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FORENSIC ASSAYS OF RICIN: DEVELOPMENT OF SNP ASSAYS TO GENERATE PRECISE GENETIC SIGNATURES FOR MIXED GENOTYPES FOUND IN RICIN PREPARATIONS

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Forensic Assays of Ricin: Development of SNP Assays to Generate Precise Genetic Signatures for Mixed Genotypes found in Ricin Preparations

Final Report

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Introduction and background

Castor beans, derived from castor plants (*Ricinus communis*) are the source of ricin, a potent toxin. Castor plants grow as ornamentals and feral populations, particularly in warmer states, and have a worldwide sub-tropical and tropical distribution. The goal of this study has been to develop methods to characterize castor and ricin samples based on the genetic signature of the beginning material. That is, to take advantage of contaminating *R. communis* DNA in ricin preparations to generate a unique genetic signature for a particular ricin preparation. Such a signature will have forensic and attribution value.

Unlike microbial forensics where the goal is often identification of a specific isolate of a pathogenic species, analysis of ricin preparations is complicated by extraction of ricin from multiple seeds, often produced by multiple, out-crossing, possibly genetically diverse plants. Thus, analysis of ricin presents a population genetics problem, not simply assignment of a specific molecular signature to a single microbial species/strain.

Extraction of DNA from ricin preparations that will support PCR shares many of the problems with DNA extraction from other beans and seeds. Polysaccharides and other materials often co-purify with DNA and it is often very difficult to obtain high quality, pure DNA from many seeds, especially oil seeds and those with high carbohydrate content. Commercial kits often do not yield pure DNA and the percentage DNA extracted is only a small fraction of the total. Before one can apply DNA-based forensic assays to ricin, one must be able to extract DNA from a variety of different ricin preparations. These range from little more than a de-fatted bean mash expected to contain a large amount of DNA to a relatively pure protein cut from the extracted castor bean mash prepared by ammonium sulfate precipitation. The amount of DNA present in the latter preparation is significantly less than that in the bean mash but the quality (as measured by size) may be better. DNA extracted from any ricin preparation must support PCR if it is to have forensic value.

An approach to characterize ricin and castor bean samples is distinct from microbial analysis in several very important ways. *R. communis* is a diploid plant that is often heterozygous at many genetic loci. None of our plant accessions are inbred lines and it is unlikely that anyone producing ricin preparations would use such inbred lines. Our results show genetic diversity even across seeds harvested from a single plant. A released microbial pathogen will likely be derived from a single genetic source (perhaps a single bacterial colony) and, consequently, all isolates in the release will be genetically indistinguishable from one another. In contrast, ricin toxin will be extracted from a population of genetically diverse beans. A single plant can produce sufficient seeds for a small ricin preparation (1–1.5 grams). A larger batch will require seeds from multiple plants. Even if the seeds to generate these plants arose from a single parent plant, there will be diversity at the different genetic loci as a result of genetic recombination, so that any genetic signature derived from a ricin preparation (and, by extension, the seeds used to make the preparation) will be a signature that describes a population, not a single entity. Given these differences, simply looking for the presence of particular genetic alleles for different loci will not provide useful information.

Allele frequencies can be used to match two mixed genotypes in much the same way two populations can be matched. The issue involves genetic distance (or conversely similarity) between populations, which is very analogous to simple correlation using

multiple variables. In this study, we used Single Nucleotide Polymorphisms (SNP's) and the more we used the more accurate the estimation of similarity. Modeling and empirical studies (Keim *et al*., 1992; Turakulov and Easteal 2003) have been used to generate "power curves" and suggest that 65 to 100 SNP loci will be optimal. However, funding constraints have required that we generate assays for a smaller number. Previous projects in these three labs and at DHS have identified over 300 SNPs in *R. communis* populations. From this resource, we have selected highly informative SNPs for assay validation. We previously demonstrated accurate SNP allele estimation in artificial mixtures of *B. anthracis* DNA. In this project, we applied this same approach to castor and ricin preparations to determine the utility and limits of the technology on these materials. We have developed protocols and procedures that provide statistically valid results, determined the statistical limits of these protocols and demonstrated reproducibility in different laboratories. Finally, we have tested different ricin toxin preparation protocols for their co-purification of DNA. We have demonstrated that sufficient DNA can be extracted and purified from ricin so that DNA-based forensic analysis is possible.

AFLP and SNP analysis of a large set of *R. communis* plants was informative. Analysis of plants derived from three seeds of each accession demonstrated genetic diversity among seeds even from the same accession. While all three seeds from the same accession mapped, with rare exceptions, to the same genetic cluster, a large number of plants are heterozygous at the different SNP loci. This result demonstrates the utility of a method that generates a population-based genetic profile. It also provided information to generate a diverse set of *R. communis* samples that could be used as a basis for determining the utility of each SNP locus and the relative frequencies of each allele in diverse populations.

We initially focused on nuclear and chloroplast SNP's. A large number of SNPs were identified in the chloroplast (cp) genome and multiple copies of this genome are present in bean cells suggesting that these could increase assay sensitivity. However, cpDNA SNPs identified at NAU and LLNL resolved *R. communis* individuals into only two major and four total groups with limited resolution. Therefore, our focus turned primarily to informative SNPs present in single-copy nuclear DNA sequences.
The goal of the proposed project has been to generate a battery of assays that, together, will provide a precise, quantitative genetic fingerprint for ricin preparations derived from a population of genetically diverse castor beans.

The results outlined in this report provide the information for needed to apply a SNP-based forensic analysis to diverse ricin preparations. The same methods could be useful in castor breeding programs that seek to reduce or eliminate ricin in oil-producing *R. communis* cultivars.

Results.

Extracting DNA from different ricin preparations.

If DNA-based forensic methods are to be effective, we first needed to demonstrate that DNA of sufficient quality to support PCR-based assays could be efficiently extracted from different ricin preparations. Demonstrating that DNA could be efficiently extracted from different ricin preparations was key to successful development of this approach. There were two challenges to meet. Many ricin preparations contain a significant number

of seed constituents and these are known to interfere with DNA isolation and to contaminated isolated DNA preparations in sufficient amounts to interfere with methods used to analyze purified DNA. Moreover, there are numerous methods of preparing ricin from castor beans. The resulting ricin preparations vary significantly in quality but the extraction methods employed to purify DNA from these must be applicable to all the different materials. The results of our efforts led to the SOP included below. The procedure provides detailed instructions for extracting DNA from different ricin preparations.

SOP Version 1.0 LLNL – *Ricinus communis* (castor) DNA extraction and purification from different ricin toxin preparations

Introduction

The purpose of this SOP is to provide a method of extracting and purifying *Ricinus communis* DNA from different ricin preparations. High quality preparations of the ricin toxin need not be free of DNA to be very effective. Therefore, there is no need to remove all DNA from such preparations. Moreover, some rather crude ricin preparations – sometimes amounting to little more than de-fatted castor bean mash – contain significant amounts of DNA. If sufficient amounts of DNA can be extracted from ricin preparations, then the DNA present in the sample can be used to generate a *R. communis* SNP-based signature that will be specific to that preparation. The first step in this process is to extract the DNA from the ricin preparation and remove the many impurities found in plant (including *R. communis)* seeds that interfere or inhibit DNA replication. Efficient DNA replication is required for the real-time PCR SNP assays.

Training requirements

There are several issues that must be addressed when working with real or putative ricin preparations. The first are regulatory. When working with ≥100 mg ricin, this toxin is considered a Select Agent (100 ≤ is exempt from the Select Agent rule). This means a single investigator may not possess amounts of ricin 100 mg or greater unless they and their institution are registered with the CDC and ricin is on their CDC permit. The individual requires a DOJ clearance and other permissions depending on the organization with which the investigator is associated. It is necessary, regardless of the amount of ricin possessed, to keep accurate records of the amount of ricin in possession and its fate. This is best done by maintaining a detailed inventory of the amount in possession, its usage and consumption in experiments and its destruction. If ricin preparations are destroyed, as happens when DNA is extracted from this, the amount of ricin used to produce the DNA must be recorded. If samples are destroyed for other purposes – for example by autoclaving, a record of this must also be maintained. Detailed inventory records must always be maintained for any Select Agent and ricin and other toxins are included in this rule.

Training requirements for possessing, handling and shipping of Select Agents differs by institution. However, at a minimum, scientists and technicians handling this toxin should have read all pertinent Select Agent rules. Such information can be obtained through the Centers for Disease Control and Prevention website (http://emergency.cdc.gov/cotper/dsat/)

Safe handling of toxic materials also requires significant training. Handling of ricin and chemicals associated with extracting DNA from the toxin preparations must complete laboratory training that addresses chemical and toxin hazards, safe laboratory practices, associated safety precautions and how to deal with emergencies (i.e., spills). Laboratory staff should read MSDS's associated with all chemicals used in this protocol, know how to store all chemicals and toxins safely and they should be familiar with use of the appropriate PPE including appropriate selection of protective gloves based on the chemicals to be used. They need to have completed training in handling, storage and disposal of hazardous and regulated wastes generated by the extraction process. Individuals who will be shipping or receiving ricin samples must receive DOT training if directly involved in shipping or they must work with someone who is certified to package and ship such materials. Competence to conduct research using toxic and hazardous materials should be tested by a combination of testing and evaluation of on-the-job performance by trainers with prior experience handling such materials. All training must be documented by the institution where the work is being performed.

Safe handling

The primary risks associated with this SOP are exposure to ricin during DNA extraction and exposure to toxic chemicals used in the extraction process. Once the DNA extraction process has begun, any active toxin in the ricin preparations will quickly be destroyed by this process (the first step in the extraction process should inactivate ricin). Many ricin recipes produce, at best, very crude toxin preparations. Some such preparations are little more than a precipitation of material the can be extracted with an aqueous buffer from de-fatted bean mash. Nevertheless, such preparations must be treated as if they contain purified ricin. Ricin samples from the FBI and NBFAC are often crude preparations similar to those expected to be generated by a terrorist organization.

There are other hazards associated with the DNA extraction process. Phenol, chloroform, isoamyl alcohol, isopropyl alcohol and ethanol are all hazardous materials and must be handled accordingly.

Engineering and PPE requirements for handling small ricin preparations are consistent with BSL-2 containment. Minimum PPE includes safety glasses or goggles, a lab coat and appropriate gloves. When possible, ricin preparations should be handled as liquids, not dried powders. If powders are provided, these should be suspended in a buffer (see procedure below) as the first step in handling the material, if possible before the vessel containing the ricin powder is opened. All ricin preparations should be handled in certified fume hood with HEPA filters or in a certified Biosafety cabinet. Biosafety cabinets, if properly chosen and maintained, are preferable to a fume hood when working toxins [Johnson, B., *et al.* (2001) Safety and health considerations for conducting work with biological toxins. Applied Biosafety 6: 117-135]. A Class II/Type B2 or better cabinet is recommended.

Destruction of ricin. Ricin can be inactivated by heat; it should be heated to 80°C for 10 minutes or to 50°C for approximately one hour at pH 7.8. A 0.1% sodium hypochlorite solution is also effective, although some authorities recommend 0.5% sodium hypochlorite ("Ricin" (2004) Center for Food Security and Public Health, College of Veterinary Medicine Iowa State University, Ames, Iowa 50011). Once experiments are completed, remaining wastes should be autoclaved or otherwise inactivated. Exposure to

moderately alkaline conditions (10% bleach, 0.5% sodium hypochlorite) will inactivate this protein toxin. Surfaces should therefore be treated with freshly prepared 10% bleach solutions. Castor bean mash is made safe for consumption by livestock by boiling the mash following extraction of the commercially valuable oil from the beans. Therefore autoclaving should destroy any remaining active toxin. All samples should be handled by strict adherence to written procedures for ricin and nucleic acid extraction. A written copy of each procedure should be used for each protocol and annotated after completion of the experiment, then included in the individual's research notebook.

Waste containing active ricin or any of the hazardous chemicals used for extraction of DNA from ricin preparations must be handled as hazardous waste and should be managed and disposed of in compliance with all federal, state and local requirements. If waste contains ricin and no other hazardous materials, the ricin should be inactivated by heating or chemical treatment before disposal.

The chemicals used in these experiments will, themselves, destroy ricin. Therefore, residual ricin in these already hazardous chemicals should not be a problem if wastes are handled, as required, to minimize exposure and discharge since other components of the waste (i.e., phenol, chloroform) will present a serious risk if not handled correctly.

Emergency procedures. All routes of exposure to ricin are dangerous. Based on animal studies, the LD_{50} in humans is ~ 1 mg for a 75 kg adult. Death can occur within 36-72 hours of exposure, depending on the route of exposure and the amount of ricin received. Symptoms of ricin poisoning depend on the route of exposure to ricin and the amount of ricin received. If ricin is ingested, symptoms occur within 2-6 hours and include nausea, vomiting, abdominal cramps and diarrhea. This can lead to severe dehydration, liver and kidney failure and death. If ricin is inhaled, symptoms occur within 8 hours and include nausea, fever, cough, chest tightness and difficulty breathing. This could lead to fluid in the lungs, respiratory failure and death. If ricin is injected, the muscles and lymph nodes near the injection site will die. This can also lead to liver and kidney failure and death. If a person lives longer than 5 days after ricin poisoning without complications, s/he will probably not die. In case of actual or suspected exposure the individual should do the following:

Immediately leave the area if a ricin preparation has been spilled or released and call 911. Be certain that no unauthorized people enter the room. In case of potential exposure carefully remove all clothing and place it into a plastic bag. Rapidly wash your entire body with large amounts of soap and water and get medical attention as quickly as possible. When removing clothing, quickly take off clothing that may have ricin in or on it. Any clothing that must be pulled over the head should be cut off the body instead of pulled over the head. Try to avoid touching contaminated areas. If your eyes are burning or vision blurred, rinse eyes with plain water for 10-15 minutes. If wearing contacts, remove these and put them with the contaminated clothing (never re-insert contacts). If eye glasses/safety glasses are contaminated, wash with soap and water. Seal the bag containing contaminated material and place in a second plastic bag. Seal this bag. Get immediate medical attention. If someone has ingested ricin, do not induce vomiting or give fluids to drink. Seek medical attention immediately. Call 911 and explain what has happened. It is most important to get immediate medical attention. Supervisors, management and E,S&H personnel should be notified only after seeking medical attention.

Reagents and Chemicals

Reagents/Chemicals needed	Suggested Vendor	Order Number	Amount
Phenol:chloroform:isoamyl alcohol (25:24:1)	Fluka BioChemika	776	500 mL
3M sodium acetate pH 5.2	Sigma/Aldrich	S-7899	100 mL
1M Tris-HCl (pH 7.8)	Sigma/Aldrich	T-2913	100 mL
5M EDTA Gibco Ultra Pure	Invitrogen Corporation	15575-038	100ml
Hexadecyltrimethlyammonium bromide (CTAB)	Sigma/Aldrich	M7635	500 gm
N-Lauroylsarcosine sodium salt (Sarkosyl NL) Sigma Ultra	Sigma/Aldrich	L5777-500G	500 gm
Sodium chloride, molecular biology grade	CalbioChem	567441	500 gm
Isopropyl alcohol	EM Science	PX1835-13	500 mL
Ethyl alcohol USP, absolute 200 proof	AAPER Alcohol and Chemical Company	N/A	3.8 L

Solutions Needed

Homogenization Solution:

5M NaCl 146.1 gm
2% (w/v) N-Lauroylsarcosine sodium salt (Sarkosyl NL) 10.0 gm
distilled water to 500 mL
It may be necessary to heat the solution to dissolve components

Extraction Buffer

100mM Tris-HCl (pH 8.0) place 60.2 gm Tris-base in 400 mL distilled water
Add 29.2 mL 0.1 M HCl – check pH/adjust to 8.0
Adjust total volume to 500 mL
20mM EDTA (disodium salt) 3.72 gm
1.4M NaCl 81.82 gm
2% (w/v) CTAB 10.0 gm
Dissolve the last three components to a final volume of 500 mL of 100 mM Tris-HCl (pH 8.0)

TE Buffer

10 mM Tris-HCl (pH 8.0)	Place 6.02 gm Tris-base in 400 mL distilled water. Add 292 µL 0.1 M HCl – check pH/adjust to 8.0
1 mM EDTA	Dissolve 186 mg of EDTA (disodium salt)

Bring volume to 500 mL with distilled water

70% (v/v) ethanol in sterile distilled water

Equipment

- Microcentrifuge
- Thermo-mixer, shaking water bath or water bath
- Fume hood for the safe handling of ricin containing materials and for handling phenol

Detailed Procedure

1. Place the ricin preparation into a 1.5 mL microcentrifuge tube.
2. Add 3X the amount of ricin (v/v or w/v) of Homogenization solution (5M NaCl, 2% Sarcosyl). Mix gently by inversion several times.
   (Note: Homogenization Solution may have to be pre-warmed prior to addition. Heating at 37^{o} C for 20 minutes generally brings the detergent into solution.)
3. Centrifuge at 4,000 x g in a microcentrifuge for 10 minutes at room temperature.
4. Transfer the supernatant to a fresh polypropylene tube.
5. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and gently mix by inversion for one minute.
6. Centrifuge at 2,000 x g for 10 minutes at room temperature.
   (Note: If the phases do not separate and a whitish precipitate is not formed at the phenol – chloroform interface, repeat centrifugation at 8200 x g.
7. Collect the upper aqueous phase to a new tube and repeat the phenol:chloroform:isoamyl alcohol extraction as described in step 5. Discard the lower phenol layer as hazardous waste.
8. Centrifuge as before for 10 minutes at 2,000 x g.
9. Collect the upper aqueous layer to a new tube and add 2x the volume of freshly prepared (less than one week old) Extraction Buffer (100mM Tris-Cl pH 7.8, 20 mM EDTA, 1.4M NaCl, and 2% CTAB). Mix gently by inversion.
10. Incubate the sample at 60^{o}C with gentle shaking for 30 minutes. If a thermo-mixer is not available, a water-bath combined with inversion by hand every five minutes is acceptable.
11. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and gently mix by inversion for one minute.
12. Centrifuge at 2,000 x g for 10 minutes at room temperature.
13. Carefully collect the upper aqueous phase to a new tube. Add 1/30th of the aqueous layer volume of 3M sodium acetate pH 5.2 and mix gently.
14. Add 0.6 volumes of isopropyl alcohol and mix gently.
15. Centrifuge at 16,000 x g for 10 minutes at room temperature to collect the precipitated DNA.

16. Gently aspirate or otherwise remove the aqueous solution (the pellet may be loose) and wash the pellet with 3 mL ice-cold 70% ethanol.
17. Centrifuge at 16,000 x g for 10 minutes at room temperature to again collect the pellet to the bottom of the tube.
18. Gently remove the supernatant taking care not to disturb the pellet.
19. Air dry the pellet.
20. Dissolve the pellet in as small a volume of sterile TE buffer (10mM Tris-Cl pH 8.0, 1 mM EDTA) as can be used.
21. Measure the DNA concentration using a picogreen assay (Quant-iT™ PicoGreen® dsDNA reagent *2000 assays* *10 x 100 µL, Cat. No. P11495 for a commercial source.
    (Note: Do NOT use an OD_{260}/OD_{280} (i.e., Nanodrop™) because it significantly over-estimates the amount of DNA present in the solution when DNA is extracted using this method).
22. Store DNA frozen (short-term -20°C; long-term -80°C). Numerous freeze/thaw cycles contribute to degrading the DNA.

This procedure was used to extract DNA from four different ricin preparations ranging from a preparation produced using a recipe available on the Internet that produces little more than de-fatted castor bean mash (ricin 1) to a preparation produced provided by the FBI that is reputed to produce high quality, highly purified ricin (ricin 4). Figure 1 shows the quality and relative quantity of DNA extracted from each ricin preparation. The results demonstrate that the quality of the DNA, as judged by average fragment size, is approximately the same from each ricin preparation. They also suggest that there are significant amounts of DNA in even relatively pure ricin preparations. We were unable to obtain sufficient amounts of DNA to conduct through PCR-based analysis from only one ricin preparation. That preparation had been passed through a hydroxyapatite, a resin previously used to study DNA. It has a very high affinity for double stranded DNA. Polyacrylamide gel electrophoresis (Figure 2) showed the presence of ricin in preparations prepared using two different methods. Even the Wu method generated a protein cut rich in ricin.

Following extraction of the DNA a method was needed to demonstrate that this DNA would support forensic analyses. To address this issue, we designed primers that amplified DNA containing different diagnostic chloroplast and chromosomal SNP's. The rationale was that, if the extracted DNA supported PCR amplification of the appropriate DNA fragments, then it was likely that such DNA would support the real-time PCR reactions used to differentiate between two SNP's at a single locus. Rather than incurring the expense of using fully functional assays to test the DNA, primers that amplify fragments containing diagnostic SNP's were designed and standard PCR was used to demonstrate that DNA extracted from different ricin preparations would support PCR. Figure 3 shows the results of amplifying DNA using primers that amplify two different nuclear DNA fragments containing informative SNP's and two different chloroplast DNA fragments containing informative SNP's.

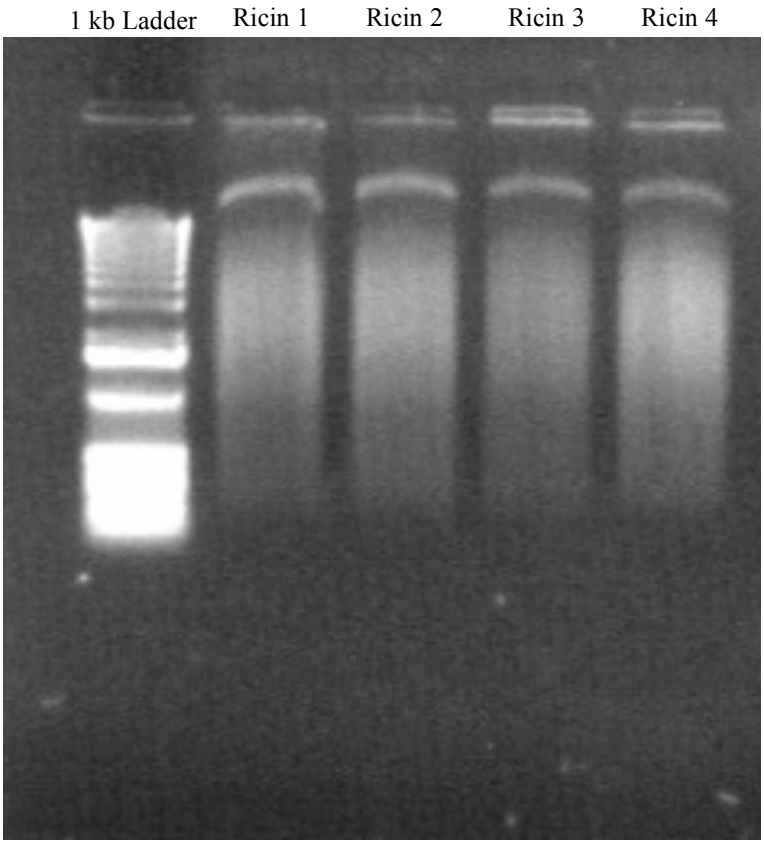
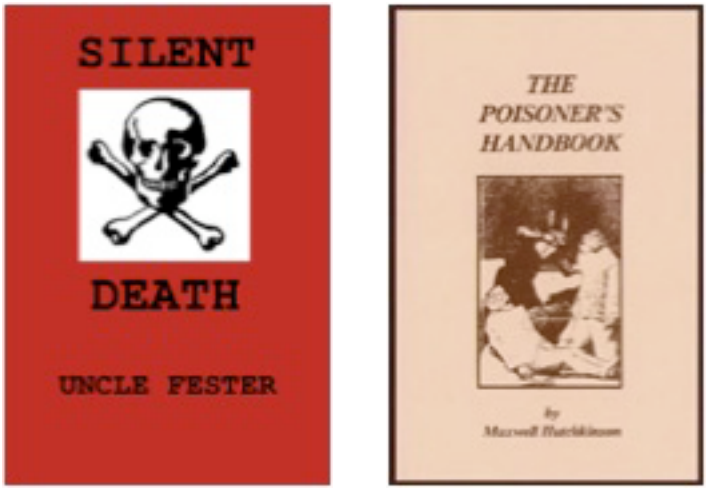


Figure 1. Agarose gel analysis of DNA extracted from four different ricin preparations. Ricin 1 contained no more than de-fatted castor bean mash while Ricin 4 was produced using a procedure provided by the FBI and reputed to produce highly purified ricin.



