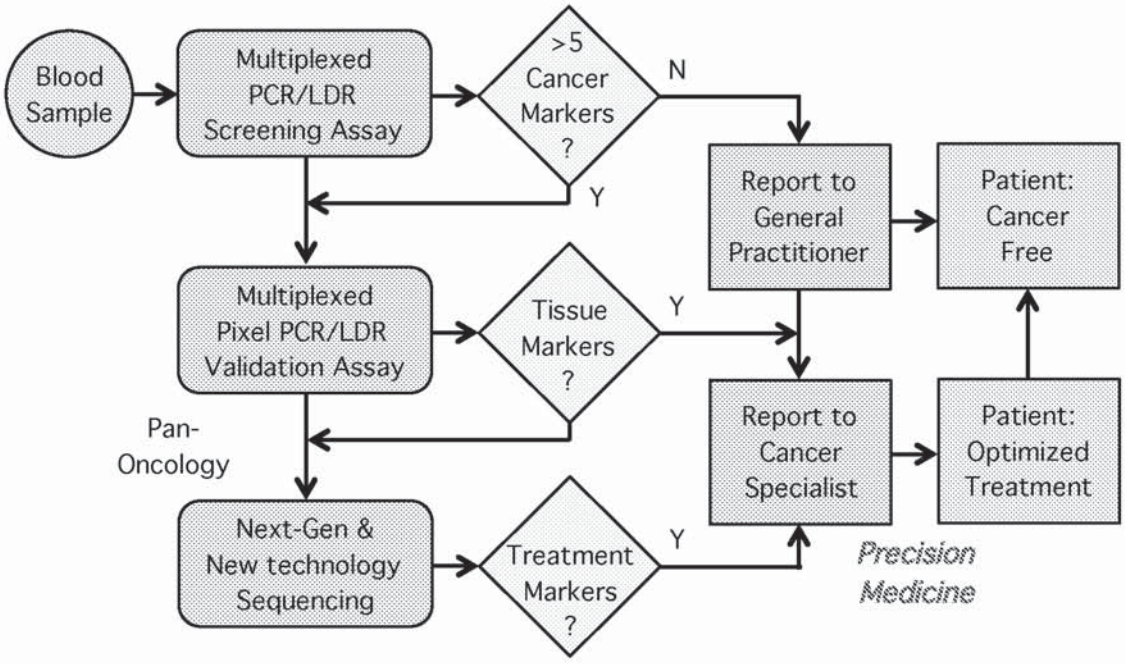

Blood Sample
Multiplexed PCR/LDR Screening Assay
>5 Cancer Markers ?
N
Y
Report to General Practitioner
Patient: Cancer Free
Multiplexed Pixel PCR/LDR Validation Assay
Tissue Markers ?
Y
Pan-Oncology
Report to Cancer Specialist
Patient: Optimized Treatment
Next-Gen & New technology Sequencing
Treatment Markers ?
Y
Precision Medicine

Figure 1

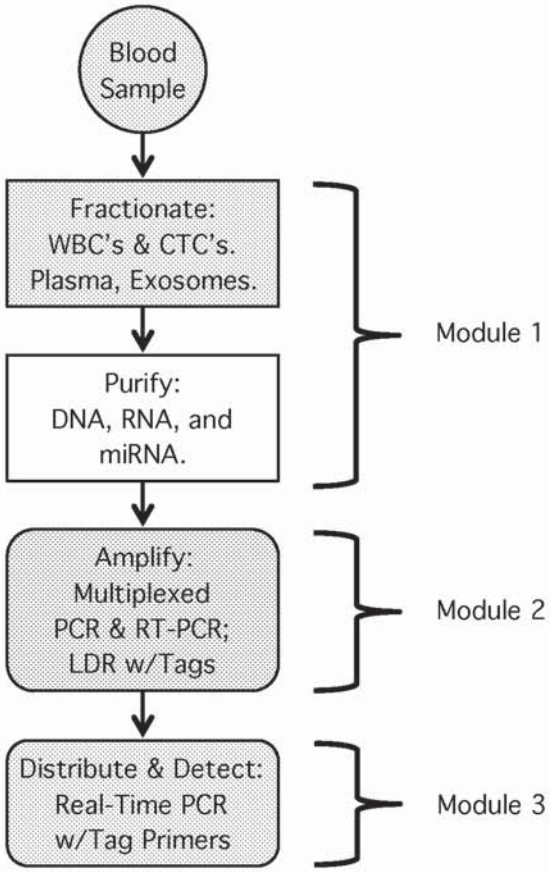

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: DNA, RNA, and miRNA.
Module 1
Amplify: Multiplexed PCR & RT-PCR; LDR w/Tags
Module 2
Distribute & Detect: Real-Time PCR w/Tag Primers
Module 3

Figure 2

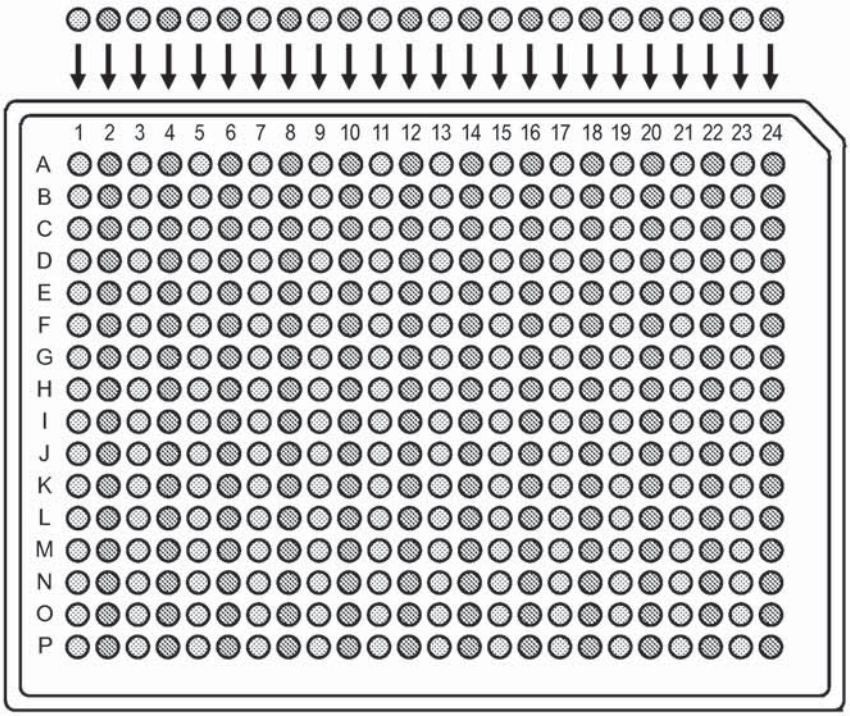

Multiplexed PCR & RT-PCR; LDR w/Tags, 24 samples.
Distribute LDR products from each sample into 16 wells.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 3

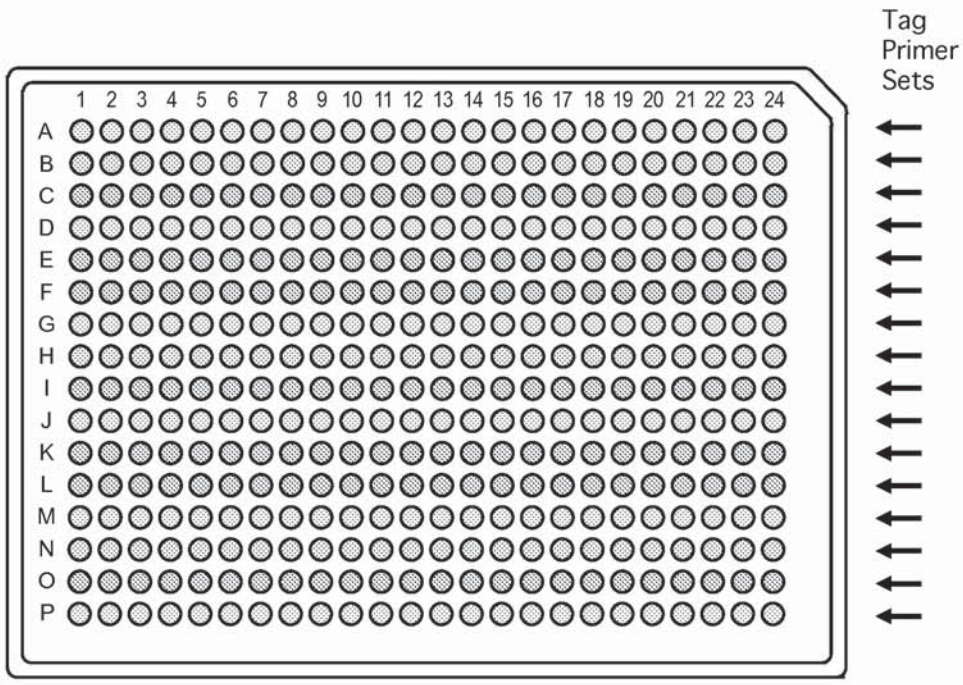
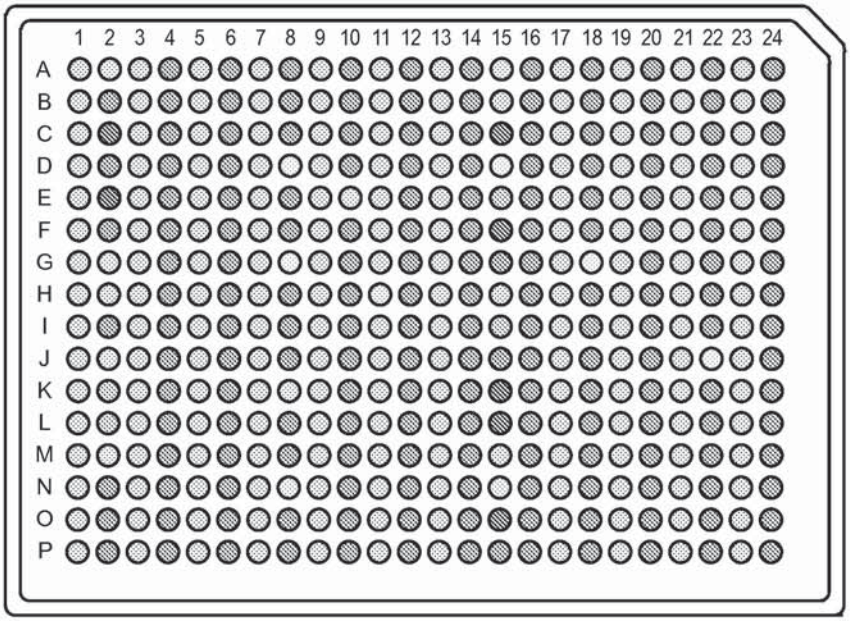

Multiplexed PCR & RT-PCR; LDR w/Tags, 24 samples
Tag
Primer
Sets
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 4

(Multiplexed PCR & RT-PCR; LDR w/Tags, 24 samples)



Real-Time PCR results, 24 samples

Figure 5

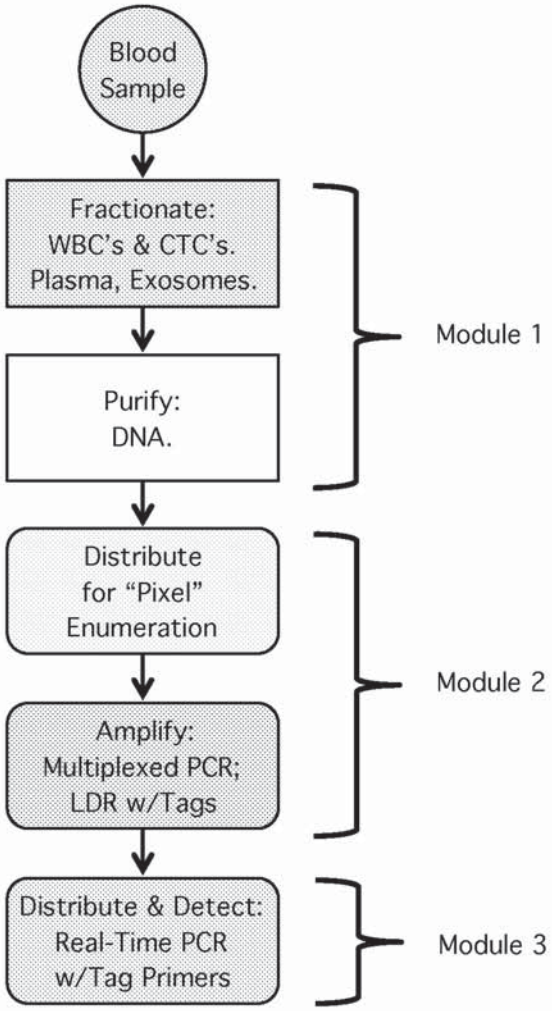

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: DNA.
Module 1
Distribute for "Pixel" Enumeration
Amplify: Multiplexed PCR; LDR w/Tags
Module 2
Distribute & Detect: Real-Time PCR w/Tag Primers
Module 3

Figure 6

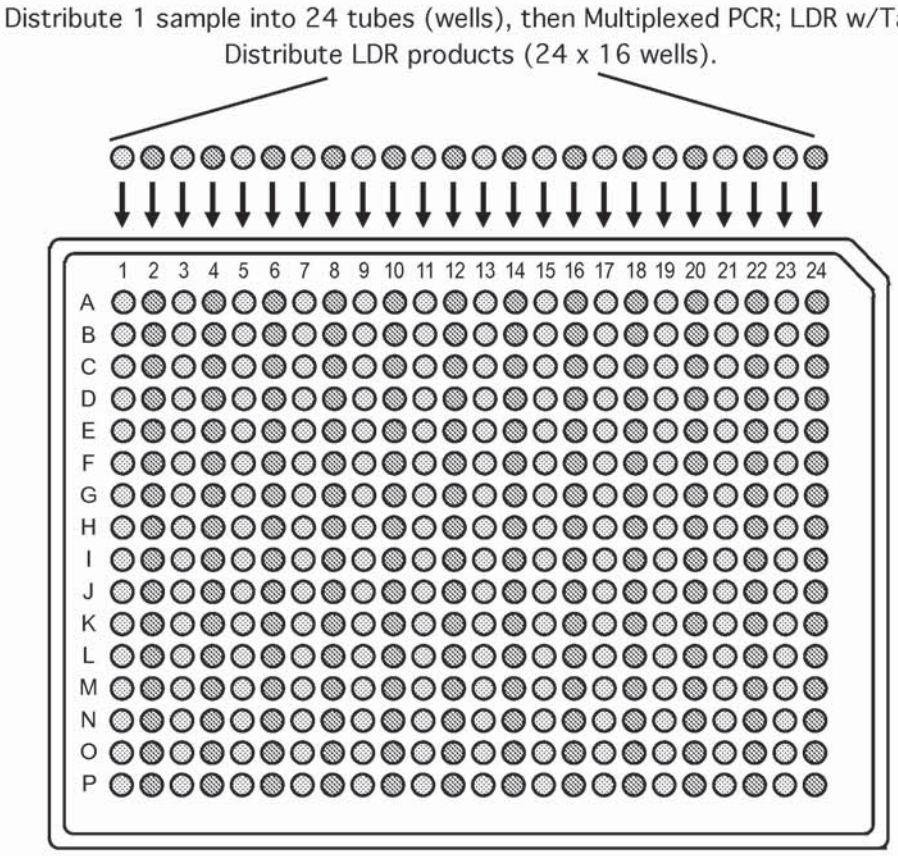

Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR; LDR w/Tags
Distribute LDR products (24 x 16 wells).
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 7

Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR; LDR w/Tags

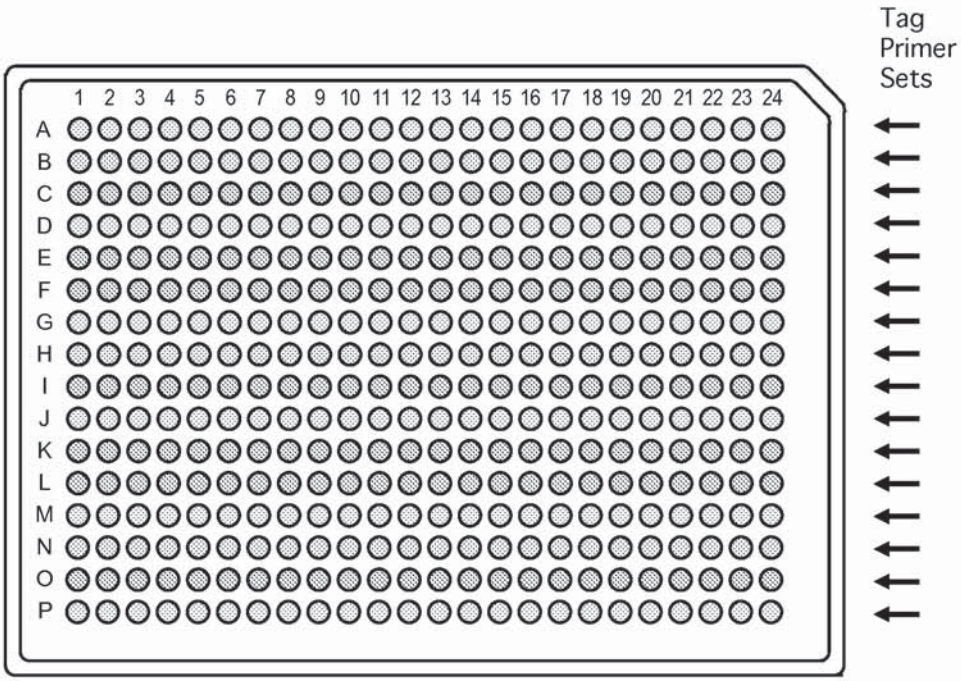
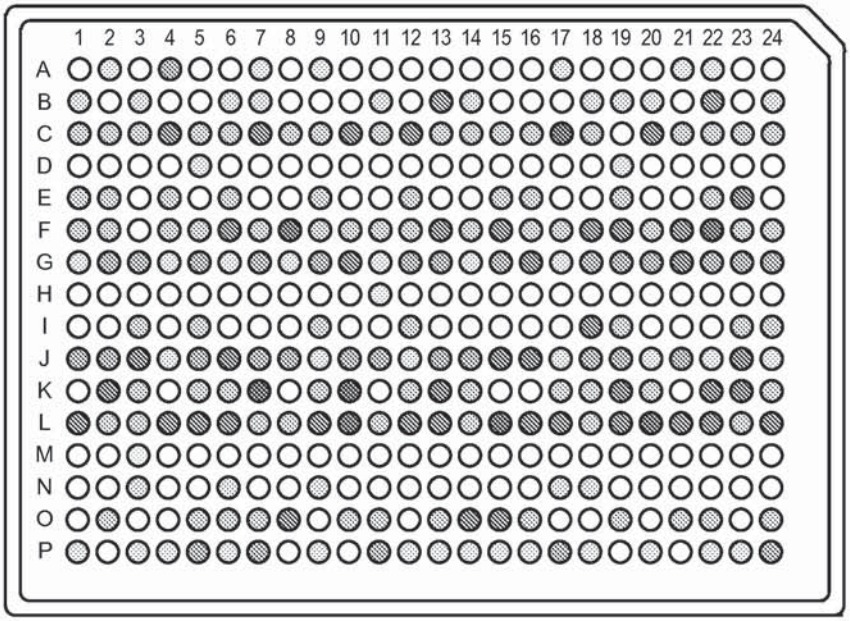


Figure 8

(Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR; LDR w/Tags)



Real-Time PCR results, 1 sample
w/ “Pixel” Enumeration

Figure 9

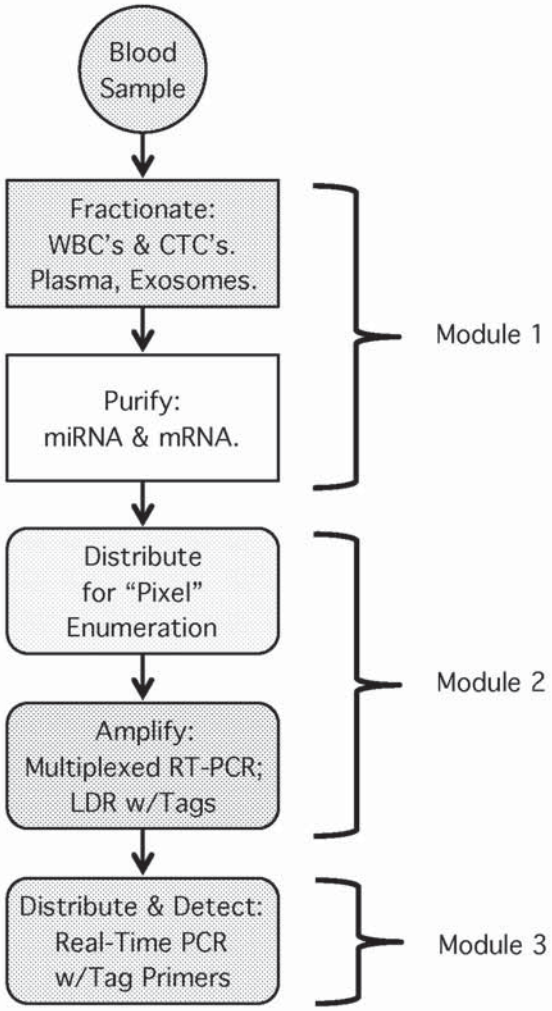

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: miRNA & mRNA.
Module 1
Distribute for "Pixel" Enumeration
Amplify: Multiplexed RT-PCR; LDR w/Tags
Module 2
Distribute & Detect: Real-Time PCR w/Tag Primers
Module 3

Figure 10

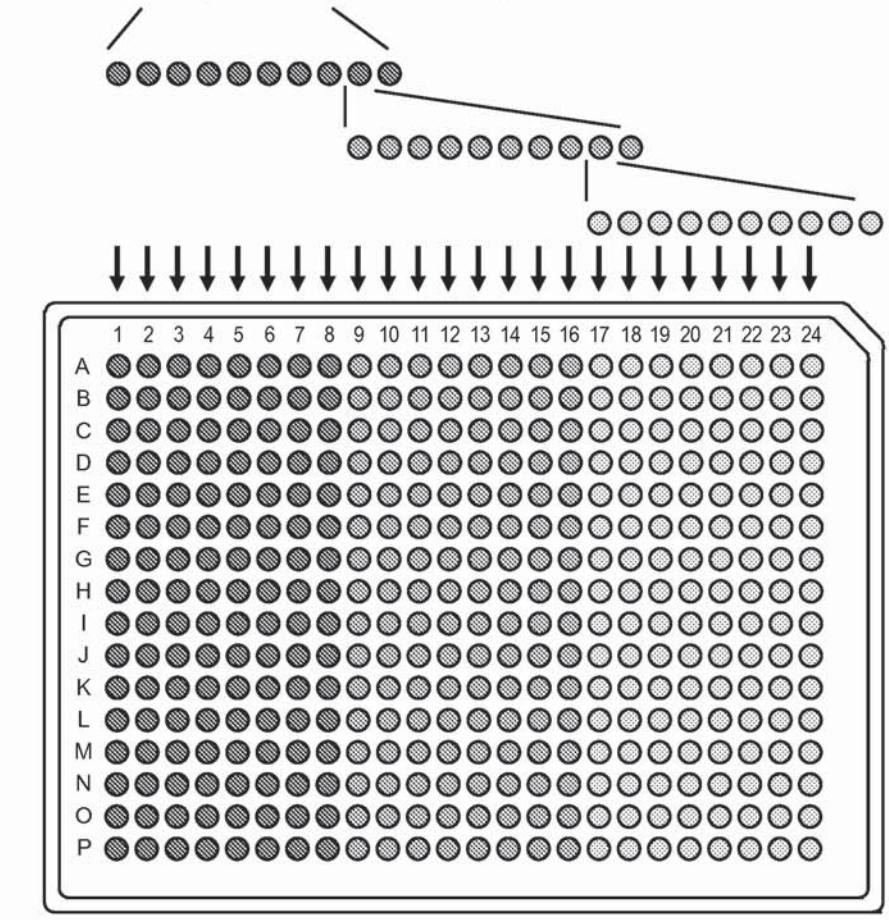

Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR; LDR w/Tags
Distribute LDR products (24 x 16 wells).
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 11

Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR; LDR w/Tags

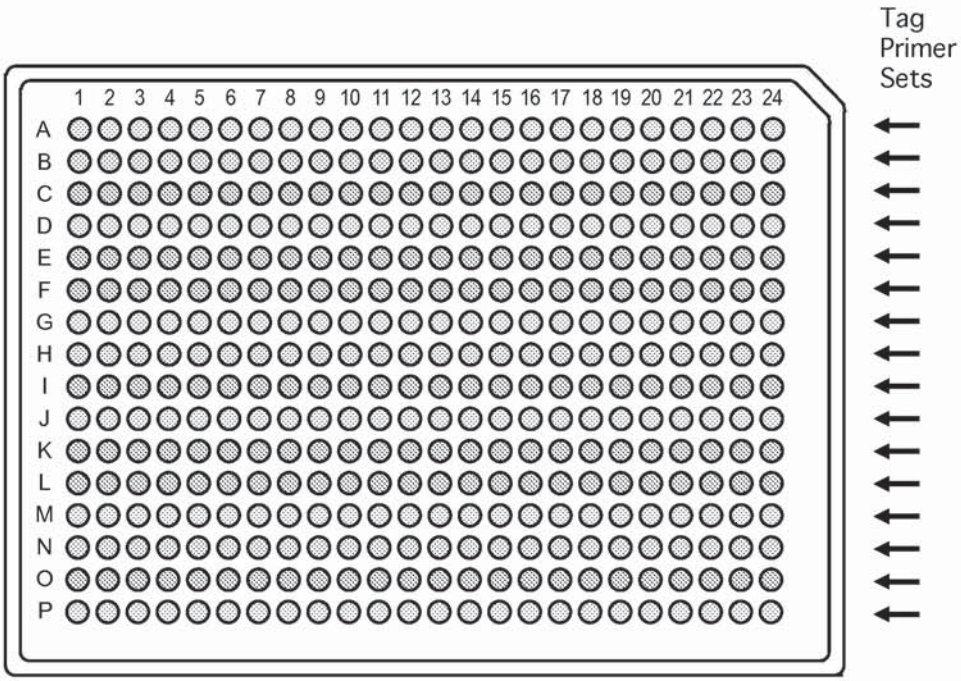
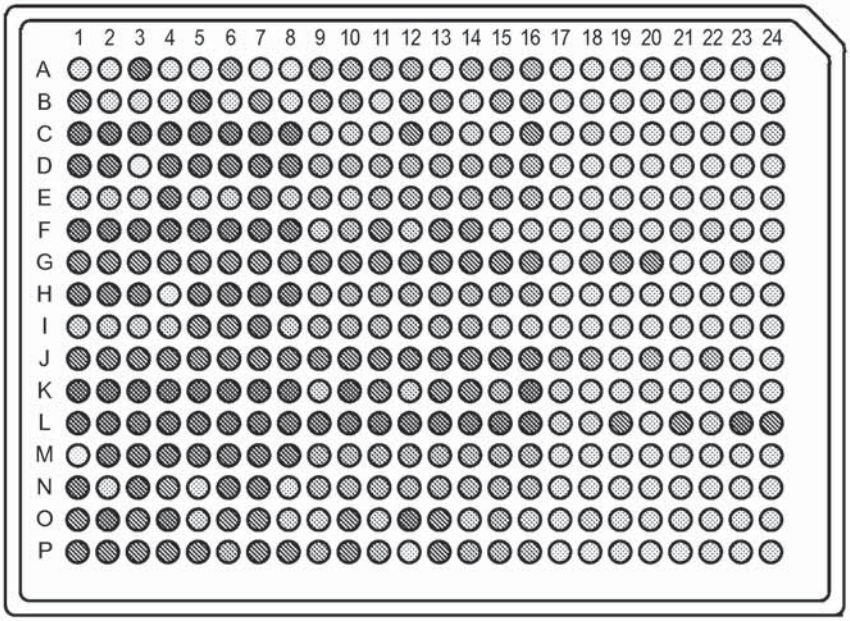


Figure 12

(Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR; LDR w/Tags)



Real-Time PCR results, 1 sample
w/ "Pixel" Enumeration

Figure 13

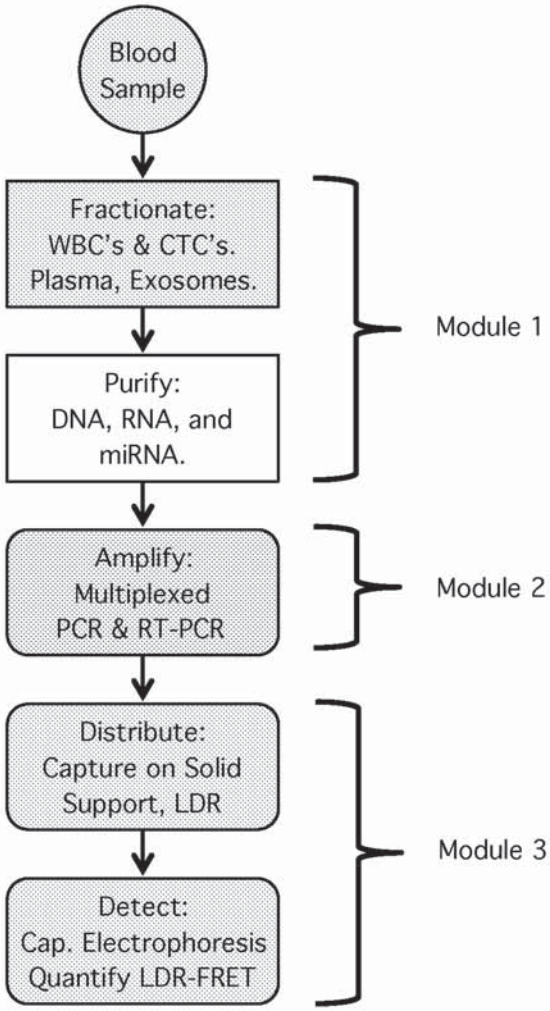

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: DNA, RNA, and miRNA.
Amplify: Multiplexed PCR & RT-PCR
Distribute: Capture on Solid Support, LDR
Detect: Cap. Electrophoresis Quantify LDR-FRET
Module 1
Module 2
Module 3

Figure 14

Multiplexed PCR & RT-PCR; 24 samples.
Distribute PCR products from each sample into 16 wells.

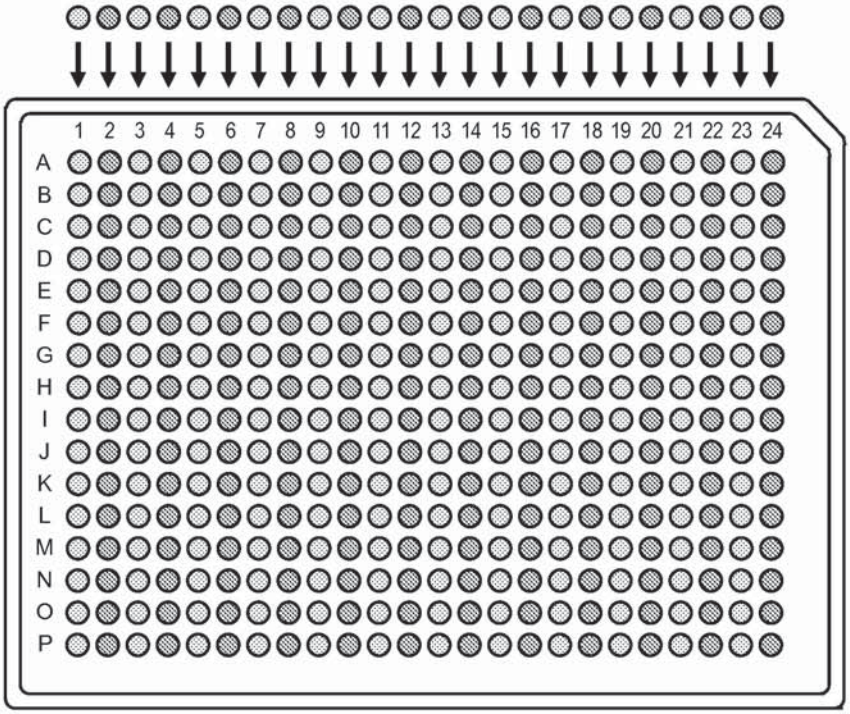
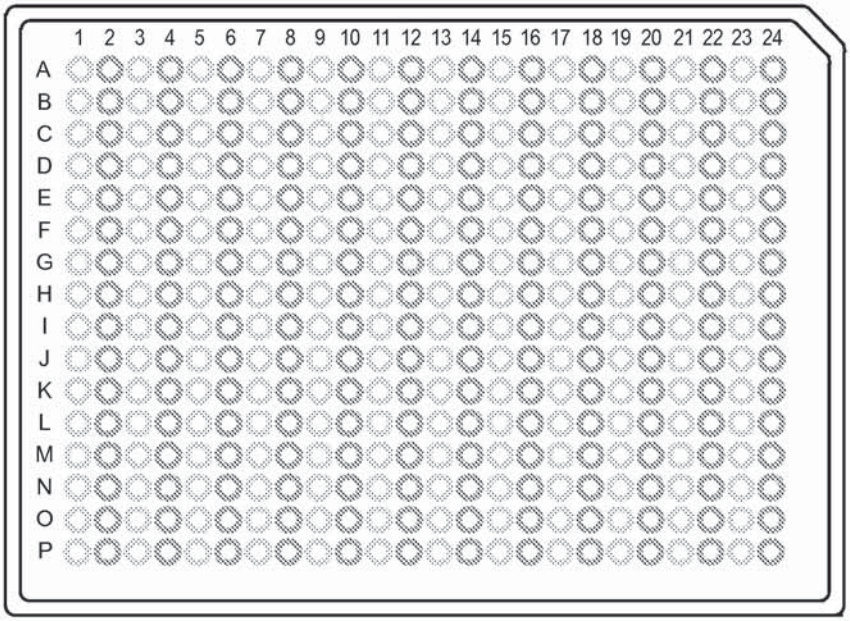


Figure 15

Multiplexed PCR & RT-PCR; 24 samples



PCR and RT-PCR Products Captured on Solid Support

Figure 16

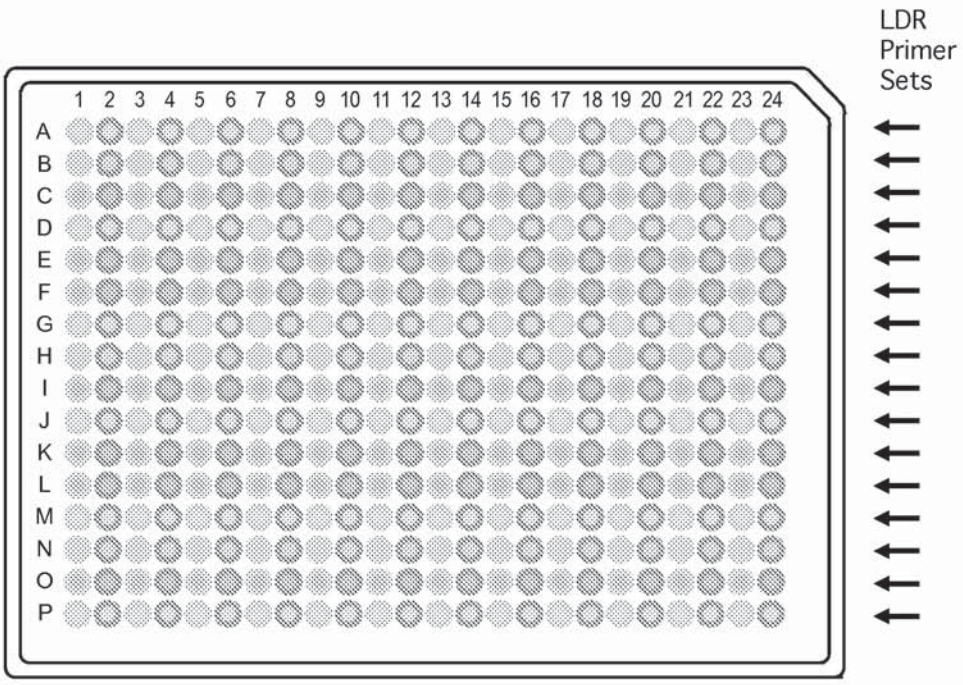
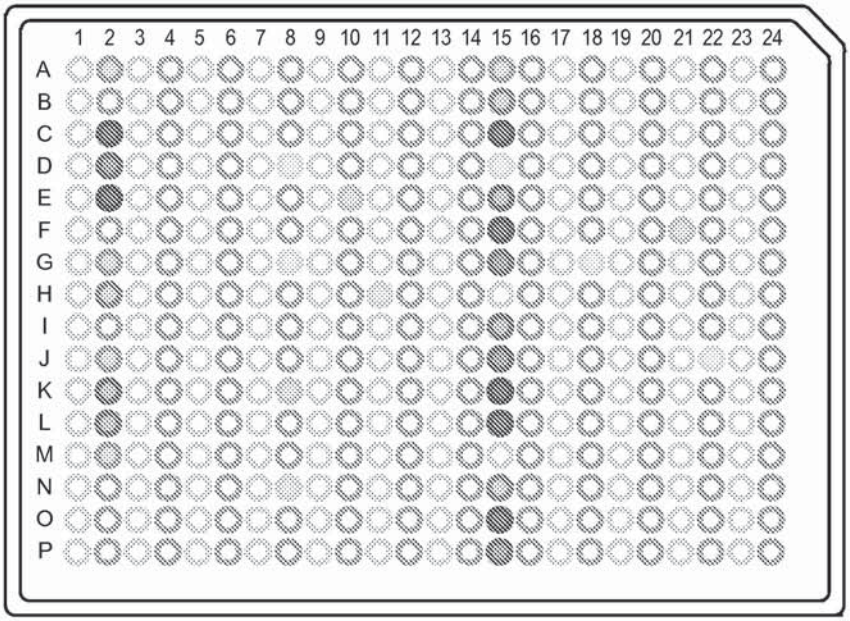

Multiplexed PCR & RT-PCR; 24 samples
LDR
Primer
Sets
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 17

(Multiplexed PCR & RT-PCR; 24 samples)



LDR-FRET results, 24 samples

Figure 18

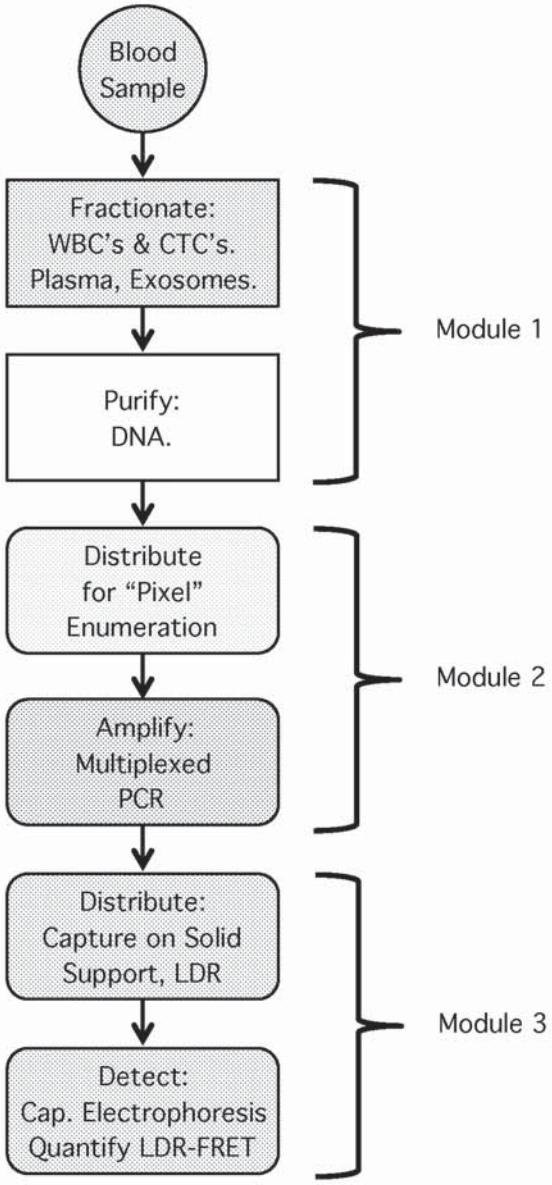

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: DNA.
Module 1
Distribute for "Pixel" Enumeration
Amplify: Multiplexed PCR
Module 2
Distribute: Capture on Solid Support, LDR
Detect: Cap. Electrophoresis Quantify LDR-FRET
Module 3

Figure 19

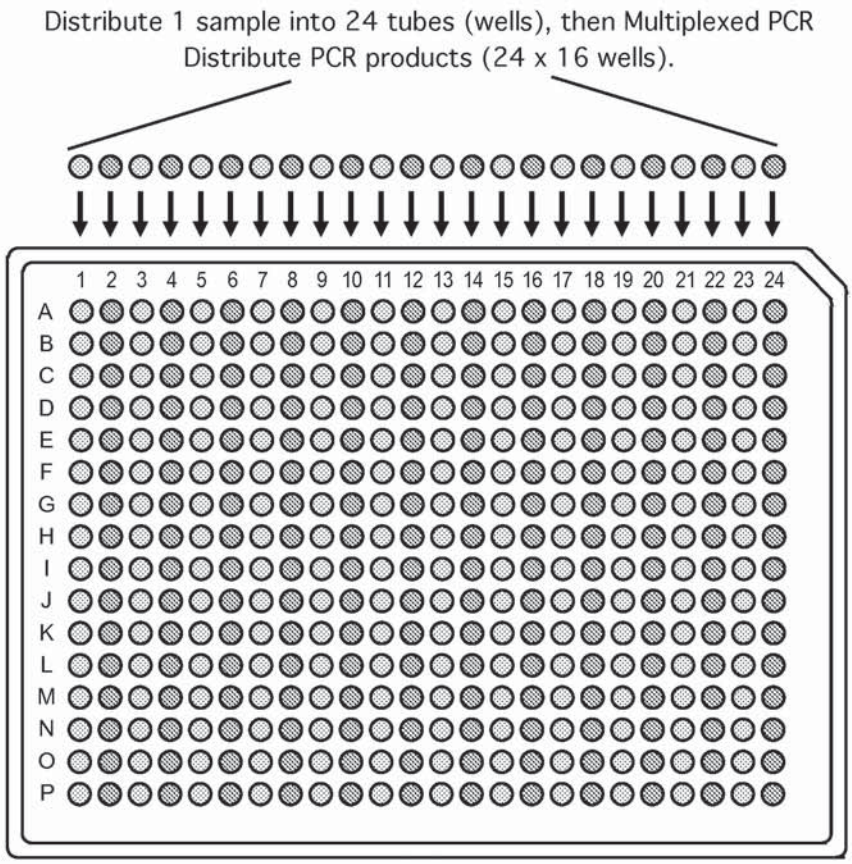
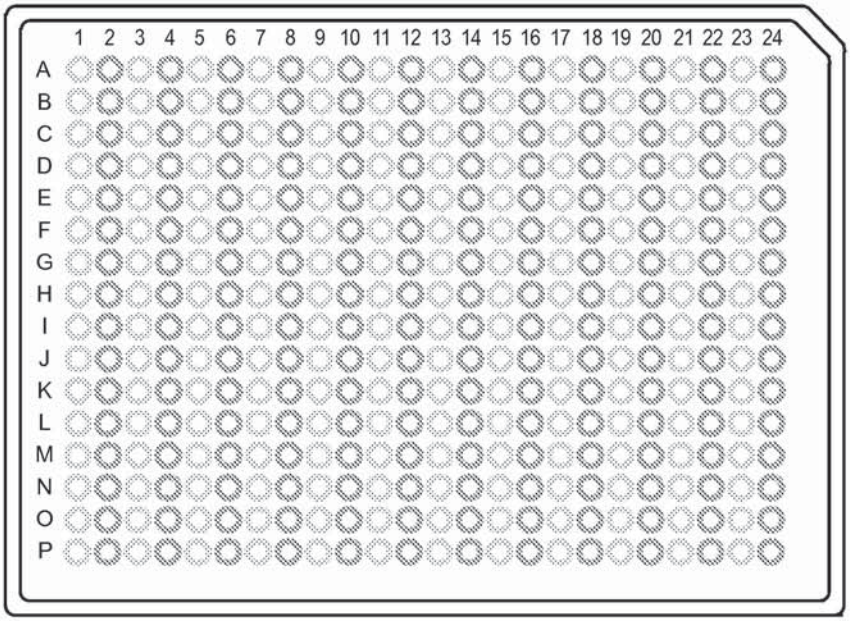

Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR
Distribute PCR products (24 x 16 wells).
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 20

Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR



PCR Products Captured on Solid Support

Figure 21

Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR

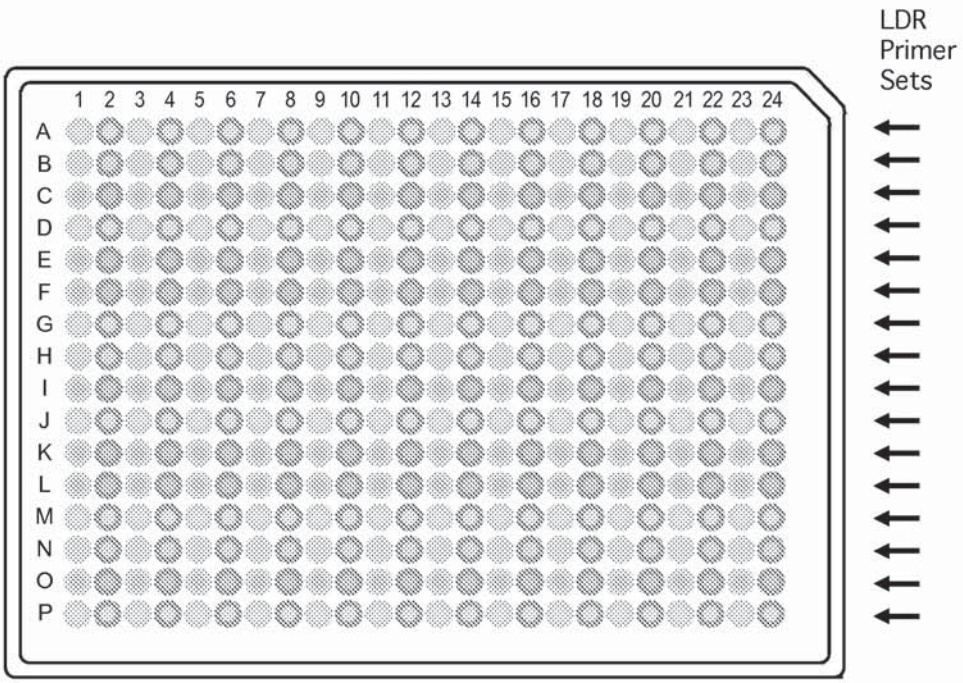
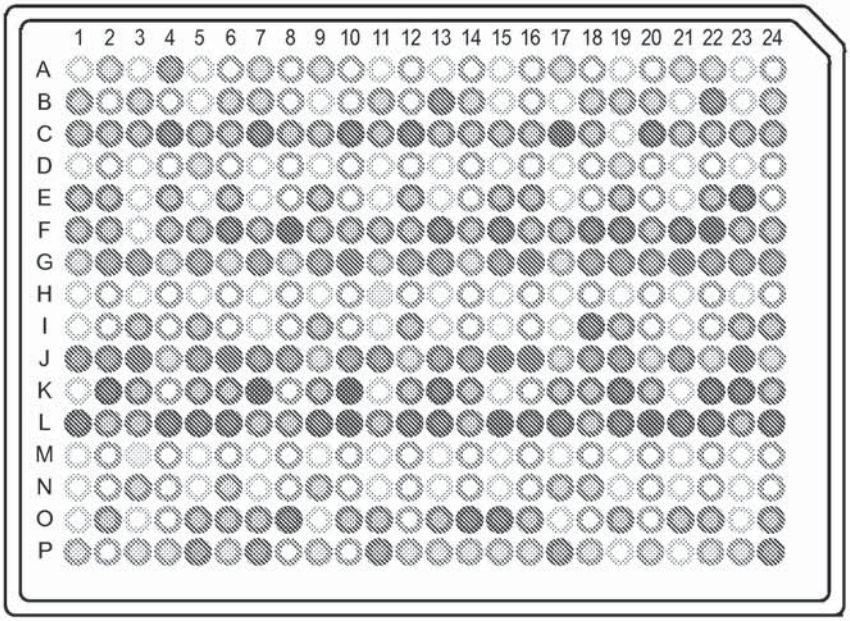


Figure 22

(Distribute 1 sample into 24 tubes (wells), then Multiplexed PCR)



LDR-FRET results, 1 sample
w/ "Pixel" Enumeration

Figure 23

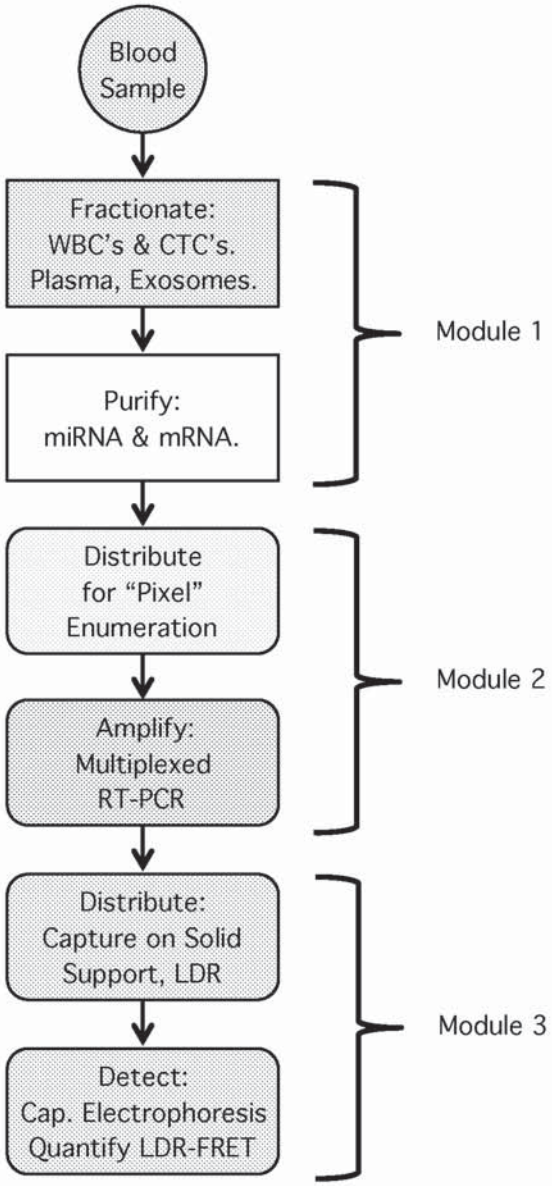

Blood Sample
Fractionate: WBC's & CTC's. Plasma, Exosomes.
Purify: miRNA & mRNA.
Module 1
Distribute for "Pixel" Enumeration
Amplify: Multiplexed RT-PCR
Module 2
Distribute: Capture on Solid Support, LDR
Detect: Cap. Electrophoresis Quantify LDR-FRET
Module 3

Figure 24

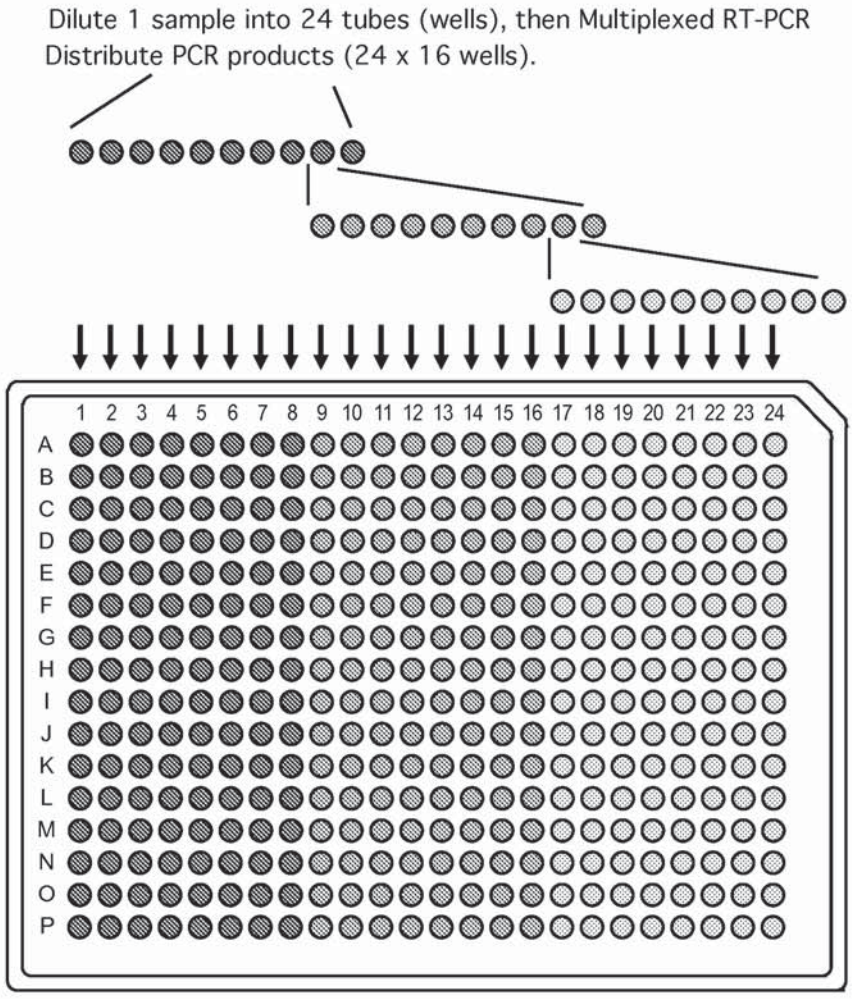
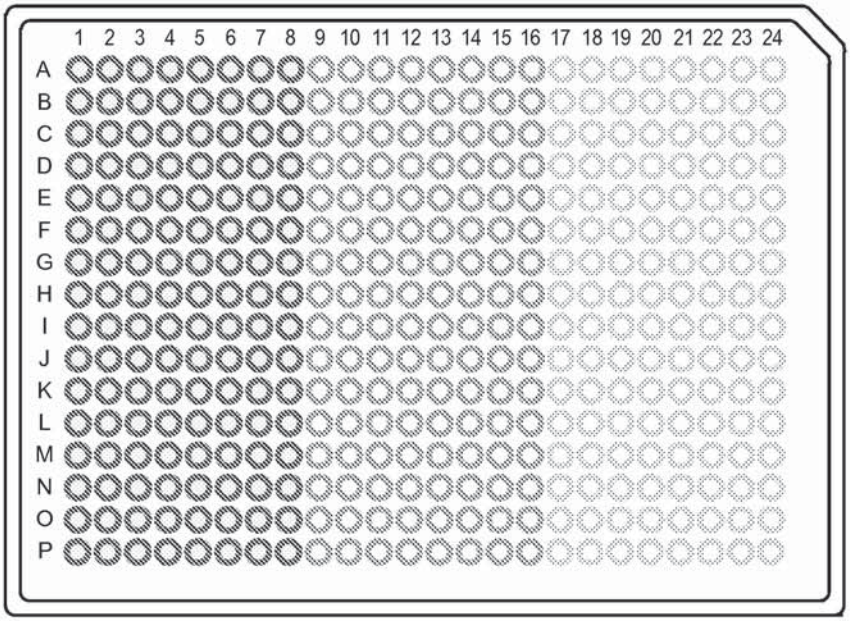

Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR
Distribute PCR products (24 x 16 wells).
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 25

Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR



RT-PCR Products Captured on Solid Support

Figure 26

Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR

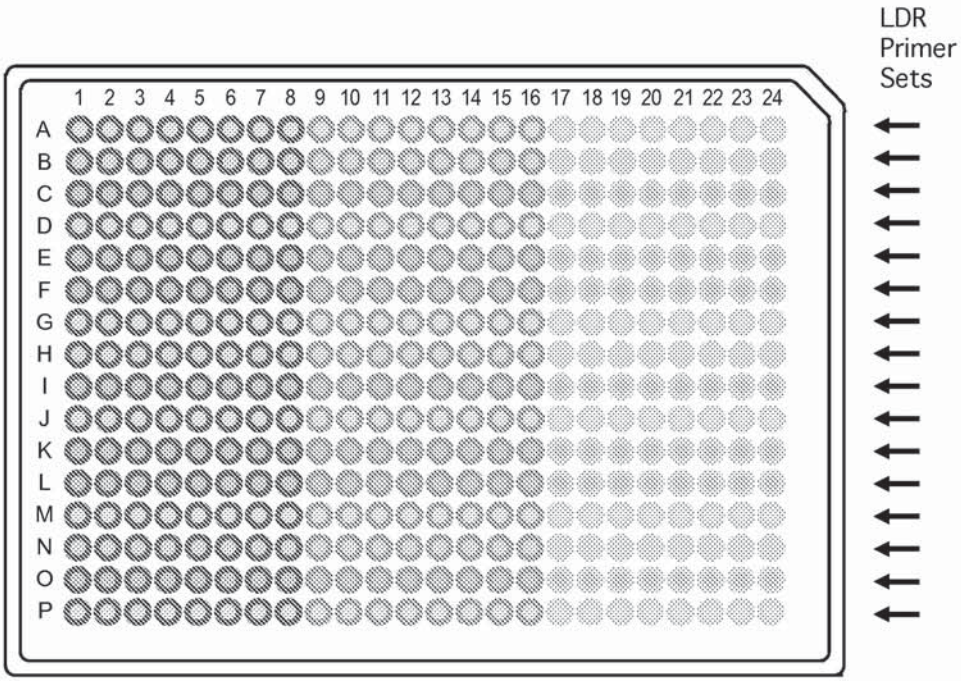
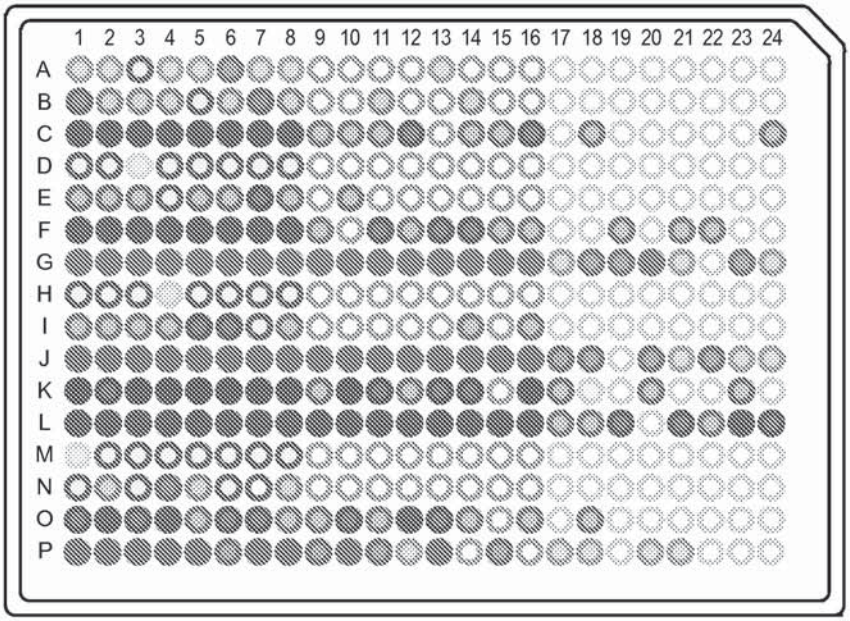


Figure 27

(Dilute 1 sample into 24 tubes (wells), then Multiplexed RT-PCR)



LDR-FRET results, 1 sample
w/ "Pixel" Enumeration

Figure 28

Poisson Distribution of 6 to 48 molecules in 24 wells

Wells with	6	12	18	24	30	36	42	48
0	19	15	11	9	7	5	4	3
1	5	7	9	9	9	8	7	6
2	1	2	3	4	5	6	6	6
3	0	0	1	1	2	3	4	4
4	0	0	0	0	1	1	2	2
5	0	0	0	0	0	0	1	1
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0

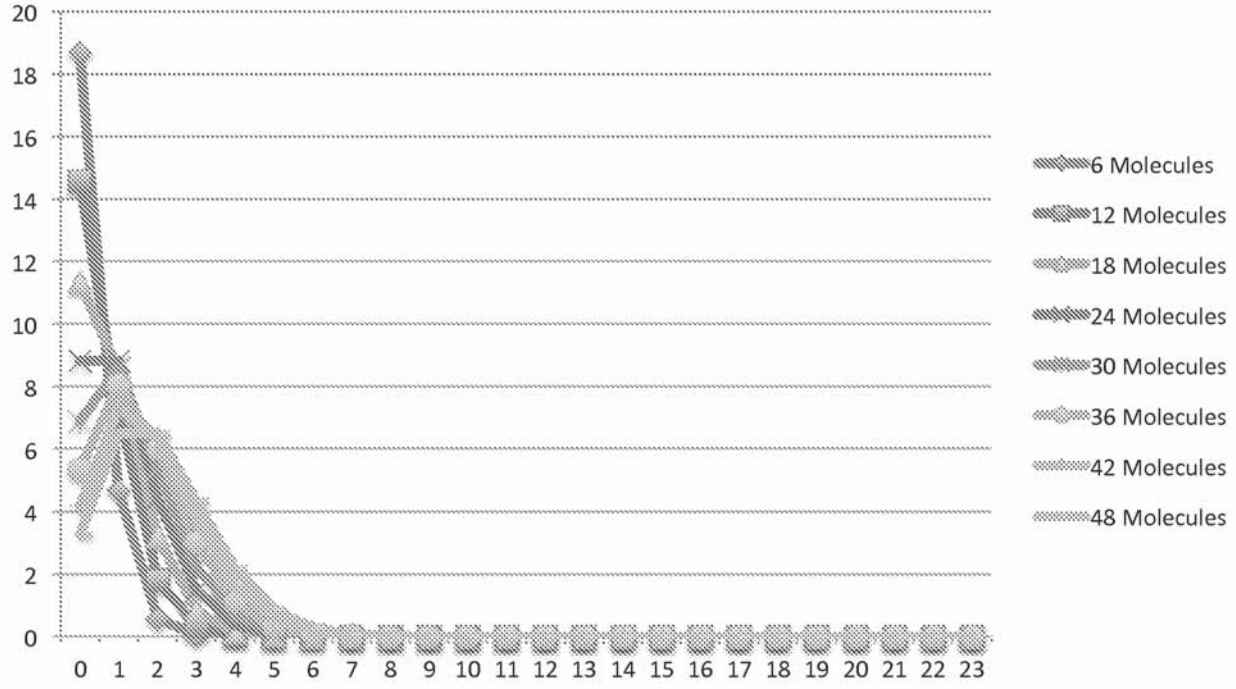


Figure 29

Poisson Distribution of 12 to 96 molecules in 24 wells

Wells with	12	24	36	48	60	72	84	96
0	15	9	5	3	2	1	1	0
1	7	9	8	6	5	4	3	2
2	2	4	6	6	6	5	4	4
3	0	1	3	4	5	5	5	5
4	0	0	1	2	3	4	5	5
5	0	0	0	1	2	2	3	4
6	0	0	0	0	1	1	2	3
7	0	0	0	0	0	1	1	1
8	0	0	0	0	0	0	0	1
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0

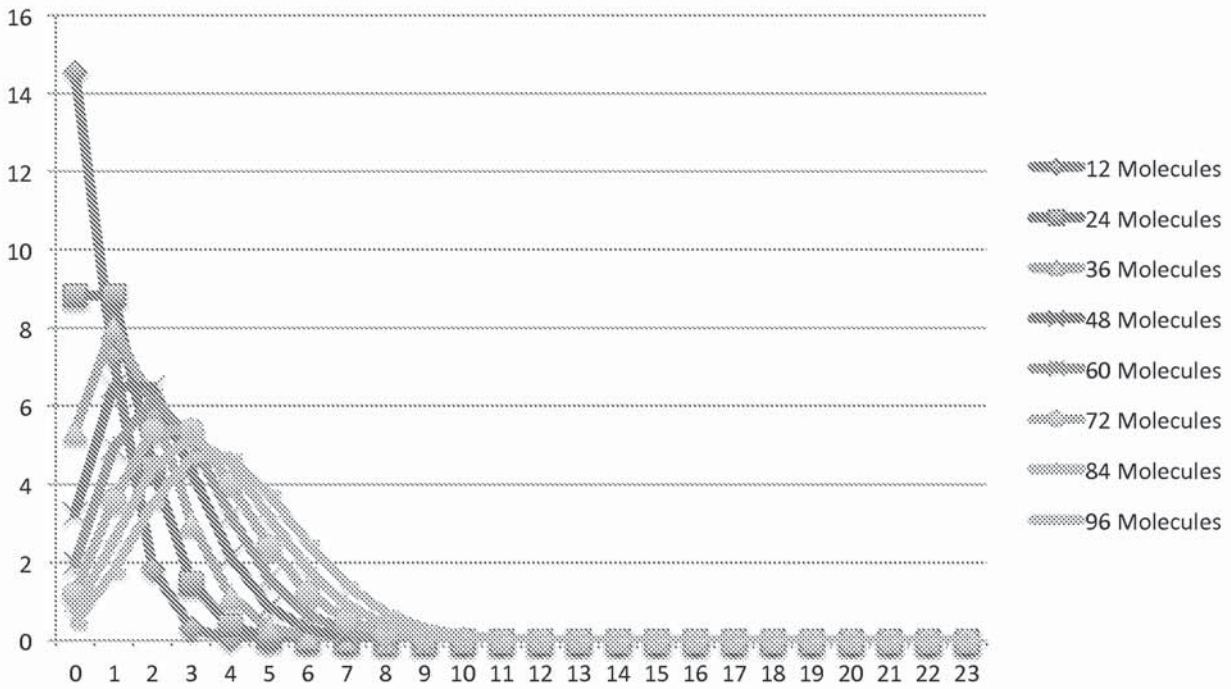


Figure 30

Poisson Distribution of 12 to 96 molecules in 48 wells

Wells with	12	24	36	48	60	72	84	96
0	37	29	23	18	14	11	8	6
1	9	15	17	18	17	16	15	13
2	1	4	6	9	11	12	13	13
3	0	1	2	3	4	6	7	9
4	0	0	0	1	1	2	3	4
5	0	0	0	0	0	1	1	2
6	0	0	0	0	0	0	0	1
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
...	...	...	...	...	...	...	...	...
47	0	0	0	0	0	0	0	0

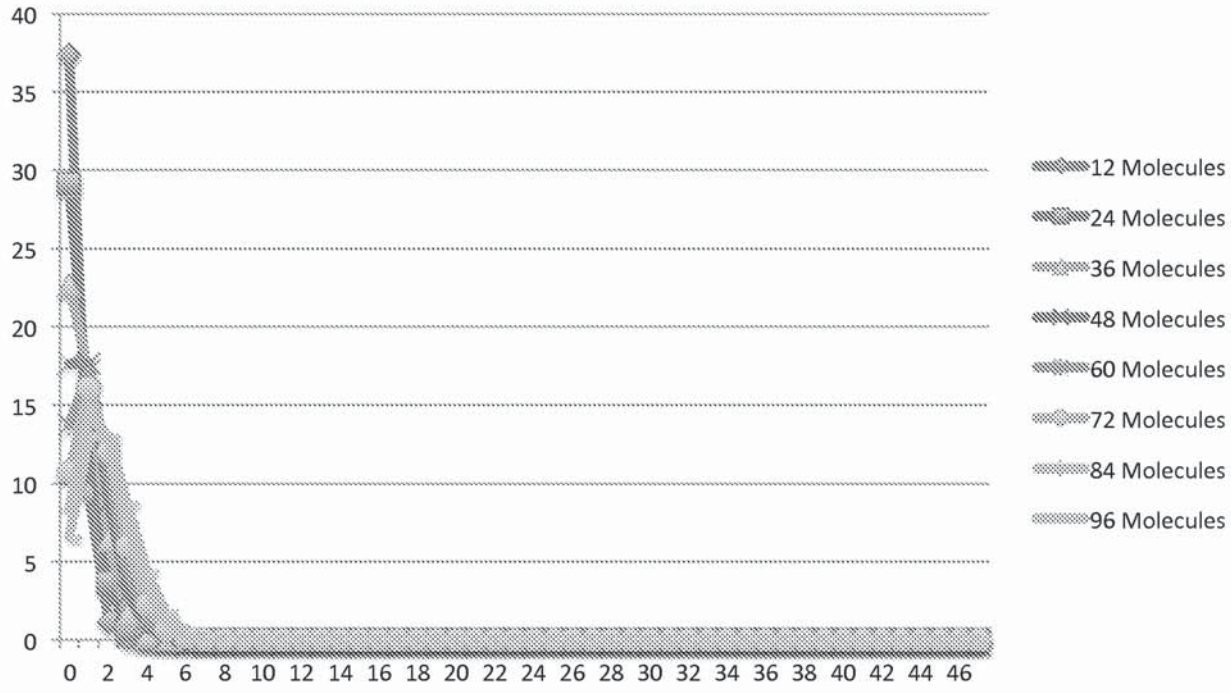


Figure 31

Poisson Distribution of 24 to 192 molecules in 48 wells

Wells with	24	48	72	96	120	144	168	192
0	29	18	11	6	4	2	1	1
1	15	18	16	13	10	7	5	4
2	4	9	12	13	12	11	9	7
3	1	3	6	9	10	11	10	9
4	0	1	2	4	6	8	9	9
5	0	0	1	2	3	5	6	8
6	0	0	0	1	1	2	4	5
7	0	0	0	0	0	1	2	3
8	0	0	0	0	0	0	1	1
9	0	0	0	0	0	0	0	1
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
...	...	...	...	...	...	...	...	...
47	0	0	0	0	0	0	0	0

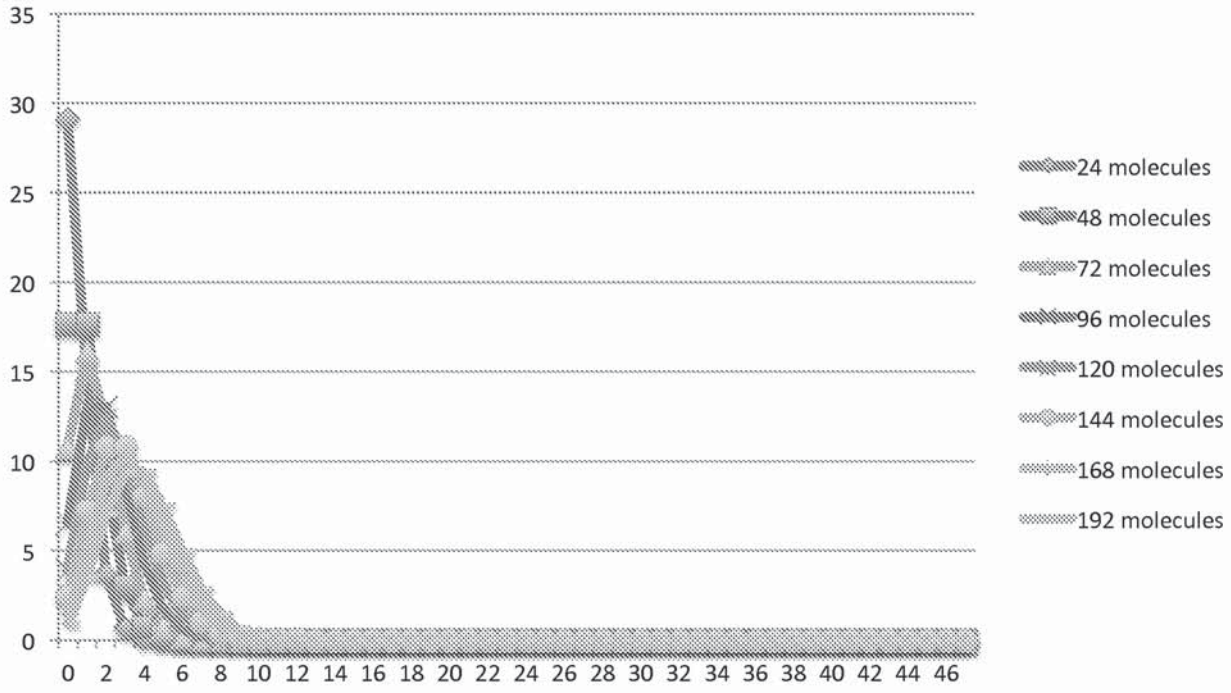


Figure 32

Poisson Distribution of 1 to 8 molecules in 8 wells

Wells with	1	2	3	4	5	6	7	8
0	7	6	5	5	4	4	3	3
1	1	2	2	2	3	3	3	3
2	0	0	0	1	1	1	1	1
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0

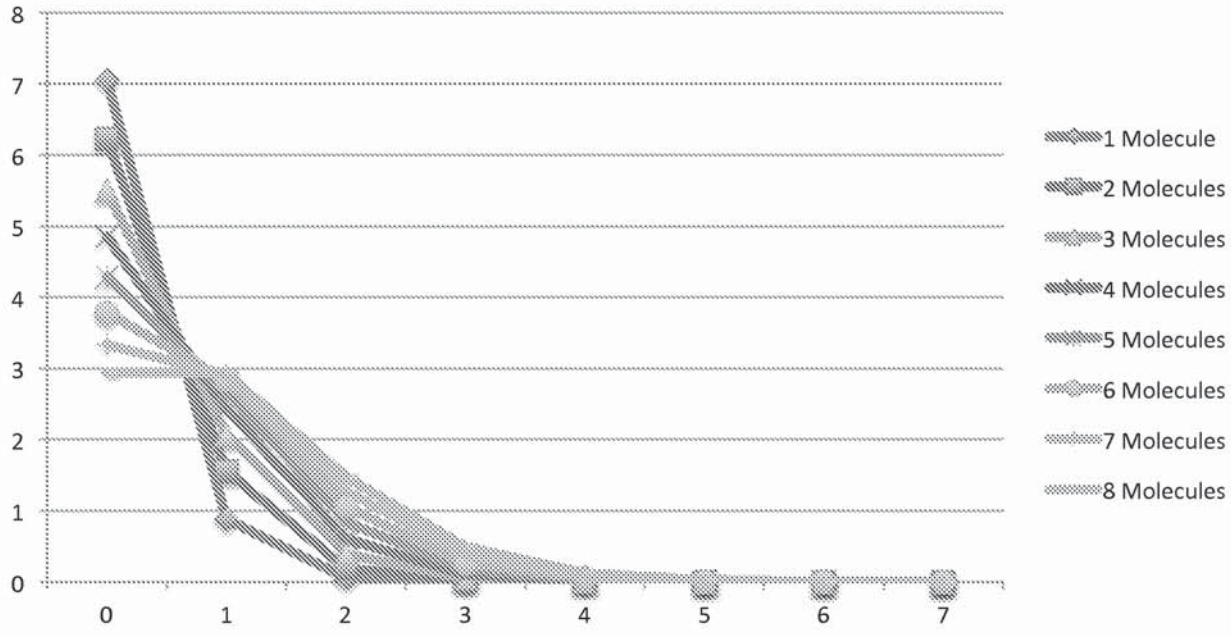


Figure 33

Poisson Distribution of 2 to 16 molecules in 8 wells

Wells with	2	4	6	8	10	12	14	16
0	6	5	4	3	2	2	1	1
1	2	2	3	3	3	3	2	2
2	0	1	1	1	2	2	2	2
3	0	0	0	0	1	1	1	1
4	0	0	0	0	0	0	1	1
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0

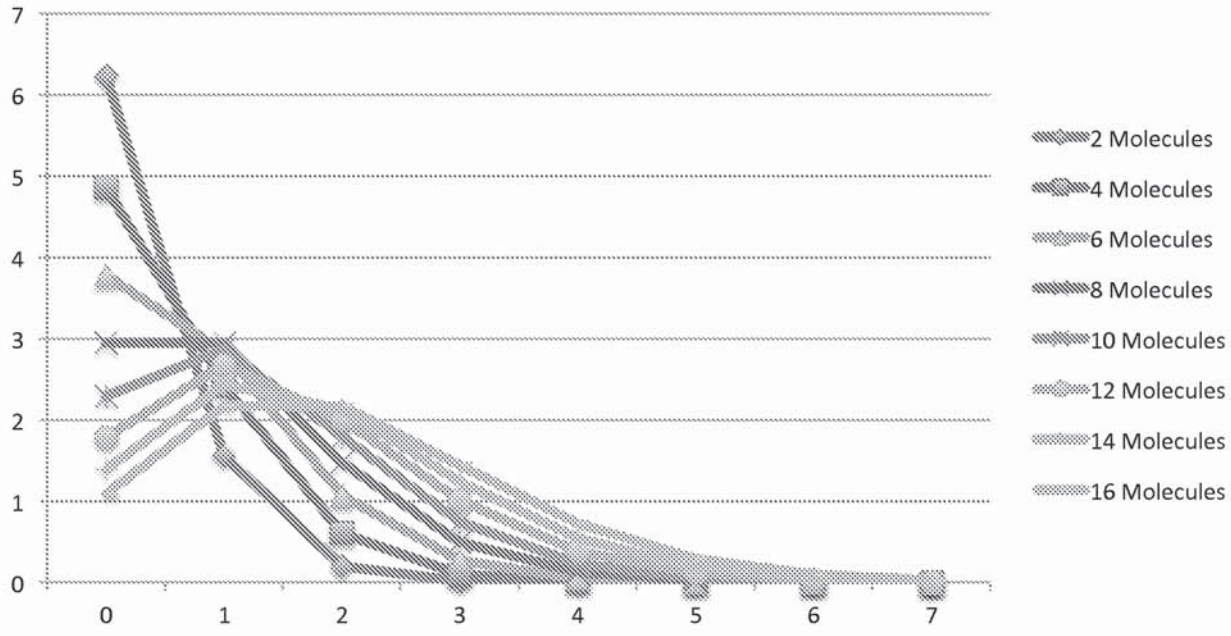


Figure 34

Poisson Distribution of 4 to 32 molecules in 8 wells

Wells with	4	8	12	16	20	24	28	32
0	5	3	2	1	1	0	0	0
1	2	3	3	2	2	1	1	1
2	1	1	2	2	2	2	1	1
3	0	0	1	1	2	2	2	2
4	0	0	0	1	1	1	2	2
5	0	0	0	0	1	1	1	1
6	0	0	0	0	0	0	1	1
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0

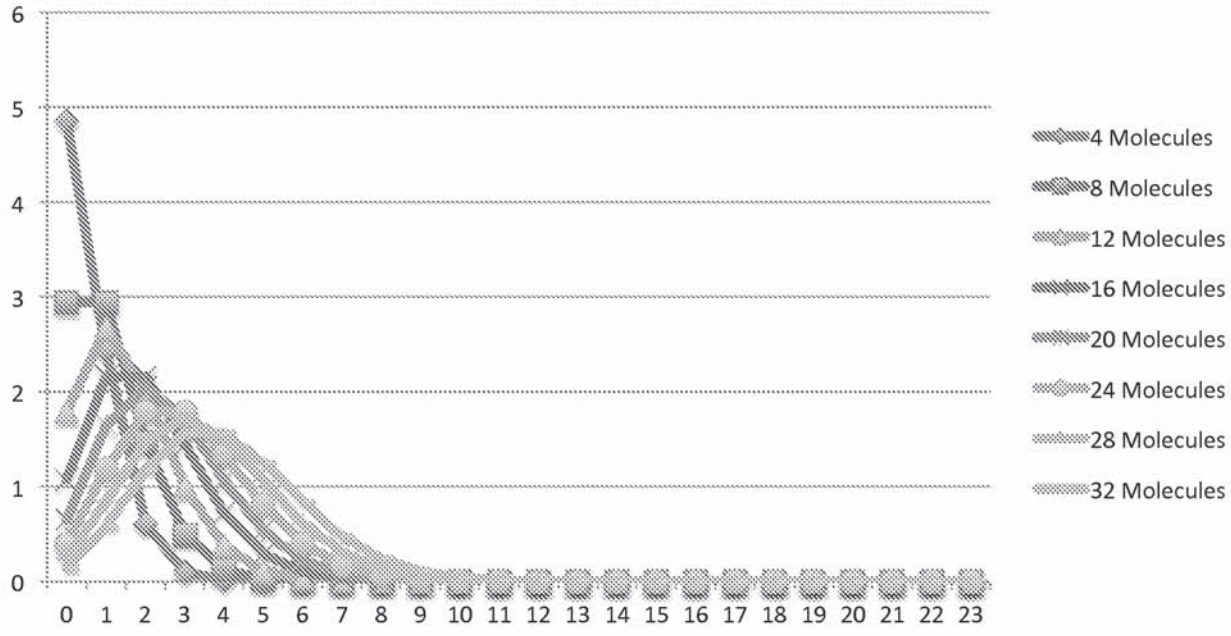


Figure 35

Poisson Distribution of 8 to 64 molecules in 8 wells

Wells with	8	16	24	32	40	48	56	64
0	3	1	0	0	0	0	0	0
1	3	2	1	1	0	0	0	0
2	1	2	2	1	1	0	0	0
3	0	1	2	2	1	1	0	0
4	0	1	1	2	1	1	1	0
5	0	0	1	1	1	1	1	1
6	0	0	0	1	1	1	1	1
7	0	0	0	0	1	1	1	1
8	0	0	0	0	1	1	1	1
9	0	0	0	0	0	1	1	1
10	0	0	0	0	0	0	1	1
11	0	0	0	0	0	0	0	1
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0

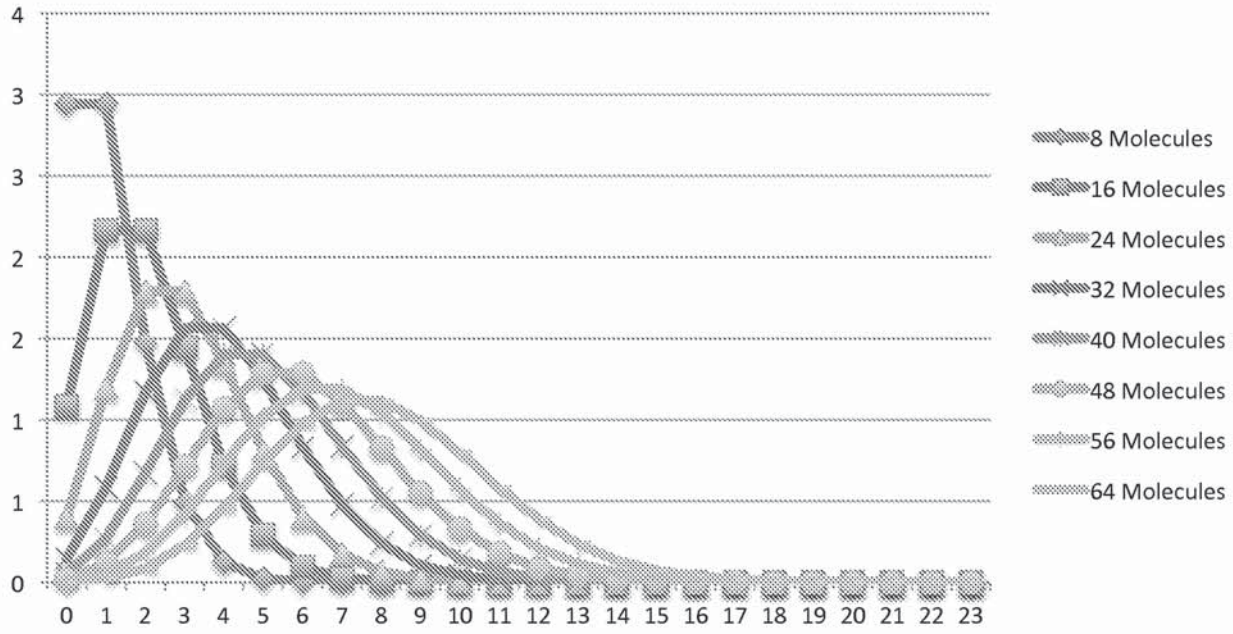


Figure 36

Poisson Distribution of 16 to 128 molecules in 8 wells

Wells with	16	32	48	64	80	96	112	128
0	1	0	0	0	0	0	0	0
1	2	1	0	0	0	0	0	0
2	2	1	0	0	0	0	0	0
3	1	2	1	0	0	0	0	0
4	1	2	1	0	0	0	0	0
5	0	1	1	1	0	0	0	0
6	0	1	1	1	1	0	0	0
7	0	0	1	1	1	0	0	0
8	0	0	1	1	1	1	0	0
9	0	0	1	1	1	1	0	0
10	0	0	0	1	1	1	1	0
11	0	0	0	1	1	1	1	0
12	0	0	0	0	1	1	1	1
13	0	0	0	0	1	1	1	1
14	0	0	0	0	0	1	1	1
15	0	0	0	0	0	1	1	1
16	0	0	0	0	0	0	1	1
17	0	0	0	0	0	0	1	1
18	0	0	0	0	0	0	0	1
19	0	0	0	0	0	0	0	1
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0

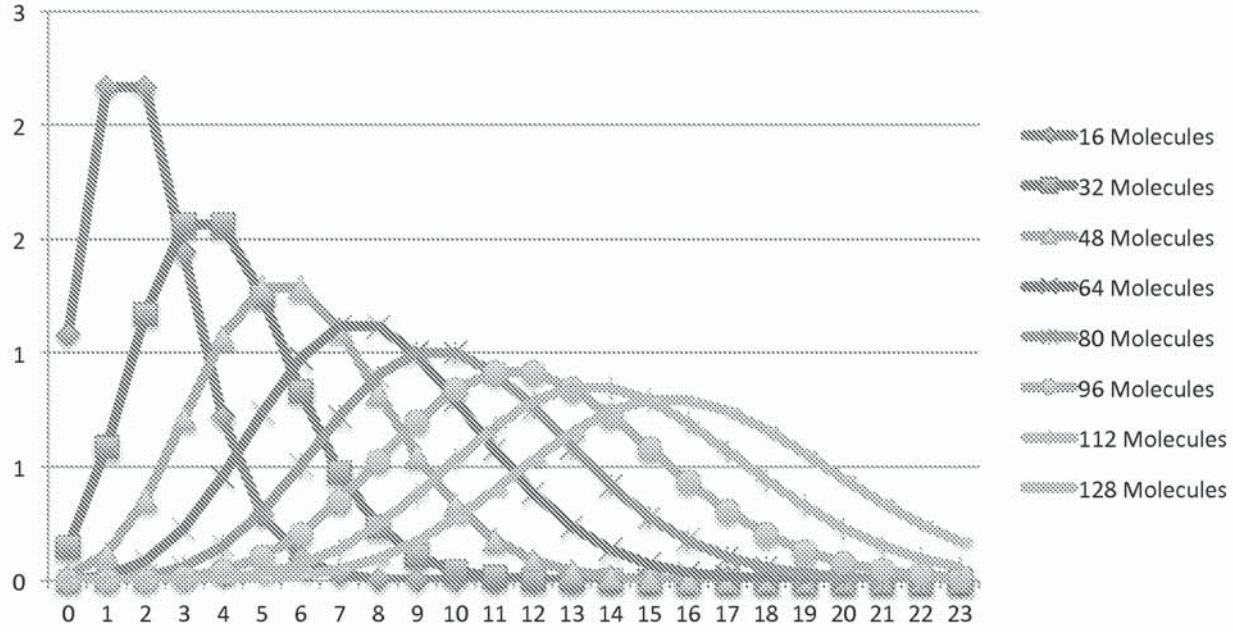


Figure 37

A. PCR-LDR-qPCR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR step.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

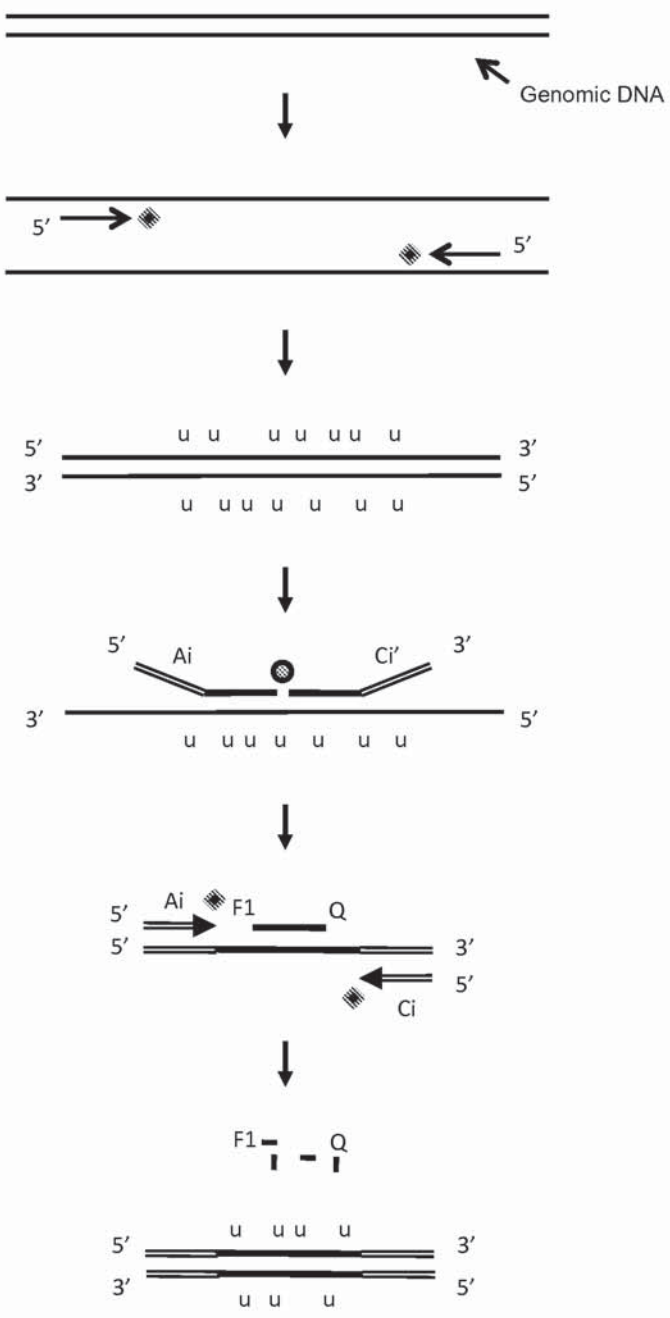


Figure 38

A. PCR-qLDR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Mutation-specific ligation oligonucleotides contain complementary tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, allowing tails to hybridize and bringing donor (D) and fluorescent group (F1) in close proximity. Then detect, and enumerate FRET signal.

Or

F. Mutation-specific ligation oligonucleotides contain complementary tails for subsequent fluorescent detection. Ligase covalently seals the two oligonucleotides together.

G. Denature product from target, allowing tails to hybridize and separate quencher (Q) from fluorescent reporter (F1). Then detect, and enumerate un-quenched fluorescent signal.

