

Allelic Discrimination Protocol
for
AOSA/SCST Protocol Proposal for AOSA Cultivar and Purity Testing Handbook
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GGT M*3 A/P Test using Allelic Discrimination TaqMan® SNP Genotyping in Ryegrass

Introduction:

Allelic discrimination (A/D) is a multiplexed end-point assay that detects variants of a single nucleic acid sequence (SNP). The GGT M*3 A/P test is an A/D protocol and is used in ryegrass as an alternative to the grow-out test (GOT) to predict growth types. The procedure is done as a purity test on individual seedlings. Either random seeds or seedlings can be chosen for analyses, or following a “Big Ugly Suspect” (BUGS) prescreening that reduces costs and provides wider test inference. It is considered a replacement to the ryegrass grow-out test and can be used in Variety Fluorescence Level (VFL) determinations. This test is a method to predict growth types of ryegrasses based on multiple SNP markers of genes involved in flowering control of grasses. Multiple markers are important because of the natural variation within the ryegrasses, which have many alleles for most genes. The two genes discussed here are the indeterminate (*LpID₁*) and one of the vernalization (*LpVrn-1*) genes, but markers to the *LpVrn2*, *LpVrn3*, and *LpCO* may also be used and DNA sequence and probe design are available on request. To reduce costs involved in conducting this TaqMan® based assay and to follow the GOT protocol format, the protocol presented below is conducted as a nested, or two stage purity test with DNA analyses conducted on individual seedlings that had fluorescent root traces (FL) in the BUGS prescreening. Non-fluorescent seedlings (NFL) and seedlings from an Italian (or annual) ryegrass cultivar should be included in the test as controls. Usually only two of each of the Italian ryegrass controls is needed, and these control seedlings do not enter into calculations. All, or as many as desired, NFL seedlings can be included, but with experience it will be found that less than 1% of these contain the flowering control markers and continued testing of them may not be warranted for the resources expended. Once allelic determination for each seedling has been made, results are reported as the number of seedlings in each of three classes: 1. Perennial-like, 2. Annual-like, and 3. Hybrid (or other) ryegrass. Formulae and calculations using the numbers of plants in each class are similar to those used in the GOT protocol, including mortality adjustments for those plants whose DNA extractions did not amplify in both markers.

Germination and prescreening:

This protocol distinguishes the potential growth types (phenotype) of BUGS seedlings, but can be done on all seedlings with the numbers obtained from the results used to modify those reported from the germination tests. It is important that proper germination and BUGS be conducted before nucleic acid determinations are made. Follow these two steps carefully:

1. As always necessary in seed testing, ensure an appropriate sample is taken from the seed lot.
2. Germinate 400 seeds and conduct a BUGS prescreening similar to the seedling root fluorescence (SRF) test according to procedures in the AOSA Rules and AOSA Cultivar Purity Handbook. The BUGS prescreening, however, does not need to go as far as a SRF test and seedlings can be harvested as soon as seedlings exhibit SRF.

Harvest and extract DNA:

3. A sample from a single leaf is harvested, kept on ice, and processed as described in step 4. Seedlings from the germination test to be tested are harvested individually. In addition to the test germination sample, ten seedlings of a standard Italian or annual ryegrass cultivar, such

as Gulf, needs to be germinated so that at least two “annual-type” control plants will be available to include in the test for comparison to test results.

4. Use the Applied Biosystems Sample-to-SNP Kit (part numbers 4403313, 4403083, 4403087, and 4403081). (Note: Other DNA extraction methods that yield clean, unsheared DNA can also be used in the PCR reaction.) Follow manufacture instructions, except 25ml extraction reactions are used instead of 50. Steps are summarized below:
 - Cut and place two 2-0mm punches of leaf tissue, or harvest a similar amount tissue ensuring that it is uniform in size for each sample. Use a 2.0mm Harris Micro-punch if available.
 - Place leaf punches in a 96-well PCR plate that is held on crushed ice.
 - Add 25µl of a thoroughly mixed Lysis Solution to each reaction well.
 - Mix the solution with the leaf tissue by pipetting up and down, or by briefly vortexing.
 - Incubate at 95°C for three minutes.
 - Thoroughly mix the DNA Stabilizing Solution without creating bubbles.
 - After incubation, add 25µl of the DNA Stabilizing Solution to each sample and pipette up and down, or briefly Vortex.

Conducting the PCR protocol:

The real-time PCR can be conducted on any qPCR system that has software to analyze for allelic discrimination using TaqMan[®] assays. Reaction volumes will depend on the type of PCR platform available. Fast systems, if associated with a Fast DNA polymerase, can use faster PCR times and smaller volumes.

5. Immediately before running the PCR, thoroughly mix the TaqMan[®] GTXpress[™] Master Mix from the Sample-to-SNP Kit and create the master reaction solutions that contain all the components below except for the sample DNA template.
 - The volume of the master reaction solution depends on the number of reactions needed. After the master reaction solutions are made, cap the tubes, vortex briefly to mix the solutions, and centrifuge briefly to eliminate air bubbles and spin down the contents.
 - Keep the solutions on ice and the TaqMan[®] SNP genotyping assay (20X) (*LpID1* or *LpVrn-1*) in the dark on ice by putting aliquots for the test in a brown tube or wrapped in tin foil.

