3. MATERIALS AND METHODS

3.1 STUDY SUBJECTS

3.1.1 Patients and Controls

All patients included in the present study were referred from various Pediatric neurology clinics and Genetic clinics across the country. They were evaluated by a clinician/pediatrician and referred to the institute for investigation of various lysosomal storage disorders (LSDs). A clinical presentation like presence of regression of milestones, neuroregression, seizures, exaggerated startle reflex, hyptonia and presence of cherry red spots on fundus examination are more likely to have GM2 gangliosidosis (Tay-Sachs disease or Sandhoff disease or GM2 activator protein deficiency). Though, neuroimaging criteria were not strictly followed, it mainly included the presence of gray/white matter disease as shown by hypointensities in T2 weighed (T2W) images of the brain computed tomography (CT scan) or magnetic resonance imaging (MRI). Clinical details were noted in a case record form (CRF) (Annexure-I). An informed written consent from parents (Annexure-II) was obtained for each recruited patient. Normal healthy controls were also enrolled in the present study for biochemical as well as molecular analysis.

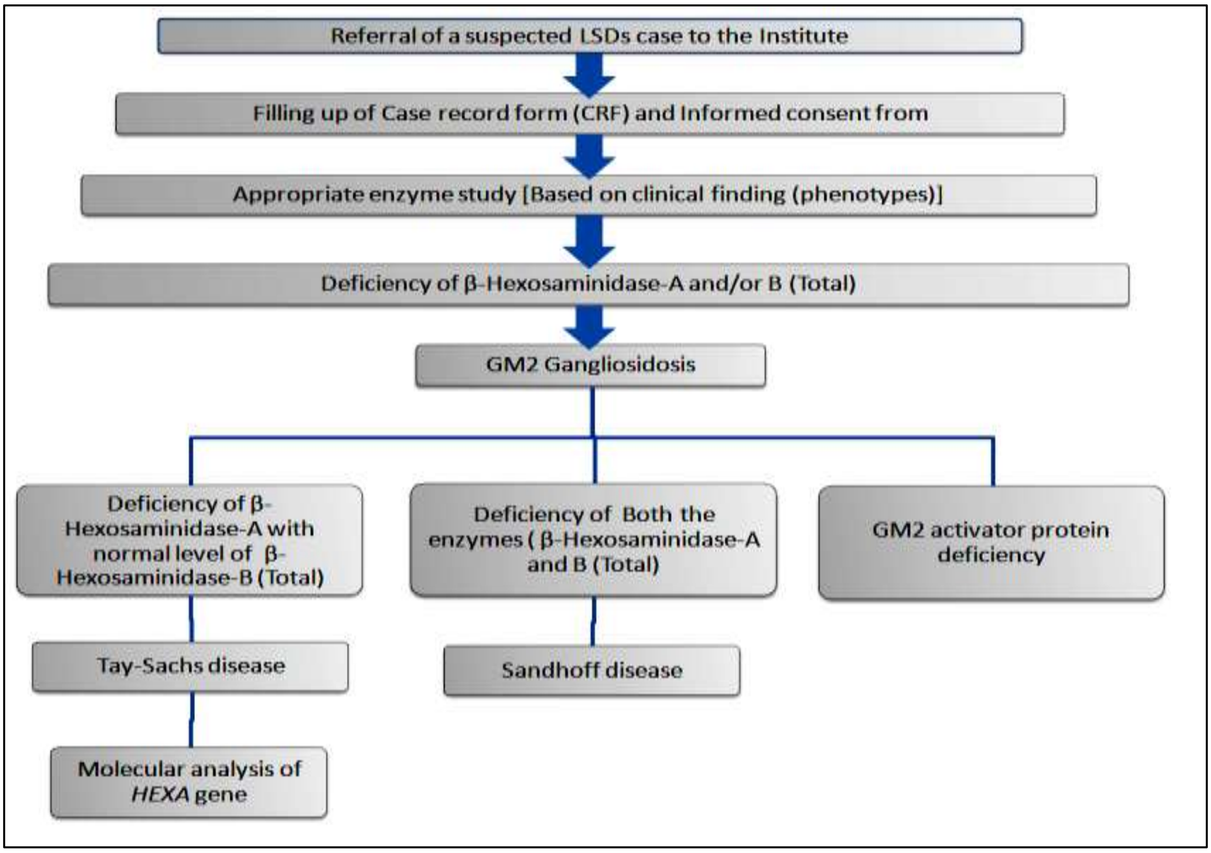
3.1.2 Sample size

FIRIGE is the national task force centre for the diagnosis of LSDs that attracted large number of referrals across the country. From the pool of 1531 suspected cases with different LSDs based on clinical presentation and neuroimaging findings referred from October 2008 to October 2015 measurement of Hex-A and total-hex enzyme activity was carried out in 409/1531 subjects. Of these, 46 cases were detected with Hex-A deficiency and normal total-hex activity suggestive of TSD. Furthermore, 31 cases were detected with deficiencies of both the enzymes (Hex-A and total-hex) suggestive of SD, while 2 cases showed normal activity of Hex-A and total-hex enzymes, but further confirmed to have GM2 activator protein deficiency. In present study, we 46 cases of classical TSD and 2 cases with AB variant form of TSD for molecular analysis of *HEXA* GM2A genes respectively. A carrier analysis was carried out in 8 parents where proband was not alive but was earlier diagnosed as TSD by enzyme study which was further confirmed by molecular analysis of *HEXA* gene. Prenatal diagnosis was carried out in 8 cases where mutation was known in index case.

3.1.3 Ethics Statement

The study protocol was approved by the Institutional ethics committee of Foundation for Research in Genetics and Endocrinology (FRIGE), Ahmedabad, Gujarat (Registration No-E/13237). (Annexure-III)

3.2 WORK PLAN



3.3 MATERIALS

3.3.1 Sample types

Four millilitre (4 ml) peripheral blood was collected in sodium heparin and/or EDTA vacutainer from all the patients for leucocytes enzyme assay and 3 ml blood in an EDTA vacutainer for DNA extraction. Chorinic villus sample (CVS) or amniotic fluid (AF) was used for prenatal diagnosis. This was carried out by a certified fetal medicine specialist after obtaining consent as per national PNDT act.

3.3.2 Buffers and Reagents

All the buffers and reagents were prepared in autoclaved sterile de-ionized water as below (*Table 3.1*). Buffer pH was adjusted, at ~25°C, with standard calibrations.

