3. MATERIALS AND METHODS

The details of the materials, equipments used and techniques adopted during the course of the present investigation are described in this chapter. The present study was conducted on 4 sheep breeds of India viz. Malpura, Mandya, Garole and Karnah (Fig. 3.1). A total of 200 unrelated animals were selected randomly from the villages in and around the natural breeding tracts of the breeds (Fig. 3.2).

3.1 BLOOD COLLECTION

10 ml of blood was collected aseptically by jugular vein puncture in a sterile vacutainer containing 15% of 0.12 ml EDTA solution (BD vacutainer). The samples were transported to the laboratory in an icebox at 4 °C. The blood was stored at -20 °C in a freezer before DNA isolation.

3.2 DNA ISOLATION FROM BLOOD AND THEIR QUALITY CHECK

Preparation of reagents

**Tris (1M) pH 8.0**

- Tris base (Trizole) 121 10 gm
- dd water 800 ml
- pH adjusted to 8.0 by adding concentrated HCl (40 ml).

The solution was allowed to cool up to room temperature before making final adjustment of pH and the volume was adjusted to 1 liter.

**Sodium chloride (5 M)**

- Sodium chloride 29.40 gm

Volume was made up to 1000 ml by dd water. It was sterilized by autoclaving.

**Sodium acetate (3 M)**

- Sodium acetate 24.60 gm
- dd water 80 ml

The pH was adjusted to 5.2 with glacial acetic acid, and volume adjusted to 100 ml and sterilized by autoclaving.
Fig. 3.1 Different sheep breeds (a. Malpura, b. Mandya, c. Garole and d. Karnah) taken for study
Fig 3.2 Sheep breed distribution locations
- The sequence of a chain of amino acids
  amino acids joined by peptide bonds

- Occurs when the amino acid sequence
  becomes linked by hydrogen bonds
  between peptides

- The folding of the amino acid chain
  occurs when disulfide bonds form
  between the R groups of the alpha
  helices and pleated sheets

A protein consisting of more than one
amino acid chain
does not occur in all proteins.

**Fig. 2.1** Prion proteins having four levels of structural organization

![Prion proteins](image)

**Fig. 2.2** A normal prion (left), compared to an aberrant, disease-causing prion (right)
Phenol equilibration (Tris saturation)

500 ml of liquefied phenol stored at -20 °C was allowed to warm to room temperature and then melted at 65 °C for 1-2 hours. 8-hydroxyquinoline was added to a final concentration of 0.1 %. To the melted phenol, an equal volume (500 ml) of 1 M Tris (pH 8.0) was added at room temperature and stirred for 4 hours on magnetic stirrer and pH repeatedly checked till it reached 8.0 and stored in amber colored bottles at 4 °C.

Proteinase-K

<table>
<thead>
<tr>
<th>Proteinase-K</th>
<th>20 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd water</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The final volume was made up to 100 ml. It was sterilized by autoclaving and stored at -20 °C.

Ethanol 70 %

<table>
<thead>
<tr>
<th>Ethanol 99.9 %</th>
<th>70 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd water</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

TE buffer (10 mM)

<table>
<thead>
<tr>
<th>Tris (1 M, pH 8.0)</th>
<th>1.00 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>200 μl</td>
</tr>
</tbody>
</table>

Final volume was made up to 100 ml. Sterilized by autoclaving and stored the buffer at room temperature.

Agarose gel 0.8 %

<table>
<thead>
<tr>
<th>Agarose</th>
<th>0.80 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE (10X)</td>
<td>10 ml</td>
</tr>
<tr>
<td>dd water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>

Agarose 1.5 %

<table>
<thead>
<tr>
<th>Agarose</th>
<th>1.50 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE (10X)</td>
<td>10 ml</td>
</tr>
<tr>
<td>dd water</td>
<td>90 ml</td>
</tr>
</tbody>
</table>
Phenol equilibration (Tris saturation)

500 ml of liquefied phenol stored at -20 °C was allowed to warm to room temperature and then melted at 65 °C for 1-2 hours. 8-hydroxyquinoline was added to a final concentration of 0.1 %. To the melted phenol, an equal volume (500 ml) of 1 M Tris (pH 8.0) was added at room temperature and stirred for 4 hours on magnetic stirrer and pH repeatedly checked till it reached 8.0 and stored in amber colored bottles at 4 °C.

Proteinase-K

Proteinase-K 20 mg
dd water 1ml

The final volume was made up to 100 ml. It was sterilized by autoclaving and stored at -20 °C.