Handbooks containing recipes to produce ricin and other toxic materials

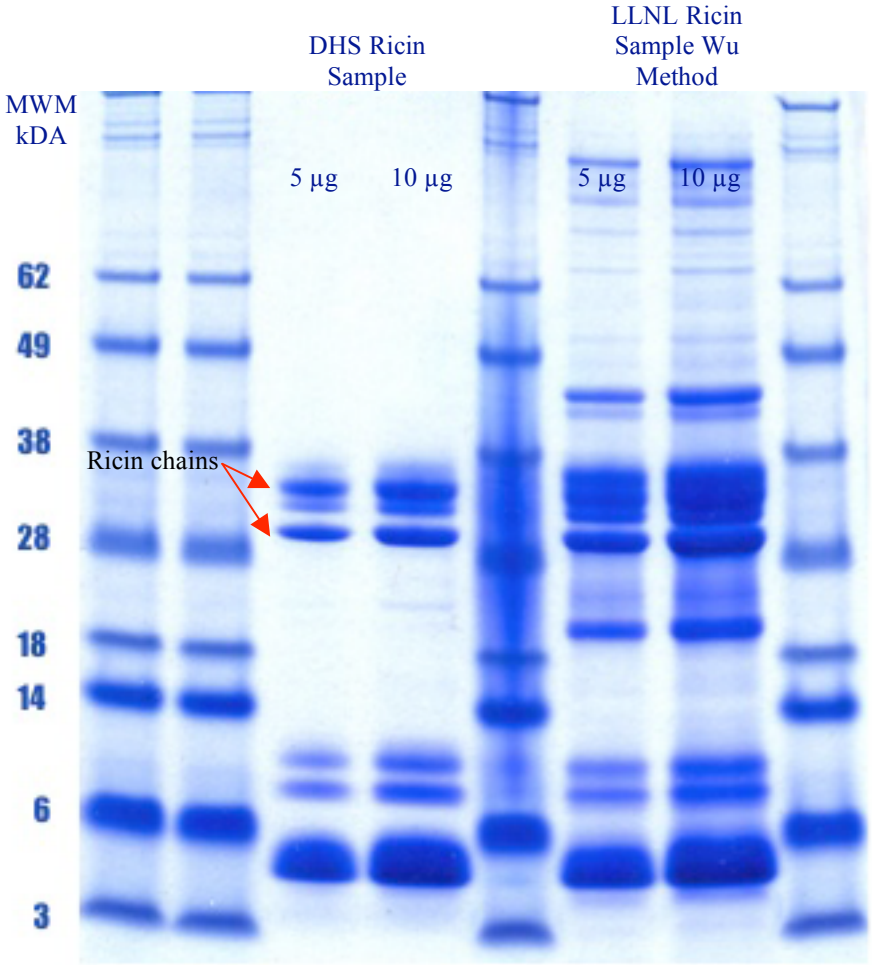


Figure 2. Polyacrylamide gel electrophoresis analysis of ricin samples prepared using different methods. Lanes 1, 2, 5 and 8; molecular weight markers. Lanes 3 and 4, ricin from a preparation provided by DHS. Lanes 6 and 7, ricin prepared using the Wu, *et al.* method. Ricin has two chains, A and B. Chain A has a molecular weight of 32 kDa (267 amino acids) while Chain B has a molecular weight of 34 kDa (262 amino acids). Note that even the relatively crude Wu, *et al*. preparation contains large amounts of ricin.

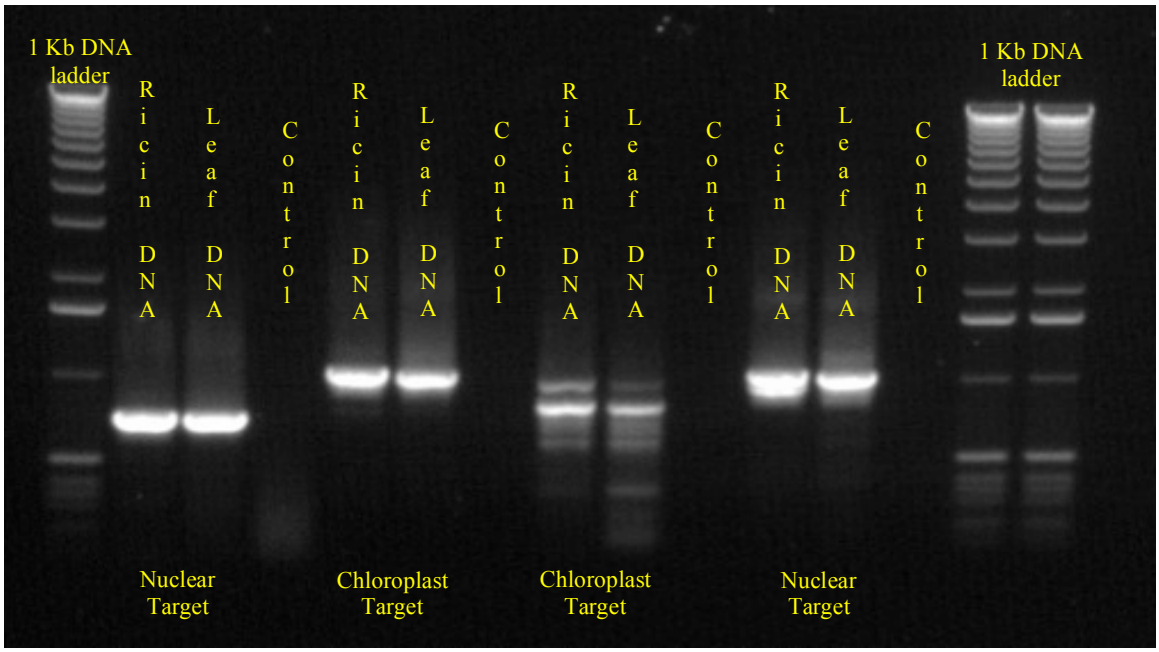


Figure 3. Demonstration that DNA purified from ricin preparations will support PCR. DNA extracted from ricin was used in PCR containing primers that amplify two different nuclear and two different chloroplast DNA fragments containing phylogenetically informative SNP's. Reactions contained DNA extracted from ricin (the first lane in each set), highly purified DNA from *R. communis* leaves (the second lane in each set) or no DNA (the third lane in each set). Approximately equal amounts of ricin or leaf DNA were included in each reaction. Results demonstrate that DNA extracted from even crude ricin preparations can be sufficiently purified and is of sufficiently high quality (length) to support PCR.

Phylogenetic analysis of a large number of different R. communis accessions

It is necessary to establish the extent of diversity within an extensive population of different castor accessions before it is possible to identify phylogenetically (and, therefore forensically) useful genetic loci. Two independent methods were used to generate phylogenetic information. Amplified Fragment Length Polymorphism (AFLP) analysis was applied to a large number of different *R. communis* accessions. DNA extracted from leaf tissue of plants grown from each of three seeds from the same accession were used in this study. Figure 4 shows an example of AFLP analysis of DNA from three different seeds of the same *R. communis* accession. The profiles are very similar to one another. However, close scrutiny of the profiles reveals differences in profiles among different seeds of the same accession. Each AFLP analysis was repeated a minimum of three times for each sample and the three different profiles were compared for each seed. Only fragments that appeared in all three profiles for the same sample were used to to generate AFLP-based phylogenetic dendrograms (Figure 5).

PI 170 686 B VS. PI 197 048 D (Otherwise known as LLNL 14B VS USDA 52D.2)
With Map Marker 1000XL Individual samples Y axis Zoom 2000

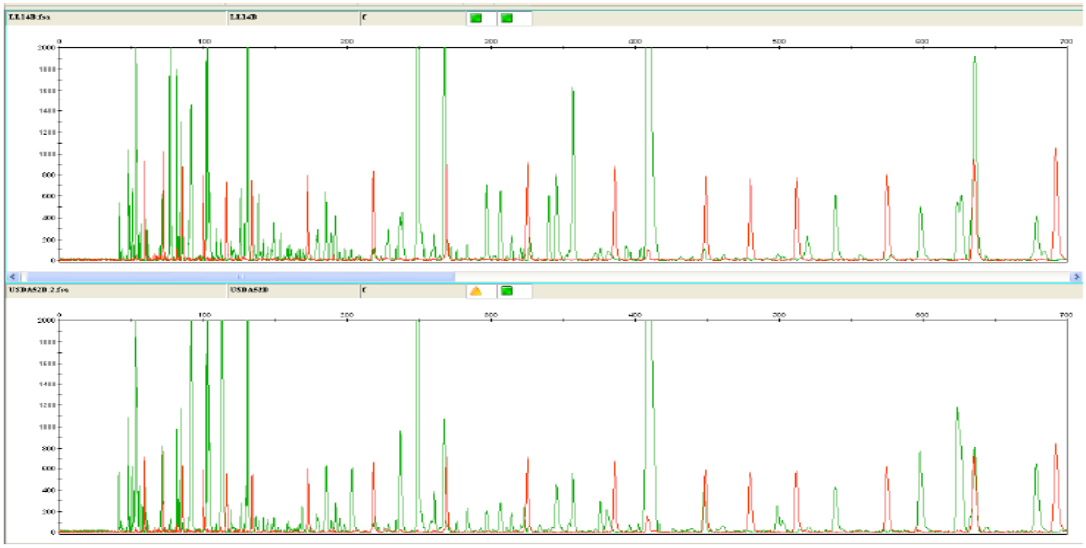


Figure 4. AFLP profiles of two different but closely related *R. communis* accessions. Red fragments are molecular weight markers used to determine the size of the green AFLP fragments. Further expansion of the dendrogram electronically reveals additional differences not visible at this resolution.

While it is not possible to resolve all of the different isolates in this tree, there area two important observations. The first is that all of the isolates resolve into two large branches. These two branches are defined by differences in their chloroplast DNA. This is not directly manifested in the tree but is observed in the SNP analyses. However, there appears to be a correlation between which chloroplast SNP set is present in a particular isolate and which branch of the AFLP the isolate maps to. The evolutionary ramifications of this are not relevant to assay development and will not be discussed here. If one generates a simplified AFLP-based dendrogram by clustering all isolates closely related on a single branch, one can better see the defining branches of the tree (Figure 6).

Figure 5. AFLP-based UPGMA dendrogram of a large collection of different *R. communis* accessions. Colors of the different isolates reflect clusters in a SNP-based phylogenetic analysis. That is, isolates that map closely together in a SNP-based phylogenetic tree have the same color. Results demonstrate that AFLP and SNP-based analyses generate very similar results.

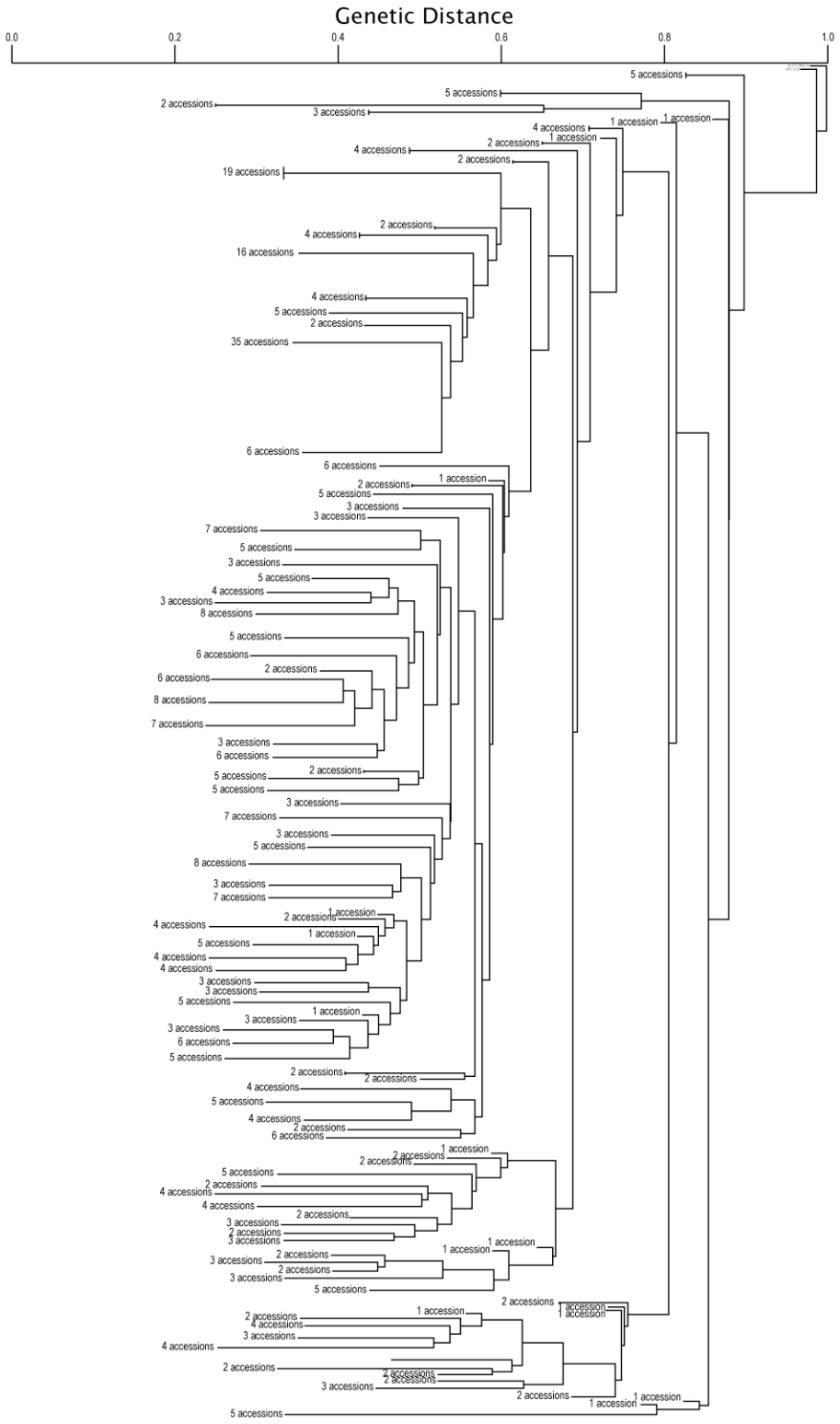


Figure 6. Condensed AFLP dendrogram revealing the major phylogenetic branches within *R. communis.* Isolates used in this study represented the major branches.

AFLP and SNP based clustering of different *R. communis* accessions				
PI240312A	PI221055A	PI244573A	PI241365C	PI203130B
PI221049B	PI221055B	PI244573B	PI240674C	PI203130C
PI217558A	PI221055C	PI244573C	PI246996A	PI215776A
PI215773A	PI221053B	PI243061B		PI215775B
	PI204324B	PI243061C	PI219773A	PI202667A
PI217539A	PI221698B	PI212115B	PI219773B	PI202667B
PI217539B	PI221698C	PI243207C	PI219773C	PI202667C
PI209132C	PI221050A	PI215770A	PI219772B	PI204323B
PI204323A	PI221050B	PI215770C	PI212115A	
PI203130A	PI221050C		PI212115C	PI173795A
PI217557A	PI240312B	PI240311B	PI174351C	PI173795B
PI241370A	PI195811A	PI209436A	PI215720B	PI173795C
PI240312A	PI195811B	PI204325B	PI215720C	PI209436B
	PI201830C		PI219775C	PI181916C
PI201830A	PI221051B	PI195811C		PI181063A
PI201830B	PI221055A	PI3BPI3A	PI243062A	
PI201830C		PI3C	PI243062B	PI208839A
PI204323C	PI221049C		PI243062C	PI197048A
PI202711A	PI217539C	PI209326A	PI241365A	PI209436C
PI204324A	PI241370B	PI209326B	PI241365B	PI209132A
PI217559A	PI241371C	PI209326C	PI219766B	PI206515B
PI217558B	PI215772A	PI215770B	PI219766C	
PI204325C	PI203661B	PI208696A	PI219775B	PI208840B
PI217557B		PI208696B	PI241362A	PI208840C
	PI240311A	PI208696C	PI241362C	PI209622B
PI241362B	PI162912B	PI240312C	PI243061A	PI209622C
PI215772B	PI208841B			PI202711B
PI243207A	PI204322B	PI241370C	PI217559B	
PI240311A	PI204322C	PI209132B	PI203126C	PI208839B
		PI204324C	PI241371A	PI208839C
PI240674A	PI215774A	PI215773B		PI176751C
PI204322A	PI215774B		PI181066C	PI173090A
PI204321C	PI215774C	PI215773C	PI176751B	PI223013C
	PI215775A	PI181066A	PI173950C	PI221051A
PI208841A	PI174351A		PI173948A	
PI181066B	PI174351B	PI173950A		PI222830C
PI215775C	PI204321A	PI173947A	PI240674B	PI217558C
	PI241371B	PI173947C	PI208841C	
PI204321B	PI215774A	PI173948B		PI222829A

			PI208842A	PI222829B
PI243207B	PI181970C	PI209622A	PI208842C	PI222829C
PI240311C	PI181970B		PI176751A	PI173091B
	PI181970A	PI222830B	PI181916A	
PI222265A			PI181916B	PI215720A
PI222265B	PI179733C	PI222745A	PI222830A	PI174350A
PI222265C	PI223013A	PI222745B		PI174350B
PI173090B	PI223408A	PI222745C	PI167287B	PI174350C
		PI223408B	PI167287C	PI173090C
PI203128B	PI241367C	PI223408C	PI207868A	PI215720A
	PI167112C	PI223013B	PI1C	
PI167238A	PI167112B	PI183076A	PI202719C	PI183468A
PI219767B	PI170686C		PI182987B	PI165446A
PI219767C	PI183143C	PI180334B	PI182987C	
	PI167288A		PI183076B	PI167238B
PI173946A	PI167288B	PI183078B	PI183347B	PI167238C
PI173946B	PI167288C	PI163162B	PI180334B	
PI170684B				PI241368A
	PI83470C	PI180334A	PI227869A	PI241368B
PI170682B		PI180334C	PI183143B	PI221053A
PI170682C	PI180335A	PI179729C	PI170682A	PI221053C
PI167238C	PI180335B	PI183076C		PI179985C
	PI180335C	PI183470A	PI227869A	PI241369A
PI183468B	PI170686B		PI227869B	PI241369B
PI183468C	PI173946C	PI179028B	PI227869C	PI241369C
PI183078C	PI183078A	PI179028C	PI241368C	PI219774C
		PI179030A	PI179733A	PI241367A
PI167342A	PI221048A	PI179030C	PI179733B	PI241367B
PI167342B	PI221048B	PI208689A	PI219774A	PI221471C
PI167342C	PI221046A	PI208689C	PI219774B	PI219776A
PI179030B	PI183471C	PI219770A	PI170686A	PI219776B
PI177537A	PI221052B	PI179733C	PI184132A	PI219770C
PI221471A	PI221052C		PI184132B	
PI221471B	PI219776C		PI170684A	
PI221052A	PI208689B		PI219770B	
PI221048C	PI203128C		PI177537B	
			PI177537C	
PI183471B			PI179028A	

			PI179985A	
			PI179985B	

Table 1. Clusters of different *R. communis* accessions that were used as a basis for selection of a phylogenetically diverse set of isolates. Note that some many clusters contain seeds from the same accession. Isolates from the largest and most diverse clusters were selected based on the amount of total leaf DNA available so that the same isolates could be shared among the three different laboratories.

The SNP data used to derive these clusters is too extensive to include in the body of this report and will be included in a separate Excel file entitled, "LLNL R communis SNP's.xls.

Development and testing of R. communis SNP assays.

The remainder of this report includes a description of the different SNP assays developed and tested at LLNL and LANL. One report containing all of the data from the LLNL studies has been merged with the data from LANL in a very similar format. At LLNL 95 *R. communis* DNA accessions were used to test 12 Castor SNP assays, (Table 2). Forty-four of the 95 DNAs were extracted at LLNL from *R. communis* plant leaves using a DNeasy Plant Mini Kit protocol (Qiagen, Valencia, Ca). The remaining 51 DNAs were received from a Florida panel of Castor accessions generated at Northern Arizona University.

At LANL 83 *R. communis* DNA Accessions were used to test 12 Castor SNP assays, (Table 2b). Fifty-one of the 83 DNAs were received from NAU and have the identifier RcFL. Thirty-two of the 83 DNAs were received from LLNL and have a 6 digit identifier.

Table 2a

Castor DNA Panel Tested with SNP Assays

RCFL4.3	RCFL6.13	RCFL1.1	RCFL10.11	RCFL12.21	PI195811	PI209622	PI203130
RCFL5.21	RCFL10.19	RCFL3.4	RCFL9.12	RCFL2.7	PI197048	PI217539	PI212115-1
RCFL7.7	RCFL10.25	RCFL3.3	RCFL9.2	RCFL3.14	PI201830	PI219767	PI222265-1
RCFL6.11	RCFL1.19	RCFL8.6	RCFL8.8	PI167238	PI203661	PI219770	PI167342-5
RCFL6.3	RCFL11.18	RCFL5.3	RCFL3.16	PI170686	PI204322	PI219776	PI215774-1
RCFL9.1	RCFL1.8	RCFL12.32	RCFL3.19	PI173946	PI206515	PI221698	PI241370-1
RCFL9.15	RCFL10.3	RCFL12.14	RCFL4.8	PI173948	PI207868	PI240312	PI243062-4
RCFL9.8	RCFL12.2	RCFL12.1	RCFL5.12	PI173950	PI208689	PI241368	PI244573-1
RCFL1.6	RCFL2.11	RCFL11.6	RCFL1.14	PI181916	PI208840	PI208839-1	PI167288-5
RCFL3.5	RCFL3.12	RCFL11.4	RCFL1.25	PI183468	PI208842	PI202667-2	PI241362
RCFL4.4	RCFL9.10	RCFL10.33	RCFL10.18	PI183470	PI209132	PI203324-2	PI179729
RCFL5.7	RCFL9.24	RCFL10.22	RCFL10.7	PI183471	PI209326	PI219773-1	

* FL DNA Panel; *LLNL DNA Panel

Table 2b

Castor DNA Panel Tested with SNP Assays

RcFL 1.1	RcFL 3.12	RcFL 6.3	RcFL 9.15	RcFL 11.6	173950	204322	219770
RcFL 1.6	RcFL 3.14	RcFL 6.11	RcFL 9.24	RcFL 11.8	179729	206515	219776
RcFL 1.8	RcFL 3.16	RcFL 6.13	RcFL 10.3	RcFL 12.2	181916	207868	221698
RcFL 1.14	RcFL 3.19	RcFL 7.7	RcFL 10.7	RcFL 12.10	183347	208689	240312
RcFL 1.19	RcFL 4.3	RcFL 8.6	RcFL 10.11	RcFL 12.14	183468	208840	241362
RcFL 1.25	RcFL 4.4	RcFL 8.8	RcFL 10.18	RcFL 12.21	183470	208842	241368
RcFL 2.7	RcFL 4.8	RcFL 9.1	RcFL 10.19	RcFL 12.32	183471	209132	
RcFL 2.11	RcFL 5.3	RcFL 9.2	RcFL 10.22	167238	195811	209326	
RcFL 3.3	RcFL 5.7	RcFL 9.8	RcFL 10.25	170686	197048	209622	
RcFL 3.4	RcFL 5.12	RcFL 9.10	RcFL 10.33	173946	201830	217539	
RcFL 3.5	RcFL 5.21	RcFL 9.12	RcFL 11.4	173948	203661	219767	
* FL DNA Panel; *LLNL DNA Panel							

PCR Analyses

At LLNL, all of the 10 genomic Castor SNP Assays (Table 3a) were designed and premixed as 40X stocks by Applied Biosystems. The VIC- and FAM-labeled probe concentrations of the assay mixture for the 40X stock were 8 µM and the forward and reverse primer concentrations were 36 µM. The real-time PCR assays contained the following components: 1X Taqman Genotyping Master Mix part # 4371355(Applied Biosystems, Foster City, CA), 0.9 µM of each custom SNP forward and reverse primers, 0.2 µM of each custom SNP VIC- or FAM-5'-labeled probe, 2 ng/µl of Castor genomic DNA, and 0.025 U/uL of Platinum Taq DNA Polymerase part # 10966-018 (Invitrogen, Carlsbad, CA).

