An Efficient Technique for Primer Development and Application that Integrates Fluorescent Labeling and Multiplex PCR

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Protocol Note

An efficient technique for primer development and application that integrates fluorescent labeling and multiplex PCR[1]

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- *Premise of the study:* Development of genetic markers can be costly and time-consuming, especially when multiple primer pairs are fluorescently labeled. This step was streamlined by combining two techniques in the same PCR reaction: (1) custom-labeling of primers by the investigator and (2) multiplexing multiple primers together in the same reaction.
- *Methods and Results:* This technique was successfully used to develop microsatellite markers in several plant species. Microsatellites amplified with this multiplexing process were identical to those generated from PCR using individual primer pairs and with traditional methods using a priori labeled fluorescent primers. Tests of PCR cycling programs revealed that conditions recommended for the commercial kit generated stronger fragment peaks than the previously recommended cycling protocol.
- *Conclusions:* This technique is an efficient and economical way to fluorescently label multiple microsatellite primers in the same reaction. It is also applicable to other markers used in PCR amplification of genetic material.

Key words: fluorescent labeling; microsatellites; multiplexing; primer testing; thermocycler conditions.

Molecular markers developed for plant and animal species have been instrumental in answering many biological questions about the natural world, including, for example, those focusing on mating systems, effective population sizes, speciation, and taxonomy. Within the past decade, microsatellites have often been the marker of choice for many of these types of studies; however, in most cases, development of these markers is typically time-consuming and expensive (e.g., Squirrell et al., 2003). There are several techniques for developing these markers, including the traditional approach using bacterial colonies containing microsatellite inserts or newer methods involving next-generation sequencing. Regardless of the initial approach, putative primers must eventually be designed and tested (Zane et al., 2002). One of the most costly steps in this process is the fluorescent labeling of individual microsatellite primers, with costs varying depending upon the dye used (e.g., 6-FAM, VIC, NED) and the manufacturer. Costs further increase both in terms of necessary consumables and labor if PCR and fragment analysis are conducted separately for each individual primer and for every single sample.

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Here we expand upon a method, first mentioned in Culley et al. (2008), to substantially increase efficiency while reducing costs during primer development by combining custom-labeling of primers by the investigator *during* the PCR reaction while also multiplexing multiple primer sets together for each sample. This is similar to the technique recently published by Blacket et al. (2012) but which uses different primers. Both methods greatly expand the technique suggested by Oetting et al. (1995), which only allowed up to five primer pairs to be multiplexed using a single universal primer. Our method also substantially advances the simplified protocol for single-dye labeling proposed by Schuelke (2000) as the "poor man's approach to genotyping." We have now extended this process to analyze multiple primers in the same reaction using four different universal primers tagged with different fluorescent dyes; this process increases speed and economy by reducing preparation time and the amount of PCR reagents. The technique also allows the investigator to alter fluorescent label colors associated with any number of primers during primer testing to develop the best possible combination of primers and associated labels within a single multiplex mix. Using this method, many different pairs of primers can be run in a single PCR reaction, with the number only limited, at least theoretically, by the number and range of fragment sizes. This method differs from previous "multiplexing" techniques (e.g., Ottewell et al., 2005; Missiaggia and Grattapaglia, 2006) in which primers were labeled in separate PCR reactions for each sample, with the resulting PCR products combined together ("pooled") by the researcher prior to fragment analysis.

Both this method and the independently derived technique of Blacket et al. (2012) involving fluorescent labeling and multiplex PCR have since been adopted by researchers (see Table 1), indicating the utility and applicability of this overall procedure across study systems. Our report adds to that of Blacket et al. (2012) in that we have expanded their conclusions by testing different thermocycler running conditions, addressing concerns that lower primer annealing temperatures in the multistep PCR recommended in earlier protocols (e.g., Oetting et al., 1995; Schuelke, 2000) could lead to spurious peaks and inconsistent intensities of amplified fragments (Shimizu and Yano, 2011; Blacket et al., 2012). We investigated the presence and effect of such spurious peaks and background noise of the two-step PCR process recommended by Schuelke (2000) compared to the single-step PCR cycling program recommended by QIAGEN, the manufacturer of the multiplexing kit used in this protocol. In addition, we tested the effect of cycle number in the PCR reaction on peak amplitude and also provide a step-by-step protocol (see Appendix 1).

METHODS AND RESULTS

In this method, forward and reverse primers are first developed for each putative microsatellite fragment (e.g., using next-generation techniques or following traditional methods such as Zane et al., 2002). To test the primer pairs, each microsatellite fragment is then amplified using three primers (a "triplet") added to the same PCR reaction: (1) a *forward tailed primer* that is tagged with a specific DNA sequence (the "tail") attached to the 5′ end; (2) a *labeled tail primer* consisting of the same tail sequence but with a fluorescent label attached on the 5′ end; and (3) the unaltered *reverse primer* (Fig. 1; Appendix 1). The fragment is amplified in the first set of PCR cycles with the forward tailed primer and the reverse primer, thus incorporating the tail sequence. In the next set of PCR cycles, the labeled tail anneals to the tail sequence and, together with the reverse primer, amplifies the fragment and in doing so incorporates the desired fluorescent label. Two elements of this protocol have been recommended by Schuelke (2000) as necessary to ensure that the forward tailed primer is incorporated before the labeled tail. First, the forward tailed primer is supplied at a reduced (1/4) concentration so it is used up in the first set of cycles. Second, a higher primer annealing temperature (57°C) is suggested for the first set of cycles to enhance binding of the forward tailed primer; the temperature is then reduced (53°C) for the second set of cycles to facilitate annealing of the larger labeled tail.

