



Modified “Allele-Specific qPCR” Method for SNP Genotyping Based on FRET

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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. In the proposed ASQ method, the fluorophores and quencher are located in separate complementary oligonucleotides. The ASQ method is based on the simultaneous presence in PCR of the following two components: an allele-specific mixture (allele-specific and common primers) and a template-independent detector mixture that contains two or more (up to four) universal probes (UP-1 to 4) and a single universal quencher oligonucleotide (Uni-Q). The SNP site is positioned preferably at a penultimate base in each allele-specific primer, which increases the reaction specificity and allele discrimination. 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. Results for SNP genotyping in the barley genes *HvSAP16* and *HvSAP8*, in which stress-associated proteins are controlled, are presented as proven and validated examples. This method is suitable for bi-allelic uniplex reactions but it can potentially be used for 3- or 4-allelic variants or different SNPs in a multiplex format in a range of applications including medical, forensic, or others involving SNP genotyping.

Keywords: allele-specific primers, fluorescence and quenching, FRET-based method, genotyping, qPCR and plate reader instruments, single nucleotide polymorphism (SNP), universal probes

INTRODUCTION

Single-nucleotide polymorphism (SNP) represents the smallest type of genetic difference in DNA between biological samples, where one nucleotide is replaced by another (Jehan and Lakhanpaul, 2006; Perkel, 2008; Jiang, 2013). Despite the simplicity of this change in the DNA, SNP analysis has emerged as one of the most powerful tools employed over a wide range of research from small-scale student-led investigations into specific SNPs, to high-throughput microarray technologies involving thousands of SNPs analyzed simultaneously (reviewed in Peatman, 2011; Thomson, 2014; Morgil et al., 2020; Zhu et al., 2021).

Many SNP genotyping platforms exist (reviewed in Kim and Misra, 2007; Schramm et al., 2019). Several use fluorescence resonance energy transfer (Didenko, 2001; Kaur et al., 2020a) as the basis for SNP visualization (**Table 1**). Such systems generally have two essential components: (1) allele-specific primers or molecular probe targeting the SNP and (2) a system of dye and quencher with at least one donor that produces a fluorescent signal and an acceptor that quenches the fluorescence when in close proximity (Chen and Sullivan, 2003; Giancola et al., 2006; Mamotte, 2006). In some methods, like Scorpions (Thelwell et al., 2000; Solinas et al., 2001) TaqMan (Schena et al., 2006; Jawhari et al., 2015) and Molecular Beacons (Hardinge and Murray, 2019), these two parts are combined in a single molecular probe.

In contrast, in other fluorescence resonance energy transfer (FRET)-based methods, such as Amplifluor, Kompetitive allele

specific PCR (KASP), and semi-thermal asymmetric reverse PCR (STARP), these components are kept separate. That is, they have: (1) non-labeled allele-specific primers (ASPs); (2) universal probes (UPs) labeled with fluorophores and quencher (Nazarenko et al., 1997; Khripin, 2006). KASP, Amplifluor, and STARP produced a very strong and maybe even “revolutionary” impact on the further development of SNP technology. Simple and cheap allele-specific primers (ASPs) can be designed and ordered for each SNP separately, while the relatively expensive UPs with fluorophores and quenchers are ordered just once in a stock that can be used over a very long time in many different SNP target analyses. The principle of ASP-UP is similar to that of Molecular Beacons with the addition of specialized identical “tags” at the 5'-end of the ASP and the 3'-end of the UP (Myakishev et al., 2001).

In these methods, a set of non-labeled ASPs includes two forward primers, acting on a competitive basis, and a single common reverse primer. Additionally, one of two corresponding UPs is included either with or without “hair-pin” FRET structures ending with either FAM or HEX/VIC fluorophores. This approach allows for great flexibility in assay design, which translates into a higher overall success rate for SNP genotyping and detection of InDels (Insertion/Deletion). This principle is used in various methods, including commercially produced Amplifluor (Millipore-Thermo Fisher Scientific, MA, USA) and KASP markers (LGC Genomics, Teddington, UK) for a fluorescent signal generation that enable bi-allelic discrimination and genotyping of SNPs or InDels.

TABLE 1 | Main FRET-based methods of genotyping.

Name	Allele-specific (AS) component	Components with fluorophore-dyes (F) and quenchers (Q)	
		Universal or not (whether separated from AS)	Probe shape, and positions of F and Q
TaqMan	Targeting SNP is located in the middle of probe	Single probe joining AS and F/Q parts together	Linear shape. F and Q are in 5'- and 3'-ends, respectively
Molecular Beacon	As above	As above	“Hair-pin” shape. F and Q are in the ends
Amplifluor, STARP (STAR-PCR)	Two non-labeled AS forward primers with “tags” and one common reverse primer	Two universal probes with the same “tags”	“Hair-pin” shape. F and Q are in 5'-end and the middle of probe, respectively
rhAmp ^a	As above ^a	Possibly two universal probes ^a	Seems to be linear, where F and Q are located at the ends ^a
KASP ^b	As above ^b	Possibly four universal probes ^b	Seems to be four linear universal probes. F and Q are in 5'- and 3'-ends of separate probes, respectively ^b
AS-qPCR	As above	Two universal probes with dye-specific “tags” and corresponding barcode sequences, and two universal probes with quenchers and barcodes	All four universal probes linear. F and Q are in 5'- and 3'-ends of separate probes, respectively
Proposed advanced AS-qPCR	As above	Two universal probes with “tags” and dye-specific barcode sequence, and one common universal probe with quencher and barcode	All three universal probes are linear. F and Q are in 5'- and 3'-ends of separate probes, respectively

^aInformation about rhAmp was taken from **Supplementary Figure S1** presented on the website of Integrated DNA Technologies (<https://sg.idtdna.com/pages/products/qpcr-and-pcr/genotyping/rhamp-snp-genotyping>).

^bInformation about KASP was taken from **Supplementary Figure S1** presented on the website of LGC Genomics (http://info.biotech.com/agrigenomics-pcr-based-kasp-genotyping?utm_campaign=SO-KASP-Targeted&utm_medium=cpc&utm_source=googleads&gclid=EAlalQobChMI1oXLhYWN8wIV-ZImAh09qAEAAAYASAAEgLJPvD_BwE).

Both Figures are presented in **Supplementary Material 1**.

Amplifluor (Rickert et al., 2004; Fuhrman et al., 2008) and, particularly, KASP markers (Wang et al., 2015; Ryu et al., 2018, 2019; Udoh et al., 2020; Brusa et al., 2021) are very successful commercial products and currently popular over a range of research areas. However, KASP technology is relatively expensive, especially for small experiments (reviewed in Kaur et al., 2020b). Other ASP-UP methods, such as Amplifluor-like and similar, are less expensive since all methods are unrestricted and published (Rickert et al., 2004; Rasheed et al., 2017; Zotova et al., 2019, 2020). The recently reported STARP or STAR-PCR method is similar but with different sequences for the length and shape of the “stem-loop” in the “hair-pin” of their UPs (Rasheed et al., 2016; Long et al., 2017; Li et al., 2019; Wu et al., 2020). In contrast, another SNP genotyping method, “rhAmp,” is based on the use of H2-dependent RNase together with UPs and ASPs (Integrated DNA Technologies, USA). During amplification cycling, polymerase extension leads to degradation of the rhAmp probe, and a fluorescent signal is released (Dobosy et al., 2011; Massa et al., 2021).

Earlier, different FRET-based methods were compared including commercially produced reagents and Master-mixes, where allele discrimination showed similar levels of accuracy with AS-PCR, TaqMan, KASP, Amplifluor, and rhAmp SNP genotyping (Giancola et al., 2006; Rosas et al., 2014; Broccanello et al., 2018; Ayalew et al., 2019; Kadirvel et al., 2020). These reports give additional options for researchers to use their products and services.

