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Genetic Monitoring of Laboratory Rodents Using Single Nucleotide Polymorphisms

Zeenat Bano Abdul Rauf Khan[1], Hemant Vira[2] and Arvind Ingle[2,3*]

[1]St. Xavier's College, 5, Mahapalika Marg, Mumbai-400001, MH, India.
[2]Advanced Centre for Treatment, Research and Education in Cancer, Kharghar, Navi Mumbai-410210 (MH), India.
[3]Homi Bhabha National Institute, Training School Complex, Anushakti Nagar, Mumbai- 400085 (MH), India.

ABSTRACT

Maintaining the genetic integrity of laboratory rodents is essential for achieving reliable and reproducible results in biomedical experiments. Genetic variations among laboratory animals can lead to flawed results, highlighting the importance of rigorous quality control testing. Among various genetic markers, single-nucleotide polymorphisms (SNPs) are the most common and provide a dependable method for genetic monitoring due to their precision and stability. These markers support efficient, cost-effective, and high-throughput genotyping strategies. This study aims to standardize SNP detection as a genetic monitoring tool in laboratory rodents using the Amplification Refractory Mutation System (ARMS) PCR. Genomic DNA was isolated from ten mouse strains: A/J, BALB/c, C3H/J, CD-1, C57BL/6, DBA/2, FVB/NJ, Swiss Webster, Swiss/Bare, and BDF1. The DNA was obtained from tail tissue samples using the phenol–chloroform extraction technique, followed by quantification with a Nanodrop spectrophotometer. DNA quality was assessed through agarose gel electrophoresis. SNP markers were chosen based on polymorphisms from the Mouse Genome Informatics (MGI) database, and primers were designed using the PRIMER1 tool. We optimized PCR conditions by systematically varying parameters like DNA concentration, PCR cycles, annealing temperature, Taq mix concentration, and reaction volume. We achieved reproducible amplification for selected SNP markers through methodical optimization, with 10 of the 19 tested markers yielding clear allele distinction across strains. The ARMS PCR approach provided straightforward, reliable genotyping without requiring restriction enzyme digestion or complex data interpretation. Our findings demonstrate that ARMS PCR can be a cost-effective tool for genetic monitoring in laboratory colonies. Future work will involve sequencing amplified products to confirm allele assignments and validate the method's accuracy.

Keywords: Single-nucleotide polymorphism, genetic monitoring, laboratory rodent.

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INTRODUCTION

The importance of mouse models in biomedical research has steadily increased over time, as reflected by the number of animals used in research. Mice are utilized across various disciplines, such as genetics, physiology, drug development, and the modelling of human diseases. (Nitzki et al., 2007). Humans and mice, both mammals, share substantial biological similarities, including analogous organs and systems. Most mouse genes are homologous to human genes, facilitating the study of mice to gain insights into human development, disease progression, and aging. The

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[*] Corresponding author.
Arvind Ingle, Advanced Centre for Treatment, Research and Education in Cancer, Kharghar, Navi Mumbai-410210 (MH), India.
Email- aingle@actrec.gov.in

availability of numerous mouse strains enables precise disease modelling, with the mouse genome being easily manipulated for specific research needs. Over a century of research has provided an extensive understanding of mouse biology and genetics (Bryda, 2013).

As per the reports, rats and mice constitute 95 to 99.3% of animals used in research (Hickman et al., 2017; Carbone, 2021). Inbred mouse strains are widely preferred due to their capacity to generate large populations of genetically uniform animals, aside from rare spontaneous mutations. As per the guidelines provided by the International Committee on Standardized Genetic Nomenclature for Mice and the Rat Genome Nomenclature Committee, a strain is considered inbred after undergoing continuous breeding, typically through brother-sister or parent-offspring mating, for a minimum of 20 generations. At this point, individuals can be traced back to a standard ancestral pair and generally display less than 2% residual heterozygosity, classifying them as isogenic. It is generally accepted that 24 generations of sibling mating are needed to reduce heterozygosity below 1%, while around 36 generations are necessary to attain complete genetic uniformity (Benavides et al., 2020).

Maintaining genetic quality, which involves monitoring inbred strains and verifying the genetic background of genetically modified animal models, is a key component of quality assurance in laboratory animal research. It is equally important as health and microbiological surveillance for validating the reliability of animal models. In the case of mice and other commonly used laboratory species, regular assessment of genetic background is necessary to preserve strain purity and consistency. Such monitoring ensures that strain characteristics remain well-defined and contributes significantly to achieving consistent and reproducible experimental outcomes across different research settings and over time (Benavides et al., 2020). Therefore, it is important that research involving laboratory rodents, especially mice and rats, is conducted using animals with well-characterized genetic backgrounds. During breeding, special care must be taken to reduce risks such as genetic drift, unexpected mutations, and accidental mixing with other strains, as these factors can compromise the reproducibility of experimental outcomes (Benavides et al., 2020). Although advanced molecular methods are available for detecting genetic contamination, they cannot identify genetic drift, where spontaneous mutations become permanently established within an inbred population. To control genetic drift, replacing the breeding stock with animals from a recognized commercial source is generally suggested after every 10 generations. This approach helps avoid the formation of genetically divergent sublines. Many breeding facilities achieve this by using frozen embryo stocks, which significantly limits genetic drift. Additionally, cryopreservation is recommended for distinct or genetically engineered strains to safeguard them from drift and potential loss due to contamination or other unforeseen events (Thorat & Ingle, 2012; Thorat et al., 2013). These strains may also be stored as embryos or sperm and reintroduced approximately every ten generations. Regular genetic monitoring is essential for the effective management of animal colonies. Although proper animal care and strong husbandry practices help minimize the risk of strain contamination, incorporating a genetic monitoring system provides an added layer of verification, particularly in facilities housing multiple mice or rats with similar coat colors, to promptly identify any cases of genetic mix-ups (Benavides et al., 2020).

