(19)

(11) **EP 1 453 978 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

Note: Bibliography reflects the latest situation

(15) Correction information:
Corrected version no 1 (W1 B1)
Corrections, see
Drawings

(48) Corrigendum issued on:
12.09.2007 Bulletin 2007/37

(45) Date of publication and mention of the grant of the patent:
28.03.2007 Bulletin 2007/13

(21) Application number: **02795451.0**

(22) Date of filing: **16.12.2002**

(51) Int Cl.:
C12Q 1/68 *(2006.01)*

(86) International application number:
PCT/NL2002/000834

(87) International publication number:
WO 2003/052142 (26.06.2003 Gazette 2003/26)

(54) **ANALYSIS AND DETECTION OF MULTIPLE TARGET SEQUENCES USING CIRCULAR PROBES**

ANALYSE UND DETEKTION VON MEHREREN ZIELSEQUENZEN UNTER VERWENDUNG VON ZIRKULÄREN PROBEN

ANALYSE ET DETECTION DE MULTIPLES SEQUENCES CIBLES A L'AIDE DE SONDES CIRCULAIRES

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SI SK TR

(30) Priority: **14.12.2001 EP 01204912**

(43) Date of publication of application:
08.09.2004 Bulletin 2004/37

(73) Proprietor: **KEYGENE N.V.**
6700 AE Wageningen (NL)

(72) Inventors:
- **VAN EIJK, Michael, Josephus, Theresia**
  NL-5373 EJ Herpen (NL)
- **HOGERS, René, Cornelis, Josephus**
  NL-6715 GR Ede (NL)

(74) Representative: **de Lang, Robbert-Jan**
Exter Polak & Charlouis B.V.,
P.O. Box 3241
2280 GE Rijswijk (NL)

(56) References cited:

WO-A-01/06012	**WO-A-95/22623**
WO-A-96/15271	**WO-A-20/04111271**
US-A- 6 156 178	**US-B1- 6 221 603**

- **THOMAS D C ET AL: "AMPLIFICATION OF PADLOCK PROBES FOR DNA DIAGNOSTICS BY CASCADE ROLLING CIRCLE AMPLIFICATION OR THE POLYMERASE CHAIN REACTION" ARCHIVES OF PATHOLOGY, AMERICAN MEDICAL ASSOCIATION, CHICAGO, IL,, US, vol. 123, no. 12, December 1999 (1999-12), pages 1170-1176, XP008001739 ISSN: 0003-9985**
- **BANER JOHAN ET AL: "More keys to padlock probes: Mechanisms for high-throughput nucleic acid analysis." CURRENT OPINION IN BIOTECHNOLOGY, vol. 12, no. 1, February 2001 (2001-02), pages 11-15, XP002244472 ISSN: 0958-1669**
- **GROSSMAN ET AL: "HIGH-DENSITY MULTIPLEX DETECTION OF NUCLEIC ACID SEQUENCES: OLIGONUCLEOTIDE LIGATION ASSAY AND SEQUENCE-CODED SEPARATION" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 22, no. 21, 1994, pages 4527-4534, XP002144258 ISSN: 0305-1048**
- **LANDEGREN U ET AL: "A LIGASE-MEDIATED GENE DETECTION TECHNIQUE" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 241, no. 4869, 26 August 1988 (1988-08-26), pages 1077-1080, XP000676556 ISSN: 0036-8075**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Printed by Jouve, 75001 PARIS (FR)

(Cont. next page)

- **SHI MICHAEL M: "Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies" CLINICAL CHEMISTRY, AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY. WINSTON, US, vol. 47, no. 2, February 2000 (2000-02), pages 164-172, XP002197957 ISSN: 0009-9147**

Description

Field of the invention

[0001] The present invention relates to the field of biotechnology. In particular the present invention provides a method for the high throughput separation and detection of nucleotide sequences, and the use of the method in the discrimination and identification of target sequence such as single nucleotide polymorphisms. The invention further provides for probes that are capable of hybridising to the target sequence of interest, primers for the amplification of ligated probes, use of these probes and primers in the identification and/or detection of nucleotide sequences that are related to a wide variety of genetic traits and genes and kits of primers and/or probes suitable for use in the method according to the invention.

Background of the invention

[0002] There is a rapidly growing interest in the detection of specific nucleic acid sequences. This interest has not only arisen from the recently disclosed draft nucleotide sequence of the human genome and the presence therein, as well as in the genomes of many other organisms, of an abundant amount of single nucleotide polymorphisms (SNP), but also from marker technologies such as AFLP. The recognition that the presence of single nucleotide substitutions (and other types of genetic polymorphisms such as small insertion/deletions; indels) in genes provide a wide variety of information has also attributed to this increased interest. It is now generally recognised that these single nucleotide substitutions are one of the main causes of a significant number of monogenically and multigenically inherited diseases, for instance in humans, or are otherwise involved in the development of complex phenotypes such as performance traits in plants and livestock species. Thus, single nucleotide substitutions are in many cases also related to or at least indicative of important traits in humans, plants and animal species.

[0003] Analysis of these single nucleotide substitutions and indels will result in a wealth of valuable information, which will have widespread implications on medicine and agriculture in the widest possible terms. It is for instance generally envisaged that these developments will result in patient-specific medication. To analyse these genetic polymorphisms, there is a growing need for adequate, reliable and fast methods that enable the handling of large numbers of samples and large numbers of (predominantly) SNPs in a high throughput fashion, without significantly compromising the quality of the data obtained.

[0004] Even though a wide diversity of high-throughput detection platforms for SNPs exist at present (such as fluorometers, DNA microarrays, mass-spectrometers and capillary electrophoresis instruments), the major limitation to achieve cost-effective high throughput detection is that a robust and efficient multiplex amplification technique for non-random selection of SNPs is currently lacking to utilise these platforms efficiently, which results in suboptimal use of these powerful detection platforms and/or high costs per datapoint. "Throughput" as used herein, defines a relative parameter indicating the number of samples and target sequences that can be analysed per unit of time.

[0005] Specifically, using common amplification techniques such as the PCR technique it is possible to amplify a limited number of target sequences by combining the corresponding primer pairs in a single amplification reaction but the number of target sequences that can be amplified simultaneously is small and extensive optimisation may be required to achieved similar amplification efficiencies of the individual target sequences. One of the solutions to multiplex amplification is to use a single primer pair for the amplification of all target sequences, which requires that all targets must contain the corresponding primer-binding sites. This principle is incorporated in the AFLP technique (EP-A 0 534 858). Using AFLP, the primer-binding sites result from a digestion of the target nucleic acid (i.e. total genomic DNA or cDNA) with one or more restriction enzymes, followed by adapter ligation. AFLP essentially targets a random selection of sequences contained in the target nucleic acid. It has been shown that, using AFLP, a practically unlimited number of target sequences can be amplified in a single reaction, depending on the number of target sequences that contain primer-binding region(s) that are perfectly complementary to the amplification primers. Exploiting the use of single primer-pair for amplification in combination with a non-random method for SNP target selection and efficient use of a high throughput detection platform may therefore substantially increase the efficiency of SNP genotyping, however such technology has not been provided in the art yet.

[0006] One of the principal methods used for the analysis of the nucleic acids of a known sequence is based on annealing two probes to a target sequence and, when the probes are hybridised adjacently to the target sequence, ligating the probes. The OLA-principle (Oligonucleotide Ligation Assay) has been described, amongst others, in US 4,988,617 (Landegren et al.) and in WO01/06012. This publication discloses a method for determining the nucleic acid sequence in a region of a known nucleic acid sequence having a known possible mutation. To detect the mutation, oligonucleotides are selected to anneal to immediately adj acent segments of the sequence to be determined. One of the selected oligonucleotide probes has an end region wherein one of the end region nucleotides is complementary to either the normal or to the mutated nucleotide at the corresponding position in the known nucleic acid sequence. A ligase is provided which covalently connects the two probes when they are correctly base paired and are located

immediately adjacent to each other. The presence or absence of the linked probes is an indication of the presence of the known sequence and/or mutation.

[0007] Abbot *et al*. in WO 96/15271 developed a method for a multiplex ligation amplification procedure comprising the hybridisation and ligation of adjacent probes. These probes are provided with an additional length segment, the sequence of which, according to Abbot *et al.,* is unimportant. The deliberate introduction of length differences intends to facilitate the discrimination on the basis of fragment length in gel-based techniques.

[0008] WO 97/45559 (Barany et al.*)* describes a method for the detection of nucleic acid sequence differences by using combinations of ligase detection reactions (LDR) and polymerase chain reactions (PCR). Disclosed are methods comprising annealing allele-specific probe sets to a target sequence and subsequent ligation with a thermostable ligase, optionally followed by removal of the unligated primers with an exonuclease. Amplification of the ligated products with fluorescently labelled primers results in a fluorescently labelled amplified product. Detection of the products is based on separation by size or electrophoretic mobility or on an addressable array.

[0009] Detection of the amplified probes is performed on a universally addressable array containing capturing oligonucleotides. These capturing oligonucleotides contain a region that is capable of annealing to a pre-determined region in the amplified probe, a so-called zip-region or zip code. Each amplified probe contains a different zip code and each zip code will hybridise to its corresponding capturing oligonucleotide on the array. Detection of the label in combination with the position on the array provides information on the presence of the target sequence in the sample. This method allows for the detection of a number of nucleic acid sequences in a sample. However, the design, validation and routine use of arrays for the detection of amplified probes involves many steps (ligation, amplification, optionally purification of the amplified material, array production, hybridisation, washing, scanning and data quantification), of which some (particularly hybridisation and washing) are difficult to automate. Array-based detection is therefore laborious and costly to analyse a large number of samples for a large number of SNPs.

[0010] The LDR oligonucleotide probes in a given set may generate a unique length product and thus may be distinguished from other products based on size. For the amplification a primer set is provided wherein one of the primers contains a label. Different primers can be provided with different labels to allow for the distinction of products.

[0011] The method and the various embodiments described by Barany *et al.* are found to have certain disadvantages. One of the major disadvantages is that the method in principle does not provide for a true high throughput process for the determination of large numbers of target sequences in short periods of time using reliable and robust methods without compromising the quality of the data produced and the efficiency of the process.

[0012] More in particular, one of the disadvantages of the means and methods as disclosed by Barany *et al.* resides in the limited multiplex capacity when discrimination is based *inter alia,* on the length of the allele specific probe sets. Discrimination between sequences that are distinguishable by only a relatively small length difference is, in general, not straightforward and carefully optimised conditions may be required in order to come to the desired resolving power. Discrimination between sequences that have a larger length differentiation is in general easier to accomplish. This may provide for an increase in the number of sequences that can be analysed in the same sample. However, providing for the necessary longer nucleotide probes is a further hurdle to be taken. In the art, synthetic nucleotide sequences are produced by conventional chemical step-by-step oligonucleotide synthesis with a yield of about 98.5% per added nucleotide. When longer probes are synthesised (longer than ca. 60 nucleotides) the yield generally drops and the reliability and purity of the synthetically produced sequence can become a problem.

[0013] These and other disadvantages of the methods disclosed in WO 97/45559 and other publications based on oligonucleotide ligation assays herein lead the present inventors to the conclusion that the methods described therein are less preferable for adaptation in a high throughput protocol that is capable of handling a large number of samples each comprising large numbers of sequences.

[0014] The specific problem of providing for longer probes has been solved by Schouten et al. (WO 01/61033). WO 01/61033 discloses the preparation of longer probes for use in ligation-amplification assays. They provided probes that are considerably longer than those that can be obtained by conventional chemical synthesis methods to avoid the problem associated with the length-based discrimination of amplified products using slab-gels or capillary electrophoresis, namely that only a small part of the detection window / resolving capacity of up to 1 kilo base length is used when OLA probes are synthesised by chemical means. With an upper limit in practice of around 100-150 bases for chemically synthesised oligonucleotides according to the current state of technology, this results in amplification products that are less than 300 base pairs long at most, but often much less (see Barany *et al.).* The difficulty of generating such long probes (more than about 150 nucleotides) with sufficient purity and yield by chemical means has been countered by Schouten *et al.,* using a method in which the probes have been obtained by an in vivo enzymatic template directed polymerisation, for instance by the action of a DNA polymerase in a suitable cell, such as an M 13 phage.

[0015] However, the production and purification of such biological probes requires a collection of suitable host strains containing M13 phage conferring the desired length variations and the use of multiple short chemically synthesised oligonucleotides in the process, thus their use is very laborious and time-consuming, hence costly and not suitable for high-throughput assay development. Furthermore, the use of relatively long probes and relatively large length differences

between the amplifiable target sequences may result in differential amplification efficiencies in favour of the shorter target sequences. This adversely affects the overall data quality, hampering the development of a true high throughput method. Thus the need for a reliable and cost-efficient solution to multiplex amplification and subsequent length-based detection for high throughput application remains.

[0016] Other solutions that have been suggested in the art such as the use of circular (padlock) probes (WO 95/22623) in combination with isothermal amplification such as rolling circle amplification (RCA) are regarded as profitable because of the improved hybridisation characteristics of circular probes and the isothermal character of RCA.

[0017] Rolling circle amplification is an amplification method wherein a first primer is hybridised to a ligated or connected circular probe. Subsequent primer elongation, using a polymerase with strand displacement activity results in the formation of a long polynucleotide strand which contains multiple representations of the connected circular probe. Such a long strand of concatamers of the connected probe is subsequently detected by the use of hybridisation probes. These probes can be labelled. Exponential amplification of the ligated probe can be achieved by the hybridisation of a second primer that hybridises to the concatameric strand and is subsequently elongated. (Exponential) Rolling Circle Amplification ((E)RCA) is described *inter alia* in US5854033, US6143495 WO97/19193, Lizardi et al, Nature genetics 19(3): 225-232 (1998).

[0018] US 5,876,924, WO98/04745 and WO98/04746 by Zhang *et al.* describe a ligation reaction using two adjacent probes wherein one of the probes is a capture probe with a binding element such as biotin. After ligation, the unligated probes are removed and the ligated captured probe is detected using paramagnetic beads with a ligand (biotin) binding moiety. Zhang also discloses the amplification of circular probes using PCR primers in a rolling circle amplification, using a DNA polymerase with strand displacement activity, thereby generating a long concatamer of the circular probe, starting from extension of the first primer. A second PCR primer subsequently hybridises to the long concatamer and elongation thereof provides a second generation of concatamers and facilitates exponential amplification. Detection is generally based on the hybridisation of labelled probes.

[0019] However, these methods have proven to be less desirable in high throughput fashion. One of the reasons is that, for a high throughput method based on length discrimination, the use of (E)RCA results in the formation of long concatamers. These concatamers are problematic, as they are not suitable for high throughput detection.

[0020] US 6,221,603 disclosed a circular probe that contains a restriction site. The probe is amplified using (E)RCA and the resulting concatamers are restricted at the restriction site. The restriction fragments are then separated by length and detected. Separation and detection is performed on a capillary electrophoretic platform, such as the MegaBACE equipment available from Molecular Dynamics Amersham-Pharmacia. For detection labelled dNTP's may be incorporated into the fragments during amplification, or the fragments may be detected by staining or by labelled detection probes. Partial digestion by the restriction enzyme may however affect the reliability of the method. Furthermore, the methods for labelling of the fragments as disclosed in US 6,221,603, do not allow to fully utilise the MegaBACE's capacity of simultaneous detection of multiple colours.

[0021] The present inventors have set out to eliminate or at least diminish the existing problems in the art while at the same time attempting to maintain the advantageous aspects thereof, and to further improve the technology. Other problems in the art and solutions provided thereto by the present invention will become clear throughout the description, the figures and the various embodiments and examples.

Description of the invention

[0022] The present invention relates to methods for high throughput separation and detection of multiple sequences. The present method resolves many of the problems previously encountered in the art. More in particular the present invention provides for a multiple ligation and amplification assay that allows for the rapid and high throughput analysis of a multiplicity of samples, preferably containing a multiplicity of sequences. The present invention also provides for a method for the high throughput discrimination and detection of a multitude of nucleotide sequences based on a combination of length differences and labels. The present invention combines the advantages of certain methods while at the same time avoids disadvantages associated with the various technique, thereby providing for an improved method for the detection of targets sequences in a reliable and reproducible manner and suitable for a high throughput detection method.

Detailed description of the invention

[0023] In a first aspect the invention relates to a method for high throughput separation and detection of a multiplicity of target sequences, optionally in a multiplicity of samples comprising subjecting each sample to a ligation-dependent amplification assay.

[0024] The method preferably is a method for determining the presence or absence of at least one target sequence (2) in a sample, wherein the method comprises the steps of:

(a) providing to a nucleic acid sample at least one circular probe (26) for each target sequence to be detected in the sample, whereby the probe has a first target specific section at its 5'-end (4) that is complementary to a first part of a target sequence (5) and a second target specific section at its 3'-end (6) that is complementary to a second part of the target sequence (7), whereby the first and second part of the target sequence are located adjacent to each other, and whereby the probe further comprises a tag section (8, 9) that is essentially non-complementary to the target sequence, whereby the tag section may comprise a stuffer sequence (10,11) and whereby the tag section comprises at least one primer-binding sequence (12, 13);
(b) allowing the first and second target specific sections of the circular probe to anneal to the first and second parts of target sequences whereby the first and second target specific sections of the probe are annealed adjacent on the target sequence;
(c) providing means for ligating the first and second target specific sections annealed adjacently to the target sequence and allowing the first and second target specific sections to be connected, to produce a ligated circular probe (28), corresponding to a target sequence in the sample;
(d) providing at least a first primer that is complementary to the primer-binding sequence (12), and a polymerase enzyme;
(e) amplifying the resulting mixture to produce an amplified sample (19) comprising amplicons (20) that are linear representations of the ligated circular probes;
(f) determining the presence or absence of a target sequence in a sample by detecting the presence or absence of the corresponding amplicon;

wherein the at least one circularisable probe contains a blocking section that comprises a blocking group that is located adjacent to the 3' end of the primer binding site, such that the blocking section stops elongation or amplification of the primer hybridised to the circularised probe, thereby generating a linear representation of the circular probe and such that the blocking group is excluded from the primer elongation or amplification.

Probe

[0025] The circular oligonucleotide probe used in the present invention is a single linear oligonucleotide probe that is provided in step (a) for each target sequence in a sample. This single linear oligonucleotide probe combines the two target specific section into a single molecule that is circularised in step (c) when the annealed complementarity sections are connected. Thus, in the single linear probe the sections of target complementarity are each present at the extreme ends of the single linear probe. The complementarity sections at the extreme ends are intervened by the sequences that may serve as primer-binding sequences and may further be intervened by stuffer sequences of variable length. An example of such an arrangement of functional groups in the circular probe is: (target-complementarity section 1 - stuffer sequence 1, primer-binding sequence 1 - primer-binding sequence 2 - stuffer sequence 2 - target-complementarity section 2). The skilled person will appreciate that the circular probes are synthesised and applied in a linear form and that they will only be circular when the two complementary sections at the extreme ends of the probe are connected (ligated) annealing to the appropriate target sequence. Thus, the term "circular probe" as used herein actually refers to a linear molecule that is circularised by target sequence dependent connection (ligation). Only the term "connected circular probe" as used herein refers to a molecule in true circular form.

[0026] The complementary sections of the oligonucleotide probes are designed such that for each target sequence in a sample a probe is provided, preferably a specific probe, whereby the probes each contain a section at both their extreme ends that is complementary to a part of the target sequence and the corresponding complementary parts of the target sequence are located essentially adjacent to each other. Within a circular oligonucleotide probe, the oligonucleotide probe has a section at its 5'-end that is complementary to a first part of a target sequence and a section at its 3'-end that is complementary to a second part of the target sequence. Thus, when the circular probe is annealed to complementary parts of a target sequence the 5'-end of the oligonucleotide probe is essentially adjacent to the 3'-end of the oligonucleotide probe such that the respective ends of the probe may be ligated to form a phosphodiester bond and hence become a circular probe.

[0027] Circular probes are advantageous in the ligation step (c) because both target-complementarity sections are contained in the same molecule. Compared with conventional linear probes such as disclosed inter alia by WO97/45559, this means that there are equimolar amounts of the two target specific sections present and in each others vicinity. Such probes are more likely to hybridise to their respective target sequences because hybridisation of the first target-complementarity section to the target facilitates hybridisation of the second one and *vice versa.* In addition, the use of circular probes reduces the chances of the formation of incorrect ligation products that result from ligation between probes of different target sequences, due to the lower number of possible combinations of ligation products that can be formed when the first and second probes are part of the same circular molecule.

[0028] For more details regarding the characteristics, design and construction, use and advantages of padlock probes

reference is made, *inter alia,* to the following documents: M. Nilsson et. al., "Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection," Science 265: 2085-88 (1994); Pickering et al. in Nucleic Acids Research, 2002, vol. 30, e60-US5854033; US5912124; WO 02/068683, WO 01/06012, WO 0077260, WO 01/57256 the contents of which are hereby incorporated by reference.

[0029] For each target sequence for which the presence or absence in a sample is to be determined, a specific oligonucleotide probe is designed with sections complementary to the adjacent complementary parts of each target sequence. Thus, in the method of the invention, for each target sequence that is present in a sample, a corresponding (specific) amplicon may be obtained in the amplified sample. Preferably, a multiplicity of oligonucleotide probes complementary to a multiplicity of target sequences in a sample is provided. An oligonucleotide probe for a given target sequence in a sample will at least differ in nucleotide sequence from probes for other target sequences, and will preferably also differ in length from probes for other targets, more preferably a probe for a given target will produce a connected probe and/or amplicon that differs in length from connected probes corresponding to other targets in the sample as described below. Alternatively, amplicons corresponding to different targets may have an identical length if they can be otherwise distinguished e.g. by different labels as described below.

Tag & Primer binding sites

[0030] The oligonucleotide probe further contains a tag that is essentially non-complementary to the target sequence. The tag does not or not significantly hybridise, preferably at least not under the above annealing conditions, to any of the target sequences in a sample, preferably not to any of the sequences in a sample. The tag preferably comprises at least one, preferably two primer-binding sites and may optionally comprises one or more stuffer sequences of variable length and/or a blocking section (see below).

Stuffers

[0031] The tag of the oligonucleotide probes may comprise one or more stuffer sequence of a variable length. The length of the stuffer varies from 0 to 500, preferably from 0 to 100, more preferably from 1 to 50. The length of the tag varies from 15 to 540, preferably from 18 to 140, more preferably from 20 to 75. The stuffer may be a unique sequence as is known as a Zip-code sequence as described by Iannone et al. (2000), Cytometry 39: pp. 131-140.

Blocking section

[0032] In an alternative embodiment, the circular probe can contain a blocking section (27). The blocking section blocks primer elongation. The blocking section is preferably located between the two primer binding sites. Preferably the blocking section is located essentially adjacent to the 3'-end of the forward primer and essentially adjacent to the 5'-end of the reverse primer binding site, see also Figure 14. An example of such an arrangement of functional groups in the circular probe is: (target-complementarity section 1 - stuffer sequence 1, primer-binding sequence 1- blocking section - primer-binding sequence 2 - stuffer sequence 2 - target-complementarity section 2). This blocking section will effectively limit the primer elongation during amplification, thereby providing linear representations of the connected circular probes. Preferably the blocking section itself is located such between the two primer binding sites that the section is excluded from the amplification. The blocking section can comprise non-nucleotide polymers such as HEG (Hexaethylene glycol). If a blocking section is present, such as a HEG group, the DNA polymerase used may have a strand displacement activity as the blocking section will prevent the formation of long concatamers.

[0033] In an alternative embodiment, the ligated or connected circular probe comprising a blocking section can also be amplified using only one primer, preferably the forward primer. This amplification will result in the linear accumulation of amplicons with each amplification round. The circular probe in this case may contain one or more primer binding sites as long as only one primer is provided.

[0034] Generating linear representations of the connected circular probes, the amplicons, the problem of long concatamers can be overcome, rendering the method suitable for true high throughput electrophoretic technologies. By amplification of only short strands of oligonucleotides, using the blocking section as described hereinabove or by using at least one primer in combination with a polymerase lacking in strand displacement activity, a set of amplicons representing a sample can be obtained wherein the amplicons are of a discrete length within a predetermined range, based on the design of the probes. Subsequent loading on a electrophoretic device will result in the swift separation of the amplicons.

Hybridisation

[0035] In step (a) a multiplicity of different target sequences, i.e. at least two different target sequences, is brought

into contact with a multiplicity of specific oligonucleotide probes under hybridising conditions. The oligonucleotide probes are subsequently allowed to anneal to the adjacent complementary parts of the multiple target sequences in the sample. Methods and conditions for specific annealing of oligonucleotide probes to complementary target sequences are well known in the art (see e.g. in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press). Usually, after mixing of the oligonucleotide probes and target sequences the nucleic acids are denatured by incubation (generally at between 94 °C and 96 °C) for a short period of time (e.g. 30 seconds to 5 minutes) in a low salt buffer (e.g. a buffer containing no salts or less salts than the ionic strength equivalent of 10 mM NaCl). The sample containing the denatured probes and target sequences is anthen allowed to cool to an optimal hybridisation temperature for specific annealing of the probes and target sequences, which usually is about 5°C below the melting temperature of the hybrid between the complementary section of the probe and its complementary sequence (in the target sequence). In order to prevent aspecific or inefficient hybridisation of one of the two probe sections, or in a sample with multiple target sequences, it is preferred that, within one sample, the sections of the probes that are complementary to the target sequences are of a similar, preferably identical melting temperatures between the different target sequences present in the sample. Thus, the complementary sections of the probes preferably differ less than 20, 15, 10, 5, or 2 °C in melting temperature. This is facilitated by using complementary sections of the probes with a similar length and similar G/C content. Thus, the complementary sections preferably differ less than 20, 15, 10, 5, or 2 nucleotides in length and their G/C contents differ by less than 30, 20, 15, 10, or 5 %. Complementary as used herein means that a first nucleotide sequence is capable of specifically hybridising to second nucleotide sequence under normal stringency conditions. A nucleotide sequence that is considered complementary to another nucleotide sequence may contain a minor amount, i.e. preferably less than 20, 15, 10, 5 or 2%, of mismatches. Alternatively, it may be necessary to compensate for mismatches e.g. by incorporation of so-called universal nucleotides, such as for instance described in EP-A 974 672, incorporated herein by reference or by the use of suitable locked nucleic acids (LNAs) and peptide nucleic acids (PNAs). Since annealing of probes to target sequences is concentration dependent, annealing is preferably performed in a small volume, i.e. less than 10 μl. Under these hybridisation conditions, annealing of probes to target sequences usually is fast and does not to proceed for more than 5,10 or 15 minutes, although longer annealing time may be used as long as the hybridisation temperature is maintained to avoid aspecific annealing.

[0036] In a preferred embodiment of the invention, excellent results have been obtained by prolonged hybridisation times such as overnight hybridisation or for more than one hour. Prolonged hybridisation times can be advantageous in these assays as the difference in signal due to different hybridisation efficiencies is reduced and it is considered desirable to achieve complete hybridisation and ligation of all probes for which a target sequence is present. Excellent results have been obtained by a combined hybridisation-ligation step using a thermostable ligase described herein. In this embodiment the hybridisation-ligation was performed by allowing the probes to hybridise during 1 hour in the presence of a thermostable ligase, followed by a denaturation step. Repeating these steps for at least 2 times provided good results. Repeating these steps 10 times provided excellent results.

[0037] To avoid evaporation during denaturation and annealing, the walls and lids of the reaction chambers (i.e. tubes or microtitre wells) may also be heated to the same temperature as the reaction mixture. In preferred oligonucleotide probes the length of the complementary section is preferably at least 15, 18 or 20 nucleotides and preferably not more than 30, 40, or 50 nucleotides and the probes preferably have a melting temperature of at least 50°C, 55°C or 60°C.

Non-hybridised probes

[0038] The probes that are not complementary to a part of the target sequence or that contain too many mismatches will not or only to a reduced extent hybridise to the target sequence when the sample is submitted to hybridisation conditions. Accordingly ligation is less likely to occur. The number of spurious ligation products from these probes in general will therefore not be sufficient and much smaller than the *bona fide* ligation products such that they are outcompeted during subsequent multiplex amplification. Consequently, they will not be detected or only to a minor extent.

Ligation

[0039] The respective 5'- and 3'-ends of the oligonucleotide probe that are annealed essentially adjacent to the complementary parts of a target sequence are connected in step (c) to form a covalent bond by any suitable means known in the art. The ends of the probes may be enzymatically connected in a phosphodiester bond by a ligase, preferably a DNA ligase. DNA ligases are enzymes capable of catalysing the formation of a phosphodiester bond between (the ends of) two polynucleotide strands bound at adjacent sites on a complementary strand. DNA ligases usually require ATP (EC 6.5.1.1) or NAD (EC 6.5.1.2) as a cofactor to seal nicks in double stranded DNA. Suitable DNA ligase for use in the present invention are T4 DNA ligase, *E. coli* DNA ligase or preferably a thermostable ligase like e.g. *Thermus aquaticus* (Taq) ligase, *Thermus thermophilus* DNA ligase, or *Pyrococcus* DNA ligase. Alternatively, chemical autoligation of modified polynucleotide ends may be used to ligate two oligonucleotide probes annealed at adjacent sites on

the complementary parts of a target sequence (Xu and Kool, 1999, Nucleic Acid Res. 27: 875-881).

[0040] Both chemical and enzymatic ligation occur much more efficient on perfectly matched probe-target sequence complexes compared to complexes in which one or both of the ends of the probe form a mismatch with the target sequence at, or close to the ligation site (Wu and Wallace, 1989, Gene 76: 245-254; Xu and Kool, *supra*). In order to increase the ligation specificity, i.e. the relative ligation efficiencies of perfectly matched oligonucleotides compared to mismatched oligonucleotides, the ligation is preferably performed at elevated temperatures. Thus, in a preferred embodiment of the invention, a DNA ligase is employed that remains active at 50 - 65˚C for prolonged times, but which is easily inactivated at higher temperatures, e.g. used in the denaturation step during a PCR, usually 90 - 100˚C. One such DNA ligase is a NAD requiring DNA ligase from a Gram-positive bacterium (strain MRCH 065) as known from WO O1/61033. This ligase is referred to as "Ligase 65" and is commercially available from MRC Holland, Amsterdam.

Gap Ligation

[0041] In an alternative embodiment, for instance directed to the identification of indels, the respective ends may be annealed such that a gap is left. This gap can be filled with a suitable oligonucleotide and ligated. Such methods are known in the art as 'gap ligation' and are disclosed *inter alia* in WO 00/77260. Another possibility to fill this gap is by extension of one end of the probe using a polymerase and a ligase in combination with single nucleotides, optionally preselected from A,T, C, or G, or di-, tri- or other small oligonucleotides.

Primers

[0042] The connected probes are amplified using a pair of primers corresponding to the primer-binding sites. In a preferred embodiment at least one of the primers or the same set of primers is used for the amplification of two or more different connected probes in a sample, preferably for the amplification of all connected probes in a sample. Such a primer is sometimes referred to as a universal primer as these primers are capable of priming the amplification of all probes containing the corresponding universal primer binding site and consequently of all ligated probes containing the universal primer binding site. The different primers that are used in the amplification in step (d) are preferably essentially equal in annealing and priming efficiency. Thus, the primers in a sample preferably differ less than 20, 15, 10, 5, or 2 ˚C in melting temperature. This can be achieved as outlined above for the complementary section of the oligonucleotide probes. Unlike the sequence of the complementary sections, the sequence of the primers is not dictated by the target sequence. Primer sequences may therefore conveniently be designed by assembling the sequence from tetramers of nucleotides wherein each tetramer contains one A,T,C and G or by other ways that ensure that the G/C content and melting temperature of the primers are identical or very similar. The length of the primers (and corresponding primer-binding sites in the tags of the probes) is preferably at least 12, 15 or 17 nucleotides and preferably not more than 25, 30, 40 nucleotides.

[0043] In a preferred embodiment, at least two of the oligonucleotide probes that are complementary to at least two different target sequences in a sample comprise a tag sequence that comprises a primer-binding site that is complementary to a single primer sequence. Thus, preferably at least one of the first and second primer in a primer pair is used for the amplification of connected probes corresponding to at least two different target sequences in a sample, more preferably for the amplification of connected probes corresponding to all target sequences in a sample. Preferably only a single first primer is used and in some embodiments only a single first and a single second primer is used for amplification of all connected probes. Using common primers for amplification of multiple different fragments usually is advantageous for the efficiency of the amplification step.

[0044] The connected probes obtained from the ligation of the adjacently annealed probe sections are amplified in step (d), using a primer set, preferably consisting of a pair of primers for each of the connected probes in the sample. The primer pair comprises primers that are complementary to primer-binding sequences that are present in the connected probes. A primer pair usually comprises a first and at least a second primer, but may consist of only a single primer that primes in both directions. Excellent results have been obtained using primers that are known in the art as AFLP primers such as described inter alia in EP534858 and in Vos et al., Nucleic Acid Research, 1995, vol. 23,4407-44014.

Selective primers

[0045] In a particular preferred embodiment, one or more of the primers used in the amplification step of the present invention is a selective primer. A selective primer is defined herein as a primer that, in addition to its universal sequence which is complementary to a primer binding site in the probe, contains a region that comprises so-called "selective nucleotides". The region containing the selective nucleotides is located at the 3'-end of the universal primer.

[0046] The principle of selective nucleotides is disclosed inter alia in EP534858 and in Vos et al., Nucleic Acid Research, 1995, vol. 23,4407-44014. The selective nucleotides are complementary to the nucleotides in the (ligated) probes that

are located adjacent to the primer sequence. The selective nucleotides generally do not form part of the region in the (ligated) probes that is depicted as the primer sequence. Primers containing selective nucleotide are denoted as +N primers, in which N stands for the number of selective nucleotides present at the 3'-end of the primer. N is preferably selected from amongst A, C, T or G.

5 **[0047]** N may also be selected from amongst various nucleotide alternatives, i.e. compounds that are capable of mimicking the behavior of ACTG-nucleotides but in addition thereto have other characteristics such as the capability of improved hybridisation compared to the ACTG-nucleotides or the capability to modify the stability of the duplex resulting from the hybridisation. Examples thereof are PNA's, LNA's, inosine etc. When the amplification is performed with more than one primer, such as with PCR using two primers, one or both primers can be equipped with selective nucleotides.
10 The number of selective nucleotides may vary, depending on the species or on other particulars determinable by the skilled man. In general the number of selective nucleotides is not more than 10, but at least 5, preferably 4, more preferably 3, most preferred 2 and especially preferred is 1 selective nucleotide.

[0048] A +1 primer thus contains one selective nucleotide, a +2 primer contains 2 selective nucleotides etc. A primer with no selective nucleotides (i.e. a conventional primer) can be depicted as a +0 primer (no selective nucleotides added).
15 When a specific selective nucleotide is added, this is depicted by the notion +A or +C etc.

[0049] By amplifying a set of (ligated) probes with a selective primer, a subset of (ligated) probes is obtained, provided that the complementary base is incorporated at the appropriate position in the desired of the probes that are supposed to be selectively amplified using the selective primer. Using a +1 primer, for example, the multiplex factor of the amplified mixture is reduced by a factor 4 compared to the mixture of ligated probes prior to amplification. Higher reductions can
20 be achieved by using primers with multiple selective nucleotides, i.e. 16 fold reduction of the original multiplex ration is obtained with 2 selective nucleotides etc.

[0050] When an assay is developed which, after ligation, is to be selectively amplified, it is preferred that the probe contains the complementary nucleotide adjacent to the primer binding sequence. This allows for pre-selection of the ligated probe to be selectively amplified.

25 **[0051]** The use of selective primers in the present invention has proven to be advantageously when developing ligation based assays with high multiplex ratios of which subsequently only a specific part needs to be analyzed resulting in further cost reduction of the ligation reaction per datapoint. By designing primers together with adjacent selective nucleotides, the specific parts of the sample that are to be amplified separately can be selected beforehand.

[0052] One of the examples in which this is useful and advantageous is in case of analysis of samples that contain
30 only minute amounts of DNA and/or for the identification of different (strains of) pathogens. For example, in an assay directed to the detection of various strains of anthrax *(Bacillus anthracis*), for each of the strains a set of representative probes is designed. The detection of the presence or absence of this set (or a characterizing portion thereof) of ligated probes after the hybridisation and ligation steps of the method of the invention may serve as an identification of the strain concerned. The selective amplification with specifically designed primers (each selective primer is linked to a
35 specific strain) can selectively amplify the various strains, allowing their identification. For instance, amplification with an +A primer selectively amplifies the ligated probes directed to strain X where a +G primer selectively amplifies the ligated probes directed to strain Y. If desired, for instance in the case of small amounts of sample DNA, an optional first amplification with a +0 primer will increase the amount of ligated probes, thereby facilitating the selective amplification.

[0053] For example, a universal primer of 20 nucleotides becomes a selective primer by the addition of one selective
40 nucleotide at its 3' end, the total length of the primer now is 21 nucleotides. See also Figure 15. Alternatively, the universal primer can be shortened at its 5' end by the number of selective nucleotides added. For instance, adding two selective nucleotides at the 3' end of the primer sequence can be combined with the absence (or removal) of two nucleotides from the 5'end of the universal primer, compared to the original universal primer. Thus a universal primer of 20 nucleotides is replaced by a selective primer of 20 nucleotides. These primers are depicted as 'nested primers' throughout this
45 application. The use of selective primers based on universal primers has the advantage that amplification parameters such as stringency and temperatures may remain essentially the same for amplification with different selective primers or vary only to a minor extent. Preferably, selective amplification is carried out under conditions of increased stringency compared to non-selective amplification. With increased stringency is meant that the conditions for annealing the primer to the ligated probe are such that only perfectly matching selective primers will be extended by the polymerase used in
50 the amplification step. The specific amplification of only perfectly matching primers can be achieved in practice by the use of a so-called touchdown PCR profile wherein the temperature during the primer annealing step is stepwise lowered by for instance 0.5 °C to allow for perfectly annealed primers. Suitable stringency conditions are for instance as described for AFLP amplification in EP 534858 and in Vos et al., Nucleic Acid Research, 1995, vol. 23, 4407-44014. The skilled man will, based on the guidance find ways tot adapt the stringency conditions to suit his specific need without departing
55 from the gist of the invention.

[0054] One of the further advantages of the selective amplification of ligated probes is that an assay with a high multiplex ratio can be adapted easily for detection with methods or on platforms that prefer a lower multiplex ratio.

[0055] One of many examples thereof is the detection based on length differences such as electrophoresis and

preferably capillary electrophoresis such as is performed on a MegaBACE or using nano-technology such as Lab-on-a-Chip.

Amplification

[0056] In step (d) of the method of the invention, the connected probes are amplified to produce (detectable) amplified connected probes (amplicons) that are linear representations of the connected circular probes by any suitable nucleic acid amplification method known in the art. Nucleic acid amplification methods usually employ two primers, dNTP's, and a (DNA) polymerase. A preferred method for amplification is PCR. "PCR" or "Polymerase Chain Reaction" is a rapid procedure for in vitro enzymatic amplification of a specific DNA segment. The DNA to be amplified is denatured by heating the sample. In the presence of DNA polymerase and excess deoxynucleotide triphosphates, oligonucleotides that hybridise specifically to the target sequence prime new DNA synthesis. It is preferred that the polymerase is a DNA polymerase that does not express strand displacement activity or at least not significantly. Examples thereof are Amplitaq and Amplitaq Gold (supplier Perkin Elmer) and Accuprime (Invitrogen). One round of synthesis results in new strands of determinate length, which, like the parental strands, can hybridise to the primers upon denaturation and annealing. The second cycle of denaturation, annealing and synthesis produces two single-stranded products that together compose a discrete doublestranded product, exactly the length between the primer ends. This discrete product accumulates exponentially with each successive round of amplification. Over the course of about 20 to 30 cycles, many million-fold amplification of the discrete fragment can be achieved. PCR protocols are well known in the art, and are described in standard laboratory textbooks, e.g. Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1995). Suitable conditions for the application of PCR in the method of the invention are described in EP-A 0 534 858 and Vos et al. (1995; Nucleic Acids Res.23: 4407- 23:4407- 4407-4407-4414), where multiple DNA fragments between 70 and 700 nucleotides and containing identical primer-binding sequences are amplified with near equal efficiency using one primer pair. Other multiplex and/or isothermal amplification methods that may be applied include e.g. LCR, self-sustained sequence replication (3SR), Q-β-replicase mediated RNA amplification, or strand displacement amplification (SDA). In some instances this may require replacing the primer-binding sites in the tags of the probes by a suitable (RNA) polymerase-binding site as long as they lead to linear amplification products as defined herein before, i.e. of discrete lengths and corresponding to the length of the circular probes.

[0057] As described herein, linear representations of the connected circular probes can be obtained by exponential amplification of the circular probe with two primers, one forward and one reverse, using a polymerase that does not or not significantly have a strand displacement activity. The first primer elongation in the amplification with the forward primer generates an oligonucleotide product until the 5'end of the forward primer is reached. There the primer elongation is terminated, due to the substantial absence of strand displacement activity of the polymerase used, leaving a elongated primer with substantially the same length as the connected circular probe. The second cycle of denaturation, primer hybridisation and primer elongation will, for the forward primer, produce the identical strand as during the first primer elongation, while the reverse primer will hybridise to the oligonucleotide product from the elongation of the first primer elongation and thereby produce the complementary strand, resulting in the exponential amplification of the circular probe to thereby produce amplicons of discrete length which are representations of the connected circular oligonucleotide probes.

Amplicons

[0058] The term 'amplicon' as used herein refers to the product of the amplification step of the connected or ligated probe. The term 'amplicon' as used herein thus refers to an amplified connected probe. After the ligation step wherein the two target specific section are connected by mean of a ligase, the connected or ligated probe is combined with one or more primers and a polymerase and amplified. The ligated probe, the primers, the polymerase and/or other parameters and variables are such that the amplification results in linear representations of the circular probe. In the present invention the amplicon is a linear oligonucleotide having a length that does not substantially exceed the length of the circular probe. The minimum length of the amplicon is at least the sum of the length of the two target complementary sections. It is preferred that the length of the amplicon corresponds to the length of the circular probe. It is more preferred that the length of the amplicon is indicative of the ligation of the corresponding circular probe. Preferably an amplicon does not contain repetitions of sections of the circular probe, i.e. is not a concatamer or a multimer of the circular probe or a multimeric representation thereof. Preferably an amplicon is a linear and monomeric representation of the connected circular probe.

[0059] The advantage obtained by the conversion from circular probes to linear amplicons is that the advantageous characteristics of the circular probe are used (improved kinetics, increased hybridisation to the target strand due to the formation of the 'padlock' conformation), while the resulting amplicons are of a discrete length an can be detected subsequently without the need for additional steps such as restriction and labelling. Figure 14 displays a schematic

representation of circular probes and amplicons. The various embodiments of the present invention will provide further detail in this respect.

Detection

[0060] Detection of the labelled separated samples is performed by a detector to result in detection data. The detector is of course dependent on the general system on which the separation is carried out (capillary electrophoresis, slab-gel electrophoresis, fixed detector-continuous gel-electrophoresis) but is also depending on the label that is present on the primer, such as a fluorescent or a radioactive label.