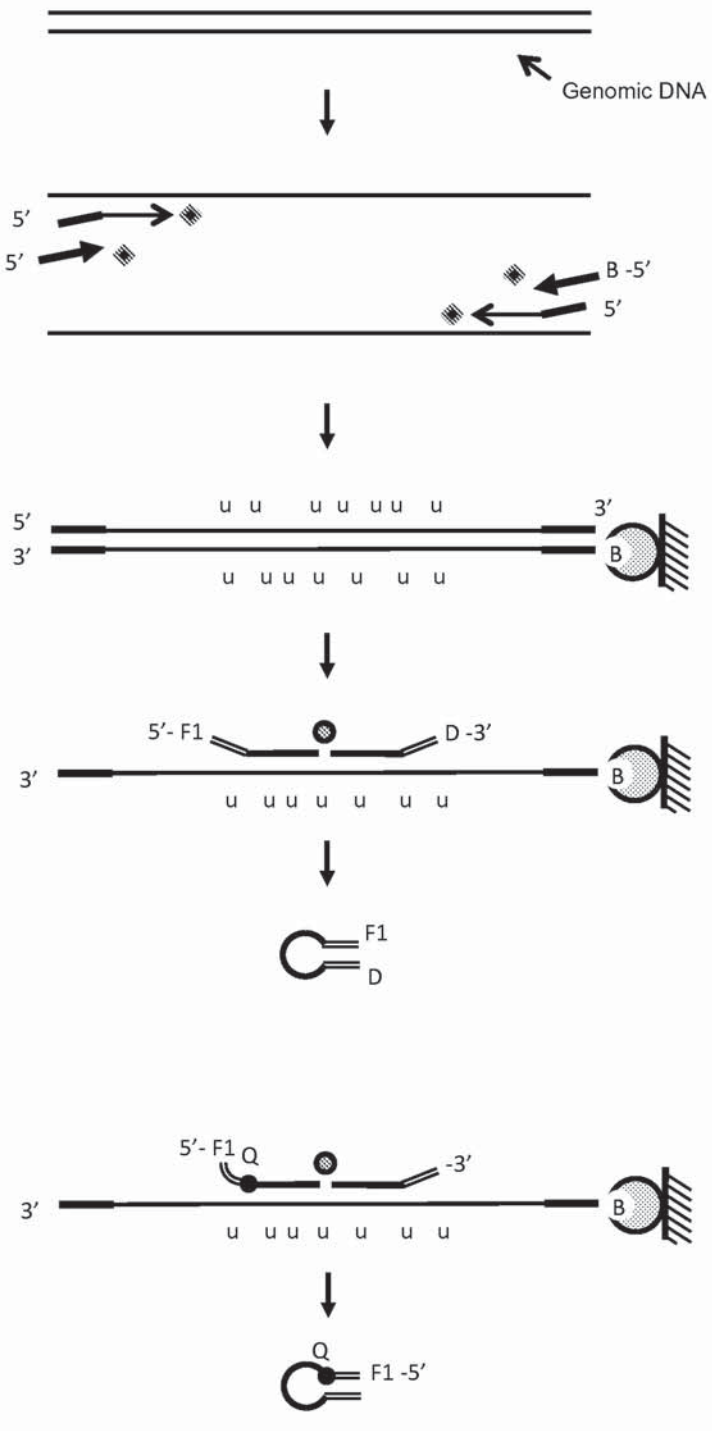


Figure 39

A. PCR-LDR-qPCR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention. Locus-specific primers are unblocked with RNaseH2 only when bound to target. Amplify mutation containing regions using PCR (and dUTP). Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

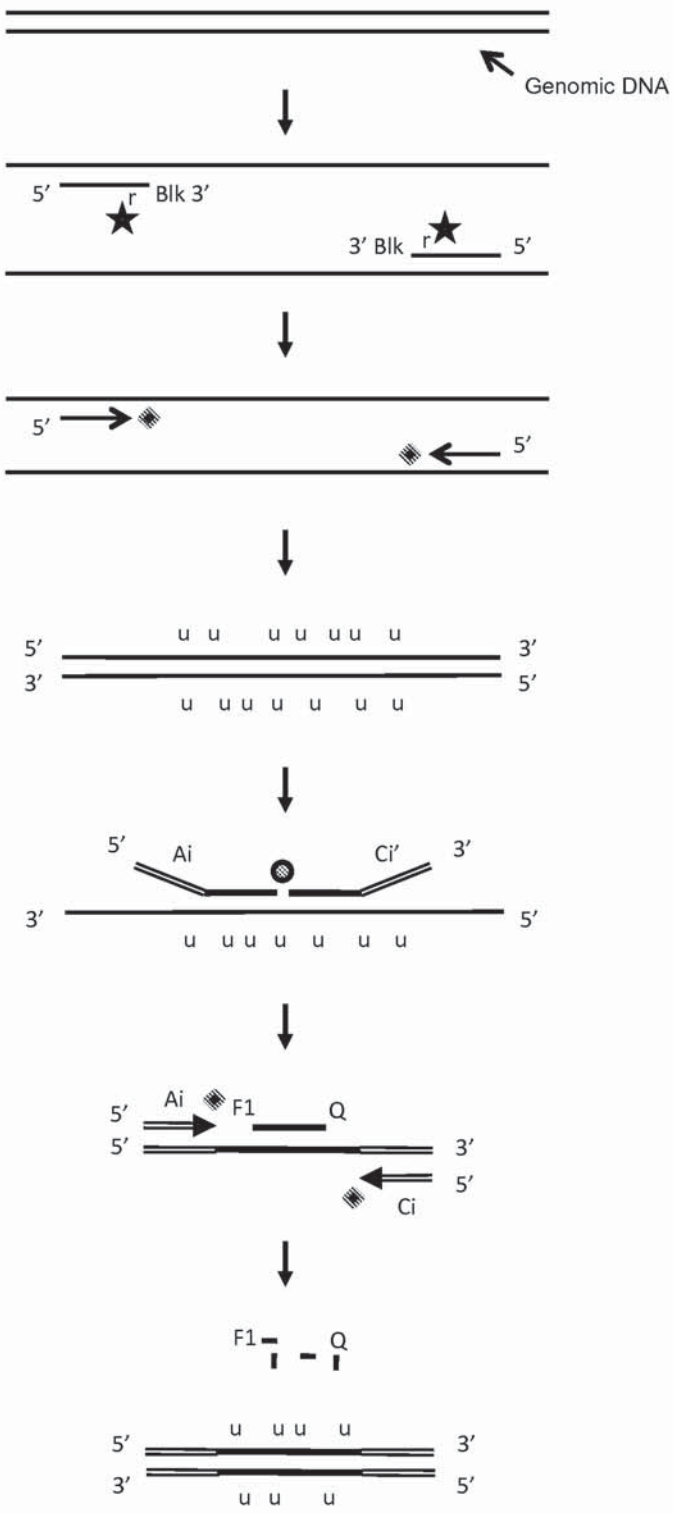


Figure 40

A. PCR-LDR-qPCR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Ci') for subsequent PCR amplification, while wild-type probe (Wt) does not. 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 upstream and phosphorylated downstream oligonucleotides together. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

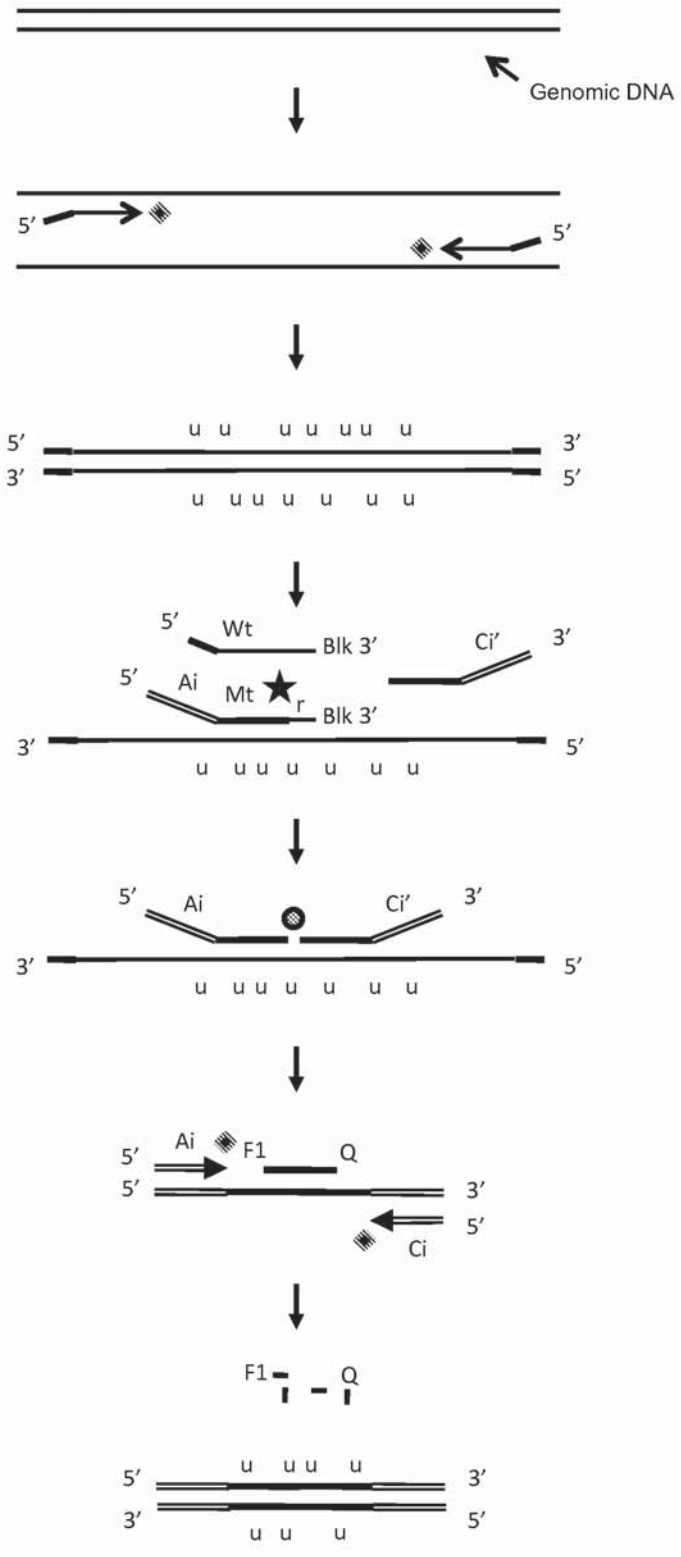


Figure 41

A. PCR-qLDR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Mutation-specific ligation oligonucleotides (Mt) contain tails for subsequent FRET detection, while wild-type probe (Wt) does not. 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 upstream and phosphorylated downstream oligonucleotides together.

F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

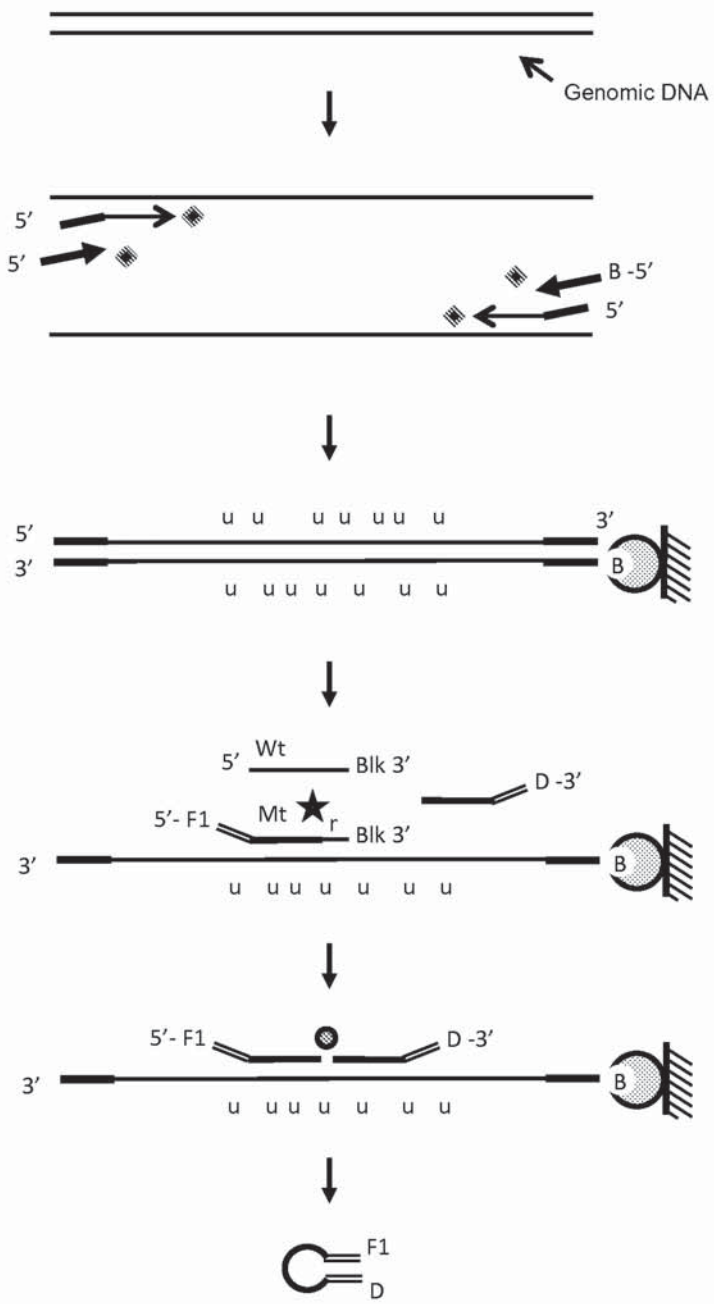


Figure 42

A. PCR-qLDR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Mutation-specific ligation oligonucleotides (Mt) overlap at the mutation base, and contain tails for subsequent FRET detection, while wild-type probe (Wt) does not. 5'-nuclease activity of polymerase cleaves off only matching 5'-overlapping base and additional flap, leaving ligation-competent 5-phosphate.

E. Ligase covalently seals the upstream and unblocked phosphorylated downstream oligonucleotides together.

F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

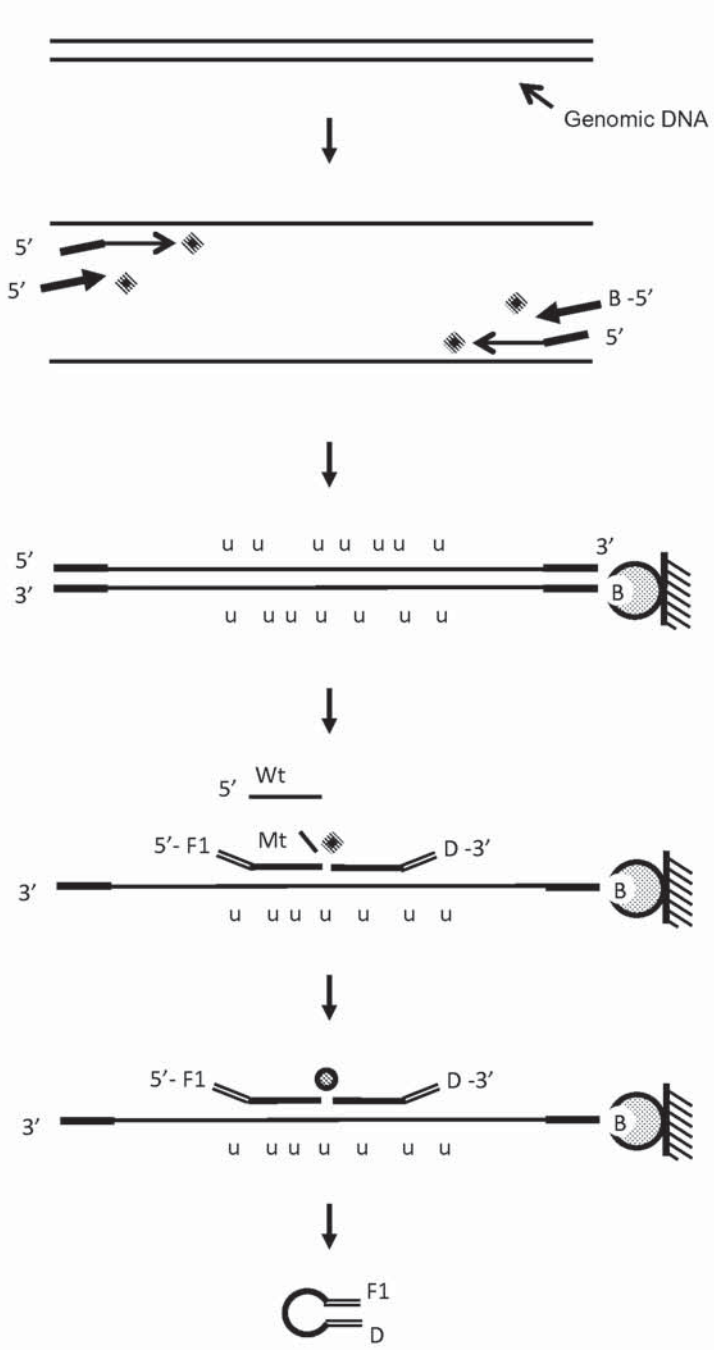


Figure 43

A. PCR-LDR-qPCR carryover prevention reaction to detect mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides contain tags (Ai, Bi'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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. (PCR products incorporate dU, allowing for carryover prevention.)

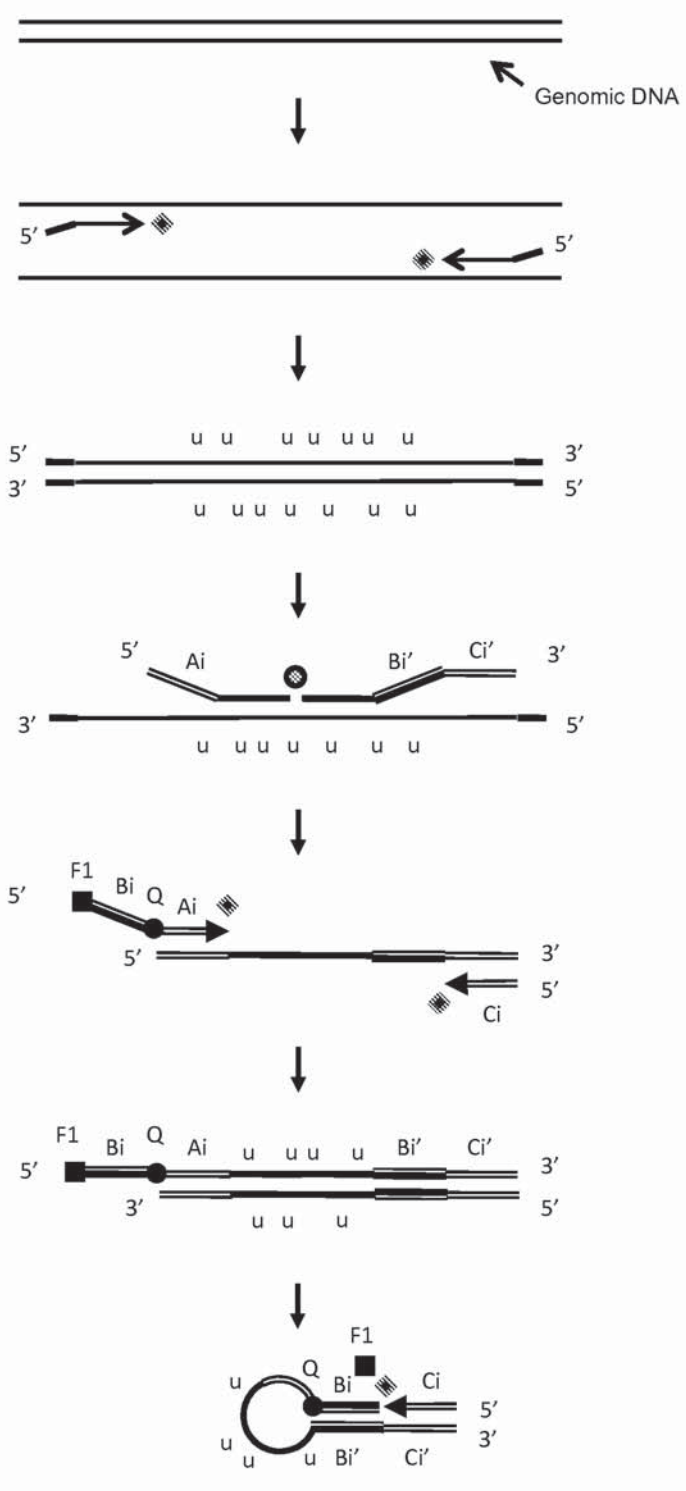


Figure 44

A. PCR-LDR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Optional: enrich for methylated DNA using antibodies. Treat with methyl-sensitive restriction endonucleases Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover.

B. Amplify methyl containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.)

D. Methyl region-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

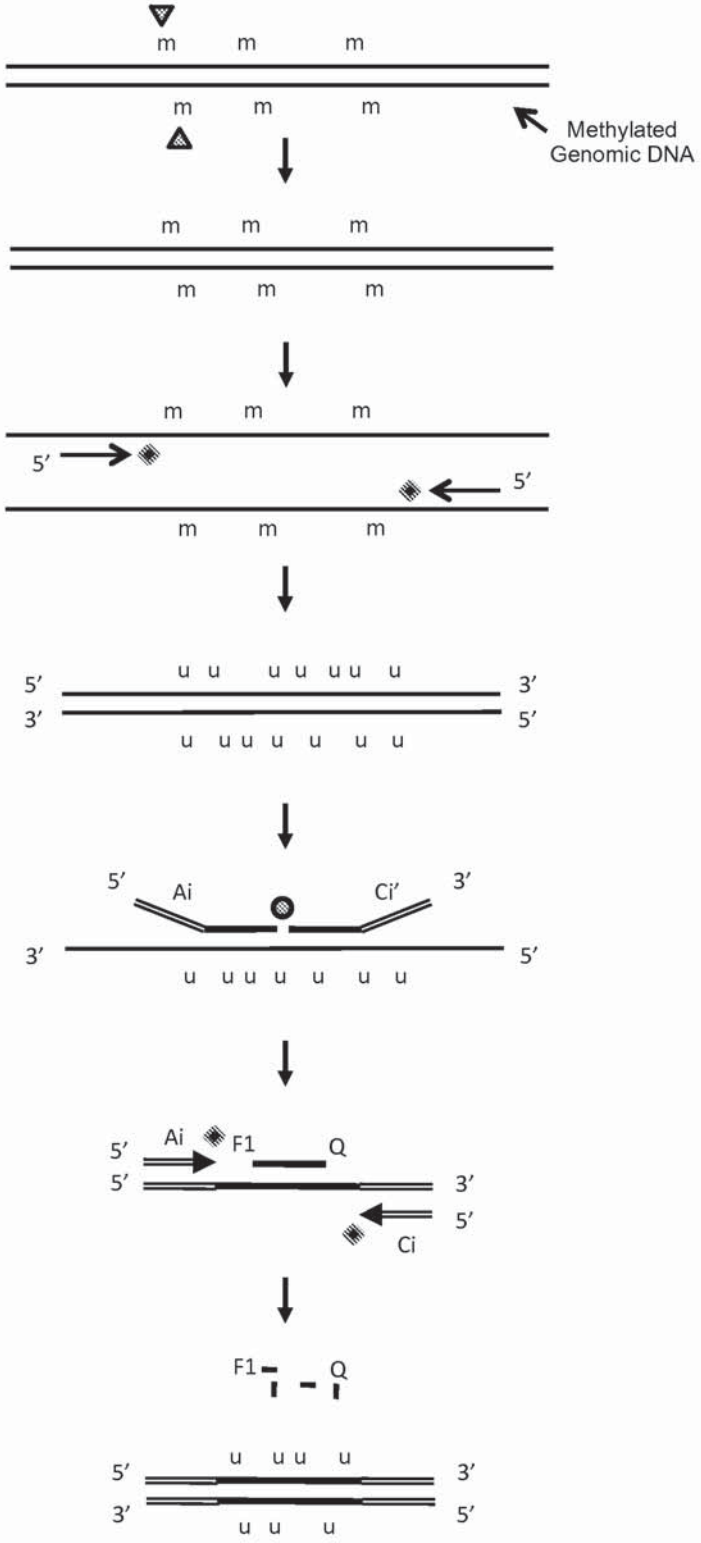


Figure 45

A. PCR-LDR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover.

B. Amplify methyl containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Methyl region-specific ligation oligonucleotides contain tags (Ai, Bi'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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. (PCR products incorporate dU, allowing for carryover prevention.)

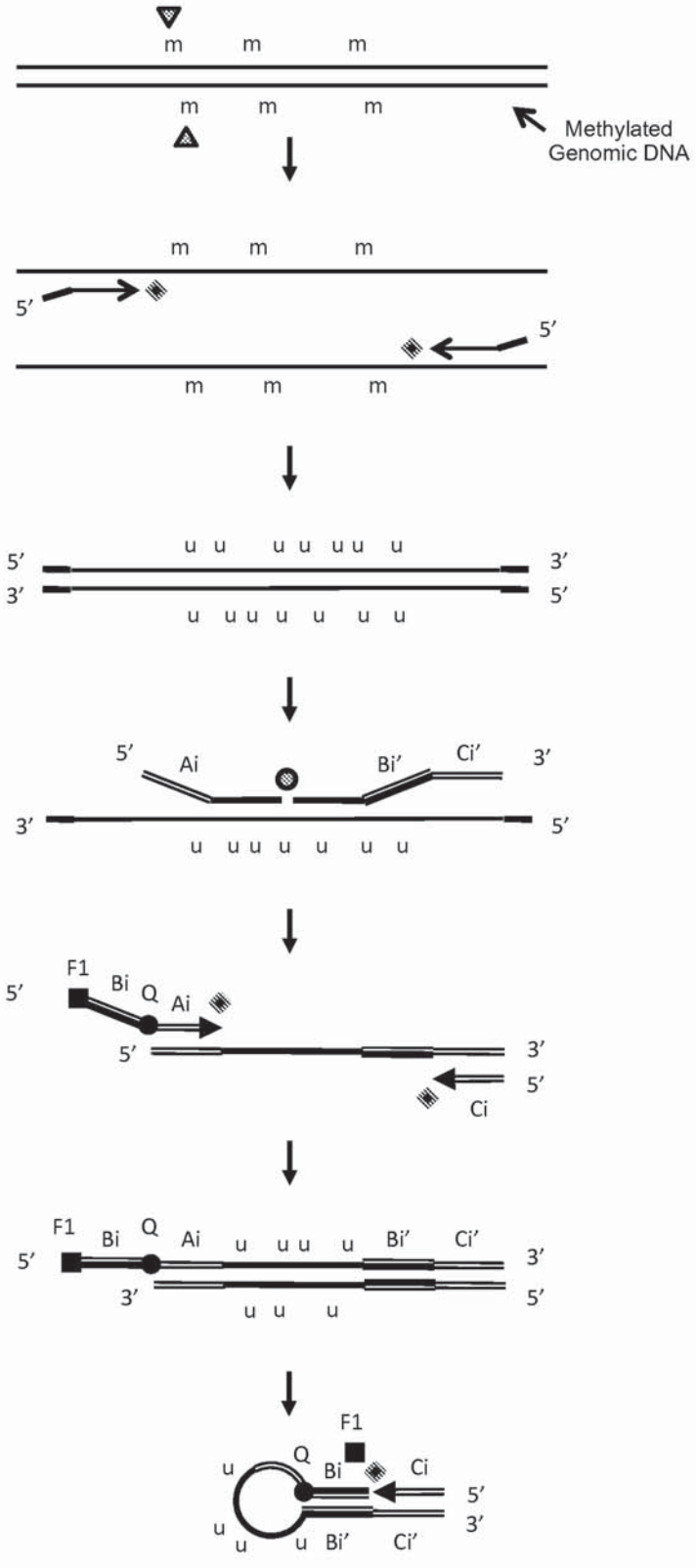


Figure 46

A. PCR-qLDR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Optional: enrich for methylated DNA using antibodies. Treat with methyl-sensitive restriction endonucleases Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover.

B. Aliquot into 12, 24, 48, or 96 wells, and amplify methyl containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.) Separate product from primers and capture biotinylated product on solid support.

D. Methyl region-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together.

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

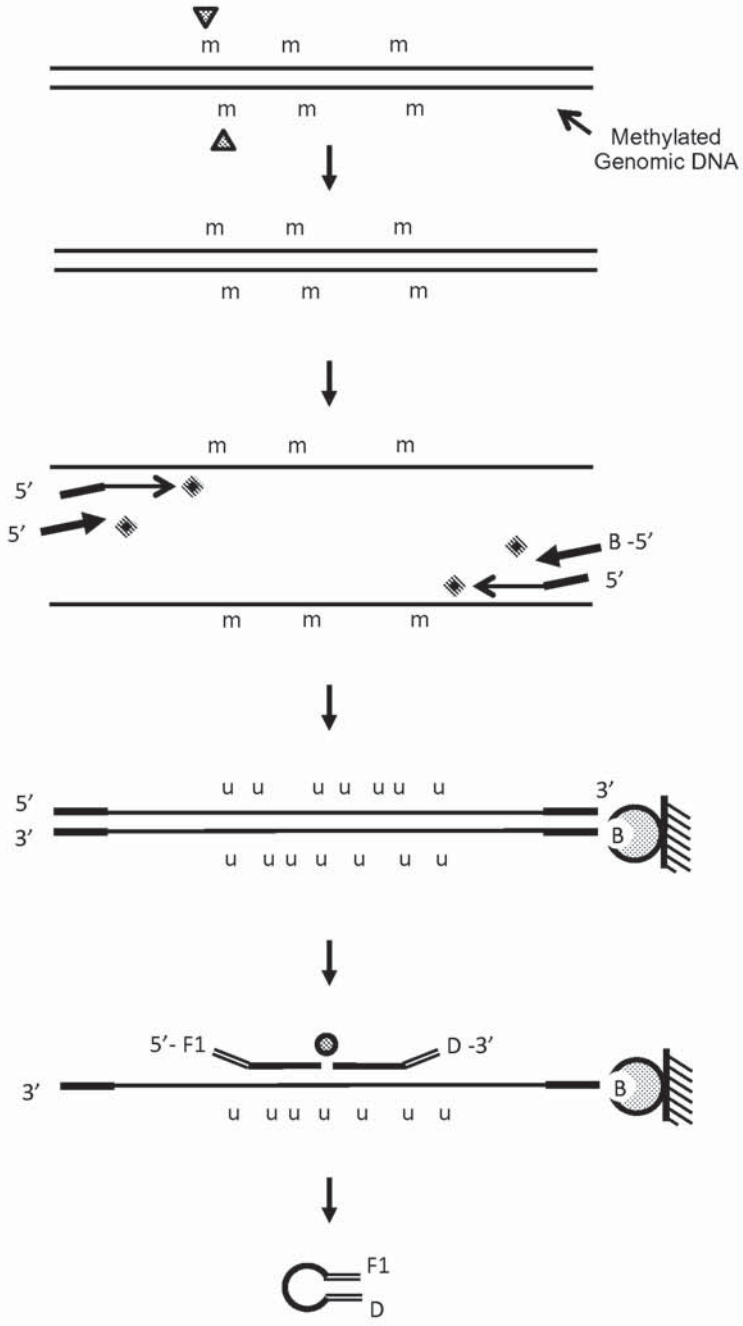


Figure 47

A. Nuclease-Ligation-PCR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with HaeIII (GG^CC) and methyl-sensitive restriction endonucleases Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover.

B. Ligate hairpin oligonucleotide containing tag sequence (Ai') to newly liberated phosphate of template strand.

C. Treat with HinP1I and Bsh1236I at 37oC, then heat kill endonucleases while activating Taq polymerase. Locus-specific primers (containing Ci tag sequence) are unblocked with RNaseH2 only when bound to target, and extended with polymerase, whose 5' nuclease activity digests 5' portion of hairpin to generate target complement with Ai' sequence. Unligated hairpin extends on itself.

D. Amplify methyl containing regions using PCR (and dUTP) with tag-primers Ai and Ci. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

E. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.) Aliquot into separate wells for Taqman detection using locus-specific primers and Taqman probe.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

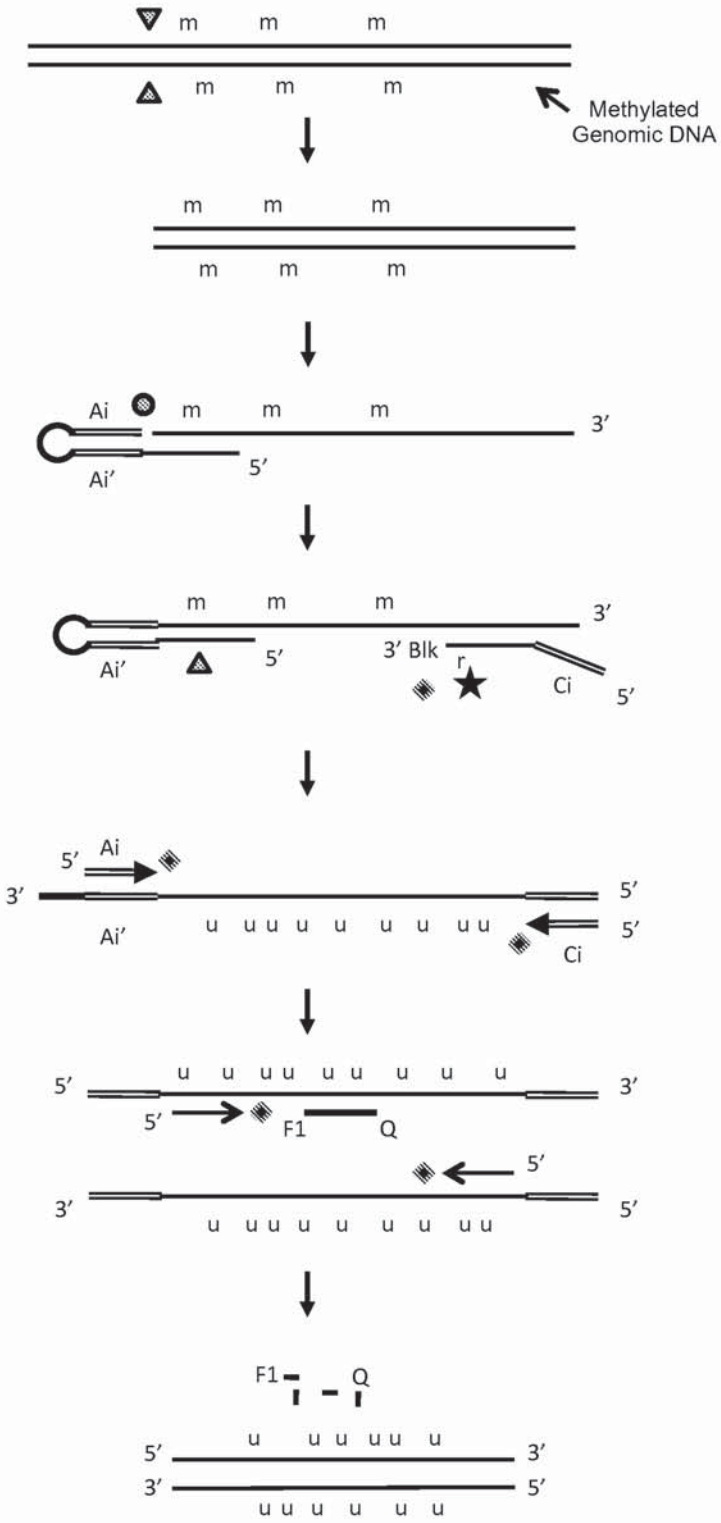


Figure 48

A. Nuclease-Ligation-PCR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with HaeIII (GG^CC), Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG.

B. Ligate hairpin oligonucleotide containing tag sequence (Ai') to newly liberated phosphate of template strand.

C. Treat with HinP1I and Bsh1236I at 37oC, then heat kill endonucleases while activating Taq polymerase. Locus-specific primers (containing Ci tag sequence) are unblocked with RNaseH2 only when bound to target, and extended with polymerase, whose 5' nuclease activity digests 5' portion of hairpin to generate target complement with Ai' sequence.

D. Amplify methyl containing regions using PCR (and dUTP) with tag-primers Ai and Ci. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

E. PCR products incorporate dU, allowing for carryover prevention. Aliquot into separate wells for UniTaq detection, and amplify using locus-specific primers tailed with Aj, and Bj-Cj, as well as Unitaq-specific primers (F1-Bj-Q-Aj, and Cj).

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 Bj and Bj'. 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.)

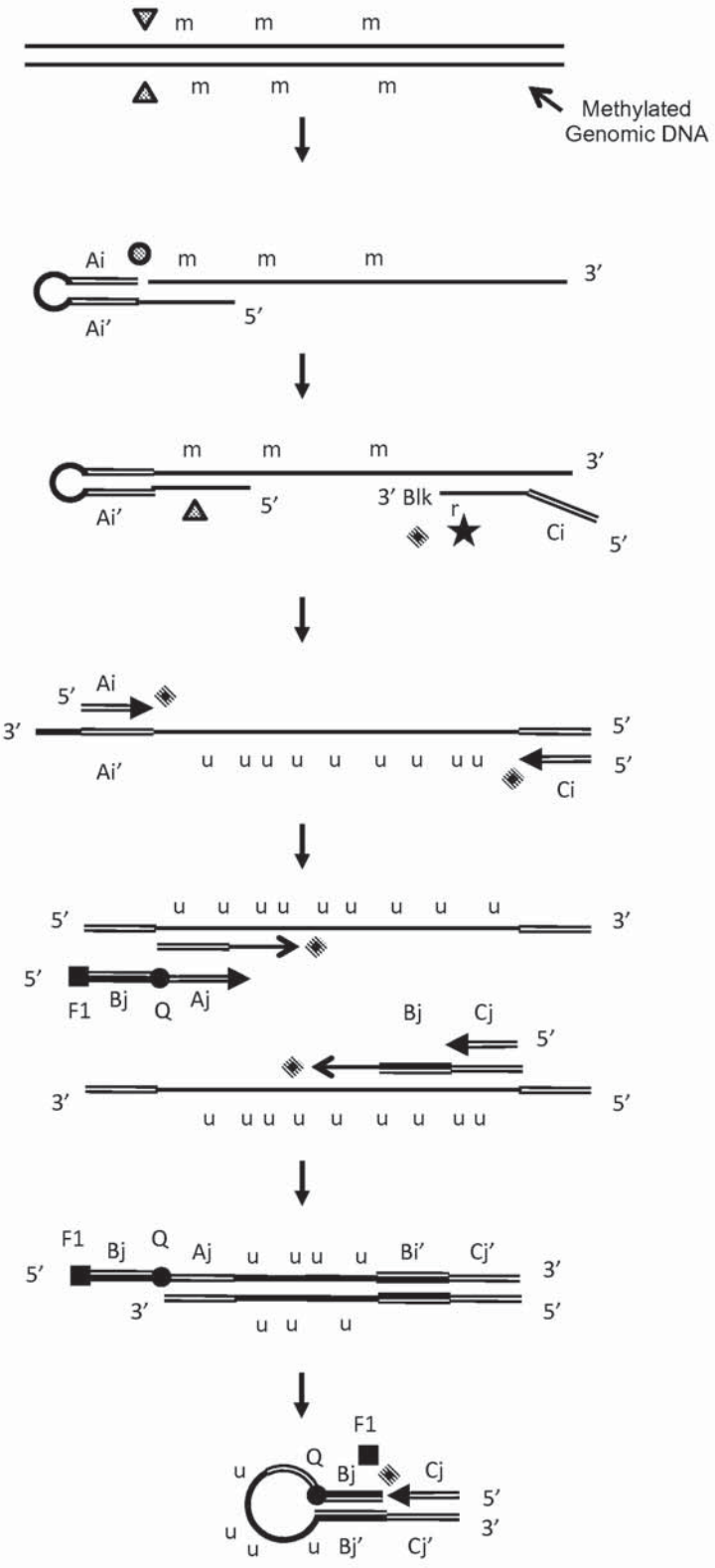


Figure 49

A. PCR-LDR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Hybridize locus-specific primers in presence of BstU1 (CG^CG), will cleave carryover DNA. Primers are unblocked with RNaseH2 only when bound to target. Amplify methyl containing regions using PCR (and dUTP). Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligo may be used to limit amplification of originally un-methylated DNA (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.)

D. Methyl region-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

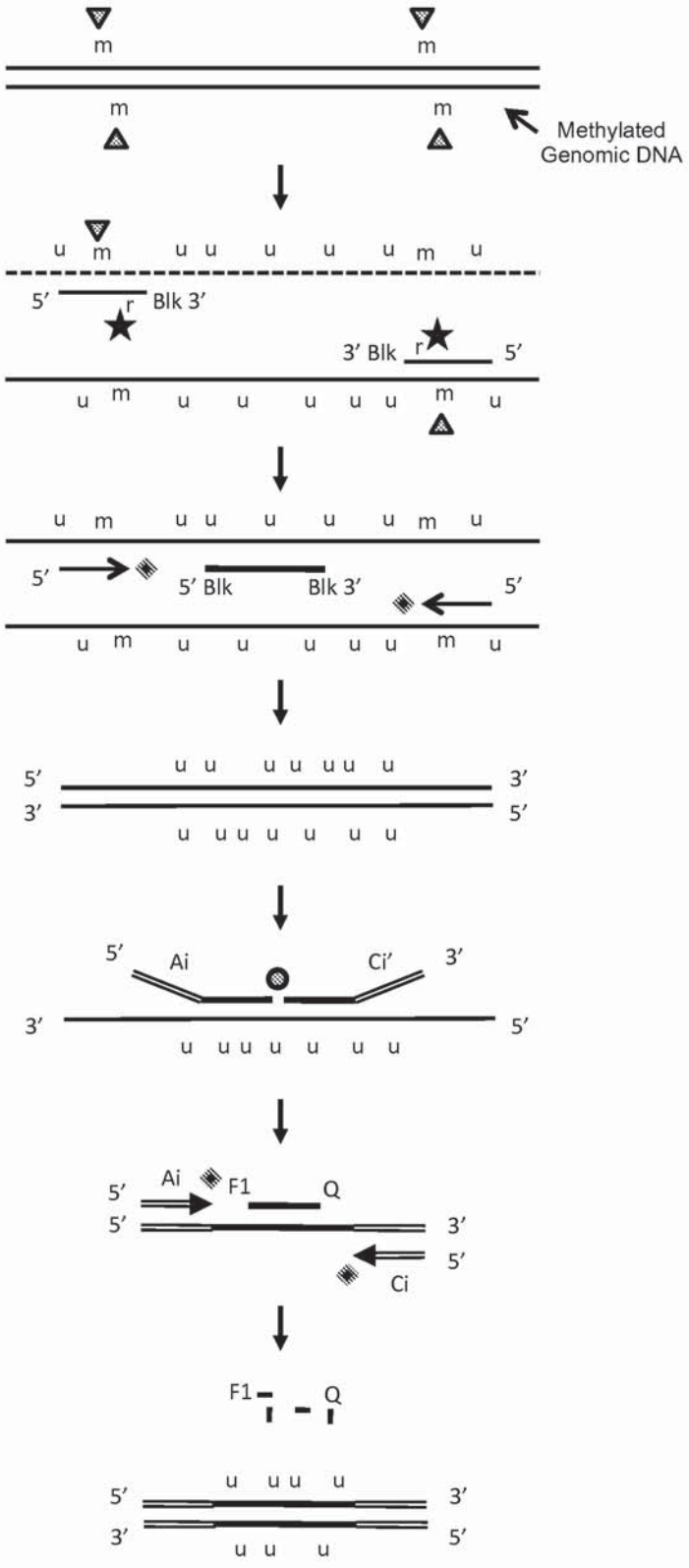


Figure 50

A. PCR-LDR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Hybridize locus-specific primers in presence of BstU1 (CG^CG), will cleave carryover DNA. Primers are unblocked with RNaseH2 only when bound to target. Amplify methyl containing regions using PCR (and dUTP). Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligo may be used to limit amplification of originally unmethylated DNA (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Methyl region-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

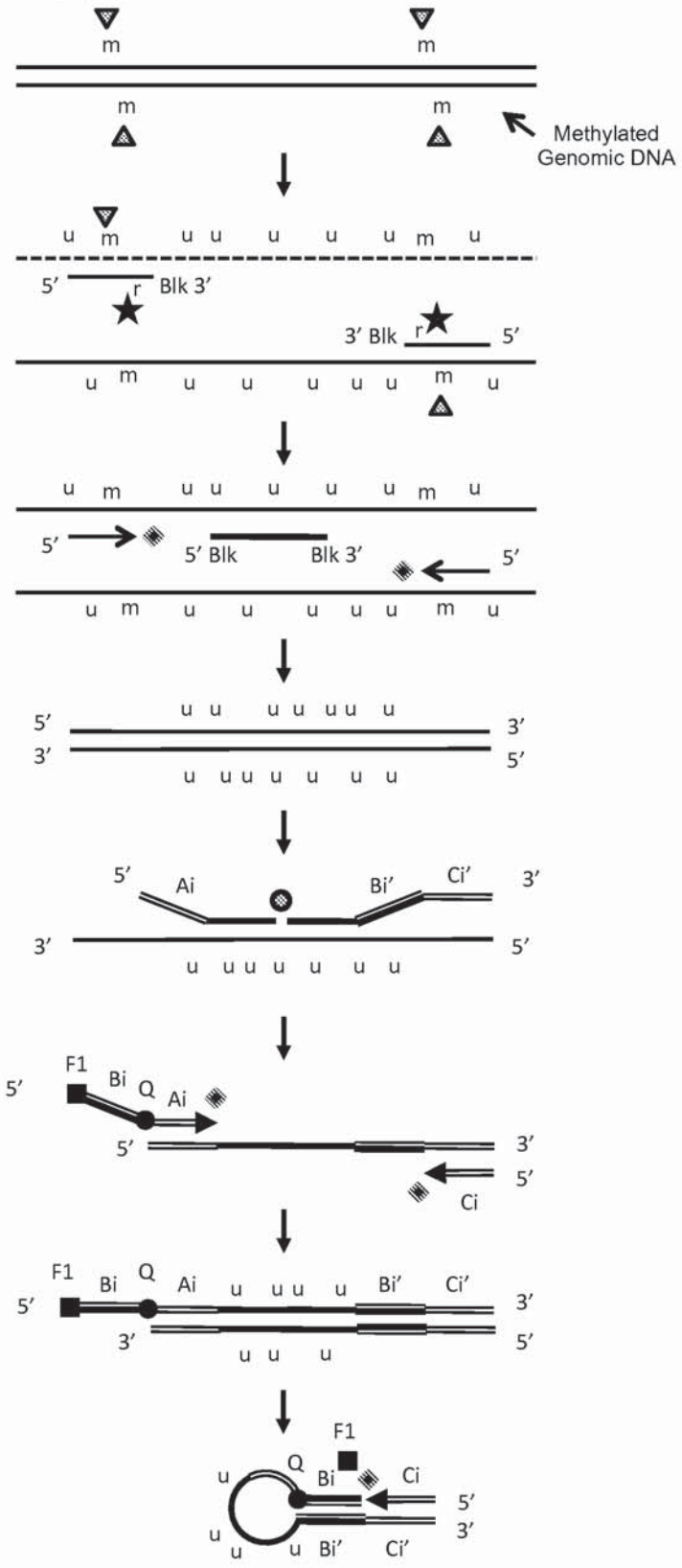


Figure 51

A. PCR-qLDR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Hybridize locus-specific primers in presence of BstU1 (CG^CG), will cleave carryover DNA. Primers are unblocked with RNaseH2 only when bound to target. Aliquot into 12, 24, 48, or 96 wells, and amplify methyl containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin. Blocking oligo may be used to limit amplification of originally unmethylated DNA.

C. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.) Separate product from primers and capture biotinylated product on solid support.

D. Methyl region-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together.

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

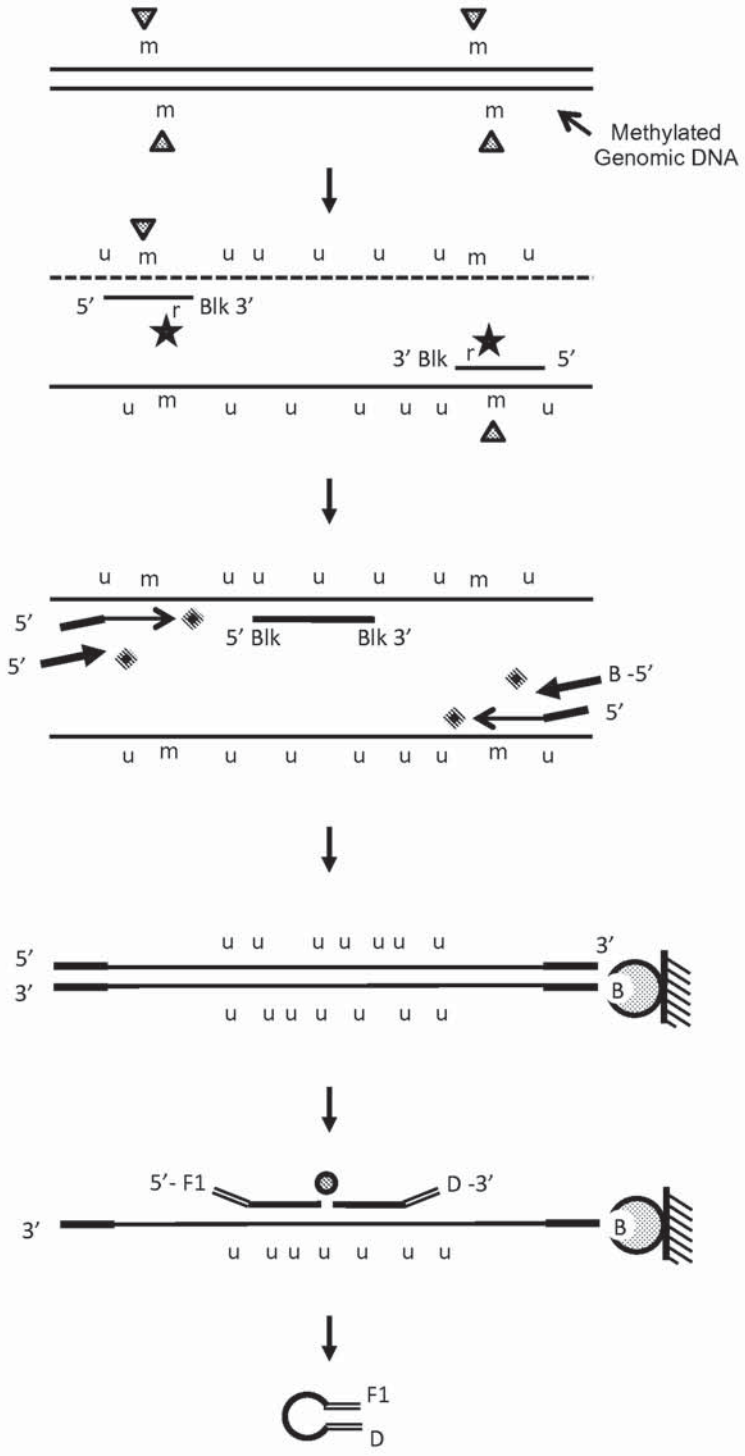


Figure 52

A. PCR-qPCR carryover prevention reaction to detect methylation. Isolate genomic or cfDNA. Treat Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Hybridize locus-specific primers in presence of BstU1 (CG^CG), will cleave carryover DNA. Primers are unblocked with RNaseH2 only when bound to target. Amplify methyl containing regions using PCR with dNTPs. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligos may be used to limit amplification of wild-type DNA. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products are unmethylated providing carryover protection.

D. Aliquot into separate wells for Taqman detection using locus-specific primers and Taqman probe. Optional blocking oligonucleotide is used to limit amplification of wild-type DNA

E. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

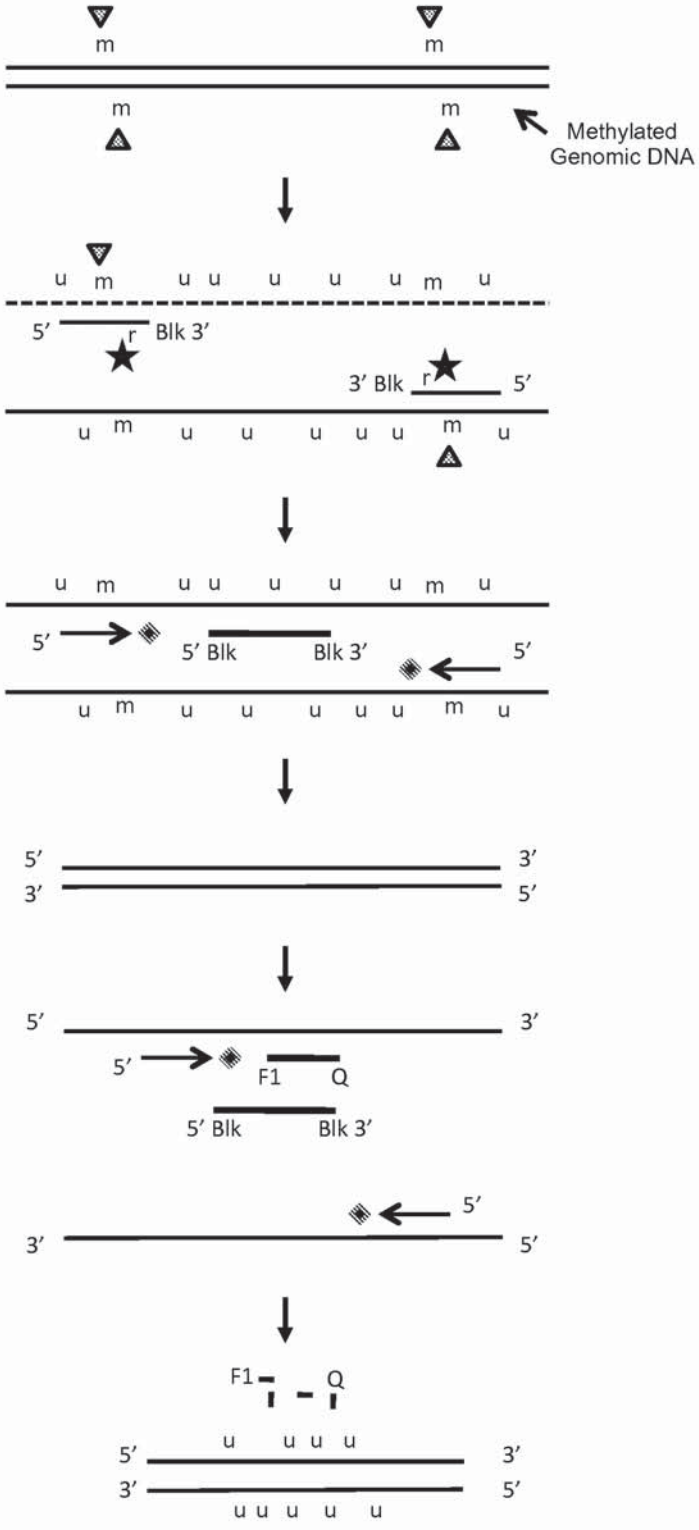


Figure 53

A. Overview: RT-PCR-LDR-qPCR carryover prevention reaction to detect translocation at the mRNA level. Illustration of translocations between two genes shown at the DNA level.

B. Examples of fusion junctions between exons 1-b, 2-b, and 3-b in mRNAs are illustrated. Isolate mRNA from whole blood cells, exosomes, or CTCs. Generate cDNA using reverse transcriptase and primer complementary to exon b. PCR amplify using forward primers to exons 1, 2, and 3. Independent of translocation breakpoint, the primers will amplify the smallest fragment containing the exon junction region.

C. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci' for individual Taqman probes; or Ai, Bi-Ci for UniTaq probes) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together, if there is perfect complementarity at the junction. Aliquot into separate wells for detection using real-time PCR.

D. When using tag-specific primers (Ai, Ci) for amplifying LDR products of the upper set, each Taqman probe spans the ligation junction, and can be scored individually. When using UniTaq-specific primers (F1-Bi-Q-Ai, Ci), for amplifying LDR products of the lower set, the same primer set scores for the given translocation, independent of the specific exon junction.

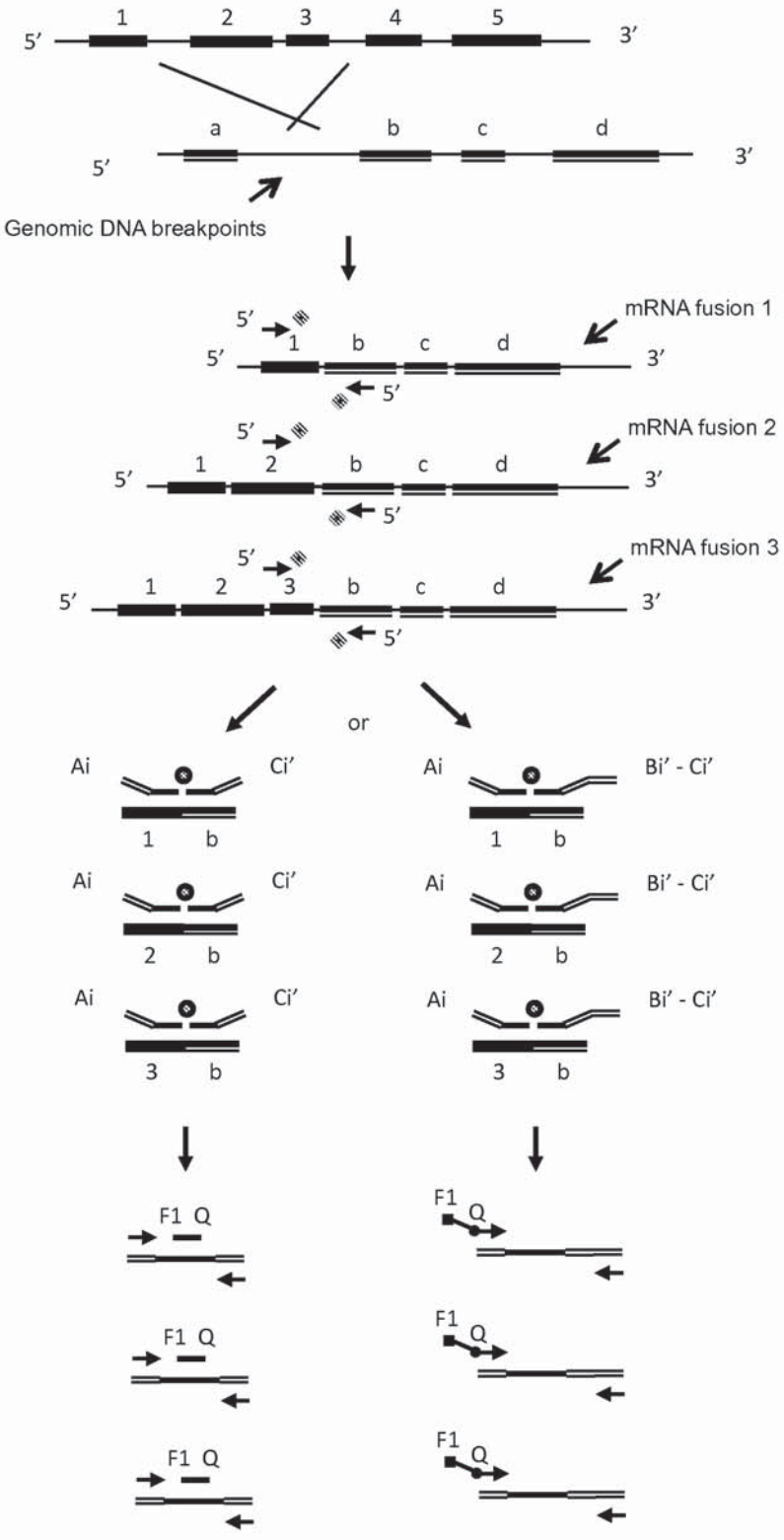


Figure 54

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect translocation at the mRNA level. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

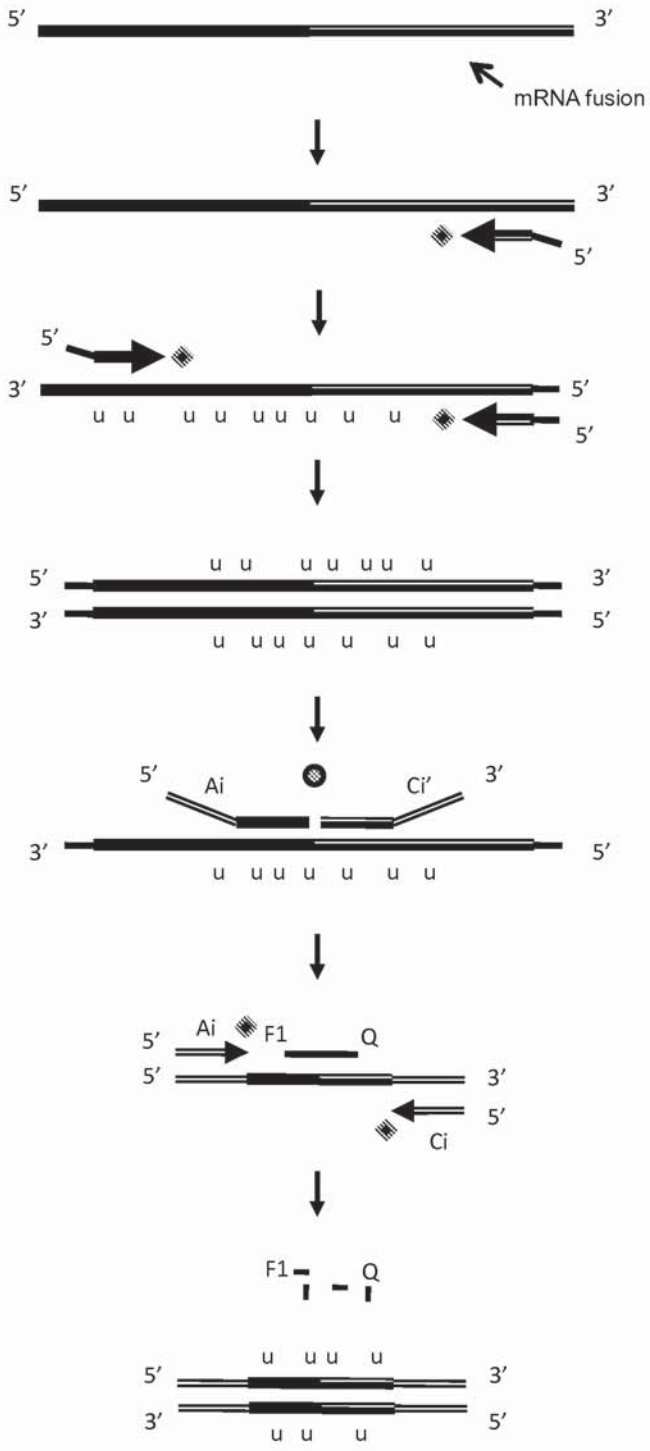


Figure 55

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect translocation at the mRNA level. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

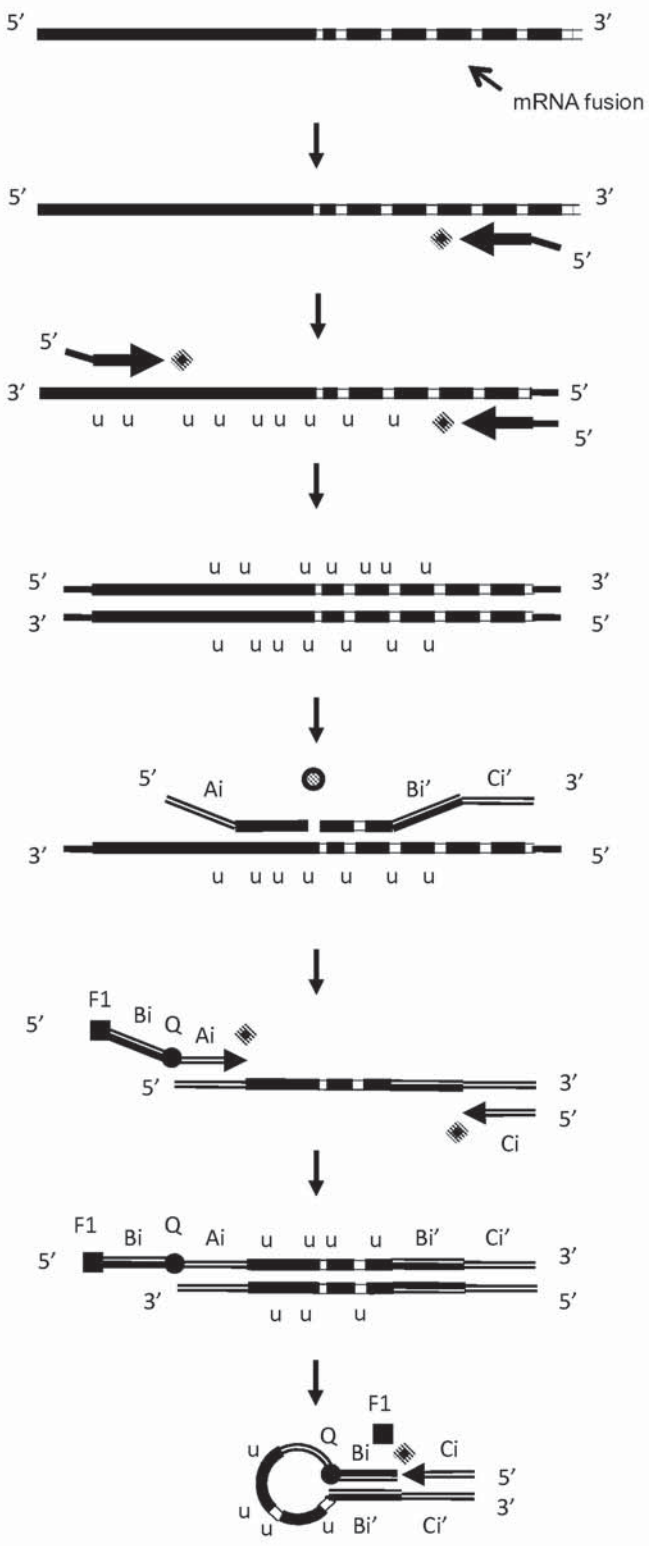


Figure 56

A. RT-PCR-qLDR carryover prevention reaction to detect translocation at the mRNA level. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

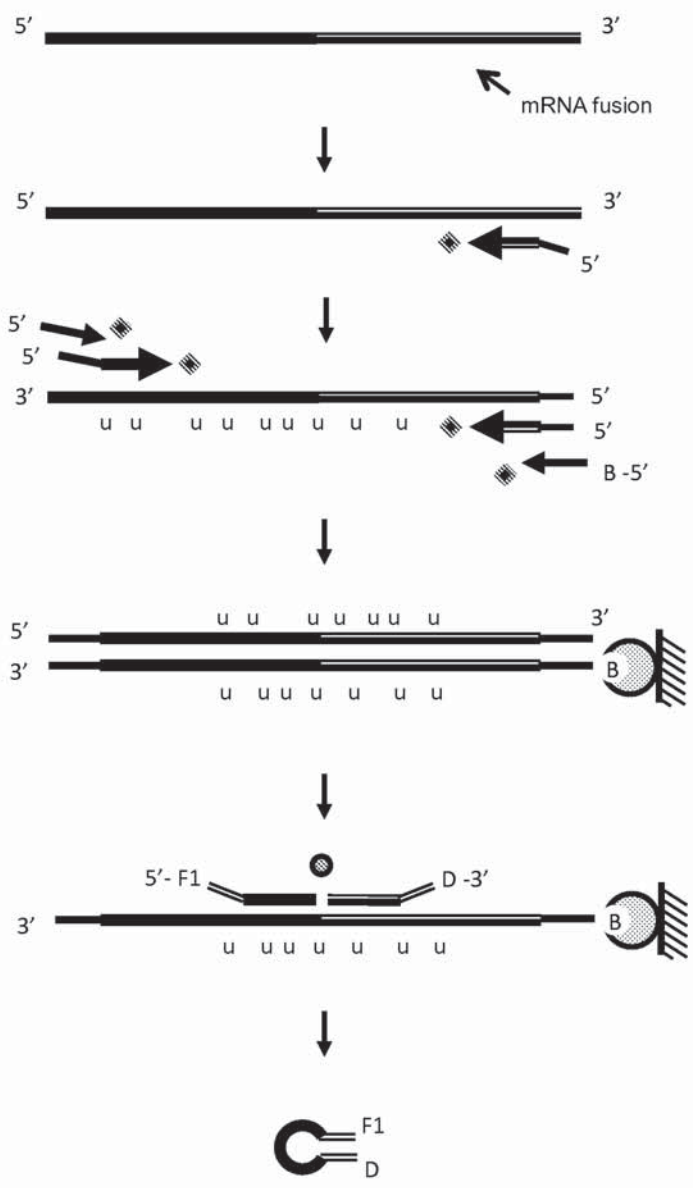


Figure 57

A. Overview: RT-PCR-LDR-qPCR carryover prevention reaction to detect alternative splicing. Illustration of gene with 5 exons shown at the DNA level.

B. Examples of normal (1-2-3a-4), alternative splice variant (1-2-3b-4) mRNAs are illustrated. Isolate mRNA from whole blood cells, exosomes, or CTCs. Generate cDNA using reverse transcriptase and primer complementary to exon 4. PCR amplify using forward primer to exon 2, generating amplicons of both splice variants.

C. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci' for individual Taqman probes; or Ai, Bi-Ci for UniTaq probes) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together, if there is perfect complementarity at the junction. Aliquot into separate wells for detection using real-time PCR.

D. When using tag-specific primers (Ai, Ci) for amplifying LDR products of the upper set, each Taqman probe spans the ligation junction, and can be scored individually. When using UniTaq-specific primers (F1-Bi-Q-Ai, Ci), for amplifying LDR products of the lower set, a given primer set scores for a given ligation junction.

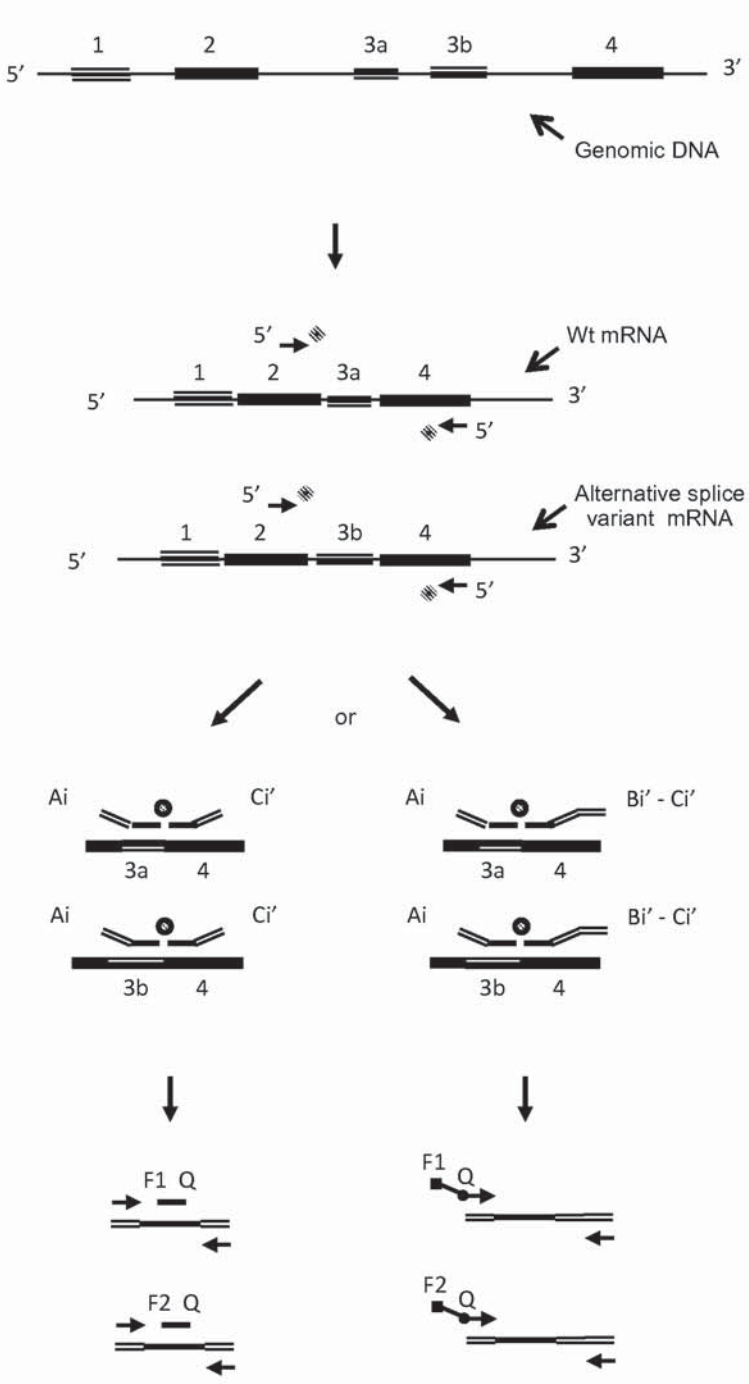


Figure 58

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

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. The relative Ct values reflects the relative amount of each transcript.

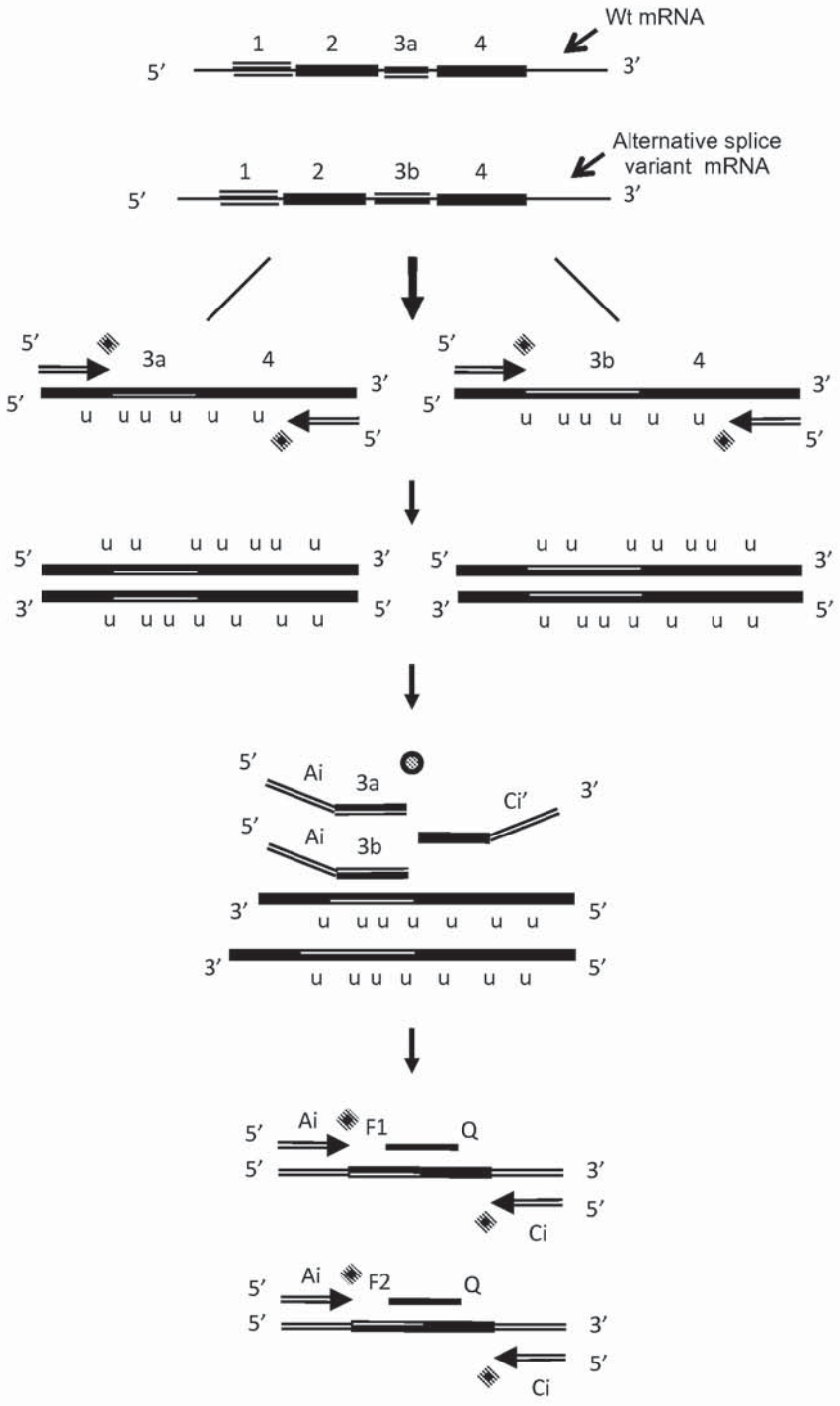


Figure 59

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

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, Ci).

F. Double-stranded DNA products of PCR (not shown); 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. The relative Ct values reflects the relative amount of each transcript.

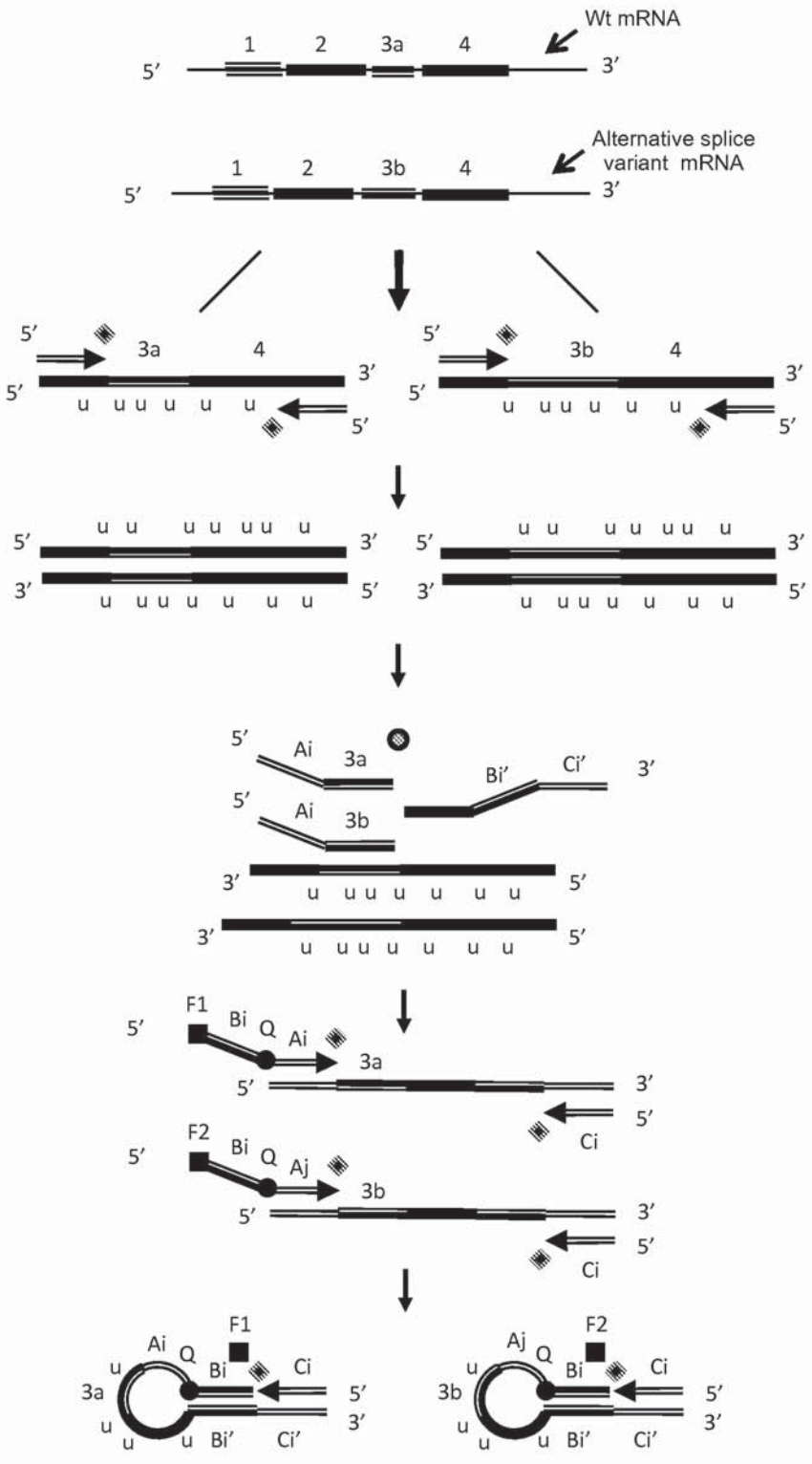


Figure 60

A. RT-PCR-qLDR carryover prevention reaction to quantify wt and alternatively spliced (3a vs. 3b) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

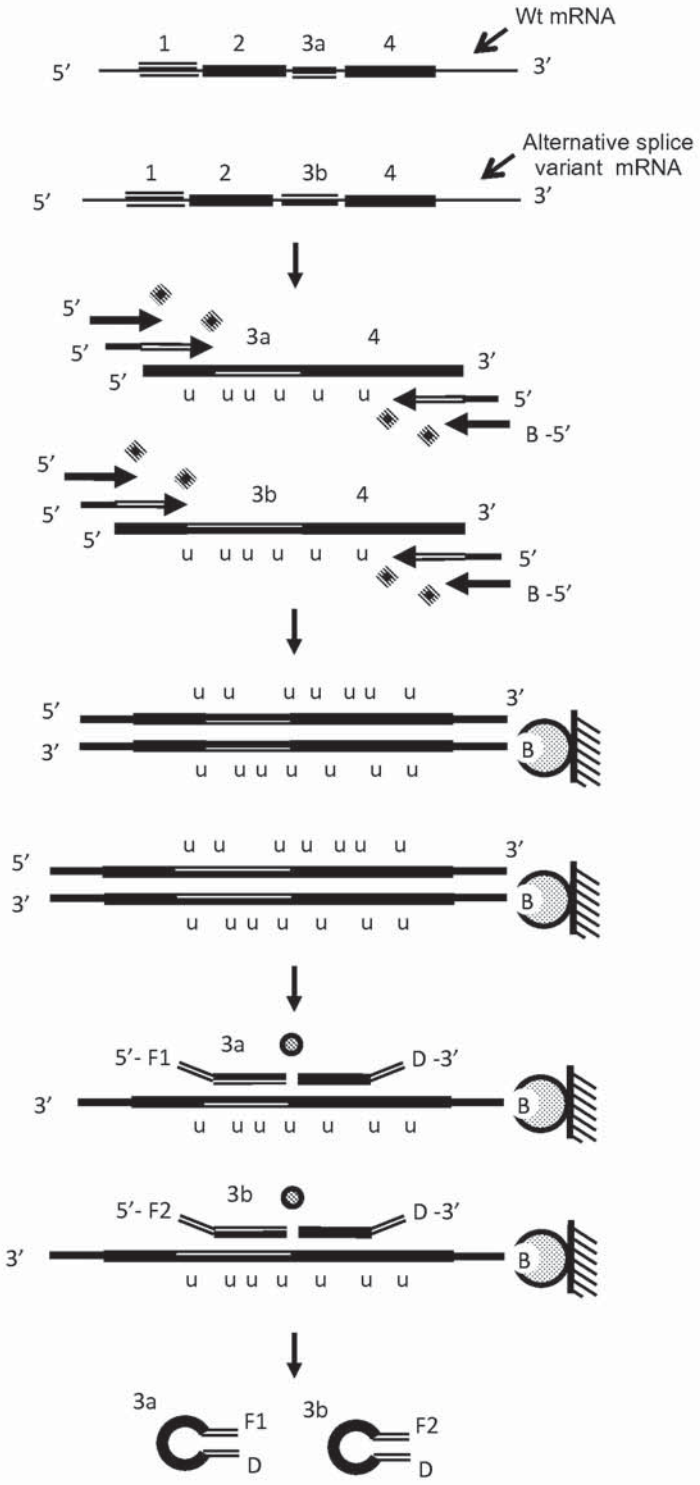


Figure 61

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low-level alternatively spliced (e.g. 3b) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Exon-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

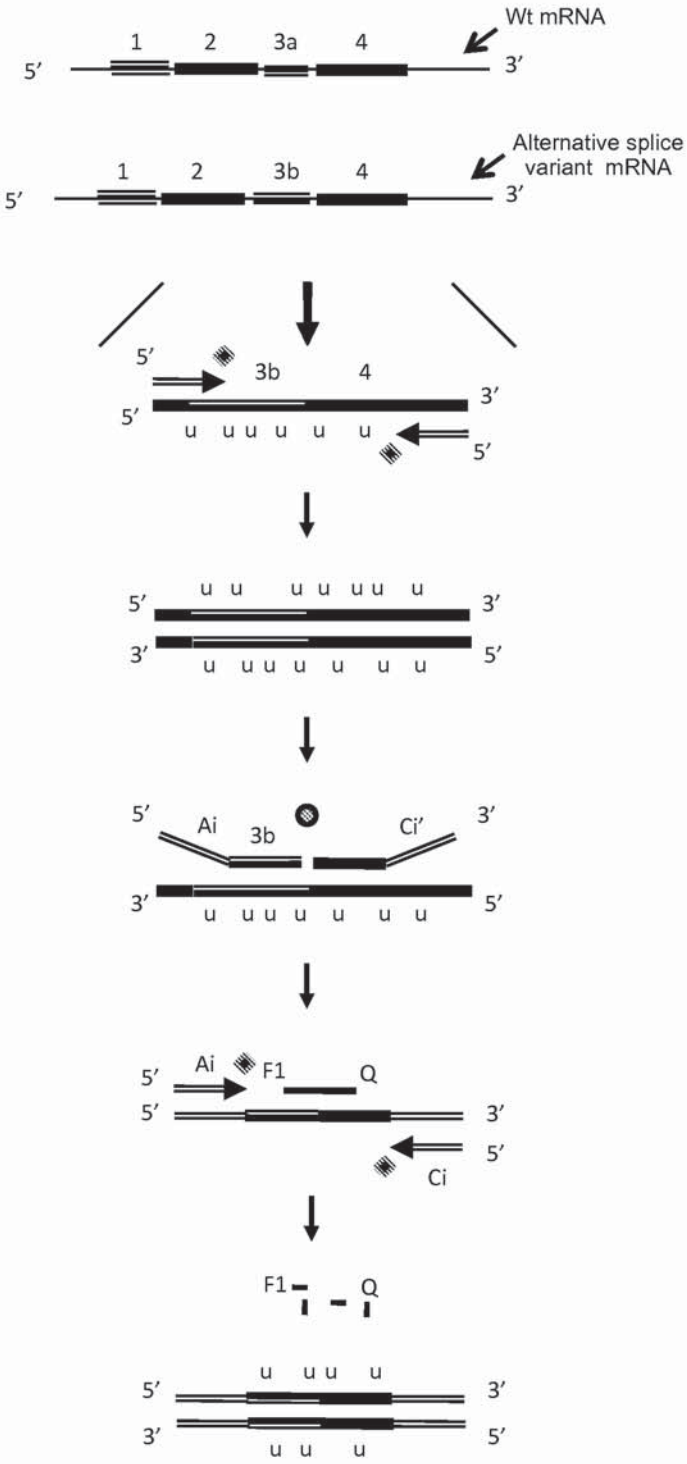


Figure 62

A. PCR-LDR carryover prevention reaction to detect low-level alternatively spliced (e.g. 3b) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Exon-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

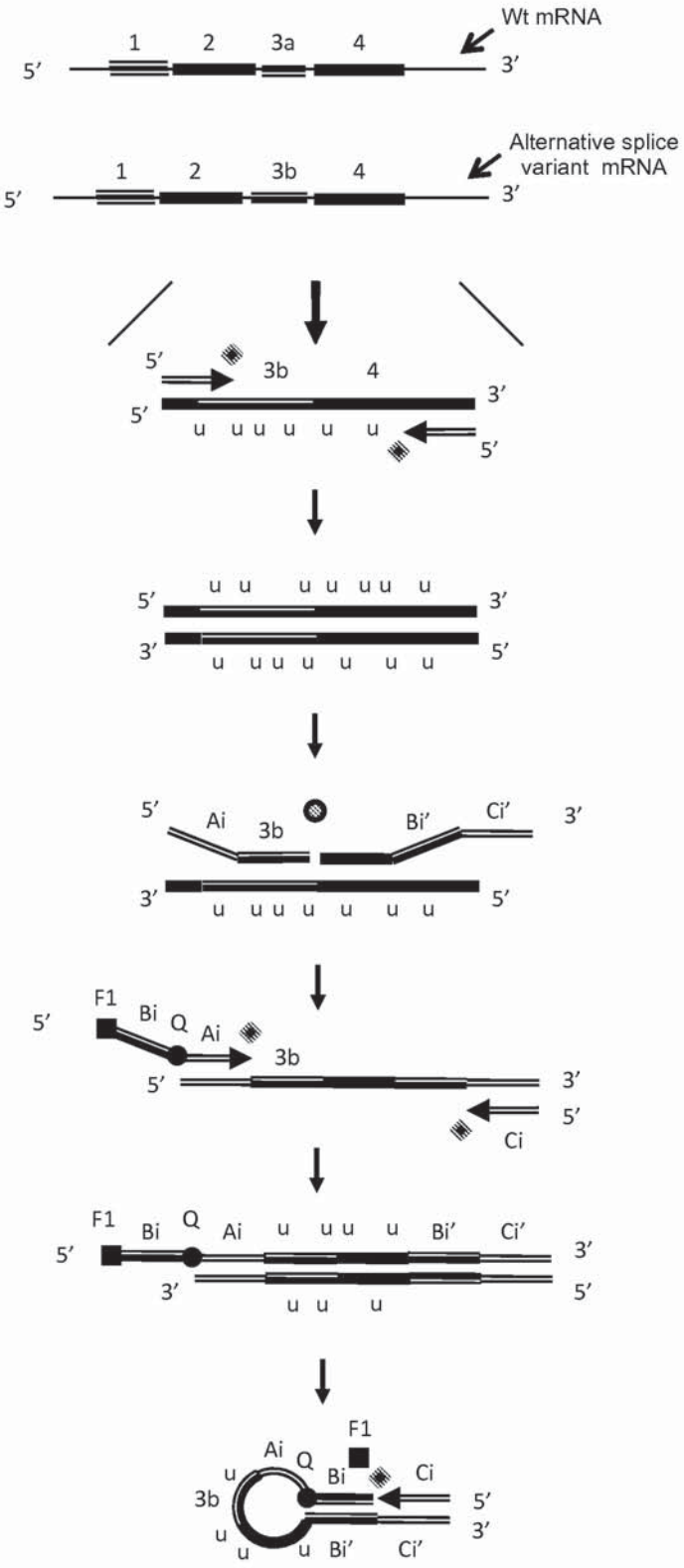


Figure 63

A. PCR-LDR carryover prevention reaction to detect low-level alternatively spliced (e.g. 3b) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

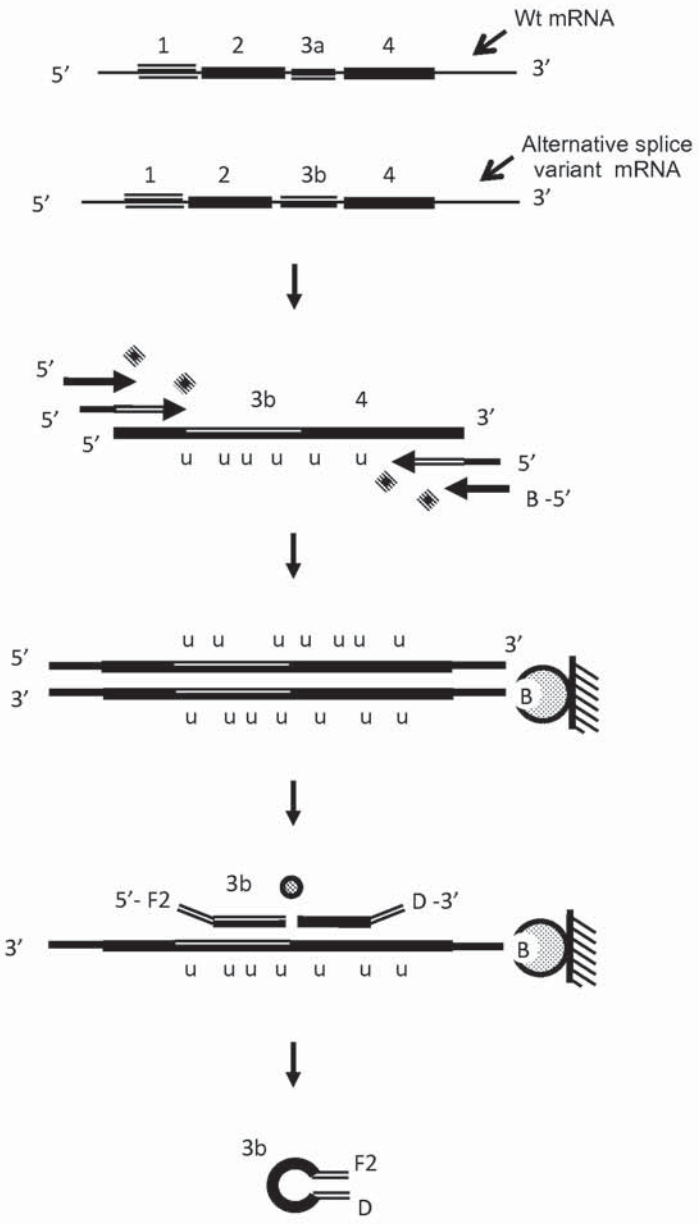


Figure 64

A. Overview: RT-PCR-LDR-qPCR carryover prevention reaction to detect alternative splicing. Illustration of gene with 3 exons, and an alternative start site and first exon, shown at the DNA level.

