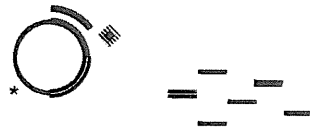


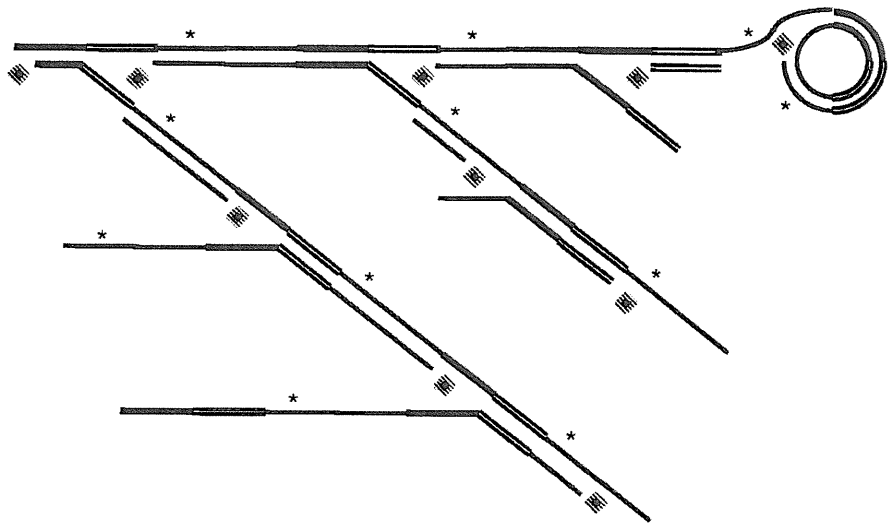
- A. Amplification of circular target with random hexamers. Using either primer or random hexamers, Phi29 DNA polymerase extends on circle template.



- B. As polymerase continues to extend, it displaces original primer to form a single-stranded tail. Additional hexamers start extending on the tail.



- C. Rolling circle amplification with random priming generates long single strands which allow for additional priming, and eventually resolve as double stranded fragments of varying length.



- D. For next generation sequencing protocols, the double-stranded DNA is fragmented such that tandem copies of the target DNA are within a single fragment. Linkers are appended to allow for standard cluster or bead amplification and paired end reads. True mutation (*) will be present 2 or 3 times, and thus distinguished from polymerase error.

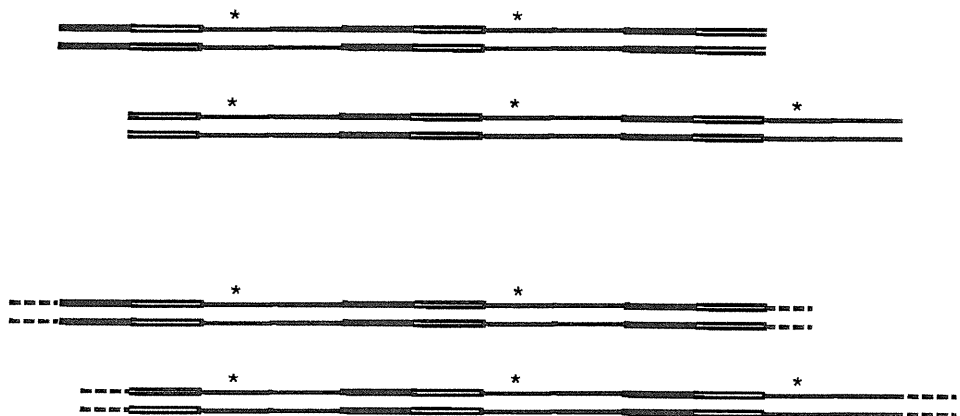
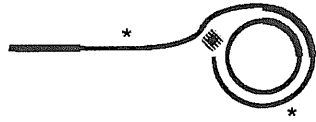


Figure 1

- A. Amplification of single stranded circle containing mutated target with defined primer.



- B. Phi29 or Bst DNA polymerase extends primer on circle template to generate single-stranded tail.



- C. The extension product is denatured from the circle. A mix of unmethylated / methylated primer is hybridized to the long single strand, and polymerase lacking 5' nuclease, 3' nuclease, and strand-displacement activity, ligase, and a 2nd restriction enzyme are added to generate tandem copies of target DNA within a single fragment. For NGS sequencing, Linkers are appended to allow for standard cluster or bead amplification and paired end reads. True mutation (*) will be present 2 or more times, and thus distinguished from polymerase error.

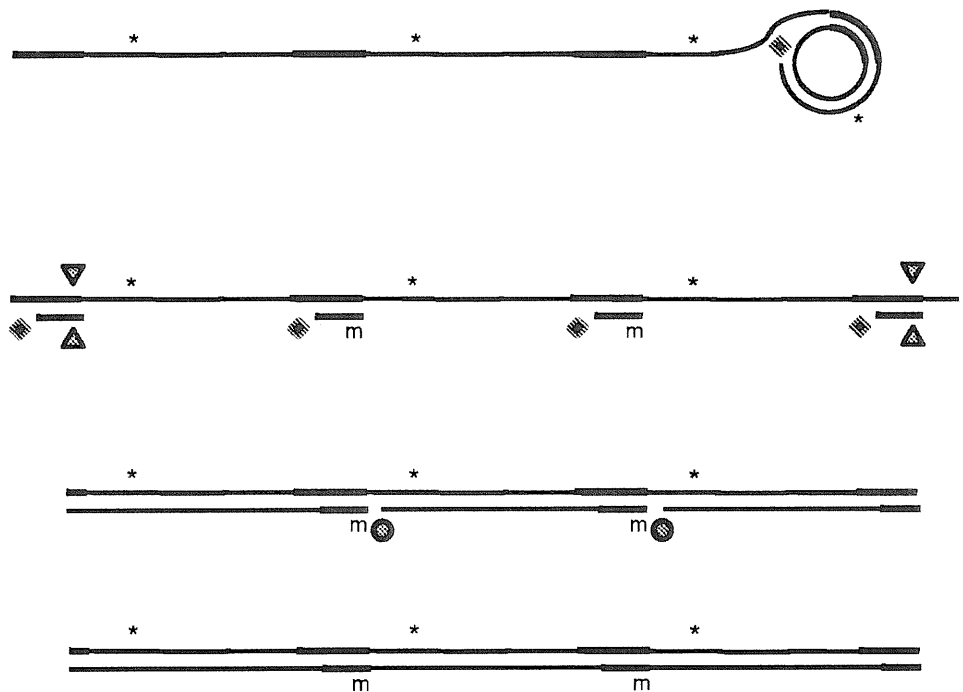
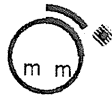
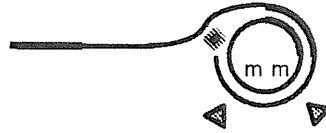


Figure 2

- A. Amplification of single stranded circle containing methylated target with defined primer.



- B. Bst DNA polymerase extends primer on circle template to generate single-stranded tail. BstU1 (CG[^]CG) will cleave dsDNA if unmethylated, but not hybrid methyl/unmethyl DNA, nor unmethylated ssDNA.



- C. The extension product is denatured from the circle (and polymerase, BstU1 heat inactivated). A mix of unmethylated / methylated primer is hybridized to the long single strand, and polymerase lacking 5' nuclease, 3' nuclease, and strand-displacement activity, ligase, and a 2nd restriction enzyme are added to generate tandem copies of target DNA within a single fragment. For NGS sequencing, Linkers are appended to allow for standard cluster or bead amplification and paired end reads.

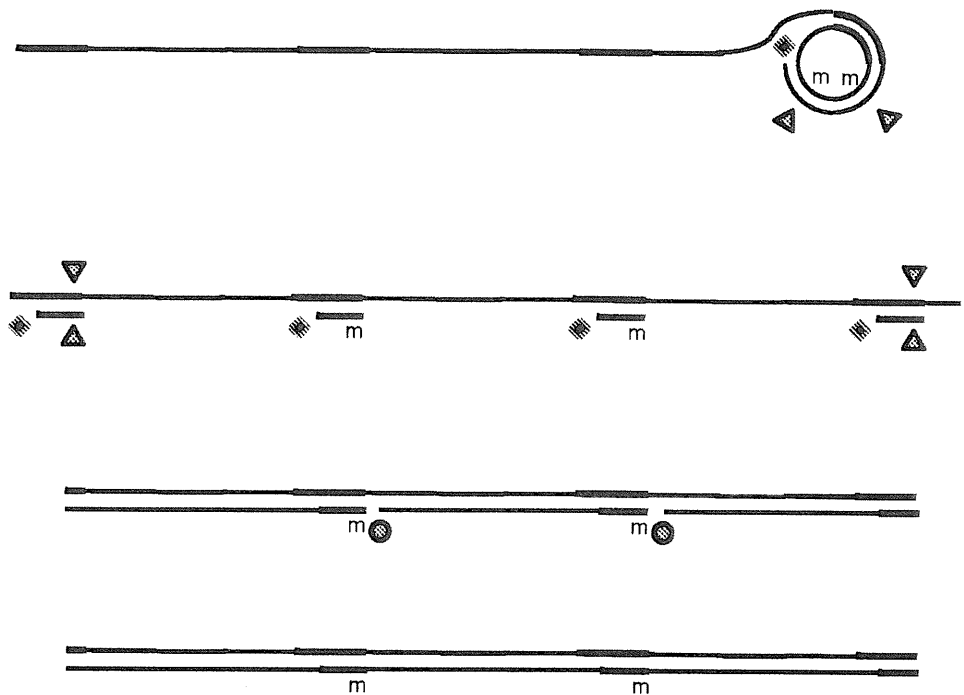
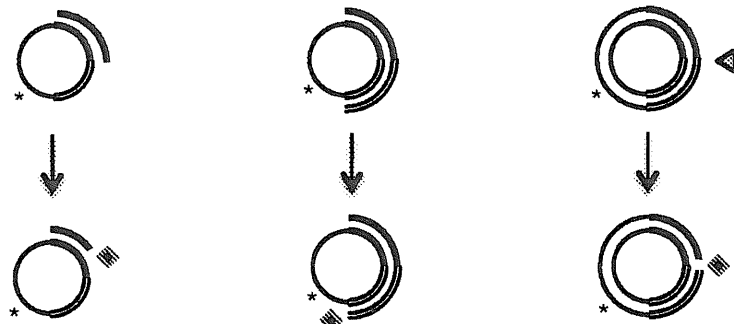
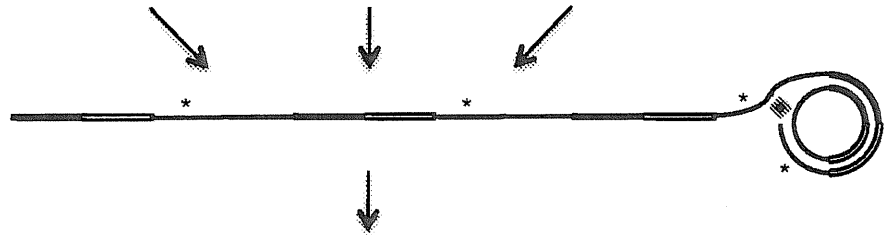


Figure 3

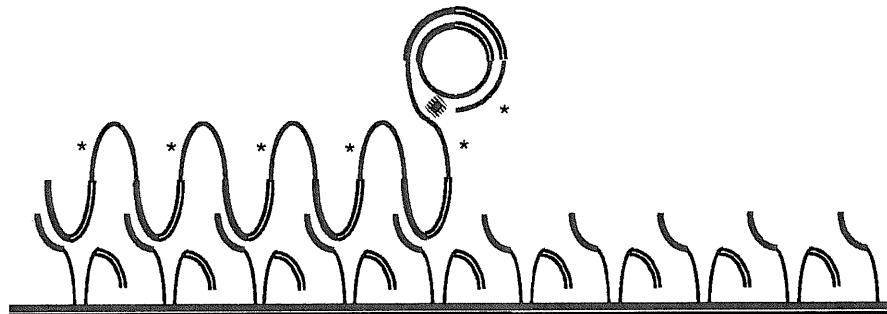
- A. Amplification of circular target with defined primer. Initial template is either primer hybridized to single-stranded circle, similar, but with hybridized coupled oligonucleotide, or double-stranded circle, that is specifically nicked.



- B. Phi29 DNA polymerase extends on circle template to generate single-stranded tail.



- C. The extension product is hybridized to primers on a solid support. As rolling circle amplification generates long single stranded tail, it gets captured locally by hybridizing to adjacent primers on the surface, creating a carpet-like structure.



- D. Alternatively, an additional sequence, suitable for capture on the solid support is already appended on the coupled oligonucleotide hybridized to the circular target. Under such conditions, the composite target / oligo may be first hybridized to the solid support, and then extended using Phi29 DNA polymerase.

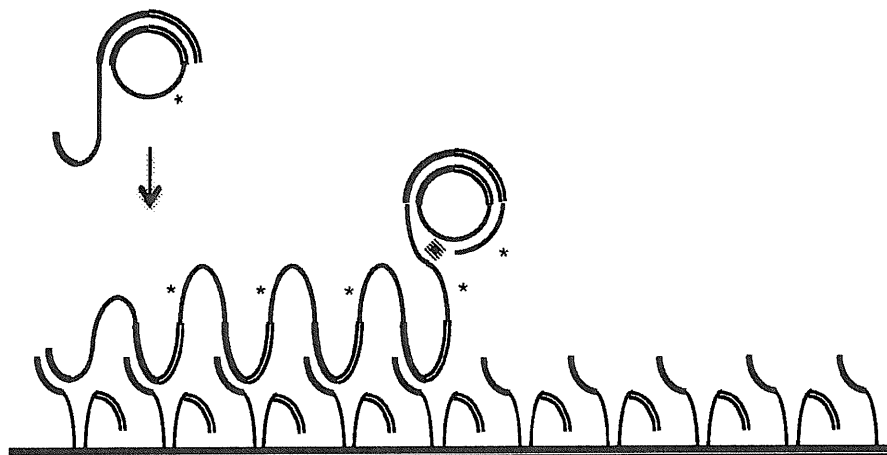
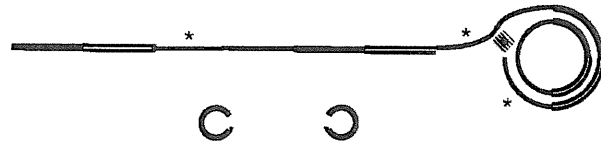
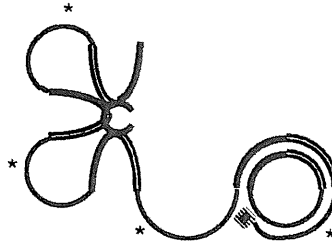


Figure 4

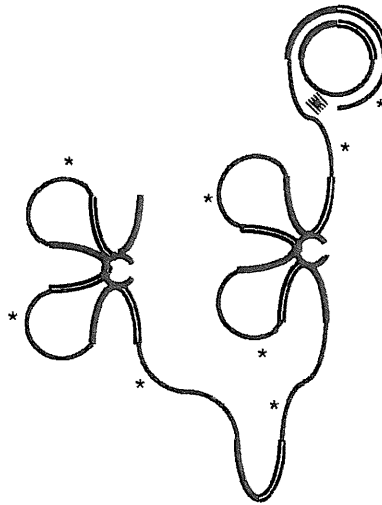
- A. Amplification of circular target with primer to form condensed structures. Phi29-DNA polymerase extends around circular target.



- B. As polymerase continues to extend, oligonucleotides containing tandem repeats of complementary sequences condense the single-stranded tail into condensed structures.



- C. Rolling circle amplification can thus generate hundreds to thousands of tandem copies of target DNA in a condensed structure.



- D. By varying the number of oligonucleotides with different complementary sequences, and the number of tandem repeats within a given oligonucleotide, the number of loops or "crosslinking nodes" can be varied, providing the opportunity to control the "compactness" of the structure.

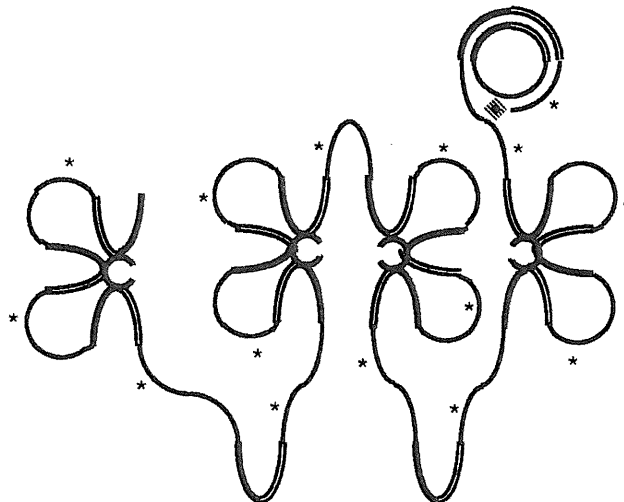
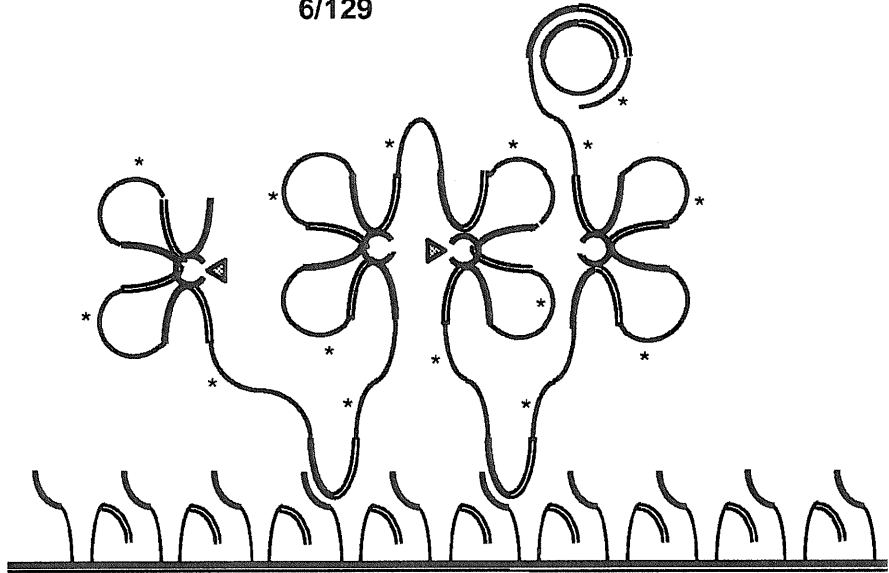
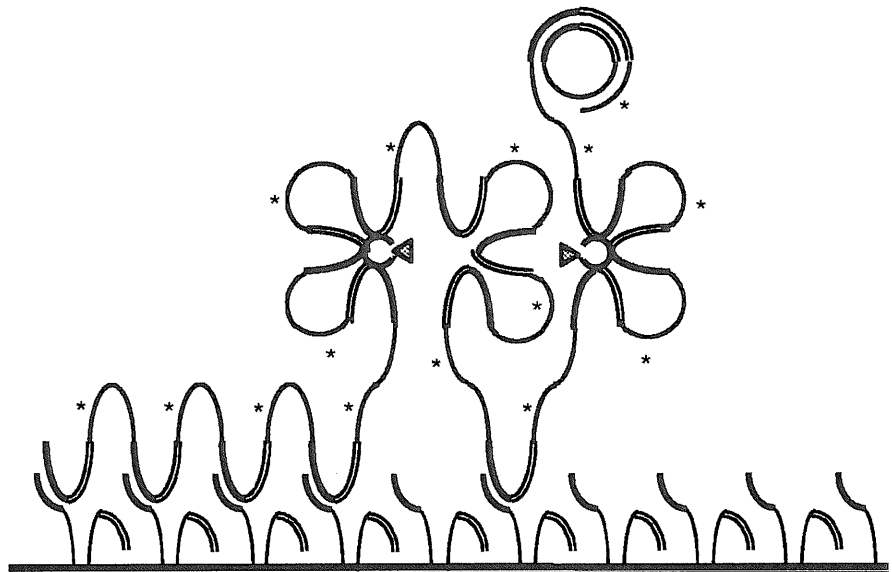


Figure 5

- E. Capture of condensed rolling circle structures on a solid support. The structures hybridize to complementary primers at a few positions. The tandem repeat complementary oligonucleotides contain cleavable linkages, that are then cleaved.



- F. As the condensed structure starts to come apart, individual loops are captured locally by hybridizing to adjacent primers on the surface, creating a carpet-like structure.



- G. This approach assures that the hundreds to thousands of tandem copies of the target are captured next to each other, and suitable for subsequent sequencing.

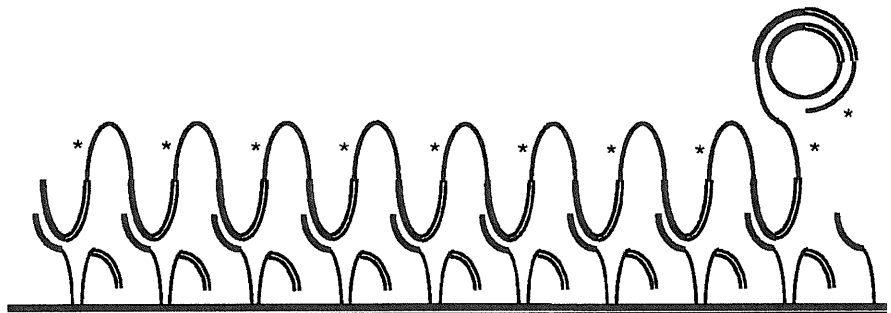
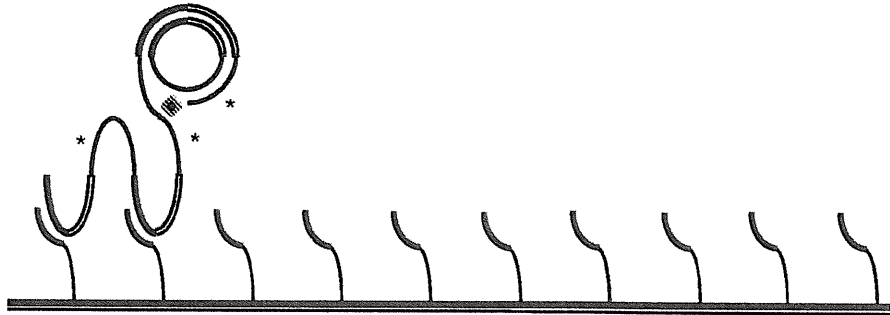


Figure 5 (continued)

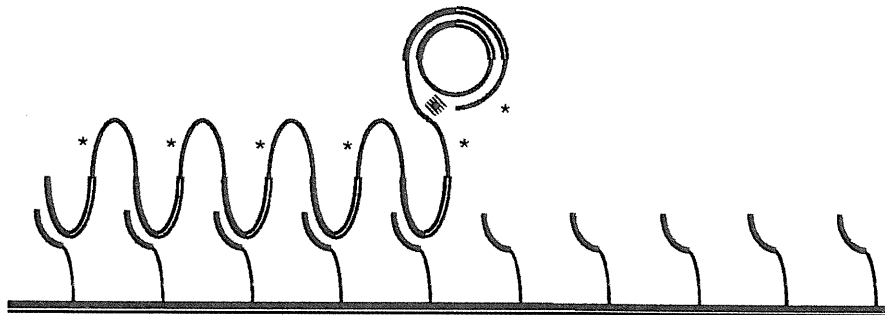
- A. Amplification of circular target for capture on a solid support with a single primer. Phi29 DNA polymerase extends on circle template to generate single-stranded primary extension product.



- B. The extension product is hybridized to first primers on a solid support.



- C. As rolling circle amplification generates a primary extension product, it is captured locally by hybridizing to adjacent first primers on the surface, creating a carpet-like structure.



- D. This approach assures that the hundreds to thousands of tandem complementary copies of the target are captured next to each other, and suitable for subsequent sequencing.

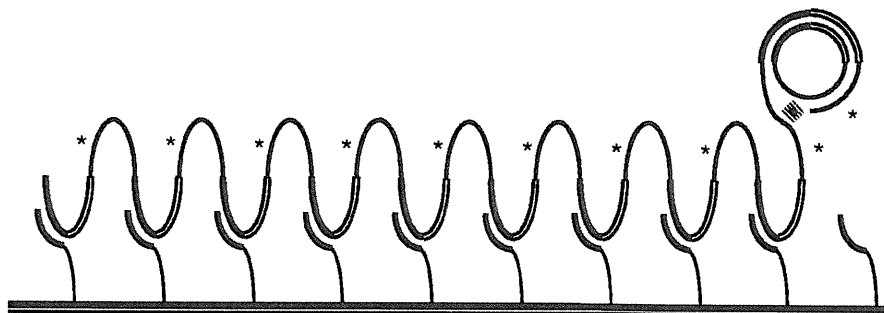
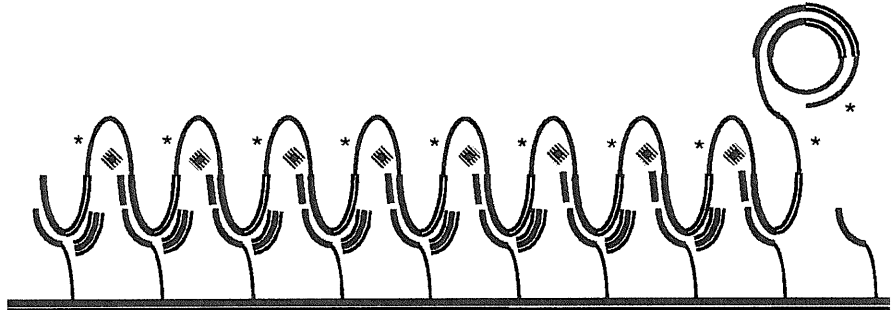
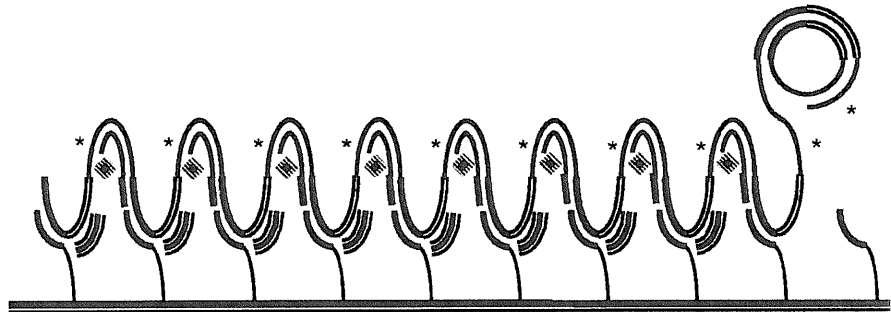


Figure 6

- E. Hybridize sequencing primer adjacent to 3' end of first oligonucleotide primer on the support, and PNA or blocking oligonucleotide adjacent to 5' end.



- F. Sequence primary extension product (complement of genomic DNA sequence) using sequencing by synthesis.



- G. Sequencing continues until extension products are unable to extend due to PNA or blocking oligonucleotide.

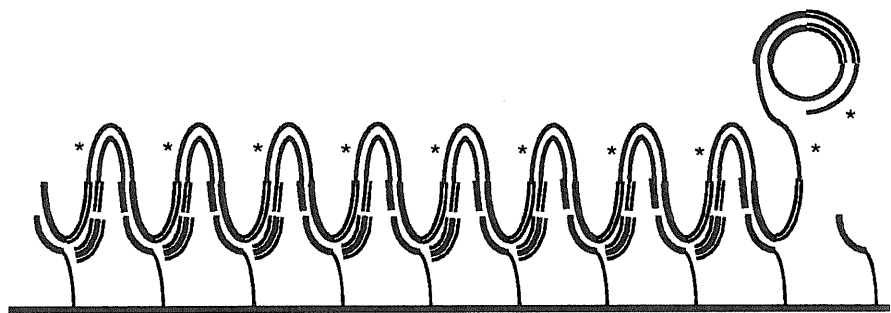
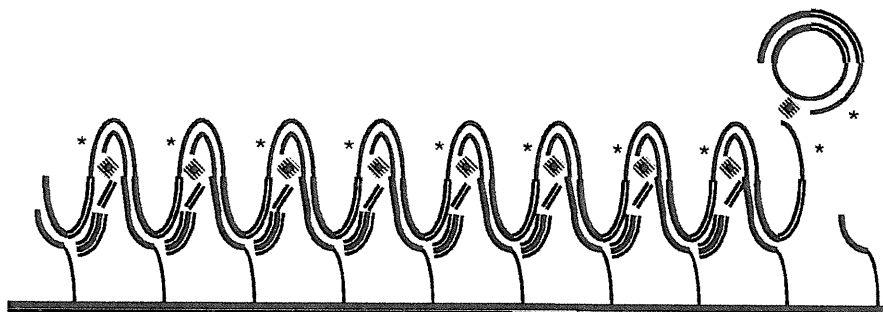
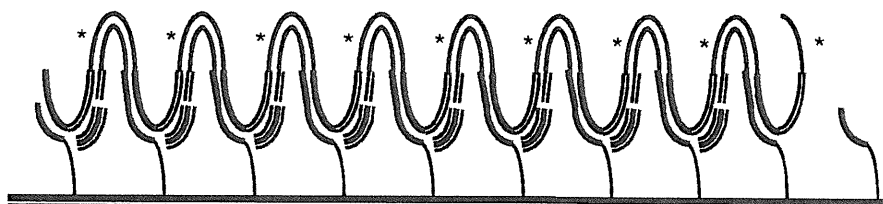


Figure 6 (continued)

- H. Unblock primer on solid support. Using polymerase with 5'-3' nuclease activity, extend tethered primer on solid support, while digesting product from sequencing by synthesis.



- I. Polymerase extends strands to form secondary extension products. Extension continues until blocked by PNA or blocking oligonucleotide. This generates uni-length copies of the template.



- J. The primary extension product and circular template are denatured and washed away. The resultant copies of the template are suitable for sequencing of the template strand.

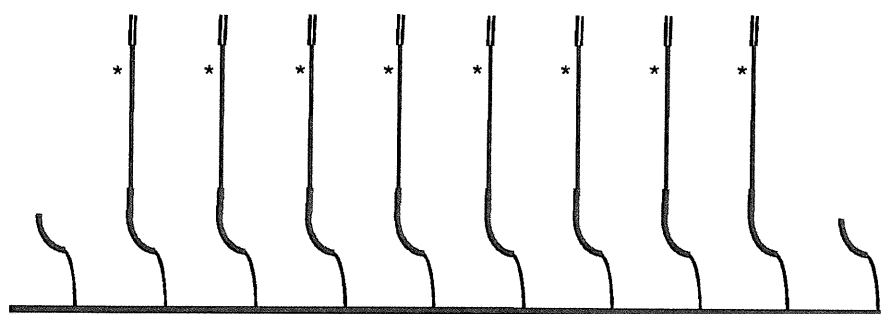
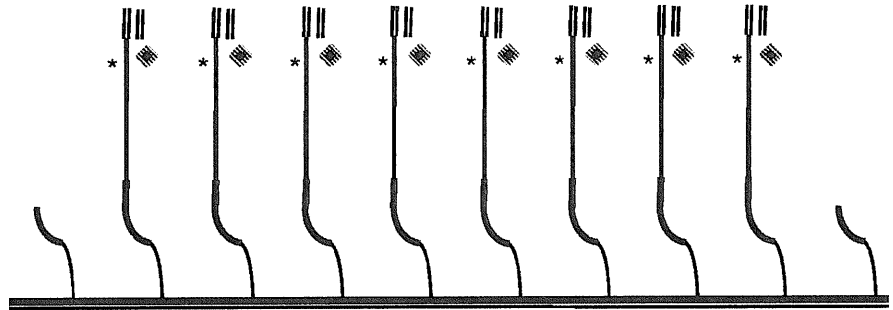
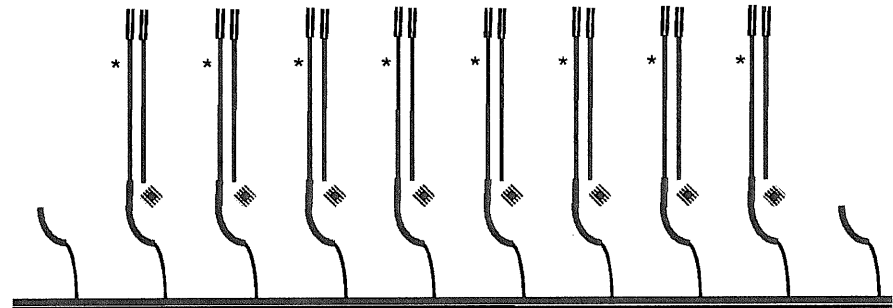


Figure 6 (continued)

- K. Hybridize sequencing primer to uni-length template strands.



- L. Sequence secondary extension product (i.e., genomic DNA strand) using sequencing by synthesis to identify mutation.



- M. Sequencing continues until extension products reach end of tethered primers.

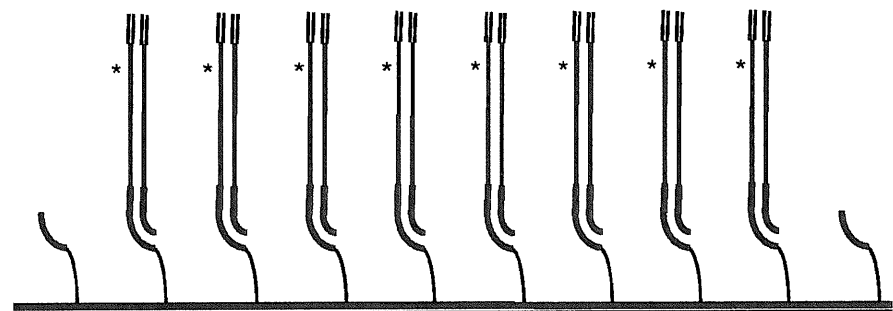
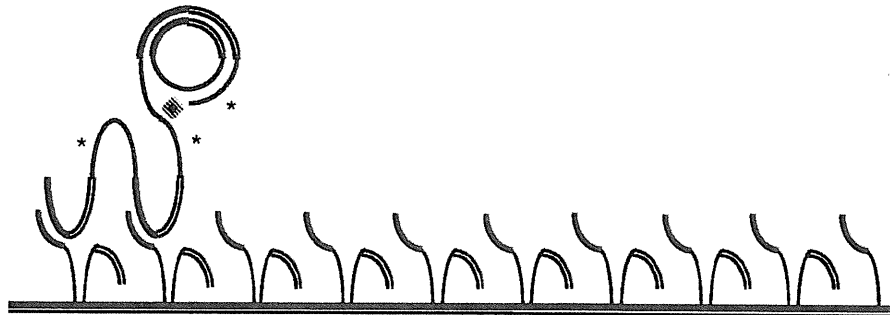


Figure 6 (continued)

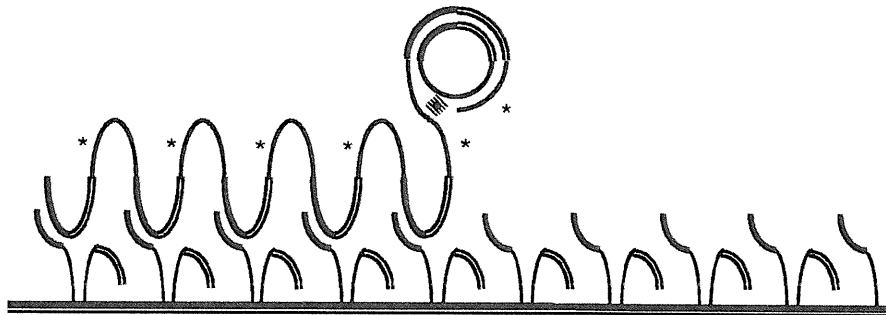
- A. Amplification of circular target for capture on a solid support with dual primers. Phi29 DNA polymerase extends on circle template to generate primary extension products.



- B. The primary extension product is hybridized to 3' blocked first primers on a solid support.



- C. As rolling circle amplification generates a growing primary extension product, it gets captured locally by hybridizing to adjacent first primers on the surface, creating a carpet-like structure.



- D. This approach assures that the hundreds to thousands of tandem complementary copies of the target are captured next to each other.

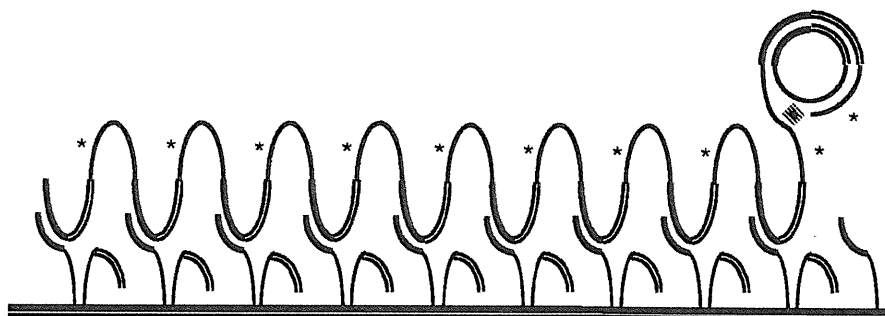
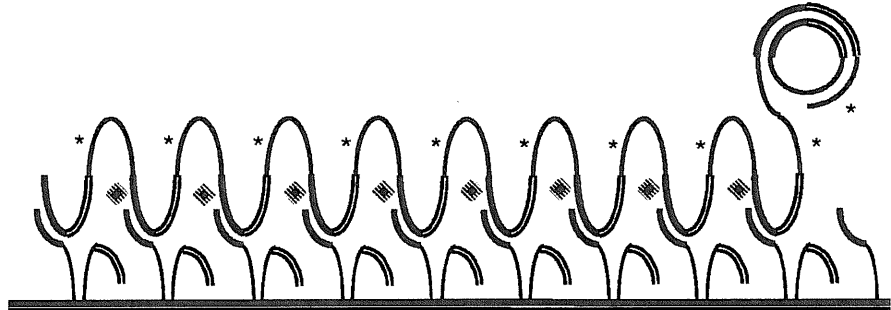
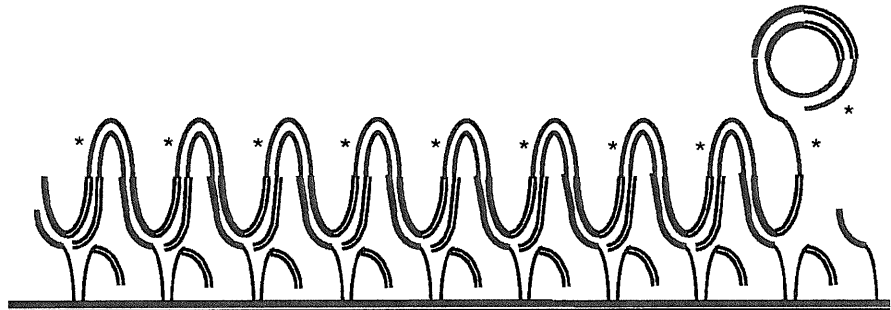


Figure 7

- E. The blocking group is removed from the first primers hybridized to template.



- F. The extension product is copied at hundreds to thousands of positions using polymerase lacking 5' - 3' nuclease activity. This generates uni-length secondary extension products.



- G. The primary extension product and circular template is denatured and washed away. The resultant secondary extension products are suitable for subsequent sequencing, as described in Figure 8.

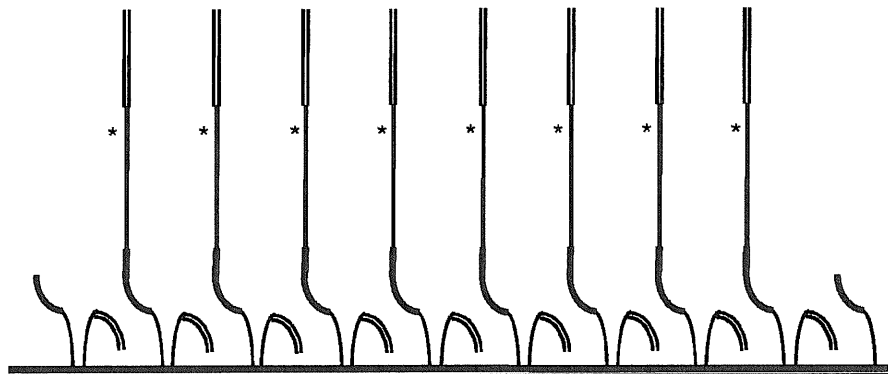
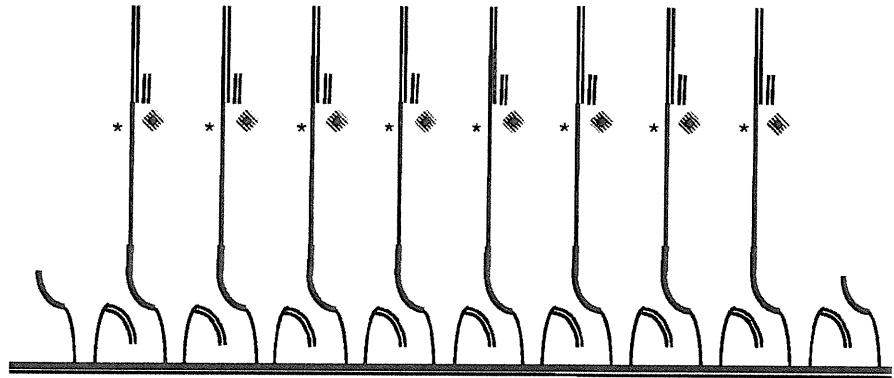
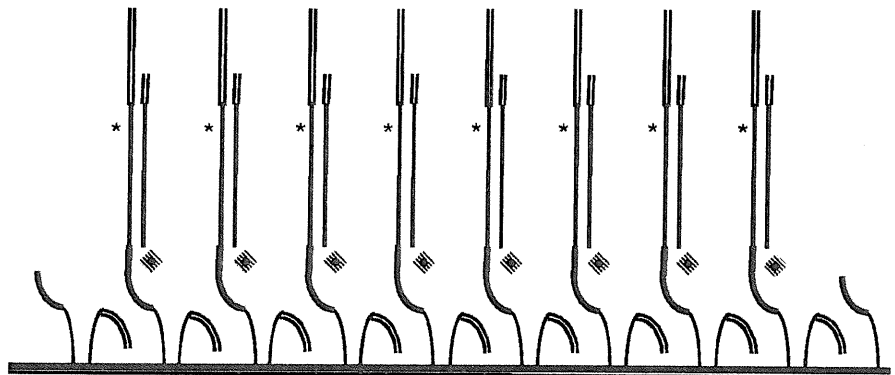


Figure 7 (continued)

- A. Sequencing of rolling circle amplified target captured on a solid support with dual primers. Hybridize sequencing primer to uni-length template strands.



- B. Sequence target DNA strand using sequencing by synthesis to identify mutation.



- C. Sequencing continues until extension products reach end of tethered primers.

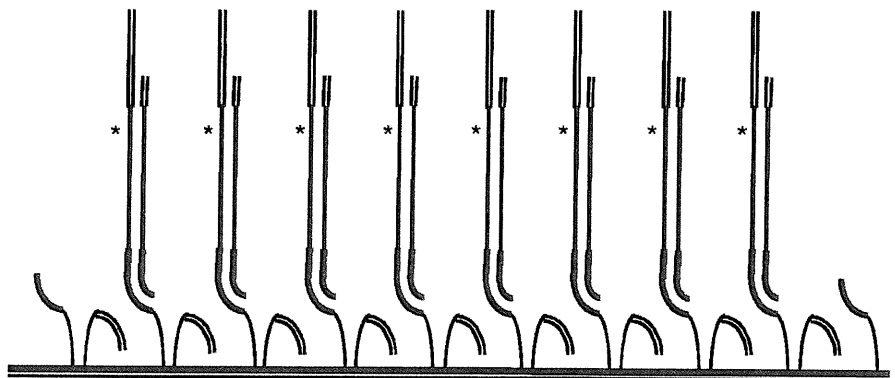
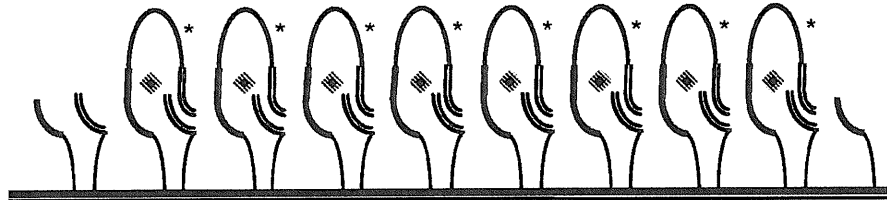
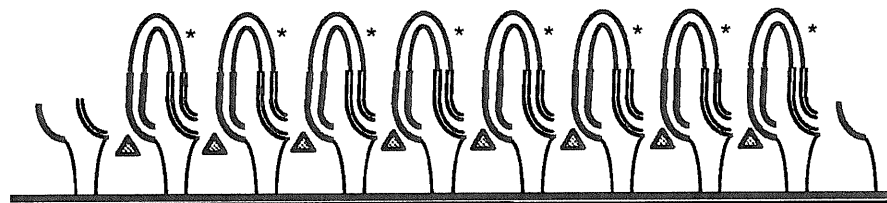


Figure 8

- D. Sequence-by-synthesis products are denatured from the tethered extension products. Single-stranded secondary extension products hybridize to the second primers on the solid support.



- E. The secondary extension products are copied by extending the second primers to generate full-length copies of the secondary extension products. The secondary extension products are cleaved.



- F. The cleaved secondary extension products are denatured and washed away. The resultant copies (tertiary extension products) are suitable for subsequent sequencing.

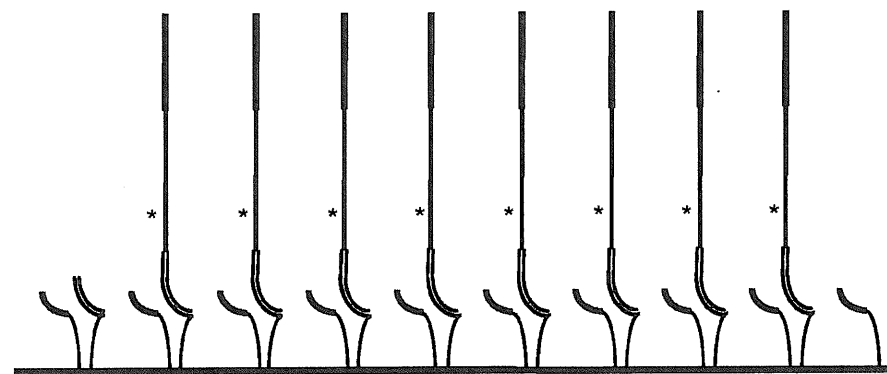
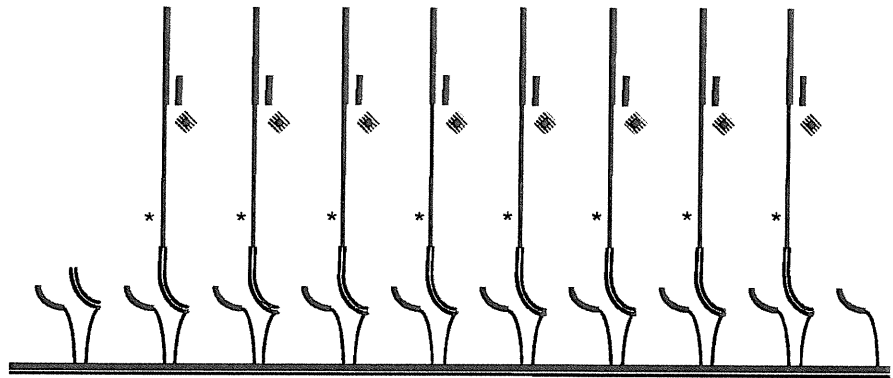
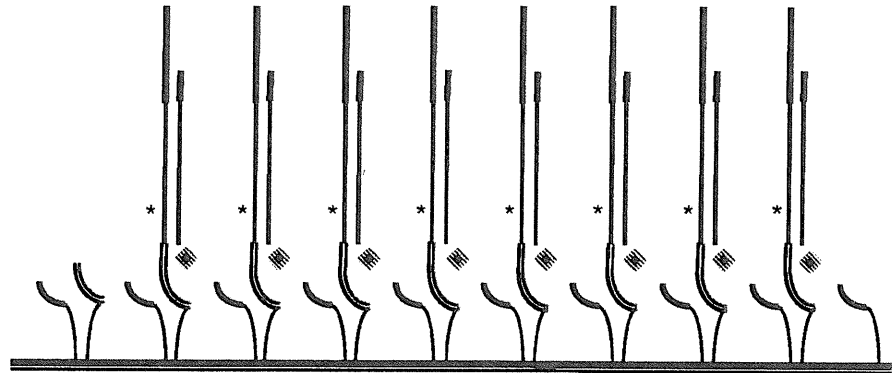


Figure 8 (continued)

- G. Sequencing of rolling circle amplified target captured on a solid support with dual primers (continued). Hybridize sequencing primer to uni-length complementary template strands.



- H. Sequence complementary target DNA strand using sequencing by synthesis to identify mutation.



- I. Sequencing continues until extension products reach end of tethered primers.

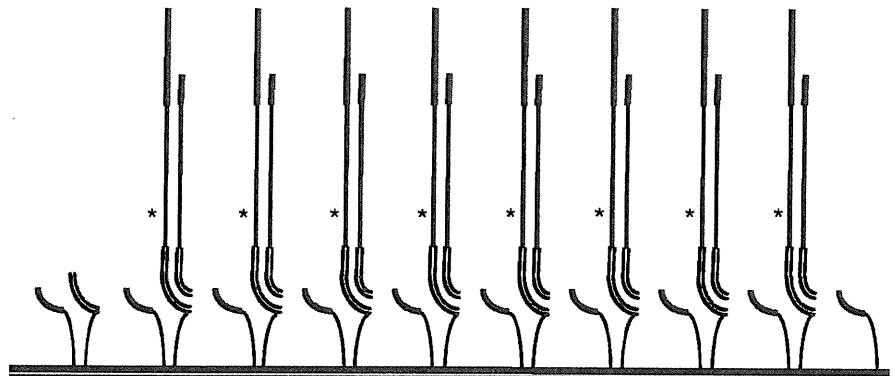
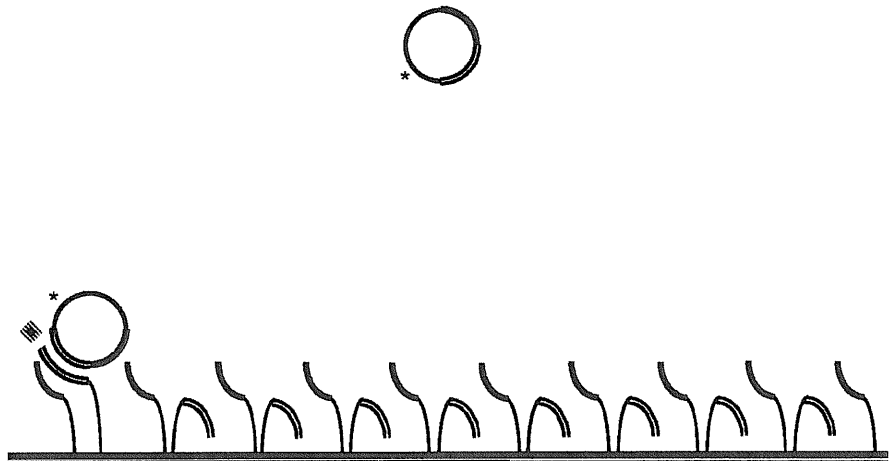
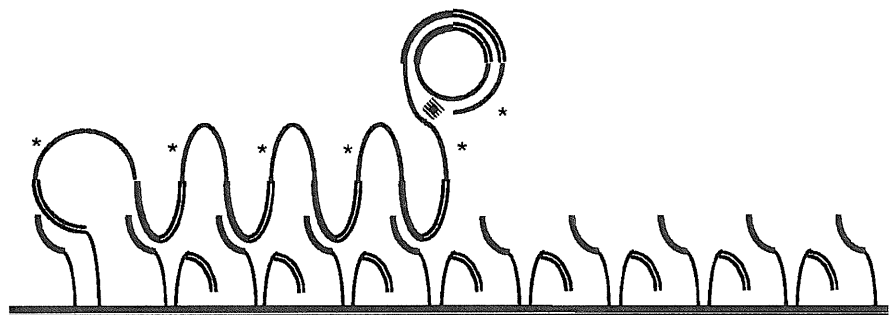


Figure 8 (continued)

- A. The circular target is hybridized to an unblocked second oligonucleotide primer, which is extended by Phi29 DNA polymerase to generate a tethered primary extension product.



- B. The single-stranded primary extension product generated by rolling circle amplification is captured locally by hybridization to adjacent first oligonucleotide primers on the surface, creating a carpet-like structure.



- C. This approach assures that the hundreds to thousands of tandem complementary copies of the target genomic sequence are captured next to each other.

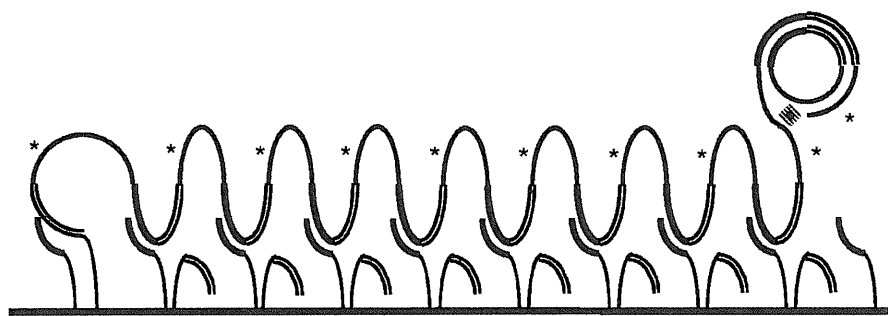
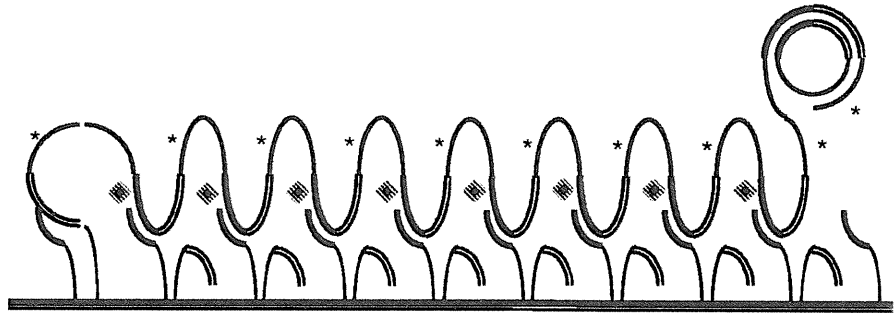
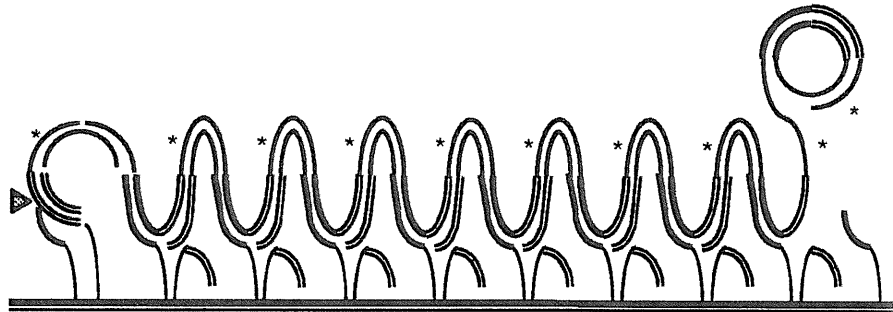


Figure 9

- D. The 3' blocking group on the first oligonucleotide primer is removed .



