# Chapter 2

# Enhancing Cohort PASA Efficiency from Lessons Assimilated by Mutant Genotyping in *C. elegans*

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## Abstract

Classical restriction fragment length polymorphism (RFLP) and sequencing are labor-intensive and expensive methods to study single base changes, whereas polymerase chain reaction amplification of specific alleles (PASA) or allele-specific polymerase chain reaction (ASPCR) is a PCR-based application that allows direct detection of any point mutation by analyzing the PCR products in an ethidium bromide-stained agarose or polyacrylamide gel. PASA is based on oligonucleotide primers containing one or more 3′ mismatch with the target DNA making it refractory to primer extension by *Thermus aquaticus* DNA polymerase lacking the 3′ to 5′ exonuclease proofreading activity because of which it is also called amplification refractory mutation system-PCR (ARMS-PCR). This technique has found application in detection of allele, mutation, single-nucleotide polymorphisms (SNPs) causing genetic and infectious diseases. This chapter describes an approach of cohort PASA in context of genotyping single and double mutant worms generated to study the process of cell migration and axon outgrowth in *C. elegans*. Single worm-based cohort PASA allows genotyping for identification of single base mutations; particularly it is convenient method to detect mutations without a visible phenotype.

**Key words** Polymerase chain reaction, Allele-specific PCR, Single worm PCR, *C. elegans*

## 1 Introduction

DNA replication is the most critical process for continuation of a species, via transmission of genetic information encoded in the DNA by replication process from mother to the daughter cells. In vivo replication is catalyzed by DNA-dependent DNA polymerases, first discovered in *E. coli* by Arthur Kornberg and colleagues [1]. Whereas, polymerase chain reaction (PCR), is an in vitro process of DNA replication, developed in the early 1980s [2, 3], which uses *Taq*, a thermostable DNA-dependent DNA polymerase. *Taq* was first isolated from the thermophilic bacteria *Thermophilus aquaticus* in 1976 [4, 5], a strain YT-1 of this bacteria was first isolated from a sample taken from mushroom springs of Yellowstone national park in 1967 [6]. The enzyme became famous for its use in PCR [7] and

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in 1989 was designated as the "Molecule of the Year" [8]. Since then the basic PCR technique has been modified to address different problems encountered in biological sciences (Table 1). However, since *Taq* DNA polymerase lacks 3′–5′ exonuclease proofreading activity and shows low replication fidelity, making it suitable for routine genotyping experiments. *Pfu* isolated from *Pyrococcus furiosus,* possessing superior thermostability and proofreading activity has been used for applications requiring precise replication [38].

Since the time of its invention, this in vitro technique producing billions of copies of DNA in a matter of couple of hours has found application in numerous areas (Table 1). This chapter discusses PASA/AS-PCR in greater details by including studies conducted in the model organism, *C. elegans.*

As the name suggests PASA is the detection of specific alleles using polymerase chain reaction. Ideally the two copies of a gene, one on each chromosome should be identical; however, differences are observed between the two gene copies at the sequence level. Although, previously allele has been strictly used to define two forms of a gene, more recently allelic variations have been observed in noncoding regions of DNA; such allelic differences regulate phenotype by modulating gene expression [39]. PASA is a technique for distinguishing two alleles either within the gene or in the extra genic region using allele-specific primers, thereby underscoring the importance of primer design. One prerequisite for PASA like any other PCR-based technique is the knowledge of DNA sequence of the target gene unlike various sequencing methods including Sanger sequencing, pyrosequencing, and next- generation sequencing (NGS), which do not require prior sequence information. This chapter primarily discusses a modified PASA technique called cohort PASA for isolating single and double mutant worms in *C. elegans.*

## 2 Advances in Primer design Strategies

Since the most important parameter for a successful and robust PASA reaction is the primers, therefore it will be most appropriate to discuss primer design and their influence on PASA-based detection of allelic variation.

### 2.1 Enhancing Specificity and Discriminatory Potential of Allele-Specific Primers

Traditionally, PASA reactions use two allele-specific primers (ASPs) and one gene-specific primer (GSP), an ASP includes an allele-specific (AS) nucleotide at the last position of the 3′-terminus. The allele specific (AS) nucleotide is present as the last base of the ASP because 3′-OH is the site of polymerase activity to add new nucleotides, therefore any mismatch at 3′ end will abolish complementary base pairing and stall the polymerase activity. Moreover, it

