Chapter III

MATERIALS AND METHODS
3.1 Materials

3.1.1 Instruments

GS-FLX whole genome analyzer (Roche, USA), Real Time PCR 7500 Fast (Applied Biosystem, USA), Bioanalyzer (Agilent, USA), Research microscope (Olympus, USA), Nanoquant spectrophotometer (Tecan, Switzerland), Nanodrop spectrophotometer (Thermo, USA), Refrigerated centrifuge (Eppendorf, Germany), Gel documentation system (Syngene, USA), Autoclave (Equitron, India), Incubator shaker (Labnet, USA), Electrophoresis system (Biorad, USA), PCR machine (Applied Biosystem, USA), Ice maker (Agion, Netherland), Ultralow deep freezer (Bluestar, India), Tissue lyser (Qiagen, USA), Weighing balance (Sartorius, USA), Cluster server (Fijitsu, Japan), Electronic cell counter (Foss, Denmark), Biosafety cabinet (Nuaire, USA), QuantiFlour ST fluorometer (Promega, USA).

3.1.2 Plasticware

All the plasticware used in this study including centrifuge tubes, petri plates, eppendorf tubes, micropipette tips, finnpipettes, PCR tubes, 96 well PCR plates and filters were procured from reputed firms viz. Corning (USA), Axygen (USA), Thermo (India), Falcon (USA) and Millipore (USA).

3.1.3 Glassware

All the glassware used during the study were procured from Borosil India. These were thoroughly washed and sterilized prior to use as per standard procedure.

3.1.4 Chemicals

Molecular Biology grade buffers and chemicals were used in the present study, which were shown in the tables below. All the primers used in this study were synthesized from MWG Biotech AG, India.
Materials and Methods

Table 3.1: List of buffers used in the study with the components.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffer Saline (PBS) (pH 7.4)</td>
<td>137.0 mM NaCl, 2.7 mM KCl, 10.1 mM NaH$_2$PO$_4$, 1.8 mM KH$_2$PO$_4$</td>
</tr>
<tr>
<td>TAE Buffer (50X)</td>
<td>2.0 M Tris-HCl, 950.0 mM Acetic acid, 50.0 mM EDTA</td>
</tr>
<tr>
<td>Tris Borate EDTA Buffer (TBE) (10x) PH 8.3</td>
<td>0.9 M Tris HCL, 0.9 M Boric Acid, 20.0 mM EDTA</td>
</tr>
<tr>
<td>10X MOPS Buffer</td>
<td>20.93 g MOPS</td>
</tr>
<tr>
<td>[3-(N-morpholino) propanesulfonic acid]</td>
<td>2.06 g Sodium acetate, 5.0 ml 0.5 M EDTA, DEPC treated water upto 200 ml</td>
</tr>
</tbody>
</table>

Table 3.2: Chemicals and stock solutions used in the study with the brand.

<table>
<thead>
<tr>
<th>Chemicals / kits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>O’RangeRuler™100 bp DNA Ladder, GeneRuler™ 1 Kb DNA Ladder, Revert Aid cDNA Synthesis kit, PCR Master Mix (2X), 6X DNA Loading Dye</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Agarose, EDTA, Glucose, Tris base, Taq DNA polymerase, Sodium chloride, Potassium chloride, Boric acid, Chloroform, Ethanol, Glycerol, Hydrochloric acid, Isopropanol, Isoamyl alcohol, Sodium hydroxide, Ammonium acetate, Cetyl trimethylammonium bromide, Sodium dodecyl sulfate, Tri reagent, Zinc chloride, Potassium permanganate</td>
<td>Sigma chemicals</td>
</tr>
<tr>
<td>RNase-A, DNase-I, Minelute PCR purification kit</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>
### Materials and Methods

Bromophenol blue, Calcium chloride, Ethidium bromide, Magnesium chloride, Potassium chloride, Nutrient agar, Sheep blood agar, McConkey’s agar, Sabouraud dextrose agar, Different antibiotic discs

Power SYBR green PCR master mix

High Sensitivity DNA Kit, RNA 6000 Nano Kit

GS Rapid Library Reagent/Adaptors Kit, GS Titanium emPCR Reagents LV (Lib-L) Kit, GS Titanium Sequencing Reagents XLR70

Mastilep Topical Herbal Gel

<table>
<thead>
<tr>
<th><strong>Target</strong></th>
<th><strong>Bacterium</strong></th>
<th><strong>Nucleotide sequence (5’-3’)</strong></th>
<th><strong>Amplicon size (bp)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td>F-TCT TCA GAA GAT GCG GAA TA</td>
<td>420</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TAA GTC AAA CGT TAA CAT ACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. agalactiae</strong></td>
<td>F-AAG GAA ACC TGC CAT TTG</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TTA ACC TAG TTT CTT TAA AAC TAG AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. dysgalactiae</strong></td>
<td>F-GAA CAC GTT AGG GTC GTC</td>
<td>264</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-AGT ATA TCT TAA CTA GAA AAA CTA TTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. uberis</strong></td>
<td>F-TAA GGA ACA CGT TGG TTA AG</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TTC CAG TCC TTA GAC CT TCT CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>F-ATC AAC CGA GAT TCC CCC AG</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-TCA CTA TCG GTC AGT CAG GAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.3:** Sets of species specific primers used in the experiment.
Table 3.4: Sets of gene specific primers used in the experiment.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Target gene</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-6 F</td>
<td>TCA TTA AGC GCA TGG TCG ACA AA</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>IL-6 R</td>
<td>TCA GCT TAT TTT CTG CCA GTG TCT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IL-8 F</td>
<td>CAC TGT GAA AAT TCA GAA ATC ATTTGTT</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>IL-8 R</td>
<td>CTT CAC AAA TAC CTG CAC AAC CTT C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IL-12 F</td>
<td>TTA TTG AGG TCG TGG TAG AAG CTG</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>IL-12 R</td>
<td>GGT CTC AGT TGC AGG TTC TTG G</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>GMCSF F</td>
<td>AGT AAT GAC ACA GAA GTC GTC TCT G</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>GMCSF R</td>
<td>GCC GTT CTT GTA CAG CTT CAG G</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>TNF-α F</td>
<td>TCT TCT CAA GCC TCA AGT AAC AAG C</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>TNF-α R</td>
<td>CCA TGA GGG CAT TGG CAT AC</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>IFN-γ F</td>
<td>TCA TTA AGC GCA TGG TCG ACA AA</td>
<td>418</td>
</tr>
<tr>
<td></td>
<td>IFN-γ R</td>
<td>TCA GCT TAT TTT CTG CCA GTG TCT</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GAPDH F</td>
<td>GGC GTG AAC CAC GAG AAG TAT AA</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>GAPDH R</td>
<td>CCC TCC ACG ATG CCA AAG T</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>β Actin F</td>
<td>CTG GAG AAG AGC TAC GAG CCT CCT G</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>β Actin R</td>
<td>AGG AAG GAA GGC TGG AAG AGA GCC T</td>
<td></td>
</tr>
</tbody>
</table>

3.2 Methods

3.2.1 Animals and preparation of milk samples

A total of 70 lactating cows comprising 30 Gir (Bos indicus), 30 Kankrej (Bos indicus) and 10 Crossbred (Bos taurus X Bos indicus) cows maintained at Livestock Research Station (LRS) and Livestock Production and Management (LPM) cattle farms in Anand town of Gujarat State, India. All these animals were lactating during the sampling period. These animals were housed, fed and managed under uniform management practices followed at farms. Milk samples of Kankrej and Gir cows were collected from LRS and samples of crossbred cattle were collected from LPM cattle farms. The udder was washed thoroughly with potassium permanganate solution (1:1000) and the teats were wiped with 70% ethyl alcohol prior to sampling. Three
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hundred milliliter of quarter milk samples from each cow were collected aseptically in sterile wide mouth glass stopper bottles on three consecutive days during the evening milking. Aliquots of 25 ml were separated and used to determine SCC using an electronic somatic cell counter and rest of the milk was used for microbiological culture examination, somatic cell isolation and total DNA extraction. Collected milk samples were transported in ice to the laboratory.

3.2.2 Microbiological cultures

All milk samples were evaluated by the 1µl calibrated loop method for microbiological growth in (a) sheep blood agar medium, (b) McConkey’s medium, and (c) Sabouraud dextrose agar medium. Inoculated sheep blood agar and McConkey’s agar plates were incubated overnight at 37° C while as Sabouraud dextrose agar plates were incubated at 28° C for 2-3 days for fungal growth. Staining and cellular morphological features of organisms were ascertained by microscopic examination of Gram-stained smears. A standard laboratory protocol of inspecting cultures daily for growth of potential pathogens was followed.