Ethanol 70 %

Ethanol 99.9 % 70 ml
dd water 30 ml

TE buffer (10 mM)

Tris (1 M, pH 8.0) 1.00 ml
EDTA (0.5 M, pH 8.0) 200 μl

Final volume was made up to 100 ml. Sterilized by autoclaving and stored the buffer at room temperature.

Agarose gel 0.8 %

Agarose 0.80 gm
TBE (10X) 10 ml
dd water 90 ml

Agarose 1.5 %

Agarose 1.50 gm
TBE (10X) 10 ml
dd water 90 ml
Phenol equilibration (Tris saturation)

500 ml of liquefied phenol stored at -20 °C was allowed to warm to room temperature and then melted at 65 °C for 1-2 hours. 8-hydroxyquinoline was added to a final concentration of 0.1 %. To the melted phenol, an equal volume (500 ml) of 1 M Tris (pH 8.0) was added at room temperature and stirred for 4 hours on magnetic stirrer and pH repeatedly checked till it reached 8.0 and stored in amber colored bottles at 4 °C.

Proteinase-K

| Proteinase-K | 20 mg |
| dd water    | 1ml   |

The final volume was made up to 100 ml. It was sterilized by autoclaving and stored at -20 °C.

Ethanol 70 %

| Ethanol 99.9 % | 70 ml |
| dd water      | 30 ml |

TE buffer (10 mM)

| Tris (1 M, pH 8.0) | 1.00 ml |
| EDTA (0.5 M, pH 8.0) | 200 μl |

Final volume was made up to 100 ml. Sterilized by autoclaving and stored the buffer at room temperature.

Agarose gel 0.8 %

| Agarose | 0.80 gm |
| TBE (10X) | 10 ml |
| dd water | 90 ml |

Agarose 1.5 %

| Agarose | 1.50 gm |
| TBE (10X) | 10 ml |
| dd water | 90 ml |
3.4 QUALITY AND QUANTITY CHECKING OF DNA

Quality of DNA was checked by electrophoresis by loading 2 µl DNA on 0.8% agarose in horizontal mini electrophoresis unit using 1xTAE as running buffer at 30 40 volts for about 90 mins. After electrophoresis, the gel was stained with ethidium bromide solution (0.5µg/ml). The gel was photographed by Gel Documentation System.

The quantity of DNA was determined by UV spectrophotometer. For this 2 µl of DNA was dissolved in 98 µl of double distilled water and loaded into a 100 µl cuvette. Optical density (O.D.) was determined at wavelengths 260 nm and 280 nm in UV-VIS spectrophotometer against distilled water as blank sample. Quantity of DNA was calculated using the following formula.

\[
\text{Quantity of DNA in } \mu \text{g/ml} = \frac{\text{OD (260 nm) x dilution factor x } 50 \ \mu \text{g/ml}}{1000}
\]

The ratio between OD 260 and OD 280 was calculated. The sample possessing a ratio of less than 1.7 and more than 2.0 was subjected to Proteinase-K digestion and DNA extracted with phenol chloroform isoamyl alcohol as described previously.

3.5 SELECTION OF PRIMERS

Details of the chosen markers are given in Table 3.1. These markers fulfilled the following criteria in sheep.

a. Had high PIC values and high heterozygosity.

b. Exhibited Mendelian inheritance.

c. Were polymorphic with large number of alleles.

d. Were unlinked.

PCR primers were designed based on published GenBank (accession No. DQ077504). The primers were synthesized by Integrated DNA Technologies, Inc. Coralville, USA).
Table 3.1 PCR-SSCP primers for PrP gene

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Gene Sequence size(bp)</th>
<th>Product size (bp)</th>
<th>Temp (°C)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>354-711 F</td>
<td>358</td>
<td>60.0</td>
<td>5'-caaggtgtagctacagtca-3'</td>
</tr>
<tr>
<td>2</td>
<td>354-711 R</td>
<td></td>
<td></td>
<td>5'-ccaacacctgtcctaaact-3'</td>
</tr>
</tbody>
</table>

3.6 PCR AMPLIFICATION

PCR reactions were carried out using a MJ Research Gradient Thermal Cycler. This locus was amplified by using the Hot start cycling programme with the annealing temperature ranging from 59 °C to 62 °C.

