CHAPTER 2

Standardization of cell culture conditions, RNA isolation and RT-PCR Techniques

Introduction

As mentioned in the objectives and scope of the present study, it becomes imperative to study the role of estradiol-17β, Protein Kinase C and Protein Kinase A on the expression pattern and regulation of AP-1 factors in Breast cancer cells. Naturally these studies would have been appropriate, if human breast tumours, or autopsy explants or primary cell culture of disintegrated autopsy samples were used. Many laboratories were successful in using explants of human breast tumours and also cells disaggregated by enzymatic trypsin digestion. Cells attached to cell culture vessel, proliferated and represent the best experimental models for *in vivo* situations. These cells have the same karyotype as the parental tissue normal or abnormal and will not undergo dedifferentiation. Although, primary cells are morphologically similar to the parental tissue, capable of dividing only to a limited number of cell divisions, later enter into a non-proliferative state called as senescence and eventually die. However, primary cell cultures of breast cancer cells suffer from following disadvantages. Autopsy samples of breast tumours are difficult to obtain and they have relatively short life span in culture and very susceptible to contamination and may not fully act like a cancer tissue due to complexity of media. After completion of the experiment, it is very difficult to get the same biopsy sample to repeat the experiments. Above all the hospitals and clinics are not ready to spare the tissue due to ethical reasons and also the state law demands the consent of the patient.

This prompted us to use immortal breast cancer MCF-7 cell lines that was demonstrated to be potential and can be subculture reasonably for good number of times. As mentioned in the introduction of chapter 1 MCF-7 cells were the source of much of current knowledge about

breast cancer [41], along with two other breast cancer cell lines, T-47D and MDA-MB-231. MCF-7 cell lines are easier to work with, attach to plates or flasks and can be easily subcultured. These cells are estrogen, progesterone receptor positive and responsive to estrogen for proliferation. The cells were capable of developing tumours with estrogen primed mice cells. These cells also suffer from disadvantage is that they might have undergone genetic alterations and their behavior *in vitro* may not represent an *in vivo* situation.

In addition we use lung A549 adenocarcinoma human alveolar basal epithelial cells as a control cells. Under *in vitro* conditions the cells grow as monolayer attaching to the culture flasks [59]. These cells are estrogen, progesterone receptor negative and estrogen non-responsive. These cells can be easily cultured under in vitro conditions can be subcultured in plates.

2.1 Culturing of Cells

Materials

Human breast cancer cells (MCF-7) and Human lung epithelial cells (A549) were purchased from NCCS (Pune, India), Fetal Bovine Serum (FBS), Penicillin, Streptomycin, Glutamine, Dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, Dulbecco's Modified Eagle Medium (DMEM), Trypsin, EDTA, Ethanol and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT**)** were purchased from Himedia (Mumbai, India), T-75 culture flasks, 6 wells, 12 wells and 96 wells plate culture plates, Sterile disposable pipettes, were purchased from Tarson (Bangalore, India). Sense and antisense primers oligonucleotides (15-20 mer) were obtained from Sigma-Aldrich (St Louis, USA), Taq DNA polymerase, Deoxynucleotide triphosphates (dNTPs), and $MgCl_2$ were purchased from Merck (Mumbai, India) SuperScript III First-Strand Synthesis System was obtained from Invitrogen BioServices India Pvt. Ltd (Bangalore, India).

Buffers:

TAE buffer (50X): 242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA pH 8.0 made up to 1 L, autoclaved and stored at 4 ^{o}C.

IX TAE buffer: 20 ml 50 X TAE Buffer and made up to 1 L by distilled water.

10X Formaldehyde Agarose Gel buffer: 200 mM 3-[N-morpholino] propane sulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0 with NaOH. Prepared in RNase-free (DEPC) water and stored at 4 ^{o}C.

1X Formaldehyde Agarose Gel Running Buffer: 100 ml 10X Formaldehyde Agarose gel buffer mixed 20 ml 37 % (12.3 M) formaldehyde and made up to 1 L by DEPC water.

RNA/DNA Gel loading dye (6X): 10 mM Tris-HCl, 50 mM EDTA, 30 % glycerol, 0.25 % Bromophenol blue and 0.25 % Xylene cyanol FF were added and prepared by mixing in DEPC water.

Ethidium bromide (EtBr) (10 mg/ml): 1 g of EtBr is added in 100 ml sterile water and stirred on magnetic stirrer for 6−8 hr for complete dissolution and stored at 4 °C in a brown bottle wrapped with aluminum foil.

2.2 Culturing of MCF-7/A549 cells and maintenance

(a) Standardization of animal cell culture

The animal cells were cultured in a basal medium containing with or without serum. The successful growth and maintenance of animal cells under *in vitro* condition requires culture environment that mimic *in vivo* condition with respect to temperature, oxygen, carbon dioxide, pH, osmolarity, and including the components that provide nutrition. Many of these requirements were met by a culture medium composed of simple low molecular weight

components generally referred to as a basal medium. The basal medium supplemented with serum (fetal bovine serum), provides growth factors that required for growth and proliferation of cells. Basal media supplemented with serum said to be complete media. The cells also require 5 % carbon dioxide, which was supplied by CO_2 cylinder attached to an incubator.