The tail can be any unique DNA sequence that is not part of the plant genome being examined and will not interfere with other primers in the reaction. Previous investigations with individual reactions have used the M13(–21) sequence (Oetting et al., 1995; Schuelke, 2000; Boutin-Ganache et al., 2001; Scott et al., 2001; Ottewell et al., 2005), human microsatellites (Missiaggia and Grattapaglia, 2006), or alterations of genome-specific primers (Neilan et al., 1997). Multiplexed approaches have also involved customized primers such as bar-coded split tags (Shimizu and Yano, 2011) or a combination of specialized universal primers with DNA adapters commonly used in 454 DNA sequencing protocols (Blacket et al., 2012). In our studies of plant species, we have successfully used M13(–21) (TGTAAAACGACGGCCAGT), T7term (CTAGTTATTGCTCAGCGGT), and two modifications of M13(–21) developed by our laboratory: M13modA (TAGGAGTGCAGCAAGCAT) and M13modB (CACTGCTTAGAGCGATGC). Each of these sequences was tagged at the 5′ end with a different color of fluorescent label (6-FAM/blue, NED/yellow, VIC/green, or PET/red). The choice of label and color is up to the investigator's discretion and the requirements of the fragment analysis facility, so that overlapping PCR products are labeled with different dye colors.

When first testing this method in a new system, it is critical for investigators to verify correct operation of the primers. Separate PCRs should use (1) unlabeled forward and reverse primers to make sure they are designed well and amplify fragments; (2) the forward tailed primer and the reverse primer only to make sure they also amplify fragments; (3) the labeled tail alone to verify that it does not amplify any product. Fragments resulting from this method should consist of the original size plus the length of the tail sequence (18–19 bp).

To expand the applicability of the earlier single-primer tagging method so investigators could fluorescently label multiple primers concurrently in the same PCR reaction and analyze multiple loci at once, we used the QIAGEN PCR Multiplexing Kit (catalog no. 206143; QIAGEN, Valencia, California,

Table 1. Species for which the dual labeling and multiplexing method was used in the primer testing step of the microsatellite development process.

Species (Family)	Habit	Total no. of primer pairs	No. of primer groups	Max no. of primer pairs per group	Repeat motif type	Size range (bp)	*A*[a] (across all loci)	Reference
Erythronium americanum Ker Gawl. (Liliaceae)	herb	10	2[b]	6	di, tri, tetra	150–270	1–8	Stokes and Culley, unpublished data
Marchantia inflexa Ness & Mont. (Marchantiaceae)	liverwort	12	2	4	di, tri	150–286	1–5	Brzyski et al., 2012
Monotropa hypopitys L. (Ericaceae)	herb	11	3	4	di, tri	176–283	1–6	Klooster et al., 2009
Pericopsis elata (Harms) Meeuwen (Fabaceae)	tree	13	2	13	di, tri	132–320	1–11	Micheneau et al., 2011
Protea punctata Meisn. (Proteaceae)	herb	10	3	5	di, tri	85–455	8–49	Prunier and Latimer, 2010
Rhamnus cathartica L. (Rhamnaceae)	shrub	15	3	6	di, tri, penta	136–276	1–14	Culley and Stewart, 2010
Santiria trimera (Oliv.) Aubrév. (Burseraceae)	tree	7	1	6[c]	di, tri	156–327	1–5	Koffi et al., 2012
Schiedea adamantis H. St. John (Caryophyllaceae)	subshrub	12	2	8	di, tri, penta, hexa	175–267	1–9	Culley et al., 2008
Spiraea virginiana Britton (Rosaceae)	shrub	11	3	4	di, tri, penta	100–312	1–4	Brzyski, 2010; Brzyski and Culley, 2011
Tiarella polyphylla D. Don (Saxifragaceae)	herb	9	1	9[d]	di	100–332	3–8	Jeong et al., 2012
Veratrum stamineum Maxim. (Melanthiaceae)	herb	10	1	10[d]	di	82–506	8–16	Kikuchi and Maki, 2011

[a] Number of alleles per locus.

[b] The PCR products from all primer groups were combined together prior to fragment analysis.

[c] Only three of these primers were developed with the current protocol but were then multiplexed together with the other four directly labeled primers.

[d] Three of the four universal primers used in this study were the same as in the current protocol.