[0061] The amplicons in a sample are preferably analysed on an electrophoretic device. The electrophoretic device preferably separates the different amplicons in an amplified sample on the basis of length, after which the separated amplicons may be detected as described below. A suitable electrophoretic device may be a gel-electrophoresis device, e.g. for conventional (polyacrylamide) slab gel-electrophoresis, or a capillary electrophoresis device such as exemplified by the MegaBACE equipment available from Molecular Dynamics Amersham-Biosciences. An alternative is the nano-sized capillary electrophoretic devices known as Lab-on-a-Chip. The electrophoretic device preferably is a multichannel device in which multiple samples are electrophoresed in multiple channels in parallel. The electrophoretic device has an application location (per channel) for application (loading) of the amplified sample to be electrophoresed, a separation area over which the fragments in the sample migrate by electrophoresis, and preferably also a detection device located at a detection location distal from the application location. The detection device will usually comprises a photomultiplier for the detection of fluorescence, phosphorescence or chemiluminescence. Alternatively, in the case of gel-electrophoresis, the separated fragments may be detected in the gel e.g. by autoradiography or fluorography.

Length discrimination

[0062] To discriminate between different target sequences in the sample preferably a difference in length of the respective corresponding amplicons is used. By separating the amplicons based on length, the presence of the corresponding target nucleotides sequences in the sample can be determined. Accordingly, in a preferred embodiment of the present invention, the discrimination between amplicons derived from different target sequences in a sample is based on a length difference between the respective amplicons corresponding to different target sequences in a sample or amplified sample.

[0063] Preferably, the length difference is provided by the length of the stuffer sequence(s) in the oligonucleotide probes. By including in each oligonucleotide probe a stuffer of a pre-determined length, the length of each amplicon in an amplified sample can be controlled such that an adequate discrimination based on length differences of the amplicon obtained in step (d) is enabled. In a preferred embodiment of a probe according to the invention, the stuffer is located between the probe's section complementary to the target sequence and a primer-binding sequence. As there are two target specific sections at both ends of the probe and two primer binding sites, two stuffer can be incorporated in the probe therein between. As such, the total length of the stuffer is provided by the combination of the length of the first stuffer and second stuffer in the probe. Accordingly, in a preferred embodiment, the oligonucleotide probe comprises two stuffers, preferably in the non target complementary tags. A graphic representation thereof can be found in Figure 14.

[0064] The length differentiation between amplicons obtained from target sequences in the sample is preferably chosen such that the amplicons can be distinguished based on their length. This is accomplished by using stuffer sequences or combinations of stuffer sequences which (together) result in clear length differences that may be distinguished on electrophoretic devices. Thus, from the perspective of resolving power, the length differences between the different amplicons, as may be caused by their stuffers, are as large as possible. However, for several other important considerations, as noted before, the length differences between the different amplicon is preferably as small as possible: (1) the upper limit that exists in practice with respect to the length of chemically synthesised probes of about 100-150 bases at most; (2) the less efficient amplification of larger fragments, (3) the increased chances for differential amplification efficiencies of fragments with a large length variation; and (4) the use of multiple injections of detection samples on the detection device which works best with fragments in a narrow length range. Preferably the length differences between the sequences to be determined and provided by the stuffers is at least sufficient to allow discrimination between essentially all amplicons. By definition, based on chemical, enzymatic and biological nucleic acid synthesis procedures, the minimal useable size difference between different amplicon in an amplified sample is one base, and this size difference fits within the resolving power of most electrophoresis devices, especially in the lower size ranges. Thus based on the above it is preferred to use multiplex assays with amplification products with differ in length by a single base(pair). In a preferred embodiment, the length difference between different amplicons in an amplified sample is at least two nucleotides. In a particularly preferred embodiment of the invention the amplicon corresponding to different target sequences in a sample have a length difference of two nucleotides.

Labels

[0065] In a preferred embodiment, at least one of the primers complementary to the primer-binding sites of the first and second oligonucleotide probes in the sample comprises a label, preferably the second primer comprises a label. The label can be selected from a large group, amongst others comprising fluorescent and/or phosphorescent moieties such as dyes, chromophores, or enzymes, antigens, heavy metals, magnetic probes, phosphorescent moieties, radioactive labels, chemiluminescent moieties or electrochemical detecting moieties. Preferably the label is a fluorescent or phosphorescent dye, more preferably selected from the group of FAM, HEX, TET, JOE, NED, and (ET-)ROX. Dyes such as FITC, Cy2, Texas Red, TAMRA, Alexa fluor 488™, Bodipy™ FL, Rhodamine 123, R6G, Bodipy 530, Alexafluor™532 and IRDyes™ by Licor as used on the NEN Glober IR^2 platform are also suitable for use in the present invention. Preferably the label may be chosen from amongst the fluorescent or phosphorescent dyes in the group consisting of FAM, TET, JOE, NED, HEX, (ET-)ROX, FITC, Cy2, Texas Red, TAMRA, Alexa fluor 488™, Bodipy™ FL, Rhodamine 123, R6G, Bodipy 530, Alexafluor™532 and IRDyes ™.

[0066] By using a primer set comprising differently labelled primers, the number of connected probes that can be discriminated in a sample and hence the number of target sequences in a sample can be doubled for each additional label. Thus, for each additional label that is used in a sample, the number of target sequences that can be analysed in a sample is doubled. The maximum number of labels that can be used in one sample in a high throughput method is governed mostly by the limitations in the detection capabilities of the available detection platforms. At present, one of the most frequently used platforms (MegaBACE, by Molecular Dynamics -Amersham-Biosciences Ltd. allows the simultaneous detection of up to four fluorescent dyes, being FAM, JOE or HEX, NED and (ET-)ROX. However, alternative capillary electrophoresis instruments are also suitable, which includes ABI310, ABI3100, ABI3700 (Perkin-Elmer Corp.), CEQ2000 XL (Beckman Coulter) and others. Nonlimiting examples of slab-gel based electrophoresis devices include ABI377 (Perkin Elmer Corp.) and the global IR^2 automated DNA sequencing system, available from LI-COR, Lincoln, Nebraska, USA.

Length and label

[0067] Throughput can be increased by the use of multiple labelled primers. One of the problems associated with the use of different labels in one sample is cross talk or residual cross talk. Cross talk or residual cross talk, as used herein, refers to the overlap between the emission spectra of different (fluorescent) labels. For instance when fluorescent dyes are used, each dye has a different emission (and absorption) spectrum. In case of two dyes in one sample, these spectra can overlap and may cause a disturbance of the signal, which contravenes the quality of the data obtained. Particularly when two nucleotide fragments to be detected in a sample are labelled with a different label and one of the fragments is present in an abundant amount whereas the other is present only in minute amounts, residual cross talk can cause that the measured signal of the fragment that is present in only minute amounts is mostly derived from the emission of another label with an overlapping emission spectrum that is abundantly contained in a fragment with identical size of another sample. The reciprocal effect of the other dye may also occur but in this example its effect is probably less because of the abundance differences between the amplicons labelled with the respective dyes.

[0068] Chehab et al. (Proc. Natl. Acad. Sci. USA, 86:9178-9182 (1989) have attempted to discriminate between alleles by attaching different fluorescent dyes to competing alleles in a single reaction tube by selecting combinations of labels such that the emission maximum of one dye essentially coincides with the emission minimum of the other dye. However, at a certain wavelength at which one dye expresses an absorption maximum, there is always also some remaining absorption from another dye present in the sample, especially when the sample contains multiple dyes.

[0069] This route to multiplex analysis was found to be limited in scale by the relatively few dyes that can be spectrally resolved. One of the major problems with the use of multiple dyes is that the emission spectra of different fluorescent labels often overlap. The resulting raw data signals have to be corrected for the contribution of similar size fragments that are detected simultaneously and are labelled with another fluorescent dye by a process called cross-talk correction. Cross-talk correction is commonly carried out by mathematical means, based on the known theoretical absorption spectra for both dyes, after "raw" data collection from the detection device. Mathematical correction is based on theoretical spectra and ignores that emission spectra of labels are sensitive and often affected by the composition of the detection sample. These sensitivities can affect the brightness and/or the wavelength of the emission. This means that parameters such as pH, temperature, excitation light intensity, non-covalent interactions, salt concentration and ionic strength strongly influence the resulting emission spectrum. In particular, it is known that the presence of residual salts in a sample affects the fluorescence signal emitted by the dye and is a critical factor in case of detection by capillary electrophoresis using electrokinetic injection because it then also affects the injection efficiency. Thus, spectral overlap is a potential source of error that negatively impacts on data quality in case of multiplex detection using different fluorescent dyes.

[0070] The present invention provides for a solution to this problem such that two (or more) labels with overlapping spectra can be used in the same sample without significantly affecting data quality. By a predetermined combination of

length differences and labels, an increase in the number of target nucleotide sequences that can be detected in sample is obtained while the quality of the data remains at least constant. In a preferred embodiment of the invention, spectral overlap between two differently labelled sequences is reduced by the introduction of a length difference between the two sequences. This label-related length difference can be provided for by the length of the stuffer sequence as described herein. The number of different labels that can be used in the same sample in the present method is at least two, preferably at least three, more preferably at least four. The maximum number of labels is functionally limited by the minimum of spectral overlap that remains acceptable, which for most applications typically amounts to less than 15 percent of the true signal, preferably less than 10 percent, more preferably lees than 5 percent and most preferably less than 1 percent of the true signal.

[0071] In order to avoid the potential influence of residual cross-talk on the data quality in case different samples are labelled with multiple fluorescent dyes with overlapping emission spectra and fragments with identical length are detected simultaneously in the same run, in a particular preferred embodiment it is preferred to choose the stuffer sequences such that amplicons differ by at least two base pairs within a multiplex set and differ by a single base pair between multiplex sets labelled with the different dyes that have overlapping spectra. By doing so, the length of the fragments labelled with the respective dyes can be chosen such that the potential influence of residual cross-talk on the quality of the data is circumvented because unique combinations of fragments size and labelling dye are defined (Figure 3).

[0072] A particular preferred embodiment of the invention is directed to a method in which a sample comprising amplicons is derived from a multiplicity of target sequences. These amplicons are differently labelled, thereby defining groups of amplicons carrying the same label. Within each group, the stuffer provided for a length difference of at least two, preferably two nucleotides. Between two groups with labels having spectral overlap, the stuffer provides a length difference of one nucleotide, effectively resulting in one group having an even number of nucleotides and one group having an odd number of nucleotides as described above.

[0073] In one aspect the present invention pertains to a method for the improved discrimination and detection of target sequences in a sample, comprising providing at least a two or more groups of oligonucleotide probes, wherein the amplicons obtained with different groups of oligonucleotide probes have different labels, wherein substantially each amplified connected probe target sequence within a group has the same label, wherein within a group of identically labelled amplicons a length difference is provided between each identically labelled probe within that group, wherein between the first and second group an additional length difference is provided such that each amplified connected probe in the amplified sample is characterised by a combination of length of the sequence and the label.

[0074] In a preferred embodiment of the method of the invention, at least two groups of oligonucleotide probes are provided to a sample, whereby each group of oligonucleotide probes has tag sequences with at least one group specific primer-binding site. The connected probes of each group are amplified from a primer pair wherein at least one of the first and second primers is complementary to the group specific primer-binding site, and whereby at least one of the first and second primers of a group comprises a group specific label. In each group, an amplicon corresponding to a target sequence in the sample differs in length from an amplicon corresponding to a different target sequence in the sample. The group specific labels are preferably such that the detection device can distinguish between the different group specific labels. The length difference is preferably provided by the length of the stuffer sequence. Preferably in this embodiment of the method of the invention, a first part of the groups has amplicons having an even number of nucleotides and a second part of the groups has amplicons having an odd number of nucleotides. Preferably, the groups of amplicons having an even number of nucleotides and the groups amplicons having an odd number of nucleotides are labelled with (fluorescent) labels, which have the least overlap in their emission spectra. Thus, two groups of amplicons, each group having an odd number of nucleotides are labelled with labels, which have the least overlap in their emission spectra. The same holds for two groups of amplicons, each group having an even number of nucleotides. Two groups of amplicons, one group having an odd number of nucleotides and the other group having an even number of nucleotides are labelled with labels that have a larger overlap in their emission spectra. The relative notions as used herein of 'the least overlap in their emission spectra' and have a larger overlap in their emission spectra' refer to a group of labels from which a selection of the labels can be made for use in the present invention. This group of labels may depend on the detection platform used to other factors such as those disclosed herein before. In a particularly preferred embodiment of this method, a first and second groups of amplicons having an even number of nucleotides are produced and a third and fourth group of connected amplified probes having an odd number of nucleotides are produced and whereby the first and second group are labelled with FAM and NED, respectively, and the third and fourth group are labelled with (ET-)ROX and either JOE or HEX, respectively; or *vice versa,* whereby the first and second group are labelled with (ET-)ROX and either JOE or HEX, respectively, and the third and fourth group are labelled with FAM and NED, respectively. Thus, in these embodiments, the fluorescent labels are chosen such that the groups of amplicons that co-migrate, because they both contain fragments with either even or odd numbers of nucleotides, have labels which have the least overlap in their emission spectra, thereby avoiding as much as possible cross-talk in the detection of amplicons in different groups (see also below).

[0075] In a preferred embodiment to avoid cross-talk it is therefore desirable to combine a difference in length with a

different label when analysing a set of amplicons in such a way that the influence of spectral overlap on the data quality is avoided by length differences between the amplicons labelled with the dyes that have overlapping emission spectra.

[0076] It is preferred that in each sample the connected probes derived from each target sequence differ from any other connected probe in the sample in length, and/or in the label or, preferably in the combination of the length and the label. To provide for an adequate separation of the amplicons of different length it is preferred that the length difference between two different connected probes is at least two nucleotides, preferably two. When detecting polymorphisms it is preferred that the difference in length between two or more (SNP) alleles of the polymorphism is not more than two, thereby ensuring that the efficiency of the amplification is similar between different alleles or forms of the same polymorphism. This implies that preferably both alleles are amplified with the same pair of primers and hence will be labelled with the same dye.

[0077] In a preferred embodiment, for example directed to the detection of different alleles of a multiplicity of loci, the distribution between odd/even lengths within a group can be designed in the following way. Two loci L1, L2 are each represented by two alleles A11, A12 for L1 and A21, A22 for L2. The lengths of the various alleles (or ligated and amplified probes representing those alleles) is such that A1 1>A12>A21>A22; A12-A1 1=2; A22-A21=2; A12-A21=3. Between groups G1 and G2 carrying labels that may have an overlap in their spectra there can be a length difference of 1 nucleotide. Thus G1(A11)-G2(A11)=1, hence the group starts with either an even or an uneven length.

[0078] This distribution has some significant advantages compared to the more densely packed distribution disclosed herein. It is known that due to conformational differences that different sequences of identical length generally differ in their electrophoretic mobility. When there is only a difference in length of one nucleotide, this may cause overlap between the peaks if the sequences are of a very different mobility. For instance the difference in mobility between two alleles of one locus (A11, A12), will be less than the difference in mobility between two alleles from different loci (A12, A21). When there is a significant difference in mobility between A12 and A21, this may lead to unreliable detection. By creating length distributions as herein disclosed this can be avoided. The lower throughput is then weighed against the reliability of the detection.

[0079] The problem of the overlap between the spectra of the different labels is then adequately avoided. This is schematically depicted in Table A.

Table A Alternative distribution scheme of labels and lengths of probes.

Length	Group 1-Label 1	Group 2-Label 2	Group 3-Label 3	Group 4-Label 4
N	G1A11		G3A11	
N+1		G2A11		G4A11
N+2	G1A12		G3A12	
N+3		G2A12		G4A12
N+4				
N+5	G1A21		G3A21	
N+6		G2A21		G4A21
N+7	G1A22		G3A22	
N+8		G2A22		G4A22
N+9				
N+10	G1A31		G3A31	
N+11		G2A31		G4A31
N+12	G1A32		G3A32	
N+13		G2A32		G4A32
N+14				
N+15	G1A41		G3A41	
N+16		G2A41		G4A41
N+17	G1A42		G3A42	
N+18		G2A42		G4A42

[0080] In an embodiment of the present invention there is provided between the amplicons within one group, a length difference of alternating two and three nucleotides, i.e. 0, 2, 5, 7, 10, 12 etc. The other group then has a length difference of 1, 3, 6, 8, 11, 13 etc.

Target sequences

[0081] In its widest definition, the target sequence may be any nucleotide sequence of interest. The target sequence preferably is a nucleotide sequence that contains, represents or is associated with a polymorphism. The term polymorphism herein refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker or site is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A polymorphic locus may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wild type form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms. A single nucleotide polymorphism occurs at a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than 1/100 or 1/1000 members of the populations). A single nucleotide polymorphism usually arises due to substitution of one nucleotide for another at the polymorphic site. Single nucleotide polymorphisms can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele. Other polymorphisms include small deletions or insertions of several nucleotides, referred to as indels. A preferred target sequence is a target sequence that is associated with an AFLP® marker, i.e. a polymorphism that is detectable with AFLP®.

DNA

[0082] In the nucleic acid sample, the nucleic acids comprising the target may be any nucleic acid of interest. Even though the nucleic acids in the sample will usually be in the form of DNA, the nucleotide sequence information contained in the sample may be from any source of nucleic acids, including e.g. RNA, polyA$^+$ RNA, cDNA, genomic DNA, organellar DNA such as mitochondrial or chloroplast DNA, synthetic nucleic acids, DNA libraries, clone banks or any selection or combinations thereof. The DNA in the nucleic acid sample may be double stranded, single stranded and double stranded DNA denatured into single stranded DNA. Denaturation of double stranded sequences yields two single stranded fragments one or both of which can be analysed by probes specific for the respective strands. Preferred nucleic acid samples comprise target sequences on cDNA, genomic DNA, restriction fragments, adapter-ligated restriction fragments, amplified adapter-ligated restriction fragments. AFLP fragments or fragments obtained in an AFLP-template preamplification.

Samples

[0083] It is preferred that a sample contains two or more different target sequences, i.e. two or more refers to the identity rather than the quantity of the target sequences in the sample. In particular, the sample comprises at least two different target sequences, in particular at least 10, preferably at least 25, more preferably at least 50, more in particular at least 100, preferably at least 250, more preferably at least 500 and most preferably at least 1000 additional target sequences. In practice, the number of target sequences is limited, among others, by the number of connected circular probes. E.g., too many different oligonucleotide probes in a sample may corrupt the reliability of the multiplex amplification step.

[0084] A further limitation is formed e.g. by the number of fragments in a sample that can be resolved by the electrophoretic device in one injection. The number can also be limited by the genome size of the organism or the transcriptome complexity of a particular cell type from which the DNA or cDNA sample, respectively, is derived.

Multiple injection

[0085] In a preferred embodiment of the invention, in order to come to a high throughput method of a multiplicity of samples, a number of samples are treated similar to thereby generate a multiplicity of amplified detection samples which can then be analysed on a multichannel device which is at least capable of detecting the labels and/or length differences. Suitable devices are described herein.

[0086] To increase throughput on electrophoretic platforms methods have been developed that are described in this

application and are commonly depicted as multiple injection. By injecting multiple samples containing fragments of discrete, pre-determined lengths, in the same electrophoretic matrix and/or in short consecutive runs, throughput can be increased. All detectable fragments preferably have a length within a specific span and only a limited number of fragments can be detected in one sample, hence the advantage of selective amplification for the reduction of the multiplex ratio by the selection of a subset of the connected probes in the amplification step resulting in a subset of amplicons.

[0087] Steps (a) to (e) of the method of the invention may be performed on two or more nucleic acid samples, each containing two or more different target nucleic acids, to produce two or more amplified samples in which is presence or absence of amplicons is analysed.

[0088] The multiplex analysis of the amplified samples following the method of the invention comprises applying at least part of an amplified sample to an electrophoretic device for subsequent separation and detection. Preferably such an amplified sample contains, or is at least suspected to contain, amplified connected probes, which is an indication that a target sequence has hybridised with the provided oligonucleotide probes and that those probes were annealed adjacently on the complementary target sequence so that they where connected, i.e. ligated. Subsequently, an amplified sample is subjected to a separating step for a selected time period before a next amplified sample is submitted.

[0089] In the method of the invention, (parts of) two or more different amplified samples are applied consecutively to the same channel of the electrophoretic device (Fig 8). Depending on the electrophoresis conditions, the time period (23) between two (or more) consecutively applied amplified samples is such that the slowest migrating amplified connected probe (19) in an amplified sample is detected at the detection location (24), before the fastest migrating amplified connected probe of a subsequently applied amplified sample is detected at the detection location (24). Thus, the time intervals between subsequent multiple injections in one channel of the device are chosen such that consecutively applied samples after separation do not overlap at a point of detection.

[0090] In a preferred embodiment the method of the invention further comprises the following steps:

(e1) repeating steps (a) to (e) to generate at least two amplified samples;

(e2) consecutively applying at least part of the amplified samples obtained in steps (e) and (e1), to an application location of a channel of an electrophoretic device, electrophoretically separating the amplicons in the amplified samples and detecting the separated amplicons at a detection location located distal from the application location of the channel; whereby the time period between the consecutively applied amplified samples is such that the slowest migrating amplified connected probe in an amplified sample is detected at the detection location before the fastest migrating amplified connected probe of a subsequently applied amplified sample is detected at the detection location.

[0091] The method according to the invention allows for the high throughput analysis of a multiplicity of samples each comprising a multiplicity of different target sequences by the consecutive injection of amplified samples, comprising amplicons corresponding to the target sequences in the samples, in a channel of a multichannel electrophoretic device such as a capillary electrophoresis device. The method according to the invention allows for the analysis of a multiplicity of target sequences in a multiplicity of samples on a multiplicity of channels, thereby significantly increasing the throughput of the number of samples that can be analysed in a given time frame compared to conventional methods for the analysis of nucleotide sequences. This method profits from samples containing amplicons to be detected that are of a discrete size range as thereby the time period (23) between the successive injections can be significantly reduced compared to methods wherein the (remains of) concatamers are present.

[0092] The selected time period prevents that consecutively applied samples after separation have an overlap of amplicons at the detection point. The selected time period is influenced by i). the length of the amplicons; ii). the length variation in amplicons; and iii). the detection device and its operating conditions. Applying samples and separating consecutively applied samples in the same channel can be repeatedly performed in one or more channels, preferably simultaneously to allow for consecutive electrophoretic separation of multiple samples in one channel and/or simultaneous analysis of multiple samples over multiple channels and/or simultaneous analysis of multiple samples over multiple channels carried out consecutively. A graphic representation thereof is given in Figure 8.

[0093] The period of time between two consecutively loaded amplified samples can be determined experimentally prior to executing the method. This period of time is selected such that, given the characteristics of an amplified sample, especially the difference in length between the shortest and the longest amplicons in an amplified sample, as well as other experimental factors such as gel (matrix) and/or buffer concentrations, ionic strength etc., the fragments in an amplified samples are separated to such extent at the detection location which is located at the opposite end (distal) from the application location where the sample was applied, that the different amplicons in a sample may be individually detected. After applying the last amplified sample, the separation can be continued for an additional period of time to allow the amplicons of the last sample to be separated and detected. The combination of the selected period of time between applying two consecutive samples and the optional additional time period is chosen such that at the detection location the different amplicons in consecutively applied samples are separated such that they may be individually

detected, despite the limited length variation that exists between the different amplicons within a single sample. Thus overlapping migration patterns are prevented when samples containing fragments of varying length are consecutively applied (injected) on the electrophoretic device.

[0094] Using the method according to the invention, it is in principle possible and preferred to continuously apply, load or inject samples. Preferably the device is able to perform such operation automatically, e.g. controlled by a programmable computer. Preferably the multichannel device is suitable for such operation or is at least equipped for a prolonged operation without maintenance such as replacement of buffers, parts etcetera. However, in practice this will generally not be the case. When a final sample is submitted it is generally needed to continue the separation for an additional time period until the last fragment of the final sample has been detected. In a preferred embodiment of the invention, the stuffers present in the tags of the oligonucleotide probes is are used to provide the length differences (i.e. 0 to 500 nucleotides, bases or base pairs) between the amplified connected probes. The total length of the amplicon and the variation in the length is governed mostly by the techniques by which these fragments are analysed. In the high throughput multiple injection method of the present invention, it is preferred that the range of lengths of amplicons in an amplified sample has a lower limit of 40, 60, 80, or 100 and an upper limit of 120, 140, 160, or 180 nucleotides, bases or base pairs, for conventional (capillary) electrophoresis platforms. It is particularly preferred that the range of lengths of the amplicons varies from 100 to 140 nucleotides. However, these numbers are strongly related to the current limits of the presently known techniques. Based on the knowledge provided by this invention, the skilled artisan is capable of adapting these parameters when other circumstances apply.

[0095] The reliability of the multiplex amplification is further improved by limiting the variation in the length of the amplified connected probes. Limitations in the length variation of amplicons is preferred to use multiple injection more efficiently and further results in reduction of the preferential amplification of smaller amplicon in a competitive amplification reaction with larger connected probes. This improves the reliability of the high throughput method of the present invention. Together with the multiple injection protocol as herein disclosed, these measures, alone or in combination provide for a significant increase in throughput in comparison with the art. A further improvement of the high throughput capacity is obtained by limiting the number of different amplicons in a sample. It is regarded as more efficient and economical to limit the multiplex capacity of the ligation/amplification step in combination with the introduction of a multiple injection protocol. One of the most advantageous aspects of the present invention lies in the combination of multiplex ligation, multiplex amplification, preferably with a single primer pair or with multiple primer pairs which each amplify multiple connected probes, repeated injection and multiplex detection of different labels. One of the further advantageous aspects of the present invention resides in the combined application of length differences with different (overlapping) labels such that each connected probe and hence each target sequence within one sample can be characterised by a unique combination of length and label. This allows for a significant improvement of the efficiency of the analysis of target sequences as well as a significant reduction in the costs for each target analysed.

[0096] The multiple injection protocol can be performed in a variety of ways. One of these is the multiple loading of two or more samples in the same matrix. This is considered as advantageously as the matrix is re-used by performing consecutive short runs, thereby increasing efficiency and throughput. Another one is the multiple loading of two or more samples in the same matrix in the same run. It is preferred to re-use the matrix by performing short consecutive runs. In this embodiment, a first sample is injected and separated. As soon as the last fragment is detected, the next sample is loaded. Preferably, between these two consecutive short runs the matrix is not replaced so that the runs are performed in the same matrix. This provides for additional efficiency and improved economics as less changes o the matrix need to occur, reducing the amount of consumables of this type of analysis (i.e. buffers etc.), reducing the cost per datapoint. Furthermore time-consuming replacements of the matrix can be avoided to a large extent, further increasing the efficiency of the method.

[0097] In itself, certain aspects of multiple loading or multiple injection have been described *inter alia* in US6156178 and WO O1/04618. The latter publication discloses an apparatus and a method for the increased throughput analysis of small compounds using multiple temporally spaced injections. The publication discloses that samples comprising primers, extended by one nucleotide (single nucleotide primer extension or SnuPE, also known as minisequencing) could be detected using multiple temporally spaced injections on a capillary electrophoresis device. Minisequencing is based on annealing a complementary primer to a previously amplified target sequence. Subsequent extension of the primer with a separately provided labelled nucleotide provides for identification of the nucleotide adjacent to the primer. Principally, the primer extension product is of a constant length. To increase throughput the use of successive injections of extension products of the same length per run is suggested. To further increase the throughput, primers of a different length can be used, varying typically from 15 to 25 nucleotides. In contrast, the present invention contemplates analysing multiplex amplification products themselves directly with a length variation typically between 50 and 150 nucleotides. This is significantly more economical than minisequencing or SnuPE as outlined hereinbefore because multiple target sequences are amplified in a single reaction, whereas with minisequencing or SnuPE amplification is carried out individually for each target sequence. Furthermore, the use of primers of a different length and complementary to the target sequence compromises the efficiency of the subsequent amplification step needed in the method of the present invention.

These applications in general do not address the problems associated with high throughput detection of highly multiplexed samples, nor provide solutions thereto.

Exonucleases

[0098] A preferred method of the invention further comprises a step for the removal of oligonucleotide probes that are not annealed to target sequences and/or that are non-connected/ligated. Removal of such probes preferably is carried out prior to amplification, and preferably by digestion with exonucleases. By removal/elimination of the oligonucleotide probes that are not connected/ligated a significant reduction of ligation independent (incorrect) target amplification can be achieved, resulting in an increased signal-to-noise ratio. One solution to eliminate one or more of the non-connected/ligated components without removing the information content of the connected probes is to use exonuclease to digest non-connected/ligated oligonucleotide probes.issensitive. sensitive. Blocking groups include use of a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone. Exonucleases include ExoI (3'-5' activity), Exo III (3'-5' activity), and Exo IV (both 5'-3' and 3'-5' activity). The circular probes of the present invention are, once ligated, insensitive to the exonuclease, as opposed to the unligated circular probes This is a further advantage of the use of padlock probes in the present invention.

[0099] An advantage of using exonucleases, for example a combination of Exo I (single strand specific) and Exo III (double strand specific), is the ability to destroy both the target sequence and the unligated oligonucleotide probes, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to amplification, the oligonucleotide probes in each set are substantially reduced, and thus hybridisation of the remaining unligated oligonucleotide probes to the original target DNA (which is also substantially reduced by exonuclease treatment) and formation of a ligation product sequence which is a suitable substrate for PCR amplification by the oligonucleotide primer set is substantially reduced, thereby improving the signal to noise ratio.

Size ladder

[0100] The sample can be supplied with a nucleotide fragment size standard comprising one or more nucleotide fragments of known length. Methods of preparing and using nucleotide size standards are well known in the art (see e.g. Sambrook and Russel, 2001, *supra).* Such a size standard forms the basis for appropriate sizing of the amplicons in the sample, and hence, for the proper identification of the detected fragment. The size standard is preferably supplied with every sample and/or with every injection. A size standard preferably contains a variety of lengths that preferably spans the entire region of lengths to be analysed. In a particular embodiment of the invention, it is considered advantageously to add flanking size standards from which the sizes of the amplicons can be derived by interpolation. A flanking size standard is a size standard that comprises at least two labelled oligonucleotide sequences of which preferably one has a length that is at least one base shorter than the shortest amplified connected probe and preferably one that is a least one base longer than the longest amplified connected probe to allow interpolation and minimise the introduction of further length variation in the sample. A preferred flanking size standard contains one nucleotide that is one nucleotide shorter the shortest amplified connected probe and one that is a least one base longer than the longest amplified connected probe and is labelled with at least one dye that is identical to the label used for labelling the amplicons contained in the sample.

[0101] A convenient way to assemble a suitable size standard is by (custom) chemical synthesis of oligonucleotides of the appropriate lengths, which are end-labelled with a suitable label. The size standard is applied with every consecutively applied sample to serve as local size references to size the loaded sample fragments. The size standard may be applied in the same channel or lane of the electrophoretic device as the sample to be analysed, i.e. together with the sample, or may be applied in a parallel channel or lane of a multichannel/lane device. The flanking size standard can be labelled with any of the labels used in the method. If the size standard is applied in the same channel of the device, the fragments of the standard are preferably labelled with a label that can be distinguished from the labels used for the detection of the amplicons in a sample.

Pooling

[0102] In a variant of the technology, the starting (DNA) material of multiple individuals are pooled such that less detection samples containing this material are loaded on the detection device, This can be advantageous in the case of Linkage Disequilibrium (LD mapping) when the objective is to identify amplified connected probes (such as those representing SNP alleles) that are specific for a particular pool of starting samples, for example pools of starting material derived from individuals which have different phenotypes for a particular trait.

Application

[0103] One aspect of the invention pertains to the use of the method in a variety of applications. Application of the method according to the invention is found in, but not limited to, techniques such as genotyping, transcript profiling, genetic mapping, gene discovery, marker assisted selection, seed quality control, hybrid selection, QTL mapping, bulked segregant analysis, DNA fingerprinting and microsatellite analysis. Another aspect pertains to the simultaneous high throughput detection of the quantitative abundance of target nucleic acids sequences. This approach is commonly known as Bulk Segregant Analysis (BSA).

Detection of single nucleotide polymorphisms

[0104] One particular preferred application of the high throughput method according to the invention is found in the detection of single nucleotide polymorphisms (SNPs). A first target complementary part of the circular oligonucleotide probes is preferably located adjacent to the polymorphic site, i.e. the single polymorphic nucleotide. A second target complementary part is designed such that its terminal base is located at the polymorphic site, i.e. is complementary to the single polymorphic nucleotide. If the terminal base is complementary to the nucleotide present at the polymorphic site in a target sequence, it will anneal to the target sequence and will result in the ligation of the two target complementary parts. When the end-nucleotide, i.e. the allele-specific nucleotide does not match, no ligation or only a low level of ligation will occur and the polymorphism will remain undetected.

[0105] When one of the target sequences in a sample is derived from or contains a single nucleotide polymorphism (SNP), in addition to the probes specific for that allele, further probes can be provided that not only allow for the identification of that allele, but also for the identification of each of the possible alleles of the SNP (co-dominant scoring). To this end a combination of target complementary parts can be provided: one complementary part is the same for all alleles concerned and one or more of the other complementary parts which is specific for each of the possible alleles. These one or more other type of complementary parts contain the basically the same complementary sequence but differ in that each contains a nucleotide, preferably at the end, that corresponds to the specific allele. The allele specific part can be provided in a number corresponding to the number of different alleles expected. The result is that one SNP can be characterised by the combination of one complementary part with four other (allele-specific) complementary parts, identifying all four theoretically possible alleles (one for A, T, C, and G), by incorporating stuffer sequences of different lengths (preferred) or different labels into the allele specific probes.

[0106] In a particular embodiment, preferably directed to the identification of single nucleotide polymorphisms, the first complementary part of the oligonucleotide probe is directed to a part of the target sequence that does not contain the polymorphic site and the second complementary part of the oligonucleotide probe contains, preferably at the end distal from first complementary part, one or more nucleotide(s) complementary to the polymorphic site of interest. After ligation of the adjacent parts, the connected probe is specific for one of the alleles of a single nucleotide polymorphism.

[0107] To identify the allele of polymorphic site in the target sequence, a set of oligonucleotide probes can be provided wherein one first complementary part is provided and one or more second complementary parts. Each second complementary part then contains a specific nucleotide at the end of the complementary sequence, preferably the 3'-end, in combination with a known length of the stuffer. For instance, in case of an A/C polymorphism, the second complementary part can contain a specific nucleotide T in combination with a stuffer length of 2 nucleotides and another second complementary part for this polymorphism combines G with a stuffer length of 0. As the primers and the complementary parts of the probes are preferably the same length, this creates a length difference of the resulting amplicons of 2 nucleotides. In case the presence and/or the absence of all four theoretically possible nucleotides of the polymorphic site is desired, the stuffer-specific nucleotide combination can be adapted accordingly. In a sample containing multiple target sequences, amplified with the same pair of amplification-primers (and hence label) or with multiple pairs of amplifications primers with labels that have overlapping emission spectra, the combined stuffer lengths are chosen such that all connected probes are of a unique length. In Figure 4 an illustration of this principle is provided of two loci and for each locus two alleles. In a preferred embodiment this principle can be extended to at least ten loci with at least two alleles per locus.

Detection of specific target sequence

[0108] The target sequence contains a known nucleotide sequence derived from a genome. Such a sequence does not necessarily contain a polymorphism, but is for instance specific for a gene, a promoter, an introgression segment or a transgene or contains information regarding a production trait, disease resistance, yield, hybrid vigour, is indicative of tumours or other diseases and/or gene function in humans, animals and plants. To this end, the first and second complementary parts of the circular probe are designed to correspond to a, preferably unique, target sequence in genome, associated with the desired information. The complementary parts in the target sequence are located adjacent

to each other. In case the desired target sequence is present in the sample, the two probes will anneal adjacently and after ligation and amplification can be detected.

Detection of AFLP markers

[0109] AFLP, its application and technology is described in Vos et al., Nucleic Acids Research, vol. 23, (1995), 4407-4414 as well as in EP-A 0 534 858 and US 6045994, all incorporated herein by reference. For a further description of AFLP, its advantages, its embodiments, its techniques, enzymes, adapters, primers and further compounds, tools and definitions used, explicit reference is made to the relevant passages of the publications mentioned hereinbefore relating to AFLP. AFLP and its related technology is a powerful DNA fingerprinting technique for the identification of for instance specific genetic markers (so-called AFLP-markers), which can be indicative of the presence of certain genes or genetic traits or can in general be used for comparing DNA, cDNA or RNA samples of known origin or restriction pattern. AFLP-markers are in general associated with the presence of polymorphic sites in a nucleotide sequence to be analysed. Such a polymorphism can be present in the restriction site, in the selective nucleotides, for instance in the form of indels or substitutions or in the rest of the restriction fragment, for instance in the form of indels or substitutions. Once an AFLP marker is identified as such, the polymorphism associated with the AFLP-marker can be identified and probes can be developed for use in the ligation assay of the present invention.

[0110] In another aspect the present invention pertains to a circular nucleic acid probe comprising a first and a second part that is capable of hybridising to corresponding parts of a target sequence and further comprising at least one, preferably two primer-binding sequence and a stuffer. Further embodiments of the probe according to the present invention are as described herein above. The invention also pertains to a set of probes comprising two or more probes wherein each probe comprises a first part and a second part that is complementary to part of a target sequence and wherein the complementary first an second parts are located essentially adjacent when hybridised to the target sequence and wherein each probe further comprises a stuffer, which stuffer is located essentially next to the complementary part and at least one, preferably two primer-binding sequence located essentially adjacent to the stuffer.

[0111] The invention in a further aspect, pertains to the use of a circular probe or set of probes in the analysis of at least one nucleotide sequence and preferably in the detection of a single nucleotide polymorphism, wherein the set further comprises at least one additional probe that contains a nucleotide that is complementary to the known SNP allele. Preferably the set comprises a probe for each allele of a specific single nucleotide polymorphism. The use of a set of probes is further preferred in a method for the high throughput detection of single nucleotide polymorphisms wherein the length of the stuffer in the probe is specific for a locus and/or allele of a single nucleotide polymorphism

[0112] Another aspect of the invention relates to the primers and more in particular to the set of primers comprising a first primer and one or more second primers, wherein each second primer contains a label and which second primer comprises a nucleotide sequence that is specific for said label.

[0113] The present invention also finds embodiments in the form of kits. Kits according to the invention are for instance kits comprising probes suitable for use in the method as well as a kit comprising primers, further a combination kit, comprising primers and probes, preferably all suitably equipped with enzymes buffers etcetera, is provided by the present invention.

[0114] The efficiency of the present invention can be illustrated as follows. When a capillary electrophoretic device with 96 channels and capable of detecting four labels simultaneously is used, allowing for 12 subsequent injections per run per channel with a empirically optimised minimum selected time period between the injections, a sample containing 20 target sequences of interest allows for the high throughput detection of 96 (channels) * 12 (injections) * 20 (targets) * 4 (labels) = 92160 target sequences, using the method of the present invention. In the case of co-dominant SNP-detection, data regarding 46080 SNPs can be detected in a single run.

Description of the Figures:

[0115] This invention is illustrated by the accompanying figures. In the figures, many of the features of the invention are demonstrated using two linear probes that hybridise adjacently. The skilled man will appreciate that most of these features also apply to other embodiments disclosed herein such as the circular probes and how to include those features in the other embodiments such as the circular probes based on the information provided in this application.

Figure 1: Schematic representation of the oligonucleotide ligation- amplification assay, resulting in amplified connected probes.
A target sequence (2) comprising a first (5) and a second (7) part to which parts first and second probes can be hybridised with sections (4) and (6) that are complementary, respectively. The probes contain a tag sequence (8,9) that is not complementary to the target sequence. The tag sequence may comprise a stuffer sequence (10,11) and a primer-binding site (12,13). After probe hybridisation and ligation the connected probe (15) can be amplified using

primers (16, 17) capable of hybridising to the corresponding primer-binding sites. At least one of the primers contains a label (L). Amplification results in an amplified sample, comprising amplicons (20)
Figure 2: Schematic representation of two connected probes, wherein

(a) only one probe contains a stuffer (10) and primer-binding sequences (12,13); and
(b) both probes contain a stuffer (10, 11) and primer-binding sequences (12,13).

Figure 3: Schematic representation of the unique combination of different lengths and labels with a schematic elution profile in one channel of a multichannel device.
Figure 4: Schematic representation of the oligonucleotide ligation-ligation assay of the present invention. The principle is represented for two loci 1 and 2 and for each locus two alleles for reasons of simplicity only, but can easily be extended to at least 10 loci with 2 alleles each. The primer set consists of one first primer (solid bold line) and one second primer (dashed bold line). The theoretically possible connected probes are schematically outlined, together with the primers. The connected probes differ in length.
Figure 5: Schematic representation of the oligonucleotide ligation-ligation assay of the present invention. The principle is represented for two loci 3 and 4 and for each locus two alleles. The primer set consists of one first primer and two second primers. The theoretically possible connected probes are schematically outlined, together with the primers. The connected probes differ in length and in label.
Figure 6: Schematic representation of the results of a sample containing 80 amplified connected probes with:

- a length difference between 135 base pairs (bp) to 97 bp for the amplified connected probes with an odd length and labelled with Label 1 and Label 3; and
- a length difference between 134 bp to 96 bp for the amplified connected probes with an even length and labelled with Label 2 and Label 4; and
- a flanking size ladder with oligonucleotides of 94/95 and 136/137 (bp) carrying label 1,2, 3 or 4

Figure 7: Schematic representation of the separation profile in one channel, submitting one sample comprising multiple amplified connected probes labelled with Label 1, 2, 3, and 4. The multiple labelled amplified connected probes are detectably separated at the point of detection.
Figure 8: Schematic representation of the multiple injection of samples in one channel, with a graphic illustration of the selected time period (23) between the injection of subsequent samples and the additional time period (25) after submitting the last sample.
Figure 9: Schematic representation of the ligation of up to 40 loci, and the subsequent amplification and detection phase of the method. Depending on the complexity and the number of loci to be analysed, the points in the procedure at which pooling can be contemplated is indicated as an optional (dotted) feature). Amplification is here carried out by using one forward primer (Forward) and for each label one (differently labelled) reverse primer (Reverse 1, 2, 3, 4). When the ligation (sub)samples are pooled, there are in principle two options for amplification. For instance if (sub)samples derived from Loci 1-10 are pooled with (sub)samples derived from Loci 11-20 prior or subsequent to ligation, the pooled (sub)sample can be amplified with the Forward primer and the Reverse primers 1 and 2 in one step or in two steps, first with Forward and Reverse 1, followed by Forward and Reverse 2 or *vice versa.* Detection can also be performed in a similar way, detecting both labels simultaneously or first label 1, followed by label 2, optionally by double injection.
Figure 10: **A** gel of a multiplex oligonucleotide ligation assay of 12 SNPs from the Colombia ecotype, the Landsberg erecta ecotype and a 50/50 mixture of the Colombia and the Landsberg erecta ecotypes.
Figure 11: **A**. Partial electropherogram of FAM labelled detection of the Colombia sample on a capillary electrophoretic device (MEGABace). The same multiplex mixture was injected. Amplified connected probes in a size range 97-134 bp and flanking sizer fragments (designated S) are 94, 95 and 137 bp. Probes and sizers are all labelled with FAM.