Procedure

a. 2.5 µl of the DNA sample was taken in the PCR tube.

b. 23 µl of cocktail/PCR mix was pipetted into each tube.

c. The tubes were centrifuged for proper mixing.

d. Tubes were then placed in PCR machine, which was programmed for specific reaction condition.

e. After completion of the PCR reaction tubes were removed from the thermal cycler.

f. Amplification of PCR product was checked in 1.5% agarose gel.

The composition of these reactions is shown in Table 3.2.

Table 3.2 Composition of PCR amplification reaction (25 µl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Component Volume</th>
<th>Final composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reaction buffer A</td>
<td>10X</td>
<td>1X</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5 pmol</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5 pmol</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>100 mM</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>3U µl</td>
<td>0.9 U</td>
</tr>
<tr>
<td>DNA sample template</td>
<td>~50 ng/µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Up to 25 µl</td>
<td></td>
</tr>
</tbody>
</table>
PCR program

The programme involved denaturation at 94 ºC for 2 mins, followed by 32 cycles of 94 ºC for 30 seconds, 60 ºC for 30 s, and 72 ºC for 30 s. This was followed by a final extension step at 72 ºC for 5 mins.

3.7 CHECKING OF PCR PRODUCT ON 1.5% AGAROSE

Following PCR amplification, the PCR products were checked on 1.5% agarose to verify the amplification of target regions. 6 µl of PCR product and 3 µl of 1x gel-loading dye were loaded on agarose along with 100 bp marker as a molecular marker and run at 100 volt for 30 minutes to conform the size of the target region.

3.8 NON-DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'-methylene bisacrylamide (BIS). The polymerization is initiated by free radical formation usually carried out with ammonium per sulfate as the initiator and N, N, N', N'-tetramethylene diamine (TEMED) as a catalyst. The length of the chain is determined by a concentration of acrylamide in the polymerization reaction. One molecule of cross-linker is included for every 29 monomers of acrylamide.

The denaturing gels are polymerized in the presence of an agent (urea or, less frequently, formamide) that suppresses base pairing in nucleic acids (alkali can not be used as denaturing agents). Denatured DNA migrates through these gels at a rate that is almost completely independent of its base composition and sequence.

3.8.1 Preparation of chemicals/buffers for SSCP

Preparation of non-denaturing gels (100 ml stock-native PAGE) 30%

Acrylamide: Bis-acrylamide

Acrylamide 29 gm; Bis-acrylamide 1 gm

Water was added to 100 ml and the solution heated at 37 ºC to dissolve chemicals.
3.8.2 Preparation of working (12% non-denaturing gels)

Table 3.3 Chemical composition for preparation of non-denaturing poly-acrylamide gels

<table>
<thead>
<tr>
<th>Chemical</th>
<th>30 ml</th>
<th>40 ml</th>
<th>60 ml</th>
<th>120 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide: Bis-acrylamide</td>
<td>12</td>
<td>16</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>10x TBE</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>dd Water</td>
<td>14</td>
<td>18.66</td>
<td>28</td>
<td>56</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.03</td>
<td>0.04</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

50x TAE(1000 ml)          10x TBE(1000 ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>242 gm</td>
<td>Tris</td>
<td>108 gm</td>
</tr>
<tr>
<td>EDTA</td>
<td>37.2 gm</td>
<td>Boric Acid</td>
<td>55 gm</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>57.1 ml</td>
<td>EDTA 0.5M</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

3.8.3 Preparation of denaturing solution (Dye)

- Formamide 95%
- NaOH 10 mM
- Xylene Cyanol 0.05 %
- Bromo phenol Blue 0.05%

3.9 SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

Rather than to obtain DNA sequence data directly, it is cheaper and faster to use techniques that estimate sequence variation. In SSCP technique this sequence variation is obtained by electrophoresis of the DNA sample through non-denaturing polyacrylamide gel. PCR is used to amplify the region of interest and the resultant DNA is separated as single-stranded molecule. A single stranded DNA folds differently from another if it differ even by a single base, and
it is believed that mutation-induced changes of tertiary structure of the DNA result in different mobility for two strands. These mutations are detected as the appearance of new band on autoradiogram, by silver staining or by use of fluorescent PCR primer, which are subsequently detected by an automated DNA SEQUENCER (non-radioactive detection). SSCP analysis is sensitive but in expensive, rapid and convenient method for determining which DNA sample in a set is differ in sequence. The major advantage of SSCP is that many individual PCR products can be screened for variation simultaneously.