- E. The primary extension product is copied at hundreds to thousands of positions using polymerase lacking 5' → 3' nuclease activity. This generates uni-length secondary extension products. The primary extension product is cleaved from the solid support.



- F. The primary extension product and circular template is denatured and washed away. The resultant copies of the template are suitable for subsequent sequencing, as described in Figure 8.

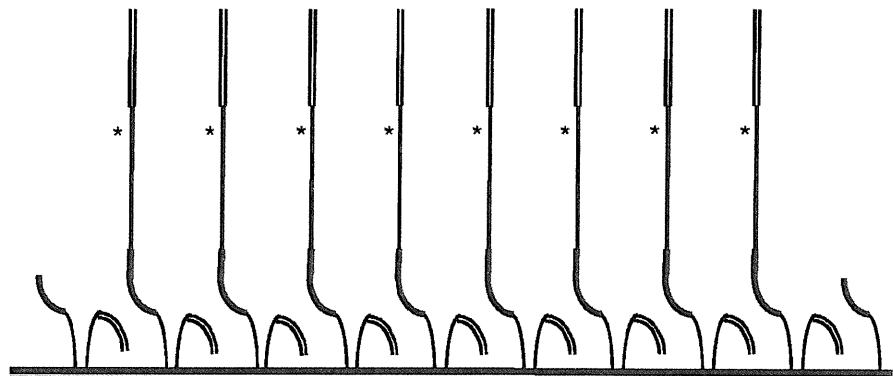
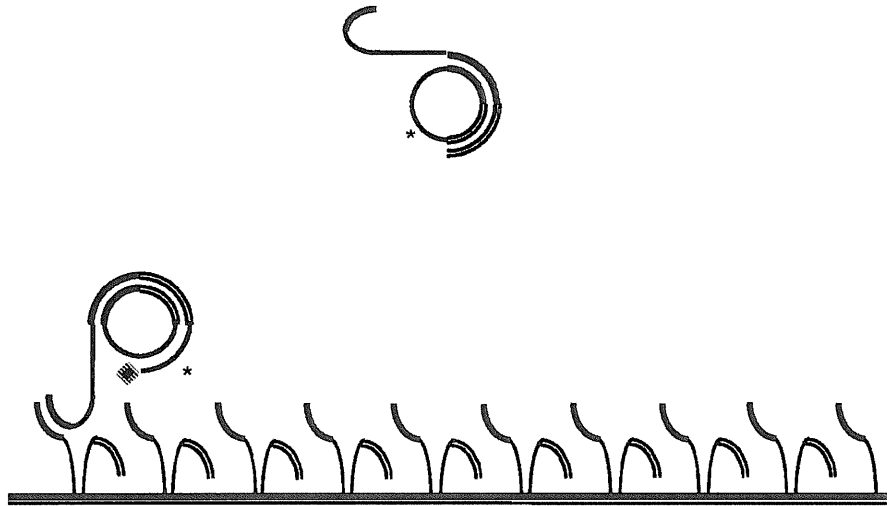
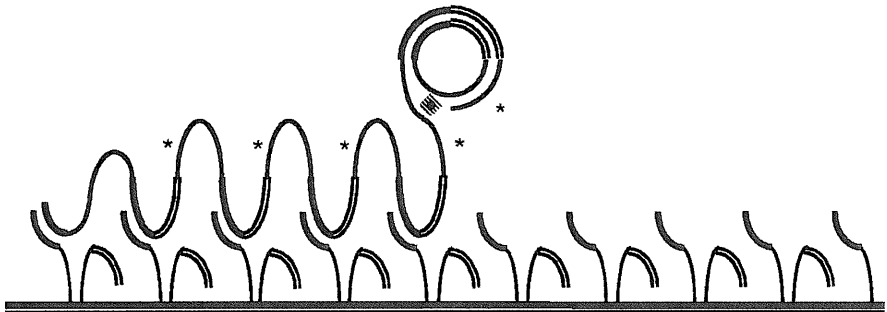


Figure 9 (continued)

- A. Amplification of circular target for capture on a solid support with dual primers. The amplification primer hybridized to the circular construct contains a further portion, that in this case, is capable of hybridizing to a 3' blocked first primer on the solid support.



- B. As rolling circle amplification of the tethered circular construct generates a single stranded primary extension product that is captured locally by hybridizing to adjacent first primers on the surface, creating a carpet-like structure.



- C. This approach assures that the hundreds to thousands of tandem copies of the target are captured next to each other.

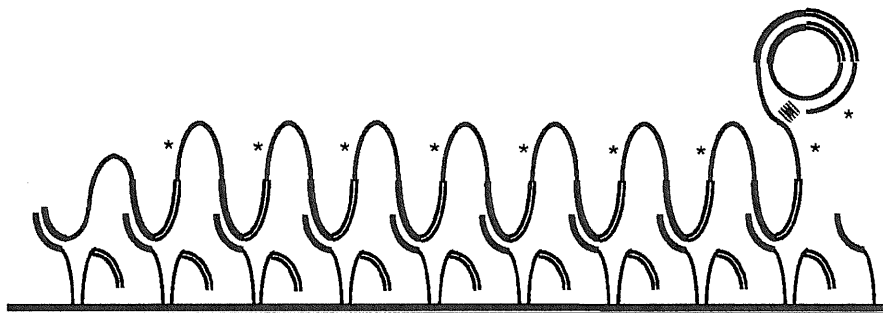
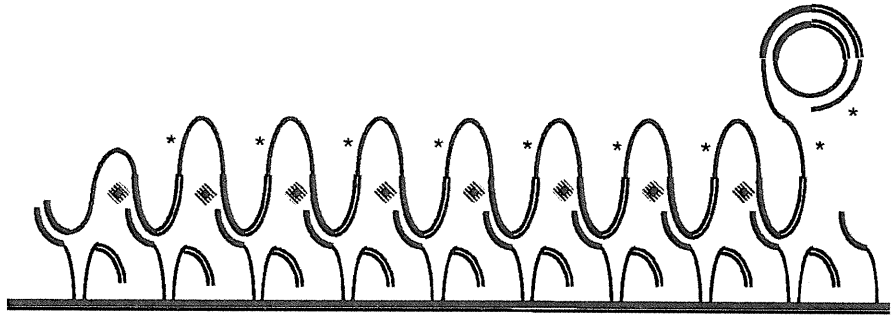
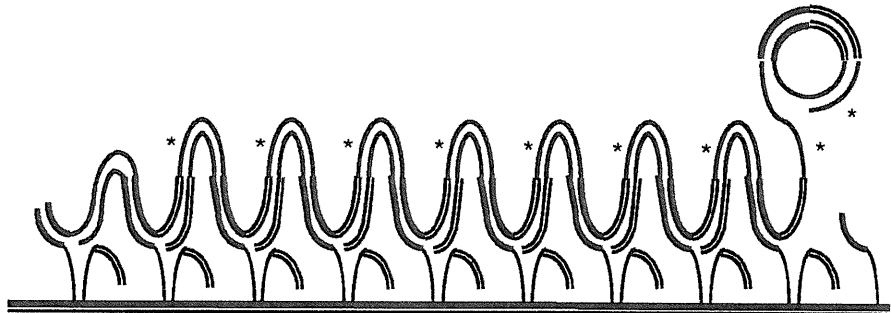


Figure 10

- D. The blocking group is removed from primers hybridized to template.



- E. The extension product is copied at hundreds to thousands of positions using polymerase lacking 5' - 3' nuclease activity. This generates uni-length copies of the template.



- F. The original extension product and circular template is denatured and washed away. The resultant copies of the template are suitable for subsequent sequencing, as described in Figure 8.

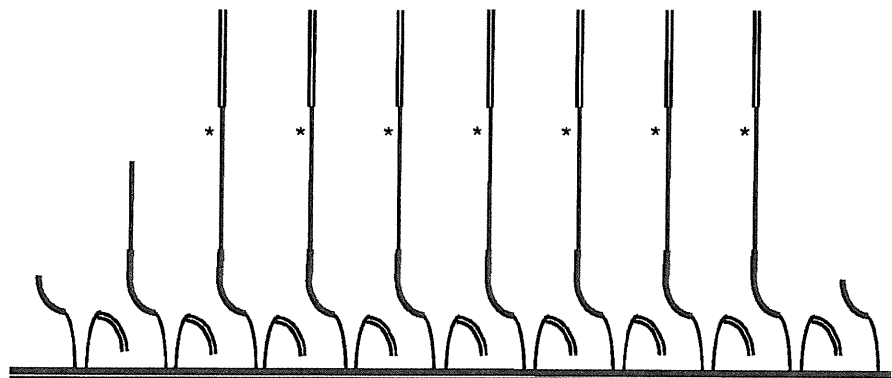
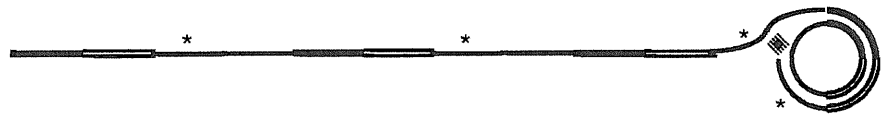
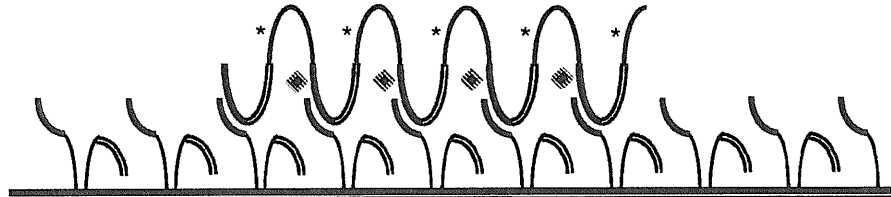


Figure 10 (continued)

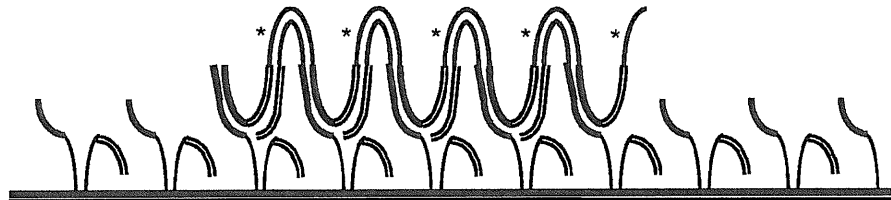
- A. Amplification of circular target for capture on a solid support with dual primers. DNA polymerase extends amplification primer on circle template to generate short primary extension product. Polymerase and extension product are denatured from circular template.



- B. The extension product is hybridized to the first primers on a solid support.



- C. The extension product is copied multiple positions using polymerase lacking 5' - 3' nuclease activity. This generates secondary extension products.



- D. The primary extension product is denatured and washed away. The secondary extension products are templates suitable for subsequent amplification.

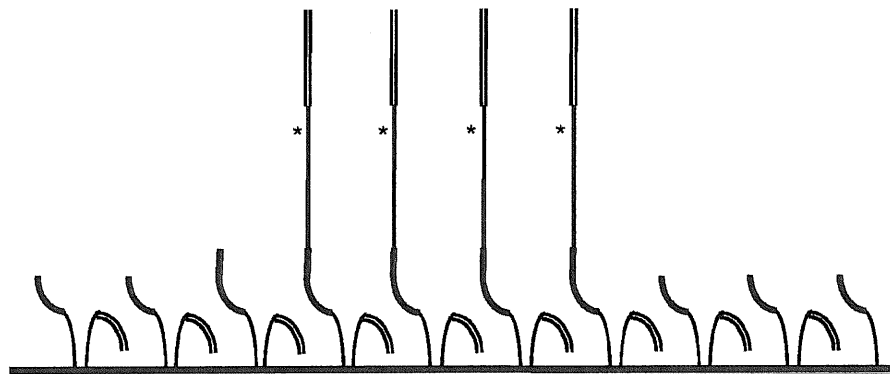
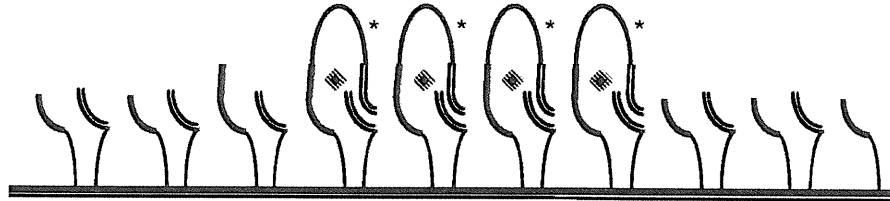
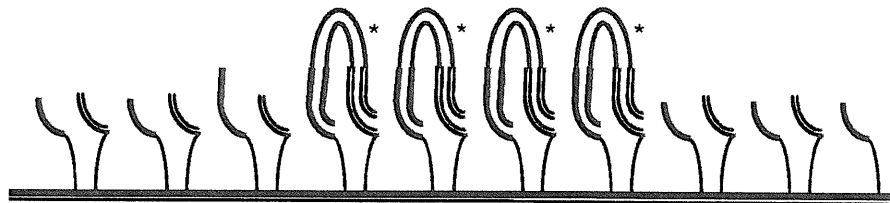


Figure 11

- E. Single-stranded secondary extension products hybridize to the second primer on the solid support.



- F. The second primers are polymerase extended to generates uni-length tertiary extension products.



- G. The extension products are denatured.

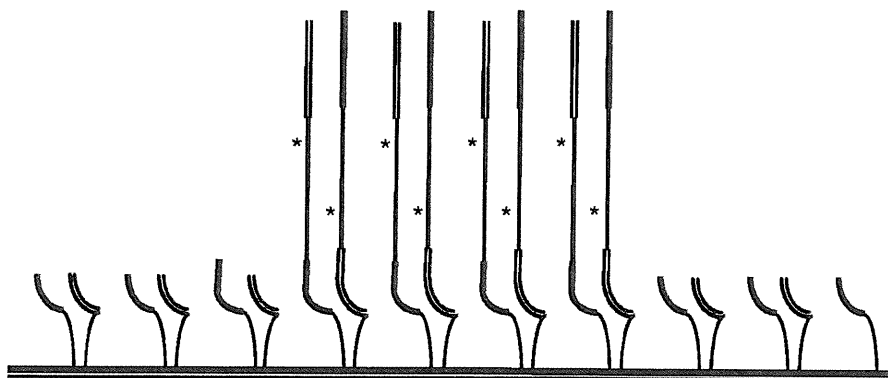
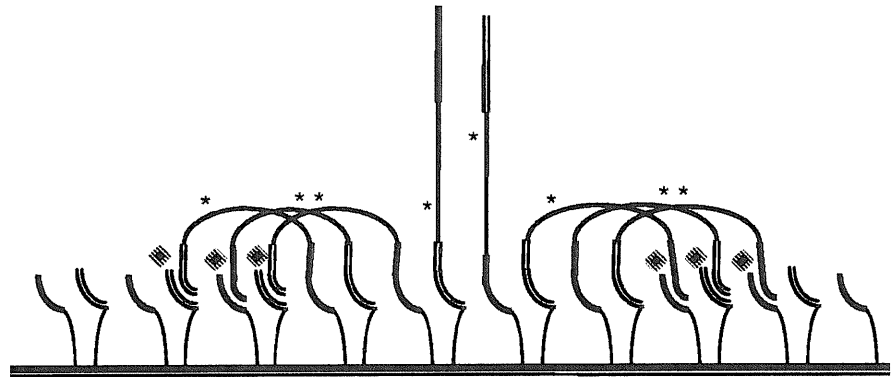
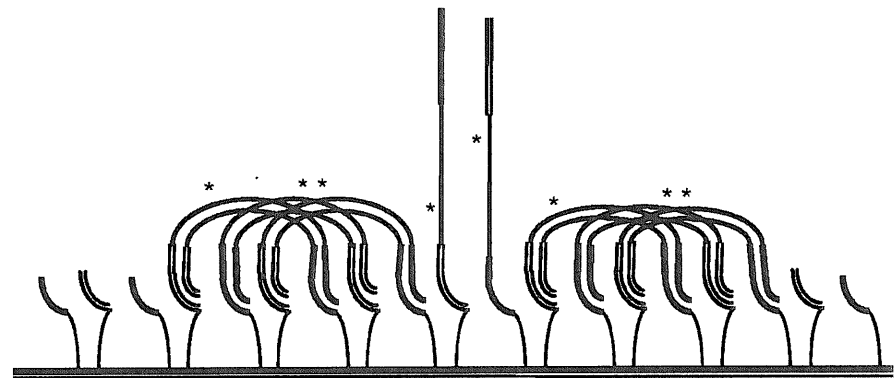


Figure 11 (continued)

- H. Newly denatured single-stranded extension products hybridize to complementary primers on the solid support.



- I. The first and second primers on the solid support are polymerase extended generating copies of the secondary and tertiary extension products.



- J. The extension products are denatured, and the process is repeated to generate enough template for sequencing by cleaving away template strands of one orientation, and proceeding as in Figure 8.

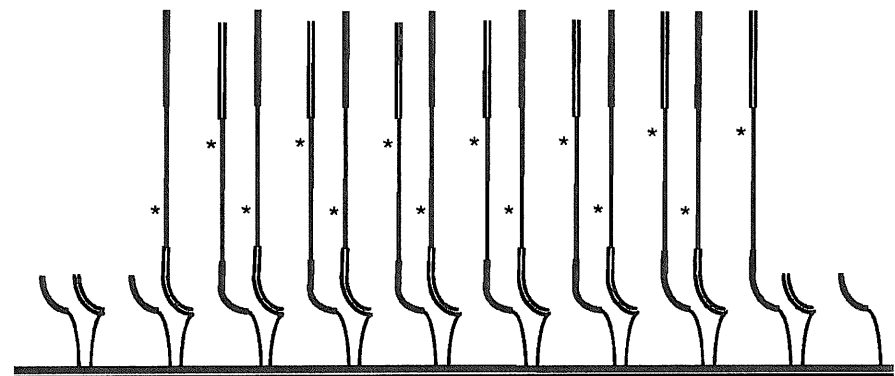


Figure 11 (continued)

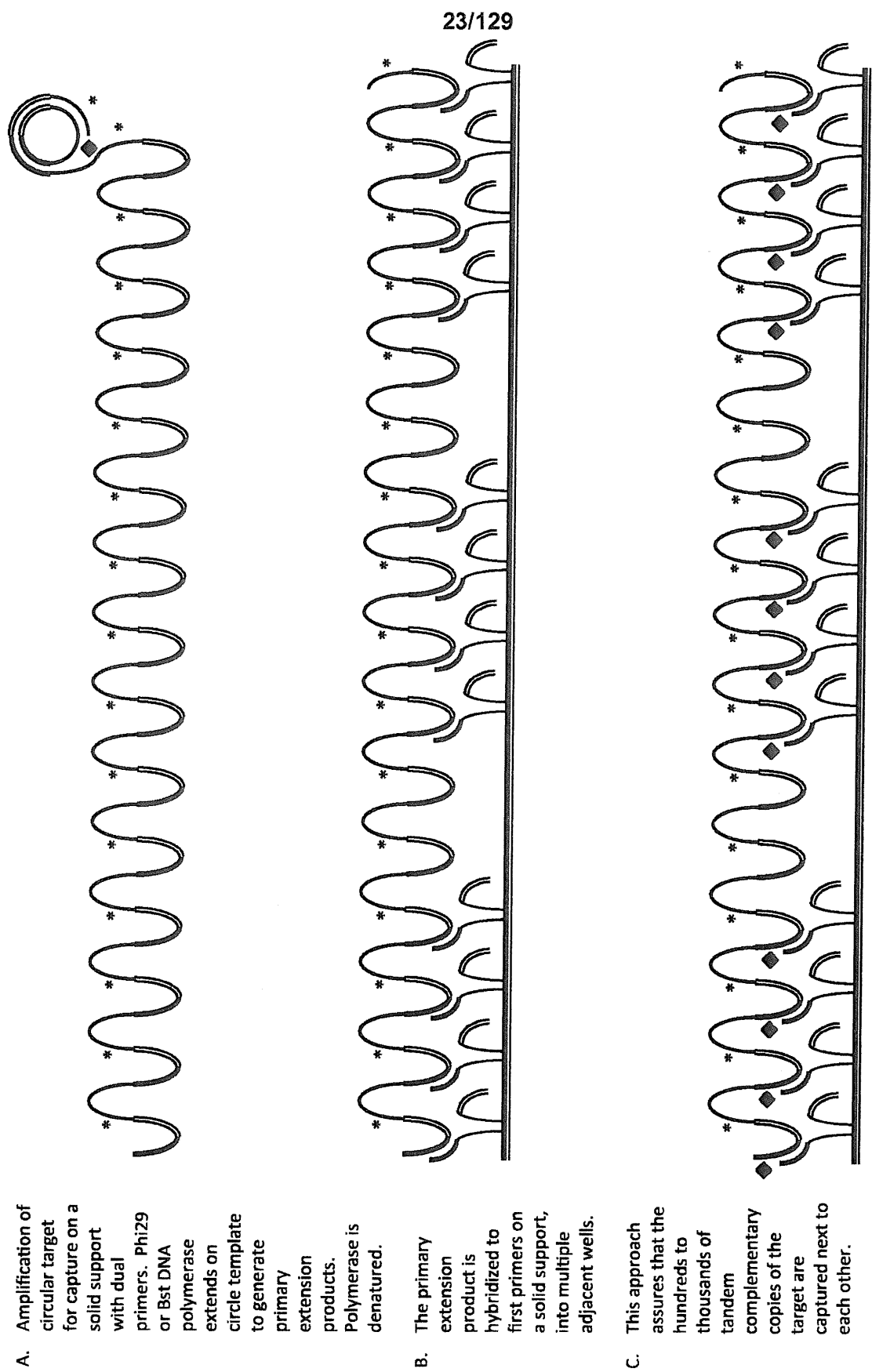


Figure 12

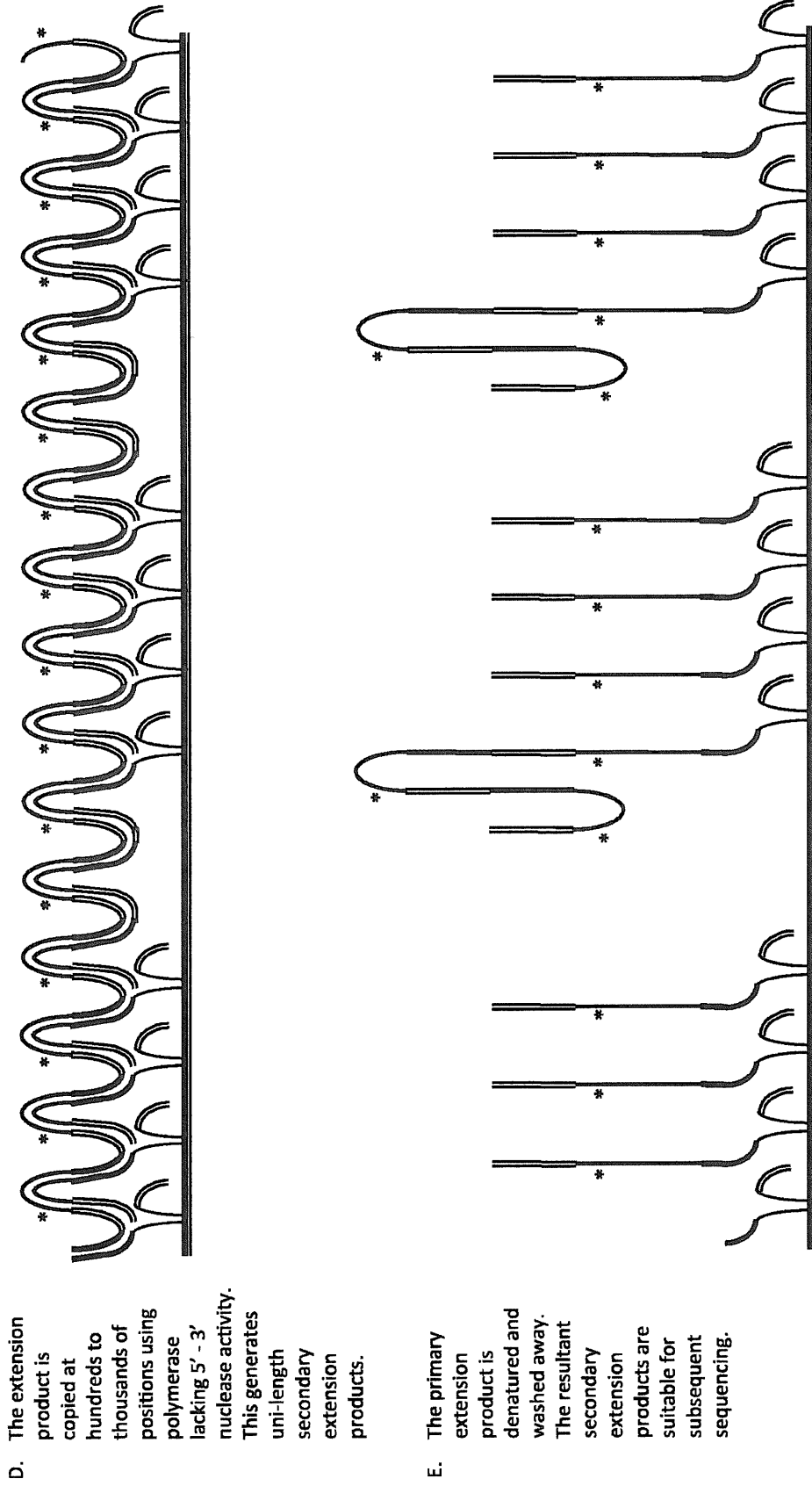


Figure 12 continued

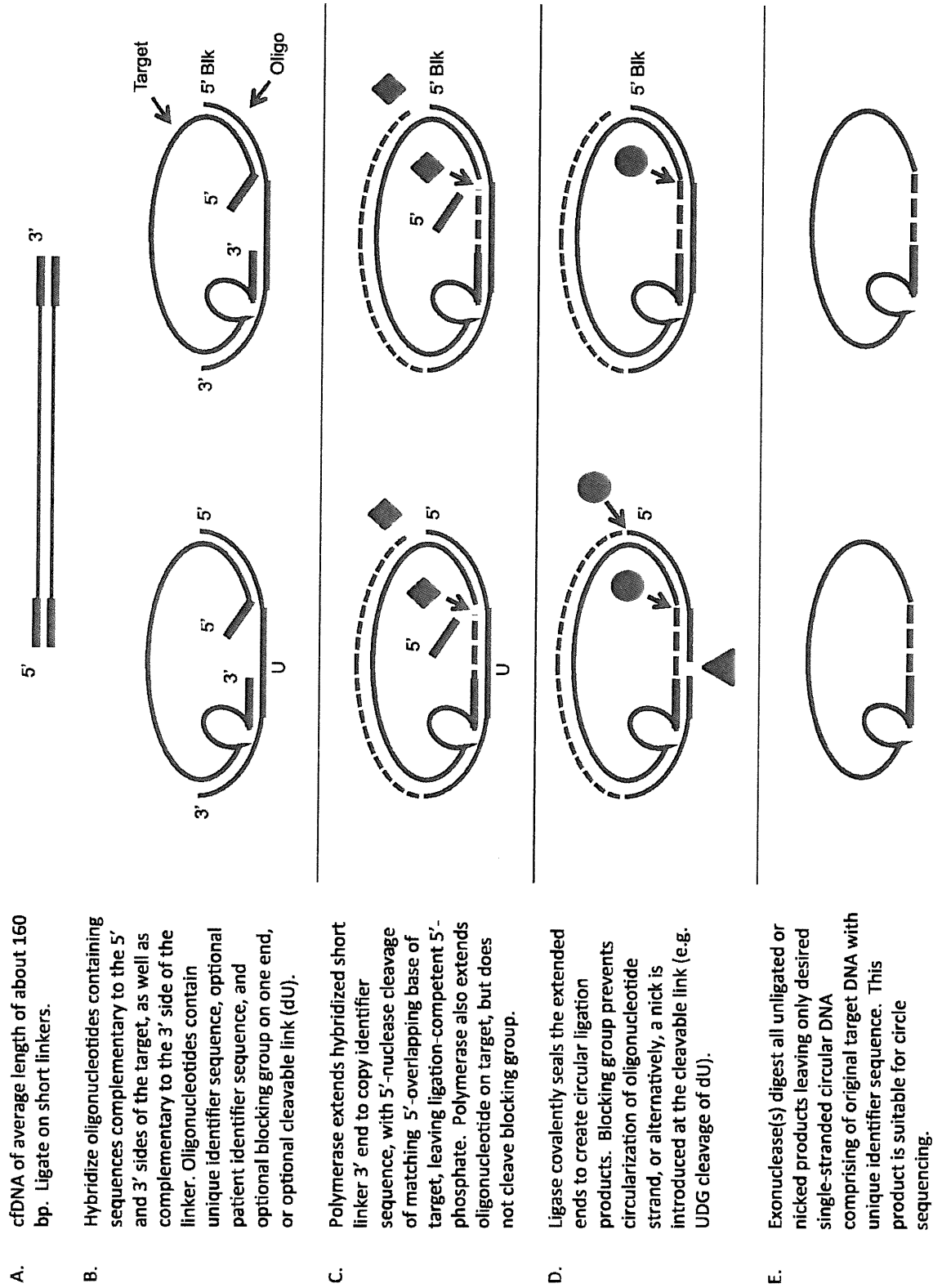


Figure 13

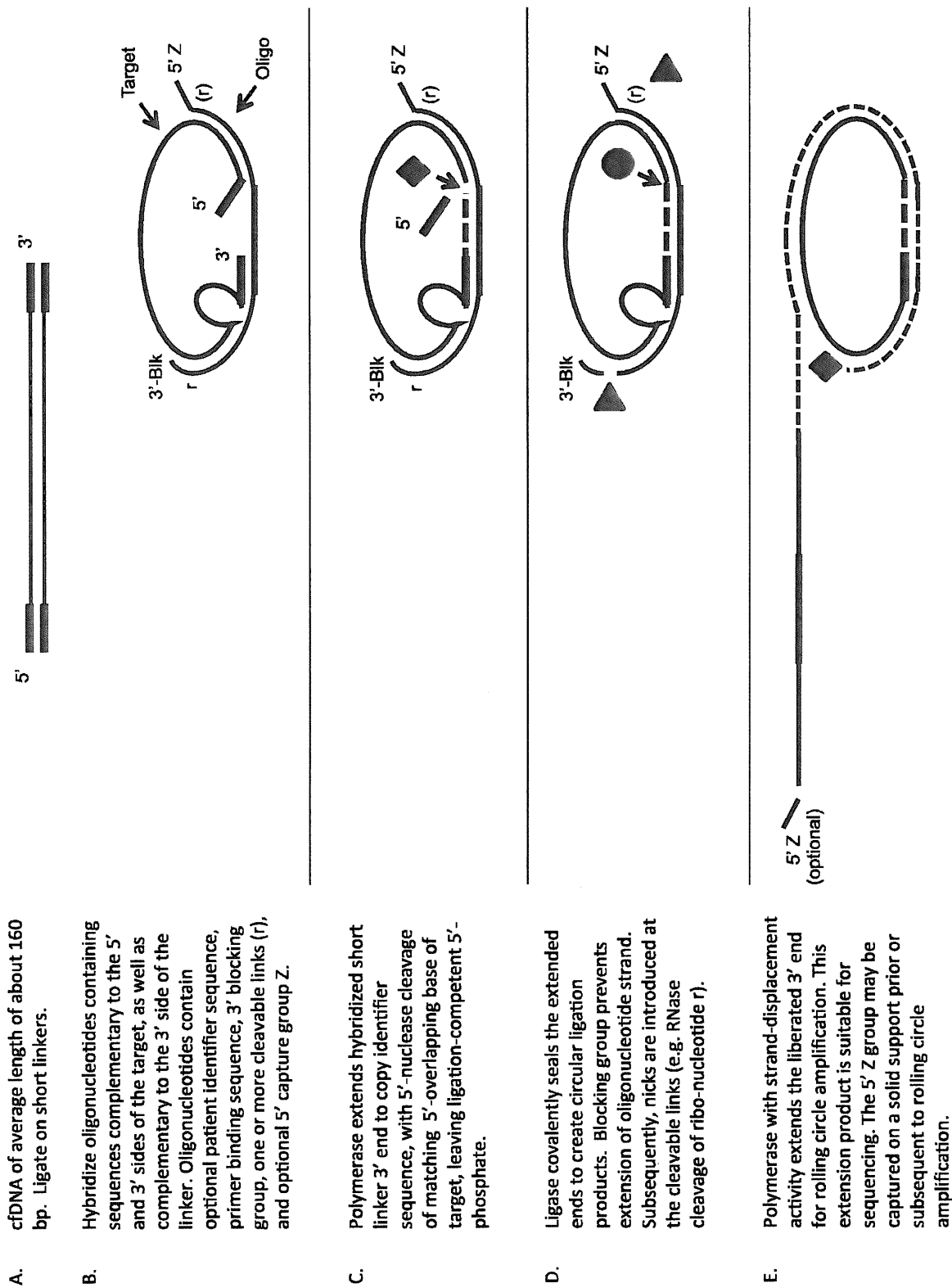


Figure 14

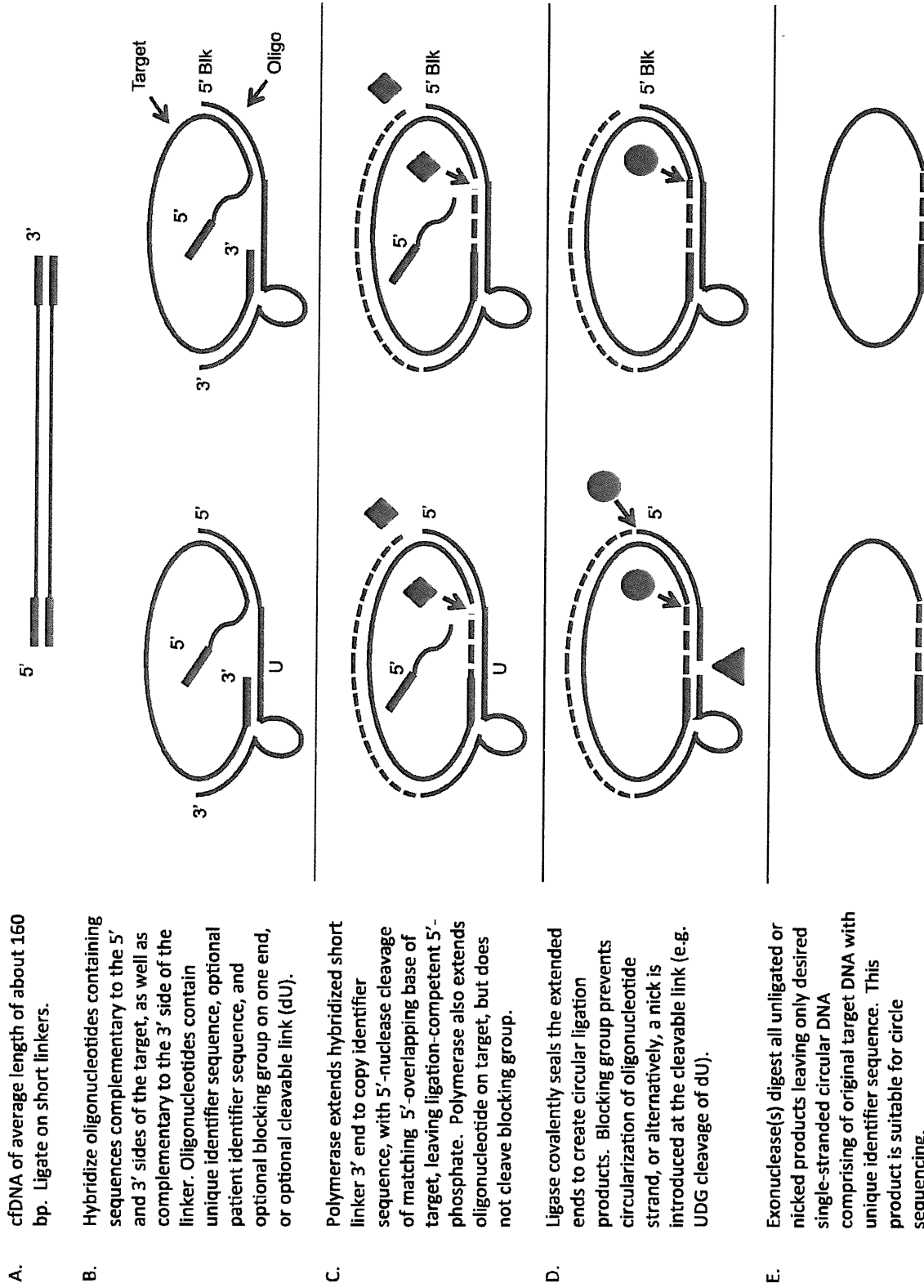


Figure 15

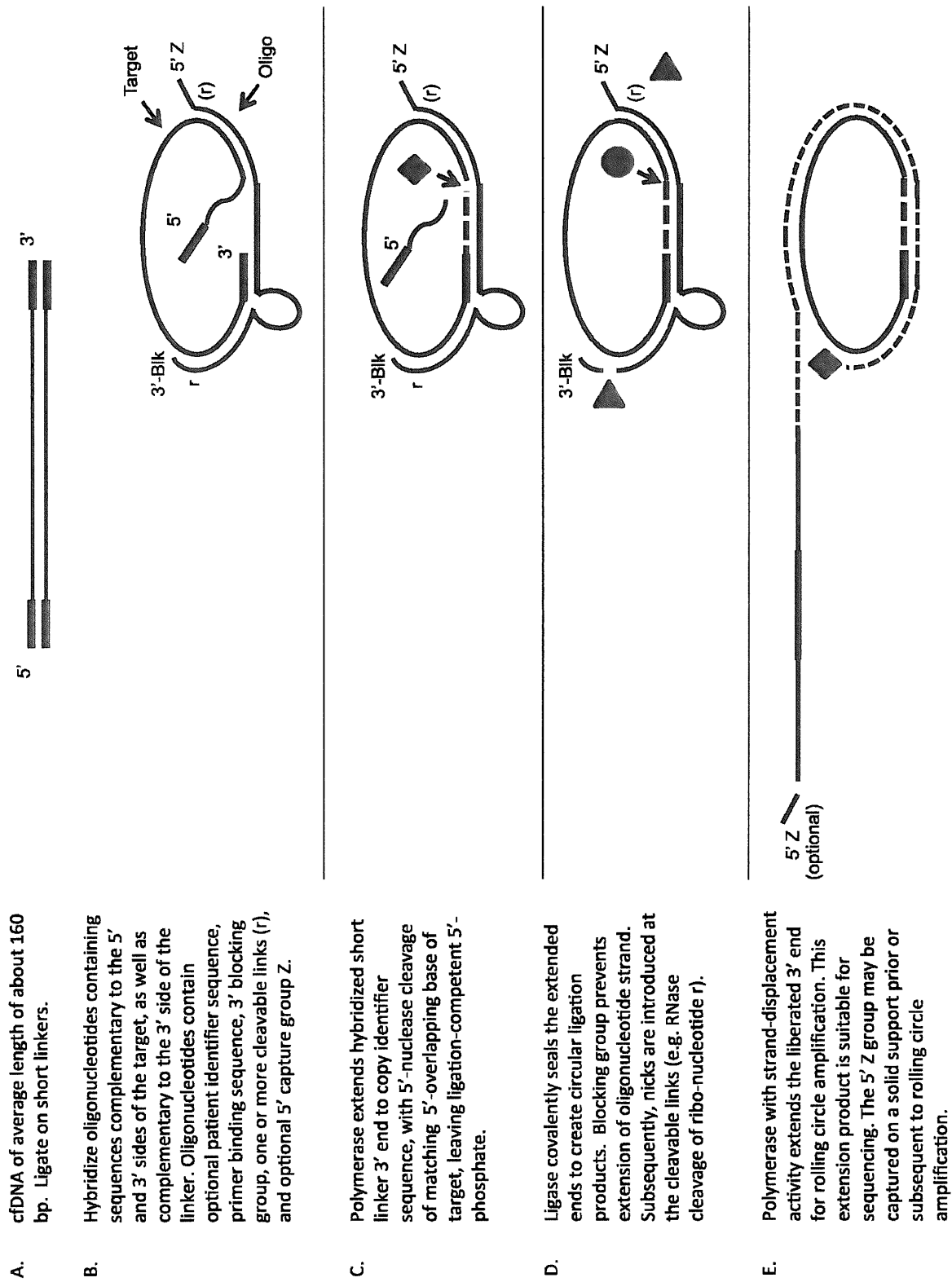


Figure 16

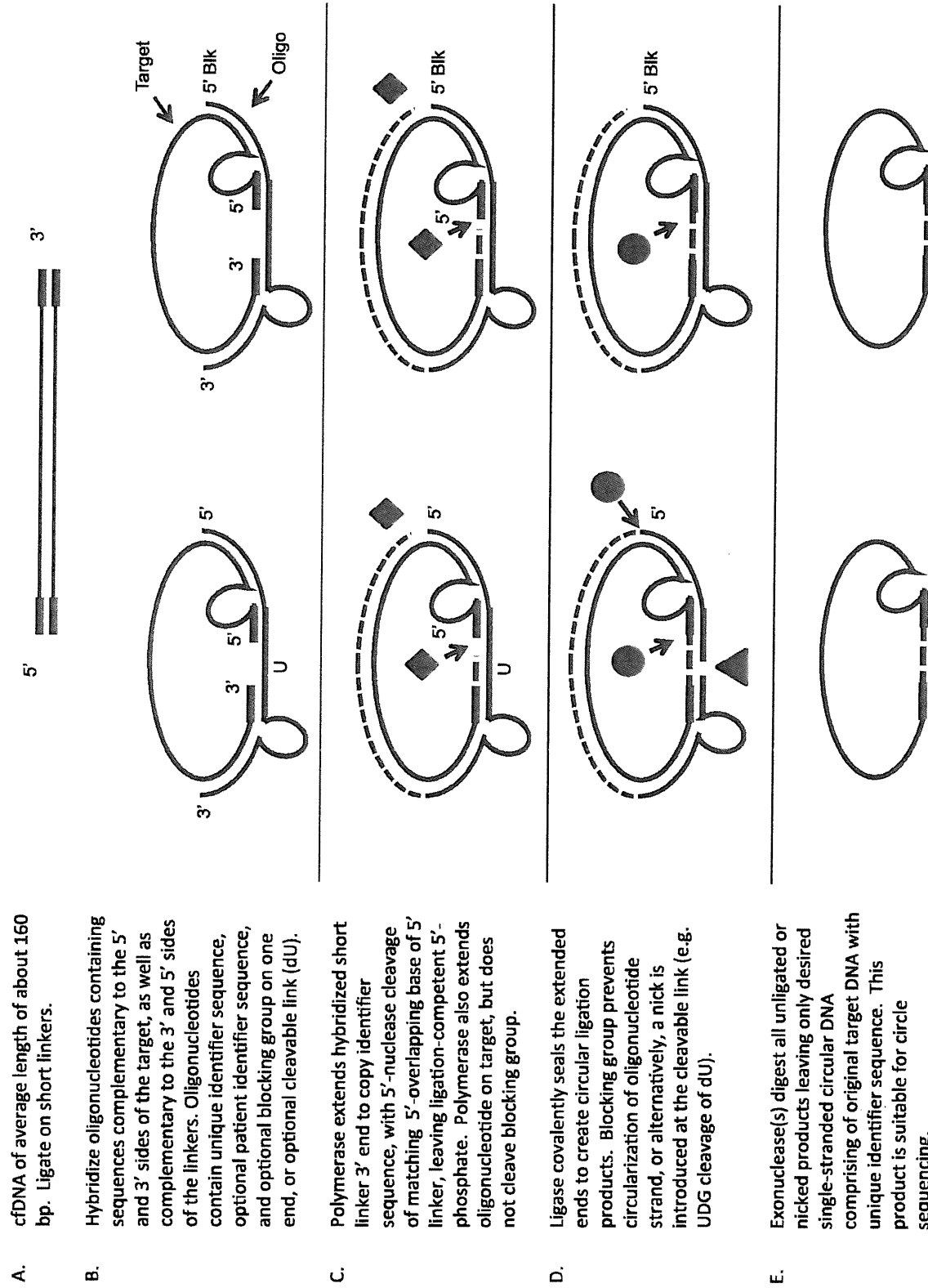


Figure 17

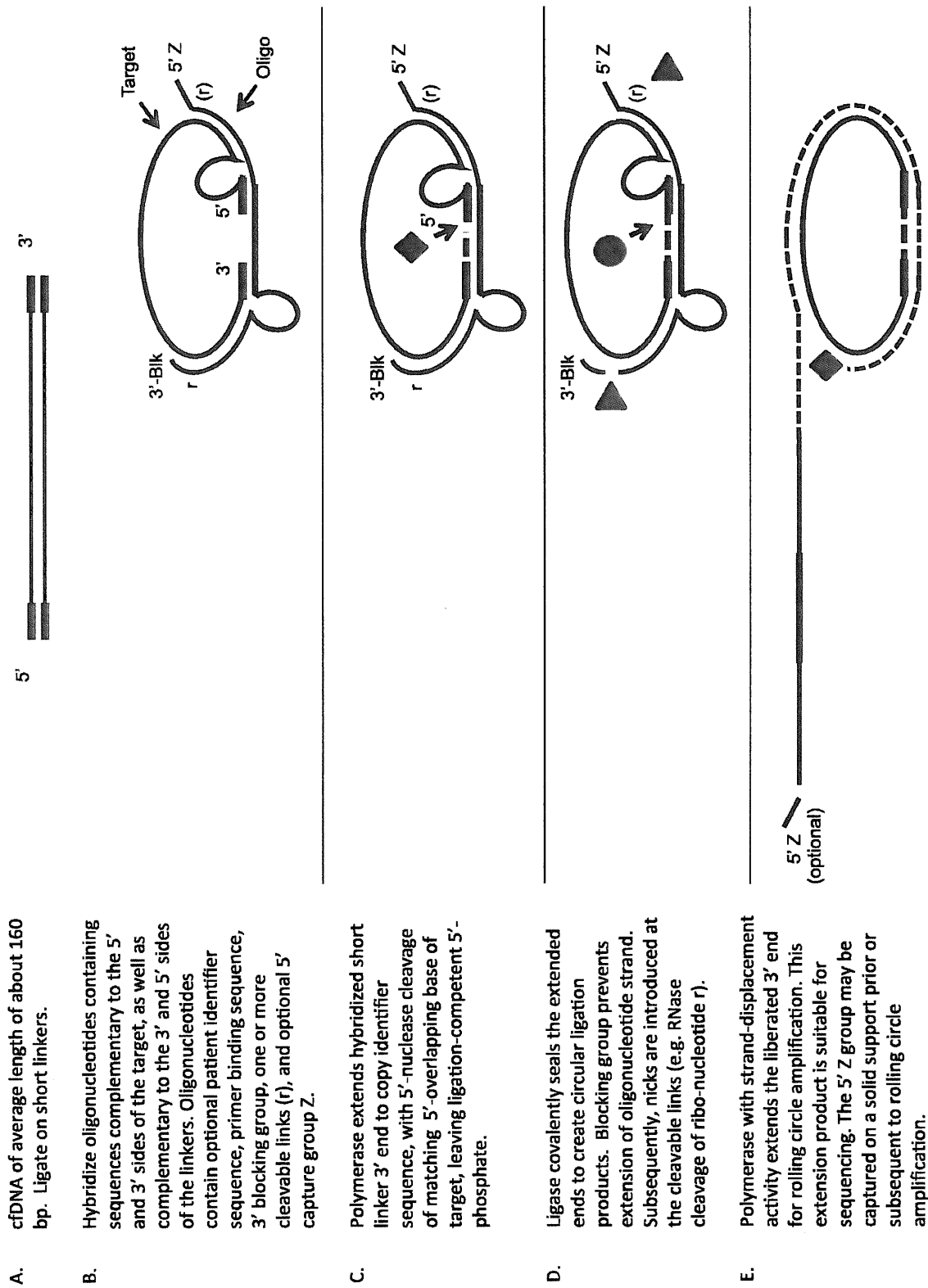


Figure 18

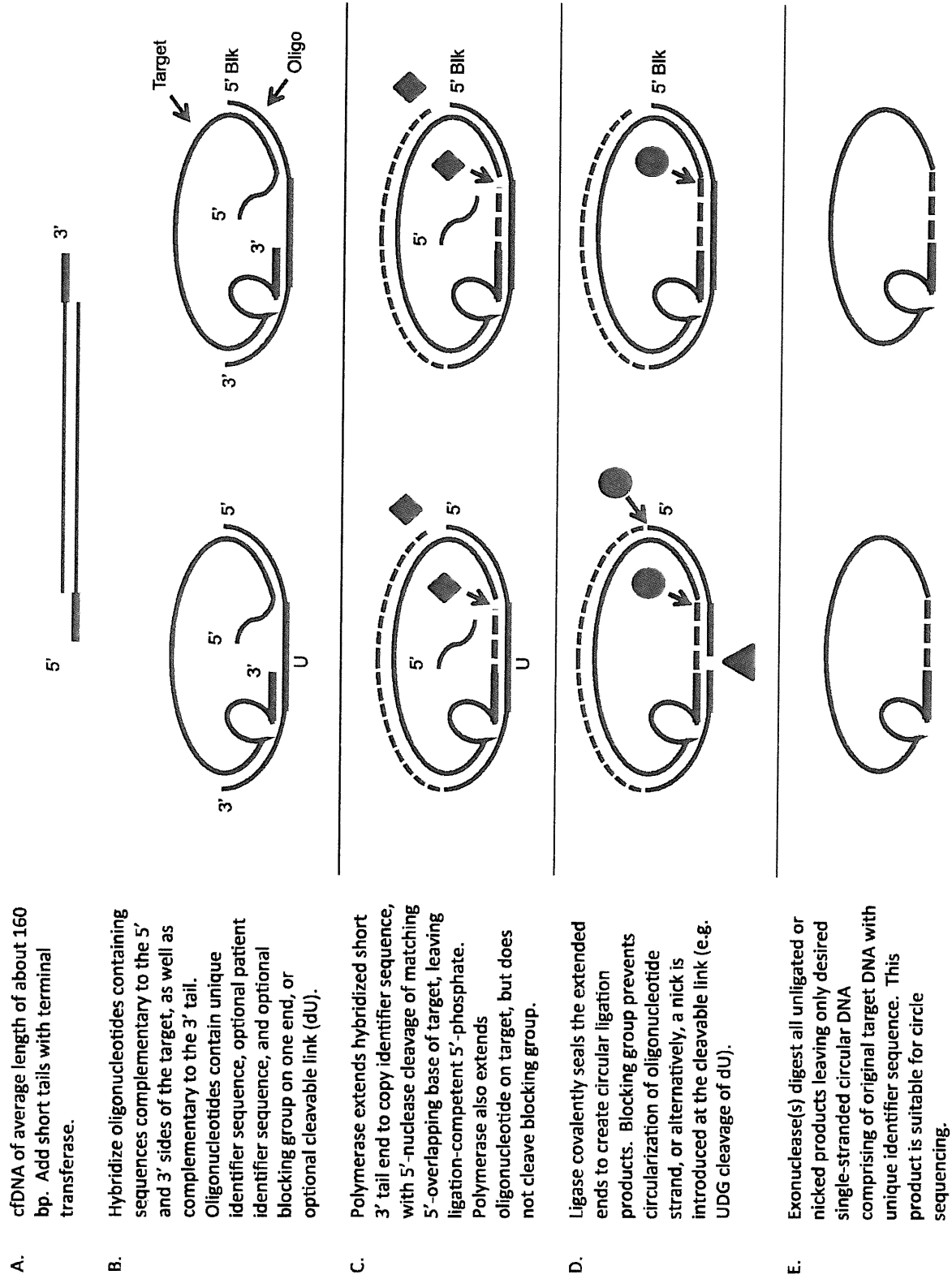


Figure 19

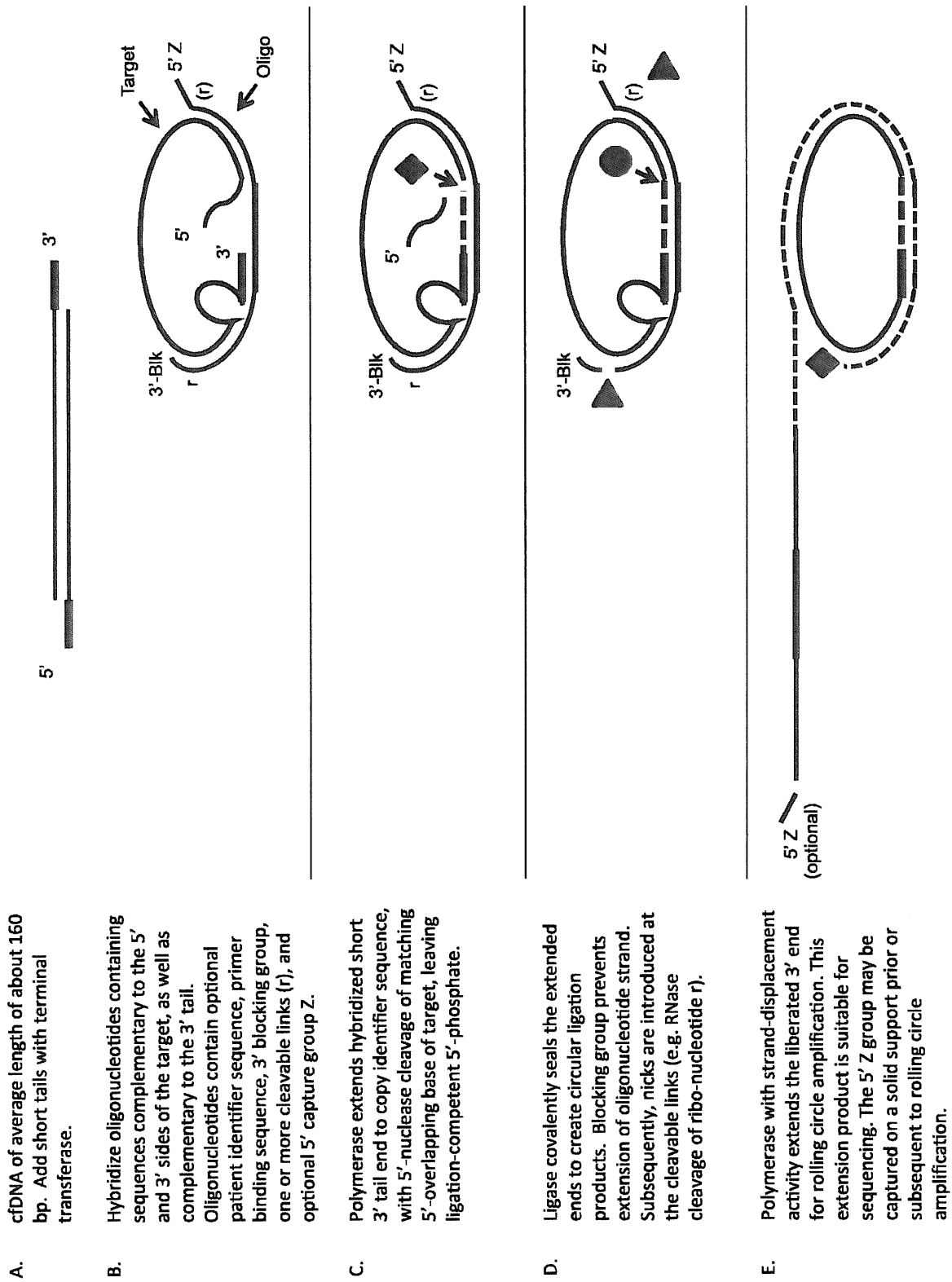


Figure 20

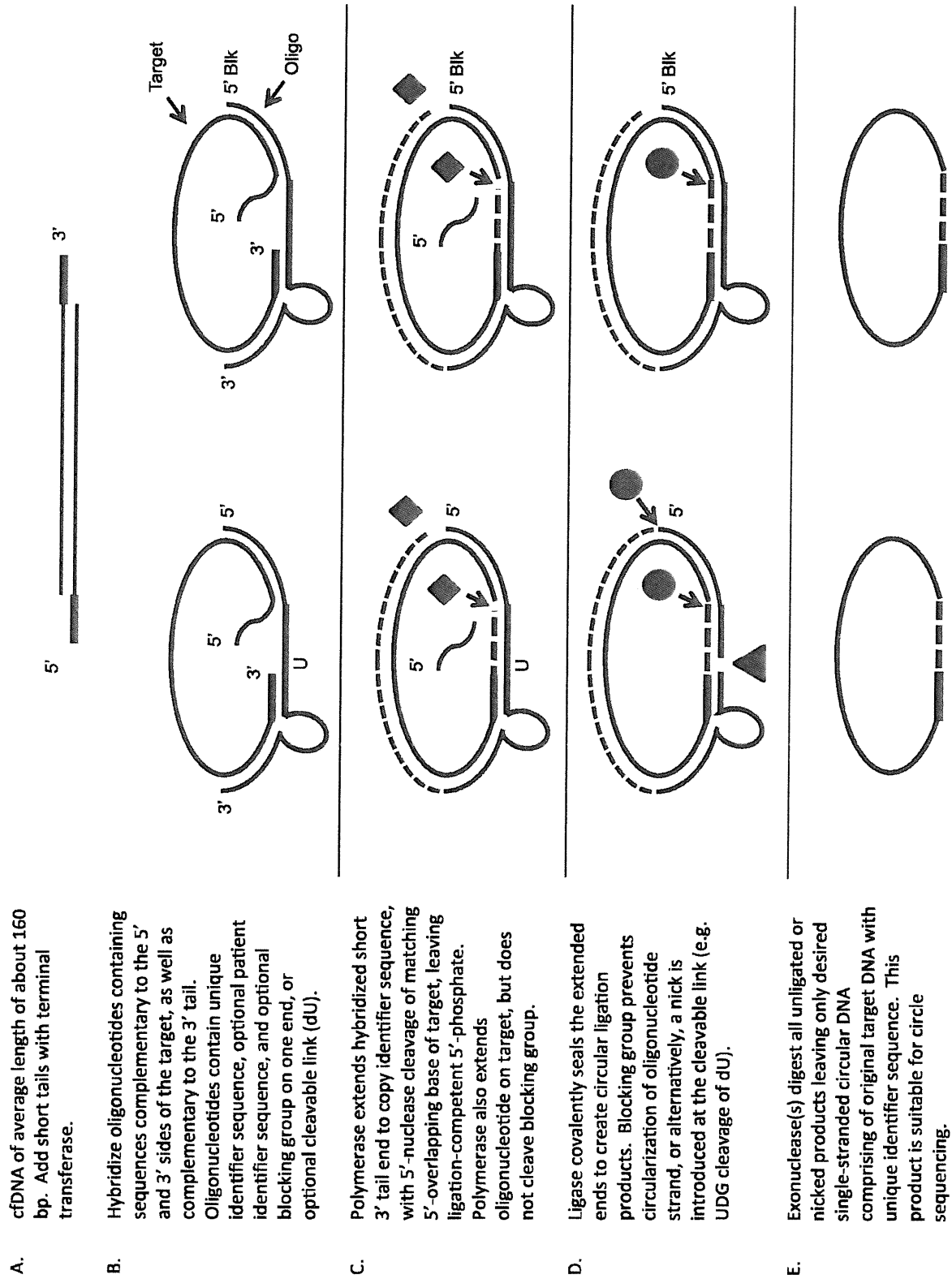


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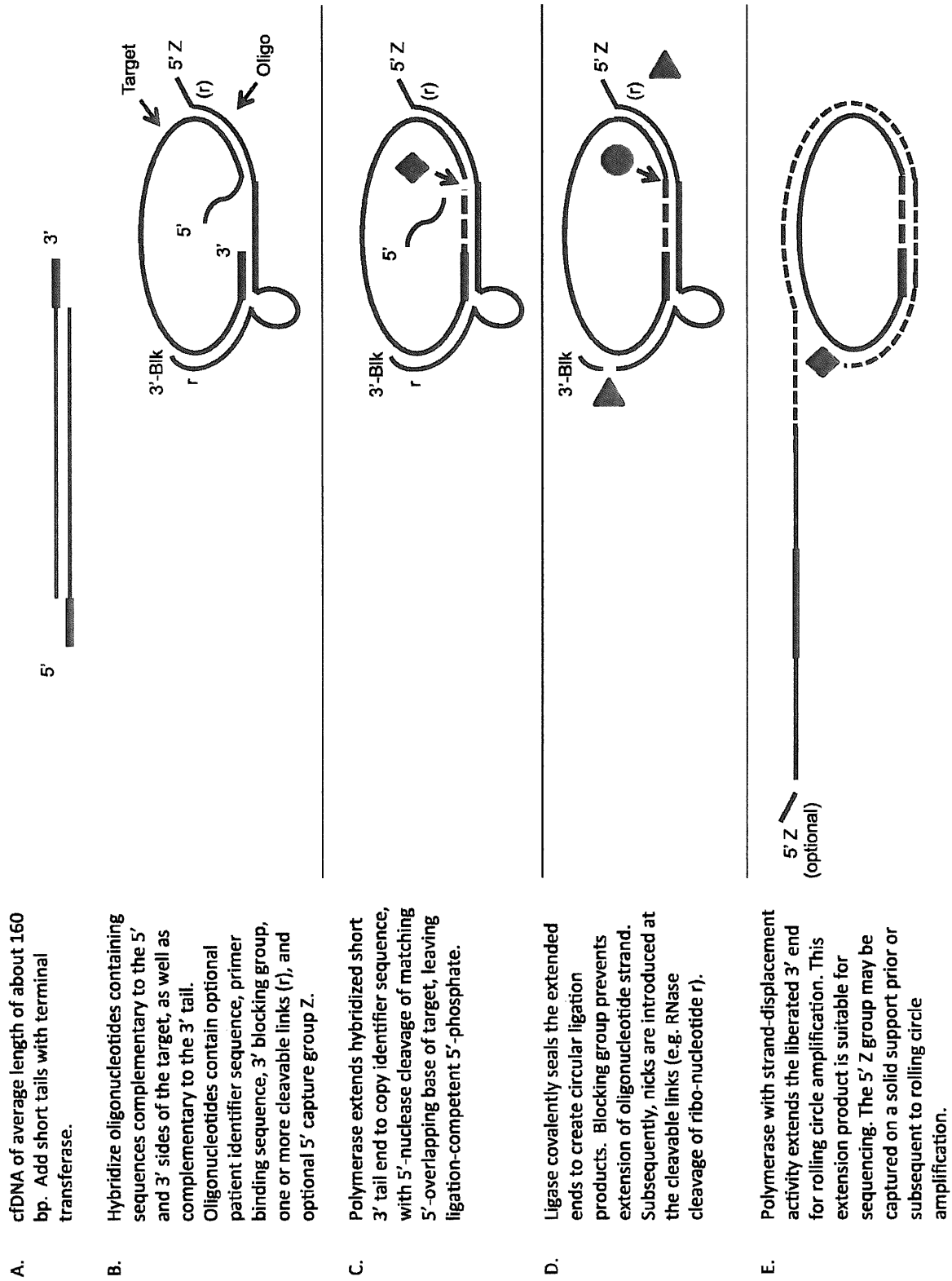