***Table 3.1**: Buffers and reagents used*

Buffers and Reagents	**Buffers and Reagents preparation**
Normal Saline (0.9% sodium chloride)-NS	Ready to use from Claris Otsuka Pvt. Ltd. (Cat. No-1050002540)
Acid-citrate-dextran solution (ACD)	Dissolve 24.5 gm anhydrous dextrose (Qualigens, Cat. No-24415, MW 180.16), 22.0 gm trisodium citrate dehydrate (Qualigens, Cat. No-14005/1, MW 294.1) and 8.0 gm citric acid monohydrate (Qualigens, Cat. No-22595, MW: 210.14) in 1L normal saline. 1L stock and a smaller aliquot in reagent bottle kept at 4°C in the laboratory refrigerator.
6% Dextran in normal saline	Dissolve 60 gm dextran (Acros Organics, Cat No. 406261000, High fraction) in 1 L normal saline.
5% D-glucose in normal saline	Dissolve 50 gm glucose (Qualigens, Cat. No-24415, MW: 180.16) in 1 L normal saline
3.6% Sodium chloride (NaCl)	Dissolve 3.6 gm NaCl (Nice Chemicals Cat. No. S12229, MW 58.44) in 100 ml water.
Bovine serum albumin (BSA) standards (10, 20, 40, 60, 80 and 100 µg/ml)	Prepare top standard by dissolving 25 mg BSA (Hazleton Biologicals; Cat. No. 84-040-025) in 250 ml water (100 µg/ml) and make dilution for 10, 20, 40, 60 and 80 µg/ml. Store in glass bottles at 4°C.
"Lowry A" solution [2% Sodium carbonate in 0.1 N sodium hydroxide pellets (NaOH)]	Dissolve 10 gm sodium carbonate, anhydrous pure (Merck; Cat. No.-MG7M571928, MW 105.99) and 2 gm sodium hydroxide pellets (Merck; Cat.No.-QK2Q622532, MW 40.0) in 500ml water. Store at 4°C.
2% Sodium potassium tartrate	Dissolve 2 gm sodium potassium tartrate, also called potassium sodium tartrate (Nice Chemicals; Cat. No. S13729, MW 282.22) in 100 ml water. Store at 4°C.
1% Copper sulphate	Dissolve 1 gm copper sulphate pentahydrate (Merck; Cat. No. QD2Q620601, MW 249.68) in 100 ml water. Store at 4°C.

Folin Ciocalteu's phenol reagent	Ready to use (Sigma; Cat. No. F9252) Store at room temperature
4-methylumbelliferone (4-MU)	2.202 mg 4-MU (Sigma; Cat. No. M1381, MW 176.17) in 0.5 ml absolute ethanol, heat it to dissolve completely (if required). Make total volume up to 500 ml with water. This will give 25 uM concentration of 4-MU. Then make serial dilution 0.5, 1.0, 1.5 and 2.5 uM. Store at -20°C.
12 mM citric acid/20 mM sodium phosphate substrate buffer, pH 4.4	Dissolve 2.5217 gm citric acid monohydrate (Qualigens; Cat. No.22595, MW 210.14) and 2.8392 gm disodium hydrogen phosphate, anhydrous (Na_2HPO_4) (Himedia; Cat No. RM257, MW 141.96) in about 950 ml water. Adjust to pH 4.4 with phosphoric acid or sodium hydroxide and make up to 1 L with water. Store frozen at -20° C in 100 ml aliquots.
0.75% Human Serum Albumin (HSA) in buffer	Dissolve 75 mg HSA (Sigma; Cat. No. A9511) in 10 ml 12mM citric acid/20mM sodium phosphate buffer. Prepare fresh on the day of a WBC assay.
3 mM 4-methylumbelliferyl-β-D-N-acetylglucosamine in substrate buffer	Dissolve 11.4 mg 4MUβglcNAc (Sigma; Cat. No. M2133, MW 379.4) in 100 ml 12mM citric acid/20mM sodium phosphate buffer. Store frozen at -20°C in 10 ml aliquots.
120 mM citric acid/160 mM Na_2HPO_4 buffer, pH 4.14	Dissolve 25.22 gm citric acid monohydrate (Qualigens, Cat. No.-22595, MW 210.14) and 22.72 gm anhydrous Na_2HPO_4 (Himedia; Cat. No.-RM257, MW 141.96) in ~ 950 ml water. Adjust to pH 3.7 with phosphoric acid or NaOH as necessary and make up to 1L with water.
6 mM 4-methylumbelliferyl-β-D-N-acetylglucosamine 6-sulphate (MUGS) in water	Dissolve 28.9 mg MUGS (Melford; Cat. No.M1189, MW 481.5) in 10 ml distilled water.
0.5M glycine-NaOH buffer, pH 10.3	Dissolve 37.53 gm glycine (Merck; Cat. No. QH3Q631809, MW 75.07) in about 800 ml water. Adjust at the pH meter to pH 10.3 using 2M NaOH (Merck; Cat. No. QK2Q622532, MW 40.0) and make up to 1 L with distilled water.
TE Buffer, pH 8.0	1 M Tris HCL (pH 8.0) (Sigma, Cat. No. T6066, MW 121.1), 0.5 M EDTA (pH 8.0) (Sigma, Cat. No. E5134, MW 186.1) in about 800 ml deionized distilled water.

	Adjust to pH 8 and make up to 1 L with distilled water.
Lysis buffer (pH=8.0)	10mM Tris HCL (pH=8.0) (Sigma, Cat. No.-T6066, MW 121.1), 5mM $Mgcl_2$ (Merck; Cat. No. MK7M561509), 1% Triton 100 (Sigma; Cat. No. X100), 0.032 M sucrose (Sigma; Cat. No. 84097, MW 342.3) in about 800 ml deionized distilled water. Adjust to pH 7.6 and make up to 1L with deionized distilled water
DNA extraction buffer (DEB) (pH=8.0)	1 M Tris HCL (pH 8.0) (Sigma, Cat. No. T6066, MW 121.1), 0.5 M EDTA (pH 8.0) (Sigma, Cat. No. E5134, MW 186.1), 10% (w/v) Sodium dodecyl sulphate (SDS) (S. D. Fine chemicals; Cat. No. 40175, MW 288.38), 10% N-Lauryl Sarcosine (Sigma; Cat. No. L5125, MW 293.38) in 1 L deionized distilled water
Proteinase-K (10mg/ml)	10 mg Proteinase-K (Sigma; Cat. No. P2308) in 1 ml deionized distilled water
5M Sodium chloride (NaCl)	29.22 gm Sodium chloride (NaCl) (Nice Chemicals Cat. No. S12229, MW 58.44)in 100 ml deionized distilled water
70% Ethanol	70 ml absolute ethanol and make up the volume to 100 ml using autoclaved deionized distilled water. Store it at 4°C
PCR buffer	First dissolve Gelatin (Gelatin from porcine skin, Type-A Cell culture tested; Sigma, Cat. No. G1890) in 3.2 ml triple distilled water (Incubate at 37°C to dissolve Gelatin), add 1.25 ml 2M KCl, 0.5 ml 1M Tris (pH 8.3) and 75 µl 1M $MgCl_2$.
Ethidium bromide ~95% (HPLC) (Etbr)	1 mg Ethidium bromide (Sigma; Cat. No. E8751, MW 394.31) in 1 ml deionized distilled water
Gel loading dye	25 mg of Xylene cyanol and 25 mg of Bromophenol blue dissolve in 6.25 ml of deionized distilled water, add 1.25 ml of 10% SDS and 12.5ml of glycerol.
TAE buffer	2 M Tris HCL (Sigma, Cat. No. T6066, MW 121.1), 50 mM EDTA (Sigma, Cat. No. E5134, MW 186.1) in about 80 ml deionized distilled water. Adjust to pH 8.0-8.3 with glacial acetic acid (Merck (approximately 5.71 ml) and make up to 100 with distilled water.

