(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 January 2008 (17.01.2008)

PCT

(10) International Publication Number
WO 2008/008501 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2007/016016

(22) International Filing Date: 13 July 2007 (13.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/830,908 14 July 2006 (14.07.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2008/008501 A2

(54) **Title:** REVERSE RESTRICTION FRAGMENT LENGTH POLYMORPHISM ASSAY AND USES THEREOF

(57) **Abstract:** The present invention presents a Reverse Restriction Fragment Length Polymorphism (RRFLP) method for the detection of the presence of an informative restriction enzyme site in a nucleotide sequence. The method includes digesting a sample with the informative restriction enzyme; performing polymerase chain reaction (PCR) on the digested sample with an oligonucleotide primer pair that flanks the informative restriction enzyme site; determining the Ct value of the sample; comparing the Ct value of the sample to the Ct value from a control sample; and calculating a ΔCt value, wherein a ΔCt value is the Ct value of the sample minus the Ct value of a control; and wherein a ΔCt value ≥ +1 indicates that the informative restriction enzyme sites is present in the nucleotide sequence.

REVERSE RESTRICTION FRAGMENT LENGTH POLYMORPHISM ASSAY AND USES THEREOF

CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 60/830,908, filed 14 July, 2006, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant Nos. 58-6612-2-219 and 10-21-RR188-174, awarded by the Agriculture Research Services, U.S. Department of Agriculture. The Government may have certain rights in this invention.

BACKGROUND

Traditional methods of genotyping ILTV isolates by multiple gene sequence analysis have many disadvantages. The genotype of a known viral isolate must be available for comparison and access to a sequencing facility is required. This method of genotyping viral isolates is time-consuming and costly. More recent methods of detecting the presence of viral infection using convention polymerase chain reaction (PCR) amplification have been developed. Although conventional PCR assays provide detection of ILTV with a high level of sensitivity and specificity, PCR assays do not give any information about the genotype of the ILTV in a particular sample. This information is important for epidemiological studies of the virus, which can be used to study the spread of the virus throughout poultry flocks. With conventional PCR, quantitative aspects are difficult and cumbersome to resolve. Furthermore, conventional PCR is prone to contamination and in some instances the interpretation of gel electrophoresis is inconclusive.

Another common molecular technique utilized for detection and differentiation of specific DNA sequences is the restriction fragment length polymorphism (RFLP) (Grodzicker *et al.*, 1974 *Cold Spring Harbor Symp. Quant.*

39:439-446; Botstein *et al*, 1980 *Am. J. Hum. Gen.* 32:314-331). In this method, restriction enzymes are used to digest target DNA (genomic or PCR amplified), which is then separated by gel electrophoresis and visualized by staining. Comparing fragment patterns with known patterns or sequence data allows the determination of the presence or absence of specific sequences within the target DNA. This type of information has been used for the detection and differentiation of many different pathogens.

Conventional PCR amplification can be combined with a standard RFLP analysis in a two step PCR-RFLP assay. In this method, a specific genomic sequence harboring a specific and identifying polymorphism is amplified by PCR. Following gel purification and isolation, the amplicon is subjected to a standard RFLP technique. Many current methods of genotyping involve gene specific PCR followed by RFLP. See, for example, Chang et al., 1997 *J Virol Meth.* 66(2):179-86; Clavijo et al., 1997 *Avian Dis.* 41(1):241-6; Creelan et al., 2006 *Avian Pathol.* 35(2):173-9; Han and Kim, 2003 *Avian Dis.* 47(2):261-71; Han and Kim, 2001 *Microbiol.* 83(4):321-31; Kirkpatrick et al., 2006 *Avian Dis.* 50(1):28-34; Sellers et al., 2004 *Avian Dis.* 48(2):430-6).

The assay methods of the present invention demonstrate an improvement over the conventional PCR, RFLP, and PCR-RFLP assays, demonstrating, for example, improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination, the simultaneous detection of viral infection and determination of the viral strain, a more objective interpretation of the RFLP analysis, utilization of small PCR products, and dramatically increased speed of the assay.

SUMMARY OF THE INVENTION

The present invention includes a method of detecting the presence of a recognition site for a restriction enzyme in a nucleotide sequence, the method including: digesting all or a portion of a sample comprising the nucleotide sequence with the restriction enzyme; performing real-time polymerase chain reaction (PCR) on the sample digested with the restriction enzyme with an oligonucleotide primer pair that flanks the restriction enzyme recognition site; determining the Ct value of the sample digested with the restriction enzyme; comparing the Ct value of the

sample digested with the restriction enzyme to the Ct value from a control sample not digested with the restriction enzyme; calculating a ΔCt value, wherein a ΔCt value is the Ct value of the sample digested with the restriction enzyme minus the Ct value of a control sample not digested with the restriction enzyme; wherein a ΔCt value $\geq +1$ indicates that the nucleotide sequence is digested by the restriction enzyme at a recognition site located between the oligonucleotide primer pair.

In some embodiments of the method, separate portions of the sample are digested with different restriction enzymes and a separate ΔCt value is calculated for each separate portion.

In some embodiments, the method can be used in the detection and/or differentiation of ILTV strains. In some embodiments of the method, a portion of the sample is digested with the restriction enzyme *Alw* 1. In some embodiments of the method, a portion of the sample is digested with the restriction enzyme *Ava* 1. In some embodiments of the method a portion of the sample is digested with the restriction enzyme *Ava* I and a portion of the sample is digested with the restriction enzyme *Alw* 1. In some embodiments of the method, the oligonucleotide primer pair flanks about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence. In some embodiments of the method, the oligonucleotide primer pair is located within nucleotide positions 2039 to 2950 of the ICP4 gene (Genebank Accession No. L32139). In some embodiments of the method, the oligonucleotide primer pair flanks nucleotide positions 2392 to 2534 of the ICP4 gene (Genebank Accession No. L32139). In some embodiments of the method, the oligonucleotide primer pair is SEQ ID NO:7 and SEQ ID NO:8, or derivatives thereof.

The present invention also includes a method of diagnosing ILTV disease, the method including digesting a sample with the restriction enzymes *Alw* 1 and/or *Ava* 1 and detecting the presence or absence of an *Alw* 1 and/or *Ava* 1 restriction enzyme recognition site located about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence.

The present invention includes oligonucleotide primers that flanks about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the Reverse Restriction Fragment Length Polymorphism (RRFLP) assay of the present invention. A DNA sample is obtained, split into separate tubes and digested with specific restriction enzyme(s). The resultant DNA is used as a template for real-time PCR using primers that flank the specific restriction enzyme sites. Ct values of sample DNA exposed to restriction enzyme and are analyzed and compared to the Ct value of sample DNA not exposed to restriction enzyme (control). A ΔCt value is calculated and the results are analyzed.

Figure 2 is a schematic representation of the standard PCR-based Restriction Fragment Length Polymorphism (PCR-RFLP) assay. A DNA sample is obtained and a specific target is amplified by PCR. The resultant amplicon is divided into separate tubes and digested with specific restriction enzyme(s). The resultant restriction fragments are loaded onto an agarose gel and the fragments are separated by gel electrophoresis, which sorts according to size. The fragments are visualized on the gel, for example, by staining with a DNA binding dye such as ethidium bromide or SYBR Green I. The banding pattern is analyzed for information.

Figure 3 is a phylogenetic analysis of ICP4 5' non-coding region encompassing nucleotide positions 2039 to 2950 (Accession number L32139). The phylogenetic tree was generated by the neighbor-joining method. The branch lengths represent the genetic distances between sequences, values are indicated in italics, in bold are bootstrap values indicated as a percentage at internal nodes (500 resamplings).

Figure 4 shows polymorphic sites of the ICP4 gene fragment targeted by the RRFLP assay. The sequences presented correspond to nucleotide positions 2392 to 2534 of the ICP4 gene sequence (Accession # L32139). Included in the alignment are the sequences of the CEO (SEQ ID NO. 1) and TCO (SEQ ID NO. 2) vaccines, the sequence of the broiler (9/C/97/BR) and broiler breeder (23/H/01/BBR) isolates identified as BR/BBR (SEQ ID NO. 3), and the sequence of backyard flock isolate (24/H/91/BCK) identified as BCK (SEQ ID NO. 4). Shaded in light gray, in the

CEO and BR/BRR sequences, is the *Alw* I enzyme recognition site. Shaded in dark gray, in the TCO sequence, is the *Ava* I enzyme recognition site. The backyard flock isolate lacked both restriction enzyme sites. Boxed nucleotides represent polymorphic sites recognized by restriction enzymes *Alw* I and *Ava* I.

Figures 5A to 5C are representative graphs obtained after RRFLP analysis. Fig. 5A presents Backyard flock isolate 24/H/91/BCK undigested C_T 25.6; digested *Alw* I C_T 25.97; digested *Ava* I C_T 25.64. Fig. 5B presents CEO vaccine undigested C_T 22.32; digested *Alw* I C_T 28.39; digested *Ava* I C_T 22.44. Fig. 5C presents TCO vaccine undigested C_T 26.01; digested *Alw* I C_T 26.05; digested *Ava* I C_T 30.58. The C_T value is calculated as the cycle number where the reaction fluorescence crosses the threshold line set at 10 units.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

The present invention presents a novel method for the detection of the presence of informative restriction enzyme sites in a nucleotide sequence. The method is a Reverse Restriction Fragment Length Polymorphism (RRFLP) method. This assay method has wide applicability, including, in particular, the field of molecular diagnostics. The method of the present invention can determine the presence or absence of an informative restriction enzyme site in a nucleotide sequence. Briefly, the method includes digesting all or a portion of a sample which includes the nucleotide sequence with the informative restriction enzyme; performing real-time polymerase chain reaction (PCR) on the sample digested with the restriction enzyme with an oligonucleotide primer pair that flanks the informative restriction enzyme site; determining the Ct value of the sample digested with the informative restriction enzyme; comparing the Ct value of the sample digested with the informative restriction enzyme to the Ct value from a control sample not digested with the informative restriction enzyme; and calculating a ΔCt value, wherein a ΔCt value is the Ct value of the sample digested with the informative restriction enzyme minus the Ct value of a control sample not digested with the informative restriction enzyme; wherein a ΔCt value $\geq +1$ indicates that the nucleotide sequence is digested by the informative restriction enzyme at a recognition site located between the oligonucleotide primer pair.

The RRFLP assay of the present invention may be applied in situations where conventional Restriction Fragment Length (RFLP) is currently used. In RFLP, restriction enzymes are used to digest target DNA (genomic or PCR amplified), which is then separated by gel electrophoresis and visualized by staining. Comparing fragment patterns with known patterns or sequence data allows the determination of the presence or absence of specific sequences within the target DNA. This type of information has been used for the detection and differentiation of many different pathogens. See, for example, Grodzicker *et al.*, 1974 *Cold Spring Harbor Symp. Quant.* 39:439-446; or Botstein *et al*, 1980 *Am. J. Hum. Gen.* 32:314-331. Conventional PCR amplification can be followed by a RFLP analysis in a two step PCR-RFLP assay. In PCR-RFLP, a specific genomic sequence harboring a specific and identifying polymorphism is amplified by PCR. Following gel purification and isolation, the amplicon is subjected to a standard RFLP technique.

The RRFLP method of the present invention has many advantages over conventional RFLP and PCR-RFLP assays. Gel electrophoresis is not required to obtain results. Instead, results are generated in real-time during a polymerase chain reaction (PCR). The RRFLP method of the present invention has wide application in molecular diagnostics, allowing for both the determination of the presence of a nucleic acid sequence in a sample and the genotyping of the nucleotide sample in the same assay. The RRFLP method of the present invention allows for a more objective interpretation that conventional RFLP analysis, utilizes small PCR products, and dramatically increases the speed of the RRFLP assay.