Figure 22

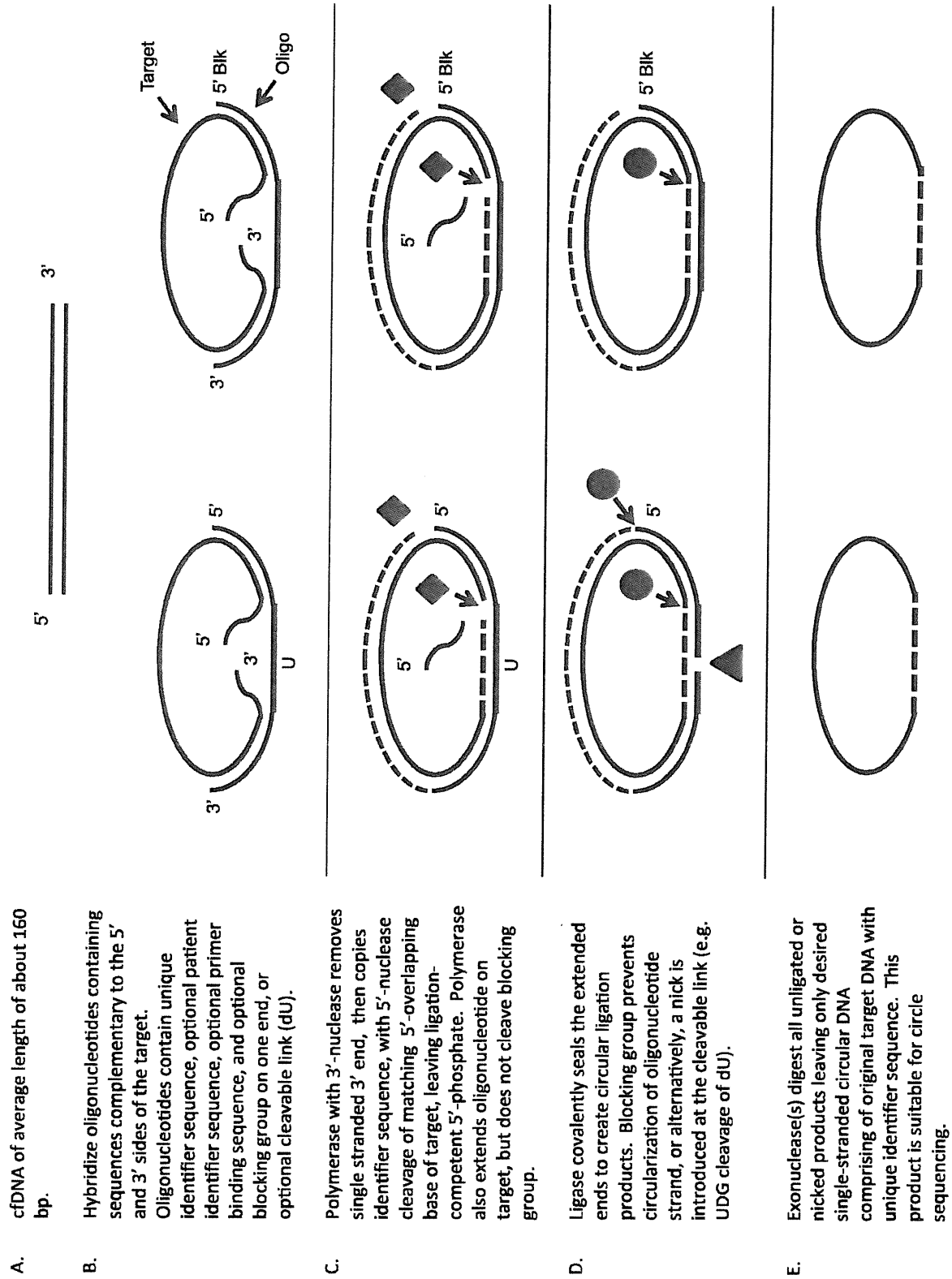


Figure 23

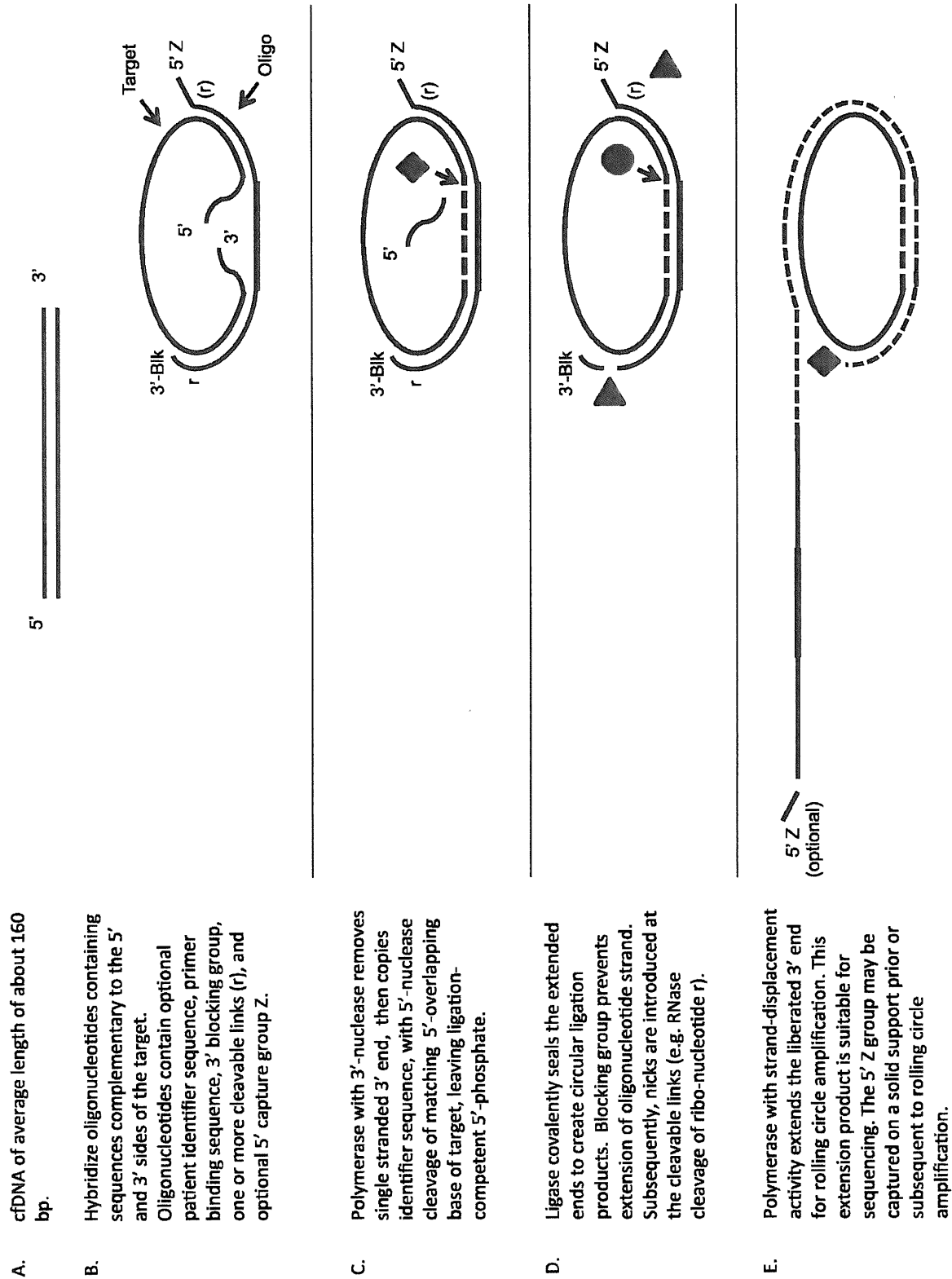


Figure 24

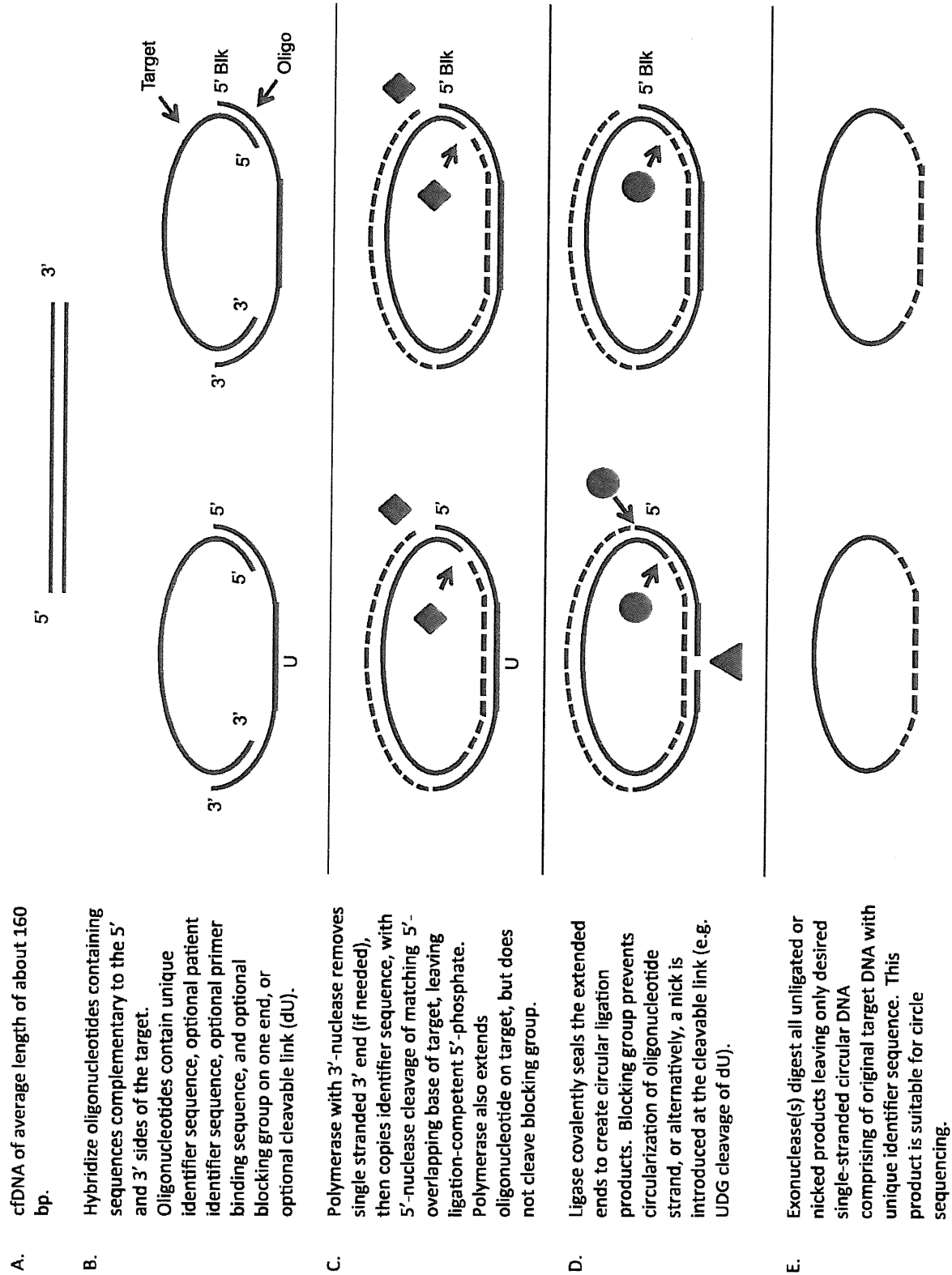


Figure 25

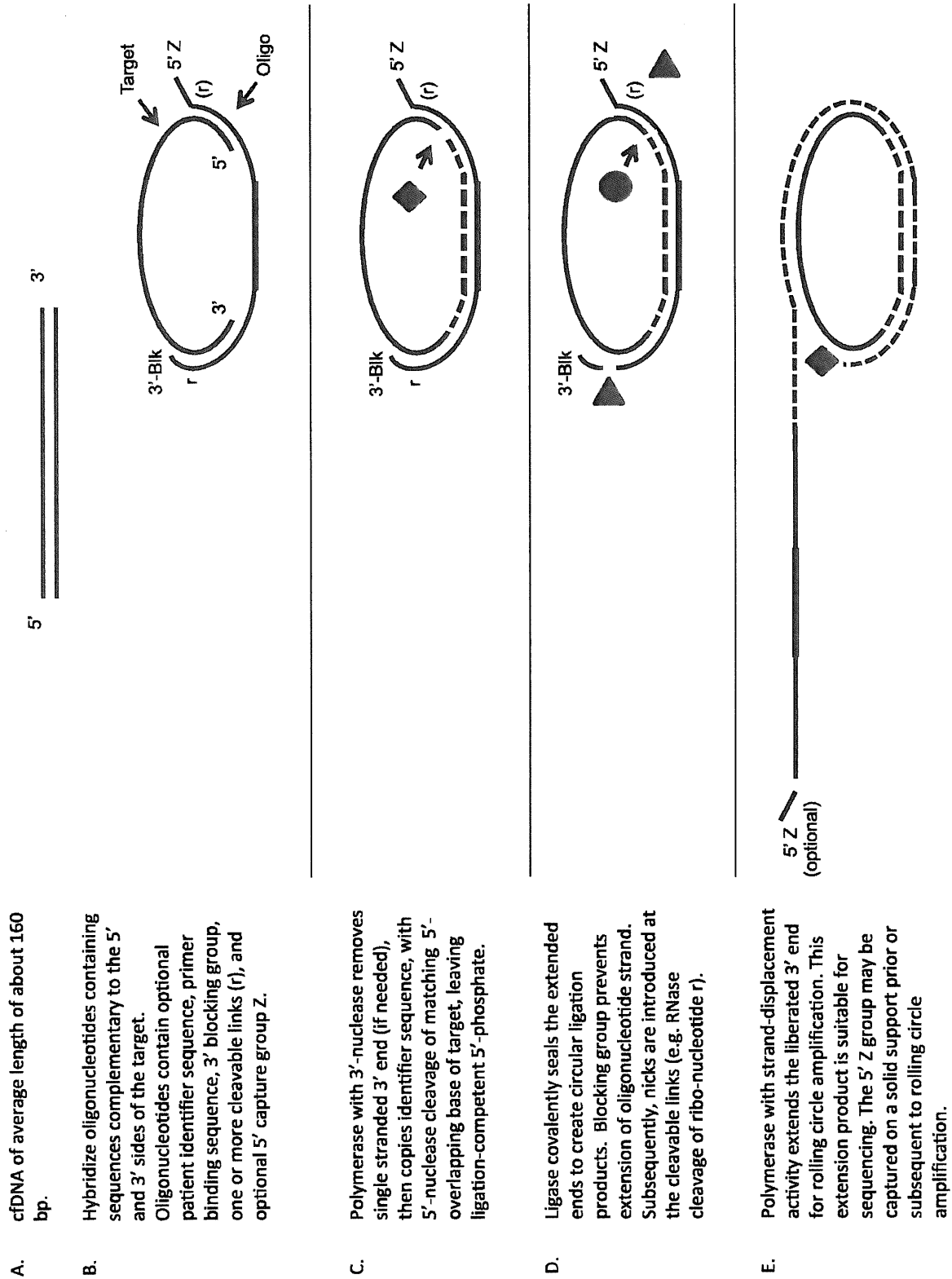


Figure 26

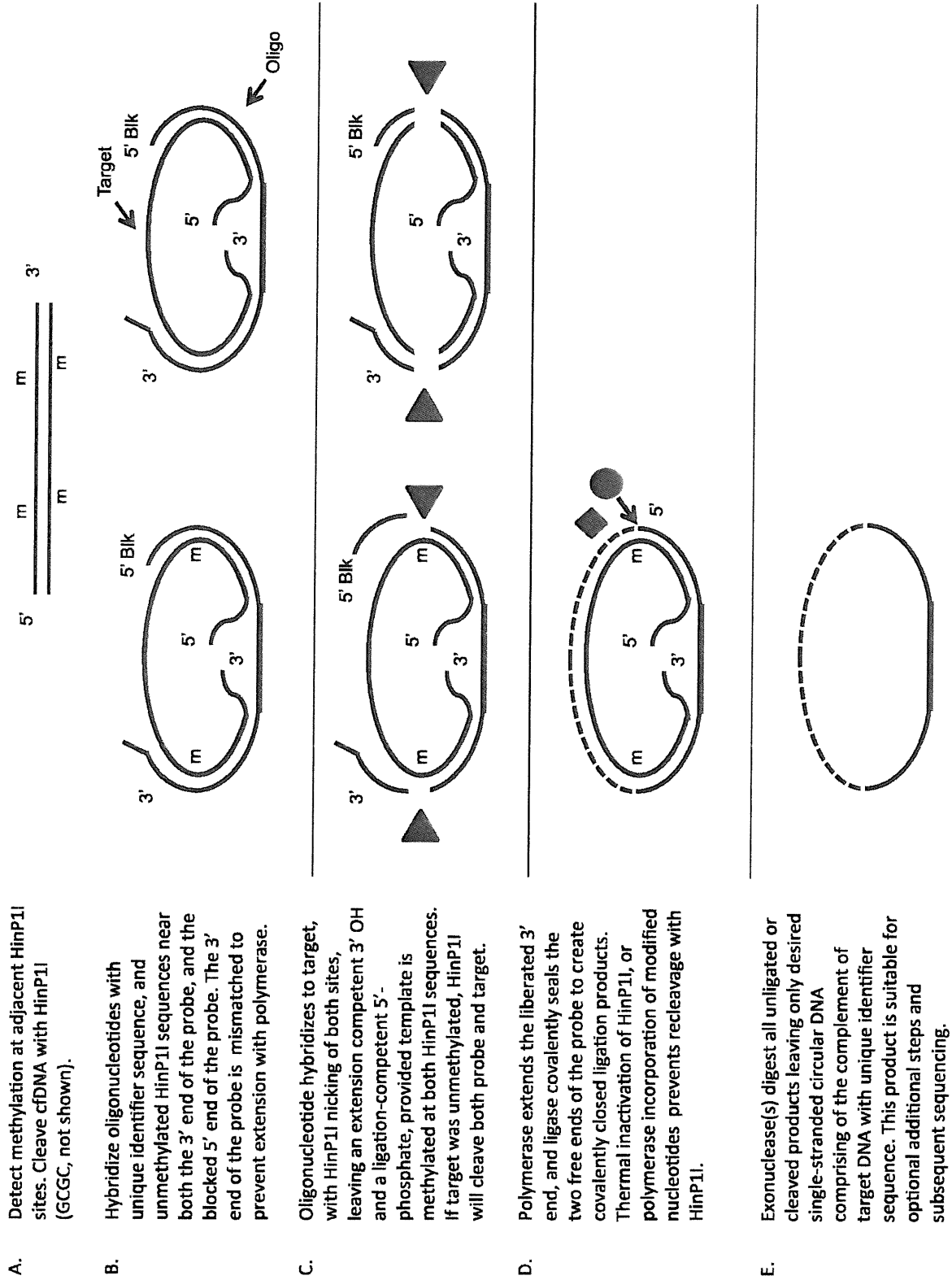


Figure 27

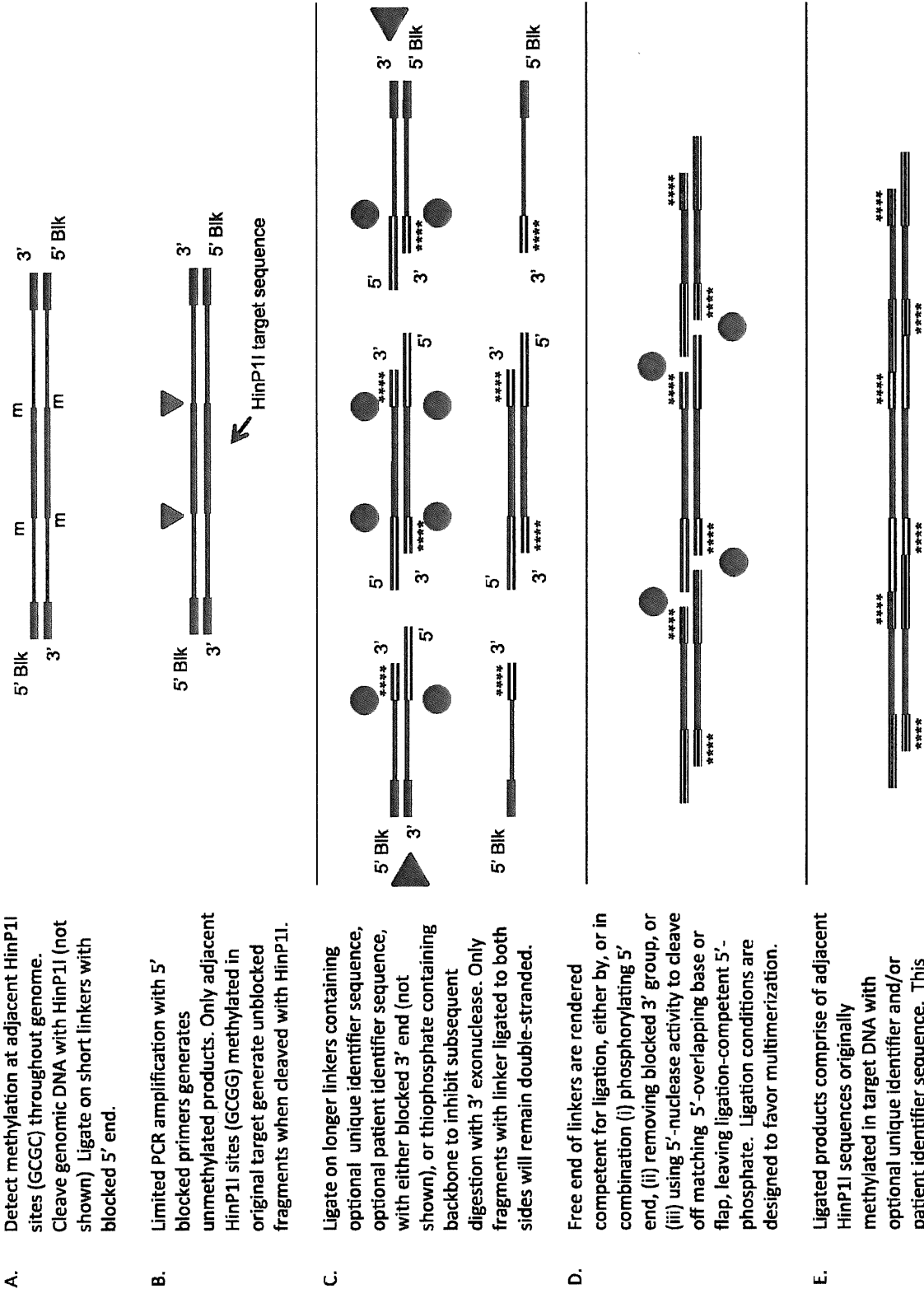


Figure 28

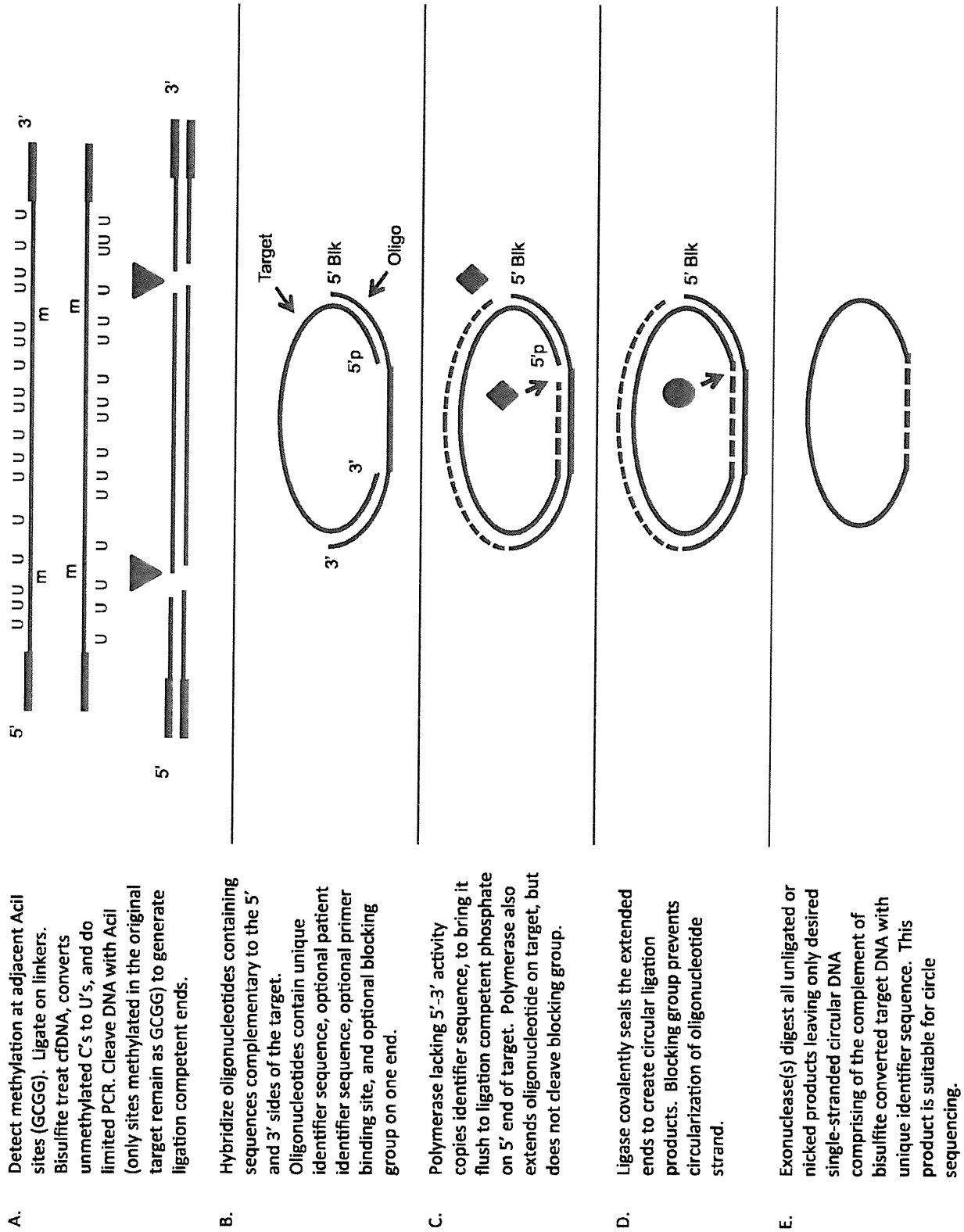


Figure 29

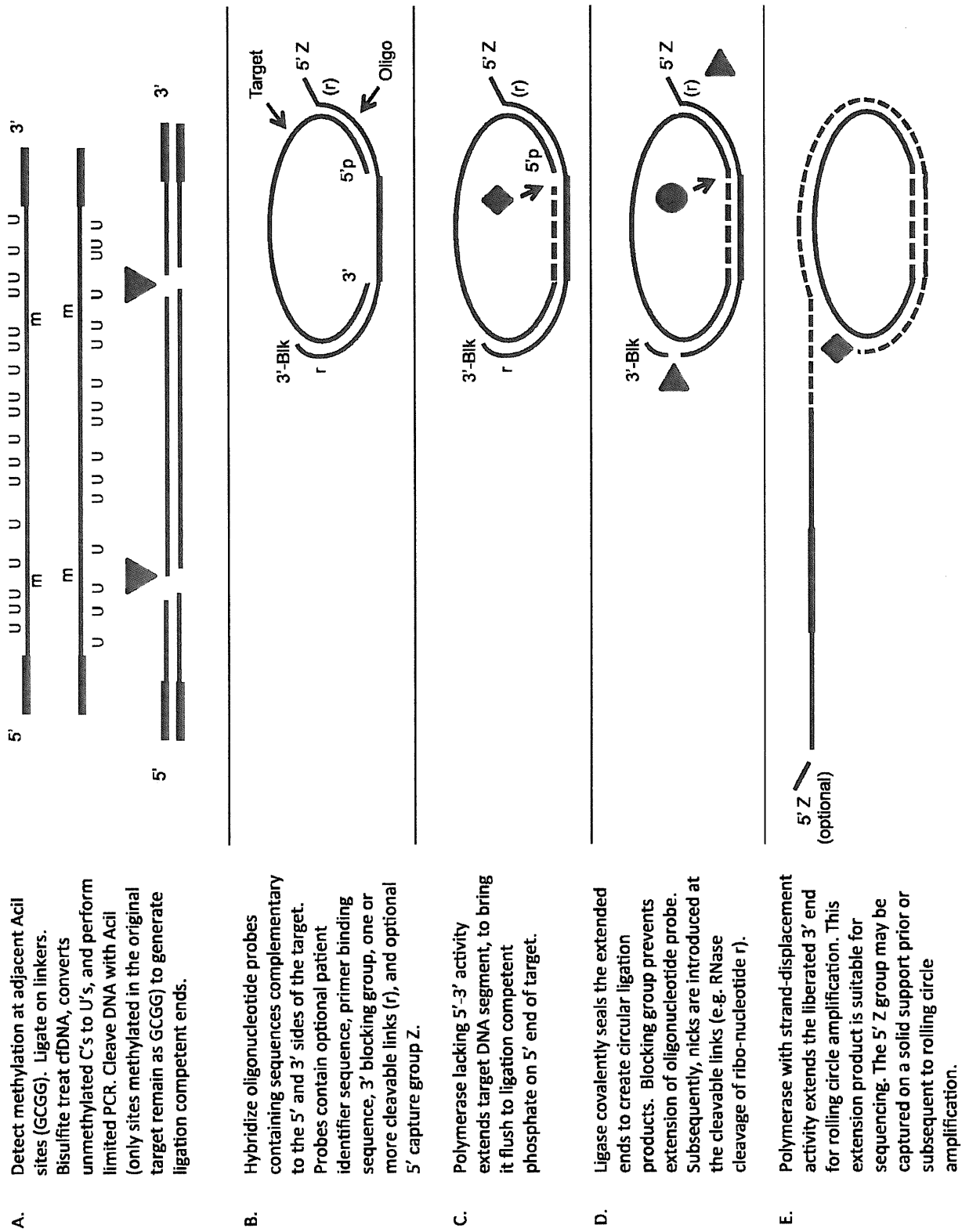


Figure 30

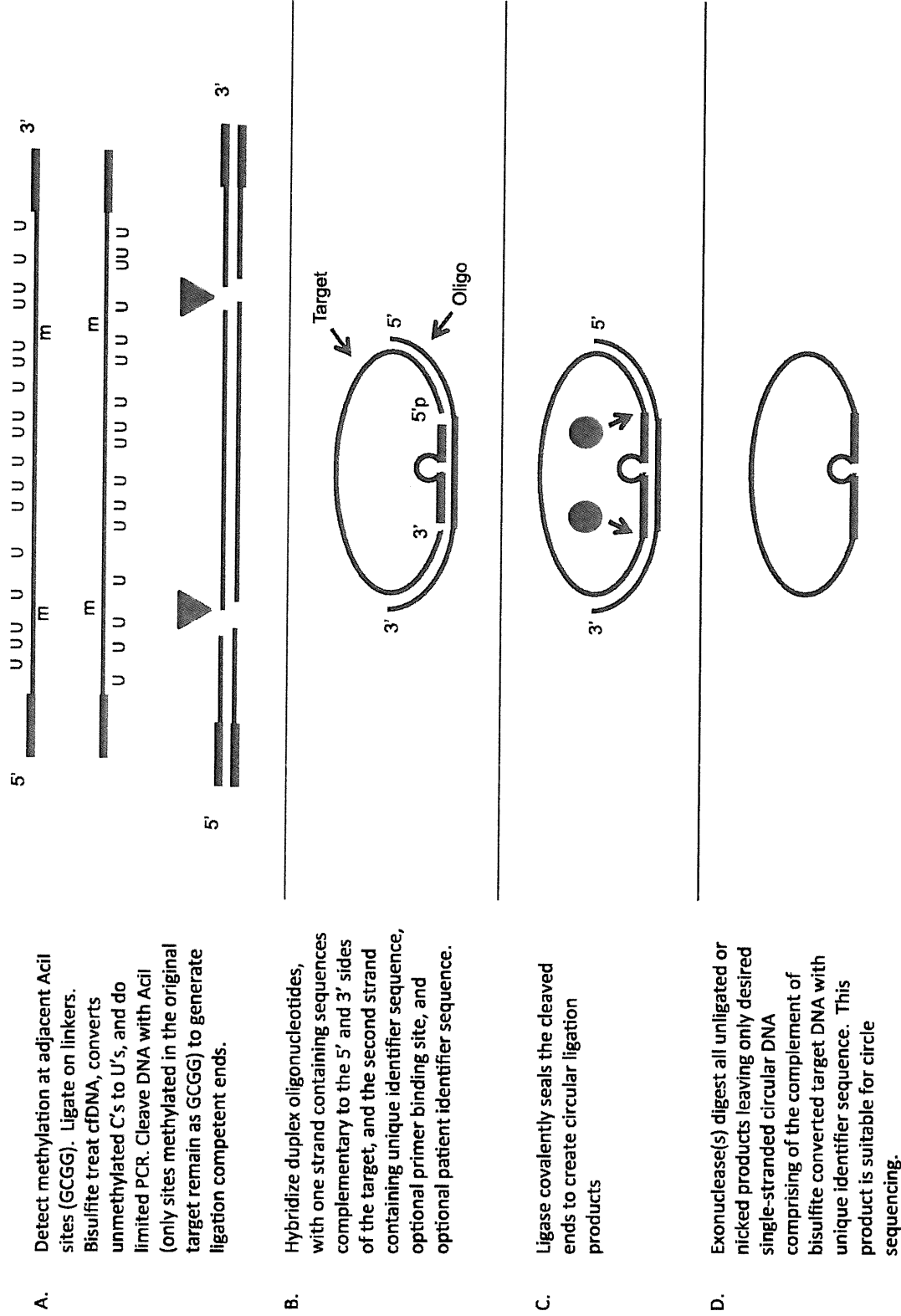


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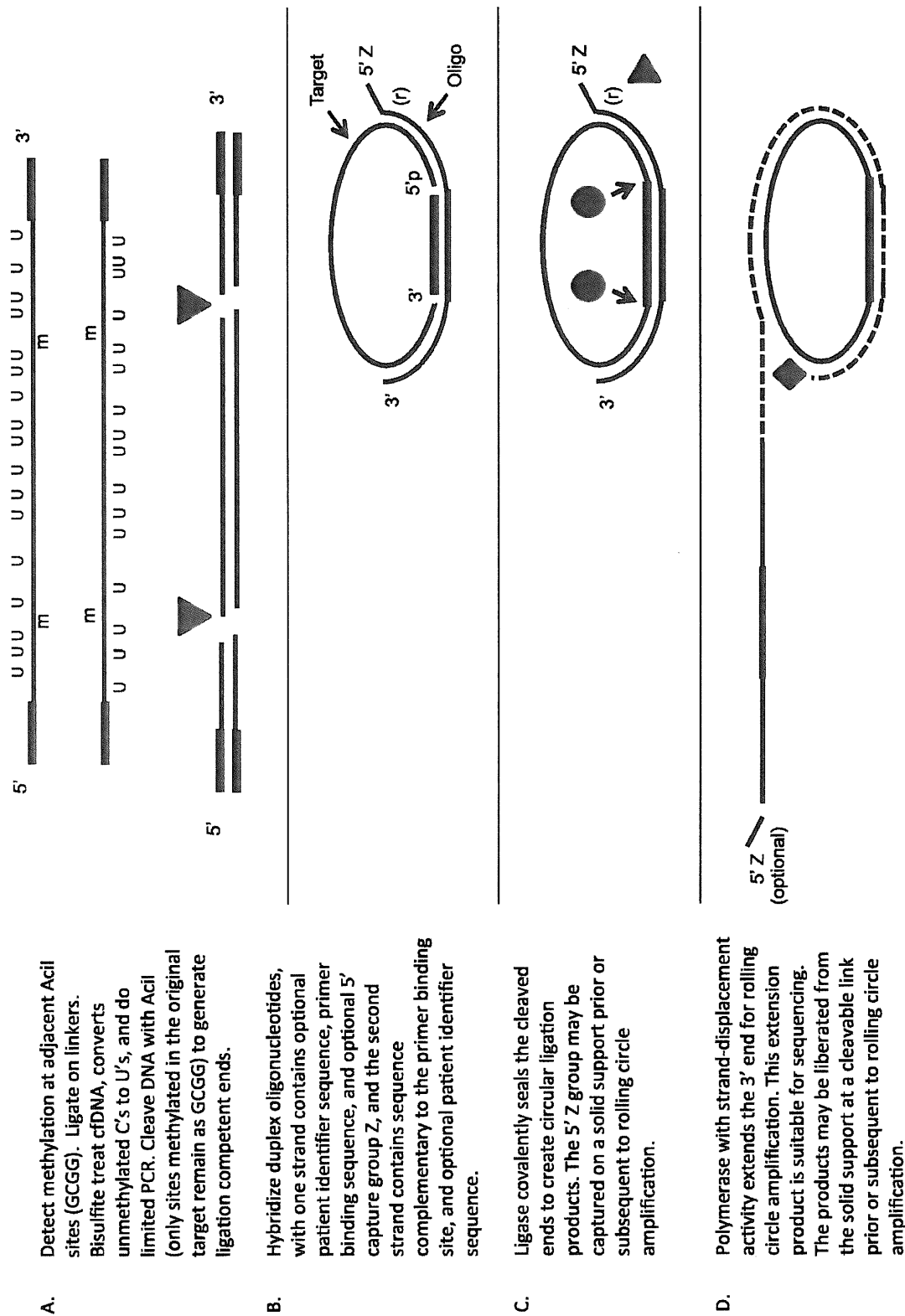


