CHAPTER 3
MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1. Selection of Patients

Patients visiting, department of neurology of various medical colleges and hospitals of North Karnataka including Neurospeciality Center, Belgaum Karnataka with the symptoms of the PD from 2009 to 2011 were included in the epidemiological investigation. A total of 557 patients belonging to both rural and urban areas were included in this study. All the subjects were interviewed with the help of a structured, pretested questionnaire. This was followed by clinical examination and relevant laboratory investigations. Standard definitions (widely accepted) were used to measure the physical activity, tremour, rigidity, bradykinesia and akinesia (139). Out of 557 patients; 100 patients were selected for molecular genetic studies with the informed consent. Along with the patients, ethnically matched 100 healthy individuals which are not related to PD patients were selected as control. Additionally 20 healthy samples from PD affected patients family were also included for the Next Generation Sequencing (NGS) study.

3.2. Clinical Sample (Blood) Collection

Consent was obtained from the subjects, who were enrolled in the study. After taking consent, 1 ml peripheral blood samples were collected in the EDTA coated vacutainers (BD367863) and stored at 4°C. Along with the patient’s blood samples, ethnically matched control blood samples were also collected.

3.3. Isolation of Genomic DNA and Quantification

From 300µl of peripheral blood, genomic DNA was isolated, with the help of commercial DNA isolation kit (Bangalore Genei, India).

3.3.1. Genomic DNA Isolation Protocol
1. In 1.5 ml EDTA coated vial 300 µl of peripheral blood was collected.
2. By adding 1 ml of 1 X solution A (provided by the kit) RBC cells were lysed.
3. At room temperature the vials were centrifuged for 5 min at 8000 RPM.
4. Until a clear white WBC pellet was obtained the above step was repeated.
5. 600µl of solution B was added (provided by the kit) to the WBC and mixed gently for clear lysis.
Molecular Characterization of SNCA Gene (α-Synuclein-1) in Parkinson's Disease

6. It was centrifuged at room temperature for 10 min at 10,000 RPM.
7. The supernatant was collected and 0.9 ml absolute cold ethanol was added to it and mixed.
8. Centrifuged at 4°C for 20 min, at 10,000 RPM.
9. Precipitate DNA was washed with the 0.5 ml of 75% ethanol.
10. Centrifuged for 5 min at 10,000 RPM.
11. 100 ul of solution C was added (provided by the kit) after air drying the DNA pellet.
12. The vial was incubated at 55°C for 10 min.
13. To remove any insoluble materials it was centrifuged at 10,000 RPM for 2 min.
14. The DNA thus obtained was stored at -20°C until further use.

The quality of the isolated DNA was checked under gel electrophoresis. 100 ml of 1% agarose gel was prepared (1 gm of Agarose + 100 ml of 1X TAE buffer). The same isolated DNA was quantified using “Nano Drop” (Quawell) and quantity and quality of the DNA was recorded.

3.4. Exon-specific intronic primer designing

Web based freely available program “Primer3” which is widely accepted was used, (http://frodo.wi.mit.edu/ primer3/input.htm) for designing PCR primers. Primer 3 is a Bioinformatics tool which helps in designing the primers for the target region in the given nucleotide sequence as per the requirement of the user or applications. The designed primers using Primer 3 were reconfirmed for the specificity for its binding site using the web based bioinformatics tool “GenomeBuild36” (https://genome.ucsc.edu/FAQ/FAQreleases.html), and for its Insilico amplification on “Insilico PCR” (http://insilico.ehu.es/PCR/). All the designed primers for our target genes or region are tabulated in table No. 1 along with the annealing temperature and amplicon size. Primers were synthesized by commercial oligo synthesizer (MWG Biotech, India).

Department of Applied Genetics, Karnatak University, Dharwad.
Table 1. Details of the primer sequences and annealing temperatures used for the amplification of all the exons of SNCA gene.

