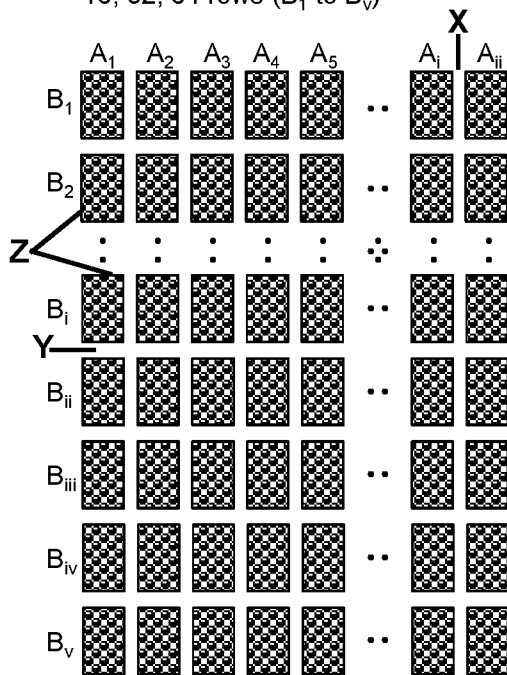
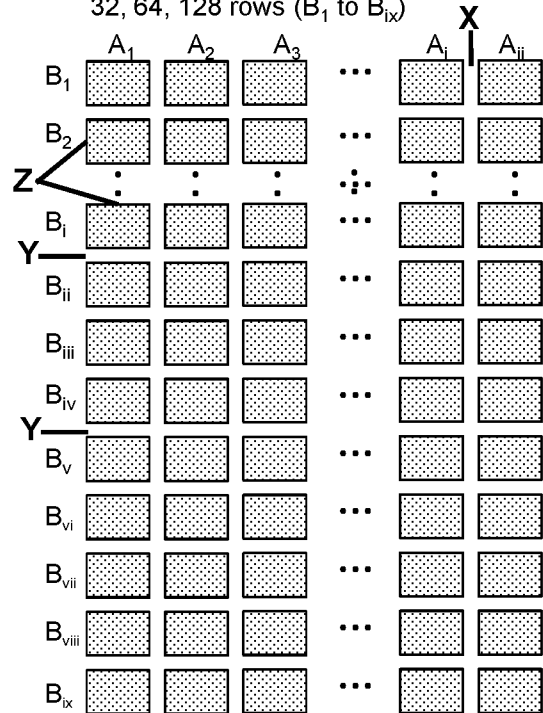


- A. 24, 48, 96 columns (A_1 to A_{ij}) x
16, 32, 64 rows (B_1 to B_v)



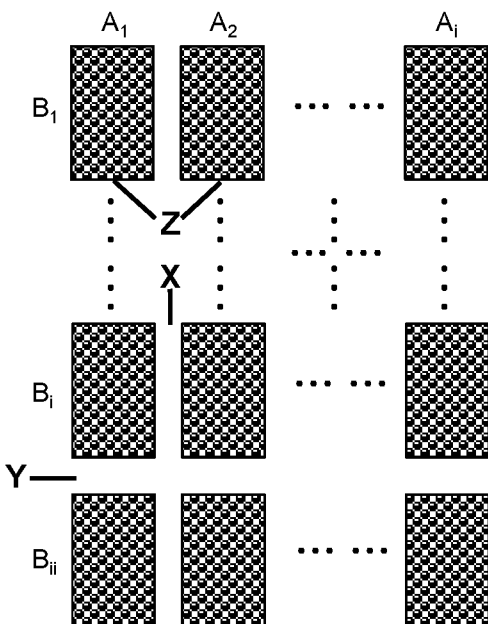
Each subdivision Z illustrated is 400 x 600 micron. Additional 100 um wide ridges X and Y between subdivisions. For Cartesian packing, a given subdivision with 50 micron pore will contain $6 \times 4 = 24$ micro-pores.

- B. 24, 48, 96 columns (A_1 to A_{ij}) x
32, 64, 128 rows (B_1 to B_{ix})



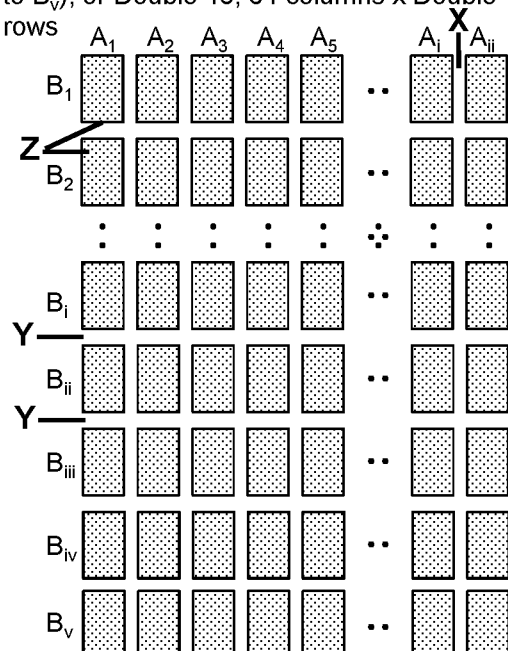
Each subdivision Z illustrated is 600 x 400 micron. Additional 100 um wide ridges X and Y between subdivisions. For hexagonal packing, a given subdivision with 5 micron pore will contain $60 \times 46 = 2,760$ micro-pores.

- C. 48, 96 columns (A_1 to A_{ij}) x
48, 96 rows (B_1 to B_{ij})



Each subdivision Z illustrated is 800 x 1,200 micron. Additional 200 um wide ridges X and Y between subdivisions. For hexagonal packing, A given subdivision with 50 micron pore will contain $12 \times 8 = 96$ micro-wells.

- D. 24, 48, 96 columns (A_1 to A_{ij}) x 16, 32, 64 rows (B_1 to B_v), or Double 48, 64 columns x Double 48, 64 rows



Each subdivision Z illustrated is 400 x 600 micron. Additional 100 um wide ridges X and Y between subdivisions. For hexagonal packing, a given subdivision with 5 micron pore will contain $60 \times 46 = 2,760$ micro-pores.

Figures 1A-1D

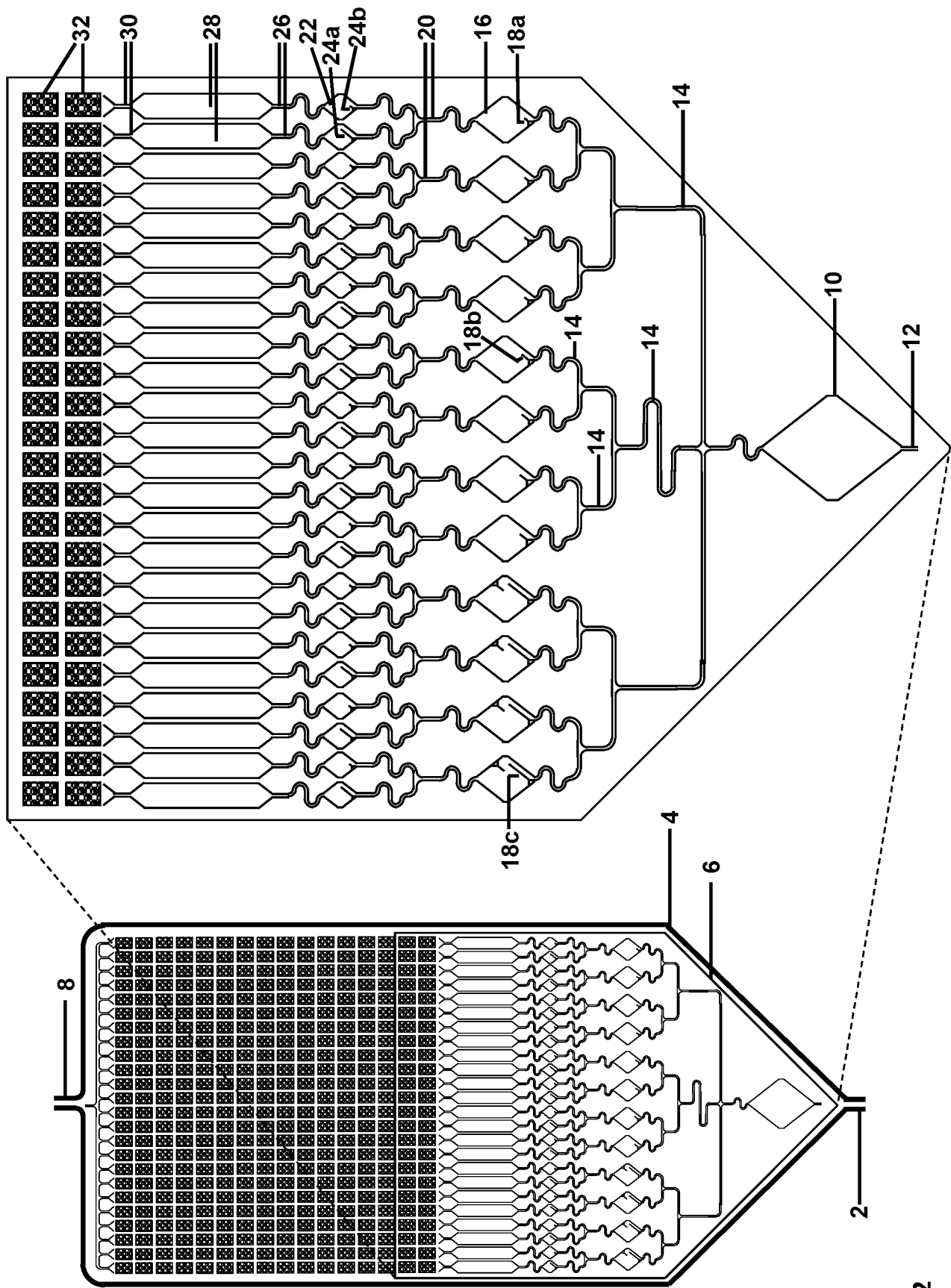


Figure 2

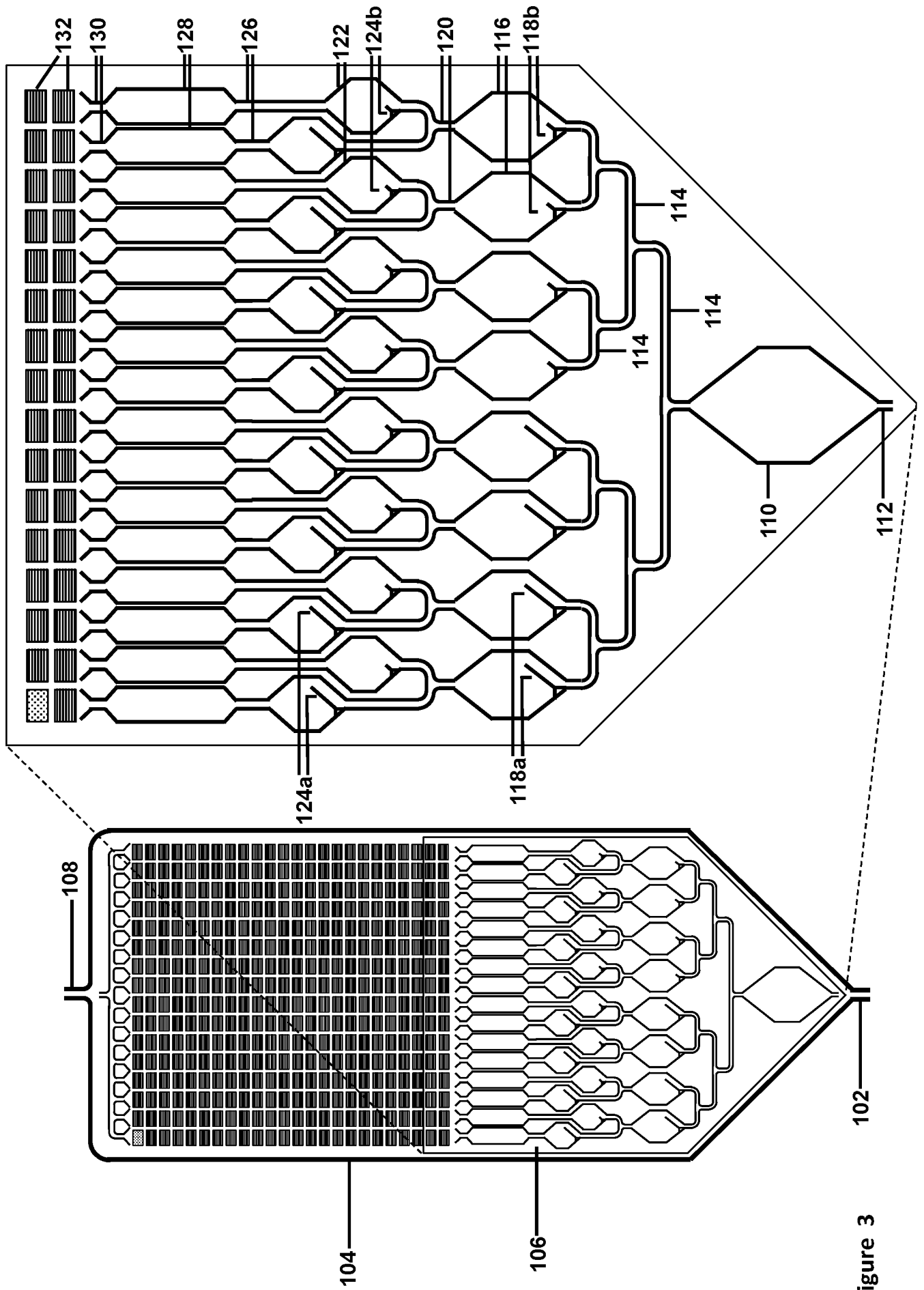
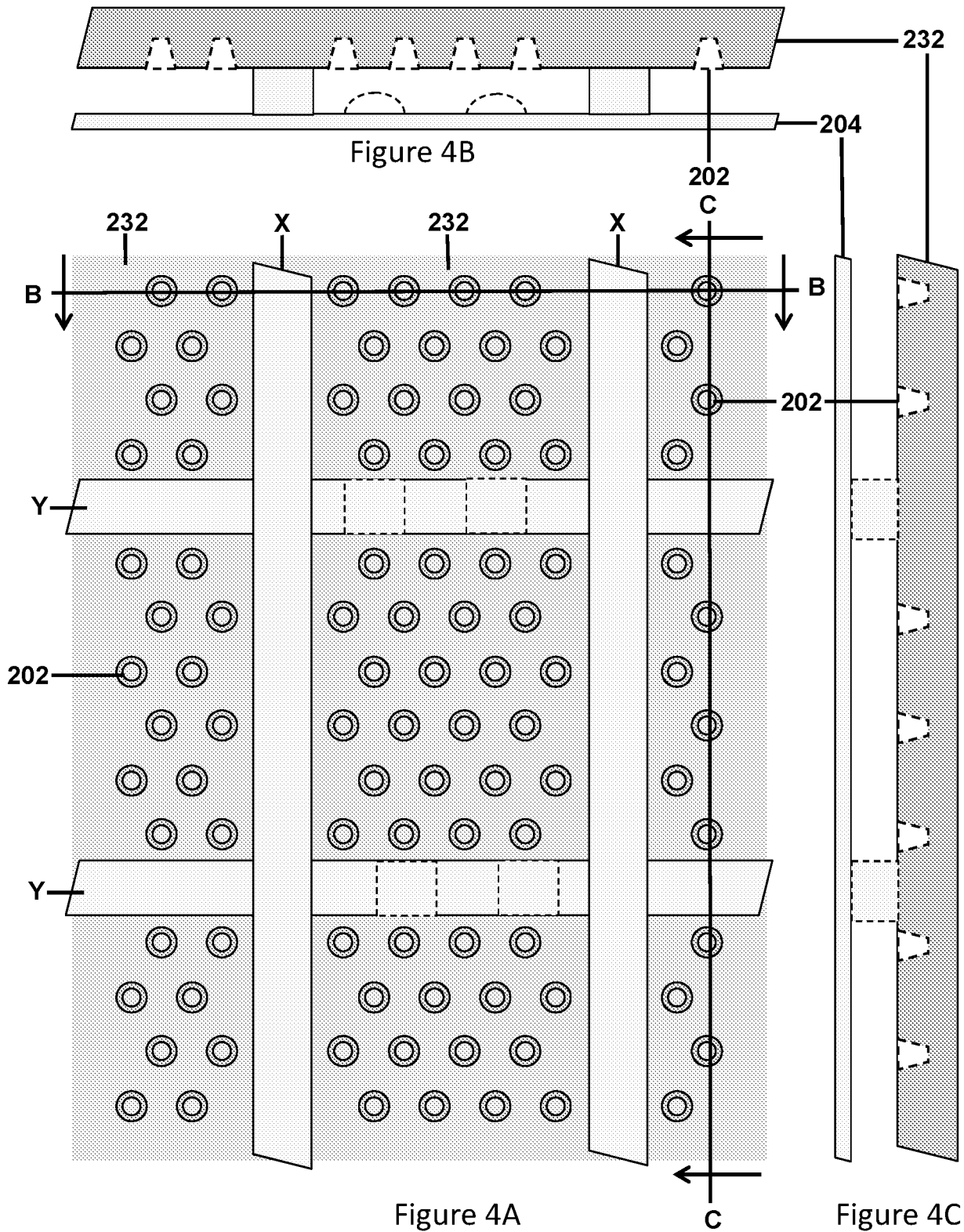
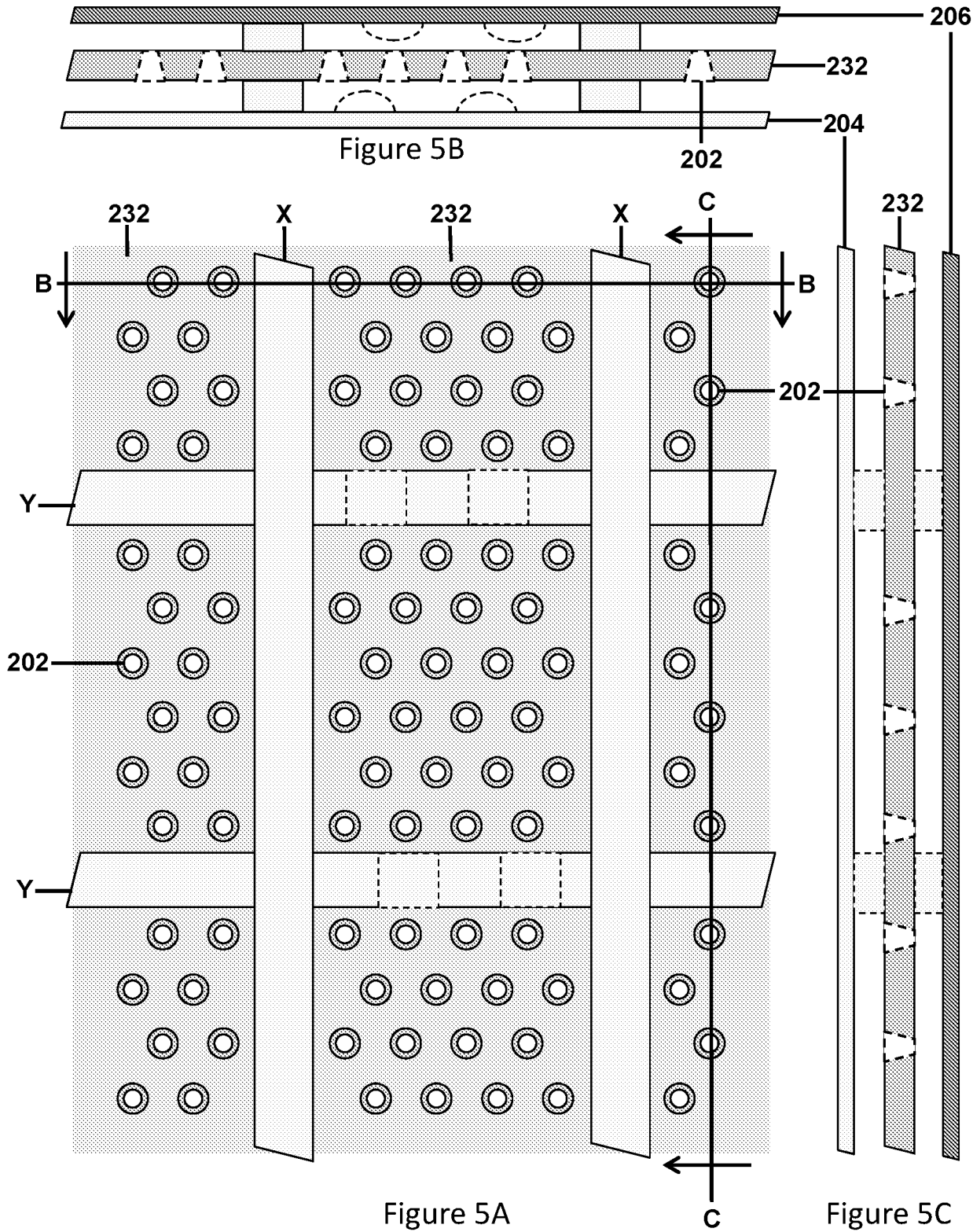