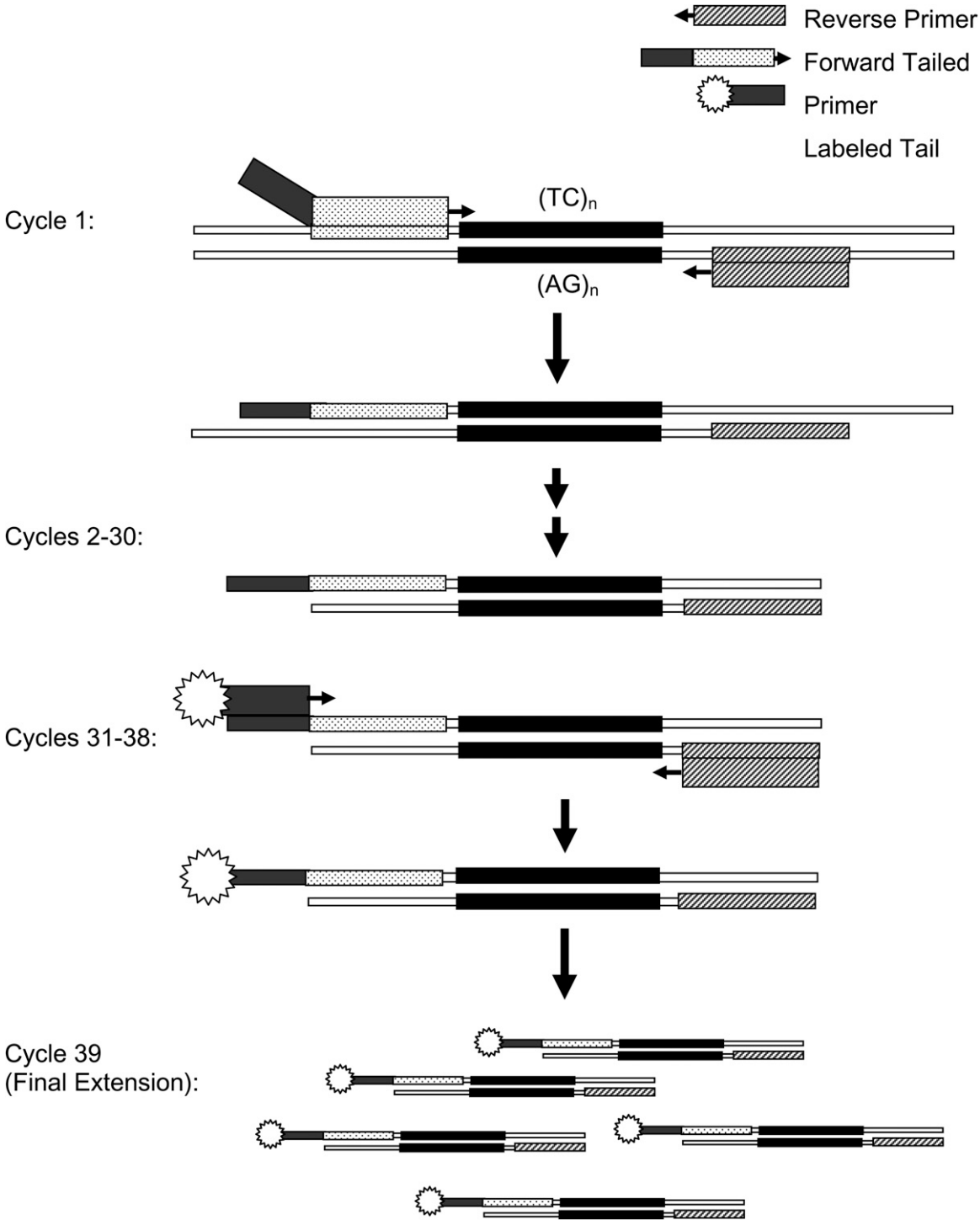
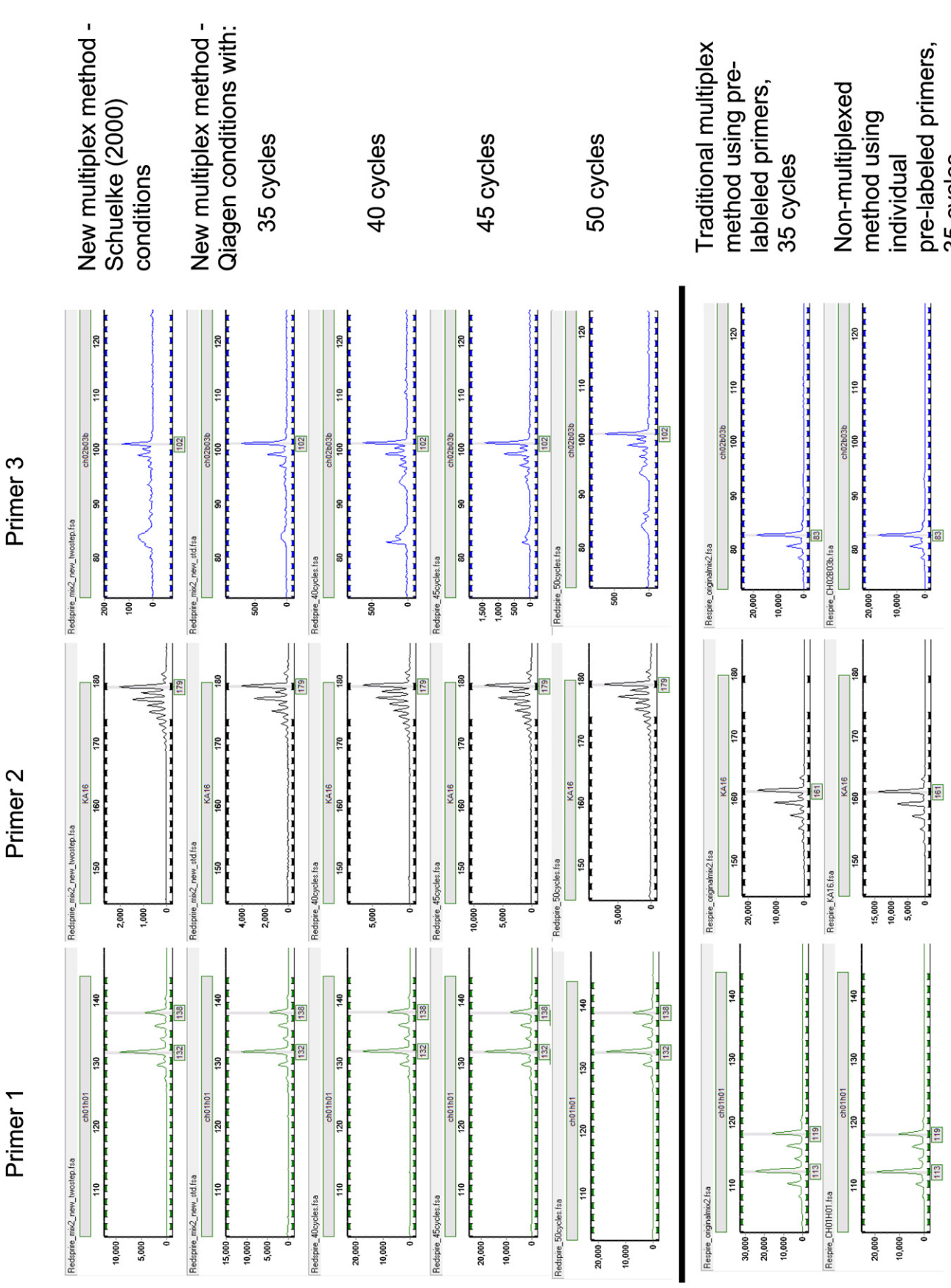