Real-time PCR (RT-PCR) may be used to monitor the formation of a double stranded DNA molecule in the RRFLP assay of the present invention. Recently, real-time PCR has become a forerunner of diagnostic detection methods. The reason for the rapid rise of real-time PCR is the extremely sensitive and specific nature of the method, along with its multiplex capabilities. First described by Higuchi et al., real-time PCR combines amplification with fluorometric detection of amplicons as the reaction occurs (Higuchi *et al.*, 1993 *Biotechnology* 11(9):1026-1030). The ability to monitor the real-time progress of PCR has revolutionized PCR-based quantitation of DNA and RNA. The process of creating quantitative assays is streamlined because the construction and characterization of such standards are no longer required. Real-time PCR allows much more precise and reproducible

quantitation of DNA and RNA than such methods as conventional PCR because it relies on CT values determined during the exponential phase of PCR rather then the endpoint. The concept of the threshold cycle (Ct) allows for accurate and reproducible quantification using fluorescence based RT-PCR. Fluorescent values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background, which is the definition of the (Ct) values. This will increase the throughput, because it is no longer necessary to analysis dilutions of each sample in order to obtain accurate results.

Any of the various means for implementing real-time PCR (RT-PCT) may be used in the present invention. For example, homogeneous detection of PCR products can be done using double- stranded DNA binding dyes, fluorogenic probes, and/or direct labeled primers. The detection of fluorescence during the thermal cycling process may, for example, be performed using Applied Biosystem's ABI Prism 7900 Sequence Detection Systems. For further discussion of real time PCR see the world wide web at ncifcrf.gov/rtp/gel/rtqpcr/WhatIs.asp; Higuchi et al., 1993 *Biotechnology* 11(9):1026-30; Mackay et al., 2002 *Nucleic Acids Res.* 30(6):1292-305; and Mackay, 2004 *Clin Microbiol Infect.* 10(3):190-212. In some embodiments, conventional PCR, rather that real-time PCR, may be used.

Real-time PCR detects products as they accumulate. A real-time system can utilize the intercalator ethidium bromide in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and detection of the resulting fluorescence with a computer-controlled cooled CCD camera. Amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles.

RT-PCR provides the ability to monitor the real-time progress of the PCR product via fluorescent detection. The point characterizes this in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR

product accumulated after a fixed number of cycles. These PCR-based fluorescent homogenous assays can be monitored by a variety of means, including, for example, labeled hybridization probe(s) (Taq Man, Molecular Beacons), labeled PCR primer (Amplifluor), and SYBR Green (Applied Biosystems).

As used herein, an informative restriction enzyme site reveals a pattern difference between the DNA fragment sizes in individual organisms after digestion with the restriction enzyme. To discover informative restriction enzyme sites, restriction enzymes (RE) are used to cut DNA at specific recognition sites. Restriction enzymes are exceedingly varied. Over 3,000 activities that have been purified and characterized. More than 250 different sequence-specificities have been discovered. Restriction Enzyme recognition sequences typically are only four to twelve nucleotides long. The recognition sequence for an informative restriction enzyme may be, for example, a four basepair sequence, a five basepair sequence, a six basepair sequence, an eight basepair sequence, or a twelve basepair sequence. A wide variety of such restriction enzymes are available. See, for example, "Restriction Endonucleases Overview," (available on the worldwide web at neb.com/nebecomm/tech_reference/ restriction_enzymes/overview.asp) and the New England Biolabs 2007-2008 catalog. Informative restriction enzyme sites can be identified by digesting a sample DNA with one or more RE's and separating the resultant fragments according to molecular size using gel electrophoresis. Alternatively, informative restriction enzyme sites can be identified by analysis of genomic DNA sequences information.

Informative restriction enzyme sites can provide the basis for a single nucleotide polymorphism (SNP). A SNP is a DNA sequence variation occurring when a single nucleotide (A, T, C, or G) in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. In this case there are two alleles, C and T. If a restriction enzyme can be found such that it cuts only one possible allele of a section of DNA (that is, the alternate nucleotide of the SNP causes the restriction site to no longer exist within the section of DNA), this restriction enzyme is an informative restriction enzyme site that can be used in the RRFLP method of the present invention.

With the RRFLP method of the present invention, the informative restriction enzyme site is flanked by a pair of primers suitable for PCR analysis. Each of the oligonucleotide primers will hybridizes to the nucleotide sequence containing the informative restriction enzyme site and serve as primers for the PCR reaction. The ability to identify an oligonucleotide primer based upon a cDNA sequence encoding a given protein, such as the ICP4 gene (Accession No. L32139), is described in, for example, Stein and Cohen (Cancer Res. 48:2659, 1988) and van der Krol et al. (BioTechniques 6:958, 1988). Oligonuclotide primers may be synthesized or may be obtained commercially.

The RRFLP method of the present invention may be used in any system in which knowledge of one or more informative polymorphic restriction enzyme sites is available. Such systems includes, but are not limited to, applications in the fields of microbiology, virology, agriculture, plant genetics and breeding, medicine and veterinary medicine, forensic identification, paternity identification, pharmacogenetics, and diagnostic assays, for example diagnostic assays the detection of infectious diseases, genetic diseases, and cancer. In addition to the application described in the Examples section included herewith, examples of the application of the RRFLP method of the present invention, include, but are not limited to, the example discussed in more detail below. In some embodiments of the present invention, separate portions of the sample may be digested with different informative restriction enzymes and a separate ΔCt value calculated for each separate portion.

The RRFLP method of the present invention may be used for the detection and characterization of infectious laryngotrachetis virus (ILTV) based on the amplification of a 222-base-pair PCR fragment using primers located in a conserved region of the infected cell protein 4 gene that encompasses a single nucleotide polymorphism restriction endonuclease *Msp*I (Creelan et al., 2006 *Avian Pathol.* 35(2):173-9).

The RRFLP method of the present invention may be used for the identification and differentiation of varicella-zoster virus (VZV) wild-type strains from the attenuated varicella Oka vaccine strain based on the PCR amplification of a VZV open reading frame (ORF) 62 region. A single specific amplicon of 268 bp with a *Sma*I enables accurate strain differentiation; there are three *Sma*I sites present

in amplicons of vaccine strain VZV, compared with two enzyme cleavage sites for all other VZV strains tested). Thus, the Oka vaccine strain can be accurately differentiated from wild-type VZV strains circulating in countries representing all six populated continents. Moreover, this informative restriction enzyme site reliably distinguishes wild-type Japanese strains from vaccine strains (Loparev et al., 2000 *J Clin Microbiol.* 38(9):3156-60).

The RRFLP method of the present invention may be used for differentiation between field isolates and live vaccine strains ts-11 and 6/85 of *Mycoplasma gallisepticum* ("MG") in Israel. PCR primers targeted to the gene mgc2, encoding a cytadherence-related surface protein are uniquely present in MG. The mgc2-PCR diagnostic primers are specific for MG in tests of all avian mycoplasmas or bacteria present in the chicken trachea and are sensitive enough to readily detect MG in tracheal swabs from field outbreaks. Differentiation of vaccine strain ts-11 is based on restriction enzyme sites in the 300-base-pair (bp) mgc2-PCR amplicon present in ts-11 and missing in MG isolates from field outbreaks in Israel. Restriction sites for the enzymes *Hae*II and *Sfa*N1 are present in the amplified region in strain ts-11 and not in twenty-eight field isolates of MG, comprising a representative cross section of all the MG isolates from the period 1997-2003; mgc2-PCR amplification and restriction of the amplicon with *Hae*II, gives a 270-bp fragment for ts-11 or no restriction for other MG strains tested. This test can also be used to identify the 6/85 vaccine strain, which yields a 237-bp product, readily differentiated from the approximately 300-bp PCR product of all other strains tested (Lysnyansky et al., 2005 *Avian Dis.* 49(2):238-45 and 2005 *Avian Dis.* 49(3):451).

The RRFLP method of the present invention may be used for the differentiation of *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. Digestion with the restriction enzyme *Sma* I is coupled with the use of a genus-specific sequence of mycoplasma for the PCR reaction. Four isolates of *M. pulmonis* contain an informative *Sma* I restriction site, while there was no digestion with *Sma* I in *M. arthritidis* (Kim et al., 2005 *Exp Anim.* 54:359-62).

The RRFLP method of the present invention may be used for the detection of a polymorphism at codon 129 of the prion protein gene that has been shown to confer genetic susceptibility to prion diseases and to influence the epidemic course

of variant Creutzfeldt-Jakob disease (de Paula et al., 2005 *Eur J Epidemiol.* 20:593-5).

The RRFLP method of the present invention may utilize any of the many RFLPs that serve as genetic markers of disease, identifying whether or not an individual possesses a particular genetic defect. There is growing list of inherited disorders where DNA probes are available to test whether or not someone is carrying a defective gene. Some examples include, but are not limited to, Duchenne muscular dystrophy where progressive muscle weakness is caused by a genetic deficiency in dystrophin protein (for further info see the world wide web at mdausa.org); cystic fibrosis where respiratory functioning is impaired and is related to genetic deficiency in a membrane ion channel protein (see the world wide web at cff.org); blood disorders such as hemophilia and sickle cell anemia caused by testable genetic defects (see the world wide web at nih.gov); and Tay-Sachs disease.

The RRFLP method of the present invention may utilize any of the various RFLPs that serve as a genetic marker for cancer. For example, breast cancer, including the BRCA1 gene, prostate cancer, and colon cancer. See, for example, Watkins, 1988 *Biotechniques* 6(4):310-319, 322. The RRFLP method of the present invention may be used for any of a variety of applications in DNA fingerprinting, such as to identify genetic diversity within breeding populations in plants and animals, to differentiate between plant species cultivars, as well as to identify plants containing a gene of interest.

The RRFLP method of the present invention may be used in human identity applications, such as forensic analysis in crimes where DNA samples of suspects are amplified for typing experiments against samples taken from the scene of the crime.

The RRFLP method of the present invention has application in the rapidly developing field of pharmacogenetics in which genetically determined propensities of individual patients to respond favorably or adversely to a given pharmacologic agent can be determined prior to administration of that drug (Cartwright, 2001 *Expert Rev Mol Diagn.* 1(4):371-6).

The RRFLP method of the present invention may be used to identify and differentiate strains of various poultry pathogens. Embodiments of the RRFLP method of the present invention may be used in a wide range of rapid diagnostic assays, including, for example assays for mycoplasmosis, infectious bronchitis, and

infectious laryngotracheitis. The RRFLP method of the present invention may be used to identify and differentiate poultry pathogens such as *Campylobacter*, infectious bursal disease virus, Newcastle disease virus, infectious bronchitis virus, *Mycoplasma gallisepticum*, fowl adenovirus, *Salmonella*, and avian parvoviruses (Ayling *et al.*, 1996 *Res. Vet. Sci.* 60(2):168-172; Jackwood and Sommer, 1997 *Avian Diseases* 41(3):627-637; Kou *et al.*, 1999 *J. Vet. Med. Sci* 61(11):1191-1195; Kwon *et al.*, 1993 *Avian Diseases* 37(1):194-202; Lysnyansky *et al.*, 2005 *Avian Diseases* 49(2):238-245; Meulemans *et al.*, 2004 *Avian Pathology* 33(2):164-170; Park *et al.*, 2001 *J. Vet. Sci.* 2(3):213-219; and Sirivan *et al.*, 1998 *Avian Diseases* 42(1):133-139).

The RRFLP method of the present invention may be used to identify and differentiate the avian pathogen infectious laryngotracheitis virus (ILTV), a member of the family *Herpesviridae*, subfamily alphaherpesviridae. Infectious laryngotracheitis (ILT) is an upper-respiratory disease of poultry of worldwide distribution (Guy and Bagust, 2003 *Diseases of Poultry*, Iowa State University Press: Ames, Iowa; pp. 121-134) characterized by acute respiratory signs, which include gasping, coughing, sneezing, depression, nasal discharge, and conjunctivitis. For severe forms of the disease, signs include labored breathing and expectoration of bloody mucous, while severe hemorrhages and mucous plugs are observable upon gross examination of the trachea. Although some severe signs of the disease are characteristic, in less severe episodes of the disease many signs are similar to other acute respiratory diseases of poultry (Linares *et al.*, 1994 *Avian Dis.* 38:188-92; Sellers et al., 2004 *Avian Dis.* 48(2):430-6) and the need for a specific differential diagnosis is essential for the rapid detection of the virus.

