To develop rapid screening method for Genetic loci of spinocerebellar ataxia

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"*To develop rapid screening method for Genetic loci of spinocerebellar ataxia*"**, submitted by Pushpendra Mani Mishra (DTU/14/M.TECH/094) in partial fulfillment of the requirement for the major project dissertation during M. Tech at Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work which is carried out by him under my guidance.

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I declare that my major project dissertation entitled — ‖ *To develop rapid screening method for Genetic loci of spinocerebellar ataxia* ‖ submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at — Genomics and Molecular Medicine Laboratory, Institute of Genomics and Integrative Biology, Council of Scientific and Industrial Research, Mall Road, New Delhi.

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TABLES OF CONTENT

ABBREVIATION

SCA	- Spinocerebellar ataxia
PCR	- Polymerase Chain Reaction
FM-PCR	- Fluorescent Multiplex PCR
Mln bp	- Miliard base pair
LINE	- Long Interspersed Nuclear Element
SINE	- Short Interspersed Nuclear Element
HERV	- Human Endogenous Retrovirus
Kbp	- kilobase pair
STR	- Simple Tandem Repeat
PP2	- Protein Phosphatase-2
MJD	- Machado Joseph Disease
dNTPs	- Deoxyribonucleotidetriphosphates
DRPLA	- Dentatorubral-pallidoluysian atrophy
HiDi	- Highly deionized formamide
Taq	- *Thermus aquaticus*
Pfu	- *Pyrococcus furiosus*
BSA	- Bovine Serum Albumin
CCD	- Charge Couple Device
M	- Marker
P	- Positive

Title -To develop rapid screening method for Genetic loci of spinocerebellar ataxia

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ABSTRACT

A heterogeneous group of disorders implicated with degeneration of neurons constituted by spinocerebellar ataxia (SCA). Lack of coordination and body imbalance while moving, cognitive dysfunction, dementia, ophthalmoplegia, pyramidal signs, pigmentary retinopathy and peripheral neuropathy are symptoms associated with spinocerebellar ataxia. SCA is considered by all or some of the above-mentioned symptoms, in addition to the progressive cerebellar ataxia. A number of genetic mutations responsible for the causation of various types of SCA, these mutation localize in diverse places of genome, most of these mutation mapped and cloned already. Growing body of evidences suggest the similarities of the physiopathology and mechanism among these disorders. Mutational basis of most of these disorder is CAG trinucleotide expansion. A toxic product formed in result to the presence of expanded polyglutamine tract in the coding region of the gene. Till date more than 30 subtypes of SCA have determined. The phenotypic overlap among the cause of SCA make its diagnosis and clinical manifestation difficult. The genetic background haven't yet determined in some of its subtypes. To elucidate the correct diagnosis, clinical findings, age of onset and geographical distribution may helpful, however absolute diagnosis necessitate molecular genetic testing. A molecular genetic diagnosis may establish based on the discovery and knowledge of numerous SCA associated gene and consequent option of predictive test and techniques. In current research wok we have determined the fastest screening method to diagnose the most frequent types of SCA in Indian population in which CAG repeat mutation occur.

INTRODUCTION

INTRODUCTION

Ataxia defined as a failure of coordination, mainly gait imbalance. Clinically, when someone diagnosed with ataxia, it is limb incoordination associated gait imbalance counting fine and gross motor control. Ataxia found in adult patient is either be genetic reason or acquired. The acquired and genetic cause of ataxia may differentiated through family history. Familial form of ataxia characterized by inevitable and slow progression, stealthy onset, degeneration of coordination. A positive result can be predicted based on the family history, gait imbalance or ataxia in a mother or father of patient's confirms the dominantly-inherited ailment. [1, 2, 3] The beginning of symptoms such as gait imbalance and appendicular ataxia subsequently demonstrate the presence of degenerative heritable ataxia. Usually, dysarthria begins soon afterward and there is chances of occurrence of visual problems. Visual problems such as diplopia, difficulty in rapidly moving the eye (saccades) and focusing problem in a moving environment. Non-cerebellar abnormalities implicated with eye movement can include ocular "stare", gaze palsies, blepharospasm, slowed saccades and ptosis. Typical cerebellar outcomes include widened stance and difficulty with gait, particularly in turning, rebound phenomena, intention tremor on finger to nose testing and dysmetria. Tandem walk testing reveal the gait imbalance in earlier course of disease in some patients. [4] A heterogeneous complex group of neurodegenerative disease, occurred in autosomal dominant manner is termed as spinocerebellar ataxia (SCA). The disease is typically implied by degeneration in cerebellum portion of brain along with is afferent and efferent connections. Other brain structures are usually affect, including the brainstem nuclei, basal ganglia, posterior column and the spinal cord anterior horn, peripheral nerves and basal ganglia. Clinical characteristic manifestation of SCA is cerebellar gait and limb ataxia such as dysarthria, nystagmus, intention tremor, dysdiadochokinesia, and dysmetria. Extra cerebellar symptoms are movement disorders (including Parkinsonism, myoclonia, chorea and dystonia), visual disorders (including pigmentary retinopathy), epilepsy, dementia, ophthalmoplegia, pyramidal signs and lower motor neuron disease and peripheral neuropathy. [5-14].

The molecular basis of disorder is nucleotide repeat within coding region, outside the coding region and various other mutations). Although the various methods such as Electrophysiological, Radiological and Biochemical available for the screening of affected brain regions and extent of damage occurs to brain in SCA disorder but these methods are not helpful in confirming the disease. Genetic Testing is the confirmatory test of SCA disorder. In present study, we sought to determine the fastest Genetic screening method for the various types of SCA.

REVIEW OF LITERATURE

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Human Genome and repetitive sequence

The human genetic material is stored in two organelles that is Mitochondria and Nucleus. The nuclear genomic content is 3.2 miliard base pair (mln bp) and packed in 22 pairs of autosome and 2 sex chromosome, Human chromosomes vary in size the smallest is chromosome 21 has size of 54 mln bp while the largest chromosome 1 has size of 249 mln bp about 5 times bigger than the smallest.

Repetitive DNA

Repetitive DNA sequence defined as the sequence which is repeated two or more than two times in a Genome or DNA molecule. The classification of repetitive DNA is based on the manner of repetition within genome. The first category of classification is interspersed repeat in which individual repeat unit found across the genome in a random manner. The second classification category is tandemly repeated DNA in which repeat unit is arranged in contiguous fashion.

Interspersed repeat

When a sequence within genome occurs at many dispersed position is called interspersed repeat.

Transposable elements

Transposable element are those interspersed repeat sequence which moves throughout genome and amplified in large copy number. The transposable element can be classified in two type based on the mode of transposition. The type one element transpose themselves by replication and involve an RNA intermediate which is reverse transcribed to DNA before reinsertion. The class one element is known as Retroelements and includes

1- LTR transposon

2- Non-LTR elements

3- Retrogenes

The class two element transpose themselves by relatively conservative cut and paste mechanism that means the donor element excised and reinsertion of these elements elsewhere in genome takes place.

Retroelements

LTR transposon

Family of retroviral-related sequences found in human genome which contain LTRs and have characteristics of encoding enzyme for retroposition. In addition, solitary LTRs of these LTR transposon may be located throughout the genome.

In human being, there is two most abundant class of repeats known as long interspersed repetitive elements (LINEs) and Short interspersed repetitive elements (SINEs). The common structure feature shared by both SINEs and LINEs is the lack of long terminal repeats (LTRs) and an A-rich 3' end. These feature separate them from related retroelements and retrovirus.

LINE (Long interspersed nuclear element)

A genome-wide repeat has frequent transposable activity. A full-length LINE also known as L1 element has size of 6.1 kbp (approximately) although many of these are truncated pseudogenes with numerous 5'ends. The reason for the numerous 5'end is due to incomplete reverse transcription. Our genome contain approximately 100000 copies of L1 sequences.

SINE (short interspersed nuclear element)

SINE is a type of widespread repeat found in genome and generally exemplified by Alu sequences, Alu sequence found in the human genomes and related mammals. The number of copy of Alu element present in human genome estimated to 500000-900000 copies representing the primary SINE family. Sequence comparisons of this Alu element to various element sequence determine its derivation from the 7SLRNA gene. Alu element possess dimeric structure and contains RNA polymerase III promoter sequences, and has flanking direct repeats and A-rich tail and its size is about 280bp.

Retrogenes

Several families of Human endogenous retrovirus (HERV) present in low abundance about 10-1000 copies in human genome and in sum total possess 1% of the genome. The size of individual element HERV varies from 6 to 10 kb.

Tandemly repeated DNA

Head to tail repeated DNA sequence motif

Microsatellites

Microsatellites is tandem repeat of di-, tri- or tetra-nucleotide unit. Also known as simple tandem repeat (STR). Throughout the genome different array of microsatellites dispersed, although they have no known function except CA/TG dinucleotide pairs which form the Z-DNA conformation in vitro, which may indicate some function of these dinucleotide repeat microsatellites. Replication slippage is the cause of repeat unit copy number variation of microsatellites. The expansion of trinucleotide repeats within gene is the cause of many genetic disorders for instance Fragile X-syndrome, Huntington disease, and others.

Minisatellites

Minisatellites are tandemly repeated sequence of DNA with size range of 1 kilobase pair (kbp) to 15kbp, the one example of such DNA is telomeric DNA sequence which contain 10-15 kb of hexanucleotide repeats, the most common is TTAGGG in Human genome present at the termini of the chromosome. Telomerase add all these sequence to ensure complete replication of the chromosome.

Macrosatellites

A very long array of tandemly repeated DNA up to hundreds of kilobases is known as Macrosatellites. [15, 16]

Repetitive DNA mutation and SCA

Genetic analysis of spinocerebellar ataxia type 1, 2, 3,6,7,12,17 demonstrate that the cause of disorder is CAG trinucleotide repeat mutation in the different gene. Each gene is associated to particular type of ataxia for instance in SCA-1, 2, 3 and 7 CAG repeat is found in ATXN1,ATXN2, ATXN3 and ATXN 7 gene respectively, In SCA-6 CAG repeat found in CACNA1A gene, In SCA-12 repeat present in PPP2R2B and in SCA-17 repeat present in TBP gene. [17]

Classification of SCA

An old system of classification done by Harding place autosomal dominant cerebellar ataxia in 4 basic categories as follows (5, 6, 18-24):

Type1-this type include symptoms like amyotrophy, ophthalmoplegia, dementia, extrapyramidal signs, optic atrophy in addition to cerebellar ataxia.

Type2-Primarily retinal degeneration extrapyramidal signs and ophthalmoplegia may present.

Type3-pure cerebellar ataxia.

Type4-Cerebellar ataxia with myoclonia and deafness.

Prevalence

1 to 5 cases per persons, among all SCA, SCA- 3 is most common type followed by SCA-1, 2, 6 and 7. [5, 6, 19-24]

Genetic aspects

Spinocerebellar ataxia numbered according to order of discovery of their genetic locus. Now this number is extended to SCA-37 as of early 2014. (25) SCA4R is the new redefined form of SCA-4. Puratrophin-1 (PLEKHG4) gene mutation of chromosome 16q22 is related to pure Japanese form of SCA-4 which is deprived of sensory axonal neuropathy. Identification of SCA-6 led to fact that its similarity to SCA-15, both are alike. The SCA-15, 29 and 19, 22 are related to one another as being the different allelic form of the same gene. Presence of an expanded and unstable polymorphic CAG trinucleotide repeat is characteristics of mutation in coding region of the disease gene, which cause dentatorubral pallidoluysian atrophy (DRPLA) and SCA types 1-3, 6, 7 and 17. The protein product of gene is known by the name of ataxin. These protein disorders is called polyglutamine disease due to presence of long stretches of polyglutamine. The mutant protein gain of toxic function stimulate the beginning of neurodegeneration. In the process of degeneration, there is formation of inclusion in Purkinje cells of cerebellar region with an involvement of Chaperons and ubiquitin-proteasome pathway. The illness of SCA cause by the mutant protein having expanded stretch of polyglutamine. Neurodegeneration of specific group of neurons and neuronal function loss takes place for each type of disease. A particular Phenomenon is associated with these SCA disorder i.e. increasingly severe nature of the clinical appearance from early onset and successive generation of the affected families. The phenomenon of anticipation is corresponding to the number of expanded CAG repeats. SCA types 8, 10, 12 included in a second group of SCA in which expanded stretches of repeats positioned outside of the coding region of genes related to disease and results in deregulated expression of gene.

CTG expansion is associated with SCA-8, repetition of pentanucleotide (ATTCT) occur in SCA-10; Deregulation in activity of protein phosphatase 2 (PP2) cause SCA-12. PP2 enzyme has an important functional role in Purkinje cells. Change in the amino acid composition of the proteins fibroblast growth factor 14 (FGF14), inositol 1,4,5-triphosphate receptor, type 1 (ITPR1), potassium channels (KCNC3), protein kinase C (PRKCG), βIII spectrin, tau tubulin kinase (TTBK2), potassium channels (KCNC3) by various mechanism cause SCA type 5, 11, 13, 14, 15 and 27 which have been included in third group of SCA. [5-7, 9-12, 24, 27-30]

SCA 1

The onset of SCA-1 begin after 20 years age, the symptoms associated with disease is ataxia (more noticeable in gait in comparison to limbs) with gait imbalance, hyperactive deep reflexes, nystagmus, dysarthria and, occasionally, ophthalmoparesis. Significant upsurge in the amplitude of saccadic eye movements is the most common irregular eye movement in SCA-1 which leads to hypermetria. SCA-1 sometimes associated with cognitive dysfunction, dystonia, chorea, bulbar paralysis and sluggish saccadic eye movements. Most affected regions are cerebellum (due to loss of cells in dentate nucleus, Purkinje cells), middle cerebellar peduncle, olives and pons reveal in pathological study. The location of SCA-1 disease is mapped to location 6p 22.3 of the chromosome 6.The mutation was described as a repeat of unstable CAG sequence repeat between 41 to 81 repeats. The sign and symptoms of disease and the duration of disease appeared in an age which has the relation with CAG repeats. A correlation has been established in some cases as in the case of paternal pattern of transmission and greater increment in the CAG repeat. In all cases of SCA the detection percent of SCA-1 is 4 to 19. It is the most common form of SCA found in some geographical locations of Japan and Italy as it represent the 50% of all the SCA cases. [6-14]

SCA 2

The characteristic feature associated with SCA-2 is cerebellar ataxia, tremor, dysarthria, hypoactive deep reflexes or areflexia of lower and upper limbs (which defines the occurrence of allied peripheral neuropathy), sluggish saccadic eye movements, fasciculations of the limbs and face. The main clinical features associated to SCA-2 is the cerebellar ataxia due to cerebellar atrophy which can be detected in neuroimaging investigations, other major symptoms are sluggish saccadic eye movements, peripheral neuropathy. Parkinsonism, myoclonia, dementia, dystonia and chorea are other clinical manifestation. The first description of SCA-2 disease given in 1971 by Swami and Wadia in India. The considerable study of disease is done by Orozoco in Cuba (Holguin) in the year 1990. SCA-2 was the most common

form in the study conducted by Salem et al., by using molecular analysis techniques, His study was based on the analysis of 42 Indian Families with SCA. In another study conducted by Basu et al., on the 9 diverse folkloric populations in India, demonstrate that the SCA-2 is most common form in India. In addition to India, study conducted in Cuba also demonstrate high prevalence of SCA-2, A study by Velazquez-Perez concluded SCA-2 positive case in 120 families out of 125 One of the highest rate of occurrence of disease reported in Baguanose city of Holguin province where 129.2 cases out of 100,000 inhabitants reported. SCA-2 reported in different part of world with varying frequency. Cerebellar atrophy, loss of granular and Purkinje cells, substantia nigra, olivary neurons and the spinal cord anterior horn are the characteristic feature of SCA-2 disease. Chromosomal location of disease is 12 (12q24.13), Trinucleotide CAG expansion in between 34 to 59 is the cause of disease. However 33 CAG repeat expansion in late onset SCA-2 is sufficient for the causation of disease. The clinical feature associated to SCA-2 is Cerebellar ataxia with tremor, dysarthria deep areflexia/ hyporeflexia of the lower and upper limbs (occurrence of associated peripheral neuropathy), characteristic sluggish saccadic eye movements, fasciculations of the face and limbs. SCA-2 is Featured by cerebellar ataxia due to cerebellar atrophy which can be clearly observe in neuroimaging examinations In addition to Cerebellar atrophy loss of Purkinje and granular cells, substantia nigra, olivary neurons and cells in the spinal cord anterior horn takes place. [7-14, 38-43]

