

# A Rapid, SuperSelective Method for Detection of Single Nucleotide Variants in *Caenorhabditis elegans*

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**ABSTRACT** With the widespread use of single nucleotide variants generated through mutagenesis screens and genome editing technologies, there is pressing need for an efficient and low-cost strategy to genotype single nucleotide substitutions. We have developed a rapid and inexpensive method for detection of point mutants through optimization of SuperSelective (SS) primers for end-point PCR in *Caenorhabditis elegans*. Each SS primer consists of a 5' "anchor" that hybridizes to the template, followed by a noncomplementary "bridge," and a "foot" corresponding to the target allele. The foot sequence is short, such that a single mismatch at the terminal 3' nucleotide destabilizes primer binding and prevents extension, enabling discrimination of different alleles. We explored how length and sequence composition of each SS primer segment affected selectivity and efficiency in various genetic contexts in order to develop simple rules for primer design that allow for differentiation between alleles over a broad range of annealing temperatures. Manipulating bridge length affected amplification efficiency, while modifying the foot sequence altered discriminatory power. Changing the anchor position enabled SS primers to be used for genotyping in regions with sequences that are challenging for standard primer design. After defining primer design parameters, we demonstrated the utility of SS primers for genotyping crude *C. elegans* lysates, suggesting that this approach could also be used for SNP mapping and screening of CRISPR mutants. Further, since SS primers reliably detect point mutations, this method has potential for broad application in all genetic systems.

**KEYWORDS** *C. elegans*; genotyping; single nucleotide variant; SuperSelective PCR; ARMS PCR

**I**N this genomic era, researchers have identified a multitude of single-base-pair substitutions, the most common type of DNA sequence variation in genome sequence data. Naturally occurring single nucleotide polymorphisms (SNPs) have been linked to human disease (Shastry 2002; Suh and Vijg 2005) and are used for gene mapping (Davis *et al.* 2005; Altshuler *et al.* 2008) and evolutionary studies (Koch *et al.* 2000). In genetic model systems, point mutants isolated through mutagenesis screens and gene editing are essential tools for discovery of gene function. Therefore, researchers working across a wide range of disciplines and systems can benefit from having a low cost, robust, and efficient method to distinguish between alleles with single nucleotide variations.

In *Caenorhabditis elegans*, many mutants have been generated in forward genetic screens, with the most commonly used chemical mutagen ethyl methanesulfonate (EMS) exhibiting a mutagenesis bias toward transition mutations (Brenner 1974; Flibotte *et al.* 2010). Over 800,000 single nucleotide substitutions (SNSs) have been identified in the million mutation project, carried out to provide the *C. elegans* research community with a resource of mutant alleles for all genes in the genome (Thompson *et al.* 2013). SNSs are now also induced by CRISPR gene editing to interrogate the function of specific amino acids (Dickinson and Goldstein 2016). To analyze the phenotype associated with a mutation and decipher gene function, genetic crosses are performed, necessitating a reliable, rapid method for routine genotyping of SNSs.

A variety of techniques for SNS genotyping are available; however, these methods are either labor intensive, expensive, or require extensive troubleshooting (Mamotte 2006). Cleaved Amplified Polymorphic Sequence (CAPS) genotyping is based on the formation or disruption of a restriction enzyme recognition site by a mutation and involves enzymatic digestion of

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DNA amplified from the target region followed by electrophoresis (Konieczny and Ausubel 1993). A modified method, dCAPS, can be used to create or remove a restriction enzyme site to distinguish between two alleles (Neff *et al.* 2002). While the CAPS method is simple, it involves extra steps beyond PCR, requires purchase of different restriction enzymes, and can lead to ambiguous results in cases of incomplete enzyme digestion. Other genotyping methods, including the TaqMan assay and melting curve analysis of FRET probes, are not labor intensive, but do require acquisition of allele-specific hybridization probes labeled with different fluorescent dyes as well as access to expensive instrumentation to allow for real-time monitoring of PCR amplification (Bernard *et al.* 1998; Livak 1999).

Allele-specific PCR, also known as Amplified Refractory Mutation System (ARMS) PCR, and the modified Simple Allele-discriminating PCR (SAP) are inexpensive genotyping methods that utilize allele-specific primers (Newton *et al.* 1989; Bui and Liu 2009; Medrano and De Oliveira 2014). Discrimination between wild-type and mutant alleles is based on a mismatch at the 3' terminal base, which prevents extension of the primer by *Taq* polymerase (Petruska *et al.* 1988; Newton *et al.* 1989; Wu *et al.* 1989; Huang *et al.* 1992). However, ARMS PCR and SAP often require extensive troubleshooting as PCR specificity must be controlled by stringent reaction conditions. Further, a lack of flexibility in primer placement can make SNS detection difficult in certain sequence contexts (Medrano and De Oliveira 2014).

To detect the presence of rare SNPs in DNA fragments found in blood samples, Vargas *et al.* (2016) developed SuperSelective (SS) primers for real-time PCR assays. A SS primer consists of a 5' anchor sequence that hybridizes to the template DNA followed by a noncomplementary bridge sequence and a short 3' foot sequence that is complementary to the target allele sequence (Vargas *et al.* 2016). Our goal was to design and optimize allele-specific primers for end point PCR genotyping based on the principle of SS primers. We probed the different regions of the primer to determine how specificity is achieved, developed simple rules for SS primer design, and validated this method for genotyping of crude *C. elegans* lysates. Our work presents SS genotyping as an advantageous alternative to existing genotyping methods that will facilitate research with genetic systems.

## Materials and Methods

### Nematode culture

*C. elegans* were maintained on Nematode Growth Medium (NGM) plates with OP50 *Escherichia coli* as a food source using standard techniques (Brenner 1974). The wild-type strain was Bristol N2. Strains and alleles used in this study were as follows: PT443 *k1p-6(sy511)* III; *him-5(e1490)* V, DM1017 *plx-2(gk2864)* II; C05B5.11(*gk2895*) III, VC40549 *cil-7(gk688330)* I, ZZ12 *lev-11(x12)* I, CB1372 *daf-7(e1372)* III, DA465 *eat-2(ad465)* II. All strains were maintained at 20° except CB1372, which was grown at 15°.