The 2 chloroplast SNP assays (Table 3) were designed at NAU so that the same primer and probe selection software would be used for all assays developed in this project and the primers and probes were separately ordered from Applied Biosystems. The final real-time PCR components and concentrations were as follows: 1X Taqman Genotyping Master Mix part # 4371355(Applied Biosystems, Foster City, CA), 0.9 µM of each custom SNP forward and reverse primers, 0.25 µM of each custom SNP VIC- or FAM-5' -labeled probe, 2 ng/uL of Castor genomic DNA, and 0.025 U/µL of Platinum Taq DNA Polymerase part # 10966-018 (Invitrogen, Carlsbad,CA).

The real time PCR assays were run in an ABI 7900 using the standard protocol setting with the following parameters: 1cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 seconds, and 40 cycles at 95°C for 15 seconds and 60°C for 1minute.

An identical protocol was followed at LANL. All of the 10 genomic Castor SNP Assays (Table 3b) were designed and premixed as 40X stocks by Applied Biosystems. The VIC- and FAM-labeled probe concentrations of the assay mixture for the 40X stock were 8 µM and the forward and reverse primer concentrations were 36 µM. The real time PCR assays contained the following components: 1X Taqman Genotyping Master Mix part # 4371355 (Applied Biosystems, Foster City, CA), 0.9 µM of each custom SNP forward and reverse primers, 0.2 µM of each custom SNP VIC- or FAM -5'-labeled probe, 2 ng/µL of Castor genomic DNA, and 0.025 U/µL of Platinum Taq DNA Polymerase part # 10966-018(Invitrogen, Carlsbad, CA).

The two chloroplast SNP assays (Table 3b) were designed at NAU and the primers and probes were separately ordered from Applied Biosystems. The final real-time PCR components and concentrations were as follows: 1X Taqman Genotyping Master Mix part # 4371355 (Applied Biosystems, Foster City, CA), 0.9 µM of each custom SNP forward and reverse primers, 0.25 µM of each custom SNP VIC- or FAM-5'-labeled probe, 2 ng/µl of Castor genomic DNA, and 0.025 U/uL of Platinum Taq DNA Polymerase part # 10966-018(Invitrogen, Carlsbad ,CA). The real-time PCR assays were run in an ABI 7900 using the standard protocol setting with the following parameters: 1cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 seconds, and 40 cycles at 95°C for 15 seconds and 60°C for 1minute.

Table 3a. LLNL genomic and chloroplast SNP assays

Genomic Assays	Allele States	Chloroplast Assays	Allele States
SNP 11	A,G	SNP Cp 19	G,T
SNP 75	A,T	SNP Cp 112	A,G
SNP84	A,T		
SNP 94	A,T		
SNP252	A,G		
SNP 262	A,G		
SNP 299	A,T		
SNP 355	A,G		
SNP 381	C,T		
SNP 389	C,T		

Table 3a. A list of the 12 LLNL SNP Castor Assays. Allele states are the nucleotides of the two possible SNPs present in each DNA.

Table 3b. LANL genomic and chloroplast SNP assays

Genomic Assays	Allele States	Chloroplast Assays	Allele States
SNP 010	G,T	SNP Cp 19	G,T
SNP 24	A,G	SNP Cp 111	C,T
SNP 26	G,T		
SNP 28	C,T		
SNP 165	G,T		
SNP 195	A,G		
SNP 270	A,G		
SNP 324	C,G		
SNP 311	A,G		
SNP 313	G,T		

Table 3b. A list of the 12 LANL SNP Castor Assays. Allele states are the nucleotides of the two possible SNPs present in each DNA.

From this point on, the LLNL and LANL results are separated for clarity.

LLNL results

Table 4. shows the sequences of the forward and reverse and SNP probes for each of the LLNL *R. communis* SNP assays.

Primers and probes

	SNP 11	SNP 75
FWD	CGCTTTATTAACACACTGAAGTTGGA	GGTTTCCCATTTGACATTGTTTACTTATTACTT
REV	GCCAGGAACTACATCAGTCATGAAA	GTTTGAAAAACCTGAACAAAATTGCCAAT
P1	CGAAGTCTTCTTTTTATACAAT	TGATGCCTTCTTTAAAGAG
P2	CGAAGTCTTCTTTTTGTACAAT	ATGCCTTCTTAAAAGAG
	SNP 84	SNP 94
FWD	TGCATTGGCAGTTGTTTTGCT	AAAGACAAGGCTTCCATAAGAAGCA
REV	TGCTATTGGAATTCTTGCCCACTTA	GGAAATCCTTAATTTAAGTTAAAACAGGTGACTT
P1	CCTACTTCTATACAAGCTTTA	AAGGGACGTTGCACTC
P2	CCTACTTCTATACTAGCTTTA	AAGGGACGATGCACTC
	SNP 252	SNP 262
FWD	TGGCACCTTCTATTGTTTCATCCAA	CGGTGGGAGGAGTTGGTT
REV	GCCCAGCCAATCAGATGATGA	TGGCTCTCTATGGCCGGATA
P1	ATCTCTAACAACTGATAAGC	AGAAATGCCGCTTAAAG
P2	TCTAACAACAGATAAGC	AGAAATGCCACTTAAAG
	SNP 299	SNP 355
FWD	CTTACACCAAAGCATATGAAGACGAAA	AGAAAGAGTACTACAGTGCATCTAGTGA
REV	ACAATGTTGACTCTTTAGCCACAAAAAA	TCCAGGTGCTGCATGCAT
P1	TGCTTGCTTGTACTTTTA	TTGATATAGACACAAGTTCA
P2	TGCTTGCTTGTTCTTTTA	TTGATATAGACACGAGTTCA
	SNP 381	SNP 389
FWD	GCCTGCCCACAATATAGTCCTTTT	GTCACCCAAGTAAGGAAGGCTTTAT
REV	TTCTGAATTTAACATGTATTACTATAGCACCTGAA	TCACCAGATGCATTATGCTTCACT
P1	TTGCTACCCCTTCCCCCTA	TTGACTCCACTGCCAAAC
P2	TTGCTACCCCTTTCCCCTA	ACTCCACCGCCAAAC
	Cp 19	Cp112
FWD	AGGTCTTGGTGCGGAACA	TGTCAGGCTATTGTTCTCCTGTTC
REV	GGAACCGTAGGACTCTATCCATTTATT	GGGAGTCCATCATGTAATCAAAAGA
P1	CAAGGTTGTGTCGAGTG	CTAAAAGTAATGAAGTAAGAC
P2	TTCAAGGTTTTGTCGAGTG	AAGTAATGGAGTAAGACATC

QPPP – specificity

The Florida and LLNL panels of 95 Castor DNAs were amplified against each of the 10 genomic and 2 chloroplast SNP assays, (Table 5). Assays that had greater than 86% of

one allelic state within both the Florida and LLNL DNA panels were not considered for further testing due to lack of diversity (Table 6).

Table 5.

Castor DNA SNP Allele Calls

FL DNAs	252	299	389	Cp112	Cp19	94	381	355	84	75	11	262
RCFL4.3	T	T	C	G	G	T	T	A	T	A	G	A
RCFL5.21	T	T	C	G	G	T	T	G	T	A	G	A
RCFL7.7	T	T	C	G	G	T	C	G	T	A	G	A
RCFL6.11	T	T	C	G	T	T	C	G	T	A	G	A
RCFL6.3	T	T	C	G	T	T	T	G	T	A	G	A
RCFL9.1	T	T	C	G	G	T	T	G	T	A	G	A
RCFL9.15	T	T	C	G	G	T	T	G	A	A	G	A
RCFL9.8	T	T	C	G	G	T	C	A	T	A	G	A
RCFL1.6	T	T	C	G	G	T	C	A	T	T	G	A
RCFL3.5	T	T	C	G	G	T	T	G	T	A	G	A
RCFL4.4	T	T	C	G	G	T	T	G	T	A	G	A
RCFL5.7	T	T	C	G	G	T	HET	HET	T	A	G	A
RCFL6.13	T	HET	C	G	G	T	T	G	T	A	G	A
RCFL10.19	A	T	C	G	G	T	T	G	T	A	G	A
RCFL10.25	T	T	C	G	G	T	C	A	T	A	G	A
RCFL1.19	T	T	C	G	G	T	C	A	T	A	G	A
RCFL11.18	T	T	C	G	G	T	T	G	T	A	G	A
RCFL1.8	T	T	C	G	G	T	T	G	T	A	G	A
RCFL10.3	T	T	C	G	NEG	T	T	G	T	A	G	A
RCFL12.2	T	T	C	A	G	T	T	G	T	A	G	A
RCFL2.11	T	T	C	G	G	T	C	A	A	A	G	A
RCFL3.12	T	T	C	G	G	T	T	G	A	A	G	A
RCFL9.10	T	T	C	G	G	T	T	G	T	HET	G	A
RCFL9.24	T	T	C	G	G	T	C	A	T	T	G	A
RCFL1.1	T	T	C	G	G	T	T	G	T	A	G	A
RCFL3.4	A	T	C	G	G	T	C	A	T	A	G	A
RCFL3.3	T	T	C	G	G	T	C	A	T	A	G	A
RCFL8.6	T	T	C	G	G	T	T	G	T	A	G	A
RCFL5.3	T	A	C	G	G	T	C	G	T	A	G	A
RCFL12.32	A	HET	C	G	G	T	C	G	T	A	G	A
RCFL12.14	T	T	C	G	G	T	C	G	T	A	G	A
RCFL12.1	T	T	C	G	G	T	T	A	T	A	G	A
RCFL11.6	T	T	C	G	G	T	T	A	T	A	G	A
RCFL11.4	T	T	C	G	G	T	T	A	T	A	G	A
RCFL10.33	T	T	C	G	G	T	T	A	T	A	G	A
RCFL10.22	T	T	C	G	G	T	T	A	T	A	G	A
RCFL10.11	T	T	C	G	G	T	T	A	T	A	G	A
RCFL9.12	T	T	C	G	G	T	T	G	T	A	G	A
RCFL9.2	HET	T	C	G	G	T	T	G	T	HET	G	A
RCFL8.8	T	T	C	G	G	T	HET	G	T	A	G	A
RCFL3.16	T	T	C	G	G	T	T	G	A	A	G	A
RCFL3.19	T	T	C	G	G	T	HET	HET	T	A	G	A
RCFL4.8	T	T	C	G	G	T	T	A	T	A	G	A
RCFL5.12	T	T	C	G	G	T	T	G	T	A	G	A
RCFL1.14	T	T	C	G	G	T	T	A	T	A	G	A
RCFL1.25	T	T	C	A	G	T	HET	G	T	A	G	A
RCFL10.18	T	T	C	G	G	T	T	G	T	A	G	A

RCFL10.7	T	T	C	G	G	T	T	A	T	A	G	A
RCFL12.21	T	T	C	G	G	T	T	A	T	A	G	A
RCFL2.7	T	T	C	G	G	T	T	G	T	A	G	A
RCFL3.14	T	T	C	G	G	T	T	G	T	A	G	A
LLNL DNAs	**252**	**299**	**389**	**Cp112**	**Cp19**	**94**	**381**	**355**	**84**	**75**	**11**	**262**
PI167238	HET	A	C	G	T	T	C	G	T	A	G	A
PI170686	A	T	C	G	T	T	C	G	A	T	G	A
PI173946	HET	T	C	G	T	T	C	G	HET	T	G	A
PI173948	HET	T	C	A	G	T	C	G	HET	T	G	A
PI173950	HET	HET	C	A	G	T	C	G	HET	T	G	A
PI181916	A	T	C	G	T	A	C	G	A	T	A	G
PI183468	A	T	C	G	T	T	C	G	HET	T	G	A
PI183470	HET	HET	C	G	T	T	C	HET	HET	T	G	HET
PI183471	T	HET	C	G	T	T	C	G	T	A	G	A
PI195811	T	HET	C	G	G	T	C	G	T	A	G	A
PI197048	T	T	C	G	G	T	T	A	T	A	G	A
PI201830	HET	T	C	G	G	T	C	A	T	A	G	A
PI203661	T	T	C	A	G	T	C	G	T	A	HET	A
PI204322	HET	T	HET	A		T	C	G	HET	T	HET	A
PI206515	T	T	C	G	G	T	C	Het	T	HET	G	A
PI207868	HET	HET	C	G	T	HET	C	G	T	HET	HET	A
PI208689	T	A	C	G	T	T	C	G	T	A	G	A
PI208840	T	T	C	G	G	T	C	G	T	T	G	HET
PI208842	A	T	C	G	G	A	Het	G	T	HET	HET	G
PI209132	T	T	C	G	G	T	C	G	T	T	A	A
PI209326	T	T	C	G	G	T	T	A	T	A	G	A
PI209622	T	HET	C	G	G	HET	C	G	HET	HET	G	HET
PI217539	T	T	C	A	G	T	C	NEG	T	A	G	A
PI219767	HET	HET	C	G	T	T	C	A	A	A	G	A
PI219770	T	A	C	G	T	T	C	G	HET	A	G	A
PI219776	T	A	C	G	T	T	C	A	T	A	HET	A
PI221698	T	A	C	G	G	T	C	G	HET	A	G	A
PI240312	T	A	C	G	G	T	T	A	T	A	HET	A
PI241368	HET	T	C	G	T	T	C	HET	HET	T	HET	G
PI208839-1	T	T	C	G	T	T	C	G	T	A	A	A
PI202667-2	HET	T	C	A	G	T	C	HET	T	A	G	A
PI203324-2	T	T	C	A	G	T	C	G	T	A	G	A
PI219773-1	A	A	C	A	G	T	C	G	T	T	A	A
PI203130-5	T	A	C	A	G	T	C	G	T	HET	G	A
PI212115-1	A	T	HET	G	T	T	C	G	A	T	A	A
PI222265-1	A	T	C	A	G	A	NEG	G	T	T	A	G
PI167342-5	HET	HET	HET	G	T	T	C	G	T	A	G	G
PI215774-1	A	T	HET	A	G	T	C	G	HET	T	A	A
PI241370-1	A	T	C	G	G	A	T	G	T	A	HET	A
PI243062-4	A	T	T	A	G	T	C	G	T	A	A	A
PI244573-1	A	T	C	G	G	T	C	G	T	A	G	G
PI167288-5	T	A	C	G	T	HET	C	G	HET	T	G	A
PI241362	HET	T	HET	A	G	T	C	G	T	A	A	A
PI179729	HET	HET	C	G	T	T	C	G	HET	HET	G	A

Table 6.

Diversity of Alleles Within Assays

Ratio of the SNP states present in the Florida and LLNL panels of Castor DNA for each Assay

	252	**299**	**389**	**Cp112**	**Cp19**	**94**
FL Panel	47/51-92.1% Allele T	48/51-94.1% Allele T	51/51-100% Allele C	49/51-96.0% Allele G	48/50-96.0% Allele G	51/51-100% Allele C
	SNP T 47	SNP A 1	SNP T 0	SNP A 2	SNP T 2	SNP T 51
	SNP A 3	SNP T 48	SNP C 51	SNP G 49	SNP G 46	SNP A 0
	SNP HET 1	SNP HET 2	SNP HET 0	SNP HET 0	SNP HET 0	SNP HET 0
LLNL Panel	**19/43-44.1% Allele T**	**25/44-56.8% Allele T**	**38/44-86.4% Allele C**	**31/44-70.4% Allele G**	**25/43-53.4% Allele G**	**37/44-84.0% Allele T**
	SNP T 19	SNP A 9	SNP T 1	SNP A 13	SNP T 18	SNP T 37
	SNP A 11	SNP T 25	SNP C 38	SNP G 31	SNP G 25	SNP A 4
	SNP HET 13	SNP HET 10	SNP HET 5	SNP HET 0	SNP HET 0	SNP HET 3

	381	**355**	**84**	**75**	**11**	**262**
FL Panel	34/51-66.6% Allele T	21/51-58.8% Allele G	47/51-92.1% Allele T	47/51-92.1% Allele A	51/51-100% Allele G	51/51-100% Allele A
	SNP C 13	SNP A 19	SNP T 47	SNP T 1	SNP A 0	SNP G 0
	SNP T 34	SNP G 30	SNP A 4	SNP A 47	SNP G 51	SNP A 49
	SNP HET 4	SNP HET 2	SNP HET 0	SNP HET 2	SNP HET 0	SNP HET 0
LLNL Panel	**39/43-90.6% Allele C**	**34/43-79.0% Allele G**	**26/43-60.0% Allele T**	**22/44-50.0% Allele A**	**27/44-61.3% Allele G**	**35/44-79.5% Allele A**
	SNP C 39	SNP A 6	SNP T 26	SNP T 16	SNP A 9	SNP G 6
	SNP T 4	SNP G 34	SNP A 4	SNP A 22	SNP G 27	SNP A 35
	SNP HET 1	SNP HET 4	SNP HET 13	SNP HET 6	SNP HET 8	SNP HET 3

Measuring Limits of Detection

All of the *R. communis* DNAs were normalized to a concentration 2 ng/uL using Pico Green to determine initial concentrations. The DNAs were diluted 10-fold in seven serial dilutions ranging from 2 ng/uL to 0.000002 ng/uL. The diluted DNAs were amplified with the Castor SNP assays 6 times each. The lowest limit of detection was determined for both allele states for the 12 assays, (Table 7). All genomic assays detected DNA quantities of 0.002 ng and above for both alleles except for SNP Assay 252 for SNP state G and SNP Assay 75 for both SNP states which could not detect less than 0.02 ng of DNA. Both chloroplast SNP assays detected 0.000002 ng or more DNA for both alleles.

Table 7. Limit of detection assays.

	Castor DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP 11 Assay						
SNP A	PI243062-5	2 ng	26.5358558	0.099452877		0.993
		.2 ng	29.9577181	0.124372314	3.4	
		.02 ng	33.697114	0.231801556	3.7	
		.002 ng	36.4159765	0.362540066	2.7	
SNP G	PI167288-5	2 ng	25.5326316	0.121421154		0.996
		.2 ng	29.0170293	0.155910622	3.5	
		.02 ng	32.7067865	0.226513486	3.7	
		.002 ng	36.2509358	0.419190093	3.5	
SNP 75 Assay						
SNP A	PI202667-2	2 ng	25.2358773	0.407951359		0.990
		.2 ng	29.6402258	0.179943915	4.4	
		.02 ng	33.8778548	0.470627261	4.2	
SNP T	PI167288-5	2 ng	26.5268103	0.122744126		0.998
		.2 ng	30.6849331	0.227302622	4.2	
		.02 ng	35.1068418	0.259132683	4.4	
SNP 84 Assay						
SNP T	PI243062-4	2 ng	25.2548133	0.154173441		0.993
		.2 ng	28.736692	0.074348187	3.5	
		.02 ng	32.0355846	0.201886424	3.3	
		.002 ng	35.8228612	0.643633874	3.8	
SNP A	LL-14	2 ng	26.7262161	0.092728288		0.990
		.2 ng	30.172598	0.197213626	3.4	
		.02 ng	33.752818	0.347015406	3.6	
		.002 ng	36.5825184	0.589512028	2.8	
SNP 94 Assay						
SNP T	PI181916	2 ng	25.3757151	0.087710052		0.995
		.2 ng	29.0585578	0.080441417	3.7	
		.02 ng	32.4642045	0.148176688	3.6	
		.002 ng	36.0514065	0.4620265	3.4	
SNP A	PI222265-1	2 ng	25.7707736	0.08136999		0.997
		.2 ng	29.364482	0.173627061	3.6	
		.02 ng	32.9542175	0.221535144	3.6	
		.002 ng	36.81152	0.282702566	3.9	

SNP 252 Assay

SNP A	PI203130	2 ng	24.0369455	0.035479083		0.978
		.2 ng	27.6838401	0.534035136	3.6	
		.02 ng	30.879618	0.233921573	3.2	
		.002 ng	34.165029	0.596773528	3.3	
		.0002 ng	36.199126	0.510204751	2	
SNP G	PI222265-1	2 ng	24.7231191	0.035136178		0.996
		.2 ng	28.2104415	0.039854224	3.5	
		.02 ng	31.5022343	0.298461063	3.3	

SNP 262 Assay

SNP A	PI167288-5	2 ng	25.9728796	1.341876314		0.988
		.2 ng	29.4994643	1.482867376	3.5	
		.02 ng	32.9629825	0.378260201	3.5	
		.002 ng	35.7669025	0.553103171	2.8	
		.0002 ng	36.798793	0.166758274	1	
SNP G	PI244573	2 ng	24.270082	0.128814487	3.5	0.991
		.2 ng	27.7824233	0.162731817	3.4	
		.02 ng	31.2127486	0.166162569	3.3	
		.002 ng	34.5066781	0.704996102	2.5	

SNP 299 Assay

SNP A	PI203130-5	2 ng	24.69536783	0.070542405		0.999
		.2 ng	28.38044067	0.0893144	3.7	
		.02 ng	31.96426417	0.229507698	3.6	
		.002 ng	35.3981868	0.415329007	3.4	
SNP T	PI222265-2	2 ng	23.633579	0.106080739		0.991
		.2 ng	26.892693	0.094452477	3.3	
		.02 ng	30.4780691	0.347239342	3.6	
		.002 ng	33.598779	0.632784568	3.1	

355 Assay

SNP G	PI215774-1	2 ng	23.82761733	0.097085353		0.998
		.2 ng	27.1933545	0.136894579	3.4	
		.02 ng	30.82901267	0.07037265	3.6	
		.002 ng	34.1060701	0.741681229	3.3	
SNP A	PI209326	2 ng	26.15050883	0.179555563		0.989
		.2 ng	29.8153575	0.14366565	3.7	

		.02 ng	33.2836445	0.213160169	3.5	
		.002 ng	36.2163528	0.680754553	2.9	

SNP 381 Assay

SNP T	PI209326	2 ng	25.7557883	0.14153336		0.997
		.2 ng	29.1204025	0.124236645	3.4	
		.02 ng	32.6820565	0.197129437	3.6	
		.002 ng	32.6820565	0.197129437	4.1	
SNP C	PI167342-5	2 ng	26.3297306	0.152380723		0.993
		.2 ng	29.7720963	0.083396146	3.4	
		.02 ng	33.1645256	0.464841851	3.4	
		.002 ng	36.5596353	0.434455633	3.4	

SNP 389 Assay

SNP T	PI243062-4	2 ng	24.3276845	0.075484088		0.987
		.2 ng	28.0628346	0.142495493	3.7	
		.02 ng	31.5131168	0.201303764	3.5	
		.002 ng	34.6127691	0.602777148	3.1	
		.0002 ng	36.8316873	0.202043445	2.2	
SNP C	PI203324-4	2 ng	24.0537497	0.092151971		0.998
		.2 ng	27.5337851	0.113271097	3.5	
		.02 ng	30.93158	0.013548217	3.4	
		.002 ng	34.359728	0.737699673	3.4	
		.0002 ng	36.4736693	0.385274528	2.1	

SNP Cp19 Assay

SNP G	PI203130-5	2 ng	14.7843741	0.051599068		0.998
		.2 ng	18.2115495	0.107376801	3.4	
		.02 ng	21.5789101	0.103113727	3.4	
		.002 ng	24.9451311	0.05866488	3.4	
		.0002 ng	28.6151753	0.366345401	3.7	
		.00002 ng	31.7813168	0.213595407	3.2	
		.000002 ng	35.6182312	0.58235941	3.8	
SNP T	PI212115-1	2 ng	15.1283138	0.114086093		0.997
		.2 ng	18.5789343	0.033431533	3.5	
		.02 ng	21.9869588	0.088470411	3.4	
		.002 ng	25.3224243	0.051014618	3.3	
		.0002 ng	28.7352211	0.145869303	3.4	
		.00002 ng	32.1541018	0.424572859	3.4	
		.000002 ng	34.8984445	0.665950966	2.7	

SNP Cp112 Assay

SNP G	PI170686	2 ng	18.7691168	0.079683678		0.987
		.2 ng	22.0055363	0.079811527	3.2	
		.02 ng	25.4455593	0.082951153	3.4	
		.002 ng	28.7958061	0.113719413	3.4	
		.0002 ng	31.9896395	0.157857477	3.2	
		.00002 ng	35.5102775	0.160697967	3.5	
		.000002 ng	36.5546533	0.464896794	1	
SNP A	PI2430632	2 ng	15.4838765	0.238798541		0.998
		.2 ng	19.0404381	0.196602268	3.6	
		.02 ng	22.4018091	0.084674692	3.4	
		.002 ng	25.7150296	0.090118132	3.3	
		.0002 ng	29.242742	0.177387412	3.5	
		.00002 ng	32.9855806	0.440932631	3.7	
		.000002 ng	36.3139118	0.577623385	3.3	

Table 7. The 12 SNP Assays amplified with 10 fold serial dilutions of DNA for each allele ranging from 2 ng to .000002 ng of DNA. Delta Ct (dCt) calculated by subtracting the average Ct value one dilution from the average Ct value of amplification of the 10fold greater dilution of DNA.
* DNA quantities which amplified an average delta Ct value outside of the 3.2-3.7 range

Linearity

The linearities were determined by using the 10-fold serial dilution assays outlined above to calculate the delta Ct values between 10-fold dilutions. The average Ct value at each dilution point was calculated and subtracted by the average Ct value of the next 10-fold dilution of that DNA for the assays. This value has been denoted the delta Ct. Delta Ct's which fell between the range of 3.2 - 3.7 were considered valid. DNA quantities that gave delta Ct's outside of this range were dismissed as being outside of the range of linearity, (Table 7).