B. Examples of normal (1-2-3), alternative splice variant (1a-2-3) mRNAs are illustrated. Isolate mRNA from whole blood cells, exosomes, or CTCs. Generate cDNA using reverse transcriptase and primer complementary to exon 2. PCR amplify using forward primers to exons 1 and 1a, generating amplicons of both splice variants.

C. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci' for individual Taqman probes; or Ai, Bi-Ci for UniTaq probes) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together, if there is perfect complementarity at the junction. Aliquot into separate wells for detection using real-time PCR.

D. When using tag-specific primers (Ai, Ci) for amplifying LDR products of the upper set, each Taqman probe spans the ligation junction, and can be scored individually. When using UniTaq-specific primers (F1-Bi-Q-Ai, Ci), for amplifying LDR products of the lower set, a given primer set scores for a given ligation junction.

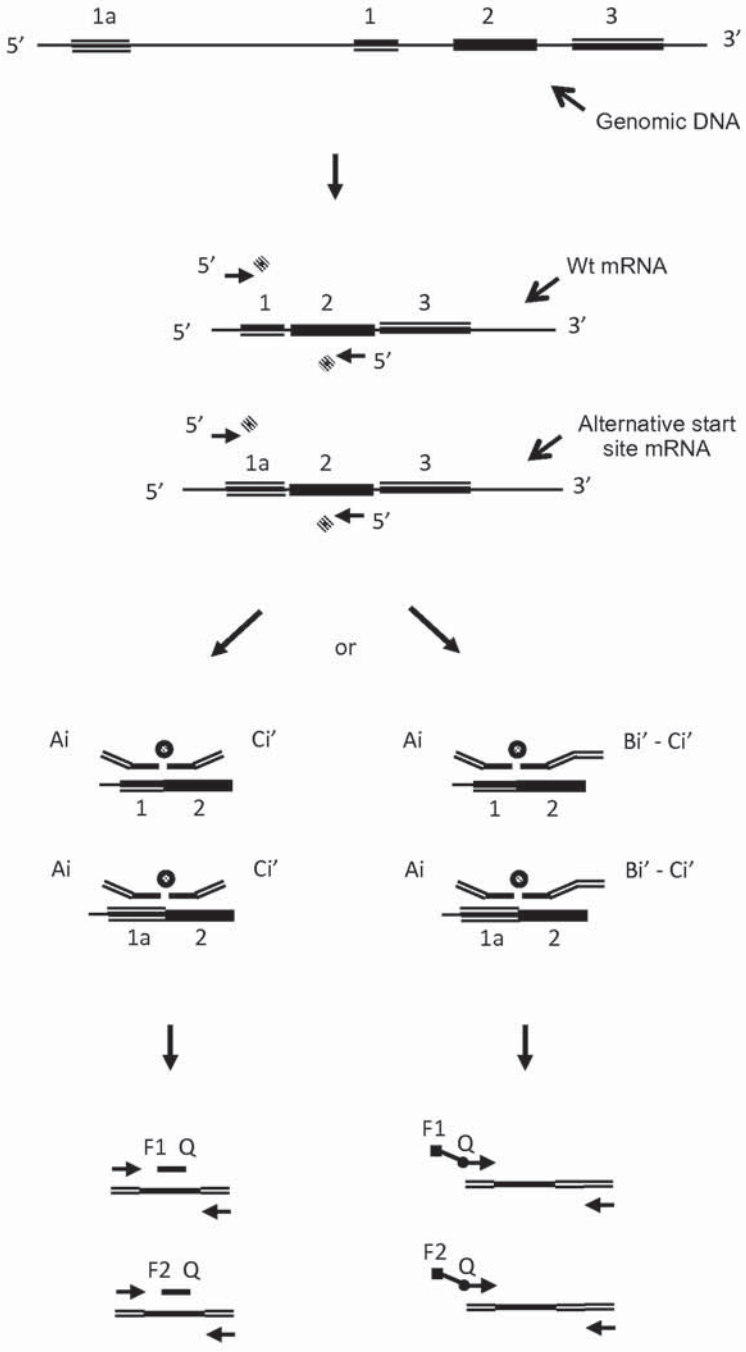


Figure 65

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. The relative Ct values reflects the relative amount of each transcript.

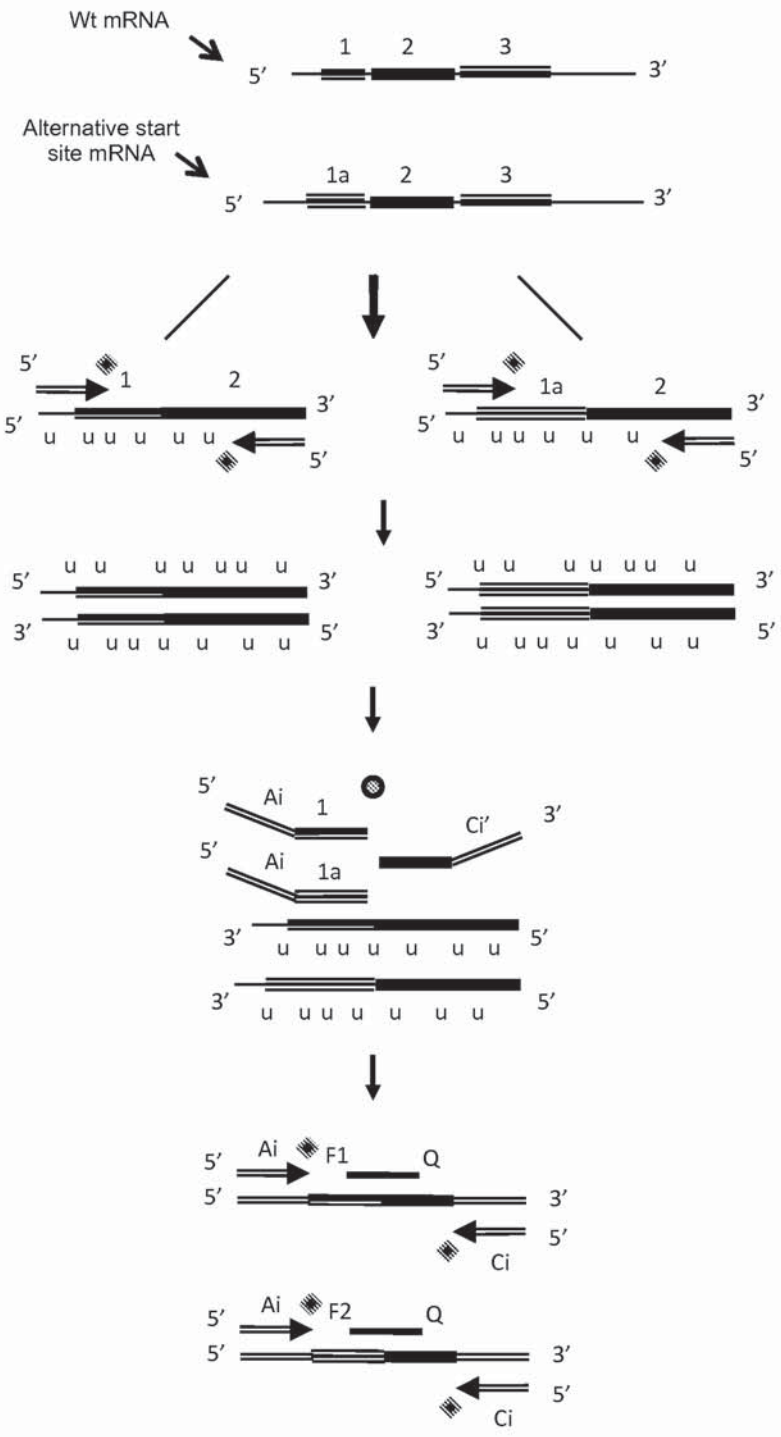


Figure 66

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. The relative Ct values reflects the relative amount of each transcript.

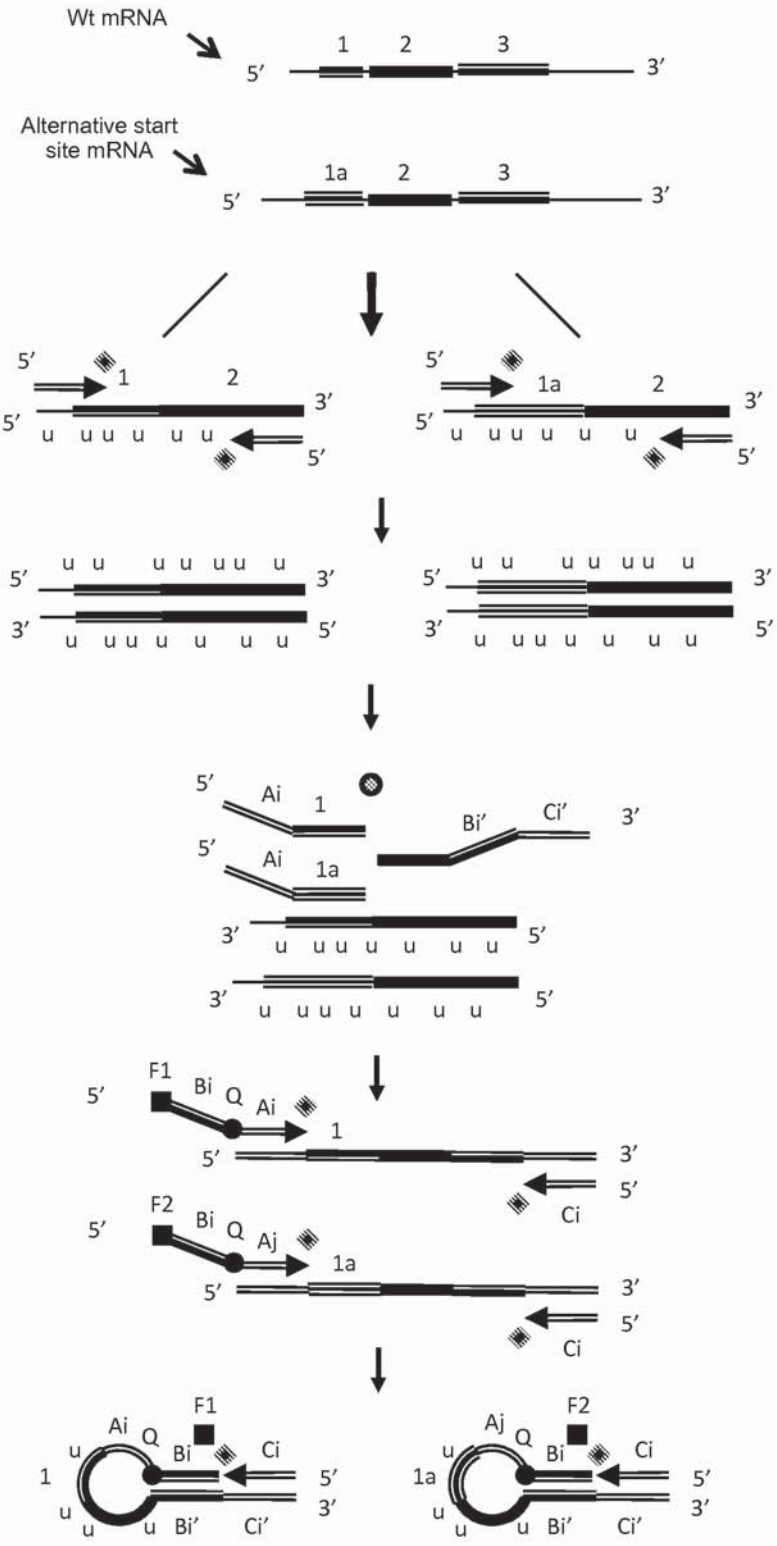


Figure 67

A. RT-PCR-qLDR carryover prevention reaction to quantify wt and alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

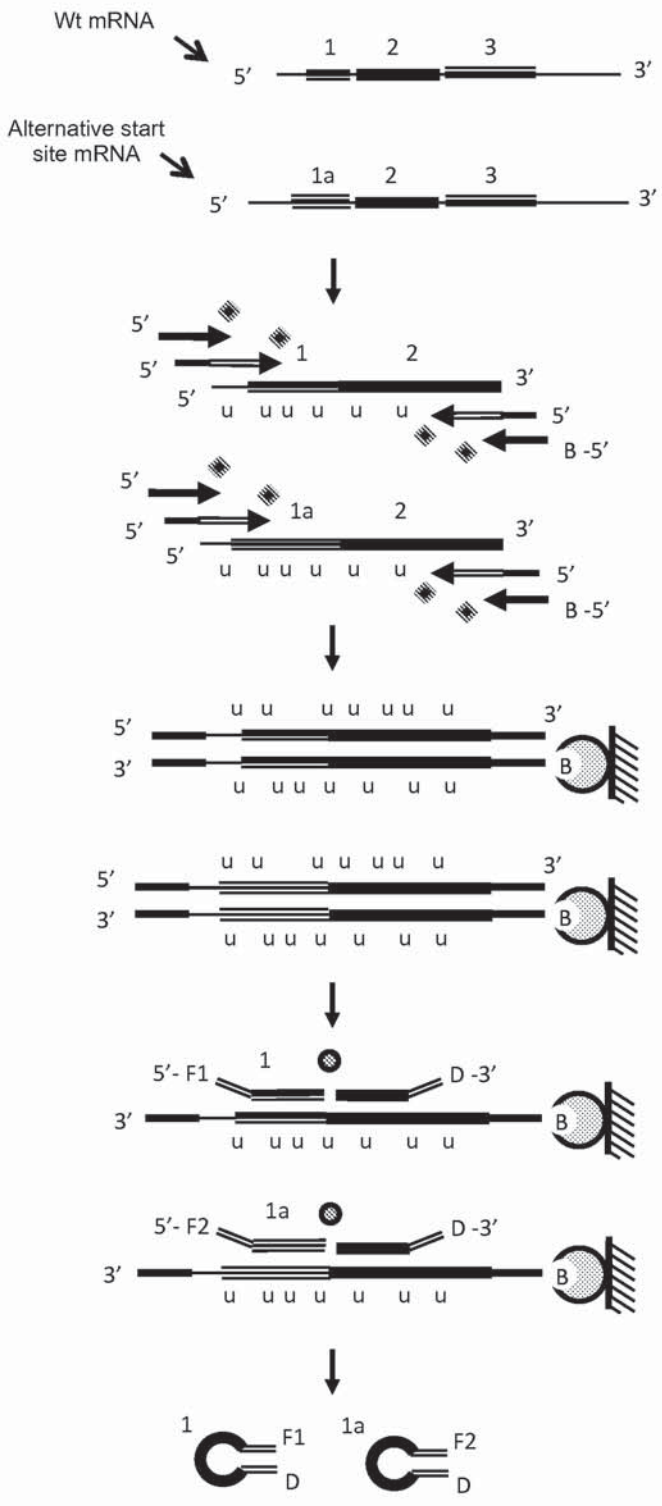


Figure 68

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low level of alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Exon-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

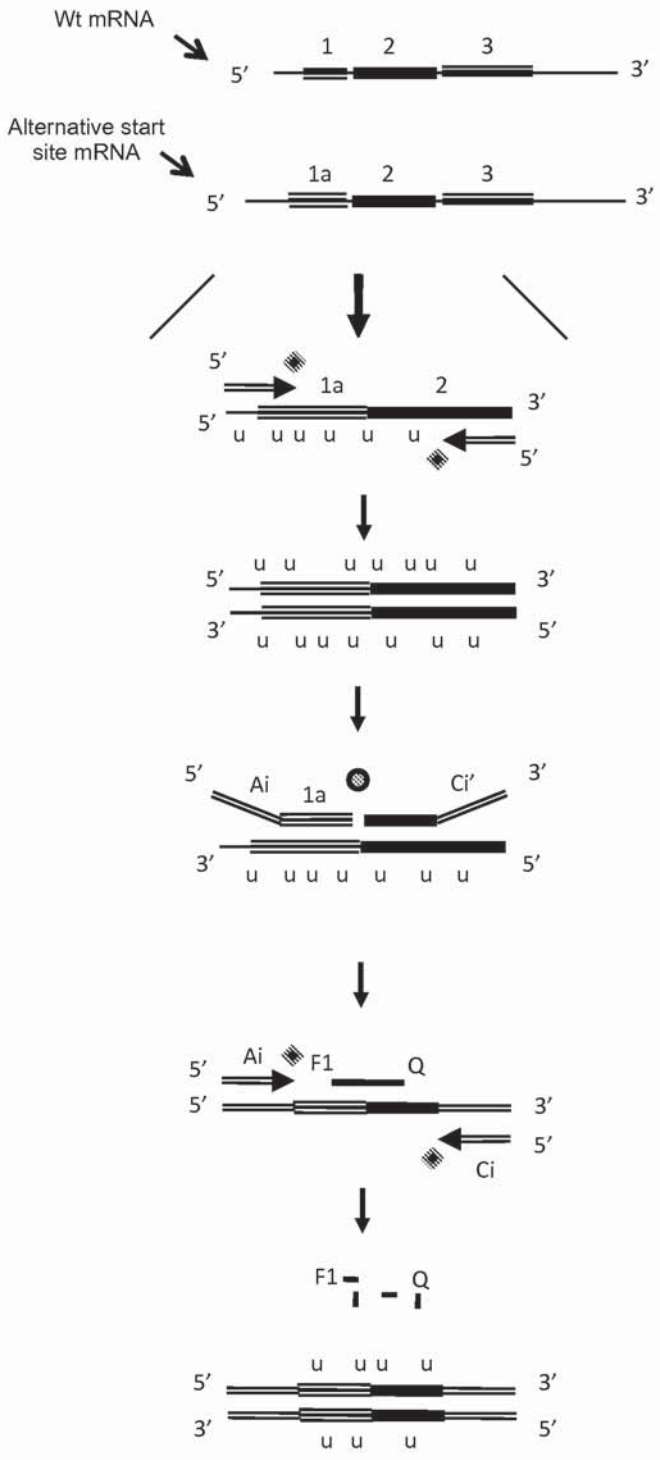


Figure 69

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low level of alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Exon-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

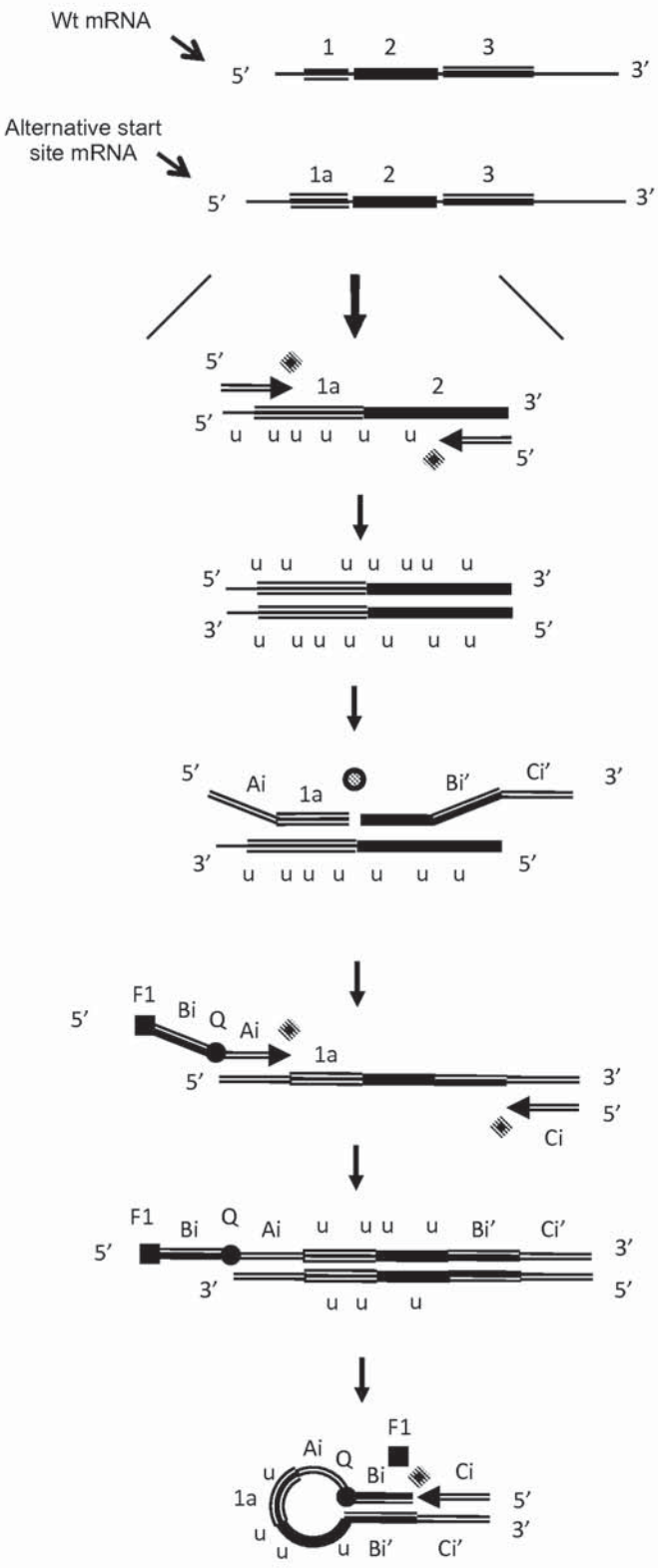


Figure 70

A. RT-PCR-qLDR carryover prevention reaction to detect low level of alternative transcript start site (1 vs. 1a). Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

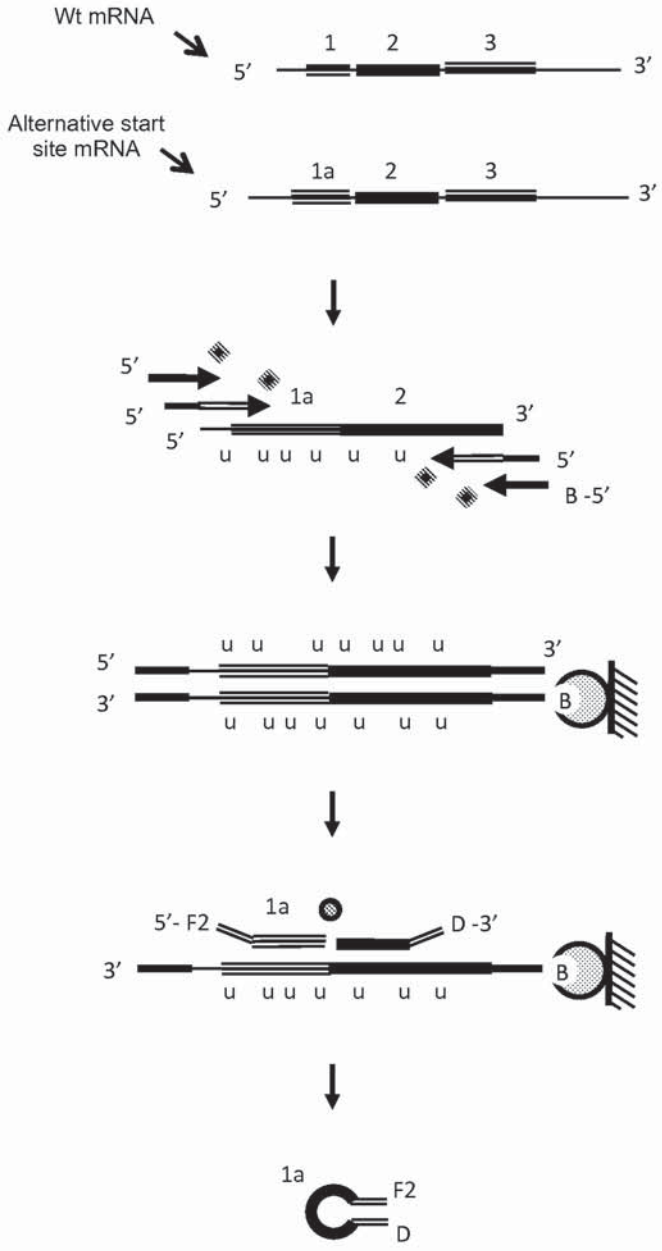


Figure 71

A. Overview: RT-PCR-LDR-qPCR carryover prevention reaction to detect exon deletion. Illustration of gene with 5 exons and 4 introns shown at the DNA level.

B. Example of normal (e1-e2-e3-e4-e5) and exon deletion (e1-e2-e3-e5) mRNAs are illustrated. Isolate mRNA from whole blood cells, exosomes, or CTCs. Generate cDNA using reverse transcriptase and primer complementary to exon e5. PCR amplify using forward primers to exons e3, and e4, generating amplicons of both splice variants.

C. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci' for individual Taqman probes; or Ai, Bi-Ci for UniTaq probes) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together, if there is perfect complementarity at the junction. Aliquot into separate wells for detection using real-time PCR.

D. When using tag-specific primers (Ai, Ci) for amplifying LDR products of the upper set, each Taqman probe spans the ligation junction, and can be scored individually. When using UniTaq-specific primers (F1-Bi-Q-Ai, Ci), for amplifying LDR products of the lower set, a given primer set scores for a given ligation junction.

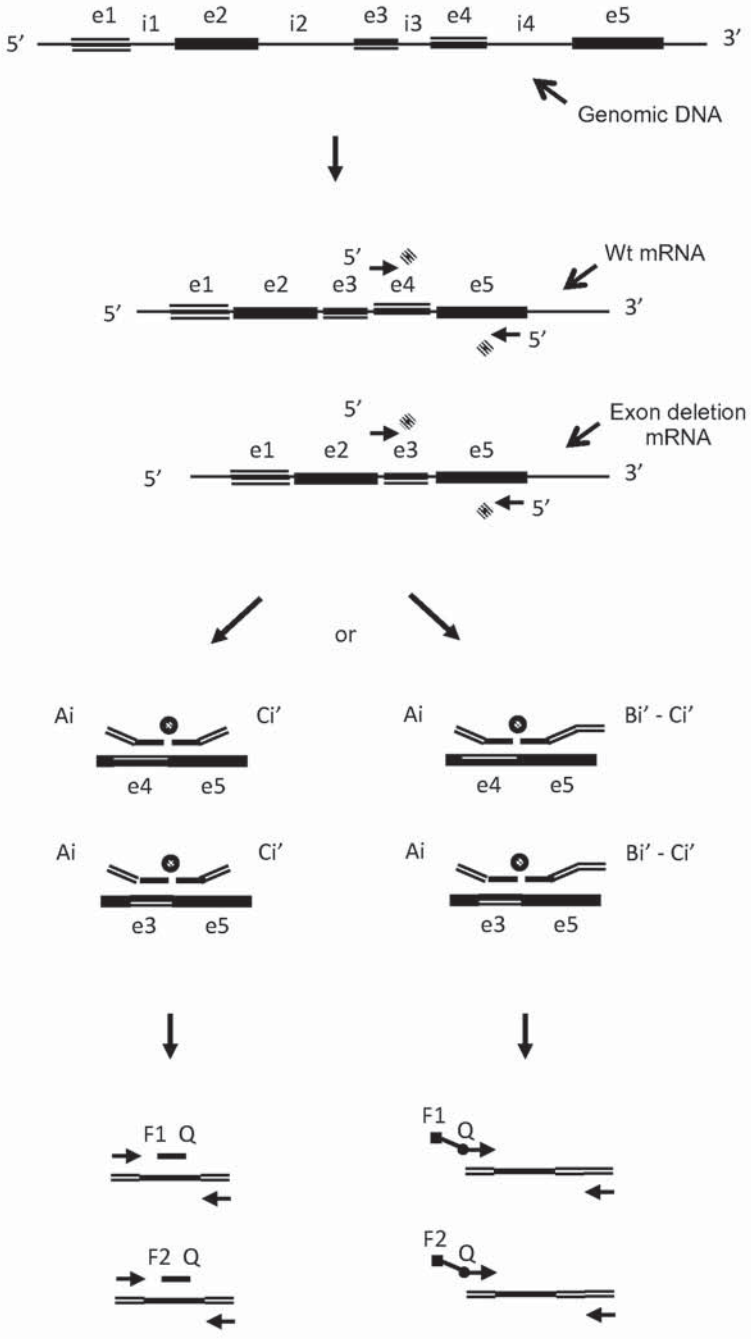


Figure 72

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. The relative Ct values reflects the relative amount of each transcript.

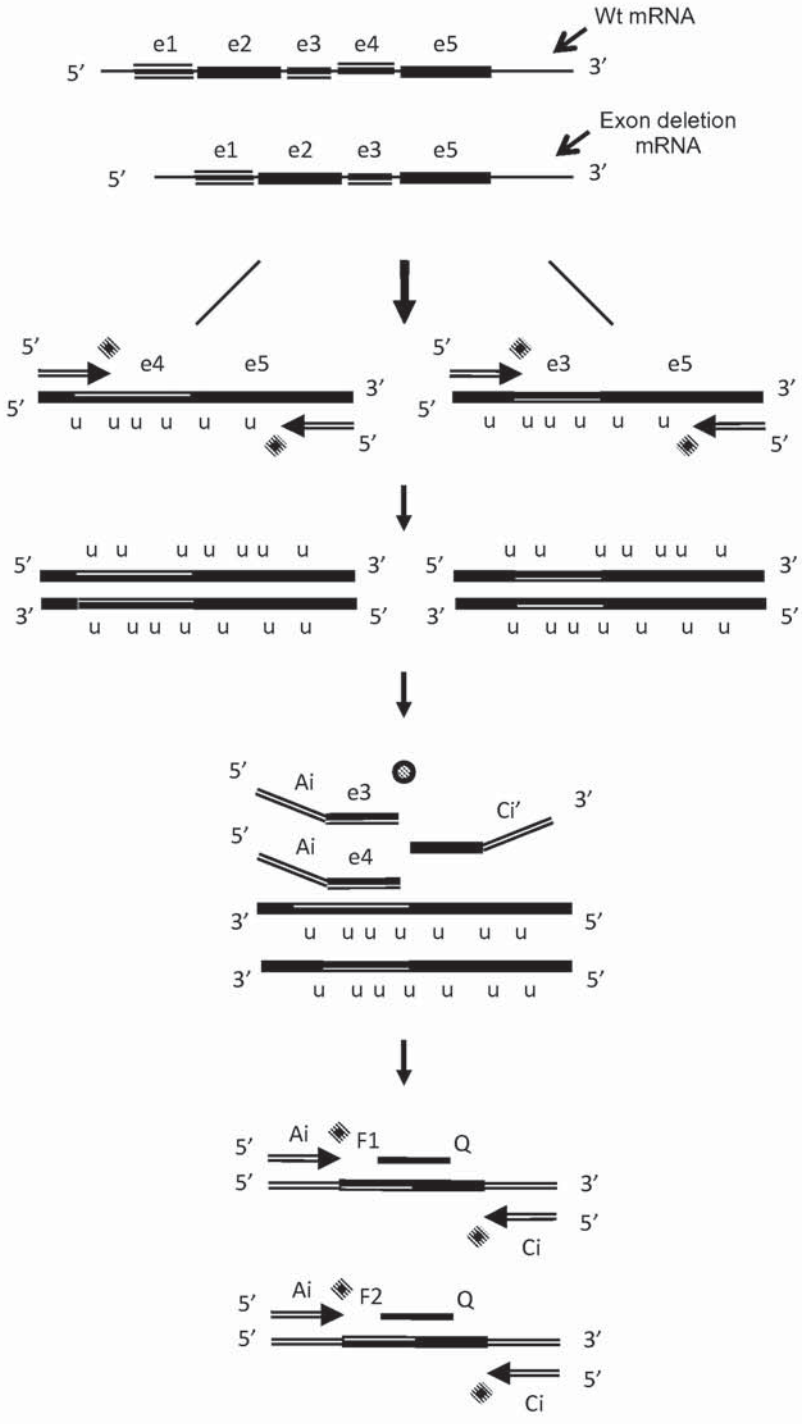


Figure 73

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, Ci).

F. Double-stranded DNA products of PCR (not shown); 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. The relative Ct values reflects the relative amount of each transcript.

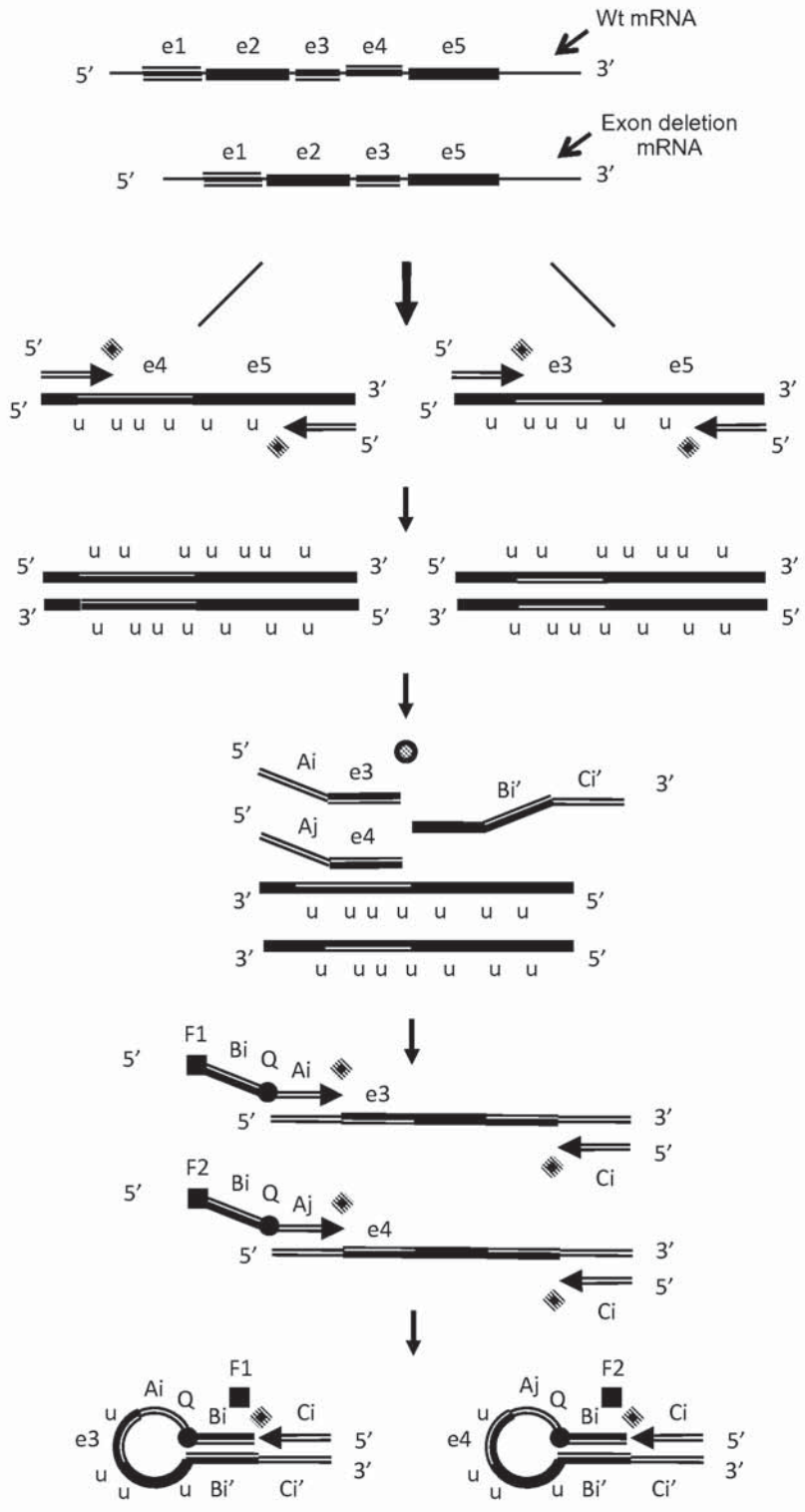


Figure 74

A. RT-PCR-qLDR carryover prevention reaction to quantify wt and alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

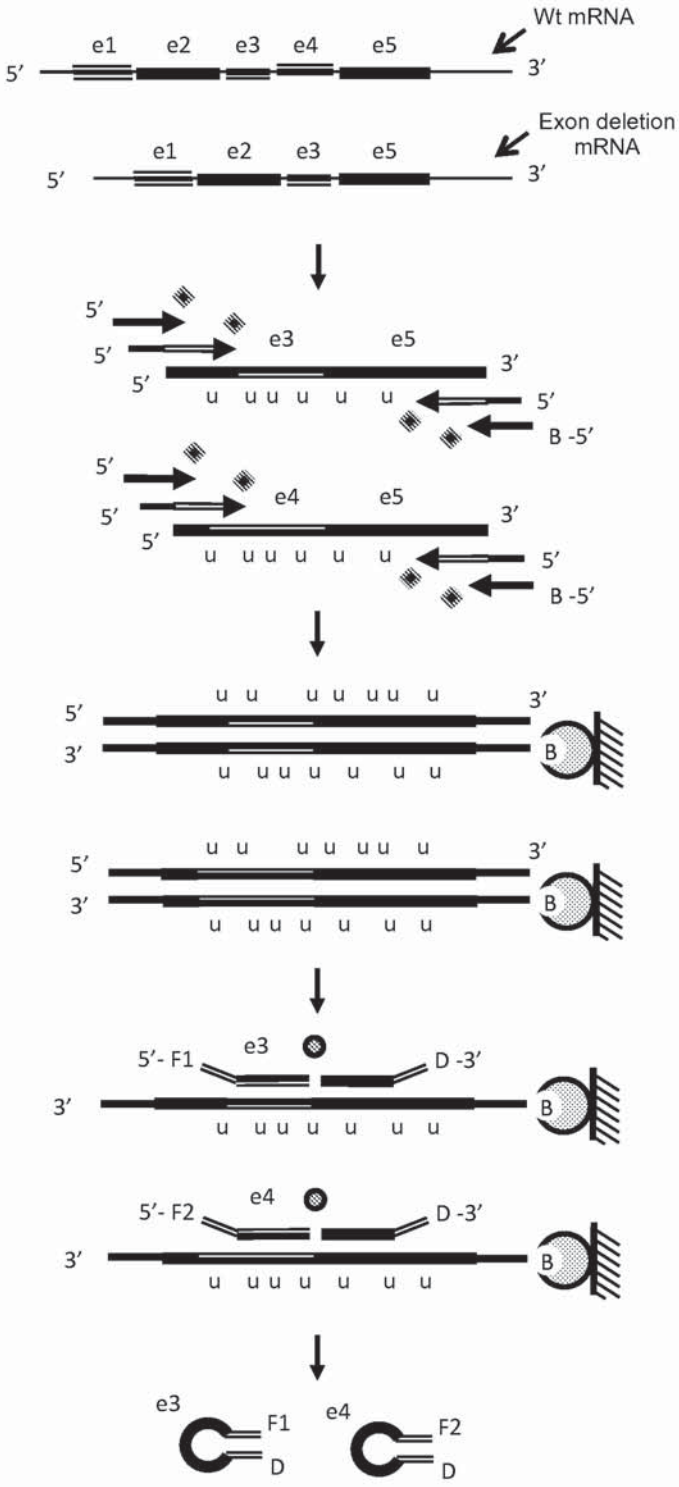


Figure 75

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low-level alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligo suppresses amplification of wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

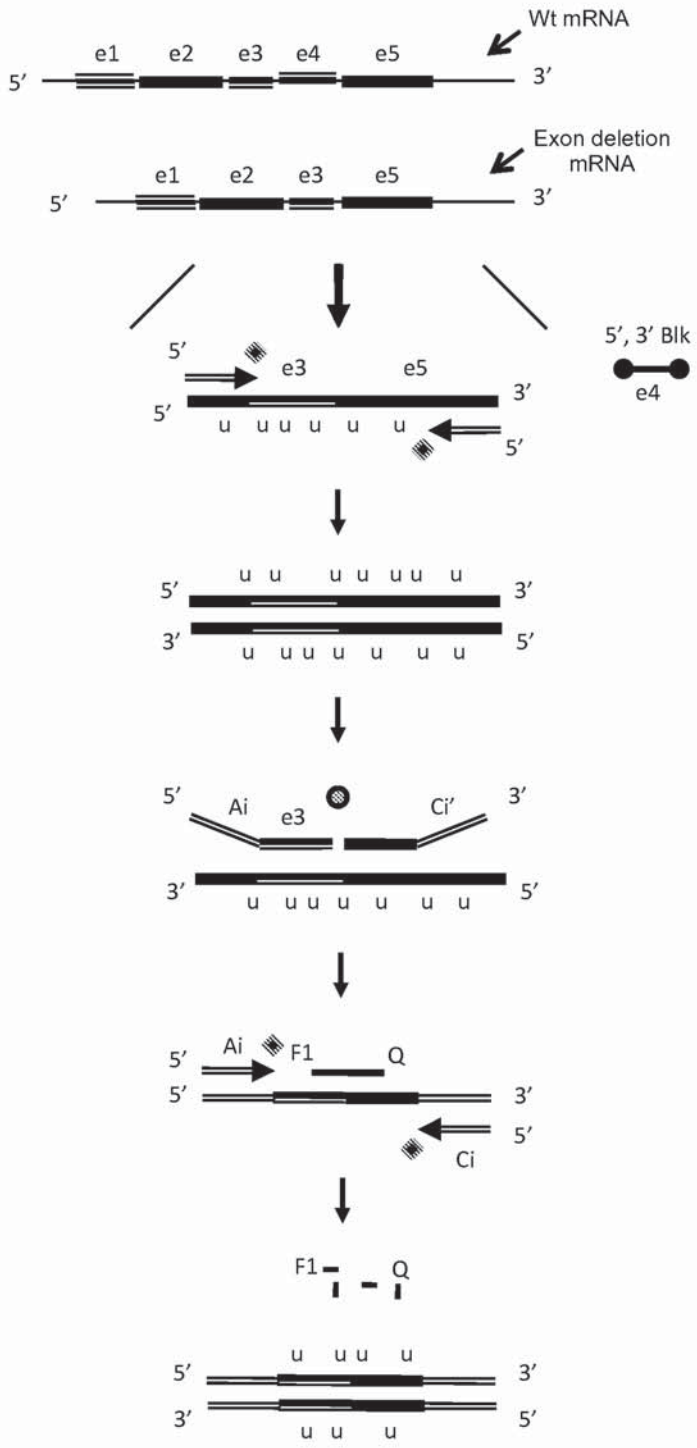


Figure 76

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low-level alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligo suppresses amplification of wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

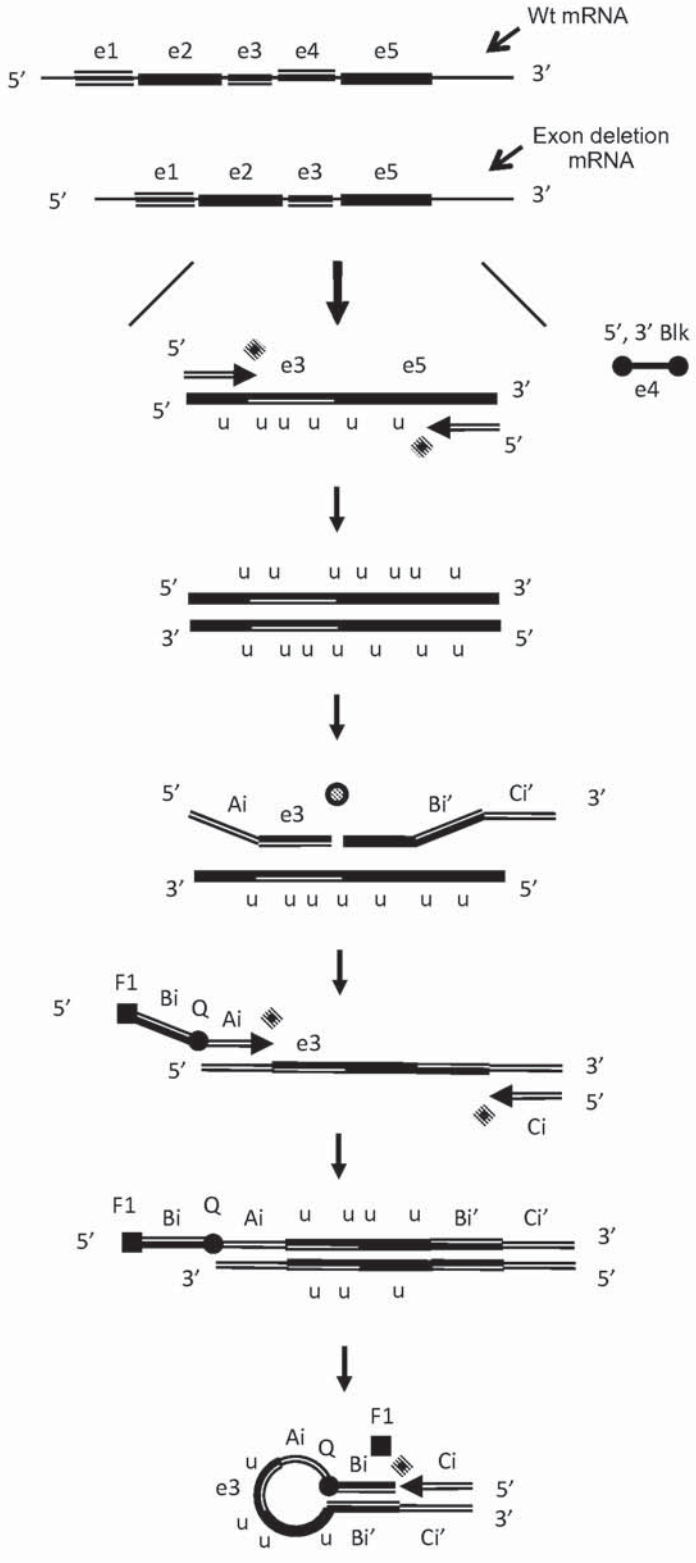


Figure 77

A. RT-PCR-qLDR carryover prevention reaction to detect low-level alternatively spliced (exon deletion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Blocking oligo suppresses amplification of wt transcript. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

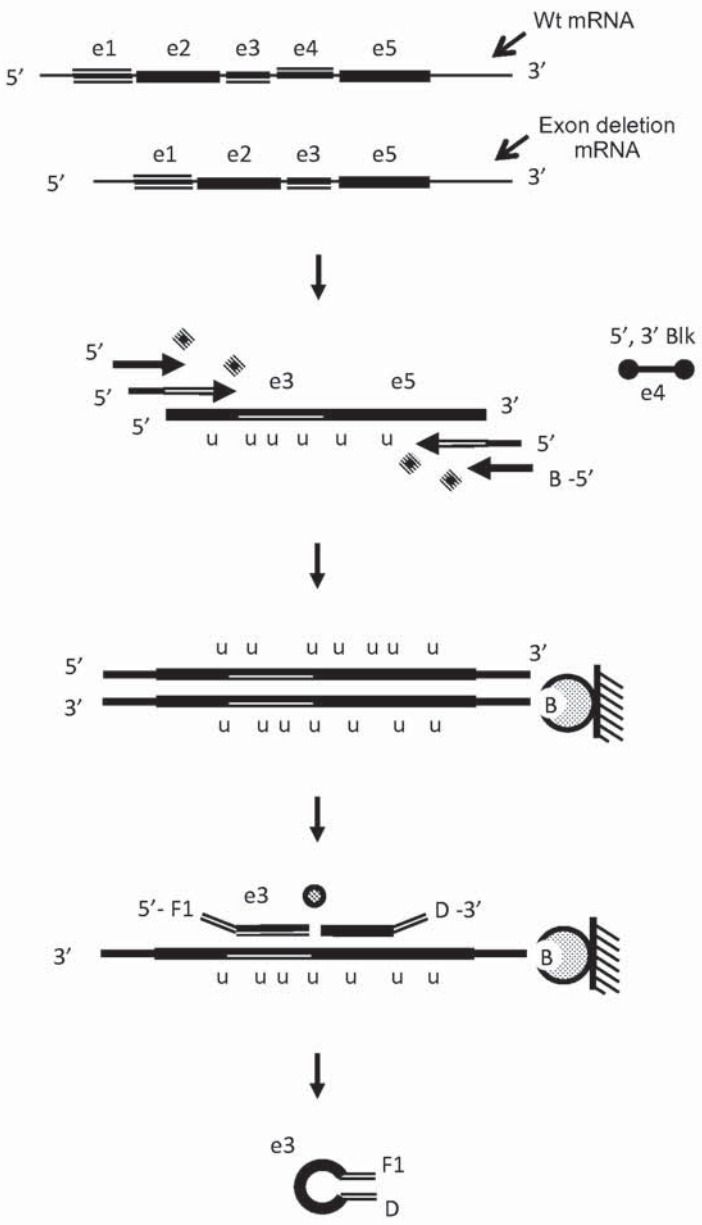


Figure 78

A. Overview: RT-PCR-LDR-qPCR carryover prevention reaction to detect alternative splicing with intron insertion. Illustration of gene with 5 exons and 4 introns shown at the DNA level.