Most researchers use SSCP to reduce the amount of sequencing necessary to detect new alleles at loci of interest. SSCP has been widely applied in medical diagnosis. The utility and convenience of SSCP is far from fully appreciated by molecular population biologist. Simple SSCP protocol helps to study variation in mitochondrial gene, nuclear intron, microsatellite and anonymous nuclear sequencing, in a range of mammalian species.

The single stranded conformation polymorphism (SSCP) method is widely used for mutation detection. The sensitivity of the method depends on several factors, most importantly on the temperature at which electrophoresis of single stranded DNA (ssDNA) takes place. The temperature has profound effect on the folded conformation of ssDNA. The temperature is predominantly determined empirically in conventional SSCP, which can be very tedious especially when a large number of different DNA sample need to be screened. For SSCP analysis of PCR products, several factors like ratio of PCR product, denaturing solution, acrylamide concentration, percentage cross linking, glycerol, voltage, running time and temperature were tested to optimize resolution and sensitivity. Setting up and casting a polyacrylamide gel using sequencing apparatus involves the following steps.

3.9.1 Preparation of glass plates

Both the glass plates were cleaned with warm water and detergent and rinsed thoroughly with deionised water to remove detergent residues and a final
95% ethanol wash of the plates was performed. Glass plates were air-dried with soft tissue paper.

3.9.2 Assembling the glass plates sandwich

The long plate was laid flat on a bench and the required spacers (0.5 mm) were arranged at each side parallel to the edges of the plate making sure that the glass plate and the spacers are clean and dry. Each side arm was placed along the appropriate side of the glass plate and plates were fixed tightly enough to hold them in position. Glass plate was sealed from bottom to prevent leakage of gel at time of casting gel. The assembled gel sandwich was placed in the casting stand and fixed there with the screw in the required direction.

3.9.3 Preparation of gel

Ultra pure reagents were used in preparing gels for denaturing PAGE. Knowing the size of the glass plates and thickness of the spacers, volume of acrylamide solution required was calculated. For preparation of 12% non-denaturing gel, 12 ml of 30% acrylamide from stock solution was taken and 14 ml MilliQ water and 3ml of 10X TBE was added. Then 300 µl of 10% APS and 30 µl of TEMED were simultaneously added to it. The solution was quickly mixed and transferred in between sandwich of glass plates avoiding any air bubble in gel.

3.9.4 Pouring the gel

PAGE mix solution was drawn into a barrel of 10 ml syringe. It was inverted to expel any trapped air bubble in the PAGE mix. Nozzle of the syringe was introduced in between the gap region of plates (space between the two glass plates) from top. PAGE mix was slowly expelled from the syringe, filling the space almost to the top.

Once the solution is filled up to the edges of the plate, appropriate comb was inserted in gel. 2-3 bulldog clips was put to keep the comb in appropriate position till the gel gets polymerized. These clips prevent formation of air bubble between the combs and glass surface as the gel polymerizes. The gel assembly was allowed to polymerize for 15 minutes.
3.9.5 Pre-run

After acrylamide has polymerized, the clamp holding the comb and casting stand was removed and comb was pulled out straight by wriggling it gently and smoothly. Any residue of unpolymerized acrylamide gel was wiped with 1X TBE buffer or de-ionized water (Dilute sufficient 10 X TBE buffer to 1X to run the gel).

Gel assembly was placed in buffer reservoir of the vertical electrophoretic unit (BioRad Protean II). Upper and lower tank was filled with 1 X TBE buffer. Buffer leakage was checked by marking the level of the buffer in the upper chamber. Safety covers and power supply was fixed on top of the upper buffer chamber to prevent evaporation of buffer. Pre run for 45-60 minutes at constant voltage (200 V) was given.

3.9.6 Preparation of sample for loading

5 ml of denaturing solution (95% formamide, 10 mM NaOH, 20 mM EDTA) and 5 ml loading dye (0.05% bromophenol 0.05% blue Xylene cyanol) was added to the samples (PCR product). All samples were denatured in a thermocycler at 85 °C for 13 mins. Denatured PCR product was immediately transferred into ice to prevent renaturation of the denatured PCR product this process is called snap cooling.

After completion of pre run the lid was removed. 10 ml to 15 ml of sample (PCR product) was loaded. Care was taken not to dislodge any air bubble that appears in tip after the solution has been ejected.

3.9.7 Running the gel

After loading the denatured samples into the wells the lid was replaced on upper buffer chamber and apparatus was attached to power pack. Gel was run at a constant voltage (375 V).

