

# A novel DNA-based diagnostic test for the detection of annual and intermediate ryegrass contamination in perennial ryegrass

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**Abstract** Perennial ryegrass (*Lolium perenne* L.) is a preferred choice for the turf grass industry due to its ability to provide a durable turf cover. Genetic or physical contamination of annual (*L. multiflorum* Lam.) or intermediate (*L. hybridum*) ryegrass species in perennial ryegrass is one of the major problems affecting the grass seed industry. At present, seedling root fluorescence (SRF), a biochemical marker, is used for the detection of annual ryegrass contamination. Due to the unreliability of the SRF test, the seed industry is seeking an alternative, more reliable and accurate detection method. Currently, there are no DNA tests available in ryegrass for detecting contamination with annual and intermediate ryegrass types. We developed a novel quantitative polymerase chain reaction (Q-PCR)-based DNA test for the detection of

annual and/or intermediate ryegrass types in perennial ryegrass. This DNA test was designed using an insertion/deletion (InDel) site in the *LpVRN2\_2* (*Vernalization 2*) gene, which is one of the several genes controlling vernalization in ryegrass. The new DNA test is more reliable, accurate and cost-effective in detecting contamination, with a high sensitivity of 0.04% in a sample size of 5,000 seeds. Use of larger sample sizes (12.5-fold higher compared to SRF test) provided additional accuracy in detecting the level of contamination. The method has produced consistent results in 68 perennial, 26 annual and 14 intermediate ryegrass lines.

**Keywords** DNA · InDel · TaqMan · PCR · Ryegrass · Root fluorescence · Vernalization · *LpVRN2*

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## Introduction

A multitude of grasses are available for lawn purposes. Depending on suitability for a particular climate, grasses are grouped as cool and warm season types. Ryegrass is the most commonly used cool season grass (Hall 1992), and was introduced from Europe and domesticated to grow throughout North America. The rye grasses are grouped into annual or Italian (*L. multiflorum* Lam.) and perennial (*L. perenne* L.) species (Hall 1992). There is an additional intermediate type generated through

genetic crossing between annual and perennial ryegrass species (*L. perenne* × *L. multiflorum* or *L. hybridum*). Perennial ryegrass, due to its over-wintering nature, does not require seeding every year and hence is the preferred type for residential lawns, sports fields and golf courses. In contrast, annual or Italian ryegrass is used mostly as a forage crop and can be used as a temporary ground cover since it does not over-winter in cold climates.

Genetic or physical contamination with annual and intermediate types in perennial ryegrass seed lots is undesirable and is one of the major problems affecting the seed industry. At present, there are few biochemical markers such as phosphoglucose isomerase (Pgi-2), superoxide dismutase (Sod1), esterase and annuloline were used to distinguish annuals from perennials. Pgi-2 (Charmet and Balfourier 1994), Sod1 (Warnke et al. 2002) and esterase (Payne et al. 1980) are isozyme markers and annuloline is a unique oxazole alkaloid secreted in roots of young annual ryegrass seedlings (Karimoto et al. 1962). Though these markers are used to detect annual ryegrass contamination, they are not specific to annual or perennial ryegrass species. They can be introgressed to annual or perennial ryegrass without affecting the annuality or perenniality nature of the ryegrass and as a result they were found in various allelic frequencies in different genetic backgrounds. Therefore, the analysis based on these biochemical markers does not provide an unambiguous estimate of seed contamination (Nyquist 1963; Charmet and Balfourier 1994; Floyd and Barker 2002; Warnke et al. 2002). However, due to the non-availability of a robust marker, the International Seed Testing Association (ISTA) currently recommends the use of an annuloline-based test called seedling root fluorescence (SRF) and/or a grow-out-based method for the estimation of annual ryegrass contamination. The SRF test which indicates the presence or absence of annuloline has proved unreliable since some perennial ryegrass varieties express the SRF trait to some degree in different environmental or SRF test conditions, leading to inaccuracy of the test results (Floyd and Barker 2002; Warnke et al. 2004). The SRF test is therefore negatively impacting the economics of the grass seed industries due to an increased number of good perennial ryegrass seed lots being rejected, or contaminated seed lots being wrongly diagnosed as pure on the basis of the presence/absence of annuloline.

The SRF test is also laborious and time-consuming: it takes 2–3 weeks to obtain the final result. The grow-out test, although reliable, is expensive and takes nearly 2 months to obtain results. There is a clear and present need for an alternative and accurate test that can distinguish annual and intermediate ryegrass from perennial ryegrass and also estimate the level of annual ryegrass seed contamination in perennial ryegrass seed lots.

In a novel attempt to develop a DNA based test, we have targeted the genetic locus which is attributed to the differential requirement of vernalization and photoperiod for over-wintering versus non-over-wintering ryegrass species (Jung et al. 1996; Jensen et al. 2005). Vernalization is the requirement of low temperature for the induction of flowering and photoperiod is the length of light requirement. While annual ryegrass does not require vernalization or photoperiod treatments for induction of flowering, perennial ryegrasses require both vernalization and short days followed by a transition to long-day photoperiodic conditions (Heide 1994). Therefore, vernalization requirement is one of the major traits that can be used to distinguish annual and perennial ryegrass types (Jung et al. 1996; Andersen and Lübberstedt 2003).