Limits of Quantification

The average Ct values and standard deviations for each DNA quantity were determined using the Ct values of the 6 amplifications at a particular DNA concentration for each assay. Standard deviations greater than 0.65 were considered outside of the limits of quantification. All genomic assays had standard deviations of less than 0.65 for DNA quantities that were within the range of linearity (delta Ct between 3.2 and 3.7). All chloroplast assays also had standard deviations at less than 0.65 for all DNA concentrations tested (Table 7).

The average Ct values were graphed on the Y axis of a scatter plot with the log of the dilution factor graphed on the X axis, (Figure 7). The R^2 value for the resulting line was calculated for each assay. All genomic and chloroplast assays gave R^2 values greater than 0.97.

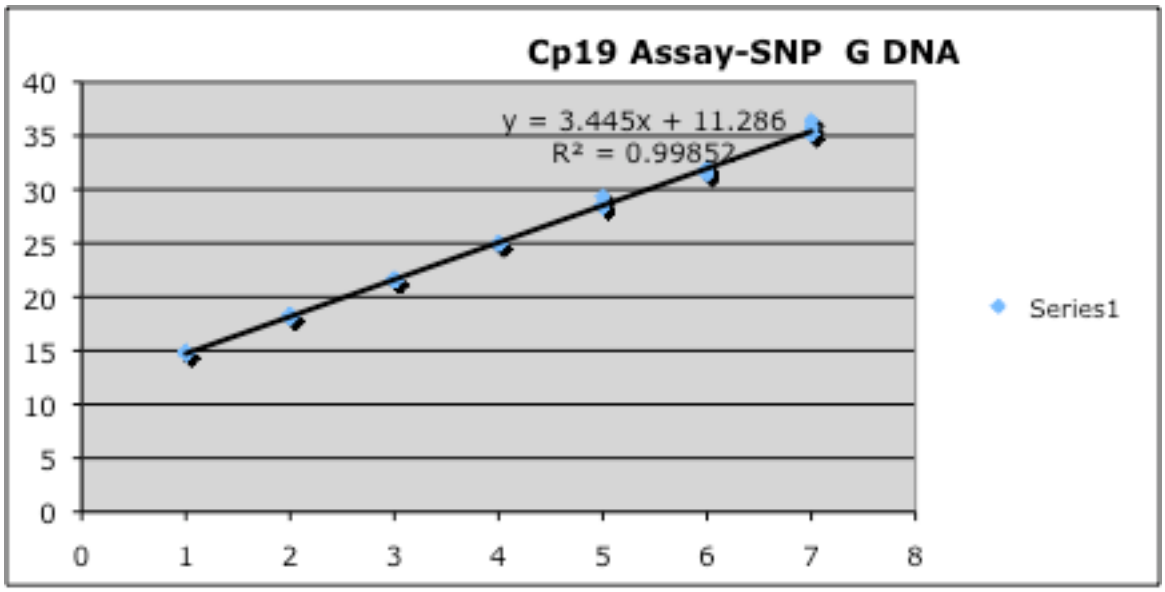


Figure 7. A graph of the Cp19 chloroplast SNP Assay amplification of SNP G DNA 10-fold serial dilution. The Ct value is graphed on the Y-axis and the log of the dilution factor is graphed on the X-axis.

Ruggedness

One representative of the genomic SNP assays (SNP 84) and one representative of the chloroplast SNP assays (Cp19) were amplified using DNAs containing each SNP state. The amplifications reactions were run six times each with 10-fold serial dilutions of the DNA. Platinum Taq has been the polymerase used to develop the Castor SNP assays. Two other polymerases were also tested: Amplitaq Gold and Hotstar Taq. In addition, a different lot of Taqman Genotyping Master Mix was tested as a fourth PCR assay (Table 8). The Platinum Taq polymerase performed better than the Amplitaq Gold or the Hotstar Taq in the genomic SNP assay. The Platinum Taq gave delta Ct's within the 3.2-3.7 range for DNA above .002 ng, while the other 2 polymerases did not give delta Ct's within that range for DNA quantities below 0.2 ng. All of the polymerases gave R^2 values above 0.97. The second lot of Taqman Genomic Master Mix performed as well as the original lot, giving comparable delta Ct's at all DNA quantities and R^2 values above 0.97. The Cp19 chloroplast SNP assay amplified equally well with the Platinum Taq and the Amplitaq Gold and somewhat less well with the Hotstar Taq for the SNP T DNA giving a delta Ct outside of the 3.2-3.7 range for DNA at less than .0002 ng. The second lot of master mix produced greater amplification than the first giving good delta Ct values at every DNA dilution.

Table 8

SNP 84 Assay	Castor DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
Platinum Taq						
SNP T	PI243062	2 ng	25.25481333	0.15417344		0.998
		.2 ng	28.736692	0.07434818	3.5	
		.02 ng	32.03558467	0.20188642	3.3	
		.002 ng	35.8228612	0.64363387	3.8	
SNP A	PI170686	2 ng	26.72621617	0.09272828		0.994
		.2 ng	30.172598	0.19721362	3.4	
		.02 ng	33.752818	0.34701540	3.6	
		.002 ng	36.5825184	0.58951202	2.8	
Amplitaq Gold						
SNP T	PI243062	2 ng	25.69344783	0.16601806		0.995
		.2 ng	29.64966833	0.13779522	3.9	
		.02 ng	33.4773695	0.43627674	3.8	
		.002 ng	36.810172	0.22064711	3.3	
SNP A	PI170686	2 ng	26.63444233	0.17727071		0.989
		.2 ng	30.42707967	0.11553462	3.8	
		.02 ng	34.26335617	0.55946648	3.8	
		.002 ng	36.7643945	0.37788139	2.5	
HotStar Taq						
SNP T	PI243062	2 ng	25.64473083	0.12090270		0.995
		.2 ng	29.251703	0.20054499	3.6	
		.02 ng	33.28607167	0.36259716	4	
		.002 ng	36.66167675	0.31004258	3.5	
SNP A	PI170686	2 ng	26.56616967	0.21558180		0.995
		.2 ng	30.438985	0.18138435	3.9	
		.02 ng	34.24387667	0.32585931	3.8	
		.002 ng	36.84689833	0.41251638	2.6	
Lot 2 Master Mix						
SNP T	PI243062	2 ng	25.75915583	0.44059062		0.989
		.2 ng	29.2197015	0.42997582	3.5	
		.02 ng	32.61835617	0.38021533	3.4	
		.002 ng	36.32353025	0.45351418	3.7	
SNP A	PI170686	2 ng	26.1936855	0.58203648		0.97
		.2 ng	29.59745867	0.75790562	3.4	
		.02 ng	32.93665617	0.77126189	3.3	
		.002 ng	36.34398275	0.59557235	3.4	

Cp19 Assay Platinum Taq	Castor DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	PI203130-5	2 ng	14.78437417	0.05159906		0.999
		.2 ng	18.2115495	0.10737680	3.4	
		.02 ng	21.57891017	0.10311372	3.4	
		.002 ng	24.94513117	0.05866488	3.4	
		.0002 ng	28.61517533	0.36634540	3.7	
		.00002 ng	31.78131683	0.21359540	3.2	
		.000002 ng	35.61823125	0.58235941	3.8	
SNP T	PI212115-1	2 ng	15.1530246	0.10811975		0.998
		.2 ng	18.5872258	0.02968957	3.4	
		.02 ng	22.0042206	0.08688499	3.4	
		.002 ng	25.3369792	0.04079562	3.3	
		.0002 ng	28.7722542	0.12771638	3.4	
		.00002 ng	32.2525052	0.39077291	3.4	
		.000002 ng	35.0719948	0.57311915	2.8	
Amplitaq Gold						
SNP G	PI203130-5	2 ng	14.47613175	0.11197370		0.997
		.2 ng	17.82937633	0.22978717	3.4	
		.02 ng	21.52031483	0.10626803	3.7	
		.002 ng	24.97283517	0.15743056	3.5	
		.0002 ng	28.627775	0.17507568	3.7	
		.00002 ng	31.87570133	0.37036615	3.2	
		.000002 ng	36.040381	0.86854585	4.2	
SNP T	PI212115-1	2 ng	15.19613967	0.08808499		0.996
		.2 ng	18.59599933	0.06927948	3.4	
		.02 ng	22.11014217	0.05619622	3.5	
		.002 ng	25.5781905	0.08661114	3.5	
		.0002 ng	29.18673167	0.20381459	3.6	
		.00002 ng	32.4613515	0.32334786	3.3	
		.000002 ng	35.1884345	0.87769542	2.7	

HotStar Taq

SNP G	PI203130-5	2 ng	14.535367	0.31056814		0.997
		.2 ng	18.181784	0.23655065	3.6	
		.02 ng	21.46082733	0.08505071	3.3	
		.002 ng	24.90165833	0.16406088	3.4	
		.0002 ng	28.6303235	0.17830183	3.7	
		.00002 ng	32.18341933	0.16070133	3.6	
		.000002 ng	34.9723605	0.76472230	2.8	
SNP T	PI212115-1	2 ng	14.52305767	0.09052805		0.996
		.2 ng	18.03191617	0.18664386	3.5	
		.02 ng	21.65697883	0.29098009	3.6	
		.002 ng	25.15726733	0.08203104	3.5	
		.0002 ng	28.890386	0.19248117	3.7	
		.00002 ng	31.8994865	0.40515067	3.0	
		.000002 ng	34.4533594	0.62486028	3.6	

Lot 2 Master Mix

SNP G	PI203130-5	2 ng	14.273202	0.21092923		0.997
		.2 ng	17.67354317	0.11586435	3.4	
		.02 ng	21.19653017	0.12432197	3.5	
		.002 ng	24.529386	0.16295281	3.3	
		.0002 ng	27.94551433	0.19264182	3.4	
		.00002 ng	31.3128985	0.48670709	3.4	
		.000002 ng	34.55333033	0.78931450	3.2	
SNP T	PI212115-1	2 ng	15.20107917	0.04822333		0.996
		.2 ng	18.40433867	0.07148518	3.2	
		.02 ng	21.8211535	0.10306516	3.4	
		.002 ng	25.27707817	0.04686275	3.5	
		.0002 ng	28.687141	0.11195194	3.4	
		.00002 ng	32.00098917	0.42557160	3.3	
		.000002 ng	34.3152838	0.70289869	2.3	

Table 8. SNP Assay 84 and SNP Assay Cp19 amplified with Platinum Taq, Amplitaq Gold, Hotstar Taq, or with a second lot of master mix with DNA representing each allele state.
* DNA quantities that amplified an average delta Ct value outside of the 3.2-3.7 range

Robustness

SNP Assay 299 and chloroplast SNP Assay Cp19 were amplified with the panel of *R. communis* DNAs using 60°C, 65°C, and 55°C annealing temperatures. These assays have been optimized to run with a 60°C annealing temperature. The assays were also run for 50 instead of 40 cycles. Both assays ran equally well using 40 or 50 cycles and called each allele correctly (Table 9). The genomic assay (SNP 299) amplified both alleles for each homozygous DNA when run using a 55°C annealing temperature. The second allele

came up much later than the first and correct allele (Figure 8). When run at a 65°C annealing temperature, SNP 299 incorrectly called several of the heterozygous DNAs as having only allele A. The chloroplast assay gave the same incorrect secondary allele amplification at 55°C as the genomic assay did. At 65°C and at 50 cycles, the allele calls matched the correct calls. At the standard 60°C annealing temperature a small number of DNAs also gave secondary allele amplification that came up much later than the correct allele.

299 Assay	**PCR Annealing Temperature**			
Sample Name	**55°C**	**65°C**	**60°C**	**60°C/50 Cycles**
PI167288-5	A	A	A	A
PI167342-5	HET	HET	HET	HET
PI202667-2	T	T	T	T
PI203130-5	A	A	A	A
PI203324-2	A	A	A	A
PI203324-4	T	T	T	T
PI208839-1	T	T	T	T
PI208839-3	HET	**A**	HET	HET
PI212115-1	T	T	T	T
PI215774-1	T	T	T	T
PI219773-1	A	A	A	A
PI219773-3	HET	HET	HET	HET
PI222265-1	T	T	T	T
PI222265-2	T	T	T	T
PI241370-1	T	T	T	T
PI243062-4	T	T	T	T
PI243062-5	T	T	T	T
PI244573-1	T	T	T	T
PI167238	A	A	A	A
PI170686	T	T	T	T
PI173946	T	T	T	T
PI173948	T	T	T	T
PI173950	HET	**A**	HET	HET
PI179729	HET	**A**	HET	HET
PI181916	T	T	T	T
PI183468	T	T	T	T
PI183470	HET	**A**	HET	HET
PI183471	HET	HET	HET	HET
PI183471	A	A	A	A
PI195811	HET	**A**	HET	HET
PI197048	T	T	T	T
PI203661	T	T	T	T
PI204322	T	T	T	T
PI206515	T	T	T	T
PI207868	HET	**A**	HET	HET
PI208689	A	A	A	A
PI208840	T	T	T	T
PI208842	T	T	T	T

PI209132	T	T	T	T
PI209326	T	T	T	T
PI209622	HET	HET	HET	HET
PI217539	T	T	T	T
PI219767	HET	HET	HET	HET
PI219776	A	A	A	A
PI221698	A	A	A	A
PI240312	A	A	A	A
PI241362	T	T	T	T
PI241368	T	T	T	T
RCFL1.1	T	T	T	T
RCFL1.14	T	T	T	T
RCFL1.19	T	T	T	T
RCFL1.25	T	T	T	T
RCFL1.6	T	T	T	T
RCFL1.8	T	T	T	T
RCFL10.11	T	T	T	T
RCFL10.18	T	T	T	T
RCFL10.19	T	T	T	T
RCFL10.22	T	T	T	T
RCFL10.25	T	T	T	T
RCFL10.3	T	T	T	T
RCFL10.33	T	T	T	T
RCFL11.18	T	T	T	T
RCFL11.4	T	T	T	T
RCFL11.6	T	T	T	T
RCFL12.1	T	T	T	T
RCFL12.14	T	T	T	T
RCFL12.2	T	T	T	T
RCFL12.32	A	A	A	A
RCFL2.11	T	T	T	T
RCFL3.12	T	T	T	T
RCFL3.16	T	T	T	T
RCFL3.19	T	T	T	T
RCFL3.3	T	T	T	T
RCFL3.4	T	T	T	T
RCFL3.5	T	T	T	T
RCFL4.3	T	T	T	T
RCFL4.4	T	T	T	T
RCFL4.8	T	T	T	T
RCFL5.12	T	T	T	T
RCFL5.21	T	T	T	T
RCFL5.3	A	A	A	A
RCFL5.7	T	T	T	T
RCFL6.11	T	T	T	T
RCFL6.13	HET	HET	HET	HET
RCFL6.3	T	T	T	T
RCFL7.7	T	T	T	T
RCFL8.6	T	T	T	T
RCFL8.8	T	T	T	T

RCFL9.1	T	T	T	T
RCFL9.10	T	T	T	T
RCFL9.12	T	T	T	T
RCFL9.15	T	T	T	T
RCFL9.2	T	T	T	T
RCFL9.24	T	T	T	T
RCFL9.8	T	T	T	T
Cp19 Assay Sample Name	**55C Annealing**	**65C Annealing**	**60C Annealing**	**60C Annealing/50 cycles**
PI167288-5	T	T	T	T
PI167342-5	T	T	T	T
PI202667-2	G	G	G	G
PI203130-5	G	G	G	G
PI203324-2	G	G	G	G
PI203324-4	G	G	G	G
PI208839-1	G	G	G	G
PI208839-3	G	G	G	G
PI212115-1	T	T	T	T
PI215774-1	G	G	G	G
PI219773-1	G	G	G	G
PI219773-3	G	G	G	G
PI222265-1	G	G	G	G
PI222265-2	G	G	G	G
PI241370-1	G	G	G	G
PI243062-4	G	G	G	G
PI243062-5	G	G	G	G
PI244573-1	G	G	G	G
PI167238	T	T	T	T
PI170686	T	T	T*	T*
PI173946	T	T	T	T
PI173948	G	G	G	G
PI173950	G	G	G	G
PI179729	T	T	T	T
PI181916	T	T	T*	T*
PI183468	T	T	T	T
PI183470	T	T	T	T
PI183471	T	T	T	T
PI183471	T	T	T	T
PI195811	G	G	G	G
PI197048	G	G	G	G
PI203661	G	G	G	G
PI204322	G	G	G	G

PI206515	G	G	G	G
PI207868	T	T	T	T
PI208689	T	T	T	T
PI208840	G	G	G	G
PI208842	G	G	G	G
PI209132	G	G	G	G
PI209326	G	G	G	G
PI209622	G	G	G	G
PI217539	G	G	G	G
PI219767	T	T	T*	T*
PI219776	T	T	T	T
PI221698	G	G	G	G
PI240312	G	G	G	G
PI241362	G	G	G	G
PI241368	T	T	T	T
RCFL1.1	G	G	G	G
RCFL1.14	G	G	G	G
RCFL1.19	G	G	G	G
RCFL1.25	G	G	G	G
RCFL1.6	G	G	G	G
RCFL1.8	G	G	G	G
RCFL10.11	G	G	G	G
RCFL10.18	G	G	G	G
RCFL10.19	G	G	G	G
RCFL10.22	G	G	G	G
RCFL10.25	G	G	G	G
RCFL10.3	G	G	G	G
RCFL10.33	G	G	G	G
RCFL11.18	G	G	G	G
RCFL11.4	G	G	G	G
RCFL11.6	G	G	G	G
RCFL12.1	G	G	G	G
RCFL12.14	G	G	G	G
RCFL12.2	G	G	G	G
RCFL12.32	G	G	G	G
RCFL2.11	G	G	G	G
RCFL3.12	G	G	G	G
RCFL3.16	G	G	G	G
RCFL3.19	G	G	G	G
RCFL3.3	G	G	G	G
RCFL3.4	G	G	G	G
RCFL3.5	G	G	G	G

RCFL4.3	G	G	G	G
RCFL4.4	G	G	G	G
RCFL4.8	G	G	G	G
RCFL5.12	G	G	G	G
RCFL5.21	G	G	G	G
RCFL5.3	G	G	G	G
RCFL5.7	G	G	G	G
RCFL6.11	T	T	T*	T*
RCFL6.13	G	G	G	G
RCFL6.3	T	T	T	T
RCFL7.7	G	G	G	G
RCFL8.6	G	G	G	G
RCFL8.8	G	G	G	G
RCFL9.1	G	G	G	G
RCFL9.10	G	G	G	G
RCFL9.12	G	G	G	G
RCFL9.15	G	G	G	G
RCFL9.2	G	G	G	G
RCFL9.24	G	G	G	G
RCFL9.8	G	G	G	G

Table 9. Alleles that are amplified when Castor SNP Assays 299 and Cp19 are amplified using 55°C, 60°C and 65°C annealing temperatures.

Heterozygous DNAs called as SNP A when amplified at 55°C annealing a temperature.

* In addition to the listed SNP state, the alternate SNP also amplifies ,but with a later Ct value.

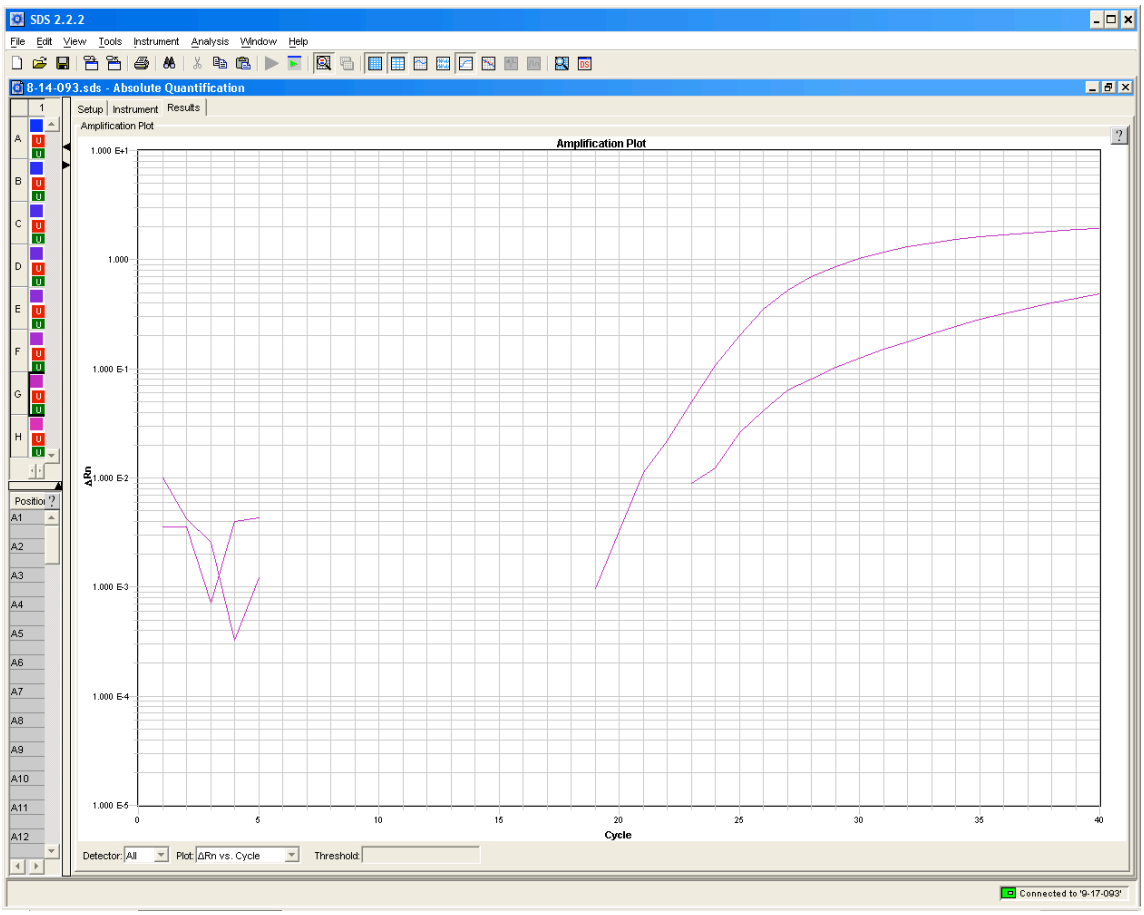


Figure 8. SNP Assay 299 amplified both alleles at 55°C annealing temperature for homozygous DNA. The second allele amplified at a delta Ct value of roughly 3 compared to the first allele. This second allele did not amplify at a 60°C or 65°C annealing temperature.

