



(86) Date de dépôt PCT/PCT Filing Date: 1998/07/10
(87) Date publication PCT/PCT Publication Date: 1999/01/28
(45) Date de délivrance/Issue Date: 2008/11/25
(85) Entrée phase nationale/National Entry: 2000/01/05
(86) N° demande PCT/PCT Application No.: FI 1998/000586
(87) N° publication PCT/PCT Publication No.: 1999/004035
(30) Priorité/Priority: 1997/07/14 (FI972992)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2006.01),
C12N 15/10 (2006.01), *C12P 19/34* (2006.01)

(72) Inventeurs/Inventors:
TENKANEN, TUOMAS, FI;
SOININEN, TIMO, FI;
SAVILAHTI, HARRI, FI;
MULTANEN, KIRSI, FI

(73) Propriétaire/Owner:
FINNZYMES OY, FI

(74) Agent: OGILVY RENAULT LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : METHODE IN VITRO D'OBTENTION DE MATRICES POUR LE SEQUENCAGE D'ADN
(54) Title: IN VITRO METHOD FOR PROVIDING TEMPLATES FOR DNA SEQUENCING

(57) **Abrégé/Abstract:**

The present invention relates to an in vitro method for providing unique templates for DNA sequencing and to a kit, which can be used, when employing the selection method of this invention. The examined DNA is subjected to a DNA transposition reaction and to an amplification reaction. The amplification reaction is carried out in the presence of a fixed primer and a selective primer having a complementary sequence to the end of a transposon DNA. A transposition reaction can be carried out also in the presence of a transposon or transposons, which have different ends enabling the design of selective primers for both ends. Each size of the amplification products represents one template for a sequencing reaction.

ABSTRACT

The present invention relates to an *in vitro* method for providing unique templates for DNA sequencing and to a kit, which can be used, when employing the selection method of this invention. The examined DNA is subjected to a DNA transposition reaction and to an amplification reaction. The amplification reaction is carried out in the presence of a fixed primer and a selective primer having a complementary sequence to the end of a transposon DNA. A transposition reaction can be carried out also in the presence of a transposon or transposons, which have different ends enabling the design of selective primers for both ends. Each size of the amplification products represents one template for a sequencing reaction.

***IN VITRO* METHOD FOR PROVIDING TEMPLATES FOR DNA SEQUENCING**

Field of the Invention

The present invention relates to the sequencing of DNA and in particular to the selection of unique templates for DNA sequencing. The selection methods of this invention can be performed fully *in vitro*. The invention further relates to a kit which can be used when employing the selection method of this invention.

Background of the Invention

DNA sequencing is a widely used method which is indispensable within modern molecular biology. One factor limiting sequencing is the shortness of the sequence to be read using one primer. Present-day techniques make it possible to read about 1000 bases starting from the primer. In practice, the range to be read covers about 400 to 800 bases. When sequencing a long DNA the examined DNA has been divided up into smaller fragments and the fragments have been separately sequenced, or the so called "DNA walk" method has been used.

In addition, it is essential that only one template is used in a single sequencing reaction. In the known methods, the problem has been solved by transforming the sequencing vector comprising the examined DNA into a bacterial cell, usually into *Escherichia coli*, and by isolating single colonies from the culture plate.

1. In the "DNA walk" method, the examined DNA is inserted into a vector and the inserted DNA is sequenced as far as possible by a known primer located in the vector. Using the obtained sequence, a new primer is designed, on which the sequencing is further continued (Itakura *et al* 1984 and Sambrook *et al*, 1989). The method is repeated until the entire sequence under examination has been exposed. The method is time-consuming and costly. The result from the previous round is needed for the next round, and new unique primers are required for each round.

2. The dividing up of the DNA into smaller, overlapping fragments has been performed by a number of different methods:

- DNA mapping using restriction enzymes and subcloning of the restriction products into sequencing vectors (Sambrook *et al*, 1989);
- the so called "shot gun" sequencing method where the DNA is randomly divided up into

fragments and cloned into sequencing vectors (Sulston *et al*, 1992), and

- the so called exodeletion method where the DNA under examination is ligated into a sequencing vector and shortened by means of an exonuclease (Sambrook *et al*, 1989 and Ausubel *et al*, 1989).

All of the above methods are laborious and in each one, the DNA must be transformed into a bacterium (usually into *E.coli*) so as to produce a unique DNA template to be sequenced.

Furthermore, various kinds of methods based on DNA transposition have been disclosed for sequencing DNA. The majority of these methods are based on *in vivo* transposition (United States Patent No. 4,716,105 and Strathmann *et al* 1991). The only known sequencing method (United States Patent No. 5,728,551) exploiting *in vitro* transposition is based on using Tyl integrase activity and even here, only the transposition reaction is carried out *in vitro*; the transposition products are transformed into *Escherichia coli* so as to obtain single sequencing templates. Related art is disclosed also in the United States Patent No. 4,683,202, No. 5,212,080, No. 5,645,991, and in Kasai *et al* (1992), Krishnan *et al* (1993), and Roach *et al* (1995).

It would be highly advantageous if the selection of single sequencing templates through a single cell culture step could be fully avoided. Even in the known, partly *in vitro* methods, the amplification products should be transformed into a bacterium and single colonies are to be picked in order to obtain unique templates for sequencing the DNA.

The present invention aims at eliminating the problems relating to the prior art and, therefore, it is an object of the present invention to provide a simple method for fragmenting larger DNA segments into pieces enabling the sequencing of whole segment by using one or a few sequencing primers.

It is another object of this invention to provide, fully *in vitro*, a unique sequencing template for a single DNA sequencing reaction.

Summary of the Invention

According to one embodiment (later called embodiments A and B) of this invention the examined DNA is subjected to a DNA transposition reaction and to an amplification reaction. The amplification reaction is carried out in the presence of a first, fixed primer, hybridizing at

a known position in the examined DNA or if the examined DNA is part of a vector for example, adjacent to the examined DNA, and a second, selective primer, hybridizing at the insertion site of a transposon. These reactions result in amplification products which can be separated on the basis of their size difference. By suitably selecting the amplification products, whose size difference is in the range of the reading capacity of the sequencing system used, normally in the range of 200 to 600 bp, overlapping DNA templates can be obtained for sequencing. The target DNA can range from a few base pairs up to 40 kilo base pairs. The only limiting factor for not choosing as target DNA even longer DNA segments is the inability of the present amplification reactions, such as PCR, to produce longer DNA segments.

According to another embodiment (later called embodiment C) of this invention a transposition reaction is carried out in the presence of the examined DNA and in the presence of a transposon or transposons which have different ends enabling the design of selective primers for both ends. The transposon ends can be part of the same molecule or separate molecules. The amplification reaction is carried out in the presence of the selective primers designed for both transposon ends. The amplification products are situated at random positions in the target DNA. To be able to cover the whole sequence of the target DNA, a sufficient number of the amplification products needs to be selected for sequencing. Very long DNA segments can be sequenced by selecting the templates according to this embodiment. The present-day amplification reactions, such as PCR, are not a limiting factor. One advantage is that no sequence data of the target DNA is needed, nor is any subcloning procedure necessary.

In another embodiment of this invention there is provided a kit which comprises a transposon recognizable by a transposase enzyme, the transposase enzyme, a fixed primer hybridizing at a known position in the examined DNA or if the examined DNA is part of a vector for example, adjacent to the examined DNA, and a selective primer including a sequence complementary to the transposon joining end.

In a further embodiment of this invention there is provided a kit which comprises a selective primer, a fixed primer, a transposon recognizable by a transposase enzyme, and the transposase enzyme, and wherein the transposon and the transposase enzyme are provided as a transposition complex.

One still further embodiment of this invention provides a kit which comprises two different transposon ends recognizable by a transposase enzyme or transposase enzymes, the transposase enzyme or transposase enzymes, and selective primers including a sequence complementary to the transposon joining ends.

These and other embodiments together with the advantages thereof over known sequencing methods and kits, which shall become apparent from the specification which follows, are accomplished by the invention as hereinafter described and claimed.

Brief Description of the Drawings

Figure 1 is a schematic representation of the principle of first (A) and second (B) embodiments of the invention.

Figure 2 is a schematic representation of the principle of a third embodiment (C) of the invention.

Figure 3 illustrates the structure of a selective primer, wherein X = additional selective nucleotide.

Figure 4 shows the result of a selective PCR reaction on an agarose gel as described in Example 1. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers correspond to the reaction numbers in Table 2.

Figure 5 shows the isolated DNA templates for sequencing on an agarose gel. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers correspond to the fragment numbers marked by arrows in Figure 4. Reaction A is performed by both primers, reaction B is performed by the selective primer only.

Figure 6 shows the results of selective PCR reactions on an agarose gel as disclosed in Example 2. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). "A" refer to the reaction A and "B" refer to reaction B. The numbers corresponds to the DNA fragments selected to the second PCR.

Figure 7 shows the results of second PCR reactions on an agarose gel as disclosed in Example 2. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo,

Finland). The lane numbers correspond to the fragment numbers marked in Figure 6. In Figure 7B a= reaction with both primers, b= reaction only with the fixed primer, c= reaction only with the selective primer.

Figure 8 shows the sequence assembly of the Example 2 results.

Figure 9 shows the results of transposition reaction on an agarose gel as disclosed in Example 3. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). Lane 1 = transposition reaction without MuA transposase. Lanes 2 to 6 = identical lanes each representing 10µl samples of transposition reaction. Lane 7 represents linearized pUC19. A = gel before cutting off. B = gel after cutting off.

Figure 10 shows the results of first PCR in Example 3. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers correspond to the reaction numbers in Table 4.

Figure 11 shows the results of second selective PCR on a 2 % agarose gel as disclosed in Example 3. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers correspond to the reaction numbers in Table 5.

Figure 12 shows the results of second selective PCR on a 4 % Metaphor agarose (FMC) gel as disclosed in Example 3. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers correspond to the reaction numbers in Table 5. The numbers at the side refer to the fragment numbers selected for the third PCR reaction.

Figure 13 shows the results of third PCR on an agarose gel as disclosed in Example 3. M = Lambda/*Hind*III + Phix174/*Hae*III DNA marker (Finnzymes Oy, Espoo, Finland). The lane numbers refer to the reaction numbers in Table 6 and to the fragment numbers in Figure 12.

Figure 14 shows the sequence assembly of the Example 3 results.

Detailed Description of the Invention

As explained above, this invention provides an *in vitro* method for selecting templates for DNA sequencing.

The term "DNA sequencing" refers to the determination of the sequence of purine and pyrimidine bases constituting a deoxyribonucleic acid segment. The purines commonly found in DNA are adenine (A) and guanine (G), the pyrimidines commonly found are cytosine (C) and thymine (T). A DNA sequence analysis results in a variation of the four bases designated
5 as ATCGTGC etc.

Embodiment A

According to the first embodiment of this invention the method comprises the following
10 steps:

- a) A transposition reaction is carried out in the presence of a target DNA, which is a circular DNA or which is part of a circular DNA and in the presence of a transposon.
- 15 A "target DNA" is a segment of the DNA of interest to be fully or partially sequenced. The target DNA can range from a few base pairs up to 40 kilo base pairs. The limiting factor for not choosing as target DNA even longer DNA segments is the inability of the present amplification reactions, such as PCR, to produce longer DNA segments. However, in the third embodiment (C) of this invention, the PCR reaction is not a limiting factor and very
20 long DNA segments can be sequenced according to that embodiment.

A "transposon" is a segment of DNA that can insert itself to a target DNA at random or at almost random locations. Transposons used in transposition reactions *in vivo* normally contain genes coding for enzymes, which are needed in a transposition reaction, but they also
25 contain DNA which is not necessary for the transposition reaction. In reactions *in vitro* it is sufficient that the transposon used contains only the necessary DNA capable of forming a functional complex with a transposase or integrase enzyme and possibly even with other enzymes needed in a transposition reaction.

- 30 It has been shown by Savilahti *et al* (1995) that a functional transposition complex can be formed using only the ends of phage Mu transposon genome and MuA transposase enzyme.

The term "transposon" is intended to mean a DNA segment or segments which is/are recognizable by a transposase or an integrase enzyme and which is/are capable of forming a

functional complex for a transposition reaction.

The term "transposase" is intended to mean an enzyme capable of forming a functional complex with a transposon or transposons needed in a transposition reaction including
5 integrases from retrotransposons and retroviruses.

A "transposition reaction" is a reaction wherein a transposon inserts into a target DNA at random or at almost random sites. Essential components in a transposition reaction are a transposon and a transposase or an integrase enzyme or some other components needed to
10 form a functional transposition complex. The method of this invention is exemplified by employing the transposition complex formed by the ends of Mu genome and MuA transposase enzyme (Savilahti *et al.*, 1995). However, the transposition method is not critical for this invention. All transposition systems capable of inserting amplification primer sites in a random or in an almost random manner are useful in this invention (Craig, 1995).
15 Examples of such systems are Ty1 (Devine and Boeke, 1994, and International Patent Application WO 95/23875), Transposon Tn7 (Craig, 1996), Tn10 and IS10 (Kleckner *et al.* 1996), Mariner transposase (Lampe *et al.*, 1996), Tc1 (Vos *et al.*, 1996, 10(6), 755-61), Tn5 (Park *et al.*, 1992), P element (Kaufman and Rio, 1992) and Tn3 (Ichikawa and Ohtsubo, 1990), bacterial insertion sequences (Ohtsubo and Sekine, 1996), retroviruses (Varmus and
20 Brown 1989) and retrotransposon of yeast (Boeke, 1989).