Fig. 1. Diagram of the dual primer labeling and multiplexing method. In the first PCR cycle, a microsatellite is amplified using a forward primer incorporating a unique tailed sequence (the forward tailed primer) and a reverse primer. PCR continues for cycles 2–30 until there are multiple copies of the same fragment. In cycles 31–38, a primer incorporating only the unique tailed sequence attached to a fluorescent tag (labeled tail) is combined with the reverse primer to label these same fragments with the fluorescent label. Cycle 39 consists of a final extension to ensure that the majority of fragments are properly amplified and labeled. (Adapted from Schuelke, 2000: fig. 1.)

USA). This kit includes HotStarTaq DNA Polymerase and a specific PCR buffer containing QIAGEN's synthetic factor MP, which is purported to stabilize bound primers and enable efficient extension of all primers in a reaction without any optimization. For our purposes, we did not use QIAGEN's Q-Solution (included in the kit), which reportedly helps amplify GC-rich templates and might be helpful in other systems. For multiplexing, we used multiple sets of the triplet primers suggested by Schuelke (2000), with each triplet specific to a different microsatellite fragment of a predicted size (obtained during primer development). If any of these triplets in the same multiplex PCR reaction were predicted to amplify similar fragments of overlapping sizes, the tail for each



triplet was selected to correspond to a unique color for that particular fragment size range (to avoid making spurious allele calls resulting from the overlapping ranges of like-colored primer labels). Multiplexing was then conducted in 10-μL reaction volumes using the QIAGEN Multiplex Kit as follows: 5 μL Multiplex PCR Master Mix, 1 μL Primer Mix (consisting of 2 μM reverse primer, 2 μM labeled tail primer, and 0.5 μM forward tailed primer per triplet set), 3.8 μL H_2O, and 0.2–0.3 μL DNA.

As with other techniques, different primers may sometimes anneal to each other during multiplexing, reducing amplification success during PCR. This occurred in a few species tested (e.g., Klooster et al., 2009; Brzyski, 2010) and was corrected by redesigning primers or running conflicting primer sets in different primer groups. Potential primer-dimer binding can also be examined a priori using primer software programs, such as AmplifX (Jullien, 2013), OLIGO (Molecular Biology Insights, Cascade, Colorado, USA; http://www.oligo.net/index.html), FastPCR (Kalendar et al., 2011), or Multiplex Manager (Holleley and Geerts, 2009).

We also compared the PCR thermocycler program suggested by Schuelke (2000) to the cycling program recommended for the multiplexing kit. First, to test Schuelke's (2000) protocol, our PCR cycling conditions were as follows: initial denaturation of 95°C for 15 min, followed by 30 cycles each of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s, and then with eight cycles each of 94°C at 30 s, 53°C for 45 s, and 72°C for 45 s. A final extension consisted of 72°C for 10 min. To test the PCR cycling program recommended for the QIAGEN kit, the thermocycler cycling conditions were as follows: initial denaturation of 95°C for 15 min, followed by 35 cycles each of 94°C for 30 s, 57°C for 90 s, and 72°C for 60 s, followed by a final extension of 60°C for 30 min. Although QIAGEN recommends 35–45 cycles for optimal results with their kit, our earlier work indicated that 35 cycles was adequate for full peak expression while minimizing the time necessary for a given PCR reaction. Because differences in peak amplitude were subsequently observed between the two reaction methods (see below), we further examined the effect of cycle number (N = 35, 40, 45, 50) on peak intensity using the QIAGEN thermocycler conditions. All fragment analysis was conducted at Cornell University's Life Sciences Core Laboratory Center on an 3730xl DNA analyzer (Applied Biosystems, Carlsbad, California, USA) using the GeneScan 500 LIZ Size Standard (Applied Biosystems). Fragments were then analyzed with GeneMarker version 1.85 (SoftGenetics, State College, Pennsylvania, USA).

To illustrate the feasibility and effectiveness of this new method of primer design, we compared it with traditional methods using primers that had already been prelabeled with the fluorescent tag and those used in (1) multiplexed reactions or (2) with each primer pair run individually. Using 24 DNA samples obtained from wild and cultivated individuals of *Pyrus calleryana* Decne. (see Culley and Hardiman, 2009) and the QIAGEN-recommended thermocycler program described above, we found that the new method consistently identified the same amplified fragments for four different loci with complete accuracy (i.e., identical allele calls were obtained as for individual PCR or the multiplexed PCR with prelabeled primers; Fig. 2). Multiplexed samples that underwent the two-step PCR program recommended by Schuelke (2000) generally exhibited weaker peaks of lower intensity (Fig. 2A) than if the same samples were subjected to the QIAGEN-recommended PCR cycling program (Fig. 2B–E). In several cases, the two-step PCR resulted in occasional spurious peaks and more background noise, as previously suggested (e.g., Shimizu and Yano, 2011; Blacket et al., 2012). However, the strongest peaks with the highest intensity (Fig. 2F) were still obtained using prelabeled fluorescent primers that were multiplexed together in the same PCR reaction, with fragment sizes 18–19 bp larger as expected.