<table>
<thead>
<tr>
<th>Name of the primer</th>
<th>Sequence</th>
<th>Amplicon Size</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA 1F</td>
<td>TCCACAAAGAGTGCTCGTGAC</td>
<td>728</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 1R</td>
<td>CTCAGATTCCAAAAAGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 2F</td>
<td>C AAGATGGATGGAGATGCT</td>
<td>468</td>
<td>60°C</td>
</tr>
<tr>
<td>SNCA 2R</td>
<td>CTCGTGACTCGACTCCACCTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 3F</td>
<td>CCCCCGTTATATCTCATTCT</td>
<td>437</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 3R</td>
<td>CTTGAATACGGGCCACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 4F</td>
<td>GGACATCTTGGGTGCTTTTG</td>
<td>407</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 4R</td>
<td>GCCGTTCACCAGTGAAGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 5F</td>
<td>GGTTTGATAACCATGTTGC</td>
<td>483</td>
<td>60°C</td>
</tr>
<tr>
<td>SNCA 5R</td>
<td>CTTCCTTCACATCTCCTCCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.1 F</td>
<td>AACTGGGAGAGTGAAAGTAGG</td>
<td>638</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 6.1 R</td>
<td>TGGGGGGAGGTACAGATACT</td>
<td></td>
<td></td>
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<tr>
<td>SNCA 6.2 F</td>
<td>GTCCTTCAATGTGCCAGTCA</td>
<td>682</td>
<td>62°C</td>
</tr>
<tr>
<td>SNCA 6.2 R</td>
<td>AGGGAATTCGAGTGGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.3 F</td>
<td>GCCATTGGAAGAGGAGGAA</td>
<td>157</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 6.3 R</td>
<td>TCTTCAACACCCCCACCTTCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.4 F</td>
<td>CTGAAGCAACACTGCCAGAA</td>
<td>606</td>
<td>62°C</td>
</tr>
<tr>
<td>SNCA 6.4 R</td>
<td>GTGACTCTGTAGTTCACACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.5 F</td>
<td>C AAGGCTCTGAGAGATGAGG</td>
<td>605</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 6.5 R</td>
<td>TGACACATGAGGTAGCTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.6 F</td>
<td>ATATGTTGGGCGCTGGTAGAC</td>
<td>661</td>
<td>62°C</td>
</tr>
<tr>
<td>SNCA 6.6 R</td>
<td>CGTGGAGTGCTATGAGGCTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNCA 6.7 F</td>
<td>C AAGTGCTGGCTTGAGGTCAAT</td>
<td>559</td>
<td>61°C</td>
</tr>
<tr>
<td>SNCA 6.7 R</td>
<td>CGTCCCAGAAGAGTGTAGAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5. Polymerase Chain Reaction (PCR)

PCR amplification was carried out in a 20μl reaction volume containing 0.5 μl of genomic DNA (75ng/μl to 150 ng/μl), 0.5μl of each primer (5pmol), 0.4μl of dNTP (10pmol), 0.2μl Taq DNA polymerases (3units/ μl), 4 μl Taq Buffer (5X) (BioRad, USA) and total volume was adjusted to 20μl using molecular biology grade water. Amplification was carried out in Mastercycler gradient (Eppendorf, Germany) under the following conditions: an initial denaturation at 98°C for 10sec, followed by 35 cycles at 98°C for 10sec (cycle denaturation). Primer annealing temperature was set depending on the annealing temperature of each primer (Table-1) for 10sec72°C for 15sec (primer
extension) and a final extension at 72°C for 5 min. PCR products were confirmed for their respective amplicon size by gel electrophoresis with standard 100bp ladder.

3.6. DNA Sequencing (Capillary Based)

PCR products were subjected for capillary based Big-Dye terminator sequencing. Prior to sequencing, the PCR products were subjected to cycle sequencing and plate processing.

3.6.1. Cycle Sequencing

As per the Sanger Sequencing protocol, Big-Dye labeling and chain termination was carried out by cycle sequencing method. To label each base, PCR amplicon was subjected to cycle sequencing reaction with single primer. Big-Dye TM terminator v3.1 was used for cycle sequencing (Applied Biosystems, USA) following the manufacturer’s guidelines. Cycle sequencing of the PCR products was carried out according to the annealing temperature of the primers.

<table>
<thead>
<tr>
<th>SL. No.</th>
<th>Constituents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Molecular Biology grade water</td>
<td>6.3</td>
</tr>
<tr>
<td>2.</td>
<td>Big Dye Buffer (5X)</td>
<td>1.3</td>
</tr>
<tr>
<td>3.</td>
<td>Big Dye</td>
<td>1.0</td>
</tr>
<tr>
<td>4.</td>
<td>Template (PCR product)</td>
<td>1.0</td>
</tr>
<tr>
<td>5.</td>
<td>Primer (Forward/Reverse)</td>
<td>0.2 µL</td>
</tr>
</tbody>
</table>

**TOTAL**               10.0

Note: Only one of the primers i.e either forward or reverse primer was used during cycle sequencing.
Table 3. The cycle sequencing conditions

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98</td>
<td>10sec.</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10sec.</td>
</tr>
<tr>
<td>Annealing (primer dependent)</td>
<td>(primer dependent)</td>
<td>10sec.</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>5min.</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Note: The annealing temperature is primer dependent and varies for each primer.