Figure 32

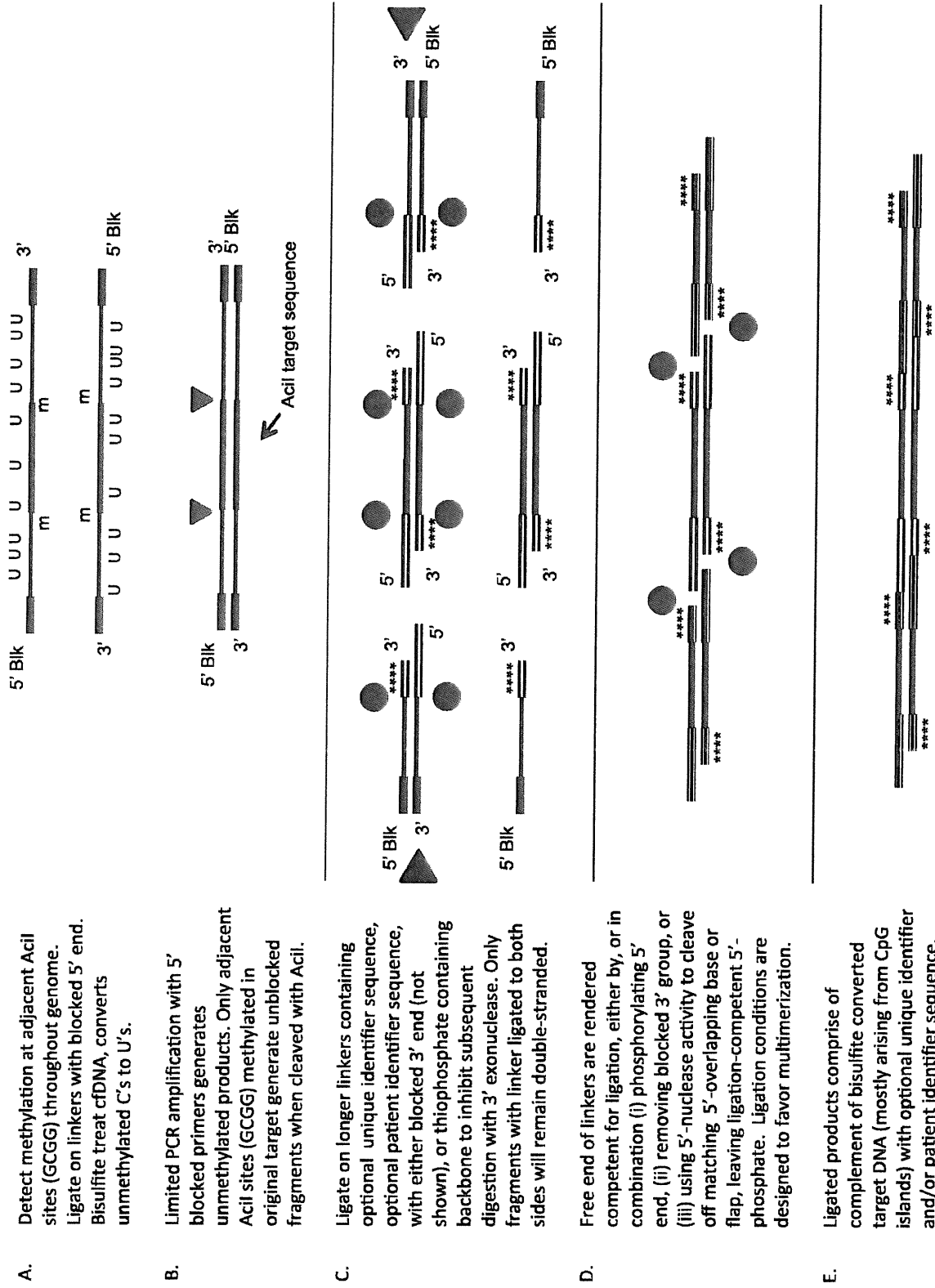


Figure 33

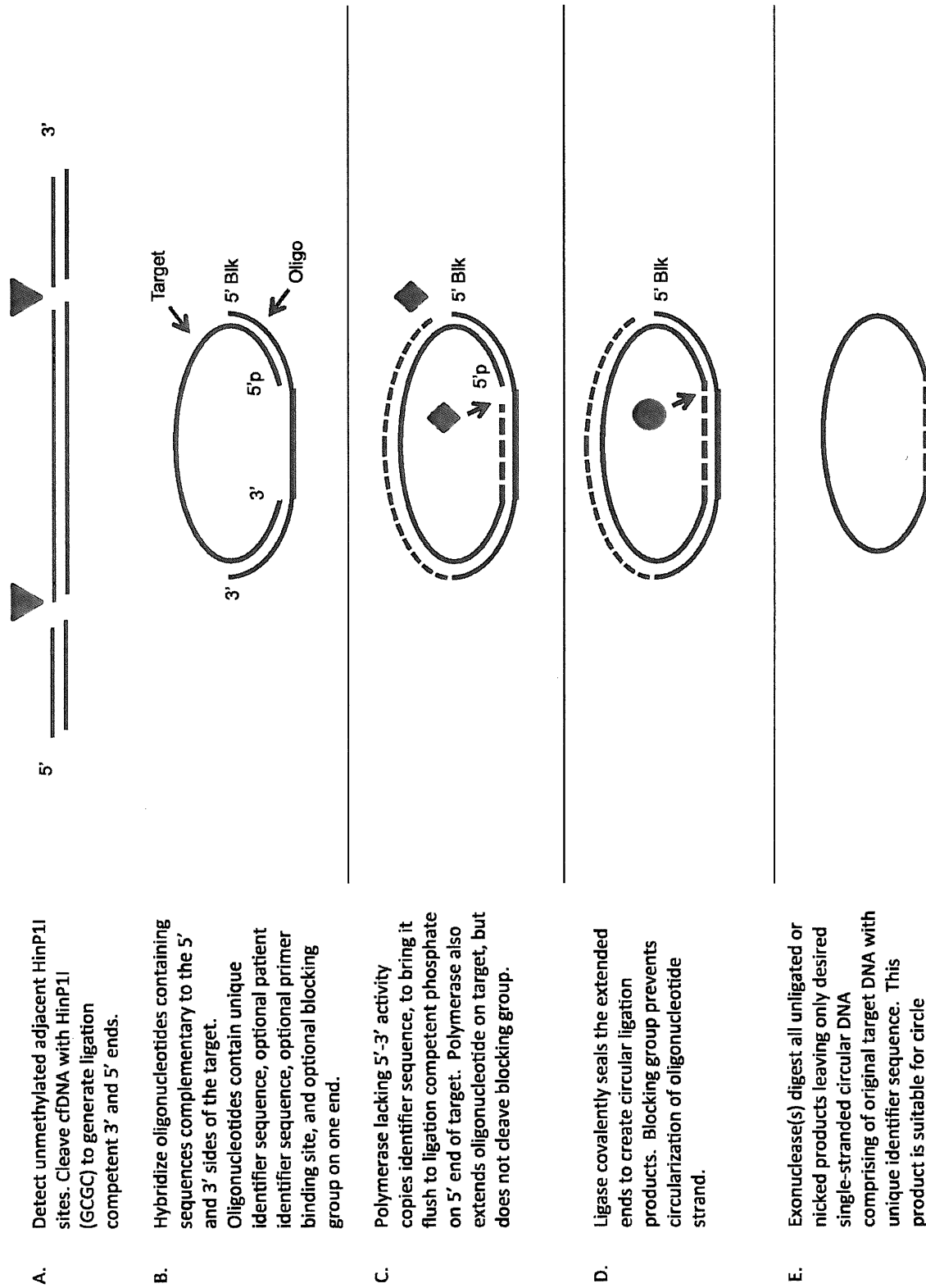


Figure 34

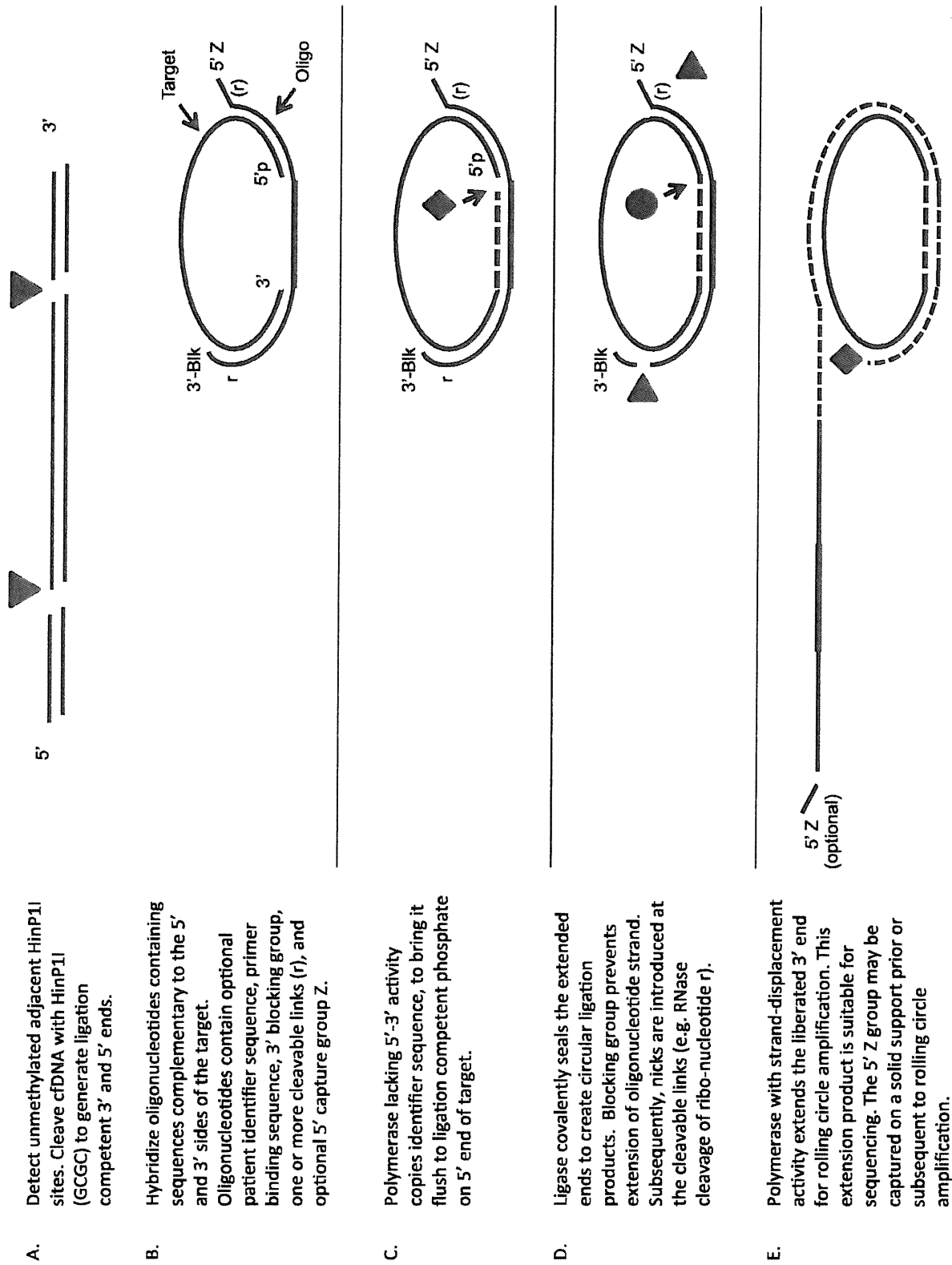


Figure 35

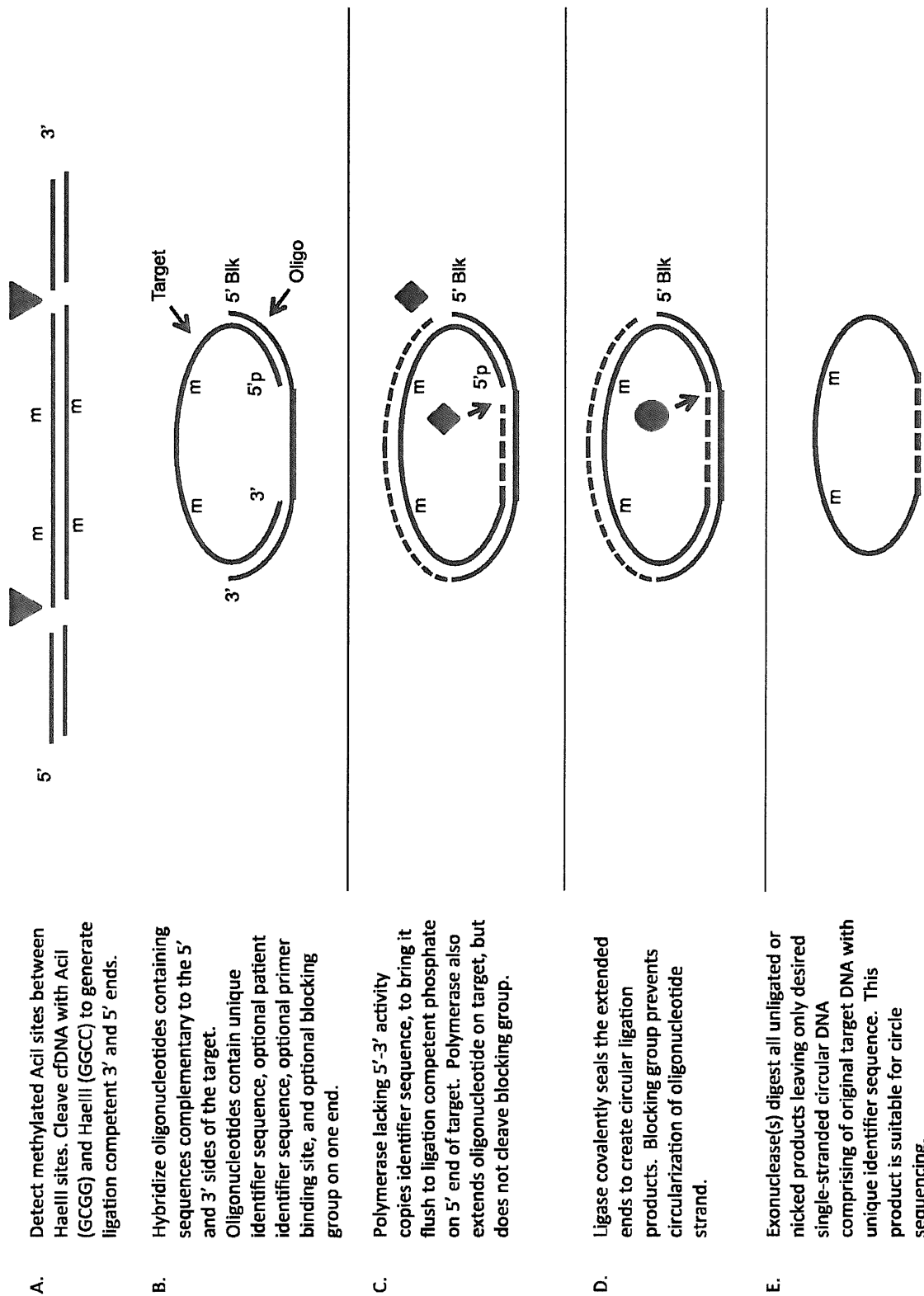


Figure 36

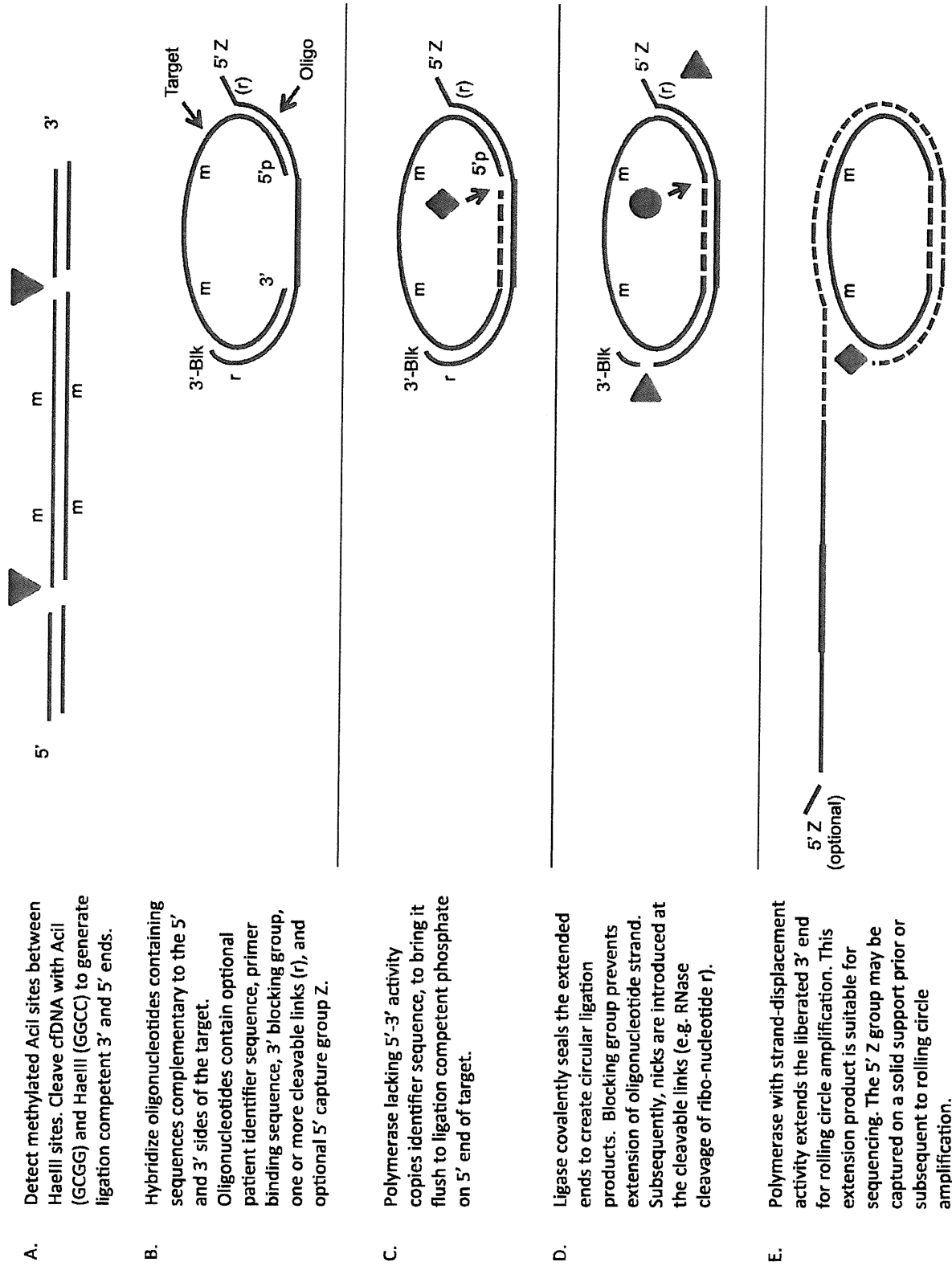


Figure 37

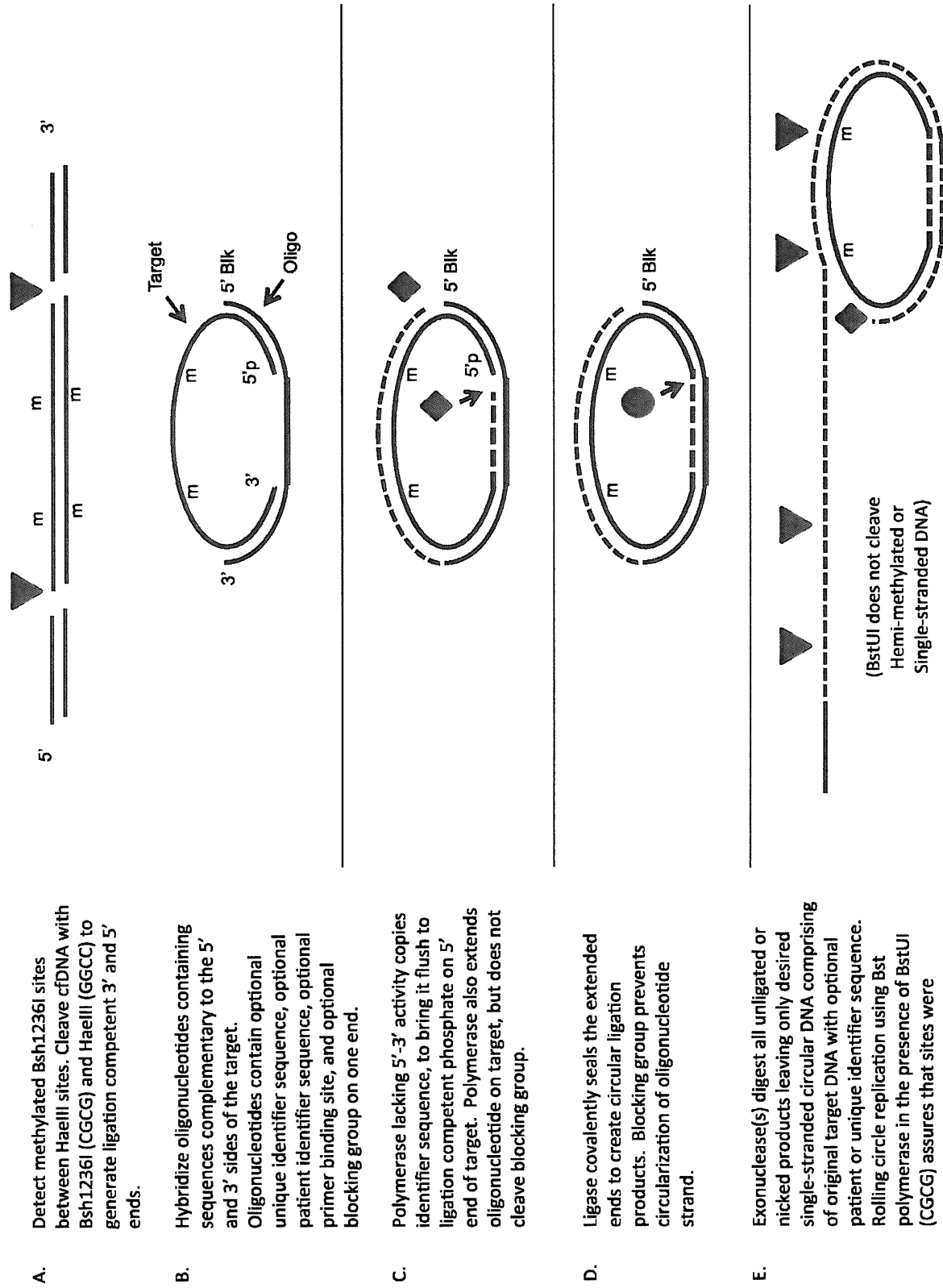


Figure 38

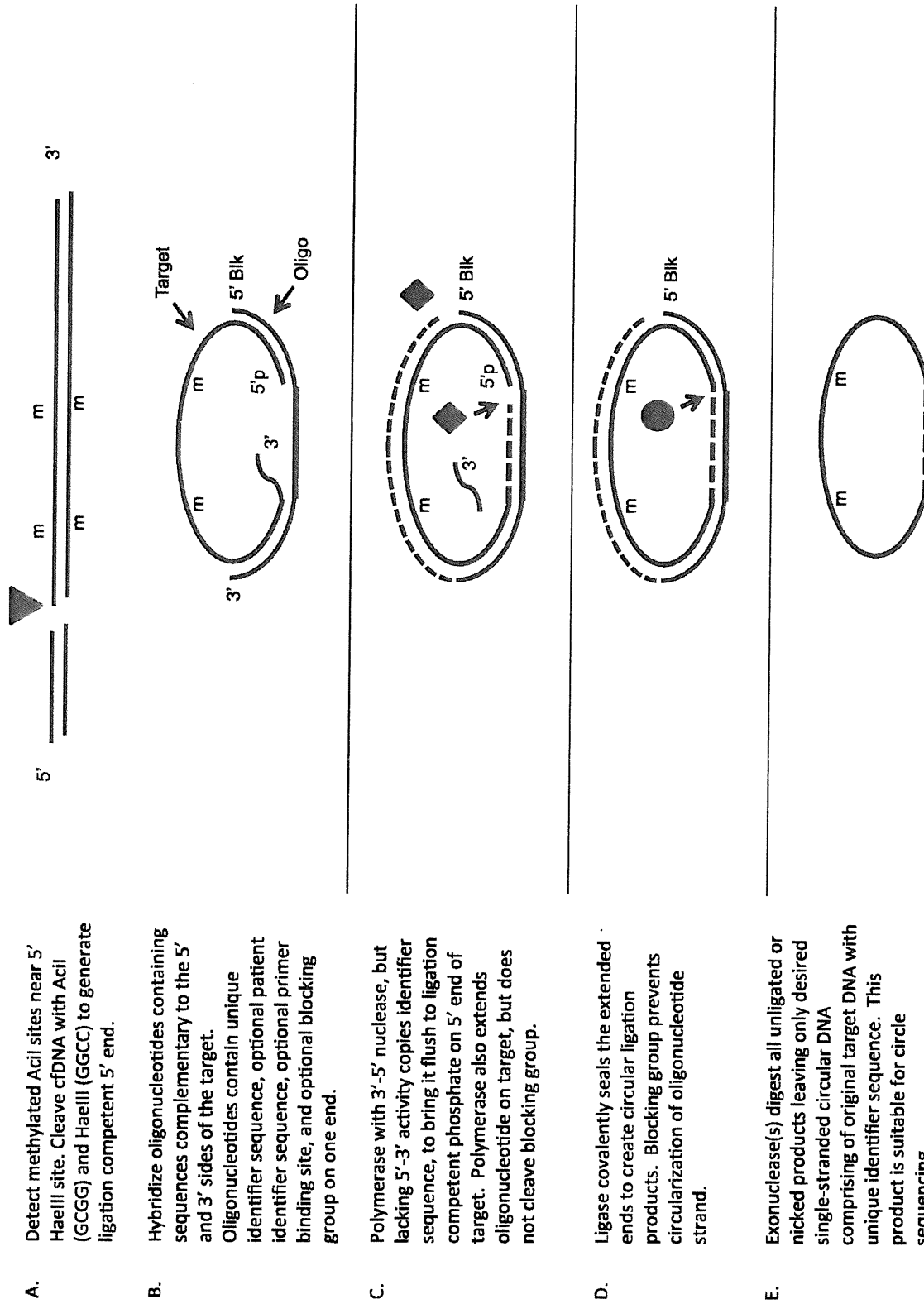


Figure 39

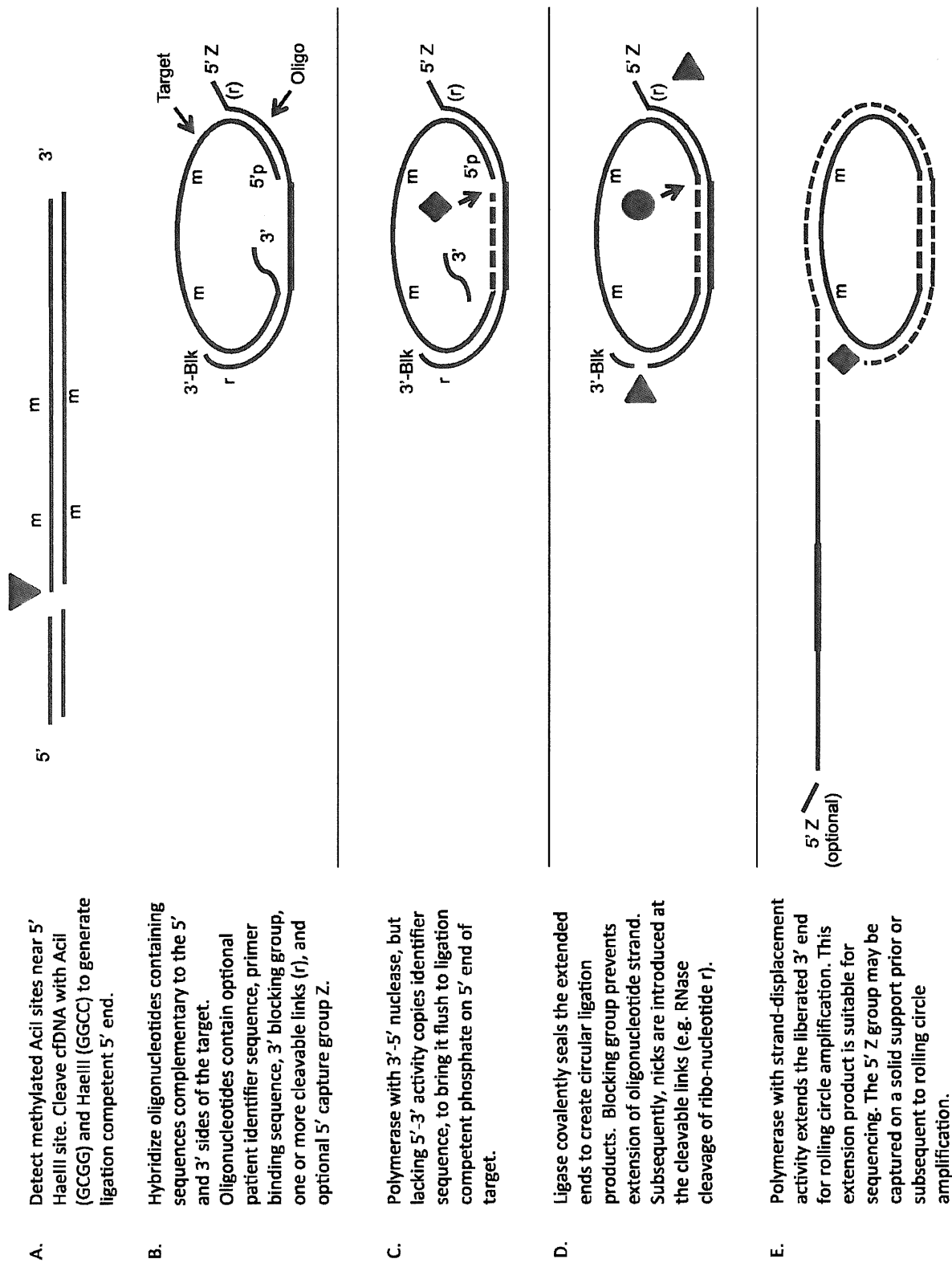


Figure 40

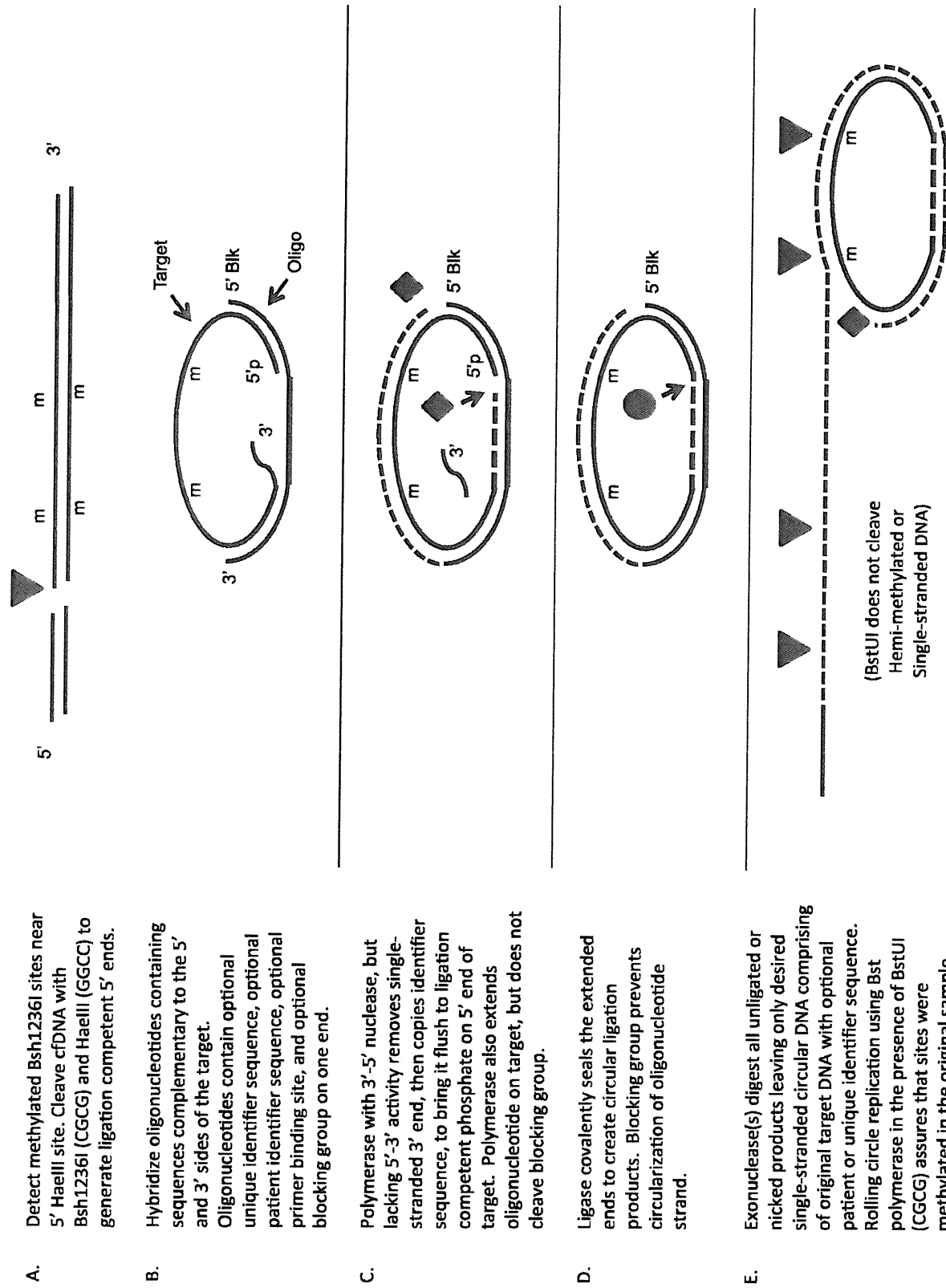


Figure 41

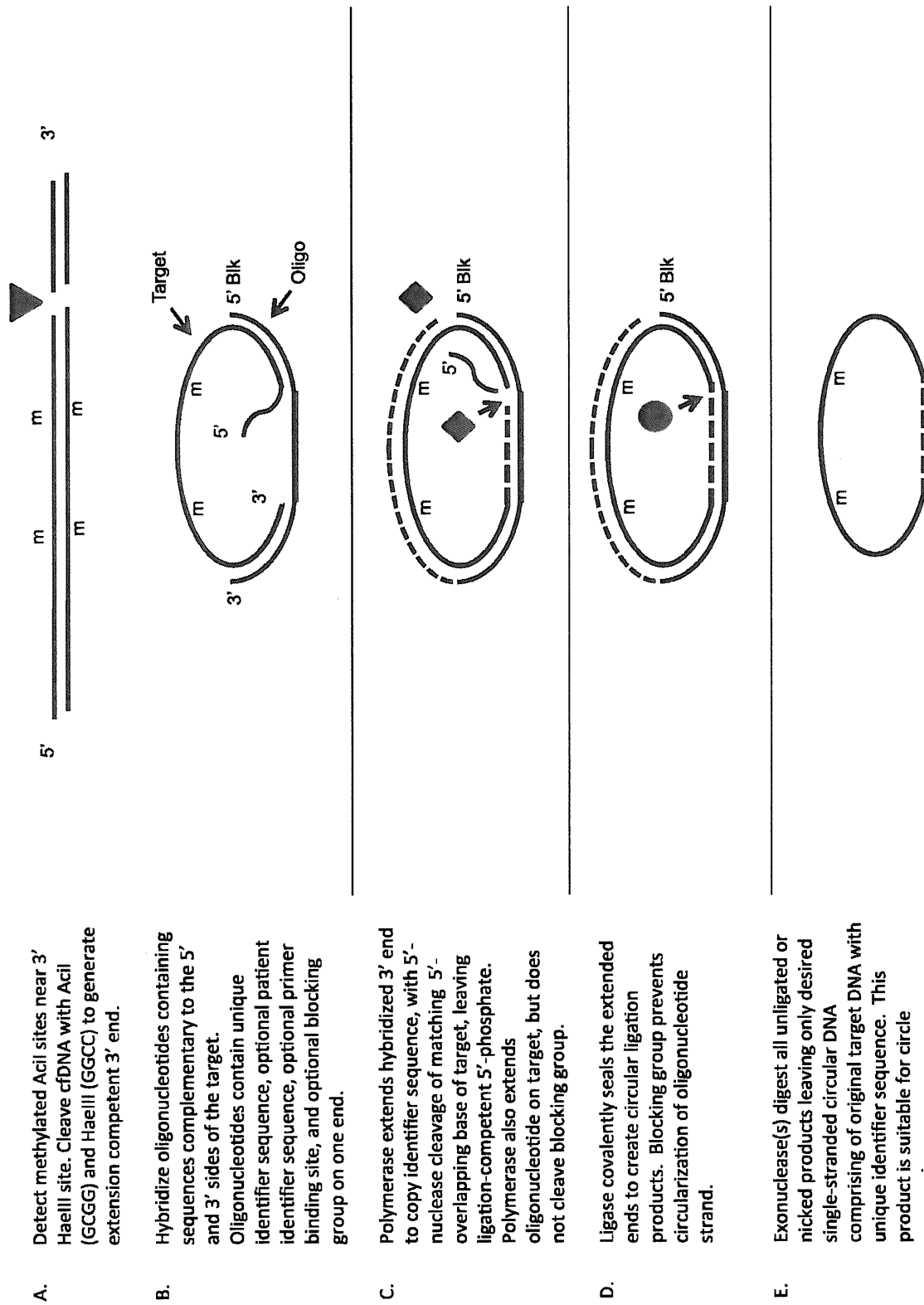


Figure 42

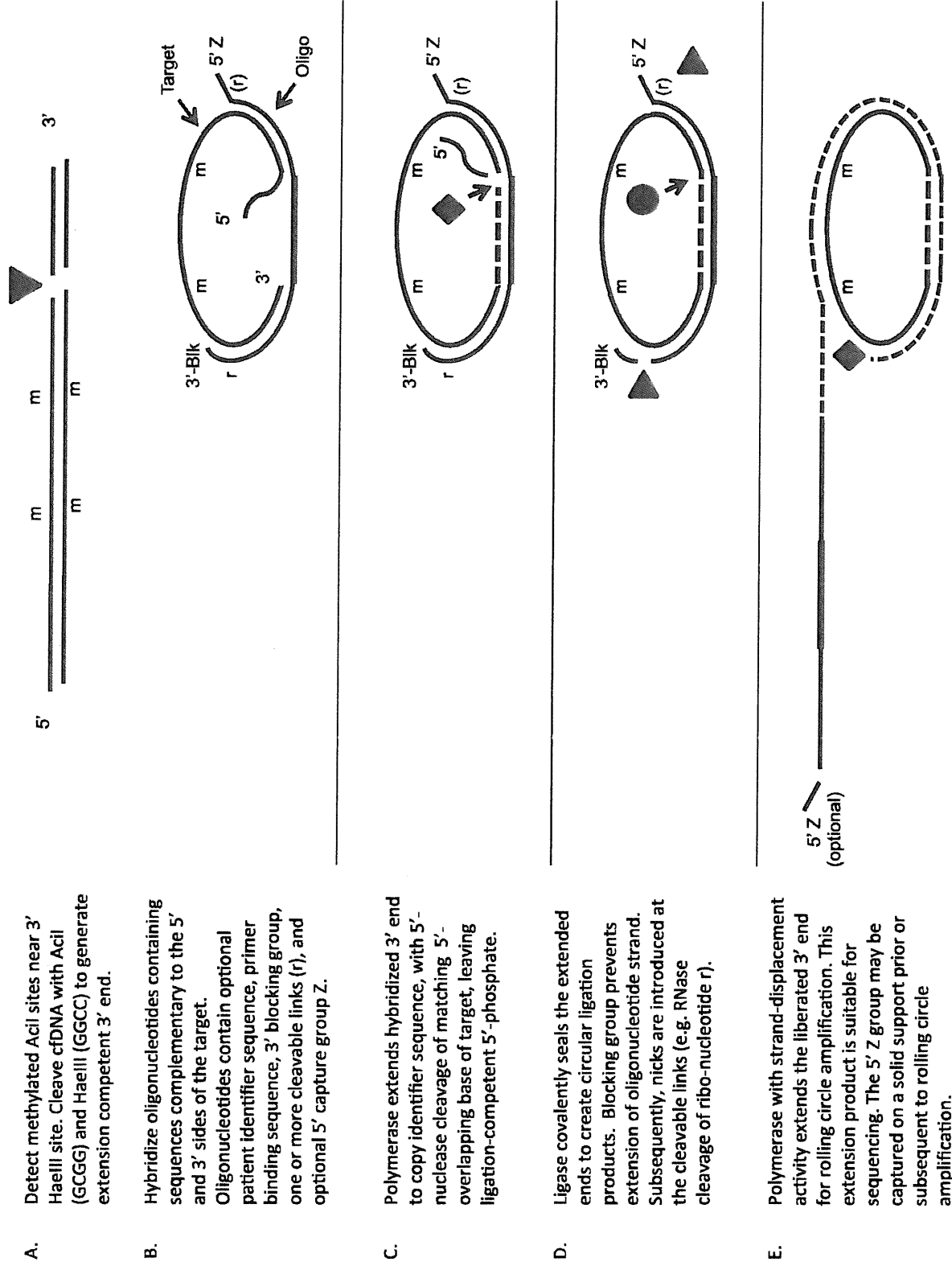


Figure 43

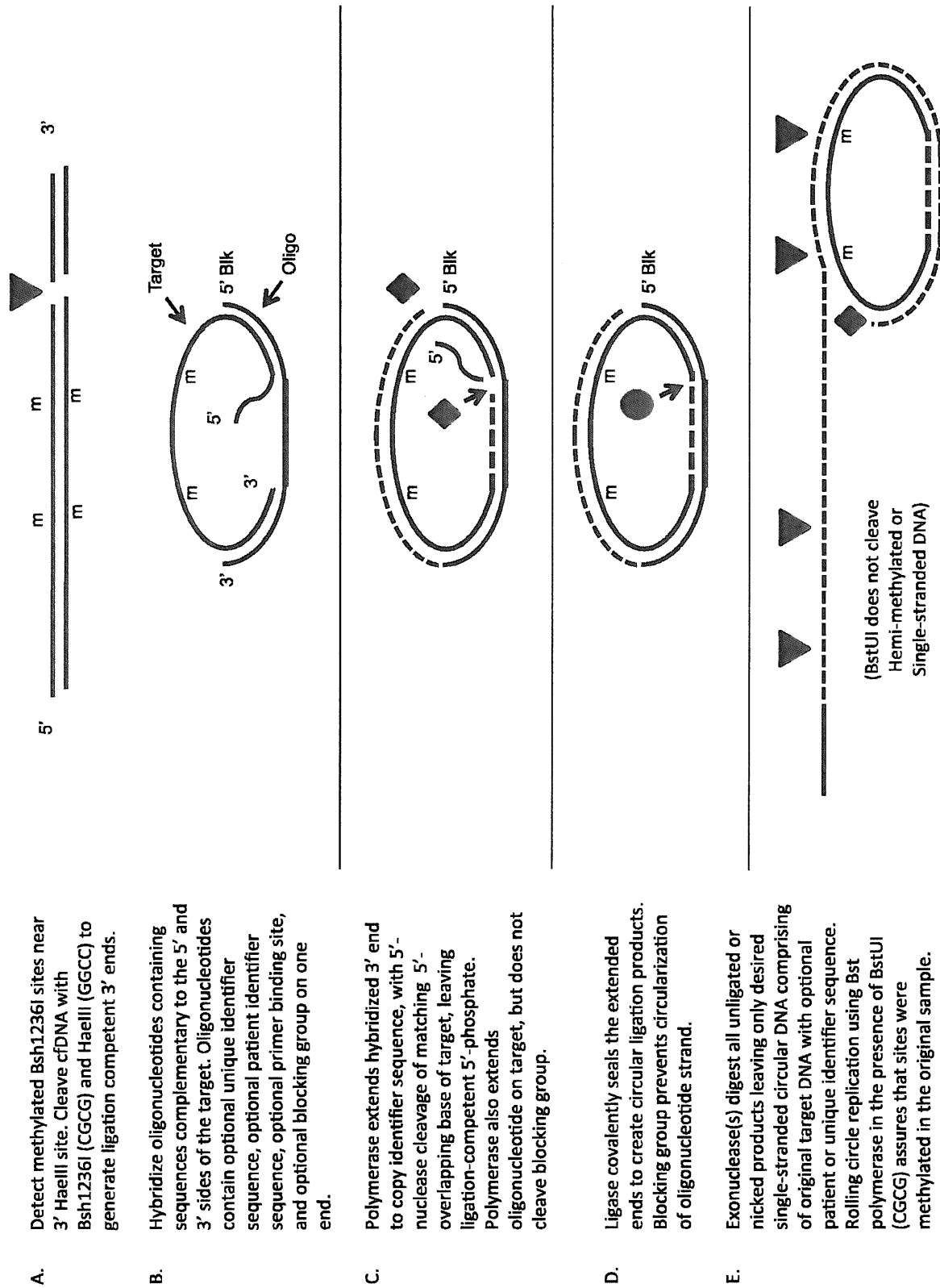
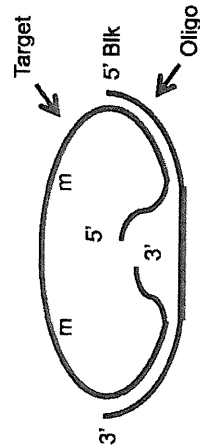


Figure 44

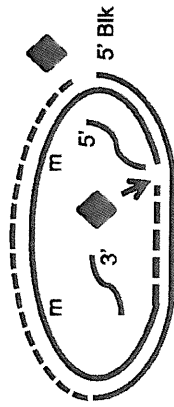
- A. Detect methylated Bsh1236I sites.
Cleave cfDNA with Bsh1236I (CGCG).



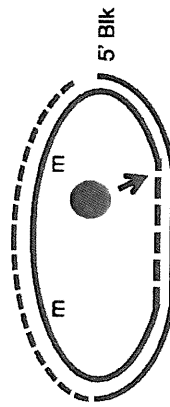
- B. Hybridize oligonucleotides containing sequences complementary to the 5' and 3' sides of the target. Oligonucleotides contain optional unique identifier sequence, optional patient identifier sequence, optional primer binding site, and optional blocking group on one end.



- C. Polymerase with 3' nuclease activity removes single-stranded 3' end, then copies identifier sequence, with 5'-nuclease cleavage of matching 5'-overlapping base of target, leaving ligation-competent 5'-phosphate. Polymerase also extends oligonucleotide on target, but does not cleave blocking group.



- D. Ligase covalently seals the extended ends to create circular ligation products. Blocking group prevents circularization of oligonucleotide strand.



- E. Exonuclease(s) digest all unligated or nicked products leaving only desired single-stranded circular DNA comprising of original target DNA with optional patient or unique identifier sequence. Rolling circle replication using Bst polymerase in the presence of BstUI (CGCG) assures that sites were methylated in the original sample.

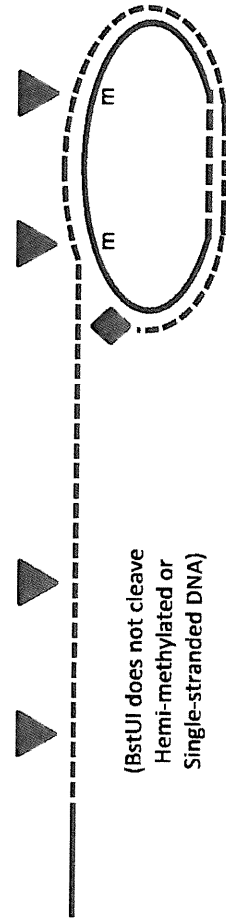


Figure 45

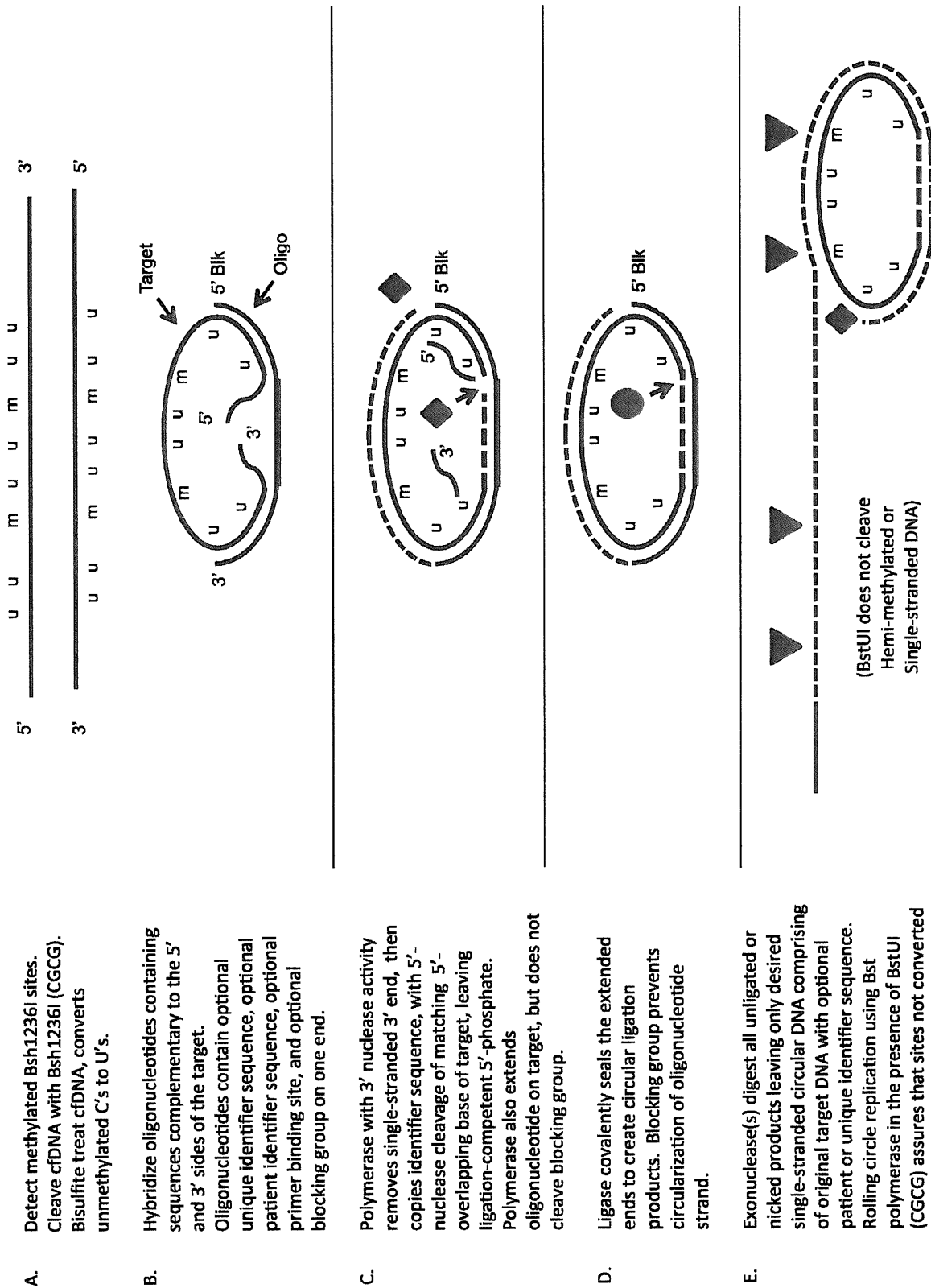


Figure 46

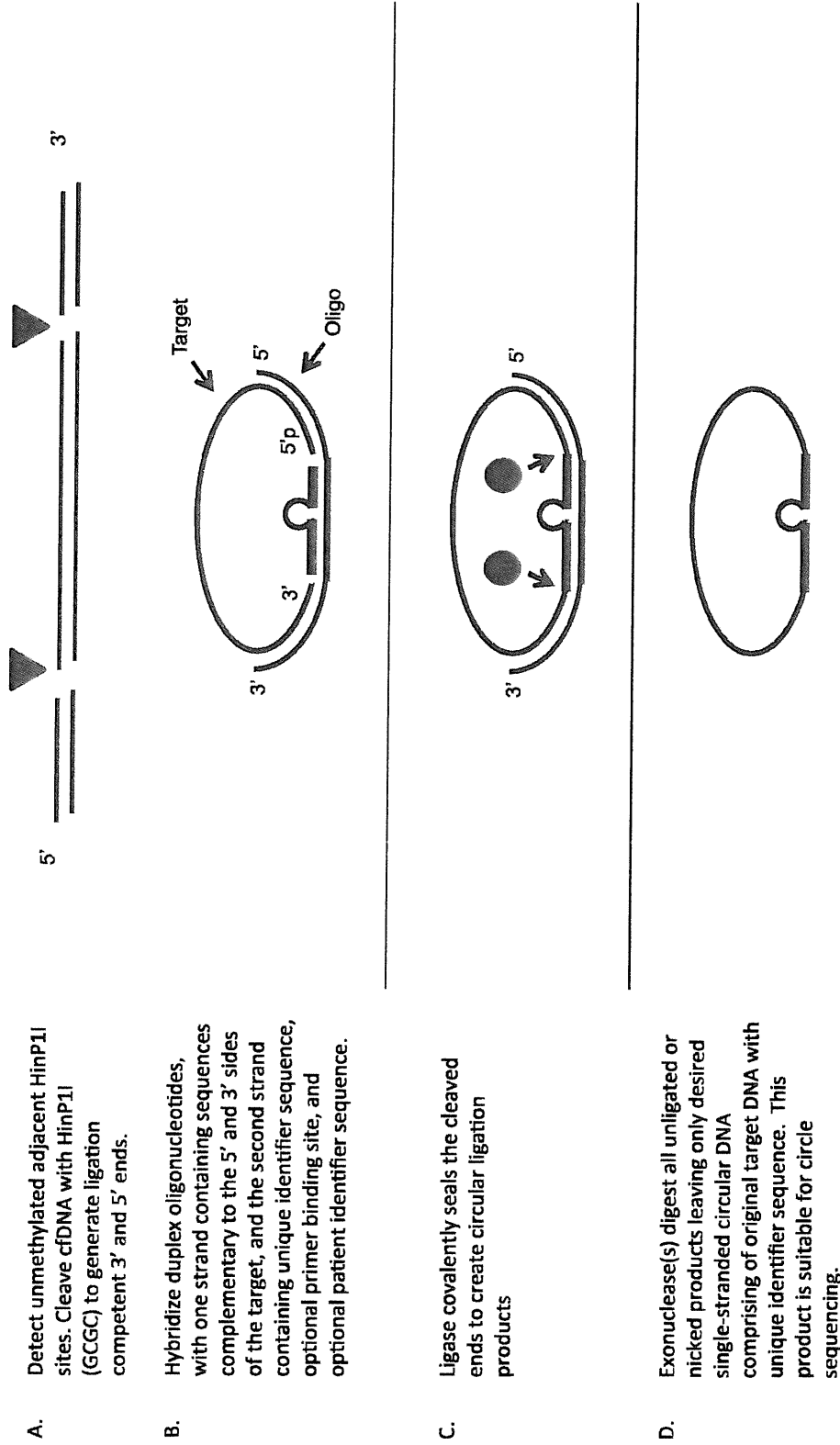
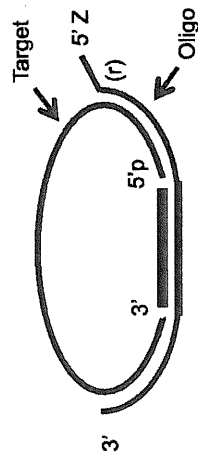


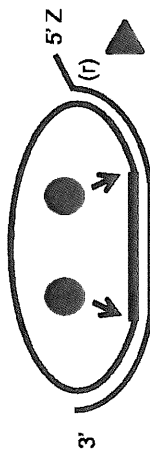
Figure 47



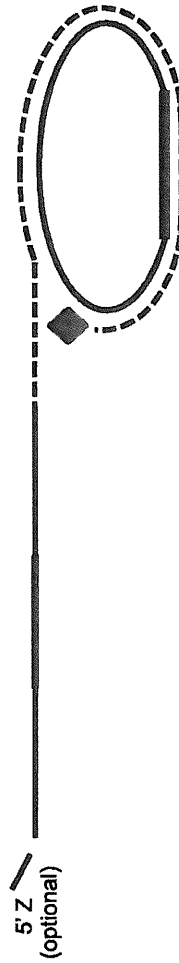
A. Detect unmethylated adjacent HinfI sites. Cleave cfDNA with HinfI (GCGC) to generate ligation competent 3' and 5' ends.



B. Hybridize duplex oligonucleotides, with one strand contains sequences complementary to the 5' and 3' sides of the target, optional patient identifier sequence, primer binding group Z, and the second strand contains sequence complementary to the primer binding site, and optional patient identifier sequence.



C. Ligase covalently seals the cleaved ends to create circular ligation products. The 5' Z group may be captured on a solid support prior or subsequent to rolling circle amplification.



D. Polymerase with strand-displacement activity extends the 3' end for rolling circle amplification. This extension product is suitable for sequencing. The products may be liberated from the solid support at a cleavable link prior or subsequent to rolling circle amplification.

Figure 48

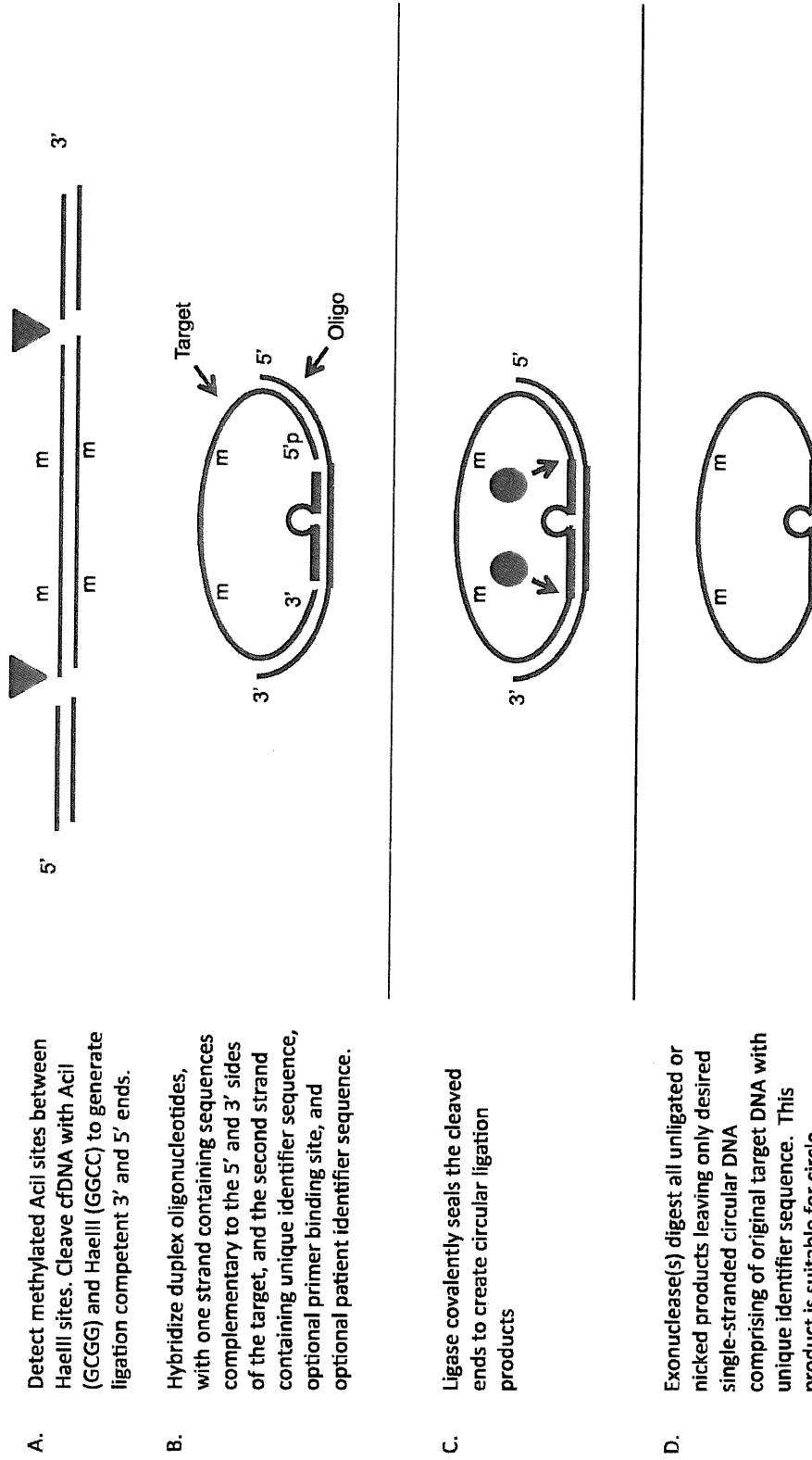


Figure 49

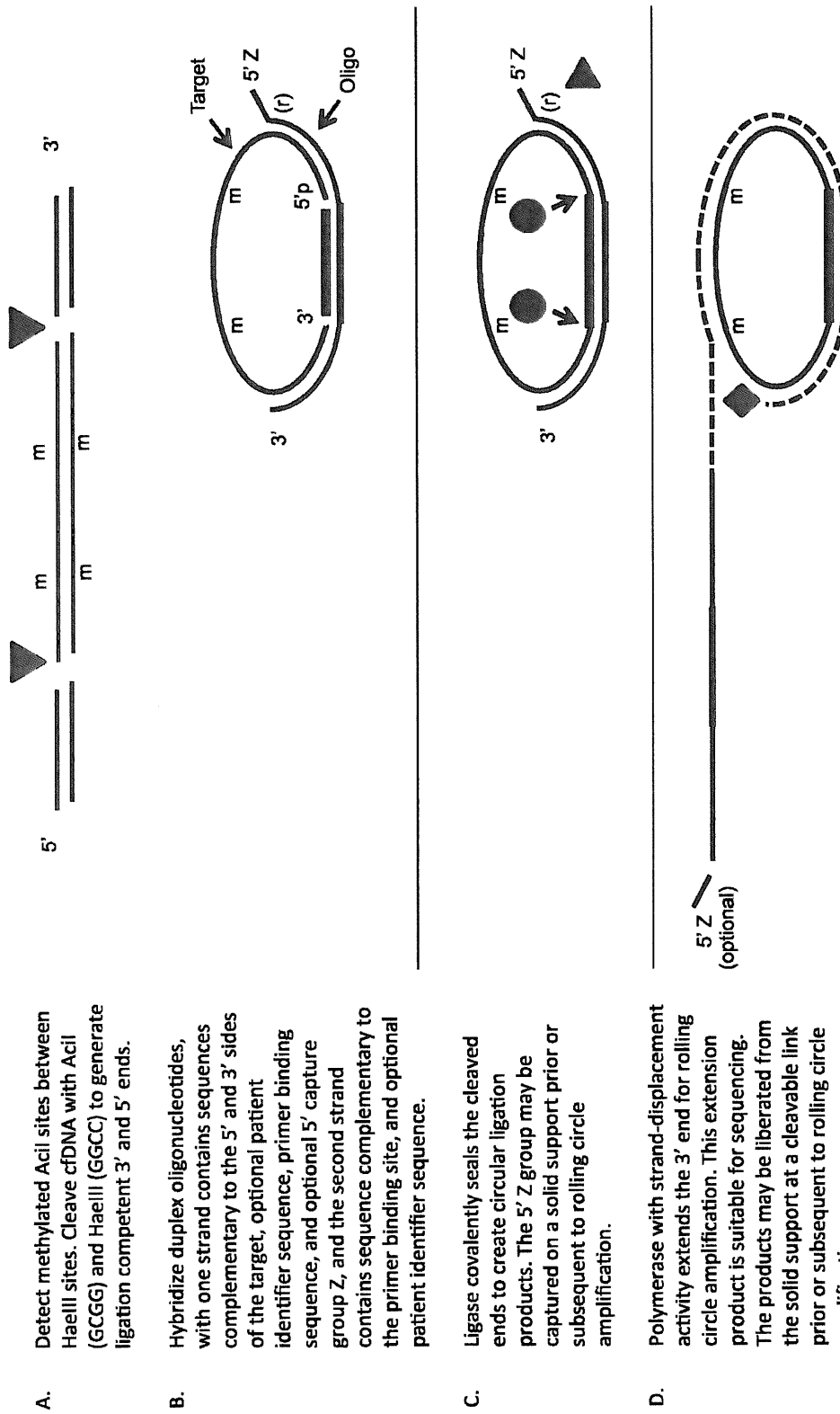


Figure 50

- A. Detect unmethylated adjacent HinP1I sites (GCGC) throughout genome. Ligate on short linkers with blocked 5' end. Cleave DNA with HinP1I (GCGC). Only adjacent HinP1I sites (GCGC) unmethylated in original target generate unblocked fragments when cleaved with HinP1I.
- B. Ligate on longer linkers containing optional unique identifier sequence, optional patient identifier sequence, with either blocked 3' end (not shown), or thiophosphate containing backbone to inhibit subsequent digestion with 3' exonuclease. Only fragments with linker ligated to both sides will remain double-stranded.
- C. Free end of linkers are rendered competent for ligation, either by, or in combination (i) phosphorylating 5' end, (ii) removing blocked 3' group, or (iii) using 5'-nuclease activity to cleave off matching 5'-overlapping base or flap, leaving ligation-competent 5'-phosphate. Ligation conditions are designed to favor multimerization.
- D. Ligated products comprise of adjacent HinP1I sequences originally unmethylated in target DNA with optional unique identifier and/or patient identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.