3.2.3 Somatic Cell Count (SCC)

Somatic cell count of quarter milk samples were carried out using an electronic somatic cell counter (Foss, Denmark). Cell counter is based on the principle of passing a very thin string of sample under a counting unit. The sample string is carried under the counting unit by a carrier fluid, which creates a very thin but well defined sample string. This is accomplished due to a diminishing diameter in the flow cuvette, and the high pressure by which it is pumped through the cuvette. The diameter of the sample string only allows one somatic cell to pass at a time. After being stained by a fluorescent dye, the milk sample is exposed to blue light in the flow cuvette. The blue light excites the fluorescent dye, making the somatic cells emit red light. This red light is magnified and can then be counted as light pulses by a photomultiplier.

Flow system description

1. The milk sample is taken by instrument. Simultaneously, the sample syringe takes in, rinsing/ sheath fluid.
2. At the same time, the dye/buffer syringe takes in dye/buffer from the mixing chamber. The mixing chamber is filled with dye concentrate from the disposal bag, with diluent from the external diluent container (ratio 1:9).

3. Milk and dye/buffer is mixed and pumped through the in-line filter. The first part of the mixture passes directly to high concentrate waste.

4. The measuring syringe is filled with rinsing/sheath fluid.

5. The measuring syringe injects milk/dye mixture in the flowcell, where the sheath fluid, constantly passing through, passes as a thin and well defined sample string under the microscope.

6. The pulse from the stained somatic cells are amplified, counted and converted into the number of somatic cells per microlitre.
   For each sample all tubes are washed through, and the in-line filter is back flushed. The flushing waste is separated in high and low concentrate waste.

3.2.4 Identification of infected quarters

By correlating the results of SCC and microbiological culture examination, infected quarters were identified following the International Dairy Federation (IDF) guidelines. (Table 3.5)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Quarter health status</th>
<th>Culturing of milk samples</th>
<th>SCC of milk samples (Cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy</td>
<td>2 times negative</td>
<td>All 3 times &lt; 5,00,000</td>
</tr>
<tr>
<td>2</td>
<td>Specific mastitis</td>
<td>2 times positive</td>
<td>Minimum 1 time &gt; 5,00,000</td>
</tr>
<tr>
<td>3</td>
<td>Non-specific mastitis</td>
<td>2 times negative</td>
<td>Minimum 1 time &gt; 5,00,000</td>
</tr>
<tr>
<td>4</td>
<td>Latent infection</td>
<td>2 times positive</td>
<td>All 3 times &lt; 5,00,000</td>
</tr>
</tbody>
</table>

(IDF, 1987)

3.2.5 Total DNA extraction from mastitic milk

Thirty ml milk sample from each selected quarters was diluted with 300 ml sterile normal saline solution (pH 7.2). The diluted samples were filtered using 3 micron
Materials and Methods

nitrocellulose filters to remove somatic cells and centrifuged at 12,000 xg for 15 min in 50 ml tubes. The pellet was washed three times with sterile normal saline solution.

**Isolation of microbial DNA by Proteinase K-SDS- Lysozyme method**

✓ **Reagents required**

- 1X TE Buffer (pH- 8.0)
- SDS (10 % w/v)
- Proteinase K (20 mg/ml)
- Lysozyme (10 mg/ml)
- 5 M NaCl
- 10 % CTAB in 0.7 M NaCl
- Tris Saturated phenol (pH-8.0)
- Chloroform
- Isoamyl alcohol
- 7.5 M Ammonium acetate
- Isopropanol
- Absolute ethanol

✓ **Protocol**

1. Pellet containing bacterial cells was resuspended in 2 ml of 1X TE buffer (pH- 8.0), 250 µl SDS (10 % w/v), 10 µl of Proteinase K solution (20 mg/ml) and 50 µl of lysozyme (10 mg/ml).
2. The suspension was mixed gently and incubated overnight in a water bath (Equitron Media Instruments, India) at 37°C.
3. Subsequently, 500 µl of 5 M NaCl and 100 µl 10% CTAB were added.
4. The suspension was then incubated in water bath at 65°C for 10 min.
5. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed effectively by inversion.
6. The solution was centrifuged at 12,000 xg for 10 min at 4°C.
7. The upper aqueous phase was then transferred to a clean 15 ml centrifuge tube.
8. To the upper aqueous phase, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed well.
9. The solution was centrifuged at 10,000 xg for 10 min at 4°C and the upper aqueous phase was transferred to a sterile 15 ml centrifuge tube.
10. The DNA was precipitated by the addition of one-tenth volume of 7.5 M Ammonium acetate and equal volume of ice cold propanol to the upper aqueous phase and mixed properly.
11. It was kept at -20°C for 2 h to facilitate precipitation.
12. DNA was pelleted by centrifugation at 12,000 xg for 10 min at 4°C and the supernatant was discarded.
13. The DNA pellet was washed twice with 70% chilled ethanol by centrifugation at 12,000 xg for 5 min.
14. Ethanol was discarded and pellet was dried by leaving the eppendorf tube with lid open in a dry bath maintained at 55°C for a few min till DNA became transparent.
15. DNA pellet was dissolved in 50 µl of autoclaved 0.3X TE buffer and kept in a water bath at 37°C for overnight.
16. The dissolved genomic DNA samples were preserved by storing at -20°C till use.

✓ Quantitation and quality assessment of DNA

The DNA stock samples was quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio of 1.8 was considered to be of good purity. Concentration of DNA was estimated using the formula.

\[
\text{Concentration of DNA (mg/ml)} = \text{OD } 260 \times 50 \times \text{Dilution factor}
\]

Quality and purity of DNA were checked by agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TBE (pH 8.0) buffer (Sambrook and Russel, 2001) was
used for submarine gel electrophoresis. Ethidium bromide (1%) was added @ 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at room temperature. DNA was visualized using gel documentation system.

3.2.6 Absolute quantification of bacterial load by Real Time PCR

Species specific PCR primers (synthesized by MWG Biotech AG) used for amplification of the target region. Total DNA extracted from the infected milk samples were used as a template for PCR reaction to quantify the bacterial load of respective organism. All PCR reactions were performed in optical 96 well plates. The amplification was carried out in a final reaction volume of 15 µl containing 1 x SYBR Green PCR master mix, 5 pmoles of each species specific forward and reverse primer and 1µl of template DNA (Table 3.6). The PCR protocol designed for 35 cycles. Fluorescence signals were measured once in each cycle at the end of the extension step.

Recombinant plasmids were diluted up to seven times (10^7) from stock plasmids (known concentration) and were used to prepare standard curve. For each sample, a dissociation curve was generated after completion of amplification to determine the specificity of PCR reaction.

Table 3.6: Components used for absolute quantification Real Time PCR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SYBR Green master mix</td>
<td>7.5</td>
<td>1X</td>
</tr>
<tr>
<td>Forward primer (10 pmoles)</td>
<td>0.5</td>
<td>5 pmoles</td>
</tr>
<tr>
<td>Reverse primer (10 pmoles)</td>
<td>0.5</td>
<td>5 pmoles</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>5.5</td>
<td>--</td>
</tr>
<tr>
<td>Template</td>
<td>1.0</td>
<td>30 ng</td>
</tr>
<tr>
<td>Total</td>
<td>15.0</td>
<td></td>
</tr>
</tbody>
</table>

✓ Visualization of PCR Product Agarose gel electrophoresis

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye buffer from each tube and electrophoresed.
Materials and Methods

along with 1 kb DNA molecular weight marker (GeneRuler, Fermentas) on 2% agarose gel containing ethidium bromide (one per cent solution at the rate of 5 µl/100 ml) at constant 80 V for 30 min in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, USA).

3.2.7 Metagenomic sequencing to analyze bacterial diversity

A. RAPID LIBRARY PREPARATION

✓ DNA fragmentation by nebulization

1. All the DNA were pooled breed wise in equimolar concentration and 500 ng of pooled DNA were taken in a 1.7 ml microcentrifuge tube.
2. TE Buffer was added to make a final volume of 100 µl.
3. Using sterile gloves, nebulizer condensor was affixed a tube around the aspiration tube.
4. Nebulizer top was assembled, with the aspiration tube pointing upwards.
5. 100 µl DNA sample was pipeted in the nebulizer cup and 500 µl of nebulization buffer was added, pipeted up and down to mix.
6. The top was assembled to the nebulizer cup and the tubing was connected to the nitrogen tank inlet.
7. A pressure of 30 psi (2.1 bar) for a minute was applied.
8. The setup was then disconnected, followed by centrifugation at 1000 xg for few seconds to concentrate the contents.
9. To the cup, 2.5 ml of PBI buffer was added followed by aspiration ensuring complete mixing.
10. Purification of the fragmented DNA was performed on the column.
11. To the column, 750µl of the sample was added and centrifuged at 10000 xg for 15 sec.
12. The flow through was discarded and the above step was repeated trice in the same column.
13. Finally centrifuged for a minute and the remaining solution was discarded.
14. Added 750µl of PE buffer, centrifuged at 13000 xg for 1 min and flow through was discarded.

15. The tubes were centrifuged for 15 sec and then rotated 180° and centrifuged for another 15 sec.

16. The column was transferred to a 1.5 ml centrifuge tube and 16 µl of elution buffer (TE buffer) was added and centrifuged for 1 min at 13000 xg.