3.3.3 Laboratory equipments used

- Cooling centrifuge (R8C, Remi, India)
- Centrifuge (R8C and R24C, Remi, India)
- Table top centrifuge (iFUGE M08VT, Neuaton, India)
- Sonicator (Q55, QSoniaca, India)
- Cyclo mixer (CM-101, Remi, India)
- Digital water bath (CIC-3, Cintexindustrial Corporation, India)
- Spectrophotometer (Shimandzu 1770 UV/VIS, USA)
- Fluorescence spectrometer (LS55, PerkinElmer, USA)
- Thermal cycler 2720 (Applied Biosystems, USA)
- Thermal cycler gradient (MJ Mini, Bio-Rad, USA)
- QIAxpert system (QIAGENE,USA)
- Gel electrophoresis apparatus (Bangalore Genei, India)
- Power pack (Bangalore Genei, India)
- Molecular E-Gel Imager system (Life technologies, USA)
- ABI PRISM ® 3130 Genetic Analyzer (Applied Biosystems, USA)

3.3.4 Online Bioinformatics and statistical tools used

- BLAST: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
- CLUSTALW: http://www.ebi.ac.uk/Tools/clustalw2/index.html
- ERNDIM: http://cms.erndimqa.nl/Home/Lysosomal-Enzymes.aspx
- GenBank (Entrez Gene): http://www.ncbi.nlm.nih.gov/Genbank/
- GeneCards version 3: http://www.genecards.org//index.php?path=/index.php
- Gene Mapper: http://www.appliedbiosystems.com/absite/us/en/home/support/software/dna-sequencing/genemapper.html
- Human Gene Mutation Database: http://www.hgmd.cf.ac.uk
- McGill University database: http://www.hexdb.mcgill.ca

- MLPA: www.mlpa.com
- Mutation Taster: http://www.mutationtaster.org/
- NEBcutter version 2.0: http://tools.neb.com/NEBcutter2/47
- NNSplice 0.9 softwares: http://www.fruitfly.org/seq_tools/splice.html
- OMIM: http://www.ncbi.nlm.nih.gov/omim/
- Primer3web version 4.0.0: http://primer3.ut.ee/
- Polyphen2 (Polymorphism Phenotyping v2): http://genetics.bwh.harvard.edu/pph2/
- Pub med: http://www.ncbi.nlm.nih.gov/pubmed/
- Quick P-value Calculator: http://www.socscistatistics.com/pvalues/Default.aspx
- Reference Sequences: http://www.ncbi.nlm.nih.gov/refseq/
- Restriction Mapper version 3 (http://www.restrctionmapper.org/).
- Sequence Analysis: http://www.ncbi.nlm.nih.gov/guide/sequence-analysis/
- SIFT (Sorting Intolerant From Tolerant): http://sift.jcvi.org/
- SNP database: http://www.ncbi.nlm.nih.gov/SNP/index.html

3.4 METHODS

3.4.1 Sample preparations

3.4.1.1 Isolation of peripheral Blood Leucocytes

Leucocytes were isolated from heparinised/EDTA peripheral blood by differential sedimentation at unit gravity in a dense isotonic dextran solution (Skoog and Beck, 1956; Magalhaes *et al.*, 1984b). The cells were washed briefly in 0.9% saline to haemolyse the red cells, and then brought back to isotonicity with 3.6% saline. Approximately 3-4 ml of blood was centrifuged at 2000 × g for 10 min at 2-8℃. The plasma was removed carefully, leaving the buffy coat undisturbed and stored. The leucocytes containing blood was transferred to 50 ml tube; add double volume of mixture containing 1.5 parts of Acid citrate dextran (ACD), 6 parts of 6% Dextran (6%) and 3.5 parts of 5% Glucose (eg. 5 ml blood sample + 10 ml mixture). Tubes were mixed gently to avoid bubbles. The tubes were allowed to stand at room temperature for 45 minutes to 1 hour. The leucocytes containing supernatant was transferred to a 15 ml tube and centrifuged at 2000 × g for about 10 min at 2-8℃. The pellet was washed (the pellet resuspended in 0.8 ml of 0.9% saline and 2.4 ml chilled distilled water, mixed gently by pipetting, after precisely 2 min, 0.8 ml of 3.6% saline was added and mixed gently before centrifuging at 2000 × g for about 10 min at 2-8℃). The pellet was washed again to obtain white cell pellet free of red cells. All the supernatant was removed and the pellet was frozen at -20℃ until assayed (usually performed within a week).

3.4.1.2 Collections and cultured CV/AF for prenatal sampling

Prenatal sampling was done depending upon the stage of pregnancy, fetal position and maternal health. CV sampling and/or amniocentesis was carried out between 10–13 and 15–18 weeks of gestation, respectively at a designated registered centered as per PNDT act. The CV samples (~10-15 mg) were collected in a sterile collection vial containing growth medium (RPMI 1640 with antibiotic). After checking for maternal cell contamination, they were thoroughly washed with sterile phosphate buffered saline (PBS) followed by distilled water. After thorough examination and washing with growth medium, CV samples were chopped and cultured in growth medium under 5 % CO_2 as per standard protocol (Lake *et al.*, 1998; Gatti *et al.*, 1985). Once cell confluence was obtained (usually within 2 weeks) CV sample was harvested carefully and protein activity was determined. Final concentration of protein was adjusted to 2-10 mg/ml and was used for enzyme study.