SCA 3 (Machado-Joseph disease)

SCA-3 is also called as Machado-Joseph disease (MJD). It is the most common form of SCA disease throughout the world. The disease is associated to CAG triplet expansion from 56-86 repeats and the chromosomal location of mutation is mapped to 14q32.12 The Neuropathological features of SCA-3 includes gliosis reaction in the structures such as dentate nucleus of the cerebellum, Substantia nigra, the pontine nuclei, the red nucleus, cells in the spinal cord anterior horn, the spinocerebellar tracts and Clarke's column and the other motor cranial nerves nuclei. The affected region may include globus pallidus although the involvement of the cerebellar, cerebellar cortex and olivary nuclei is uncommon. Neuroimaging studies demonstrate the presence of pontocerebellar atrophy, typically without presence of the olives. However magnetic resonance imaging study conducted by Murata et al. demonstrate presence of pontocerebellar atrophy, atrophy of frontal and temporal lobe and globi pallidi. The clinical features of MJD is presence of pyramidal sign such as nystagmus, ophthalmoparesis, peripheral amyotrophy, bulging eyes, and dystonia, Parkinsonism, and fasciculations of the face, tongue with occasional cerebellar ataxia. The diagnostic criteria proposed by Lima and Coutinho in 1980 for MJD detection were mode of inheritance: autosomal dominant, Major neurological signs:

Cerebellar ataxia, extrapyramidal signs and amyotrophy pyramidal signs, minor neurological signs: fasciculations and bulging eyes, dystonia, and progressive external ophthalmoplegia. Paula Coutinho a doctoral student in 1992 proposed the diagnostic criteria for the diagnosis of MJD in her PhD thesis as given below: Onset in adult life, autosomal dominant transmission, presence of ataxia, supranuclear ophthalmoparesis and pyramidal and extrapyramidal signs, with involvement of the peripheral nervous system, Minor signs include fasciculations and bulging eyes, normal higher cortical functions, mean survival of 21 years,. [5-7, 9-12, 19-24, 26, 31-35]

SCA 6

Pure cerebellar ataxia is the characteristic feature associated to SCA type 6. Which can be accompanied by symptoms such as nystagmus, dysphagia, dysarthria, dystonia and loss of proprioception. Intense episode of vertigo is common in most of the patients before the onset of ataxia, while in some of the patients intermittent ataxia episode (corresponding to episodic ataxia type 2) in parallel to the sign and symptoms of slowly progressive cerebellar ataxia. There is slow progressive evolution of SCA-6 disease. The appearance of clinical symptoms takes place around the age of 50 year. Neuroimaging study and pathological examination of SCA-6 disease reveals the cerebellar atrophy, gliosis of the inferior olivary complex and a loss of Purkinje cells in the cerebellar cortex. The mutation associated to SCA-6 is CAG repeat expansion between 21 and 31 units long and mapped to the chromosome location 19p13.133, the responsible gene is CACNA1A4 which is voltage-dependent calcium channel know as alpha 1A. SCA-6 alone is responsible for 10 to 30 % of total SCA cases. It is the second most common form of SCA in some geographical location of Japan while in the certain regions of Japan, for instance, the district of Kinski it is the most common form. It is noteworthy from the clinical point of view that the SCA-6 mutation is allelic with sporadic or episodic SCA type 2 and hemiplegic migraine. [5, 7, 8, 44]

SCA 7

When Cerebellar ataxia present with visual deficit which is progressive in nature and caused by degeneration of retina (macular dystrophy). The other features which accompanied the disease includes slow saccadic movements, Parkinsonism, and ophthalmoplegia. The neuro associated pathological features in this disorder is degeneration of olivopontocerebellar degeneration pigmentary macular dystrophy and loss of retinal ganglion cells. The chromosomal location of SCA-7 is mapped to 3p14.1 region of chromosome 3. The CAG repeats in pathological alleles have between 36 to 306 repeats. The mutant protein ataxin 7 expressed in many tissues of the body. The function of this

mutant protein is unknown but it cause selective neuronal cell death in the brain region of central nervous system. The appearance of clinical features have taken place from early infant age to end of the fifth decades, progression is very quickly in case of early onset condition. The anticipation may present in the family with reported case of SCA-7. Despite being the less common form among all SCA, SCA-7 is reported in many countries and is most common form in Finland and Sweden. [7-10, 36, 37]

SCA 12

SCA-12 is first of all described by Holmes et al. in the year 1999 in a family of German origin named as R. The appearance of clinical features usually begins in the fourth decades of life with gait ataxia, tremor in the upper limbs, head tremor, hyperactive deep reflexes, dysmetria, abnormal eye movements and dementia at later stage. MRI analysis reveals the cerebellar and cerebral cortex atrophy. The mutation behind the disease is expansion of a CAG trinucleotide repeat in the PPP2R2B region which codes for a subunit of the protein phosphatase PP2A3 and present in the 5' region of the gene complex. The location of PPP2R2B region mapped to 5q32 region of chromosome 5. The case of SCA-12 is mostly reported in Indian family as Fujigasaki et al. in the year 2001 reported an Indian family with SCA-12 subsequently Srivastava et al. reported five more cases of SCA-12 in Indian families. A number of American families with SCA studied by chofin et al. but they failed to find the SCA-12 positive case. [7-14, 45]

SCA 17

An expansion of CAG triplet repeat in the TBP gene region cause SCA-17. TBP gene which is located in chromosome 6q27 and codes for a transcription initiation factor 1-4, called as TATA binding protein. SCA-17 disease first time described in a 14-year-old female in Japan. The clinical feature in the 14-year-old patient observed at the age of 6 years and it was included gait ataxia later which followed by Parkinsonism, intellectual deterioration, and hyperactive deep reflexes as disease progression takes place. Till date, the number of cases of SCA-17 with different clinical demonstration have been described. The various cases of SCA-17 have a phenotypic similarity to Huntington's disease and with the focal dystonia accompanied by dementia and cerebellar ataxia. [5-8, 46-49]

Genetic Testing

The popularity of Genetic testing for SCA has gained in last decade. The testing facility available in many commercial labs and they provide facility for the testing of more than a dozen genes of SCA and these number growing continuously. The genetic testing serves various purpose to the clinician as it is useful in the prenatal testing, carrier testing, and risk factor assessment in addition to predictive testing. The current available commercial genetic test is for SCA type 1, 2, 3, 5, 6, 7, 8, 10, 11, 12, 13, 14 and 17 which comprise 75 percent of known gene of SCA. The positive Genetic test result in the diagnosis while a negative genetic test does not exclude hereditary ataxia on confirmatory basis. In case a person demonstrate clinical features and their ataxia progressed slowly over 10 years very likely has a genetic basis despite the fact that the genetic panel test result is negative. [50-53]

Polymerase Chain Reaction

PCR method is a versatile and rapid method of amplification of targeted DNA sequence. First of all the technique was formulated by Kerry Mullis in 1983. A PCR reaction consist of various components such as template DNA, Primers, deoxyribonucleotide triphosphates (dNTPs), Thermostable DNA polymerase, reaction buffer, and water. Template DNA contain the specific target DNA sequence which is amplified by the PCR. Primers are synthesize as nucleotide sequence (18-30 bp) same sequence as the 5' end of each target strand use as forward and reverse primer. NTPs are building blocks of DNA. Equal molar mixture of all four deoxyribonucleotides triphosphates that is dATP, dCTP, dGTP, dTTP should be used for PCR. PCR reaction require denaturation of template DNA at 94^{o}C during every cycle. Therefore, for automated PCR reaction only thermostable DNA polymerase which can withstand high temperature can be used. Taq DNA polymerase and Pfu DNA polymerase are the most popular thermostable DNA polymerase used in PCR. Reaction buffer used in PCR reaction consist of KCl, Tris-Cl, $MgCl_2$ and Gelatin/BSA. KCl provides proper ionic strength for the buffer, Tris-Cl act as a buffering agent, $MgCl_2$ act as cofactor required for the activity of DNA polymerase.

Gelatin or BSA act as a stabilizing agent for the DNA polymerase. Buffer is normally supplied as 5X or 10X and it is added to a final concentration of 1X. PCR amplification takes place in following different steps:

1-Denaturation-Denaturation is carried out at 94^{o}C for 30 Sec.

2-Annealing-Annealing is carried out for 30 Sec at 37-72^{o}C for Taq polymerase and at 37-68^{o}C for Pfu polymerase.

3-Extension-During this step, the primer is extended by adding complementary bases at its 3' hydroxyl

end. Extension is carried out at 72°C for Taq Polymerase and 68°C for Pfu polymerase.

4-PCR cycle-Three consecutive steps of denaturation, Annealing, and Extension constitute one PCR cycle. The extension product is again denatured to begin the next PCR cycle. Likewise about 25 to 35 PCR cycles are carried out to get desired quantity of PCR product that can be visualize by agarose gel electrophoresis and Ethidium bromide staining.

There is the various factors which affect the PCR reaction these factors related to the quality and quantity of various PCR reaction components such as Template DNA, Primer, dNTPs, Thermostable DNA Polymerase and Buffer. [54, 55]

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a separation method regularly used to analyze DNA and RNA. The principle of agarose gel electrophoresis is as follows: The agarose gel consist of microscopic pore that act as a molecular sieve, DNA, and RNA sample can be loaded into the well made in the gel. When an electric field is applied due to strong negative charge on DNA and RNA molecule due to its phosphate backbone, it migrate from negative to positive electrode. During this movement, the pores in the gel separate the DNA or RNA molecules according to their size and shape. The rate of movement of linear DNA molecules in agarose gel electrophoresis is inversely proportional to the log10 of their molecular weight. An electrophoresis unit consist of a tank and a power supply unit. The electrophoresis tank is just a box with electrodes at the ends. Platinum wire is used as electrode for its good conductivity and long life. The black wire electrode at which electrons enter in the box in which gel is placed from the power supply is called the cathode and is negative (-). The red wire electrode at which electron leave the gel box and re-enter the power supply is called the anode and is positive (+). A potential energy difference between the electrodes due to flow of the electrons, which is measured in Volts (V). An electric field is established through which the ion in electrophoresis buffer migrate. The migration of ions creates electrical current, which is measured in milli-amperes (mA).The typical application of agarose electrophoresis include the visual determination of quality of DNA preparations, Quantification of DNA, Size determination of DNA and purification of DNA or RNA fragments. [56, 57]

DNA Fragment Analysis by ABI 3130XL Genetic Analyzer

Analysis of microsatellite present in DNA sequence is done by Genetic analyzer in which capillary electrophoresis is the method of detection of fluorescently labelled PCR product, ABI 3130XL Genetic analyzer is a highly automated machine, Multiplex STR amplification products keep in 96 or 384 well tray which sequentially injected into an array of 16 capillary in less than 30 seconds and get separated. While moving past the laser detection window near the end of the capillary the fluorescent PCR product is illuminated by laser-induced fluorescence and detected by CCD camera. CCD camera detects all wavelength in visible range. Fresh polymer POP-7 is loaded into capillary between each injections automatically. The software used for Fragment analysis is Gene Mapper. [58-61]

Fluorescent Multiplex PCR Assay

Dorschner et al. developed the rapid detection of five SCA type (SCA 1, 2, 3, 6, and 7) by Fluorescent Multiplex PCR (FM-PCR) method. They specifically use the five sets of chimeric primer to amplify the multi-locus, the chimeric primers were designed from locus specific sequences and tagged with sequence from M13mp18 (gcggtcccaaaagggtcagt). [62] FM-PCR based detection of four SCA types (SCA 1, 2, 3, and 7) developed by Bauer et al., they used the concept of touchdown multiplex PCR to overcome the problem of varying annealing temperature and to amplify the multi-locus by four set of primers. The result of their experiment was successful with an equivalent amount of obtained PCR products. [63]

MATERIAL AND METHOD

MATERIAL AND METHOD

The starting material for the analysis of Multiplex PCR reaction was stored DNA and Fluorescent Primers. The DNA was extracted from the blood of patients who were clinically suspected for SCA, The blood was collected from The All India Institute of Medical Science. Salting out method is applied for the extraction of DNA from the blood, the reagents used in the salting out method is illustrated in Appendix-2. The extracted DNA used for the Screening of suspected SCA type by the use of Primers enlisted in Appendix-1 as a Table-20. The primers were designed from the use of Primer 3 tools, One primer from each pair was labelled on its 5' end with fluorescent dye, Hex (Green) is tagged with SCA-1 primer and FAM (Blue) is tagged with SCA-2, 3, and 12 Primers. To determine the success rate of Multiplex PCR and detail analysis of the obtained result, SCA screened and stored DNA and Fluorescent primers used for the multiplex PCR Experiment -1, the result was successful for the amplification of locus of four SCA types namely SCA-1, 2, 3, and 12 but the amount of amplification was not uniform it was less in case of SCA-2 and 3. The next approach was to make availability of more binding site for the SCA primers 1, 2, 3, and 12 for this purpose the DNA fragmentation Experiment with Heat and snap chilling method is carried out. In DNA fragmentation Experiment, three DNA sample is heated for 5, 10 and 20 minute respectively at 95^{o} C and snap chilling for 5 minute is given by transferring the DNA contained tube to the Icebox. The less fragmented DNA which was heated for 5 minute is used as a template in Multiplex PCR Experiment-3, the result of Multiplex PCR Experiment-3 was the amplification of three targeted locus by primer SCA-12, 2, and 1 in near uniform amount, and primer SCA-3 couldn't amplify its one allele of targeted locus in Experiment-3 due to partial loss of primer binding site in DNA fragmentation experiment. Despite the uniform amplification we couldn't use optimize condition of Experiment-3 in routine screening of SCA-12, 2 and 1 due to overlap in size range and color code of SCA-2 and 12 as it may produce the false result.

We designed the seven set of new primer pairs (Chimeric Primers) with the help of Primer 3 tool and to overcome the problem of Size overlap we added the sequence from M13mp18 (gcggtcccaaaagggtcagt). The number of M13 bases that was added varied to approximate a melting temperature (Tm) of ~80°C and a similar GC content between primer pairs and also among the seven loci. The three different fluorescent dye HEX (Green), FEM (Blue), and NED (Black) is tagged to 5' end of either forward or Reverse Primer of each primer pair. The new set of primer is shown in Table-21 of Appendix-1. Each primer is checked individually for its proper working, first each one is used to amplify the DNA locus by the PCR later the PCR products of each one checked on the 2% Agarose gel followed by checking in ABI 3130XL Genetic Analyzer. We optimize the

different Multiplex PCR reaction condition in Experiment-3 to 6 for the amplification of different targeted locus in a single tube reaction, for instance Experiment-3 condition is optimized for the amplification of targeted locus of primer SCA-2, 12 and 17, Experiment-4 condition is optimize for primer SCA-1, 6, 12, and 17 targeted locus, Experiment-5 condition is optimize for amplification of Primer SCA-6, 12, and 17 targeted locus and Experiment-6 condition optimize for the amplification of primer SCA-6 and 17 targeted locus. These optimized conditions applied to obtain the Multiplex PCR products, 5-10 µl of these products checked on the 2% Agarose gel by Agarose Gel Electrophoresis, after checking the products on Agarose Gel, DNA fragment analysis of these products is performed by ABI 3130 XL Genetic Analyzer. To perform the DNA fragment analysis, first the 1 µl of product is mix with 0.4 µl ROX 550 marker and 8.6 µl of highly deionized formamide (HiDi) in a 96 or 384 well plate and later heat denatured by heating for 95^{o}C for 5 minute followed by snap chilling on Ice for 5 minute. The function of HiDi is to maintain DNA in denatured condition, ROX 550 marker used as a standard marker in DNA fragment analysis, it contain the 14 PCR amplified products of size 70 bp, 85bp, 115bp, 150bp, 185bp, 225bp, 250bp, 285bp, 350bp, 375bp, 435 bp, 475 bp, 530bp and 550bp. The denatured Multiplex PCR products containing plate linked to the Genetic analyzer in which sample is injected into an array of 16 capillaries within 30 seconds and separated within flowable polymer matrix, which is automatically loaded into capillaries prior to each run and flushed out at the end of run. The laser beam illuminate the capillaries and Fluorescence light is detected from DNA fragment by Charge couple device (CCD) camera and data is transferred to the device computer for further processing and analysis. Details of all the techniques used in the development of Multiplex PCR methods and its analysis is illustrated in Appendix-2.