17. The eluted sample was transferred to 200 µl PCR tubes.

✔ **Fragment end repair**

End repair mix was prepared as shown in Table 3.7

---

**Table 3.7: Preparation of fragment end repair mix.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL 10× PNK buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>RL ATP</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>RL dNTPs</td>
<td>1 µl</td>
</tr>
<tr>
<td>RL T4 Polymerase</td>
<td>1 µl</td>
</tr>
<tr>
<td>RL PNK</td>
<td>1 µl</td>
</tr>
<tr>
<td>RL Taq Polymerase</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9 µl</strong></td>
</tr>
</tbody>
</table>

1. To the PCR tubes containing the samples, 9 µl of end repair mix was added.

2. The tubes were vortexed followed by centrifugation at 10000 xg for few seconds.

3. The samples were run in the PCR machine with the parameters-

✔ 25° C for 20 min

✔ 72° C for 20 min

✔ 4° C on hold

✔ **Bead preparation**

1. The AMPure beads were vortexed for 20 sec.

2. Fractionated 125µl of the beads to a 1.5 ml centrifuge tubes.
3. The tubes were placed in a magnetic particle concentrator (MPC) until all the beads get pelleted to the side of the magnet.
4. The supernatant was then pipetted out carefully.
5. To the tubes, 73 µl of TE buffer was added and vortexed for 5 sec.
6. To the beads, 500 µl of sizing solution was added, vortexed for 5 sec and centrifuged for 2 sec.
7. Tubes were then placed on ice for further use.
8. In a separate tube, 5 ml of 70% ethanol was prepared by mixing 3.5 ml of absolute ethanol and 1.5 ml of molecular biology grade water.

✓ Adapter ligation
1. To the tubes 1 µl of adapter and 1 µl of Ligase were added, after the completion of the end repair programmed cycles.
2. The tubes were incubated at 25° C for 10 min.

✓ Small fragment removal
1. To the bead solution, adaptor ligated samples were added. Samples were vortexed and incubated at room temperature for 5 min.
2. After incubation the tubes were placed on the MPC until all the beads were pelleted to the side of the tubes. Carefully the supernatant was discarded.
3. To the tubes, 100 µl of TE buffer was added. Vortexed for 5 sec and 500 µl of sizing solution was added and again vortexed for 5 sec.
4. The tubes were then incubated at room temperature for 5 min.
5. They were then placed on the MPC till all the beads pelleted on the wall and discard the remaining supernatant.
6. The above steps (3-5) were repeated twice.
7. The tubes were placed on the MPC and the beads were washed twice with 70% ethanol.
8. The tubes were then air dried for 2-5 min at room temperature.
9. To the tubes, 53 µl of TE buffer was then added vortexed for 5 sec and centrifuged for 2 sec in a micro centrifuge.
10. The tubes were placed on the MPC, the beads were pelleted and 50 µl of supernatant which contained the library was transferred to a new centrifuge tube.

✓ **Library quantitation**

Library quantitation was performed by using a single cuvette QuantiFlour ST fluorometer (Promega, USA).

✓ **Preparation of standard**

For generation of standard curve, 8 tubes were labeled as (1-8).

1. Tube 1: 2.5 \times 10^9 \text{ molecule/µl} solution of RL standard was prepared by mixing 90 µl of RL standard with 90 µl of TE Buffer.
2. The remaining 7 tubes (2-8) were filled with 60 µl of TE buffer.
3. From tube 1 to tube 2, 120 µl of solution was transferred.
4. The tube was vortexed for 5 sec and spun in a mini centrifuge for 2 sec.
5. From tube 2 to tube 3 120 µl of solution was transferred.
6. The tube was vortexed for 5 sec and spun in a mini centrifuge for 2 sec.
7. The serial dilution was preceded as above for the remaining 5 tubes.

✓ **QuantiFlour ST fluorometer**

1. To 8 cuvettes, 50 µl of the 8 dilution of the RL standard were transferred.
2. As the blank, 50 µl of TE buffer was taken.
3. The fluorometer was set on the blue channel with the blue cuvette holder insert.
4. The standard value was set to 250
5. The fluorometer was calibrated with the blank and 2.5 \times 10^9 \text{ molecule/µl} solution RL standard.
6. The relative fluorescence units (RFU) was read and recorded for each dilution.
7. To a cuvette 50 µl of the sample library were transferred. The fluorescence was read and recorded.
8. The sample library was transferred back to its tube with 20 µL pipette.
To generate the standard curve of the fluorescence readings and to calculate the library sample concentration, the Rapid Library Quantitation Calculator was used.

**Library quality assessment**

1. To assess the quality of library, 1 µl aliquot of the DNA library was run on an Agilent Bioanalyzer High Sensitivity DNA chip.
2. The quality of the DNA library was assessed by smear analysis (average fragment length should be between 600-900 bp).

**B. EMULSION PCR**

**Preparation of reagents**

1. The emPCR reagents were thawed at room temperature except the enzymes which were stored at -15º C to -25º C.
2. Vortex the thawed reagents.
3. EmPCR additive was first vortexed and heated at 55º C for 5 min to dissolve the precipitate.
4. All the kit components including the enzymes were centrifuged at 10,000 xg for 10 sec in a mini centrifuge.
5. The enzymes were stored at -15º C to -25º C and the others were maintained on ice.

**Preparation of emulsion oil**

1. The cups of emulsion oil were securely placed in the tissue lyser and shaken at 28 Hz for 2 min.
2. The cups were removed from the tissue lyser after the completion of the time.

**Preparation of mock amplification mix and pre-emulsion**

1. Working solution was prepared by diluting 2 ml of 5X mock amplification mix with 8 ml of molecular biology grade water.
2. To each cup of emulsion oil, 5 ml of the mock amplification mix was added.
3. The cups mixed by inverting and placed in the tissue lyser, secured firmly.
4. The cups were shaken in the tissue lyser at 28 Hz for 5 min.
5. After the time, the emulsions were removed from the tissue lyser.

✓ **Preparation of live amplification mix**

1. The live amplification mix was prepared accordingly to the table given below (Table 3.8).
2. The amplification mix was vortexted for 5 sec and stored on ice.

**Table 3.8: Preparation of live amplification mix.**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular biology grade water</td>
<td>2400</td>
</tr>
<tr>
<td>EmPCR additive</td>
<td>3000</td>
</tr>
<tr>
<td>5x Amplification mix</td>
<td>1560</td>
</tr>
<tr>
<td>Amplification primer</td>
<td>460</td>
</tr>
<tr>
<td>EmPCR enzyme mix</td>
<td>400</td>
</tr>
<tr>
<td>PPiase</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>7830</strong></td>
</tr>
</tbody>
</table>

✓ **DNA library capture**

1. Capture bead wash buffer (1X) was prepared by mixing 1 ml of 10X capture bead wash buffer with 9 ml of molecular biology grade water.
2. The DNA capture beads were vortexted and pelleted in a minifuge by spinning for 10 sec, rotating the tube 180° and again spinning for 10 sec.
3. The supernatant was decanted carefully.
4. The beads were washed twice with 1 ml of 1X capture bead wash buffer. Vortexted to resuspend the beads and the supernatant was discarded after each wash.
5. The DNA prepared with Rapid library methods was heat denatured in a thermocycler with the following parameters:
   - 95° C for 2 min and 4° C on hold.
6. To the washed DNA capture beads correct volume of DNA library that would provide optimum concentration was added.

7. The volume of DNA library to be added was calculated as follows:

\[
\mu l \text{ of library per tube} = \frac{\text{Desired molecules per bead} \times 35 \times 10^6 \text{ beads per tube}}{\text{Library concentration (in molecules/}\mu l)}
\]

8. The tubes were vortexed for 5 sec to mix their contents.

9. DNA capture beads with library DNA mixes were prepared for individual emulsion mixtures.
   - The captured library mixes were transferred to 15 ml tubes.
   - To each 15 ml tube, 3.75 ml of live amplification mix was added.
   - To the DNA capture beads containing the captured library, 1 ml of the mix was transferred.
   - Repeated the above steps twice and vortexed.

✓ **Emulsification**

1. The contents from 15 ml centrifuge tubes were transferred to the prepared emulsion cup.
2. The cups were inverted thrice to mix.
3. They were placed in the tissue lyser at 12 Hz for 5 min.

✓ **Dispersion of the emulsions**

1. After the emulsification process has completed, the amplification mixes (100 \(\mu l\)) were dispensed into 96 well PCR plates. The contents of each tube approximately filled 10 wells.
2. The wells were capped and sealed firmly.

✓ **Amplification reaction**

The plates having the emulsified amplification mixes were placed in the thermocycler. The PCR parameters were set as 4 min at 94° C, 50 cycles of 30 sec at
Materials and Methods

94°C, 4.5 min at 58°C and 30 sec at 68°C followed by 10°C on hold. The total time to complete the process was 6 h.