B. Partial electropherogram of FAM labelled detection of the Landsberg erecta sample on a capillary electrophoretic device (MEGABace). The same multiplex mixture was injected. Amplified connected probes in a size range 97-134 bp and flanking sizer fragments (designated S) are 94, 95 and 137 bp. Probes and sizers are all labelled with FAM.

Figure 12: **A**: Raw trace file of a sample containing a 120 bp ET-ROX labelled fragment and a 124 bp NED -labelled fragments. Note the FAM and JOE labels from other labelled fragments in the sample with the same length. FAM and JOE have overlapping fluorescence spectra (ET-ROX and FAM, JOE and NED), resulting in overlapping signals (cross-talk) with sequences of equal length.

B: Mathematical cross-talk correction resulting in a processed, cross-talk corrected trace file. Cross talk is reduced, but remains of the overlapping spectra (FAM, JOE) are present, resulting in false positive (or negative) signals.
C, D, E, F: single label plots illustrate the presence of remnants (D, E) of the mathematical correction, compared to the positive signals (C, F)

Figure 13 A: Representation of the effect of incomplete removal of cross-talk of a 120 bp ET-ROC fragment and a 124 bp NED fragment, resulting in incorrect scored data, compared to theoretically expected data.

B: Representation of the effect of the use of cross-talk correction by length-label combinations. Scored data and expected data are correctly interpreted and false-positive or negative data are eliminated.

Figure 14: Representation of a circular probe with primer binding sites, primers and an optional blocking section and their relative positioning in the circular probe. After amplification amplicons are formed that are representations of the circular probe.
Figure 15: Representation of the design of the selective or nested primers used in the selective amplification of a sample of connected circular probes. The connected circular probe is schematically drawn with one primer binding site and adjacent nucleotides denoted as N. For a 24-plex ligation assay, the selective amplification with one selective nucleotide is used to visualise the reduction to 6-plex amplification and detection assays.
Figure 16: Amplification with primer Eook+T5'-JOE of a 10 plex ligation product of set 4 on sample 2. Signal of Joe channel is shown.

A. Cross-talk in the NED channel caused by the amplification of the 10 plex ligation of set 4 on sample 2with primer Eook+T5'-JOE (see A). NED signal has been omitted.
B. Signal in the NED channel caused by the amplification with primer Eook+T5'-JOE and a NED labelled E00k amplification of a 10 plex ligation of set 4 on sample 2 (see A). Because 5'+T E00k-Joe signal in NED differs 1bp, this two peaks can be distinguished. X means cross-talk of the Joe fluorescent dye in Ned channel (corresponds to signal in B).
C. Amplification of a 10-plex ligation of set 4 on sample 2 was carried out using a NED labelled E00k amplification primer and a 5' +T E00k JOE labelled primer and the reaction products were combined for detection on the MegaBACE. Unprocessed signal in the NED channel is shown. Because the JOE labelled products differ by one bp in length, the peaks from NED and JOE can be distinguished in the NED channel.
D. The same reaction products shown in C but after processing of the raw data, i.e. after cross talk removal. The 1 bp size difference of the 5'T E00k JOE products prevent miss-scoring caused by cross-talk of JOE signals into the NED channel as show in Figures 16 A, B and C.

All signals of A, B, C and D are obtained after processing by Genetic Profiler version 1 software from Molecular Dynamics. Signal shown in D is corrected for cross talk and hence shows processed signals. The signals in A, B, and C are raw data and are not corrected for cross talk.
Figure 17:

A. Analysis of 5'+T Joe and FAM labelled E00k amplification of ligation products of set 4 for sample5 (capillary G05) and 6 (capillary G06). Run time was 40 minutes.
B. Second analysis of 5'+T Joe and FAM labelled E00k amplification of ligation products of set 4 for sample5 (capillary G05) and 6 (capillary G06). This run was performed directly after the one shown in A, on the same matrix. Run time was 40 minutes.

Figure 18:
Selective amplification of 3 sets out of one 40-plex ligation for sets 1, 2, 4 and 5 from sample 3.

A. Selective amplification of set 1 with E01k-Ned and M01k.
B. Selective amplification of set 2 with E03k-5'+T-JOE and M04k.
C. Selective amplification of set 5 with E04k-Fam and M03k.

[0116] All channels are visible. It is clear that it is possible to amplify a specific set out of a multiplex ligation product for more sets.

Examples

I. Design of the stuffer sequences

[0117] In order to prevent cross-hybridisation between the amplification products, it is preferred that the sequences of the stuffer sequences are different and do not form hairpins. In the tables 1-5, stuffer sequences are presented which can be used for the development of probes for each fluorescent dye, and have been verified for the absence of hairpins using Primer Designer version 2.0 (copyright 1990,1991, Scientific and Educational software) The stuffer sequences are assembled from randomly chosen tetramer blocks containing one G, C, T and A, and have therefore by definition a 50% GC content. The stuffer sequence in the forward OLA probe for the two SNP alleles are kept identical to avoid preferential SNP allele amplification.

Table 1: Lengths of stuffer sequences

ET-ROX and JOE probes.			FAM and NED probes.		
Total stuffer length	Stuffer length 1st type probe	Stuffer length 2nd type probe	Total stuffer length	Stuffer length 1st type probe	Stuffer length 2nd type probe
0	0	0	1	1	0
2	0	2	3	1	2
4	4	0	5	5	0
6	4	2	7	5	2
8	8	0	9	9	0
10	8	2	11	9	2
12	12	0	13	13	0
14	12	2	15	13	2
16	16	0	17	17	0
18	16	2	19	17	2
20	20	0	21	21	0
22	20	2	23	21	2
24	24	0	25	25	0
26	24	2	27	25	2
28	28	0	29	29	0
30	28	2	31	29	2
32	32	0	33	33	0
34	32	2	35	33	2
36	36	0	37	37	0
38	36	2	39	37	2

Table 2: Stuffer sequences for ET-ROX probes (5'-3').

Stuffer length	
1st type probe	2nd type probe
0	0
0	2 CA
4 TGCA	0
4TGCA	2CA

(continued)

Stuffer length	
1st type probe	2nd type probe
8 ACGT TACG	0
8 ACGT TACG	2CA
12 TAGC GTCA GCAT	0
12 TAGC GTCA GCAT	2 CA
16 CATG GCAT ACGT TACG	0
16 CATG GCAT ACGT TACG	2 CA
20 GATC GCTA ACGT TACG GCAT	0
20 GATC GCTA ACGT TACG GCAT	2 CA
24 TCGA GATC ACGT CATG CTGA GCAT	0
24 TCGA GATC ACGT CATG CTGA GCAT	2 CA
28 CAGT TCAG GCAT TCGA CTAG CGTA TACG	0
28 CAGT TCAG GCAT TCGA CTAG CGTA TACG	2 CA
32 GTCA ATCG GACT CTGA GACT CATG CGAT GACT	0
32 GTCA ATCG GACT CTGA GACT CATG CGAT GACT	2 CA
36 GATC CGAT CGAT ATCG ACGT AGCT GCAT CGTA ATCG	0
36 GATC CGAT CGAT ATCG ACGT AGCT GCAT CGTA ATCG	2 CA

Table 3: Stuffer sequences for JOE probes (5'-3').

Stuffer length	
First type probe	2nd type probe
0	0
0	2 TG
4 ACTG	0
4 ACTG	2 TG
8 GCAT CAGT	0
8 GCAT CAGT	2 TG
12 ATCG GCAT TACG	0
12 ATCG GCAT TACG	2 TG
16 TACG GCAT AGTC ACGT	0
16 TACG GCAT AGTC ACGT	2 TG
20 GATC GCTA ACGT TACG GCAT	0
20 GATC GCTA ACGT TACG GCAT	2 TG
24 CTAG ATGC TCAG GCTA TCGA CATG	0
24 CTAG ATGC TCAG GCTA TCGA CATG	2 TG
28 GTAC CGAT ACGT TAGC GACT TAGC CGTA	0
28 GTAC CGAT ACGT TAGC GACT TAGC CGTA	2 TG
32 CGTA ATCG GATC CGTA ACGT GCAT ATGC CAGT	0

(continued)

Stuffer length	
First type probe	2nd type probe
32 CGTA ATCG GATC CGTA ACGT GCAT ATGC CAGT	2 TG
36 GACT TCGA GATC TGCA ACGT ACGT CGTA AGCT GCTA	0
36 GACT TCGA GATC TGCA ACGT ACGT CGTA AGCT GCTA	2 TG

Table 4: Stuffer sequences for FAM probes (5'-3').

Stuffer length	
First type probe	2nd type probe
1C	0
1 C	2 GA
5 C GACT	0
5 C GACT	2 GA
9 C CGAT TAGC	0
9 C CGAT TAGC	2 GA
13 C ATCG GATC AGCT	0
13 C ATCG GATC AGCT	2 GA
17 C ATGC TAGC ACGT ACTG	0
17 C ATGC TAGC ACGT ACTG	2 GA
21 C GTAC CAGT CATG GATC CGAT	0
21 C GTAC CAGT CATG GATC CGAT	2 GA
25 C GATC ATCG ACTG GTAC TACG GACT	0
25 C GATC ATCG ACTG GTAC TACG GACT	2 GA
29 C GTAC GCAT GCTA ACGT TACG GACT ATCG	0
29 C GTAC GCAT GCTA ACGT TACG GACT ATCG	2 GA
33 C CGTA GCAT CGAT ATCG GTCA ACTG GATC AGCT	0
33 C CGTA GCAT CGAT ATCG GTCA ACTG GATC AGCT	2 GA
37 C GTAC CATG TCGA CGTA GATC CGTA TAGC ACTG AGTC	0
37 C GTAC CATG TCGA CGTA GATC CGTA TAGC ACTG AGTC	2 GA

Table 5: Stuffer sequences for NED probes (5'-3').

Stuffer length	
First type probe	2nd type probe
1C	0
1 C	2 TC

(continued)

Stuffer length	
First type probe	2nd type probe
5 C GTAC	0
5 C GTAC	2 TC
9 C GCAT TCGA	0
9 C GCAT TCGA	2 TC
13 C ATCG GCAT GACT	0
13 C ATCG GCAT GACT	2 TC
17 C GTCA ATGC ACGT TACG	0
17 C GTCA ATGC ACGT TACG	2 TC
21 C GCAT CGAT AGCT CTGA ACGT	0
21 C GCAT CGAT AGCT CTGA ACGT	2 TC
25 C GCAT ATCG GATC GATC GCAT ACGT	0
25 C GCAT ATCG GATC GATC GCTA ACGT	2 TC
29 C ATCG GATC CATG CGTA GCAT ATCG ACGT	0
29 C ATCG GATC CATG CGTA GCAT ATCG ACGT	2 TC
33 C TGCA AGTC CGAT TACG ATCG ACGT GCTA TGCA	0
33 C TGCA AGTC CGAT TACG ATCG ACGT GCTA TGCA	2 TC
37 C AGCT CAGT ATCG AGTC GACT ACGT TGCA TACG GATC	0
37 C AGCT CAGT ATCG AGTC GACT ACGT TGCA TACG GATC	2 TC

II. EXAMPLES MULTIPLEX LIGATION ASSAY AND DETECTION

Example 1. Description of biological materials and DNA isolation.

[0118] Recombinant Inbred (RI) lines generated from a cross between the *Arabidopsis* ecotypes Colombia and Landsberg *erecta* (Lister and Dean, Plant Journal, 4, pp 745-750, (1993) were used. Seeds from the parental and RI lines were obtained from the Nottingham Arabidopsis Stock Centre.

[0119] DNA was isolated from leaf material of individual seedlings using methods known *per se,* for instance essentially as described in EP-0534858, and stored in IX TE (10 mM Tris-HCl pH 8.0 containing 1 mM EDTA) solution. Concentrations were determined by UV measurements in a spectrophotometer (MERK) using standard procedures, and adjusted to 100 ng / μl using 1X TE.

Example 2. Selection of Arabidopsis SNP's.

[0120] The Arabidopsis SNP's that were selected from *The Arabidopsis Information Resource* (TAIR) website: http://www.arabidopsis.org/SNPs.html:, are summarised in Table 6 in

Table 6. Selected SNPs from *Arabidopsis thaliana.*

	SNP	SNP alleles*	RI Map position
1	SGCSNP1	G/A	chr. 2; 72,81
2	SGCSNP20	A/C	chr. 4; 15,69
3	SGCSNP27	T/G	chr. 3; 74,81
4	SGCSNP37	C/G	chr 2; 72,45

(continued)

	SNP	SNP alleles*	RI Map position
5	SGCSNP39	T/C	chr.5;39,64
6	SGCSNP44	A/T	not mapped
7	SGCSNP55	C/A	chr. 5; 27,68
8	SGCSNP69	G/A	chr. 1; 81,84
9	SGCSNP119	A/T	chr. 4; 62,06
10	SGCSNP164	T/C	chr. 5; 83,73
11	SGCSNP209	C/G	chr. 1; 70,31
12	SGCSNP312	G/T	chr. 4; 55,95
* For all SNP's the allele preceding the backslash is the Colombia allele.			

Example 3. Oligonucleotide probe design for oligonucleotide ligation reaction

[0121] The oligonucleotide probes (5'-3' orientation) were selected to discriminate the SNP alleles for each of the twelve SNP loci described in Example 2. PCR binding regions are underlined, stuffer sequences are double underlined. Reverse primers are phosphorylated at the 5' end:. p indicates phosphorylated. The sequences are summarised in Table 7.

TABLE 7 Oligonucleotide probes for detection of Colombia and Landsberg SNPs

SEQ.ID	Code	Nucleotide sequence
	SCGSNP1	
1	SNPfwd001 (G allele)	CGCCAGGGTTTTCCCAGTCACGACTTCAGGACTAGTCTATACCTTGAG
2	SNPfwd002 (A allele)	CGCCAGGGTTTTCCCAGTCACGACGACTTCAGGACTAGTCTATACCTTGAA
3	SNPrev001 (Common reverse SNP001)	pCTATGTGAACCAAATTAAAGTTTACTCCTGTGTGAAATTGTTATCCGCT
	SGCSNP20	
4	SNPfwd003 (A-allele)	CGCCAGGGTTTTCCCAGTCACGACCTGCTCTTTCCTCGCTAGCTTCAGA
5	SNPfwd004 (C-allele)	CGCCAGGGTTTTCCCAGTCACGACGACTGCTCTTTCCTCGCTAGCTTCAGC
6	SNPrev002 (common reverse SNP20):	pAGATTCGGACCTTCTCTCATAATCCGACTTCCTGTGTGAAATTGTTATCCGCT
	SGCSNP27	
7	SNPfwd005 (T-allele)	CGCCAGGGTTTTCCCAGTCACGACGAAGAGGAGAGTGGCTACGAACTCT
8	SNPfwd006 (G-allele)	CGCCAGGGTTTTCCCAGTCACGACGAGAAGAGGAGAGTGGCTACGAACTCG
9	SNPrev003 (common reverse SNP27)	pGCGATAACTGCTCTGTAGAAAGACCCGATTAGCTCCTGTGTGAAATTGTTATCCGC T
	SGCSNP37	
10	SNPfwd007 (C-allele)	CGCCAGGGTTTTCCCAGTCACGACAATCGGCCTAAGCAAGCTTGTTTTC
11	SNPfwd008 (G-allele)	CGCCAGGGTTTTCCCAGTCACGACGAAATCGGCCTAAGCAAGCTTGTTTTG
12	SNPrev004 (common reverse SNP37)	PTGCTATTGATATCTCTGTGCAACTCATCGGATCAGCTTCCTGTGTGAAATTGTTATC CGCT
	SGCSNP39	
13	SNPfwd009 (T-allele	CGCCAGGGTTTTCCCAGTCACGACGACCGGAAAGATATCGGAGCTCCTT
14	SNPfwd010 (C-allele) .	CGCCAGGGTTTTCCCAGTCACGACGAGATCGGAAAGATATCGGAGCTCCTG

5 10 15 20 25 30 35 40 45 50 55

(continued)

TABLE 7 Oligonucleotide probes for detection of Colombia and Landsberg SNPs		
SEQ.ID	Code	Nucleotide sequence
15	SNPrev005 (common reverse SNP39)	pGTCGGTGTCAACCGATCCACGGCGCATGCTAGCACGTACTGTCCTGTGTGAAATTG TTATCCGCT
	SGCSNP44	
16	SNPfwd011 (A-allele)	CGCCAGGGTTTTCCCAGTCACGACGAACTGGCATCAATCAGGCCTCCAA
17	SNPfwd012 (T-allele)	CGCCAGGGTTTTCCCAGTCACGACGAGAACTGGCATCAATCAGGCCTCCAT
18	SNPrev006 (common reverse SNP44)	pCCTTAATGCAAGGGCTTATTACGTCGTACCAGTCATGGATCCGATTCCTGTGTGAA ATTGTTATCCGCT
	SGCSNP55:	
19	SNPfwd013 (C-allele)	CGCCAGGGTTTTCCCAGTCACGACGGACTCCAAGGTATTGTTAGGCGCC
20	SNPfwd014 (A-allele)	CGCCAGGGTTTTCCCAGTCACGACGAGGACTCCAAGGTATTGTTAGGCGCA
21	SNPrev007 (common reverse SNP55)	pAACCACCAAGATCAGTCTCATCTTCGATCATCGACTGGTACTACGGACTTCCTGTG TGAAATTGTTATCCGCT
	SGCSNP69	
22	SNPfwd015 (G-allele)	CGCCAGGGTTTTCCCAGTCACGACCATCTCTTGCGCCTTCTCAGTGTTG
23	SNPfwd016 (A-allele)	CGCCAGGGTTTTCCCAGTCACGACGACATCTCTTGCGCCTTCTCAGTGTTA
24	SNPrev008 (common reverse SNP69)	pTGACGTCCGTCGAAGAATAGGTAACGTACGCATGCTAACGTTACGGACTATCGTC CTGTGTGAAATTGTTATCCGCT
	SGCSNP 119	
25.	SNPfwd017 (A-allele).	CGCCAGGGTTTTCCCAGTCACGTACAGTTTCAAAACCCATGACGCTTCTA
26	SNPfwd018 (T-allele)	CGCCAGGGTTTTCCCAGTCACGACGAAGTTTCAAAACCCATGACGCTTCTT

5 10 15 20 25 30 35 40 45 50 55

(continued)

TABLE 7 Oligonucleotide probes for detection of Colombia and Landsberg SNPs

SEQ.ID	Code	Nucleotide sequence
27	SNPrev009 (common reverse SNP119)	pGTGATAGCTGAAAAGACCCATTCTCCGTAGCATCGATATCGGTCAACTGGATCAG CTTCCTGTGTGAAATTGTTATCCGCT
	SGCSNP164	
28	SNPfwd019 (T-allele)	CGCCAGGGTTTTCCCAGTCACGACATACTCCAATTGCTCAGGCACAGTT
29	SNPfwd020 (C-allele)	CGCCAGGGTTTTCCCAGTCACGACGAATACTCCAATTGCTCAGGCACAGTC
30	SNPrev010 (common reverse SNP164)	pCTCCTTGTCCCACGAAGATAGTTCCGTACCATGTCGACGTAGATCCGTATAGCACT GAGTCTCCTGTGTGAAATTGTTATCCGCT
	SGCSNP209	
31	SNPfwd021 (C-allele)	CGCCAGGGTTTTCCCAGTCACGACGTAGAGGCTCTAAACAGCTGCTTCC
32	SNPfwd022 (G-allele).	CGCCAGGGTTTTCCCAGTCACGACGAGTAGAGGCTCTAAACAGCTGCTTCG
33	SNPrev011 (common reverse SNP209)	pCTTGTTTATGCTAAGGGCCGGCTCCTCCTGTGTGAAATTGTTATCCGCT
	SGCSNP312	
34	SNPfwd023 (G-allele)	CGCCAGGGTTTTCCCAGTCACGACTAAGTCAGCTCCTAAGCTTCCATCG
35	SNPfwd024 (T-allele)	CGCCAGGGTTTTCCCAGTCACGACGATAAGTCAGCTCCTAAGCTTCCATCT
36	SNPrev012 (common reverse SNP312)	pAAGCCACTTCCTCCTGCTCAAGCGCGACTTCCTGTGTGAAATTGTTATCCGCT
All oligonucleotides were purchased from MWG, Ebersberg, Germany. The concentration of the oligonucleotides was adjusted to 1μM		

Example 4. Design of the PCR amplification primers

[0122] The sequences of the primer used for PCR amplification were complementary to the PCR primer binding regions incorporated in the ligation probes described in Example 3. The sequences represent the so called M1 3 forward and M13 reverse primers. Usually the forward primer is labelled with FAM or □33P-dATP depending on the detection platform. The sequence of the primers in 5'-3' orientation are:

M13 forward: CGCCAGGGTTTTCCCAGTCACGAC [SEQ ID No. 37]
M13 reverse: AGCGGATAACAATTTCACACAGGA [SEQ ID No.38]

[0123] The concentration of these oligo's was adjusted to 50 ng / μl.

Example 5. Buffers and Reagents

[0124] The composition of the buffers was: Hybridisation buffer (1X), 20 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$,100 mM KCl, 10 mM DTT, 1 mM NAD^+ Ligation buffer (1X) 20 mM Tris-HCl pH 7.6, 25 mM Kac, 10 mM $MgAc_2$,10 mM DTT, 1 mM NAD^+, 0.1 % Triton-X100.PCR buffer (10X):10x PCR buffer (contains 15 mM $MgCl_2$). (Qiagen, Valencia, United States of America).No additions were used in the PCR

Example 6. Ligation and Amplification

Ligation reactions:

[0125] Ligation reactions were carried out as follows: 100 ng genomic DNA (1 μl of 100 ng / μl) in 5 μl total volume was heat denatured by incubation for 5 minutes at 94 ˚C and cooled on ice. Next 4 fmol of each OLA forward and reverse probes described in Example 3 (36 oligonucleotides in total) were added, and the mixture was incubated for 16 hours at 60 ˚C. Next, 1 unit of Taq Ligase (NEB) was added and the mixture was incubated for 15 minutes at 60 ˚C.

[0126] Next, the ligase was heat-inactivated by incubation for 5x minutes at 94 ˚C and stored at -20˚C until further use.

PCR amplification:

[0127] PCR reactions mixture contained 10 μl ligation mixture, 1 μl of 50 ng/μl (FAM or ^{33}P) labelled M13 forward and reverse primer (as described in Example 4), 200 μM of each dNTP, 2.5 Units HotStarTaq Polymerase Qiagen, 5 μl 10X PCR buffer in a total volume of 50 μl.

[0128] Amplifications were carried out by thermal cycling in a Perkin Elmer 9700 thermo cycler (Perkin Elmer Cetus, Foster City, United States of America), according to the following thermal cycling profile:

Profile 1: Initial denaturation/enzyme activation 15 min at 94 ˚C, followed by 35 cycles of: 30 sec at 94 ˚C, 30 sec at 55 ˚C, 1 min at 72 ˚C, and a final extension of 2 min at 72 ˚C, 4 ˚C, forever.
Profile 2: Initial denaturation/enzyme activation 15 min at 94 ˚C, followed by 35 cycles of: 5 se

[0129] In case a ^{33}P end-labelled M13 forward PCR primers was used, the labelling was carried out by kination as described in Vos et al., 1995 (Nucleic Acids Research, vol. 23: no. 21, pp. 4407-4414, 1995 and patent EP0534858).

Example 7. Radioactive detection of 12-plex SNPWave products

[0130] Figure 10 shows an electrophoretic gel from a multiplex oligonucleotide ligation assay of the 12 Arabidopsis SNPs listed in Example 2. Following the procedures described here-in before, using DNA of the Colombia ecotype (C), Landsberg erecta ecotype (L) or a mixture of equal amount of both ecotype (C+L) as the starting material.

[0131] Figure 10 shows that the appropriate alleles of SNP's SNP SGCSNP164, SGCSNP119, SGCSNP69, SGCSNP29, SGCSNP27 and SGCSNP1 are clearly observed in the Colombia sample, , and the appropriate SNP alleles of SNP loci SGCSNP164, SGCSNP119, SGCSNP69, SGCSNP29, SGCSNP27 and SGCSNP1 are clearly observed in the Landsberg sample and that all these SNP alleles together are observed in the mixture of both samples.

[0132] This Example illustrates that at least six SNP's can be simultaneously ligated and amplified using the multiplex ligation / amplification procedure. This example further illustrates that at least 12 SNPs can be detected in one sample. The results are represented in Table 8

Table 8

	SNP Name	Length	Allele	Result
Lan	SGCSNP164	136	C	Yes
Col	SGCSNP164	134	T	Yes
Lan	SGCSNP119	132	T	Yes
Col	SGCSNP119	130	A	Yes
Lan	SGCSNP69	128	A	Yes
Col	SGCSNP69	126	G	Yes
Lan	SGCSNP55	124	A	No
Col	SGCSNP55	122	C	yes
Lan	SGCSNP44	120	T	No
Col	SGCSNP44	118	A	No
Lan	SGCSNP39	116	C	No
Col	SGCSNP39	114	T	yes
Lan	SGCSNP37	112	G	Yes
Col	SGCSNP37	110	C	No
Lan	SGCSNP27	108	G	Yes
Col	SGCSNP27	106	T	Yes
Lan	SGCSNP20	104	C	Ns*
Col	SGCSNP20	102	A	Ns
Lan	SGCSNP312	104	T	Ns
Col	SGCSNP312	102	G	Ns
Lan	SGCSNP209	100	G	Yes
Col	SGCSNP209	98	C	Yes
Lan	SGCSNP1	100	A	Yes
Col	SGCSNP1	97	G	Yes
*; not scored; Col: Colombia allele, Lan: Landsberg allele				

Example 8. Gel electrophoresis

[0133] Gel electrophoresis was performed as described in Vos et al., Nucleic Acids research 23(21),(1995), 4407-4414. After exposure of the dried gel to phospho-imaging screens (Fuji Photo Film Co., LTD, Type BAS III) for 16 hours, an image was obtained by scanning using the Fuji scanner (Fuji Photo Film Co., LTD, Fujix BAS 2000) and stored in digital form.

Example 9. Oligonucleotide sizers for capillary electrophoresis

sizer 94 bp:

[0134]

5'fam-
ACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTC
GACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCG [SEQ ID No. 39]

sizer 95 bp:

[0135]

5'fam-
ACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTC
GACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGG [SEQ ID No.40]

sizer 137 bp:

[0136]

5'fam-
ACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTC
GACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGC
GGGGCATGACTATCGTCGCCGCACTTATGACTGTC [SEQ ID No.41]

Example 10. Purification and dilution of amplified connected probes

[0137] In case of detection using the MegaBACE 1000 capillary sequencing instrument, desalting and purification of the PCR reactions mixtures was carried in 96-well format, using the following procedure:

A. Preparation of the 96-well Sephadex purification plates

[0138] Dry Sephadex™ G-50 superfine (Amersham Pharmacia Biotech, Uppsala, Sweden) was loaded into the wells of a 96-well plate (MultiScreen®-HV, Millipore Corporation, Bedford, MA, USA), using the 45 microliter column loader (Millipore Corporation) as follows:

1. Sephadex G-50 superfine was added to the column loader.
2. Excess Sephadex was removed from the top of the column loader with a scraper.
3. The Multiscreen-HV plate was placed upside-down on top of the Column Loader.
4. The Multiscreen-HV plate and the Column Loader were both inverted.
5. The Sephadex G-50 was released by tapping on top or at the side of the Column Loader.

[0139] Next, the Sephadex G-50 was swollen en rinsed as follows:

6. 200 μl Milli-Q water was added per well using a multi-channel pipettor.
7. A centrifuge alignment frame was placed on top of a standard 96-well microplate, the Multiscreen-HV plate was place on top and the minicolumns were packed by centrifugation for 5 min at 900 g.
8. The 96-well plate was emptied and placed back.

9. Steps 5-7 were repeated once.
10. 200 μl Milli-Q water was added to each well to swell the Sephadex G-50 and incubated for 2-3 hours. Occasionaly, at this stage the Multiscreen-HV plates with swollen mini-columns of Sephadex G-50 superfine were tightly sealed with parafilm and stored a refrigerator at 4 °C until further use.
11. A centrifuge alignment frame was placed on top of a standard 96-well microplate, the Multiscreen-HV plate was placed on top of the assembly and the minicolumns were packed by centrifugation for 5 min at 900 g.
12. The 96-well microplate was removed.
13. The mixtures containing the amplified connected probes were carefully added to the centre of each well.
14. Using the centrifuge alignment frame, the Multiscreen-HV plate was placed on top of a new standard U-bottom microtitre plate and centrifugation was carried out for 5 min at 900 g.
15. The eluate in the standard 96-well plate (approximately 25 μl per well) contains the purified product.

B. Dilution of the purified products

[0140] Purified samples were diluted 25-75 fold in Milli-Q water before injection.

Example 11. Capillary electrophoresis on the MegaBACE

Preparation of the samples:

[0141] A 800-fold dilution of ET-900 Rox size standard (Amersham Pharmacia Biotech) was made in water. 8 μl diluted ET-900 Rox was added to 2 μl purified sample. Prior to running, the sample containing the sizing standard was heat denatured by incubation for 1 min at 94 °C and subsequently put on ice.

Detection on the MegaBACE:

[0142] MegaBACE capillaries were filled with 1X LPA matrix (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Parameters for electrokinetic injection of the samples were as follows: 45 sec at 3 kV. The run parameters were 110 min at 10 kV. Post-running, the cross-talk correction, smoothing of the peaks and cross-talk correction was carried out using Genetic Profiler software, version 1.0 build 20001017 (Molecular Dynamics, Sunnyvale, CA, USA), and electropherograms generated.

Example 12. Repeated injection on the MegaBACE.

[0143] The minimum time interval for adequate separation between two consecutively injected samples was determined by injecting the sizer sample as described in Example 8. The resulting time interval was used, with a small additional margin, when injecting the purified amplified connected probes from the oligonucleotide assay. The results are presented in Fig 11.

A. Partial electropherogram of FAM labelled detection of the Colombia sample on a capillary electrophoretic device (MegaBACE). The same multiplex mixture was injected twice. Amplified connected probes (size range 97-134 bp) and flanking sizer fragments (94, 95 and 137 bp) are all labelled with FAM
B. Partial electropherogram of FAM labelled detection of the Landsberg erecta sample on a capillary electrophoretic device (MegaBACE). The same multiplex mixture was injected twice. Amplified connected probes (size range 97-134 bp) and flanking sizer fragments (94, 95 and 137 bp) are all labelled with FAM.

Example 13. Cross-talk reduction using stuffer sequences of different lengths

[0144] In this experiment the use of different length-label combinations to avoid the negative influence of incomplete cross-talk removal on the quality of a dominantly scored (presence /absence) dataset of SNP markers is demonstrated. Stuffer lengths were chosen such that ET-ROX and JOE-labelled fragments have identical sizes, and that FAM and NED fragments have identical sizes, but differing by 1 basepair from those of ET-ROX and JOE-labelled fragments. The result is that even in case of incomplete cross-talk removal between dyes with overlapping emission spectra, the observed signal will not result in incorrect scoring because the expected sizes of the amplification products are known for every label. Hence length-label combinations define the expectance patterns for genuine signals are signals originating from incomplete cross-talk correction. The results are presented in Fig 12 and 13.
[0145] The example shows in Figure 13:

A). The effect of incomplete cross talk removal on the data quality in case of a sample that contains a ET-ROX labelled fragment of 120 basepair and a NED labelled fragment of 124 basepairs in a situation where fragments of a particular size can be observed in combination with all labels. In this case, incomplete cross-talk of ET-ROX signal into the FAM Channel at 120 bp removal leads to the incorrect scoring of a FAM fragment of 120 basepairs (in reality an ET-ROX labelled fragment of 120 basepairs).Similarly, incomplete cross-talk correction removal of NED signal into JOE at 124 bp leads to incorrect scoring of a JOE fragment of 124 basepairs (in reality a NED labelled fragment of 124 basepairs), in addition to the correct fragments.

B). The effect of the use of cross-talk-optimised length-label combinations such that ET-ROX- and FAM-labelled fragments of the same length are not avoided by choosing different stuffer lengths, because their emission spectra overlap. Similarly, same-size amplified connected probe fragments labelled with JOE and NED are avoided. In case of a hypothetical sample containing a 120 bp ET-ROX -labelled fragment and a 124 bp NED labelled fragment (identical to the that described above in A), the small but detectable signals (peaks) of FAM at 120 bp and of JOE at 124 bp that remain after incomplete (mathematical) cross-talk correction will not be scored because they are known to originate from cross talk of ET-ROX and NED signals, respectively. Hence, they have no impact on the data quality and both fragments are scored correctly.

Example 14. Identification of SNPs

[0146] The selected SNPs are identified and summarized in Table 9.

Table 9. Selected SNP sequences and position of the SNP.

SEQ ID #					W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C; H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T
Set 1	Fragment code	Locus nr	Length	SNP position.	SEQUENCE
75	9651- f06	1	735	174	CCKCGGAGAAWTGAAGAAGTATGCTGTGTCATCCGGTGTTGGCTCACACTCAGGTACTGTTAGACCCATCT CATGCTTAACAATKKGATTCTTTGAGCGTTACCTAKTGAACTAGTATATTTTKGGTGTGCTCACTTACTGC CTCAAGTTATGTGATGGTTTCTAATTKTGACTTTAATTATAAATCATGCACATCTTATATAAATCAGATTT CCAAAGCTGCTGTATATTGGTTCAGTAGATAATATGGTTTTATCTCTTAACTGGTTATATCTGCAGTCATT TTTTGGTTATACCTCTTTCATAGTCCTGATTAAAGGATTTTGAGTTATTTTCAATGTCTCTTTGTAAACAA AGATTATACTAGAATCAATCTAATGTTTTCTTTCCTTTAAATAAATTACAGATAAGGAAGATGAAGGGTTT GAAACAGAAGAAAGCCCATTTGATGGAGATCCAGGTTAATGGAGGATCAATTGCTCAGAAGGTTGACTTCG CATATGGTTTCTTTGAGAAGCAGGTTCCAGTTGATGCTGTTTTCCAGAAGGATGAGATGATTGACATCATT GGTGTCACCAAGGGTAAGGGTTATGAAGGTGTYGTAACTCGTTGGGGTGTGACACGTCTTCTCGCAAAACC CACGGGGTCTACGTAAGGTGCTGTTGGGGC
76	9372- d11	2	561	475	GCYTGGGGACTAGTTCTTTTCAGAATCATATCATCTGTAGAGAAATCAGCTGCTTTCCTGAATGTTCCTCG TCCGAAATCTAGGTTGTAAGAGTCTGTAAGACCTTCCACCAGATCAAAATCAGGTTTCCATCCTAGCTCAG CCTTTGACTTCTCGATGGATGTAAAGAAATGCTGTAGAAATTCGATGTTAAAACCAACGAGAAGACATAGA TAGACTAGTGTTGGACAAGAATCCGATATTAAACAGACAAGCTAACAACTTCAACAGAGGAAATAAACCAT ATTTCTTGTAGTATTTCGTTTGGACTACGATTGATTGTACAAAAATGTGTGTTAATTTTAGTGAGCATACT GATGTGTGTTTTAGGAAGGGACTAGGATAAGAGGCGGTGAACATGTTGTGAATCTTCACTGATGATTCATT TAGTTTGATCATATCATTTGATTCTTTGATAAAGAATGTCTCGAATTTCAATATGAATGGTAAACAACTGA AATCAACACACTAATATTTACCTGGTCACGGAATGGGAATGCTTTCTTCTTGCCAAAAT

(continued)

SEQ ID #					W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C; H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T
Set 1	Fragment code	Locus nr	Length	SNP position.	SEQUENCE
77	9371-d06 6	6	827	164	AGNAHKYYCVAGGCTCACDASCAGGTTGGAAAAATCATTTTGATACARAARTTGCATTTTCTGGTTATTCA GGTGATTTCCCTTCTATATGTCAAACTTATTGAAACGAGTCTTCTGAAAAATAAATGGAAAGTTATATGGA AAAASATTTCCAGGATATTGCTTAGTTTCTCATAAGTATAAAGCTTTATATGTGAACCAATTCAACAGGTA CATATATCAGAGGCCCGGGTTTCTGCTGCTTTAGATAAGCTAGCTTACATGGAAGAATTGGTTAACGATAG GCTTCTGCAAGAGAGAAGCACAGTAGAATCAGAATGCACGTCTTCCTCTGCAAGCACGTCAACAGGATTAT TAGACACTCCAAAAAGCAAGCAACCACGAAGAACGCTGAATGTCTCAGGTCCTGTCCAAGATTACAGTTCC CGTTTGAAGAACTTTTGGTACCCTGTTGCATTCTCCGCAGATCTTAAGAATGACACCATGGTGAGTCAATT ATCSTCATATCTGCCAGTCTCTTTAACCTAAAAGAAAGAAAACATTTGATCTAAAACACAGAAAACCATGT AGATGCAAAATTATGATGCCAAAACAAATTAACAAGCTATATGATCTACGCTCCTACTTTATGGTCTTCCA TGTATATTCTTKGGGATCTTCTAATTGATGACTGTTAACTGTATCTTTGTAGTTACCGATTGATTGCTTGC AGACACACCGGGGGTRA
78	9651-b06 5	3	363	187	AGGGAGAHTAGAMCCAGAAGTGTCACCAAGAACCTATCTTCAAGAACTACAGCTTGCCTCCTAATAAATGT GGATACCCTGGTGGTATTTTCAACCCACTCAACTTTGCACCAACTGAAGAGGCCAAGGAGAAGGAACTTGC TAATGGTAAGTGGATGTTCACTTTCTCTAAATGAYTTTATATACCTGAACCAGGCTAATTATTTTAGGTGG ATAATTTGCAGGGAGATTGGCTATGTTGGCATTTTTGGGATTTATAGTGCAGCACAATGTGACTGGGAAGG GACCTTTTGACAACCTTCTGCAGCACCTCTCTGACCCATGGCACAACACCATCATCCAAACACTCA
79	9651- d02	5	247	125	GACTMCTGGCTKTAATGTTGCATTGGTAGCCAAGTGACACCCCTGTTGCTCATTGCTTGAAGGTTTGGCTG ATTTGGAAGTTGCAGCTTGTCTTTGCACTGCCATTAAGGCTAATGTACTTGGGATTGTCAAATTAGATATT CCTGTTGCTCTTAGTGCTTTGGTTAGTGCTTGTGCTAAGAAAGTTCCCACAGGTTTCAAGTGTGGTTAATT AGAGTATTAATTAGCCAAGGGTGGGGA

5 10 15 20 25 30 35 40 45 50 55

(continued)

SEQ ID #					W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C; H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T
Set 1	Fragment code	Locus nr	Length	SNP position.	SEQUENCE
80	9861- c03	4	390	90	MAAAKCTAAAYYAAGGCTTKATTTKGACCAACCCTKGTAATCCATTAGGTACCATTTTAGATAGGGACACA CTTAAAAAAATCTCCACYTTCACTAACGAACATAATATCCATCTTGTTTGCGACGAAATATATGCTGCTAC CGTRTTCAATYCTCCAAAATTCGTTAGCATCGCTGAAATTATCAACGAAGATAATTGTATCAATAAAGATT TAGTACACATTGTGTCTAGTCTTTCCAAGGACTTAGGTTTTCCAGGATTTCGAGTGGGAATTGTGTACTCR TTCAACGATGATGTTGTTAACTGTGCTAGAAAAATGTCGAGTTTKGGGTCTTGTTTCGACTCAGACACAAC ATTTGCTAGCTTTCATGTTGTCTGACGATGAATT
81	9861- c03	7	390	285	MAAAKCTAAAYYAAGGCTTKATTTKGACCAACCCTKGTAATCCATTAGGTACCATTTTAGATAGGGACACA CTTAAAAAAATCTCCACYTTCACTAACGAACATAATATCCATCTTGTTTGCGACGAAATATATGCTGCTAC CGTRTTCAATYCTCCAAAATTCGTTAGCATCGCTGAAATTATCAACGAAGATAATTGTATCAATAAAGATT TAGTACACATTGTGTCTAGTCTTTCCAAGGACTTAGGTTTTCCAGGATTTCGAGTGGGAATTGTGTACTCR TTCAACGATGATGTTGTTAACTGTGCTAGAAAAATGTCGAGTTTKGGGTCTTGTTTCGACTCAGACACAAC ATTTGCTAGCTTTCATGTTGTCTGACGATGAATT
82	9703- a03	10	491	267	AGACGACMCCAMGCTAAAGGAGAAACACAAGAAGCATTTAAAAAGAACATTGAAGCAGCAACTAAGTTTCT TTTGCAAAAGATCAAGGACTTGCAATTGTATGTCCATTTTAAATTGTTTTATGACATTGTCTAAGCTATTT CTTACTGAAGTTGAATGTGTTTTGTTTTCCTTCTACTTCATACCTGGCACCTTTAATAGAAACTGATACTA TTTGTGTGTGTGCTGGCAGCTTTGTTGGTGAGAGCATGCATGATGATGGCGCCCTGGTGTTTGCGTACTAC AAGGAGGGTTCAGCTGATCCTACCTTTTTGTACATTGCACCTGGTTTGAAGGAGATCAAGTGCTAGATGTC TGGTGGAGTGCTTCTGCTAGAAGTTTTGCATTCGAGATTATGTTTCATGTAGTTTTTAATATTTGGTCTTT TTTGCTTATTTATGTCTGGTGTTTCTTCTAAACCTTGGGTACTTGCTGTGACCAGTACCGGAA

(continued)

SEQ ID #					W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C; H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T
Set 1	Fragment code	Locus nr	Length	SNP position.	SEQUENCE
83	9651- f04	9	842	182	TGGGGGYYCATTACACAAAACAAGAACTTCAGCCATTGTGTGTTGTTCAAACCAAACCCCGTGGTTTCTAA TTCAACAGAGGAAAGTTCTTCTTCATTAAAGGCATTCTCTGCAGCACTTGCGTTTGTCTTCTATTCTTTTG TCAGCACCAGTTCTTCCAGCTTCTGCTGACATCTCTGGCTTACACCTTGCAAGGACTCAAAACAGTTTGCT AAAAGGGAGAAGCAACAGATCAAGAAGCTTCAAAATTCTTTGAAACTTTATGCACCTGATAGCGCCCCTGC ACTTGCTATCAATGCCACTATTGAGAAAACTAAACGCAGGTTTGCCTTCAGTATCTTTCTTCACAATTTTC AAAAAGTTTTACTTCTTATTTGCCTATTTKKCCCTAGTTGATCATTTTTTTATTGTGTACTAGATAGAGAG TACTTATAGTTAAGATTTGCGGGATTCTAATCAATTTTGTTAGGGGTTTACAAATTAAAATACATAGTACA AATATAGGGTCTATGGAAAAGCTACTGAATTCGTTCGAACCCATGTTAGGAGTAGGAGTAGAAGAAGAGCT AAAAGTATTCTTKTACGAATGAAAGCATACTGTACATTAMCATTTGCTTATCAGAGAAAAGCAGATTGTTC AACTTTTCCTKGGCATATGCCGTTGAGATTAGACTAGGAAACTCCACATWGAACATACATATACCSKTTGA TACTCGAGTAAGTAAAAGTTTAATYCMTCAGACGTCCCNCACTA
84	9371- f08	8	257	179	ACACCGWGAGARGAAGATAGCTTTTACAATTCTTCGCCATGACAGGAATCTTCTTCTGAGTATGAGATCGC TTGGGCAAAAGTACCGCATAAACGACCTCGAGGAGGATAACGCCGCGCTCAAGGAAGAACAAGAAGGGCTC GTTCACCGAATGAACCATATCAAGCAAAGTCTACTTGCTGAAGCTGCTAGTGAGCCCACTGGTGCCTTTGC TTCCCGTCTTCGCCGCCTCTTTGGTGATGAAAGCTGAA