Consistent genetic monitoring establishes unique genetic profiles for each strain, aiding in detecting potential genomic aberrations in inbred animal colonies (Naseem et al., 2022). Historically, this monitoring was carried out using biochemical or immunological markers and assays, which were known to be time-consuming (Bryda & Riley, 2008). Various approaches are used for genetic monitoring, from assessments based on observable traits to advanced DNA-based methods. Phenotypic evaluations typically include examining physical characteristics, skeletal measurements, reproductive traits, skin grafting outcomes, and protein content, often with the help of PCR. In contrast, DNA-based strategies rely on genetic markers to track and assess genetic integrity (Naseem et al., 2022). Molecular markers are powerful tools for studying genetic differences and are frequently used to link phenotypic traits to underlying genetic variations (Kalia et al., 2011). These markers are specific DNA fragments with known positions within the genome found in all individuals of a particular species or strain. Molecular methods can identify changes in the nucleotide sequence within these regions. A range of polymorphism functions as genetic markers, including single-nucleotide polymorphisms (SNPs), simple sequence length polymorphisms (SSLPs), as well as minisatellites (also known as variable number of tandem repeats or VNTRs), and microsatellites (short tandem repeats or STRs) (Antony et al., 2022). Single-nucleotide polymorphisms (SNPs) present in mice are genetic variations involving a change in a single nucleotide. These variations are widespread across the genome, though their occurrence may not be completely random. Typically, SNPs are biallelic, indicating that each marker generally appears in two allele forms (Bryda & Riley, 2008). SNPs are widely available, functionally significant, exhibit low mutation rates, and allow swift and highly automated genotyping (Liu et al., 2005). Advanced genotyping on a large scale is primarily performed using techniques such as TaqMan assays, DNA microarrays, MALDI-TOF mass spectrometry, and pyrosequencing. Despite their effectiveness, these approaches often demand costly instruments and specialized reagents, which can become a limiting factor (Ruan and Camila, 2024).

Establishing standardized PCR procedures is essential for achieving consistent and reproducible outcomes across multiple experiments and research facilities. However, ensuring such consistency is often difficult due to materials, equipment, and user handling differences. This study seeks to develop a dependable and affordable SNP genotyping strategy tailored to our facility's laboratory rodents' needs. Most currently used SNP genotyping methods are built upon PCR, but they usually require post-PCR steps like using radioactive labels, restriction enzymes, or conducting multiple amplification rounds. These extra processes tend to raise both time and financial costs. Although PCR–RFLP has yielded significant genetic data, it is generally slower and restricted to variants that affect specific enzyme recognition sites.

To support progress in both fundamental research and clinical studies, it is vital to develop genotyping techniques that are more accessible. Innovations that enhance speed, affordability, and overall efficiency are key. ARMS-PCR (Allele-Specific PCR) meets these modern genomic research needs, as it overcomes many limitations of older methods and allows for rapid, reliable, and cost-effective SNP analysis (Ruan & Camila, 2024).

In this study, ARMS-PCR was chosen as the preferred method. This technique is based on allele-specific amplification, where primers are designed with intentional mismatches at their 3′ ends to selectively bind to a particular SNP allele while avoiding amplification of the alternative allele. DNA polymerase will initiate extension only when the primer's 3′ end aligns perfectly with the template sequence, ensuring that amplification occurs only when the sequence match is exact. This allows the generation of a specific PCR product, enabling precise genotyping of the target DNA based on the presence or absence of the expected amplicon. This technique, employing the two-tube format, offers significant advantages regarding reagent economy and simplicity, requiring two reactions-one for each SNP allele (Petkov et al., 2004). Additionally, the results can be easily visualized using standard PCR followed by gel electrophoresis, eliminating the need for high-end equipment (Ruan & Camila, 2024). Nonetheless, refining the method can demand considerable manual effort and may take a substantial amount of time. By focusing on this method, we aim to establish a standardized protocol that can be widely adopted for genetic monitoring in laboratory rodents at ACTREC, ensuring consistent and accurate results.

MATERIAL AND METHODS

Sample Collection and DNA Extraction

Two animals were selected at random from each of the following strains: A/J, BALB/c, C3H/J, CD-1, C57BL/6, DBA/2, FVB/NJ, Swiss Webster, Swiss/Bare, and BDF1, as part of a genetic monitoring quality control process. They were euthanized humanely using a carbon dioxide chamber. Tail samples were collected under aseptic conditions and transferred into sterile 1.5 ml Eppendorf tubes for subsequent DNA isolation. Genomic DNA was isolated from tail tissues using the Proteinase K–phenol–chloroform protocol, resulting in samples with high purity suitable for analysis. DNA quantification was conducted with the ND-1000 Spectrophotometer, assessing concentration and purity via the 260/280 nm absorbance ratio. To verify DNA quality, 5 µl from each preparation was loaded onto a 1% agarose gel containing ethidium bromide and electrophoresed at 120V for 30 minutes. Band visualization was performed under UV light. After integrity confirmation, each DNA sample was diluted to 100 ng/µl in 1X TE buffer, ensuring standardized concentration for further experiments.

SNP Marker Selection and Preparation

Genetic markers were chosen using the Mouse Genome Informatics resource (http://www.informatics.jax.org/marker) with BALB/c as the reference strain, ensuring coverage across all chromosomes (**Table 1**: Critical Subset of SNP Markers). Markers showing polymorphism between the strains were selected, with preference for markers that could differentiate more than one strain from the others. Flanking sequences were obtained from the Ensembl Genome Browser (https://www.ensembl.org/Mus_musculus/Info/Index), and forward primers were created using the PRIMER1 software tool (http://primer1.soton.ac.uk/primer1.html). Reverse primers were generated by obtaining the reverse complement (https://www.bioinformatics.org/sms/rev_comp.html) of the selected sequences. MilliporeSigma synthesized primers, desalted, and prepared at an initial concentration of 0.03 µM (OD 0.025). Working primer solutions were made by diluting the stock 1:10 with 1X TE buffer, resulting in a final concentration of 0.003 µM for PCR reactions. **Table 2. A and B** provides detailed information on the primers designed for each SNP across all 19 chromosomes.