C. BEAD RECOVERY

✓ Vacuum assisted emulsion breaking-up

1. The enhancing fluid XT and the annealing buffer XT were retrieved and kept at room temperature.
2. To each of the two lids of the emPCR breaking kit LV/MV, 50 ml conical tube was attached.
3. The blue connector was inserted into the top opening of the 8-pronged transpette.
4. The other end of the tubing was connected to the vaccum source.

![Image of vacuum assisted emulsion breaking set-up]

Figure 3.1: Vacuum assisted emulsion breaking set-up.

✓ Emulsion collection and initial washes

1. The emulsion from each emulsion cup were aspirated, 8 wells at a time and were collected in the two 50 ml collection tubes.
2. The wells were rinsed twice with 100 of isopropanol per well.
3. An additional 5 ml of isopropanol was aspirated to collect the remaining beads in the tubing.
4. The vacuum was turned off and the tubes containing the amplified DNA beads were capped.
Bead washes and recovery

1. Isopropanol was added in each tube to make the final volume to 40 ml and vortexed.
2. The beads were pelleted in centrifuge at 1000 xg for 5 min and the supernatant was carefully removed.
3. To the tubes, 35 ml of enhancing fluid XT was added and vortexed well to resuspend the beads.
4. The tubes were centrifuged as in above step and the supernatant was discarded.
5. To the tubes, 35 ml of Isopropanol was added and vortexed well.
6. The tubes were centrifuged and the supernatant was discarded.
7. Step 5-6 were repeated one more time.
8. To the tubes, 35 ml of absolute ethanol was added and vortexed well.
9. The tubes were centrifuged and the supernatant was discarded.
10. To the tubes, 35 ml of enhancing fluid XT was added and vortexed well.
11. The supernatant was discarded by approximately leaving 2 ml of enhancing fluid XT.
12. The DNA bead suspension was transferred using a 1000 µL pipette in two 1.7 ml tubes for each emulsion cup.
13. The tubes were centrifuged at 10,000 xg for 15 sec and the supernatant was discarded.
14. Repeat the above two steps until the entire DNA bead suspension were transferred.
15. To rinse each of the 50 ml collection tubes, 600 µl of enhancing fluid XT was used and it was transferred to the 1.7 ml tubes. Spin-rotate-spin was carried out and the supernatant was decanted.
16. Each pellet bead was rinsed twice with 1 ml of enhancing XT. Spin-rotate-spin was carried out and the supernatant was decanted.
17. To each bead pellet, 1 ml of enhancing fluid XT was added and vortexed.

Preparation for DNA library bead enrichment

1. The heating dry block was set at 65° C.
2. The melt solution was prepared by mixing 125 µl of NaOH (10N) in 9.875 ml of molecular biology grade water.

3. The tubes were spin-rotate-spin and the supernatant was decanted.

4. Per tube of beads, 1 ml of melt solution was added and vortexed.

5. The tubes were incubated for 2 min at room temperature.

6. Spin-rotate-spin was carried out and the supernatant was discarded.

7. Repeated the step 4-6 one more time.

8. Per tube of beads, 1 ml of annealing buffer XT was added and vortexed.

9. Spin-rotate-spin was carried out and the supernatant was discarded.

10. Repeated step 8-9 one more time.

11. Per tube, 45 µl of annealing buffer XT and 25 µl of enrichment primer were added per tube, and vortexed.

12. The tubes were placed in the heat block at 65º C for 5 min and immediately cooled on ice for 2 min.

13. Per tube, 800 µl of enhancing fluid XT was added and vortexed.

14. Spin-rotate-spin was performed and the supernatant was discarded.

15. Per tube, 1 ml of enhancing fluid XT was added and vortexed.

16. Supernatant was discarded.

17. Repeated step 15-16 two more times.

18. Per tube, 800 µl of enhancing fluid XT was added and vortexed.

✓ Preparation of enrichment beads

1. The tube of brown enrichment beads was vortexed for 1 min to resuspend its contents completely.

2. Of the total volume of emPCR, 160 µL was used and the rest of the contents were stored at +2 to +8° C.

3. The enrichment beads were pelleted by using a MPC.

4. The supernatant was decanted and care was taken that the enrichment beads were not drawn along with the supernatant.

5. To the tube, 1 ml of enhancing fluid XT was added and vortexed.

6. The enrichment beads were pelleted using MPC.
7. The supernatant was discarded.
8. Repeated the above three steps one more time.
9. The tubes were removed from the MPC after the supernatant was discarded.
10. For 2 emulsion cups 320 µL (160 µL for 1 emulsion cup) of enhancing fluid XT was added and vortexed.

**Enrichment of the DNA carrying beads**

1. To each amplified DNA beads, 80 µL of washed enrichment beads was added and vortexed.
2. The tubes were rotated on the rotator at room temperature (+15 to +25° C) for 5 min.
3. The tubes were placed in the MPC to allow the beads to pellet.
4. The supernatant was decanted carefully using a 1000 µL pipette. Care was taken that the beads were not collected along with the supernatant.
5. The beads were washed with enhancing fluid XT until there are no visible beads remaining in the supernatant.
6. The washing step was repeated 6 to 10 times until the white DNA beads are no longer aspirated.

**Collection of enriched DNA beads**

1. The tubes of enriched beads were removed from the MPC and each bead pellet was resuspended in 700 µL of melt solution.
2. The tubes were vortexed for 5 sec and were placed in the MPC till the brown beads have been pelleted.
3. The supernatant containing the enriched beads were transferred into a single 1.7 ml collection tube for each sample.
4. The 1.7 ml collection tubes were spin-rotated-spin and the supernatants were discarded.
5. Again 700 µL of melt solution was added to the enrichment tubes.
6. The tubes were vortexed for 5 sec and placed in the MPC for few minutes to allow the brown beads to pellet.
7. The supernatant containing the enriched DNA beads were transferred to the same collection tubes.

8. The enrichment tubes were then discarded.

9. The 1.7 ml collection tubes were spin-rotated-spin and the supernatant was discarded.

10. Per collection tube, 1 ml of annealing buffer XT was added and vortexed for 5 sec.

11. Spin-rotate-spin was done and the supernatant was discarded.

12. The above two steps were repeated twice.

13. Each bead pellet was then resuspended in 200 µL of annealing buffer XT.

✓ Sequencing primer annealing

1. To each collection tube, 50 µL of sequencing primer was added and vortexed.

2. The collection tubes were placed in the heat block for 5 min at 65° C and then promptly placed on ice.

3. Per collection tube, 800 µL of annealing buffer XT was added and vortexed for 5 sec. Spin-rotate-spin and the supernatant was discarded.

4. The above step was repeated two times with 1 ml annealing buffer XT.

5. To each pellet, 1 ml of annealing buffer XT was added and vortexed.

6. The % bead enrichment was calculated by the following formula:

\[
% \text{Bead Enrichment} = \frac{\text{Number of enriched beads}}{35 \times 10^6 \text{ beads/cup}} \times 100
\]

7. The beads were stored at +2 to +8° C.

D. PYROSEQUENCING

✓ Pre wash run

To ensure optimal performance, it is important to thoroughly rinse the Genome Sequence FLX Instrument before initiating each sequencing Run. Under normal conditions of continuous operation, the instrument is kept running after a run completed. Therefore, the Pico Titer Plate (PTP) device from the previous run should still be in
place in the instrument’s PTP cartridge, within the camera door. If there is no PTP device on the cartridge, a PTP device must be installed before proceeding with the prewash. Next, a pre-wash is performed to flush residual reagents, remaining from the previous Run, out of the fluidics system. Pre-wash is performed by discarding the spent reagents and cleaning the reagents cassette.

- Open the exterior fluidics door and raise the sipper manifold completely.
- Slide the reagents cassette out (Fig. 3.2).

*Figure 3.2: Removing the reagents cassette (from the previous run).*

- The fluid remaining in the container was discarded and the spent bottles and tubes were thrown away.
- With warm tap water the empty reagent cassette was rinsed thoroughly.
- Used sipper tubes were removed and discarded.
- The sipper tubes were replaced and gloves were changed to avoid contamination of other components.
- The pre wash cassette was prepared by placing the GS FLX Pre-wash tube holder on top of the reagents cassette and 11 small tubes and 4 large tubes were inserted in the cassette (Fig. 3.3).
- The tubes were filled with prewash buffer and the cassette was placed in the instrument. The fluidics door was then closed.
**Figure 3.3:** The Pre-wash cassette; loading before a pre-wash.

- **Pre-wash launch**
  1. In the Global action area, start button was selected which opens the run wizard’s first window.
  2. The procedure “pre-wash” was chosen and the “next” button leads to the run wizard’s second window. “Start Pre-wash” (Fig. 3.4).

- **Preparation of bead buffer**
  1. To 200 ml bottle of pre-chilled titanium bead buffer, 1.2 ml of titanium supplement CB was added.
  2. Re-capped and gently mixed by inverting the bottle.