In the original allele-specific quantitative PCR (ASQ) method (Lee et al., 2016), fluorophores and quenchers are on separate linear fragments with complementary sequences but without a “hair-pin” structure as used in Amplifluor (Rickert et al., 2004; Fuhrman et al., 2008). This original ASQ method was previously carried out only in medical research (Lee et al., 2016) and it was chosen as the “prototype” for the modified technique used in our present study on plant analysis.

The method we propose here is suitable for uniplex (and theoretically for multiplex) application and includes elements previously used in various approaches for SNP genotyping. The method requires two separate components: (1) *allele-specific part*; two or more AS primers target the SNP with identity in the penultimate positions of the 3'-end and specific tags in the 5'-end; (2) *universal part*; two or more universal probes (UPs) with corresponding tags and different fluorescent dyes in the 5'-end, and a single common universal probe with a quencher in the 3'-ends (Uni-Q), complementary to all UP tags.

Our proposed method can potentially be used for any type of polymorphic site target, with any SNP/InDel, as well as for multiplex PCR for different DNA targets or both cases simultaneously. The flexibility of the method is due to the universal part, which is the task-independent UPs with a single Uni-Q. Each UP can potentially be used for any task where differential and multiplex analysis of two or more targets is required.

The aim of this report was to demonstrate the functionality of the proposed ASQ method, the development of all components

and conditions, and to show examples of their application for SNP genotyping in barley with comparisons to Amplifluor and KASP methods.

MATERIALS AND METHODS

Computer Software and Primer Design

The FastPCR software with a computer-based calculator (<https://primerdigital.com/fastpcr.html>) was used for the design of ASQ assays and calculation of all parameters for ASPs and universal probes (UPs), including their common tags and fluorescence-specific parts (Kalendar et al., 2017; Kalendar, 2022). The melting temperature (T_m) for primers and probes was calculated using FastPCR software, as indicated above, and presented in basic values. The T_m was calculated using a formula based on a Nearest neighbor thermodynamic theory with unified Gibbs free energy (dG), with entropy and enthalpy parameters (dS and dH, respectively) (Allawi and Santalucia, 1997; SantaLucia, 1998). Melting temperature was calculated for oligonucleotides at a concentration of 100-500 nM, depending on the specific oligonucleotide, and for standard PCR reaction buffer with 50 mM K^+ and 3 mM Mg^{2+} , or without magnesium for ASP sequences calculated without a tag. The FastPCR program was used to arrange the SNP preferably in the penultimate position at the 3'-end of the ASP. If no suitable penultimate position of the InDel site could be found, the program recommended an optimal variant of the design in one of three last nucleotides at 3'-end of ASP, which increases the reaction specificity and allele discrimination. InDels (Insertion-Deletion polymorphism) allow more options for automatic design of SNP site at the 3'-end of the ASP using the FastPCR program.

Plant Material and DNA Extraction

Cultivated barley (*Hordeum vulgare* L.) was used, comprising the two feed varieties: (1) Natali. Origin: Russia (Catalog No: K-30957 at Vavilov Research Institute of Plant Industry, St.-Petersburg, Russia) (Zlotina et al., 2013) and (2) Auksiniai-2. Origin: Lithuania (Catalog EBDB ID: 36534 at ECPGR European Barley Database, Gatersleben, Germany; <https://ebdb.ipk-gatersleben.de/apps/ebdb>). Barley plants were grown in irrigated pots with soil in open natural conditions at the Nur-Sultan city Campus of KATU (S.Seifullin Kazakh AgroTechnical University), Nur-Sultan, Kazakhstan, in 2018 and 2019. At least 10–12 individual plants from each cultivar were used for DNA extraction but various numbers of biological replicates (from 3–4 to 10–12) were used in different tests for SNP genotyping, while 42 and 58 plants were used from their hybrids.

For PCR-fragment sequencing, one young, fully developed leaf was collected from each of the 1-month-old plants. Leaf samples were placed separately in 10 ml plastic tubes (not bulked), frozen, and kept at $-20^{\circ}C$ until DNA extraction. A phenol-chloroform method of DNA extraction was used as described earlier (Shavrukov et al., 2016; Zotova et al., 2018). The extracted DNA pellet was dissolved in 1/10 diluted TE buffer, and DNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA), with quality assessed using 1 mg of DNA visualized after running

in a 1% agarose gel. DNA samples were split in two, with one half used at Kazakh AgroTechnical University (KATU) and the other transported to Flinders University (Australia) for sequencing analysis.

For SNP genotyping, two pieces of the leaf at about 3 cm in length, were collected from individual plants as described above and placed in 1.1 ml collecting tubes in a 96-hole rack-box, frozen at -80°C for at least 2 h and used for freeze-dried DNA extraction following the original protocol (Shavrukov et al., 2010). The extracted DNA pellet was dissolved in 200 μl of sterile water. DNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA), and adjusted to a concentration of 20 $\text{ng}/\mu\text{l}$ for use in PCR with a known gene for quality control.

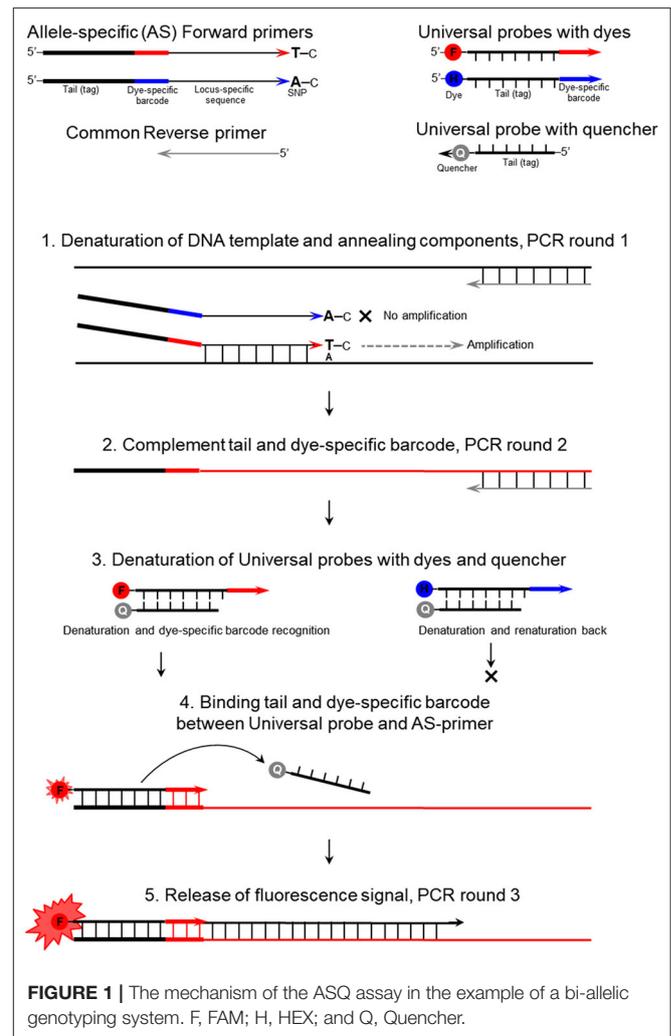
Sequencing of the Target Gene in Barley

Genes *HvSAP16* (MLOC_52196 = AK360983) and *HvSAP8* (MLOC_43986 = AK372340), encoding stress-associated proteins in barley, were identified earlier (Baidyussen et al., 2020), and amplified PCR products were Sanger sequenced with subsequent capillary separation using the Applied Biosystems 3730/3730xl DNA Analyzer (Thermo Fisher Scientific, USA) for SNP identification in this study. Information about the primers used and sequences of the target gene, conditions of PCR, purification of PCR products, and sequencing is presented in **Supplementary Material 2**. The sequence fragment with the identified SNP in *HvSAP16* with designed ASPs is provided in the Results section. All information related to *HvSAP8* including sequences of the identified second SNP developed primers, which are T_m for both 3 mM Mg^{2+} and basic parameters, is presented in **Supplementary Material 3**.