Table 1: Critical Subset for SNP Markers.

Chromosome No.	**RS ID**	**Map Position**	**Gene involved**	**SNP**	**Product size (bp)**	**BALB/c**	**A/J**	**C3H/J**	**C57BL/6**	**DBA/2**	**FVB/NJ**
1	rs3022801	Chr1: 56874303	Satb2	A/G	220	G	A	G	G	G	A
2	rs3022883	Chr2: 37641262	Intergenic	A/C	226	A	C	C	A	C	C
3	rs3022960	Chr3: 65237234	Kcnab1	G/A	223	G	A	A	A	G	G
4	rs3023026	Chr4: 141089362	Gm13076	A/G	227	A	A	A	A	G	G
5	rs3023040	Chr5: 32824140	Yes1	A/G	191	A	A	A	G	G	A
6	rs3023064	Chr6: 17723651	St7	G/T	202	T	T	T	G	T	T
7	rs3023148	Chr7: 90803415	Dlg2	C/T	174	C	C	C	T	C	C
8	rs3023176	Chr8: 14809093	Dlgap2	A/G	203	G	G	G	A	G	G
9	rs3023202	Chr9: 13349912	Maml2	A/G	177	G	G	G	A	G	A
10	rs3023233	Chr10: 28472043	Ptprk	A/G	200	G	G	A	G	A	A
11	rs3023249	Chr11: 11121143	Vwc2	G/T	176	G	G	G	T	G	G
12	rs3023339	Chr12: 24854819	2900060N12Rik	A/C	224	C	C	C	A	C	C
13	rs3023379	Chr13: 18471247	Pou6f2	A/G	168	G	G	G	A	G	G
14	rs3023409	Chr14: 50064173	Gm8417	A/G	266	A	A	A	G	A	G
15	rs3023415	Chr15: 10820589	Gm19276	A/G	174	G	A	G	G	G	A
16	rs3023432	Chr16: 10558776	Clec16a	A/T	141	A	A	T	A	T	A
17	rs3022791	Chr17: 72316195	Alk	C/T	200	C	C	T	T	T	T
18	rs3023470	Chr18: 70000392	Intergenic	A/C	209	C	A	A	A	A	A
19	rs3023481	Chr19: 20395311	1500015L24Rik	G/A	235	A	A	G	G	G	A

{**Source:** Mouse Genome Informatics (http://www.informatics.jax.org)}

Table 2. A: Primer Sequences for Successfully Standardized SNP Markers (Chromosomes: 2, 3, 4, 5, 6, 10, 11, 13, 18, and 19)

Chr. No.	SNP ID		Primer sequences 5'<-----Sequence----->3'
2	rs3022883	rs3022883C	TATTCAGAGAGTTAATTTCCTCTGCAAAC
		rs3022883A	TATTCAGAGAGTTAATTTCCTCTGCAAAA
		rs3022883R	GCTATAACTCCATCCTGTTTCTGGGTCT
3	rs3022960	rs3022960C	CAGAAAAACGGAAGAATGAACACTTGAGAC
		rs3022960T	CAGAAAAACGGAAGAATGAACACTTGAGAT
		rs3022960R	AAAACAAGACATCCAAATCCATAAGGGC
4	rs3023026	rs3023026A	CGTCTGCCACACCTCCACTATTATAAATCA
		rs3023026G	CGTCTGCCACACCTCCACTATTATAAATCG
		rs3023026R	TTCTCCAAACTTCCTGTAAGATGCTCAC

5	rs3023040	rs302340A	CCAGCTCACCTTTAAAACTGTGGTATA
		rs302340G	CCAGCTCACCTTTAAAACTGTGGTATG
		rs302340R	CAAGAAAAACTACCAGTGTCAGAAATGT
6	rs3023064	rs3023064G	TTCTAAGATATTCAGTCTCATTATCATTAG
		rs3023064T	TTCTAAGATATTCAGTCTCATTATCATTAT
		rs3023064R	TTTGTCTCTTTCTCTATGTGTCTGTATG
10	rs3023233	rs3023233G	AAAAAGGACTTGATCAGTTTCTGTGAATG
		rs3023233A	AAAAAGGACTTGATCAGTTTCTGTGAATA
		rs3023233R	AAGAGAGGGAAATGCCACTTACAAAGTG
11	rs3023249	rs3023249G	GCCCTTTTGACCTTGCTGAGAACCAG
		rs3023249T	GCCCTTTTGACCTTGCTGAGAACCAT
		rs3023249R	AGGTGCTGAGGACAGTCAGCTGCATAAG
13	rs3023379	rs3023379A	ATTTATCTCACTGTGAAGTCTGCCGAA
		rs3023379G	ATTTATCTCACTGTGAAGTCTGCCGAG
		rs3023379R	CACACATCGAGTAAGTGTGAAAACAGTG
18	rs3023470	rs3023470A	TTGACTTGCTGTCTGTTAGTCAGTCCA
		rs3023470C	TTGACTTGCTGTCTGTTAGTCAGTCCC
		rs3023470R	TATGAATGAACCCATTTGCAGACATAAG
19	rs302381	rs3023481G	AGGAGACTATATATCTACTTTATGTGTAG
		rs3023481C	AGGAGACTATATATCTACTTTATGTGTAA
		rs3023481R	AGATAGATAACTATATTTAACAAGCTCC

