CHAPTER-IV

METHODOLOGY
Methodology:

4.1: Patient enrolment:
The present study was carried out at the Department of Biotechnology, Gauhati University in collaboration with Gynecology Department of Gauhati Medical College and Hospital in between Jan’ 2010 to June’ 2014. The study was undertaken after approval from the Institutional ethics committee of Gauhati University vides order no: GU/Ethical/2012/3991.

4.1.1. Sample collection:
Patient attending the Gynecology OPD of Gauhati Medical College and Hospital from June 2010 onwards up to June 2014 were evaluated clinically, and women who fulfilled the below mentioned selection criteria were selected and enrolled with informed consent.

4.1.1. A: Inclusion criteria:
Age group of 18-60 years showing at least any of the following risk factors:
- Early marriage.
- Early pregnancy (teenage pregnancy)
- Sexual activity at an early age.
- Multiparity
- Multiple sexual partners.
- Women with sexually transmitted disease (STD)
- Leucorrhea
- Irregular vaginal bleeding

4.1.1. B: Exclusion criteria:
- Patient’s with history of previous treatment for cervical pre-cancerous lesion.
- Patients who didn’t gave consent.
4.1.1. C: Procedure for selection of patients:
The selected patients after clinical examination underwent visual inspection with acetic acid (VIA) and visual inspection with lugols iodine (VILLI) test. Only VIA and or VILLI positive cases were selected for the study. They were called for cervical biopsy and blood collection for molecular examination. Patients’ case histories including age, marital status, gravida, parity, socio-economic status, personal hygiene etc. parameters were investigated and recorded.

**Basis of visual inspection with acetic acid (VIA):**
Acetic acid dissolves mucus, induces intracellular dehydration and causes coagulation of protein. So, in dysplasia and cancer cervix cells with increased nuclear cytoplasm ratio (N/C) ratio, nuclear density, and chromosomal aneuploidy become opaque.

**Basis of visual inspection with lugol’s iodine (VILLI):**
Normal cell of the cervix containing glycogen take up iodine and turn mahogany brown and abnormal area of the cervix remains unstained.

**Supplies and equipments required for VIA and VILLI:**
- Examination gloves
- Examination table
- Vaginal Speculum (preferably Cusco’s or Sims)
- Cotton tipped swabs
- Freshly prepared 5% acetic acid
- Lugol’s iodine solution
- Focusing light (with halogen bulb preferred)/torch/flashlight
- Rubber/plastic sheets
- Small bowl to hold acetic acid and lugol’s iodine
- A screen or confidential room to provide privacy
- VIA forms and Registers
Preparation of 5% acetic acid for VIA: Dissolve 5gms of glacial acetic acid in 95 ml of distilled water, shake it. It can be used for 7 days.

![Acetic acid preparation](image)

**Fig 4.1: Acetic acid preparation**

Preparation of lugol’s iodine for VILLI: Dissolve 10 gm potassium iodide in 100 ml of distilled water. Slowly add 5 gm iodine crystals, while shaking. Filter and store in a tightly Stoppard brown bottle. It can be stored for one (1) month.

Method of VIA test: - After clinical examination, cervix is washout with normal saline. 5% acetic acid solution is applied on the cervix and kept for 1 minute. After 1 minute changes in the cervix is noted.

Method of doing VILI: After doing VIA, Lugol’s iodine solution is applied over the cervix for 30 seconds and changes is noted.

VIA positive: Sharp, distinct, dense aceto-white lesion with definite raised or non-raised margins closer to the squamocolumner junction (SCJ) in the transformation zone (TZ) and not far away from the external os.

VILLI positive findings are-- Neoplastic area of cervix remains unstained.