(b) Preparation of Basal medium

The powdered DMEM (9.6 g) was dissolved in 900 ml of tissue culture grade water with constant, gentle stirring. The pH of the medium was adjusted to 7.0. Sodium bicarbonate (49.5 ml of 7.5 %) and 20 ml of 200 mM L-Glutamine solution was added to the medium. The medium was autoclaved at 121 ºC at 15 psi for 15 min. The volume was made up to 1 L with tissue culture grade water. The medium was cooled to room temperature. Filter sterilized antibiotics Streptomycin and Penicillin (to a final concentration of 100 Units) were added before use.

In addition, commercially available complete, sterile RPMI-1640 media is also used in our studies. The complete sterile media is mixed with heat inactivated FBS to a final concentration of 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine were added.

(c) Reviving of frozen cells (Thawing of cells) for cell culture

MCF-7 and A549 cells were revived as per the protocol described earlier [184]. A frozen vial containing cells was taken out of liquid nitrogen and allowed to thaw at 37 °C. Cells were resuspended in 1 ml of RPMI-1640 and centrifuged at 3000 rpm to remove traces of DMSO. Pellet containing cells was suspended in RPMI-1640 medium and transferred to T-25 culture flask and incubated at 37 °C in a CO_2 incubator. The medium in culture flask was changed every three days, till they become 90 % confluent and ready for subculture. Sufficient number of cells were frozen in vials and stored as per the protocol discussed in 2.1(d) for the subsequent studies.

(d) Freezing of cells for long time storage

Both MCF-7 and A549 cells in 90 % confluent T-75 flasks were treated with 3 ml of 0.25 % trypsin in RPMI-1640 medium for 1 min by swirling up and down, scrapped using scrapper and carefully resuspended and diluted in 30 ml of RPMI-1640 medium to minimize the action of trypsin and centrifuged at 1500 rpm, washed twice to remove traces of trypsin. Cell pellet was resuspended in RPMI-1640 medium containing 10 % FBS and 15 % DMSO and transferred to freezing vials with proper labeling. Vials were kept on ice for 30 min, transferred to –20 ^{o}C freezer and kept overnight. Next day the cells were transferred to -70 ^{o}C freezer and kept for 48 hr and finally transferred to liquid nitrogen container for long storage.

(e) Sub culturing of cells in microplates for treatment

Both MCF-7 and A549 cells were subcultured in culture plates for the treatment. The medium was removed from 90 % confluent T 75 flask, washed with sterile Dulbecco's phosphate buffer saline (DPBS) and treated with 3 ml of 0.25 % trypsin in RPMI-1640 medium for 1 min to dislodge the cells, scrapped using the sterile scrapper and carefully transferred to 50 ml Falcon tubes. The cells were diluted with 30 ml RPMI-1640 medium, washed twice with the medium to remove last traces of trypsin and counted using cell counting chamber. The cells were suspended in RPMI-1640 medium containing 10 % FBS. Medium containing (2×10^5 cells/wells) were seeded into 6 wells plate for treatment and RNA isolation or 2 to (4×10^3 cells/wells) were seeded into 96 wells plate for treatment and MTT assay. The cells in the plates were incubated in carbon dioxide incubator for different time intervals and processed. The cells were subcultured (1:3/1:4 ratio) in a T-75 tissue culture flask if large number of cells required for the treatment. Media were changed every three

days and after 90 % confluence the flasks were ready for subculture. Animal cell culture laboratory is considered as a sterile zone and hence was fumigated quite often, with the limited accessibility to avoid cross contamination. Good laboratory practice was followed using all precautions and aseptic conditions for the maintenance and sub-culturing of cell lines in the laboratory. The temperature, humidity and pressure were monitored in the incubator on day to day basis and maintained.

f) Study of cell morphology, proliferation and validation

The normal cell morphology and confluency of cells in cell culture plates/T-25/T-75 flasks were observed using inverted microscope. Contaminated plates/flasks were discarded.

A549 cells were cultured in different plates using RPMI-1640 medium supplemented with antibiotics, then the cells were incubated in CO_2 incubator for different periods (1-6 days) and every day number of cells present were determined by trypan blue counting method using Neubauer counting chamber.

g) Treatment of culturing of cells

TheMCF-7 cells or A549 cells were grown in RPMI-1640 medium with 10 % Fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-Gslutamine with phenol red in a humidified atmosphere at 37 °C by passing 5 % CO_2. The cells seeded into culture plates were incubated overnight for the attachment and later treated with or without modulators in the RPMI-1640 medium containing without phenol red. The phenol red was avoided in cultures during treatment as it appears to exhibit weak estrogen activity. The plate containing cells were further incubated for different time intervals as indicated in different sections and processed further for MTT/RNA isolation.

2.3 Cell growth and viability Assay

Cancer cells (MCF-7/A549) following the treatment trypsinized, washed with PBS and suspended in a known volume of PBS. Cell growth and viability was analyzed by cell counting method and MTT assay as per the protocol described below.