### Molecular biology

Primers were designed as described in the results section and obtained from Integrated DNA Technologies (IDT). A complete list of primers used is in Supplemental Material, Table S1. Clean genomic DNA (gDNA) was isolated with the Genra Puregene Tissue Kit (Qiagen Cat. No. 158667) following the manufacturer's instructions, diluted to 5 ng/ $\mu$ l, aliquoted, and stored at -20°; the same preparations were used for all experiments. Crude genomic DNA used in Figure 1, G-I was extracted by incubating 30 worms in 60  $\mu$ l of 1 $\times$  lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl<sub>2</sub>, 0.45% NP-40, 0.45% Tween-20, and 1 mg/ml of Proteinase K) for 1 hr at 60° followed by 95° for 15 min. For single worm lysis experiments in Figure 7, each individual animal was lysed in 10  $\mu$ l of lysis buffer. When preparing crude lysates, a freeze-thaw step was not performed before lysis as we found that this does not affect PCR performance (data not shown).

Gradient PCR was performed by aliquoting a master mix containing GoTaq DNA Polymerase (Promega Cat. No. M3008), 500 nM of each primer, and 5 ng of clean gDNA into 15  $\mu$ l reactions. Instead of 1  $\mu$ l clean gDNA, 1  $\mu$ l of crude lysate was used for Figure 1, G-I. For SS genotyping of individual animals in Figure 7, each single worm lysate was divided into two separate 20  $\mu$ l reactions. The following PCR protocol was performed: 98° for 30 sec (cycle one only), 98° for 10 sec, annealing temperature for 15 sec, and 72° for 30 sec for 30 cycles. Annealing temperatures were determined for the anchor of SS primers using the New England Biolabs  $T_m$  calculator and are indicated in Table S1. Gradient temperatures were across 10°;  $T_m$  minus 5° (lowest temperature) to  $T_m$  plus 5° (highest temperature) except in Figure 6, a 45° to 55° gradient was used. PCR products were resolved on 1% agarose gels and visualized with SYBR safe (Thermo Fisher Cat. No. S33101). All gradient PCR experiments were performed at least twice, and a representative image is displayed in the figures.

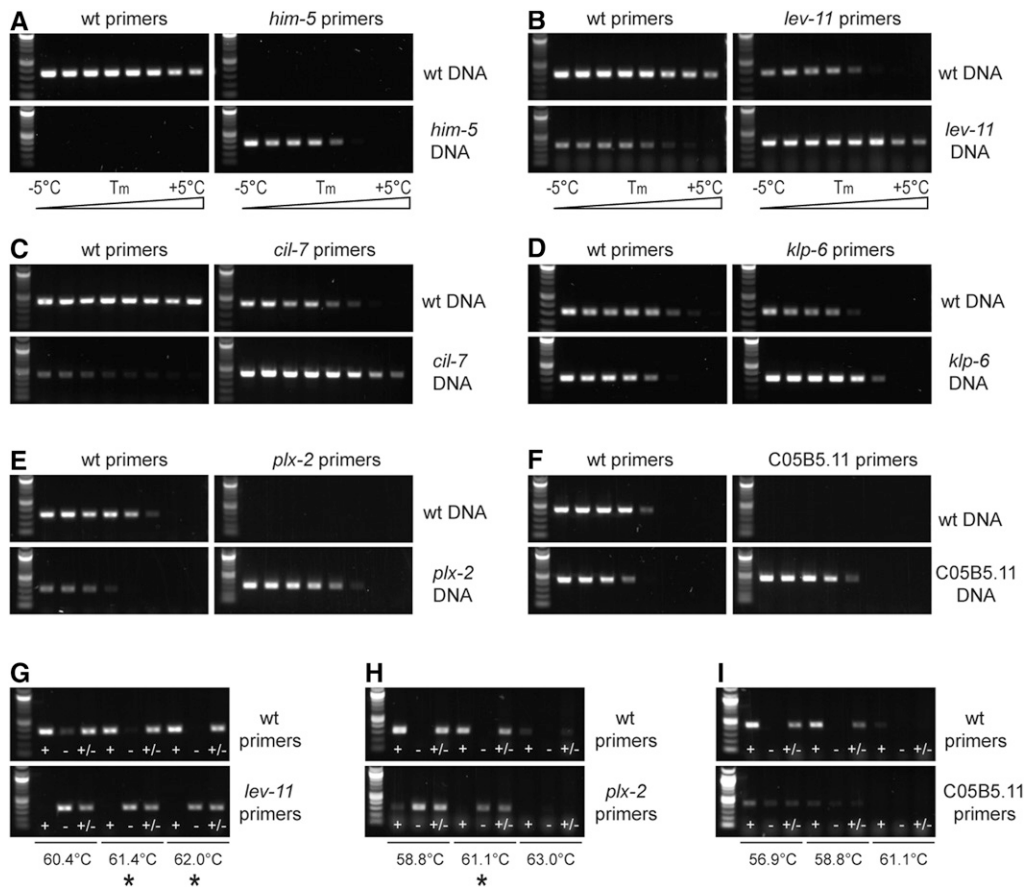
### Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All data and methods required to confirm the conclusions of this work are within the article, figures, and supplemental materials. Supplemental material available at figshare: <https://doi.org/10.25386/genetics.12814565>.

## Results

### Limitations of ARMS PCR for *C. elegans* genotyping

We have been performing genetic crosses with point mutants that do not cause visible phenotypes or change restriction sites for multiple ongoing projects. To discriminate between wild-type and mutant alleles, we sought to use ARMS PCR genotyping, which is based on the principle that a mismatch at the 3' terminal base of a primer results in inefficient amplification (Petruska *et al.* 1988; Wu *et al.* 1989; Huang *et al.* 1992) as the absence of exonuclease activity in *Taq* DNA polymerase



**Figure 1** Genotyping with ARMS primers. (A–F) Gradient PCR shows varying specificity of ARMS primers in distinguishing (A) *him-5*(*e1490*), (B) *lev-11*(*x12*), (C) *cil-7*(*gk688330*), (D) *klp-6*(*sy511*), (E) *plx-2*(*gk2864*), and (F) C05B5.11(*gk2895*) mutant alleles from the wild type (wt). Gradient temperatures in (A–F) were across 10°;  $T_m$  minus 5° (lowest temperature) to  $T_m$  plus 5°C (highest temperature) as shown in (A) and (B). The  $T_m$  for each primer is indicated in Table S1. (G–I) PCR performed on crude *C. elegans* lysate from wild type (+), mutant (–), and heterozygous animals (+/–) at three temperatures optimal for specificity based on the gradient PCR. The wild type can be distinguished from (G) *lev-11*(*x12*) and (H) *plx-2*(*gk2864*) at temperatures indicated with asterisks, but not (I) C05B5.11(*gk2895*).