Invasive: - Clinically visible ulcero-proliferative or cauliflower like growth in of the cervix

4.1.1. D: Study sample:

Cervical tissue biopsies from the both the cancerous region as well as the non- cancerous control area of the cervix, and whole blood (3ml) were collected from all the study subjects. A portion of the collected tissue samples were formalin fixed, paraffin embedded and was used for routine histopathological examination and immunohistochemistry based analysis. The tissue samples were collected either
in PBS or RNA later solution in vials and stored in -80°C or liquid nitrogen till further use. Retrospectively collected paraffin embedded biopsy cases from patients pre-cancerous lesions (CIN I-III) were also included for the present study for comparative analysis. Whole blood (2ml) was also collected from age matched voluntary female community controls (n=50), the plasma was separated, and stored at -20°C for cytokine analysis.

4.1.2: Extraction of total DNA from cervical cancer tissue sample:
DNA extraction was done from the collected cervical cancer tissue as well as adjacent non neoplastic tissue samples by the cell-lysis protocol. The DNA is then used of HPV detection and HPV genotyping using specific primers by PCR amplification. This extracted DNA samples are stored at 4°C till analysis is completed.

**Principle:**
Tissue lysis buffer is generally used for the cells and Proteinase K is used to bring up the denaturation of the protein. For purification the cellular extract is centrifuged at low speed to remove the debris that pellets out at the bottom of the tube. The supernatant is collected and treated with phenol that precipitates protein at the interface between the organic and aqueous
layers. Chloroform stabilizes the phenol-chloroform solution and prevents emulsification as proteins react with phenol. DNA remains intact during the degustation process.

**Chemicals:**

a) Tissue lysis buffer,
b) Proteinase K,
c) Nacl,
d) Isopropanol,
e) Ethanol and
f) TE buffer.

**Procedure:**

2 mm³ of cervical tissue was taken in a mortar and crushed in to powder with the help of liquid nitrogen by pestle

The powdered tissue was transferred to a fresh eppendorf tube and 800μl of TLB (tissue lysis buffer) and 9μl of Proteinase K (20μg/μl) was added to it.

The tube was taken kept for overnight incubation at 37°C or at 55°C for 2 hr

The tubes were next centrifuge @13000rpm for 10 minutes

The supernatant was transferred to a new tube and 180μl of 5M NaCl was added to it

The tube was centrifuge@ 13,000rpm for 5 minutes, and again transferred the supernatant to a fresh tube and added 500μl of isopropanol to it.

The components in the tube were mixed by swirling from 2-3 minutes and centrifuged @ 13000 rpm for 15 minutes

The pellet thus formed as precipitate was retained after discarding the supernatant
The pellet was washed by addition of 500 μl 70% ethanol and centrifuging @ 13000 rpm for 10 minutes.

The precipitate pellet was air dried after discarding the supernatant and re-suspended in TE buffer and stored for further use.

**Fig: 4.2: Flowchart explaining the steps involved in extraction of DNA from tissue.**

**4.1.3: Extraction of total RNA from tissue samples:**
The RNA was extracted from the cervical cancer tissue as well as adjacent non cancer tissue samples by the Trizol method and then stored at -20°C for cDNA preparation.

**Principle:**
The trizol method involves use of trizol reagent which maintains the integrity of the RNA while disrupting cell and dissolving cell components. Addition of the chloroform followed by centrifugation separates the solution into aqueous phase and inorganic phase. RNA remains extensively in the aqueous phase. After transfer of the aqueous phase, RNA is recovered by precipitation with isopropanol.

**Chemicals required:**
- a) Liquid nitrogen
- b) Trizol reagent
- c) Chloroform
- d) Isopropanol
- e) Ethanol
- f) DEPC treated water or nuclease free water.

**Procedure for RNA isolation from tissue:**
Tissue was cut into small pieces, and crushed into powder with the help of liquid nitrogen using mortar and pestle.