The disease is common in areas of intense poultry production and during severe outbreaks it causes great economic losses due to high bird morbidity and moderate mortality. The disease is mainly controlled by vaccination with live attenuated vaccines. Traditionally in the U.S. two types of live-attenuated vaccines have been widely utilized. One type of vaccine is attenuated by multiple passages in embryonated eggs (chicken embryo origin "CEO") (Samberg *et al.* 1971 *Avian Dis.* 15:413-417). In the second type, vaccine is generated by multiple passages in tissue culture of chicken cells (tissue culture origin "TCO") (Gelenczei & Marty, 1965 *Avian Dis.* 14:44-56).

The virus can be isolated from field material in specific pathogen free (SPF) chicken embryos (CE) inoculated via the chorioallantoic (CAM) route or by isolation in primary chicken embryo kidney (CEK), chicken embryo liver (CELi), or in chicken kidney (CK) cells. Although sensitive, virus isolation may take three to four weeks and several cell culture passages are required before the cytopathic effect (CPE) caused by ILTV replication appears in the cell culture systems. Therefore, rapid assays for detection of the virus are usually performed in combination with virus isolation to accelerate the diagnosis of the disease. Histopathology examination remains the standard method for the rapid diagnosis of ILT. Characteristic lesions of ILT include syncytial cell formation of the tracheal epithelial cells with intranuclear inclusion bodies, necrosis, and hemorrhage. Inclusion bodies are present only during the early stages of infection (one to seven days post infection) and disappear as infection progresses as a result of the necrosis and desquamation of epithelial cells. And in cases of mild forms of the disease the differential diagnosis based on histopathological differentiation can be difficult due to infrequent number of intranuclear inclusions found with or without syncytial cell formation. Therefore other rapid assays have been utilized for the detection of ILTV such as the use of fluorescently-labeled polyclonal antibodies (FA) as immunoprobes to detect viral antigens intracheal and conjunctival smears. Monoclonal antibodies have been used to detect viral antigens in frozen tracheal sections by an indirect immunoperoxidase method, and in an antigen capture ELISA.

Earlier experimental evidence demonstrated that live attenuated vaccine strains could easily revert to virulence after bird-to-bird passage (Guy *et al.*, 1991 *Avian Dis.* 35(2):348-355), or after reactivation from latency (Hughes *et al.*, 1991 *Arch. Virology* 121:213-218). Once vaccine strains have been introduced in the field the identification of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses (Guy and Bagust, 2003 *Diseases of Poultry*, Iowa State University Press: Ames, Iowa; pp. 121-134). Initial attempts to differentiate among ILTV strains in the U.S. were achieved by restriction fragment length polymorphism (RFLP) analysis of the viral genome (Leib *et al.*, 1986 *Avian Dis.* 30:835-837; Guy *et al.*, 1989 *Avian Dis.* 33:316-323; Andreasen *et al.*, 1990 *Avian Dis.* 34:646-656; Keller *et al.*, 1992 *Avian Dis.* 36:575-581; Keeler *et al.*, 1993 *Avian Dis.* 37:418-426). However, routine use of RFLP analysis of the viral

genome for epidemiological purposes was limited due to the difficulties in obtaining high yields of pure viral DNA. With the advent of the polymerase chain reaction, restriction fragment length polymorphism of PCR products (PCR-RFLP) has greatly facilitated the differentiation of ILTV strains. PCR-RFLP and sequencing analysis of single and multiple viral genes and genome regions has permitted the differentiation of ILTV isolates from vaccine strains in different parts of the world (Chang *et al.*, 1997 *J Virol. Meth.* 66:179-186; Clavijo & Nagy, 1997 *Avian Dis.* 41(1):241-246; Graham *et al.*, 2000 *Avian Pathology* 29:57-62; Han & Kim, 2001 *Vet. Microbiol.* 4:321-331; Han & Kim, 2003 *Avian Dis.* 47:261-271; Kirkpatrick *et al.*, 2006 *Avian Dis.* 50:28-34; Creelan *et al.*, 2006 *Avian Pathology* 35(2):173-179). The use of multiple gene sequence analysis and multiple PCR-RFLP has been essential to identify informative single nucleotide polymorphism (SNP) appropriate for the discrimination of isolates from a particular region (Kirkpatrick *et al.*, 2006 *Avian Dis.* 50:28-34, Ojkic *et al.*, 2006 *Avian Pathology* 35(4):286-292, Oldoni and García 2007 *Avian Pathology* 36(2):167-176). PCR-RFLP analysis of informative SNPs has been utilized to differentiate field isolates form vaccine strains (Creelan *et al.*, 2006 *Avian Pathology* 35(2):173-179).

The RRFLP assay of the present invention may be applied as a method for the detection of ILTV infected birds. Indeed, PCR has already proven to be an effective and rapid test to detect ILTV infected birds in severe (Williams et al., 1992 *J Gen Virol.* 73(9):2415-20) and mild outbreaks (Sellers et al., 2004 *Avian Dis.* 48(2):430-6) of the disease. And, PCR has been successfully used to detect ILTV DNA from trachea scrapings of experimentally and naturally infected chickens, from extra-tracheal sites such as the conjuctiva and from the trigeminal ganglia and from formalin-fixed, paraffin-embedded tissues as well.

The RRFLP assay of the present invention may be applied as a method to differentiate between the two different types of infectious laryngotracheitis virus attenuated live vaccines. Sequence analysis of ILTV vaccines revealed an informative polymorphic site in the 5' non-coding region of the infected cell protein (ICP4) recognized by restriction enzymes *AvaI* and *AlwI* present in the tissue culture origin (TCO) and chicken embryo origin (CEO) attenuated vaccines, respectively. These two informative polymorphic sites were used in a RRFLP assay to rapidly and

reproducibly genotype ILTV attenuated live vaccines, outbreak related isolates, and clinical isolates.

The methods of the present invention may be used in the detection and/or differentiation of ILTV strains. In some embodiments of the present invention, for the detection and/or differentiation of ILTV strains, and/or a portion of the sample may be digested with the restriction enzyme *Alw* 1 and a portion of the sample may be digested with the restriction enzyme *Ava* I and the oligonucleotide primer pair flanks about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence (as shown in Fig. 4).

Application of the RRFLP method of the present invention include, but are not limited to, those described in more detail in the Examples included herewith. As described in Examples 1 to 6, the RRFLP assay of the present invention can be used as a novel diagnostic assay for the differentiation of infectious laryngotracheitis virus isolates. The method of the present invention has commercial applicability, for example, in the poultry diagnostic laboratory as a rapid means of differentiating an ILTV isolate into one of three categories; 1) wild type, 2) CEO vaccine or 3) TCO vaccine.

The present invention provides a kit for detecting or differentiating ILTV, the kit including oligonucleotide primers that flank an informative restriction enzyme site in the ICP4 gene (Accession No. L32139). Accession No. L32139 is the complete nucleotide sequence, bases 1 to 8364, of the major immediate early protein (ICP4) gene of infectious laryngotracheitis virus (gallid herpesvirus 1) (version L32139.1, February 27, 1996). The sequence is available on the world wide web at ncbi.nlm.nih.gov/entrez/ viewer.fcgi?db=nuccore&id =493597. See also, Johnson et al., 1995 *Virus Res.* 35:193-204. The informative restriction enzyme site may located between about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, as shown in Figure 4. The oligonucleotide primer pair may be located within nucleotide positions 2039 to 2950 of the ICP4 gene (Accession No. L32139). The oligonucleotide primer pair may flank nucleotide positions 2392 to 2534 of the ICP4 gene (Genebank Accession No. L32139). The oligonucleotide primers may flank about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, as shown

in Figure 4. One of the oligonucleotide primers may include SEQ ID NO: 7, may be SEQ ID NO:7, or may be a sequence derived from SEQ ID NO:7. One of the oligonucleotide primers may include SEQ ID NO:8, may be SEQ ID NO:7, or may be a sequence derived from SEQ ID NO:8. A kit of the present invention may also include the restriction enzyme *Alw* 1, and/or the restriction enzyme *Ava* 1, and/or restriction buffers for the *Alw* 1 and/or *Ava* 1 restriction enzymes. The kit may include printed instructions, one or more positive controls, one or more negative controls, and/or reagents used in the performance of PCR, such as, for example, 10X buffers and/or polymerase enzymes.

The present invention includes isolated oligonucleotide primers for use in the methods of the present invention. Oligonucleotide primers of the present invention may flank the informative *Alw* 1 or *Ava* 1 restriction enzymes sites found within nucleotide positions 2039 to 2950 of the ICP4 gene (Accession No. L32139), as shown in Fig. 4. Oligonucleotide primers of the present invention include, but are not limited to, any of the oligonucleotide primers described herein. Oligonucleotide primers of the present invention may be found within nucleotide positions 2039 to 2950 of the ICP4 gene (Accession No. L32139), or be complementary to a sequence within nucleotide positions 2039 to 2950 of the ICP4 gene (Accession No. L32139). An oligonucleotide primer of the present invention may flank nucleotide positions 2392 to 2534 of the ICP4 gene (Genebank Accession No. L32139). An oligonucleotide primer of the present invention may flank about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, as shown in Fig. 4. An oligonucleotide primer of the present invention may include SEQ ID NO: 7, may be SEQ ID NO:7, or may be a sequence derived from SEQ ID NO:7. An oligonucleotide primer of the present invention may include SEQ ID NO:8, be SEQ ID NO:7, or may be a sequence derived from SEQ ID NO:8, or a derivative thereof. The present invention also includes an isolated oligonucleotide primer having the sequence ACG GTA ATG GTA TGC TGG G (SEQ ID NO:5), CTC ACA GCG GTT GTT TTC TC (SEQ ID NO:6), TAC TAC TCC CCA CCA GAA AG (SEQ ID NO:7), or CGT CGA GGA ATC AGA GGA CAT (SEQ ID NO:8). The present invention also includes an isolated oligonucleotide primer selected from the group consisting SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

An isolated primer of the present invention may be a nucleic acid sequence that hybridizes under stringent conditions to one or more of: nucleotides 2039 to 2950 of the ICP4 gene (Accession No. L32139); SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; the direct complement of nucleotides 2039 to 2950 of the ICP4 gene (Accession No. L32139); the direct complement of SEQ ID NO:1; the direct complement of SEQ ID NO:2; the direct complement of SEQ ID NO:3; or the direct complement of SEQ ID NO:4. As used herein, stringent hybridization conditions may be 50% formamide, 5X SSC, 50 M sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml, 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC and 50% formamide at 55°C, followed by a wash comprising of 0.1X SSC containing EDTA at 55°C. The ability to derive an oligonucleotide primer based upon a cDNA sequence encoding a given protein, such as the ICP4 gene (Accession No. L32139), is described in, for example, Stein and Cohen (Cancer Res. 48:2659, 1988) and van der Krol et al. (BioTechniques 6:958, 1988).

An oligonucleotide primer of the present invention may be about 5 to about 50 nucleotides in length, about 14 nucleotides to about 30 nucleotides in length, about 17 to about 23 nucleotides in length, about 18 to about 22 nucleotides in length, about 19 to about 21 nucleotides in length. It may be about 5 bases in length, about 6 bases in length, about 7 bases in length, about 8 bases in length, about 9 bases in length, about 10 bases in length,
about 11 bases in length, about 12 bases in length, about 13 bases in length, about 14 bases in length, about 15 bases in length, about 16 bases in length, about 17 bases in length, about 18 bases in length, about 19 bases in length, about 20 bases in length, about 21 bases in length, about 22 bases in length, about 23 bases in length, about 24 bases in length, about 25 bases in length, about 30 bases in length, about 35 bases in length, about 40 bases in length, about 45 bases in length, or about 50 bases in length.