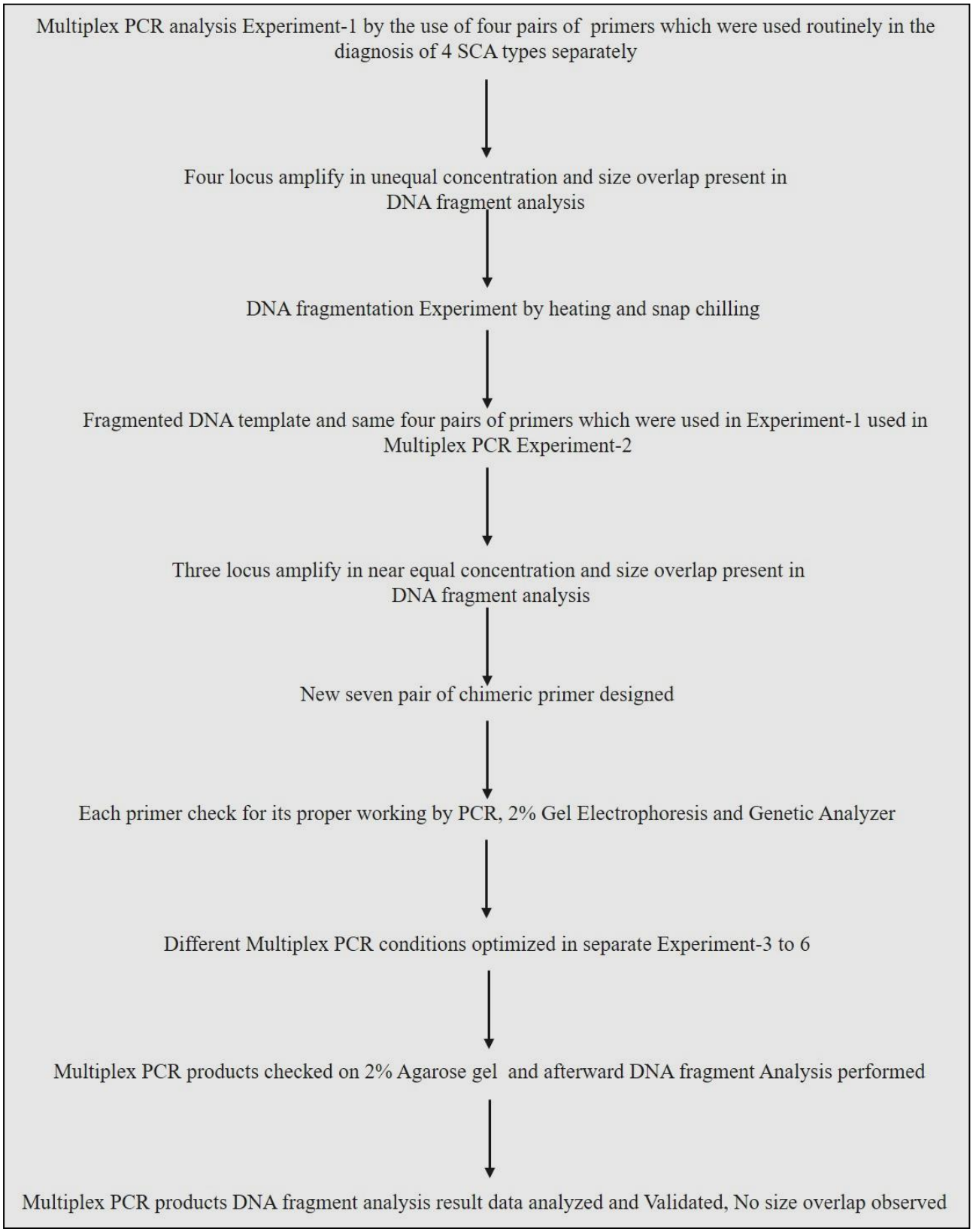


Figure-1 Flow diagram of strategies developed in the rapid screening of Genetic loci of SCA

RESULTS

RESULT

DNA Extraction from Blood and its concentration adjustment

DNA is isolated from the blood sample of clinically suspected spinocerebellar ataxia patients and normal healthy individual by salting out method (method detail description in Appendix-2). The quantification and purity of DNA find out with the help of Nanodrop machine by considering the absorbance or optical density ratio 260/280 of extracted DNA. The aliquots of clinically suspected patients DNA in concentration of 50 ng/µl prepared for PCR and Genetic analyzer based screening of SCA disorder. The screened DNA samples which were positive for SCA disorders used as a sample DNA and DNA sample from normal healthy individual used as a control DNA in Aliquots of 50 ng/µl and 100 ng/µl in the Multiplex PCR Experiment 1 to 6 and the aliquots were prepared from the stock of each DNA sample.

Multiplex PCR Experiment-1

In Multiplex PCR Experiment-1 four sets of primers (Table-20) used to amplify the targeted four-locus of DNA sample (Control and SCA positive). Optimized Multiplex PCR reaction mix and cyclic condition of Experiment-1 is demonstrated in Table-1 and 2, these conditions used to amplify the four-locus of DNA. The four locus amplify but the PCR products not obtained in equivalent amount. The amplified PCR products separated on 2% agarose gel and the gel picture is shown in figure-2. To determine the number of amplified locus and size of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-3 and the amplified allelic regions by Primer SCA-1, 2, 3 and 12 and their size is demonstrated in Table-13.

Temperature and snap chilling based fragmentation of DNA

The purpose of temperature and snap chilling based DNA fragmentation was to increase the primer binding site and to obtain the equivalent amount of amplified PCR products. Although the equivalent amount of three amplified regions is obtained but it also leads to the partial loss of one binding site. The fragmented DNA at temperature of 95^{o}C for different length of time followed by snap chilling for 5 minute is shown in figure-4.

Multiplex PCR Experiment-2

Multiplex PCR Experiment-2 is carried out by using the temperature and snap chilling based fragmented DNA and the same four set of primers as used in Experiment-1 to amplify the four-locus of Control and SCA positive DNA. The optimized Multiplex PCR reaction mix and cyclic condition of Experiment-2 is demonstrated in Table-3 and 4. In Multiplex PCR Experiment-2 three locus amplify in equivalent proportion (SCA-1, 2, and 12 primer targeted locus) but due to partial loss of binding site of a locus (SCA-3 primer targeted), we couldn't obtained the amplification of an allele of a locus. The amplified PCR products separated on 2% agarose gel, the gel picture is shown in figure-5. To determine the number of amplified locus and size of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-6 and the amplified allelic regions by Primer SCA-1, 2, 3 and 12 and their size is demonstrated in Table-14.

Efficiency check and analysis of newly designed Primers

Due to size overlap of amplified PCR products and same tagged color coding of three primers we designed the new seven set of chimeric primers (Table-21). These chimeric primer designed by combining locus specific sequence and universal sequence from M13mp18 (gcggtcccaaaagggtcagt). To find out the proper working and efficiency yield of these primers, we used each primer pairs to amplify the particular single locus of control and SCA positive DNA and later we analyzed the amplified PCR products by Genetic analyzer. Agarose gel picture of PCR products of Primer SCA-1, 2, and 3 are displayed in Figure-7, and DNA fragment analysis picture shown in Figure-8, 9 and 10. Agarose gel and DNA fragment analysis picture of PCR products of Primer SCA-6 is shown in Figure-11 and 12. Similarly Agarose gel picture of PCR products of Primer SCA-7, 12 and 17 are shown in Figure-13, and DNA fragment analysis picture shown in Figure-14, 15 and 16.

Multiplex PCR Experiment-3

In Multiplex PCR Experiment-3 newly designed seven sets of primers (SCA 1, 2, 3, 6, 12 and 17) used to amplify the three-locus of Control and SCA positive DNA. Multiplex PCR reaction mix and cyclic condition of Experiment-3 is demonstrated in Table-5 and 6. These conditions used to amplify the three-locus (primer SCA-2, 12, and 17 targeted locus). Three locus amplify but the PCR products not obtained in equivalent amount. The amplified PCR products separated on 2% agarose gel and the gel picture is shown in figure-17. To determine the number of amplified locus and size

of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-18 and the amplified allelic regions by Primer SCA-2, 12, and 17 and their size is demonstrated in Table-15.

Multiplex PCR Experiment-4

In Multiplex PCR Experiment-3 the same primer pairs used which had been used in Experiment-3 to amplify the four-locus of Control and SCA positive DNA. The optimized Multiplex PCR reaction mix and cyclic condition of Experiment-4 is demonstrated in Table-7 and 8. These conditions used to amplify the four-locus (primer SCA-1, 6, 12, and 17 targeted locus).The equivalent amount of PCR products from four targeted locus is obtained in this experiment. The amplified PCR products separated on 2% agarose gel and the gel picture is shown in figure-19. To determine the number of amplified locus and size of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-20 and the amplified allelic regions by Primer SCA-1, 6, 12 and 17 and their size is demonstrated in Table-16.

Multiplex PCR Experiment-5

Same new sets of primers used in Multiplex PCR Experiment-5 as used in Multiplex PCR Experiment 3 and 4 to amplify the three-locus of control and SCA positive DNA. Multiplex PCR reaction mix and cyclic condition of Experiment-5 is demonstrated in Table-9 and 10. These conditions used to amplify the three-locus (primer SCA-6, 12, and 17 targeted locus). The three locus amplify but the PCR products not obtained in equivalent amount. The amplified PCR products separated on 2% agarose gel and the gel picture is shown in figure-21. To determine the number of amplified locus and size of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-22 and the amplified allelic regions by Primer SCA-6, 12, and 17 and their size is demonstrated in Table-17.

Multiplex PCR Experiment-6

We used the same primer pairs as used in Multiplex PCR Experiment-3, 4 and 5 to amplify the two-locus of Control and SCA positive DNA. The optimized Multiplex PCR reaction mix and cyclic condition of Experiment-6 is demonstrated in Table-11 and 12. These conditions used to amplify

the two-locus (primer SCA-6 and 17 targeted locus). Two locus amplify but the PCR products not obtained in equivalent amount. The amplified PCR products separated on 2% agarose gel and the Agarose gel picture is shown in figure-23. To determine the number of amplified locus and size of allele the DNA fragment analysis of amplified products by ABI 3130 XL Genetic analyzer is performed. The DNA fragment analysis data displayed in figure-24 and the amplified allelic regions by Primer SCA-6 and 17 and their size is demonstrated in Table-18.

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (5x)	4 µl
2	$MgCl_2$ (25mM)	1.6 µl
3	dNTPs (2mM)	3 µl
4	Forward Primer (10pmol/µl)	3.2 µl (0.8 µl of each Primer SCA-1, 2, 3, and12)
5	Reverse Primer (10pmol/µl)	3.2 µl (0.8 µl of each Primer SCA-1, 2, 3, and12)
6	Taq polymerase (5U/µl)	0.5 µl
7	Betaine (5M)	1.5 µl
8	Milli Q water	2 µl
9	DNA (50ng/µl)	6 µl
	Total	**25 µl**

Table-1 PCR reaction mix for Multiplex PCR Experiment-1

PCR program		
Initial denaturation	95°C for 5 Min.	
Denaturation	95°C for 45 Sec.	
Annealing	62°C for 45 Sec.	10 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 45 Sec.	
Annealing	58°C for 45 Sec.	10 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 45 Sec.	
Annealing	56°C for 45 Sec.	15 Cycle
Extension	72°C for 45 Sec.	
Hold	72°C for 10 Min.	
	4°C for 10 Min.	

