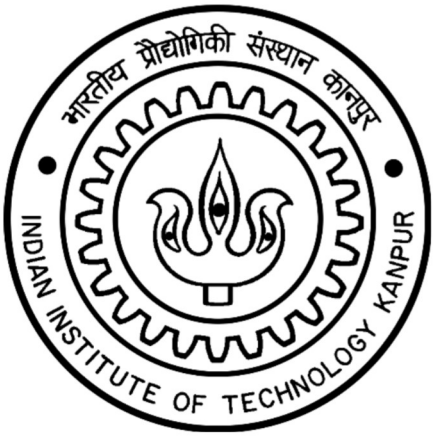

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# Acknowledgement

The training opportunity I had with **Indian Institute Of technology, Kanpur** was a great chance for learning and professional development. Therefore, I consider myself as a very lucky individual as I was provided with an opportunity to be a part of it. I am also grateful for having a chance to meet so many wonderful people and professionals who led me though this internship period.

Bearing in mind previous I am using this opportunity to express my deepest gratitude and special thanks to the **Prof. S Ganesh, Department of Biological Sciences and Bioengineering, IIT- Kanpur** who in spite of being extraordinarily busy with his duties, took time out to hear, guide and keep me on the correct path and allowing me to carry out my project at their esteemed organization and extending during the training.

I express my deepest thanks to **Dr. Rashmi Parihar and Miss Saloni Agarwal** for taking part in useful decision & giving necessary advices and guidance and arranged all facilities to make it easier. I choose this moment to acknowledge their contribution gratefully.

I perceive as this opportunity as a big milestone in my career development. I will strive to use gained skills and knowledge in the best possible way, and I will continue to work on their improvement, in order to attain desired career objectives. Hope to continue cooperation with all of you in the future.

**I would also like to thank my family, especially my mother for her support.**

Thank you for everything.

# Introduction

## About SSBP1

SSBP1 is a housekeeping gene involved in mitochondrial biogenesis. It is also a subunit of a single-stranded DNA (ssDNA)-binding complex involved in the maintenance of genome stability. It is of utmost importance in RNA binding, chromatin binding, protein binding and single-stranded DNA binding and plays important role in DNA replication, mitochondrial DNA replication, mitochondrion morphogenesis and organisation and positive regulation of helicase activity.

To study any gene, it is the first step to make many copies of it, which is done by molecular cloning.

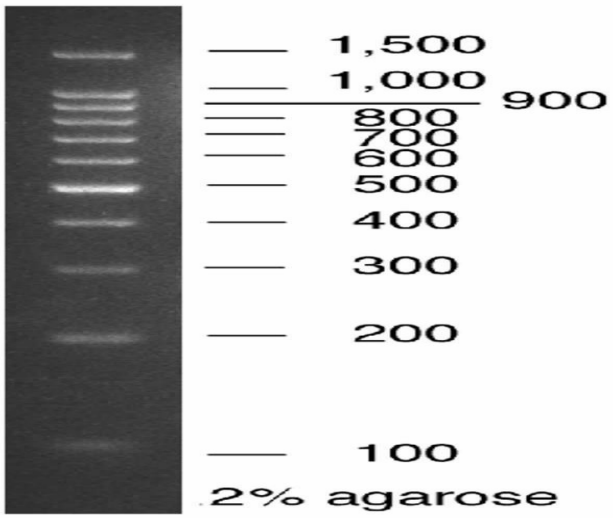
Molecular Cloning is a set of experimental methods in molecular biology that are used to assemble recombinant DNA molecules and to direct their replication within host organisms.

In this, the DNA to be cloned is obtained from an organism of interest, then treated with enzymes in the test tube to generate smaller DNA fragments. Subsequently, these fragments are then combined with vector DNA to generate recombinant DNA molecules. The recombinant DNA is then introduced into a host organism. This will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. Because they contain foreign DNA fragments, these are transgenic or genetically modified microorganisms (GMO). This process takes advantage of the fact that a single bacterial cell can be induced to take up and replicate a single recombinant DNA molecule. This single cell can then be expanded exponentially to generate a large number of colonies of bacteria, each of which contain copies of the original recombinant molecule. Thus, both the resulting bacterial population, and the recombinant DNA molecule, are commonly referred to as "clones".