Trypan blue dye exclusion and cell count

Trypan blue dye exclusion test is a simplest and economical method used to assess and measure the number of viable cells present in a cell culture suspension following the treatment. It is based on the principle that all the live cells contain intact cell membrane and exclude trypan blue, while dead cells take up the dye. Live and dead cells were counted in a Neubauer counting chamber. The counting chamber is a modified microscope slide with a polished chamber with known depth and displays ruled grid. The grid consists of nine primary squares 1 mm each side (area 1 mm^2) having two-three closely spaced lines (2.5 µm apart). These lines help the cells to be counted. The primary square consisted of more lines that help to align the microscope field to the chamber to be counted. The plane of grid lies 0.1 mm below two ridges which support the coverslip. The depression on the outer edge of each polished surface helps to add cell suspension. The liquid will be drawn into the chamber by capillary action and the cells were counted on all the four corner side squares (64 squares) present in the chamber. The cells in culture plates after trypsin treatment and PBS wash, suspended in known volume of PBS (pH 7.4). Cell suspension was mixed with 0.4 % trypan blue solution in 1:1 ratio, gently mixed and incubated for 2 min. The dye treated cells were filled into a Neubauer counting chamber and allowed for 2 min to distribute the suspension uniformly in the cavity of the chamber. Cells were visually examined under inverted microscope using 10 X objective to confirm whether cells were taken up or excluded the dye. Viable cells look clear with transparent cytoplasm, whereas nonviable cells look blue. Trypan blue dye solution was filtered to remove suspended particles before use, as the particles may disturb the counting process. The cells were counted immediately and a

prolonging counting (>30 min) may result in false counting due to cell death by dye toxicity [185].

The viable cell number/ml of cell suspension was calculated using the formula:

$$C = n/v$$

Where C = cell concentration, n = number of cells counted, v = volume (ml) represented by the grid.

$V = 1 \times 1 \times 0.1\ mm^3$

$= 0.1 \times 10^{-3} ml$

No. of viable cells/ml = Total no. of cells counted × Dilution factor

2.4 MTT assay

MTT assay is a simple assay that measures the reduction of yellow 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial NADH + H^+ and succinate dehydrogenase. Following the treatment the MTT crosses the cell membrane and later enters into mitochondria. In the mitochondria they are reduced to insoluble, dark purple coloured formazan. The product formazan were solubilised in DMSO and measured spectrophotometrically at 540 nm. Reduction of MTT occurs only with metabolically active cells and hence OD is a measure of viability of cells.

(a) Standardization of number of cells required for MTT assay

To measure and validate the conversion of MTT by mitochondrial succinate dehydrogenase to blue formazan, we seeded different number of MCF-7 cells ($1x10^3$–3.2×10^4 cells/wells; n = 8) in 200 µl of RPMI-1640 medium into different wells of 96-wells plate, allowed to grow overnight in the carbon dioxide incubator. Then freshly prepared 0.5 % MTT (20 µl) in PBS was added to each well and incubated further for 4 hr at 37 °C in CO_2 incubator. The blue formazan products formed in cells were dissolved in 200 µl DMSO and

spectrophotometrically measured at 540 nm. The number of viable cells were calculated and represented graphically.

(b) MTT assay of treated cells

Based on the results obtained in section (Fig. 2.4 a), the number of cells (MCF-7/A549 cells) used for all the subsequent treatments for MTT assay was found to be between 2000 and 4000 cells. MCF-7 or A549 cells (3.5×10^3 cells/well) in 200 µl of RPMI-1640 were seeded into 96-wells plate and incubated overnight at 37 °C with the supply of 5 % CO_2. The cells were treated with and without modulators, incubated further for 48/72 hr in carbon dioxide incubator. Cells were washed with PBS and treated with 0.5 % MTT (20 µl) in PBS, incubated further for 4 hr at 37 °C in CO_2 incubator. The blue formazan products formed in cells were dissolved in 200 µl DMSO and spectrophotometrically measured at 540 nm. The proliferative effect/toxicity effect of modulators were calculated and represented graphically.

2.5 RNA isolation

(a) Procedure

Isolation of total RNA is an initial step and critical for performing subsequent molecular techniques such as c-DNA synthesis, RT-PCR, sequencing, northern analysis etc. There are three basic methods used for isolating total RNA from cells and tissues namely, Guanidium-CsCl method, Guanidium acid phenol method and non-phenol glass fibers method. Although initially we tried Guanidium thiocynate which was found to be cumbersome, considering the processing of large sample number and to reduce laborious procedure, RNA isolation using TRIzol reagent was adapted. This method is quick and reasonably high quality total RNA can be obtained, compared to above conventional methods. TRIzol RNA isolation method involves three major steps, the extraction, precipitation and finally the purification. RNA is

highly unstable, subjected to degradation by RNases, and hence requires extra care during processing including storage. We followed all aseptic conditions and good lab practice to culture and isolate the good quality of RNA from cancer cell lines.

(b) Standardization of RNA Isolation

All the glasswares and eppendorff tubes were treated with DEPC treated water and autoclaved before used for RNA isolation to avoid RNA degradation. TRIzol reagent manufactures protocol was followed for isolation of total RNA with minor modification. In brief, overnight cultures of MCF-7 cells or A549 cells (3×10^5 cells/well) were seeded into 6-wells plate and treated with or without modulators for 48 hr. Both control and treated MCF-7/A549 cells overnight cultures were washed twice with sterile PBS and TRIzol reagent (1 ml) was added. The plates were incubated in ambient temperature for 1.5 min and mixed by rotating the plates ensuing complete dissolution. The content of each well was transferred quantitatively to separate Eppendorf tubes. Chloroform:Phenol (1:1) mixture (1 ml) was added, mixed by gentle vortexing for 15 sec. The tubes were incubated for 5 min at room temperature, and centrifuged at 12,000 rpm for 15 min at 4 °C in a refrigerated centrifuge. To the supernatant aqueous phase 0.5 ml of isopropanol was added, mixed and incubated for 3 min. Tubes were centrifuged at 10,000 rpm for 8 min at 4 °C. To the RNA pellet 0.5 ml of ethanol and 100 µl of 1.3 M sodium acetate was added and incubated for 2 min at room temperature. The tubes were centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet was washed with 70 % ethanol by centrifuging at 2,000 rpm for 5 min. The pellet was air dried for 15 min and dissolved in 50 µl of DEPC treated MQ water. RNA present in the samples was quantitated by spectrophotometry and the quality of RNA was analysed by Formaldehyde gel electrophoresis. RNA was stored at –80 °C until further use.

