

Real-Time Polymerase Chain Reaction method to differentiate Annual from Perennial Ryegrass Species

Introduction:

Genotyping, DNA molecular markers, and the Polymerase Chain Reaction (PCR):

The genotype of an individual, tissue, or cell is its underlining genetic makeup. The term genotype is also used to describe a group of individuals that share the same genetic makeup. Genotyping therefore, is the process through which the genotype is determined. The ultimate genotyping will be the determination of the DNA sequence of a genome; the cost of such an endeavor however is still prohibitively high, therefore, genotyping is done by different methods of sampling the genome. The sampling is performed by the use of various DNA molecular markers. Molecular marker in general describes “any biological molecule that is found in an organism that can be used as a signal (thus marker) of the state of a trait or a process in the organism that cannot be assessed directly” (<http://www.cancer.gov/dictionary/?CdrID=579630>). In the narrower context of genotyping; a DNA molecular marker will be any polymorphic DNA segment that its nucleotide sequence can be repeatedly and consistently be probed. Many DNA molecular markers systems are available, some assay the variation of single or low copy number target sequences, for example Restriction Fragment Length Polymorphisms (RFLP), or, Expressed Sequence Tags (EST), other targets multi loci, or repetitive DNA sequences such as Simple Sequence Repeats (SSR, also known as microsatellites), further still, some probe arbitrary sequences such as Randomly Amplified Polymorphic DNA (RAPD), or Amplified Fragment Length Polymorphisms (AFLP). Many genotyping methods and platforms were developed and are employed to conduct these analyses; presently, the prevailing methodologies are different applications of the Polymerase Chain Reaction (PCR).

PCR is a method of *in-vitro* amplification of DNA sequences. It takes advantage of the properties of a naturally occurring enzyme; DNA polymerase I (abbreviated: Taq), that is active *in-vivo* in processes that require amplification of cellular DNA content, such as mitosis and meiosis. The isolation of a thermo-stable version of the enzyme (from the hot springs bacteria *Thermus aquaticus*) enabled the automation of the in-vitro reaction. These PCR reactions are run on thermocyclers and involve three key steps:

Denaturation: DNA is subjected to high temperatures (95°C), disassociating the double stranded DNA into single strands, in order to successfully allow primers (short stretch of oligo-nucleotides) to bind at the target regions.

Annealing: Temperature is lowered (to a specific value for the target). During this step primers bind to the complementary regions in the DNA template strands and the DNA re-associates to form double strands. For a given single reaction typically there is a forward primer that binds to the 5' end and a reverse primer that binds to the 3' end of the DNA segment of interest.

Extension: In this step the temperature is raised to 72°C which is the optimal temperature for the activity of Taq Polymerase; selective regions are extended between the primer binding sites through incorporation of nucleotides by Taq polymerase enzyme.

These steps are repeated in sequence (cycles) typically for ~40 times. Thus, a target DNA sequence that is present as a single copy in the template DNA used in a PCR reaction will theoretically be amplified to $2^{(40)}$ copies by the end of the reaction. Many applications and variation on this general theme exist, for example, a PCR reaction can target one Gene Of Interest (GOI) or more (multiplexed reactions), or can be a three step reaction as described above, or two steps, where annealing and extension are combined to one step. Generally PCR reactions can be divided into qualitative, conventional PCR, or quantitative, real-time PCR where the thermocycler is also engineered to monitor the rate of amplification in real-time and thus provide a quantitative estimate of the number of target DNA molecules in the original DNA sample – such methodology is used in the assay described herein.

PurePRG¹ testing, general:

The general outline of the testing process involves a number of steps (Fig.1):

- Sample preparation – each sample and control is prepared as a pool of approximately 3000 seed in triplicates.
- DNA Isolation: The seed is ground and DNA is isolated.
- Thermocycling: The DNA samples are utilized in real-time PCR reactions.
- Analysis: The real-time PCR data obtained is transformed to relative quantities and % annual contamination.
- Report.

