Chapter 2

Materials and Methods

2.1 Materials:

2.1.1 Chemicals and Reagents:

All the chemicals used for routine molecular biology work were procured from Sigma-Aldrich Chemicals (St Louis, MO, USA) unless otherwise mentioned. Taq polymerase for PCR and standard DNA markers and protein markers were purchased from MBI Fermentas. Tissue Culture materials like DMEM medium (for A549), Ham's F-12 medium (for HPLD), Opti-MEM medium, 0.5% trypsin-EDTA, 100X antibiotic-antimycotic, freezing medium, fungizone, 200mM L-glutamine, fetal bovine serum (FBS), Lipofectamin-2000 and TRIzol were obtained from GIBCO BRL (Gaithersburg, Maryland, USA). CMRL medium was purchased from ICN laboratories. M-MLV reverse transcriptase, RNase inhibitor, dNTPs and $MgCl_2$ were obtained from Invitrogen Corporation (Carlsbad, CA). ECL western detection kit and Hybond™- P were purchased from Amersham biosciences (GE Healthcare, UK).

2.1.2 Antibodies:

Monoclonal antibody against KRAS were purchased from Merck Research Laboratories, phospho p44/42 (ERK1/2) and total p44/42 (ERK1/2) antibodies were purchased from Cell Signaling Technologies. Anti tubulin antibody was obtained Sgima-Aldrich Chemicals. HRP conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Bangalore Genei Pvt. Ltd.

2.1.3 Cell Lines:

A549 (human lung adenocarcinoma epithelial) and murine fibroblast cell line NIH3T3 was purchased from American Type Culture Collection. Murine lung epithelial cell line E-9 and E-10 were a kind gift from Dr. L.M.Anderson, (NCI-FCRDC, Frederick, Maryland).

Human lung epithelial type II cells (HPLD) were a kind gift from Dr. T Takahashi, Japan. All the cell lines were maintained in 5% CO2 with the recommended media containing 10% FBS (3% FBS for HPLD) and following the standard guidelines.

2.2 Buffers and Media:

Whole cell lysis buffer:

20mM Tris (P^H 7.5)

150mM NaCl

1mM EDTA

1mM EGTA

1 % triton X 100

2.5mM sodium pyrophosphate

1mM β-glycerophosphate

1mM Na_3VO_4

1µg/ml aprotinin, 1µg/ml leupeptin and 1µ.ml pepstatin

SDS-PAGE:

Stacking Gel Mix (4ml, 5%):

380µl of 1M Tris-Cl (pH 6.8)

500µl of 30% acrylamide ; bisacrylamide (29:1) Mix

15 µl of 20% SDS

2.1 ml of milli-Q water

30 µl of 10% APS

5 µl of TEMED.

12% Resolving Gel Mix (10ml):

2.5 ml of 1.5 M Tris-Cl (pH 8.8)

4.0 ml of 30% acrylamide; bisacrylamide (29:1) mix

50.0 µl of 20% SDS

3.35 ml of milli-Q water

100 µl of 10% APS

10.0 µl of TEMED.

2X SDS loading Buffer:

130 mM Tris-Cl (pH 8.0)

20% (v/v) glycerol

4.6% (w/v) SDS

0.02% bromophenol blue

2% DTT

SDS PAGE Running Buffer:

25mM Tris base,

0.2M glycine

1% SDS

Western Blot:

1 x Blotting Buffer (2Litres):

25mM tris base,

0.2M glycine

20% methanol

Phosphate Buffer saline (PBS):

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

Tris Buffer Saline (TBS) (10X):

12.1gm Trizma Base

40.0gm NaCl

Adjust PH to 7.6; make up the volume to 1 lit with milli Q water.

HEPES Buffer Saline:

20 mM HEPES (pH 7.5)

150 mM NaCl

Blocking Buffer:

5% fat free milk or 2% BSA in PBST or TBST.

Stripping Buffer:

100 mM β-mercaptoethanol

2% (w/v) SDS

62.5 mM Tris-HCl (pH6.7)

Luria Broth:

10g tryptone

10g NaCl

5g yeast extract, make up the volume to 1 lit with water.