3.6.2. Sequencing Cleanup (Plate Processing)

To remove the unbounded fluorescent DTNP's from terminator sequencing reaction, 2µl of 3M sodium acetate, 50µl of 100% ethyl alcohol were added to each sample and incubated at room temperature for 15 minutes to precipitate the DNA. The samples were centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was discarded and the reaction plate was centrifuged in reverse manner at 300 rpm for 20 seconds. 100µl of 75% alcohol was added to each sample and centrifuged at 4000 rpm for 15 minutes at 25°C. The supernatant was discarded and plate was centrifuged in reverse manner at 300 rpm for 20 seconds to remove alcohol completely. The plate was dried at room temperature until the last drop of alcohol dripped off.

10µl of Hi-Di Formamide was added to each well of the sample plate. The samples were heated to 96°C for 5 minutes and immediately cooled to 4°C to denature and linearise the cycle sequencing products. The processed products were loaded in the sequencer for sequencing.

3.6.3. Sequencing Run

Sample information sheets which contain analysis protocols along with the sample details were prepared and imported into the data collection software. Prepared samples were analyzed on ABI 3730 genetic analyzer (Applied Biosystems, USA) to generate DNA sequences or electropherograms. After completion of sequencing reaction the quality of generated sequence was checked by using Sequencing Analysis v5.4 software (Applied Biosystems, USA).
3.6.4. Sequence Alignment

The generated sequences were aligned to their respective reference sequences with the use of Variant reporter software (ABI v1.1). Variant reporter is one of the compatible softwares of Applied Biosystems designed for automated sequence data analysis. It performs sequence comparisons for novel mutations, known variants, insertions and deletions. It allows analysis of the resequenced data, comparing the consensus sequences to a known reference sequence. The results of the variant reporter were tabulated in PDF format as default programme of the software.

3.7. Next Generation (NGS) Sequencing and Mutation Analysis

Today's complex genomic research questions demand a depth of information beyond the capacity of traditional DNA sequencing technologies. Next-generation sequencing has filled that gap and has become a regular research tool to address these questions. With its unprecedented throughput, scalability, and speed, next-generation sequencing (NGS) enables researchers to study biological systems at a level never before possible.

There are three major NGS platforms (Illumina/ Solexa, Roche-454, ABI-SOLiD) which have been widely used by scientists and published extensively since 2007(140). In our study of Targeted Re-sequencing of Human DNA, Illumina-Solexa platform has been used, more specifically the GAIIx instrument. Illumina next-generation sequencing utilizes a fundamentally different approach from the classic Sanger chain-termination method. It leverages sequencing by synthesis (SBS) technology – tracking the addition of labeled nucleotides as the DNA chain is copied – in a massively parallel fashion. 72bp singleton sequence reads were generated and data analyzed including alignment, assembly, and Variation Discovery.

Figure 2: Illumina Next Generation Sequencer (NGS)
Next-gen sequencing generates masses of DNA sequence data that's richer and more complete than is imaginable with Sanger sequencing. Illumina's sequencing systems can deliver data output ranging from 300 kilobases up to 1 terabase in a single run, depending on instrument type and configuration.

**Figure 3: Systematic workflow of NGS**

Next generation sequencing technologies of Illumina involves an approach, where in before sequencing itself, the sequencing libraries are generated via PCR amplification of the DNA templates. In Illumina, preparation of the sequencing library is done by bridge PCR, while the sequencing is done by a technology referred to as cyclic reversible termination.

In the bridge PCR, primers and the DNA template are immobilized to the 2-dimensional surface. The primers are designed to target the adaptors of the DNA fragments so that the fragments bind to primers in their neighborhood. Within each PCR cycle the fragments build so called bridges and the following denaturation leaves single
stranded templates anchored to the surface. The copies remain local and form dense clusters. To sequence the clusters a universal primer is hybridized to the adaptor sequence of the DNA fragments.