(continued)

Set 2	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
85	9861-e05	11	544	342	AAYGGSTGTGGAYCTGGCTGCAGTGCGGCAGTGGCTGTGGAGGGTAAGTTCTTCCTAAAATATTTATATGT TACATAAATATATATAACGACTTTCATTTAAAAAAAAATCATAGAATCGAGATGATCTAGTTTACAGTTTAAT TTATTCCTTTCACTAAATTTAATTATCTAAATTCTTGATTTTGTATAATTAATTGCAGATGTGGGATGTAC CCCGACTTGGAGAGCACCACTACCTTTACCATCATTGAGGGTGTTGCACCTATGAAGAAGTTAGTCTAATT TTAACATAAAAGACTTTTTCTACATTTGTTATATATGATCGGAATGATTACGAAGTAATTTTAGAATTCAT TAACAAAATTAAGAAGTTTCACTCTCGAAATTTGAATTATAACACATAAATTGAAACAGGTCACCTAAAAG ATAACTATAATGTTAGAATTAATAATATTGAAACACATAACACGTTCTATTAATATGAATTTTGTTTACCA TATTAAAGTGTATATATATATATAATTTACATGAATTAATTGCG
86	3348.2	12	596	126	TGGTAAGATGTGCTTATGAGGTCTGTCGATATTCCCTTCTGAAAAGATCTTCAATCCCACTTGAAATCATA CCCATTAAACAATCAGAGTTAAGAGAAAAAGGGTTATACTGGCGTGRAAGAGGGAAATTAGAAAGCACTGA GTTTTCATTTACTCGTTTTTTGACACCCCATTTGGCTAATTTTGAAGGATGGGCTATGTATTGTTGATTGT GATTTCTTGTATTTAGGGGATATTAAGGAATTGAGGGATATGGTGGATGATAAATATGCTTTAATGTGTGT ACAACATAATTATGCTCCTAAAGAAACTACTAAAATGGATGGGGCAGTACAAACTGTGTATCCTAGGAAGA ATTGGTCATCCATGGTTCTTCTATAATTGTGCGCATCCAAAGAATAAGGTCTTGACACCTGAKAKTTGTCA ATACTGAAACTGGGGCATTTTCTCCATAAGCTTTACTATGGTTGGAAKATGAGGAGATTGGGGAAGTTCCG TTCGTTKGGAACTTCCGTCGATCG

(continued)

Set 2	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
87	9572- f05	13	660	177	AARGGAGYAAGTGKGATYCTCGAATMCATTGACGAGACATTTGAAGGCCCTTCCATCTTACCTAAAGACCC TTATGATCGAGCTTTAGCTCGTTTCTGGGCTAAATTCTTCGAAGATAAGGTATATCGACTCCTTAACTTGT CTCTACTCTGTTAATTGAATATTCTAACTTAWAAATGATCAACTATACATCTCCAAAATTTATGTGGCATG TCATGAGGTGTCTACGAGACATGTTAAAGAGTTGGAGTGCTTAATTGTTAATTGAGACCAAATATTTAGAT ATGCACATTCAAAGTTAGAGTACTTATTATCGGATACAACCAAGTCAGAATGTCATTTTATATATATTATA TGTCTTGTGTAAAATTGGACTAAAGTAATAAAATATCACATTGCCAACAATAACTTATTTGTGACTGACTA ATGTACTTCTATTGTTGTAGATTTATATCTTTAAAATTTTGTTGAATTYAAGTTCCAATTGTTATGTAGTG GCCATCAATGATGAAAAGTCTATTTTTCAAAGGAGAGGAGCAAGAGAAAGGTACMGAGGAAGTTAATGAGA TGTTGAAAATTCTTGATAATGAGCTCAGGGACGRAMAGTTTTTTGTTGGTAACAACTTTGGATTGKTGATG TTGTGCAATGCTGTA
88	9682-a05	14	370	201	AAAGKGGCAGAATTAGAACCAGGAAGTGTCACCAAGACCTATCTTCAAGAACTACAGCTTGCCTCCTAATA AATGTGGATACCCTGGTGGTATTTTCAACCCACTCAACTTTGGCACCAAMCTGAAGAGGCCAAGGAGAAGG AACTTGCTAATGGTAAGTGGATGTTCACTTTCTCTAAATGAYTTTATATACCTGAACCAGGCTAATTATTT TAGGTGGATAATTTGCAGGGAGATTGGCTATGTTGGCATTTTTGGGATTTATAGTGCAGCACAATGTGACT GGGAAGGGACCTTTTGACAACCTTCTGCAGCACCTCTCTGACCCATGGCACAACACCATCATCCAAACACA A

(continued)

Set 2	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
89	9651-b05	15	879	387	GRAAGGAGGATCTGATGCTTCTGGCACAATGGGTTTAGTTTTSGCAAATTTTTGTATATCAAAAATTTACT AAATTTTTATACRCACTCTTTTCTTTTTAATCTGTTATAAAAATAATTACTTATACAATTTTATCAYTAAT CATGACATGCTCTTAATGTCACGTGTCATATTTAAGACCATGATTTTTATTAGATATACTTTTGATATATC GTAAAACTCTTTATATTGTCTAATTTCATGTTCATTCAAATATTCTACGAAATTAGAATTTGAAACTTTTG ATTTTTTTGTAGTTTTAGTCTTTTTGAGTCATCAGATTCTAAATTGATGGTATATATTAAATAAATTTGGT TGAGTCGAATATAAARTATTAGTCAAATTAGTGAATTCTGTCAAACTCGCTTCTTATCTTTTAGCTTTATC TATCCCTTCGTAAAATAATAGTGAAACATATATGAATTTTTTTAATAGTCTAAATTTTATTTTCACGAAA ATTTTTATGCTCAATCAAATACTGTTTTACGAAATAAGATAGAAGGATAGTTATAATGACATGAATTCTGA TTATTAACAATGATTGTCTGGAACAGGGCGGTGCTTGTGGCTATGGGAACTTGTACTCAAACAGGTTATGG TACAAACACTGCTGCATTAAGTACTGCCTTTGTTCAATGATGGAGCATCATGTGGTCATGTTCCCMTTTGT GTGATTMTCATCCGATCMAWKKGTSYMTRGGRACTCYTTACATTTMCGCCTATTTGYCCCCAWKYKHYCHC KGCMCCCCBSBSSBBMHHMAHHWMHAACAACAACAAAACAATA
90	9572-g11	16	400	361	AAAGCACAGAAACAGAGATTATGAACAACATACAACCCAATTAGCCAAAAGTTCTTAGTTCTGGTTGACAT GTCAAATAAGATCCTAGGGACATAATAAATTCCAGAACACTGGTCAAATCACATCAGAATCAAACCCCAAC TACAAATAATGGATAATAAAGAAGGGAAACACAATTAATGATGTAAATTGAGTTAGACCTAACAAGTTACA CCAATGCAATGCTGCTCTCACCACCTGGAGGCTTGCGAACCCCGCCATAGAAGTCTCGAGATTCTACTTTC CCATCTGCAAATATATTGCTTCCACTCATTTCTCGCAACTTTGCTGAACTCAGGTGCTTCTCAGCTGATGT TGGAGGATTATCGCCCTTGAATATGTCATTTCCTGTGGAA
91	9703- f12	17	310	145	TTTGAACCGTTTGTRCCACYGACTTACWTTTKKGAMAAGASMCMACCAAGAGTTGAGGCTTTCTTGCRGCC ATTGCCAGTAAGGTYCTCAAAGACTACTTCAGCATCAAAACCACCAAAGTTTCAAGTGAAGGCTTCGCTTA AGSAGAAAGCTTTGACAGGACTGACAGCAGCTGCACTCACTGCTTCCATGGTCATGCCTGATGTAGCCGAA GCAGCAGAGAGTGTTTCACCATCCCTAAAGAACTTTTTGCTCAGCATTTCTGCAGGTGGAGTTGTGCTTGC TGCAATTCTTGGCGCTATAATTGGTGTTTANNAN

(continued)

Set 2	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
92	9782-c03	18	610	161	CACGASAGAGGGTTGACAGTACGGATGATTTTTTTCAAAAACAGGATATTTTTTTCGATTCACTAAAGAAA ATAAAAGTGCTTTTAACCAAGTGGTTCCTGATTTTGGAGCCGTAACGAGAATGATATCATTATCTTGAGCT TGATATTGTCGTTGACATGCAATCACCCCTTGGATAAGTCTTGGTAATGCCCAAAAGCCTTGATAATTATA CACATAAGATCCAACCCATCCTCTTTCTTTTGGTAGGGTAGAAAGCAATTTCTTACAATCTTCACTTACAT CATCTTCTTGTAAATATTTRTGAGGAGTTGGTGAAGAGGTTTGAGAAAGGGCTCGCAACAGAAACCAGCCG CGATGCGGCGTCGGACCAGGGGCAAGAGCACCCCAGCGAACGCATCACAACGGCCCCCTCGCNCACAATAA CAACAGNACAACACTCACACGCGGCGWAGATCCCGCCATCCCAACAACGCCCACCAANAATACAACCCCCC CCAGACCACCTTCACTACCCCACTCCACSCTTCACGGCCAACCACACACAANCAATCGAAACCACCCGGTC CACAAACGCACAAACACAACGACACCA
93	9782-b11	19	340	245	CAGAGAAGAYTTTGCACATTCAGCTCCCKGGTGAGGKGCACAGTAGAAAGTGTAAGTTCCTTTCTCACTCA AAGTGACACTGTATGTCTCTCCTGCTGCATTCAGAAGATCCTCTTCAGAACATGGAAATCTTACTAGCATC CACACCAGCTGGGATTTCATCTTCATCAAATACGACGTTGTGTGGGAACCCTGCATTGTTCTTGAATGTAA TTTTCTCACCAGCACTAACGCTGAAGTTCCCAGGAATAAAAGCTAGACTCCCATCATCACCACCAAGCAAC ACTTCAAGTGCCATGGCATTGCTAGCAAGCATCGCGCTAACAGCGGTGGCASMAAAAA
94	9652- f0	20	443	370	GAGAATGWWCTAATCATCCCATTCCAATGGTTTATAACAACTGGCCATAAAATAAAAAACTAAAATATACG AAGGAGCATATTCCCAGAGAGTATGACATGCTCTGATCCAAGAACAAGATAAAGACATTCTAAAACTTACA ACCATCATCACTCAGAACGATTGGCATACCTCTCCACCTTTTCATCAAGATTGATTCCAACCATAGCCTCA CCAAGCCCACAGCTAATTTCAGCCAGCAATTGTGGGTCACTGTAATGAGTCACTGCTTGCACGATGGCACG TCCCCTCTTTGCAGGGTCACCACTCTTGAAGATACCAGAACCCACGAACACGCCGTCACATCCCAACTGCA TCATAAGCGCTGCATCTGCTGGTGTCGCCACCCCACCTGCTGCAAAGTGAACCACAGGGAGCCTACCAAGT TGCTTTGT

(continued)

Set 4	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
95	43F	31	472	246	TATCCACTCAGGTCTCCGCAAGCCAGAAATGGGATATACACCTTGTTACGACCYTCAAGCCATCCACTACT GCAATCTGTCATGTCACAGATGTTCGGAAGATAATGTATAAGTACAACTATATAGTCGGAWTTGCATCTAG TCTAGCATTCGGAAAATGGAAGCCATGCTACTTCTAGCATAAAAAACAGCAGCTAGAAATCGTAACTCCAA TGATACGAGGAAGTATTCAGAGTTTAGAGTGAWGTACAATGCAATTTAGAGAACAAGCATCTGCACATCRA AGTTACCTAGGTCCTCAGCGCCTGATGGACTTCCAACTTGTTCAAGAAGGCGATAAAGGTCTTTCTCATTG AATCCTTCAGGTGGAGAGTAGTTTTCACAAACTGCAAATGCCTCTGCACAGCGGAAAGATTGAATTAGATT TATGTTATATAGCCATTCTAGTCTTGCTTTAATGGATCTTTCTCGA
96	61F	32	222	175	CCACAGTTTCATGCTGCACCTACATGTGTAAGCAACTATCATAGCAAGTCTCGGAACAATTGGTAGGAAAA AATCMYKTAAGGATATGAAACATACTGTYCTTTCTTCATCTGAGTCTGYAGAGTTAATTTTTAACTCTTGG GATAAATGCAAAGAWTTAGACATGGAKGAGTYCTTAACACGTCCAGACAAGAGGCGTAACACAGGTACACC TTTTCTCGA
97	64F	33	133	121	TTGTGCTTGATGAATTGTAGGTCCAGTGCAGGTTTGCTTCTAAAACAGGGAGCACTTTGCAAGTGGTGAAA GTTCTATTAGCTGGGAAAGTGTAGTTTGAGCAGTTTTGAGCTGARTTAACAAGAAAAATCGA
98	75F	34	250	47	CCGCCACTGGGTAATTGAGTTTCATATTGATGGTTTTGTTTTTGTTRACGCTTCTTCCTTGTTGAGAGGGT TCAATGGAGAGATTCTATCTCGTCCTCCATTAGTTGAAGCTATTGCCTTTGATCCTATCCTTTCAAAGGYC AAGATGATTGCAGATAATTGGAATCCATTAACCAATGATTCTACGGAAAATTTATTCCCTCACTGGAGGAG ATGGGCAGAGATAAATATGAGATTTTGTGATGACAT
99	92R	35	284	84	TCGAGTAAGGCGGATGGATATGGAACAAGCCATTTCAAGGAGCAATTTCCCAGGATTTTCAGCTTTGCAAC AGCAGAAGTGTAYCTCTGCAGAGATAGATCATAACCTTTGGAAAGGTGTAGTAATTGTCAAAGGGAGGAAT GAGCCAGGAAACTGATAGACTATGTTGCGAAAATAAGCTATACTTCACTAAAAAAAGGCTAGACGTTTGAG AAATGAAGCAAGAACTAACACCTCTCACCAATTGCATCATTTTCTTAGTTCAGTTGATGTGATGAGCTTGT

(continued)

Set 4	Fragment code	Locus nr.	Length	SNP position	SEQUENCE
100	28R	36	320	31	TCGATATCCWCTCTTGTTTGTTGCAGGAGCWGAACTATAAATTGCTTGCAGGAACCTTGACATATGCTTTC TGTTGAGACTTGAATCACCAGCATGGATTTGAATGCCTTGCCACAGCCAGAGGATGACGAYGAGATTTTTG GACAACAATTAGAAGATGAACCACAAGAACCTATTTTACGTAGTGATGAGCSTGCAGATTATGTCACGAGT GCTGTAGAGATTTCACGTCGCGTATGTTTCTGCTTATACTGCTCGCTGTATCAACTATTGAACYGTACTAC TACTTGARCTTGCTCGTTTATTGGATATTTCTTTTT
101	14446E1 0	40	193	159	GAATTCACACTASGTTCGATGAAATTGAAACGTTCTCTTTCTGAAGAAKATACACAAGAAAAAATCTTATA GTCCTCAACAATATTCTTCTTCGTAACAGAAAACACGGAAGAAAATCTCTTCTGAAAATCCCTATAATCAC TGGCTGGAACTTCTCCSAACTCTCAATTTTTCAACCTTCTCTATGTTAA
102	14447C0 6	38	291	89	CTGCAGAADTACTGTTTGTTCAGGACTTACTAAATATCCTAAACAAAATTGATGATAGAGCCAATAATGTA TGCATGATTGGCGGTCCRTTCTTTTGTTATAGCAAGAGCTTGAAGCTAATTTTGTTTGTCATAATGGCCGC ACTAATTGTTTATTATCTCAGAATGAACAAAAAGAAGCAAGTCAGAAGCTTTSTACTCTATACTGAACAAC TTTGGAATTGGAACTATGTACTTATCTAGCCACGCCTCATAGATCTTTGTGGTTTAGGAGTGTTAA
103	14446E0 1	39	337	122	GAATTCACAATGAAAAAKGKDGTAAAAACACGAAATCAATCAAGCATGCAAGAGATAATGTTGTCCATCCA GTTGTTGTTGATGTTTCGGTATTGTATGTGTGTTGGGAGGAGTTATCTGGRCAGCAAGTCGAGGTTTGAAC GTCAAAAAGGTATGGGTTGTCTTCTCTCTTTGTCCCTTTTCGAAGAGACCCCTAAGGTTCAGACGAATCTA TTCCAAAAACTAGGGTTGTTCCTTGTTGCATCTCCTTKTCACAAGCTCCCATCGCATCATAAGTAGGGTAT GTTTGATGGTAGAATTTACGGATGTAATTTACTTTTGAAATGATTATGTTAA
104	14157A0 4	37	373	63	AGAGAGACGAGAGCTCGACTAGTGATAGTGTTATGTGCAACAGTTGAATAGAAAGATGYACACGAGCCTCG GATCAATGGCAGGGAAAGAGGCGTGGTGCTACGAACCATAAAGGCAAGGTTGAGCTTTCCTTTACAGAGTA CATCGCCTATTCCATACTCCGCTGATACTCTTTGATAAATCAAAATCTGTGGTGATCTCGTAGTTCTTGGG GATCCCAGCCAAAACCACCTTCGAGGTTCAACACAACATAGACAGTATGGCAGAATATCAAGACAATGACT GCTCGAAACTGCTGATGGCATTATGTGCAACCGTTGAATAGAGAGATGTACACGAGTCTCGGATCAATGGC AGGAAAAGAGAGTGCTTG

(continued)

Set 5	Fragment code	Locus nr.	Length	SNP position	
105	14446E0 1	41	337	252	GAATTCACAATGAAAAAKGKDGTAAAAACACGAAATCAATCAAGCATGCAAGAGATAATGTTGTCCATCCA GTTGTTGTTGATGTTTCGGTATTGTATGTGTGTTGGGAGGAGTTATCTGGRCAGCAAGTCGAGGTTTGAAC GTCAAAAAGGTATGGGTTGTCTTCTCTCTTTGTCCCTTTTCGAAGAGACCCCTAAGGTTCAGACGAATCTA TTCCAAAAACTAGGGTTGTTCCTTGTTGCATCTCCTTKTCACAAGCTCCCATCGCATCATAAGTAGGGTAT GTTTGATGGTAGAATTTACGGATGTAATTTACTTTTGAAATGATTATGTTAA
106	15091F1 2	42	264	215	CTGCAGAATTTGACTTACATTTTCCTAATGAATCTGATGATAAGGTGCTAGATGATCYTASTKTGTATCAG AAGCTAGTAGGAAGGTTGCTTTATCTGACAATAACAAGACCAGACATAGYTTTYGYAGTGYAGCTCTTGAG TCAGTTCATGCATAGTCCTAAAGCATCTTACATGSAAGCTGMAATGRRGGTGGTAAGATATGTCAAGCAGG CACCAGGACTGGGTATACTTATGGCAGCCAATACAACTGATCAGTTAA
107	15089D0 6	43	451	393	CTGCAGATGGTGGTGACATTACAGGAGGTGGTGCAACCAGCCCAAAAGGCGGGATCGTAATGTTATGATCA CAAGGTGGAGGCACAGGAAGACTGGTATTATTATGTTCAGATGGCAAAGTGGCACCTTCCAGGACTTGATC AATGCCATGGCATCTGATGGAAGCACTTTTAGTGCAGATATTTGGACTAACGACTCGAGCATHGTTGAGAA ACAAATGCATGTATCCAGAATTGTTGACTCTGAGGAAAAGGTCAGGTTTTGAAGTTGGTATCATGGATCCT GTTGGGAAGTGTTGGAGGTGGTCAAAAAGCAACGGTGATGGTAAGGAATGTCGTTGCAAGAAATCTACCAC GTTGTTTTCTTGTATTAGTGTTTGGGACAGTGTCTTRTCTCTTGGCATCAAGAAAGTGATGTTTCCTTTTA CAAGGTCATCAGGGGCCATGTTAA
108	14447D0 1	44	124	98	CTGCAGAASCAGTACATAGGTTGTATTGAMACCTGTATTTACAATAAGGAGACTCTARTGATACCGACCTA TCCCTATAATGAGTCTAAGACATCAAYGATAGAGAYGRTACCATTAGAGTTAA
109	257F	45	149	50	GACAAGTAATGGTTCTAAGTTGAGGGTGTTGATGTGCTAYGAAATATTGRGACATTTGATGTTTGATAAGT ATAAGTATGAACTAATACTAAATTAAGTGAAGTTTTTATGATTTGRTATTTTTGTTGAATGTGTAAGCAAA ATCTCGA

5 10 15 20 25 30 35 40 45 50 55

(continued)

Set 5	Fragment code	Locus nr.	Length	SNP position	
110	15090H0 6	46	267	90	CTGCAGAAAGTGATTCGGTTGGAGATGCAGTTACACGAAGCACTCTTACATCGGCTTCTGCTGGGGTAGAC AAATATGCTTCGACTAACTGTCCACATTCTGCTTCTTCATTTGATTATGTTGTCAGTACATTTGATGAGGG ACATCATCAGACAAAAGTCTTCAGCTCTTTGGATTGTCACAAGGAGTCAAAAATATCTAATACTAACAAGA AAAGGAGACGGTCTGGTGATAGTCATAAGCCCAGACCACGAGATAGGCAGTTAA
111	5091H0 2	47	211	168	CTGCAGAAGTCACACTGAASTCATACCAAAGACCATTTCAACTGCTAACATTAGACTAGAAGAGAACCTTC CATGACTGCCACAGCTTTCCCTCTCAGAMATACCCTCTGCTTCTCATCGTCTAGATGCAGTTTCACGACGC CACCTCTAGGTGAGGCCTGGACCAYAATACAATAAAATCAATAGGGCAAAAGAGAACTATGAGGTTAA
112	5091A1 0	48	165	113	CTGCAGAAAGATATAGCCAGAGGAAGGTGGAGCAATTTCATGTGGATAGGWTGCATAATGCATGTTCTTWC TTTATTTCGTATCTTGGTGAAGCATAGATATAGACAGATCAMAGAAGCACATYGGGATCTACCACCTACCA AGATGCTCTCATTTTACAGTTAA
113	4157A0 4	49	373	63	AGAGAGACGAGAGCTCGACTAGTGATAGTGTTATGTGCAACAGTTGAATAGAAAGATGYACACGAGCCTCG GATCAATGGCAGGGAAAGAGGCGTGGTGCTACGAACCATAAAGGCAAGGTTGAGCTTTCCTTTACAGAGTA CATCGCCTATTCCATACTCCGCTGATACTCTTTGATAAATCAAAATCTGTGGTGATCTCGTAGTTCTTGGG GATCCCAGCCAAAACCACCTTCGAGGTTCAACACAACATAGACAGTATGGCAGAATATCAAGACAATGACT GCTCGAAACTGCTGATGGCATTATGTGCAACCGTTGAATAGAGAGATGTACACGAGTCTCGGATCAATGGC AGGAAAAGAGAGTGCTTG
114	5091F0 9	50	312	47	CTGCAGAATGGATATTTCAATCTTTGCCATCAAATACTGGCTAGATCGTTGCAATCGCTCCTTGAATTGAA CAAACTCAATAACCTAAAAAAGTTCACAGATGAAGATTTTGTTACCATTGGGCTAGCTCATTGTATGATTA CTAATTTATCTTTTCGTTCACAAAKGGAACCATTAGTATTTGAAATGATCCTAAGAGAGAATCGTCATGAT AAGCAAYGTAAGTTTCTACACCAGAAAATAAATAATTGCTCCAACAAATACCCACTCAAGACTCACTTCGC AAGAACTAAGTTGTCCAGAAACAGTTAA

Example 15. Oligonucleotide probe design for oligonucleotide ligation reaction

[0147] The circular oligonucleotide probes (5'-3' orientation) were selected to discriminate the SNP alleles for each of the SNP loci described in Example 14. PCR binding regions are underlined, stuffer sequences are double underlined. Reverse primers are phosphorylated at the 5' end:. p indicates phosphorylated. The sequences are summarised in Table 10.

Table 10. Oligonucleotide probes for detection of SNPs from Table 9.

SEQ ID #					
Set 1 AFLP + 1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
115	02W561	9651- fU6	1	124	CACATACTTGAGGCAGTAAGTGAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAGGCA TTCGACTAGCGTATACGCAGATCCGATCGATTTATAATTAAAGTCAAATTAGAAACCA
116	02W562	9651- f06		122	CACATACTTGAGGCAGTAAGTGAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAGGCA TTCGACTAGCGTATACGGATCCGATCGATTTATAATTAAAGTCAAATTAGAAACCT
117	02W563	9372- d11	2	119	AAATTCGAGACATTCTTTATCAAAGGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGCATCG ACTGGTACTACGGACTCAGATCCGATGATTTCAGTTGTTTACCATTCATATTG
118	02W564	9372- d11	2	117	AATATGAATGGTAAACAACTGAAATCGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGCATC GACTGGTACTACGGACTGATCCGATCTTTGATAAAGAATGTCTCGAATTTT
119	02W565	9371- d06	6	114	ATTTCCAGGATATTGCTTAGTTTCTGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAGT CATGGATCCGATCAGATCCGAAATAAATGGAAAGTTATATGGAAAAAC
120	02W566	9371- d06	6	112	ATTTCCAGGATATTGCTTAGTTTCTGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAGT CATGGATCCGATGATCCGAAATAAATGGAAAGTTATATGGAAAAAG
121	02W567	9651- b06	3	109	TTTATATACCTGAACCAGGCTAATTAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCTA ACGTTACGGCATGATCCGTGGATGTTCACTTTCTCTAAATGAC
122	02W568	9651- b06	3	107	TTTATATACCTGAACCAGGCTAATTAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCTA ACGTTACGGCATGATCTGGATGTTCACTTTCTCTAAATGAT
123	02W569	9651- d02	5	104	CTTGGGATTGTCAAATTAGATATTCCGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTGAT CAGCTGATCCGATCTGCACTGCCATTAAGGCTAATGTA
124	02W570	9651- d02	5	102	CTTGGGATTGTCAAATTAGATATTCCGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTGAT CAGCTGATCCGATGCACTGCCATTAAGGCTAATGTG
125	02W571	9861- c03	4	99	TTCACTAACGAACATAATATCCATCTGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAT ACGTTACGGACACACTTAAAAAAATCTCCACC

(continued)

SEQ ID #					
Set 1 AFLP + 1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
126	02W572	9861- c03	4	97	TTCACTAACGAACATAATATCCATCTGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTCAT ACGTTAGGACACACTTAAAAAAATCTCCACT
127	02W573	9861- c03	7	94	TTCAACGATGATGTTGTTAACTGTGGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTAGTC AGATTTTCGAGTGGGAATTGTGTACTCG
128	02W574	9861- c03	7	92	TTCAACGATGATGTTGTTAACTGTGGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTAGTG ATTTTCGAGTGGGAATTGTGTACTCA
129	02W575	9703- a03	10	89	GCCCTGGTGTTTGCGTACTACAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTTCAGCAT GTGAGAGCATGCATGATGATGGC
130	02W576	9703- a03	10	87	CCATCATCATGCATGCTCTCACGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTTCAGCTG TAGTACGCAAACACCAGGGCA
131	02W577	9651- f04	8	84	AAGCTGGAAGAACTGGTGCTGGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGTAGGTGTAA GCCCAGAGATGTCAGCAG
132	02W578	9651- f04	9	82	TGCTGACATCTCTGGGCTTACACGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGGCAGCAC CAGTTCTTCCAGCTTG
133	02W579	9371- f08	8	79	CTACTTGCTGAAGCTGCTAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGCGAATGAACCA TATCAAGCAAAGT
134	02W580	9371- f08	8	77	CTACTTGCTGAAGCTGCTAGTGAATTGGTACGCAGTCGATGAGTCCTGAGTAAAGAATGAACCATA TCAAGCAAAGC

(continued)

Set 2 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
135	02W581	9861- e05	11	124	AGTAATTTTAGAATTCATTAACAAAATTACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGC GATTAGCGATACGTTAGCGACTTAGCCGTACTGTTATATATGATCGGAATGATTACGA
136	02W582	9861- e05	11	122	AGTAATTTTAGAATTCATTAACAAAATTACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGA TTAGCGATACGTTAGCGACTTAGCCGTACTGTTATATATGATCGGAATGATTACGT
137	02W583	3348.2	12	119	AAGAGGGAAATTAGAAAGCACTGACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCATTCG AAATCGGACTCTGAGACTCATGCGATGACTGAAAAAGGGTTATACTGGCGTGA
138	02W584	3348.2	12	117	AAGAGGGAAATTAGAAAGCACTGACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGTTCGAA ATCGGACTCTGAGACTCATGCGATGACTGAAAAAGGGTTATACTGGCGTGG
139	02W585	9572f05	13	114	AAATGATCAACTATACATCTCCAAAACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGATCA GTCCAGTCATGGATCCGATCACTCTGTTAATTGAATATTCTAACTTAT
140	02W586	9572f05	13	112	AAATGATCAACTATACATCTCCAAAACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCAGT CCAGTCATGGATCCGATCACTCTGTTAATTGAATATTCTAACTTAA
141	02W587	9682a05	14	109	TTTATATACCTGAACCAGGCTAATTACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCGAC TTCGCTAACGTTACGGCATGGATGTTCACTTTCTCTAAATGAC
142	02W588	9682a05	14	107	TTTATATACCTGAACCAGGCTAATTACCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGACTT CGCTAACGTTACGGCATGGATGTTCACTTTCTCTAAATGAT
143	02W589	9651- b05	15	104	TATTAGTCAAATTAGTGAATTCCGTCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGACTG CGGATCAGCTAAATAAATTTGTTGAGTCGAATATAAAG
144	02W590	9651- b05	15	102	TATTAGTCAAATTAGTGAATTCCGTCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGTGCG GATCAGCTAAATAAATTGGTTGAGTCGAATATAAAA
145	02W591	9572g11	16	99	TGGAGGATTATCGCCCTTGAATATCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGTACTGG CATACGTTACGTCAGGTGCTTCTCAGCTGATGC
146	02W592	9572g11	16	97	TGGAGGATTATCGCCCTTGAATATCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCTGGCA TACGTTACGTCAGGTGCTTCTCAGCTGATGT

(continued)

Set 2 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
147	02W593	9703f12	17	94	AGAAAGCTTTGACAGGACTGACAGCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGGTGGAT CAGCTTCAAGTGAAGGCTTCGCTTAAGC
148	02W594	9703f12	17	92	AGAAAGCTTTGACAGGACTGACAGCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGGGATCA GCTTCAAGTGAAGGCTTCGCTTAAGG
149	02W595	9782c03	18	89	ATTGTCGTTGACATGCAATCACCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCTCAAAT GATATCATTATCTTGAGCTTGAA
150	02W596	9782c03	18	87	ATTGTCGTTGACATGCAATCACCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCCAATGA TATCATTATCTTGAGCTTGAT
151	02W597	9782b11	19	84	CCAGGAATAAAAGCTAGACTCCCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCACACCA GCACTAACGCTGAAGTTC
152	02W598	9782b11	19	82	CCAGGAATAAAAGCTAGACTCCCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGCACCAGC ACTAACGCTGAAGTTT
153	02W599	9652- f04	20	79	CGCTGCATCTGCTGGTGTCCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGGTCACATCCCA ACTGCATCATAAG
154	02W600	9652- f04	20	77	CGCTGCATCTGCTGGTGTCCGAATTGGTACGCAGTCGATGAGTCCTGAGTAATGTCACATCCCAAC TGCATCATAAA
Set 4 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
155	02W601	43F	31	124	GTACAATGCAATTTAGAGAACAAGCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCTGA CCGATCGATATCGACGTAGCTGCATCGTAATCGGGAAGTATTCAGAGTTTAGAGTGAA
156	02W602	43F	31	122	GTACAATGCAATTTAGAGAACAAGCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCATCC GATCGATATCGACGTAGCTGCATCGTAATCGGGAAGTATTCAGAGTTTAGAGTGAT
157	02W603	61F	32	119	CTTAACACGTCCAGACAAGAGGCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCACCATG TCGACGTAGATCCGTATAGCACTGAGTCGCAAAGAATTAGACATGGATGAGTT
158	02W604	61F	32	117	CTTAACACGTCCAGACAAGAGGCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCCATGT CGGATCAGCTAAATAAATTTGTTGAGTCGAATATAAAG

(continued)

Set 4 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
159	02W605	64F	33	114	TTAACAAGAAAAATCGGTCAGGACTGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCGTA CGCATGCTAACGTTACGGACTATCTAGTTTGAGCAGTTTTGAGCTGAA
160	02W606	64F	33	112	TTAACAAGAAAAATCGGTCAGGACTGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCTACG CATGCTAACGTTACGGACTATCTAGTTTGAGCAGTTTTGAGCTGAG
161	02W607	75F	34	109	ACGCTTCTTCCTTGTTGAGAGGGGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCGATGC TCAGGCTATCGACATGTTCATATTGATGGTTTTGTTTTTGTTA
162	02W608	75F	34	107	ACGCTTCTTCCTTGTTGAGAGGGGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCATGCTC AGGCTATCGACATGTTCATATTGATGGTTTTGTTTTTGTTG
163	02W609	92R	35	104	CTCTGCAGAGATAGATCATAACCTGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCATCAC GTCATGCTGAGCATAGCTTTGCAACAGCAGAAGTGTAT
164	02W610	92R	35	102	CTCTGCAGAGATAGATCATAACCTGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCACGT CATGCTGAGCATAGCTTTGCAACAGCAGAAGTGTAC
165	02W611	28R	36	99	GAACTATAAATTGCTTGCAGGAACCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCTCGC TAACGTTACGCTCTCTTGTTTGTTGCAGGAGCA
166	02W612	28R	36	97	GAACTATAAATTGCTTGCAGGAACCGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCGCTA ACGTTACGCACTCTTGTTTGTTGCAGGAGCT
167	02W613	14446E10	40	94	AACTCTCAATTTTTCAACCTTCTCTAGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCGTC ATTCGAATCACTGGCTGGAACTTCTCCC
168	02W614	14446E10	40	92	AACTCTCAATTTTTCAACCTTCTCTAGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCAT TCGAATCACTGGCTGGAACTTCTCCG
169	02W615	14447CO6	38	89	TTCTTTTGTTATAGCAAGAGCTTGAAGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCCG ATGTATGCATGATTGGCGGTCCA
170	02W616	14447CO6	38	87	TTCTTTTGTTATAGCAAGAGCTTGAAGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCCAT GTATGCATGATTGGCGGTCCG
171	02W617	14446E01	39	84	TCACAAGCTCCCATCGCATCATGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCTGTTGTT CCTTGTTGCATCTCCTTT
172	02W618	14446E01	39	82	TCACAAGCTCCCATCGCATCATGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGGTTGTTCC TTGTTGCATCTCCTTG

(continued)

Set 4 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
173	02W619	14157A04	37	79	ACACGAGCCTCGGATCAATGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGTGCAACAGTTG AATAGAAAGATGT
174	02W620	14157A04	37	77	ACACGAGCCTCGGATCAATGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAACGCAACAGTTGAA CGACGTAGATCCGTATAGCACTGAGTCCAAAGATTTAGACATGGAGGAGTC
Set 5 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
175	02W621	14446E01	41	124	TCACAAGCTCCCATCGCATCATAGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGACTCGT ACCATGTCGACGTAGATCCGTATAGCACTGAGTCGTTGTTCCTTGTTGCATCTCCTTG
176	02W622	14446E01	41	122	TCACAAGCTCCCATCGCATCATAGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCTCGTAC CATGTCGACGTAGATCCGTATAGCACTGAGTCGTTGTTCCTTGTTGCATCTCCTTT
177	02W623	15091F12	42	119	GCACCAGGACTGGGTATACTTATGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGATCCGA TCGATATCGACGTAGCTGCATCGTAATCGGAGGTGGTAAGATATGTCAAGCAG
178	02W624	15091F12	42	117	GCACCAGGACTGGGTATACTTATGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTCCGATC GATATCGACGTAGCTGCATCGTAATCGAGGGTGGTAAGATATGTCAAGCAA
179	02W625	15089D06	43	114	TCTCTTGGCATCAAGAAAGTGATGGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTATCGA GTCGACTACGTTGCATACGGATCTATTAGTGTTTGGGACAGTGTCTTA
180	02W626	15089D06	43	112	TCTCTTGGCATCAAGAAAGTGATGGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTCGAGT CGACTACGTTGCATACGGATCTATTAGTGTTTGGGACAGTGTCTTG
181	02W627	14447D01	44	109	GATAGAGATGGTACCATTAGAGTTAGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGTAGA TCCGTATAGCACTGAGTCCCTATAATGAGTCTAAGACATCAAC
182	02W628	14447DOI	44	107	GATAGAGACGATACCATTAGAGTTAGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGTAGA TCCGTATAGCACTGAGCCTATAATGAGTCTAAGACATCAAT
183	02W629	257F	45	104	GACATTTGATGTTTGAAGTATAAGTATGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTCG ACGTGCTATGCAGGTGTTGATGTGCTATGAAATATTGA
184	02W630	257F	45	102	GACATTTGATGTTTGAAGTATAAGTATGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCGA CGTGCTATGCAGTGTTGATGTGCTACGAAATATTGG

(continued)

Set 5 AFLP+1	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
185	02W631	15090H06	46	99	GTCCACATTCTGCTTCTTCATTTGGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGTGCAT ATGCCAGTGTAGACAAATATGCTTCGACTAACT
186	02W632	15090H06	46	97	GTCCACATTCTGCTTCTTCATTTGGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGCATAT GCCAGTGTAGACAAATATGCTTCGACTAACC
187	02W633	15091H02	47	94	AATACAATAAAATCAATAGGGCAAAAGGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCTA CGGACTCTCTAGGTGAGGCCTGGACCAT
188	02W634	15091H02	47	92	AATACAATAAAATCAATAGGGCAAAGAGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCACG GACTCTCTAGGTGAGGCCTGGACCAC
189	02W635	15091A10	48	89	AGAAGCACATCGGGATCTACCACGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCCGATTG AAGCATAGATATAGACAGATCAC
190	02W636	15091A10	48	87	AGAAGCACATTGGGATCTACCACGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGATTGAA GCATAGATATAGACAGATCAA
191	02W637	14157A04	49	84	ACACGAGCCTCGGATCAATGGCGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGGTGCAAC AGTTGAATAGAAAGATGT
192	02W638	14157A04	49	82	ACACGAGCCTCGGATCAATGGCGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTGCAACAG TTGAATAGAAAGATGC
193	02W639	15091F09	50	79	GTTGCAATCGCTCCTTGAATTGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGCCATCAAA TACTGGCTAGATC
194	02W640	15091F09	50	77	GTTGCAATCGCTCCTTGAATTGAGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCATCAAATA CTGGCTAGATT
Set 4 AFLP+0	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
195	02R123	43F	31	120	GTACAATGCAATTTAGAGAACAAGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGGATCCGA TCGATATCGACGTAGCTGCATCGTAATCGGGAAGTATTCAGAGTTTAGAGTGAA
196	02R124	43F	31	118	GTACAATGCAATTTAGAGAACAAGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGTCCGATC GATATCGACGTAGCTGCATCGTAATCGGGAAGTATTCAGAGTTTAGAGTGAT

5 10 15 20 25 30 35 40 45 50 55

(continued)

Set 4 AFLP+0	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
197	02R125	61F	32	116	CTTAACACGTCCAGACAAGAGGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGACCATGTCG ACGTAGATCCGTATAGCACTGAGTCGCAAAGAATTAGACATGGATGAGTT
198	02R126	61F	32	114	CTTAACACGTCCAGACAAGAGGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCATGTCGA CGTAGATCCGTATAGCACTGAGTCCAAAGATTTAGACATGGAGGAGTC
199	02R127	64F	33	112	TTAACAAGAAAAATCGGTCAGGACTGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGTACGC ATGCTAACGTTACGGACTATCGTAGTTTGAGCAGTTTTGAGCTGAA
200	02R128	64F	33	110	TTAACAAGAAAAATCGGTCAGGACTGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGTACGCAT GCTAACGTTACGGACTATCGTAGTTTGAGCAGTTTTGAGCTGAG
201	02R129	75F	34	108	ACGCTTCTTCCTTGTTGAGAGGGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTAGATGCT CAGGCTATCGACATGTTCATATTGATGGTTTTGTTTTTGTTA
202	02R130	75F	34	106	ACGCTTCTTCCTTGTTGAGAGGGGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGAGATGCTCA GGCTATCGACATGTTCATATTGATGGTTTTGTTTTTGTTG
203	02R131	92R	35	104	CTCTGCAGAGATAGATCATAACCTGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGGAGATCAC GTCATGCTGAGCATAGCTTTGCAACAGCAGAAGTGTAT
204	02R132	92R	35	102	CTCTGCAGAGATAGATCATAACCTGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGGATCACGT CATGCTGAGCATAGCTTTGCAACAGCAGAAGTGTAC
205	02R133	28R	36	100	GAACTATAAATTGCTTGCAGGAACCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGTCGCTAA CGTTACGGCATCTCTCTTGTTTGTTGCAGGAGCA
206	02R134	28R	36	98	GAACTATAAATTGCTTGCAGGAACCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGGCTAACG TTACGGCATCACTCTTGTTTGTTGCAGGAGCT
207	02R135	14157A04	37	96	ACACGAGCCTCGGATCAATGGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGTGCTAGCACG TACTGGTGCAACAGTTGAATAGAAAGATGT
208	02R136	14157A04	37	94	ACACGAGCCTCGGATCAATGGCGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTAGCACGTA CTGGTGCAACAGTTGAATAGAAAGATGC
209	02R137	14447C06	38	92	TTCTTTTGTTATAGCAAGAGCTTGAAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCGATT AGCATGTATGCATGATTGGCGGTCCA

(continued)

Set 4 AFLP+0	Padlock nr.	Fragment code	Locus nr.	Length (bp)	5'-PH -3'
210	02R138	14447C06	38	90	TTCTTTTGTTATAGCAAGAGCTTGAAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCCGTAG CATGTATGCATGATTGGCGGTCCG
211	02R139	14446E01	39	88	TCACAAGCTCCCATCGCATCATAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCGTTACGGT TGTTCCTTGTTGCATCTCCTTT
212	02R140	14446E01	39	86	TCACAAGCTCCCATCGCATCATAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGCTACGGTTG TTCCTTGTTGCATCTCCTTG
213	02R141	14446E10	40	84	AACTCTCAATTTTTCAACCTTCTCTAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGGTATCA CTGGCTGGAACTTCTCCC
214	02R142	14446E10	40	82	AACTCTCAATTTTTCAACCTTCTCTAGGAATTGGTACGCAGTCGATGAGTCCTGAGTAAGATCACT |GGCTGGAACTTCTCCG

Example 16. Design of the PCR amplification primers

[0148] The sequence of one of the primers used for PCR amplification was complementary to the PCR primer binding regions incorporated in the ligation probes described in Example 15. The sequence of the second PCR primer matched the PCR primer binding region of the probe. Usually the forward primer is labelled. The concentration of the oligonucleotides was adjusted to 50 ng / µl. The sequence of the primers in 5'-3' orientation are depicted in Table 11.

