MATERIALS AND METHODS

3.1 Collection of plant material

Forty Six different cultivars of apple were collected from farm at IHBT Palampur. The leaf samples were collected on the basis of visual symptoms.

These collected samples were subjected for screening against apple stem grooving virus using the detection techniques which includes serological techniques, nucleic acid based detection techniques (RT-PCR based diagnostics). Further confirmation was made by sequencing of PCR amplified products.

3.2. Serological detection (DAS-ELISA) of ASGV

Samples were checked for the presence of virus at different stages of plant growth at bud stage and fully opened and mature leaf stage. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was performed which is described below.

3.2.1. Principle of ELISA

To detect antigen, purified antibody specific for antigen is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells to which they bind nonspecifically; residual sticky sites on the plastic are blocked by adding irrelevant proteins. The labeled antibody is then added to the wells under conditions where nonspecific binding is prevented, so that only binding to antigen causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction. This assay allows arrays of wells known as micro titer plates to be read in

ELISA reader (BIO-RAD) greatly speeding the assay. Modifications of this basic assay allow antibody or antigen in unknown samples to be measured

In DAS-ELISA antibodies are coated to the surface of a Polystyrene plate to capture the antigens. The presence of antigen is detected using specific antibodies coupled with enzyme alkaline phosphatase. Finally the addition of the substrate solution (available with kit) induces a yellow product, detectable at 405 nm, when the antigen is present. This yellow product is formed due to reaction of alkaline phosphatase with 4-nitrophenyl phosphate yielding free 4-nitrophenol present in substrate solution.

3.2.2. Protocol of DAS-ELISA:

3.2.2.1 Coating of Antibodies

a) Coating antibodies were diluted 200 times in coating buffer (Appendix), as per manufacture's instructions. 200 µl of diluted antibodies were added to each well. The plate was incubated for 4 hr at 37oC.

b) After incubation, 4 washings were given with 1X washing buffer to remove unbound antibodies.

3.2.2.2 Sample preparation and loading

a) About 100mg of each sample was crushed in 2 ml of Conjugate/sample buffer (Appendix) using pre-sterilized mortar pestle. The sample was homogenized properly and centrifuged at 12,000rpm for 1min. 100 µl of supernatant was loaded per well .

3.2.2.3. Coating of conjugate antibodies

a) Conjugate Antibody alkaline phosphate conjugate was also diluted 200 times in conjugate buffer (AppendixI), and coated on to wells as 100 µl per well

b) Positive and negative controls were also loaded

c) The plate was incubated for overnight at 4oC

d) After overnight incubation 4 washings were given with 1X washing buffer to remove unbound antigen.

3.2.2.4. Addition of substrate and recording of absorbance values:

a) Wells were filled with 200 µl of substrate solution (Dissolve 1 substrate tablet in 20ml substrate buffer1x) immediately prior to use. Provided with kit.

b) Substrate was added in dark as it is light sensitive.

c) Plate was kept at 37oC for1hr. Then absorbance at 405 nm was taken for complete ELISA plate with a Flow ELISA micro plate reader (BIO-RAD). The reaction was considered positive if absorbance was observed to be at least two to three times absorbance of negative control.

3.3 Detection of ASGV by RT-PCR

3.3.1 Isolation of total RNA from leaf samples

The total RNA was extracted from virus-infected apple using the two methods: RNA aqueous (Ambion, USA) and QIAGEN RNeasy Plant mini kit (Germany)

3.3.1.1 Total RNA isolation using QIAGEN RNeasy Plant mini kit (Germany)

All steps were carried out at room temperature.

a) The infected leaves were crushed to fine powder in liquid nitrogen using baked mortar and pestle.

b) The tissue powder (100 mg) was immediately transferred to an eppendorf containing 450 µl of RLT Buffer or RLC Buffer solution and vortex-vigorously

c) The homogenate was incubated at 56C for 1-3 min. which help to disrupt the tissue.

d) The lysate is Transfer to a QIAShredder spin column and placed in a 2ml collection tube and centrifugate for 2 min at full speed. The supernatant is carefully transfer to a new micro centrifuge tube without disturbing the cell-debris pellet in the collection tube.