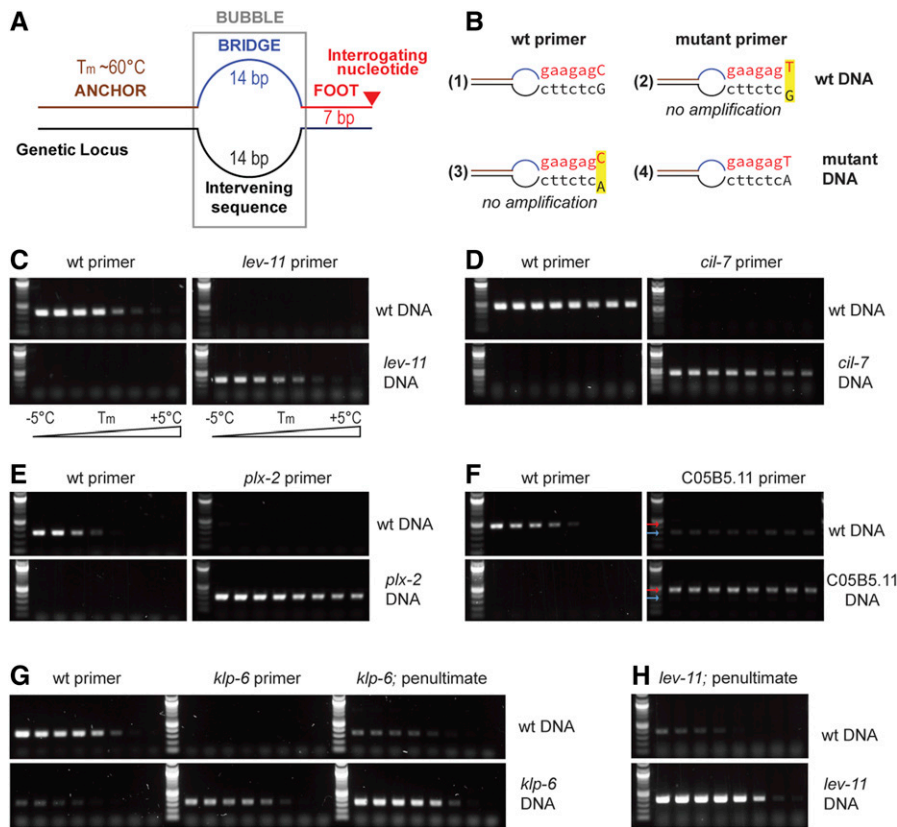
prevents primer-template mismatch repair (Tindall and Kunkel 1988). We designed two allele-specific primers that hybridized to the variant base in either the wild-type or mutant template. Each allele-specific primer was paired with a common primer and gradient PCR reactions were performed to determine the optimal temperature for discriminatory power. As most existing *C. elegans* mutations are transitions due to EMS mutagenesis bias (Flibotte *et al.* 2010), we first focused on differentiating between guanine/cytosine (G/C) to adenine/thymine (A/T) substitutions. While all primers had similar melting temperatures, we found that genetic sequence affected specificity (Figure 1, A–D). ARMS primers discriminated between wild type and *him-5*(*e1490*) across the entire gradient (Figure 1A). However, primers designed to distinguish between wild type and the *lev-11*(*x12*), *cil-7*(*gk688330*), and *klp-6*(*sy511*) alleles were discriminatory only at the highest annealing temperatures when identical concentrations of clean genomic DNA were used (Figure 1, B–D). We also tested the capability of ARMS primers to distinguish between other variants such as T to A in *plx-2*(*gk2864*) and G to T in C05B5.11(*gk2895*). While the primers that detect the *plx-2* and C05B5.11 mutant alleles were specific across the entire temperature range, the primers detecting wild type exhibited only weak selectivity at high annealing temperatures (Figure 1, E and F).

We next determined if ARMS primers could be used for genotyping with crude DNA lysates, which are of lower quality

and contain PCR inhibitors. Using annealing temperatures optimal for specificity based on the gradient PCRs, the wild type could be distinguished from the *lev-11* and *plx-2* mutants as well as heterozygotes over a small temperature interval (Figure 1, G and H). However, it was not possible to distinguish the C05B5.11 mutant from the wild type or heterozygote because at temperatures required for specificity, amplification efficiency was low (Figure 1I). These results demonstrate that ARMS PCR genotyping requires extensive experimentation to identify the optimal annealing temperature and cannot always be used to distinguish between alleles. Furthermore, there is no flexibility in the placement of ARMS primers, which can prevent use of this method for genotyping alleles in regions that contain single nucleotide repeats, A/T rich sequences, or tandem repeats.

### SS primers exhibit discriminatory power for PCR genotyping

We searched for an alternative genotyping method for point mutants and discovered SS primers, which had previously been used for detection of rare variants in qPCR assays (Vargas *et al.* 2016). A SS primer contains a long 5' sequence termed the “anchor” that anneals to the template and is separated from a short 3' “foot” sequence complementary to the region around the mismatch by a “bridge,” which is noncomplementary to the template intervening sequence (Figure 2, A and B). When the primer is hybridized to the template, the bridge



**Figure 2** SS primers exhibit specificity across a broad range of annealing temperatures. (A) Each SS primer contains a 5' anchor (brown), a bridge sequence (blue) noncomplementary to the template (black), and a 3' foot (red) that is perfectly complementary for one allele, but contains a mismatch at the interrogating nucleotide (red triangle) for the nontarget allele. The bridge and intervening sequences form a bubble (gray box). (B) Four annealing schematics illustrate different SS primers used to detect the wild-type vs. the mutant allele in the presence of wild-type and mutant DNA; base pairs in the foot are from the *cil-7(gk688330)* locus. In (1) and (4) there is perfect complementarity between the primer and template. In (2) and (3) there is a mismatch (offset, yellow) at the interrogating nucleotide (capital letter). (C–F) SS primers with an anchor  $T_m$  of  $\sim 60^\circ$ , 14 bp bridge and 7 bp foot discriminate between wild type and (C) *lev-11(x12)*, (D) *cil-7(gk688330)*, (E) *plx-2(gk2864)*, and (F) *C05B5.11(gk2895)* across the entire gradient PCR. In (F), *C05B5.11(gk2895)* mutant (red arrow) and nonspecific (blue arrow) amplification are indicated. (G) The SS primer to detect the wild-type allele from the *klp-6(sy511)* mutant allele is not completely selective; the *klp-6(sy511)* mutant SS primer exhibits perfect selectivity. Placement of the interrogating nucleotide at the penultimate position increases PCR efficiency, but decreases specificity of (G) *klp-6(sy511)* and (H) *lev-11(x12)* mutant SS primers. For (C–H), gradients were across  $10^\circ$ ;  $T_m \pm 5^\circ\text{C}$  as shown in (C).

and intervening sequence in the template form a bubble that separates the anchor from the foot. The terminal 3' nucleotide in the foot, termed the “interrogating nucleotide,” distinguishes the allele variant. Because the foot is short, even a single mismatch can destabilize binding and prevent primer extension.