As used herein an "isolated" nucleic acid molecule or primer is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature.

Samples that can be used in the methods of the present invention can be obtained from any source. Samples include, but are not limited to, environmental or food samples and medical or veterinary samples. Samples may be liquid, solid, or semi-solid. Samples may be swabs of solid surfaces. Samples may include environmental materials, such as the water samples, airborne particles such as pollen and dust, and filters from air samples. Samples may be of meat, poultry, processed foods, milk, cheese, or other dairy products. Samples may be foodstuffs, beverages, cosmetic products, pharmaceutical products, healthcare products, or surfaces such as floors and tables. Medical or veterinary samples include, but are not limited to, blood, blood products, tissue, ascites, culture media, body fluids, skin, pus, urogenital specimens, feces, sputum, cerebrospinal fluid, fecal samples, and different types of swabs. A sample may be obtained from a clinical isolates, for example, and isolate obtained from skin or soft tissue infections. A sample may be obtained from a swab of a body site, for example, from the nose, including, but not limited to, the anterior nares, the throat, the perineum, the axilla, or the skin. Samples may be used directly in the methods of the present invention, without preparation or dilution. Samples may be diluted or suspended in solution, which may include, but is not limited to a buffered solution or a bacterial culture medium. A sample that is a solid or semi-solid may be suspending in a liquid by mincing, mixing or macerating the solid in the liquid.

With the present invention, DNA for RRFLP analysis may be prepared by any of a variety of methods, including, but not limited to any of those described herein. For example, extraction by a standard procedure such as that described in Ausubel, F. M., R. Brent, R. E. Kingston, B. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York, N.Y. may be used.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example 1

Genotyping Infectious Laryngotracheitis Virus (ILTV) by Multiple Gene Sequencing and Reverse Restriction Fragment Length Polymorphism (RRFLP)

In this example, the development of a novel Reverse Restriction Fragment Length Polymorphism method for the identification of infectious laryngotracheitis virus genotype is described. In order to understand the ***R***everse ***R***estriction ***F***ragment ***L***ength ***P***olymorphism (RRFLP) assay of the present invention (Fig. 1), it is constructive to compare the conventional ***R***estriction ***F***ragment ***L***ength ***P***olymorphism (RFLP) assay for determining viral genotypes (Fig. 2).

Infectious laryngotracheitis virus (ILTV) is an acute respiratory disease of chickens that affects poultry worldwide. Waves of the disease are observed in US one or twice a year particularly in areas of dense broiler production. In an effort to better understand the origin of these outbreaks typing of outbreak-related isolates has been conducted by multiple viral gene sequencing. The construction of a database of viral sequences from vaccine strains, backyard flock isolates, broiler and breeder isolates led to the differentiation of viral strains and the identification of genome sites that can be utilized as makers to trace isolates in the field. Using this multiple gene sequence typing approach, 27 ILTV isolates originated between the years 1991 to 2005 from different regions in the US, from broilers, layers, broiler-breeders and backyard flocks were characterized (Table 1). Sequencing analysis of multiple genes has allowed the differentiation of US ILTV isolates into three main groups CEO-like (23 isolates), TCO-like (1 isolate), and field or backyard-flock isolates (3 isolates).

Although conventional polymerase chain reaction (PCR) assays provide detection of ILTV with a high level of sensitivity and specificity, such assays do not give any information about the genotype of the ILTV in a particular sample. This information is important for epidemiological studies of the virus, which can be used to study the spread of the virus throughout poultry flocks. Current methods of genotyping involve the two steps of gene specific PCR followed by RFLP (Chang et al., 1997 *J Virol Methods* 66(2):179-86; Clavijo et al., 1997 *Avian Dis.* 41(1):241-6; Creelan et al., 2006 *Avian Pathol.* 35(2):173-9; Han and Kim, 2003 *Avian Dis.*

47(2):261-71; Han and Kim, 2001 *Microbiol.* 83(4):321-31; Kirkpatrick et al., 2006 *Avian Dis.* 50(1):28-34; Sellers et al., 2004 *Avian Dis.* 48(2):430-6) or sequence analysis (Chang et al., 2000 *Avian Dis.* 44(1):125-31; and Han and Kim, 2001 *Microbiol.* 83(4):321-31).

Table 1. Genotype of United States ILTV strains by multiple gene sequencing analysis.

ISOLATES	ORIGIN
648-88	BYF[A]
S2816-02	BYF
8954-97	BRF[B]
9030-97	BRF
7227-03	BRF
7253-03	BRF
7271-03	BRF
7399-03	BRF
32396-03	BRF
32650-03	BRF
18138-04	BRF
JEL-04	BRF
K0400820-04	BRF
L6172-04	BRF
L6811B-04	BRF
L6811C-04	BRF
L15505-04	BRF
S1687A-04	BRF
S1687B-04	BRF
CL1-04	BRF
CL2-04	BRF
FA1-04	LYF[C]
1219-00	BBF[D]
6902-02	BBF
Chicken embryo origin (CEO)	Vaccine
Tissue culture origin (TCO)	Vaccine
USDA	Challenge strain

[A]BYF-backyard flock; [B]BRF- broiler flock;
[C]BBF- broiler breeder flock; [D]layer flock

However, with conventional PCR, quantitative aspects are difficult and cumbersome to resolve. Furthermore, conventional PCR is prone to contamination and in some instances the interpretation of gel electrophoresis is inconclusive. As the second generation of nucleic amplification methods, real-time PCR has received wider acceptance in the diagnosis of viral diseases due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Mackay et al., 2002 *Nucleic Acids Res.* 30(6):1292-305). In addition, the capability of real time PCR to quantify viral DNA has added clinical value to the molecular diagnostic of viral diseases.

Rather than PCR-RFLP, a novel technique named Reverse Restriction Fragment Length Polymorphism (RRFLP) assay was utilized for analyses. The RRFLP assay is a novel technique where the viral DNA is initially digested with restriction enzymes followed by real-time polymerase chain reaction (PCR) amplification of the target genome region. The analysis of the cycle threshold (ΔC_T) differences obtained from digested and undigested template DNA provides the genotype of the sample. The Ct value is the PCR cycle number where a particular reaction crosses a predefined threshold level of fluorescence. To calculate the ΔCt value, the following formula is used:

ΔCt value = (Ct value of reaction exposed to restriction enzyme) – (Ct value of reaction not exposed to restriction enzyme).

For any sample, a ΔCt value $\geq +1$ after treatment with a specific restriction enzyme means that the sample DNA is susceptible to cleavage by the specific restriction enzyme at a site between the flanking primer set. Therefore (Fig. 1), the sample was digested by Enzyme B between the flanking primers, which meant that there was a site between the flanking primer set susceptible to restriction enzyme B and this information differentiated the sample into a particular genotype.

Example 2

Identification of a polymorphic site within the ICP4 gene

In this example, a polymorphic site was identified in the non-coding promoter region of the ICP4 gene that allowed for the differentiation of ILTV isolates from the United States into three groups, CEO-like, USDA/TCO-like, and wild-type. Natural restriction enzyme sequences at the polymorphic site were used to rapidly genotype ILTV positive samples from infected birds using the RRFLP assay as described in Example 1.

Materials and Methods

DNA extraction. DNA extraction from viral isolates, and trachea and eye conjunctiva samples was performed using the Qiamp Mini kit (Qiagen, Valencia, CA) with modifications from the manufacturer's recommendations. Briefly, 100 µl of the swab suspension was incubated with 10 µl of proteinase K and 400 µl of lysis buffer (AL) at 56° C for 10 minutes. After incubation, 100 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Amplification and sequence analysis of the ICP4 gene fragment. A 1,246 base pair fragment of the ICP4 gene, encompassing part of the gene non-coding region and the 5' coding region, was amplified and sequenced for six CEO vaccines, the TCO vaccine and ten ILTV isolates. The amplification and sequencing reactions were performed using the set of primers listed in Table 2. The PCR reaction setup was as follows: 28.5 µl of water, 5 µl of 10X PCR buffer, 4 µl of 25 mM $MgCl_2$, 5 µl of 1 mM dNTPs, 1 µl of 5 µM of ICP4 non-coding primer, 1 µl of 5 µM ICP4 coding primer, 0.5 µl of Taq polymerase (5U/µl), and 5 µl of template. The reaction was cycled in a conventional thermocycler using a program of one cycle of 94° C, two minutes; 35 cycles of 94° C, one minute; 53° C, one minute; 72° C, 1.5 minutes; and one cycle of 72° C, 12 minutes. After amplification, the PCR products were separated by gel electrophoresis on a 1.0 % agarose gel and visualized by UV trans-illumination. Amplification reactions producing the expected 1,246 base pair fragment were purified with a QIAquick PCR purification kit (Qiagen, Valencia,

CA). Purified amplification products were sequenced at the Integrated Biotech Labs (IBT, The University of Georgia, Athens, GA) using the same primers (Table 2). Raw sequence data was edited using SeqMan and aligned using MegAlign programs from DNASTAR software version 5.5 (DNASTAR, Inc. Madison, WI). Percentage sequence identity of the ICP4 sequences was determined by clustal method MegAlign and phylogenetic analysis was performed using the neighbor joining group method and 500 boostrap resamplings with the PAUP Version 4 program (Sinauer Associates, Inc. Publishers).

Table 2. ILTV ICP4 gene primer sequences.

Primer name	Sequence 5' to 3'	Nucleotide positions[a]
ICP4 non-coding[b]	ACG GTA ATG GTA TGC TGG G (SEQ ID NO. 5)	1807 - 1825
ICP4 coding[b]	CTC ACA GCG GTT GTT TTC TC (SEQ ID NO. 6)	3052 - 3033
ICP4 polymorphic site F[c]	TAC TAC TCC CCA CCA GAA AG (SEQ ID NO. 7)	2389 - 2408
ICP4 polymorphic site R[c]	CGT CGA GGA ATC AGA GGA CAT (SEQ ID NO. 8)	2534 - 2514

[a] Primer position according to ICP4 sequence accession number L32139
[b] Sequencing primers
[c] Real-time PCR primers

Results

Sequencing analysis of ICP4 gene fragment. Sequence analysis corresponding to nucleotide positions 2039 to 2950 of the ICP4 gene (Accession number L32139) was performed for CEO vaccines, TCO vaccine strain, and 10 field isolates (Table 3). Sequences segregated as shown in the phylogenetic tree in two main lineages (Fig. 3), a lineage that includes the six commercial CEO vaccines and eight outbreak related isolates with 99.9 to 100% sequence similarity. A second lineage containing the TCO vaccine, one back-yard flock isolate (24/H/91/BCK), and one broiler-breeder isolate (13/E/03/BBR). The breeder isolate (13/E/03/BBR) sequence was 100% similar to the TCO vaccine and share 99.8% similarity with the back-yard flock isolate (24/H/91/BCK) sequence.

An informative polymorphic site was identified in the 5' non-coding region of the ICP4 gene (Fig. 4). Within this region, the CEO vaccine strains and isolates, 9/C/97/BR, 10/C/97/BR, 23/H/01/BBR, 15/E/03/BR, 26/I/03/BR, 11/C/05/BR,

21/G/05/BR, 314/K/BR/04 (identified in figure 4 as BR/BBR) contained a unique *Alw* I restriction site. The TCO vaccine strain contained a unique *Ava* I restriction site, and the backyard flock strain 24/H/91/BCK lacked either restriction enzyme site

Table 3. Infectious laryngotracheitis virus outbreak related isolates.

Isolates[a]	Age[b]	Vaccination
24/H/91/BCK	183 to 548	NV[c]
9/C/97/BR	56	NV
10/C/97/BR	53	CEO
23/H/01/BBR	392	NV
13/E/03/BBR	441	TCO
15/E/03/BR	35	NV
26/I/03/BR	40	NV
11/C/05/BR	42	NV
21/G/05/BR	42	NV
314/K/BR/04	33	NV

[a]Sample number/State/Year of isolation/Bird type: States of origin are indicated by letters (C, E, G, H, I) - each isolate with the same letter originated in the same state; bird type: from commercial poultry broiler (BR), broiler breeder (BBR), from backyard flock (BCY)
[b]Bird age expressed in days;
[c]Flocks non-vaccinated against ILTV.

