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Canadian Intellectual Property Office

CA 3020587 C 2023/03/07

(11)(21) **3 020 587**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(22) **Date de dépôt/Filing Date:** 2003/09/12

(41) **Mise à la disp. pub./Open to Public Insp.:** 2004/03/25

(45) **Date de délivrance/Issue Date:** 2023/03/07

(62) **Demande originale/Original Application:** 2 498 746

(30) **Priorité/Priority:** 2002/09/12 (US10/243,367)

(51) **Cl.Int./Int.Cl.** ***C12Q 1/6844*** (2018.01), ***C12P 19/34*** (2006.01), ***C12Q 1/6834*** (2018.01), ***C12Q 1/6837*** (2018.01), ***C40B 40/06*** (2006.01), ***C40B 50/06*** (2006.01)

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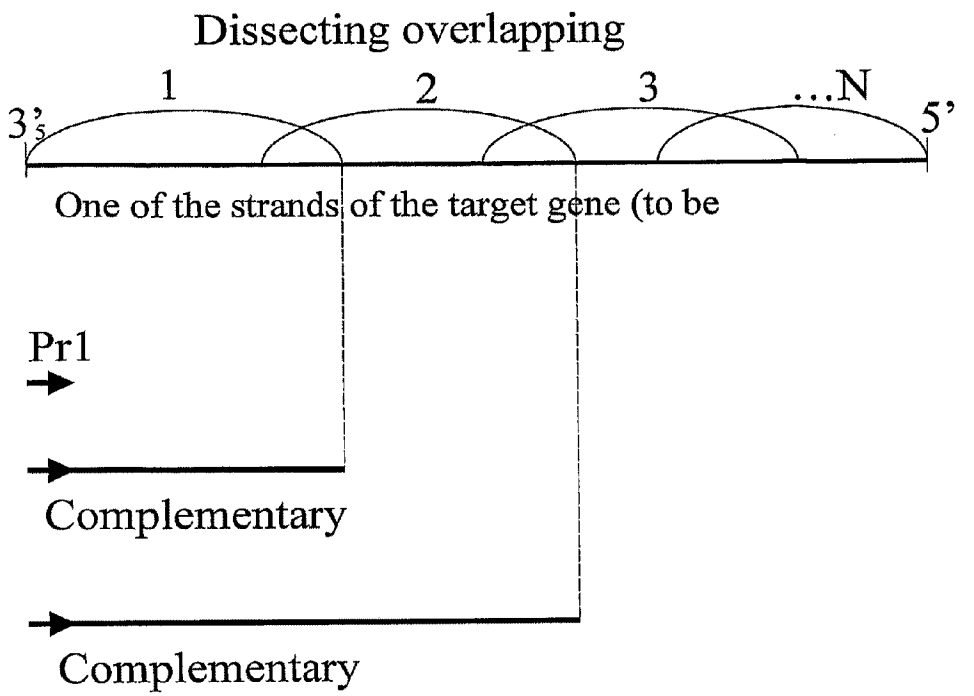
(54) **Titre : SYNTHESE ET ASSEMBLAGE EN MICRORESEAUX DE POLYNUCLEOTIDES DE LA LONGUEUR D'UN GENE**
(54) **Title: MICROARRAY SYNTHESIS AND ASSEMBLY OF GENE-LENGTH POLYNUCLEOTIDES**



(57) **Abrégé/Abstract:**
There is disclosed a process for in vitro synthesis and assembly of long, gene-length polynucleotides based upon assembly of multiple shorter oligonucleotides synthesized in situ on a microarray platform. Specifically, there is disclosed a process for in situ synthesis of oligonucleotide fragments on a solid phase microarray platform and subsequent, "on device" assembly of larger polynucleotides composed of a plurality of shorter oligonucleotide fragments.

Abstract

There is disclosed a process for *in vitro* synthesis and assembly of long, gene-length polynucleotides based upon assembly of multiple shorter oligonucleotides synthesized *in situ* on a microarray platform. Specifically, there is disclosed a process for *in situ* synthesis of oligonucleotide fragments on a solid phase microarray platform and subsequent, "on device" assembly of larger polynucleotides composed of a plurality of shorter oligonucleotide fragments.

MICROARRAY SYNTHESIS AND ASSEMBLY OF GENE-LENGTH POL YNUCLEOTIDES

This application is a divisional application of Canadian Application No. 2,498,746, filed September 12, 2003.

Technical Field of the Invention

The present invention provides a process for *in vitro* synthesis and assembly oflong, gene-length polynucleotides based upon assembly of multiple shorter oligonucleotides synthesized *in situ* on a microarray platform. Specifically, the present invention provides a process for *in situ* synthesis of oligonucleotide sequence fragments on a solid phase microarray platform and subsequent, "on chip" assembly of larger polynucleotides composed of a plurality of smaller oligonucleotide sequence fragments.

Background of the Invention

In the world of microarrays, biological molecules *(e.g.,* oligonucleotides, polypeptides and the like) are placed onto surfaces at defined locations for potential binding with target samples of nucleotides or receptors. Microarrays are miniaturized arrays of biomolecules available or being developed on a variety of platforms. Much of the initial focus for these microarrays have been in genomics with an emphasis of single nucleotide polymorphisms (SNPs) and geno:i;nic DNA detection/validation, functional genomics and proteomics (Wilgenbus and Lichter, *J. Mol. Med.* 77:761, 1999; Ashfari et al., *Cancer Res.* 59:4759, 1999; Kurian et al., *J. Pathol.* 187:267, 1999; Hacia, *Nature Genetics* 21 suppl.:42, 1999; Hacia et 20 al., *Mol. Psychiatry* 3:483, 1998; and Johnson, *Curr. Biol.* 26:Rl 71, 1998).

There are, in general, three categories ofmicroarrays (also called "biochips" and "DNA Arrays" and "Gene Chips" but this descriptive name has been attempted to be a trademark) having oligonucleotide content. Most often, the oligonucleotide microarrays have a solid surface, usually silicon-based and most often a glass microscopic slide. Oligonucleotide microarrays are often made by different techniques, including (1) "spotting" by depositing single nucleotides for *in situ* synthesis or completed oligonucleotides by physical means (ink jet printing and the like), (2) photolithographic techniques for *in situ* oligonucleotide synthesis (see, for example, Fodor U.S. Patent '934 and the additional patents that claim priority from this priority document, (3) electrochemical *in situ* synthesis based upon pH based removal of blocking chemical functional groups (see, for example, Montgomery U.S. Patent 6,092,302 and Southern U.S. Patent 5,667,667), and (4) electric field attraction/repulsion of fully-formed oligonucleotides (see, for example, Hollis et al., U.S. Patent 5,653,939 and its duplicate Heller U.S. Patent 5,929,208). Only the first three basic techniques can form oligonucleotides *in situ* that are, building each oligonucleotide, nucleotide-by-nucleotide, on the microarray surface without placing or attracting fully formed oligonucleotides.

With regard to placing fully formed oligonucleotides at specific locations, various micro-spotting techniques using computer-controlled plotters or even ink-jet printers have been developed to spot oligonucleotides at defined locations. One techniques loads glass fibers

having multiple capillaries drilled through them with different oligonucleotides loaded into each capillary tube. Microarray chips, often simply glass microscope slides, are then stamped out much like a rubber stamp on each sheet of paper of glass slide. It is also possible to use "spotting" techniques to build oligonucleotides *in situ*. Essentially, this involves "spotting" relevant single nucleotides at the exact location or region on a slide (preferably a glass slide) where a particular sequence of oligonucleotide is to be built. Therefore, irrespective of whether or not fully formed oligonucleotides or single nucleotides are added for *in situ* synthesis, spotting techniques involve the precise placement of materials at specific sites or regions using automated techniques.

Another technique involves a photolithography process involving photomasks to build oligonucleotides *in situ*, base-by-base, by providing a series of precise photomasks coordinated with single nucleotide bases having light-cleavable blocking groups. This technique is described in Fodor et al., U.S. Patent 5,445,934 and its various progeny patents. Essentially, this technique provides for "solid-phase chemistry, photolabile protecting groups, and photolithography . . . to achieve light-directed spatially-addressable parallel chemical synthesis."

The electrochemistry platform (Montgomery U.S. Patent 6,092,302) provides a microarray based upon a semiconductor chip platform having a plurality of microelectrodes. This chip design uses Complementary Metal Oxide Semiconductor (CMOS) technology to create high-density arrays of microelectrodes with parallel addressing for selecting and controlling individual microelectrodes within the array. The electrodes turned on with current flow generate electrochemical reagents (particularly acidic protons) to alter the pH in a small "virtual flask" region or volume adjacent to the electrode. The microarray is coated with a porous matrix for a reaction layer material. Thickness and porosity of the material is carefully controlled and biomolecules are synthesized within volumes of the porous matrix whose pH has been altered through controlled diffusion of protons generated electrochemically and whose diffusion is limited by diffusion coefficients and the buffering capacities of solutions. However, in order to function properly, the microarray biochips using electrochemistry means for *in situ* synthesis has to alternate anodes and cathodes in the array in order to generated needed protons (acids) at the anodes so that the protons and other acidic electrochemically generated acidic reagents will cause an acid pH shift and remove a blocking group from a growing oligomer.

Gene Assembly

The preparation of arbitrary polynucleotide sequences is useful in a "post-genomic" era because it provides any desirable gene oligonucleotide or its fragment, or even whole genome material of plasmids, phages and viruses. Such polynucleotides are long, such as in excess of 1000 bases in length. *In vitro* synthesis of oligonucleotides (given even the best yield conditions of phosphoramidite chemistry) would not be feasible because each base addition reaction is less than 100% yield. Therefore, researchers desiring to obtain long

polynucleotides of gene length or longer had to turn to nature or gene isolation techniques to obtain polynucleotides of such length. For the purposes of this patent application, the term "polynucleotide" shall be used to refer to nucleic acids (either single stranded or double stranded) that are sufficiently long so as to be practically not feasible to make *in vitro* through single base addition. In view of the exponential drop-off in yields from nucleic acid synthesis chemistries, such as phosphoramidite chemistry, such polynucleotides generally have greater than 100 bases and often greater than 200 bases in length. It should be noted that many commercially useful gene cDNA's often have lengths in excess of 1000 bases.

Moreover, the term "oligonucleotides" or shorter term "oligos" shall be used to refer to shorter length single stranded or double stranded nucleic acids capable of *in vitro* synthesis and generally shorter than 150 bases in length. While it is theoretically possible to synthesize polynucleotides through single base addition, the yield losses make it a practical impossibility beyond 150 bases and certainly longer than 250 bases.

However, knowledge of the precise structure of the genetic material is often not sufficient to obtain this material from natural sources. Mature cDNA, which is a copy of an mRNA molecule, can be obtained if the starting material contains the desired mRNA. However, it is not always known if the particular mRNA is present in a sample or if the amount of the mRNA might be too low to obtain the corresponding cDNA without significant difficulties. Also, different levels of homology or splice variants may interfere with obtaining one particular species of mRNA. On the other hand many genomic materials might be not appropriate to prepare mature gene (cDNA) due to exon-intron structure of genes in many different genomes.

In addition, there is a need in the art for polynucleotides not existing in nature to improve genomic research performance. In general, the ability to obtain a polynucleotide of any desired sequence just knowing the primary structure, for a reasonable price, in a short period of time, will significantly move forward several fields of biomedical research and clinical practice.

Assembly of long arbitrary polynucleotides from oligonucleotides synthesized by organic synthesis and individually purified has other problems. The assembly can be performed using PCR or ligation methods. The synthesis and purification of many different oligonucleotides by conventional methods (even using multi-channel synthesizers) are laborious and expensive procedures. The current price of assembled polynucleotide on the market is about $12-25 per base pair, which can be considerable for assembling larger polynucleotides. Very often the amount of conventionally synthesized oligonucleotides would be excessive. This also contributes to the cost of the final product.

Therefore, there is a need in the art to provide cost-effective polynucleotides by procedures that are not as cumbersome and labor-intensive as present methods to be able to provide polynucleotides at costs below $1 per base or 1-20 times less than current methods. The present invention was made to address this need.

