



Application of PCR to detect varietal purity in barley malt
by Debra Kay Habernicht

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Agronomy

Montana State University

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Abstract:

Genetic markers are used to detect inherited physical or molecular characteristics that differ among individuals. The field of forensic science has been revolutionized by the ability to extract and type DNA, from biological fluids left at a crime scene, using molecular techniques. These same techniques can be applied to the area of plant and seed identification. Currently, barley cultivar identification is based on morphological characteristics. The genetic diversity within the two-row and six-row germplasm is narrow and limits the usefulness of morphological characteristics. Also, once the barley is malted most of these morphological traits are undetectable. The North American Barley Genome Mapping Project (NABGMP) has generated large quantities of molecular markers for mapping. Most of the NABGMP clones have been sequenced and sequence-tagged-site (STS) specific primers developed for polymerase chain reaction (PCR)-based analysis. We used the STS-PCR approach to identify DNA fragments that differ in structure among genotypes. These primers with the use of endonucleases, provide a rapid analysis of DNA from young leaf tissue, harvested grain, or malted barley. Molecular markers were identified that differentiated cultivars within and between the two-row and six-row malting germplasm. We modified current DNA isolation protocols to have practical application in industry. These methods are described in detail. Using these techniques, cultivars were characterized for heterogeneity. Mixtures of cultivars were described and quantified. Bulk DNA extractions were used to detect if an allotment of malt was contaminated with an off-type cultivar. The limit of resolution was as low as 1% for some cultivars using specific primer sets. Single malt kernel extractions were used to quantify the mixture. These DNA isolation protocols and PCR-based technique are rapid, relatively inexpensive, and are more accurate than morphological traits. The use of molecular techniques and DNA typing can be used to estimate the brewing value of the malt based on varietal performance and ensure that finished malt meets customer specifications.

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APPROVAL

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Debra Kay Habernicht

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Genetic markers are used to detect inherited physical or molecular characteristics that differ among individuals. The field of forensic science has been revolutionized by the ability to extract and type DNA, from biological fluids left at a crime scene, using molecular techniques. These same techniques can be applied to the area of plant and seed identification. Currently, barley cultivar identification is based on morphological characteristics. The genetic diversity within the two-row and six-row germplasm is narrow and limits the usefulness of morphological characteristics. Also, once the barley is malted most of these morphological traits are undetectable. The North American Barley Genome Mapping Project (NABGMP) has generated large quantities of molecular markers for mapping. Most of the NABGMP clones have been sequenced and sequence-tagged-site (STS) specific primers developed for polymerase chain reaction (PCR)-based analysis. We used the STS-PCR approach to identify DNA fragments that differ in structure among genotypes. These primers with the use of endonucleases, provide a rapid analysis of DNA from young leaf tissue, harvested grain, or malted barley. Molecular markers were identified that differentiated cultivars within and between the two-row and six-row malting germplasm. We modified current DNA isolation protocols to have practical application in industry. These methods are described in detail. Using these techniques, cultivars were characterized for heterogeneity. Mixtures of cultivars were described and quantified. Bulk DNA extractions were used to detect if an allotment of malt was contaminated with an off-type cultivar. The limit of resolution was as low as 1% for some cultivars using specific primer sets. Single malt kernel extractions were used to quantify the mixture. These DNA isolation protocols and PCR-based technique are rapid, relatively inexpensive, and are more accurate than morphological traits. The use of molecular techniques and DNA typing can be used to estimate the brewing value of the malt based on varietal performance and ensure that finished malt meets customer specifications.

INTRODUCTION

The U.S. public, usually indifferent to matters scientific, has suddenly become obsessed with DNA (Lander and Budowle, 1994). The new-found fascination with nucleic acids does not stem from recent breakthroughs in genetic screening for breast cancer susceptibility or progress in gene therapy — developments which will indeed effect the lives of millions. Rather, it focuses on the murder case against the former U.S. football star, O. J. Simpson, and the use of DNA markers in genotype analysis.

DNA fingerprinting (typing) has become an important research tool in a broad range of disciplines. Paternity testing, diagnostic medicine, population genetics, animal and plant evolution research, archeology, conservation biology, wildlife and human forensic analysis, have all benefitted from improved techniques of genotypic analysis.

In contrast, U.S. Plant Varietal Protection (PVP) standards for crops are primarily based on plant and seed morphology. A Certificate of Protection is awarded to an owner of a cultivar after an examination shows that it is new, distinct from other cultivars, and genetically uniform and stable through successive generations (Stanton, 1994). Alan Atchley (personal communication, 1997), the Barley Examiner, explains that uniformity and stability are usually evaluated in barley cultivars by morphological characteristics. Cultivars that express under 5% variants are usually accepted as uniform, and stability must be observed over more than two generations. A distinction must be made between

the applicant's cultivar and any cultivar the PVP office can not tell apart from it based on their limited descriptions. Also, a clear difference must be made between the applicant's cultivar and a "most similar" cultivar. In crops other than barley, such as soybeans, distinction is sometimes made on molecular evidence. To date, issued barley PVP certificates have not been based on molecular differences; individual genes for disease resistance are the only molecular differences cited. This did not include pending certificates, such as Chinook. Some chemical properties (e.g. diastatic power, alpha-amylase activity) have been used to identify distinctness. No molecular markers have been used as such, even as supporting differences to some morphological difference.

DNA fingerprinting is a technique that identifies individual DNA samples. Just as no two individuals have the same fingerprint pattern, this technique produces a unique banding pattern exposing variation in one's DNA; identical twins are the exception. Genetic markers are the fundamental tool used in DNA fingerprinting.

Polymerase chain reaction (PCR) is a process by which a minute portion of DNA from an organism is selectively synthesized. If that synthesized portion of DNA differs in structure between two barley cultivars, that structure difference can provide the basis for varietal identification. The purpose of this work was to determine if sequence-tagged-site (STS) primers, derived from low-copy restriction fragment length polymorphism (RFLP) markers, amplified by PCR show differences in the DNA sequence among malted barley (*Hordeum vulgare* L.) cultivars. If so, could these markers be used to describe mixtures? The goal of this study was to determine the limits of resolution of varietal identification in malted barley.

This study was designed to have practical application in industry. Results from this work can be utilized by the maltster to determine the varietal purity of incoming barley. Protocols herein, may also be used as a method of quality control to ensure that finished malt meets customer specifications. The brewer can adopt these procedures as a method of quality assurance, to estimate the brewing value of the malt.

LITERATURE REVIEW

Genetic Markers

Any inherited physical or molecular characteristic that differs among individuals and is easily detectable is a potential genetic marker (Casey, 1992). Markers must be polymorphic to be informative. That is alternative forms must exist among individuals. Polymorphisms are variations in DNA sequence that occur in the nuclear, mitochondrial, or chloroplast genomes. Genetic markers can be analyzed at the level of morphology, protein variation, secondary metabolite variation, and at the level of DNA sequence variation.

Genomes of diploid organisms carry two copies of every chromosome, one copy derived from each parent. Genes (expressed DNA regions) are located at a particular position (locus) on the chromosome, but they may exist in alternate forms, termed alleles, which differ at the DNA sequence level. Proteins are encoded by genes on chromosomes. Different alleles may give rise to proteins that exhibit different properties, or there may be no detectable difference. An individual possessing two identical copies of a particular gene, or copies that give rise to a single protein type, is said to be homozygous at that locus. An individual carrying different alleles at a particular locus is said to be heterozygous (Reynolds et al., 1991). Cultivars of self-pollinated crops, such as barley, are primarily homozygous for all loci, but depending on the breeding strategy, may be

heterogeneous at a particular locus. The goal of genetic marker typing is to determine which alleles are present in specific individuals at genetically variable loci. Variations within exon sequences can lead to observable changes, such as differences in eye color, blood type, disease susceptibility, aleurone color, or rachilla hair length. These physical characteristics whose inheritance can be directly monitored are morphological markers. Variations in DNA sequence along a genome are common (Jeffreys, 1979). Most genomic DNA variation occurs within segments that have no known coding function. The majority of the known DNA sequence variations are apparently due to base-pair (bp) changes that create or destroy a cleavage site for a specific restriction enzyme, causing a change in the length of a DNA fragment (Nakamura et al., 1987). One clear advantage of genetic marker typing at the DNA level is the potential to detect genetic variation beyond that which results in a detectable change in a physical property of a protein (Reynolds et al., 1991) or the gross phenotype of an individual. These types of genetic markers are being discovered rapidly.

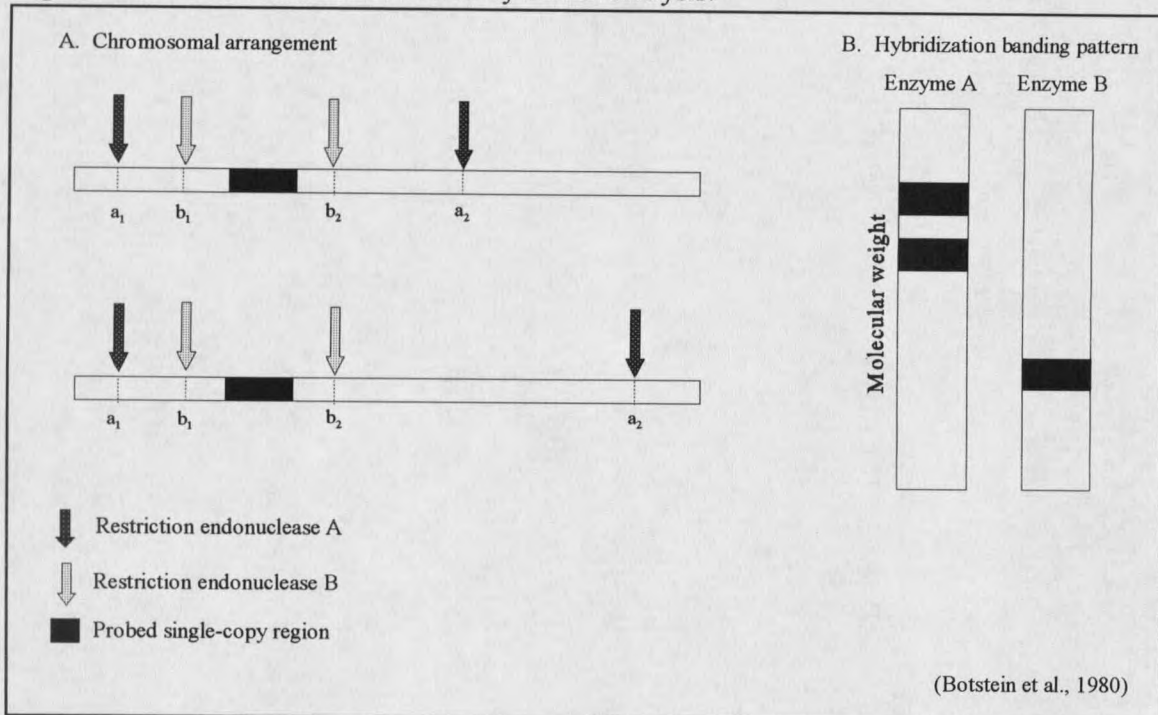
Molecular Marker Techniques

Restriction fragment length polymorphism (RFLP)

The first step toward the analysis of DNA sequence variation was the use of restriction fragment length polymorphism (RFLP) analysis (Jeffreys, 1979; Botstein et al., 1980). This was the first DNA-based method applied to problems of individual identification and the basis for constructing the genetic linkage map in man. Shin et al.

(1990) and Kleinhofs et al. (1993) also used this technique in developing the map of the barley genome.

DNA restriction enzymes recognize specific sequences in DNA and catalyze endonucleolytic cleavages, yielding fragments of defined lengths (Botstein et al., 1980). Restriction fragments may be displayed by electrophoresis in agarose gels, separating the fragments according to their molecular size (Figure 1). Differences among individuals in the lengths of a particular restriction fragment could result from many kinds of genotypic differences: one or more individual bases could differ, resulting in a loss of a cleavage site or formation of a new one; alternatively, insertion or deletion of blocks of DNA within a fragment could alter its size. These genotypic changes can all be recognized by the altered mobility of restriction fragments on agarose gel electrophoresis. The fragments are transferred to a nylon membrane, and incubated with a radioactive probe. The probe hybridizes to complementary sequences in the DNA fragments attached to the membrane, and the bound probe is visualized by autoradiography (Southern, 1975). The fragments to which the probe hybridizes vary in size between individuals.

Figure 1. Genetic variation revealed by RFLP analysis.

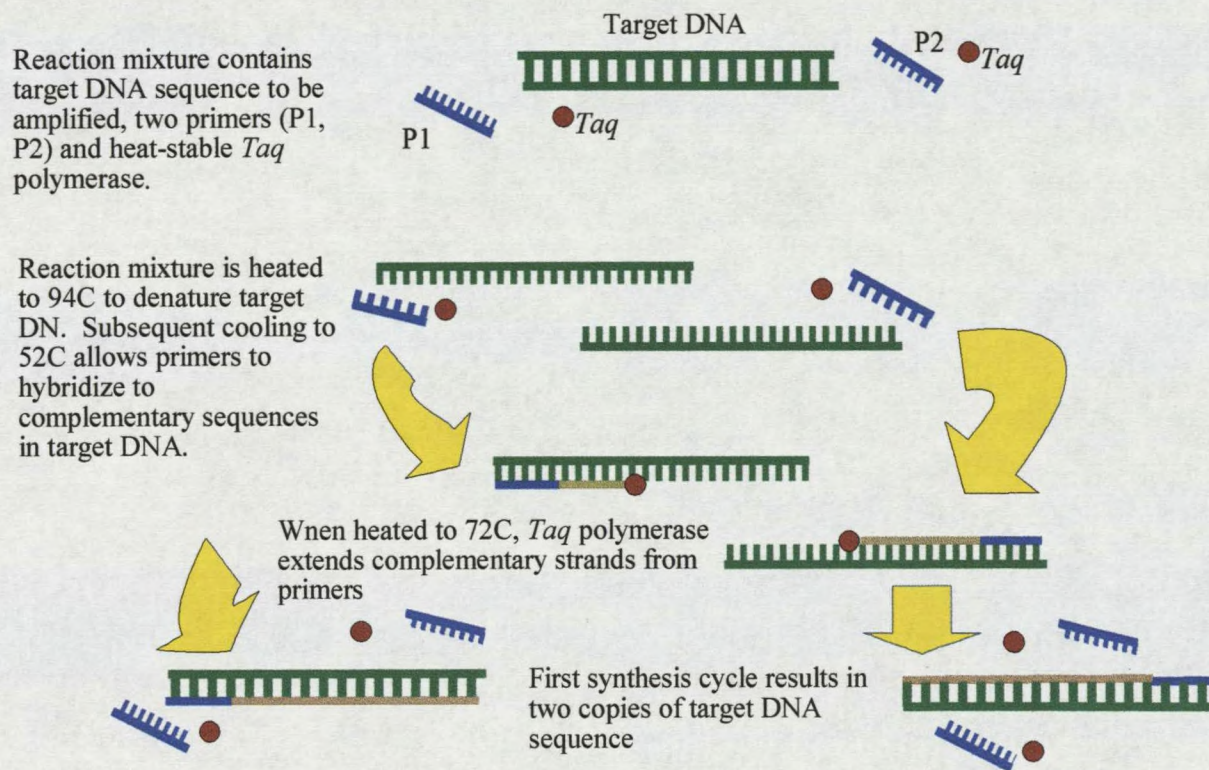
This method, while powerful in its ability to differentiate individuals, is limited by quantity and quality of DNA required for an unambiguous result by the amount of time it takes to obtain a result (Jeffreys et al., 1988; Reynolds et al., 1991). The method that overcame these limitations, devised in 1983 (Mullis and Faloona, 1987), and a molecular breakthrough in 1989 (Saiki et al., 1988; Eckert and Kunkel, 1990; Mullis, 1990) revolutionized the field of molecular biology. The method, polymerase chain reaction (PCR); the molecule voted the “Molecule of the Year” by *Science* magazine in 1989 (Guyer and Koshland, 1989) — the DNA polymerase molecule that drives the reaction. With PCR, tiny bits of embedded, often hidden, genetic information can be amplified into large quantities of accessible, identifiable, and analyzable material. A single cell provides enough material for analysis (Honghua et al., 1988); a single hair (Higuchi et al., 1988)

can be used to identify an individual (Guyer and Koshland, 1989).