The DNA fragments co-migrate with the tracking dyes, bromophenol blue and xylene cyanol. After completion of electrophoresis, the gel plates were removed from the side arms and the gel plates were transferred in water carefully. Gels were recovered by inserting a flat edged spatula between the
edges of the glass plates, slowly separating the plates taking care of the gel. The gel was fixed in 10% ethanol (800 ml) for 30 minutes to overnight.

3.10 SILVER STAINING

3.10.1 Principle

The silver staining procedure is based on a photo chemically-derived silver stain, originally designed for the staining of proteins, in which silver nitrate is the impregnation uses relatively low concentration of silver in a solution containing formaldehyde. Typically, gels are fixed in acetic acid, washed with distilled water, impregnated with silver, quickly rinsed with distilled water, and developed at 8-12 °C until optimum image contrast is obtained. Image development is stopped with fixative, and gels are washed with water. The procedure is fast, has relatively few steps and reagents, and produces the least number of staining artifacts.

3.10.2 Preparation of reagents

Fixative (10% acetic acid)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>dd water</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

Staining solution (2000 ml)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nitrate</td>
<td>2 gm</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3 ml</td>
</tr>
<tr>
<td>dd water</td>
<td>1500 ml</td>
</tr>
</tbody>
</table>

Developer (1500 ml)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate</td>
<td>45 gm</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>2.25 ml</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>300 µl</td>
</tr>
</tbody>
</table>

Stop solution (10% acetic acid) 1 liter

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>dd water</td>
<td>900 ml</td>
</tr>
</tbody>
</table>
Procedure

a. The gels were taken out from the assembly and fixed in acetic acid for 30 mins.
b. This was followed by washing the gels twice with dd water for 3 mins.
c. The gels were kept in staining solution for 30 mins.
d. The gels were washed with dd water for few seconds.
e. The gels were kept in developer till the bands developed properly.
f. The reaction was stopped with stop solution.
g. The stained gels were washed with dd water for 3 mins.
h. The gels were then placed between two cellophane sheets and dried.

3.10.3 Gel drying

a. The silver stained gel was transferred in between a blotting paper and cellophane paper sheet, avoiding any wrinkles as far as possible.
b. Extra cellophane sheet (if any) was trimmed around the gel, just leaving not more than one inch around the filter paper.
c. The gel was placed into gel dryer (Model 583, Biorad) and dried for permanent record and scoring.

3.11 SCORING OF SSCP GELS

Identified different banding patterns of the amplified gene or gene fragment in dried gel, these variants or haplotypes (because of their conformational changes due to insertion, deletion, transversion, etc of the nucleotide sequence) were marked, score and again cross checked by SSCP and finally found typical gene variants were used for further study or analysis like sequencing or association.

Procedure

a. The gel was superimposed with transparency sheet.
b. The bands were marked with thin OHP marker pens. Upper most part of each band was marked and this criterion was followed uniformly for all gels.

c. To distinguish different variants from one another.

d. The gel is scored for different patterns.

e. Each SSCP pattern was labeled as A, B, C, D, E, F, G, H and I.

3.12 SEQUENCING OF PCR PRODUCT

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by electrophoresis and the bases at the end are extracted by data collection software, recreating the original sequence of the DNA. The most obvious application of DNA sequencing technology is the accurate sequencing of genes and genome. Another one application is the typing of single nucleotide polymorphism.

3.12.1 Template

Template purity and the optimum concentration are crucial in obtaining the good quality of DNA sequence. Template preparation is the most crucial part of the automated DNA sequencing.

3.12.2 PCR product

PCR product should be clear of dimers and non-specific amplifications. After PCR amplified DNA was purified for the removal of excess reaction components. It is done mainly by following methods.

3.12.3 Exo-SAP digestion of PCR product

Exo SAP is used to clean up the PCR products by using following composition.
Table 3.4 Chemical composition for purification of PCR product

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Components</th>
<th>Amount for sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Exo I</td>
<td>0.025 µl (0.5 units)</td>
</tr>
<tr>
<td>2</td>
<td>SAP</td>
<td>0.5 µl (0.5 units)</td>
</tr>
<tr>
<td>3</td>
<td>PCR buffer</td>
<td>0.1 µl (10X)</td>
</tr>
<tr>
<td>4</td>
<td>Milli Q</td>
<td>0.375 µl</td>
</tr>
<tr>
<td>5</td>
<td>Total volume</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Program for Exo-SAP digestion

PCR products in 96 well plate were treated with shrimp alkaline phosphatase (1 unit/µl) and Exonuclease I (10 units/µl) (Roche Laboratories) using a volume equaling 5% of the PCR product, incubated at 37 °C for 30 mins followed by 80 °C for 10 minutes and kept at -20 °C before sequencing.