Table 11. PCR amplification primers

SEQ ID #		Primer nr	5'- 3'	
215	MseI+0:	93E40	GATGAGTCCTGAGTAA*	M00k
216	EcoRI+0	93L01	GACTGCGTACCAATTC*	E00k
217	EcoRI+1	93LO2	GACTGCGTACCAATTCA	E01K NED
218	EcoRI+1	93LO4	GACTGCGTACCAATTCG	E03K 5'+T Joe
219	EcoRI+1	93LO5	GACTGCGTACCAATTCT	E04K FAM
*Multiple labels possible				

Example 17. Ligation and amplification

[0149] 9 samples (samples1-9) of homozygous tomato lines (Example 14) were subjected to a multiplex oligonucleotide ligation reaction using a mixture of 20 padlock probes (set 4). Conditions used were 1x Taq DNA ligase buffer (NEB), 0.2 U/µl Taq DNA ligase, and 0.05 fmol/µl of each probe in a volume of 10 µl. Ligation was performed in a thermocycler (Perkin Elmer) with the following cycling conditions: 2 minutes at 94 ˚C + 10*(15 seconds at 94 ˚C + 60 minutes at 60˚C) + 4 ˚C continuously. Following ligation, the 10 µl ligation product was diluted with 30 µl 1x Taq DNA ligase buffer. The 40 µl of each reaction was used to perform 4 amplification reactions using 4 different labelled E00k primers each combined with M00k. The E00k primer labelled with ET-ROX and JOE were designed with an extra 1 bp in comparison with E00k labelled with FAM and NED length, to prevent possible crosstalk between fluorescent labels when analysing these products on the MegaBACE. Conditions used were 30 ng labelled E00k primer and 30 ng M00k primer, 1x Accuprime buffer I, 0.4 ul Accuprime polymerase (Invitrogen) on 10 µl diluted ligation product in a 20 µl PCR reaction. PCR was performed in a thermocycler with the following cycling conditions: 2 minutes at 94 ˚C + 35 *(15 seconds at 94 ˚C + 30 seconds at 56 ˚C + 60 seconds at 68 ˚C) +4 ˚C continuously. PCR product was purified using Sephadex 50 and diluted 80 times with MQ. Diluted PCR product was analysed on the MegaBACE. The different fluorescent-labelled products were run separately and in different combinations (2, 3 and 4 fluorescent dyes). The results are presented in Fig 16.

Example 18: Use of length/dye combinations and the principle of repeated injection in combination with reuse of the LPA matrix.

[0150] The amplification products of set 4 were analysed using consecutive runs without replacement of the LPA matrix between runs. Samples of the amplification products were injected after a run period of 40 minutes without changing the matrix. Results are presented in Fig 17. Consecutive runs can be performed without changing the matrix and without significant loss of data quality.

Example 19: Selective amplification of a multiplex ligation sample

[0151] This experiment demonstrates the possibility of a higher multiplex of oligonucleotide ligation, in combination with the selective amplification of a subset of the formed ligation products using (AFLP) amplification primers with selective nucleotides.

[0152] Using the 4 designed probe sets, primers are based on set 1,2,4 and 5 but with additional selective nucleotides located immediately 3' of the primer binding sites in the probes.

[0153] Each set was ligated separately, and in combination with other sets, up to a multiplex of 40 based on the 4 sets together. AFLP+1/+1 amplifications using different labelled E00k primers were performed using the scheme depicted below.

Ligation set	Amplification			
	Label	Primers	Selective bases	Set
1	NED	E01k/M01k	+A/ +A	1
2	JOE	E03k/M04k	+G/ +T	2
5	FAM	E04k/M03k	+T/ +G	5
1+4	NED	E01k/M01k	+A/ +A	1
2+4	JOE	E03k/M04k	+G/ +T	2
4+5	FAM	E04k/M03k	+T/ +G	5
1+2+4+5	JOE	E03k/M04k	+G/ +T	2
1+2+4+5	NED	E01k/M01k	+A/ +A	1
1+2+4+5	FAM	E04k/M03k	+T/ +G	5

[0154] Conditions used were 1x Taq DNA ligase buffer (NEB), 0.2 U/μl Taq DNA ligase, and 0.05 fmol/μl of each probe in a volume of 10 μl. Ligation was performed in a thermocycler (Perkin Elmer) with the following cycling conditions: 2 minutes at 94 ˚C+10*(15 seconds at 94 ˚C; 60 minutes at 60 ˚C)+ 4 ˚C continuously. Following ligation, the 10 μl ligation product was diluted with 30 μl 1x Taq DNA ligase buffer. Conditions used were 30 ng labelled E0k primer and 30 ng M00k primer, 1x Accuprime buffer (Invitrogen) I, 0.4 ul Accuprime polymerase (Invitrogen) on 10 μl diluted ligation product in a 20 μl PCR reaction. PCR was performed in a thermocycler with the following cycling conditions: 2 minutes at 94 ˚C+35*(15 seconds at 94 ˚C + 30 seconds at 56 ˚C + 6 minutes at 68 ˚C)+ 4 ˚C continuously. PCR product was purified using Sephadex 50 and diluted 80 times with MQ. Diluted PCR product was analysed on the Megabace. The different fluorescent-labelled products were run in separate capillaries. The results are presented in Fig 18.

Buffer compositions:

1x Taq DNA ligase buffer

[0155]

20 mM Tris-HCl
25 mM potassium acetate
10 mM Magnesium acetate
10 mM DTT
1 mM NAD
0.1% Triton X-100
(pH 7.6@ 25˚C)

1xAccuPrime Taq DNA polymerase buffer

[0156]

20 mM Tris-HCl (pH8.4)
50 mM KCl
1.5 mM $MgCl_2$
0.2 mM dGTP, dATP, dTTP and dCTP
thermostable AccuPrime™ protein
10% glycerol

SEQUENCE LISTING

[0157]

<110> Keygene N.V.

<120> Analysis and detection of multiple target sequences using circular probes

<130> P205833PCT

<140> P205833PCT
<141> 2002-12-16

<150> EP 01204912.8
<151> 2001-12-14

<160> 219

<170> PatentIn version 3.1

<210> 1
<211> 48
<212> DNA
<213> artificial

<400> 1

cgccagggtt ttcccagtca cgacttcagg actagtctat accttgag
48

<210> 2
<211> 51
<212> DNA
<213> artificial

<400> 2

cgccagggtt ttcccagtca cgacgacttc aggactagtc tataccttga a
51

<210> 3
<211> 49
<212> DNA
<213> artificial

<400> 3

ctatgtgaac caaattaaag tttactcctg tgtgaaattg ttatccgct
49

<210> 4
<211> 49
<212> DNA
<213> artificial

<400> 4

cgccagggtt ttcccagtca cgacctgctc tttcctcgct agcttcaga
49

5 <210> 5
<211> 51
<212> DNA
<213> artificial

10 <400> 5

cgccagggtt ttcccagtca cgacgactgc tctttcctcg ctagcttcag c
51

15

<210> 6
<211> 53
<212> DNA
<213> artificial

20

<400> 6

agattcggac cttctctcat aatccgactt cctgtgtgaa attgttatcc gct
25 53

<210> 7
<211> 49
<212> DNA
30 <213> artificial

<400> 7

35 cgccagggtt ttcccagtca cgacgaagag gagagtggct acgaactct
49

<210> 8
<211> 51
40 <212> DNA
<213> artificial

<400> 8

45

cgccagggtt ttcccagtca cgacgagaag aggagagtgg ctacgaactc g
51

<210> 9
50 <211> 57
<212> DNA
<213> artificial

<400> 9

55

gcgataactg ctctgtagaa agacccgatt agctcctgtg tgaaattgtt atccgct
57

<210> 10
<211> 49
<212> DNA
<213> artificial

<400> 10

cgccagggtt ttcccagtca cgacaatcgg cctaagcaag cttgttttc
49

<210> 11
<211> 51
<212> DNA
<213> artificial

<400> 11

cgccagggtt ttcccagtca cgacgaaatc ggcctaagca agcttgtttt g
51

<210> 12
<211> 61
<212> DNA
<213> artificial

<400> 12

tgctattgat atctctgtgc aactcatcgg atcagcttcc tgtgtgaaat tgttatccgc
60

t
61

<210> 13
<211> 49
<212> DNA
<213> artificial

<400> 13

cgccagggtt ttcccagtca cgacgatcgg aaagatatcg gagctcctt
49

<210> 14
<211> 51
<212> DNA
<213> artificial

<400> 14

cgccagggtt ttcccagtca cgacgagatc ggaaagatat cggagctcct c
51

<210> 15
<211> 65
<212> DNA
<213> artificial

<400> 15

gtcggtgtca accgatccac ggcgcatgct agcacgtact gtcctgtgtg aaattgttat
60

ccgct
65

<210> 16
<211> 49
<212> DNA
<213> artificial

<400> 16

cgccagggtt ttcccagtca cgacgaactg gcatcaatca ggcctccaa
49

<210> 17
<211> 51
<212> DNA
<213> artificial

<400> 17

cgccagggtt ttcccagtca cgacgagaac tggcatcaat caggcctcca t
51

<210> 18
<211> 69
<212> DNA
<213> artificial

<400> 18

ccttaatgca agggcttatt acgtcgtacc agtcatggat ccgattcctg tgtgaaattg
60

ttatccgct
69

<210> 19
<211> 49
<212> DNA
<213> artificial

<400> 19

cgccagggtt ttcccagtca cgacggactc caaggtattg ttaggcgcc
49

5

<210> 20
<211> 51
<212> DNA
<213> artificial

10

<400> 20

cgccagggtt ttcccagtca cgacgaggac tccaaggtat tgttaggcgc a
51

15

<210> 21
<211> 73
<212> DNA
<213> artificial

20

<400> 21

aaccaccaag atcagtctca tcttcgatca tcgactggta ctacggactt cctgtgtgaa
60

25

attgttatcc gct
73

30

<210> 22
<211> 49
<212> DNA
<213> artificial

35

<400> 22

cgccagggtt ttcccagtca cgaccatctc ttgcgccttc tcagtgttg
49

40

<210> 23
<211> 51
<212> DNA
<213> artificial

45

<400> 23

cgccagggtt ttcccagtca cgacgacatc tcttgcgcct tctcagtgtt a
51

50

<210> 24
<211> 77

55

<212> DNA
<213> artificial

<400> 24

tgacgtccgt cgaagaatag gtaacgtacg catgctaacg ttacggacta tcgtcctgtg
60

5

tgaaattgtt atccgct
77

<210> 25
<211> 49
10
<212> DNA
<213> artificial

<400> 25

15

cgccagggtt ttcccagtca cgacagtttc aaaacccatg acgcttcta
49

20
<210> 26
<211> 51
<212> DNA
<213> artificial

25
<400> 26

cgccagggtt ttcccagtca cgacgaagtt tcaaaaccca tgacgcttct t
51

30

<210> 27
<211> 81
<212> DNA
35
<213> artificial

<400> 27

40
gtgatagctg aaaagaccca ttctccgtag catcgatatc ggtcaactgg atcagcttcc
60

tgtgtgaaat tgttatccgc t
81

45

<210> 28
<211> 49
<212> DNA
<213> artificial

50

<400> 28

cgccagggtt ttcccagtca cgacatactc caattgctca ggcacagtt
49

55

<210> 29
<211> 51

<212> DNA
<213> artificial

<400> 29

5

cgccagggtt ttcccagtca cgacgaatac tccaattgct caggcacagt c
51

10

<210> 30
<211> 85
<212> DNA
<213> artificial

15

<400> 30

ctccttgtcc cacgaagata gttccgtacc atgtcgacgt agatccgtat agcactgagt
60

20

ctcctgtgtg aaattgttat ccgct
85

25

<210> 31
<211> 49
<212> DNA
<213> artificial

30

<400> 31

cgccagggtt ttcccagtca cgacgtagag gctctaaaca gctgcttcc
49

35

<210> 32
<211> 51
<212> DNA
<213> artificial

40

<400> 32

cgccagggtt ttcccagtca cgacgagtag aggctctaaa cagctgcttc g
51

45

<210> 33
<211> 49
<212> DNA
<213> artificial

50

<400> 33

55

cttgtttatg ctaagggccg gctcctcctg tgtgaaattg ttatccgct
49

<210> 34
<211> 49
<212> DNA
<213> artificial

<400> 34

cgccagggtt ttcccagtca cgactaagtc agctcctaag cttccatcg
49

<210> 35
<211> 51
<212> DNA
<213> artificial

<400> 35

cgccagggtt ttcccagtca cgacgataag tcagctccta agcttccatc t
51

<210> 36
<211> 53
<212> DNA
<213> artificial

<400> 36

aagccacttc ctcctgctca agcgcgactt cctgtgtgaa attgttatcc gct
53

<210> 37
<211> 24
<212> DNA
<213> artificial

<400> 37

cgccagggtt ttcccagtca cgac
24

<210> 38
<211> 24
<212> DNA
<213> artificial

<400> 38

agcggataac aatttcacac agga
24

<210> 39
<211> 4
<212> DNA

<213> artificial

<400> 39

5

tgca
4

<210> 40
10 <211> 8
<212> DNA
<213> artificial

<400> 40
15

acgttacg
8

20 <210> 41
<211> 12
<212> DNA
<213> artificial

25 <400> 41

tagcgtcagc at
12

30
<210> 42
<211> 16
<212> DNA
<213> artificial
35
<400> 42

catggcatac gttacg
40 16

<210> 43
<211> 20
<212> DNA
45 <213> artificial

<400> 43

50 gatcgctaac gttacggcat
20

<210> 44
<211> 24
55 <212> DNA
<213> artificial

<400> 44

tcgagatcac gtcatgctga gcat
24

5 <210> 45
<211> 28
<212> DNA
<213> artificial

10 <400> 45

cagttcaggc attcgactag cgtatacg
28

15

<210> 46
<211> 32
<212> DNA
<213> artificial

20

<400> 46

gtcaatcgga ctctgagact catgcgatga ct
25 32

<210> 47
<211> 36
30 <212> DNA
<213> artificial

<400> 47

35

gatccgatcg atatcgacgt agctgcatcg taatcg
36

<210> 48
40 <211> 4
<212> DNA
<213> artificial

<400> 48

45

actg
4

50 <210> 49
<211> 8
<212> DNA
<213> artificial

55 <400> 49

gcatcagt
8

5 <210> 50
<211> 12
<212> DNA
<213> artificial

10 <400> 50

atcggcatta cg
12

15
<210> 51
<211> 16
<212> DNA
<213> artificial
20
<400> 51

tacggcatag tcacgt
25 16

<210> 52
<211> 20
<212> DNA
30 <213> artificial

<400> 52

35 gatcgctaac gttacggcat
20

<210> 53
<211> 24
40 <212> DNA
<213> artificial

<400> 53

45
ctagatgctc aggctatcga catg
24

<210> 54
50 <211> 28
<212> DNA
<213> artificial

<400> 54
55

gtaccgatac gttagcgact tagccgta
28

<210> 55
<211> 32
<212> DNA
<213> artificial

<400> 55

cgtaatcgga tccgtaacgt gcatatgcca gt
32

<210> 56
<211> 36
<212> DNA
<213> artificial

<400> 56

gacttcgaga tctgcaacgt acgtcgtaag ctgcta
36

<210> 57
<211> 5
<212> DNA
<213> artificial

<400> 57

cgact
5

<210> 58
<211> 9
<212> DNA
<213> artificial

<400> 58

ccgattagc
9

<210> 59
<211> 13
<212> DNA
<213> artificial

<400> 59

catcggatca gct
13

<210> 60
<211> 17
<212> DNA

<213> artificial

<400> 60

5

catgctagca cgtactg
17

<210> 61
10 <211> 21
<212> DNA
<213> artificial

<400> 61
15

cgtaccagtc atggatccga t
21

20 <210> 62
<211> 25
<212> DNA
<213> artificial

25 <400> 62

cgatcatcga ctggtactac ggact
25

30
<210> 63
<211> 29
<212> DNA
<213> artificial
35
<400> 63

cgtacgcatg ctaacgttac ggactatcg
40 29

<210> 64
<211> 33
<212> DNA
45 <213> artificial

<400> 64

50 ccgtagcatc gatatcggtc aactggatca gct
33

<210> 65
<211> 37
55 <212> DNA
<213> artificial

<400> 65

cgtaccatgt cgacgtagat ccgtatagca ctgagtc
37

<210> 66
<211> 5
<212> DNA
<213> artificial

<400> 66

cgtac
5

<210> 67
<211> 9
<212> DNA
<213> artificial

<400> 67

cgcattcga
9

<210> 68
<211> 13
<212> DNA
<213> artificial

<400> 68

catcggcatg act
13

<210> 69
<211> 17
<212> DNA
<213> artificial

<400> 69

cgtcaatgca cgttacg
17

<210> 70
<211> 21
<212> DNA
<213> artificial

<400> 70

cgcatcgata gctctgaacg t
21

<210> 71
<211> 25
<212> DNA
<213> artificial

5

<400> 71

cgcatatcgg atcgatcgca tacgt
25

10

<210> 72
<211> 29
<212> DNA

15

<213> artificial

<400> 72

20

catcggatcc atgcgtagca tatcgacgt
29

<210> 73
<211> 33

25

<212> DNA
<213> artificial

<400> 73

30

ctgcaagtcc gattacgatc gacgtgctat gca
33

35

<210> 74
<211> 37
<212> DNA
<213> artificial

40

<400> 74

cagctcagta tcgagtcgac tacgttgcat acggatc
37

45

<210> 75
<211> 669
<212> DNA
<213> Lycopersicon esculentum

50

<400> 75

55

cckcggagaa wtgaagaagt atgctgtgtc atccggtgtt ggctcacact caggtactgt 60

tagacccatc tcatgcttaa caatkkgatt ctttgagcgt tacctaktga actagtatat 120

tttkggtgtg ctcacttact gcctcaagtt atgtgatggt ttctaattkt gactttaatt 180

ataaatcatg cacatcttat ataaatcaga tttccaaagc tgctgtatat tggttcagta 240

gataatatgg ttttatctct taactggtta tatctgcagt catttttttgg ttatacctct 300

ttcatagtcc tgattaaagg attttgagtt attttcaatg tctctttgta aacaaagatt 360

atactagaat caatctaatg ttttctttcc tttaaataaa ttacagataa ggaagatgaa 420

gggtttgaaa cagaagaaag cccatttgat ggagatccag gttaatggag gatcaattgc 480

tcagaaggtt gacttcgcat atggtttctt tgagaagcag gttccagttg atgctgtttt 540

ccagaaggat gagatgattg acatcattgg tgtcaccaag ggtaagggtt atgaaggtgt 600

ygtaactcgt tggggtgtga cacgtcttct cgcaaaaccc acggggtcta cgtaaggtgc 660

tgttggggc 669

<210> 76
<211> 556
<212> DNA
<213> Lycopersicon esculentum

<400> 76

```
gcytggggac tagttctttt cagaatcata tcatctgtag agaaatcagc tgctttcctg
60

aatgttcctc gtccgaaatc taggttgtaa gagtctgtaa gaccttccac cagatcaaaa
120

tcaggtttcc atcctagctc agcctttgac ttctcgatgg atgtaaagaa atgctgtaga
180

aattcgatgt taaaaccaac gagaagacat agatagacta gtgttggaca agaatccgat
240

attaaacaga caagctaaca acttcaacag aggaaataaa ccatatttct tgtagtattt
300

cgtttggact acgattgatt gtacaaaaat gtgtgttaat tttagtgagc atactgatgt
360

gtgttttagg aagggactag gataagaggc ggtgaacatg ttgtgaatct tcactgatga
420

ttcatttagt ttgatcatat catttgattc tttgataaag aatgtctcga atttcaatat
480

gaatggtaaa caactgaaat caacacacta atatttacct ggtcacggaa tgggaatgct
540

ttcttcttgc caaaat
556
```

<210> 77
<211> 727
<212> DNA
<213> Lycopersicon esculentum

<220>
<221> misc_feature
<222> (1)..(727)
<223>

<220>
<221> misc_feature
<222> (1)..(727)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<400> 77

agnahkyycv aggctcacda scaggttgga aaaatcattt tgatacaraa rttgcatttt
60

ctggttattc aggtgatttc ccttctatat gtcaaactta ttgaaacgag tcttctgaaa
120

aataaatgga aagttatatg gaaaaasatt tccaggatat tgcttagttt ctcataagta
180

taaagcttta tatgtgaacc aattcaacag gtacatatat cagaggcccg ggtttctgct
240

gctttagata agctagctta catggaagaa ttggttaacg ataggcttct gcaagagaga
300

agcacagtag aatcagaatg cacgtcttcc tctgcaagca cgtcaacagg attattagac
360

actccaaaaa gcaagcaacc acgaagaacg ctgaatgtct caggtcctgt ccaagattac
420

agttcccgtt tgaagaactt ttggtaccct gttgcattct ccgcagatct taagaatgac
480

accatggtga gtcaattatc stcatatctg ccagtctctt taacctaaaa gaaagaaaac
540

atttgatcta aaacacagaa aaccatgtag atgcaaaatt atgatgccaa aacaaattaa
600

caagctatat gatctacgct cctactttat ggtcttccat gtatattctt kgggatcttc
660

taattgatga ctgttaactg tatctttgta gttaccgatt gattgcttgc agacacaccg
720

ggggtra
727

<210> 78
<211> 350
<212> DNA
<213> Lycopersicon esculentum

<400> 78

agggagahta gamccagaag tgtcaccaag aacctatctt caagaactac agcttgcctc 60

ctaataaatg tggataccct ggtggtattt tcaacccact caactttgca ccaactgaag 120

aggccaagga gaaggaactt gctaatggta agtggatgtt cactttctct aaatgayttt 180

atatacctga accaggctaa ttattttagg tggataattt gcagggagat tggctatgtt 240

ggcatttttg ggatttatag tgcagcacaa tgtgactggg aagggacctt ttgacaacct 300

tctgcagcac ctctctgacc catggcacaa caccatcatc caaacactca 350

<210> 79
<211> 240
<212> DNA
<213> Lycopersicon esculentum

<400> 79

gactmctggc tktaatgttg cattggtagc caagtgacac ccctgttgct cattgcttga 60

aggtttggct gatttggaag ttgcagcttg tctttgcact gccattaagg ctaatgtact 120

tgggattgtc aaattagata ttcctgttgc tcttagtgct ttggttagtg cttgtgctaa 180

gaaagttccc acaggtttca agtgtggtta attagagtat taattagcca agggtgggga 240

<210> 80
<211> 389
<212> DNA
<213> Lycopersicon esculentum

<400> 80

maaakctaaa yyaaggcttk atttkgacca accctkgtaa tccattaggt accattttag 60

atagggacac acttaaaaaa atctccacyt tcactaacga acataatatc catcttgttt 120

gcgacgaaat atatgctgct accgtrttca atyctccaaa attcgttagc atcgctgaaa 180

ttatcaacga agataattgt atcaataaag atttagtaca cattgtgtct agtctttcca 240

aggacttagg ttttccagga tttcgagtgg gaattgtgta ctcrttcaac gatgatgttg 300

ttaactgtgc tagaaaaatg tcgagtttkg ggtcttgttt cgactcagac acaacatttg 360

ctagctttca tgttgtctga cgatgaatt 389

<210> 81
<211> 389
<212> DNA
<213> Lycopersicon esculentum

<400> 81

maaakctaaa yyaaggcttk atttkgacca accctkgtaa tccattaggt accattttag 60

atagggacac acttaaaaaa atctccacyt tcactaacga acataatatc catcttgttt 120

gcgacgaaat atatgctgct accgtrttca atyctccaaa attcgttagc atcgctgaaa 180

ttatcaacga agataattgt atcaataaag atttagtaca cattgtgtct agtctttcca 240

aggacttagg ttttccagga tttcgagtgg gaattgtgta ctcrttcaac gatgatgttg 300

ttaactgtgc tagaaaaatg tcgagtttkg ggtcttgttt cgactcagac acaacatttg 360

ctagctttca tgttgtctga cgatgaatt 389

<210> 82
<211> 489
<212> DNA
<213> Lycopersicon esculentum

<400> 82

agacgacmcc amgctaaagg agaaacacaa gaagcattta aaaagaacat tgaagcagca
60

actaagtttc ttttgcaaaa gatcaaggac ttgcaattgt atgtccattt taaattgttt
120

tatgacattg tctaagctat ttcttactga agttgaatgt gttttgtttt ccttctactt
180

catacctggc acctttaata gaaactgata ctatttgtgt gtgtgctggc agctttgttg
240

gtgagagcat gcatgatgat ggcgccctgg tgtttgcgta ctacaaggag ggttcagctg
300

atcctacctt tttgtacatt gcacctggtt tgaaggagat caagtgctag atgtctggtg
360

gagtgcttct gctagaagtt ttgcattcga gattatgttt catgtagttt ttaatatttg
420

gtctttttg cttatttatg tctggtgttt cttctaaacc ttgggtactt gctgtgacca
480

gtaccggaa
489

<210> 83
<211> 754
<212> DNA
<213> Lycopersicon esculentum

<220>
<221> misc_feature
<222> (749)..(799)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C; H= A, C or T; B= C, G or T; V= A, C or G; D= G or T; N= A, C, G or T

<400> 83

tgggggyyca ttacacaaaa caagaacttc agccattgtg tgttgttcaa accaaacccc 60

gtggtttcta attcaacaga ggaaagttct tcttcattaa aggcattctc tgcagcactt 120

gcgtttgtct tctattcttt tgtcagcacc agttcttcca gcttctgctg acatctctgg 180

cttacacctt gcaaggactc aaaacagttt gctaaaaggg agaagcaaca gatcaagaag 240

cttcaaaatt ctttgaaact ttatgcacct gatagcgccc ctgcacttgc tatcaatgcc 300

actattgaga aaactaaacg caggtttgcc ttcagtatct ttcttcacaa ttttcaaaaa 360

gttttacttc ttatttgcct atttkkccct agttgatcat ttttttattg tgtactagat 420

agagagtact tatagttaag atttgcggga ttctaatcaa ttttgttagg ggtttacaaa 480

ttaaaataca tagtacaaat atagggtcta tggaaaagct actgaattcg ttcgaaccca 540

tgttaggagt aggagtagaa gaagagctaa aagtattctt ktacgaatga aagcatactg 600

tacattamca tttgcttatc agagaaaagc agattgttca acttttcctk ggcatatgcc 660

gttgagatta gactaggaaa ctccacatwg aacatacata taccskttga tactcgagta 720

agtaaaagtt taatycmtca gacgtcccnc acta 754

<210> 84
<211> 251
<212> DNA
<213> Lycopersicon esculentum

<400> 84

acaccgwgag argaagatag cttttacaat tcttcgccat gacaggaatc ttcttctgag
60

tatgagatcg cttgggcaaa agtaccgcat aaacgacctc gaggaggata acgccgcgct
120

caaggaagaa caagaagggc tcgttcaccg aatgaaccat atcaagcaaa gtctacttgc
180

tgaagctgct agtgagccca ctggtgcctt tgcttcccgt cttcgccgcc tctttggtga
240

tgaaagctga a
251

<210> 85
<211> 539
<212> DNA
<213> Lycopersicon esculentum

<400> 85

aayggstgtg gayctggctg cagtgcggca gtggctgtgg agggtaagtt cttcctaaaa
60

tatttatatg ttacataaat atataacgac tttcatttaa aaaaaaatca tagaatcgag
120

atgatctagt ttacagttta atttattcct ttcactaaat ttaattatct aaattcttga
180

ttttgtataa ttaattgcag atgtgggatg taccccgact tggagagcac cactaccttt
240

accatcattg agggtgttgc acctatgaag aagttagtct aattttaaca taaaagactt
300

tttctacatt tgttatatat gatcggaatg attacgaagt aattttagaa ttcattaaca
360

aaattaagaa gtttcactct cgaaatttga attataacac ataaattgaa acaggtcacc
420

taaaagataa ctataatgtt agaattaata atattgaaac acataacacg ttctattaat
480

atgaattttg tttaccatat taaagtgtat atatatataa tttacatgaa ttaattgcg
539

<210> 86
<211> 521
<212> DNA
<213> Lycopersicon esculentum

<400> 86

tggtaagatg tgcttatgag gtctgtcgat attcccttct gaaaagatct tcaatcccac 60

ttgaaatcat acccattaaa caatcagagt taagagaaaa agggttatac tggcgtgraa 120

gagggaaatt agaaagcact gagttttcat ttactcgttt tttgacaccc catttggcta 180

attttgaagg atgggctatg tattgttgat tgtgatttct tgtatttagg ggatattaag 240

gaattgaggg atatggtgga tgataaatat gctttaatgt gtgtacaaca taattatgct 300

cctaaagaaa ctactaaaat ggatggggca gtacaaactg tgtatcctag gaagaattgg 360

tcatccatgg ttcttctata attgtgcgca tccaaagaat aaggtcttga cacctgakak 420

ttgtcaatac tgaaactggg gcattttctc cataagcttt actatggttg gaakatgagg 480

agattgggga agttccgttc gttkggaact tccgtcgatc g 521

<210> 87
<211> 654
<212> DNA
<213> Lycopersicon esculentum

<400> 87

aarggagyaa gtgkgatyct cgaatmcatt gacgagacat ttgaaggccc ttccatctta 60

cctaaagacc cttatgatcg agctttagct cgtttctggg ctaaattctt cgaagataag 120

gtatatcgac tccttaactt gtctctactc tgttaattga atattctaac ttawaaatga 180

tcaactatac atctccaaaa tttatgtggc atgtcatgag gtgtctacga gacatgttaa 240

agagttggag tgcttaattg ttaattgaga ccaaatattt agatatgcac attcaaagtt 300

agagtactta ttatcggata caaccaagtc agaatgtcat tttatatata ttatatgtct 360

tgtgtaaaat tggactaaag taataaaata tcacattgcc aacaataact tatttgtgac
420

tgactaatgt acttctattg ttgtagattt atatctttaa aattttgttg aattyaagtt
480

ccaattgtta tgtagtggcc atcaatgatg aaaagtctat ttttcaaagg agaggagcaa
540

gagaaaggta cmgaggaagt taatgagatg ttgaaaattc ttgataatga gctcagggac
600

gramagtttt ttgttggtaa caactttgga ttgktgatgt tgtgcaatgc tgta
654

<210> 88
<211> 356
<212> DNA
<213> Lycopersicon esculentum

<400> 88

aaagkggcag aattagaacc aggaagtgtc accaagacct atcttcaaga actacagctt
60

gcctcctaat aaatgtggat accctggtgg tattttcaac ccactcaact ttggcaccaa
120

mctgaagagg ccaaggagaa ggaacttgct aatggtaagt ggatgttcac tttctctaaa
180

tgaytttata tacctgaacc aggctaatta ttttaggtgg ataatttgca gggagattgg
240

ctatgttggc atttttggga tttatagtgc agcacaatgt gactgggaag ggaccttttg
300

acaaccttct gcagcacctc tctgacccat ggcacaacac catcatccaa acacaa
356

<210> 89
<211> 824
<212> DNA
<213> Lycopersicon esculentum

<400> 89

graaggagga tctgatgctt ctggcacaat gggtttagtt ttsgcaaatt tttgtatatc
60

aaaaatttac taaattttta tacrcactct tttcttttta atctgttata aaaataatta
120

cttatacaat tttatcayta atcatgacat gctcttaatg tcacgtgtca tatttaagac
180

catgattttt attagatata cttttgatat atcgtaaaac tctttatatt gtctaatttc
240

atgttcattc aaatattcta cgaaattaga atttgaaact tttgattttt ttgtagtttt
300

agtctttttg agtcatcaga ttctaaattg atggtatata ttaaataaat ttggttgagt
360

cgaatataaa rtattagtca aattagtgaa ttctgtcaaa ctcgcttctt atcttttagc
420

tttatctatc ccttcgtaaa ataatagtga aacatatatg aatttttttt aatagtctaa
480

attttatttt cacgaaaatt tttatgctca atcaaatact gttttacgaa ataagataga
540

aggatagtta taatgacatg aattctgatt attaacaatg attgtctgga acagggcggt
600

gcttgtggct atgggaactt gtactcaaac aggttatggt acaaacactg ctgcattaag
660

tactgccttt gttcaatgat ggagcatcat gtggtcatgt tcccmtttgt gtgattmtca
720

tccgatcmaw kkgtsymtrg gractcytta catttmcgcc tatttgyccc cawkykhych
780

ckgcmccccb sbssbbmhhm ahhwmhaaca acaacaaaac aata
824

<210> 90
<211> 395
<212> DNA
<213> Lycopersicon esculentum

<400> 90

aaagcacaga aacagagatt atgaacaaca tacaacccaa ttagccaaaa gttcttagtt
60

ctggttgaca tgtcaaataa gatcctaggg acataataaa ttccagaaca ctggtcaaat
120

cacatcagaa tcaaacccca actacaaata atggataata aagaagggaa acacaattaa
180

tgatgtaaat tgagttagac ctaacaagtt acaccaatgc aatgctgctc tcaccacctg
240

5 gaggcttgcg aaccccgcca tagaagtctc gagattctac tttcccatct gcaaatatat
300

tgcttccact catttctcgc aactttgctg aactcaggtg cttctcagct gatgttggag
360

10

gattatcgcc cttgaatatg tcatttcctg tggaa
395

<210> 91
15 <211> 318
<212> DNA
<213> Lycopersicon esculentum

<220>
20 <221> misc_feature
<222> (315)..(315)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

25 <220>
<221> misc_feature
<222> (316)..(316)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

30

<220>
<221> misc_feature
<222> (318)..(318)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
35 H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<400> 91

40 tttgaaccgt ttgtrccacy gacttacwtt tkkgamaaga smcmaccaag agttgaggct
60

ttcttgcrgc cattgccagt aaggtyctca aagactactt cagcatcaaa accaccaaag
120

45

tttcaagtga aggcttcgct taagsagaaa gctttgacag gactgacagc agctgcactc
180

actgcttcca tggtcatgcc tgatgtagcc gaagcagcag agagtgtttc accatcccta
240

50

aagaactttt tgctcagcat ttctgcaggt ggagttgtgc ttgctgcaat tcttggcgct
300

55 ataattggtg tttannan
318

<210> 92

<211> 595
<212> DNA
<213> Lycopersicon esculentum

<220>
<221> misc_feature
<222> (418)..(418)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<220>
<221> misc_feature
<222> (433)..(433)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<220>
<221> misc_feature
<222> (484)..(484)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<220>
<221> misc_feature
<222> (549)..(549)
<223> W= A or T; M= A or C; R= A or G; Y= C or T; K= G or T; S= G or C;
H= A, C or T; B= C, G or T; V= A, C or G; D= A, G or T; N= A, C, G or T