**Table 1**
**Modifications of basic PCR technique finds application in solving various biological problems**

| PCR Technique | Principle and Application | References |
|---|---|---|
| PCR-restriction fragment length polymorphism (PCR-RFLP) | PCR amplification followed by RFLP for detection of DNA polymorphism, mutations, single-nucleotide polymorphism (SNP). | [9] |
| Methylation-specific-PCR (MSP-PCR) | Assessing methylation status of any group of CpG sites within a CpG island by using sodium bisulfite, converting all unmethylated but not methylated cytosine to uracil and subsequent amplification with primers specific for methylated versus unmethylated DNA. | [10, 12] |
| Differential display-PCR (DD-PCR) | Used to identify changes in gene expression at mRNA level by reverse transcribing mRNA into cDNA to create cDNA library followed by PCR. | [13] |
| Random amplified polymorphic DNA-PCR (RAPD-PCR) | Random primers are used to amplify stretches of DNA of unknown sequences to trace the phylogeny of plant and animal species. | [13] |
| Quantitative PCR (qPCR)/real-time PCR/quantitative real-time PCR | Simultaneous amplification and product quantification of a target DNA as the process takes place in real-time, absolute quantification of initial copy number, and relative quantification to reference target. | [14–17] |
| Microchip PCR | Miniaturizes conventional PCR systems and reduces operation time and cost. | [18, 19] |
| Repetitive element sequence based-PCR (Rep-PCR) | Used as an effective method for bacterial strain typing. | [20] |
| In silico PCR | A complementary method to ensure primer specificity for an extensive range of PCR applications. | [21] |
| Gap-PCR | Detection of deletions in DNA, otherwise not detectable by sequencing. | [22] |
| Droplet digital-PCR (ddPCR) | For determining original concentration of template by analysis of droplets formed in a water-oil emulsion to determine the fraction of PCR-positive droplets in the original sample. | [23] |
| CO-amplification at lower denaturation temperature-PCR (COLD-PCR) | Selectively amplifies mutations by performing single PCR at a critical temperature at which the mutation containing DNA is preferentially melted. | [24] |
| Multiplex PCR | Detection of multiple target DNA in single reaction using multiple primer pairs. Used in molecular diagnostics and species determination. | [25] |

(continued)

**Table 1**
**(continued)**

| PCR Technique | Principle and Application | References |
|---|---|---|
| PCR-Reverse Dot Blot (PCR-RDB) | Genotyping polymorphisms and identifying heterogeneity in genes by amplification of target DNA, hybridization of the biotinylated amplicon to oligonucleotide probes immobilized on a membrane, followed by color development using streptavidin-conjugated alkaline phosphate. | [26] |
| Quantitative fluorescence-PCR (QF-PCR) | Determination of aneuploidy associated with autosomes. | [27] |
| PCR-melting profile (PCR-MP) | Used for distinguishing different lineages or varieties within a species by doing qPCR followed by melting curve analysis. | [28] |
| Nested-PCR | Used for increasing sensitivity and specificity of PCR requiring two sets of primers for amplifying low-abundant cDNA. | [29] |
| Arbitrary primer sequence-based PCR (AP-PCR) | Used in conjunction with pulsed field gel electrophoresis for molecular typing of strains causing nosocomial outbreaks. | [30] |
| PCR-based denaturing high-performance liquid chromatography (PCR-DHPLC) | Species identification by DHPLC based on length and sequence of amplicons. | [31] |
| Degenerate-PCR | Degenerate-PCR primer towards target amplification and sequencing is a useful technique when a population of organisms under investigation is evolving rapidly or is highly diverse. | [32] |
| Reverse transcription-PCR (RT-PCR) | Used for gene expression analysis. | [33] |
| PCR-Enzyme linked immunosorbent assay (PCR-ELISA) | PCR products are biotinylated and captured on streptavidin-coated microtiter plates and detected by using an antidigoxigenin Fab-peroxidase conjugate. Used for diagnostic purpose. | [34] |
| Chromatin-immunoprecipitation-PCR (ChIP-PCR) | Determine and quantitate if the ChIP actually enriched the DNA sequences that are associated with the target protein. | [35, 36] |
| Polymerase chain reaction amplification of specific alleles (PASA) or allele-specific polymerase chain reaction (ASPCR) | Detection of point mutation. | [37] |

has been observed that ASP's allelic determination is often hampered by cross-hybridization between the defined genotypic ASP and the alternative allele's template giving rise to non-specific amplification. Therefore, to improve specificity, an artificial mismatch is introduced either in the penultimate (second last nucleotide) or antipenultimate (third last nucleotide) at the 3′ terminus of an ASP [40]. Single-nucleotide polymorphisms (SNPs) are a cause of various disorders and studied using restriction fragment length polymorphism (RFLP), high-resolution melting (HRM) and real-time PCR using hydrolysis probes. A study was conducted on 15 SNPs and 15 clinically relevant somatic mutations using double mismatch allele-specific primers (DMAS) for qPCR assay (DMAS-qPCR). DMAS primer included a mismatch at fourth nucleotide from the 3′ terminus and was used for qPCR assays [41]. It was concluded from this study that the artificial mismatch at fourth position enhanced the discriminatory power of the DMAS primer and was independent of the nucleotide type.

Although, it has been observed that inclusion of artificially mismatched nucleotides does enhance specificity and priming of ASPs, these cannot always accurately discriminate between different alleles, thereby leading to false-positive results. A study conducted to detect an oncogenic mutation, *BRAF* V600E in *B-raf* (BRAF) proto-oncogene, using Taqman-based qPCR assays devised two additional strategies besides introduction of mismatch in the penultimate nucleotide, to circumvent the nonspecific amplification against nonmatched alleles. The first strategy included a competitive external allele-specific controller (CEAC) to allele-specific (AS) -PCR (cAS-PCR) and the second approach included a referenced internal positive controller (RIPC), in the cAS-PCR (rcAS-PCR) [40]. The CEAC plasmid shares the same binding sequences as in ASP, satisfying the requirement for the thermodynamic driving force of DNA polymerase, thereby eliminating nonspecific amplification observed in AS-PCR. Whereas, RIPC, human leptin gene, in rcAS-PCR monitored the initial amount of input sample genomic DNA (gDNA) to avoid false-negative results [40]. Another study for detection of codon 600 mutation in *BRAF* kinase gene used sense and antisense allele-specific primers in conjunction with Taqman probe. Two Taqman probes specific to sense and antisense strands were used [42].