SNP Genotyping Comparison Using Three Different Methods: ASQ, Amplifluor-Like, and KASP

Experiments were carried out both in S. Seifullin Kazakh AgroTechnical University, Nur-Sultan (Kazakhstan), and Flinders University, Adelaide (Australia). Design of ASP and UP and PCR conditions for SNP genotyping in the proposed ASQ method are presented in the Results section, while the information for Amplifluor-like and KASP methods are provided in **Supplementary Material 4**. ASPs and UPs were obtained from “DNA Synthesis” (<https://oligos.ru>; Moscow, Russia) and Sigma-Aldrich (Sydney, Australia), respectively. KASP Master-mix was kindly provided by the University of Adelaide (Australia).

A QuantStudio-7 Real-Time PCR instrument (Thermo Fisher Scientific, MA, USA) and CFX96 Real-Time PCR Detection System (Bio-Rad, USA) were used in Kazakhstan and Australia, as described earlier (Khassanova et al., 2019; Sweetman et al., 2020). These instruments have detection systems with filters for FAM (Fluorescein) and VIC (2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein)/HEX (Hexachlorofluorescein) fluorophores and the conditions are described in the Results section. SNP identity calls were made automatically using software accompanying the instruments, but amplification



curves were checked for each genotype manually for final allele discrimination. SNP genotyping experiments used at least three to eight biological replicates and were repeated three times (three technical replicates).

Identical microplate reader instruments, the FLUOstar Omega, Model 415-103 (BMG LabTech, Germany) were used at both S. Seifullin KATU and Flinders University, with FAM and HEX filters with absorption/emission ratio for 485/520 and 544/590 nm, respectively.

RESULTS

Functional Principles of the ASQ Method—First Component: Allele-Specific Primers

The mechanism of the proposed ASQ assay is presented in **Figure 1** and initially appears similar to those used in Amplifluor and KASP methods. The non-labeled ASPs are developed for each SNP under study and are separate from the second part of the method. The primer design for the *HvSAP16* gene of barley,

TCCTTTCTTTTCGCTC AAAATGTATTCAATCCTTCTGGACTTCTGACTTTCTTAATATGAAA
 ATAAGATTGCACATTTGTACTGAGGATGGTAGTTATTTATAGCGATTAGTAAAGATTGCACGC
 GATTAG [W] GAAGCTTGTCCAACATCGTCTGAGATGGTTTGGGCATATTCAGCGCAGGCCTCC
 AGAAGCCCCAGTGCATAGCGGACGGCTAAAGCATTGTTGATAATGTCAAGAGAGGCCAGGGTA
[W] = [A/T]

Primers:

HvSAP16-F1: 5'-ccagctgaacggtTCGACCAGACGATGTTGGACAAGCTTCTC-3'
HvSAP16-F2: 5'-ccagctgaacggtACCTGCAGACGATGTTGGACAAGCTTCAAC-3'
HvSAP16-R: 5'-GCACATTTGTACTGAGGATGGTAG-3'

FIGURE 2 | Example of fragment sequence in the promoter region of the *HvSAP16* gene of barley (*Hordeum vulgare* L.) with targeted SNP and designed ASPs for the proposed genotyping method. Forward and reverse primers are highlighted in pink and yellow, respectively. The SNP is designated as “[W]” in red and may be either “A” or “T” nucleotides. The design of two forward primers in the reverse-complement sequence is shown in the lower part of the Figure. The uncoloured 19-bp tag containing a 13-bp identical sequence (in lower case letters) and 6-bp unique “barcode” fragment (in Capital Italic letters) is shown. The common reverse primer (in yellow) is the same as present in the gene sequence.

TABLE 2 | Allele-specific primers and universal probes with either fluorescent dyes or a quencher.

Primer ID	Sequence (5'-3')	T _m (°C) ^a	T _m (°C) ^b	dG (kcal /mole)	
(A) Allele-specific primers (ASPs)					
HvSAP16-F1	ccagctgaacggtTCGACC-AGACGATGTTGGACAAGCTTCTC	63.5	55.7	-28.4	
HvSAP16-F2	ccagctgaacggtACCTGC-AGACGATGTTGGACAAGCTTCAAC	64.5	56.3	-28.7	
HvSAP16-R	GCACATTTGTACTGAGGATGGTAG	64.9	56.4	-28.2	
Probe ID	Sequence (5'-3')	T _m (°C) ^c	T _m (°C) ^d	dG (kcal/mole)	Fluorescent dye and quencher
(B) Universal probes (UPs)					
UP-1	FAM-ccagctgaacggtTCGACC	66.5	58.2	-25.7	5'-FAM
UP-2	HEX-ccagctgaacggtACCTGC	66.2	57.9	-25.5	5'-HEX, VIC or JOE
UP-3	Cy3-ccagctgaacggtTGCAGG	67.1	58.8	-25.9	5'-Cy3 or TAMRA
UP-4	Cy5-ccagctgaacggtAGGTCG	65.6	57.3	-25.3	5'-Cy5 or Liz
Uni-Q	accgttcagctgg-Q	53.7	46.1	-16.7	3'-Quencher (Eclipse, Dabsyl, BHQ1, TQ2 or BBQ-650)

(A) Allele-specific primers for the example of the *HvSAP16* gene in barley which contains a common sequence (Upper case) but differs at the SNP position (Bold, underlined). A tag contains an identical 13-bp part (Lower case) followed by a 6-bp unique “barcode” fragment (Upper case, Italics).

(B) universal molecular probes are shown with four different fluorescent dyes (UP-1 to UP-4) and the single universal probe with a quencher (Uni-Q). Identical 13-bp parts of the tag and 6-bp unique barcode fragments are the same as in the ASPs. The universal molecular probe Uni-Q has a 13-bp tag in complement (Lower case, Italics).

^aMelting temperature (T_m), calculated for ASPs, oligonucleotide concentration of 100 nM (500 nM for a common reverse primer oligonucleotide), 50 mM K⁺ with 3 mM Mg²⁺, for ASP sequences calculated without tag.

^bT_m calculated for ASPs, oligonucleotide concentration of 100 nM (500 nM for a common reverse primer oligonucleotide), 50 mM K⁺, in the absence of Mg²⁺.

^cT_m calculated for UPs, an oligonucleotide concentration of 300 nM (500 nM for Uni-Q oligonucleotide), 50 mM K⁺ with 3 mM Mg²⁺.

^dT_m calculated for an oligonucleotide concentration of 300 nM (500 nM for Uni-Q oligonucleotide), 50 mM K⁺, in the absence of Mg²⁺.

with an amplicon size of 83-bp, is presented in **Figure 2**. ASPs consist of a set of three oligonucleotides: two forward primers and one common reverse primer, each around 22 bp in length, as usual for PCR primers (**Table 2A**). As in other methods, e.g., Amplifluor and KASP, the two forward primers target the alternative nucleotides at the SNP site and so act competitively.

In our proposed method, the base in the ASPs that targets the SNP is placed penultimately at the 3'-end of each of two forward ASPs rather than at the terminal position as in other methods (Bui and Liu, 2009). Based on our prediction of the stereochemical outcome, the penultimate position in the 3'-end of the ASP provides greater allele discrimination.

The second difference is that the two forward primers have 19-bp tags added at the 5'-end. The first 13-bp from the 5'-end of the primer are identical in all forward ASPs, while a unique 6-bp “barcode” fragment is located the next, making the full length generally about 41-bp (22 + 19 bp). Therefore, the two forward ASPs differ from each other in the single nucleotide matching the SNP in the penultimate positions and the unique 6-bp barcode fragments. The two ASPs can be developed to either the forward or reverse strand of the target sequence (and this may also be done with KASP markers). The melting temperature of the ASP oligonucleotides with the complementary sequence is ~55–56°C (in the absence of Mg²⁺). The common reverse primer, which has no tag, is designed to be with a similar melting point to that

of the forward primer and is positioned such that an amplicon of 50–100 bp (Table 2A).