To test if SS primers could be used for end point PCR to distinguish *lev-11(x12)* from wild type, we designed two allele-discriminating forward primers, one for wild type and the other for the *lev-11* mutant following the rules described by Vargas *et al.* (2016). Each SS primer had an anchor with a melting temperature ( $T_m$ ) of  $\sim 60^\circ$ , a 14 base pair (bp) bridge, and a 7 bp foot with the interrogating nucleotide located at the 3' end. As performed with the ARMS primers, we prepared two sets of PCR reactions in parallel for each genomic DNA. One set of PCR reactions contained the wild-type allele-specific primer with a common reverse primer, while the other set of reactions contained the mutant allele-specific primer with the common reverse primer. We observed a dramatic increase in discriminatory ability of SS primers compared to the ARMS primers as the SS primers that detected the wild-type and *lev-11* mutant alleles were perfectly selective across a wide temperature range (Figure 2C; compare to Figure 1B). *cil-7*, *plx-2*, and *C05B5.11* mutants, which were also poorly distinguished from the wild type with ARMS primers, were successfully discerned with

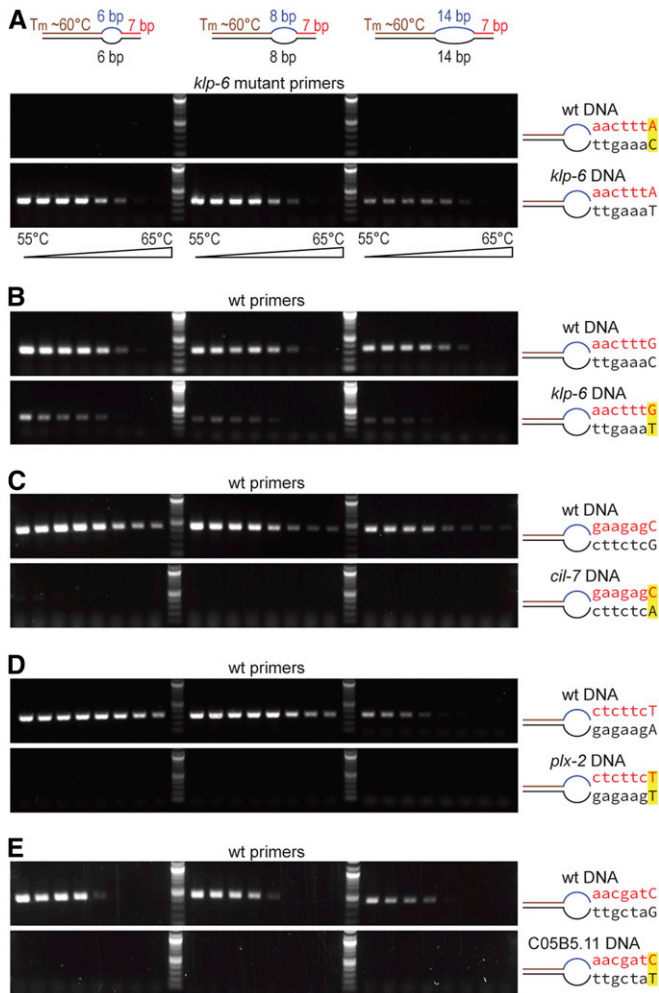
SS primers across all annealing temperatures (Figure 2, D–F). While the SS primer used to distinguish the wild type from the *klp-6* mutant allele did not exhibit complete specificity (Figure 2G), there was significant improvement compared to the ARMS primer (Figure 1D). These results show that SS primers can be used to detect SNSs in different genetic contexts over a broad range of annealing temperatures.

*Taq* polymerase exhibits less efficient amplification when primers contain an A or T at the 3' end instead of a G or C. Since Vargas *et al.* (2016) found that positioning the interrogating nucleotide at the penultimate position did not affect specificity, we added a C, complementary to the template, to the 3' end of the *klp-6* and *lev-11* mutant primers, which have an A and T at the interrogating nucleotide, respectively. While this increased amplification efficiency, it reduced discriminatory power (Figure 2, G and H). Thus, for SS genotyping, the mismatch should be placed at the 3' terminal nucleotide.

### Manipulating the bridge region of the SS primer increases efficiency

Having established the use of SS primers for end point PCR genotyping, we next sought to probe different regions of the primer to develop simple rules for design. All SS primers in Figure 2 contained a 14-bp bridge, with the corresponding intervening sequence also 14 bp, forming a symmetrical





**Figure 3** A decrease in bubble circumference increases PCR efficiency. (A–E) Gradient PCR ( $T_m$  55°–65°) with SS primers to detect the (A) *klp-6*(*sy511*) mutant allele and wild-type alleles at the (B) *klp-6*(*sy511*), (C) *cil-7*(*gk688330*), (D) *plx-2*(*gk2864*), and (E) C05B5.11(*gk2895*) loci. SS primers have an anchor  $T_m$  of  $\sim 60^\circ$ , 7 bp foot and varying bridge sequence length (6 bp, 8 bp, and 14 bp); primer schematics at the top of the figure correspond to all panels. In the primer schematics on the right, the capital letter is the interrogating nucleotide; yellow highlight indicates a mismatch.

bubble. To determine how bubble circumference impacts SS primer efficiency we designed SS primers to distinguish the wild type and *klp-6* mutant allele, each with an anchor  $T_m$  of  $\sim 60^\circ$  and 7 bp foot sequence, but different symmetrical bubbles. Comparison of SS primers with 6, 8, and 14 bp bridge sequences for both the wild-type and *klp-6* mutant alleles showed that the smallest bubble circumference resulted in the greatest amplification efficiency (Figure 3, A and B). However, the wild-type SS primer containing the 6 bp bridge sequence was nonspecific across all annealing temperatures (Figure 3B). SS primers with 6, 8, and 14 bp bridge sequences maintained specificity in distinguishing the wild type from the *cil-7*, *plx-2*, and C05B5.11 mutants, with the SS primers containing a 6 bp bridge exhibiting the greatest efficiency (Figure 3, C and E). This suggests that, irrespective of the 3'

mismatch and sequence context, smaller bubble circumference corresponds with an increase in amplification.