TB buffer for preparation of competent cells:

10 mM PIPES (free acid)

15 mM $CaCl_2.2H_20$

250 mM KCl

55 mM $MnCl_2.4H_20$

50 x TAE (1 litre):

242 g of tris base

57.1 ml of glacial acetic acid

100 ml of 0.5 M EDTA

Alkaline Lysis Solution 1:

50mM tris-HCl (pH 8.0)

10.0 mM EDTA

50 mM glucose

Alkaline Lysis Solution 2:

0.2M NaOH

1% SDS

Alkaline Lysis Solution 3:

3.0M Potassium acetate

2.3 Experimental Procedures:

2.3.1 Ultra Competent Cells Preparation:

All the salts (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2.2H_2O$) except $MnCl_2$ were dissolved in milliQ water and pH was adjusted to 6.7 with 1N KOH. $MnCl_2$ was dissolved separately in mill Q water. $MnCl_2$ was added drop wise while stirring ($MnCl_2$ if added directly will give a brown color to the solution and precipitate out, hence it needs to be dissolved separately). Solution was then filter sterilized and

stored. To prepare competent cells pre-inoculum was prepared. A single bacterial colony was picked from LB agar plate, inoculated into 3 ml LB medium, and incubated overnight at 37°C temperature with shaking at 200 rpm. 1% of this pre-inoculums was sub cultured in 100 ml LB-broth and incubated at 18°C with shaking until OD at 600nm reached 0.5 - 0.6 (approx.). Culture was kept on ice for 10 min. with constant shaking. Cells were pelleted by centrifugation at 2000 g at 4°C for 8 min. Pellet was resuspended in 40 ml of ice-cold TB buffer. Bacterial suspension was kept on ice for 30 min, re-spun at 2000 g at 4°C for 8 min. Pellet was resuspended in 8 ml of TB buffer in which final concentration of DMSO was 7% and left on ice for 10 min. 100 µl aliquots were made and snap frozen in liquid nitrogen and stored at -80°C.

2.3.2 Preparation of Plasmid DNA by alkaline lysis:

Overnight Grown culture was pelleted by centrifugation at 10,000g at 4°C for 3 min and the supernatant was discarded. Pellet was resuspended in 250 µl of ice-cold alkaline lysis solution 1. 300 µl of alkaline solution 2 was then added and the tube was inverted gently 3-4 times and incubated at room temperature for 5 min. 350 µl of ice cold solution 3 was added and mixed by inverting the tube rapidly for 3 or 5 times. Suspension was incubated on ice for 10 min. Bacterial lysate was spun at 10,000g for 12 min at 4°C. Supernatant was transferred to a fresh tube. 0.4 volume of phenol: chloroform was added to the supernatant and the contents mixed. It was then spun at 10,000g at 4°C for 12 min. Aqueous phase was taken out in a fresh tube and 0.6 volume of isopropanol was added, mixed properly and incubated at room temperature for half an hour followed by spinning at 10,000g at RT for 20 min. Supernatant was discarded. Pellet was washed with 70% ethanol. The tube was stored at room temperature until the

ethanol has dried. The pellet was resuspended in 20 µl of milliQ water and 20 µg/ml RNase added. The tube was incubated at 50°C for 45 min. the tube was vortexed for few seconds. Quality of the plasmid DNA was then accessed by running 1% agarose gel.

2.3.3 Spectro-photometric estimation of nucleic acids

The quantity and purity of nucleic acids was determined by measuring the absorbance at 260 and 280 nm. The concentration of nucleic acids was calculated by taking 1 OD $_{260}$= 50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The purity of nucleic acids was checked by their A_{260}/A_{280} ratio.

2.3.4 Transient transfections in adherent cells

Transient transfection of plasmid DNA in culture cells was performed using Lipofectamine 2000 according to manufacturer's protocol. Briefly, forty million cells were seeded in a 35mm tissue culture dish, one day before transfection. Transfection was performed 18-24 hrs after seeding the cells. 4µg DNA was mixed in 50µl of Opti-MEM in one eppendorf tube. In another tube, 5µl of Lipofectamine 2000 was diluted in 50µl Opti-MEM and incubated at room temperature for 5 minutes. After five minutes, DNA and Lipofectamine 2000 were mixed together and complexes, incubated for 30 minutes at room temperature. Meanwhile, the adherent cells were washed twice with PBS and 1ml of Opti-MEM was added. 100µl of complexes were then added to each dish containing cells and medium. After 6hrs, the medium containing complexes was removed and complete medium was added and transgene expression was accessed 24-48 hrs after transfection.