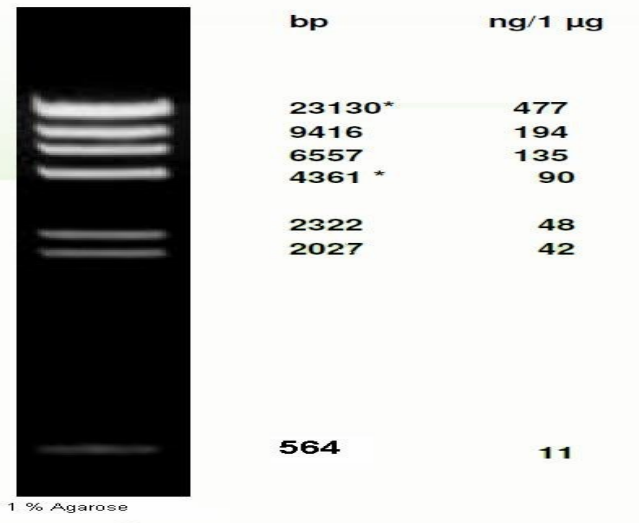
**Primary Aim**: To successfully clone Single-stranded DNA binding protein 1 (SSBP1)

**Secondary Aim**: To understand the common methods and techniques required.

**100bp DNA Ladder**




**λ Hind III**



# Objective

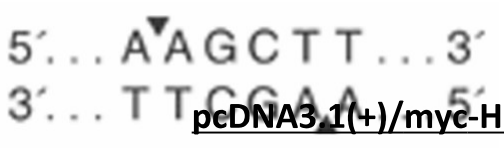
As discussed, SSBP1 is of utmost importance and to understand its functioning and perform various experiments on it, it is very important to make multiple copies if it, which is done by molecular cloning.

Molecular cloning is a complex procedure consisting of many steps:

- HeLa Cells➔RNA isolation ➔RT PCR ➔ cDNA ➔ PCR ➔ SSBP1 / SSBP1 ΔMTS amplification ➔ Restriction digestion (1)
- pcDNA transformation ➔ plasmid isolation ➔ Restriction digestion (2)
- Digested product (1) and (2) ➔ Ligation ➔ Transformation ➔ Plating ➔ Screening

