AOSA RULE PROPOSAL – Identification of ryegrass types

Appendix I

Rule Proposal Supporting Evidence:

Submitted by Reed E. Barker and Sharon Davidson

The following supporting evidence applies to item 5.2 d. (1) **Genetic Purity**, and is implemented as an Allelic Discrimination (A/D) assay. This test is a method to predict growth types of ryegrasses based on multiple genetic markers of genes involved in flowering control of grasses. Multiple markers are important because of the natural variation within the ryegrasses. This group of grasses has many alleles of the genes for which the markers are based. The two genes discussed here are the indeterminate ($LpID_1$) and one of the vernalization (*LpVrn-1*) genes, but markers to the *LpVrn2*, *LpVrn3*, and *LpCO* are also available and may be used in the test following the same principles discussed here. 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 a Big UGly Suspect (BUGS) prescreening. Markers for the $LpID_1$ and either the *LpVrn-1* or the *LpVrn2* flowering control genes may be used in these assays. They can be used in combination on individual seedlings as a Genetic Purity test, or as individual markers on bulk seed as an impurity test.

The ryegrasses are a complex set of subspecies with a range of plant types (Figure 1). The overlap of plant morphology types is created by the natural variation within. Tyler et al. (1987) considered the perennial ryegrass (*Lolium perenne* L.) group of subspecies as a "huge hybrid swarm" with the Italian and perennial types representing the opposite extremes. Clearly, the variation and phenotype overlap raises concerns for a completely accurate classification.

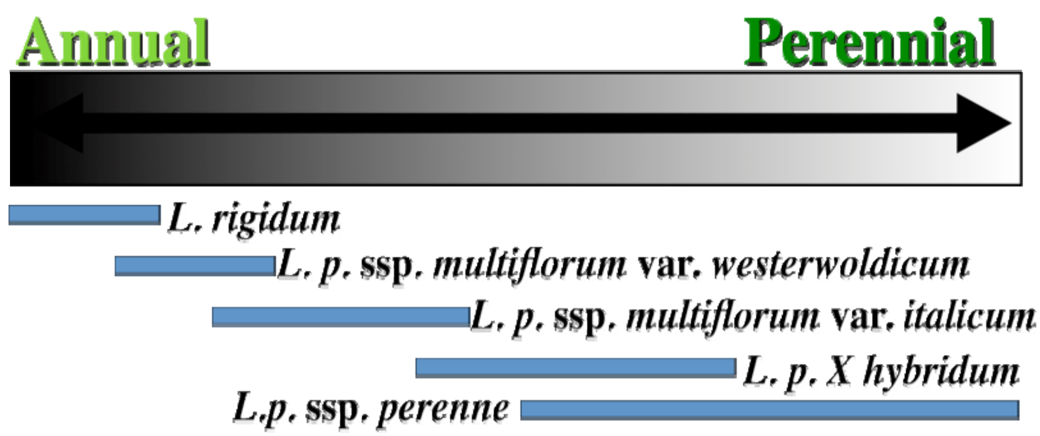

Figure 1. Diagrammatic representation of growth type among the main out-breeding ryegrasses. Types range from truly annual to fully perennial. This relationship is based on reported intercrossing, plant morphology, and molecular data. Taxonomic classification from USDA, NRCS. 2004. The PLANTS Database, Version 3.5 <http://plants.usda.gov>. National Plant Data Center, Baton Rouge, LA 70874-4490 USA.

Seed analysts have known for years that the Seedling Root Fluorescence Test (SRF) in ryegrass has been a problem (Davidson 2010, see Appendix IV). Generally, germinating Italian (or annual) ryegrass seedlings fluoresce under ultraviolet light and perennial ryegrass does not. False positives occur due to lack of complete test standardization, age of seed, seed analyst subjectivity in scoring results, and plant biology (Floyd and Barker 2002). This false-positive bias has been costly to grass seed growers who receive lowered seed payments and costly to seed companies because of inappropriately labeled seed being sold to the consumer.

An accurate test that detects both physical (mixtures) and genetic (pollen flow) contamination of Italian ryegrass in perennial ryegrasses cultivars is necessary, especially if the seed is for permanent turf. Contamination of perennial turf with annual type ryegrasses detracts from the aesthetically pleasing appearance of perennial ryegrass with dark-green color and fine turf texture. Other than a physical plant grow-out test for each seed lot (Barker et al. 2002, Nittler and Kenny 1972), SRF is the only accepted test for detecting the presence of Italian ryegrass in seed lots of perennial ryegrass. Tolerances, however, are not applied to test results from year to year, or field to field.

To alleviate the income loss to seed growers, a maturity grow-out test (GOT) was developed and implemented for labeling seed in 2001. After several years experience with the GOT, we found that the GOT is also causing problems in the grass seed industry. Recent research showed that it might underestimate annual-type plants in the SRF seedlings (Appendix I Figure 1 and 2). Problems with the GOT include: the implemented GOT is conducted for too short a time, there is no clearly defined end point, artificial lighting conditions are not standardized at high enough intensity levels, and many labs are not equipped to conduct the GOT.

A new DNA-based genetic test, (referred to here as Allelic Discrimination (A/D)) is an additional test that can be used to determine the number of seedlings predicted to be annual- or perennial-type. The A/D test is implemented similar to the GOT, but is intended to replace the ryegrass grow-out test and can be used in VFL determinations. The A/D test is a method to predict growth types of individual ryegrass plants based on multiple genetic markers of genes involved in flowering control of grasses. Once "annual- or perennial-like" determination for each seedling has been made, the numbers of plants for each class are reported similar to the GOT.

For the past 13 to 14 years, Barker, while with USDA/ARS in cooperation with Oregon State University, Agri Seed Testing and others (see References in Appendix I), worked on a PCR test that is faster and more accurate than either the SRF test or the GOT. An Allelic Discrimination (A/D) test was developed based on single nucleotide polymorphisms (SNPs) to major genes involved in the flowering control process in grasses. A SNP is an allele, which is an alternate form of a gene. SNPs of the indeterminate gene ($LpID_1$) and one of the vernalization genes (*LpVrn-1*) are used in the A/D test as described here (Appendix I Figure 2). Implementation of this rule will be provide more accurate results than GOT for ryegrass because it is based on actual flowering control genes, it will remove the bias to SRF tests, lessen the burden placed on seed growers, and provide answers six weeks earlier than a GOT and at similar costs for low contamination seed lots.

A. Current data: Following the initial discovery studies, twenty-two seed lots chosen for their wide range of SRF values were examined to verify that the A/D tests could be conducted on a commercial scale. Agri Seed Testing provided the seed test data and GGT conducted the A/D analyses (Appendix I Table 2, and Table 1). Seed for each seed lot were germinated, a SRF conducted, and a grow-out test started according to AOSA protocols. During the grow-out, a leaf from each plant was harvested, DNA extracted, and an A/D conducted for both *LpVrn-1* and $LpID_1$.

From the A/D test, individual plants were determined to be "annual-like" or "perennial-like." This determination was made using a multi-point decision process. Seedlings with fluorescing root traces (FL+) were declared annual-like if either *LpVrn-1* or $LpID_1$ had an "annual" allele. For non-fluorescing plants (FL-), two "annual" alleles were needed to declare it annual-like. This is a "two of three" marker determination (called GGT M*3 AR/PR test). Multiple markers are more robust than one marker alone, unless the marker is the actual "annuality" gene, if there is one.

Numbers from Appendix I Table 2 were used as described in the AOSA Cultivar Purity Testing Handbook "Grow-Out of Fluorescent Ryegrass Seedling to Differentiate Between Annual and Perennial Types" to calculate actual "annual-type" contamination (%) (Table 1). Eight of the seed lots (in bold) were tested for VFL. Lots 61490 to 61493 were from the cultivar Prosport 2 and 61494 to 61497 were from Pacesetter II. The VFL using GOT values were 1.87 for Prosport 2 and 0.99 for Pacesetter II. Using DNA marker values were 1.64 for Prosport 2, and 0.90 for Pacesetter II. Results for actual VFL are similar between GOT and molecular markers, but M*3 values are obtained six weeks earlier than possible with GOT.