Subsequent tests altering the number of cycles in the QIAGEN method (Fig. 2B–E) indicated that peak amplitude generally increased with cycle number (or remained high if substantial already), with the optimal condition obtained at 45 cycles (Fig. 2D); peak amplitude typically dropped for most samples at 50 cycles (Fig. 2E). Peak amplitude at 45 cycles often approached that of samples multiplexed with prelabeled primers (Fig. 2F) or when primers were run individually (Fig. 2G). In general, samples run at 45 cycles (Fig. 2D) exhibited slightly more background noise than when samples were multiplexed using prelabeled primers (Fig. 2F), but all peaks were still readily discernable.

To date, we have successfully used this method to develop microsatellite markers in several plant species from a variety of plant families representing different habits; it has also been used in a number of other taxa in other laboratories (Table 1). In each of the species analyzed, seven to 15 microsatellite markers were developed and successfully run in one to three primer groups in multiplexed reactions (with a maximum of four to 13 primer pairs per multiplexed primer group). The microsatellite fragments were of varying size and composition, ranging from dinucleotide to hexanucleotide repeats. In some cases, the applicability of the technique was straightforward without any modification (as with *Schiedea adamantis* H. St. John [Culley et al., 2008] and *Rhamnus cathartica* L. [Culley and Stewart, 2010]), but in other cases (*Monotropa hypopitys* L. [Klooster et al., 2009] and *Spiraea virginiana* Britton [Brzyski, 2010]), certain primer sets were found not to amplify together and had to be run individually or placed in different primer groups. In addition, there were isolated cases in which a primer with especially strong amplification appeared as small false peaks for other loci in the same reaction (i.e., peak carry-over), which can also happen with traditional labeling of primers. Therefore, some troubleshooting may be required to ensure that all primers amplify properly. In addition to its use in microsatellite development, this technique has also been used to label nonmicrosatellite primers, such as those that amplify self-incompatibility alleles in *Pyrus calleryana* (Culley and Hardiman, unpublished data) and *Spiraea virginiana* (Brzyski and Culley, unpublished data).

CONCLUSIONS

The dual labeling and multiplexing method presented here has been successfully applied to several plant species using primarily microsatellite markers, although it should be applicable to other types of markers as well. In addition, the method offers flexibility in tagging primers with different fluorescent dyes and/or colors during the early stages of primer development. Like Blacket et al. (2012), we found that using triplet sets of primers containing unique universal tail sequences provided an effective way to amplify multiple loci in the same PCR reaction. Furthermore, we also showed that the amplitude and fluorescent intensity of the labeled fragments generated during multiplexing will also depend in part upon the thermocycler conditions during the PCR process. We discovered that the two-step thermocycler program suggested by Schuelke (2000) previously used by our laboratory (e.g., Culley et al., 2008; Klooster et al., 2009) did not yield the best results. Instead, the thermocycler conditions optimized by QIAGEN for their Multiplex PCR Kit resulted in cleaner fragment peaks with higher intensity (Fig. 2), especially when the cycle number was increased to 45, the maximum recommended by QIAGEN. Consequently, the major objection to using M13-based primers (i.e., that they require a lower primer annealing temperature; Blacket et al., 2012) was not borne out by our study as the primer(s) performed even better when a higher standard annealing temperature was used (57°C). We therefore recommend the QIAGEN-recommended thermocycler protocol with 45 cycles if the QIAGEN Multiplexing Kit will be used by the investigator.

←

Fig. 2 (see p. 4). The effect of multiplex PCR thermocycler programs during the dual labeling process on the amplitude and signal strength of fragment peaks. Shown are electropherograms from microsatellite loci KA16, ch0203b, and ch01h01 in the 'Redspire' cultivar of *Pyrus calleryana* when the thermocycler cycling program was that (A) suggested by Schuelke (2000) as two series of cycles or recommended by the QIAGEN Multiplexing Kit for (B) 35 cycles, (C) 40 cycles, (D) 45 cycles, and (E) 50 cycles. For comparison, the same samples are presented (F) following traditional multiplexed PCR with prelabeled primers and (G) when analyzed individually with a single prelabeled primer pair; in both of these cases, the normal QIAGEN-recommended protocol was used with 35 cycles.

When applied correctly, the technique reported here can reduce the expense of primer development by decreasing the amount of time and labor spent in preparing and running multiple PCRs and by reducing the direct cost of consumable supplies and services. We found that the expense of the new method is typically less than half of the cost of the traditional technique of individually labeling each primer prior to nonmultiplexed PCR reactions (Appendix S1). For example, testing 10 primer pairs in two primer groups using 50 samples with the new method is less than half the cost of the traditional technique (estimated currently at US$639 compared to US$1750; Appendix S1). These cost savings will increase as more primers are incorporated into the multiplex PCR reaction with fewer sets of primer mixes. Consequently, this technique would be particularly advantageous for laboratories on a limited budget. However, researchers must balance the cost savings and flexibility of this new method with the observation that multiplex PCR using prelabeled fluorescent primers, which yielded strong peaks in our tests, would require less time in terms of constructing primer mixes because one less primer per set is involved. One approach employed in our laboratory is to first use the dual labeling method to fine-tune the dyes associated with different primer mixes before ordering the prelabeled forward primers direct from a manufacturer for the full analysis; this is particularly effective for large-scale studies involving hundreds of individuals. In smaller studies, we continue to use the dual labeling method exclusively to maximize dye flexibility and minimize overall costs of primer development and application. To assist researchers, a step-by-step protocol of the dual labeling technique described above is provided as Appendix 1, which contains all necessary details as well as answers to frequently asked questions.

