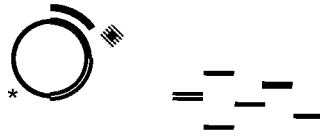


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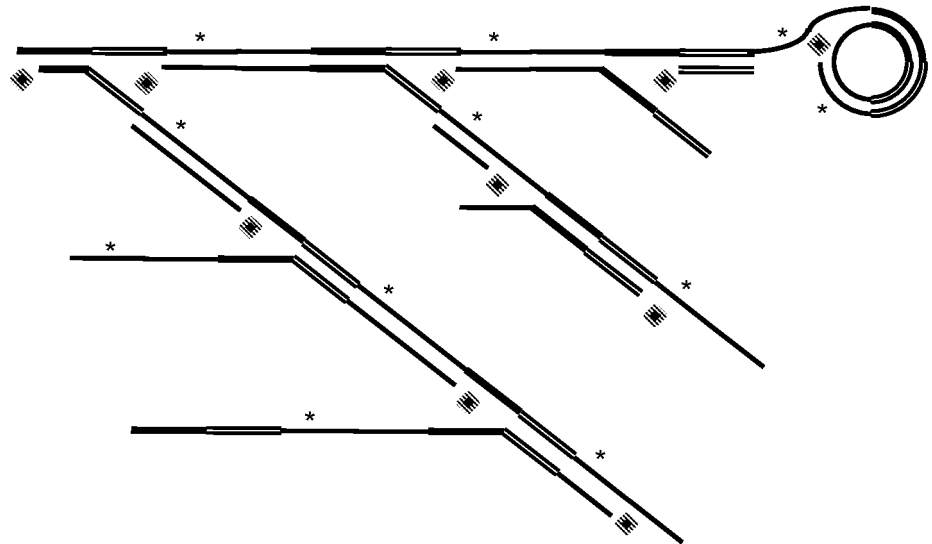
- A. Amplification of circular target with random hexamers. Using either primer or random hexamers, Phi29 DNA polymerase extends on circle template.



- 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.

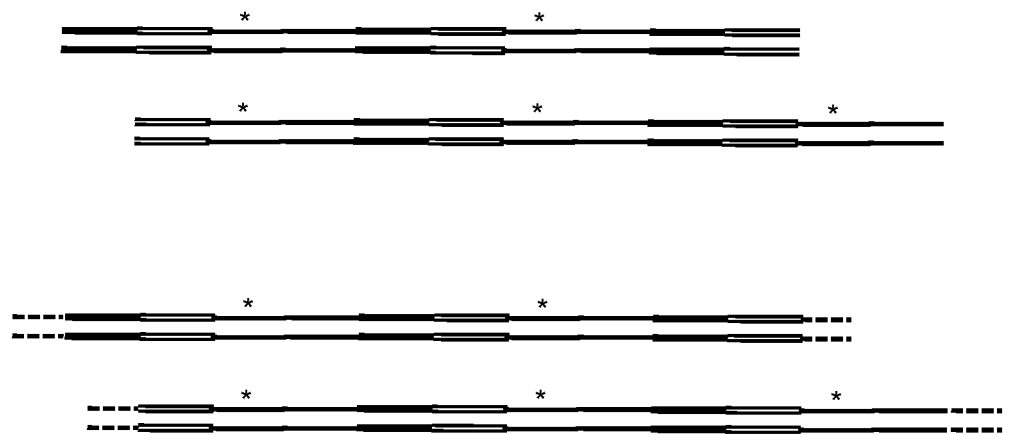


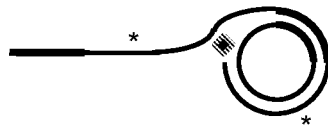
Figure 1

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- 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.

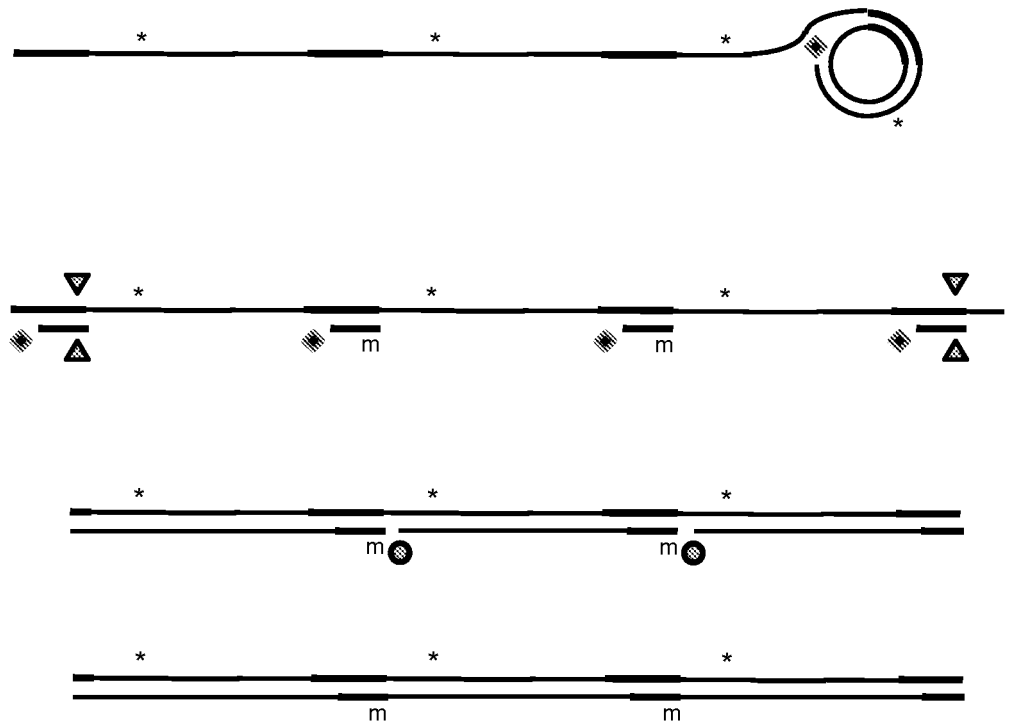


Figure 2

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- 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. BstUI (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, BstUI 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.

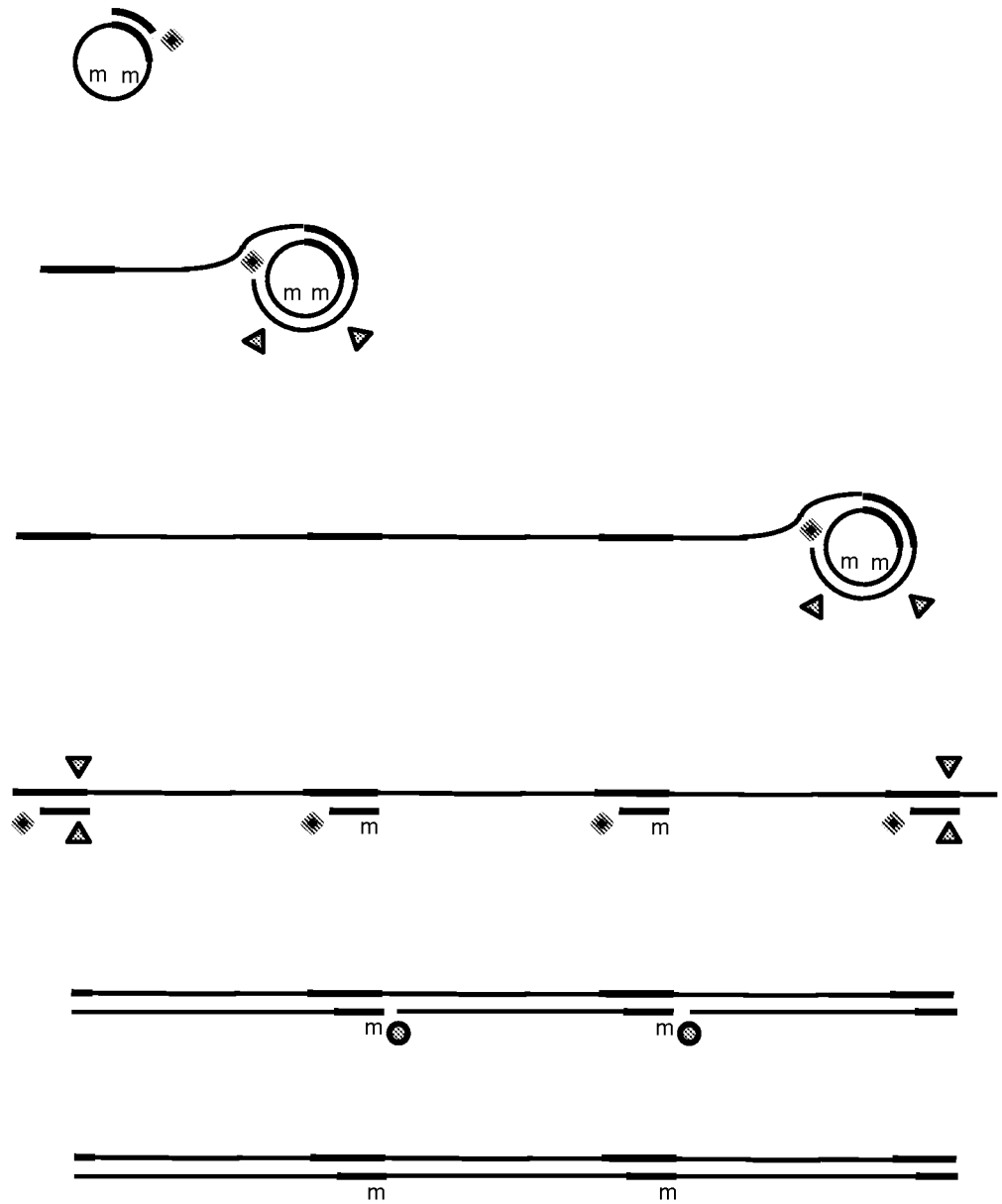
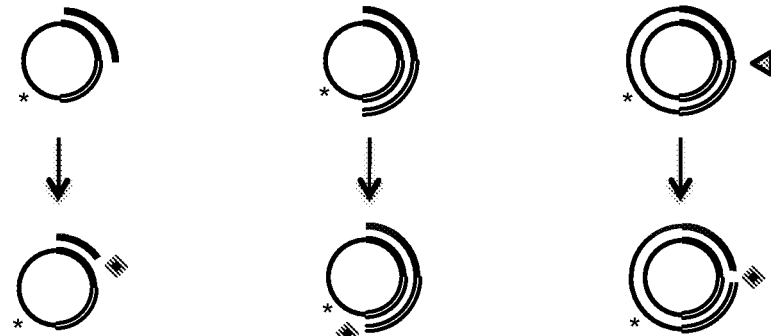


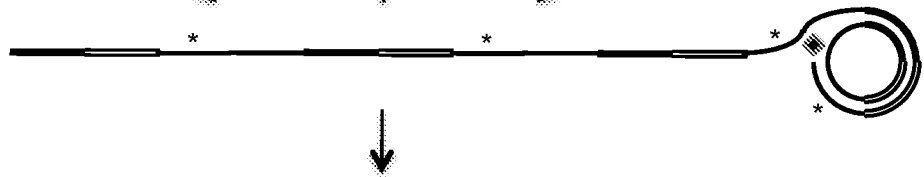
Figure 3

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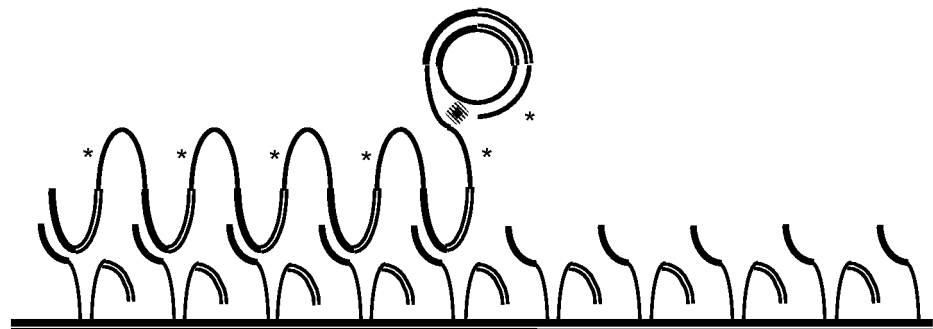
- A. Amplification of circular target with defined primer. Initial template is either primer hybridized to single-stranded circle, similar, but with hybridized coupled oligonucleotide, or double-stranded circle, that is specifically nicked.



- 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.

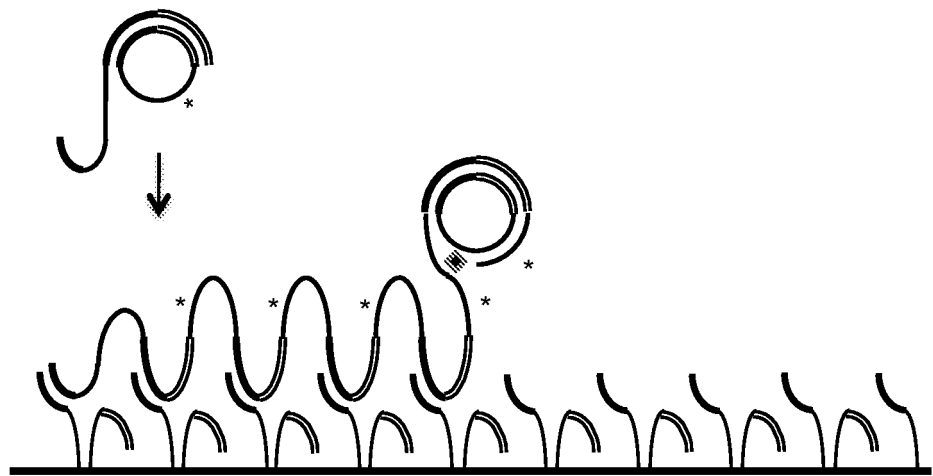
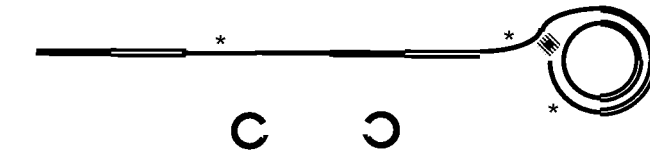


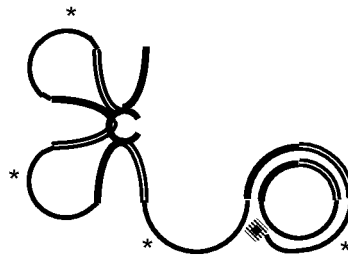
Figure 4

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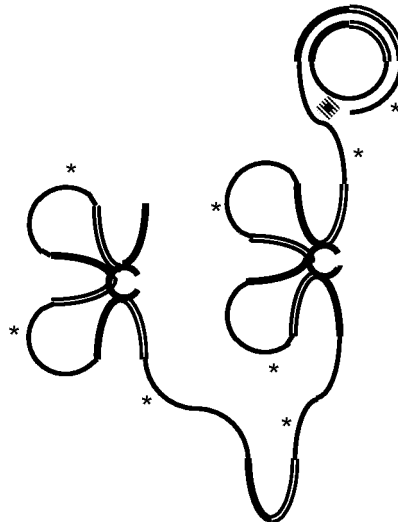
- A. Amplification of circular target with primer to form condensed structures. Phi29-DNA polymerase extends around circular target.



- 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.

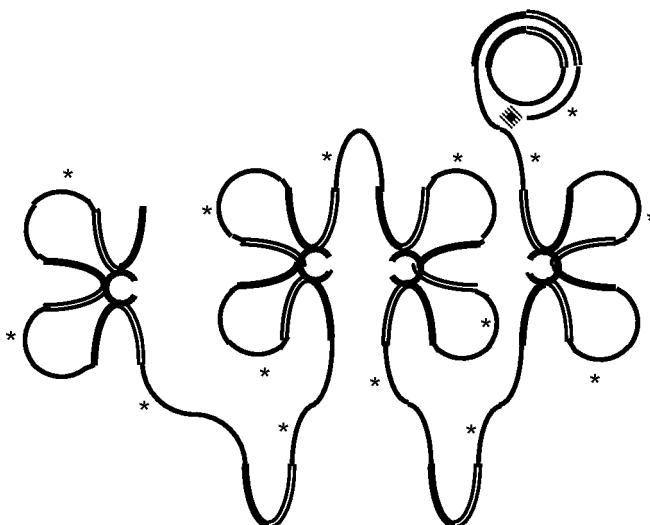
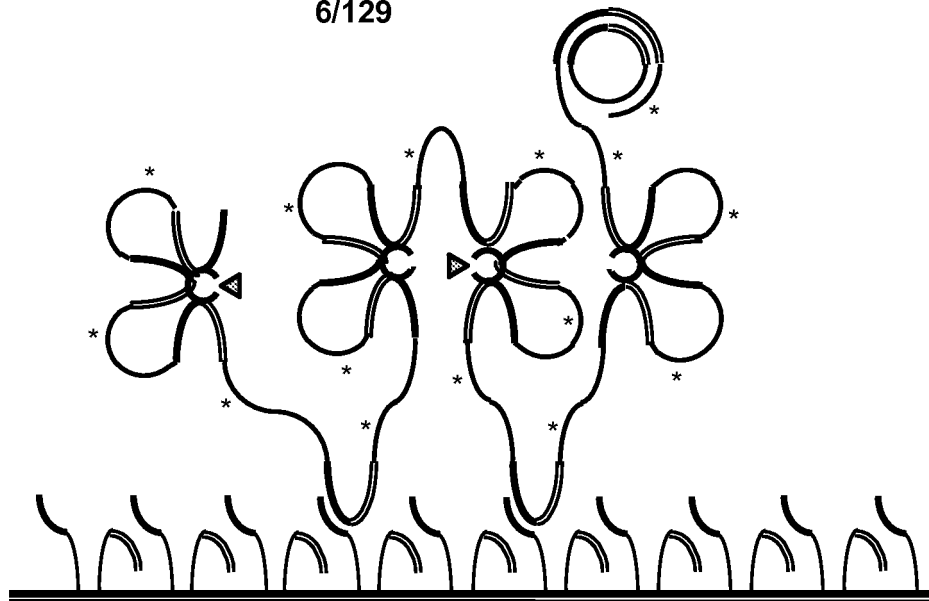


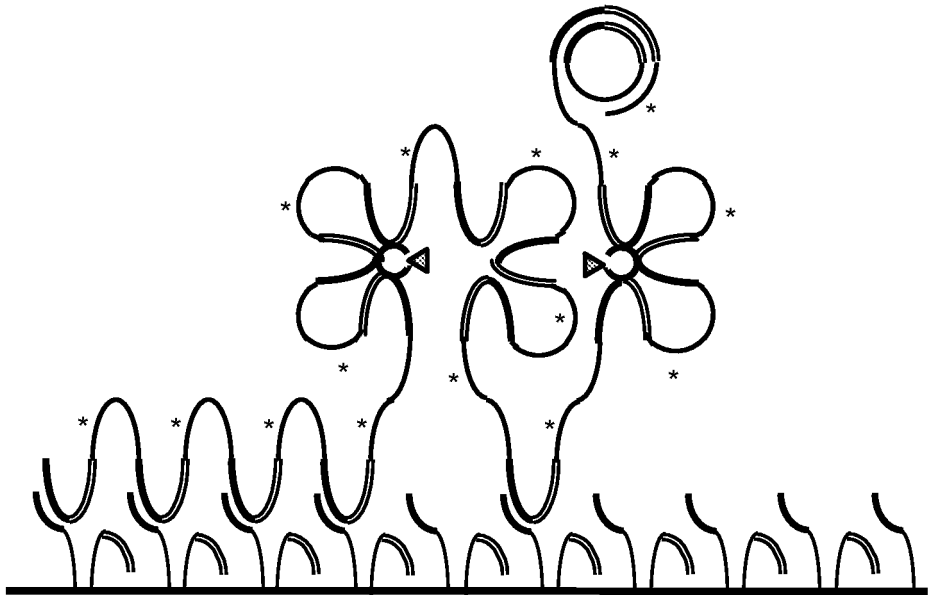
Figure 5

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- 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.

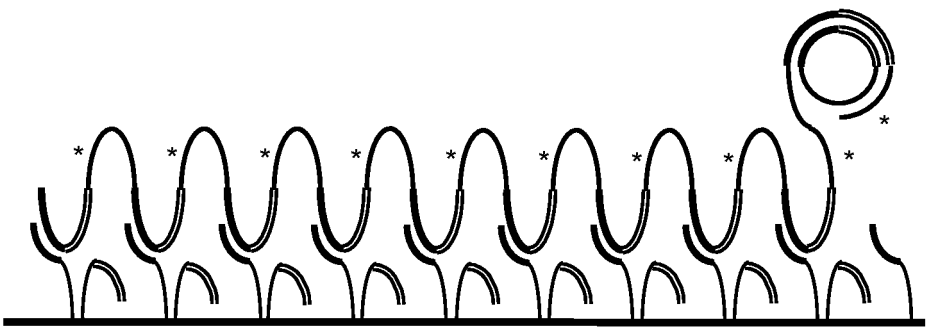
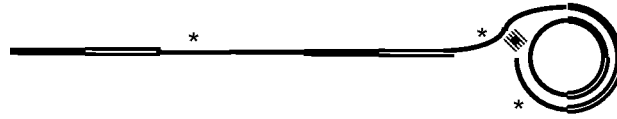


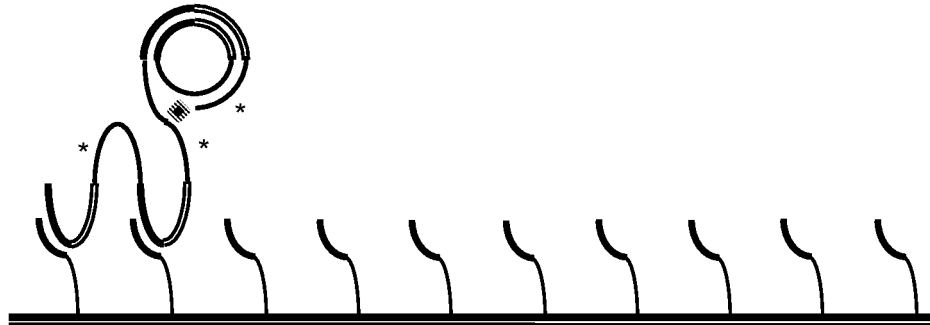
Figure 5 (continued)

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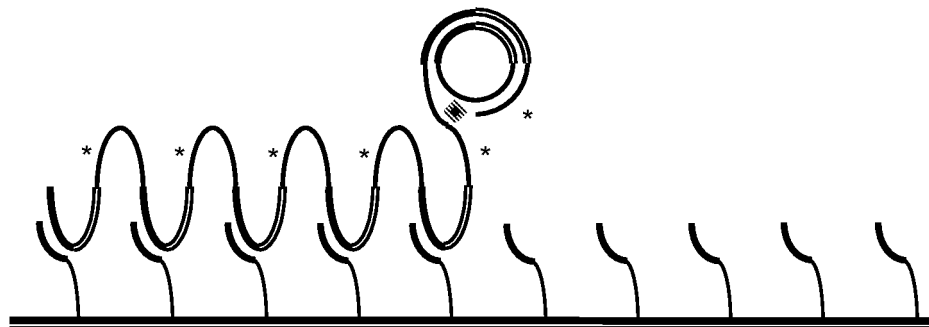
- A. Amplification of circular target for capture on a solid support with a single primer. Phi29 DNA polymerase extends on circle template to generate single-stranded primary extension product.



- 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.

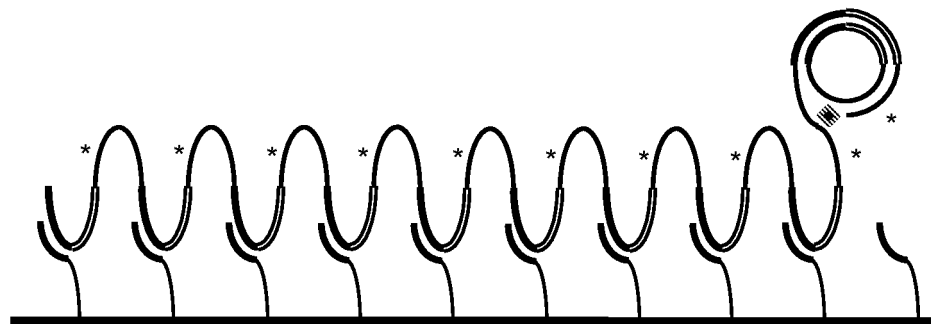
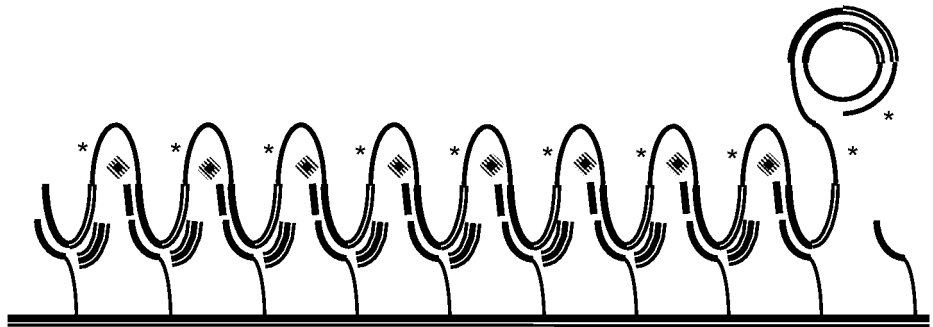


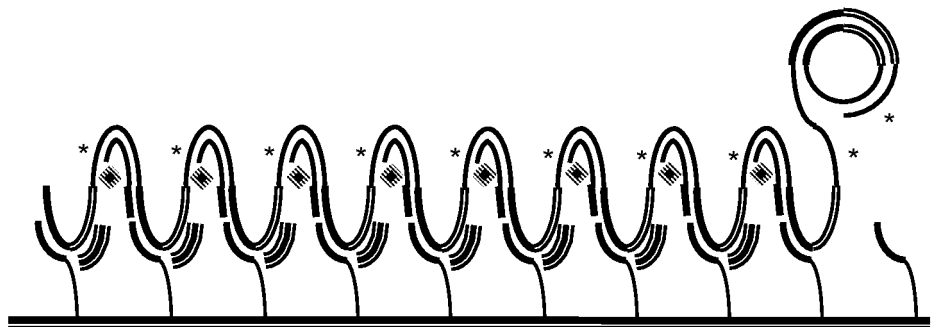
Figure 6

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- 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.

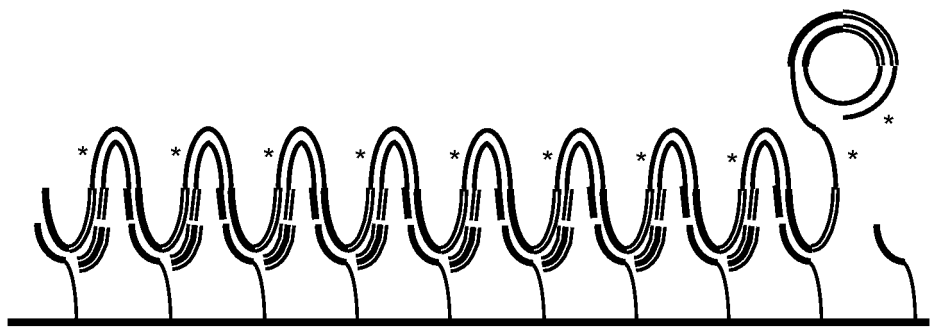
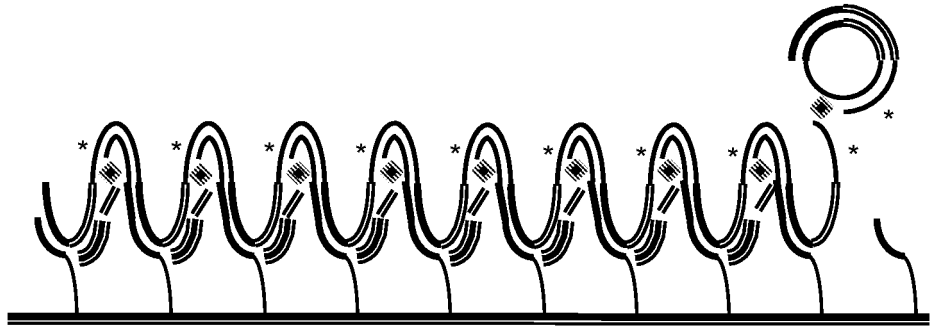


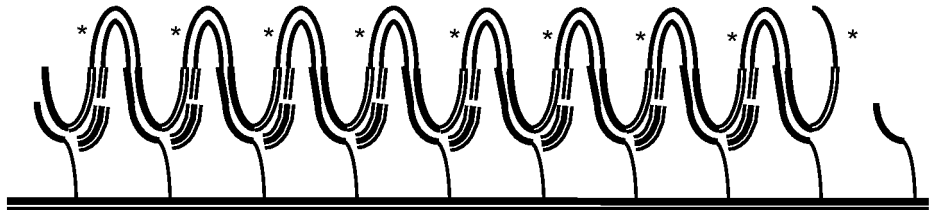
Figure 6 (continued)

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- 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.

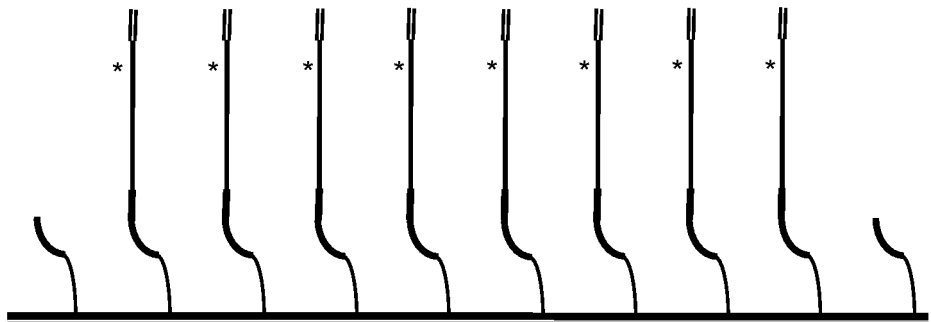
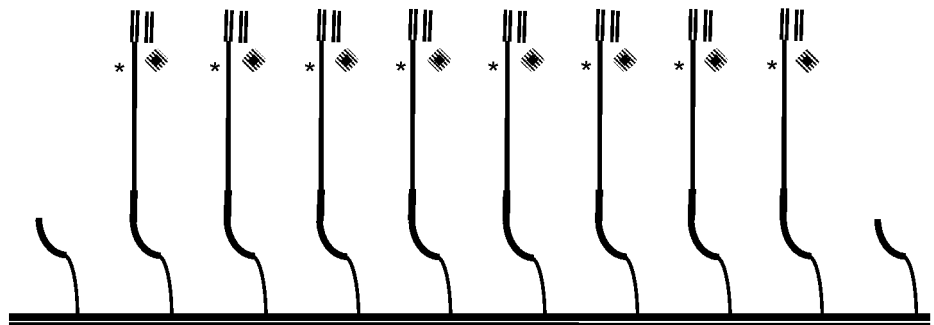


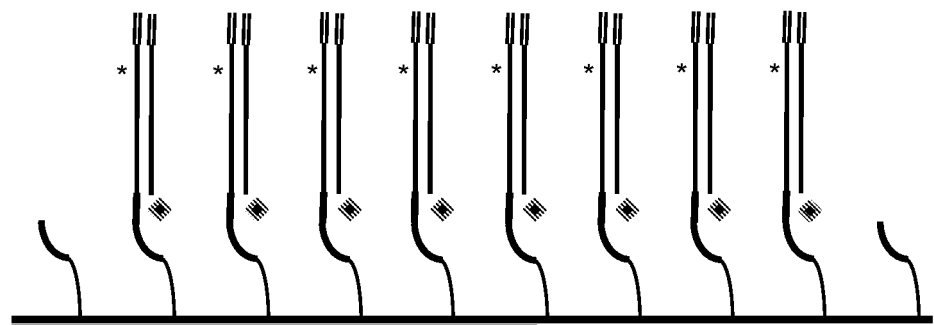
Figure 6 (continued)

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- 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.

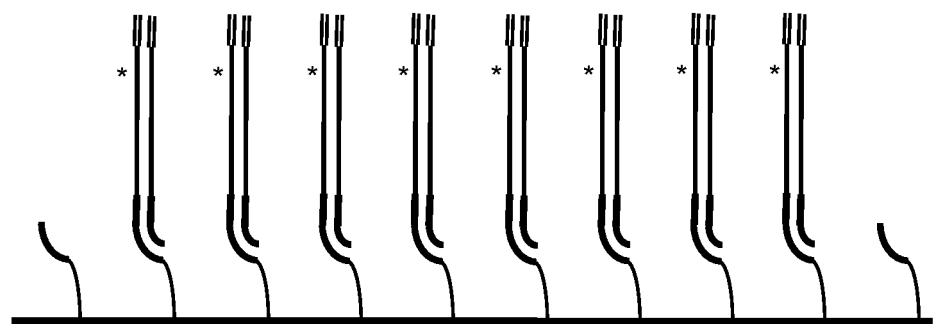
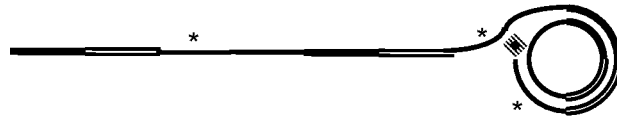


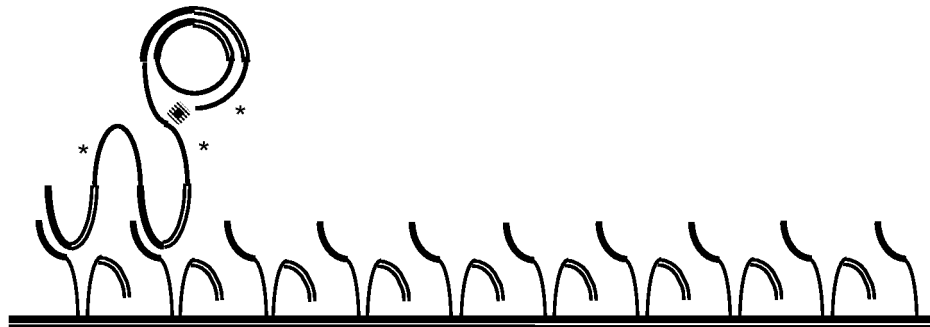
Figure 6 (continued)

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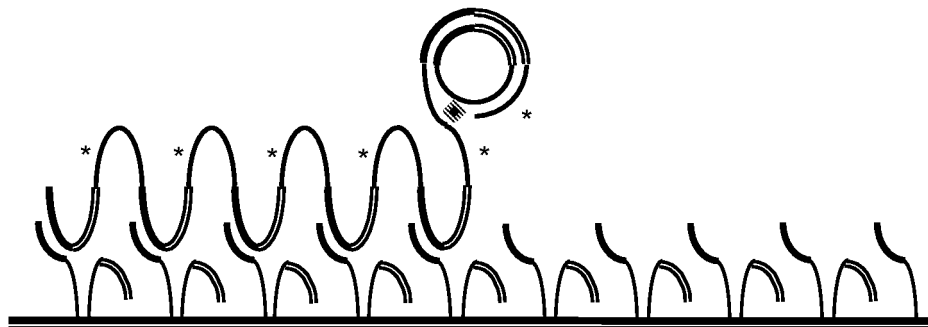
- A. Amplification of circular target for capture on a solid support with dual primers. Phi29 DNA polymerase extends on circle template to generate primary extension products.



- 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.

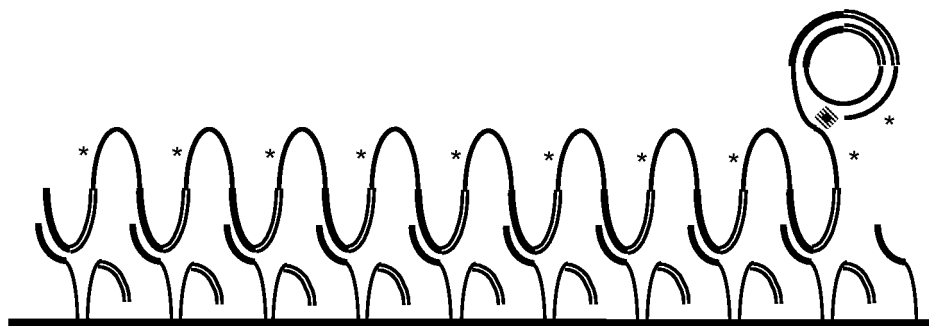
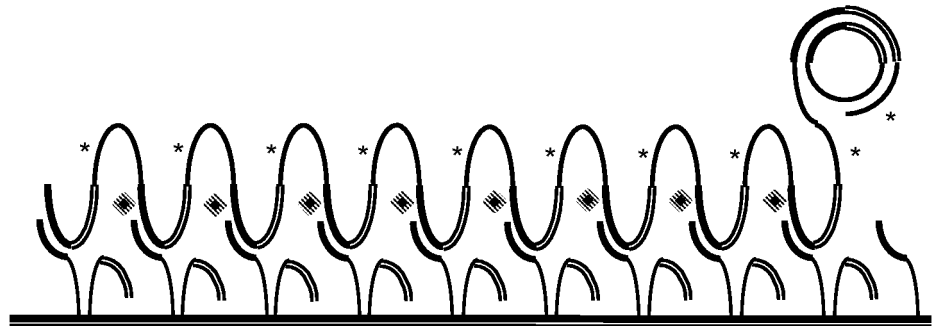


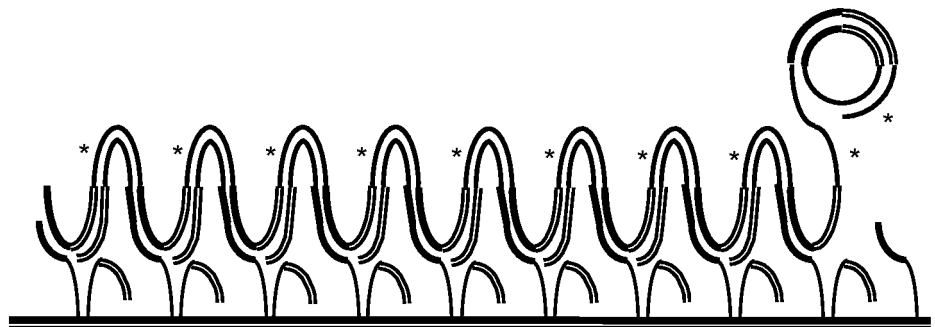
Figure 7

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- 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.

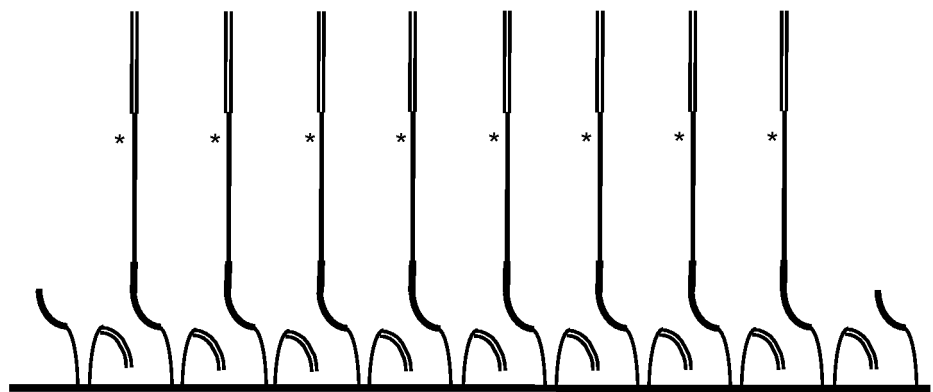
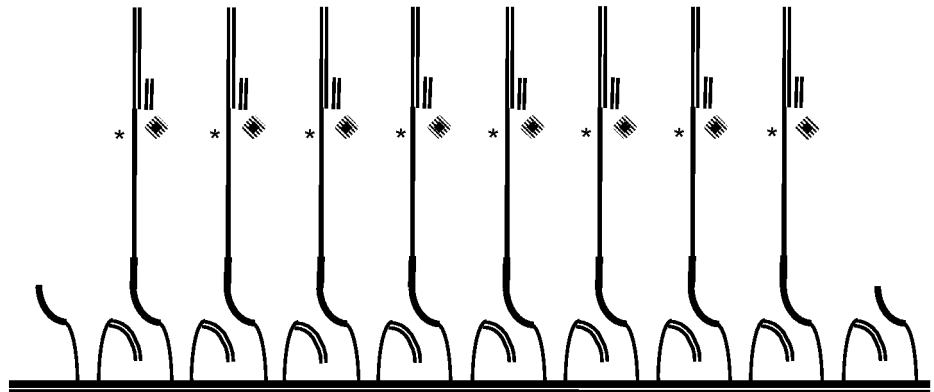


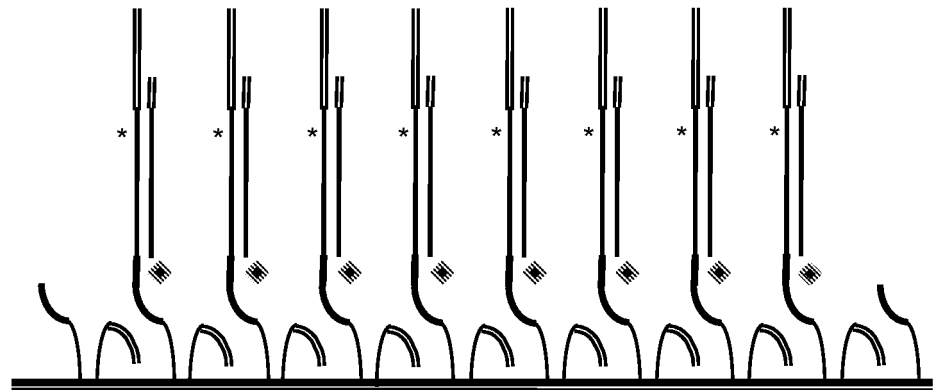
Figure 7 (continued)

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- 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.

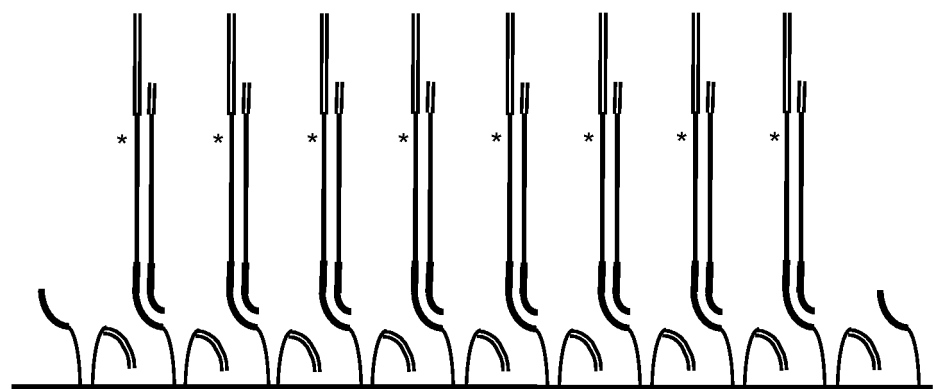
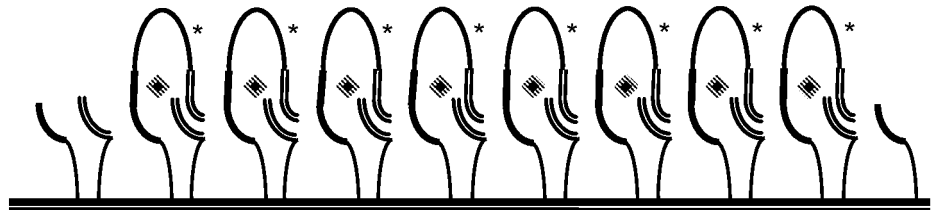


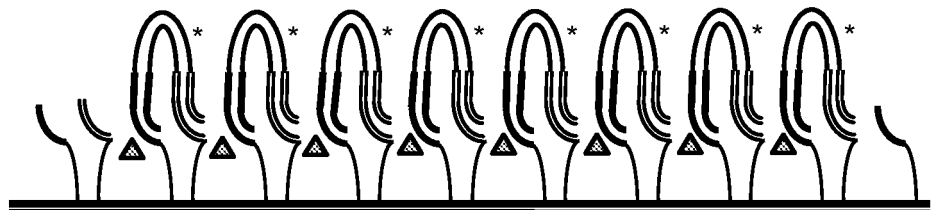
Figure 8

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- 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.

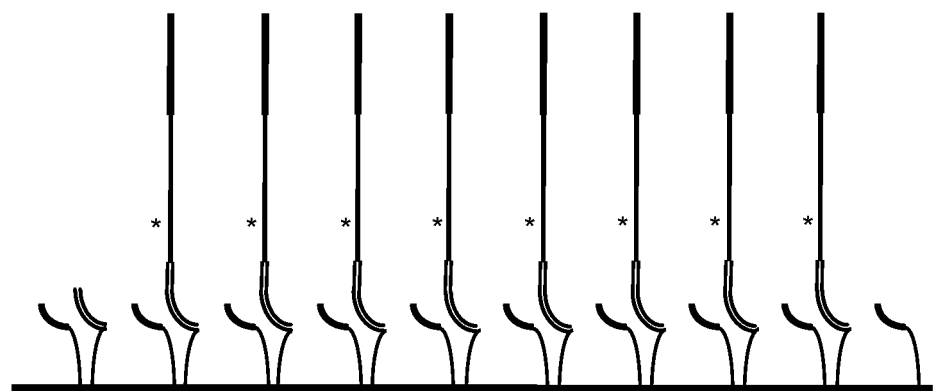
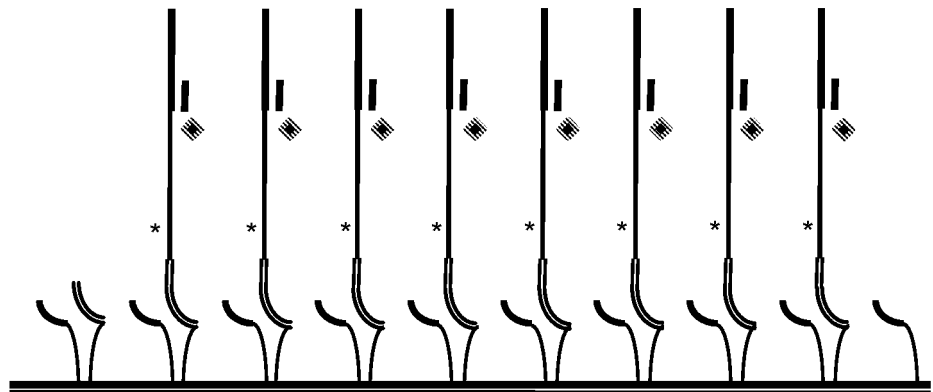


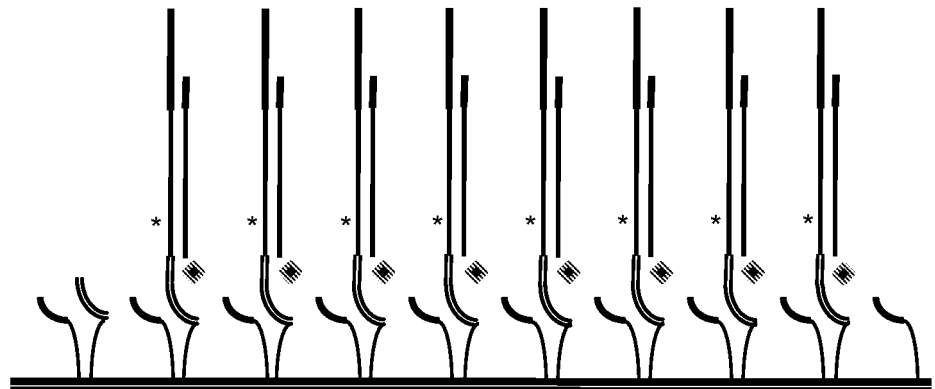
Figure 8 (continued)

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- 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.

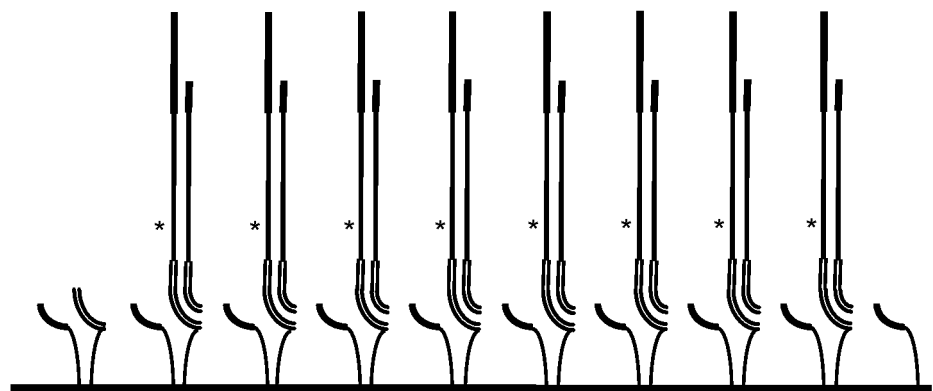
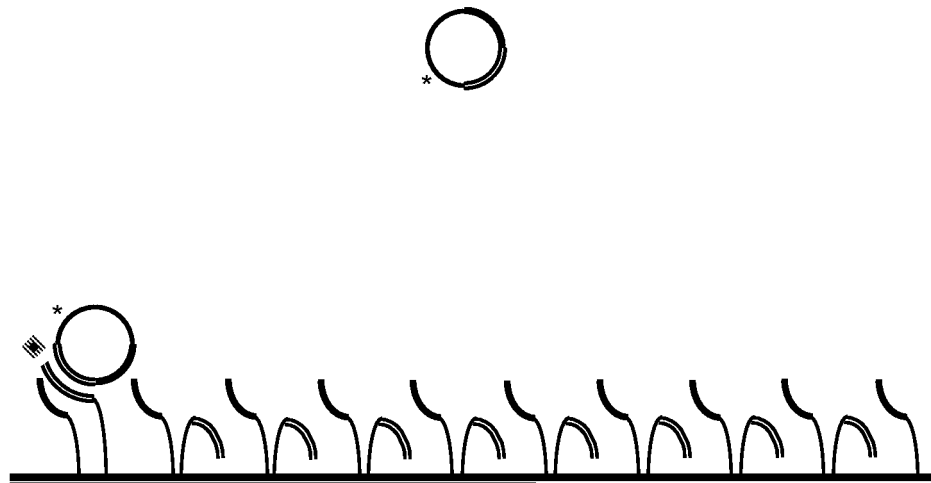


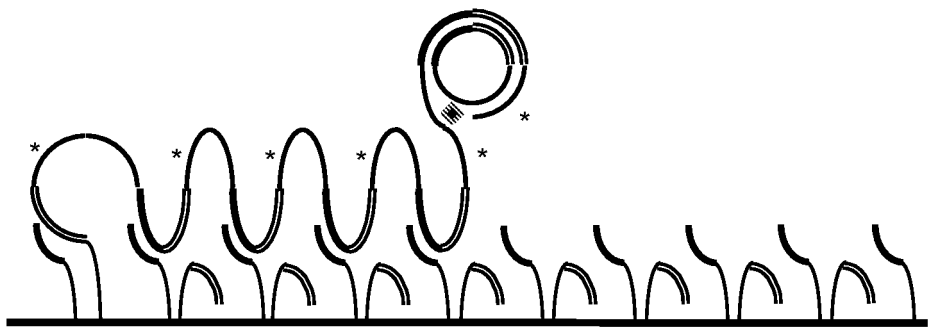
Figure 8 (continued)

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- 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.

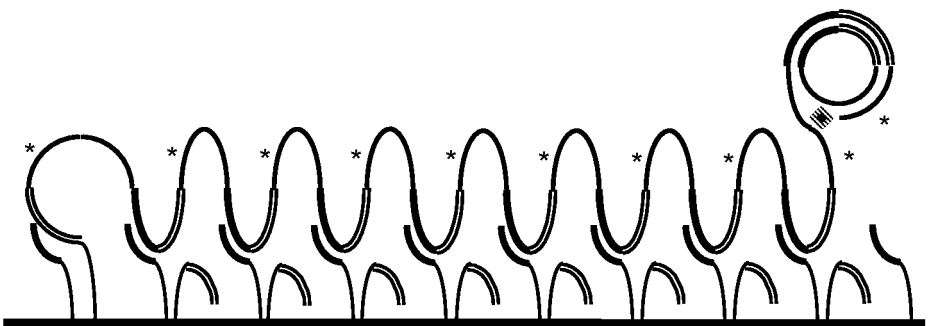
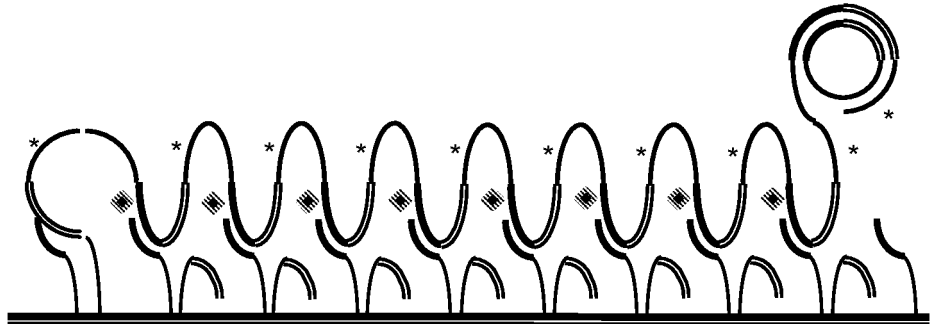


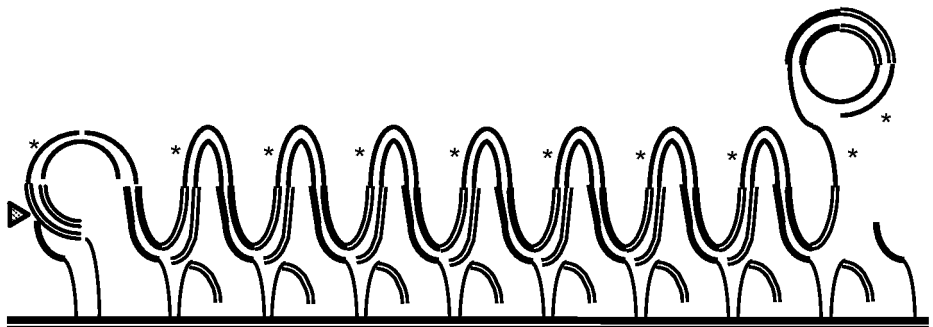
Figure 9

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- 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.