About 15-20 ml of amniotic fluid was collected (devoid of maternal blood contamination). The cells were cultured in two different growth mediums following centrifugation. After the cell confluence was attained, the grown fibroblasts were studied for different lysosomal enzymes after protein estimation (Lake *et al,* 1998; Gatti *et al,* 1985).

3.4.1.3 Extraction of cells

Cell pellets, (leukocytes or cultured CV/AF), were suspended in 100-400 µl double distilled water. They were further subjected to sonication with 20 amplitudes of 5 second, 50 watts, with pauses of 3 second (QSONICA Sonicator Q55). The homogenate was kept for 30-45 min at room temperature and the supernatant (referred to as extract) was collected. All these procedures were performed at 4°C. The resulting supernatant, referred to as homogenate, served as the source of enzyme in all assays.

3.4.1.4 Protein concentration in leucocytes using Lowry method

Protein was measured according to modified Lowry's method (Lowry *et al.*, 1951; Herbert *et al.*, 1974). A series of Bovine serum albumin (BSA) standards (0.1 mg/ml to 1.0 mg/ml) was used. Final sample protein concentration was adjusted to 2–10 mg/ml and was employed for enzyme studies.

3.4.1.5 Measurement of Hexosaminidase-A (Hex-A) and Tolal-Hexosaminidase (total-hex) activity using LS55 fluorescence spectrometer

Fluorometric assays were performed using 4-methylumbelliferone (4-MU) derivative artificial substrates, essentially according to procedures described by Galjaard (1980). Enzyme activity was determined by fluorimetric method using specific synthetic substrate (Shapria *et al.,* 1989). Total-Hex activity was measured from the hydrolysis of synthetic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine (MUG) that releases fluorescent 4-methylumbelliferone when acted upon by β-hexosaminidase. The β-Hexosaminidase-B (Hex-B) was determined as the activity left over after incubating the sample for three hours at 50°C. This procedure led to the loss of heat-labile Hex-A but not of Hex-B or intermediate isoenzyme. Therefore, Hex-A activity was obtained after subtracting Hex-B activity from the total-Hex activity. Hex-A can also be assayed with the more specific substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine-6-sulphate (MUGS) (Wendeler and Sandhoff 2009). Hex-A activity (obtained from MUGS lysis) was expressed as percentage of total activity (obtained from MUG) lysis.

<u>3.4.1.5.1 Assay of hexosaminidase-A (Hex-A) enzyme activity</u>

All the samples were tested in duplicates. The blanks were freshly prepared at each run. For cell extracts (Leucocytes for postnatal diagnosis; AF cells/ CT cells for prenatal diagnosis) mix equal volumes (120 µl) of buffer (120 mM citric acid/160 mM Na_2HPO_4 buffer pH 4.14) and substrate (6 mM MUGS), in all 'test' and 'blank' tubes. Then add leucocytes sample (10 µl) for postnatal diagnosis while CT cells (10 µl) and/or AF cells (10 µl) for prenatal diagnosis into the 'test' tube and 10 µl distilled water into the 'blank' tube. Incubate the tubes exactly for 30 min at 37℃. The reaction was stopped by the addition of 3 ml glycine-NaOH buffer (pH 10.7). The relative fluorescence of each tubes was read on the Perkin-Elmer LS-55 fluorescence spectrometer with excitation wavelength/slit width (366 nm/25 nm), and the emission wavelength/slit width (446/2.5 nm). Standards used for the calibration curve were prepared from a solution of 0.5-2.5 nmol of 4-MU. The relative fluorescence readings (RF) were corrected by subtracting the blank reading from each tube and enzyme activity calculated as nanomoles per hour per milligram of protein using the following equation.

$$\textbf{Hex-A activity} = \text{Sample-Blank} \times \frac{\text{Standard concentration (2.5 nmol)}}{\text{Volume of standard used (100 µl)}} \times \frac{\text{Volume of termination buffer (3 ml) + Assay Volume (60 µl Substrate + 60 µl buffer + 10 µl sample)}}{\text{Volume of termination buffer (3 ml) + Volume of standard (100 µl)}} \times \frac{\text{1 hour (60 min)}}{\text{Incubation time at 37℃ (30 min)}} \times \frac{1000}{\text{Protein value (P.V)} \times \text{volume of sample (10µl)}}$$

$$\textbf{Hex-A activity} = \text{Sample-blank} \times \frac{2.5}{100} \times \frac{3.0+0.1}{3.0+0.1} \times \frac{60}{30} \times \frac{1000}{\text{P.V} \times 10}$$

<u>3.4.1.5.2 Assay for total-hexosaminidase (total-Hex) activity</u>

All the samples were tested in duplicates. The blanks were freshly prepared at each run. For cell extract (Leucocytes for postnatal diagnosis; AF cells/ CT cells for prenatal diagnosis) make 1:10 dilution with 0.75% HSA (1 part sample plus 9 part 0.75% HSA). Diluted samples (25 µl) were added in the 'test' tubes and 25 µl 0.75 HSA in 'blank' tube. Then add 3mM 4-methylumbelliferyl-N-acetyl-β-D-glucosamine (MUG) substrate (50 µl; Dissolve 11.4 mg 4-methylumbelliferyl-N-acetyl-β-D-glucosamine in 100 ml 12 mM citric acid/20 mM sodium phosphate buffer: pH 4.4) and the tubes were incubated for exactly 60 min at 37℃. The reaction was stopped by the addition of 3 ml glycine-NaOH buffer (pH 10.7). The relative fluorescence of each tubes was read on the Perkin-Elmer LS-55 spectropfluorimeter with excitation wavelength/slit width (366 nm/25 nm), and the emission wavelength/slit width (446/2.5 nm). Standards used for the calibration curve were prepared from a solution of 0.5-2.5 nmol. The relative fluorescence readings (RF) were corrected by subtracting the blank reading from each tube and enzyme activity calculated as nanomoles per hour per milligram of protein using the following equation.

$$\textbf{Total-hex activity} = \text{Sample-Blank} \times \frac{\text{Standard concentration (2.5nmol)}}{\text{Volume of standard used (100µl)}} \times \frac{\text{Volume of termination buffer (3ml) + Assay Volume (50µl Substrate + 25µl diluted sample)}}{\text{Volume of termination buffer (3ml) + Volume of standard (100µl)}} \times \frac{\text{1 hour (60 min)}}{\text{Incubation time at 37℃ (60 min)}} \times \frac{1000}{\text{Protein value} \times A}$$

Where, A= Sample volume (25µl)/dilution (1:10)

A=25/10=0.25

$$\textbf{Total-hex activity} = \text{Sample-blank} \times \frac{2.5}{100} \times \frac{3.0+0.75}{3.0+0.1} \times \frac{60}{60} \times \frac{1000}{\text{P.V} \times 2.5}$$

3.4.1.6 Quality control

An external quality control system was set up with ERNDiM quality control program for lysosomal enzymes study. Six unknown affected and/or normal samples every year were received from ERNDiM quality control program; the samples were run with test batches where possible. All our testing complied with ERNDiM quality reporting.