LITERATURE CITED

Blacket, M. J., C. Robin, R. T. Good, S. F. Lee, and A. D. Miller. 2012. Universal primers for fluorescent labelling of PCR fragments—An efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources* 12: 456–463.

Boutin-Ganache, I., M. Raposo, M. Raymond, and C. F. Deschepper. 2001. M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques* 31: 25–28.

Brzyski, J. R. 2010. Isolation and characterization of microsatellite markers in the rare clonal plant, *Spiraea virginiana* (Rosaceae). *American Journal of Botany* 97: e20–e22.

Brzyski, J., and T. M. Culley. 2011. Genetic variation and clonal structure of the rare, riparian shrub *Spiraea virginiana* (Rosaceae). *Conservation Genetics* 12: 1323–1332.

Brzyski, J. R., K. J. Adams, C. M. Walter, K. H. Gale, and D. N. McLetchie. 2012. Characterization of 12 polymorphic microsatellite markers in the liverwort *Marchantia inflexa* (Marchantiaceae). *American Journal of Botany* 99: e440–e442.

Culley, T. M., S. G. Weller, A. K. Sakai, and K. A. Putnam. 2008. Characterization of microsatellite loci in the Hawaiian endemic shrub, *Schiedea adamantis* (Caryophyllaceae), and amplification in related genera. *Molecular Ecology Resources* 8: 1081–1084.

Culley, T. M., and N. A. Hardiman. 2009. The role of intraspecific hybridization in the evolution of invasiveness: A case study of the ornamental pear tree *Pyrus calleryana. Biological Invasions* 11: 1107–1119.

Culley, T. M., and J. R. Stewart. 2010. Microsatellite primers in *Rhamnus cathartica* (Rhamnaceae) and applicability in related taxa to assess hybridization events. *American Journal of Botany* 97: e7–e10.

Holleley, C. E., and P. G. Geerts. 2009. Multiplex Manager 1.0: A crossplatform computer program that plans and optimizes multiplex PCR. *BioTechniques* 46: 511–517.

Jeong, K. S., M. Masayuki, and J.-H. Pak. 2012. Isolation and characterization of microsatellite markers from *Tiarella polyphylla* (Saxifragaceae). *American Journal of Botany* 99: e255–e257.

Jullien, N. 2013. AmplifX 1.6.1. Centre de Recherche en Neurobiologie et Neurophysiologie de Marseille (CNRS), Aix-Marseille Université, Marseille, France. Website http://crn2m.univ-mrs.fr/pub/amplifx-dist [accessed 23 January 2013].

Kalendar, R., D. Lee, and A. H. Schulman. 2011. Java web tools for PCR, *in silico* PCR, and oligonucleotide assembly and analysis. *Genomics* 98: 137–144. Software available at: http://primerdigital.com/fastpcr.html [accessed 4 September 2013].

Kikuchi, R., and M. Maki. 2011. Characterization of polymorphic microsatellite markers isolated from the relict perennial *Veratrum stamineum* var. *micranthum* Satake (Melanthiaceae). *Conservation Genetics Resources* 3: 507–509.

Klooster, M. R., A. W. Hoenle, and T. M. Culley. 2009. Characterization of microsatellite loci in the myco-heterotrophic plant *Monotropa hypopitys* (Ericaceae) and amplification in related taxa. *Molecular Ecology Resources* 9: 219–221.

Koffi, K. G., H. Heuertz, R. Jans, O. J. Hardy, G. G. Vendramin, and J. Duminil. 2012. Characterization of new microsatellite loci isolated from *Santiria trimera* (Burseraceae). *American Journal of Botany* 99: e334–e336.

Micheneau, C., G. Dauby, N. Bourland, J.-L. Doucet, and O. J. Hardy. 2011. Development and characterization of microsatellite loci in *Pericopsis elata* (Fabaceae) using a cost-efficient approach. *American Journal of Botany* 98: e268–e270.

Missiaggia, A., and D. Grattapaglia. 2006. Plant microsatellite genotyping with 4-color fluorescent detection using multiple-tailed primers. *Genetics and Molecular Research* 5: 72–78.

Neilan, B. A., A. N. Wilton, and D. Jacobs. 1997. A universal procedure for primer labelling of amplicons. *Nucleic Acids Research* 25: 2938–2939.

Oetting, W. S., H. K. Lee, D. J. Flanders, G. L. Wiesner, T. A. Sellers, and R. A. King. 1995. Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. *Genomics* 30: 450–458.

Ottewell, K. M., S. C. Donnellan, G. F. Moran, and D. C. Paton. 2005. Multiplexed microsatellite markers for the genetic analysis of *Eucalyptus leucoxylon* (Myrtaceae) and their utility for ecological and breeding studies in other *Eucalyptus* species. *Journal of Heredity* 96: 445–451.