Equipment

Instruments:

1. Pipettes: P10, P100, P1000, 8-Channel 0.5-100 μ L (or equivalent)
2. Analytical balance
3. Centrifuge
4. Real time PCR instrument
5. Rocket Blenders with short cups and flat blades (or equivalent)

Reagents and consumables:

1. Genotyping Master Mix
2. HPLC Grade Water
3. 96-well PCR plate
4. Optically clear adhesive film
5. Reagent Grade Ethanol
6. 96- deep well plate DNA Isolation

1. Patent pending. Method can be licensed from BioDiagnostics Inc.

Sample Preparation:

- A. Weigh 5.1 g of seed (equivalent to 3000 seeds) per sample.
- B. Grind the sample to a fine powder.
- C. From each sample, sub-sample three **replications** of 0.5ml flour (ground seeds) into 96 well plate.
- D. Include three replication of each of the following controls (Table 1):
 - a) **Negative Control** (pure perennial).
 - b) Calibrators at **1%, 5% and 10 %** annual rye grass in perennial.
 - c) Limit of Detection controls (LOD) (0.03%) for every test.

DNA Isolation:

Isolation of seed DNA can be performed using any one of a variety of protocols or commercial kits; the following are some examples:

DNA isolation kits:

- 1) NucleoSpin (<http://www.mn-net.com>)
- 2) DNeasy (<http://www.qiagen.com>)

Protocols:

- 1) Dellaporta, S.L., J. Wood and J.B. Hicks, 1983. A plant DNA Mini-preparation: version II. Plant Mol. Biol. Rep., 1: 19–21
- 2) Doyle, J.J. and J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. Focus, Vol. 12, pp. 11–5

Regardless of the method it is essential that the DNA will be of high quality (devoid of RNA and proteins) and integrity and at a reasonable concentration.

Real-Time Quantitative PCR

1. Program your real-time thermocycler as follows:
 - Set the instrument to read FAM (BDirye-LM probe) and NED (BDiryeIC probe).
 - Choose relative quantification ($\Delta\Delta Ct$) program. Quantification will be done relative to the endogenous gene control (NED-BDiryeIC) and using the 1% control as Calibrator.
 - Program the following amplification profile

	Step	Temp	Time
Initial Cycles	UNG Activation (Optional)	50°C	2 min
	DNA Polymerase activation	95°C	10 min
Cycle 40 times {	Denature	95°C	15sec
	Anneal/Extend	60°C	1min

2. Design a PCR plate map that will include the desired replication per DNA sample (minimum of 2) and all the necessary controls and calibrator/s (Fig. 2).
3. Prepare a multiplexed PCR reaction master mix according to the following table:

PCR Reagents	Stock Concentration	Final Concentration (μM)	1X (μL)
Water			7.0
PCR Buffer mix	2X	X	12.5
BDirye-LM fwd primer	100 μM	0.6	0.15
BDirye-LM rev primer	100 μM	0.6	0.15
BDirye-LM probe (FAM-BHQ1)	100 μM	0.2	0.05
BDirye-IC fwd primer	100 μM	0.2	0.05
BDirye-IC rev primer	100 μM	0.2	0.05
BDirye-IC probe (NED-BHQ)	100 μM	0.2	0.05
DNA template	20ng/ μl	4ng/ μl	5
Total Volume (μL)			25

4. Dispense 20 μL master mix to each well according to your PCR plate map
5. Dispense DNA to wells according to your PCR plate design.
6. Seal plate with optically clear adhesive film.
7. Briefly centrifuge to a speed of 3000 rpm.
8. Load plate into the real-time PCR instrument. Identify targets and wells according to manufacturer instructions (various instruments/manufactories require different user interface).
9. Initiate PCR program.
10. At the end of the run perform following tasks:
 - a) Set 1% control as Calibrator.
 - b) Export Relative Quantification (RQ) values to an Excel spreadsheet and save.
 - c) Determine if the CT values of your controls fall within the range as described in Table 1.

