Chapter-3

Methodology

3.1 Materials

3.1.1 Survey and Collection of Samples

Blood samples were collected from patients at the Cachar Cancer Hospital and Research Centre, Silchar (CCHRC) and Silchar Medical College and Hospital, Silchar, with informed consent. Samples were also collected from healthy volunteers without a history of cancer, with informed consent. Data was collected from patients and healthy volunteers in response to a questionnaire. Patients included in the study were those suffering from breast cancer. This study was approved by CCHRC institutional review board (IRB).

3.1.2. Chemicals

Molecular biology grade chemicals were used for all experiments. Most of the chemicals were from SRL Company. Dehydrate Ethanol and Rectified spirits were supplied by Bengal Chemical and Pharmaceuticals Works Ltd. Kolkata. *Taq* DNA polymerase, dNTPs Master mix, Reagents kit, Buffer, etc. were purchased from Fermentas, Germany; Bioline, U.K; Applied Biosystem, USA (Table 3.1).

Sl no.	Experiments	Chemicals
1	**Hematoxylin and Eosin staining**	Harris hematoxylin, eosin, acetone, xylene, formalin, egg albumin, DPX mount, Graded series of alcohol (50%, 70%, 90% and absolute alcohol), HCl, NaCl, NFW, paraffin.
2	**Immunohistochemistry**	Primary antibodies, secondary antibodies, Tris, HCl, Nacl, citric acid, citrate, Acetone, NaoH pellet, $MgCl_2$, Twin-20, xylene, Bovine serum Albumin, BCIP, NBT, Graded series of

		alcohol, NFW.
3	**DNA Isolation**	Phenol, chloroform, isoamyl alcohol, absolute alcohol, Proteinase K, RNase solution, SDS, NaCl, HCl, Tris, Sodium acetate, EDTA, Nuclease free water (NFW).
4	**PCR Amplification**	Taq polymerase, Master mix, Tris, HCl, $MgCl_2$, Gelatin, Primers, dNTP mix, DMSO, NFW.
5	**Gel Electrophoresis**	Bromophenol blue, Agarose, Ethidium bromide, DNA ladder, NFW, sucrose, glycerol.
6	**Quantification in Biophotometer**	Nuclease free water and DNA.
7	**Elution of PCR Amplicon**	Sodium iodide, Elution kit.

Table 3.1 Chemical lists for the respective experiments.

3.1.3 Preparation Reagents and Buffer

All reagents and chemicals used were of molecular grade, that is, they were DNase, RNase and protease free. Frequently used solutions were commonly prepared as stock solutions of higher, recommended strengths. The stock solutions were diluted to obtain the required volume of the working solution. The solvent used for preparation of the stock solution was also used as the diluent for preparation of working solution. For appropriate dilution, the formula followed was

$$V1 \times S1 = V2 \times S2$$

Where,

V1 is the volume to be taken from stock solution

S1 is the strength of the stock solution

V2 is the required volume of working solution
S2 is the required strength of the working solution

3.1.3.1 Reagents

1. Tris (hydroxymethyl aminomethane)-HCl or Tris-HCL- 10ml (1 M, pH- 8):

[a] Tris Base	1.211 gm
[b] Nuclease free water	10 ml

Prepared by dissolving 1.211 gm of Tris base (hydroxymethyl aminomethane) in 8 ml of Nuclease free water. The pH was adjusted to 8 with concentrated HCl, and the final volume rose to 10 ml with water. (Stored at room temperature, RT)

2. Ethylenediamine tetrachloro acetic acid (EDTA), (0.5 M, pH 8): 10 ml

[a] EDTA	1.862 gm
[b] Nuclease free water	10 ml

Prepared by dissolving 1.862 gm of Ethylenediamine tetrachloro acetic acid (EDTA) in 8 ml of water. The pH was adjusted to 8 with sodium hydroxide pellets, and the final volume rise to 10 ml with nuclease free water. (Stored at RT)

3. Sodium chloride (NaCl) - 10 ml (5M) –

[a] NaCl	2.422 gm
[b] Nuclease free water	10 ml

NaCl 2.422 gm was added in 10 ml of nuclease free water and dissolved properly with an autoclaved stirrer and then autoclaved at 15 lb for 10 mins and kept at 22^0C.