Several studies investigating the genetic basis of vernalization have reported five quantitative trait loci (QTL) controlling the trait. These QTLs have been mapped to linkage groups (LG) 2, 4, 6 and 7 (Armstead et al. 2004; Jensen et al. 2005; Andersen et al. 2006). A candidate gene, *LpVRN1* (GenBank accession no. AY198326) on LG 4 was found to be an ortholog of wheat *TmVRN1* (Jensen et al. 2005). Two candidate genes on LG 7 controlling vernalization in ryegrass, namely *LpVRN2\_2* (GenBank accession no. DQ202716) and *LpVRN2\_3* or *LpCO* (GenBank accession no. DQ202717) were subsequently identified and shown to have amino acid sequence similarity to *TmVRN2* of diploid wheat and *Hd1* (*Heading date 1*) of rice, respectively (Martin et al. 2004; Andersen et al. 2006). To map the candidate genes *LpVRN2\_2* and *LpCO*, the perennial ryegrass lines *Veyo* and *Falster* were used because of their contrasting primary and secondary flowering induction responses (Andersen et al. 2006). Both genes are predicted to be required for the increased induction of *LpVRN1* transcription and influence average number of days to heading (Andersen et al. 2006).

Here we report development of a new DNA test based on a conserved polymorphic insertion/deletion (InDel) site in the gene sequence of *LpVRN2\_2*. The method is more efficient and accurate than the SRF test and effectively distinguishes annual or intermediate types from perennial ryegrass. The method has been tested using several pure annual, intermediate and perennial ryegrass lines and perennial ryegrass lines with different levels of annual ryegrass seed contamination.

## Materials and methods

### Plant materials

Genetically pure as well as contaminated seed stocks of perennial and annual ryegrass varieties were obtained from various sources including two universities (University of Minnesota, MN and Oregon State University, OR, USA) and three private grass seed companies (Pennington Seeds Inc., Northern Excellence and American Grass Seed Producers). A total of 32 pure perennial, 26 pure annual and 2 intermediate lines were used to test the specificity of the new DNA marker. In addition, 41 perennial lines with known levels of annual seed contamination were used to test percent accuracy of the DNA test in estimating percent contamination.

### Grow-out test

Standard grow-out protocol was followed as set forth by Oregon State University Seed Laboratory, except that all the seedlings were used for transplanting without prior selection on the basis of SRF. It is done to eliminate the bias in sampling due to root-fluorescence. The test was performed in a Conviron plant growth chamber (Conviron, Manitoba, Canada) at BioDiagnostics Inc, River Falls, WI, maintaining adequate growth conditions.

### DNA extraction

Each ryegrass seed sample was manually counted to 1,000 and weighed. An appropriate seed weight equivalent to 5,000 seeds was subsequently used for the DNA extraction. Each sample was ground in a half-pint (c. 240 ml) jar using clean blenders.

Approximately 0.5 ml of the finely ground seed material was sampled in a 96-well matrix plate. Four replications of ground seeds per sample were collected for DNA extraction. The DNA extraction was carried out using Qiagen's Genra Puregene extraction kit according to the manufacturer's instructions (Catalog nos. 158908 and 158912) using BioMek FX liquid handling robotic platform (Beckman Coulter, Fullerton, CA, USA). For individual plant DNA extractions, the plants were germinated on paper towels following the standard germination protocol. Seedlings 2–3 weeks old were individually sampled into a 96-well plate and DNA was extracted from at least four plants per line using Qbiogene's FastDNA<sup>®</sup> kit (Qbiogene, Carlsbad, CA, USA) following the manufacturer's instructions.

### Quantitative real-time PCR

A TaqMan-based quantitative real-time PCR (Q-PCR) was designed to simultaneously amplify the annual ryegrass-specific *LpVRN2\_2* gene marker (referred hereafter as BDIRyeInDel3) and *β-tubulin*, a house-keeping gene common to both annual and perennial ryegrass, as an internal control (referred hereafter as BDIRyeIC). The PCR reaction was carried out using an ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA) in 'ddCt (Relative Quantitation) plate' mode. Detailed steps for PCR set up were given in the ABI manual (part no. 4347824 Rev. C). Each optimized PCR reaction (25 µl) contained 1× ABI gene expression master mix (Catalog no. 4369510), 600 nM of BDIRyeInDel3 forward and reverse primers, 200 nM of BDIRyeIC forward and reverse primers, 200 nM of BDIRyeInDel3 TaqMan probe (annual- or perennial-specific), 200 nM of BDIRyeIC TaqMan probe and 5 µl (25 ng/µl) of total genomic DNA. The primer and probe sequences used for the analysis are provided in Table 1. The thermal cycling parameters include 2 min at 50°C for Amp-erase UNG activation followed by denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. After the PCR, RQ (relative quantitation) values of the test samples were obtained by running 'ddCt (Relative Quantitation) study' mode using averages of eight Ct values of a calibrator (refer the manual for detailed protocol). In our study, we used control samples representing various percentage contaminations such

**Table 1** Primer and probe sequences used for the analysis

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan (MGB) probe
BDIRyeInDel3	GCTCAACGCCATCAGTAATGG	CAGCTACATACACCTGCCTTCA	Annual-specific probe: FAM-TGACCCTCCTCC Perennial-specific probe: VIC-TGACCCCTCCTC
BDIRyeIC	AAGCTCGCCCTCCTCCAGTTCTA	TCCTTCCTGAGCCTGGTGACCTT	NED-AAGTTCGACGACGGCACC