<400> 92

cacgasagag ggttgacagt acggatgatt tttttcaaaa acaggatatt tttttcgatt 60

cactaaagaa aataaaagtg cttttaacca agtggttcct gattttggag ccgtaacgag 120

aatgatatca ttatcttgag cttgatattg tcgttgacat gcaatcaccc cttggataag 180

tcttggtaat gcccaaaagc cttgataatt atacacataa gatccaaccc atcctctttc 240

ttttggtagg gtagaaagca atttcttaca atcttcactt acatcatctt cttgtaaata 300

tttrtgagga gttggtgaag aggtttgaga aagggctcgc aacagaaacc agccgcgatg 360

cggcgtcgga ccaggggcaa gagcacccca gcgaacgcat cacaacggcc ccctcgcnca 420

caataacaac agnacaacac tcacacgcgg cgwagatccc gccatcccaa caacgcccac 480

caanaataca accccccca gaccaccttc actaccccac tccacscttc acggccaacc 540

acacacaanc aatcgaaacc acccggtcca caaacgcaca aacacaacga cacca 595

<210> 93
<211> 342
<212> DNA
<213> Lycopersicon esculentum

<400> 93

cagagaagay tttgcacatt cagctccckg gtgaggkgca cagtagaaag tgtaagttcc 60

tttctcactc aaagtgacac tgtatgtctc tcctgctgca ttcagaagat cctcttcaga 120

acatggaaat cttactagca tccacaccag ctgggatttc atcttcatca aatacgacgt 180

tgtgtgggaa ccctgcattg ttcttgaatg taattttctc accagcacta acgctgaagt 240

tcccaggaat aaaagctaga ctcccatcat caccaccaag caacacttca agtgccatgg 300

cattgctagc aagcatcgcg ctaacagcgg tggcasmaaa aa 342

<210> 94

<211> 434
<212> DNA
<213> Lycopersicon esculentum

5

<400> 94

gagaatgwwc taatcatccc attccaatgg tttataacaa ctggccataa aataaaaaac
60

10

taaaatatac gaaggagcat attcccagag agtatgacat gctctgatcc aagaacaaga
120

taaagacatt ctaaaactta caaccatcat cactcagaac gattggcata cctctccacc
180

15

ttttcatcaa gattgattcc aaccatagcc tcaccaagcc cacagctaat ttcagccagc
240

aattgtgggt cactgtaatg agtcactgct tgcacgatgg cacgtcccct ctttgcaggg
300

20

tcaccactct tgaagatacc agaacccacg aacacgccgt cacatcccaa ctgcatcata
360

25

agcgctgcat ctgctggtgt cgccacccca cctgctgcaa agtgaaccac agggagccta
420

30

ccaagttgct ttgt
434

35

<210> 95
<211> 472
<212> DNA
<213> Lycopersicon esculentum

40

<400> 95

45

50

55

tatccactca ggtctccgca agccagaaat gggatataca ccttgttacg accytcaagc
60

catccactac tgcaatctgt catgtcacag atgttcggaa gataatgtat aagtacaact
120

atatagtcgg awttgcatct agtctagcat tcggaaaatg gaagccatgc tacttctagc
180

ataaaaaaca gcagctagaa atcgtaactc caatgatacg aggaagtatt cagagtttag
240

agtgawgtac aatgcaattt agagaacaag catctgcaca tcraagttac ctaggtcctc
300

agcgcctgat ggacttccaa cttgttcaag aaggcgataa aggtctttct cattgaatcc
360

ttcaggtgga gagtagtttt cacaaactgc aaatgcctct gcacagcgga aagattgaat
420

tagatttatg ttatatagcc attctagtct tgctttaatg gatctttctc ga
472

<210> 96
<211> 222
<212> DNA
<213> Lycopersicon esculentum

<400> 96

ccacagtttc atgctgcacc tacatgtgta agcaactatc atagcaagtc tcggaacaat
60

tggtaggaaa aaatcmykta aggatatgaa acatactgty ctttcttcat ctgagtctgy
120

agagttaatt tttaactctt gggataaatg caaagawtta gacatggakg agtycttaac
180

acgtccagac aagaggcgta acacaggtac accttttctc ga
222

<210> 97
<211> 133
<212> DNA
<213> Lycopersicon esculentum

<400> 97

ttgtgcttga tgaattgtag gtccagtgca ggtttgcttc taaaacaggg agcactttgc
60

5 aagtggtgaa agttctatta gctgggaaag tgtagtttga gcagttttga gctgarttaa
120

caagaaaaat cga
133

10

<210> 98
<211> 249
<212> DNA
15 <213> Lycopersicon esculentum

<400> 98

20 ccgccactgg gtaattgagt ttcatattga tggttttgtt tttgttracg cttcttcctt
60

gttgagaggg ttcaatggag agattctatc tcgtcctcca ttagttgaag ctattgcctt
120

25 tgatcctatc ctttcaaagg ycaagatgat tgcagataat tggaatccat taaccaatga
180

ttctacggaa aatttattcc ctcactggag gagatgggca gagataaata tgagattttg
240

30

tgatgacat
249

<210> 99
35 <211> 284
<212> DNA
<213> Lycopersicon esculentum

<400> 99

40

tcgagtaagg cggatggata tggaacaagc catttcaagg agcaatttcc caggattttc
60

agctttgcaa cagcagaagt gtayctctgc agagatagat cataaccttt ggaaaggtgt
45 120

agtaattgtc aaagggagga atgagccagg aaactgatag actatgttgc gaaaataagc
180

50 tatacttcac taaaaaaagg ctagacgttt gagaaatgaa gcaagaacta acacctctca
240

ccaattgcat cattttctta gttcagttga tgtgatgagc ttgt
284

55

<210> 100
<211> 320

<212> DNA
<213> Lycopersicon esculentum

<400> 100

5

tcgatatccw ctcttgtttg ttgcaggagc wgaactataa attgcttgca ggaaccttga
60

10

catatgcttt ctgttgagac ttgaatcacc agcatggatt tgaatgcctt gccacagcca
120

gaggatgacg aygagatttt tggacaacaa ttagaagatg aaccacaaga acctatttta
180

15

cgtagtgatg agcstgcaga ttatgtcacg agtgctgtag agatttcacg tcgcgtatgt
240

ttctgcttat actgctcgct gtatcaacta ttgaacygta ctactacttg arcttgctcg
300

20

tttattggat atttcttttt
320

25

<210> 101
<211> 191
<212> DNA
<213> Lycopersicon esculentum

30

<400> 101

gaattcacac tasgttcgat gaaattgaaa cgttctcttt ctgaagaaka tacacaagaa
60

35

aaaatcttat agtcctcaac aatattcttc ttcgtaacag aaaacacgga agaaaatctc
120

ttctgaaaat ccctataatc actggctgga acttctccsa actctcaatt tttcaacctt
180

40

ctctatgtta a
191

45

<210> 102
<211> 279
<212> DNA
<213> Lycopersicon esculentum

50

<400> 102

55

ctgcagaadt actgtttgtt caggacttac taaatatcct aaacaaaatt gatgatagag 60

ccaataatgt atgcatgatt ggcggtccrt tcttttgtta tagcaagagc ttgaagctaa 120

ttttgtttgt cataatggcc gcactaattg tttattatct cagaatgaac aaaaagaagc 180

aagtcagaag ctttstactc tatactgaac aactttggaa ttggaactat gtacttatct 240

agccacgcct catagatctt tgtggtttag gagtgttaa 279

<210> 103
<211> 336
<212> DNA
<213> Lycopersicon esculentum

<400> 103

gaattcacaa tgaaaaakgk dgtaaaaaca cgaaatcaat caagcatgca agagataatg 60

ttgtccatcc agttgttgtt gatgtttcgg tattgtatgt gtgttgggag gagttatctg 120

grcagcaagt cgaggtttga acgtcaaaaa ggtatgggtt gtcttctctc tttgtccctt 180

ttcgaagaga cccctaaggt tcagacgaat ctattccaaa aactagggtt gttccttgtt 240

gcatctcctt ktcacaagct cccatcgcat cataagtagg gtatgtttga tggtagaatt 300

tacggatgta atttactttt gaaatgatta tgttaa 336

<210> 104
<211> 373
<212> DNA
<213> Lycopersicon esculentum

<400> 104

agagagacga gagctcgact agtgatagtg ttatgtgcaa cagttgaata gaaagatgya 60

cacgagcctc ggatcaatgg cagggaaaga ggcgtggtgc tacgaaccat aaaggcaagg 120

ttgagctttc ctttacagag tacatcgcct attccatact ccgctgatac tctttgataa 180

atcaaaatct gtggtgatct cgtagttctt ggggatccca gccaaaacca ccttcgaggt 240

tcaacacaac atagacagta tggcagaata tcaagacaat gactgctcga aactgctgat 300

ggcattatgt gcaaccgttg aatagagaga tgtacacgag tctcggatca atggcaggaa 360

aagagagtgc ttg 373

<210> 105
<211> 336
<212> DNA
<213> Lycopersicon esculentum

<400> 105

gaattcacaa tgaaaaakgk dgtaaaaaca cgaaatcaat caagcatgca agagataatg 60

ttgtccatcc agttgttgtt gatgtttcgg tattgtatgt gtgttgggag gagttatctg 120

grcagcaagt cgaggtttga acgtcaaaaa ggtatgggtt gtcttctctc tttgtccctt 180

ttcgaagaga cccctaaggt tcagacgaat ctattccaaa aactagggtt gttccttgtt 240

gcatctcctt ktcacaagct cccatcgcat cataagtagg gtatgtttga tggtagaatt 300

tacggatgta atttactttt gaaatgatta tgttaa 336

<210> 106
<211> 261
<212> DNA
<213> Lycopersicon esculentum

<400> 106

ctgcagaatt tgacttacat tttcctaatg aatctgatga taaggtgcta gatgatcyta
60

stktgtatca gaagctagta ggaaggttgc tttatctgac aataacaaga ccagacatag
120

ytttygyagt gyagctcttg agtcagttca tgcatagtcc taaagcatct tacatgsaag
180

ctgmaatgrr ggtggtaaga tatgtcaagc aggcaccagg actgggtata cttatggcag
240

ccaatacaac tgatcagtta a
261

<210> 107
<211> 450
<212> DNA
<213> Lycopersicon esculentum

<400> 107

ctgcagatgg tggtgacatt acaggaggtg gtgcaaccag cccaaaaggc gggatcgtaa
60

tgttatgatc acaaggtgga ggcacaggaa gactggtatt attatgttca gatggcaaag
120

tggcaccttc caggacttga tcaatgccat ggcatctgat ggaagcactt ttagtgcaga
180

tatttggact aacgactcga gcathgttga gaaacaaatg catgtatcca gaattgttga
240

ctctgaggaa aaggtcaggt tttgaagttg gtatcatgga tcctgttggg aagtgttgga
300

ggtggtcaaa aagcaacggt gatggtaagg aatgtcgttg caagaaatct accacgttgt
360

tttcttgtat tagtgtttgg gacagtgtct trtctcttgg catcaagaaa gtgatgtttc
420

cttttacaag gtcatcaggg gccatgttaa
450

<210> 108
<211> 124
<212> DNA
<213> Lycopersicon esculentum

<400> 108

ctgcagaasc agtacatagg ttgtattgam acctgtattt acaataagga gactctartg
60

ataccgacct atccctataa tgagtctaag acatcaayga tagagaygrt accattagag
120

ttaa
124

<210> 109
<211> 149
<212> DNA
<213> Lycopersicon esculentum

<400> 109

gacaagtaat ggttctaagt tgagggtgtt gatgtgctay gaaatattgr gacatttgat
60

gtttgataag tataagtatg aactaatact aaattaagtg aagtttttat gatttgrtat
120

ttttgttgaa tgtgtaagca aaatctcga
149

<210> 110
<211> 267
<212> DNA
<213> Lycopersicon esculentum

<400> 110

ctgcagaaag tgattcggtt ggagatgcag ttacacgaag cactcttaca tcggcttctg
60

ctggggtaga caaatatgct tcgactaact gtccacattc tgcttcttca tttgattatg
120

ttgtcagtac atttgatgag ggacatcatc agacaaaagt cttcagctct ttggattgtc
180

acaaggagtc aaaaatatct aatactaaca agaaaaggag acggtctggt gatagtcata
240

agcccagacc acgagatagg cagttaa
267

<210> 111
<211> 210
<212> DNA
<213> Lycopersicon esculentum

<400> 111

ctgcagaagt cacactgaas tcataccaaa gaccatttca actgctaaca ttagactaga
60

agagaacctt ccatgactgc cacagctttc cctctcagam ataccctctg cttctcatcg
120

tctagatgca gtttcacgac gccacctcta ggtgaggcct ggaccayaat acaataaaat
180

caatagggca aaagagaact atgaggttaa
210

<210> 112
<211> 165
<212> DNA
<213> Lycopersicon esculentum

<400> 112

ctgcagaaag atatagccag aggaaggtgg agcaatttca tgtggatagg wtgcataatg
60

catgttcttw ctttatttcg tatcttggtg aagcatagat atagacagat camagaagca
120

catygggatc taccacctac caagatgctc tcattttaca gttaa
165

<210> 113
<211> 373
<212> DNA
<213> Lycopersicon esculentum

<400> 113

agagagacga gagctcgact agtgatagtg ttatgtgcaa cagttgaata gaaagatgya
60

cacgagcctc ggatcaatgg cagggaaaga ggcgtggtgc tacgaaccat aaaggcaagg
120

ttgagctttc ctttacagag tacatcgcct attccatact ccgctgatac tctttgataa
180

atcaaaatct gtggtgatct cgtagttctt ggggatccca gccaaaacca ccttcgaggt
240

tcaacacaac atagacagta tggcagaata tcaagacaat gactgctcga aactgctgat
300

ggcattatgt gcaaccgttg aatagagaga tgtacacgag tctcggatca atggcaggaa
360

aagagagtgc ttg
373

<210> 114
<211> 312
<212> DNA
<213> Lycopersicon esculentum

<400> 114

ctgcagaatg gatatttcaa tctttgccat caaatactgg ctagatcgtt gcaatcgctc
60

cttgaattga acaaactcaa taacctaaaa aagttcacag atgaagattt tgttaccatt
120

gggctagctc attgtatgat tactaattta tcttttcgtt cacaaakgga accattagta
180

tttgaaatga tcctaagaga gaatcgtcat gataagcaay gtaagtttct acaccagaaa
240

ataaataatt gctccaacaa atacccactc aagactcact tcgcaagaac taagttgtcc
300

agaaacagtt aa
312

<210> 115
<211> 124
<212> DNA
<213> artificial

<400> 115

cacatacttg aggcagtaag tgagtgaatt ggtacgcagt cgatgagtcc tgagtaaagt 60

caggcattcg actagcgtat acgcagatcc gatcgattta taattaaagt caaattagaa 120

acca 124

<210> 116
<211> 122
<212> DNA
<213> artificial

<400> 116

cacatacttg aggcagtaag tgagtgaatt ggtacgcagt cgatgagtcc tgagtaaagt 60

caggcattcg actagcgtat acggatccga tcgatttata attaaagtca aattagaaac 120

ct 122

<210> 117
<211> 119
<212> DNA
<213> artificial

<400> 117

aaattcgaga cattctttat caaaggtgaa ttggtacgca gtcgatgagt cctgagtaaa 60

gcatcgactg gtactacgga ctcagatccg atgatttcag ttgtttacca ttcatattg 119

<210> 118
<211> 117
<212> DNA
<213> artificial

<400> 118

aatatgaatg gtaaacaact gaaatcgtga attggtacgc agtcgatgag tcctgagtaa 60

agcatcgact ggtactacgg actgatccga tctttgataa agaatgtctc gaatttt 117

<210> 119
<211> 114

<212> DNA
<213> artificial

<400> 119

atttccagga tattgcttag tttctgtgaa ttggtacgca gtcgatgagt cctgagtaaa
60

gtcagtcatg gatccgatca gatccgaaat aaatggaaag ttatatggaa aaac
114

<210> 120
14 <211> 112
<212> DNA
<213> artificial

<400> 120

atttccagga tattgcttag tttctgtgaa ttggtacgca gtcgatgagt cctgagtaaa
60

gtcagtcatg gatccgatga tccgaaataa atggaaagtt atatggaaaa ag
112

<210> 121
<211> 109
<212> DNA
<213> artificial

<400> 121

tttatatacc tgaaccaggc taattagtga attggtacgc agtcgatgag tcctgagtaa
60

agtctaacgt tacggcatga tccgtggatg ttcactttct ctaaatgac
109

<210> 122
<211> 107
<212> DNA
<213> artificial

<400> 122

tttatatacc tgaaccaggc taattagtga attggtacgc agtcgatgag tcctgagtaa
60

agtctaacgt tacggcatga tctggatgtt cactttctct aaatgat
107

<210> 123
<211> 104
<212> DNA
<213> artificial

<400> 123

cttgggattg tcaaattaga tattccgtga attggtacgc agtcgatgag tcctgagtaa
60

agtgatcagc tgatccgatc tgcactgcca ttaaggctaa tgta
104

<210> 124
<211> 102
<212> DNA
<213> artificial

<400> 124

cttgggattg tcaaattaga tattccgtga attggtacgc agtcgatgag tcctgagtaa
60

agtgatcagc tgatccgatg cactgccatt aaggctaatg tg
102

<210> 125
<211> 99
<212> DNA
<213> artificial

<400> 125

ttcactaacg aacataatat ccatctgtga attggtacgc agtcgatgag tcctgagtaa
60

agtcatacgt tacgggacac acttaaaaaa atctccacc
99

<210> 126
<211> 97
<212> DNA
<213> artificial

<400> 126

ttcactaacg aacataatat ccatctgtga attggtacgc agtcgatgag tcctgagtaa
60

agtcatacgt taggacacac ttaaaaaaat ctccact
97

<210> 127

<211> 94
<212> DNA
<213> artificial

5 <400> 127

ttcaacgatg atgttgttaa ctgtggtgaa ttggtacgca gtcgatgagt cctgagtaaa
60

10 gtagtcagat tttcgagtgg gaattgtgta ctcg
94

<210> 128
15 <211> 92
<212> DNA
<213> artificial

<400> 128
20

ttcaacgatg atgttgttaa ctgtggtgaa ttggtacgca gtcgatgagt cctgagtaaa
60

25 gtagtgattt tcgagtggga attgtgtact ca
92

<210> 129
<211> 89
30 <212> DNA
<213> artificial

<400> 129

35
gccctggtgt ttgcgtacta cagtgaattg gtacgcagtc gatgagtcct gagtaaagtt
60

cagcatgtga gagcatgcat gatgatggc
89
40

<210> 130
<211> 87
<212> DNA
45 <213> artificial

<400> 130

50 ccatcatcat gcatgctctc acgtgaattg gtacgcagtc gatgagtcct gagtaaagtt
60

55 cagctgtagt acgcaaacac cagggca
87

<210> 131
<211> 84
<212> DNA
<213> artificial

<400> 131

aagctggaag aactggtgct ggtgaattgg tacgcagtcg atgagtcctg agtaaagtag
60

gtgtaagccc agagatgtca gcag
84

<210> 132
<211> 82
<212> DNA
<213> artificial

<400> 132

tgctgacatc tctgggctta cacgtgaatt ggtacgcagt cgatgagtcc tgagtaaagg
60

cagcaccagt tcttccagct tg
82

<210> 133
<211> 79
<212> DNA
<213> artificial

<400> 133

ctacttgctg aagctgctag tgaattggta cgcagtcgat gagtcctgag taaagcgaat
60

gaaccatatc aagcaaagt
79

<210> 134
<211> 77
<212> DNA
<213> artificial

<400> 134

ctacttgctg aagctgctag tgaattggta cgcagtcgat gagtcctgag taaagaatga
60

accatatcaa gcaaagc
77

<210> 135
<211> 124

<212> DNA
<213> artificial

<400> 135

agtaatttta gaattcatta acaaaattac cgaattggta cgcagtcgat gagtcctgag
60

taatgcgatt agcgatacgt tagcgactta gccgtactgt tatatatgat cggaatgatt
120

acga
124

<210> 136
<211> 124
<212> DNA
<213> artificial

<400> 136

agtaatttta gaattcatta acaaaattac cgaattggta cgcagtcgat gagtcctgag
60

taatgcgatt agcgatacgt tagcgactta gccgtactgt tatatatgat cggaatgatt
120

acga
124

<210> 137
<211> 119
<212> DNA
<213> artificial

<400> 137

aagagggaaa ttagaaagca ctgaccgaat tggtacgcag tcgatgagtc ctgagtaatg
60

cattcgaaat cggactctga gactcatgcg atgactgaaa aagggttata ctggcgtga
119

<210> 138
<211> 117
<212> DNA
<213> artificial

<400> 138

aagagggaaa ttagaaagca ctgaccgaat tggtacgcag tcgatgagtc ctgagtaatg
60

ttcgaaatcg gactctgaga ctcatgcgat gactgaaaaa gggttatact ggcgtgg
117

<210> 139
<211> 114
<212> DNA
<213> artificial

<400> 139

aaatgatcaa ctatacatct ccaaaaccga attggtacgc agtcgatgag tcctgagtaa
60

tgatcagtcc agtcatggat ccgatcactc tgttaattga atattctaac ttat
114

<210> 140
<211> 112
<212> DNA
<213> artificial

<400> 140

aaatgatcaa ctatacatct ccaaaaccga attggtacgc agtcgatgag tcctgagtaa
60

tgcagtccag tcatggatcc gatcactctg ttaattgaat attctaactt aa
112

<210> 141
<211> 109
<212> DNA
<213> artificial

<400> 141

tttatatacc tgaaccaggc taattaccga attggtacgc agtcgatgag tcctgagtaa
60

tgcgacttcg ctaacgttac ggcatggatg ttcactttct ctaaatgac
109

<210> 142
<211> 107
<212> DNA
<213> artificial

<400> 142

tttatatacc tgaaccaggc taattaccga attggtacgc agtcgatgag tcctgagtaa 60

5 tgacttcgct aacgttacgg catggatgtt cactttctct aaatgat 107

<210> 143
10 <211> 104
<212> DNA
<213> artificial

<400> 143
15

tattagtcaa attagtgaat tccgtcccga attggtacgc agtcgatgag tcctgagtaa 60

20

tgactgcgga tcagctaaat aaatttgttg agtcgaatat aaag 104

25

<210> 144
<211> 102
<212> DNA
30 <213> artificial

<400> 144

35 tattagtcaa attagtgaat tccgtcccga attggtacgc agtcgatgag tcctgagtaa 60

tgtgcggatc agctaaataa attggttgag tcgaatataa aa 102

40

<210> 145
<211> 99
<212> DNA
<213> artificial
45
<400> 145

tggaggatta tcgcccttga atatccgaat tggtacgcag tcgatgagtc ctgagtaatg 60
50

tactggcata cgttacgtca ggtgcttctc agctgatgc 99

55 <210> 146
<211> 97
<212> DNA
<213> artificial

<400> 146

tggaggatta tcgcccttga atatccgaat tggtacgcag tcgatgagtc ctgagtaatg
60

ctggcatacg ttacgtcagg tgcttctcag ctgatgt
97

<210> 147
<211> 94
<212> DNA
<213> artificial

<400> 147

agaaagcttt gacaggactg acagccgaat tggtacgcag tcgatgagtc ctgagtaatg
60

gtggatcagc ttcaagtgaa ggcttcgctt aagc
94

<210> 148
<211> 92
<212> DNA
<213> artificial

<400> 148

agaaagcttt gacaggactg acagccgaat tggtacgcag tcgatgagtc ctgagtaatg
60

ggatcagctt caagtgaagg cttcgcttaa gg
92

<210> 149
<211> 89
<212> DNA
<213> artificial

<400> 149

attgtcgttg acatgcaatc accccgaatt ggtacgcagt cgatgagtcc tgagtaatgc
60

tcaaatgata tcattatctt gagcttgaa
89

<210> 150
<211> 87
<212> DNA
<213> artificial

<400> 150

attgtcgttg acatgcaatc accccgaatt ggtacgcagt cgatgagtcc tgagtaatgc
60

caatgatatc attatcttga gcttgat
87

<210> 151
<211> 84
<212> DNA
<213> artificial

<400> 151

ccaggaataa aagctagact cccccgaatt ggtacgcagt cgatgagtcc tgagtaatgc
60

acaccagcac taacgctgaa gttc
84

<210> 152
<211> 82
<212> DNA
<213> artificial

<400> 152

ccaggaataa aagctagact cccccgaatt ggtacgcagt cgatgagtcc tgagtaatgc
60

accagcacta acgctgaagt tt
82

<210> 153
<211> 79
<212> DNA
<213> artificial

<400> 153

cgctgcatct gctggtgtcc cgaattggta cgcagtcgat gagtcctgag taatggtcac
60

atcccaactg catcataag
79

<210> 154
<211> 77
<212> DNA
<213> artificial

<400> 154

cgctgcatct gctggtgtcc gaattggtac gcagtcgatg agtcctgagt aatgtcacat
60

cccaactgca tcataaa
77

<210> 155
<211> 124
<212> DNA
<213> artificial

<400> 155

gtacaatgca atttagagaa caagcgggaa ttggtacgca gtcgatgagt cctgagtaac
60

gctgatccga tcgatatcga cgtagctgca tcgtaatcgg gaagtattca gagtttagag
120

tgaa
124

<210> 156
<211> 122
<212> DNA
<213> artificial

<400> 156

gtacaatgca atttagagaa caagcgggaa ttggtacgca gtcgatgagt cctgagtaac
60

gcatccgatc gatatcgacg tagctgcatc gtaatcggga agtattcaga gtttagagtg
120

at
122

<210> 157
<211> 119
<212> DNA
<213> artificial

<400> 157

cttaacacgt ccagacaaga ggcgggaatt ggtacgcagt cgatgagtcc tgagtaacgc
60

accatgtcga cgtagatccg tatagcactg agtcgcaaag aattagacat ggatgagtt
119

<210> 158

<211> 117
<212> DNA
<213> artificial

5 <400> 158

cttaacacgt ccagacaaga ggcgggaatt ggtacgcagt cgatgagtcc tgagtaacgc 60

10 ccatgtcgac gtagatccgt atagcactga gtccaaagat ttagacatgg aggagtc 117

<210> 159
15 <211> 114
<212> DNA
<213> artificial

<400> 159
20

ttaacaagaa aaatcggtca ggactgggaa ttggtacgca gtcgatgagt cctgagtaac 60

25 gccgtacgca tgctaacgtt acggactatc tagtttgagc agttttgagc tgaa 114

<210> 160
<211> 112
30 <212> DNA
<213> artificial

<400> 160

35

ttaacaagaa aaatcggtca ggactgggaa ttggtacgca gtcgatgagt cctgagtaac 60

gctacgcatg ctaacgttac ggactatcta gtttgagcag ttttgagctg ag
40 112

<210> 161
<211> 109
45 <212> DNA
<213> artificial

<400> 161

50

acgcttcttc cttgttgaga gggggaattt ggtacgcagt cgatgagtcc tgagtaacgc 60

cgatgctcag gctatcgaca tgttcatatt gatggttttg tttttgtta
55 109

<210> 162
<211> 107

<212> DNA
<213> artificial

<400> 162

acgcttcttc cttgttgaga ggggggaatt ggtacgcagt cgatgagtcc tgagtaacgc
60

atgctcaggc tatcgacatg ttcatattga tggttttgtt tttgttg
107

<210> 163
<211> 104
<212> DNA
<213> artificial

<400> 163

ctctgcagag atagatcata acctgggaat tggtacgcag tcgatgagtc ctgagtaacg
60

catcacgtca tgctgagcat agctttgcaa cagcagaagt gtat
104

<210> 164
<211> 102
<212> DNA
<213> artificial

<400> 164

ctctgcagag atagatcata acctgggaat tggtacgcag tcgatgagtc ctgagtaacg
60

ccacgtcatg ctgagcatag ctttgcaaca gcagaagtgt ac
102

<210> 165
<211> 99
<212> DNA
<213> artificial

<400> 165

gaactataaa ttgcttgcag gaaccgggaa ttggtacgca gtcgatgagt cctgagtaac
60

gctcgctaac gttacgctct cttgtttgtt gcaggagca
99

<210> 166
<211> 97

<212> DNA
<213> artificial

<400> 166

gaactataaa ttgcttgcag gaaccgggaa ttggtacgca gtcgatgagt cctgagtaac
60

gcgctaacgt tacgcactct tgtttgttgc aggagct
97

<210> 167
<211> 94
<212> DNA
<213> artificial

<400> 167

aactctcaat ttttcaacct tctctaggga attggtacgc agtcgatgag tcctgagtaa
60

cgcgtcattc gaatcactgg ctggaacttc tccc
94

<210> 168
<211> 92
<212> DNA
<213> artificial

<400> 168

aactctcaat ttttcaacct tctctaggga attggtacgc agtcgatgag tcctgagtaa
60

cgccattcga atcactggct ggaacttctc cg
92

<210> 169
<211> 89
<212> DNA
<213> artificial

<400> 169

ttcttttgtt atagcaagag cttgaaggga attggtacgc agtcgatgag tcctgagtaa
60

cgcccgatgt atgcatgatt ggcggtcca
89

<210> 170
<211> 87
<212> DNA
<213> artificial

<400> 170

ttcttttgtt atagcaagag cttgaaggga attggtacgc agtcgatgag tcctgagtaa
60

cgccatgtat gcatgattgg cggtccg
87

<210> 171
<211> 84
<212> DNA
<213> artificial

<400> 171

tcacaagctc ccatcgcatc atgggaattg gtacgcagtc gatgagtcct gagtaacgct
60

gttgttcctt gttgcatctc cttt
84

<210> 172
<211> 82
<212> DNA
<213> artificial

<400> 172

tcacaagctc ccatcgcatc atgggaattg gtacgcagtc gatgagtcct gagtaacggt
60

tgttccttgt tgcatctcct tg
82

<210> 173
<211> 79
<212> DNA
<213> artificial

<400> 173

acacgagcct cggatcaatg ggaattggta cgcagtcgat gagtcctgag taacgtgcaa
60

cagttgaata gaaagatgt
79

<210> 174
<211> 77

<212> DNA
<213> artificial

<400> 174

5

acacgagcct cggatcaatg ggaattggta cgcagtcgat gagtcctgag taacgcaaca
60

10 gttgaataga aagatgc
77

<210> 175
<211> 124
15 <212> DNA
<213> artificial

<400> 175

20

tcacaagctc ccatcgcatc atagagaatt ggtacgcagt cgatgagtcc tgagtaagcg
60

actcgtacca tgtcgacgta gatccgtata gcactgagtc gttgttcctt gttgcatctc
25 120

cttg
124

30 <210> 176
<211> 122
<212> DNA
<213> artificial

35 <400> 176

tcacaagctc ccatcgcatc atagagaatt ggtacgcagt cgatgagtcc tgagtaagcc
60

40

tcgtaccatg tcgacgtaga tccgtatagc actgagtcgt tgttccttgt tgcatctcct
120

tt
45 122

<210> 177
<211> 119
50 <212> DNA
<213> artificial

<400> 177

55

gcaccaggac tgggtatact tatgagaatt ggtacgcagt cgatgagtcc tgagtaagcg
60

atccgatcga tatcgacgta gctgcatcgt aatcggaggt ggtaagatat gtcaagcag
119

<210> 178
<211> 117
<212> DNA
<213> artificial

<400> 178

gcaccaggac tgggtatact tatgagaatt ggtacgcagt cgatgagtcc tgagtaagct
60

ccgatcgata tcgacgtagc tgcatcgtaa tcgagggtgg taagatatgt caagcaa
117

<210> 179
<211> 114
<212> DNA
<213> artificial

<400> 179

tctcttggca tcaagaaagt gatggagaat tggtacgcag tcgatgagtc ctgagtaagc
60

tatcgagtcg actacgttgc atacggatct attagtgttt gggacagtgt ctta
114

<210> 180
<211> 112
<212> DNA
<213> artificial

<400> 180

tctcttggca tcaagaaagt gatggagaat tggtacgcag tcgatgagtc ctgagtaagc
60

tcgagtcgac tacgttgcat acggatctat tagtgtttgg gacagtgtct tg
112

<210> 181
<211> 109
<212> DNA
<213> artificial

<400> 181

gatagagatg gtaccattag agttagagaa ttggtacgca gtcgatgagt cctgagtaag 60

cgtagatccg tatagcactg agtccctata atgagtctaa gacatcaac 109

<210> 182
<211> 107
<212> DNA
<213> artificial

<400> 182

gatagagacg ataccattag agttagagaa ttggtacgca gtcgatgagt cctgagtaag 60

cgtagatccg tatagcactg agcctataat gagtctaaga catcaat 107

<210> 183
<211> 104
<212> DNA
<213> artificial

<400> 183

gacatttgat gtttgaagta taagtatgag aattggtacg cagtcgatga gtcctgagta 60

agctcgacgt gctatgcagg tgttgatgtg ctatgaaata ttga 104

<210> 184
<211> 102
<212> DNA
<213> artificial

<400> 184

gacatttgat gtttgaagta taagtatgag aattggtacg cagtcgatga gtcctgagta 60

agccgacgtg ctatgcagtg ttgatgtgct acgaaatatt gg 102

<210> 185
<211> 99
<212> DNA
<213> artificial

<400> 185

gtccacattc tgcttcttca tttggagaat tggtacgcag tcgatgagtc ctgagtaagc
60

gtgcatatgc cagtgtagac aaatatgctt cgactaact
99

<210> 186
<211> 97
<212> DNA
<213> artificial

<400> 186

gtccacattc tgcttcttca tttggagaat tggtacgcag tcgatgagtc ctgagtaagc
60

gcatatgcca gtgtagacaa atatgcttcg actaacc
97

<210> 187
<211> 94
<212> DNA
<213> artificial

<400> 187

aatacaataa aatcaatagg gcaaaaggag aattggtacg cagtcgatga gtcctgagta
60

agcctacgga ctctctaggt gaggcctgga ccat
94

<210> 188
<211> 94
<212> DNA
<213> artificial

<400> 188

aatacaataa aatcaatagg gcaaaaggag aattggtacg cagtcgatga gtcctgagta
60

agcctacgga ctctctaggt gaggcctgga ccat
94

<210> 189
<211> 89
<212> DNA
<213> artificial

<400> 189

agaagcacat cgggatctac cacgagaatt ggtacgcagt cgatgagtcc tgagtaagcc 60

5

cgattgaagc atagatatag acagatcac 89

10 <210> 190
<211> 87
<212> DNA
<213> artificial

15 <400> 190

agaagcacat tgggatctac cacgagaatt ggtacgcagt cgatgagtcc tgagtaagcg 60

20

attgaagcat agatatagac agatcaa 87

<210> 191
25 <211> 84
<212> DNA
<213> artificial

<400> 191

30

acacgagcct cggatcaatg gcgagaattg gtacgcagtc gatgagtcct gagtaagcgg 60

35 tgcaacagtt gaatagaaag atgt 84

<210> 192
<211> 82
40 <212> DNA
<213> artificial

<400> 192

45

acacgagcct cggatcaatg gcgagaattg gtacgcagtc gatgagtcct gagtaagctg 60

caacagttga atagaaagat gc 82

50

<210> 193
<211> 79
<212> DNA
55 <213> artificial

<400> 193

gttgcaatcg ctccttgaat tgagaattgg tacgcagtcg atgagtcctg agtaagcgcc
60

5 atcaaatact ggctagatc
79

<210> 194
<211> 77
10 <212> DNA
<213> artificial

<400> 194

15

gttgcaatcg ctccttgaat tgagaattgg tacgcagtcg atgagtcctg agtaagccat
60

caaatactgg ctagatt
77
20

<210> 195
<211> 120
<212> DNA
25 <213> artificial

<400> 195

30 gtacaatgca atttagagaa caagcggaat tggtacgcag tcgatgagtc ctgagtaagg
60

atccgatcga tatcgacgta gctgcatcgt aatcgggaag tattcagagt ttagagtgaa
120

35

<210> 196
<211> 118
<212> DNA
<213> artificial
40

<400> 196

gtacaatgca atttagagaa caagcggaat tggtacgcag tcgatgagtc ctgagtaagt
45 60

ccgatcgata tcgacgtagc tgcatcgtaa tcgggaagta ttcagagttt agagtgat
118

50 <210> 197
<211> 116
<212> DNA
<213> artificial

55 <400> 197

cttaacacgt ccagacaaga ggcggaattg gtacgcagtc gatgagtcct gagtaagacc 60

5 atgtcgacgt agatccgtat agcactgagt cgcaaagaat tagacatgga tgagtt 116

<210> 198
<211> 114
10 <212> DNA
<213> artificial

<400> 198

15 cttaacacgt ccagacaaga ggcggaattg gtacgcagtc gatgagtcct gagtaagcca 60

tgtcgacgta gatccgtata gcactgagtc caaagattta gacatggagg agtc 114
20

<210> 199
<211> 112
<212> DNA
25 <213> artificial

<400> 199

30 ttaacaagaa aaatcggtca ggactggaat tggtacgcag tcgatgagtc ctgagtaagc 60

gtacgcatgc taacgttacg gactatcgta gtttgagcag ttttgagctg aa
35 112

<210> 200
<211> 110
40 <212> DNA
<213> artificial

<400> 200

45 ttaacaagaa aaatcggtca ggactggaat tggtacgcag tcgatgagtc ctgagtaagt 60

acgcatgcta acgttacgga ctatcgtagt ttgagcagtt ttgagctgag 110
50

<210> 201
<211> 108
<212> DNA
55 <213> artificial

<400> 201

acgcttcttc cttgttgaga gggggaattg gtacgcagtc gatgagtcct gagtaagcta
60

gatgctcagg ctatcgacat gttcatattg atggttttgt ttttgtta
108

<210> 202
<211> 106
<212> DNA
<213> artificial

<400> 202

acgcttcttc cttgttgaga gggggaattg gtacgcagtc gatgagtcct gagtaagaga
60

tgctcaggct atcgacatgt tcatattgat ggttttgttt ttgttg
106

<210> 203
<211> 104
<212> DNA
<213> artificial

<400> 203

ctctgcagag atagatcata acctggaatt ggtacgcagt cgatgagtcc tgagtaagga
60

gatcacgtca tgctgagcat agctttgcaa cagcagaagt gtat
104

<210> 204
<211> 102
<212> DNA
<213> artificial

<400> 204

ctctgcagag atagatcata acctggaatt ggtacgcagt cgatgagtcc tgagtaagga
60

tcacgtcatg ctgagcatag ctttgcaaca gcagaagtgt ac
102

<210> 205
<211> 100
<212> DNA
<213> artificial

<400> 205

gaactataaa ttgcttgcag gaaccggaat tggtacgcag tcgatgagtc ctgagtaagt
60

5 cgctaacgtt acggcatctc tcttgtttgt tgcaggagca
100

<210> 206
<211> 98
10 <212> DNA
<213> artificial

<400> 206

15

gaactataaa ttgcttgcag gaaccggaat tggtacgcag tcgatgagtc ctgagtaagg
60

ctaacgttac ggcatcactc ttgtttgttg caggagct
98
20

<210> 207
<211> 96
<212> DNA
25 <213> artificial

<400> 207

30 acacgagcct cggatcaatg gcggaattgg tacgcagtcg atgagtcctg agtaagtgct
60

agcacgtact ggtgcaacag ttgaatagaa agatgt
96

35

<210> 208
<211> 94
<212> DNA
<213> artificial
40

<400> 208

acacgagcct cggatcaatg gcggaattgg tacgcagtcg atgagtcctg agtaagctag
45 60

cacgtactgg tgcaacagtt gaatagaaag atgc
94

50 <210> 209
<211> 92
<212> DNA
<213> artificial

55 <400> 209

ttcttttgtt atagcaagag cttgaaggaa ttggtacgca gtcgatgagt cctgagtaag
60

5

ccgattagca tgtatgcatg attggcggtc ca
92

10

<210> 210
<211> 90
<212> DNA
<213> artificial

15

<400> 210

ttcttttgtt atagcaagag cttgaaggaa ttggtacgca gtcgatgagt cctgagtaag
60

20

ccgtagcatg tatgcatgat tggcggtccg
90

25

<210> 211
<211> 88
<212> DNA
<213> artificial

30

<400> 211

tcacaagctc ccatcgcatc ataggaattg gtacgcagtc gatgagtcct gagtaagcgt
60

35

tacggttgtt ccttgttgca tctccttt
88

40

<210> 212
<211> 86
<212> DNA
<213> artificial

45

<400> 212

tcacaagctc ccatcgcatc ataggaattg gtacgcagtc gatgagtcct gagtaagcta
60

50

cggttgttcc ttgttgcatc tccttg
86

<210> 213
<211> 84

55

<212> DNA
<213> artificial

<400> 213

aactctcaat ttttcaacct tctctaggaa ttggtacgca gtcgatgagt cctgagtaag
60

5

gtatcactgg ctggaacttc tccc
84

10 <210> 214
<211> 82
<212> DNA
<213> artificial

15 <400> 214

aactctcaat ttttcaacct tctctaggaa ttggtacgca gtcgatgagt cctgagtaag
60

20

atcactggct ggaacttctc cg
82

<210> 215
25 <211> 16
<212> DNA
<213> artificial

<400> 215

30

gatgagtcct gagtaa
16

35 <210> 216
<211> 16
<212> DNA
<213> artificial

40 <400> 216

gactgcgtac caattc
16

45

<210> 217
<211> 17
<212> DNA
<213> artificial

50

<400> 217

gactgcgtac caattca
17

55

<210> 218
<211> 17

<212> DNA
<213> artificial

<400> 218

```
gactgcgtac caattcg
17
```

<210> 219
<211> 17
<212> DNA
<213> artificial

<400> 219

```
gactgcgtac caattct
17
```

Claims

1. A method for determining the presence or absence of a target sequence in a nucleic acid sample, wherein the method comprises the steps of :

(a) providing to a nucleic acid sample at least one circularisable probe for each target sequence to be detected in the sample, whereby the probe has a first target specific section at its 5 '-end that is complementary to a first part of a target sequence and a second target specific section at its 3' -end that is complementary to a second part of the target sequence, whereby the first and second part of the target sequence are located adjacent to each other, and whereby the probe further comprises a tag section that is essentially non-complementary to the target sequence, whereby the tag section may comprise a stuffer sequence and whereby the tag section comprises at least one primer-binding sequence;
(b) allowing the first and second target specific sections of the circular probe to anneal to the first and second parts of target sequences whereby the first and second target specific sections of the probe are annealed adjacent on the target sequence;
(c) providing means for ligating the first and second target specific sections annealed adjacently to the target sequence and allowing the first and second target specific sections to be connected, to produce a ligated circular probe, corresponding to a target sequence in the sample;
(d) providing at least a first primer that is complementary to the primer-binding sequence, and a polymerase enzyme;
(e) amplifying the resulting mixture to produce an amplified sample comprising amplicons that are linear representations of the ligated circular probes;
(f) determining the presence or absence of a target sequence in a sample by detecting the presence or absence of the correspondihg amplicon;

wherein the at least one circularisable probe contains a blocking section that comprises a blocking group that is located adjacent to the 3' end of the primer binding site, such that the blocking section stops elongation or amplification of the primer hybridised to the circularised probe, thereby generating a linear representation of the circular probe and such that the blocking group is excluded from the primer elongation or amplification.

2. A method according to claim 1, wherein an amplicon in an amplified sample corresponding to a target sequence in a sample differs in length from an amplicon in the amplified sample corresponding to a different target sequence in the sample, wherein the difference in length is provided by stuffer sequences of different length incorporated in the probe.

3. A method according to claims 1-3, wherein the amplicon has a length that corresponds to the length of the connected

circular probe.

4. A method according to claims 1-3, wherein the length difference is provided by the length of the stuffer sequence.

5. A method according to any one of claims 1-4, wherein two different circular probes contain the same primer-binding site capable of hybridising to a single primer sequence.

6. A method according to any one of claims 1-5, wherein the primer comprises a label.

7. A method according to claim 1-6, wherein the primer contains a universal primer having a section that is complementary to the primer binding sequence and further contains at least one selective nucleotide at its 3' -end.

8. A method according to claim 1, wherein:

(a) at least two groups of circularisable oligonucleotide probes are provided to a sample, whereby each group of circularisable oligonucleotide probes has tag sequences with at least one group-specific primer-binding site;
(b) the ligated circular probes of each group are amplified from a first primer wherein at least one primer is complementary to the group-specific primer-binding site, and whereby at least one of the primers of a group comprises a group-specific label; and,
(c) in each group, an amplified ligated probe corresponding to a target sequence in the sample, differs in length from an amplified ligated probe corresponding to a different target sequence in the sample;

wherein the difference in length is provided by the use of stuffer sequences incorporated in the probe of different length.

9. A method according to claim 8, whereby in a first part of the groups of amplified ligated probes are produced having an even number of nucleotides and in a second part of the groups of amplified ligated probes are produced having an odd number of nucleotides.

10. A method according to claim 9, wherein the groups of ligated amplified probes having an even number of nucleotides and the groups of ligated amplified probes having an odd number of nucleotides are labelled with fluorescent labels which have the least overlap in their emission spectra.

11. A method according to claim 10, wherein a first and second groups of ligated amplified probes having an even number of nucleotides are produced and a third and fourth group of ligated amplified probes having an odd number of nucleotides are produced and whereby the first and second group are labelled with FAM and NED, respectively, and the third and fourth group are labelled with (ET-) ROX and either JOE or HEX, respectively; or whereby the first and second group are labelled with (ET-)ROX and either JOE or HEX, respectively, and the third and fourth group are labelled with FAM and NED, respectively.

12. A method according to any one of the preceding claims, wherein the circularisable probe further comprises a second primer-binding site.

13. A method according to claim 12, wherein the blocking section stopping the primer elongation comprises a blocking group, and wherein the blocking group is located between the two primer binding sites, adjacent to the 3'end of the forward primer binding site and adjacent to the 5'-end of the reverse primer binding site such that the blocking group is excluded from the primer elongation or amplification.

14. A method according to any of the preceding claims, wherein the polymerase does not express significant strand displacement activity.

15. Method according to claim 1, further comprising the following steps:

(a) (e1) repeating steps (a) to (e) to generate at least two amplified samples;
(b) (e2) consecutively applying at least part of the amplified samples obtained in steps (e) and (e1), to an application location of a channel of an electrophoretic device, electrophoretically separating the amplicons in the amplified samples and detecting the separated amplicons at a detection location located distal from the application location of the channel; whereby the time period between the consecutively applied amplified samples

is such that the slowest migrating amplified connected probe in an amplified sample is detected at the detection location before the fastest migrating amplified connected probe of a subsequently applied amplified sample is detected at the detection location.

16. A method according to any one of the preceding claims, wherein the target nucleotide sequence contains a polymorphism, preferably a single nucleotide polymorphism.

17. A method according to any one of the preceding claims, wherein the target nucleotide sequence is a DNA molecule selected from the group consisting of: cDNA, genomic DNA, restriction fragments, adapter-ligated restriction fragments, amplified adapter-ligated restriction fragments and AFLP fragments.

18. Use of a method as defined in any of the preceding claims, for high throughput detection of a multiplicity of target nucleotide sequences.

19. Use of a method according to claim 18, for the detection of polymorphisms, preferably single nucleotide polymorphisms.

20. Use of a method according to claim 19, for transcript profiling.

21. Use according to claim 18, for the detection of the quantitative abundance of target nucleic acid sequences.

22. Use according to claim 18, for genetic mapping, gene discovery, marker assisted selection, seed quality control, hybrid selection, QTL mapping, bulked segregant analysis, DNA fingerprinting and for disclosing information relating to traits, disease resistance, yield, hybrid vigour, and/or gene function.

23. Circularisable nucleic acid probe comprising a first target specific section at its 5' -end that is complementary to a first part of a target sequence and a second target specific section at its 3'-end that is complementary to a second part of the target sequence, whereby the probe further comprises a tag section that is essentially non-complementary to the target sequence, whereby the tag section may comprise a stuffer sequence and whereby the tag section comprises at least one primer-binding sequence, wherein the taq-section further contains a blocking section that comprises a blocking group that is located adjacent to the 3' end of the primer binding site such that the blocking section is able to stop elongation or amplification of any primer hybridised to said circularizable nucleic acid probe.

24. A circularisable oligonucleotide acid probe according to claim 23 for use in a method as defined in claims 1-16.

25. A set of two or more circularisable oligonucleotide probes according to claim 23, for use in a method as defined in claims 1-16.

26. Use of a set of two or more oligonucleotide probes as defined in claim 23, wherein the set comprises a probe for each allele of a single nucleotide polymorphism.

27. A set of primers, together with a probe or set of probes as defined in claims 23-26 for use in a method according to any one of claims 1-16, the set of primers comprising a first primer and one or more second primers, wherein each second primer contains a label.

28. A kit comprising oligonucleotide probes as defined in claims 23, 24 or 25, suitable for use in a method as defined in claims 1-16.

29. A kit according to claim 28, further comprising primers for use in a method as defined in claims 1-16.

Patentansprüche

1. Ein Verfahren zur Bestimmung des Vorhandenseins oder des Fehlens einer Zielsequenz in einer Nukleinsäureprobe, wobei das Verfahren die folgenden Schritte umfasst:

a) das Ausstatten einer Nukleinsäureprobe mit wenigstens einer zirkularisierbaren Sonde für jede in der Probe nachzuweisende Zielsequenz, wobei die Sonde einen ersten zielspezifischen Abschnitt an ihrem 5'-Ende auf-

weist, der zu einem ersten Teil einer Zielsequenz komplementär ist, sowie einen zweiten zielspezifischen Abschnitt an ihrem 3'-Ende aufweist, der zu einem zweiten Teil der Zielsequenz komplementär ist, wobei der erste und der zweite Teil der Zielsequenz benachbart zueinander positioniert sind, und wobei die Sonde zusätzlich einen Tag-Abschnitt umfasst, der im Wesentlichen zu der Zielsequenz nicht komplementär ist, wobei der Tag-Abschnitt eine Füllsequenz umfassen kann und wobei der Tag-Abschnitt wenigstens eine primerbindende Sequenz umfasst;

b) das Ermöglichen, dass die ersten und zweiten zielspezifischen Abschnitte der zirkulären Sonde an die ersten und zweiten Teile der Zielsequenzen binden, wobei die ersten und zweiten zielspezifischen Abschnitte der Sonde benachbart zueinander auf der Zielsequenz binden;

c) das Bereitstellen von Mitteln zum Ligieren der ersten und zweiten zielspezifischen Abschnitte, die zueinander benachbart an die Zielsequenz gebunden sind, und das Ermöglichen, dass die ersten und zweiten zielspezifischen Abschnitte miteinander verbunden werden, um eine ligierte zirkuläre Sonde zu produzieren, die mit einer Zielsequenz in der Probe korrespondiert;

d) das Bereitstellen wenigstens eines ersten Primers, der zu der primerbindenden Sequenz komplementär ist, sowie eines Polymeraseenzyms;

e) das Amplifizieren der resultierenden Mischung zur Herstellung einer amplifizierten Probe, die Amplikons umfasst, die lineare Verkörperungen der ligierten zirkulären Sonden sind;

f) das Bestimmen des Vorhandenseins oder des Fehlens einer Zielsequenz in einer Probe durch den Nachweis des Vorhandenseins oder des Fehlens des korrespondierenden Amplikons;

wobei die wenigstens eine zirkularisierbare Sonde einen blockierenden Abschnitt enthält, der eine blockierende Gruppe umfasst, die benachbart zu dem 3'-Ende der primerbindenden Stelle so positioniert ist, dass der blockierende Abschnitt die Verlängerung oder Amplifikation des Primers verhindert, der an die zirkularisierte Sonde hybridisiert ist, wodurch eine lineare Verkörperung der zirkulären Sonde generiert wird, und derart, dass die blockierende Gruppe von der Primerverlängerung oder Amplifikation ausgeschlossen ist.