4. SDS solution in water (10 %) –

[a] SDS	10 gm
[b] Nuclease free water	100 ml

Prepared by dissolving 10 g of SDS (sodium dodecyl sulphate/ sodium lauryl sulphate) in 100 ml of water and kept at 37^0C for some time. (Stored at RT)

5. Ethidium bromide (EtBr) (10 mg/ml)

[a] Ethidium bromide	10 mg
[b] Nuclease free water	1 ml

Supplied by the manufacturer in the form of solution/ powder. The powder was dissolved in water to obtain a stock solution of concentration 10 mg/ ml, which is stored at 4 °C. Final staining solution is prepared at a concentration of 0.3 µg/ ml by dissolving the stock solution, in appropriate volume of dissolved agarose gel/ 1 X TAE buffer.

6. Bromophenol blue solution - This solution had the following composition:

[a] Sucrose	40 % (w/v)
[b] Bromophenol blue	0.25 %

To prepare 10 ml of loading solution, 4 gm of sucrose and 20 mg of bromophenol blue were dissolved in 10 ml of Millipore water. This solution was stored refrigerated at 4°C.

7. Tris Saturated Phenol (pH 7.5) - Solid phenol was liquefied at 68° C in water bath and equal volume of 1 M Tris HCL (pH 7.5) was added. Discarded the upper aqueous phase and repeated the extraction procedure until the pH of the aqueous phase became 7.5. After final extraction an equal volume of TE was added to it and stored in a bottle wrapped with aluminum foil at 4° C.

8. Chloroform isoamyl alcohol solution (24:1) – 24 parts of chloroform were added with 1 part of isoamyl alcohol and mixed properly and stored at 4° C.

9. Proteinase K solution (20 mg ml^{-1})- This was prepared by dissolving 20 mg of Proteinase K in 1 ml of sterilized Millipore water in a sterile microcentrifuge tube. The Proteinase K solution was divided into small aliquots and stored refrigerated at -20 °C.

10. RNase solution (5 mg ml^{-1})- This was prepared by dissolving 5 mg of DNase free ribonuclease in 1 ml of sterilized Millipore water in a sterile microcentrifuge tube. The RNase solution was divided into small aliquots and stored refrigerated at -20 °C.

11. Sodium acetate solution, (pH 5.2, 3 M)- To prepare 100 ml of sodium acetate solution, 26.4 g of sodium acetate was dissolved in Millipore water and the pH was adjusted to 5.2 with glacial acetic acid. The final volume was made up to 100 ml with Millipore water. This solution was sterilized by autoclaving and was stored at RT.

12. BCIP/NBT Solution - The premixed solution contains 0.48 mM Nitro Bluetetrazolium (NBT), 0.56 mM 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), 10mM Tris Hcl, pH 9.2 and 59.3mM $MgCl_2$.

3.1.3.2 Buffer

1. TES buffer (10ml): This buffer consisted of the following components:

[a] Tris-HCl,	50 mM
[b] EDTA	25 mM
[c] NaCl	150 mM
[d] Millipore water	

EDTA was prepared as a 500 mM stock solution by dissolving 14.61 g of EDTA in Millipore water and adjusting the pH to 8 with pellets of NaOH. The final volume was then raised to 100 ml with Millipore water. To prepare 100

ml of Tris-Cl buffer, 1.21 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. To prepare 100 ml of extraction buffer, 6.38 g of sorbitol and 1 ml of EDTA stock were dissolved in 100 ml of Tris-Cl buffer. The extraction buffer was sterilized by autoclaving and stored at RT.

2. Lysis Buffer:

This buffer consisted of the following components

[a] Tris –Cl M (pH 8.0): 1ml (10mM)
[b] EDTA 0.5M (pH 8.0): 20ml (100mMM)
[c] SDS: 500mg (0.5%)
[d] Add distilled water up to 100ml.