(c) Quantification of RNA by spectroscopic measurement

Although there are several methods available to quantify RNA present in solutions, the most easy and reasonably accurate quantification can be done by UV spectroscopy. Majority of the laboratories across the globe are using this method and is based on the property of absorption of UV light by bases present in RNA. Nucleic acids also can be quantified by measuring the UV-induced emission of fluorescence from intercalated ethidium bromide. This method is useful only when there are no enough DNA/RNA samples available to quantify using spectrophotometer. Spectrophotometric technique for quantification of DNA/RNA is based on the principle of Lambert Beer's law. RNA sample is preferably treated with DNase before quantification as DNA gives the maximum absorbance at 260 nm wavelength. Absorbance of RNA is taken at two wavelengths i.e. 260 nm and 280 nm. OD at 260 nm gives the quantity of RNA present/ml sample and the ratio 260/280 gives the idea about the quality of the RNA sample. Freshly prepared RNA (1 µl) is diluted with 499 µl sterile DEPC treated water (1:500 dilution) in quartz cuvette, absorbance was taken at 260 nm and also at 280 nm and sterile DEPC treated water was used as a blank.

1 OD at 260 nm for RNA = 40 µg of RNA/ ml.

The OD at 280 nm gives the amount of protein present in the sample. Pure preparation of DNA/RNA will have ratio of OD_{260}/OD_{280} value of 2.0. If the samples show contamination with protein/ phenol, the ratio will be decreased to 1.7 and the samples which show less than 1.7 and any value significantly less than 1.7 considered to be contaminated with protein and the RNA samples must be subjected further to chloroform phenol extraction or RNA samples have to be discarded.

(d) Quality assessment of total RNA by formaldehyde gel electrophoresis

Integrity of isolated total RNA is best determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. Formaldehyde gel electrophoresis was carried out

as per the protocol described earlier [186]. RNA has the tendency to form both secondary and tertiary structures that may hamper the separation of different species of RNA by electrophoresis. As it is known that, identical species of RNA exhibit varying degrees of intra-molecular base- pairing migrates at different rates and result in smear of distinct RNA molecules. Consequently, the electrophoresis of RNA needs to be performed under denaturing conditions. Heat denaturation of the RNA sample prior to electrophoresis is not sufficient, as secondary structure will reform, unless a denaturing system is used in the agarose gel and in electrode buffer. Hence successful electrophoresis of RNA is accomplished by denaturing the RNA prior to electrophoresis and also during electrophoresis by the addition of formaldehyde and formamide to gel as well as electrode buffer for superior resolution. Formaldehyde is a good denaturing agent, denatures RNA, RNA resolution is better in formaldehyde gel and MOPS buffer.

The formaldehyde agarose (1 %) gel was prepared by using 10X Formaldehyde agarose gel buffer. (200 mM 3-[N-morpholino] propane sulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0). Equal quantity of RNA (4 µl) samples was taken in a Eppendorff tube and mixed with 1 µl of 5X loading buffer (16 µl saturated aqueous bromophenol blue solution, 80 µl 500 mM EDTA, pH 8.0, 720 µl 37 % formaldehyde, 2 ml glycerol, 3.84 µl formamide, 4 ml of 10X Formaldehyde Agarose gel buffer made up to 10 ml with RNase-free water) and incubated for 3–5 min at 65 °C, chilled on ice, and loaded on to equilibrated 1.2 % Formaldehyde Agarose gel. Electrophoresis was carried out in 1X Formaldehyde Agarose Gel Running Buffer, at 60 volts for 2 hr.

For standardization of formaldehyde gel electrophoresis conditions three RNA samples were separated on both formaldehyde gel as well as 1 % normal agarose gel. For the electrophoresis of normal agarose 1X TAE buffer was used. Both formaldehyde gel and agarose gel were stained with 0.005 % ethidium bromide and RNA bands were visualized in

UV transilluminator. Photographs were taken in Alpha Imager Gel Documentation ssystem (CA, USA).

2.6 Standardization of semi quantitative RT-PCR

(a) cDNA synthesis

The cDNA synthesis was carried out using SuperScript III First-Strand Synthesis System obtained from Invitrogen life technologies as per the protocol provided by manufacture to synthesize cDNA. The SuperScript III First-Strand Synthesis System for RT-PCR is optimized to synthesize first-strand cDNA from purified total RNA. The amount of total RNA used varies from 1–5 µg. The SuperScript III Reverse Transcriptase used was a version of M-MLV RT that was engineered by manufacturer to reduce RNase H activity and provide increased thermal stability. The enzyme used to synthesize cDNA was at a temperature range between 42–55 °C and provides increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. Because SuperScript III RT is not significantly inhibited by ribosomal and transfer RNA, enzyme was used to synthesize first-strand cDNA from a total RNA. The Superscript III First-Strand Synthesis System contains the following components, Oligo (dT), random hexamers, RT-Buffer, MgCl2, DTT, dNTP mix, Super Script III RT, RNase OUT, *E.coli* RNase H, DEPC treated water.