2.3.5 Total RNA isolation from cultured mammalian cells:

Total RNA was isolated by TRIzol method using the manufacturer's protocol. Briefly, medium was removed, from 35mm dish and 1ml to TRIzol was added directly to the dish and kept at room temperature for 5 minutes. The cells were harvested by pipetting up and down three four times and transferred to a 1.5ml microfuge tube. For each 1ml TRIzol, 200µl of chloroform was added and tubes were shaken vigorously for 10 seconds to completely dissociate the nucleoprotein complexes, followed by vortexing for about 30 seconds. The mixture was kept for 3-5 minutes at room temperature and then centrifuged at maximum speed for 10 minutes. The upper aqueous phase was transferred into a fresh microcentrifuge tube and 500 µl of isopropanol was added and this was incubated at -20°C for 1 hour. The RNA was pelleted by centrifugation at maximum speed for 30 minutes at 4°C. The supernatant was decanted and the pellet washed with 1ml of 70% ethanol followed by a second wash with 1ml of 90% ethanol and centrifugation at maximum speed for 10 minutes. The supernatant was removed and the pellet air-dried for about 5 minutes and re-solubilized in 30-50 µl RNase free deionized (DEPC-treated Milli-Q) water and aliquots were stored at -70°C.

2.3.6 Semi-quantitative RT-PCR:

1 µg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MLV reverse transcriptase (Invitrogen) according to manufacturer's protocol. Briefly, a 20µl reaction volume was made for 1µg of RNA. In a microcentrifuge tube, 1µl oligo (dT)(500µg/ml) , 1µg total RNA, 1µl 10mM dNTP mix and sterile water was added to a final volume of 13µl. The mixture was then incubated at 65°C for 5 minutes ad quickly chilled on ice. To this mixture were added 4µl of 5X first strand buffer and 2µl of 0.1M

DTT. Then contents were then mixed and 1µl (200 units) of M-MLV was added. The mixture was then incubated at 37°C for 50 minutes. The reaction was stopped by incubating the mixture at 70°C for 15 minutes. The cDNA thus prepared was then used as a template for PCR. The expression of the investigated genes was determined by normalizing their expression against the expression of actin or GAPDH gene.

2.3.7 Lysate Preparation for Immuno- blotting:

For adherent cells from which lysates have to be prepared , culture medium was removed and cells were washed with ice cold 1X PBS twice and then scraped with cell scraper in Cell Lysis buffer. Cells were rotated at 4°C for 30min at cold room and centrifuged at 13000 rpm for 10min at 4°C. The supernatant was collected and protein concentration was estimated using BCA assay. For standard western, 50-70µg of protein was loaded on to the gel.

2.3.8 Immunobloting :

The proteins were resolved using denaturing SDS-PAGE gel and after completion of the run, the gel was over laid on a nitrocellulose paper cut to the size of gel and kept in the blotting cassette in the presence of blotting buffer. Finally the cassette was put in the mini transblot apparatus (Bio Rad) and blotting was done for 4 hours at a constant voltage of 60 V. Then the membrane was taken out and rinsed in PBS containing 0.1% Tween - 20 (PBST) for 5 minutes by gentle shaking. Later the membrane was immersed in 5% non-fat milk solution in PBST with gentle shaking for 1 hour at 37°C. The membrane was washed off from the traces of the fat free milk with PBST and the membrane was over laid with primary antibody diluted in PBST for 3 hours at 4°C with shaking. After incubation the membrane was washed with PBST and layered with

appropriate secondary antibody (conjugated with horse-radish peroxidase) diluted in 5% fat free milk solution (in PBST) and incubated for 45 minutes at room temperature. After incubation the membrane was washed and processed for the detection of protein bands using ECL-plus detection reagent (Amersham Biosciences) followed by detection of signal on X-ray film (Hyperfilm-ECL, Amersham Biosciences).

2.3.9 Site Directed Mutagenesis

The following K-Ras-ras mutants were generated by site directed mutagenesis according to the protocol described in QuickChange site directed mutagenesis kit (Stratagene). The primers used are shown in the following table:-

Mutation	**Primers**
Codon12(Asp) GGT→ GAT	GTT GGA GCT GAT GGC GTA GGC GCC TAC GC CAT CAG CTC CAA C
Codon12 (Cys) GGT→ TGT	GTA GTT GGA GCT TGT GGC GTA GG CCT AC GC CAC AAG CTC CAA CTAC
Codon 13 (Val) GGC→ GTC	GGA GCT GGT GTC GTA GGC AAG CTT GCC TAC GAC ACC AGC TCC
Codon13 (Cys) GGC→ TGC	GGA GCT GGT TGC GTA GGC AAG CTT GCC TAC GAC ACC AGC TCC
Codon 61 (Arg) CAA→CGA	CAC AGC AGG TCG AGA GGA GTAC GTA CTC CTC TCG ACC TGC TGT G

The reaction mix included 2µl of PSKll(39+) (50ng) containing wild type *K-Ras* cDNA , 5µl 10x buffer, 20pmoles of primers , 1µl of 10mM dNTP mix and 1µl of deep vent polymerase (NEB).