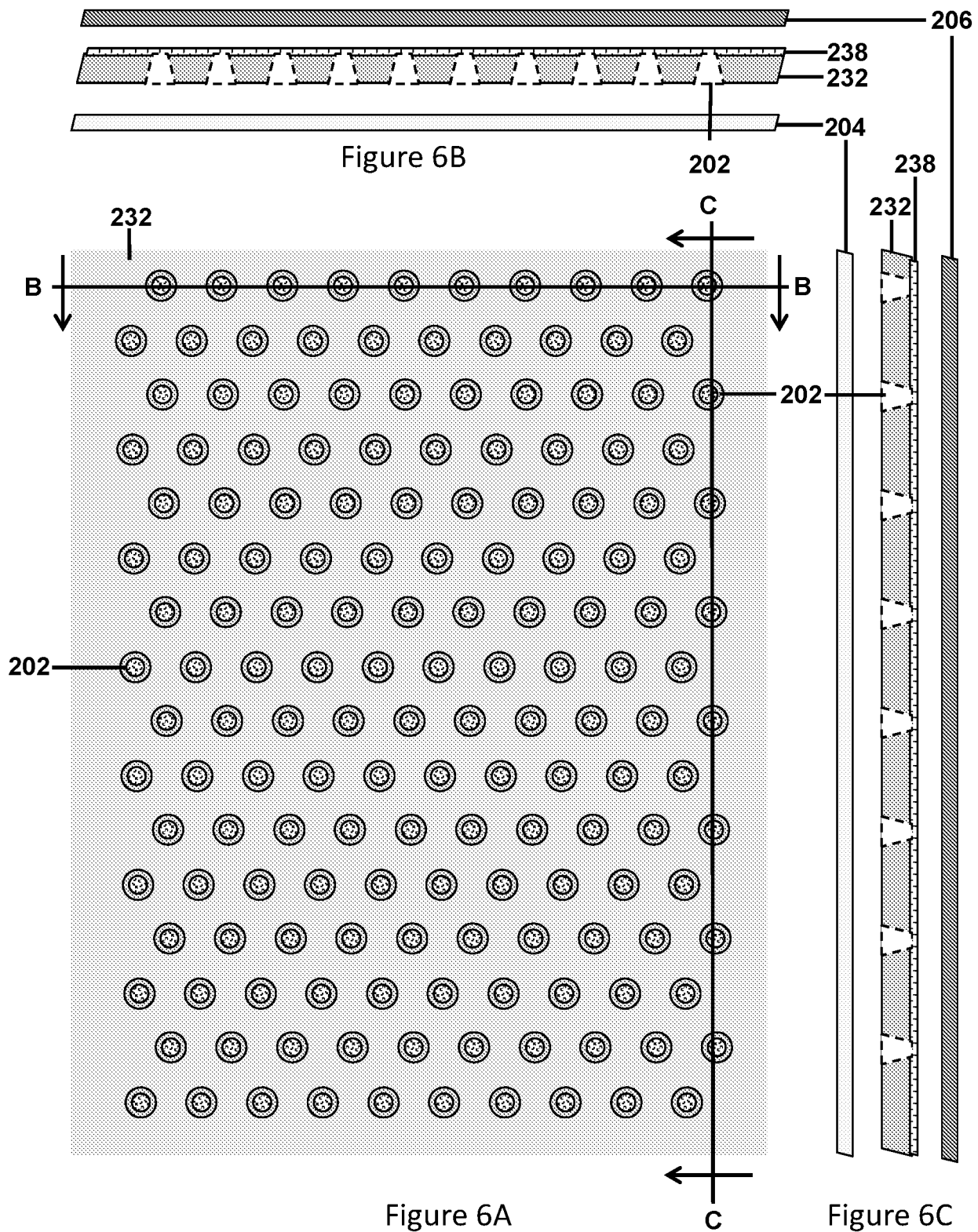
Figure 3



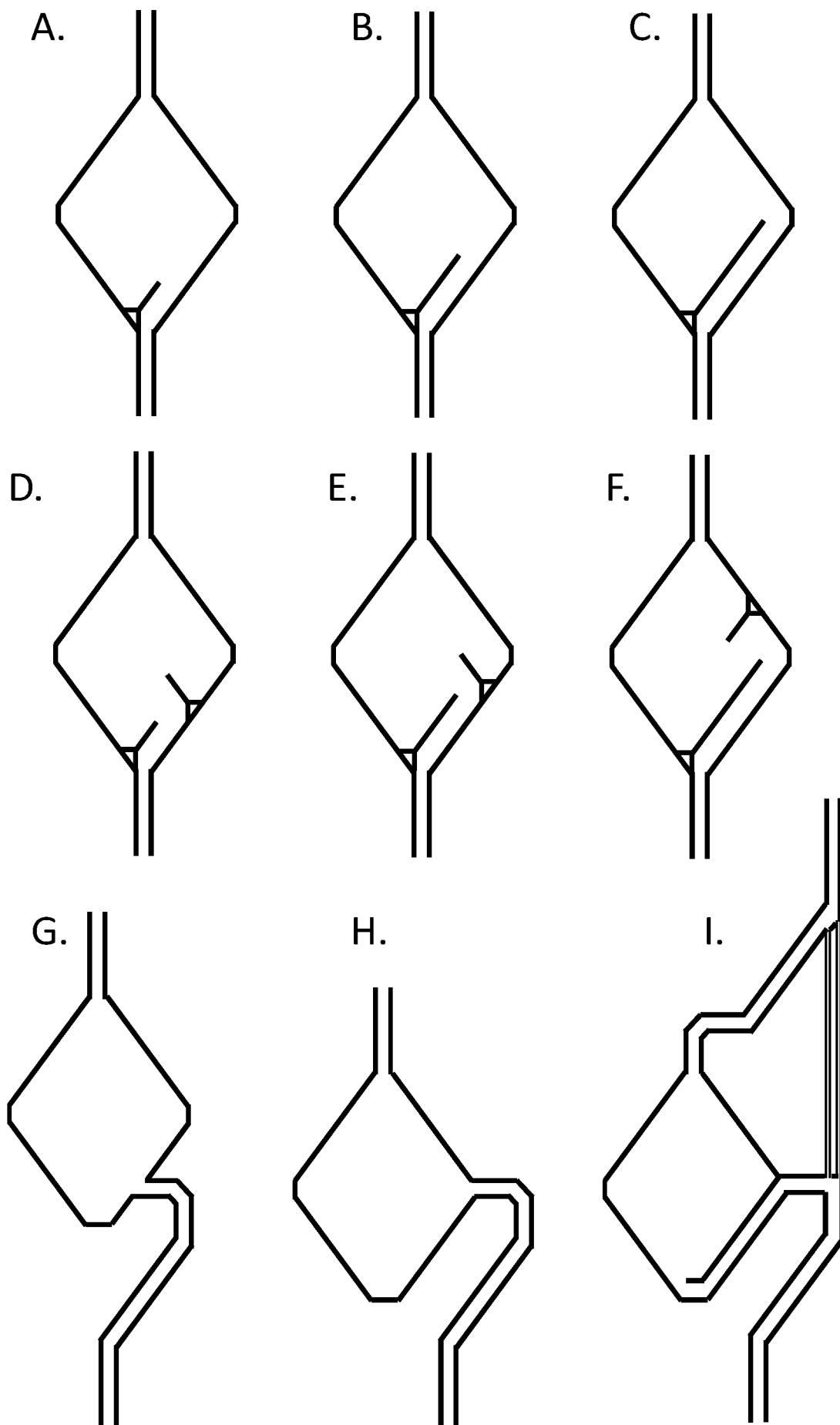
Figures 4A-4C



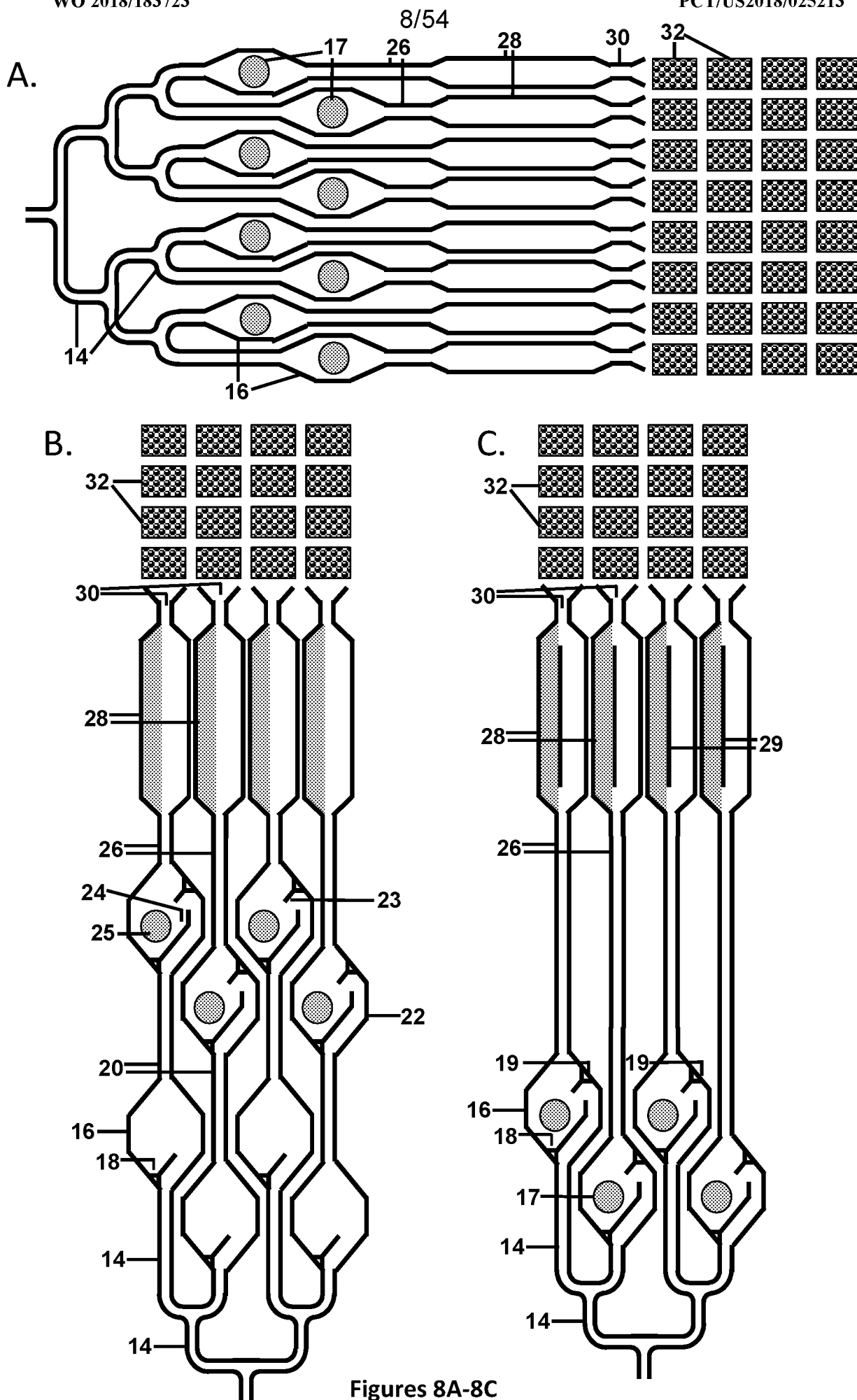
Figures 5A-5C



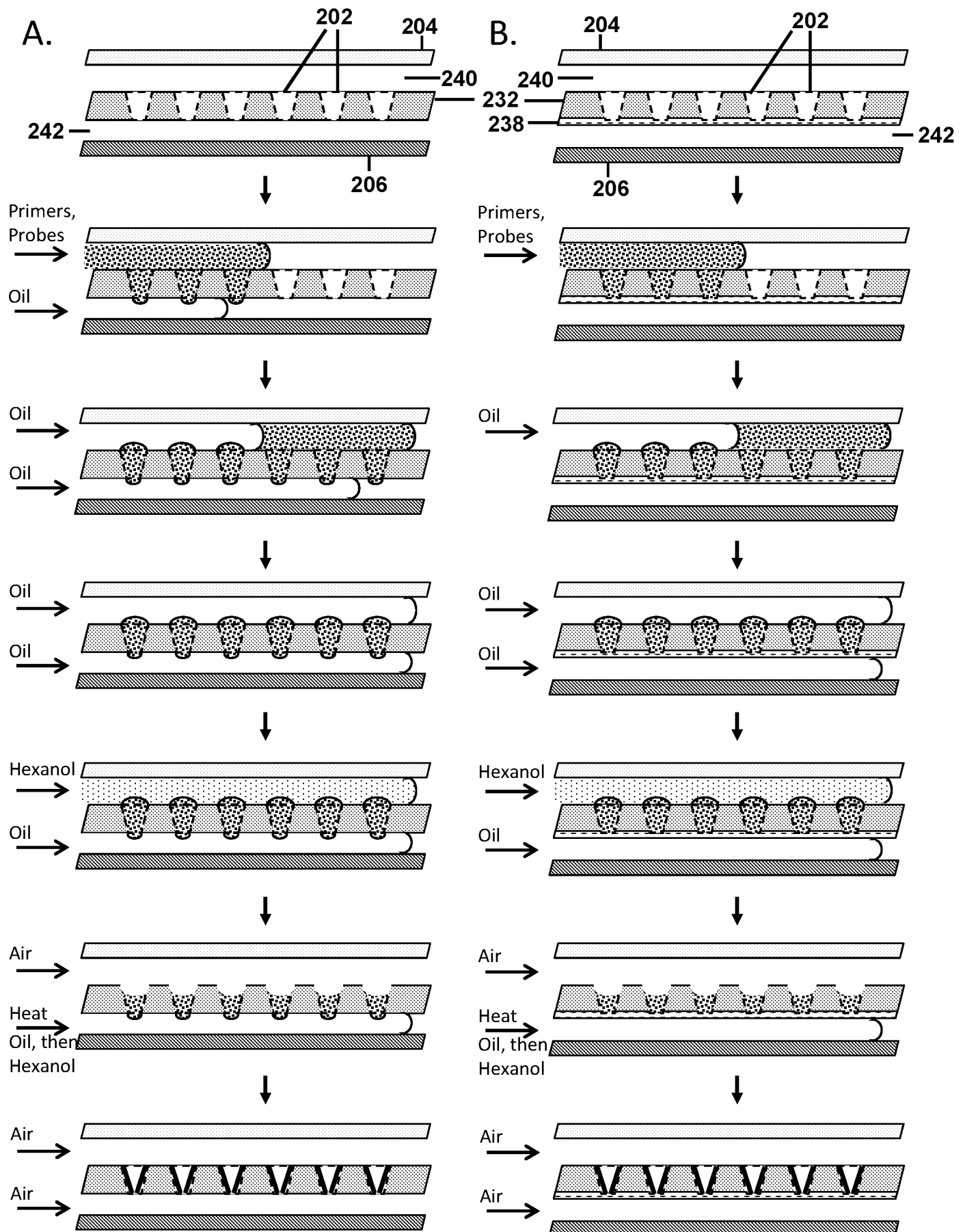
Figures 6A-6C



Figures 7A-7I



Figures 8A-8C



Figures 9A-9B

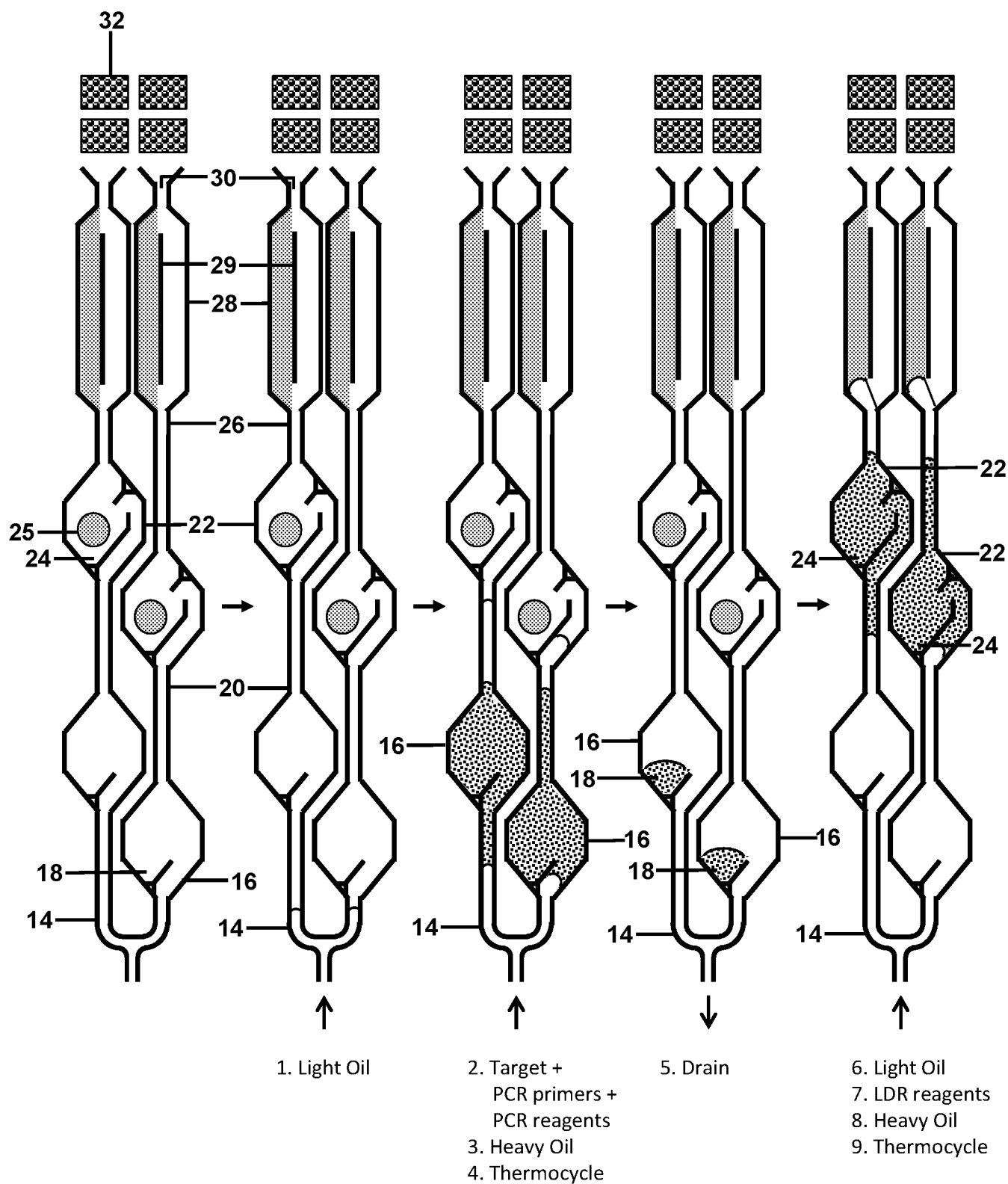
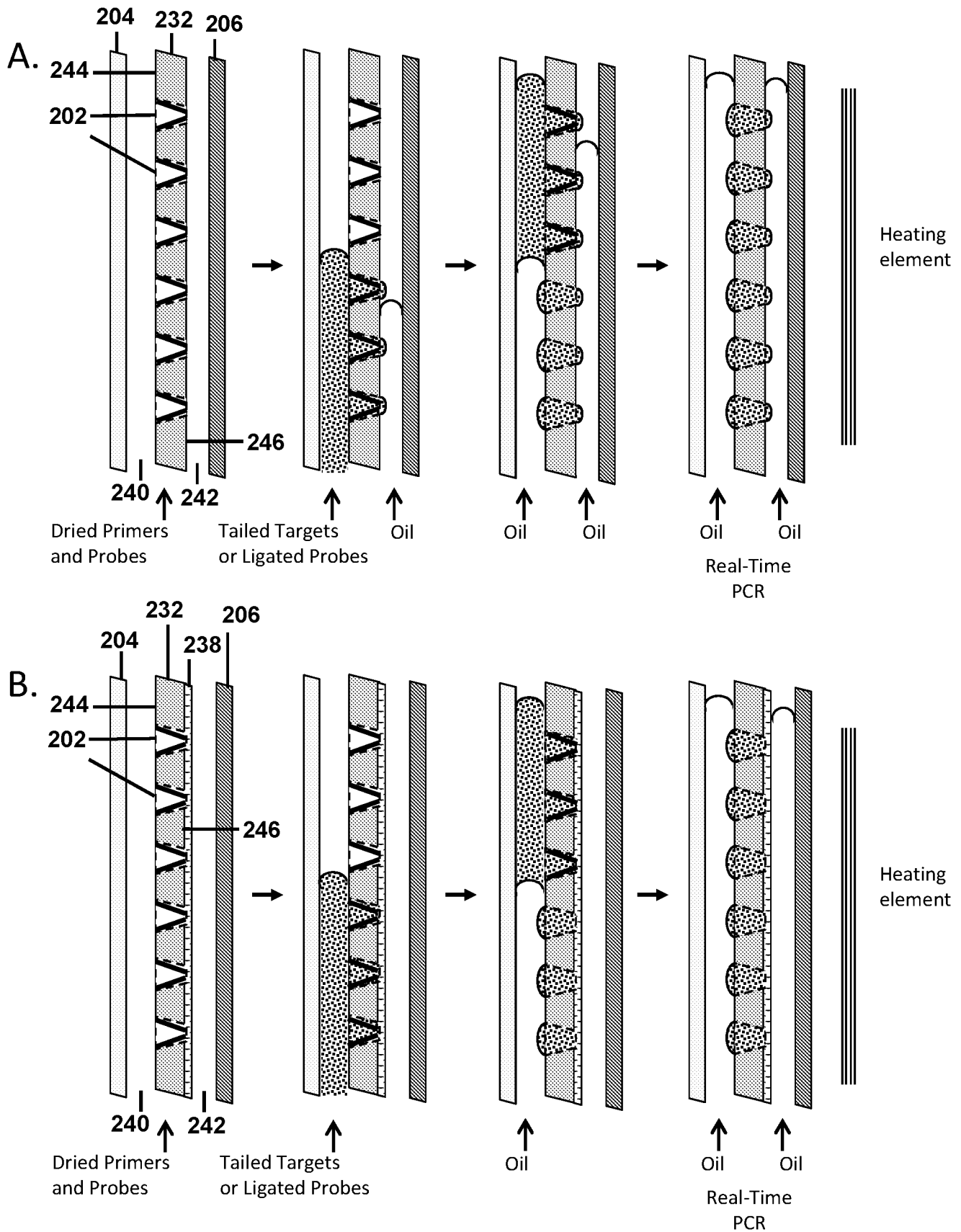


Figure 10



Figures 11A-11B

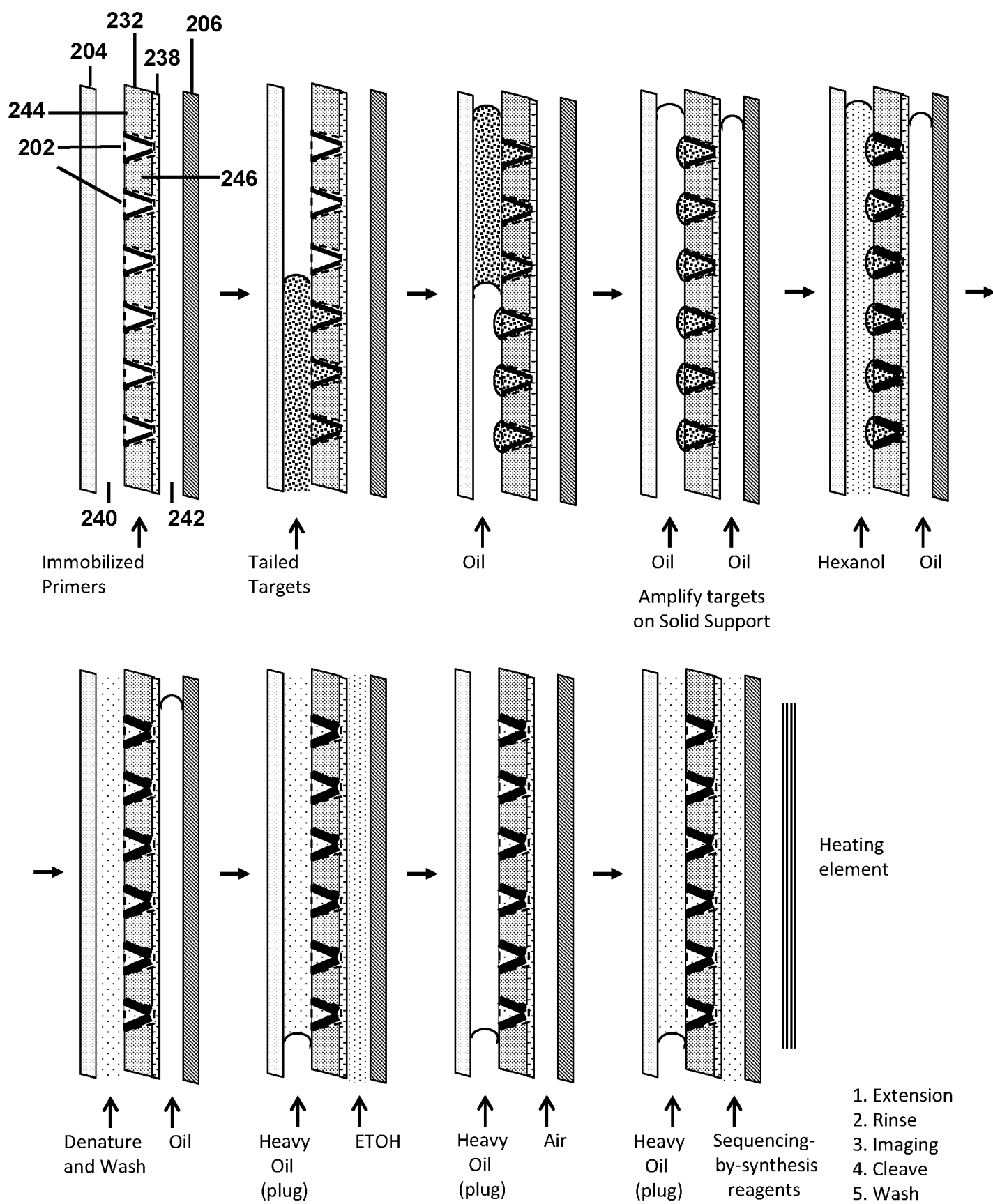
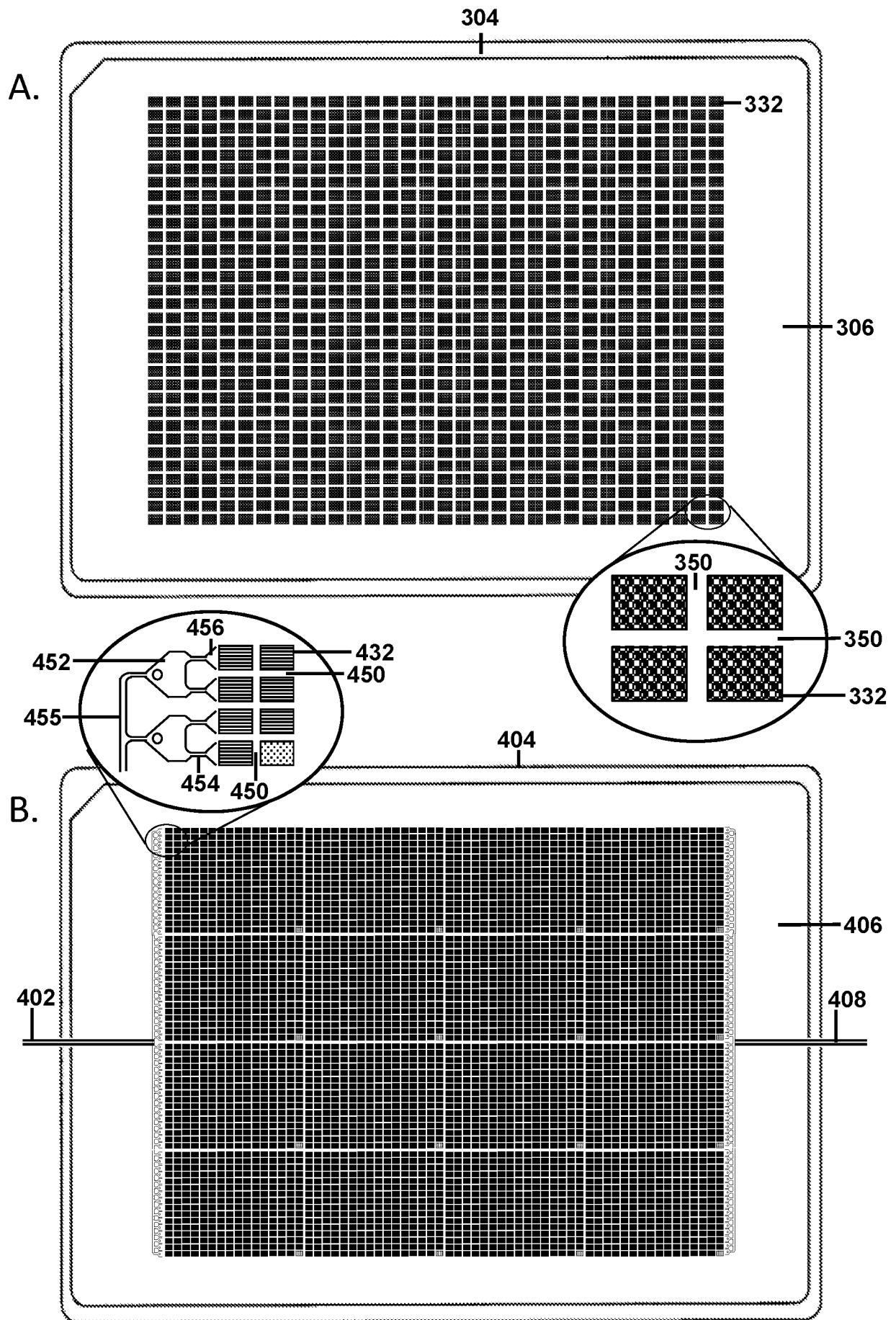


Figure 12



Figures 13A-13B

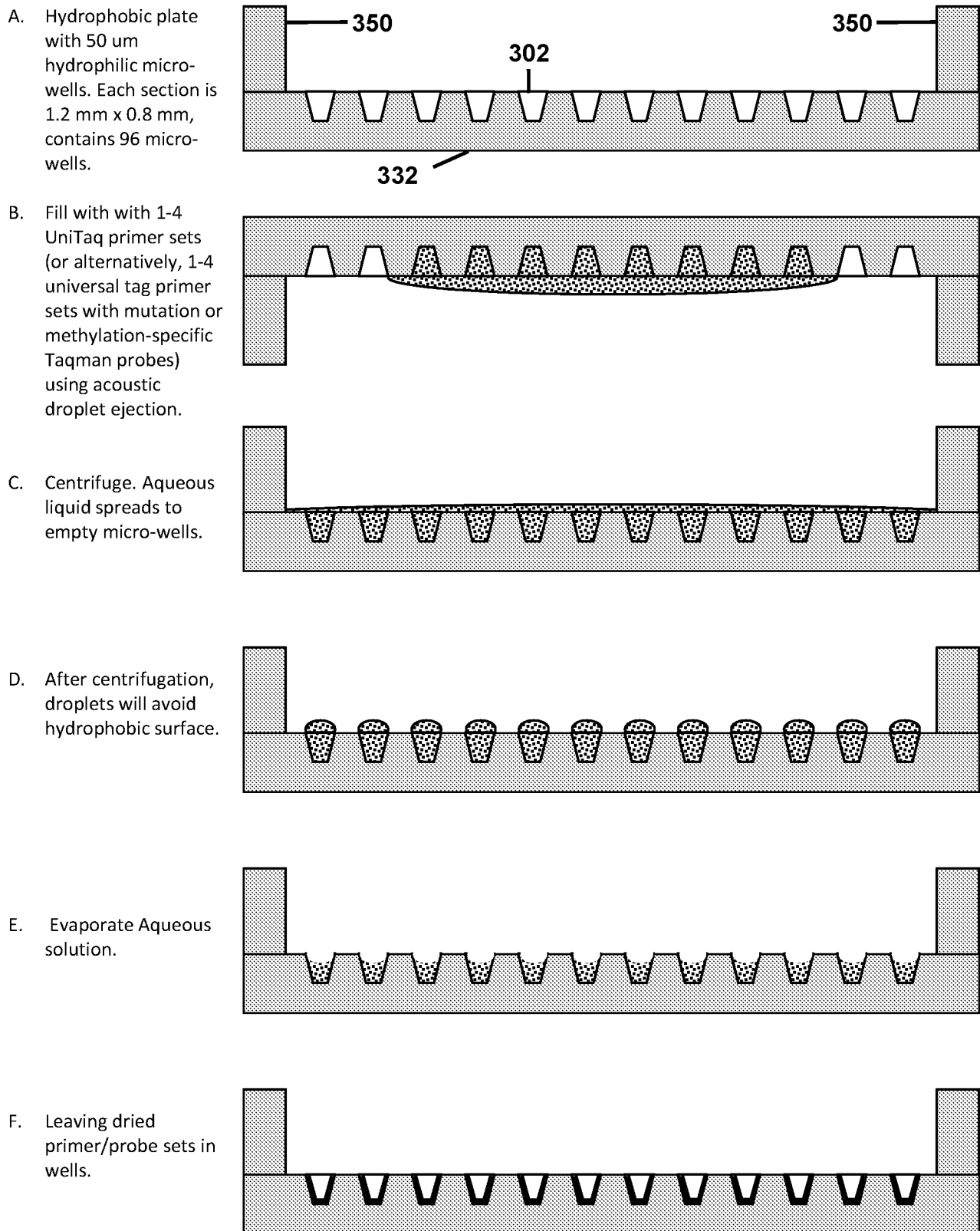
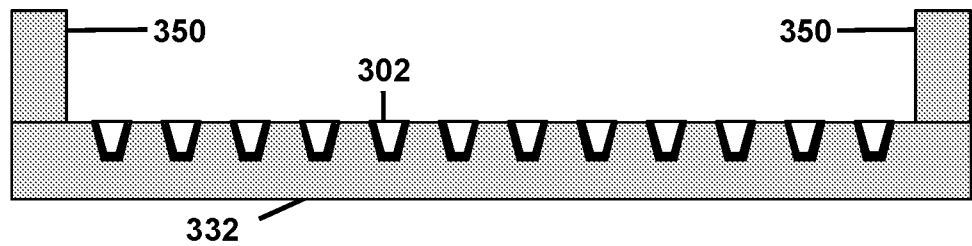
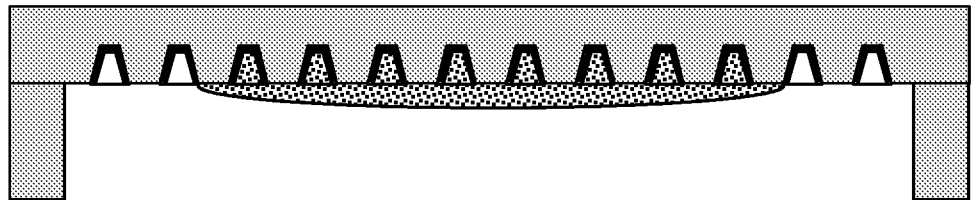


Figure 14

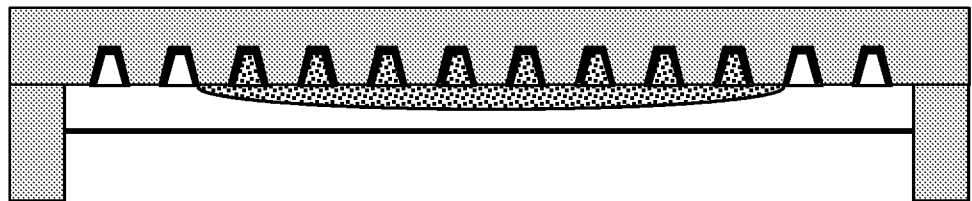
- A. Hydrophobic plate with 50 μm hydrophilic micro-wells. Each section is 1.2 mm x 0.8 mm, contains 96 micro-wells.