3.4.1.7 Statastic analysis

Quick P-value Calculator (http://www.socscistatistics.com/pvalues/Default.aspx) was used for calculation of p value.

3.4.2 Molecular analysis

3.4.2.1 Genomic DNA Extraction

DNA was isolated by standard salting out method (Miller *et al.*, 1988). The cell suspension was lysed by detergents (SDS) in the presence of high concentrations of chelating agent EDTA. Deproteinization was obtained with Proteinase-K treatment. Ethanol was used to precipitate DNA and dissolved in TE buffer.

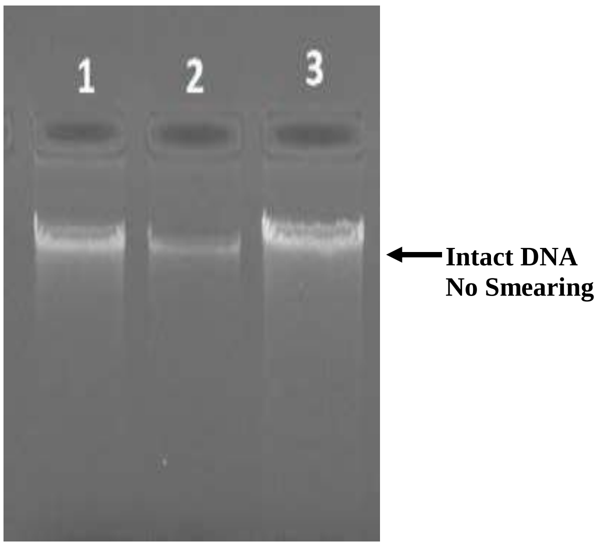
3.4.2.2 Determination of purity and concentration of isolated DNA

Purity of the isolated DNA was measured by taking ratio of optical density [OD or also known as Absorbance (A)] at 260 nm and 280 nm. If OD_{260} /OD_{280} ratio was within 1.6 to 1.8, then the DNA was considered as pure and was used for further experiments.

Concentration was measured by taking standard as OD_{260} =1 for 50µg/ml dsDNA (Double stranded DNA). Thus the DNA concentration in µg/ml was calculated by the formula = A 260 of experimental DNA × dilution factor × 50.

3.4.2.3 Integrity of DNA

Agarose gel electrophoresis of DNA: High molecular weight DNA was checked for its integrity. DNA was run on 2% ethidium bromide (EtBr) stained agarose gel to check its integrity. Electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook *et al.*, (1989). DNA samples (200 ng) were mixed with 1.0 µl of the DNA gel loading buffer. The gel was run under constant voltage (100 V) for 30 minutes. The bands were viewed under ultra violet (UV) light in gel documentation system (*Figure 3.1*).



Lanes 1- 3 showing yield of intact DNA

Figure 3.1: *Agarose gel of genomic DNA extracted from whole blood to check integrity*

3.4.2.4 Common mutations screening

3.4.2.4.1 The Polymerase chain reaction (PCR)

Primary screening of the common mutations c.1278insTATC, c.1421+1 G.C, 7.6 kb deletion, the pseudo-deficiency alleles (p.R247W and p.R249W) was performed by amplification refractory mutation system - polymerase chain reaction (ARMS PCR). This technique uses distinct 3' specific end primer complementary to either the mutant or normal allele described in *3.2* and PCR conditions were used as shown in *table 3.3*.(Kaplan *et al.*, 1991; Stockley and Ray, 2003). All PCR reactions were performed in thermal cycler 2720 (Applied Biosystem, USA) using 0.2 ml thin-walled PCR tubes, the PCR reaction were carried out in final volume of 15-20 µl containing the reagents as listed with negative control included.

All the patients who were found negative after screening using these PCR based techniques were later analyzed by quantitative analysis using MLPA techniques to rule out the presence of large deletion/duplications which cannot be analyzed using PCR based techniques.

Table 3.2: *Primers used for Tay-Sachs common mutation*

Tay-sach's common mutations			
Primer No	**Mutation**	**Primer Sequence 5' – 3'**	**Amplicon Size**
166	TATC1278 **M** (EXON 11)	CTGCCCCCTCGTACCTGAACCGTGTATCT	100
167	TATC1278 **N** (EXON 11)	CTGCCCCCTCGTACCTGAACCGTGTAT**C**CT	96
168	TATC1278 **CF** (EXON 11)	GCTCTCTGCTTTCACCTTCAAATGCC	-
169	1421+1G>C **M** (INTRON 12)	CACCTCCCCCCCGAAAACCCTTAG	160
170	1421+1G>C **N** (INTRON 12)	CACCTCCCCCCCGAAAACCCTTAC	160
171	1421+1G>C **CF** (INTRON 12)	AGTTACCCCACCATCACCAGACTGT	-
172	805G>A **M** (EXON 7)	GTCCCTCTCGTCCCAGACATCATTCTTACT	197
173	805G>A **N** (EXON 7)	GTCCCTCTCGTCCCAGACATCATTCTCACC	197
174	739C>T **M** (EXON 7)	CAAGCACACGGATACCCCCGAGCCA	126
175	739C>T **N** (EXON 7)	TGCAAGCACATGGATACCCCGGAGTCG	128
176	745C>T **M** (EXON 7)	TGTCAAACTCTGTAAGCACACGGATACCTCA	138

177	745C>T **N** (EXON 7)	TGTCAAACTCTGCAAGCACACAGATACTCCG	138
178	Common F. Primer for 805G>A, 739C>T and 745C>T	AAGTGTGAACCTGAAGAGTGTCTTGTGCC	-
179	7.6 Kb del **M** (EXON 1)	TATCAGCTTTAGGATAATAA	328
180	7.6 Kb del **N** (EXON 1)	GTAGCCTGAATAAGACATAC	168
181	7.6 Kb del **CF** (EXON 1)	GGCATCCAATGTGAAATAAGC	-