### 2.2 Enhancing Sensitivity of PASA-Based Approach

Molecular tests on DNA mutation detection have been used by clinical laboratories and physicians for better understanding of diseases in patients. Several probe-blocking methods have been introduced in real-time AS-PCR to block amplification of wild-type templates and to increase detection sensitivity and specificity for the mutant allele [43]. However, these methods have a limited sensitivity (no better than 1%) and are complex in the design of blockers, and thus cannot be readily adapted for different mutation

assays. The AS-nonextendable primer blocker (NEPB)-PCR method amplifies only mutant allele-specific DNA, whereas wild-type (*wt*) DNA is blocked from amplification by a modified NEPB. This method was tested for three mutations in cancer; *K-Ras*, *B-Raf*, and *EGFR*, and with three different types of modified blockers (phosphate, inverted dT, and amino-C7), resulting in a detection limit of 0.1% [42].

### 2.3 Web-Based Applications for Primer Design

Various softwares are available for designing primers with greater accuracy including http://bioinfo.biotec.or.th/WASP, www.primerxl.org, Oligo Primer Analysis Software v7 from Molecular Biology Insights (Cascade, CO), PRIMER 3 software (http://frodo.wi.mit.edu/primer3).

## 3 Advances in Detection Techniques of PASA

Traditionally, qualitative detection of PASA products has been done by size separation using agarose gel electrophoresis. However, newer techniques have been used to reduce cost, time, and sensitivity of detection.

### 3.1 Increased Sensitivity of Detection

In a study to detect point mutation in *K-Ras* gene using allele-specific PCR, a gold nanoparticle (AuNP)-DNA tag was covalently attached to the 5′-end of each primer by a nine-carbon linker to produce a sticky end. Therefore, one of the sticky ends of the PCR products was bound to gold nanoparticles, while the other sticky end was captured onto a nitrocellulose membrane of lateral flow strips. The lateral flow strip showed a great sensitivity, which detected mutations in as low as 10 tumor cells [43].

### 3.2 Reduced Cost and Time

Allele-specific amplification, combined with TaqMan probe-based real-time polymerase chain reaction (real-time AS-PCR) has been widely used for detecting genetic variants, single-nucleotide polymorphisms, and genetic mutations. Use of TaqMan probe-based methods makes detection process easy and eliminates step involving agarose gel electrophoresis [42].

A melting curve-based allele-specific PCR method was developed to genotype two single-nucleotide polymorphisms (SNPs) of Apolipoprotein E (APOE) locus. The method involved the PCR Tm shift method. In this method, two allele-specific forward PCR primers were tagged with two different lengths of GC tail, respectively which generated two allele-specific PCR amplicons with different sizes. As melting temperature of PCR amplicon is size-dependent the two different sizes of allele-specific amplicons resulted in two distinct melting temperatures in dissociation curve analysis. Hence, this method has a high resolution in the determination of the SNP genotype of APOE. Further experiments showed that DNA dissolved from blood collected on Guthrie filter paper

and total blood cell lysate without DNA extraction can be used in the melting curve-based allele-specific PCR method. Thus, it was suggested to be fast, accurate, and robust APOE genotyping method with a flexible throughput and suitable for DNA template from different preparations [44].

## 4 Cohort PASA, a Case Study in *C. elegans*

In a laboratory culture, *C. elegans* majorly exists as a hermaphrodite; however to study function of genes and interaction between genes, males are artificially induced and crosses are performed with worms harboring different mutations. Graphic of the workflow of a typical cross involving two mutations to generate a double mutant is depicted in Fig. 1. Briefly, male and female worms (P0) were put together on a mating plate. Individual F1 progenies obtained from the cross were placed onto separate fresh nematode growth medium (NGM)-containing plates and allowed to lay eggs comprising F2 generation. While all the F1 progenies exhibited a wild-type phenotype because they were heterozygous for the mutations, after undergoing self-fertilization and segregation produced F2's, which were either homozygous for the wild-type or mutant allele or heterozygous for one or both mutations. Genotyping using cohort PASA was performed on 8 gravid F2 worms picked from a single plate (Fig. 1). Once the plate homozygous for mutation was identified, individual worms were picked and transferred on to fresh plate, followed by second round of genotyping to confirm presence of the mutations (Fig. 1).