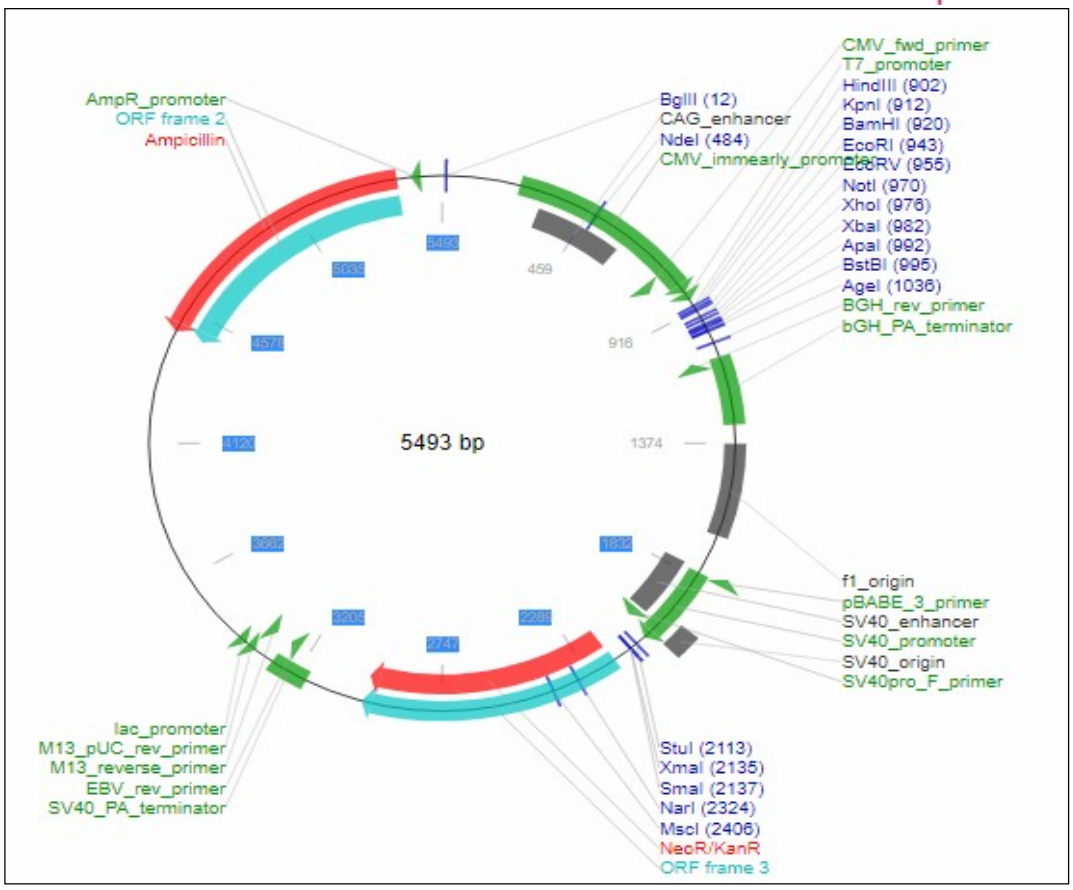
**Restriction Enzymes:**

**Hind III :**




**Xba I**


**pcDNA3.1(+)/myc-His A**



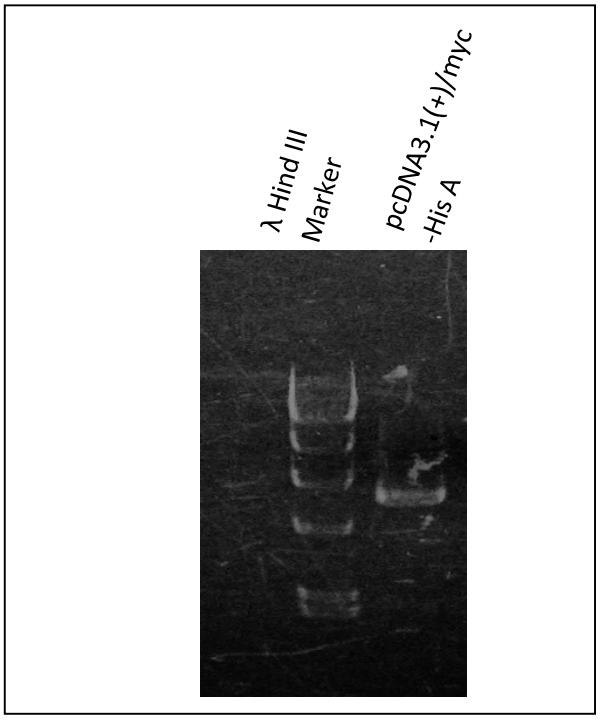

## Results

The cloning of SSBP1 consist of various steps:

**Plasmid isolation:**

The vector plasmid (pcDNA3.1(+)/myc-His A) was isolated and Gel electrophoresis was done on 1.5% Agarose Gel.

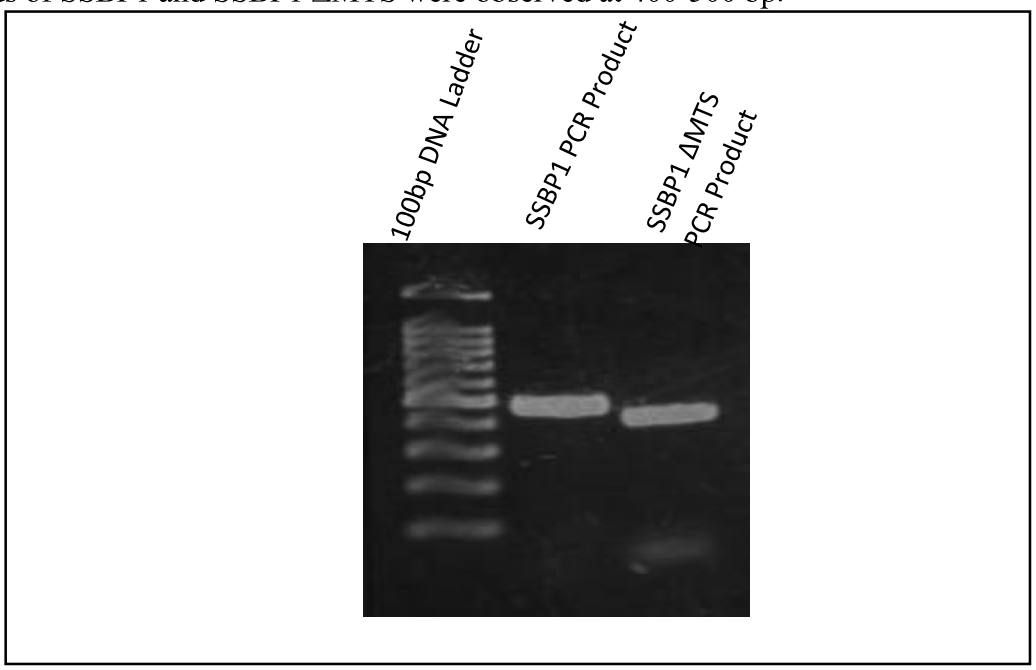
The band was observed between 3rd (6557bp) and 4th (4361bp) band of λ Hind III marker, which is our vector (5493bp).