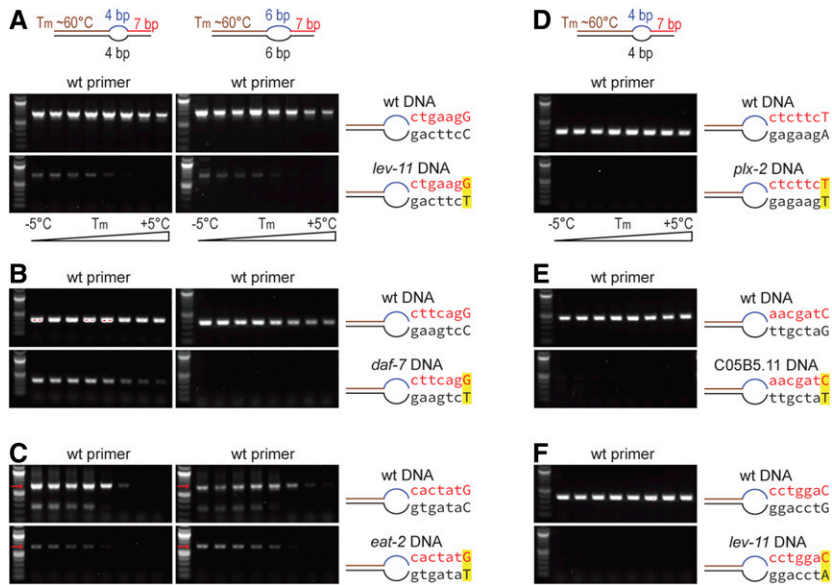
### Primer–template mismatch at the interrogating nucleotide impacts specificity

The wild-type SS primer, which forms a G-T mismatch with the *klp-6* mutant sequence was less specific than all other SS primers tested (Figure 3). This purine-pyrimidine mismatch has similar geometry to G-C and A-T base pairings, causing only a weak destabilizing effect, which enables it to be extended more efficiently by *Taq* polymerase than any other mismatch (Huang *et al.* 1992; Rejali *et al.* 2018). To determine whether the nonspecificity of the SS primer used to detect the wild-type allele at the *klp-6* locus was due to the weak G-T primer-template mismatch or the flanking sequence, we designed SS primers with short bridge sequences to distinguish wild type from the *lev-11*(*x12*), *daf-7*(*e1372*), and *eat-2*(*ad465*) G to A transition mutants. SS primers with a 6 bp bridge sequence specifically detected the wild-type allele across all annealing temperatures at the *daf-7*, but not *lev-11* and *eat-2* mutant loci. Specificity worsened when the bridge sequence was shortened to 4 bp for all G-T primer-template mismatches (Figure 4, A–C). SS primers used to discriminate the wild-type allele from *plx-2*(*gk2864*), C05B5.11(*gk2895*) and *lev-11*(*x12*), which result in T-T, C-T and C-A primer-mutant template mismatches respectively, were specific even with a short 4 bp bridge (Figure 4, D–F). These results demonstrate that the limited specificity of the primer detecting the wild type allele from *klp-6*(*sy511*) was likely due to the G-T primer–template mismatch. Further, these data show that the minimum circumference of the bubble needed to achieve specificity depends on the mismatch at the interrogating nucleotide, and that additional considerations may need to be taken when designing SS primers that form a G-T mismatch with the nontarget template.

### The foot region of the SS primer can be manipulated to increase specificity

We next investigated how the length of the foot impacts efficiency and specificity using SS primers that detect the wild-type allele at the *klp-6* locus. The initial wild-type detecting primer contained a 7 bp foot sequence (Figure 2G). We discovered that shortening the foot to 5 or 6 bp decreased efficiency without affecting specificity (Figure 5A). A SS primer with a 4 bp foot did not produce any product (Figure 5A) even at a low 45° annealing temperature (data not shown). These results show that shortening the foot to  $<7$  bp provides no benefit in terms of specificity and hinders amplification efficiency.

Since shortening the foot sequence had an undesirable effect on amplification, we sought to determine if additional mismatches in the foot could be used to increase SS genotyping specificity. For three SS primers that previously lacked specificity (Figures 3B and 4, B and C), we introduced an



**Figure 4** Guanine-thymine primer-template mismatches are the least discriminatory. (A–C) SS primers with an anchor  $T_m$  of  $\sim 60^\circ$ , 7 bp foot, and either 4 bp or 6 bp bridge sequence cannot perfectly distinguish the wild type from (A) *lev-11(x12)*, (B) *daf-7(e1372)*, and (C) *eat-2(ad465)* mutant alleles. Schematics at the top of the figure correspond to all panels below. In (A–C), the SS primer-template mismatch at the interrogating nucleotide is G–T (yellow); intervening sequence length is equal to bridge sequence length (4:4 and 6:6). In (C), the red arrow indicates the band at the expected size. (D–F) SS primers with a 4 bp bridge sequence exhibit complete specificity when the interrogating nucleotide mismatch is (D) T–T in the *plx-2(gk2864)* mutant, (E) C–T in the *C05B5.11(gk2895)* mutant, and (F) C–A in the *lev-11(x12)* mutant. The SS primer to detect the wild-type from *lev-11(x12)* mutant allele in (F) anneals to the opposite strand compared to (A). Gradient temperatures in (A–F) were  $T_m \pm 5^\circ$  as shown in (A) and (D).

additional mismatch at the penultimate position to the interrogating nucleotide, which we designate the (–1) position. Placing a G–A mismatch, which has a strong destabilizing effect (Rejali *et al.* 2018), at the (–1) site prevented amplification of the wild-type allele at the *kfp-6(sy511)* locus, while introduction of a weak C–A purine-pyrimidine mismatch at the same position resulted in allele-specific amplification across all annealing temperatures (Figure 5B). Introduction of a weak A–C purine-pyrimidine mismatch at the (–1) position in SS primers that previously could not distinguish wild type from *daf-7* and *eat-2* mutant alleles also led to specificity across the entire gradient (Figure 5, C and D). Likewise, introduction of a purine-pyrimidine mismatch terminal to the interrogating nucleotide also generated specificity (Figure 5, C and D). This demonstrates that placement of an additional weak destabilizing mismatch either penultimate (–1) or terminal (+1) to the interrogating nucleotide increases discriminatory power.