The PCR parameters were as follows:

Segment	Cycles	Temperature	Time
1	1	95^0C	30 seconds
2	14	95^0C	30 seconds
		55^0C/58^0C	1 minute
		68^0C	7. 30 minutes (2 minutes/Kb of plasmid length)
3	1	68^0C	2 minutes

After PCR, 1 µl of Dpn1 enzyme (10U/µl) was added to the amplification mix and incubated at 37°C for 6hours. After that, 10µl of the amplification mix was taken to transform Dh5α cells. Positive clones were selected after confirming the sequence of plasmid DNA.

2.3.10 Growth Curves:

Growth curves were prepared for various cell lines using the modified method adopted by Serrano et al, 1997. Briefly, 10, 000 cells were seeded in a 24 well plate in quadruples. At the indicated times, cells were washed once with PBS and fixed in 10% formalin for 20 minutes and rinsed with distilled water. Cells were stained with 0.05% crystal violet for 30 minutes, rinsed extensively and dried. Cell associated dye was extracted with 1.0ml acetic acid. Aliquots were diluted 1:4 with water and transferred to 96 well microtitre plates and the optical density at 590nm determined. Values were

normalized to the optical density at day 0 for the appropriate cell type. Growth curve was determined twice.

2.3.11 β-gal assay in transfected cells:

2.3.11.1 Solutions:

• Lysis Buffer: 0.1% Triton X-100/0.1 M Tris-HCl (pH 8.0).

450 ml distilled water

50 ml 1M Tris-HCl (pH 8.0)

0.5 ml Triton X-100 detergent

• 100X Mg^{++} solution:

0.1 M magnesium chloride

4.5 M 2-mercaptoethanol

Stored at 4°C.

• 0.1 M sodium phosphate (pH 7.5)

41 ml 0.2 M Na2HPO4

9 ml 0.2 M Na H2PO4

50 ml distilled water

• 4 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside) in

0.1 M sodium phosphate (pH 7.5) containing 2 mM β-mercaptoethanol,

Stored at –20°C.

• 0.1 mg/ml β-gal standard: 0.1 mg/ml β-gal in 0.1 M sodium

phosphate (pH 7.5) containing 2 mM 2-mercaptoethanol

Stored at 4°C.

• 1 M sodium carbonate in water

2.3.11.2 Procedure:

β- galactosidase assay was performed in a 96 well format. Briefly, 4000-5000 cells were plated in 96 well tissue culture coated plate. Cells were transfected with reporter plasmid after 18 -24 hrs and after 48 hrs the cells were washed once with D-PBS. 50µl of lysis buffer was added to the well and cells were lysed by freezing plate at -70°C and thawing at 37°C. Cells were pipette up and down and then the plate was centrifuged at 9000 X g for 5 minutes. The supernatant from each plate was transferred to clean eppendorf tube.

Immediately prior to assay the **ONPG cocktail** was prepared as below:

47 µl 0.1 M sodium phosphate (pH 7.5)

22 µl 4 mg/ml ONPG

1 µl 100X Mg solution

30µl of each well extract was added to microtitre well plate and 70µl of ONPG cocktail was added to each well. The plate was kept on ice throughout the procedure.

After addition of ONPG cocktail the plate was transferred to 37°C and the development of colour was monitored every 10 minutes for development of color. After development of yellow colour, the reaction was stopped by addition of 150µl of 1M sodium carbonate to each well.

2.3.12 *In situ* β-gal staining of Transfected Cells

This protocol is for the detection of β-gal expression in fixed cells. It was performed on 96-well plates for initial screening of tTA transfected clone, and is a modification of Sanes *et al.,* 1986.

2.4.12.1Stock Solutions:

20 mg/ml X-gal in dimethylformamide

Solution A as 40 mM potassium ferricyanide.