The mixture from the mortar is transferred into a new eppendorf tube, vortexed, incubated in room temperature for 15 mints.
280µl of chloroform was added and kept in room temperature for 5 minutes, vortexed (curdy appearance)

Kept at room temperature for 5 minutes

Centrifuged at 13,000 rpm at 4°C for 15 minutes

The supernatant was collected (Upper aqueous phase)

600µl of ice cool isopropanol was added

Incubated at -20°C for 20 minutes

Centrifuged at 13,000 rpm for 20 minutes

500µl of 70% ethanol was added and centrifuged at 13,000 rpm for 10 minutes

Alcohol was discarded and the pellet was air dried

The pellet was suspended with 20µl of DEPC treated or nuclease free water

Fig 4.3: Flowchart explaining the steps involved in extraction of RNA from tissue

4.1.4. Agarose Gel electrophoresis of the extracted DNA and RNA:
In order to check the quality of the DNA and RNA isolated from the respective samples, the DNA and RNA obtained was subjected to agarose gel electrophoresis in a 3% and 1.2% agarose gel respectively and stained with Ethidium bromide and observed in a transilluminator.
Chemicals:

a) Agarose
b) Ethidium bromide (EtBr, 10mg/ml)
c) 6X gel loading buffer.
d) Electrophoresis buffer [1X Tris acetate EDTA (TAE)]
e) DNA sample

Procedure:

The mould for the gel along with comb was prepared

Appropriate percentage of agarose is calculated weighed and added to required volume of 1X TAE buffer in a conical flask

It was heated in microwave until agarose gel dissolved completely

It was cooled to 40°C and then poured into the gel mould and allowed to caste

The gel tank was prepared and connects to an electrophoresis power pack

Then required volume of 1X TAE buffer was poured into the gel tank

The comb was removed carefully and then the tape around the gel was removed

Then it was placed along with the gel plate into tank until the surface of the gel was submerged in the buffer

5µl of the sample DNA or RNA is mixed with 1µl of loading dye and loaded in the appropriate well of the gel

Electrophoresis was carried out at 80V and 400 mili Amp current for 30 minutes

The gel was observed under u.v. transilluminator

Fig.4.4: Flowchart explaining the steps involved in the agarose gel electrophoresis.
4.1.5.: Preparation of cDNA:

Intact, pure poly (A+) RNA is essential for the synthesis of high-quality cDNA. Since RNA is very unstable, so for relative quantification or mRNA based expression study of the target gene it should be converted to the stable form. Therefore, cDNA of the mRNA (present in the RNA extracted) is prepared with the help of Reverse Transcriptase and stored at -20°C.

Requirements:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>6 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Random Hexamer</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

**Table 4.1: Master Mix 1**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>dTT</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

**Table 4.2: Master Mix 2**

Procedure:

1. **Mastermix 1**
   - PCR condition: 70°C, 10 minutes
   - Snap freeze on ice

2. Add **Mastermix 2**
   - PCR condition: 25°C, 15 minutes
Snap freeze on ice

2µl of RT (20 units/µl) was added

PCR condition: 42˚C-1hr/72˚C-10min/4˚C -hold

cDNA (store at -20˚C)

Figure 4.5: Flowchart showing the preparation of cDNA from the mRNA

4.2: HPV detection and genotyping by using general PCR methodology:
HPV detection was done in the DNA extracted from the cervical cancer tissue samples. As the viral DNA is found to be integrated in the host genome, whole genome extraction from the tissue was done. Presence of HPV was detected by PCR amplification of the DNA by MY09/11 consensus primer of HPV. The presence of HPV was confirmed by a bright band of 450bp size, after running the PCR product on agarose gel electrophoresis.

As global data and regionally available data is indicative of the presence of either HPV 16 or 18 in majority of the HPV infected cervical cancer cases especially in squamous cell carcinoma, therefore screening for HPV 16 and 18 genotypes was done using type specific PCR in our studied cohort in which all the cervical cancer cases were clinically and pathologically proved squamous cell carcinoma.

Principle of Polymerase Chain Reaction:
PCR is a molecular biological technique used for amplification of small portion of DNA using specific oligonucleotide primers. Basic PCR consist of three steps – thermal denaturation of the target DNA, primer annealing to the target region on the DNA and extension at the annealed primers by DNA polymerase. The three steps are then repeated in a number of times, each time approximately doubling the product number.