The Second Component: Universal Probes (UPs) With Individual Dyes or Quencher

The second universal part consists of fluorescently-labeled universal probes (UPs) (Table 2B). These oligonucleotides contain a common 19-bp tag identical to those in the 5'-end of the corresponding ASP tags, where the unique 6-bp barcode partly matches one of four corresponding UPs with different fluorophores (in this example, UP-1 and 2) (Table 2B). A minimum of two UPs are required in this case for bi-allelic SNP analysis in uniplex PCR, but up to three or four ASP-UP combinations potentially can be used for more complex SNP differences in the polymorphic position or using multiplex PCR for multiple target detection, subject to further experimental proof.

A single quencher oligonucleotide Uni-Q is complementary to the UPs' 5'-tags. Also, the quencher oligonucleotide is complementary to the 5'-proximal sequence in fluorescent probes (Uni-1 to Uni-4). The quencher oligonucleotide at the 3'-terminus carries a universal (meaning compatible with different dyes) wide-range quencher, that efficiently quenches various fluorescent dyes in the blue to red emission spectra, including, for example, Eclipse, Dabsyl, BBQ-650, BHQ1-2, or Tide Quencher 3. Differences in the tag sequences are determined by a short barcode 6-bp fragment that is not part of the universal tag sequence. The identical sequence of the 13-bp tag-fragment in each UP (UP-1 to 4) fully complements those in the Uni-Q (Table 2B).

Following the FRET principle, the fluorescent dye attached to the 5'-end of the UPs (UP-1 to 4) is located in close proximity to a quencher at the 3'-end of the Uni-Q probe due to the full complementarity of the 13-bp DNA-duplex. In this configuration, fluorescent emissions efficiently quench various fluorescent dyes in the emission spectra (Table 2B).

Design of Tags and Barcode Sequences Specific for SNP Alleles in ASPs and Fluorescent Dye in UPs

For the design of the 6-bp barcode sequence, 348 highest-ranked at linguistic sequence complexity (LC) variants from 4,096 possible combinations of nucleotides were selected (Supplementary Material 5). There are several possible combinations and the most suitable 6-bp barcode sequences with similar thermodynamic characteristics were selected (Table 2B). The main criteria for the selection were based on (1) maximal differences for 4-bp between each possible pair combination of UPs (UP-1 to UP-4); (2) similar and as close as possible melting temperature (T_m) and Gibbs free energy (dG) (Table 2B). Additionally, extra-6-bp barcode sequences provide at least 10°C higher T_m for all 19-bp UPs with fluorophores compared to 13-bp Uni-Q quencher sequences.

Uni-Q is designed in such a way that it does not inhibit the binding of UPs to the target amplification products. Therefore, the T_m for Uni-Q was significantly lower than all T_m values of all oligonucleotides used. At a T_m value of 60°C or higher, Uni-Q cannot bind to any of the UPs. This is a theoretical assumption based on the T_m values for Uni-Q and UPs. However, after the synthesis of the target product is completed, the detection of the product generated is performed by binding all free UPs with free Uni-Q.

ASPs and UPs Have Similar Melting Temperatures but Differ in Annealing Access for Amplification

The ASPs targeting the SNP in the template and UPs with corresponding tags are designed to have a similar range of melting temperature (T_m) between 55–56°C. However, ASPs will bind the SNP region of single-stranded target DNA after denaturation and in increasing numbers of the corresponding regions in PCR products following each amplification cycle. In contrast, UPs have a delay in their activity due to binding only PCR products with fragments complementary to the tags. As the PCR cocktail includes a mixture of both ASPs and UPs, their initial concentrations are very important for optimal genotyping results. In the case of equal concentrations, ASPs and UPs will have similar access in terms of their binding abilities to the amplified PCR products, which produces a low level of fluorescence signals. An even less effective process takes place with higher concentrations of ASPs than UPs, with minimal or no fluorescence.

The specificity of the binding of ASPs to the DNA target can be increased by lowering their concentration. Decreasing the concentration of the oligonucleotide reduces the T_m value of the oligonucleotide while increasing its effective binding could only be to the fully complementary site.

So, only lower ASPs and higher UPs, with a 2- to 3-fold difference in oligonucleotide concentration was successful. For example, in our optimal case, ASPs (both forward) and UPs (UP-1 and UP-2) had final concentrations of 0.1 and 0.3 μM , respectively. The use of a slightly higher annealing temperature of 60–62°C encourages preferential binding and amplification with UPs at the second round of PCR (Table 4). Consequently, a lower annealing temperature of 55°C with a higher concentration of UPs gives more opportunity to bind the 13-bp of single-stranded Uni-Q, but not the single-stranded amplified PCR product.

Higher Concentration of Universal Probe With a Quencher (Uni-Q) Provides Better Fluorescence Background

Close interaction between the 5'-fluorophore and 3'-quencher occurs in the tags of UPs and Uni-Q. It is important that, in this situation, all UPs with fluorophores are bound by Uni-Q, and fluorescence is quenched completely. After progressive amplification and introgression of ASPs with specific tags to target amplicons, increasing numbers of corresponding UPs are bound and incorporated in the PCR product. At 55°C,

all unused UPs renature and anneal with Uni-Q and so are quenched completely.

Quencher Requirements

The common quencher of the Uni-Q must show broad-spectrum absorbance. This enables simultaneous quenching of multiple fluorophores with diverse spectra, from green FAM to red ROX used for uniplex, and all four dyes in multiplex PCR. The best choices may be Eclipse followed by Black Hole Quenchers (BHQ), which effectively quench a broad group of fluorescent dyes with emissions of 390–625 nm. Any available quencher could be used as a common universal quencher if it adequately quenches the fluorescence of all existing fluorophores. In the design of both Uni-Q and any of UP-1 to UP-4, the quencher and fluorophores are located in close proximity due to full complementary and hybridization. Additionally, the location of the quencher in the 3'-end of the Uni-Q oligonucleotide acts as an effective "stop-block" for Taq-polymerase and eliminates Uni-Q from primer-elongation.

General Conditions, the Composition of PCR Cocktail Mix, and Optimized Level of Magnesium Mg²⁺

The PCR cocktail mix composition was 5 μ l or 10 μ l reaction volume, and the latter case is presented in **Table 3**. It is recommended that two pre-mixes are prepared, one for ASPs and the second for UPs but this can be re-calculated for each component separately. For ASPs, the final concentration of both forward ASPs should be about 0.1 μ M, with the common reverse primer present in at least a 3- to 5-fold higher concentration (0.5 μ M). The UPs pre-mix should be adjusted only once in preliminary tests, and aliquots of each component from 100 μ M stock solutions can be mixed, used, wrapped in foil or dark-plastic tubes, and kept at -20°C for a very long time. The optimal concentration of UPs with fluorophores should be about 0.3 μ M, and in a combination of two for bi-allelic SNP determination in uniplex format, while three or four UPs with different dyes can be used in multiplex format with three or four designated SNP alleles. The Uni-Q must be present in at least the same or double the concentration compared to the sum of all UPs containing 5' fluorophores.

The concentration of magnesium Mg²⁺ is critical for optimal SNP genotyping using the proposed ASQ method. Best performance for most brands of standard Taq-polymerase requires a high Mg²⁺ concentration (3 mM), which is 1.7- to 2-fold higher than the concentration used in the Amplifluor-like method. However, at least one preliminary test is required, since lowering the optimal magnesium concentration will result in poor or non-specific amplification with incorrect allele discrimination (**Figure 3A**). Higher Mg²⁺ concentration will increase the efficiency of processive DNA Taq-polymerase but a magnesium concentration higher than optimal can lead to non-specific amplification.

A decrease in magnesium concentration reduces the efficiency of polymerase complexing with the DNA-duplex, which leads to a decrease in the amplification yield. Magnesium plays more

of a role as a factor in the binding of the polymerase to the DNA-duplex than as a factor in PCR specificity. On amplification specificity, magnesium concentration within optimal limits (1.5–3 mM) makes no difference. As magnesium concentration decreases from 3 mM, PCR product formation shifts toward more distant PCR cycles. For this reason, we calculate T_m values for oligonucleotides with and without magnesium concentration. Because the presence of magnesium in solution has a diverse function in PCR, and the stability of the DNA-duplex in the presence of magnesium is more complex in nature than the presence of monovalent cations.