Table 1. Annual-type contamination in perennial ryegrass seed lots (%) as determined by the GOT formulae for both the GOT and molecular markers.

Test#	VFL	TFL	From grow-out	Using molecular markers
		- - - - - - - - - - % - - - - - - - - - -		
59216	0.77	5.39	0.60	0.90
59337	0.12	5.29	0.28	1.11
59669	0.77	6.50	0.00	0.89
60079	5.88	8.84	1.26	4.42
60080	5.88	9.83	2.53	6.65
60660	VNS	11.65	1.36	6.78
61248	4.83	4.01	0.27	1.07
61249	4.83	7.22	1.67	4.16
61490	1.87	0.75	0.00	0.00
61491	1.87	1.16	0.00	0.00
61492	1.87	0.83	0.00	0.09
61493	1.87	1.24	0.00	0.31

(Table 1. Continued)

Test#	VFL	TFL	From grow-out	Using molecular markers
		- - - - - - - - - - % - - - - - - - - - -		
61494	0.99	0.94	0.00	0.00
61495	0.99	2.56	0.09	0.28
61496	0.99	1.10	0.00	0.00
61497	0.99	2.90	0.00	0.68
61761	11.53	22.04	3.54	14.59
62408	1.84	8.99	0.00	4.17
62409	1.84	6.29	0.00	0.39
62411	1.84	4.31	0.00	2.16
62476	7.31	13.67	1.09	6.27
62477	7.31	10.93	0.00	0.33
Mean	**3.15**	**6.20**	**.58**	**2.51**

Test # followed by the same VFL are from different seed lots of the same cultivar and the TFL, GOT and M*3 tests are variable among seed lots indicating the natural variation seen in the out-crossing ryegrasses. Correlations among the three tests, and with VFL should be viewed with caution because VFL is based on faulty TFL and GOT. The VFL should not be the ultimate determinator.

B. References

Barker, R.E., S.E. Warnke, S.G. Elias, A.E. Garay, and R.L. Cook. 2002. Ryegrass grow-out tests in relation to seedling root fluorescence. p. 90-95. *In* W.C. Young III (ed.). 2001 Seed Production Research. Dept. Crop and Soil Sci. Ext/CrS 121, 4/02.

Davidson, S.K. 2010. I Hate Fluorescence. Presentation at the Annual Meetings of AOSA/SCST, 2010 St. Louis, MO. The Seed Technologist Newsletter, Volume 84, Number 2.

Floyd, D.J., and R.E. Barker. Change of ryegrass seedling root fluorescence expression during three generations of seed increase. Crop Sci. 42:905-911. 2002.

Nittler, L.W., and T.J. Kenny. 1972. Distinguishing annual from perennial ryegrass. Agron. J. 64:767–768.

Tyler, B.F., K.H Chorlton, and I.D. Thomas. 1987. Collection and field-sampling techniques fro forages. Pp. 3-10. *In* B.F. Tyler, Ed. Collection , Characterization and Utilization of Genetic Resources of Temperate Forage Grass and Clover. IBPGR Training Courses: Lecture series 1. Intl. Board Pl. Gen. Resources, Rome.

C. Referee data:

Ten seed samples were examined in three labs by the A/D test. After adjustment for differences among labs for TFL variation, there were no within or among lab differences for the results of the A/D test. Details are provided as Appendix III.

Background Information:

In 1938, Dr. H.H. Rampton (1938, 1966) wrote in the Agronomy Journal, **"The fluorescence test is useful in the classification of the general run of domestic ryegrass seed and in making approximate determinations… It cannot be used as an infallible guide in classifying questionable lots of ... ryegrass seed."** Yet the grass seed industry and seed testing authorities made the SRF test more exacting by employing the Variety Fluorescence Level for new ryegrass varieties (AOSA 1991, 2009). Implementation of an A/D test can put an end to the biased and improper abuses of the SRF test and the GOT. These new molecular tools are available to provide more accurate results in less time than the GOT and the SRF tests. The A/D results can be available in as little as two days after SRF is determined.

In developing the A/D test presented below, the protocol has been conducted at Oregon State University (OSU) and Grass Genomic Testing, Inc. (GGT) using different real-time PCR (qPCR) machines and different personnel yielding essentially the same results. The following includes the background data on SRF, GOT and A/D results obtained from a few of our studies.

Several reports have been made regarding the inadequacies of the SRF test (Barker et al. 1997, Floyd and Barker 1997 and 2002, Rampton 1938). Barker reported to AOSA and AASCO on an ISTA sampling study where 60 samples were drawn from one 55lb seed lot. He had SRF tests conducted on each of the 60 samples after they were drawn and analyzed for germination, then had SRF tested again the following year on the same samples (Table 2). TFL ranged from 1.57 to 5.40% in 1998, but TLFs increased the second year tests were conducted and the range increased. Differences between the two test years were large, and ranged from -1.92 to 3.21, demonstrating that SRF has more variation associated with it than tolerance tables indicate. SRF determinations are biased upwards toward "annual-type" presence (Barker 2010, Barker and Cooper 2010).

Table 2. Variation among TFL results from 60 samples drawn from one 55lb seed lot of Repell II perennial ryegrass that has a VFL of 1.56% and an original TFL of 1.83%.

	Statistics over the 60 samples (as TFL %)		
Year	**Average**	**Minimum**	**Maximum**
1998	2.86	1.57	5.04
1999	3.60	1.83	5.85
Difference	0.77	-1.92	3.21

After several years experience with the GOT, we at USDA/ARS, OSU, and AST found that the GOT has problems as it is applied in the grass seed industry similar to those of the SRF test. Research showed that the GOT might be underestimating the number of "annual-type" plants found among the SRF seedlings. This may be because the implemented GOT is not conducted for long enough time, there is not a consistent end point, and artificial lighting conditions were not standardized at high enough intensity levels. As implemented by AOSA, the GOT ends at or before week 6, but plants continue to head until at least 12wk in continuous light at 25°C. We examined this GOT bias by examining 20 commercial seed lots in 2005 and repeated the test again in 2006. Data were published in The Seed Technologist Newsletter, May 2010, and only totals or means from those data will be presented here. In 2006, a 84-day GOT in a high light intensity (PAR 425 $\mu mol\ m^{-2}s^{-1}$) growth chamber with lights on continuously was conducted on those seedlings that showed fluorescence root traces (FL+) plus an equal, or more number of seedlings that did not show fluorescence (NFL). Growth rate (accumulated number of plants headed) of both the FL+ and FL- plants increased over time (Figure 2). The differential rate to heading of the seedlings from the two classes is what makes the GOT effective in many situations, but a six-week test is an arbitrary end point and results will change with the changing conditions in which the plants were grown.