Estimation of % contamination using SeedCalc

1. Open 'Seed Calc7.1' software program.
2. Open 'Quant Impurity Estimation' sheet.
3. Enter '3000' against # of seeds per pool, '3' against # flour sub samples, '# of PCR replications used' against # measurements per sample and '1' against # pools per sample. If there are any changes made with respect to any of the above parameters enter the values accordingly.
4. Format RQ values to percentages.

5. Enter 6 RQ values obtained per individual seed sample in 'Measurements' columns. Enter data from 1st PCR replication for a sample in the first row and data from 2nd PCR replication in the second row appropriately (Fig 3).
6. The software automatically calculates the % estimations. Note down the value that appear against 'Estimated % Impurity', 'Measurement CV' and 'Flour Std Dev' values. **This is the % contamination estimation for a sample.**

Important Note: The estimated value directly corresponds to % contamination **ONLY when 1% control was used** as Calibrator during 'ΔΔCt (Relative Quantification) Study'. If a control other than 1% was used, then **multiply** the 'Estimated % Impurity' values with the respective % control value (for example, multiply by 5 if 5% control was used). This gives the actual % contamination.

By default, use 1% Control for 'initial' contamination estimation.

- a) Use 1% control as calibrator for sample contamination <2.5%
 - b) Use 5% control as calibrator for sample contamination 2.5 to 7.5%
 - c) Use 10% control as calibrator for sample contamination >7.5%
7. Repeat steps 4-6 for every sample.
 8. Report the results (Fig 4).

Appendix:

Figure 1:

Bonafide BDI Pure PRG Flow Chart: Schematic representation of the work flow of the Bonafide BDI – PurePRG testing process