In contrast, the most favorable Mg²⁺ concentration shows much better differences in SNP genotyping (**Figure 3B**), where sub-sets of genotypes with different SNP alleles showed their clear and adequate amplification with subsequent correct allele discrimination (**Figures 3C,D**).

The remaining general components include a standard mixture of dNTPs and any suitable Taq-polymerase with its given buffer (**Table 3**). The brand and choice of Taq-polymerase is not critical and depends on the preferences of researchers and their own experiences in working with them. The suggested range of 0.04–0.05 units/ μ l of the enzyme in final concentration was optimal in our experiments. A lower concentration of Taq-polymerase can result in stronger background interference while too many Taq-polymerase results in rapid amplification with very narrow a window for clear and correct SNP allele discrimination. In general, a Hot-start Taq-polymerase usually required fewer cycles, but ultimately there seems to be little difference in SNP genotyping compared to regular Taq-polymerase.

Application of DMSO for PCR improvement is possible but it makes sense only in cases where no effective allele discrimination was found in regular conditions for SNP genotyping. However, it is important to note that the application of DMSO will affect all used oligonucleotides, including UPs with dyes and a quencher (Uni-Q), and it can result in a less effective quenching process. Therefore, added DMSO can be used in some cases.

Moving the SNP-site to the penultimate position significantly increases the differentiation of each allele, including moving the SNP-site to the third position from the 3'-end of the ASP. This is due to the stability and geometry of the hybridization DNA-duplex, and the nucleotide composition surrounding the SNP site. Depending on the geometry of the hybridization DNA-duplex, this complex can be either stable or not. Therefore, even in the penultimate position, SNP detection for some sequences will also not be effective. In this case, moving the SNP-site to the third position from the 3'-end of the ASP is required. The first 12 nucleotides from the 3'-end of the primers are critical for binding the primer to the DNA target. Therefore, within these 12 nucleotides, the SNP site from the 3'-end of the ASP can potentially be moved and preferably, the destabilization of the hybridization DNA-duplex should be maximal for each alternative allele close to the 3'-end of the ASP.

The recommended total volume of PCR for SNP genotyping using the modified ASP-UP method is from 5 to 10 μ l per reaction in 384/96-well microplate formats, which yielded very good scoring results. A smaller volume of PCR with ASP-UP is more economical but requires greater accuracy in pipetting

TABLE 3 | The composition of PCR cocktail mix for the proposed ASQ method of SNP genotyping.

Component	Concentration	Volume (μ l)	Final concentration
GoTaq Buffer (clear, without MgCl ₂)	5×	2.0	1×
MgCl ₂	25 mM	1.2	3.0 mM
dNTP	2 mM	1.0	0.2 mM
ASPs pre-mix			
ASP-F1	1 μ M	1.0	0.1 μ M
ASP-F2	1 μ M		0.1 μ M
ASP-R	5 μ M		0.5 μ M
UPs pre-mix			
UP-FAM:	3 μ M	1.0	0.3 μ M
UP-HEX/VIC	3 μ M		0.3 μ M
Uni-Q	6 μ M		0.6 μ M
Go-Taq polymerase	5 units/ μ l	0.08	0.04 units/ μ l
Sterile water	–	1.72	–
Master mix	–	8.0	–
DNA template	10 ng/ μ l	2.0	20 ng/ μ l
Total	–	10.0	–

TABLE 4 | PCR running protocol for genotyping using advanced ASQ method for SNP genotyping.

Step	Temperature	Duration	Notes
1	94°C	2 min	Initial denaturation. Can be variable depending on Taq-polymerase
2	94°C	10 s	First-round denaturation
3	55°C	20 s	First-round annealing
4	68°C	20 s	First-round extension
5			10 cycles repeat for steps 2–4
6	94°C	10 s	Second-round denaturation
7	62°C	20 s	Second-round annealing-extension
8	68°C	20 s	Second-round extension (optional)
9	55°C	30 s	Cooling, extension and Microplate read
10			30 cycles repeat for steps 6–9

and manual operation, with a reasonable speed in loading PCR Master-mix and DNA template on ice to prevent evaporation. A robotic system of loading is obviously extremely accurate but also very expensive for small laboratories. In this regard, digital pipettes for loading Master-mix and multi-channel pipettes for the addition of templates can be very useful tools for accurate PCR preparation.

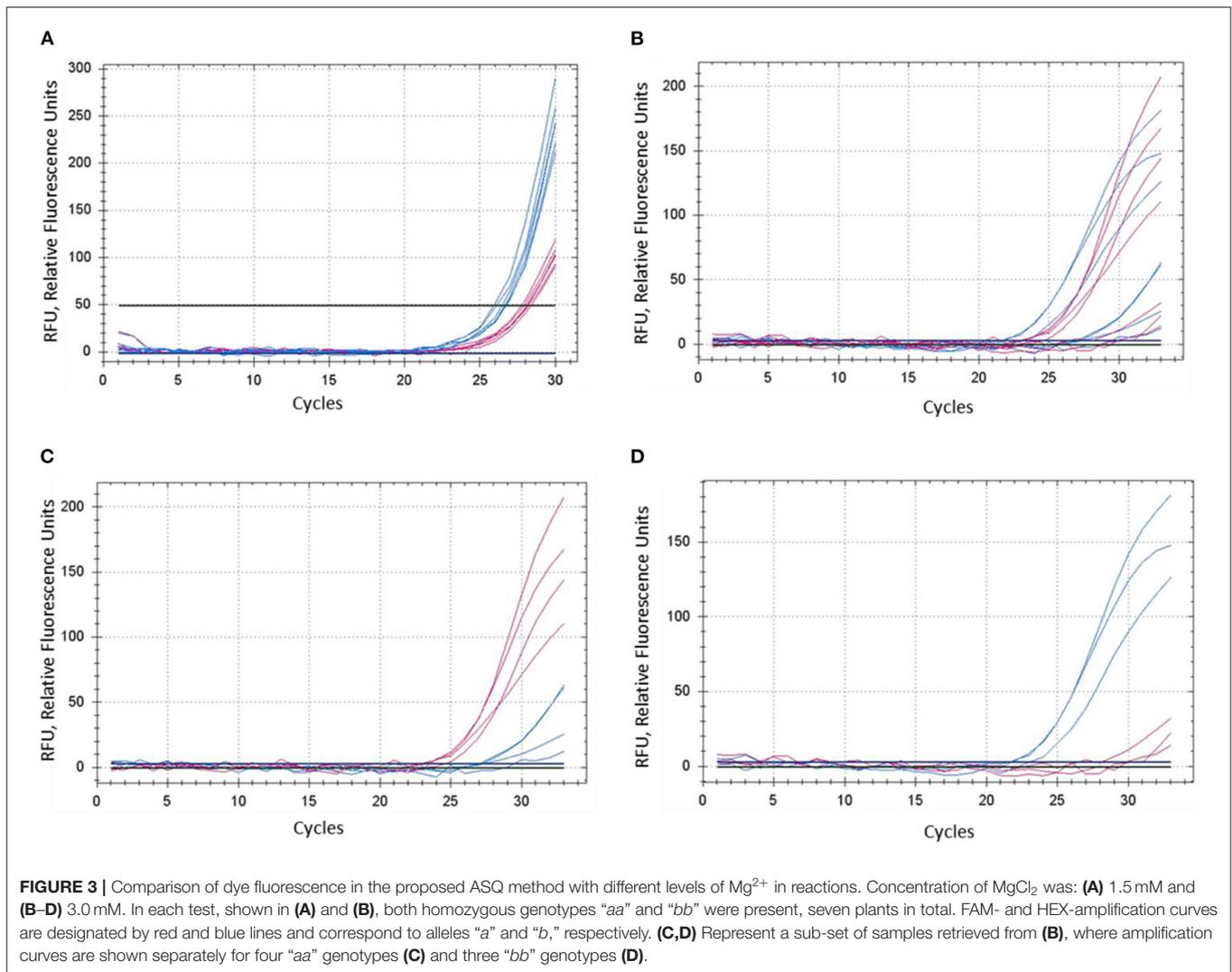
Template DNA extracted by any appropriate method with suitable quality control may be used for SNP genotyping if the Taq-polymerase is not inhibited by contaminants (Kalendar et al., 2021). DNA can be diluted and used in quite a wide range of concentrations from 5 to 50 nM, where 10 or 20 nM for 5 or 10 μ l reactions, respectively, might be estimated as optimal for most plant species.