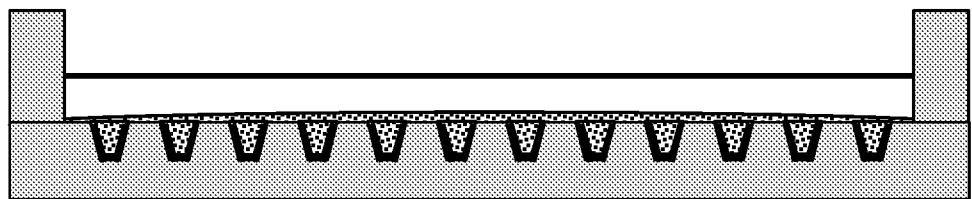
- B. Fill TaqMan reaction with target DNA using acoustic droplet ejection.



- C. Overlay with mineral oil.



- D. Centrifuge. Denser aqueous liquid spreads to empty micro-wells.



- E. Move plate to thermocycler. Droplets separate. Amplify.

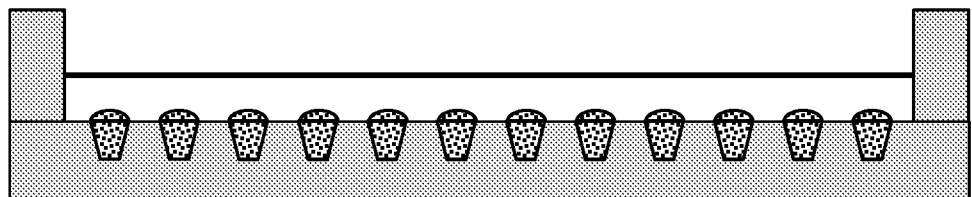


Figure 15

- A. PCR-PCR-qPCR (Taqman) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial limited cycle multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers.
- C. Distribute initial PCR products into 24, 36, or 48 Primary PCR Reaction Chambers. Perform limited cycle nested PCR using primers with Universal tag tails. Primers are unblocked with RNaseH2 only when bound to correct target.
- D. PCR products comprise of Ai tag sequence, pathogen-specific sequence, and Ci' tag sequence. Distribute products of each Primary PCR Reaction Chamber into 384 or 768 micro-pores.
- E. Amplify authentic pathogen-specific products with Tag-specific primers (Ai and Ci). Each Taqman probe spans a unique pathogen-specific sequence.
- F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal.

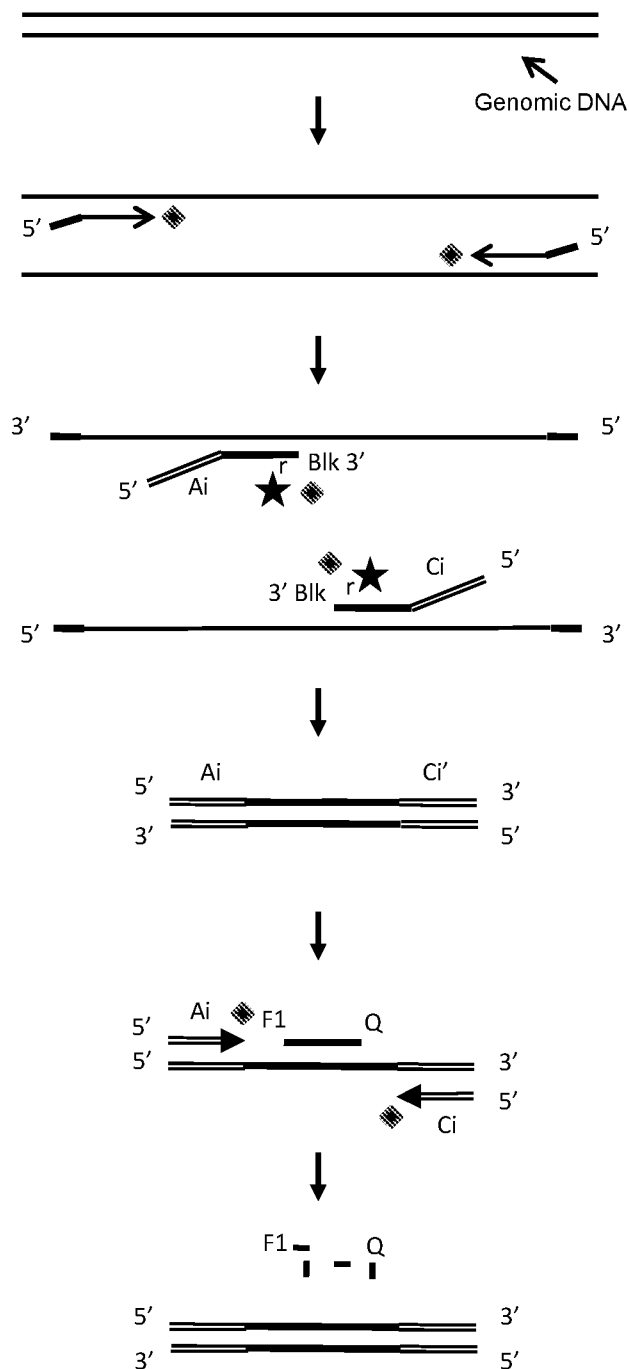


Figure 16

- A. PCR-PCR-qPCR (UniTag) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial limited cycle multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers.
- C. Distribute initial PCR products into 24, 36, or 48 Primary PCR Reaction Chambers. Perform limited cycle nested PCR using primers with UniTag tails. Primers are unblocked with RNaseH2 only when bound to correct target.
- D. PCR products comprise of Ai tag sequence, pathogen-specific sequence, Bi', and Ci' tag sequence. Distribute products of each Primary PCR Reaction Chambers into 384 or 768 micro-pores.
- E. Amplify authentic pathogen-specific products with UniTag-specific primers (F1-Bi-Q-Ai and Ci).
- F. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- G. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'→3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal.

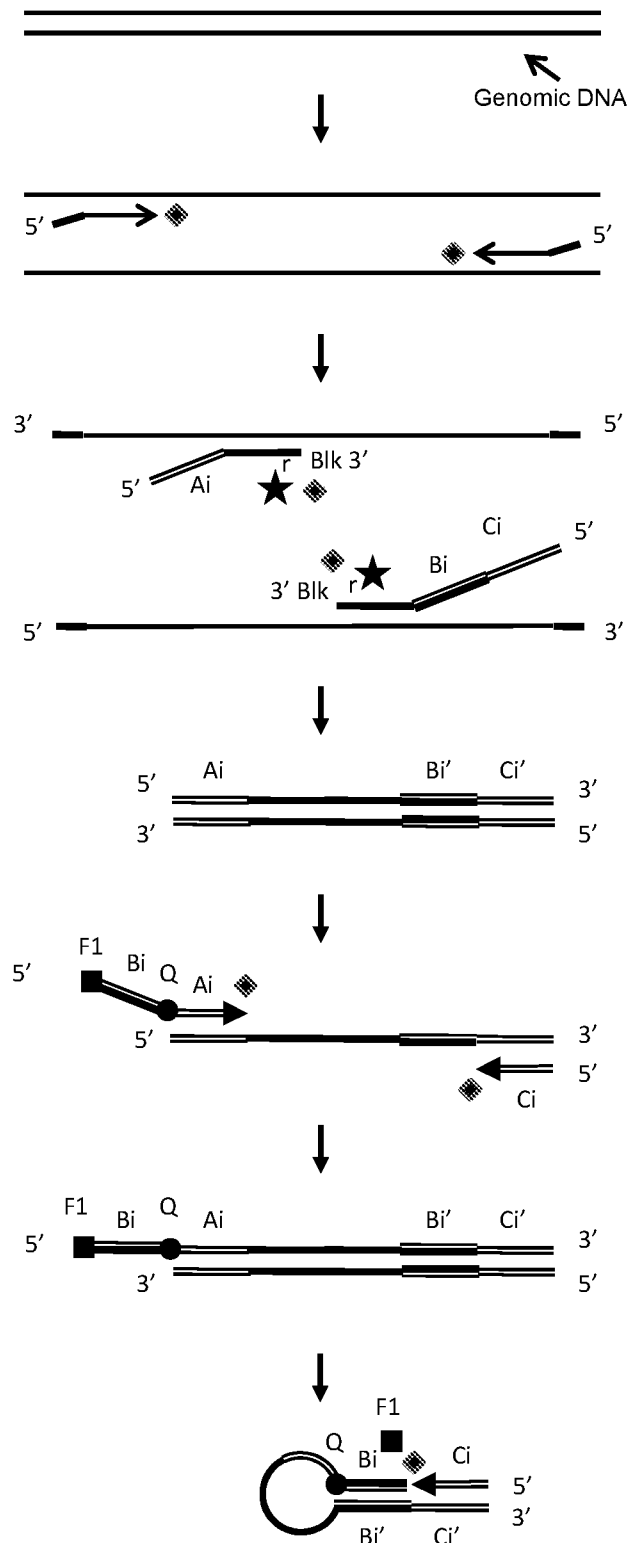


Figure 17

- A. PCR-PCR-qPCR (UniRq) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial limited cycle multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers.
- C. Distribute initial PCR products into 24, 36, or 48 Primary PCR Reaction Chambers. Perform limited cycle nested PCR using primers with UniTaq tails. Primers are unblocked with RNaseH2 only when bound to correct target.
- D. PCR products comprise of Ai-Bi'-ti' tag sequence, pathogen-specific sequence with ti-tj, and tj'-Bj'-Ci' tag sequence. Distribute products of each Primary PCR Reaction Chambers into 384 or 768 micro-pores.
- E. Amplify authentic pathogen-specific products with UniTaq-specific primers (F1-r-Bj,Bi-Q-Ai, and Ci), using polymerase with strand-displacing activity, and lacking 5'-3' nuclease activity.
- F. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- G. After the denaturation step, as the temperature decreases, 4 hairpins form between pathogen-specific sequences (ti & ti'; tj & tj'), Bi & Bi', and Bj & Bj'. This renders the ribose base in the Bj sequence double-stranded, enabling RNaseH2 to liberate the fluorescent group and generate signal. Primer dimer lacks pathogen-specific sequences, would not form all hairpins, and thus RNaseH2 would not liberate signal.

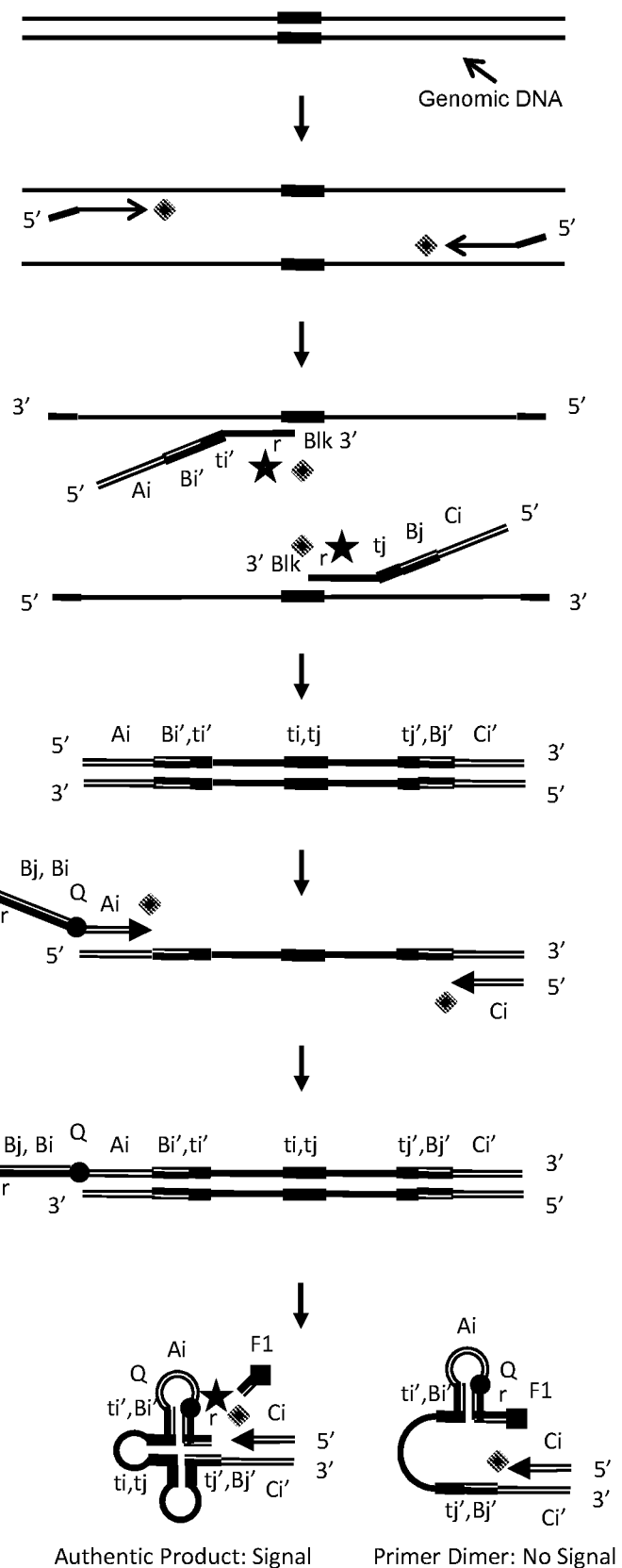
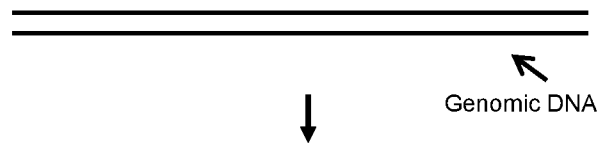
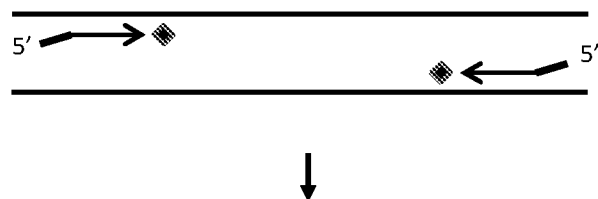


Figure 18

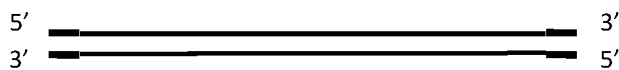
- A. PCR-LDR-qPCR (Taqman) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.



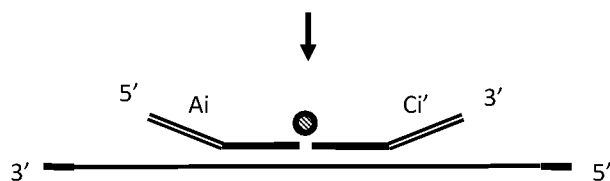
- B. Perform initial multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers



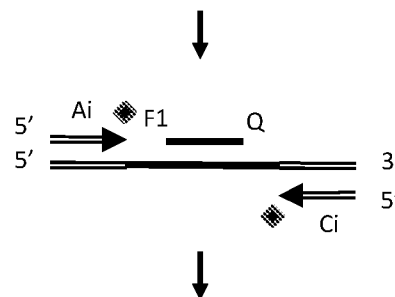
- C. Distribute initial PCR products into 24, 36, or 48 Primary LDR Reaction Chambers.



- D. Pathogen-specific ligation oligonucleotides contain tags (Ai and Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together.



- E. Distribute ligation products of each Primary LDR Reaction Chamber into 384 or 768 micro-pores. Amplify authentic pathogen-specific products with Tag-specific primers (Ai and Ci). Each Taqman probe spans a unique pathogen-specific sequence.



- F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal.

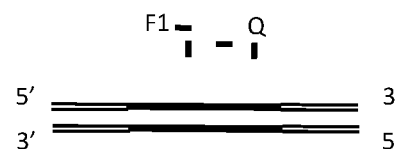


Figure 19

- A. PCR-LDR-qPCR (UniTaq) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers
- C. Distribute initial PCR products into 24, 36, or 48 Primary LDR Reaction Chambers.
- D. Pathogen-specific ligation oligonucleotides contain tags (Ai, Bi'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together.
- E. Distribute ligation products of each Primary LDR Reaction Chamber into 384 or 768 micro-pores. Amplify authentic pathogen-specific products with UniTaq-specific primers (F1-Bi-Q-Ai and Ci).
- F. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- G. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'->3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal.

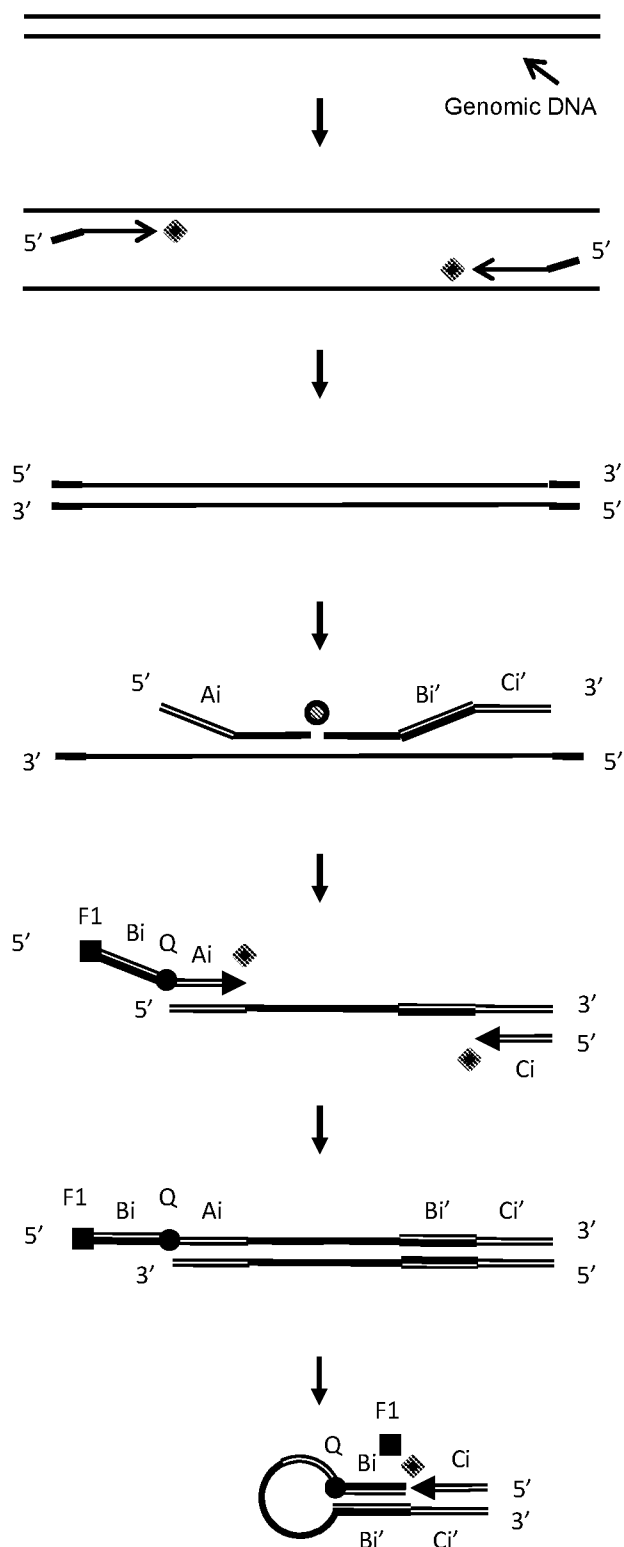
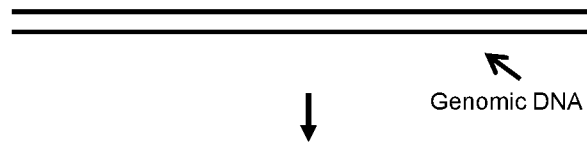


Figure 20

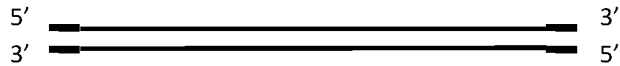
- A. PCR-LDR-qPCR (UniSpTq) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.



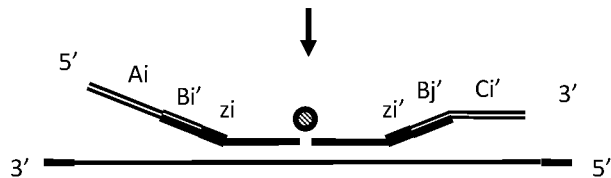
- B. Perform initial multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers



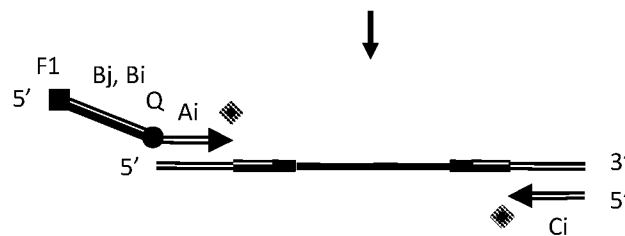
- C. Distribute initial PCR products into 24, 36, or 48 Primary LDR Reaction Chambers.



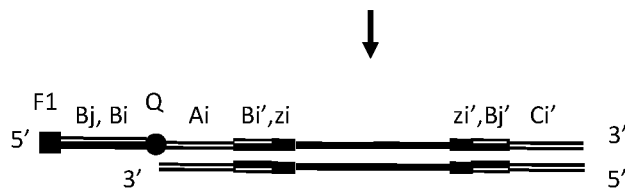
- D. Pathogen-specific ligation oligonucleotides contain tags (Ai-Bi'-zi; zi'-Bj'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together.



- E. Distribute ligation products of each Primary LDR Reaction Chamber into 384 or 768 micro-pores. Amplify authentic pathogen-specific products with UniTaq-specific primers (F1-Bj, Bi-Q-Ai and Ci).



- F. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.



- G. After the denaturation step, as the temperature decreases, 3 hairpins forms between Bi & Bi', zi & zi', and Bj & Bj'. The 5'->3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal. As soon as polymerase has traversed Bj', the short zi-zi' stem falls apart and polymerase continues extending to create the dsDNA product.

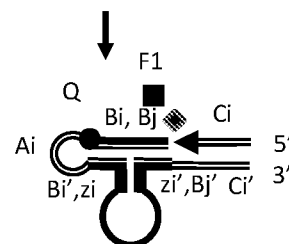


Figure 21

- A. PCR-qLDR (UniLDq) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers
- C. Distribute initial PCR products into 384 or 768 micro-pores.
- D. Pathogen-specific ligation oligonucleotides have tags ($Bi'-ti'$; $tj'-Bj'$) for subsequent detection. (When detecting specific SNPs or mutations, blocking LNA or PNA Wt probes suppress ligation to Wt sequence.) Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the two oligonucleotides together.
- F. In the presence of probe ($F1-r-Bj, Bi-Q$), and after the denaturation step, as the temperature decreases, 4 double-stranded stems form between probe and pathogen-specific sequences (ti & ti' ; tj & tj'), Bi & Bi' , and Bj & Bj' . This renders the ribose base in the Bj sequence double-stranded, enabling RNaseH2 to liberate the fluorescent group and generate signal. The cleaved probe dissociates from the product and new probe can hybridize to generate additional signal. Unligated LDR primers would not form all hairpins, and thus RNaseH2 would not liberate signal.