Summary of the Invention

The present invention provides a process for the assembly of oligonucleotides synthesized on microarrays into a polynucleotide sequence. The desired target polynucleotide sequence is dissected into pieces of overlapping oligonucleotides. In the first embodiment these oligonucleotides are synthesized *in situ*, in parallel on a microarray chip in a non-cleavable form. A primer extension process assembles the target polynucleotides. The primer extension process uses starting primers that are specific for the appropriate sequences. The last step is PCR amplification of the final polynucleotide product. Preferably, the polynucleotide product is a cDNA suitable for transcription purposes and further comprising a promoter sequence for transcription.

The present invention provides a process for assembling a polynucleotide from a plurality of oligonucleotides comprising:

(a) synthesizing or spotting a plurality of oligonucleotide sequences on a microarray device or bead device having a solid or porous surface, wherein a first oligonucleotide is oligo 1 and a second oligonucleotide is oligo 2 and so on, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein the first oligonucleotide sequence has an overlapping sequence region of from about 10 to about 50 bases that is the same or substantially the same as a region of a second oligonucleotide sequence, and wherein the second oligonucleotide sequence has an overlapping region with a third oligonucleotide sequence and so on;

(b) forming complementary oligo 1 by extending primer 1, wherein primer 1 is complementary to oligo 1;

(c) disassociating complementary oligo 1 from oligo 1 and annealing complementary oligo 1 to both oligo 1 and to the overlapping region of oligo 2, wherein the annealing of complementary oligo 1 to oligo 2 serves as a primer for extension for forming complementary oligo 1+2;

(d) repeating the primer extension cycles of step (c) until a full-length polynucleotide is produced; and

(e) amplifying the assembled complementary full length polynucleotide to produce a full length polynucleotide in desired quantities.

Preferably, the solid or porous surface is in the form of a microarray device. Most preferably, the microarray device is a semiconductor device having a plurality of electrodes for synthesizing oligonucleotides *in situ* using electrochemical means to couple and decouple nucleotide bases. Preferably, the primer extension reaction is conducted through a sequential process of melting, annealing and then extension. Most preferably, the primer extension reaction is conducted in a PCR amplification device using the microarray having the plurality of oligonucleotides bound thereto.

The present invention further provides a process for assembling a polynucleotide from a plurality of oligonucleotides comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence has an overlapping region corresponding to a next oligonucleotide sequence within the sequence and further comprises two flanking sequences, one at the 3' end and the other at the 5' end of each oligonucleotide, wherein each flanking sequence is from about 7 to about 50 bases and comprising a primer region and a sequence segment having a restriction enzyme cleavable site;

(b) amplifying each oligonucleotide using the primer regions of the flanking sequence to form double stranded (ds) oligonucleotides;

(c) cleaving the oligonucleotide sequences at the restriction enzyme cleavable site; and

(d) assembling the cleaved oligonucleotide sequences through the overlapping regions to form a full length polynucleotide.

Preferably, the flanking sequence is from about 10 to about 20 bases in length. Preferably, the restriction enzyme cleavable site is a class II endonuclease restriction site sequence capable of being cleaved by its corresponding class II restriction endonuclease enzyme. Most preferably, the restriction endonuclease class II site corresponds to restriction sites for a restriction endonuclease class II enzyme selected from the group consisting of Mly I, BspM I, Bae I, BsaX I, Bsr I, Bmr I, Btr I, Bts I, Fok I, and combinations thereof. Preferably, the flanking sequence further comprises a binding moiety used to purify cleaved oligonucleotides from flanking sequences. Preferably, the process further comprises the step of labeling the flanking sequence during the amplification step (b) using primer sequences labeled with binding moieties. Most preferably, a binding moiety is a small molecule able to be captured, such as biotin captured by avidin or streptavidin, or fluorescein able to be captured by an anti-fluorescein antibody.

The present invention further provides a process for assembling a polynucleotide from a plurality of oligonucleotides comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence has an overlapping region corresponding to a next oligonucleotide sequence within the sequence, and further comprises a sequence segment having a cleavable linker moiety;

(b) cleaving the oligonucleotide sequences at the cleavable linker site to cleave each oligonucleotide complex from the microarray or bead solid surface to form a soluble mixture of oligonucleotides, each having an overlapping sequence; and

(c) assembling the oligonucleotide sequences through the overlapping regions to form a full length polynucleotide.

Preferably, the cleavable linker is a chemical composition having a succinate moiety bound to a nucleotide moiety such that cleavage produces a 3'hydroxy nucleotide. Most preferably, the cleavable linker is selected from the group consisting of 5'-dimethoxytrityl-thymidine-3'succinate, 4-N-benzoyl-5'-dimethoxytrityl-deoxycytidine-3'-succinate, 1-N-benzoyl-5'-dimethoxytrityl-deoxyadenosine-3'-succinate, 2-N-isobutyryl-5'-dimethoxytrityl-deoxyguanosone-3'-succinate, and combinations thereof.

The present invention further provides a process for assembling a polynucleotide from a plurality of oligonucleotides comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence has a flanking region at an end attached to the solid or porous surface, and a specific region designed by dissecting the polynucleotide sequence into a plurality of overlapping oligonucleotides, wherein a first overlapping sequence on a first oligonucleotide corresponds to a second overlapping sequence of a second oligonucleotide, and wherein the flanking sequence comprises a sequence segment having a restriction endonuclease (RE) recognition sequence capable of being cleaved by a corresponding RE enzyme;

(b) hybridizing an oligonucleotide sequence complementary to the flanking region to form a double stranded sequence capable of interacting with the corresponding RE enzyme;

(c) digesting the plurality of oligonucleotides to cleave them from the microarray device or beads into a solution; and

(d) assembling the oligonucleotide mixture through the overlapping regions to form a full length polynucleotide.

Preferably, the flanking sequence is from about 10 to about 20 bases in length. Preferably, the restriction enzyme cleavable site is a class II endonuclease restriction site sequence capable of being cleaved by its corresponding class II restriction endonuclease enzyme. Most preferably, the restriction endonuclease class II site corresponds to restriction sites for a restriction endonuclease class II enzyme selected from the group consisting of Mly I, BspM I, Bae I, BsaX I, Bsr I, Bmr I, Btr I, Bts I, Fok I, and combinations thereof. Preferably, the process further comprises a final step of amplifying the polynucleotide sequence using primers located at both ends of the polynucleotide.

The present invention further provides a process for creating a mixture of oligonucleotide sequences in solution comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence further comprises two flanking sequences, one at the 3' end and the other at the 5' end of each oligonucleotide, wherein each flanking sequence is from about 7 to

about 50 bases and comprising a primer region and a sequence segment having a restriction enzyme cleavable site;

(b) amplifying each oligonucleotide using the primer regions of the flanking sequence to form a double stranded (ds) oligonucleotides; and

(c) cleaving the double stranded oligonucleotide sequences at the restriction enzyme cleavable site.

Preferably, the flanking sequence is from about 10 to about 20 bases in length. Preferably, the restriction enzyme cleavable site is a class II endonuclease restriction site sequence capable of being cleaved by its corresponding class II restriction endonuclease enzyme. Most preferably, the restriction endonuclease class II site corresponds to restriction sites for a restriction endonuclease class II enzyme selected from the group consisting of Mly I, BspM I, Bae I, BsaX I, Bsr I, Bmr I, Btr I, Bts I, Fok I, and combinations thereof. Preferably, the flanking sequence further comprises a binding moiety used to purify cleaved oligonucleotides from flanking sequences. Preferably, the process further comprises the step of labeling the flanking sequence during the amplification step (b) using primer sequences labeled with binding moieties. Most preferably, a binding moiety is a small molecule able to be captured, such as biotin captured by avidin or streptavidin, or fluorescein able to be captured by an anti-fluorescein antibody.

The present invention further provides a process for creating a mixture of oligonucleotide sequences in solution comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence has a sequence segment having a cleavable linker moiety;

(b) cleaving the oligonucleotide sequences at the cleavable linker site to cleave each oligonucleotide sequence from the microarray or bead solid surface to form a soluble mixture of oligonucleotides.

Preferably, the cleavable linker is a chemical composition having a succinate moiety bound to a nucleotide moiety such that cleavage produces a 3'hydroxy nucleotide. Most preferably, the cleavable linker is selected from the group consisting of 5'-dimethoxytrityl-thymidine-3'succinate, 4-N-benzoyl-5'-dimethoxytrityl-deoxycytidine-3'-succinate, 1-N-benzoyl-5'-dimethoxytrityl-deoxyadenosine-3'-succinate, 2-N-isobutyryl-5'-dimethoxytrityl-deoxyguanosone-3'-succinate, and combinations thereof.

The present invention further provides a process for creating a mixture of oligonucleotide sequences in solution comprising:

(a) synthesizing *in situ* or spotting a plurality of oligonucleotide sequences on a microarray device or bead device each having a solid or porous surface, wherein the plurality of oligonucleotide sequences are attached to the solid or porous surface, and wherein each oligonucleotide sequence has a flanking region at an end attached to the solid or porous

surface, and a specific region, wherein the flanking sequence comprises a sequence segment having a restriction endonuclease (RE) recognition sequence capable of being cleaved by a corresponding RE enzyme;

(b) hybridizing an oligonucleotide sequence complementary to the flanking region to form a double stranded sequence capable of interacting with the corresponding RE enzyme;

(c) digesting the plurality of oligonucleotides to cleave them from the microarray device or beads into a solution.

Preferably, the flanking sequence is from about 10 to about 20 bases in length. Preferably, the restriction enzyme cleavable site is a class II endonuclease restriction site sequence capable of being cleaved by its corresponding class II restriction endonuclease enzyme. Most preferably, the restriction endonuclease class II site corresponds to restriction sites for a restriction endonuclease class II enzyme selected from the group consisting of Mly I, BspM I, Bae I, BsaX I, Bsr I, Bmr I, Btr I, Bts I, Fok I, and combinations thereof.

Brief Description of the Drawings

Figure 1 shows a schematic of gene assembly on a microarray device surface or porous matrix. In Figure 1A, the target gene sequence is dissected into number of overlapping oligonucleotides. The 3' and 5' are the ends of the shown strand. Figure 1A also shows, relative to the target sequence, primer Pr1; extension product of primer Pr1, which is complementary to oligonucleotide 1; and extension product of complementary oligonucleotide 1, which is complementary to oligonucleotides 1+2. Figure 1B illustrates one embodiment of the initial steps of an assembly process. In step 1 of assembly, Primer Pr1 is annealed to oligonucleotide 1 and extended by appropriate polymerase enzyme into product complementary to oligonucleotide 1. The second step is melting, re-annealing and extension (*i.e.*, amplification) to lead to production of larger amount of Pr1 extension product (complementary oligonucleotide 1), re-association of the complementary oligonucleotide 1 with oligonucleotide 1, and to annealing of the complementary oligonucleotide 1 with oligonucleotide 2 followed by its extension into product complementary to oligonucleotides 1+2. Figure 1C shows a continuation of the assembly process from Figure 1B. Specifically, step 3 of the process (*i.e.*, melting, re-annealing and extension) leads to the same products as step 2 plus a product complementary to oligonucleotides 1+2+3. Cycles (steps) are repeated until a full-length complementary polynucleotide is formed. The final step is preparation of the final target polynucleotide molecule in desirable amounts by amplification (*i.e.*, PCR) using two primers complementary to the ends of this molecule (PrX and PrY).

Figure 2 shows a second embodiment of the inventive gene assembly process using oligonucleotides synthesized *in situ* onto a microarray device, each having a flanking sequence region containing a restriction enzyme cleavage site, followed by a PCR amplification step and followed by a REII restriction enzyme cleavage step.