Table 2. B: Primer Sequences for SNP Markers That Could Not Be Standardized.
(Chromosomes: 1, 7, 8, 9, 12, 14, 15, 16, and 17)

Chr. No	SNP ID		Primer sequences 5'<-----Sequence----->3'
1	rs3022801	rs3022801A	CAGAAAAACGGAAGAATGAACACTTGAGAA
		rs3022801G	CAGAAAAACGGAAGAATGAACACTTGAGAG
		rs3022801R	ACAAGACATCCAAATCCATAAGGGCACT
7	rs3023148	rs3023148C	ACAGGCATCCATTGTGATTCATCATAAC
		rs3023148T	ACAGGCATCCATTGTGATTCATCATAAT
		rs3023148R	AACATATTTCCCATATATGTGCTGGCCT
8	rs3023176	rs3023176A	TCTCACCCTGTAGCCTTTCTAGTGCA
		rs3023176G	TCTCACCCTGTAGCCTTTCTAGTGCG
		rs3023176R	ACATGACAGTCTGACAGTGACTGTGGAA
9	rs3023202	rs3023202A	AACTTTTAGGATTAAATGAGAAACGA
		rs3023202G	AACTTTTAGGATTAAATGAGAAACGG
		rs3023202R	AATGACAAACTTTTAGTACTTTTAGGGT
12	rs3023339	rs3023339A	CAGAAAGGTCCGGCTACATCTCA
		rs3023339C	CAGAAAGGTCCGGCTACATCTCC
		rs3023339R	TAAGTTTGGCTGAAGTCTTCAGACACAA
14	rs3023409	rs3023409A	GTGATATGTGGAAGTTATATAAGCTGCA
		rs3023409G	GTGATATGTGGAAGTTATATAAGCTGCG
		rs3023409R	TCAGTTGTGCCTAATGTTCACATACTTA

15	rs3023415	rs3023415A	ACTGCTCATTGGAACAAGTGAAATCA
		rs3023415G	ACTGCTCATTGGAACAAGTGAAATCG
		rs3023415R	ATAAACTTTCCTGGAGTGGTTATGAGCA
16	rs3023432	rs3023432A	ATCATTGAAAAGCAAGTGGCCTGTAA
		rs3023432T	ATCATTGAAAAGCAAGTGGCCTGTAT
		rs3023432R	CCTCCACCCAATGTGGAATTTTTAGTAT
17	rs3022791	rs3022791C	AAGGTCACGCATGTAGGATGGCGACCC
		rs3022791T	AAGGTCACGCATGTAGGATGGCGACCT
		rs3022791R	AGGGGCGATATTTGCCTGTCACAAGTTA

Optimization of PCR Conditions for SNP Marker Amplification

Optimal PCR conditions for each SNP marker were determined through a systematic optimization process, beginning with gradient PCR to estimate suitable annealing temperatures. Due to non-specific amplification with specific markers, additional parameters were further adjusted, including DNA template concentration, Taq polymerase mix concentration, reaction volume, annealing temperature, and cycle number. This multifactorial approach enabled specific and reproducible amplification for each SNP marker. Initial standardization was performed on smaller gels using DNA from 2-3 strains to optimize PCR conditions. Once the optimal conditions were achieved, the whole panel was applied as shown in the Results section. Statistical validation (e.g., coefficient of variation or repeatability) was not conducted for this phase, as the focus was on optimizing conditions and minimizing resource usage before running the complete panel.

Reaction Volume and PCR Program for Final Panel

Various volumes were tested to determine the optimal reaction volume, but no significant differences were observed. Consequently, a consistent reaction volume of 13 µl was used for all PCR assays, which helped reduce each reaction's overall cost. For these reactions, 1 µl of DNA sample (containing 100 ng of DNA) was added to each PCR tube for some markers, while 1.5 µl was used for others, depending on marker-specific needs. A reaction mixture was assembled using a 2X Taq polymerase mix and nuclease-free water obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai. This mixture also included a primer pair consisting of an allele-specific forward primer and a universal reverse primer. Each PCR tube was filled with 11 µl of this master mix to achieve a final reaction volume of 13 µl. The thermal cycler was programmed as outlined in Table 3 to carry out the PCR reactions.

Table 3: Thermal cycling conditions for ARMS PCR reactions. Annealing temperature and number of cycles were optimized and thus varied for each marker.

Cycle no.	Process	Temperature	Time
Cycle 1,	Initial denaturation,	94°C,	5 mins.,
Cycle 2,	Denaturation,	94°C,	20 mins.,
Cycle 3,	Annealing,	Temp. varied for each marker,	30 mins.,
Cycle 4,	Extension,	72°C,	20 mins.,
Back to cycle 2x No. of cycles, (No. of cycles varied for each marker)			
Cycle 5,	Final extension,	72°C,	3 mins.,
Cycle 6,	Hold.	4°C.	Infinite time.

Out of the total PCR reaction mixture of 13 µL, 10 µL was loaded onto a 2% gel made with agarose. The gel was cast and electrophoresed using 1X TAE buffer. The electrophoresis was carried out at 120 V for 40 minutes to 1 hour, depending on the number of PCR cycles optimized for each specific marker.

RESULTS

In this study, we attempted the amplification of 19 SNP markers located across all 19 autosomes (**Table 1**) in laboratory mice using the ARMS (Amplification Refractory Mutation System) technique. Of these markers, 10 were successfully standardized (**Table 4. A & B**), producing reproducible amplification across various inbred mice strains maintained at the Laboratory Animal Facility, ACTREC (Figs. 1 and 2). Successful amplification was achieved for most markers, though several markers exhibited variability in amplification. Specifically, markers with high GC content (e.g., rs3023148 on chromosome 7 and rs3022791 on chromosome 17) showed poor amplification, likely due to the challenges associated with GC-rich regions. In contrast, rs3022801 on chromosome 1 and rs3023202 on chromosome 9 failed to amplify in all samples. Additionally, discrepancies were observed for marker rs3023176 on chromosome 8, where strain C57BL/6 showed unexpected band patterns, while CD-1 failed to show any amplification.