Example 3

Use of Reverse Restriction Fragment Length Polymorphism to Identify Infectious Laryngotracheitis Virus Strain by ICP4 genotype

In this example, two informative polymorphic sites identified in the non-coding region of the ICP4 gene (see Example 2) were used for the differentiation of ILTV live attenuated vaccines (CEO and TCO). Rather than using conventional PCR followed by RFLP analysis, the Reverse Restriction Fragment Length Polymorphism (RRFLP) assay of the present invention was utilized for analysis of the ICP4 gene. In the RRFLP assay, viral DNA is initially digested with restriction enzymes followed by real-time polymerase chain reaction (PCR) amplification of the target genome region. The analysis of the cycle threshold (ΔC_T) differences obtained from digested and undigested template DNA provides the genotype of the sample.

Materials and Methods

Real-Time ILTV PCR assay. Before RRFLP analysis, the viral genome copy number $\log_{10}$ per sample was determined with Real-Time PCR ILTV assay as previously described (Callison *et al.*, 2007 *J. Virol. Meth.* 139:31-38). Briefly, the primers and probe for the Real-Time PCR ILTV assay are located in the viral glycoprotein C and were synthesized by IDT (Coralville, IA), and BioSearch Technologies (Novato, CA). The final reaction volume was 25 µl including; 12.5 µliters of 2X master mix (Quantitect Probe PCR kit, Qiagen, Valencia, CA), primers were utilized to a final concentration of 0.5 µM, probe to a final concentration of 0.1 µM, 1 µl of HK-UNG (Epicentre, Madison, WI), 2 µl of water, and 5 µl of DNA template. The tubes were closed and cycled in a Smart Cycler thermocycler (Cepheid, Sunnyvale, CA) using a thermocycle program of 50° C, 2 minutes; 95° C, 15 minutes; and 40 cycles of 94° C, 15 seconds; 60° C, 60 seconds with optics ON. To determine the genome copy number $\log_{10}$ per sample, the standard curve equation previously reported by Callison *et al.* (2007 *J. Virol. Meth.* 139:31-38) was utilized to quantify the copy number of viral genomes found per sample.

Results

Reverse Restriction Fragment Length Polymorphism (RRFLP). The RRFLP technique was developed around the two informative polymorphic sites identified upstream of the ICP4 gene (Fig. 4). One CEO vaccine, the TCO vaccine, and the backyard flock isolate 24/H/91/BCK were selected for initial analysis. As shown in Fig. 5A, the RRFLP analysis showed that the backyard flock isolate (24/H/91/BCK) lacked both restriction enzyme sites as estimated by the ΔC_T values of 0.32 for *Alw* I and -0.01 for *Ava* I. The CEO strain was susceptible to *Alw* I digestion (ΔC_T of 6.07) and resistant to *Ava* I digestion (ΔC_T value of 0.12) indicating that the CEO type viruses has the *Alw* I site and lacks the *Ava* I site upstream of the ICP4 gene (Fig. 5B). The TCO strain was susceptible to the *Ava* I digestion (ΔCt value of 4.57) while resistant to *Alw* I digestion (ΔC_T value of 0.04) indicating that the viral nucleic acid has the *Ava* I site and lacks the *Alw* I site(Fig. 5C).

Example 4

Testing the Stability of the ICP4 Polymorphic Site and the Reproducibility of the Reverse Restriction Fragment Length Polymorphism Assay

In this example, the quality of the novel RRFLP assay was assessed by analyzing the stability of the polymorphic ICP4 site and by examining the reproducibility of the assay itself. In order to evaluate the reproducibility of the RRFLP analysis, two separate experiments with CEO and TCO vaccinated, and vaccine contact exposed chickens were performed.

Materials and Methods

Animals. Ninety-six white leghorn specific pathogen free (SPF) chickens were obtained from Merial (Gainesville, GA) for each experiment. Chickens were housed in stainless steel cages in the isolation room with filtered-air and positive-pressure at the Poultry Diagnostic and Research Center (PDRC, Athens, GA), and fed a standard diet and water *ad libitum*.

Vaccination experiments. At four weeks of age, birds were divided in four groups of twenty-four chickens per cage, 12 of which were vaccinated, and 12 were contact-exposed to the vaccinated birds. Wing bands were used in to identify contact-exposed birds. Chickens were vaccinated by eye-drop with the TCO and CEO live attenuated vaccine during two separate experiments using the recommended dose (33 µl per chicken). Larynx/trachea and eye conjunctiva swabs were collected from two vaccinated and two contact-exposed chickens at different time pointes post-vaccination. Eye conjunctiva swabs were collected from both eyes and resuspended in 1 ml of sterile phosphate buffered saline solution (PBSS) containing antibiotic-antimycotic (Gibco, Grand Island, NY) and 2% newborn calf serum (Gibco Grand Island, NY). Chickens were euthanized by CO_2 gas inhalation (Institutional Animal Care and Use Committee). During necropsy, the larynx and the trachea were dissected. The larynx and trachea were open longitudinally and the inside epithelium was scraped. Larynx and trachea scrapings were resuspended in 1 ml of PBSS. Both eye conjunctiva and tracheal samples were stored at – 80° C until further PCR processing.

Results

The stability of the polymorphic site within the ICP4 gene (Fig. 4) was assessed by testing different commercially available ILTV vaccines from the bottle and vaccines after several *in vivo* and *in vitro* passages by RRFLP and sequence analysis (Table 4). In total, six different CEO vaccines, four different CEO passages, one TCO vaccine, and five different passages were analyzed. The polymorphic site was stable in each vaccine tested.

The stability of the informative region upstream of the ICP4 gene was further assessed using samples collected from CEO and TCO vaccinated birds. Eye conjunctiva and trachea samples were collected from CEO and TCO vaccinated and contact exposed chickens at different days post vaccination. The results for eye conjunctiva samples are summarized in Table 5.

Table 4. Reproducibility of Reverse RFLP on commercially available ILTV vaccines and vaccine *in vivo* and *in vitro* passages

Isolate	ΔCt value for AlwI[a]	ΔCt value for AvaI[b]	RRFLP interpretation[c]	Sequencing interpretation[d]
CEO 1	5.42	0.36	CEO	CEO
CEO 2	4.01	0.04	CEO	CEO
CEO 3	3.00	0.49	CEO	CEO
CEO 4	4.13	0.09	CEO	CEO
CEO 5	4.20	-0.22	CEO	CEO
CEO 6	3.40	-0.32	CEO	CEO
CEO 4 (5 DPV)[e]	6.13	-0.41	CEO	CEO
CEO 4 (9 DPV)[e]	6.25	-0.09	CEO	CEO
CEO 4 (p1)[f]	3.36	-0.80	CEO	CEO
CEO 4 (p6)[f]	4.48	-0.53	CEO	CEO
TCO	0.16	3.63	TCO/USDA	TCO/USDA
TCO 5 (DPV)[e]	0.43	3.74	TCO/USDA	ND[g]
TCO 9 (DPV)[e]	0.18	4.33	TCO/USDA	ND
TCO (p1)[f]	0.26	3.59	TCO/USDA	ND
TCO (p2)[f]	0.19	3.54	TCO/USDA	ND
TCO (p3)[f]	0.41	3.80	TCO/USDA	ND

[a]ΔCt value for *Alw* I = (Ct value of sample DNA cut with *Alw* I)-(Ct value of uncut DNA sample)
[b]ΔCt value for Ava I = (Ct value of sample DNA cut with *Ava* I)-(Ct value of uncut DNA sample)
[c]RRFLP interpretation - a sample with ΔCt values ≤ 1 for either enzyme was interpreted as wild-type (WT); a sample with a ΔCt value for *Alw* I ≥ 1 and a ΔCt value for *Ava* I ≤ 1 was interpreted as chicken origin embryo (CEO) like isolate; a sample with a ΔCt value for *Alw* I ≤ 1 and a ΔCt value for *Ava* I ≥ 1 was interpreted as USDA or tracheal culture origin (TCO) like strain (USDA/TCO)
[d]Sequence interpretation – as determined by the phylogenetic relationship of the ICP4 gene fragment analyzed.
[e] Vaccine recovered from vaccinated birds 5 and 9 days post vaccination (DPV).
[f]Vaccine after consecutive passages (p #) in chicken embryo kidney (CEK) cells.
[g]Not done.

RRFLP analysis accurately and reproducibly identified each vaccine type. The RRFLP analysis was reproducible and consistent for each type of vaccine at the different time point tested. RRFLP analysis of CEO vaccinated and contact-exposed chickens produced $\Delta C_T \geq 1$ for *Alw* I digestion reactions and for TCO vaccinated and contact exposed chickens $\Delta C_T \geq 1$ were observed for *Ava* I digestions (Table 5). Similar RRFLP results were observed for tracheal swab samples collected from vaccinated and contact exposed chickens.

The limit of detection for the RRFLP assay was determined by testing 10-fold serial dilutions of DNA extracted from a CEO vaccine. The dilution representing 10^3 genomic copies (as determined by Real-Time PCR ILTV assay) was the last dilution positive for the RRFLP assay.

Table 5. Reverse RFLP analysis on eye conjunctiva samples collected from CEO and TCO vaccinated and contact-exposed birds

	Re Ti - PCR C_T[b]	RRFLP Undigested C_T	RRFLP *Alw* I C_T	RRFLP *Ava* I C_T	ΔC_T *Alw* I	ΔC_T *Ava* I	Interpretation[c]
CEO vaccinated[a]							
4	19.77	24.81	29.24	24.14	4.43	-0.67	CEO
6	24.19	33.11	37.93	32.78	4.82	-0.33	CEO
CEO contact-exposed[a]							
9	24.44	30.22	36.01	30.84	5.79	0.62	CEO
TCO vaccinated[a]							
4	25.44	33.40	34.10	38.20	0.70	4.80	TCO
6	25.70	32.84	31.87	36.16	-0.97	3.32	TCO
TCO contact-exposed[a]							
9	23.57	30.79	31.12	35.78	0.33	4.99	TCO

[a] Days post-vaccination; RRFLP interpretation - values ≤ 1 for either as a wild-type (WT) ΔC_T value for *Alw* I ≥ 1 I ≤ 1 was genotyped as sample with a ΔC_T a ΔC_T value for *Ava* I $\geq$ TCO like isolate

[b] Callison et al., 2007; [c] a sample with ΔC_T enzyme was genotyped strain, a sample with a and a ΔC_T value for *Ava* a CEO like isolates, a value for *Alw* I ≤ 1 and 1 was genotyped as

Example 5

Genotyping of Infectious Laryngotracheitis Virus in Field Isolates

In this example, outbreak-related field isolates of ILTV were analyzed by RRFLP to determine the genotype of the infectious virus.

Materials and Methods

Samples. Six commercially available chicken embryo origin (CEO) ILTV vaccines were obtained from Intervet America (Millsboro, DE, USA), Lohman Animal Health (Winslow, MA, USA), Schering-Plough Animal Health (Omaha, NE, USA), and Fort Dodge (Fort Dodge, IA, USA). The tissue culture origin (TCO) ILTV vaccine was obtained from Schering-Plough Animal Health (Omaha, NE, USA), and the USDA reference strain of ILTV was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All vaccines were resuspended in 10 ml of sterile phosphate buffered saline (PBS) and aliquots were stored at – 80° C as stocks for further propagation. Each vaccine strain was propagated and passed three consecutive times in chicken kidney (CK) cells prepared as previously described (Tripathy, 1998 *A Laboratory Manual for the Isolation and Identification f Avian Pathogens 4th Ed.*; American Assoc. of Avian Pathologists: Kennett Square, PA; pp. 111-115.)