The cDNA synthesis was carried out using total RNA primed with oligo (dT) or, random primers and it involves following steps, denaturation, annealing, cDNA synthesis and termination of reaction including removal of RNA. Total RNA (1 µg-5 µg), primers (10 pm) and dNTP's (10 mM) were taken in a total volume of 10 µl in eppendorf tube and incubated for 65 °C for 5 min for denaturation. Immediately eppendorff tube containing above components was placed on ice for 1 min. Then cDNA synthesis mix (10 µl) was added and incubated for 50 °C for 50 min. The reaction was terminated by incubating at 85 °C for 5 min

and RNA removed by adding 1 µl of RNase H at 37 °C, incubated for 20 min. The cDNA product can be stored at 4 °C until further use.

(b) Standardization of Polymerase Chain Reaction:

Polymerase chain reaction (PCR) is the powerful method used for *in vitro* DNA synthesis for cloning genes and for semi quantification of mRNA expression. Large amount of specific targets of DNA with defined length and sequence can be synthesized from a small amount of DNA template. PCR is a rapid, sensitive and less expensive procedure for amplifying DNA of specific interest. The principle of PCR is simple and involves enzymatic amplification of a DNA fragment flanked by two primers (oligonucleotides) hybridized to opposite strands of the template with the 3′ ends facing each other. DNA polymerase synthesizes new DNA starting from 3′ end of each primer. Repeated PCR cycles of heat denaturation of the template, annealing of the primers and extension of the annealed primers by DNA polymerase results in amplification of the specific DNA fragment. The extension product of each primer can serve as a template for the other primer resulting in essentially doubling the amount of the DNA fragment in each cycle. Final result would be amplification and exponential increase in the amount of specific DNA fragment defined by the 5′ ends of the primers [187].

(c) The cycling reactions

There are three major steps (denaturation, annealing and extension) in PCR and steps to be repeated 30 or 40 times called as cycles. PCR was carried out on an automated thermocycler, which heats and cools the tubes containing reaction mixture rapidly. During denaturation at 94 ^{o}C the double stranded DNA melts to single stranded. Annealing temperature varies and different for different primers, usually 3 °C less than Tm of primers used, which forms

Watson crick base pairing between complimentary sequences of primer and template DNA. The polymerase attaches and starts copying the template in the extension step at 72 °C

(d) Semi quantitative PCR

The cDNA was subjected to 30 cycles of PCR in a gradient Eppendorf thermocycler using different forward and reverse primers of modulator genes with different specific annealing temperatures. The β-actin primer was used separately to amplify actin and used as a positive control for normalization. First-strand cDNA (1 µl) were mixed with 2 µl of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 µl 50 mM $MgCl_2$, 1 µl each of 10 pico moles of forward and reverse primers, 1 µl 10 mM dNTP's and 1 unit Taq DNA polymerase in a final 20 µl reaction volume. PCR amplification conditions used were initial denaturation at 94 °C for 5 min followed by 30 cycles with 94 °C for 1 min, annealing temperatures (different but specific for different set of primers) for 1 min, elongation at 72 °C for 50 sec, and a final extension cycle at 72 °C for 7 min. Amplified PCR products were analyzed by electrophoresis using 1 % agarose gels and 1X TAE Buffer.

(e) Standardization of annealing temperature and PCR cycles for amplification of β-actin gene

Total RNA isolated was used to set the RT-PCR reaction for amplification β-actin gene as above and the reaction mixture taken in different tubes were kept at different temperatures (56, 58, 60 and 62 ^{o}C). After 30 cycles the tubes were removed from PCR machine and processed on 1 % agarose gel electrophoresis. Similarly the different tubes were set to incubate the tubes at different PCR cycles (25, 30 and 35 cycles) at annealing temperature of 60 °C and the tubes were analyzed on 1 % agarose gel as per the protocol described in 2.4.f section.

(f) Agarose gel electrophoresis

Principle

Electrophoresis is a most widely used technique in biochemistry to analyze proteins/nucleic acids based on its size and charges present on the molecule. In contrast to protein, that may have either a net positive or negative charge, the nucleic acids have a negative charge imparted by phosphate backbone, migrates towards positively charged anode.

Procedure

Agarose powder (1 g) was taken in a 250 ml conical flask. Agarose (1 %) gel was prepared using 1X TAE and 0.05 % ethidium bromide. Gel was placed in the electrophoresis tank and sufficient TAE buffer (1X) was added to the tank to cover the gel. Gel loading dye (5 µl of 6X) was added to each of 30 µl PCR reaction completed tube. The order of each sample loaded on to the gel was recorded. Carefully each sample (7 µl) was pipetted along with the loading dye and loaded on to separate wells in the gel. DNA ladder standard (2 µl) was loaded on to one of the wells along with the dye. The electrode wires of the tank were connected to power pack with positive and negative cords. The power supply was turned on and maintained at 100 volts. Electrophoresis of DNA was continued until the dye front approaches all most bottom of the gel. Carefully the gel was removed and analyzed using transilluminator using ultraviolet light.