**Figure 3.4:** The Run Wizard’s first window (Choose a procedure), with the Pre-wash option selected.
3. The tube of Apyrase enzyme was removed from the sequencing kit and thawed.
4. The components were kept on ice.
5. To the bottle of titanium bead buffer plus the titanium supplement CB, 34 µl of Apyrase solution was added.
6. Re-capped and labeled as “bead buffer 2”.
7. Mixed and placed on ice.

✓ Preparation of PTP and Bead Deposition Devices (BDD)
1. The PTP device was retrieved from the shipping tray.
2. The PTP device was then completely submerged in the BB2 for 10 min at room temperature.
3. The remaining BB2 was kept on ice.
4. The bead loading cassette was washed by gently shaking the gasket and sealed for 30 sec in sparkleen solution or nanopure water.
5. Rinsed thoroughly with nanopure water and dried on a paper towel.
6. The BDD was washed with a soft bristle brush and sparkleen solution.
7. Rinsed thoroughly and the device was dried on a paper towel.

✓ Preparation of different beads
   The Genome Sequencer FLX System contains 4 kinds of microparticles (beads), each type of bead must undergo a specific preparation procedure, as described below (Table 3.9).

✓ Preparation of packing beads
1. The packing beads were washed three times in 1 ml of BB2, centrifuged at 10,000 xg for 5 min.
2. After the third wash, 550µl of BB2 was added per tube.
3. Resuspended and placed on ice.
Table 3.9: Four types of beads used in the GS FLX titanium system chemistry.

<table>
<thead>
<tr>
<th>Beads</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Beads</td>
<td>DNA Beads carry the DNA library to be sequenced (sample). These samples were prepared using the appropriate Genome Sequencer System procedures and kits. Before use, the samples DNA Beads were spiked with Control DNA Beads, which serve as an internal control for the sequencing reaction.</td>
</tr>
<tr>
<td>Enzyme Beads</td>
<td>Enzyme Beads carry the immobilized enzyme components of the chemiluminescence system (Sulfurylase and Luciferase).</td>
</tr>
<tr>
<td>PPIase Beads</td>
<td>PPIase Beads scavenge inorganic pyrophosphate (PPI) to reduce well-to-well crosstalk and interference during each nucleotide flow, as well as residual background noise after and between flows.</td>
</tr>
<tr>
<td>Packing Beads</td>
<td>Packing Beads stabilize and maintain all the immobilized components of the system within the wells of the PTP device, throughout the sequencing run.</td>
</tr>
</tbody>
</table>

✓ Preparation of DNA beads (sample and control)

1. The number of sample DNA beads required was determined as shown in Table 3.10 (column 3).
2. The volume of DNA library beads to be sequenced based on the number of beads needed and the concentration of the library, in beads/µl was calculated.
3. The DNA library beads were vortexed and appropriate amounts of beads were transferred into clean tubes of appropriate size.
4. Appropriate volume of control DNA beads suspension to each DNA library bead tube was added (column 4).
5. The beads were centrifuged for 1 min at 10,000 xg. Rotated 180° and centrifuged again for 1 min.
6. The volume of supernatant to be removed that would leave the volume indicated in column 5 was calculated. This volume will vary depending on the volume of DNA library beads used, which in turn depends on the concentration of the DNA library.

7. The appropriate amount of supernatant was discarded carefully by not disturbing the bead pellet.

---

**Table 3.10: Preparation of sample and control beads.**

<table>
<thead>
<tr>
<th>Loading region size</th>
<th>PTP size</th>
<th>Number of DNA library beads to load per region</th>
<th>Volume of control DNA beads (µl)</th>
<th>Target final volume after centrifugation (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 70×75mm</td>
<td>70,000(×8)</td>
<td>10(×4)</td>
<td>30(×4)</td>
<td></td>
</tr>
</tbody>
</table>

---

**✓ DNA Bead Incubation Mix (DBIM) preparation**

The DBIM was prepared separately in a 15 ml tube as shown in the Table 3.11. Vortexed gently to ensure that the polymerase co-factor and DNA polymerase are thoroughly mixed.

**Table 3.11: Preparation of the DNA Bead Incubation Mix.**

<table>
<thead>
<tr>
<th>Loading region size</th>
<th>PTP size</th>
<th>BB2 (µl)</th>
<th>Polymerase cofactor (µl)</th>
<th>DNA Polymerase (µl)</th>
<th>Total volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sizes 70×75mm</td>
<td>1570</td>
<td>150</td>
<td>300</td>
<td></td>
<td>2020</td>
</tr>
</tbody>
</table>

---

1. The DBIM mix was added to each tube of DNA beads as per Table 3.12. Vortexed after addition.

2. The samples were incubated at room temperature for 15 min on the lab rotator.
Table 3.12: Dilution of DNA beads in DNA Bead Incubation Mix.

<table>
<thead>
<tr>
<th>Loading region size</th>
<th>PTP size</th>
<th>DNA beads (µl)</th>
<th>DBIM (µl)</th>
<th>Total volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>70x75mm</td>
<td>30 (×4)</td>
<td>320 (×4)</td>
<td>350 (×4)</td>
</tr>
</tbody>
</table>

✓ Preparation of Enzyme beads and PPiase beads (bead layer 1, 3 and 4)

Both the beads were prepared simultaneously and care was taken that the pipette tips were changed to ensure no cross contamination.

1. The enzyme as well as the PPiase beads were vortexed and pelleted on the MPC for 30 sec. The tubes were inverted and allowed to pellet again for another 30 sec.
2. The supernatants were decanted and the tubes were removed from the MPC.
3. The enzyme and PPiase beads were washed thrice with 1 ml of BB2. The tubes were vortexed and washed.
4. After the third wash, 1 ml of BB2 for enzyme beads and 500 µl of BB2 for PPiase beads were added, vortexed and placed on ice.
5. The beads for layers 1, 3 and 4 were prepared separately in labeled 15 ml tubes. It was prepared by diluting the enzyme and PPiase beads according to the Table 3.13.
6. The tubes were vortexed to get a uniform suspension before transferring.

Table 3.13: Dilution of the Enzyme and PPiase beads for the bead layers 1, 3, and 4.

<table>
<thead>
<tr>
<th>Bead layer</th>
<th>Kit</th>
<th>BB2 (µl)</th>
<th>Enzyme beads (µl)</th>
<th>PPiase beads (µl)</th>
<th>Total volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer 1</td>
<td>XLR 70</td>
<td>3250</td>
<td>550</td>
<td>----</td>
<td>3800</td>
</tr>
<tr>
<td>Layer 3</td>
<td>XLR 70</td>
<td>2500</td>
<td>1300</td>
<td>----</td>
<td>3800</td>
</tr>
<tr>
<td>Layer 4</td>
<td>XLR 70</td>
<td>3340</td>
<td>----</td>
<td>460</td>
<td>3800</td>
</tr>
</tbody>
</table>
✓ **Combining the DNA and Packing beads**

1. After the completion of DNA beads incubation, appropriate volume of washed packing beads and BB2 were transferred to the tubes containing the DNA beads according to the Table 3.14.
2. The DNA and packing beads were vortexed and rotated for 5 min at room temperature.
3. The unused packing beads were discarded.

<table>
<thead>
<tr>
<th>DNA beads (µl)</th>
<th>Packing beads (µl)</th>
<th>BB2 (µl)</th>
<th>Total volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>350 (×4)</td>
<td>100 (×4)</td>
<td>210 (×4)</td>
<td>660 (×4)</td>
</tr>
</tbody>
</table>

✓ **Assembly of the BDD with the PTP device and Bead loading gasket**

1. The PTP device was removed from the BB2 bath. The excess BB2 was removed.
2. The back of the PTP device was wiped dry with kimwipe carefully to ensure wells do not get dried.
3. The PTP device was placed on to the BDD base, aligning the notched corners of the PTP device and the BDD base (Fig. 3.5).
4. The washed, dried bead loading gasket was secured to the base of BDD by laying it on to the top of the PTP device.
5. The notched corners of the bead loading gasket and the BDD base were then aligned.
6. The BDD was carefully placed such that the port holes and air vents lined up with the loading regions defined by the gasket.
7. The top of the BDD were pressed down and the two latches from the BDD base were rotated into the groves securing the setup.

✓ **Deposition of the four layers of beads on the PTP device**

1. The beads were deposited onto the PTP device by injecting the bead suspension through the holes of the assembled BDD.
2. The beads were settled onto the bottom of the plate by applying centrifugal force.

3. The above process was repeated for each of the four layers.
   - Bead layer 1: Enzyme beads pre-layer
   - Bead layer 2: DNA and Packing beads layer
   - Bead layer 3: Enzyme beads post-layer
   - Bead layer 4: PPiase bead layer.

Figure 3.5: Assembly of the Bead Deposition Device.