Sequencing of clusters generated via bridge PCR is done by a technique called cyclic reversible termination. Thereafter, the polymerase extension is performed with reversible terminators. These are deoxynucleotides carrying a fluorophore and a blocking group. The 4 nucleotides have different fluorophores attached. The polymerase incorporates just 1 labeled nucleotide as the blocking group terminates DNA synthesis. Unincorporated nucleotides are washed away and the array is imaged to determine the identity of the incorporated nucleotide. This is followed by a cleavage step which removes the blocking group and the fluorophore. The resulting 4-colour images are used to decode the sequence.

3.7.1. Capture Array Design

To capture the coding regions of target genes of interest, 1 x 1 Million Agilent capture array comprising of 60 mer tiling probes were designed. From UCSC data base, all the possible transcripts for the genes of interest were downloaded. For the coding regions of the genes of interest, exonic BED files were created. At the upstream and downstream to each coding exon in the BED file, 70 b flanking regions were added. The overlapping regions were merged into a unique target region. Known repeat regions and gaps overlapping with the unique target region were identified. Probes were not designed in these repeat regions; however probes were allowed to have a maximum of 10 bases into repeat regions to avoid losing coverage on borders.

3.7.2. Library Construction and Array Capture

Materials for DNA sequencing
- TruSeq DNA Sample Preparation kit (Illumina, # FC-121-1001 or FC-121-1002)
- Agencourt AMPURE XP beads (Beckman Coulter, #A63881)
- 7500 Bioanalyzer Chips High Sensitivity Bioanalyzer Chips (Agilent, #5067-4626)
- Oligo aCGH Hybridization kit (Agilent, # 5188-5380)
- Microarray slide backings (Agilent, # G2534-60005)

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3.7.3. Preparation of Samples for Next Generation Sequencing

1. Out of 100 samples included for the study 20 PD samples, 20 control samples which are not related to PD patients and 20 control from the PD family were selected for NGS analysis depending on the quality and quantity. For our convenience samples are grouped as "A – Control from the same family", "B – ethnically matched control", "C – PD patients".

2. 20 samples from each set A, B and C were pooled in equal amounts to get ~5 micrograms of genomic DNA per set and then deep sequencing was carried.

3. The genomic DNA was concentrated to 100 ul (Vacuum Concentrator) and sonicated (60 pulses on high at 30s ON and 30s OFF) to fragment DNA into sizes ranging between 100 to 400 bp (Bioruptor).

Protocol for Sonication of DNA

The Bioruptor uses a gentle method of sonication to retain the integrity of DNA and/or biological complexes, including chromatin, protein-protein binding, protein-DNA complexes, and other biochemical and biological assay systems. The Bioruptor Sonication System uses a water bath to generate these indirect sonication waves, which emanate from an ultrasound element below the water tank. The Bioruptor sonication system uses ultrasound to create focused mechanical stress to lyse cells or shear DNA or chromatin. The ultrasound waves pass through the sample, expanding and contracting liquid. During expansion, negative pressures pull the molecules away from one another and form a cavity or bubble in a process called cavitation. The bubble continues to absorb energy until it can no longer sustain itself and then implodes, producing intense focused shearing forces, which disperses or breaks biomolecules. Also, the fragmentation of DNA takes place as a consequence of this mechanical stress or shear from the bubbles. With the Bioruptor, the entire volume
of water present in the waterbath is exposed to ultrasound, allowing the samples in the tubes to be efficiently sonicated.

- Prior to sonication, human DNA samples were dissolved in a sonication buffer of TE (10 mM Tris, 1 mM EDTA), pH 8 with a DNA concentration of 0.01 μg/μl and a final volume of 100 μl.
- All samples were vortexed and centrifuged with Costar 0.65 ml Low Binding Microcentrifuge Tube (Diagenode, Cat. No. COR-3206) and Tube holder for 12 x 0.5 ml tubes (Diagenode, Cat. No. UCD-pack 0.5) before shearing in the Bioruptor sonication system.
- After shearing, 30 to 40 cycles were carried out to get a 100-400bp DNA fragment, which is recommended by Illumina GAIIx.