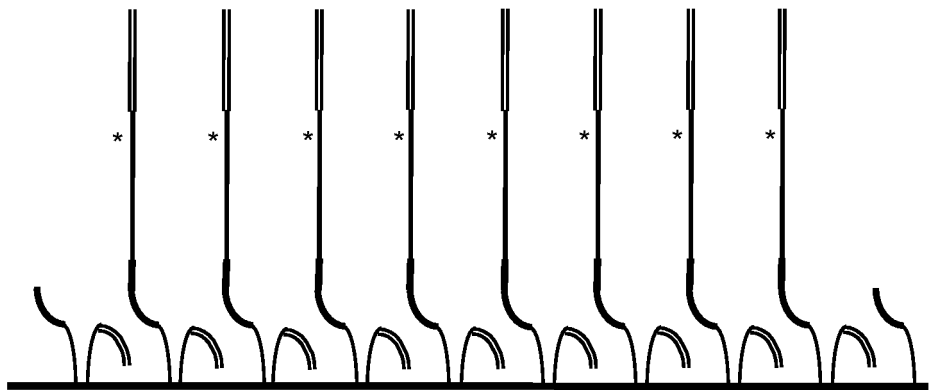
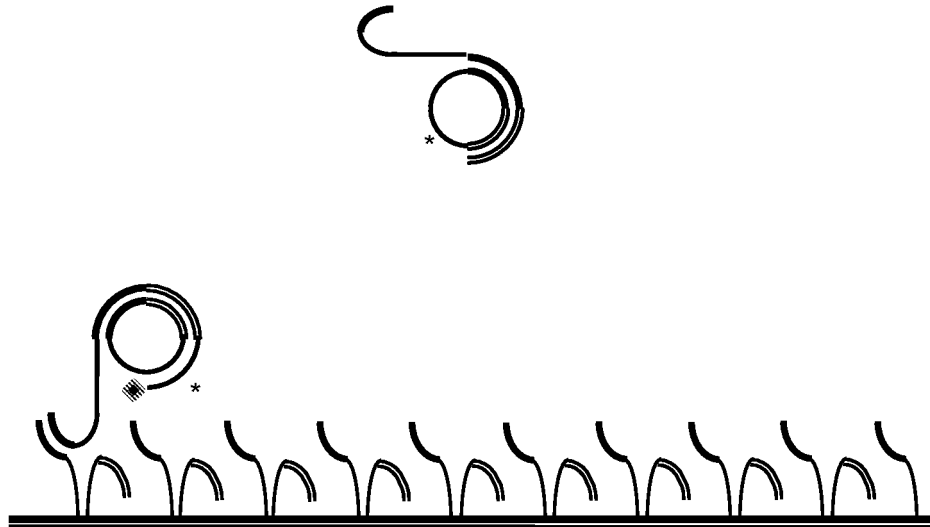


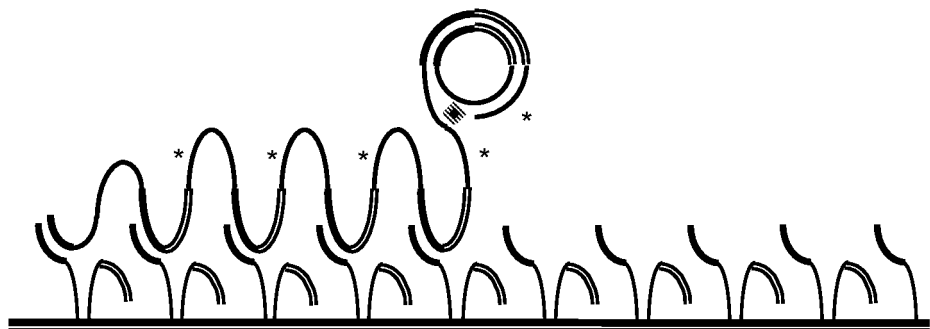
Figure 9 (continued)

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- 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.

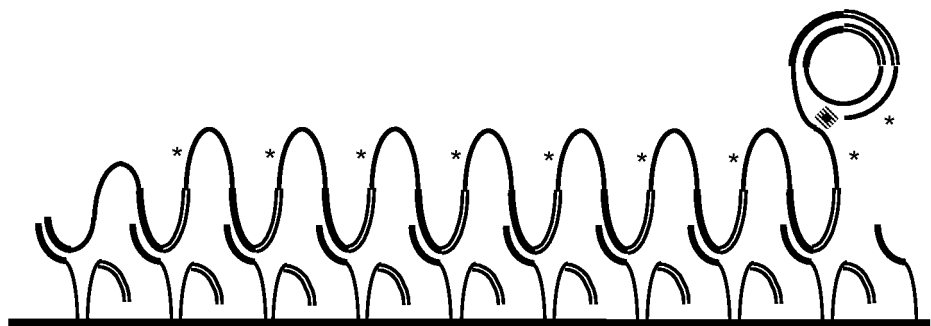
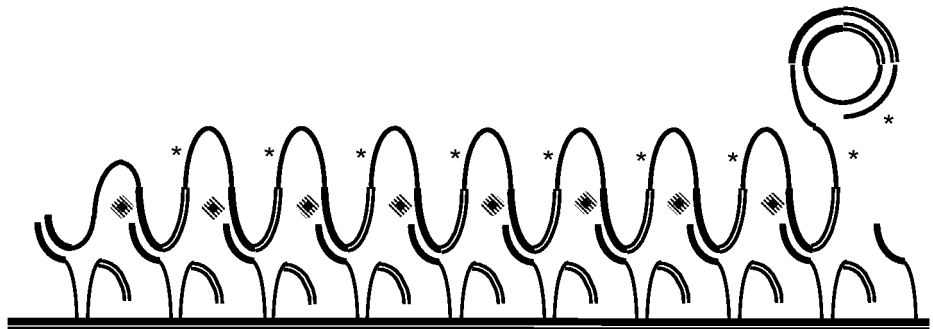


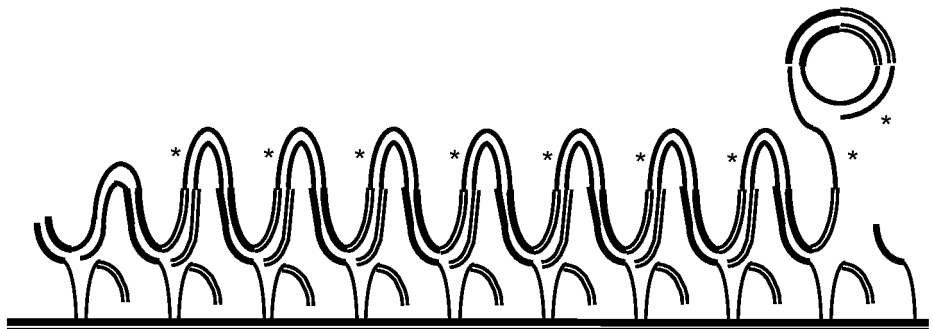
Figure 10

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- 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.

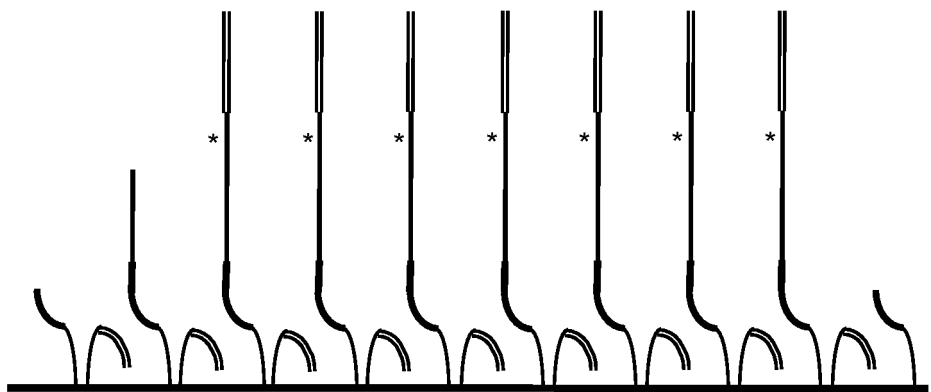
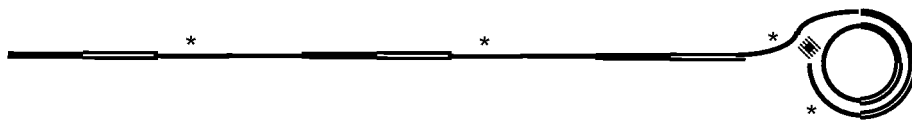


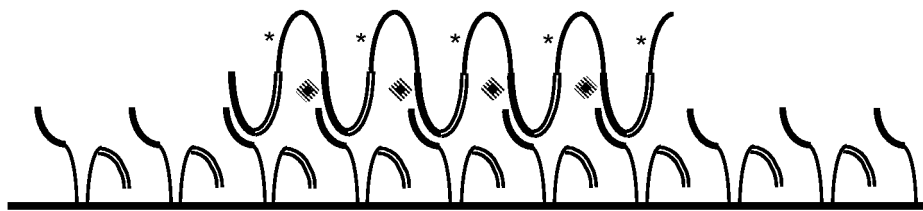
Figure 10 (continued)

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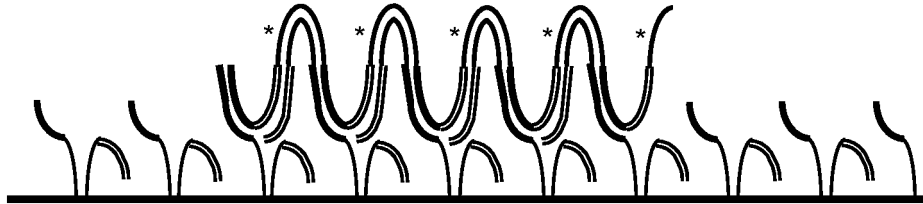
- A. Amplification of circular target for capture on a solid support with dual primers. DNA polymerase extends amplification primer on circle template to generate short primary extension product. Polymerase and extension product are denatured from circular template.



- 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.

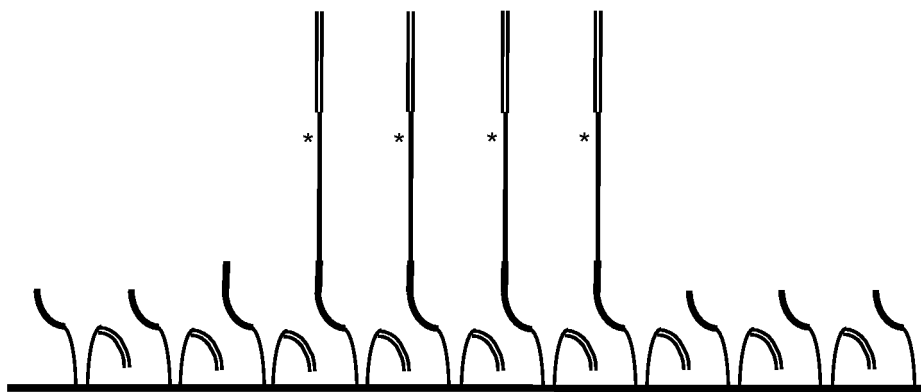
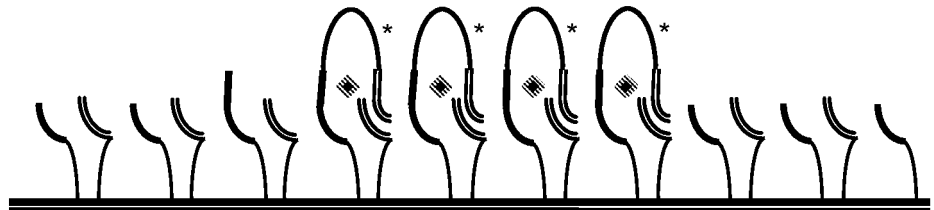


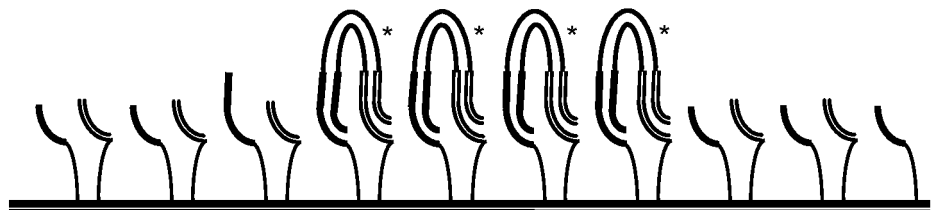
Figure 11

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



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



- G. The extension products are denatured.

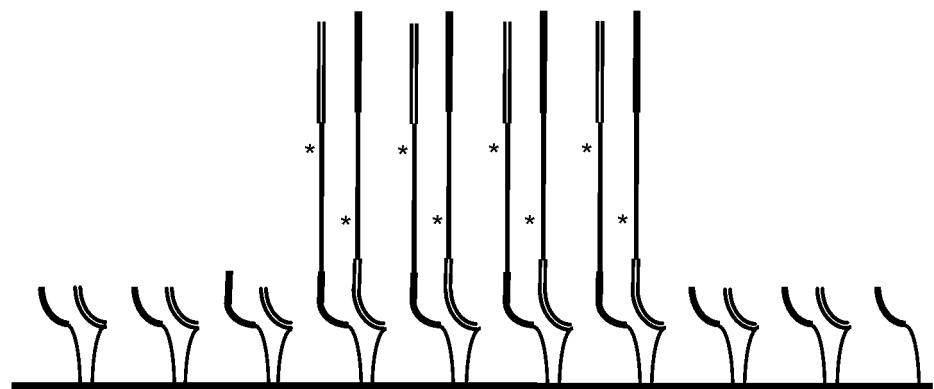
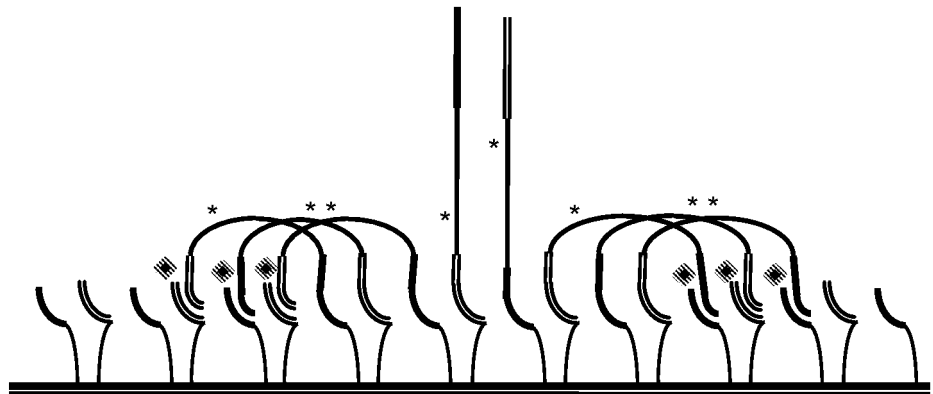


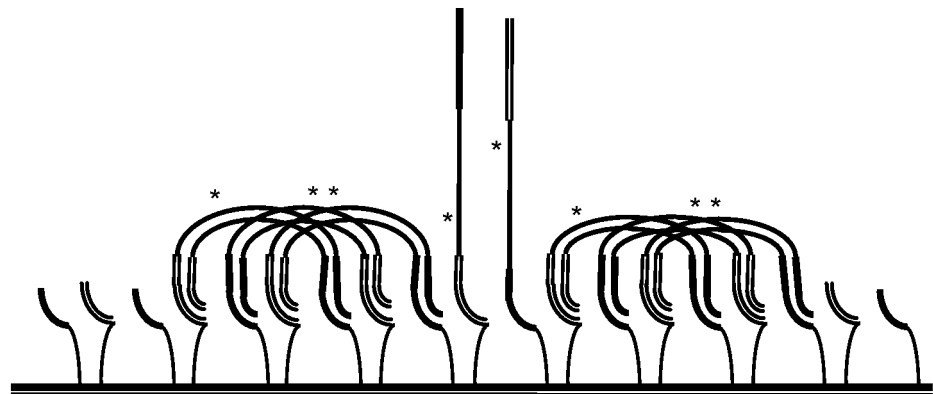
Figure 11 (continued)

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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.

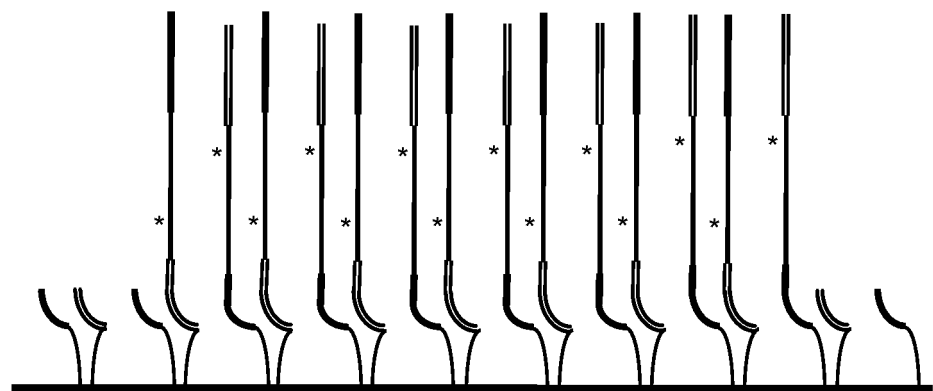


Figure 11 (continued)

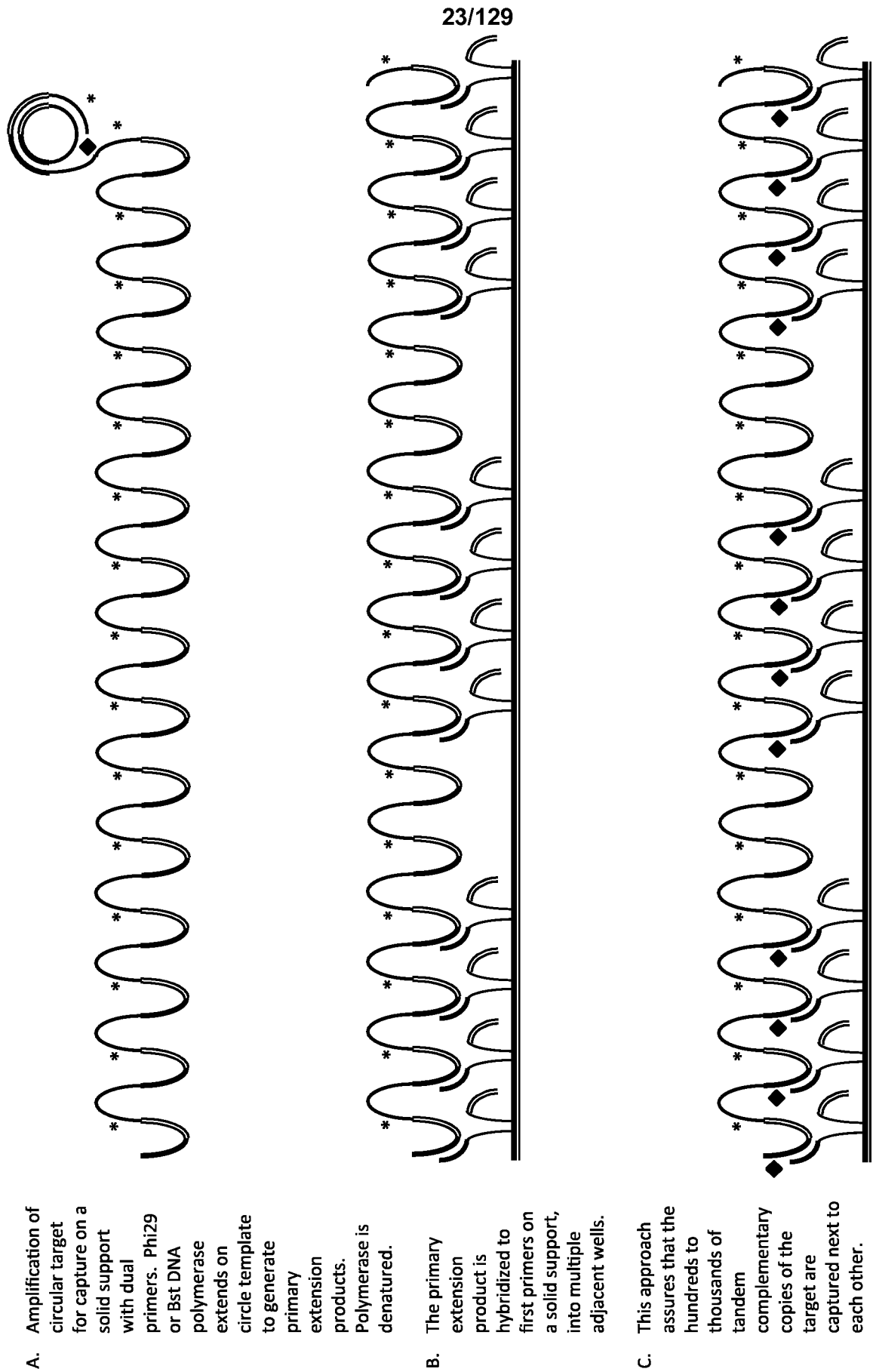


Figure 12

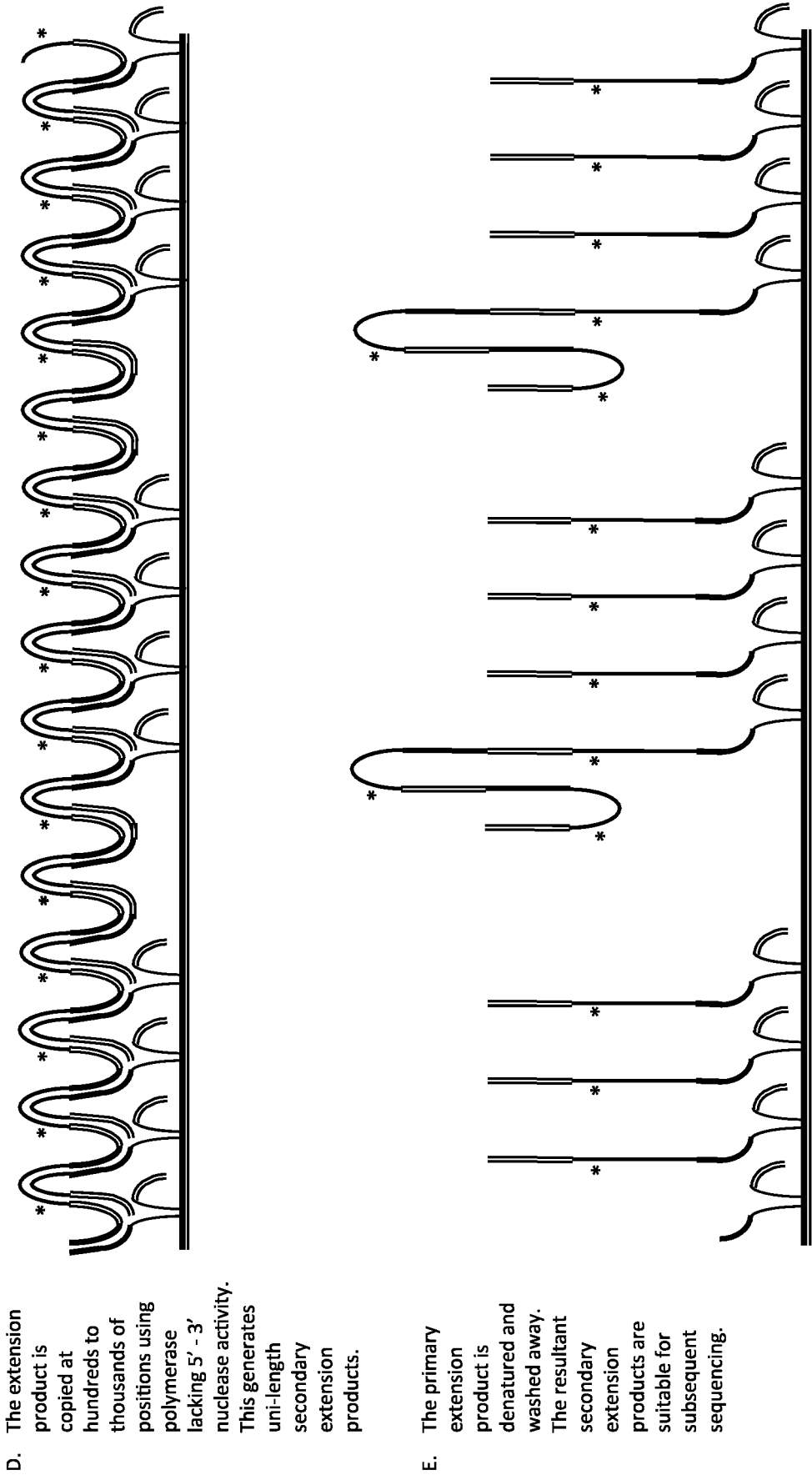


Figure 12 continued

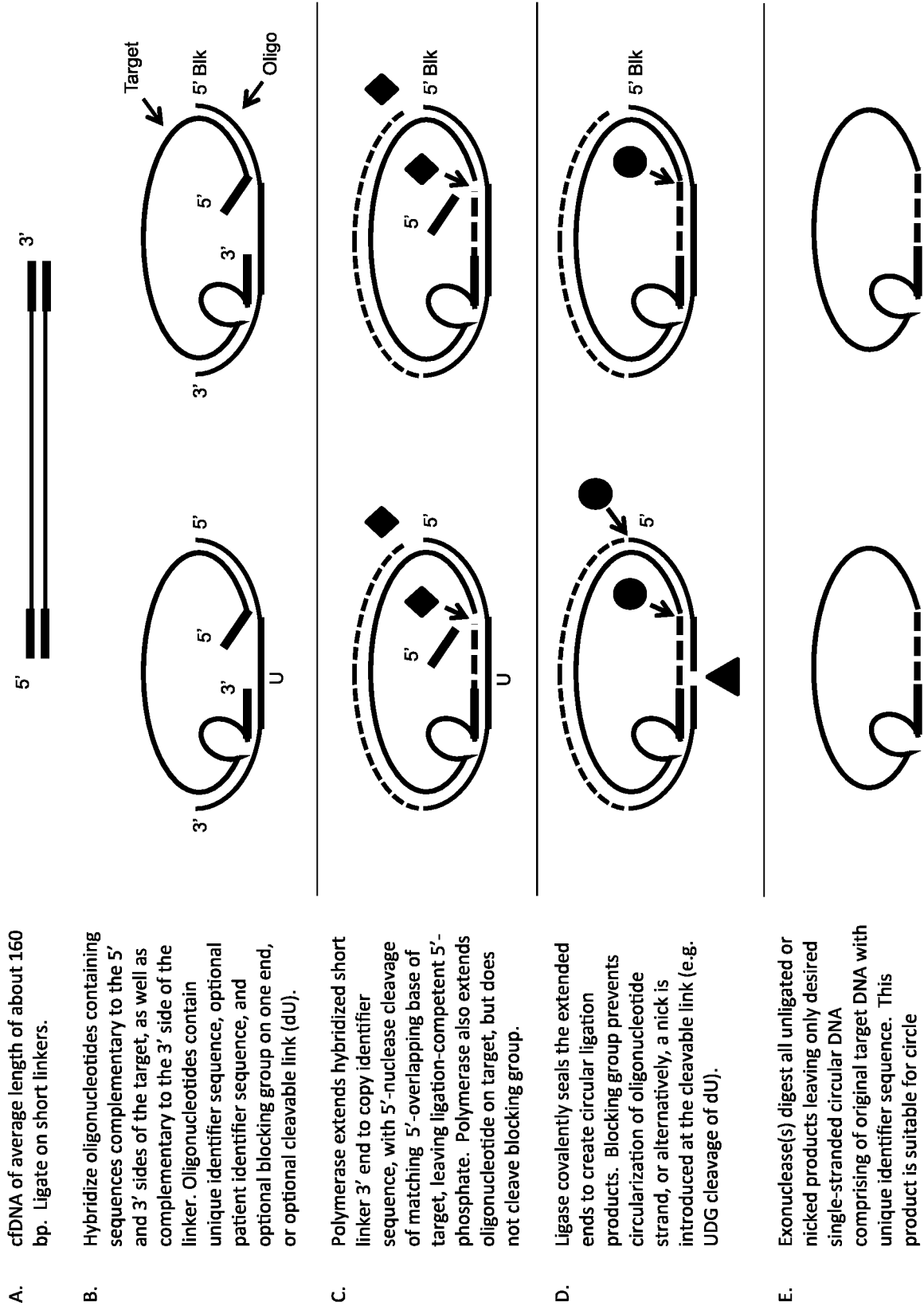


Figure 13

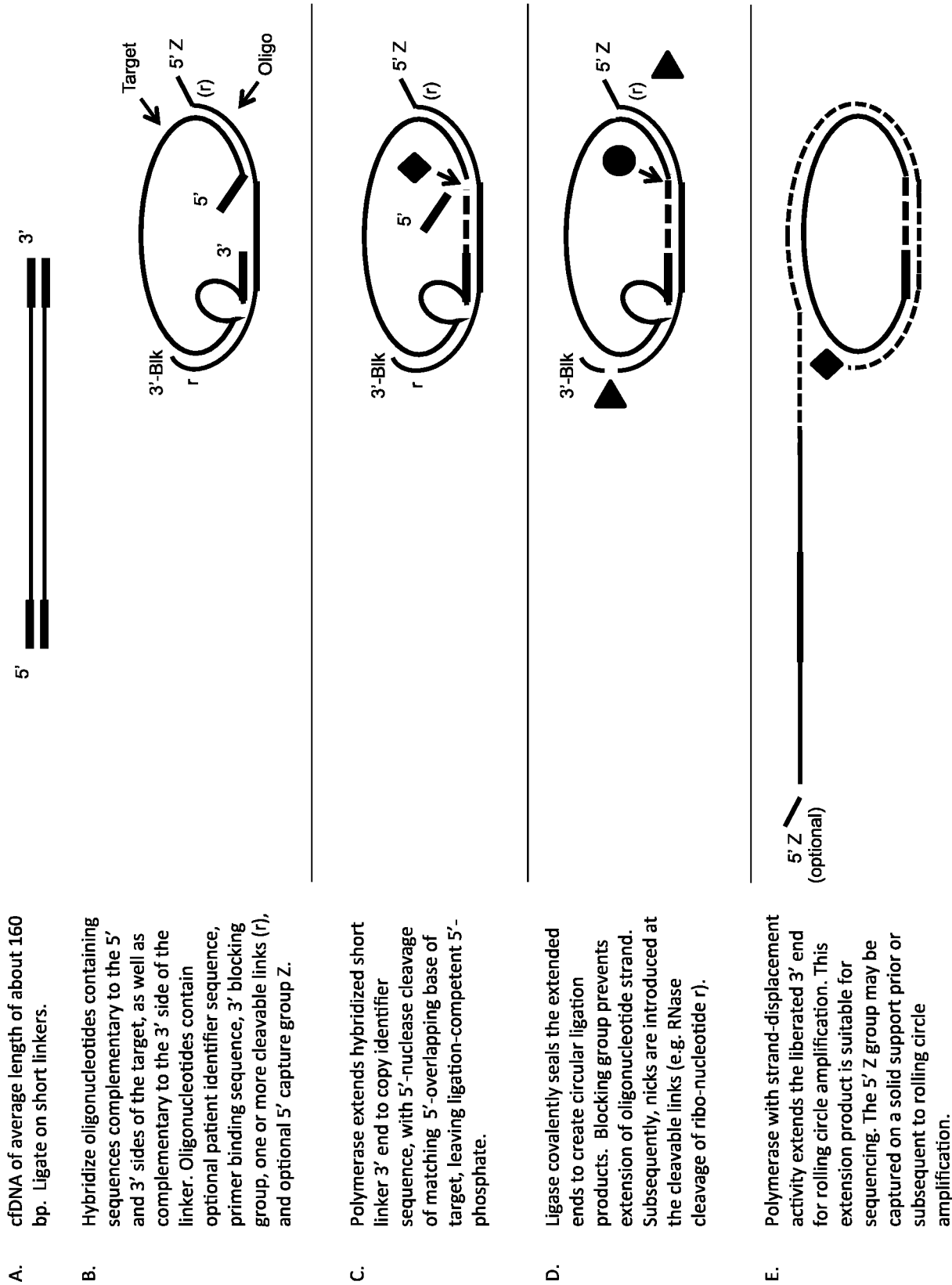


Figure 14

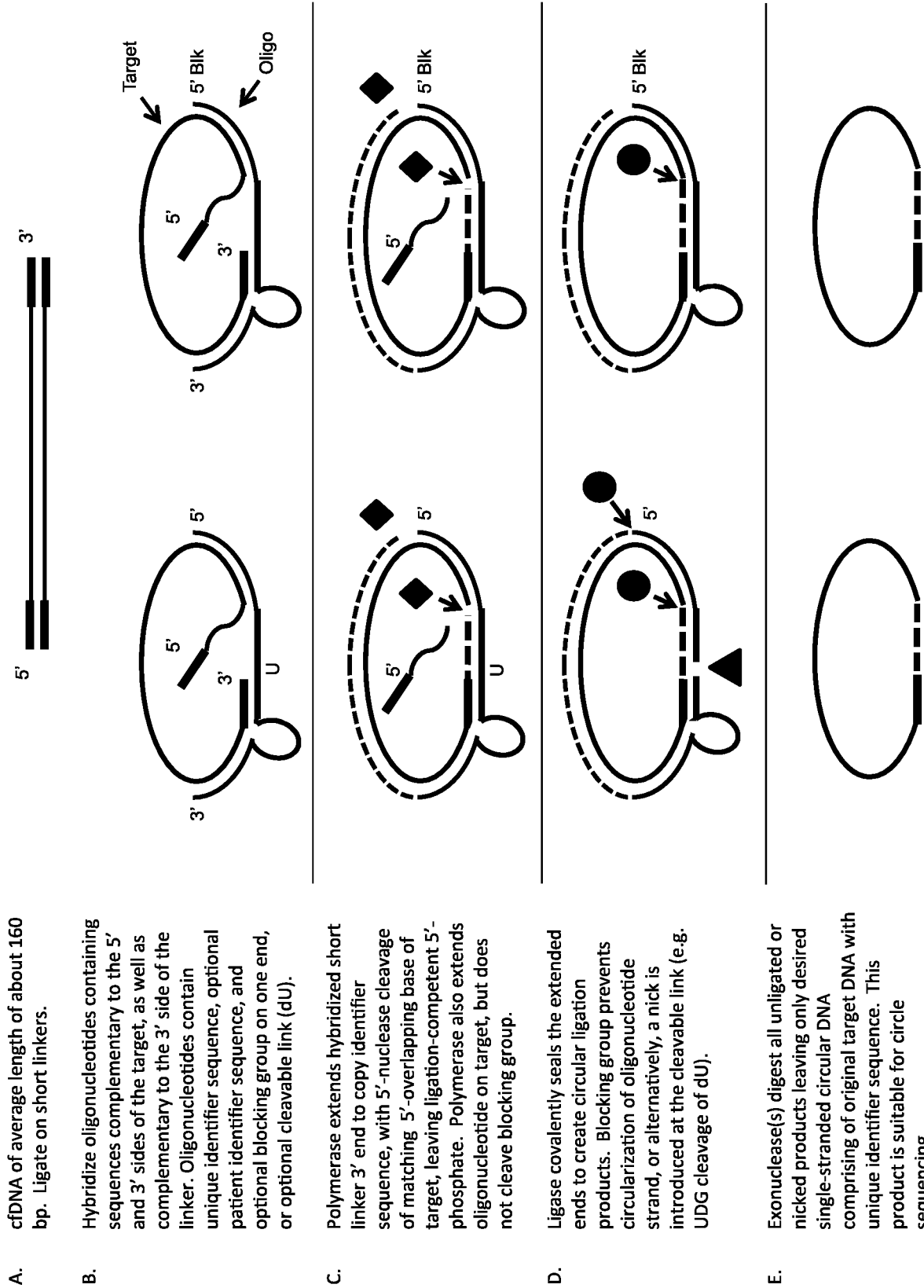


Figure 15

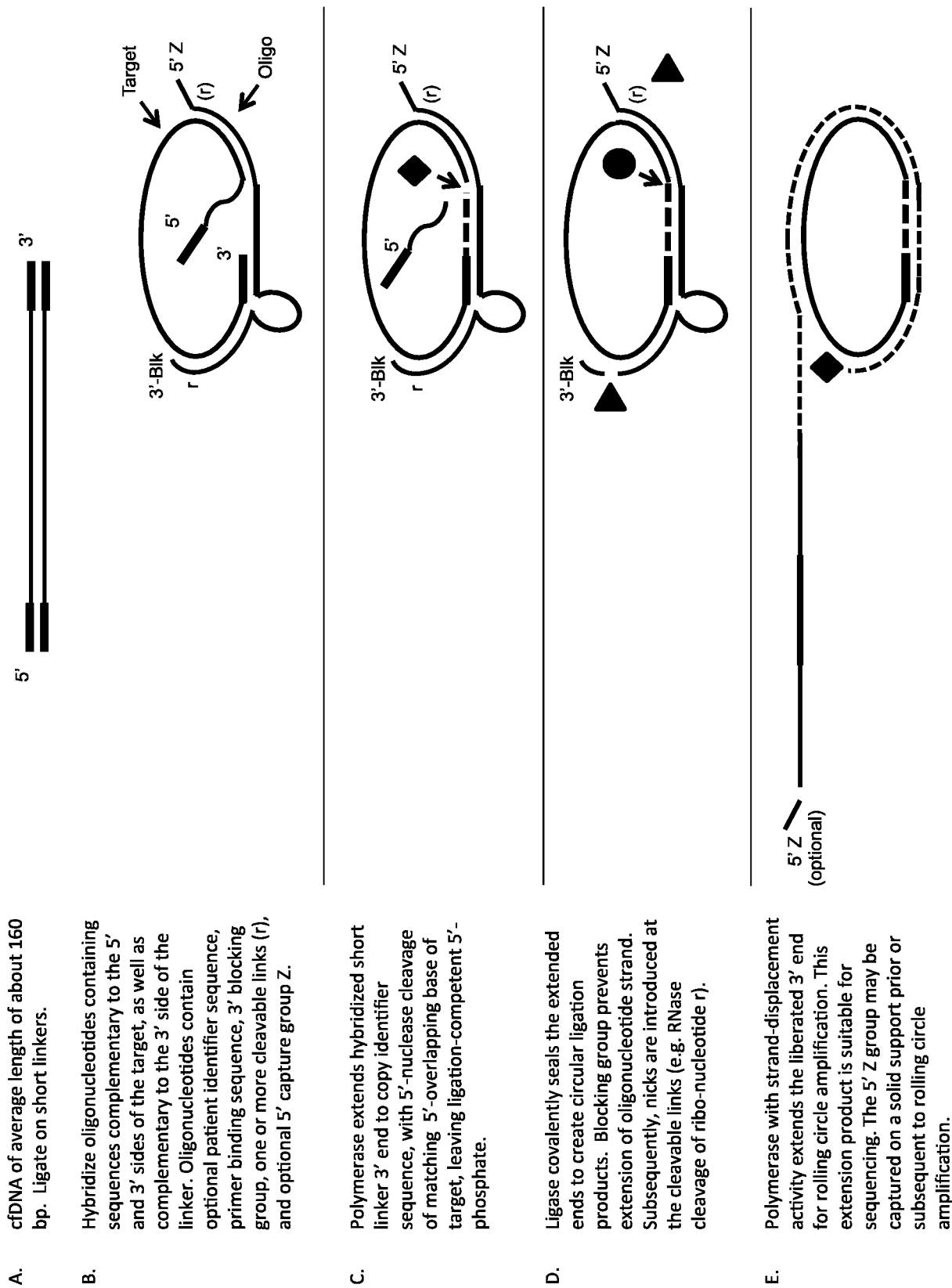


Figure 16

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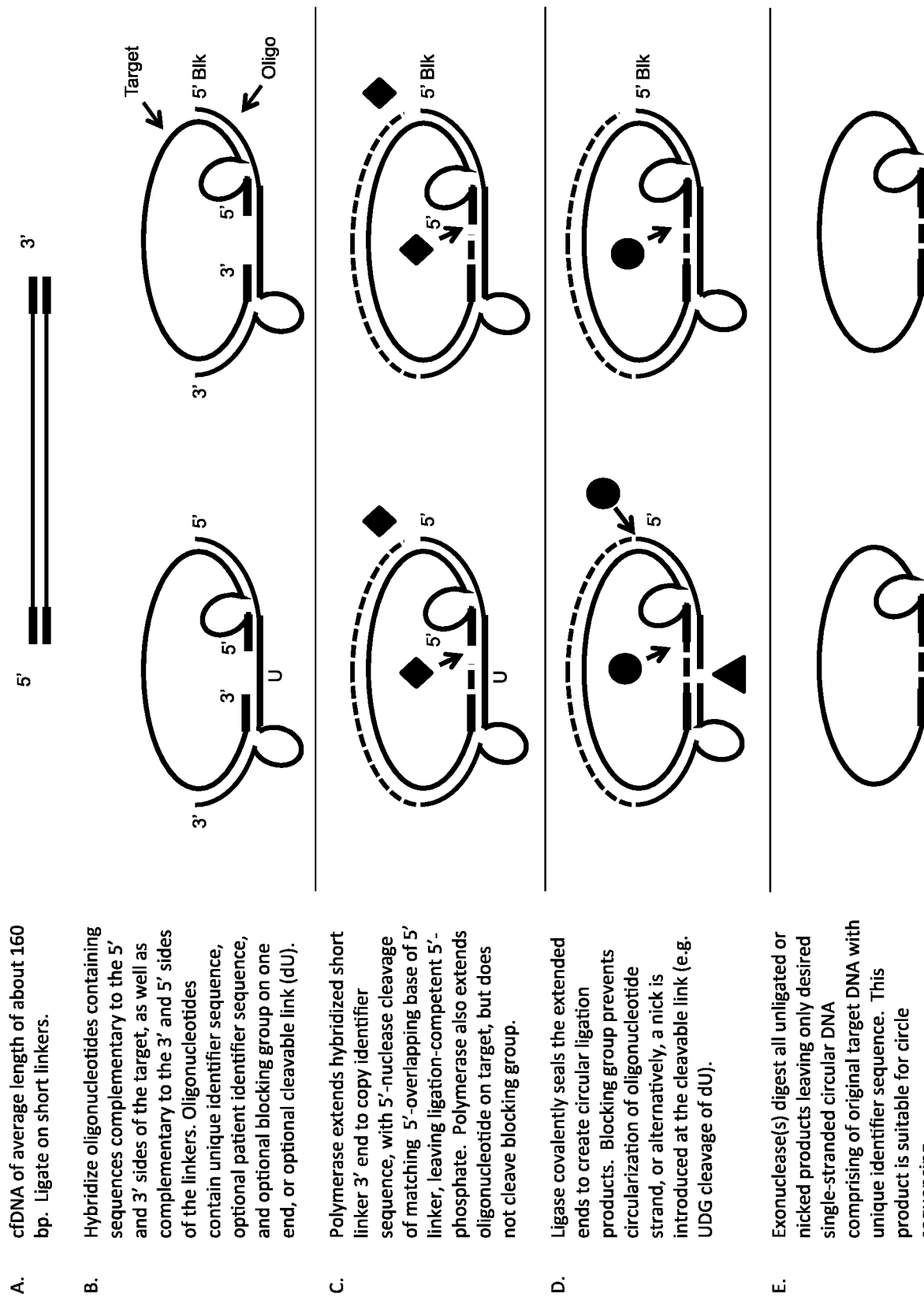


Figure 17

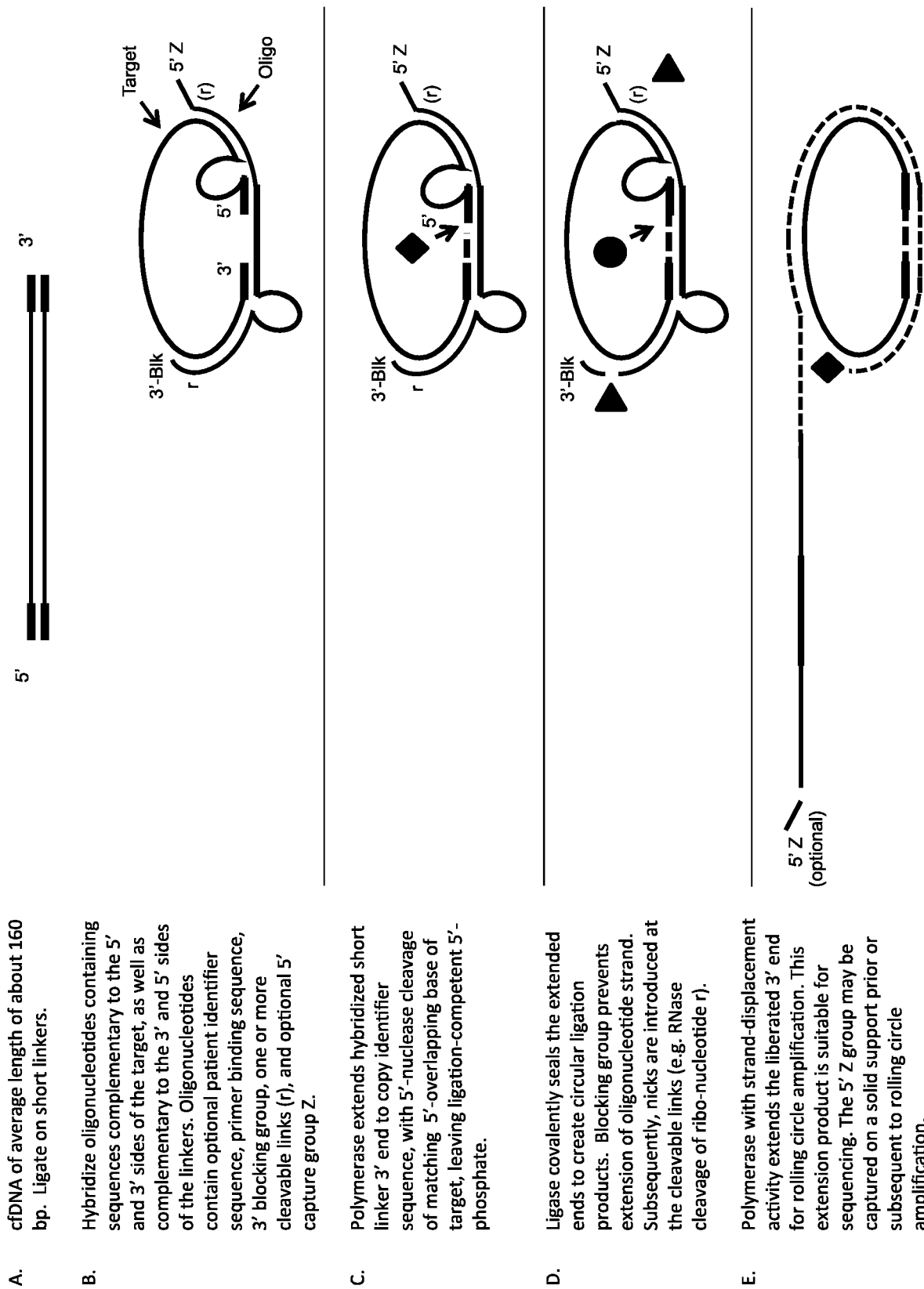


Figure 18

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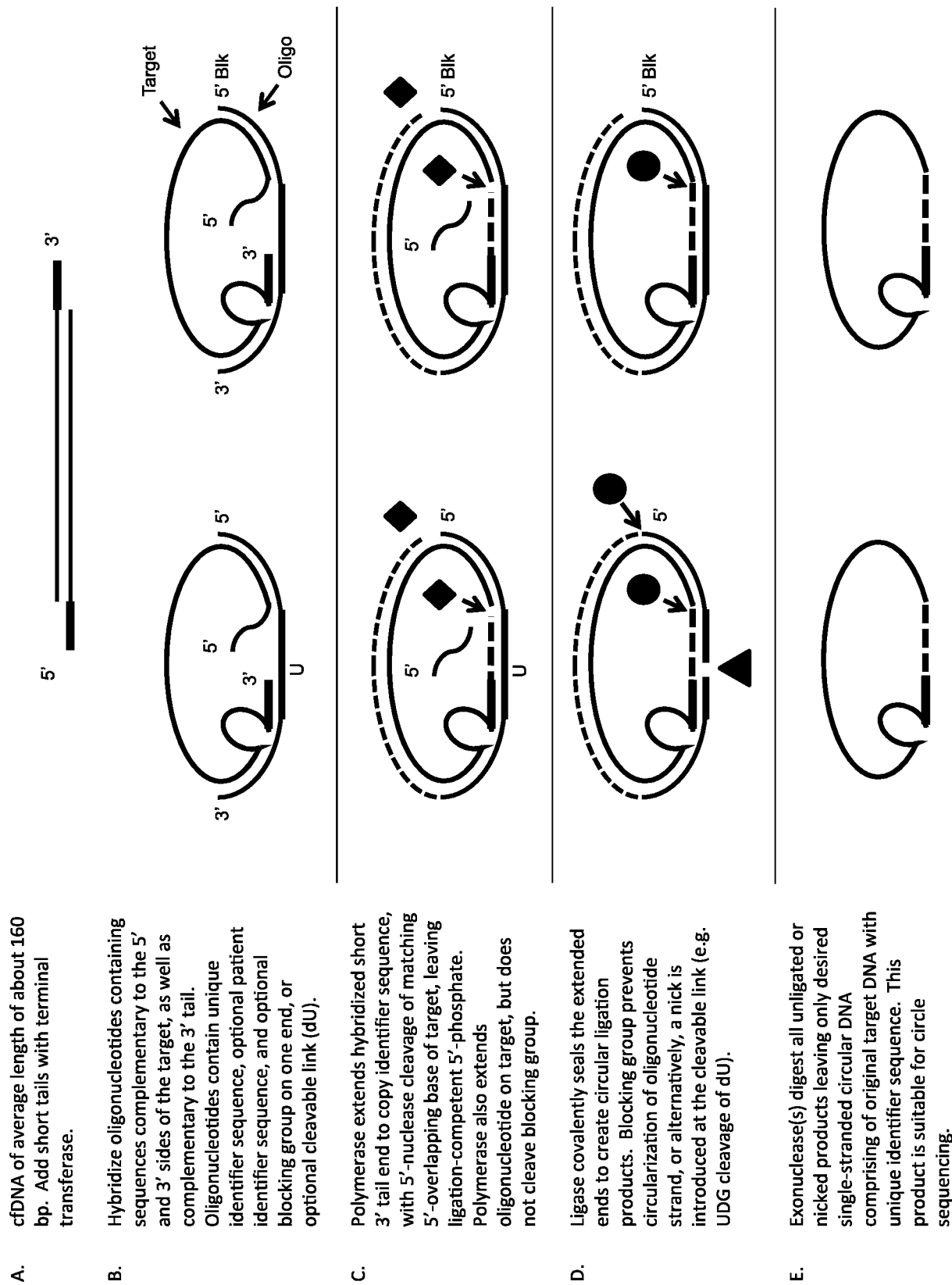


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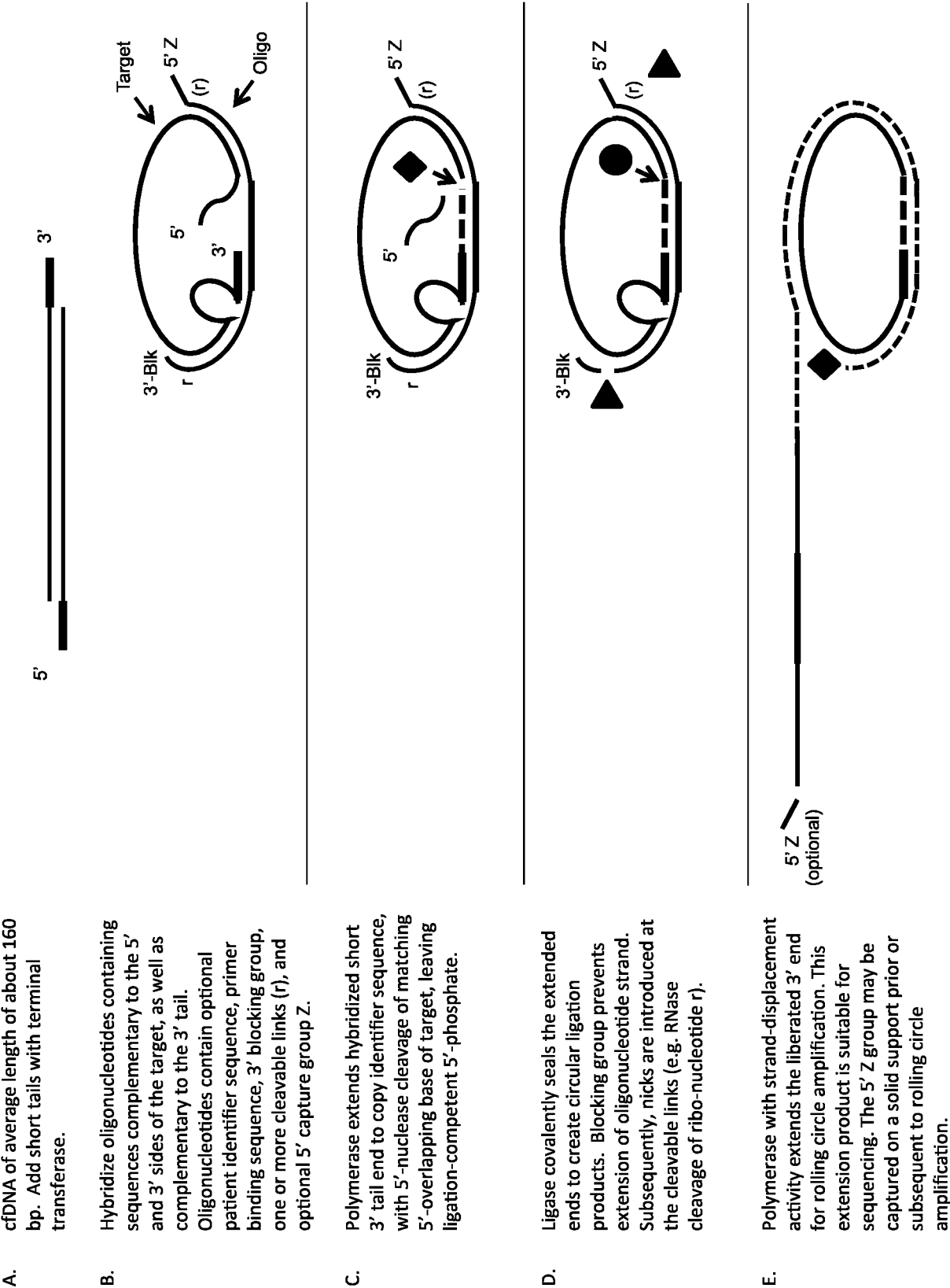
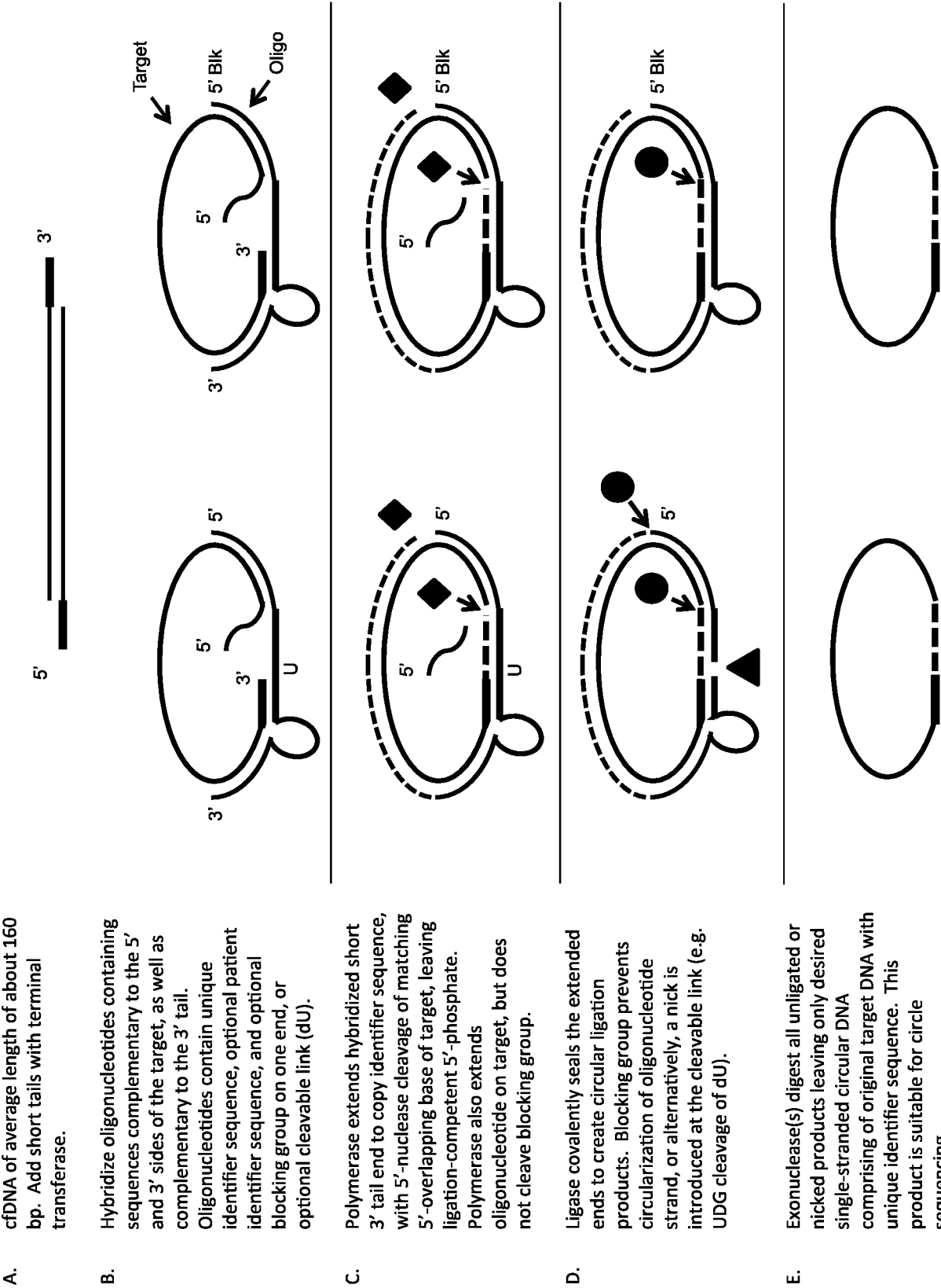