Figure 3 shows a schematic for gene assembly using oligos synthesized and then cleaved from a microarray device. Specifically, in the upper panel marked "A",

oligonucleotide sequences are connected to the microarray device through a cleavable linker (CL) moiety. An example of a cleavable linker moiety is provided in Figure 3A. The cleavable linkers are molecules that can withstand the oligonucleotide synthesis process (*i.e.*, phosphoramidite chemistry) and then can be cleaved to release oligonucleotide fragments. Chemical cleavage at cleavable linker CL recreates usual 3' end of specific oligos 1 through N. These oligonucleotides are released into a mixture. The mixture of oligonucleotides is subsequently assembled into full-length polynucleotide molecules. In the lower panel marked "B" of Figure 3, oligonucleotide sequences are connected to the microarray device through additional flanking sequence containing a restriction enzyme (RE) sequence site. Another oligonucleotide sequence, complementary to the flanking sequence region, is hybridized to the oligonucleotides on the microarray device. This recreates a "ds" or double-stranded oligonucleotide structure, each having a RE sequence recognition region in the flanking sequence region. Digestion of this ds oligonucleotides with the corresponding RE enzymes at the RE recognition sites in the flanking sequence regions releases the specific oligonucleotides 1 through N. When assembled, oligonucleotide sequences 1 through N form a full-length polynucleotide molecule.

Figure 4 shows the assembly of a polynucleotide from three oligonucleotide fragments wherein each oligonucleotide fragment was synthesized *in situ* on a microarray device. The fully assembled polynucleotide was 172 mers in length, a length not practically achievable by *in situ* synthesis. The first embodiment inventive process was used in this example.

Figure 5 shows the oligonucleotide sequences used to assemble the 172-mer polynucleotide of Figure 4. The sequences of primers X and Z are underlined. The *Hpa* II restriction site is indicated by italic underlined letters.

Figure 6 shows a scheme for preparing the sequences of flanking regions and primers used for preparation of specific oligonucleotide for assembly using the REII enzyme *MlyI*. Primer 1 is complementary to the oligonucleotide strand on a microarray device and contains a Biotin-TEG (triethylene glycol) moiety. Primer 2 is the same strand as the oligonucleotide strand on microarray device and contains Biotin-TEG moiety. Any sequence between the primers can be used and is just designated by a string of N's.

Figure 7 shows the results of PCR and *Mly*I digestion of an oligonucleotide sequence as described in Figure 6. The clean bands show the ability to obtain pure oligonucleotides using the second embodiment of the inventive process to cleave off oligonucleotide sequences using appropriate restriction enzymes.

Figure 8 shows the sequences from nine oligonucleotides fragments (consecutively numbered 1-9) used to assemble a 290 bp polynucleotide. The flanking regions are shown in bold and underlined. The process used for polynucleotide assembly was the second embodiment. The overlapping regions further contained a cleavable site as the *Mly*I recognition site for the *Mly*I class II restriction endonuclease.

Figure 9 shows a schematic in the top panel for assembling a polynucleotide from nine oligonucleotides. Nine oligonucleotide sequences, shown in Figure 8, were amplified by PCR using primers 1 and 2 (as described in Figure 6) into ds DNA fragments containing the same flanking regions and specific overlapping sequences, digested with *Mly*I enzyme to remove flanking sequences, and used for assembly of 290 bp DNA fragment. The columns in the gel shown are M – markers, 1 – negative control, assembly without primers FP1 and FP2, 2 – negative control, assembly without specific oligos, 3 – assembly of 290 bp fragment from specific oligos plus amplification with FP1 and FP2 primers. The band in column 3 shows a high efficiency of the inventive polynucleotide assembly process.

Figure 10 shows a sequence of an assembled polynucleotide in Example 4, broken down into its component oligonucleotides.

Detailed Description of the Invention

The present invention describes the preparation of a polynucleotide sequence (also called "gene") using assembly of overlapping shorter oligonucleotides synthesized or spotted on microarray devices or on solid surface bead devices. The shorter oligonucleotides include sequence regions having overlapping regions to assist in assembly into the sequence of the desired polynucleotide. Overlapping regions refer to sequence regions at either a 3' end or a 5' end of a first oligonucleotide sequence that is the same as part of the second oligonucleotide and has the same direction (relative to 3' to 5' or 5' to 3' direction), and will hybridize to the 5' end or 3' end of a second oligonucleotide sequence or its complementary sequence (second embodiment), and a second oligonucleotide sequence to a third oligonucleotide sequence, and so on. In order to design or develop a microarray device or bead device to be used for polynucleotide assembly, the polynucleotide sequence is divided (or dissected) into a number of overlapping oligonucleotides segments, each with lengths preferably from 20 to 1000 bases, and most preferably from 20 to 200 bases (Figure 1A). The overlap between oligonucleotide segments is 5 or more bases, preferably 15 – 25 bases to that proper hybridization of first to second, second to third, third to fourth and so on occurs. These oligonucleotides (or oligos) are preferably synthesized on a microarray device using any available method (*i.e.*, electrochemical *in situ* synthesis, photolithography *in situ* synthesis, ink-jet printing, spotting, etc.). The direction of synthesis relative to the microarray device surface or porous matrix covering a microarray device can be from 3' to 5' or from 5' to 3'. Preferably, *in situ* synthesis is done in the 3' to 5' direction.

In the first embodiment the inventive gene/polynucleotide assembly process uses oligonucleotides immobilized on a microarray device. The microarray device itself or a porous reaction layer with immobilized oligonucleotides can be used for the inventive gene/polynucleotide assembly process.

With regard to Figure 1B, the process comprises several repeated steps of melting, annealing and extension (Figure 1B), which can be performed in any thermal cycler instrument. The cycling program is similar to the programs used for PCR. At the first step of

gene/polynucleotide assembly, primer Pr1 is added and anneals to oligonucleotide 1 on the microarray device and then extends by appropriate polymerase enzyme into product complementary to oligonucleotide 1 (called complementary oligonucleotide 1). At the second step of the process the product complementary to oligonucleotide 1 is melted from oligonucleotide 1, primer Pr1 is annealed again to the oligonucleotide 1 as well as product complementary to oligonucleotide 1 is partially re-anneals to oligonucleotide 1 and partially anneals to oligonucleotide 2 due to an overlapping sequence region between oligonucleotide 1 and oligonucleotide 2. Extension of Pr1 leads to production of an additional amount of Pr1 extension product (complementary oligonucleotide 1). The annealing of the complementary oligonucleotide 1 to oligonucleotide 2 followed by its extension leads to product complementary to oligonucleotides 1+2 (called complementary oligonucleotides 1+2). Similarly, at step 3 of the process melting, re-annealing and extension lead to the same products as at step 2 plus a product complementary to oligonucleotides 1+2+3. These cycles of melting, annealing and extension are repeated until full-length polynucleotide is formed. The number of cycles should be equal or more than the number of oligos on microarray device. After formation, the final target polynucleotide molecule is amplified by a PCR process with two primers complementary to the ends of this molecule to the desirable amounts.

In a second embodiment, a plurality of oligonucleotides that together comprise (with overlapping regions) the target polynucleotide sequence are synthesized on a microarray device (or can be synthesized on beads as a solid substrate), wherein each oligonucleotide sequence further comprises flanking short sequence regions, wherein each flanking sequence region comprises one or a plurality of sequence sites for restriction endonuclease, preferably endonuclease class II (ERII) enzymes. Each oligonucleotide is amplified by PCR using appropriate oligonucleotide primers to the flanking sequence regions to form a preparation of a plurality of oligonucleotides. The preparation of oligonucleotides is treated then with appropriate REII enzyme(s) (specific to the restriction sequences in the flanking sequence regions) to produce flanking fragments and overlapping oligonucleotides that, together comprise the desired polynucleotide sequence. Flanking fragments and PCR primers are removed from the mixture, if desired, by different methods based on size or specific labeling of the PCR primers. The oligonucleotides resembling the desired target polynucleotide then assembled into the final target polynucleotide molecule using repetition of the primer extension method and PCR amplification of the final molecule.

Specifically, in the second embodiment, the assembly process initially uses oligonucleotides immobilized on a microarray device or beads, via immobilization techniques, such as spotting or ink-jet printing or by direct *in situ* synthesis of the microarray device using various techniques, such as photolithography or electrochemical synthesis. The overlapping oligonucleotide sequences are designed having an overlapping region and one or two flanking sequence regions comprising a restriction class II recognition site (Figure 2A). The assembled oligonucleotides together comprise the target polynucleotide sequence.

The length of flanking sequences is at least the length of REII recognition site. The flanking sequences are designed to have minimal homology to the specific oligonucleotide sequences regions on the microarray device. The flanking sequences can be the same for each oligonucleotide fragment, or be two or more different sequences. For example, a pair of appropriate primers, called Pr1 and Pr2, was designed to amplify each oligonucleotide on a microarray device (Figure 2) by PCR. Each primer may contain a binding moiety, such as biotin, that does not affect their ability to serve as primers. After PCR amplification the amplified ds copy of each oligonucleotide was present in the reaction mixture. This reaction mixture was treated with the appropriate REII enzyme or enzymes specific for the restriction sites in the flanking sequence regions. The digestion sites for REII were designed, after cleavage, to produce the desired specific oligonucleotide sequence fragments that, when assembled will form the target polynucleotide sequence. As a result of digestion a mixture of specific double stranded (ds) overlapping oligonucleotide sequence fragments resembling the structure of desired target polynucleotide, and ds flanking sequences were formed. If desired, these flanking sequences and residual primers are removed from the mixture using specific absorption through specific moieties introduced in the primers (such as, for example, by absorption on avidin beads for biotin-labeled primers), or based on the size difference of the specific oligos and flanking sequences and primers. The mixture of specific oligonucleotide sequences resembling target gene sequence is used to assemble the final target polynucleotide molecule using repeated cycles of melting, self-annealing and polymerase extension followed by PCR amplification of the final target polynucleotide molecule with appropriate PCR primers designed to amplify. This final PCR amplification step is routinely done in the art and described in, for example, Mullis et al., *Cold Spring Harb. Symp. Quant. Biol.* 51 Pt 1:263-73, 1986; and Saiki et al., *Science* 239:487-91, 1988. PCR amplification steps generally follow manufacturer's instructions. Briefly, A process for amplifying any target nucleic acid sequence contained in a nucleic acid or mixture thereof comprises treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers and extending the primers with a thermostable enzyme to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence. The amplified sequence can be readily detected. The steps of the reaction can be repeated as often as desired and involve temperature cycling to effect hybridization, promotion of activity of the enzyme, and denaturation of the hybrids formed.

In another embodiment for the assembly step, oligonucleotide sequences that together comprise the target polynucleotide molecule are assembled using a ligase chain reaction as described in Au et al., *Biochem. Biophys. Res. Commun.* 248:200-3, 1998. Briefly, short oligonucleotides are joined through ligase chain reaction (LCR) in high stringency conditions to make "unit fragments" (Fifty microliters of reaction mixture contained 2.2 mM of each oligo, 8 units Pfu DNA ligase (Stratagene La Jolla, CA) and reaction buffer provided with the enzyme. LCR was conducted as follows: 95 °C 1 min; 55 °C 1.5 min, 70 °C 1.5 min, 95 °C 30

sec for 15 cycles; 55 °C 2 min; 70 °C 2 min, which are then fused to form a full-length gene sequence by polymerase chain reaction.

In another embodiment the ds oligonucleotide sequences are assembled after preparation by chain ligation cloning as described in Pachuk et al., *Gene* 243:19-25, 2000; and U.S. Patent 6,143,527. Briefly, chain reaction cloning allows ligation of double-stranded DNA molecules by DNA ligases and bridging oligonucleotides. Double-stranded nucleic acid molecules are denatured into single-stranded molecules. The ends of the molecules are brought together by hybridization to a template. The template ensures that the two single-stranded nucleic acid molecules are aligned correctly. DNA ligase joins the two nucleic acid molecules into a single, larger, composite nucleic acid molecule. The nucleic acid molecules are subsequently denatured so that the composite molecule formed by the ligated nucleic acid molecules and the template cease to hybridize to each. Each composite molecule then serves as a template for orienting unligated, single-stranded nucleic acid molecules. After several cycles, composite nucleic acid molecules are generated from smaller nucleic acid molecules. A number of applications are disclosed for chain reaction cloning including site-specific ligation of DNA fragments generated by restriction enzyme digestion, DNAse digestion, chemical cleavage, enzymatic or chemical synthesis, and PCR amplification.