Method:
HPV detection is done by using L1 consensus primer MY09/11
My09: 5’CGTCCMARRGGAWACTGATC3’
My11: 5’GCMCAGGGWCATAAYAATGG3’
M=A or C, W=A or T, Y=C or T, R=A or G
HPV genotyping is done by using type specific primer for HPV16 and HPV18

**Primers used for HPV16**
F: 5’TCAAAAGCCACTGTGTCCTGA3’
R: 5’GGTGTTCTTGATGATCTGCAA3’

**Primers used for HPV18**
F: 5’CCGAGCACGACAGGAACGACT3’
R: 5’TCGTTTTCTTCTCTGAGTCGCTT3’

**Reagents used for PCR amplification using MY09/11 primer**
For 10 µl of PCR reaction mixture

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Chemicals used</th>
<th>Amount used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclease free water</td>
<td>6.3 µl</td>
</tr>
<tr>
<td>2</td>
<td>(10x) PCR buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>3</td>
<td>(25mM) MgCl2</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>4</td>
<td>(10mM) dNTP</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>5</td>
<td>(10ρm/μl) primer forward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>6</td>
<td>(10ρm/μl) primer backward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>7</td>
<td>(3 unit/μl) Taq polymerase</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>8</td>
<td>Template</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Table 4.3: PCR master mix composition for my09/11 primer (10µl)

The amplification was carried out under the following reaction conditions for 35 cycles.
Initial denaturation at 94°C—5mins/ denaturation 94°C—30secs/ annealing 55°C—30secs/ extension 72°C—45secs/ final extension 72°C—7mins/ hold at 4°C for ∞ (infinite), 5 µL of the PCR product was analyzed on 1.5% of agarose gel.

**Reagents used for PCR amplification of DNA using HPV16 primer**

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Chemicals used</th>
<th>Amount used</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Nuclease free water</td>
<td>6.72 µl</td>
</tr>
<tr>
<td>2</td>
<td>(10x) PCR buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>3</td>
<td>(25mM) MgCl2</td>
<td>0.6 µl</td>
</tr>
<tr>
<td>Sl no.</td>
<td>Chemicals used</td>
<td>Amount used</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>4</td>
<td>(10mM) dNTP</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>5</td>
<td>(10ρm/µl) primer forward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>6</td>
<td>(10ρm/µl) primer backward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>7</td>
<td>(3 unit/µl) Taq polymerase</td>
<td>0.08 µl</td>
</tr>
<tr>
<td>8</td>
<td>Template</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Table 4.4: PCR master mix composition for HPV 16 (10µl)

The amplification was carried out in the following reaction conditions. Initial denaturation at 94°C—5mins/ denaturation 94°C—30secs/ annealing 55°C—30secs/ extension 72°C—45secs/ final extension72°C—7mins/ hold at 4°C for ∞, 5µl of the PCR product was analyzed on 3% agarose gel.

Reagents used for PCR amplification of DNA using HPV18 primer

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Chemicals used</th>
<th>Amount used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclease free water</td>
<td>6.3 µl</td>
</tr>
<tr>
<td>2</td>
<td>(10x) PCR buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>3</td>
<td>(25mM) MgCl2</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>4</td>
<td>(10mM) dNTP</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>5</td>
<td>(10ρm/µl) primer forward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>6</td>
<td>(10ρm/µl) primer backward</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>7</td>
<td>(3 unit/µl) Taq polymerase</td>
<td>0.08 µl</td>
</tr>
<tr>
<td>8</td>
<td>Template</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Table 4.5: PCR master mix composition for HPV18 (10µL)

The amplification was carried out in the following reaction conditions. Initial denaturation at 94°C—5mins/ denaturation 94°C—30secs/ annealing 59°C—30secs/ extension 72°C—45secs/ final extension72°C—7mins/ hold at 4°C for ∞, 5µl of the PCR product was analyzed on 3% agarose gel.