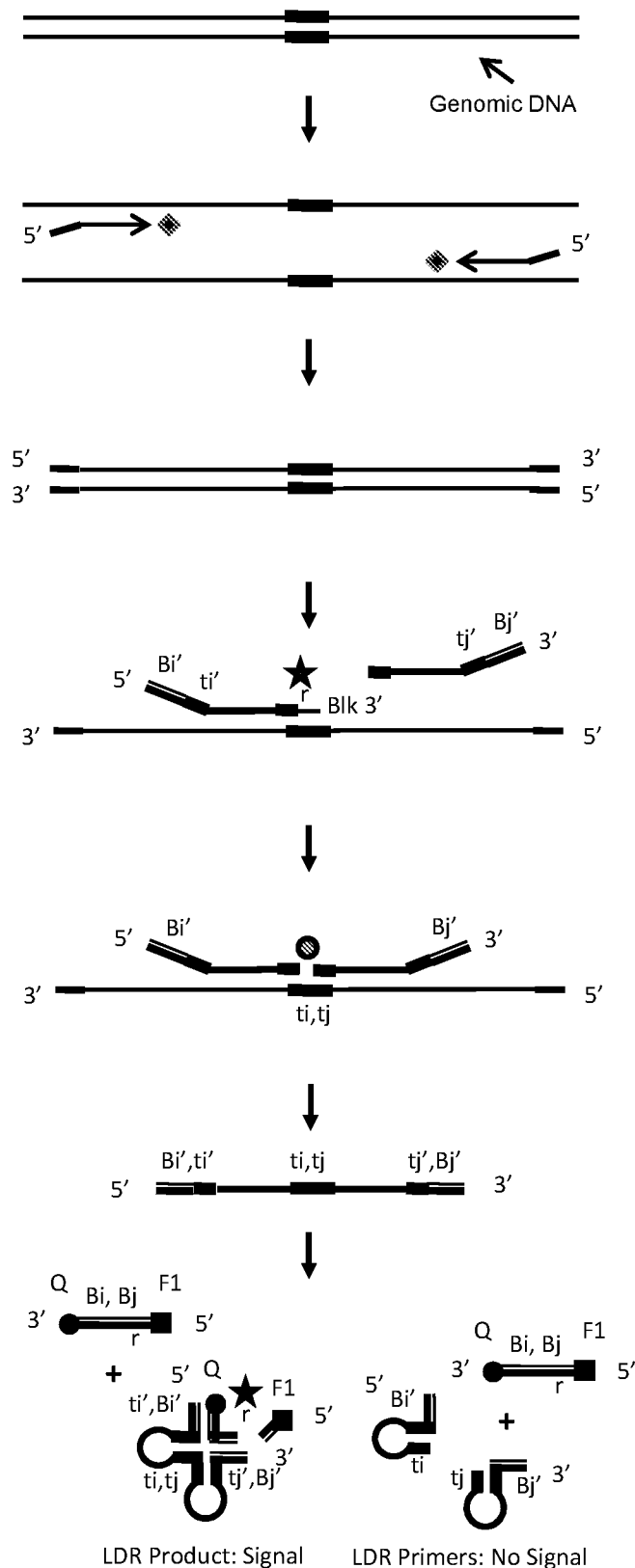


Figure 22

- A. PCR-qLDR (TsLDq) for unknown pathogen identification and quantification. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA.
- B. Perform initial multiplexed amplification of pathogen-specific regions in an Initial Reaction Chamber. Primers contain identical 8-11 base tails to prevent primer dimers
- C. Distribute initial PCR products into into 384 or 768 micro-pores.
- D. Pathogen-specific ligation oligonucleotides have tags (Bi' and tj') for subsequent detection. (When detecting specific SNPs or mutations, blocking LNA or PNA Wt probes suppress ligation to Wt sequence.) Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the two oligonucleotides together.
- F. In the presence of probe (F1-r-pathogen sequence-Bi-Q), and after the denaturation step, as the temperature decreases, 2 double-stranded stems form between pathogen-specific sequences (ti,tj & ti',tj'), and Bi & Bi'. This renders the ribose base in the pathogen sequence double-stranded, enabling RNaseH2 to liberate the fluorescent group and generate signal. The cleaved probe dissociates from the product and new probe can hybridize to generate additional signal. Unligated LDR primers would not form both stems, and thus RNaseH2 would not liberate signal.

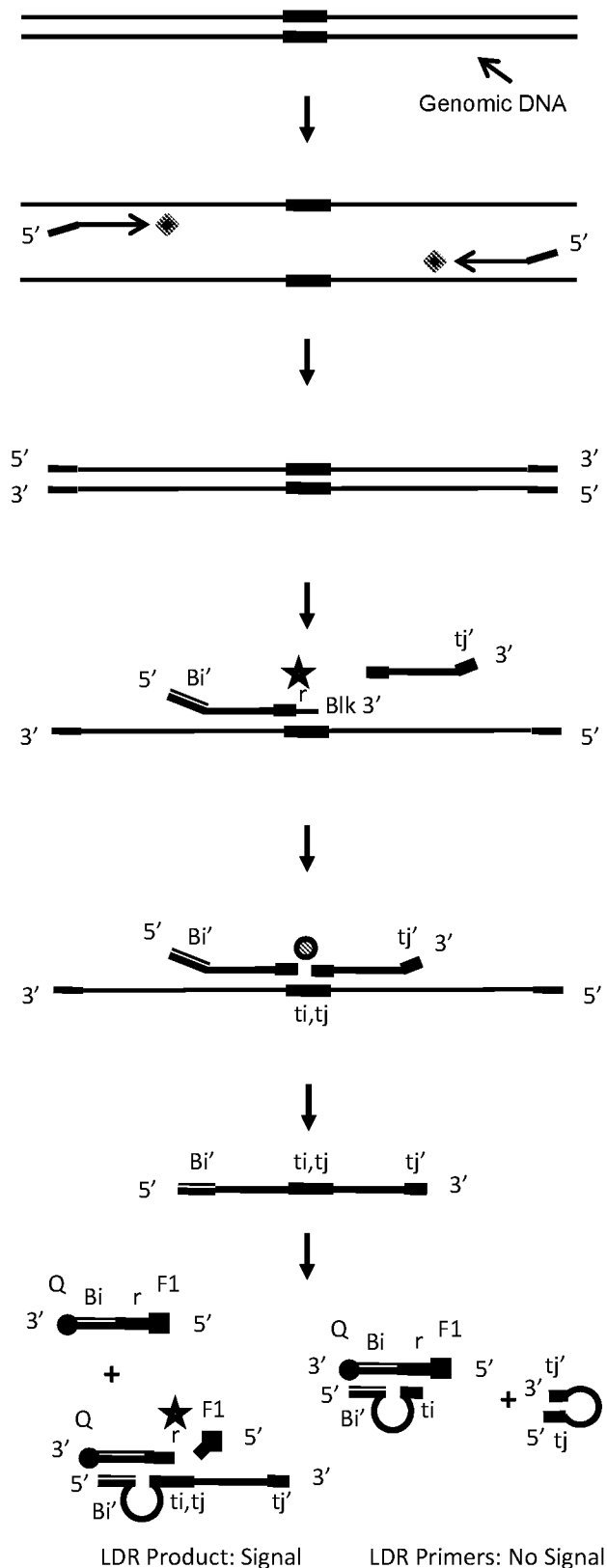


Figure 23

- A. Unknown pathogen identification and quantification; using Multiplexed PCR - Nested PCR - UniTaq detection. (Alternatively, Multiplexed PCR – Nested PCR - Real-time-PCR with target-specific Taqman probes.)
 - B. Preparation: Rows are pre-filled with 1-4 UniTaq primer sets (or alternatively, 1-4 universal tag primer sets with target-specific Taqman probes). Primers are distributed into each row and dried down into individual micro-pores.
 - C. Preparation: Pre-fill pre-chambers to columns with nested PCR primer sets with either UniTaq or universal tag sequences on their 5' ends, and dry down.
 - D. Multiplexed PCR amplification (or reverse-transcription-PCR for RNA targets) for 9 cycles in an Initial Reaction Chamber to generate 512 copies of each original target.
 - E. Distribute initial multiplex reaction into 24 Primary PCR Reaction Chambers, with average distribution of 20 copies of each original pathogen in each Primary PCR Reaction Chamber. Perform 5 cycles of nested PCR using target-specific primers with UniTaq or universal tags in groups of 16, 32, or 64 primer sets, to generate an average of 640 copies of each pathogen-specific target per Primary PCR Reaction Chamber.
 - F. Distribute nested PCR products of each Primary PCR Reaction Chamber into mixing chambers and then into 384 or 768 micro-pores of each column. Universal or UniTaq primers in each row will amplify only those products from each column with the correct tags. Ct values across micro-pores will provide approximate copy information for pathogen-specific targets.
-
- A. For Unknown pathogen identification and typing; using Multiplexed PCR - LDR- UniTaq detection or - Real-time-PCR.
 - B. Prepare rows as above, and pre-fill chambers to columns with LDR probe sets with either UniTaq or universal tag sequences on their non-ligating 5' (upstream) and 3' (downstream) ends, and dry down.
 - C. Multiplexed PCR amplification (or reverse-transcription-PCR for RNA targets) for 30 cycles to amplify original target in an Initial Reaction Chamber.
 - D. Distribute initial multiplex reaction into 24 Primary LDR Reaction Chambers, while diluting 10-fold. Perform 20 cycles of LDR using allele-specific probes with UniTaq or universal tags in groups of 16, 32, or 64 probe sets.
 - E. Distribute LDR products of each Primary LDR Reaction Chamber into mixing chambers and then into 384 or 768 micro-pores of each column. Universal or UniTaq primers in each row will amplify only those products from each column with the correct tags. Ct values across micro-pores will provide approximate copy information for pathogen-specific targets initially present at high abundance.

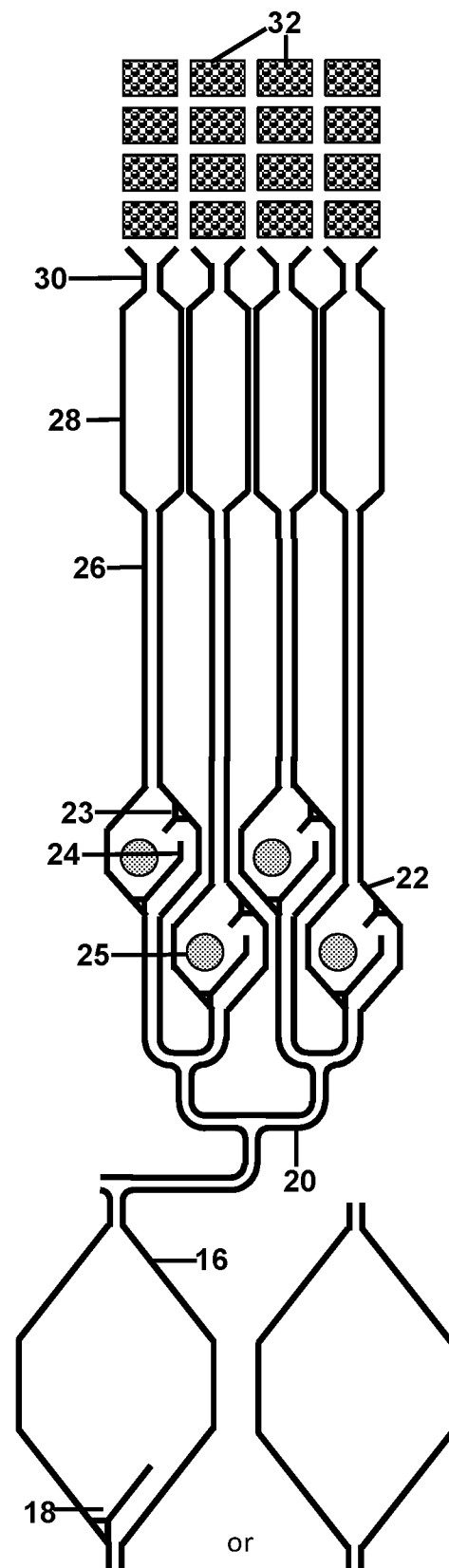
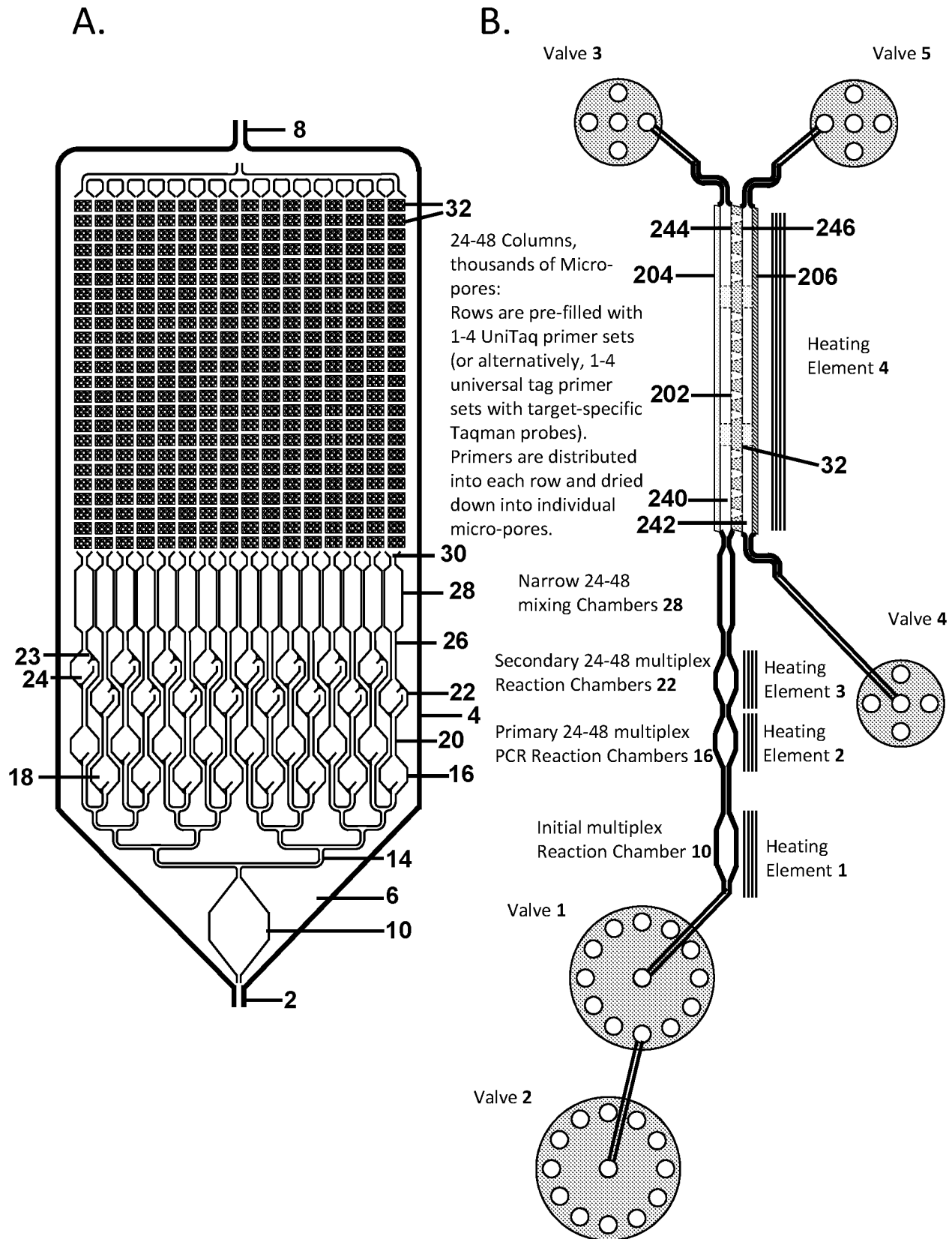


Figure 24



Figures 25A-25B

- A. PCR-PCR-qPCR (Taqman) for unknown bacterial pathogen identification directly from blood. Isolate genomic DNA.
- B. Distribute initial sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns). Perform initial multiplexed amplification of pathogen-specific regions. Use tandem or more PCR primer sets. Primers contain identical 8-11 base tails to prevent primer dimers.
- C. Perform nested PCR using primers with Universal tag tails in Secondary PCR Reaction Chambers. Primers are unblocked with RNaseH2 only when bound to correct target.
- D. PCR products comprise of Ai tag sequence, pathogen-specific sequence, and Ci tag sequence. Distribute products of each Secondary PCR Reaction Chamber into 384 or 768 micro-pores (16 or 32 rows). Universal tag primers in each row will amplify only those products from each Secondary PCR Reaction Chamber (columns) with the correct tags.
- E. Amplify authentic pathogen-specific products with Tag-specific primers (Ai and Ci). Each Taqman probe spans a unique pathogen-specific sequence.
- F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal.

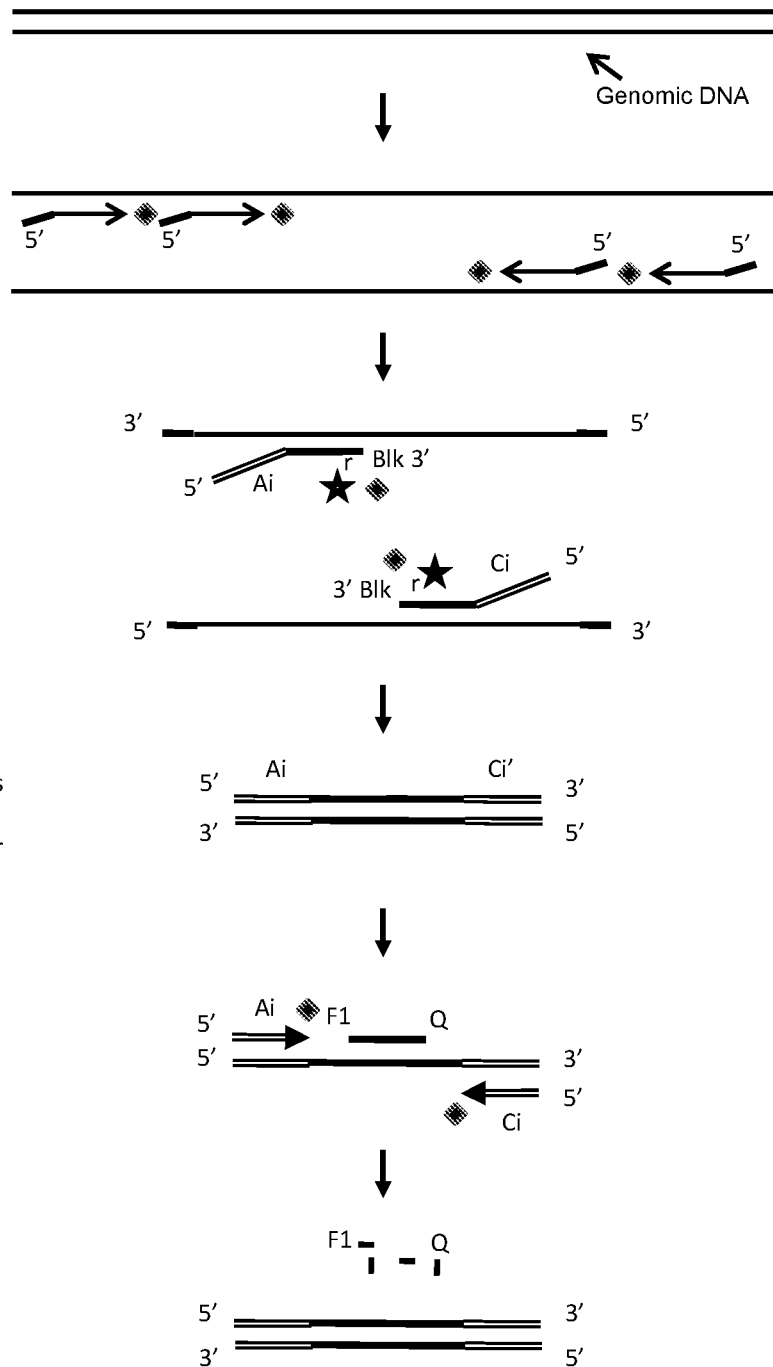


Figure 26

- A. PCR-PCR-qPCR (UniTag) for unknown bacterial pathogen identification directly from blood. Isolate genomic DNA.
- B. Distribute initial sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns). Perform initial multiplexed amplification of pathogen-specific regions. Use tandem or more PCR primer sets. Primers contain identical 8-11 base tails to prevent primer dimers.
- C. Perform nested PCR using primers with Universal tag tails in Secondary PCR Reaction Chambers. Primers are unblocked with RNaseH2 only when bound to correct target.
- D. PCR products comprise of Ai tag sequence, pathogen-specific sequence, Bi', and Ci tag sequence. Distribute products of each Secondary PCR Reaction Chamber into 384 or 768 micro-pores (16 or 32 rows). UniTag primers in each row will amplify only those products from each Secondary PCR Reaction Chamber (columns) with the correct tags.
- E. Amplify authentic pathogen-specific products with UniTag-specific primers (F1-Bi-Q-Ai and Ci).
- F. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- G. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'→3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal.

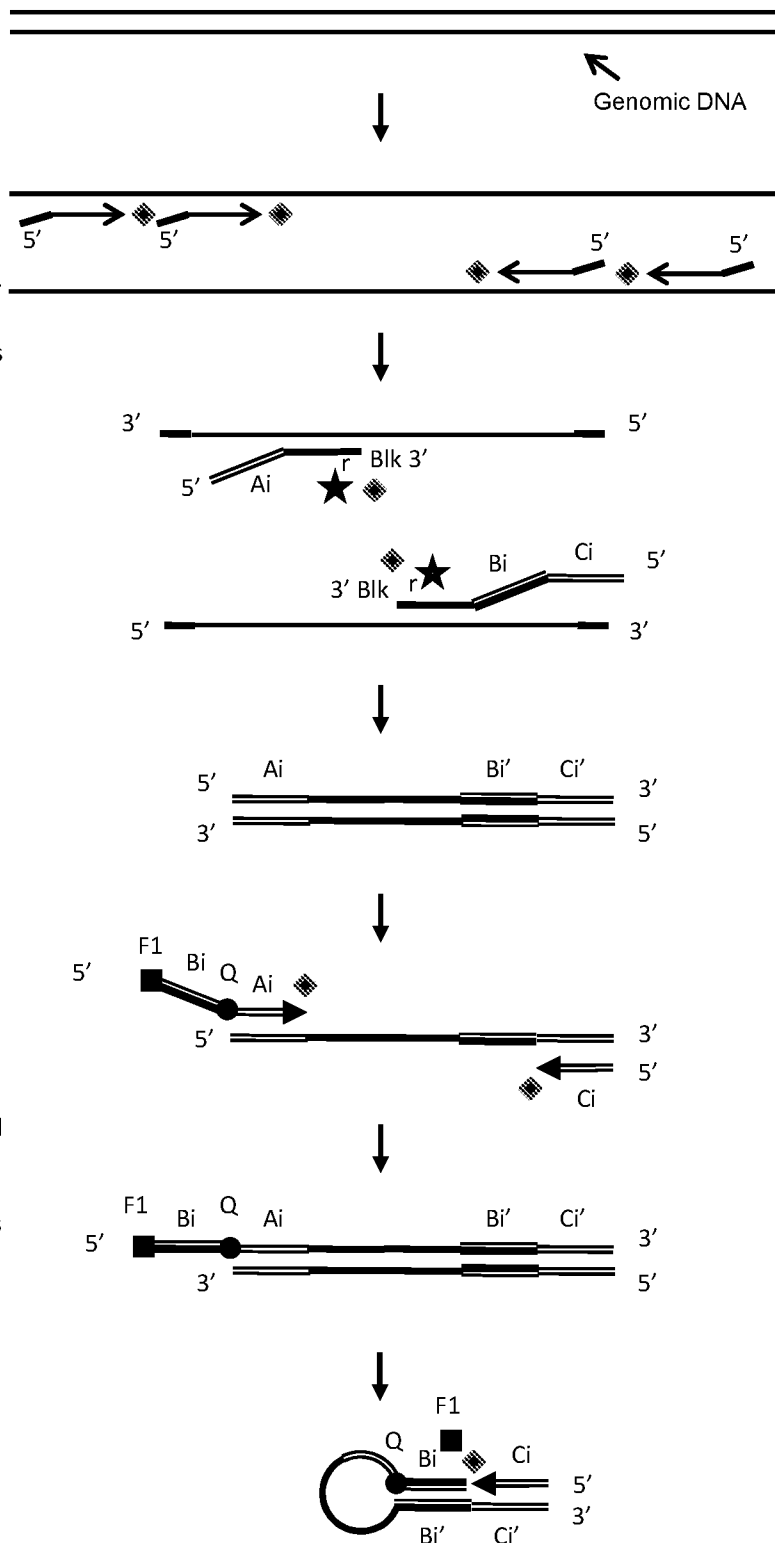


Figure 27

- A. PCR-LDR-qPCR (Taqman) reaction (with optional carryover prevention) to detect low-level mutation. Isolate genomic or cfDNA.
- B. Distribute initial sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns). Treat with UDG for carryover prevention. Upstream locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Blocking LNA or PNA Wt probes facilitate preferential amplification of mutation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers.
- C. PCR products incorporate dU, allowing for carryover prevention. Distribute products into Secondary LDR Reaction Chambers.
- D. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Ci') for subsequent PCR amplification. Blocking LNA or PNA Wt probes suppress ligation to Wt sequence. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the unblocked mutation-specific upstream and phosphorylated downstream oligonucleotides together in each Secondary LDR Reaction Chamber.
- F. Add Taqman mastermix with UDG for carryover prevention, which also destroys original target amplicons. Distribute products of each Secondary LDR Reaction Chamber into 384 or 768 micro-pores. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci'). Each Taqman probe spans the ligation junction.
- G. The 5'→3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

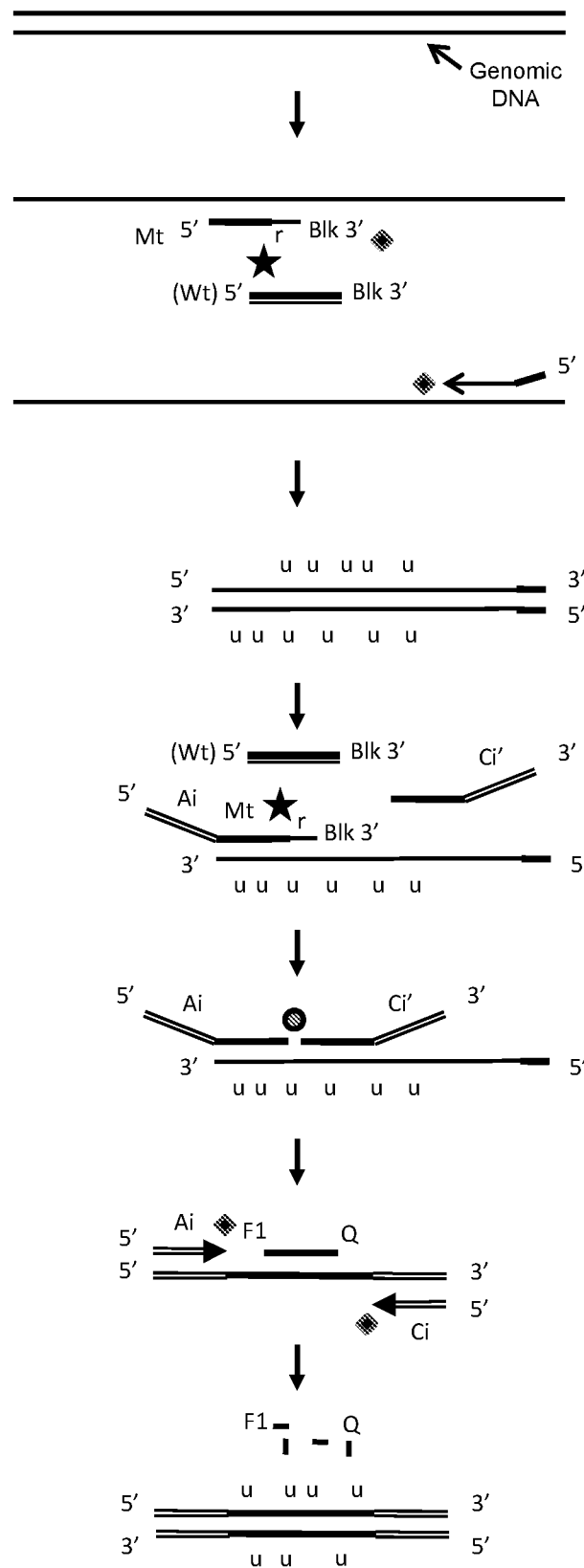


Figure 28

- A. PCR-LDR-qPCR (UniTa_q) reaction (with optional carryover prevention) to detect low-level mutation. Isolate genomic or cfDNA.
- B. Distribute initial sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns). Treat with UDG for carryover prevention. Upstream locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Blocking LNA or PNA Wt probes facilitate preferential amplification of mutation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers.
- C. PCR products incorporate dU, allowing for carryover prevention. Distribute products into Secondary LDR Reaction Chambers.
- D. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Bi', Ci') for subsequent PCR amplification. Blocking LNA or PNA Wt probes suppress ligation to Wt sequence. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the unblocked mutation-specific upstream and phosphorylated downstream oligonucleotides together in each Secondary LDR Reaction Chamber.
- F. Add Taqman mastermix with UDG for carryover prevention, which also destroys original target amplicons. Distribute products of each Secondary LDR Reaction Chamber into 384 or 768 micro-pores. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, Ci).
- G. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- H. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'→3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