Prunier, R., and A. Latimer. 2010. Microsatellite primers in the white proteas (*Protea* section *Exsertae*, Proteaceae), a rapidly radiating lineage. *American Journal of Botany* 97: e1–e3.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Scott, I. A. W., C. M. Hayes, J. S. Keogh, and J. K. Webb. 2001. Isolation and characterization of novel microsatellite markers from the Australian tiger snails (Elapidae: *Notechis*) and amplification in the closely related genus *Hoplocephalus. Molecular Ecology Notes* 1: 117–119.

Shimizu, T., and K. Yano. 2011. A post-labeling method for multiplexed and multicolored genotyping analysis of SSR, indel, and SNP markers in single tube with bar-coded split tag (BStag). *BMC Research Notes* 4: 161.

Squirrell, J., P. M. Hollingsworth, M. Woodhead, J. Russell, A. J. Lowe, M. Gibby, and W. Powell. 2003. How much effort is required to isolate nuclear microsatellites from plants? *Molecular Ecology* 12: 1339–1348.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. Strategies for microsatellite isolation: A review. *Molecular Ecology* 11: 1–16.

Appendix 1. Protocol for concurrent primer labeling and multiplexing.

Developed by the Culley Laboratory, University of Cincinnati
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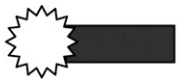
In this method, a generic sequence (the "Tail") incorporating a fluorescent label is attached to each forward primer *during the PCR process*, instead of labeling the primer itself prior to PCR. Along with the unlabeled reverse primer, this set of PCR primers can then be combined with others in a multiplex reaction using the QIAGEN Multiplexing Kit (catalog no. 206143; QIAGEN, Culver City, California, USA). This kit is typically sold in 50-µL PCR reactions, but we have reduced this down to 10-µL reactions for further cost savings. The protocol below first describes the case of a single primer pair but can be applied to any number of primer pairs:

1) Order the Labeled Tail primer(s) in any combination of colors that are needed. We have developed four Tails, three of which are either the standard M13(–21) sequence or derived from that. Theoretically, other Tails would be developed and used, as long as they do not amplify plant DNA. Each tail could be preordered with any color; typical colors are indicated below.

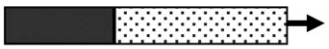

(Labeled Tail)

Tail name	Primer sequence	Size	Color
M13(–21)	TGT AAA ACG ACG GCC AGT	18 bp	6-FAM/blue
M13 modified A	TAG GAG TGC AGC AAG CAT	18 bp	NED/yellow
M13 modified B	CAC TGC TTA GAG CGA TGC	18 bp	PET/red
T7term	CTA GTT ATT GCT CAG CGG T	19 bp	VIC/green

2) Forward and reverse primers are developed as usual for each target microsatellite region, using your desired method. Order and test the unlabeled forward and reverse primers in PCR reactions, and run out on 1% agarose gels to make sure they amplify a fragment of the desired size.


(Reverse Primer)

3) Order the forward primer with the desired tail sequence attached to the 5′ end:


(Forward Tailed Primer)

NOTE: When first testing this labeling method in a new system, it is critical to run PCR tests on a subsample to first verify that everything is operating correctly. Separate PCRs should use (1) unlabeled Forward and Reverse primers to make sure they are designed well and amplify fragments; (2) the Forward Tailed primer and the Reverse primer only to make sure they amplify fragments; (3) the Tail alone to verify that it does not amplify. If the Tail itself amplifies a fragment, you must check for contamination or use a different generic sequence that is completely unique to the species under investigation.

4) All three primers are then combined together in one PCR reaction using the QIAGEN Multiplexing Kit (although this could be adapted to traditional PCR as well). The

Forward Tailed primer is supplied at a reduced concentration to ensure that it is used up in the first set of cycles so that the Labeled Tail is then incorporated into all subsequent reactions:

Primer	Ratio	Example amount
Reverse	1	2 μM
Forward Tailed	1/4	0.5 μM
Labeled Tail	1	2 μM

To use the QIAGEN kit, all three primers (the "primer set") for each microsatellite must be combined into a Primer Mix. Thus if you wanted to amplify four microsatellite fragments, you would need to include 12 primers in your Primer Mix (four primer sets). The QIAGEN kit suggests that primers be at a concentration of 2 μM each, which we comply with *except for the Forward Tailed primer which needs to be at a lower concentration.* Typically, 0.5 μM works well.

Example (using 200 μM stock solutions):

Reverse: $C_1V_1 = C_2V_2 \rightarrow (200\ \mu M)(V_1) = (2\ \mu M)(50\ \mu L) \rightarrow V_1 = 0.5\ \mu L$

Forward Tailed: $C_1V_1 = C_2V_2 \rightarrow (200\ \mu M)(V_1) = (0.5\ \mu M)(50\ \mu L) \rightarrow V_1 = 0.125\ \mu L$

**NOTE: This small amount is often beyond the capacity of most pipettors, so a working solution of 20 μM is often used.*

Labeled Tail: $C_1V_1 = C_2V_2 \rightarrow (200\ \mu M)(V_1) = (2\ \mu M)(50\ \mu L) \rightarrow V_1 = 0.5\ \mu L$

For a single set of primers:	5.0 μL	Reverse primer (20 μM working soln)
(using working solutions)	5.0 μL	Labeled Tail primer (20 μM working soln)
	1.25 μL	Forward Tailed primer (20 μM working soln)
	38.75 μL	dH_20

If multiplexing with multiple primer sets, it may be necessary to use stock solutions. Otherwise, the added primer amount will exceed the required quantity of primer mix.