Reagents used for PCR amplification of DNA for expression of (β-actin):

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Chemicals used</th>
<th>Amount(in µ1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclease free water</td>
<td>7.08</td>
</tr>
<tr>
<td>2</td>
<td>(10X) PCR Buffer</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Table 4.6: PCR master mix composition for β-actin (10 µl)

<table>
<thead>
<tr>
<th></th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>(25mM) MgCl₂</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>(10mM) dNTP</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>(10pm/µl) primer forward</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>(10pm/µl) primer backward</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>(3 unit/µl) Tag polymerase</td>
<td>0.12</td>
</tr>
<tr>
<td>8</td>
<td>Template</td>
<td>1</td>
</tr>
</tbody>
</table>

The amplification was carried out in the following conditions:
Initial denaturation at 94°C for 5 mins/ Denaturation 94°C for 30 secs/ Annealing at 58°C for 30 secs/ Extension at 72°C for 45 secs/ Final extension at 72°C for 7 mins/ hold at 4°C for 5 µl of the PCR product was analyzed on 3% of agarose gel.

4.3: TH1/TH2 expression profile:
The TH1/TH2 profile was analyzed from serum by Magpix multiplex ELISA method using customized magnetic bead based kit and Xpotent software based analysis (Merck Millipore).

Principle:
An antibody reacts with the concerned antigen in a highly specific manner, i.e., only with the specific determinant or region of an antigen; this produces an Antigen-Antibody complex. Multiplex MAP is based on the Luminex MAP technology which is capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex™C microspheres. Luminex uses proprietary techniques to internally color code microspheres with two fluorescent dyes. Through accurate concentrations of two dyes, 100 distinctly coloured bead sets can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by a bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin PE conjugate, the reporter molecule to complete the reaction. The microspheres are allowed to pass rapidly through a laser, which excites the internal dyes marking the microsphere set. A second later excites PE, the fluorescent dye on the reporter molecule. Finally, high speed digital-signal processors identify each individual microsphere and quantify the result of the bioassay, based on fluorescent reporter signals. The capability of adding multiple conjugated beads to each sample results in the ability to
obtain multiple results from each sample thus reducing time, labor and costs over traditional methods.

**Immunoassay procedure:**

- 200 µl of wash buffer was added into each well of the plate. Sealed and mixed on a plate shaker for 10 minutes at room temperature.
- Wash buffer was decanted and the residual amount from all wells was removed by inverting the plate and tapping it smartly onto absorbent towels several times.
- 25 µl of each standard and quality control was added into the appropriate wells. Assay buffer should be used for 0 pg/ml standard (background).
- 25 µl of assay buffer was added to the sample wells.
- 25 µl of appropriate matrix solution was added to the background, standards and control wells. While assaying plasma used the serum matrix provided in the kit.
- 25 µl of plasma sample was added into the appropriate sample wells.
- Premixed beads bottle were vortexed and 25 µl of beads was added to each well. (Note: During addition of beads, shooked bead bottle intermittently to avoid settling)
- The plate was plated with a plate sealer. The plate was wrapped with foil and incubated with agitation on a plate shaker overnight at 4°C.
- Well contents were gently removed and the plate was washed 2 times with 200 µl wash buffer.
- 25 µl of detection antibodies was added into each well (prior to addition, the detection antibodies were allowed to warm to room temperature).
- The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Do not aspirate after incubation.
- 25 µl Streptavidin-Phycoerythrin was added to each well containing the 25 µl of detection antibodies.
- The plate was sealed, covered with foil and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
- Well contents were gently removed and the plate was washed 2 times with 200 µl wash buffer.
- 150 µl of Sheath fluid (or Drive fluid if using MAGPIX) was added to all wells. The beads were re suspended on a plate shaker for 5 minutes.
- The plate was run on Luminex MAGPIX with Xponent software.
The Median Fluorescent Intensity (MFI) data was saved and analyzed using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokine concentration in samples.

4.4: Real Time PCR (RT PCR):
PCR or the Polymerase Chain Reaction has become the cornerstone of modern molecular biology the world over. Real-time PCR is a bespoke from the Polymerase Chain Reaction that maximizes the potential of the technique.

Principle:
RT PCR is also known as quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are:

Non-specific fluorescent dyes that intercalate with any double-stranded DNA, and Sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. Frequently, real-time PCR is combined with reverse transcription to quantify messenger RNA and Non-coding RNA in cells or tissues.