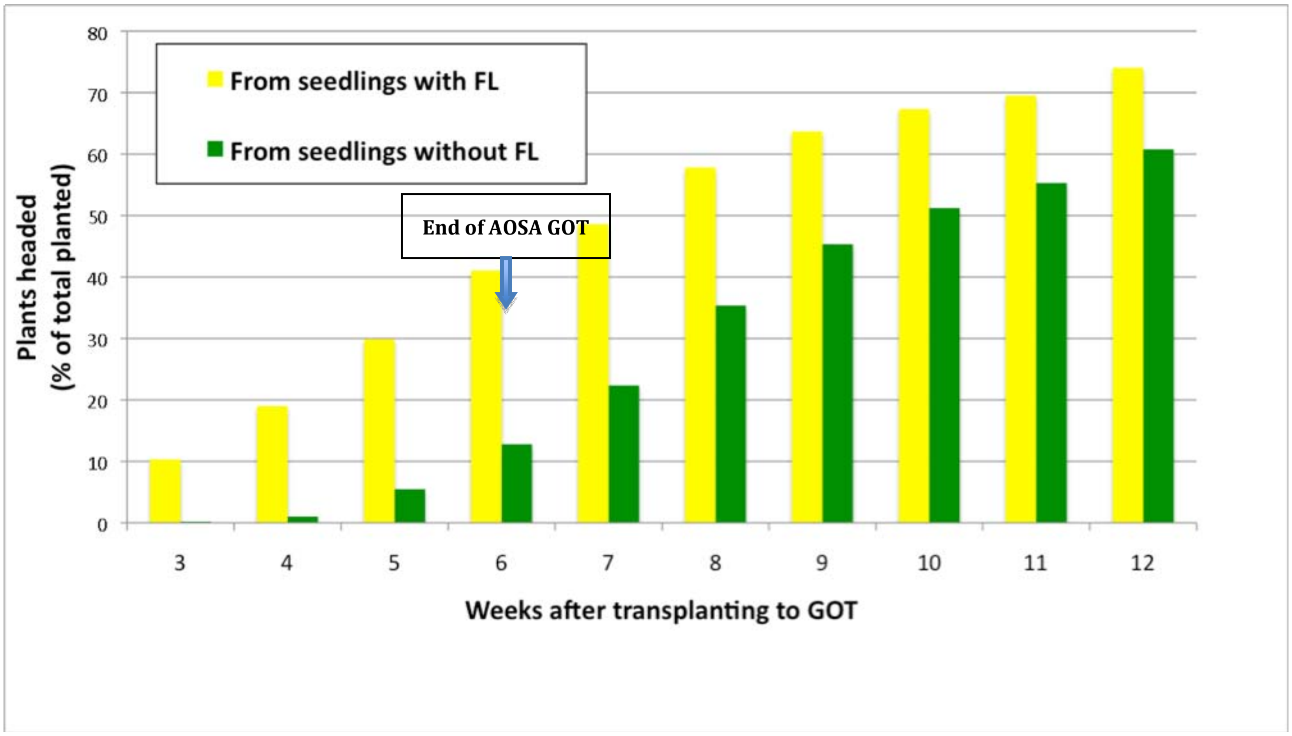

Figure 2. Percent of plants headed after each week of a 12-wk GOT. Yellow bars are for plants that had fluorescence root traces as seedlings and the green bars are from plants that did not fluorescence as seedlings.

Number of plants from FL+ seedlings, totaled over all 20 cultivars, for accumulated numbers of headed plants was reported (Figure 3). Of 441 FL+ seedlings, 359 survived the GOT with 118 of these having molecular marker determination for “annual-like” or “hybrid” alleles. By the end of the GOT 94 of the FL+ seedlings had not headed. Only two of the plants from NFL seedlings had DNA markers present.

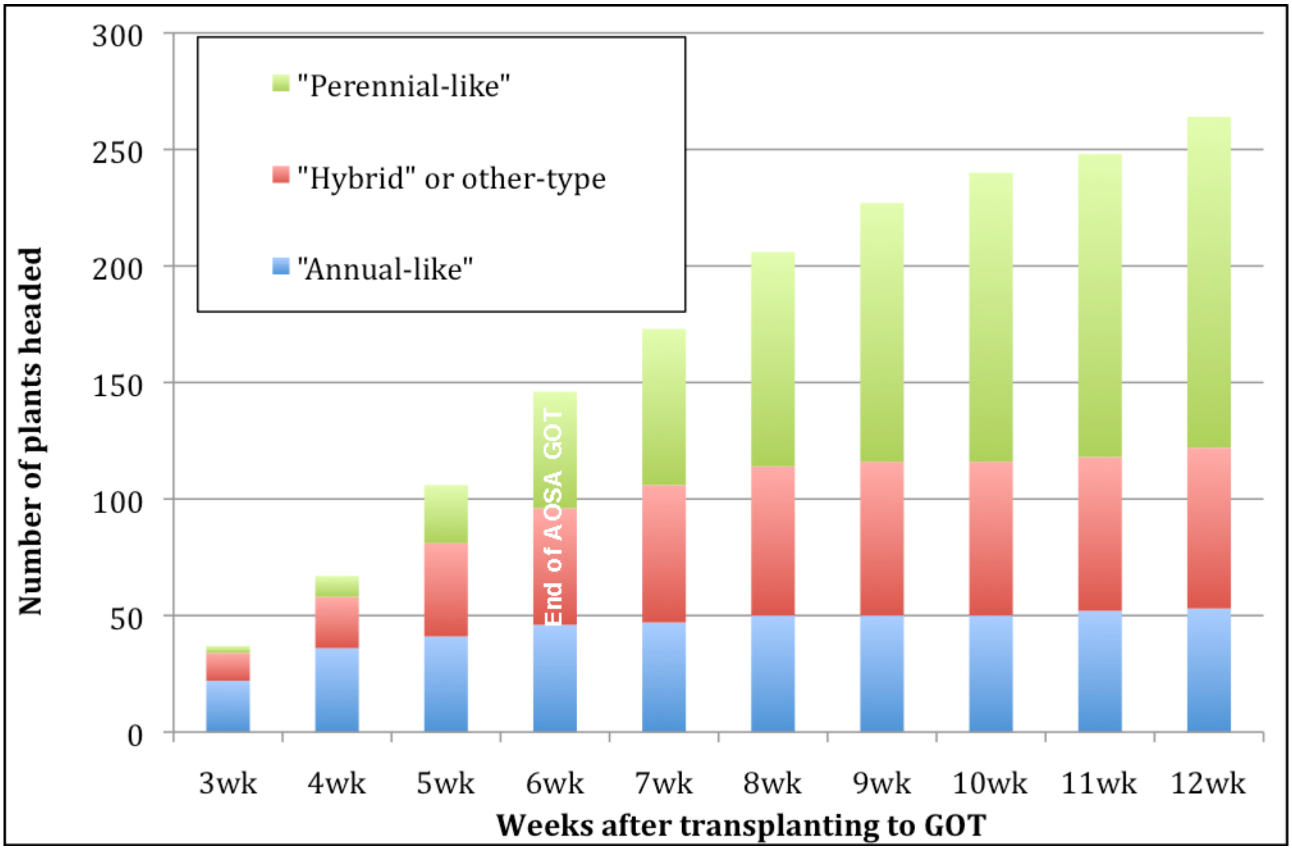

Figure 3. Accumulated heading of FL+ seedlings in a GOT grown for 84 days under continuous light in a high light intensity growth chamber using 20 ryegrass cultivars. The GOT is usually terminated before or at week 6. The SRF test and either of two molecular markers, $LpID_1$ and *LpVrn-1* to determine annual types were measured on seedlings before the GOT started and the tested seedlings were grown to heading. Markers from seedlings before transplanting to the GOT can predict results equivalent to a 10-wk or more GOT.

Bars in Figures 2 and 3 indicate accumulated heading, i.e. those heading during the one-week interval were added to the previous weeks total. The GOT was conducted according to AOSA rules, but was conducted for twelve weeks in a high intensity growth chamber (continuous light at 425 µmol $m^{-2}s^{-1}$ and 25˚C). SRF and the molecular markers were measured on the seedlings before transplanting to the GOT. Numbers of seedlings that were determined to "perennial-like," "annual-like," or "hybrid" are the segments of the SRF seedling bars in Figure 3. Results from the two molecular markers (considered together) indicate the reason for the SRF inaccuracies. Percent SRF of the plants that headed in each one week increment never got below 30%, while use of multipoint molecular markers dropped to less than 4%, equivalent to a more than nine week GOT.