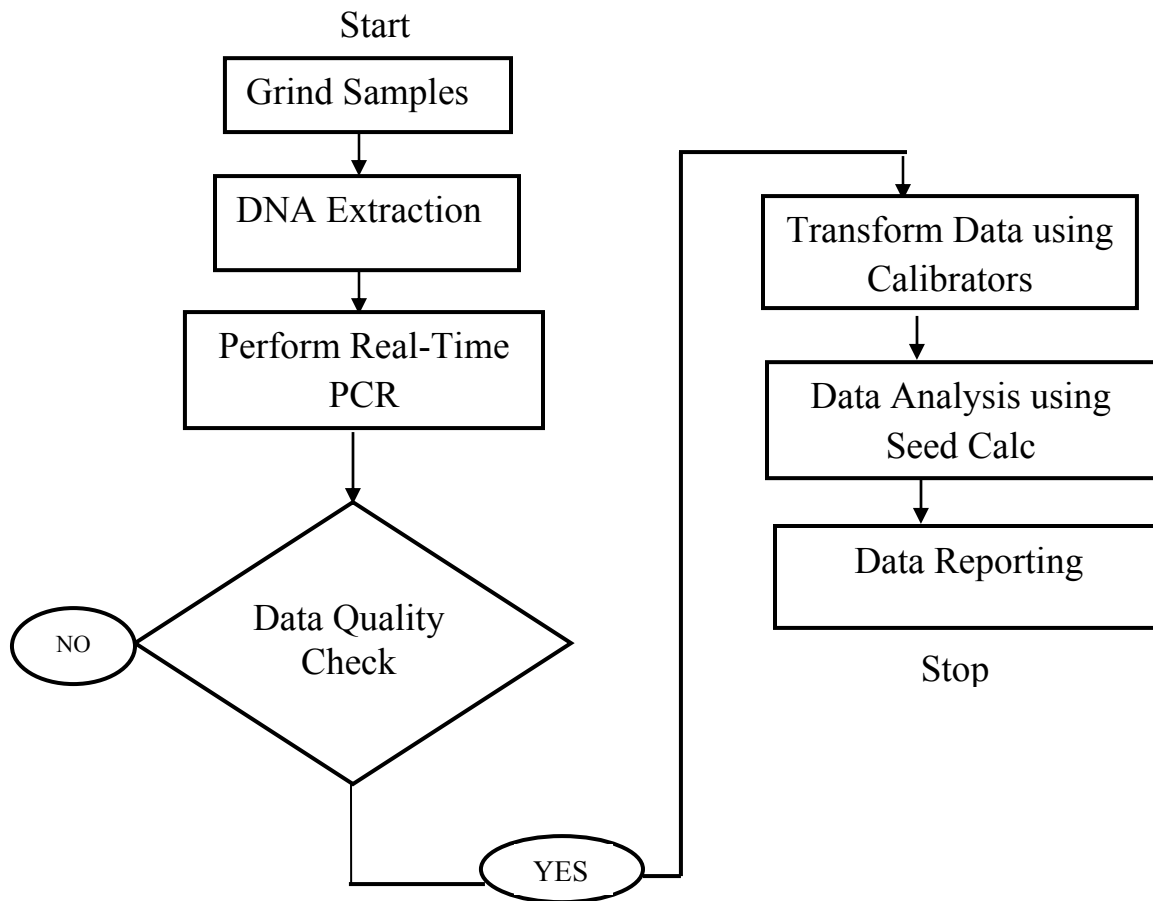


Figure 2:
PCR plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk1.1	Unk1.2	Unk1.3									
B												
C												
D												
E												
F												
G							Unk 27.1	Unk 27.2	Unk 27.3	0.03% (LOD)	0.03% (LOD)	1%
H	1%	1%	5%	5%	5%	10%	10%	10%	NTC Ctrl	NTC Ctrl	NEG Ctrl	NEG Ctrl

Unk: Unknown test samples; NTC: No template Control; Pos ctrl: Positive control (calibrator control); Neg Ctrl: Negative control; LOD: Limit of detection control