Polymerase Chain Reaction (PCR)

PCR amplification involves two oligonucleotide primers that flank the DNA segment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase (Figure 2) (Mullis, 1990; Saiki et al., 1988). These primers hybridize to opposite strands of the target sequence and are oriented so DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover, since the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment, approximately 2^n , where n is the number of cycles.

Figure 2. Graphic representation of the first cycle of PCR.



(Mullis, 1990)

PCR-based Molecular Marker Typing

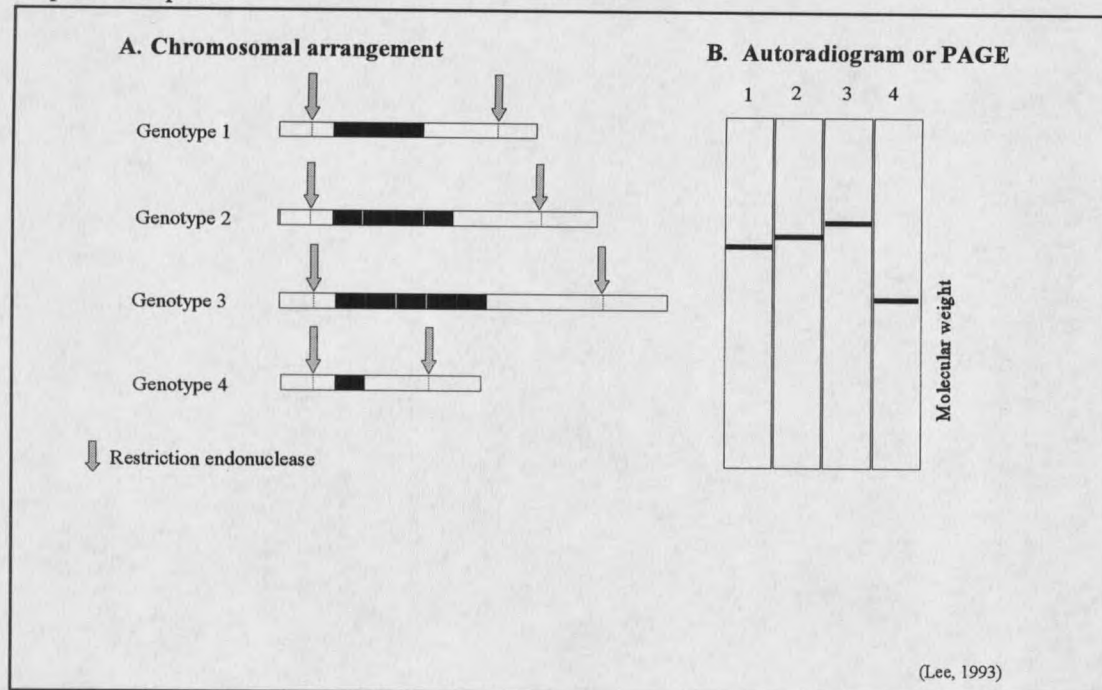
A sequence tagged site (STS) is a short, unique DNA sequence sequenced from a clone that defines a landmark on a chromosome (Olson et al., 1989). RFLPs, and other landmarks on a chromosome, can be used to design oligonucleotide primers for PCR based analysis (Mullis and Faloona, 1987; Saiki et al., 1985). A PCR assay for a STS could be implemented simply by synthesizing two short (~20 nucleotides) oligodeoxynucleotides chosen to be complementary to opposite strands and opposite ends of the sequence tract (Olson et al., 1989). STS-PCR provides an easier and quicker means to perform DNA typing and, it eliminates the use of radioactivity.

Genetic analysis is also simplified by probes and primers becoming available for hypervariable regions of DNA showing multiallelic variation and correspondingly high heterozygosities (Jeffreys et al., 1985a). This variable region in the genome, consists of tandem repeats of a short sequence (or 'minisatellite') and polymorphism results from allelic differences in the number of repeats, arising presumably by mitotic or meiotic unequal exchanges or by DNA slippage during replication. The resulting minisatellite length variation can be detected using any restriction endonuclease which does not cleave the repeat unit and provides for such loci a set of stable inherited genetic markers (Jeffreys et al., 1985a). A genetic sequence that contains tandem repeats but represents only a single locus, are identified as variable number of tandem repeats — VNTRs (Nakamura et al., 1987). Minisatellites are made of arrays of a core oligonucleotide sequence of 10 to 60 base pairs. Jeffreys et al. (1991) developed two multilocus DNA fingerprinting probes termed 33.6 and 33.15 that have been extensively applied to civil and criminal casework,

as well as resolving immigration disputes. These probes hybridize to multiple variable minisatellite fragments of human DNA to produce a highly individual-specific DNA fingerprint. Nelke et al. (1993) used Jeffreys' probes to detect variation in the red clover genome and distinguished individual-specific genotypes in seven cultivars.

Microsatellites (simple sequence repeats — SSR), composed of mono-, di-, tri-, and tetranucleotide repeats, are more randomly distributed, very frequent and can be scored by PCR (Weber and May, 1989; Rongwen et al., 1995). Hypervariable regions can be amplified by PCR, using primers from DNA flanking the mini- or microsatellite to amplify the entire block of tandem repeat units (Jeffreys et al., 1988). Microsatellites are well suited for searching for large numbers of alleles at a defined locus. The high levels of heterozygosity and the ease of automating PCR makes them ideal markers for DNA fingerprinting in human DNA. However, the mutation rate of microsatellites is unknown and may vary from locus to locus.

Figure 3. Graphical representation of variable number of tandem repeats or simple sequence repeats.



There are a number of fingerprinting techniques that require no prior investments in terms of sequence analysis, primer synthesis or characterization of DNA probes. There are a number of techniques that meet this requirement, two of which are random amplified polymorphic DNA (RAPD) and AFLPs. The name AFLP, was chosen based on the resemblance with the RFLP technique and should not be used as an acronym, because the technique will display presence or absence of restriction fragments rather than length differences (Vos et al., 1995).

RAPD involves the amplification of DNA segments using random sequence primers, generally of ten bases, to find polymorphic regions within the genome defined by the primer of arbitrary nucleotide sequence (Williams et al., 1990). The annealing

temperature in the thermal cycling profile is kept below 40°C to allow arbitrary primers to bind to the template DNA. This can cause some problems in later cycles of PCR, the random primers may attach to areas within the target DNA and amplify various sections of the template. Overlapping sets of DNA fragments may also occur. The products formed are analyzed by electrophoresis.

AFLP is a DNA fingerprinting technique that detects genomic restriction sites by PCR and resembles in that respect the RFLP technique, with the major difference that PCR amplification instead of Southern hybridization is used for detection of restriction fragments (Vos et al., 1995). This PCR-based method is a new time savings alternative for generating large numbers of polymorphic bands on polyacrylamide gels. This technique involves restriction of the DNA with two restriction endonucleases simultaneously (e.g., *MseI* and *EcoRI*), ligation of oligonucleotide adapters, selective amplification of sets of restricted fragments, and gel analysis of the amplified fragments.

Forensic Aspects of Molecular Marker Analysis

Biological fluids are key elements in the investigation of many kinds of crimes (Gill et al., 1985). The goal of the forensic serologist is to determine, by genetic marker typing, whether the biological evidence could have originated from a particular individual, either victim or suspect.

The ability to extract and type DNA from forensic evidentiary samples, has revolutionized the field of forensic serology (Jeffreys et al., 1985a). Forensic DNA

fingerprinting, represents perhaps the greatest advance in forensic science since the development of ordinary fingerprints in 1892, and is soundly rooted in molecular biology (Lander and Budowle, 1994; Marx, 1988).

Previously, genetic marker typing was limited to the analysis of blood group markers and soluble polymorphic protein markers (Gill et al., 1985; Reynolds et al., 1991). The best known and most venerable of the genetic markers used in forensic testing is the ABO blood group system; the ABO markers are quite stable and can be detected in most types of biological evidence. Since the discovery of the ABO blood group polymorphism in 1900, a large number of genetic variants have been analyzed in man (Jefferys, 1979). During the 1970's, the forensic genetic typing tools were expanded to include the electrophoretic detection of enzymes and other protein markers. Such analyses have demonstrated the existence of extensive polymorphisms which occur at the structural loci of many human proteins. Of the many protein markers found in blood, approximately 10 proved to be sufficiently robust to be typed in bloodstains. Unfortunately, only a few protein markers are present in other kinds of biological evidence such as semen. At this time there was almost no information on how much variation exists at the level of DNA sequence in man, and on what types of DNA sequence variants might occur in human populations.

Forensic biology was introduced into U.S. courts in 1988 (Lee, 1993; Lander, 1989; Lander and Budowle, 1994). Using minisatellite sequences in combination with single locus probes, Alec Jeffreys demonstrated that DNA could be extracted from virtually all forms of biological evidence. This greatly expanding the potential for

individual identification (Jeffreys et al., 1985b). Of particular importance it was found that the DNA from sperm could be differentially extracted from the DNA of other cell types, and typed independently. This extraction method greatly benefits the typing of sexual assault evidence which typically contains a mixture of sperm from the assailant and epithelial cells from the victim. This distinction was not possible with the blood and protein markers (Gill et al., 1985).

Closely following the introduction of RFLP analysis for typing forensic samples was the introduction of a PCR-based typing system suitable for the analysis of forensic samples (Reynolds et al., 1991). Consequently, DNA extracted from single hairs (Higuchi et al., 1988), small blood, semen (Honghua et al., 1988), and saliva stains, organ tissue, and even fragments of bone (Hagelberg et al., 1991) and teeth has been typed using PCR (Gill et al., 1985; Reynolds et al., 1991).

In the rush to use the tremendous power of DNA fingerprinting as a forensic tool, more than 80 criminal trials in the U.S. used DNA fingerprinting as evidence applying the admissibility of novel scientific evidence defined in *U.S. v. Frye* (Lander, 1989). Named after this 1923 ruling by the U.S. Court of Appeals for the District of Columbia Circuit (Lander, 1989; Lee, 1993), the Frye test requires a new scientific technique to have won general acceptance in the relevant scientific community before it can be used in court. In a challenge to "generally accepted", the National Research Council (NRC) ran a 3-year study to lay to rest the intense debate and scrutiny over DNA fingerprinting used as court evidence (Lander and Budowle, 1994). The controversy was not over using DNA fingerprinting as a powerful tool for forensic identification, it was the lack of standards

used and the over optimistic probabilities of an alleged match occurring at random (Lander, 1989). One such report, estimated a frequency of 1 in 738,000,000,000,000, based on a four-locus match. The NRC committee established rigorous lab practices and protocols as well as "the ceiling principle" (Lander and Budowle, 1994). The ceiling principle is founded on a theoretical model based on population genetics and the product rule of probability. Applying the conservative ceiling principle, a four-locus match performed by forensic labs could still provide odds of 6,250,000:1. If this were not enough, two additional loci could increase the odds to more than 15,000,000,000:1. The controversy is over, there is no scientific reason to doubt the accuracy of forensic DNA fingerprinting results, provided that the testing laboratory and the specific tests are on par with currently practiced standards in the field (Lander and Budowle, 1994).

Molecular Marker Analysis in Varietal Identification

Just as DNA typing has revolutionized forensic analysis, it may have a similar effect in the area of plant identification. Our particular interest, is varietal identification in barley and malted barley. Because barleys, and malt produced from them, differ in their processing behavior, maltsters and brewers have sought effective means of varietal identification (Huston et al., 1988; Bamforth and Barclay, 1993). Intermixing of even two malting barley cultivars can cause severe problems, in that both cultivars may not process the same during malting. For example, one cultivar may be water sensitive and the other require a longer steeping schedule. The cultivar that is water sensitive will drown, if the

maltster chooses to use a long steep cycle. The cultivar which requires a longer steep, if subjected to a short steep, will be under-modified as finished malt. For the brewer, high levels of non-germinating barley can result in reduced malt extract yield, poor starch conversion and run-off or filtration difficulties (Thomas and Palmer, 1994). Since traditional American Society of Brewing Chemists (ASBC), Institute of Brewing (IOB), and European Brewing Congress (EBC) malt analyses are performed on a homogenized, ground sample of malt, these differences in malt quality are not always detectable. Therefore, varietal purity is essential for processing in both the malthouse and brewhouse.

In some cases, morphological characteristics such as row-type, aleurone color or rachilla hair length are sufficient to identify barley cultivars. The increasing number of morphologically similar seeds and the fact most morphological characteristics are indistinguishable after the barley has been malted, has required other techniques to determine varietal purity in an allotment of barley or malt. Biochemical techniques that permit the correct classification of barley cultivars are rapidly evolving.

The development of more complex analytical methods included the electrophoretic examination of barley storage proteins. Gebre et al. (1986) cataloged 40 barley cultivars into 17 groups based on unique hordein protein banding patterns. The most extensive standard classification system published to date (White and Cooke, 1992), classifies 353 barley cultivars based on their B- and C-hordein composition following polyacrylamide gel electrophoresis (PAGE). Huston et al. (1988), found that isozyme electrophoresis on starch gels stained specifically for esterase activity provided clear differentiation between malting-type barley cultivars Robust and Morex. Jones and Heisel (1991) differentiated

seeds of several U.S. malting barleys, either by seed morphology, hordein, or esterase electrophoregrams. Isozyme electrophoresis was effective because each gel could be stained for many enzymes. However, the seeds must be germinated for four days, which is not practical for routine analysis in industry. Black and Durig (1995) used isoelectric focusing of three separate protein fractions. They successfully identified twenty-one barley cultivars based on banding patterns of hordeins, albumins/globulins, and peroxidase isoenzymes. Although several biochemical techniques have been found useful in barley varietal identification, none provides sufficient resolution to discriminate among all cultivars likely to be found in a mixture (Chee et al., 1993). Also, it has not been determined if these techniques will work using malted barley.