A 10µl and 20µl will both be considered here. Mix reaction wells in a new qPCR plate necessary for the available qPCR platform available according to the following table:

Table 1. Reaction mix recipes for TaqMan® SNP genotyping assay.

Reaction Component	Volume (µl) /Reaction	
	96-well Fast-plate	96-well Plate
TaqMan® GTXpress™ Master Mix (2X)	5	10
TaqMan® SNP genotyping assay (20X) (<i>ID₁</i> or <i>Vrn-1</i>)*	0.5	1.0
Sample DNA template	2	4
Nuclease-free water	2.5	5
Total volume	10µl	20µl

*Note: TaqMan® SNP genotyping assays (20X) (*LpID₁* or *LpVrn-1*) are considered proprietary by Applied Biosystems (AB) and it is necessary to obtain order information for the two assays from Grass Genomic Testing, Inc. in advance. Both the *ID₁* and *Vrn-1* assays need to be run on each sample separately if using AB systems.

6. Add two wells for each of the control cultivars (i.e. NFL and Gulf controls) and for the No Template Control (NTC). The NTC has all of the ingredients except the sample DNA template.
7. Using different pipette tips for each transfer, place the Sample DNA template in each well near the bottom, and then add the master reaction solutions to each well and pipette up and down to mix.
8. Use component volumes according to the table above. Seal the PCR plate with the optical sealing film necessary for the qPCR platform to be used and briefly centrifuge the plate to further mix and spin down the contents.
9. The PCR protocol for the TaqMan® GTXpress™ Master Mix in a Fast PCR system includes 95°C for 20 sec to activate the Taq enzyme, followed by 40 cycles of 3 sec at 95°C to denature, and 30 sec at 60°C for the annealing/extension steps combined.

Data Analysis—Assigning calls and exporting the data:

Data output will depend on the qPCR platform used in the A/D PCR analysis and the instructions for Allelic Discrimination for that platform should be followed. The DNA quantitation window or file will have a series of sigmoid curves similar to those generated for GMO analysis. There will be one curve for each plant, or reaction well.

The A/D window or files will look similar to Figure 1 (conducted on a Bio-Rad CFX96 qPCR at the GGT lab). Homozygous alleles are located in the top left and bottom right quadrants, depending on which dye (Fam or Vic) is attached to which TaqMan® allele probe. The heterozygotes (one copy from each allele) will be located in the upper right quadrant. The NTCs or any samples with no DNA amplification should be located in the lower left quadrant.

For the *LpID₁* assay, the perennial-like allele (MM) has the FAM dye and the annual-like allele (FF) has the VIC dye. The assignment of the dyes for *LpVrn-1* assay were designed opposite to the *LpID₁* assay with the perennial-like allele (MM) having VIC and the annual-like allele (FF) having FAM.

Normalized Relative Fluorescence Units (NRFU) for each allele (dye) plotted against the opposite allele is presented in Figure 1. Relative Fluorescence Units (RFU), however, is usually used to collect the data for individual plants. Normalization is accomplished relative to the NTC wells. Numbers of plants in each classification can either be counted directly from the computer screen by highlighting data from each marker on an individual plant, or data can be exported to an Excel

spreadsheet for further analysis and reporting. Exported data will be labeled Allele 1, Allele 2, Heterozygote, or None depending on dyes used in the probes. These labels may be replaced in the exported spreadsheet by the appropriate allele names if desired. Remember that Allele 1 and Allele 2 are labeled opposite each other between the *LpID₁* and *LpVrn-1* assays.