Due to the high sensitivity of the known amplification methods, such as PCR (United States Patent No. 4,683,195, No. 4,683,202 and No. 4,965,188), the transposition reaction in this embodiment need not be very efficient. Preferably, transposons are inserted into the target
25 DNA in conditions allowing insertion only into a fraction of target molecules to minimize the possibility of having the target DNA with two or more transposon inserts. In case amplification products have sites for selective primer at both ends they are unsuitable for use as sequencing templates.

30 The transposition reaction is preferably carried out by using a transposition complex comprising only the ends of a transposon DNA and a transposase enzyme resulting in a complete cleavage of the target DNA after the insertion of the transposon.

One transposon insertion linearizes the target DNA, whereas two or more insertions shorten

the target DNA giving a smear below the linearized target DNA on an agarose gel. If 5% or less of the target DNA molecules become linearized, the result is sufficient but does not limit the subsequent selective amplification reactions.

- 5 b) The target DNA is amplified by using as amplification primers a first primer, the fixed primer, at a known location, and a second primer, the selective primer, at the transposon insertion site.

The "fixed primer" is intended to mean a DNA primer having a sequence complementary to
 10 the known sequence in the target DNA or if the target DNA is part of a vector, complementary to the known sequence in the vector adjacent to the insertion site of the target DNA. The vector may be any sequencing vector such as the vectors of the pUC series or their derivatives.

- 15 The "selective primer" is intended to mean a primer having a sequence complementary to the joining end of the transposon DNA (complementary to that strand having 5' end at the joining). The selective primer is in 5' → 3' direction towards the target DNA and has 0 to 10, preferably 0 to 5 additional nucleotides of known identity at its 3' end (Figure 3).

- 20 The term "transposon joining end" means that end of the double stranded transposon DNA, which joins to the target DNA at the insertion site. The transposon joining end does not refer to the strand actually ligating to the target DNA. For example the 3' end of the transposon MuA joining end ligates to the 5' end of the target DNA leaving a gap between the 3' end of the target DNA and 5' end of transposon joining end.

25

- A selective amplification reaction, such as PCR, is carried out by using the fixed primer and the selective primer as amplification primers. The additional known nucleotides at the 3' end of the selective primer cause selection of the amplification products. An increasing amount of additional nucleotides improves the selection. In the absence of any additional nucleotides at
 30 the 3' end of the selective primer, the amplification product is a mixture of products of varying sizes making the isolation of products of a specific size very difficult. This is because of the random nature of the transposition reaction *in vitro*. By adding one additional nucleotide to the 3' end of the selective primer, the number of possible templates can be decreased to one-fourth compared to a PCR reaction performed without selection. Two

additional nucleotides decrease the amount of templates to one-sixteenth, three additional nucleotides to one-sixty-fourth, and so on. A similar approach can be found in Liang P. and Pardee B. (1992) in differential display of mRNA. However, no one has previously suggested the insertion of PCR primer sites to target DNA and the production of sequencing templates
5 for PCR by using selective primers according to the principle disclosed here.

The use of selective primers in the amplification reaction results in specific amplification products varying in size, each size representing one template for a sequencing reaction.

10 It is preferable to minimize the unspecific PCR products in the selective PCR by performing a "Hot Start PCR". "Hot Start" can be done by any method known in the art including the addition of DNA polymerase to the reactions after a preheating of samples, isolation of template from DNA polymerase with a wax or using an inactive DNA polymerase that is activated by heat treatment (AmpliTaq Gold, Perking Elmer, USA). Specificity can also be
15 increased by performing an additional PCR before the selective PCR using non selective primer pairs located just before the primers used in selective PCR and transferring small amount of that product into the selective PCR. The method is known as "nested PCR". Unspecific products can, however, also be used for sequencing if they represent only one DNA template.

20

To get large PCR products it is preferable to use DNA polymerase mixtures designed for long PCR. Such mixtures, however, often contain 3' - 5' exonuclease activity also called proof reading activity that can cleave the selective nucleotides from the 3' ends of the selective primers. This problem can also occur if proof reading enzymes like "Vent" or "Deep
25 Vent" from New England Biolabs, USA or Pfu DNA polymerase from Stratagene, USA are used. This phenomenon can be avoided by using phosphorothioate linkages on the last two 3' bases of the selective primers (De Noronha, C. M. C. and Mullins, J. I., 1992).

c) The amplification products are separated on the basis of their size by methods known in
30 the art such as gel electrophoresis, capillary electrophoresis, HPLC or other well-known methods.

The specific amplification products varying in size are separated on the basis of their size difference by running them on an agarose gel for example, and by picking the separate

"bands" into a new amplification reaction, such as PCR, by means of, e.g., a needle or a stick and performing a PCR using the same primers as in the selective amplification. Reaction products of different sizes are obtained, each size corresponding to a separate transposition reaction. The exact position of each PCR product is known in the studied DNA.

5

By suitably selecting PCR products whose size difference is in the range of the reading capacity of the used sequencing system, normally in the range of 200 to 600 bp, overlapping DNA templates are obtained for sequencing. By using the same fixed primer, it is even possible to sequence a larger DNA segment at one go.

10

One possible method for selecting suitable templates for sequencing is to use differently labeled fixed and selective primers and to test that both labels are present in the purified amplification product. The labels can be for example radioactive labels or fluorescent labels and the testing can be performed with an appropriate equipment depending on the labels.

15 From the amplification products are selected for use as templates in sequencing those which have both labels.

d) The target DNA is sequenced by using as templates amplification products overlapping each other and varying in size from each other.

20

The sequencing method can be any sequencing method known in the prior art like the nucleotide specific chemical reaction and cleavage reactions of Maxam and Gilbert (1977) and the primer extension reactions in the presence of nucleotide specific terminators as disclosed by Sanger *et al* (1977) preferably by using the cycle sequencing method.

25

Embodiment B

According to a second embodiment of this invention the target DNA is a linear DNA or part of a linear DNA. A partial sequence of the target DNA or a DNA adjacent to the insertion
30 site of the target DNA is known enabling the design of a fixed primer having a sequence complementary to the known partial sequence. Accordingly, the position of the fixed primer is known within the target DNA or within the linear DNA comprising the target DNA.

The embodiment B comprises the same method steps as the embodiment A except that there

is no linearization step of the target DNA as in the embodiment A.

Embodiment C

According to the third embodiment of this invention the method comprises the following
5 steps (Figure 2):

- a) Two different sites for amplification primers are inserted to a target DNA by transposition. The transposition reaction is carried out by using transposons that have different ends enabling the design of different selective primers for both ends. The transposon ends can be
10 parts of the same molecule or parts of two separate transposon molecules (Savilahti *et al*, 1995). In this embodiment it is preferable to have a more efficient transposition reaction than in the embodiments A and B in order to ensure a sufficient number of molecules having two transposon hits.
- 15 If required, the other DNA strand can be filled in by DNA polymerase and DNA ligase. Most of the transposition systems leave a nick or gap in one of the strands at their joining site to the target DNA.
- b) A selective amplification reaction, such as a PCR reaction, is carried out by using two
20 selective primers as amplification primers. One (first) primer is complementary to one (first) transposon end and the other (second) primer is complementary to the other (second) transposon end. In addition both selective primers have additional nucleotides of known identity at their 3' end .
- 25 The use of selective primers in the amplification reaction results in specific amplification products varying in size, each size representing one template for a sequencing reaction. An increasing amount of additional nucleotides improves the selection.

The selectivity can be accomplished by adding selective nucleotides into the end of only one
30 of the primers. The selectivity can be still further improved by adding selective nucleotides at the end of both primers.

c) The amplification products are separated on the basis of their size by methods known in the art such as gel electrophoresis, capillary electrophoresis, HPLC or other well known

methods.

Suitable templates for sequencing can be selected by exposing the amplification products to three parallel amplification reactions. In one of the reactions only one of the selective primers is used, in the other the second selective primer and in the third reaction both selective primers. Only those templates, which are the result of an amplification reaction by both primers are suitable templates for sequencing. However, a control PCR reaction with only one primer sometimes results in a product the same size as a product correctly produced by using both primers. Another method to select suitable templates is to use differently labeled primers and to test that both labels are present in the purified PCR product. The labels can be for example radioactive labels or fluorescent labels and the testing can be performed with an appropriate equipment depending on the labels.

d) The amplification products are situated at random positions at the target DNA. To be able to cover the whole sequence of the target DNA, a sufficient amount of specific amplification products are selected for sequencing templates.

The sequencing method can be any sequencing method known in the prior art like the nucleotide specific chemical reaction and cleavage reactions of Maxam and Gilbert (1977) and the primer extension reactions in the presence of nucleotide specific terminators as disclosed by Sanger *et al* (1977) preferably using cycle sequencing.

One of the advantages of the third embodiment (C) is that very long DNA segments can be sequenced by selecting the templates according to this embodiment. In this embodiment the present-day amplification reactions, such as PCR, are not a limiting factor, although more sequencing work is needed as compared to the first two embodiments (A and B). Another advantage is that no sequence data of the target DNA is needed nor any subcloning procedure is necessary.

One further embodiment of the present invention is to provide a kit for selecting templates for DNA sequencing.

According to one embodiment of the invention the kit comprises:

- a transposon comprising a DNA sequence which is recognizable by a transposase enzyme,

said transposon DNA and said transposase enzyme being capable of forming a functional complex needed in a transposition reaction,

- a transposase enzyme for carrying out the transposition reaction,
- a fixed primer having a complementary sequence to the known sequence in the target DNA
- 5 or if the target DNA is part of a vector, complementary to the known sequence in the vector adjacent to the insertion site of the target DNA, and
- a selective primer having a sequence complementary to the transposon joining end and having 1 to 10, preferably 1 to 5 additional nucleotides of known identity at its 3' end.

10 According to another embodiment of the invention the kit comprises:

- a transposition complex comprising a transposon and a transposase enzyme, said transposon comprising a DNA sequence which is recognizable by said transposase enzyme,
- a fixed primer having a complementary sequence to the known sequence in the target DNA
- 15 or if the target DNA is part of a vector, complementary to the known sequence in the vector adjacent to the insertion site of the target DNA, and
- a selective primer having a sequence complementary to the transposon joining end and having 1 to 10, preferably 1 to 5 additional nucleotides of known identity at its 3' end.

According to one further embodiment of the invention the kit comprises:

- 20 - at least one transposon having two different joining ends comprising a DNA sequence or sequences which is/are recognizable by a transposase enzyme, said transposon and said transposase enzyme being capable of forming a functional complex needed in a transposition reaction;
- a transposase enzyme for carrying out a transposition reaction, and
- 25 - a first selective primer, having a sequence complementary to the first joining end and having 0 to 10, preferably 0 to 5 additional nucleotides of known identity at its 3' end, and a second selective primer, said second primer having a sequence complementary to the second joining end and having 0 to 10, preferably 0 to 5 additional nucleotides of known identity at its 3' end, wherein at least one selective primer, said first or said second selective primer has
- 30 1 to 10, preferably 1 to 5 additional nucleotides at its 3' end.

The kit may also comprise other components needed in the transposition reaction. Such components may be other enzymes involved in the transposition reaction, or buffers, salts and/or stabilizers which have been chosen depending on the transposase/integrase system.

According to a preferred embodiment of this invention the transposon comprises the Mu transposon ends, recognizable by the MuA transposase.

The following non-limiting examples illustrate the invention in more detail.

5

Example 1

Transposition reaction

10

The target DNA is pUC19 plasmid (GeneBank access no. X02514).

The transposition reaction has been carried out as essentially as described in Savilahti *et al* (1995) using a double stranded transposon DNA of the following sequence (SEQ ID 1):

15 5'TGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACAA
GCTTTTCCATCTCTCCTCCCCCCTG 3'

The 3' end of the complementary strand of the above sequence is joined to the target DNA in a transposition reaction.

20

The reaction conditions were the following:

10 nM Transposon DNA (donor DNA)

1.60 nM pUC19 DNA

25 15 % DMSO

120 mM NaCl

0,05 % Triton X-100

10 mM MgCl₂

25 mM Tris, pH 8.0

30 100 µg/ml BSA

15 % Glycerol

8.7 µg/ml MuA

The reaction volume was 25 µl.

The reaction mixture was incubated at 30 °C for 1 h and frozen after the reaction.

Selective PCR reaction

5

A selective PCR reaction was carried out by using primer 1 and 2 as the fixed PCR primers and primers 3, 4 or 5 as the selective PCR primers according to Table 2. The sequences of the primers are shown in Table 1.

10

Table 1. DNA sequences of the primers

Primer number	Sequence	SEQ ID
15 1	AGCTGGCGAAAGGGGGATGTG	2
2	TTATGCTTCCGGCTCGTATGTTGTGT	3
3	GTTTTTCGTGCGCCGCTTCA	4
4	GTTTTTCGTGCGCCGCTTCAG	5
5	GTTTTTCGTGCGCCGCTTCAGA	6
20 6	GTTTTTCGTGCGCCGCTTCAGAG	7
7	GTTTTTCGTGCGCCGCTTCAGAGT	8
8	GTTTTTCGTGCGCCGCTTCAGATCT	9
9	CGGCGGGAGTTTGGGAGGTT	10
10	GCGGCGGGAGTTTGGGAG	11
25 11	CGCTTTCGTACTTCAAGTGAATCAATACA	12
12	CGCTTTCGTACTTCAAGTGAATCAATACAG	13
13	CGCTTTCGTACTTCAAGTGAATCAATACAGA	14
14	CGCTTTCGTACTTCAAGTGAATCAATACAGAG	15
15	CGCTTTCGTACTTCAAGTGAATCAATACAGAGT	16

30 wherein

1 is a fixed primer in forward direction of pUC19

2 is a fixed primer in reverse direction of pUC19

3 to 8 are selective primers with 0 to 5 selective nucleotides complementary to donor A

9 and 10 are primers complementary to donor B adjacent to the nonjoining end

11 to 15 are selective primers with 0 to 4 selective nucleotides complementary to donor B

Table 2.