Table-2 PCR cyclic condition for Multiplex PCR Experiment-1

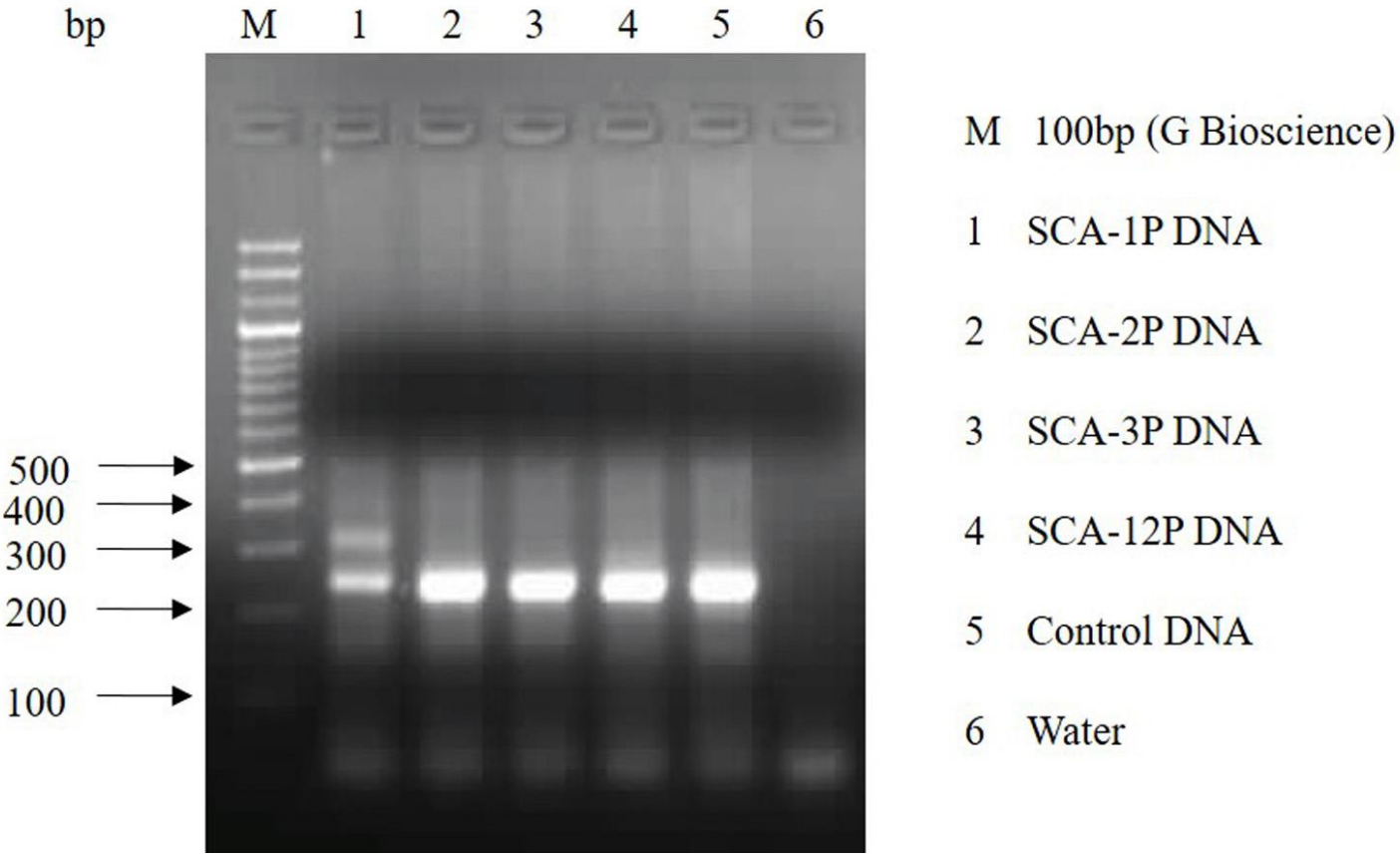


Figure-2 Agarose gel electrophoresis of Multiplex PCR Experiment-1 Products

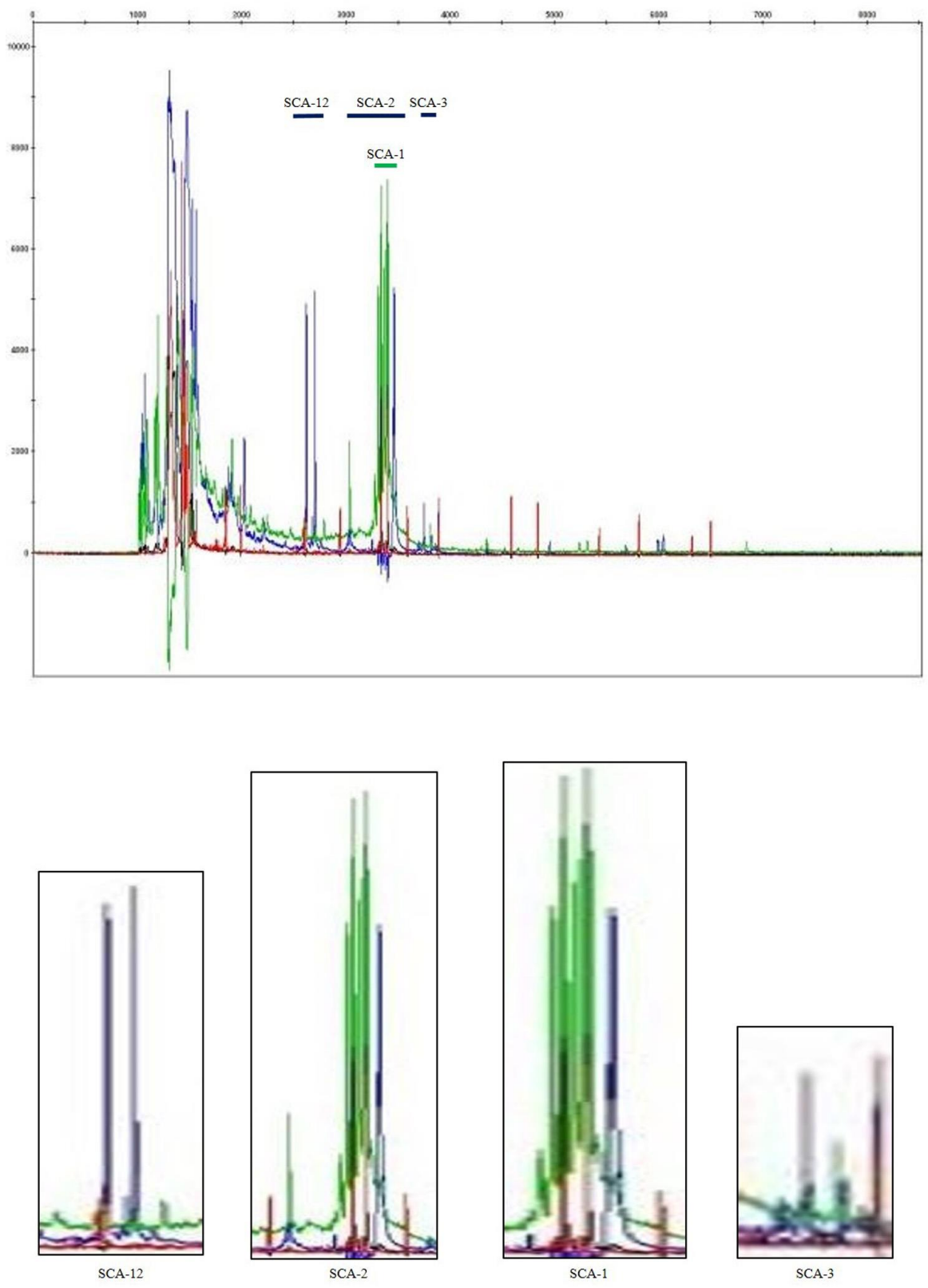
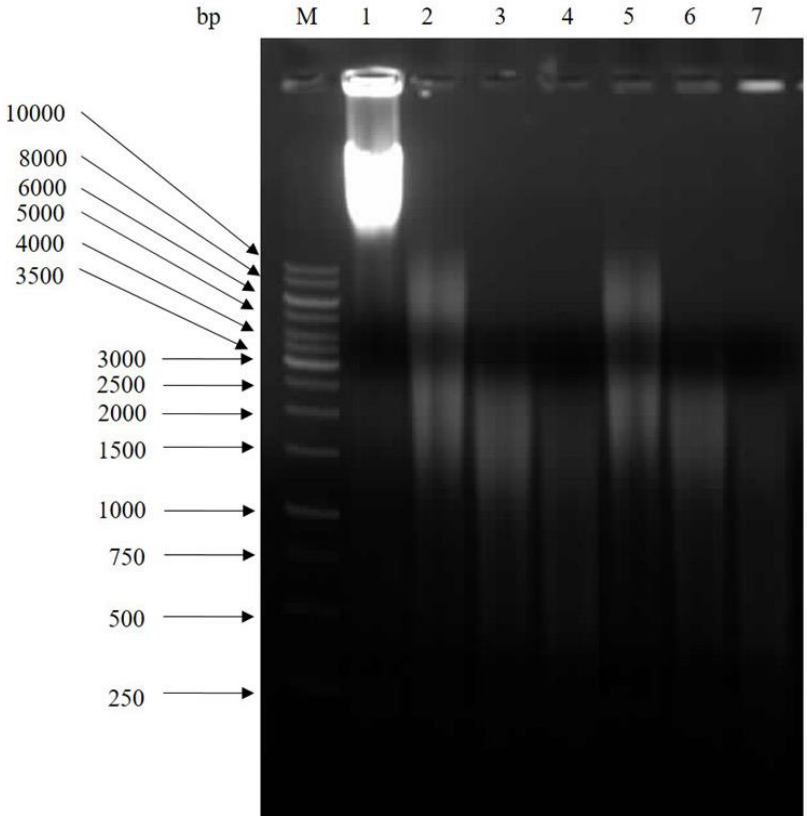


Figure-3 DNA fragment analysis raw data of Multiplex PCR Experiment-1 Products and Zoomed view of amplified locus by Primer SCA-1, 2, 3 and 12



M 1 Kb marker (Thermoscientific)

1 Control DNA

2 Fragmented DNA (95°C heat for 10 min followed by 5 min snap chilling)

3 Fragmented DNA (95°C heat for 20 min followed by 5 min snap chilling)

4 Fragmented DNA (95°C heat for 30 min followed by 5 min snap chilling)

5 Fragmented DNA (95°C heat for 10 min without snap chilling)

6 Fragmented DNA (95°C heat for 20 min without snap chilling)

7 Fragmented DNA (95°C heat for 30 min without snap chilling)

Figure 4-Agarose gel electrophoresis of DNA fragmentation Experiment

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (5x)	4 µl
2	$MgCl_2$ (25mM)	1.6 µl
3	dNTPs (2mM)	3 µl
4	Forward Primer (10pmol/µl)	3.2 µl (0.8 µl each Primer SCA-1, 2, 3, and 12)
5	Reverse Primer (10pmol/µl)	3.2 µl (0.8 µl each Primer SCA-1, 2, 3, and 12)
6	Taq polymerase (5U/µl)	0.5 µl
7	Betaine (5M)	1.5 µl
8	Milli Q water	2 µl
9	DNA (50ng/µl)	6 µl
	Total	**25 µl**

Table-3 PCR reaction mix for Multiplex PCR Experiment-2

PCR program		
Initial denaturation	95°C for 5 Min.	
Denaturation	95°C for 45 Sec.	
Annealing	62°C for 45 Sec.	10 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 45 Sec.	
Annealing	58°C for 45 Sec.	10 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 45 Sec.	
Annealing	56°C for 45 Sec.	15 Cycle
Extension	72°C for 45 Sec.	
Hold	72°C for 10 Min.	
	4°C for 10 Min.	

Table-4 PCR cyclic condition for Multiplex PCR Experiment-2

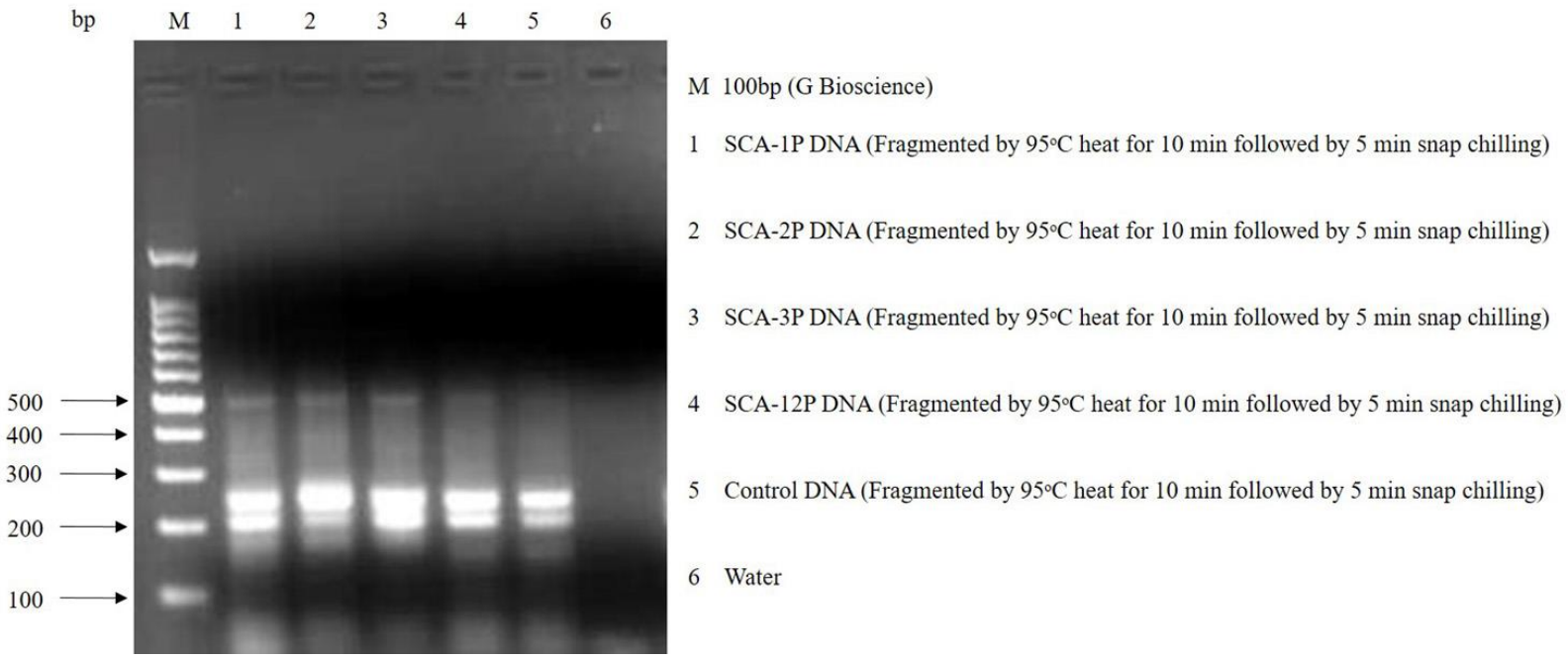


Figure-5 Agarose gel electrophoresis of Multiplex PCR Experiment-2 Products

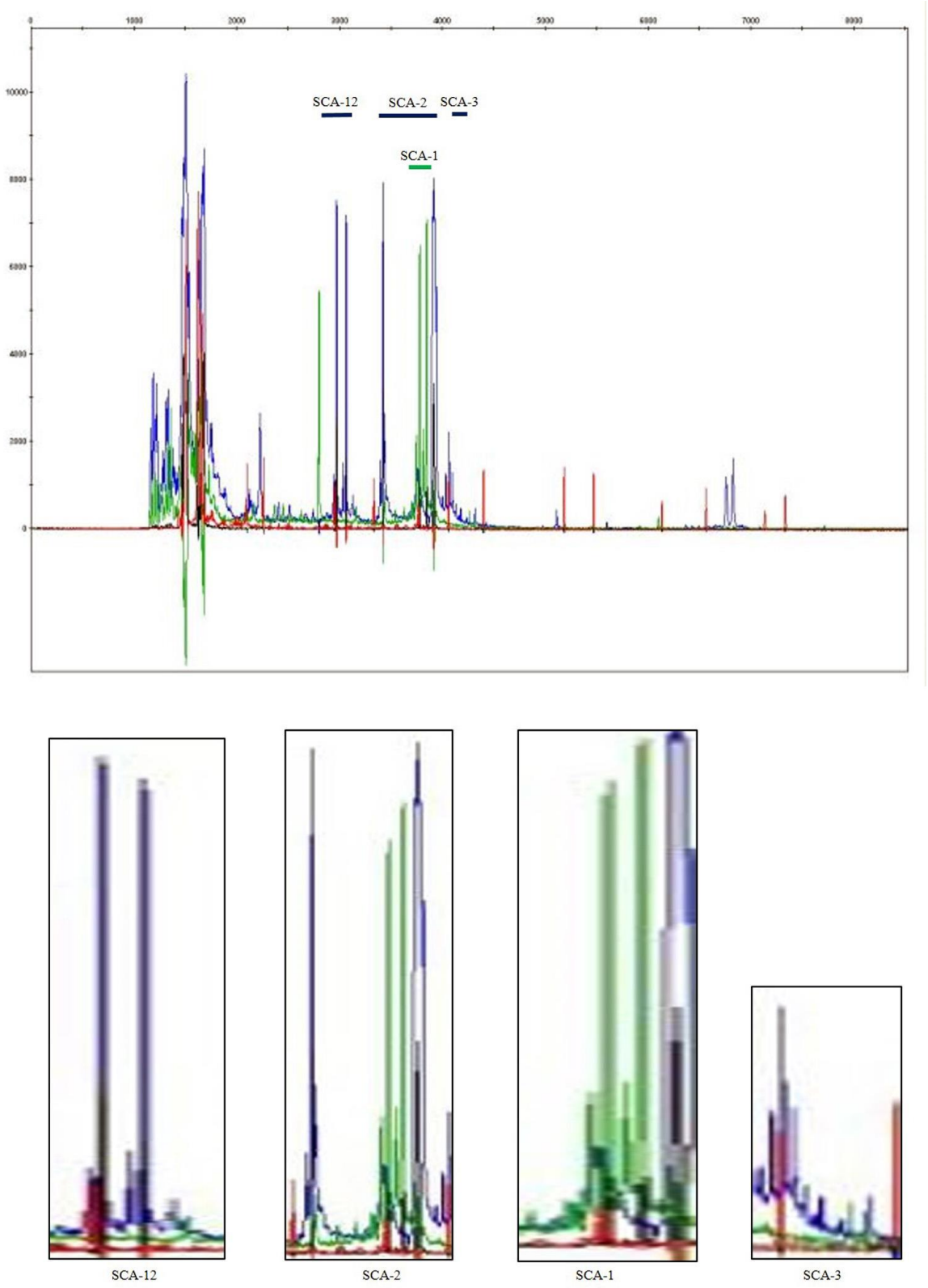


Figure-6 DNA fragment analysis raw data of Multiplex PCR Experiment-2 Products and Zoomed view of amplified locus by Primer SCA-1, 2, 3 and 12

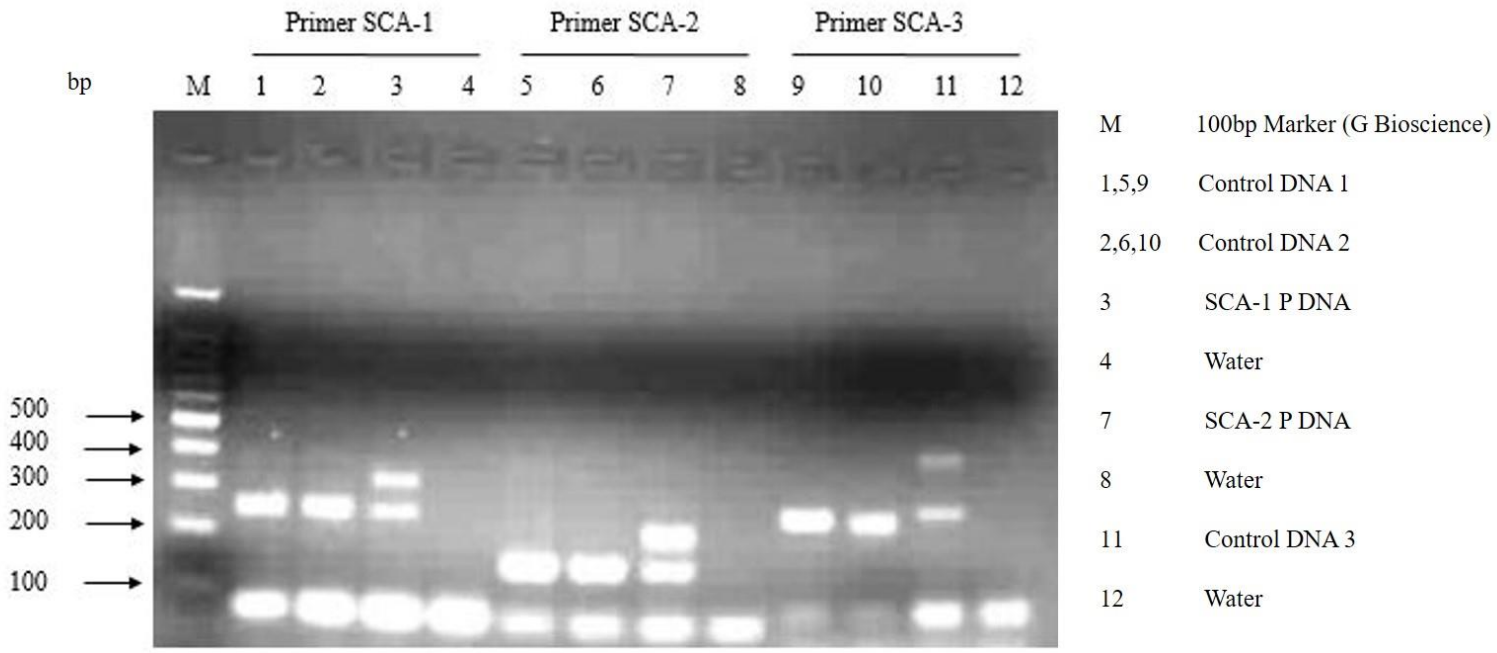


Figure-7 Agarose gel electrophoresis of PCR Products of newly designed Primer SCA-1, 2, and 3