Precision

The 12 *R. communis* SNP assays were tested against a panel of 22 *R. communis* DNAs including a negative no-template control. The 22 DNAs contained representatives of each allele state for the 12 assays. The panel was blinded and each real time assay was run ten times. The resulting allele states were compared against the known genotypes. All assays amplified the correct allele state for each previously tested *R. communis* DNA (Table 10).

DNA	11 Assay		75 Assay		84 Assay		94 Assay	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
PI 202667-2	G	G	A	A	T	T	T	T
PI 203324-4	G	G	A	A	T	T	T	T
PI 167342-5	G	G	A	A	T	T	T	T
PI 219773-1	A	A	T	T	T	T	T	T
PI181916	A	A	T	T	A	A	A	A
PI 212115-1	A	A	T	T	A	A	T	T
PI 243062-5	A	No Data	A	No Data	A	No Data	T	No Data
Neg. Control	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
PI 240312	HET	HET	A	A	T	T	T	T
PI 244573-1	G	G	A	A	T	T	T	T
PI 203324-2	G	G	A	A	T	T	T	T
PI 209326	G	G	A	A	T	T	T	T
PI 222265-1	A	A	T	T	T	T	A	A
PI 241370-1	HET	HET	A	A	T	T	A	A
PI 167288-5	G	G	T	T	HET	HET	HET	HET
PI 208839-3	G	G	T	T	T	T	A	A
PI 208839-1	A	A	A	A	T	T	T	T
PI 243062-4	A	A	A	A	T	T	T	T
PI 222265-2	G	No Data	T	No Data	T		A	No Data
PI170686	G	G	T	T	A	A	T	T
PI217539	G	G	A	A	T	T	T	T
PI 215774-1	A	A	T	T	HET	HET	T	T
PI 219773-3	HET	HET	HET	HET	T	T	T	T

DNA	252 Assay		262 Assay		299 Assay		355 Assay	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
PI 202667-2	HET	HET	A	A	T	T	HET	HET
PI 203324-4	T		A	A	T	T	HET	HET
PI 167342-5	HET	HET	G	G	HET	HET	G	G
PI 219773-1	A	A	A	A	A	A	G	G
PI181916	A	A	G	G	T	T	G	G
PI 212115-1	A	A	A	A	T		G	G
PI 243062-5	A	No Data	A	No Data	T	T	G	No Data
Neg. Control	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
PI 240312	T	T	A	A	A	A	A	A
PI 244573-1	A	A	G	G	T	T	G	G
PI 203324-2	T	T	A	A	A	T	G	G
PI 209326	T	T	A	A	T	T	A	A
PI 222265-1	A	A	G	G	T	T	G	G
PI 241370-1	A	A	A	A	T	T	G	G
PI 167288-5	T	T	A	A	A	A	G	G
PI 208839-3	T	No Data	G	G	HET	No Data	HET	HET
PI 208839-1	T	T	A	A	T	T	G	G

PI 243062-4	A	A	A	A	T	T	G	G
PI 222265-2	A	No Data	G	No Data	T	T	G	No Data
PI170686	A	A	A	A	T	T	G	G
PI217539	T	T	A	A	T	T	NEG	NEG
PI 215774-1	A	A	A	A	T	T	G	G
PI 219773-3	A	No Data	A	A	HET	No Data	G	G
DNA	**381 Assay**		**389 Assay**		**Cp19 Assay**		**Cp112 assay**	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
PI 202667-2	C	C	C	C	G	G	A	A
PI 203324-4	HET	HET	C		G	No Data	A	
PI 167342-5	C	C	HET	HET	T	T	G	G
PI 219773-1	C	C	C	C	G	G	A	A
PI181916	C	C	C	C	T	T	G	G
PI 212115-1	C	C	HET	HET	T	T	G	G
PI 243062-5	C	No Data	T	No Data	G	No Data	A	No Data
Neg. Control	NEG	NEG	NEG	NEG	NEG	No Data	NEG	NEG
PI 240312	T	T	C	C	G	No Data	G	G
PI 244573-1	C	C	C	C	G	G	G	G
PI 203324-2	C	C	C	C	G	Not Tested	A	A
PI 209326	T	T	C	C	G	G	G	G
PI 222265-1	NEG	NEG	C	C	G	G	A	A
PI 241370-1	T	T	C	C	G	G	G	G
PI 167288-5	C	C	C	C	T	T	G	G
PI 208839-3	HET	HET	C	No Data	G	No Data	G	No Data
PI 208839-1	C	C	C	C	G	T	G	G
PI 243062-4	C	C	T	T	G	G	A	A
PI 222265-2	C	No Data	C	No Data	G	No Data	A	No Data
PI170686	C	C	C	C	T	T	G	G
PI217539	C	C	C	C	G	G	A	A
PI 215774-1	C	C	HET	HET	G	G	A	A
PI 219773-3	C	C	C	No Data	G	No Data	A	No Data

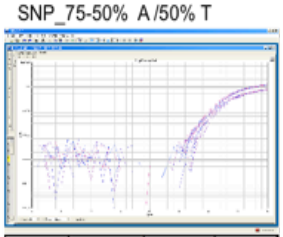
Table 10. 12 SNP assays amplified blindly and compared to actual allele states.

Selectivity

DNAs representing each allele state were normalized to equivalent concentrations using an assay for which each DNA contained the same allele. The equivalent DNA dilutions were mixed together in the following ratios of DNAs: 100/0, 99/1, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 5/95, 1/99, and 0/100. Each mixture was then amplified 8 times with the SNP assay that contained different alleles for each DNA. The delta Ct's were calculated by subtracting the average Ct of 1 SNP amplification from the average Ct of the second SNP amplification in each reaction for each mixture ratio. Mixtures that contained 100% of 1 DNA and 0% of the second DNA

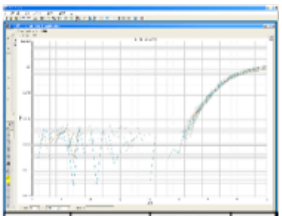
amplified only the allele corresponding to the first DNA. Mixtures that contained 50% of each DNA amplified each allele at fairly equal Ct values. Mixtures that contained 90% of 1 DNA and 10% of the second DNA gave a higher delta Ct value than DNAs that contained only 80% of the first DNA and 20% of the second DNA. This trend of delta Ct's increasing with the amount of the first DNA was present in all assays through the 90/10 and 10/90 mixtures, (Figure 9). The genomic assays did not amplify mixtures of 95/5 or 99/1 efficiently. Either the delta Ct's did not increase at these mixture ratios or the second allele did not amplify at all when the DNA amount was less than 10% of the total DNA, (Table 11). The trend of delta Ct's increasing and decreasing according to DNA mixture ratios held up on all genomic assays through the 90/10 and 10/90 ratios. The chloroplast assays were able to amplify both alleles for all mixture ratios. The delta Ct trend also continued through the 1/99 and 99/1 mixture ratio with both of the chloroplast assays.

SNP_75-50% A /50% T



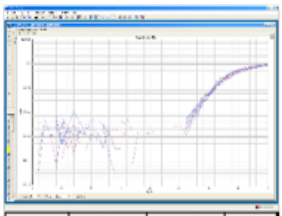
	AVG	ST DEV	dCt
SNP T	28.964	0.2242	0.83
SNP A	29.799	0.3405	

SNP_75-60% A/ 40% T



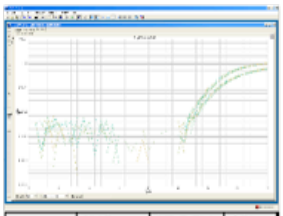
	AVG	ST DEV	dCt
SNP T	29.222	0.3036	0.2
SNP A	29.43	0.2241	

SNP_75-70% A/30% T



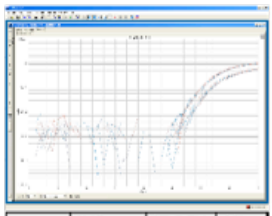
	AVG	ST DEV	dCt
SNP T	29.674	0.279	0.31
SNP A	29.357	0.2382	

SNP_75-80% A/20% T



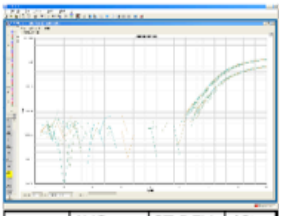
	AVG	ST DEV	dCt
SNP T	30.248	0.2346	1.09
SNP A	29.166	0.2215	

SNP_75-90%A/10%T



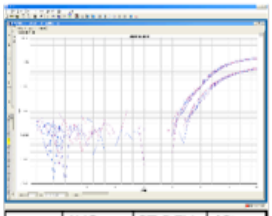
	AVG	ST DEV	dCt
SNP T	30.237	0.2594	1.41
SNP A	28.833	0.2039	

SNP_75-40% A/60% T



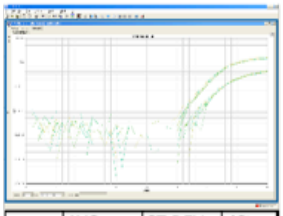
	AVG	ST DEV	dCt
SNP T	28.703	0.1954	1.3
SNP A	30.008	0.2521	

SNP_75-30%A/70%T



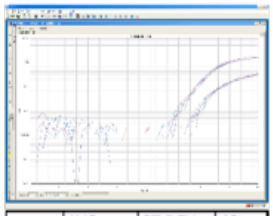
	AVG	ST DEV	dCt
SNP T	28.475	0.2644	1.95
SNP A	30.429	0.2755	

SNP_75-20%A/80%T



	AVG	ST DEV	dCt
SNP T	28.222	0.2199	2.69
SNP A	30.909	0.1877	

SNP_75-10% A/90% T



	AVG	ST DEV	dCt
SNP T	27.92	0.2243	3.51
SNP A	31.428	0.21152	

Figure 9. SNP assay 75 amplified with DNAs representing both alleles at mixture ratios of 100/0, 99/1.95/5,90/10, 80/20,70/30, 60/40, 50/50,40/60, 30/70, 20/80, 10/90, 5/95,1/99,and 0/100. All PCRs repeated 8 times, dCt calculated by subtracting the average Ct of one SNP DNA from the average Ct of the second SNP DNA.

11 Assay	SNP	AVERAGE Ct	Standard Deviation	dCt
0% SNP A/ 100% SNP G	SNP A			
0% SNP A/ 100% SNP G	SNP G	27.26971938	0.135468456	
1% SNP A/ 99% SNP G	SNP A			
1% SNP A/ 99% SNP G	SNP G	25.1051675	0.093351796	
5% SNP A/ 95% SNP G	SNP A			
5% SNP A/ 95% SNP G	SNP G	25.19415738	0.076450734	
10% SNP A/ 90% SNP G	SNP A	29.46959475	0.121838503	-2.01
10% SNP A/ 90% SNP G	SNP G	27.46115413	0.206002703	
20% SNP A/ 80% SNP G	SNP A	28.94231325	0.112823232	-1.33
20% SNP A/ 80% SNP G	SNP G	27.61368263	0.157891341	
30% SNP A/ 70% SNP G	SNP A	28.63667963	0.12999984	-0.72
30% SNP A/ 70% SNP G	SNP G	27.91431863	0.191793613	
40% SNP A/ 60% SNP G	SNP A	28.20224125	0.121081123	-0.32
40% SNP A/ 60% SNP G	SNP G	27.88251163	0.123442749	
50% SNP A/ 50% SNP G	SNP A	27.99388288	0.257700359	0.13
50% SNP A/ 50% SNP G	SNP G	28.12498813	0.243440002	
60% SNP A/ 40% SNP G	SNP A	27.88444025	0.101295415	0.53
60% SNP A/ 40% SNP G	SNP G	28.41940688	0.122345188	
70% SNP A/ 30% SNP G	SNP A	27.14395171	1.351499524	1.68
70% SNP A/ 30% SNP G	SNP G	28.82182583	0.078069597	
80% SNP A/ 20% SNP G	SNP A	27.46124963	0.234029706	1.8
80% SNP A/ 20% SNP G	SNP G	29.24041813	0.202659493	
90% SNP A/ 10% SNP G	SNP A	27.35421063	0.230536203	2.74
90% SNP A/ 10% SNP G	SNP G	30.0920386	0.193342239	
95% SNP A/ 5% SNP G	SNP A	25.24182788	0.120489731	
95% SNP A/ 5% SNP G	SNP G			
99% SNP A/ 1% SNP G	SNP A	25.14442788	0.095813258	
99% SNP A/ 1% SNP G	SNP G			
100% SNP A/ 0% SNP G	SNP A	27.1625515	0.266525473	
100% SNP A/ 0% SNP G	SNP G			

75 Assay				
0% SNP A/ 100% SNP T	SNP T	27.7930815	0.158358081	
0% SNP A/ 100% SNP T	SNP A			
1% SNP A/ 99% SNP T	SNP T	24.86592338	0.242484769	
1% SNP A/ 99% SNP T	SNP A			
5% SNP A/ 95% SNP T	SNP T	24.7480615	0.19086281	
5% SNP A/ 95% SNP T	SNP A			
10% SNP A/ 90% SNP T	SNP T	27.91966	0.224298177	3.51
10% SNP A/ 90% SNP T	SNP A	31.42778288	0.211517047	
20% SNP A/ 80% SNP T	SNP T	28.221832	0.219917473	2.69
20% SNP A/ 80% SNP T	SNP A	30.9091715	0.1877236	
30% SNP A/ 70% SNP T	SNP T	28.47515438	0.264358606	1.95
30% SNP A/ 70% SNP T	SNP A	30.429483	0.27549119	
40% SNP A/ 60% SNP T	SNP T	28.70342575	0.195423906	1.3
40% SNP A/ 60% SNP T	SNP A	30.00753988	0.252102417	
50% SNP A/ 50% SNP T	SNP T	28.964463	0.224175538	0.83
50% SNP A/ 50% SNP T	SNP A	29.7985965	0.340547946	

60% SNP A/ 40% SNP T	SNP T	29.22230188	0.303618492	0.21
60% SNP A/ 40% SNP T	SNP A	29.42994038	0.224131639	
70% SNP A/ 30% SNP T	SNP T	29.674478	0.279038583	-0.32
70% SNP A/ 30% SNP T	SNP A	29.35698538	0.238160014	
80% SNP A/ 20% SNP T	SNP T	30.247934	0.234573747	-1.09
80% SNP A/ 20% SNP T	SNP A	29.16589938	0.221483779	
90% SNP A/ 10% SNP T	SNP T	30.23730275	0.259374452	-1.41
90% SNP A/ 10% SNP T	SNP A	28.83330575	0.203949308	
95% SNP A/ 5% SNP T	SNP T			
95% SNP A/ 5% SNP T	SNP A	25.55097575	0.288067147	
99% SNP A/ 1% SNP T	SNP T			
99% SNP A/ 1% SNP T	SNP A	25.51968988	0.171510526	
100% SNP A/ 0% SNP T	SNP T			
100% SNP A/ 0% SNP T	SNP A	28.70394025	0.14301594	

84 Assay				
0% SNP T/100% SNP A	SNP A	28.05652413	0.092079686	
0% SNP T/100% SNP A	SNP T			
1% SNP T/ 99% SNP A	SNP A	25.18313213	0.088316923	
1% SNP T/ 99% SNP A	SNP T			
5% SNP T/ 95% SNP A	SNP A	25.49487375	0.107244167	
5% SNP T/ 95% SNP A	SNP T			
10% SNP T/ 90% SNP A	SNP A	28.19976063	0.1196596	3.03
10% SNP T/ 90% SNP A	SNP T	31.23069763	0.437488664	
20% SNP T/ 80% SNP A	SNP A	28.32056875	0.124054665	1.65
20% SNP T/ 80% SNP A	SNP T	29.974066	0.419707351	
30% SNP T/ 70% SNP A	SNP A	28.36164013	0.095391727	1.01
30% SNP T/ 70% SNP A	SNP T	29.37545888	0.178051157	
40% SNP T/ 60% SNP A	SNP A	28.635769	0.137156382	0.64
40% SNP T/ 60% SNP A	SNP T	29.27556513	0.242997701	
50% SNP T/50% SNP A	SNP A	28.72086675	0.207802603	0.16
50% SNP T/50% SNP A	SNP T	28.880559	0.225485664	
60% SNP T/40% SNP A	SNP A	28.9522875	0.088168825	-0.25
60% SNP T/40% SNP A	SNP T	28.70620013	0.181521499	
70% SNP T/30% SNP A	SNP A	29.13816913	0.221469734	-0.75
70% SNP T/30% SNP A	SNP T	28.38389825	0.240346601	
80% SNP T/20 % SNP A	SNP A	29.527668	0.204054316	-1.31
80% SNP T/20 % SNP A	SNP T	28.216837	0.25440604	
90% SNP T/10% SNP A	SNP A	29.98178425	0.300870063	-1.87
90% SNP T/10% SNP A	SNP T	28.11194375	0.100267152	
95% SNP T/ 5% SNP A	SNP A			
95% SNP T/ 5% SNP A	SNP T	25.15227625	0.102859336	
99% SNP T/ 1% SNP A	SNP A			
99% SNP T/ 1% SNP A	SNP T	25.00736425	0.130419237	
100% SNP T/ 0% SNP A	SNP A			
100% SNP T/ 0% SNP A	SNP T	28.00957225	0.211652169	

94 Assay			
0% SNP T/100% SNP A	SNP A	27.632176	0.128422622

0% SNP T/100% SNP A	SNP T			
1% SNP T/ 99% SNP A	SNP A	25.71341113	0.078017469	
1% SNP T/ 99% SNP A	SNP T			
5% SNP T/ 95% SNP A	SNP A	25.756776	0.11586865	
5% SNP T/ 95% SNP A	SNP T			
10% SNP T/ 90% SNP A	SNP A	27.6527245	0.148562018	5.18
10% SNP T/ 90% SNP A	SNP T	32.82834275	3.111121	
20% SNP T/ 80% SNP A	SNP A	27.85053671	0.161111224	2.38
20% SNP T/ 80% SNP A	SNP T	30.23443143	0.18807308	
30% SNP T/ 70% SNP A	SNP A	27.96255063	0.132625099	1.64
30% SNP T/ 70% SNP A	SNP T	29.5999095	0.192553141	
40% SNP T/ 60% SNP A	SNP A	28.217158	0.139995742	1.16
40% SNP T/ 60% SNP A	SNP T	29.37744363	0.265614673	
50% SNP T/50% SNP A	SNP A	28.3775515	0.169365371	0.61
50% SNP T/50% SNP A	SNP T	28.99031013	0.19364756	
60% SNP T/40% SNP A	SNP A	28.5612235	0.17966488	0.14
60% SNP T/40% SNP A	SNP T	28.70272125	0.214144729	
70% SNP T/30% SNP A	SNP A	28.82509138	0.107277044	-0.38
70% SNP T/30% SNP A	SNP T	28.43985438	0.239109063	
80% SNP T/20 % SNP A	SNP A	29.2855865	0.176852971	-0.84
80% SNP T/20 % SNP A	SNP T	28.43808363	0.163554916	
90% SNP T/10% SNP A	SNP A	29.811423	0.16735368	-1.53
90% SNP T/10% SNP A	SNP T	28.27878825	0.106426698	
95% SNP T/ 5% SNP A	SNP A			
95% SNP T/ 5% SNP A	SNP T	26.21462188	0.124365761	
99% SNP T/ 1% SNP A	SNP A			
99% SNP T/ 1% SNP A	SNP T	25.98782238	0.161909166	
100% SNP T/ 0% SNP A	SNP A			
100% SNP T/ 0% SNP A	SNP T	28.037042	0.159744198	

252 Assay				
0% SNP T/100% SNP A	SNP A	24.22733113	0.066477769	
0% SNP T/100% SNP A	SNP T			
1% SNP T/99% SNP A	SNP A	21.801284	0.079915752	3.64
1% SNP T/99% SNP A	SNP T	25.44845471	1.128100026	
5% SNP T/95% SNP A	SNP A	21.84304514	0.118683949	2.89
5% SNP T/95% SNP A	SNP T	24.729699	0.545663608	
10% SNP T/ 90% SNP A	SNP A	24.40007363	0.101748608	1.98
10% SNP T/ 90% SNP A	SNP T	26.37818388	0.472724947	
20% SNP T/ 80% SNP A	SNP A	24.80224063	0.13119519	0.99
20% SNP T/ 80% SNP A	SNP T	25.7905805	0.254673696	
30% SNP T/ 70% SNP A	SNP A	24.749056	0.122121526	0.71
30% SNP T/ 70% SNP A	SNP T	25.4593645	0.228714243	
40% SNP T/ 60% SNP A	SNP A	24.84557263	0.105312038	0.2
40% SNP T/ 60% SNP A	SNP T	25.05009338	0.375672843	
50% SNP T/50% SNP A	SNP A	25.060446	0.173347277	0.11
50% SNP T/50% SNP A	SNP T	24.94673213	0.199062777	
60% SNP T/40% SNP A	SNP A	25.46895588	0.174913848	-0.82
60% SNP T/40% SNP A	SNP T	24.6516025	0.157093011	

70% SNP T/30% SNP A	SNP A	25.86095388	0.06919796	-1.29
70% SNP T/30% SNP A	SNP T	24.56303288	0.191910079	
80% SNP T/20 % SNP A	SNP A	26.45930638	0.178864183	-2.1
80% SNP T/20 % SNP A	SNP T	24.358597	0.238092601	
90% SNP T/10% SNP A	SNP A	27.83715475	0.299259599	-3.48
90% SNP T/10% SNP A	SNP T	24.35329338	0.173782956	
95% SNP T/5% SNP A	SNP A	24.425898	0.70658948	-1.15
95% SNP T/5% SNP A	SNP T	23.27008829	0.050066824	
99% SNP T/1% SNP A	SNP A	25.80407329	1.050309481	-2.48
99% SNP T/1% SNP A	SNP T	23.31961357	0.07777564	
100% SNP T/ 0% SNP A	SNP A			
100% SNP T/ 0% SNP A	SNP T	24.16980413	0.344689404	

262 Assay				
0% SNP A/ 100% SNP G	SNP A			
0% SNP A/ 100% SNP G	SNP G	25.60826325	0.203853092	
1% SNP A/ 99% SNP G	SNP A	29.69058675	0.981575279	-5
1% SNP A/ 99% SNP G	SNP G	24.68765163	0.175104751	
5% SNP A/ 95% SNP G	SNP A	26.75736913	0.575548345	-2.08
5% SNP A/ 95% SNP G	SNP G	24.67118313	0.175829571	
10% SNP A/ 90% SNP G	SNP A	29.69129225	0.64216864	-3.96
10% SNP A/ 90% SNP G	SNP G	25.72562875	0.123032743	
20% SNP A/ 80% SNP G	SNP A	28.33218188	0.481282149	-2.44
20% SNP A/ 80% SNP G	SNP G	25.84410975	0.137411623	
30% SNP A/ 70% SNP G	SNP A	27.69202438	0.46147221	-1.57
30% SNP A/ 70% SNP G	SNP G	29.122664	0.438423363	
40% SNP A/ 60% SNP G	SNP G	26.11773625	0.159845106	-1.16
40% SNP A/ 60% SNP G	SNP A	27.22087663	0.349193105	
50% SNP A/ 50% SNP G	SNP G	26.05671475	0.102957915	-0.22
50% SNP A/ 50% SNP G	SNP A	26.61742	0.310451758	
60% SNP A/ 40% SNP G	SNP G	26.40194338	0.141938746	0.49
60% SNP A/ 40% SNP G	SNP A	26.51422363	0.330539304	
70% SNP A/ 30% SNP G	SNP G	27.00592838	0.15801515	0.99
70% SNP A/ 30% SNP G	SNP A	26.31669888	0.209293664	
80% SNP A/ 20% SNP G	SNP G	27.3116965	0.249258671	1.67
80% SNP A/ 20% SNP G	SNP A	26.1461855	0.197710657	
90% SNP A/ 10% SNP G	SNP G	27.81683338	0.420115152	3.16
90% SNP A/ 10% SNP G	SNP A	25.95733488	0.17285968	
95% SNP A/ 5% SNP G	SNP A	24.14244725	0.210713488	2.34
95% SNP A/ 5% SNP G	SNP G	26.4792345	0.607892994	
99% SNP A/ 1% SNP G	SNP A	25.36410675	0.380396546	7.48
99% SNP A/ 1% SNP G	SNP G	32.8393255	2.076287838	
100% SNP A/ 0% SNP G	SNP A	25.83455988	0.217830088	
100% SNP A/ 0% SNP G	SNP G			