The loading pattern for all gels (**Figs. 1 and 2)** was as follows: Each sample is represented by two lanes corresponding to two different alleles. The numbering corresponds to specific rodent strains, with two samples per strain as shown below. A 100 bp DNA ladder and 'no template controls' (-ve) for each gel are included. The product size for each SNP marker is indicated above the corresponding band in the gel images (**Figs. 1 and 2**).

Loading pattern:

Sample 1 & 2: A/J, Sample 3 & 4: BALB/c, Sample 5 & 6: C3H/J, Sample 7 & 8: CD-1, Sample 9 & 10: C57BL/6, Sample 11 & 12: DBA/2, Samples 13 & 14: FVB/NJ, Sample 15 & 16: Swiss Webster, Samples 17 & 18: Swiss/ Bare, Sample 19 & 20: BDF1

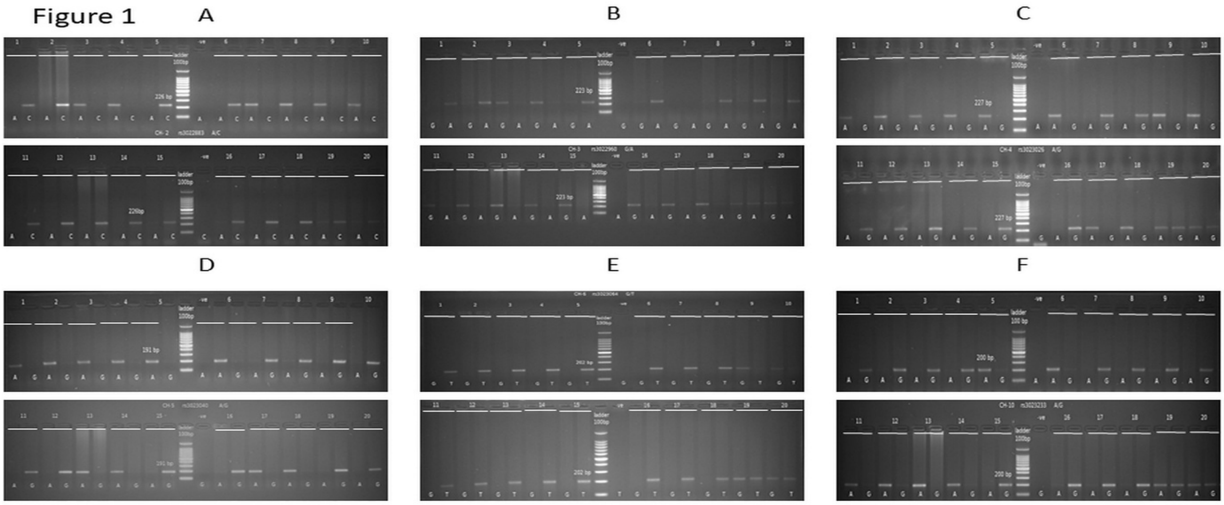

Figure 1 A: Genotyping results for SNP on Chromosome 2, Marker rs3022883, Alleles A/C.
Figure B: Genotyping results for SNP on Chromosome 3, Marker rs3022960, Alleles G/A.
Figure C: Genotyping results for SNP on Chromosome 4, Marker rs3023026, Alleles A/G
Figure D: Genotyping results for SNP on Chromosome 5, Marker rs3023040, Alleles A/G.
Figure E: Genotyping results for SNP on Chromosome 6, Marker rs3023064, Alleles G/T.
Figure F: Genotyping results for SNP on Chromosome 10, Marker rs3023233, Alleles A/G.

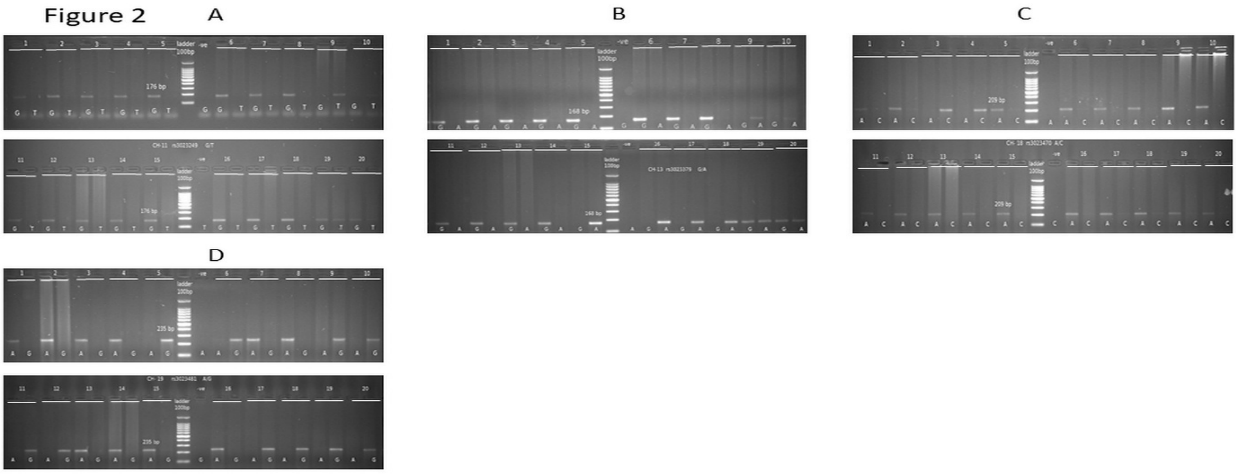

Figure 2A: Genotyping results for SNP on Chromosome 11, Marker rs3023249, Alleles G/T.
Figure B: Genotyping results for SNP on Chromosome 13, Marker rs3023379, Alleles G/A.
Figure C: Genotyping results for SNP on Chromosome 18, Marker rs3023470, Alleles A/C.
Figure D: Genotyping results for SNP on Chromosome 19, Marker rs3023481, Alleles A/C.