For the past 13 or more years, Barker and cooperators at USDA/ARS, OSU Seed Lab, Agri Seed Testing, and others (see References) worked on a PCR test that is faster and more accurate than either the SRF test or the GOT. An Allelic Discrimination (A/D) test was developed and proof of concept determined based on single nucleotide polymorphisms (SNPs) to two major genes involved in the flowering control process in grasses (Figure 4). A SNP results in an allele, which is an alternate form of a gene. SNPs of the "indeterminate" gene ($LpID_1$) and one of the vernalization genes (*LpVrn-1*) are used in the A/D test. **Work at OSU was done on an Applied Biosystems (ABI) 7500 qPCR system.**

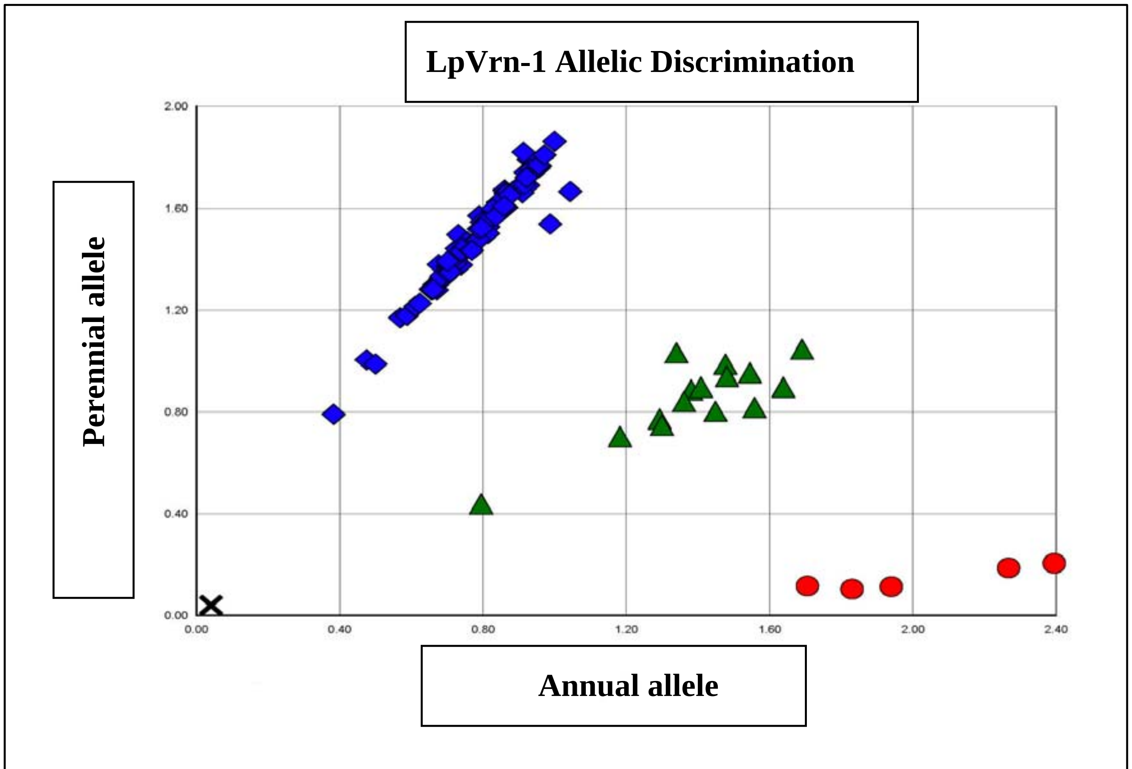

Figure 4. Allelic discrimination analysis of a perennial ryegrass cultivar for *LpVrn-1* as measured on an ABI7500 real-time PCR machine. Diamonds are perennial types, circles are annual types, and triangles are "hybrids" or heterozygotes.

Data from the Barker USDA lab:

We used published gene sequences to design gene-specific PCR primers and amplify fragments of the homologous genes from ryegrass. We then sequenced the fragments and compared the gene sequences from Italian and perennial types. Sequence differences were the basis for single nucleic polymorphic (SNP) markers, which can be detected as a TaqMan® assay using real-time PCR and probes labeled with fluorescent reporter dyes. We successfully sequenced most of the *LpVrn-1* homologue from both Italian and perennial ryegrasses, and identified several SNPs that distinguish the Italian ryegrass types from perennial ryegrass. We developed a genetic linkage maping population and the population plants were screened for the *LpID₁* and *LpVrn-1* SNPs, allowing us to place them on our linkage map. *LpVrn-1* is located on linkage group 4, and *LpID₁* on linkage group 5 syntenic to similar genes on the wheat and barley maps. The *Vrn-1* gene controls plant response to vernalization and is epistatic to photoperiod; expression is increased following vernalization (cold treatment) in perennial ryegrass. The *LpCO* gene was found on linkage group 7 and is the same or correspons to the *LpVrn2* gene, epistatic to *LpVrn-1*. The A/D test for *LpVrn-1* and *LpID₁* in our initial studies was confirmed.

The SRF gene was found on linkage group 1 and confirmed the statement in the AOSA Cultivar Purity Testing Handbook (2009) that SRF is not genetically linked to other growth type genes. It is, however, on the same linkage group as the *earliness per se* gene that is a secondary genetic system for flower control and explains why the association discovered by

A validation study was conducted on 20 perennial ryegrass cultivars, comprising 880 total plants. The molecular markers and SRF were determined on the plants before transplanting to a GOT. Distribution of fluorescence (FL+ for present, FL- for absent) is shown in the first two bars of Figure 5. Over 40% of the plants tested were FL+ and about 60% were FL-. These two fluorescence classes were further subdivided into classes based on GOT and the molecular markers. About the same ratio of flowering in 42 days (the usual length of a commercial GOT) as they appeared in the FL classes. Molecular markers (*LpVrn-1* and *LpID1*) were developed primarily for their ability to detect the annual growth-type, so larger numbers of plants were found in the molecular marker classes for the FL+ class. Very few of the annual-type markers were found in the FL- class, and if both markers were considered together as is done with the proposed A/D test, molecular markers would misidentify less than 0.05% of the perennial-type plants. Single markers alone misclassify more of the non-fluorescing seedlings, which is a danger for single marker tests.

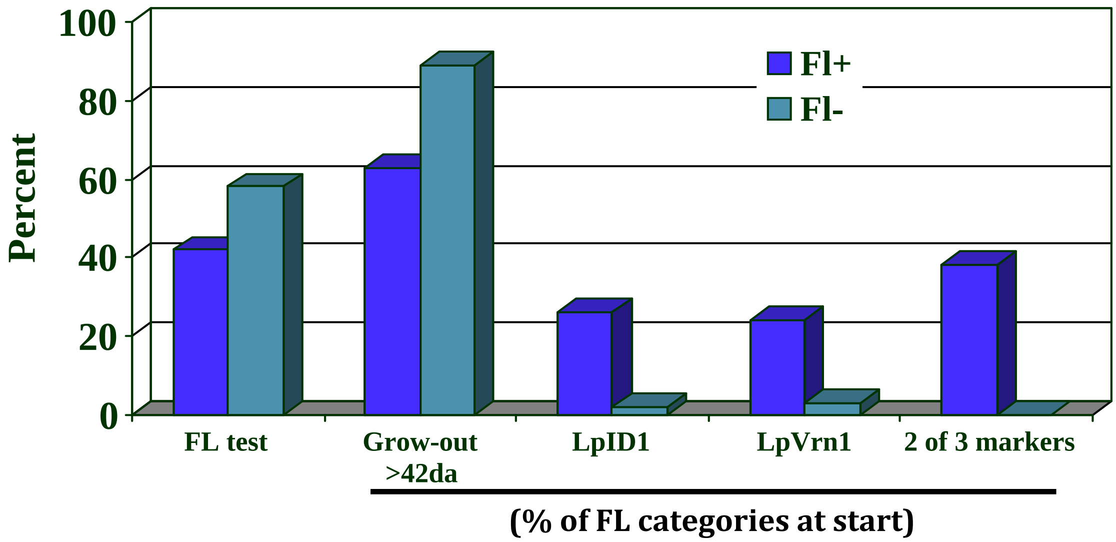

Figure 5. Characteristics for FL and the percent of plants in each of those classes that had "annual" alleles based on grow-out, *LpVrn-1*, *LpID1*, and both *LpVrn-1* and *LpID1* considered together from 20 Perennial Ryegrass Cultivars.