3. Tris Acetate Buffer (TAE)

This buffer had the following composition:

[a] Tris-acetate, pH 8, 40 mM
[b] EDTA, pH 8, 1 mM

The buffer was prepared and stored as a stock solution of 50 X, at RT. To prepare 1 litre of 50 X TAE buffer, 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 500 mM EDTA, pH 8 were dissolved and the final volume made up to 1 liter with Millipore water. This stock solution was diluted to 1 X with Millipore water before use.

4. Tris Buffered Saline (TBS)

The buffer consisted of the following components

[a] Tris-HCL, pH 7.5, 10mM
[b] NaCl, 500mM

To prepare 100ml of Tris-HCL buffer, 0.12 gm of tris base was dissolved in 100 ml of Millipore water. The pH was adjusted to 7.5 with concentrated HC

and the final volume was adjusted to 100 ml with Millipore water. TBS buffer was prepared by dissolving the required amount of NaCl in Tris-HCl Buffer.

5. Tween-20-Tris Buffered Saline (TTBS)

This solution consisted of 0.05% Twin-20 (v/v) in TBS Buffer. To prepare 1 litre of TTBS solution, 50 ul of Twin-20 was dissolved in 1 litre of TBS buffer by continuous stirring.

6. Tris-EDTA (TE) Buffer

[a] Tris-HCl 10 mM
[b] EDTA 1 mM

Genomic and Plasmid DNA can be stored in TE Buffer at 4^0C for short term use, or -20^0C to -80^0C for long term storage. The buffer consisted of the following components

7. Citrate Buffer

The buffer consisted of the following components

a) 0.1 M citric acid (1.92 mg citric acid anhydrous to 100 ml glass distilled water).
b) 0.1 M Na Citrate, dehydrate (14.7gm Na Citrate dehydrate to 500 ml glass distilled water).

8. Alkaline Phosphatase Buffer

The buffer consisted of the following components

[a] 100 mM tris-HCl
[b] 100 mM NaCl
[c] 5 mM $MgCl_2$

3.1.4 Primers:

Several primer pairs were used designed to amplify regions of interest of the *BRCA1* and *p53* genes in this study. Primers used for amplification of *GSTT1*, *GSTM1* and *CYP1* primers were as previously reported (Mondal et al., 2012). We have designed all primers using OligoCalc, Sequence Manipulation Suite and Primer3. The primer efficiency was checked through various Bioinformatic tools which is listed in Table 3.2. All the primer pairs were to amplify less than 200 base pare of respective genes except low penetrance candidate genes. All the sequence are partial gene sequence from human breast cancer samples. Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mishybridization to a similar sequence nearby. A commonly used method is BLAST search whereby all the possible regions to which a primer may bind can be seen. Both the nucleotide sequence as well as the primer itself can be BLAST searched. The free NCBI tool Primer-BLAST integrates primer design tool and BLAST search into one application, so does commercial software product such as Primer3, SMS, OligoCalc, Beacon Designer (Table 3.2). Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.

Sl no.	Tool Name	Description	Website
1	OligoCalc	Calculating primer nucleotide	http://www.basic.northwestern.edu/biotools/OligoCalc.html
2	Sequence manipulation suite	The SMS is a collection of JavaScript programs for generating, formatting, and analyzing short DNA and protein sequences.	http://www.bioinformatics.org/sms2/

3	Primer3	Comprehensive PCR primer and hybridization probe design tool	www.basic.nwu.edu/biotools/primer3
4	DoPrimer	Easily design primers for PCR and DNA sequencing	www.doprimer.interactiva.de
5	Primer selection	Select PCR primers from nucleotide sequence	www.alces.med.umn.edu/rawprimer
6	Web Primer	Allow alternative design of primers for either PCR or sequencing purposes	www.genome-www2.stanford.edu/cgi-bin/SGD/web-primer
7	PCR Primer Designer	An application that designs primers for PCR	www.pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
8	RawPrimer	A tool of selection of PCR primers	www.alces.med.umn.edu/rawprimer.html
9	PrimerQuest	A primer design tool	www.idtdna.com/biotools/primer_quest.asp

Table 3.2 Online software tools used for primer designing.

