ALLELE-SPECIFIC QPCR METHOD FOR SNP AND INDEL GENOTYPING BASED ON FRET

Ruslan Kalendar, Ulykbek Kairov

Center for Life Sciences, National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan

INTRODUCTION

Polymerase chain reaction (PCR) is a simple and rapid method for detecting nucleotide polymorphisms and sequence variations in basic research applications, agriculture, and medicine. Variants of PCR, collectively known as allele-specific PCR (AS-PCR), use a competitive reaction in the presence of allele-specific primers to preferentially amplify only certain alleles.

The proposed method is a modified and improved version of the existing "Allele-specific q-PCR" (ASQ) method for genotyping of single nucleotide polymorphism (SNP) based on fluorescence resonance energy transfer (FRET). This method is similar to frequently used techniques like Amplifluor and Kompetitive allele specific PCR (KASP), as well as others employing common universal probes (UPs) for SNP analyses. The proposed ASQ method is advanced in providing a very clear and effective measurement of the fluorescence emitted, with very low signal background-noise, and simple procedures convenient for customized modifications and adjustments. Importantly, this ASQ method is estimated as two- to ten-fold cheaper than Amplifluor and KASP, and much cheaper than all those methods that rely on dual-labeled probes without universal components, like TaqMan and Molecular Beacons.

We developed a bioinformatic tool for designing probe sequences for PCR-based genotyping assays. Probe sequences are designed in both directions, and both single-nucleotide polymorphisms (SNPs) and (uniquely amongst software published to date) insertion-deletions (InDels) can be targeted. In addition, the tool allows discrimination of up to four allelic variants at a single SNP site. To increase both reaction specificity and the discriminative power of SNP genotyping, each allele-specific primer is designed so that the penultimate base before the primer's 3' end base is positioned at the SNP site. The tool allows designing custom Fluorescence Resonance Energy Transfer (FRET) cassette reporter systems for fluorescence-based assays. Using the FastPCR environment and the tool for designing AS-PCR provides unparalleled flexibility for developing genotyping assays and specific and sensitive diagnostic PCR-based tests, which translates into a greater likelihood of research success.

Conclusion

Here we describe a convenient alternative to originally described fluorescent reporters (FRET cassettes) that employs one universal quencher oligonucleotide. We describe a tool working in the FastPCR environment for developing PCR-based genotyping assays. The tool helps design assays for detection of SNPs and InDels. The FastPCR software was originally created with the intention to allow great flexibility when designing PCR assays. Now with KASP capabilities, FastPCR allows for even more sophisticated genotyping assays, which translates into a higher overall success rate and provides the unique ability to genotype mix-type SNP/InDel polymorphisms.

REFERENCES

1. Kalendar R, Shustov AV, Akhmetollaev IA, Kairov U **2022**. Designing allele-specific competitive-extension PCR-based assays for high-throughput genotyping and gene characterization. ***Frontiers Molecular Biosciences***, 9: 773956. DOI: 10.3389/fmolb.2022.773956
   WoS IF_{2020}=5.246 Q2; Scopus $CiteScore_{2020}$=4.1 SCR Q1 72th percentile

2. Kalendar R, Baidyussen A, Serikbay D, Zotova L, Khassanova G, Kuzbakova M, Kurishbayev A, Jatayev S, Hu Y-G, Schramm C, Anderson PA, Jenkins CLD, Soole KL, Shavrukov Y **2022**. Modified 'Allele-specific qPCR' method for SNP genotyping based on FRET. ***Frontiers in Plant Science***, 12: 747886. DOI: 10.3389/fpls.2021.747886.
   WoS IF_{2020}=5.753 Q1; Scopus $CiteScore_{2020}$=8.2 SCR Q1 95th percentile