Procedure:
After performing the reverse transcription reaction the RT-PCR using SYBR green is performed.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O (Sterilized distilled water)</td>
<td>6.2 μl</td>
</tr>
<tr>
<td>PCR Reverse Primer (respective genes)</td>
<td>0.15 μl</td>
</tr>
<tr>
<td>PCR Forward Primer (respective genes)</td>
<td>0.15 μl</td>
</tr>
<tr>
<td>Syber green</td>
<td>7.5 μl</td>
</tr>
<tr>
<td>Template (cDNA)</td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>

Table 4.7: Preparation of the RT-PCR reaction mixture

4.4.1: Relative Quantification using the Real Time RT-PCR:

Relative quantification also known as the comparative threshold method (2-ΔΔct method) is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (calibrator being e.g. tissue from non-neoplastic area). In the Real Time PCR (RT-PCR), if the amplification efficiency of a reference gene (endogenous housekeeping gene) is the same as the efficiency of the target gene, the amount of normalized target gene relative to the normalized calibrator can be found out by the 2-ΔΔct method. The value of 2-ΔΔct is the level of expression change for the target gene.

Principle:

The Ct is the basic principle of the relative quantification in the Real-Time PCR used for producing accurate and reproducible data. Ct is the “threshold cycle” or the cycle number after which the Real Time PCR starts detecting the increase in fluorescence with each exponential increase in the amount of template. The Ct value depends on starting amount of the template. Any expression change in the target gene, we can detect it by comparing the Ct value of the target gene in a sample with that in the calibrator.

Method:

Expression of the mRNA transcriptions of IL-10, IL-12 and TNF-α, and NF-κB isolated from the cervical cancer tissue samples was determined by the Real Time PCR which allows continuous measurement of the PCR product amount by means of SYBR Green fluorescent dye. The mRNA levels of the target genes are normalized to the transcript level of the
housekeeping gene β-actin. For relative quantification analysis, the expression of mRNA transcripts of the target genes from normal cervical cancer tissues was also determined.

i) **mRNA analysis of the IL-10 transcript:** IL-10 expression was studied using the mRNA specific primers-
   F: 5’ TCT TGC AAA ACC AAA CCA Ca 3’
   R: 5’ ACT CTG CTG AAG GCA TCT CG 3’

ii) **mRNA analysis of the IL-12 transcript:** IL-12 expression was studied using the mRNA specific primers:-
   F: 5’ AAG GAG GCG AGG TTC TAA GC 3’
   R: 5’ GCA GGT GAA ACG TCC AGA AT 3’

iii) **mRNA analysis of the TNF-α transcripts:** TNF-α expression was studied using the mRNA specific primers-
    F: 5’ CGC TCC CCA AGA AGA CAG 3’
    R: 5’ GCC AGA GGG CTG ATT AGA GA 3’

iv) **mRNA analysis of the NF-kB transcripts:** The differential mRNA expression for NF-kB was studied in HPV16 positive affected and non-neoplastic control areas using Syber green chemistry and with the primers-
   F: 5’CC TATGTGGAG ATCATTG3’
   R: 5’GGTGGGTCTTGGTGGTATCT3’

   The annealing temperature is 59°C, and β-actin being used for internal normalization.

v) **mRNA analysis of β-actin** as internal control: primer sequence is
   F: 5’ AGATAGTGGATCAGCAAGCAG 3’
   R: 5’ GCGAAGTTAGGTTTTGTCA 3’

β-actin was used as the internal control. The Real Time PCR conditions for β-actin were-95°C-5 mins/95°C-30 secs/58°C-30 secs/72°C-45 secs.
Melt curve conditions: 95°C-15 mins/60°C-1 mins/95°C-15 secs.