Figure 3:
Screen shot of Data entry to Seed Calc. 7.1

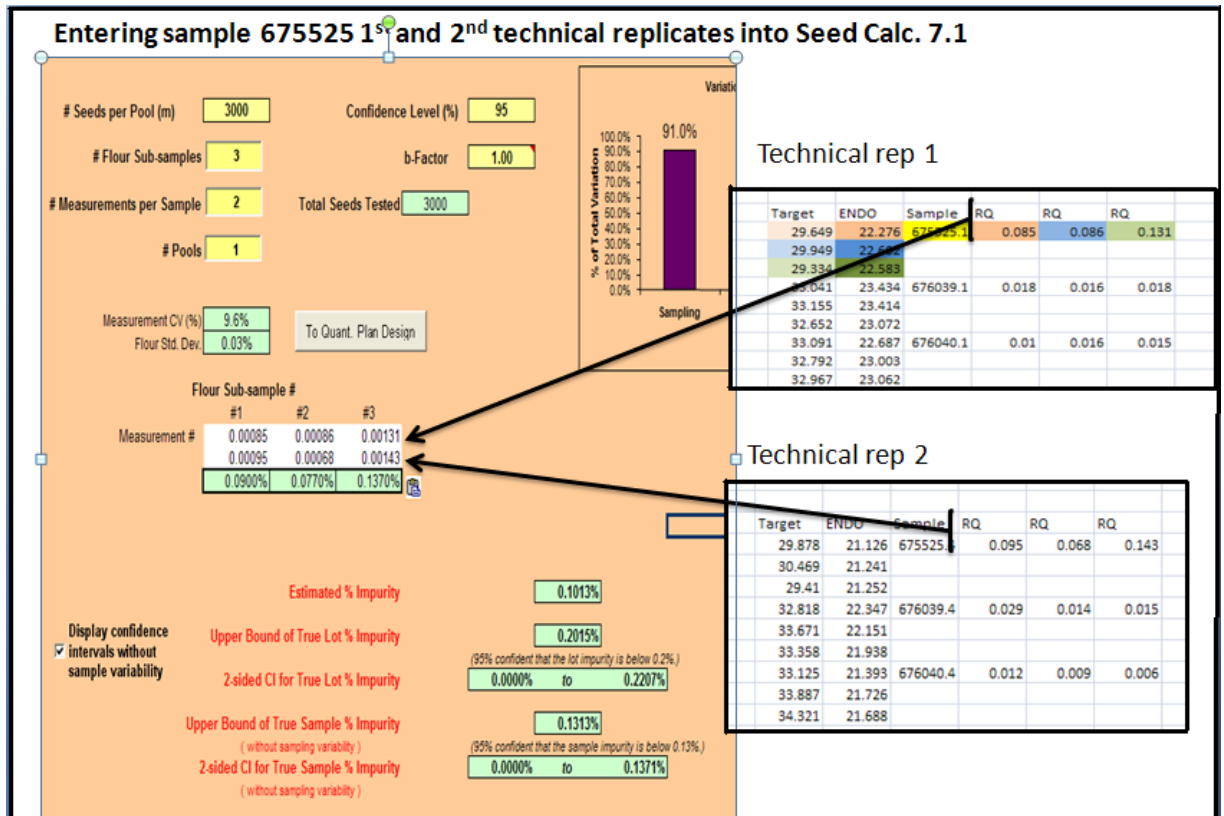


Figure 4:

BioDiagnostics PurePRG Report:

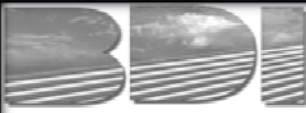
		BioDiagnostics, Inc. 507 Highland Drive 715-426-0246 715-426-0251 www.biodiagnostics.net				
Bonafide BDI™ Pure PRG™ Analysis Report						
Customer Name:	XXX				Seed Kind:	Perennial Ryegrass
Address:	XXX				Genus/Species:	<i>Lolium perenne</i>
Phone:	XXX				Date Received:	August 10, 2009
Fax:	-				Date Completed:	August 20, 2009
Email:	-				Date Reported:	August 21, 2009
Test Protocol Used: Bonafide BDI™ Pure PRG™ Assay						
<i>Sample Information</i>						
Lab Sample No.	Class	Variety	Lot #			
681256	Non-Certified	Silver Dollar	RB3245			
<i>Results Analysis</i>						
The test for the presence of annual ryegrass (<i>Lolium multiflorum</i>) was performed according to the Bonafide BDI™ Pure PRG™ Assay. Basis: 3,000 seed test.						
# Seeds Tested	# Pools	# DNA Extractions per Pool	# PCR Reps / DNA Extraction	Estimated Annual Ryegrass %	Upper Limit %	Confidence Level
3,000	1	3	2	0.73%	1.04%	95%
Issued By Seed Analyst			Authorized Signature			
<p><u>Testing Lab</u> warrants that it uses due care when performing tests for its customers. However, testing results are only reflective of the sample submitted by the customer. <u>Testing Lab</u> does not warrant the sample is representative of the seed lot from which the sample is drawn. Except for the testing services provided, <u>Testing Lab</u> makes no warranties or guarantees of any kind, expressed or implied, with respect to the services rendered.</p>						

Table 1:

Control Type	Description	CT values
NTC(no template control)	A sample devoid of any template. It provides a means of measuring a contamination that could give a false positive signal	<=40 cycles
Positive Control	In the current protocol, the calibrator controls serve as a positive control. Give proper PCR conditions both the target and the endogenous gene should amplify in +Ve control samples	26-30 cycles
Negative Control	Negative sample can show an amplification for the endogenous gene, however, should not amplify the target gene. Pure perennial ryegrass is used as a negative control.	<40
LOD	The lowest amount of target that can be statistically detected in a sample, but not necessarily quantified. The LOD for the current protocol is (0.03%)	30-32