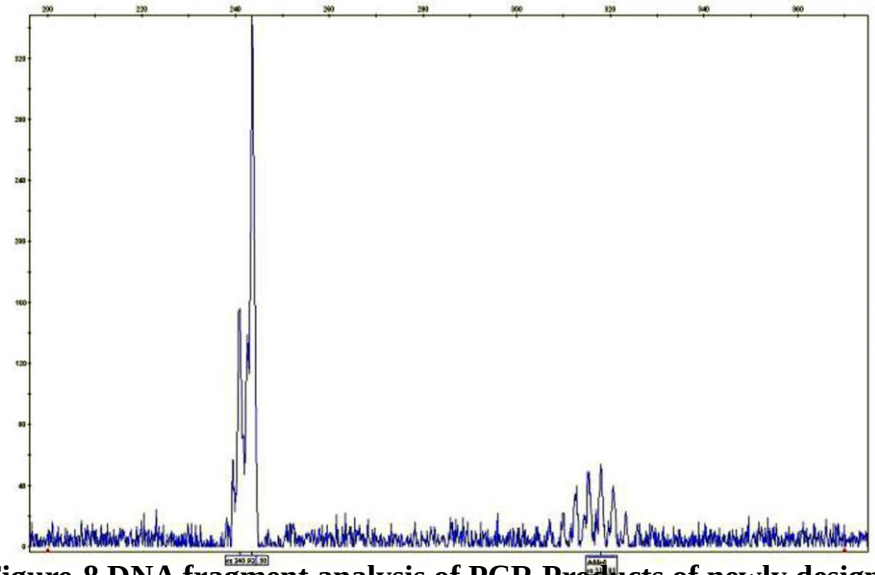


Figure-8 DNA fragment analysis of PCR Products of newly designed Primer SCA-1

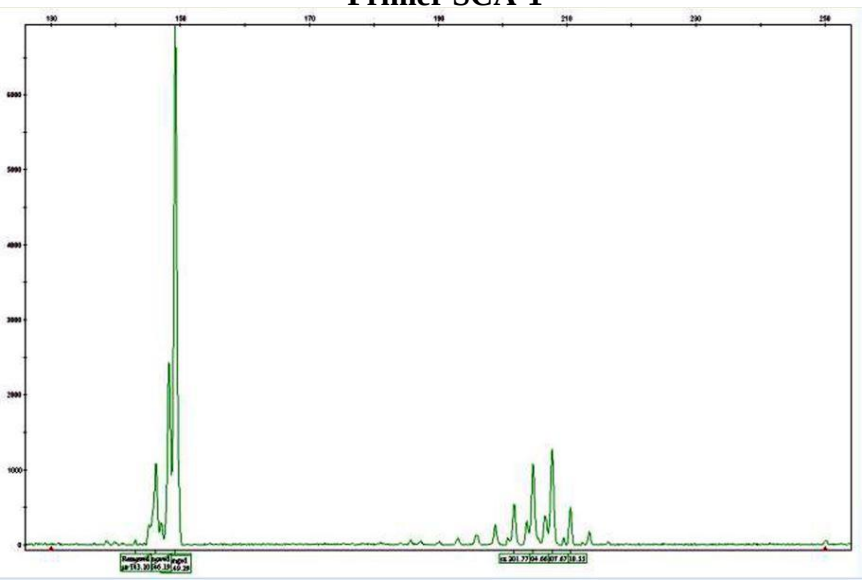


Figure-9 DNA fragment analysis of PCR Products of newly designed Primer SCA-2

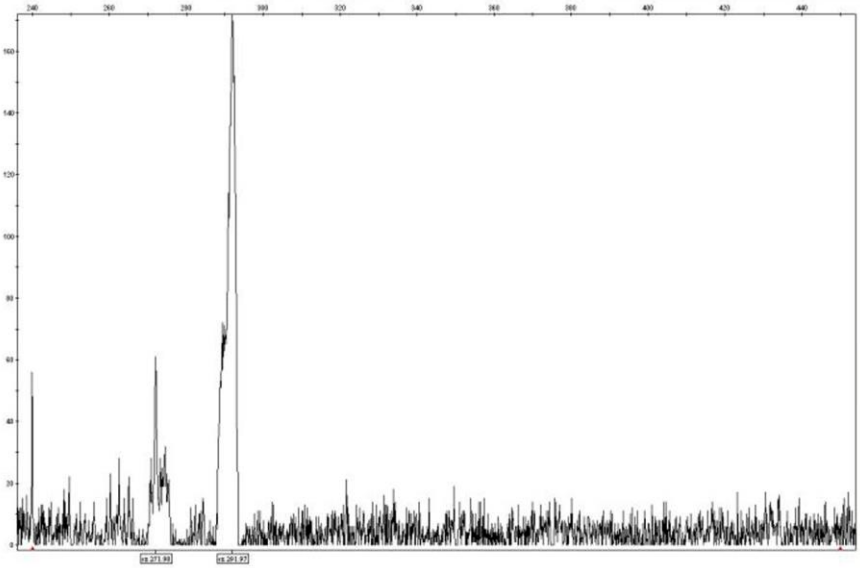


Figure-10 DNA fragment analysis of PCR Products of newly designed Primer SCA-3

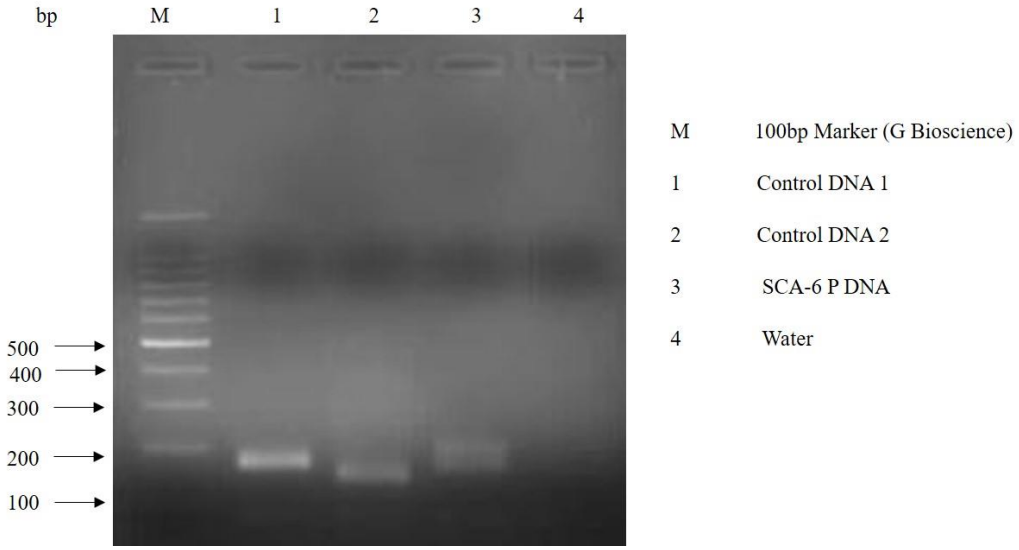


Figure-11 Agarose gel electrophoresis of PCR Products of newly designed Primer SCA-6

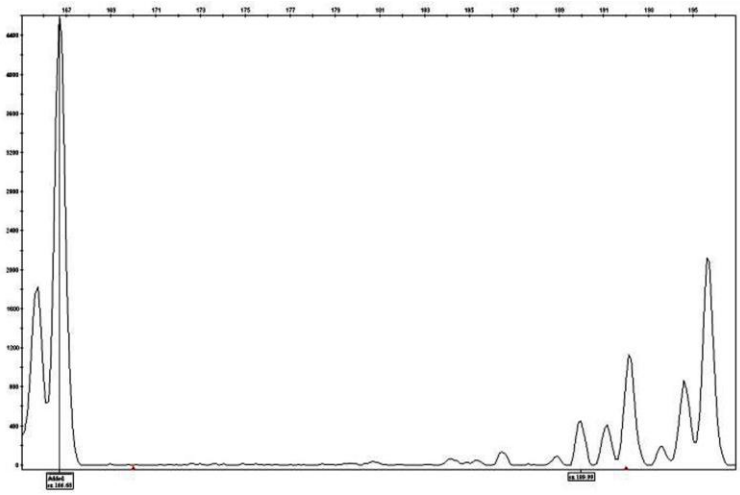


Figure-12 DNA fragment analysis of PCR Products of newly designed Primer SCA-6

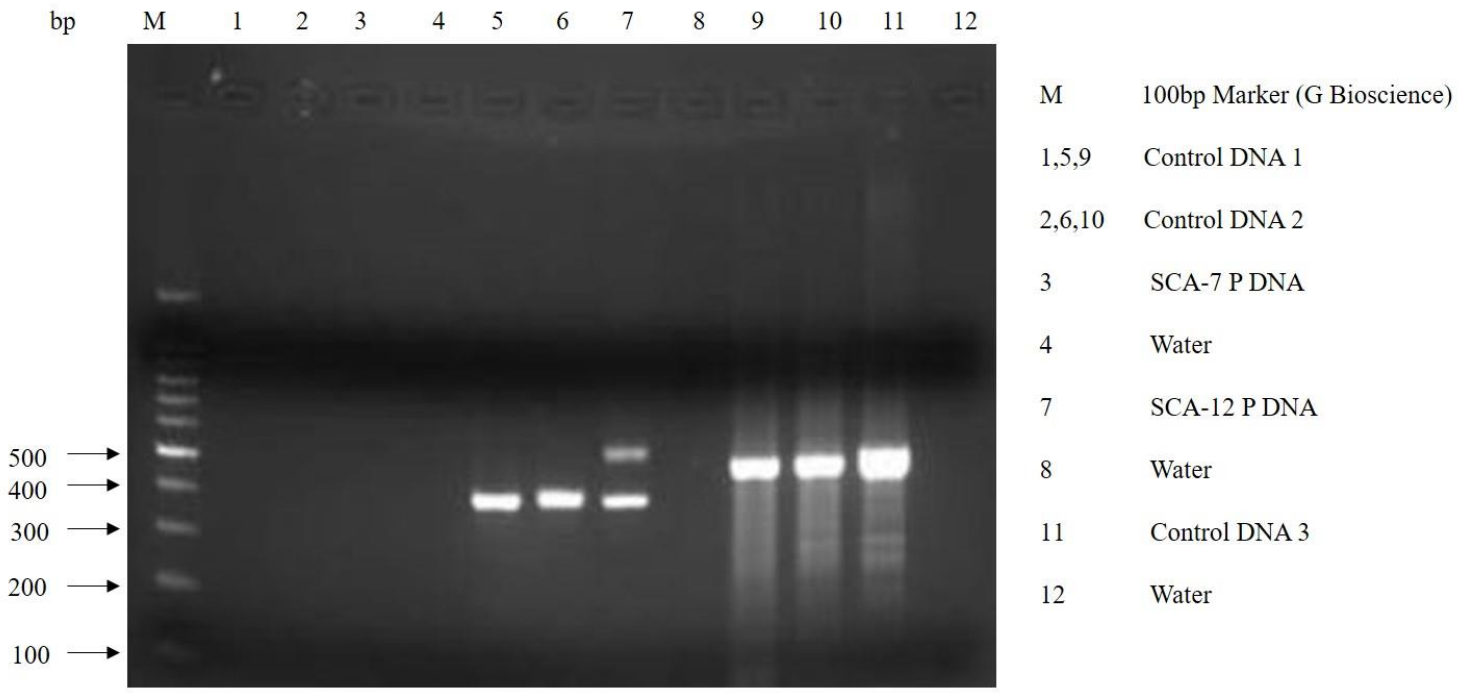


Figure-13 Agarose gel electrophoresis of PCR Products of newly designed Primer SCA-7, 12, and 17

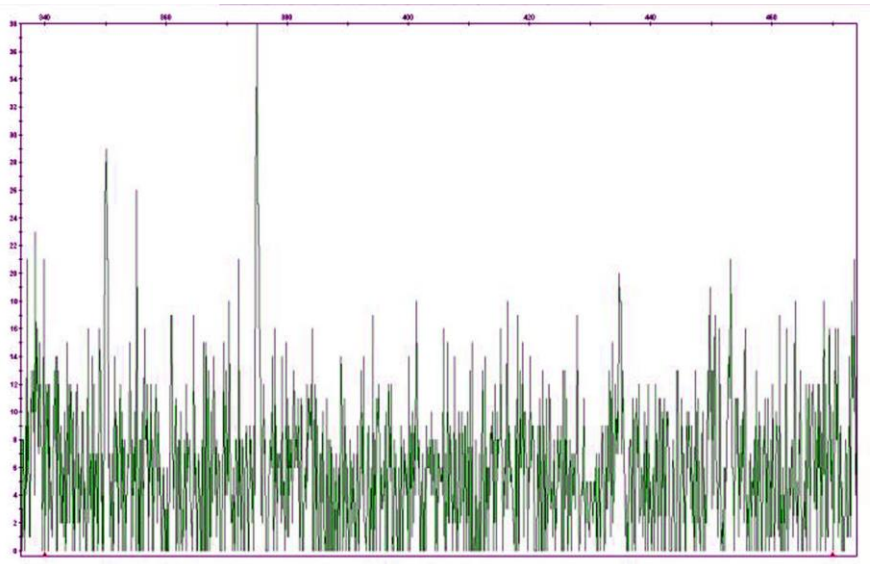


Figure-14 DNA fragment analysis of PCR Products of newly designed Primer SCA-7

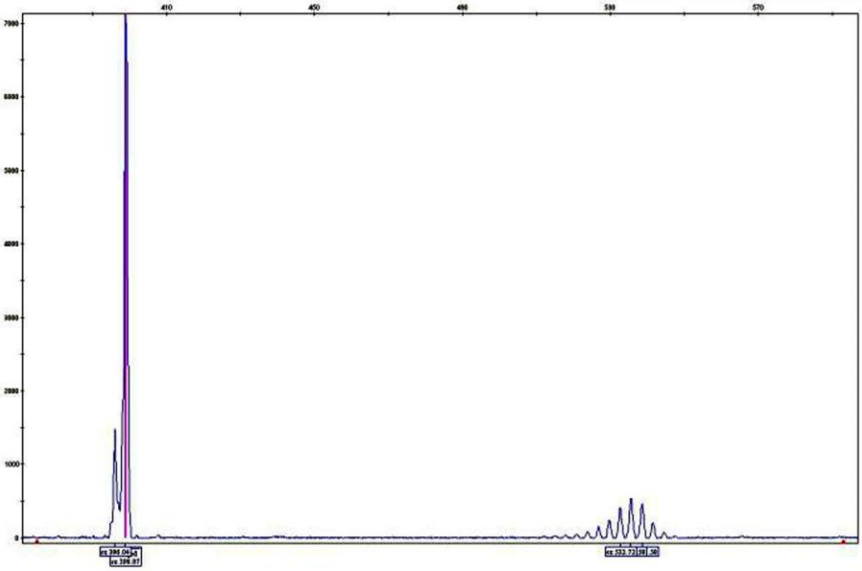


Figure-15 DNA fragment analysis of PCR Products of newly designed Primer SCA-12

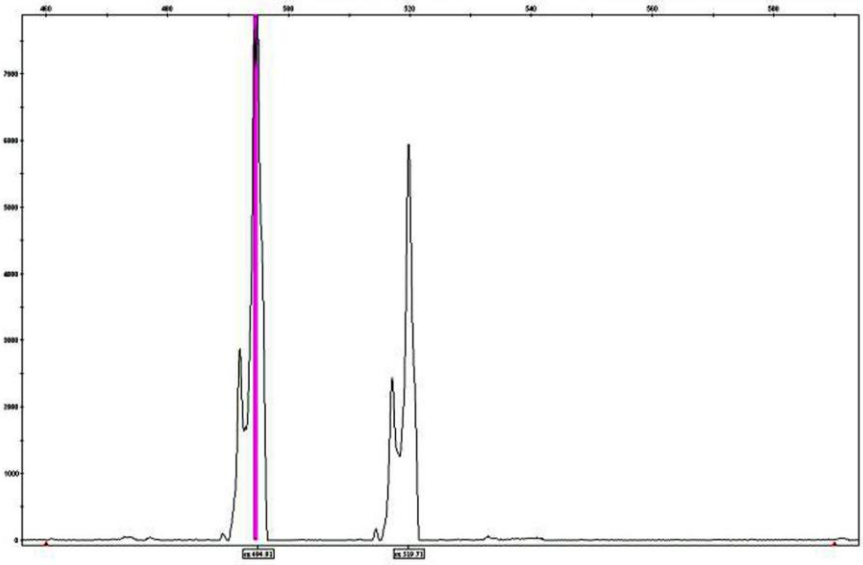


Figure-16 DNA fragment analysis of PCR Products of newly designed Primer SCA-17

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (5x)	4 µl
2	$MgCl_2$ (25mM)	1.5 µl
3	dNTPs (2mM)	3 µl
4	Forward Primer (10pmol/µl)	5.6 µl (0.8 µl each of Primer SCA-1, 2, 3, 6, 7, 12 and 17)
5	Reverse Primer (10pmol/µl)	5.6 µl (0.8 µl each of Primer SCA-1, 2, 3, 6, 7, 12 and 17)
6	Taq polymerase (5U/µl)	0.5 µl
7	Betaine (5M)	1 µl
8	MilliQ water	0.8 µl
9	DNA (100ng/µl)	3 µl
	Total	**25 µl**

Table-5 PCR reaction mix for Multiplex PCR Experiment-3

PCR program		
Initial denaturation	95°C for 5 Min.	
Denaturation	95°C for 30 Sec.	
Annealing	70°C for 1 Min.	35 Cycle
Extension	72°C for 1 Min.	
Hold	72°C for 10 Min.	
	4°C for 10 Min.	

