APPENDIX 1

EXPERIMENTAL PROTOCOLS

A. Extraction of genomic DNA from whole blood

Extraction from 5ml whole blood:

RBC Lysis

1. 5ml of whole blood was added to a tube containing 15 ml of RBC lysis solution, mixed well and incubated at room temperature for ten minutes by inverting once during the incubation.
2. The mix was then centrifuged at 13,000 x g for 20 seconds and the supernatant was removed leaving behind the visible white cell pellet.
3. The tube was then vortexed vigorously to resuspend the white blood cells In the residual supernatant.

Cell Lysis

1. About 5ml of cell lysis solution was added to the tube containing the resuspended cells and pipetted up and down gently to lyse the cells.
2. The mixture was then incubated at 37 ºC until the solution became homogenous.

RNase Treatment

25µl of RNase solution was added to the cell lysate and incubated at 37ºC for 15 minutes.

Protein precipitation

1. The samples were cooled to room temperature and 1.67 ml of protein precipitation solution was added to the cell lysate.
2. The protein precipitation solution was mixed well with the cell lysate by vortexing at high speed for 20 seconds.
3. The proteins were precipitated as a dark brown pellet by centrifuging at 13,000 x g for 3 minutes.

DNA Precipitation

1. The supernatant containing the DNA was added to sterile tube containing 5ml of chilled 100% isopropanol.

2. The sample was mixed by gently inverting the tube until white threads of DNA formed a visible clump, which was then centrifuged at 13,000 x g for 1 minute to get a small white pellet.
3. The supernatant was poured off and 5 ml of 70 % ethanol was added to wash the DNA pellet.
4. This was then centrifuged at 13,000 x g for 1 minute and the supernatant was carefully poured off without dislodging the DNA pellet
5. The sample was then allowed to air dry for 15 minutes.

DNA Hydration

The isolated DNA was then dissolved in Tris-EDTA buffer (pH) .

Quantitation of DNA

The extracted DNA was then quantitated by measuring the optical density at 260nm using a spectrophotometer. The concentration of the DNA was the adjusted to 100µg/ ml and stored at 4ºC until further analysis was done.

Reagents

RBC Lysis Solution

Cell Lysis Solution

Protein Precipitation Solution

RNase

Tris-EDTA buffer (pH 7.6)

10mM Tris Cl (pH 7.6)

1 mM EDTA (pH 8.0)

100% isopropanol

70% ethanol

B. Agarose gel electrophoresis of genomic DNA

1. Sufficient amount of 1X electrophoresis buffer (1XTBE) was prepared to fill the electrophoresis tank and to prepare the gel. The correct amount of powdered agarose was added as per the requirement to a measured quantity of 1X electrophoresis buffer.

2. The agarose was melted in a microwave oven and the solution was cooled to 60^0C. Ethidium bromide (from a stock solution of 10mg/ml in water) was added to the cooled solution to a final concentration of 0.5µg/ml and mixed thoroughly.
3. The comb was positioned 0.5-1 mm above the gel casting plate and the warm agarose solution was poured into the mould by taking care to avoid air bubble formation.
4. The gel (3mm-5mm thick) was completely set after 30 minutes at room temperature and it was mounted in the electrophoresis tank after carefully removing the comb.
5. Electrophoresis buffer was added enough to cover the gel to a depth of 1mm.
6. 10µl of the PCR product was mixed with 2µl of 6X gel loading buffer and the mixture was loaded into the slots of the submerged gel using a disposable micropipette.
7. The lid of the gel tank was closed and the electrical leads were attached in such a way that the DNA will migrate toward the anode. A voltage of 1-5 V/cm was applied. The gel was run until the bromophenol blue and the xylene cyanol FF had migrated the appropriate distance through the gel.
8. The electric current was turned off and the gel was examined under ultraviolet light using a UV transilluminator.
9. The gel was then photographed using the Kodak Digital Science Gel Documentation System.

Reagents

1. 1X TBE
   0.04M Tris-borate
   0.01 M EDTA
2. 6X Gel loading buffer
   0.25% bromophenol blue

0.25% xylene cyanol FF

40% sucrose in water

3. Agarose
4. Ethidium Bromide (0.5µg/ml)

C. Polymerase Chain Reaction (PCR)

Polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of known sequence.

Components of Polymerase Chain Reaction:

Oligonucleotides

Oligonucleotides used for priming the PCR reaction should be preferably 20-24 nucleotides in length.

Buffer

The standard buffer for PCR reaction contains 50 mM KCl, 10 mM Tris Cl (pH 8.3) and 1.5 mM $MgCl_2$The presence of divalent cations (Mg ++) is critical.

Taq DNA Polymerase

Taq DNA polymerase is the native thermostable enzyme purified from *Thermus aquaticus* which carry a 5'-3' polymerization dependant exonuclease activity and lack a 3'-5' exonuclease activity.

Deoxyribonucleoside Triphosphates

dNTPs are used at saturating concentrations (200 µM for each dNTP).

Target Sequences

DNA containing the target sequences can be added to the PCR mixture in the single or double stranded form.