With regard to the second embodiment of the inventive process (illustrated in Figure 2), a target polynucleotide gene sequence (either strand) is divided into number of overlapping oligonucleotide sequences by hand or with a software program, as shown in Figure 1. These oligonucleotide sequences, plus flanking sequences A and B (having one or a plurality of restriction enzyme sites in the flanking region sequence), are synthesized (*in situ*) on a microarray device, or on a bead solid surface using standard *in situ* synthesis techniques, or spotted (pre-synthesized) onto a microarray device using standard oligonucleotide synthesis procedures with standard spotting (*e.g.*, computer-aided or ink jet printing) techniques. The oligonucleotide sequences are amplified, preferably using a PCR process with a pair of primers (Pr1 and Pr2). The primers are optionally labeled with specific binding moieties, such as biotin. The resulting amplified mixture of different amplified oligonucleotide sequences are double stranded (ds). The mixture of ds oligonucleotide sequences are treated with an appropriate restriction enzyme, such as an REII restriction enzyme (*e.g.*, Mly I enzyme), to produce mixture of different double stranded (ds) overlapping oligonucleotide sequences that can be assembled into the structure of the desired polynucleotide (gene) and ds flanking sequences. Optionally, the flanking sequences and residual primers are removed from the ds oligonucleotide sequence mixture, preferably by a process of specific absorption using specific binding moieties introduced in the primers (*e.g.*, biotin), or by a process of size fractionation based on the size differences of the specific oligonucleotide sequences and flanking sequences. The mixture of specific oligonucleotide sequences is assembled, for example, by a process of repeated cycles of melting, self-annealing and polymerase extension followed by PCR

amplification of the final molecule with appropriate PCR primers designed to amplify this complete molecule (*e.g.*, as described in Mullis et al., *Cold Spring Harb. Symp. Quant. Biol.* 51 Pt 1:263-73, 1986; and Saiki et al., *Science* 239:487-91, 1988).

In yet another embodiment of the inventive process (illustrated in Figure 3), the oligonucleotide sequences comprising the target polynucleotide sequence are synthesized on a microarray device or bead solid support, each oligonucleotide having a cleavable linker moiety synthesized within the sequence, such that after synthesis, oligonucleotides can be cleaved from the microarray device into a solution. Examples of appropriate cleavable linker moieties are shown in Figure 3A. In addition to this method of cleavage, a sequence containing RE enzyme site can be synthesized at the ends of oligonucleotides attached to the microarray device. These oligonucleotides on the microarray device then hybridize with an oligonucleotide complementary to this additional flanking sequence and treated with an RE enzyme specific for the RE enzyme site. This process releases oligonucleotide fragments resembling the structure of the target polynucleotide. This set of oligonucleotides then can be assembled into the final polynucleotide molecule using any one of the methods or combination of the methods of ligation, primer extension and PCR.

In a third embodiment of the inventive process, a plurality of oligonucleotides that can be assembled into a full length polynucleotide are synthesized on a microarray device (or beads having a solid surface) having specific cleavable linker moieties (Figure 3A) or capable of being cleaved from the solid support of the microarray device or beads by a chemical treatment. The net effect is to recreate the functional 3' ends and 5' ends of each specific oligonucleotide sequence. After treatment to cleave them, the oligonucleotides (each having overlapping regions) are released into a mixture and used for full-length polynucleotide gene assembly using any of the gene assembly processes described herein.

Specifically, in the third embodiment and as illustrated in Figure 3, a target polynucleotide sequence is dissected into number of overlapping oligonucleotide sequences by a software program or on paper, but not necessarily physically in a laboratory. These oligonucleotide sequences are physically synthesized on a microarray device. In alternative A, the oligonucleotide sequences are connected to the microarray device through cleavable linker moiety. Chemical cleavage under basic conditions (*e.g.*, through addition of ammonia), at cleavable linker CL recreates the usual 3' end of the specific oligonucleotide sequences 1 through N. Oligonucleotide sequences 1 through N are released into a mixture. The mixture of oligonucleotide sequences is used for polynucleotide assembly.

In alternative B, oligonucleotide sequences are connected to a microarray device through additional flanking sequence regions containing a restriction enzyme (RE) sequence site. A second oligonucleotide fragment, complementary to the flanking sequence, is hybridized to the oligonucleotides on the microarray device. This recreates a ds structure at the flanking sequence region, including the RE recognition site. Digestion of this ds DNA structure with RE enzyme specific to the RE recognition site in the flanking sequence region

will release specific oligonucleotides 1 through N into a mixture solution. The oligonucleotides 1 through N are able to assemble into a polynucleotide molecule in solution.

In another example of alternative B, oligonucleotides that together assemble into the polynucleotide are synthesized on a microarray device, each having a flanking sequence on the microarray side. The flanking sequence further comprises a restriction endonuclease (RE) recognition site (see Figure 3B). Oligonucleotides complementary to the flanking sequence region are added and hybridized to the oligonucleotides on microarray device. After hybridization a RE (restriction enzyme specific to the RE sequence in the flanking region) is added to the microarray device. Specific oligonucleotide sequences are released from the microarray device as a result of RE digestion into a mixture. The mixture of specific oligonucleotide sequences assembled into the full-length polynucleotide sequence.

Example 1

This example illustrates assembly of 172-mer polynucleotide sequence from non-cleavable oligonucleotide sequences synthesized on a microarray device according to the first embodiment inventive process (Figures 4 and 5). Three oligonucleotides (sequences shown in Figure 5) were synthesized *in situ* on a microarray device according to an electrochemical process (see U.S. Patent 6,093,302. The oligonucleotide sequences synthesized were amplified by a PCR reaction with primers X (complementary to the strand of oligo#1) and Z (same strand as oligo#3) (Figure 5). After 45 cycles of PCR using a PCR kit with AmplyGold® enzyme (Applied Biosystems) a correct DNA fragment of 172 bp was synthesized (Figure 4). Its subsequent digestion confirmed the specificity of this enzyme with *Hpa*II producing two fragments of 106 bp and 68 bp.

Example 2

This example illustrates the second embodiment of the inventive process for preparing oligonucleotides for assembly into full-length polynucleotides by PCR and REII (restriction enzyme) digestion. A single oligonucleotide sequence was synthesized on a microarray device according to the procedure in Example 1 (see Figures 2 and 6). The oligonucleotide sequence further comprised 2 flanking sequences, each having a recognition site for a *Mly*I restriction enzyme. This microarray device was subject to a PCR (25 cycles) reaction with two primers (shown in Figure 7) to produce an amplified PCR fragment mixture. The amplified PCR fragment obtained was digested by *Mly*I restriction enzyme and purified by a PCR purification kit (Qiagen) to produce specific oligonucleotides ready for assembly (Figure 7). Similarly, this specific oligonucleotide was purified from the flanking sequences by absorption of the digestion mixture by Streptavidin-agarose (Sigma).

Example 3

This example illustrates the assembly of a 290 bp polynucleotide sequence from 9 oligonucleotide sequences, each having flanking sequences containing a *Mly*I restriction site.

Each of the nine different oligonucleotide sequences was synthesized on a microarray device through an *in situ* electrochemistry process as described in example 1 herein.

The microarray device containing the nine specific oligonucleotide sequences (with flanking sequences as shown in Figure 8) was used for PCR amplification of each oligonucleotide sequence using two primers, Primer 1 and 2, described in Figure 6 to form a mixture of ds oligonucleotide sequences. The primers were complementary to the flanking sequences. The mixture of the amplified ds oligonucleotide sequences was digested by *Mly*I enzyme. Specific ds oligonucleotide sequences were purified and then assembled into the final 290 bp polynucleotide sequence in two steps as described in Figure 2 and shown schematically in Figure 9. At the first step of assembly 20 cycles of melting-annealing-extension were used. The final product was amplified using two primers FP1 and FP2 (Figure 9) in 25 cycles of PCR into a 290 bp polynucleotide DNA.

Example 4

This example illustrates the creation of a cDNA polynucleotide sequence capable of coding on expression for fusion protein MIP-GFP-FLAG (Macrophage Inflammation Protein – Green Fluorescence Protein – FLAG peptide) using thirty-eight overlapping oligonucleotide sequences (Figure 10). The 38 oligonucleotides were synthesized on a microarray device using an electrochemical *in situ* synthesis approach, as described in example 1. Each oligonucleotide sequence contained a cleavable linker moiety (see Figure 3A) at their 3' end. After simultaneous deprotection and cleavage of these oligonucleotide sequences by concentrated ammonia, the mixture of oligonucleotide sequences was purified by gel-filtration through the spin column. The purified oligonucleotide sequences were assembled into a polynucleotide by a process shown schematically in Figure 3. The resulting DNA polynucleotide was 965 bp and contained both a T7 RNA-polymerase promoter and a coding sequence for MIP-GFP-FLAG fusion protein. The polynucleotide assembled in this example was used in a standard transcription/translation reaction and produced the appropriate MIP-GFP-FLAG fusion protein. The translated protein was purified from the reaction mixture using anti-FLAG resin (Sigma). The functional protein possessed green fluorescence signal in appropriate blue light. Accordingly, this experiment demonstrated that the inventive gene assembly process provided the correct DNA sequence coding for the functional protein.

CLAIMS:

1. A method for producing a polynucleotide comprising a target sequence, the method comprising:

(a) providing a plurality of double-stranded oligonucleotides, each double-stranded oligonucleotide comprising:

(i) an internal sequence identical to a portion of the target sequence, wherein the internal sequence, at one or both ends, comprises an overlapping sequence region that:

is identical to an overlapping sequence region of another double-stranded oligonucleotide in the plurality of double-stranded oligonucleotides; and

comprises a Type II restriction endonuclease cleavage site; and

(ii) one or more flanking sequences, wherein each flanking sequence comprises a Type II restriction endonuclease recognition site corresponding to a Type II restriction endonuclease cleavage site of the internal sequence;

(b) digesting, in a single pool, the plurality of double-stranded oligonucleotides with a Type II restriction endonuclease that recognizes the Type II restriction endonuclease recognition site ; and

(c) ligating the digested oligonucleotides to produce the polynucleotide comprising the target sequence.

2. The method of claim 1, wherein each double-stranded oligonucleotide in (a) comprises a flanking sequence at each end.

3. The method of claim 1, wherein the plurality of double-stranded oligonucleotides are digested in (b) with a Type IIS endonuclease.

4. The method of claim 3, wherein the Type IIS restriction endonuclease includes at least one of MylI, BspMI, BsaXI, BsrI, BmrI, BtsI, and Fok1.

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5. The method of claim 1, wherein the method does not comprise PCR amplification prior to step (c).

6. The method of claim 1, further comprising:
purifying the digested oligonucleotides prior to ligation in (c);
amplifying the polynucleotide comprising the target sequence; or
a combination thereof.

7. The method of claim 1, wherein the plurality of double-stranded oligonucleotides are synthesized from a plurality of single-stranded oligonucleotides, wherein each oligonucleotide of the plurality of single-stranded oligonucleotides is bound to a surface.

8. A method for producing a polynucleotide comprising a target sequence, the method comprising:

(a) providing a first plurality of single-stranded oligonucleotides, each single-stranded oligonucleotide of the first plurality comprising:

(i) an internal sequence identical to or complementary to a portion of the target sequence, wherein the internal sequence, at one or both ends, comprises an overlapping sequence region that is identical to or complementary to an overlapping sequence region of another single-stranded oligonucleotide in the plurality of single-stranded oligonucleotides; and

(ii) a flanking sequence comprising a Type II restriction endonuclease recognition site; and

(b) contacting the first plurality of single-stranded oligonucleotides with a second plurality of single-stranded oligonucleotides, each single-stranded oligonucleotide of the second plurality comprising a sequence having a region that complements a flanking sequence of a single-stranded oligonucleotide of the first plurality to produce a plurality of double-stranded oligonucleotides;

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(c) digesting, in a single pool, the plurality of double-stranded oligonucleotides with a Type II restriction endonuclease that recognizes the Type II restriction endonuclease recognition site; and

(d) ligating the digested oligonucleotides to produce the polynucleotide comprising the target sequence.