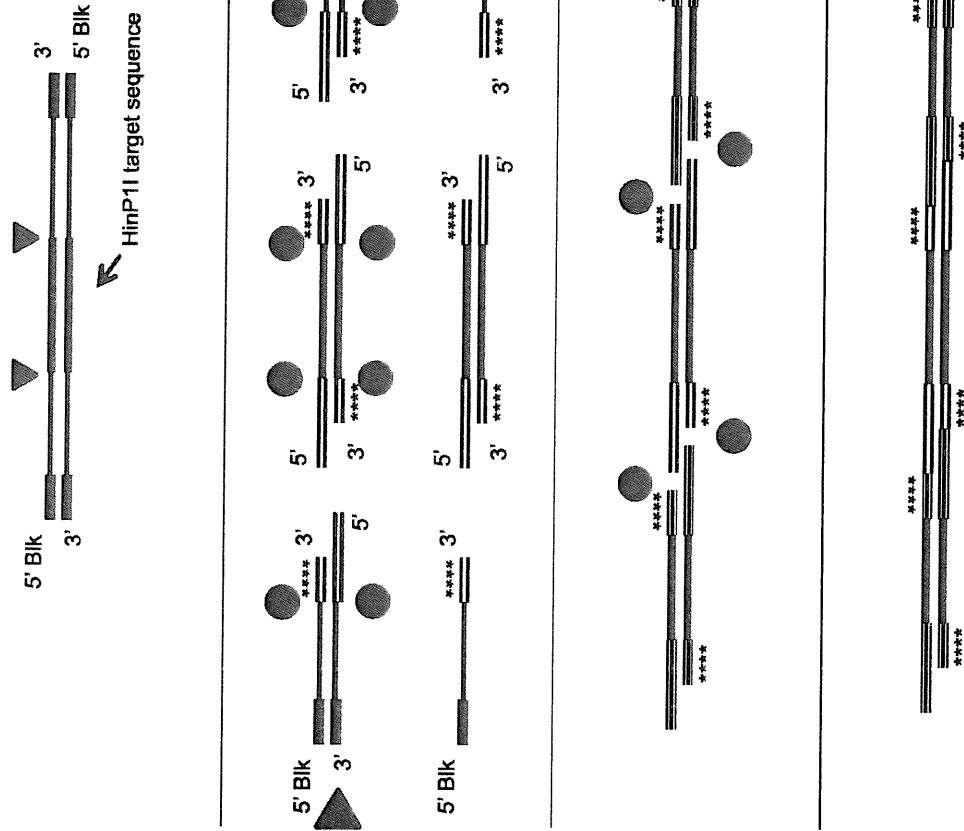
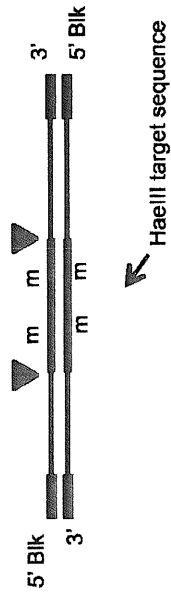
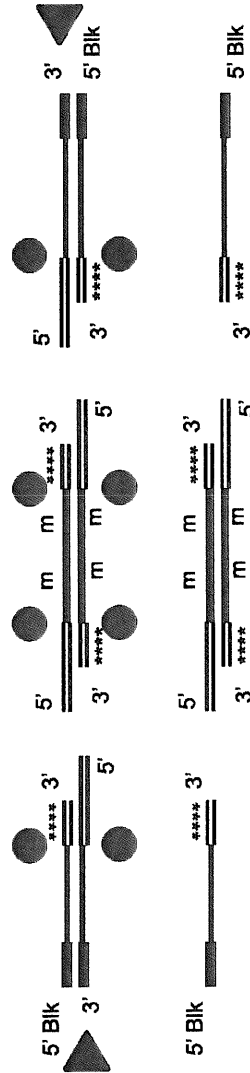


Figure 51

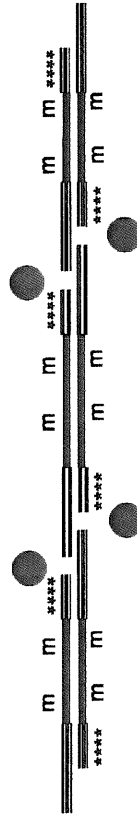
A. Detect methylated adjacent AclI sites (GCGG) throughout genome. Cleave with AclI. Ligate on short linkers with blocked 5' end. Cleave DNA with HaeIII (GGCC). Only adjacent HaeIII sites (GGCC) with methylated AclI sites in original target generate unblocked fragments when cleaved with HaeIII.



B. Ligate on longer linkers containing optional unique identifier sequence, optional patient identifier sequence, with either blocked 3' end (not shown), or thiophosphate containing backbone to inhibit subsequent digestion with 3' exonuclease. Only fragments with linker ligated to both sides will remain double-stranded.



C. Free end of linkers are rendered competent for ligation, either by, or in combination (i) phosphorylating 5' end, (ii) removing blocked 3' group, or (iii) using 5'-nuclease activity to cleave off matching 5'-overlapping base or flap, leaving ligation-competent 5'-phosphate. Ligation conditions are designed to favor multimerization.



D. Ligated products comprise of adjacent HaeIII sequences originally methylated at AclI sites in target DNA with optional unique identifier and/or patient identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.



Figure 52

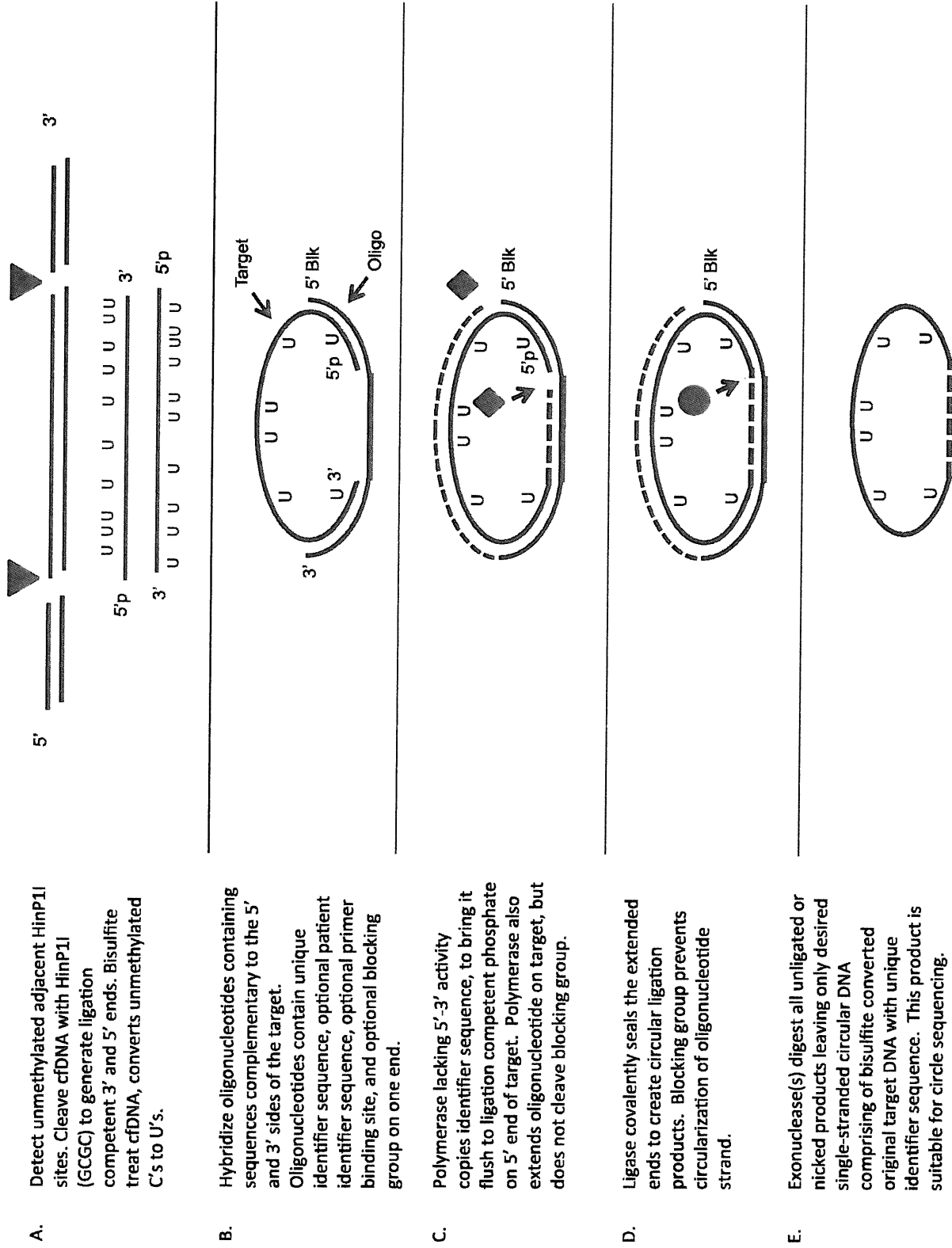


Figure 53

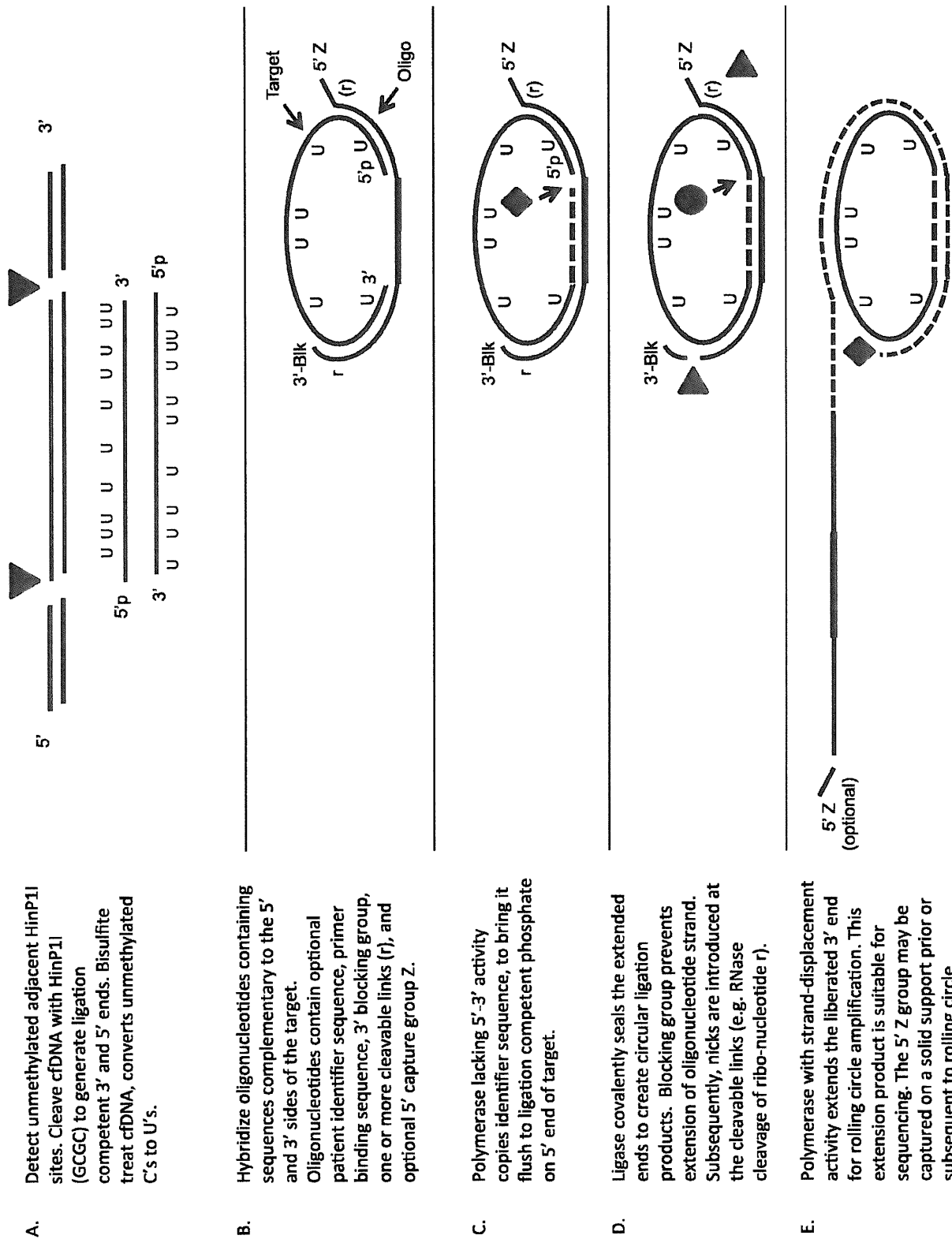


Figure 54

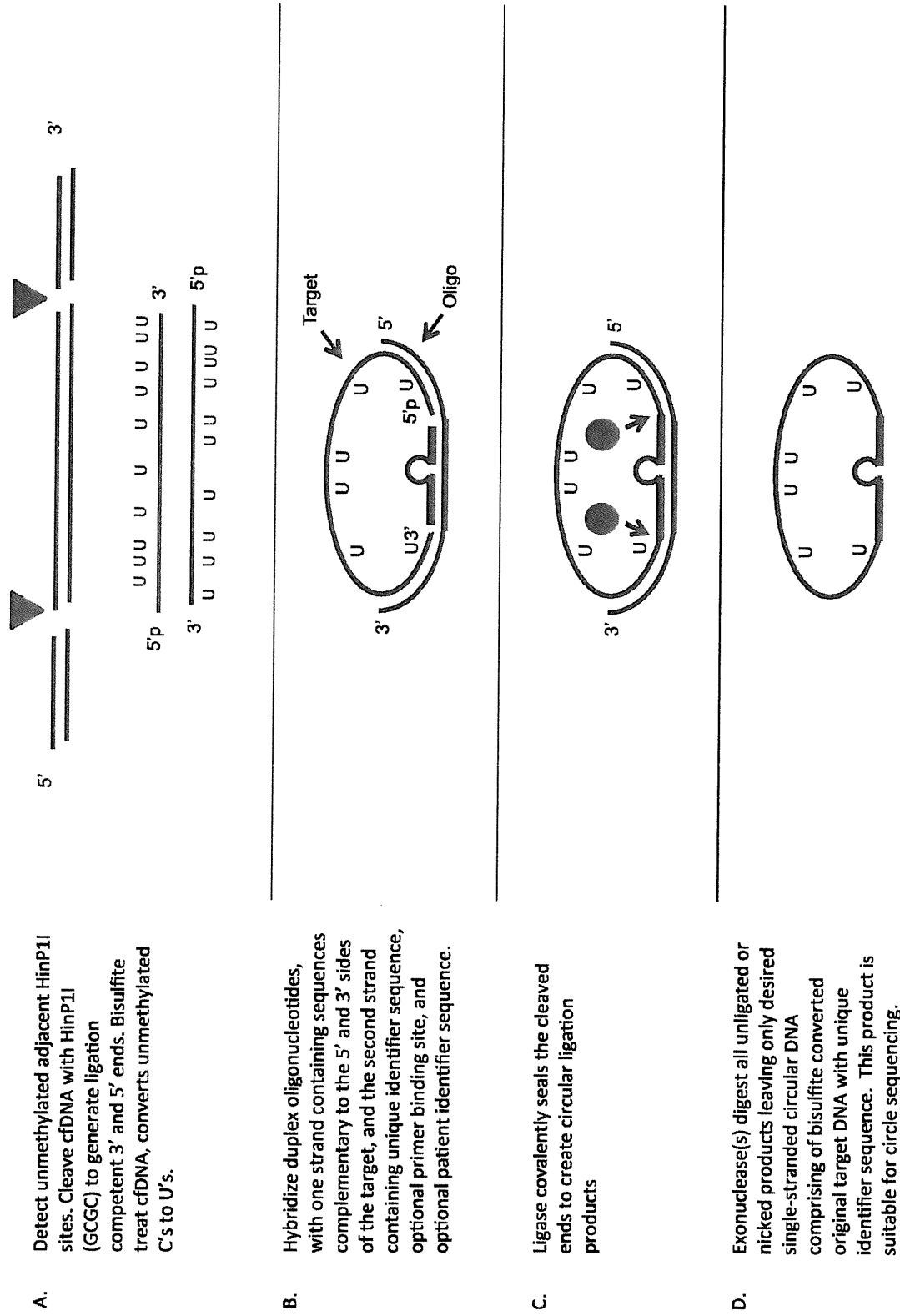
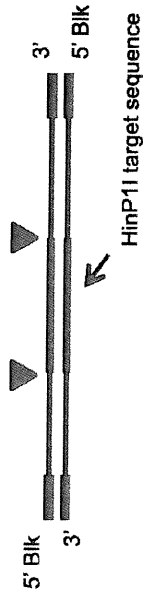
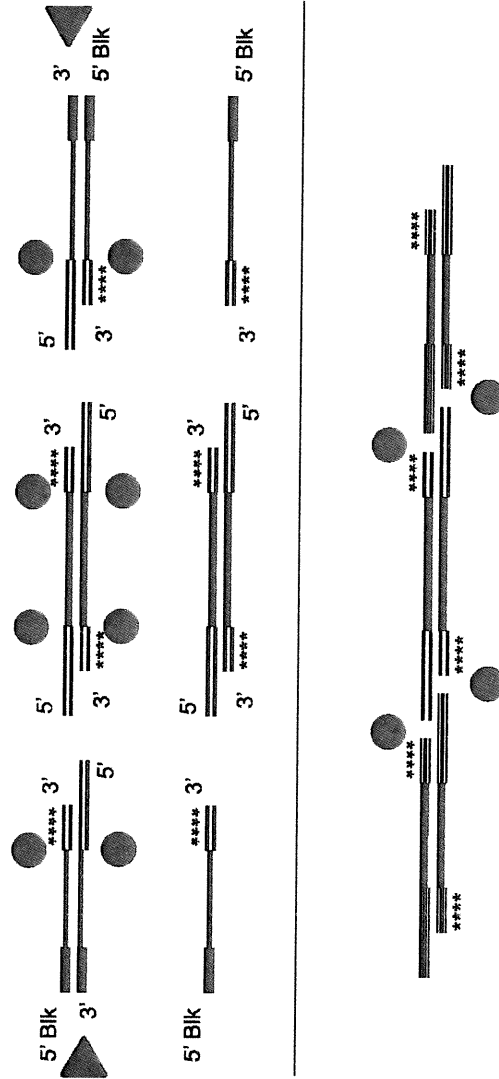


Figure 55

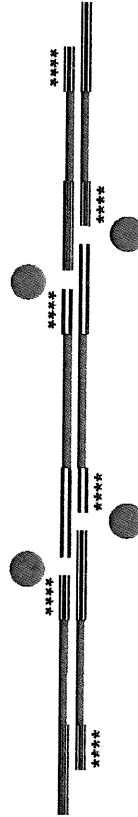
- A. Detect unmethylated adjacent HinP1I sites (GCGC) throughout genome. Ligate on short linkers with blocked 5' end. Cleave cfDNA with HinP1I (GCGC). Only adjacent HinP1I sites (GCGG) unmethylated in original target generate unblocked fragments when cleaved with HinP1I.



- B. Ligate on longer linkers containing optional unique identifier sequence, optional patient identifier sequence, with either blocked 3' end (not shown), or thiophosphate containing backbone to inhibit subsequent digestion with 3' exonuclease. Only fragments with linker ligated to both sides will remain double-stranded.



- C. Free end of linkers are rendered competent for ligation, either by, or in combination (i) phosphorylating 5' end, (ii) removing blocked 3' group, or (iii) using 5'-nuclease activity to cleave off matching 5'-overlapping base or flap, leaving ligation-competent 5'-phosphate. Ligation conditions are designed to favor multimerization.



- D. Bisulfite treat cfDNA, converts unmethylated C's to U's. Ligated products comprise bisulfite converted DNA, within adjacent HinP1I sequences originally unmethylated in target DNA with optional unique identifier and/or patient identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.

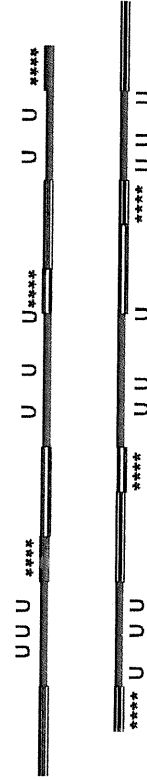
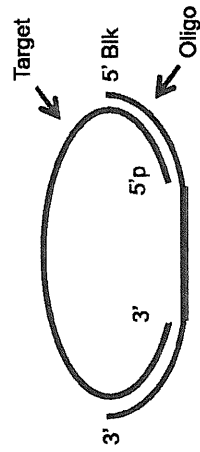


Figure 57

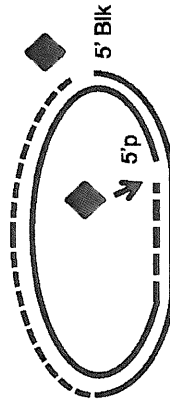
- A. Detect unmethylated adjacent "HphI" sites. Ligate on linkers. Bisulfite treat cDNA, converts unmethylated C's to U's, and do limited PCR. Cleave DNA with HphI (only unmethylated GGCGA → GGTGA) to generate ligation competent ends.



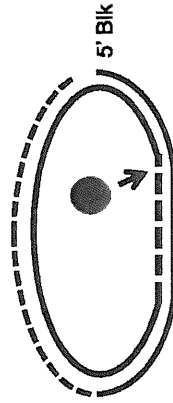
- B. Hybridize oligonucleotides containing sequences complementary to the 5' and 3' sides of the target. Oligonucleotides contain unique identifier sequence, optional patient identifier sequence, optional primer binding site, and optional blocking group on one end.



- C. Polymerase lacking 5'-3' activity copies identifier sequence, to bring it flush to ligation competent phosphate on 5' end of target. Polymerase also extends oligonucleotide on target, but does not cleave blocking group.



- D. Ligase covalently seals the extended ends to create circular ligation products. Blocking group prevents circularization of oligonucleotide strand.



- E. Exonuclease(s) digest all unligated or nicked products leaving only desired single-stranded circular DNA comprising of the complement of bisulfite converted target DNA with unique identifier sequence. This product is suitable for circle sequencing.

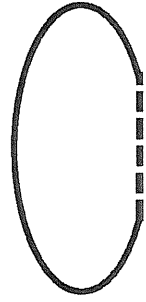
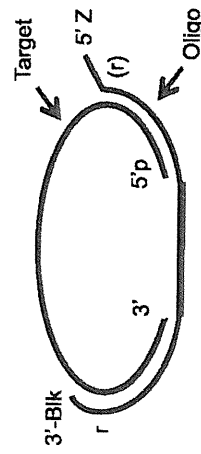


Figure 58

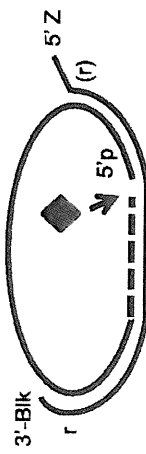
A. Detect unmethylated adjacent "HphI" sites. Ligate on linkers. Bisulfite treat cDNA, converts unmethylated C's to U's, and do limited PCR. Cleave DNA with HphI (only unmethylated GGCGA → GGTGA) to generate ligation competent ends.



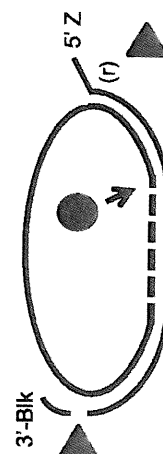
B. Hybridize oligonucleotides containing sequences complementary to the 5' and 3' sides of the target. Oligonucleotides contain optional patient identifier sequence, primer binding sequence, 3' blocking group, one or more cleavable links (r), and optional 5' capture group Z.



C. Polymerase lacking 5'-3' activity copies identifier sequence, to bring it flush to ligation competent phosphate on 5' end of target.



D. Ligase covalently seals the extended ends to create circular ligation products. Blocking group prevents extension of oligonucleotide strand. Subsequently, nicks are introduced at the cleavable links (e.g. RNase cleavage of ribo-nucleotide r).



E. Polymerase with strand-displacement activity extends the liberated 3' end for rolling circle amplification. This extension product is suitable for sequencing. The 5' Z group may be captured on a solid support prior or subsequent to rolling circle amplification.

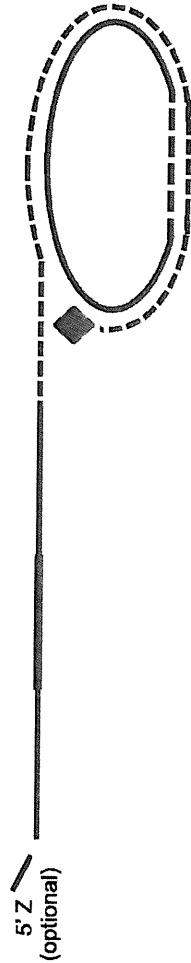


Figure 59

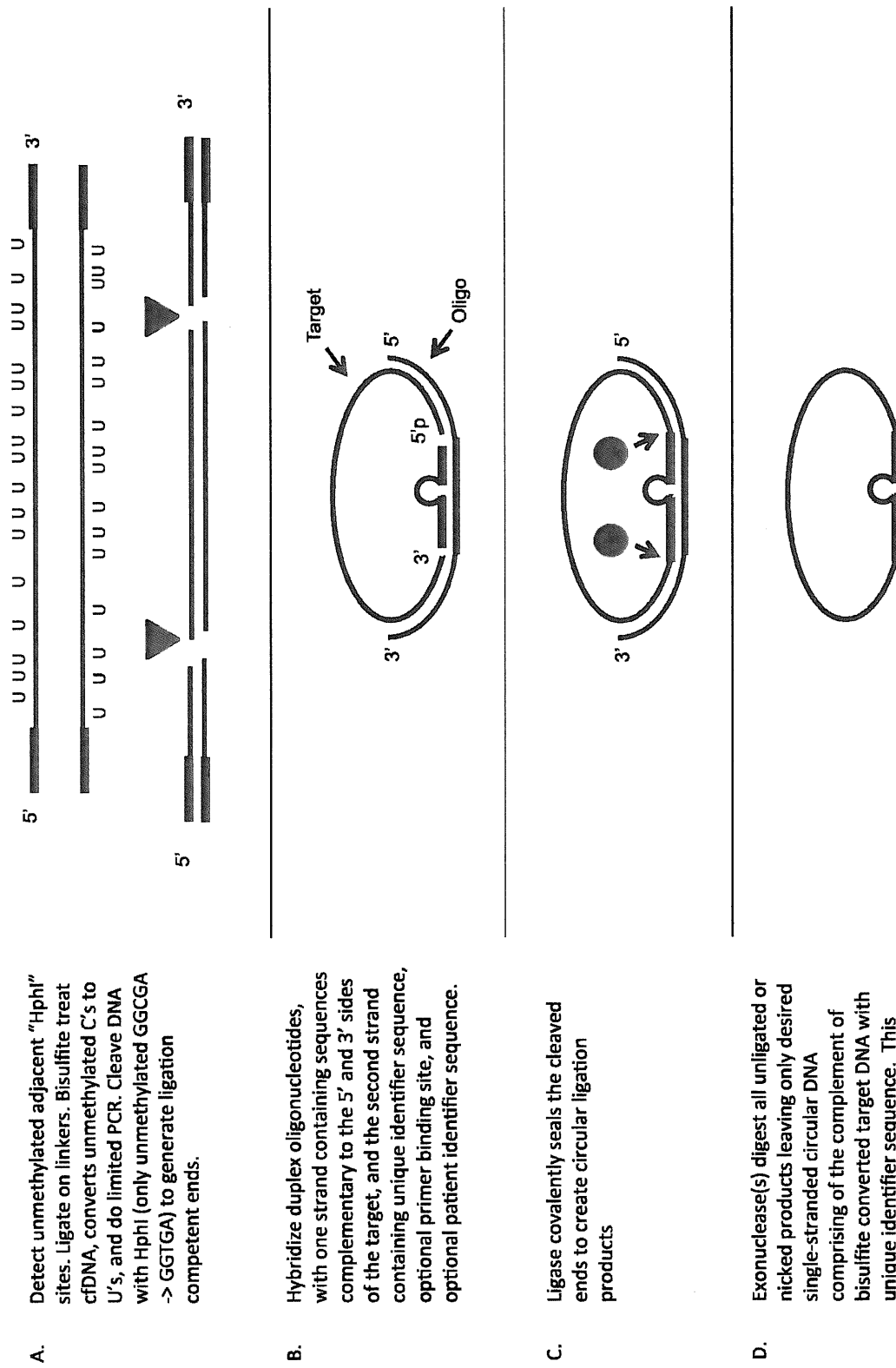


Figure 60

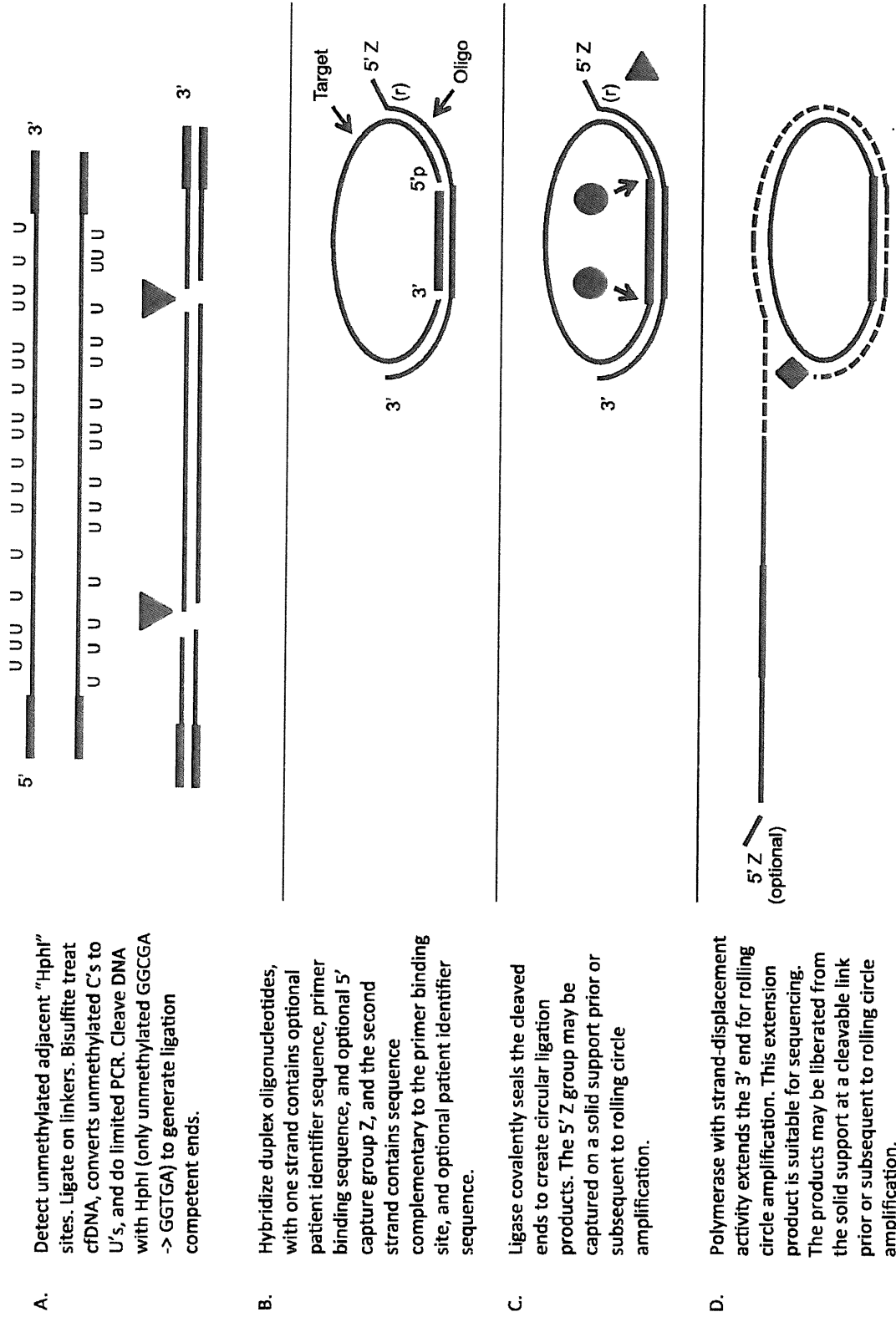
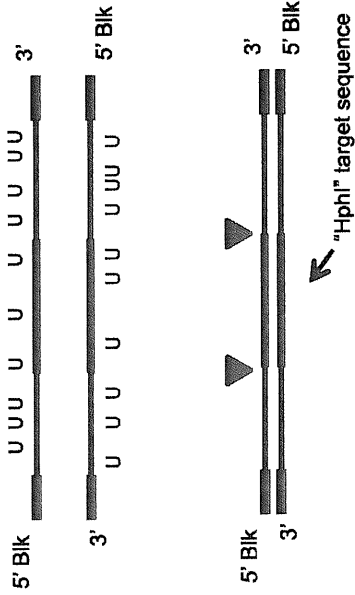
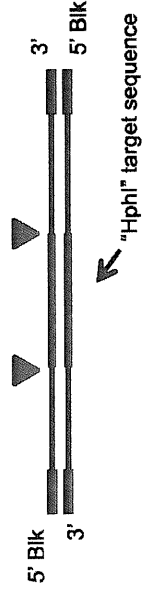


Figure 61

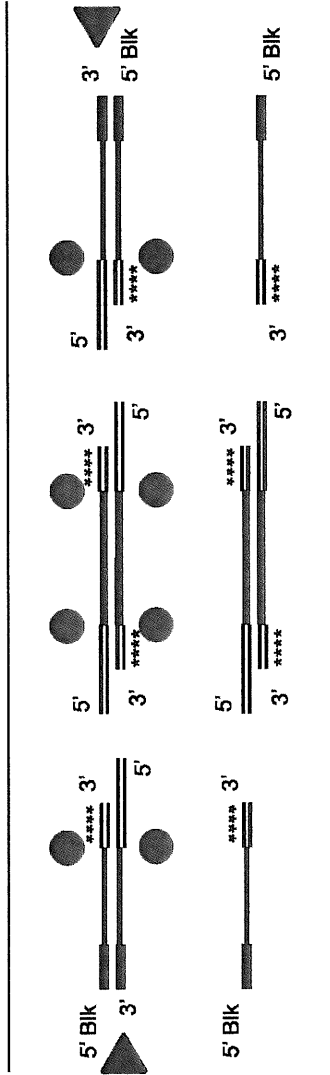
A. Detect unmethylation at adjacent "HphI" sites throughout genome. Ligate on linkers with blocked 5' end. Bisulfite treat cDNA, converts unmethylated C's to U's.



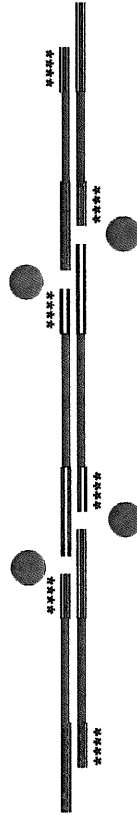
B. Limited PCR amplification with 5' blocked primers generates unmethylated products. Only adjacent HphI sites (unmethylated GGCGA → GGTGA) unmethylated in original target generate unblocked fragments when cleaved with HphI.



C. Ligate on linkers containing optional unique identifier sequence, optional patient identifier sequence, with either blocked 3' end (not shown), or thiophosphate containing backbone to inhibit subsequent digestion with 3' exonuclease. Only fragments with linker ligated to both sides will remain double-stranded.



D. Free end of linkers are rendered competent for ligation, either by, or in combination (i) phosphorylating 5' end, (ii) removing blocked 3' group, or (iii) using 5'-nuclease activity to cleave off matching 5'-overlapping base or flap, leaving ligation-competent 5'-phosphate. Ligation conditions are designed to favor multimerization.



E. Ligated products comprise complement of bisulfite converted DNA, within adjacent "HphI" sequences originally unmethylated in target DNA with optional unique identifier and/or patient identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.



Figure 62

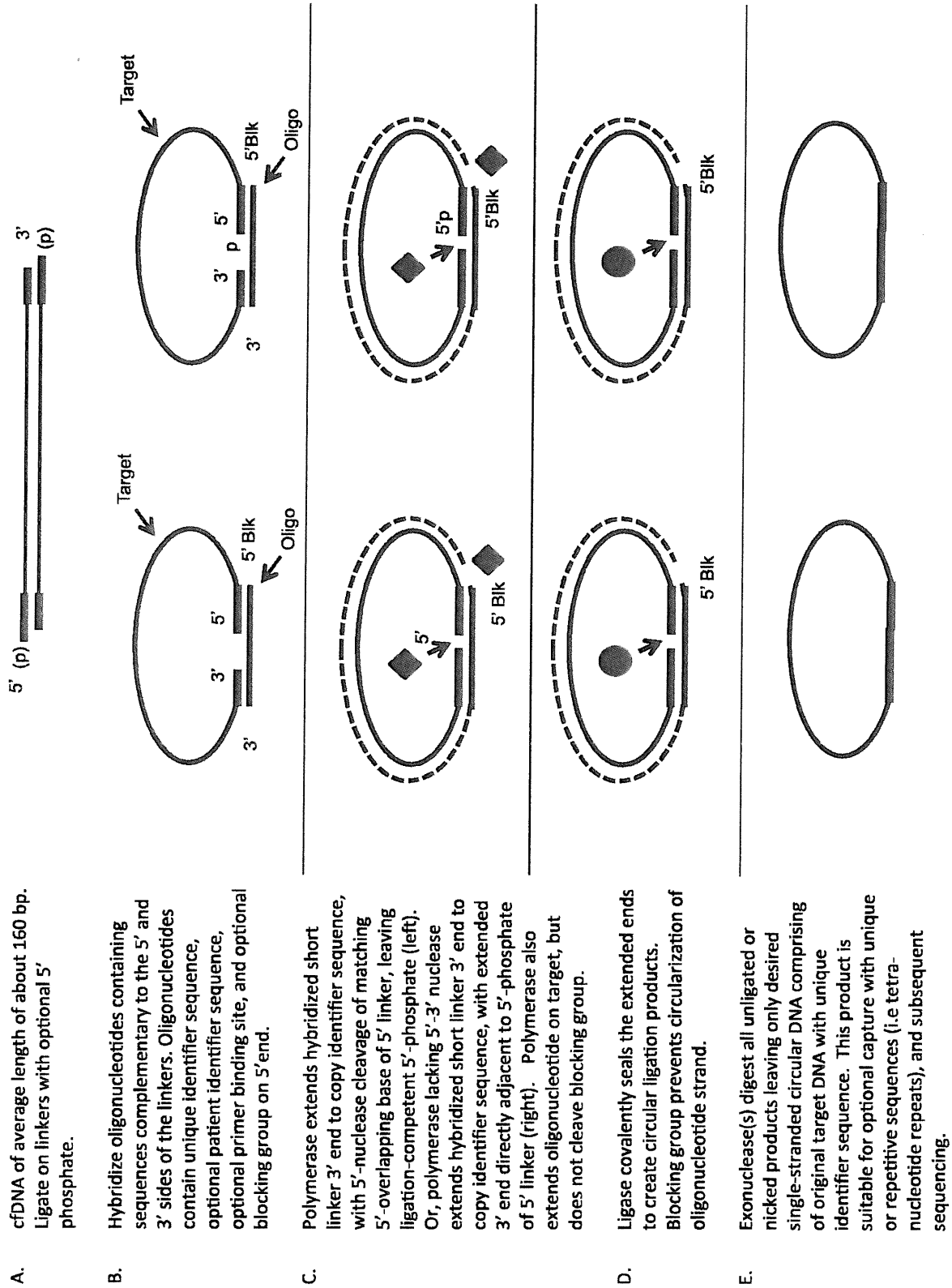
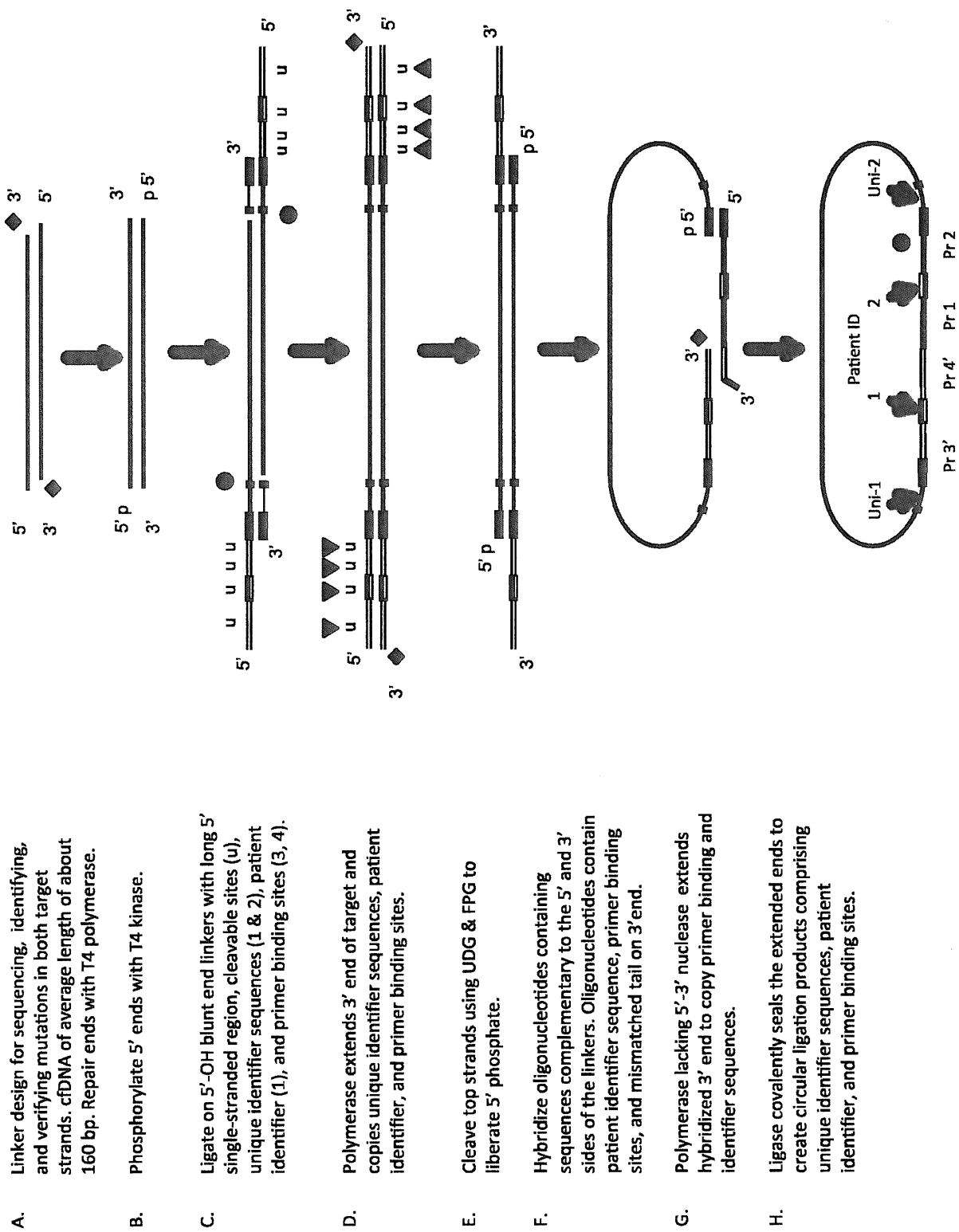