Protocol

1. A reaction mixture containing the appropriate concentrations of oligonucleotides, dNTPs, buffer, Taq DNA polymerase, and the template DNA was added as per the specific protocol to a sterile 0.2 ml microfuge tube and made upto 50 µl.
2. The PCR amplification reactions were carried out in a thermal cycler with the appropriate PCR conditions consisting of an initial denaturation,

followed by the desired number of cycles of denaturation, annealing and extension. This was followed by a final extension step.

3. 10 µl of the PCR product was used to analyze the amplified DNA by agarose gel electrophoresis.

D. Restriction Fragment Length Polymorphism (RFLP)

The following is the procedure for a typical reaction volume of 20µl.

1. 10 µl of the purified PCR product was taken in a sterile microfuge and mixed with sufficient water to give a volume of 18 µl.
2. 2 µl of the appropriate 10X restriction enzyme digestion buffer was added to the above mix.
3. 10-20 units of the appropriate restriction enzyme was then added and mixed thoroughly by tapping the tube.
4. The mixture was then incubated at the appropriate temperature for the required period of time
5. The reaction was then stopped by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10mM.
6. 6 µl of gel loading buffer was then added to the reaction mix and vortexed briefly.
7. The digestion mix was then loaded into gel slots and electrophoresis was carried out. The fragments were visualized under a UV transilluminator and photographed using a gel documentation system.

Polymorphism Analyzed	Restriction Enzyme	Incubation
XPD codon 751	*Mbo II*	37 ºC for 1 hr
ERCC1 codon 118	*HpyCH4*	37 ºC for 3 hr
ERCC4	*Xmn I*	37 ºC overnight
XRCC codon 194	*Pvu I I*	37 ºC for 1 hr
XRCC codon280	*Rsa I*	37 ºC for 1 hr
XRCC codon399	*Msp* I	37 ºC for 1 hr
XRCC3 codon241	*NlaIII*	37 ºC for 3 hr

Reagents

Restriction enzyme buffer 1x

BSA

Restriction Enzyme (1 Unit)

E. Sequencing

Preparation of samples for Sequencing

The PCR products were run on electrophoresed on 2% agarose gels. The bands were cut using sterile surgical blade and then eluted and purified.

Elution from agarose gel using GFX gel band purification kit

1. Sample capture

A 1.5ml eppendorf tube was weighed and weight recorded.

The tube was again weighed after putting the cut band in it.

The weight of the sliced band was calculated.

10micrlitre of capture buffer type 2 was added for each 10mg of gel slice and mixed by inversion.

The tube was incubated at 60^0C, until all agarose was completely dissolved. The tube was mixed by inversion every 3 minutes.

A GFX microspin was placed into a collection tube.

2. Sample binding

The sample mix was briefly centrifuged and 600 microlitre of the mix was transferred into the column.

The tubes were incubated at room temperature for 3 minutes.

The tubes were centrifuged at 12000 rpm for 3 minutes.

The flow through in the collection tube was discarded.

3. Wash

Wash buffer type I (500 micrlitre) was added to the column.

The tubes were centrifuged 10,000 rpm for 1 minute.

The collection tube was then discarded and the column transferred to a fresh 1.5 ml tube.

4. Elution

25 micrlitre elution buffer type 6 was added to the centre of the membrane in the column and incubated at room temperature for 2 minutes.

The tubes were then centrifuged at 10,000 rpm for 1 minute to recover the purified DNA.

The tubes were stored at -20^{0}C

DNA Sequencing

The purified products were then sequenced using an automated sequencer ABI 3730 capillary sequencer (Lab India).

F. Cytokinesis Block Micronucleus Assay

Separation of lymphocytes

1. 4 ml of blood was taken into autoclaved EDTA blood tubes and mixed well.
2. The blood was diluted with 1X PBS in equal amounts in a centrifuge tube.
3. 3 ml of Ficoll was added into a centrifuge tube and 9 ml of blood aws layered on top of this Ficoll.
4. Tubes were centrifuged at 1600 rpm for 20 min. The separated lymphocyte layer was transferred to another tube.
5. Cells were washed twice with RBC lysis solution and once with plain RPMI media.

Culturing of lymphocytes

6. Cells were then resuspended in 2 ml of 10% RPMI media
7. Cells were counted using a hemocytometer and $2X10^6$ cells /ml were seeded in 10% RPMI.
8. Cells were then incubated in 5% CO2 incubator
9. After 44 hours of incubation, cell division was arrested using Cytochalasin B(4.5ug/ml)
10. Cells were mixed thouroughly and incubation continued for another 28 hours.

Staining of cells

11. Cells were centrifuged at 2000 rpm for 10 minutes, washed with PBS and 1 ml of methanol was added for fixation.
12. Cells were stained with acridine orange and ethidium bromide ((6uL+ 4uL in 15 ml 1X PBS) and observed directly under 100X (Carl-Zeiss) microscope.

The assay was coded and set up in duplicates. 1000 binucleate cells were counted and number of MN counted and recorded.