Table 3.3: *PCR conditions for common mutations*

Mutations	**PCR conditions**	**PCR cycles**
+TATC 1278	Denaturation 94° C , 30 s Annealing 62° C , 30 s Extension 72° C , 30 s	30 cycle
+1421 +1 G>C	Denaturation 94° C , 30 s Annealing 61.5° C , 30 s Extension 72° C , 30 s	30 cycle
739 C>T	Denaturation 94° C , 30 s Annealing 61° C , 30 s Extension 72° C , 30 s	25 cycle
745 C>T	Denaturation 94° C , 30 s Annealing 55° C , 30 s Extension 72° C , 30 s	25 cycle
7.6 Kb del.	Denaturation 94° C , 1 min Annealing 55° C , 1 min Extension 72° C , 3 min	32 cycle

3.4.2.4.2 Electrophoresis of PCR amplicons

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook *et al.,* (1989). Electrophoresis of all PCR amplicon was carried out on 2.5% agarose gel to separate PCR amplicon by molecular size. The agarose was dissolved in 1X TAE buffer by microwaving on the high setting for 1-2 min followed by cooling to approximately 50° C. Etbr (100 µg/l) was added and the gel left to set for about 30 min before the PCR products were loaded. The PCR products (10 µl) were mixed with 1 µl gel loading dye to monitor the movement of amplicons in the gel system. This dye runs at approximately 40 bp and therefore did not obscure the amplicon being tested. The electrophoresis was carried out at 100 V in a Bangalore Genei gel electrophoresis system (Bangalore Genei, India) for 30 min and the gel was visualised using a UV transillumination (λ=302nm) and the image captured on E-Gel imager system (Life technologies, USA).

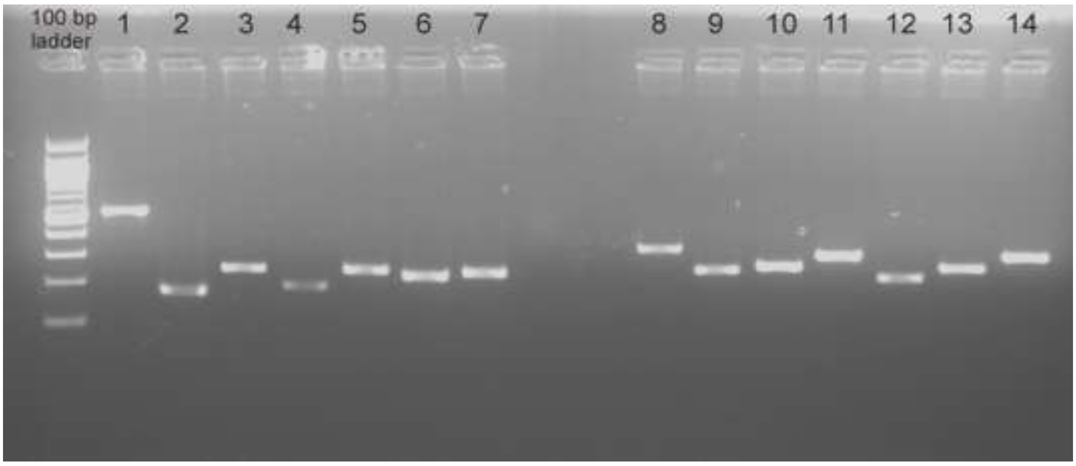
3.4.2.5 DNA sequencing of HEXA gene (Hexosaminidase-A)

DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of template using specific targeted forward and reverse DNA primers DNA. In essence, the DNA is used as a template to generate a set of fragments that differ in length from each other by a single base. The fragments are then separated by size, and the bases at the end are identified, recreating the original sequence of the DNA. The most commonly used method of sequencing DNA - the dideoxy or chain termination method - was developed by Fred Sanger in 1977. The key to the method is the use of modified bases called dideoxy bases; when a piece of DNA is being replicated and a dideoxy base is incorporated into the new chain, it stops the replication reaction.

The exonic and intronic flanking sequences of the *HEXA* gene were PCR amplified into 14 fragments using the primer pairs as described earlier (Trigss Raine et al., 1991) (*Table 3.4*). The amplification was carried out by using Taq polymerase (from invitrogen), dNTPs, PCR buffer and exon specific forward and reversed primers. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA) for 30 cycles, each consisting of 30 s denaturation at 94° C, 30 s annealing at 64° C, and 90 s extension at 72° C and PCR products were run on agarose gel to look amplified PCR product visualized under UV transillumination (λ=302nm) and the image captured on a Geldoc system (E-Gel imager, Life technologies, USA). Sequencing of PCR amplicon required purification of the amplicon using Exo-sap treatment. Exo-sap was used to remove extra dNTPs and primer dimmer. This is based on the utilization of two hydrolytic enzymes, Exonuclease I and shrimp alkaline phosphatase which removes unwanted dNTPs and primers. These clean-up PCR products ranging in size from less than 100bp up to over 20kb. The DNA is now ready for direct sequencing using manual or automated methods.

Table 3.4: *Primers for sequencing of HEXA Gene*

Exon No	**Primer Sequence (5'→3')**	**Annealing temperature**	**Fragment Size (bp)**
Exon 1	F.P: CTCACCTGACCAGGGTCTCACGTT R.P: CTCCTGATTGAACCGTAGTCCTA	61° C	502
Exon 2	F.P: GCTTCTAATGGCAGGTTGGCCGCA R.P: GCTCTTCTAAGACAGGGAACAGGA	61° C	166
Exon 3	F.P: GAATATCTGGTCTATAATCTGAG R.P: TGAGCAGGGACTGGGTTACTGCA	61° C	226
Exon 4	F.P: TCTGCTACATTGAGAACCTTCC R.P: GATCCAACCCCAGAGATGAA	61° C	182
Exon 5	F.P: ATCTCCCTGTGCCCCCATAGTAA R.P: TGCTCCATCACCCTAGAACTCTTA	61° C	230
Exon 6	F.P: TGAAACCGGAGAGACTGTGATG R.P: GCCACAGCCAGATTCAGACATTG	61° C	211
Exon 7	F.P: GCTAGCTTTCAGGAAGTGTGAACC R.P: TAACAAGCAGAGTCCCTCTGGT	61° C	221
Exon 8	F.P: TGCGTGGGGGTTTATGTATT R.P: GCTAACTTCTATTCTGAGTAAGCAAC	61° C	298
Exon 9	F.P: CAGGCATTAGGCTTTCAGGATGTT R.P: CAAGCAGGGCCTGACTCGGTATG'	61° C	230
Exon 10	F.P: GTCTAGAACCCATCTGAGCTAAG R.P: AAGCCCAATCCAAACCAGGAGGA	61° C	237
Exon 11	F.P: CCAGGAATCTCCTCAGCTTTGTGT R.P: AGCCTCCTTTGGTTAGCAAGG	61° C	276
Exon 12	F.P: AGTTACCCCACCATCACCAGACTG R.P: TTGGGTCTCTAAGGGAGAACTCCT	61° C	203
Exon 13	F.P: ACCTTTGCAGGGAATACAGGGC R.P: TGTCTTCCTCTCTCTAAGGGGTTC	61° C	235
Exon 14	F.P: TGACTGGTGTGAAAAGTGTTGCTG R.P: CCTTTCTCTCCAAGCACAGG	61° C	273