Figure 63



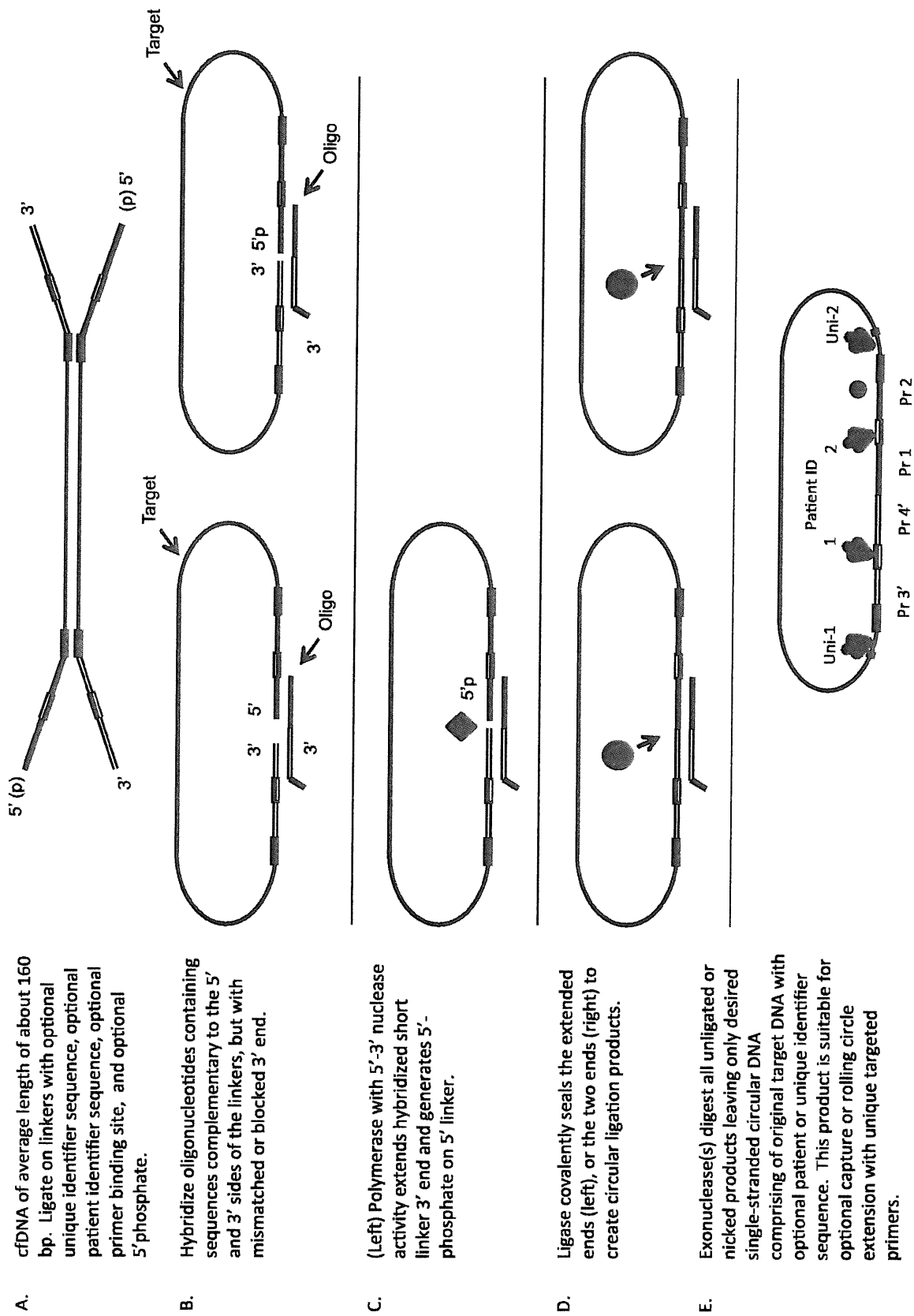


Figure 65

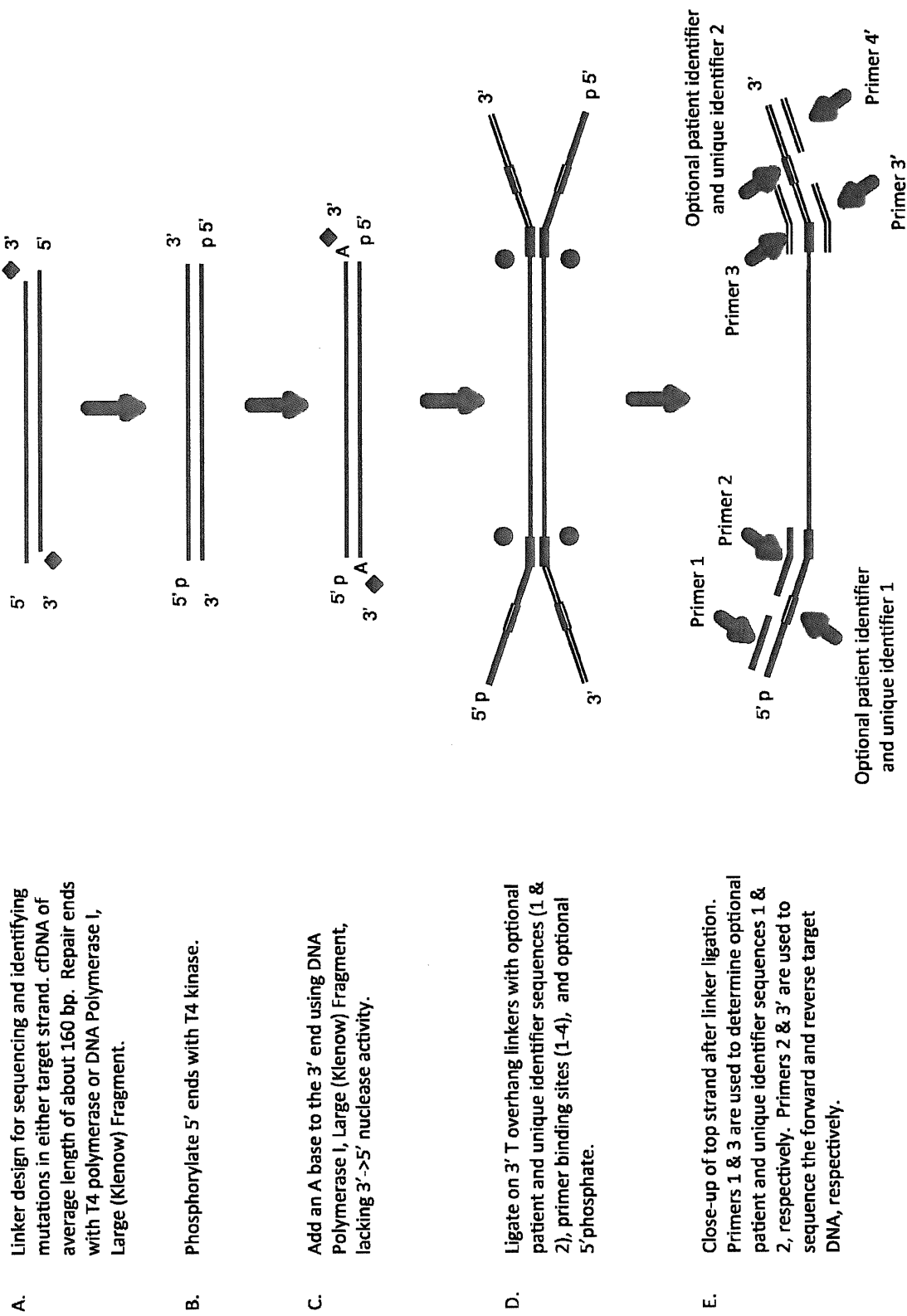


Figure 66

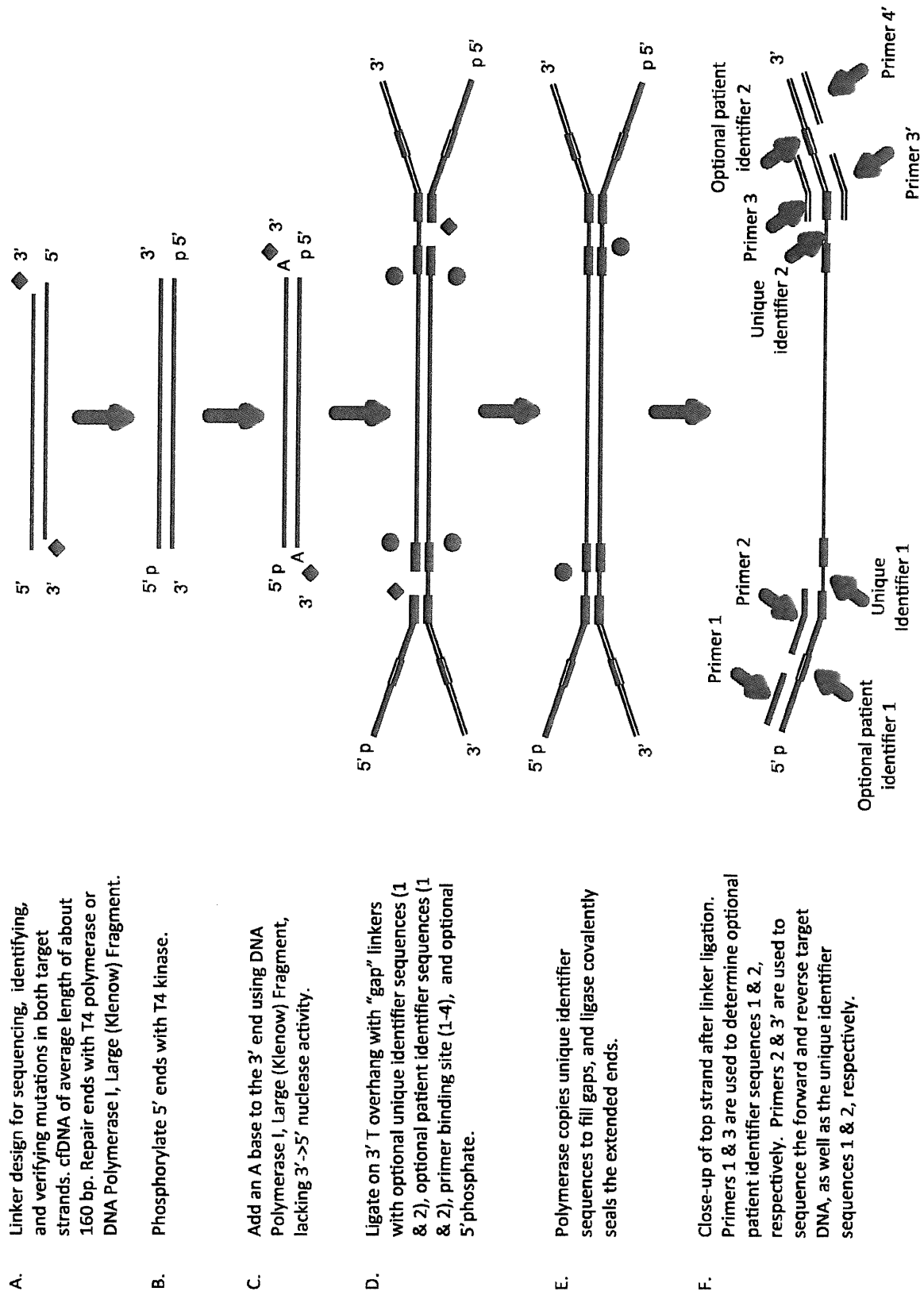


Figure 67

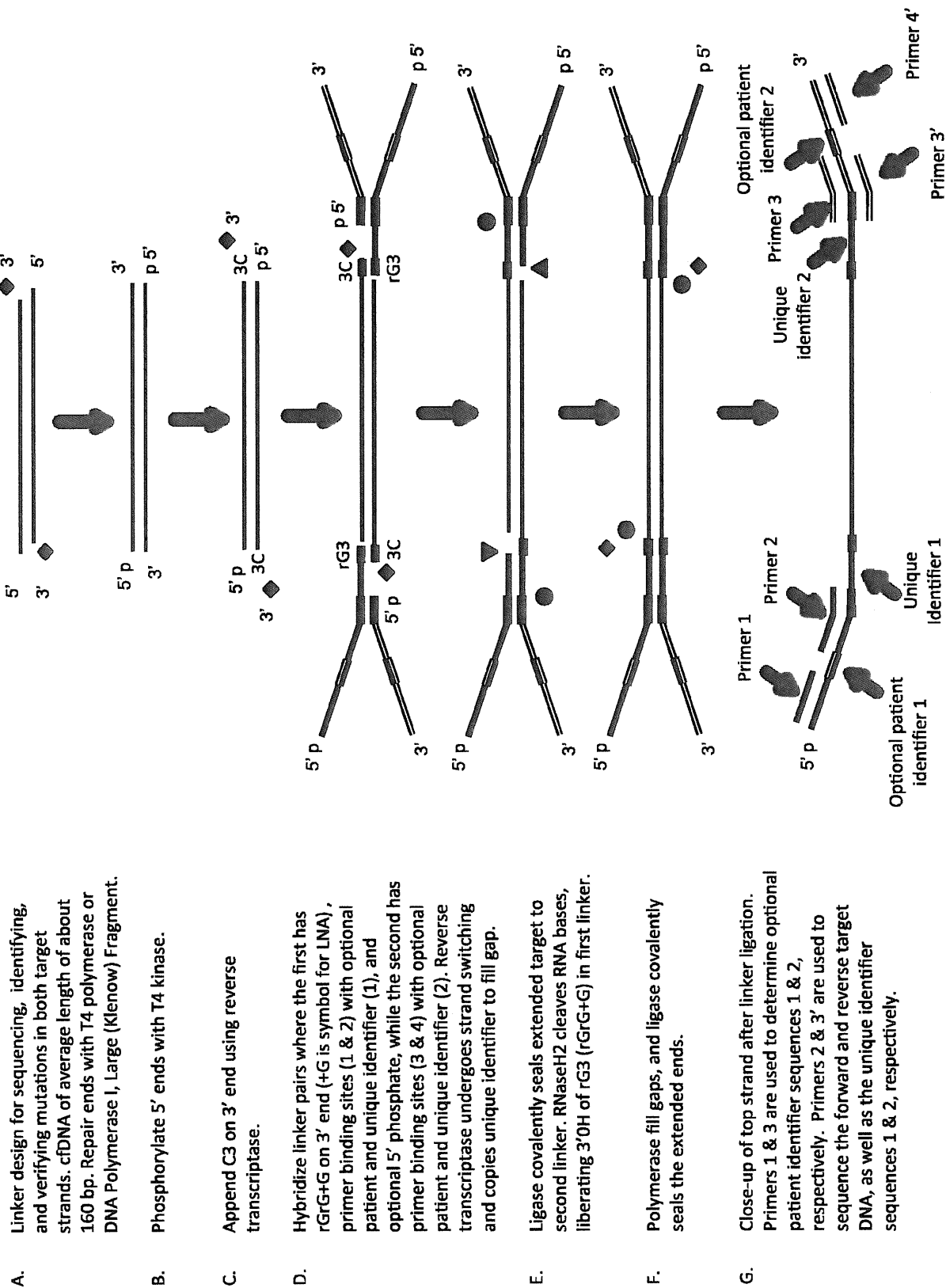


Figure 68

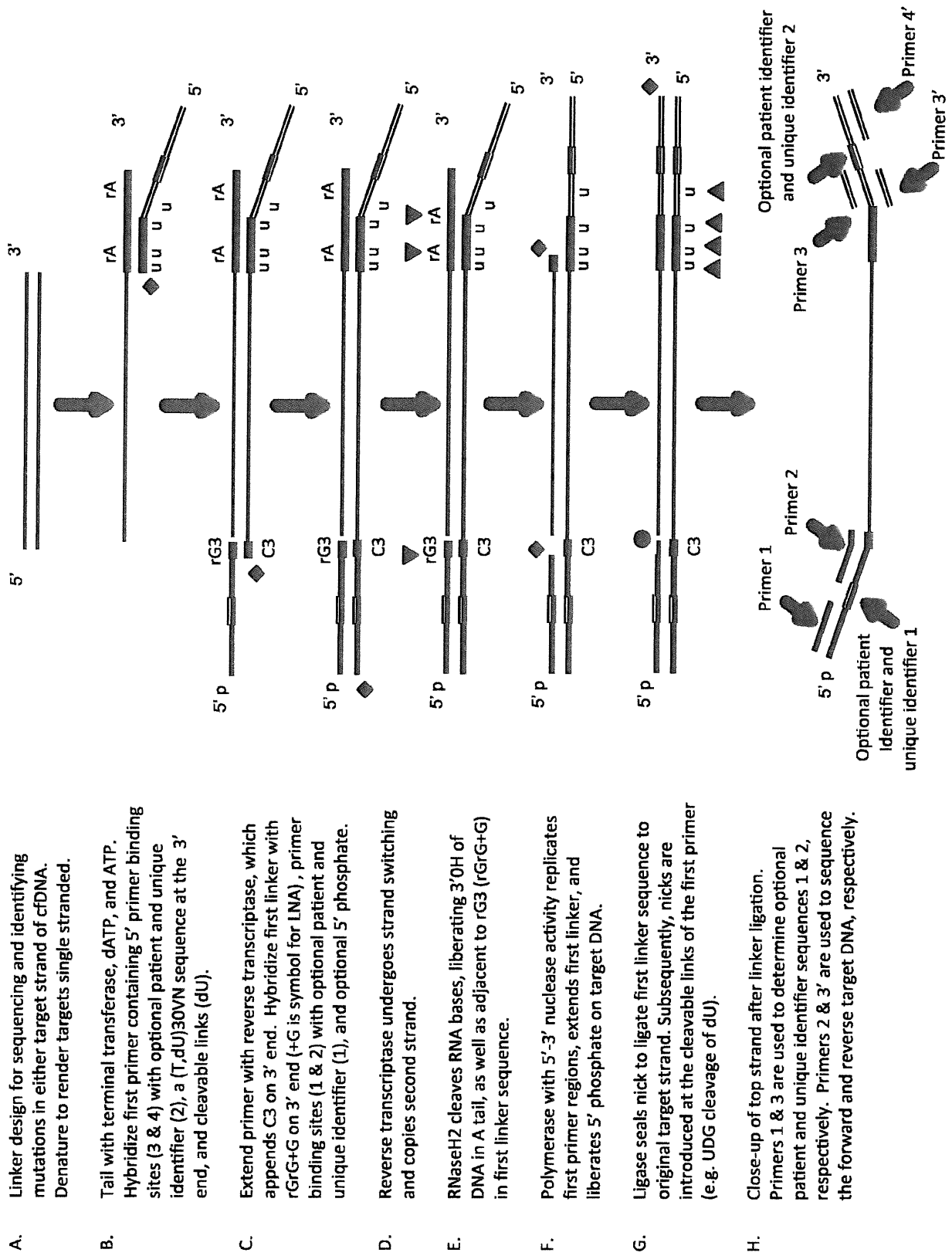


Figure 69

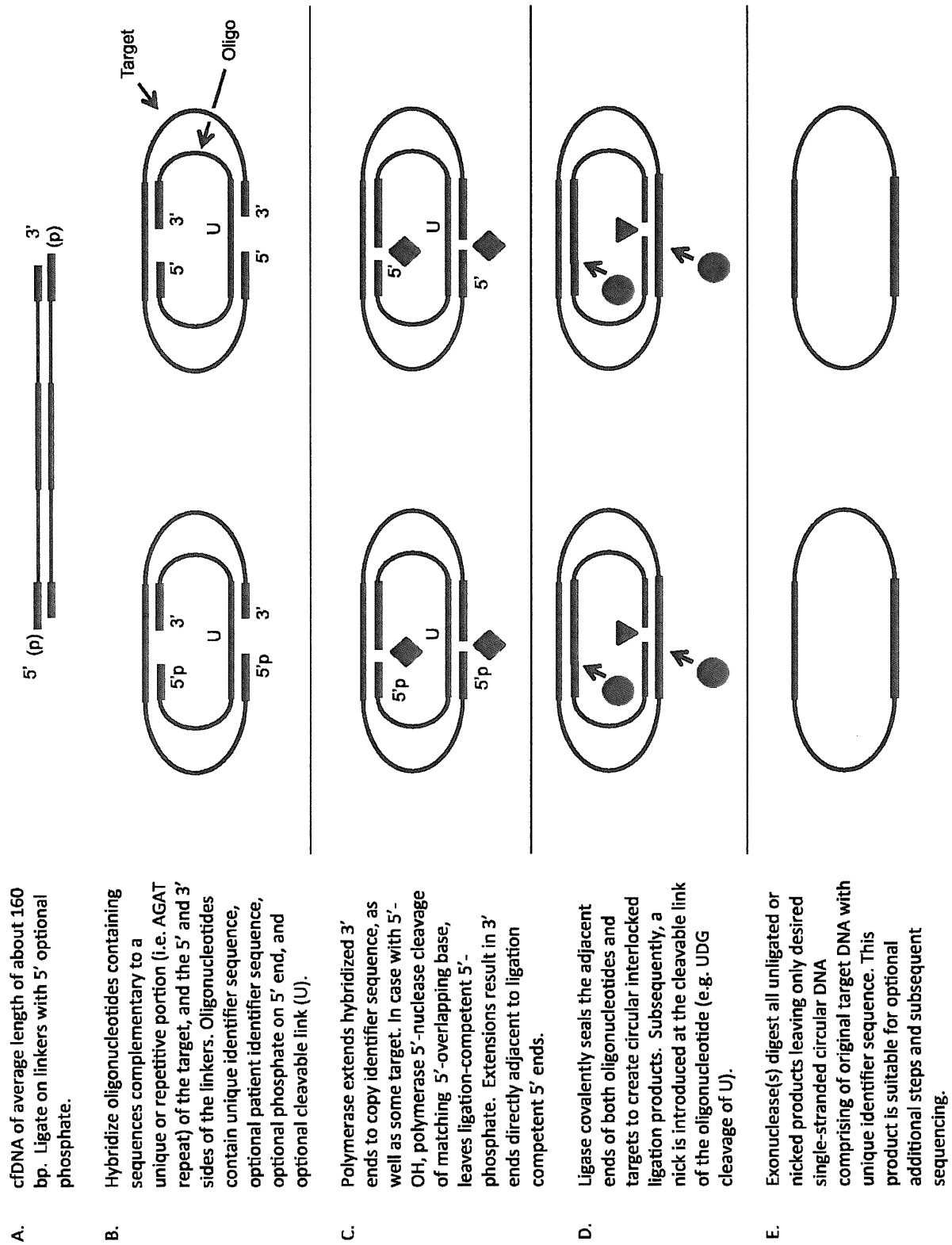


Figure 70

- A. cfDNA of average length of about 160 bp. Blunt ends, extend 3' end with a single A base, and ligate on A:T rich linkers with single-base T overhang and 5' phosphate on other end.
- B. Hybridize oligonucleotides containing sequences complementary to a unique portion of the target, and the 5' and 3' sides of the linkers. Oligonucleotides contain optional unique identifier sequence, optional patient identifier sequence, optional phosphate on 5' end, a blocked 3' end, and optional cleavable link (dU).
- C. Oligonucleotides are unblocked with RNaseH2 only when bound to target. Polymerase extends hybridized 3' linker and oligonucleotide ends to copy identifier sequence, as well as some target. In case with 5'-OH, polymerase 5'-nuclease cleavage of matching 5'-overlapping base, leaves ligation-competent 5'-phosphate. Extensions result in 3' ends directly adjacent to ligation competent 5' ends.
- D. Ligase covalently seals the adjacent ends of both oligonucleotides and targets to create circular interlocked ligation products. Subsequently, a nick is introduced at the cleavable link of the oligonucleotide (e.g. UDG cleavage of dU).
- E. Exonuclease(s) digest all unligated or nicked products leaving only desired single-stranded circular DNA comprising of original target DNA with optional patient or unique identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.

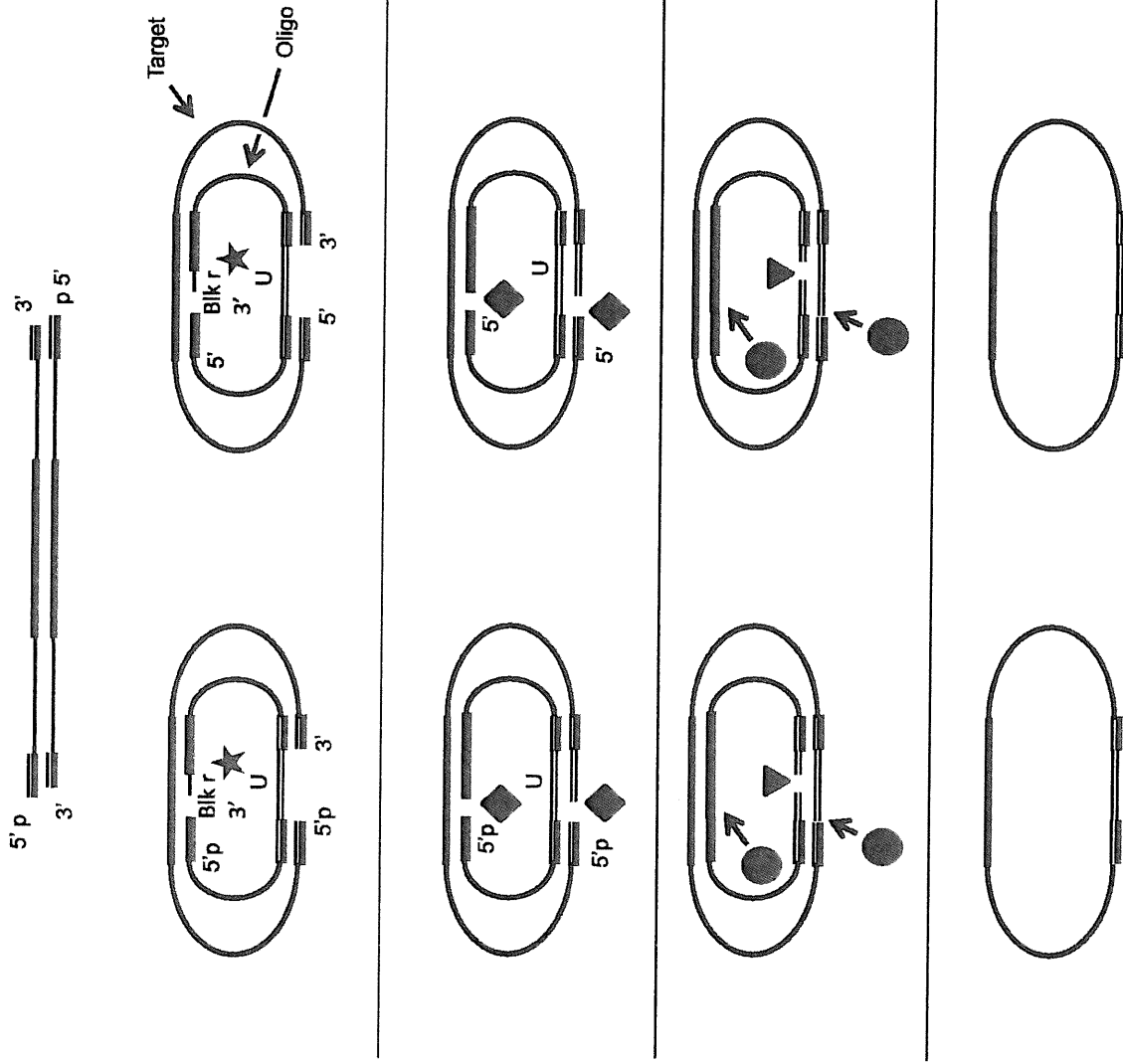


Figure 71

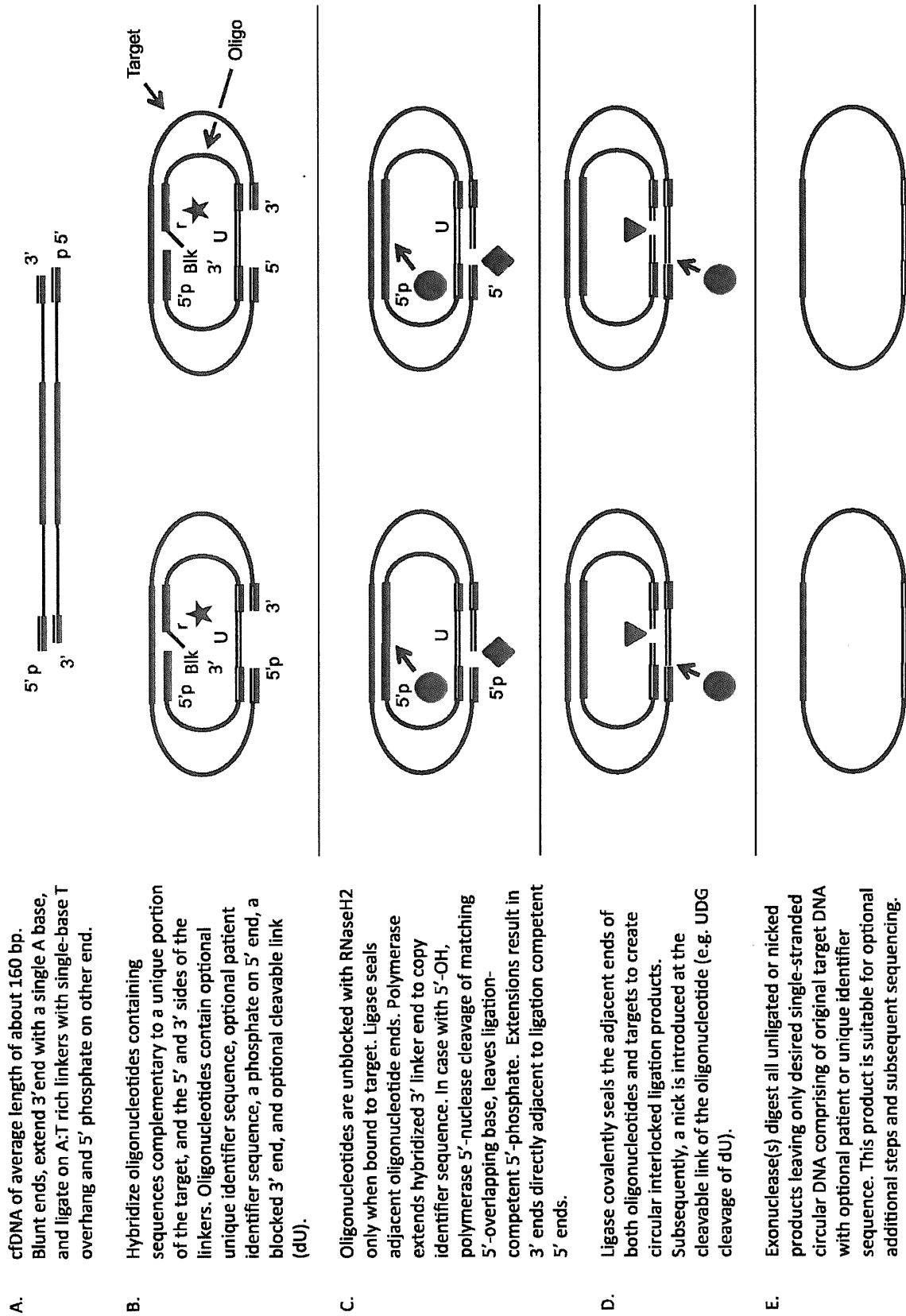


Figure 72

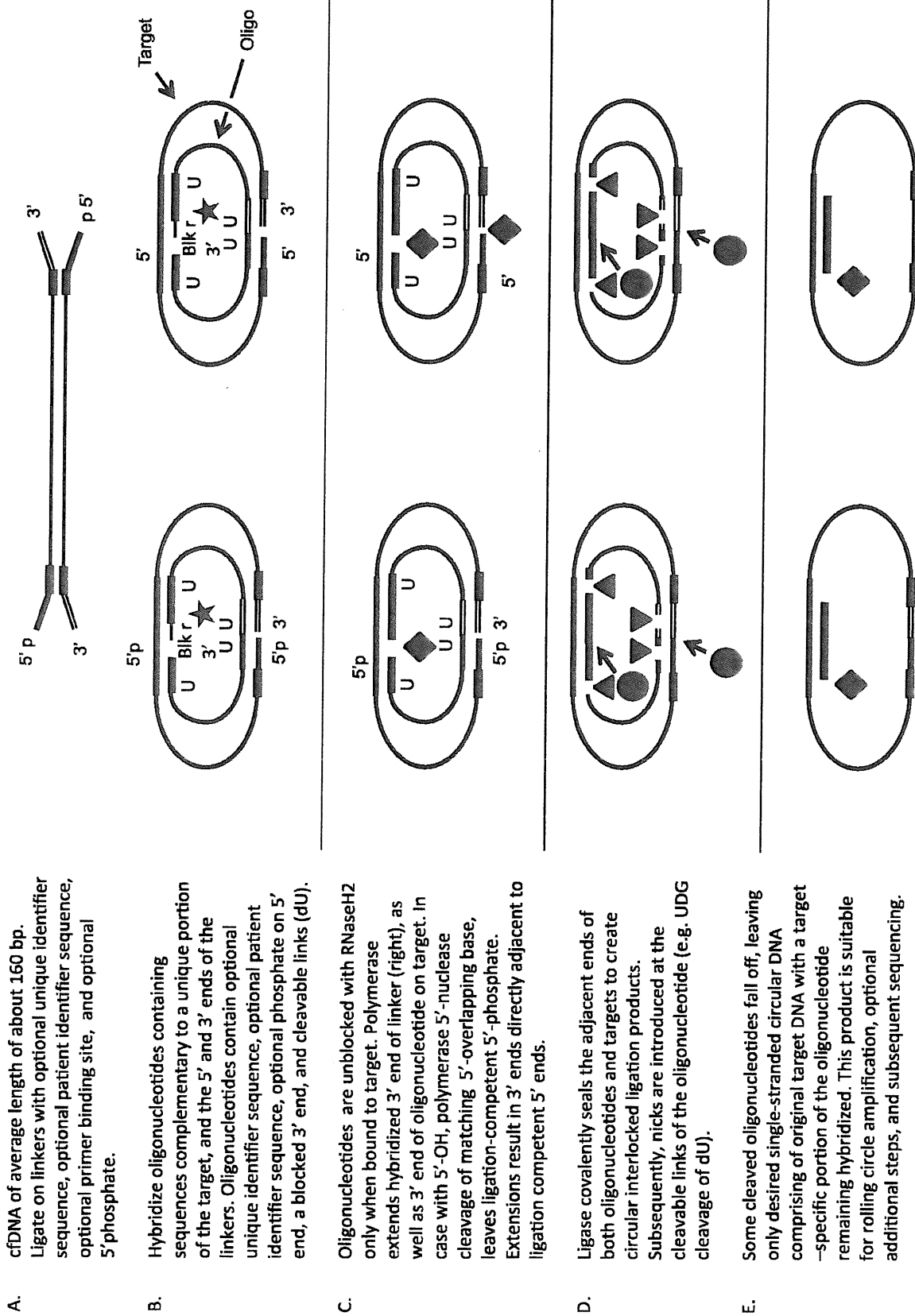


Figure 73

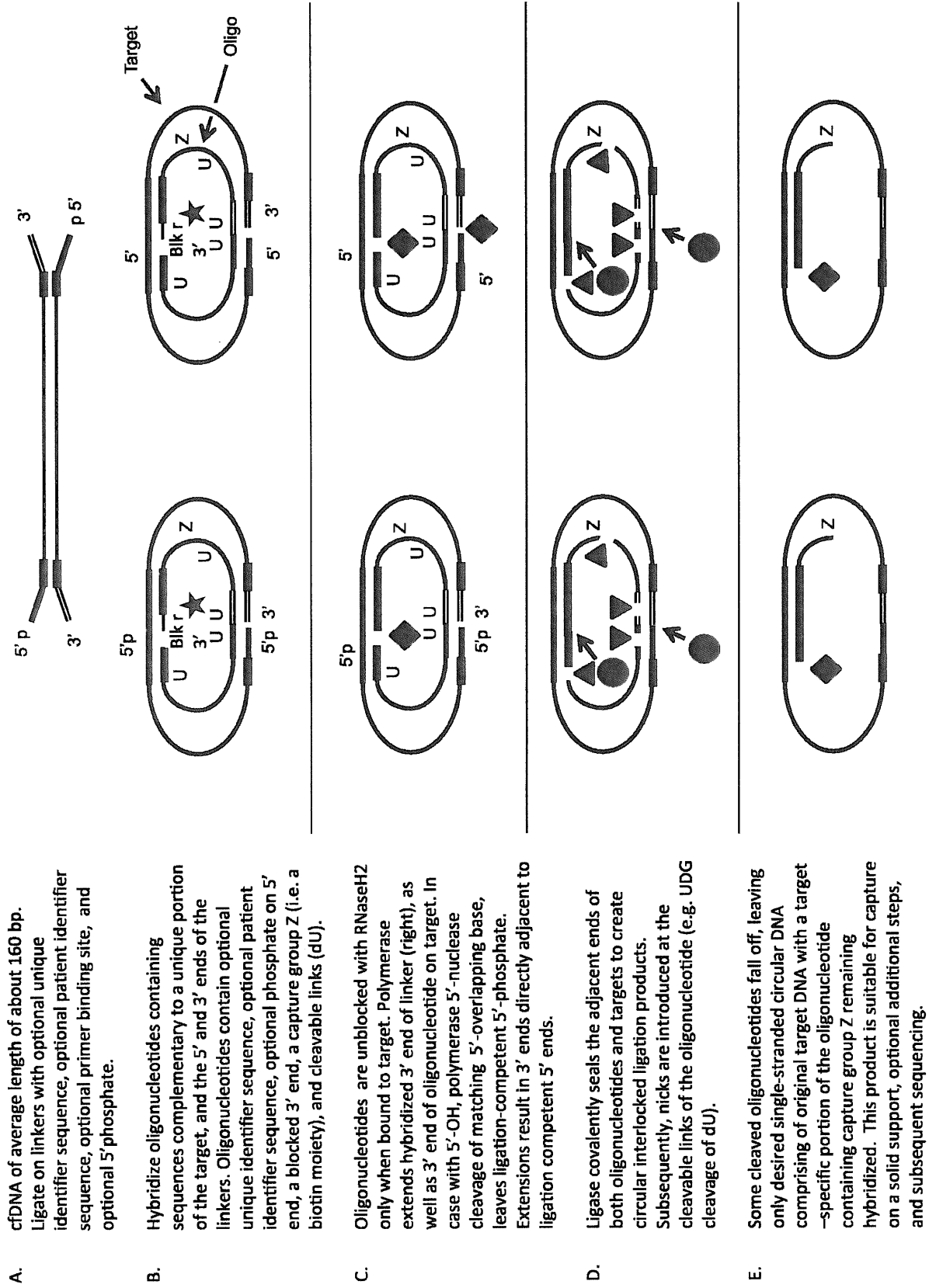


Figure74

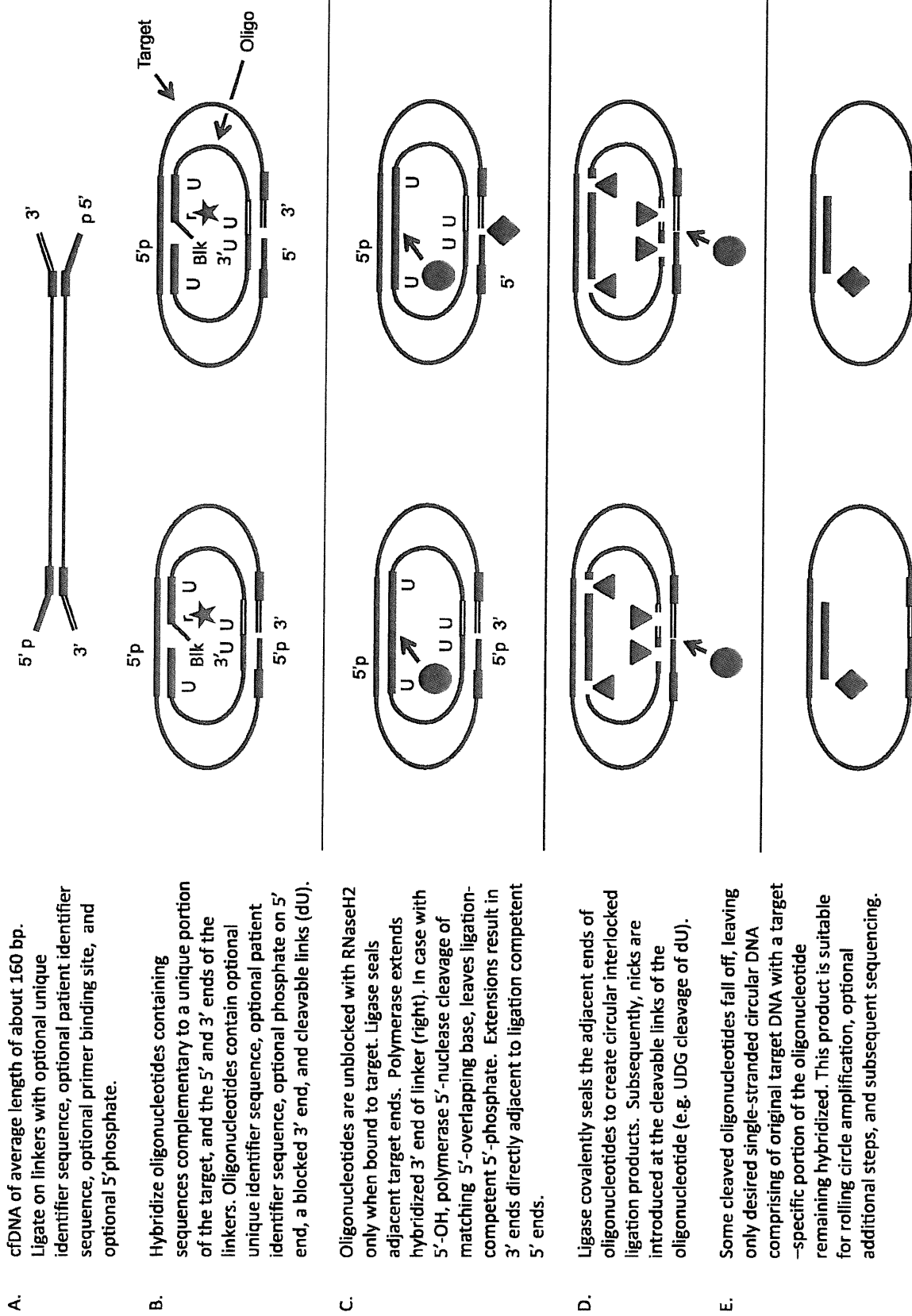


Figure 75

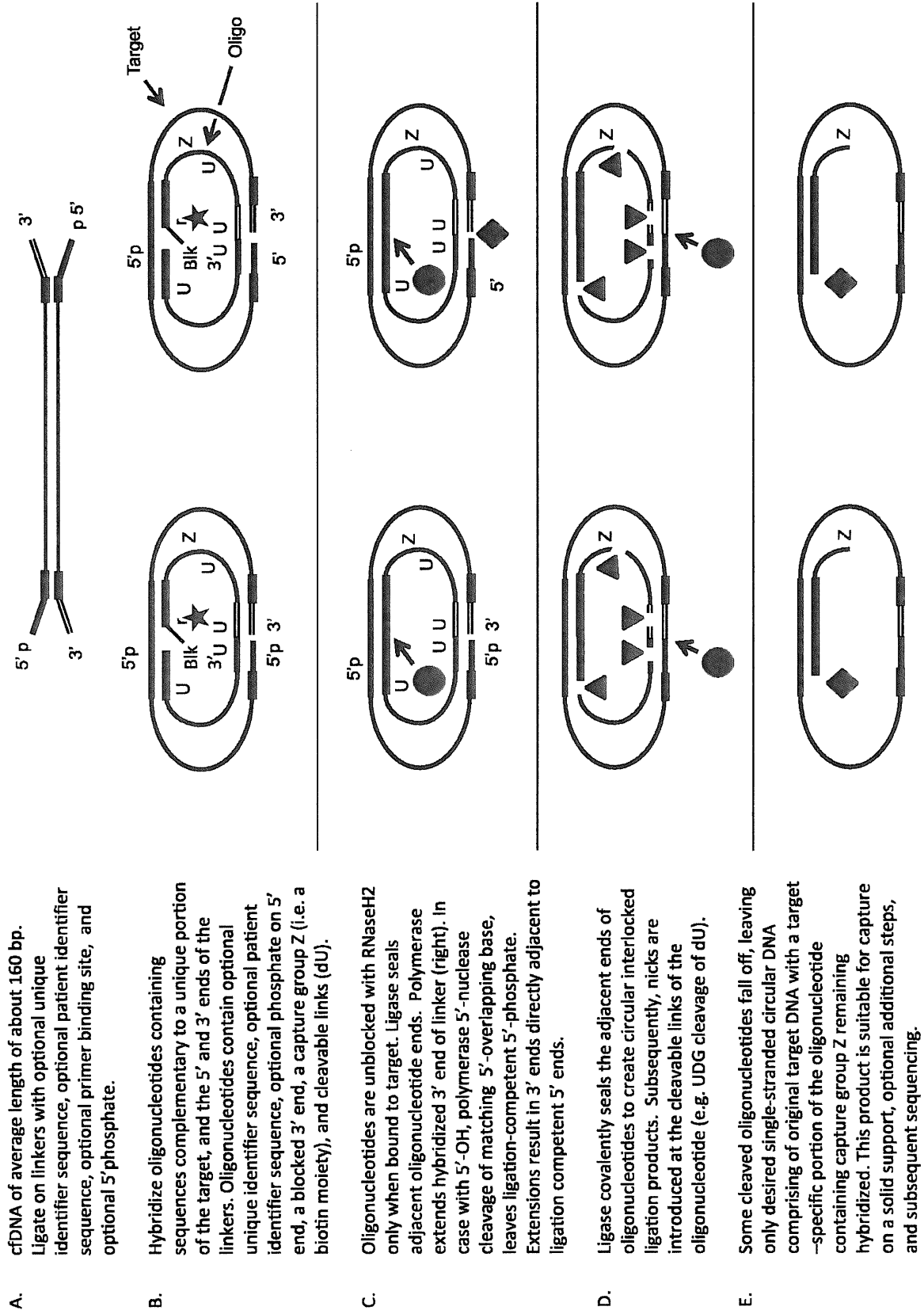


Figure 76

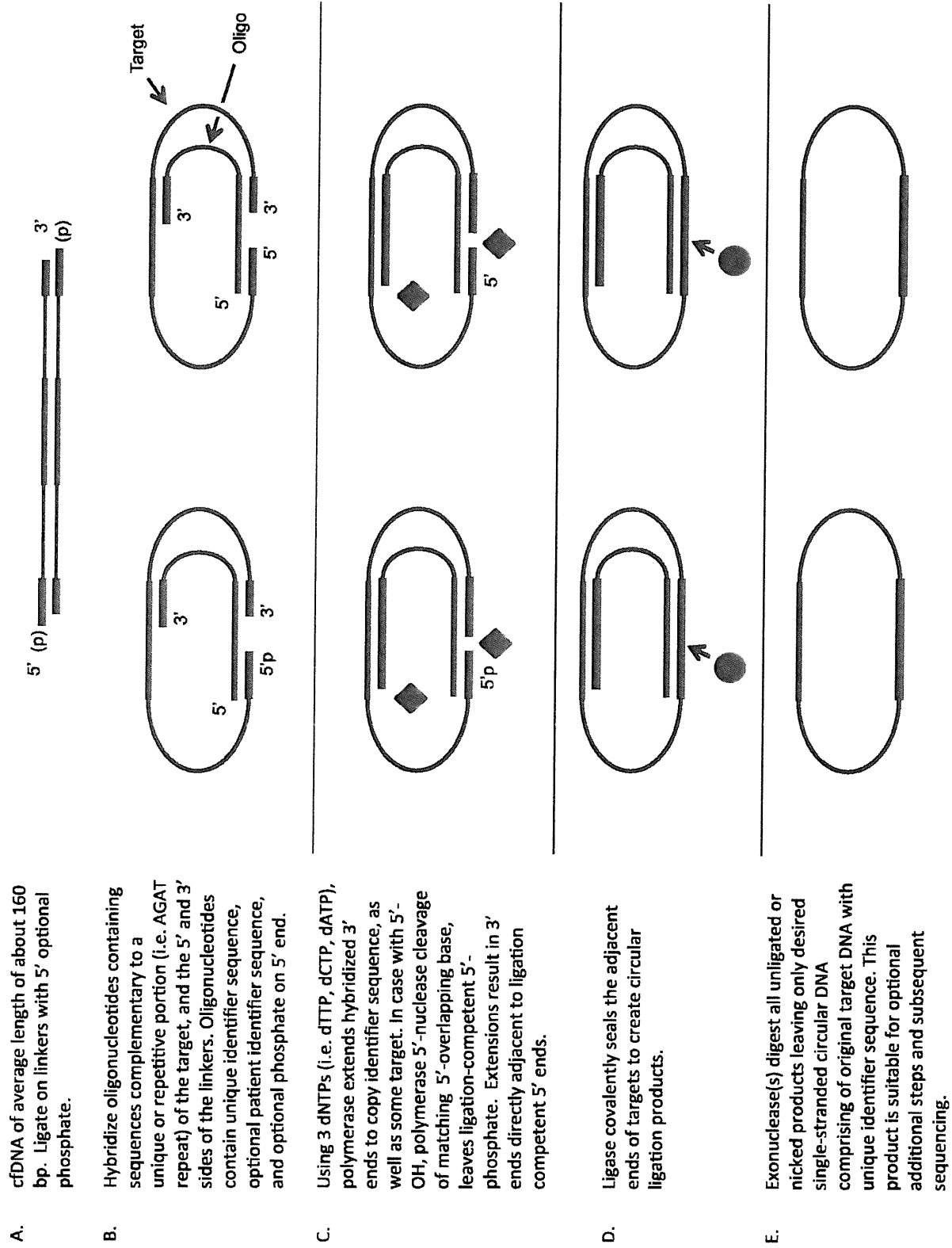


Figure 77

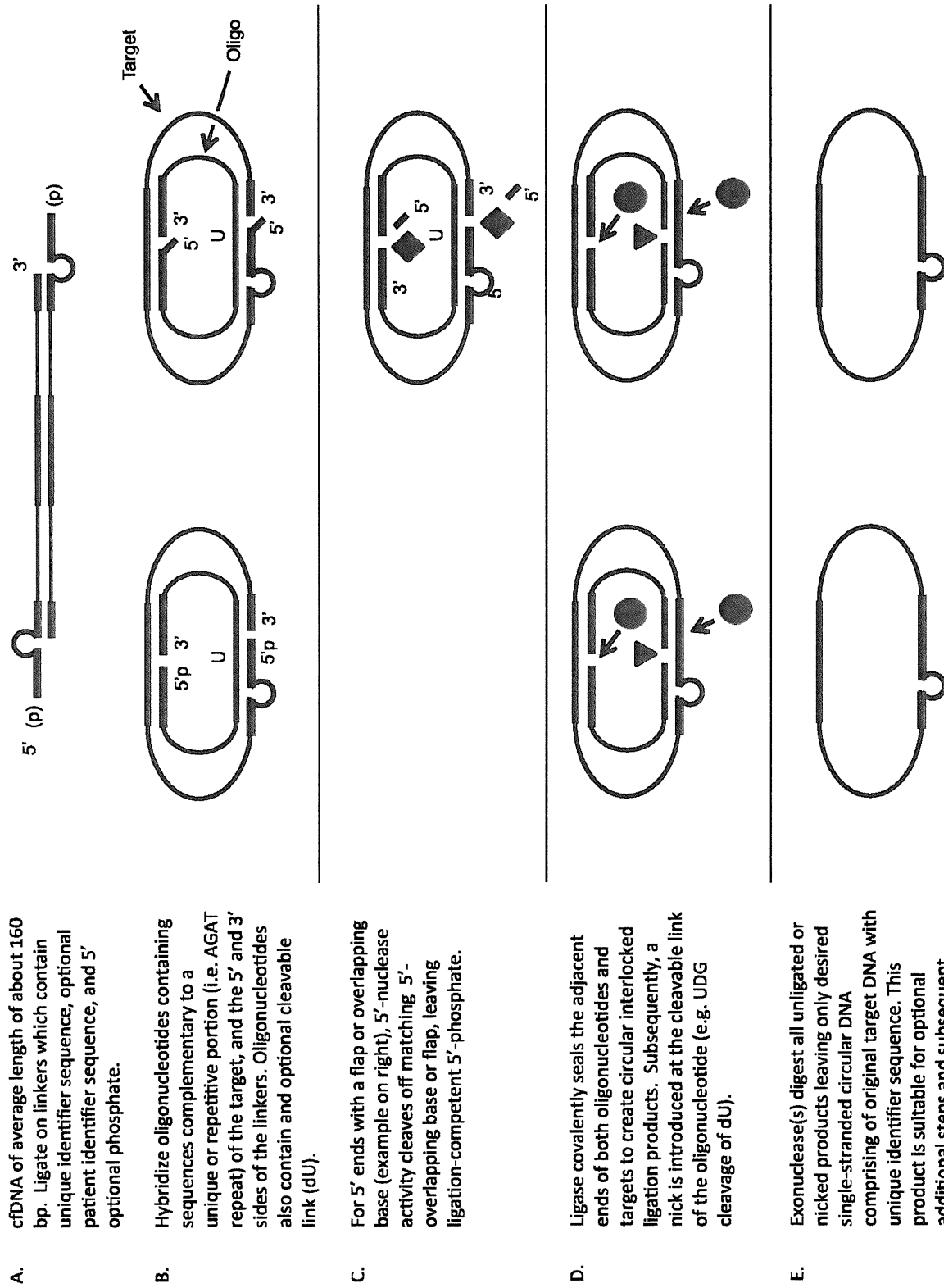
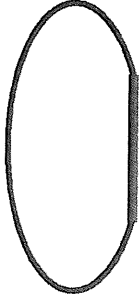
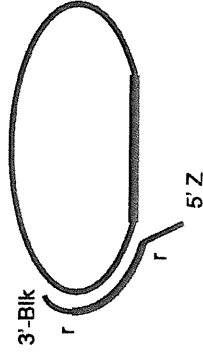


Figure 78

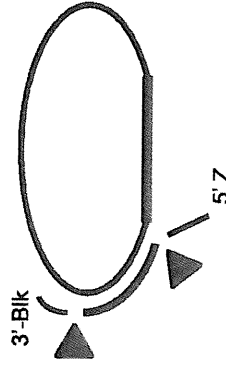
- A. (Single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.)



- B. Hybridize oligonucleotides containing sequences complementary to the target. Oligonucleotides contain 3' blocking group with one or more cleavable links (*r*), and optional 5' capture group Z (i.e. a biotin moiety). Target-specific circular targets are enriched by capturing onto a solid support via the Z group.



- C. Non-target containing circles are removed by washing. Nicks are introduced at the cleavable links (e.g. RNase cleavage of ribo-nucleotide *r*) to liberate the target-containing circles from the solid support, and generate a 3'-OH group.



- D. Polymerase with strand-displacement activity extends the liberated 3' end for rolling circle amplification. This extension product is suitable for sequencing.

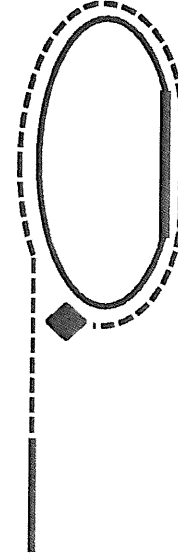
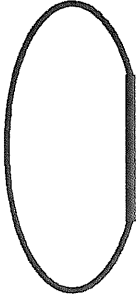
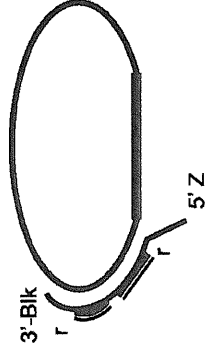


Figure 79

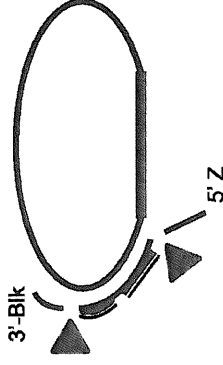
A. (Single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.)



B. Hybridize oligonucleotides containing a 5' 15-20 base anchor sequence, 3-5 mismatched bases, 6-10 matched bases, complementary to the target. Oligonucleotides contain 3' blocking group with cleavable links (r), and optional 5' capture group Z (i.e. a biotin moiety). Target-specific circular targets are enriched by capturing onto a solid support via the Z group.



C. Non-target containing circles are removed by washing. Nicks are introduced at the cleavable links (e.g. RNase cleavage of ribo-nucleotide r) to liberate the target-containing circles from the solid support, and generate a 3'-OH group.



D. Polymerase with strand-displacement activity extends the liberated 3' end for rolling circle amplification. This extension product is suitable for sequencing.

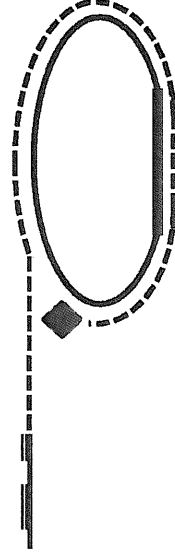
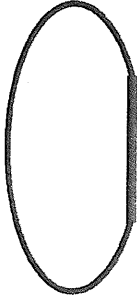
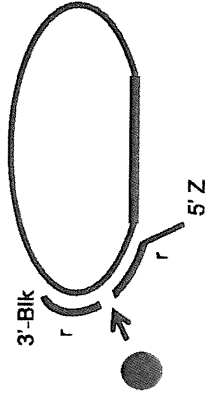


Figure 80

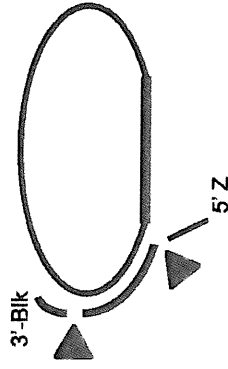
A. (Single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.)



B. Hybridize adjacent oligonucleotides containing sequences complementary to the target. Upstream oligonucleotides contains optional cleavable link and 5' capture group Z (i.e. a biotin moiety). Downstream oligonucleotides contain 5' phosphate and optional 3' blocking group with cleavable link (r). In the presence of target, ligase covalently links the oligonucleotides. Target-specific circular targets are enriched by capturing onto a solid support via the Z group.



C. Non-target containing circles are removed by washing. Nicks are introduced at the cleavable links (e.g. RNase cleavage of ribo-nucleotide r) to liberate the target-containing circles from the solid support, and generate a 3'-OH group.



D. Polymerase with strand-displacement activity extends the liberated 3' end for rolling circle amplification. This extension product is suitable for sequencing.

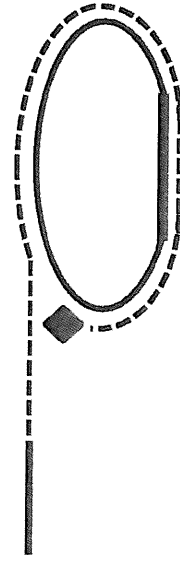
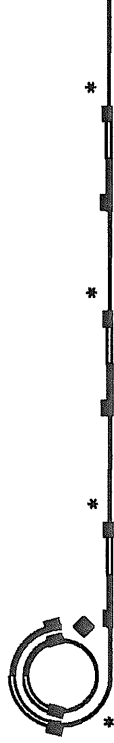


Figure 81

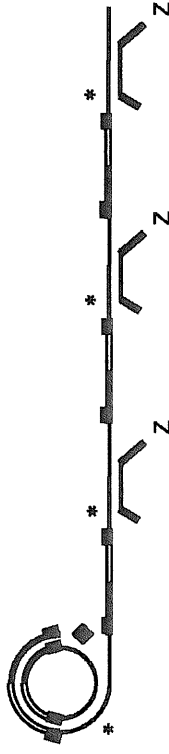
- A. Start with product comprising hybridized target-specific primer, or hybridize such primer to single-stranded circular target DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.



- B. Extend primer using strand-displacement polymerase to generate single strands of tandem copies.



- C. Hybridize oligonucleotides complementary to the extension product. Oligonucleotides are optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-specific tandem-repeat extension product on solid support and wash away other nucleic acids.



- D. Denature tandem repeat extension product from capture oligonucleotides on the solid support. This extension product is suitable for sequencing.

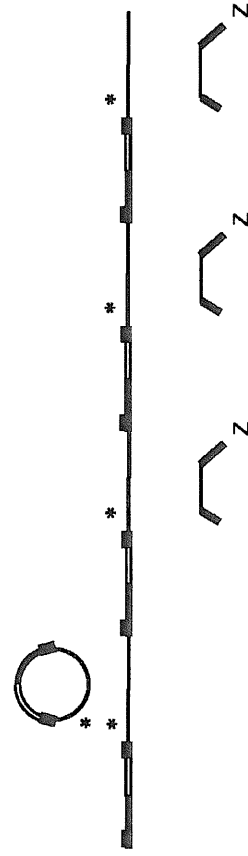


Figure 82

- A. Start with product comprising hybridized target-specific primer, or hybridize such primer to single-stranded target DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site. Primer contains capture group Z (i.e. a biotin moiety) for capture on a solid support.



- B. Wash away other nucleic acids. Extend primer using polymerase with 5'-3' nuclease activity, to generate nicked circles that are liberated from the solid support. Heat kill polymerase.



- C. Extend nicked circles using strand-displacement polymerase to generate single strands of tandem copies containing target DNA. This extension product is suitable for sequencing.



Figure 83

A. Start with product comprising hybridized target-specific primer, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.



B. Hybridize oligonucleotides complementary to the target DNA. Oligonucleotides are optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-containing circular DNA on solid support and wash away other nucleic acids.



C. Extend primer using strand-displacement polymerase to generate single strands of tandem copies of target DNA, and liberate extension product from the solid support. This extension product is suitable for sequencing.

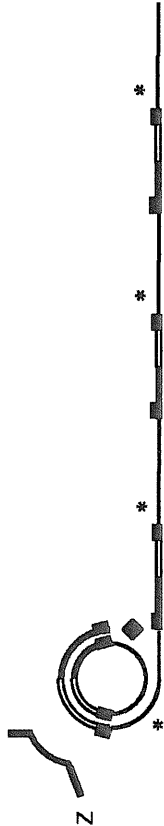
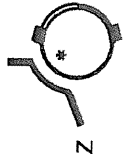


Figure 84

A. Start with product comprising hybridized target-specific oligonucleotide, or hybridize such oligonucleotide to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.



B. Oligonucleotides are optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-containing circular DNA on solid support and wash away other nucleic acids. Hybridize target-specific primers either before or after capture.



C. Extend primer using strand-displacement polymerase to generate single strands of tandem copies of target DNA, and liberate extension product from the solid support. This extension product is suitable for sequencing.

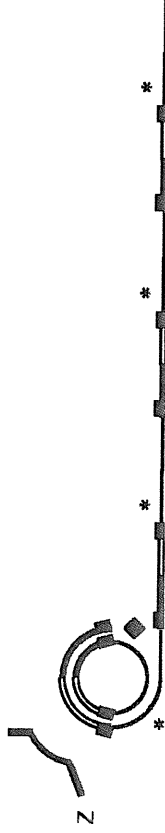
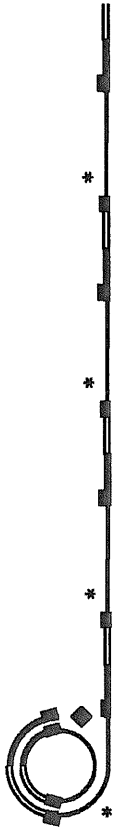


Figure 85

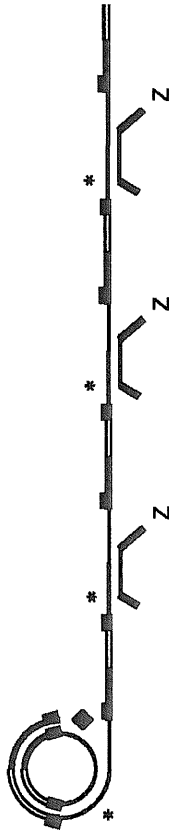
A. Start with product comprising primer hybridized to primer binding site, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, and primer binding site.



B. Extend primer using strand-displacement polymerase to generate single strands of tandem copies.



C. Hybridize oligonucleotides complementary to the extension product. Oligonucleotides are optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-specific tandem-repeat extension product on solid support and wash away other nucleic acids.



D. Denature tandem repeat extension product from capture oligonucleotides on the solid support. This extension product is suitable for sequencing.

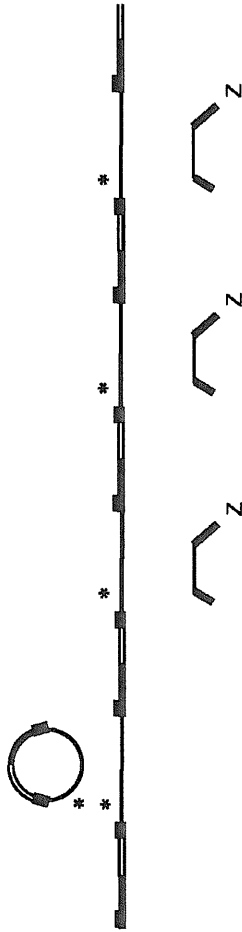
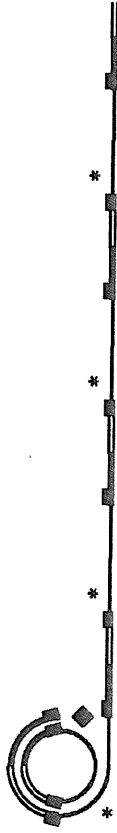


Figure 86

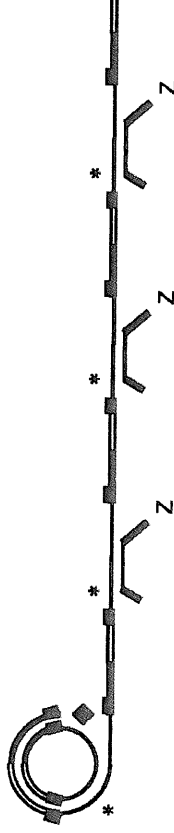
- A. Start with product comprising primer hybridized to primer binding site, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, and primer binding site.



- B. Extend primer using strand-displacement polymerase to generate single strands of tandem copies.



- C. Hybridize oligonucleotides complementary to the extension product. Oligonucleotides are optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-specific tandem-repeat extension product on solid support and wash away other nucleic acids.



- D. Hybridize primers complementary to the extension products. Extend the extension products. Extend primers using strand-displacement polymerase to liberate tandem repeat extension product from capture oligonucleotides on the solid support. This extension products and their complements are suitable for sequencing.