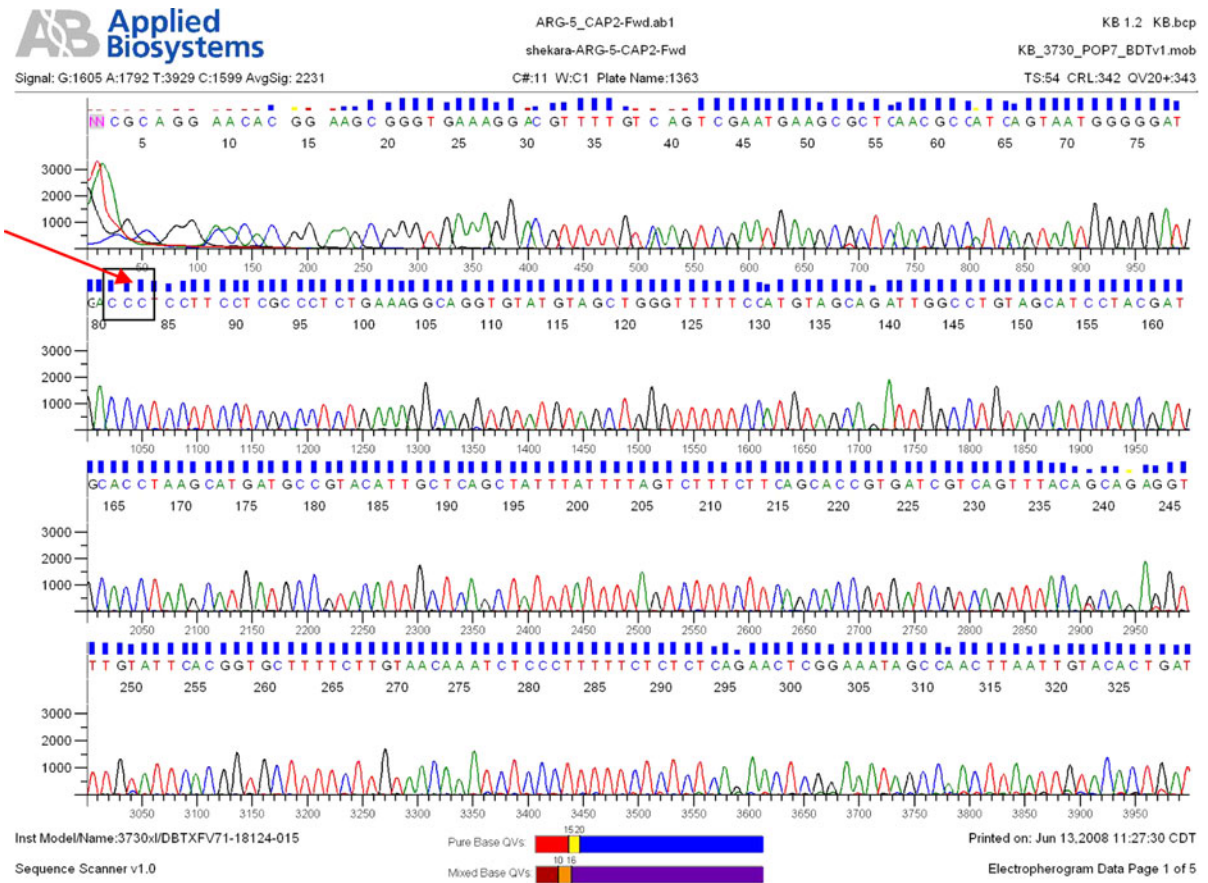
as 0.1, 0.5, 1, 2, 5, 10 and 20. Calibrator samples were generated by physically spiking appropriate annual ryegrass seeds (Gulf variety) into pure perennial seeds. Finally, percentage annual seed contamination was calculated using the eight RQ values for each sample (four DNA replications  $\times$  two PCR replications) using ISTA (International Seed Testing Association) Seed Calc7.1 statistical program.

## Results and discussion

Identification of conserved DNA sequence polymorphism between annual and perennial ryegrass varieties

In an effort to develop an accurate and sensitive test to detect annual and/or intermediate ryegrass contamination in perennial ryegrass seed lots, we made an extensive literature search to identify phenotypic traits that are specific to annual ryegrass. We tested microsatellite or simple sequence repeat (SSR) markers (Studer et al. 2006) and chloroplast specific genes *psbA* (*herbicide protein binding D1*) and intergenic spacer *trnT-trnL* as tools to distinguish annual ryegrass species from perennial ryegrass. One of the few SSR markers (LM28) identified by Studer et al. (2006) was shown to distinguish the two species. However, since the SSR assay involves individual plant testing, genotyping is expensive and does not lend itself to high-throughput processing even with capillary-based SSR screening methods. Therefore, to identify single nucleotide polymorphism (SNP) sequences for the development of a TaqMan-based SNP assay around the LM28 marker, we sequenced the DNA in the proximity of the LM28 locus. Based on the sequencing data, no conserved SNPs were found in this region (data not shown). The extensive use in phylogenetic analyses of the chloroplast-