### SS primers enable flexibility in anchor placement

In primer design, it is important to avoid single nucleotide repeats, A/T rich domains, tandem repeats, and sequences that form secondary structure. Lack of flexibility in the positioning of the primer can make detection of certain point mutations by ARMS PCR difficult. We considered that increasing template intervening sequence length would allow for anchor placement flexibility, provided that a lower annealing temperature was used to accommodate the topological restraint caused by an asymmetric bubble. To determine how an asymmetric bubble affects amplification, we designed a SS primer with a 6 bp bridge to a 24 bp intervening sequence (6:24) and a 7 bp foot to distinguish wild type from the *lev-11* mutant and observed specific amplification across the entire gradient (Figure 6A). However, we saw little amplification when SS primers with 6:24 and 6:30 asymmetric bubbles

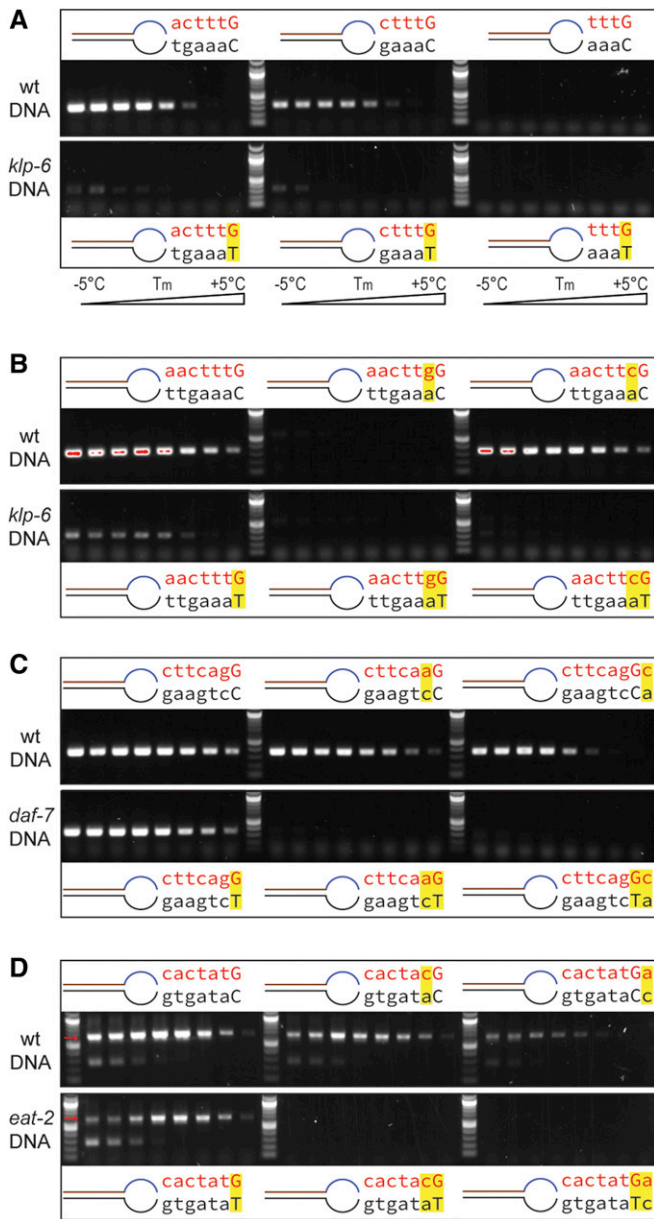
were used for detection of the wild-type allele at the *kfp-6* and *cil-7* loci, respectively (Figure 6, B and C). Increasing foot length to 8 bp, with a C in the 3' terminal position improved the efficiency of these SS primers without affecting discriminatory power (Figure 6, B and C). Since the sequence surrounding *daf-7(e1372)* is A/T rich, we created a SS primer that forms an extremely asymmetric 6:51 bubble, and this primer perfectly discriminated the wild-type from the *daf-7* mutant allele across all gradient temperatures. Although increasing the length of the intervening sequence required an increase in foot length for efficient amplification, these results demonstrate that the anchor of SS primers can be moved to enable genotyping in difficult sequence contexts.

### SS primers can be used to genotype crude *C. elegans* DNA lysates

In order for SS genotyping to be used routinely, it must work not only on the pure lysates that were used to optimize primer design, but also on crude DNA preparations. We performed single worm lysis, and, for every sample, we set up two PCR reactions, each with a different allele-specific primer and the common reverse primer (Figure 7A). The allele-specific SS primers were able to clearly distinguish homozygous wild-type animals from *kfp-6(sy511)*, *eat-2(ad465)*, *cil-7(gk688330)*, and *lev-11(x12)* mutants and heterozygotes (Figure 7, B–E). This robust differentiation of the genotypes, even when using crude single-worm lysate, demonstrates the power of this method for routine genotyping.

### Discussion

We developed a rapid, low-cost method for detection of point mutants by optimizing SS primers for end-point PCR. Our analyses using seven different genes and eight different types of primer–template mismatches show that SS primers can be



**Figure 5** SS primer foot length and sequence influence efficiency and specificity. (A) Decreasing foot length from 6 bp (left) to 5 bp (middle) decreases efficiency; a 4 bp foot (right) eliminates amplification. In (A and B) primers detect the wild-type allele at the *klp-6*(*sy511*) locus; bridge is 6 bp. (B) The wild-type detecting SS primer is not completely selective (left). A strong G-A primer-template mismatch at the penultimate (−1) position to the interrogating nucleotide prevents amplification (middle), whereas a weak C-A mismatch at (−1) improves specificity. (C and D) Introduction of a weak purine-pyrimidine primer-template mismatch at the (−1) (middle) or (+1) position (right) increases specificity of SS primers with a 4 bp bridge that detect the wild-type allele at the (C) *daf-7*(*e1372*) and (D) *eat-2*(*ad465*) loci. In (D), the red arrow indicates the expected amplicon size. For (A–D) schematics, the interrogating nucleotide is capital and mismatches are highlighted in yellow; gradient PCR temperatures were  $T_m \pm 5^\circ$  as shown in (A).

used universally for genotyping over a broad range of annealing temperatures. We discovered that balancing stabilizing vs. destabilizing factors in the foot region affects specificity,

while decreasing bridge length increases efficiency. Amplification occurs even when the SS primer bridge and intervening template sequence form an asymmetric bubble, allowing for flexibility in anchor placement. Our work demonstrates the power of SS primers for routine genotyping on crude worm lysates, and we propose that this method could also be used for SNP mapping and screening of CRISPR mutants.