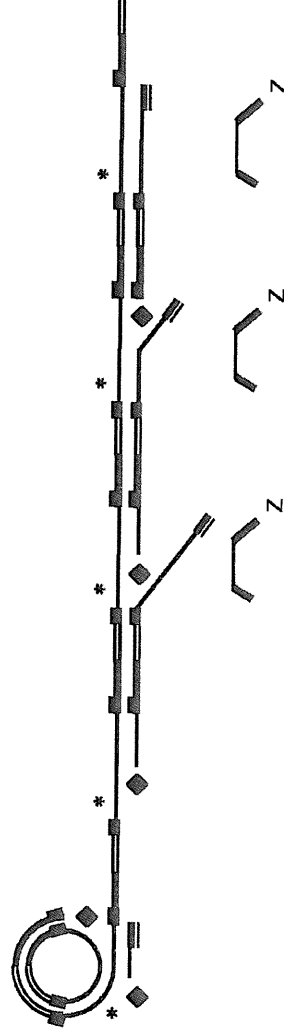
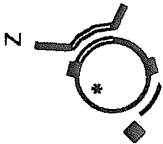


Figure 87

A. Start with product comprising hybridized target-specific primer, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site.



B. Hybridize oligonucleotide complementary to primer binding site. Oligonucleotide is optionally blocked or mismatched at the 3' end and contain capture group Z (i.e. a biotin moiety) on the 5' end. Capture target-containing circular DNA on solid support and wash away other nucleic acids.



C. Extend target-specific primer using strand-displacement polymerase to generate single strands of tandem copies of target DNA, and liberate extension product from the solid support. This extension product is suitable for sequencing.