specific *psbA* and *trnT-trnL* regions (Mummenhoff et al. 2001; Yang and Boo 2004) lead us to test these regions in ryegrass. Based on analysis of CAPS markers (Characterized Amplified Polymorphic Sequence; Konieczny and Ausubel 1993), no conserved SNPs were present in either chloroplast DNA region. In a novel attempt, we further investigated the possibility of using genes involved in vernalization as molecular markers to distinguish annual from perennial ryegrass. Since there were no reports comparing vernalization genes such as *LpVRN1*, *LpVRN2\_2* or *LpCO* between annual and perennial ryegrass varieties, we compared the DNA sequences of *LpVRN1*, *LpVRN2\_2* and *LpCO* genes in ten perennial and five annual varieties (data not shown). For the DNA sequencing, we used the primer sequences provided in Andersen et al. (2006). It is important to note that while the objective of the study carried out by Andersen et al. was to identify genes involved in vernalization, our investigation concerned to the use of vernalization genes as a tool in seed testing to distinguish annual from perennial ryegrass. The DNA sequencing result for vernalization genes indicated that there were 11 SNPs and two InDel (Insertion/Deletion) sites at *LpVRN2\_2*. There were no conserved polymorphisms found at *LpCO* between annual and perennial varieties. Initially we tested two of the 11 SNPs at *LpVRN2\_2* using TaqMan-based real-time PCR. These SNP markers (BDIRyeSNP1 and BDIRyeSNP2; data not shown) distinguished some but not all of the annual and perennial varieties tested. Finally, a TaqMan probe designed to detect one of the InDel polymorphisms at *LpVRN2\_2* resulted in successful differentiation from perennial varieties of all annual and intermediate ryegrass varieties tested. The conserved InDel site has two cytosine nucleotide insertions in perennial ryegrass or two cytosine nucleotide deletions in annual ryegrass. The DNA sequence used for designing annual and perennial



**Fig. 1** The DNA sequence of *LpVRN2\_2* in annual ryegrass indicating two cytosine nucleotide deletion sites as compared to perennial ryegrass. Arrow indicates the InDel site

specific probes is provided in Fig. 1. Importantly, the published DNA sequence polymorphism between two perennial varieties, *Veyo* and *Falster*, having contrasting vernalization requirements (Andersen et al. 2006) was found to be completely different from the polymorphism we observed between annual and perennial ryegrass varieties.

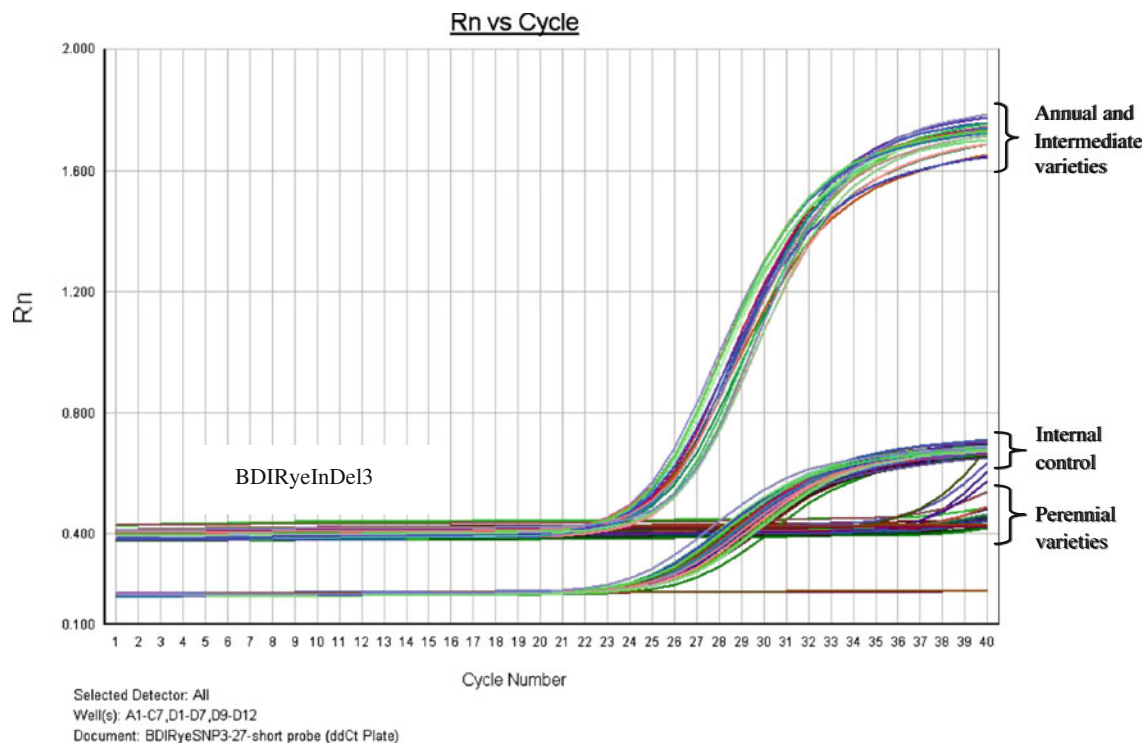
A new DNA marker is specific to annual ryegrass

To determine whether the new DNA marker (BDIRyeInDel3) can specifically detect annual and intermediate varieties, we tested 32 pure perennial, 26 annual and two intermediate lines on an individual plant basis using a qualitative PCR method. The study also included an additional nine intermediate plants found as contaminants in a perennial ryegrass plot. As expected, the marker could effectively detect all the annuals and intermediates and there was no

detection of any perennial lines (Fig. 2). Similarly the BDIRyeInDel3 marker with perennial ryegrass-specific TaqMan probe could detect all perennial lines and few intermediates, and none of the annual lines were detected (data not shown). The intermediate ryegrass plants, although always distinguishable from perennials, could however, be distinguished from annual plants only in the F1 generation.