Figure 20



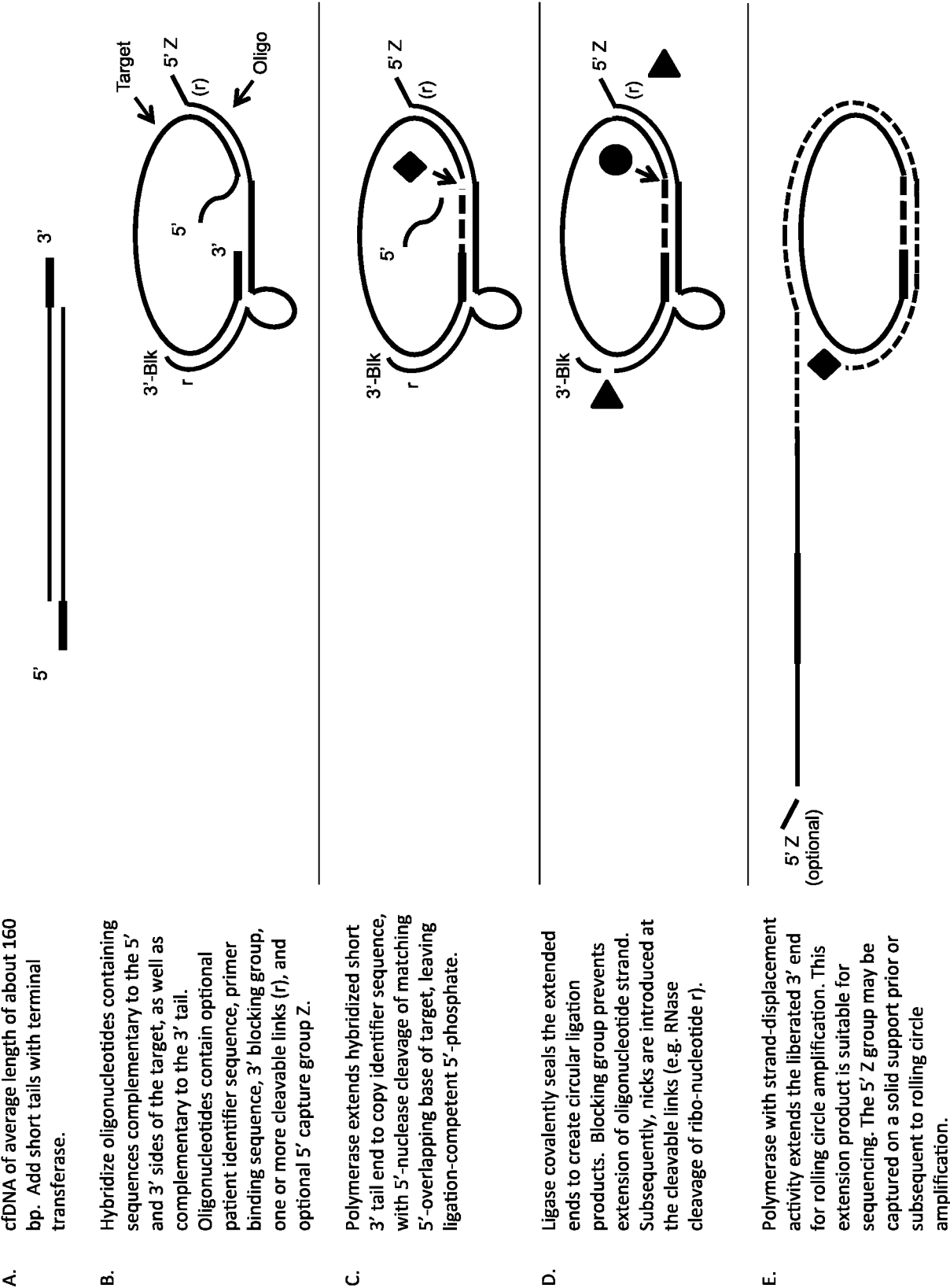
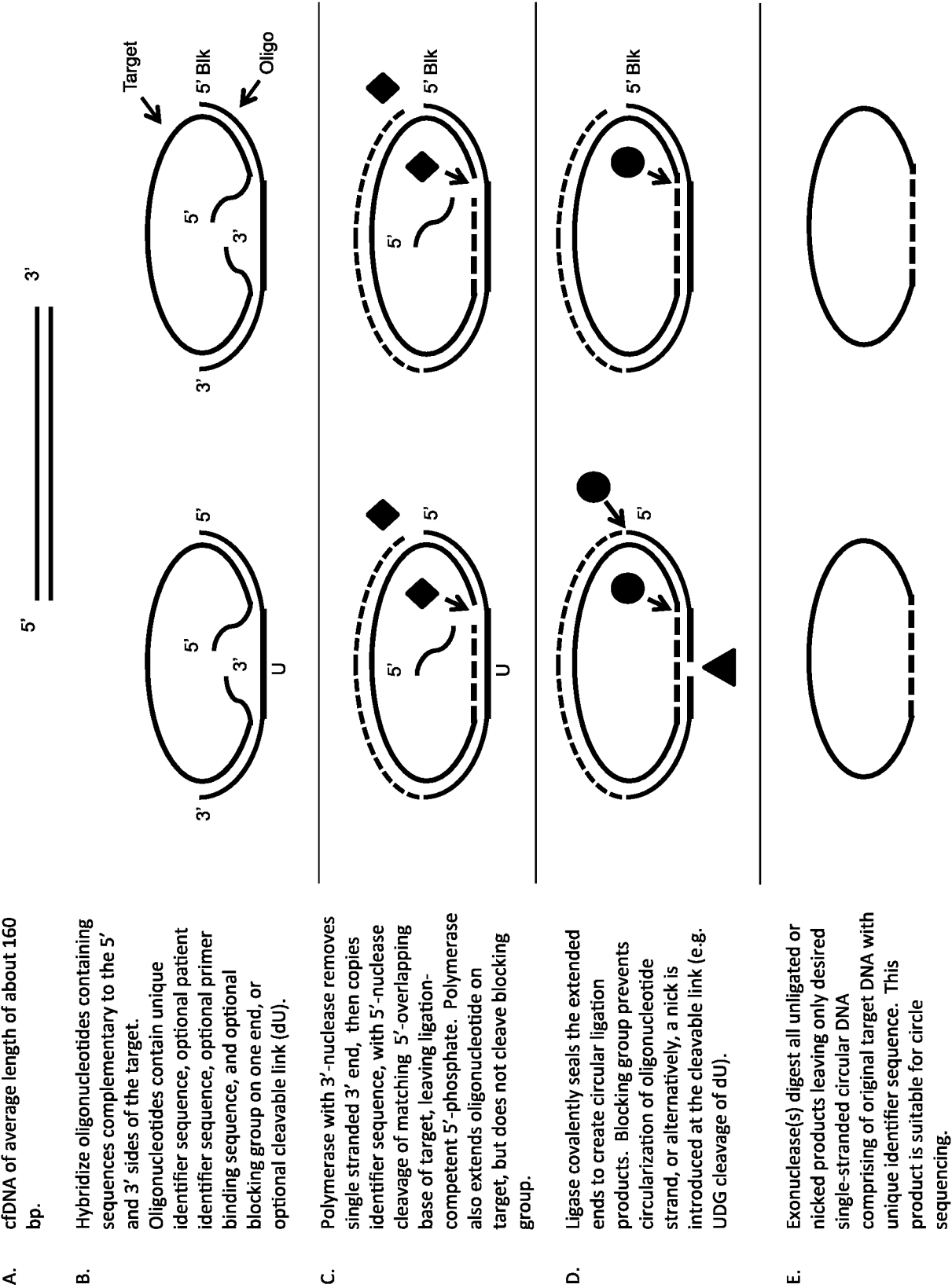
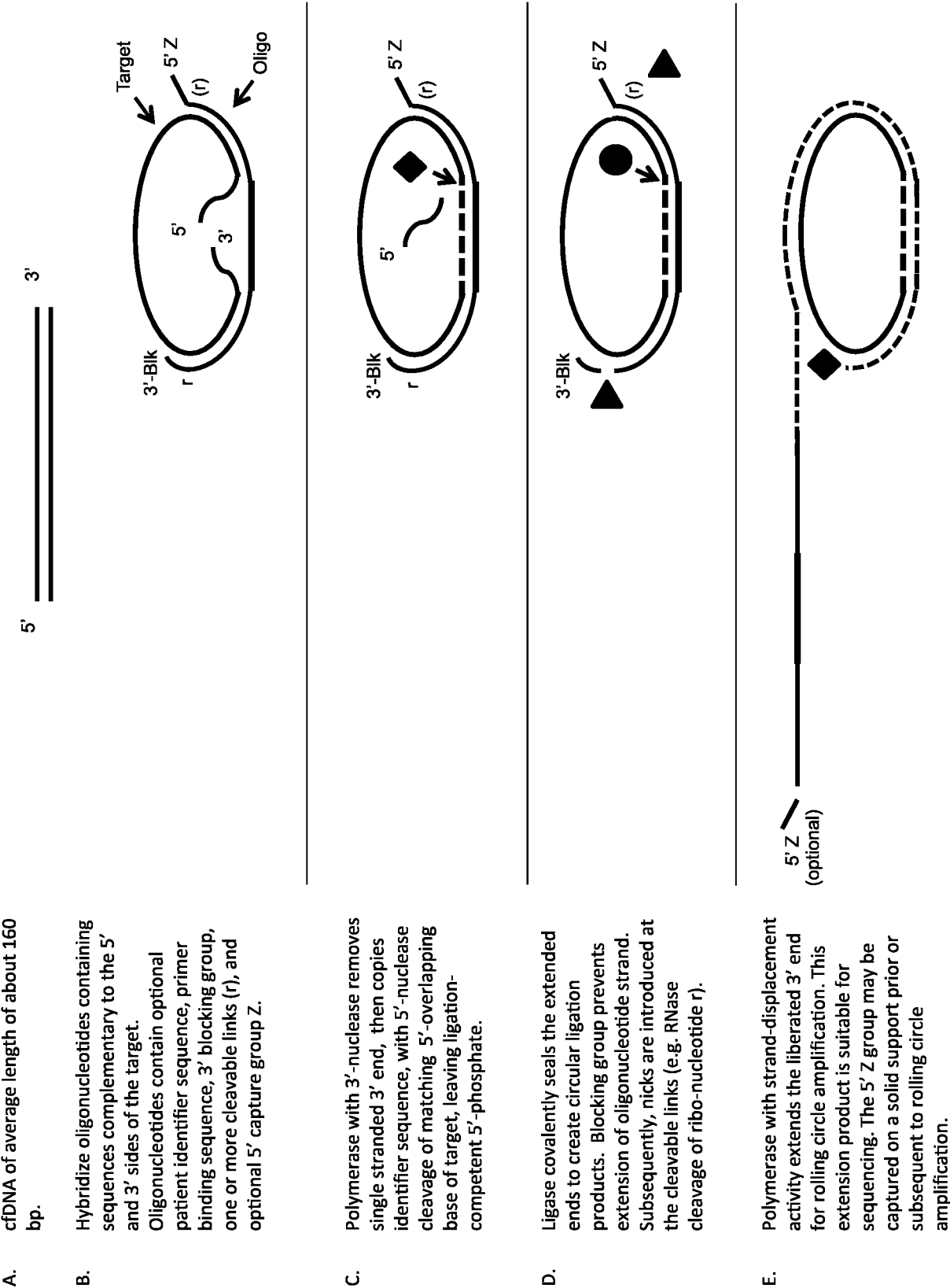
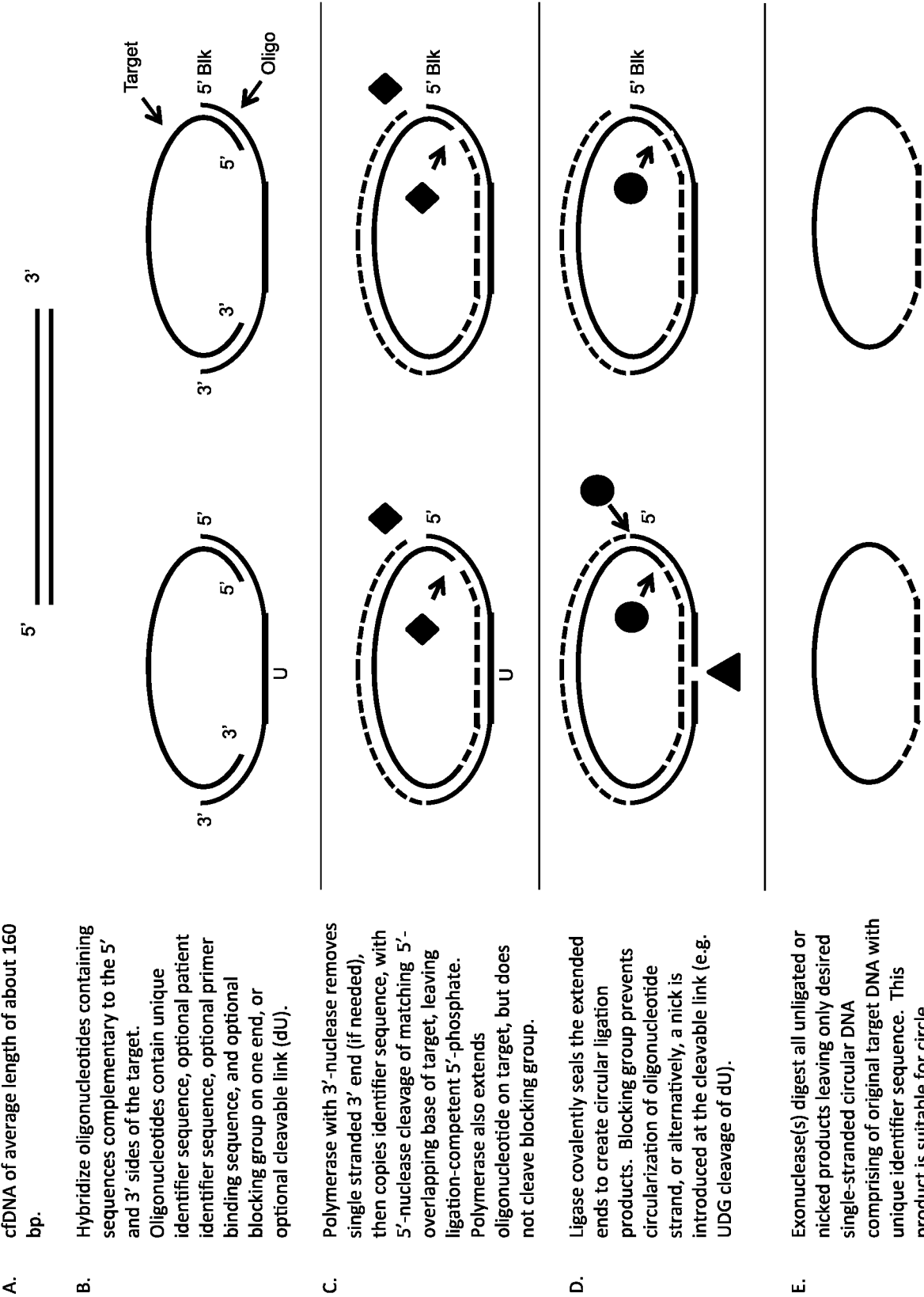
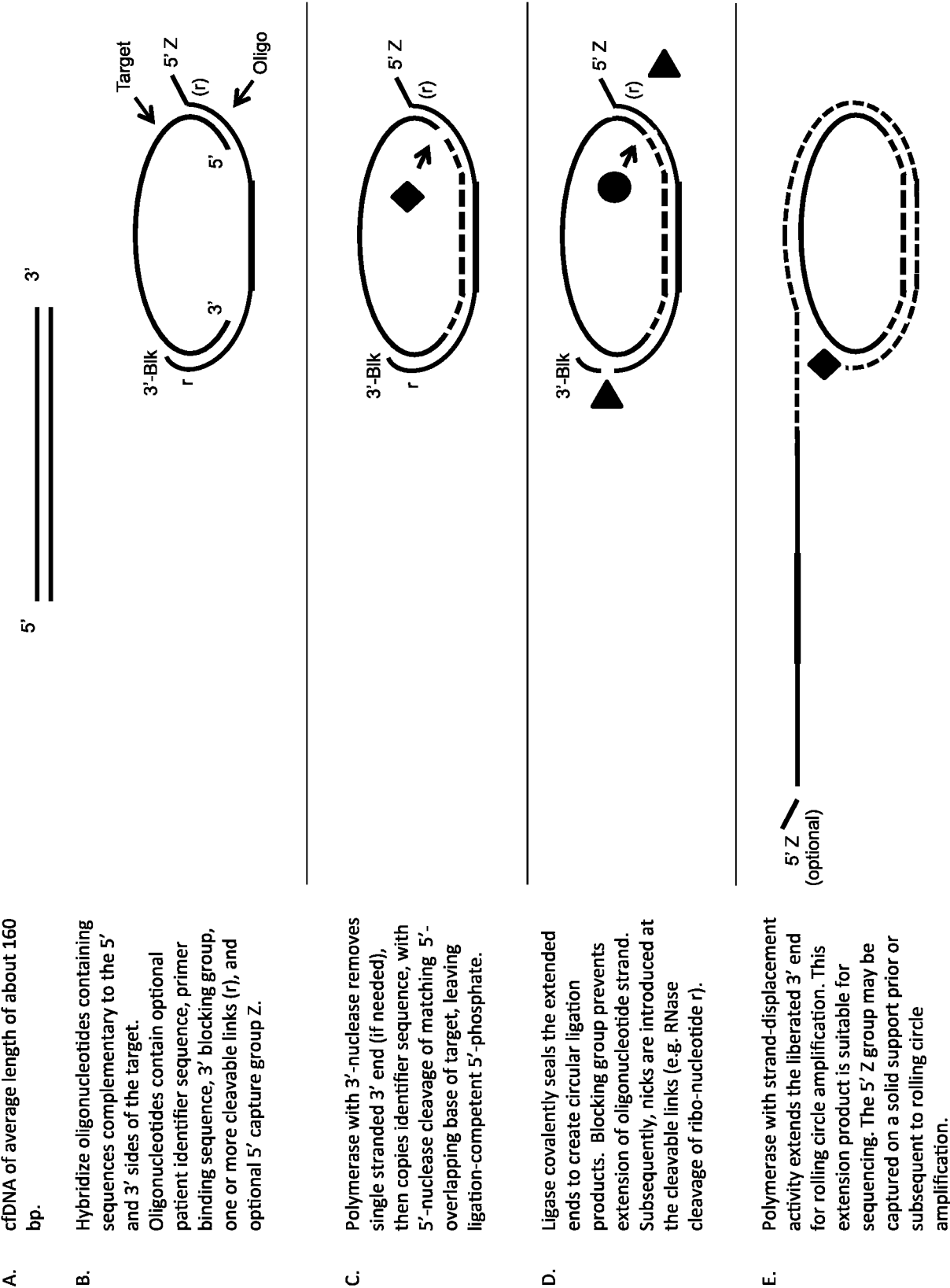


Figure 22









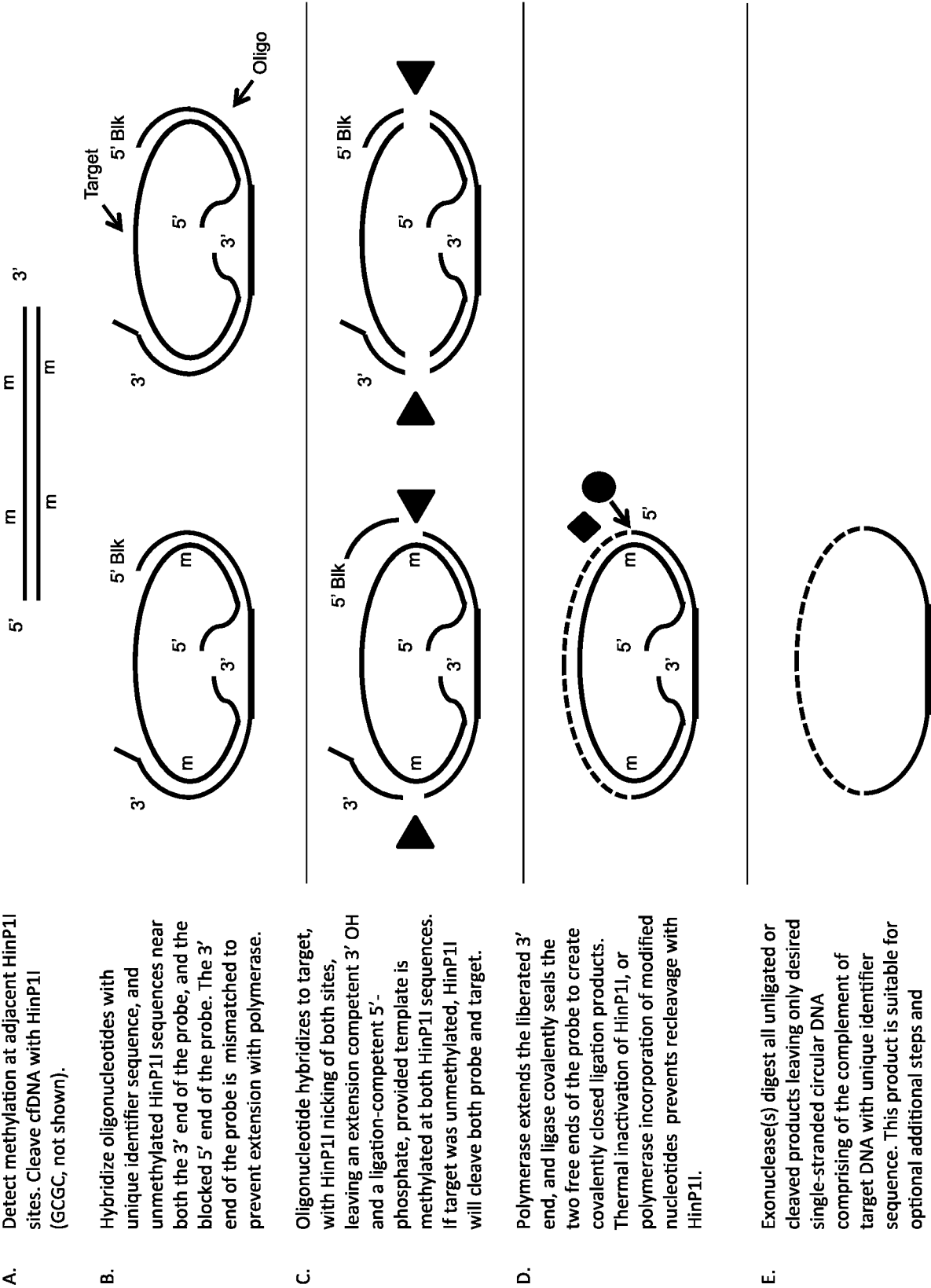


Figure 27

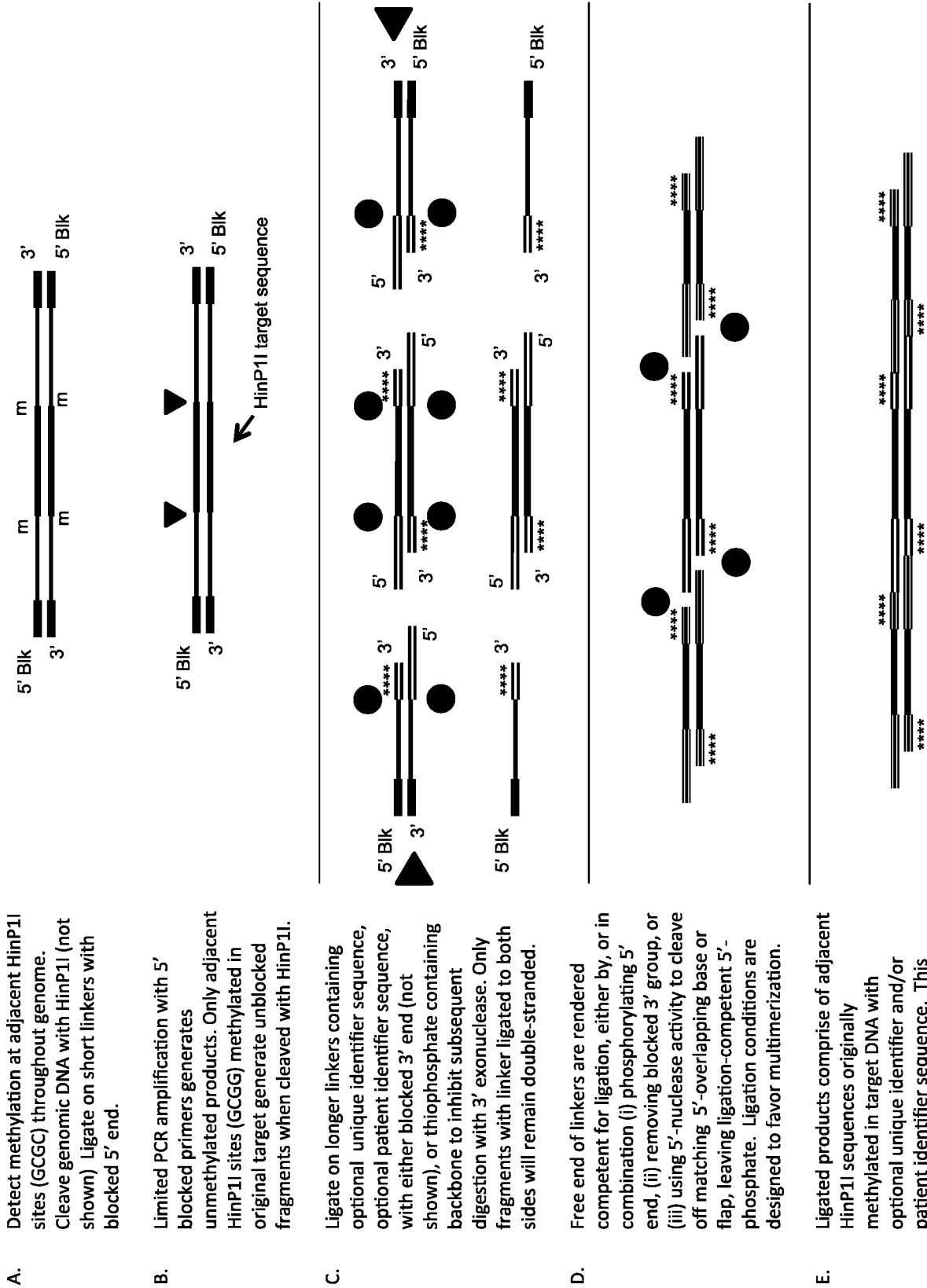


Figure 28

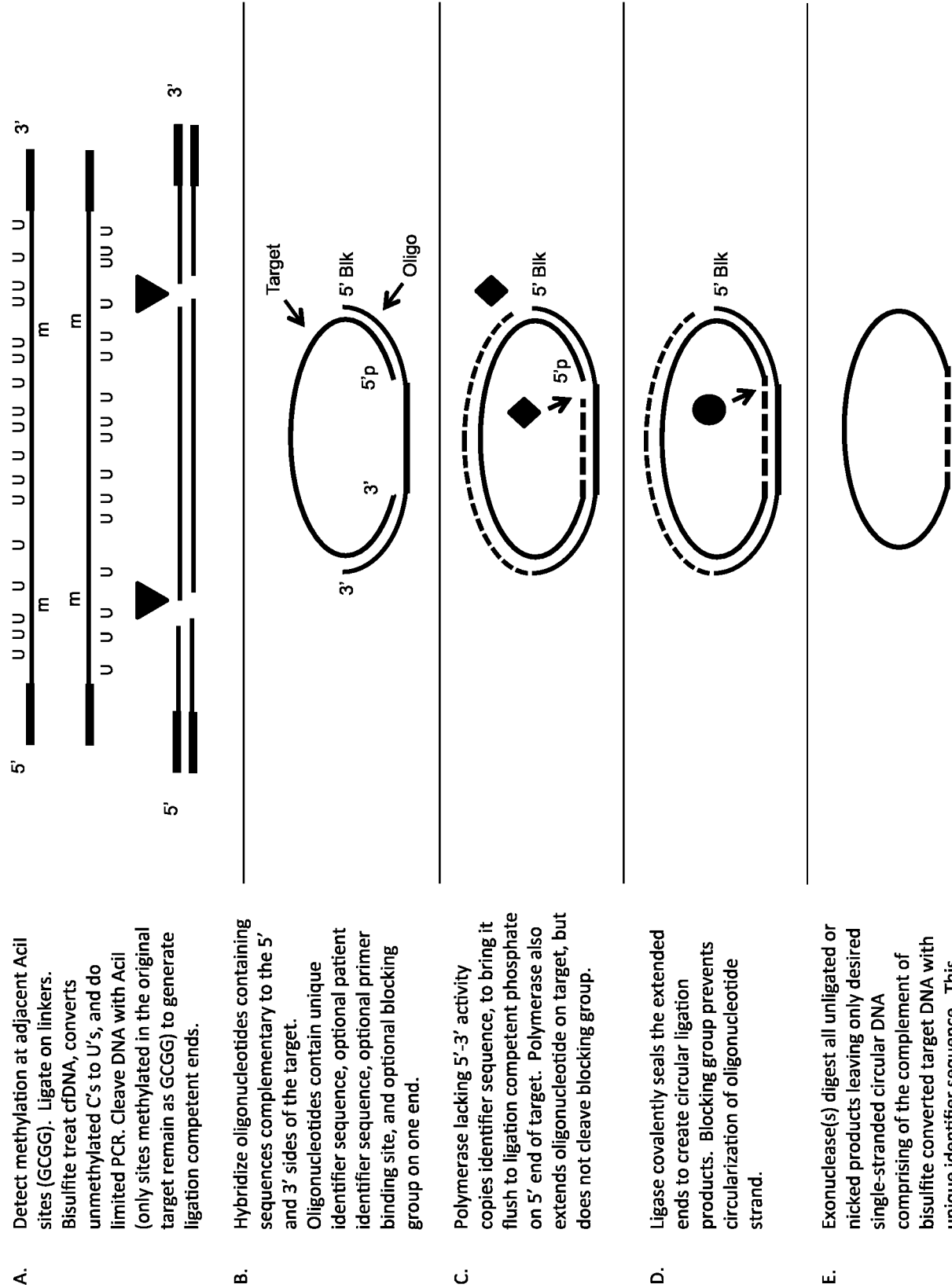
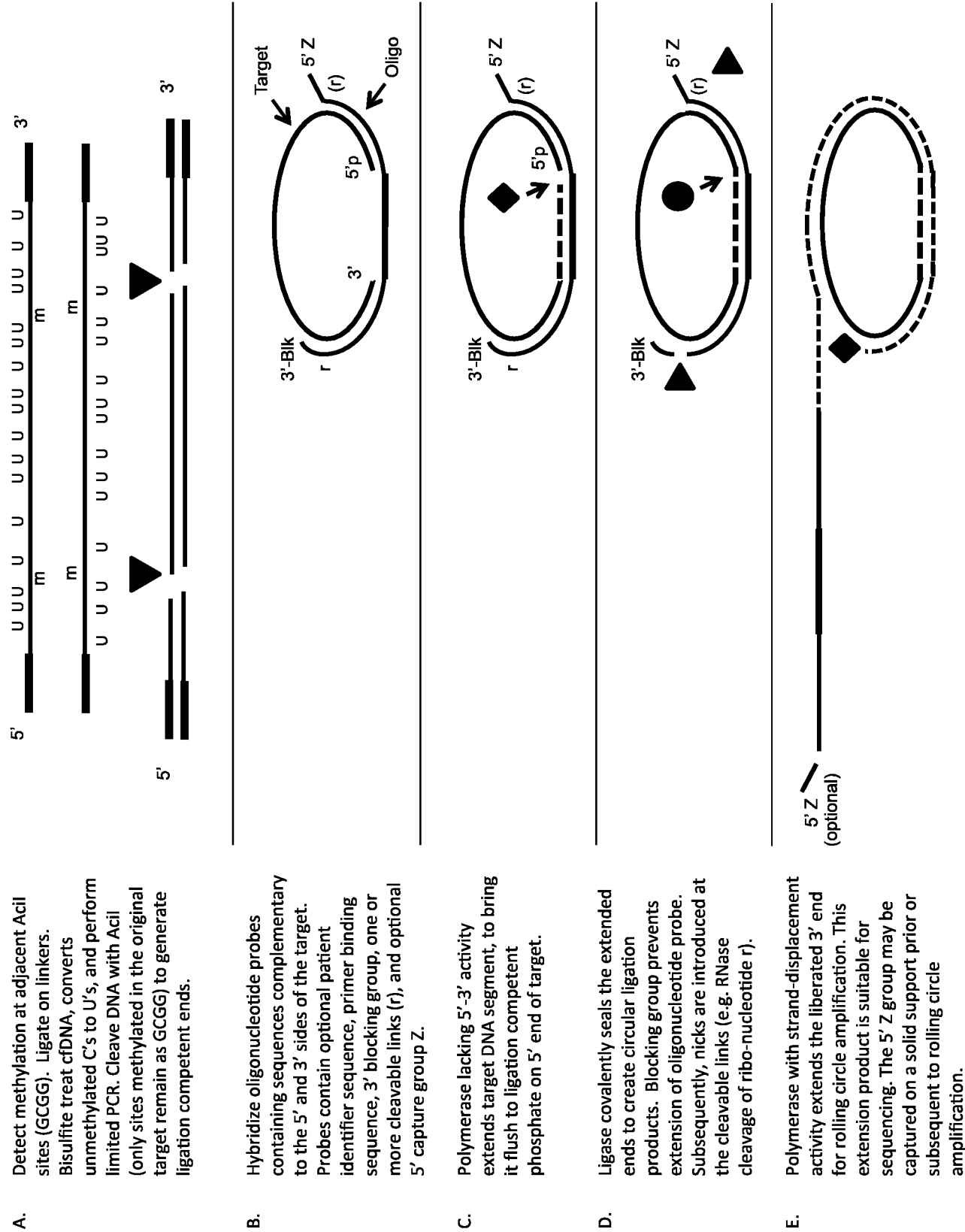