2. Ein Verfahren gemäß Anspruch 1, wobei ein Amplikon in einer amplifizierten Probe, das mit einer Zielsequenz in einer Probe korrespondiert, sich in der Länge von einem Amplikon in der amplifizierten Probe, das mit einer unterschiedlichen Zielsequenz in der Probe korrespondiert, unterscheidet, wobei der Unterschied in der Länge durch Füllsequenzen von unterschiedlicher Länge bereit gestellt wird, die in die Sonde eingebaut sind.

3. Ein Verfahren gemäß den Ansprüchen 1 - 3, wobei das Amplikon eine Länge aufweist, die mit der Länge der verbundenen zirkulären Sonde korrespondiert.

4. Ein Verfahren gemäß den Ansprüchen 1 - 3, wobei der Längenunterschied durch die Länge der Füllsequenz bereit gestellt wird.

5. Ein Verfahren gemäß einem der Ansprüche 1 - 4, wobei zwei unterschiedliche zirkuläre Sonden die gleiche Primerbindungsstelle enthalten, die in der Lage ist, an eine einzelne Primersequenz zu hybridisieren.

6. Ein Verfahren gemäß einem der Ansprüche 1 - 5, wobei der Primer einen Marker umfasst.

7. Ein Verfahren gemäß den Ansprüchen 1 - 6, wobei der Primer einen Universalprimer mit einem Abschnitt enthält, der zu der Primerbindungssequenz komplementär ist und zusätzlich wenigstens ein selektives Nukleotid an seinem 3'-Ende enthält.

8. Ein Verfahren gemäß Anspruch 1, in dem

a) eine Probe mit wenigstens zwei Gruppen der zirkularisierbaren Oligonukleotidsonden ausgestattet wird, wobei jede Gruppe der zirkularisierbaren Oligonukleotidsonden Tag-Sequenzen mit wenigstens einer gruppenspezifischen Primerbindungsstelle aufweist;

b) die ligierten zirkulären Sonden von jeder Gruppe mit einem ersten Primer amplifiziert werden, wobei wenigstens ein Primer komplementär zu der gruppenspezifischen Primerbindungsstelle ist und wobei wenigstens einer der Primer aus einer Gruppe einen gruppenspezifischen Marker umfasst; und

c) in jeder Gruppe eine amplifizierte ligierte Sonde, die mit einer Zielsequenz in der Probe korrespondiert, sich in der Länge von einer amplifzierten ligierten Sonde, die mit einer unterschiedlichen Zielsequenz in der Probe korrespondiert, unterscheidet,

wobei der Unterschied in der Länge durch die Verwendung von Füllsequenzen in verschiedener Länge bereit gestellt wird, die in die Sonde eingebaut sind.

9. Ein Verfahren gemäß Anspruch 8, wobei in einem ersten Teil der Gruppen der amplifizierten ligierten Sonden diese mit einer geradzahligen Anzahl von Nukleotiden hergestellt werden und in einem zweiten Teil der Gruppen der amplifizierten ligierten Sonden diese mit einer ungeraden Anzahl von Nukleotiden hergestellt werden.

10. Ein Verfahren gemäß Anspruch 9, wobei die Gruppen der ligierten amplifzierten Sonden mit einer geradzahligen Anzahl von Nukleotiden und die Gruppen der ligierten amplifizierten Sonden mit einer ungeraden Anzahl von Nukleotiden mit Fluoreszenzmarkern markiert sind, die die geringste Überlappung in deren Emissionsspektren aufzeigen.

11. Ein Verfahren gemäß Anspruch 10, wobei eine erste und zweite Gruppe von ligierten amplifizierten Sonden mit einer geradzahligen Anzahl von Nukleotiden hergestellt werden und eine dritte und vierte Gruppe von ligierten amplifizierten Sonden mit einer ungeraden Anzahl von Nukleotiden hergestellt werden und wobei die erste und zweite Gruppe jeweils mit FAM und NED markiert werden und die dritte und vierte Gruppe jeweils mit (ET-) ROX und entweder JOE oder HEX markiert werden; oder wobei die erste und zweite Gruppe mit (ET-)ROX und entweder JOE oder HEX markiert werden und die dritte und vierte Gruppe jeweils mit FAM und NED markiert werden.

12. Ein Verfahren gemäß einem der vorangegangenen Ansprüche, wobei die zirkularisierbare Sonde zusätzlich eine zweite Primerbindungsstelle umfasst.

13. Ein Verfahren gemäß Anspruch 12, wobei der blockierende Abschnitt, der die Primerverlängerung stoppt, eine blockierende Gruppe umfasst und wobei die blockierende Gruppe zwischen den zwei Primerbindungsstellen benachbart zu dem 3'-Ende der Bindungsstelle des Vorwärtsprimers und benachbart zu dem 5'-Ende der Bindungsstelle des Rückwärtsprimers so positioniert ist, dass die blockierende Gruppe von der Primerverlängerung oder Amplifikation ausgeschlossen ist.

14. Ein Verfahren gemäß einem der vorangegangenen Ansprüche, wobei die Polymerase keine wesentliche Strangverdrängungsaktivität exprimiert.

15. Ein Verfahren gemäß Anspruch 1, das zusätzlich die folgenden Schritte umfasst:

a) (e1) die Wiederholung der Schritte (a) bis (e) zur Generierung von wenigstens zwei amplifizierten Proben;
b) (e2) das nacheinander Auftragen wenigstens eines Teils der amplifizierten Proben, die in den Schritten (e) und (e1) erhalten wurden, auf eine Auftragsposition eines Kanals einer elektrophoretischen Vorrichtung, das elektrophoretische Trennen der Amplikons in den amplifizierten Proben und das Nachweisen der getrennten Amplikons an einer Nachweisposition, die distal von der Auftragsposition des Kanals positioniert ist; wobei der Zeitraum zwischen den nacheinander aufgetragenen amplifizierten Proben derart ist, dass die am langsamsten wandernde amplifizierte gebundene Sonde in einer amplifizierten Probe an der Nachweisposition nachgewiesen wird, bevor die am schnellsten wandernde amplifizierte gebundene Sonde einer danach aufgetragenen amplifizierten Probe an der Nachweisposition nachgewiesen wird.

16. Ein Verfahren gemäß einem der vorangegangenen Ansprüche, wobei die Zielnukleotidsequenz einen Polymorphismus enthält, vorzugsweise einen Einzelnukleotidpolymorphismus.

17. Ein Verfahren gemäß einem der vorangegangenen Ansprüche, wobei die Zielnukleotidsequenz ein DNA-Molekül ist, das aus der Gruppe ausgewählt ist, die aus: cDNA, genomischer DNA, Restriktionsfragmenten, Adapter-ligierten Restriktionsfragmenten, amplifizierten Adapter-ligierten Restriktionsfragmente und AFLP-Fragmenten besteht.

18. Verwendung eines Verfahrens, wie es in einem der vorangegangenen Ansprüche definiert wird, für den Nachweis einer Vielzahl von Zielnukleotidsequenzen mit hohem Durchsatz.

19. Verwendung eines Verfahrens gemäß Anspruch 18 zum Nachweis von Polymorphismen, vorzugsweise von Einzelnukleotidpolymorphismen.

20. Verwendung eines Verfahrens gemäß Anspruch 19 zur Erstellung eines Transkriptprofils.

21. Verwendung gemäß Anspruch 18 zum Nachweis der quantitativen Häufigkeit von Zielnukleinsäuresequenzen.

22. Verwendung gemäß Anspruch 18 zur Genkartierung, Entdeckung von Genen, zur Marker-unterstützten Selektion, zur Qualitätskontrolle von Aussaat, Hybridselektion, QTL-Kartierung, Massensegregantenanalyse, für DNA-Fingerabdrücke und zur Offenbarung von Information, die in Bezug zu Merkmalen, Krankheitsresistenz, Ausbeute, Hybridlebensfähigkeit und/oder Genfunktion steht.

23. Zirkularisierbare Nukleinsäuresonde, umfassend einen ersten zielspezifischen Abschnitt an ihrem 5'-Ende, der zu einem ersten Teil einer Zielsequenz komplementär ist, sowie einen zweiten zielspezifischen Abschnitt an ihrem 3'-Ende, der zu einem zweiten Teil der Zielsequenz komplementär ist, wobei die Sonde zusätzlich einen Tag-Abschnitt umfasst, der im Wesentlichen zu der Zielsequenz nicht komplementär ist, wobei der Tag-Abschnitt eine Füllsequenz umfassen kann und wobei der Tag-Abschnitt wenigstens eine primerbindende Sequenz umfasst,
wobei der Tag-Abschnitt zusätzlich einen blockierenden Abschnitt enthält, der eine blockierende Gruppe umfasst, die benachbart zu dem 3'-Ende der Primerbindungsstelle lokalisiert ist, so dass der blockierende Abschnitt in der Lage ist, die Verlängerung oder Amplifikation von jeglichem Primer zu stoppen, der an die zirkularisierbare Nukleinsäuresonde hybridisiert ist.

24. Eine zirkularisierbare Oligonukleotidsäuresonde gemäß Anspruch 23 zur Verwendung in einem Verfahren, wie es in den Ansprüchen 1 - 16 definiert wird.

25. Eine Gruppe von zwei oder mehr zirkularisierbaren Oligonukleotidsonden gemäß Anspruch 23 zur Verwendung in einem Verfahren, wie es in den Ansprüchen 1 - 16 definiert wird.

26. Verwendung einer Gruppe von zwei oder mehr Oligonukleotidsonden, wie sie in Anspruch 23 definiert werden, wobei die Gruppe eine Sonde für jedes Allel eines Einzelnukleotidpolymorphismus umfasst.

27. Ein Gruppe von Primern zusammen mit einer Sonde oder einer Gruppe von Sonden, wie sie in den Ansprüchen 23 - 26 definiert werden, zur Verwendung in einem Verfahren gemäß einem der Ansprüche 1-16, wobei die Gruppe von Primern einen ersten Primer und einen oder mehrere zweite Primer umfasst, wobei jeder zweite Primer einen Marker enthält.

28. Ein Kit, der Oligonukleotidsonden umfasst, wie sie in den Ansprüchen 23, 24 oder 25 definiert werden, der zur Verwendung in einem Verfahren geeignet ist, wie es in den Ansprüchen 1 - 16 definiert wird.

29. Ein Kit gemäß Anspruch 28, der zusätzlich Primer zur Verwendung in einem Verfahren umfasst, wie es in den Ansprüchen 1-16 definiert wird.

Revendications

1. Procédé destiné à déterminer la présence ou l'absence d'une séquence cible dans un échantillon d'acide nucléique, lequel procédé comprend les étapes consistant à :

(a) fournir à un échantillon d'acide nucléique au moins une sonde circularisable pour chaque séquence cible à détecter dans l'échantillon, de sorte que la sonde ait une première section spécifique de la cible au niveau de son extrémité 5' qui est complémentaire d'une première partie d'une séquence cible et une seconde section spécifique dé la cible au niveau de son extrémité 3' qui est complémentaire d'une seconde partie de la séquence cible, de sorte que la première et seconde partie de la séquence cible soient situées l'une à côté de l'autre; et de sorte que la sonde comprenne en outre une section étiquette qui est essentiellement non complémentaire de la séquence cible, de sorte que la section étiquette puisse comprendre une séquence de remplissage et de sorte que la section étiquette comprenne au moins une séquence se liant à l'amorce ;
(b) permettre aux première et seconde sections spécifiques de la cible de la sonde circulaire de s'anneler aux premières et secondes parties des séquences cibles de sorte que les première et seconde sections spécifiques de la cible de la sonde soient annelées à côté de la séquence cible ;
(c) fournir des moyens pour ligaturer les première et seconde sections spécifiques de la cible annelées de manière adjacente à la séquence cible et permettre aux première et seconde sections spécifiques de la cible d'être connectées, pour produire une sonde circulaire ligaturée, correspondant à une séquence cible dans l'échantillon;

(d) fournir au moins une première amorce qui est complémentaire de la séquence se liant à l'amorce, et une enzyme polymérase ;
(e) amplifier le mélange résultant pour produire un échantillon amplifié comprenant des amplicons qui sont des représentations linéaires des sondes circulaires ligaturées ;
(f) déterminer la présence ou l'absence d'une séquence cible dans un échantillon en détectant la présence ou l'absence de l'amplicon correspondant ;

dans lequel la sonde circularisable contient une section d'arrêt qui comprend un groupe d'arrêt qui est situé à côté de l'extrémité 3' du site de liaison de l'amorce, de sorte que la section d'arrêt stoppe l'élongation ou l'amplification de l'amorce hybridée avec la sonde circulaire, générant de ce fait une représentation linéaire de la sonde circulaire et de sorte que le groupe d'arrêt est exclu de l'élongation ou de l'amplification de l'amorce.

2. Procédé selon la revendication 1, dans lequel un amplicon dans un échantillon amplifié correspondant à une séquence cible dans un échantillon diffère en longueur d'un amplicon dans l'échantillon amplifié correspondant à une séquence cible différente dans l'échantillon, dans lequel la différence en longueur est fournie par des séquences de remplissage de longueurs différentes incorporées dans la sonde.

3. Procédé selon les revendications 1 à 3, dans lequel l'amplicon a une longueur qui correspond à la longueur de la sonde circulaire connectée.

4. Procédé selon des revendications 1 à 3, dans lequel la différence de longueur est fournie par la longueur de la séquence de remplissage.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel deux sondes circulaires différentes contiennent le même site se liant à l'amorce capable de s'hybrider à une unique séquence d'amorce.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel l'amorce comprend un marqueur.

7. Procédé selon les revendications 1 à 6, dans lequel l'amorce contient une amorce universelle ayant une section qui est complémentaire de la séquence se liant à l'amorce et contient en outre au moins un nucléotide sélectif au niveau de son extrémité 3'.

8. Procédé selon la revendication 1, dans lequel :

(a) au moins deux groupes de sondes d'oligonucléotides circularisables sont fournis à un échantillon, de sorte que chaque groupe de sondes d'oligonucléotides circularisables a des séquences étiquettes avec au moins un site se liant à l'amorce d'un groupe spécifique ;
(b) les sondes circulaires ligaturées de chaque groupe sont amplifiées à partir d'une première amorce, au moins une amorce étant complémentaire du site se liant à l'amorce d'un groupe spécifique, et de sorte qu'au moins une des amorces d'un groupe comprend un marqueur spécifique d'un groupe ; et,
(c) dans chaque groupe, une sonde ligaturée amplifiée correspondant à une séquence cible dans l'échantillon, diffère en longueur d'une sonde ligaturée amplifiée correspondant à une séquence cible différente dans l'échantillon ;

dans lequel la différence de longueur est fournie par l'utilisation de séquences de remplissage incorporées dans la sonde de longueur différente.

9. Procédé selon la revendication 8, par lequel dans une première partie des groupes des sondes ligaturées amplifiées ayant un nombre pair de nucléotides sont produites et dans une seconde partie des groupes des sondes ligaturées amplifiées ayant un nombre impair de nucléotides sont produites.

10. Procédé selon la revendication 9, dans lequel les groupes de sondes ligaturées amplifiées ayant un nombre pair de nucléotides et les groupes de sondes ligaturées amplifiées ayant un nombre impair de nucléotides sont marqués avec des marqueurs fluorescents dont les spectres d'émission ont le moins de chevauchement.

11. Procédé selon la revendication 10, dans lequel un premier et deuxième groupes de sondes amplifiées ligaturées ayant un nombre pair de nucléotides sont produits et un troisième et quatrième groupes de sondes amplifiées ligaturées ayant un nombre impair de nucléotides sont produits et de sorte que le premier et deuxième groupes

sont marqués avec FAM et NED, respectivement, et le troisième et quatrième groupes sont marqués avec (ET-)ROX et soit JOE soit HEX, respectivement ; ou de sorte que le premier et deuxième groupes sont marqués avec (ET-)ROX et soit JOE soit HEX, respectivement, et le troisième et quatrième groupes sont marqués avec FAM et NED, respectivement.

12. Procédé selon l'une quelconque des revendications précédentes, dans lequel la sonde circularisable comprend en outre un second site se liant à l'amorce.

13. Procédé selon la revendication 12, dans lequel la section d'arrêt stoppant l'élongation de l'amorce comprend un groupe d'arrêt, et dans lequel le groupe d'arrêt se situe entre les deux sites de liaison d'amorces, à côté de l'extrémité 3' du site de liaison d'amorce sens et à côté de l'extrémité 5' du site de liaison d'amorce anti-sens de sorte que le groupe d'arrêt est exclu de l'élongation ou de l'amplification de l'amorce.

14. Procédé selon l'une quelconque des revendications précédentes, dans lequel la polymérase n'exprime pas d'activité de déplacement de brin significative.

15. Procédé selon la revendication 1, comprenant en outre les étapes suivantes :

(a) (e1) répéter les étapes (a) à (e) pour générer au moins deux échantillons amplifiés ;
(b) (e2) appliquer consécutivement au moins une partie des échantillons amplifiés obtenus dans les étapes (e) et (e1), à un endroit d'application d'un canal d'un dispositif électrophorétique, séparant électrophorétiquement les amplicons dans les échantillons amplifiés et détectant les amplicons séparés à un endroit de détection situé de manière distale de l'endroit d'application du canal ; de sorte que la période de temps entre les échantillons amplifiés appliqués consécutivement est telle que la sonde reliée amplifiée migrant le plus lentement dans un échantillon amplifié est détectée à l'endroit de détection avant que la sonde reliée amplifiée migrant le plus rapidement d'un échantillon amplifié appliqué plus tard soit détectée à l'endroit de détection.

16. Procédé selon l'une quelconque des revendications précédentes, dans lequel la séquence de nucléotides cibles contient un polymorphisme, de préférence un polymorphisme simple nucléotide.

17. Procédé selon l'une quelconque des revendications précédentes, dans lequel la séquence de nucléotides cibles est une molécule d'ADN choisie dans le groupe constitué de : l'ADNc, l'ADN génomique, les fragments de restriction, les fragments de restriction ligaturés à un adaptateur, les fragments de restriction amplifiés ligaturés à un adaptateur et les fragments d'AFLP.

18. Utilisation d'un procédé tel que défini dans l'une quelconque des revendications précédentes, pour la détection à haut débit d'une multiplicité de séquences nucléotidiques cibles.

19. Utilisation d'un procédé selon la revendication 18, pour la détection de polymorphismes, de préférence des polymorphismes simple nucléotide.

20. Utilisation d'un procédé selon la revendication 19, pour l'analyse de la transcription.

21. Utilisation selon la revendication 18, pour la détection de l'abondance quantitative des séquences d'acides nucléiques cibles.

22. Utilisation selon la revendication 18, pour la cartographie génétique, la découverte de gènes, la sélection assistée par marqueurs, le contrôle de la qualité des semences, la sélection d'hybride, la cartographie QTL, l'analyse de ségrégation en mélange, l'empreinte de fragments de restriction d'ADN et pour décrire une information relative aux traits, à la résistance à la maladie, au rendement, à la vigueur de l'hybride, et/ou à la fonction des gènes.

23. Sonde d'acide nucléique circularisable comprenant une première section spécifique de la cible au niveau de son extrémité 5' qui est complémentaire d'une première partie d'une séquence cible et une seconde section spécifique de la cible au niveau de son extrémité 3' qui est complémentaire d'une seconde partie de la séquence cible, de sorte que la sonde comprend en outre une section étiquette qui est essentiellement non complémentaire de la séquence cible, de sorte que la section étiquette puisse comprendre une séquence de remplissage et de sorte que la section étiquette comprend au moins une séquence se liant à l'amorce, dans laquelle la section étiquette contient en outre une section d'arrêt qui comprend un groupe d'arrêt qui est situé à côté de l'extrémité 3' du site de liaison

de l'amorce de sorte que la section d'arrêt puisse stopper l'élongation ou l'amplification de toute amorce hybridée avec ladite sonde d'acide nucléique circularisable.

24. Sonde d'acide d'oligonucléotide circularisable selon la revendication 23 pour une utilisation dans un procédé tel que défini dans les revendications 1 à 16.

25. Ensemble de deux ou plusieurs sondes d'oligonucléotides circularisables selon la revendication 23, pour une utilisation dans un procédé tel que défini dans les revendications 1 à 16.

26. Utilisation d'un ensemble de deux ou plusieurs sondes d'oligonucléotides telles que définies dans la revendication 23, dans laquelle l'ensemble comprend une sonde pour chaque allèle de polymorphisme simple nucléotide.

27. Ensemble d'amorces, conjointement à une sonde ou un ensemble de sondes tel que défini dans les revendications 23 à 26 pour une utilisation dans un procédé selon l'une quelconque des revendications 1 à 16, l'ensemble d'amorces comprenant une première amorce et une ou plusieurs secondes amorces, dans lesquelles chaque seconde amorce contient un marqueur.

28. Kit comprenant des sondes d'oligonucléotides telles que définies dans les revendications 23, 24 ou 25, appropriées pour une utilisation dans un procédé tel que défini dans les revendications 1 à 16.

29. Kit selon la revendication 28, comprenant en outre des amorces pour une utilisation dans un procédé tel que défini dans les revendications 1 à 16.

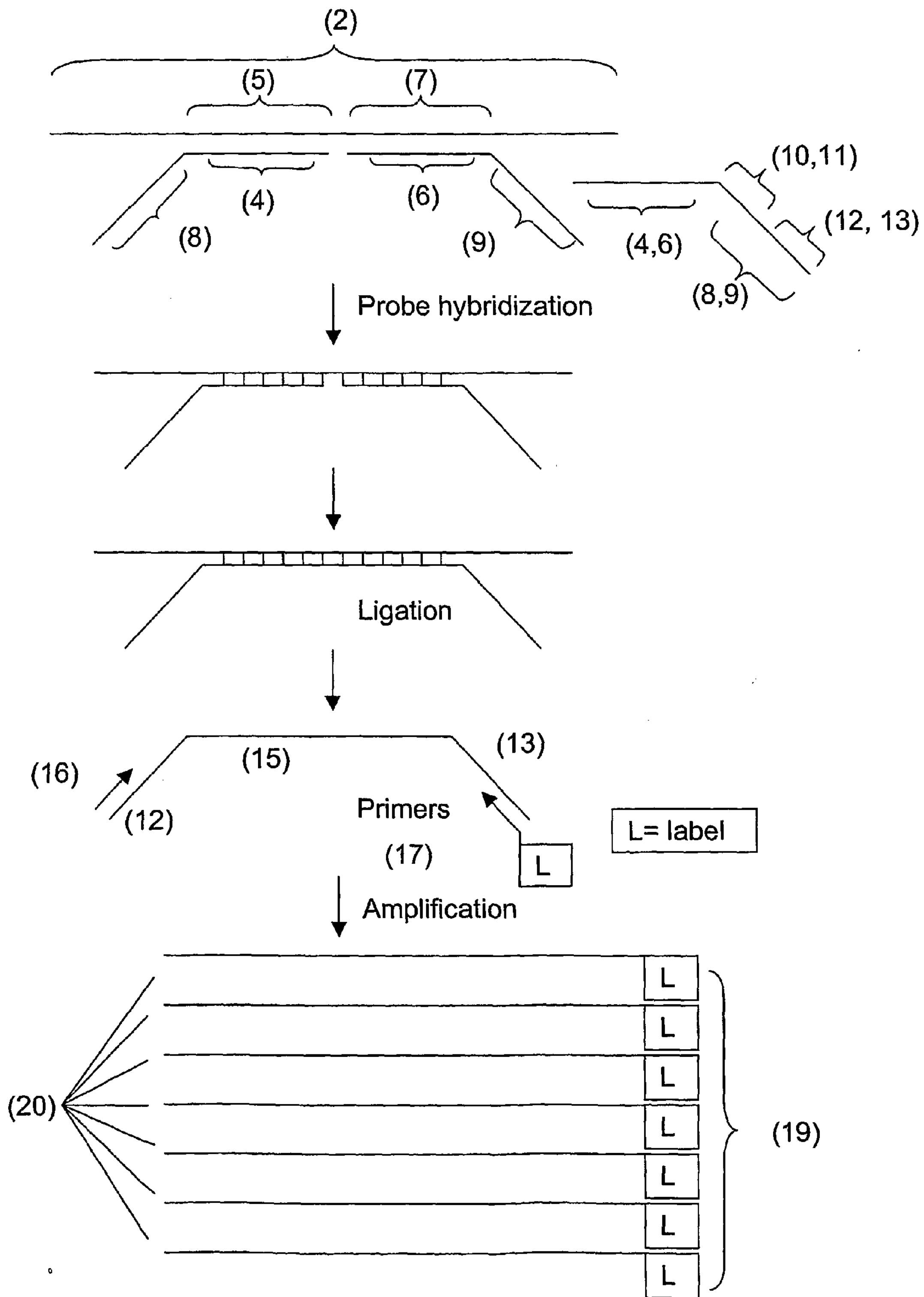


FIG 1
(2)
(5)
(7)
(4)
(6)
(8)
(9)
(10,11)
(12, 13)
(4,6)
(8,9)
Probe hybridization
Ligation
(16)
(15)
(13)
(12)
Primers
(17)
L
L= label
Amplification
(20)
(19)
L

Fig 2

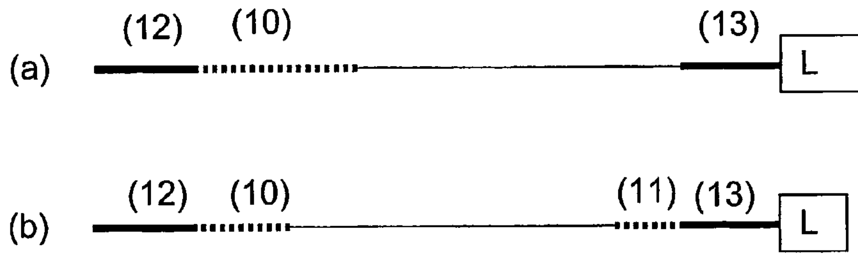
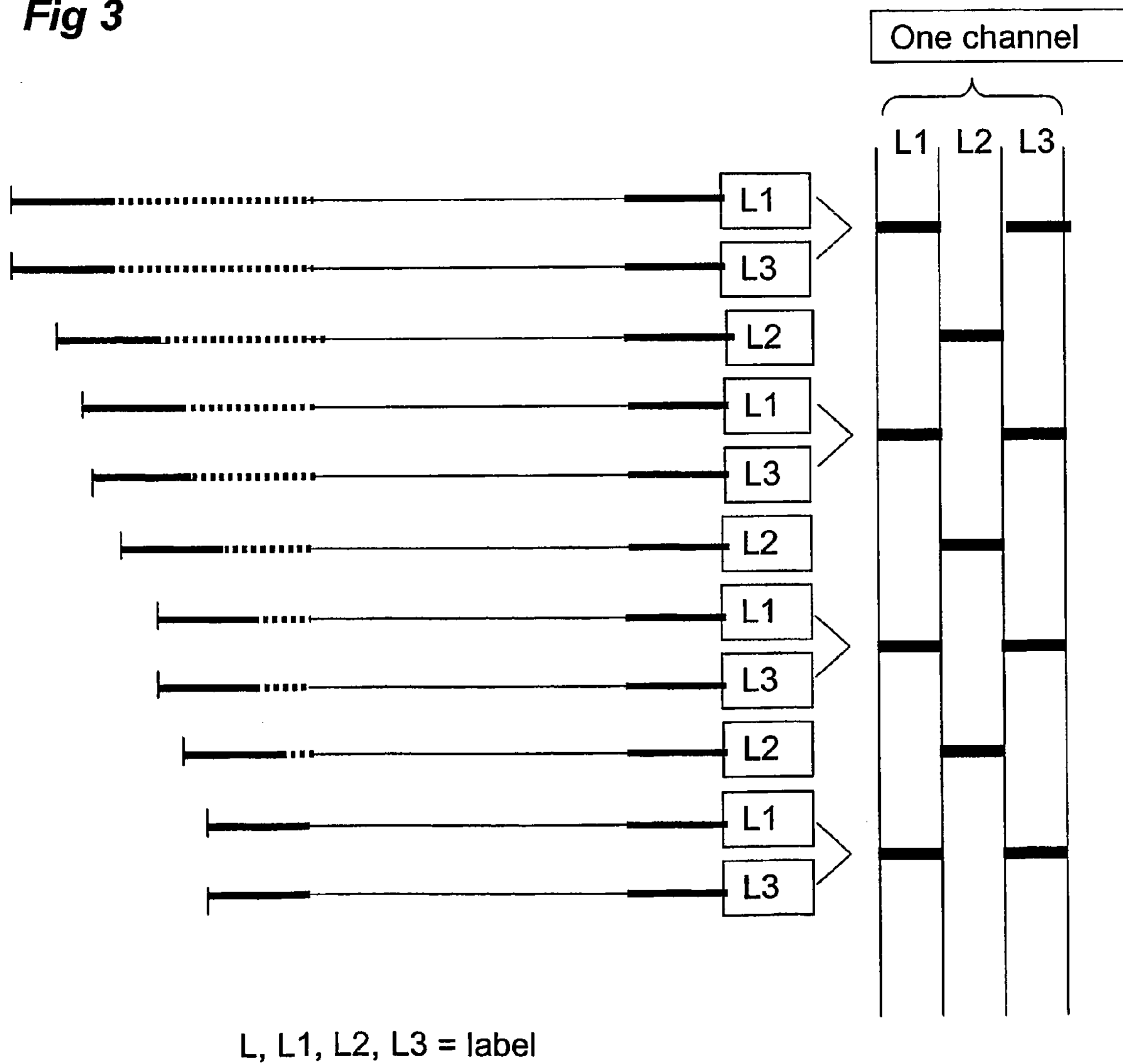


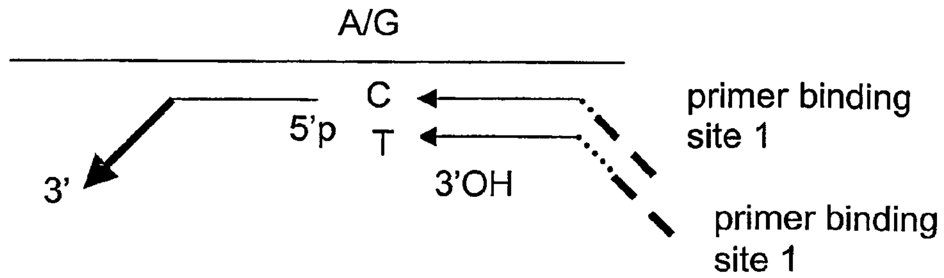
Fig 3



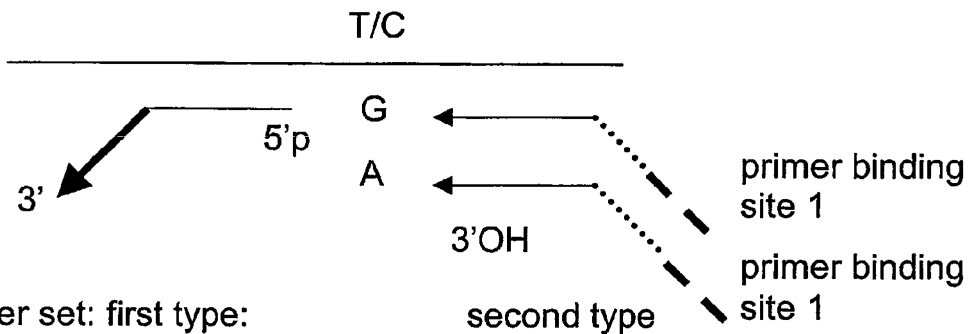
L, L1, L2, L3 = label

Fig 4

Locus 1



Locus 2



Primer set: first type: second type

Connected probes with annealed primers:

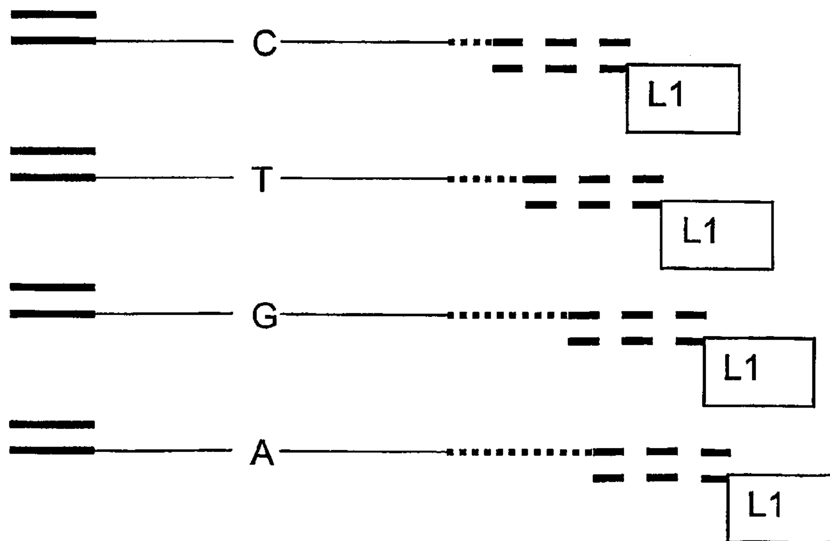


Fig 5

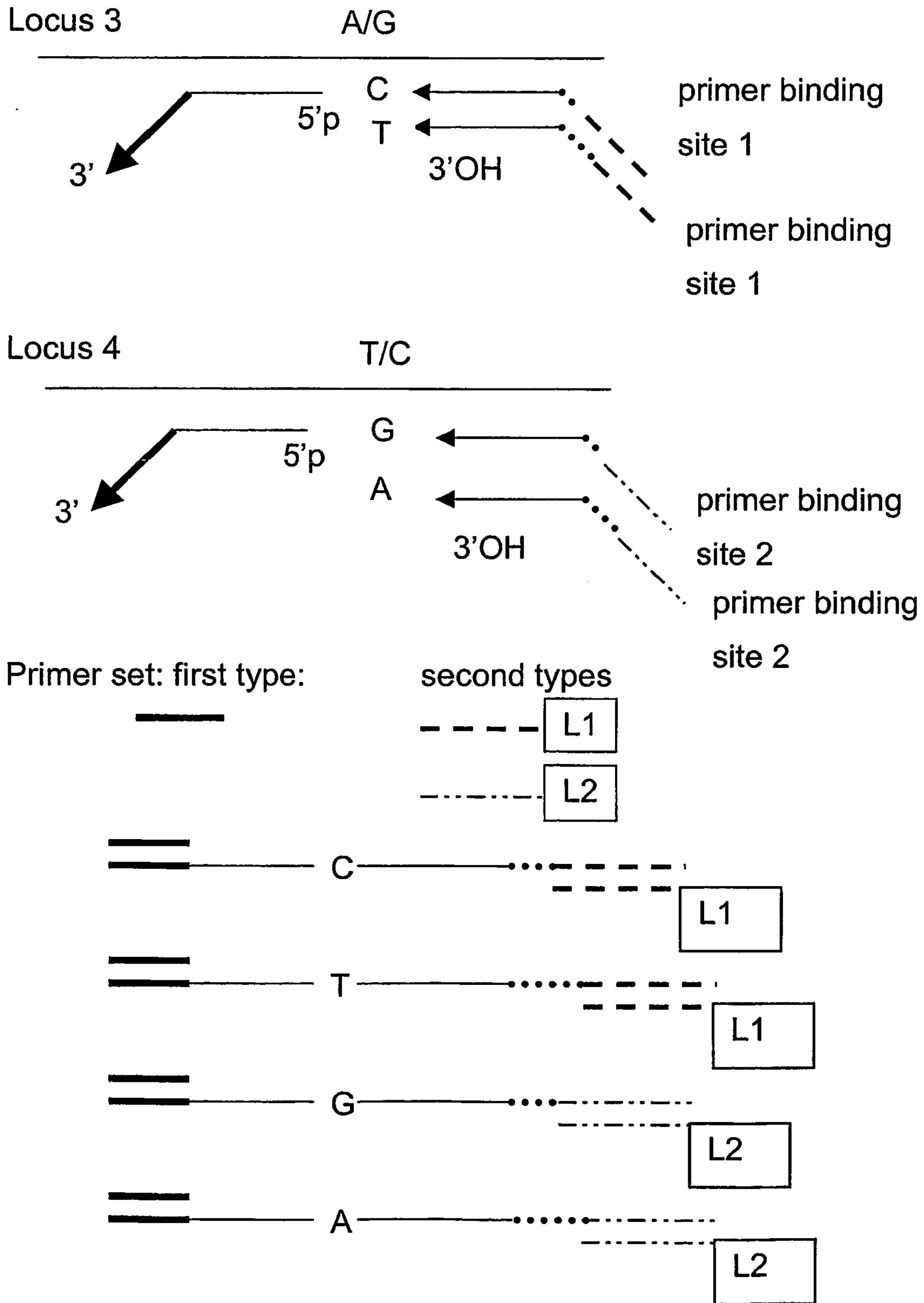


Fig 6

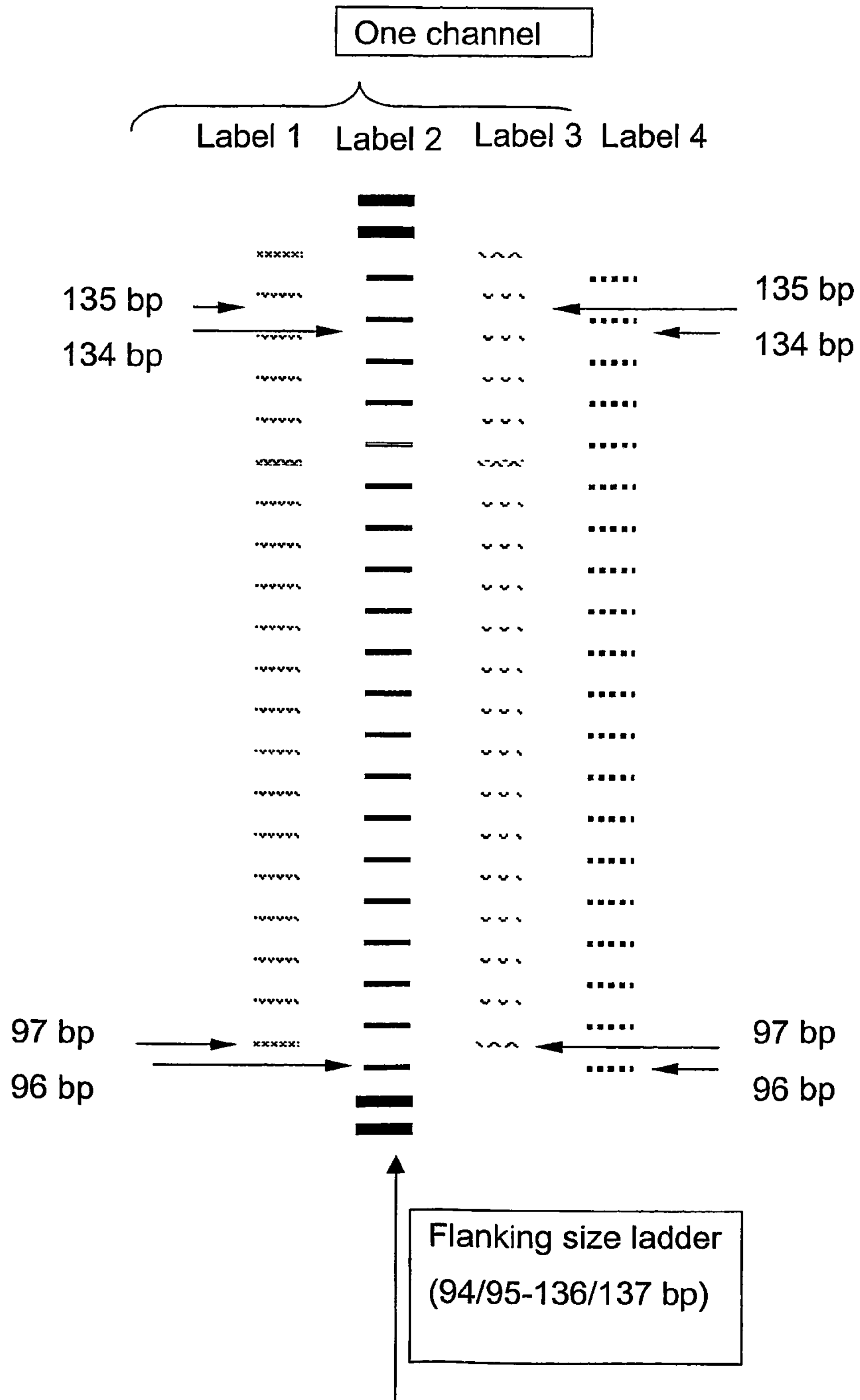


Fig 7

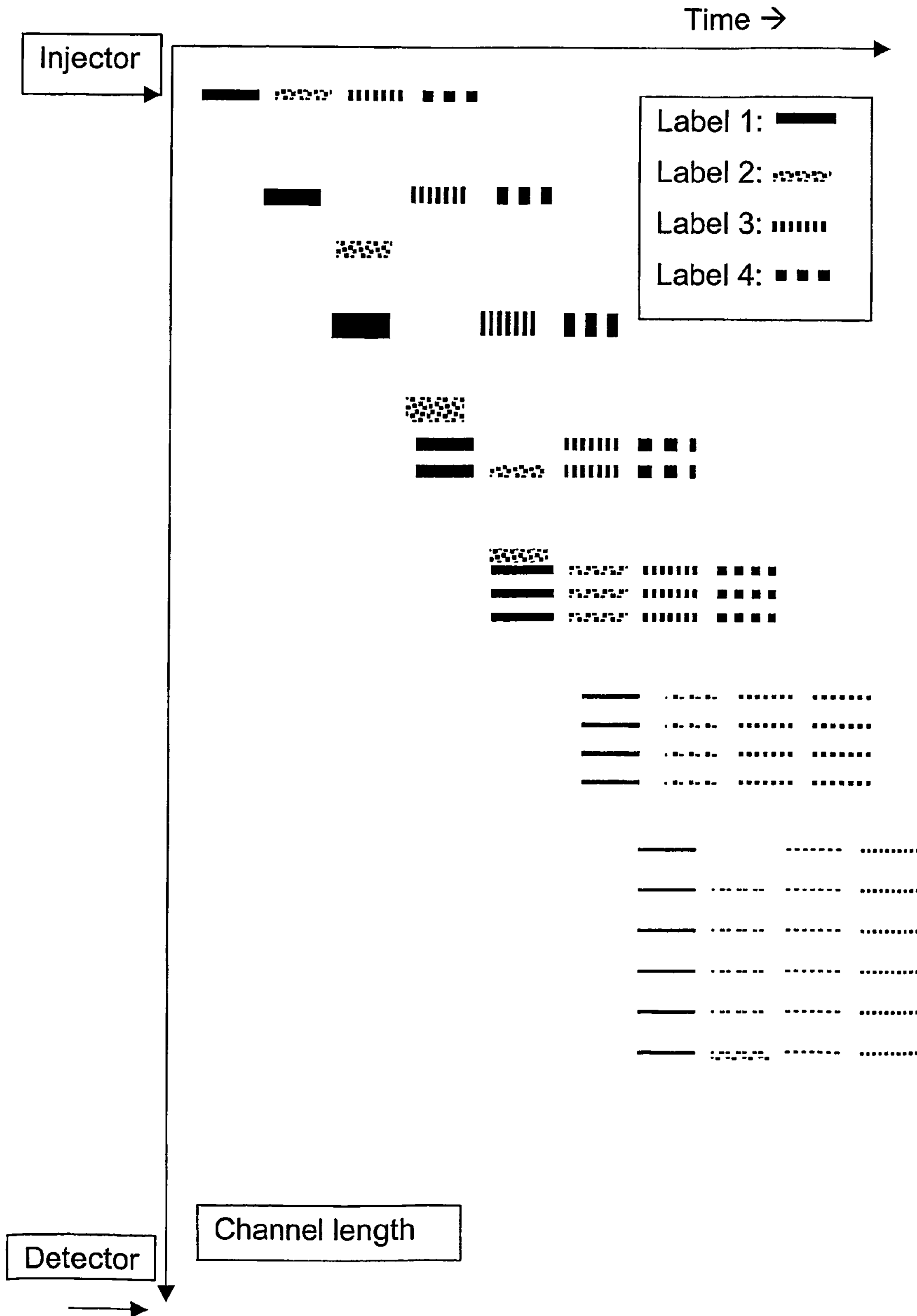
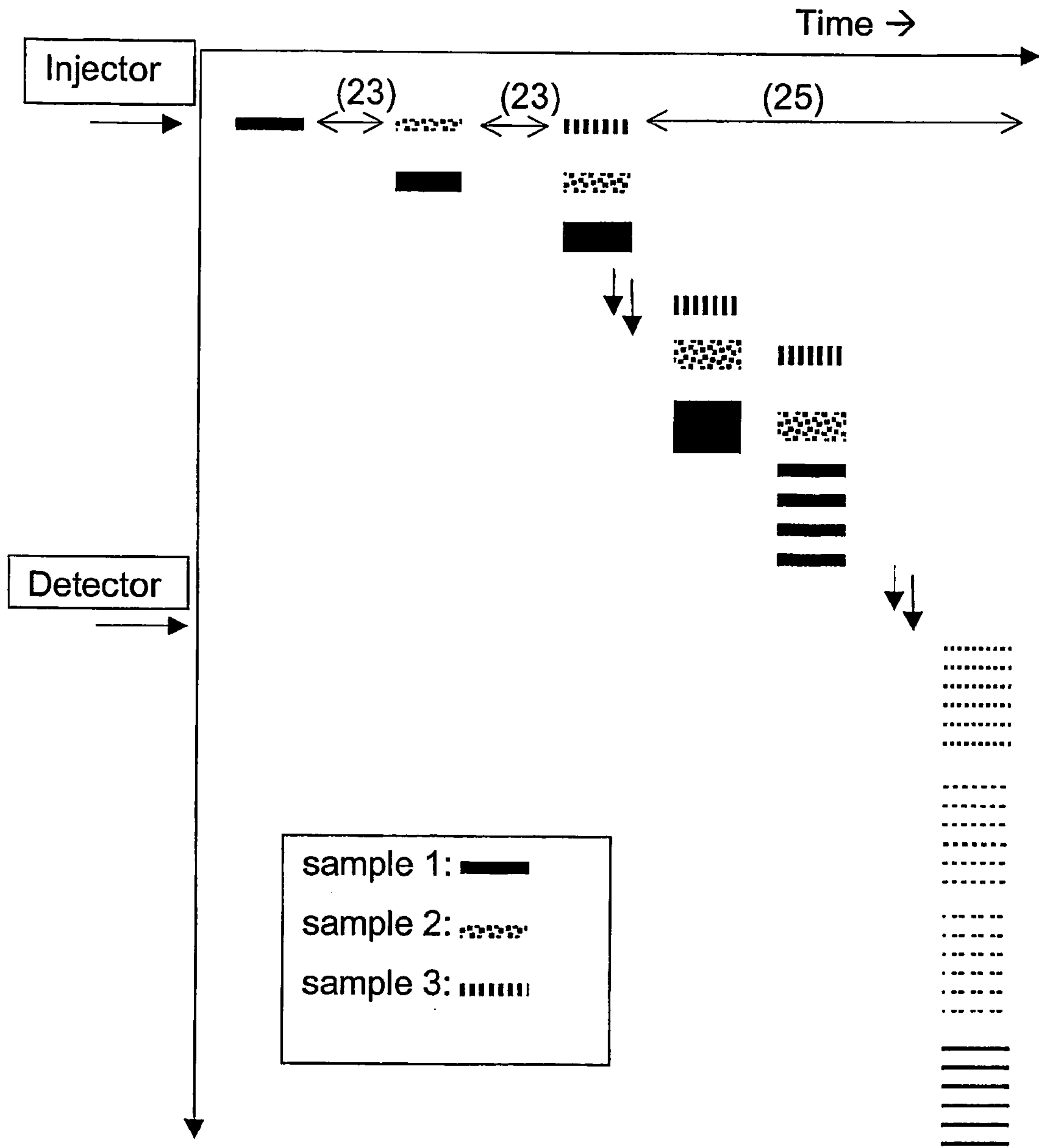