Results

Standardization of culture conditions for MCF-7 and A549 cells:

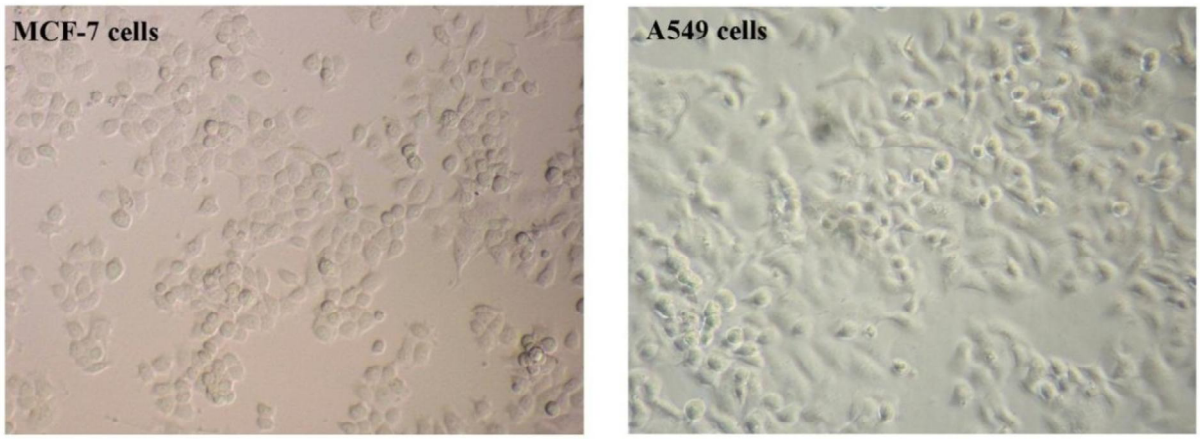


Fig. 2.1 Microscopic photograph of (a) MCF-7 cells (b) A 549 cells

The microscopic picture of MCF-7 (Fig. 2.1a) and A549 cells (Fig. 2.1b) show normal cell morphology

The conditions were standardized for MCF-7 and A549 cells and the results show that both the cells were grown in RPMI-1640 medium. MCF-7 cells require 4–5 days and A549 cells require 2–3 days for subculturing. The culture conditions were also standardized to grow both the cells without phenol red and FCS, and all the subsequent experiment were carried out without phenol red and FCS. Table 2.1 shows some important properties exhibited by MCF-7 cells and A549 cells.

Table 2.1 Some of the properties exhibited by MCF-7 and A549 cells

SL No	Cell property	MCF-7 cells	A549 cells
1	Cell type	Breast	Lung
2	Characteristics	Epithelial	Epithelial
3	Tissue origin	breast ductal carcinoma	Adenocarcinomic human alveolar
4	Media	RPMI-1640	RPMI-1640
5	Morphology	Irregular Epithelial	Epithelial
6	Doubling time	32 hr	22 hr
7	Subculturing	3-4 days	2-3 days
8	Culture conditions	Temp: 37 °C; 95% air; 5 % CO2	Temp: 37°C; 95% air; 5% CO2

Cell growth validation of A549 cells

Results show that the A549 cells were increased every day and doubled every 24 hr and suggested that the A549 cells are growing normally and can be used for studying the effect of different modulators (Fig. 2.2).

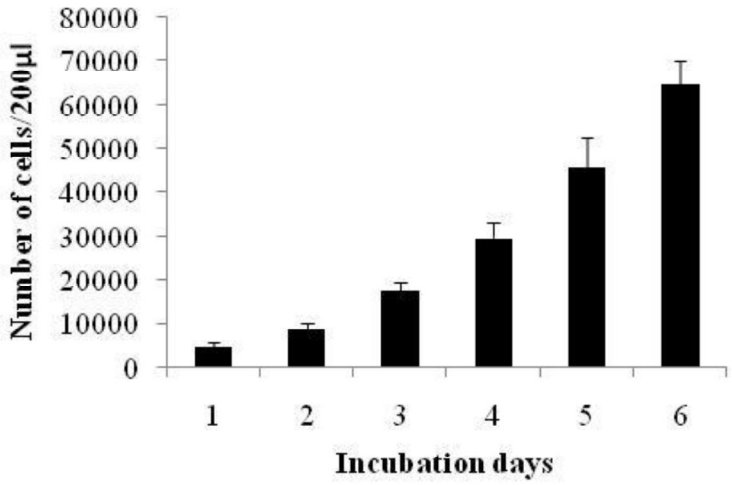


Fig. 2.2 Study of effect of time (days) on normal cell growth of A549 cells

A549 cells were cultured in different plates for different periods (1-6 days) and every day number of cells present were determined by trypan blue counting method.

Validation of MTT assay of MCF-7 cells

The number of cells (1×10^3–3.2×10^4 cells/wells, n = 8) in 96-wells plate grown overnight in carbon dioxide incubator treated with 0.5 % MTT (20 µl) in PBS, incubated further for 4 hr at 37 °C in CO_2 incubator. Blue formazan products formed in cells were dissolved in 200 µl DMSO and OD was measured at 540 nm in a spectrophotometer. OD obtained was represented graphically against number of cells using bar diagram. Results show that the OD increases with increase in number of cells and maximum OD 0.45 was obtained with (3.2×10^4 cells/wells). Based on the results (4×10^3 cells/wells) were used in subsequent experiments (Fig. 2.3).