Viral isolates selected for this study were obtained from outbreaks in broiler, broiler-breeders, and one backyard flock from the different regions of the U.S. (See Table 3, Example 2). Viral isolates were propagated in the chorioallantoic membrane (CAM) of chicken embryos and the second passage in CAM was utilized for viral DNA extraction.

Results

A total seven vaccines, 10 field isolates, and two CK cells passages of CEO and TCO vaccine were analyzed by the RRFLP and sequencing of the ICP4 5' non-coding region. The RRFLP technique correctly differentiated each vaccine type and agreed with sequencing results (See Fig. 3 as discussed in Example 2). For the

outbreak related isolates, the RRFLP technique typed 8 isolates (9/C/97/BR, 10/C/97/BR, 23/H/01/BBR, 15/E/03/BR, 26/I/03/BR, 11/C/05/BR, 21/G/05/BR, 314/K/BR/04) as CEO, isolate 24/H/91/BCY as wild type, and isolate 13/E/03/BBR as TCO. RRFLP typing showed a perfect agreement with the sequencing analysis of the ICP4 5' non-coding region when compared to the percentage of sequence similarities (Table 6).

Table 6. Reverse RFLP and Sequencing analysis ILTV vaccines (CEO and TCO) and outbreak related isolates

Vaccine/ Isolate	Real Time PCR C_T[c]	RRFLP Undigested C_T	RRFLP *Alw* I C_T	RRFLP *Ava* I C_T	ΔC_T *Alw* I	ΔC_T *Ava* I	RRFLP Interpretation[d]	Sequencing Analysis [f]
CEO VAC 1[a]	25.32	30.83	33.87	29.97	3.04	-0.86	CEO	CEO (100%)
CEO VAC 2[a]	24.94	29.99	34.70	29.93	4.71	-0.06	CEO	CEO (100%)
CEO VAC 3[a]	25.08	29.98	34.92	29.85	4.94	-0.13	CEO	CEO (100%)
CEO VAC 4[a]	24.51	29.98	34.95	29.92	4.97	-0.06	CEO	CEO (100%)
CEO VAC 5[a]	23.21	30.86	34.95	30.37	4.09	-0.49	CEO	CEO (100%)
CEO VAC 6[a]	23.38	30.39	34.79	29.92	4.40	-0.47	CEO	CEO (100%)
CEO VAC 4 (p3)[b]	15.51	18.32	22.80	18.71	4.48	0.39	CEO	CEO (100%)
TCO VAC	19.07	24.48	25.27	29.16	0.79	4.68	TCO	TCO (100%)
TCO VAC (p3)[b]	18.70	24.00	24.70	27.34	0.70	3.34	TCO	TCO (100%)
24/H/91/BCY	22.44	25.77	25.94	25.56	0.17	-0.21	WT[e]	TCO (99.8%)
9/C/97/BR	12.47	17.14	21.86	17.26	4.72	0.12	CEO	CEO (100%)
10/C/97/BR	12.68	15.68	18.94	15.72	3.26	0.04	CEO	CEO (100%)
23/H/01/BBR	23.70	27.43	28.86	26.77	1.43	-0.66	CEO	CEO (100%)
13/E/03/BBR	23.54	25.97	26.12	30.72	0.15	4.75	TCO	TCO (100%)

Table 6 (continued)

15/E//03/BR	20.14	22.90	27.34	23.20	4.44	0.30	CEO	CEO (100%)
26/I/03/BR	18.27	20.76	26.15	21.14	5.39	0.38	CEO	CEO (100%)
11/C/05/BR	11.84	18.14	22.46	18.37	4.32	0.23	CEO	CEO (100%)
21/G/05/BR	21.09	27.50	30.50	27.25	3.00	-0.25	CEO	CEO (100%)
314/K/BR/04	14.23	19.39	23.98	19.40	4.59	0.01	CEO	CEO (100%)

[a] A 1:100 dilutions of the vaccines were utilized in the RRFLP analysis

[b] Third passages in chicken kidney cells of CEO and TCO vaccines

[c] Callison *et al.*, 2007 *J Virol. Meth* 139:31-38

[d] A sample with ΔCT values ≤ 1 for either enzyme was genotyped as wild-type (WT); A sample with a ΔCT of ≥ 1 for *Alw* I and a $\Delta Ct \leq 1$ for *Ava* I was genotyped as chicken origin embryo (CEO); a sample with a $\Delta CT \leq 1$ for *Alw* I and a $\Delta CT \geq 1$ for *Ava* I was genotyped as tissue culture origin (TCO)

[e] Wild type strain

[f] Sequence analysis of nucleotide 2039 to 2950 of the ICP4 gene (Accession number L32139), indicated the strain with the highest nucleotide sequence similarity and in parenthesis the percentage of sequence similarity.

Example 6

Genotyping of Infectious Laryngotracheitis Virus in Clinical Samples

In this example, tracheas were collected from flocks affected by three recent ILTV outbreaks in broiler flocks and genotyped.

Materials and Methods

Clinical Samples. A total of 32 cases from three independent outbreaks were processed. Briefly, an average of three tracheas were received per case and processed separately. The mucosa of the trachea and larynx were scraped with a scalpel and the scrapings were resuspended in 1 ml of sterile 1X PBS containing 100 µg/ml of gentamicin (Invitrogen, Carlsbad, CA) and 5 µg/ml of amphotericin B (Invitrogen, Carlsbad, CA). Samples were vortexed and stored at - 80° C until further analysis.

Genotyping of clinical samples by RRFLP assay. Tracheal samples from three recent outbreaks of ILTV were analyzed by RRFLP and the results confirmed by sequence analysis of the ICP4 gene. All samples were genotyped as CEO-like virus by both methods (Table 7).

Table 7. Reverse RFLP and sequencing analysis on tracheal samples from ILTV outbreaks

Tracheal Samples		ΔCt value for Alw I[a]	ΔCt value for Ava I[b]	RRFLP interpretation[c]	Sequencing interpretation[d]
Outbreak	1A	5.37	0.32	CEO	CEO
	1B	4.28	0.01	CEO	CEO
	1C	9.88	0.45	CEO	CEO
	1D	7.32	0.42	CEO	CEO
	1E	7.28	0.48	CEO	CEO
	1F	8.05	0.12	CEO	CEO
	1G	5.61	0.11	CEO	CEO
	1H	5.55	0.10	CEO	CEO
	1I	7.75	0.46	CEO	CEO
	1J	5.60	0.45	CEO	CEO
	1K	6.60	0.76	CEO	CEO
	1L	7.19	0.61	CEO	CEO
	1M	5.37	0.32	CEO	CEO
	1N	6.11	0.41	CEO	CEO
	1O	5.00	0.20	CEO	CEO
	1P	6.18	-0.16	CEO	CEO
	1Q	4.89	-0.38	CEO	CEO
	1R	4.37	-0.04	CEO	CEO
	1S	6.76	0.15	CEO	CEO
	1T	4.21	0.69	CEO	CEO
	1U	6.70	0.39	CEO	CEO
	1V	4.85	0.23	CEO	CEO
Outbreak	2A	5.53	0.14	CEO	CEO
	2B	6.88	-0.05	CEO	CEO
	2C	7.04	0.48	CEO	CEO
	2D	5.24	-0.36	CEO	CEO
Outbreak	3A	5.91	0.03	CEO	CEO
	3B	4.60	-0.40	CEO	CEO
	3C	2.67	-0.33	CEO	CEO
	3D	5.34	0.27	CEO	CEO
	3E	4.98	0.25	CEO	CEO
	3F	5.60	0.40	CEO	CEO

[a]ΔCt value for *Alw* I = (Ct value of sample DNA cut with *Alw* I)-(Ct value of uncut DNA sample)
[b]ΔCt value for Ava I = (Ct value of sample DNA cut with *Ava* I)-(Ct value of uncut DNA sample)
[c]RRFLP interpretation - a sample with ΔCt values ≤ 1 for either enzyme was interpreted as wild-type (WT); a sample with a ΔCt value for *Alw* I ≥ 1 and a ΔCt value for *Ava* I ≤ 1 was interpreted as chicken origin embryo (CEO) like isolate; a sample with a ΔCt value for *Alw* I ≤ 1 and a ΔCt value for *Ava* I ≥ 1 was interpreted as USDA or tracheal culture origin (TCO) like strain (USDA/TCO)
[d]Sequence interpretation – as determined by the phylogenetic relationship of the ICP4 gene fragment analyzed.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified. Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

SEQUENCE LISTING FREE TEXT

SEQ ID NO:1	Basepairs 2392 to 2534 of the ICP4 gene sequence as found in the chicken embryo origin (CEO) vaccine.
SEQ ID NO: 2	Basepairs 2392 to 2534 of the ICP4 gene sequence as found in the tissue culture origin (TCO) vaccine.
SEQ ID NO:3	Basepairs 2392 to 2534 of the ICP4 gene sequence as found in broiler (9/C/97/BR) and broiler/breeder (23/H/01/BBR) isolates, identified as BR/BBR.
SEQ ID NO:4	Basepairs 2392 to 2534 of the ICP4 gene sequence as found in the backyard flock isolate(24/H/91/BCK) identified as BCK.
SEQ ID NO:5	ILTV ICP4 non-coding region synthetic oligonucleotide gene primer.
SEQ ID NO:6	ILTV ICP4 coding region synthetic oligonucleotide gene primer.
SEQ ID NO:7	ILTV ICP4 polymorphic site forward synthetic oligonucleotide gene primer.
SEQ ID NO:8	ILTV ICP4 polymorphic site reverse synthetic oligonucleotide gene primer.

What is claimed is:

1. A method of detecting the presence of a recognition site for a restriction enzyme in a nucleotide sequence, the method comprising:

digesting all or a portion of a sample comprising the nucleotide sequence with the restriction enzyme;

performing real-time polymerase chain reaction (PCR) on the sample digested with the restriction enzyme with an oligonucleotide primer pair that flanks the restriction enzyme recognition site;

determining the Ct value of the sample digested with the restriction enzyme;

comparing the Ct value of the sample digested with the restriction enzyme to the Ct value from a control sample not digested with the restriction enzyme;

calculating a ΔCt value, wherein a ΔCt value is the Ct value of the sample digested with the restriction enzyme minus the Ct value of a control sample not digested with the restriction enzyme;

wherein a ΔCt value $\geq +1$ indicates that the nucleotide sequence is digested by the restriction enzyme at a recognition site located between the oligonucleotide primer pair.

2. The method of claim 1 wherein separate portions of the sample are digested with different restriction enzymes and a separate ΔCt value is calculated for each separate portion.

3. The method of claim 1 used in the detection and/or differentiation of ILTV strains.

4. The method of claim 3, wherein a portion of the sample is digested with the restriction enzyme *Alw* 1.

5. The method of claim 3, wherein a portion of the sample is digested with the restriction enzyme *Ava* 1.

6. The method of claim 3, wherein a portion of the sample is digested with the restriction enzyme *Ava* I and a portion of the sample is digested with the restriction enzyme *Alw* 1.

7. The method of claims 3 to 6, wherein the oligonucleotide primer pair flanks about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence.

8. The method of claims 3 to 6, wherein the oligonucleotide primer pair flanks nucleotide positions 2039 to 2950 of the ICP4 gene (Genebank Accession No. L32139).