The North American Barley Genome Mapping Project (NABGMP) generated large quantities of molecular markers for developing linkage maps, using RFLP analysis (Shin et al., 1990; Graner et al., 1991; Jahoor, 1991; Pecchioni et al., 1993; Kleinhofs et al., 1993). Shin et al. (1990) characterized one locus with allelic variation. They sequenced the region around the insertion or deletion event, flanking oligonucleotide priming sets were identified, and PCR was performed using oligonucleotide primers. Larson et al. (1996) also demonstrated the conversion of RFLPs to a more practically useful PCR-based markers that were co-dominant and allelic to the RFLP markers from which they were derived. Blake et al. (1996b) described and released 115 STS-PCR primer sets which direct the amplification of 135 products from barley which can be unambiguously distinguished from their wheat counterparts by simple gel electrophoresis (Talbert et al., 1994). PCR technology and markers converted to their sequence-tagged-

site, is a technique that is suitable for mapping (Tragoonrung et al., 1992), fingerprinting or purity testing. Chee et al. (1993) selected 14 cultivars important to the malting and brewing industry and without error, uniquely identified each of them. Ko and Henry (1994) had similar success using primer sequences from alpha-amylase genes to distinguish between morphologically similar malting and feed barley cultivars. They also (Ko et al., 1994) investigated cereal cultivars and species using PCR with arbitrary primers (RAPD) and primers specific to the 5S ribosomal RNA spacer. Weining and Langridge (1991) also found polymorphisms in cereals using PCR, useful for identification and mapping.

Saghai Maroof et al. (1994) found extraordinarily polymorphic microsatellite DNA in barley. Becker and Heun (1995) surveyed barley sequence data from GenBank and EMBL to determine which simple sequence repeat (SSR) motif prevailed in barley. Nearly all types were found. SSR DNA markers were evaluated using 96 diverse soybean cultivars to distinguish uniqueness for purposes of plant variety protection (Rongwen et al., 1995). Only two genotypes, having similar pedigrees, had similar SSR allelic profiles. Plaschke et al. (1995) used only a small number of microsatellites to distinguish closely related elite wheat breeding material, to carry out phylogenetic studies, and to select cultivars for highest genetic diversity. Because of the higher resolving power of microsatellites, a co-dominant inheritance pattern, their distribution throughout the entire genome, an absence of radioactive nucleotides, and a less demanding technique, microsatellites may become the marker of choice.

METHODS AND MATERIALS

Genetic Stock

Barley Cultivars

The eight barley cultivars screened (Table 1) were selected based on their importance in the malting and brewing industry. B1202 was developed at Busch Agricultural Resources, Inc. (BARI). Chinook was developed by the Montana Agricultural Experiment Station (Blake et al., 1996a). Harrington (Harvey and Rossnagel, 1984) and Manley were developed at the Crop Science Department, University of Saskatchewan. Excel (Rasmusson et al., 1991), Morex (Rasmusson and Wilcoxson, 1979), Robust (Rasmusson and Wilcoxson, 1983) and Stander (Rasmusson et al., 1993) were developed by the Minnesota Agricultural Experiment Station. Approximately 300 plants of each cultivar were grown from breeders' seed (produced by the barley breeding program) at Bozeman, MT, with the exception of B1202 which was donated from BARI. Seeds were planted 2.54 cm apart in flats filled with moist Sunshine® potting mixture. Cultivars were harvested after two weeks of growth under greenhouse conditions.

Barley Malt

Micromalting was conducted using a custom built Webber micromalting unit.

Samples (500 g) of the eight cultivars were steeped at 15°C for six hours and air rested eleven hours in three cycles for a total steep schedule of 18/22 hours. Moisture of steeped grain was maintained at 45% by adding water. Seeds were germinated for four days at 15°C and 90% relative humidity. For kilning, temperature was held at 65°C for 24 hours. Samples were cleaned to resemble commercial malt.

Table 1. Pedigree, date released, and head type of barley cultivars used in this study.

Cultivar	Pedigree	Released	Head Type
B1202	RPB70-268/2B75-1223//‘Klages’	1989	Two-row
Chinook	‘Hector’/‘Klages’	1995	Two-row
Harrington	‘Klages’/3/‘Gazelle’/‘Betzes’//‘Centennial’	1981	Two-row
Manley	‘Norbert’//‘Hector’/‘Klages’	1990	Two-row
Excel	‘Cree’/‘Bonanza’//‘Manker’/3/2‘Robust’	1990	Six-row
Morex	‘Cree’/‘Bonanza’	1978	Six-row
Robust	‘Morex’/‘Manker’	1983	Six-row
Stander	‘Excel’//‘Robust’/‘Bumper’	1993	Six-row

DNA Isolation

Leaf Tissue or Bulk Malt Kernel

Genomic DNA was prepared using a modified version of the protocol described in Dellaporta et al. (1983). For detailed procedure, see Appendix.

Young leaf tissue was ground to a fine powder under liquid nitrogen; malt was ground to a fine powder using a Micro-mill, and incubated at 65°C in extraction buffer (100 mM Tris, pH 7.6-8.0, 50 mM EDTA, pH 8.0, 100 mM NaCl, 1% SDS, 10 mM mercaptoethanol) for 60 min. Weights and volumes for each method are listed in Table 2. All extracts were transferred to a tube or bottle and 5 M potassium acetate was added. Samples incubated on ice for 60 min, to remove proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulfate precipitate, and centrifuged for 20 min. Extract was filtered through a Miracloth filter into a clean tube or bottle containing chilled 2-propanol and 5 M ammonium acetate and incubated for 20 minutes at -20°C. Precipitated DNA was hooked out using a curled glass pasteur pipet and air dried. Dried DNA was redissolved in TE. DNA extracted from tissue was cleaned up by adding 3M sodium acetate, pH 7.0 and chilled 2-propanol. DNA was spun for 30 seconds in a microfuge. Supernatant was poured off and tubes were left at room temperature until dry. Pellet was redissolved in 50 µl TE. Concentrated DNA was diluted 1:20 for PCR. Concentrated DNA from bulk malt kernel extractions was used for PCR. The isolated DNA was stored at -20°C until use.

Table 2. Unit requirements for extracting DNA from plant tissue and 100 malt kernels.

Genetic Stock	Leaf Tissue	Barley Malt (Bulk)
Weight	0.40 g young leaf tissue	4 g malt flour (100 kernels)
Extraction Buffer	5 ml	50 ml
Bottle/Tube Size	6.5 ml centrifuge tube	250 ml polypropylene bottle
5M Potassium Acetate	1.67 ml	16.7 ml
Centrifuge Speed	5,000 x G	10,000 x G
Bottle/Tube Size	17 x 100 mm culture tube	125 ml polystyrene bottle
2-propanol	4 ml	40 ml
5M Ammonium Acetate	330 μ l	3.30 ml
Sterile Tube	1.7 μ l Eppendorf tube	6.5 ml culture tube
TE	230 μ l	300 μ l
3M Sodium Acetate	25 μ l	-
2-propanol	167 μ l	-
TE	50 μ l	-

Single Malt Kernel

Genomic DNA samples from a single malt kernel was obtained using the protocol described by Edwards et al. (1991). A single cleaned malt kernel was crushed between weigh paper, using a set of pliers or hammer. The crushed kernel was collected in sterile Eppendorf tubes. 400 μ l of extraction buffer (200 mM Tris HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and each sample vortexed for 5 seconds. The extracts were centrifuged at 13,000 rpm for one minute and 300 μ l of the supernatant transferred to a fresh Eppendorf tube. This supernatant was mixed with 300 μ l of chilled

2-propanol and left at room temperature for 2 minutes. The samples were centrifuged a second time for 5 minutes and left at room temperature until all traces of liquid had evaporated. Samples were dissolved in 100 μ l TE. Concentrated DNA was used for PCR. The isolated DNA was stored at -20°C until use.

Molecular Analysis

All amplifications were done in 50 μ l, in 200- μ l microfuge tubes under one drop (approximately 50 μ l) of mineral oil. Each reaction mix included 0.25 units Taq polymerase, 0.2 μ M primers, approximately 50 ng DNA, 0.1 mM each dNTP, 1.5 mM MgCl₂ and 10 x reaction buffer. Reactions were performed in either: 1) in a Coy model 110S-96 thermocycler, using the following program: Following initial denaturation (92°C, 5 min), samples were subjected to 35 cycles of 1 min at 92°C, 1 min at 50°C, 1 min at 72°C, followed by a final extension reaction of 5 min at 72°C; or 2) in a Perkin-Elmer model 9600 thermocycler, using the following program: Following initial denaturation (94°C, 4 min), samples were subjected to 35 cycles of 1 min at 94°C, 1 min at 52°C, 1.2 min at 72°C, followed by a final extension reaction of 5 min at 72°C.

All samples were analyzed in 7% polyacrylamide gels (29:1 ratio of monomer to crosslinker) in 0.5 x TBE. Gels were run for 2.0 hours at 225 volts, stained with ethidium bromide and photographed using a CCD camera and thermal printer.

RESULTS AND DISCUSSION

Table 3 provides a list of primer sets selected for this experiment, which were selected based on allelic variation among the cultivars selected. The six clones used to design STS-PCR primers included three derived from genomic DNA RFLP (ABG) and three derived from cDNA RFLP (ABC) obtained from the North American Barley Genome Mapping Project (NABGMP) (Kleinhofs et al., 1993). The allelic states for each genotype are characterized in Table 4. Figure 4 - 9 demonstrates the clarity of amplified restriction site polymorphisms and allelic variation due to a difference in amplified fragment size.

Table 3. Primer sequences and chromosomal location for the six primer sets selected.

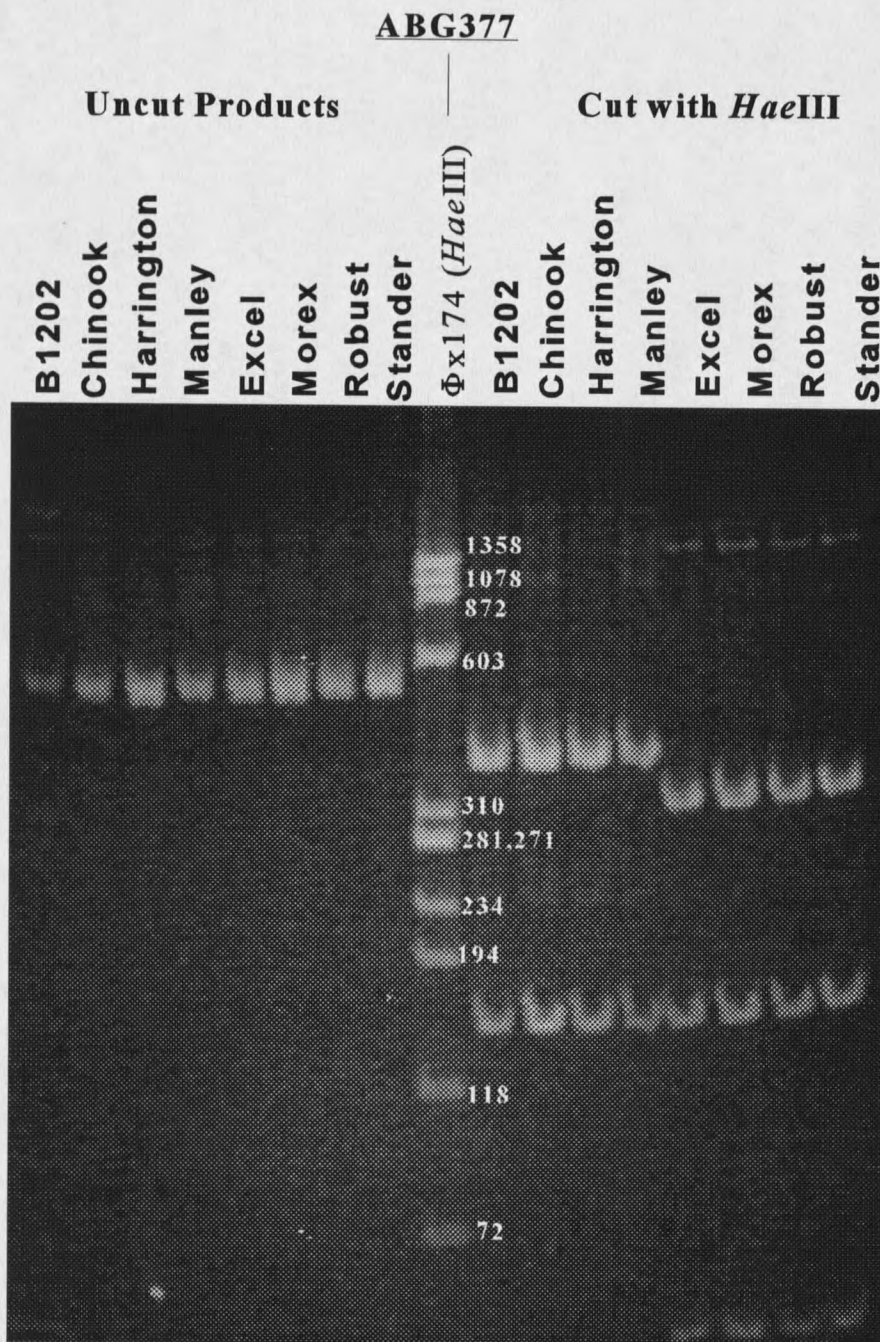
Primer Set	Primer Sequence	Chromosomal Location
ABG 380	5'CACGAACAGAAGGAGACTAA3' 5'TGGTATGAAACAGGTGAATA3'	1
ABC 454	5'GCGTGCGAGGGGAAGGAGAA3' 5'TTCAACGAGGCATCAATCTT5'	2
ABG 070	5'GGACCAAGCAAATATCTCAG3' 5'AACACGAGTTTGAATTTTAC3'	3
ABG 377	5'GCTGCTATGAGGAGAGAACC3' 5'TGGTATGAAACAGGTGAATA3'	3
ABC 717	5'CAATACGGCAACAAATAACA3' 5'CCCCACCAAATTACCAGTC3'	5
ABC 253	5'GCATGGTGACAGATTTCAA3' 5'AGGGAATGCAGATCTCACAC3'	7

Table 4. Summary of the allele states for primer sets and endonucleases used to determine the correct genotype for each cultivar.