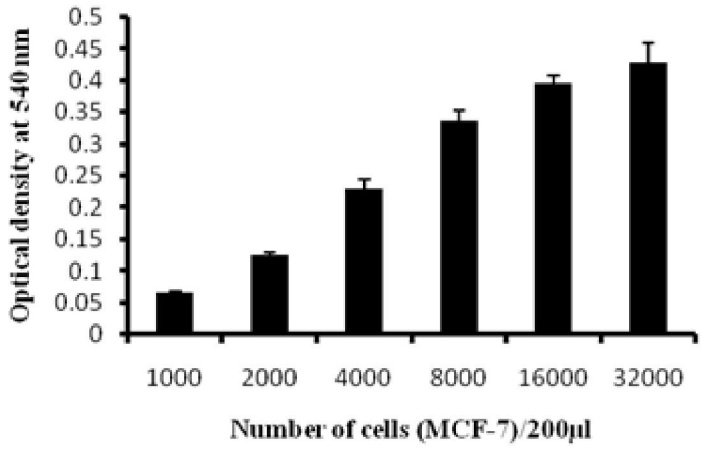


Fig 2.3 Validation of MTT assay for MCF-7 using different number of cells

To measure and validate the conversion of MTT by mitochondrial succinate dehydrogenase to blue formazan, the different number of MCF-7 cells in 200 µl of RPMI-1640 medium into different wells of 96-wells plate. Viability of the cells were analyzed by MTT assay.

Quantification of RNA

The quantification of DNA/RNA is based on the principle of absorbance of nucleic acid that gives maximum absorbance at 260 nm wavelength. Absorbance of RNA is taken at two wavelengths 260 nm and 280 nm to determine the ratio 260/280 nm gives the idea about the quality of the RNA sample and OD at 260nm gives the quantity of RNA present/ml sample.

1 OD at 260 nm for RNA = 40 µg of RNA/ ml.

Pure preparation of DNA/RNA will have ratio of OD_{260}/OD_{280} value of 1.7 to 2.0.

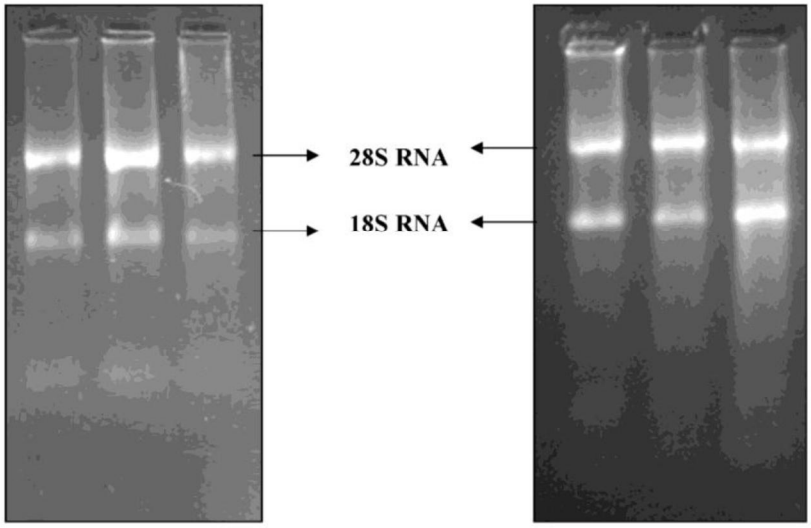
The results show that all the samples found to have all most same OD values at 260 nm and the ratio was found to be above 1.7 at 260/280 nm. The RNA isolated appears to be clean; the cells treated with E2/TMX have no effect on RNA concentration and can be used in subsequent experiments (Table 2.2).

Table 2.2 Absorbance of different RNA samples isolated in one of the typical experiment, listed as a representative table.

Sl. No.	RNA Samples	O.D at 260 nm	O.D at 280 nm	OD_{260}/OD_{280}
1.	Control-1	0.236	0.125	1.89
2.	Control-2	0.238	0.126	1.88
3.	Control-3	0.235	0.126	1.86
4.	E_2Treated- 1	0.238	0.125	1.90
5.	E_2Treated -2	0.236	0.124	1.90
6.	E_2Treated- 3	0.234	0.127	1.84
7.	TMX Treated -1	0.230	.126	1.82
8.	TMX Treated -2	0.232	0.124	1.8
9.	TMX Treated -3	0.236	0.126	1.9

Quality of RNA: Formaldehyde agarose gel electrophoresis

To check the quality of the RNA the RNA samples were separated on formaldehyde agarose gel electrophoresis as well as normal agarose gel electrophoresis of RNA as per the protocol discussed in materials and methods. Results show that the quality of all the three RNA samples were found to be good, as the RNA gave 28 S and 18 S RNA and there was no DNA in the samples as there were no smears (Fig. 2.4b). The bands obtained on formaldehyde gel was sharper and clear (Fig. 2.4b) compared to the gel run on normal agarose gel electrophoresis (Fig. 2.4a). Further RNA samples I and III were found to be degraded and hence difficult to interpret about the quality of RNA samples isolated. Hence all RNAs isolated were checked for purity by using formaldehyde gel electrophoresis.



(a) **(b)**

Fig. 2.4 Electrophoresis of RNA Samples using (a) Agarose (1 %) gel, (b) MOPS-formaldehyde gel (1 % agarose) showing 28 s RNA and 18 s RNA. RNA isolated was found to be intact.