Cohort PASA data were analyzed from genetic crosses conducted in *C. elegans* for investigating signaling modules regulating the process of axon outgrowth and cell migration [45]. The study provided supporting data for genetic interaction between *unc-53* and rac GTPases; *ced-10* and *mig-2;* and the netrin receptor *unc-5* in regulating distal tip cell (DTC) migration [46]. Additionally, interaction between known regulators of cell migration and axon outgrowth process including *rpm-1* and *unc-73* with *unc-53* was also studied (A. Pandey et al., unpublished data). *C. elegans* is an excellent model organism to study gene mutation because generally mutation in a gene in *C. elegans* results in a phenotype such as the mutant *unc-53* has an Unc (*unc*oordinated) phenotype, therefore it is convenient to microscopically identify mutant worms. However, not all gene mutations result in a visible phenotype such as *mig-2* (*mu28*), *rpm-1*( *ju41*), *ced-10(n1993*) and *ced-10*(*n3246*) to name a few genes, underscoring the need for an alternative approach for isolating mutant worms after performing genetic crosses. The studies conducted in *C. elegans* show PASA as an excellent method for genotyping because it is convenient and sensitive for differentiating mutant and wild-type alleles in single and double mutant worms. Moreover, a single gravid worm containing 10–14 eggs,

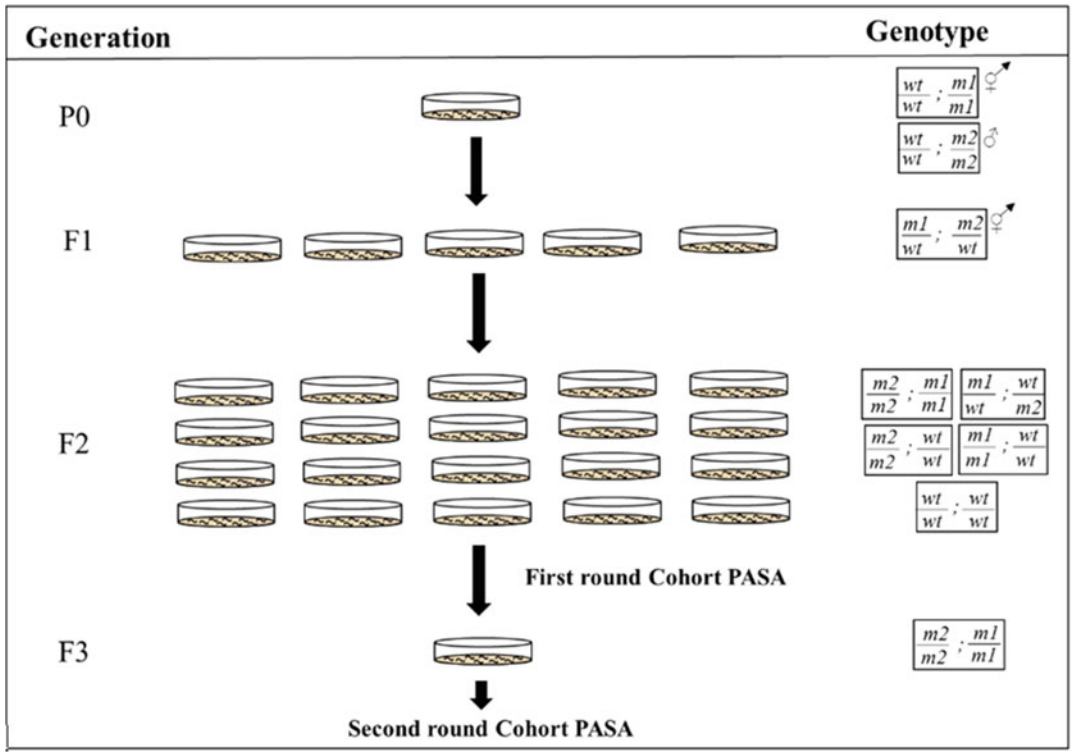


**Fig. 1** Work flow of cohort PASA: Male and hermaphrodite worms were crossed harboring mutations (*m1* and *m2*) representing the P0 generation. F1 progenies with heterozygous for mutation were picked and transferred to fresh separate NGM plates and allowed to lay eggs, which represented the F2 generation comprising worms homozygous for wild-type or mutant (*m1* or *m2*) alleles and heterozygous or one or both mutations. First round of cohort PASA was performed with gDNA extracted from eight worms of F2 generation picked from an individual plate until the plate homozygous for both mutation was identified. Solo worm from plate containing double mutant was transferred to fresh NGM plate and a second round of cohort PASA was performed to confirm the double mutant genotype

representing a cohort of gravid F2 progenies is used for gDNA extraction, subsequently a modified PASA called cohort PASA was performed. By using gravid F2 worms, this technique genotyped F2 and F3 generation in a single step. This book chapter is based on analysis of cohort PASA data obtained from genotyping of single and double mutants using allele-specific primers to detect point mutations. The study also provides useful insights for future development of sensitive and specific genotyping using allele-specific primers (ASPs). The present study is based on analysis of six mutations in genes including *rpm-1*, *ced-10*, *mig-2*, *unc-73*, and *unc-5*, sequences for the mutant (*m*) and wild-type (*wt*) alleles were obtained from WormBase (Fig. 2). A universal system of gene nomenclature followed in *C. elegans* will be adhered to in the chapter, which includes gene name in italics followed by mutation in the parenthesis, for example *ced-10*(*n1993*).

