Version 2

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Protocol for the detection of rare alleles in bulk seed samples V.2

Anthony Brusa[1], Eric Patterson[2]

[1]University of Minnesota - Twin Cities; [2]Michigan State University

1 *Works for me* dx.doi.org/10.17504/protocols.io.brtam6ie

Anthony Brusa

ABSTRACT

This protocol was developed for the detection of *Amaranthus palmeri* seeds in mixed pools with other *Amaranthus sp.* seeds. This is a bulk testing protocol; each pool of seeds generates **one** data point, the presence or absence of *A. palmeri* in that sample. Testing pools should be assembled based on the relevant information (e.g. location, population, seed lot, seed supplier, etc.). Controls are extremely important for KASP based assays; this protocol requires positive, negative, and no-template controls. We strongly recommend running **8 controls** for the rare allele being detected (e.g. *A. palmeri*) to aid in data analysis.

The protocols may be extended to any species for which appropriate species-specific KASP markers have been developed. DNA extraction protocols were performed using the MPBio FastDNA Plant Kit, but other methods may be substituted.

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WHAT'S NEW

Corrected a typographical error

KEYWORDS

Amaranthus palmeri; Palmer amaranth; genotyping; diagnostic assay; species identification; seed genetic testing; invasive plants; weed identification

IMAGE ATTRIBUTION

Image © Anthony Brusa

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GUIDELINES

The tissue disruption step is critial to the accuracy of this protocol. **Ensure that all seeds have been properly disrupted.** Depending on seed size this may take upwards of 6 minutes total grinding time.

Do not use standard software on lightcyclers for rare allele calling. Refer to section 4 of this protocol for data interpretation.

MATERIALS TEXT

MATERIALS

KASP 2x Mastermix **LGC biosearch**

Customer primers (2 forward 1 reverse) **Contributed by users**

FastDNA™ Spin Kit for Plant and Animals Tissues **MP Biologicals Catalog #116540800**

Deionized water **Contributed by users**

SAFETY WARNINGS

Refer to tissuelyser manual for mechanical safety warnings.
Refer to DNA extraction kit for chemical warnings.

BEFORE STARTING

Prepare seed samples by sorting into pools of up to 200 seeds. Each pool should correspond to either commercial seed lot, seed supplier, natural population, or other biologically relevant grouping.

Tissue Disruption

1 Tissue disruption

1.1 Assemble samples to be tested, partition into pools of an appropriate number of seeds (200 seeds for Amaranthus sp. testing).

1.2 Place seeds and grinding bead into a tube suitable for tissue disruption. Avoid conical bottom tubes as this will lead to incomplete tissue disruption.

1.3 Add 1mL lysis buffer (800 µL CLS-VF and 200 µL PPS in MPBio kit) and ensure that all seeds are submerged in buffer.

1.4 Incubate overnight to soften seed coat for easier disruption.

1.5 ⚠

Disrupt seeds using Qiagen Tissuelyser II (or similar equipment). Recommend grinding at 30hz in 90 second intervals. Visually inspect tube after each interval to determine if further grinding is needed. Ensure that all seeds have been properly disrupted. Depending on seed size this may take upwards of 6 minutes total grinding time.

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DNA extraction

2 DNA extraction

Perform the remainder of the DNA extraction using instructions from the kit/protocol of your choice.

PCR

3 PCR

3.1 Create 10nmol solutions of your KASP primers. (There will be three total. Two forward primers and one reverse)

3.2 Create **150 µl** of primer mix according to the following:

3 µl of **10 Micromolar (µM)** forward primer (not A. palmeri)

20 µl of **10 Micromolar (µM)** forward primer (A. palmeri specific)

45 µl of **10 Micromolar (µM)** reverse primer

82 µl of DI water

3.3 Create your final reaction solution by combining **432 µl** of KASP 2x Mastermix with **12 µl** of primer mix.

3.4 

Plate **4 µl** of reaction solution into a standard 96-well PCR plate.

3.5 

Add **4 µl** of DNA (approximately 10-20 ng/µL) solution to each well. Include positive controls, negative controls and No Template Controls (NTCs). We recommend running 8 negative controls to assist in the data interpretation step below.

3.6 

Run on a Lightcycler or RT-PCR machine with the following program:
15 minutes @ **94 °C**

Touchdown program for 9 cycles at:
20 sec @ **94 °C**
60 sec @ **61 °C** to **55 °C** (increment with each cycle)

Followed by 30 cycles of:
20 sec @ **94 °C**

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60 sec @ 51 °C

30 sec @ 28 °C

Take an endpoint fluorescence read at 465-510 and 533-580nm for 30 seconds @ 28 °C

3.7 ⚠

Note: 465-510 and 533-580nm correspond to the optimal fluorescence peaks of the FAM and HEX dyes respectively. At the time of writing KASP mastermix is only available for these two dyes. If your mastermix uses different dyes change the fluorescence readings as appropriate.

Data interpretation

4 ⚠

Data interpretation

*Note: Standard software on lightcyclers is **not** designed for rare allele calling. Use the following steps instead.*

4.1 Export raw 465-510nm and 533-580nm reads from lightcycler software as a .csv file. Import into your statistical software of choice (R, SPSS, SAS, Mathematica, etc.)

4.2 Perform an arctan transform on the ratio of 465-510nm to 533-580nm reads. This converts your Cartesian coordinates into datapoint trajectories (angle of the datapoint relative to the origin).

4.3 Generate a box-and-whisker plot using the appropriate steps in your software.

4.4 A statistical comparison between the negative control and test sample will determine if a given sample contains at least one A. palmeri seed.

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