Cultivar	Primer and Endonuclease					
	ABG 380 <i>DpnII</i>	ABC 454 uncut	ABG 070 <i>HinFI</i>	ABG 377 <i>HaeIII</i>	ABC 717 <i>HaeIII</i>	ABC 253 <i>DdeI</i>
BA 1202	▲	▲	▲	▲	▲	▲
Chinook	▲	▲	●	▲	▲	●
Harrington	●	▲	●	▲	●	▲
Manley	●	●	▲	▲	▲	●
Excel	■	▲	▲	●	●	■
Morex	●	▲	▲	●	▲	● / ■
Robust	■	▲	▲	●	▲	■
Stander	■	●	▲	●	●	■

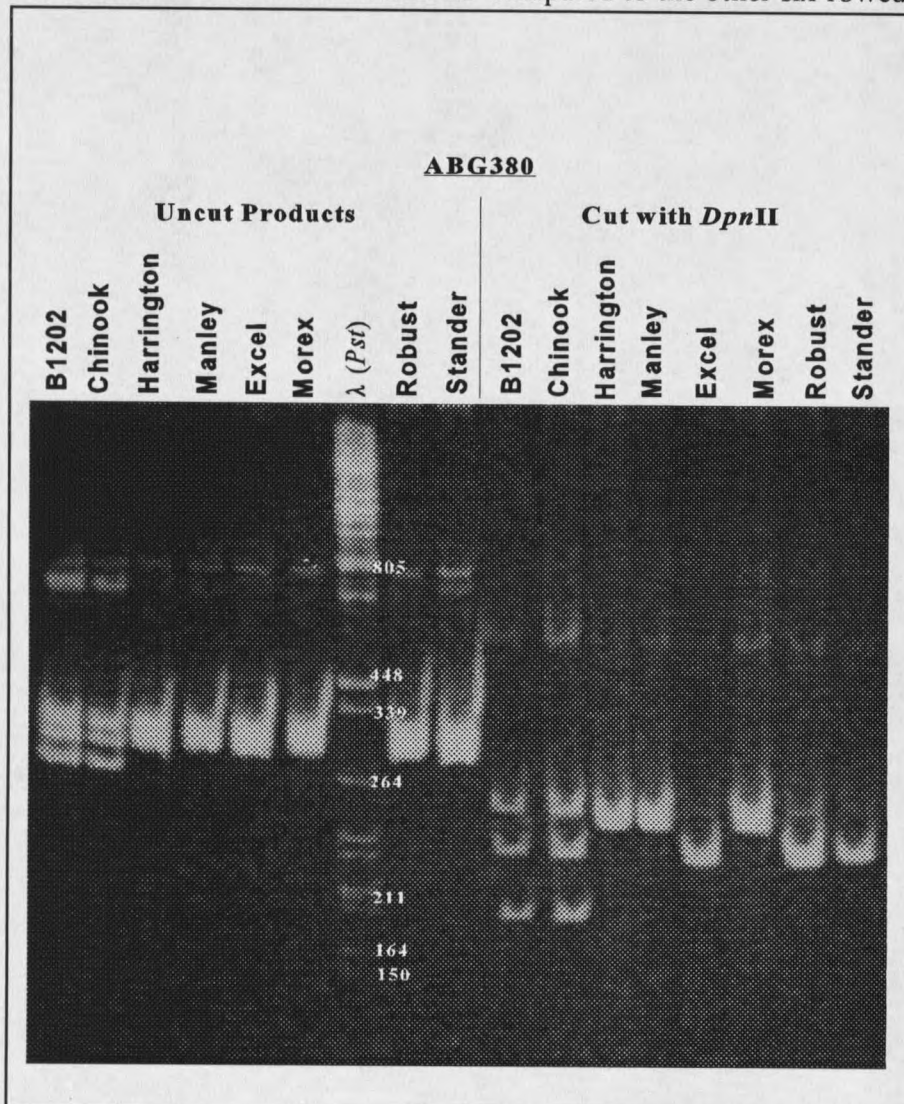
A single 595 bp product is produced using ABG377 primer set, which when cut with *HaeIII* clearly distinguishes six-rowed cultivars from two-rowed cultivars (Figure 4). ABG377 resides on barley chromosome-3 and happens to be very close to one of the genes important for yield, especially in six-row backgrounds (Larson et al., 1996). The allele found in the six-rowed cultivars in this study, is particularly unfavorable, resulting in a 10% reduction when compared to lines carrying the alternative allele.

Figure 4. ABG377 uncut and cut with endonuclease *Hae*III. The cut products distinguish two-rowed from six-rowed cultivars.



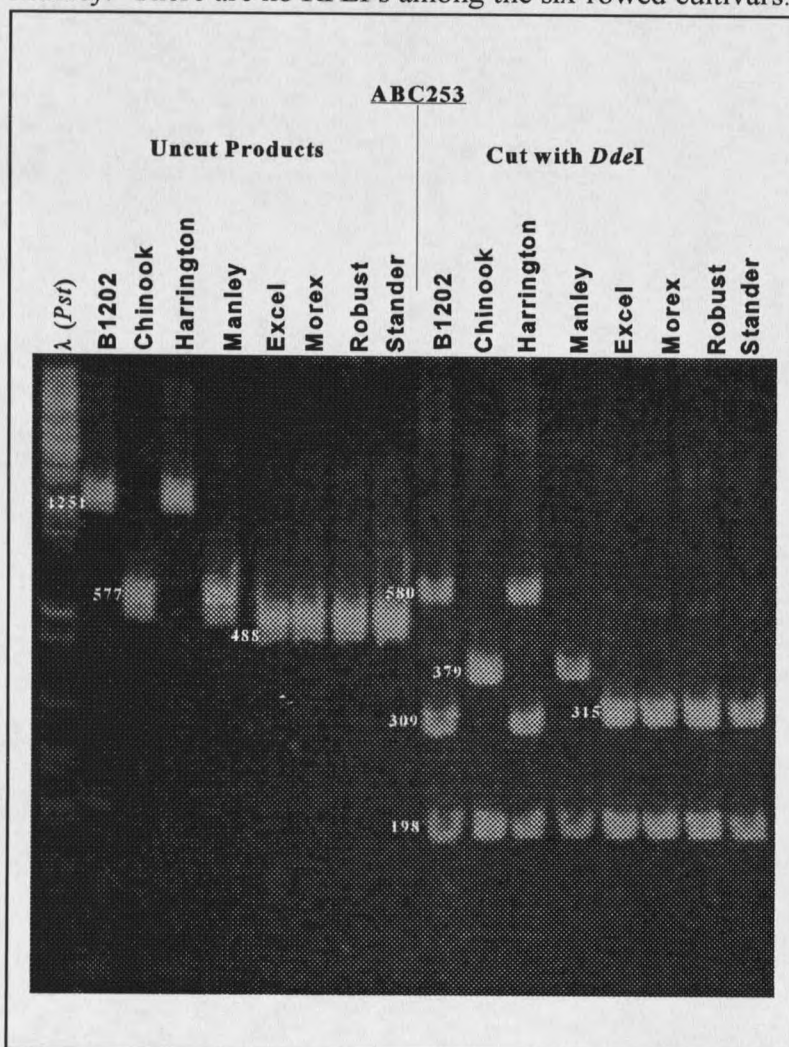
Only two primer sets are necessary to distinguish between the two-rowed barleys. ABG380 is located on barley chromosome-1 and maintains a high level of sequence variation among the two-rowed cultivars, in regards to fragment size difference (Figure 5). B1202 and Chinook have three products (262 bp, 224 bp, 187 bp), while Harrington and Manley have a single 252 bp product. When cut with *DpnII*, this marker also distinguishes Morex (252 bp) from the other six-rows (220 bp).

Figure 5. ABG380 uncut and cut with endonuclease *DpnII*. Among the two-rowed, Harrington and Manley are distinguished from B1202 and Chinook. Morex shows a RFLP compared to the other six-rows.



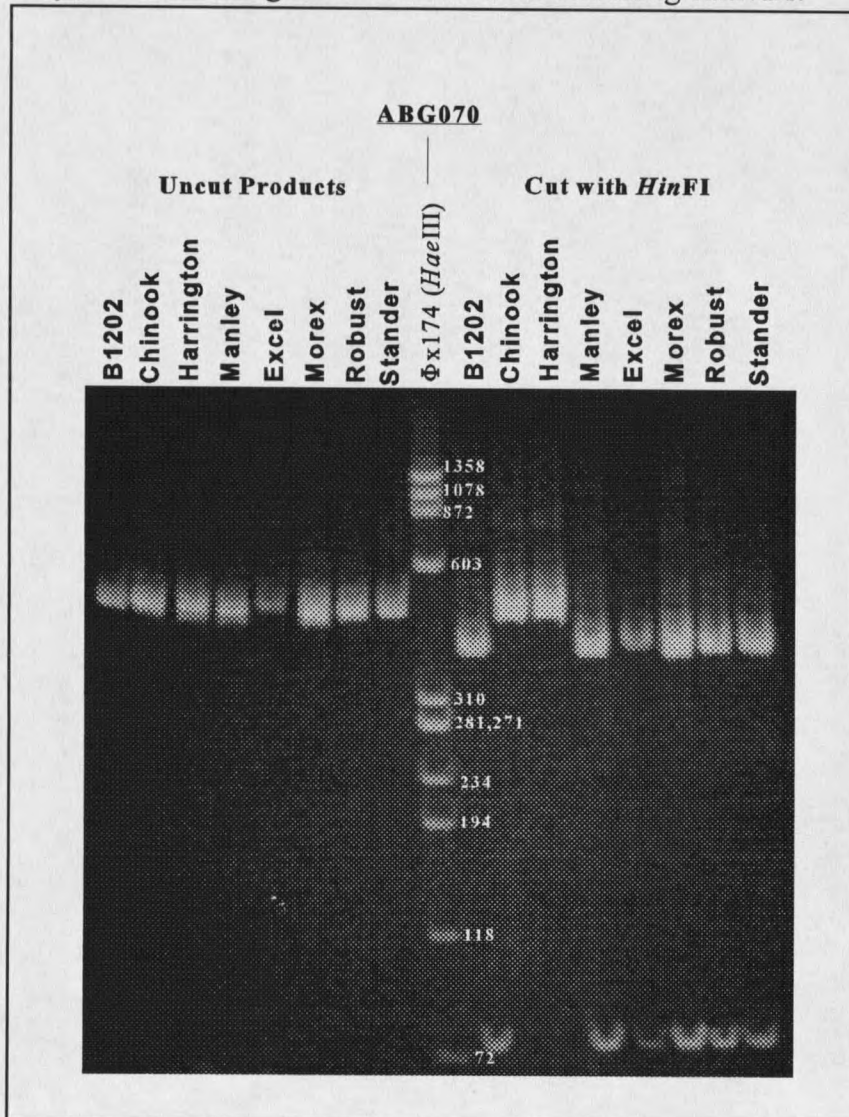
ABC253 also shows a high level of variation in product sizes. This marker resides on barley chromosome-7. B1202 and Harrington have a larger single product (1251bp), with at least two restriction sites for *Dde*I, while Chinook and Manley have only one restriction site in their smaller product fragment of 577bp (Figure 6). Six-rowed cultivars have a single product size of 488bp using this marker and one restriction site for this enzyme.

Figure 6. ABC253 uncut and cut with endonuclease *Dde*I. B1202 and Harrington vary in product size from Chinook and Manley. There are no RFLPs among the six-rowed cultivars.



ABG070 is located near the distal end on barley chromosome 3 and is known to co-segregate with barley chromosome-3 telomere sequences (Larson et al., 1996). It produces a single product consisting of 453bp (Figure 7). When cut with endonuclease *HinFI*, a 354bp product can be seen in all the cultivars except for Chinook and Harrington. Their products are not digested by *HinFI*.

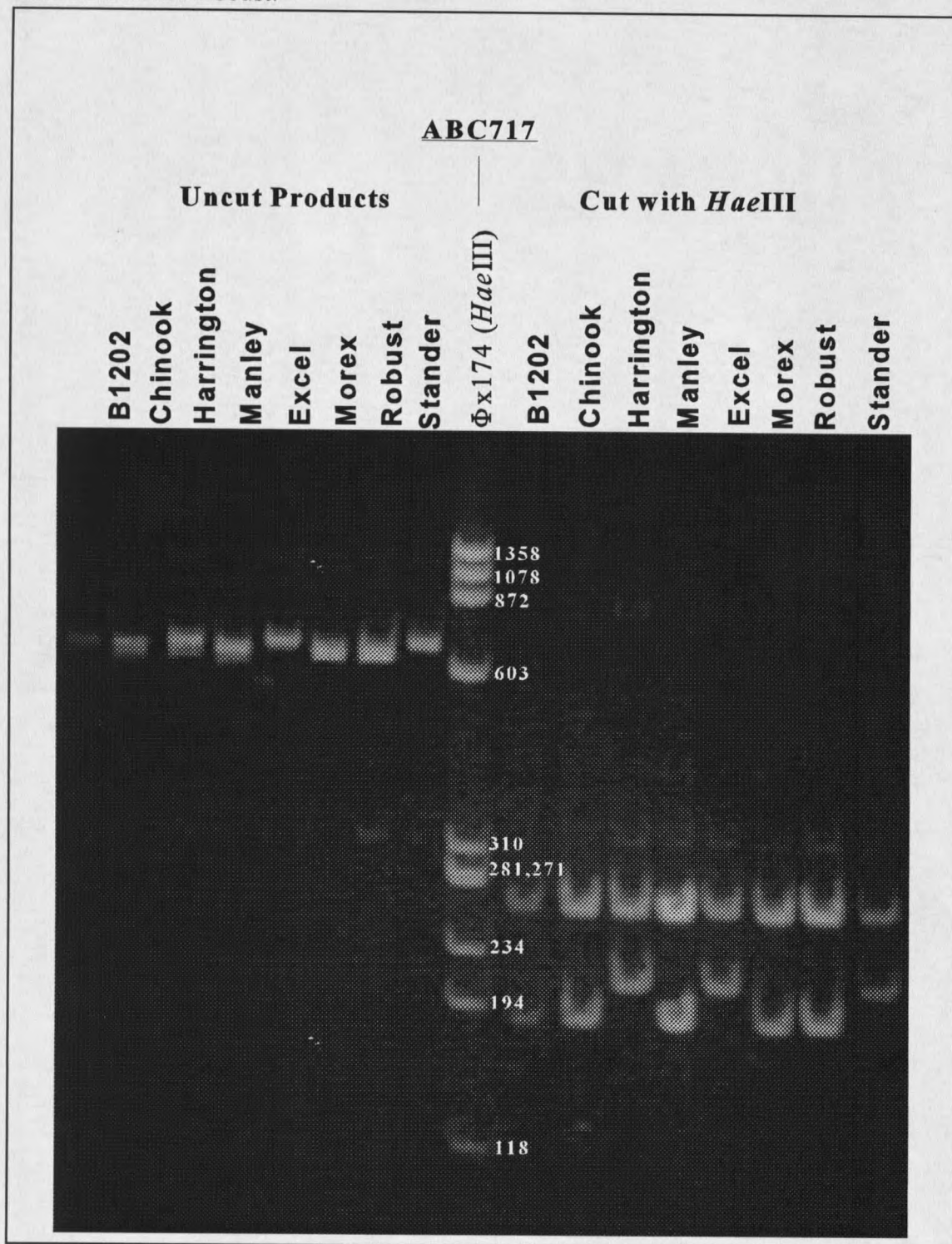
Figure 7. ABG070 uncut and cut with endonuclease *HinFI*. Chinook and Harrington do not have a restriction site for this enzyme. This distinguishes them from the remaining cultivars.



Even though ABG070 was not required to distinguish further among the two-rowed cultivars, we kept it in the screening because the polymorphisms in banding patterns were due strictly to variation in endonuclease restriction sites. This can be an advantage when screening for varietal purity, because the PCR technique prefers to amplify smaller products over larger ones, therefore both products have equal chance of being amplified. However, if the off-type cultivar has the smaller product, the preference in amplifying the smaller product will increase sensitivity in the test.