299 Assay				
0% SNP T/100% SNP A	SNP T			
0% SNP T/100% SNP A	SNP A	28.14464475	0.160931839	
1% SNP T/99% SNP A	SNP T			

1% SNP T/99% SNP A	SNP A	23.87441143	0.125620873	
5% SNP T/95% SNP A	SNP T	27.08341567	0.135577863	-3.2
5% SNP T/95% SNP A	SNP A	23.87715729	0.074404483	
10% SNP T/ 90% SNP A	SNP T	30.2957175	0.152692619	-2
10% SNP T/ 90% SNP A	SNP A	28.29880888	0.136967999	
20% SNP T/ 80% SNP A	SNP T	29.56270675	0.132523437	-1.22
20% SNP T/ 80% SNP A	SNP A	28.34683475	0.06975447	
30% SNP T/ 70% SNP A	SNP T	29.20797188	0.118132801	-0.65
30% SNP T/ 70% SNP A	SNP A	28.55389975	0.070789302	
40% SNP T/ 60% SNP A	SNP T	29.04146075	0.105235387	-0.26
40% SNP T/ 60% SNP A	SNP A	28.7783515	0.203867579	
50% SNP T/50% SNP A	SNP T	28.62338413	0.180191596	0.33
50% SNP T/50% SNP A	SNP A	28.95534838	0.148857199	
60% SNP T/40% SNP A	SNP T	28.416405	0.141222348	0.66
60% SNP T/40% SNP A	SNP A	29.07172525	0.115421595	
70% SNP T/30% SNP A	SNP T	28.23669025	0.140901934	1.25
70% SNP T/30% SNP A	SNP A	29.49022463	0.183603999	
80% SNP T/20 % SNP A	SNP T	28.10664013	0.17223651	1.65
80% SNP T/20 % SNP A	SNP A	29.75770213	0.171774893	
90% SNP T/10% SNP A	SNP T	28.01024038	0.168669048	2.66
90% SNP T/10% SNP A	SNP A	30.6745545	0.221530887	
95% SNP T/5% SNP A	SNP T	23.63608914	0.038239791	3.4
95% SNP T/5% SNP A	SNP A	27.03925243	0.158619411	
99% SNP T/1% SNP A	SNP T	23.63710771	0.048346398	4.81
99% SNP T/1% SNP A	SNP A	28.4426976	0.953909438	
100% SNP T/ 0% SNP A	SNP T	27.78155163	0.086942913	

355 Assay				
0% SNP G/100% SNP A	SNP G			
0% SNP G/100% SNP A	SNP A	27.425995	0.321785592	
1% SNP G/99% SNP A	SNP G			
1% SNP G/99% SNP A	SNP A	23.62703738	0.153257581	
5% SNP G/95% SNP A	SNP G			
5% SNP G/95% SNP A	SNP A	23.61940275	0.111038286	
10% SNP G/90% SNP A	SNP G	29.9474705	0.951030365	-2.14
10% SNP G/90% SNP A	SNP A	27.8008725	0.754837577	
20% SNP G/80% SNP A	SNP G	29.02267763	0.339195947	-1.39
20% SNP G/80% SNP A	SNP A	27.63722488	0.284641421	
30% SNP G/70% SNP A	SNP G	28.53619413	0.374142116	-0.67
30% SNP G/70% SNP A	SNP A	27.86935713	0.230597481	
40% SNP G/60% SNP A	SNP G	28.25751975	0.306742158	-0.16
40% SNP G/60% SNP A	SNP A	28.0999505	0.23172536	
50% SNP G/50% SNP A	SNP G	27.9837485	0.34674843	0.26
50% SNP G/50% SNP A	SNP A	28.24572925	0.218545845	
60% SNP G/40% SNP A	SNP G	27.83630413	0.185758299	0.72
60% SNP G/40% SNP A	SNP A	28.554751	0.226926156	
70% SNP G/30% SNP A	SNP G	27.7868695	0.44574886	1.12
70% SNP G/30% SNP A	SNP A	28.91126588	0.238243088	
80% SNP G/20% SNP A	SNP G	27.85780663	0.880705219	1.98

80% SNP G/20% SNP A	SNP A	29.83508363	0.886607913	
90% SNP G/10% SNP A	SNP G	27.760515	0.763118817	3.26
90% SNP G/10% SNP A	SNP A	31.02462888	0.855081221	
95% SNP G/5% SNP A	SNP G	23.68937738	0.116119159	
95% SNP G/5% SNP A	SNP A			
99% SNP G/1% SNP A	SNP G	23.60243275	0.152553579	
99% SNP G/1% SNP A	SNP A			
100% SNP G/0% SNP A	SNP G	27.218454	0.189697791	
100% SNP G/0% SNP A	SNP A			

381 Assay				
0% SNP C/ 100% SNP T	SNP T	29.27294888	0.138205148	
0% SNP C/ 100% SNP T	SNP C			
1% SNP C/ 99% SNP T	SNP T	25.01839775	0.174536186	
1% SNP C/ 99% SNP T	SNP C			
5% SNP C/ 95% SNP T	SNP T	25.0235355	0.239655297	
5% SNP C/ 95% SNP T	SNP C			
10% SNP C/ 90% SNP T	SNP T	29.24212425	0.184879527	6.58
10% SNP C/ 90% SNP T	SNP C	35.82638371	2.968930247	
20% SNP C/ 80% SNP T	SNP T	28.99973029	0.142231763	2.21
20% SNP C/ 80% SNP T	SNP C	31.21277629	2.955200401	
30% SNP C/ 70% SNP T	SNP T	29.58340938	0.252423509	0.79
30% SNP C/ 70% SNP T	SNP C	30.38092375	0.229635253	
40% SNP C/ 60% SNP T	SNP T	29.65336763	0.181953825	0.12
40% SNP C/ 60% SNP T	SNP C	29.77662938	0.224433279	
50% SNP C/ 50% SNP T	SNP T	29.79302613	0.143414134	-0.37
50% SNP C/ 50% SNP T	SNP C	29.4266095	0.229926428	
60% SNP C/ 40% SNP T	SNP T	29.99204875	0.189747007	-0.66
60% SNP C/ 40% SNP T	SNP C	29.32946063	0.105819242	
70% SNP C/ 30% SNP T	SNP T	30.35909575	0.146193077	-1.19
70% SNP C/ 30% SNP T	SNP C	29.16722225	0.142125277	
80% SNP C/ 20% SNP T	SNP T	30.60828314	0.227937321	-1.85
80% SNP C/ 20% SNP T	SNP C	28.7596345	0.11459303	
90% SNP C/ 10% SNP T	SNP T			
90% SNP C/ 10% SNP T	SNP C	28.78485438	0.151450575	
95% SNP C/ 5% SNP T	SNP T			
95% SNP C/ 5% SNP T	SNP C	25.22549888	0.148228544	
99% SNP C/ 1% SNP T	SNP T			
99% SNP C/ 1% SNP T	SNP C	25.15818388	0.182864735	
100% SNP C/ 0% SNP T	SNP T			
100% SNP C/ 0% SNP T	SNP C	28.55537675	0.208544823	

389 Assay				
0% SNP C/ 100% SNP T	SNP C			
0% SNP C/ 100% SNP T	SNP T	25.4199555	0.250122643	
1% SNP C/ 99% SNP T	SNP C	25.99989063	0.884405478	-1.81
1% SNP C/ 99% SNP T	SNP T	24.18657125	0.085557027	
5% SNP C/ 95% SNP T	SNP C	24.70710188	0.341519459	-0.59
5% SNP C/ 95% SNP T	SNP T	24.11315138	0.072651947	

10% SNP C/ 90% SNP T	SNP C	27.6779555	0.60928184	-1.87
10% SNP C/ 90% SNP T	SNP T	25.8062205	0.796032311	
20% SNP C/ 80% SNP T	SNP C	26.73494563	0.234375868	-1.06
20% SNP C/ 80% SNP T	SNP T	25.66673738	0.27234728	
30% SNP C/ 70% SNP T	SNP C	26.28048588	0.288033687	-0.47
30% SNP C/ 70% SNP T	SNP T	25.811475	0.127210626	
40% SNP C/ 60% SNP T	SNP C	26.18571338	0.276979549	-0.18
40% SNP C/ 60% SNP T	SNP T	26.00093188	0.276442883	
50% SNP C/ 50% SNP T	SNP C	25.47316075	0.211780863	0.73
50% SNP C/ 50% SNP T	SNP T	26.20396175	0.10790637	
60% SNP C/ 40% SNP T	SNP C	25.28015738	0.224250929	0.106
60% SNP C/ 40% SNP T	SNP T	26.3408125	0.204086535	
70% SNP C/ 30% SNP T	SNP C	24.96314088	0.199235371	1.59
70% SNP C/ 30% SNP T	SNP T	26.55599713	0.075462586	
80% SNP C/ 20% SNP T	SNP C	25.12738163	1.035308942	2.02
80% SNP C/ 20% SNP T	SNP T	27.1527945	0.718648861	
90% SNP C/ 10% SNP T	SNP C	24.86366413	0.778281391	2.86
90% SNP C/ 10% SNP T	SNP T	27.7202655	0.532147515	
95% SNP C/ 5% SNP T	SNP C	22.47890463	0.099151505	2.9
95% SNP C/ 5% SNP T	SNP T	25.37806888	0.176415304	
99% SNP C/ 1% SNP T	SNP C	22.40897513	0.142021072	3.52
99% SNP C/ 1% SNP T	SNP T	25.92636463	0.232360676	
100% SNP C/ 0% SNP T	SNP C	24.47783775	0.114038953	
100% SNP C/ 0% SNP T	SNP T			

Cp19 Assay				
0% SNP G/ 100% SNP T	SNP T	17.688359	0.053156505	
0% SNP G/ 100% SNP T	SNP G			
1% SNP A/99% SNP G	SNP G	15.54628521	0.093928928	7.26
1% SNP A/99% SNP G	SNP A	22.81048286	0.503250612	
5% SNP A/95% SNP G	SNP G	15.61834836	0.122417292	4.82
5% SNP A/95% SNP G	SNP A	20.44780386	0.292306756	
10% SNP G/ 90% SNP T	SNP T	17.76634025	0.059750538	3.14
10% SNP G/ 90% SNP T	SNP G	20.9098005	0.092603687	
20% SNP G/ 80% SNP T	SNP T	21.35831138	6.437093602	1.87
20% SNP G/ 80% SNP T	SNP G	23.2319885	5.672528442	
30% SNP G/ 70% SNP T	SNP T	18.047677	0.050498672	1.54
30% SNP G/ 70% SNP T	SNP G	19.5905325	0.033018143	
40% SNP G/ 60% SNP T	SNP T	18.13943575	0.065348878	1.19
40% SNP G/ 60% SNP T	SNP G	19.25855713	0.08577245	
50% SNP G/ 50% SNP T	SNP T	18.43470363	0.045511345	0.39
50% SNP G/ 50% SNP T	SNP G	18.82131263	0.072018507	
60% SNP G/ 40% SNP T	SNP T	18.740225	0.044701661	-0.07
60% SNP G/ 40% SNP T	SNP G	18.67472513	0.04531069	
70% SNP G/ 30% SNP T	SNP T	19.04531838	0.033432882	-0.74
70% SNP G/ 30% SNP T	SNP G	18.30920513	0.080777528	
80% SNP G/ 20% SNP T	SNP T	19.50301713	0.054838213	-1.28
80% SNP G/ 20% SNP T	SNP G	18.21895788	0.043315055	
90% SNP G/ 10% SNP T	SNP T	20.60016188	0.095557625	-2.57

90% SNP G/ 10% SNP T	SNP G	18.02680863	0.05500052	
95% SNP A/5% SNP G	SNP G	19.06609786	0.178348307	-2.99
95% SNP A/5% SNP G	SNP A	16.07117214	0.103968487	
99% SNP A/1% SNP G	SNP G	20.54564443	0.142581945	-4.49
99% SNP A/1% SNP G	SNP A	16.05601043	0.136448972	
100% SNP G/ 0% SNP T	SNP T			
100% SNP G/ 0% SNP T	SNP G	17.9079205	0.061420378	

Cp112 Assay				
0% SNP A/ 100% SNP G	SNP G	17.43285838	0.089558637	
0% SNP A/ 100% SNP G	SNP A			
1% SNP G/99% SNP T	SNP T	16.352801	0.237892781	12.78
1% SNP G/99% SNP T	SNPG	29.124194	1.550771885	
5% SNP G/95% SNP T	SNP T	16.29041143	0.108429942	8.36
5% SNP G/95% SNP T	SNPG	24.653952	0.520604467	
10% SNP G/90% SNP T	SNP T	16.46023071	0.240923919	5.76
10% SNP G/90% SNP T	SNPG	22.22458914	0.298674095	
20% SNP A/ 80% SNP G	SNP G	17.68004525	0.119522176	1.89
20% SNP A/ 80% SNP G	SNP A	19.57197013	0.227441824	
30% SNP A/ 70% SNP G	SNP G	17.73780075	0.080738321	1.41
30% SNP A/ 70% SNP G	SNP A	19.14484738	0.162285455	
40% SNP A/ 60% SNP G	SNP G	17.7828035	0.081247955	0.86
40% SNP A/ 60% SNP G	SNP A	18.63824188	0.205723584	
50% SNP A/ 50% SNP G	SNP G	18.11120413	0.131675786	0.57
50% SNP A/ 50% SNP G	SNP A	18.67911838	0.189076935	
60% SNP A/ 40% SNP G	SNP G	18.4091155	0.078320143	0.16
60% SNP A/ 40% SNP G	SNP A	18.57135175	0.099232172	
70% SNP A/ 30% SNP G	SNP G	18.40730113	0.106661782	-0.23
70% SNP A/ 30% SNP G	SNP A	18.1751775	0.128093306	
80% SNP A/ 20% SNP G	SNP G	18.90904625	0.090282271	-0.76
80% SNP A/ 20% SNP G	SNP A	18.15063163	0.112320829	
90% SNP A/ 10% SNP G	SNP G	18.92363457	0.253263393	-0.97
90% SNP A/ 10% SNP G	SNP A	17.94992386	0.208640094	
95% SNP G/5% SNP T	SNP G	19.72574713	0.117393434	-1.67
95% SNP G/5% SNP T	SNP A	18.05406963	0.120270041	
99% SNP G/1% SNP T	SNP G	19.97732871	0.242084577	-2.08
99% SNP G/1% SNP T	SNP A	17.89615143	0.073201338	
100% SNP A/ 0% SNP G	SNP G			
100% SNP A/ 0% SNP G	SNP A	18.00296525	0.137353576	

* DNA mixtures that did not amplify each allele or did not fall within the trend of delta Ct's .

Table 11. Delta CT values for different concentrations of different SNP alleles. Results demonstrate the range over which these assays give dependable and statistically relevant results.

LANL Results

Table 12 provides the forward and reverse primer and probe sequences for each of the LANL *R. communis* SNP assays tested.

	SNP 010	SNP 24
FWD	TGTTTCCCCACCTTCAAACAGT	CTGACTTATGTGATGTAACTTCTCTAATGAAAGT
REV	TGGTTTATGTAAGTATATGCAAAGGGTTGT	GCACACACAGAAAAGAAGAAGAAGA
P1	AGGCCACTGTCTGAG	ACGAACCATCTAGTCAAA
P2	AGGCCACTGGCTGAG	ACGAACCATCTGGTCAAA
	SNP 26	SNP 28
FWD	TCATTGGCAGCTTTCCACCTAAAA	ACTTCTAGAGCTAGAAATGTATGAAGATGTCT
REV	CTAAAAGATCTTAATACTAGAGAACAGTACAAAAGCT	GGTCATCAACACGGATAAGTTTGC
P1	AACATAAGATACATAGAATAT	AAAGATCAATTACAAAAAG
P2	ACATAAGATACAGAGAATAT	CAAAGATCAATTATAAAAAG
	SNP 165	SNP 195
FWD	GGGCTGCTCTTATCAATATTATAAAAATTAGCT	GAATGCAATTTAGCAGTTCAATGTTACCT
REV	ACTCATCCATTTTTTAGGCACTCACA	ACACTTCTTTTTTCCTTGTCTTTCTTTTTGT
P1	CATACACTCATAATAAG	CCCTTTTGTTCACTAGCT
P2	CATACACTCAGAATAAG	CCCTTTTGTTCGCTAGCT
	SNP 270	SNP 311
FWD	ACACAGATGCTCTCATATGTTCATTTGAT	GAGCATCACCCAAGAATGTAAAGAC
REV	CTGGAAGAGATTCCCTTGACAAGTT	GCGACAAGGGTCCTGCAT
P1	AAAGATGACAGAGAAGTT	AGTCTCAAAACAAATTATTA
P2	AAGATGACGGAGAAGTT	TGAGTCTCAAAACAAGTTATTA
	SNP 313	SNP 324
FWD	TCAAGTAACAAGTGCATTGAACAAACAG	TCTCAAATCATGCAGAGCTTTCTGT
REV	GCTGTTTTTGCATCTTTTTGTTTTCCA	GCCGACCACATATGCATTGG
P1	CTACAGATACTTTTTTTTTT	CATCCAGGGCTAATG
P2	CTACAGATACTTTGTTTTTT	ACATCCAGCGCTAATG
	Cp19	Cp111
FWD	AGGTCTTGGTGCGGAACA	GATTGATTGGCTGATGTTTCAAAA
REV	GGAACCGTAGGACTCTATCCATTTATT	AAAGAAACGTCTGTATTCAGCTACAAAG
P1	CAAGGTTGTGTCGAGTG	ATACCCAAAGCTCCCA
P2	TTCAAGGTTTTGTCGAGTG	ATACCCAAAGTTCC

Table 11. Primers and probe sequences for LANL *R. communis* SNP assays.

QPPP – Specificity

The Florida and LLNL panels of 83 *R. communis* DNAs were amplified using each of the ten genomic and two chloroplast SNP assays (Table 13).

	R. communis SNPs											
DNAs	010	24	26	28	165	195	270	311	313	324	Cp19	Cp111
RcFl 1.1	G	A	G	C	G	H	A	A	T	H	G	C
RcFl 1.6	G	A	G	T	G	H	A	G	T	H	G	C
RcFl 1.8	T	G	G	T	NO	H	A	G	T	H	G	C
RcFl 1.14	G	H	G	C	G	G	A	A	T	H	G	C
RcFl 1.19	G	A	G	C	G	G	A	A	T	H	G	C
RcFl 1.25	G	A	G	C	T	G	A	A	T	H	G	H
RcFl 2.7	G	A	G	T	G	A	A	A	T	H	G	C
RcFl 2.11	G	A	G	C	G	G	A	A	T	H	G	C
RcFl 3.3	T	A	T	T	T	A	H	G	T	H	G	C
RcFl 3.4	G	A	T	C	T	A	A	G	T	H	G	C
RcFl 3.5	T	A	T	C	T	A	G	G	G	H	G	C
RcFl 3.12	T	A	T	C	T	A	G	G	G	G	G	C
RcFl 3.14	G	H	G	C	G	H	A	A	T	H	H	C
RcFl 3.16	G	H	G	C	G	A	A	A	T	H	H	C
RcFl 3.19	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 4.3	G	H	G	C	G	G	A	A	T	H	H	C
RcFl 4.4	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 4.8	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 5.3	G	H	G	C	G	H	A	A	T	H	G	C
RcFl 5.7	G	A	G	H	T	H	A	A	T	H	H	C
RcFl 5.12	G	H	G	C	G	H	A	A	T	H	H	C
RcFl 5.21	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 6.3	G	A	G	T	T	A	G	A	T	H	T	C
RcFl 6.11	G	A	G	T	G	H	G	H	H	H	T	C
RcFl 6.13	G	A	G	H	H	A	A	A	T	H	G	C
RcFl 7.7	G	A	G	T	G	H	A	A	T	H	H	C
RcFl 8.6	G	A	G	T	G	H	A	A	T	H	G	C
RcFl 8.8	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 9.1	G	A	G	C	G	H	A	A	T	H	G	C
RcFl 9.2	H	A	G	H	G	H	H	A	T	H	G	C
RcFl 9.8	T	H	G	C	G	A	A	A	T	H	H	C
RcFl 9.10	T	A	G	C	G	A	H	A	T	H	H	C
RcFl 9.12	G	A	G	C	G	H	A	A	T	H	H	C
RcFl 9.15	H	A	G	C	G	H	A	A	G	H	H	C
RcFl 9.24	H	A	G	C	G	A	G	G	G	G	G	C
RcFl 10.3	H	A	G	C	G	H	A	A	T	H	G	H
RcFl 10.7	H	A	G	C	G	H	A	A	T	H	G	C
RcFl 10.11	G	G	T	C	G	G	G	A	T	G	G	C
RcFl 10.18	H	A	G	C	G	G	A	A	T	H	H	C
RcFl 10.19	H	G	T	C	G	H	G	H	H	G	G	H
RcFl 10.22	H	G	G	C	G	H	G	A	T	G	G	C
RcFl 10.25	H	A	G	C	G	A	A	A	T	H	G	C
RcFl 10.33	H	A	G	C	G	H	A	A	T	H	G	C

RcFl 11.4	H	H	G	C	NO	H	A	A	T	H	H	C
RcFl 11.6	H	A	G	C	G	G	A	A	T	H	G	C
RcFl 11.18	H	G	G	H	G	A	G	G	T	H	H	C
RcFl 12.2	H	A	G	T	G	G	A	G	T	H	G	C
RcFl 12.10	H	A	G	T	G	G	A	G	T	H	G	C
RcFl 12.14	H	A	G	C	G	G	A	A	T	H	G	C
RcFl 12.21	H	A	G	T	G	A	A	A	T	H	G	C
RcFl 12.32	H	G	T	C	G	G	A	A	T	H	H	C
167 238	H	H	H	T	G	A	A	H	H	H	T	C
170 686	T	A	T	C	T	A	G	G	T	H	T	C
173 946	T	A	T	T	T	A	G	H	T	H	T	C
173 948	T	A	H	C	T	A	G	G	T	H	G	C
173 950	H	A	G	C	T	A	G	H	T	H	G	C
179 729	T	A	G	H	H	H	H	H	H	H	T	C
181 916	G	G	T	C	T	A	G	G	G	H	H	T
183 347	T	H	T	C	G	A	G	G	T	H	T	C
183 468	T	A	T	C	T	A	G	G	T	H	T	C
183 470	H	A	G	H	T	A	G	H	T	H	T	C
183 471	H	A	H	T	G	G	A	G	G	H	T	C
195 811	G	A	G	C	G	H	A	G	G	G	G	C
197 048	G	A	G	H	G	H	G	A	T	H	G	C
201 830	T	A	H	T	G	A	G	A	T	H	G	C
203 661	H	A	G	T	T	H	A	G	G	H	H	C
204 322	H	A	G	H	T	A	G	A	T	G	H	C
206 515	H	A	G	H	T	G	H	H	T	H	H	C
207 868	G	H	H	T	H	A	H	G	G	H	T	C
208 689	T	G	G	T	G	G	A	G	G	H	T	C
208 840	H	A	G	C	G	G	G	H	H	G	H	C
208 842	H	G	H	C	G	G	H	G	T	H	G	C
209 132	G	A	G	H	G	H	G	A	T	G	G	C
209 326	H	A	G	T	G	H	A	A	T	H	G	C
209 622	H	H	G	C	G	H	H	G	G	H	G	C
217 539	H	A	G	T	T	A	A	A	T	H	G	C
219 767	T	A	H	T	H	H	A	A	T	H	T	C
219 770	G	G	G	H	G	G	A	A	H	H	T	C
219 776	H	A	G	T	G	H	A	A	T	H	T	H
221 698	H	A	G	T	G	G	G	G	G	H	G	C
240 312	G	G	G	T	H	G	A	G	G	H	G	C
241 362	H	A	G	C	H	A	H	A	T	H	G	C
241 368	H	G	H	H	H	A	G	H	H	G	T	C

Table 13. *Ricinus communis* allele states for the different accessions. A letter designation of "H" refers to a heterozygous allele. "NO" indicates that no amplification was observed.