Sequence Name	**Primers**	**Product**	**Tc**
BRCA1-185delAG-F	ATT GGA ACA GAA AGA AAT GG	185bp	85.5
BRCA1-185delAG-R	CCT AGT ATG TAA GGT CAA TTC T		
BRCA1-1014delGT-F	ACA GCA TGA GAA CAG CAG	195bp	86
BRCA1-1014delGT-R	CAC AGG GGA TCA GCA TTC AGA		
BRCA1-3889delAG-F	TCT ACT AGG CAT AGC ACC GTT	192bp	85.5
BRCA1-3889delAG-R	CTT CCA ATT CAC TGC ACT GTG		
GSTT1-F	TTC CTT ACT GGT CCT CAC ATC TC	480bp	----
GSTT1-R	TCA CGG GAT CAT GGC CAG CA		
GSTM1-F	GAA CTC CCT GAA AAG CTA AAG C	220bp	----

GSTM1-R	GTT GGG CTC AAA TAT ACG GTG G		
CYP1-F	GAA CTG CCA CTT CAG CTG TCT	312bp	----
CYP1-R	GCT GCA TTT GGA AGT GCT C		
P53-E8-F	GCT TCT CTT TTC CTA TCC TG	167bp	81
P53-E8-R	CTT ACC TCG CTT AGT GCT		

Table 3.3 Primer sequence of different portion from the BRCA1, p53, GSTT1, GSTM1 and CYP1 gene.

3.2 Methods

3.2.1 Processing of tissue and blood

3.2.1.1 Hematoxylin and Eosin staining

Sections of tissue were prepared through a series of steps described as follows

Fixation: - Tissues from breast were cut into 2 mm thick pieces and fixed in 10 % formalin overnight. The following day the tissue sections were washed in tap water.

Dehydration: - Tissue sections were dehydrated through different grades of ethanol as follows- (a) 70 % ethanol for 12 hours (b) 90 % ethanol for 12 hours (c) 2 changes of 100 % ethanol for 2 hours each. This was followed by washing with 2 changes of acetone for 1 hour each and 2 changes of xylene for 45 min each.

Embedding: - The tissue sections were embedded in paraffin wax maintained at 58-60°C in an oven, with 3 changes of wax for 1 hour each.

Block making: - Tissue sections were transferred to paper moulds containing paraffin wax maintained at 60-62°C. The wax was allowed to cool at RT.

Sectioning: - Tissue blocks were cut into 5 -7 µm thick sections with the help of a microtome, transferred to albumin coated glass slides, and left to dry at RT.

Deparaffinization: - Tissue sections were deparaffinized by pressing the glass slides over a hot plate till the paraffin just melts. The slodes are immediately dipped in xylene and kept immersed for 20 min.

Drying and rehydration: - The glass slide was blotted dry and then transferred through serial dilutions of ethanol, i.e., 100 % ethanol for 1 min, 90 % ethanol for 1 min, 70 % ethanol for 1 min, and 50 % ethanol for 1 min. This was followed by immersion in tap water for 2 min.

Staining: - Staining was performed following a stepwise procedure. The sections were first stained with Harris hematoxylin for 10 min, and dipped in acid water/ ethanol mixture, followed immediately by washing under running tap water for 10 min. At this stage the sections were viewed under a microscope for staining of nuclei, and staining with hematoxylin was repeated for 5 min if the nuclei were not distinct. Sections stained with hematoxylin were dipped in acid water/ alcohol mixture and removed immediately. The sections were then stained with 1 % aqueous solution of eosin for 30 sec. These sections are referred to as H & E stained sections.

Dehydration: - The H & E stained sections were dehydrated through serial grades of ethanol, i.e. 70 % ethanol for 1 min, 90 % ethanol for 1 min and absolute alcohol for 1 min.

Mounting: - The H & E stained sections were dried in an incubator maintained at 37° C and dipped in xylene for 5 min. A drop of DPX mountant was then taken on a cover slip and pressed firmly over the sections, taking care to avoid trapping air bubbles. Excess DPX was wiped off, and the slides were left to dry O/N at RT.

Imaging: - The H & E sections were viewed and photographed with the help of Olympus Bright field Microscope at a magnification of 400 X.