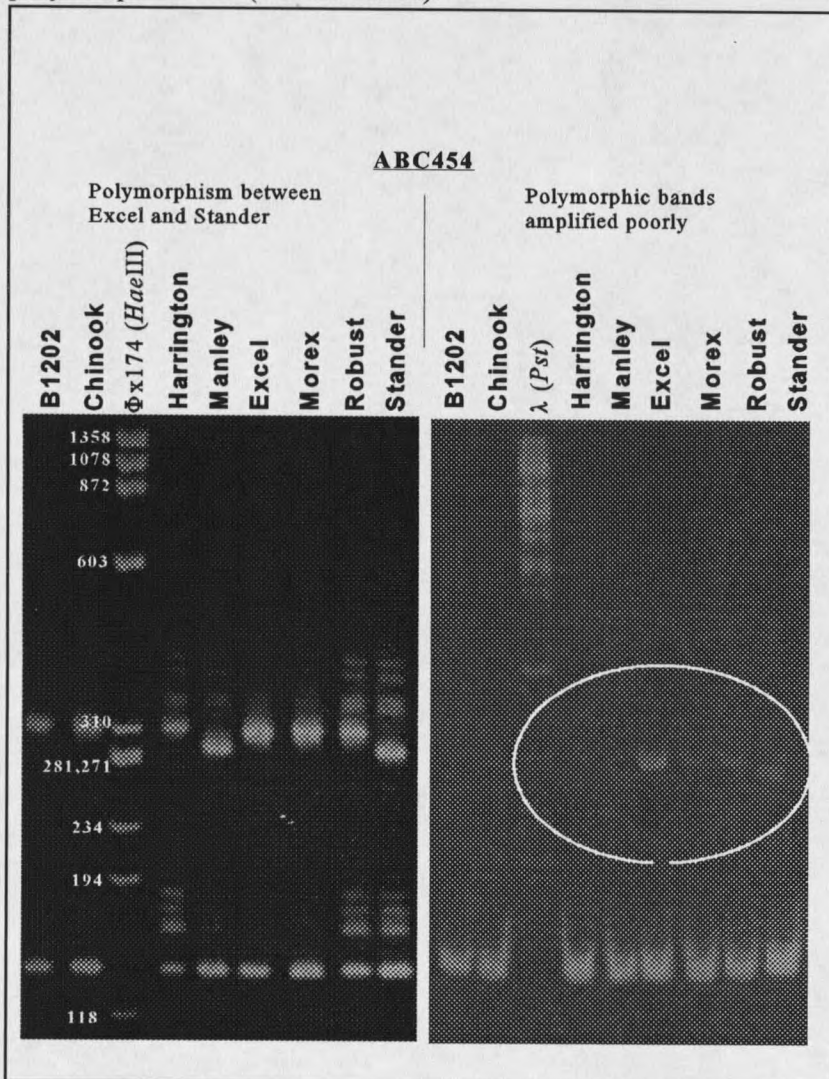
Among the six-rowed barleys, more primer sets are required to differentiate among them due to the small genetic base used in crossing these cultivars for malting quality. As mentioned, ABG380 cut with *DpnII*, distinguishes Morex from Excel, Robust, and Stander. Morex was found to be heterogeneous at the locus for ABC253. ABC717 has a single product around 650bp and is located on barley chromosome-5 (Figure 8). It utilizes *HaeIII* to reveal a 24bp difference between alleles of Excel and Stander (263 bp, 192 bp, 73 bp) versus Morex and Robust (263 bp, 168 bp, 73 bp). This primer set also distinguishes Harrington from the other two-rowed cultivars, carrying the same allele found in Excel and Stander.

Figure 8. ABC717 uncut and cut with endonuclease *Hae*III. Harrington is distinguished among the two-rowed, the same allele is seen in Excel and Stander, which identify them from Morex and Robust.



ABC454, located on barley chromosome-2, does not require an endonuclease to revealed a small 18bp variation between Excel (305 bp) and Stander (287 bp) (Figure 9). However, using a standard PCR program, this primer set prefers to amplify the small 142bp product over the polymorphic fragments. Therefore this primer set is not reliable for these cultivars.

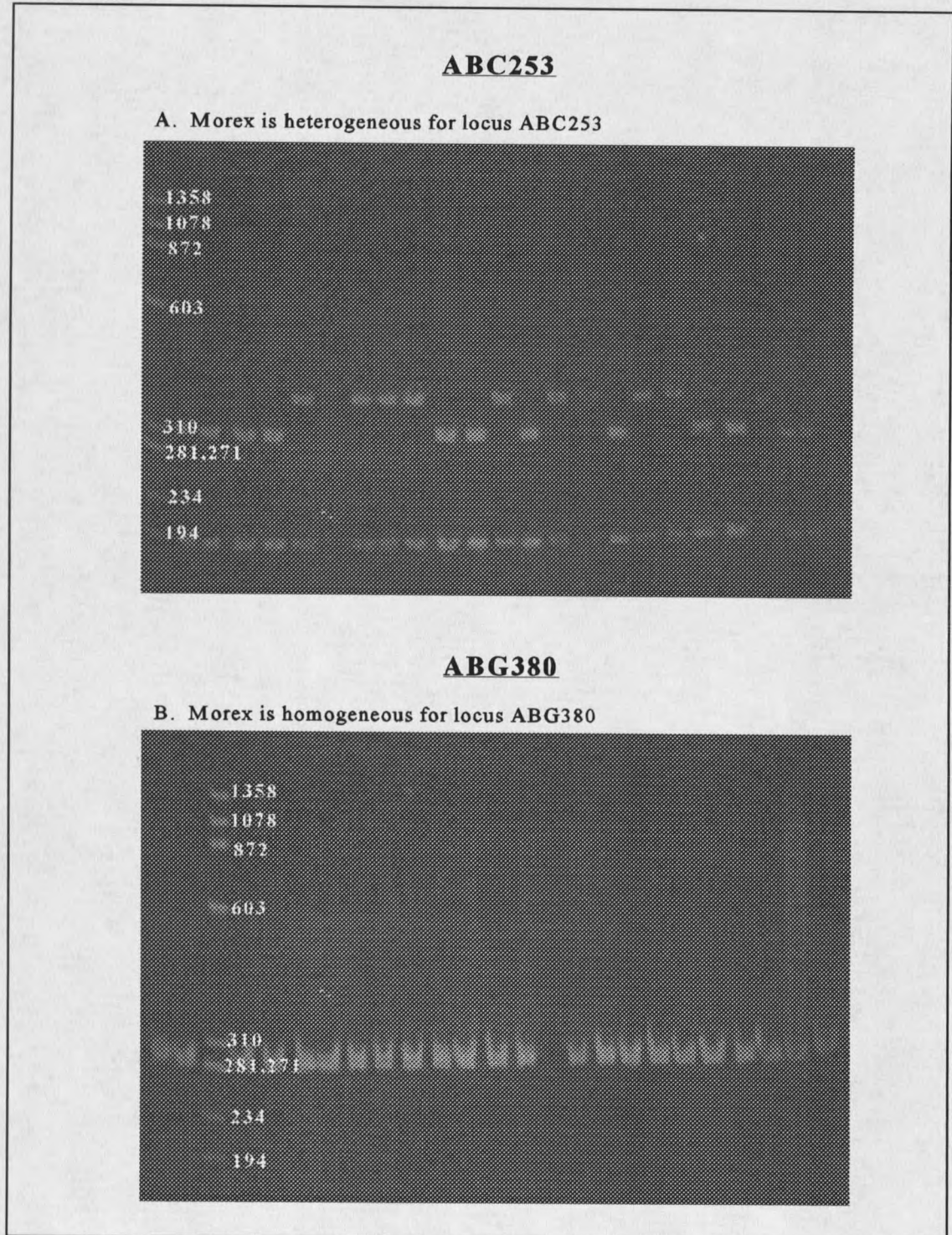
Figure 9. ABC454 uncut products distinguish Excel from Stander, Manley has the same allele as Stander. This primer is not reliable for these cultivars because under a standard program, the non-polymorphic bands amplified over the larger polymorphic ones (circled bands).



PCR-based molecular markers are a powerful approach to the characterization of barley cultivars. In contrast to Black and Durig (1995), who doubted the use of this technique for routine purity analysis, we believe this methodology is practical and may be adopted into an industrial quality control laboratory. We agree with Black and Durig in the fact that a minimum of 100 seeds are required to determine the purity of an allotment of barley. We introduce a modification to the DNA extraction method that we labeled the "bag technique". This technique renders high quality DNA yields for up to 96 samples per run. This is an appropriate number, since it coincides with the capacity of a standard thermocycler. Genomic DNA extracted from this procedure is suitable for PCR and AFLP (data not shown). This technique can be used for tissue, seeds or malted barley.

Before mixtures could be described, the cultivars had to be characterized for heterogeneity. DNA isolated from 96 plants per cultivar was tested to determine if the cultivar was homogeneous at each loci. With the exception of Morex, all cultivars were homogeneous at the locus necessary to distinguish that particular cultivar (data not shown). Morex was the only one found to be heterogeneous, with a frequency of approximately 0.5, at the locus for ABC253 (Figure 10A) but homogeneous at the locus for ABG380 (Figure 10B).

Figure 10. A. Morex is heterogeneous using ABC253; B. Morex is homogeneous with primer set ABG380.



Bulk DNA extractions can be used to detect if an allotment of malt is contaminated with an off-type cultivar. Duplicate blends of 100 kernels of Manley:Harrington malt were made. Proportion of Manley ranged from 1% to 95%. Manley in Harrington can be detected at levels as low as 1%, using primer set ABC253 (Figure 11), because the small fragment size of Manley is exaggerated due to preference of *Taq* polymerase to amplify a smaller fragment. Harrington in blends with Manley, using this primer set, can be detected at a level of 15-20%.

ABG070 can also be used to describe this mixture (Figure 12). The limit of resolution is between 5 and 20% for detecting a mixture. At levels above 25%, there is no question of having a mixture.

Figure 11. Bulk blends of Manley and Harrington are amplified using primer set ABC253. Manley in Harrington can be described as low as 1%; Harrington in Manley can be detected around 15%. Duplicate samples are shown.