Figure 29



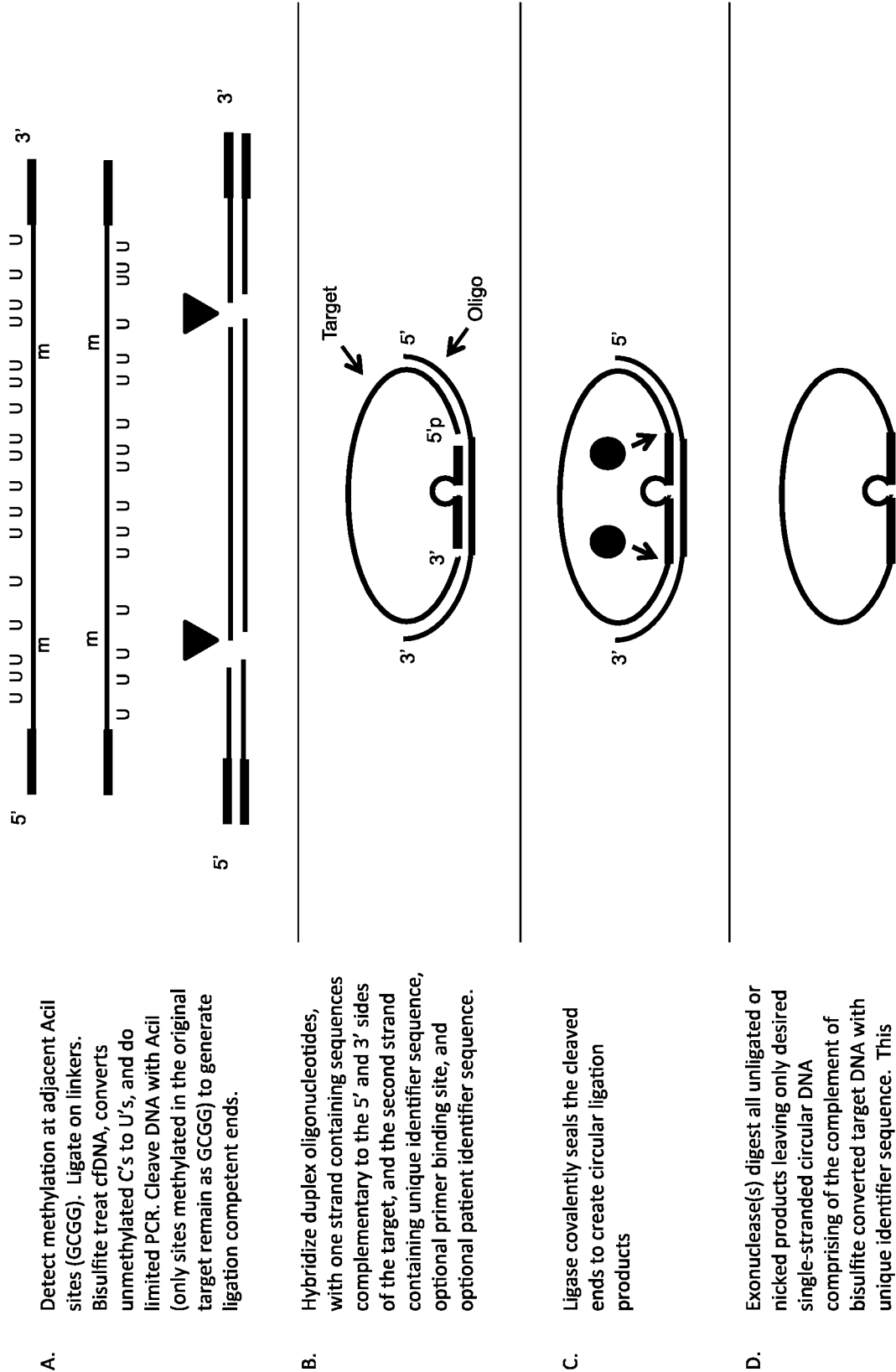


Figure 31

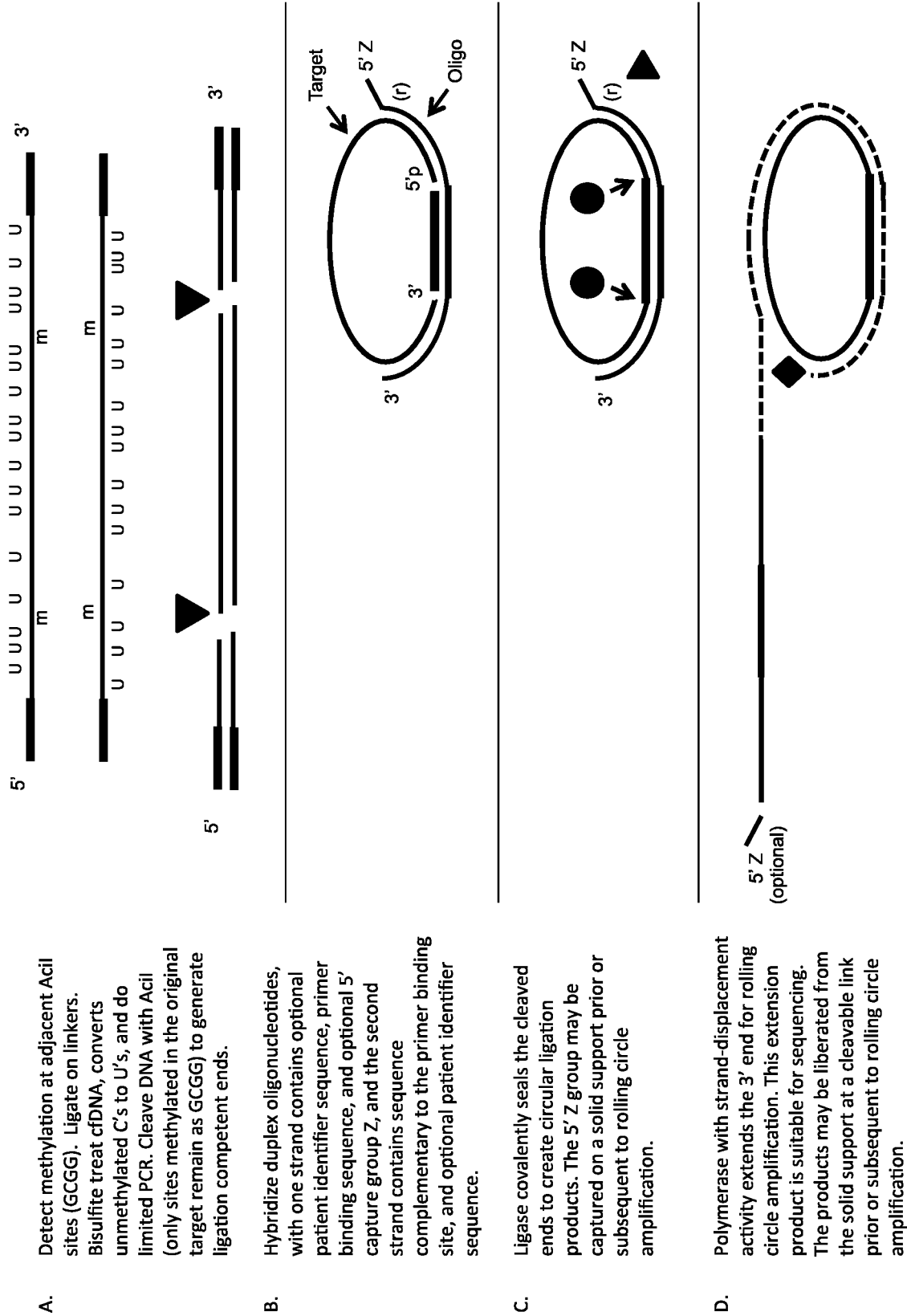


Figure 32

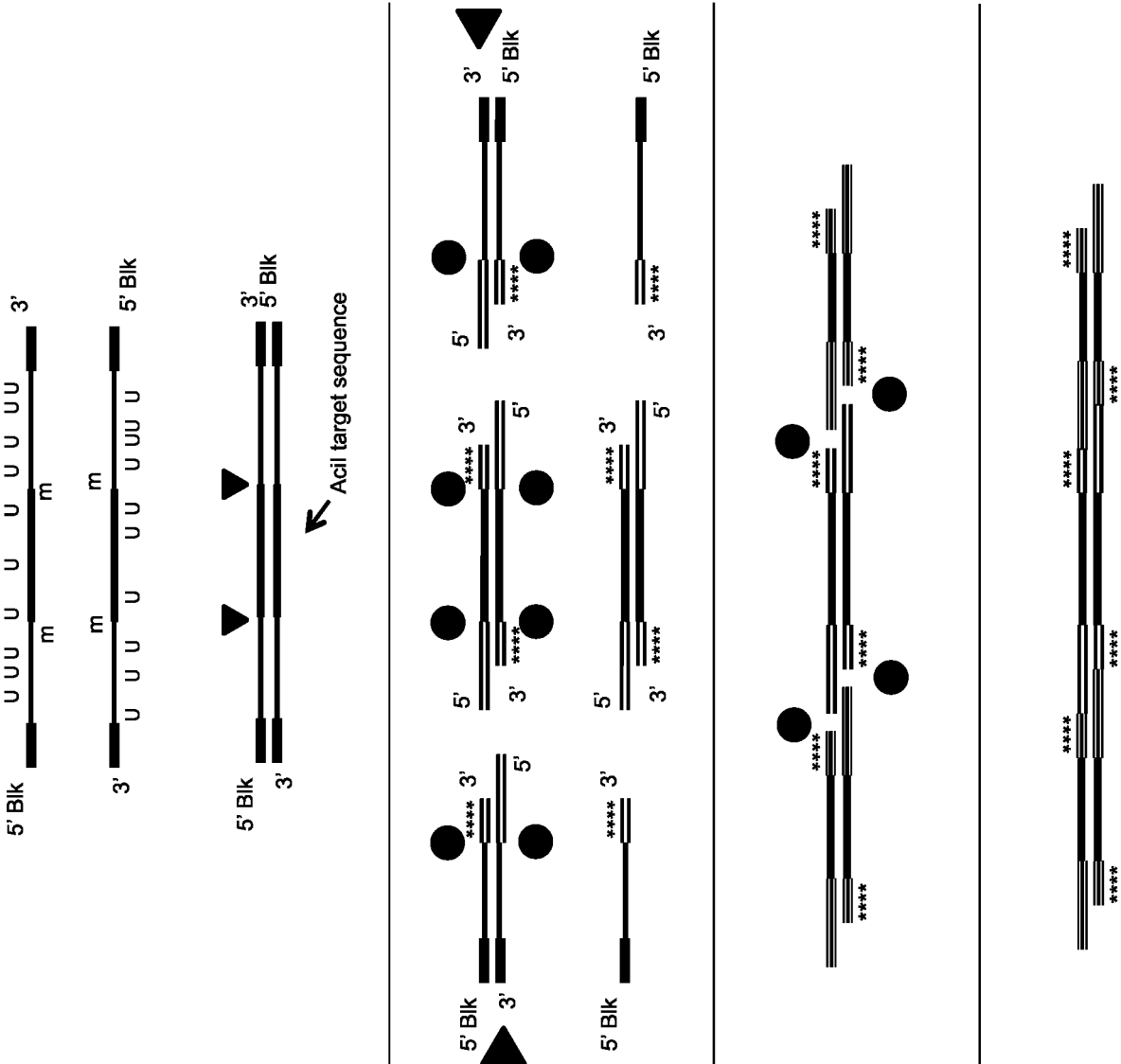


Figure 33

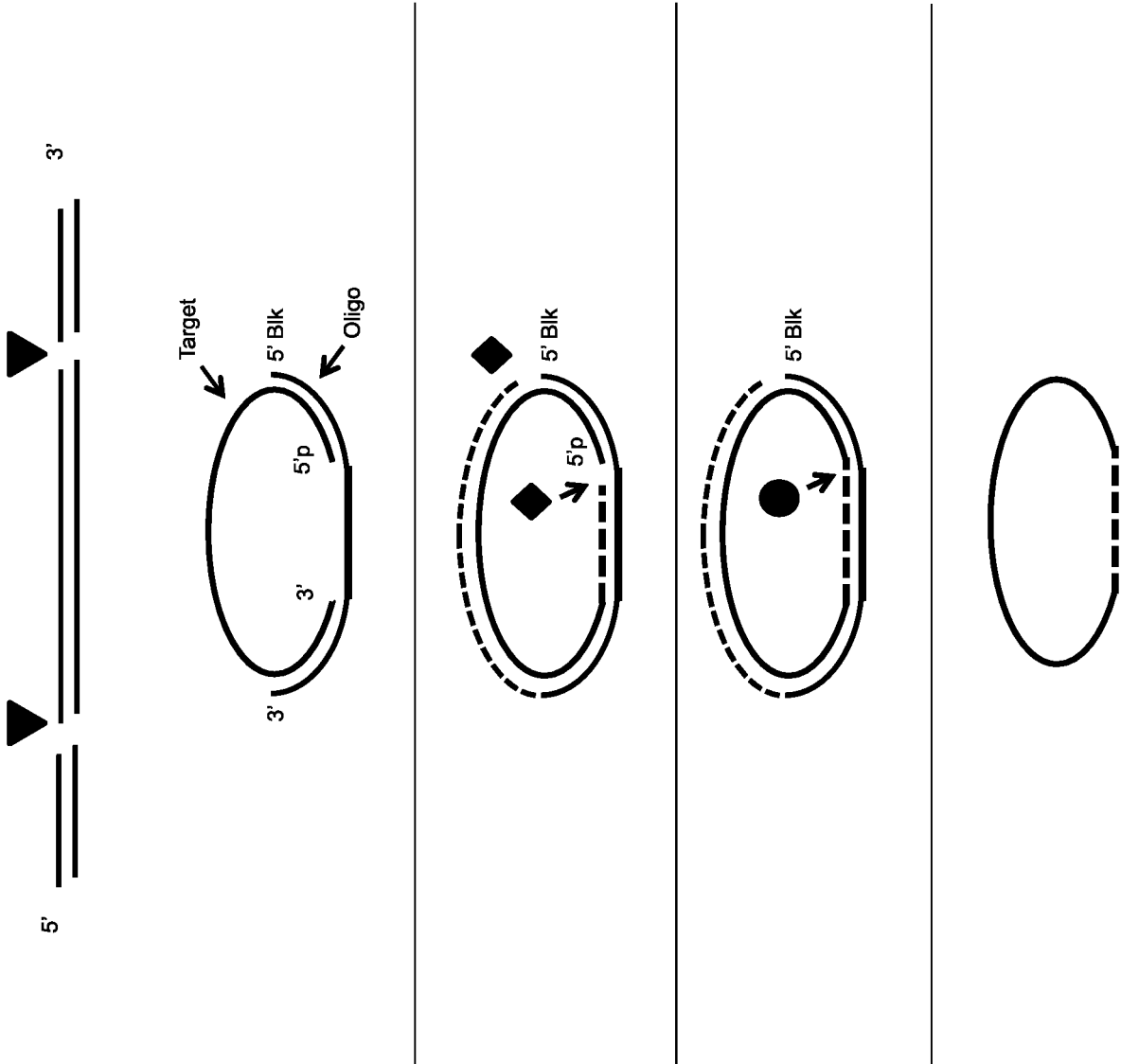


Figure 34

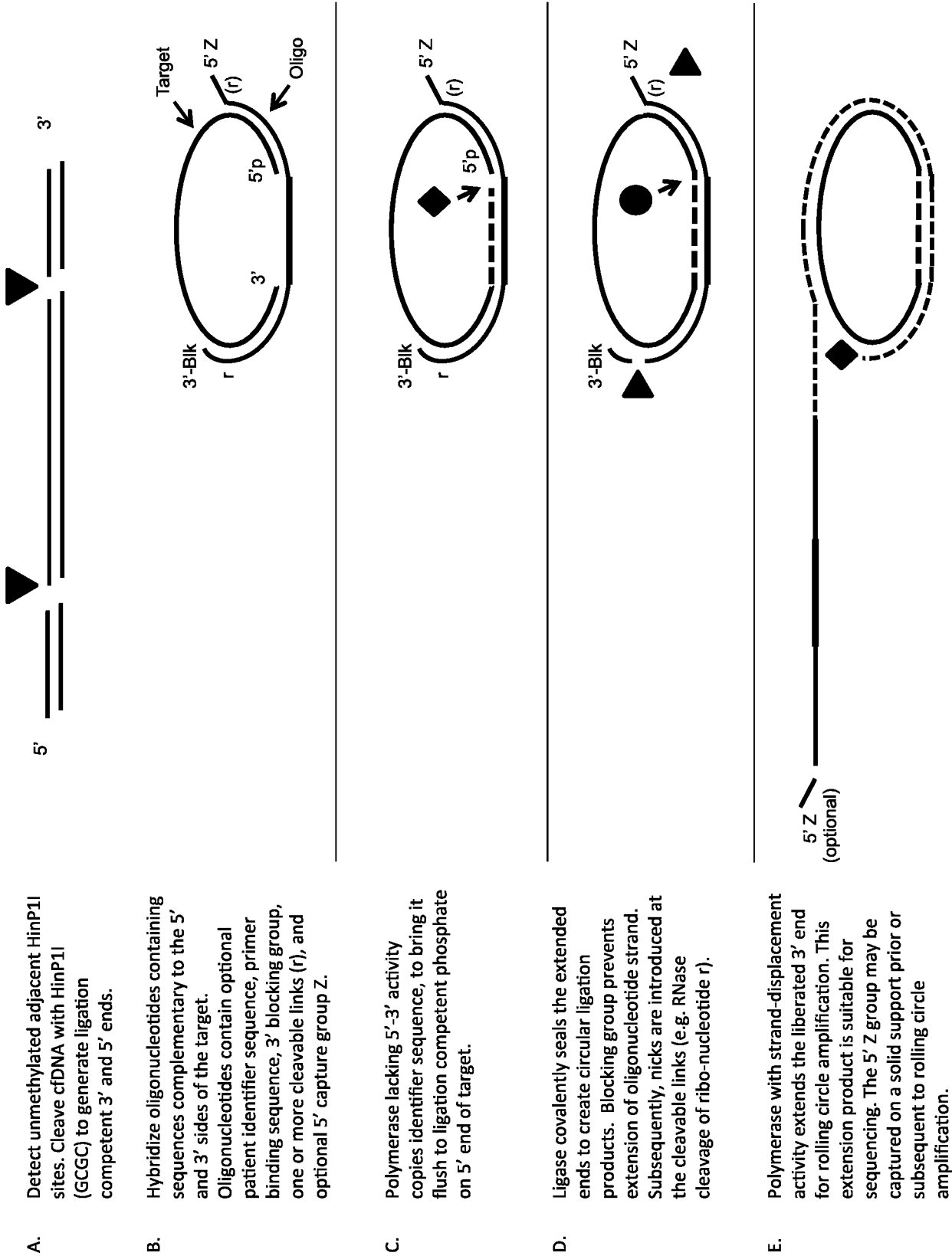


Figure 35

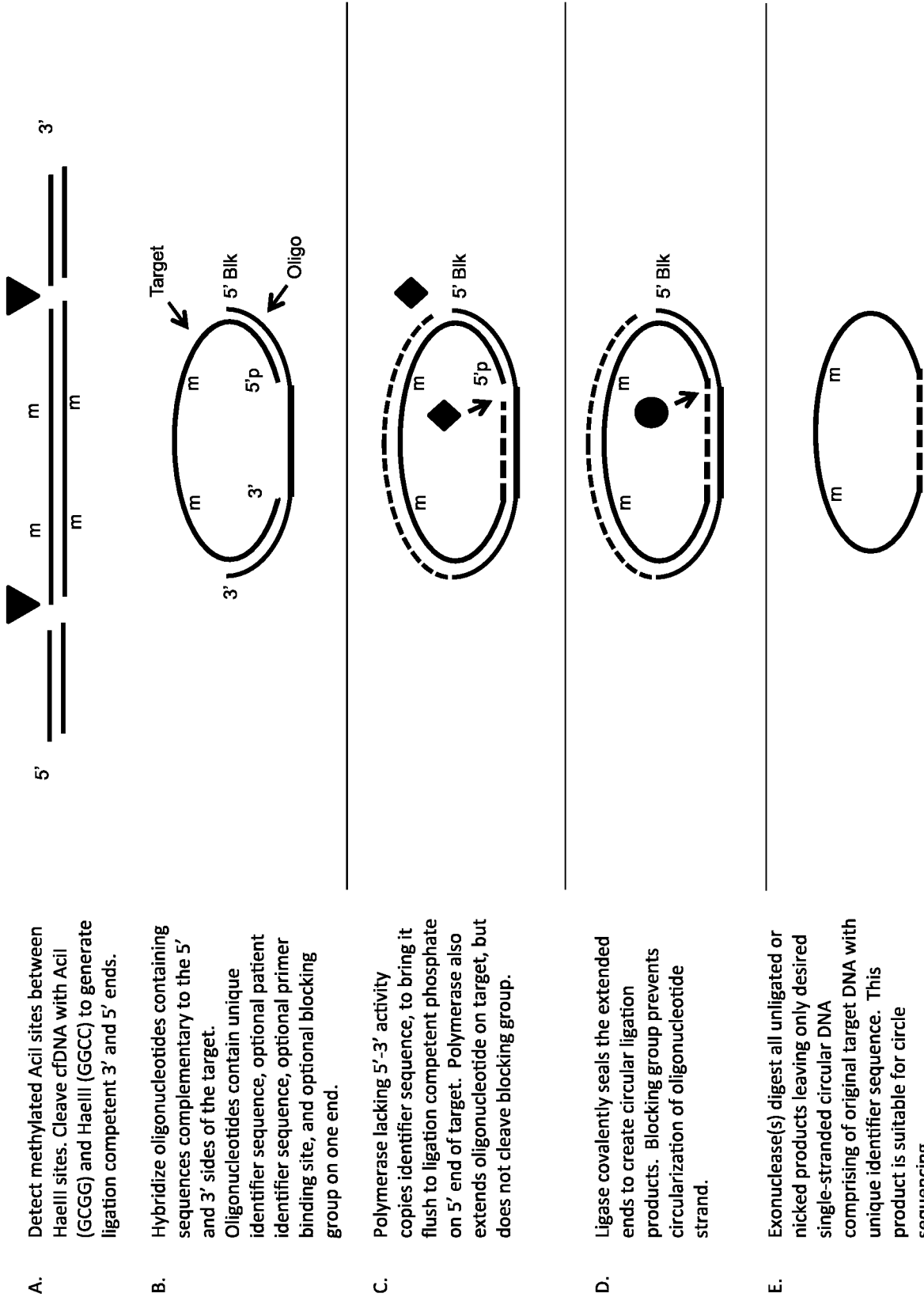


Figure 36

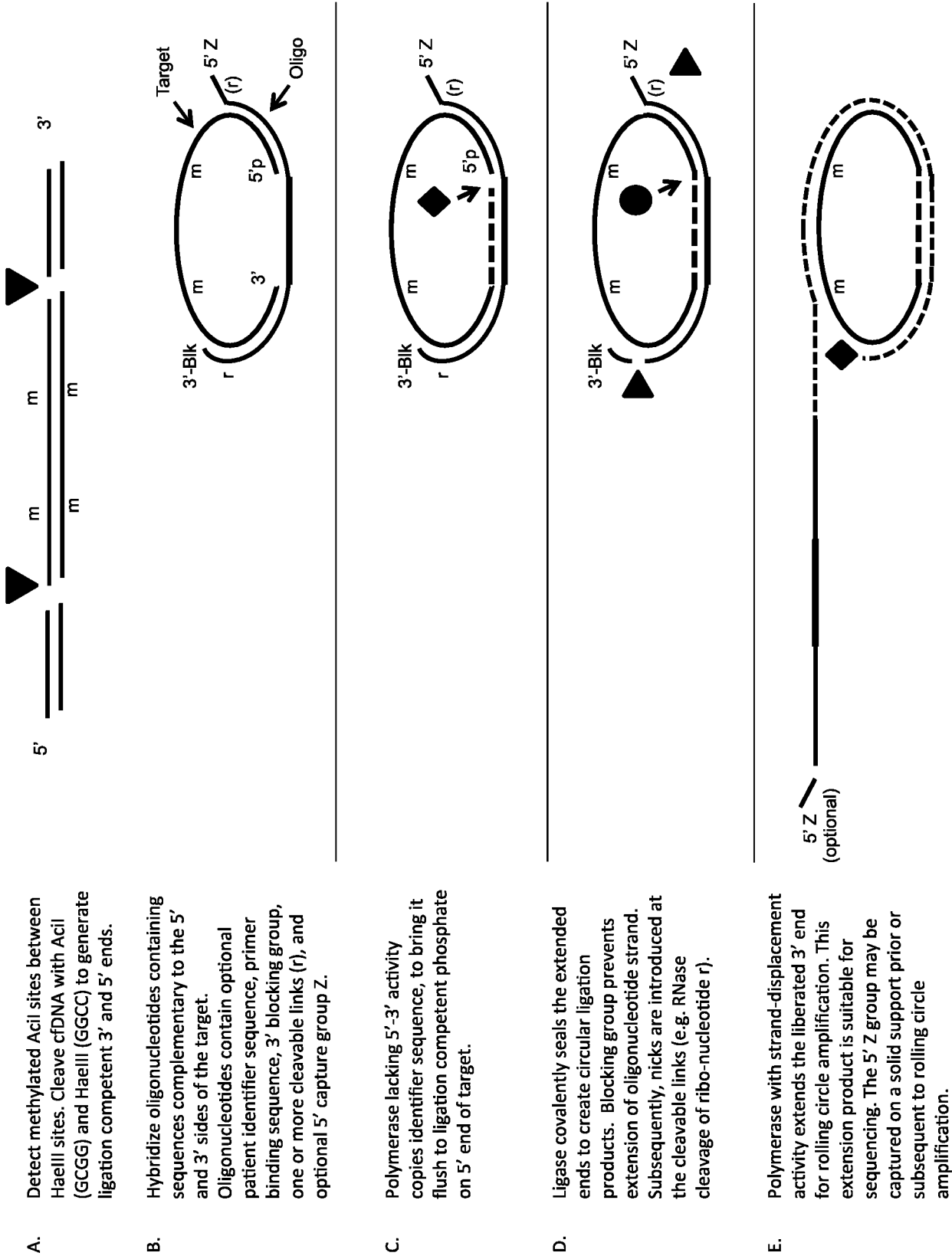


Figure 37

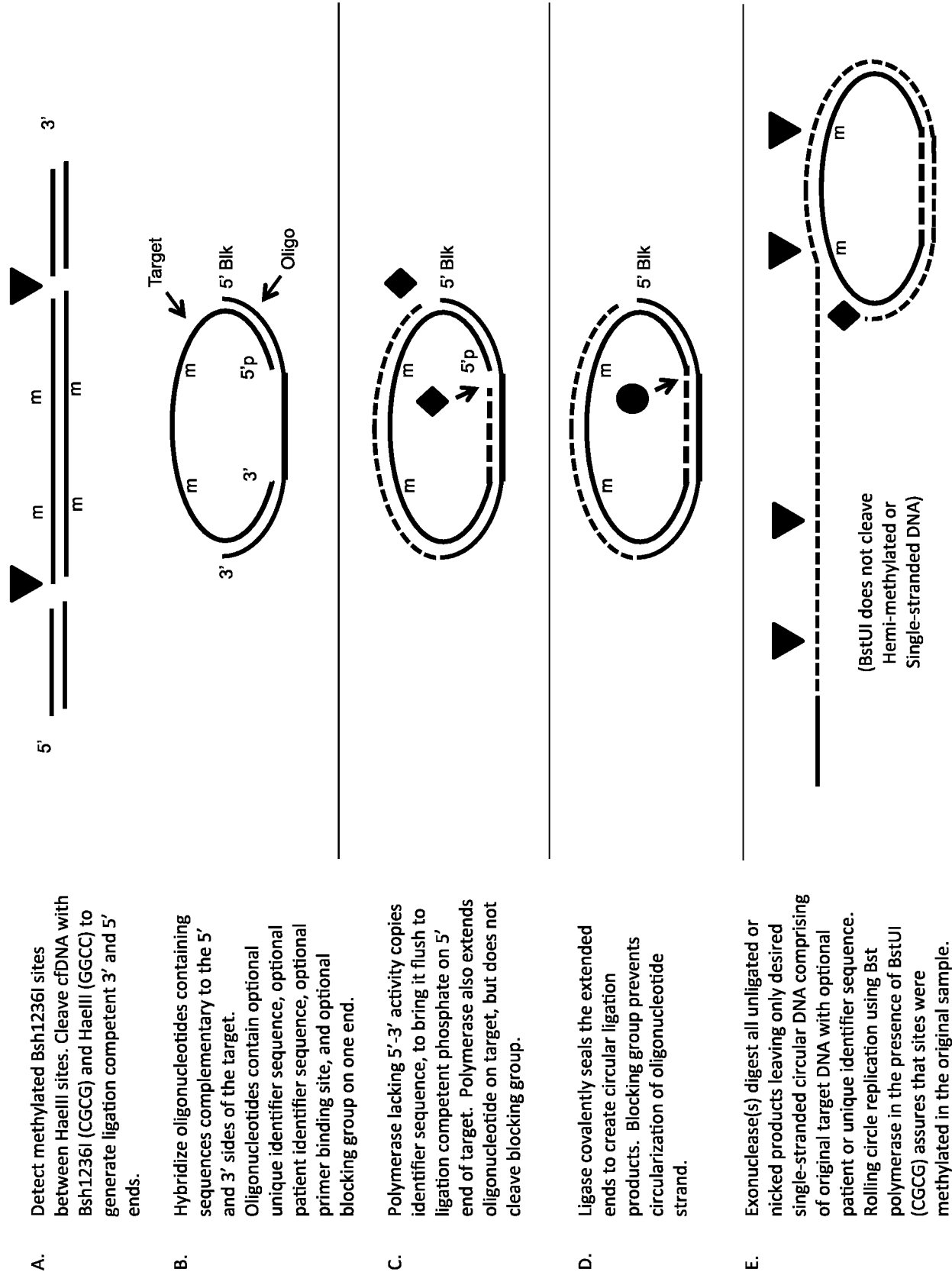
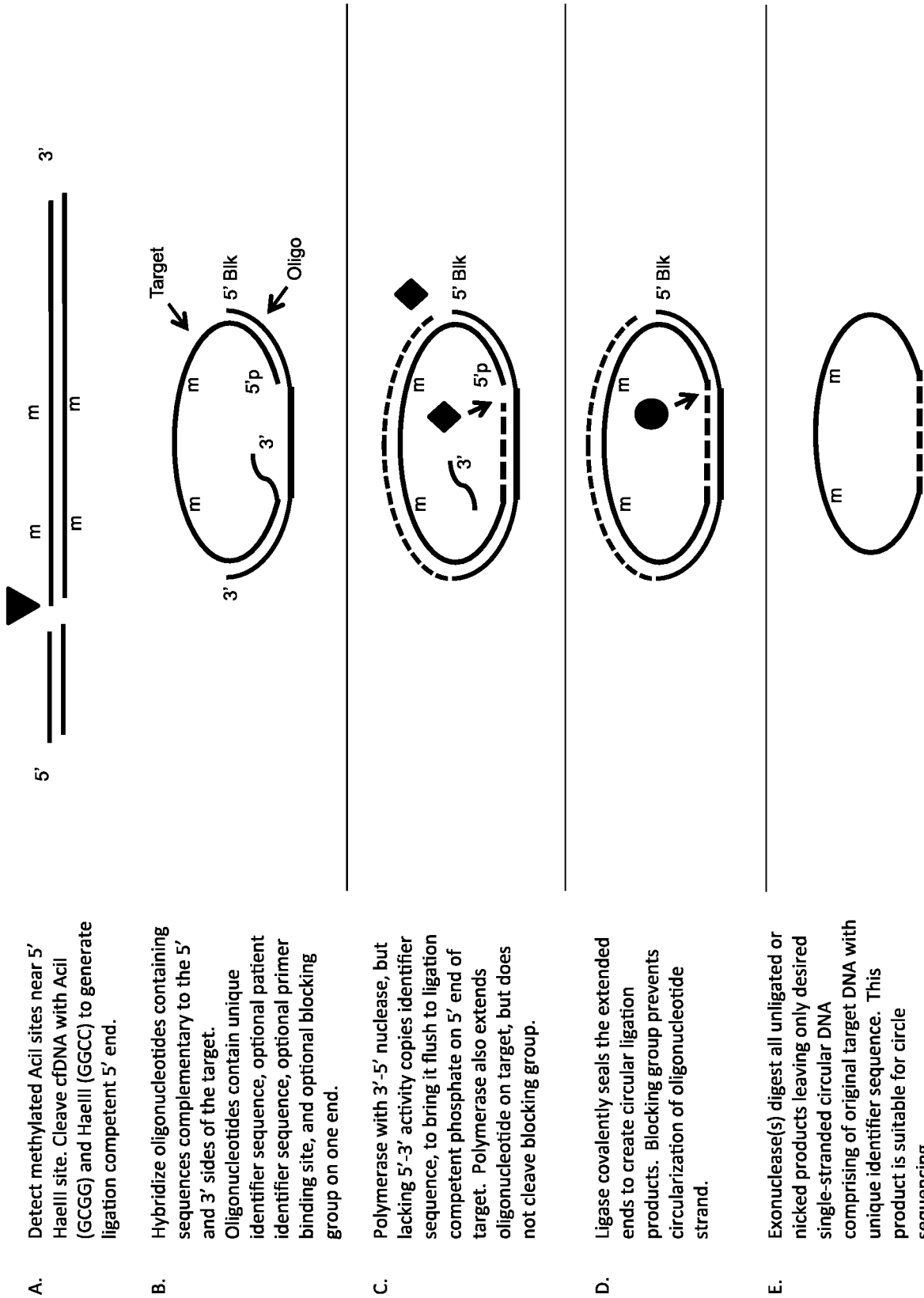


Figure 38



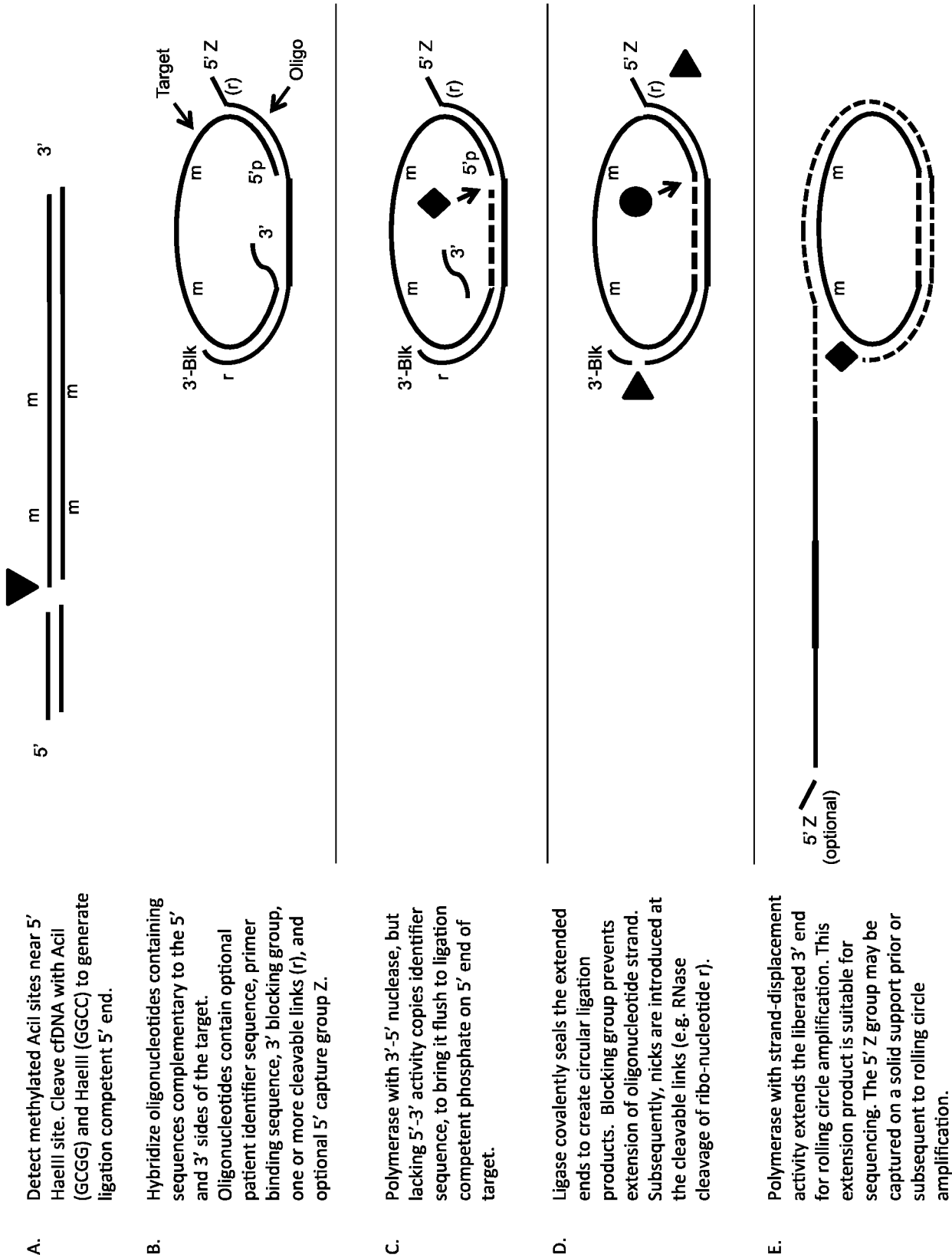


Figure 40

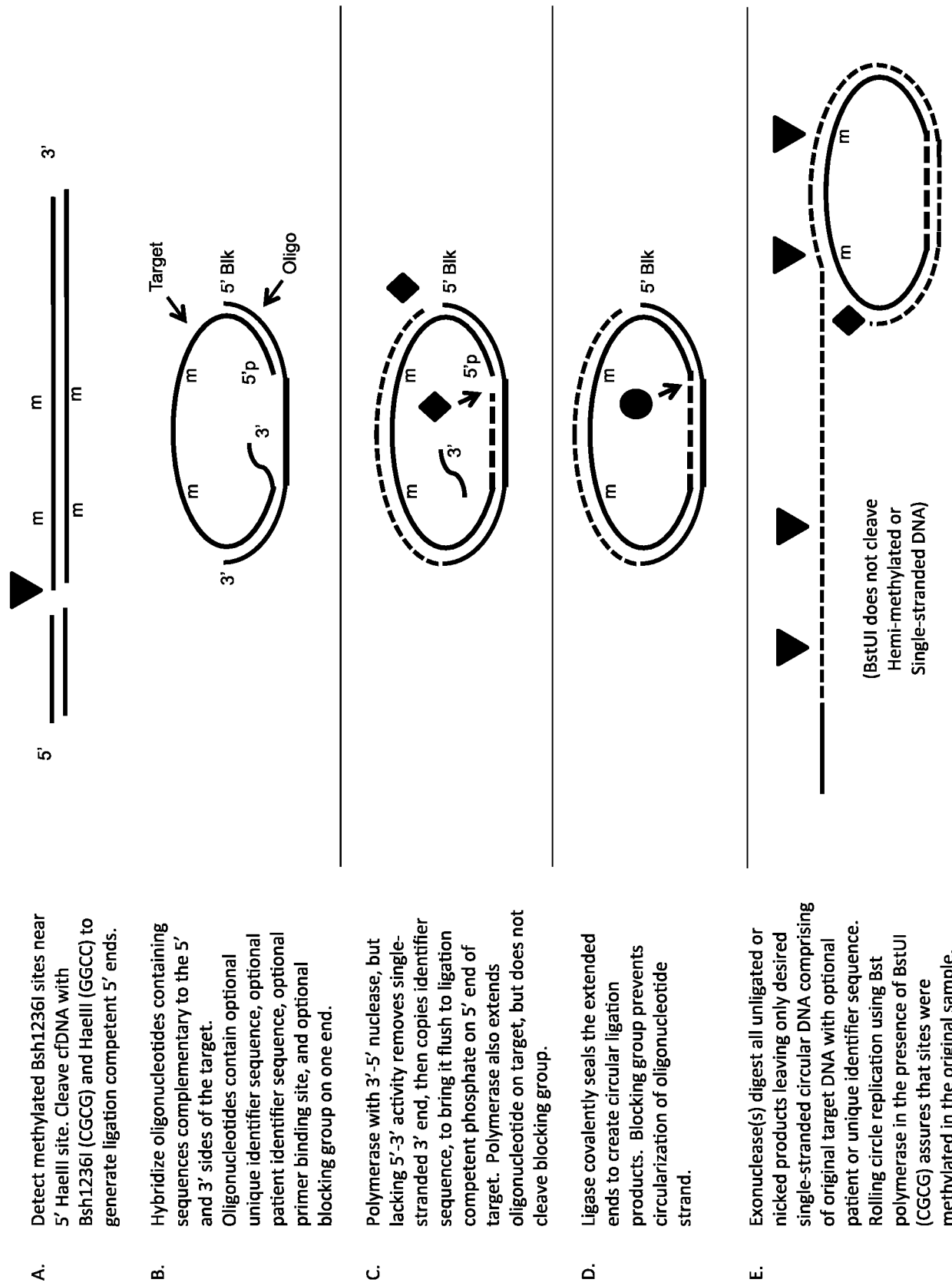
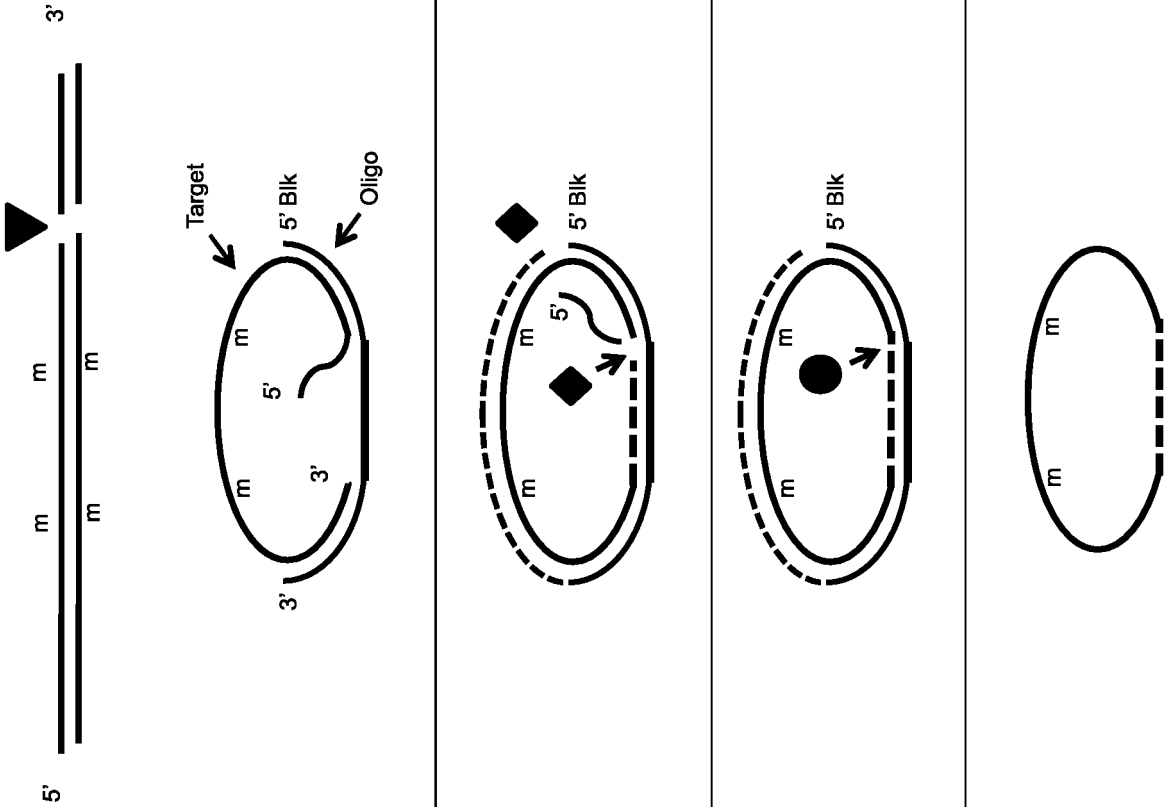


Figure 41



- A. Detect methylated Acil sites near 3' HaeIII site. Cleave cfDNA with Acil (GCGG) and HaeIII (GGCC) to generate extension competent 3' end.
- 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 extends hybridized 3' end to copy 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 unique identifier sequence. This product is suitable for circle sequencing.

Figure 42

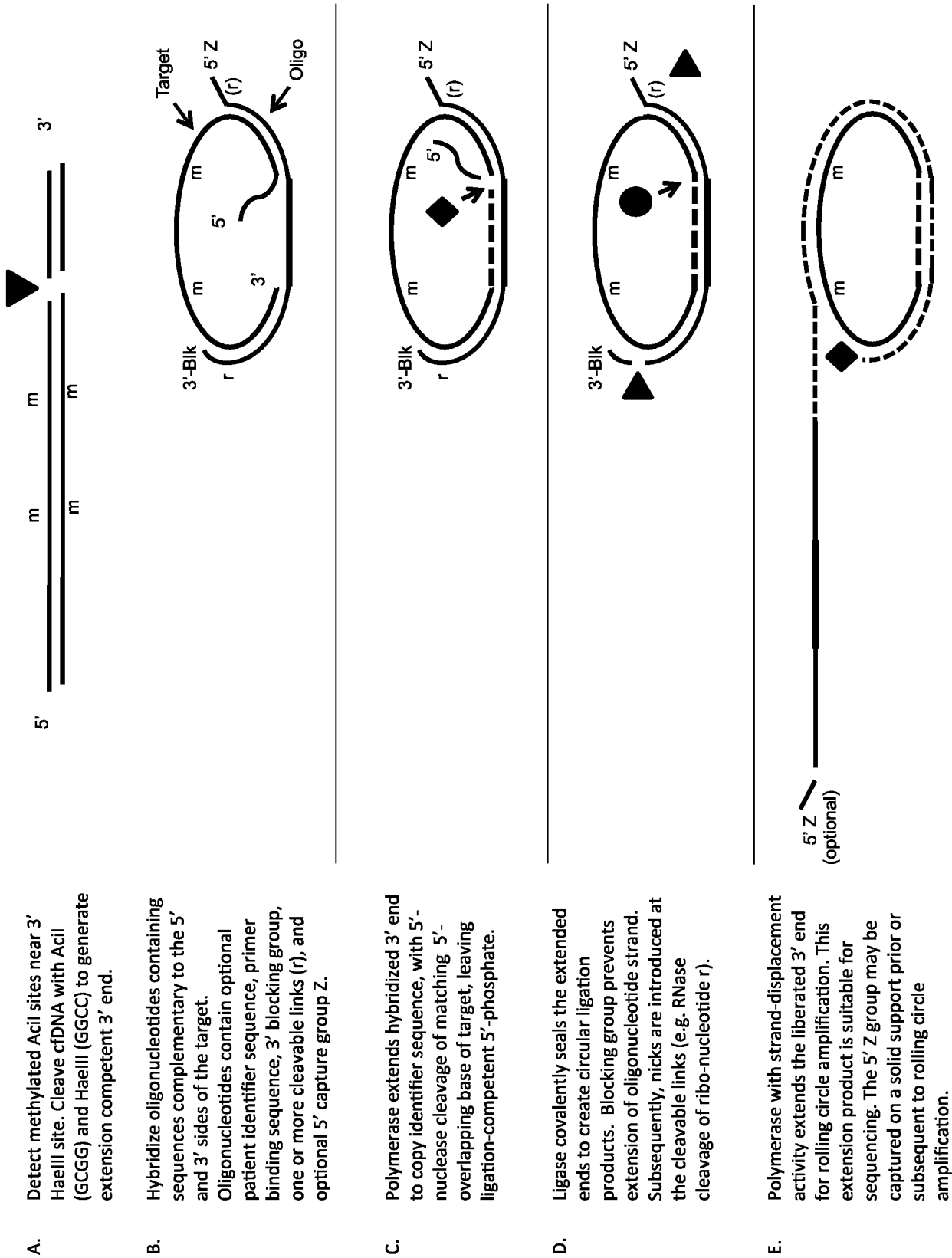


Figure 43

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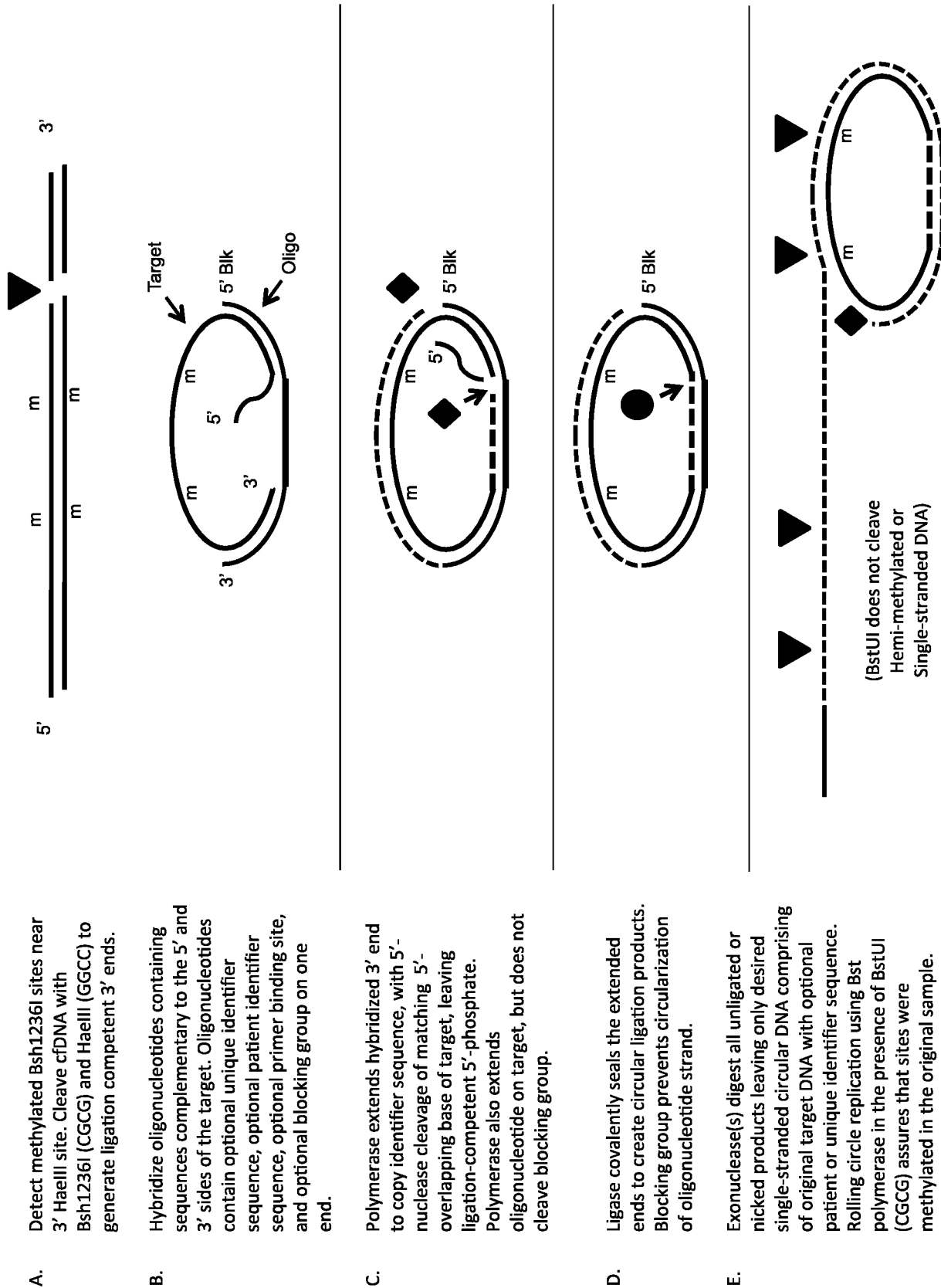


Figure 44

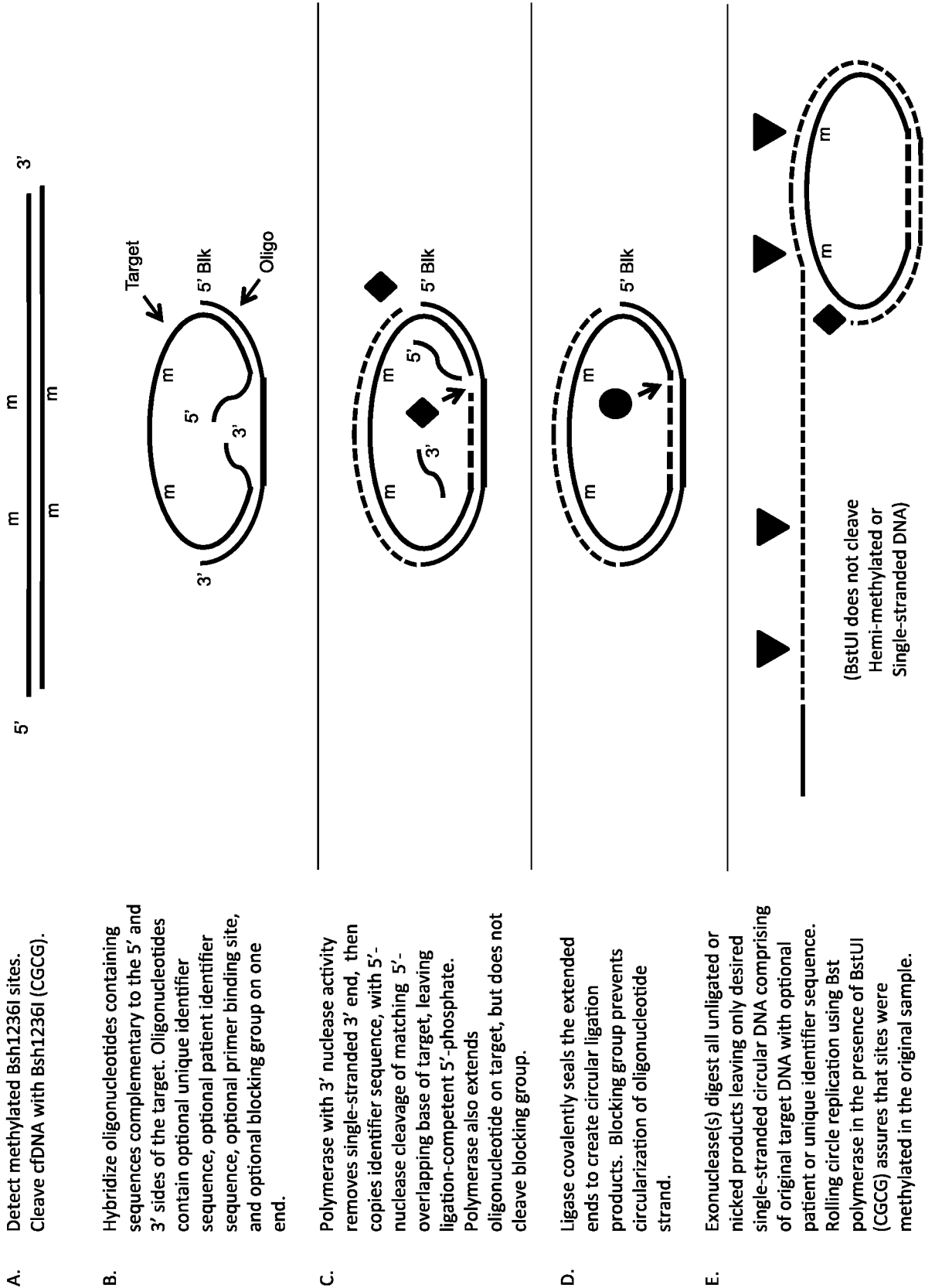


Figure 45

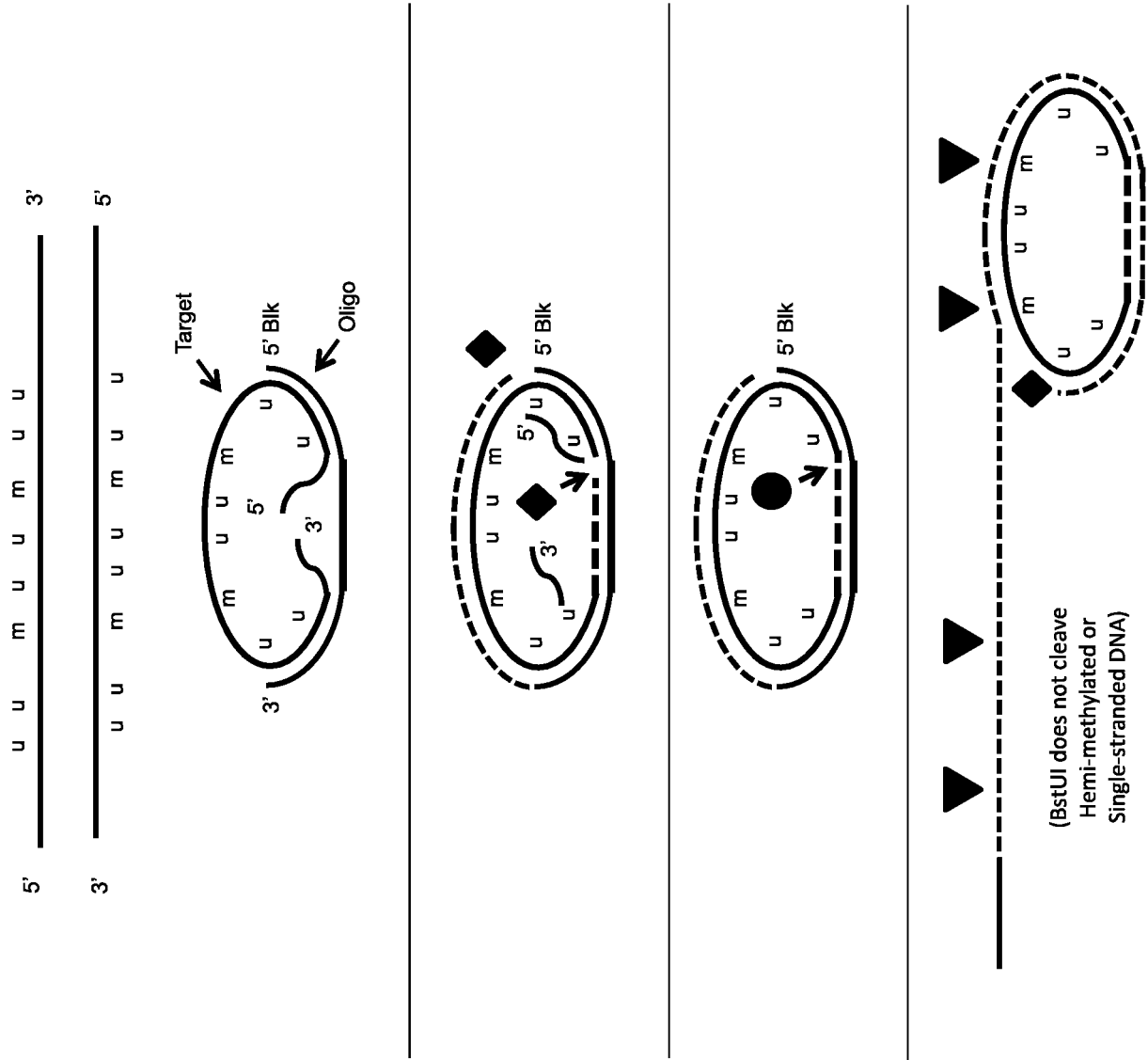


Figure 46

- A. Detect methylated Bsh12361 sites. Cleave cfDNA with Bsh12361 (CGCG). Bisulfite treat cfDNA, converts unmethylated C's to U's.
- 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 not converted by bisulfite were methylated in the original sample.

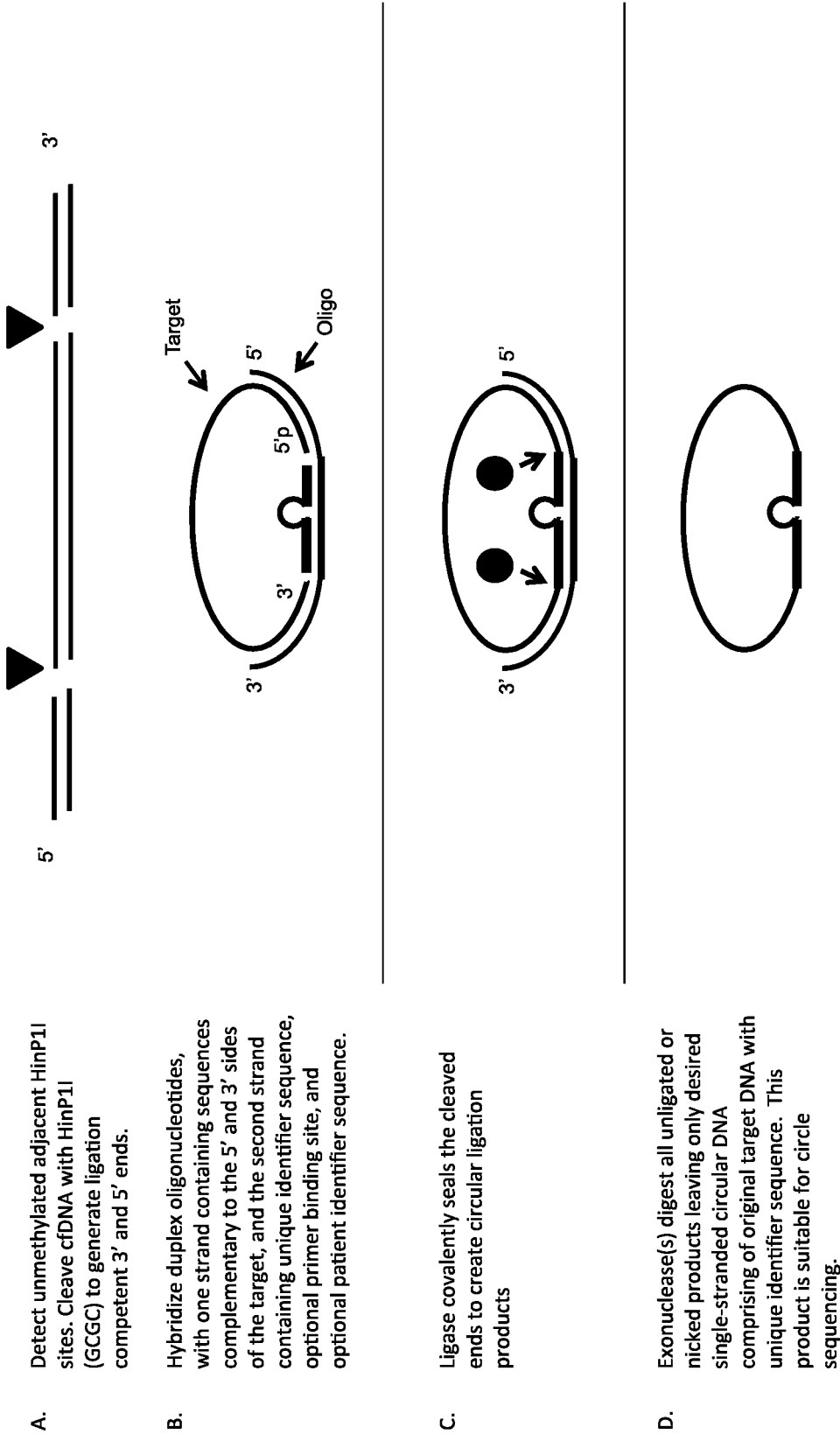


Figure 47

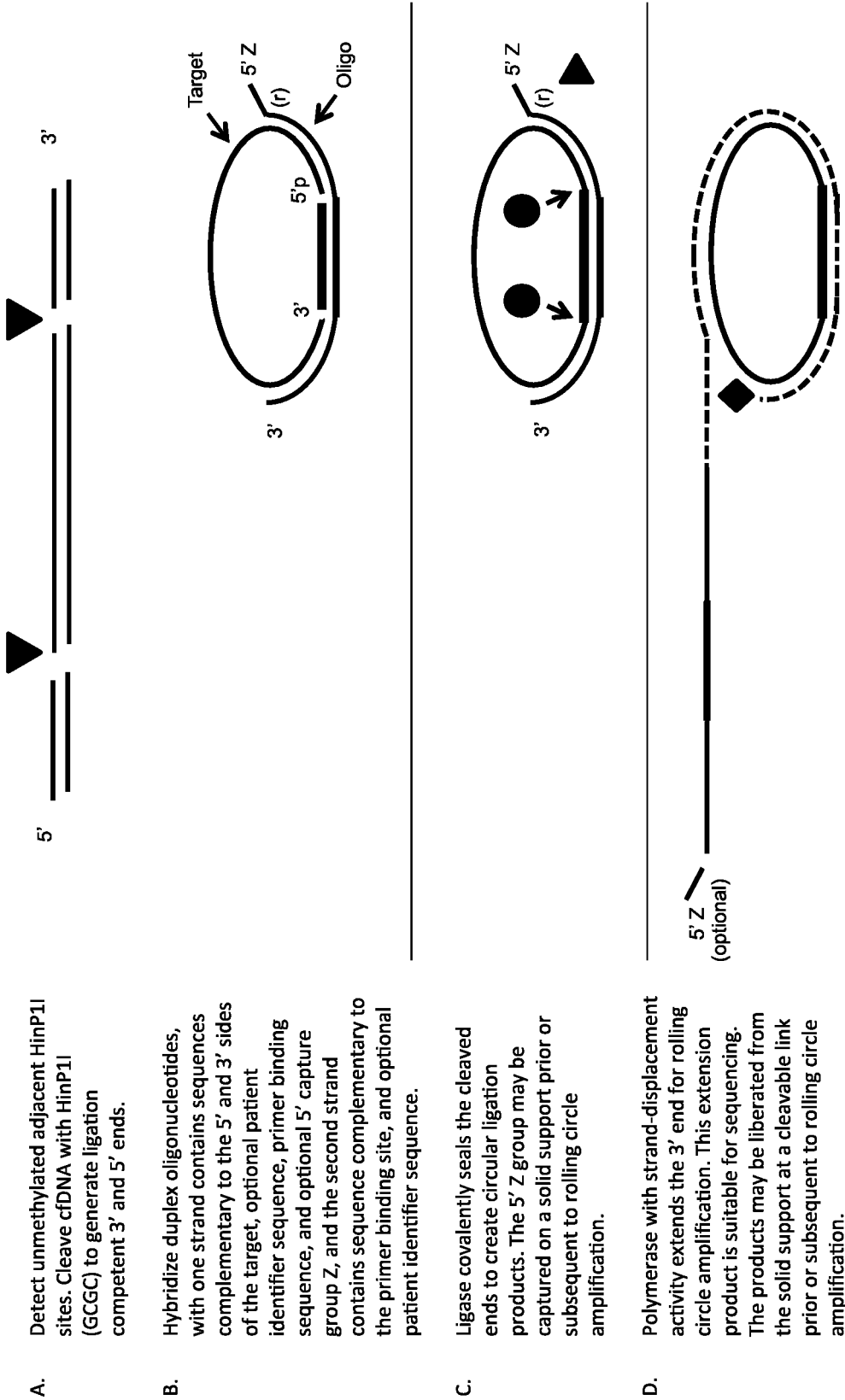
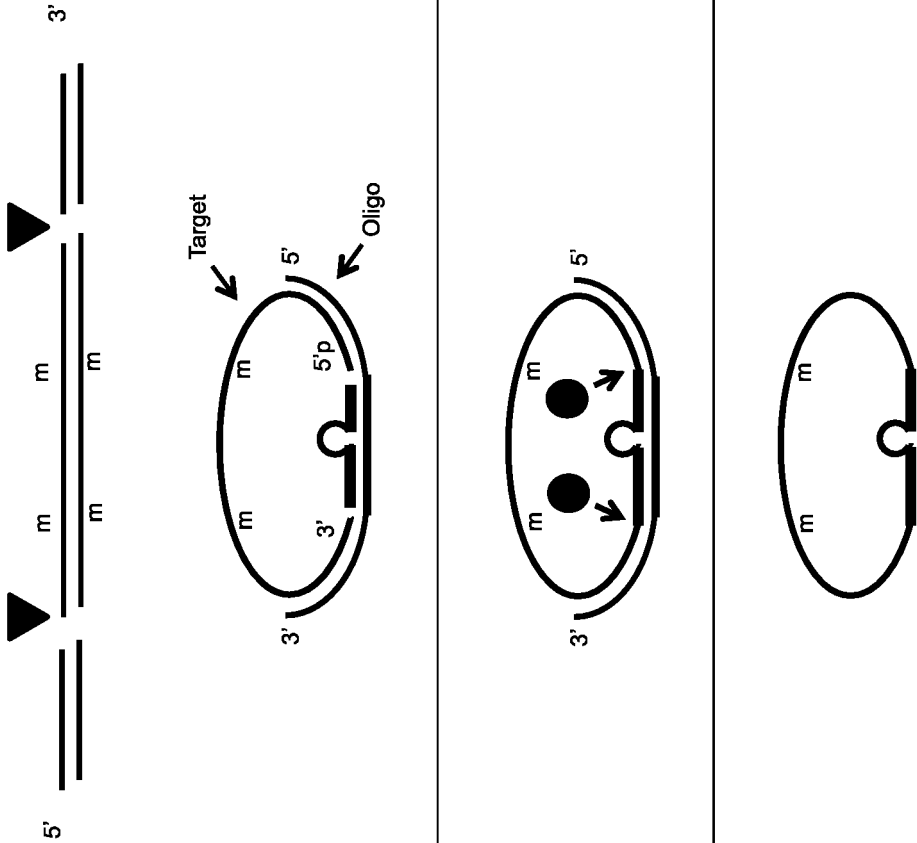


Figure 48



- A. Detect methylated Acil sites between HaeIII sites. Cleave cfDNA with Acil (GCGG) and HaeIII (GGCC) to generate ligation competent 3' and 5' ends.
- B. Hybridize duplex oligonucleotides, with one strand containing sequences complementary to the 5' and 3' sides of the target, and the second strand containing unique identifier sequence, optional primer binding site, and optional patient identifier sequence.
- C. Ligase covalently seals the cleaved ends to create circular ligation products
- D. Exonuclease(s) digest all unligated or nicked products leaving only desired single-stranded circular DNA comprising of original target DNA with unique identifier sequence. This product is suitable for circle sequencing.