The DNA test can accurately estimate percentage annual seed contamination

In order to make the new DNA test more robust and less expensive, we tested its accuracy and sensitivity of detection of annual seed contamination in a pool of 5,000 perennial seeds. For this validation, we physically spiked pure annual ryegrass seeds (Gulf variety; purity was confirmed by grow-out as well as SRF tests) into pure perennial seeds at various levels with a



**Fig. 2** Amplification of annual and intermediate varieties by BDIRyeInDel3 marker with annual-specific probe. Both BDIRyeInDel3 and BDIRyeIC were bplexed in a single reaction

minimum of one annual seed per 5,000 perennial seeds. Interestingly, the percent estimation of annual seed contamination was accurate, consistent and highly sensitive. Accuracy of the assay was estimated using 0.02–50% level of contamination using 21 perennial seed lots which included validation samples from other seed companies as part of the external validation (Table 2 and Fig. 3). The result indicated that estimations were more accurate in the 0.04–20.0% range ( $R^2 = 0.99$ ), moderate in the 20–50% range and less accurate above 50% contamination level (Table 2). The moderate to low accuracy at the high percent contamination level could be due to saturation of the fluorescent signal. Importantly, high accuracy of estimation at low levels of contamination (0–20%) is critical for the seed industry and this test is designed mainly to address that problem.

#### Comparison of DNA and SRF test results

The percentage contamination estimated by the new DNA test was different from the SRF test estimations for most of the samples tested (Table 3). This

difference was expected since the SRF test is known to be inaccurate because the SRF trait is not specific to annual types. To confirm that our DNA-based test results represent the actual level of contamination, we carried out grow-out test for a perennial seed lot, BDI-25 (see Table 3), which showed the greatest difference in estimated values between DNA (7.55%) and SRF (44.91%) tests. The grow-out test was conducted using 76 plants of BDI-25 without pre-selection based on root fluorescence. Of the 76 plants, flowering was observed in four plants (5.3%). Therefore, the grow-out test result indicated that the DNA test is more accurate in estimating annual ryegrass contamination. The relatively low level of contamination indicated by the grow-out test as compared to the DNA test may be due to fewer plants (76) tested as against the 66-fold higher sample size (5,000 seeds) used in DNA analysis.

The DNA test is time- and cost-effective

The DNA test was designed to be suitable for high-throughput testing to obtain accurate results in a time-

and cost-effective manner. The detailed advantages of the DNA test over the SRF test were provided in Table 4. Since the DNA test was carried out on seeds, it greatly reduced the time required for testing. Also, it does not involve the laborious plant germination and UV-based individual root screening required for the SRF test. In addition, the 12.5-fold higher sample size (5,000 seeds) compared to the SRF test (400 seedlings) will provide more accurate estimation of contamination levels and is not influenced by the

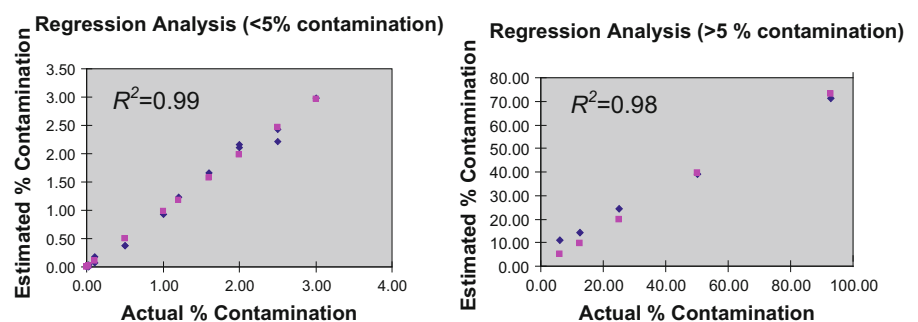
**Table 2** Accuracy and sensitivity of new DNA test in estimating annual seed contamination in perennial seed lots

Samples	Estimated contamination (%)	±SE (%)	Actual contamination (%)
1	0.00	0.00	0.00
2	0.01	0.01	0.00
3	0.04	0.01	0.02
4	0.00	0.00	0.02
5	0.17	0.01	0.10
6	0.08	0.01	0.10
7	0.37	0.00	0.50
8	0.37	0.04	0.50
9	0.93	0.11	1.00
10	1.24	0.09	1.20
11	1.67	0.09	1.60
12	2.16	0.08	2.00
13	2.11	0.21	2.00
14	2.42	0.00	2.50
15	2.21	0.23	2.50
16	2.99	0.25	3.00
17	10.99	0.15	6.25
18	14.46	0.61	12.50
19	24.14	0.62	25.00
20	39.08	0.01	50.00
21	71.19	1.36	93.00