Lane 1-14: Exon 1-14 respectively

Figure 3.2: *Agarose gel of amplified PCR product for HEXA gene (Exons 1-14)*

Sequencing reaction was performed using 2-5 µl purified PCR product, 3 pmol primer forward or reversed) and 4 µl of ABI Dye-2 Terminator mix in a total volume of 20 µl with nuclease free water. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA) for 35 cycles of 94° C for 10 s, 50° C for 10 s, 60° C for 4 min. Sequencing product was cleaned up by using sodium acetate and ethanol, dry final pallet at 56° C in DNA dryer, dissolved in formamide and run in sequencer (Model-3110 from Applied Bio-system). Sequenced alignment was performed using the Blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul *et al.*, 1997).

The nucleotide numbers are derived from *HEXA* cDNA sequences (RefSeq NM_000520.4). Putative mutations were confirmed in two separate PCR products from the patient's DNA. Heterozygosity for these mutations was confirmed in parents. The mutations identified were then looked up in public domain of Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), SNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) and McGill University database (http://www.hexdb.mcgill.ca). Novel variants were further confirmed for their pathogenicity by studying 100 control individuals with normal leucocytes enzyme results.

3.4.2.6 Multiplex Ligation dependent Probe Amplification (MLPA)

Copy number changes in targeted regions of the *HEXA* gene were identified by hybridizing with MLPA (Multiplex Ligation dependent Probe Amplification) probes kit from MRC Holland. Each MLPA probe consists of two hemi-probes that bind to adjacent sites on the target sequence. Upon ligation and subsequent PCR amplification, each distinct MLPA probe (specific to distinct target regions) generates an amplicon with a unique length which are separated and quantified by capillary electrophoresis. Heterozygous deletions within target sequences will prevent efficient probe binding and give a 35-50% reduced relative peak area of the amplification product specific to that probe set. Copy number differences of various exons between test and control DNA samples can be detected by analyzing the MLPA peak patterns.

The *HEXA* gene comprises 14 exons, spanning more than 33 kb of genomic DNA on chromosome 15q23, about 73 Mb from the p-telomere. This P199 HEXA probemix contains 18 MLPA probes for *HEXA* gene sequences. Two of these probes are specific for certain frequent point mutations: c.1278insTATC at 166 nt (also known as c.1274_1277dupTATC (p.Tyr427Ilefs) and c.1421+1G>C at 172 nt (also known as IVS12, G-C, +1). The c.1278insTATC mutation is found in 80% carriers of TSD from the Ashkenazi Jew population. A deletion of 7.5 kb, including all of exon-1, is the major mutation found in Tay-Sachs disease carriers in the French-Canadian population. In addition, 11 reference probes are included in this probemix detecting several different autosomal chromosomal locations.

MLPA analysis was carried out using P199-B2 HEXA P probe mix (MRC-Holland, Amsterdam, The Netherlands). The procedures were carried out according to the manufacturer's recommendations (www.mlpa.com). 100 ng DNA was denatured and hybridized overnight at 60º C with the SALSA probemix P199-B2 (*HEXA* exons 1-14). Samples were then treated with Ligase for 15 min at 54º C. The reactions were stopped by incubation at 98º C for 5 min. Finally, PCR amplification was carried out with the specific SALSA FAM PCR primers. Amplification products were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) with the following modules: capillaries 36 cm, Polymer POP-4, run temperature 60º C, capillary fill volume 184 steps, pre-run voltage 15 kV, pre-run time 180 sec, injection voltage 1.6 kV, injection time 10-30 sec, run voltage 15 kV, data delay time 1 sec, run time 1800 sec. The obtained data were analysed by using Gene mapper Software from Applied biosystems. Three healthy controls without family history of TSD were analyzed as controls.

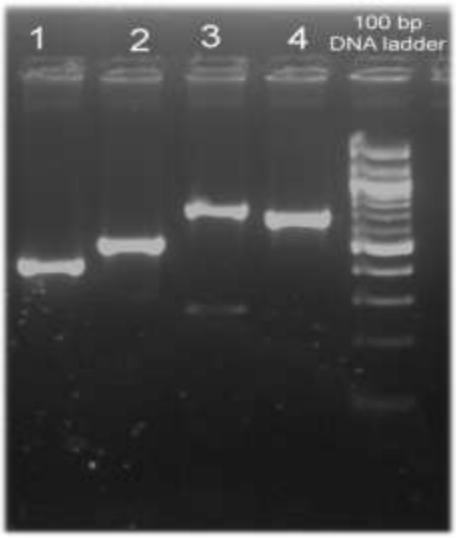
3.4.2.7 DNA sequencing of GM2A gene

The exonic and intronic flanking sequences of the *GM2A* gene were PCR amplified into 4 fragments using the primer pairs as described earlier (Chen *et al.*, 1999) (*Table 3.5*). Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA) for 43 cycles, each consisting of 30 s denaturation at 94° C, 30 s annealing at 54° C, and 30 s extension at 72° C and PCR products were run on agarose gel to look amplified PCR product visualized under UV transillumination (λ=302nm) and the image captured on a Gel doc system (E-Gel imager, Life technologies, USA) (*Figure 3.3*). The DNA sequencing was carried out using aforementioned DNA sequencing method (refer section 3.4.2.5).

The nucleotide numbers are derived from *GM2A* c.DNA sequences (RefSeq NM_000405.4). Putative mutations were confirmed in two separate PCR products from the patient's DNA. Heterozygosity for these mutations was confirmed in parents. The mutations identified were then looked up in public domain of Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), SNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) and McGill University database (http://www.hexdb.mcgill.ca).