9. The method of claim 8, wherein each single-stranded oligonucleotide in the plurality of single-stranded oligonucleotides comprises flanking sequences, one at each end of the internal sequence, each flanking sequence comprising: (i) a primer binding site; and (ii) a Type II restriction endonuclease recognition site.

10. The method of claim 9, wherein (b) further comprises amplifying the plurality of double-stranded oligonucleotides.

11. The method of claim 8, wherein the first plurality of single-stranded oligonucleotides has been synthesized on a surface.

12. The method of claim 11, wherein the surface is a microarray or wherein the surface comprises beads.

13. The method of claim 8, wherein the plurality of double-stranded oligonucleotides are digested in (c) with a Type IIS restriction endonuclease.

14. The method of claim 13, wherein the Type IIS restriction endonuclease includes at least one of MylI, BspMI, BsaXI, BsrI, BmrI, BtsI, and Fok1.

15. The method of claim 8, wherein the method does not comprise PCR amplification prior to step (c).

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16. The method of claim 8, further comprising:

purifying the digested oligonucleotides prior to ligation in (d);

amplifying the polynucleotide comprising the target sequence; or

a combination thereof.

17. A method for producing a polynucleotide comprising a target sequence, the method comprising:

(a) synthesizing on a surface a plurality of single-stranded oligonucleotides, each single-stranded oligonucleotide comprising:

(i) an internal sequence identical to or complementary to a portion of the target sequence, wherein the internal sequence, at one or both ends, comprises an overlapping sequence region that is identical to or complementary to an overlapping sequence region of another single-stranded oligonucleotide in the plurality of single-stranded oligonucleotides; and

(ii) flanking sequences, one at each end of the internal sequence, each flanking sequence comprising a primer binding site, and at least one flanking sequence further comprising a Type II restriction endonuclease recognition site;

(b) amplifying the plurality of single-stranded oligonucleotides;

(c) digesting, in a single pool, the amplified oligonucleotides with a Type II restriction endonuclease that recognizes the Type II restriction endonuclease recognition site; and

(d) ligating the digested oligonucleotides to produce the polynucleotide comprising the target sequence.

18. The method of claim 17, wherein each single-stranded oligonucleotide in the plurality of single-stranded oligonucleotides comprises flanking sequences, one at each end of the internal sequence, each flanking sequence comprising: (i) a primer binding site; and (ii) a Type II restriction endonuclease recognition site.

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19. The method of claim 17, wherein the surface is a microarray or wherein the surface comprises beads.

20. The method of claim 17, wherein the amplified oligonucleotides are digested in (c) with a Type IIS restriction endonuclease.

21. The method of claim 20, wherein the Type IIS restriction endonuclease includes at least one of MylI, BspMI, BsaXI, BsrI, BmrI, BtsI, and Fok1.

22. The method of claim 17, further comprising:
purifying the digested oligonucleotides prior to ligation in (d);
amplifying the polynucleotide comprising the target sequence; or
a combination thereof.

23. A method for producing a polynucleotide comprising a target sequence, the method comprising:

(a) providing a plurality of oligonucleotides, each oligonucleotide comprising:

(i) an internal sequence identical to or complementary to a portion of the target sequence, wherein the internal sequence, at one or both ends, comprises an overlapping sequence region that is identical to or complementary to an overlapping sequence region of another oligonucleotide in the plurality of oligonucleotides; and

(ii) flanking sequences, one at each end of the internal sequence, each flanking sequence comprising a primer binding site, and at least one flanking sequence further comprising a Type II restriction endonuclease recognition site;

(b) amplifying the plurality of oligonucleotides;

(c) digesting, in a single pool, the amplified oligonucleotides with a restriction endonuclease that recognizes the Type II restriction endonuclease recognition site; and

(d) ligating the digested oligonucleotides to produce the polynucleotide comprising the target sequence.

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24. The method of claim 23, wherein each oligonucleotide in the plurality of oligonucleotides comprises flanking sequences, one at each end of the internal sequence, each flanking sequence comprising: (i) a primer binding site; and (ii) a Type IIS restriction endonuclease recognition site.

25. The method of claim 23, wherein the plurality of oligonucleotides has been synthesized on a surface.

26. The method of claim 25, wherein the surface is a microarray or comprises beads.

27. The method of claim 23, further comprising:
purifying the digested oligonucleotides prior to ligation in (d);
amplifying the polynucleotide comprising the target sequence; or
a combination thereof.

28. The method of claim 23, wherein the amplified oligonucleotides are digested in (c) with a Type IIS restriction endonuclease.

29. The method of claim 28, wherein the Type IIS restriction endonuclease includes at least one of MylI, BspMI, BsaXI, BsrI, BmrI, BtsI, and Fok1.

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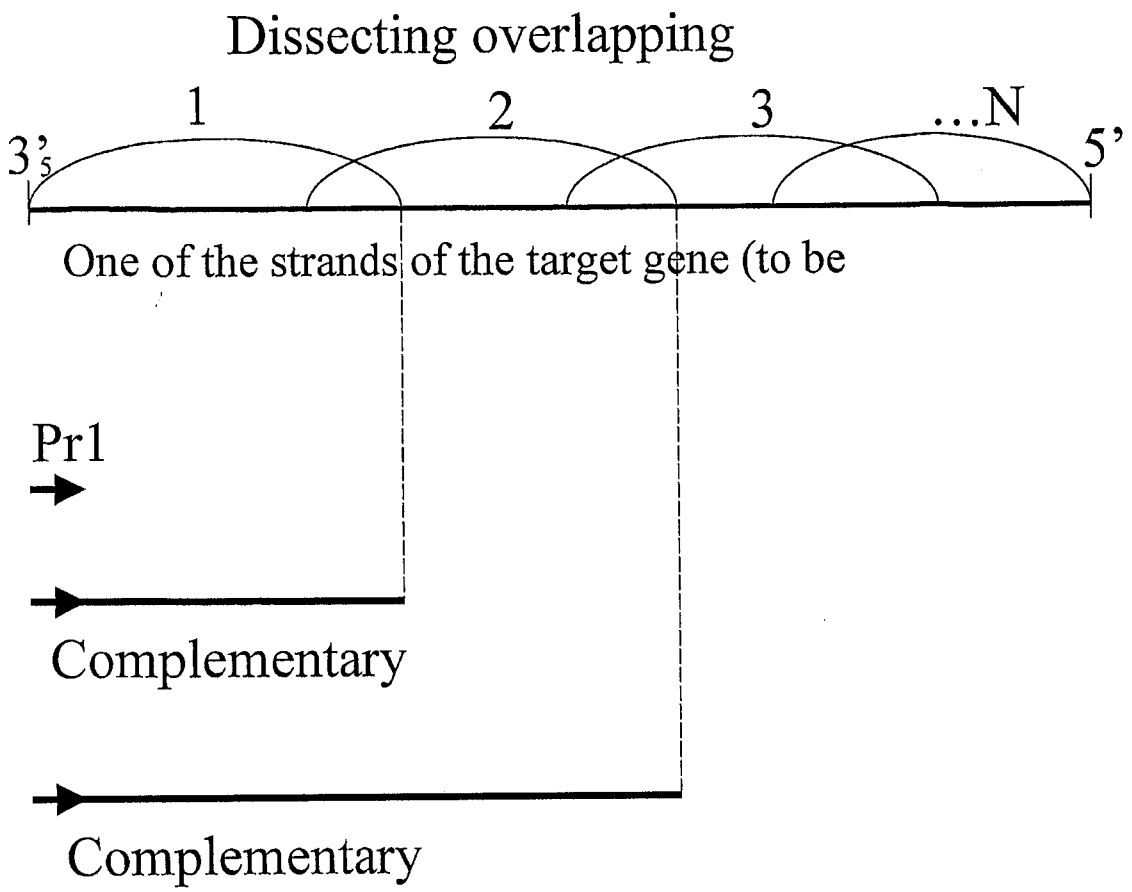

Dissecting overlapping
1
2
3
...N
3'
5'
One of the strands of the target gene (to be
Pr1
Complementary
Complementary

Figure 1A

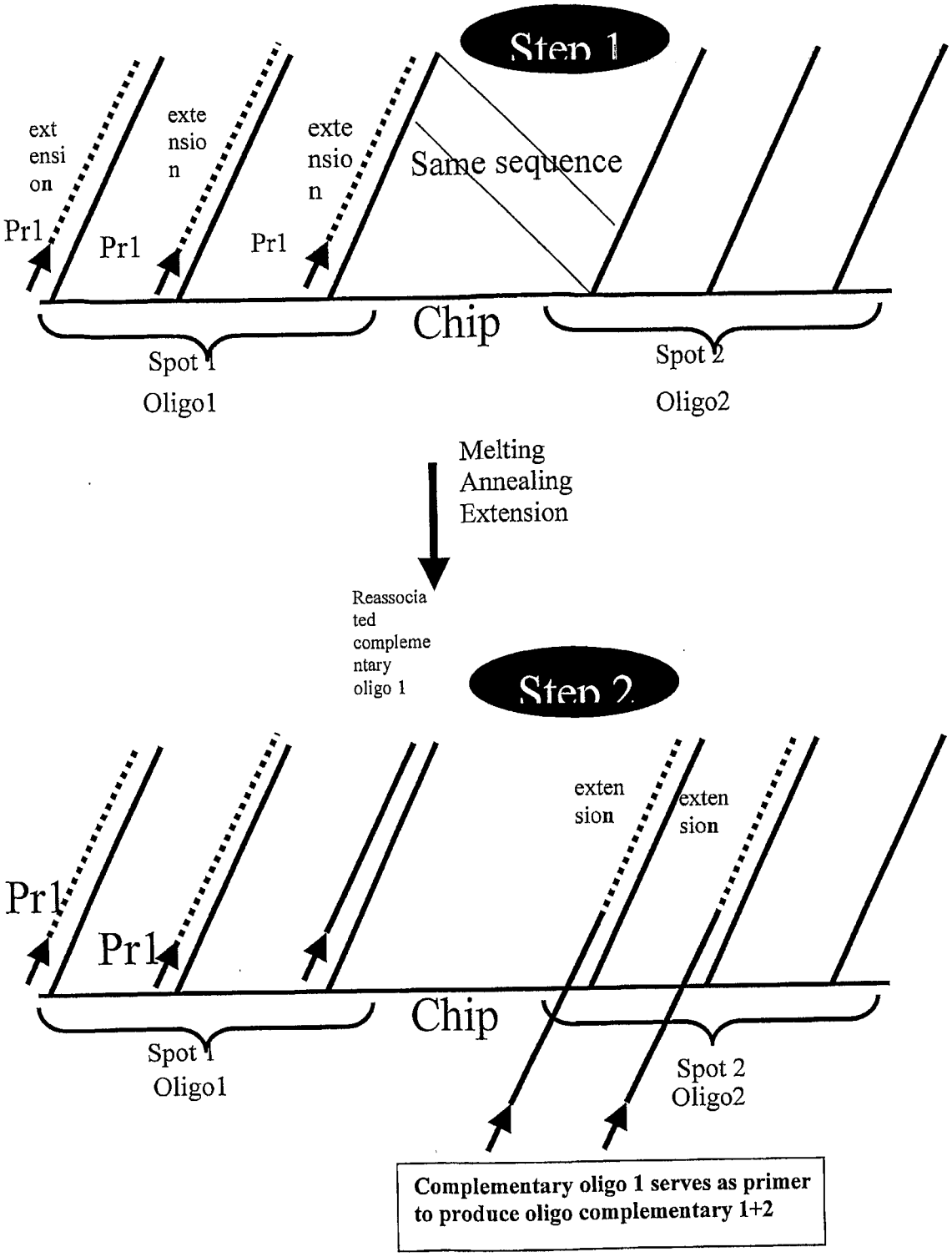

Step 1
ext ensi on
exte nsio n
exte nsio n
Same sequence
Pr1
Pr1
Pr1
Chip
Spot 1
Oligo1
Spot 2
Oligo2
Melting
Annealing
Extension
Reassocia ted compleme ntary oligo 1
Step 2
exten sion
exten sion
Pr1
Pr1
Chip
Spot 1
Oligo1
Spot 2
Oligo2
Complementary oligo 1 serves as primer to produce oligo complementary 1+2