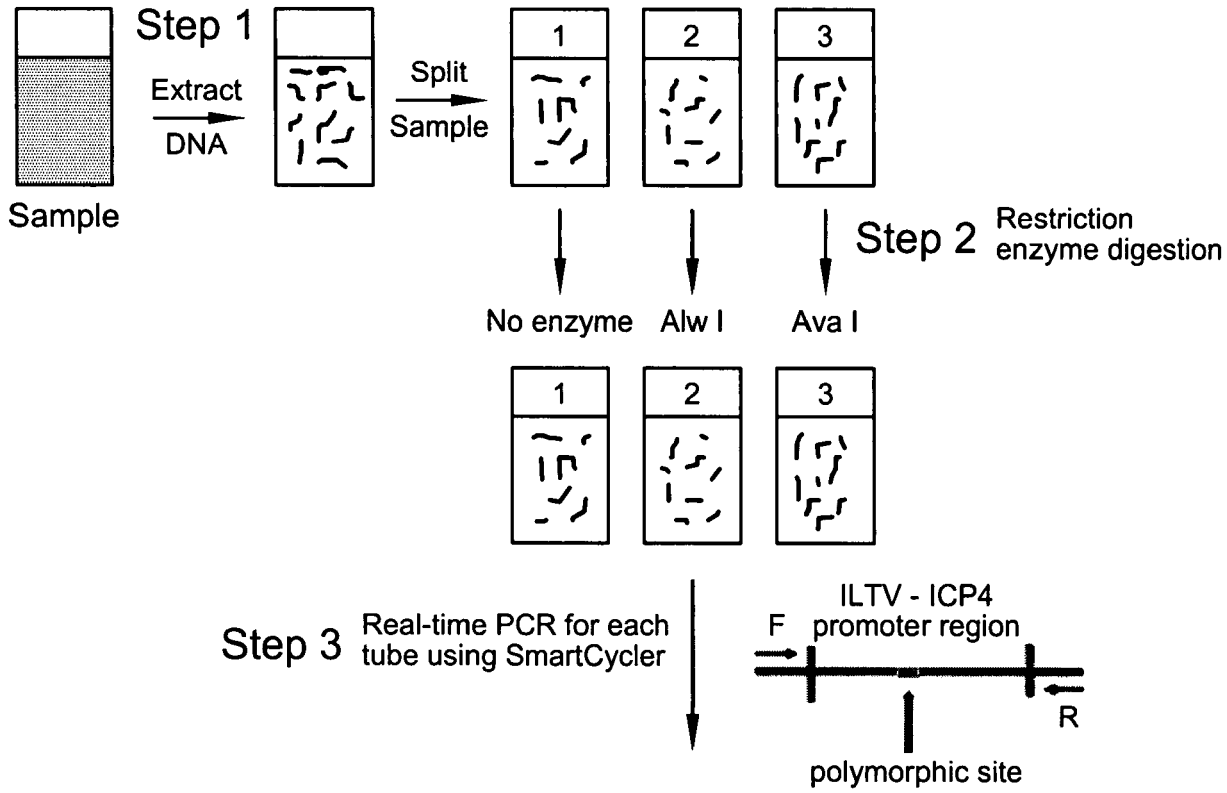
9. The method of claims 3 to 6, wherein the oligonucleotide primer pair flanks nucleotide positions 2392 to 2534 of the ICP4 gene (Genebank Accession No. L32139).

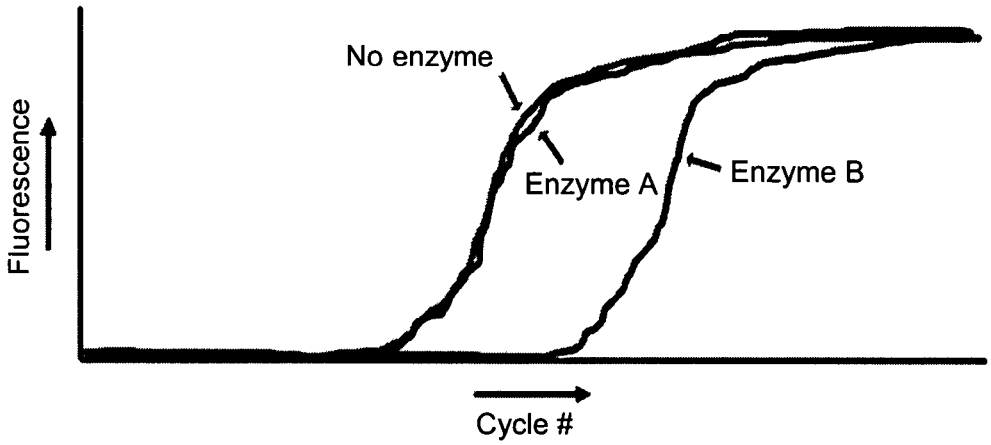
10. The method of claims 3 to 6, wherein the oligonucleotide primer pair is SEQ ID NO:7 and SEQ ID NO:8.

11. A method of diagnosing ILTV disease, the method comprising digesting a nucleotide sample comprising about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence with the restriction enzymes *Alw* 1 and/or *Ava* 1.

12. An oligonucleotide primer pair that flanks about nucleotide 60 to about nucleotide 80 of the ICP4 gene promoter sequence.

Fig. 1

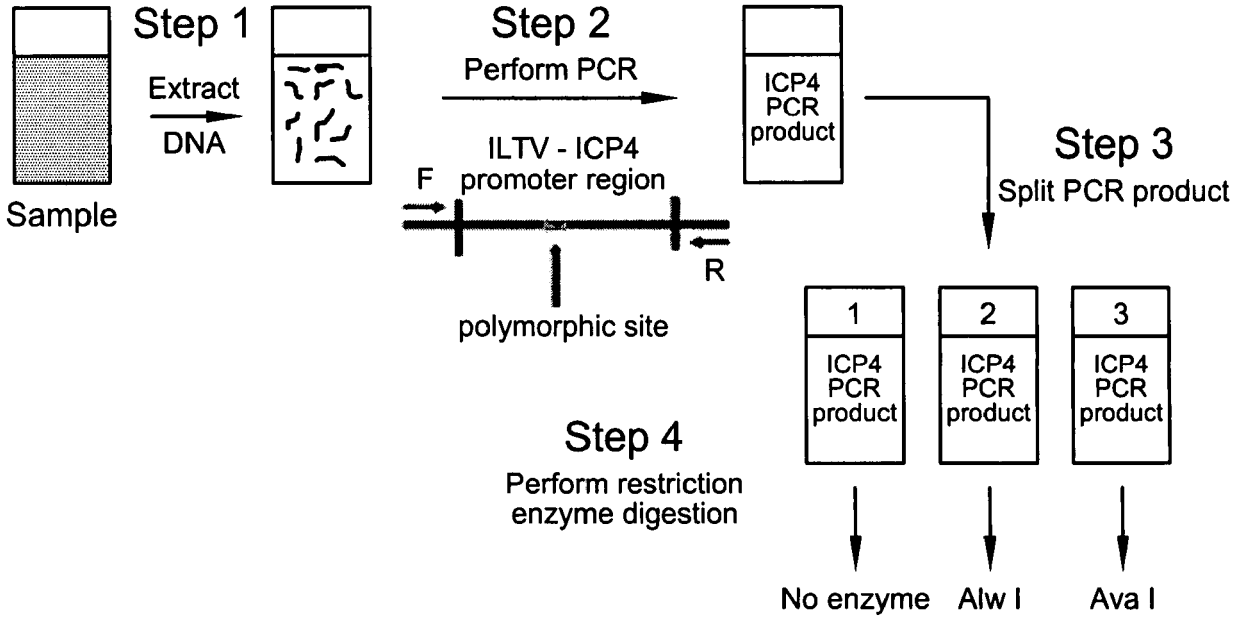




Treatments	Ct Value	Δ Ct Value[A]
No enzyme	21.23	-
Enzyme A	21.45	0.22 (no digestion)
Enzyme B	27.84	6.66 (digestion)

[A] Δ Ct Value = Ct enzyme – Ct no enzyme

Fig. 2


Step 1
Extract
DNA
Sample
Step 2
Perform PCR
ILTV - ICP4
promoter region
F
R
polymorphic site
ICP4
PCR
product
Step 3
Split PCR product
1
2
3
ICP4
PCR
product
ICP4
PCR
product
ICP4
PCR
product
Step 4
Perform restriction
enzyme digestion
No enzyme
Alw I
Ava I

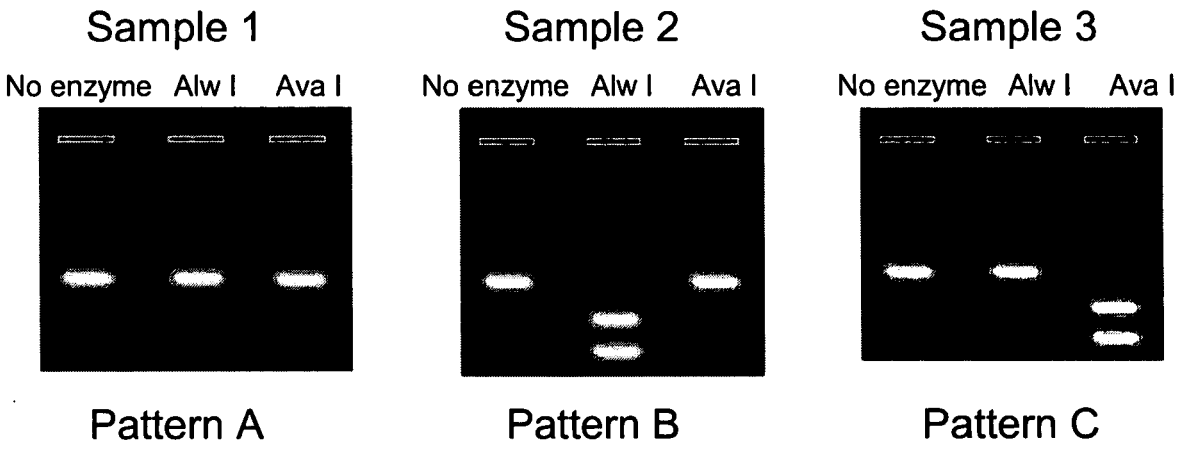

Sample 1
No enzyme Alw I Ava I
Sample 2
No enzyme Alw I Ava I
Sample 3
No enzyme Alw I Ava I
Pattern A
Pattern B
Pattern C