5 Reaction number	Fixed primer according to Table 1	Selective primer according to Table 1
1	1	3
2	1	4
3	1	5
4	2	3
10 5	2	4
6	2	5

The PCR reaction conditions were the following:

15

1 µl of ten-fold diluted transposition reaction mixture

0.4 µM of each primer 1 or 2 and primer 3, 4 or 5 according to Table 2

200 µM dNTP (per each nucleotide)

1.5 mM MgCl₂

20 10 mM Tris-HCl pH 8.3

50 mM KCl

2U Dynazyme II DNA polymerase (F-501, Finnzymes Oy, Finland)

The reaction volume was 50µl. Prior to adding the primers the reaction mixture was

25 incubated 10 min at 72 °C to fill in the gap at the insertion site between the 3' end of the target DNA and the 5' end of the transposon DNA.

Cycling conditions:

30 Step	Time	T
1	1 min 30 s	96 °C
2	45 s	96 °C
3	2 min	72 °C
4	29 times to step 2	

5	5 min	72 °C
6	kept until removed	4 °C

The PCR equipment was a DNA Engine from MJ-Research, MA, USA.

5

PCR reactions were loaded and run on regular 1,8 % agarose gel. The result is shown in Figure 4.

Using an 18 G needle small samples of fragments from reactions 3 and 6 from the gel were transformed into new PCR reactions and PCR was performed using the same primers and conditions as in the previous PCR reaction. In addition, a control PCR using only the selective primer was done (see Figure 5, reaction B). The new PCR reactions were loaded and run on 1.8 % agarose gel resulting in separate DNA fragments of different sizes. Primers and nucleotides were removed from each reaction using an AGTC purification system according to the manufacturer's instructions (Advanced Genetic Technologies Corporation, MD, USA). Purified DNA fragments served as sequencing templates (Figure 5).

The sequencing was done using Thermo Sequenace cycle sequencing kit according to the manufacturer's instructions using P³³ labeled primers (Amersham Life Science, UK). The sequencing reactions were run on a 6 % Gene Page Plus polyacrylamide gel (Amresco, Ohio, USA) using a single loading.

Table 3

25 Fragment #	Reaction #	Size	Sequence location	Sequence length
			at pUC19	read
1	3	162 bp	318 to 469	151
2	3	374 bp	457 to 681	224
3	3	646 bp	670 to 953	283
4	4	347 bp	188 to 325	137
30 5	5	503 bp	32 to 202	170

It was possible to sequence 921 bp of pUC19 in one run. The pUC19 was chosen as target DNA because the sequence was known. The experiment shows that the method works and could be employed to select templates for sequencing longer DNA segments. The only

limiting factor is the capacity of the PCR reaction to produce longer DNA fragments.

5 Example 2

The target DNA to be sequenced is the 6557 bp DNA fragment from Lambda DNA-*Hind*III digestion inserted into pUC19 at the *Hind*III site.

- 10 The transposition reaction was carried out as described in Savilahti *et al* (1995) using a double stranded transposon DNA (donor A) of the following sequence (SEQ ID 1):

5'TGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACAA
GCTTTTCCATCTCTCCTCCCCCCTG3'

15

The 3' end of the complementary strand of the above sequence is joined to the target DNA in a transposition reaction.

The reaction conditions were the following:

20

50 nM Transposon DNA (donor A)

1.6 nM (10 ng/μl) pUC19 DNA having 6557 bp insert from lambda phage

15 % DMSO

120 mM NaCl

25 0.05 % Triton X-100

10 mM MgCl₂

25 mM Tris, pH 8.0

100 μg/ml BSA

15 % Glycerol

30 8.7 μg/ml MuA

The reaction volume was 50 μ l.

The reaction mixture was incubated at 30 °C for 1 h and frozen after the reaction.

5 Selective PCR reaction

Two different selective PCR reactions were carried out by using forward primer 1 (reaction A) or reverse primer 2 (reaction B) as the fixed PCR primer and primer 7 as the selective primer. The sequences of the primers are shown in Table 1 in Example 1.

10

The PCR reaction conditions were the following:

- 1 μ l of ten-fold diluted transposition reaction mixture
- 15 0.4 μ M of primer 1 (reaction A) or primer 2 (reaction B)
- 0.4 μ M primer 7
- 360 μ M dNTP (per each nucleotide)
- 1.8 mM MgCl₂
- 50 mM Tris-HCl (pH 9.0 at 25 °C)
- 20 15 mM (NH₄)₂SO₄
- 0.1 % Triton X-100
- 1 U Dynazyme EXT DNA polymerase (F-501, Finnzymes Oy, Finland)
- H₂O added to 50 μ l

25

Cycling conditions for reaction A:

Step	Time	T
1	1 min 30 s	96 °C
30 2	45 s	96 °C
3	8 min	72 °C
4	29 times to step 2	
5	8 min	72 °C

20

6 kept until removed 4 °C

Cycling conditions for reaction B:

5	Step	Time	T
	1	1 min 30 s	96 °C
	2	45 s	96 °C
	3	1 min	68 °C
	4	5 min	72 °C
10	5	29 times to step 2	
	6	10 min	72 °C
	7	kept until removed	4 °C

The PCR instrument was a PTC-200 DNA Engine from MJ-Research, MA, USA.

15

PCR reactions were loaded and run on 1 % Seakem LE agarose gel (FMC) in TBE buffer. The gel was stained after the run with 1:10,000 diluted SYBR Green gel stain (FMC) for 30 minutes in a low speed shaker at room temperature. SYBR Green gel stain is more sensitive than ethidium bromide and facilitates sampling of especially large lower intensity PCR products from the gel. The results are shown in Figure 6.

20

Using a 10 µl disposable pipette tip samples of specific fragments from reactions A (17 fragments) and B (16 fragments) were picked from the gel and transferred into 20 µl TE buffer. One micro liter of samples were then used in new PCR reactions. PCR was performed using the same primers and conditions as in the previous PCR reaction except that the extension time of A samples was shortened from 8 minutes to 5 minutes at 72 °C. In addition, a control PCR using only the selective primer or fixed primer was done for B samples. The new PCR reactions were loaded and run on 1.0 % agarose gel resulting in separate DNA fragments of different sizes. Results are shown in Figure 7.

25

30

Reactions A3, A4, A5, A8, B5 - B16 had several fragments in the same reaction. The desired fragments were isolated and purified from the gel using Qiaex II Gel Extraction Kit (Qiagen, USA) according to manufacturer's instructions.

Reactions A6, A9, A12, A13, A14, A15, A16 and A17 showed only one main band and they were purified directly by Qiaquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions.

- 5 The purified DNA templates were sequenced by using SequiTherm EXCEL II Long-Read DNA Sequencing Kit (Epicentre Technologies, USA) according to their Cycle Sequencing Protocol for Li-Cor automated DNA sequencer. The used amount of each DNA template was 7.8 µl. The sequencing primer was IRD800 labeled primer 3 (Table 1).

- 10 The cycling parameters were:

Step	time	temperature
1	5 min	95 °C
2	30 sec	95 °C
15 3	15 sec	67 °C
4	1 min	70 °C
5	29 times to step 2	
6	kept until removed	4 °C

- 20 The automated DNA sequencing apparatus was a Li-Cor Long Read IR 4200. The used gel length was 66 cm and the gel was 4 % Long Ranger (FMC).

The sequencing data were assembled with Lasergene Seqman software (Dnastar, WI, USA). Data are shown in Figure 8.

25

- Of the 33 selected fragments 17 sequencing templates were received and sequenced. With received sequence data it was possible to cover the full sequence of the 6557 bp insert of lambda phage. The insert is in the vector at the position from 450 to 7007. The accuracy of the sequence from position 450 to 4200 was better than 99 % and the accuracy of the
 30 sequence from 4970 to 7050 was better than 98 % compared to the known sequence of lambda DNA.

This example shows that according to the method of this invention it is possible to rapidly sequence *in vitro* a larger DNA insert in a vector. To get more complete sequence it is

preferable to use several different selective primers to produce more templates to be sequenced.

Example 3

5

Transposition reaction

The target DNA was pUC19 plasmid.

The transposition reaction was carried out essentially as described in Savilahti *et al* (1995) using a mixture of two different double stranded transposon DNAs (donor A and donor B) of the following sequences (SEQ ID 1 and SEQ ID 17) capable of forming a functional transposon complex with MuA transposase:

Donor A (SEQ ID 1)

15 5'TGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACAA
GCTTT TCCATCTCTCCTCCCCCCTG 3'

Donor B (SEQ ID 17)

5'TGTATTGATTCACTTGAAGTACGAAAGCGTTTCACGAAAAACGCGAAAGCAGA
20 TCTAACCTCCCAAACCTCCCGCCGC 3'

The 3' end of the complementary strand of the above sequences is joined to the target DNA in a transposition reaction.

25 The reaction conditions were the following:

250 nM Transposon DNA (donor A DNA) SEQ ID 1

250 nM Transposon DNA (donor B DNA) SEQ ID 17

5.7 nM (10 ng/μl) pUC19

30 15 % DMSO

120 mM NaCl

0.05 % Triton X-100

10 mM MgCl₂

25 mM Tris, pH 8.0

100 µg/ml BSA
 15 % Glycerol
 87 µg/ml MuA
 H₂O added to 50 µl

5

The reaction mixture was incubated at 30 °C for 4 h. 10 µl of MuA stop solution, 2.5 % SDS, 25 % Ficoll 400, 0.025% bromophenol blue and 50 mM EDTA were added to the reaction. 10 µl samples of the reaction were loaded in to 5 lanes on a 0.8 % Seaplaque agarose gel (FMC) and run on a standard horizontal gel apparatus in TAE buffer.

10

The first transposon hit to the circular pUC19 plasmid causes the linearization of the plasmid. Two or more transposon hits to the same plasmid molecule result in different random size products that are seen on the Figure 9 as a smear under the linearized plasmid DNA. That DNA was isolated by cutting off that part of the gel and solubilized and extracted twice with an equal volume of phenol solution and once with an equal volume of chloroform/isoamylalcohol (24:1). Sodium acetate was added to 300 mM final concentration and DNA was precipitated by adding two volumes of ethanol. After drying the DNA was dissolved in 120 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

15

20 **Selective PCR reaction**

Selective PCR was done in two steps. In the first step the PCR was done using primer 3 or primer 4 and primer 9 or 10 according to the Table 1 and 4. Primer 3 and 4 are complementary to the joining end of donor A having 0 or 1 selective nucleotide. Primer 9 and 10 are complementary to the non joining end of donor B having no selective nucleotides. Control reactions having only one primer were also done.

25

The PCR reaction conditions were the following:

30 1 µl of gel isolated transposition reaction mixture
 0.4 µM of each primer according to Table 4
 200 µM dNTP (per each nucleotide)
 1.5 mM MgCl₂

WO 99/04035

PCT/FI98/00586

24

10 mM Tris-HCl pH 8.3
 50 mM KCl
 0.01 % (w/V) gelatin
 2.5 U AmpliTaq Gold DNA polymerase (Perking Elmer, USA)
 5 H₂O added to 50 µl

PCR Cycling conditions:

	Step	Time	T
10	1	9 min	95 °C
	2	1 min 30 s	96 °C
	3	45 s	96 °C
	4	1 min	68 °C
	5	2 min	72 °C
15	6	29 times to step 3	
	7	5 min	72 °C
	8	kept until removed	4 °C

Table 4

20	Reaction number	Donor A primer according to Table 1	Donor B primer according to Table 1
	1	3	9
	2	No primer	9
	3	3	10
25	4	No primer	10
	5	3	No primer
	6	4	9
	7	4	10
	8	4	No primer

30

The PCR instrument was a PTC-200 DNA Engine from MJ-Research, MA, USA.

9 µl of each reaction were run on regular 2 % agarose gel electrophoresis stained with ethidium bromide. The results are shown in Figure 10.

The PCR reaction number 6 showed a good distribution of different size PCR products.

- 5 Reaction 6 was used in the second step PCR. The aim was to study what selection strength is needed.

Selective primers were now used at both sides (Donor A and Donor B) according to Table 5.

10 The PCR conditions and cycles and the electrophoresis of the samples were the same as in the previous PCR. The results are shown in Figure 11.

Table 5

	Reaction number	Primer A	Primer B	Selective nucleotides at Donor A side	Selective nucleotides at Donor B side
15	1	5	11	GA	-
	2	no primer	11	-	-
	3	5	12	GA	G
	4	no primer	12	-	G
	5	5	13	GA	GA
20	6	no primer	13	-	GA
	7	5	14	GA	GAG
	8	no primer	14	-	GAG
	9	5	15	GA	GAGT
	10	no primer	15	-	GAGT
25	11	5	no primer	GA	-
	12	6	11	GAG	-
	13	6	12	GAG	G
	14	6	13	GAG	GA
	15	6	14	GAG	GAG
30	16	6	15	GAG	GAGT
	17	6	no primer	GAG	-
	18	7	11	GAGT	-
	19	7	12	GAGT	G
	20	7	13	GAGT	GA
35	21	7	14	GAGT	GAG
	22	7	15	GAGT	GAGT
	23	7	no primer	GAGT	-
	24	8	11	GATCT	-
	25	8	12	GATCT	G
40	26	8	13	GATCT	GA
	27	8	14	GATCT	GAG
	28	8	15	GATCT	GAGT
	29	8	no primer	GATCT	-

Different selections in the PCR resulted in different specific DNA bands on agarose gel electrophoresis. Control reactions with only one primer showed that some of the PCR products have Donor A at both ends.