It is assumed that markers for flowering control are closely associated with growth-type, and they should be. Flowering control, however, is very complicated and no one gene can be expected to predict growth-type by itself. If we knew the exact gene that causes annual vs. perennial growth-type, that would be the most accurate. All other markers are still **associated** predictors. Flowering is controlled by at least three families of vernalization genes, two families of photoperiod genes, and genes in several other biological pathways. Use of the flowering control genes indicated here are still better predictors than the SRF and GOT tests.

In 2008, 22 additional seed lots were chosen based on a wide range of SRF values and molecular markers were examined on them to verify that the A/D tests could be conducted on a commercial scale. Agri Seed Testing provided the seed test data and GGT conducted the A/D analyses (Table 2, and Tables 1 and 2 in the AOSA Proposal). Seed for each seed lot were germinated and a SRF conducted, and a grow-out test conducted according to AOSA protocols. During the grow-out, a portion of the leaf of each plant was harvested, DNA extracted, and an A/D conducted for both *LpVrn-1* and *LpID1* (Table 2).

A/D tests at GGT were done on a Bio-Rad CFX96 qPCR system with different personnel from those conducted at OSU.

Annual-like and perennial-like using the markers was determined using a multi-point decision.

Plants that were FL+ were declared annual-like if either LpVrn-1 or LpID1 had an "annual" allele. For FL- plants, two "annual" alleles were needed to declare it annual-like. This is the "two of three" marker determination (GGT M*3 AR/PR Test).

Table 3. Percentage of plants from twenty perennial ryegrass cultivars for test fluorescence level (TFL) studied in a grow-out (GOT) and with the two molecular markers, *LpVrn-1* and *LpID*$_1$. Total number of plants in each category was recorded from the GOT and for the *LpVrn-1* and *LpID1* markers combined for decision making.

						Grow-out results			Using both molecular markers		
Seed Test#	**VFL (%)**	**Total Seed Tested**	**No. Norm. Germ**	**Germ (%)**	**Norm. FL (%)**	**"A"-Like***	**"P"-Like***	**Mor-tality**	**"A"-Like***	**"P"-Like***	**No DNA amp**
59216	0.77	400	371	92.75	20	2	16	2	3	15	0
59337	0.12	400	378	94.50	20	1	18	1	4	15	0
59669	0.77	400	369	92.25	24	0	23	1	3	19	1
60079	5.88	400	362	90.50	32	4	24	4	14	14	0
60080	5.88	400	356	89.00	35	9	26	0	23	11	1
60660	VNS	400	369	92.25	43	5	38	0	25	18	0
61248	4.83	400	374	93.50	15	1	14	0	4	11	0
61249	4.83	400	388	97.00	28	6	20	2	15	11	0
61490	1.87	1200	1067	88.92	8	0	8	0	0	7	1
61491	1.87	1200	1036	86.33	12	0	11	1	0	8	3
61492	1.87	1200	1090	90.83	9	0	9	0	1	8	0
61493	1.87	1200	1047	87.25	13	0	12	1	2	6	4
61494	0.99	1200	1063	88.58	10	0	9	1	0	8	1
61495	0.99	1200	1094	91.17	28	1	27	0	3	24	1
61496	0.99	1200	996	83.00	11	0	11	0	0	11	0
61497	0.99	1200	1070	89.17	31	0	30	1	7	22	0
61761	11.53	400	372	93.00	82	13	68	1	47	24	10
62408	1.84	400	356	89.00	32	0	31	1	13	15	3
62409	1.84	400	334	83.50	21	0	18	3	1	15	2
62411	1.84	400	348	87.00	15	0	12	3	6	6	0
62476	7.31	400	373	93.25	51	4	46	1	22	26	2
62477	7.31	400	375	93.75	41	0	38	3	1	32	5

*** "A"-like = Annual-like types and "P"-like = Perennial-like types.**

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Appendix II

Allelic Discrimination Protocol

AOSA/SCST Protocol Proposal for AOSA Cultivar and Purity Testing Handbook
Reed E. Barker and Sharon Davidson

GGT M*3 A/P Test using Allelic Discrimination TaqMan® SNP Genotyping in Ryegrass

The protocol is attached as a separate standalone document.

Appendix III

Referee Data

Raw data:

Ten seed samples with identifications concealed were distributed to three labs for conducting the GGT M*3 AR/PR tests. Test numbers 6149* came from four different lots of the same cultivar that was being examined for VFL determination and were not "true" replications. For purposes of comparisons here, the "Annual-types" and "Hybrids" were combined (Table 1). Initial impressions were that the M*3 test separated the test entries very well, but further statistical analyses were needed because of result correlation with TFL that indicated that the M*3 test is dependent on conducting an optimized SRF test.

Table 1. Raw data summary results from referee test that include study structure, "Annual-type" content according to the GGT M*3 AR/PR Allelic Discrimination test, and the SRF test reported as TFL.

		"Annual-type" DNA markers (%)				TFL (%)		
		Testing Lab				Testing Lab		
Seed lot	**Rep**	**OR**	**MD**	**IL**		**OR**	**MD**	**IL**
61761	1	16.71	8.83	6.36		22.04	15.76	10.79
	2	13.33	8.39	7.64		22.48	14.69	11.60
53666	1	6.63	2.94	5.82		14.13	12.07	14.40
	2	7.05	4.38	5.36		15.57	11.34	13.67
62409	1	1.14	0.00	1.80		6.29	4.19	4.19
	2	2.17	1.45	1.19		9.21	6.38	3.86
6149*	1	0.00	0.28	0.00		1.16	0.83	1.16
6149*	2	0.09	0.26	0.00		0.83	0.51	1.90
6149*	3	0.41	0.00	0.00		1.24	0.28	0.85
6149*	4	0.00	0.00	0.28		0.75	0.28	1.39

Analyzed results:

The referee study was organized so that it could be statistically analyzed in an analysis of variance (ANOVA). In the statistical model, all effects except reps were considered fixed. Differences among Reps were trivial and not significant indicating that **within** Lab variation is not a problem for conducting the M*3 test. Further, all interactions with reps were not significant and means over Labs and Entries could be presented directly (Table 2).

As expected, there were significant differences for the main effects of Labs and Entries for "Annual-type" contamination. The OR lab had the highest test results and were significantly different from both the MD and IL labs. This difference is mainly a reflection among labs for ability to get maximum SRF expression. When average results for each lab were adjusted for the TFL results in an analysis of covariance, there were no significant differences among labs for "Annual-type" contamination. Thus, there are no significant differences **among** and **within** labs for conducting the GGT M*3 AR/PR DNA-based purity ryegrass test and it is determined that it is a viable alternative to distinguish ryegrass growth type differences.

Table 2. Seed lot and lab means from the referee test on ten ryegrass samples. “Annual-type” content according to the GGT M*3 AR/PR Allelic Discrimination test combined the “annual-type” marker with the “hybrids” for this comparison.

		“Annual-type” DNA markers (%)			
		Testing Lab			
Seed lot	**VFL**	**OR**	**MD**	**IL**	**Mean**
61761	11.53	15.02	8.61	7.00	10.21 a
53666	5.88	6.84	3.66	5.59	5.36 b
62409	1.84	1.66	0.73	1.50	1.29 c
6149a	1.87	0.05	0.27	0.00	0.11 d
6149b	1.87	0.21	0.00	0.14	0.12 d
Mean		4.75 a	2.65 b	2.85 b	
Adjusted LS Mean		3.79 a	3.07 a	3.39 a	

It is the SRF test, however, that creates variation among labs. In 2009, Sharon Davidson (AST inc.) and DaNell Jamieson (BioDiagnostics, Inc.) completed a study among ten labs for their ability to conduct the SRF test on seven ryegrass seed lots. They found extreme variation among the labs for TFL results (personal communication, 2010). For TFL, one lot varied 38% to 97% across the ten labs, while another varied from 0.27% to 2.15%. Lots with highest contamination varied more than those with low contamination.