Figure 49

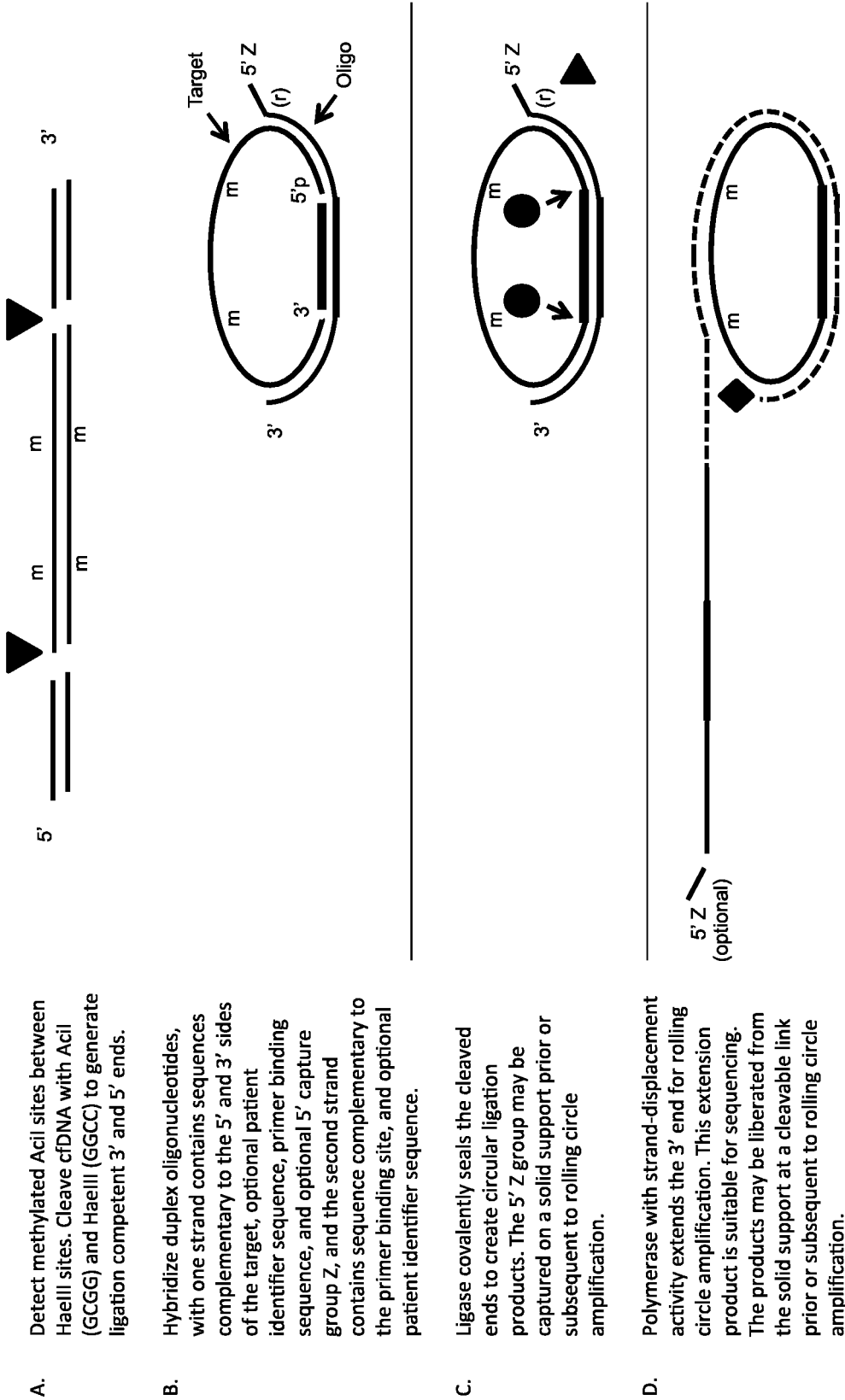
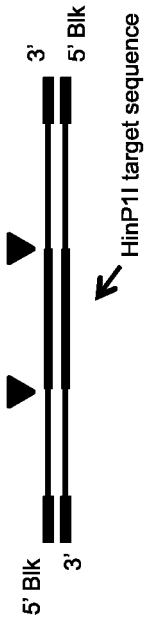
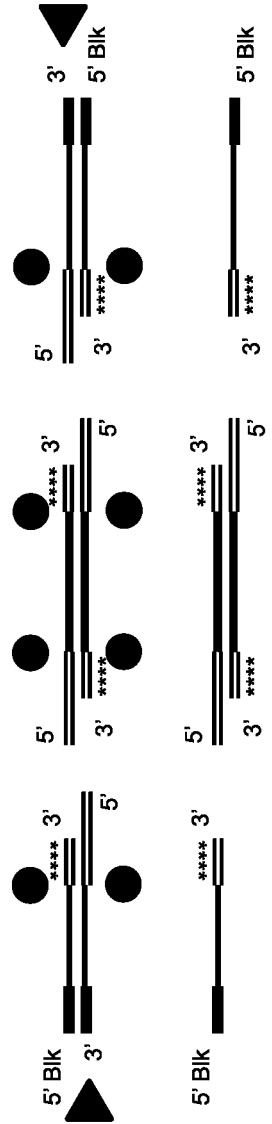


Figure 50

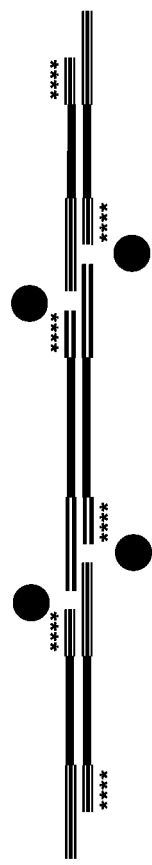
A. Detect unmethylated adjacent HinP1I sites (GCGC) throughout genome. Ligate on short linkers with blocked 5' end. Cleave DNA with HinP1I (GCGC). Only adjacent HinP1I sites (GCGC) unmethylated in original target generate unblocked fragments when cleaved with HinP1I.



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.

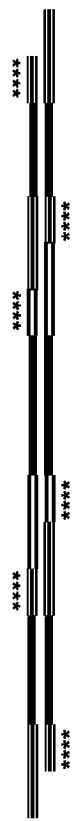
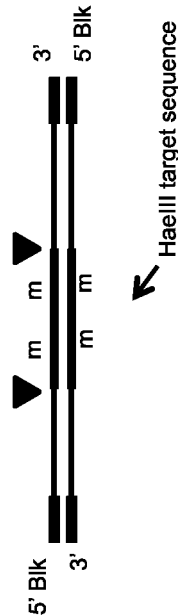
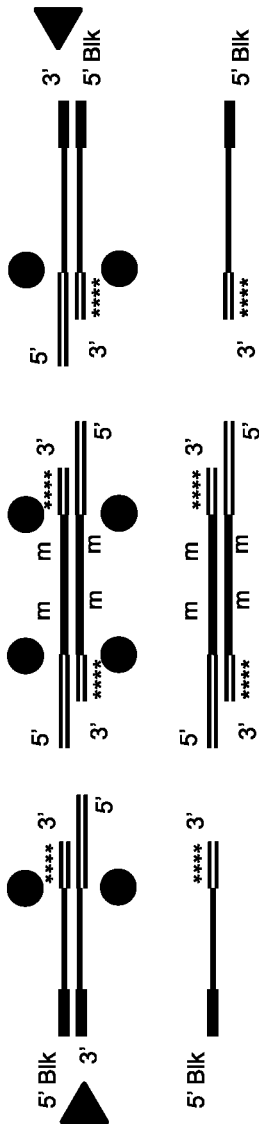


Figure 51

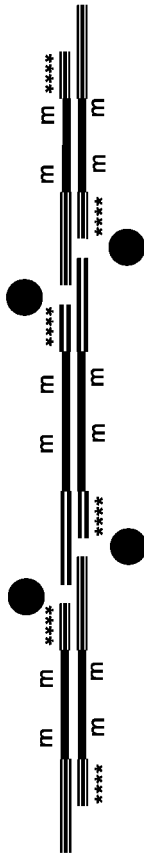
A. Detect methylated adjacent Acil sites (GCGG) throughout genome. Cleave with Acil. Ligate on short linkers with blocked 5' end. Cleave DNA with HaeIII (GGCC). Only adjacent HaeIII sites (GGCC) with methylated Acil sites in original target generate unblocked fragments when cleaved with HaeIII.



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 Acil sites in target DNA with optional unique identifier and/or patient identifier sequence. This product is suitable for optional additional steps and subsequent sequencing.

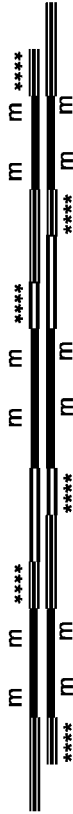


Figure 52

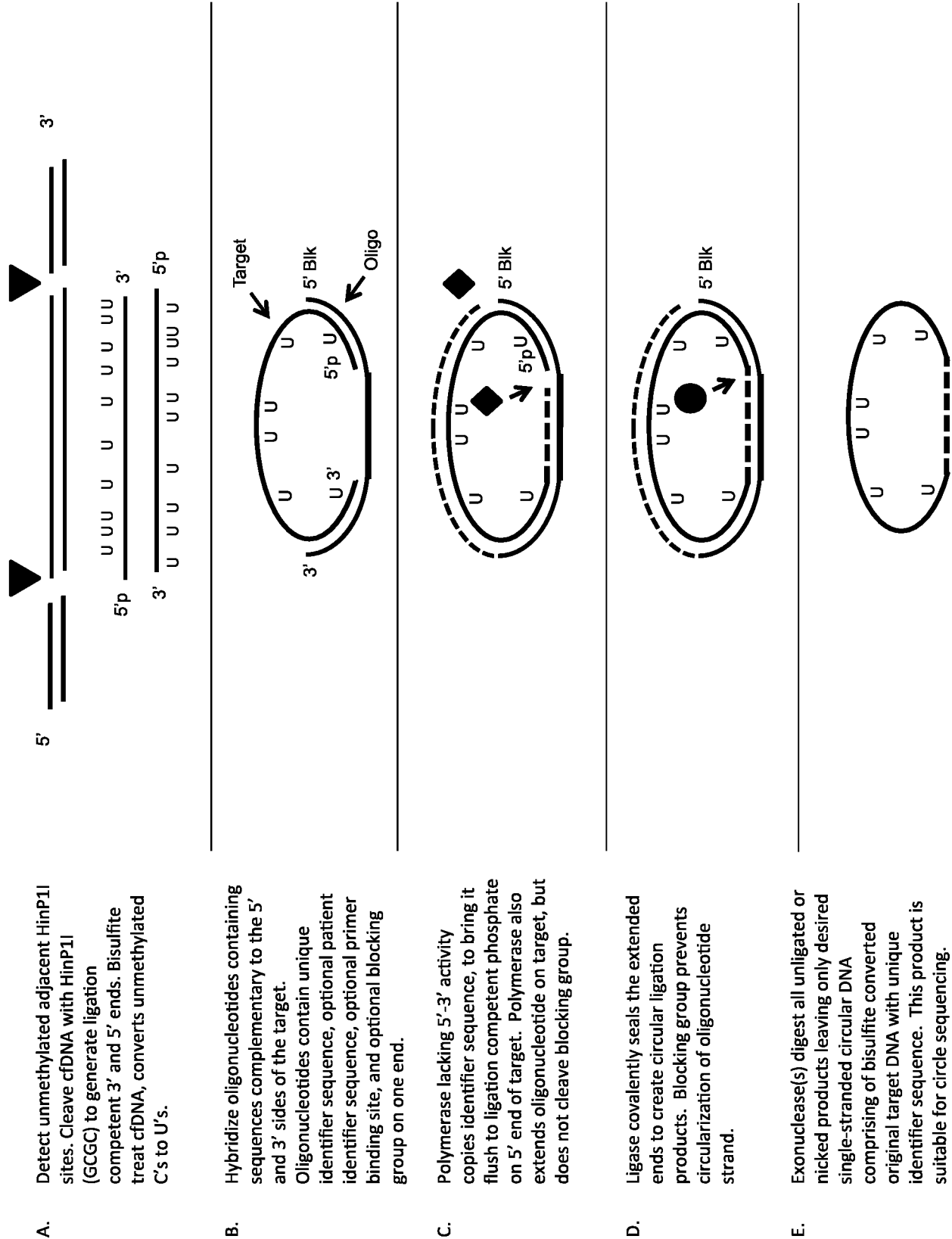


Figure 53

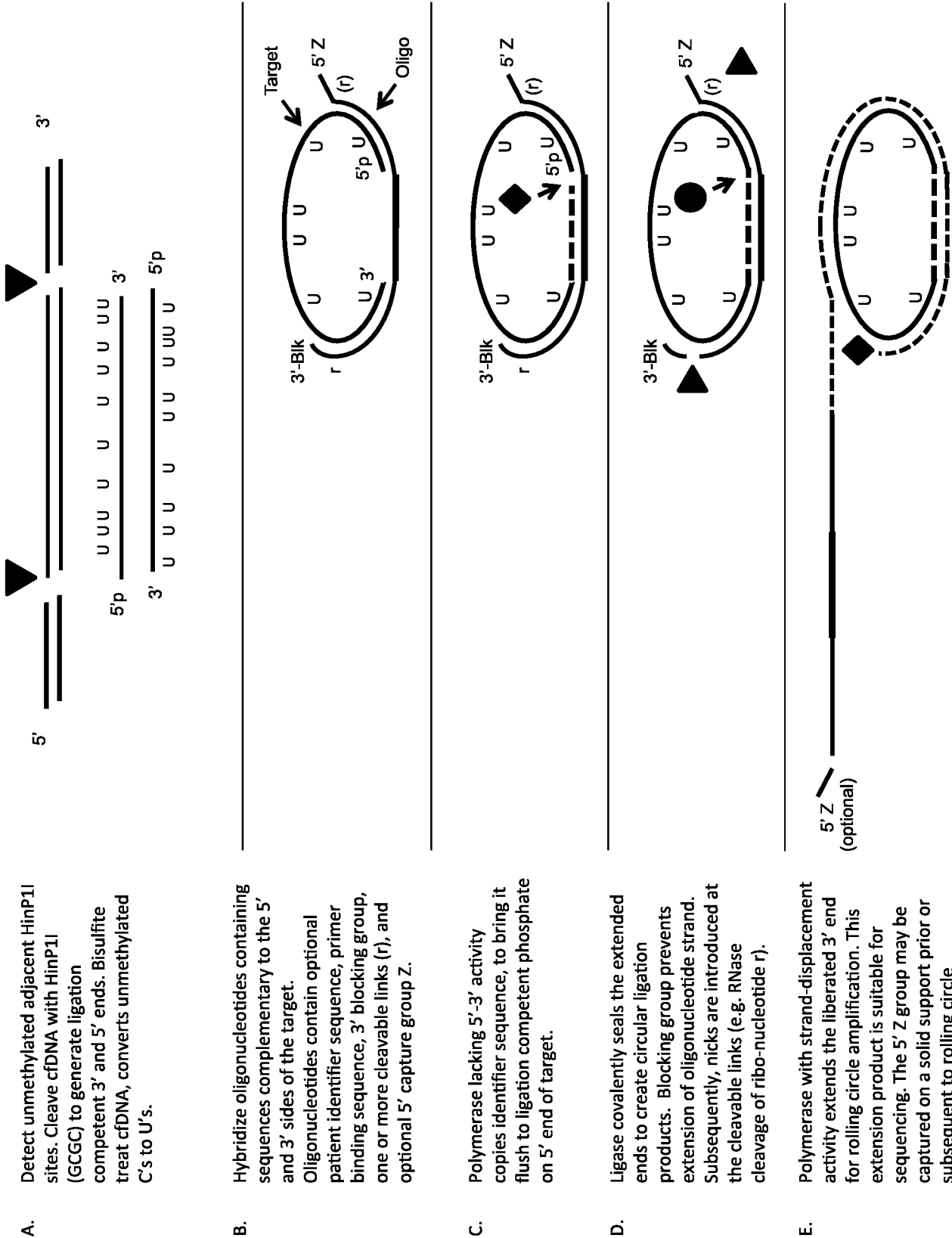
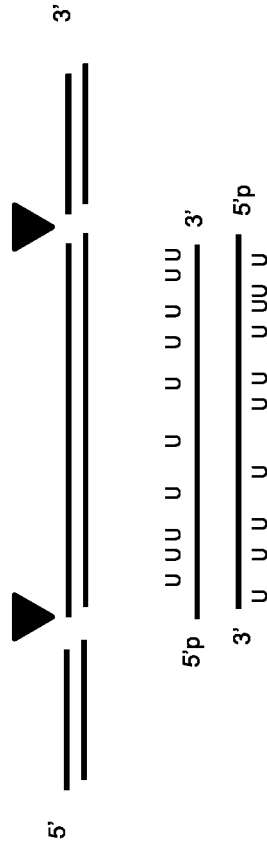
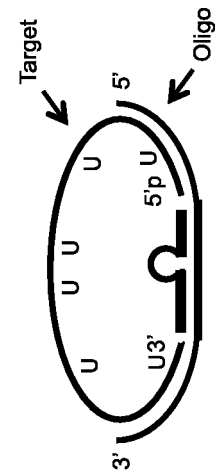


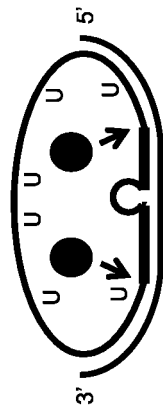
Figure 54



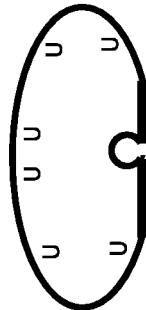
A. Detect unmethylated adjacent HinP1I sites. Cleave cDNA with HinP1I (GCGC) to generate ligation competent cDNA, converts unmethylated treat cDNA, converts unmethylated C's to U's.



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



C. Ligase covalently seals the cleaved ends to create circular ligation products



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

Figure 55

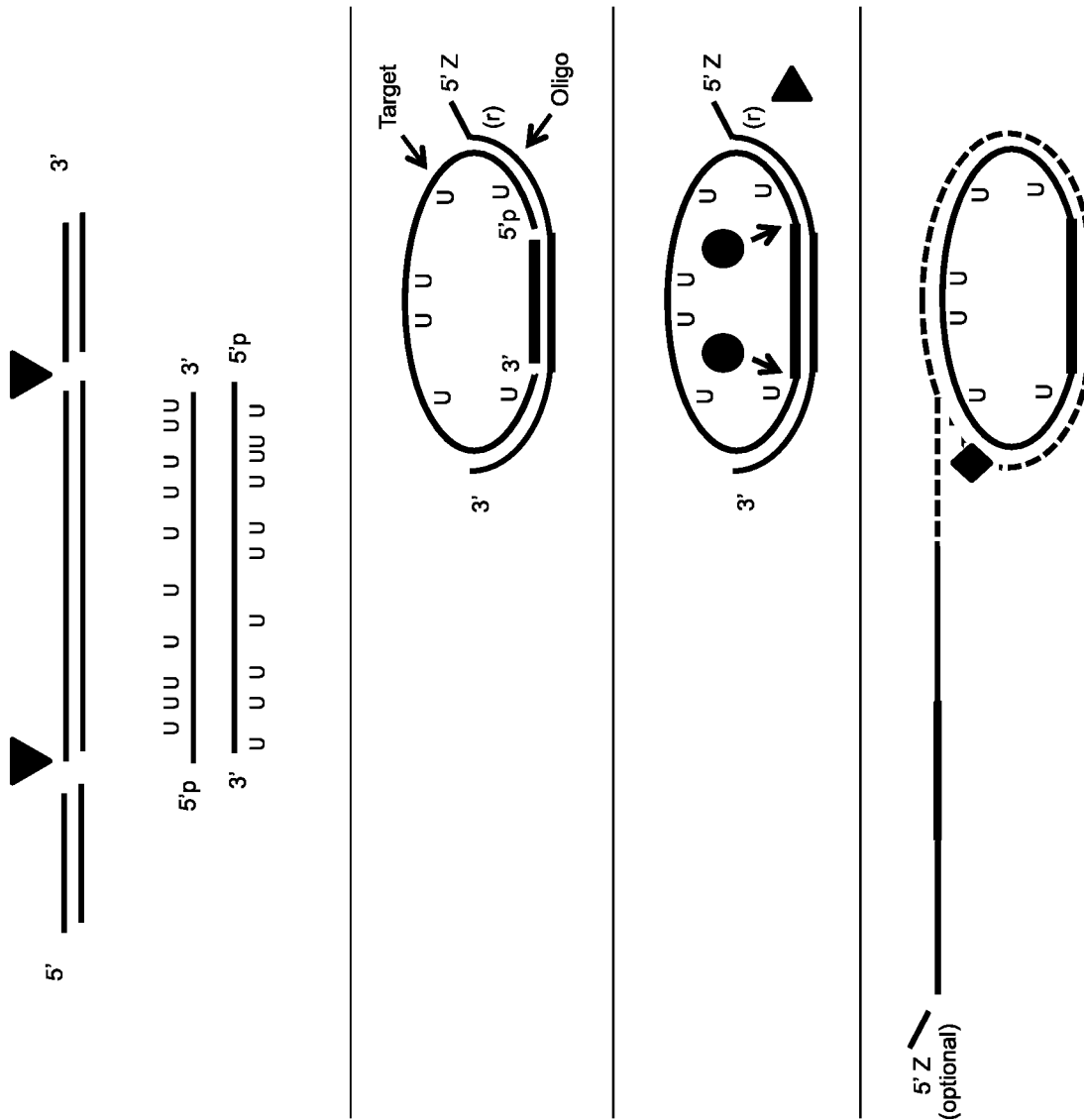
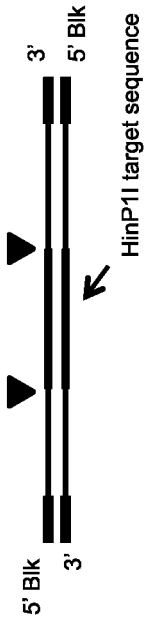
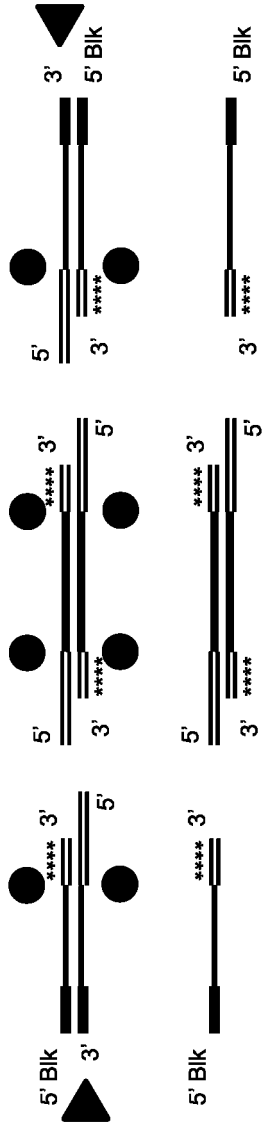


Figure 56

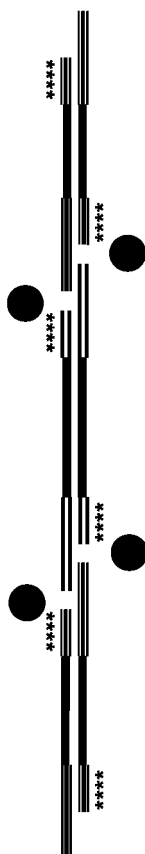
A. Detect unmethylated adjacent HinP1I sites (GCGC) throughout genome. Ligate on short linkers with blocked 5' end. Cleave cfDNA with HinP1I (GCGC). Only adjacent HinP1I sites (GCGG) unmethylated in original target generate unblocked fragments when cleaved with HinP1I.



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.

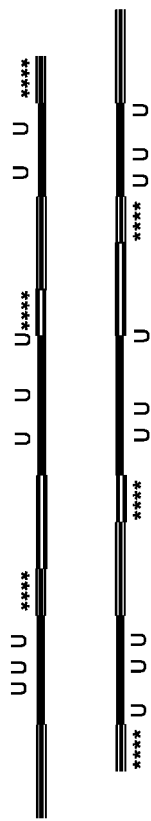


Figure 57

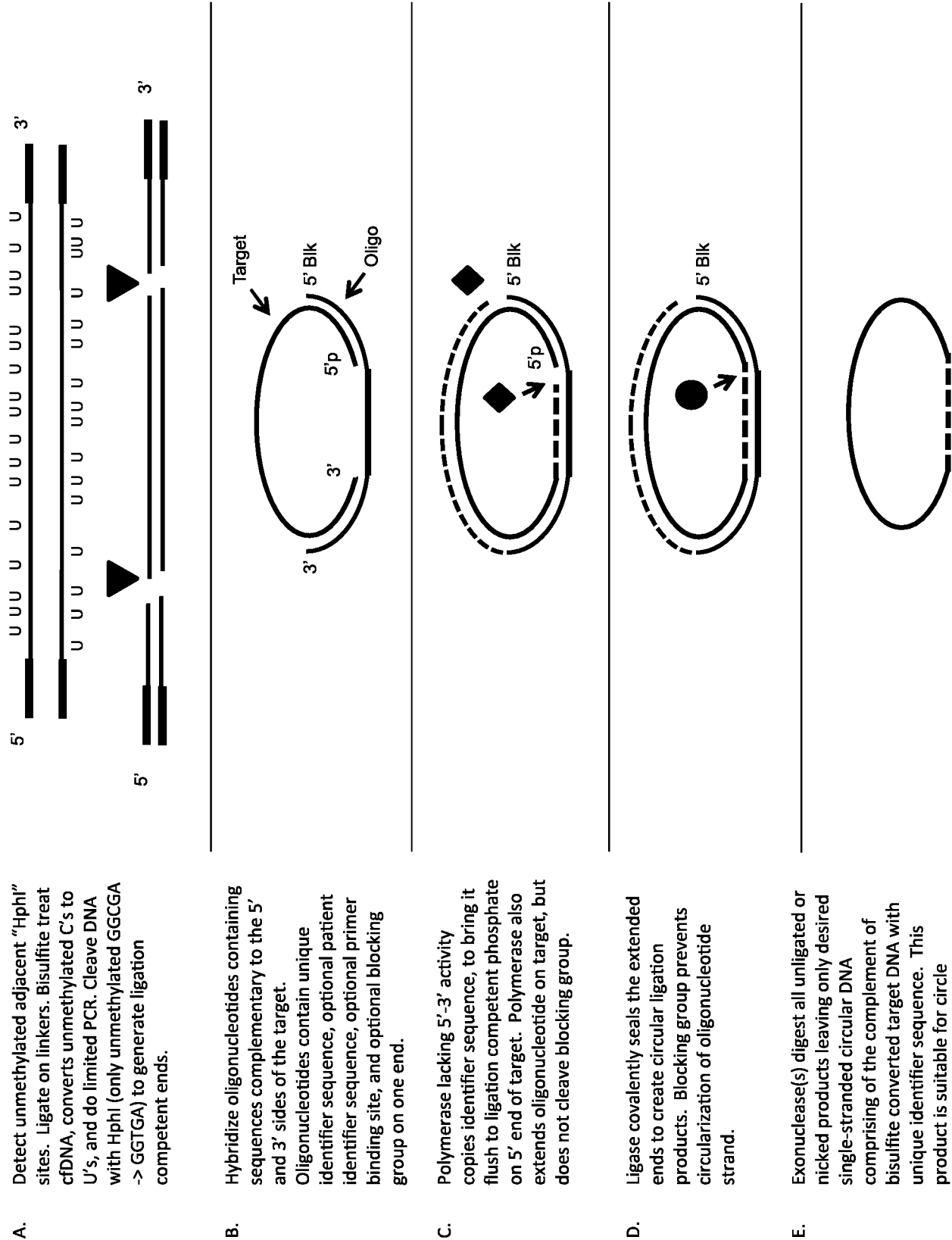


Figure 58

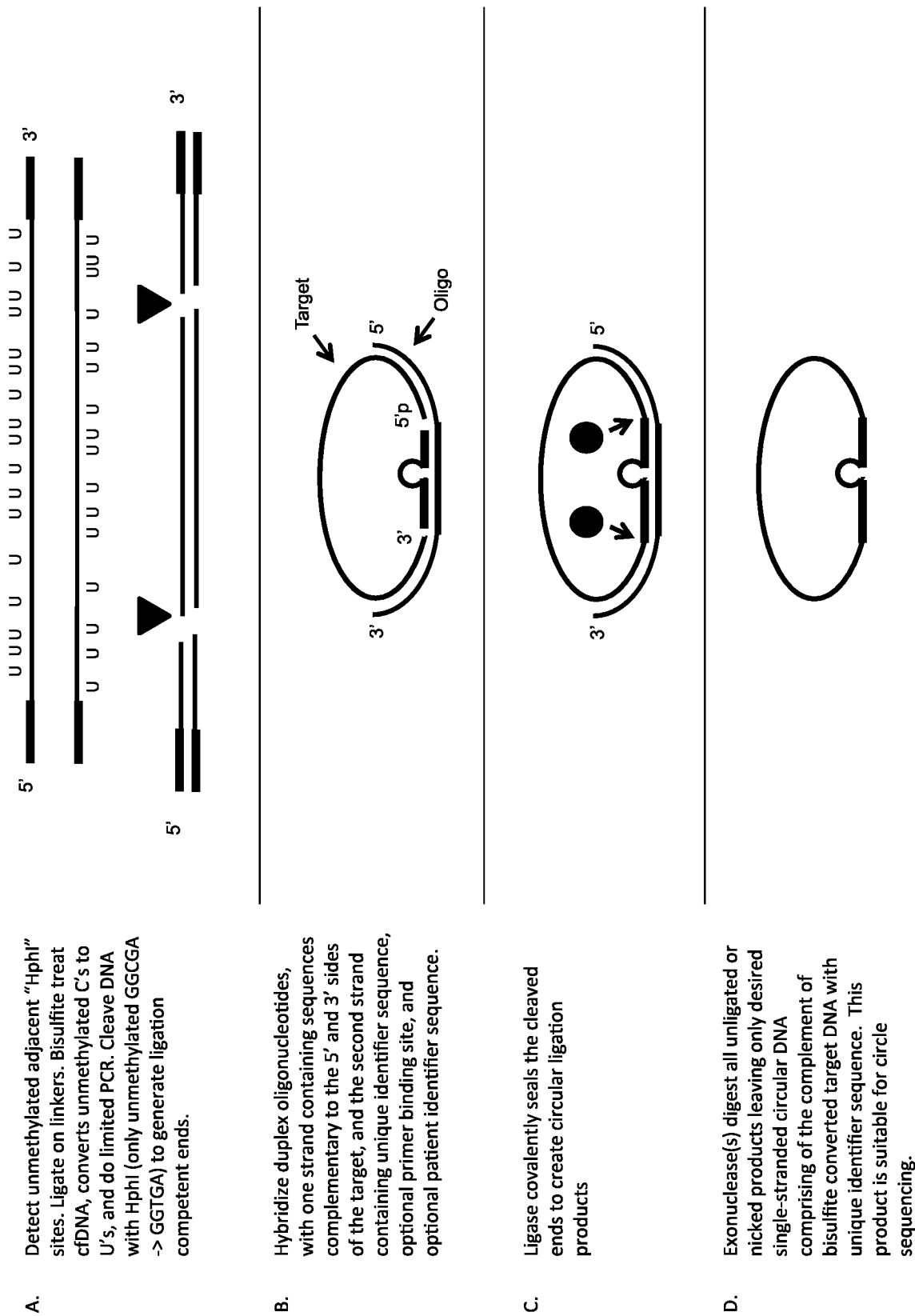


Figure 60

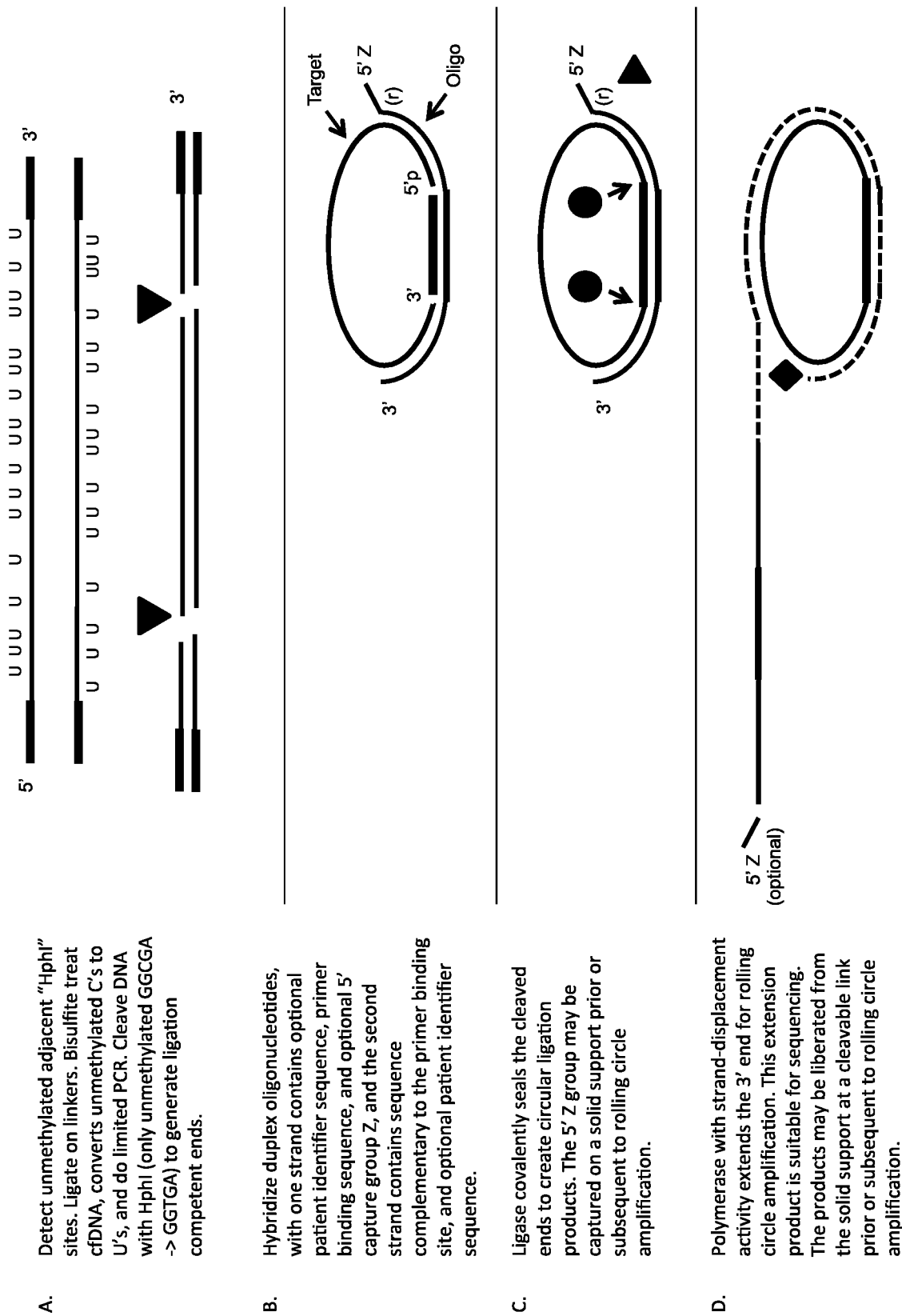


Figure 61

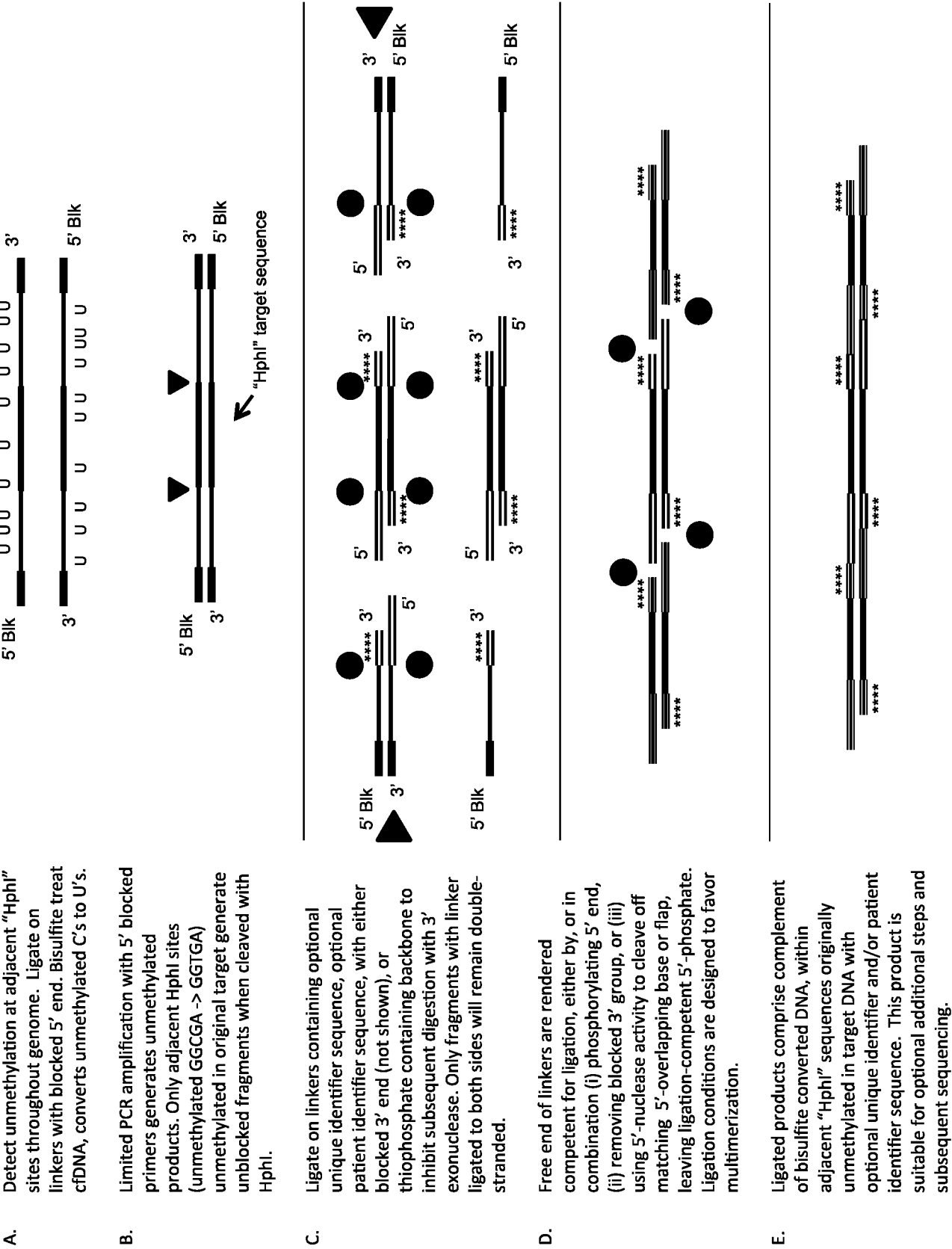


Figure 62

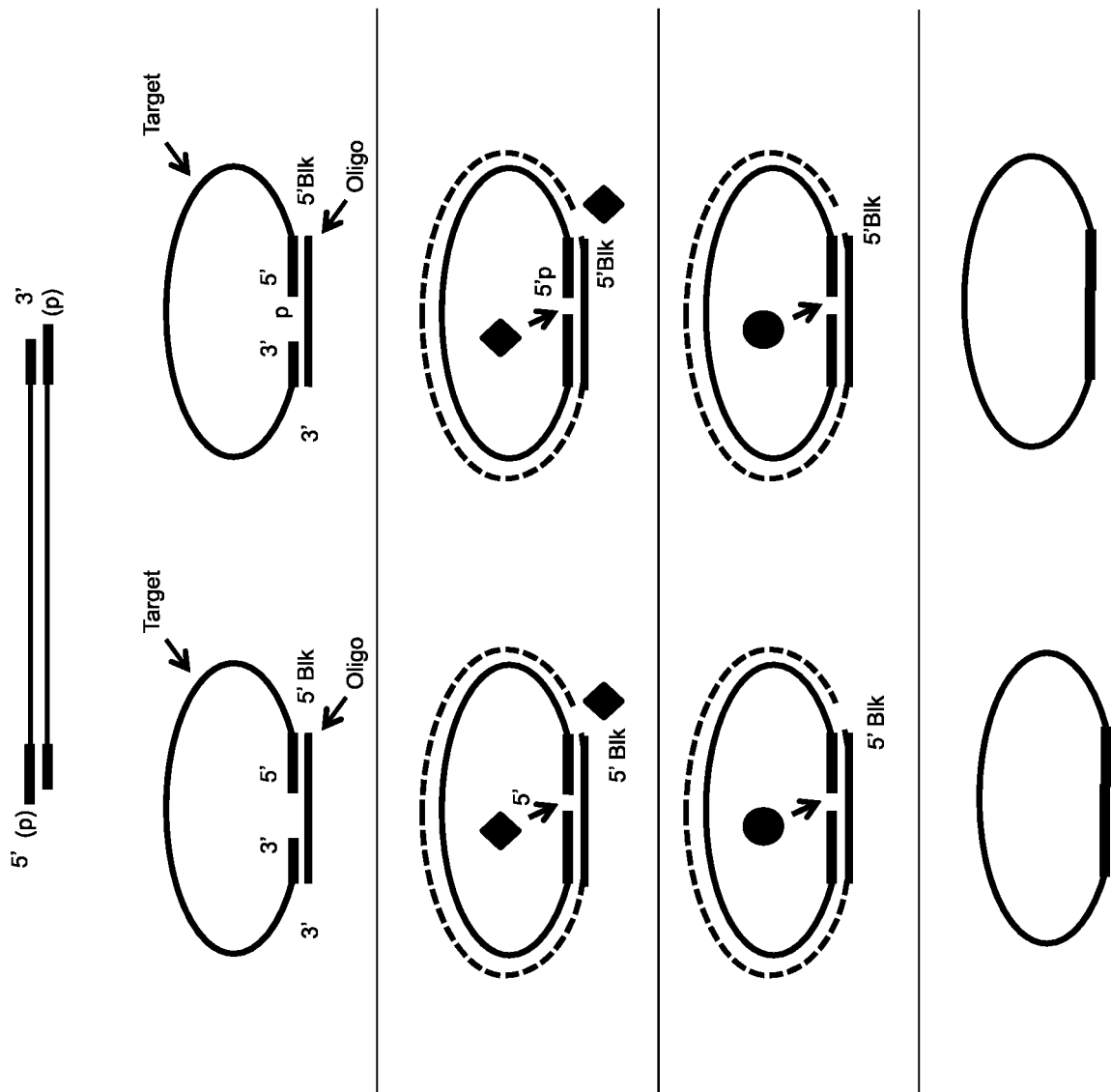


Figure 63

- A. cfDNA of average length of about 160 bp. Ligate on linkers with optional 5' phosphate.
- B. Hybridize oligonucleotides containing sequences complementary to the 5' and 3' sides of the linkers. Oligonucleotides contain unique identifier sequence, optional patient identifier sequence, optional primer binding site, and optional blocking group on 5' end.
- C. Polymerase extends hybridized short linker 3' end to copy identifier sequence, with 5'-nuclease cleavage of matching 5'-overlapping base of 5' linker, leaving ligation-competent 5'-phosphate (left). Or, polymerase lacking 5'-3' nuclease extends hybridized short linker 3' end to copy identifier sequence, with extended 3' end directly adjacent to 5'-phosphate of 5' linker (right). 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 unique identifier sequence. This product is suitable for optional capture with unique or repetitive sequences (i.e. tetra-nucleotide repeats), and subsequent sequencing.

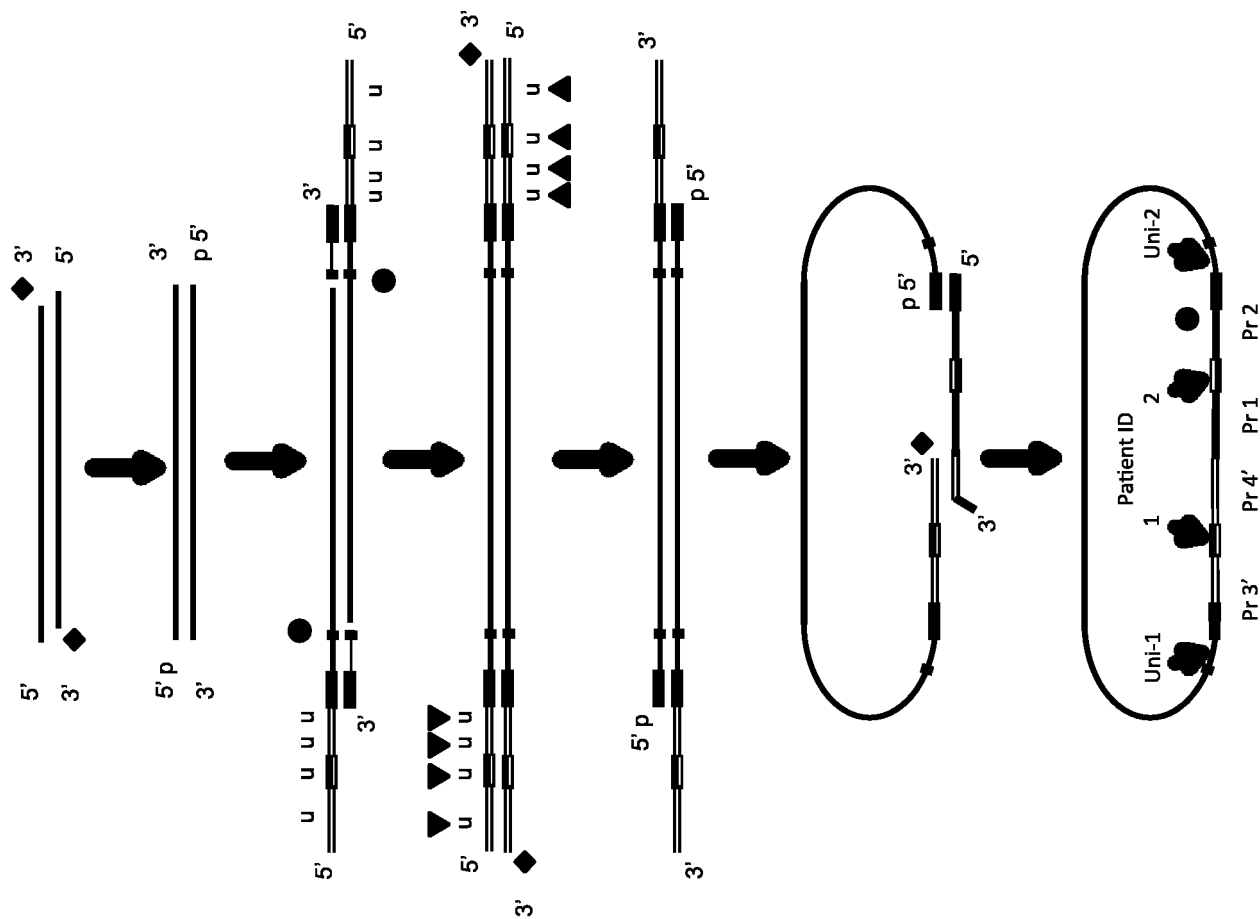


Figure 64

- A. Linker design for sequencing, identifying, and verifying mutations in both target strands. cfDNA of average length of about 160 bp. Repair ends with T4 polymerase.
- B. Phosphorylate 5' ends with T4 kinase.
- C. Ligate on 5'-OH blunt end linkers with long 5' single-stranded region, cleavable sites (u), unique identifier sequences (1 & 2), patient identifier (1), and primer binding sites (3, 4).
- D. Polymerase extends 3' end of target and copies unique identifier sequences, patient identifier, and primer binding sites.
- E. Cleave top strands using UDG & FPG to liberate 5' phosphate.
- F. Hybridize oligonucleotides containing sequences complementary to the 5' and 3' sides of the linkers. Oligonucleotides contain patient identifier sequence, primer binding sites, and mismatched tail on 3' end.
- G. Polymerase lacking 5'-3' nuclease extends hybridized 3' end to copy primer binding and identifier sequences.
- H. Ligase covalently seals the extended ends to create circular ligation products comprising unique identifier sequences, patient identifier, and primer binding sites.

