Appendix 1. Protocol for concurrent primer labeling and multiplexing.

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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.

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.

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

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.