- 5 Samples of reactions 12, 13, 14, 17, 18, 19, 20, 23, 24, 25, 26 and 29 were run again on a 4 % Metaphor agarose (FMC) electrophoresis in TBE-buffer. The gel was stained after the run with 1:10,000 diluted SYBR Green gel stain (FMC) for 30 minutes in a low speed shaker at room temperature. The results are shown in the Figure 12.
- 10 Using 10 µl disposable pipette tips 15 samples of different specific fragments were picked and transferred into 20 µl TE buffer. Those samples were then used as a template in the next PCR to amplify the specific products.

The PCR reaction conditions were the following:

15

1 µl of template

0.4 µM of each primer according to Table 6

200 µM dNTP (per each nucleotide)

1.5 mM MgCl₂

20 10 mM Tris-HCl pH 8.3

50 mM KCl

1 U Dynazyme II DNA polymerase (F-501, Finnzymes Oy, Finland)

H₂O added to 50 µl

25

Table 6

	Reaction number	Primer A	Primer B
30	1	6	11
	2	6	11
	3	6	11
	4	6	11
	5	7	11
35	6	7	11
	7	7	11
	8	7	11
	9	7	11

WO 99/04035

PCT/FI98/00586

27

5	10	8	11
	11	8	11
	12	8	11
	13	8	11
	14	8	11
	15	8	11

Cycling conditions for reactions :

10

Step	Time	T
1	1 min 30 s	96 °C
2	45 s	96 °C
3	1 min	68 °C
15 4	2 min	72 °C
5	29 times to step 2	
6	5 min	72 °C
7	kept until removed	4 °C

20 5 µl samples of the reactions were run on a 2 % standard agarose gel electrophoresis stained with ethidium bromide. The results are shown in Figure 13. Reactions 6, 7, 8, 10, 13 and 15 showed only one main fragment and were purified by Qiaquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. Two fragments from reaction 4 and one fragment from each of reactions 9 and 14 were isolated and purified from the gel using Qiaex 25 II Gel Extraction Kit (Qiagen, USA) according to the manufacture's instructions.

Cycle Sequencing of the isolated DNA templates

The purified DNA templates were sequenced by using the Thermo Sequenase cycle sequencing kit according to the manufacturer's instructions using IRD800 labeled primer 3 (Table 1).

The cycling parameters were:

35	Step	time	temperature
	1	30 sec	95 °C

28

- | | | |
|---|--------------------|-------|
| 2 | 30 sec | 67 °C |
| 3 | 1 min 30 sec | 72 °C |
| 4 | 60 times to step 2 | |
| 5 | kept until removed | 4 °C |

5

The automated DNA sequencing apparatus was a Li-Cor Long Read IR 4200. The used gel length was 41 cm and the gel was 6 % Gene Page (Amresco, USA).

The sequencing data were assembled with Lasergene Seqman software (Dnastar, WI, USA) and compared to the known sequence of the pUC19 plasmid. Data are shown in Figure 14.

The experiment shows that the method works and could be employed to select templates for sequencing of an unknown DNA without any subcloning and completely *in vitro*.

15

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., 1989, Current Protocols in Molecular Biology 1.

20

Boeke J. D. 1989. Transposable elements in *Saccharomyces cerevisiae* in Mobile DNA. Berg D. E. and Howe M. M. eds. American society for microbiology, Washington D. C. pp. 335-374.

25 Craig N. L. 1995. Unity in transposition reactions. Science 270: 253-254.

Craig N. L. 1996. Transposon Tn7. Curr. Top. Microbiol. Immunol. 204: 27-48.

De Noronha, Carlos M. C. and Mullins, James I., PCR Methods Appl. ,1992, 2(2): 131-6.

30

Devine, S.E. and Boeke, J.D., Nucleic Acids Research, 1994, 22(18): 3765-3772.

Ichikawa H. and Ohtsubo E., J. Biol. Chem., 1990, 265(31): 18829-32.

Itakura, K., Rossi, J.J. and Wallace, R.B., *Ann. Rev. Biochem.* 1984, 53: 323-356.

Kasai, H., Isono, S., Kitakawa, M., Mineno, J., Akiyama, H., Kurnit, D.M., Berg, D.E. and Isono, K. *Nucleic Acids Research*, 1992, 20 (24): 6509-6515

5

Kaufman P. and Rio D.C., *Cell*, 1992, 69(1): 27-39.

Kleckner N., Chalmers R. M., Kwon D., Sakai J. and Bolland S. *Tn10 and IS10*

Transposition and chromosome rearrangements: mechanism and regulation in vivo and in
10 vitro. *Curr. Top. Microbiol. Immunol.*, 1996, 204: 49-82.

Krishnan, R. et al., *Methods in Enzymology*. Transposon-based and polymerase chain
reaction-based sequencing of DNAs cloned in a phage, 1993, Vol. 218.

15 Lampe D.J., Churchill M.E.A. and Robertson H.M., *EMBO J.*, 1996, 15(19): 5470-5479.

Liang P. and Pardee B., *Science*, 1992, 257: 967-971.

Maxam and Gilbert, *PNAS*, 1977, 74:560.

20

Ohtsubo E. & Sekine Y. Bacterial insertion sequences. *Curr. Top. Microbiol. Immunol.*,
1996, 204: 1-26.

Park B.T., Jeong M.H. and Kim B.H., *Taehan Misaengmul Hakhoechi*, 1992, 27(4): 381-9.

25

Roach, J.L., Boysen, C., Wang, K. and Hood, L., 1995, 26(2):345-53.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular Cloning A Laboratory Manual*,
Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

30

Sanger, F., Nicklen, S., and Coulson, A.R., *Proc. Natl. Acad. Sci. USA*, 1977, 74: 5463-
5467.

Savilahti, H., Rice, P.A. and Mizuuchi, K., *The EMBO Journal*, 1995, 14(19): 4893-4903.

Strathmann M., Hamilton B. A., Mayeda C. A., Simon M. I., Meyerowitz E. M. and Palazzolo M. J., Proc. Natl. Acad. Sci. USA, 1991, 88:1247-1250.

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Nature 1992, 356: 37-41.

5

Varmus H and Brown. P. A. 1989. Retroviruses. in Mobile DNA. Berg D. E. and Howe M. M. eds. American society for microbiology, Washington D. C. pp.53-108.

Vos J.C., Baere I. And Plasterk R.H.A., Genes Dev., 1996,10(6): 755-61.

10

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Finnzymes Oy
- (B) STREET: Riihitontuntie 14 B
- (C) CITY: ESPOO
- (E) COUNTRY: FINLAND
- (F) POSTAL CODE (ZIP): 02200
- (G) TELEPHONE: +358 9 584121

(ii) TITLE OF INVENTION: In vitro method for providing templates for DNA sequencing

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: 2,295,968

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: FI 972992
- (B) FILING DATE: 14-JUL-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "transposon"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Savilahti, H
Rice, P A
Mizuuchi, K
- (C) JOURNAL: EMBO J.
- (D) VOLUME: 14
- (F) PAGES: 4893-4903

(G) DATE: 1995

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGAAGCGGCG CACGAAAAAC GCGAAAGCGT TTCACGATAA ATGCGAAAAC AAGCTTTTCC 60
ATCTCTCCTC CCCCCTG 77

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGCTGGCGAA AGGGGGATGT G 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTATGCTTCC GGCTCGTATG TTGTGT 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTTTTTCGTG CGCCGCTTCA

20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTTTTTCGTG CGCCGCTTCA G

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTTTTTCGTG CGCCGCTTCA GA

22

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTTTTTCGTG CGCCGCTTCA GAG

23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTTTTCGTG CGCCGCTTCA GAGT

24

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTTTTTCGTG CGCCGCTTCA GATCT

25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGGCGGGAGT TTGGGAGGTT

20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGGCGGGAG TTTGGGAG

18

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGCTTTCGTA CTTCAAGTGA ATCAATACA

29

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGCTTTCGTA CTTCAAGTGA ATCAATACAG

30

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CGCTTTCGTA CTTCAAGTGA ATCAATACAG A

31

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGCTTTCGTA CTTCAAGTGA ATCAATACAG AG

32

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGCTTTCGTA CTTCAAGTGA ATCAATACAG AGT

33

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "transposon"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TGTATTGATT CACTTGAAGT ACGAAAGCGT TTCACGAAAA ACGCGAAAGC AGATCTAACC	60
TCCCAAACCTC CCGCCGC	77

WHAT IS CLAIMED IS:

1. An *in vitro* method for providing templates for DNA sequencing, comprising the steps of
 - carrying out a transposition reaction in the presence of a target DNA and in the presence of a transposon, said transposition reaction resulting in at least one insertion into said target DNA;
 - performing an amplification reaction by using as primers a first, fixed primer hybridizing at a known location in said target DNA or if the target DNA is part of a vector adjacent to said target DNA, and a second, selective primer hybridizing at the transposon insertion site, said selective primer having a sequence complementary to the joining end of said transposon and having additional nucleotides of known identity at its 3' end;
 - separating the amplification products on the basis of their size, and
 - recovering said amplification products suitable for use as templates in sequencing said target DNA.
2. The method according to claim 1, wherein the selective primer has 1-10 additional nucleotides at its 3' end.
3. The method according to claim 1, wherein the amplification products useful as templates overlap each other and differ in size from each other.
4. The method according to claim 1, 2 or 3 wherein the first, fixed primer and the second, selective primer are differently labeled and said target DNA having both labels is selected from the amplification products.
5. The method of claim 4, wherein the first, fixed primer and the second, selective primer are differently labeled by labels selected from the group consisting of radioactive labels and fluorescence labels.
6. The method according to any one of claims 1 to 5, wherein said amplification reaction is a PCR reaction.

7. The method according to any one of claims 1 to 6, wherein said target DNA is a circular DNA or part of a circular DNA.
8. The method according to any one of claims 1 to 6, wherein said target DNA is a linear DNA or part of a linear DNA.
9. The method according to any one of claims 1 to 8, wherein said target DNA is inserted into a vector and said first, fixed primer, has a sequence complementary to the vector sequence adjacent to the target insertion site.
10. The method according to any one of claims 1 to 9, wherein the transposon comprises a DNA sequence which is recognizable by a transposase enzyme and said transposon DNA and said transposase enzyme form a functional complex needed in the transposition reaction.
11. The method according to claims 1 to 10, wherein the transposon comprises the Mu transposition ends, recognizable by the MuA transposase.
12. An *in vitro* method for providing templates for DNA sequencing, comprising the steps of:
- carrying out a transposition reaction in the presence of at least one transposon having two different joining ends, said joining ends differing from each other by at least one nucleotide;
 - performing an amplification reaction by using as primers a first, selective primer, having a sequence complementary to the first joining end and a second, selective primer, said second primer having a sequence complementary to the second joining end, wherein at least said first or said second selective primer has additional nucleotides of known identity at its 3' end;
 - separating said amplification products on the basis of their size;
 - selecting templates for DNA sequencing among the amplification products which have different ends and which are produced only by both primers together, and

- recovering said amplification products suitable for use as templates in sequencing said target DNA.

13. A method as defined in claim 12, wherein at least one of said first and second primers has 1-10 additional nucleotides at its 3' end.

14. The method according to claim 11, wherein the first primer and the second primer are differently labeled and from the amplification products are selected for use as templates in sequencing those which have both labels.

15. The method of claim 14 wherein the first primer and second primer are differently labeled by labels selected from the group consisting of radioactive labels and florescence labels.

16. The method according to any one of claims 14 and 15, wherein the amplification reaction is a PCR reaction.

17. The method according to any one of claims 12 to 16, wherein the transposon or transposons is/are recognizable by a transposase enzyme, said transposon DNA and said transposase enzyme being capable of forming a functional complex needed in the transposition reaction.

18. The method according to any one of claims 12 to 17, wherein the transposon comprises the Mu transposon ends, recognizable by the MuA transposase.

19. A kit for providing templates for DNA sequencing, comprising

- a transposon comprising a DNA sequence which is recognizable by a transposase enzyme, said transposon DNA and said transposase enzyme being capable of forming a functional complex needed in a transposition reaction;
- a transposase enzyme for carrying out the transposition reaction;
- a fixed primer having a sequence complementary to a known sequence in the target DNA or if the target DNA is part of a vector complementary to a vector sequence adjacent to inserted target DNA, and

- a selective primer having a sequence complementary to the joining end of the transposon and the 3' end of said primer having additional nucleotides of known identity.

20. A kit for providing templates for DNA sequencing, comprising

- a transposition complex containing a transposon and a transposase enzyme, said transposon comprising a DNA sequence which is recognizable by said transposase enzyme,
- a fixed primer having a sequence complementary to a known sequence in the target DNA or if the target DNA is part of a vector complementary to a vector sequence adjacent to inserted target DNA, and
- a selective primer having a sequence complementary to the joining end of the transposon and the 3' end of said primer having additional nucleotides of known identity.

21. A kit as defined in either of claims 19 or 20, wherein said selective primer has from 1-10 additional nucleotides at its 3' end.

22. A kit for providing templates for DNA sequencing, comprising

- at least one transposon having two different joining ends comprising a DNA sequence or sequences, which is/are recognizable by a transposase enzyme, said transposon and said transposase enzyme being capable of forming a functional complex needed in a transposition reaction;
- a transposase enzyme for carrying out the transposition reaction;
- a first selective primer, having a sequence complementary to the first joining end and a second selective primer, said second selective primer having a sequence complementary to the second joining end, wherein at least said first or said second selective primer has additional nucleotides of known identity at its 3' end.