**Table 3** Comparison of percent estimation of annual ryegrass contamination in perennial ryegrass by DNA and SRF tests

Samples	Estimations based on DNA test (%)	±SE (%)	Estimations based on SRF test (%)
BDI-1	0.00	0.00	1.29
BDI-2	0.02	0.01	0.27
BDI-3	0.03	0.07	0.00
BDI-4	0.04	0.05	0.80
BDI-5	0.04	0.00	0.53
BDI-6	0.10	0.04	0.00
BDI-7	0.13	0.16	0.00
BDI-8	0.42	0.20	1.01
BDI-9	0.47	0.20	1.08
BDI-10	0.93	0.24	2.43
BDI-11	1.21	0.61	3.84
BDI-12	1.28	0.65	1.84
BDI-13	1.29	0.83	1.55
BDI-14	1.38	0.45	2.43
BDI-15	1.58	0.41	3.08
BDI-16	1.65	0.19	2.73
BDI-17	1.80	0.64	2.09
BDI-18	2.11	0.60	1.81/0.94
BDI-19	2.24	0.48	1.70
BDI-20	2.33	0.71	6.25
BDI-21	2.79	0.00	2.68
BDI-22	4.04	0.00	1.02
BDI-23	4.41	1.22	1.68
BDI-24	5.74	1.12	1.34
BDI-25	7.55	0.12	44.91
BDI-26	36.12	0.00	90 (Intermediate forage)
BDI-27	50.09	4.46	90 labeled 10 perennial
BDI-28	63.95	11.12	99.97
BDI-29	65.87	17.05	99.00

**Fig. 3** Regression analysis of estimated percentage contamination using DNA-based test. The diamond and square data points represent actual and estimated contamination percentages, respectively



**Table 4** Comparison between DNA and SRF tests

Particulars	DNA test	SRF test
Reliable estimation of annual ryegrass seed contamination considering grow-out test estimates as a reference	High ( $R^2 = 0.98$ )	Low (Floyd and Barker 2002)
Environmental influence	No	Yes
Sample size	5,000 seeds	400 seedlings
Time required for completion of test	2 days	14–21 days
Distinguish intermediates from annual ryegrass	Possible	No
Seed germination	Not required	Required
Seed grinding	Required	Not required

environmental conditions. Due to high-throughput processing, it also reduces the cost per assay. However, the DNA test provides a high level of accuracy at <20% contamination but is less accurate at high contamination levels (perennial ryegrass with >50% annual seed contamination). Furthermore, the calculation of percent contamination by the DNA test assumes that the perennial seed lot is contaminated by annual ryegrass (not intermediates), which in fact represent the major type of contamination. Therefore, estimation of contamination levels by the DNA test can be higher than actual seed contamination if the perennial seed lot is contaminated by intermediates or in different proportions of annual and intermediate type of seeds.

## Conclusion

The economic viability of turf grass industry was greatly affected by the inability to accurately detect annual and/or intermediate ryegrass seed contamination in seedlots of perennial ryegrass. The SRF test originally designed to detect annual ryegrass contamination is unreliable. Our new DNA-based test offers a highly sensitive, accurate, time- and cost-effective alternative method for an adventitious presence test in perennial ryegrass.

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## Results from Ryegrass Fluorescence Survey - September 2009 – January 2010

### 1. Do you pre-chill perennial ryegrass germination tests? (All questions refer to samples that will also have a fluorescence test)

13 – Yes

3 – No

### 2. If, yes what is your criteria for when to pre-chill or not to pre-chill?

5 – Fresh seed

3 – 0-3 Months

2 – 0-6 Months

1 – 0-12 Months

Always, unless we know for sure it was older seed.

### 3. If yes, what temperature do you use for the pre-chill?

3 – 5c

10 – 10c

### 4. What germination media did you use?

8 – Filter paper

5 – Blotters

4 – Filter paper and blotters

### 5. Do you tilt the planting boxes?

13 – Yes

3 – No

### 6. Do you use a solution on your media?

10 – KNO<sub>3</sub>

1 – Distilled water

4 – Water

### 7. Do you use light in test?

13 – 8 Hours

1 – 12 Hours

2 – 16 Hours

### 8. If you use lights what is the intensity of your germinator lights?

2 – Fluorescence tubes / 3800 lumens each (85-90 ft.-c)

1 – 75 ft.-c

1 – 100 ft. -c

1 - 700 Lux

1 – 750-1250 Lux

1 - Don't know. Four 40 watt fluorescence light tubes is our source.

1 – 30 Watt daylight fluorescence

1 – 6 - 40 Watt

### 9. Please describe your UV light setup:

9 – Dark rooms

5 – Dark boxes

2 – Enclosed cabinet

**10. What is the distance between the UV light and the planting box at which you evaluate?**

- 1 – 5 Inches
- 6 – 6 Inches
- 2 – 8 Inches
- 5 – 12 Inches
- 1 – 18 Inches

**11. When do you do a first count?**

- 11 – 7 Days
- 2 – 10 Days
- 1 – 10 Days for annual/14 days for perennial
- 1 – None – Only if customer requests 7 days

**12. When do you do a final count?**

- 16 – 14 Days

**13. Do you remove all seedlings at final count from the media?**

- 5 – Yes
- 11 – No

**14. Do you look underneath roots to see a path of fluorescence?**

- 6 – Yes
- 4 – No
- 4 – Only if they glow
- 1 – From the underside of the box without moving seedlings

**15. In fluorescence evaluation do you discriminate based on intensity of fluorescence?**

- 15 – No
- 1 - Yes

**16. Do you feel that you can determine fungal fluorescence from seedling root fluorescence?**

- 12 – Yes
- 4 - No

**17. If a root fluoresces does it matter to you how much of the root is fluorescing? Please explain.**

- 10 – For any part of root
- 1 – For any part of root – exception fungal growth
- 1 – For any part of root – sometimes not all of the root is touching the paper
- 1 – For any part of root – we do not check under the root for faint fluorescence left on paper
- 1 – For any part of root – AV lab does not perform many ryegrass tests. Most of the ones seen already have a test
- 1 – Primary root
- 1 – All of root

**18. Do you feel that fluorescence should be performed on samples without a pre-chill due to the stress factor of a pre-chill?**

- 4 – Yes
- 7 - No
- 1 – No – Some years the seed can be more dormant than other years so a pre-chill is needed to get the best germination.
- 1 – No – I don't think a short pre-chilling would be that stressful to a vigorous seed lot.
- 1 - I don't believe there is a stress factor
- 1 – I have not seen any recordable difference outside of increased total germination & standard variance within that.