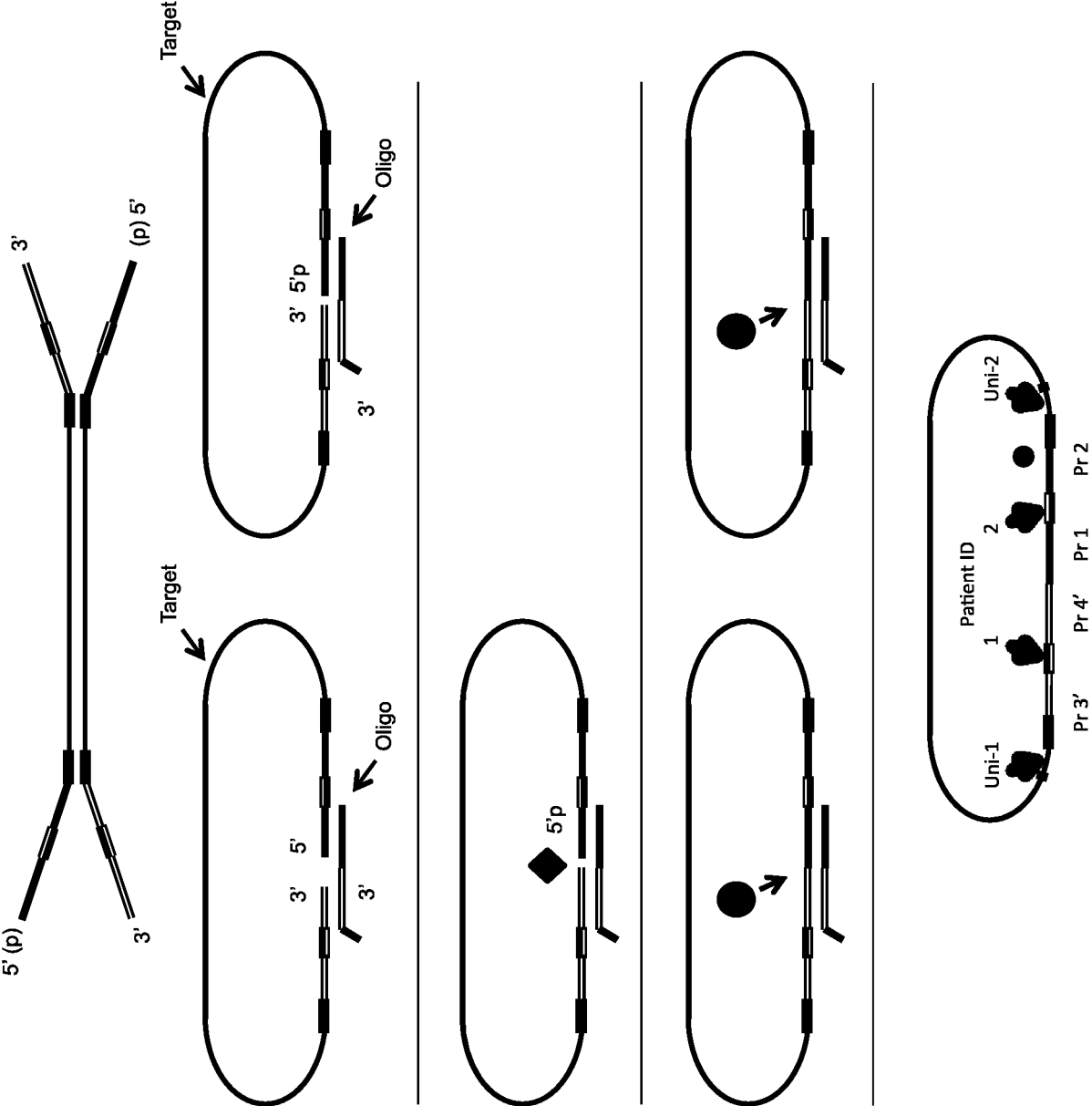


Figure 65

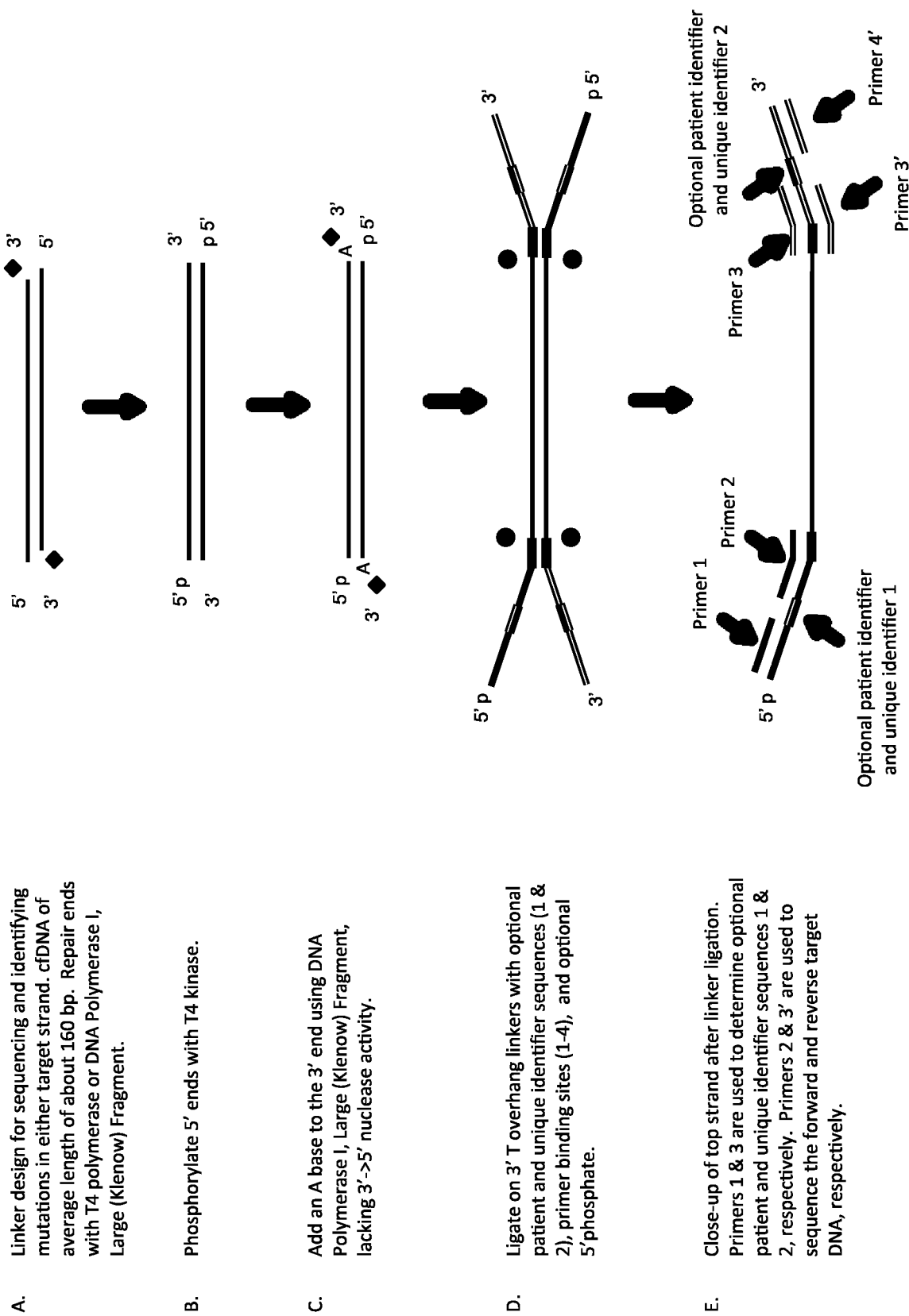


Figure 66

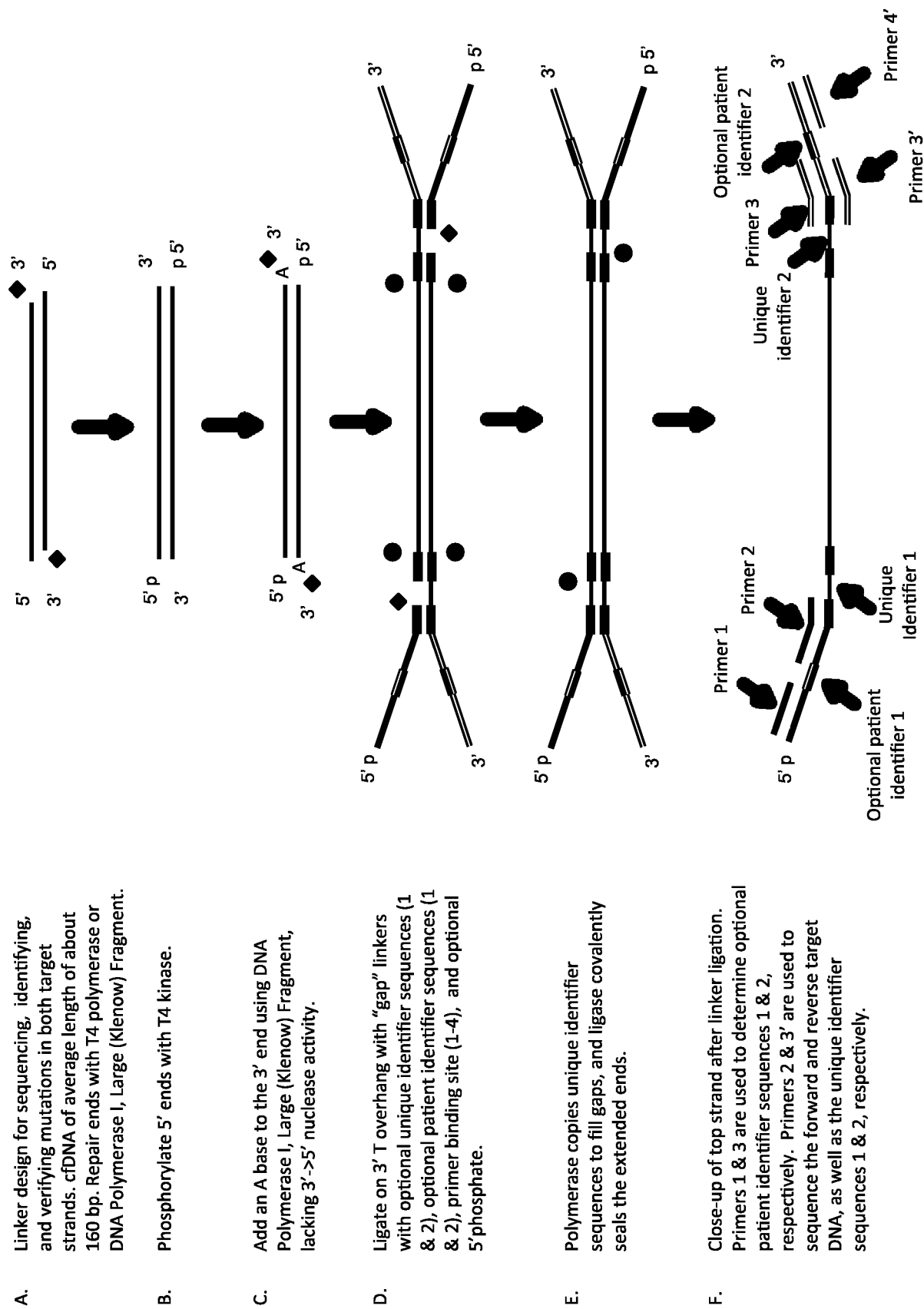


Figure 67

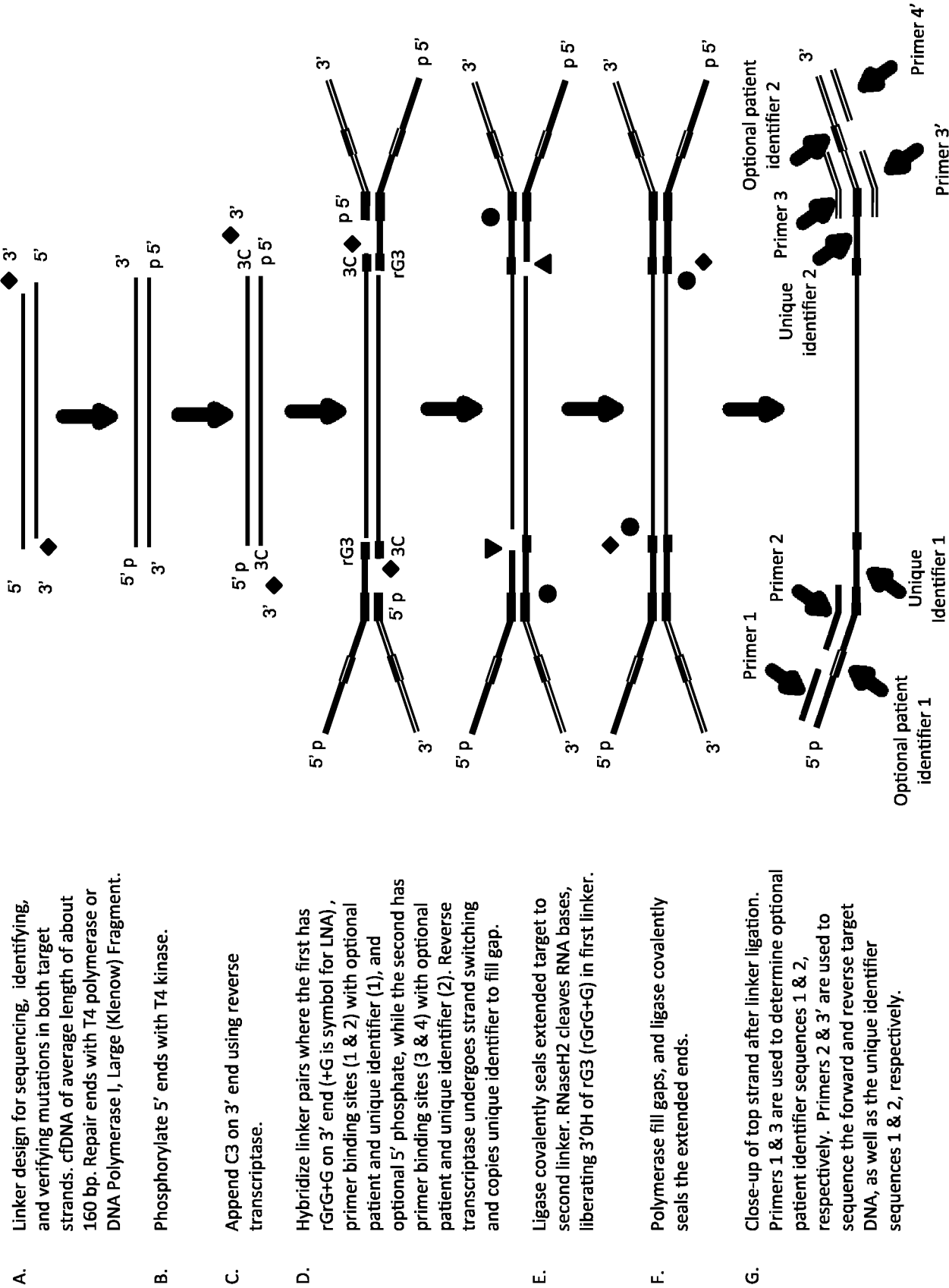


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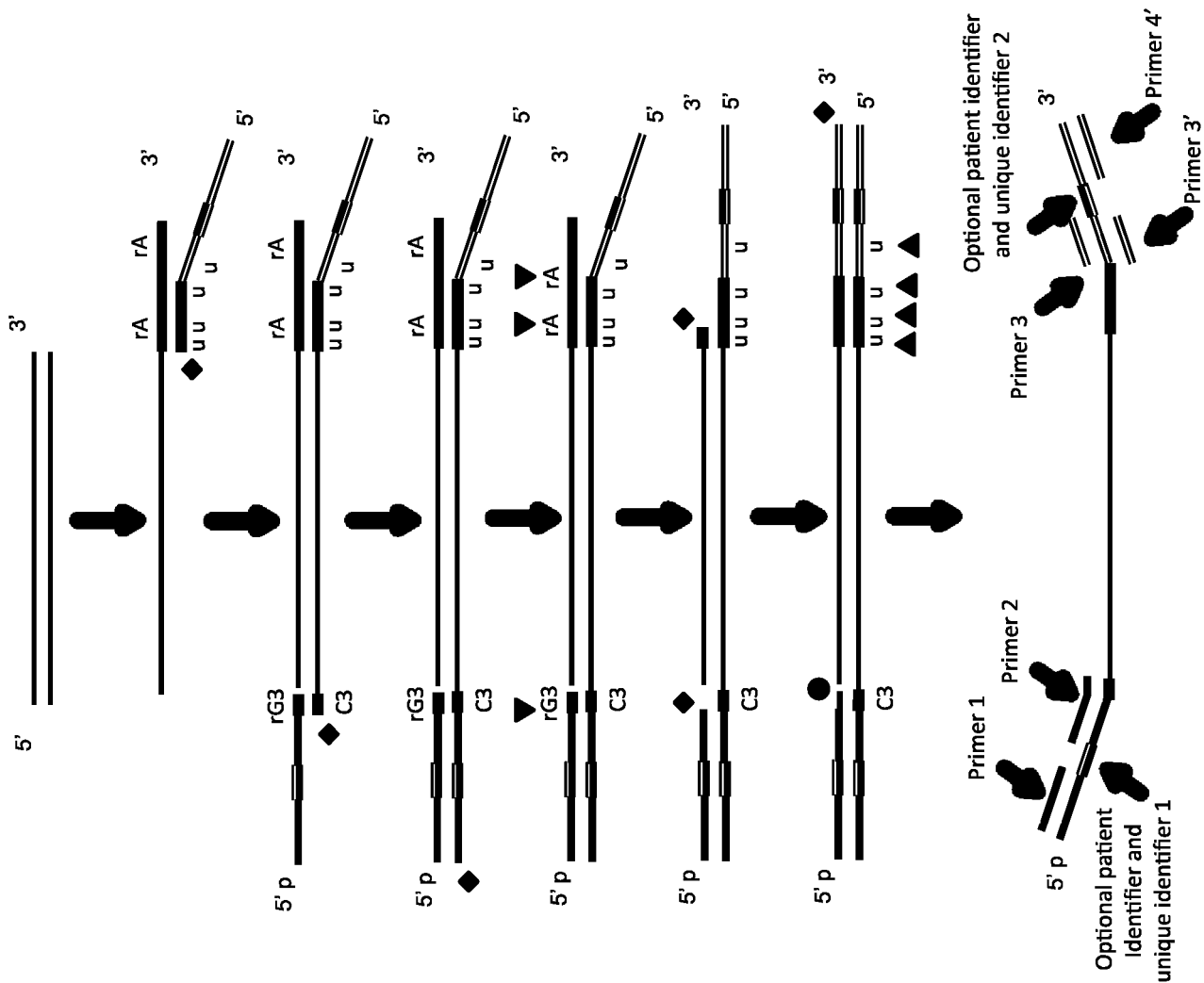


Figure 69

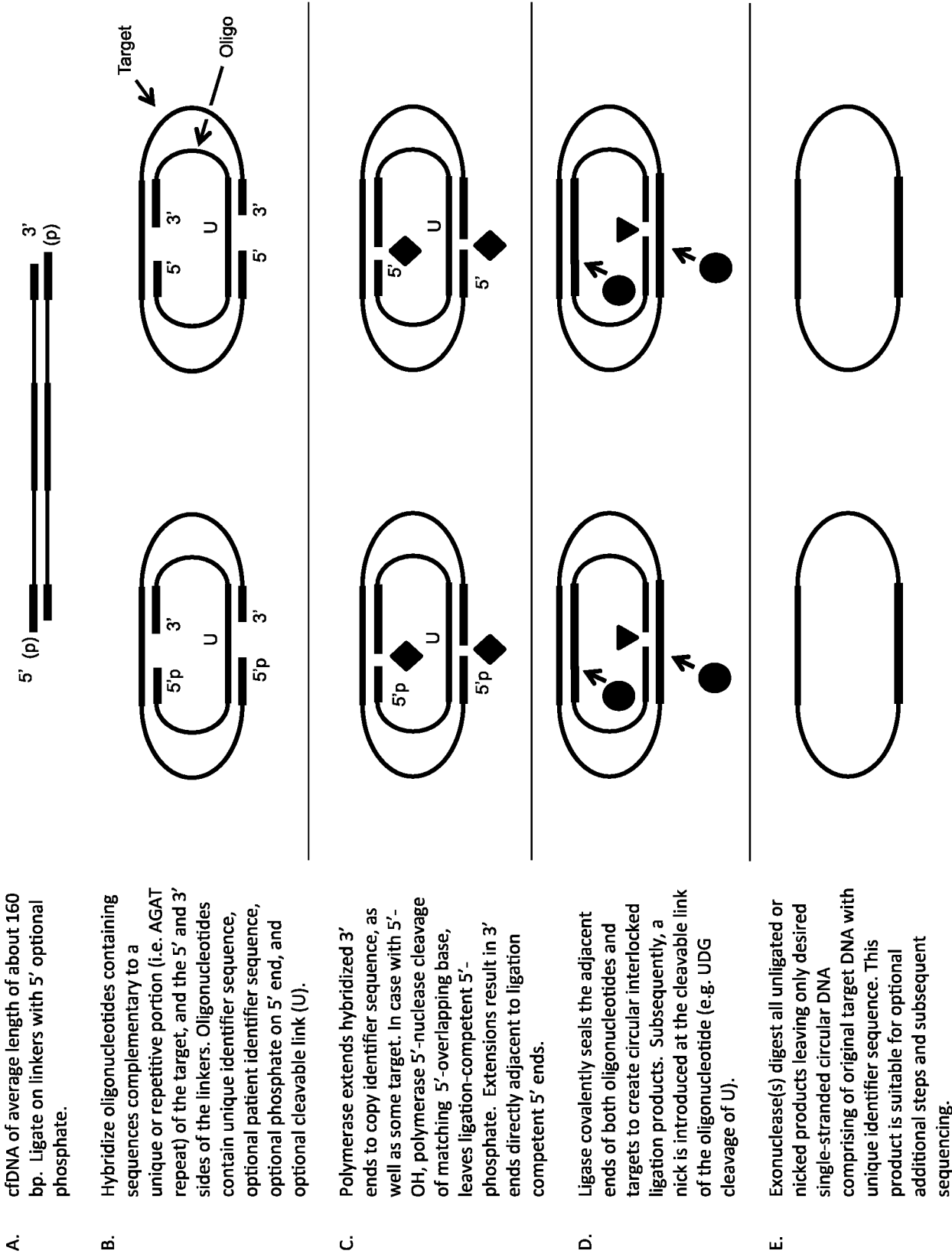
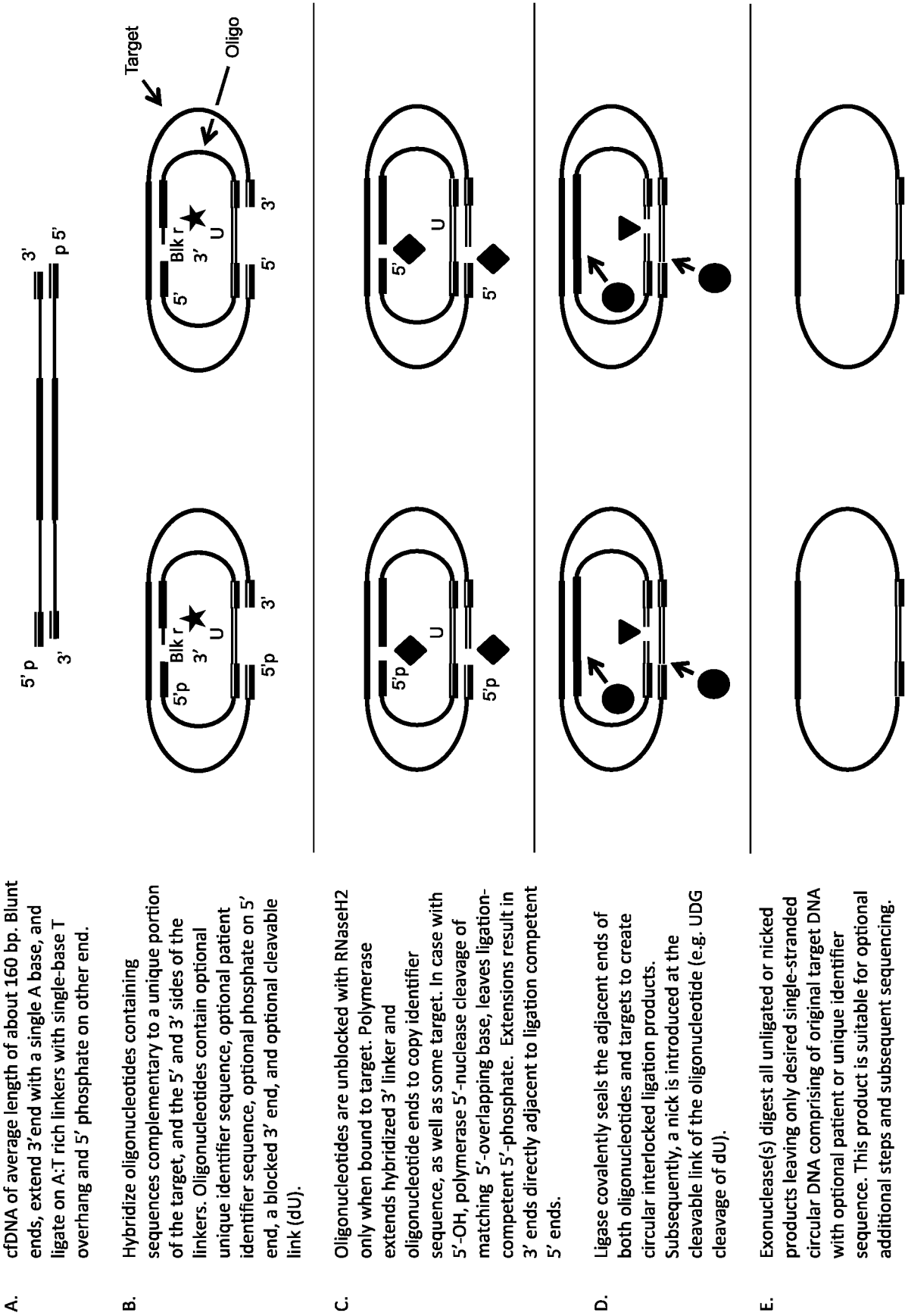


Figure 70



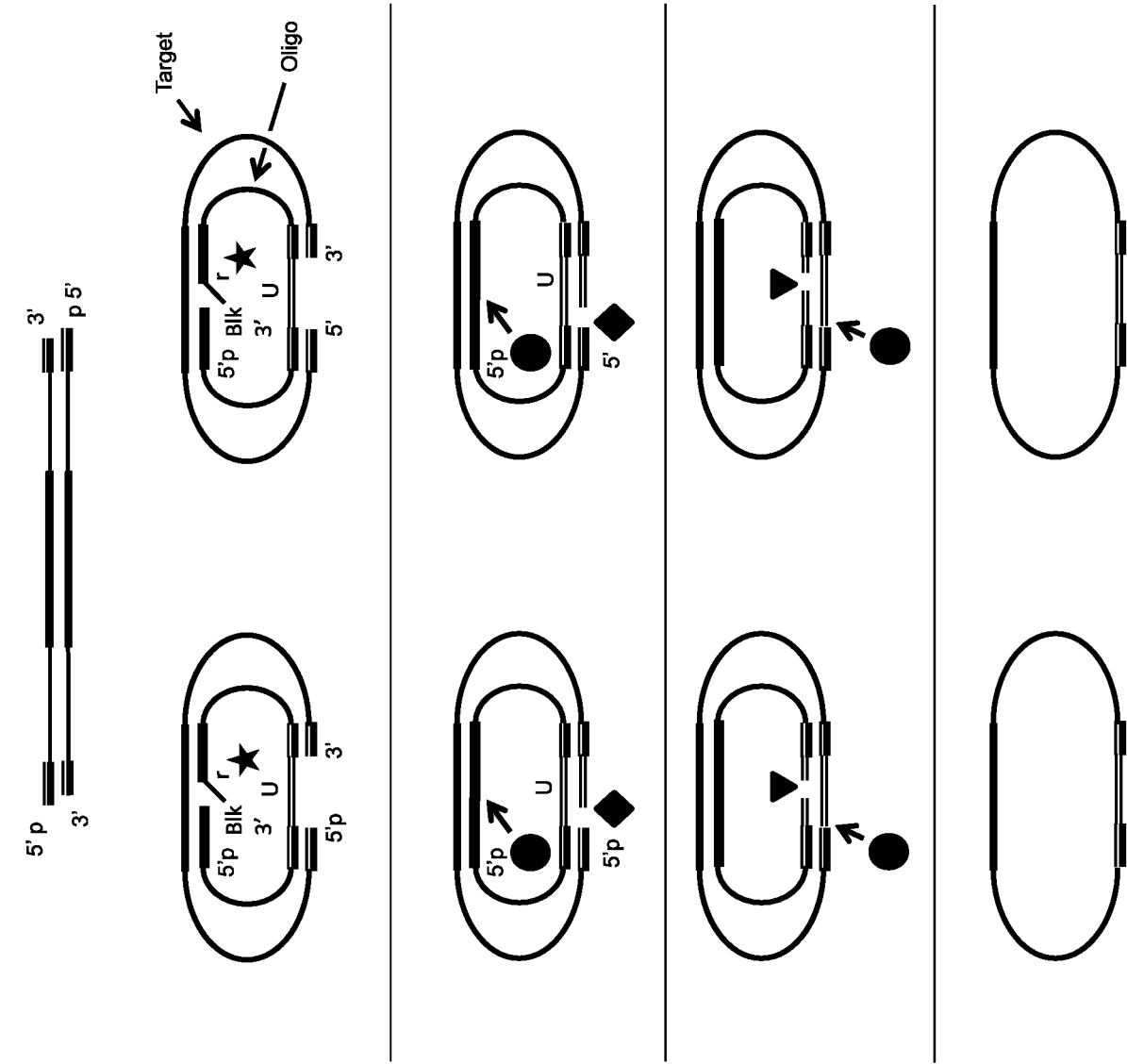


Figure 72

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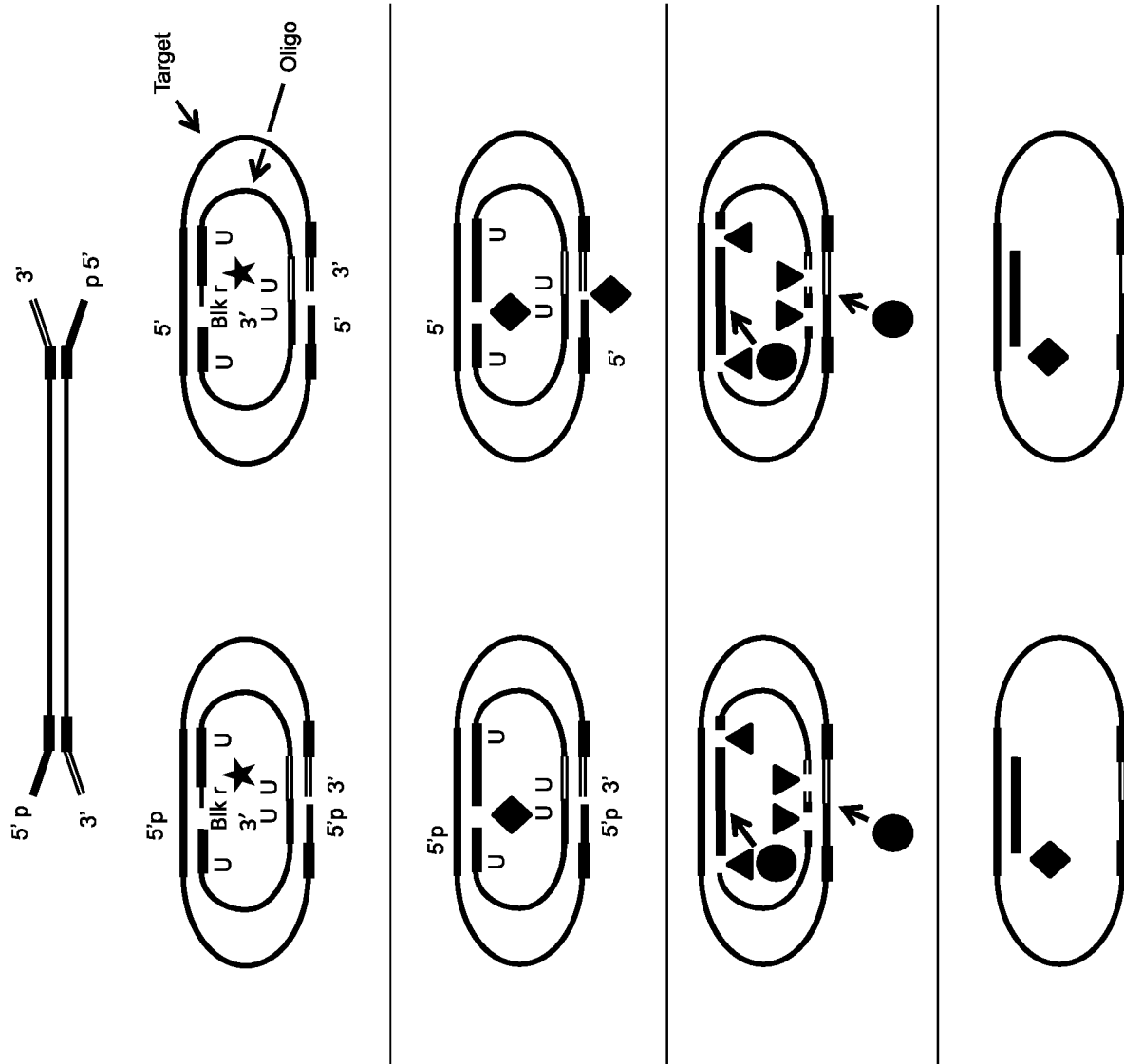


Figure 73

- A. cfDNA of average length of about 160 bp. Ligate on linkers with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site, and optional 5'phosphate.
- B. Hybridize oligonucleotides containing sequences complementary to a unique portion of the target, and the 5' and 3' ends of the linkers. Oligonucleotides contain optional unique identifier sequence, optional patient identifier sequence, optional phosphate on 5' end, a blocked 3' end, and cleavable links (dU).
- C. Oligonucleotides are unblocked with RNaseH2 only when bound to target. Polymerase extends hybridized 3' end of linker (right), as well as 3' end of oligonucleotide on 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, nicks are introduced at the cleavable links of the oligonucleotide (e.g. UDG cleavage of dU).
- E. Some cleaved oligonucleotides fall off, leaving only desired single-stranded circular DNA comprising of original target DNA with a target-specific portion of the oligonucleotide remaining hybridized. This product is suitable for rolling circle amplification, optional additional steps, and subsequent sequencing.

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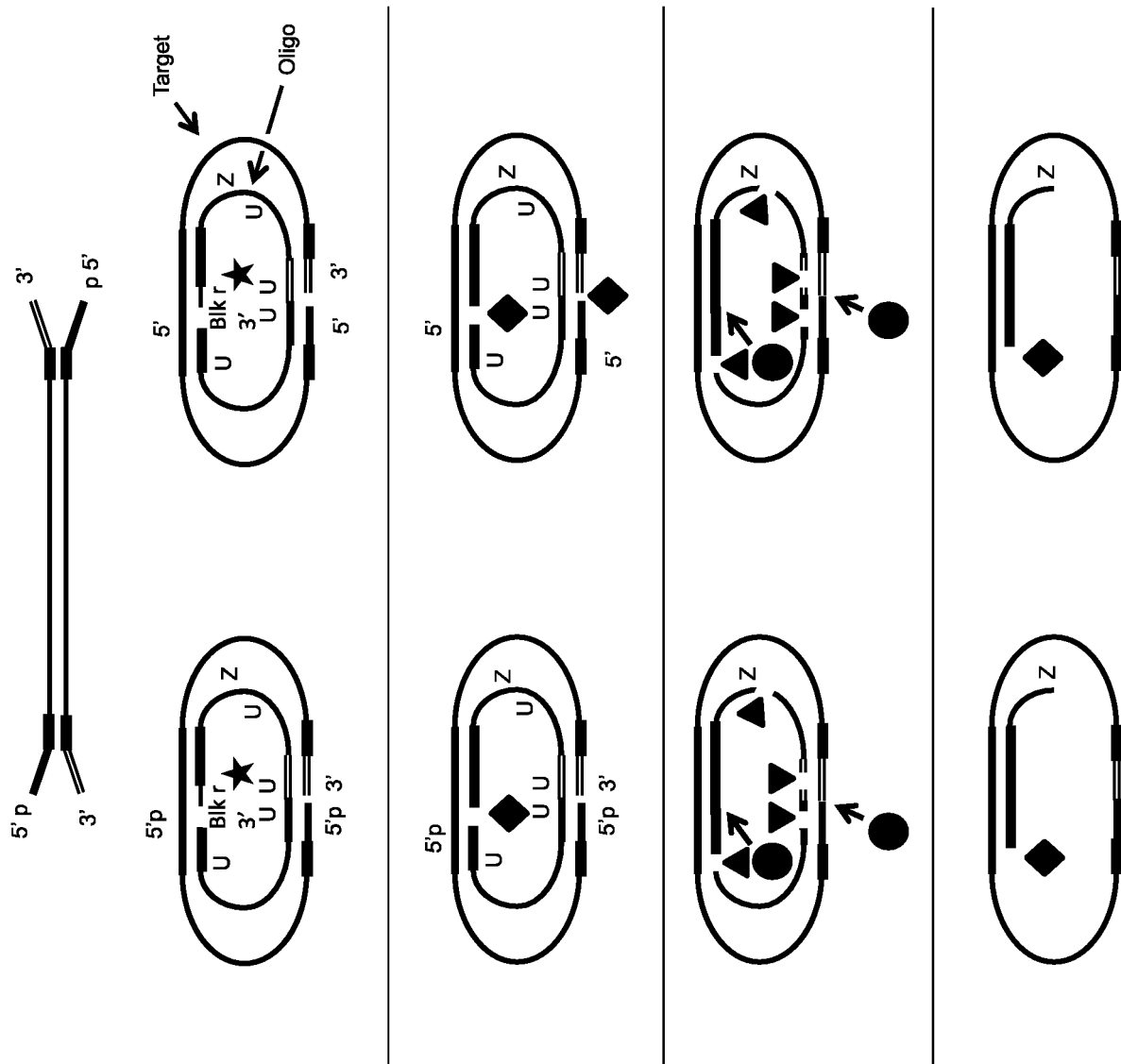


Figure 74

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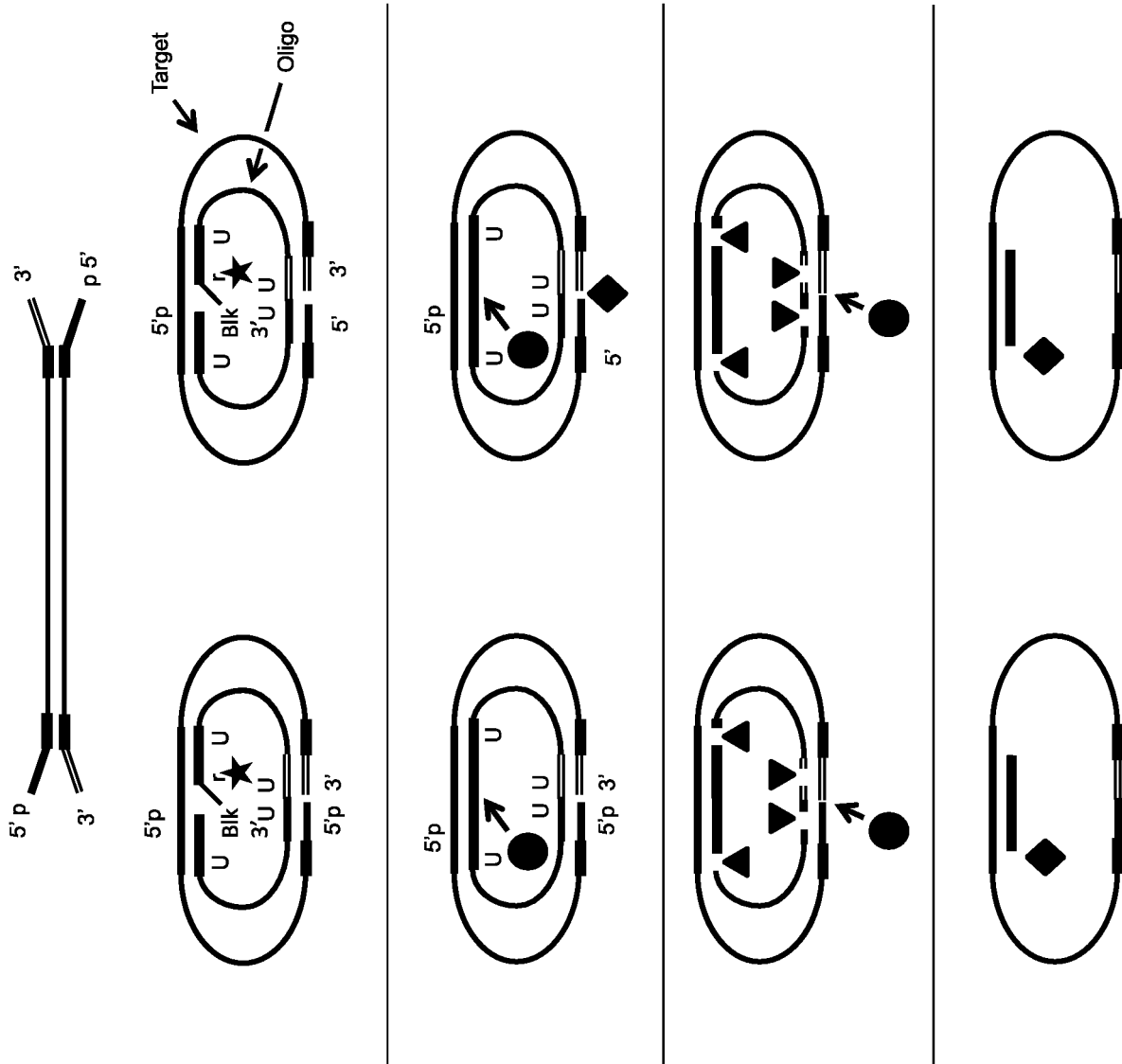


Figure 75

- A. cfDNA of average length of about 160 bp. Ligate on linkers with optional unique identifier sequence, optional patient identifier sequence, optional primer binding site, and optional 5' phosphate.
- B. Hybridize oligonucleotides containing sequences complementary to a unique portion of the target, and the 5' and 3' ends of the linkers. Oligonucleotides contain optional unique identifier sequence, optional patient identifier sequence, optional phosphate on 5' end, a blocked 3' end, and cleavable links (dU).
- C. Oligonucleotides are unblocked with RNaseH2 only when bound to target. Ligase seals adjacent target ends. Polymerase extends hybridized 3' end of linker (right). 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 oligonucleotides to create circular interlocked ligation products. Subsequently, nicks are introduced at the cleavable links of the oligonucleotide (e.g. UDG cleavage of dU).
- E. Some cleaved oligonucleotides fall off, leaving only desired single-stranded circular DNA comprising of original target DNA with a target-specific portion of the oligonucleotide remaining hybridized. This product is suitable for rolling circle amplification, optional additional steps, and subsequent sequencing.

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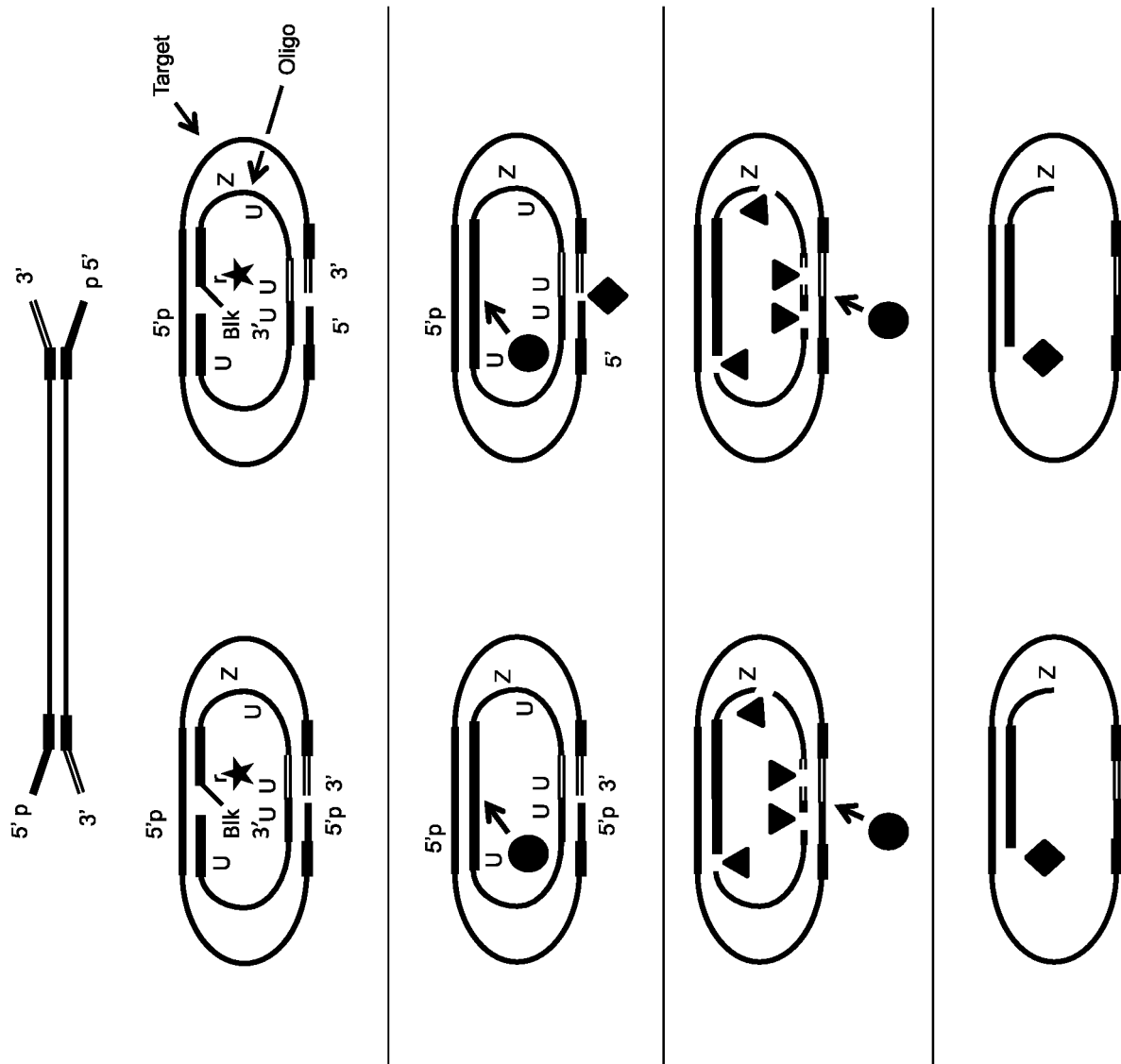


Figure 76

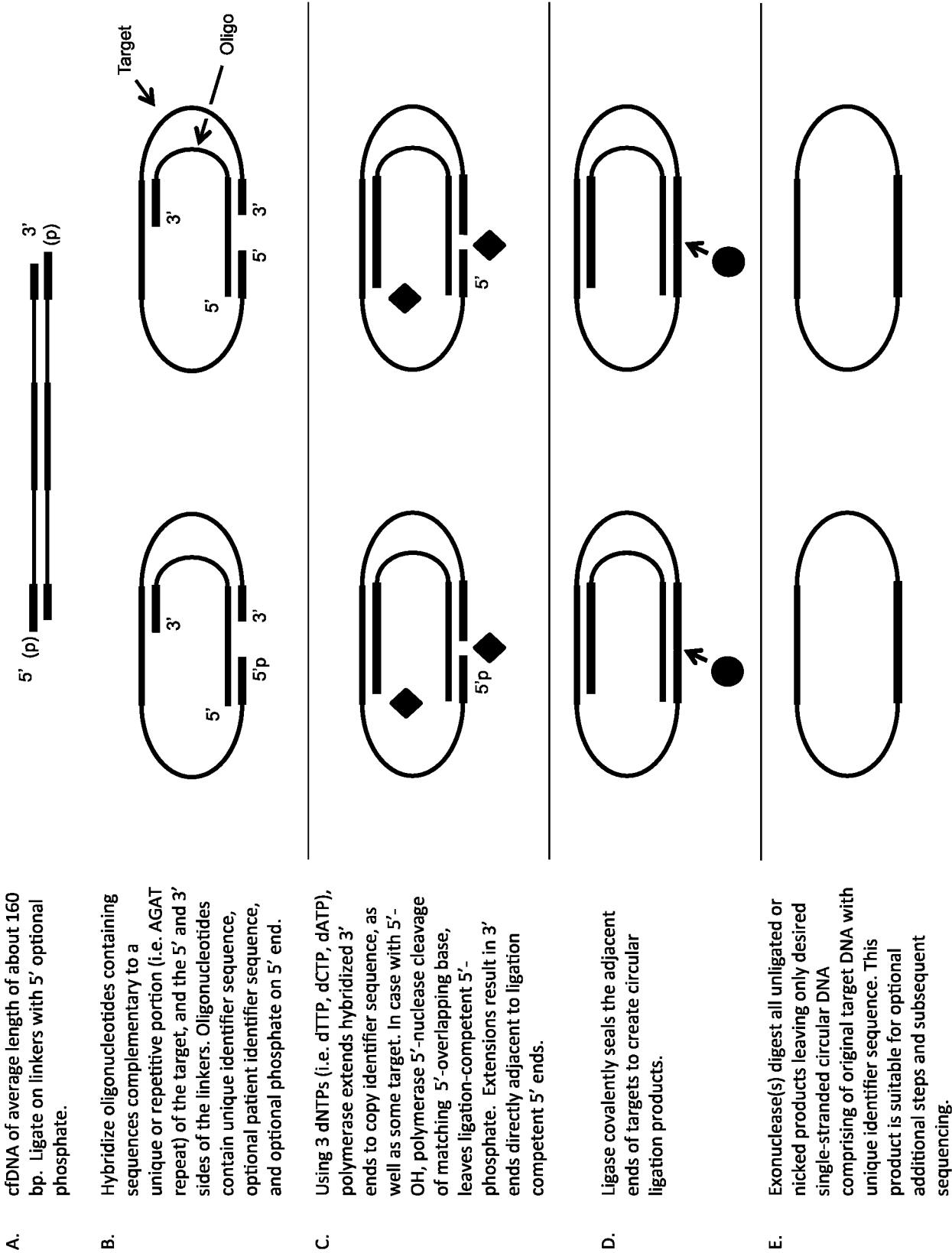


Figure 77

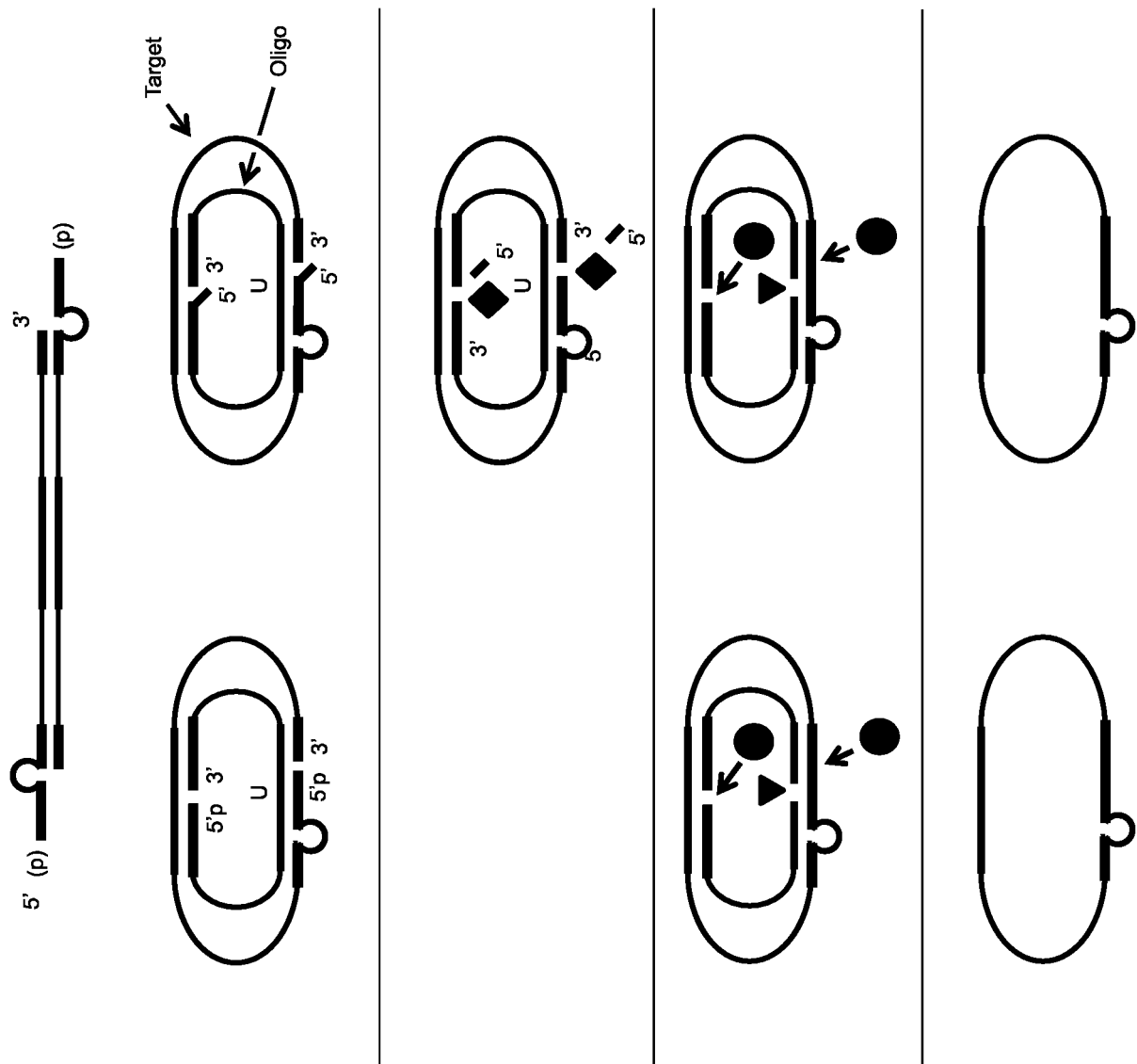


Figure 78

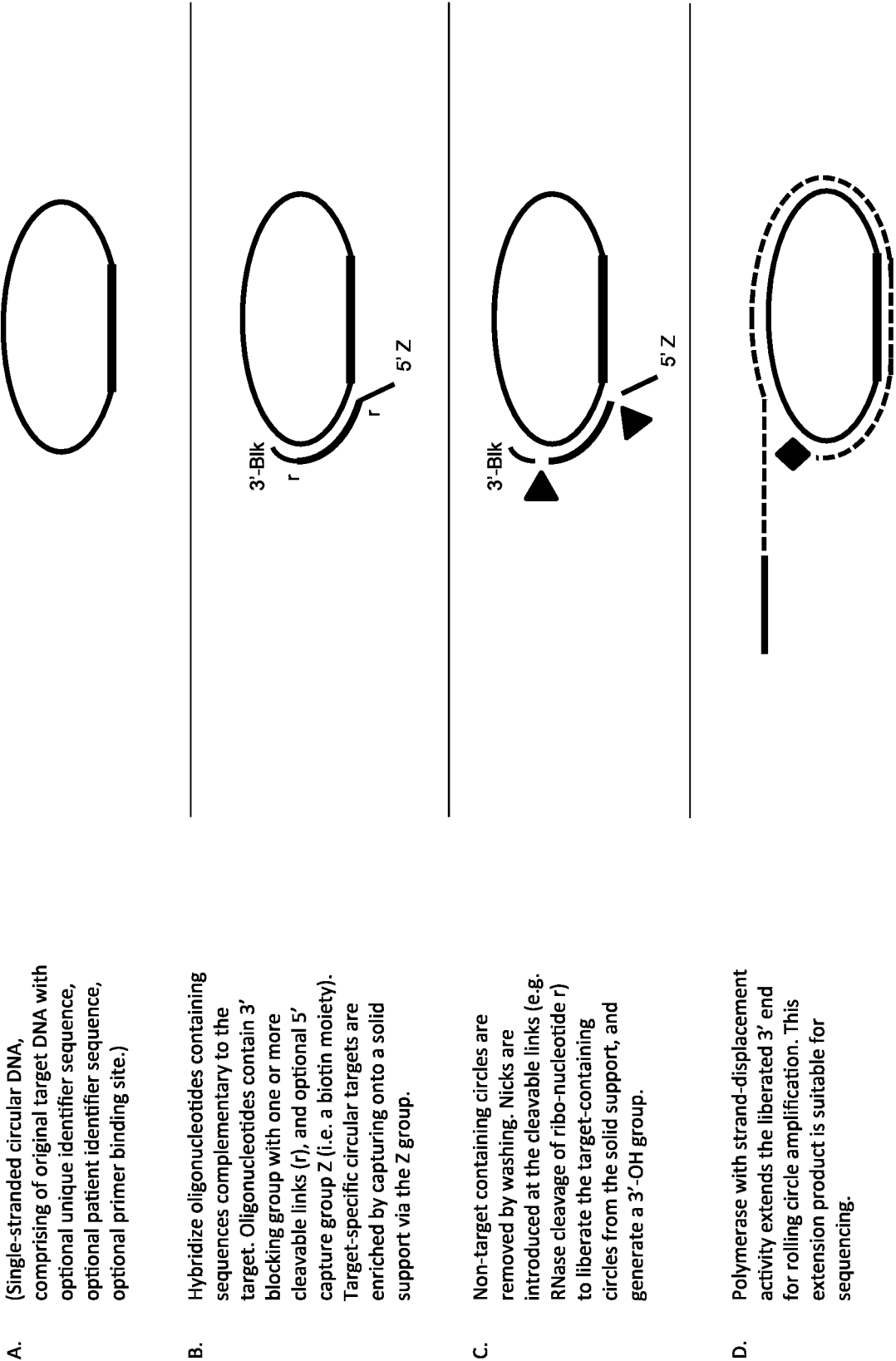
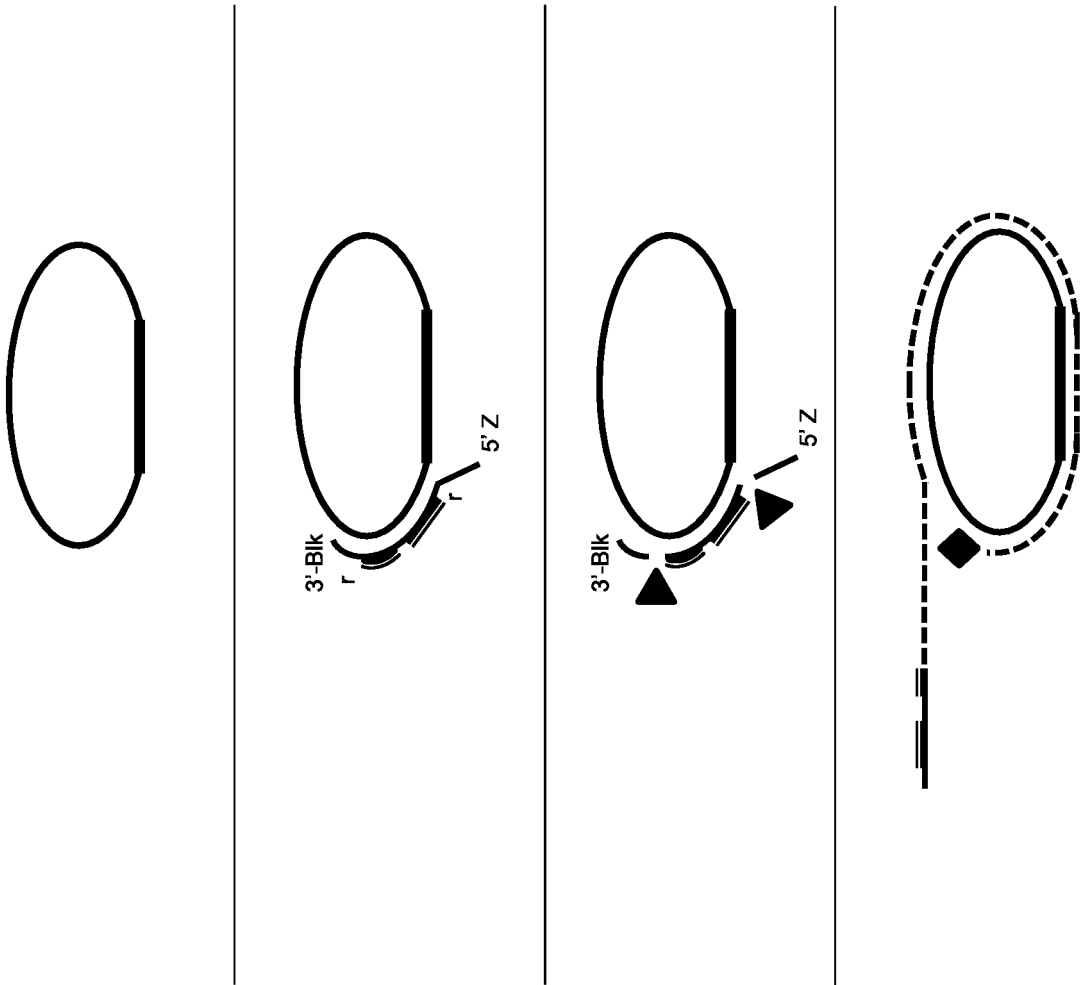


Figure 79



- 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

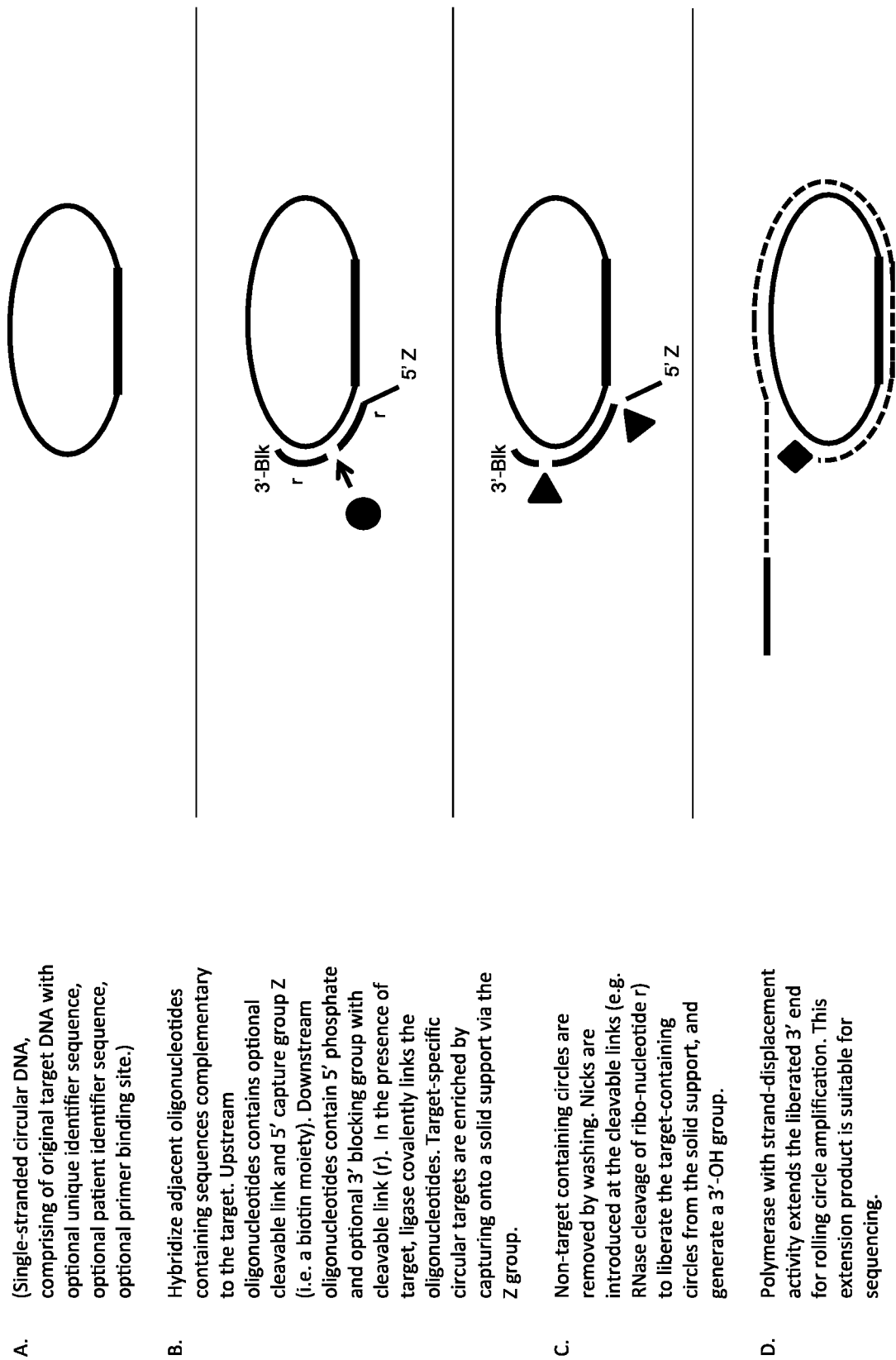


Figure 81

- 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.