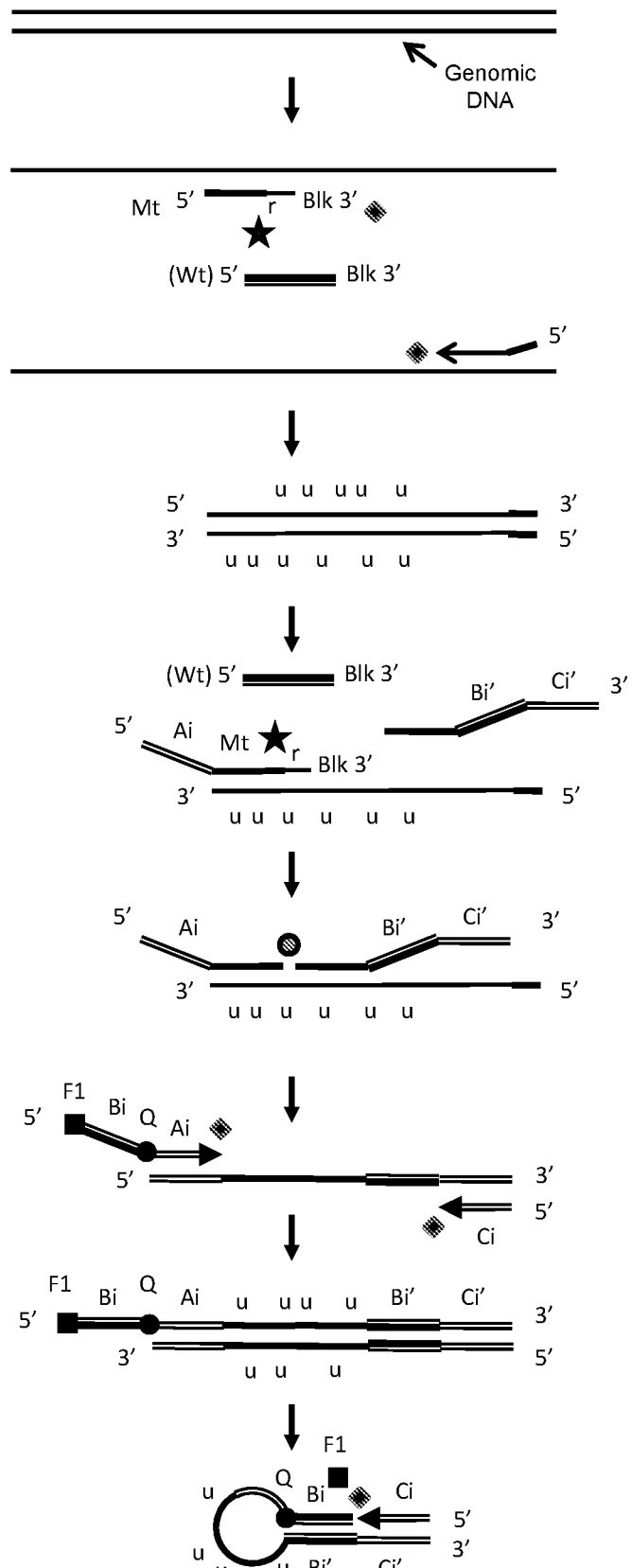


Figure 29

- A. Mutations and Methylations at low-level in plasma; using Multiplexed PCR - LDR- UniTaq detection. (Alternatively, Multiplexed PCR – LDR- Real-time-PCR with mutation or methylation-specific Taqman probes.)
- B. Preparation: Rows are pre-filled with 1-4 UniTaq primer sets (or alternatively, 1-4 universal tag primer sets with mutation or methylation-specific Taqman probes). Primers are distributed into each row and dried down into individual micro-pores.
- C. Preparation: Pre-fill pre-chambers to columns with LDR primer sets with either UniTaq or universal tag sequences on their non-ligating 5' (upstream) and 3' (downstream) ends, and dry down. (Alternatively, when using identical LDR primer sets in each pre-chamber, they may be added after the PCR step.)
- D. Digest half of cfDNA with Bsh1236I to cleave unmethylated DNA in the Initial Reaction Chamber. Treat with Bisulfite. Re-purify DNA strands.
- E. Distribute initial cfDNA and Bsh1236I/bisulfite treated cfDNA into 24 Primary PCR Reaction Chambers. Highest level of DNA in plasma = 10,000 genome equivalents. After Bsh1236I/bisulfite treatment, about 200 genome equivalents. On average, 400 copies of each untreated target, and 8 copies of each Bsh1236I/bisulfite treated target per Primary PCR Reaction Chamber, with at most 1 mutation or methylated fragment per Primary PCR Reaction Chambers. Perform 30-40 cycles of locus-specific PCR with blocking PNA or LNA to reduce amplification of wild-type DNA (for mutation detection, optional for methylation detection).
- F. Dilute products of each Primary PCR Reaction Chamber into pre-dried LDR primers and buffers of each Secondary LDR Reaction Chamber. Perform 20 cycles of LDR using mutation-specific or methylation-specific primers with UniTaq tags, in groups of 16, 32, or 64 primer sets. LDR primers for different methylation regions, i.e. top and bottom strand of the same promoter region may be designed to give the same signal in the same Secondary LDR Reaction Chamber.
- G. Add Taqman master-mix with UDG and distribute LDR products into each mixing chamber, and into 384 or 768 micro-pores of each column. Universal or UniTaq primers in each row will amplify only those products from each column with the correct tags. Presence or absence of specific mutations or methylations in each of the 24 columns allows for enumerating the number of low-level mutations and specific methylation in cfDNA from plasma.

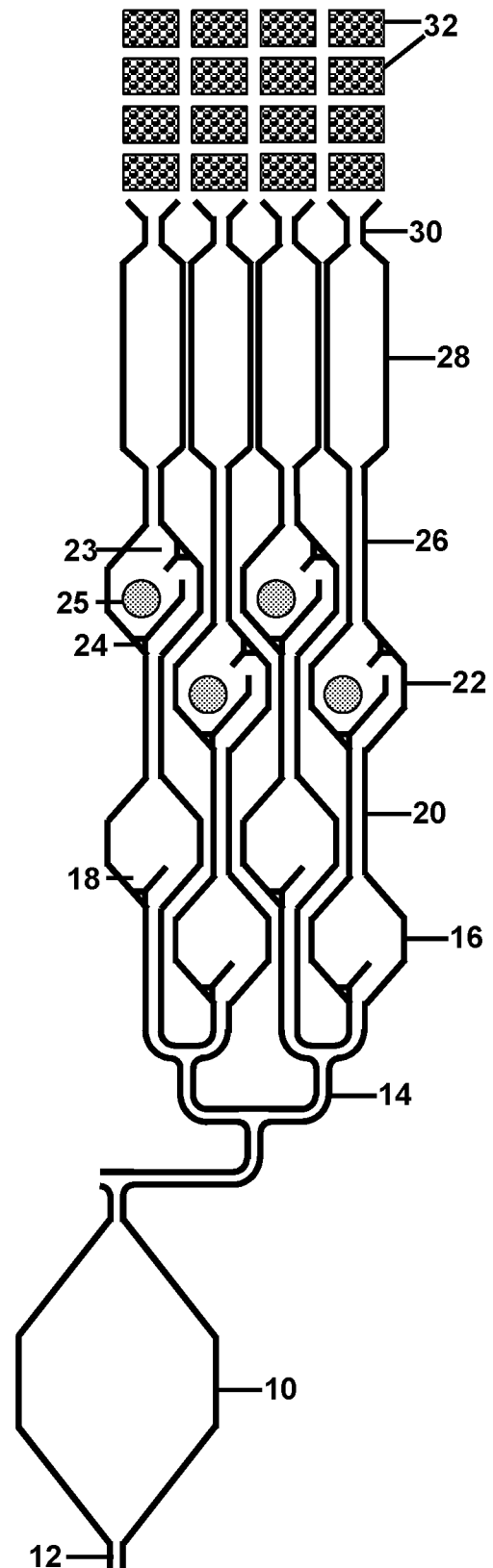


Figure 30

- A. PCR-LDR-qPCR (Taqman) reaction (with optional carryover prevention) to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG[^]CG) and UNG (37°C, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover in an Initial Reaction Chamber. Treat with bisulfite, which renders the strands non-complementary.
- B. Distribute bisulfite treated sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns) and PCR amplify. Upstream locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Downstream primers contain identical 8-11 base tails to prevent primer dimers.
- C. PCR products incorporate dU, allowing for carryover prevention. Distribute products into Secondary LDR Reaction Chambers
- D. Methylation-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the unblocked mutation-specific upstream and phosphorylated downstream oligonucleotides together in each Secondary LDR Reaction Chamber.
- F. Add Taqman mastermix with UDG for carryover prevention, which also destroys original target amplicons. Distribute products of each Secondary LDR Reaction Chamber into 384 or 768 micro-pores. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci'). Each Taqman probe spans the ligation junction.
- G. The 5'→3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

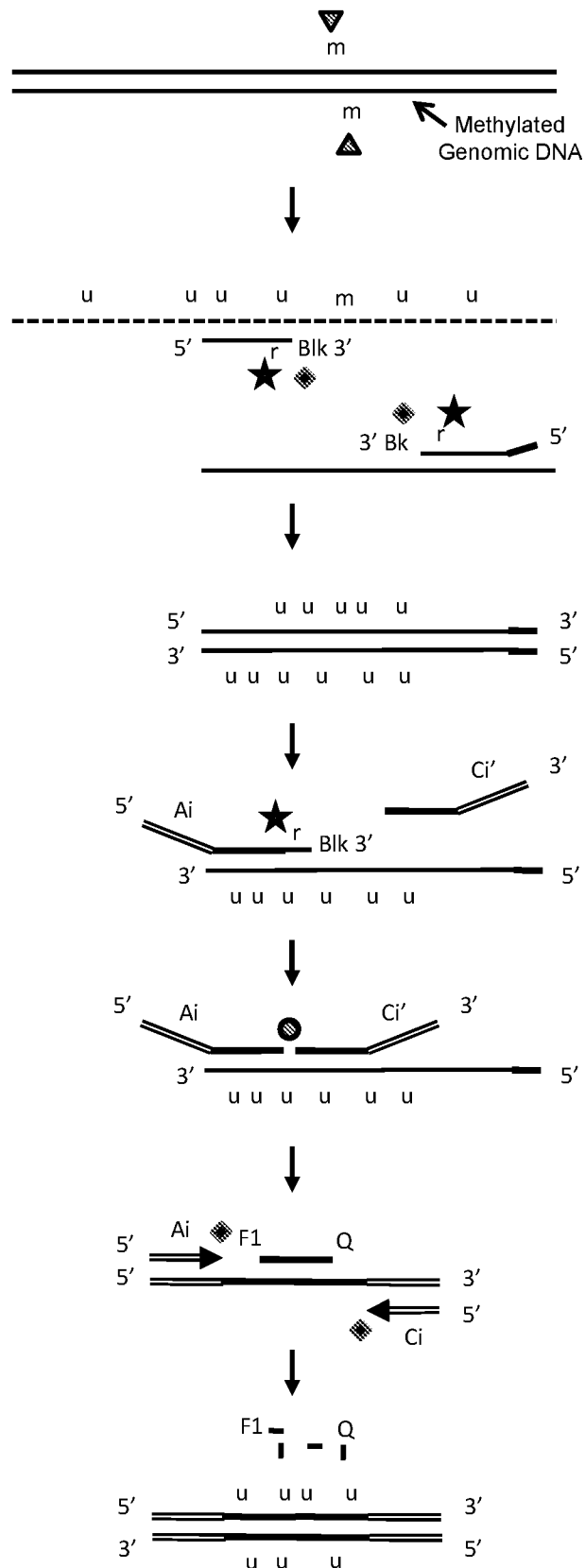


Figure 31

- A. PCR-LDR-qPCR (UniTaQ) reaction (with optional carryover prevention) to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG[^]CG) and UNG (37°C, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover in an Initial Reaction Chamber. Treat with bisulfite, which renders the strands non-complementary.
- B. Distribute bisulfite treated sample into 24, 36, or 48 Primary PCR Reaction Chambers (columns) and PCR amplify. Upstream locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Downstream primers contain identical 8-11 base tails to prevent primer dimers.
- C. PCR products incorporate dU, allowing for carryover prevention. Distribute products into Secondary LDR Reaction Chambers.
- D. Methylation-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target.
- E. Ligase covalently seals the unblocked mutation-specific upstream and phosphorylated downstream oligonucleotides together in each Secondary LDR Reaction Chamber.
- F. Add Taqman mastermix with UDG for carryover prevention, which also destroys original target amplicons. Distribute products of each Secondary LDR Reaction Chamber into 384 or 768 micro-pores. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, Ci).
- G. Double-stranded DNA products of PCR; the polymerase blocker stops extension of the bottom strand.
- H. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'→3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal.

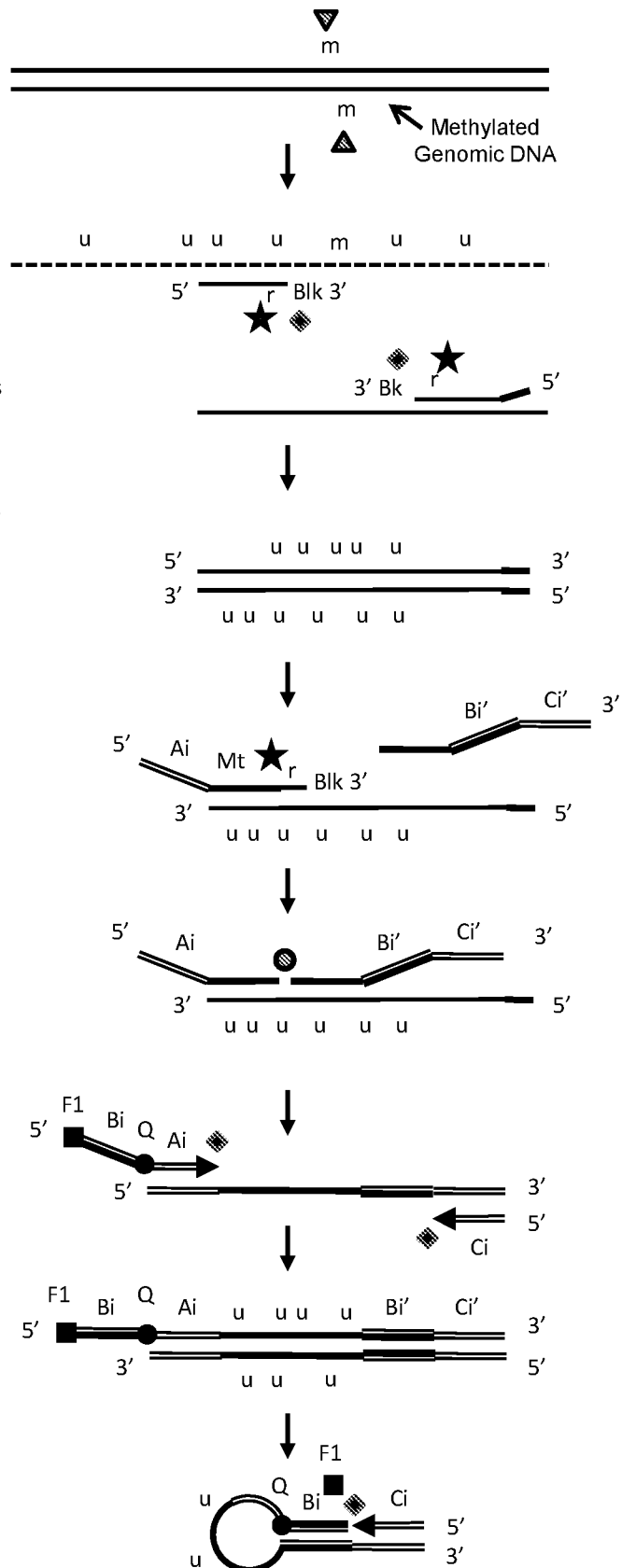
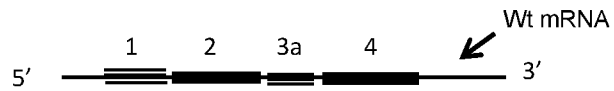
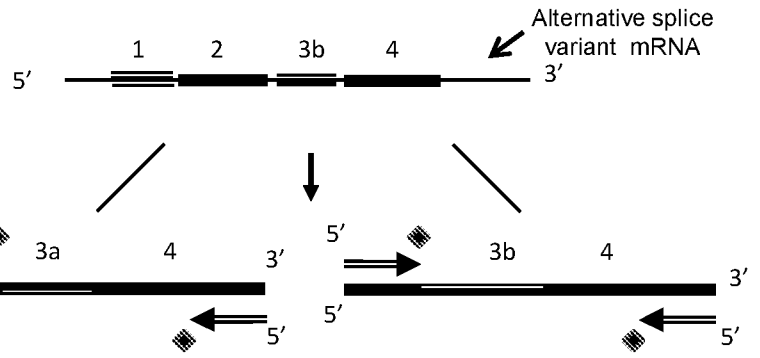


Figure 32

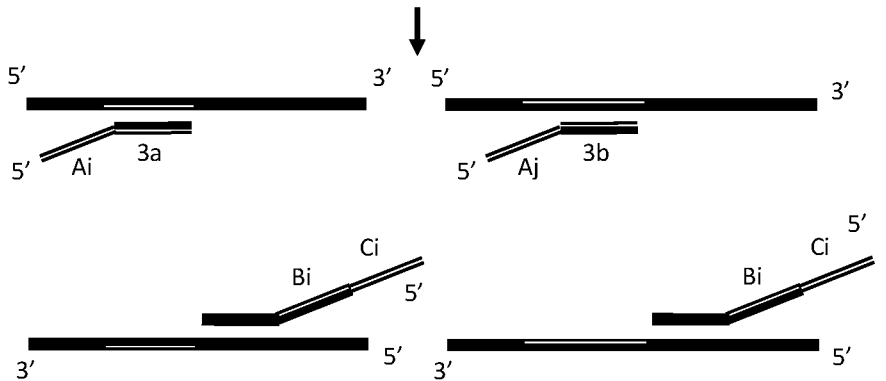
- A. RT-PCR-PCR-qPCR (Taqman) to quantify wt and alternatively spliced (3a vs. 3b) transcript. Isolate mRNA.



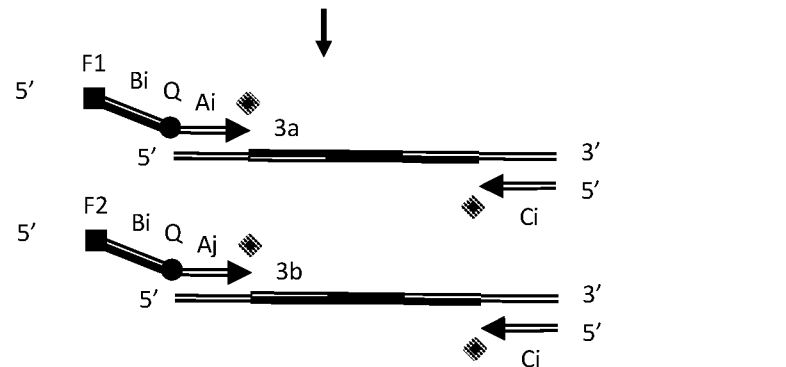
- B. Use reverse-transcriptase to make cDNA copy with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (i.e. 7 cycles) to maintain relative ratios of different amplicons in an Initial Reaction Chamber. Primers contain 5' identical 8-11 base tails to prevent primer dimers. Distribute initial multiplexed products into 6 Primary PCR Reaction Chambers.



- C. Perform nested PCR (i.e. 10 cycles) using exon junction-specific primers with Universal tag tails (Ai; Aj; and Bi-Ci). Differentially dilute products.



- D. PCR products comprise of Ai or Aj tag sequence, exon-junction-specific sequence, Bi', and Ci tag sequence. Differentially dilute products from each of the 6 Primary PCR Reaction Chambers into 4 Secondary PCR Reaction Chambers for a total of 24 chambers. Distribute products of each Secondary PCR Reaction Chamber into 384 or 768 micro-pores (16 or 32 rows). UniTaq primers in each row will amplify only those products from each Secondary PCR Reaction Chamber (columns) with the correct tags.



- E. Double-stranded DNA products of PCR (not shown); the polymerase blocker stops extension of the bottom strand.

- F. After the denaturation step, as the temperature decreases, a hairpin forms between Bi and Bi'. The 5'→3' nuclease activity of Taq polymerase extends primer Ci and liberates the fluorescent group to generate signal.

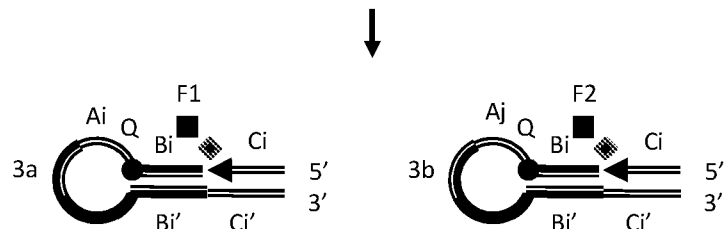


Figure 33

- A. Exact enumeration of both rare and over-expressed lncRNA, mRNA, or splice variants; using Multiplexed RT-PCR - Nested PCR - UniTaq detection. (Alternatively, Multiplexed RT-PCR - Nested PCR - Real-time-PCR with target-specific Taqman probes.)
- B. Preparation: Rows are pre-filled with 1-4 UniTaq primer sets (or alternatively, 1-4 universal tag primer sets with target-specific Taqman probes). Primers are distributed into each row and dried down into individual micro-pores.
- C. Preparation: Pre-fill reaction chambers to columns with nested PCR primer sets with either UniTaq or universal tag sequences on their 5' ends, and dry down.
- D. Multiplexed reverse-transcription PCR amplification for 7 cycles to generate 128 copies of each original target in an Initial Reaction Chamber.
- E. Distribute initial multiplex reaction into 6 Primary PCR Reaction Chambers, with average distribution of 20 copies in each Primary PCR Reaction Chamber of each original transcript. Perform 10 cycles of nested PCR using target-specific primers with UniTaq or universal tags in groups of 16, 32, or 64 primer sets. Each Primary PCR Reaction Chamber is designed to retain a certain percentage of liquid volume after draining. Perform 3 cycles of filling and draining to differentially dilute products.
- F. Dilute products from each of 6 Primary PCR Reaction Chambers into 4 Secondary Chambers each (= 24 total). Each Secondary Chamber is designed to retain a certain percentage of liquid volume after draining. Perform 3 cycles of filling and draining to differentially dilute products.
- G. Distribute nested PCR products into each mixing chamber and then into 384 or 768 micro-pores of each column. Universal or UniTaq primers in each row will amplify only those products from each column with the correct tags. Poisson distribution in micro-pores will enumerate low-copy, medium-copy, and high-copy lncRNA, mRNA, or splice variants.

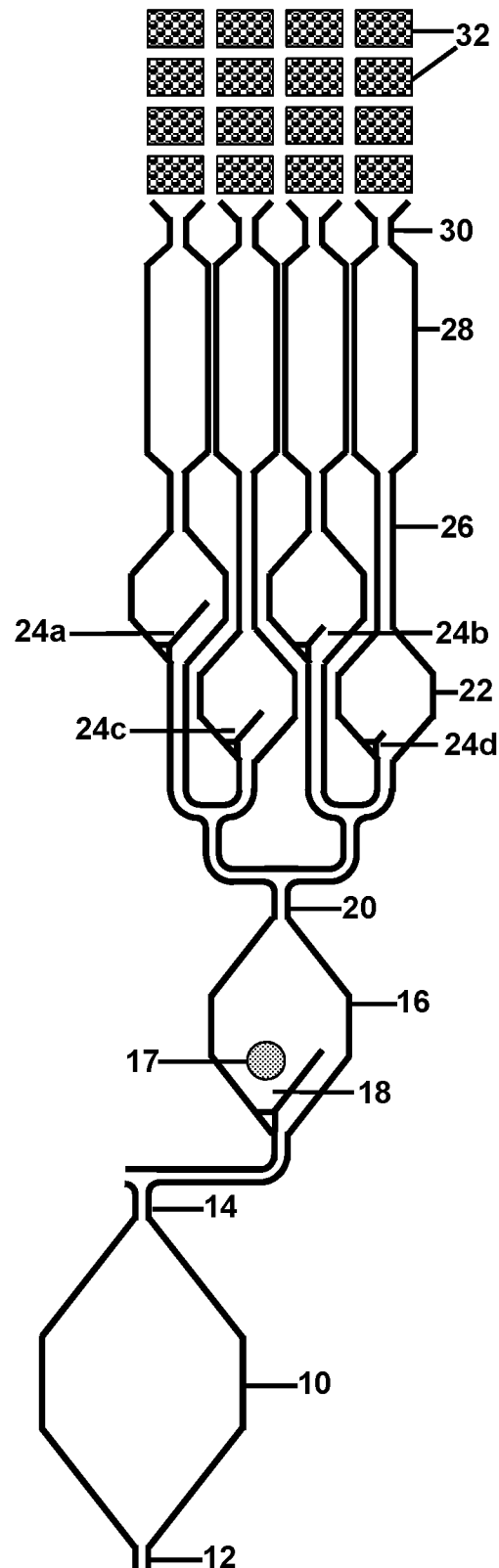
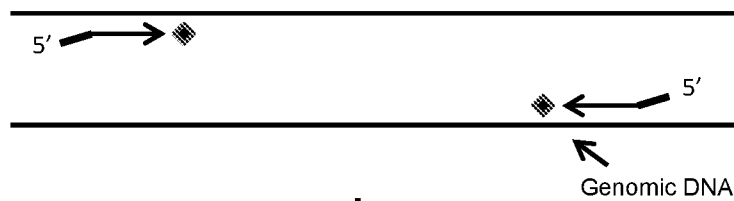
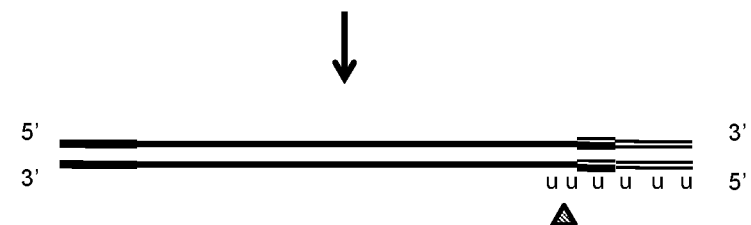
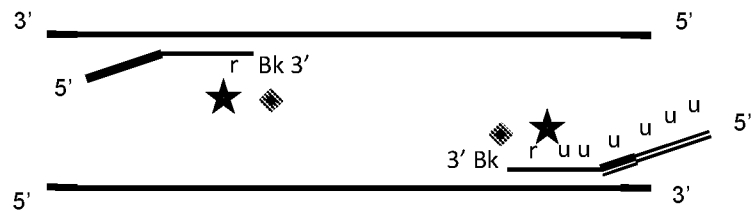


Figure 34

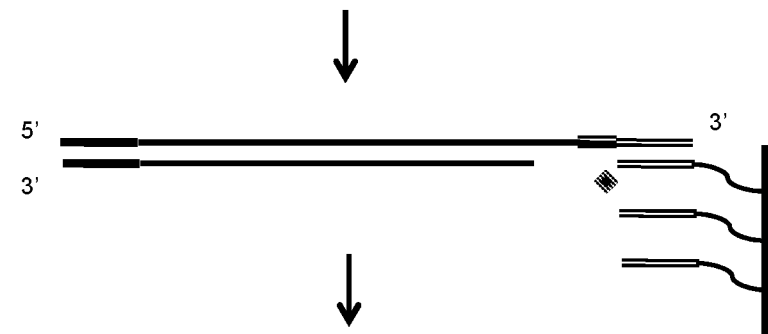
- A. Primer design for sequencing and identifying mutations in one target strand in unknown pathogens. Isolate genomic DNA. For RNA viruses, an initial reverse-transcriptase step generates cDNA. PCR amplify target regions in an Initial Reaction Chamber.



- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Nested, locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence.



- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.



- D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

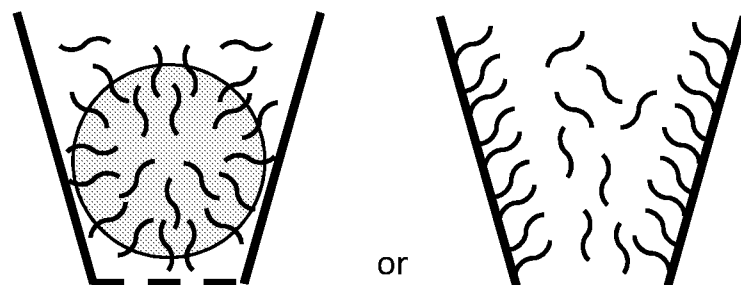
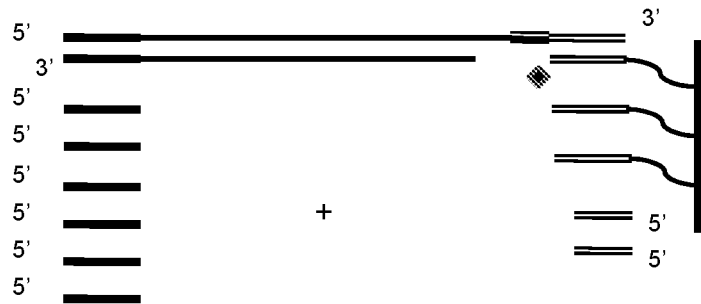
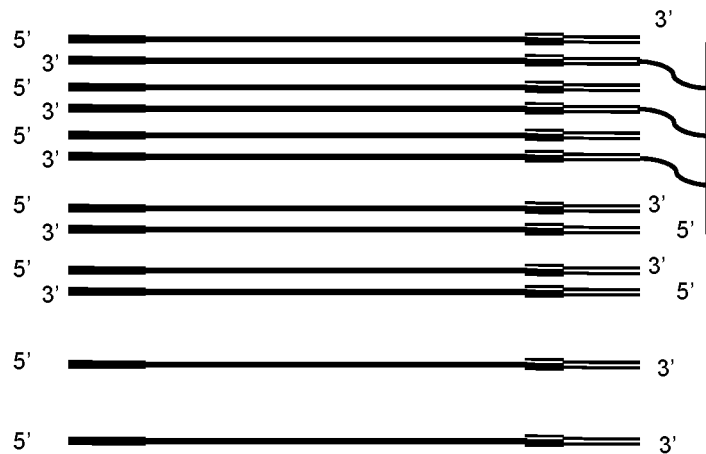


Figure 35

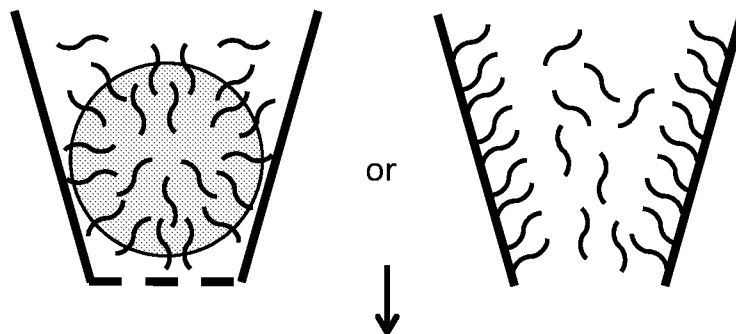
- A. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions.



- B. In solution first tag primer is in excess of both second tag primers. Immobilized primer is longer than in solution second tag primer. Optionally, at end of PCR cycles, hybridization temperature are above T_m of shorter tag primer to favor synthesis of single stranded products to hybridize to immobilized primer and drive extension of such primers to completion.



- C. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.



- D. A gene-specific (GS) primer may be used for sequencing. Alternatively, a first set of 8-16 sequencing primers may comprise a common 5' sequence (16 bases), and variable 3' sequences (8 bases). Or, a second set of 64-256 sequencing primers may comprise a common 5' sequence (8 bases), a variable middle sequence (8 bases, 8-16 variants) and hyper-variable 3' sequences (8 bases, 64-256 variants).

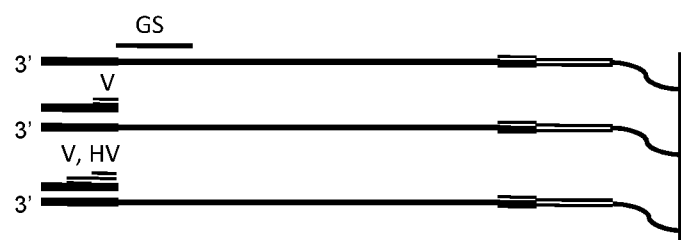
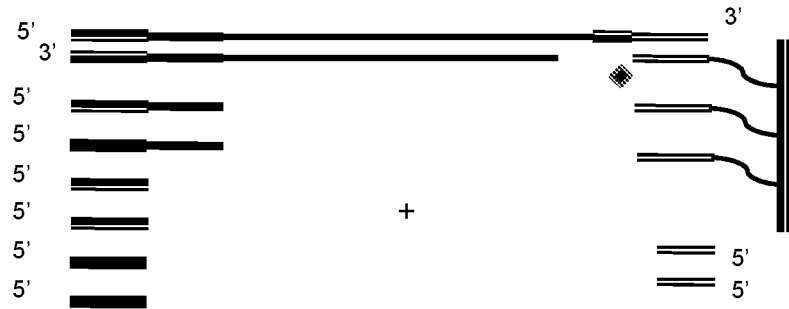
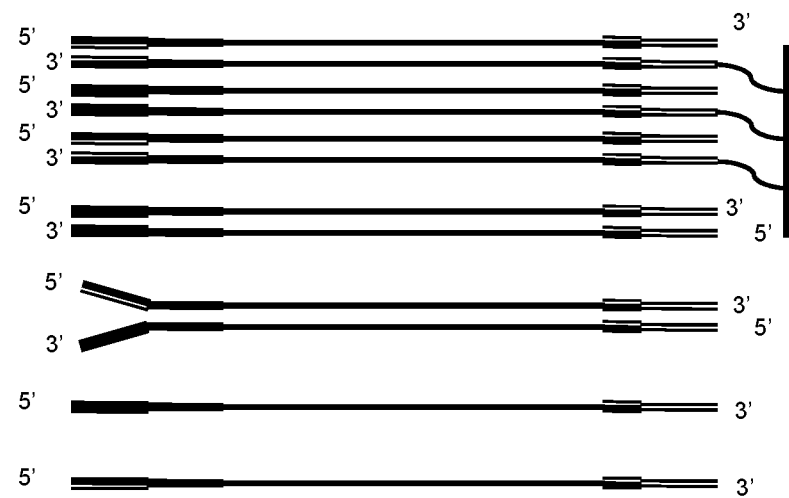


Figure 36

- A. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions.



- B. In solution first tag primers comprise two different 5' portions, and with added 5' portion primers, which are in excess of both second tag primers. Immobilized primer is longer than in solution second tag primer. Using strand-displacement polymerase lacking 5'-3' nuclease activity, perform combined isothermal and thermo-cycling amplification. Re-annealing of products with different 5' portions enables strand displacement amplification and helps drive extension of immobilized primers to completion.



- C. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

- D. A gene-specific (GS) primer may be used for sequencing. Alternatively, a first set of 8-16 sequencing primers may comprise a common 5' sequence (16 bases), and variable 3' sequences (8 bases). Or, a second set of 64-256 sequencing primers may comprise a common 5' sequence (8 bases), a variable middle sequence (8 bases, 8-16 variants) and hyper-variable 3' sequences (8 bases, 64-256 variants).

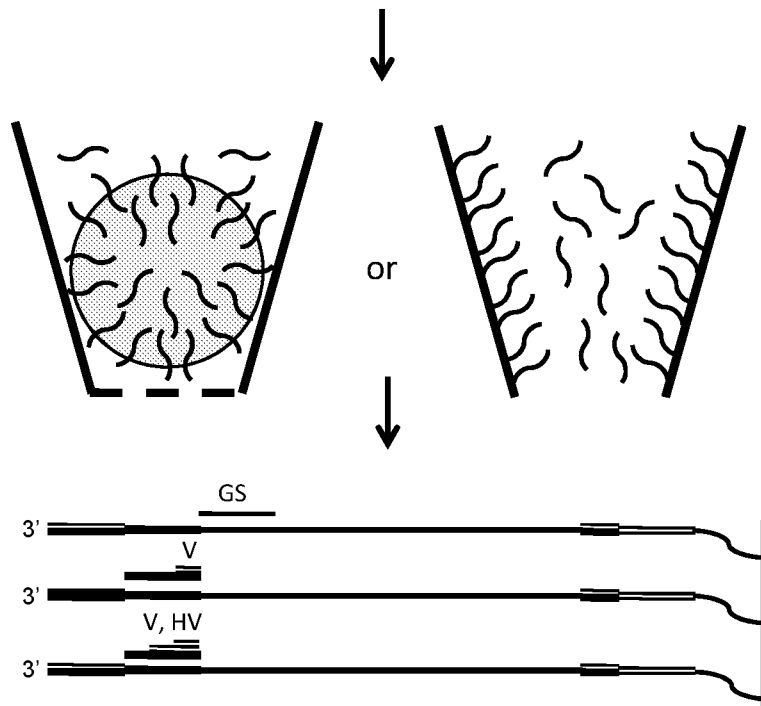
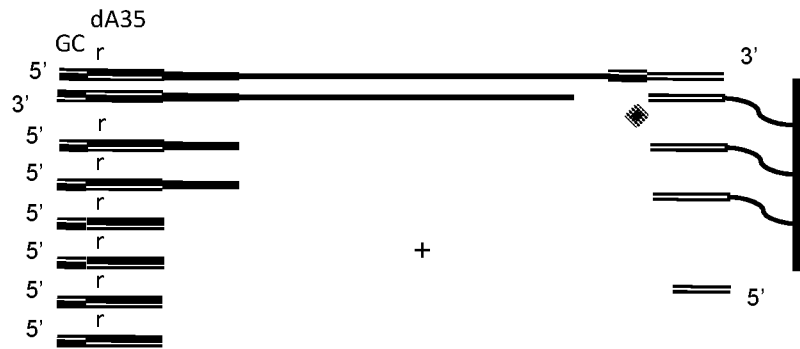
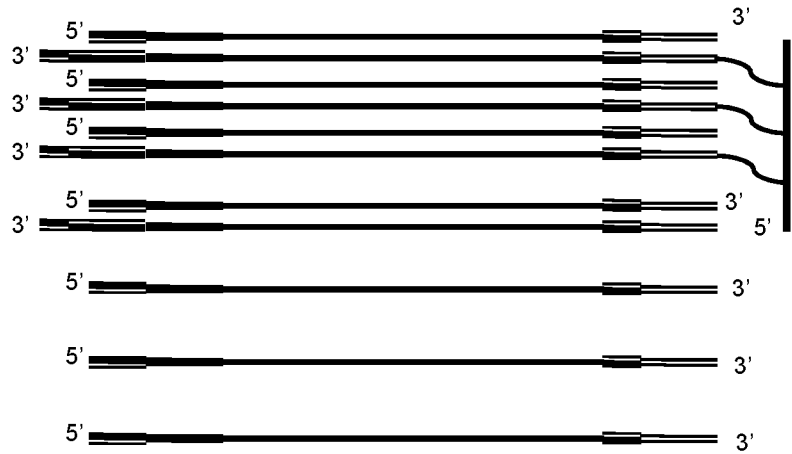


Figure 37

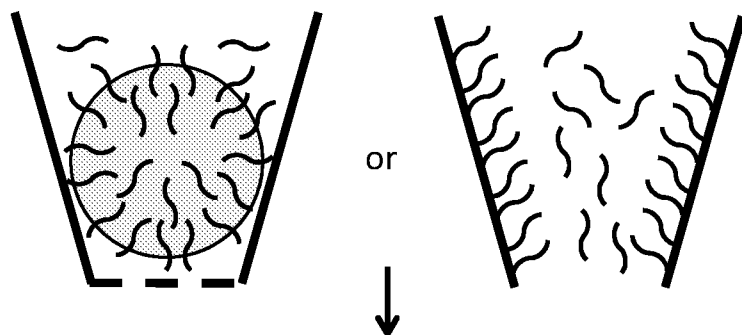
- A. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions.



- B. In solution first tag primer comprises dA35, and with added dA35 with GC rich toehold primer, are in excess of both second tag primers. Immobilized primer is longer than in solution second tag primer. Using strand-displacement polymerase lacking 5'-3' nuclease activity, perform isothermal and/or thermo-cycling amplification. Primer toehold is released with RNaseH2 only when bound to target. Excess single-stranded product hybridizes to immobilized primer and helps drive extension of immobilized primers to completion.



- C. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.



- D. A gene-specific (GS) primer may be used for sequencing. Alternatively, a first set of 8-16 sequencing primers may comprise a common 5' sequence (16 bases), and variable 3' sequences (8 bases). Or, a second set of 64-256 sequencing primers may comprise a common 5' sequence (8 bases), a variable middle sequence (8 bases, 8-16 variants) and hyper-variable 3' sequences (8 bases, 64-256 variants).

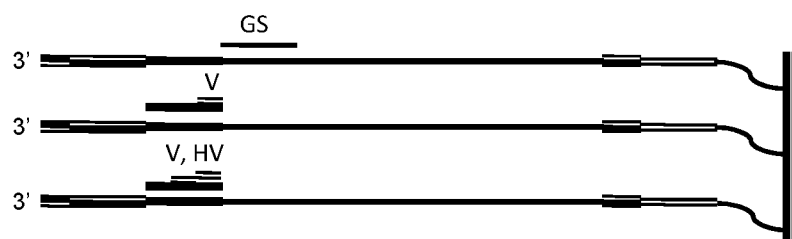


Figure 38

- A. Unknown pathogen identification, quantification and genotyping; using Multiplexed PCR - Nested PCR – sequencing.
- B. Preparation: Rows are pre-filled with one or more universal tag primer sets, where one primer is immobilized and the other primer is bound, but released at higher temperature. Primers are distributed into each row and dried down into individual micro-pores.
- C. Preparation: Pre-fill pre-chambers to columns with nested PCR primer sets with universal tag sequences on their 5' ends, and dry down.
- D. Multiplexed PCR amplification (or reverse-transcription-PCR for RNA targets) for 10 cycles to generate 1,024 copies of each original target in an Initial Reaction Chamber.
- E. Distribute initial multiplex reaction into 48 Primary PCR Reaction Chambers, with average distribution of 20 copies in each Primary PCR Reaction Chamber of each original pathogen. Perform 5 cycles of nested PCR using target-specific primers with universal tags in groups of 32, or 64 primer sets, with average of 640 copies of each pathogen. Remove universal primer sequence from product with UDG/APE1, facilitates hybridization to immobilized primer in micro-pore.
- F. Distribute nested PCR products with single-stranded tails into mixing chambers and then into micro-pores of each column. PCR amplify one or more products in each micro-pore and melt off non-anchored strand. Universal primers in each row will amplify only those products from each column with the correct tags.
- G. Add either target-specific, or universal tag-specific sequencing primers. Perform sequencing-by-synthesis. Poisson distribution in micro-pores will enumerate target sequences, while direct sequence information will identify variant pathogens.
- H. Alternative approach, where all micro-pores are pre-filled with a single universal primer, which is immobilized, and micro-pores are dried.
- I. Preparation: Pre-fill pre-chambers to columns with nested PCR primer sets with 8-12 unique tag sequence on one primer of the set, and universal sequences on their 5' ends, and dry down.
- J. Initial multiplexed PCR amplification, distribution into 48 Primary PCR Reaction Chambers, nested PCR using target-specific primers, distribution into mixing chambers and then into micro-pores, and PCR amplification in micro-pores is as described above.
- K. Add either target-specific, or universal primers with unique tag-specific portions as sequencing primers. Perform sequencing-by-synthesis. Poisson distribution in micro-pores will enumerate target sequences, while direct sequence information will identify variant pathogens

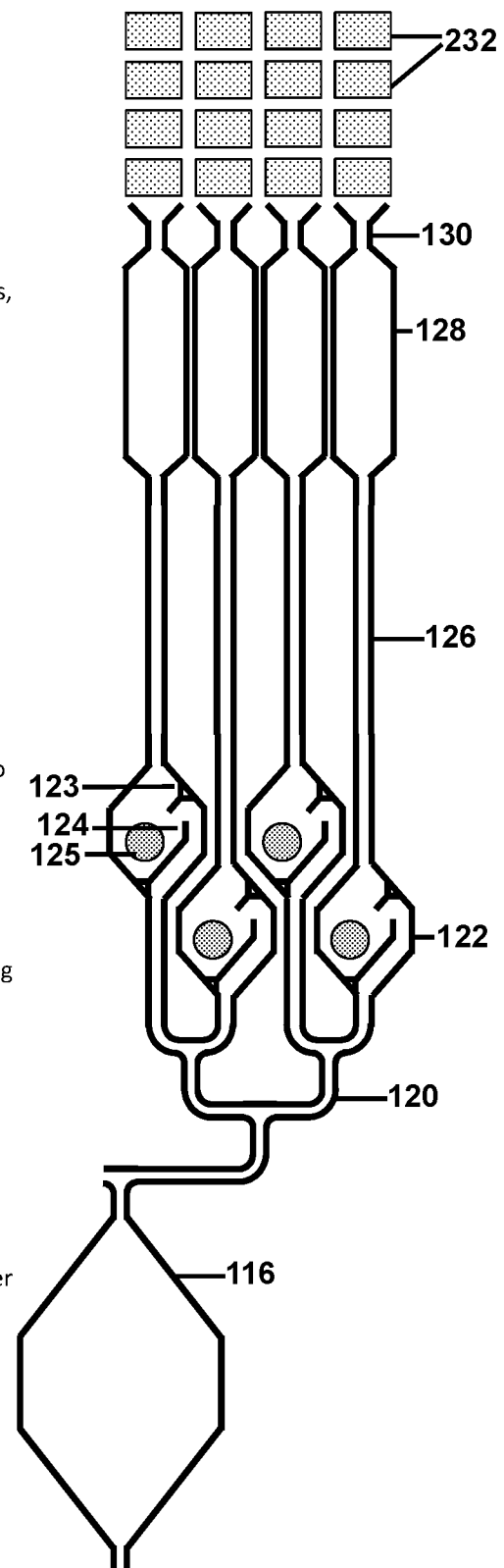


Figure 39

- A. Primer design for sequencing and identifying mutations in one target strand. cfDNA of average length of about 160 bp.
- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.
- D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

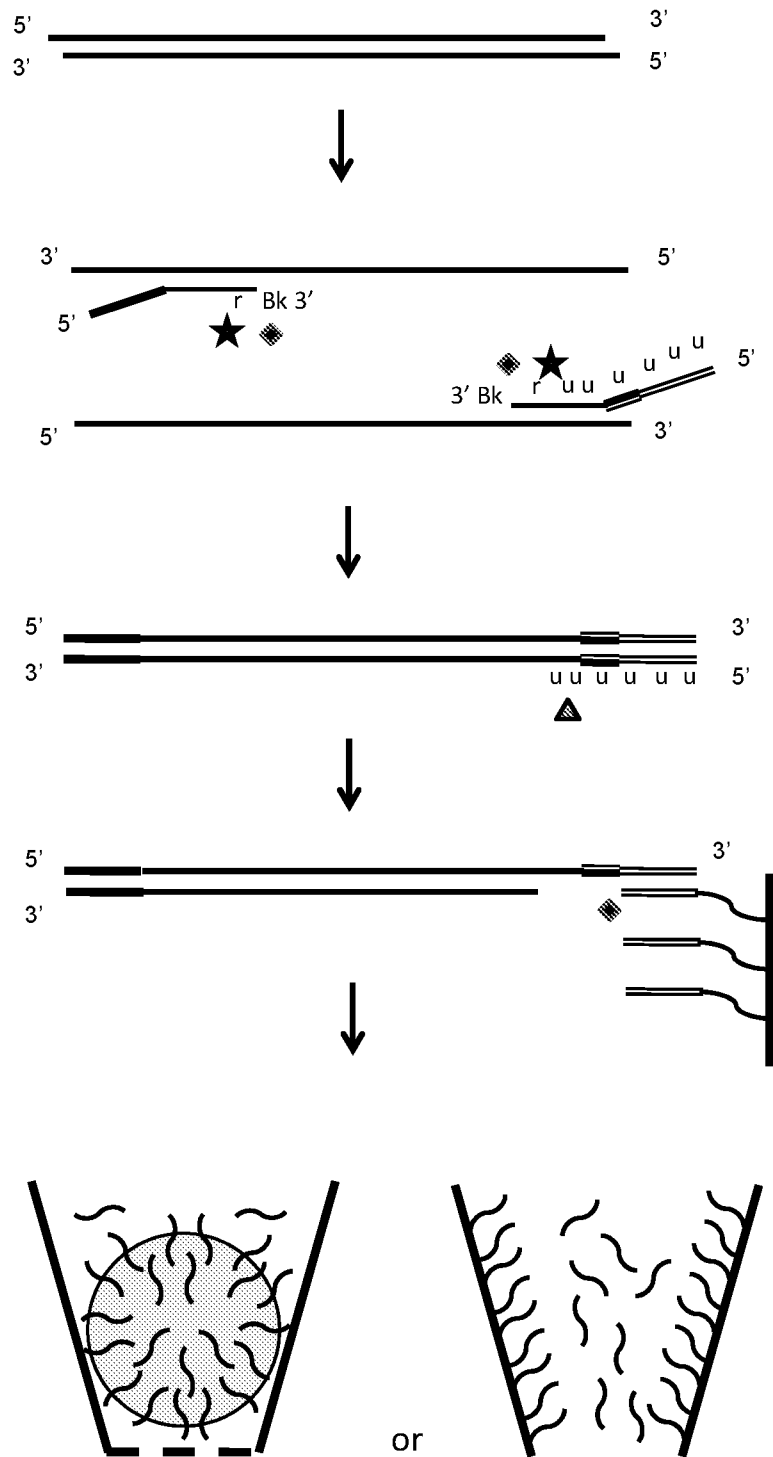


Figure 40

- A. Primer design for sequencing and identifying mutations in one target strand, across overlapping fragments. cfDNA of average length of about 160 bp.
- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence, which differs slightly from first tag sequence.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.
- D. Anneal biotinylated primer containing second tag sequence. Strand displacement polymerase extends to form full length double-stranded copy of fragment.
- E. Both extended and free biotinylated primers are captured on streptavidin coated beads to be distributed in micro-pores, or directly on streptavidin coated micro-pores. In the presence of both first and second tag primers, longer products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region. Shorter products form panhandles and do not amplify. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

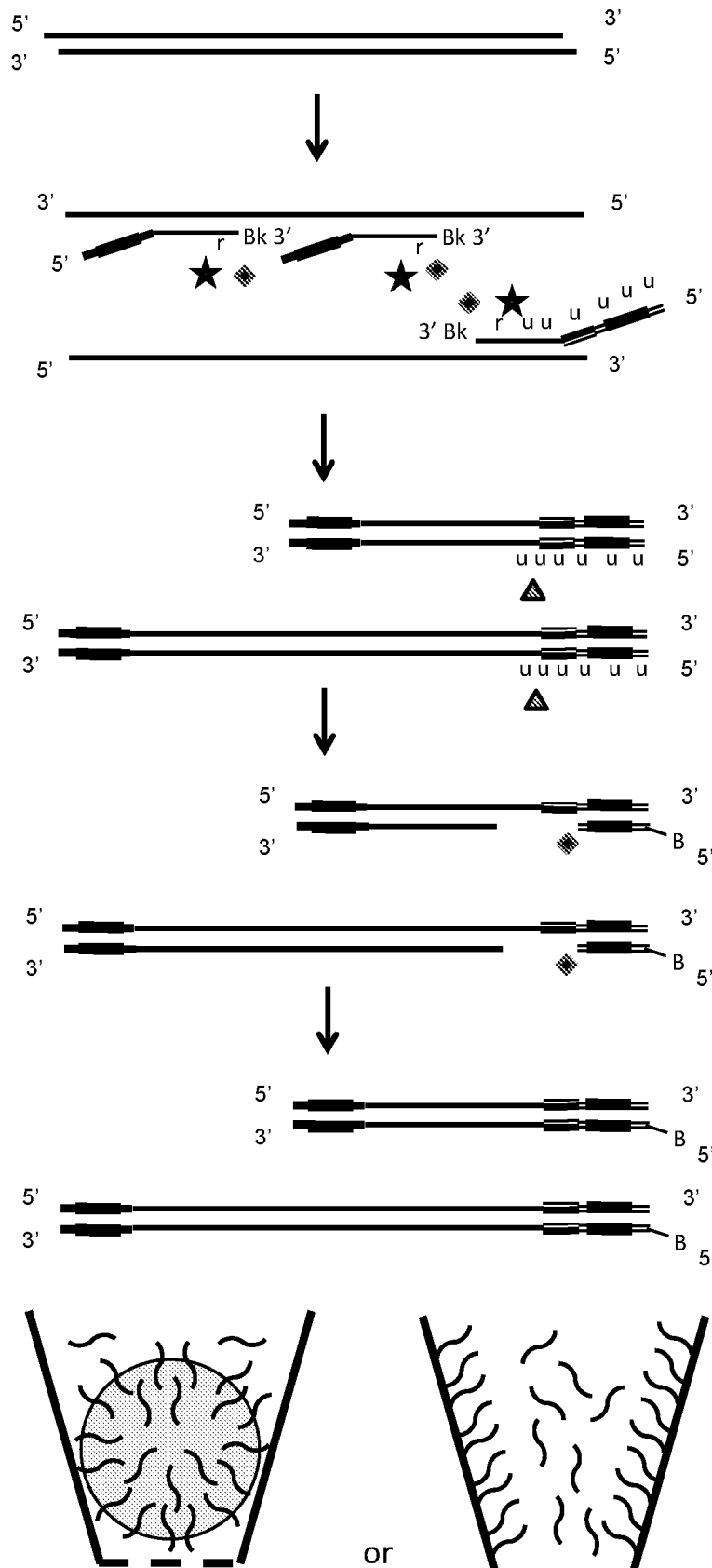


Figure 41

A. Primer design for sequencing and identifying mutations in one target strand, across overlapping fragments. cfDNA of average length of about 160 bp.