| | | | | |
|---|---|---|---|---|
| *ced-10* | TTGTTTGCTC | CAATTTCCCC | ATCTTAGAGC | ACCGTACACT |
| *ced-10(1993)* | TTGTTTGCTC | CAATTTCCCC | ATCTTAGAGC | CCCGTACACT |
| *ced-10* | TCTTCCTGTC | CAGCTGTATC | CCAGAGCCCG | AGATTTATCG |
| *ced-10(n3246)* | TCTTCCTGTC | TAGCTGTATC | CCAGAGCCCG | AGATTTATCG |
| *rpm-1* | GTTCAATAGG | TTGAATGCAC | AATGGACACT | GCATAAAACG |
| *rpm-1(ju41)* | GTTCAATAGG | TTGAATGCAC | AATGGACACT | ACATAAAACG |
| *mig-2* | TAGGATTGTG | GGATACTGCT | GGACAGGAGG | ATTATGATCG |
| *mig-2(mu28)* | TAGGATTGTG | AGATACTGCT | GGACAGGAGG | ATTATGATCG |
| *unc-5* | AACTCGAAGA | GCAAGCACTC | CAATCACTCC | ATGAACTCCA |
| *unc-5(e53)* | AACTCGAAGA | GCAAGCACTC | TAATCACTCC | ATGAACTCCA |
| *unc-73* | ATCCCGTTCG | GATTGAATAA | GCTCTCGCAT | TGGCTCAAGC |
| *unc-73(rh40)* | ATCCCGTTCG | AATTGAATAA | GCTCTCGCAT | TGGCTCAAGC |

**Fig. 2** Target sequences of the mutations analyzed by cohort PASA: Sequences of wild-type (*wt*) alleles including *ced-10, rpm-1, mig-2, unc-5, unc-73,* and mutant (*m*) allele including *ced-10*(*n1993*), *ced-10* (*n3246*), *rpm-1*( *ju41*), *mig-2*(*mu28*), *unc-5*(*e53*), *unc-73*(*rh40*) were obtained from WormBase. Bases undergone point mutation are highlighted in red and the region highlighted in blue shows the primer sequence. Forward primer sequence was identical to highlighted sequence, whereas the reverse primer sequence was complementary to highlighted sequence. All the primers included the mutation in the last base at the 3′ terminus

### *4.1 Mechanism Involved in Cohort PASA*

Since a DNA polymerase cannot synthesize a nascent DNA strand de novo, therefore all types of PCR use a short sequence of DNA complementary to the target sequence called the primer. Additionally, complementary base pairing and hydrogen bonding between the primer and target sequence at the 3′ terminus are absolutely essential for the polymerase activity because the new nucleotides are added at the 3′-OH of the primer. In a PASA technique when a mismatch is introduced at the antipenultimate base at 3′ terminus, the primer will form stable hydrogen bond only when the terminal and penultimate bases are complementary to the target (Fig. 3). However, if the terminal base and the antipenultimate bases are not complementary to target sequence, hydrogen bonding will not occur and polymerase activity will be stalled resulting in no subsequently amplification (Fig. 3).

### *4.2 Primer Design Strategy*

The optimal primer length was taken between 18 and 25 bp, providing adequate specificity to bind to the target sequence and anneal at required temperature. Shorter primer lengths are desired as they show higher binding specificity at required annealing temperature. Besides length, primer sequence also determined success of a cohort PASA reaction; in general sequences with either high A T or G C content should be avoided. Regions of the target sequence with repetitive sequences were avoided wherever possible during designing primers. GC clamp was observed, where out of the five bases present at the 3′ terminus not more than 3 bases were taken as G or C to enhance target binding. Specificity of the primers