e) 0.5 vol of 96% ethanol is added to cleared lysate and mixed with vigorously pipetting and precipitate are formed. f) The sample including any precipitate is transferred to an RNeasy spin column (pink) placed in a 2ml collection tube(supplied).Lid is closed and centrifuge for 15s at 10,000 rpm and flow through is discarded. Collection tube is reused for further steps.

f) RW1 Buffer 700ul is added to RNeasy spin column and centrifuge for 15s at 10,000rpm to wash spin membrane and flow through is discarded.

g) RPE Buffer 500ul was added to column and same step g) is repeated. i) After discarding the wash solution, empty spin column was given at 14,000rpm for 1 minute to remove any residual ethanol.

h) RNeasy spin column was inserted into a fresh collection tube and RNA was eluted by applying 50 µl of elution solution or ddH20 (preheated to 70-80ºC) to the center of the filter. And recover elute by centrifugate at 10,000rpm for 1min at room temperature.

3.3.1.2 Total RNA isolation by RNA aqueous (Ambion, USA)

a) For RNA isolation lysate was prepared by crushing the sample thoroughly in liquid nitrogen to powder form and put in a collection tube which already contained lysis solution (binding solution) of about 700 µl.

b) The mixture was vortexed and centrifuged at 14000 rpm for 5 min. Supernatant was taken in another tube.

c) Equal volume of 64% ethanol was added to the supernatant and mixed thoroughly by vortexing or inverting the tube several times.

d) Filter cartridge was inserted into a 2ml collection tube to prepare binding column.

e) Mixture of lysate and ethanol was pipetted onto filter cartridge (700 µl at one time)

f) Filter cartridge was centrifuged for 30 sec to 1 min at 12000 rpm and flow through was discarded. The step was repeated for rest of the lysate.

g) *Washing*: Filter cartridge was applied with 700 µl of wash solution-I. Then centrifuged for 30sec to 1min at 12000 rpm. Flow through was discarded.

h) Similarly filter cartridge was washed with 500 µl of wash solution-II. Then centrifuged for 30sec to1min at 12000 rpm. Flow through was discarded. The whole step was repeated.

i) After discarding the wash solution, empty spin was given at 14000rpm for 3 minutes to remove any residual ethanol.

j) Filter cartridge was inserted into a fresh collection tube and RNA was eluted by applying 50 µl of elution solution or ddH20 (preheated to 70-80ºC) to the center of

the filter. And recover elute by centrifugate at 14000rpm for 30seconds at room temperature

l) The amount of elution solution was considered as the amount of RNA and was stored at -80ºC.

Checking integrity of RNA:

Agarose gel (0.1%) was prepared in 1X TAE buffer. (Appendix) RNA (5 ul) were loaded with 1X RNA loading dye (Appendix) and electrophoresed at 80 V for 1-2 hr. After the run, gel was stained with ethidium bromide (0.5 mg/ml) (Appendix) and visualized in U.V. Transilluminator.

3.3.2 Detection of ASGV by Reverse transcription-Polymerase chain reaction (RT-PCR)

PCR is an efficient means of *in vitro* amplification of specific DNA sequence by using a pair of oligonucleotides binding to the opposite strands of DNA. PCR was devised and named by Mullis *et al*. (1986). In general, PCR is a very useful tool in the detection and diagnosis of viruses and other pathogens specifically.

Standardization of RT-PCR

Tm (melting temperature) of the primer was calculated using the above formulae, annealing temperature was calculated i.e. annealing temperature (oC) =[Tm (oC) -5]. Based on this several annealing temperatures were tested for amplification. 60

3.3.2.1 Reverse transcription (RT)

Reverse transcription is the process by which cDNA is synthesized from RNA. This was done with the help of reverse transcriptase enzyme, which is RNA dependent DNA polymerase and it catalyses the conversion of RNA into cDNA.