B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence, which differs slightly from first tag sequence.

C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.

D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, longer products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region. Shorter products form panhandles and do not amplify. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

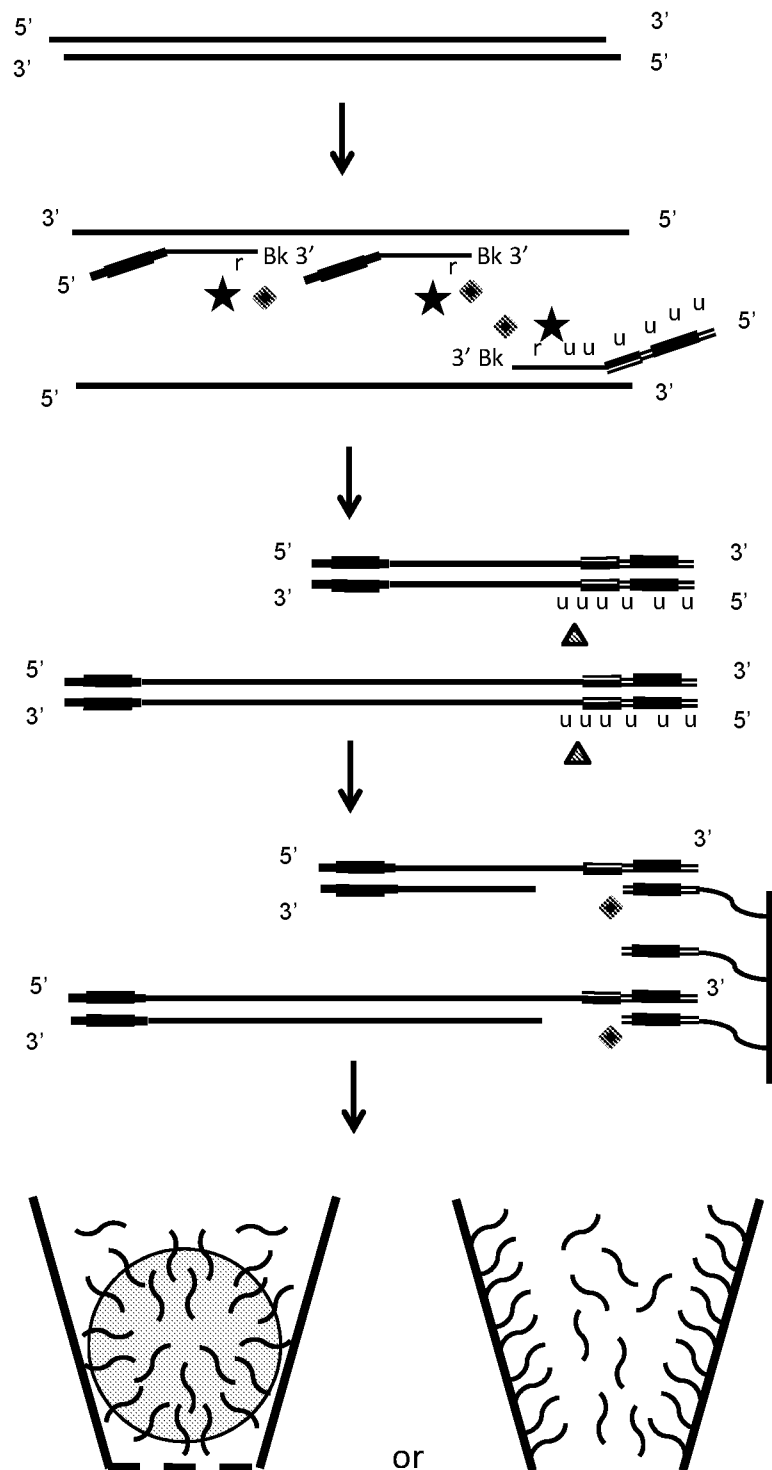
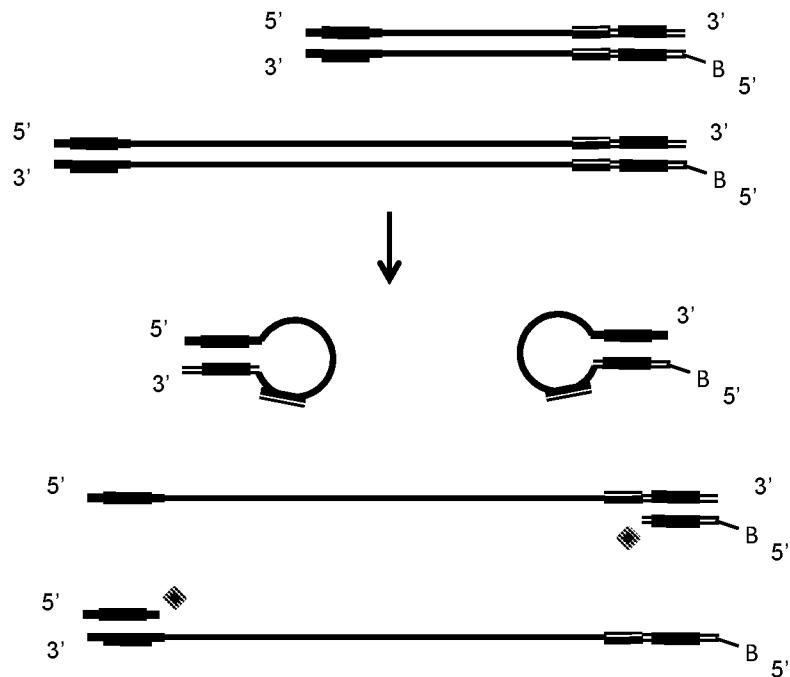


Figure 42

- A. Close-up illustrating primer design where shorter amplicons form panhandles, which do not amplify, while the desired longer products amplify on the solid support. Illustrated with capture of biotin primer.



- B. Close-up illustrating primer design where shorter amplicons form panhandles, which do not amplify, while the desired longer products amplify on the solid support. Illustrated with primers immobilized to the solid support.

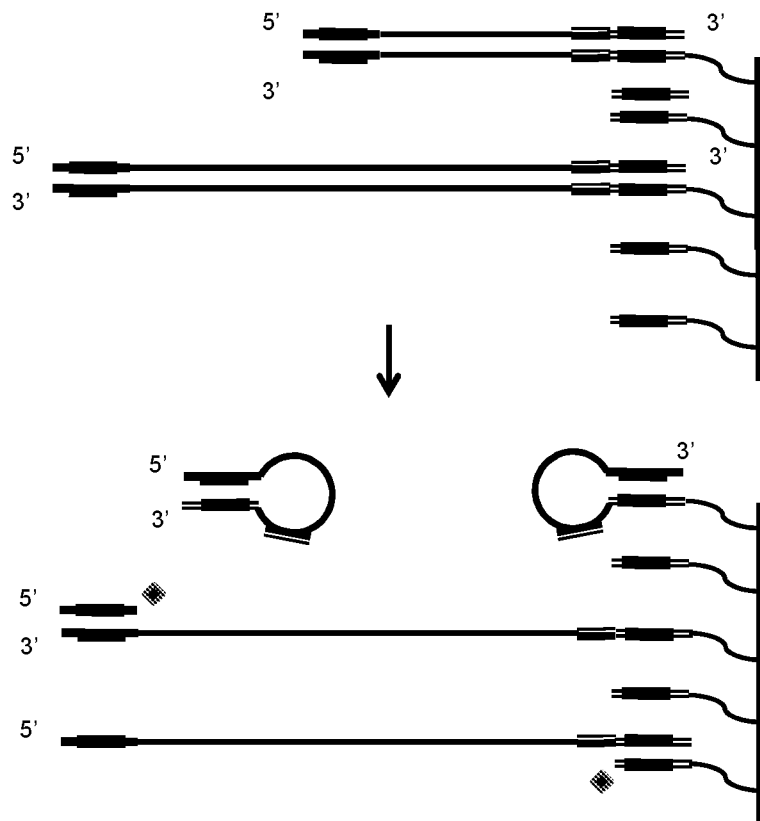


Figure 43

- A. Primer design for sequencing and identifying mutations in one target strand, across overlapping fragments. cfDNA of average length of about 160 bp.

- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence.

- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.

- D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, longer products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region. Shorter products form panhandles, or are missing a second tag sequence and do not amplify. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

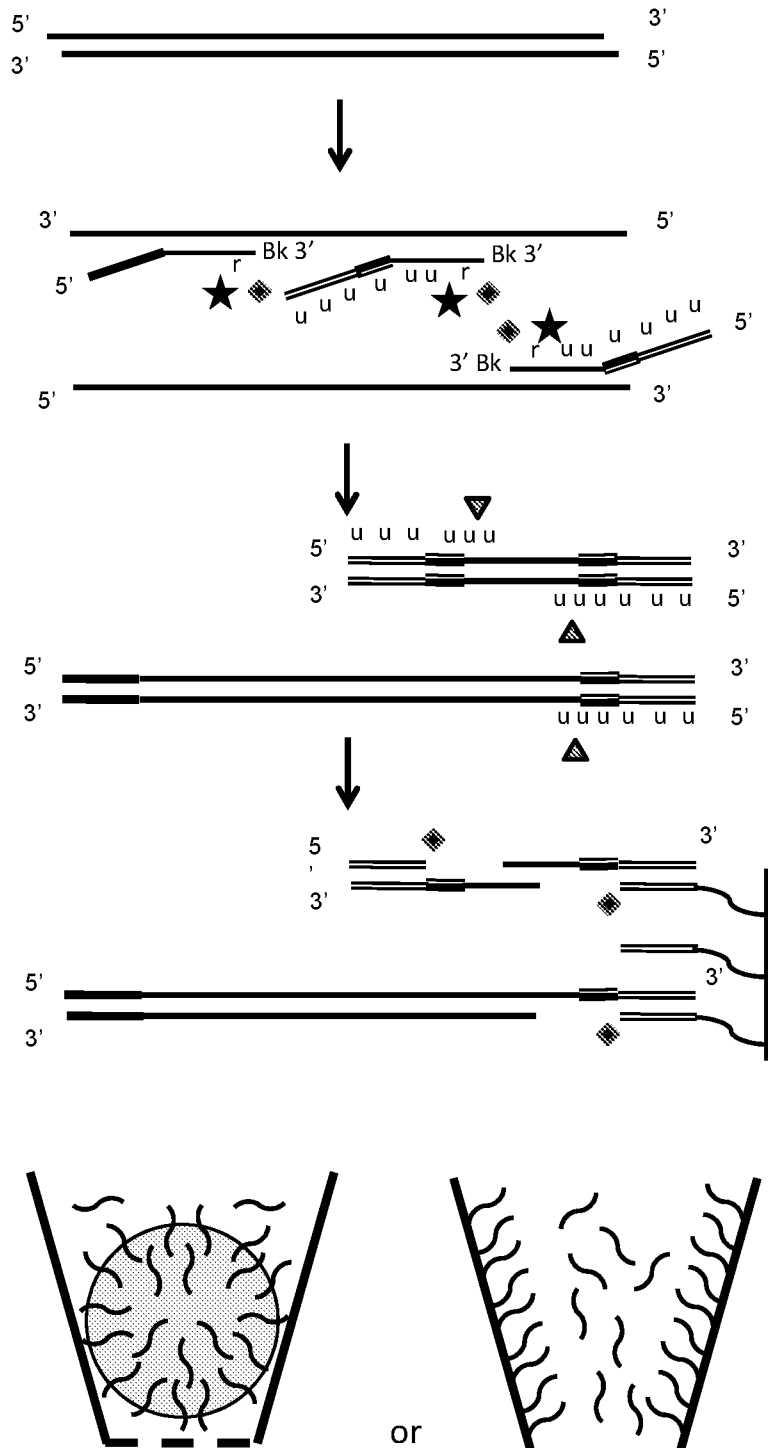


Figure 44

- A. Primer design for sequencing and identifying mutations in one target strand, across overlapping fragments. cfDNA of average length of about 160 bp.
- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.
- D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, longer products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region. Shorter products form panhandles, or are missing a second tag sequence and do not amplify. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

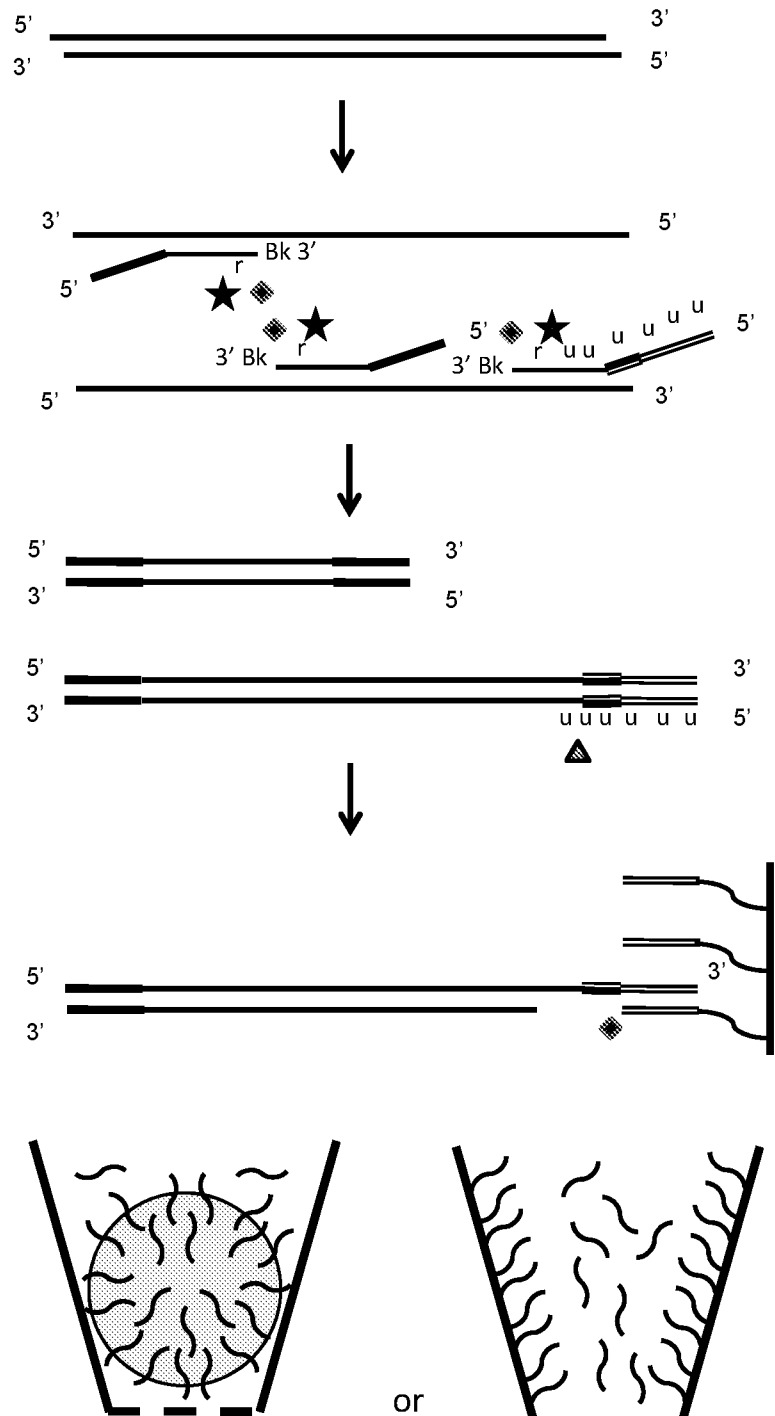


Figure 45

- A. Primer design for sequencing and identifying mutations in both target strands, across overlapping fragments. cfDNA of average length of about 160 bp.
- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2-3 cycles of amplification with Taq polymerase. All primers contain dU, target-specific region, fragment identifier, and the same first tag sequences. 5'-3' proofreading activity will destroy most smaller fragments.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in both short and long products.
- D. Distribute products into wells or beads into wells containing immobilized first tag sequence primers. Strand displacement polymerase extends to form double-stranded copy of both strands.
- E. Nested, target-specific primers are unblocked with RNaseH2 only when bound to target. Perform 1-2 cycles of amplification with strand-displacement thermostable polymerase. Nested target-specific primers contain second tag sequence.
- F. In the presence of both first and second tag primers, longer products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

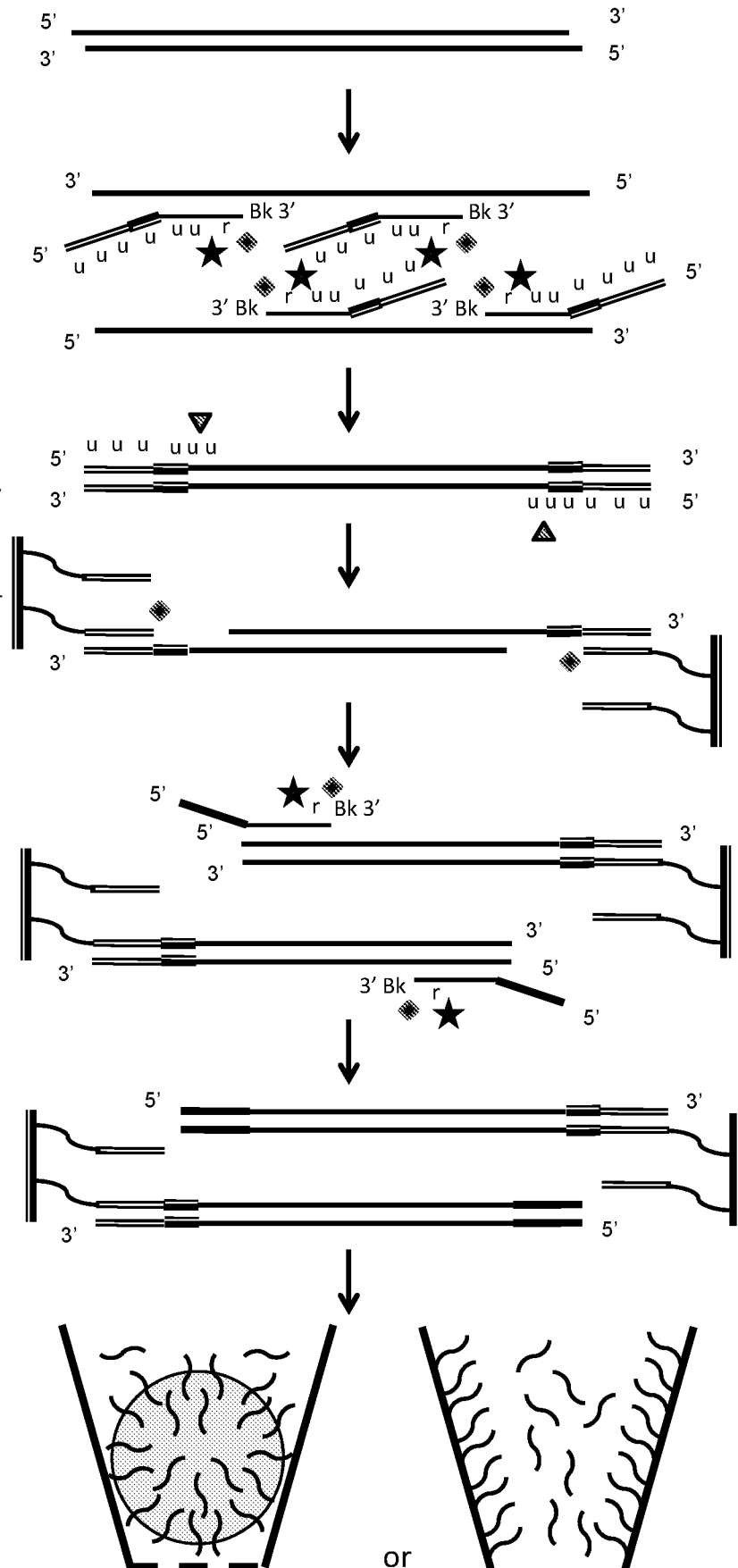


Figure 46

- A. Primer design for identifying SNPs and enumerating copy number of both locus-specific strands. cfDNA of average length of about 160 bp.
- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 3 cycles of amplification with strand-displacement thermostable polymerase. Both primers contain dU, target-specific region, fragment identifier, and the same first tag sequences.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.
- D. Distribute products into wells or beads into wells containing immobilized first tag sequence primers. Strand displacement polymerase extends to form double-stranded copy of fragments.
- E. Nested, target-specific primers are unblocked with RNaseH2 only when bound to target. Perform 1-2 cycles of amplification. Nested primers contain second tag sequence, with optional variable regions.
- F. In the presence of both first and second tag primers, products are PCR amplified in micro-pores. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

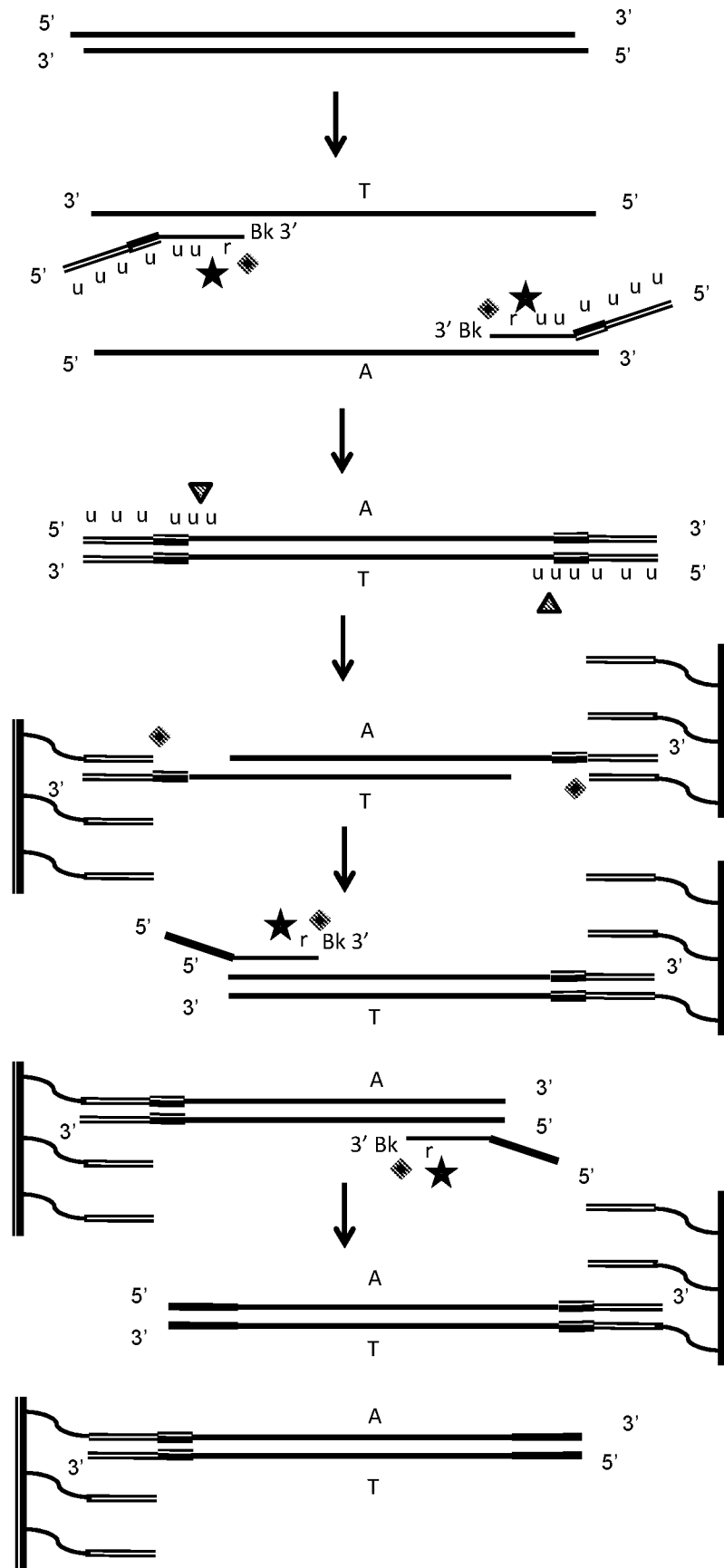


Figure 47

- A. For identifying unknown mutations at low-abundance in plasma; using Fragment identifier PCR - sequencing.
- B. Preparation: All micro-pores are pre-filled with a single universal primer, which is immobilized, and micro-pores are dried.
- C. Distribute initial sample into 48 Primary PCR Reaction Chambers. Highest level of DNA in plasma = 10,000 genome equivalents. On average, 200 copies of each target per Primary PCR Reaction Chamber, with at most 1 mutation. Perform 3 cycles of fragment identifier PCR for both strands, each strand covering slightly different sequences. Yields 4 copies of top strand, and 4 copies of bottom strand.
- D. Treat with UDG/APE1 and distribute Fragment identifier PCR products with single-stranded tails into mixing chambers and then micro-pores of each column. PCR amplify one or more products in each micro-pore using nested target-specific primers and universal primers and melt off non-anchored strand.
- E. Add either target-specific, or universal primers with unique tag-specific portions as sequencing primers. Perform sequencing-by-synthesis. Generate about 80 bases of sequence information, plus 10 bases of unique fragment identifier barcode, for accurate enumeration of each mutation, with verification on both strands.
- A. For non-invasive prenatal testing of trisomy in plasma; using Fragment identifier PCR - sequencing.
- B. Preparation: All micro-pores are pre-filled with a single universal primer, which is immobilized, and micro-pores are dried.
- C. Adjust DNA in plasma/ sample to 2,000 genome equivalents. Distribute initial sample into 48 Primary PCR Reaction Chambers. On average, 40 copies of each locus per Primary PCR Reaction Chamber, with different SNPs. Perform 3 cycles of fragment identifier PCR for both strands, each strand covering slightly different sequences. Yields 4 copies of top strand, and 4 copies of bottom strand.
- D. Treat with UDG/APE1 and distribute Fragment identifier PCR products with single-stranded tails into mixing chambers and then micro-pores of each column. PCR amplify one or more products in each micro-pore using nested locus-specific primers and universal primers and melt off non-anchored strand.
- E. Add either target-specific, or universal primers with unique tag-specific portions as sequencing primers. Perform sequencing-by-synthesis. Generate about 50 bases of sequence information, plus 10 bases of unique fragment identifier barcode, for accurate enumeration of each SNP and chromosomal copy number, with verification on both strands.