3.2.1.2 Immunohistochemistry for Anti-BRCA1 (Ab-1423) protein was performed following the method

1. The paraffin of the tissue slides were melted at 90^0C-100^0C in hot plate. Melted paraffin slides were kept in xylene for 20 minutes.
2. After that slides were kept in 100% alcohol for 1 min followed by 90% of alcohol for 1 min, 70%, 50% alcohol for 1 minute.
3. For 2 minute slides were dipped in tap water containing 4 NaoH pellets. Then after slides were dipped in citrate buffer for 10 minute at 70^0C.
4. Applying blocking solution to slides were kept for 2-3 hours at room temperature.
5. Primary antibody (1:500) was applied to the slides and left it for overnight incubation.
6. Primary antibody (1^0) was discarded and subjected to washing stage with TTBS for 5 minutes for twice.
7. Then after secondary antibody was applied to the slides with blocking solution and incubated for two hours at 37^0 C.
8. Secondary antibody (1:8000) (2^0) was discarded and washed through TTBS twice per 5 min.
9. TBS washing stage was followed by TTBS washing stage for 10 min and then slides were subjected for ALP buffer for 10 minutes.
10. BCIP/NBT solution was exposed for 10 min after discarding ALP buffer from the slides.
11. Then the slides were dipped in distilled water for a while and dried in room temperature.
12. The last step was DPX mounting of slides through DPX.

3.2.2 Isolation and Quantification of DNA

Preparation of DNA was according to the method described by Ausubel et al., (1995), with some modifications which were incorporated from the method to increase the yield of DNA in our laboratory.

3.2.2.1 Isolation of DNA from blood

1. Blood samples typically were obtained as 1 ml of whole blood stored in EDTA vacutainer tubes frozen at 4^0C.
2. The blood sample was pipetted in a sterilized micro-centrifuge tube containing 500 μl of Lysis buffer.
3. To the above sample, 50 μl of 10% SDS was added and thoroughly mixed for few minutes and 2-3 μl of Proteinase k was added.
4. The sample was mixed thoroughly by inverting the micro-centrifuge tube for few minutes and it was then incubated at 56^0C for 30 minutes.
6. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed for few minutes.
7. Then the sample was centrifuged for 10 minutes at 12,000 rpm in centrifuge machine.
8. Carefully aqueous layer was removed to a new sterilized micro centrifuge tube, 500 ml of Chloroform: Isoamyl alcohol (24:1) was added and mixed thoroughly by repeated inverting the micro-centrifuge tube for few minutes.
9. Mixture was centrifuged at 12,000 rpm for 10 minutes in a centrifuge machine.
10. Upper aqueous layer was transferred in a fresh sterilized micro-centrifuge tube and double the volume of Chilled Absolute ethanol was added.
11. The above sample was kept at -20^0C for overnight for precipitation.
12. The above sample was centrifuged at 10,000 rpm for 10 minutes.
13. After centrifugation, supernatant was decanted.

14. To the pellet, 500 μl of 70% ethanol was added and again centrifugation at 7000 rpm for 10 minutes was done and supernatant was decanted.
15. The pellet was kept for air dry under laminar air flow.
16. The pellet was suspended in Nuclease free water or 1x TE buffer and stored in -20^{0}C or- 86^{0}C for long preservation.

3.2.2.2 Isolation of DNA from tissue

1. Tissue was collected properly and kept on ice. For longer use of tissue it was kept in -20^{0}C.
2. Tissue was cut into smaller pieces.
3. Tissue was kept in to a pre-cooled (dry ice) mortar and homogenized gently in 2 volume (w/v) cold TES buffer. Then the volume was adjusted to the volume (500μl) with TES buffer.
4. To the above sample, 50 μl of 10% SDS was added followed by 5-10 μl of 20mg/ml of Proteinase K, incubated it in 56^{0}C 1-18 hours until the tissue is totally dissolve.
5. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed it thoroughly for few minutes.
6. The samples were centrifuged for 10 minutes with 12000 rpm.
7. Upper phase was transferred to new 1.5 ml tube and equal volume of chloroform: isoamylalcohol was added and centrifuged with 12000 rpm for 10 minutes.
8. The step 7 was repeated up to transferring aqueous phase and double the volume, chilled absolute alcohol was added.
9. The above sample was kept at -20^{0}C for overnight for precipitation.
10. The above sample was centrifuged at 10,000 rpm for 10 minutes and supernatant was decanted and pellet was retained in the centrifuged tube.
11. To the pellet, 500 μl of 70% ethanol was added and again centrifuge at 7000 rpm for 10 minutes was done and then after supernatant was decanted.
12. The pellet was kept for air dry under laminar air flow.