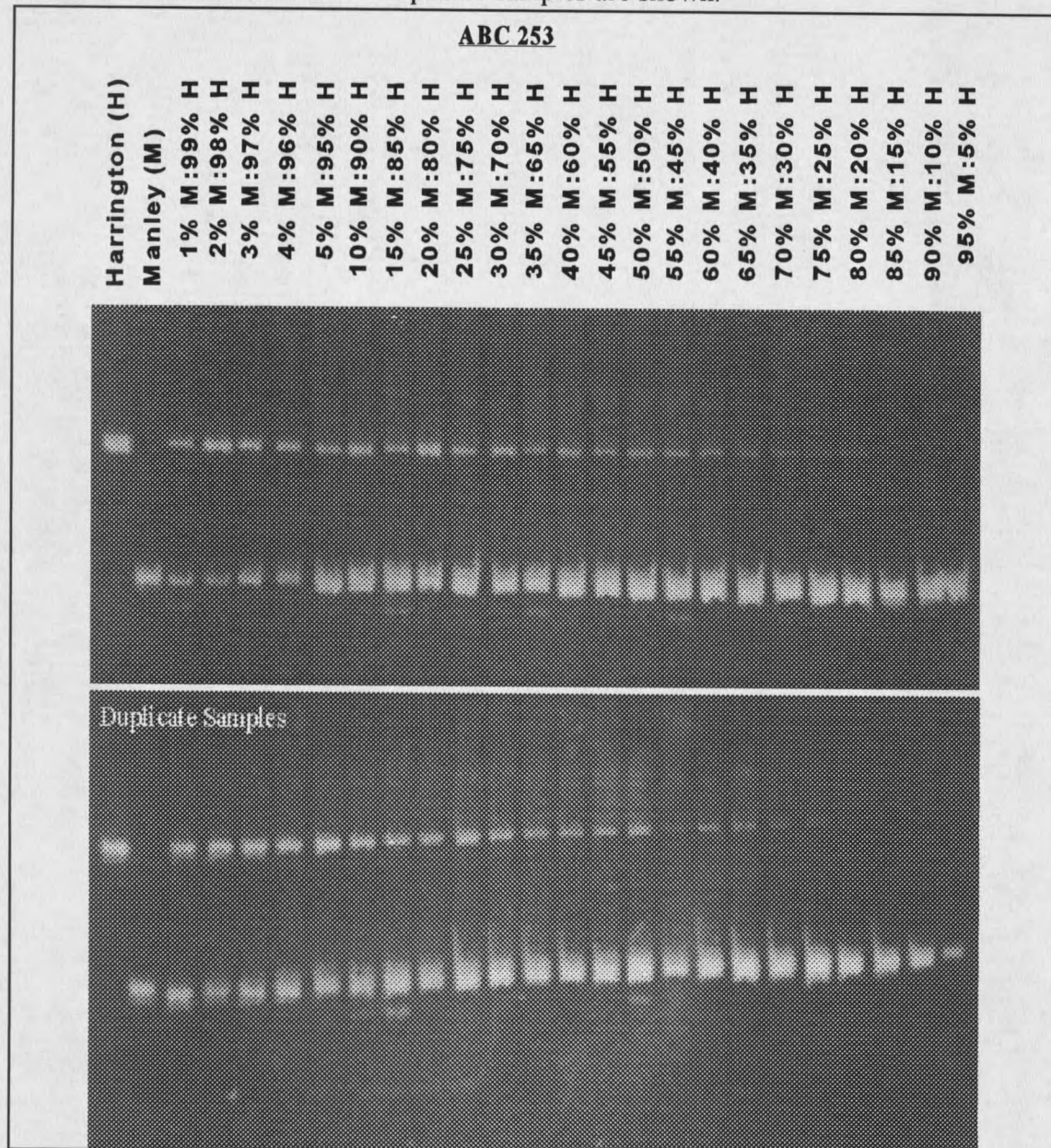
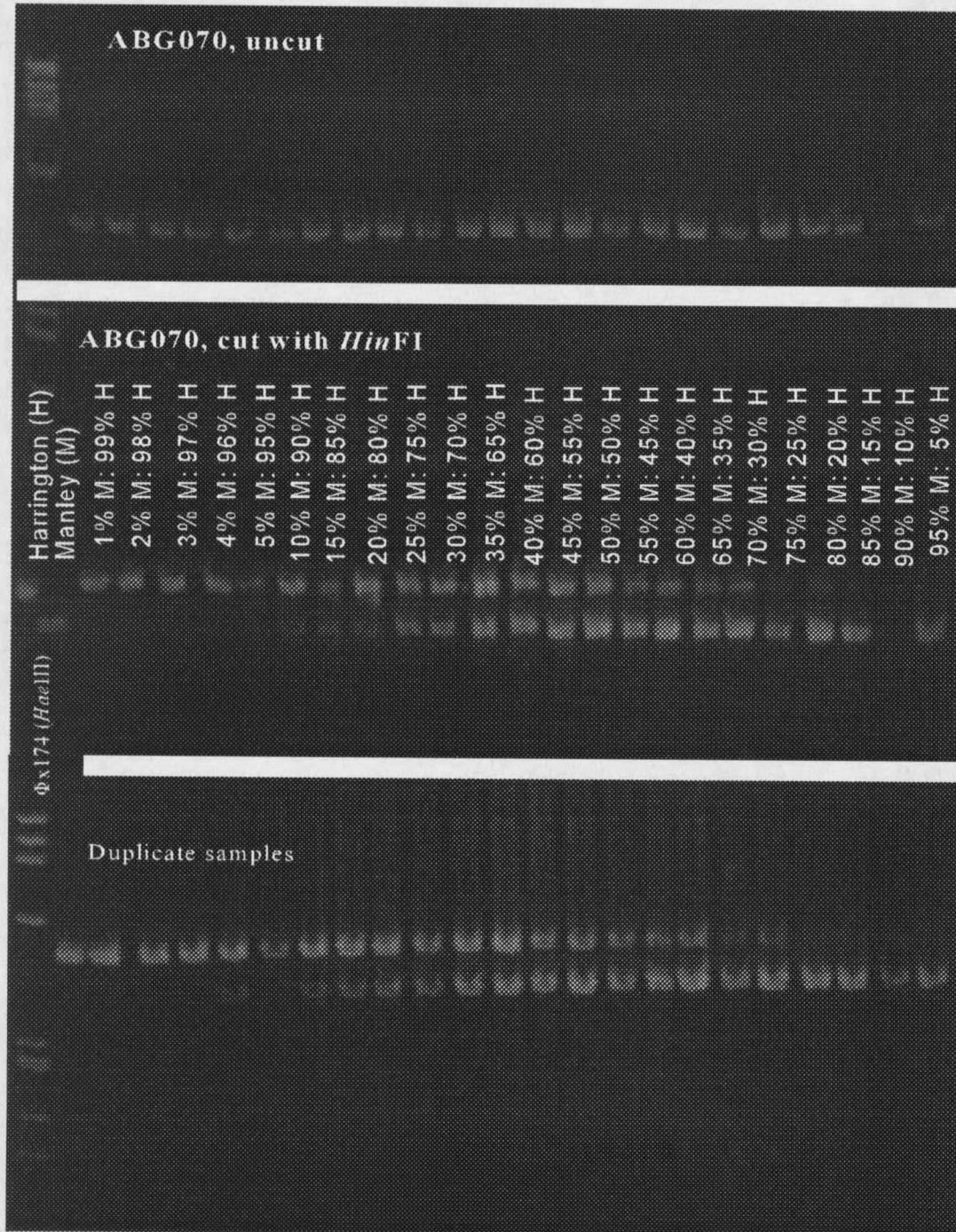
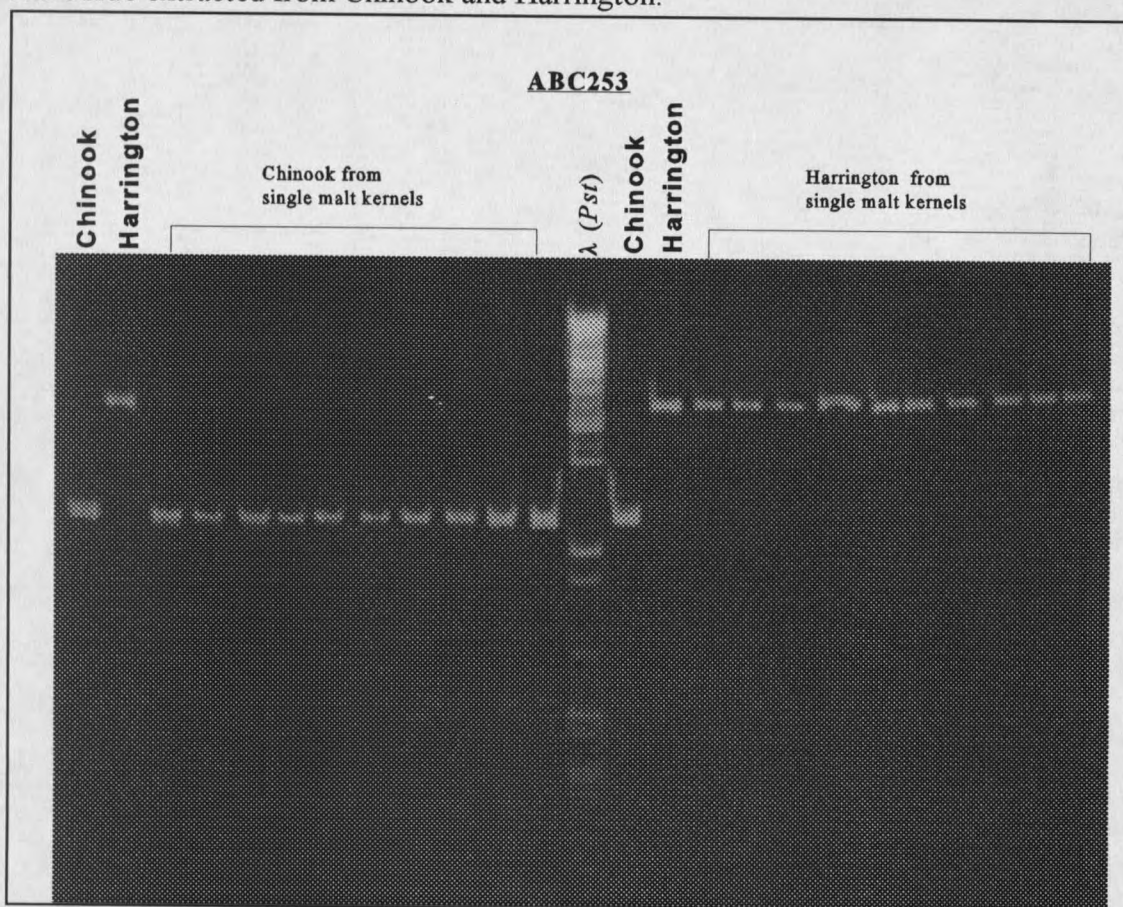


Figure 12. ABG070 provides a single product. Cut with *HinFI*, blends of Manley and Harrington can be described with a limit of resolution between 5% - 20%. Duplicate samples are shown.



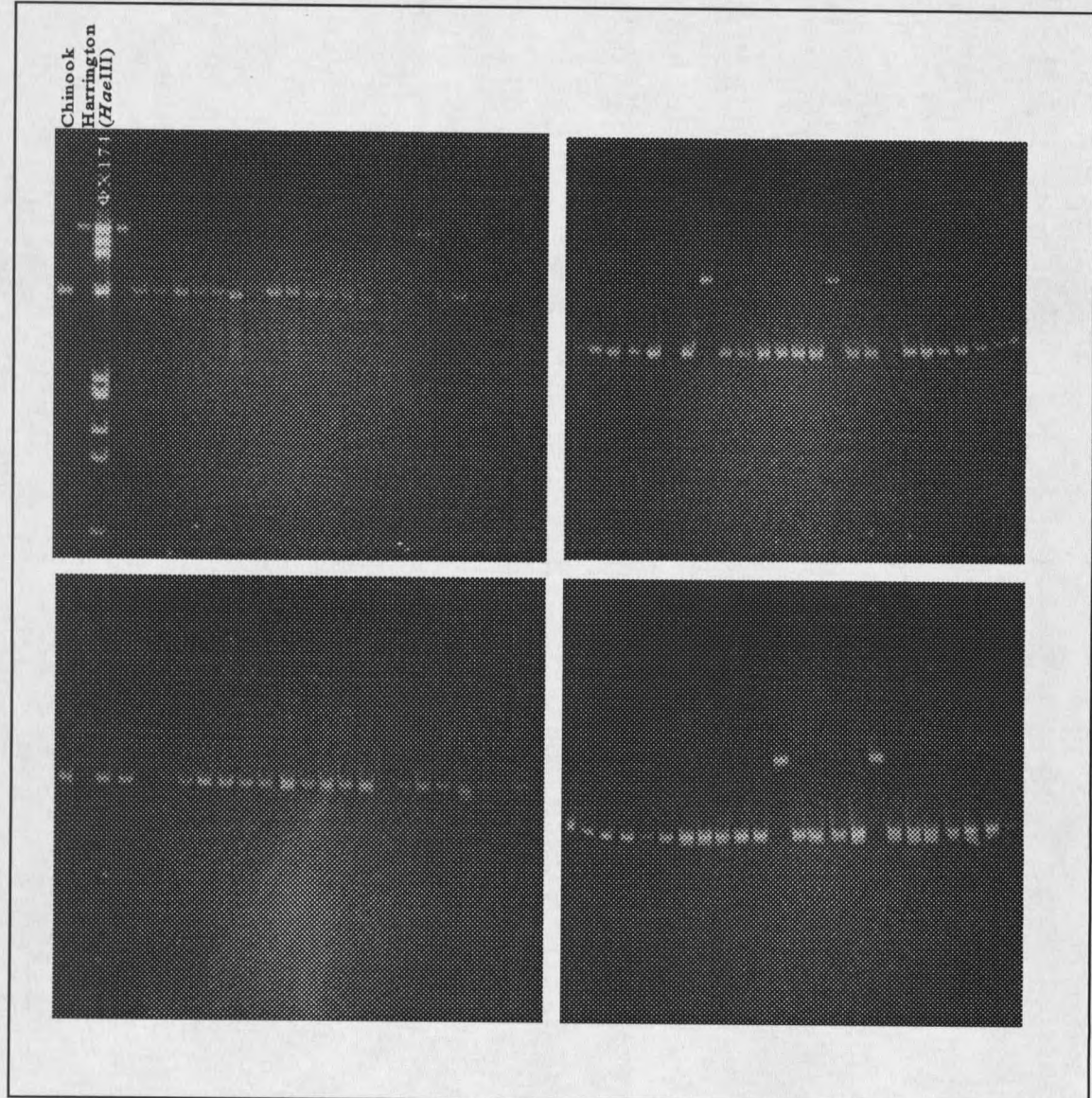
Single kernel malt extractions can be used to quantify the mixture. Ten kernels of both Chinook and Harrington were extracted and amplified using ABC253 (Figure 13). Tissue samples of each cultivar were used as controls. The single kernel extractions amplified as well as the tissue extractions. This method can be used to estimate mixtures of cultivars in a single reaction.

Figure 13. DNA extracted from ten single kernels of malt amplify as well as DNA from leaf tissue extracted from Chinook and Harrington.



We selected 96 kernels from a 10:90 blend of Harrington:Chinook. Genomic DNA was extracted from the single kernels and subjected to PCR using ABC253 as the primer set. Seven kernels out of 94 showed the Harrington allele (Figure 14), or 7.4% contamination was detected; which is within the 95% confidence limits of 3.9% to 16.1%. Therefore, this method is reliable in detecting mixtures.

Figure 14. A mixture of 10% Harrington : 90% Chinook, can be quantified using single malt kernel extractions. Seven Harrington kernels were detected out of 94 kernels, or 7.4% contamination.



VARIETAL PURITY AND QUALITY ASSURANCE

Sampling Strategy

Binomial Distribution

Industrial laboratories routinely make recommendations regarding quality control. These recommendations are based on the statistical inferences about population distributions. Binomial distributions are an important class of discrete probability distributions. These distributions are important in statistics when one wishes to make inferences about the proportion p of "successes" in the population (Moore and McCabe, 1993). For example, consider an allotment of malt containing mostly Chinook and a small amount of Harrington. Harrington is considered to be a contaminant, since the customer wanted pure Chinook. How can one estimate the population of the contaminants in a batch of malt? Kernels are pulled at random, and the probability theory allowed us to calculate the probability of obtaining a sample (n) containing 'x' number of Harrington kernels. The binomial distribution can be used to estimate these probabilities. It is important to note that industry specification for accepting an off-type or contaminating cultivar is 2% ($p = 0.02$). For practical purposes, we assume the act of removing one kernel, or sample of kernels, has no effect on the proportion of the off-type kernels remaining. We assume that the count 'x' of Harrington kernels in the sample has the

binomial distribution $B(96, 0.02)$. A normal approximation is used for $np(1-p) > 25$. Figure 15-18, are theoretical distributions for $p = 0.02, 0.03, 0.04$ and 0.05 for detecting exactly 'x%' off-type kernels, given the proportion of contaminants. Probabilities were calculated on the nearest integer; for example, 3% of 384 is 11.52 or 12 kernels.

Figure 15. Binomial probability distributions for $n = 96, 192, 288$ and 384 , given the proportion of contaminating cultivar is $p = 0.02$.

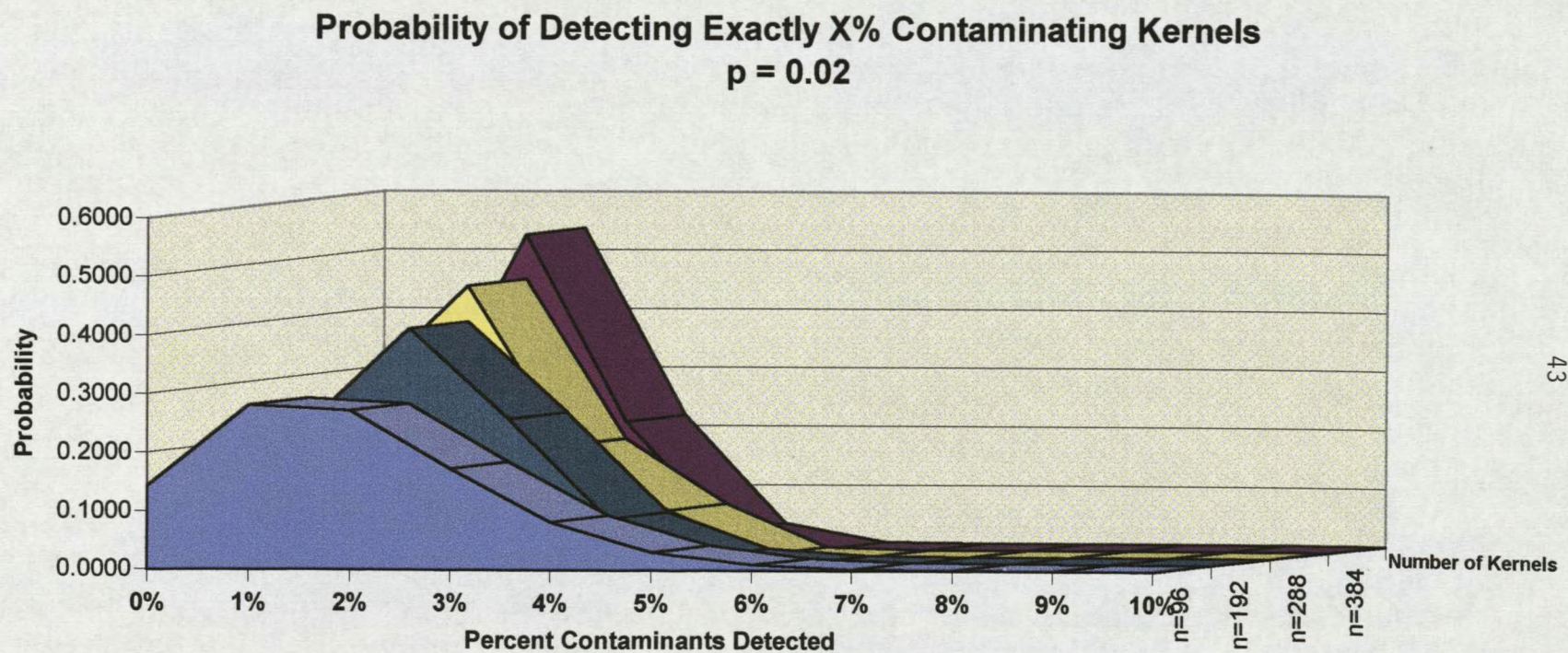


Figure 16. Binomial probability distributions for $n = 96, 192, 288$ and 384 , given the proportion of contaminating cultivar is $p = 0.03$.