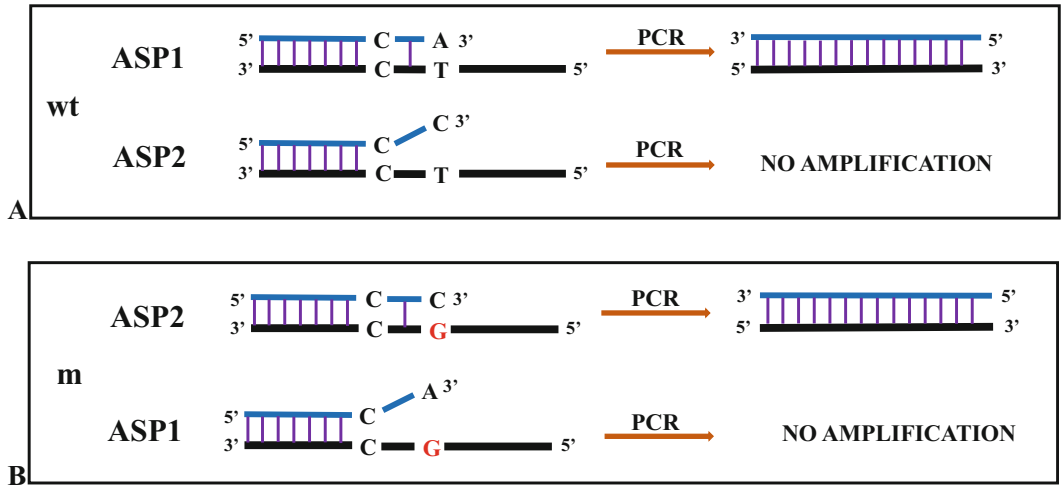


**Fig. 3** Mechanism of action of allele-specific primer: The primer with a mismatch in the antipenultimate base can only bind efficiently to target sequence, which shows complementary base pairing to terminal nucleotide, resulting in amplification. (**a**) Wild-type allele (*wt*) binds to the ASP1 primers specific to *wt* allele because of complementary base pairing at the 3′ terminal base and undergoes PCR amplification, whereas ASP2, the mutant allele (*m*)-specific primer does not bind to *wt* allele due to mismatch at two positions at the 3′ terminus attenuating amplification. (**b**) Mutant allele (*m*) undergoes complementary base pairing at the terminal base with ASP2 primers and undergoes PCR amplification, whereas the ASP1 primer does not bind to mutant allele due to mismatch at two positions and shows no PCR amplification

was analyzed using the following link https://wormbase.org/tools/blast_blat, to avoid secondary annealing leading to generation of secondary amplicons. All the primers were further analyzed by IDT OligoAnalyzer™ tool and NEB Tm calculator for assessing secondary structure formation and annealing temperature. For genotyping to isolate single and double mutants, three primers were designed for each mutant genotype including two primers each targeting the mutant and the wild-type allele, labeled as ASP2 and ASP1 and one common primer targeting the gene, labeled GSP (data not shown). A mismatch was introduced in the antipenultimate base (in parenthesis) of the primers specific to the wild-type and mutant allele (Table 2).

While introducing the mismatched bases, the hydrogen bonding capacity was not altered from that shown by the original base, for example Adenine was replaced with Thymine (both form two hydrogen bonds) and Cytosine was replaced with Guanine (both form three hydrogen bonds).

In general, the GC content ranged between 40% and 60% for stronger hydrogen bond formation, determining the overall stability of the primers. However, a high GC content can also result in formation of primer dimers hindering amplification. For this study percent GC content was taken as 50% and melting temperatures

**Table 2**
**Gene/allele investigated in the present study and genotyped using cohort PCR amplification of specific alleles**

| Gene/ Allele | Primer Name | Primer sequence | %GC | Tm | Size (bp) | Cal. Ann Tm | Exp. Ann. Tm |
|---|---|---|---|---|---|---|---|
| *ced-10* | ASP-1 | gctccaatttccccatcttaga(g/C)cA | 48 | 69°C | 440 | 66°C | 65°C |
| *ced-10* (*n1993*) | ASP-2 | gctccaatttccccatcttaga(g/C)cC | 52 | 70°C | | | |
| *ced-10* | ASP-1 | ctcgggctctgggatacag(c/G)tG | 64 | 72°C | 459 | 66°C | UD* |
| *ced-10* (*n3246*) | ASP-2 | ctcgggctctgggatacag(c/G)tA | 59 | 70°C | | | |
| *mig-2* | ASP-1 | atcctcctgtccagcagta(t/A)cC | 55 | 69°C | 150 | 66°C | 65°C |
| *mig-2* (*mu28*) | ASP-2 | atcctcctgtccagcagta(t/A)cT | 50 | 67°C | | | |
| *rpm-1* | ASP-1 | ggttgaatgcacaatggaca(c/G)tG | 48 | 67°C | 460 | 68°C | 64°C |
| *rpm-1* (*ju41*) | ASP-2 | ggttgaatgcacaatggaca(c/G)tA | 43 | 65°C | | | |
| *unc-5* | ASP-1 | tcgaagagaaagcac (t/A)cC | 50 | 62°C | 243 | 66°C | UD* |
| *unc-5* (*e53*) | ASP-2 | tcgaagagaaagcac (t/A)cT | 44 | 60°C | | | |
| *unc-73* | ASP-1 | ccaatgcgagagcttattca(a/T)tC | 43 | 64°C | 522 | 65°C | UD* |
| *unc-73* (*rh40*) | ASP-2 | ccaatgcgagagcttattca(a/T)tT | 39 | 63°C | | | |

[a]Undetermined

were kept at 65 °C and above. Interestingly, unlike PCR where the annealing temperature is generally 4–5 °C below the calculated annealing temperature, the annealing temperature for cohort PASA determined by a gradient cohort PASA was found to be around 65 °C, very close to the calculated Tm of the individual primers.

### 4.3 Genomic DNA Extraction

Genomic DNA was extracted using single worm lysis method [47]. Briefly, for each allele, wild-type (*wt*) and mutant (*m*), solo gravid worms were placed in each tube of a strip PCR tube containing 10μl worm lysis buffer. Worm lysis to release genomic DNA (gDNA) was carried out in a thermal cycler under following conditions; 60 °C for 60 min, 95 °C for 15 min. Thereafter, 1μl of the worm lysate was used for PCR using two sets of primers in a total reaction mixture of 20μl.