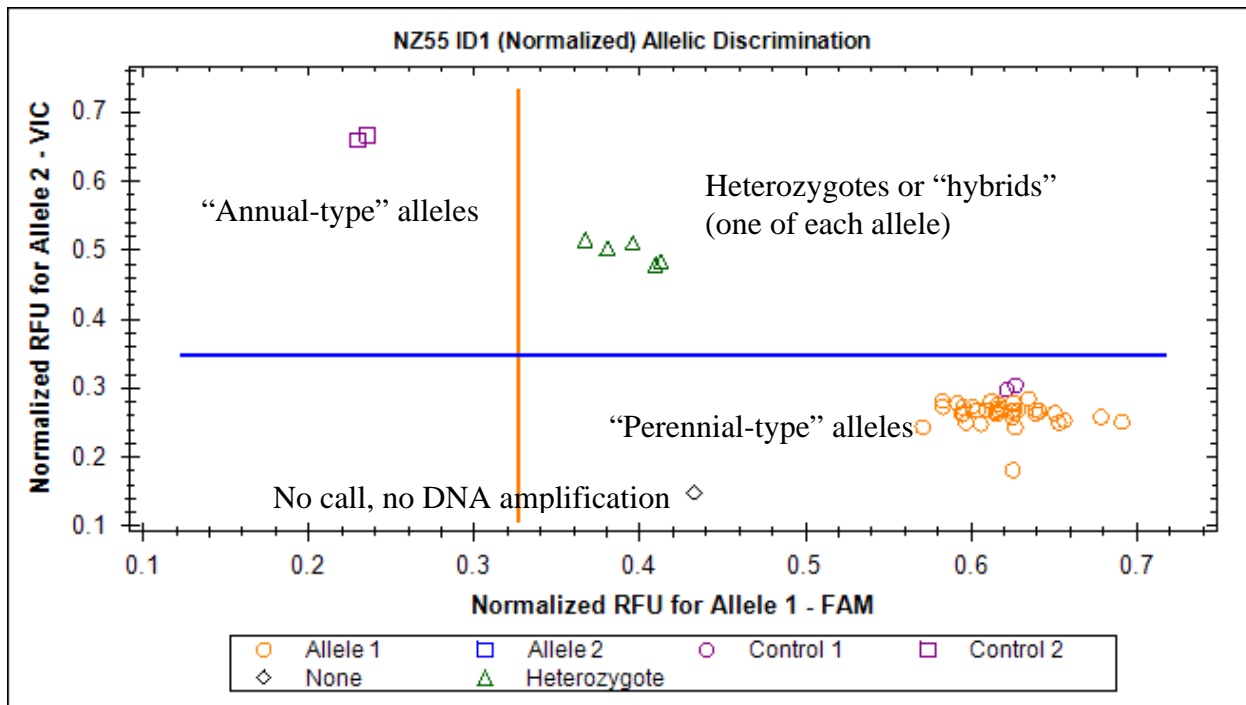


Figure 1. This annotated computer screen view is similar to what the A/D graph should look like after analysis. This analysis was conducted on a Bio-Rad CFX96 qPCR and data were normalized based on the NTC results. Non-normalized results will be similar, but spread more.

Making the final “call” and reporting: The final designation of “annual-like”, “perennial-like” and hybrid is made using a three-of-five marker approach, which allows placing plants in the respective growth type classes (Table 2). The five markers are FL [fluorescence presence (+, 1, or Yes) or absence (-, 0, or No)], with an allele for each *LpID₁* and *LpVrn-1* (Tables 2 and 3). For seedlings with fluorescent root traces (FL+) (Table 2), calls are weighted more heavily for *LpID₁* than for *LpVrn-1*. If one or the other marker is missing, it is treated as a missing value and ignored in making final determinations. In practice for most seed testing needs, the heterozygote class may be added to the annual class. For breeding purposes, the status of markers at each allele is useful.

Table 2. Decision table for seedlings WITH fluorescent root traces. P=perennial-like, A=annual-like, and H=heterozygote, or hybrid.

Seedlings with Fluorescence		
ID1	Vrn-1	Call
MM	MM or MF	P
MM	FF	A
MF	MM or MF	H
MF	FF	A
FF	MM, MF, or FF	A

Table 3. Decision table for seedlings WITHOUT fluorescent root traces. P=perennial-like, A=annual-like, and H=heterozygote, or hybrid.

Seedlings without Fluorescence		
ID1	Vrn-1	Call
MM	MM or MF	P
MM	FF	H
MF	MM or MF	H
MF	FF	A
FF	MM	H
FF	MF or FF	A

10. **Calculations.** Exported data are used to count the number of plants in each plant-type grouping. Only the final numbers are used in calculations. The numbers for “annual-like” and “perennial-like” are inserted into the calculations and formulae below. Number of heterozygote, or hybrid, is a third classification and can either be reported as “other or hybrid ryegrass” or combined with the “annual-like” class. For test comparison purposes, confidence intervals based on sampling error rates may be calculated using the Qual Purity Estimation tab in SeedCalc8. SeedCalc is available to download from the ISTA website: http://www.seedtest.org/en/stats_tool_box_content---1--1143.html. The No DNA amplifications labeled “None” in the output are treated as missing values and ignored for a single marker, but treated as a mortality seedling if both markers are missing. Reporting of ryegrass purity is finalized as:

$$\%Annual\ ryegrass = \frac{Number\ of\ seedlings\ with\ "A"\ scores}{Total\ number\ of\ normal\ seedlings\ X\ Survival\ factor} \times 100$$

$$\%Other\ ryegrass = \frac{Number\ of\ seedlings\ with\ "H"\ scores}{Total\ number\ of\ normal\ seedlings\ X\ Survival\ factor} \times 100$$

$$Survival\ factor = \frac{No.\ of\ normal\ seedlings - No.\ of\ normal\ seedlings\ that\ died\ or\ did\ not\ amplify}{No.\ of\ normal\ seedlings}$$

$$\% Perennial\ ryegrass = \% Pure\ ryegrass - \% Annual\ ryegrass - \% Other\ ryegrass$$

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