✓ Deposition of bead layer 1: Enzyme beads pre-layer
   1. The bead suspension was vortexed for layer 1 for 5 sec to obtain a homogenous solution.
   2. Six hundred sixty micro liter of bead suspension was drawn with the help of a pipette.
   3. The beads were resuspended completely. The bead suspension was loaded in the first region of the PTP device through the port hole on the BDD top (Fig. 3.6).
   4. Care was taken that a smooth dispersion and even distribution over the entire region was maintained.
   5. The above steps were repeated for the other loading regions of the PTP device.
6. The assembled BDD and the counter weight BDD were placed into the centrifuge swinging baskets and centrifuged for 5 min at 1600 xg.

✓ **Deposition of bead layer 2: DNA and packing beads**

1. The BDD was removed from the centrifuge and the BDD port seals were discarded.
2. With a pipette, the supernatants were removed through the port holes on the BDD top.
3. The tubes having bead suspension of layer 2 were removed and briefly spun to concentrate the materials.
4. Six hundred sixty micro liter of bead suspension was taken.
5. The beads were resuspended by pipetting up and down and then were loaded onto the first region of the PTP device through the port hole.
6. The same procedure was carried out for all the other loading regions.
7. The loading ports were sealed.

The loaded PTP device was centrifuged for 10 min at 1600 xg.

*Figure 3.6: Filling the loading regions of the assembled BDD.*
✓ **Deposition of Bead Layer 3: The Enzyme Beads Post-Layer**

1. BDD from the centrifuge was gently taken out and BDD port seals that cover the loading ports and air vents were discarded.
2. Using a pipette, the supernatant from the centrifuged bead layer 2 was gently drawn out, through the port holes on the BDD top.
3. The bead layer 2 supernatant was collected separately, for troubleshooting purposes in case the sequencing Run does not produce the expected number of reads.
4. The bead suspension for layer 3 was gently vortexed for 5 sec to obtain a uniform suspension.
5. Using a pipette and tip of the proper size, 660 µl of bead suspension for the loading regions was used.
6. Pipetted up and down three times to resuspend the beads completely and the bead suspension was loaded onto the first region of the PTP device, through the port hole on the BDD top.
7. Steps 4 to 6 were repeated for all loading regions of the PTP device.
8. The loading ports and the vent holes were covered with new BDD port seals.
9. The loaded PTP device in the BDD was centrifuged for 5 min at 1600 ×g.

✓ **Deposition of bead layer 4: PPIase beads**

1. BDD from the centrifuge was gently taken out and BDD port seals that cover the loading ports and air vents were discarded.
2. Using pipettor, the supernatant from the centrifuged bead layer 3 was gently drawn out, through the port holes on the BDD top.
3. The bead suspension for layer 4 was gently vortexed for 5 sec to obtain a uniform suspension.
4. Using a pipette and tip of the proper size, 660 µl of bead suspension for the loading regions was used.
5. Pipetted up and down three times to re-suspend the beads completely and the bead suspension was loaded onto the first region of the PTP device, through the port hole on the BDD top.
6. Steps 4 to 6 were repeated for all loading regions of the PTP device.
7. The loading ports and the vent holes were covered with new BDD port seals.
8. The loaded PTP device in the BDD was centrifuged for 5 min at 1600 xg.

✓ Removal of pre-wash cassette and cleaning the fluidics area deck
1. Exterior fluidics door was opened and the sipper manifold was raised completely to remove the pre-wash tubes and the GS FLX pre-wash tube holder from the reagents cassette.
2. The contents of the pre-wash tubes was emptied and discarded.
3. Outside surfaces of the reagents cassette was wiped completely with a paper towel.
4. The fluidics area deck (inside the instrument) was wiped with 50% ethanol and a paper towel, and allowed to air dry completely.

✓ Preparation and loading of sequencing reagents cassette
1. To each bottle of Titanium buffer CB, 6.6 ml of Titanium Supplement CB and 1000 µl of DTT were added.
2. The bottles were recapped and mixed by inverting it 2 to 3 times.
3. Sequencing reagents tray was placed to the right hand side of the reagents cassette (Fig. 3.7).
4. In a 1.7 ml tube, 5 µl of the PPiase reagent in 45 µl Inhibitor TW reagent was diluted and vortexed.
5. The tubes were inverted 20 times to uniformly mix the contents of all tubes and to make sure that there are no undissolved particulates in any of the reagents.
6. Supplemented sequencing reagents with Sodium chlorite, PPiase, Apyrase, and dATP as shown in the Table 3.15.
### Table 3.15: Supplementing sequencing reagents with Sodium chlorite, PPiase, Apyrase, and dATP.

<table>
<thead>
<tr>
<th>Sodium Chlorite tablet to add to the tube of “Post run wash”</th>
<th>Volume of diluted PPiase to add to “Inhibitor TW”</th>
<th>Volume of Apyrase to add to “Buffer for Apyrase”</th>
<th>Volume of dATP to add to “Buffer for dATP (A)”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 tablet</td>
<td>13.2 µl</td>
<td>260 µl</td>
<td>3000 µl</td>
</tr>
</tbody>
</table>

**Figure 3.7:** Loading the reagents cassette, with the sequencing reagents tray in the proper orientation.

7. The Reagents cassette was loaded onto the instrument (Fig. 3.8).
8. The caps from all bottles and tubes were removed and the sipper was carefully lowered, and exterior fluidics door was closed gently.
Cleaning the PTP cartridge and the camera faceplate

1. The camera door was unlocked by clicking on Unlock button from the instrument tab and then opened by pulling the camera door gently using the handle (Fig. 3.9).

2. The spent PTP device from the PTP cartridge was removed gently by first pressing the PTP frame spring latch to lift the frame from the cartridge, and then the used PTP device was removed (Fig. 3.10).
3. The PTP frame was closed and then using a pair of plastic forceps, PTP cartridge seal from the PTP cartridge was removed.
4. Using Kimwipe with 50% ethanol, the surface of the cartridge was wiped to remove any bead and reagent residue.
5. Using a new Zeiss pre-moistened cleaning tissue, the camera faceplate was wiped gently and allowed to air dry completely.
6. Using Kimwipe with a 10% user-prepared solution of Tween-20, the surface of the PTP cartridge was wiped completely.

✓ **Loading and starting the run script**
1. Instrument computer was switched on; the GS Sequencer main window is displayed, with the Instrument tab active.
2. “Start button” in the global action area was clicked, which opens the Run Wizard’s first window: **Choose a procedure** (Fig. 3.11).
3. In the Run Wizard’s first window, the Sequencing Run option was selected, and the next button guides to the Run Wizard’s second window: **Enter ids and barcodes**.
4. The barcode of the PTP device to be used in the run was entered. The next button guides to the Run Wizard’s third window: **Enter Run name and Run Group.**

![Figure 3.11: The Run Wizard’s first window: Choose a procedure.](image1)

![Figure 3.12: The Run Wizard’s fourth window: Choose sequencing kit.](image2)
5. A specific, unique name for this run was entered. Run group list was then selected. Next button guides to the Run Wizard’s fourth window: **Choose sequencing kit** (Fig. 3.12).

6. The type of sequencing kit to be used in this run was selected.

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**Figure 3.13:** The Run Wizard’s fifth window: Choose Pico Titer Plate type.

**Figure 3.14:** Run Wizard’s sixth window: Choose number of cycles.
7. For the GS FLX Titanium chemistry, the only valid choice is XLR70Ti. This opens the Run Wizard’s fifth window: Choose Pico Titer Plate type (Fig. 3.13).

8. The bead loading gasket to be used in this run was selected, and click next this opens the Run Wizard’s sixth window: Choose number of cycles (Fig 3.14).

✔ Loading a PTP device into the cartridge

1. PTP was loaded into the PTP frame cartridge, then the PTP frame was closed and made sure it is properly secured by the latch

2. The back of the PTP device was wiped with a Kimwipe, and the camera door was closed gently. The “Start” button in the Run wizards was clicked.

![Image of instrument tab](image_url)

**Figure 3.15:** The Instrument tab of the GS Sequencer application main window during a sequencing run.

The progress of the sequencing run can be monitored periodically. The instrument status and the data images captured by the camera can be viewed (Fig. 3.15). After the successful completion of the sequencing run, a message appears in the status bar of the application window.

All the images were transferred to the cluster attached with the GS FLX instrument. Using ‘GS Run Browser’ software, all the images were converted into sequence reads by the process called image processing.
3.2.8 Analysis of sequence reads by Metagenome Rapid Annotation using Subsystem Technology (MG-RAST)

1. The MG-RAST was used to analyze the metagenomic datasets of the present study.
2. The raw reads were subjected to MG-RAST and based on the homology they get aligned to sequences in the subsystem and are classified into metabolic and phylogenetic profiles.
3. Metabolic profile (using SEED subsystem technology) provides with an output based on homology comparison.
4. Phylogenetic profile (using RDP, SEED, SILVA LSU, SILVA SSU and GREENGENES Databases) provides with taxonomical distribution obtained based on homology of the sequences with each database and are given out respectively.
5. In addition, comparative analysis was also provided via the tools such as Heatmap, Recruitment plot and KEGG map, each gives an output on comparing all the 3 samples sequences with the entire database.
6. The domain distribution data was calculated for the metagenomes of Kankrej, Gir and crossbred cattle and these were compared to SEED (The cooperative effort on the development of the comparative genomics environment: www.theseed.org) using a maximum e-value of 0.001, a minimum identity of 85 per cent and a minimum alignment length of 20 bp.
7. All the outputs were obtained and converted into a graphical representation.