B. Examples of normal (e1-e2-e3-e4-e5) and intron insertion (e1-i1-e2-e3-e4-e5) mRNAs are illustrated. Isolate mRNA from whole blood cells, exosomes, or CTCs. Generate cDNA using reverse transcriptase and primer complementary to exon e2. PCR amplify using forward primers to exon e1, generating amplicons of both splice variants.

C. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci' for individual Taqman probes; or Ai, Bi-Ci for UniTaq probes) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together, if there is perfect complementarity at the junction. Aliquot into separate wells for detection using real-time PCR.

D. When using tag-specific primers (Ai, Ci) for amplifying LDR products of the upper set, each Taqman probe spans the ligation junction, and can be scored individually. When using UnTtaq-specific primers (F1-Bi-Q-Ai, Ci), for amplifying LDR products of the lower set, a given primer set scores for a given ligation junction.

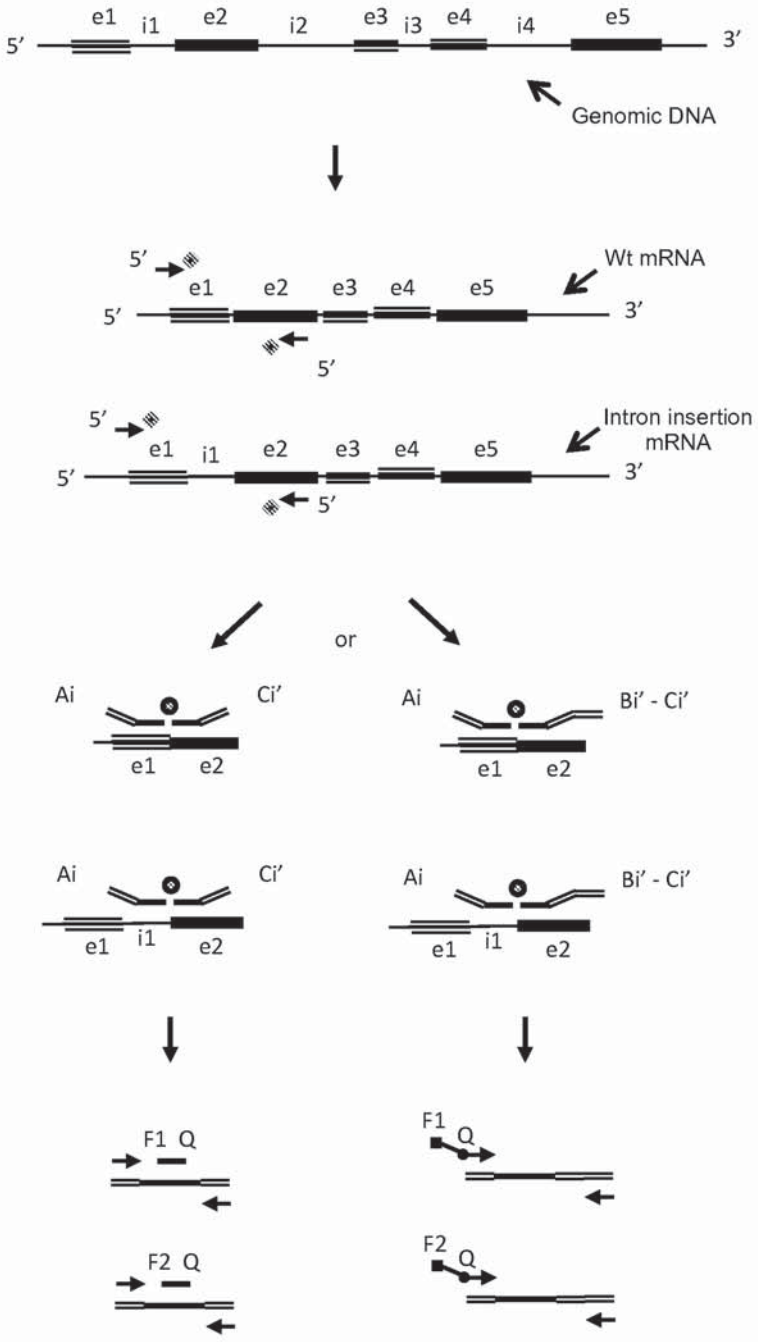


Figure 79

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. The relative Ct values reflects the relative amount of each transcript.

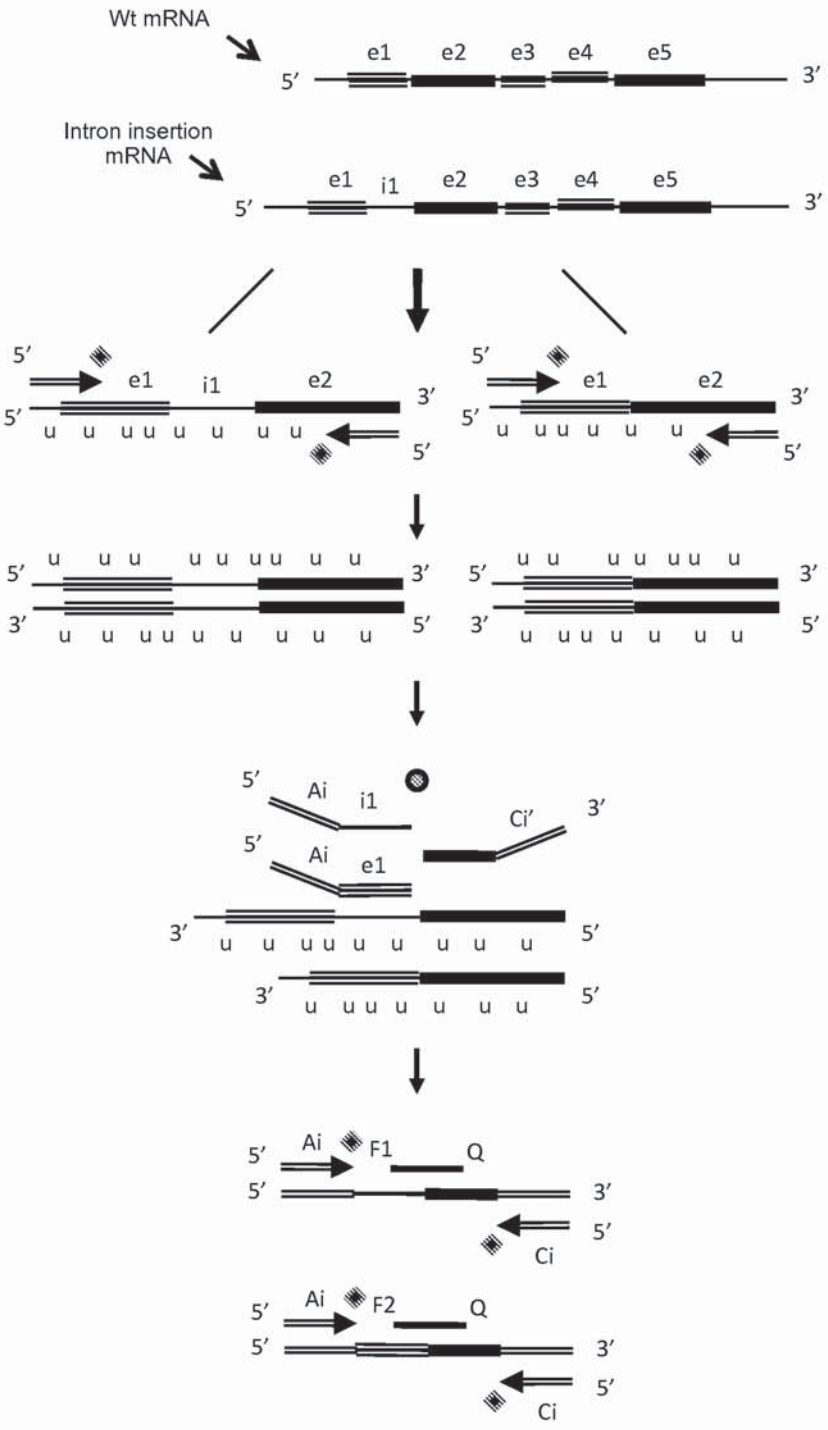


Figure 80

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify wt and alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, Ci).

F. Double-stranded DNA products of PCR (not shown); 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. The relative Ct values reflects the relative amount of each transcript.

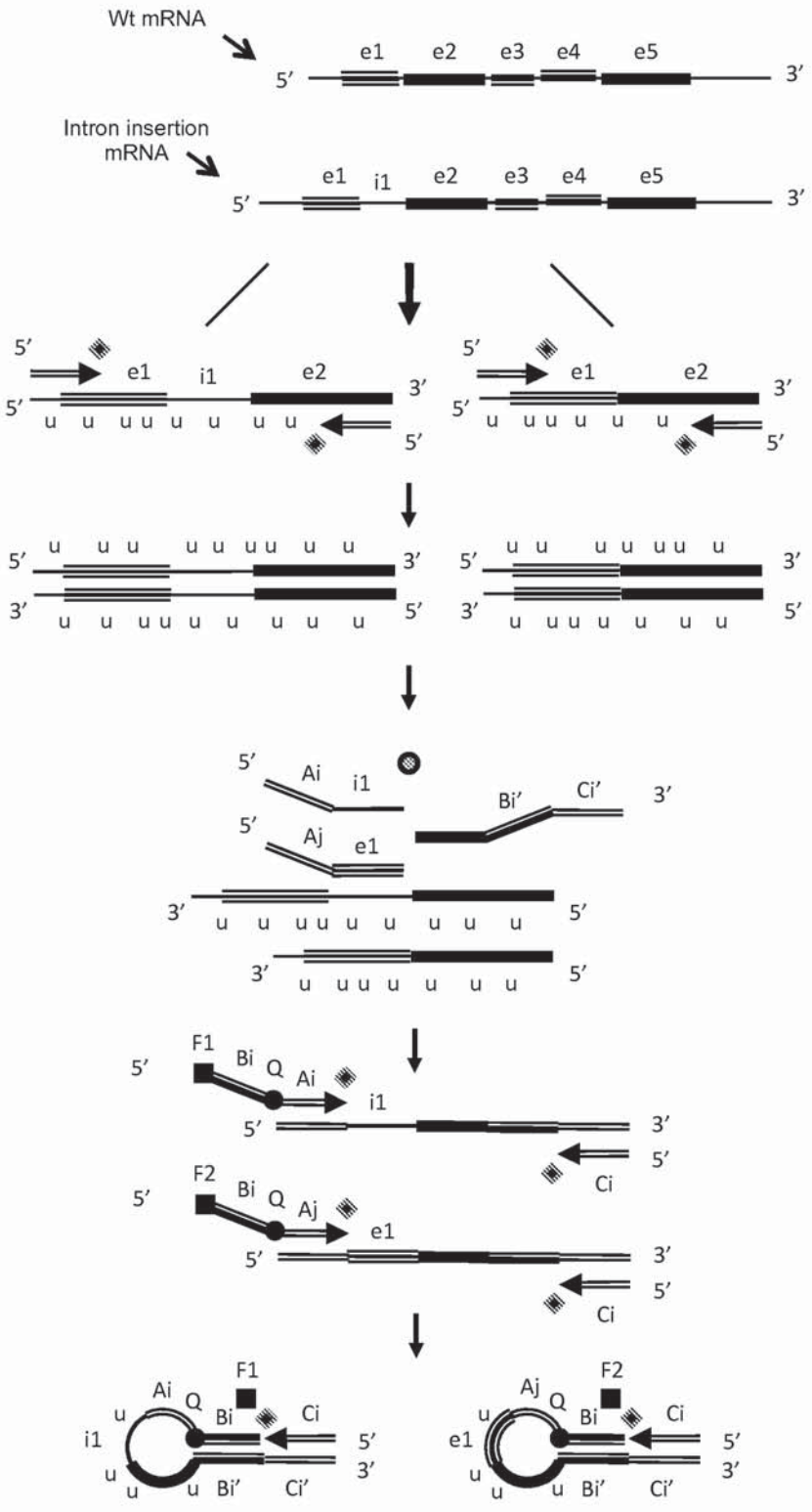


Figure 81

A. RT-PCR-qLDR carryover prevention reaction to quantify wt and alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

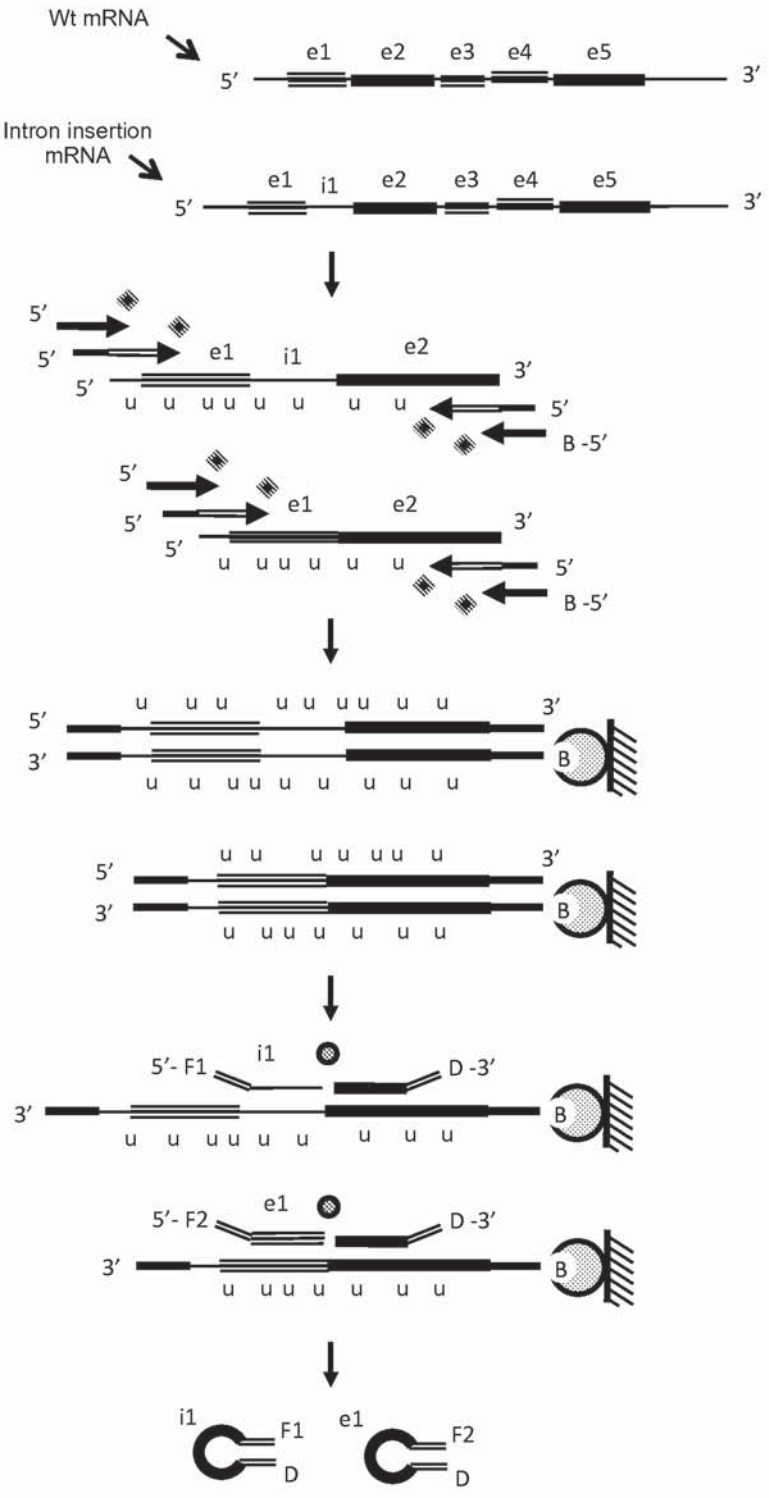


Figure 82

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low-level alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Intron-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

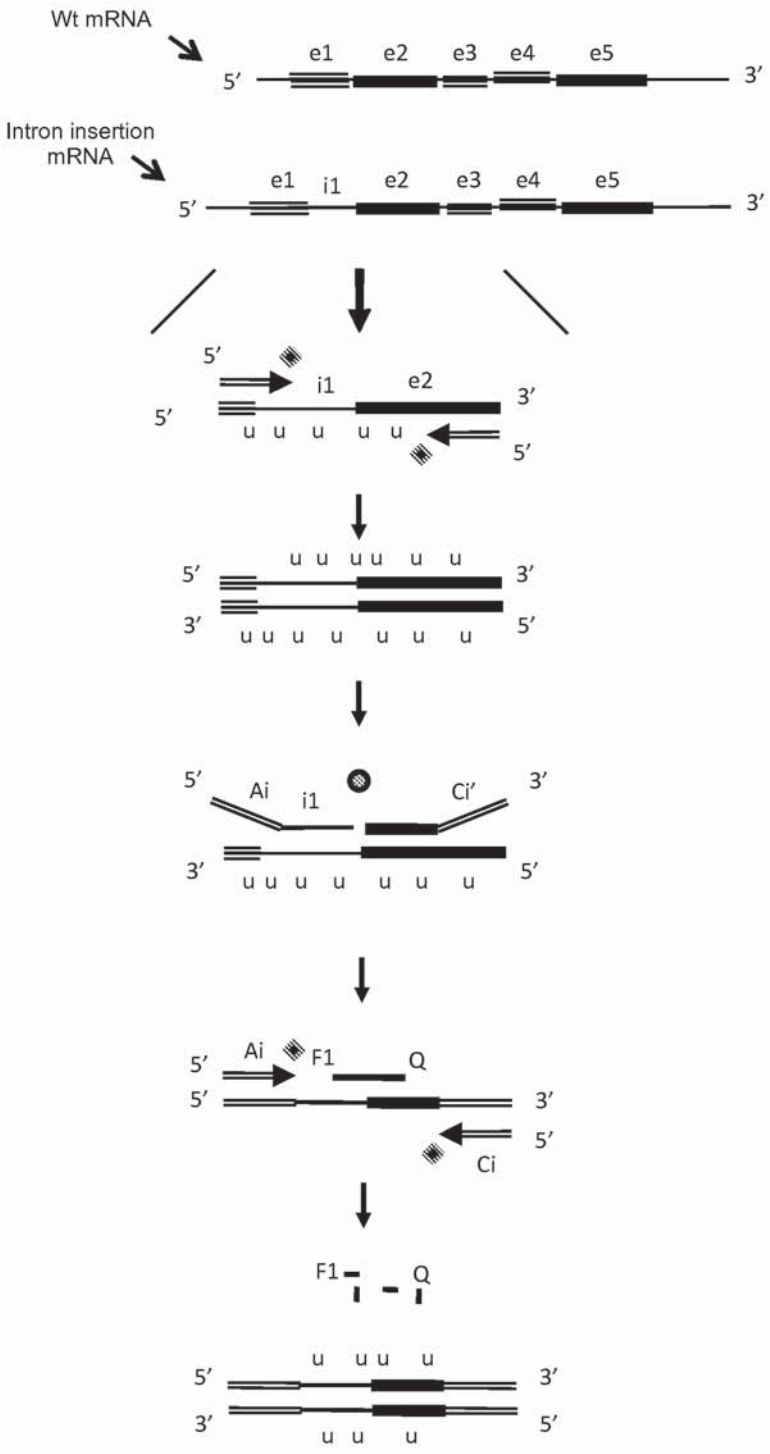


Figure 83

A. RT-PCR-LDR-qPCR carryover prevention reaction to detect low-level alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Intron-specific primer does not amplify wt transcript. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Exon junction-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

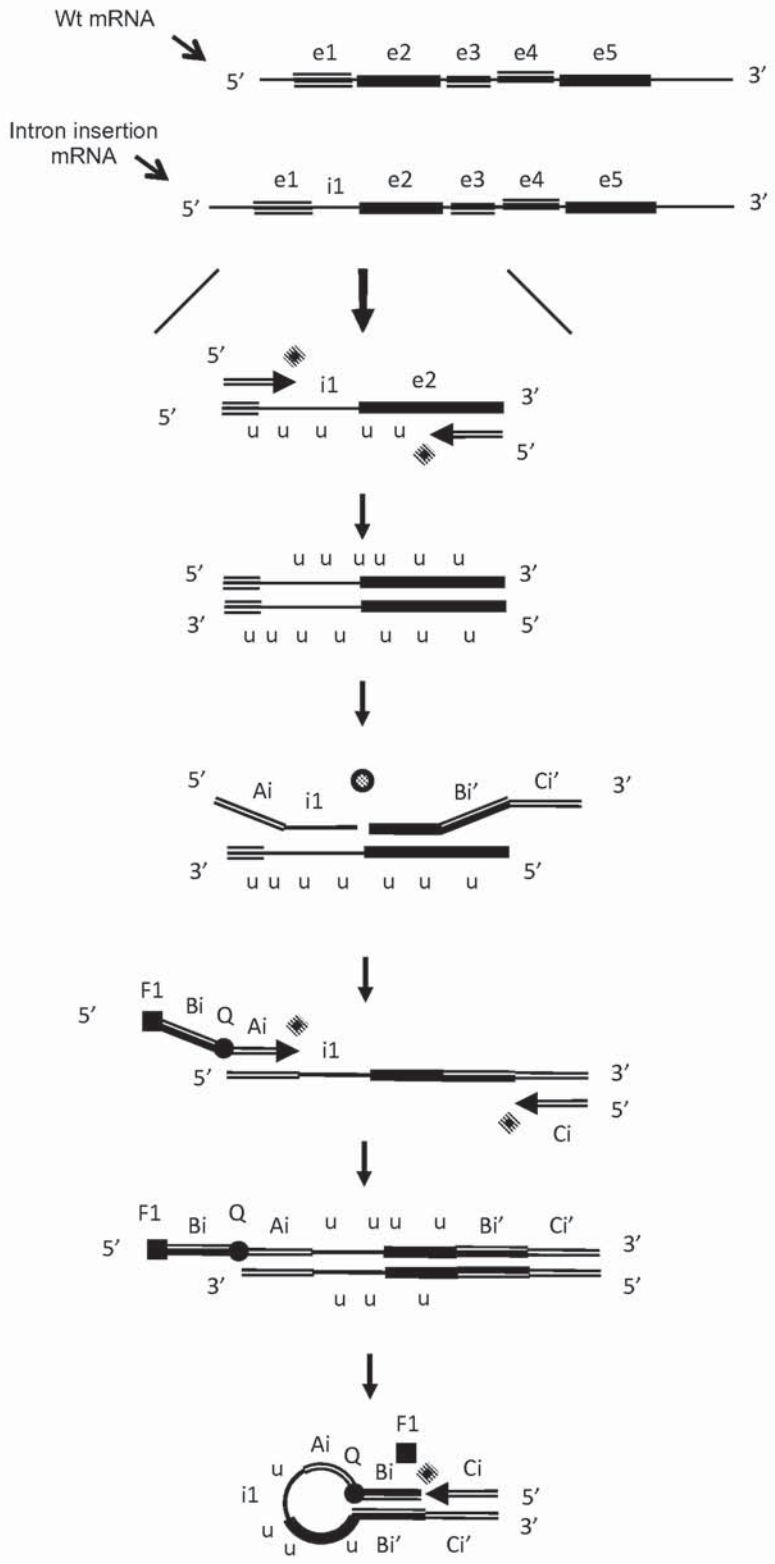


Figure 84

A. PCR-LDR carryover prevention reaction to detect low-level alternatively spliced (intron insertion) transcript. Isolate mRNA.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Intron-specific primer does not amplify wt transcript. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Exon junction-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

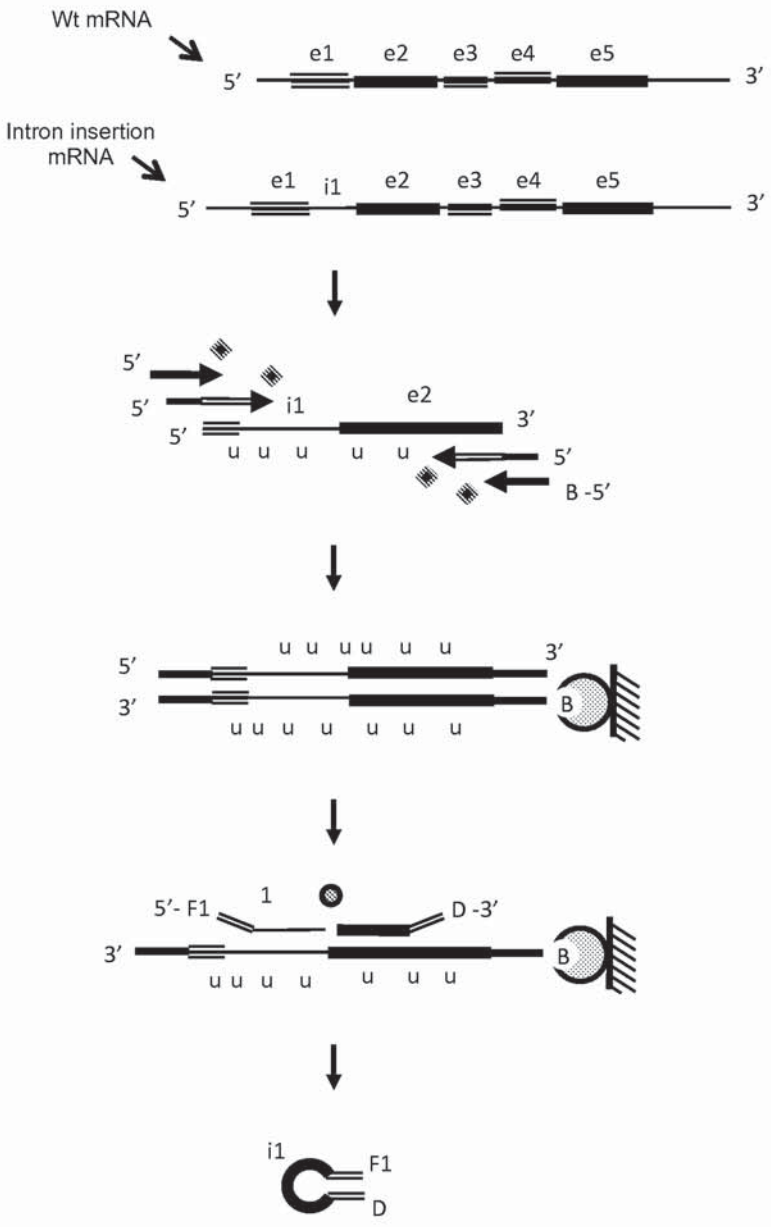


Figure 85

A. PCR-LDR-qPCR carryover prevention reaction to enumerate DNA copy number. Isolate tumor DNA from CTCs or tumor-specific exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and amplify chromosomal regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. For accurate enumeration, aliquot into 12, 24, 48, or 96 wells prior to PCR.

C. PCR products incorporate dU, allowing for carryover prevention.

D. Locus-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. Determine copy number based on signal from wells where original distribution was one copy/ well.

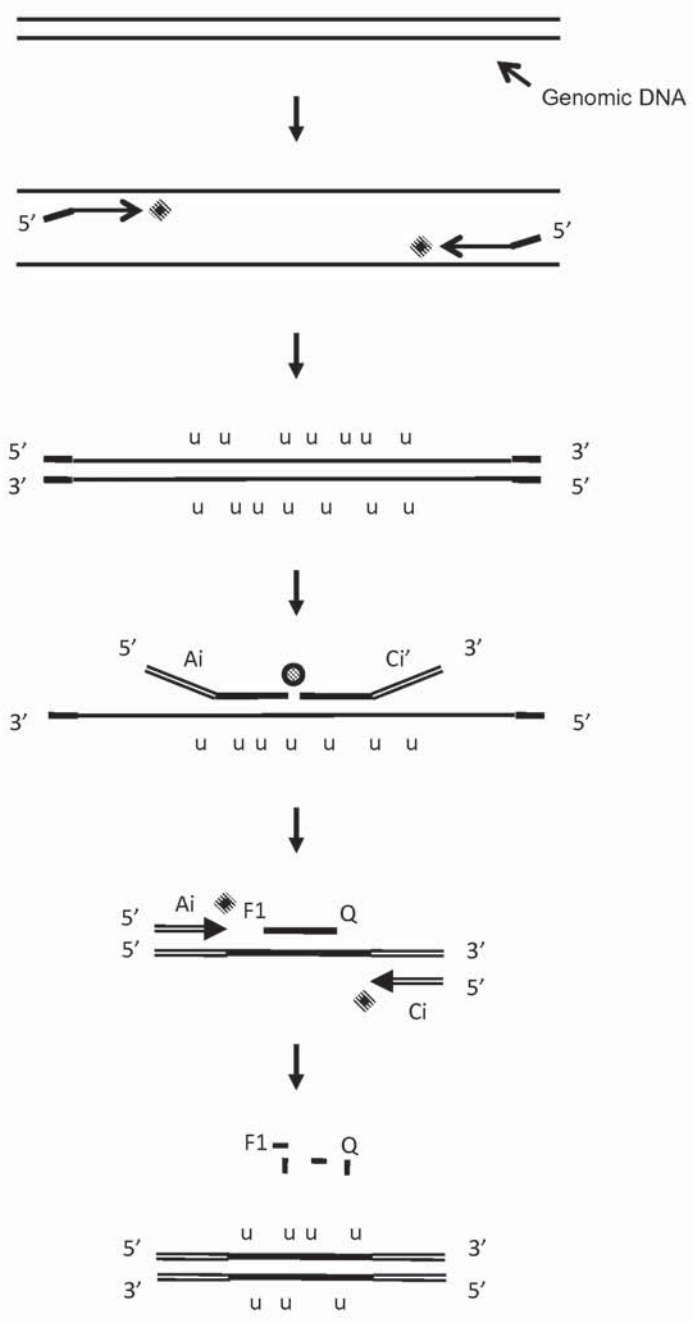


Figure 86

A. PCR-LDR-qPCR carryover prevention reaction to enumerate DNA copy number. Isolate tumor DNA from CTCs or tumor-specific exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and amplify chromosomal regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. For accurate enumeration, aliquot into 12, 24, 48, or 96 wells prior to PCR.

C. PCR products incorporate dU, allowing for carryover prevention.

D. Locus-specific ligation oligonucleotides contain tags (Ai, Bi'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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. Determine copy number based on signal from wells where original distribution was one copy/well.

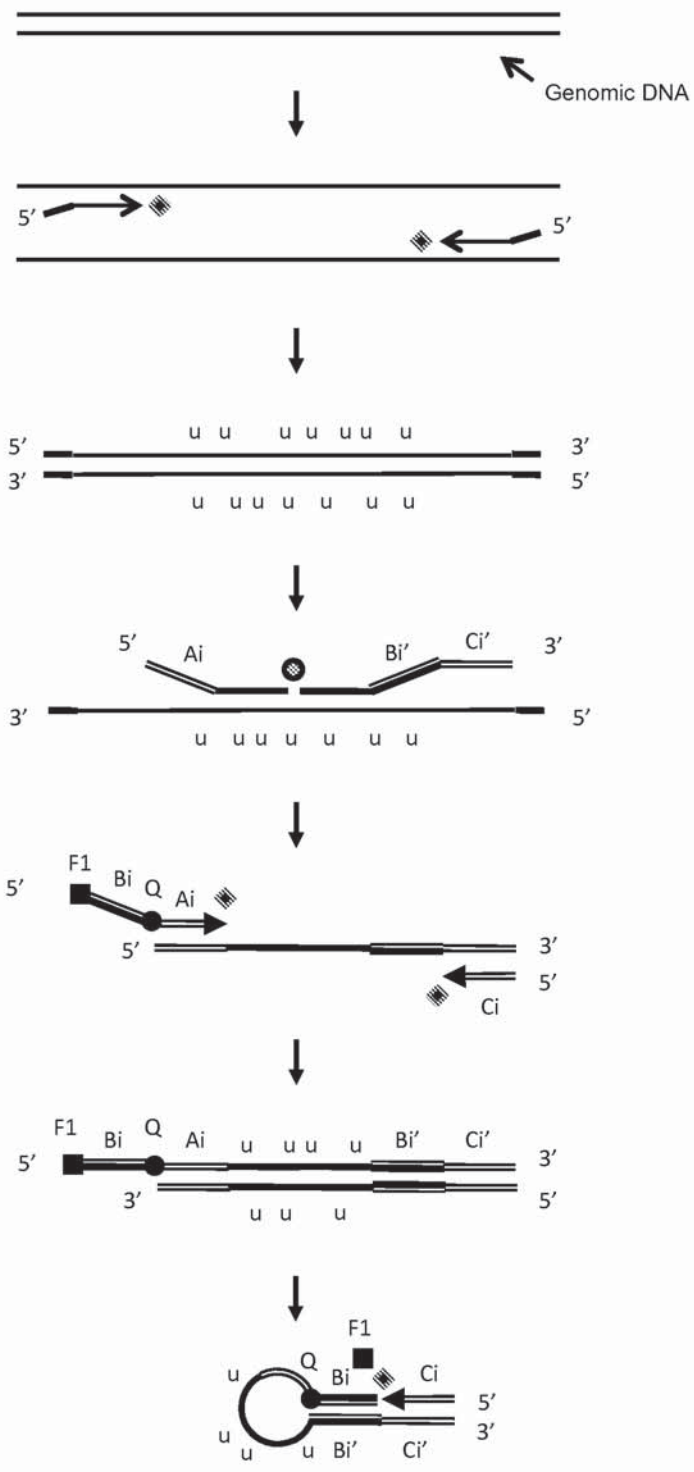


Figure 87

A. PCR-qLDR carryover prevention reaction to enumerate DNA copy number. Isolate tumor DNA from CTCs or tumor-specific exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, aliquot into 12, 24, 48, or 96 wells, and amplify mutation containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5' biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Locus-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal. Determine copy number based on signal from wells where original distribution was one copy/well.

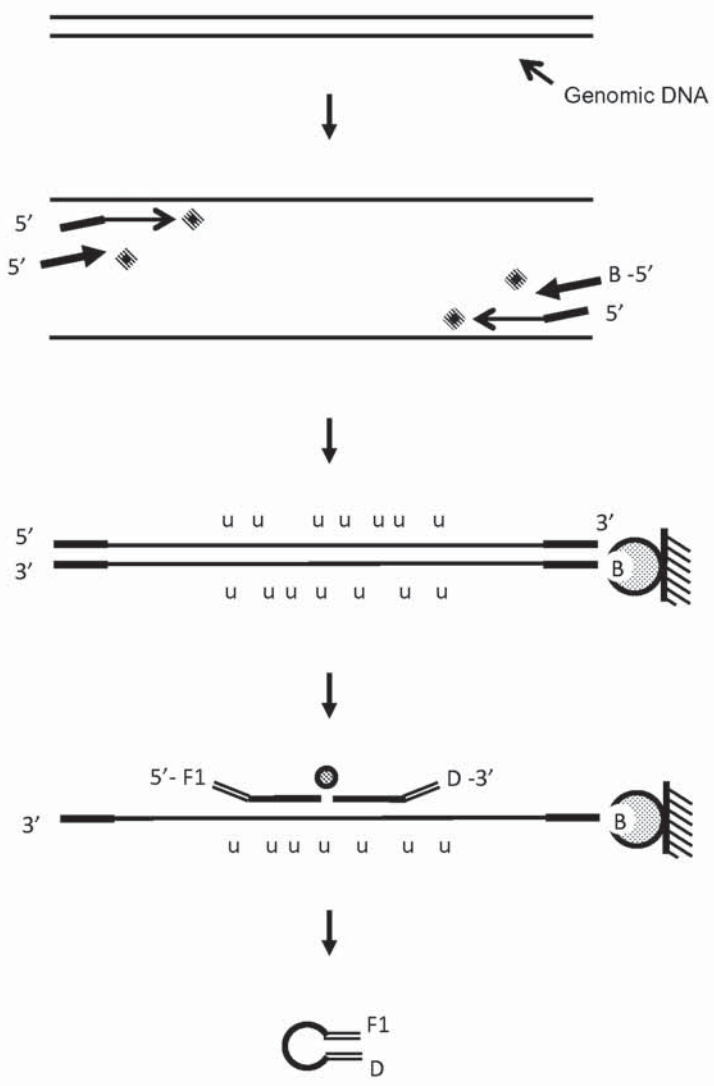


Figure 88

A. RT-PCR-LDR-qPCR carryover prevention reaction to enumerate RNA copy number. Isolate RNA from whole blood cells, exosomes, or CTCs..

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. For accurate enumeration, aliquot into 12, 24, 48, or 96 wells prior to PCR.

C. PCR products incorporate dU, allowing for carryover prevention.

D. Locus-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. Determine copy number based on signal from wells where original distribution was one copy/well.

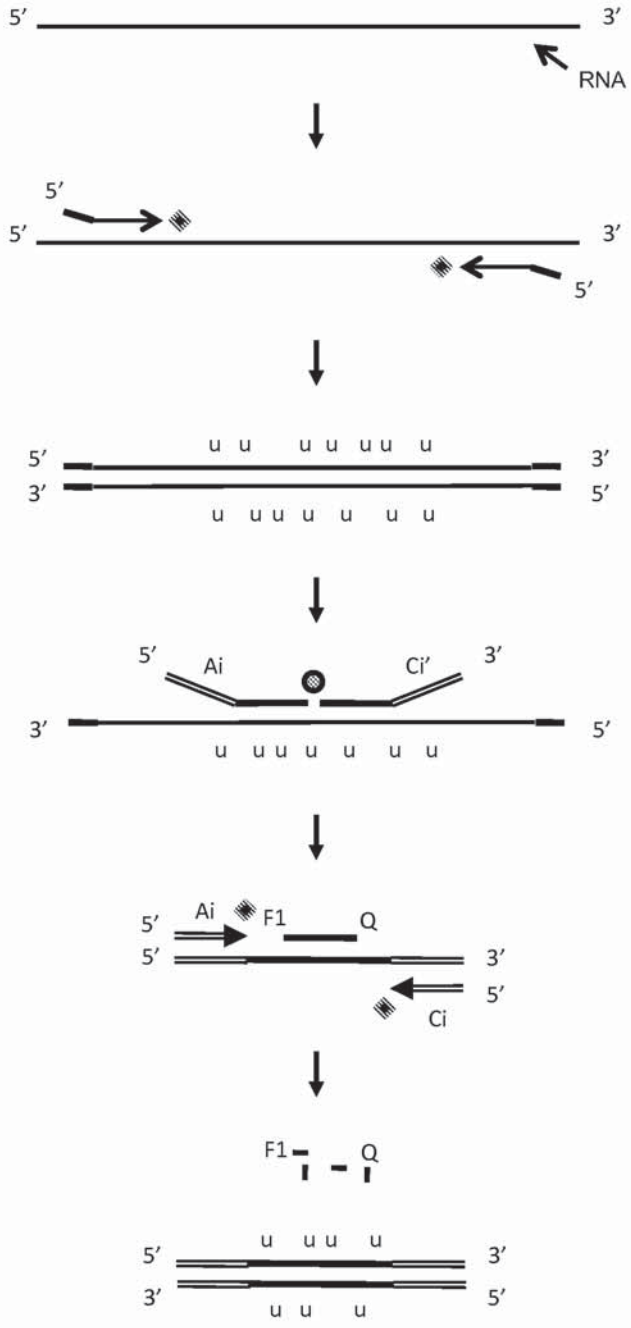


Figure 89

A. RT-PCR-LDR-qPCR carryover prevention reaction to enumerate RNA copy number. Isolate RNA from whole blood cells, exosomes, or CTCs.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3' transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. For accurate enumeration, aliquot into 12, 24, 48, or 96 wells prior to PCR.