***R. communis* SNP Assays**						
	010	**24**	**26**	**28**	**165**	**195**
RcFL	26/51-51% Allele G	37/51-73% Allele A	44/51-86% Allele G	35/51-69% Allele C	41/49-84% Allele G	25/51-49% Allele A/G
	G= 26	A= 37	G= 44	C= 35	G= 41	H= 25
	T= 6	G= 6	T= 4	T= 11	T= 7	A= 14
	H= 19	H= 8	H= 0	H= 4	H= 1	G= 16
	010	**24**	**26**	**28**	**165**	**195**
LLNL	16/32-50% Allele G/T	24/32-75% Allele A	19/32-59% Allele G	14/32-44% Allele T	15/32-47% Allele G	15/32-47% Allele A
	H= 16	A= 24	G= 19	T= 14	G= 15	A= 15
	T= 9	G= 6	T= 5	C= 11	T= 11	G= 8
	G= 7	H= 2	H= 8	H= 7	H= 6	H= 9

***R. communis* SNP assays**						
	270	**311**	**313**	**324**	**Cp 19**	**Cp 111**
RcFL	39/51-76% Allele A	39/51-76% Allele A	45/51-88% Allele T	46/51-90% Allele C/G	30/51-59% Allele G	48/51-94% Allele C
	A= 39	A= 39	T= 45	H= 46	G= 30	C= 48
	G= 9	G= 10	G= 4	G= 5	T= 2	T= 0
	H= 3	H= 2	H= 2	C= 0	H= 19	H= 3
	270	**311**	**313**	**324**	**Cp 19**	**Cp 111**
LLNL	15/32-47% Allele G	14/32-44% Allele G	20/32-63% Allele T	27/32-84% Allele C/G	14/32-44% Allele T	30/32-94% Allele C
	G= 15	G= 14	T=20	H= 27	T= 14	C= 30
	A= 11	A= 10	G= 9	G= 5	G= 13	T= 1
	H= 6	H= 8	H= 3	C= 0	H= 5	H= 1

Table 14. Diversity of alleles within each assay.

Limits of detection.

Each of the *R. communis* DNAs were normalized to a concentration 2 ng/µL using Pico Green to determine initial concentrations. The DNAs were diluted 10-fold in 7 serial dilutions ranging from 2 ng/µL to 0.000002 ng/µL. The diluted DNAs were amplified with the Castor SNP assays 6 times each. The lowest limit of detection was determined for both allele states for the 12 assays and is shown in Table 15. The limits of detection varied for the different assays. Data for chloroplast SNP assays were more sensitive than the genomic SNPs assays.

010 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	RcFL 1.1	2 ng	27.908	1.419		0.990
		0.2 ng	31.960	1.441	4.05	
		0.02 ng	35.205	1.316	3.24	
		0.002 ng	38.844	0.861	3.64	
		0.0002 ng	39.846	0.134	1.00	
SNP T	RcFL 3.12	2 ng	27.742	1.460		0.999
		0.2 ng	31.397	1.593	3.65	
		0.02 ng	35.269	1.418	3.87	

24 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP A	RcFL 1.1	2 ng	29.075	0.917		0.998
		0.2 ng	32.286	0.539	3.21	
		0.02 ng	36.128	0.570	3.84	
SNP G	181 916	2 ng	27.512	0.619		0.998
		0.2 ng	31.015	0.429	3.50	
		0.02 ng	34.440	0.368	3.42	
		0.002 ng	37.851	0.538	3.41	

26 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	RcFL 1.1	2 ng	27.051	0.084		0.998
		0.2 ng	30.595	0.098	3.54	
		0.02 ng	34.388	0.727	3.79	
		0.002 ng	37.928	0.468	3.54	
SNP T	RcFL 3.12	2 ng	27.546	0.088		0.999
		0.2 ng	30.784	0.172	3.23	
		0.02 ng	34.452	0.538	3.67	
		0.002 ng	37.750	1.204	3.30	

28 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP C	RcFL 1.1	2 ng	27.752	0.425		0.998
		0.2 ng	30.888	0.146	3.14	
		0.02 ng	34.719	0.232	3.83	
		0.002 ng	38.607	0.501	3.89	
SNP T	RcFL 6.3	2 ng	27.972	0.176		0.993
		0.2 ng	31.280	0.262	3.31	
		0.02 ng	34.799	0.115	3.52	
		0.002 ng	38.948	0.442	4.15	

165 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	RcFL 1.1	2 ng	27.711	0.397		0.995
		.2 ng	30.843	0.213	3.13	
		.02 ng	34.220	0.207	3.38	
		.002 ng	38.153	0.505	3.93	
SNP T	RcFL 3.12	2 ng	27.777	0.143		0.999
		0.2 ng	31.178	0.181	3.40	
		0.02 ng	34.857	0.223	3.68	
		0.002 ng	38.213	0.492	3.36	

195 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	RcFL 1.1	2 ng	28.033	0.150		0.989
		0.2 ng	32.163	1.337	4.13	
		0.02 ng	35.449	0.197	3.29	
		0.002 ng	39.011	0.127	3.56	
SNP A	181 916	2 ng	26.498	0.285		0.998
		0.2 ng	29.518	0.136	3.02	
		0.02 ng	33.175	0.218	3.66	
		0.002 ng	37.238	0.667	4.07	
		0.0002 ng	38.415	0.972	1.18	

270 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP A	RcFL 1.1	2 ng	26.109	0.279		0.996
		.2 ng	29.540	0.180	3.43	
		.02 ng	32.986	0.328	3.46	
		.002 ng	36.557	0.492	3.57	
		0.002 ng	38.484	0.572	1.92	
SNP G	RcFL 3.12	2 ng	25.784	0.146		0.999
		.2 ng	29.210	0.321	3.43	
		.02 ng	32.578	0.234	3.37	
		.002 ng	35.974	0.572	3.37	

311 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP A	RcFL 1.1	2 ng	27.284	0.182		0.994
		0.2 ng	30.451	0.192	3.17	
		0.02 ng	33.904	0.295	3.45	
		0.002 ng	37.951	0.791	4.05	
SNP G	RcFL 3.12	2 ng	27.198	0.271		0.983
		0.2 ng	30.457	0.249	3.26	
		0.02 ng	33.436	0.342	2.98	
		0.002 ng	38.008	0.818	4.57	
		0.002 ng	38.859	0.478	0.85	

313 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP T	RcFL 1.1	2 ng	25.867	0.203		0.996
		0.2 ng	29.573	0.610	3.71	
		0.02 ng	33.510	0.536	3.94	
		0.002 ng	37.841	1.034	4.33	
SNP G	RcFL 3.12	2 ng	26.280	0.419		0.995
		0.2 ng	29.878	0.498	3.60	
		0.02 ng	33.645	0.574	3.77	
		0.002 ng	37.945	0.764	4.30	
		0.0002 ng	39.072	0.031	1.13	

324 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP C	RcFL 1.1	2 ng	24.869	0.319		0.993
		0.2 ng	27.898	0.048	3.03	
		0.02 ng	31.950	0.345	4.05	
		0.002 ng	36.538	0.841	4.59	
SNP G	RcFL 3.12	2 ng	25.271	0.451		0.992
		0.2 ng	28.952	0.516	3.68	
		0.02 ng	32.266	0.283	3.31	
		0.002 ng	36.633	0.811	4.37	
		0.0002 ng	38.619	0.994	1.99	

Cp19 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP G	RcFL 1.1	2 ng	15.275	0.315		0.997
		0.2 ng	18.724	0.475	3.45	
		0.02 ng	22.355	0.271	3.63	
		0.002 ng	25.978	0.676	3.62	
		0.0002 ng	30.954	1.127	4.98	
		0.00002 ng	33.969	1.544	3.01	
SNP T	RcFL 6.3	2 ng	18.280	0.502		0.992
		0.2 ng	21.489	0.580	3.21	
		0.02 ng	25.279	0.268	3.79	
		0.002 ng	29.602	0.292	4.32	
		0.0002 ng	32.393	0.420	2.79	
		0.00002 ng	34.016	0.604	1.62	
		0.000002 ng	34.194	0.616	0.18	
		0.0000002 ng	33.896	0.622	-0.30	

Cp111 Assay	DNA	DNA Amount	Average Ct	Standard Deviation	dCt	R^2
SNP C	RcFL 1.1	2 ng	20.183	1.767		0.970
		0.2 ng	21.921	0.386	1.74	
		0.02 ng	25.445	0.327	3.52	
		0.002 ng	30.000	0.478	4.56	
		0.0002 ng	32.711	0.433	2.71	
		0.00002 ng	33.841	0.677	1.13	
		0.000002 ng	33.960	0.712	0.11	
		0.0000002 ng	35.870	1.05	1.91	
SNP T	181 916	2 ng	20.611	0.831		0.999
		0.2 ng	23.915	0.546	3.30	
		0.02 ng	27.266	0.559	3.35	
		0.002 ng	30.802	0.502	3.54	
		0.0002 ng	34.482	0.506	3.68	
		0.00002 ng	38.368	0.982	3.89	

Table 15. The 12 SNP Assays amplified with 10-fold serial dilutions of DNA for each allele ranging from 2 ng to .000002 ng of DNA. Delta Ct (dCt) calculated by subtracting the average Ct value one dilution from the average Ct value of amplification of the 10-fold greater dilution of DNA.
* DNA amounts that amplified an average delta Ct value outside of the 3.2-3.7 range

Linearity

The linearities were determined by using the 10-fold serial dilution assays to calculate the delta Ct values between 10-fold dilutions. The average Ct value at each dilution point was calculated and subtracted by the average Ct value of the next 10-fold dilution of that DNA for the assays. This value has been denoted the delta Ct. Delta Ct's that fell within the range of 3.2-3.7 are considered valid. DNA quantities that gave delta Ct's outside of this range were dismissed as being outside of the range of linearity in Table 15.

Limits of Quantification

The average Ct values and standard deviations for each DNA quantity were determined using the Ct values of the six amplifications at a particular DNA quantity for each assay. The average Ct values from six replicate experiments ranged from 15 to 29 for the different assays. Standard deviations greater than 0.65 were considered outside of the limits of quantification. The standard deviation for SNP 010 show that this is the poorest performing assay (Table 15).

The average Ct values were graphed on the Y-axis of a scatter plot along with the log of the dilution factor graphed on the X axis, (Figure 10). The R^2 value for the resulting line was calculated for each assay. All genomic and chloroplast assays gave R^2 of greater than 0.97.

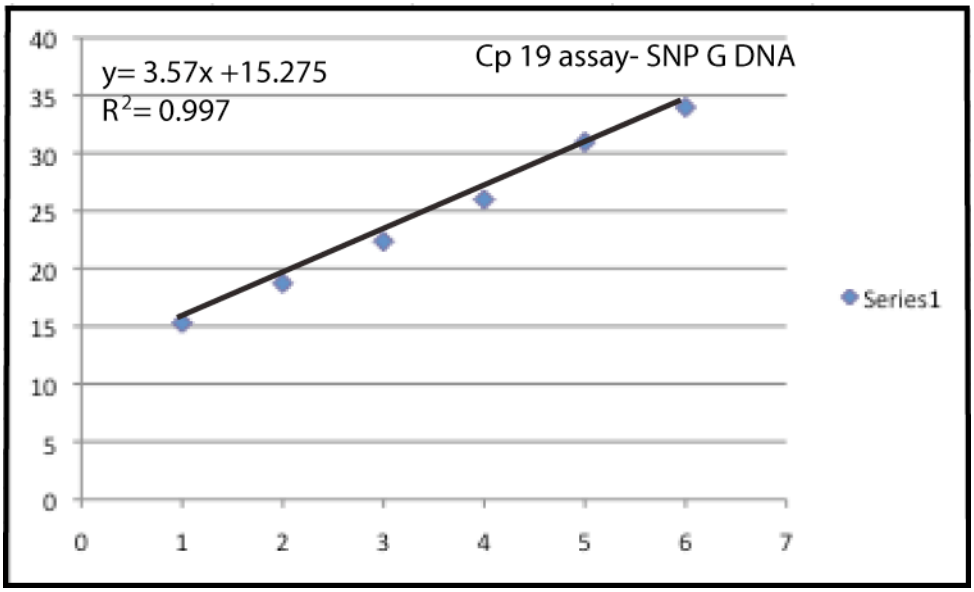


Figure 10. A graph of the Cp19 chloroplast SNP assay amplification of SNP G DNA 10-fold serial dilutions. The Ct value is graphed on the Y axis and the log of the dilution factor is graphed on the X axis

Precision

The 12 *R. communis* SNP assays were tested against a panel of 22 *R. communis* DNAs including a negative no-template control. The 22 DNAs contained representatives of each allele state for the 12 assays. The panel was blinded and each real time assay was run 10 times. The resulting allele states were compared against the known genotypes. All assays amplified the correct allele state for each previously tested *R. communis* DNAs (Table 16).

DNA	**010 Assay**		**24 Assay**		**26 Assay**		**28 Assay**	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
RcFL 1.1	G	G	A	A	G	G	C	C
RcFL 1.6	G	G	A	A	G	G	T	T
RcFL 1.19	G	G	A	A	G	G	C	C
RcFL 1.25	G	G	A	A	G	G	C	C
RcFL 2.7	G	G	A	A	G	G	T	T
RcFL 3.3	T	T	A	A	T	T	T	T
RcFL 3.5	T	T	A	A	H	T	C	C
RcFL 3.12	T	T	A	A	H	T	C	C
RcFL 3.19	G	G	A	A	G	G	C	C
RcFL 4.4	G	G	A	A	G	G	C	C
RcFL 5.7	G	G	A	A	G	G	H	H
RcFL 5.12	G	G	A	H	G	G	C	C
RcFL 6.3	G	G	A	A	G	G	T	T
RcFL 6.11	G	H	A	A	G	G	T	T
RcFL 6.13	G	G	A	A	G	H	C	H
RcFL 7.7	G	G	A	A	G	G	T	T

RcFL 8.8	G	G	A	A	G	T	C	C
RcFL 9.10	T	T	A	A	G	T	C	C
RcFL 10.3	G	H	A	A	G	T	C	C
167 238	H	H	A	H	H	G	T	T
173 948	T	T	A	A	G	T	C	C
181 916	G	G	G	G	T	T	C	C
NTC	NO	NO	NO	NO	NO	NO	NO	NO
DNA	**165 Assay**		**195 Assay**		**270 Assay**		**311 Assay**	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
RcFL 1.1	G	G	G	H	A	A	A	A
RcFL 1.6	G	G	G	H	A	A	G	G
RcFL 1.19	T	G	G	G	A	A	A	A
RcFL 1.25	G	T	G	G	A	A	A	A
RcFL 2.7	G	G	A	A	A	A	A	A
RcFL 3.3	T	T	A	A	H	H	G	G
RcFL 3.5	T	T	A	A	G	G	G	G
RcFL 3.12	T	T	A	A	G	G	G	G
RcFL 3.19	G	G	G	H	A	A	A	A
RcFL 4.4	G	G	G	H	A	A	A	A
RcFL 5.7	T	T	G	H	A	A	A	A
RcFL 5.12	G	G	G	H	A	A	A	A
RcFL 6.3	T	T	A	A	G	G	A	A
RcFL 6.11	G	G	H	H	G	G	H	H
RcFL 6.13	H	H	A	A	A	A	A	A
RcFL 7.7	G	G	G	H	A	A	A	A
RcFL 8.8	G	G	G	H	A	A	A	A
RcFL 9.10	G	G	A	A	A	H	A	A
RcFL 10.3	G	G	G	H	A	A	A	A
167 238	G	G	A	A	A	A	H	H
173 948	T	T	A	A	G	G	G	G
181 916	T	T	A	A	G	G	G	G
NTC	NO	NO	NO	NO	NO	NO	NO	NO
DNA	**313 Assay**		**324 Assay**		**Cp19 Assay**		**Cp111 assay**	
	Blind	Correct	Blind	Correct	Blind	Correct	Blind	Correct
RcFL 1.1	T	T	H	H	G	G	C	C
RcFL 1.6	T	T	H	H	G	G	C	C
RcFL 1.19	T	T	H	H	G	G	C	C
RcFL 1.25	T	T	G	H	G	G	C	H
RcFL 2.7	T	T	H	H	G	G	C	C
RcFL 3.3	T	T	H	H	G	G	C	C

RcFL 3.5	G	G	G	H	G	G	C	C
RcFL 3.12	G	G	G	G	G	G	C	C
RcFL 3.19	T	T	H	H	G	H	C	C
RcFL 4.4	T	T	H	H	G	H	C	C
RcFL 5.7	T	T	H	H	G	H	C	C
RcFL 5.12	T	T	H	H	G	H	C	C
RcFL 6.3	T	T	H	H	T	T	C	C
RcFL 6.11	H	H	H	H	T	T	C	C
RcFL 6.13	T	T	H	H	G	G	C	C
RcFL 7.7	T	T	H	H	G	H	C	C
RcFL 8.8	T	T	H	H	G	H	C	C
RcFL 9.10	T	T	H	H	G	H	C	C
RcFL 10.3	T	T	H	H	G	H	C	H
167 238	H	H	H	H	T	T	C	C
173 948	T	T	G	H	G	H	C	C
181 916	G	G	G	H	T	H	T	T
NTC	NO	NO	NO	NO	NO	NO	NO	NO

Table 16. Twelve SNP assays amplified in blind study and compared to actual allele states.

Selectivity

DNAs representing each allele state were normalized to equivalent concentrations using an assay for which each DNA contained the same allele. The equivalent DNA dilutions were mixed together in the following ratios of DNAs: 100/0, 99/1, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 5/95, 1/99, and 0/100. Each mixture was then amplified 8 times with the SNP assay that contained different alleles for each DNA. The delta Ct's were calculated by subtracting the average Ct of 1 SNP amplification from the average Ct of the second SNP amplification in each reaction for each mixture ratio.

Mixtures that contained 100% of 1 DNA and 0% of the second DNA amplified only the allele corresponding to the first DNA. Mixtures that contained 50% of each DNA amplified each allele at fairly equal Ct values. Amplification plots are shown in Figure 11 and the delta Ct's for the mixture ratios are shown in Table 17. The trend of delta Ct's increasing and decreasing according to DNA mixture ratios held up on all genomic assays through the 90/10 and 10/90 ratios. The chloroplast assays were able to amplify both alleles for all mixture ratios. The delta Ct trend also continued through the 1/99 and 99/1 mixture ratio with both of the chloroplast assays.

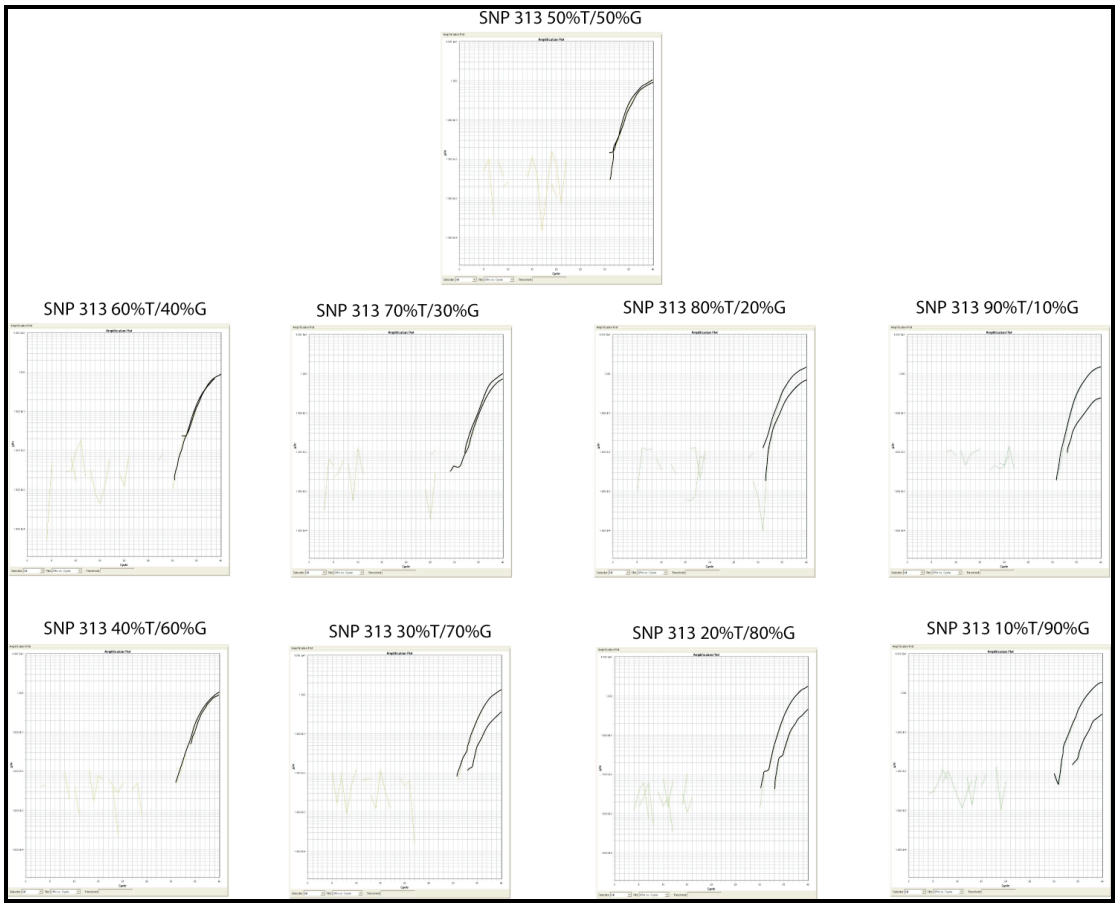


Figure 11. SNP assay 313 amplified with DNAs representing both alleles at mixture ratios of 100/0, 99/1, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 5/95, 1/99 and 0/100. All PCR experiments were repeated 8 times, the dCt calculated by subtracting the average Ct of one SNP from the average Ct of the second SNP DNA.