Table 4. A: SNP Results for a Panel of 10 Markers under Standardized Conditions.

Chromosome No.	SNP ID	Alleles	Product size (bp)	A/J	BALB/c	C3H/J	CD-1	C57BL/6	DBA/2	FVB/NJ	Swiss Webster	Swiss/Bare	BDF1
2	rs3022883	A/C	226	C	A	C	*A	A	C	C	*C	*C	BOTH
3	rs3022960	G/A	223	A	G	A	*A	A	G	G	*G	*G	BOTH
4	rs3023026	A/G	227	A	A	A	*G	A	G	G	*G	*A	BOTH
5	rs3023040	A/G	191	A	A	A	*G	G	G	A	*G	*A	G
6	rs3023064	G/T	202	T	T	T	*T	G	T	T	*T	*T	BOTH
10	rs3023233	A/G	200	G	G	A	*G	G	A	A	*G	*G	BOTH
11	rs3023249	G/T	176	G	G	G	*G	T	G	G	*G	*G	BOTH
13	rs3023379	A/G	168	G	G	G	*G	A	G	G	*A	*A	BOTH
18	rs3023470	A/C	209	A	C	A	*A	A	A	A	*A	*A	A
19	rs3023481	G/A	235	A	A	G	*A	G	G	A	*A	*G	G

Table 4. B: Optimized PCR Conditions for Successfully Standardized SNP Markers.

Chromosome No.	**SNP ID**	**Alleles**	**Product size(bp)**	**Template DNA**	**Annealing Temp**	**No. of Cycles**
2	rs3022883	A/C	226	100 ng	60ºC	25
3	rs3022960	G/A	223	100 ng	62ºC	22
4	rs3023026	A/G	227	100 ng	66ºC	24
5	rs3023040	A/G	191	100 ng	65ºC	24
6	rs3023064	G/T	202	150 ng	62ºC	34
10	rs3023233	A/G	200	100 ng	61ºC	26
11	rs3023249	G/T	176	100 ng	71ºC	24
13	rs3023379	A/G	168	150 ng	67ºC	34
18	rs3023470	A/C	209	100 ng	66.5ºC	23
19	rs3023481	G/A	235	100 ng	60ºC	25

DISCUSSION

Genetic variation in laboratory animals can lead to flawed experimental outcomes, potentially resulting in the wasted use of animals. To address this, maintaining genetic consistency is crucial for reducing the number of animals required in scientific research. This approach aligns with the ‘Reduction’ principle of the 3Rs (Replacement, Reduction, and Refinement) first introduced by Russell and Burch in 1959. Nonetheless, occasional breeding errors may modify the genetic characteristics of a strain, highlighting the need for stringent control over the breeding of different mouse strains. Ensuring the genetic integrity of research animals while keeping costs manageable has become increasingly critical in recent years (Cui et al, 2012).

Traditional genetic quality control methods, reliant on physical and behavioral traits like biochemical markers and histocompatibility haplotyping, often suffer from reduced accuracy, environmental sensitivity, and lack of coverage of all genetic loci. DNA molecular markers, particularly SNP genotyping, offer significant advantages (Cui et al., 2012). They provide precise, stable, and minimally invasive genetic monitoring, with broader applicability and faster processing than traditional methods. The ability of SNP genotyping to detect a wide range of genetic variations makes it a superior tool for ensuring genetic purity and distinguishing between inbred strains (Cui et al., 2012). Numerous SNP genotyping methods exist, with new techniques continually emerging to reduce costs and enhance throughput (Kalendar et al., 2022).

We have employed the PCR (polymerase chain reaction) technique to identify single-nucleotide polymorphisms (SNPs) as part of a genetic monitoring approach in laboratory rodents.

The ARMS (Amplification Refractory Mutation System) technique in this study allowed for efficient genotyping through the direct visualization of PCR products using agarose gel electrophoresis. This method is simple, consistent, and does not require radioactive labelling, eliminating the need for enzymatic digestion, allele-specific probes, or conventional sequencing of PCR amplicons (Newton et al., 1989).

ARMS-PCR offers several advantages over other commonly used SNP genotyping methods, such as TaqMan and pyrosequencing. One of the key benefits of ARMS-PCR is its cost-effectiveness, as it relies on standard PCR reagents and does not require expensive probes or specialized equipment, unlike TaqMan assays, which involve probes and fluorescent reporters. Regarding sensitivity, ARMS-PCR is effective for detecting SNP variations, but it provides qualitative results based on gel electrophoresis, whereas TaqMan assays offer higher sensitivity with quantitative capabilities. Pyrosequencing provides high sensitivity and precision but requires more complex and costly equipment, making it less accessible for laboratories with limited resources. Regarding high throughput, ARMS-PCR allows for efficient screening of multiple markers. However, methods like TaqMan and pyrosequencing may be better suited for large-scale, high-throughput applications due to their automation potential. Overall, while ARMS-PCR may not match the high throughput or quantitative capabilities of TaqMan or pyrosequencing, it remains a reliable, affordable, and efficient choice for SNP genotyping, especially in studies where cost, simplicity, and moderate throughput are priorities (Ruan & Camila, 2024). First, we selected one SNP marker per autosome, strategically positioned across all 19 autosomes (**Table 1**). This approach ensures comprehensive genome coverage and precise detection. This approach was implemented to monitor the genotypes of 10 different inbred mouse strains maintained at the Laboratory Animal Facility, ACTREC (**Table 4. A**).