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.

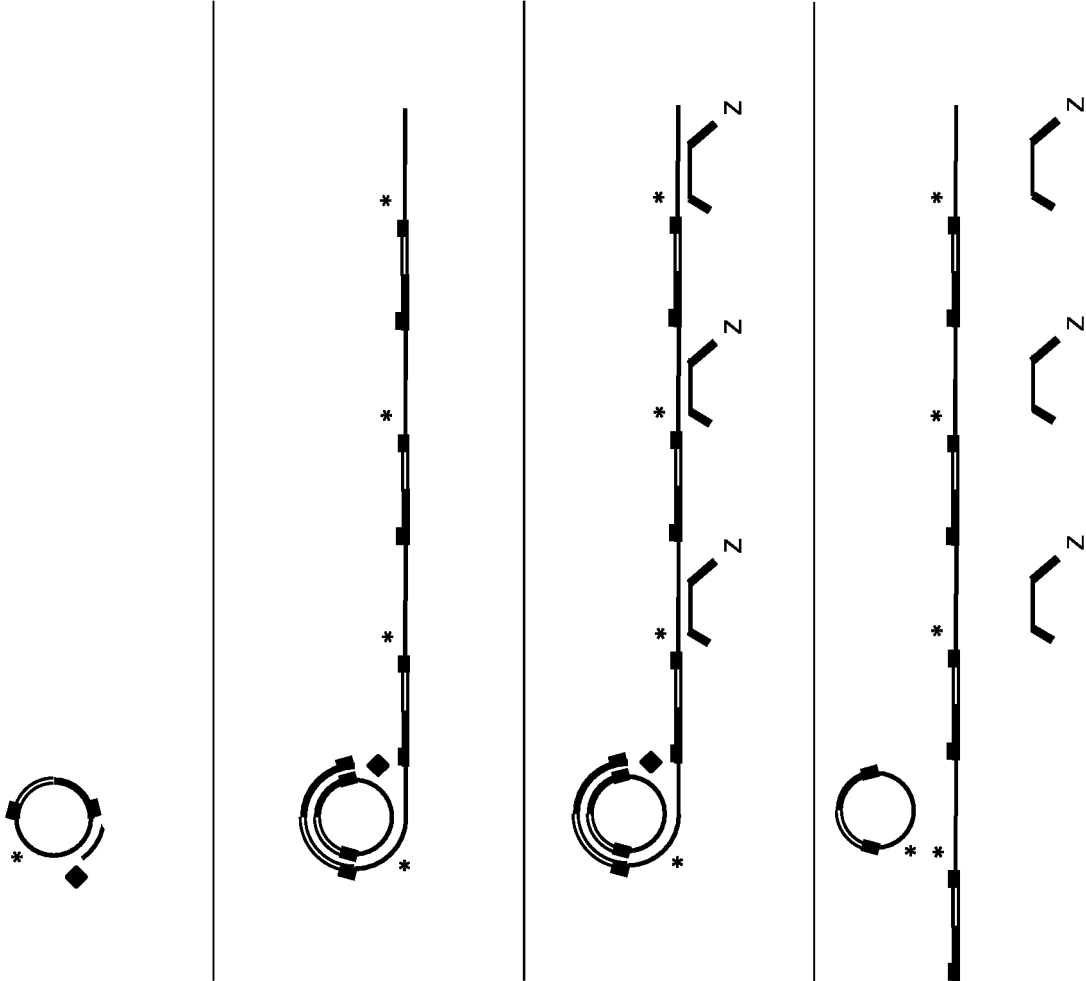


Figure 82

- A. Start with product comprising hybridized target-specific primer, or hybridize such primer to single-stranded circular 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.

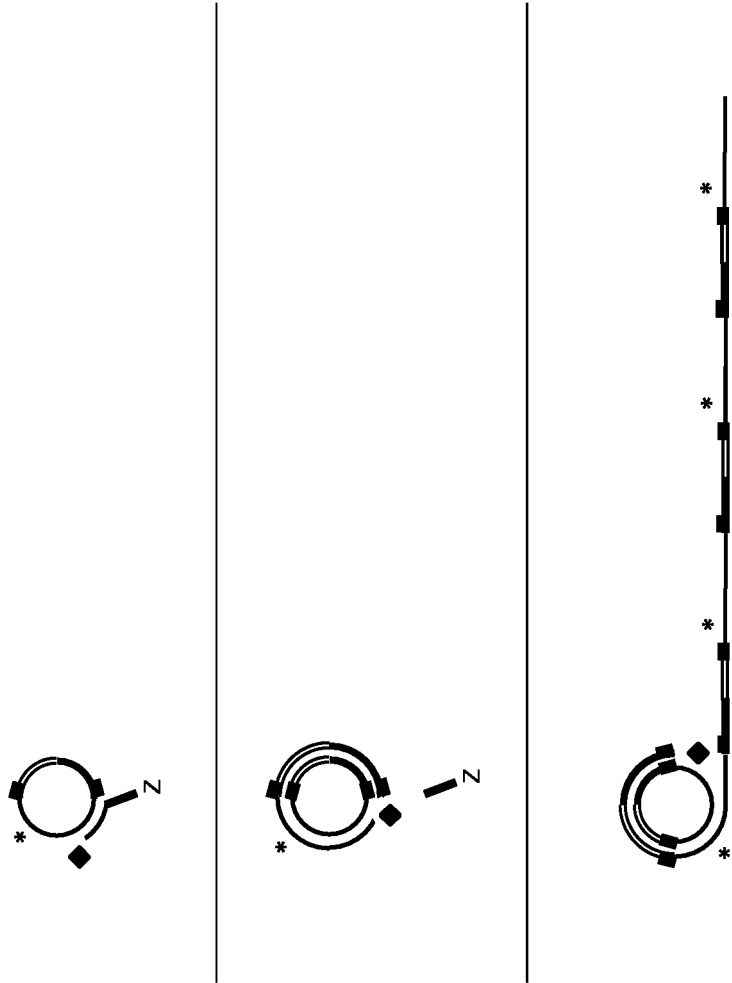


Figure 83

- 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.

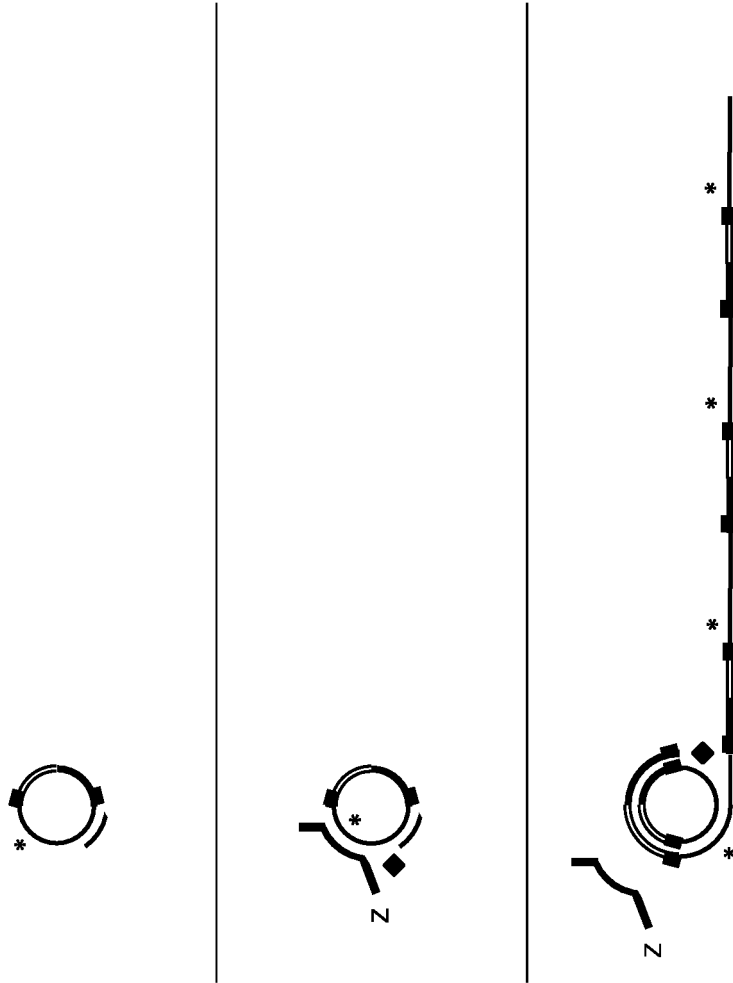


Figure 84

- 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.

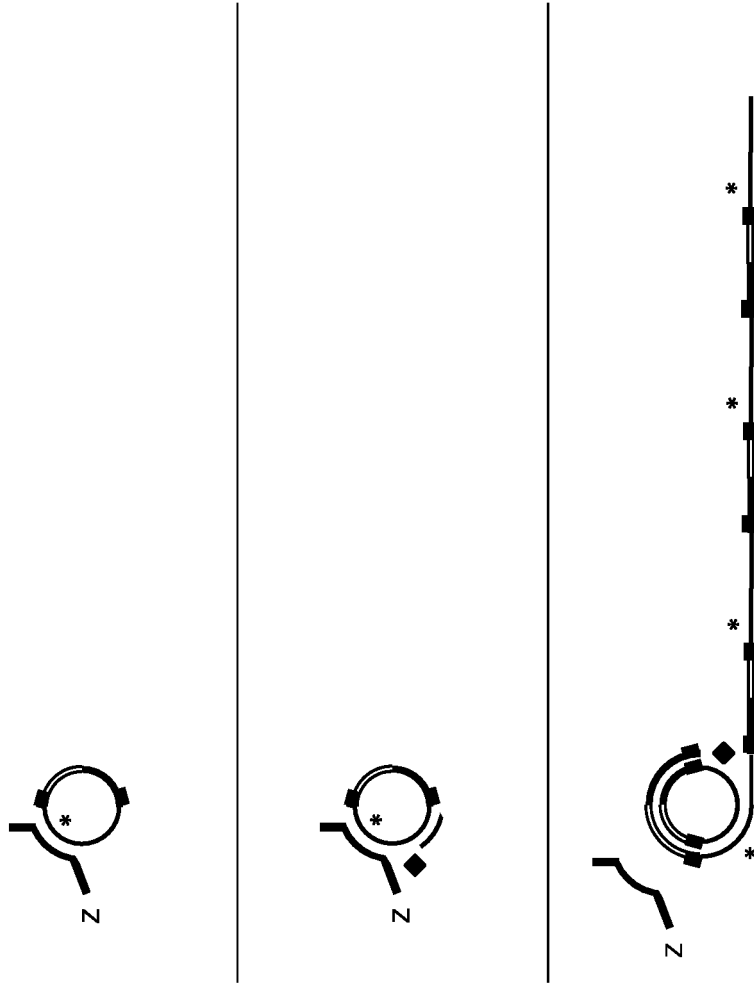


Figure 85

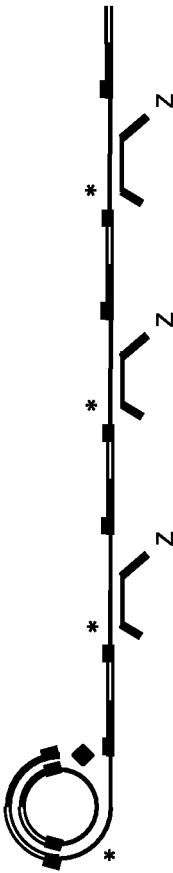
A. Start with product comprising primer hybridized to primer binding site, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, and primer binding site.



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.

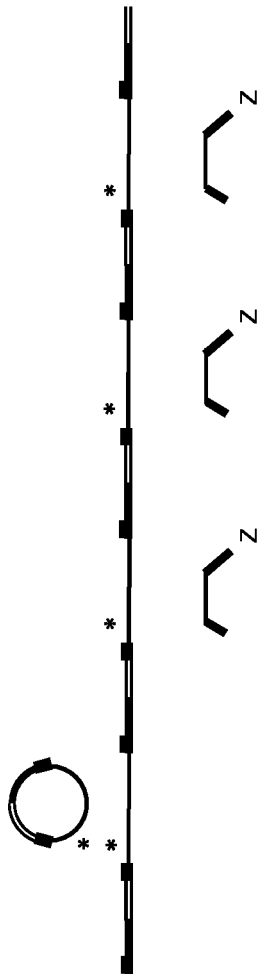


Figure 86

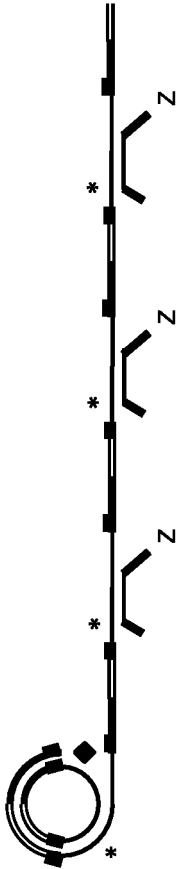
A. Start with product comprising primer hybridized to primer binding site, or hybridize such primer to single-stranded circular DNA, comprising of original target DNA with optional unique identifier sequence, optional patient identifier sequence, and primer binding site.



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 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.

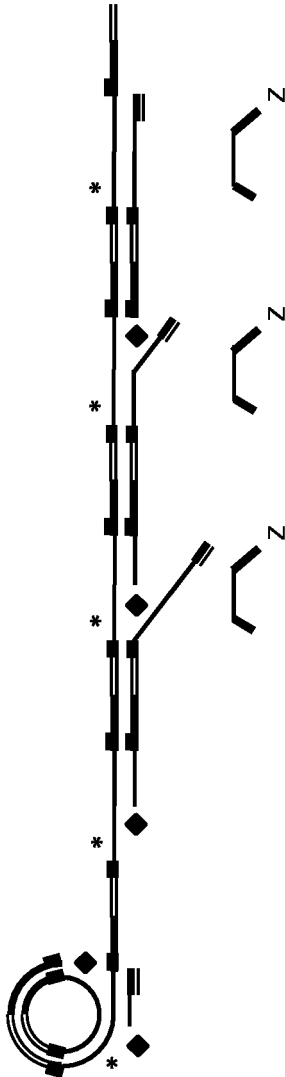


Figure 87

- 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.

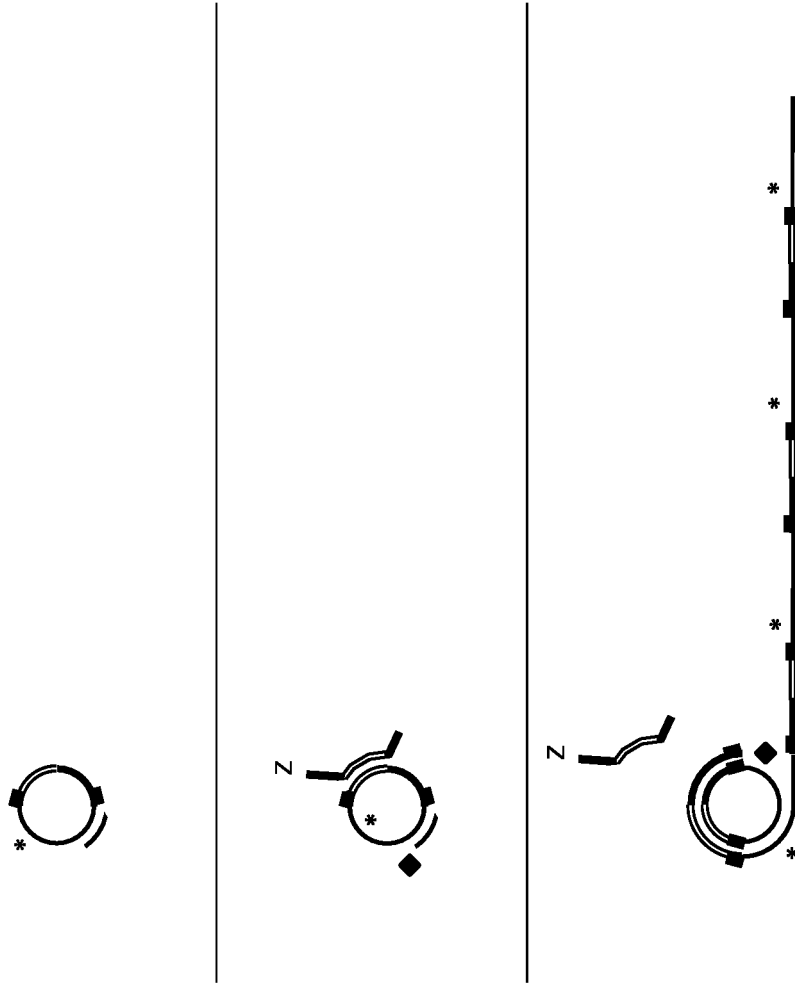


Figure 88

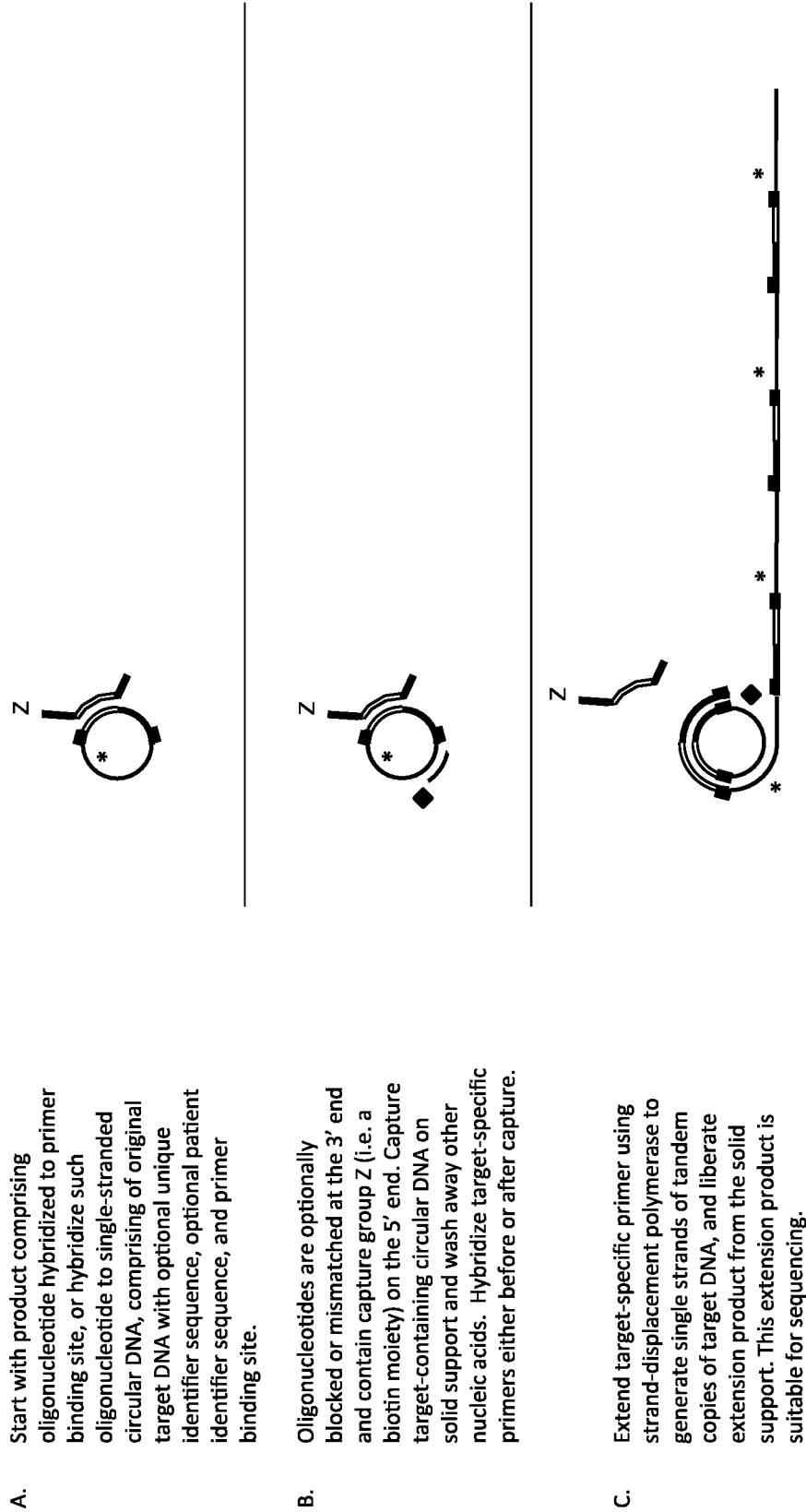


Figure 89

Figure 90

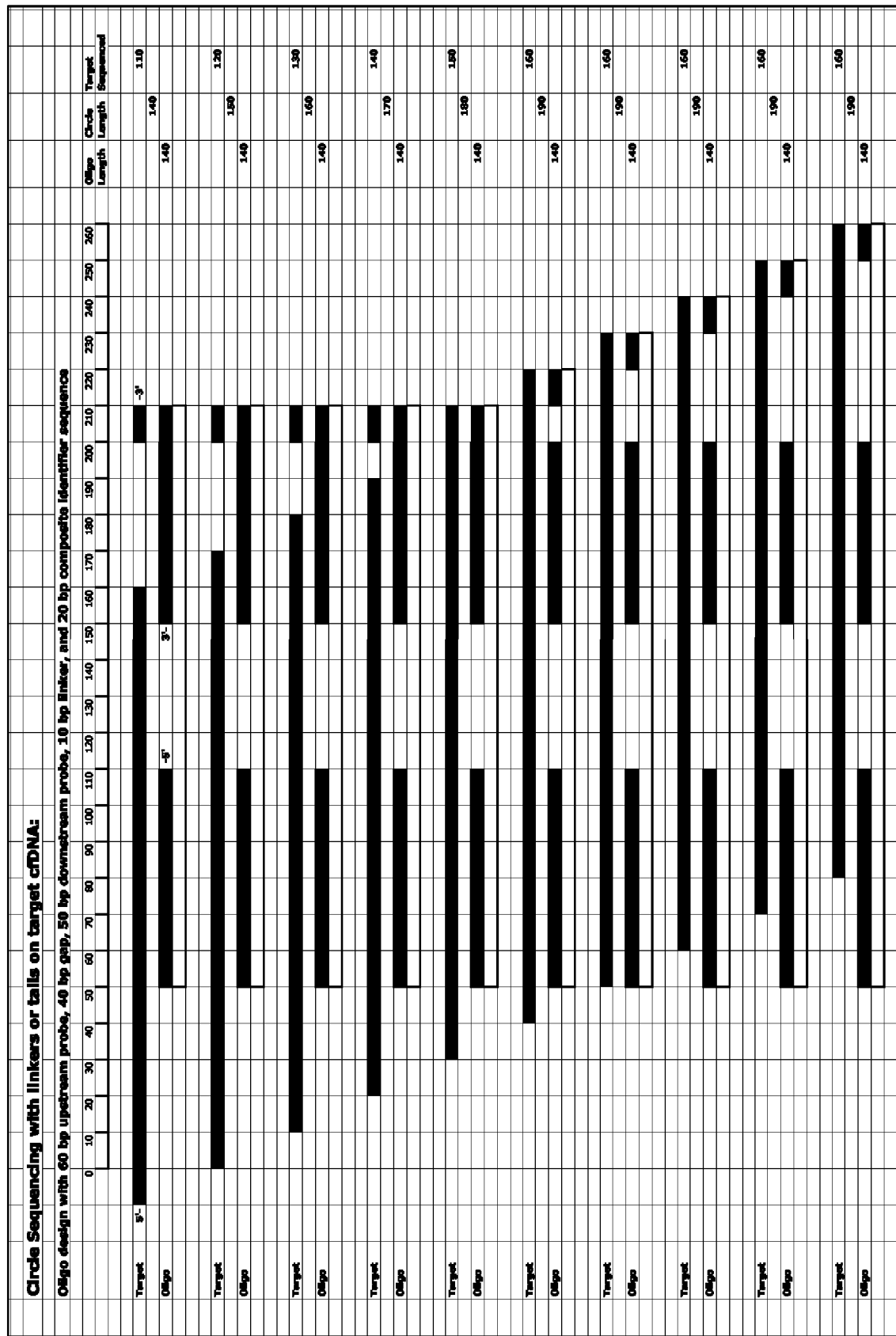


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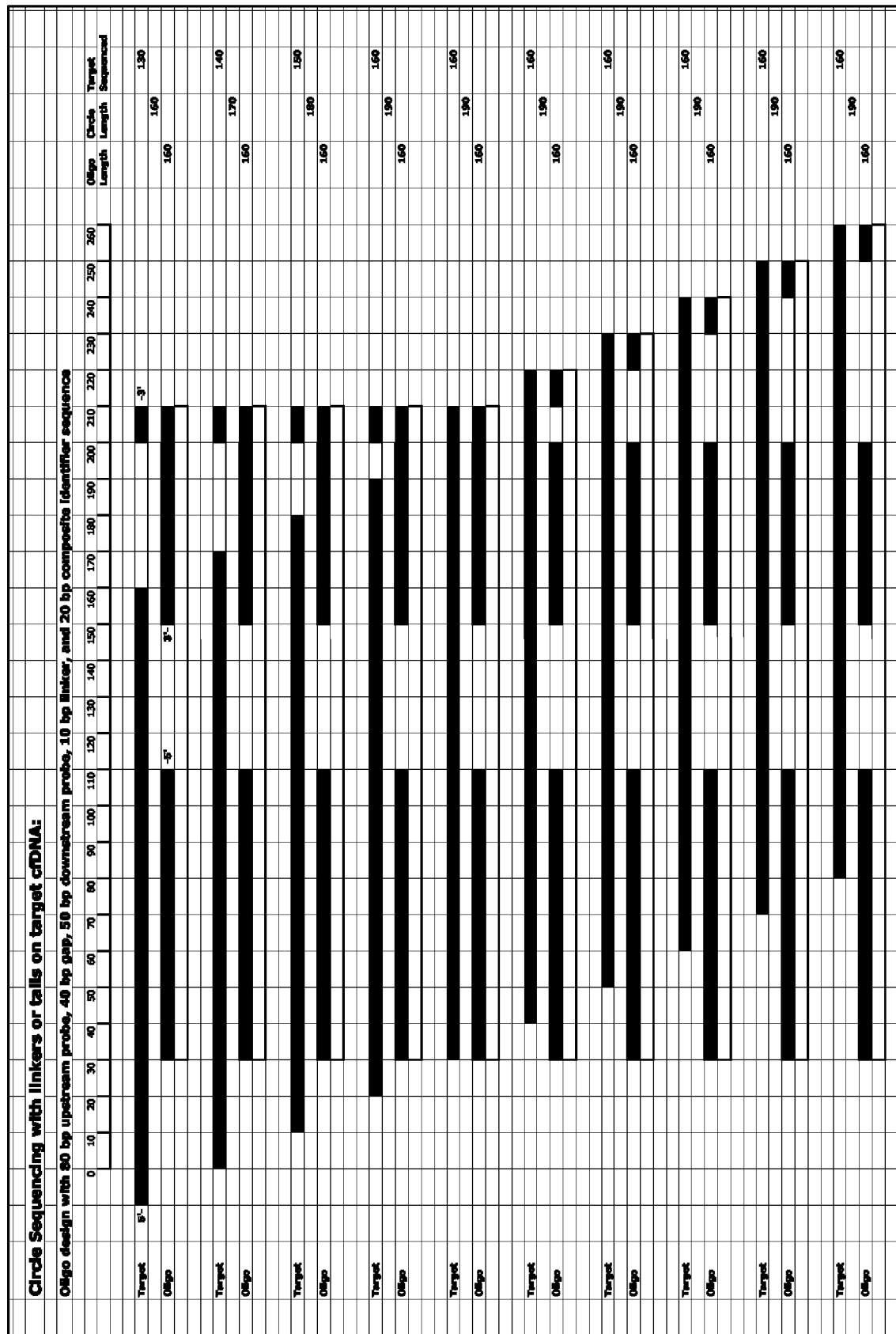


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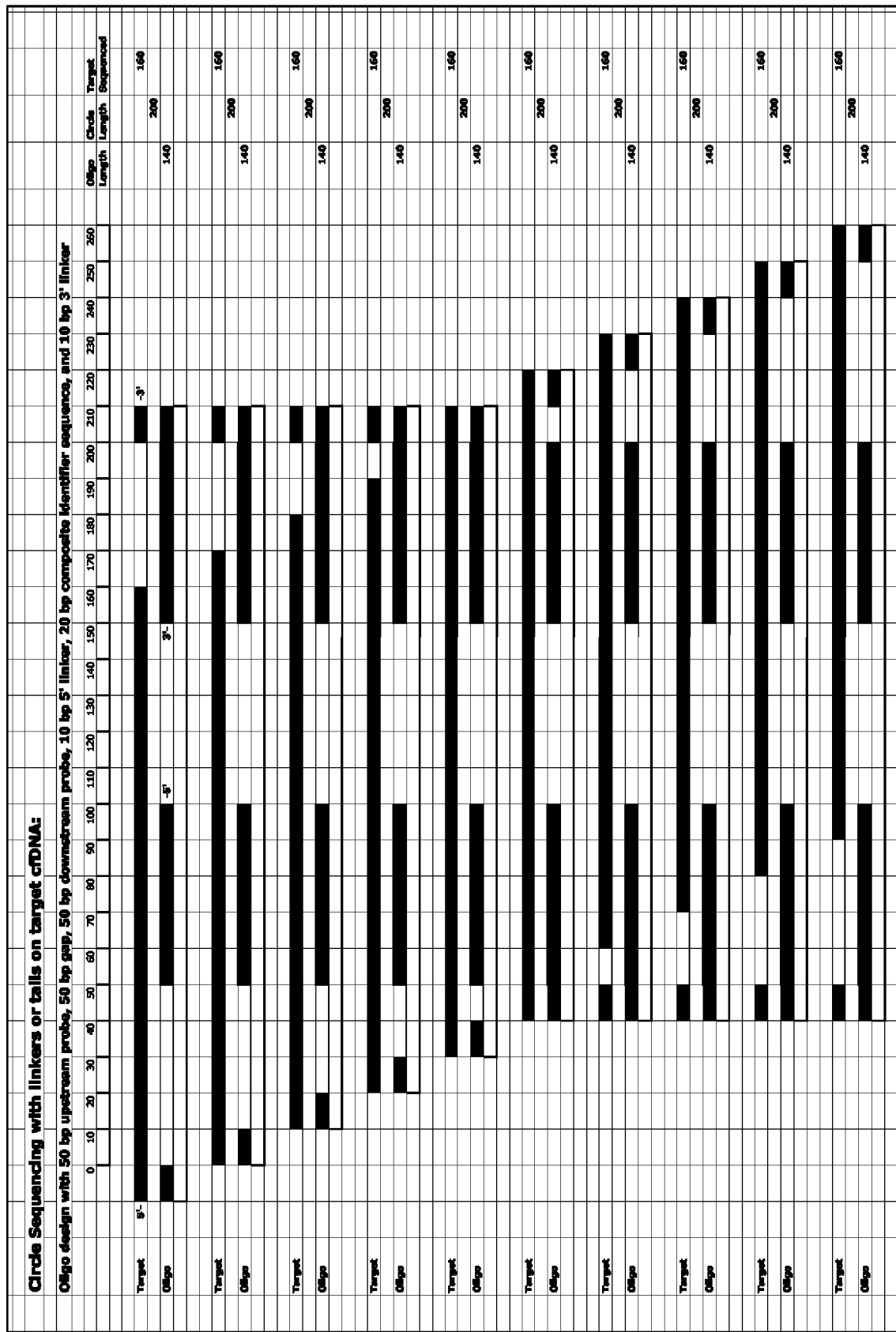


Figure 94

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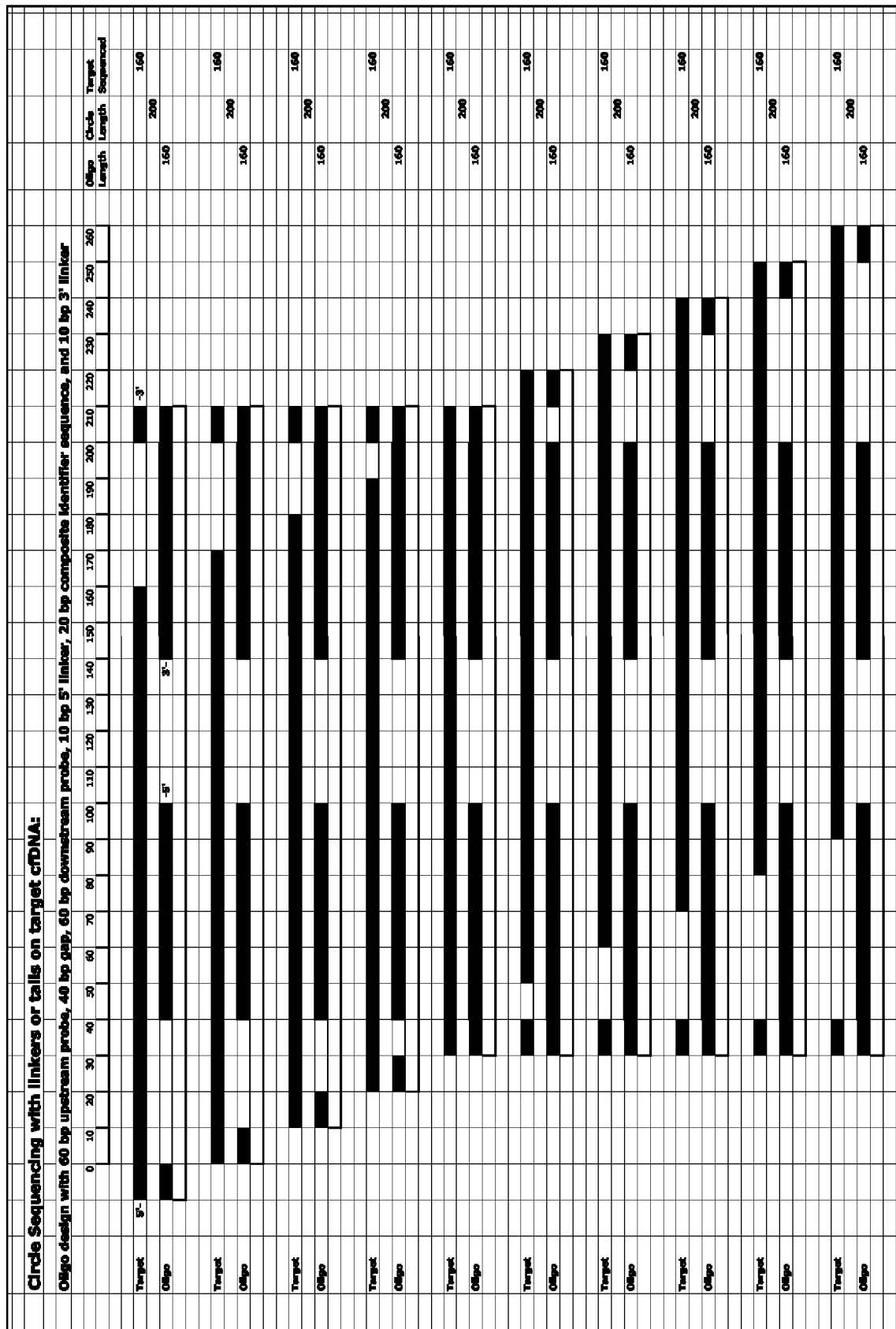
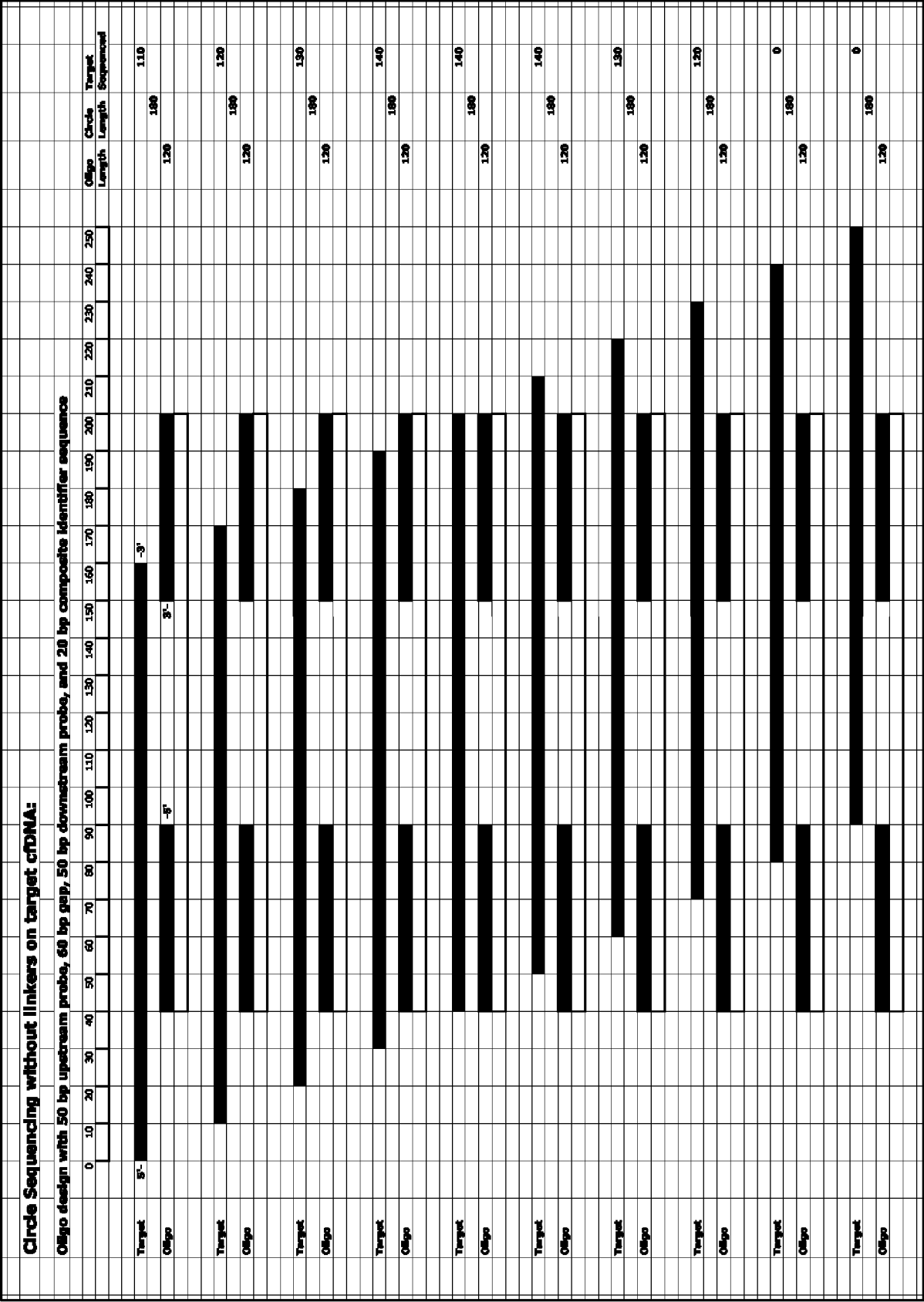


Figure 95



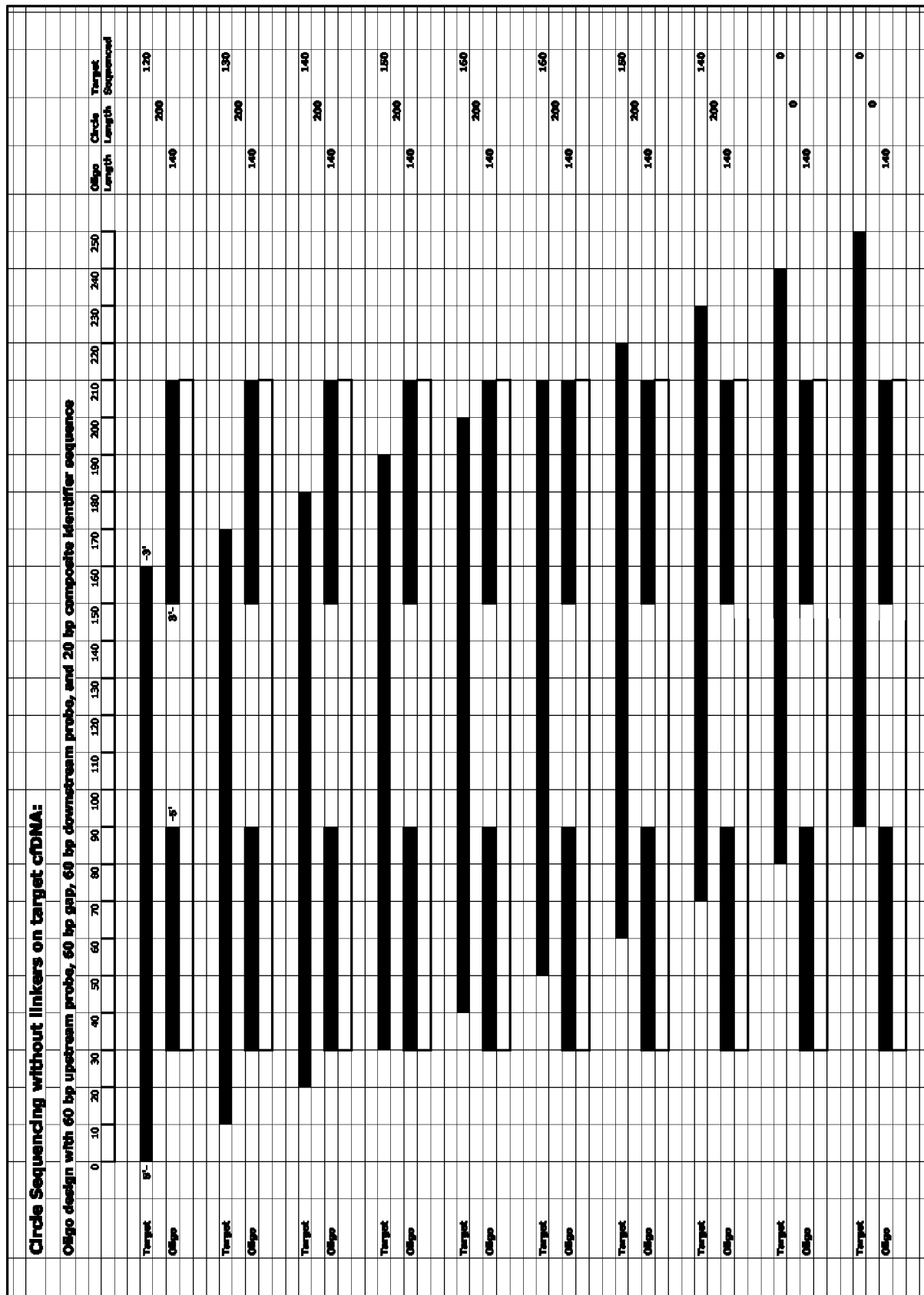
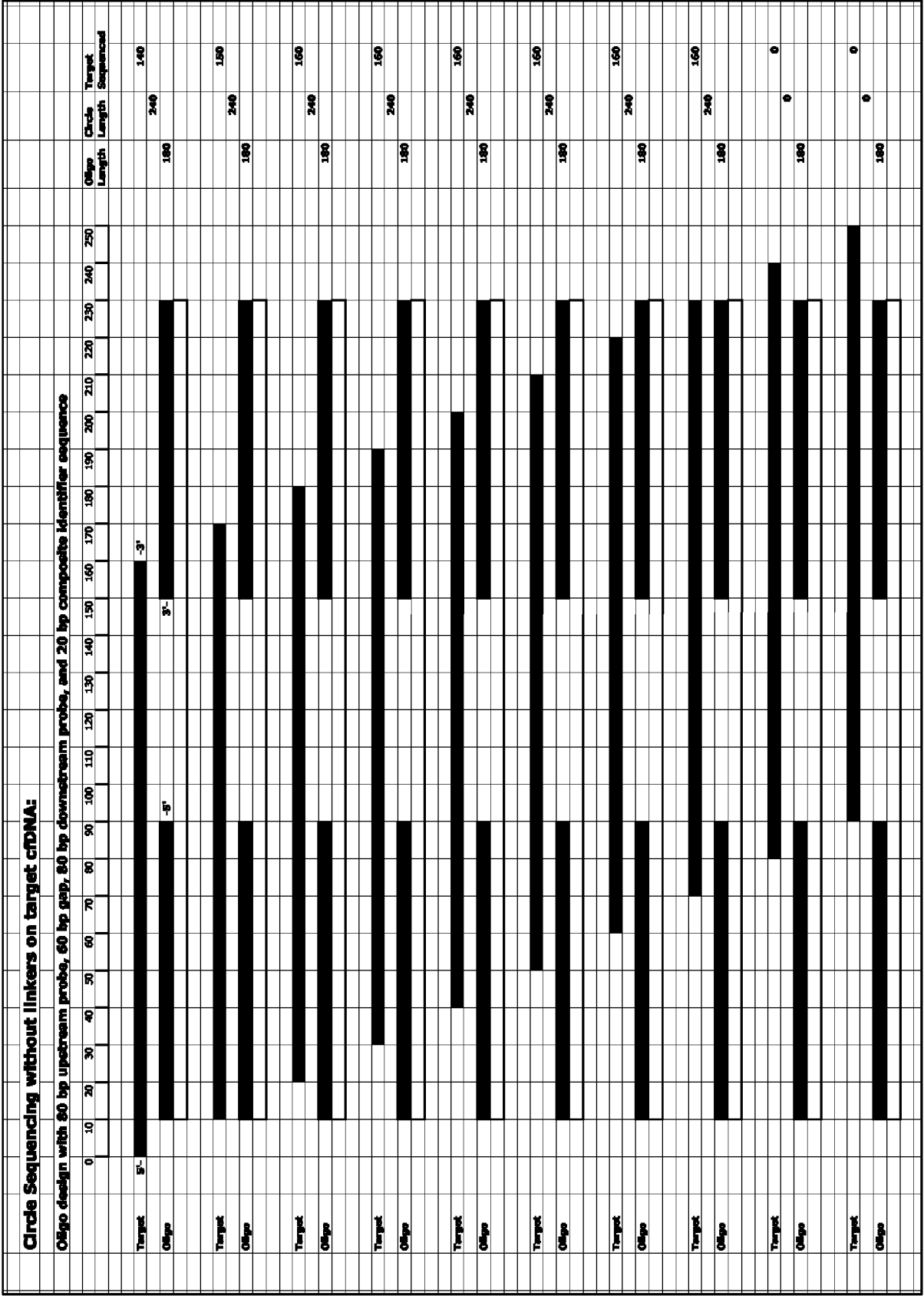


Figure 97



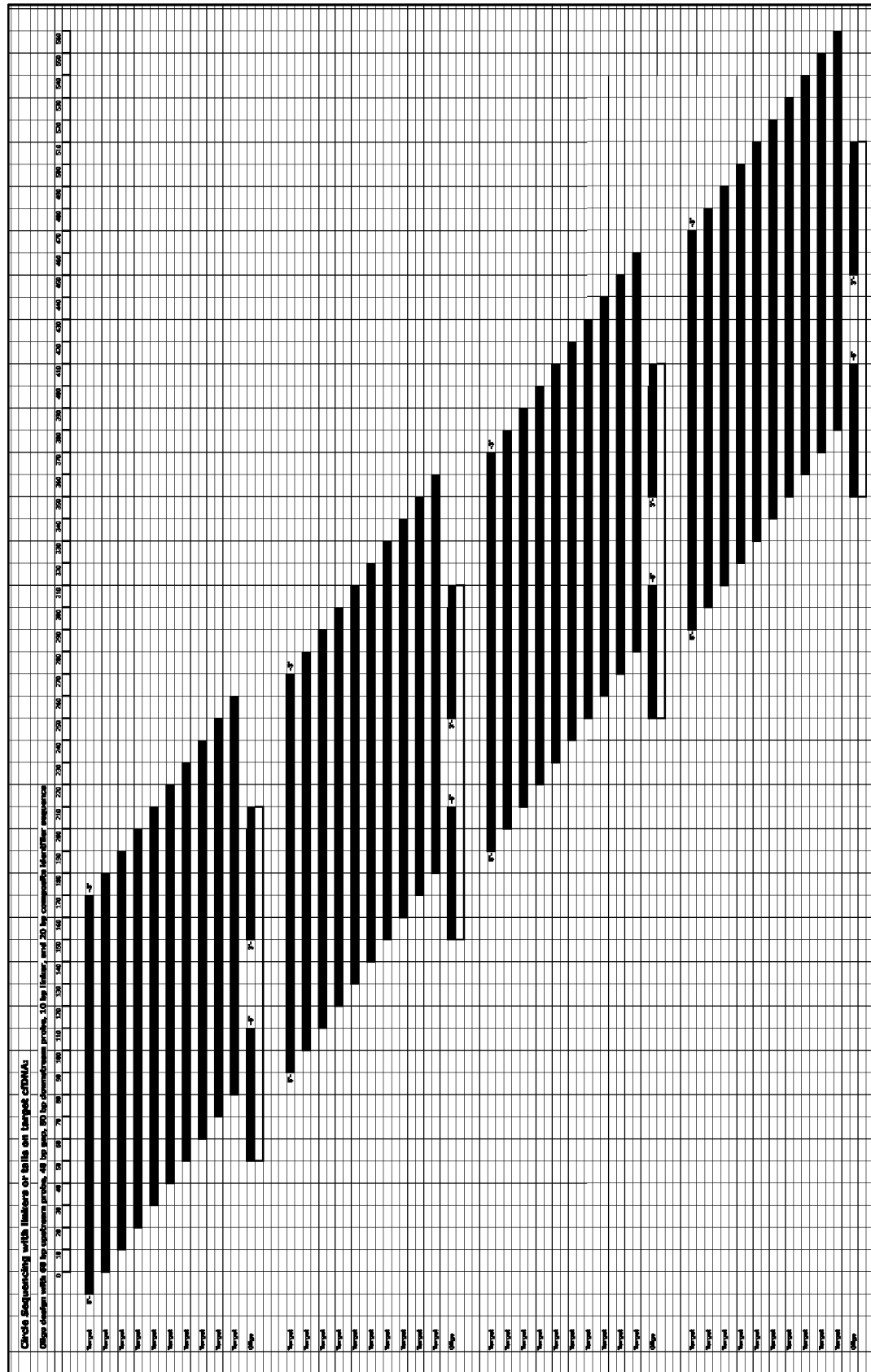


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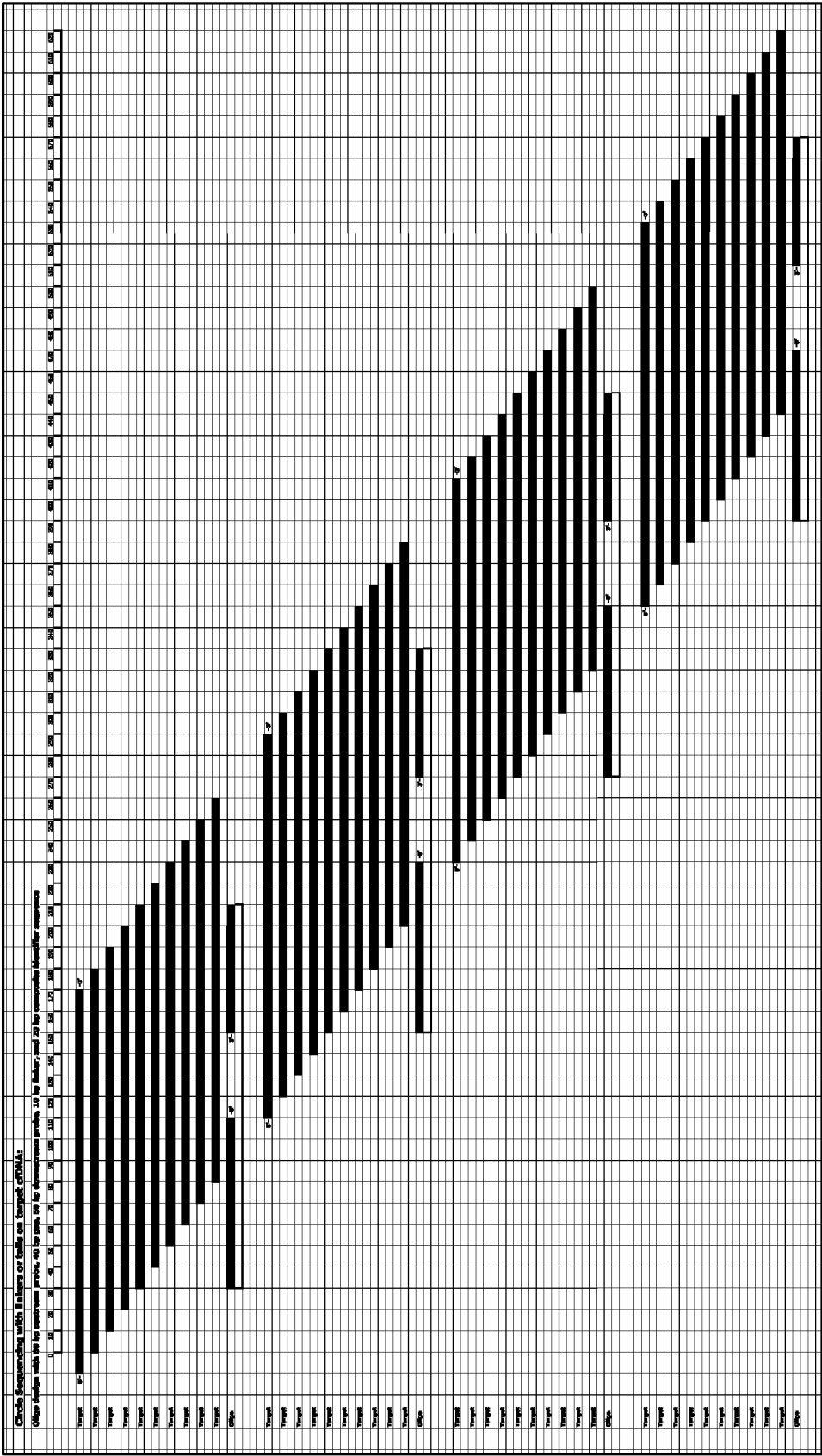