23. The sequencing kit according to any one of claims 19 to 22, wherein the transposon comprises the Mu transposon ends, recognizable by the MuA transposase.

24. The sequencing kit according to claim 22, wherein at least one of said first and second primers has 1-10 additional nucleotides at its 3' end.

1/15

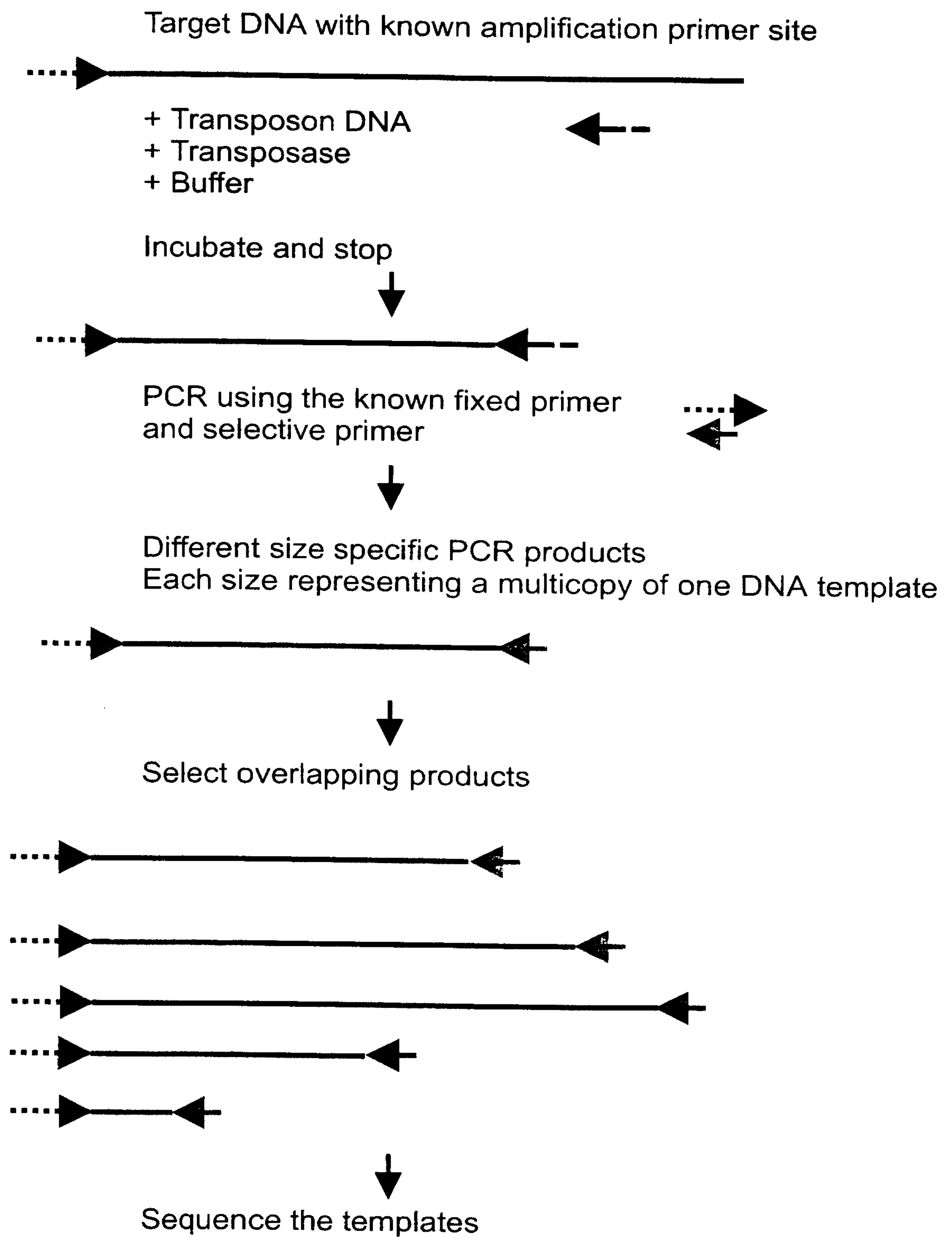


FIG. 1

2/15

Target DNA to be "Shot Gun" sequenced

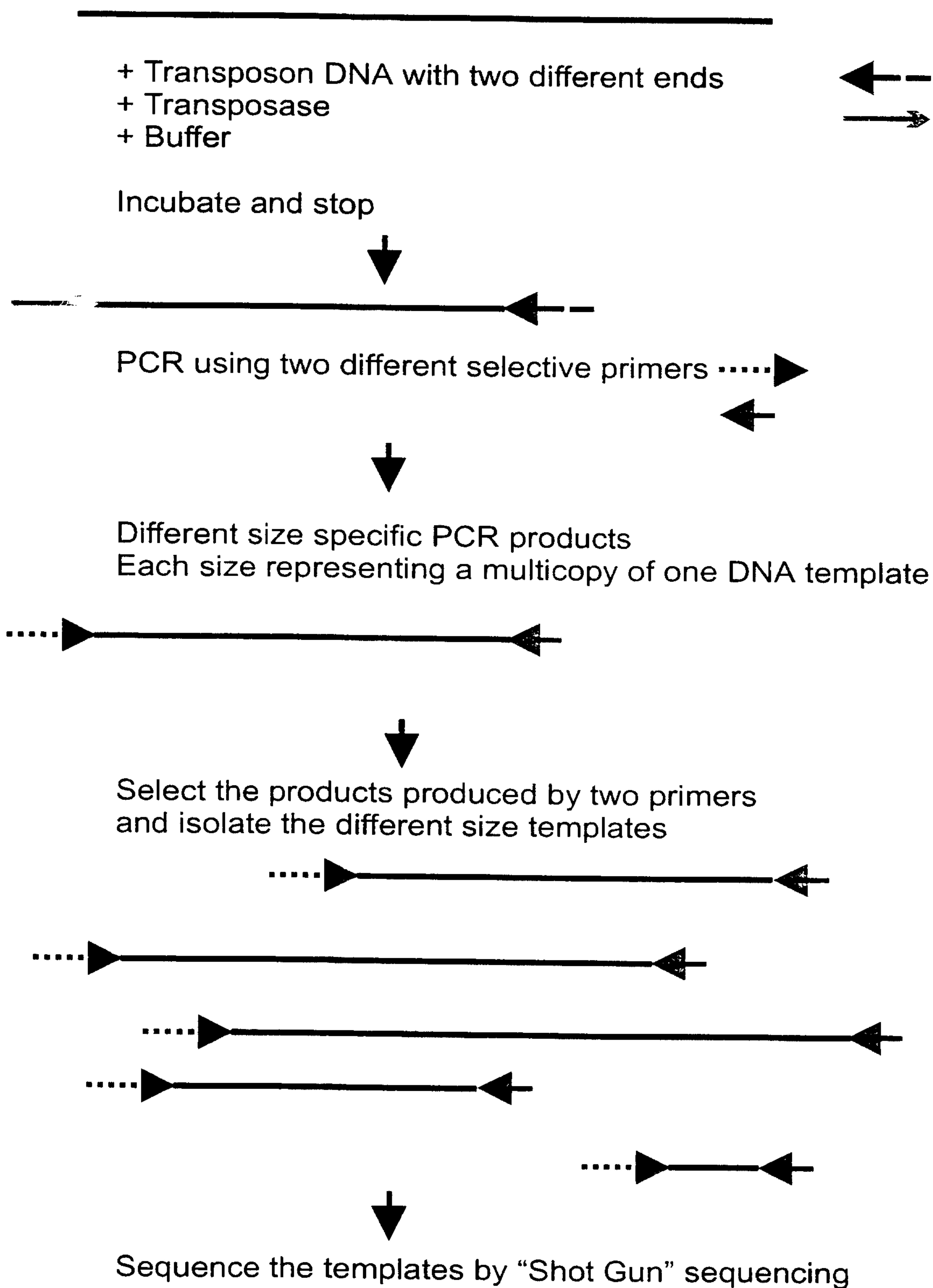


FIG. 2

WO 99/04035

PCT/FI98/00586

3/15

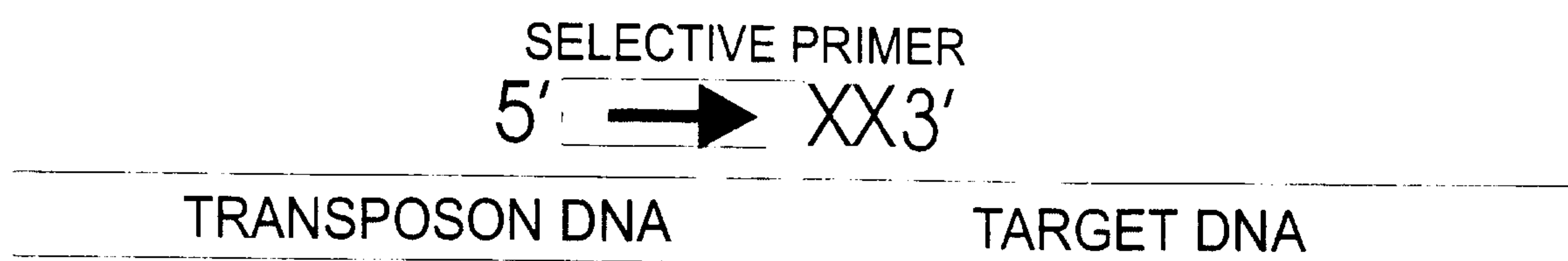


FIG. 3

4/15

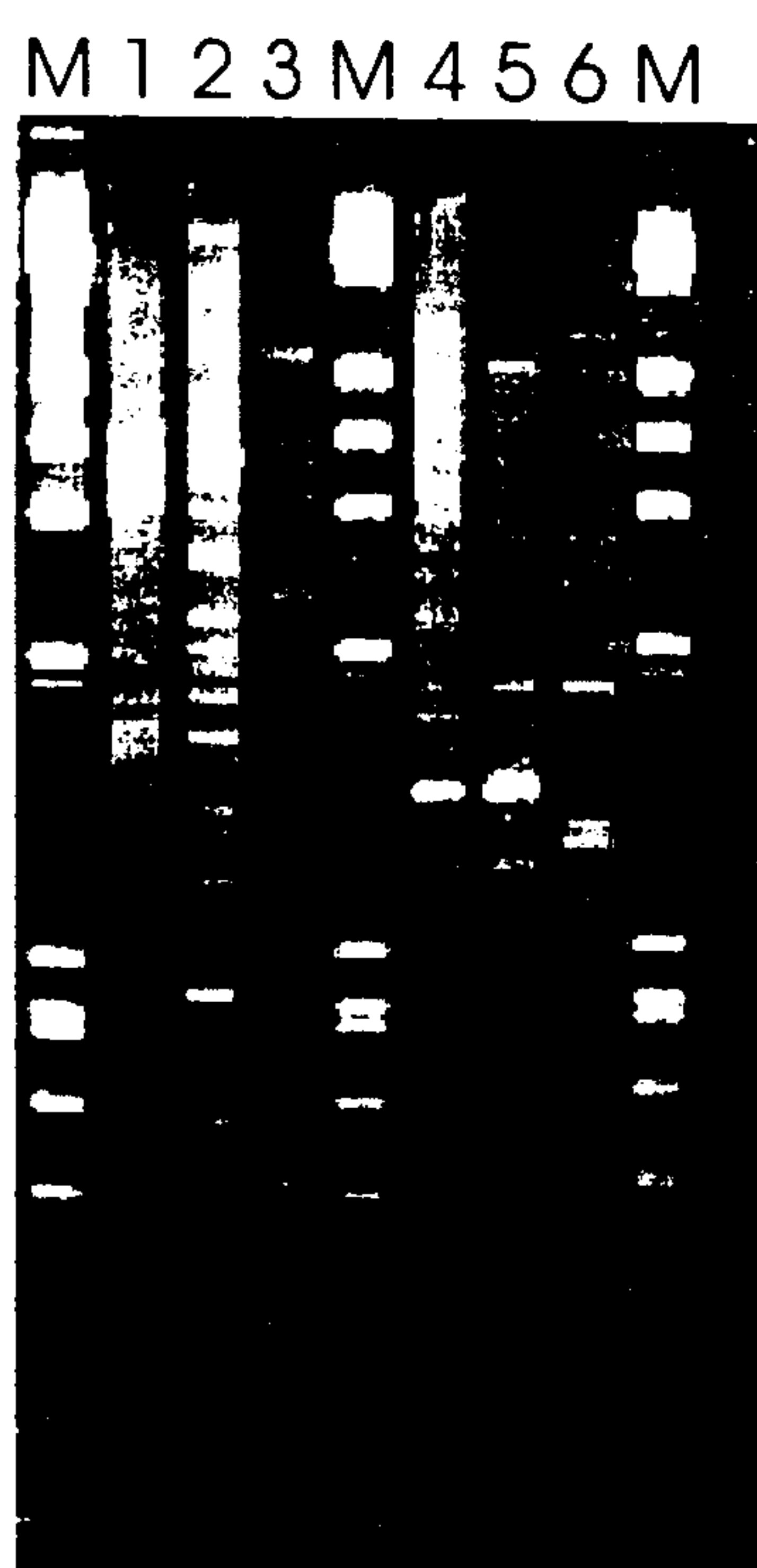


FIG. 4

5/15

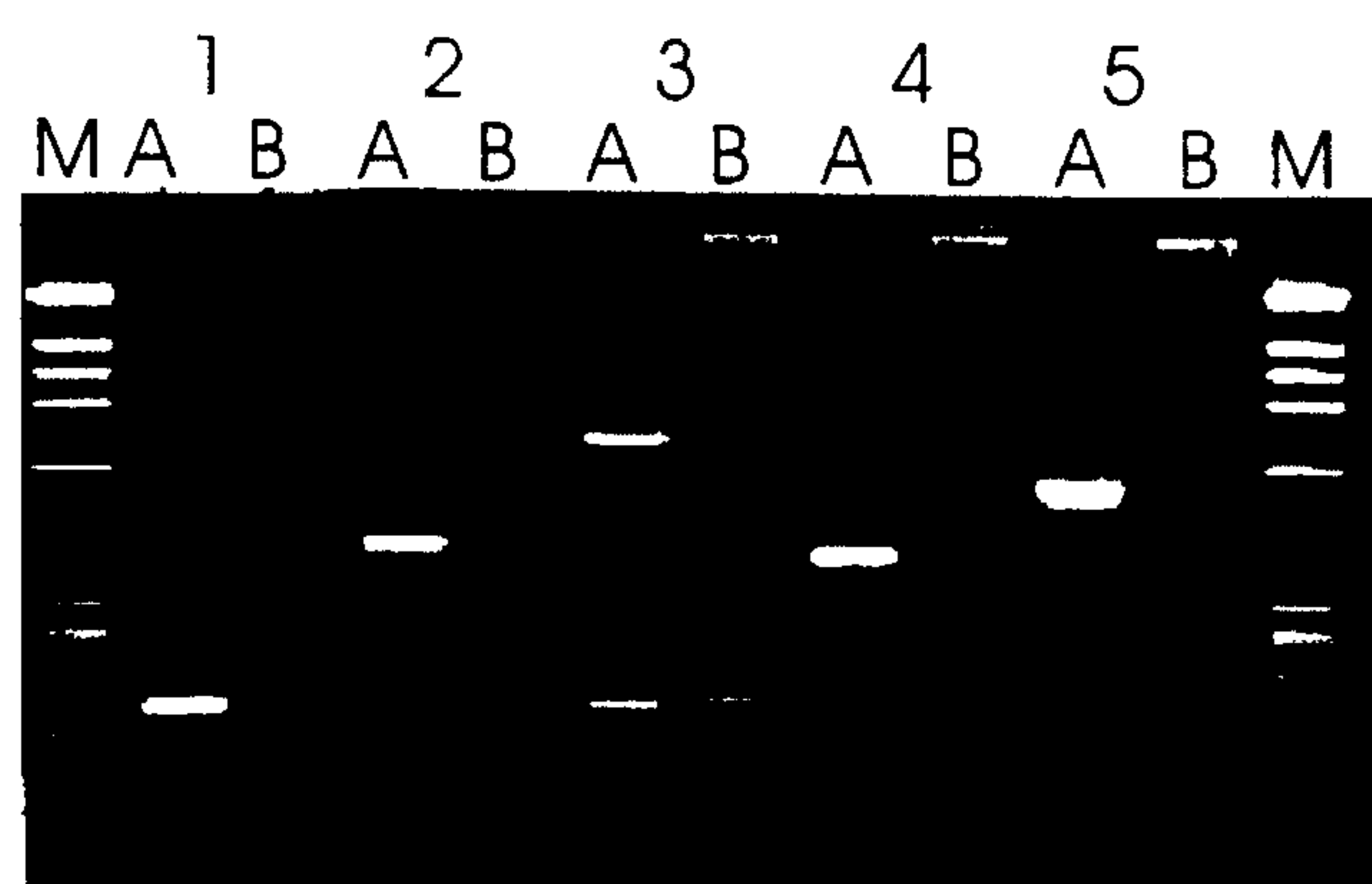
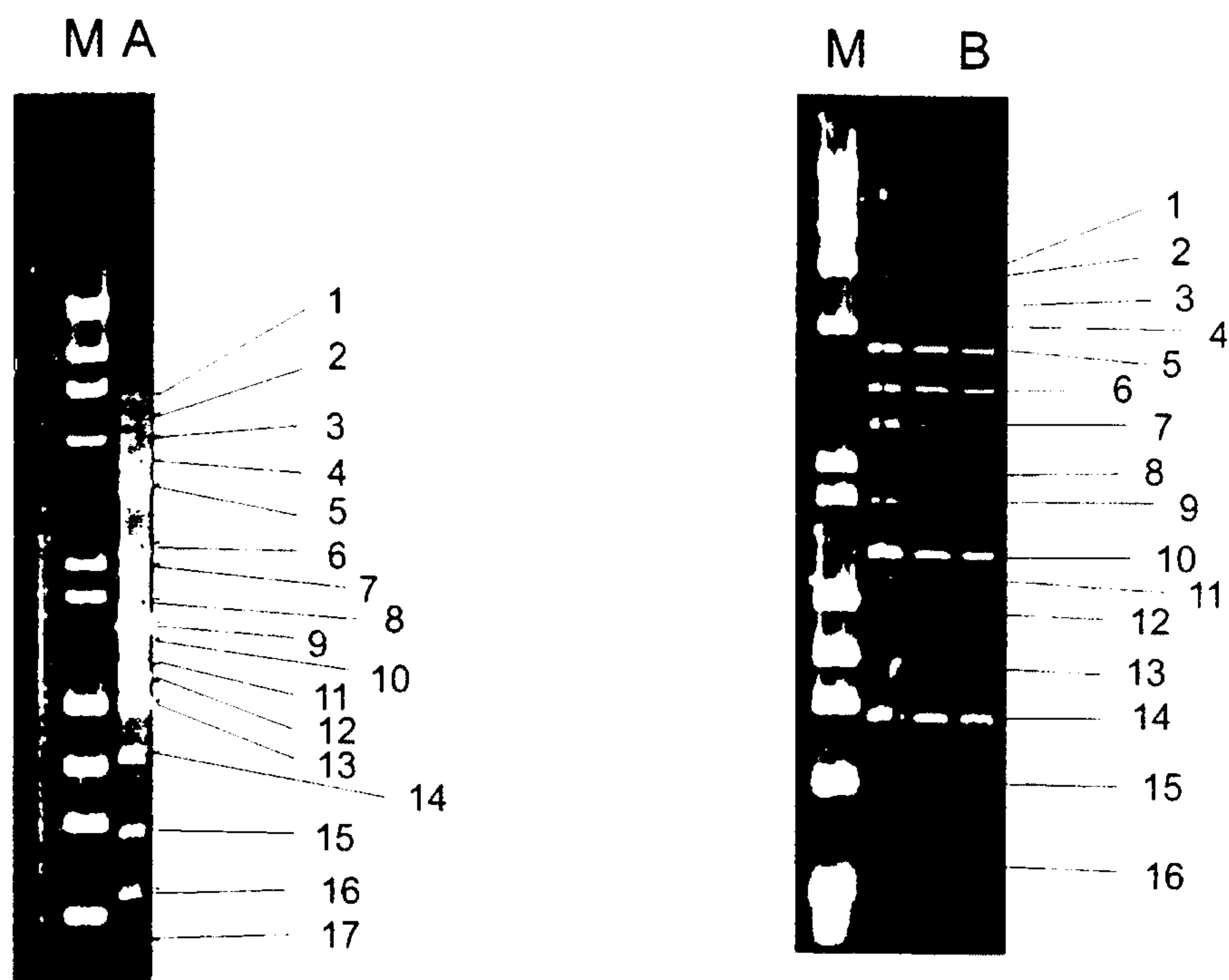