Composition of RT reaction

Contents	**Quantity**
RT buffer (5X)	5.0 µl
dNTP mix (40 mM)	1.5 µl
Primer (downstream) (200 ng/µl)	1.0 µl
RNase inhibitor (40 u/µl)	0.3 µl
RNA sample	7.0 µl
Reverse transcriptase (MMLV-RT, 200 u/µl)	0.4 µl
H2O	9.8µl
Total	25 µl

The mixture was then mixed by pipetting up and down and centrifuged briefly to collect the mixture at the bottom of the tube (0.2 ml). RT mixture was same for both gene and group specific PCR except for primers used. cDNA synthesis was carried out under following temperature conditions in a Thermal Cycler (Eppendorf, Gene Amp);

Steps	**Temp (°C)**	**Time (min)**

1. For RT work	37 C	75 min.
2. Elongation	70 C	5 min

The pcr tubes were then immediately placed on ice or in refrigerator at -20 C.

3.3.2.2.1 Amplification of cDNA using ASGV specific primers

PCR reaction mix prepared was as follows:

Contents	**Quantity**
Taq buffer (10X)	5.0 µl
dNTP mix (10 mM)	1.5 µl
Primer (forward, 200 ng/µl)	1.0 µl
Primer (reverse, 200 ng/µl)	1.0 µl
cDNA	7.0 µl
Gene I Taq DNA polymerase (5 u/µl)	0.3 µl
H2O	34.2µl
Total	50 µl

We have also added MgCl2 as one of the component when we used Taq DNA polymerase and Taq buffer of other manufacturer instead of Gen I. Gen I Taq buffer already have MgCl2.

The reaction was carried out under following temperature conditions:

Steps	*Temp*(**°C**)	*Time*(**min**)
Intitation Denaturation	*94*	*30*
Denaturation	*94*	*10*

Annealing	*60*	*35*
Elongation	*72*	*40*
Final Elongation	*72*	*10*

Steps 2-4 were repeated 30 times and a final elongation was carried out at 72oC for 10 min.

3.3.3 Agarose gel electrophoresis of PCR product

After completion of amplification, the PCR product (20 µl) mixed with 4 µl of gel 6X loading dye and was loaded on 1% agarose gel and electrophoresed in TAE buffer at 80 V for 1-2 h. DNA marker (100 bp DNA ladder, Fermentas, USA) was loaded in one well for size comparison. After run, the gel was stained in ethidium bromide (0.5 mg/ml) and viewed under UV transilluminator and photographed in Alpha DigidocTM (Alpha Innotech Corporation).

3.3.4 Elution of DNA from agarose gel

The PCR amplified product was eluted from the gel using **AuPrep TM Gel x** Gel Extraction Kit as per the following procedure;

a) The band was excised from the agarose gel using clean, sharp blade and trimmed to minimize the extra gel weight.

b) Gel was weighted in colorless tarred tube; about 50-200mg was placed in to a sterile 1.5 or 2 ml centrifuge tube. Then 500 µl of GEX buffer was added.

c) The gel was completely dissolved by incubating it at 60 C on water bath for 5-10 min. Gel mixture was cooled down at room temperature.

d) Then placed Gel x column on to a 2 ml collection tube and 700 µl of dissolved gel mixture was added to it.

e) Centrifuged the column for 30-60 sec at 12000-14000 rpm. Flow through was discarded.

f) For washing column was applied with 500 µl of WF Buffer. Centrifuged the column for 30-60 seconds. Flow through was discarded.

g) Then column was washed with 700 µl of WS Buffer. Centrifuged for 30-60 seconds. Flow through was discarded.

h) Then column was centrifuged at 14000rpm for 3 min to remove residual ethanol.

i) Column was transferred to the fresh collection tube.

j) DNA was eluted by applying 50 µl of elution solution or double distilled water (preheated to 65 oC) to the centre of column.

k) Incubated for 1min at room temperature and centrifuged for 1min at 12000rpm.