C. PCR products incorporate dU, allowing for carryover prevention.

D. Locus-specific ligation oligonucleotides contain tags (Ai, Bi'-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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. Determine copy number based on signal from wells where original distribution was one copy/well.

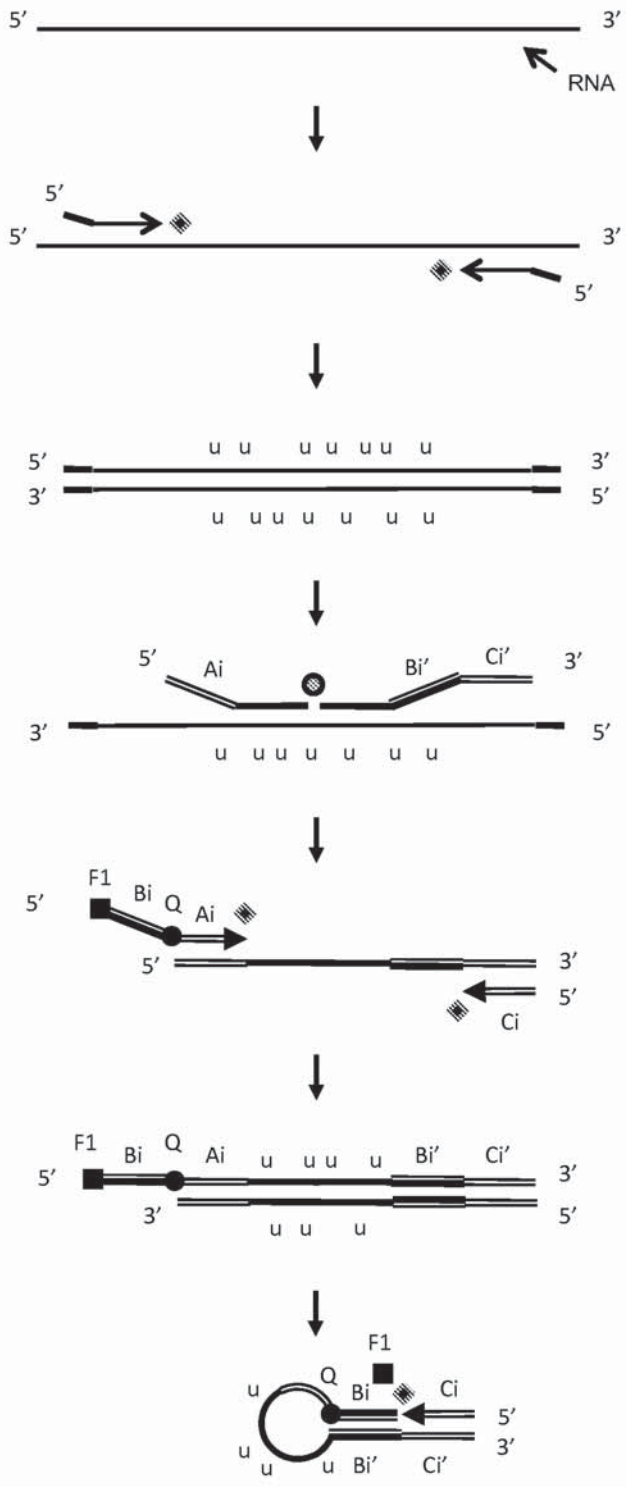


Figure 90

A. RT-PCR-qLDR carryover prevention reaction to enumerate RNA copy number. Isolate RNA from whole blood cells, exosomes, or CTCs.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and aliquot into 12, 24, 48, or 96 wells. Use reverse-transcriptase to make cDNA copy (using dUTP) with 3′ transcript-specific primers. Activate Taq polymerase and perform limited cycle PCR amplification (12-20) to maintain relative ratios of different amplicons. Primers contain universal tails (with identical 8-11 bases to prevent primer dimers), also enabling universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Locus-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal. Determine copy number based on signal from wells where original distribution was one copy/well.

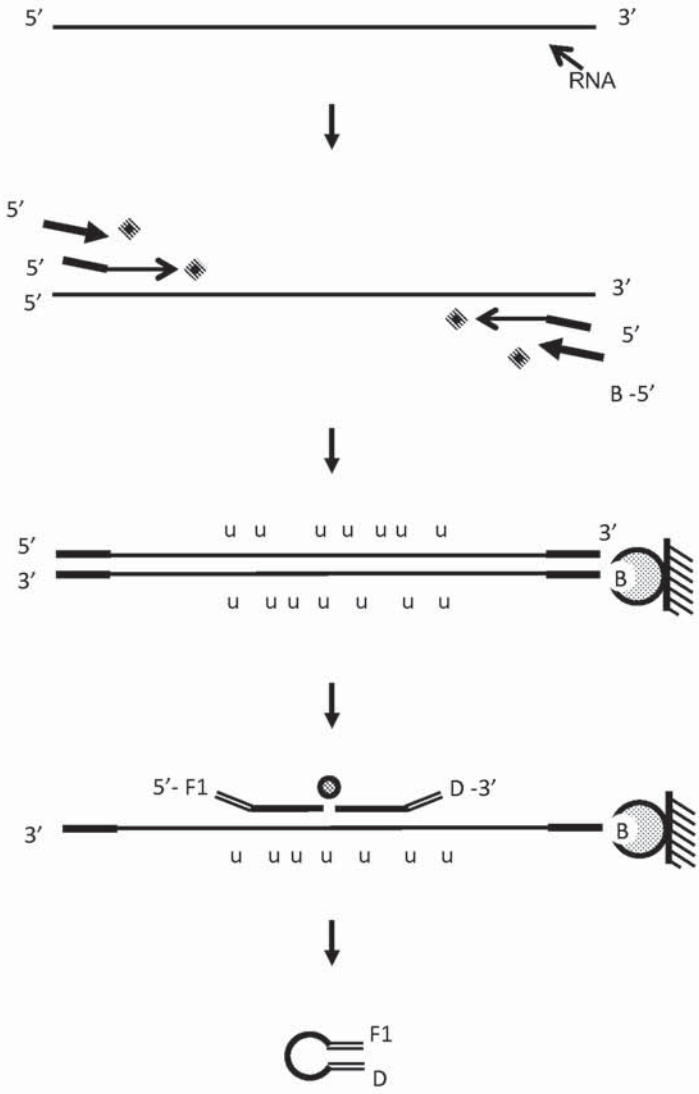


Figure 91

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Hybridize probe complementary to 3′ end of target miRNA, containing a stem-loop, tag (Tj), and blocking group. Extend 3′ end of loop primer with reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Ci′) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5′->3′ nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

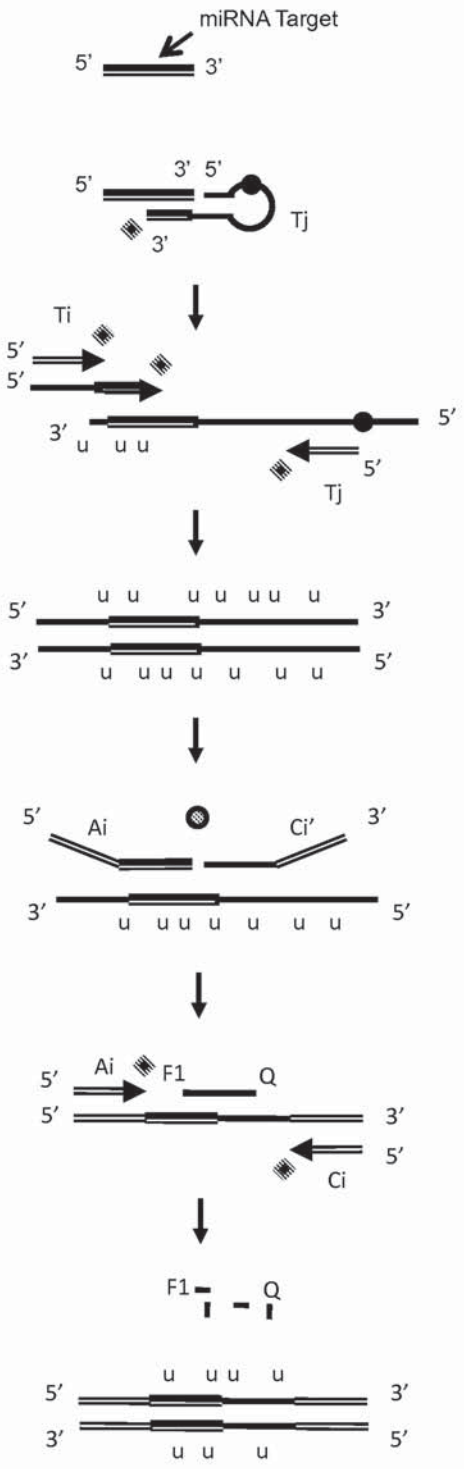


Figure 92

A. RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Hybridize probe complementary to 3' end of target miRNA, containing a stem-loop, tag (Tj), and blocking group. Extend 3' end of loop primer with reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

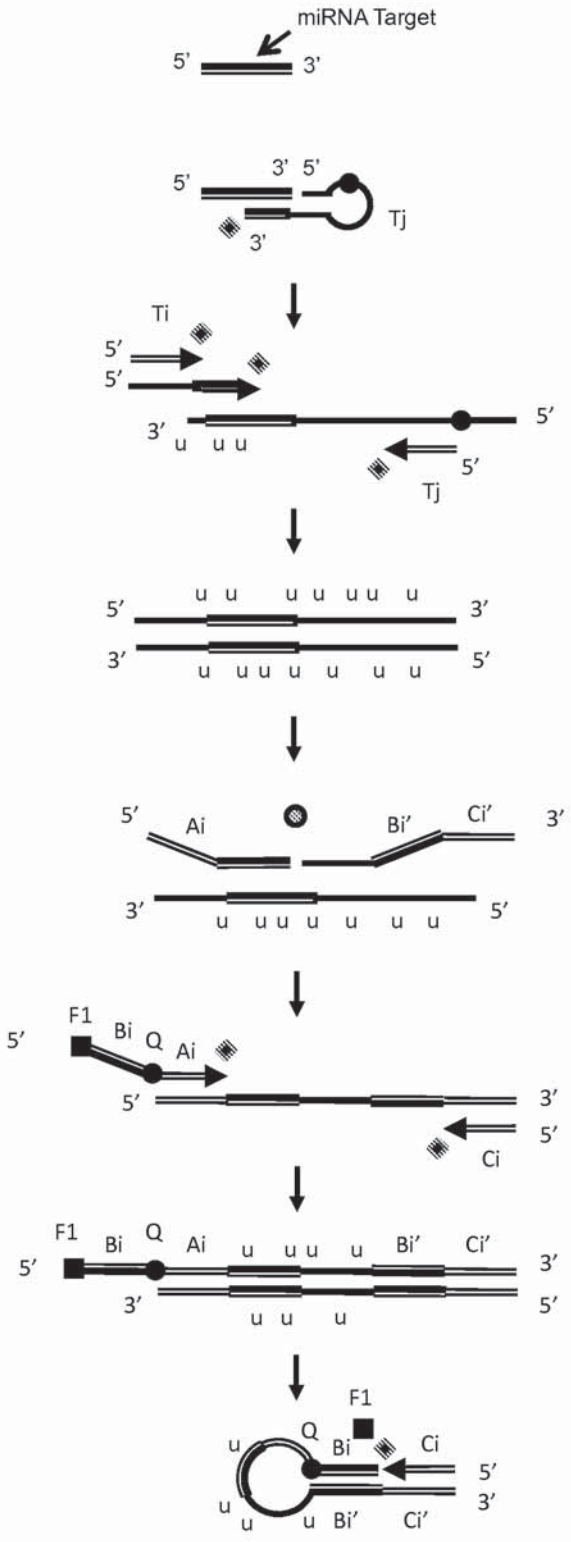


Figure 93

A. RT-PCR-qLDR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and aliquot into 12, 24, 48, or 96 wells. Hybridize probe complementary to 3′ end of target miRNA, containing a stem-loop, tag (Tj), and blocking group. Extend 3′ end of loop primer with reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers, with one primer containing a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. miRNA-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

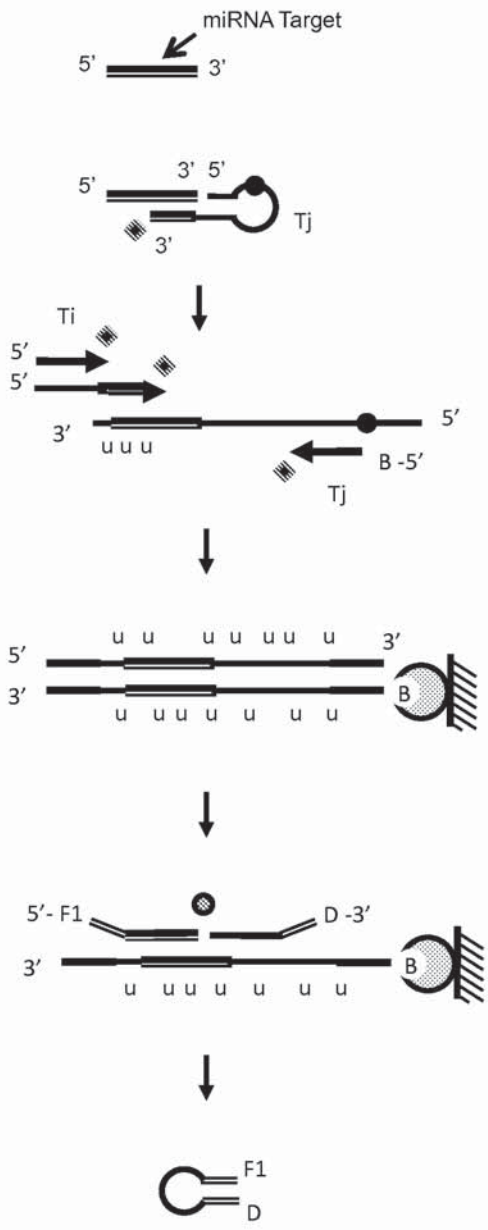


Figure 94

A. Ligation-RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Ligate probe complementary to 3' end of target miRNA, containing a stem-loop, Tag (Tj'), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci). Each Taqman probe spans the ligation junction.

F. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

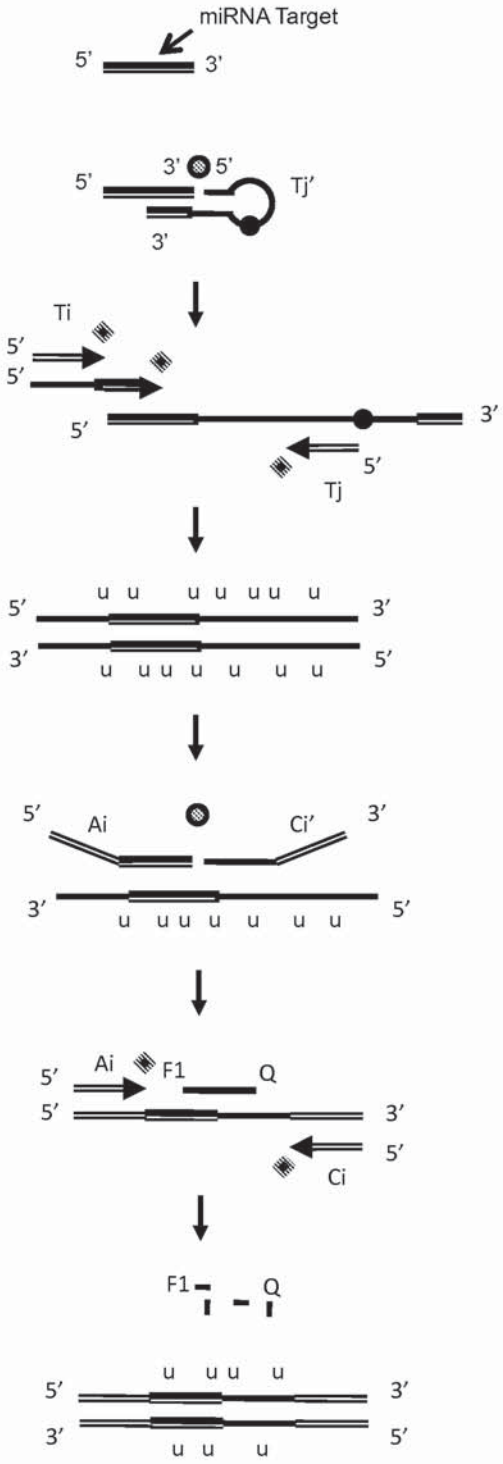


Figure 95

A. Ligation-RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Ligate probe complementary to 3' end of target miRNA, containing a stem-loop, Tag (Tj'), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Bi-Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

E. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with Unitaq-specific primers (F1-Bi-Q-Ai, 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.

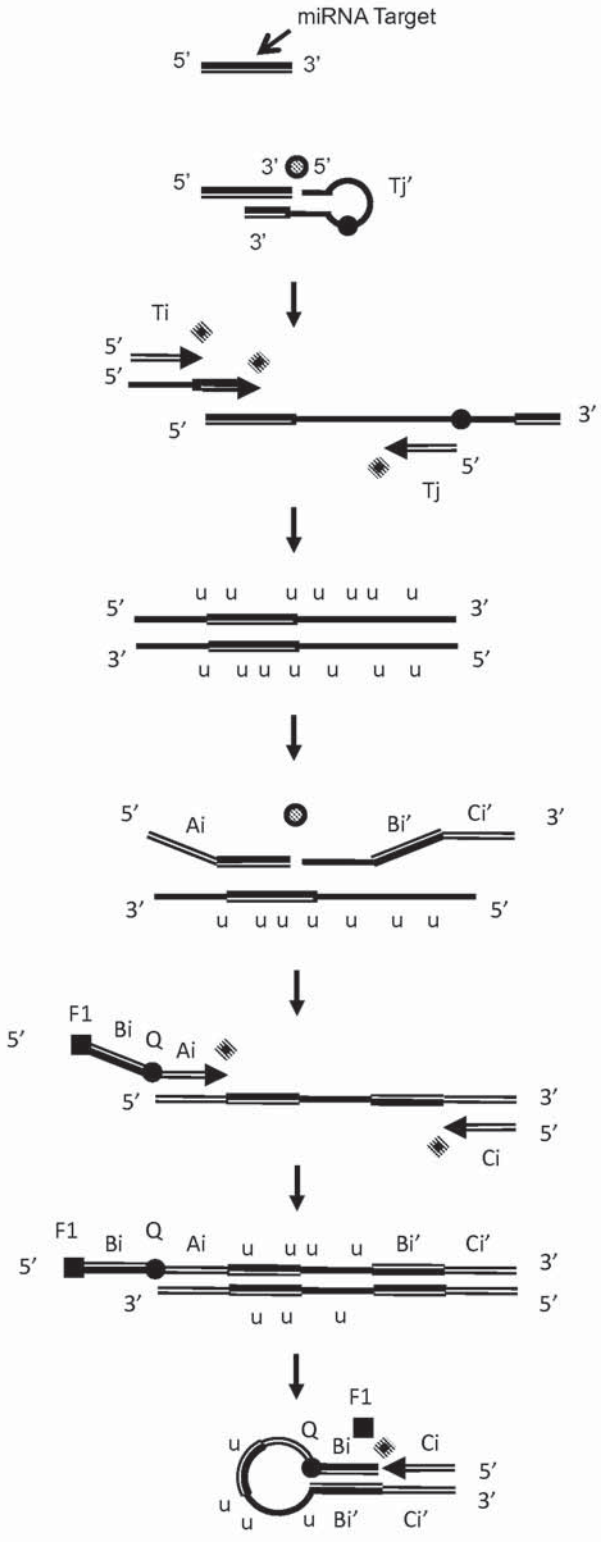


Figure 96

A. Ligation-RT-PCR-qLDR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and aliquot into 12, 24, 48, or 96 wells. Ligate probe complementary to 3′ end of target miRNA, containing a stem-loop, Tag (Tj′), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP). Activate Taq polymerase and perform limited cycle PCR amplification (12-20), using bridge and tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers, with one primer containing a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. miRNA-specific ligation oligonucleotides contain tails for subsequent FRET detection. Ligase covalently seals the two oligonucleotides together. (LDR products are not amplified by either original PCR or LDR primers, providing additional protection.)

E. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

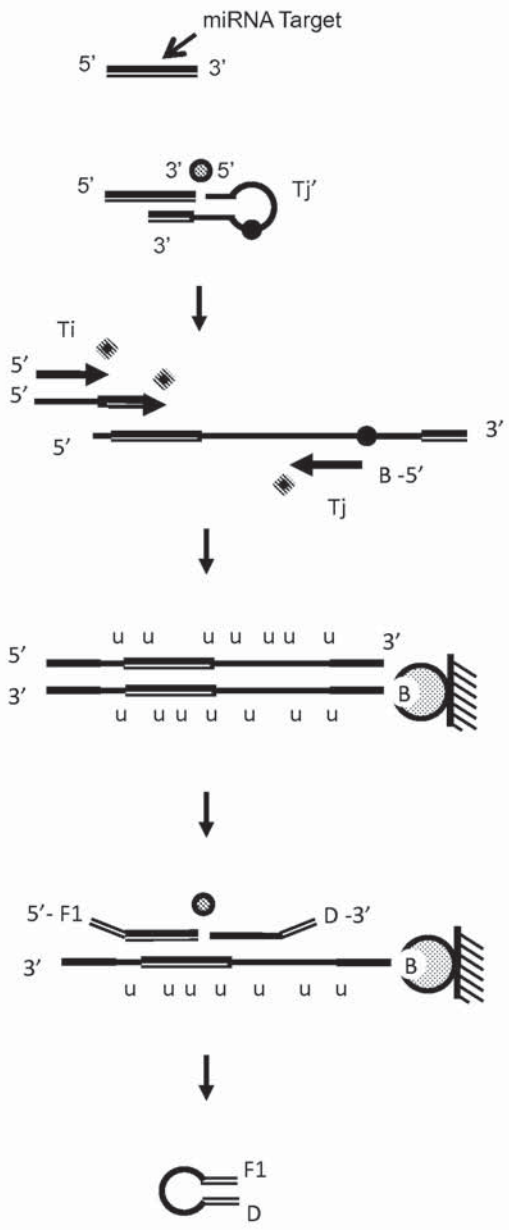


Figure 97

A. Ligation-RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Ligate probe complementary to 3' end of target miRNA, containing a stem-loop, Tag (Tj'), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP).

C. miRNA-specific bridge primers are unblocked with RNaseH2 only when bound to target. Perform limited cycle PCR amplification (12-20), using tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

D. PCR products incorporate dU, allowing for carryover prevention.

E. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Ci') for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

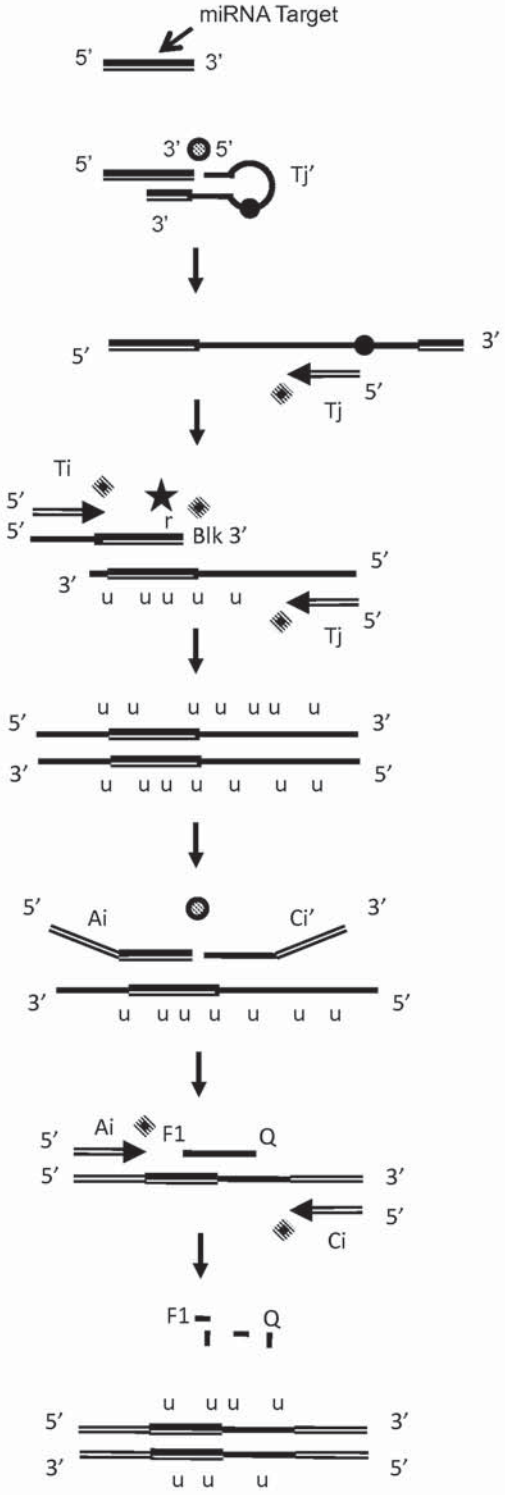


Figure 98

A. Ligation-RT-PCR-LDR-qPCR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, heat kill. Ligate probe complementary to 3′ end of target miRNA, containing a stem-loop, Tag (Tj′), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP).

C. miRNA-specific bridge primers are unblocked with RNaseH2 only when bound to target. Perform limited cycle PCR amplification (12-20), using tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

D. PCR products incorporate dU, allowing for carryover prevention.

E. miRNA sequence-specific ligation oligonucleotides contain tags (Ai, Bi-Ci′) for subsequent PCR amplification. Ligase covalently seals the two oligonucleotides together. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.

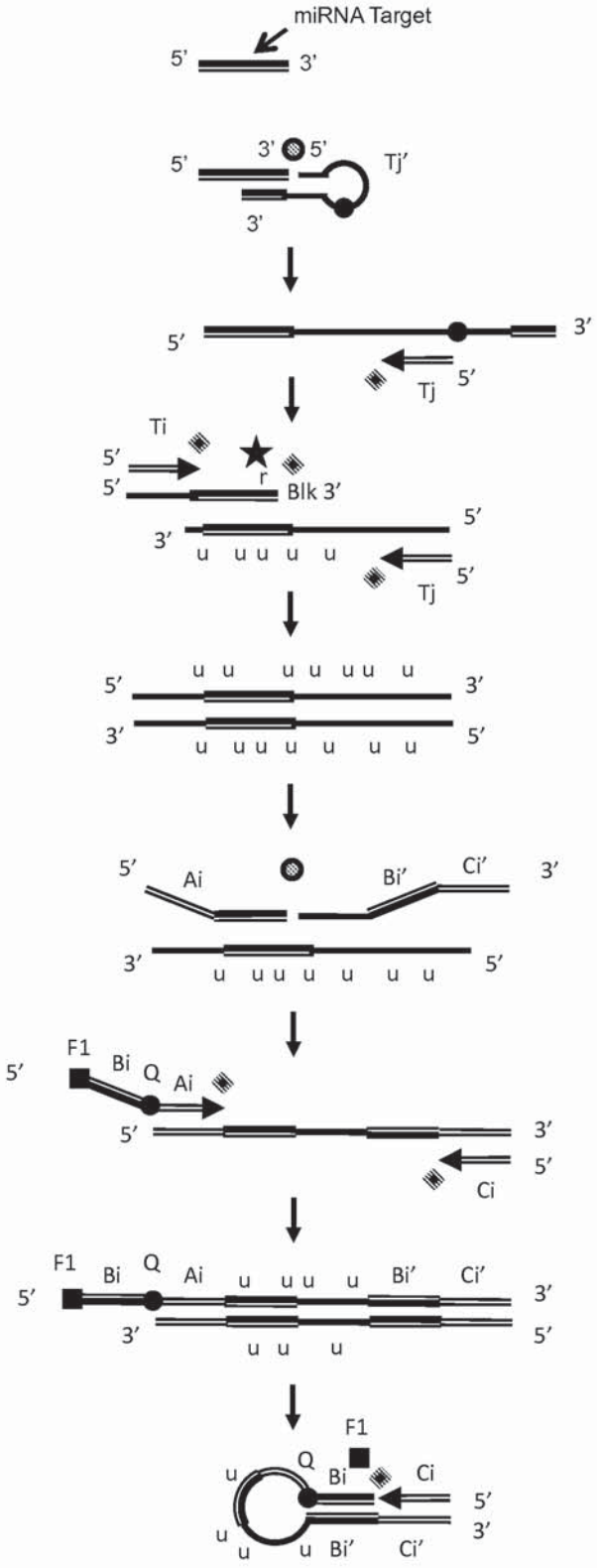


Figure 99

A. Ligation-RT-PCR-qLDR carryover prevention reaction to quantify miRNA. Isolate miRNA from exosomes.

B. Treat with UDG (uracil DNA glycosylase) for carryover prevention, and aliquot into 12, 24, 48, or 96 wells. Ligate probe complementary to 3′ end of target miRNA, containing a stem-loop, Tag (Tj′), and blocking group. Extend with primer Tj and reverse transcriptase (using dUTP).

C. miRNA-specific bridge primers are unblocked with RNaseH2 only when bound to target. Perform limited cycle PCR amplification (12-20), using tag primers (Ti, Tj), to maintain relative ratios of different amplicons. Primers contain identical 8-11 base tails to prevent primer dimers, with one primer containing a 5′ biotin.

D. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

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F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

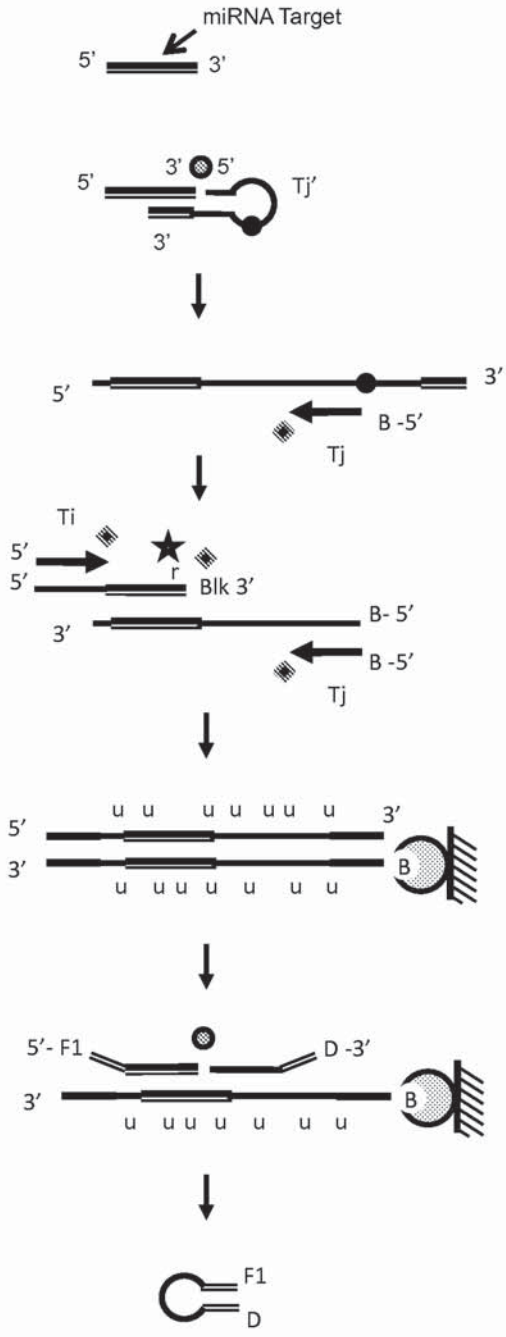
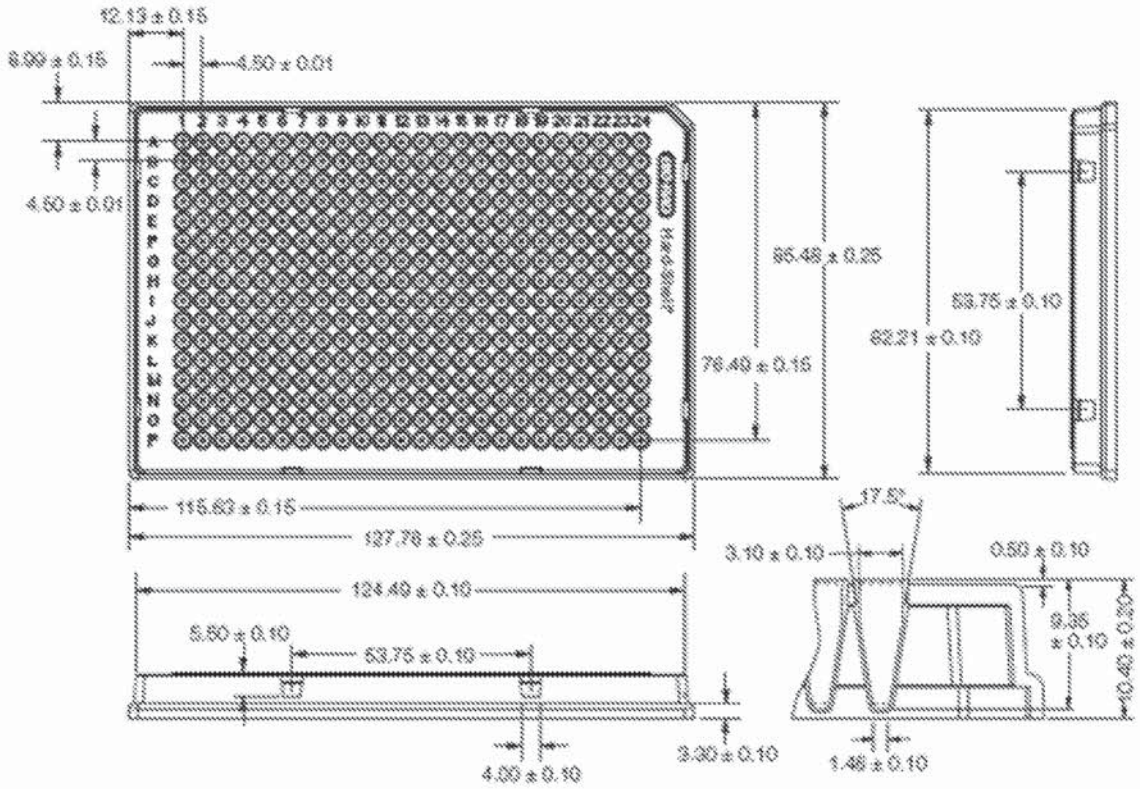


Figure 100



Not drawn to scale.
All dimensions in mm, unless otherwise noted.

Figure 101

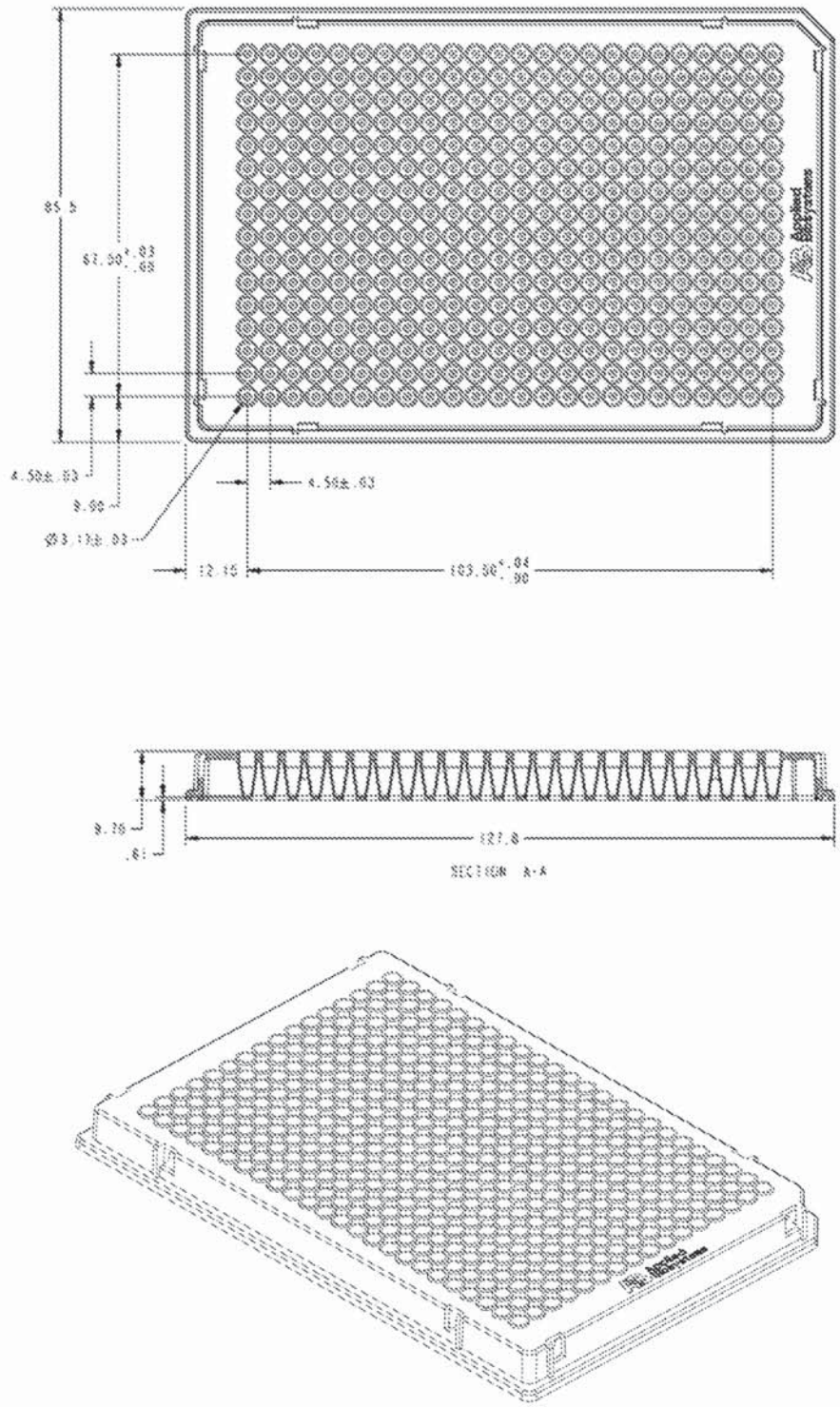


Figure 102

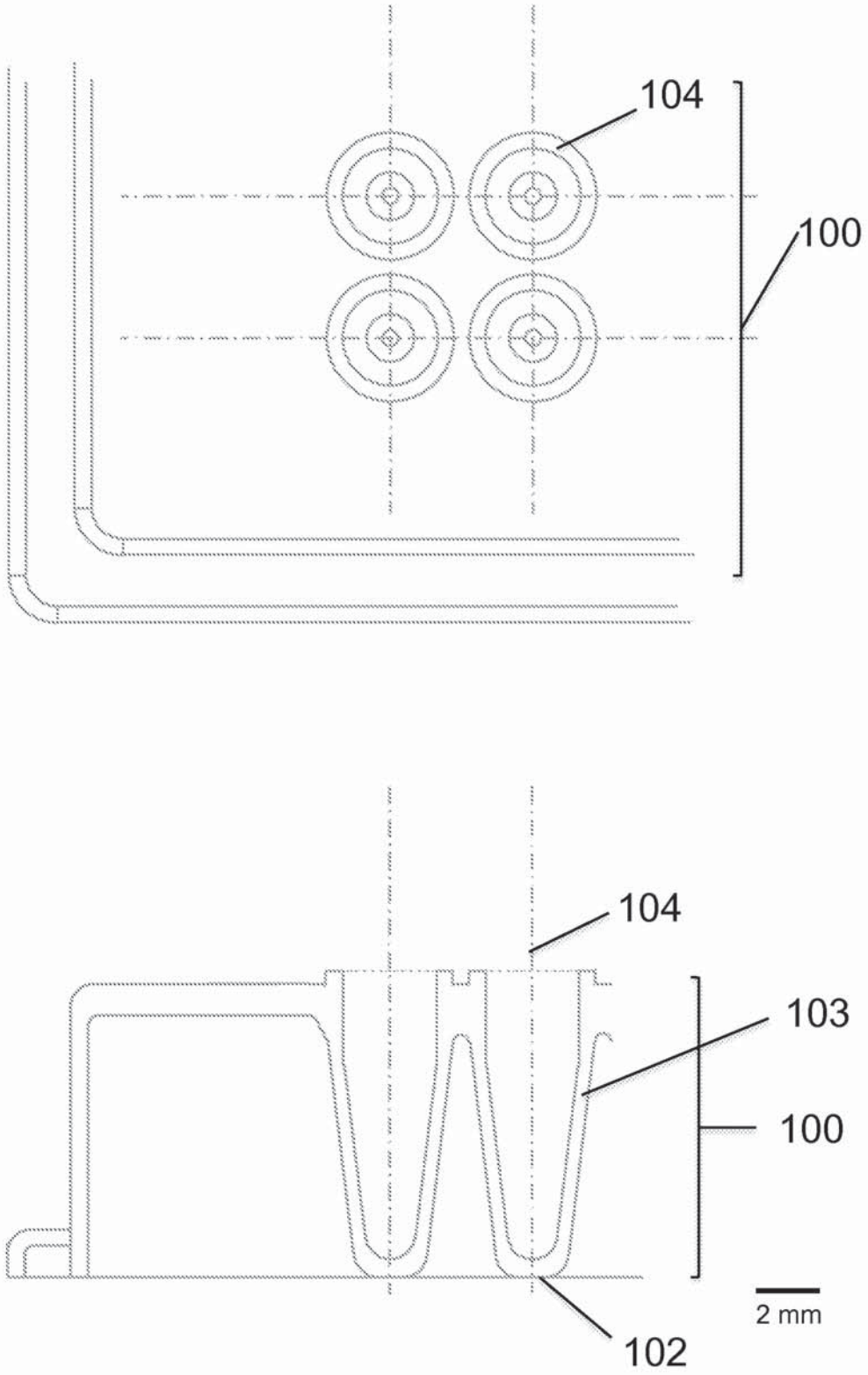


Figure 103

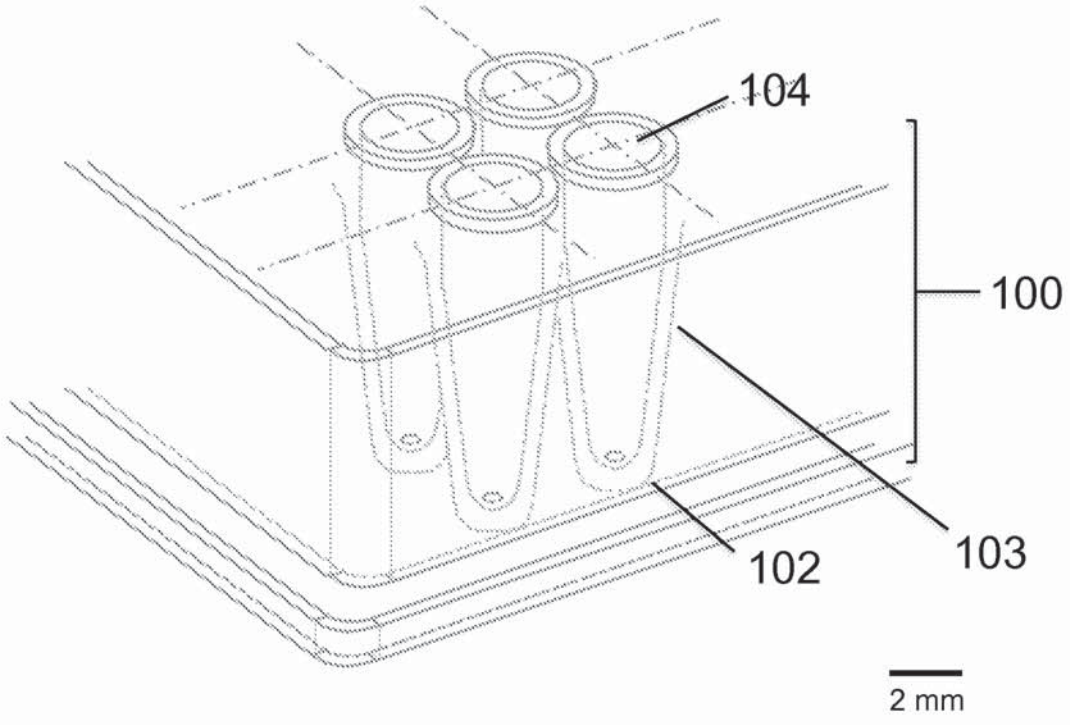
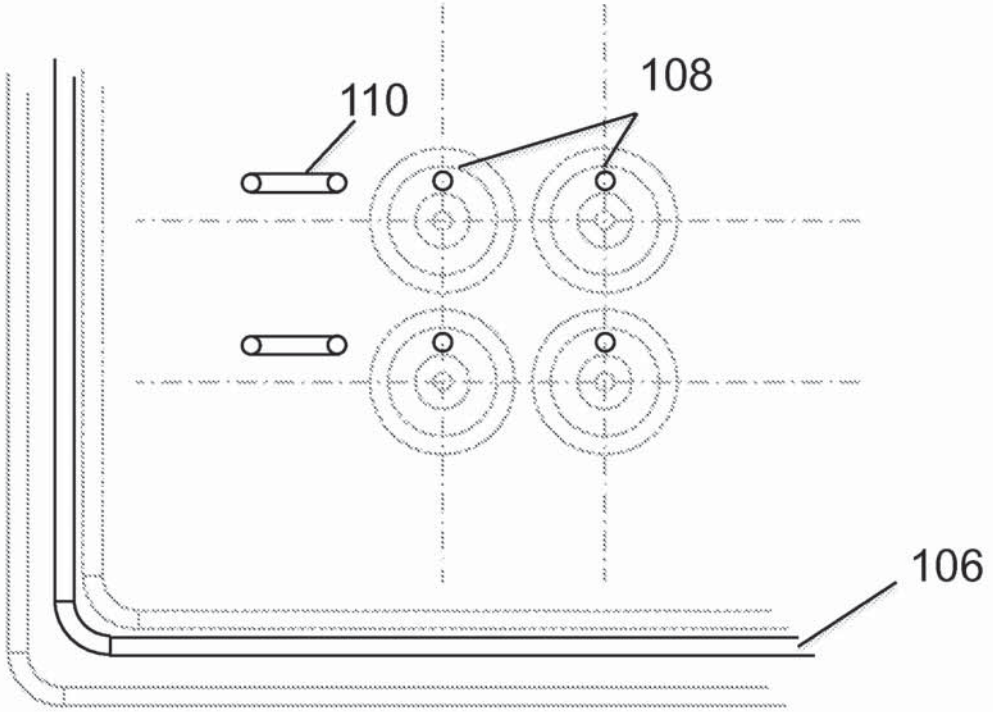