The literature is full of references on how to optimize the SRF test, yet many labs are not following these conditions and optimizing their lab’s ability to maximize SRF expression as found in the Jamieson & Davidson survey report (2009). This lack of within lab optimization and among lab standardization is specifically what has led to the failure of SRF testing in the past. The natural variation within an outcrossed grass also contributes to the variation. SRF expression is based on a chemical (annuloline) being exuded from a seedling germinating on white filter paper. Annuloline is a by-product of lignin biosysthesis and any conditions that affect lignin production also affects SRF expression. Further, while we follow the model for flowering control in wheat and barley, the genetic systems for ryegrass are far more complicated because of more modifier genes (multiple alleles) being present.

Results from a GGT M*3 AR/PR test will be affected by the inability to properly screen for maximum expression of SRF, but statistical adjustments can be made to the results. Further, more FL- plants can be tested to determine the amount of plants not detected by SRF prescreening. The DNA-based test is available and can be implemented to satisfy many needs in grass seed analysis.

Appendix IV

I Hate Fluorescence

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Introduction and overview

Originally the fluorescence test was used as a "Kind" determination for Annual or Perennial Ryegrass. It was believed that only annual seedling roots would exude annuoline, which produces a bluish fluorescent line on white filter paper when examined under ultraviolet black light.

In the late 70's a seed company decided to use fluorescence in their marketing plan. Their varieties, grown in the North Valley, had little or no fluorescence; in marketing lingo this meant no annual contamination. This caused some havoc for the companies that used growers in other parts of the valley. In the late 80's a committee was formed, new calculations were derived and the Test Fluorescence (TFL) and Varietal Fluorescence (VFL) were born in 1991.

The growers were still saying that this was not an accurate reflection of their production. New research showed the problem was higher fluorescing perennials occurring than previously thought and the grow out test was developed and implemented into the Cultivar Purity Handbook in 2001.

And with the addition of calculations into the rules we started digging our hole.

The problems:

In the beginning:

Annual	Perennial
Wide leaves	Fine leaves
awns	no awns
short glumes	long glumes
rolled vernation	folded vernation
light green	dark green
tall	short
root fluorescence	no root fluorescence
larger seed size	smaller seed size
rust resistance	susceptible to rust
jointed	not jointed

Remember, originally the fluorescence test was used as a "Kind" determination for Annual or Perennial Ryegrass. The fluorescence gene was thought to be stable and unique to annual ryegrass.

Then plant breeders started to play by using annual ryegrass as a parent in new perennial varieties, 'knowing' that the genes that control fluorescence were stable, trying to improve seed size, seed production and disease resistance. They found that the fluorescence gene is not stable and is not 'hooked' to annuality.

Besides plant breeders we know that the environment plays a role in higher or lower TFL's each year. On a dry year we expect to see higher TFL. Seems like stress to the plant before, during or directly after pollination, causes higher TFL. We have not been able to find the trigger.

40 years ago, Dad would drop us kids off at the edge of a 100 acre field and tell us to pull all the plants with 'hairs' on them. 30 years ago, in college, I learned the 'hairs' were awns and they indicated annual ryegrass. 20 years ago, as a seed analyst, I started seeing perennial ryegrass seeds with an awn! The awn gene can transfer as well as the fluorescent gene does! The most frustrating thing for a grower is to have this beautiful, uniform perennial ryegrass field and the test report comes back with annual ryegrass on it. Certification did not report annual ryegrass in the field inspections, the grower did not see any because the kids pulled out all the tall plants, and the company field rep did not see any annual plants.

We know that the 'wild' pollen blows in and causes problems. Especially in the south valley, where the annual ryegrass production has been for all our production lives, the pollen blows the same time the perennial ryegrass is accepting pollen. In the north valley, annual pollen usually blows earlier than perennial pollen. But we do have our years of pollen tornados. So, the field can look perennial, but the seed produced by those plants can have mixed pollen resulting in perennial, annual or hybrid fluorescing seedlings.

Another environmentally induced issue could be the change in chemicals. We now apply growth regulators to our fields. Shorten up the perennial ryegrass plants so that they will stand longer for better pollen reception. Also allows for more adventitious pollen to land.
And we also are applying stronger herbicides, for annual bluegrass control, to the outside rounds of a field; it stresses the perennial plants almost to the point of death. We know stressing the plant causes higher fluorescence occurrence, could this be working in the same manner? We also spray our fields for rust control, something we didn't do 15 years ago. Could chemicals be altering the plant gene base to express themselves differently in the produced seed?

We can influence fluorescence levels in the lab as well. A study done in the crop science department at OSU found that fluorescence intensity was brightest when seedlings were grown in the dark. Fluorescence intensity decreased with each successive increase in light intensity in the germinator. They also found that the higher the temperature, the more intense the fluorescence, lowest levels were at 15C and highest were at 30C. No difference was noted when seeds were germinated in KNO_3 vs. water. Fluorescence intensity was considerably brighter on wet filter paper than after the paper dried, however we do find more fluorescence occurrence when the substrate dries out during the test.

By manipulating the light intensity, light duration, temperature or moisture we can have differences between results. Fluorescence tests performed on seedlings grown under sub-optimal germination conditions, or with faulty equipment, could conceivably have different results than tests conducted on the same seed germinated under ideal conditions.

As we analysts get older, eye sight becomes an issue and intensity of fluorescence can make a difference. My lab had a comment from a plant breeder that when he submits lots for a VFL test, his TFL's come back lower than when the same lot was submitted for a regular test. That didn't sound right, so I randomly took 1 box of 100 seedlings from different samples one day and blind labeled them. I then had each analyst that performs the fl test in my lab, and one person who does not, read each box and record their findings on a separate sheet of paper. What I learned was we had a bit of a swing between analysts. We conducted a workshop and had all 7 people look at each of the boxes as a group. A few months later we repeated the exercise and the readings were much closer. This is a good exercise to do in your own labs.

One of the old wives tales amongst the growers is that fluorescence is always lower in the spring. I thought we might take a look at that and for 8 months I evaluated 55 different samples of perennial ryegrass. What I found was not consistent but did show that a sample can vary up to 6% in TFL over a period of less than one year. I found that 28% of the samples had their lowest TFL in April, 18% were lowest in March, 16% in December. Maybe a little bit of truth to what they are saying, nearly 2/3's of the samples had their lowest TFL in the spring. Could it be because annual ryegrass has less dormancy and germinates right after harvest showing higher fl levels sooner after harvest and then as the perennial starts to break dormancy the TFL level is lower just because of the math? Or is there a time trigger we can't find? Whatever the reason, the growers with over 3% other crop due to fluorescence usually wait until the spring and run another test so they are not dinged the 5 – 10 cents a pound.

To develop the VFL for a new variety a minimum of three lots from two different years are used to find a minimum of 30 fluorescent seedlings in a maximum of 10,000 seeds. All the fluorescent seedlings, 25 non-fluorescent seedlings of the new variety and 25 annual seedlings are planted in soil and grown out following the CPH method. At the end of six weeks the seedlings are classified and a VFL is calculated.

The VFL is then used as a 'fudge factor' against the TFL. Seed growers are 'docked' on price if the fluorescence is greater than 3% over the VFL. While this is an improvement, they were losing more money without the VFL allowance, this still is not accurate. The false positives found in the fluorescence test can further be misclassified in the grow-out test.

Back to the plant breeders, another way they can play the system is with the VFL. When submitting the lots for the VFL procedure they can pull samples from lots with high fluorescence levels to get a higher 'fudge factor'. The higher the fudge factors the easier to get a grower to produce it. We know the TFL can vary from year to year because of environmental factors and maybe cultural ones. The lots selected make a difference in the outcome, north valley has less adventitious pollen, south valley has more. Lower germination lots can show a higher TFL due to the mathematical equation.

Interesting side note, in 1991 there were only 19 perennial ryegrass varieties with VFL. Now there are over 300. And at least 10% of those described have been redescribed with a double or triple VFL outcome.