Figure 1B

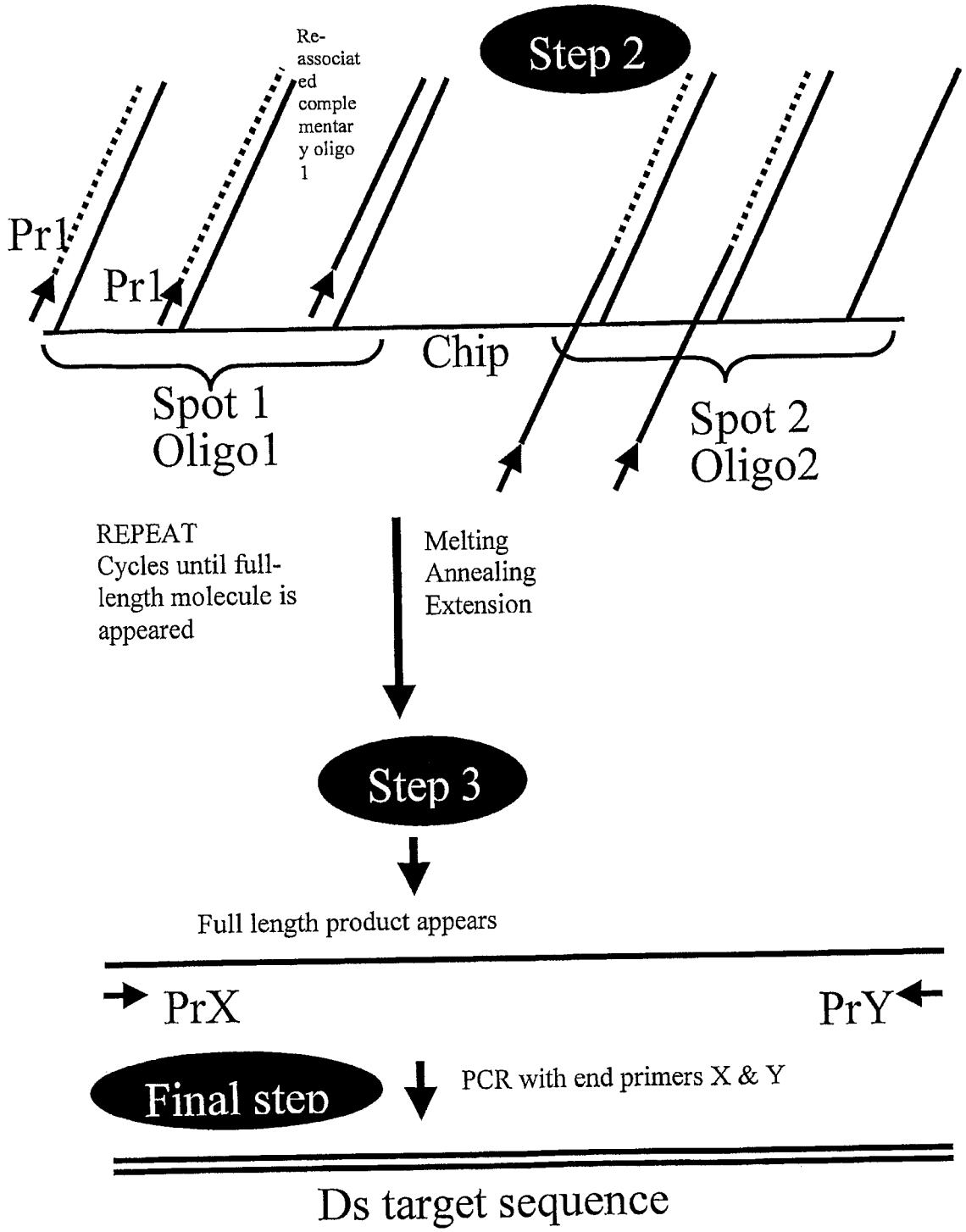
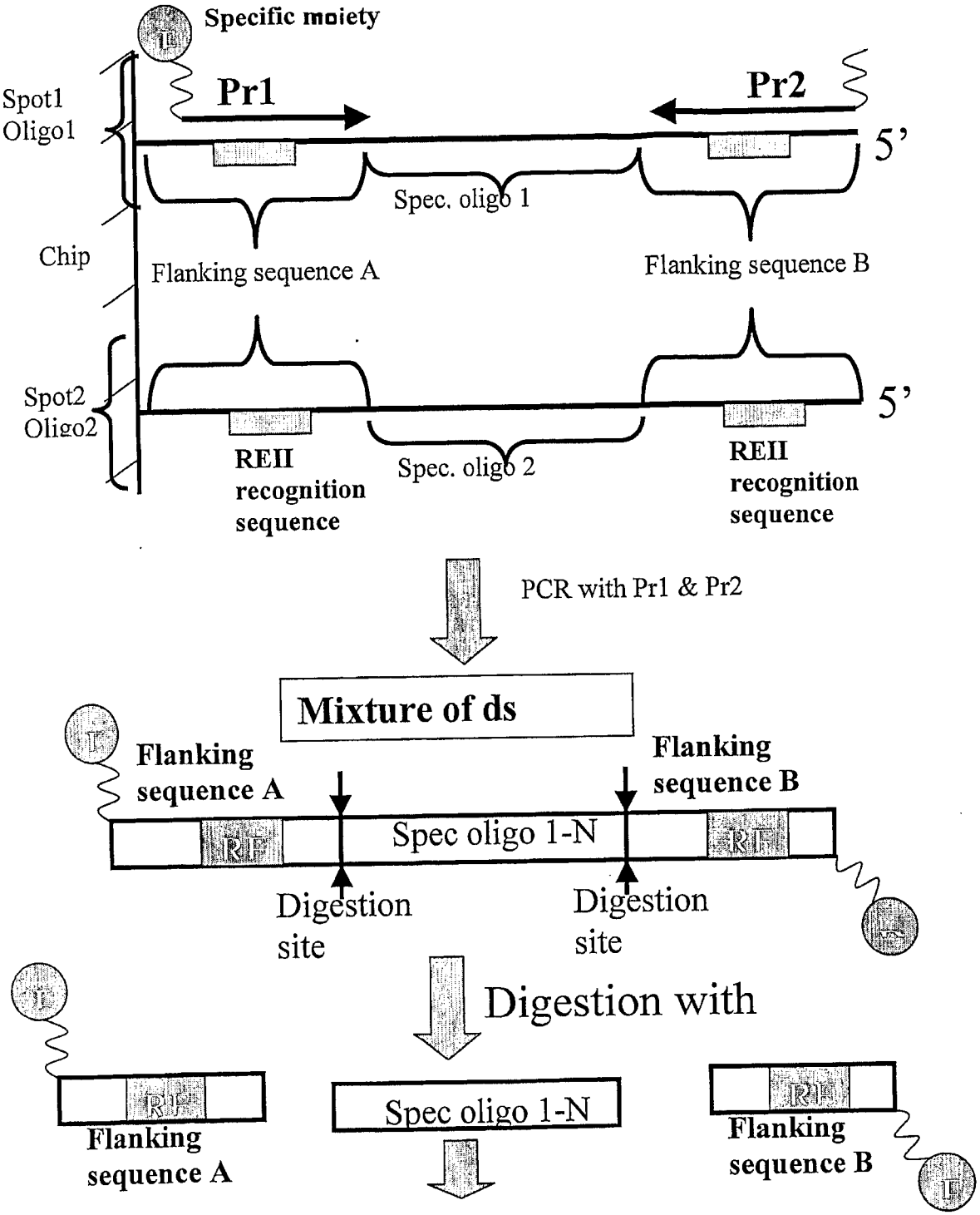

Step 2
Re-
associat
ed
comple
mentar
y oligo
1
Pr1
Pr1
Chip
Spot 1
Oligo1
Spot 2
Oligo2
REPEAT
Cycles until full-
length molecule is
appeared
Melting
Annealing
Extension
Step 3
Full length product appears
PrX
PrY
Final step
PCR with end primers X & Y
Ds target sequence

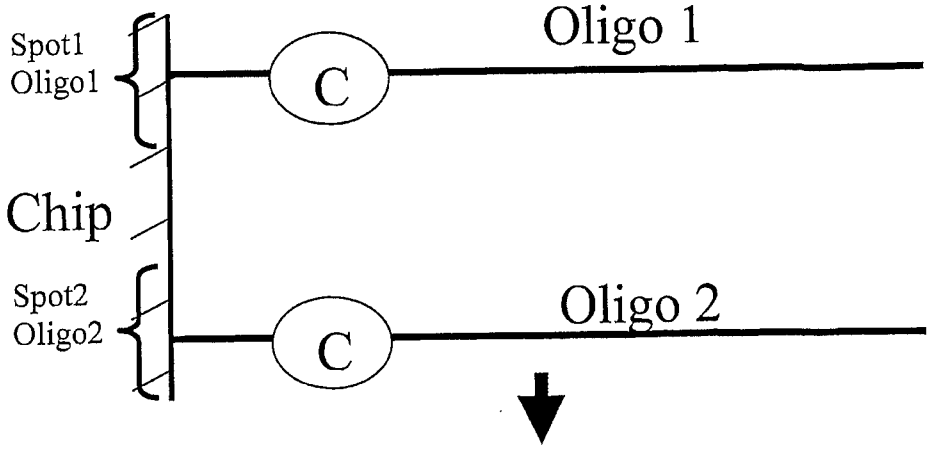
Figure 1C



Assemble into target sequence by melting, annealing, extension followed by PCR

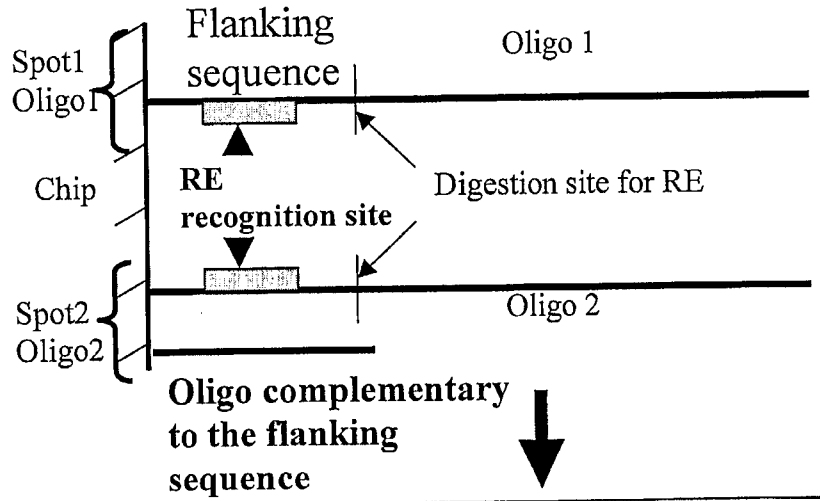
Figure 2

A



Chemical cleavage at cleavable linker CL recreate 5' or 3' end of specific oligos (1, 2, ...N). Oligos are released into a mixture and used for assembly.