3. Kalendar R, Shustov A, Schulman A **2021**. Palindromic sequence-targeted (PST) PCR, version 2: an advanced method for high-throughput targeted gene characterization and transposon display. ***Frontiers in Plant Science***, 12: 691940. doi: 10.3389/fpls.2021.691940.
   WoS IF_{2020}=5.753 Q1; Scopus $CiteScore_{2020}$=8.2 SCR Q1 95th percentile

4. Kalendar R., Kospanova D., Schulman A. **2021**. Transposon-based tagging in silico using FastPCR software. ***Methods in Molecular Biology***, 2250: 245-256. doi: 10.1007/978-1-0716-1134-0_23.
   Scopus $CiteScore_{2021}$=2.7, SJR_{2020}=0.711, 25th percentile; SCR=Q3

5. Kalendar R **2022**. A guide to using FASTPCR software for PCR, in silico PCR, and oligonucleotide analysis. ***Methods in molecular biology***, 2392: 223-243. DOI:10.1007/978-10716-1799-1_16
   Scopus $CiteScore_{2021}$=2.7, SJR_{2020}=0.711, 25th percentile; SCR=Q3

RESULTS

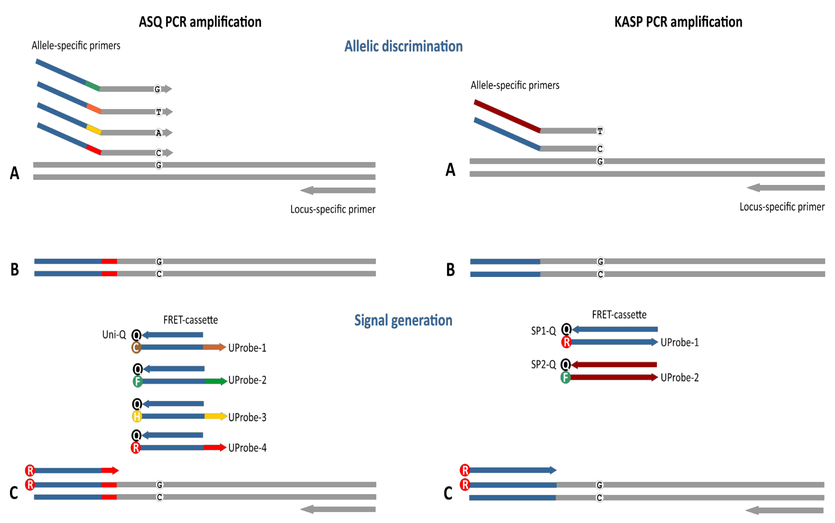


Figure 1. A four-plex fluorescent ASQ assay genotyping system compared with the standard two-plex KASP technology (LGC Biosearch Technologies). The main differences in these approaches are associated with the potential number of simultaneously detectable polymorphic sites (four in ASQ, two in KASP) and the structure of the primers that compose the FRET cassette. For ASQ, the FRET cassette consists of two or more of allele-specific primers (ASP) and a fluorescently-labeled universal probe (UP) with a single universal quencher probe (Uni-Q). Differences in the tail sequence ASPs and UPs are determined by a unique 6-nt barcode sequence that is not part of the universal tail of the Uni-Q sequence. KASP technology includes two variants of ASPs and fluorescently-labeled UPs (UP-1/2), with each UP requiring a specific quencher (SP1/2-Q). In addition, the ability to design an allele-specific primer with the SNP site at the penultimate or antepenultimate 3' base of each ASP is characteristic of the ASQ method. (A) Both ASPs query the SNP locus. Denaturation of the DNA template and annealing ASP to the target, PCR round 1. (B) Formation of a PCR product containing a specific tail sequence that is complementary to allele-specific primers. This PCR product will be used in subsequent PCR cycles as a template for amplification using a specific fluorescently-labeled UP (C). During the first two amplification cycles, a tail sequence is incorporated into the amplicon that is subsequently recognized by a universal, probe-based reporter system.