Solution B as 40 mM potassium ferrocyanide.

Solution C as 200mM magnesium chloride.

10X fixative (20% formaldehyde; 2% glutaraldehyde in 10X PBS)

10X PBS as 0.017 M KH_2PO4, 0.05 M Na_2HPO4, 1.5 M NaCl, pH 7.4

2.4.12.2 Working Solutions:

1X PBS diluted in distilled water

1X fixative solution diluted in distilled water

2.4.12.3 Staining Solution

25 µl Solution A

25 µl Solution B

25 µl Solution C

125 µl 20 mg/ml X-gal in DMF

2.3 ml 1X PBS

2.3.12.4 Procedure**:**

$4x10^3$-$5x10^3$ cells were plated in 96 well plate, well. Cells were transfected with reporter plasmid 18 -24 hrs after plating. After 48 hrs, cells were washed once with PBS

and fixed with 100µl of fixative solution per well, for 10 minutes at room temperature. The cells were then washed twice with PBS and 100µl of staining solution was added to each well. The plate was kept at 37° C, until the color development.

2.3.13 Clonogenic Assay

For clonogenic assays, $1x10^3$ (A549) or $2x10^3$ (E-10) cells were seeded per well of a six well tissue culture plate and grown for 15 days. For identification of signaling pathways various inhibitors were used *viz,* PI3K inhibitor LY294002 (10µM), MEK inhibitor PD98059 (10µM) or p38 inhibitor SB203580 (10µM). Cells were grown in the presence of inhibitor for seven days following which fresh medium was added. For staining, cells were washed twice with PBS and fixed in 10% formalin for 10 minutes, washed extensively with water and stained with 0.25% crystal violet prepared in 25% methanol for 4hrs at 4°C. Plates were then washed with milli Q water and dried before scanning.

2.3.14 Soft Agar Assay

For soft agar assays $2x10^{4,}$ (A549) or $1x10^5$ cells (E-10) were used in 1.5ml top agar. For preparing bottom agar plates (0.64% final con. of agar), a following mix was prepared for five dishes.

1.	2X media with FBS, L-glutamine and Pen-Strep	10 ml
2.	fetal bovine serum	5 ml
3.	sterile water	1 ml
4.	noble agar 1.8% (1.8 g/100mLs)	9 ml
5.	Total	25 ml

Agar solution was prepared in a sterile 50ml Schott Duran Bottle and boiled in microwave until fully dissolved and kept at 55°C to 65°C. Master Mix with the rest of the components of bottom agar was made in a sterile corning 50ml tube prewarmed at 55°C and agar solution was added. The solution was once vortex briefly and then added (2ml) carefully to each well avoiding air bubbles. The plates were left undisturbed in laminar flow hood until the agar set fully. Two days before final assay, the bottom agar plates were kept in tissue culture incubator for equilibration.

On the day of assay the following mix was prepared for Top Agar

		4 dishes	5 dishes
1.	media with FBS, L-glutamine and Pen-Strep	4.8 ml	6 ml
2.	fetal bovine serum	1.8 ml	2.5 ml
3.	sterile water	1.8 ml	2.5 ml
4.	agar 1.8% (1.8 g/100mLs)	1.8 ml	2.5 ml
5.	cell suspension 1.0 X 10^5/ dish	100 to 350 µl	100 to 350 µl
6.	Total	10.2 ml	13.5 ml

Top agar mix without cells was first prepared and kept at 42°C. The cells were then trypsinized and re-suspended after counting in final volume of 100µl to 200 µl. Cells were then mixed with top agar and solution was quickly poured over the bottom agar.

The plates were kept in incubator gently and the colony formation was monitored every week. Media (500µl) was added to the plates every 4^{th}-5^{th} day to avoid drying. Colonies formed in soft agar photographed were taken without staining, under a microscope in light field.

2.3.15 Flow Cytometry

Cells growing in culture medium were harvested by trypsinization and washed twice with ice cold PBS. Cells were fixed by adding ice cold 70% ethanol and stored at 4°C. Before harvesting cells were washed twice with PBS and re-suspended in adequate amount of PBS containing Propidium Iodide (PI) to a final concentration of 50µg/ml and RNase to a final concentration of 10µg/ml. Thereby the cell suspension was incubated at 37°C for 30 minutes in dark. Analysis was done by running the samples in BD FACS Vantage System according to the standard procedures after calibration of instrument with Calibrite beads.