### Effect of specific primer–template mismatches on PCR specificity

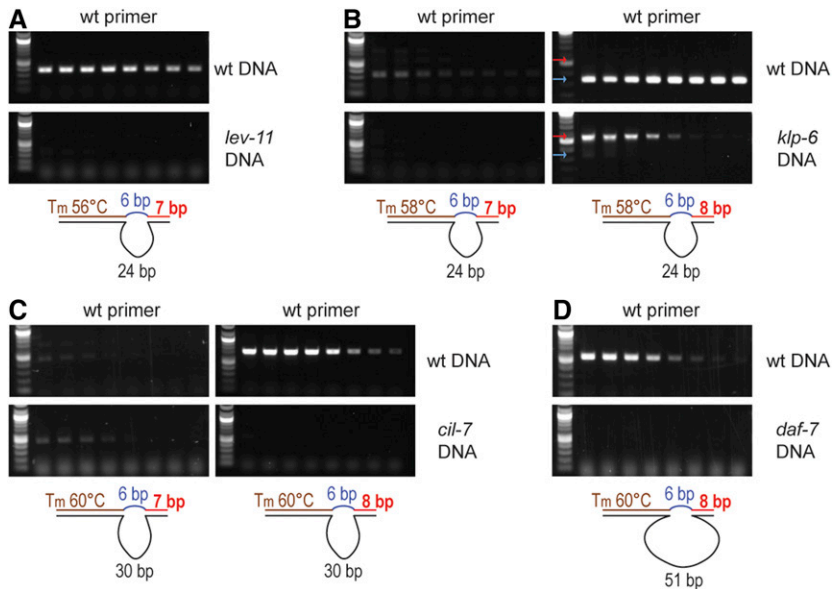
A single 3' terminal mismatch destabilizes primer–template interaction, and, as *Taq* DNA polymerase does not possess 3' to 5' exonuclease activity for mismatch repair, this mismatch reduces extension efficiency when compared with a primer perfectly complementary to the template (Petruska *et al.* 1988; Tindall and Kunkel 1988; Huang *et al.* 1992; Rejali *et al.* 2018). While this serves as the foundation for SS genotyping, PCR amplification is also influenced by the specific primer–template mismatch, with purine-purine mismatches being the most inhibitory, and purine-pyrimidine mismatches being the least inhibitory (Huang *et al.* 1992; Rejali *et al.* 2018). EMS, the primary chemical mutagen used for forward genetic screens in *C. elegans*, exhibits a mutagenesis bias toward G/C to A/T transitions (Flibotte *et al.* 2010). When genotyping EMS-generated alleles, a G at the interrogating nucleotide of the wild-type-detecting primer mismatches with a T in the mutant template. Here, we found that primers with a G-T mismatch were less selective than those with T-T, C-T, and C-A mismatches, consistent with the G-T mismatch being the least inhibitory (Huang *et al.* 1992; Rejali *et al.* 2018). As previously reported for extension rate (Huang *et al.* 1992), we also observed that sequence context influenced end-point PCR genotyping for G-T mismatches.

To decrease extension efficiency, and thus improve PCR specificity, an additional mismatch can be introduced either penultimate or terminal to the interrogating nucleotide (Ugozzoli and Wallace 1991; Bui and Liu 2009). Some purine-purine penultimate mismatches such as G-A inhibit extension efficiency even more than a 3' terminal G-T mismatch (Rejali *et al.* 2018). In fact, we found that a G-A mismatch at the penultimate position in the SS primer to detect the wild-type allele at the *klp-6* locus prevented amplification. Thus, if introducing an additional mismatch at the penultimate position, strong G-A, G-G, A-A, and C-C primer-template mismatches should be avoided, while weak G-T and C-A mismatches are tolerated.

### SS genotyping offers distinct advantages compared to other methods

Here, we consider how SS primers compare with other existing allele discrimination methods. Mutations that result in creation or disruption of a restriction site can be detected by amplification of the template from the target region





**Figure 6** Asymmetric bubble design allows for flexibility in anchor placement. (A) A SS primer with a 6 bp bridge, which forms an asymmetric bubble with a 24 bp template intervening sequence (6:24), detects the wild-type allele at the *lev-11(x12)* locus. (B and C) Asymmetric bubbles result in poor amplification of the wild-type allele at the *klp-6(sy511)* and *cil-7(gk688330)* loci (left panels); efficiency is improved by increasing foot length to 8 bp. In (B) wild-type (blue arrow) and nonspecific (red arrow) amplification are indicated. (D) A SS primer which forms a 6:51 asymmetric bubble with the template detects the wild-type allele at the *daf-7(e1372)* locus. For (A–D) gradient PCR  $T_m$  45°–55°.

followed by enzymatic digestion of the DNA and electrophoresis. However, genotyping of many different alleles by this method requires a large collection of different restriction enzymes, and a suitable restriction enzyme or artificial restriction site cannot be introduced at all locations. SS genotyping can be used to distinguish between single nucleotide variants in all sequence contexts and does not require reagents or effort beyond PCR. Further, unlike single-base extension genotyping (Sauer *et al.* 2000; Treweek *et al.* 2011), the 5' fluorogenic nuclease Taqman assay (Livak *et al.* 1995; Callegaro *et al.* 2006), and melting curve analysis of FRET probes (Livak 1999; Combrinck *et al.* 2013), expensive equipment and specialized training are not required for design and use of SS primers for allele detection.

Similar to SS genotyping, ARMS PCR and modified simple allele-discriminating PCR are inexpensive methods that utilize allele-specific oligonucleotide primers (Bui and Liu 2009; Medrano and De Oliveira 2014). However, when genotyping *C. elegans* point mutants, we found that ARMS PCR required extensive troubleshooting to determine optimal annealing temperature and could not always be used to distinguish between alleles. While the specificity of ARMS PCR can be increased by introducing an additional mismatch one to three bases upstream of the 3' mismatch, extensive experimentation is required to determine which position and mismatch will allow for allele discrimination without inhibiting amplification (Bui and Liu 2009). Here, we have shown that addition of a second purine-pyrimidine mismatch at the (–1) position improved specificity without impacting efficiency for multiple SS primers that create a weak G-T mismatch with the nontarget allele template, suggesting that this is a simple rule to follow when designing SS primers. Finally, the placement of ARMS primers is restricted by the position of the mutation, which makes allele detection difficult in certain sequence locations (Medrano and De Oliveira 2014). The flexibility in the positioning of the anchor