Fig 8



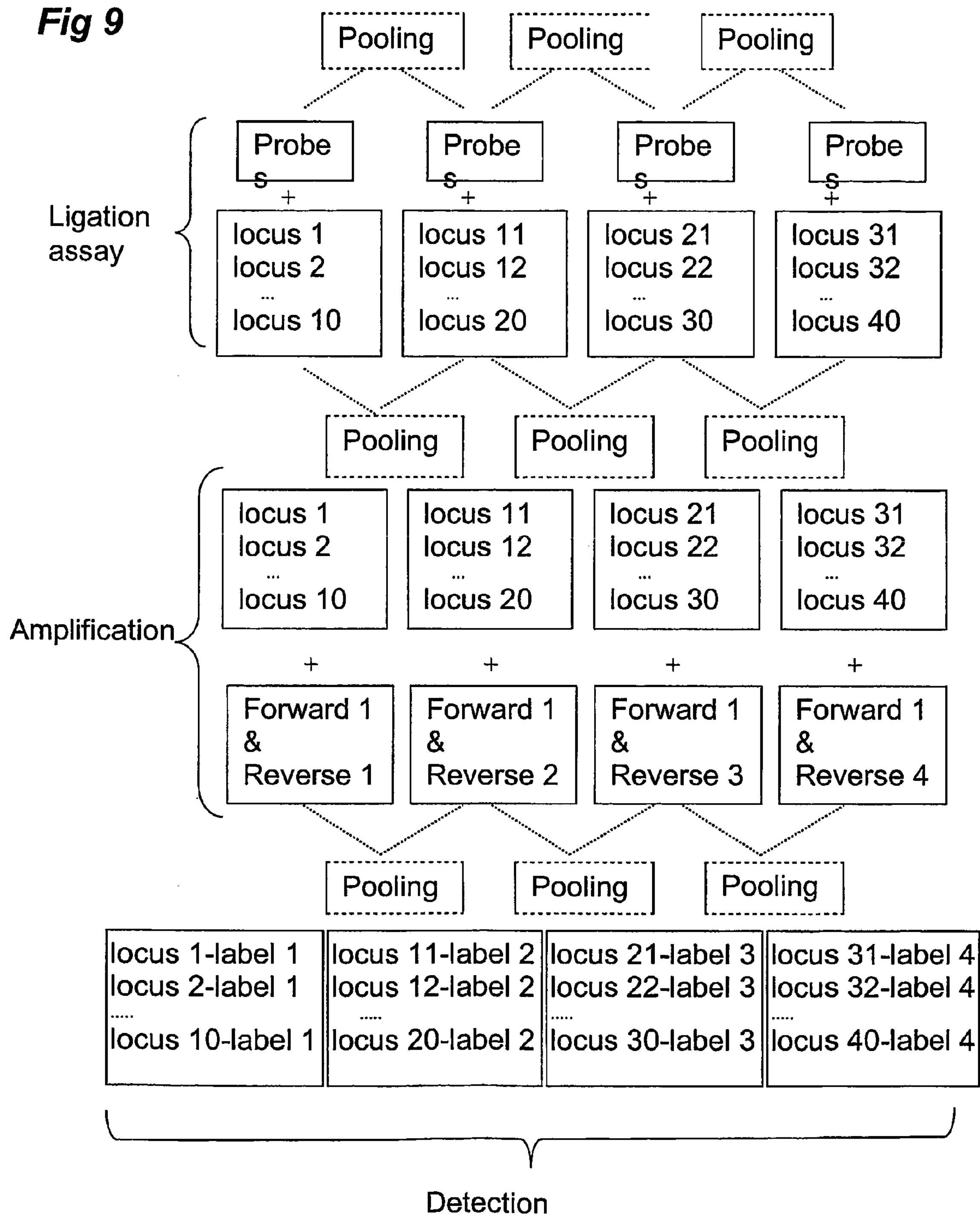
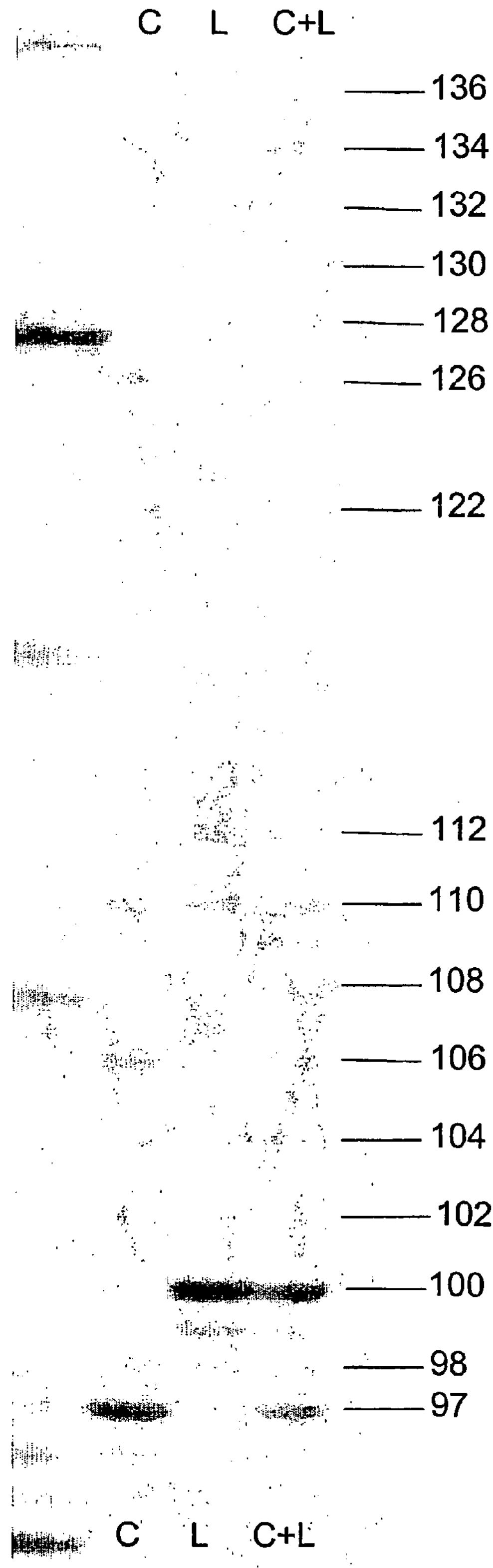


Fig 9
Pooling
Pooling
Pooling
Ligation assay
Probes
+
locus 1
locus 2
...
locus 10
locus 11
locus 12
...
locus 20
locus 21
locus 22
...
locus 30
locus 31
locus 32
...
locus 40
Amplification
Forward 1 & Reverse 1
Forward 1 & Reverse 2
Forward 1 & Reverse 3
Forward 1 & Reverse 4
locus 1-label 1
locus 2-label 1
.....
locus 10-label 1
locus 11-label 2
locus 12-label 2
.....
locus 20-label 2
locus 21-label 3
locus 22-label 3
.....
locus 30-label 3
locus 31-label 4
locus 32-label 4
.....
locus 40-label 4
Detection

FIG 10



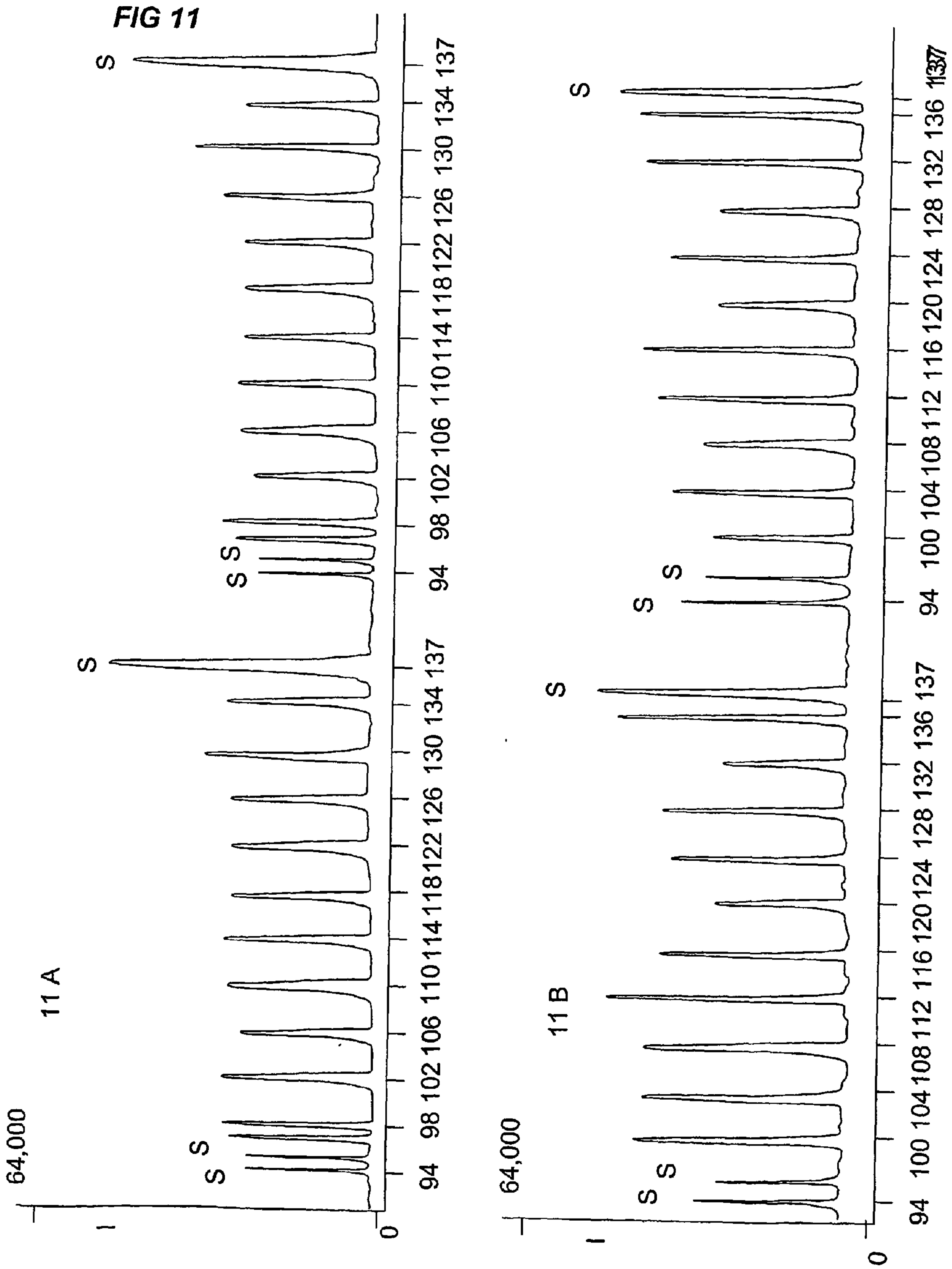


FIG 11
11 A
11 B
64,000
0
S
94 98 102 106 110 114 118 122 126 130 134 137
94 100 104 108 112 116 120 124 128 132 136 137

FIG 12

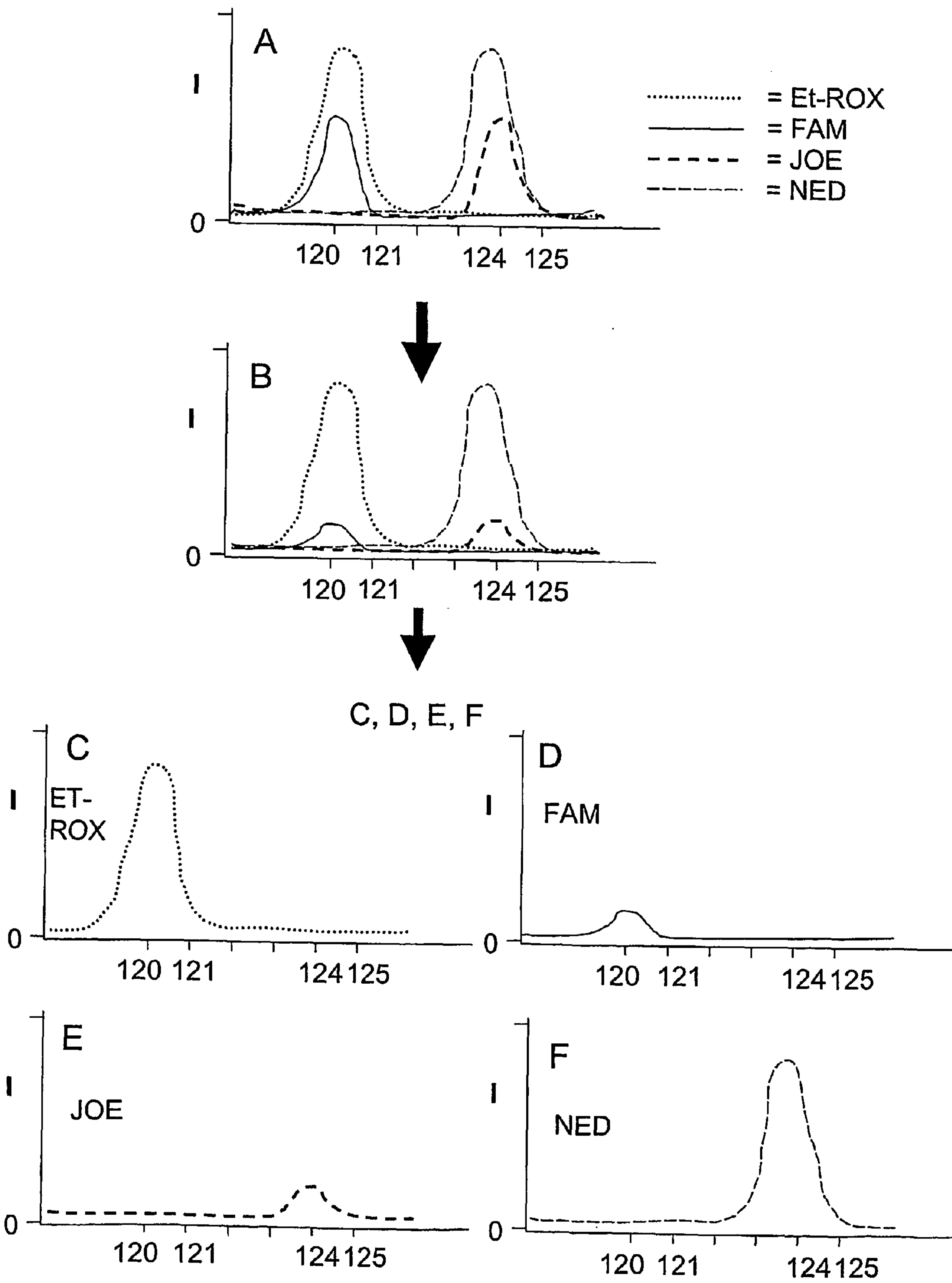


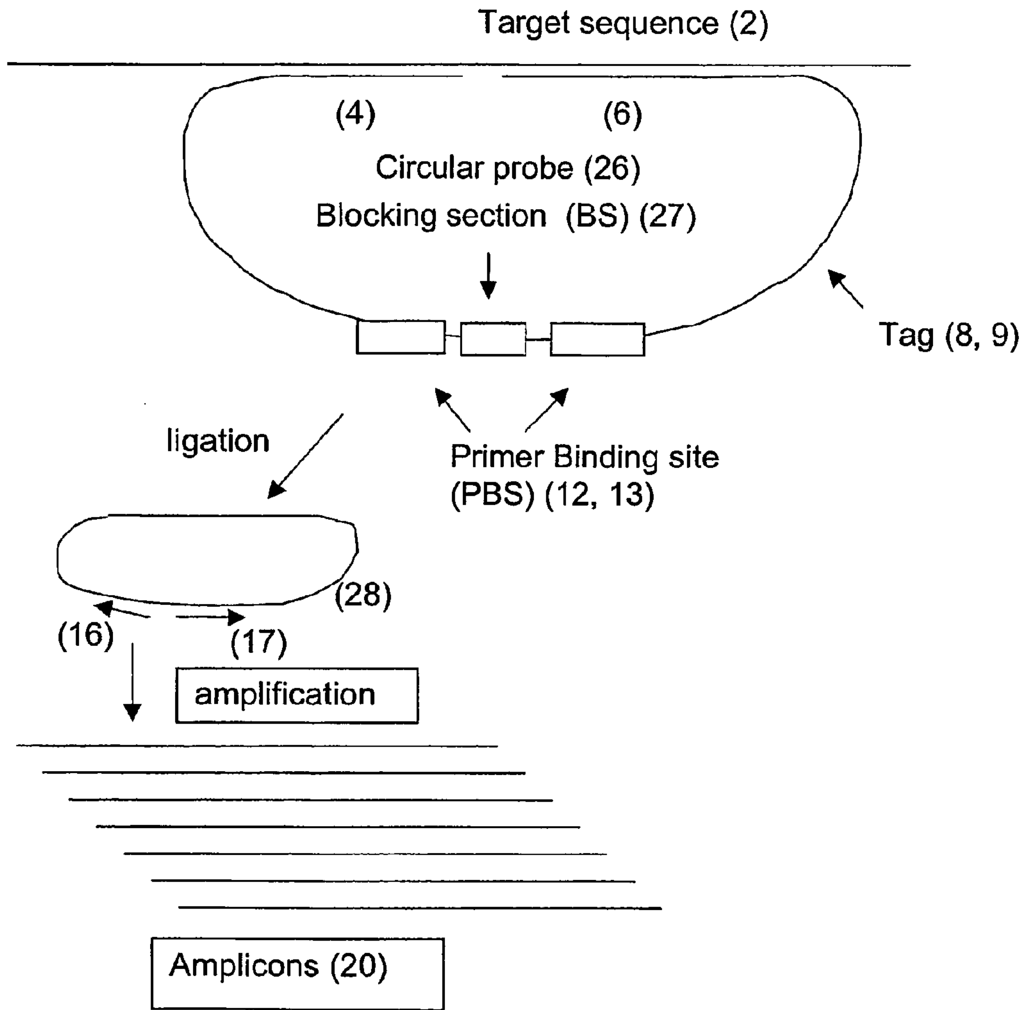
FIG 13

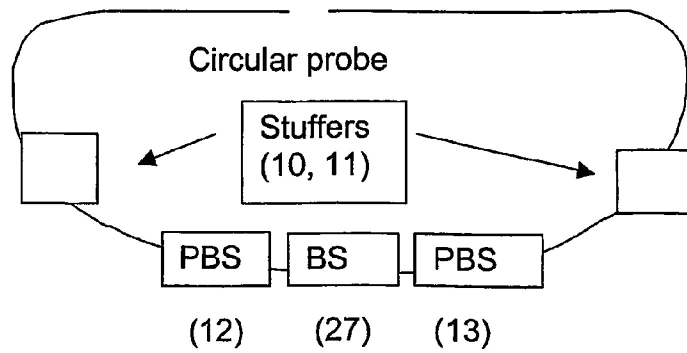
A	Observed data		Scored data		Expected data	
bp	**120**	**124**	**120**	**124**	**120**	**124**
ET-ROX	+	-	+	-	+	-
FAM	+	-	Θ	-	-	-
JOE	-	+	-	Θ	-	-
NED	-	+	-	+	-	+

B	Observed data				Scored data				Expected data			
bp	**120**	**121**	**124**	**125**	**120**	**121**	**124**	**125**	**120**	**121**	**124**	**125**
ET-ROX	+	-	-	-	+		-		+		-	
FAM	+	-	-	-		-		-		-		-
JOE	-	-	+	-		-		-		-		-
NED	-	-	+	-	-		+		-		+	

Legend	
+	Present
-	Absent
Θ	False (Present/Absent)
	Signal ignored

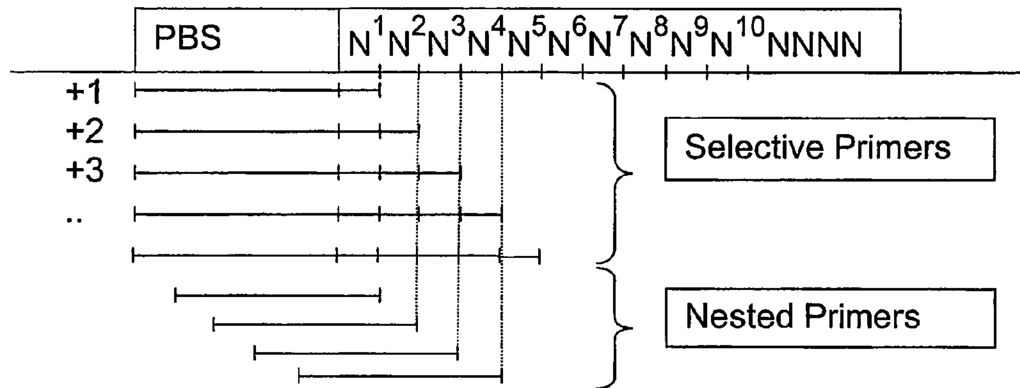
Fig 14



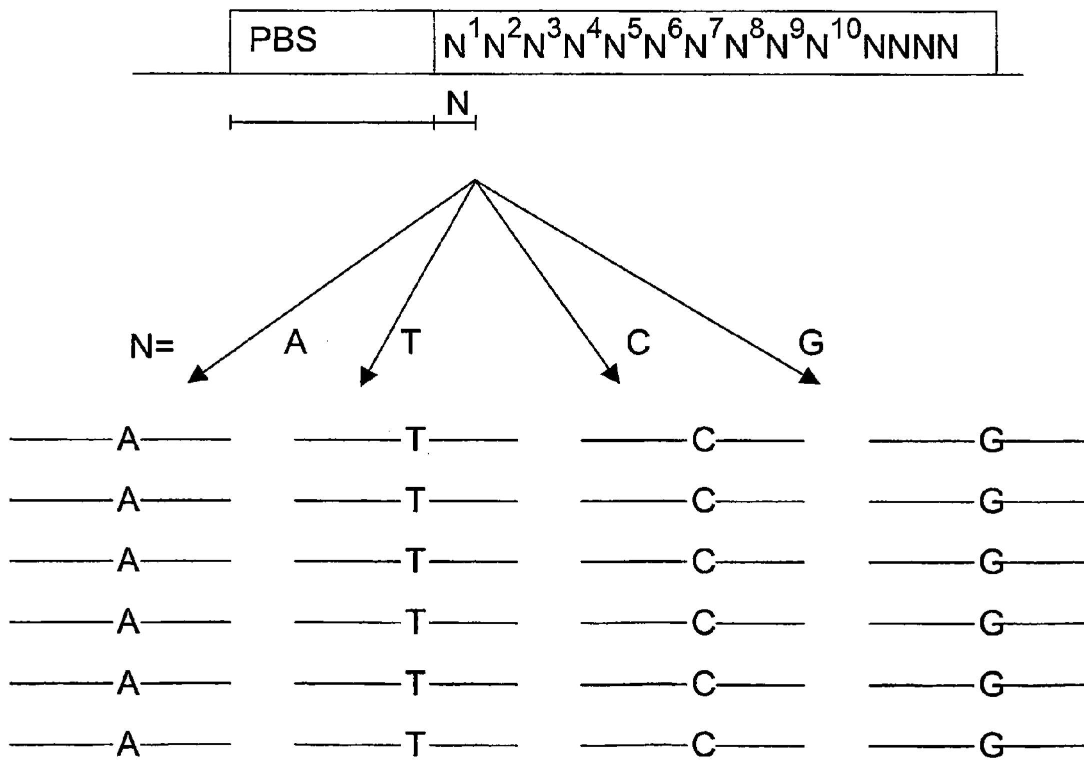


amplification

Fig 15

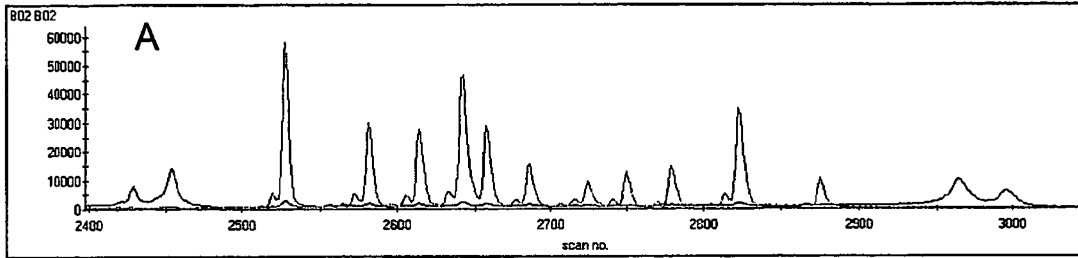


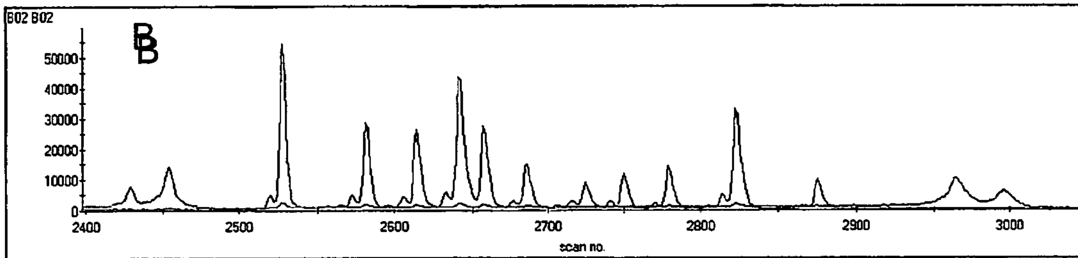
24-PLEX

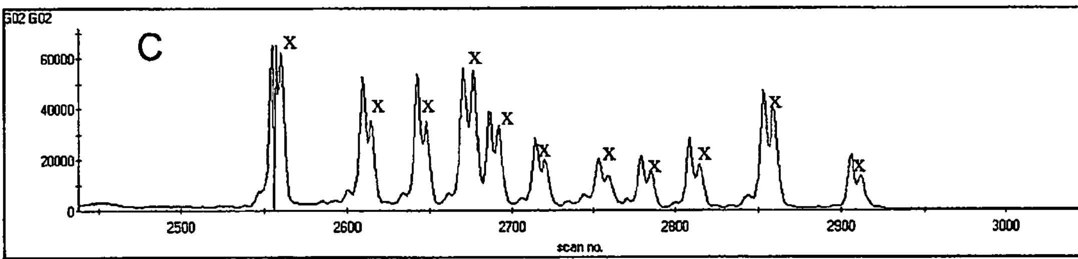


6-PLEX

Fig 16







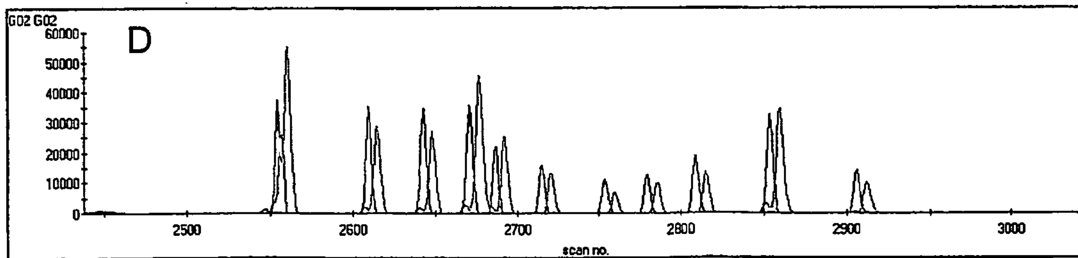


Fig 17

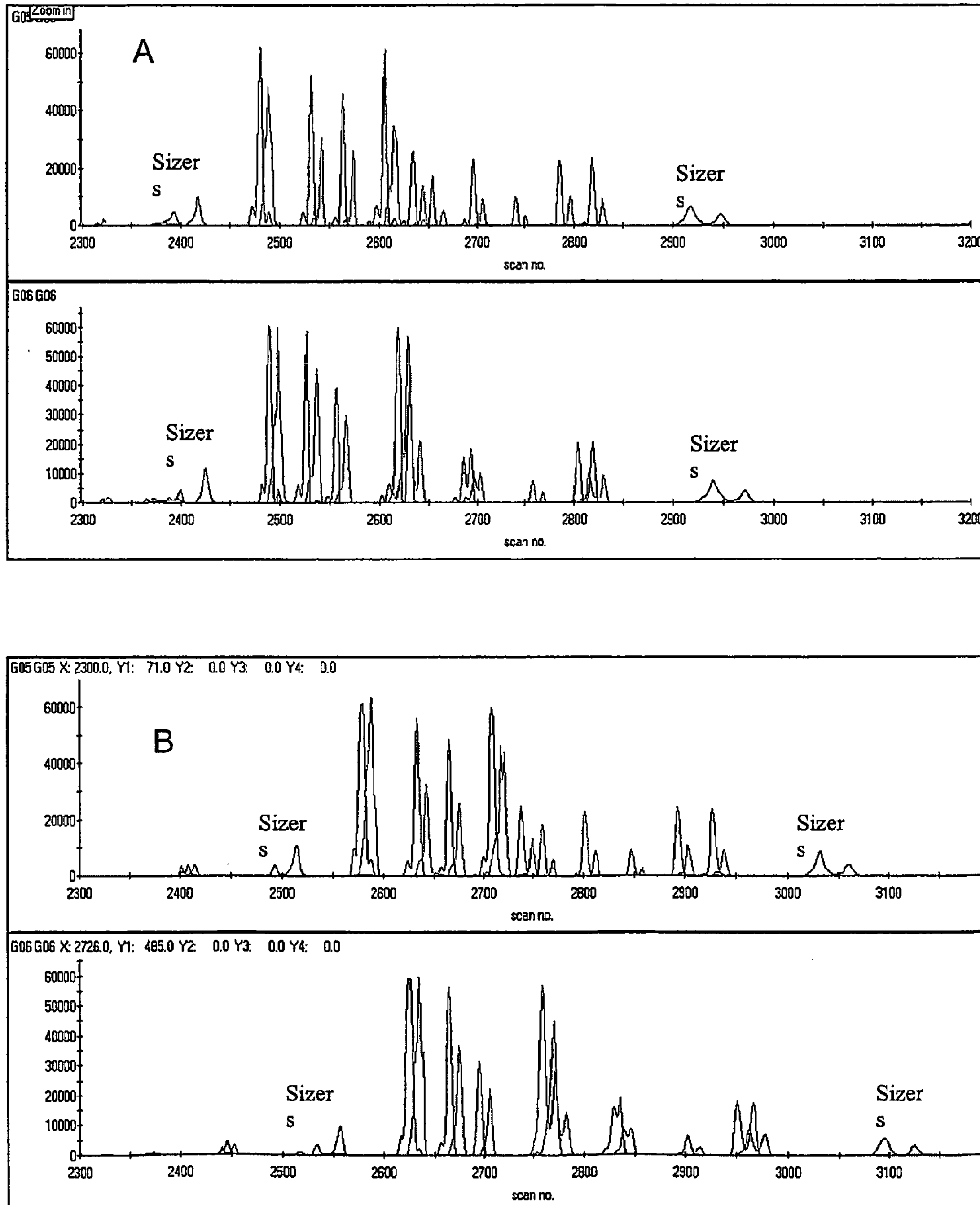
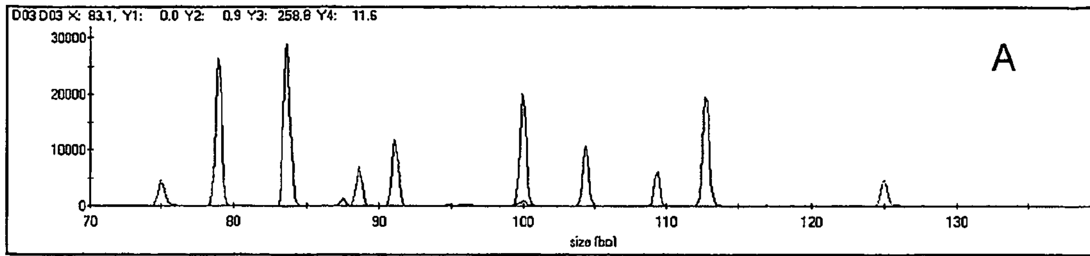
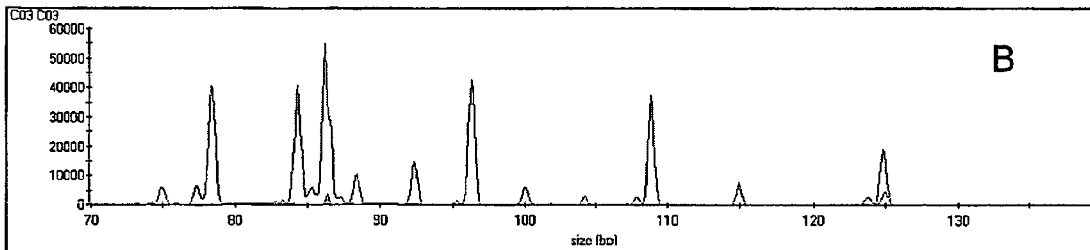
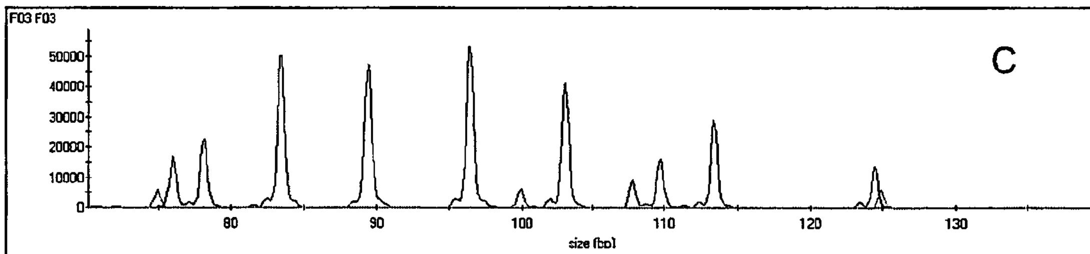


Fig 18







REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- EP 0534858 A **[0005] [0056] [0109] [0119] [0129]**
- US 4988617 A, Landegren **[0006]**
- WO 0106012 A **[0006] [0028]**
- WO 9615271 A **[0007]**
- WO 9745559 A, Barany **[0008] [0013] [0027]**
- WO 0161033 A, Schouten **[0014] [0014]**
- WO 9522623 A **[0016]**
- US 5854033 A **[0017] [0028]**
- US 6143495 A **[0017]**
- WO 9719193 A **[0017]**
- US 5876924 A **[0018]**
- WO 9804745 A **[0018]**
- WO 9804746 A, Zhang **[0018]**
- US 6221603 B **[0020] [0020]**
- US 5912124 A **[0028]**
- WO 02068683 A **[0028]**
- WO 0077260 A **[0028] [0041]**
- WO 0157256 A **[0028]**
- EP 974672 A **[0035]**
- WO O161033 A **[0040]**
- EP 534858 A **[0044] [0046] [0053]**
- US 6156178 A **[0097]**
- WO O104618 A **[0097]**
- US 6045994 A **[0109]**
- EP 01204912 A **[0157]**

Non-patent literature cited in the description

- **LIZARDI et al.** *Nature genetics,* 1998, vol. 19 (3), 225-232 **[0017]**
- **M. NILSSON.** Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection. *Science,* 1994, vol. 265, 2085-88 **[0028]**
- **PICKERING et al.** *Nucleic Acids Research,* 2002, vol. 30 **[0028]**
- **IANNONE et al.** *Cytometry,* 2000, vol. 39, 131-140 **[0031]**
- Molecular Cloning: A Laboratory Manual. **SAMBROOK ; RUSSEL.** Cold Spring Harbor Laboratory. Cold Spring Harbor Laboratory Press, 2001 **[0035]**
- **XU ; KOOL.** *Nucleic Acid Res.,* 1999, vol. 27, 875-881 **[0039]**
- **WU ; WALLACE.** *Gene,* 1989, vol. 76, 245-254 **[0040]**
- **VOS et al.** *Nucleic Acid Research,* 1995, vol. 23, 4407-44014 **[0044] [0046] [0053]**
- **AUSUBEL et al.** Current Protocols in Molecular Biology. John Wiley & Sons, 1995 **[0056]**
- **VOS et al.** *Nucleic Acids Res.,* 1995, vol. 23, 4407, 4414 **[0056]**
- **CHEHAB et al.** *Proc. Natl. Acad. Sci.,* 1989, vol. 86, 9178-9182 **[0068]**
- **VOS et al.** *Nucleic Acids Research,* 1995, vol. 23, 4407-4414 **[0109]**
- **LISTER ; DEAN.** *Plant Journal,* 1993, vol. 4, 745-750 **[0118]**
- **VOS et al.** *Nucleic Acids Research,* 1995, vol. 23 (21), 4407-4414 **[0129]**
- **VOS et al.** *Nucleic Acids research,* 1995, vol. 23 (21), 4407-4414 **[0133]**