Fig. 3

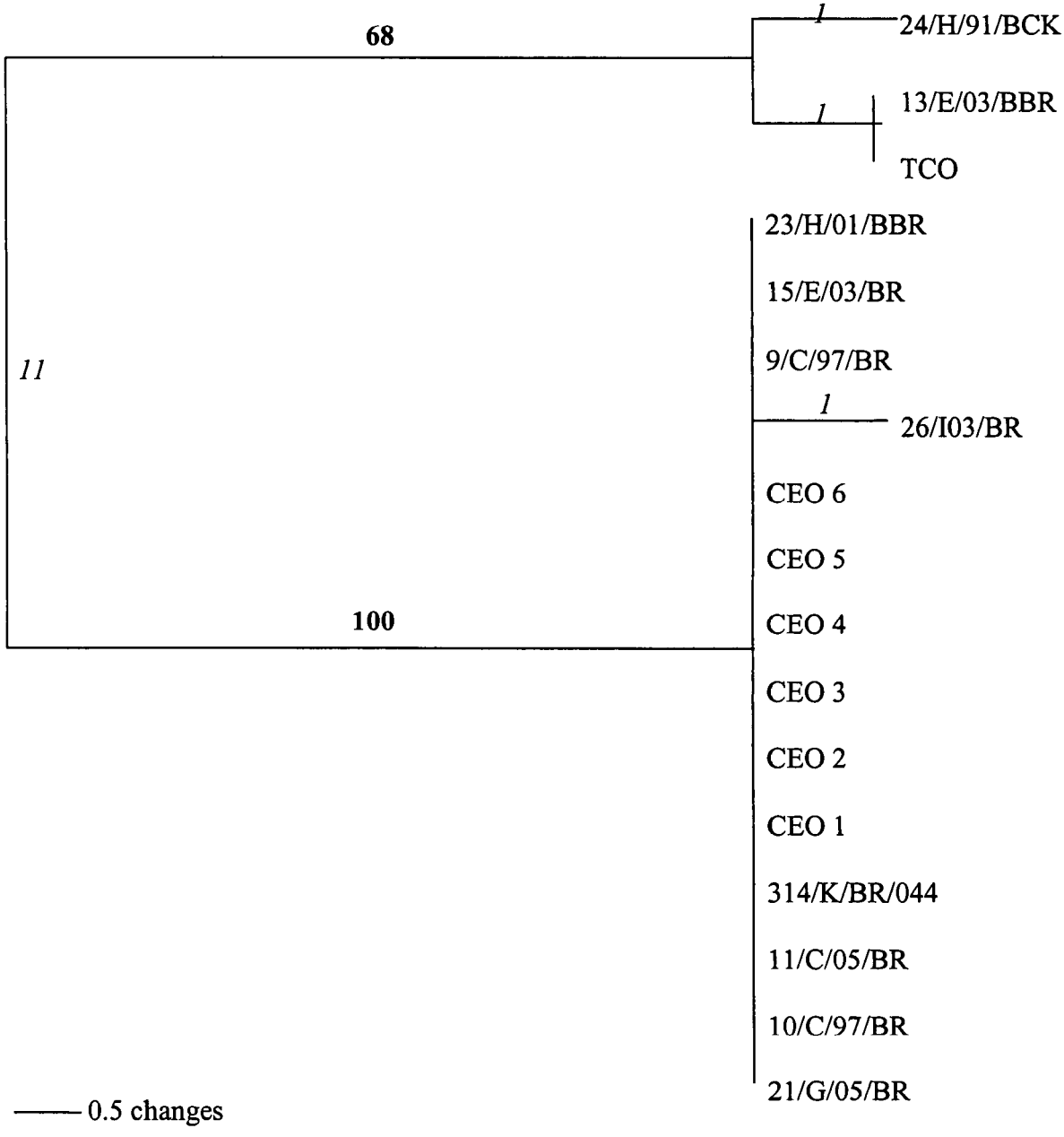

68
1
24/H/91/BCK
1
13/E/03/BBR
TCO
23/H/01/BBR
15/E/03/BR
9/C/97/BR
11
1
26/I03/BR
CEO 6
CEO 5
100
CEO 4
CEO 3
CEO 2
CEO 1
314/K/BR/044
11/C/05/BR
10/C/97/BR
21/G/05/BR
0.5 changes

Fig. 4

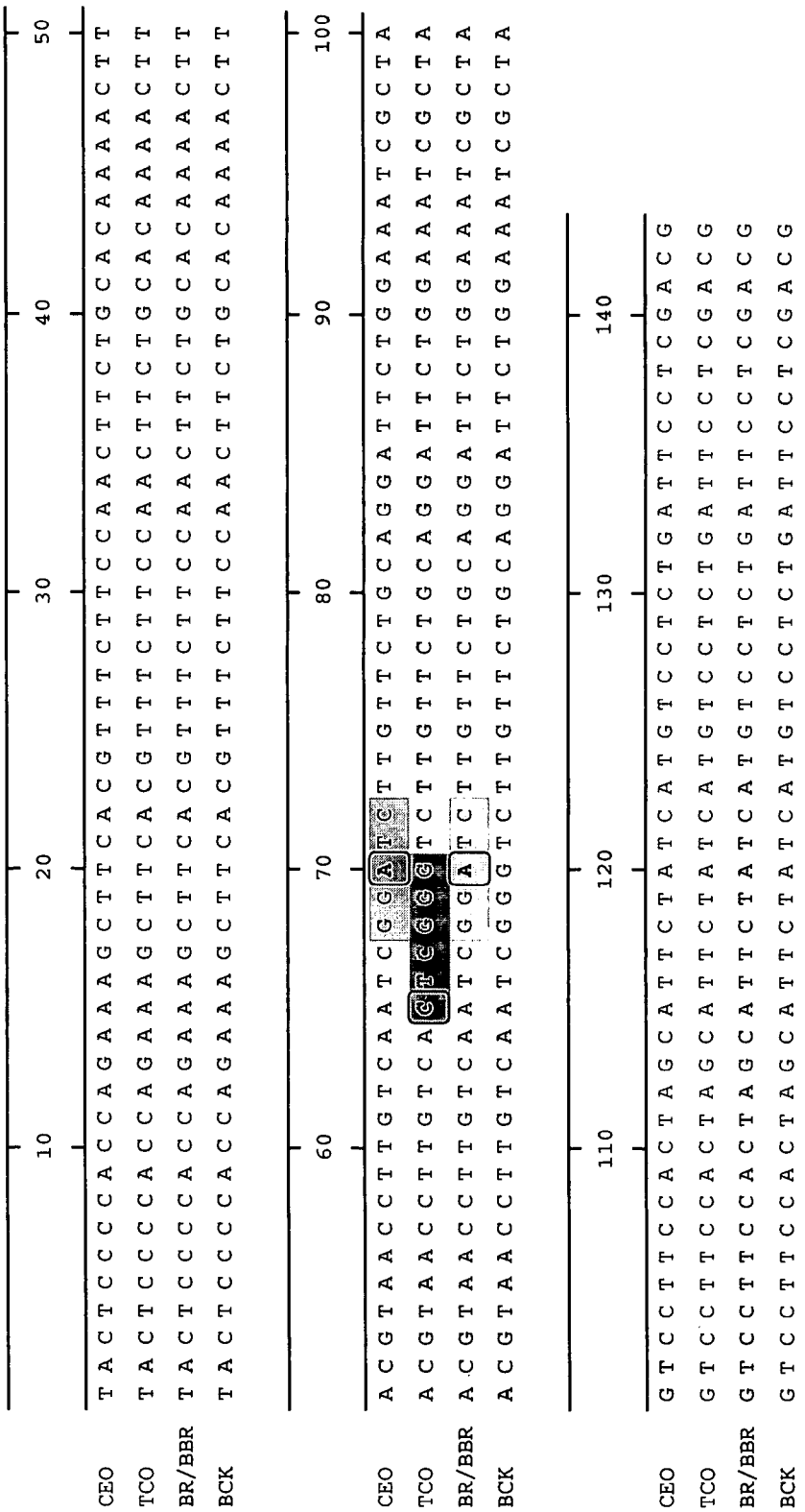

10
20
30
40
50
60
70
80
90
100
110
120
130
140
CEO TACTTCCCCCACCCAGAAAAGCTTTCACGTTTTCCTTCCAACTTCTGCACAAAACTT 50
TCO TACTTCCCCCACCCAGAAAAGCTTTCACGTTTTCCTTCCAACTTCTGCACAAAACTT 50
BR/BBR TACTTCCCCCACCCAGAAAAGCTTTCACGTTTTCCTTCCAACTTCTGCACAAAACTT 50
BCK TACTTCCCCCACCCAGAAAAGCTTTCACGTTTTCCTTCCAACTTCTGCACAAAACTT 50
CEO ACGTAACCTTGTCAATCGGATCTTGTTCTGCAGGAATTCTGGAAATCGCTA 100
TCO ACGTAACCTTGTCAGTTCTTGTTCTGCAGGAATTCTGGAAATCGCTA 100
BR/BBR ACGTAACCTTGTCAATCGGATCTTGTTCTGCAGGAATTCTGGAAATCGCTA 100
BCK ACGTAACCTTGTCAATCGGGTCTTGTTCTGCAGGAATTCTGGAAATCGCTA 100
CEO GTCCTTCCACTAGCATTCTATCATGTCCTCTGATTCCTCGACG 143
TCO GTCCTTCCACTAGCATTCTATCATGTCCTCTGATTCCTCGACG 143
BR/BBR GTCCTTCCACTAGCATTCTATCATGTCCTCTGATTCCTCGACG 143
BCK GTCCTTCCACTAGCATTCTATCATGTCCTCTGATTCCTCGACG 143

Fig. 5A

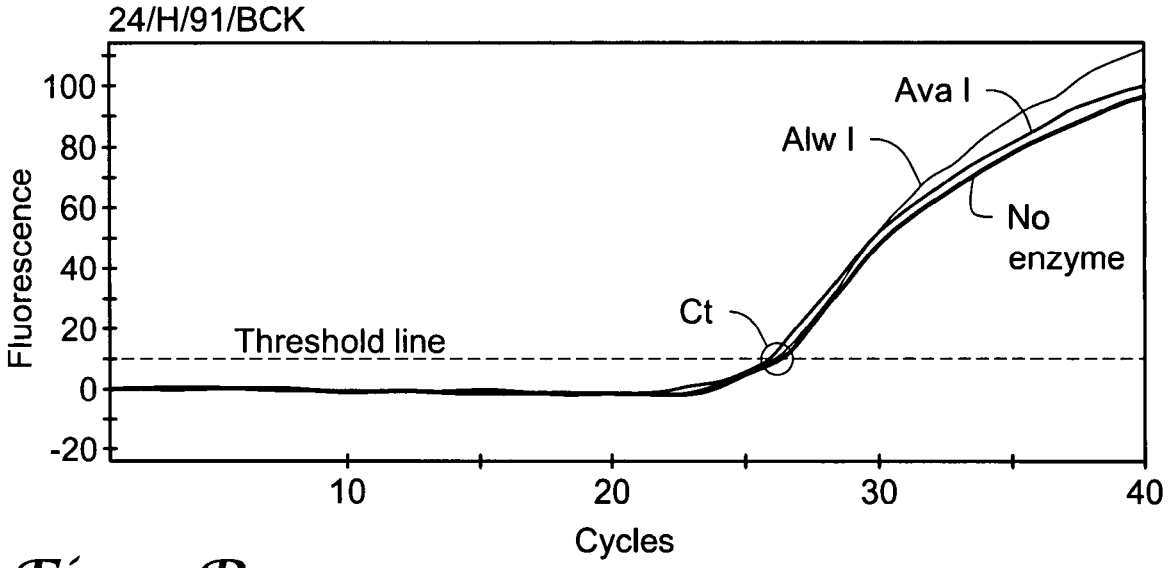


Fig. 5B

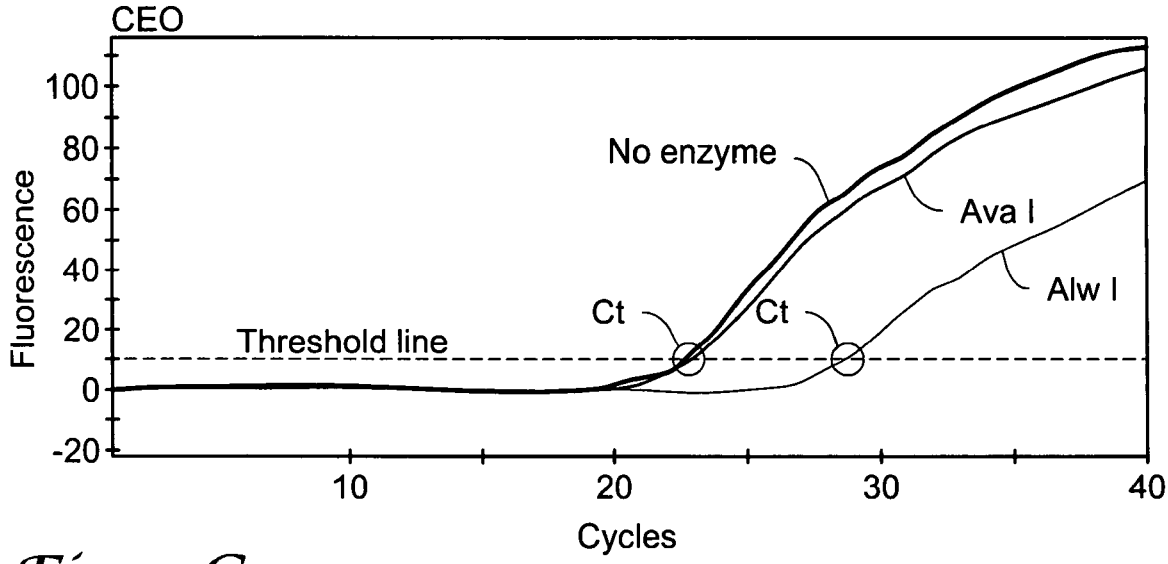


Fig. 5C