**RNA Isolation:**

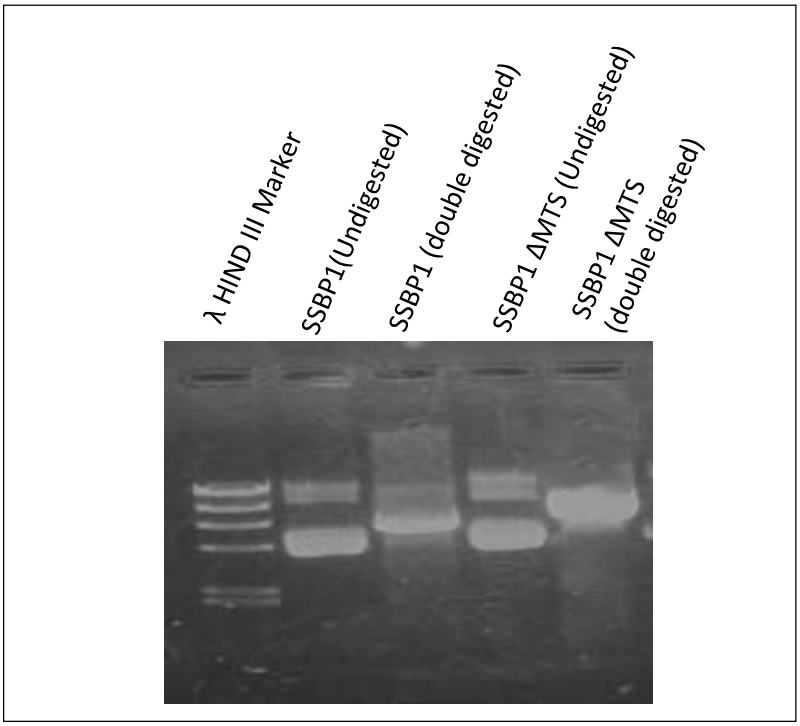
RNA of SSBP1 and SSBP1 ΔMTS was isolated. Further cDNA was obtained form which was amplified and product was run through gel electrophoresis (1.5% agarose gel).

Bands of SSBP1 and SSBP1 ΔMTS were observed at 400-500 bp.

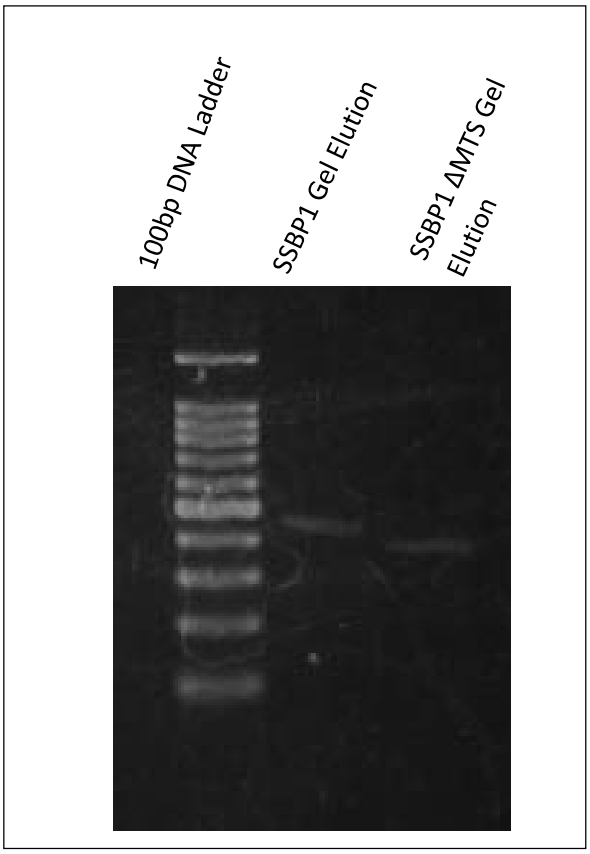


**Double Digestion:**

The vector DNA and cDNA of SSBP1 and SSBP1 ΔMTS was double digested using Xba I and Hind III and the digested product were run through gel electrophoresis and their respective bands were obtained.