Modified PCR Protocol for SNP Genotyping

The proposed PCR protocol for the modified ASQ method of SNP genotyping has some steps very similar to other FRET-based methods like Amplifluor or KASP, but there are important differences (Table 4). During the two rounds of PCR, the first

one with 10 cycles had an annealing temperature of 55°C (Step 3) which allowed specific binding of ASPs to the template DNA in the region of the SNP. The extension temperature of 60–68°C (Step 4) is recommended for regular PCR. There were no differences found in experiments using “touch-down” annealing temperatures reduced by 0.6–1.0°C per cycle as recommended in the KASP method (Jatayev et al., 2017). Therefore, a fixed annealing temperature was used in the first round of amplification.

More changes were made in the second round of PCR, where the annealing temperature of 62°C (Step 7) was optimal for binding of UPs to the target amplicons produced from the previous PCR round. This will make the PCR process more specific, particularly for binding of single-stranded amplified fragments with UPs and their corresponding fluorophores. The signal detection step takes place at Step 9, where the temperature was decreased from elongation (Step 8) at 68–55°C. The T_m value for Uni-Q is lower than all T_m values of all other oligonucleotides used. At T_m values of 60°C and higher, Uni-Q cannot bind to any of the UPs because it is in a totally free coil state.



After completion of amplicon synthesis (Step 8), Uni-Q effectively binds all free UPs at 55°C. At higher temperatures (Step 7), stable complexes between UPs and Uni-Q are not possible or poorly stable. Therefore, Uni-Q does not inhibit target amplification throughout amplification.

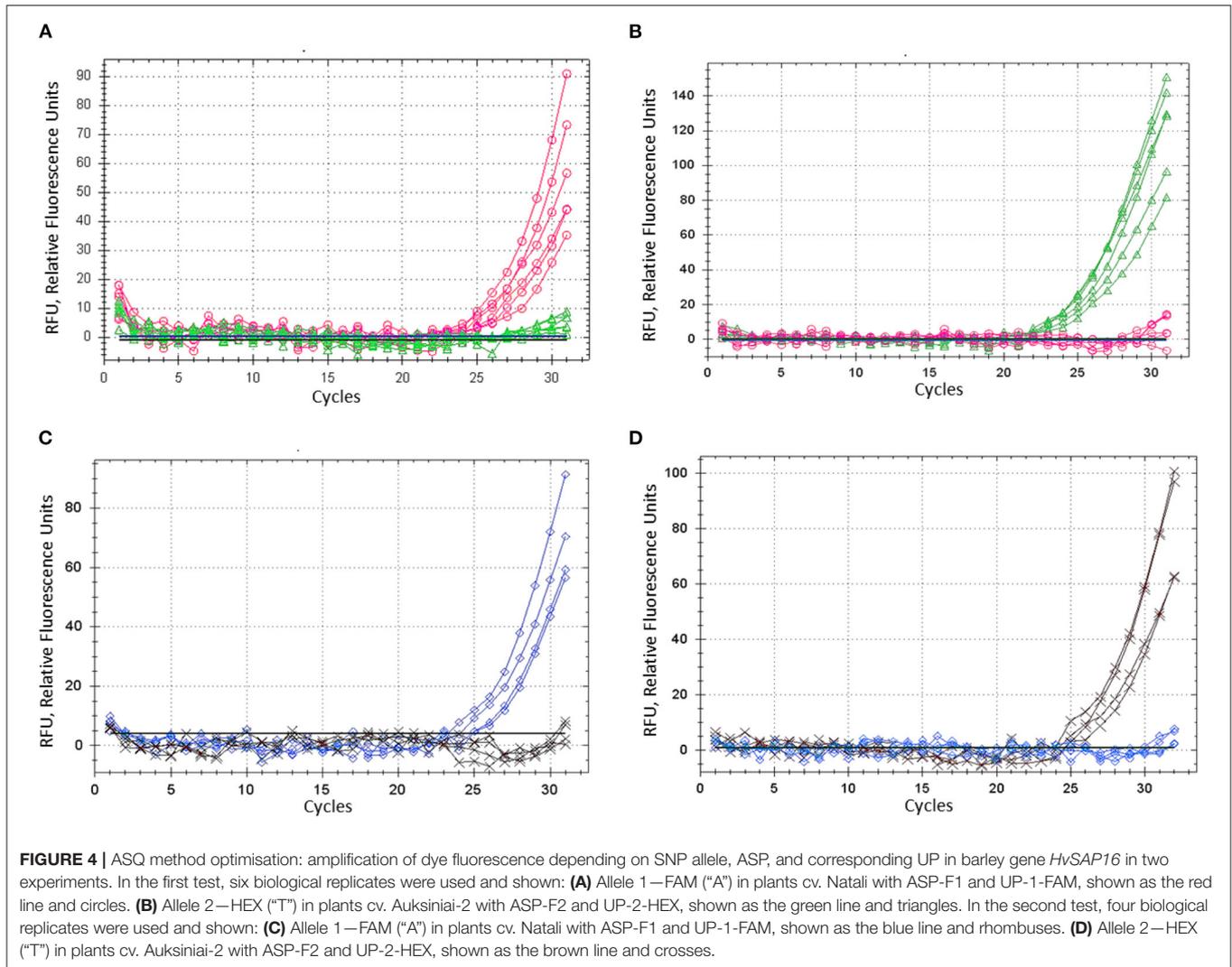
This additional annealing step provides a dramatic reduction of the second-step extension temperature by 13–17°C and will cause strong binding and renaturation of all amplified fragments and UPs with fluorophores. This temperature drop has an enormous impact on microplate reading results with dye fluorescence. The Uni-Q with quencher has a shorter fragment by 13 bp (Table 2) but its higher concentration (Table 3) presents the opportunity to bind all non-used UPs with fluorophores and quench all background fluorescence completely.

Instruments for qPCR and End-Point Microplate Reader

The proposed ASQ method for SNP genotyping can be used with two types of equipment. Real-time qPCR instruments detect changes in fluorescence level after each cycle of

amplification, which shows dynamic changes in a given dye or mixture of dyes. Amplification curves for fluorophores can be observed in real-time in each cycle. However, as expected, the detection of fluorescence is registered after at least 20–25 cycles depending on modifications and adjustments applied. The released fluorescence is directly related to several consequential processes, including the incorporation of ASPs in the binding of the SNP targeted region, amplification of the complementary PCR products, engagement of UPs with fluorophores, and finally detachment from Uni-Q and freedom from quenching. Comparison with a no-template control (sterile water, NTC) provides either manual or automatic normalization of Relative fluorescence units (RFU). Therefore, allele discrimination for dye fluorescence can be calculated at any cycle of the microplate reading. The presented SNP genotyping results will differ depending on the brand of qPCR instruments and associated computer software.

Alternatively, a simple “end-point” approach can be used with a PCR cycler and Microplate Reader with appropriate



filters matching the wavelength of the fluorophores used. After completing the amplification protocol (Table 4), usually, in 96-well plate format, PCR products may be diluted and used directly for fluorescence measurements in the Microplate Reader. Allele discrimination can be simply calculated based on the ratio between the fluorescence of the dyes used, either manually or *via* the instrument software. An example of scoring results for FAM and HEX fluorescence units with end-point detection in a part of 96-well microplate with SNP genotyping using the proposed ASQ method is presented in Supplementary Material 6.