13. The pellet was suspended in Nuclease free water or 1x TE buffer and stored in -20^0C or- 86^0C for long preservation.

3.2.2.3 Visualization of DNA using Agarose Gel Electophoresis

Agarose gel electrophoresis was performed according to standard protocol (Ausubel et al., 1995). The procedure is described below.

Gel preparation: -

A 0.8 % gel was used for electrophoresis of genomic DNA, and 2 % gel for electrophoresis of PCR amplicons. Accordingly, the gel was prepared by taking 0.8 g or 2 g of agarose in 100 ml of 1 X TAE buffer, and heating in a microwave oven with frequent swirling until the agarose flakes had completely dissolved, and, a clear solution was obtained. The agarose solution was allowed to cool to 40^0C, and was poured into a gel casting tray, following which a comb of appropriate size was inserted into the gel. Care was taken to avoid trapping air bubbles within the gel. The gel was left undisturbed at RT for 20 min, in order to polymerize. The comb was then removed, and the gel was submerged in 1 X TAE buffer in an electrophoresis tank.

Sample preparation: -

For AGE of genomic DNA, a constant amount of 3 µg of each DNA sample was loaded in the agarose gel. For AGE of PCR amplicon, a constant volume of 5 µl of each PCR product was loaded in the agarose gel. The 100 bp DNA ladder was used for determination of size of PCR amplicon. Sample was prepared for electrophoresis by mixing the appropriate volume of DNA solution, PCR product or 100 bp DNA solution with $1/10^{th}$ volume of sample loading solution. This mixture was then directly loaded into the wells of the submerged agarose gel.

Electrophoresis and staining: -

The samples loaded in to wells of the submerged agarose gel were subjected to electrophoresis at constant voltage, using a gel electrophoresis apparatus. EB is intercalated between adjacent nucleotides of a nucleic acid, and fluoresces under UV-irradiation, thereby enabling the nucleic acids to be visualized. Thus, on completion of electrophoresis, the agarose gels were stained with a 0.3 μg ml^{-1} solution of EB in 1 X TAE buffer for 10 min, with gentle shaking on a rocker, followed by destaining with Millipore water for 5-10 min in order to remove excessive stain and obtain a clear background. The EB-stained agarose gels were visualized and photographed on a gel doc.

3.2.3 PCR amplification of selected genes

In order to perform a PCR reaction for amplification of a target region, the PCR mix was prepared in a sterile hood by mixing all the components of 10X amplification buffer, dNTP mix, Forward Primer, Reverse Primer, Taq DNA polymerase, DNA Sample and sterile Millipore water in a sterile PCR tube. The contents of the tube were briefly spun down by centrifugation (10000 x *g* for 30 sec at RT), and the tube was then transferred to a thermal cycler in order to perform the PCR reaction. The thermal cycling conditions used for amplification of each target region were programmed into the thermal cycler. These thermal cycling conditions are listed in Table 3.4. For amplification of the selected region of BRCA1 gene, a *fast* COLD PCR protocol was used, in order to enrich the sequence chromatogram.

Normal PCR Cycling Conditions		
STEPS	**TEMPERATURE**	**TIME**
Initial Step		
Initial Denaturation	95 ^{0}C	120 s

30 Cycle		
Denaturation	94 ^{0}C	15 s
Primmer annealing	55 ^{0}C	30 s
Extension	72 ^{0}C	60 s
Final Step		
Final Extension	83.5 ^{0}C	3 s
Hold	4 ^{0}C	∞

Table 3.4 Normal PCR cycling conditions.