3.2.9 Isolation of milk somatic cells

Aliquots of twenty five ml milk were used to determine SCC using an electronic somatic cell counter. The remainder of the milk was centrifuged in 50 ml tubes at 1000 xg for 15 min at room temperature. Fat layer and the supernatant were discarded and the cell pellets were washed twice in 50 ml phosphate buffered saline.
3.2.10 RNA extraction

Total RNA from the milk somatic cell pellets were extracted by TRI Reagent®, following manufacturer’s instructions. In brief,

1. The pellet suspension (150 µl) was taken in a 2.0 ml centrifuge tube, to which 750 µl of TRI Reagent® (Sigma, USA) was added and mixed by passing the suspension several times through a pipette tip.
2. The homogenate was stored for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes.
3. The lysate was supplemented with 200 µl chloroform per 750 µl of TRI Reagent®, and the centrifuge tube was tightly capped and then shaken vigorously for 15 sec. The resulting mixture was kept at room temperature for 15 min.
4. The mixture was then centrifuged at 12,000 xg for 15 min at 4°C, to separate it into a lower red phenol-chloroform phase, inter phase and the colorless upper aqueous phase.
5. The aqueous phase was carefully transferred to a fresh tube and RNA was precipitated from the aqueous phase by mixing with isopropanol at the rate of 600 µl of isopropanol per 750 µl of TRI Reagent® used for the initial homogenization.
6. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 xg for 10 min at 4°C, to obtain RNA precipitate in the form of a gel like or white pellet at the side and bottom of the tube.
7. The RNA pellet was washed twice with one ml of 75% ethanol by vortexing and subsequent centrifugation at 12,000 xg for 15 min at 4°C. In cases, the RNA pellet got accumulated at the side of the tube with a tendency to float; ethanol wash was performed at 12,000 xg.
8. The RNA pellet was air dried for 15-30 min. A pellet was dissolved in 30 µl of DEPC treated/ RNase free water and stored at -40°C for further use.
3.2.10.1 Quantitation of RNA

RNA was quantified by spectrophotometric analysis using the convention that one absorbance unit at 260 nm wavelength equals 40 µg RNA per ml. The ultra violet absorbance was checked at 260 and 280 nm for determination of RNA concentration and purity. Purity of RNA was judged on the basis of optical density ratio at 260:280 nm. The samples with acceptable purity (i.e. ratio 1.65-2.0) were quantified. RNA integrity was verified by 1% denaturing agarose gel electrophoresis and Bioanalyzer.

3.2.10.2 Qualitative analysis of RNA by denaturing agarose gel electrophoresis

✓ Gel running buffer

250 ml 10X MOPS gel running buffer was prepared by adding 20.93g N-morpholino propane sulfonic acid (MOPS) to 200 ml DEPC treated water. It was then followed by addition of 2.06g sodium acetate. The mixture was stirred until it was completely dissolved. Then 5 ml DEPC treated 0.5M EDTA added followed by adjusting to pH 7.0 with 10N NaOH. Finally the volume was made to 250 ml with DEPC treated water.

✓ RNA loading buffer

2.5 ml pure glycerol was added to 2.5 ml DEPC treated water. Then 0.25 mg ethidium bromide was added, followed by addition of 0.02 mg bromophenol blue. Finally 10µL of 0.5 M EDTA was added.

✓ Denaturing agarose gel electrophoresis

For preparing 60 ml 1% HCHO agarose gel, 0.6 gm agarose was taken and to it 40 ml DEPC treated water added. It was then placed in microwave to dissolve the agarose. Solution was cooled to 60 °C and 6 ml 10x MPOS was added to it. Ten ml HCHO (40%) was added at last and final volume was made 60 ml. It was then poured in gel caster and once the gel got solidified, it was transferred to electrophoresis tank filled with 1X gel running buffer. The level of buffer was kept at least 1 cm above the gel.
Table 3.16: Sample preparation for denaturing agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (up to 1-2 µg)</td>
<td>2 µL</td>
</tr>
<tr>
<td>10 X MOPS</td>
<td>2 µL</td>
</tr>
<tr>
<td>HCHO (40%)</td>
<td>3.5 µL</td>
</tr>
<tr>
<td>Formamide</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

The mixture shown in Table 3.16 was incubated in water bath at 65° C for 15 min and then chilled on ice for 5 min. To this 2 µL of HCHO gel loading dye was added. The wells were carefully charged with RNA mixed in gel loading dye. The electrophoresis was carried out in submarine gel electrophoresis tank at 80 Volts. On completion of electrophoresis, the gel was visualized under UV trans-illuminator and documented by gel documentation system (Syngene, U.K.).

3.2.10.3 Qualitative and quantitative analysis of RNA using Agilent 2100 Bioanalyzer

✓ Decontaminating the Electrodes
1. The 350 µl RNaseZAP was filled in one of the wells of an electrode cleaner.
2. The lid was opened and electrode cleaner was placed in the Agilent 2100 bioanalyzer, the lid was closed and after 1 min it was removed.
3. Then 350 µl RNase-free water was slowly filled in one of the wells of another electrode cleaner, the lid was closed and after 10 sec it was removed.
4. The lid was kept open for 10 sec to evaporate water from the electrode.

✓ Preparing the Gel
1. All reagents were allowed to equilibrate to room temperature for 30 min before use.
2. Five hundred fifty µl of Agilent RNA 6000 Nano gel matrix was placed into the top receptacle of a spin filter and the spin filter was placed in a microcentrifuge and spun for 10 min at 1500 xg.
3. Sixty five µl filtered gel was aliquoted into 0.5 ml RNase-free microfuge tubes that were included in the kit. The aliquots were stored at 4° C for future use.

✓ Preparing the Gel-Dye Mix
   1. All reagents were allowed to equilibrate to room temperature for 30 min before use. The dye concentrate vial was protected from light.
   2. RNA 6000 Nano dye concentrate was vortexed for 10 sec and spun down and 1 µl of RNA 6000 Nano dye concentrate was added.
   3. The tube was capped, vortexed and spun for 10 min at 13000 xg.

✓ Loading the Gel-Dye Mix
   1. The gel-dye mix was allowed to equilibrate to room temperature for 30 min before use and the gel-dye mix was protected from light during this time.
   2. A new RNA Nano chip was taken out of its sealed bag and placed on the chip priming station.
   3. Nine µl of the gel-dye mix was pipetted at the bottom of the well marked G and the gel-dye mix was dispensed.
   4. Before chip priming station was closed, it was made sure that the plungers were positioned at 1 ml. The lock of the latch clicked when the priming station was closed correctly.
   5. The plunger of the syringe was pressed down until it was held by the clip.
   6. After waiting for exactly 30 sec, the plunger was released with the clip release mechanism, wait until plunger moved back at least to the 0.3 ml mark than after waiting for 5 sec, the plunger was slowly pulled back to the 1 ml position.
   7. Nine µl of the gel-dye mix was pipetted in each of the wells marked C.

✓ Loading RNA 6000 Nano marker
   Pipette 5 µl of the RNA 6000 Nano marker into the well marked with the ladder symbol and each of the 12 sample wells.
✓ **Loading the ladder and samples**

1. Before use, ladder aliquots were thawed and kept on ice.
2. To minimize secondary structure, the samples were heat denatured (70° C, 2 min) before loading on the chip.
3. One μl of the RNA ladder was pipetted into the well marked with the ladder symbol and 1μl of each sample was pipetted into each of the 12 sample wells.
4. The chip was placed horizontally in the adapter of the IKA vortex mixer without damaging the buldge that fixed the chip during vortexing. Any liquid spill at the top of the chip was carefully removed with a tissue.
5. It was vortexed for 60 sec at 2400 rpm.

✓ **Inserting a chip in the Agilent 2100 Bioanalyzer**

1. It was checked that the electrode cartridge was inserted properly and the chip selector was in position.
2. The chip was placed carefully into the receptacle as the chip fits one direction.
3. The lid was carefully closed.

✓ **Starting the chip run**

1. In the instrument context, the eukaryote total RNA Nano Series II assay was selected from the assay menu.
2. At the time of feeding of details of samples, the results file storage location and the number of samples that were to be analyzed was customized.
3. Run was started by applying current.
4. The raw signals trace was reviewed by returning to the Instrument context.
5. After the chip run is finished, the chip was removed from the receptacle of the Bioanalyzer and disposed according to good laboratory practices.