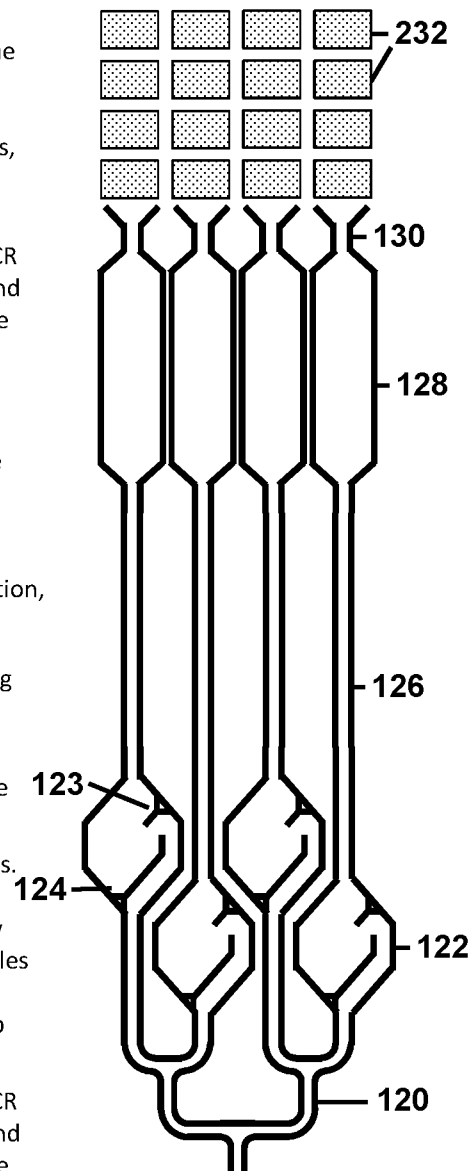
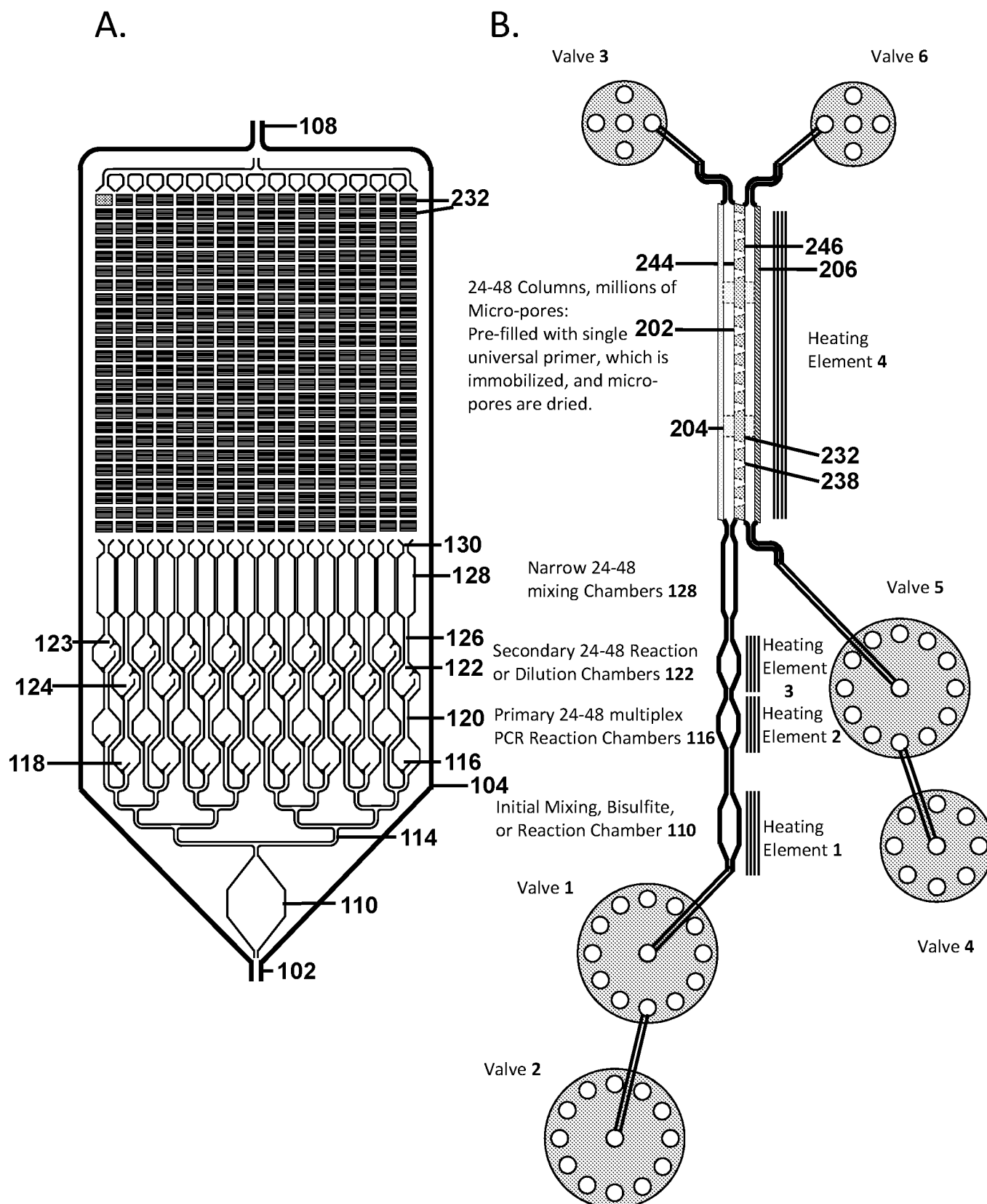


Figure 48



Figures 49A-49B

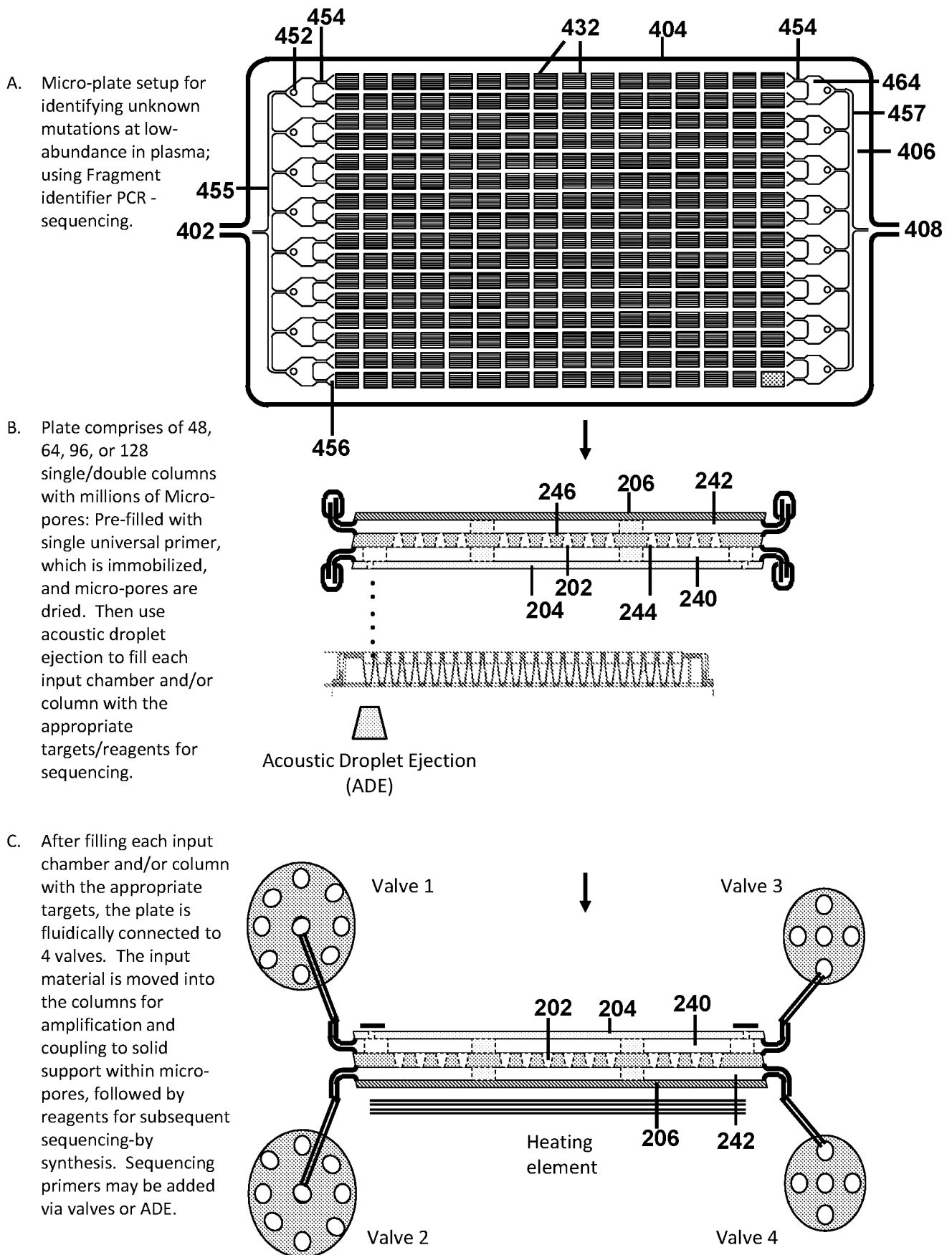


Figure 50

- A. Primer design for sequencing and identifying methylation in one target strand. cfDNA of average length of about 160 bp. Treat with Bsh1236I (CG[^]CG) to completely digest unmethylated DNA in an Initial Reaction Chamber. Treat with bisulfite, which renders the strands non-complementary.

- B. Distribute sample into 24, 36, or 48 Primary PCR Reaction Chambers. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a target-specific region and first tag sequence, the second primer contains a target-specific region, fragment identifier, dU, and a second tag sequence.

- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.

- D. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

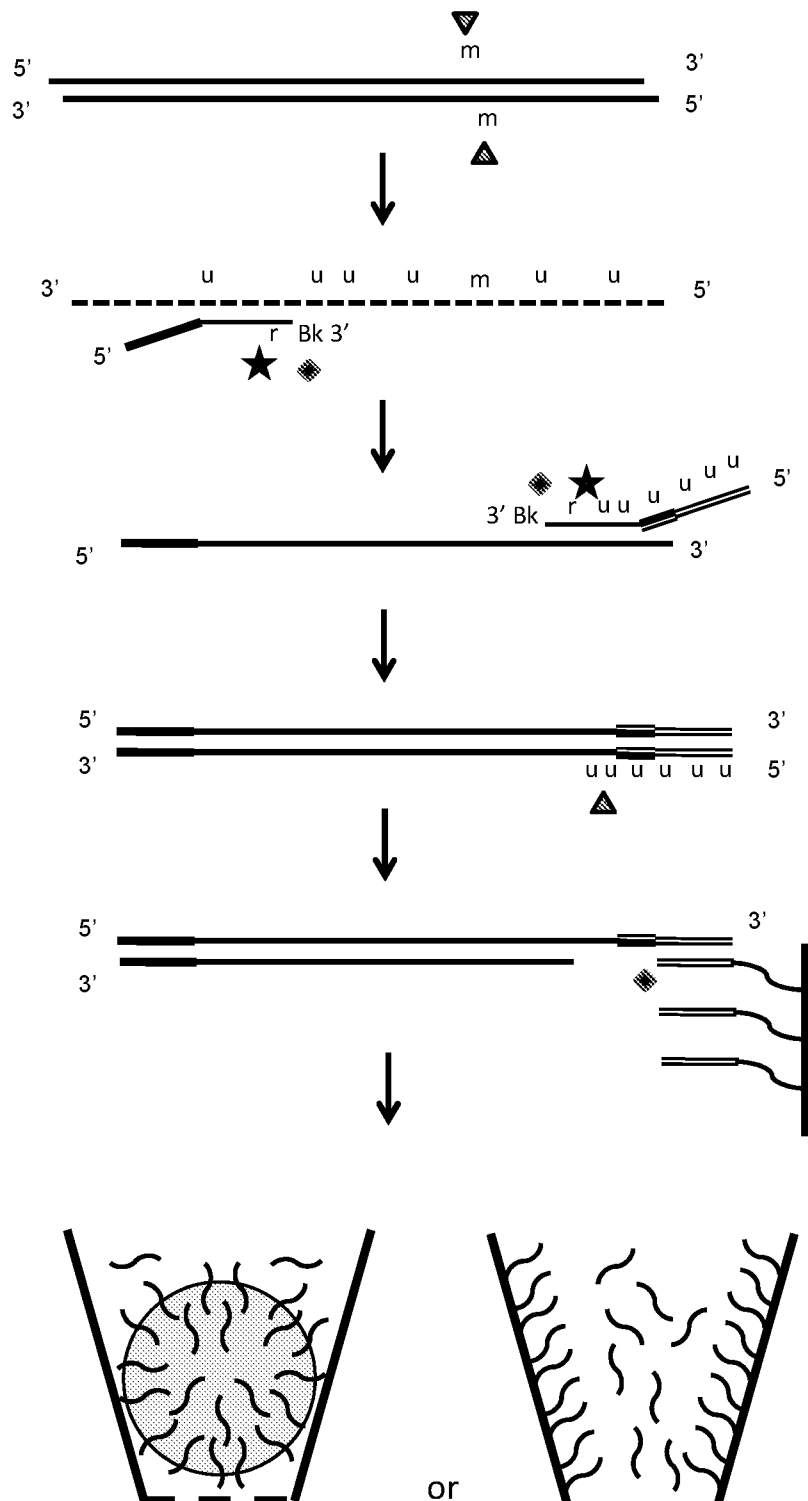


Figure 51

- A. For identifying low-abundance unknown mutations and methylations in plasma; using Fragment identifier PCR - sequencing.
- B. Preparation: All micro-pores are pre-filled with a single universal primer, which is immobilized, and micro-pores are dried.
- C. Divide initial sample in half, and mix other half with buffer, primers, reagents, and polymerase, and distribute into first set of 48 Primary PCR Reaction Chambers. Highest level of DNA in plasma = 10,000 genome equivalents. On average, 200 copies of each target per Primary PCR Reaction Chamber, with at most 1 mutation. Perform 3 cycles of fragment identifier PCR for both strands, each strand covering slightly different sequences. Yields 4 copies of top strand, and 4 copies of bottom strand.
- D. Treat with UDG and distribute Fragment identifier PCR products with single-stranded tails into 48 Secondary Chambers, while draining and washing out the Primary PCR Reaction Chambers with several washes.
- E. Digest second half of sample with Bsh1236I in an Initial Reaction Chamber. Treat with Bisulfite. Re-purify DNA strands.
- F. Mix with primers, reagents, and polymerase, and distribute bisulfite treated sample into first set of 48 Primary PCR Reaction Chambers. On average, after endonuclease treatment, 4 copies of each target per Primary PCR Reaction Chamber, with at most 1 is methylated. Perform 3 cycles of fragment identifier PCR for both strands, each strand covering slightly different sequences. Yields 4 copies of top strand, and 4 copies of bottom strand of originally methylated DNA.
- G. Treat with UDG/APE1, combine with earlier mutation-specific products in set of 48 Secondary Reaction Chambers, add fresh reagents, polymerase and nested primers, mix in 48 mixing chambers, and distribute Fragment identifier PCR products into micro-pores of each column. PCR amplify one or more products in each micro-pore and melt off non-anchored strand.
- H. Add either target-specific, or universal primers with unique tag-specific portions as sequencing primers. Perform sequencing-by-synthesis. Generate about 80 bases of sequence information, plus 10 bases of unique fragment identifier barcode, for accurate enumeration of each mutation or methylated region, with verification on both strands.

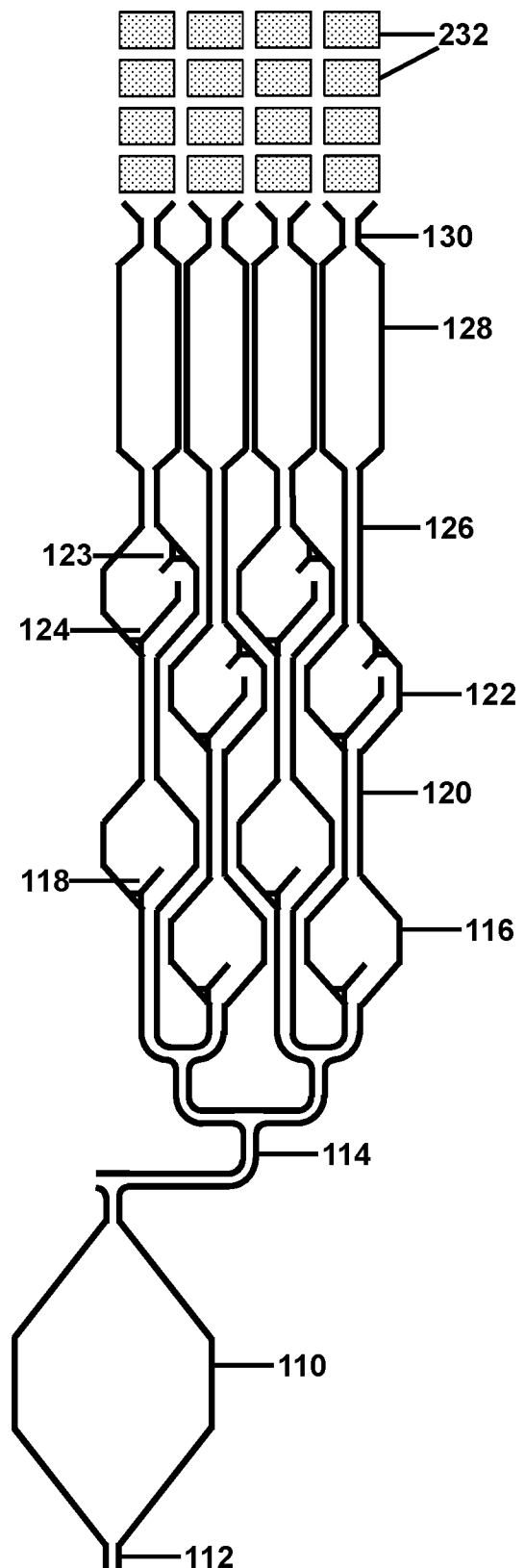


Figure 52

- A. Primer design for sequencing low- and medium-abundance lncRNA, mRNA, and splice-site variants, isolated from CTC's or exosomes. Use reverse-transcriptase to make cDNA copies with 3' transcript-specific primers in an Initial Reaction Chamber.
- B. Distribute sample into 24 Primary PCR Reaction Chambers. Transcript-specific primers are unblocked with RNaseH2 only when bound to cDNA or complement. Perform 2 - 3 cycles of amplification with strand-displacement thermostable polymerase. One primer contains a transcript-specific region and first tag sequence, the second primer contains a transcript-specific region, transcript identifier, dU, and a second tag sequence.
- C. Treat with UDG (uracil DNA glycosylase) and optionally, APE1 (human apurinic endonuclease) to destroy original primers and portion of primers in products.
- D. Differentially dilute products from each of the 24 Primary PCR Reaction Chambers into two Secondary Chambers = 48 total. Distribute products into micro-pores or beads into micro-pores containing immobilized second tag sequence primers. In the presence of both first and second tag primers, products are PCR amplified in micro-pores such that a given micro-pore generally contains zero or one clonal amplification of a given region, but may contain multiple clonal amplicons from different regions. After denaturation, and removal of unbound fragments, remaining tethered single-stranded target DNA is suitable for primer-directed sequencing.

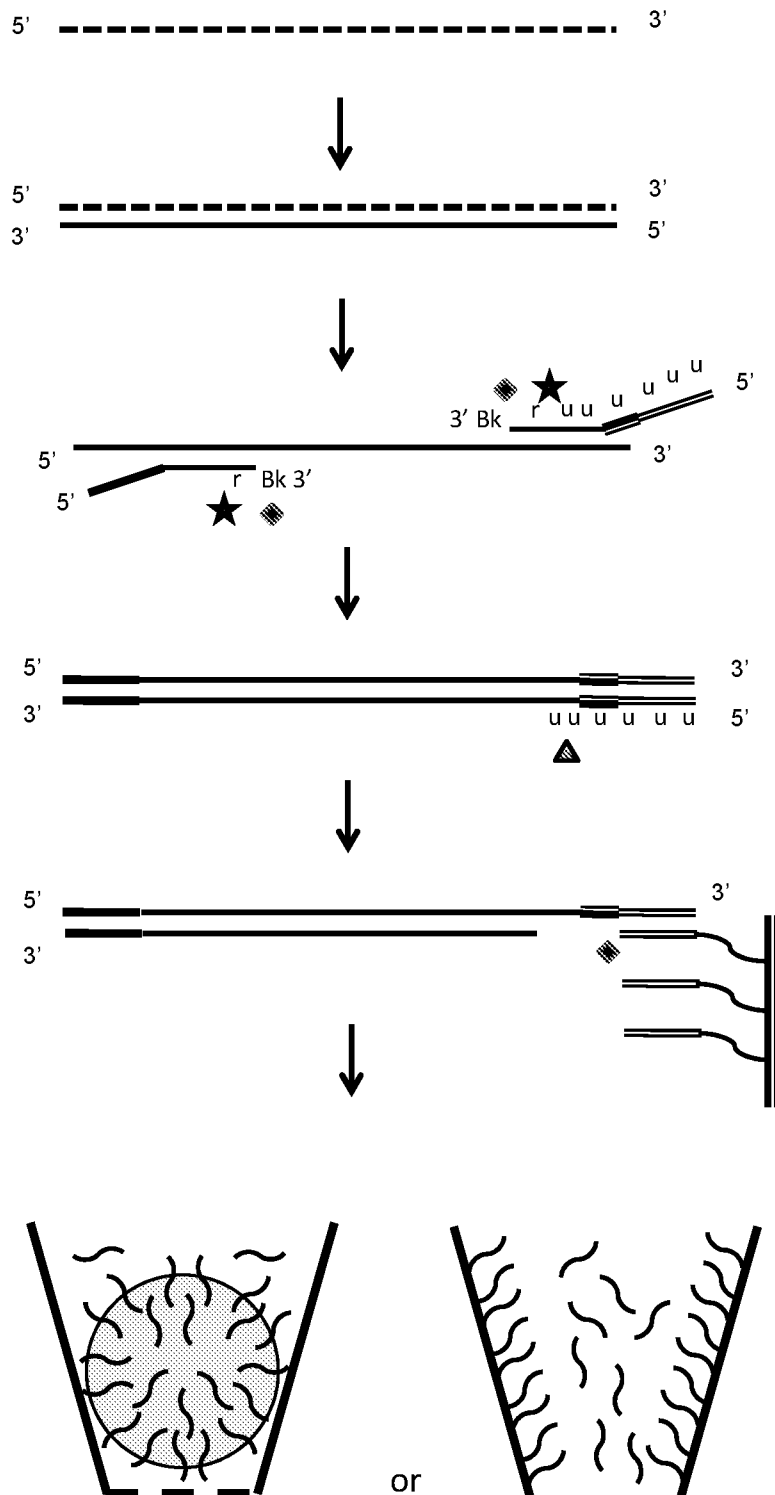


Figure 53

- A. Exact enumeration of low-abundance, medium-abundance, and over-expressed lncRNA, mRNA, splice variants, or gene-fusions; using Multiplexed RT-PCR - Nested PCR - UniTaq detection. (Alternatively, Multiplexed PCR – Nested PCR - Real-time-PCR with transcript-specific Taqman probes.)
- B. Preparation: Rows are pre-filled with 1-4 UniTaq primer sets (or alternatively, 1-4 universal tag primer sets with target-specific Taqman probes). Primers are distributed into each row and dried down into individual micro-pores.
- C. Preparation: Pre-fill reaction chambers to columns with nested PCR primer sets with either UniTaq or universal tag sequences on their 5' ends, and dry down.
- D. Initial multiplexed reverse-transcription PCR amplification for 9 cycles to generate 512 copies of each original transcript in an Initial Reaction Chamber.
- E. Distribute initial multiplex reaction into 24 Primary PCR Reaction Chambers, with average distribution of 20 copies in each Primary PCR Reaction Chamber of each original transcript. Perform 10 cycles of nested PCR using transcript-specific primers with UniTaq or universal tags in groups of 16, 32, or 64 primer sets. Each Primary PCR Reaction Chamber is designed to retain a certain percentage of liquid volume after draining. Perform 3 cycles of filling and draining to differentially dilute products.
- F. Dilute products from each of 24 Primary PCR Reaction Chambers into 2 Secondary Reaction Chambers (= 48 total). Each Secondary Reaction Chamber is designed to retain a certain percentage of liquid volume after draining. Perform 2 cycles of filling and draining to differentially dilute products.
- G. Distribute nested PCR products into mixing chambers and then into micro-pores of each column. Universal or UniTaq primers in each row will amplify only those products from each column with the correct tags. Poisson distribution in micro-pores will enumerate low-abundance, medium-abundance, and over-expressed lncRNA, mRNA, splice variants, or gene-fusions.

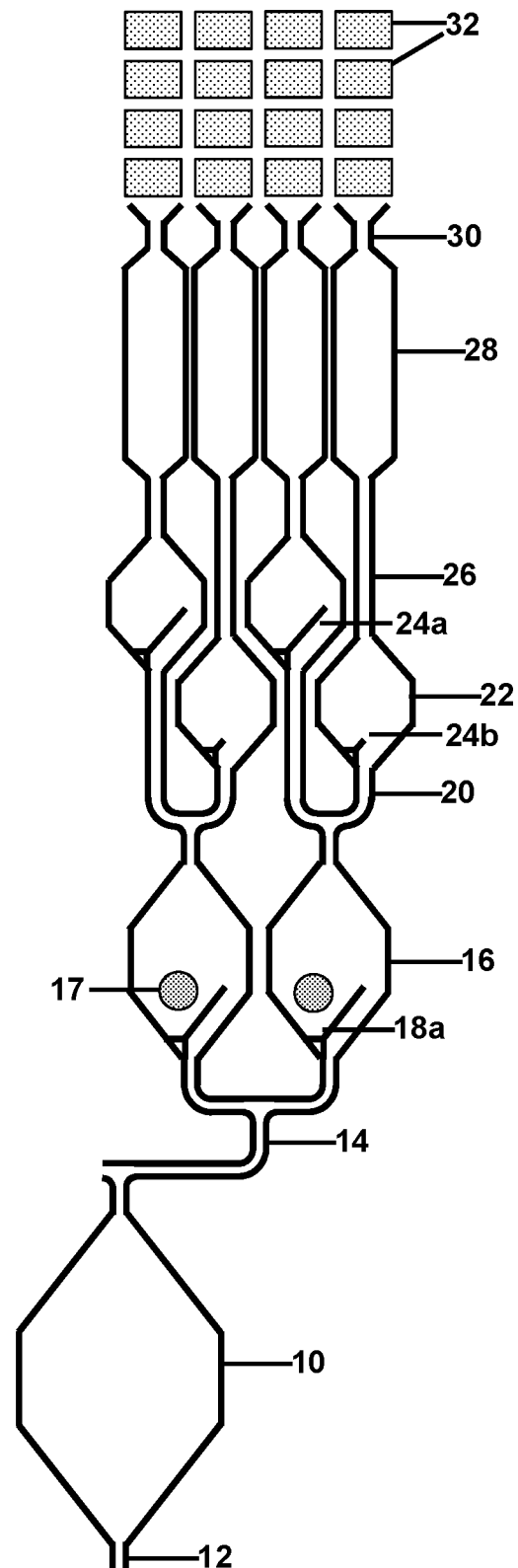


Figure 54