3.12.4 Setting of cycle sequencing reaction

Reaction was set as per recommendation of ABI using 1 µl of ready reaction mix (mixture of labeled dNTPs, DNA polymerase and buffers) in addition to template and primer.

Table 3.5 Ready reaction composition for sequencing reaction of purified PCR product

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Components</th>
<th>Amount for sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR Product</td>
<td>2 µl</td>
</tr>
<tr>
<td>2</td>
<td>Ready reaction mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>3</td>
<td>5 X sequencing Buffer</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>4</td>
<td>Primer</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>5</td>
<td>Milli Q water</td>
<td>5.2 µl</td>
</tr>
</tbody>
</table>
Mixed the contents briefly and kept it in a thermal cycler set at following reaction conditions.

**Program for sequencing**

The programme involved denaturation at 96 °C for 2 mins, followed by 30 cycles of 96 °C for 10 s and 60 °C for 4 minutes. For short PCR products, number of cycles can be reduced (e.g. 20 cycles for a 300-bp or smaller fragment).

If the Tm of a primer is >60 °C, the annealing step can be eliminated. If the Tm of a primer is <50 °C, increase the annealing time to 30 seconds or decrease the annealing temperature. For templates with high GC content (>70%), heat the tubes at 98 °C for 5 minutes before cycling to help denature the template.

### 3.13 PURIFICATION OF THE SEQUENCING PRODUCT

After the sequencing reaction the products were purified by the following protocol.

a. Added 2 µl of 125 m M EDTA to stop the reaction and mixed well.

b. Added 2 µl for 3M Sodium acetate (pH 4.6) to each reaction well.

c. Ensured proper mixing of the contents.

d. Added 50 µl of 95% ethanol to each well and incubated at room temperature for 15 minutes.

e. Spun at a speed of 1650 g for 45 minutes at room temperature.

f. Inverted the plate on paper towel and gave a short spin at 180 g for removing the supernatant.

g. 200 µl of 75% ethanol was added and spun at 1650 g for 5 minutes.

h. Inverted the plate on paper towel and centrifuged at 180 g for 1 minute.

### 3.14 DENATURATION

Added 10 µl of Hi Di Formamide and denatured at 95 °C for 5 minutes and chilled it on ice immediately for 5 minutes.
3.15 SEQUENCING

a. The plate was then loaded in automated DNA sequencer. (ABI 3100 Avant – Automated DNA Sequencer)

b. Four capillaries were provided with different array sizes (22 cm, 36 cm, 50 cm and 80 cm). The large surface area of a capillary allows heat generated during electrophoresis to be dissipated efficiently, allowing high-voltage electrophoresis. The result was rapid, high-resolution separation of DNA fragments.

c. Polymer POP-6 was used for sequencing (Performance Optimized Polymer 6, medium used to separate DNA fragments).

d. Plate record was loaded with appropriate model number and sequencing chemistry.

e. 96 well plate was linked and started the run.

3.15.1 Data collection and extraction

After the completion of the electrophoresis (run) chromatogram drawn by data collection software was used to extract the sequence data.

**Nucleotide data retrieval from chromatogram software**

Sequence obtained after sequencing of variants was edited using Chromas 2.13 Software.

**Sequence editing**

The software was used to resolve ambiguous bases in the chromatogram obtained after sequencing.

**Establishment of gene identity**

Edited sequence was used for BLAST analysis to confirm sequence gene identity.
Generated nucleotide data analysis

The nucleotide data obtained after sequencing and sequence editing was used for further analysis. All available sequence of sheep at Gene Bank database was analyzed by multiple sequence alignment software (CLUSTAL-W) to find SNPs in our sequence.

CLUSTAL-W ANALYSIS was carried out to find out whether the three patterns obtained from the each primer are the same or different. The BLAST analysis was also used to find the percent homology of the sequences that has been obtained in the study and with all other sequences of the other species.

The nucleotide sequences of the other sheep breeds (Gene Bank) were compared with our sequences to find out the nucleotide changes as well as amino acid production between them.