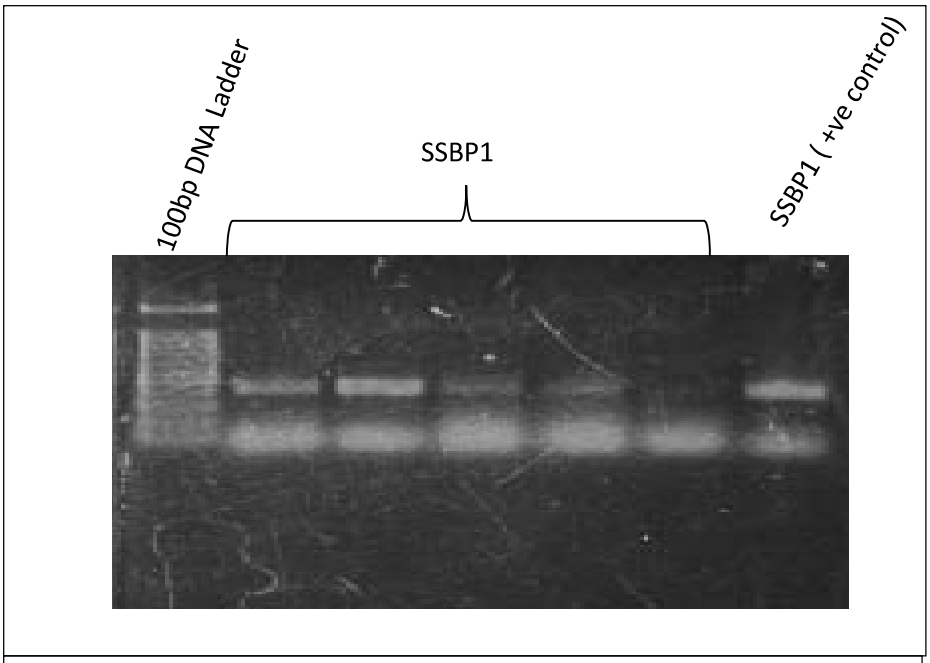


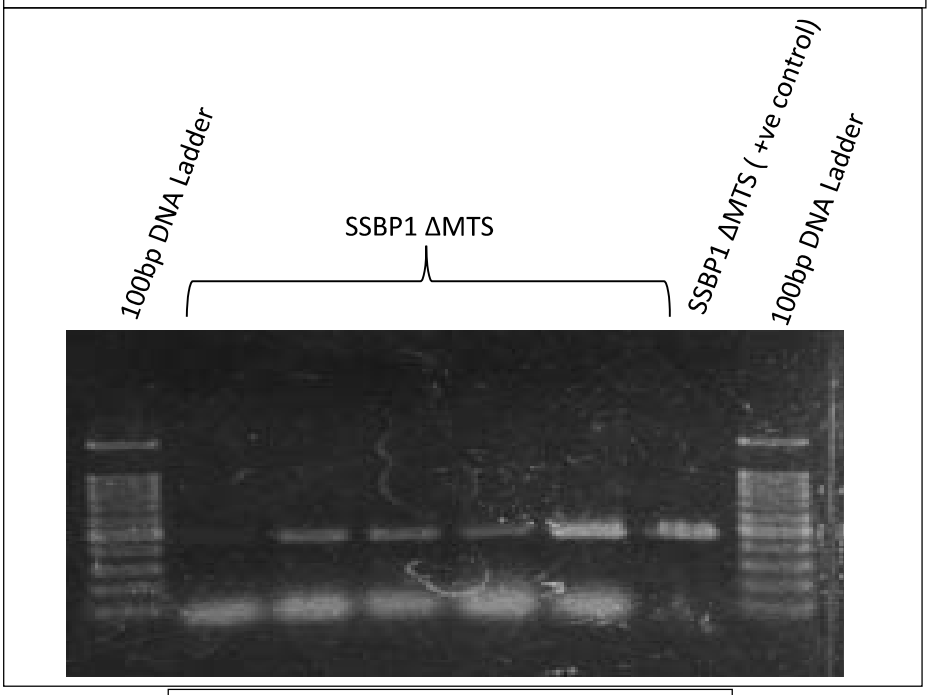

After the digestion, gel elution was done of the products obtained and eluted products were run through gel electrophoresis and their bands were observed.