**Figure 4: Work flow of library preparation for NGS**

4. The resulting fragmented DNA was cleaned up using Ampure XP SPRI beads (Beckman Coulter).

**Protocol for DNA Clean up Using Ampure XP SPRI Beads**

The SPRI works Fragment Library System I uses the patented Agencourt SPRI paramagnetic particle-based technology to generate fragment libraries from fragmented or sheared input DNA. The SPRI works Fragment Library System I automated method is performed on the SPRI-TE Nucleic Acid Extractor, using pre-measured reagents in a sealed reagent cartridge, and enables the construction of three library types: no size selection; 200-400 bp size selection; and 300-600 bp size selection. The SPRI works Fragment Library System I method begins with the off-line shearing of input DNA by nebulization or other means. Fragmented DNA is then loaded onto the SPRI-TE instrument which performs the subsequent
steps in the library construction process. The SPRI works Fragment Library System I employs enzymatic reactions, SPRI-based reaction purifications, and optional SPRI-based size selection to automatically generate fragment libraries. All reagents required to construct a single library are contained within the reagent cartridge. Additional reagents and primers are required for subsequent enrichment of the library. The method can generate from one to ten libraries simultaneously per 5-hour run. After processing on the SPRI-TE instrument, libraries are further amplified and used as templates for sequencing on the Illumina Genome Analyzer.

5. The size distribution was checked by running an aliquot of the sample on Agilent 7500 Bioanalyzer Chip.

Protocol for Agilent 7500 Bioanalyzer Chip

Agilent DNA kits contain chips and reagents designed for sizing and analysis of DNA fragments. Each Agilent DNA contains an interconnected set of microchannels that are used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. Agilent DNA kits are designed for use with the Agilent 2100 bioanalyzer only. Agilent DNA 7500 and DNA 12000 kits are designed for the sizing and quantitation of double-stranded DNA fragments from 100 to 7500 base pairs (DNA 7500) and 100 to 12000 bp (DNA 12000 respectively). These kits can be used to analyze, for example, PCR and RT-PCR products as well as restriction digests. For accurate determination of DNA concentration, the total amount of DNA in sample must be between 0.5–50 ng/μl. If concentration is excessively high, dilute to 0.5–50 ng/μl.

Sample Preparation

- DNA dye concentrate and DNA gel matrix were allowed to equilibrate at room temperature for 30 min.
- 25 μl of DNA dye concentrate was added to a DNA gel matrix vial.
- Solution was well vortexed and spin down. Transferred to spin filter.
- Centrifuged at 1500 g ± 20 % for 15 min. Solution was protected from light. Stored at 4°C.
- The gel-dye mix was allowed to equilibrate at room temperature for 30 min before use.
- A new DNA chip was put on the chip priming station.
- 9.0 μl of gel-dye mix was pipetted in the well marked for the purpose.
- Closed the chip priming station.
- Plunger was pressed until it is held by the clip.
- Waited for exactly 30s, and then released the clip.
- Waited for 5s, than slowly pulled back plunger to 1ml position.
- Chip priming station was opened and pipetted 9.0 μl of gel-dye mix in the wells marked for the purpose.
- Pipetted 5 μl of marker in all 12 samples and ladder wells. No wells were left empty.
- 1 μl of DNA ladder was pipetted in the well marked for the ladder.
- In each of the 12 sample wells, 1 μl of sample (used wells) or 1 μl of deionized water (unused wells) was pipetted.
- Chip was put in the adapter horizontally and vortexed for 1 min at the indicated setting (2400 rpm).
- Chip was analysed in the Agilent 2100 bioanalyzer within 5 min.

Figure 5: Agilent bioanalyzer chip

6. Subsequently, DNA was subjected to a series of enzymatic reactions as per manufacturer's instruction, that repair frayed ends to phosphorylate the fragments, and add a single nucleotide ‘A’ overhang and ligate adaptors (Illumina’s TruSeq DNA sample preparation kit).
A' Overhang and Ligate Adaptors Protocol

A single ‘A’ nucleotide is added to the 3’ ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single ‘T’ nucleotide on the 3’ end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