**19. When would you decide to retest?**

- 2 - Anytime results of 1-4 reps is "unexpected"/out of tolerance on germ or fluo. per kind, variety/qual
- 6 - If the test is out of tolerance
- 1 - If replications are very different or results don't match up with variety submitted.
- 1 - If the pure seed and other crop are out of tolerance with label as a result of the fluorescence test, then a retest is done before a stop-sale is issued.
- 1 - Low germination versus TZ test; out of tolerance; other variables too numerous to mention.
- 1 - Reps are out of tolerance. Or if results cause the pure seed % to be out of tolerance of label.
- 1 - If analyst judges test inaccurate due to environmental conditions or other suspected error.
- 1 - When customer requests

**20. If you are using the guidelines in the Cultivar Purity Handbook, what is the date of revision you are using?**

- 2 - 1998
- 1 - 2003
- 1 - 2006
- 1 - 2007
- 5 - 2008
- 1 - Latest
- 1 - 2008-09 (Latest per SCST/AOSA Executive Director)

**21. Do the analysts that conduct the fluorescence test have their eyes examined for night blindness? Or do they wear protective UV glasses? Please make any comments that you feel are important about eye sight.**

- 1 - Analysts are encouraged to have basic eye exam once/yr (lab expense)
- 1 - UV Glasses, my Eye Doctor recommends them to prevent cataracts.
- 3 - Protective UV glasses
- 1 - They have not been tested, and have not required them to wear UV protection.
- 1 - Not examined for night blindness. Not wearing protective UV glasses.
- 2 - I have 20/20 Vision
- 3 - No, no

**22. How many samples of ryegrass do you test per year?**

- 1 - 1,750 (=,-)
- 1 - 100
- 1 - 5-10
- 1 - Several Hundred
- 1 - 200
- 2 - 400+
- 1 - Not sure somewhere around 5,000
- 1 - 3 to 5 fluorescence tests per year.
- 2 - 2000-3000
- 1 - one or two
- 1 - Less than 10
- 2 - 1000
- 1 - 800-1000 per year

**24. How experienced are you with ryegrass testing?**

- 3 - Very experienced- ryegrass is my life
- 9 - Experienced- totally comfortable testing this species
- 3 - Intermediate- somewhat experienced, becoming comfortable w/species

**Table 1.** Analysis of variance (ANOVA) for the effect pre-chilling treatment, samples, and laboratory variation on final germination percent and fluorescence test of seven perennial ryegrass samples tested in eight laboratories.

Source of Variation	df	Germination	Fluorescence
Rep (R)	3	*	ns
Labs (L)	7	***	***
Chill/No Chill (C)	1	ns	***
(L) x (C)	7	**	***
Samples (S)	6	***	***
(L) x (S)	42	***	***
(C) x (S)	6	**	***
(L) x (C) x (S)	42	***	***

\*, \*\*, \*\*\* Significant at the 0.05, 0.01, and 0.001 probability levels, respectively; NS, not significant at 0.05 probability level.