Table 3.5: *Primers for sequencing of GM2A Gene*

Exon No	**Primer Sequence (5'→3')**	**Annealing temperature**	**Fragment Size (bp)**
Exon 1	F.P: GGAAGGCATTTAAAGGACCTCT R.P: AAGGCTGTCTGCATTTTCACTC	54° C	426
Exon 2	F.P: CCCAGGTTCATAGGTATGGAGT R.P: GCTGGCGCTGACATCAGAGATT	54° C	528
Exon 3	F.P: GATTATTAATGTCTGCCATGGG R.P: GCTAGGATTGCAGCCATGAACT	54° C	716
Exon 4	F.P: CATGTCTCTGGATTTGTAAGCC R.P: GGCTATCAAGAACTGTCCAACT	54° C	665



Lane 1-4: Exon 1-4 respectively

Figure 3.3: *Agarose gel of amplified PCR product for GM2A gene (Exons 1-4)*

3.4.3 Bioinformatics analysis

Various bioinformatics software for prediction of effect of sequence variations on the structure and function of protein were employed. Various softwares are available which allow prediction of changes in DNA structure as a consequence of sequence variations as well predict their effect on protein structure and sequenced based on function. The sequence variations are predicted as deleterious or not depending upon their nature e.g. affecting folding, structure, function or conservation. The various methods used for analyzing sequence variations are as follows:

3.4.3.1 Prediction tools (bioinformatics softwares) predicting non-synonymous and splice site sequence variations.

Prediction of functional effects of non-synonymous single nucleotide substitutions (nsSNPs) was done using various software programs; SIFT (Sorting Intolerant From Tolerant) (available from: http://sift.jcvi.org/), Polyphen2 (Polymorphism Phenotyping v2) (available from: http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (available from: http://www.mutationtaster.org/) (Kumar *et al.*, 2009; Adzhubei *et al.*, 2010; Schwarz *et al.*, 2010) to know the effect of variants on protein function.

3.4.3.2 Multiple sequence alignment of HEXA gene

Evolutionary conservation of the amino acid residues of *HEXA* was analyzed using ClustalW program (available from: http://www.uniprot.org/help/sequence-alignments). Using this program, the sequence of Human *HEXA* gene was aligned with *HEXA* gene of different species.

3.4.3.3 Protein modeling and molecular docking studies

3.4.3.3.1 Protein preparation

The mutant (αD322N, αD322Y and αE462V) were built using build mutant protocol of Accelrys Discovery studio 2.0 using the crystallographic structure of Hex-A (PDB ID: 2GJX) as the template. The wild type protein and mutant energy minimized using CHARMm force field and 200 cycles of steepest descent algorithm.

3.4.3.3.2 Ligand preparation

The structural coordinates of GM2 ganglioside was retrieved from PubChem database (CID 9898635). The GlcNAc (N-acetyl galactosamine) protein of the GM2 ganglioside was considered for docking on the active site of Hex-A as reported previously (Lemiaux *et al.,* 2006). It was prepared using the Prepare Ligand protocols of Accelrys Discovery studio 2.0.

3.4.3.3.3 Molecular Docking

Structural analysis revealed that residues R178, D207, H262, E323, D322, W373, W392, Y421, N423, R424 and E462 constitute the active site of αHex-A. This information was used to define the binding site and docking studies were performed using the Ligand Fit protocol of Accelrys Discovery studio 2.0. The potential energy and Root Mean Square Deviation (RMSD) of the mutant structure with respect to the wide-type structure were calculated. RMSD values more than 0.15 were considered as a significant structure perturbations that could have functional implication for the protein (Ohno *et al.,* 2008)

3.4.4 Haplotype analysis

Haplotype analysis was performed by sequencing the introns 1, 5, 12, 13 and a 417 bp region 3' to *HEXA* gene which contained several polymorphic markers, in all TSD patients and 30 ethnic controls.

3.4.5 Established simple cost effective PCR based methods for common mutations

Herewith, we have designed simple ARMS-PCR and RFLP-PCR method for two common mutations E462V and D322Y respectively identified in *HEXA* gene during the present study.

3.4.5.1 Established ARMS-PCR for E462V mutation

<u>Primer designing</u>

Primers were designed using online Primer3 software and manually modified as mutation specific primer, wild type specific primer and common forward primer as shown below

Forward primer: 5'-agttaccccaccatcaccagactg-3' (same as exon-12 forward primer)

Reverse Primer: 5'-ccaggtttgtgttgtccacatatt-3' (wild type specific primer)

5'-ccaggtttgtgttgtccacatata-3' (mutation specific primer)

All primers selected were checked for all essential parameter as per universal standard guideline (e.g. primer length, GC content, Tm, complimentary of primer etc.) (Abd-Elsalam, 2003) and use for PCR consistency.

ARMS-PCR

PCR amplification was performed for each mutation separately in a 15 µl final reaction volume containing 500 ng genomic DNA, 10× Cetus buffer, 2.0 mM dNTPs, 1 U Taq polymerase, 5 pmol of each primers and 1.5 µl 10X Cetus buffer. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA). The thermo cycling conditions were 10 min at 94° C, followed by 25–32 cycles of amplification consisting 30 s - 60 s at 94° C, 30 s - 60 s at 55-63° C and 30 s - 3 min at 72° C, and a final elongation for 10 min at 72° C and PCR product were run on 2% agarose and visualized under UV transillumination (λ=302nm) and the image captured on E-Gel imager (Life technologies, USA).

3.4.5.2 Established PCR-RFLP for D322Y mutation

Primer designing:

Primers were designed using online softwere Primer3web version 4.0.0 (http://primer3.ut.ee/). All primers selected were checked for all essential parameter as per universal standard guideline (e.g. primer length, GC content, Tm, complimentary of primer etc.) (Abd-Elsalam, 2003) and use for PCR consistency.

PCR

The amplification of Expn-8 (*HEXA* gene) was carried out using 10× Cetus buffer, 2.0 mM dNTPs, 1U Taq DNA polymerase, 10pmol exon specific forward and reversed primers as shown in table-2.4 and 250ng genomic DNA. Amplification was performed in a thermal cycler 2720 (Applied Biosystems, CA, USA) for 30 cycles, each consisting of 30 s denaturation at 94°C, 30 s annealing at 64°C, and 90 s extension at 72°C and PCR products were run on agarose gel to look amplified PCR product visualized under UV transillumination (λ=302 nm) and the image captured on E-Gel imager (Life technologies, USA).

Restriction enzyme (RE) selection

The *BseR1* restriction enzyme (RE) was selected using online softwere NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) and/or Restriction Mapper version 3 (http://www.restrctionmapper.org/).