3.2.4 Sequence enrichment through COLD-PCR

Co-amplification at lower denaturation temperature-PCR (COLD-PCR) is a new technology discovered by Jin Li et al., (2009). It is a single-step method that results in the enhancement of both known and unknown minority alleles during PCR, irrespective of mutation type and position. This approach is based on critical denaturation temperature (Tc) for a given DNA sequence, that the percent of denaturation becomes sensitive to the exact DNA sequence, such that even point mutations make a substantial difference (Mancinni et al., 2010).

In *fast* COLD-PCR (for enrichment of Tm-reducing mutations), preferential amplification of mutations via COLD-PCR is so pronounced that, for the majority of point mutations, mutant enrichment occurs even without performing the intermediate cross-hybridization step at 70^{0}C. Thus, rapid PCR amplification performed at the Tc instead of at 94^{0}C discriminates strongly toward the lower-Tm allele (Li and wang, 2008). Breast cancer isolated DNA was demonstrated by performing COLD PCR with bidirectional primers. Each amplification reaction mixture (Total Volume of 25µL) contained Biomix (buffer, Mg2+, dNTPs, enzyme), sets of Primers (reverse and forward),

Isolated DNA from different Breast cancer samples. Amplification was carried out in the Thermal Cycler (Table 3.5).

The basic steps of PCR were- It had three cycles, but most important was the third cycle. And its steps were: -

Initiation step: - This step consists of heating the reaction to a temperature of 94 ^{0}C which is held for 2 min.

Denaturation step: - This step also consists of heating the reaction to a temperature of 94 ^{0}C for 45 sec. This step denatures the double stranded DNA into a single stranded.

***fast* COLD-PCR Cycling Conditions**		
	TEMPEARTURE	**TIME**
1st CYCLE x 1		
Initial Denaturation	95^{0}C	120 s
2nd CYCLE X 10		
Denaturation	95^{0}C	15 s
Primmer annealing	55^{0}C	30 s
Extension	72^{0}C	60 s
3rd CYCLE X 30		
Critical temperature	83.5^{0}C	3 s
Primmer annealing	55^{0}C	30 s
Extension	72^{0}C	60 s

Table-3.5 *fast* COLD PCR cycling conditions.

Intermediate step: - This is the step to form a heteroduplex structure in the PCR reaction mixture. This step plays a major role in critical denaturation step.

Critical denaturation step: - Here is the step where the specific temperature was given to amplify the specific target site in the ratio of wild and mutant type.

Annealing step: - This step is the primer annealing step during which the PCR primers find their complementary targets and attach themselves to those sequences. The choice of temperature is largely determined by the melting temperature (Tm) of the two PCR primers. Here temperature is maintained at 58^{0}C for 1 min.

Primer extension step: - In this step temperature is maintained at 72^{0}C for 1 min. In this step the DNA polymerase accelerates the polymerization process of primers and therefore extends the primers resulting in synthesis of copies of target DNA sequence.

3.2.5 Purification of PCR amplicons

Amplification of the targeted region of exons of BRCA1 and p53 gene yielded multiple amplification products was therefore eluted out from the gel. The procedure is described below.

a) The DNA band was excised from the gel with the help of the sterilized blade, and the gel piece was weighed. Assuming the specific gravity of the gel to be 1, 2.5 volumes of sodium iodide solution were then added to the gel, in a microcentrifuge tube.

b) The gel was solubilized by incubation in a water bath at 55^{0}C for 7-8 min, and thoroughly mixing the contents, until the agarose gel was completely dissolved.

c) The glass solution was vortexed until it formed a homogenous mixture, and 20 µl was then added to the solubilized sample. The contents were mixed thoroughly and left at RT for 10 min, with occasional mixing.

d) The mixture was centrifuged at 15000 x *g* for 30 sec, and the supernatant was discarded.

e) The pellet was washed twice by adding 250 µl of wash buffer and vortexing, followed by centrifuging at 15000 x *g* for 30 sec. The supernatant was discarded each time.

f) After the final washing step, the tube was kept at 55^0C for 10 min in a water bath, in order to remove all traces of wash buffer.

g) The pellet was suspended in 30-40 µl of sterile water by mild vortexing, and incubation at 55^0C for 5 min in a water bath, in order to elute the DNA.

h) The suspension was centrifuged at 15000 x *g* for 30 sec, and the supernatant was collected in a fresh tube.

i) The elution of the desired band was verified by subjecting part of the supernatant to AGE alongside a 100 bp DNA ladder as molecular weight marker.