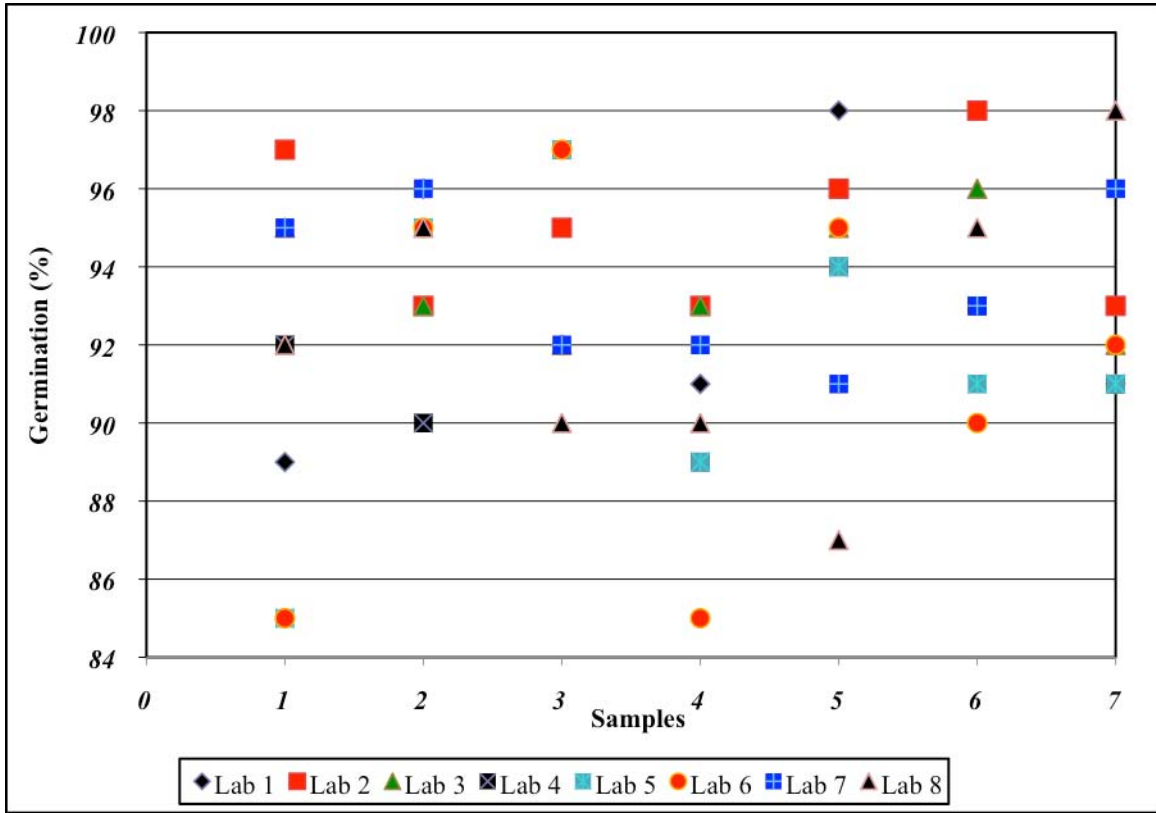


Figure 1. Germination percentage of seven perennial ryegrass samples tested in eight laboratories. The germination percentage of each sample is out of tolerance within lowest and highest laboratory results.

**Table 2.** Germination percentage of seven perennial ryegrass samples tested in eight laboratories. The germination percentage of each sample is out of tolerance within lowest and highest laboratory results.

Sample	Labs								Min Germ	Max Germ	Tolerance			
	1	2	3	4	5	6	7	8			Avg.	Diff max-Min	Tolerance value	Within Tolerance
	Germination %													
<b>1</b>	89†	97	95	92	85	85	95	92	85	97	91	12	5	No‡
<b>2</b>	96	93	93	90	95	95	96	95	90	96	93	6	5	No
<b>3</b>	92	95	92	97	97	97	92	90	90	97	94	7	4	No
<b>4</b>	91	93	93	89	89	85	92	90	85	93	89	8	6	No
<b>5</b>	98	96	95	94	94	95	91	87	87	98	93	11	5	No
<b>6</b>	98	98	96	93	91	90	93	95	90	98	94	8	4	No
<b>7</b>	91	93	92	91	91	92	96	98	91	98	95	7	4	No

† Each value represents and average of four replications of 100 seeds each.

‡ According to Table 13 J of the AOSA Rules for Testing Seeds, 2009.

**Table 3.** Fluorescence percentage of seven ryegrass samples tested in eight laboratories. Samples were chilled before the germination test.

Sample	Cultivar	VFL	% Annual type by		Labs								Sd	Sd without outliers	
					1	2	3	4	5	6	7	8			OR
			GOT	DNA	% Fluorescent seedlings										
1	Blackcat	0.0	0.01	0.95	0.0	4.0	0.5	1.0	0.0	0.0	0.0	0.0	2.2	1.4	
2	Big green	N/A	0.01	19.4	0.5	0.3	0.0	10.3	10.8	0.8	0.0	0.0	59.1	4.8	
3	Satukibare	N/A	no GOT	61.7	0.8	0.0	0.0	47.5	1.3	0.3	0.0	0.0	99.7	16.7	
4	Silver Dollar	0.04	0.0	0.1	0.8	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.6	0.4	
5	Prelude	1.72	0.01	1.8	94.3	13.0	0.0	2.0	0.8	0.0	0.0	0.3	2.9	32.8	0.8
6	Arctic Green	0.22	0.0	0.2	96.3	2.8	1.0	0.0	0.3	0.0	0.0	4.8	0.3	33.6	1.8
7	Silver Dollar	0.04	no GOT	0.1	0.3	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.0	0.5	

**Table 4.** Fluorescence percentage of seven ryegrass samples tested in eight laboratories. Samples did not receive prechilling treatment before the germination test.

Sample	Cultivar	VFL	% Annual type by		Labs								Sd	Sd without outliers	
					1	2	3	4	5	6	7	8			OR
			GOT	DNA	% Fluorescent seedlings										
1	Blackcat	0.0	0.01	0.95	0.3	2.0	0.3	0.8	0.8	0.0	0.3	0.0	1.4	0.7	
2	Big green	N/A	0.01	19.4	0.3	0.0	0.3	21.5	17.0	33.8	0.3	0.0	56.9	13.2	
3	Satukibare	N/A	no GOT	61.7	0.0	0.0	0.5	6.8	11.5	3.5	0.0	0.0	98.5	4.3	
4	Silver Dollar	0.04	0.0	0.1	0.3	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.3	
5	Prelude	1.72	0.01	1.8	80.0	28.3	0.3	1.3	3.0	0.8	0.0	0.3	5.1	28.3	1.1
6	Arctic Green	0.22	0.0	0.2	98.3	7.8	8.5	0.0	0.0	0.3	3.3	6.5	0.6	33.6	3.8
7	Silver Dollar	0.04	no GOT	0.1	0.3	0.0	0.0	0.0	0.3	0.0	2.3	1.0	0.0	0.8	