- Removed one tube of A-Tailing Mix and, when using the A-Tailing Control, one tube of A-Tailing Control per 48 reactions from -15°C to -25°C storage and thawed them at room temperature.
- Briefly centrifuged the thawed ALP plate to 280xg for 1 minute, and then removed the adhesive seal from the plate.
- 2.5 µl of A-Tailing Control was added to the bottom of each well of the ALP plate.
- 12.5 µl of A-Tailing Mix was added to the bottom of each well of the ALP plate.
- Adjusted the single channel pipette to 30 µl and gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
- Sealed the ALP plate with a Microseal ‘B’ adhesive seal.
- Incubated the ALP plate on the thermal cycler, with the lid closed, for 30 minutes at 37°C.
- Immediately removed the ALP plate from the thermal cycler, and then proceeded immediately to Ligate Adapters.
- Removed the appropriate DNA Adapter Index tubes, and one tube each of Ligase Control and Stop Ligase Mix per 48 reactions from -15° to -25°C storage and thawed them at room temperature.
- Removed the AMPure XP beads from storage and were allowed to stand for at least 30 minutes to bring them to room temperature.
- Applied a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- Applied a SSP barcode label to a new 96-well 0.3 ml PCR plate.
• Briefly centrifuged the thawed DNA Adapter Index tubes, Ligase Control, and Stop Ligase Mix tubes to 600xg for 5 seconds.
• Immediately before use, removed the DNA Ligase Mix tube from -15°C to -25°C storage.
• Removed the adhesive seal from the ALP plate.
• 2.5 µl of Ligase Control was added to each well of the ALP plate.
• 2.5 µl of DNA Ligase Mix was added to each well of the ALP plate.
• The DNA Ligase Mix tube was kept back to -25°C storage immediately after use.
• 2.5 µl of each thawed DNA Adapter Index was added to each well of the ALP plate.
• Adjusted the single channel pipette to 37.5 µl and gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
• Sealed the ALP plate with a Microseal ‘B’ adhesive seal.
• Incubated the ALP plate on the thermal cycler, with the lid closed, for 10 minutes at 30°C.
• Removed the ALP plate from the thermal cycler.
• Removed the adhesive seal from the ALP plate.
• 5 µl of Stop Ligase Mix was added to each well of the ALP plate to inactivate the ligation.
• Adjusted the single channel pipette to 42.5 µl and gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
• Vortexed the AMPure XP Beads until they are well dispersed then added 42.5 µl of mixed AMPure XP Beads to each well of the ALP plate using a single channel pipette.
• Adjusted the single channel or multichannel pipette to 85 µl and gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
- Incubated the ALP plate at room temperature for 15 minutes.
- Placed the ALP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- Removed and discarded 80 µl of the supernatant from each well of the ALP plate using a single channel pipette. Some liquid may remain in each well. Taken care not to disturb the beads. Changed the tip after each sample.
- With the ALP plate remaining on the magnetic stand, added 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.
- Incubated the ALP plate at room temperature for at least 30 seconds, then removed and discarded all of the supernatant from each well using a single channel pipette. Taken care not to disturb the beads. Changed the tip after each sample.
- The ALP plate was allowed to stand at room temperature for 15 minutes to dry and then removed the plate from the magnetic stand.
- Re-suspended the dried pellet in each well with 52.5 µl Re-suspension Buffer. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
- Incubated the ALP plate at room temperature for 2 minutes.
- Placed the ALP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
- Transferred 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode using a single channel pipette. Changed the tip after each sample.
- Vortexed the AMPure XP Beads until they are well dispersed then added 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up using a single channel pipette.
- Adjusted the single channel pipette to 100 µl and gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
- Incubated the CAP plate at room temperature for 15 minutes.
Placed the CAP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

Removed and discarded 95 μl of the supernatant from each well of the CAP plate using a single channel pipette. Some liquid may remain in each well. Taken care not to disturb the beads. Changed the tip after each sample.

With the CAP plate remaining on the magnetic stand, added 200 μl of freshly prepared 80% EtOH to each well without disturbing the beads.

Incubated the CAP plate at room temperature for at least 30 seconds, then removed and discarded all of the supernatant from each well using a single channel pipette. Taken care not to disturb the beads. Changed the tip after each sample.

Allowed the CAP plate to stand at room temperature for 15 minutes to dry and then removed the plate from the magnetic stand.

Re-suspended the dried pellet in each well with 22.5 μl Re-suspension Buffer. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.

Incubated the CAP plate at room temperature for 2 minutes.

Placed the CAP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

Transferred 20 μl of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the SSP barcode using a single channel pipette. Changed the tip after each sample.

7. After ligation, multiple PCR amplifications were performed on the ligated products (enrichment PCR) so as to obtain ~15-17 micrograms of library per set.

Enrichment PCR & Quality Control Protocol

- 5 μl of thawed PCR Primer Cocktail was added to each well of the PCR plate using a single channel pipette. Changed the tip after each sample.

- 25 μl of thawed PCR Master Mix was added to each well of the PCR plate using a single channel pipette. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.
• Amplified the PCR plate in the pre-programmed thermal cycler, with the lid closed, as follows:
  i. 98°C for 30 seconds
  ii. 10 cycles of:
      98°C for 10 seconds
      60°C for 30 seconds
      72°C for 30 seconds
  iii. 72°C for 5 minutes
  iv. Hold at 4°C

• Vortexed the AMPure XP Beads until they are well dispersed, then added 50 μl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 μl of the PCR amplified library using a single channel pipette. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each column.