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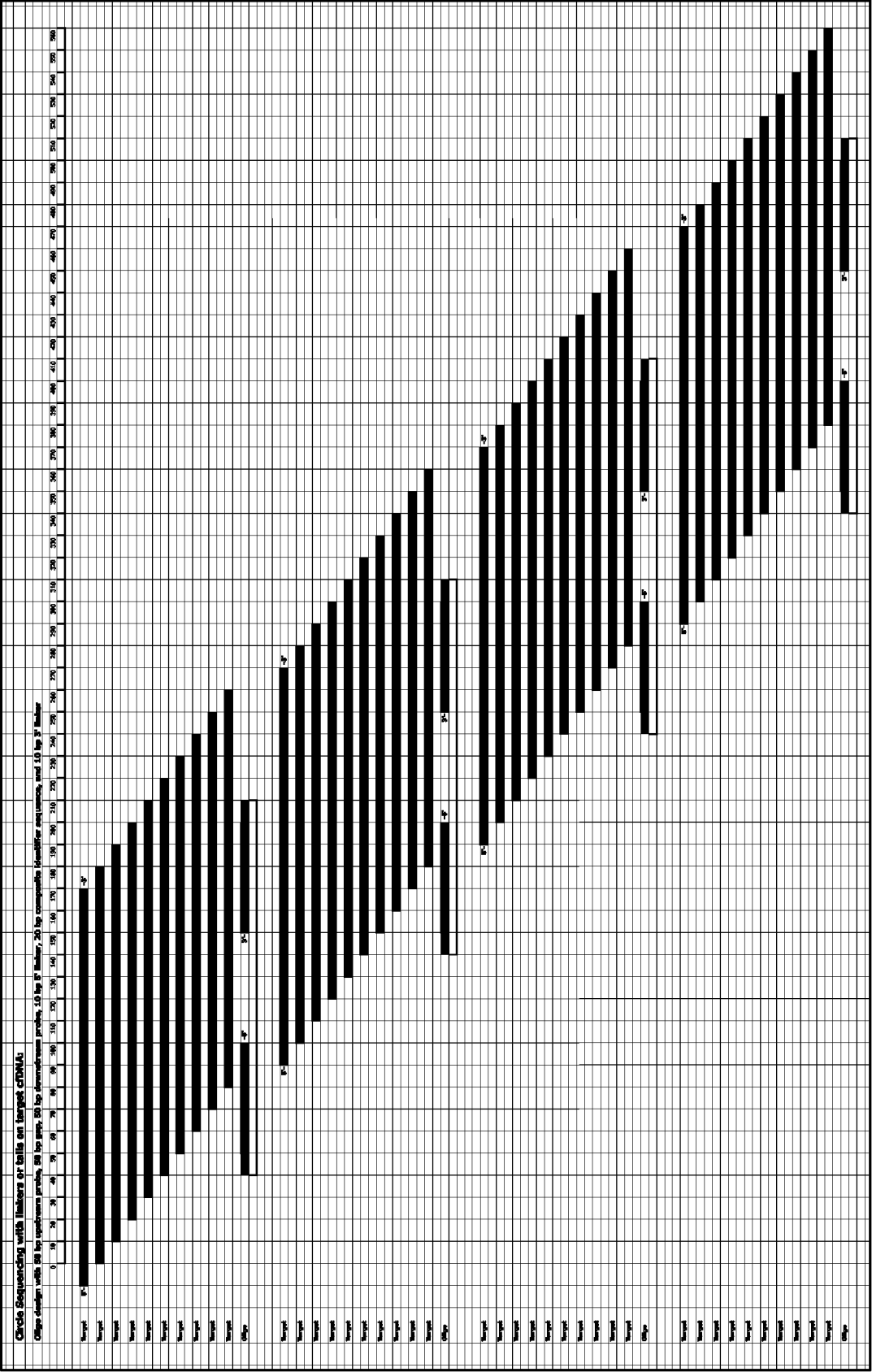


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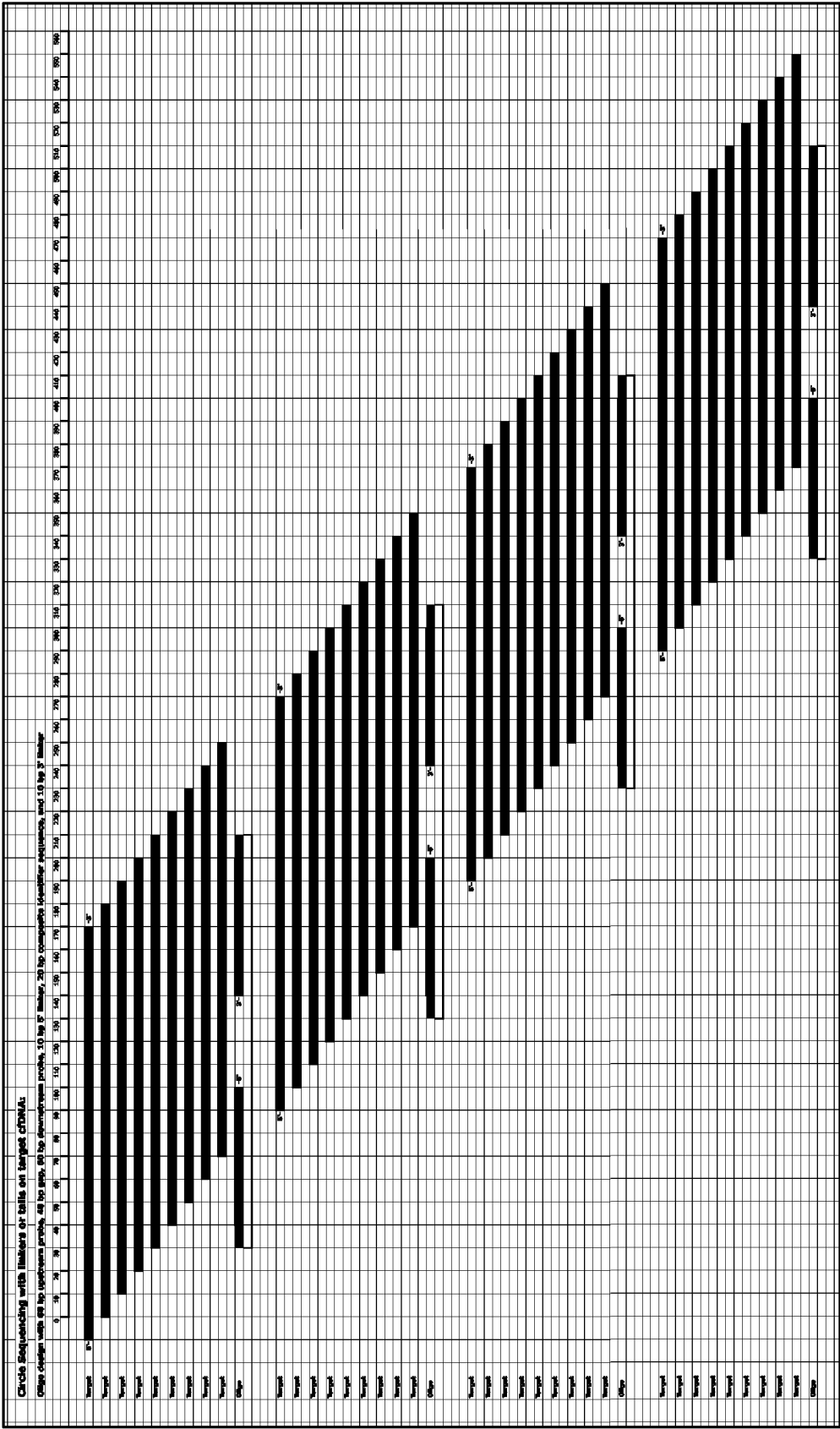


Figure 103

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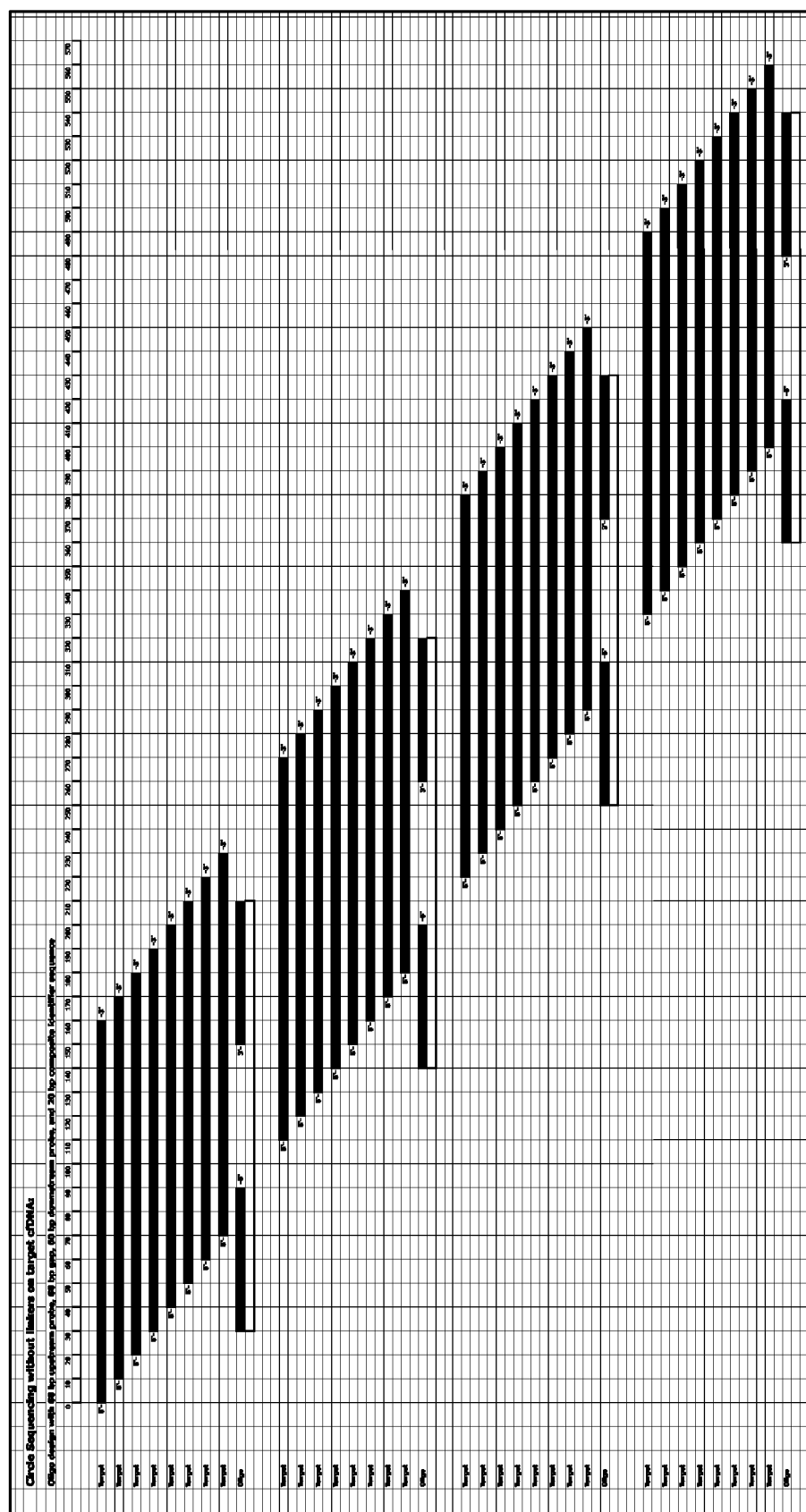


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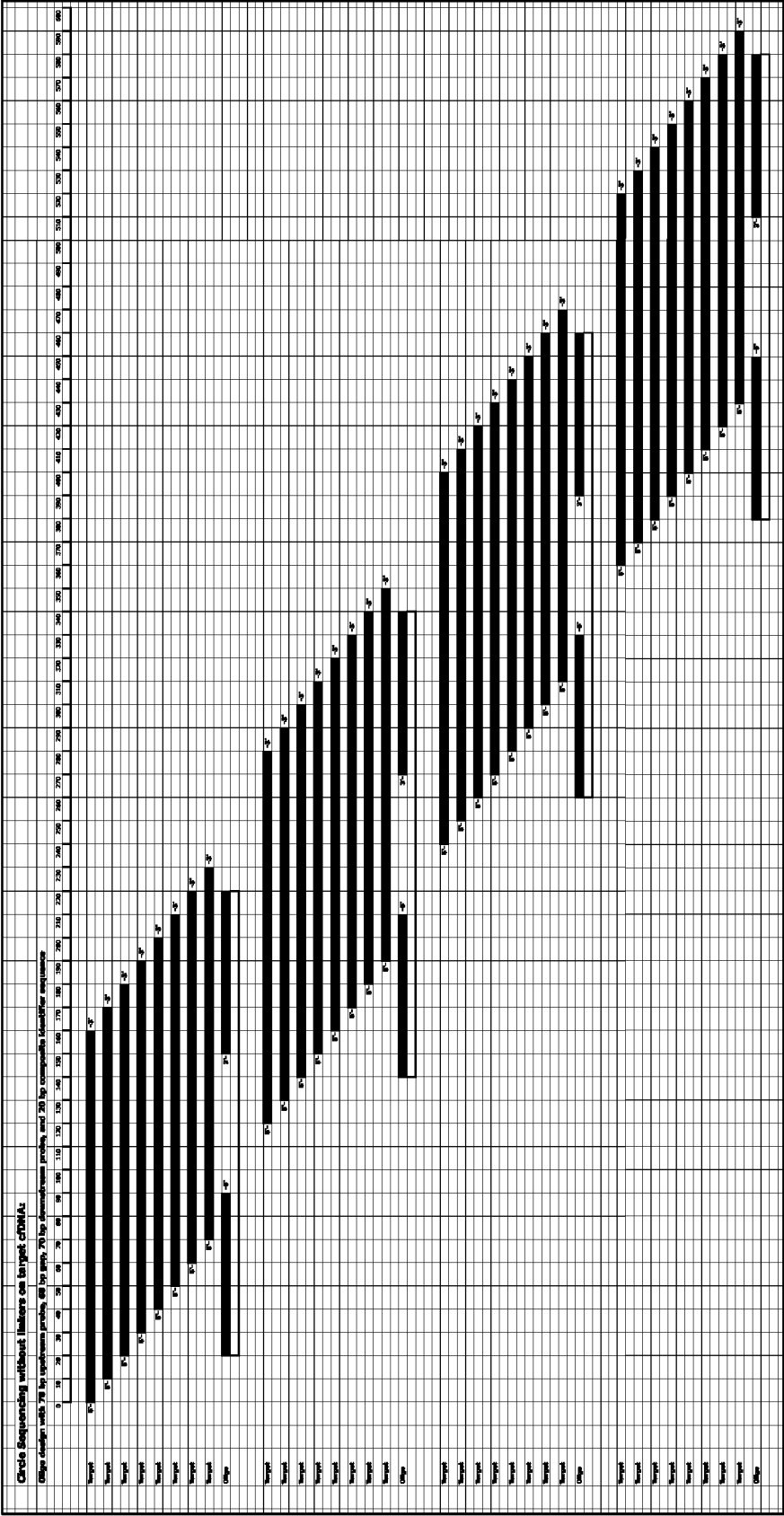


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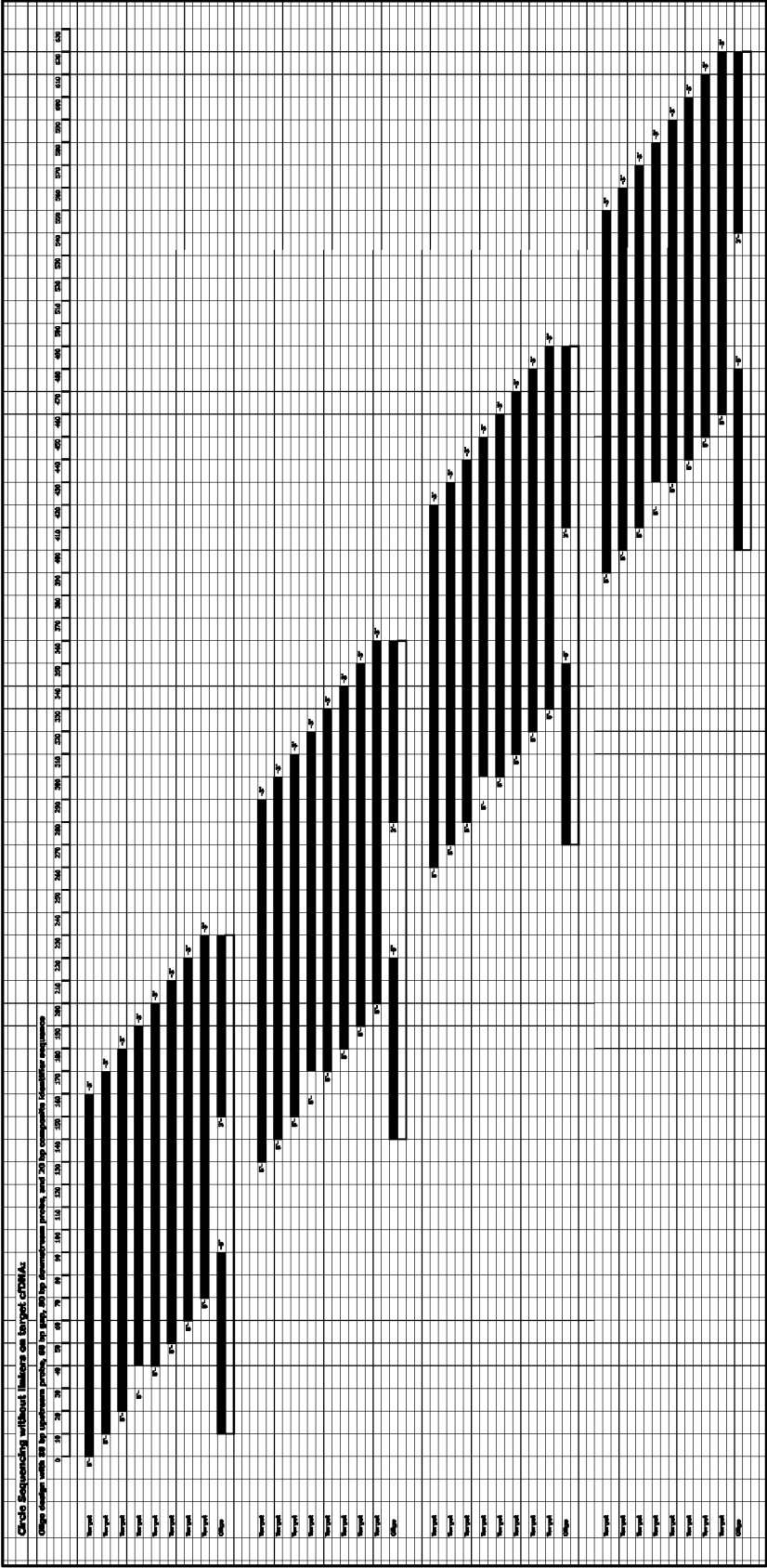


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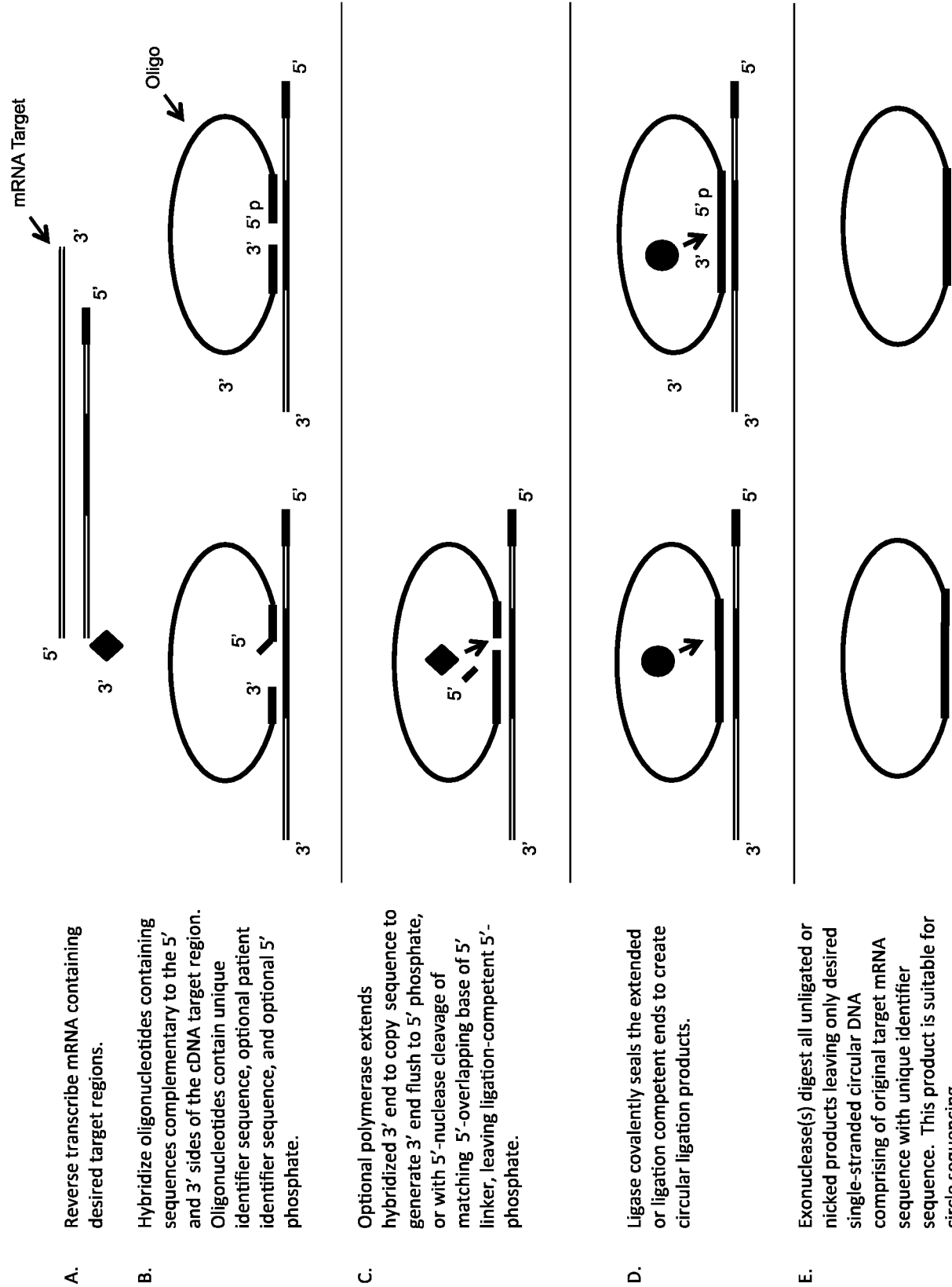


Figure 108

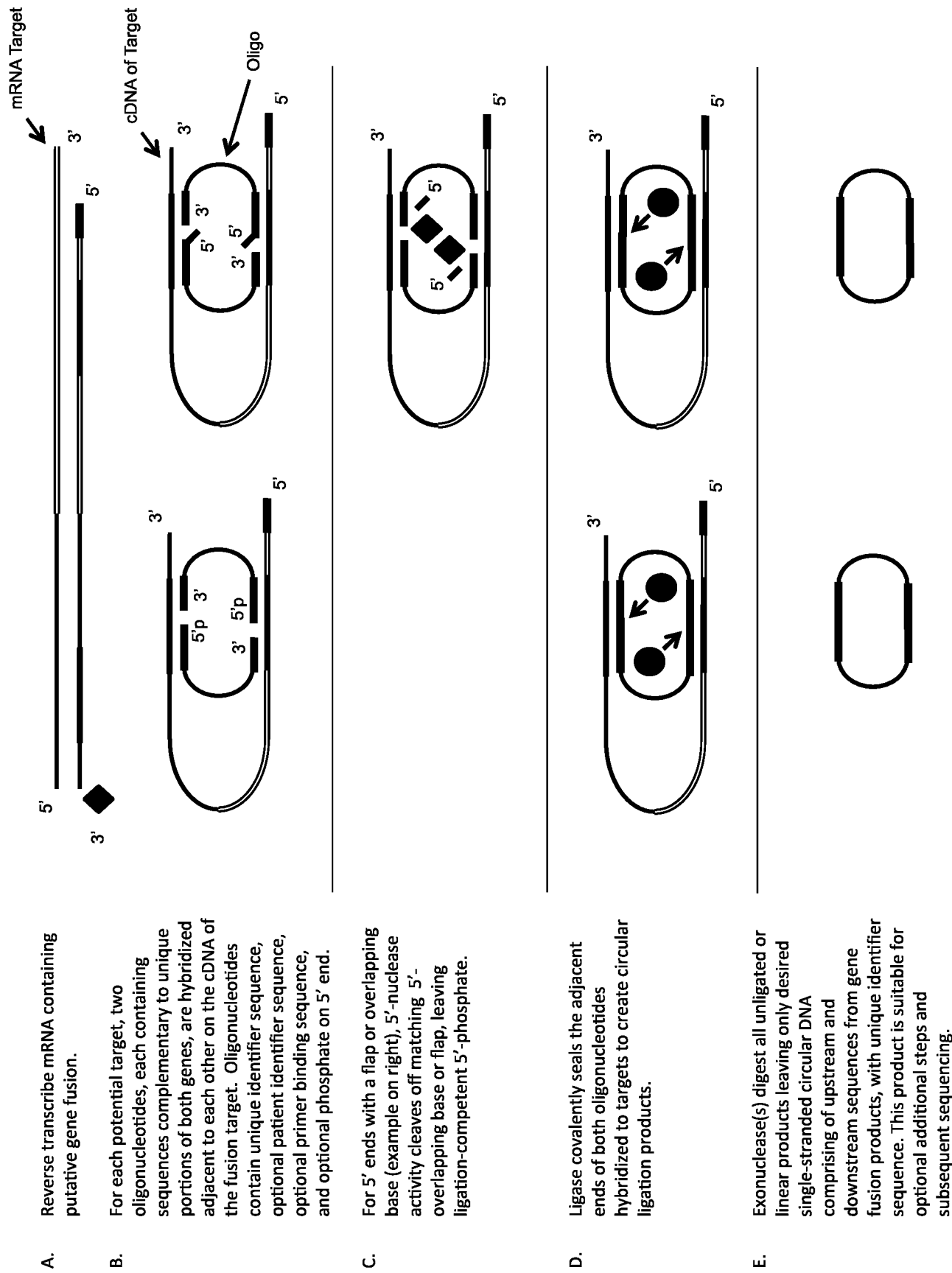


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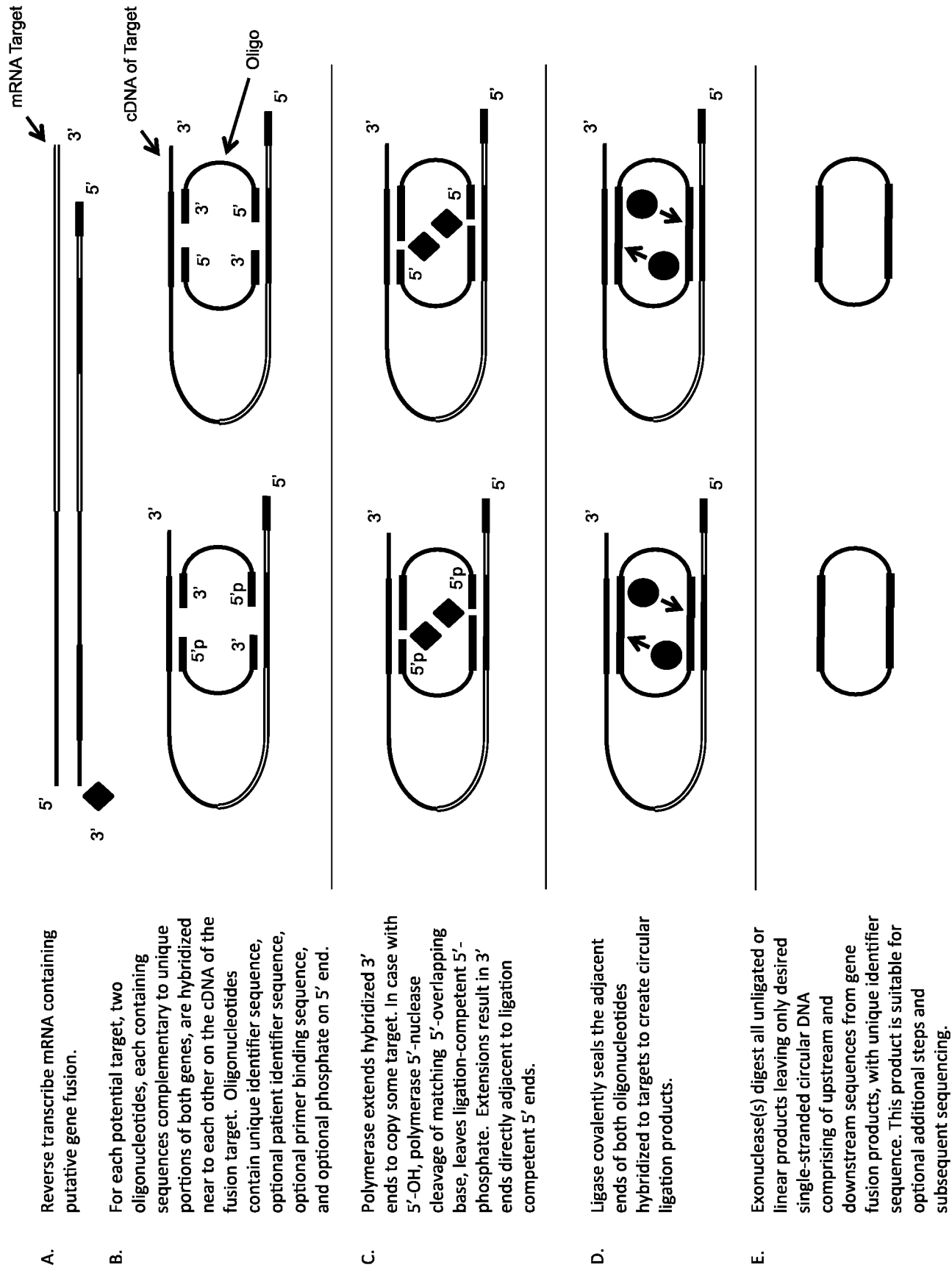


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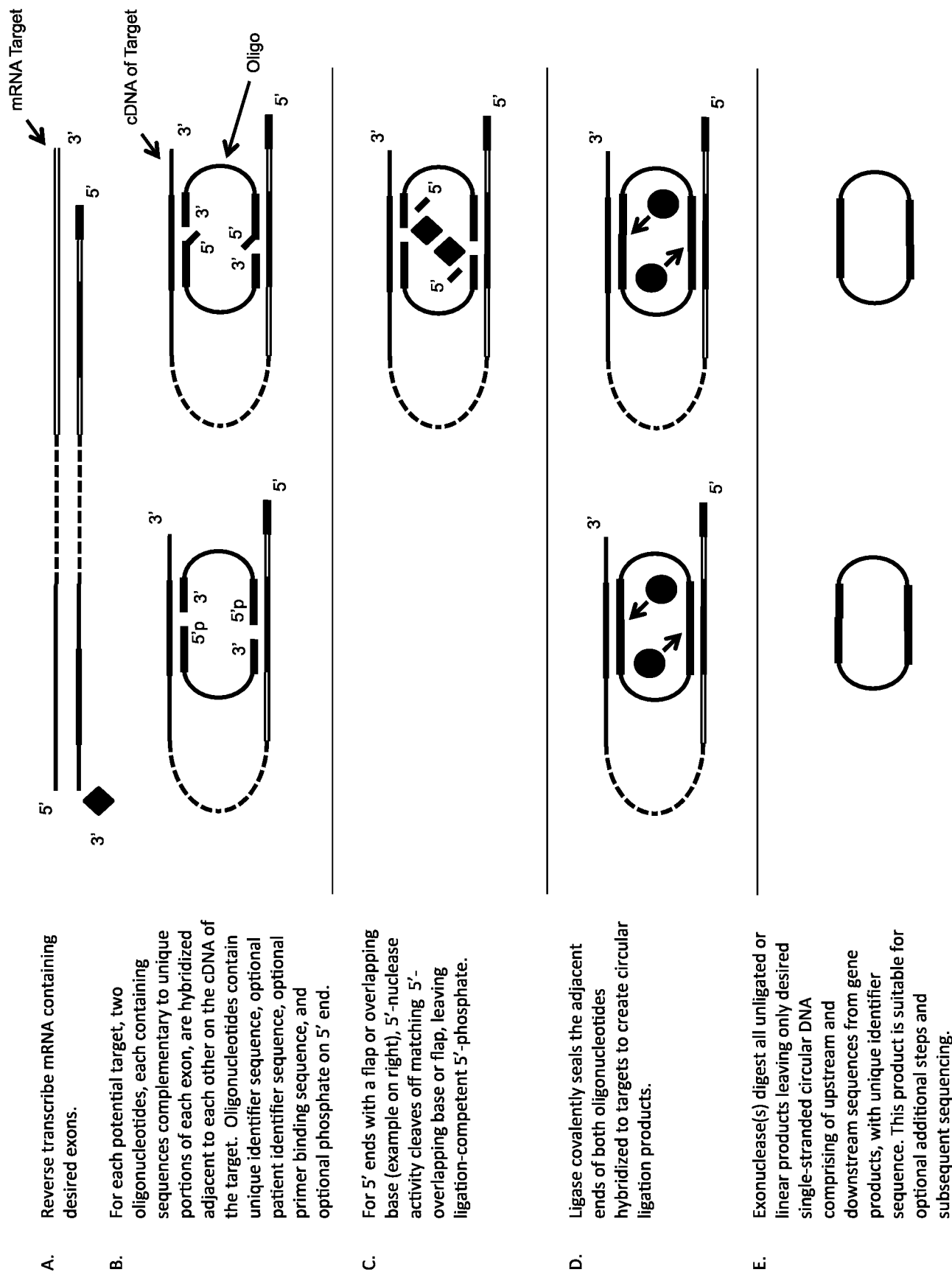


Figure 111

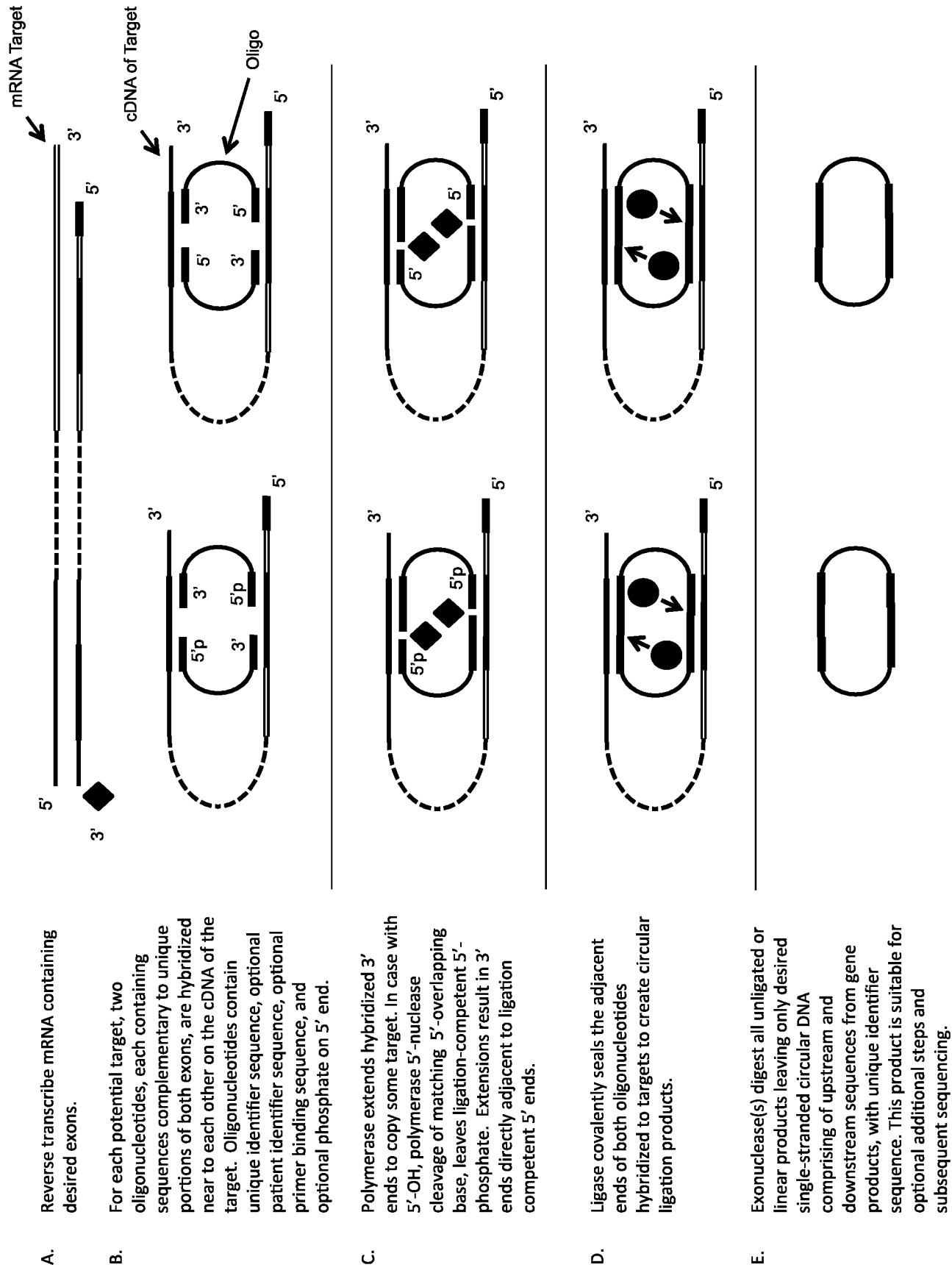


Figure 112

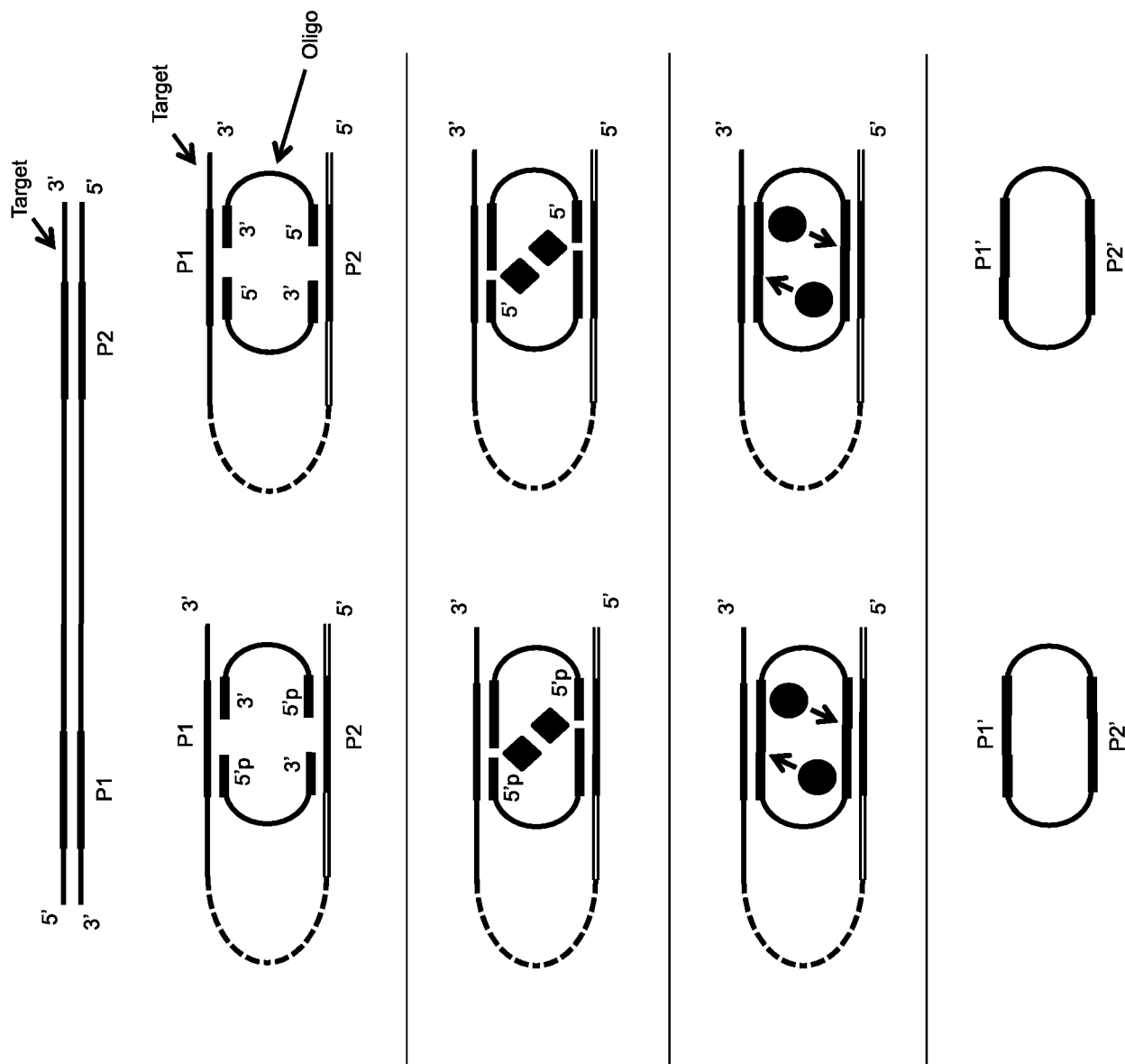
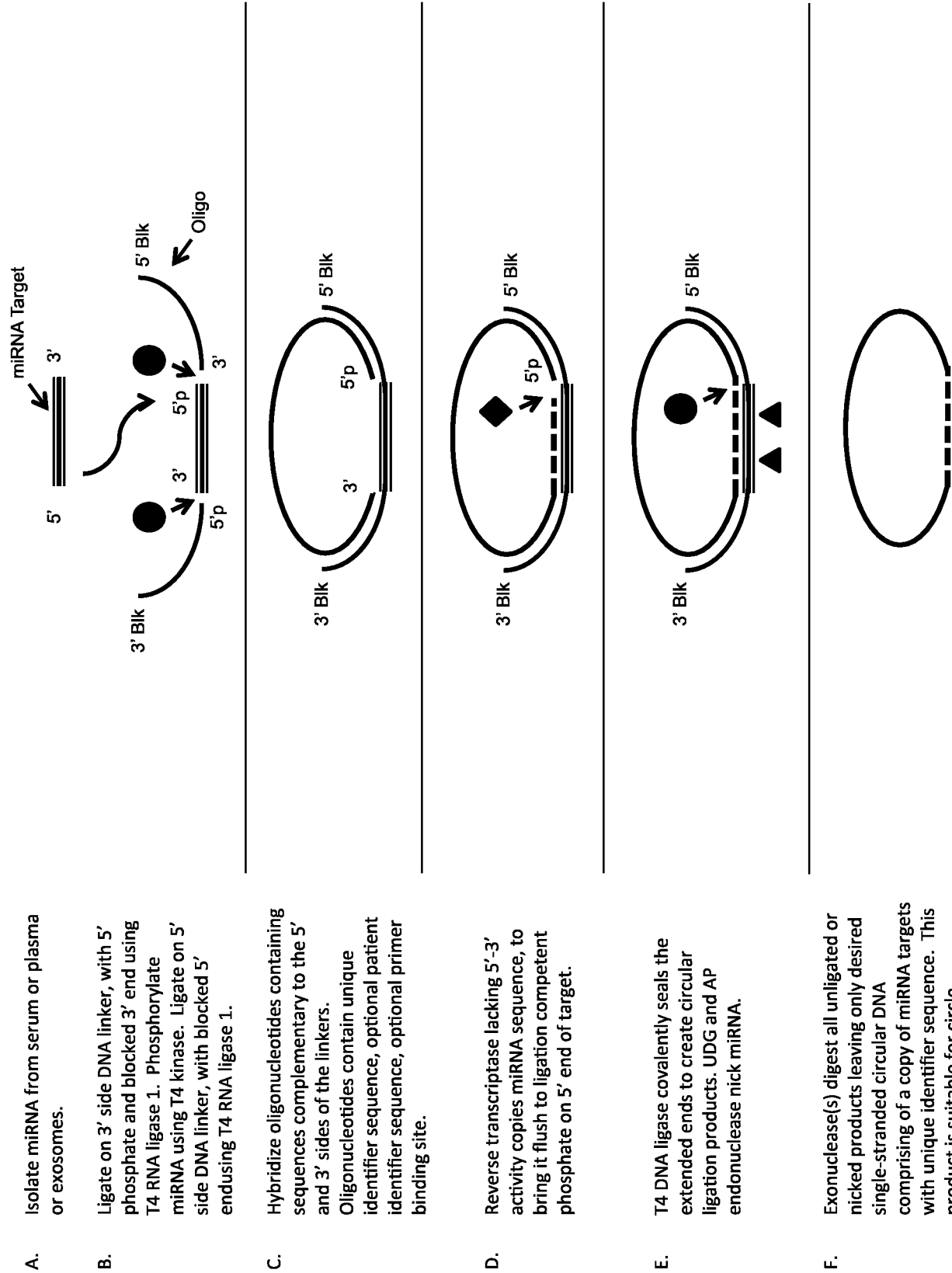
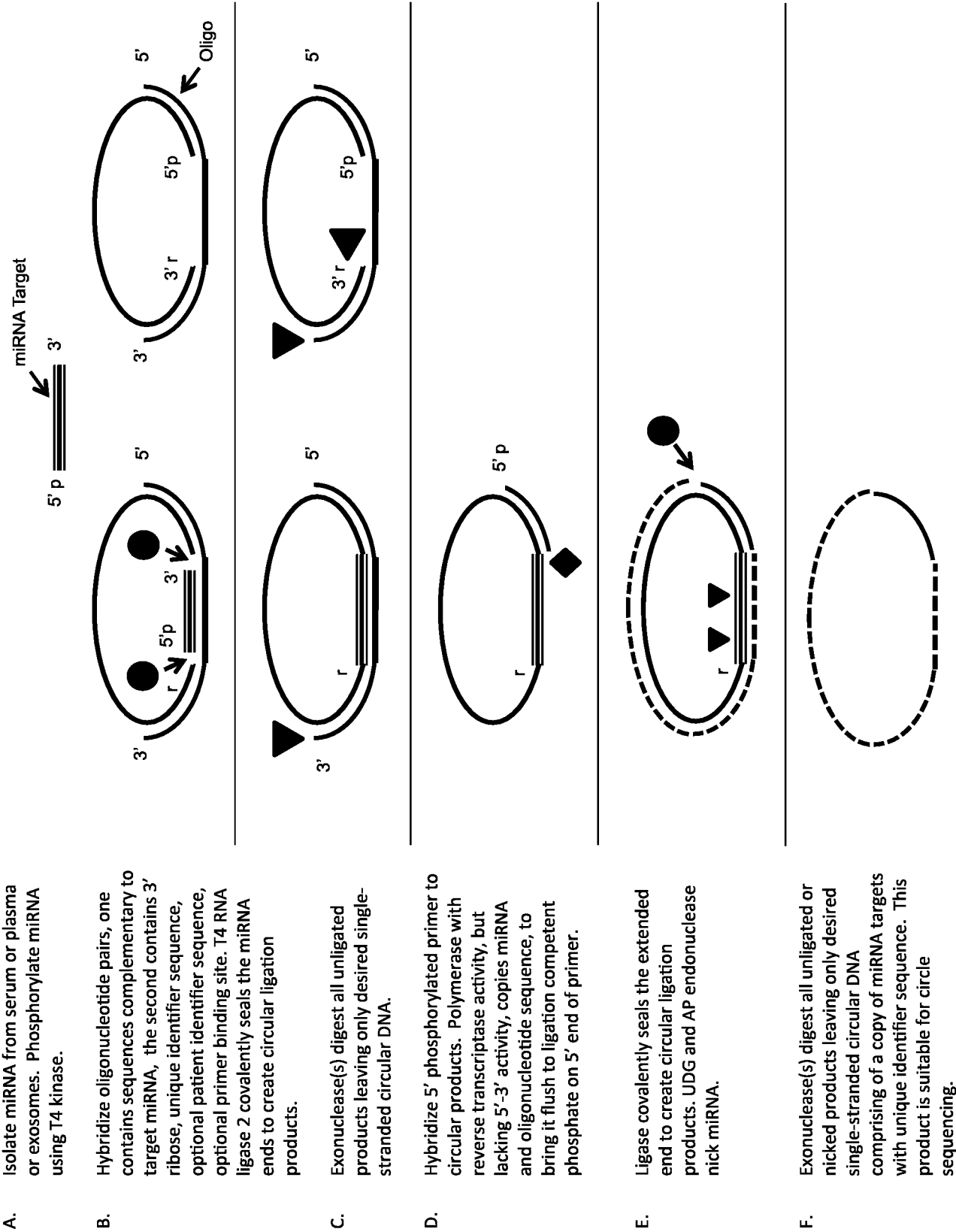


Figure 113





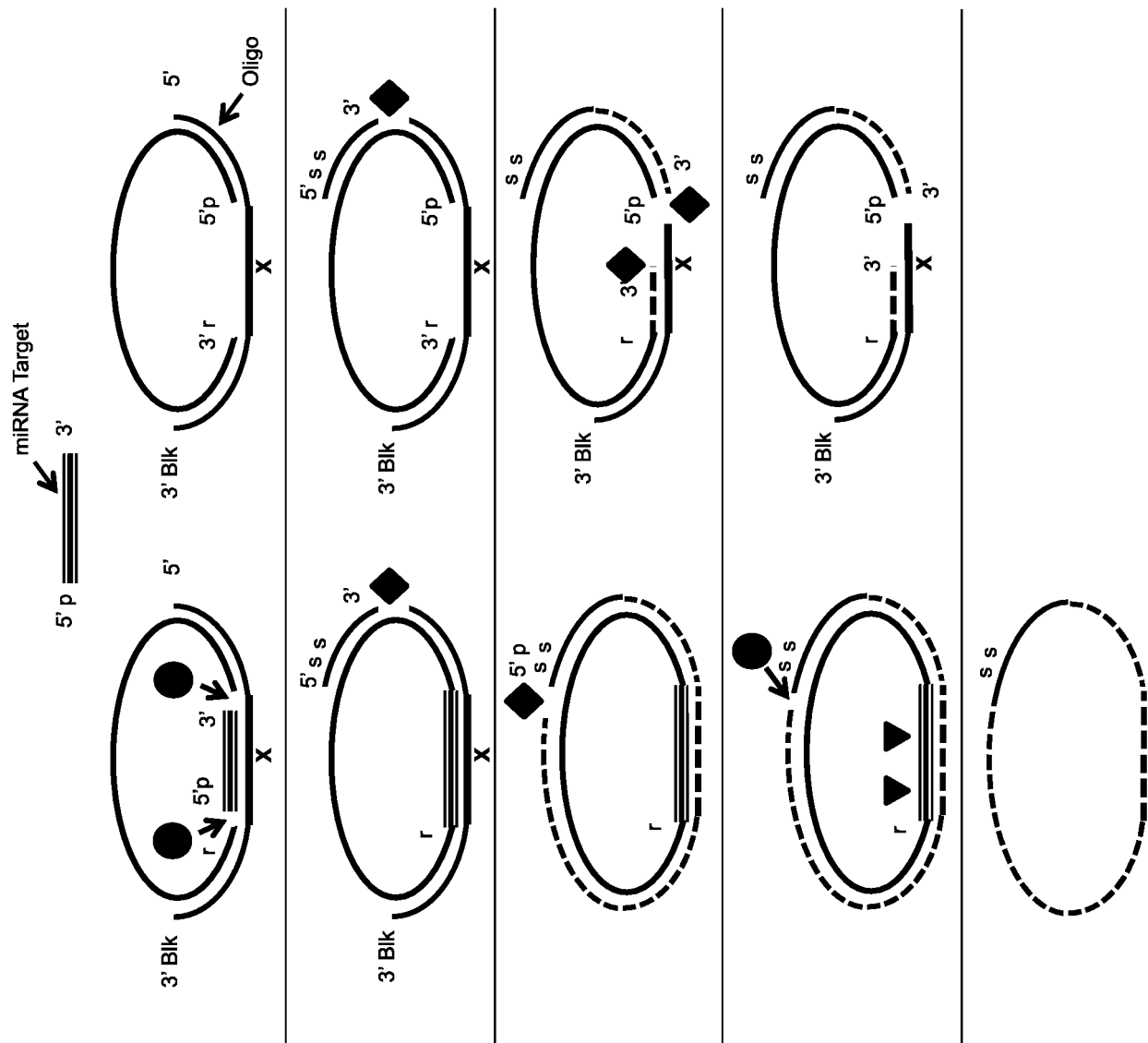


Figure 116