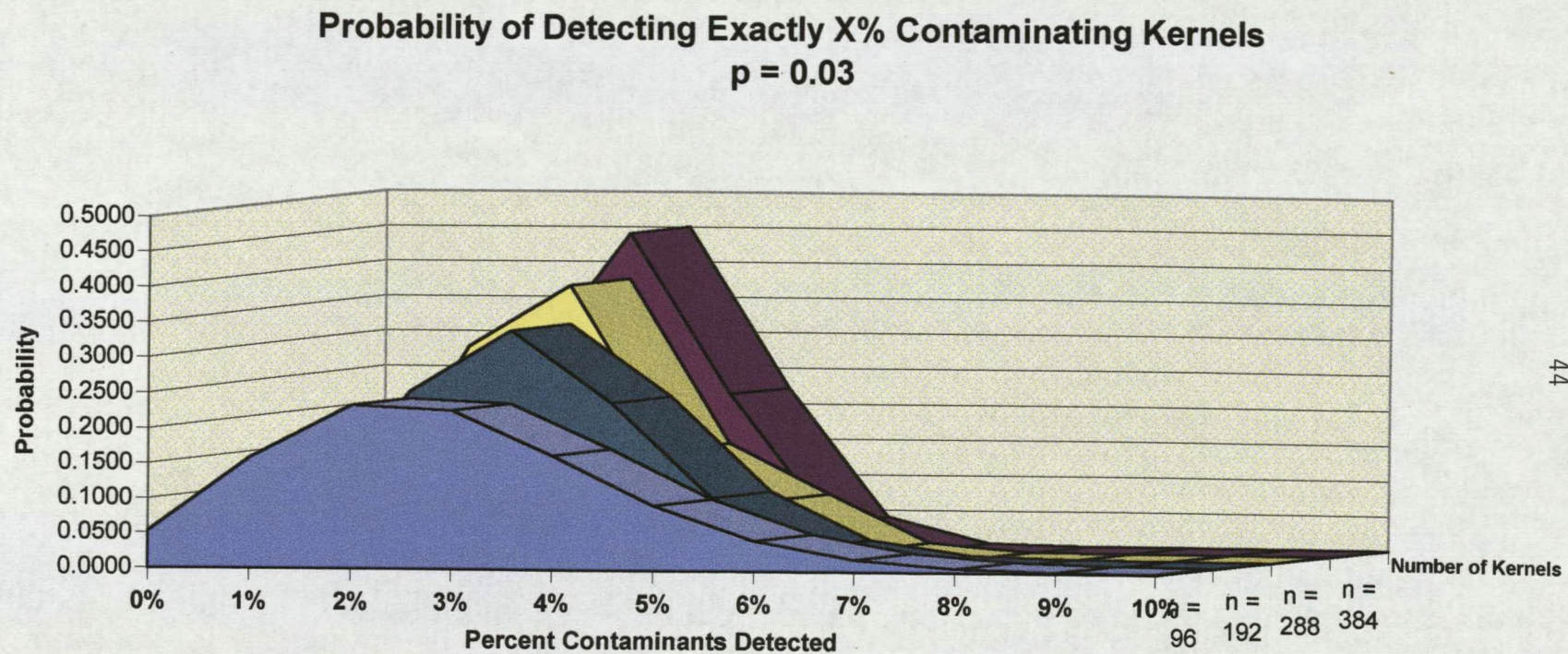


Figure 17. Binomial probability distributions for $n = 96, 192, 288$ and 384 , given the proportion of contaminating cultivar is $p = 0.04$.

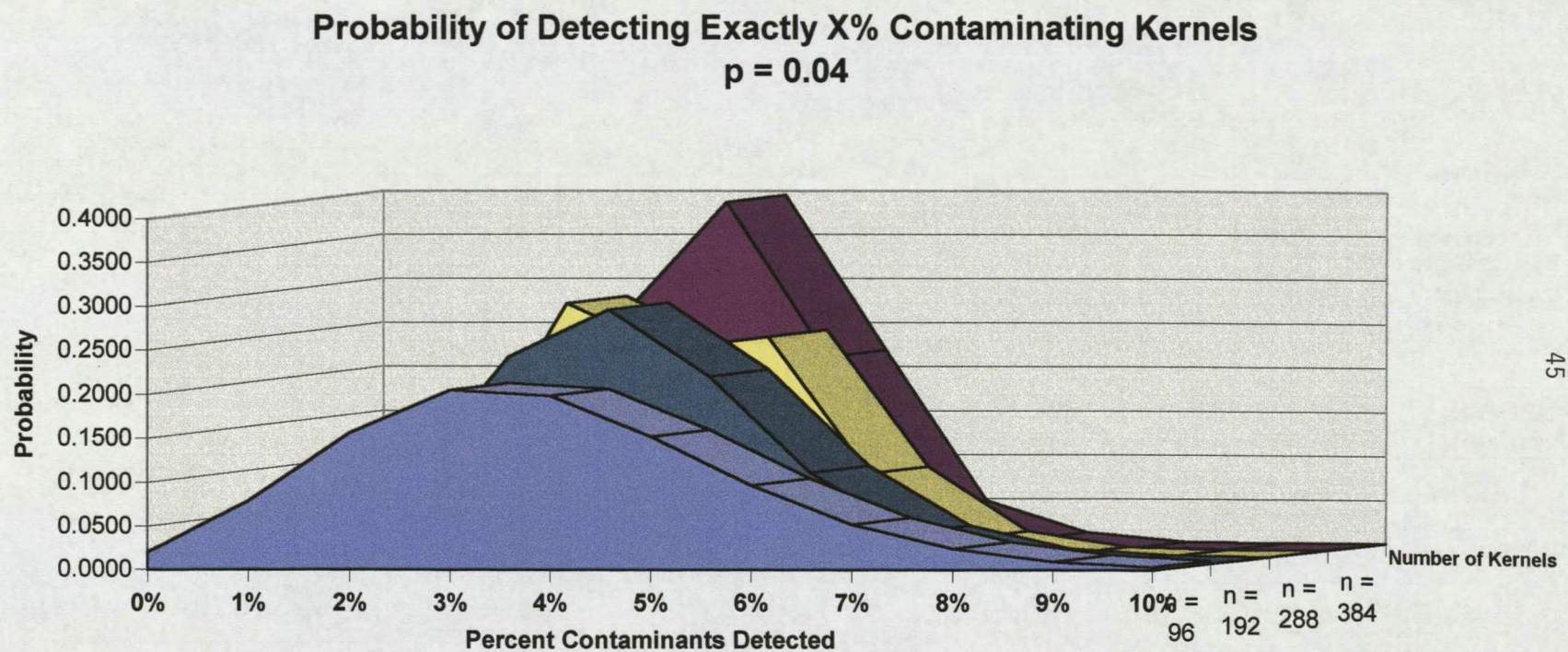


Figure 18. Binomial probability distributions for $n = 96, 192, 288$ and 384 , given the proportion of contaminating cultivar is $p = 0.05$.

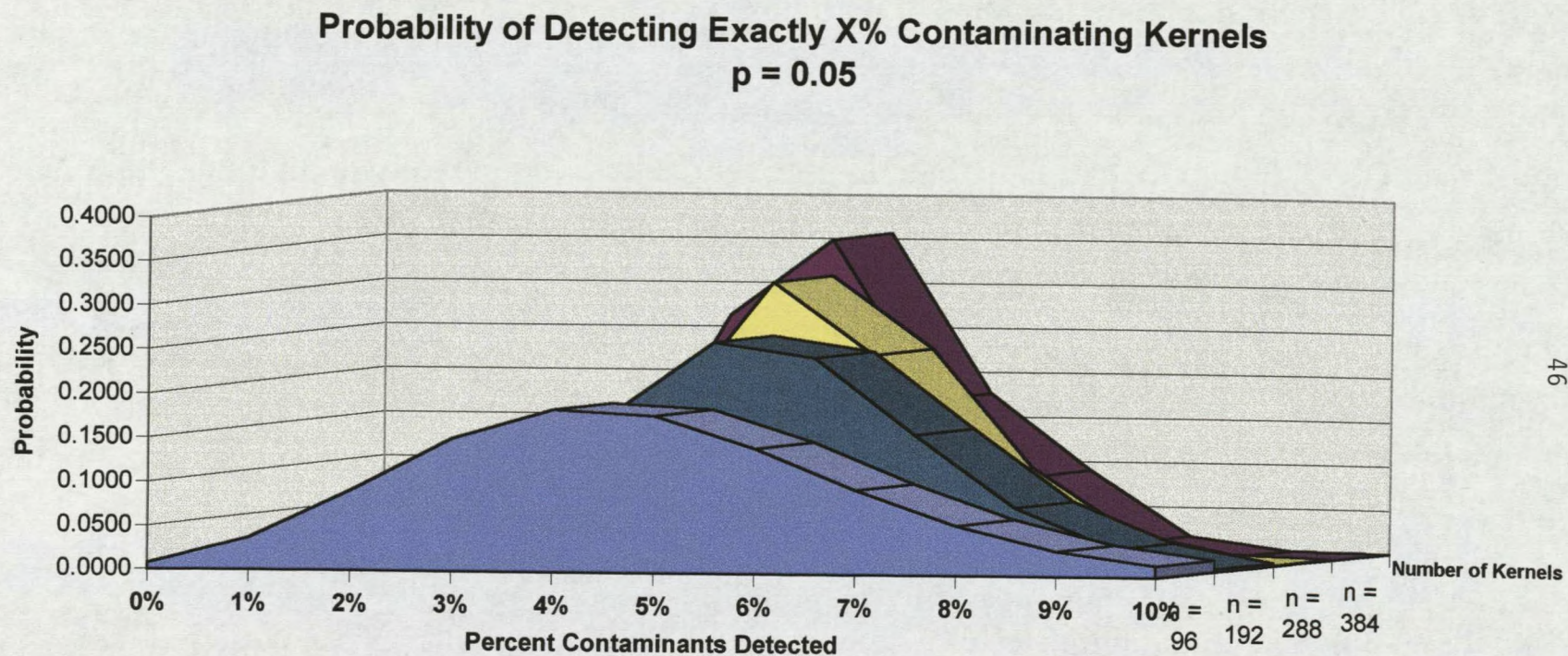


Table 5, lists the probability of 'x' or less than, greater than 'x', for a binomial distribution, having 'n' trials and proportion parameter 'p'. Note 'x' was rounded to the nearest integer, where $np=x$.

Table 5. Probability of less than 'x' number of kernels, and 'x' or greater number of kernels, for a binomial distribution having 'n' trials, and proportion parameter 'p'.

n	p	x	x or less than	greater than x
96	0.02	2	0.6985	0.3015
	0.03	3	0.6745	0.3255
	0.04	4	0.6607	0.3393
	0.05	5	0.6520	0.3480
192	0.02	4	0.6604	0.3396
	0.03	6	0.6454	0.3546
	0.04	8	0.6381	0.3619
	0.05	10	0.6343	0.3657
288	0.02	6	0.6452	0.3548
	0.03	9	0.6355	0.3645
	0.04	12	0.6318	0.3682
	0.05	14	0.5270	0.4730
384	0.02	8	0.6376	0.3624
	0.03	12	0.6315	0.3685
	0.04	15	0.5305	0.4695
	0.05	19	0.5419	0.4581

The probability of detecting less than 'x' number of off-type kernels, is the probability that one will correctly described the mixture. The probability of detecting 'x' number of off-type kernels or greater, is the chance one will fail to detect the correct number of contaminating kernels. The chance of correctly describing a mixture depends on the number of kernels sampled. Table 6 contains a list of confidence intervals and their relationship to sample size. The limits were calculated using the following equation:

$p \pm Z_{\alpha/2} \sqrt{p(1-p)/n}$, where p = proportion of off-type kernels, n = number of kernels sampled, and $\alpha = 0.05$ for significance level. For example, if an allotment of malt contained 2% off-type kernels, and 96 kernels were sampled, we are 95% confident that the number of off-type kernels detected would fall between 0 and 5%. However, if we sample 384 kernels we can be 95% confident that fewer than 11 (3%) of the kernels would be of the "off-type". For practical purposes, confidence limits should be rounded to the nearest integer; getting a partial kernel is not realistic.

Table 6. Confidence intervals (Lower Limits and Upper Limits) for detecting an off-type cultivar given: $\alpha = 0.05$, n = number of kernels, $p = 2\%$ contaminants.

Number of kernels sampled, n	Lower Limit, %	Upper Limit, %
96	0.00	4.80
192	0.02	3.98
288	0.38	3.62
384	0.60	3.40
480	0.75	3.25
576	0.86	3.14
672	0.94	3.06
768	1.01	2.99
864	1.07	2.93
960	1.11	2.89
1056	1.16	2.84
1152	1.19	2.81
1248	1.22	2.78
1344	1.25	2.75
1440	1.28	2.72
1536	1.30	2.70
1632	1.32	2.68
1728	1.34	2.66
1824	1.36	2.64
1920	1.37	2.63
2016	1.39	2.61

BASIC EQUIPMENT AND SUPPLIES

The purpose of this section is to provide the technician with information on the basic equipment and supplies necessary to perform the methods and techniques described. The materials and costs listed are those used in isolating DNA from barley malt (detailed DNA isolation from plant tissue is described in Appendix), setting up and running a PCR, and detecting the PCR product on a polyacrylamide gel (Tables 7-12). The product information on each item is to provide a reference and estimated cost for the technician, substitutions that serve the same purpose may be used.

Table 7. Reagents for genomic DNA isolation procedure (s).

Reagent	Use in protocol	Fisher Scientific, product no.	Cost*
Potassium acetate, $C_2H_3KO_2$	Facilitates ethanol precipitation of DNA	BP364-500, 500 g	\$16.20
2-propanol	Precipitates DNA	A417-1, 1 liter	\$20.50
Ammonium acetate, CH_3COONH_4	Facilitates ethanol precipitation of DNA	BP326-500, 500 g	\$22.20
Sodium acetate, $CH_3COONa \cdot 3H_2O$	Facilitates ethanol precipitation of DNA	BP334-500, 500 g	\$17.80
Tris, $C_4H_{11}NO_3$	Buffer	BP152-1, 1 kg	\$52.90
EDTA, $C_{10}H_{14}O_8Na_4 \cdot 2H_2O$	Minimizes metal ion impurities in rxn buffer	BP120-500, 500 g	\$36.70
Sodium Hydroxide, NaOH	Adjusts pH	BP359-500, 500 g	\$14.00
Sodium chloride, NaCl	Buffer	BP358-1, 1 kg	\$16.50
SDS, $CH_3(CH_2)_{11}OSO_3Na$	Denatures proteins	BP166-500, 500 g	\$97.95
2-Mercaptoethanol, C_2H_6OS	Reduces disulfide linkages in proteins and peptides	BP176-100, 100g	\$21.80

*Prices are from the 1997 Fisher Scientific Catalog.