G. Host Cell Reactivation Assay

PLASMID TRANSFORMATION

Plasmids: pGL2SV40luc, pRTKluc

Antibiotic: Ampicillin

Bacterial strain: E.coli JM109

Medium: LB broth (2g/100ml)

1. LB broth was prepared 25ml each in 4 conical flasks and autoclaved

2. 50ul of Ampicillin (stock 50mg/ml) was added to each flask
3. 100ul of glycerol stock of both plasmids was added to 2 respectively labeled flasks.
4. The flasks were incubated at 37C o/n in a shaker incubator.

PLASMID EXTRACTION USING QIAGEN MIDIPREP

- RNAse A sol was added to buffer P1
- Check buffer P2 for SDS precipitation
- Prechill buffer P3

A. Bacterial culture harvest and lysis.

1. Pellet 25ml o/n LB culture at 10,000rpm 15min 4C
2. homogenously resuspend the bacterial pellet in 4 ml buffer P1
3. Added 4 ml buffer P2, mix thoroughly by inverting 4-6 times and incubate at room temp for 5 min.
4. Added 4 ml buffer P3 mix thoroughly by vigorously inverting 4-6 times. Incubate in ice 15 min.

B. Bacterial lysate clearing

1. Centrifuge at 15000 rpm for 30 min at 4C
2. Recentrifuge the supernatant at 15000rpm 15 min 4C

C. Bind wash and elute plasmid DNA on Qiagen tip

1. Equilibrate a qiagen tip 100 by applying 4ml buffer QBT and allow column to empty by gravity flow
2. Apply the supernatant to the tip and allow it to enter the resin by gravity flow
3. Wash the tip with 2X20 ml buffer QC
4. Elute DNA with 5 ml buffer QF into clean 15 ml tubes

D. Precipitate wash and redissolve plasmid DNA

1. Precipitate DNA by adding 3.5 ml isopropanol (room temp) to the eluted DNA and mix. Centrifuge 15000rpm for 30 min 4C. Carefully decant supernatant.
2. Wash DNA pellet with 2 ml, 70% ethanol (rt) and centrifuge at 15000 rpm , 10 min 4C. Carefully decant supernatant
3. Airdry pellet for 5- 10 min and redissolve DNA in 150 ul of buffer(Nuclease free water)

ISOLATION OF LYMPHOCYTES

1. Collected 10 ml of blood in EDTA. Dilute in equal volume of 1X PBS
2. 4 ml of Ficoll was taken in a 15 ml test tube and layer 5 ml blood onto it.
3. Centrifuged at 1600 rpm for 20 min at 4C.
4. Separated buffy coat into a fresh tube and add three times volume of RBC lysis sol. Centrifuge at 2000 rpm for 10 min at 4C
5. Pellet was resuspended in 20%RPMI and transferred to a T25 flask and incubated for 3 hrs at 37C.

PLASMID IRRADIATION

1. Plasmids (pNFkBluc and pRTKluc) were thawed in ice and 100ul of each in two 35mm petri dishes were exposed to UV (254nm) for 3 min and 20 sec.
2. Damaged plasmids were then transferred to 1.5 ml tubes and stored in ice.

TRANSFECTION

1. Plasmid + Lipofectamine Cocktail:
    A. 74 uL optimem+ 12uL pGL2 promoter vector+ 12uL pGL2control vector+2 uL pRTK vector
    B. 74 uL optimem+ 12uL damaged pGL2 promoter vector+ 12uL pGL2control vector+2 uL pRTK vector
    1. 92 uL optimem+ 8 uL lipofectamine
    2. 92 uL optimem+ 8 uL lipofectamine

Mix A to 1 and B to 2 and incubate at room temp for 45 min.

2. Centrifuge the cells at 2000 rpm for 10 min and resuspend the pellet in 2 ml OptiMEM.
3. Count the cells and seed $1X10^6$ cells each in 35mm petri dishes.
4. Contents from the respective cocktails were added to the Petri plates with the same labels.
5. The cultures were then incubated at 37C for 5 hrs to allow transfection to occur.
6. The cells from each plate were washed with 20%RPMI twice (2000rpm, 10 min) and incubated with 20%RPMI for 48 hrs at 37C.

LUCIFERASE ASSAY

Dual luciferase reporter assay: Promega

Reagent preparation

1. 1XPLB: 1 volume of 5X passive lysis buffer (PLB) was added to 4 volumes distilled water.
2. LAR II: The lyophilized luciferase assay substrate was resuspended in Luciferase assay buffer II.
3. Stop and Glo Reagent: 2.1 ml of 50X Stop and Glo substrate was added to 105 ml of stop and glo reagent. Stored at -20^0C

Cell Lysis

1. Media was removed from cultured cells.
2. The cells were washed in 1X PBS.
3. 250 uL of 1X PLB was added to the tubes.
4. The tubes were placed on a shaker bath for 15 minutes.

Assay with Luminometer

1. 100uL of LAR II was predispensed into the Luminometer tube as blank/control
2. 20uL of PLB lysate was transferred and firefly reading was measured

3. 100 uL of Stop and Glo reagent was dispensed and renilla luciferase activity was also measured.
4. DRC% was calculated as ratio of damaged plasmid value to undamaged plasmid value X 100.