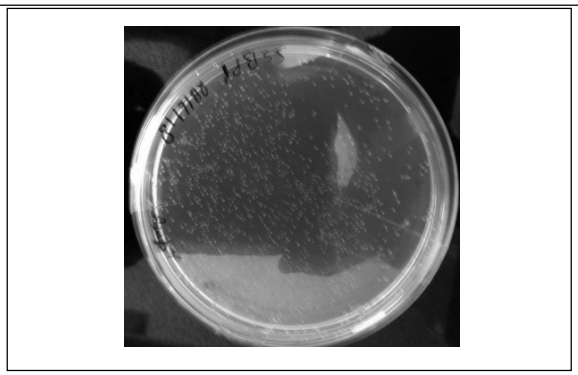



**Screening:**

After the digestion ligation was done and then transformation of the ligated products was done. Afterwards plating was done and then they were isolated at 37C for a day and then they were screened for positive colonies for which 5 random colonies were picked, colony PCR was done and they were run through gel electrophoresis. The screening showed positive result. **Thus, cloning of SSBP1 and SSBP1ΔMTS was successfully done.**



## Materials and Methods

## RNA Isolation

1. HeLa cells were taken and centrifuged at 4°C at 12000 RPM for 35 minutes.
2. The medium(supernatant) was carefully removed and 1ml TRIzol was added and left for 5 minutes.
3. It was then mixed and transferred into a 1.5 ml Eppendorf tube and left for 5 min.
4. 200µL of chloroform was added and tube was shaken vigorously for 20 seconds and left at Room temperature for 5 minutes.
5. It was then centrifuged at 12000 RPM for 15 minutes at 4° C.
6. At this point, there were three layers in each tube:
   **Top layer:** aqueous
   **Middle layer/interphase:** white precipitated DNA
   **Bottom layer:** pink organic phase
7. The aqueous phase was taken out which was fresh RNA, without disturbing other layers.
8. 500 µL isopropanol was added and solution was mixed and left at RT for 10 min.
9. It was then centrifuged at 12000 RPM for 10 min at 4° C and 1 mL chilled alcohol (75%) was added.
10. Then it was centrifuged at 7500 RPM for 5 minutes and wash was discarded.
11. 75% alcohol was added and RNA pellet was stored at -80° C for further use.

## Plasmid Isolation

1. The culture was vortexed and transferred to 2 mL Eppendorf tubes.
2. Then they were centrifuged at 12000RPM for 1 minute and supernatant was removed.
3. 200 µL resuspension buffer was added and was vortexed for 1 minute.
4. 200µL freshly prepared Lysis buffer was added very slowly and slowly inverted for 5-6 times.
5. 350µL neutralization buffer was added and tube was inverted for 5-6 times.
6. Then it was centrifuged for 10 minutes for 12000 RPM.
7. The supernatant was taken and transferred to spin-win columns which were incubated earlier with QBT.
8. Then they were centrifuged at 12000 RPM for 1 minute, the flow through was removed.
9. 700µL wash buffer was added and then they were centrifuged at 12000RPM for 1 minute and flow through was removed.
10. Then they were centrifuged for 2 minutes at 12000RPM and flow through was removed.
11. Then the columns were transferred to 2 mL Eppendorf and 80µL elution buffer was added and incubated for 30 minutes.
12. Then they were centrifuged at 12000RPM for 2 mins and the plasmids were stored at -20°C.
13. Check on 1.5 x Agarose gel.

**Preparation of silica column:**

1. The column was taken and 700µL of QBT was added and left for 10 minutes.
2. Then they were centrifuged at 12000 RPM for 1 min and flow through was discarded.