Table-6 PCR cyclic condition for Multiplex PCR Experiment-3

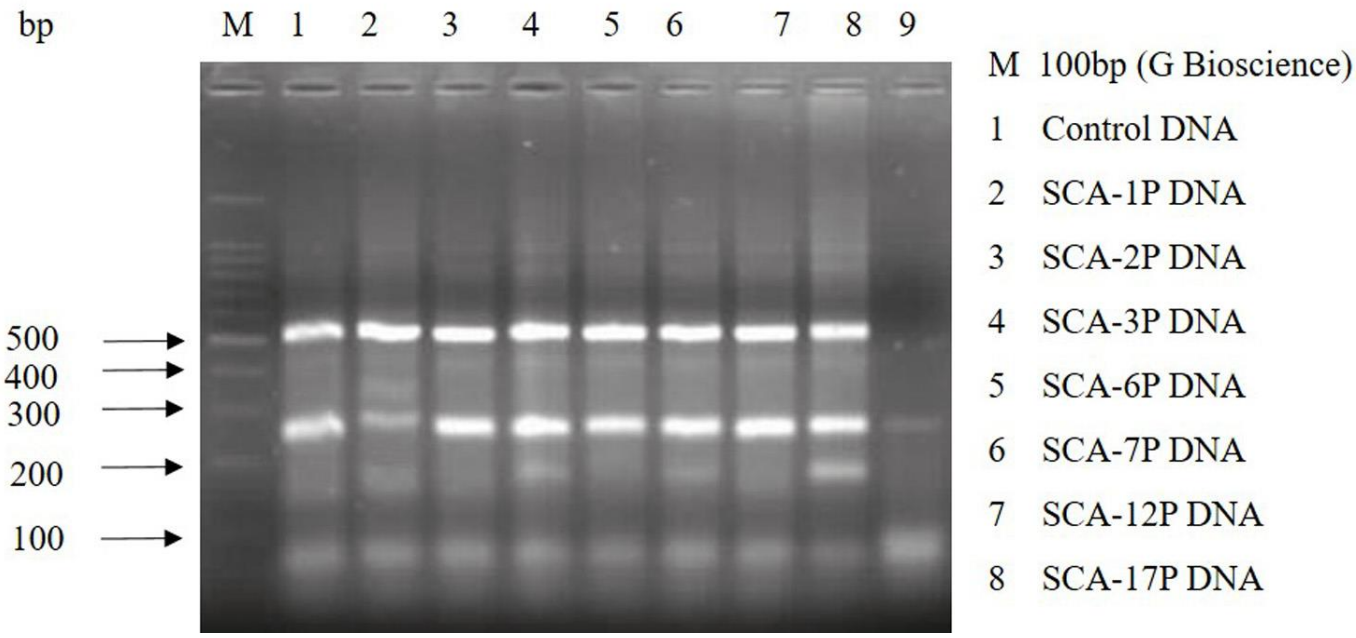


Figure-17 Agarose gel electrophoresis of Multiplex PCR Experiment-3 Products

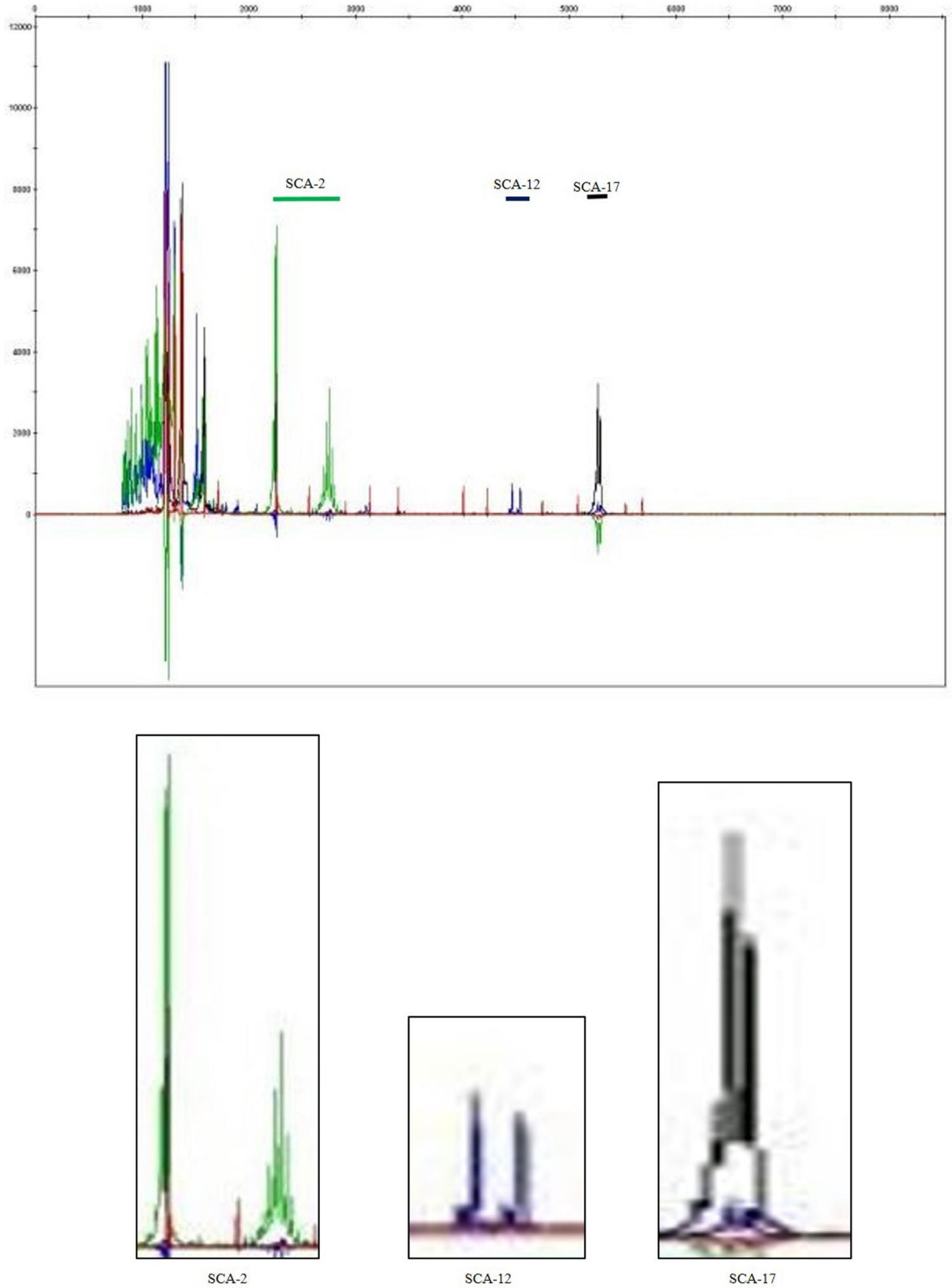


Figure-18 DNA fragment analysis raw data of Multiplex PCR Experiment-3 Products and Zoomed view of amplified locus by Primer SCA-2, 12 and 17

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (5x)	3 µl
2	$MgCl_2$ (25mM)	1.5 µl
3	dNTPs (2mM)	4.7 µl
4	Forward Primer (10pmol/µl)	7 µl{(0.8*3 µl Primer SCA-7,12,17)+(1*1µl Primer SCA-6)+(1.2*3 Primer SCA-1,2,3)}
5	Reverse Primer (10pmol/µl)	7 µl{(0.8*3 µl Primer SCA-7,12,17)+(1*1µl Primer SCA-6)+(1.2*3 Primer SCA-1,2,3)}
6	Taq polymerase (5U/µl)	0.5 µl
7	Betaine (5M)	1 µl
8	MilliQ water	2.3 µl
9	DNA (100ng/µl)	3 µl
	Total	**30 µl**

Table-7 PCR reaction mix for Multiplex PCR Experiment-4

PCR program		
Initial denaturation	95°C for 5 Min.	
Denaturation	95°C for 30 Sec.	
Annealing	65.3°C for 45 Sec.	8 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 30 Sec.	
Annealing	53.9°C for 45 Sec.	10 Cycle
Extension	72°C for 45 Sec.	
Denaturation	95°C for 45 Sec.	
Annealing	50.5°C for 45 Sec.	20 Cycle
Extension	72°C for 45 Sec.	
Hold	72°C for 10 Min.	
	4°C for 10 Min.	

Table-8 PCR cyclic condition for Multiplex PCR Experiment-4

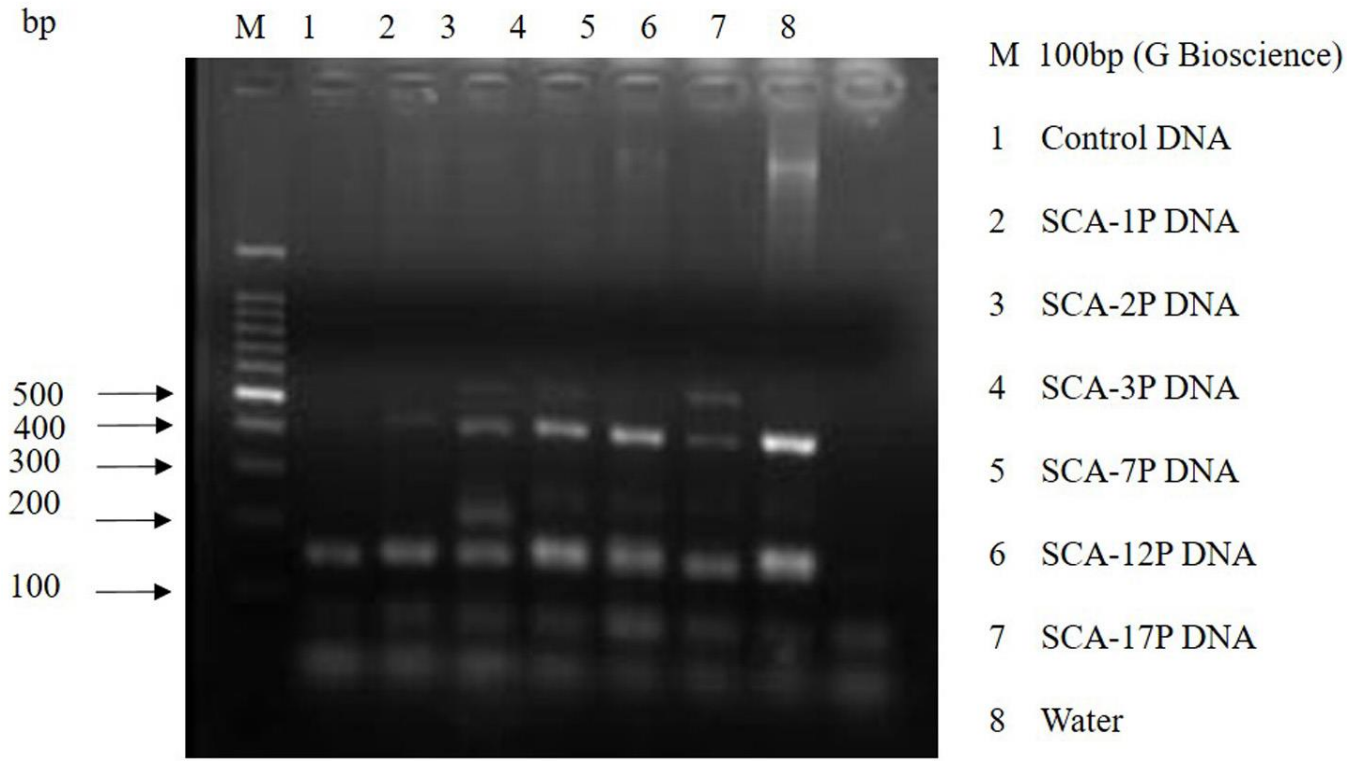


Figure-19 Agarose gel electrophoresis of Multiplex PCR Experiment-4 Products

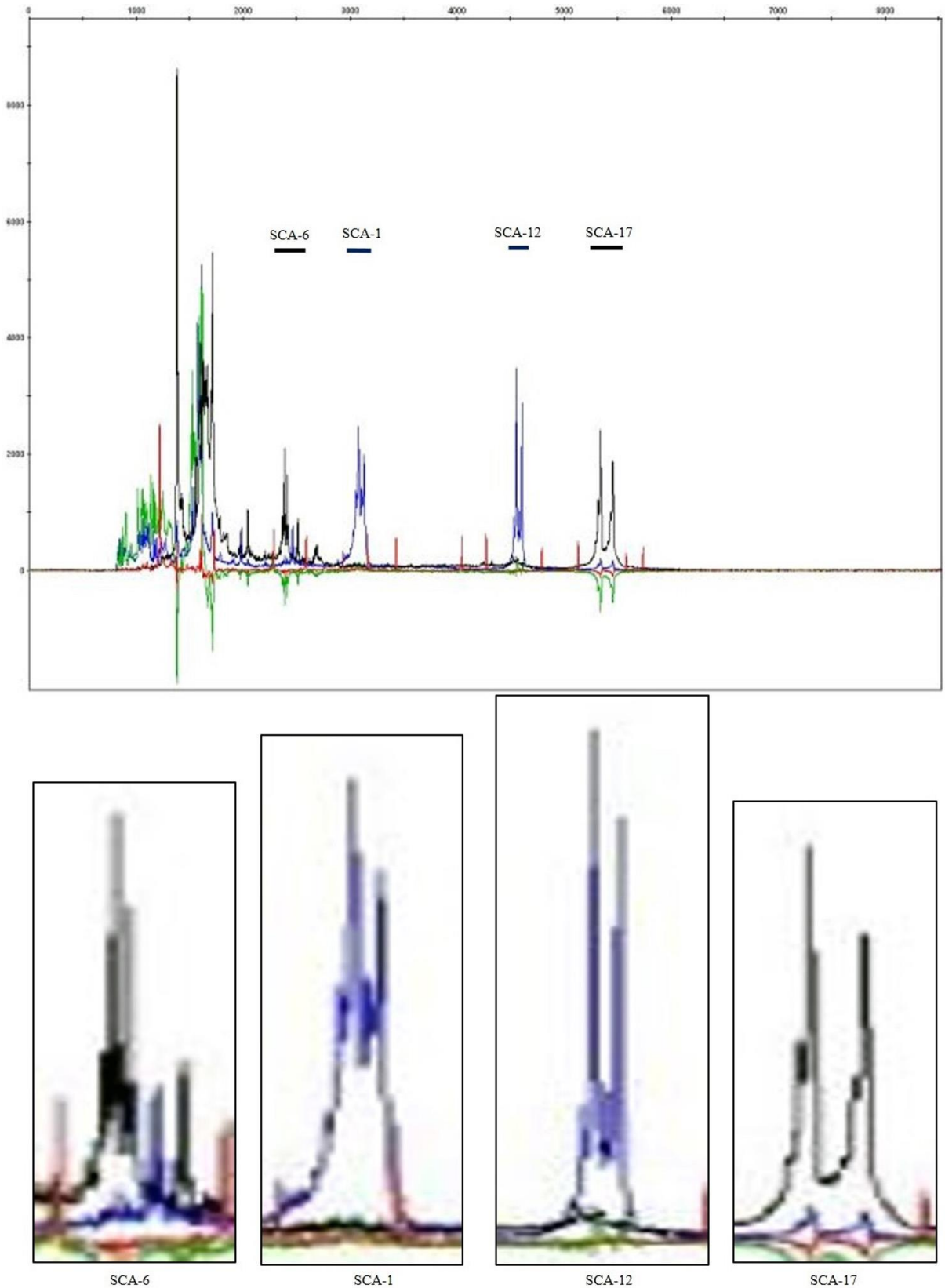


Figure-20 DNA fragment analysis raw data of Multiplex PCR Experiment-4 Products and Zoomed view of amplified locus by Primer SCA-1, 6, 12 and 17