B



Hybridization to oligo complementary to the flanking sequence

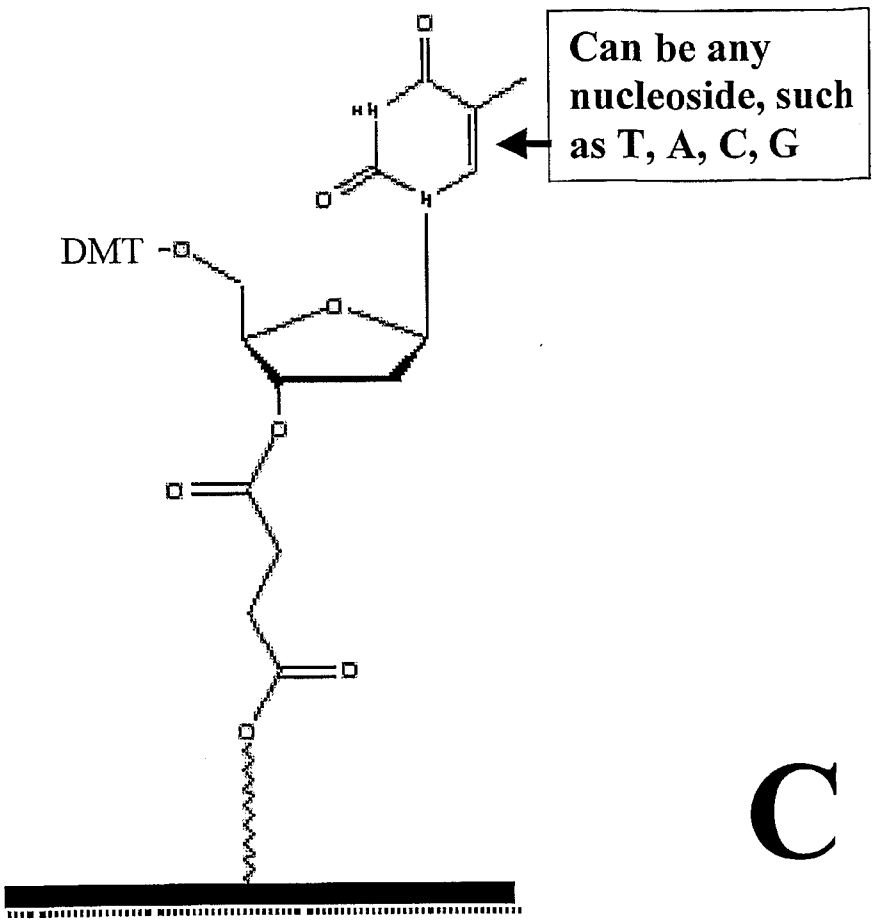
Digestion with RE releases specific oligos into a mixture. This mixture is used for final gene assembly.

Figure 3

Figure 3A

Cleavable linker for oligonucleotide

5



C

On-chip DNA assembly: three non-cleavable oligos

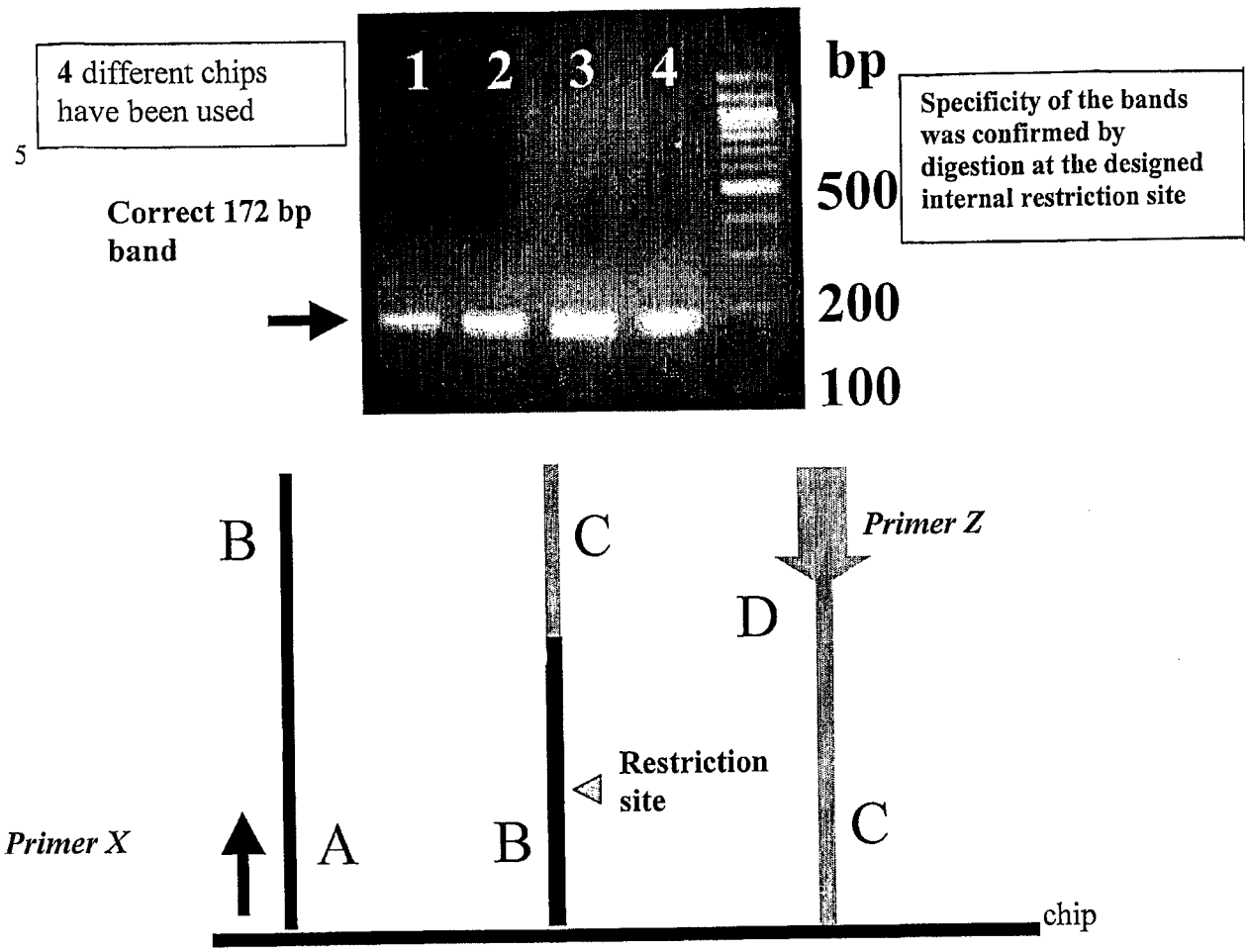


Figure 4

#1
Chip- Primer X
3'TAATTATGCTGAgTGATATCCCTTTCTACCTGTGCGGCTGGCGGACGACGAAGT
CGAATGTGGAGGGCCGTCTAAGGTGTCT5' (82mer)

#2
Chip- HpaII-site
3'GGACGACGAAGTCGAATGTGGAGGGCCGTCTAAGGTGTCTTAAAGTATCGA
CTGATGAAACTCTGCTCGTCGGTCACGAGGTTC-5' (84mer)

#3
CHIP-
3'GTATCGACTGATGAAACTCTGCTCGTCGGTCACGAGGTTCCCTCGACCACCG
CATGATGTTTCTGCTACTGCTGTTCACGATTATC-5' (86mer)
Primer Z

Final product: 172mer (one of two strands is shown, direction is 3' to 5' for convenience)

3'TAATTATGCTGAgTGATATCCCTTTCTACCTGTGCGGCTGGCGGACGACGAAGT
CGAATGTGGAGGGCCGTCTAAGGTGTCTTAAAGTATCGACTGATGAAACTCTG
CTCGTCGGTCACGAGGTTCCCTCGACCACCGCATGATGTTTCTGCTACTGCTGTT
CACGATTATC5'

Figure 5

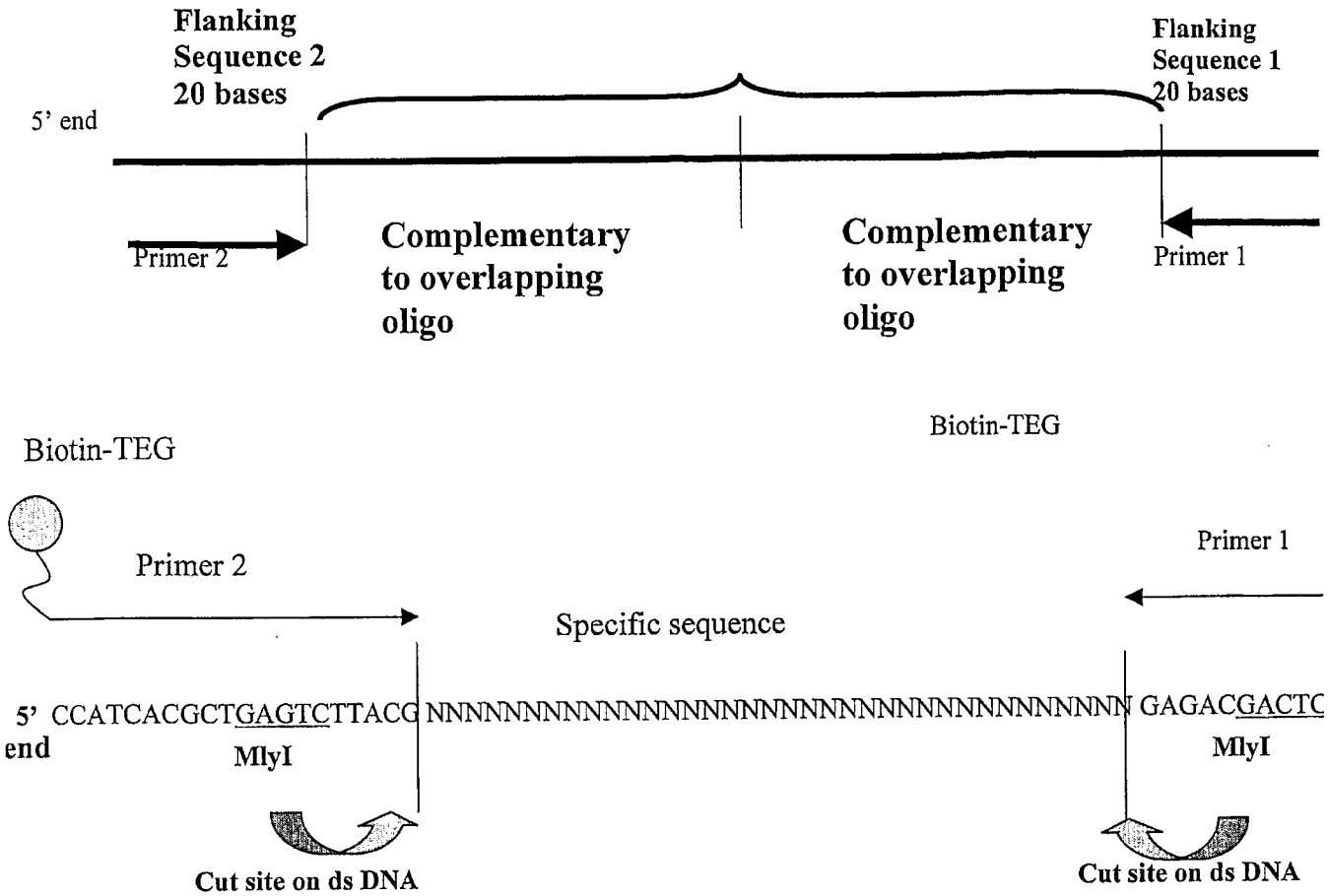

Flanking
Sequence 2
20 bases
Flanking
Sequence 1
20 bases
5' end
Primer 2
Complementary
to overlapping
oligo
Complementary
to overlapping
oligo
Primer 1
Biotin-TEG
Biotin-TEG
Primer 2
Primer 1
Specific sequence
5' CCATCACGCTGAGTCTTACG NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN GAGACGACTC
end
MlyI
MlyI
Cut site on ds DNA
Cut site on ds DNA