We systematically varied key parameters to optimize the PCR conditions, including template DNA concentration, PCR cycle number, annealing temperature, Taq mix concentration, reaction volume, and run duration (**Table 3**). This methodical optimization identified the optimal conditions for achieving consistent and reproducible amplification of the selected SNP markers.

BDF1 strain is a hybrid of C57BL/6 and DBA/2 inbred mice. However, since both the strains bear similar alleles for the SNP IDs on chromosomes nos. 5, 18, and 19 studied here, this amplified only one allele (Table 4). While our current results are promising, sequencing can be conducted to confirm further and validate the allele assignments made using this method. This will involve excising the relevant bands from the gel, extracting the DNA, and sequencing the samples to ensure exact allele identification.

Among the 19 selected markers (**Table 1**), 10 were standardized (Figs. 1 and 2) with promising results, though further refinement may be needed. The current panel of 10 markers (**Table 4. A**) may be sufficient for detecting genetic contamination in mice strains at the Laboratory Animal Facility, ACTREC, as per the critical subset of the markers.

Several studies highlight the limitations of ARMS PCR, emphasizing that achieving reliable results can be challenging without optimal conditions. Traditional optimization methods, such as adjusting primer and reagent concentrations, annealing temperatures, cycling conditions, and adding destabilizing mismatches, are designed to improve specificity but often result in a narrow range of optimal conditions and reduced PCR yield. This balancing act between avoiding nonspecific amplification and unsuccessful results can make ARMS PCR less attractive compared to more costly and complex methods.

We encountered similar challenges during our optimization process and employed traditional approaches to address them. Despite these difficulties, our results indicate that ARMS PCR is effective for our research scope, i.e., genetic monitoring in laboratory rodents.

With careful and precise optimization, ARMS PCR has proven to be a viable and reliable method for SNP detection in our studies. For laboratories lacking access to advanced methods, ARMS PCR remains a practical and effective option for SNP detection and genetic monitoring. Thus, despite the noted limitations, our findings support the continued use of ARMS PCR in contexts where more sophisticated techniques are unavailable.

One of the main challenges we encountered while optimizing ARMS PCR was non-specific amplification. Through careful analysis, we found that three factors were crucial in addressing this issue: template DNA concentration, annealing temperature, and, most importantly, the number of PCR cycles. For most markers, a cycle range between 22 and 26 proved optimal for minimizing non-specific amplification. However, reducing the number of cycles, while effective in preventing non-specific bands, naturally resulted in lower band intensity than higher cycle numbers.

Interestingly, we also observed that some markers did not produce non-specific bands, even at 34 cycles (Figs. 1E and 2B). This suggests that while adjusting the number of cycles is generally essential, specific markers may inherently exhibit higher specificity, allowing for greater flexibility in cycle numbers without compromising the accuracy of the results.

It should be noted that other factors, such as variations in specific PCR reagents like $MgCl_2$, Taq polymerase, dNTPs, and buffer, might also influence the outcome. Our study used a Taq Mix (2X) from SRL Biolit, Mumbai, which included these components in fixed concentrations. As a result, varying individual reagent concentrations was not feasible. Consequently, the impact of these variations on non-specific amplification could not be fully assessed and may warrant further investigation.

Additionally, literature suggests that alternative PCR techniques, such as touch-down PCR and HotStar PCR, could help reduce non-specific amplification. Touch-down PCR involves a gradual decrease in annealing temperature over several cycles, which enhances specificity by allowing more stringent binding of primers. HotStar PCR, in contrast, utilizes a specially modified polymerase that is inactive at low temperatures, helping to minimize non-specific amplification by delaying enzyme activation until the reaction reaches elevated temperatures (Korbie & Mattick, 2008; Lee et al., 2023). Our study did not explore these methods but could be considered for future work to improve specificity.

PCR of G-C rich regions (GC content >60%) poses considerable challenges due to the formation of stable secondary structures, often leading to poor amplification. As noted in the results section, some SNP markers, specifically markers rs3023148 on chromosome 7 and rs3022791 on chromosome 17, exhibited poor amplification, likely due to their high GC content, which complicates the PCR process and reduces amplification efficiency.

Although the literature suggests that various additives, such as Dimethyl sulfoxide (DMSO), formamide, 7-deaza-2'-deoxyguanosine 5'-triphosphate (dc7GTP), and betaine, can be employed to mitigate the challenges of amplifying G-C rich regions (Harris & Jones, 1997), no attempts were made to use these additives in this study. These additives can enhance amplification by disrupting base pairing, improving primer annealing, or reducing the dependence of melting temperature on nucleotide composition. However, incorporating these additives introduces additional complexity to the reaction setup and may affect the concentration of other reagents.

During the standardization of the SNP marker rs3023415 on chromosome 15, primer dimer formation and faint bands of the specific product were observed (data not included here). Unwanted primer-primer interactions typically arise when primers bind to themselves or one another, resulting in a short fragment of under 100 base pairs in length, which becomes visible during agarose gel electrophoresis. To address this, the ratio of template to primer was adjusted, as an excess of primer concentration relative to the template increases the likelihood of primers annealing to themselves or each other instead of the DNA template. However, this adjustment did not resolve the issue. Although literature suggests that adding DMSO and using a hot start thermal cycling method can be beneficial, neither approach was applied in this study. When these strategies do not work, it is recommended that new primers be designed, which may be preferred in future efforts.

For SNP marker rs3023409 on chromosome 14, amplification of one allele was successful, but the other allele did not amplify as per the expected product size (data not included here). The primers designed for the second allele consistently failed to produce the expected amplification products in any samples. This suggests that the primer design or reaction conditions may require further optimization to achieve reliable amplification of both alleles.