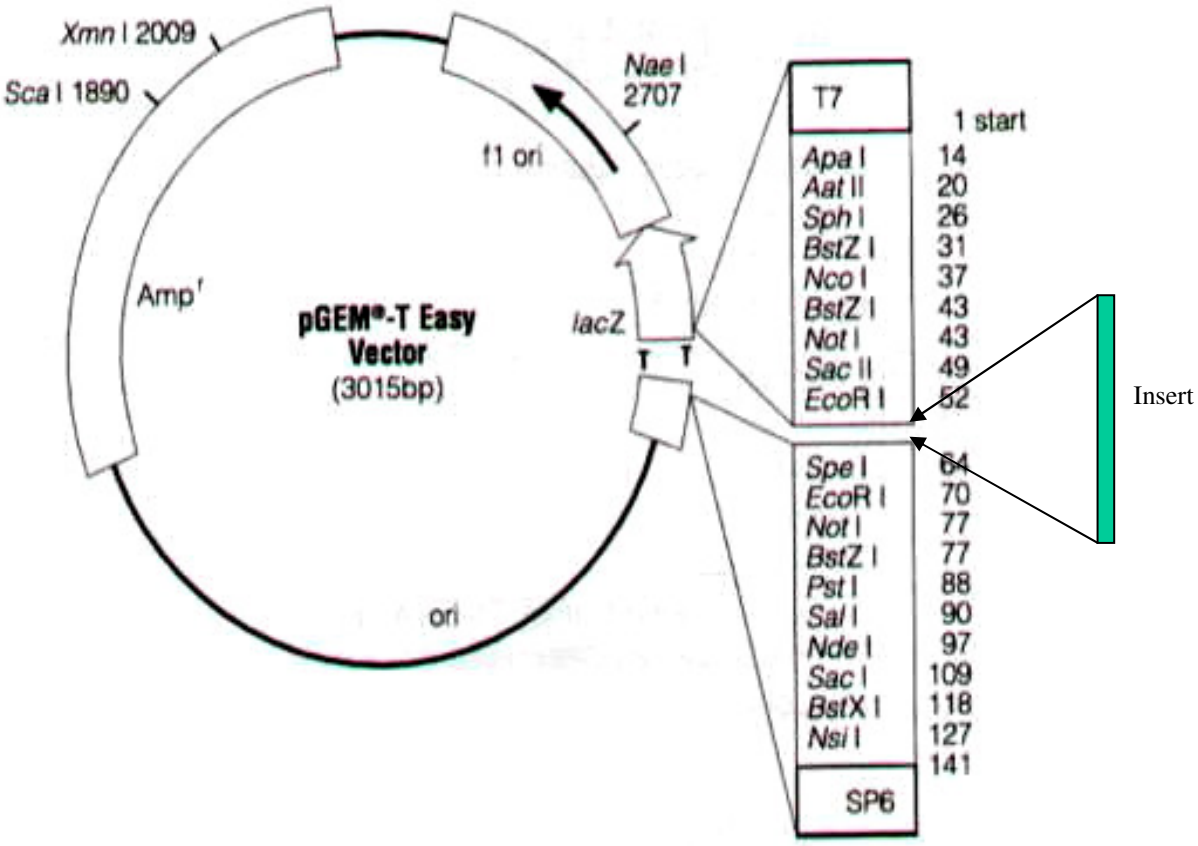
l) Stored the eluted product as DNA at -20 oC for future use

Checking of eluted DNA:

Agarose gel (1%) was prepared in 1X TAE buffer. DNA (5 ul) were loaded with 1X DNA loading dye and electrophoresed at 80 V for 1-2 hr. After the run, gel was stained with ethidium bromide (0.5 mg/ml) and visualized in U.V. transilluminato**3.2.4**

Cloning and Transformation

The eluted RT-PCR amplified products (~650 bp) were cloned in pGEM-T Easy vector (Promega, USA) (Fig. 3.1).



3.3.4.1 Ligation of DNA in to pGEM-T Easy vector (Promega, USA)

The pGEM-T Easy vector has a unique 3' terminal thymidine to both ends preventing the re-circularization of the vector and providing complete overhangs for PCR

product, which has 5' adenine to both ends. PCR amplified products (~300bp) were ligated in pGEM-T Easy vector system.