Proposed ASQ Method for SNP Genotyping Analysis of Barley Gene *HvSAP16*

Barley plants from cvs. Natali and Auksiniai-2 with previously identified SNP alleles of the *HvSAP16* gene were used to confirm the accuracy of the modified ASQ method. This accuracy and specificity are based on the comparison of allele discrimination of known genotypes of the barley cultivars used, with SNP presence confirmed by Sanger sequencing. Several rounds with minor optimisation adjustments of the method were carried

out, and amplification results for each of two fluorophores used (FAM and HEX) are shown for bi-allelic SNP identification in *HvSAP16* (Figure 4). RFU increased significantly in FAM and HEX after 24–25 and 22–23 cycles, respectively, in the amplification with studied barley genotypes. In each test, RFU of a single fluorescence only was recorded until about 30 cycles when RFU from the second fluorophore was practically negligible. However, even beyond 30 cycles, the difference in amplification of one fluorophore over the other was strong in all analyses, which reflects perfect discrimination of alleles in a wide range of “real-time” and “end-point,” after 25–33 cycles and beyond (Figure 4).

Comparative Analysis of the *HvSAP16* Gene Using Amplifluor and KASP Methods and Validation of the ASQ Method Assessing SNP in *HvSAP8*

The proposed ASQ method was compared with two other popular ones, Amplifluor-like and KASP. In addition, the same barley genotypes were tested using two different brands of qPCR

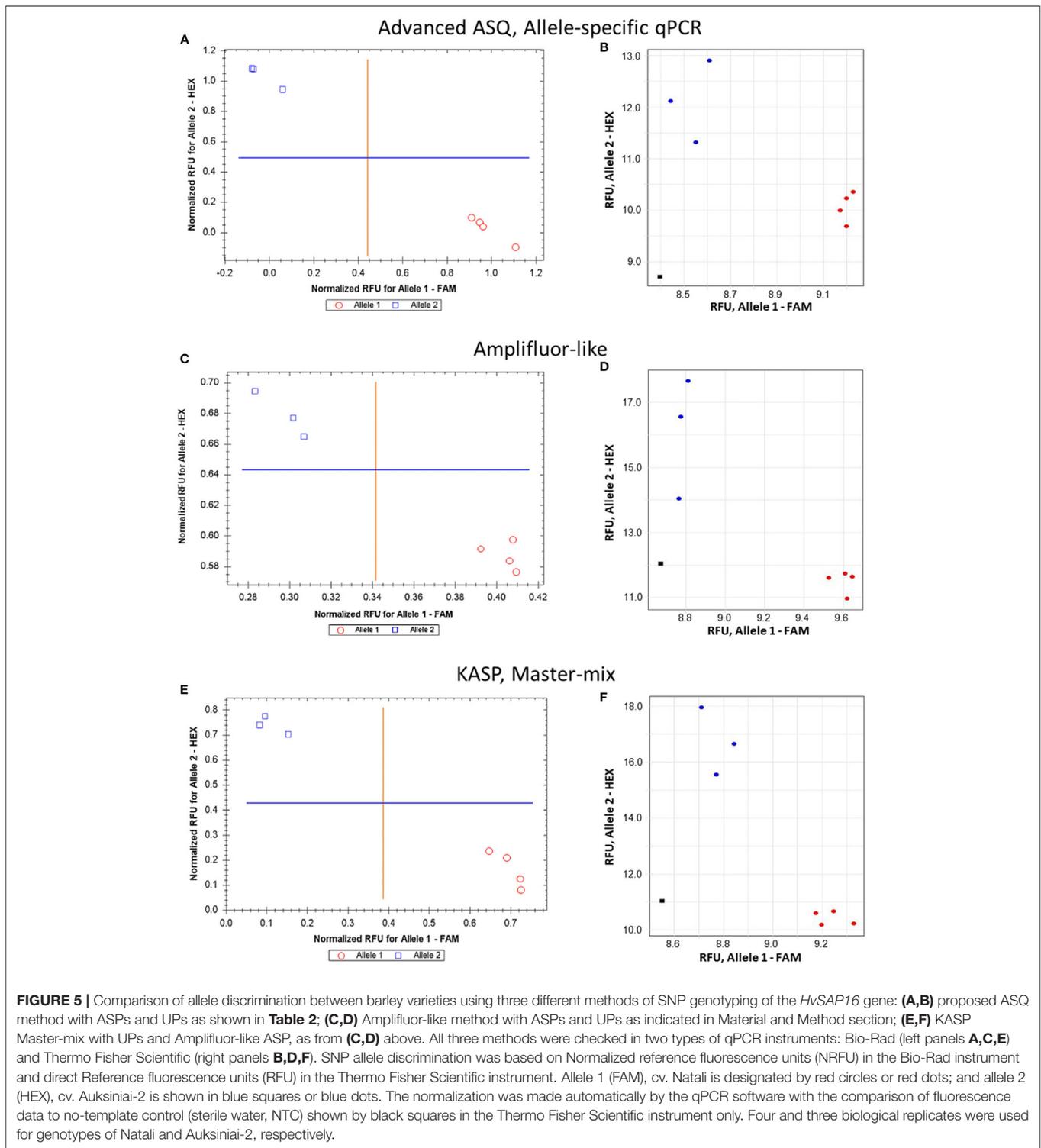
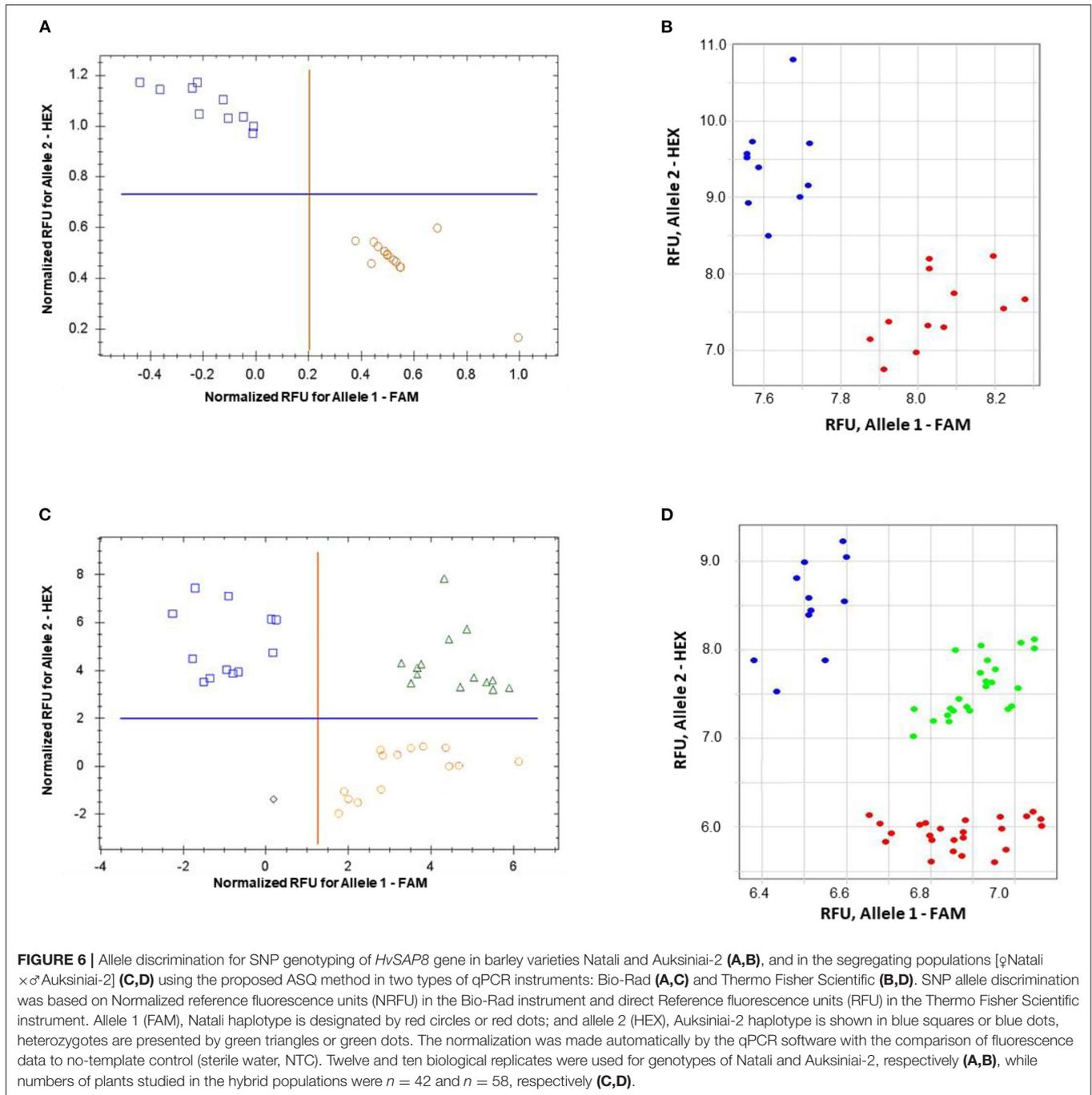