For the marker rs3023176 on chromosome 8, strain C57BL/6 exhibited a band corresponding to an allele different from what was expected. In contrast, strain CD-1 did not show bands for either allele (data not included here). However, these two strains did show expected product sizes in both alleles for the other 10 markers (Figs. 1 and 2). These discrepancies in strain C57BL/6 could be attributed to early strain development, where specific alleles became fixed. Additionally, fixed random mutations or genetic influences from outcrossing may have contributed to these variations. In the case of strain CD-1, the absence of bands may be due to genetic variations or deletions that affect the primer binding sites or the target region, preventing successful amplification.

For marker rs3022801 on chromosome 1, the corresponding sequence did not amplify in any samples. Marker rs3023202 on chromosome 9 showed poor amplification of both alleles across all samples.

Additionally, for markers rs3023339 on chromosome 12 and rs3023432 on chromosome 16, the amplification of both alleles was inconsistent across the samples (data not included here). Amplification occurred in the opposite allele rather than the expected one, resulting in variability across the samples. This inconsistency suggests that the amplification process deviated from the expected pattern, potentially due to primer specificity, suboptimal annealing temperatures, or non-specific binding.

The reaction conditions or primer design may not have been fully optimized for these markers, leading to non-specific amplification in the unintended allele. Additionally, cross-reactivity between alleles or polymorphisms in the primer-binding regions may have contributed to the inconsistencies. To address this, further optimization may be required, including adjustments to PCR conditions or redesigning primers, to achieve consistent and reliable amplification for these markers.

The successful optimization of this method suggests that ARMS-PCR, despite its challenges, can be a cost-effective alternative for genetic monitoring in research settings where access to more advanced techniques is limited. Once standardized, laboratories can readily adopt this protocol for more effective genetic monitoring. This method allows researchers to streamline the genotyping process and achieve more accurate and efficient genetic assessments.

This standardized SNP protocol has significant potential for advancing genotyping methods and enhancing genetic monitoring in laboratory rodents. It offers a foundational approach for future development of genotyping kits, currently unavailable in our region, which could streamline genetic monitoring across labs. SNPs are valuable genetic markers with applications in personalized medicine, particularly for tracking drug responses and monitoring diseases like diabetes, cancers, and metabolic disorders. Notably, our protocol could be adapted to detect clinically important mutations in EGFR, KRAS, and TP53 genes that contribute to diagnostic assessments, predict clinical outcomes, and guide **targeted treatment strategies** (Matsuda, 2017).

Our method may also enhance **the verification of cell line identities**. **Although STR profiling is currently the** accepted standard, SNP profiling could more accurately distinguish between closely related cell lines, providing a reliable alternative for research accuracy (Almeida & Korch, 2004).

When combined with microsatellite markers, our SNP protocol can support ‘speed congenics’ by reducing the time needed to transfer genetic mutations between strains, enhancing the precision of congenic line development (Andrews et al., 2021). This combined SNP-microsatellite method may also play a role in developing humanized mouse models by facilitating the integration and functional study of human SNPs in cancer research.

To build on this foundation, future studies must focus on expanding the SNP panel by selecting multiple markers per chromosome at equal genomic intervals. This will enhance genome-wide coverage and increase the resolution of genetic analyses. Although the current study included only two mice per strain, increasing the sample size in subsequent research will support downstream applications such as pedigree assessment and genetic distance evaluation. The expanded marker set may also facilitate the development of SNP array chips, enabling high-throughput genotyping that is both time- and cost-effective.

Additionally, further work will involve optimizing newly selected SNP markers to create a comprehensive genotyping panel. Once fully standardized, the SNP genotyping protocol will be integrated into the genetic monitoring workflow at the Laboratory Animal Facility at ACTREC, complementing the existing microsatellite-based system. This combined non-lethal approach, requiring only tail samples, will align with the ethical principles of the 3Rs while enhancing the reliability of genetic monitoring. The facility also plans to offer this dual-marker genotyping service to external researchers, strengthening genetic quality assurance in laboratory rodents across institutions.

CONCLUSION

The standardized SNP genotyping protocol attempted in this study highlights the utility of ARMS PCR as a cost-effective method for genetic monitoring in laboratory rodents. By optimizing critical parameters, we achieved reproducible amplification for most markers, demonstrating the practicality of this approach in maintaining genetic integrity and reducing experimental variability. Out of the 19 markers tested, 10 were successfully standardized, covering a significant portion of the mouse genome, and were able to differentiate between various inbred mouse strains reliably. While challenges such as non-specific amplification and inconsistent marker performance were observed, systematic optimization significantly improved most markers’ results. This protocol lays a foundation for broader applications, including developing genotyping kits, enhancing precision in genetic monitoring, and facilitating advancements in personalized medicine and model organism research. Future efforts will focus on refining this method and expanding its scope to overcome limitations and further improve its applicability in research settings.

LIST OF ABBREVIATIONS

SNP: Single-nucleotide polymorphisms, ARMS: Refractory Mutation System, MGI: Mouse Genome Informatics, PCR: Polymerase chain reaction, SSLP: Simple sequence length polymorphisms, VNTR: Variable number of tandem repeats, STR: Short tandem repeats, TE: Tris EDTA, TAE: Tris-acetate EDTA, µM: Micro molar, bp: Base pair, µL: Micro Liter, ID: Identity, DNA: Deoxyribonucleic acid, ACTREC: Advanced Centre for Treatment, Research and Education in Cancer, $MgCl_2$: Magnesium chloride, DMSO: Dimethylsulfoxide

ETHICS APPROVAL

The proposal to use the animals for quality control was approved by the Institutional Animal Ethics Committee of ACTREC, Navi Mumbai

AUTHORS' CONTRIBUTIONS

ZK and AI have made substantial contributions to the conception and design of the work; ZK, HV, and AI have made substantial contributions to the acquisition and analysis; ZK and AI have made substantial contributions to the interpretation of data; ZK and AI have drafted the work and substantively revised it.

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CONFLICT OF INTEREST

All authors declare no conflict of Interest.

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