FIG. 5

6/15

**FIG. 6**

7/15

A-REACTIONS

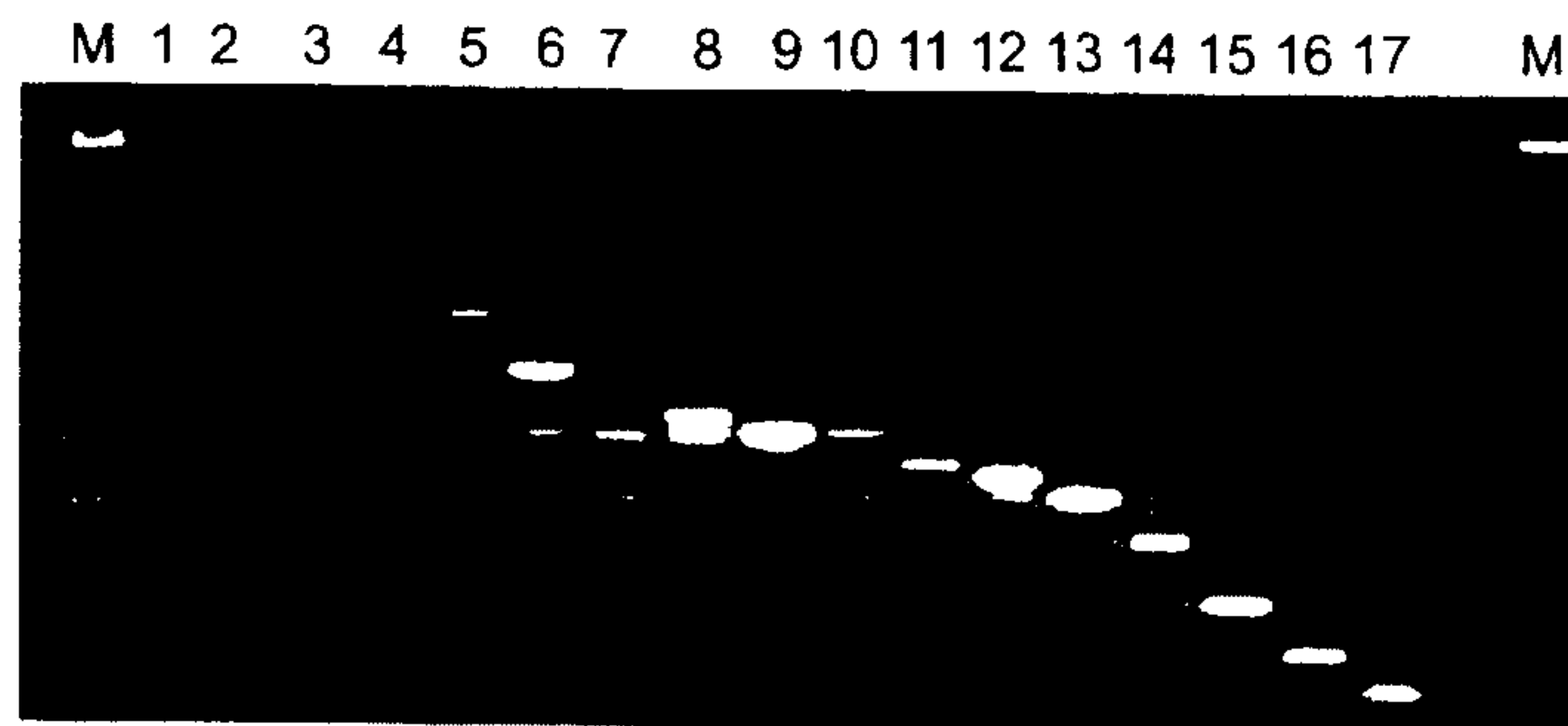


FIG. 7A

8/15

B-REACTIONS

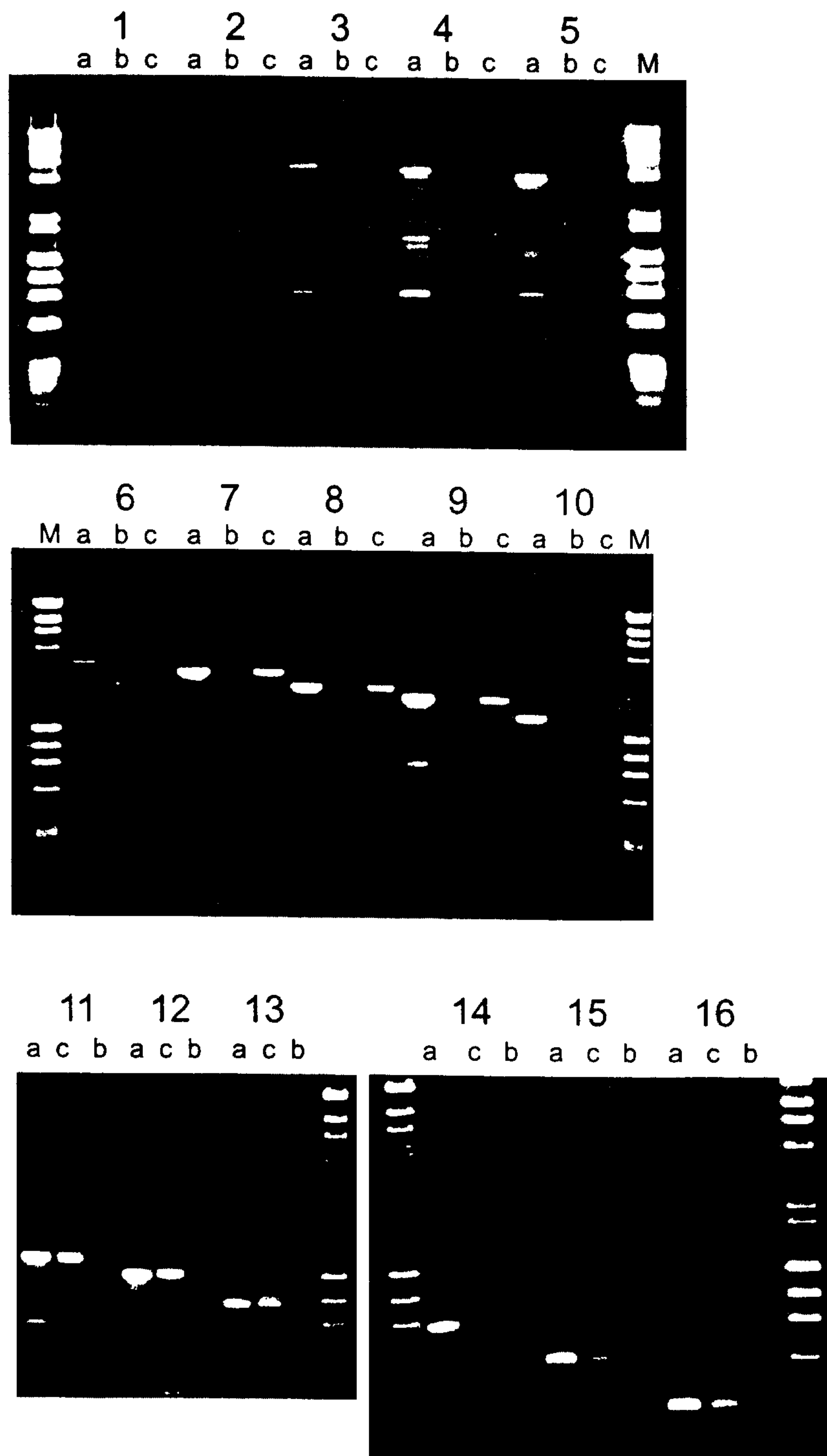
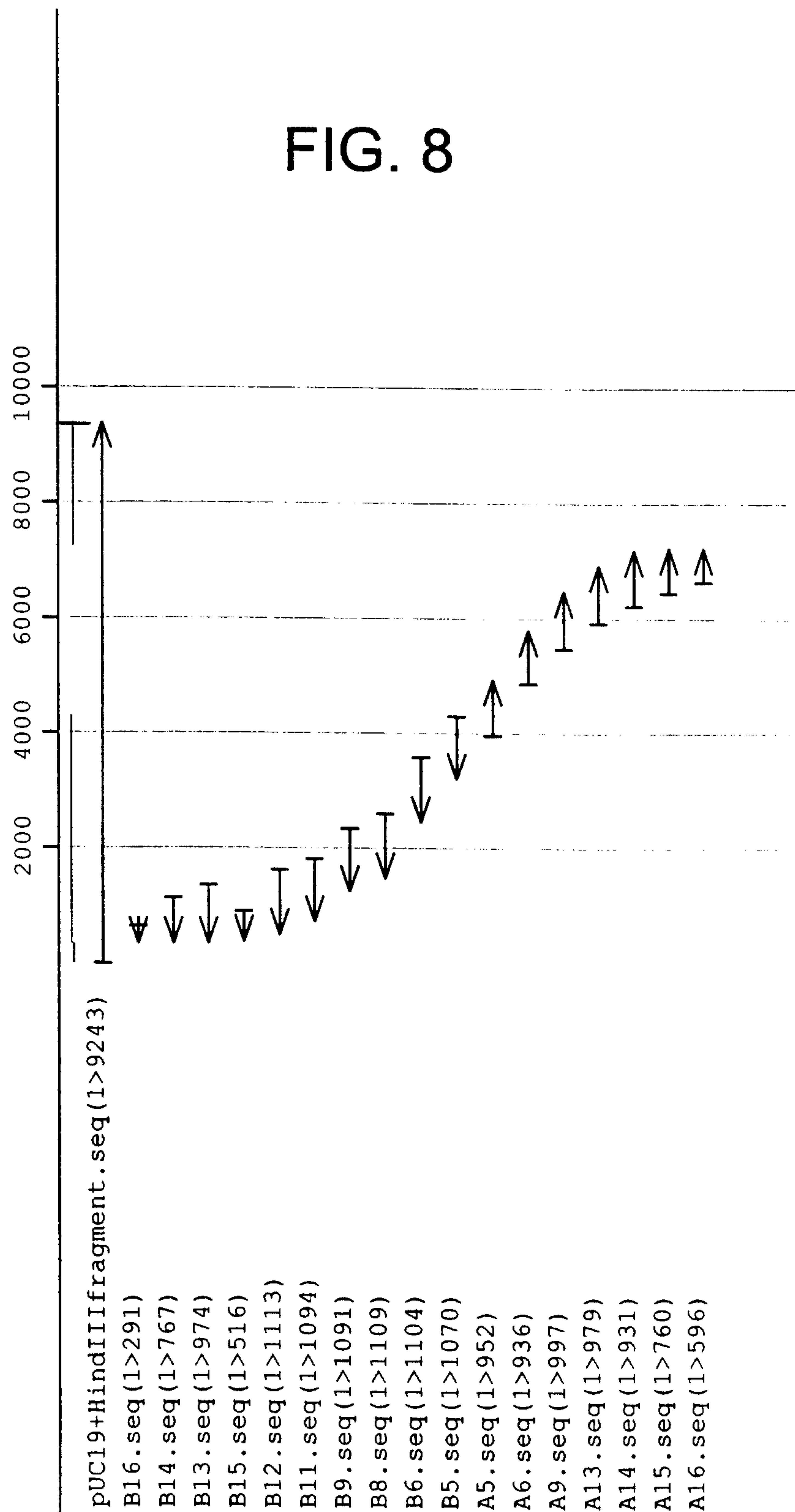