It's no secret, the grass seed industry sucks right now due to the economy, no new houses, fewer golfers and water issues for homeowners. We have banks screaming at growers to sell some seed to 'downsize' the operating loan. We have growers screaming at seed companies to pay them for their production so they can ease the banker calls. The seed companies can't move the seed so they release it to the grower and tell them good luck sell it if you can but not with our variety name. Grower calls us to delete variety name and wah lah – that fudge factor disappears and there is a contamination problem that the grower didn't expect. And is it a contamination problem? Maybe this could be stretched to mean truth in labeling, but it is definitely not truth in representation. It is the same seed in the bag as it was with a variety name, but we can't use the VFL because the name came off. It's just not right. And who is liable? The grower loses money, the buyer doesn't get what he thinks he bought – All because the AOSA Rules require calculations!

Cultivar Purity Handbook issues

Although the AOSA Cultivar Purity Handbook (CPH) has methods for conducting fluorescence and grow-out testing for ryegrass, it states in the beginning paragraphs that "research indicates that there is no linkage between the gene or genes controlling seedling fluorescence and the genes controlling growth habit or other morphological characteristics." We all agree. Never the less, the AOSA Rules require a fluorescence test to be made with all ryegrass purity tests in which percentages of perennial and/or annual ryegrass are reported, any fluorescence over the VFL is reported as annual ryegrass contamination instead of other ryegrass.

Step 6 of the procedure for fluorescence testing is how to final the test at 14 days. In part it states "Remove and record fluorescence for dead seeds or abnormal seedlings." This is confusing and causes inexperienced analysts some misunderstandings. Maybe a better wording would be "remove and note", the intent is not to include fl of dead or abnormal seedlings into the final calculation.

In 1990 we helped with some research to eliminate the lifting of seedlings to find hidden fluorescence. In all there were 190 seedlings, from 32 varieties, that showed fluorescence only after they were removed from the media. These seedlings were divided and grown out in green houses at two different locations. Each location found only 1 seedling with annual characteristics. All seedlings were grown out until they had headed, the 188 remaining seedlings showed perennial characteristics. The study was then expanded and over 1,000 seedlings with hidden fluorescence were grown out. This study showed that lifting increased the annual ryegrass contamination due to fluorescence content by only .02%.

In 1991 the research data was submitted to AOSA by Rodger Danielson and Don Grabe and accepted. The Cultivar Purity Handbook was revised in 1994 deleting the sentences "Then lift all non-fluorescent seedlings, observing the path of each root, since some faint fluorescence will show up only after the root is pulled off the paper. Mark any additional fluorescent seedlings found by lifting the roots. All fluorescent root traces are to be counted, regardless of intensity of fluorescence." In the 2003 electronic version of the CPH, an old version was used to burn the CD and the sentences were put back in the method. This was corrected again in the 2008 printing of the CPH. In a survey conducted by BDI last year, 5 of the 12 labs responded that they are using old versions of the CPH. The production laboratories are not lifting, but some consumer laboratories are. This is causing higher fluorescent levels to be reported on the consumer end leading to some red tag or stop sales to occur.

One little study that came from all the Bright and Light fluorescence seedlings investigations was some work a breeder thought he wanted to do to create a totally fluorescent perennial ryegrass as a joke. We were checking his breeder block plants for fluorescence levels. Out of the 50 plants we found one plant that exhibited fluorescence at 20%. The breeder took the 5 bright fluorescent seedlings and planted them in a secluded nursery surrounded by fir trees. He harvested the seed as a batch lot because we "knew" the fluorescent gene was dominant, and expected all of the harvested seeds to fluoresce; instead we again found 20% fluorescence. We did not know if that 20% came from one plant or if all 5 plants contributed. We were going to look at that part of the puzzle the next harvest year but the little secluded nursery turned out was in the path of some trees destined for logging and our plants were skidded over. We still learned that fluorescing seeds do not necessarily parent fluorescing seeds.

We participated in another study to prove that fluorescence is not connected to annuality. We used 11 varieties, collected data over three years. Total seeds tested were 69,400, total seeds with normal germination were 63,996 with 970 of those exhibiting fluorescence. We tracked the bright and hidden fluorescent seedlings through a grow-out and what we found was of the 480 bright seedlings that survived only 7 had morphological characteristics of annual plants, of the 418 hidden fluorescent seedlings only 1 displayed annual characteristics.

And with that we dug a deeper hole by adding the Growout test to the rules.

The Grow-out test method as described in the CPH also has some problems, besides the fact that any one can change, delete or manipulate the procedure without informing those of us that use the procedure, but that is another argument for another day. This method involves growing and evaluating seedlings and plants in a controlled uniform environment. This approach insures that all seedlings and plants are grown under the same conditions and that cultivar differences have a genetic basis.

On page 46 of the CPH it states that ryegrass can be evaluated as early as 35 days, that is about 2 weeks too soon. It also says that you should maintain 25C, unless you have a greenhouse that can fluctuate +/-8 C and that doesn't really matter. Then you also should have 1200 foot candles, because increasing the light intensity speeds up flowering and heading, but again if you have a green house on a cloudy day it does not appear to reduce the growth or heading. These statements seem to contradict the opening statement of controlled uniform enviornment. So much for consistency!

The classification criteria are vague at best. No mention of phenotypical markers except heading, nothing about vernation, jointed vs. not. There is mention of color, which should never be used, and wider leaf blades for annuals; however we do have forage perennials with pretty wide leaf blades – and we now have turf type annual ryegrass. There have been several lawsuits that have been settled out of court due to inaccurate Growout classifications? As a result, some seed companies in Oregon will not pay growers based on a grow-out.

We argued for a third category for classification since we all know that ryegrass is a continuum with annual on one end and perennial on the other and the gray in the middle. The seedlings resulting from adventitious pollen are the gray area seedlings. They usually are 'big uglies', not heading, not jointed, limey green, not rolled or folded but kind of rolling folded, hugeish. I do not call these true intermediates, I use the term hybrid. The committee disbanded because of this argument and the rule proposal went forward against our wishes.

To readdress this third category, in December 2009 a group of plant breeders, seed analysts and production companies met at the OSU greenhouses to review the grow out classifications. Some of you will probably see this as a 10? Well this is my 10! Everything is subjective, same thing with the evaluations, not even the plant breeders could agree on what to call a certain plant. If ryegrass plant breeders can't agree what to call perennial or annual, what the heck are seed analysts supposed to be doing? Some analysts are treating the grow out as a trueness to variety test, and any seedling that does not look like the non fl control plants is classified as an annual. Some analysts are evaluating strictly on phenotype, if it heads, is rolled and jointed than it is annual. And we have no place to put the ones in the middle, some classify as annuals because they don't look like control non fluorescence, some as perennials because they have not headed and are not jointed. We are now seeing more often that the non fl control plants are heading, I have even seen some of the fl plants that look perfectly like the non fl control and are heading. So, evidently that gene can cross also. We decided not to change any thing in the CPH, I think, and the industry folks decided to stick with not accepting the grow out test.

Genetic testing
There are DNA based test now available to help determine if the seed is annual or perennial. Since there is so much grey area between annual and perennial this was harder than they first thought. Should they try and tie it to the fluorescent gene, day length to flowering gene or what since it seems all the genes readily cross from annual to perennial? Is there a Big Ugly Gene we don't even know about? Will this test be economically feasible on a per lot basis? The DNA test can be run in a matter of hours vs. 14 days for the fluorescence test. However in a production state where we receive 200 samples a day what is the cost for a lab to be equipped to handle the load? Since the equipment for this type of testing is expensive will regulatory agencies be able to accept this kind of labeling in lieu of a black light and a dark room? Of course the more we use it, the more research goes into it, the less expensive it will be. AOSCA says we can't use molecular testing until it is in the AOSA Rules. Politics kept it from being accepted as a rule proposal this year. Since we have molecular testing available to us now, we should make it an option to the industry who wants to use it.

What do we really need?
I think we need to determine what information we really want here. Do we really want to know if the plant will set seed and die in one cycle = annual? Or do we want to know the big uglies in a planting = hybrids or annuals? Or do we need to know as much as we can for proper marketing and use of the seed?
Are we just looking for an indicator test to settle contract payments?
I know the production companies pay the grower based on the quality of the seed, and fluorescence is used as one indication of quality. The false positives however result is millions of dollars lost to the growers.
The buyer/seller contracts use the same quality indicator as a standard. There can be loss to the seed company of 50 – 60% margin due to false positives and in the case of a grow-out based sale, an unknown loss to end users to replace undesirable seed product.
Certification uses fluorescence as a quality standard to issue a blue tag or not.