FIGURE 5 | Comparison of allele discrimination between barley varieties using three different methods of SNP genotyping of the *HvSAP16* gene: **(A,B)** proposed ASQ method with ASPs and UPs as shown in **Table 2**; **(C,D)** Amplifluor-like method with ASPs and UPs as indicated in Material and Method section; **(E,F)** KASP Master-mix with UPs and Amplifluor-like ASP, as from **(C,D)** above. All three methods were checked in two types of qPCR instruments: Bio-Rad (left panels **A,C,E**) and Thermo Fisher Scientific (right panels **B,D,F**). 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), cv. Natali is designated by red circles or red dots; and allele 2 (HEX), cv. Auksiniiai-2 is shown in blue squares or blue dots. The normalization was made automatically by the qPCR software with the comparison of fluorescence data to no-template control (sterile water, NTC) shown by black squares in the Thermo Fisher Scientific instrument only. Four and three biological replicates were used for genotypes of Natali and Auksiniiai-2, respectively.

instruments, Bio-Rad and Thermo Fisher Scientific, with their supplementary software analyses. As shown in **Figure 5**, similar accuracy and quite distinctive results were received for SNP allele discrimination with all three methods regardless of the qPCR instruments used.

The validation of the proposed ASQ method was made using SNP in the barley *HvSAP8* gene. SNP allele discrimination for 12 and 10 biological replicates in Natali and Auksiniyai-2 genotypes, respectively, and for the segregating populations between these cultivars with $n = 42$ and $n = 58$, are presented in **Figure 6**. These



results for SNP genotyping in both *HvSAP16* and *HvSAP8* genes in barley validated our conclusion that the proposed ASQ method is very accurate and effective for any SNP genotyping in barley, and we expect in other plant species and beyond.

DISCUSSION

Many SNP genotyping methods, especially TaqMan and Molecular Beacons, where allele-specific and dual-labeled parts

are combined in a single molecular probe, are too expensive to be adopted by smaller laboratories (Edenberg and Liu, 2009; Kumar et al., 2012; You et al., 2018; Zhao et al., 2019; Zhu et al., 2021). The latest generation of SNP genotyping methods, for example, Amplifluor, KASP, rhAmp, and STARP, use universal molecular probes which are not related to any particular SNP and, therefore, can be applied to different SNP analyses (Rasheed et al., 2016; Long et al., 2017; Broccanello et al., 2018; Ayalew et al., 2019; Li et al., 2019; Wu et al., 2020). Such SNP technologies

are significantly cheaper than the previous methods, but they still remain relatively expensive for laboratories with a limited budget.

The ASQ “prototype” (Lee et al., 2016) and the method proposed here had some differences compared to other methods with known UP structures. Although these methods use similar UPs, ASQ does not include a “hair-pin” loop. Moreover, the uncoupling of UPs into two independent units with a separate fluorophore and quencher in the ASQ method may give the appearance of a more complicated system but it results in a reduced cost per SNP, much greater flexibility, and easier adjustment of the method to any conditions.

More specifically, adding a single Uni-Q at 2- to 3-fold higher concentration and annealing at a lower temperature of 55°C (Step 8 of the amplification protocol, **Table 4**), ensures complete quenching of all non-incorporated UPs and thus a very low signal to noise ratio. Furthermore, the UPs and the Uni-Q differ in size (19- and 13-bp, respectively) and, therefore, have different annealing temperatures. This favors UP binding to amplified fragments and thus the generation of the signal.

The proposed ASQ method is based on a prototype that was used in medical research only (Lee et al., 2016) and it is unclear why it received so little attention outside the area. Our main modification of the method was the use of a single Uni-Q, which improves upon the original prototype and helps to address this oversight. In the proposed method, we completely redesigned the 19 bp tags, and their complements in the UPs were added to the ASPs. This resulted in a significant reduction in overall cost as only a single, universal quencher is then used. Although we did not show this experimentally, our modification with a single, common Uni-Q opens the way to assaying three or four allelic variants at a single SNP locus.

Although ASQ is similar to Amplifluor and KASP with regard to the use of two allele-specific forward primers, it differs in the positioning of the allele-specific nucleotide. In our protocol, the SNP is placed in the penultimate position rather than at the 3' end. We believe this to be optimal because it allows for the use of proof-reading polymerases, which is important where template DNA quality may be poor.

Furthermore, the possibility of artificial mismatches added to the 5'-end of ASP in various methods can be considered (Liu et al., 2012; Choi et al., 2017; Han et al., 2017; Lu et al., 2020). Despite some possible increased discrimination between alleles (Hirotzu et al., 2010), the artificial mismatches in ASP can cause a reduction of total PCR efficiency, or inhibit the extension of the product by Taq polymerase (Ke and Wartell, 1993; Wu et al., 2009). Terminal mismatches can stabilize the DNA duplex structure, whereas internal mismatches can cause destabilization due to unfavorable helical constraints that prevent the formation of optimal base stacking and H-bond geometry (Stadhouders et al., 2010). Therefore, based on our estimations, artificial mismatches could improve allele discrimination but not in all cases.

There was an additional benefit of our protocol. Theoretically, the use of a single, common oligonucleotide to quench the fluorescence of all UPs could be extended for use in other protocols, such as quantitative and qualitative PCR. As an example, multiplex qPCR could be used to detect different

targets simultaneously. Thus, for each individual target, the forward primer would contain a unique tag sequence for the corresponding UPs. The number of UPs could be expanded to equal the number of detection channels available on the device in any particular laboratory. The authors did not test this idea but suggested the potential extension of this method. An example of such applications would be the diagnosis of pathogens where simultaneous detection of multiple DNA targets and internal control was required. We can see no theoretical or practical difficulties in implementing this approach, including adapting to existing applications (Fors et al., 2000; Kreuzer et al., 2001; La Paz et al., 2007; Ragoussis, 2009; Gašparič et al., 2010; Löfström et al., 2015; Weck et al., 2021).

The presented results for the modified ASQ method are compared with those obtained from other methods. We estimated that our protocol was 2- to 10-fold cheaper than KASP or Amplifluor, based on cost per SNP (**Supplementary Material 7**), and even cheaper than other methods. The synthesis and purification of dual- or single-labeled fluorogenic oligonucleotides were the most expensive part of all methods. The use of Uni-Q with a common quencher in our method allows a significant reduction in overall cost. This improvement made our modified ASQ genotyping method a more adaptable, universal, cost-effective, and high-throughput genotyping alternative, and thus applicable to laboratories with a limited budget for the cost of SNP genotyping.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

RK conceived the idea and provided bioinformatics analyses to design primers, tags and barcode sequences. AB, DS, LZ, GK, and MK generated results in Kazakhstan and carried out data analysis. SJ, Y-GH, and KS supervised the project and provided financial support for the research. CS, PAA, and CLDJ helped with illustrations, previous publications and the manuscript editing; YS coordinated all groups and prepared initial draft of the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.747886/full#supplementary-material>

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