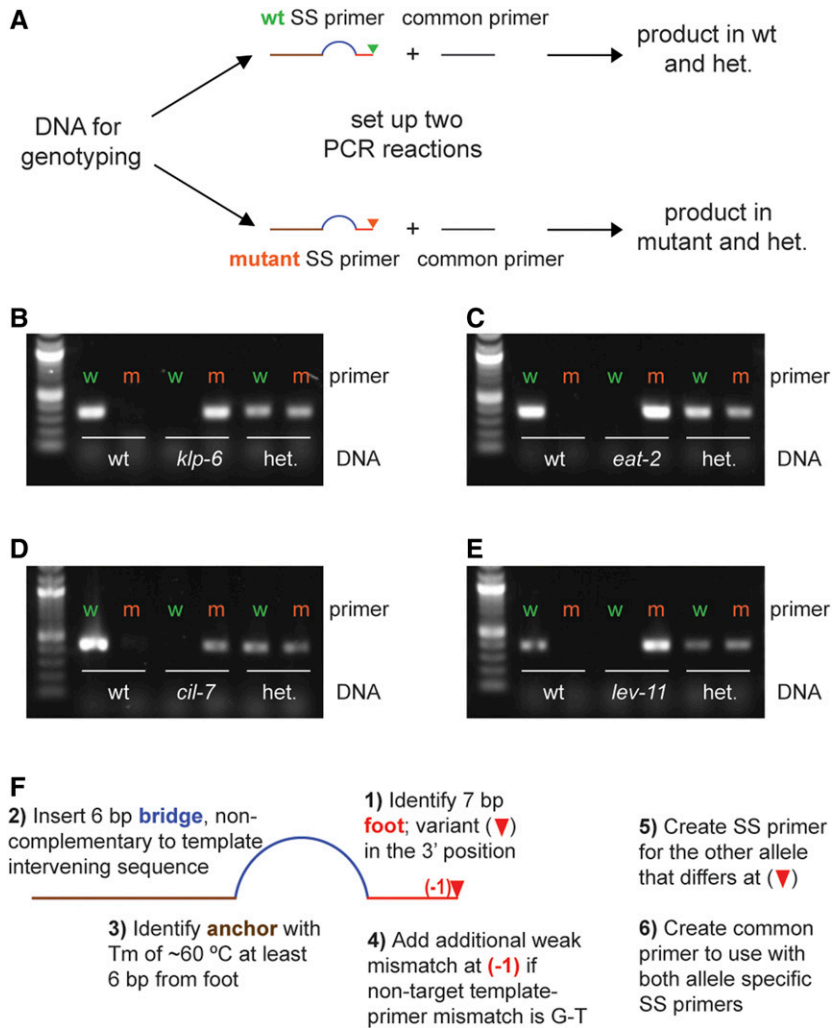
by creating an asymmetric bubble enables SS primers to be used for genotyping in all regions, even those with sequences that are challenging for standard primer design.

Tetra-primer ARMS PCR utilizes two allele-specific internal primers and two external primers that are different distances from the SNP to generate allele-specific fragments of distinct sizes. This method has been utilized successfully in *C. elegans* and presents a distinct advantage as wild type, mutant, and heterozygous animals can be distinguished in a single PCR reaction (Sullenberger and Maine 2018). However considerable time is required to establish the reaction conditions for tetra-primer ARMS PCR as both the wild type and mutant primer sets must first be tested on worm lysates across a temperature gradient. Even after an optimal temperature is identified, one product may be preferentially amplified in the tetra-primer reaction, which can complicate genotyping analyses. We performed extensive troubleshooting of tetra-primer SS PCR, but observed preferential amplification of certain products, nonspecific bands, and primer dimers, all of which were likely due to the complexity of the internal SS primers (data not shown). Nevertheless, we have shown that two SS PCR reactions can be performed easily on a single lysed worm, demonstrating that this method can be used for routine genotyping. Further, since SS genotyping is specific across a broad temperature range, in most cases there is no need to optimize the annealing temperature.

### Simple instructions for SS primer design

We have defined several important factors to consider for SS genotyping (Figure 7F, Supplementary Figure S1). To design a SS primer, first identify a 7-bp foot with the interrogating nucleotide in the 3' position. Second, identify a 5' anchor sequence with ~50% G/C content and a  $T_m$  of 50–60° that is at least 6 bp away from the foot. In many cases, a symmetric bubble consisting of a 6 bp bridge between the anchor and foot in the primer that is noncomplementary to the





**Figure 7** SS genotyping of crude *C. elegans* lysates. (A) Two PCR reactions are set up for each DNA lysate; each reaction contains an allele specific primer and common reverse primer. (B–E) Single worm lysates, each split across two PCR reactions, differentiate between homozygous wild type, homozygous mutant, and heterozygotes animals at the (B) *klp-6*(*sy511*), (C) *eat-2*(*ad465*), (D) *cil-7*(*gk688330*), and (E) *lev-11*(*x12*) loci. In (B–E) the allele-specific primer used in each reaction, either the wild type (w, green) or the mutant (m, orange), is indicated;  $T_m$  50° for all PCR reactions. (F) Simple instructions for SS primer design; additional guidelines can be found in Figure S1.

corresponding 6 bp intervening sequence in the template provides both good efficiency and specificity. The intervening sequence length can be increased to enable placement of the anchor in a more favorable position; however, for primers with extremely asymmetric bubbles, the foot must be extended to increase amplification efficiency. Next, consider the mismatch between the primer and nontarget allele at the interrogating nucleotide. A G-T mismatch will reduce capability to distinguish between alleles. To decrease undesired stability between the primer and nontarget template, a second G-T or C-A mismatch can be introduced either penultimate or terminal to the interrogating nucleotide. Finally, design another SS primer to detect the other allele as well as a common reverse primer, and make sure that the primers do not have secondary structure using the IDT OligoAnalyzer. Additional primer design considerations and troubleshooting guidelines can be found in Figure S1.

While we have used gradient PCR to examine the properties of SS primers, given that specificity is generally observed across a broad range of annealing temperatures, we recommend using the anchor sequence annealing temperature minus 5° for routine genotyping. No more than 30 cycles should be performed since

the number of amplicons produced by the perfectly matched primer should reach plateau by this point and undesired products will continue to be amplified exponentially in additional cycles (Saiki *et al.* 1988). Finally, *Taq* polymerase must be used since proofreading enzymes such as Phusion will correct the 3' mismatch, resulting in a loss of discriminatory power.

In conclusion, SS genotyping is (1) low cost, (2) does not require special equipment, (3) works over a broad range of annealing temperatures, (4) allows for flexibility in primer placement, and (5) can be performed on crude DNA lysates. SS primers can theoretically be utilized for genotyping in other organisms and for any laboratory applications that require discernment between alleles.

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