I know the lawn and turf industries would like to know the big ugly population in a lot and to avoid those lots that have them.
The forage and over seeders want to know how much of their planting is going to die in one cycle or due to heat or cold stress. Neither care about the fluorescence number per se.

Regardless we need to find a better informational way to label these products. The fluorescence test does not do this, the grow-out test does not do it.
The most important educational thing we can do is to make everyone aware that fluorescence does not equate to annuality. Fluorescence should never be considered a 'kind' test; it should not be used to label seed lots. We have too many fluorescent perennials for this to be an accurate test. And we don't know what makes fluorescence happen.

Fl does not mean annual anymore. The mandatory calculations do not give fair representation of the seed in the bag.

At the end of the Day….

Let's bury the calculations.

Thank you for listening.

Appendix V

Letters of Support

1. Brad Dozler, Lewis Seed Company, Shedd, OR

2. Skip Coville, Pennington Seed Oregon, Lebanon, OR

   These two letters and letters from the Oregon Seed Trade Association and Oregon Ryegrass Seed Growers Commission are on file.

DNA based purity testing of ryegrass – a background study

Through cooperative research between the USDA-ARS and OSU, a maturity Grow-Out Test (GOT) was developed based on vernalization requirements. Beginning with the 2002 crop year, the SRF test was augmented by the GOT test for seed labeling purposes (Barker *et al.* 2002). When the GOT test was officially implemented, the industry financial loss to growers each year because of payment discounts was temporarily alleviated (Personal communication, Oregon Ryegrass Seed Testing Committee, 2001). The addition of the GOT results is a lower estimate of contamination levels, which benefits growers, but the GOT is time-consuming to conduct and may not be beneficial to seed companies. Further, the GOT *per se* does not fully estimate growth-type, but overly predicts perennial-type plants and under estimates those that are actually annual. The results can be altered by even minor changes in the conditions under which the plants are grown (Barker *et al.* 2003, Barker 2010, and Barker and Cooper 2010).

A GOT usually lasts six weeks, but heading continues at least up to twelve weeks (Figure 1). Barker and cooperators conducted a study on seedlings from 20 commercially grown cultivars. Germination, SRF, and molecular marker (*LpVrn-1* and *$LpID_1$* TaqMan® assay) tests were conducted on the seedlings, and the seedlings were transplanted to a GOT. All seedlings with fluorescent root traces (FL+) along with an equal number of seedlings without fluorescent root traces (FL-) for a total of 880 seedlings were used in the GOT. The study was terminated after 84 days (12 weeks) in a high intensity growth chamber. This study was a growth analysis from seedlings that had markers predetermined on them. In the results, FL+ plants were compared to the FL- plants with markers considered within each group. In traditional SRF testing followed by GOT, only results from FL+ plants are reported. The FL+ plants started to produce heads before the FL- plants and continued to head well after the normal 6-wk end of the GOT. This continued heading demonstrates why GOT results vary from lab to lab. There is no clear, standard cut off point and growth analyses continue in the linear phase of the growth curve. Heading in the FL- plants were delayed as compared with the FL+ plants, but the linear growth was much more rapid.

Plant type was predicted using *$LpID_1$* and *LpVrn-1* TaqMan® assays on seedling tissues were measured as perennial-type ("P"), annual-type ("A"), or heterozygote/hybrid ("H"). All the FL+ seedlings that had "A" markers headed in the first 8wk of the GOT and all of the "H" markers by 9wk (Figure 1). Thus, purity as determined by molecular markers can be completed in as little as two days from harvest of seedling tissue and provide similar results as a 9wk GOT. This is a huge savings of both time and potential loss of income to seed producers. The effectiveness of molecular markers is faster and more accurate than either the SRF or GOT tests. **There was only one seedling of FL- plants that showed "H" markers indicating that fewer FL-plants probably need to be tested in purity analyses.**

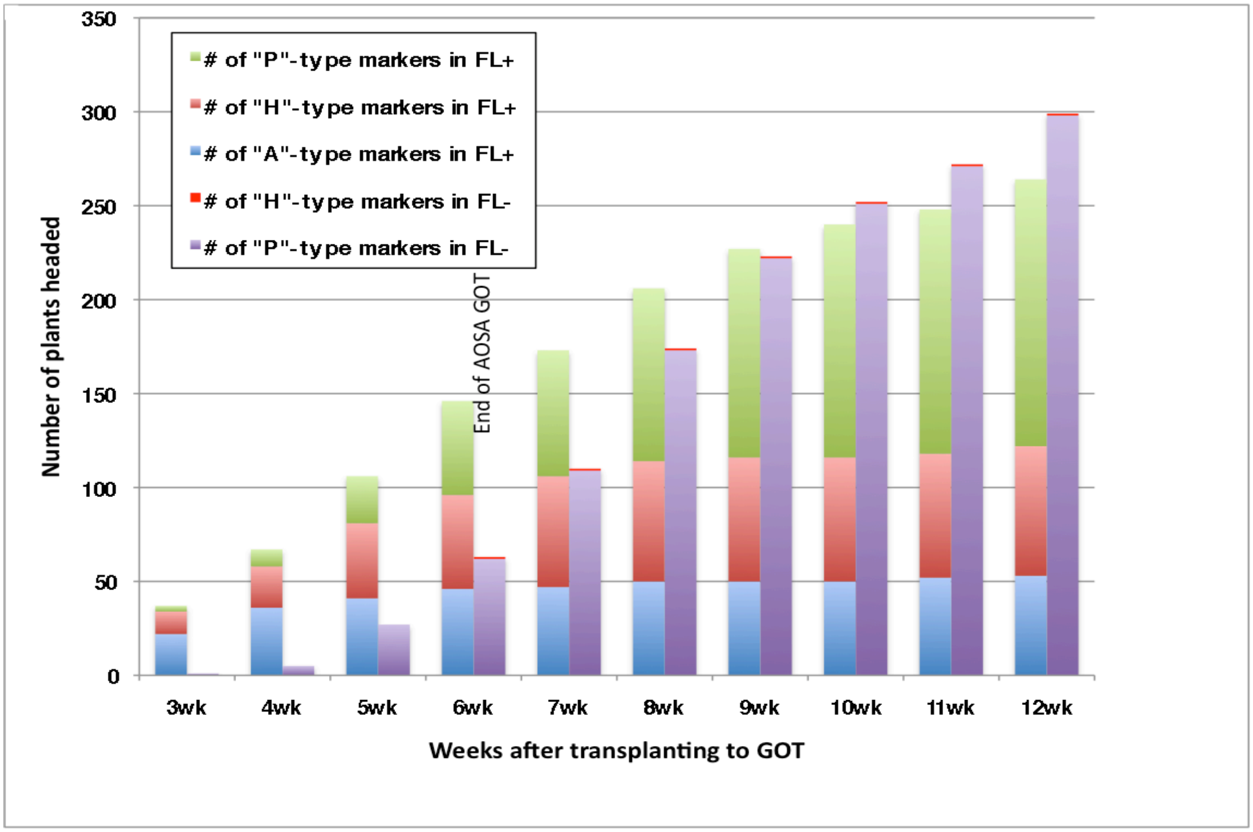

Figure 1. Accumulated heading in a GOT grown for 84 days under continuous light in a high light intensity growth chamber using 20 ryegrass cultivars totaling over 880 total plants (vertical bars). Molecular marker assays and SRF tests were done on seedlings before transplanting to the GOT. The GOT is usually terminated before or at week 6. Seedlings that had FL+ markers are represented in the first of the paired bars (multicolored) and the FL- seedlings in the second of the paired bars (purple). Markers can predict ryegrass growth type equivalent to a 9 to 10-wk GOT.