### 3.2.11 DNase treatment

All the samples were then treated with DNase I to remove any possible DNA contamination. Mixture was prepared as shown in Table 3.17 and incubated at 37° C for 60 min. followed by 65° C for 10 min.
Table 3.17: Components used for DNase treatment.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µl)</th>
<th>Additional Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>25.0</td>
<td>Incubated at 37° C for 60 min.</td>
</tr>
<tr>
<td>DNase I (Fermentas)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>DNase I buffer</td>
<td>6.0</td>
<td>followed by 65° C for 10 min</td>
</tr>
<tr>
<td>DEPC treated water</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

3.2.12 Reverse Transcription/cDNA preparation

RevertAid™ cDNA synthesis kit having following contents (Table 3.18).

Table 3.18: Reaction mixture for reverse transcription.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer RT</td>
<td>2.0 µL</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs mix (25 µM each)</td>
<td>2.0 µL</td>
<td>50.0 µM each</td>
</tr>
<tr>
<td>Ribonuclease inhibitor</td>
<td>1.0 (10U/µL)</td>
<td>10.0U</td>
</tr>
<tr>
<td>RT enzyme</td>
<td>1.0 µL</td>
<td>4.0 U</td>
</tr>
<tr>
<td>Random hexamer (2µM)</td>
<td>2.0 µL</td>
<td>4.0 µM</td>
</tr>
<tr>
<td>RNA</td>
<td>2.0 µg</td>
<td>2000 ng</td>
</tr>
<tr>
<td>RNase free water</td>
<td>Upto 20 µL</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20.0 µL</td>
<td></td>
</tr>
</tbody>
</table>

1. Template RNA obtained from the samples was thawed on ice. The primer solutions, 10X buffer RT, dNTPs mix and RNase-free water were thawed at room temperature and then stored on ice immediately.

2. A fresh reaction mixture as detailed above was prepared on ice, thoroughly mixed by vortexing for five sec and spun briefly to collect residual liquid from the sides of the tube and was stored on ice.

3. The template RNA (2 µg) was added to the individual tubes containing the reaction mixture and mixed thoroughly and carefully by vortexing for 5 sec. It was then centrifuged briefly to collect residual liquid from the sides of the tube.
4. The tubes were incubated in a thermal cycler for 10 min at 25°C followed by 60 min at 42°C for cDNA formation.

5. The cDNA samples were heated at 70°C for 10 min to inactivate the enzyme. The cDNA formed was stored at -40°C until used for PCR.

3.2.13 Real Time quantitative Polymerase Chain Reaction

Expression of cytokines mRNA was quantified by Real Time PCR and analyzed using Applied Biosystems 7500 Fast SDS software. All PCR reactions were performed in optical 96 well plates. The amplification was carried out in a final reaction volume of 15 µl containing 1X Quanti Tect SYBR Green PCR master mix, 5 pmol of each gene specific primer and 1µl of cDNA template (Table 3.19). A two step PCR protocol was designed as initial denaturation at 95°C 10 min, and 40 cycles of 95°C 15sec, 60°C for 1 min. For each sample, a dissociation curve was generated after completion of amplification to determine the specificity of PCR reaction. Fluorescence signals were measured once in each cycle at the end of the extension step. For each gene of interest, negative control was used. Negative control were the samples in which cDNA was not added. For each sample a dissociation curve was generated after completion of amplification and analyzed in comparison to negative control, to determine the specificity of PCR reaction. For improved visualization of melting temperature, melting peaks were derived by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT vs T).

Table 3.19: Composition of reaction mix.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-RNase free water</td>
<td>4.50 µl</td>
<td>--</td>
</tr>
<tr>
<td>2X PCR master mix</td>
<td>7.50 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Forward Primer (5 pmole/µl)</td>
<td>1.00 µl</td>
<td>5 pmole</td>
</tr>
<tr>
<td>Reverse Primer (5 pmole/µl)</td>
<td>1.00 µl</td>
<td>5 pmole</td>
</tr>
<tr>
<td>cDNA Template</td>
<td>1.00 µl</td>
<td>--</td>
</tr>
<tr>
<td>Total</td>
<td>15.00 µl</td>
<td></td>
</tr>
</tbody>
</table>
Visualization of PCR Products

To confirm the targeted PCR amplification, five µl of PCR product for each amplified target tube was mixed with 1 µl of 6X gel loading dye and electrophoresed along with 100 bp DNA molecular weight marker (O’RangeRuler™, Fermentas) on 2.0% agarose gel containing ethidium bromide (0.5 µg/ml) at constant 80 V for 30 min in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, UK).

Relative quantification

Cytokine quantification was achieved using the Ct (Cycle threshold) comparative method, and is expressed as “n-fold up regulation of cytokine transcription” in relation to a calibrator which is represented by the smallest signal detectable for that specific cytokine. The expression of each gene was analyzed using the relative quantification method described by Pfaffl (2001). For relative quantitation by the comparative CT method, values are expressed relative to a reference sample, called the calibrator (Samples from healthy cattle). The CT for the target gene and the CT for the internal control were determined for each sample and the calibrator. The expression of selected cytokine genes was calibrated by that of the reference gene, bovine GAPDH and β Actin, at each time point and converted to the relative expression ratio (fold of induction), where,

\[
\text{Fold of Induction} = \frac{E \text{ (target)} \cdot \Delta CP \text{ target (Mean control- Mean Samples)}}{E \text{ (reference)} \cdot \Delta CP \text{ reference (Mean control- Mean Samples)}}
\]

Where,

- E (target) is real time efficiency of the target gene transcript
- E (reference) is the real time PCR efficiency of reference gene transcript
- ΔCP target is the CP deviation of control-sample of target gene transcript
- ΔCP reference is the CP deviation of control-sample of reference gene transcript
Determination of amplification efficiency

For the comparative CT method (ΔΔCT method) to be valid, the amplification efficiencies of the target and the endogenous control must be approximately equal. To determine the amplification efficiencies of GAPDH and the cytokine cDNAs, two methods were employed-

I. **Standard curve method** - Five dilutions of cDNA preparations were amplified in duplicate. The resulting CT values plotted against the dilution of input total RNA and the regression line was calculated. The slopes of the regression line for each cytokine gene were input in the REST software version 384 and efficiencies calculated. The amplification efficiency \((E)\) was calculated based on the slope, where \(E = 10^{[-1/\text{slope}]}\).

II. **Increase in fluorescence** - The amplification efficiency of every individual reaction was carried out by method of amplification efficiency estimation based on absolute fluorescence increase in single reaction kinetics data as suggested by Liu and Saint (2002). In this method, the portion of the data array believed to be exponentially behaving was taken, log transformed and plotted. The slope of the regression line was considered the amplification efficiency. The amplification fluorescence raw data were available by data export from ABI Prism Sequence Detection System software (Livak et al., 2001) and the efficiency estimation was based on these data. Once the beginning and the end of the exponential phase are defined, the exponential model is plotted over these data (equation): 

\[ f = \gamma 0 + \alpha \times E_n \]

The fluorescence value is represented by \(f\), \(\gamma 0\) is the upward shift due to ground fluorescence, \(\alpha\) is the fluorescence due to the nucleic acid input, \(n\) is the cycle number and \(E\) is the efficiency of amplification in the early exponential phase of Real Time PCR.

3.2.14 Therapeutic studies

✓ **Treatment of subclinical intramammary infection**

Therapeutic drug trials were conducted to evaluate the efficacy of herbal product *Mastilep* Topical Herbal Gel (Dabur Ayurved Ltd., Ghaziabad, India). Each 10gm of
Mastilep contained *Eucalyptus globulus*- 0.20gm, *Glycyrrhiza glabra*- 0.20gm, *Curcuma longa*- 0.04gm, *Cedrus deodara*- 1.00gm, *Paedaria foetida*- 0.04gm and Sulphur- 1.00gm in a gel base. Approximately 5gm herbal gel was topically applied over the affected udder quarter by gentle massaging including the teats, after the morning and evening milking for five consecutive days.

**Post-treatment observation and laboratory evaluations**

After application of the drug for specified period, quarter milk samples were collected and their microbiological culture examination, electronic somatic cell count and expressions profiles of different cytokines i.e. IL-6, IL-8, IL-12, GM-CSF, IFN–γ and TNF-α were carried out on day 0, and 5 and 21 post-last treatment to assess the efficacy of tested drugs.

**3.2.15 Statistical analysis**

The data were analyzed using Statistical Analysis System (SAS) v4.1 (SAS institute, Inc., USA) software. Least square means of transcriptional activity of targeted cytokines, SCC and total bacterial count in the milk of Kankrej, Gir and crossbred cattle on day 0, and day 5 and 21 post-treatment were analyzed using two-factor analysis of variance (ANOVA) and Pearson's correlation analysis between and within cytokine transcriptional activity, total bacteria and SCC were adopted to study effect on various parameters used in the present investigation. The continuous by nominal/ordinal scatter plot with means diamonds were also plotted between cytokines transcriptional activity, SCC and total bacterial load using SAS software.