For a combined Primer Mix of four primer sets:

Reverse primer:	0.5 μL of each 200 μM stock soln × 4 = 2 μL
Labeled Tail primer:	0.5 μL of each 200 μM stock soln × 4 = 2 μL
Forward Tailed primer:	1.25 μL of each 20 μM working soln × 4 = 5 μL
dH_20:	41.0 μL

NOTE: If you will be multiplexing with multiple primer sets in a single reaction, you must be sure that any overlapping PCR products generated by the primers are labeled with different color dyes. For example, we typically have a group of primers that amplify fragments ranging from 150 to 180 bp labeled separately with VIC, NED, PET, and 6-FAM. Then, a next group of fragments 190–220 are labeled with the four dyes again, as are a third group of fragments 230–260 bp (or larger). It is often helpful to use a program such as Multiplex Manager (www.multiplexmanager.com) or create an Excel spreadsheet listing all primer pairs along with their fragment size. These can then be sorted according to fragment size and then organized into groups of four sets for labeling.

5) Use the QIAGEN Multiplexing Kit (catalog no. 206143) for PCR as directed, except adjust for 10-μL reactions instead of the suggested 50 μL. Combine the Master Mix, dH_2O, and Primer Mix in a cocktail. Add DNA (0.2–0.4 μL, depending on concentration) and be sure to shield from light within aluminum foil whenever possible to maintain the integrity of the fluorescent dyes:

For a single sample:	5 μL	Master Mix
	1 μL	Primer Mix (as described above)
	3.8 μL	dH_20
	0.2 μL	dH_20

6) There are two possible PCR conditions that can be used: the single set of cycles advocated by QIAGEN for use with their kit and the two sets of cycles recommended by Schuelke (2000). We have had the best success with the QIAGEN approach (see above) but both should be tested in other plant species to determine the best method.

PCR Method for a Single Set of Cycles
This method has been fine-tuned for the multiplexing kit, which includes HotStarTaq polymerase. The thermocycler conditions are as follows:

Initial denature: 95°C for 15 min
45 cycles of (kit recommends 30–45 cycles):
Denature: 94°C for 30 s
Anneal: 57°C for 90 s (kit recommends 57–63°C)
Extension: 72°C for 60 s
Final extension: 60°C for 30 min

PCR Method for Two Sets of Cycles (Schuelke, 2000)
Initial incorporation of the Forward Tailed primer is promoted by increasing the annealing temperature for the first set of cycles of the PCR reaction. The annealing temperature is subsequently reduced in the next set of cycles to facilitate annealing of the Labeled Tail. Thermocycler conditions are as follows:

Initial denature: 95°C for 15 min
30 cycles: Denature: 94°C for 30 s
Anneal: 57°C for 45 s
Extension: 72°C for 45 s
8 cycles: Denature: 94°C for 30 s
Anneal: 53°C for 45 s
Extension: 72°C for 45 s
Final extension: 72°C for 10 min

7) Following PCR, products can be run on a 1.2–4% agarose gel for verification (using 2 μL of each PCR product). Samples should then be prepared for fragment analysis, as normally done.

Frequently Asked Questions

How many generic tail sequences do I need?
Typically, you may wish to have one generic tail sequence for each color of fluorescent dye that you use in your research. This is especially important if you plan on truly multiplexing all primer pairs together in a single reaction before PCR. (*Note that some authors use the term "multiplexing" even though they are running PCRs separately for each primer before combining PCR products afterward for fragment analysis—also known as "pool-plexing."*) If you plan on only running PCR with single reactions involving one primer pair at a time, only one color is needed and therefore only one generic tail sequence.

What generic tail sequence(s) should I use?
The actual sequence that you use is optional, with caveats that it not already be part of the plant genome under scrutiny and that it not interfere with other primers in the reaction. It should be a completely unique sequence. Previous investigations have used the M13(–21) sequence or human microsatellites (see text for citations). In our lab, we have had success with M13 and various modifications we created, as well as the T7term sequence (see above).

How do I know that the generic tail sequence is being incorporated into the fragment?
This is a critical question and can be easily answered by looking at the size of the fragments following PCR. The fragments should consist of the original size as indicated by the unlabeled primers plus the length of the generic tail sequence. For example, if a set of unlabeled forward and reverse primers is known to amplify a fragment of 220 bp and then the M13 generic tail sequence is used (i.e., 18 bp), the final fragment size should be 238 bp. Alternatively, the fragment could be sequenced to verify that it contains the generic tail.

What colors of fluorescent dyes should I use?
The choice of dye color is up to the investigator's discretion, but they must be consistent with the filter set installed on a given ABI sequencer. Consequently, users should check with their fragment analysis facility first to see which dyes are allowed before labeled tags are ordered. We prefer 6-FAM as our top choice because it performs well and is not proprietary to any single company and it is thus less expensive. For the 3730xl Capillary Genetic Analyzer, we also typically use VIC, NED, and PET, which are only available from Applied Biosystems. Note that we have the most difficulty with NED because the yellow dye can emit brighter than other colors, causing alleles to peak off the electropherogram and thus rendering the rest of the loci unable to be scored. Some core facilities have reported that NED produces weak amplification and can be difficult to use in multiplex reactions. Some dyes and their representative colors are described in the following table:

Dye[a]	Approx. max emission (nm)	Color
6-FAM	520	blue/green
TET	539	green
VIC*	553	green
HEX	555	yellow/green
NED*	575	yellow
TAMARA	583	red
PET*	590	red
ROX*	608	orange
LIZ*	650	orange

[a] Note that LIZ and ROX are reserved for size standards.
* Proprietary and available only from Applied Biosystems, now Life Technologies.