Table 8. Equipment for DNA isolation from barley malt (bulk and single kernel).

Item	Estimated Cost
Seal-a-Meal, bag sealer	\$20.00
Plastic bag material	\$5.50/roll
Micro-mill	\$950.00
Waterbath, to 65°C	\$1000.00
Scale, to 0.01	\$1000.00
Polypropylene bottles, 250ml	\$2.00 ea.
Refrigerated centrifuge	\$10,000.00
Miracloth	\$38.00/roll
Funnels, 55 mm	\$25.00/100
Freezer, to -20°C	\$3500.00
Polyethylene bottles, 125 ml	\$0.30 ea.
Glass pasteur pipets	\$17.00/250
Bunsen burner	\$19.00
Dispensers	\$200.00 ea
Pipets, 10 ml and 25 ml	\$5.00 ea.
pH meter	\$250.00
Vortex mixer	\$200.00
Culture tubes, 12 x 75 mm	\$70.00/500
1.7µl microfuge tubes	\$25.00/500
Graduated cylinders	\$30.00
Beakers	\$25.00
Latex gloves	\$10.00/box

Table 9. Reagents for PCR and endonucleases.

Item	Use in PCR	Cost*
<i>Taq</i> DNA polymerase, 50 μ l with MgCl and 10 x buffer	Catalyzes primer extension	\$90.00
dNTPs (dATP, dCTP, dGTP, dTTP) HPLC grade, 100 μ l	Deoxynucleotide triphosphates for enzymatic extension	\$210.00
Oligonucleotide primers	Initiate enzymatic extension of DNA template	Price varies
Light mineral oil, 1l	Prevent evaporation	\$24.60
Endonuclease(s), price per unit	Restricts amplified DNA fragments	Price varies

*Prices are from the 1997 Promega Catalog.

Table 10. Equipment for PCR

Item	Cost
Thermocycler, 96-well Perkin-Elmer	\$6000.00
0.2 ml PCR tubes, 120 strips of 8	\$280.00
Pipettors, adjustable to deliver 1 - 1000 μ l Rainin P-10, P-200, P-1000	\$250.00ea.
Tips	\$38.00/1000

Table 11. Reagents for polyacrylamide gel electrophoresis (PAGE).

Reagent	Use in protocol	Fisher Scientific, product no.	Cost*
Acrylamide, $\text{CH}_2\text{:CHCONH}_2$	Gel forming reagent	BP170-500, 500 g	\$112.50
Bis-acrylamide $\text{CH}_2\text{:CHCONHCH}_2\text{NHCOCH:CH}_2$	Cross-linker	BP171-100, 100g	\$46.20
Tris, $\text{C}_4\text{H}_{11}\text{NO}_3$	Buffer	BP152-1, 1 kg	\$52.90
Boric acid, H_3BO_3	Buffer	BP168-1, 1 kg	\$33.75
TEMED, $(\text{CH}_3)_2\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$	Catalyst for the polymerization for acrylamide/bis-acrylamide	BP150-20, 20 g	\$18.50
Ammonium persulfate, $(\text{NH}_4)_2\text{S}_2\text{O}_8$	Initiator of acrylamide polymerization	BP179-25, 25 g	\$11.25
Ethidium Bromide, $\text{C}_{21}\text{H}_{19}\text{N}_3\text{Br}$	Intercalating dye used to visualize DNA in gel	BP1302-10, 10 ml	\$26.30
Bromphenol blue-xylene cyanal dye kit, 5 ml	Loading solution	Sigma, Mol. Biol.	\$5.25
Marker, Phix174, 50 μl	Base pair marker	NEB 302-1S	\$50.00

*Prices are from the 1997 Fisher Scientific Catalog, unless otherwise noted.

Table 12. Equipment for PAGE.

Item	Cost
Electrophoresis gel box and casting chamber, BioRad Protean IIXi Cell w/o spacers/comb	\$875.00
Spacer, 0.75 mm, set of four	\$55.00
Comb, 25 teeth, 0.75 mm	\$40.00
Electrophoresis power supply, BioRad Power Pac 1000	\$1150.25
Hamilton syringe, 50 μ l	\$26.00
Fisher Scientific 14-824-32	
Staining Tray	\$20.00
UV light box	\$300.00
CCD camera and thermal printer (optional)	\$3000.00

SUMMARY AND CONCLUSIONS

DNA fingerprinting and PCR techniques have become important research tools in a broad range of disciplines. This is a powerful approach to characterize barley cultivars, and can be used for absolute identification for protection under the Plant Variety Protection Act. The use of bulk and single kernel malt extractions provide the brewing industry with a single technology that can be used to discriminate among genotypes and to describe mixtures in an allotment of barley malt. The bulk DNA extraction can identify questionable shipments. They can then be subjected to the single kernel extraction technique, to estimate the degree of lot contamination. The use of this rapid, single kernel malt extraction technique is reliable and provides sufficient amplification quantities of DNA for several PCR reactions.

Application of this technology is limited to facilities set up for molecular techniques. The equipment to set up such a lab is a capital expense. Estimated cost is \$20,000 to \$30,000, depending on current laboratory equipment and supplies. However, the cost per reaction is fairly low, approximately \$0.50. Therefore the number of samples needed to determine the percentage of a contaminated shipment of barley malt (384 kernels) is estimated to cost a technician one working day and \$200 in supplies.

Forensic laboratories have been quick to realize the potential of the new DNA technologies. The Federal Bureau of Investigation (FBI) geared up for forensic DNA typing in 1988, and instituted a program for training forensic scientists from state crime

laboratories around the country. The FBI wants to maintain a data bank of from persons and crime scenes in different jurisdictions (Marx, 1988). The United States Department of Agriculture could benefit from a data base that included DNA patterns and the comparison of DNA samples from agricultural crops as evidence to provide legal protection under the PVPA to developers of new varieties of plants which are sexually reproduced by seed, or are tuber-propagated.

Commercial companies have found forensic DNA typing to be a lucrative business. It may be that these companies broaden their genetic stock to include that which would benefit the field of agriculture. Or perhaps a centralized laboratory could offer a DNA typing service that could support agricultural businesses as well as the PVPA. Until these services are available, the malting and brewing industry can adopt these procedures in-house to ensure barley and malt meet customer specifications.

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APPENDIX

GENOMIC DNA ISOLATION

Scope

This method is designed to render high quality DNA yields for up to 96 samples per run. Genomic DNA extracted from this procedure is suitable for PCR or AFLPs. This technique can be used for tissue, seed, or malted barley. *Note: this procedure is written for extracting 0.4 g plant tissue, adjust volumes and increase container sizes if using a greater amount of genomic stock.*

Principle

Genomic stock is ground with extraction buffer. After incubation in 65°C potassium acetate is added to facilitate ethanol precipitation of DNA, and solution is iced to remove proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulfate precipitate. DNA is isolated via ethanol precipitation.

Reagents

- a. *Liquid nitrogen* - for extracting DNA from plant tissue.
- b. *Extraction buffer*, Combine the following solutions:

100mM Tris, pH 7.6-8.0	1M Tris (reagent h)	50 ml
50mM EDTA, pH 8.0	0.5M EDTA (reagent i)	50 ml
100mM NaCl	4M NaCl (reagent j)	12.5 ml
1% SDS	10% SDS (reagent k)	50 ml
10mM Mercaptoethanol	Dilute solution to 500 ml with sterile water.	
	Add 14.4 M ME (reagent l)	347 µl
- c. *Potassium acetate* ($C_2H_3KO_2$), 5M, enzyme grade
Dissolve 245.4 g of potassium acetate in double distilled water and dilute solution to 500 ml.
- d. *Isopropanol* or *2-propanol*
- e. *Ammonium acetate*, (CH_3COONH_4), 5M, enzyme grade
Dissolve 77.08 g of ammonium acetate in double distilled water and dilute solution to 200 ml.
- f. *TE*
10mM Tris
1mM EDTA
Dilute 1.0 ml 1M Tris (reagent h) and 200 µl 0.5M EDTA (reagent l) to 100 ml double distilled water.
- g. *Sodium acetate*, ($CH_3COONa \cdot 3H_2O$), 3M, enzyme grade
Dissolve 40.8 g of sodium acetate in double distilled water. Adjust the pH to 7.0 using glacial acetic acid, and dilute solution to 100 ml.

- h. *Tris*, pH 7.6-8.0, ($C_4H_{11}NO_3$), 1M, molecular biology grade
Dissolve 121.1 g Tris base in 800 ml of double distilled water. Adjust the pH to 7.6 to 8.0 by adding concentrated HCl. Dilute solution to 1 liter.
- i. *Disodium ethylene diamine tetraacetate-H₂O (EDTA)*, 0.5M, pH 8.0, ($C_{10}H_{14}O_8Na_4 \cdot 2H_2O$), electrophoresis grade
Add 186.1 g pf EDTA tp 800 ml of double distilled water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dilute solution to 1 liter.
NOTE: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.
- j. *Sodium Chloride*, (NaCl), 4M, enzyme grade
Dissolve 46.8 g NaCl in double distilled water and dilute solution to 200 ml.
- k. *Sodium Dodecyl Sulfate (SDS)*, ($CH_3(CH_2)_{11}OSO_3Na$), 10%, electrophoresis grade
Dissolve 100 g SDS in 900 ml double distilled water. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 liter. NOTE: Wear a mask when weighing SDS.
- l. *2-Mercaptoethanol*, (C_2H_6OS), 14.4M, electrophoresis grade

Apparatus

- a. Bag sealer, Scotchpak® Pouch Sealer
- b. Plastic bag material, Micro-Seal, Model 50358, (8 in. X 20 ft.)
- c. Thermo-flask to carry liquid nitrogen.
- d. Latex gloves
- e. Waterbath, 65°C
- f. Scale, Mettler PC 400
- g. Pestle, Coors No. 60323
- h. Centrifuge tubes, 6.5 ml (13 x 100 mm) with closures
- i. Ice
- j. Beckman, Model J-6 Refrigerated centrifuge
- k. Miracloth, 3x3 in. squares
- l. Funnel, 55 mm
- m. Incubator, -20°C
- n. Sterile culture tubes, 17 x 100 mm
- o. Glass pasteur pipets
- p. Bunsen burner
- q. Microfuge tubes, 1.5 ml
- r. Dispensers,
0.2 to 0.8 ml, 0.8 to 3.0 ml, 3.0 to 10.0 ml with 1% accuracy.
- s. Pipets,
5 ml serological pipet assembled with Drummond Pipet-aid
Gilson Pipetman P1000
Rainin 1000 ml electronic digital pipet
- t. Microfuge, Survall® Instruments, Microspin 24S

u. Speed Vac, Savant Speed Vac Condenser

Procedures

Before you begin the procedure, prepare the following:

- ▶ Make bags. Cut 26 cm strips of plastic pouch material. Seal both cut ends and three seams that make four 6 cm pockets if using 0.40 g plant tissue. Note: "Bag" is referring to a set of four pockets.
 - ▶ Turn on 65°C waterbath and refrigerated centrifuge.
 - ▶ Obtain liquid nitrogen, 7 to 10 pounds.
 - ▶ Collect plants.
 - ▶ Make Extraction Buffer, place in waterbath.
 - ▶ Make any necessary reagents.
- Minimum volumes required to extract 96 samples:
- | | |
|-----------------------------|---------------------------|
| 500 ml Extraction buffer | 35 ml 5M Ammonium acetate |
| 165 ml 5M Potassium acetate | 30 ml TE |
| 400 ml Isopropanol | 3 ml 3M Sodium acetate |
- ▶ Plug in bag sealer.
 - ▶ Get ice bucket to keep plant tissue cool until use.
 - ▶ Prepare Miracloth filters.
 - ▶ Curl ends of pasteur pipets, using a Bunsen burner.

1. Cut one tracking edge off of the bags. Hold bag using a test tube peg rack.
2. Collect 0.4 ± 0.02 g of leaf tissue in each pocket. A glass rod can be used to hold pocket open. Push is down into the pocket using a glass rod. Place tissue on ice until use. Note: Cover ice with plastic to keep bags dry. Grinding over ice crystals may cause holes in pockets.

NOTE: Wear gloves during the entire procedure and keep tissue cool until use.

3. After all plant tissue is collected, dip one tissue filled bags into liquid nitrogen, open end up, for approximately 20-30 seconds. Remove and crush tissue, through the plastic, using a large pestle. Don't allow tissue to thaw.
4. Dispense 5.0 ml warm extraction buffer through the open end of pocket, in sets of four. Seal the open end of bag. Grind any whole tissue by rubbing fingers together across bag. Note: This will also allow you to detect any leaks. Seal out any leaks if possible, otherwise prepare an other pocket or bag. Place bag in waterbath. Note time first and last bag goes into waterbath. Repeat steps 3-5 until all plant tissue have been ground.
5. Incubate bags for approximately 45 minutes. Start pulling the first bags in, out and proceed to next step. This way each bag is in the bath for about the same time.
6. Cut pockets apart. Press contents of bag from one end and cut it open. Using a funnel, pour contents into 6.5 ml centrifuge tubes.
7. After all pockets have been transferred to tubes, dispense 1.67 ml 5M potassium acetate to each tube. Fit closure and mix well.
8. Incubate tubes on ice for 60 minutes. Make sure tubes are at least one inch apart

- to ensure the tube gets chilled.
9. Dry tubes and place in refrigerated centrifuge. Spin tubes at 5,000xG (4,000rpm-Beckman) for 20 minutes. While tubes are spinning, prepare tubes used in step 11.
 10. If tubes are clean, pour directly into prepared tubes. If the tube has junk on the top of supernatant, pour it through a Miracloth filter into a sterile 17x100mm culture tube containing 4ml cold 2-propanol and 330 μ l 5M ammonium acetate. Do not invert tubes. Incubate tubes at -20°C for a minimum of 20 minutes. If you cannot see any DNA, gently invert the tube and place it back in the freezer for 20 additional minutes.
 11. Hook DNA with glass hooks and redissolve it in 230 μ l TE in a 1.5 ml microfuge tube.
 12. Add 26 μ l 3M sodium acetate, pH 7.0, and 168 μ l cold 2-propanol. Mix well and pellet the clot of DNA for 30 seconds in a microfuge.
 13. Pour off supernatant and dab top of microfuge tube with a kimwipe. Place in Speed Vac or on counter top until dry.
 14. Redissolve in 50 μ l TE. Dilute 1:20 for PCR.
 15. Store at -20°C until use.

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