Figure 6

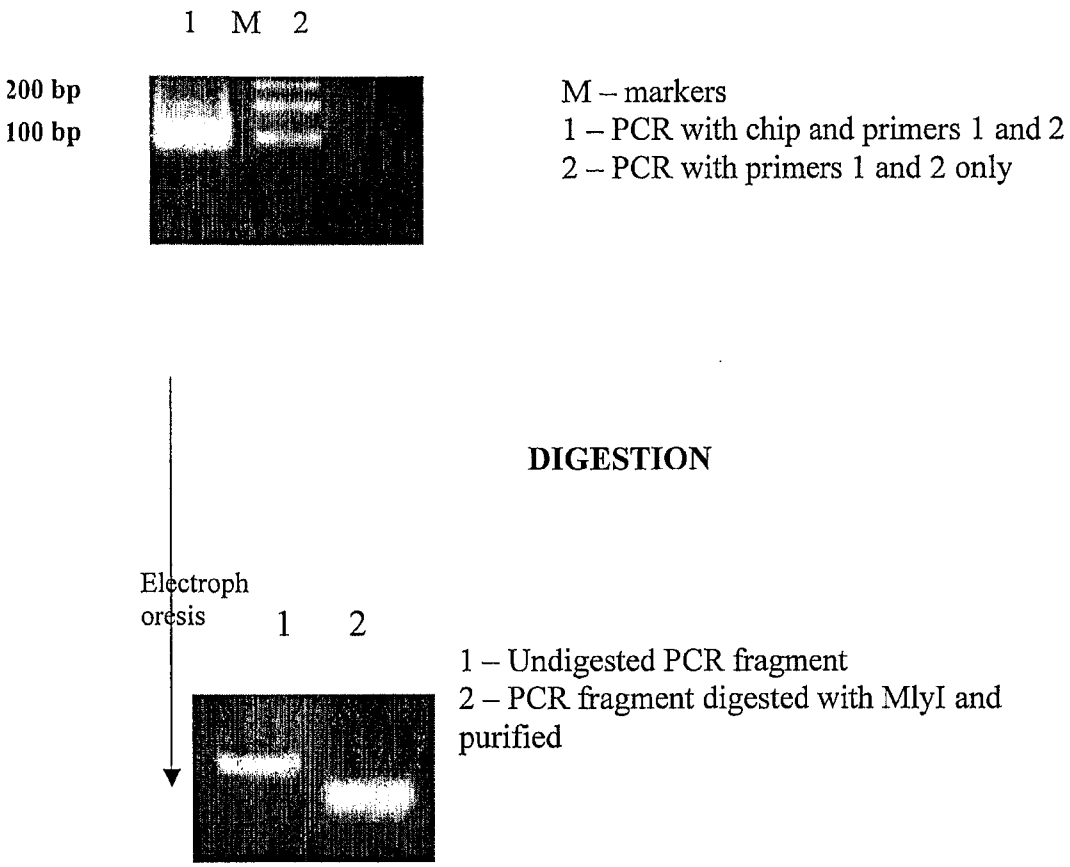
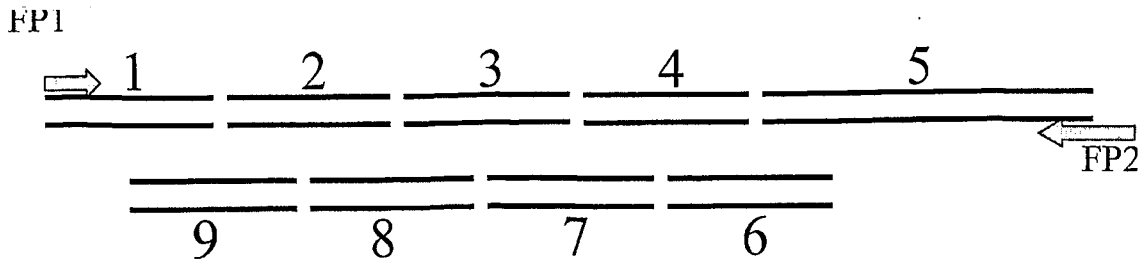

1 M 2
200 bp
100 bp
M – markers
1 – PCR with chip and primers 1 and 2
2 – PCR with primers 1 and 2 only
DIGESTION
Electroph
oresis
1 2
1 – Undigested PCR fragment
2 – PCR fragment digested with MlyI and
purified

Figure 7

1:
CCATCACGCTGAGTCTTACGTACGTAATACGACTCACTATAGGGAAAGTCGCCACCATG
GACACGCCGAC**GAGACGACTCCTAATCGAA**
2:
CCATCACGCTGAGTCTTACGCGCCTGCTGCTTCAGCTACACCTCCCGGCAGATTCCACAG
AATTTC**GAGACGACTCCTAATCGAA**
3:
CCATCACGCTGAGTCTTACGATAGCTGACTACTTTGAGACGAGCAGCCAGTGCTCCAAG
CCCGGTGTC**GAGACGACTCCTAATCGAA**
4:
CCATCACGCTGAGTCTTACGATCTTCCTAACCAAGCGAAGCCGGCAGGTCTGTGCTGACC
CC**GAGACGACTCCTAATCGAA**
5:
CCATCACGCTGAGTCTTACGCAGGCACTCAGCTCTACGGGGCCGTCGCCGATGGGGGTG
TTCTGCTGGTAGTGGTCGGCGAGCTGCATATTTCTGGACCCACTCCTCACT**GAGACGACTC**
CTAATCGAAC

6:
CCATCACGCTGAGTCTTACGATATTTCTGGACCCACTCCTCACTGGGGTCAGCACAGACC
TGCC**GAGACGACTCCTAATCGAA**
7:**CCATCACGCTGAGTCTTACG**GGCTTCGCTTGGTTAGGAAGATGACACCGGGCTTGGAG
CACTGGC**GAGACGACTCCTAATCGAA**
8:**CCATCACGCTGAGTCTTACG**TGCTCGTCTCAAAGTAGTCAGCTATGAAATTCTG
TGGAATCTGCC**GAGACGACTCCTAATCGAA** 9:
CCATCACGCTGAGTCTTACGGGGAGGTGTAGCTGAAGCAGCAGGCGGTCGGCGTGTCCA
TGGTGGCGAC**GAGACGACTCCTAATCGAA**

FIGURE 8



FP1 and FP2 are final primers 1 and 2 used to PCR the target sequence

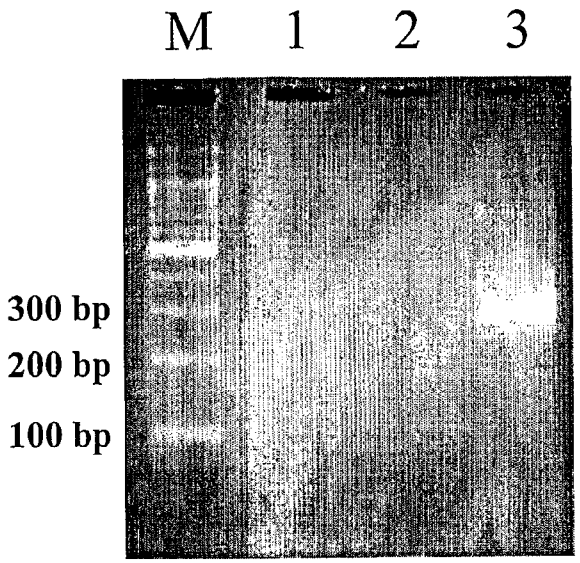


Figure 9

1: TACGTAATACGACTCACTATAGGGAAAGTCGCCACCATGGACACGCCGAC

2: CGCCTGCTGCTTCAGCTACACCTCCCGGCAGATTCCACAGAATTTC

3: ATAGCTGACTACTTTGAGACGAGCAGCCAGTGCTCCAAGCCCGGTGTC

4: ATCTTCCTAACCAAGCGAAGCCGGCAGGTCTGTGCTGACCCC

5: AGTGAGGAGTGGGTCCAGAAATATGTCAGCGACCTAGAGCTGAGTGC

6: ATATTTCTGGACCCACTCCTCACTGGGGTCAGCACAGACCTGCC

7: GGCTTCGCTTGGTTAGGAAGATGACACCGGGCTTGGAGCACTGGC

8: TGCTCGTCTCAAAGTAGTCAGCTATGAAATTCTGTGGAATCTGCC

9: GGGAGGTGTAGCTGAAGCAGCAGGCGGTCGGCGTGTCCATGGTGGCGAC

1F: GGTGAACAGCTCCTCGCCCTTGCTCACCATGGCACTCAGCTCTAGGTCGCTGAC

2F: CATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTC

3F: *TTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCC*

4F: GAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC

5F: *TTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAAC*

6F: GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC

7F: *CAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGC*

8F: ACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGC

9F: *GGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAGGGTGGGC*

10F: GTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTC

11F: *GTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTC*

12F: TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTC

13F: *GGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGAC*

14F: AAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC

15F: *AGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGC*

16F: GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGAC

FIGURE 10

17F: *TCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTC*

18F: GGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTC

19F: *GTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTAC*

20F: TATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATC

21F: *ACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCC*

22F: CGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGC

23F: *ACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGC*

24F: AGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC

25F: *TTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGC*

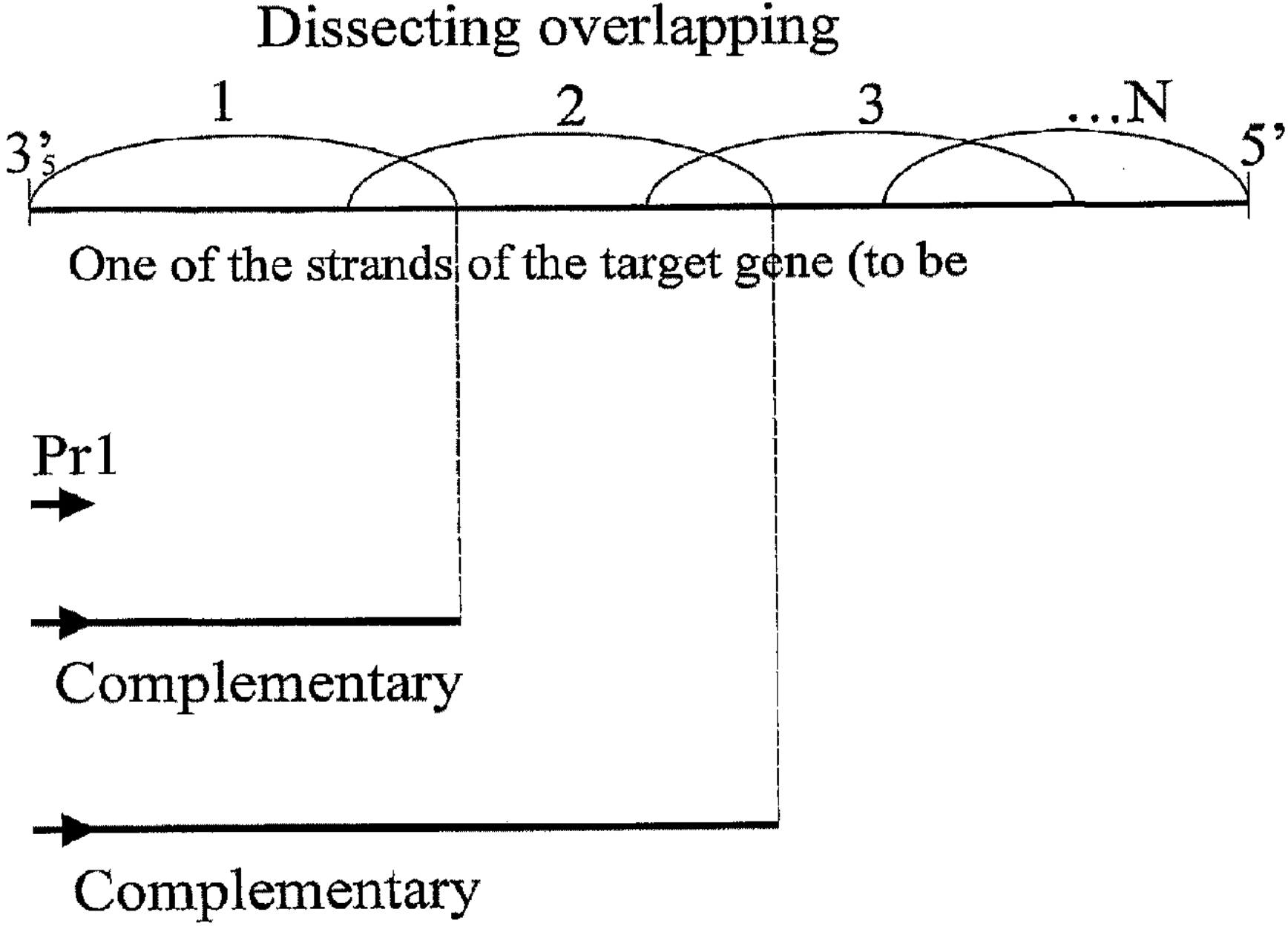
26F: TGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCAC

27F: *GGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTC*

28F: ATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGAC

29F: *GGCGGCCGCTTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC*

Figure 10
(continuation)


Dissecting overlapping
1
2
3
...N
3'
5'
One of the strands of the target gene (to be
Pr1
Complementary
Complementary