KASP primers assay design tool

Allele-Specific PCR (AS-PCR) - KASP™ (Kompetitive Allele Specific PCR) or PACE™ (PCR Allele Competitive Extension) or Allele-Specific Quantitative PCR (ASQ) based genotyping assay design for multiallelic discrimination of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci. The application is based on FastPCR software and provides professional facilities for genotyping assays design for SNP/InDel-specific KASP assay targeting primers (KASP Assay Mix) and the universal Master Mix.

Upload or paste sequence(s) in FASTA format:

Choose File No file chosen

```
>1
tgaactacggcgtgcgactaccgcggataaacctgtgtaaagaatataagtgttactcg [R]
tcaattccgcgatctagttaatccgcttcgttagtcgtattcatgggacagaaattatggacgct

>2
tgtccatatgtcgaacggtcagagaccgctgacactagtgca [/ATAGACGTCGATCGT]
gttgctccacatggcagaaagaaatgaggatgagtggaaaatccataatcacgatgaac

>3
tgggcagcattagtagaagaaagtacaagaccgtgtgtagaggatactct [GATATACTTGAG/CAGTCC]
agcagatagcgttggataggcgacaggattattggagcgccgtcgagaac
```

Primer design:		
Minimal length (nt):	18	☑ Non-specific priming control
Maximal length (nt):	28	☐ Overlaping primers
Minimal Tm (°C):	60	☐ C>>T bisulfite conversion
Maximal Tm (°C):	62	
Minimal Linguistic Complexity (%):	70	
SNP position on the 3'-terminus (1-12):	2	

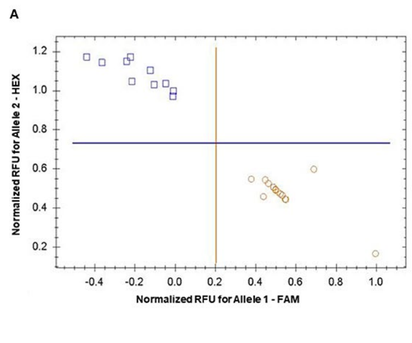
Generate Clear

To export the results: select all (Ctrl-A), copy (Ctrl-C) and paste (Ctrl-V) to Excel sheet.

Primer list | AS-PCR primers pair sets

PrimerID	Sequence(5'-3')	Length(nt)	Tm(°C)	CG(%)	Linguistic_Complexity(%)	Fragment_Size(bp)/Tm(°C)
1:						
F_32-62_ASP1	cctgtgtaaagaatataagtgttactcgat	30	61.6	33.3	81	
F_34-62_ASP2	tgtgtaaagaatataagtgttactcggt	28	60.9	32.1	80	
R_107-86	ccatgaatacgactaacgaagc	22	60.3	45.5	82	76/67.2
F_32-62_ASP1	cctgtgtaaagaatataagtgttactcgat	30	61.6	33.3	81	
F_34-62_ASP2	tgtgtaaagaatataagtgttactcggt	28	60.9	32.1	80	
R_110-89	gtcccatgaatacgactaacga	22	60.9	45.5	85	79/67.2
F_32-62_ASP1	cctgtgtaaagaatataagtgttactcgat	30	61.6	33.3	81	
F_34-62_ASP2	tgtgtaaagaatataagtgttactcggt	28	60.9	32.1	80	
R_111-90	tgtcccatgaatacgactaacg	22	61.1	45.5	87	80/68.4
F_32-62_ASP1	cctgtgtaaagaatataagtgttactcgat	30	61.6	33.3	81	
F_34-62_ASP2	tgtgtaaagaatataagtgttactcggt	28	60.9	32.1	80	
R_113-91	tctgtcccatgaatacgactaac	23	60.8	43.5	90	82/68.4

Figure 2. This figure shows examples for performing an AS-PCR task. The examples are shown here demonstrate the design capabilities of the AS-PCR set both single nucleotide polymorphisms (SNPs) and insertion-deletions (InDels) may be targeted. In addition, specific tags set can be selected for a specific polymorphism (target).