### 4.4 Determining Experimental Annealing Temperature for Cohort PASA

Genomic DNA from both the wild-type and mutant animals was genotyped using both sets of primer, i.e., primers specific to wild-type allele and mutant allele, respectively. Initially, a gradient cohort PASA was performed for all primer pairs taking the calculated annealing temperature as the midpoint of the gradient, as shown for *rpm-1* (Fig. 4). Qualitative assessment was performed by size separation of PCR products on a 0.8% agarose gel and imaged using gel documentation system. An amplicon of expected size was observed for *rpm-1* (Fig. 4) and *ced-10* and *mig-2* (data not shown). Moreover, the experimentally obtained annealing temperature was found to be independent of the length of amplicon

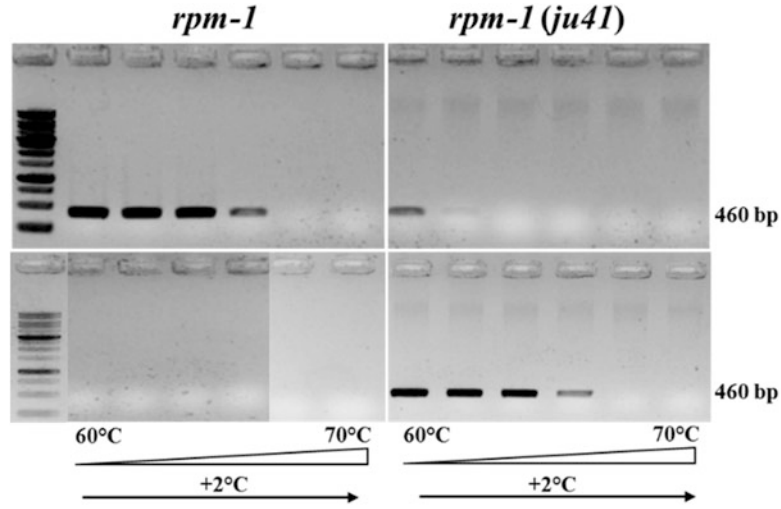


**Fig. 4** Gradient cohort PASA for detection of *rpm-1* and *rpm-1*(*ju41*) alleles: A gradient of 60 °C to 70 °C was performed with a rise of 2 °C for each reaction (from left to right). The cohort PASA products were size separated using agarose gel electrophoresis and the sizes were determined by using 1 kb DNA ladder as a reference. An experimental annealing temperature of 64 °C was used for subsequent studies

amplified for example *rpm-1*, *ced-10,* and *mig-2* had the same annealing temperatures even though the length of the amplicons is different (Table 2).

Interestingly, for the primers targeted to *unc-73*, wild-type and mutant alleles, the annealing temperature could not be determined by gradient cohort PASA. For *m* allele, an amplicon of expected size was observed only for ASP2; however ASP1 primers specific to *wt* allele amplified a nonspecific amplicon of similar size in both wild-type and *rh40* alleles (Fig. 5a), therefore these primer sets were not used for further genotyping analysis. These observations could be explained either due to a very high GC content or high Tm observed for ASP1 *unc-73* primers (Table 2). Similarly, while the ASP1 *ced-10* primers showed amplification in *wt* worm lysates, the ASP2 primers did not exhibit robust amplification for any of the temperatures used in the gradient for *n3246* worm lysates (Fig. 5b). Primer analysis revealed a high GC content and high Tm for both ASP1 and ASP2 primers, which could result in primer dimer formation, subsequently resulting in reduced or no amplification. Similarly, experimental annealing temperature could not be determined for *unc-5* (data not shown). *unc-5* primer analysis revealed a low Tm and less than 50% GC content, which could contribute to nonspecific amplification observed in the gradient cohort PASA (Data Not Shown).

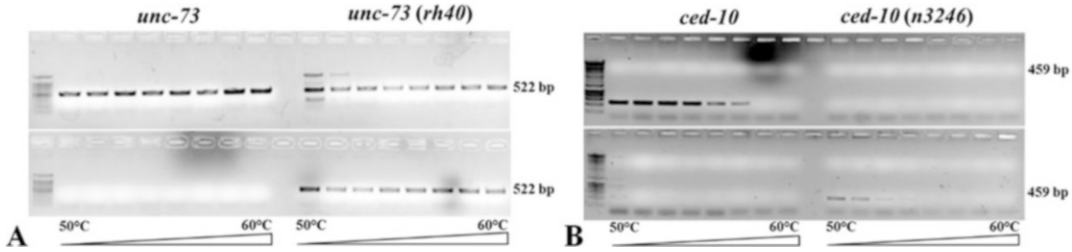


**Fig. 5** Gradient cohort PASA to determine annealing temperature for *unc-73* and *ced-10*, *wt* and *m* alleles: (**a**) A gradient cohort PASA was performed for wild-type and *rh40* alleles of *unc-73* followed by size separation of amplified products using agarose gel electrophoresis. The upper gel shows nonspecific amplification in mutant gDNA with ASP1 primers; however ASP2 primers showed amplification only for *m* gDNA (lower gel). (**b**) A gradient cohort PASA was performed for wild-type and *n3246* alleles of *ced-10* followed by size separation of amplicons using agarose gel electrophoresis. The upper gel shows specific amplification in *wt* gDNA with ASP1 primers, whereas the ASP2 primers did show robust amplification at any of the temperatures used for *m* gDNA (lower gel). 1 kb ladder was used as a reference to determine amplicon size