Contents **Volume**
2x rapid ligation buffer 5.0 µl
pGEM-T Easy vector (50ng) 1.0 µl
Eluted DNA (insert) 3.0 µl
T4 DNA ligase (3 Weiss units) 1.0 µl
Total 10 µl

The reaction mixture was mixed by pipetting, centrifuged briefly and incubated overnight at 4oC in refrigerator.

3.3.4.2 Preparation of competent cells

Competent cells can be prepared either freshly for each use or can be premade and stored at -70oC. For the transformation experiments either DH5α or XL1-Blue (tetR) strains of *E.coli* were used for transformation. Single colony of the DH5α strain of the bacteria was streaked on to LB agar plate with appropriate antibiotics. This was incubated at 37oC for 12-16hr. These freshly grown colony of *E.coli* cells were used for preparation of competent cells or culture was grown overnight at 37oC and a part of it was used to inoculate larger volumes of the medium.

a) Procedure for preparing competent cells, which can be stored at -70oC

250ml LB was inoculated with 1ml of overnight culture of *E.coli* and grown at 37oC on a shaker till OD at 600nm was around 0.5.

Culture was cooled on ice immediately and cells were harvested by centrifugation at 6,000 rpm for 5 min at 4oC.

Supernatant was discarded carefully and traces of it were removed by inverting the centrifuge tubes on filter paper. However, the tubes were never removed from ice for long.

The bacterial pellet was resuspended in 70 ml of ice cold, sterile 0.1 M CaCl2 (sterilized by autoclaving) and incubated on ice for 30 min.

The cells were recovered by centrifugation at 6,000 rpm for 5 min at 4oC.

Supernatant was discarded and pellet was resuspended in 70 ml cold and sterile 0.1 M MgCl2 and incubated on ice for 30 min.

Centrifuged at 6,000 rpm for 5 min at 4oC

The cells were finally recovered and resuspended in 10 ml of 0.1M CaCl2 containing 10% glycerol.

Aliquots of 200 µl each were prepared and immediately stored at -70oC.

b) Competent cells which can be used fresh each time

1. 50 ml of LB was inoculated with 50 µl overnight culture of *E. coli* DH5α and allowed to grow at 37oC on a shaker till OD at 600nm was around 0.5.
2. Culture was cooled on ice and cells were pelleted down by centrifuging at 5,000 rpm for 5 min at 4ºC.
3. Supernatant was completely removed. The pellet was resuspended in 10 ml of ice cold 0.1M CaCl2 and incubated on ice for 15 min.
4. Cells were recovered by centrifugation at 5,000rpm for 5 min at 4oC.
5. Cell pellet was resuspended in 2 ml of ice cold 0.1M CaCl2 and aliquots of 200 µl each were prepared to be used for single transformation reaction.
6. These competent cells can be stored on ice for not more than 12-16 h.

   This treatment results in increase of competence as well.

During the preparation of competent cells all the solutions were chilled, pipette tips cooled and cells always kept on ice.

3.3.5 Transformation of competent cells (*E. coli* strain DH 5α) with ligated products

Transformation is the process of uptake of foreign DNA by bacterial cells (*E. coli*). From the medium this uptake and retention of foreign DNA molecules is generally detected by examining the expression of the gene carried by the foreign DNA (Brown, 1995). For example, if foreign DNA contains the gene for resistance against certain antibiotics, to which normal bacterial cells are sensitive, the transformed cells become resistant to those antibiotics. These cells are checked for the expression of the antibiotic resistance by growth on a selective medium containing the antibiotics. The cells, which are not transformed, perish in such medium.

Protocol

Frozen competent cells were thawed on ice for 10 min or freshly prepared cells were taken for transformation. Ligated product 10 µl (or DNA to be transformed) was mixed with competent cells and incubated on ice for 30 min.

Heat shock was given at 42oC for 90 sec and immediately transferred to ice for 5 min.

To this, 800 µl of Luria broth without any antibiotics was added to the mixture and the tube was incubated at 37oC for 1 h on a shaker.

The tube contents were centrifuged at 12,000 rpm for 30 sec and supernatant was discarded leaving approximately 100 µl of culture in the tube.

The pellet was resuspended by pipetting culture and plated on LB agar plates contained 100 µl ampicillin (100 mg/ml stock). Cells were spread uniformly with glass beads.