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (5x)	6 µl
2	$MgCl_2$ (25mM)	1.8 µl
3	dNTPs (2mM)	4.7 µl
4	Forward Primer (10pmol/µl)	8.4 µl (1.2 µl each of Primer SCA-1, 2, 3, 6, 7, 12, 17)
5	Reverse Primer (10pmol/µl)	8.4 µl (1.2 µl each of Primer SCA-1, 2, 3, 6, 7, 12, 17)
6	Taq polymerase (5U/µl)	0.7 µl
7	Betaine (5M)	1.5 µl
8	MilliQ water	5 µl
9	DNA (100ng/µl)	3.5 µl
	Total	**40 µl**

Table-9 PCR reaction mix for Multiplex PCR Experiment-5

PCR program		
Initial denaturation	95°C for 5 Min.	
Denaturation	95°C for 30 Sec.	
Annealing	68.8°C for 45 Sec.	35 Cycle
Extension	72°C for 45 Sec.	
Hold	72°C for 10 Min.	
	4°C for 10 Min.	

Table-10 PCR cyclic condition for Multiplex PCR Experiment-5

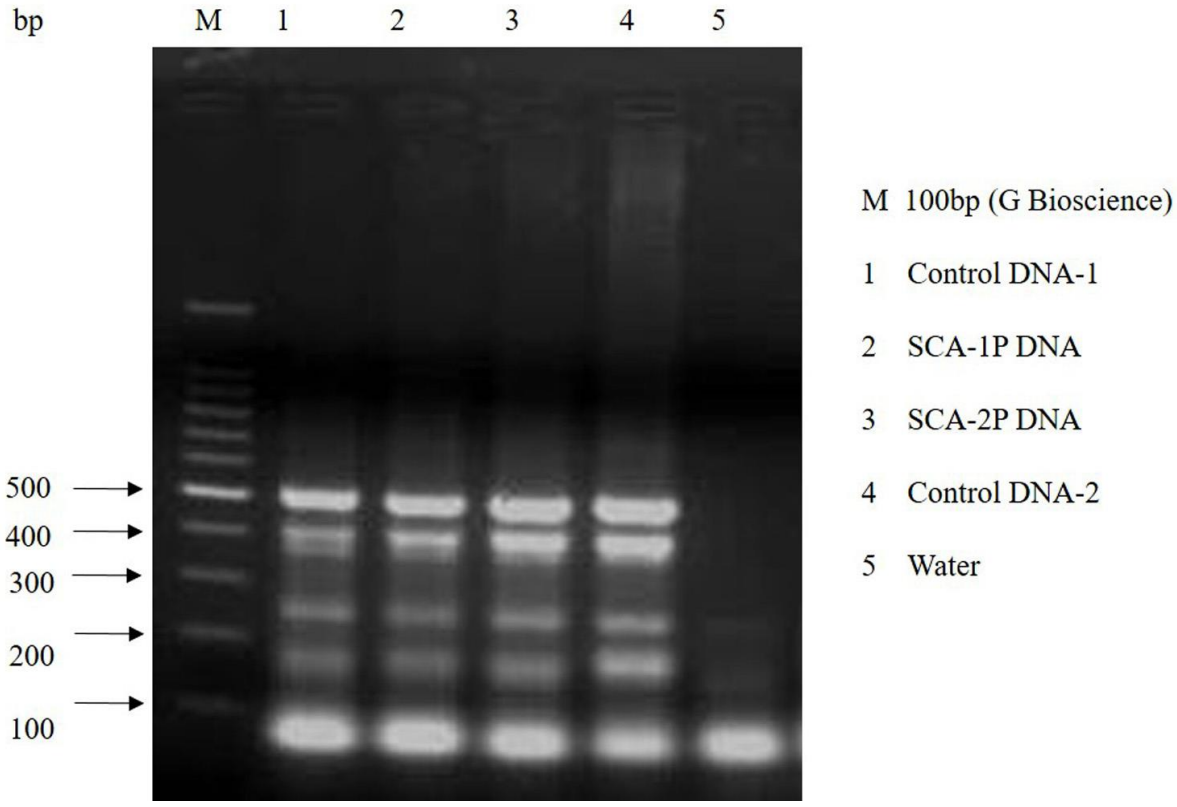


Figure-21 Agarose gel electrophoresis of Multiplex PCR Experiment-5 Products

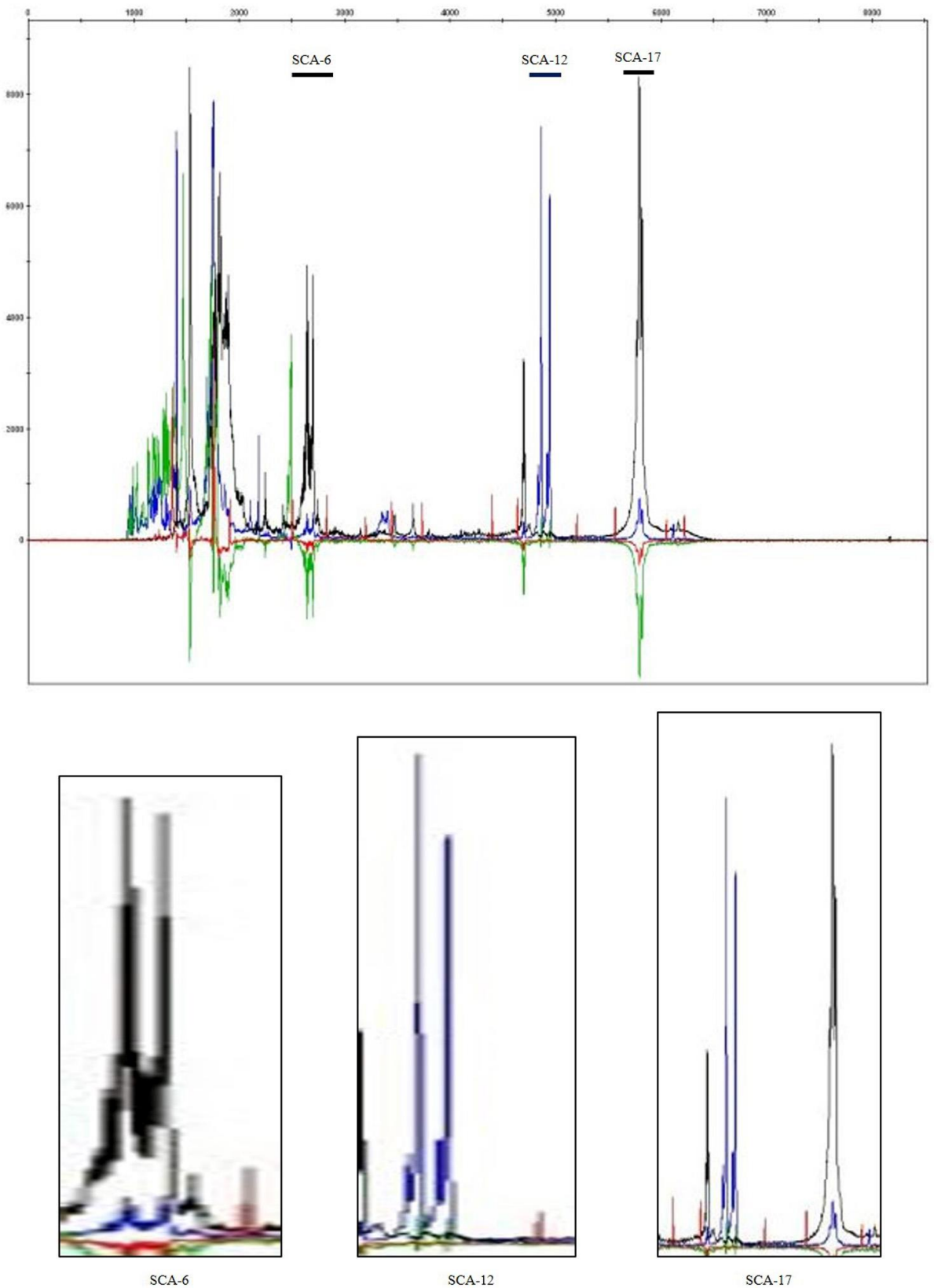


Figure-22 DNA fragment analysis raw data of Multiplex PCR Experiment-5 Products and Zoomed view of amplified locus by Primer SCA-6, 12, and 17

S. No.	PCR reaction mix	Volume (µl)
1	Buffer (10x)	2.5 µl
2	dNTPs (2mM)	0.6 µl
3	Forward Primer (10pmol/µl)	4µl{0.6*5 µl (Primer SCA-1,2,3,7,12)+(0.5*2µl Primer SCA-6,17)}
4	Reverse Primer (10pmol/µl)	4µl{0.6*5 µl (Primer SCA-1,2,3,7,12)+(0.5*2µl Primer SCA-6,17)}
5	Betaine (5M)	1 µl
6	Pfu ultra polymerase (5U/µl)	0.3 µl
7	MilliQ water	5.2 µl
8	DNA (100ng/µl)	8 µl
	Total	**25 µl**

Table-11 PCR reaction mix for Multiplex PCR Experiment-6

PCR program		
Initial denaturation	95°C for 3 Min.	
Denaturation	95°C for 1 Min.	
Annealing	60°C for 1 Min.	35 Cycle
Extension	68°C for 1.5 Min.	
Hold	68°C for 10 Min.	
	4°C for 10 Min.	

Table-12 PCR cyclic condition for Multiplex PCR Experiment-6

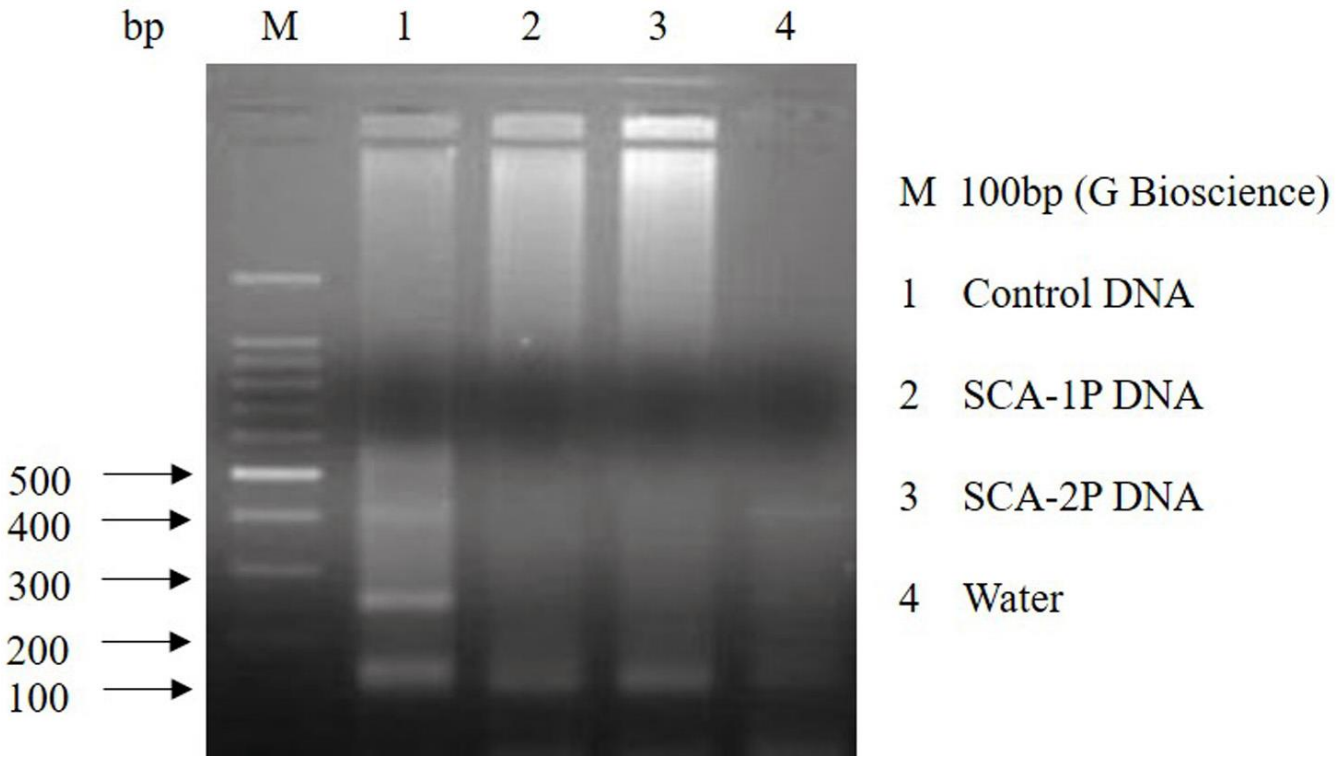


Figure-23 Agarose gel electrophoresis of Multiplex PCR Experiment-6 Products

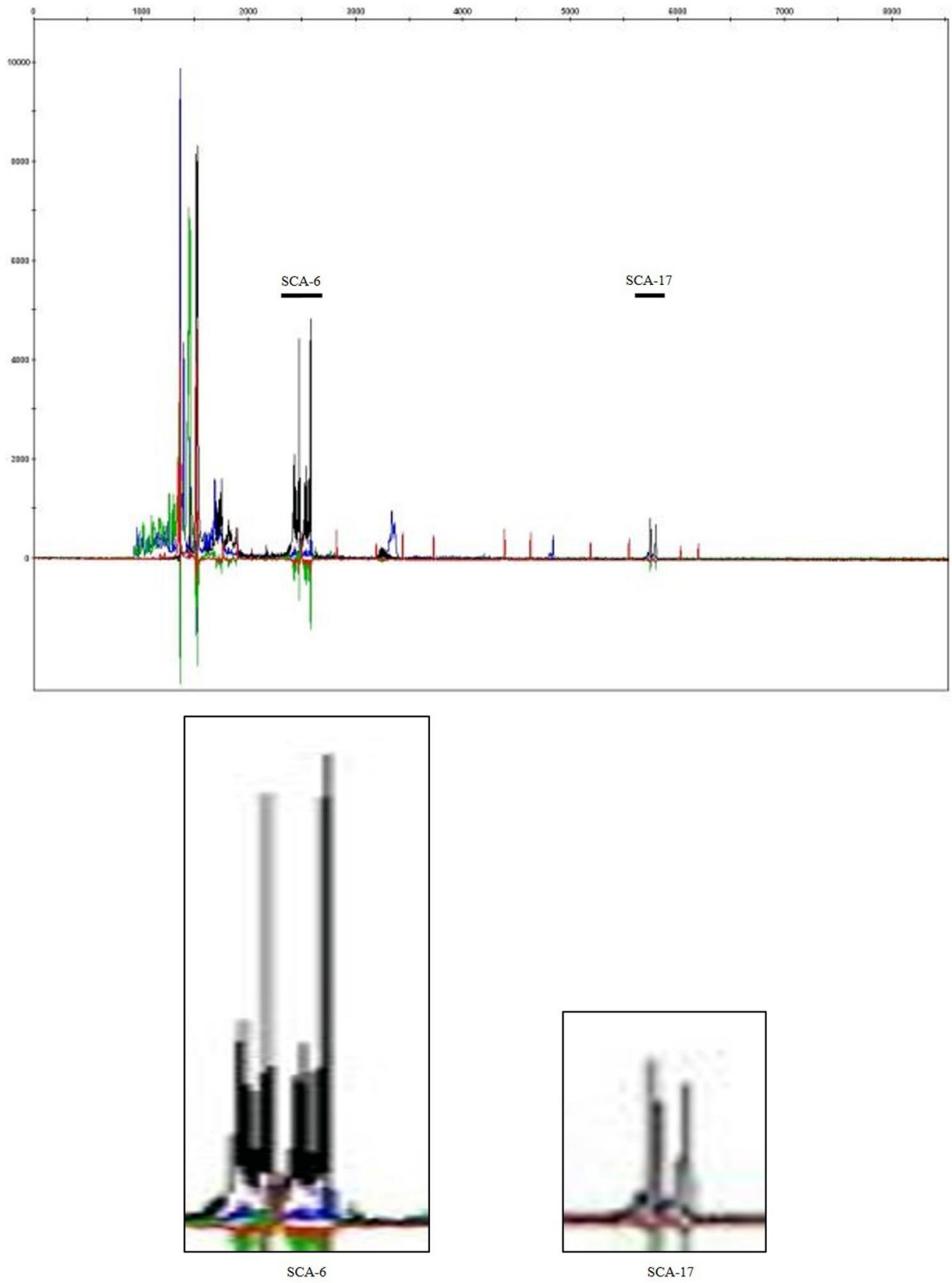


Figure-24 DNA fragment analysis raw data of Multiplex PCR Experiment-6 Products and Zoomed view of amplified locus by Primer SCA-6 and 17