**Lysis Buffer: For 2mL**

10% SDS : 200µL | 2 N NaOH : 200µL | ADW : 1600µL

**Composition of other buffers:**

**Resuspension buffer**: 50mM Tris HCl, 10mM EDTA, RNase

**Neutralisation buffe**:4 M Guanidine Hydrochloride, 0.5M Potassium acetate

## Transformation

1. Competent cells (*E. coli* DH5α) were taken and were placed on ice (to avoid temperature stress).
2. 1.5 mL Eppendorf tube was taken with about 500ngDNA (6µL).
3. 60µL of competent cells were added to the to the tube and mixed slowly to avoid any stress.
4. Then tube was incubated on ice for 20 minutes.
5. Heat shock was given for 90 seconds at 42°C.
6. The tube was again placed on ice for 20 minutes.
7. 600µL Lysogeny Broth was added and tube was kept 37°C on shaking incubator at 90 RPM for 1 hour.
8. Then it was centrifuged at 12000 RPM for 1 minute.
9. The supernatant was removed but small amount was left.
10. Then it can either be plated or incubated into Lysogeny Broth for overnight.
11. For Incubate: 10 ml LB + 1 ml Ampicillin.
12. For Plating: 100ml ADW + 1.5 Agar + 2.5 LB ➔ autoclave ➔ Add ampicillin and Cool at RT ➔ Do plating.

## Restriction Digestion

For 20µL using Hind III and Xba I:

| | | | |
|---|---|---|---|
| DNA | 1µg | : | 5µL |
| Enzyme | 1 Unit | : | .1 µL for each |
| Buffer (Tango Buffer) | 10x ➔ 1x | : | 1µL |
| ADW | | : | 13.8µL |

- Incubate at 37 °C for overnight.
- Check on 1x Agarose gel.

## DNA Precipitation

1. Add 1/10$^{th}$ of $CH_3COONa$ in total volume of DNA, 5µl for 50 µL.
2. 2µL Glycogen (stored at -20°c) was added and the tube was vortexed.
3. 500µL absolute ethanol was added and mixture was left at -20°C for half an hour.
4. Then it was centrifuged at 12000 RPM for 30 minutes at 4°C and supernatant was discarded.
5. 500µL of 70% Ethanol was added and then it centrifuged for 10 minutes at 12000 RPM.
6. The alcohol was aspirated and incubated for 20 minutes and DNA was dissolved in 50µL 1xTE.

## Gel Elution

1. The gel favourable part of gel was taken out.
2. The mass of 1.5 ml Eppendorf tube was noted and the gel was transferred into it and mass was again noted, and mass of gel was calculated.
3. 200µL of Binding buffer was added per 100mg of Gel and gel was dissolved in buffer using dry bath at 50°C.
4. The solution was transferred into spin win column and incubated at 15 minutes and was centrifuged at 12000 RPM for 1 min.
5. The flow through was poured again in column and centrifuged for 1 minute at 12000RPM and this step was repeated for 3 times.
6. At last the flow through was discarded and 700 µL Wash buffer was added and centrifuged at 1 minute for 12000 RPM and flow through was discarded.
7. Then it was centrifuged at 12000RPM for 2 minutes.
8. The column was transferred into 2 ml Eppendorf and then eluted with 50 µL ADW and incubated for 30 min. Then it was centrifuged for 1 min at 12000RPM and DNA was collected.

## Ligation

For 20 µL Reaction:

| | | SSBP1 | SSBP1ΔMTS | Dynamitin |
|---|---|---|---|---|
| Vector DNA | 13 ng | 5µL | 5 µL | 5 µL |
| Insert DNA | calculated | 3 µL | 3 µL | 3 µL |
| Water | | 11 µL | 11 µL | 11 µL |
| T4 DNA Ligase | | 1 µL | 1 µL | 1 µL |
| | | | | |

- Then the reaction mixture was left at 16°C for overnight.
- Then it was dry heated at 65°C for 10 minutes.
- Then it was transformed and plated.

## Polymerase Chain Reaction

**Reaction Mixture for 20 mL RT PCR:**

| | |
|---|---|
| RNA | 5 µL |
| Primer | 1µL |
| RT enzyme | 1 µL |
| RiboLock | 0.5 µL |
| dNTP (10mM) | 2 µL |
| Buffer | 4 µL |
| ADW | 6.5 µL |

**Thermo regulation for RT PCR:**

| | |
|---|---|
| 25°C | 5 minutes |
| 42°C | 90 minutes |
| 70°C | 5 minutes |
| 4°C | ∞ |

**Reaction Mixture for 20 µL PCR:**

| | |
|---|---|
| cDNA | 2 µL |
| Forward Primer | 0.5 µL |
| Reverse Primer | 0.5 µL |
| dNTP(2mM) | 1 µL |
| *Taq* Enzyme | 0.1 µL |
| Buffer | 1 µL |
| ADW | 4.8 µL |

**Thermo Regulation for PCR:**

| | | |
|---|---|---|
| 95°C | 5 minutes | |
| 95°C | 30 minutes | X 34 |
| 60°C | 30 minutes | |
| 72°C | 1.5 minutes | |
| 72°C | 5 | |
| 12°C | ∞ | |

# Extra Methods and techniques Learned

## SDS- PAGE

1. Stand was settled up and the Resolution buffer was made and poured.
2. When the buffer was solidified, stacking buffer was made and poured and slit maker was inserted.
3. Loading dye was added into the cells and cells were given water bath for 5 minutes.
4. Then the cells were loaded and transfer buffer was poured into SDS-PAGE.
5. SDS-PAGE ran for about 1.5 hours at 30mA.
6. Then western blotting was done and blot was prepared.