Standardization of RT-PCR

As a representative for standardization of annealing temperature and PCR cycles in qRT-PCR we used β-actin gene. The results show that 60 °C is the optimum temperature required for amplification, however, lower temperatures (56 and 58 °C) gave less intense/diffused bands (Fig. 2.5a). While standardizing the different cycles the 30 cycle numbers gave reasonably sharp band (Fig. 2.5b) and hence 30 cycles were used in our studies. Using equal quantity of three different RNA samples, when analyzed for PCR amplification of actin, all the three samples gave good results (Fig. 2.5c).

The β-actin gene can be amplified at 60 °c and 30 PCR cycles.

For all the other genes studied each RNA made for these genes were standardized using the above technique and results of such a study were listed in table 2.3. For all the genes we used final concentration of 0.5 mM dNTPs and 10 pM of sense and antisense primers and 2.5 mM $MgCl_2$ and 1 U of Taq polymerase as per the instruction given in the kit.

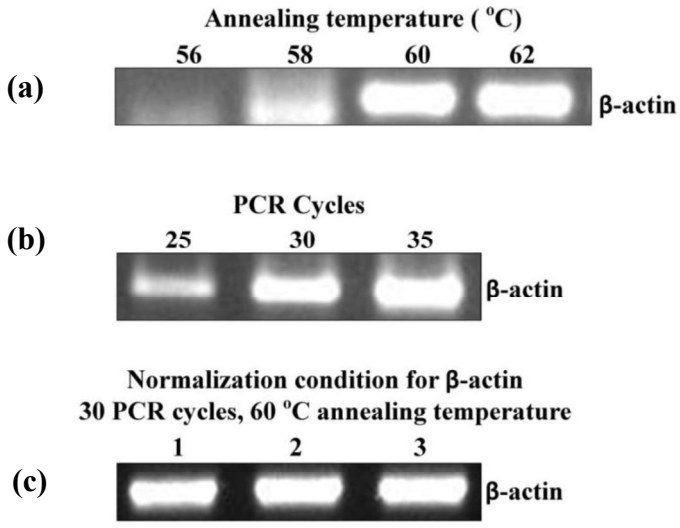


Fig. 2.5 Standardization of annealing temperature and PCR cycles for β-actin gene amplification by qRT-PCR

Table 2.3 Standardization of annealing temperature and PCR cycles for different gene amplified by RT-PCR

Sl. No.	Name of the gene	Annealing temperature (^{o}C)	Amplicon length (bp)	PCR cycles
1.	**c-Jun**	64	525	30
2.	**Jun-D**	64	500	30
3.	**Jun-B**	58	257	30
4.	**c-Fos**	58	577	30
5.	**Fra-1**	60	497	30
6.	**Fra-2**	60	518	30
7.	**FosB**	58	400	35
8.	**ER-α**	61	650	35
9.	**ER-β**	60	528	35
10.	**Cyclin D1**	58	574	35
11.	**Cyclin E1**	60	415	35
12.	**CDK4**	60	912	30
13.	**P53**	56	375	35
14.	**Bcl-2**	62	365	30
15.	**Bax**	61	366	30
16.	**Caspase 8**	58	366	35
17.	**PKCε**	61	449	30
18.	**β-actin**	62	516	30

Discussion

Our objectives of the present study is to understand the role of estradiol-17β, protein kinase C and protein kinase A on the expression pattern of AP-1 factors in Breast cancer cells. Hence we used breast cancer MCF-7 cell lines which are estrogen, progesterone receptor positive and responsive to estrogen for proliferation. MCF-7 cells attach to plates or flasks shows good growth and can be easily sub cultured. In addition we used lung A549 human alveolar lung epithelial cells as control cells as these cells are estrogen, progesterone receptor negative and estrogen non-responsive. Both the cells grow as a monolayer attaching to the culture flasks. The conditions required for culturing of both, MCF-7 and A549 cells were also standardized to grow in RPMI-1640 medium with or without serum and phenol red. In our study, we use estradiol-17β as one of the modulator and therefore conditions have to be standardized for growing cells in media without phenol red. The phenol red acts as a weak estrogen and hence results obtained with estradiol-17β may mask the effect. When the culture flasks become confluent methods were standardized for long time storage of cells in freezing vials in liquid nitrogen or subcultured in 96-wells and 6-wells microplates for treatment with modulators. Methods were also standardized for number of cells to be seeded into 96-wells plate (2 to 4×10^3 cells/well) or 6 wells plate (2×10^5 cells/ well). Trypan dye exclusion method and MTT assay were standardized for measuring the proliferation and cell viability of MCF-7 cells and A549 cells. TRIzol reagent was used for isolation of total RNA and quantification was done by spectroscopic measurement by taking OD at 260 and 280 nm and the quality assessment of total RNA was checked by formaldehyde gel electrophoresis. PCR conditions were standardized using β-actin as standard for annealing temperatures and number of cycles used for optimum results. The β-actin was used for normalization of all the genes used in our studies. Finally the 1 % agarose gel electrophoresis was used to separate different amplified products and photographed using gel doc system.

Summary

MCF-7 and A549 cells culture conditions were standardized for subculturing in flasks, plates and for treatments with and without FBS and phenol red.

Using MCF-7/A549 cells MTT assay, conditions for isolation of total RNA, quantification, RT-PCR techniques were standardized for all the genes used in our study.