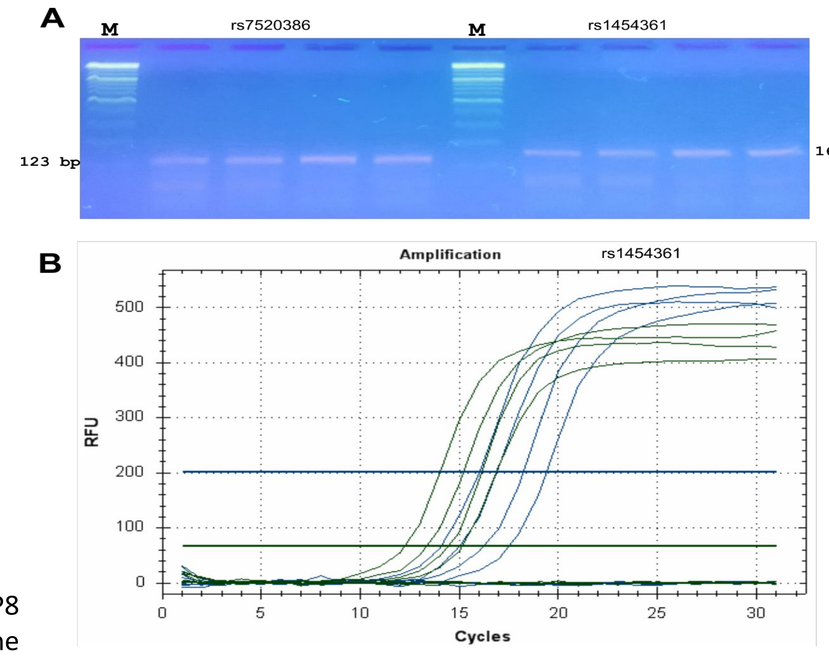


Figure 4. Allele discrimination for SNP genotyping of HvSAP8 gene in barley varieties Natali and Auksiniai-2 (A,B), and in the segregating populations [♀Natali × ♂Auksiniai-2] (C,D) using the proposed ASQ method in two types of qPCR instruments: Bio-Rad (A,C) and Thermo Fisher Scientific (B,D). SNP allele discrimination was based on Normalized reference fluorescence units (NRFU) in the Bio-Rad instrument and direct Reference fluorescence units (RFU) in the Thermo Fisher Scientific instrument. Allele 1 (FAM), Natali haplotype is designated by red circles or red dots; and allele 2 (HEX), Auksiniai-2 haplotype is shown in blue squares or blue dots, heterozygotes are presented by green triangles or green dots. Twelve and ten biological replicates were used for genotypes of Natali and Auksiniai-2, respectively (A,B), while numbers of plants studied in the hybrid populations were n = 42 and n = 58, respectively (C,D).

Figure 3. Validation and testing of the ASQ method. Probes for ASQ assays were designed using FastPCR to human SNPs (rs7520386 and rs1454361) that were employed for SNP genotyping of humans in forensic studies, and PCR products from a series of PCR reactions using these primers were examined by agarose gel electrophoresis without staining (A). M – Thermo Scientific GeneRuler DNA Ladder Mix (100-10,000 bp) stained with SYBR Green I. PCR bands of the correct size (123 bp for rs7520386 and 168 bp for rs1454361, respectively) were obtained from each qPCR. (B) qPCR amplification plot for SNP (rs1454361). FAM plot (green) shows amplifications of an A-allele, whereas HEX plot (blue) shows amplifications of a T-allele.

Funding

This work was supported by Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan (BR18574184).

Availability

The software is available at **https://primerdigital.com/tools/kasp.html**

Ruslan Kalendar, PhD, Laboratory of Bioinformatics and Systems Biology, National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan
Tel: +7(7172)709312, e-mail: ruslan.kalendar@nu.edu.kz, http://www.nu.edu.kz