### 4.4.2: Calculation of ΔCt, ΔΔCt and 2-ΔΔCT values:

The readings of Ct value for the target genes, internal control and calibrator (normal) were obtained and from this the ΔCt, ΔΔCt and 2-ΔΔCt was calculated from the following formula-

\[
\Delta C_t(t) = C_t(t) - C_t(\text{ref})
\]

\[
\Delta C_t(n) = C_t(n) - C_t(\text{ref})
\]

Where:

- \( C_t(\text{target}) \) = Ct (target)
- \( C_t(\text{reference}) \) = Ct (reference)
\[ \Delta \Delta C_t = \Delta C_t (t) - \Delta C_t (n) \quad \text{Ct} (n) = \text{Ct (calibrator)} \]

Expression of the target gene normalized the reference gene and relative to the calibrator – \(2^{-\Delta \Delta C_t}\). the value of 2\(\Delta \Delta C_t\) indicates the fold change in expression of the target gene compared to that in the normal (reference).

4.5: Immunohistochemical (IHC) study of TNF-\(\alpha\):

**Principle:**
The demonstration of antigens in tissues and cells by immunostaining is a two-step process. First step is the binding of an antibody to the antigen of interest, and the second step is the detection and visualization of bound antibody by one of a variety of enzyme chromogenic systems. The choice of detection systems will dramatically impact the sensitivity, utility and ease-of-use of the method. The Super Sensitive™ One-Step Polymer-HRP Detection System is a novel detection system using a non-biotin polymeric technology that makes use of only one major component: a polymer-HRP reagent. Tissues or cell preparations are frozen or fixed, sectioned, and attached to slides. The sections are then dewaxed if paraffin-embedded, treated with an antigen retrieval solution if required, blocked with a proteinaceous blocking solution and then incubated with a primary antibody. The bound primary antibody is detected by the addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate. When adequate color development is seen, the slides are washed in water to stop the reaction, counterstained, and covered with a mounting medium.

**Procedure:**
CIN I-III grade cases (n=25) and randomly selected HPV-16 infected squamous cell carcinoma (SSC) cases along with control areas (n=35) were taken for immunohistochemical study of TNF-\(\alpha\) protein expression in cervical tissues using the Super Sensitive™ One-Step Polymer-HRP Detection System (Biogenex). Slides with 3-5 \(\mu\) tissue segments were prepared for the test. Since HPV-16 was the predominant genotype in our cohort, therefore CIN cases (n=25) who were screened positive for HPV-16 genotype were only included for immunohistochemistry based analysis. The slides were examined and graded for TNF-\(\alpha\) expression by a senior pathologist.
- **Pre-treatment of the prepared slides:**
  - Dew axing of the slides was done by treating the slides with Xylene twice, each time for 5 minutes.
  - The slides were then washed with graded alcohol thrice, first with 50% alcohol, second with 70% and finally with 90%.
  - The slides were then washed in running water for 5 minutes.

- **Antigen retrieval:**
  Antigen retrieval was done by using Tris-EDTA buffer (pH=9)
  - The slides were then washed with wash buffer i.e. Tris (pH=7) thrice.

- **Staining procedure:**
  - **Endogenous Peroxide Block:**
    Peroxide Block was applied to cover the specimen according to tissue size or auto staining slide parameters. The slides were then incubated at room temperature for 10 minutes. After incubation, the liquid was drained and gently blotted around the section.
  - **Power Block:**
    Power Block was applied to cover the section and then the slides were incubated for 5 minutes at room temperature. It was then drained and gently blotted.
  - The slides were then washed with wash buffer 3 times, each time for 1 minute.
  - **Application of primary antibody:**
    Appropriate amount of primary antibody (TNF-α, at a dilution of 1:200) was added to cover the specimen according to the tissue size. The slides were incubated for 30 minutes.
  - **Application of Polymer-HRP Reagent:**
    Appropriate volumes of Polymer-HRP Reagent to the specimen and incubated for 15 minutes at room temperature.
    - The slides were then washed with wash buffer for 1 minute, thrice.
  - **Application of DAB Substrate solution:**
    - Appropriate volume of Substrate solution was added and the slides were then incubated at room temperature for 5 minutes.
The slides were then washed in running water.