The plates were then incubated at 37oC for overnight for development of transformed colonies.

3.3.6 Checking of transformed colonies

About 10 colonies, growing on the LB agar plates were picked up, cultured separately on fresh LB agar plates as well as in LB containing ampicillin and incubated at 37o C for overnight. After overnight incubation the plates were stored at 4o C.

The plasmid DNA containing the gene of interest was purified from the bacterial cells cultured in tubes.

3.3.6.1 Plasmid DNA isolation by Boiling Miniprep method (Holmes and Quingley, 1981)

Principle

Bacterial plasmids DNA are widely used as cloning vehicles in recombinant research. Method denatures high molecular weight chromosomal DNA while covalently closed circular DNA (cDNA) remains double stranded. There is a narrow range of pH (12-12.5) within which denaturation of the linear DNA occurs but cDNA does not.

Spin at 10,000 rpm for 2 min. Cells are lysed completely with NaOH and SDS. By choosing the ratio of cell suspension to NaOH solution carefully, a reproducible alkaline pH value is obtained without the need for monitoring the pH. Further, pH control is obtained by including glucose as a pH buffer. Chromosomal DNA, still in a very high molecular weight form is selectively denatured and when the lysate is neutralized by

acidic NaOH (CH3COONa), chromosomal DNA renatures and aggregates to form insoluble network. Simultaneously, high concentration of Sodium acetate causes precipitation of protein-SDS complexes and of high molecular weight RNA. Thus, the three major contaminants are co-precipitated and removed by a single centrifugation. Plasmid DNA and residual low molecular weight RNA are recovered from supernatant by ethanol precipitation

a. Overnight grown culture (1.5 ml) was pelleted down in 1.5ml eppendorf tube by centrifugation. Supernatant was discarded.

b. Pelleted cells were resuspended in 110 µl of STET buffer.

c. Fresh lysozyme (in 10 mM Tris HCl, pH 8.0) was prepared to a final concentration of 1 mg/100µl.From this stock 10-12 µl of lysozyme was added to eppendorf tube containing cells suspended in STET buffer.

d. The tubes were incubated at room temperature for 5 min. These were then incubated in boiling water bath for 40-45 sec.

e. The suspension was centrifuged at 14000 rpm for 20 min.

f. Pellet containing the cell debris was removed with the help of a sterile tooth prick. DNA in supernatant was precipitated by one volume of isopropanol (100-110 µl).

g. Precipitated DNA was collected immediately by centrifugation for 25 min at full speed.

h. Supernatant was removed completely and the pellet was dried to remove traces of isopropanol. DNA pellet was dissolved in sterile water (30-200 µl) and used as such for further manipulations.

3.3.6.2 Checking of recombinant clones

Several methods are used to check the recombinant clones viz. restriction digestion, insertional inactivation, colony hybridization, colony PCR, and antibiotic selection. Among these we used restriction digestion method for checking of ASGV partial CP gene clones**.**

Restriction digestion

pGEM-T® Easy Vectors contain multiple restriction sites within the multiple cloning regions. These restriction sites allow for the release of the insert by restriction digestion with a single restriction enzyme. pGEM-T® Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes *Eco* RI, *Bst* Z1, *Not* 1, *Pvu* II. The single enzyme can therefore be used for release of insert that is further proved by running the digested product on gel electrophoresis.

Before going to digest the isolated plasmid, RNase treatment was given to degrade RNA. (1 ul of concentrated Ribonuclease A was taken and diluted to 50 times by adding 49 ul of ddwater. From this 1 ul RNase was used for entire Plasmid DNA and incubated at 37oC for 1hr in water bath)

a) The following reaction mixture was prepared in eppendorf tube on ice as shown in table to check the insertion of gene of insert isolated in the plasmid vector in transformed *E. coli* cells.