010 Assay	SNP	Average Ct	Standard Deviation	dCt
0% SNP G/ 100% SNP T	SNP T	34.576	0.376	
0% SNP G/ 100% SNP T	SNP G			
1% SNP G/ 99% SNP T	SNP T	35.309	0.6927	
1% SNP G/ 99% SNP T	SNP G			
5% SNP G/ 95% SNP T	SNP T	34.987	0.351	
5% SNP G/ 95% SNP T	SNP G			
10% SNP G/ 90% SNP T	SNP T	34.809	0.817	-4.074
10% SNP G/ 90% SNP T	SNP G	38.883	0.709	
20% SNP G/ 80% SNP T	SNP T	35.498	0.532	-2.893
20% SNP G/ 80% SNP T	SNP G	38.391	0.867	
30% SNP G/ 70% SNP T	SNP T	35.802	0.632	-2.537
30% SNP G/ 70% SNP T	SNP G	38.339	1.380	
40% SNP G/ 60% SNP T	SNP T	37.196	1.028	0.044
40% SNP G/ 60% SNP T	SNP G	37.152	0.461	
50% SNP G/ 50% SNP T	SNP T	36.608	0.989	0.047
50% SNP G/ 50% SNP T	SNP G	36.561	0.919	
60% SNP G/ 40% SNP T	SNP T	37.036	0.874	0.283
60% SNP G/ 40% SNP T	SNP G	36.753	0.988	

70% SNP G/ 30% SNP T	SNP T	37.675	0.911	1.52
70% SNP G/ 30% SNP T	SNP G	36.155	0.592	
80% SNP G/ 20% SNP T	SNP T	38.258	0.822	2.504
80% SNP G/ 20% SNP T	SNP G	35.754	0.568	
90% SNP G/ 10% SNP T	SNP T			
90% SNP G/ 10% SNP T	SNP G	35.863	0.6181	
95% SNP G/ 5% SNP T	SNP T			
95% SNP G/ 5% SNP T	SNP G	35.502	0.271	
99% SNP G/ 1% SNP T	SNP T			
99% SNP G/ 1% SNP T	SNP G	35.490	0.486	
100% SNP G/ 0% SNP T	SNP T			
100% SNP G/ 0% SNP T	SNP G	35.026	0.235	

24 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/ 100% SNP A	SNP A	36.359	1.134	
0% SNP G/ 100% SNP A	SNP G			
1% SNP G/ 99% SNP A	SNP A	36.302	1.023	
1% SNP G/ 99% SNP A	SNP G			
5% SNP G/ 95% SNP A	SNP A	36.306	0.762	
5% SNP G/ 95% SNP A	SNP G			
10% SNP G/ 90% SNP A	SNP A	36.333	0.865	
10% SNP G/ 90% SNP A	SNP G			
20% SNP G/ 80% SNP A	SNP A	36.807	0.959	
20% SNP G/ 80% SNP A	SNP G			
30% SNP G/ 70% SNP A	SNP A	36.807	0.959	-0.949
30% SNP G/ 70% SNP A	SNP G	37.756	1.353	
40% SNP G/ 60% SNP A	SNP A	37.935	1.127	-0.182
40% SNP G/ 60% SNP A	SNP G	38.117	1.867	
50% SNP G/ 50% SNP A	SNP A	38.070	1.346	1.07
50% SNP G/ 50% SNP A	SNP G	37.000	1.514	
60% SNP G/ 40% SNP A	SNP A	38.420	1.408	1.786
60% SNP G/ 40% SNP A	SNP G	36.634	1.310	
70% SNP G/ 30% SNP A	SNP A			
70% SNP G/ 30% SNP A	SNP G	36.559	1.592	
80% SNP G/ 20% SNP A	SNP A			
80% SNP G/ 20% SNP A	SNP G	35.927	1.295	
90% SNP G/ 10% SNP A	SNP A			
90% SNP G/ 10% SNP A	SNP G	35.773	1.195	
95% SNP G/ 5% SNP A	SNP A			
95% SNP G/ 5% SNP A	SNP G	35.741	1.195	
99% SNP G/ 1% SNP A	SNP A			
99% SNP G/ 1% SNP A	SNP G	35.520	1.330	
100% SNP G/ 0% SNP A	SNP A			
100% SNP G/ 0% SNP A	SNP G	35.538	1.477	

26 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/100% SNP T	SNP T	35.535	0.787	
0% SNP G/100% SNP T	SNP G			
1% SNP G/ 99% SNP T	SNP T	35.404	0.371	

1% SNP G/ 99% SNP T	SNP G			
5% SNP G/ 95% SNP T	SNP T	35.292	0.208	
5% SNP G/ 95% SNP T	SNP G	38.284	0.965	
10% SNP G/ 90% SNP T	SNP T	35.326	0.507	-2.992
10% SNP G/ 90% SNP T	SNP G	37.968	0.804	
20% SNP G/ 80% SNP T	SNP T	35.731	0.706	-2.642
20% SNP G/ 80% SNP T	SNP G	36.875	0.876	
30% SNP G/ 70% SNP T	SNP T	36.308	0.443	-1.144
30% SNP G/ 70% SNP T	SNP G	36.256	0.450	
40% SNP G/ 60% SNP T	SNP T	37.029	0.900	0.052
40% SNP G/ 60% SNP T	SNP G	36.246	0.718	
50% SNP G/50% SNP T	SNP T	37.664	1.103	0.783
50% SNP G/50% SNP T	SNP G	35.776	0.313	
60% SNP G/40% SNP T	SNP T	37.322	0.945	1.888
60% SNP G/40% SNP T	SNP G	35.727	0.657	
70% SNP G/30% SNP T	SNP T	38.159	0.884	1.595
70% SNP G/30% SNP T	SNP G	35.442	0.670	
80% SNP G/20 % SNP T	SNP T	38.845	1.008	2.717
80% SNP G/20 % SNP T	SNP G	35.847	0.512	
90% SNP G/10% SNP T	SNP T			
90% SNP G/10% SNP T	SNP G	35.400	0.477	
95% SNP G/ 5% SNP T	SNP T			
95% SNP G/ 5% SNP T	SNP G	35.374	0.655	
99% SNP G/ 1% SNP T	SNP T			
99% SNP G/ 1% SNP T	SNP G	35.361	0.350	
100% SNP G/ 0% SNP T	SNP T			
100% SNP G/ 0% SNP T	SNP G	35.246	0.626	

28 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP T/100% SNP C	SNP C	35.570	0.948	
0% SNP T/100% SNP C	SNP T			
1% SNP T/ 99% SNP C	SNP C	35.460	0.642	
1% SNP T/ 99% SNP C	SNP T			
5% SNP T/ 95% SNP C	SNP C	35.365	0.686	
5% SNP T/ 95% SNP C	SNP T			
10% SNP T/ 90% SNP C	SNP C	35.924	1.170	
10% SNP T/ 90% SNP C	SNP T			
20% SNP T/ 80% SNP C	SNP C	36.066	0.563	
20% SNP T/ 80% SNP C	SNP T			
30% SNP T/ 70% SNP C	SNP C	36.003	0.787	
30% SNP T/ 70% SNP C	SNP T	38.231	1.548	
40% SNP T/ 60% SNP C	SNP C	36.480	0.904	-2.228
40% SNP T/ 60% SNP C	SNP T	38.576	0.529	
50% SNP T/50% SNP C	SNP C	36.791	0.786	-2.096
50% SNP T/50% SNP C	SNP T	37.830	1.120	
60% SNP T/40% SNP C	SNP C	36.907	1.231	-1.039
60% SNP T/40% SNP C	SNP T	37.136	1.108	
70% SNP T/30% SNP C	SNP C	38.536	0.454	-0.229
70% SNP T/30% SNP C	SNP T	36.862	1.239	

80% SNP T/20 % SNP C	SNP C			
80% SNP T/20 % SNP C	SNP T	36.401	1.060	
90% SNP T/10% SNP C	SNP C			
90% SNP T/10% SNP C	SNP T	36.241	0.826	
95% SNP T/ 5% SNP C	SNP C			
95% SNP T/ 5% SNP C	SNP T	36.086	0.692	
99% SNP T/ 1% SNP C	SNP C			
99% SNP T/ 1% SNP C	SNP T	36.260	1.030	
100% SNP T/ 0% SNP C	SNP C			
100% SNP T/ 0% SNP C	SNP T	36.402	1.395	

165 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/100% SNP T	SNP T	36.202	0.332	
0% SNP G/100% SNP T	SNP G			
1% SNP G/99% SNP T	SNP T	36.378	0.523	
1% SNP G/99% SNP T	SNP G			
5% SNP G/95% SNP T	SNP T	36.389	0.391	
5% SNP G/95% SNP T	SNP G			
10% SNP G/ 90% SNP T	SNP T	36.716	1.090	
10% SNP G/ 90% SNP T	SNP G			
20% SNP G/ 80% SNP T	SNP T	37.238	0.833	-1.367
20% SNP G/ 80% SNP T	SNP G	38.605	0.563	
30% SNP G/ 70% SNP T	SNP T	37.194	0.557	-0.711
30% SNP G/ 70% SNP T	SNP G	37.905	0.804	
40% SNP G/ 60% SNP T	SNP T	37.772	0.883	0.447
40% SNP G/ 60% SNP T	SNP G	37.325	0.938	
50% SNP G/50% SNP T	SNP T	38.366	1.104	1.336
50% SNP G/50% SNP T	SNP G	37.030	1.062	
60% SNP G/40% SNP T	SNP T	38.647	0.815	2.19
60% SNP G/40% SNP T	SNP G	36.457	0.543	
70% SNP G/30% SNP T	SNP T	38.771	1.006	2.576
70% SNP G/30% SNP T	SNP G	36.195	0.589	
80% SNP G/20 % SNP T	SNP T			
80% SNP G/20 % SNP T	SNP G	35.920	0.350	
90% SNP G/10% SNP T	SNP T			
90% SNP G/10% SNP T	SNP G	35.948	0.287	
95% SNP G/5% SNP T	SNP T			
95% SNP G/5% SNP T	SNP G	35.765	0.431	
99% SNP G/1% SNP T	SNP T			
99% SNP G/1% SNP T	SNP G	35.738	0.415	
100% SNP G/ 0% SNP T	SNP T			
100% SNP G/ 0% SNP T	SNP G	35.477	0.335	

195 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/ 100% SNP A	SNP A			
0% SNP G/ 100% SNP A	SNP G	35.940	0.645	
1% SNP G/ 99% SNP A	SNP A	38.536	1.601	2.106
1% SNP G/ 99% SNP A	SNP G	36.430	0.951	
5% SNP G/ 95% SNP A	SNP A	39.031	0.966	2.602

5% SNP G/ 95% SNP A	SNP G	36.429	1.011	
10% SNP G/ 90% SNP A	SNP A	37.537	1.466	1.208
10% SNP G/ 90% SNP A	SNP G	36.329	0.740	
20% SNP G/ 80% SNP A	SNP A	37.250	0.819	0.622
20% SNP G/ 80% SNP A	SNP G	36.628	0.653	
30% SNP G/ 70% SNP A	SNP A	36.839	0.737	0.151
30% SNP G/ 70% SNP A	SNP G	36.688	0.522	
40% SNP G/ 60% SNP A	SNP G	36.722	1.315	-0.410
40% SNP G/ 60% SNP A	SNP A	37.132	0.647	
50% SNP G/ 50% SNP A	SNP G	35.788	0.793	-1.695
50% SNP G/ 50% SNP A	SNP A	37.483	0.791	
60% SNP G/ 40% SNP A	SNP G	35.644	0.978	-2.073
60% SNP G/ 40% SNP A	SNP A	37.717	0.869	
70% SNP G/ 30% SNP A	SNP G	35.444	0.811	-2.940
70% SNP G/ 30% SNP A	SNP A	38.384	1.061	
80% SNP G/ 20% SNP A	SNP G	35.161	1.093	-3.663
80% SNP G/ 20% SNP A	SNP A	38.824	0.591	
90% SNP G/ 10% SNP A	SNP G	34.875	0.979	
90% SNP G/ 10% SNP A	SNP A			
95% SNP G/ 5% SNP A	SNP A	34.858	0.895	
95% SNP G/ 5% SNP A	SNP G			
99% SNP G/ 1% SNP A	SNP A	34.611	0.795	
99% SNP G/ 1% SNP A	SNP G			
100% SNP G/ 0% SNP A	SNP A	34.710	0.856	
100% SNP G/ 0% SNP A	SNP G			

270 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/100% SNP A	SNP A			
0% SNP G/100% SNP A	SNP G	33.772	0.413	
1% SNP G/99% SNP A	SNP A			
1% SNP G/99% SNP A	SNP G	34.024	0.457	
5% SNP G/95% SNP A	SNP A			
5% SNP G/95% SNP A	SNP G	34.024	0.336	
10% SNP G/ 90% SNP A	SNP A			
10% SNP G/ 90% SNP A	SNP G	34.136	0.348	-4.206
20% SNP G/ 80% SNP A	SNP A	38.342	1.067	
20% SNP G/ 80% SNP A	SNP G	34.228	0.167	-2.868
30% SNP G/ 70% SNP A	SNP A	37.096	0.906	
30% SNP G/ 70% SNP A	SNP G	34.317	0.461	-2.576
40% SNP G/ 60% SNP A	SNP A	36.893	1.365	
40% SNP G/ 60% SNP A	SNP G	34.836	0.630	-1.121
50% SNP G/50% SNP A	SNP A	35.957	1.441	
50% SNP G/50% SNP A	SNP G	34.989	0.665	-0.273
60% SNP G/40% SNP A	SNP A	35.262	0.360	
60% SNP G/40% SNP A	SNP G	35.221	0.799	0.104
70% SNP G/30% SNP A	SNP A	35.117	0.402	
70% SNP G/30% SNP A	SNP G	35.688	0.476	0.937
80% SNP G/20 % SNP A	SNP A	34.751	0.480	
80% SNP G/20 % SNP A	SNP G	36.186	0.578	1.615

90% SNP G/10% SNP A	SNP A	34.571	0.493	
90% SNP G/10% SNP A	SNP G	38.089	1.037	3.692
95% SNP G/5% SNP A	SNP A	34.397	0.475	
95% SNP G/5% SNP A	SNP G	37.657	1.594	3.519
99% SNP G/1% SNP A	SNP A	34.138	0.517	-4.206
99% SNP G/1% SNP A	SNP G			
100% SNP G/ 0% SNP A	SNP A	34.176	0.410	
100% SNP G/ 0% SNP A	SNP G			

311 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/100% SNP A	SNP A			
0% SNP G/100% SNP A	SNP G	35.840	0.370	
1% SNP G/99% SNP A	SNP A	38.946	0.502	3.826
1% SNP G/99% SNP A	SNP G	35.120	0.437	
5% SNP G/95% SNP A	SNP A	38.891	1.395	3.815
5% SNP G/95% SNP A	SNP G	35.076	0.415	
10% SNP G/90% SNP A	SNP A	37.248	0.763	2.068
10% SNP G/90% SNP A	SNP G	35.180	0.424	
20% SNP G/80% SNP A	SNP A	37.912	0.910	2.563
20% SNP G/80% SNP A	SNP G	35.349	0.512	
30% SNP G/70% SNP A	SNP A	36.706	0.589	1.746
30% SNP G/70% SNP A	SNP G	34.960	0.396	
40% SNP G/60% SNP A	SNP A	36.347	0.470	1.021
40% SNP G/60% SNP A	SNP G	35.326	0.452	
50% SNP G/50% SNP A	SNP A	36.042	0.484	-0.025
50% SNP G/50% SNP A	SNP G	36.067	0.551	
60% SNP G/40% SNP A	SNP A	36.144	0.729	-0.012
60% SNP G/40% SNP A	SNP G	36.156	0.840	
70% SNP G/30% SNP A	SNP A	35.321	0.455	-0.737
70% SNP G/30% SNP A	SNP G	36.058	0.456	
80% SNP G/20% SNP A	SNP A	35.345	0.592	-1.072
80% SNP G/20% SNP A	SNP G	36.417	0.392	
90% SNP G/10% SNP A	SNP A	34.933	0.516	-2.001
90% SNP G/10% SNP A	SNP G	36.934	0.537	
95% SNP G/5% SNP A	SNP A	35.113	0.561	-2.45
95% SNP G/5% SNP A	SNP G	37.563	0.747	
99% SNP G/1% SNP A	SNP A	34.985	0.690	-3.406
99% SNP G/1% SNP A	SNP G	38.391	0.844	
100% SNP G/0% SNP A	SNP A	34.872	0.321	-3.368
100% SNP G/0% SNP A	SNP G	38.240	0.429	

313 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/ 100% SNP T	SNP T	39.131	0.3569	4.237
0% SNP G/ 100% SNP T	SNP G	34.894	0.645	
1% SNP G/ 99% SNP T	SNP T	36.918	0.416	2.093
1% SNP G/ 99% SNP T	SNP G	34.825	0.270	
5% SNP G/ 95% SNP T	SNP T	37.588	0.933	2.584
5% SNP G/ 95% SNP T	SNP G	35.004	0.559	
10% SNP G/ 90% SNP T	SNP T	37.468	1.104	2.65

10% SNP G/ 90% SNP T	SNP G	34.818	0.218	
20% SNP G/ 80% SNP T	SNP T	36.622	0.650	1.418
20% SNP G/ 80% SNP T	SNP G	35.204	0.457	
30% SNP G/ 70% SNP T	SNP T	35.938	0.871	0.659
30% SNP G/ 70% SNP T	SNP G	35.279	0.290	
40% SNP G/ 60% SNP T	SNP T	35.600	0.425	0.315
40% SNP G/ 60% SNP T	SNP G	35.285	0.389	
50% SNP G/ 50% SNP T	SNP T	35.338	0.561	-0.465
50% SNP G/ 50% SNP T	SNP G	35.803	0.966	
60% SNP G/ 40% SNP T	SNP T	35.038	0.401	-0.711
60% SNP G/ 40% SNP T	SNP G	35.749	0.317	
70% SNP G/ 30% SNP T	SNP T	34.768	0.294	-1.958
70% SNP G/ 30% SNP T	SNP G	36.726	0.778	
80% SNP G/ 20% SNP T	SNP T	34.778	0.505	-2.114
80% SNP G/ 20% SNP T	SNP G	36.892	0.576	
90% SNP G/ 10% SNP T	SNP T	34.439	0.262	-3.588
90% SNP G/ 10% SNP T	SNP G	38.027	1.155	
95% SNP G/ 5% SNP T	SNP T	34.549	0.384	-3.757
95% SNP G/ 5% SNP T	SNP G	38.306	1.099	
99% SNP G/ 1% SNP T	SNP T	34.379	0.471	-4.981
99% SNP G/ 1% SNP T	SNP G	39.360	0.503	
100% SNP G/ 0% SNP T	SNP T	34.186	0.262	-4.849
100% SNP G/ 0% SNP T	SNP G	39.035	0.562	

324 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP C/ 100% SNP G	SNP G	32.975	0.327	-1.869
0% SNP C/ 100% SNP G	SNP C	34.844	0.605	
1% SNP C/ 99% SNP G	SNP G	33.113	0.484	-2.298
1% SNP C/ 99% SNP G	SNP C	35.411	0.845	
5% SNP C/ 95% SNP G	SNP G	33.381	0.412	-1.816
5% SNP C/ 95% SNP G	SNP C	35.197	1.018	
10% SNP C/ 90% SNP G	SNP G	33.277	0.575	-1.278
10% SNP C/ 90% SNP G	SNP C	34.555	0.687	
20% SNP C/ 80% SNP G	SNP G	33.743	0.479	-0.573
20% SNP C/ 80% SNP G	SNP C	34.316	0.412	
30% SNP C/ 70% SNP G	SNP G	33.907	0.297	-0.506
30% SNP C/ 70% SNP G	SNP C	34.413	0.514	
40% SNP C/ 60% SNP G	SNP G	34.125	0.511	0.266
40% SNP C/ 60% SNP G	SNP C	33.859	0.474	
50% SNP C/ 50% SNP G	SNP G	34.801	0.999	1.015
50% SNP C/ 50% SNP G	SNP C	33.786	0.503	
60% SNP C/ 40% SNP G	SNP G	34.683	1.113	0.808
60% SNP C/ 40% SNP G	SNP C	33.875	0.571	
70% SNP C/ 30% SNP G	SNP G	34.550	0.356	0.906
70% SNP C/ 30% SNP G	SNP C	33.644	0.442	
80% SNP C/ 20% SNP G	SNP G	35.300	0.988	1.548
80% SNP C/ 20% SNP G	SNP C	33.752	0.579	
90% SNP C/ 10% SNP G	SNP G	36.520	1.357	2.898
90% SNP C/ 10% SNP G	SNP C	33.622	0.445	

95% SNP C/ 5% SNP G	SNP G	36.897	0.989	3.285
95% SNP C/ 5% SNP G	SNP C	33.612	0.346	
99% SNP C/ 1% SNP G	SNP G	35.990	0.381	2.470
99% SNP C/ 1% SNP G	SNP C	33.520	0.343	
100% SNP C/ 0% SNP G	SNP G	38.007	0.368	4.852
100% SNP C/ 0% SNP G	SNP C	33.155	0.370	

Cp19 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP G/ 100% SNP T	SNP T			
0% SNP G/ 100% SNP T	SNP G	26.247	1.420	
1% SNP A/99% SNP G	SNP G			
1% SNP A/99% SNP G	SNP A	26.985	1.331	
5% SNP A/95% SNP G	SNP G			
5% SNP A/95% SNP G	SNP A	26.842	1.353	
10% SNP G/ 90% SNP T	SNP T			
10% SNP G/ 90% SNP T	SNP G	26.703	1.387	
20% SNP G/ 80% SNP T	SNP T	27.851	1.890	0.641
20% SNP G/ 80% SNP T	SNP G	27.210	1.206	
30% SNP G/ 70% SNP T	SNP T	26.375	1.611	-1.936
30% SNP G/ 70% SNP T	SNP G	28.311	1.959	
40% SNP G/ 60% SNP T	SNP T	25.742	1.550	-3.53
40% SNP G/ 60% SNP T	SNP G	29.272	2.171	
50% SNP G/ 50% SNP T	SNP T	25.084	1.405	-6.308
50% SNP G/ 50% SNP T	SNP G	31.392	0.624	
60% SNP G/ 40% SNP T	SNP T	24.541	1.287	
60% SNP G/ 40% SNP T	SNP G			
70% SNP G/ 30% SNP T	SNP T	24.445	1.180	
70% SNP G/ 30% SNP T	SNP G			
80% SNP G/ 20% SNP T	SNP T	23.769	1.158	
80% SNP G/ 20% SNP T	SNP G			
90% SNP G/ 10% SNP T	SNP T	23.791	1.277	
90% SNP G/ 10% SNP T	SNP G			
95% SNP A/5% SNP G	SNP G	23.469	1.077	
95% SNP A/5% SNP G	SNP A			
99% SNP A/1% SNP G	SNP G	23.788	0.322	
99% SNP A/1% SNP G	SNP A			
100% SNP G/ 0% SNP T	SNP T	23.256	1.067	
100% SNP G/ 0% SNP T	SNP G			

Cp111 Assay	**SNP**	**Average Ct**	**Standard Deviation**	**dCt**
0% SNP T/ 100% SNP C	SNP C	27.727	1.416	
0% SNP T/ 100% SNP C	SNP T			
1% SNP T/99% SNP C	SNP C	28.487	1.792	
1% SNP T/99% SNP C	SNP T			
5% SNP T/95% SNP C	SNP C	27.806	1.386	
5% SNP T/95% SNP C	SNP T			
10% SNP T/90% SNP C	SNP C	27.822	1.567	
10% SNP T/90% SNP C	SNP T			
20% SNP T/ 80% SNP C	SNP C	27.836	1.297	

20% SNP T/ 80% SNP C	SNP T			
30% SNP T/ 70% SNP C	SNP C	28.338	1.608	
30% SNP T/ 70% SNP C	SNP T			
40% SNP T/ 60% SNP C	SNP C	28.792	1.494	
40% SNP T/ 60% SNP C	SNP T			
50% SNP T/ 50% SNP C	SNP C	29.361	1.553	
50% SNP T/ 50% SNP C	SNP T			
60% SNP T/ 40% SNP C	SNP C	30.031	1.866	-1.494
60% SNP T/ 40% SNP C	SNP T	31.525	1.604	
70% SNP T/ 30% SNP C	SNP C	30.282	1.703	-0.145
70% SNP T/ 30% SNP C	SNP T	30.427	1.204	
80% SNP T/ 20% SNP C	SNP C	31.879	0.689	2.375
80% SNP T/ 20% SNP C	SNP T	29.504	0.808	
90% SNP T/ 10% SNP C	SNP C			
90% SNP T/ 10% SNP C	SNP T	28.906	1.091	
95% SNP T/5% SNP C	SNP C			
95% SNP T/5% SNP C	SNP T	28.857	1.039	
99% SNP T/1% SNP C	SNP C			
99% SNP T/1% SNP C	SNP T	28.947	0.992	
100% SNP T/ 0% SNP C	SNP C			
100% SNP T/ 0% SNP C	SNP T	28.946	0.808	

* DNA mixtures that did not amplify each allele or did not fall within the trend of delta Ct's .

Table 17. Delta CT values for different concentrations of different SNP alleles. Results demonstrate the range over which these assays give dependable and statistically relevant results.

Summary.

The results presented in this final report demonstrate the utility of using quantitative real-time SNP assays to generate quantitative SNP profiles for different *R. communis* accessions and isolates. By extension, these same assays can be applied to residual DNA found in almost all ricin preparations. We have also demonstrated that it is possible to extract sufficient high quality DNA from a variety of ricin preparations to use in these assays and we have provided recipes and protocols that can be used for such DNA isolation. Together, LANL and LLNL investigators have generated 23 SNP assays – twenty that detect genomic SNP's and three that detect chloroplast SNP's. We have subjected these to a battery of QPPP tests and demonstrated the limits of their utility. Such assays should be able to unequivocally demonstrate whether two or more ricin sources came from the same preparation and large differences in the frequencies of some alleles will be an indication that different preparations probably did not come from the same castor bean source.

These assays also have definite utility in plant breeding studies and have already been requested by a group that is attempting to breed *R. communis* plants that produce castor beans with high oil content but no ricin.

It is now up to the sponsor to determine what will become of they assays, the reagents and the large numbers of DNA samples generated during the course of this study.