FIG. 7B

9/15

FIG. 8



10/15

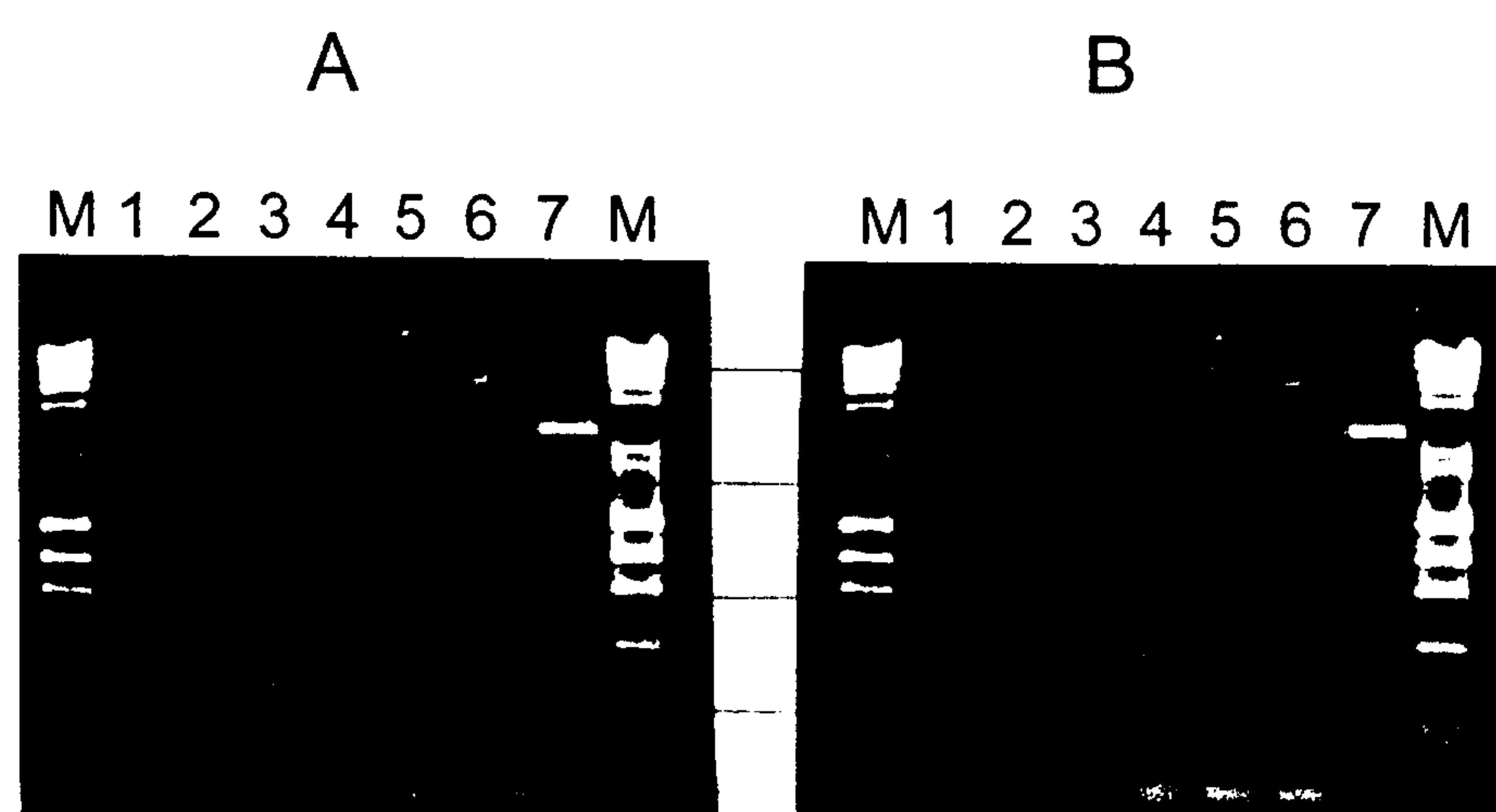


FIG. 9

11/15

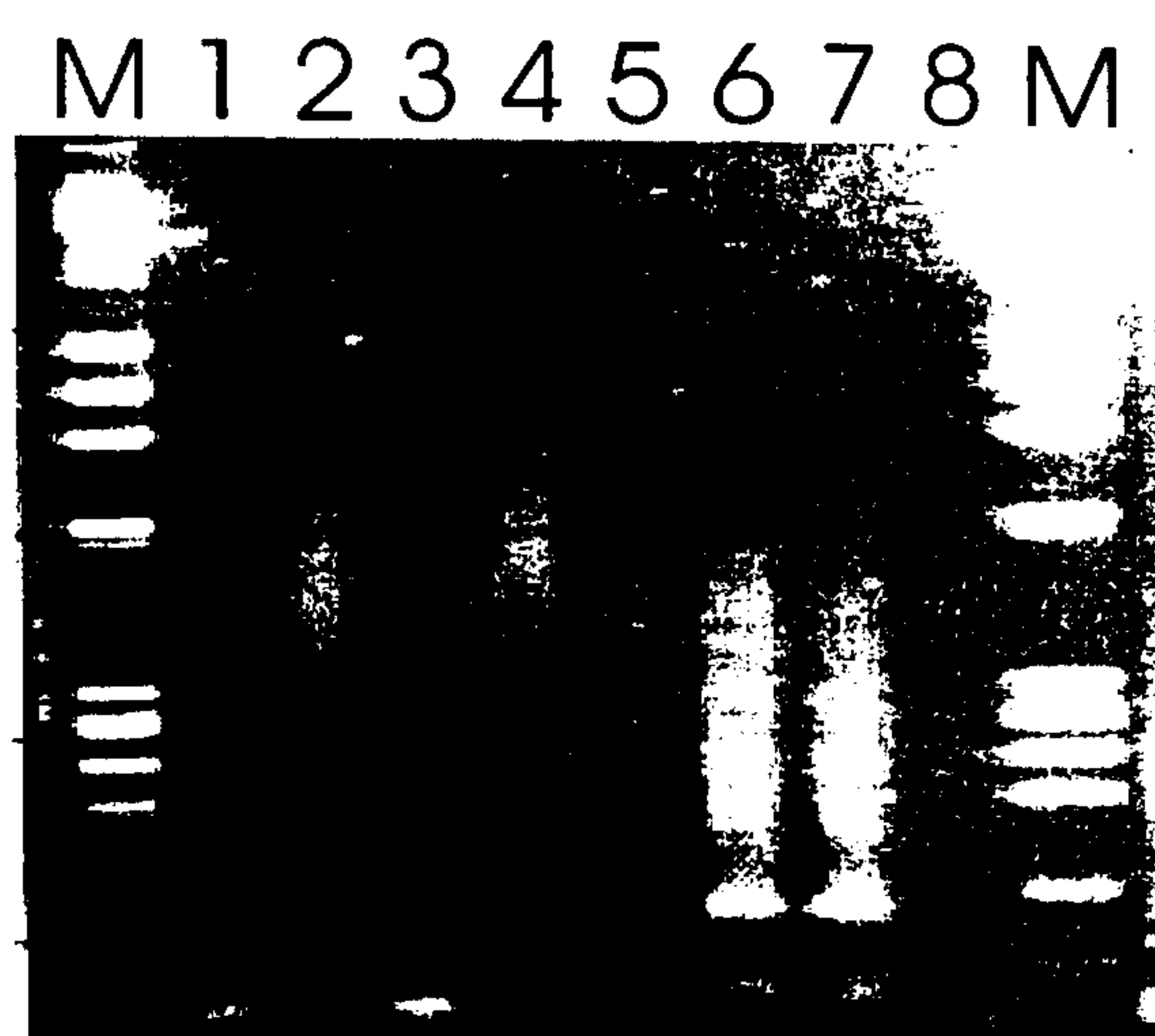


FIG. 10

12/15

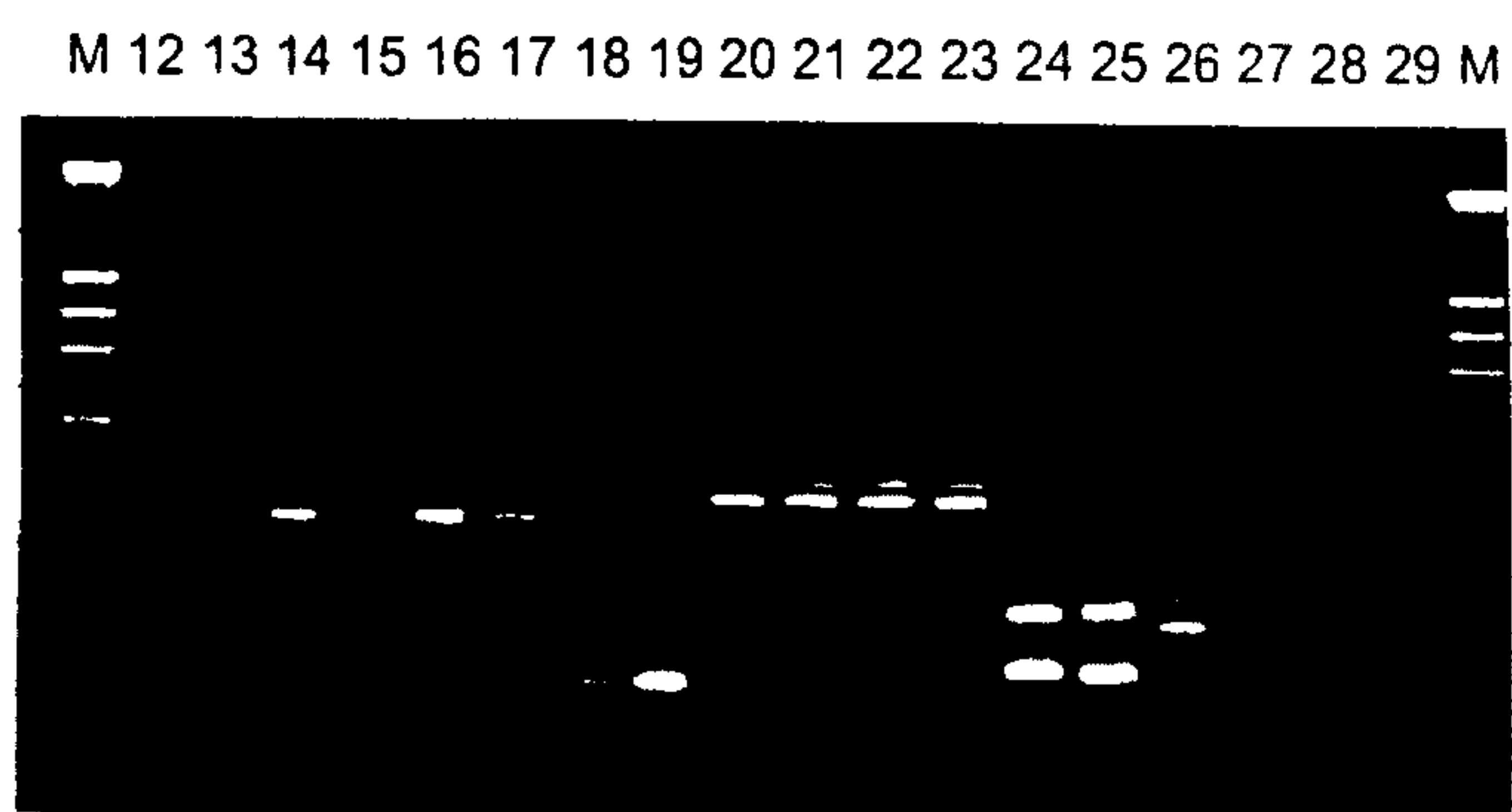