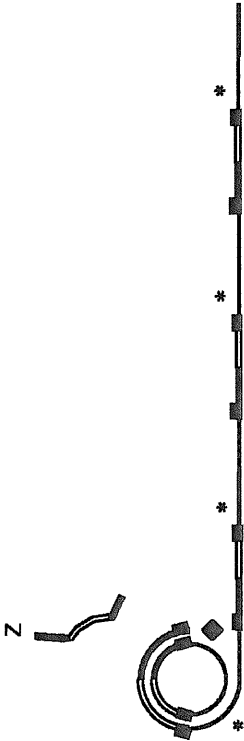


Figure 88

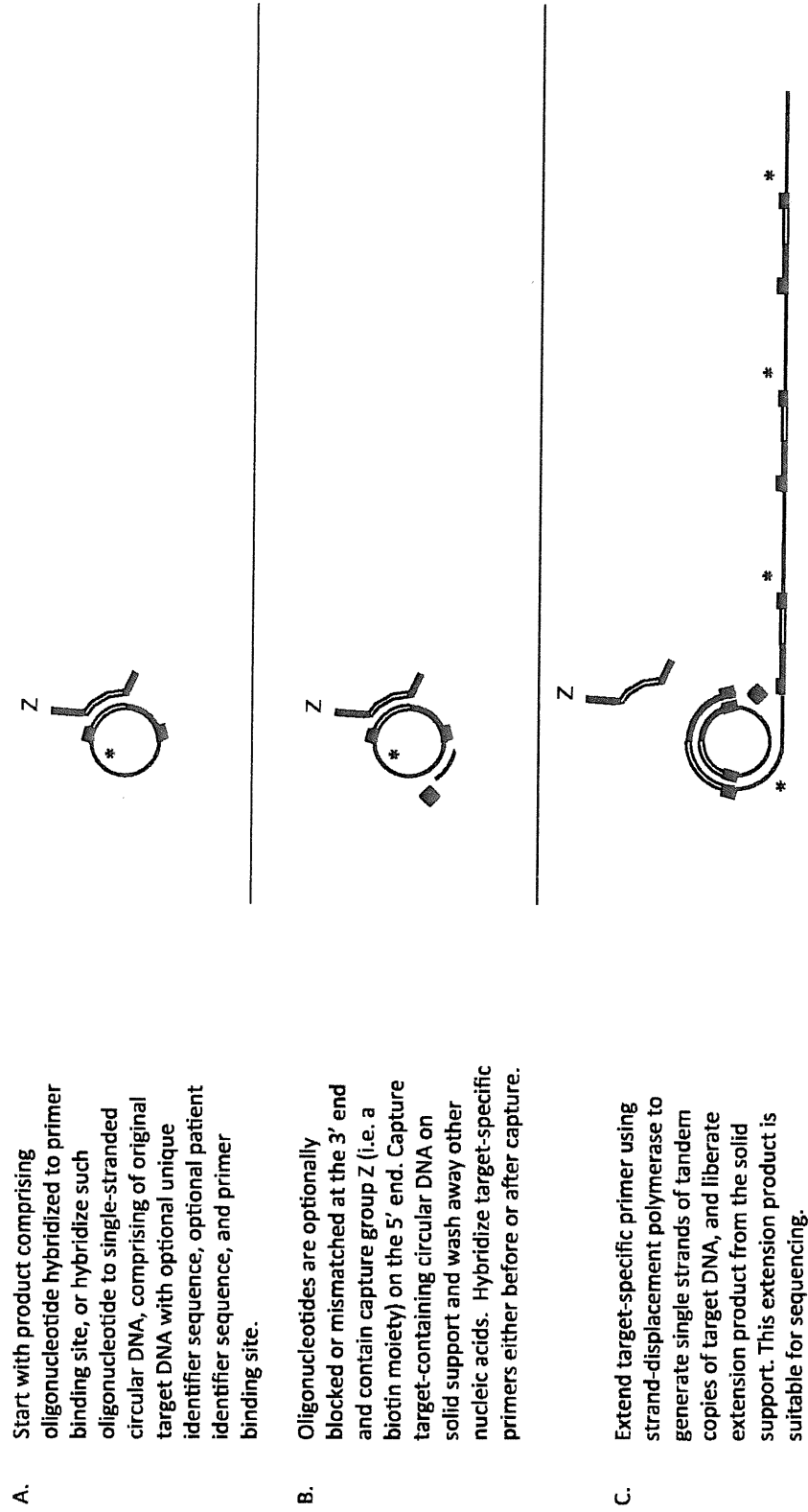


Figure 89

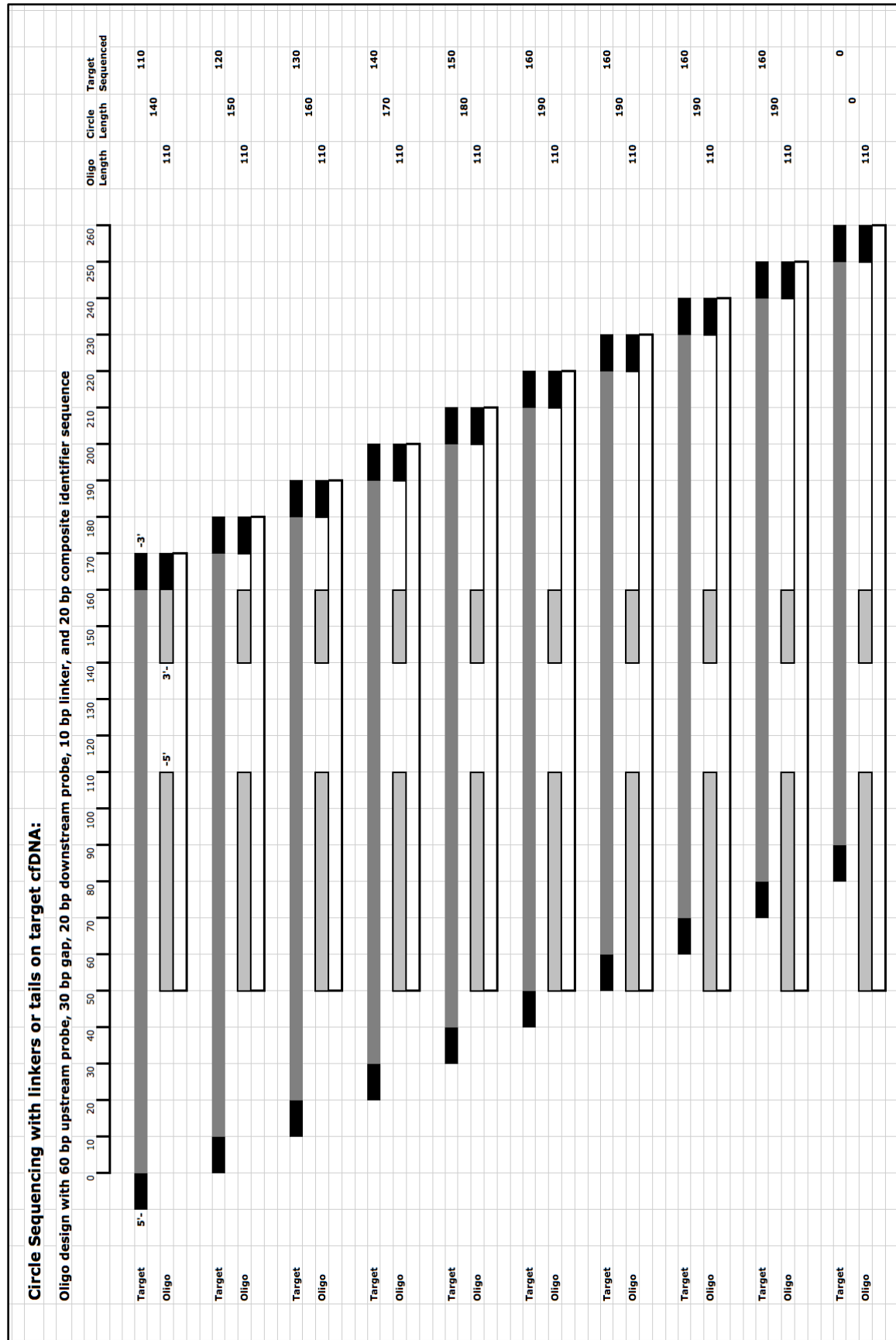


Figure 90

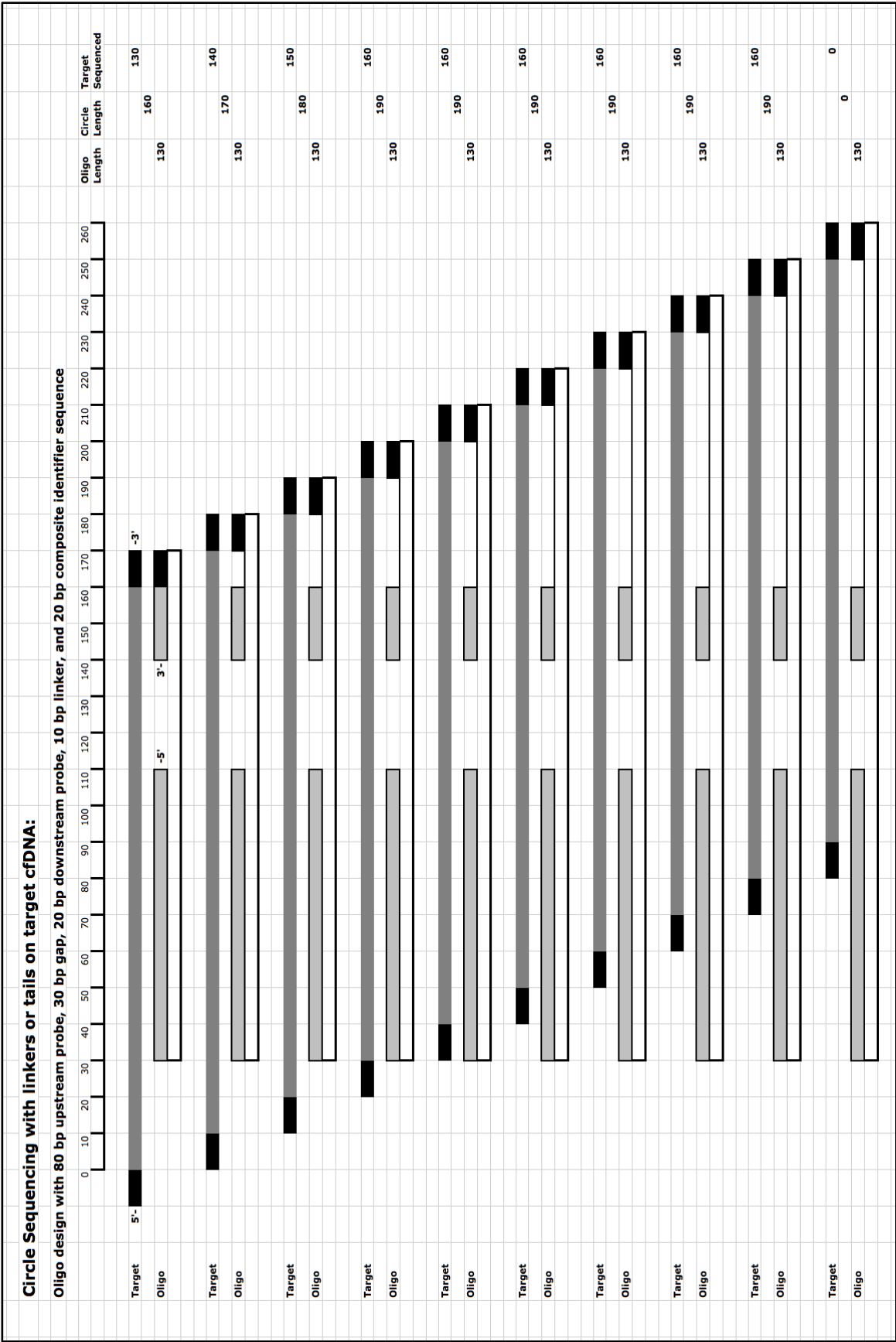


Figure 91

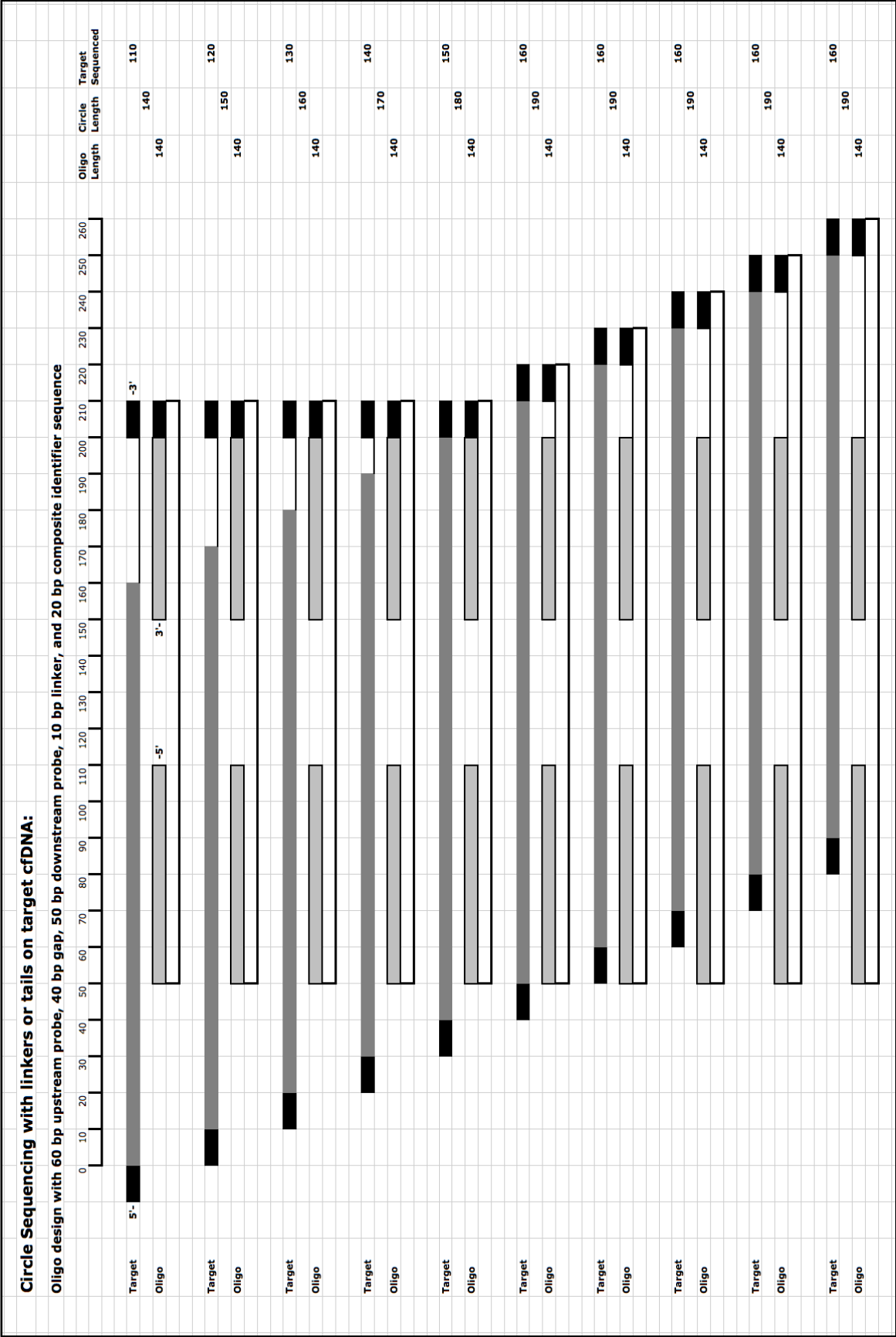


Figure 92

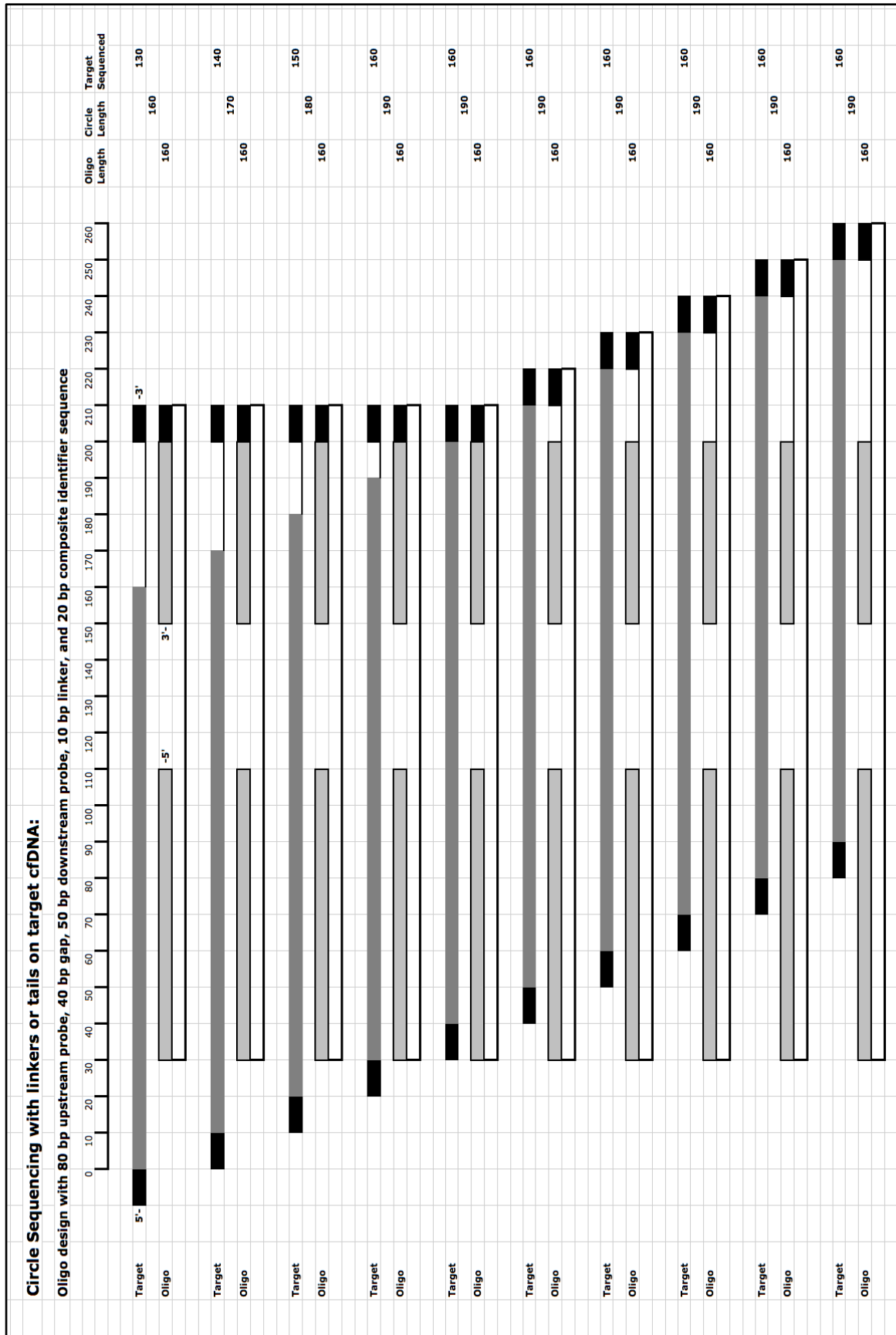


Figure 93

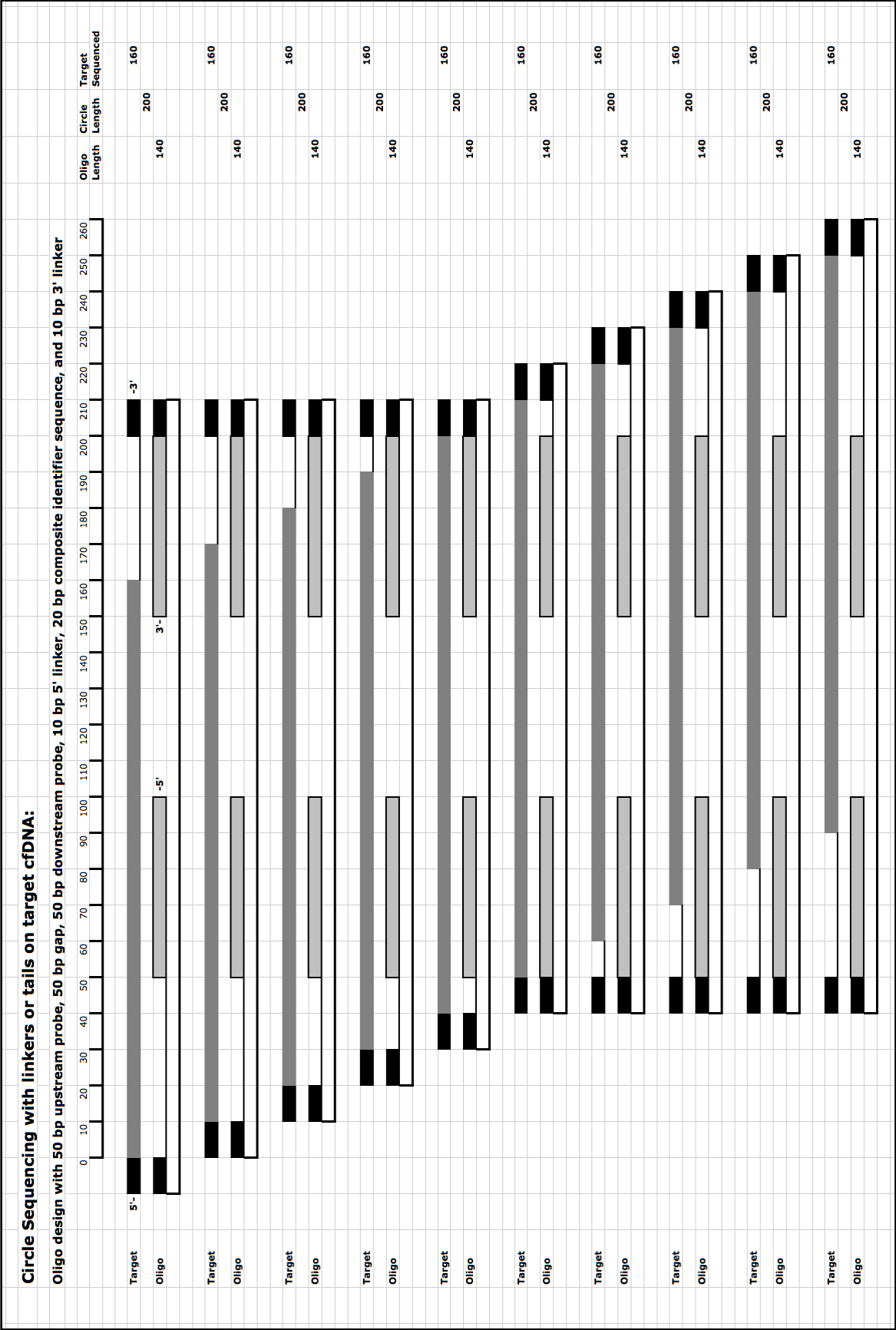


Figure 94

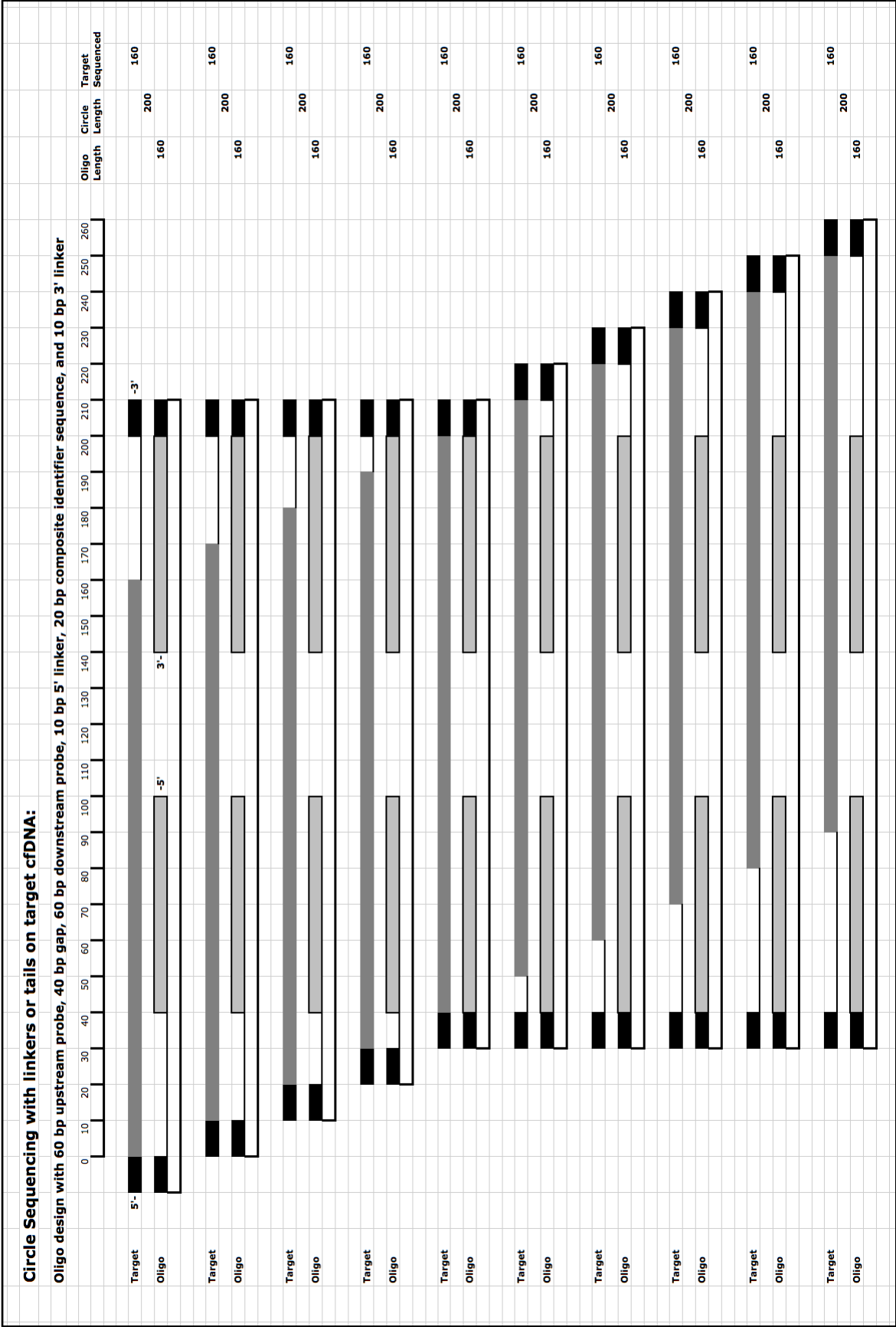


Figure 95

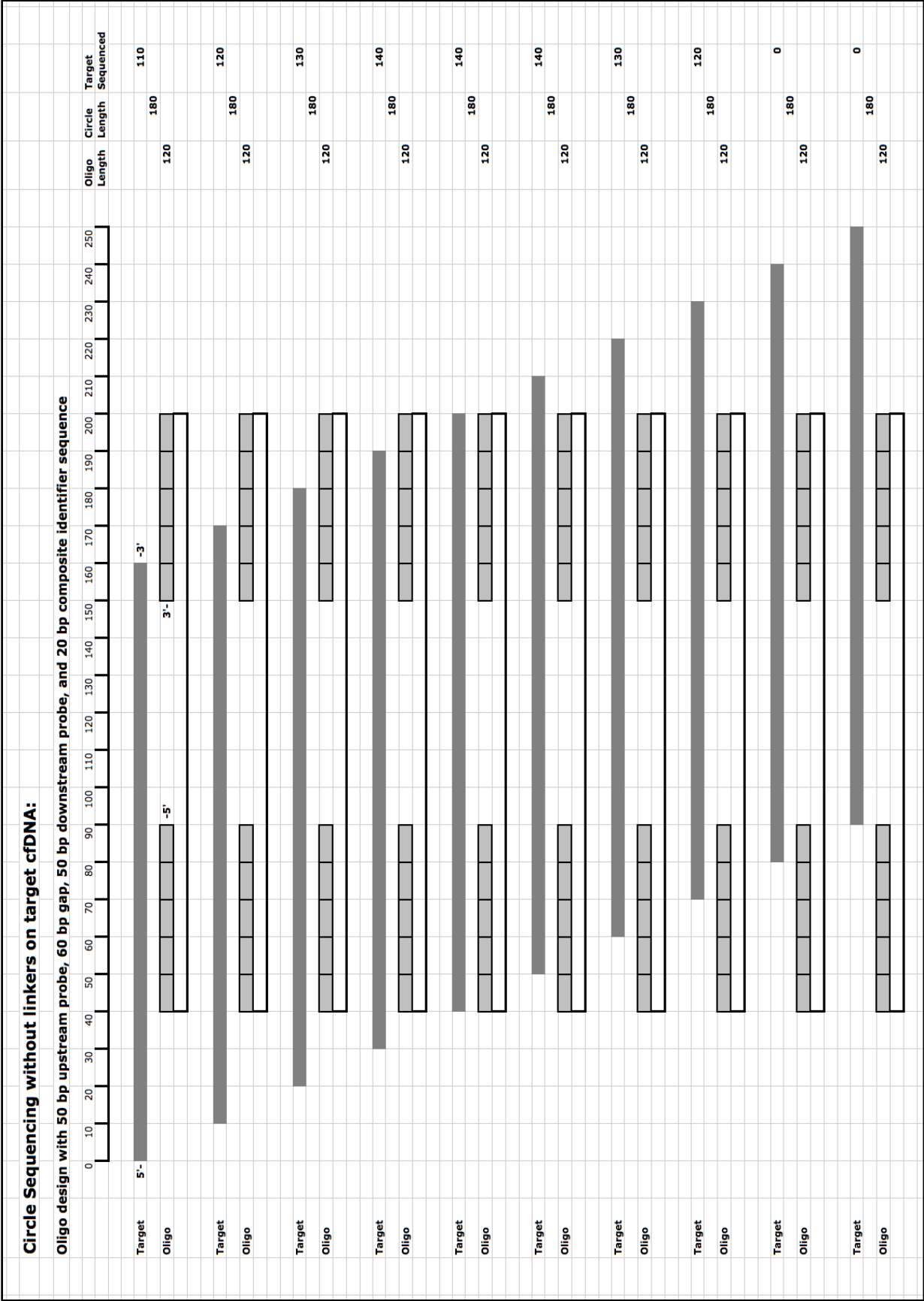


Figure 96

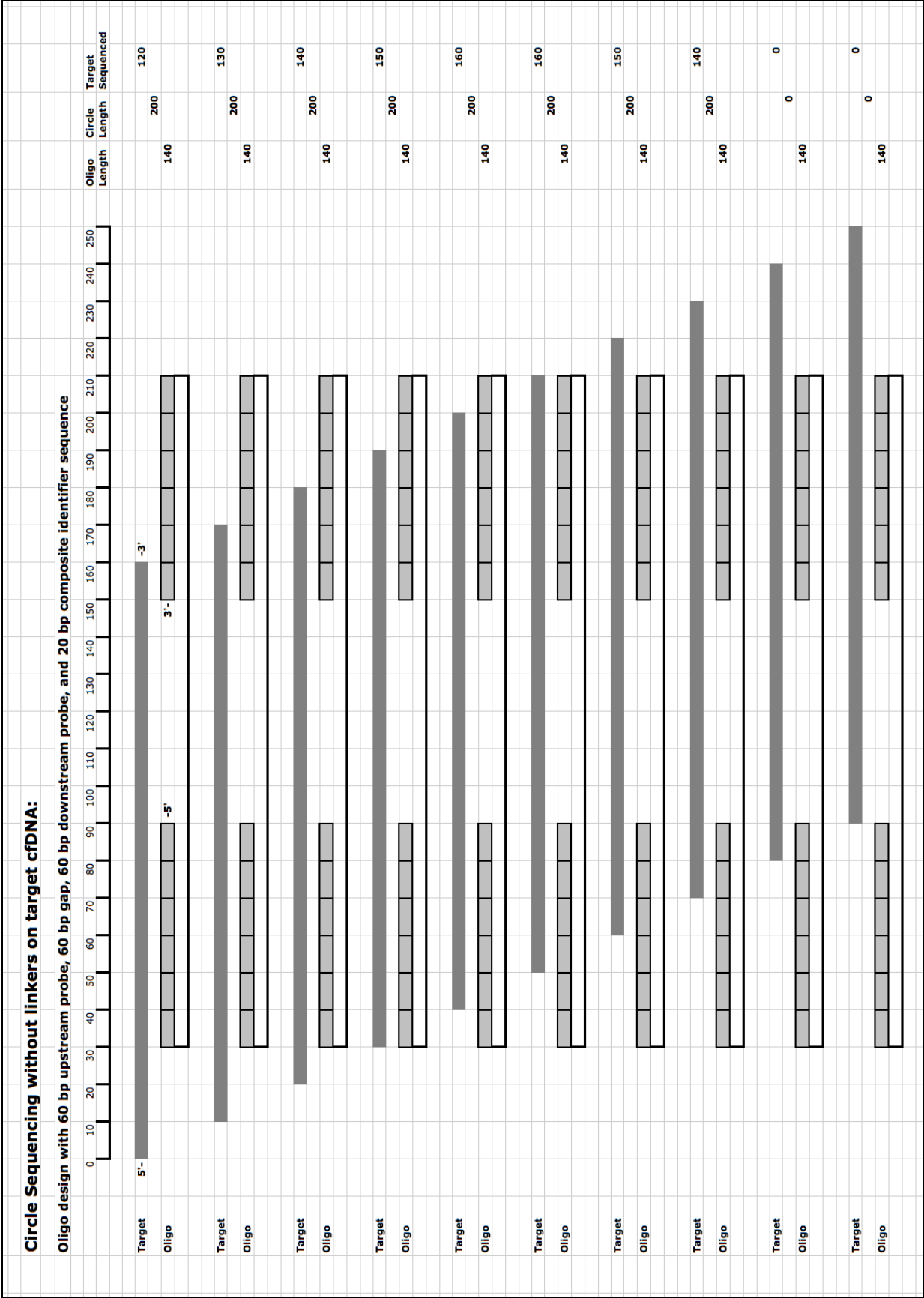


Figure 97

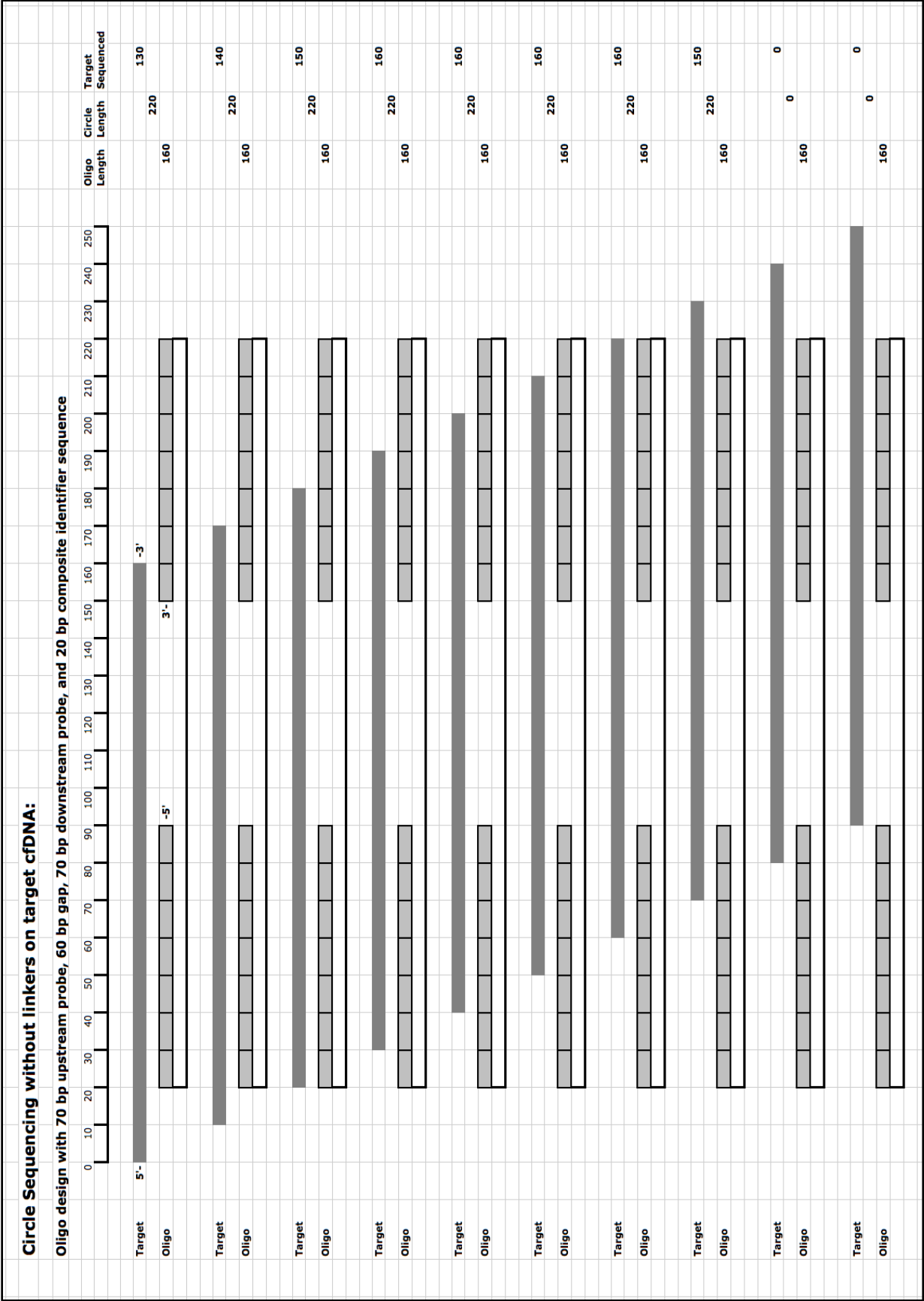


Figure 98

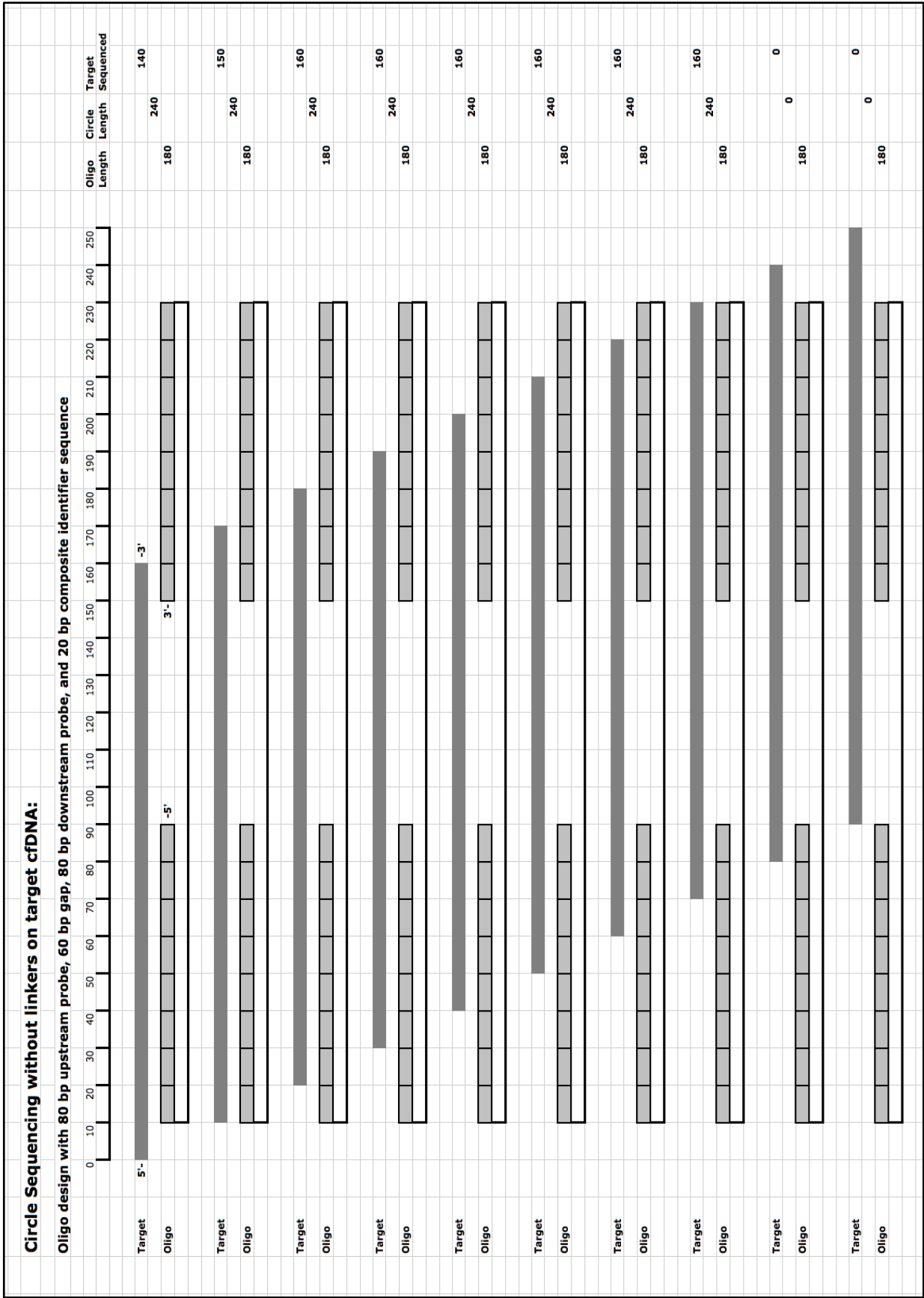


Figure 99

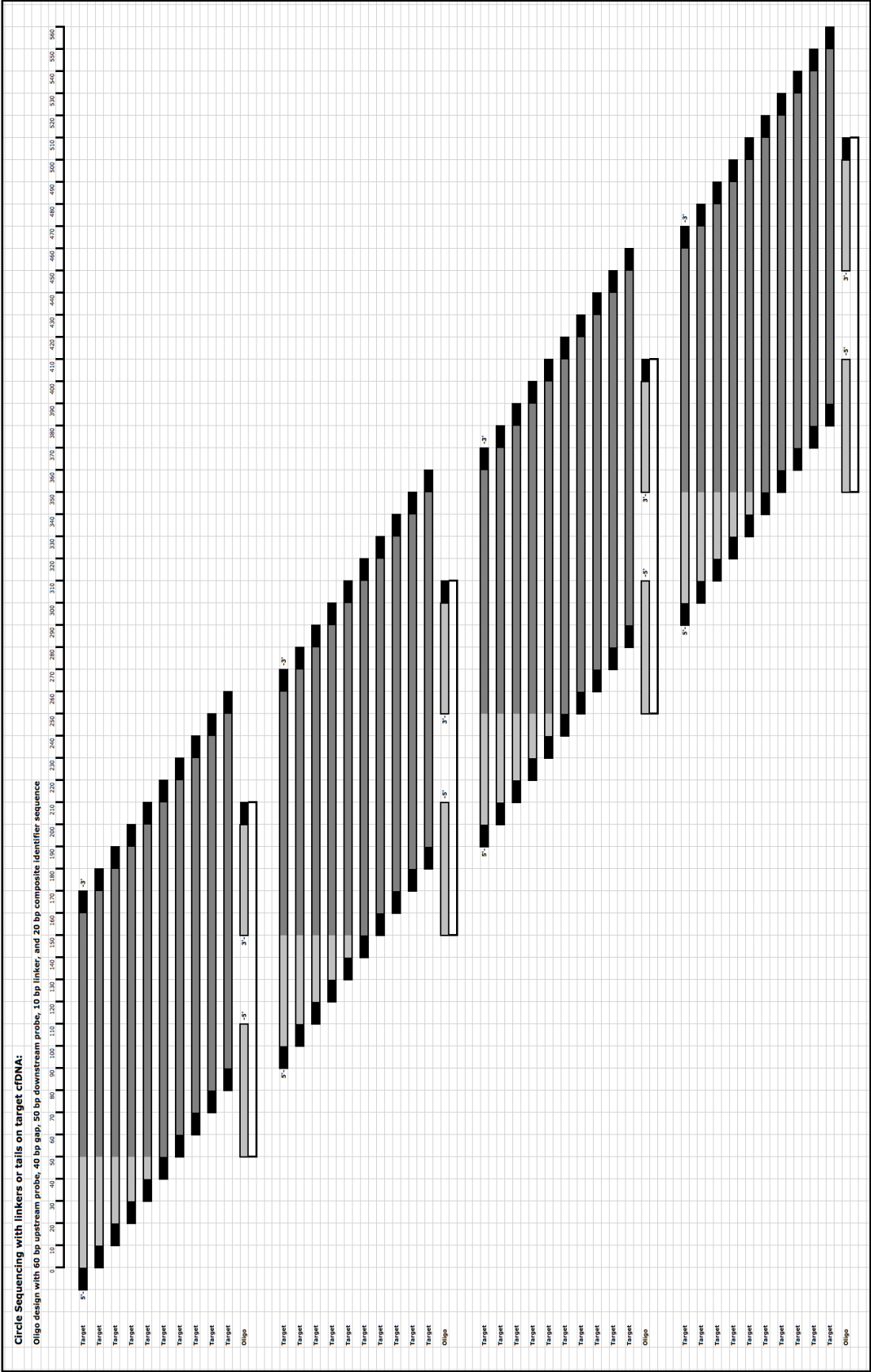


Figure 100

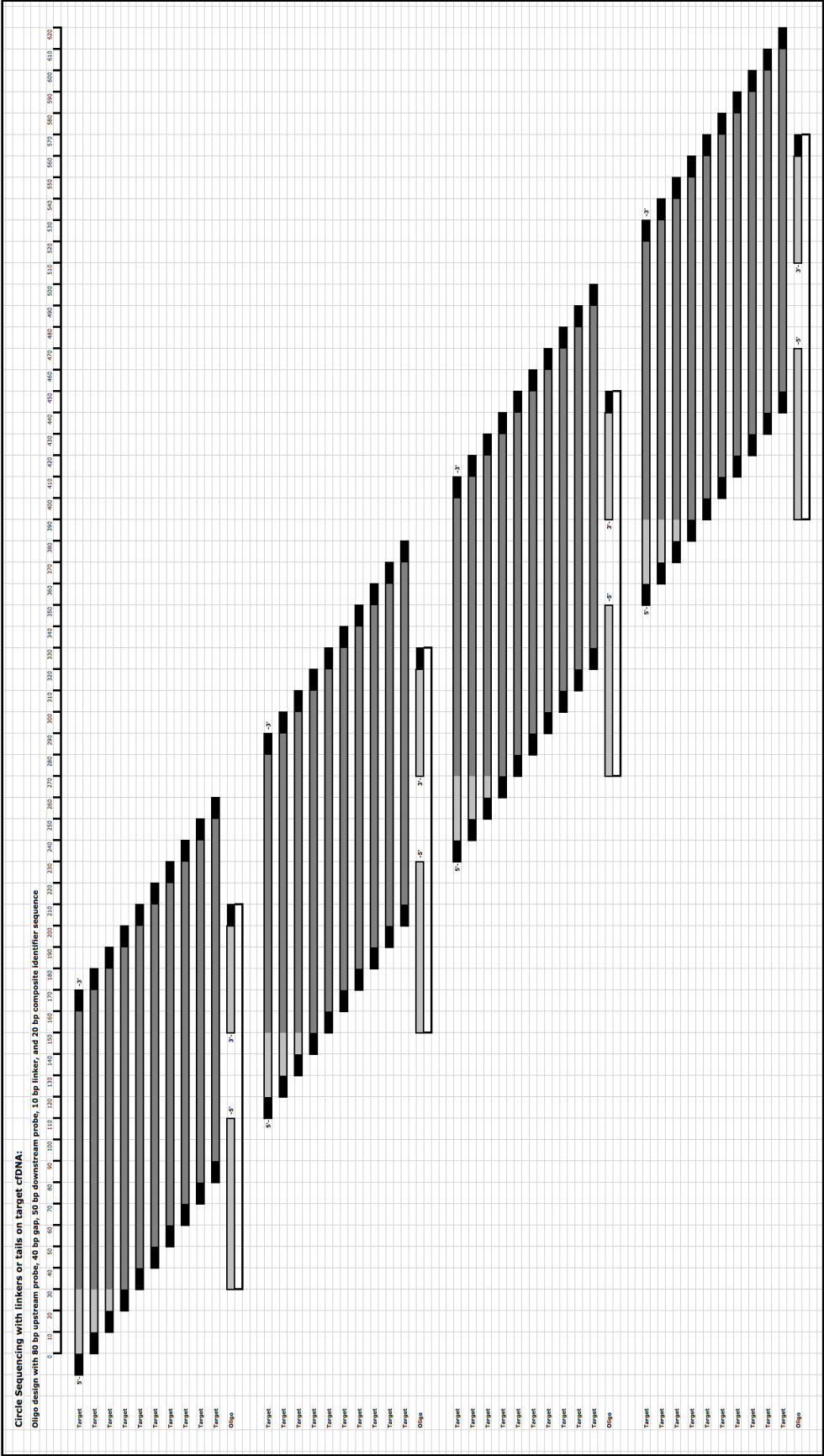


Figure 101

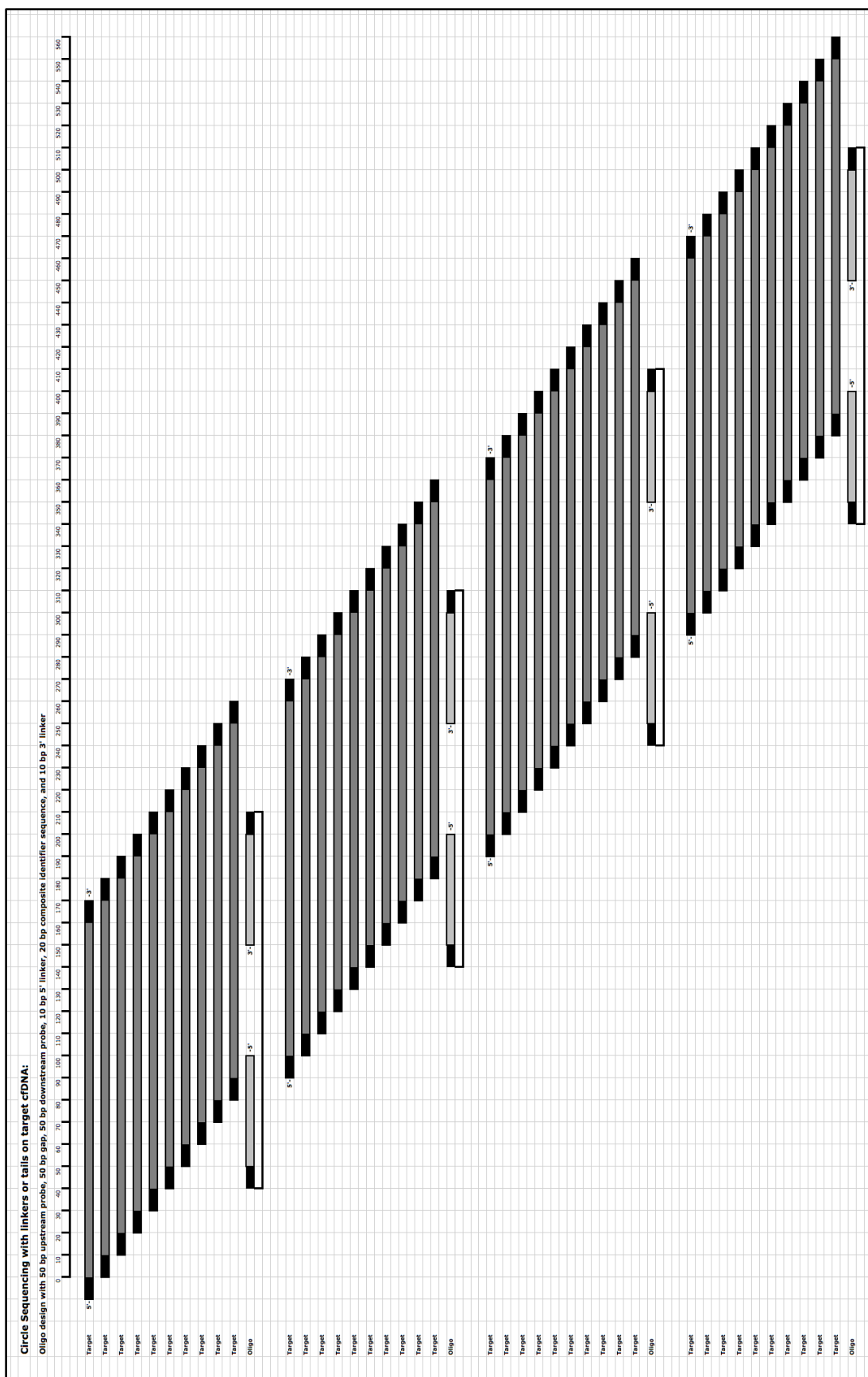


Figure 102

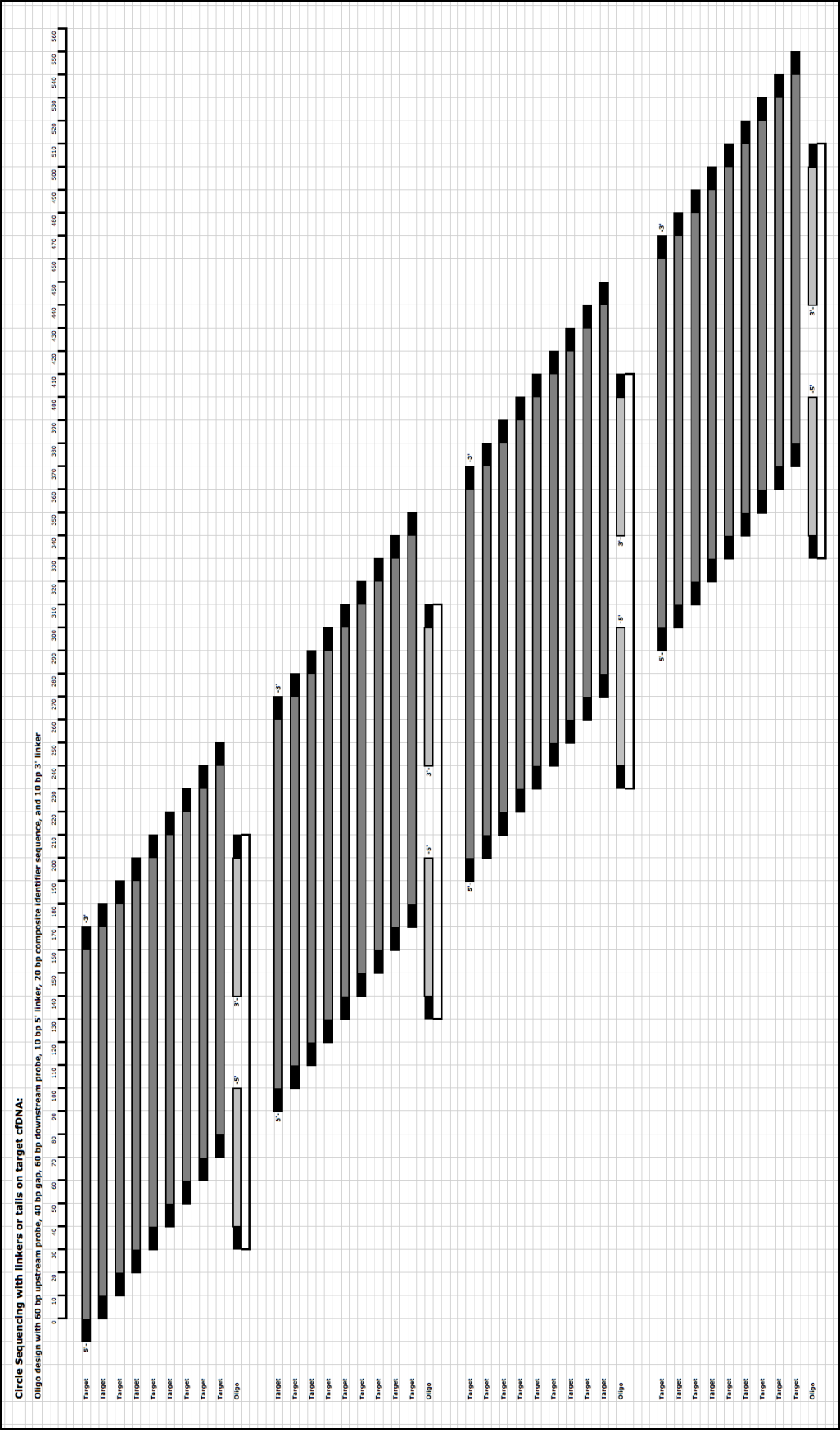


Figure 103

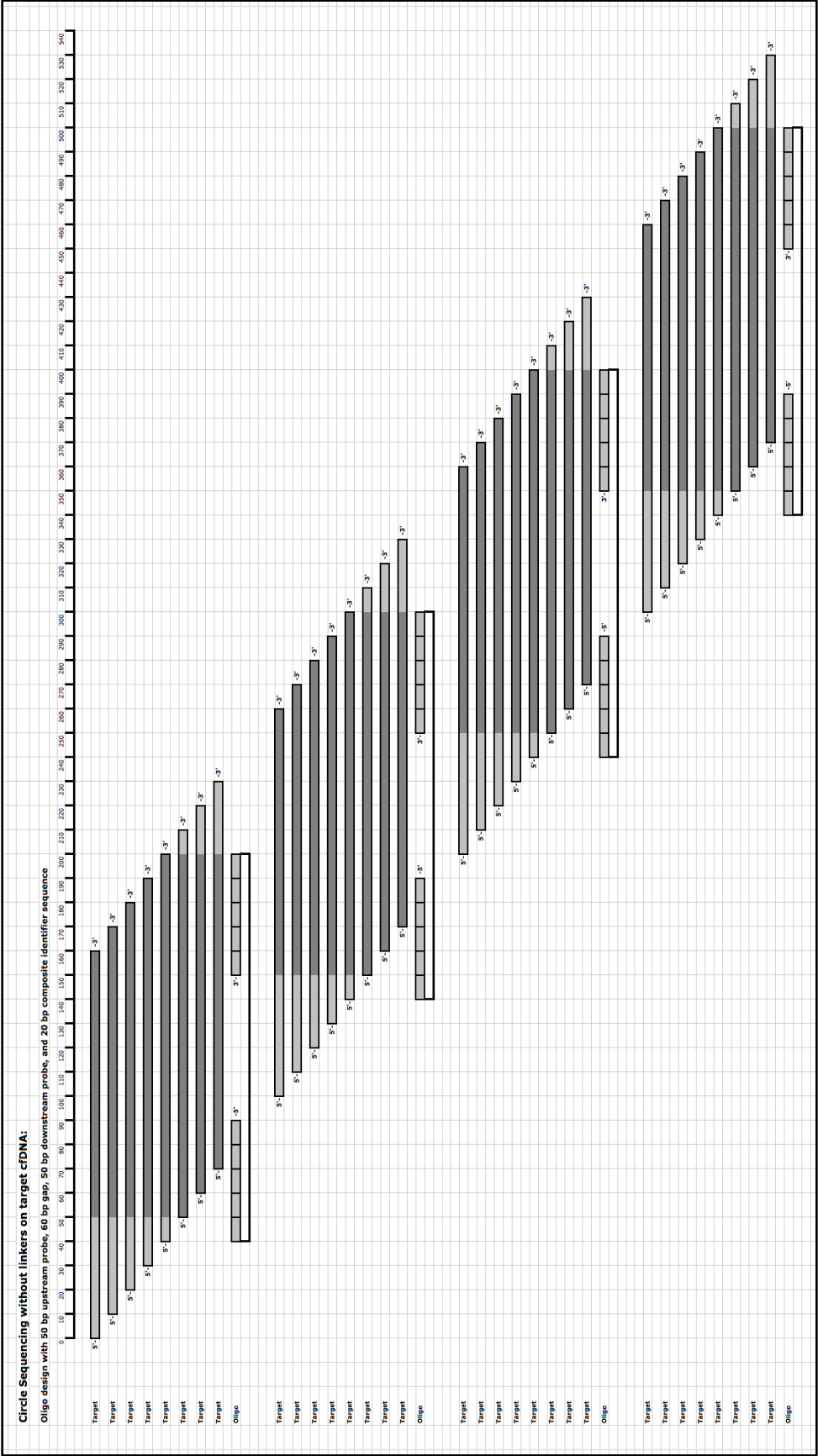


Figure 104

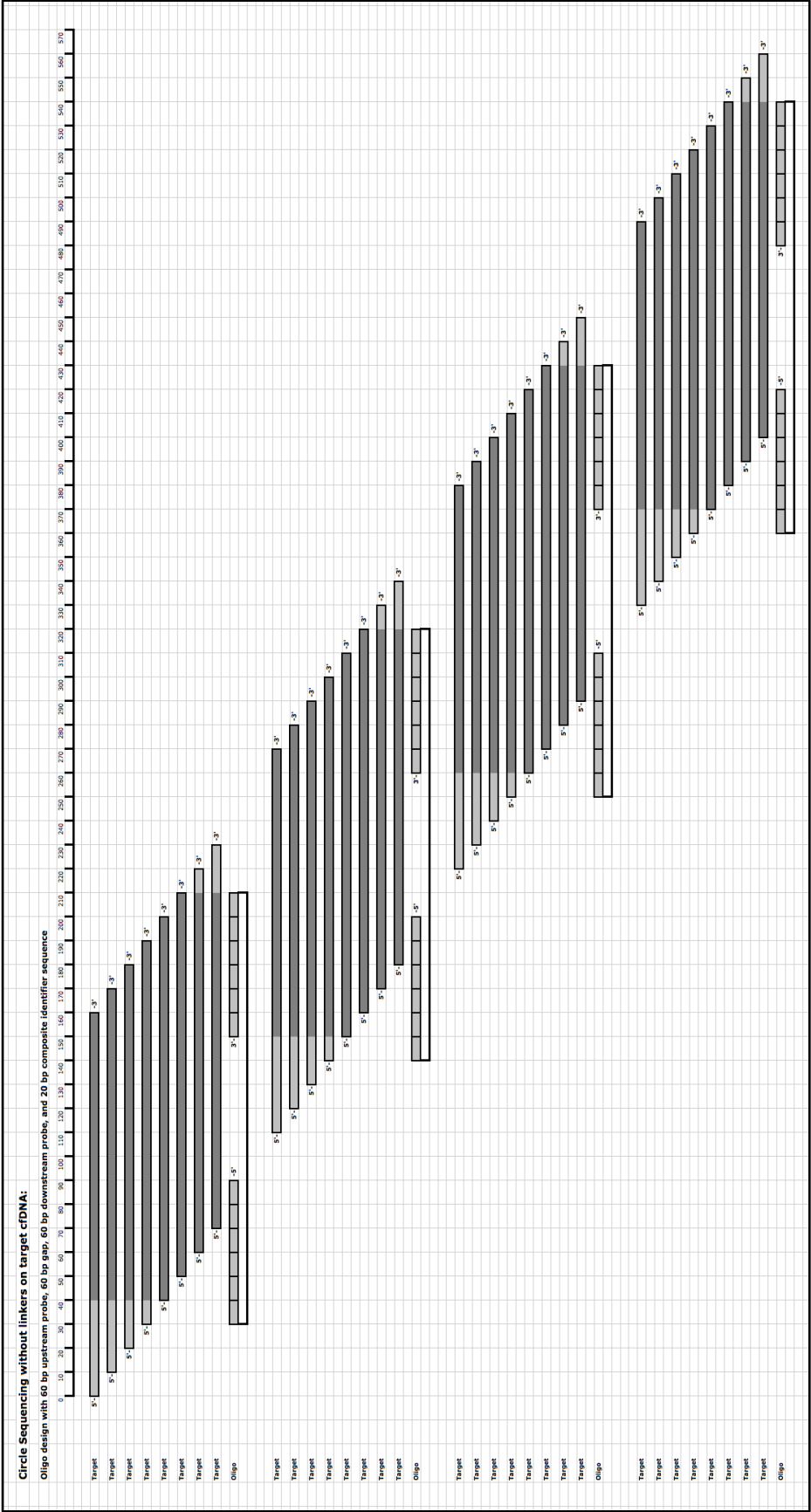


Figure 105

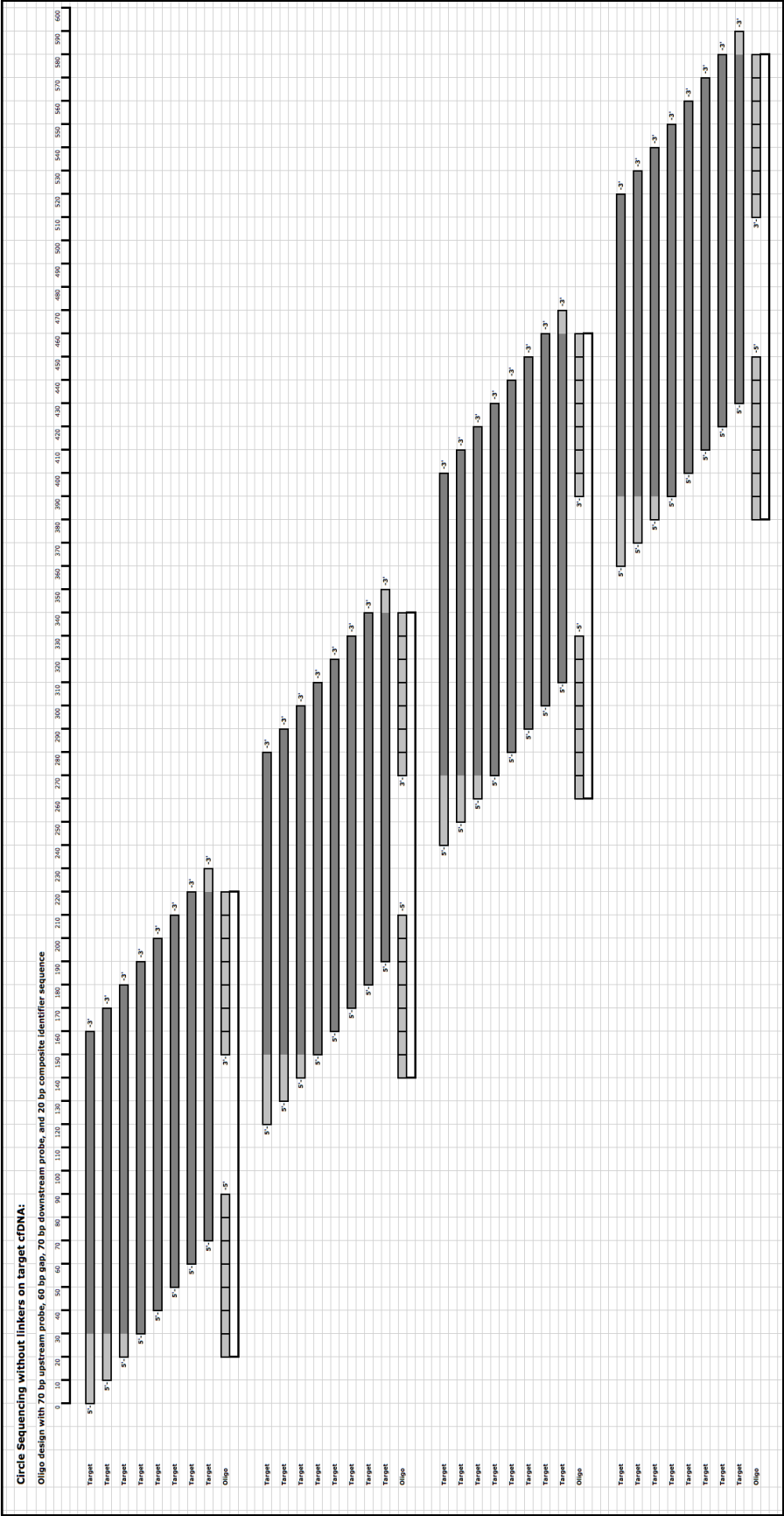


Figure 106

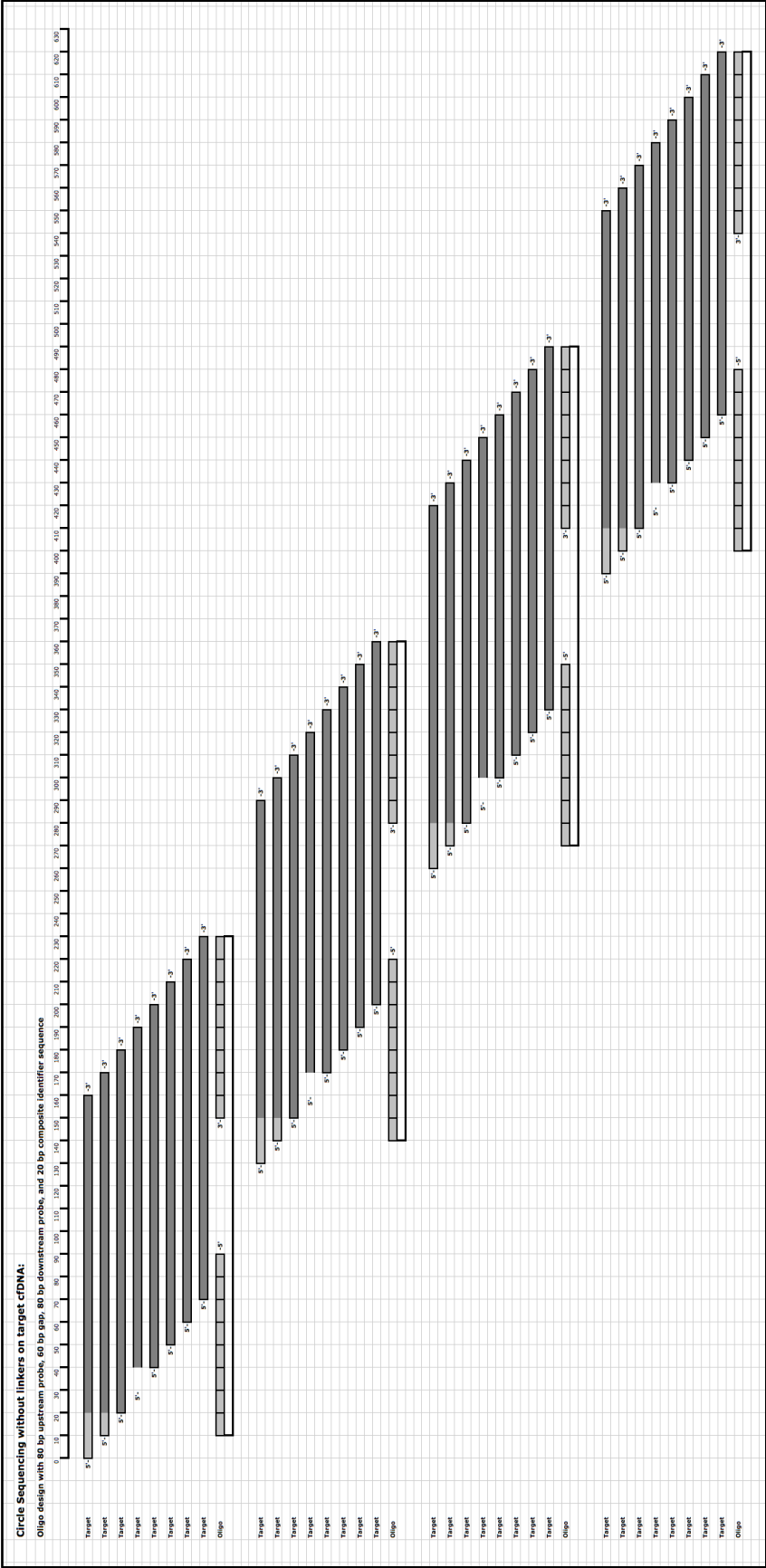


Figure 107

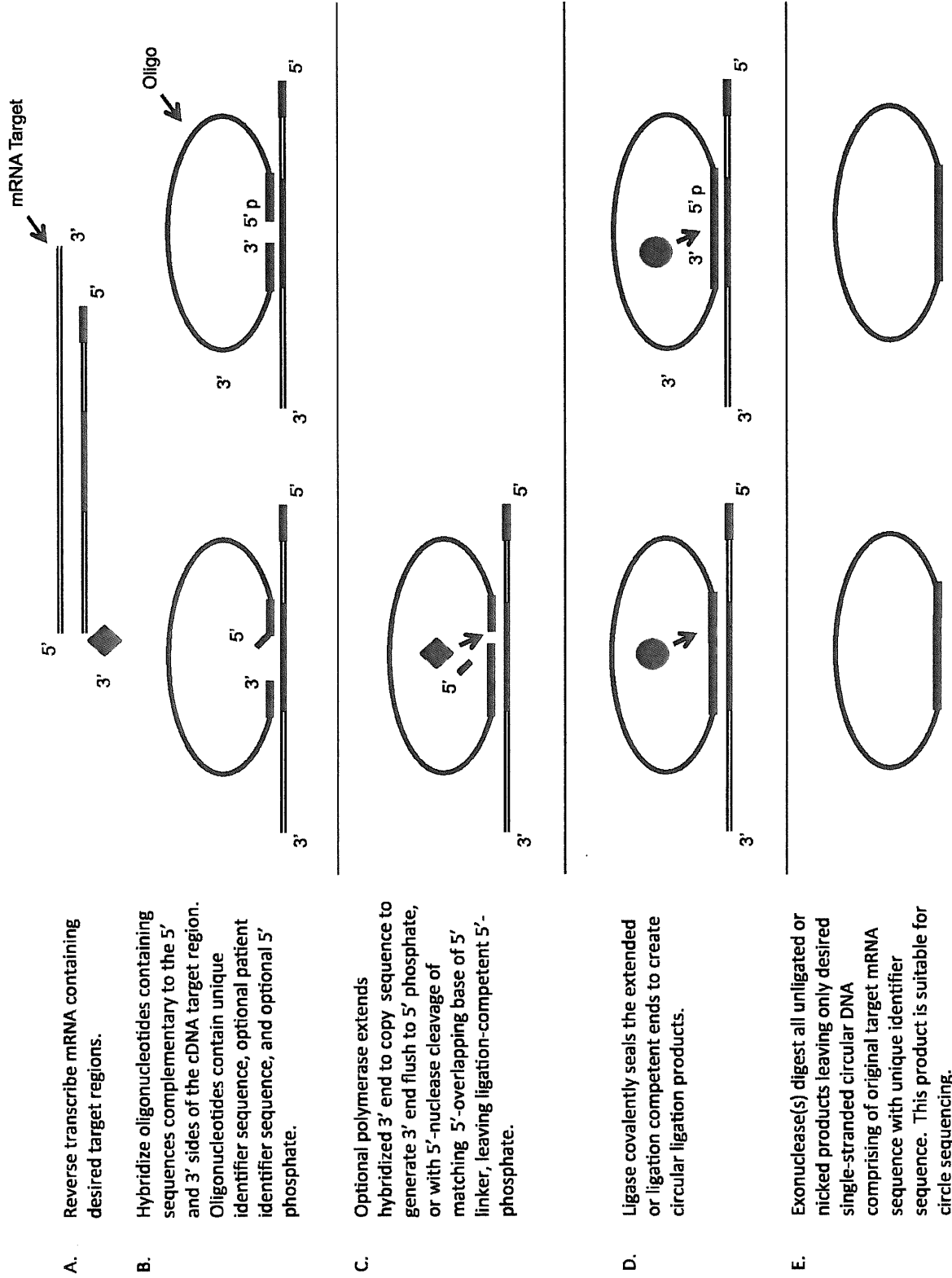


Figure 108

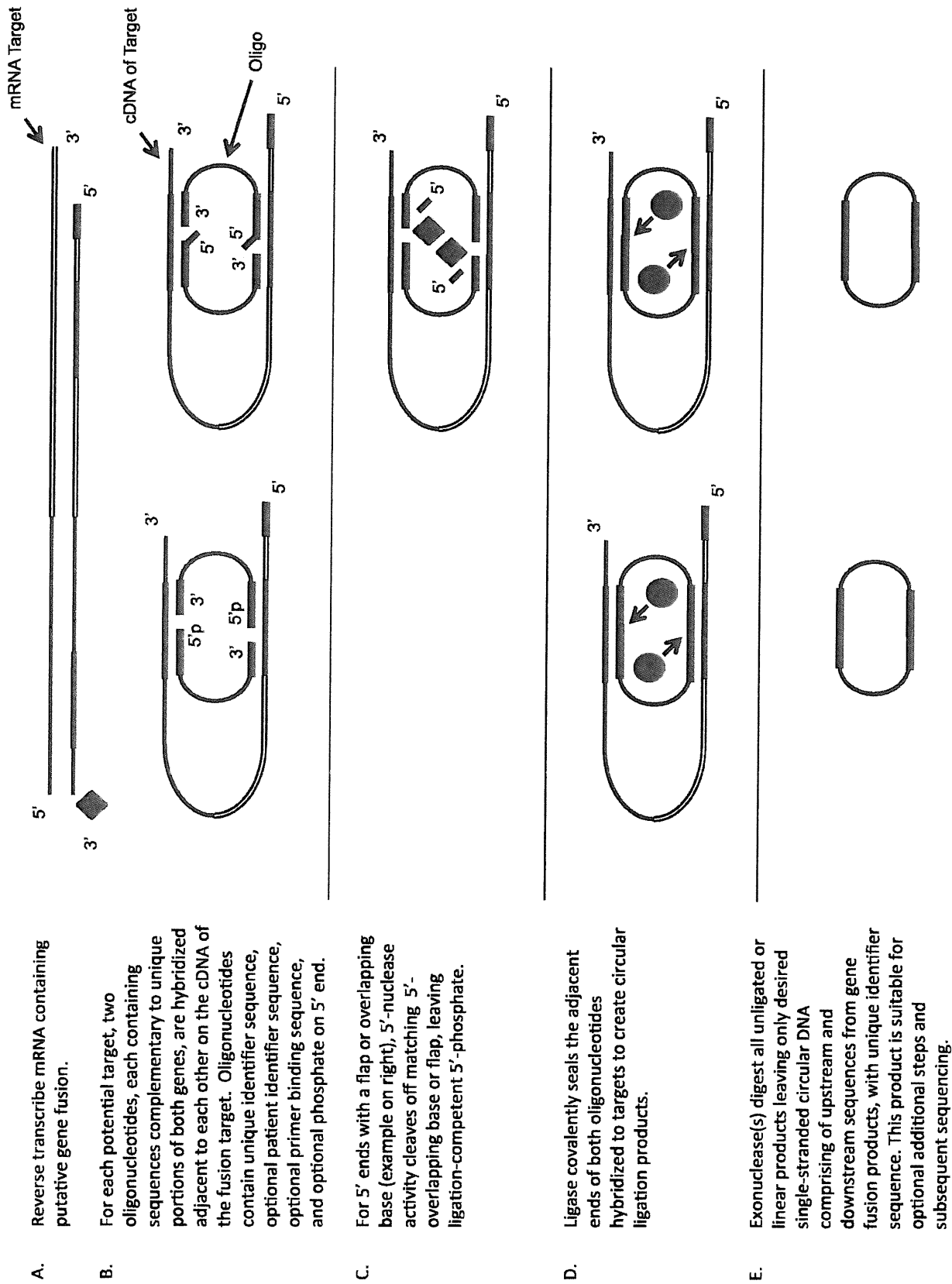


Figure 109

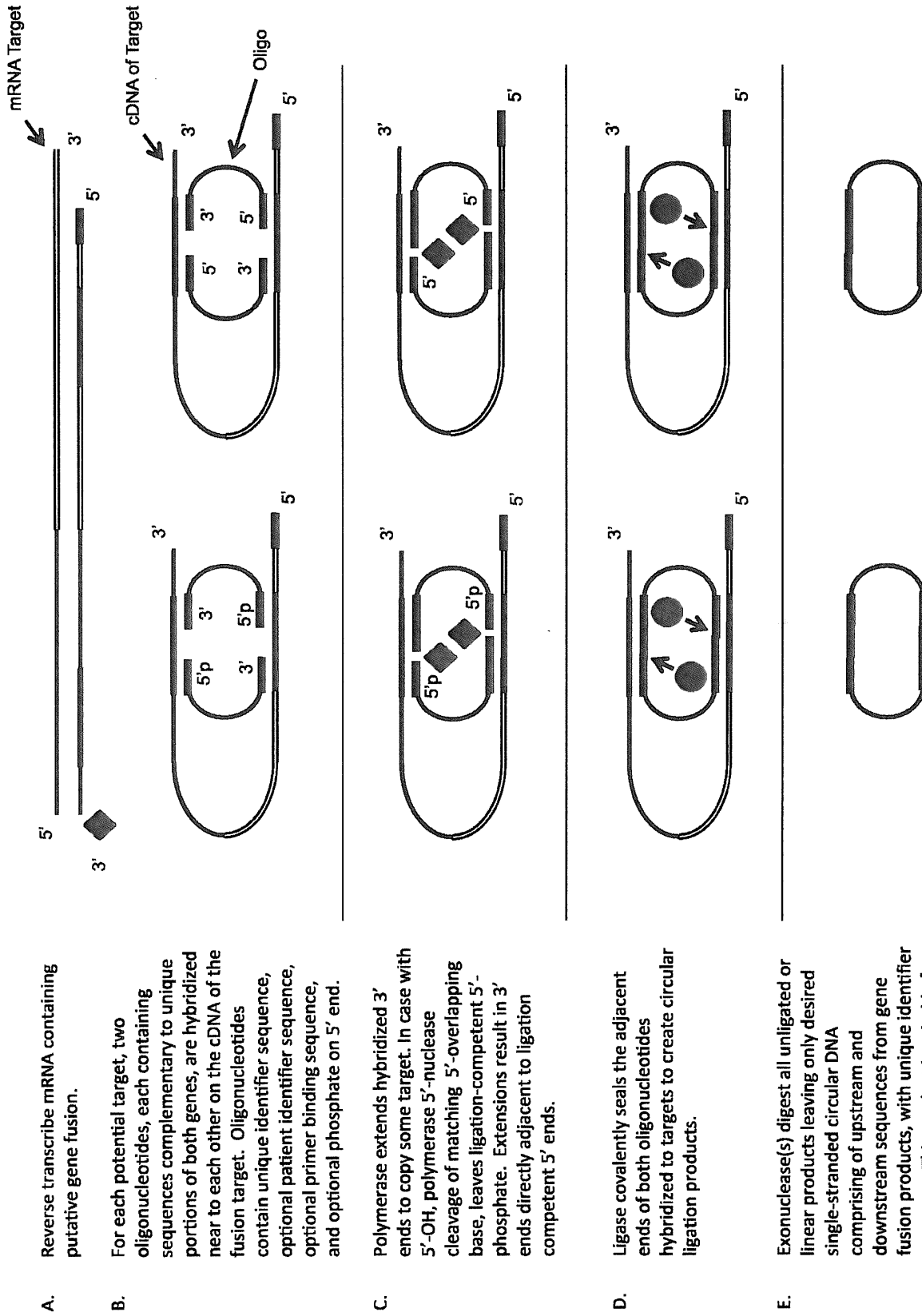


Figure 110

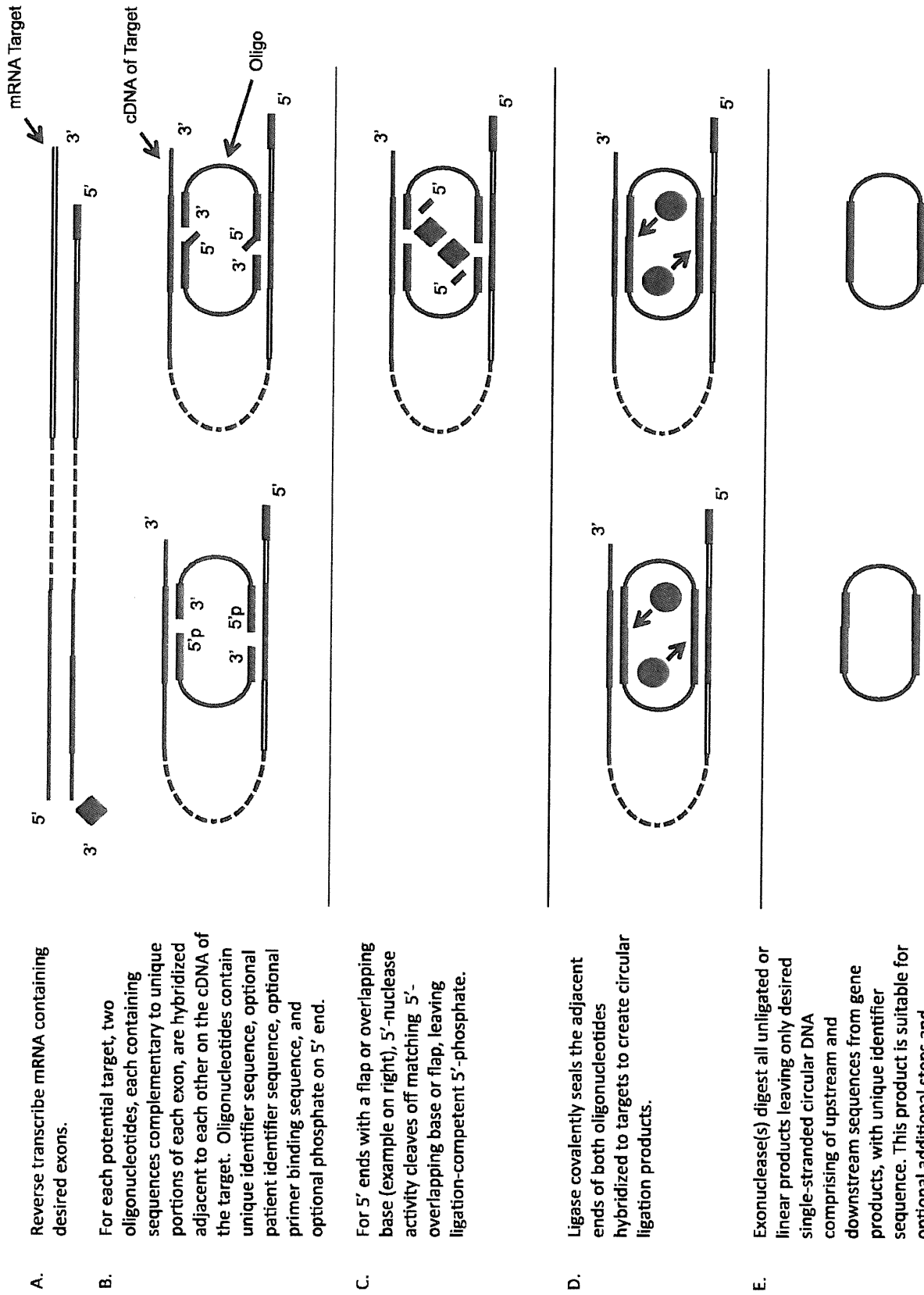


Figure 111

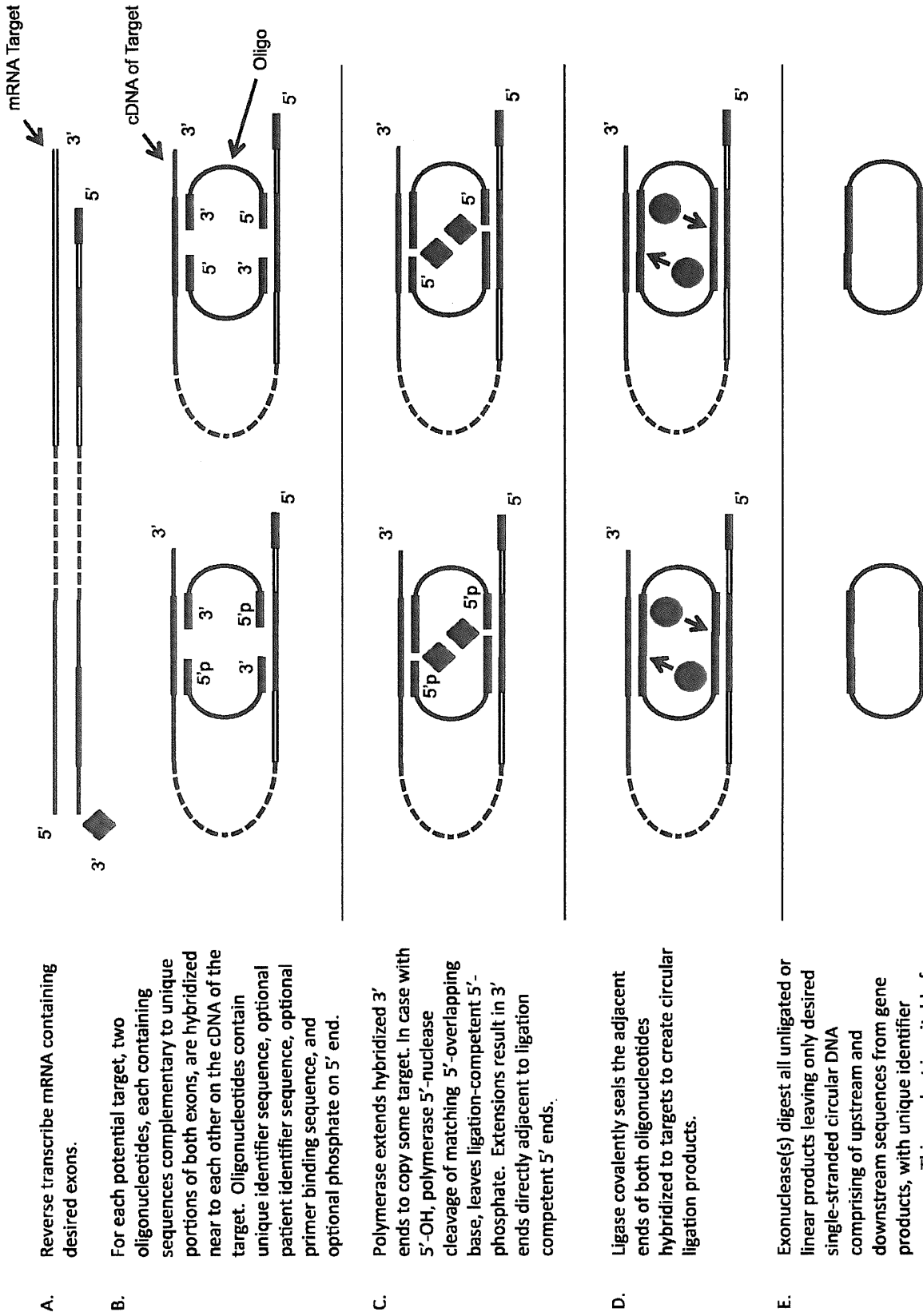


Figure 112

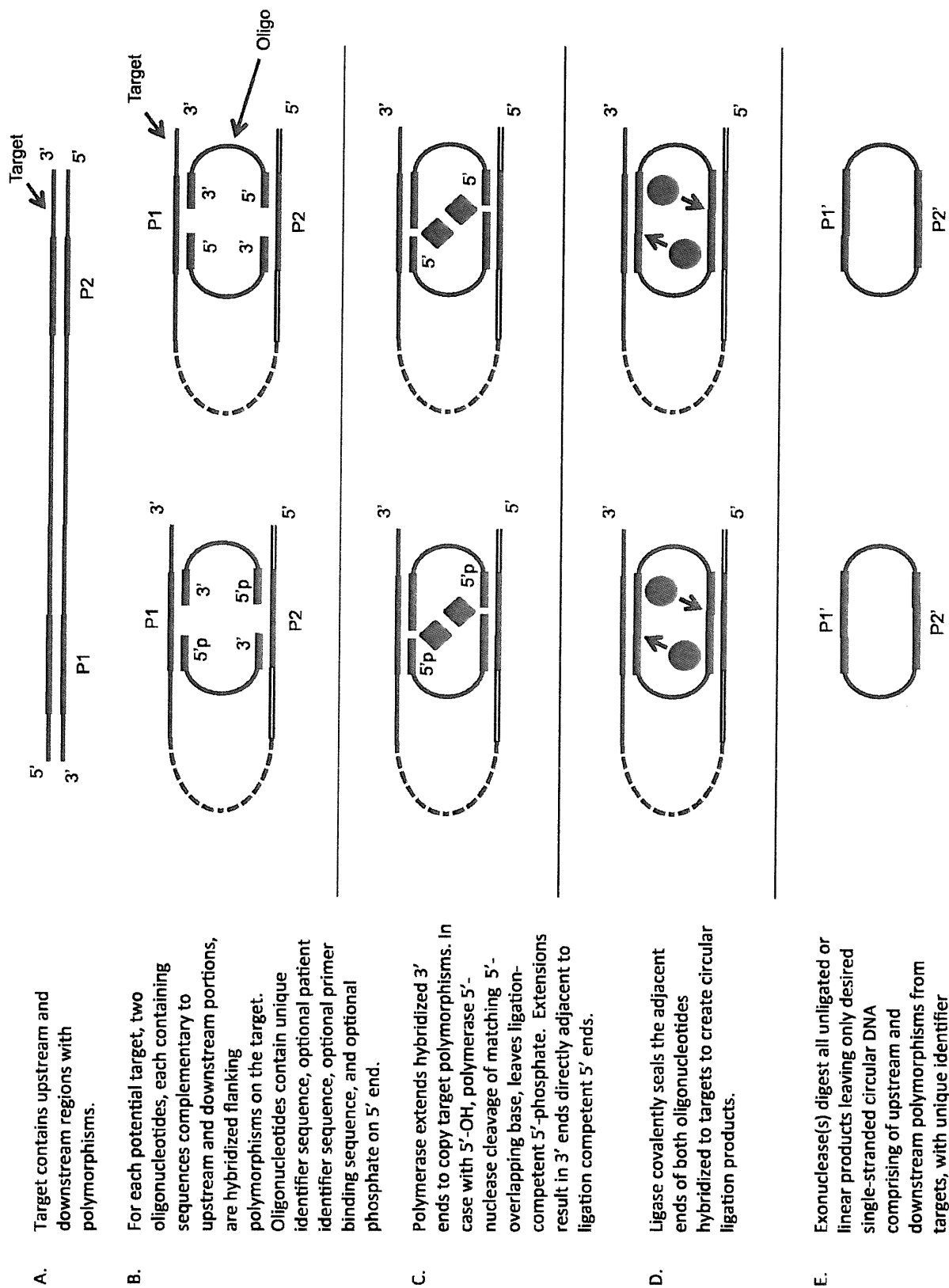


Figure 113

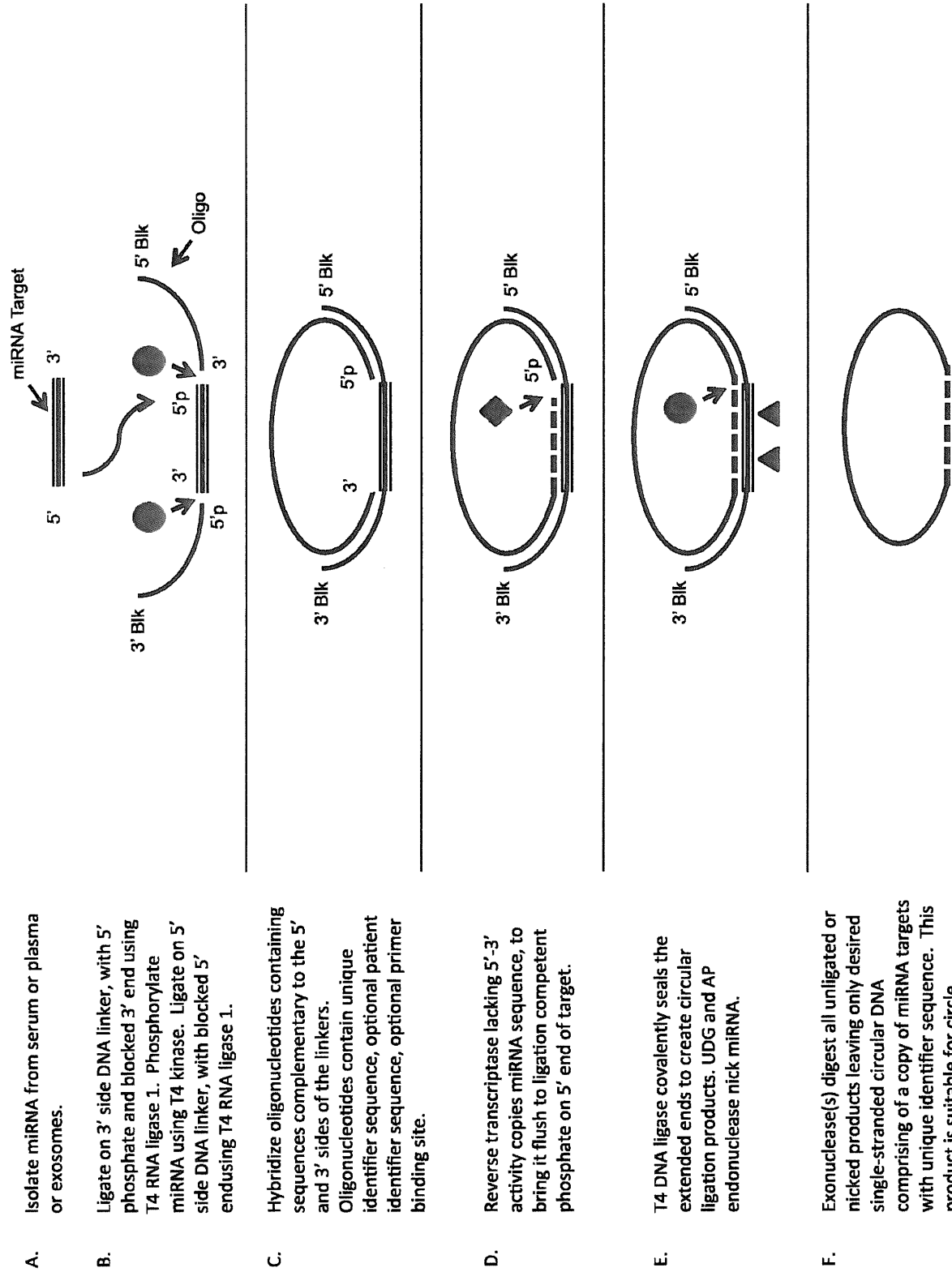


Figure 114

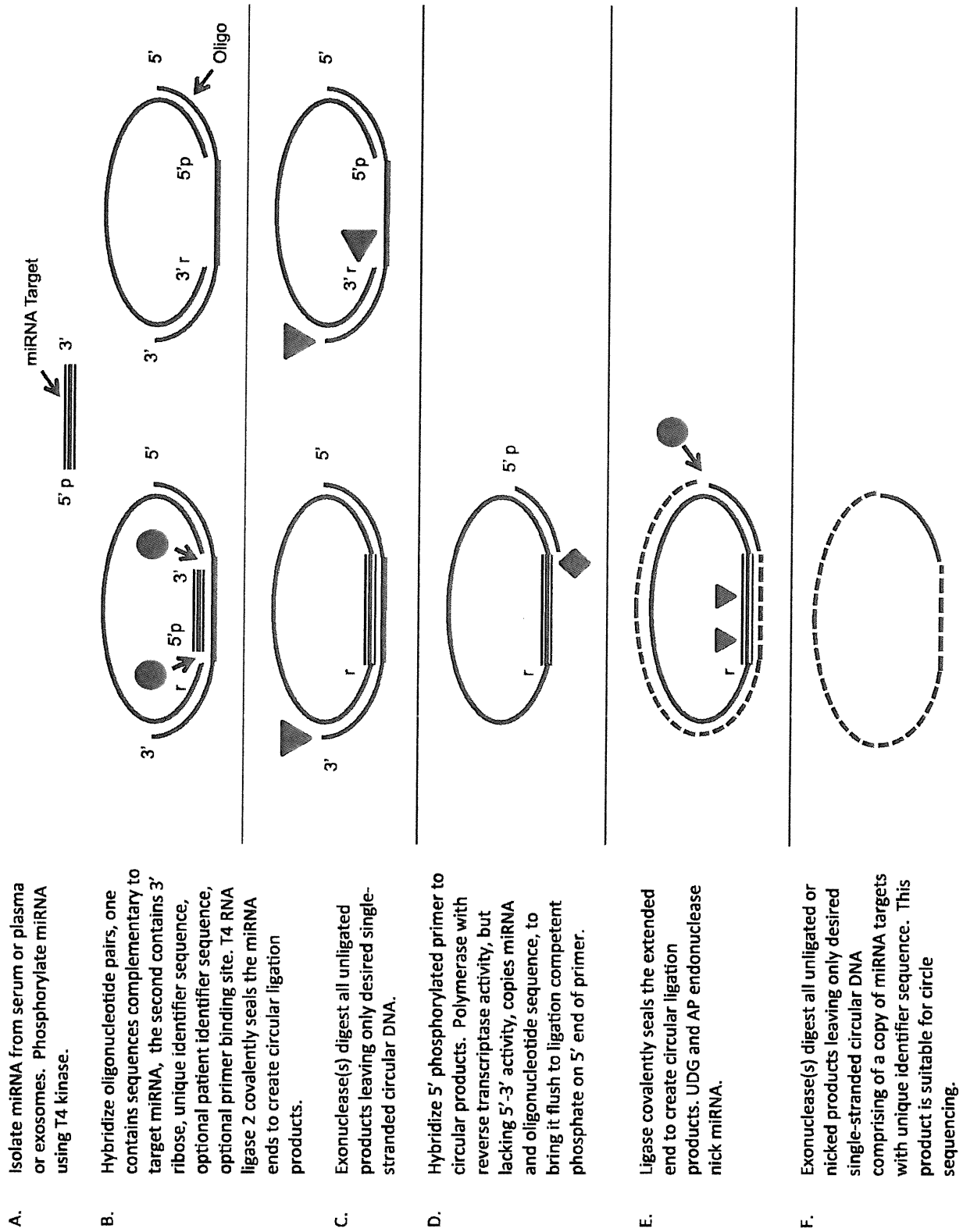


Figure 115

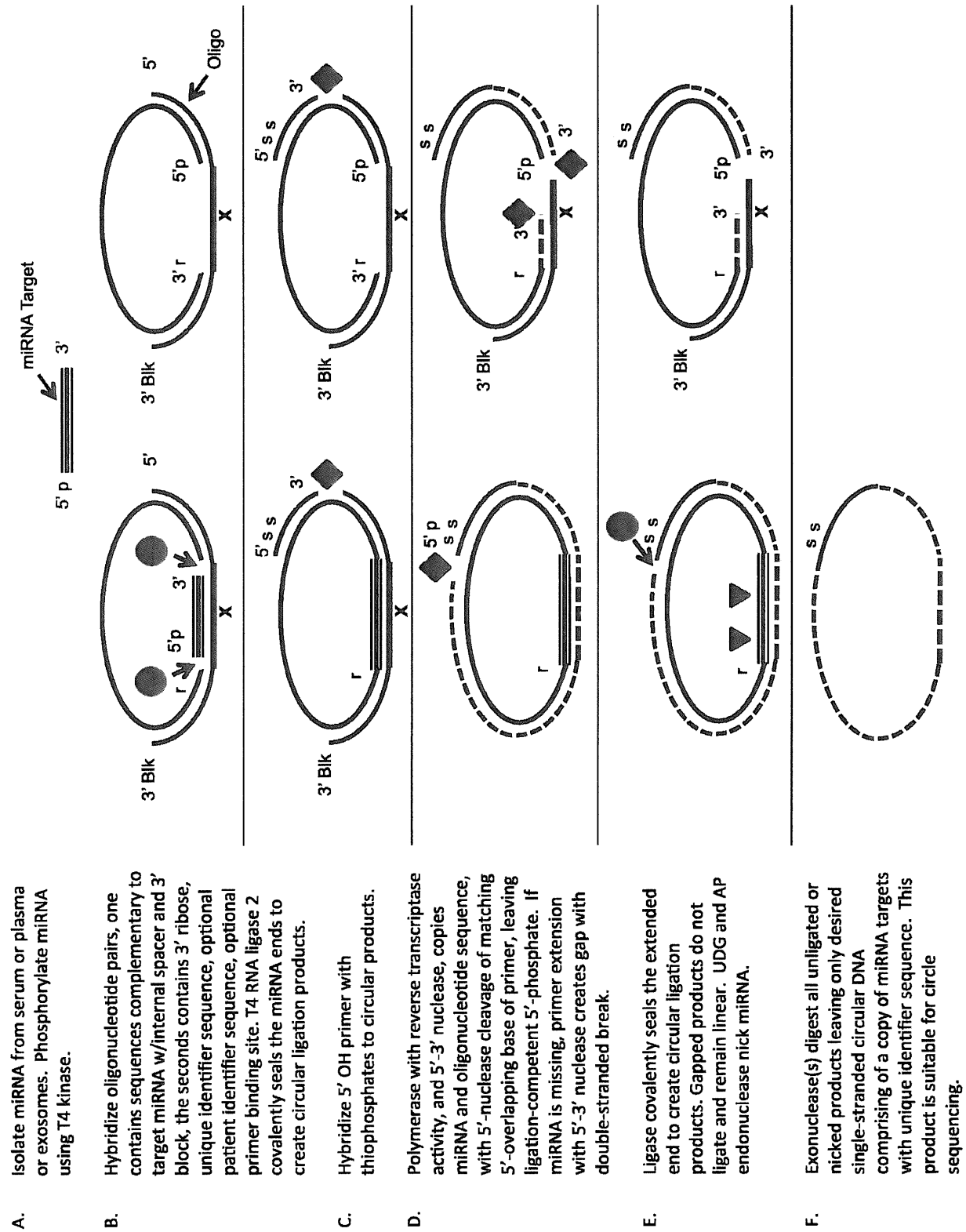


Figure 116

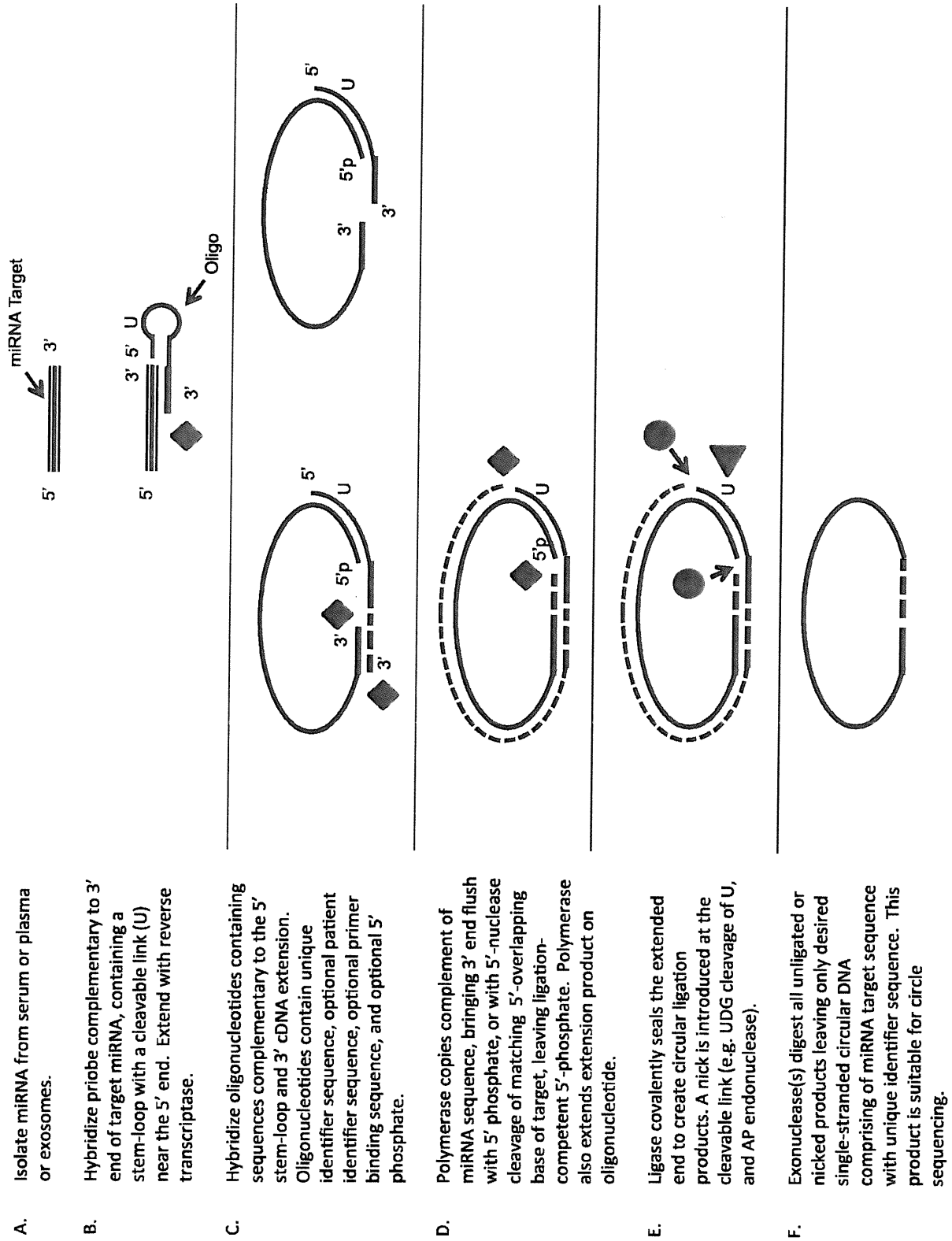


Figure 117