• Incubated the PCR plate at room temperature for 15 minutes.

• Placed the PCR plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

• Removed and discarded 95 μl of the supernatant from each well of the PCR plate using a single channel pipette. Some liquid may remain in each well. Taken care not to disturb the beads. Changed the tip after each sample.

• With the PCR plate remaining on the magnetic stand, added 200 μl of freshly prepared 80% EtOH to each well without disturbing the beads.

• Incubated the PCR plate at room temperature for at least 30 seconds, then removed and discarded all of the supernatant from each well using a single channel pipette. Taken care not to disturb the beads. Changed the tip after each sample.

• Removed the PCR plate from the magnetic stand and allowed the plate to stand at room temperature for 15 minutes to dry.

• Re-suspended the dried pellet in each well with 32.5 μl Re-suspension Buffer using a single channel pipette. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Changed the tip after each sample.

• Incubated the PCR plate at room temperature for 2 minutes.
• Placed the PCR plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.
• Transferred 30 μl of the clear supernatant from each well of the PCR plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode using a single channel pipette. Changed the tip after each sample.
• Transferred 10 μl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode using a single channel pipette. Changed the tip after each sample.
• Normalized the concentration of sample library in each well of DCT plate to 10 nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.
• Gently pipetted the entire normalized sample library volume up and down 10 times to mix thoroughly.
• Proceeded to cluster generation.

Preparing Sample DNA for Cluster Generation
• Template DNA was denatured with 2 N NaOH to a final DNA concentration of 0.5 nM and final NaOH concentration of 0.1 N. This is suitable for performing the hybridization step on the Cluster Station at a DNA concentration up to 4 pM.
• Vortexed the template solution.
• Pulse centrifuged the solution.
• Incubated for 5 minutes at room temperature to denature the template into single strands.
• Followed the onscreen instructions to load reagents and samples.
• Removed the flow cell from the Cluster Station.
• The flow cell was ready to be sequenced on the Genome Analyzer.
8. ~15-17 micrograms of DNA from each pool was hybridized on to separate Agilent 1*1 million probe microarrays (AMADID: G3358A_034851) following standard protocol recommended by Agilent for 65h at 65°C.

9. After standard washing procedures, the slides were reassembled with nuclease free water (Ambion), exposed to high temperature (95°C for 10 min) and the eluted DNA in nuclease free water was recovered using a syringe.

10. PCR was carried out with the eluted DNA and cleaned up using Ampure XP SPRI beads (Beckman Coulter).

11. An aliquot of the captured library was run on an Agilent High Sensitivity Bioanalyzer Chip.

12. Real time PCR validation was performed with pre and post capture libraries to observe the capture efficiency.

3.7.4. qPCR Based Capture Validation

Real Time PCR validation was performed with pre and post capture libraries to observe the capture efficiency. As per the instructions of the Genotypic Technologies, PVT. LTD Bangalore the qPCR was carried out.
PCR primers were designed for the following regions for capture validation:

**Target Primers:**

- **Target 1 (T1):** Chr5-172195695-6205-81F : GAATGCTGGAGAAGGGTGTT
  Chr5-172195695-6205-231R : GTTGGGAGAGATGATGCCTTCG
- **Target 2 (T2):** Chr5-174868692-0172-505F : TGTGGCATGGACCTTGCTTG
  Chr5-174868692-0172-655R : GGCATATGTCTCTGAGGC
- **Target 3 (T3):** Chr9-124534816-5844-84F : ACCCGGTACCGTCTCCAC
  Chr9-124534816-5844-236R : CATGGAGAGCCTTTGACC

**Non-Target Primers:**

- **Non-Target 1 (NT-1):** Chr18-29202209-5724-177F : CACAATGCTGGATCATAAACTTG
  Chr18-29202209-5724-328R : AGGGAGGACGGGTGAGAGAA
- **Non-Target 2 (NT-2):** Chr3-178741526-3013-33F : ATCCACGGTGATCTGGCCAT
  Chr3-178741526-3013-183R : CATTCCTGTACCCTGTTCAGAC

Sample enrichment was confirmed by qPCR, and was subjected to sequencing on genome analyzer.