FIG. 11

13/15

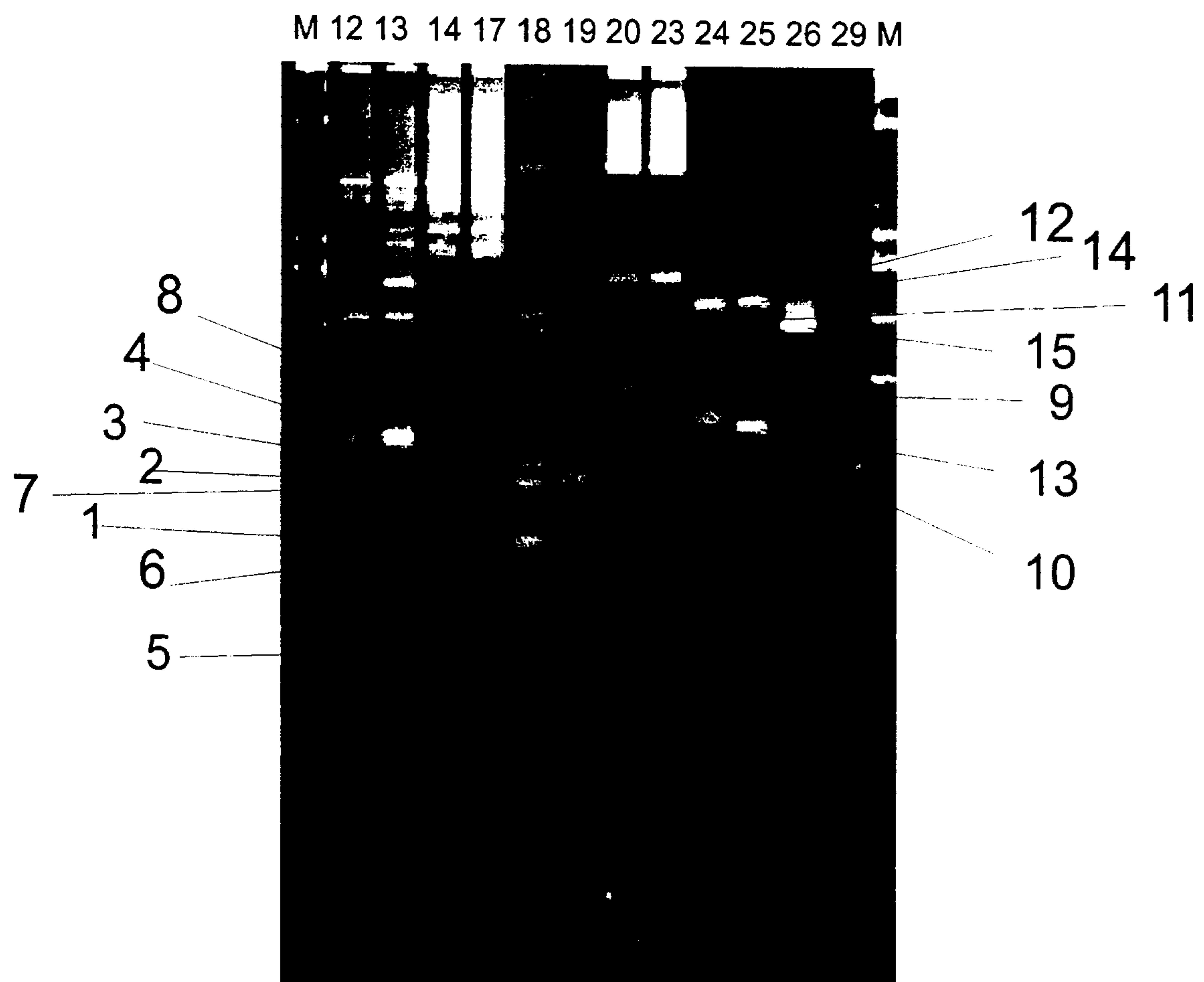


FIG. 12

14/15

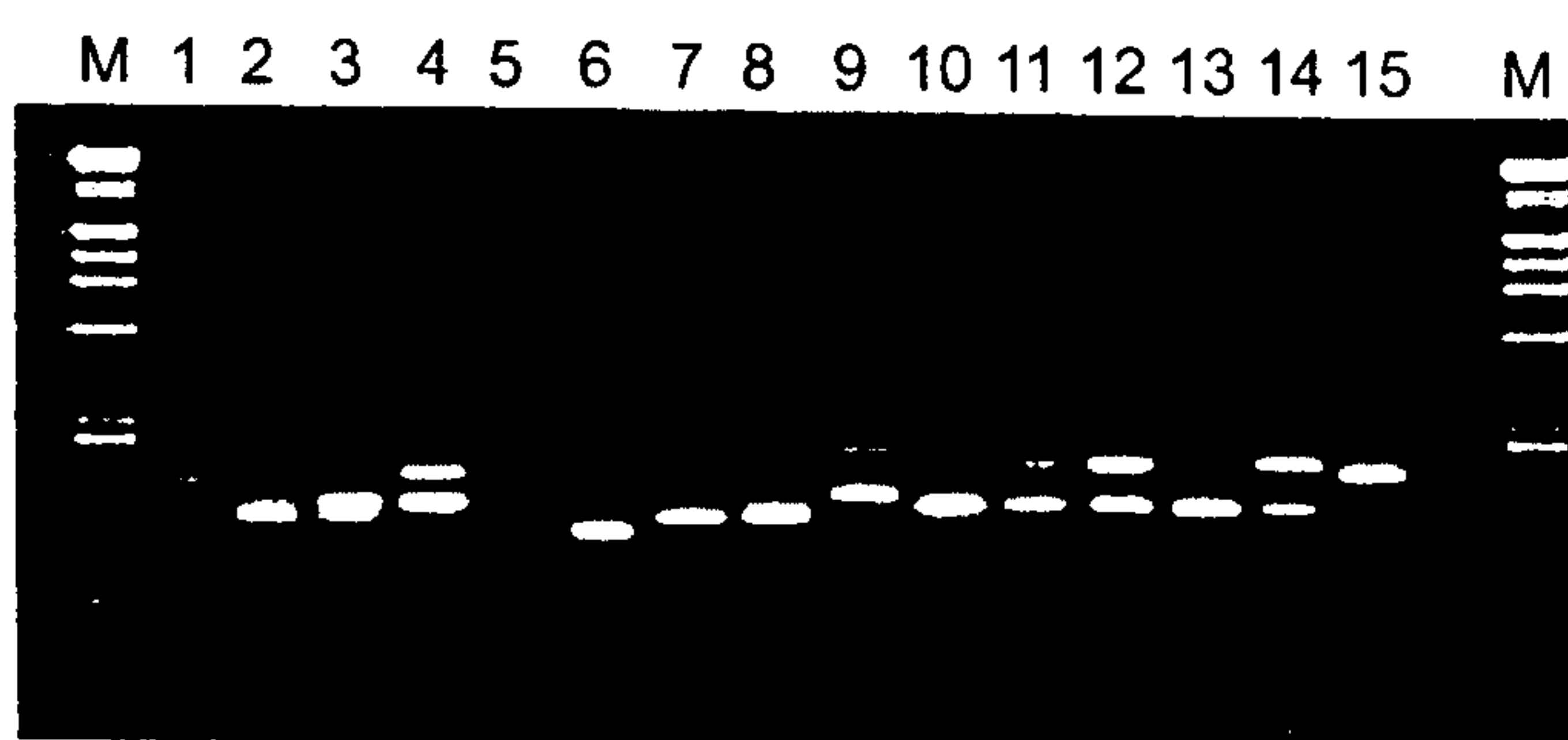


FIG. 13

15/15

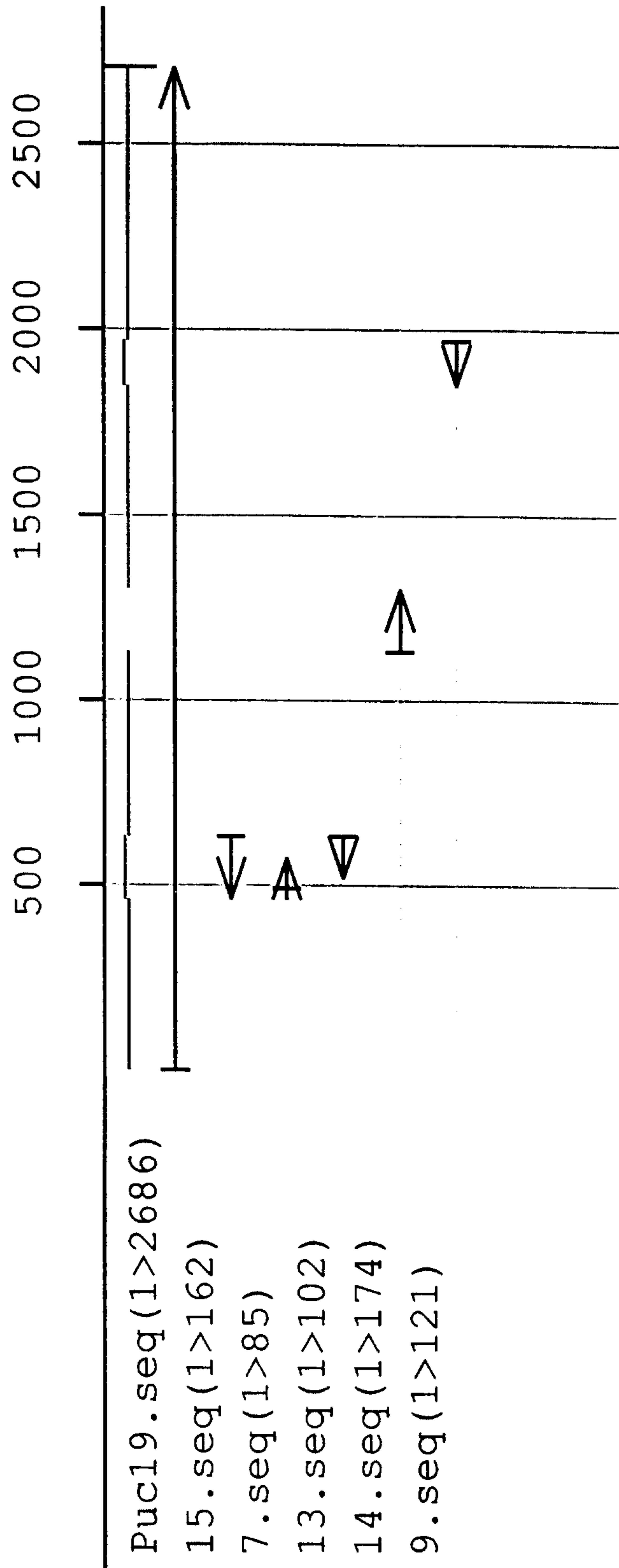


FIG. 14