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-1	225.39	230.62
2	SCA-2	194.72	231.01
3	SCA-3	268.41	284.76
4	SCA-12	151.57	160.11

Table-13 Size of amplified allele in Multiplex PCR Experiment-1

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-1	225.92	231.73
2	SCA-2	193.89	250.61
3	SCA-3	237.7	500.05
4	SCA-12	151.61	160.2

Table-14 Size of amplified allele in Multiplex PCR Experiment-2

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-2	150.24	207.59
2	SCA-12	401.9	410.75
3	SCA-17	497.62	500.45

Table-15 Size of amplified allele in Multiplex PCR Experiment-3

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-1	240.57	246.31
2	SCA-6	165.37	176.85
3	SCA-12	407.44	413.32
4	SCA-17	314.36	500.37

Table-16 Size of amplified allele in Multiplex PCR Experiment-4

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-6	165.25	171.06
2	SCA-12	398.5	407.24
3	SCA-17	500.24	503.07

Table-17 Size of amplified allele in Multiplex PCR Experiment-5

S. No.	Primer	Allele 1 Size (bp)	Allele 2 Size (bp)
1	SCA-6	165.22	183.63
2	SCA-17	497.51	503.12

Table-18 Size of amplified allele in Multiplex PCR Experiment-6

DISCUSSION

DISCUSSION

The concept of FM-PCR based detection method is developed due to the overlap in clinical features of many SCA types. The advantage of FM-PCR is the rapid and time-saving detection of particular SCA type from many clinically suspected one. Initially to explore the method and finding the success rate of the experiment, a four set of primers used (Table-20) that were routinely used before to screen Individual SCA types. The outcome of this experiment was successful but due to same tagged fluorescent color of three primers namely SCA-1, 3 and 12 and an overlap between the size ranges of amplified products of these primers (fragments), we ordered new seven set of primers (Table-21) in which a distinction is made among the PCR amplified products of fluorescent tagged primers (fragments) based on their color code and size. The size range among amplified products of new sets of primer is developed due to difference in CAG repeat, in particular, SCA type in Normal and disease condition. Table-19 demonstrate the Number of CAG repeat in normal and disease condition in SCA type 1, 2, 3, 6, 7, 12, and, 17 [64] For the multiplex PCR experiment we set up our experimental condition near the protocol followed in the literature [62, 63]. We had used the two different type of polymerases (Pfu, Taq) and their PCR components in separate reactions with many PCR cyclic conditions (Table-1 to 12). The initial success of multiplex experiment with new set of primers and Taq polymerase was the amplification of three targeted region with primers pairs of SCA-2, 12 and 17 which is shown in the figure-18 After the change in amount of PCR reaction components and cyclic condition we have obtained the PCR condition at which four targeted regions of DNA get amplify in near equal amount with primer pairs of SCA-1, 6, 12, and 17. The PCR condition is illustrated in Table-7 and 8 and result is illustrated in Figure-20 respectively and size of amplified product is shown in Table-16. The multiplex PCR experiment with Pfu polymerase leads to very less amplification of targeted regions and the concentration of amplified product was anomalous. In comparative analysis of our Multiplex PCR experiments with both polymerase till date, it was found that the optimization of multiplex PCR primer with Taq polymerase is relatively easy and it yields near equal amount of amplified products at optimized reaction mix and cyclic condition.

S. No.	SCA Type	CAG Repeats	
		Normal	Expanded
1	SCA-1	6-39	40-82
2	SCA-2	14-31	33-64
3	SCA-3	12-42	54-86
4	SCA-6	4-18	19-30
5	SCA-7	4-27	37-200
6	SCA-12	7-32	55-78
7	SCA-17	25-44	47-63

Table-19 SCA Types and their CAG repeats in Normal and Expanded Condition

CONCLUSION

CONCLUSION

We have developed the Multiplex PCR condition for the rapid detection of four most common types of SCA (SCA 1, 6, 12, and 17). Our method reduce the time of diagnosis in cost effective manner and utilize less human and material resource.

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APPENDIX

APPENDIX-1

Reagents used:

Reagents used in salting out method of DNA extraction:

RBC lysis buffer (10x)

NH_4Cl 8.20 gm

$NaHCO_3$ 0.84 gm

EDTA 0.37 gm

Dissolve in 100ml of distilled water, autoclaved and stored at 40C.

Working dilution (1x)

For 500 ml 1x RBC lysis buffer - 50ml RBC lysis buffer (10x) + 450 ml autoclaved water.

Nucleus Lysis Buffer (1X)

1M Tris HCL (pH 8.0) – 4ml

5 M NaCl – 32ml

0.5 M EDTA (pH 8.0) – 1.6ml

Final volume made up to 400 ml (Autoclaved and stored at room temperature)

Proteinase K solution (20 mg / ml) – It inhibit DNase and RNase activity.

20 mg of Proteinase K was dissolved in 1 ml autoclaved distilled water.

Store at 40C.

10% SDS (sodium dodecyl acetate)

For 100 ml – 10 gm of SDS was dissolved in autoclaved distilled water.

Final volume made up to 100 ml (stored at room temperature).

6M saturated NaCl solution

NaCl – 35.064 gm.

Dissolve in distilled water and make final volume up to 100 ml.

5M saturated NaCl solution

NaCl – 29.22 gm.

Dissolve in distilled water and make final volume up to 100 ml.

TE Buffer (200ml) – it dissolve DNA

10 mM Tris (pH 8.0)

1 mM EDTA (pH 8.0)

Autoclave and store at 40C.

Multiplex PCR reagents (Promega)

1-PCR buffer (5x)

2-$MgCl_2$ (25mM)

3-dNTPS (2mM)

4-Primers forward (10pmol/µl)

5-Primers reverse (10pmol/µl)

6- Betaine (5M)

7-Milli Q water

8-Taq polymerase (5U/µl)

Multiplex PCR reagents (Agilent)

1-PCR buffer (10x)

2-dNTPS (25mM)

3-Primers forward (10pmol/µl)

4-Primers reverse (10pmol/µl)

5- DMSO

6-Milli Q water

7-Pfu polymerase (5U/µl)

Name	Fluorochrome	Sequences (5'→ 3)	Amplicon (bp)
SCA1F	HEX	CAACATGGGCAGTCTGAG	148+(CAG)n
SCA1R		GGTGCGGCCGGTGTTCTG	
SCA2F	FAM	GGGCCCCTCACCATGTCG	177+ (CAG)n
SCA2R		GTGGCCGAGGACGAGGAGAC	
SCA3F	FAM	CCAGTGACTACTTTGATTCG	217+(CAG)n
SCA3R		CTTACCTAGATCACTCCCAA	
SCA12F	FAM	TGCTGGGAAAGAGTCGTG	122+(CAG)n
SCA12R		GCCAGCGCACTCACCCTC	

Table-20 Primers used in the multiplexing of SCA 1, 2, 3, and 12 in Multiplex PCR Experiment-1 and 2

Name	Fluorochrome	Sequences (5'→ 3)	Amplicon (bp)
SCA1F		gcggtcccaaaagggtcagtAACTGGAAATGTGGACGTAC	162+(CAG)n
SCA1R	FAM	ggtcccaaaagggtcagtCAACATGGGCAGTCTGAG	
SCA2F		aaaagggtcagtGGGCCCCTCACCATGTCG	84+ (CAG)n
SCA2R	HEX	caaaagggtcagtCGGGCTTGCGGACATTGG	
SCA3F	NED	gcggtcccaaaagggtcagtCCAGTGACTTTGATTCG	201+(CAG)n
SCA3R		gcggtcccaaaagggtcagtTGGCCTTTCACATGGATGTGAA	
SCA6F		caaaagggtcagtCAGGTGTCCTATTCCCCTGTGATCC	126+(CAG)n
SCA6R	NED	aaagggtcagtTGGGTACCTCCGAGGGCCGCTGGTG	
SCA7F	HEX	gcggtcccaaaagggtcagtTGTTACATTGTAGGAGCGGAA	314+(CAG)n
SCA7R		gtcccaaaagggtcagtCACGACTGTCCCAGCATCACTT	
SCA12F	FAM	caaaagggtcagtAGATGGCAGGGACAGGATTC	353+(CAG)n
SCA12R		aaagggtcagtACCTGAACCTGCAAGCTAGTC	
SCA17F	NED	aaaagggtcagtACCTGCTGTTCCACCAAGAAAG	366+(CAG)n
SCA17R		caaaagggtcagtGTGGCAGGAGTGATGGGG	

†Upper case, SCA locus-specific primer sequence; lower case, sequence from M13mp18 (gcggtcccaaaagggtcagt).

Table-21 Primers used in the multiplexing of SCA 1, 2, 3, 6, 7, 12 and 17 in Multiplex PCR Experiment-3 to 6

Agarose Gel Electrophoresis Reagents

1-TAE buffer (1X)

TAE buffer (50X) 1000 ml preparation	
Tris Base	242 gm
Glacial Acetic Acid	57.1 ml
0.5 M EDTA (pH-8)	100 ml
Milli Q water	Adjust to the final volume 1000 ml

2-Agarose

3-Ethidium Bromide (EtBr)

4-6X Sample Loading Buffer

5-DNA ladder standard

6-Electrophoresis chamber

7-Power supply

8-Gel casting tray and combs

9-Gloves

10-Pipette and tips

DNA Fragment Analysis Reactions components

1- Highly deionized Formamide (HiDi)

2-Marker (Rox-550)

APPENDIX-2

Method and Techniques employed:

Salting out Method of DNA Extraction from Blood

- Collect blood sample from patients in a tube containing ACD buffer
- Take 5-10 ml of blood and make the volume 50 ml using RBC lysis buffer (1X) in falcon tube of volume 50ml
- Mix the suspension by inverting the tubes several times till it become translucent.
- Keep the tube at room temperature for about 20min with gentle shaking till the lysis action of buffer completed.
- Centrifuge the lysed blood to 2500 rpm for 10 min at room temperature.
- Discard the supernatant in hypochlorite and water solution.
- Add 15 ml of RBC lysis buffer to the pellet and mix it by brief vortexing.
- Centrifuge again at 1000 rpm for 10 min at room temperature.
- Discard the supernatant in hypochlorite and water solution.
- Add 12 ml of nucleus lysis buffer (NLB) to pellet and mix it by vortexing.
- Add 0.8ml of 10% SDS and 50 µl of proteinase-k (20mg/ml) and mix it well.
- Incubate the tubes at 65^{o}C temperature for 2 hrs in a water bath.
- Add 4ml of saturated NaCl (6M) sol. and shake virgously for 15 sec.
- Immediately spin the tube at 3500 rpm for 35 minute at room temperature.

- Take the supernatant carefully with not disturbing pellet and make twice volume (i.e. make the volume up to 50ml) of absolute ethanol (i.e. 100%) and keep it at room temperature.

- Precipitate (ppt) DNA by inverting 10-20 times very slowly (or keep it overnight at -20^{o}C).

- Transfer the Precipitated DNA using a pipette tip to a micro centrifuge tube containing 1 ml of 70% ethanol.

- Vertex briefly the tubes and centrifuge at 13000 rpm for 10 min.

- Carefully remove the supernatant without disturbing the pellet.

- Air dry the pellet for 1-2 hrs and re-suspend in 1 ml of TE buffer (Tris + EDTA) pH 8.0

- Dissolve the DNA by keeping it 65^{o}C temp for 2 hrs and store at (-20) ^{o}C temperature.

Adjustment of DNA concentration to 50ng/µl and 100ng/µl

Nano drop machine is used to quantify the DNA purity and concentration, the final concentration of DNA is made to 50 ng/µl and 100 ng/µl with addition of MilliQ water.

Temperature and snap chilling based fragmentation of DNA

DNA is heated at 95^{o} for different length of time such as 10, 20 and 30 minutes followed by snap chilling on ice for 5 minutes.

Multiplex PCR

Many PCR conditions with varying mixture of reaction components and PCR cycle is used to amplify the DNA, Table 3(a, b), 4(a, b), 5(a, b), 6(a, b), 7(a, b) and 8(a, b) demonstrate the PCR reaction components and the PCR cycling conditions used respectively for different experiments named as Multiplex PCR Experiment 1-6. The primers used in Multiplex PCR Experiment 1 and 2 is enlisted in Table-1 and Table-2 the different set of primers used in Experiment 3-6 which is tabulated in Table-2. The temperature fragmented DNA is used as a template in Multiplex PCR Experiment-2.

Agarose Gel Electrophoresis:

Preparing the agarose gel of 2%

- Measure 2 gm of powder of Agarose and put it into a 100 ml TAE buffer in a 500 ml flask
- Melt the agarose in microwave oven or hot water bath till the solution become clear.
- Wait until the solution temperature reach to 45-50^{o}C temperature, whirling the flask occasionally to reduce temperature evenly.
- Add the 5µl of Ethidium bromide (EtBr) to the melted agarose.
- Seal the end of tray by cello tape
- Keep the combs in the casting tray of agarose gel.
- Decant the melted agarose solution into the casting tray and let it cool until it is solidify (it should appear milky white).
- Carefully take out the combs and take away the tape.
- Place the gel in the electrophoresis chamber.
- Add sufficient TAE Buffer so that there is about 3-4mm of buffer over the gel.

Loading the gel

- Add 2 µl of 6X DNA Loading Buffer to each 10 µl products of PCR reaction.
- Carefully pipette 12 µl of each sample and Sample Loading Buffer mixture into the separate wells in the gel.
- Pipette 5 µl of the standard DNA ladder into at least one well of each row on the gel.

Running the gel

- Place the lid on the gel box, connecting the electrodes.
- Connect the wires to power supply
- Turn on the power supply to about 100 volts, it should not exceed 5 volts/ cm between Anode and Cathode.
- Let the power supply on until the blue loading dye reach to the end of the gel.
- Turn off the power.
- Disconnect the wires from the power supply.
- Remove the lid of the electrophoresis chamber.
- Using gloves, carefully remove the tray and gel.
- Observe and analyze gel under UV Transilluminator gel-doc analyzer.

DNA Fragment Analysis

- Add the Marker (Rock 550), Hidi and PCR product in the well of fragment analyzer tray given as follows:
    - Marker: 0.4 µl
    - Hidi: 8.6 µl
    - PCR Product: 1µl
- Heat denature the PCR products (DNA) for 95^{o} for 5 minutes followed by snap chilling on ice for 5 minutes.
- For the analysis of product load the tray in machine

- Analyze the result by using software gene mapper version 4.