**Counterstaining:**
- The slides were immersed in a bath of hematoxylin for 1 minute, depending on the strength of hematoxylin used, and then rinsed with tap water.
- The slides were then washed with graded alcohol thrice, first with 90% alcohol, then with 70% and finally with 50% alcohol.
- The slides were then again treated with xylene twice, each time for 5 minutes.
- Finally, the slides were mounted using 1-2 drops of DPX.

**4.6: Differential expression analysis for NF-kB at mRNA and protein level:**

**4.6.1. Differential mRNA expression analysis:**
The mRNA based differential expression analysis was performed by Real-Time PCR in the HPV16 infected affected area compared to control area of the same paired samples, using β-actin as internal control.

**4.6.2 Extraction of total protein from cervical cancer tissue sample:**

**Principle:** The total protein content inside the cell is extracted out by lysing the cells or cell membranes using magnesium lysis buffer, and stabilizing the protein by safeguarding against external proteases. The cell debris is precipitated out by centrifugation and the protein content in the supernatant part is quantified and used for further investigations.

**Reagents:**

a) Magnesium containing lysis buffer
   - \( \text{NH}_4\text{Cl- 155mM} \)
   - \( \text{KHCO}_3^- 10 \text{mM} \)
   - \( \text{Na}_2\text{EDTA-0.1mM} \)
   - Fill to 1000 ml with distilled water
   Adjust to pH 7.4 with 1M HCl or NaOH for each use.

b) Addition of 10 ug/ml Leupeptin, 10 ug/ml Aprotinin, 5ug/ml PMSF as protease inhibitors just before use.
c) Specimen: 2 cm$^3$ of tissue sample

d) Ice

e) Centrifuge

f) Homogenizer

g) Rocker

h) Micropipettes

i) Eppendorf tubes

j) Autoclaved tips

k) Falcon tubes

**Procedure:**

Measuring out a 2 cm$^3$ of freshly isolated or properly stored tissue sample

- Putting the tissue in a 2 ml eppendorf tube or 15 ml falcon tubes

- Addition of 1 ml of MLB buffer to the sample and homogenize using a mechanical homogenizer

- Putting the homogenized sample on ice for 5 minute

- Place in a rotating rocker in a cold room/ cold condition for at least 20-30 minute

- Centrifuge the tubes at 10,000 rpm for 5 min at 4$^\circ$ C

- Cellular debris as precipitate

- Collect the supernatant containing the protein in fresh eppendorf tube

- Store at -20$^\circ$ C or quantify the protein by Folin Lowry method

**4.6.3: Differential NF-kBp65 protein expression analysis**

The expression of NF-kB at tissue protein level was analyzed by Abcam’s NF-kB in vitro Simple Step ELISA kit (catalogue no.ab176648, Abcam) following manufacturer’s instructions.
4.6.4: Correlation analysis of NF-kB expression with TNF-α

The differential expression pattern of NF-kB was correlated with the differential TNF-α expression at both mRNA and protein levels using Pearson correlation and Spearman’s rho based statistical analysis using the SPSSv13.0 software.

4.7: Statistical Evaluation:

Data were expressed as means ± standard deviations (SD). Statistical analyses were performed using a Wilcoxon signed rank test for categorical variables and Mann–Whitney’s non-parametric test appropriate. Differences were considered significant if P value is less than 0.05. The statistical analysis software used was SPSS, version 13.0. The differential expression pattern was correlated by using Pearson correlation and Spearman’s rho based statistical analysis.
Fig 4.8: WORK DESIGN

Patient’s enrolment & collection of sample

Cervical tissue (cancerous & non neoplastic)

Whole blood

Stored in liquid N2

Formalin fixed & paraffin

DNA

Total RNA

Protein

IHC for TNF-alpha

Serum separation

cDNA

Differential NFkB expression analysis by ELISA

Th1 & Th2 profile by Multiplex ELISA

HPV detection by

HPV genotyping for HPV16/18 by type Specific PCR

RT PCR based analysis for differential expression of Th1 & Th2 markers & NF-kB

Statistical analysis Using SPSS software