Restriction digestion mixture

Reaction mix components For pGEM-T Easy vector

Reaction mix components	**For pGEM-T® Easy vector**

Eco RI (10 U/µl) enzyme	.8 µl
RNAse	1 µl
Eco RI Buffer(10X)	5 µl
Plasmid DNA	10 µl
Water	33.2 µl
Total	50µl

b) RNase and plasmid was firstly mixed and kept for 1hr incubation at 37°C on water bath.

c) After incubation add other components and this mixture was incubated at 37°C for 3 h on water bath.

d) After incubation absolute alcohol 2.5 volumes (125 µl) was added to mixture and incubated at -20° C for overnight or at -80° C for 60 min.

e) It was centrifuged at 14,000 rpm for 25 min at 4°C.

f) The supernatant was discarded and pellet was dissolved in 12-14 µl of millipore H2O and stored at -20°C for further use.

g) DNA (10 µl) mixed with 4 µl of gel loading dye (1x) was loaded on agarose gel (1%). Electrophoresis was carried out at 80 V for 2-3 h in TAE buffer.

h) The gel was stained in ethidium bromide (0.5 mg/ml) for 2 min and visualized in UV transilluminator.

3.3.7 Purification of plasmid DNA for sequencing

One of the colonies containing the gene of interest ligated with vector was chosen for further downstream reaction. Plasmid DNA was isolated and purified for sequencing as the procedure given below using AuPrepTM Spin Plasmid Isolation kit.

Transformed *E.coli* grown overnight culture (3-5 ml) was centrifuged at 12000-13000 rpm for 1-2 min. Supernatant was discarded and pellet was resuspended in 250 µl of MX1 Buffer, vortexed it. Then 250 µl of MX2 Buffer was added, gently mixed to lyse the cells until the lysate becomes clear. Incubated the mixture for 5 min at room temperature. After incubation, 350 µl of MX3 buffer was added gently mixed to neutralize the lysate. A white precipitate was formed. Centrifuged for 5-10 min. Supernatant was kept. SPIN column was placed onto a collection tube. Supernatant was transferred on to column. Centrifuged for 30-60 seconds at 12000rpm, flow through was discarded. Column was washed once with 500ul WF buffer and 700 µl of WS Buffer. Centrifuged, flow through was discarded. Column was centrifuged at full speed for 3 min to remove residual ethanol. Column was placed onto a new centrifuge tube. 50 µl of Elution Buffer was applied onto the center of the membrane and incubated at room temperature for 1 min. Then centrifuged at 14,000 rpm for 1 min to elute plasmid DNA in the tube. The eluted product was purified plasmid DNA. Stored at -20° C or 4° C. DNA (2 µl) was loaded onto 1% agarose gel for checking of concentration of DNA.

3.3.8 Quantification of purified DNA

Absorbance in spectrophotometer helps to quantify of purified DNA. Absorbance of DNA was recorded in UV range from 260/280 nm. Values of A max, A min (A260/A280) were calculated to know the appropriate concentration of DNA.

3.3.9.1 Automated sequencing

The primer T7 (forward) and SP6 (reverse) were used in sequencing of clones in pGEM-T Easy vector. PCR was carried out to amplify DNA for extension through the primers in a 0.2 ml PCR tubes.

PCR reaction was setup as follows

Sequencing Buffer	**1ul**
Big dye Terminator(rxn mixture)	**1ul**
Primer T7	**.5ul**
Plasmid DNA(50ng/ul)	**2.5ul**
Total	**5ul**

The reaction mixture was mixed properly, centrifuged briefly to collect the mixture at the bottom of the tube and incubated in Thermocycler (Gene amp 9700). The cycling condition of reaction was as follows:

(i) 95oC for 10sec Denaturation. (ii) 50oC for 40 sec Annealing(iii) 60oC for 4 min Extension These steps were repeated for 25 times.

The volume of PCR extension reaction was raised to 100 µl by adding water and DNA was precipitated by adding 10 µl of 3 M sodium acetate (pH 4.6) and 250 µl of absolute alcohol.

The reaction contents were centrifuged at 14,000 rpm for 25 min at room temperature. Supernatant was removed carefully and pellet was washed twice with 250 µl 70% alcohol at room temperature.

After washing the pellet was air-dried.

The pellet was resuspended in 15 µl of Template Suppression Reagent (TSR).Then product was given to Genei Bangalore for sequence.