3.2.6 Sequencing of PCR amplicons

Following PCR amplification and AGE of PCR amplicons, the amplicons whose sizes were determined to be equal to the expected size were eluted from the agarose gel by the help of QIA kit® (Cat. No. 28704, USA) and processed for bidirectional Sanger sequencing at Bose Institute, India.

3.2.7 Bioinformatric Analysis

After Sanger sequencing the raw chromatograms was assembled in Chromas and Applied Biosystems Sequence Scanner v1.0 (Applied Biosystem, Inc. USA) and sequence with greater than 2% ambiguous bases were discarded, using quality Value of 40 for bidirectional reads. Manual editing of raw traces and subsequent alignments of forward and reverse sequences enabled us to

assign edited sequences for most species. In some cases of discrepancy, both the sequences were reviewed and quality values of the sequences were considered to determine the most likely nucleotide. Then the 3' and 5' terminals were clipped to generate consensus sequences for each sample. Finally, each of the sequences was compared in NCBI through BLASTN. The sequences were translated using the online software ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and aligned through BLASTP (Altschul et al., 1990) to examine whether the partial amino acid codes were coherent with the respective gene frame and without any stop codon. In this way, the generated sequences were confirmed to be the fragments of chloroplast gene. All the analyzed sequences were then deposited in GenBank.

GenBank -This format consists of a mixture of compressed and uncompressed ASCII text files, containing sequence data and indices that cross reference author names, journal citations, gene names and keywords to individual GenBank records. For convenience, the GenBank records are partitioned into divisions according to source organism or type of sequence. Records within the same division are packaged as a set of numbered files so that records from a single division may be contained in a series of many files.

FASTA - The format begins with single line sequence description proceed by ">". On the next line, the actual sequence is represented in the standard IUB\IUPAC amino acid or nucleic acid code. Most of the programs can read or import FASTA format.

BLAST - Bioinformaticians have developed so called 'heuristic' algorithms, which allow searching a database in considerably less time. The most popular one is Basic Local Alignment Search Tool (BLAST). The Percentage similarity of the resulting DNA or protein sequences was analyzed through BLAST (Altschul et al., 1997); http://www.ncbi.nlm.nih.gov/blast/), a choice is offered between the different BLAST programs through different hyperlinks (nucleotide blast, protein blast, blastx, tblastn, tblastx etc). In this study, we

were searched nucleotide (nucleotide blast) and protein sequences (protein blast).

BioEdit - BioEdit is a mouse-driven, easy-to-use sequence alignment editor and sequence analysis program. BioEdit is intended to supply a single program that can handle most simple sequence and alignment editing and manipulation functions that researchers are likely to do on a daily basis, as well as a few basic sequences analyses (Hall 1999).

Sequence Manipulations Suite - The Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/) is written in JavaScript 1.5, which is a lightweight, cross-platform, object-oriented scripting language. JavaScript is now standardized by the ECMA (European Computer Manufacturers Association). The first version of the ECMA standard is documented in the ECMA-262 specification. The ECMA-262 standard is also approved by the ISO (International Organization for Standards) as ISO-16262. JavaScript 1.5 is fully compatible with ECMA-262, Edition 3. Sequences submitted to the Sequence Manipulation Suite instead manipulated by the web browser, which executes the JavaScript. The Sequence Manipulation Suite was written by Paul Stothard (University of Alberta, Canada). Short descriptions of the programs which were used in this study:

ORF Finder – Search for open reading frames (ORFs) in the DNA sequence was performed using ORF Finder program. The program returned the range of each ORF, along with its protein translation. ORF Finder supports the entire IUPAC alphabet and several genetic codes. Here, bacterial genetic code was selected in chloroplast matK sequences. ORF Finder was used to search newly sequenced DNA for potential protein encoding segments.

Pairwise Align DNA - accepted two DNA sequences and determined the optimal global alignment. Pairwise Align DNA was used to look for conserved sequence regions.