### *4.5 Differentiating Homozygotes from Heterozygotes*

Cohort PASA assays used in this study could differentiate between homozygotes and heterozygotes. While genotyping to obtain *ced-10*(*n1993*) worms, it was found that most of the animals used for the assay had both *wt* and *m* allele and were heterozygous (Fig. 6a). The ASP1 and ASP2 primers showed amplification in the gDNA extracted from 8 gravid worms picked from few of the plates during genotyping to isolate *ced-10*(*n1993*) mutant worms. Although, not all the 8 worms showed amplification with both sets of primers (Fig. 6a). Cohort PASA technique was successfully employed for isolation of double mutant worms (Fig. 6b). *ced-10*(*n1993*); *mig-2* (*mu28*) double mutants were isolated by performing cohort PASA using allele-specific primers. Five of the eight worms showed amplification with ASP2 primers targeted to *ced-10* and *mig-2* (Fig. 6b). Amplification was not observed for three worms probably because of inhibition of amplification due to various factors including quality and quantity of gDNA.

## 5 Conclusions

This study is based on parsing of cohort PASA data obtained from genotyping of various *C. elegans* mutations studied for their role in axon outgrowth and cell migration ([46]; A. Pandey et al., unpublished data). PASA also called AS-PCR is a technique particularly useful for identification of point mutations instead of using laborious techniques such as RFLP, SNP, and sequencing. Cohort PASA technique used for this study uses a cohort of worms composed of F2 gravid adult harboring F3 eggs to isolate single and double mutant worms. The technique was successfully employed for genotyping of single and double mutants and differentiation of

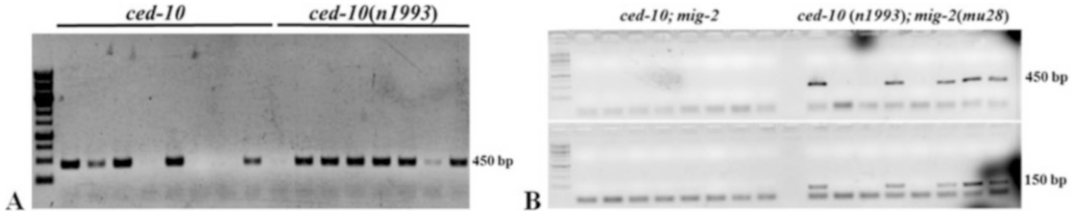


**Fig. 6** Differentiation of homozygotes and heterozygotes and isolation of double mutant worms using cohort PASA: (**a**) A gradient cohort PASA was performed on 8 gravid worms for wild-type and mutant (*n1993*) alleles of *ced-10* followed by size separation of amplicons using agarose gel electrophoresis. The first 8 wells show amplification with ASP1 primers specific to wild-type allele, whereas the following 8 wells show amplification with ASP2 primers specific to mutant allele. (**b**) A gradient cohort PASA was performed for isolation of *ced-10* (*n1993*) and *mig-2*(*mu28*) double mutant followed by size separation of amplicons using agarose gel electrophoresis. Upper gel shows amplification with *n1993*-specific primers only, whereas the lower gel shows amplification with *mu28*-specific primers only. No amplicon was observed with ASP1 primers targeted to *wt* allele of both genes. Size of the amplicons was determined using 1 kb ladder used as a reference

homozygous from heterozygous worms using agarose gel electrophoresis as an end point analysis. After analysis of the qualitative data, this study provides some useful insights, which can contribute to enhancing sensitivity and specificity and decreasing cost and time for genotyping of mutations using PASA technique. The study concludes that primers should be designed with a GC content of around 50%, supported by nonspecific amplification or no amplification observed for primer sets with lower and higher GC contents, respectively. Since the usually recommended primer length is 18–25 bp, which is also the length of primers used in this study, therefore the relationship between primer length and cohort PASA efficiency could not be assessed. Although, the hydrogen bonding capacity of the mismatched base was kept same as that observed for the original base, it will be interesting to investigate the effect of a mismatched base with altered hydrogen bonding capacity on cohort PASA efficiency. It was also observed if primers had repeat sequences of either single base or more, the amplification efficiency and specificity was affected, as observed for *ced-10*(*n3246*) and *unc-73*(*rh40*) specific primers, therefore primers with repeat sequences should be avoided. Moreover, the study supports to test primers for specificity for target gene using blast tool, which results in elimination of secondary amplicons. As a general rule, a GC clamp of 2–3 bases in terminal 5 bases at the 3′ end was observed to enhance stability of hydrogen bond formation at 3′ terminus, which is an absolute requirement for polymerase activity. In conclusion, cohort PASA is a convenient method for genotyping and its specificity and sensitivity are determined by the primers designed.

The study further proposes strategies to reduce time and cost and increase throughput efficiency of cohort PASA technique. The first approach could be to use SYBR green-based end point analysis system. SYBR green binds to dsDNA and the fluorescent signal

generated can be detected in a real-time machine thereby eliminating use of agarose gel electrophoresis to analyze results. qPCR can also be performed by using different sizes of amplicons for wild-type and mutant allele and performing melting curve analysis to identify the cohort, which is homozygous for the mutant allele. Colorimetric end point analysis can be also an approach eliminates laborious agarose gel electrophoresis step.

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