3.7.5. **Next Generation Sequencing (NGS)**

The Genome Analyzer sequences clustered template DNA using a robust four-color DNA Sequencing-by-Synthesis (SBS) technology that employs reversible terminators with removable fluorescence. Illumina's sequencing by synthesis (SBS) technology is the most successful and widely-adopted next-generation sequencing platform worldwide. TruSeq technology supports massively parallel sequencing using a proprietary reversible terminator-based method that enables detection of single bases as they are incorporated into growing DNA strands. A fluorescently-labeled terminator is imaged as each dNTP is added and then cleaved to allow incorporation of the next base. Since all four reversible terminator-bound dNTPs are present during each sequencing cycle, natural competition minimizes incorporation bias. The end result is true base-by-base sequencing that enables the most accurate data for a broad range of applications. This approach provides a high degree of sequencing accuracy even through homopolymeric regions. High sensitivity fluorescence detection is achieved using laser excitation and total internal reflection optics. Short sequence reads are aligned against a
reference genome and genetic differences are called using a specially developed data pipeline (141).

**Figure 7:** Four-color DNA sequencing by synthesis using cleavable fluorescent nucleotide reversible terminators

1. The DNA from prepared libraries was denatured and sequenced on the Illumina GAIIx by Sequencing by synthesis method to read 100 bases paired end.
2. DNA library fragments were diluted, denatured and hybridized to a lawn of oligonucleotides immobilized on the flow cell surface.
3. Hybridized DNA template was amplified using immobilized oligonucleotides as primers.
4. Each hybridized template through the process of isothermal bridge amplification resulted in the formation of clusters comprised of roughly 1000 clonal copies.
5. Sequencing was performed by synthesis (SBS) technology using four fluorescently labeled nucleotides to sequence each cluster on the flow cell surface in parallel.
6. During each sequencing cycle, a single labeled deoxynucleotide triphosphate (dNTP) was added and clusters were imaged.
7. The fluorescent dye and blocker was cleaved off and the next complementary base was added to the nucleic acid chain and imaged. 100 such cycles were performed.

8. Individual bases were called directly from signal intensity measurements during each cycle. These cycles comprised Read 1 of the sequencing run.

9. Once Read 1 was completed, a second set of cluster generation took place on the Genome Analyzer. Once the clusters were generated, sequencing was performed again on the reverse strand (Read 2).

10. Once sequencing was completed, the raw data was extracted from the server using the Illumina pipeline software to obtain FASTQ files. Quality check of raw data was performed using SeqQC.

3.7.6. Illumina Sequences

Paired-end, 100bp reads (37million per pair) Illumina reads sequenced using Illumina GAIIx Analyzer.

3.8. Bioinformatics

NGS Analysis

3.8.1. QC of Illumina Sequences

QC was performed for the Illumina sequenced reads using Genotypic’s in-house QC tool SeqQC. Using 20ph red quality for high quality cutoff, we found that 88-90% of the bases were of high quality. 82-95% of reads had more than 70% high quality bases across three pools, which were used for further analysis.

3.8.2. Reference Sequence

Human Genome (HG19) was used as reference sequence. Sequence was downloaded from UCSC (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/)

3.8.3. Genome Information

Size: 3.1 GB; Haploid chromosomes: 22 + X, Y;
3.8.4. Gapped Alignment

- Alignment software BWA (version 0.5.9rc1) 3 was used to perform gapped alignment of Illumina sequences against the reference sequence. Following parameters were used for alignment:
  - Maximum number or fraction of gap opens – 2
  - Maximum number of gap extensions – 10
  - Maximum occurrences for extending a long deletion – 5

3.8.5. Ungapped Alignment

- Alignment software bowtie (version 0.12.7) 3 was used to perform ungapped alignment of Illumina sequences against the reference sequence.
- In order to overcome possibilities of false variations induced by allowing gaps, we call variations from both gapped and ungapped alignments. Variations which are reported in both the alignments are expected to be of higher confidence.

3.8.6. SNP (Single Nucleotide Polymorphism) Calling

Samtools (versionsamtools-0.1.7a) 2 was used for calling SNPs.

3.8.7. Indel (Insertions & Deletions) Detection

Indel's are detected using Samtools (versionsamtools-0.1.7a) 2 from the gapped alignment performed using BWA3.

3.9. Mutation Analysis

The sequencing data was pooled in Microsoft Excel 2007 and compared between all the three pools to identify the mutations specific for PD patient's sample.