Resolution Buffer: for 15 mL 12%

| | |
|---|---|
| ADW | 5.9ml |
| 30% Acrylamide mix | 5.0 mL |
| 1.0 M Tris pH 8.8 | 3.8mL |
| 10%SDS | 0.15mL |
| 10% Ammonium persulfate | 0.15mL |
| TEMED | 0.006mL |

Stacking Buffer: 8 mL

| | |
|---|---|
| ADW | 5.5mL |
| 30% Acrylamide mix | 1.3mL |
| 2.0 M Tris pH 6.8 | 1.0mL |
| 10%SDS | 0.08 |
| 10% Ammonium persulfate | 0.08 |
| TEMED | 0.008 |

Transfer Buffer: for 800 mL

| | |
|---|---|
| ADW | 640mL |
| Methanol | 160mL |
| Glycine | 11.2g |
| Tris Base | 2.4g |
| SDS | 0.8g |

## Developing Blot

1. The blot was taken from the transfer assembly and washed with 1xTBST for 5 minutes.
2. Then it was blocked in milk blocking for 1 hour at 37℃ at 34 RPM.
3. Then blot was treated with Tubulin for 1 hour at room temperature.
4. Then it was washed with 1xTBST for 5 minutes for 2 times and one time with milk blocking for 5 minutes.
5. Then the blot was treated with 2° anti-body for 1 hour at room temperature.
6. Then it was washed with 1x TBST 2 times for 7 minutes and one time with 1xTBS.
7. Then the blot was developed.

**1x TBST: 1L**

100 ml 10xTBS + 900 mL ADW ➔ 1x TBS

1xTBS + 1 ml Twee20 ➔ 1x TBST

**5% Milk Blocking:**

5g Milk Blocking

100mL 1xTBST

## Designing Primer

**>NM_001256510.1:436-882 Homo sapiens single stranded DNA binding protein 1 (SSBP1), transcript variant 1, mRNA**

ATG TTT CGA AGA CCT GTA TTA CAG GTA CTT CGT CAG TTT GTA AGA CAT GAG TCC GAA ACA ACT ACC AGT TTG GTT CTT GAA AGA TCC CTG AAT CGT GTG CAC TTA CTT GGG CGA GTG GGT CAG GAC CCT GTC TTG AGA CAG GTG GAA GGA AAA AAT CCA GTC ACA ATA TTT TCT CTA GCA ACT AAT GAG ATG TGG CGA TCA GGG GAT AGT GAA GTT TAC CAA CTG GGT GAT GTC AGT CAA AAG ACA ACA TGG CAC AGA ATA TCA GTA TTC CGG CCA GGC CTC AGA GAC GTG GCA TAT CAA TAT GTG AAA AAG GGG TCT CGA ATT TAT TTG GAA GGG AAA ATA GAC TAT GGT GAA TAC ATG GAT AAA AAT AAT GTG AGG CGA CAA GCA ACA ACA ATC ATA GCT GAT AAT ATT ATA TTT CTG AGT GAC CAG ACG AAA GAG AAG GAG TAG

Yellow highlighted region is mitochondrial targeting sequence (MTS).

Forward primer 5’AAGCTTGCCATGTTTCGAAGA3’ (Hind III enzyme)

Reverse primer 5’TCTAGACTCCTTCTCTTTCGT3’ (Xba I)

SSBP1 ΔMTS

Forward primer 5’ AAGCTTGCCATGGAGTCCGAAACA3’

Green highlighted regions are restriction sites.

## Vector

**>pcDNA3.1(+)/myc-His A**

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCA
TAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAAT
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AATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACAT
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TTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCT
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GCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGT
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TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCC
ACCTGACGTC

Green highlighted regions are restriction sites.

Blue highlighted region is myc tag.

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