Figure 104



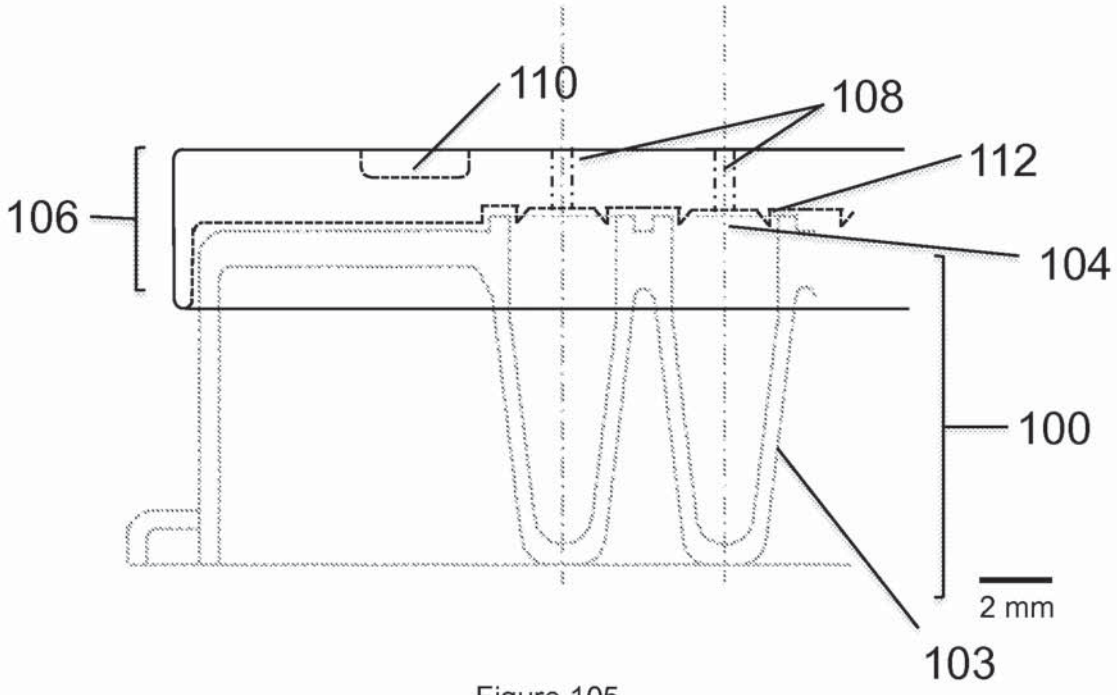


Figure 105

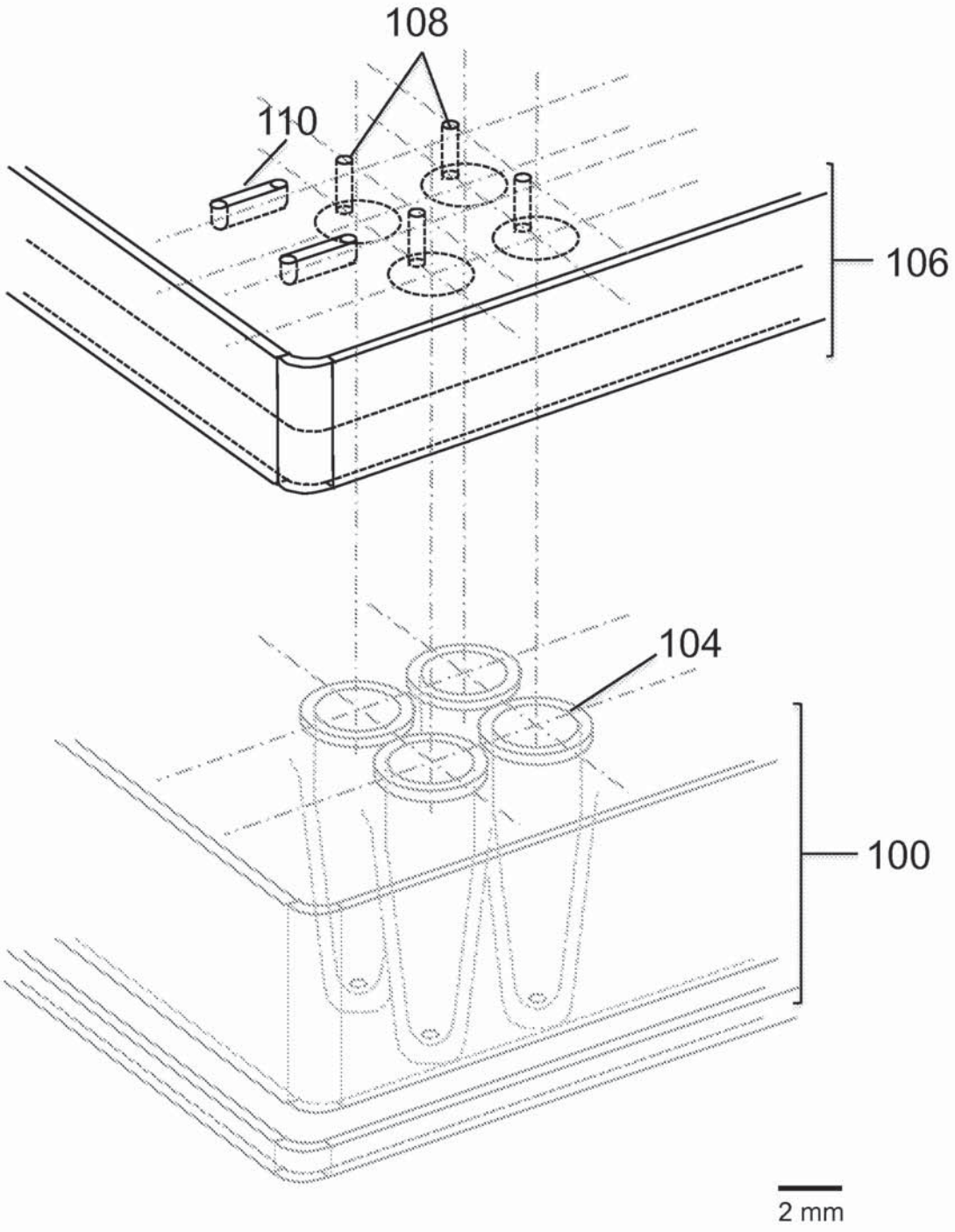


Figure 106

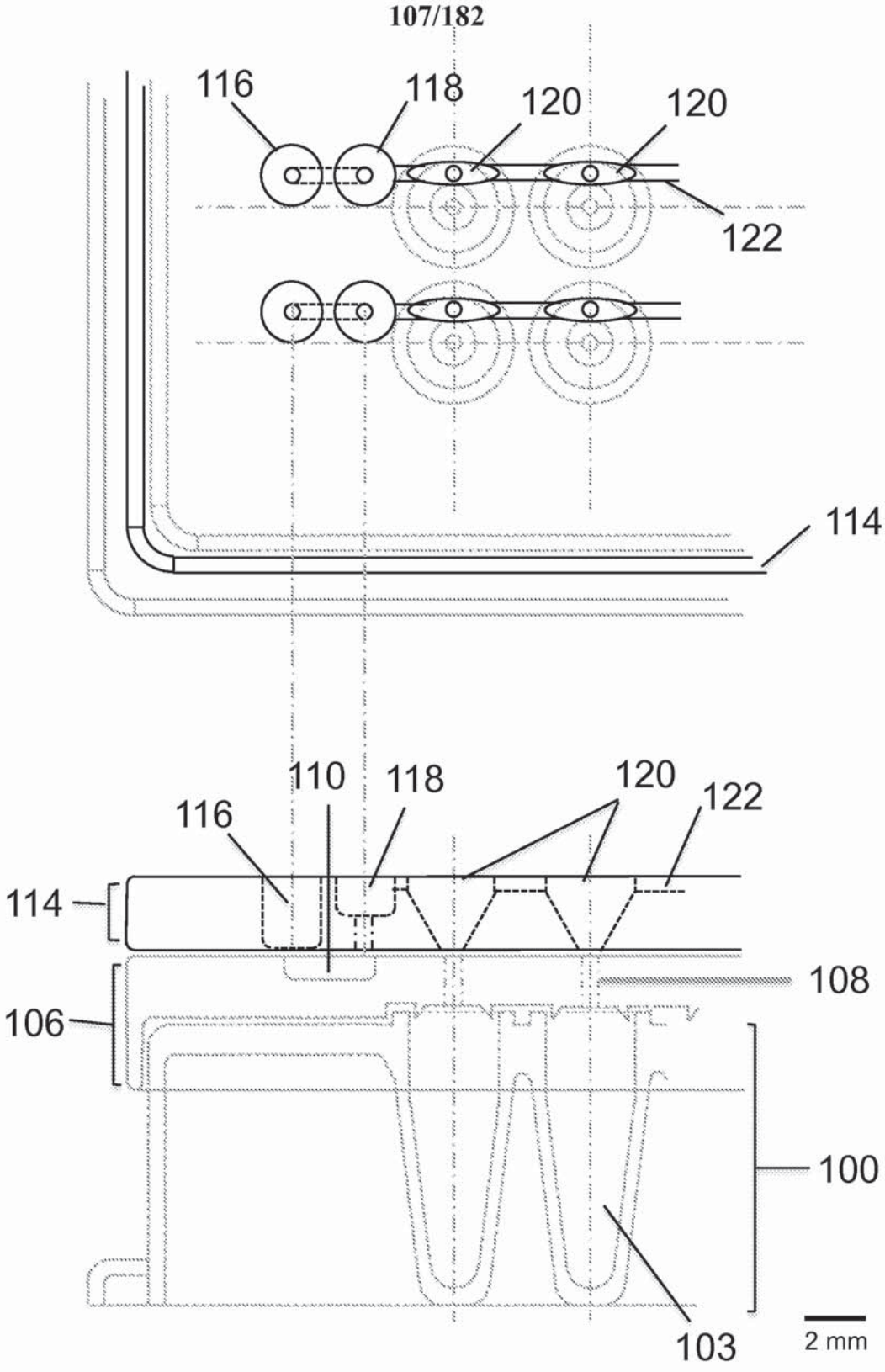

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Figure 107

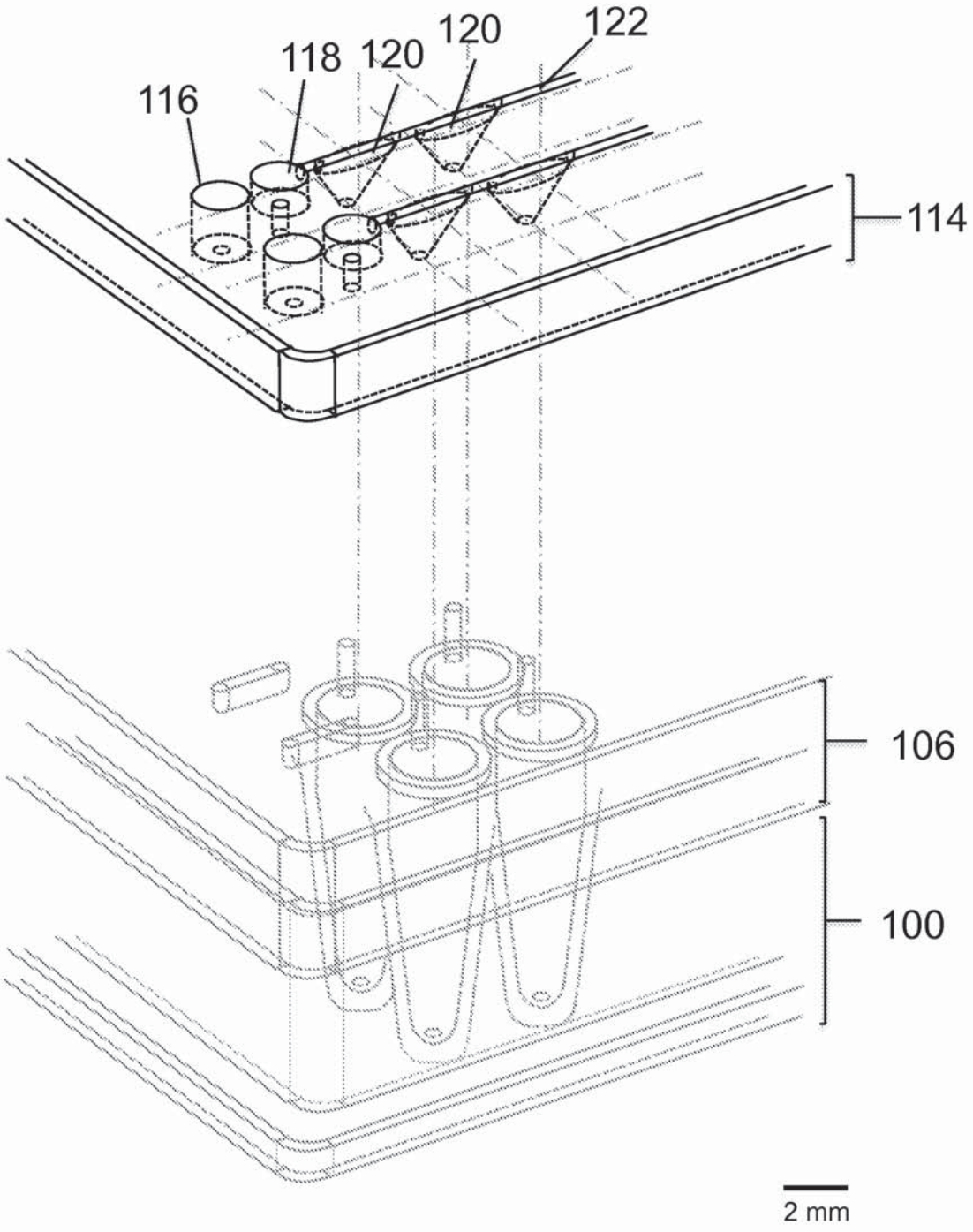
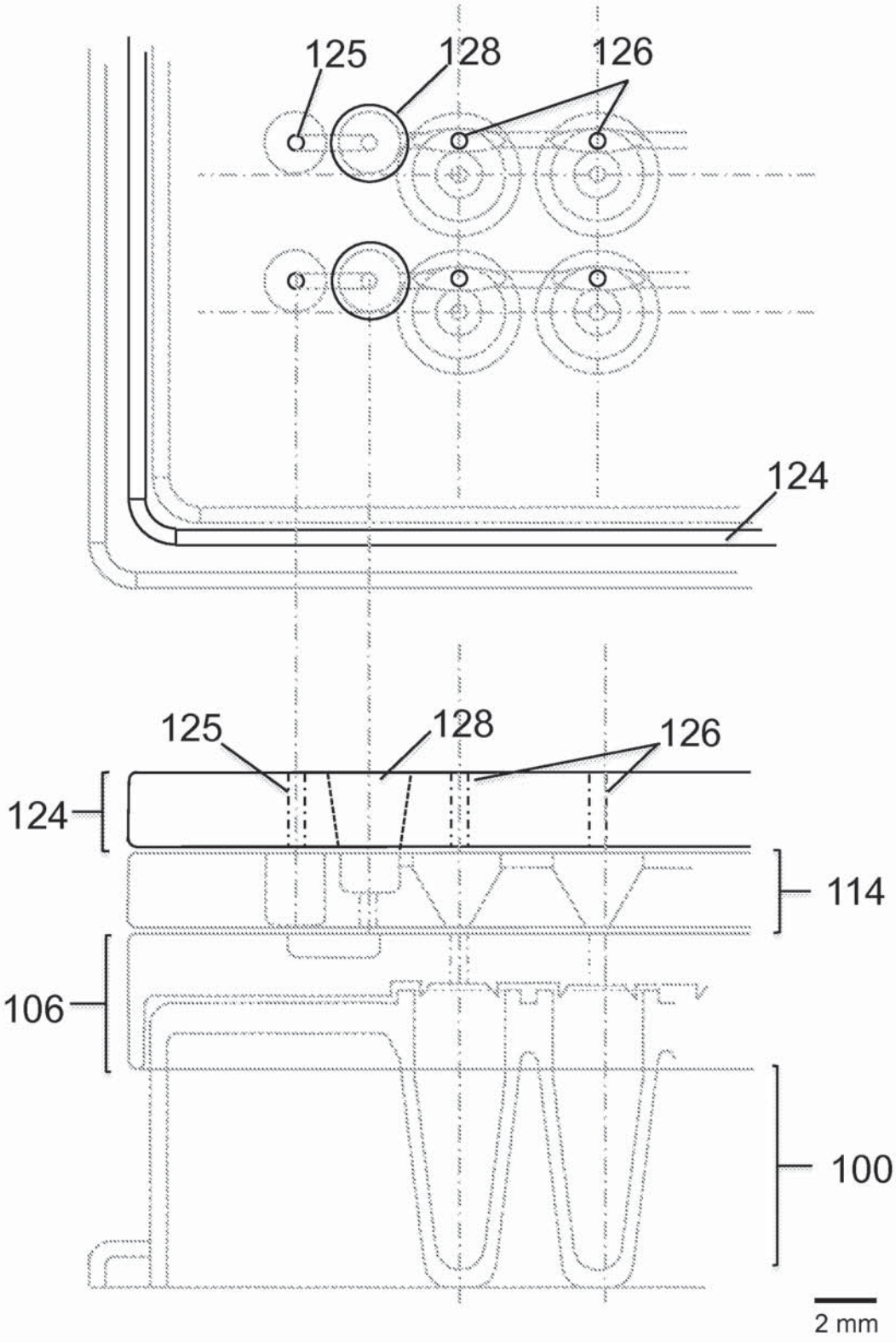


Figure 108



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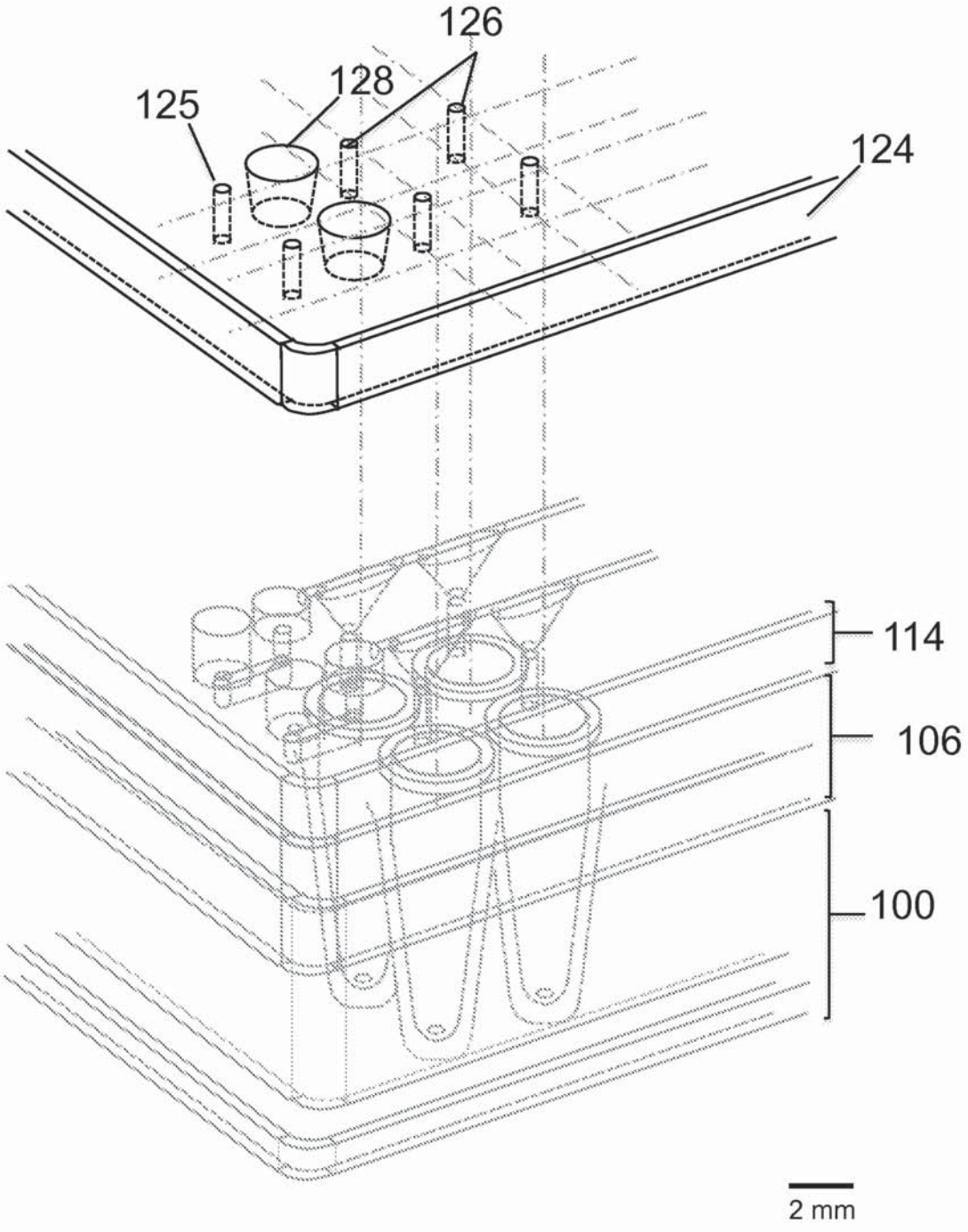

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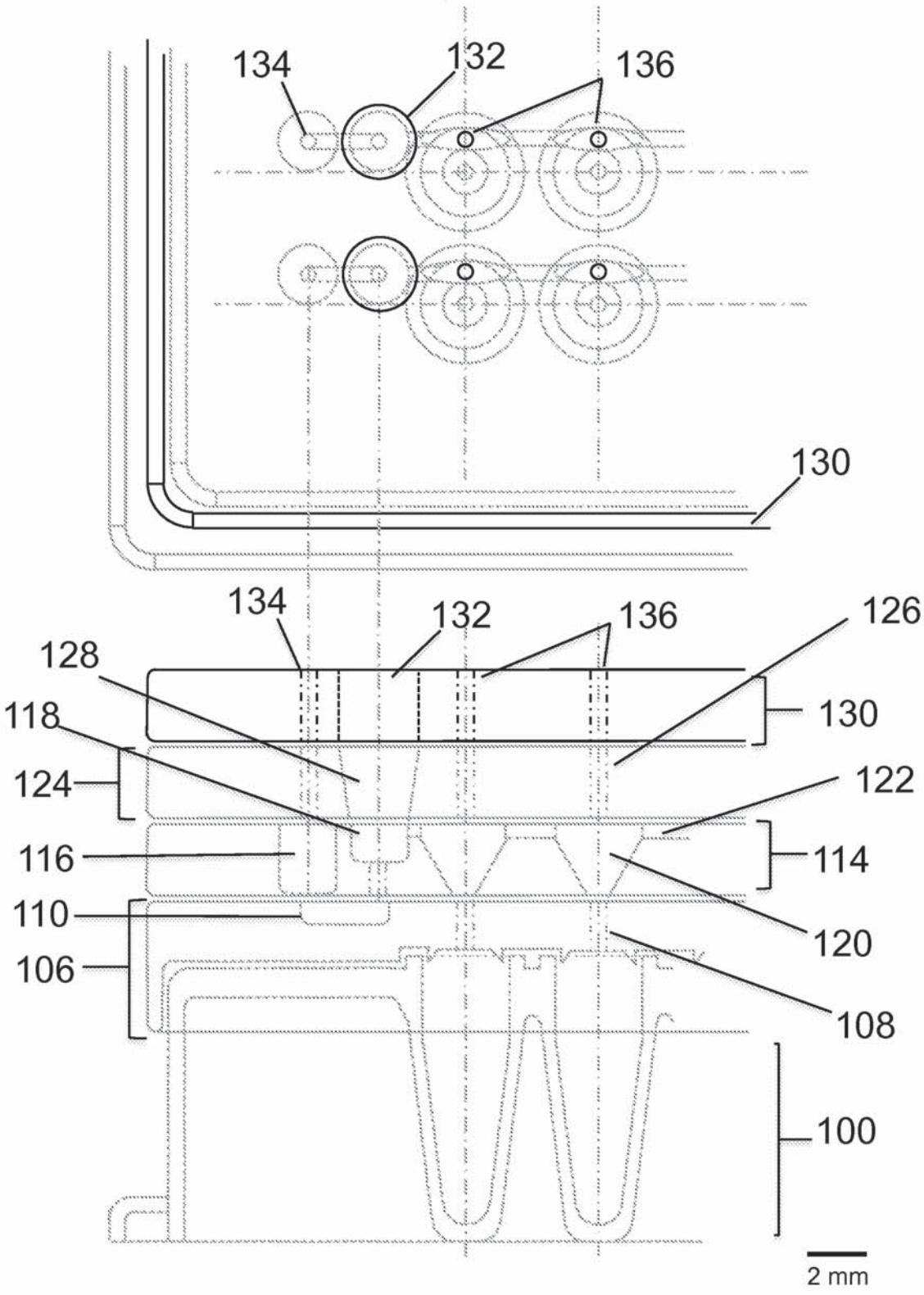
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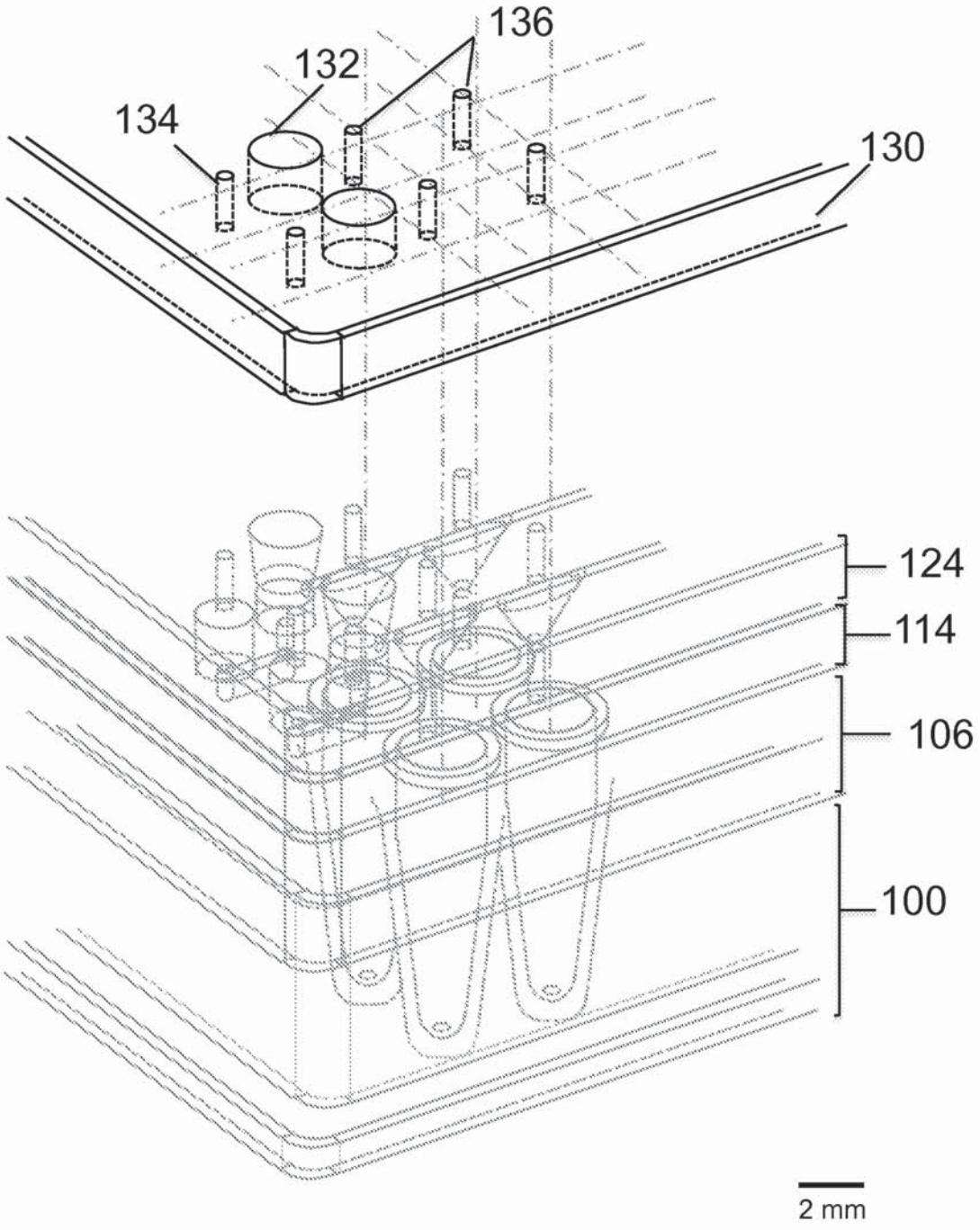
Figure 111



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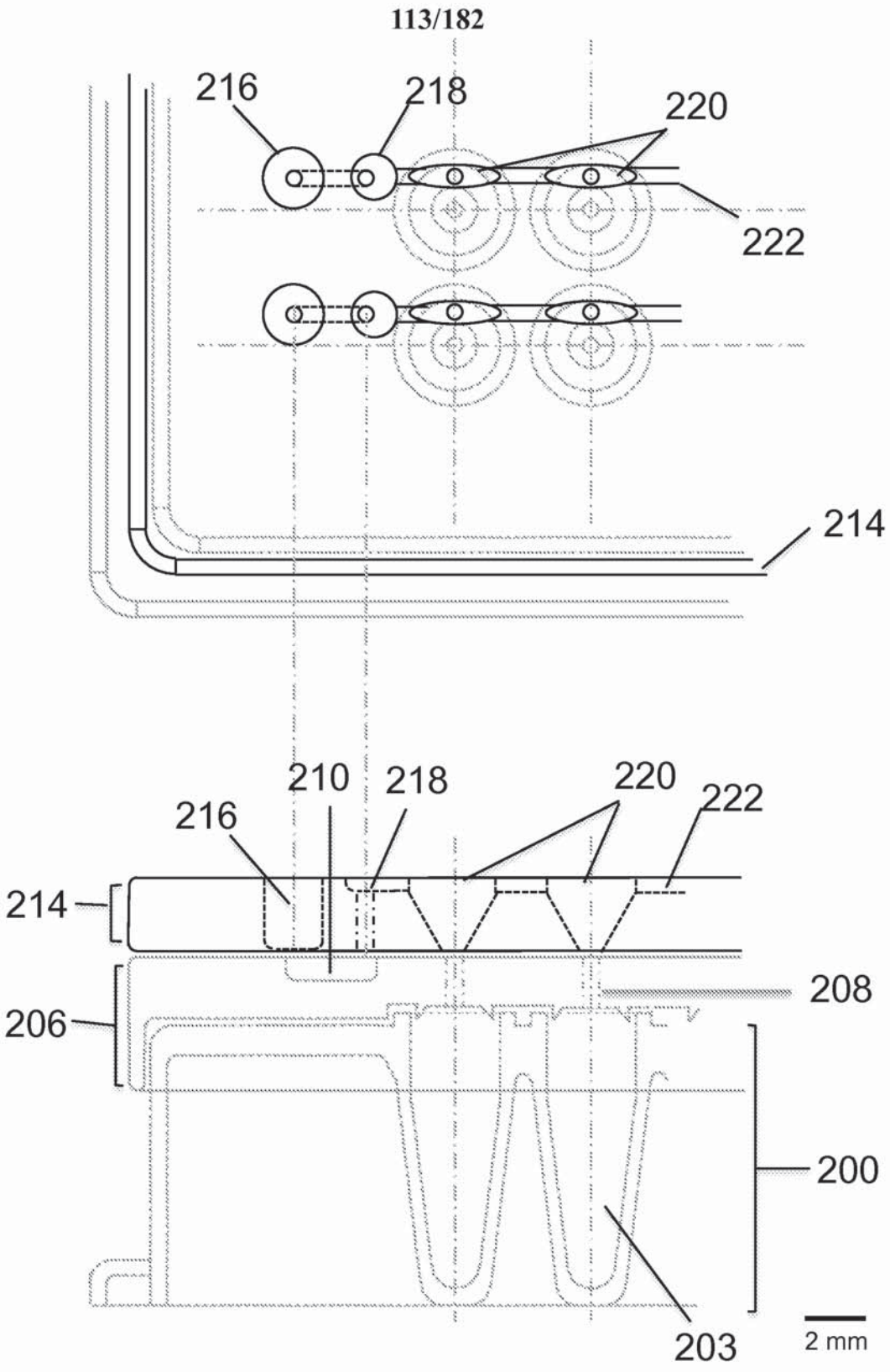

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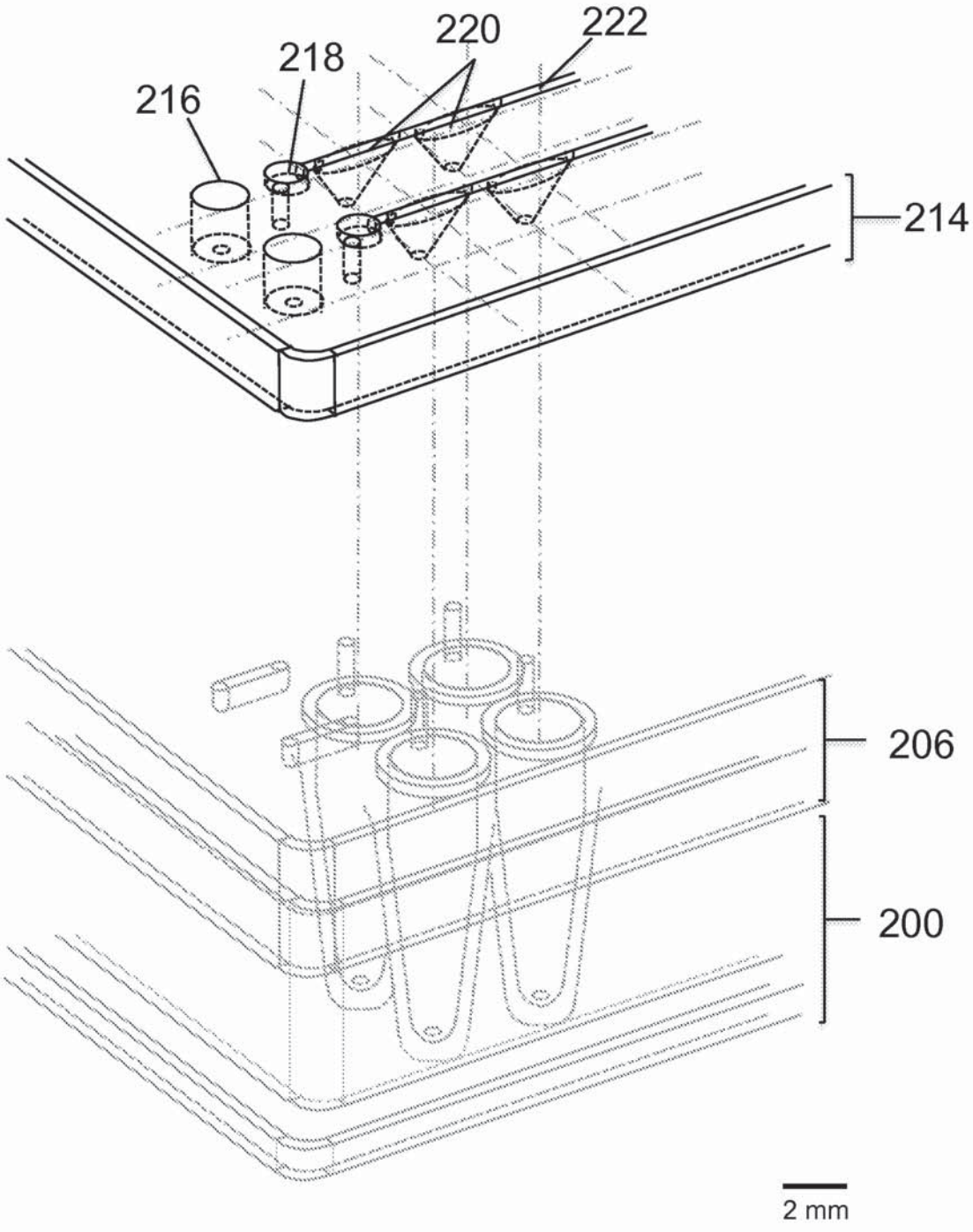

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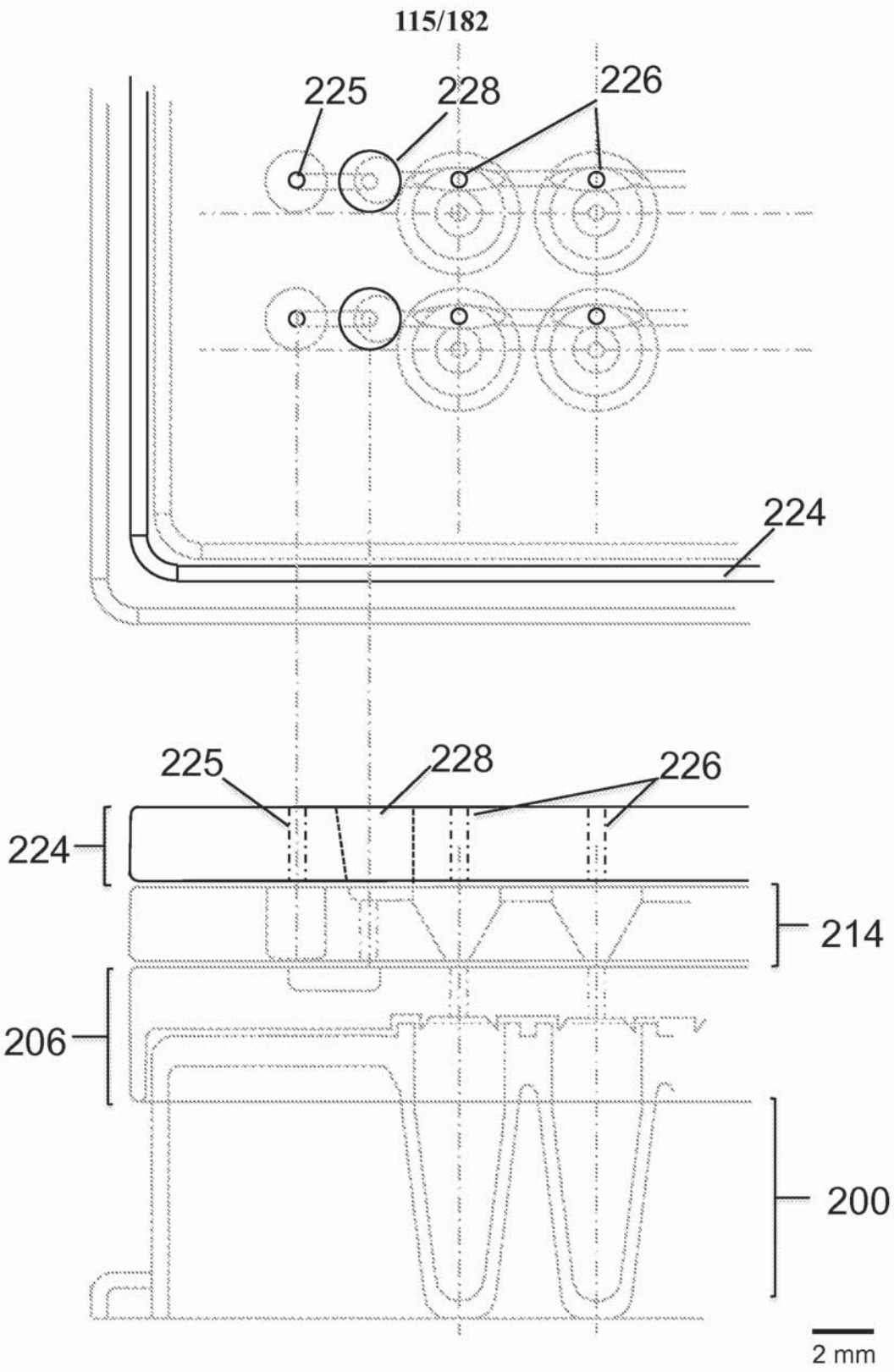
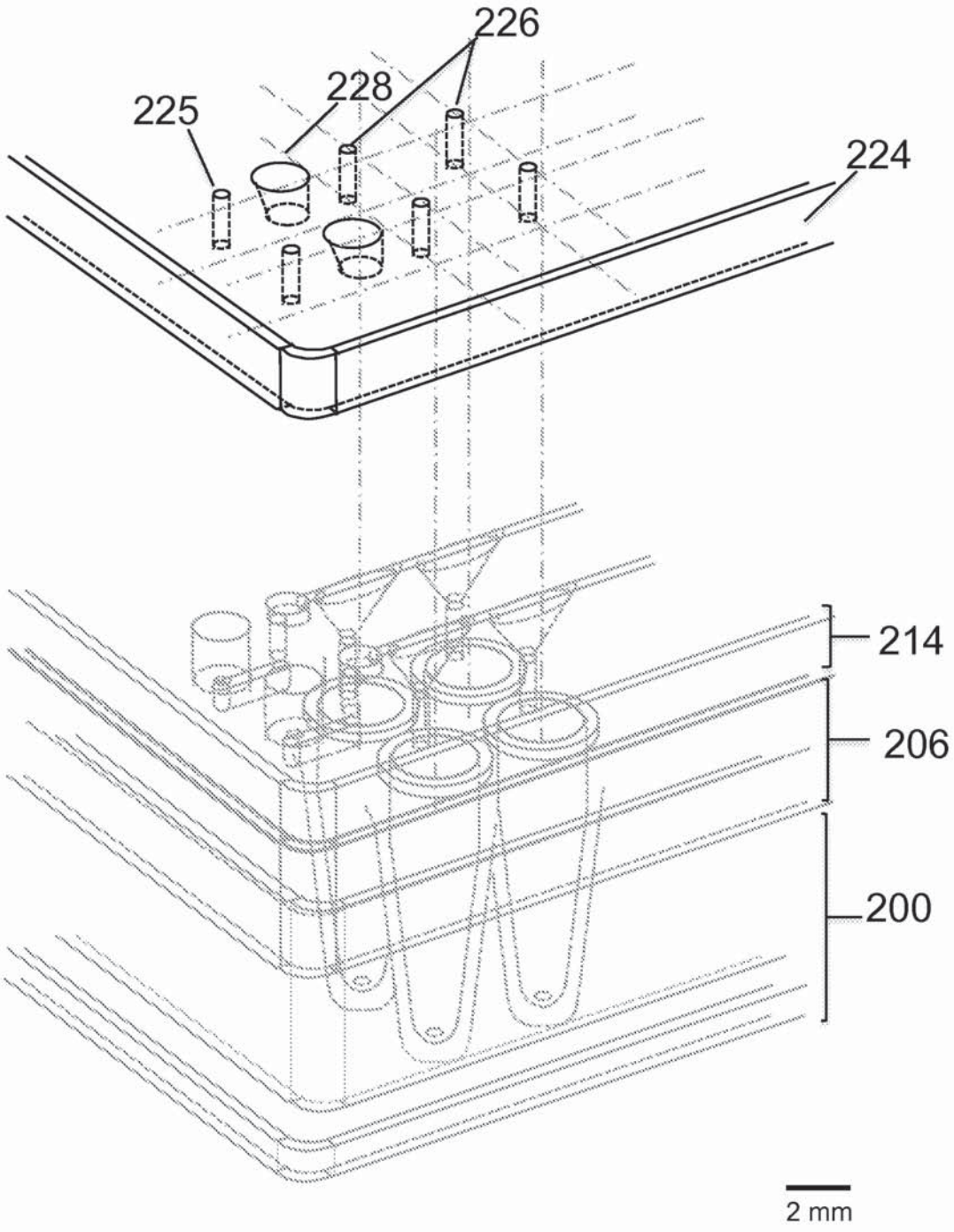


Figure 115



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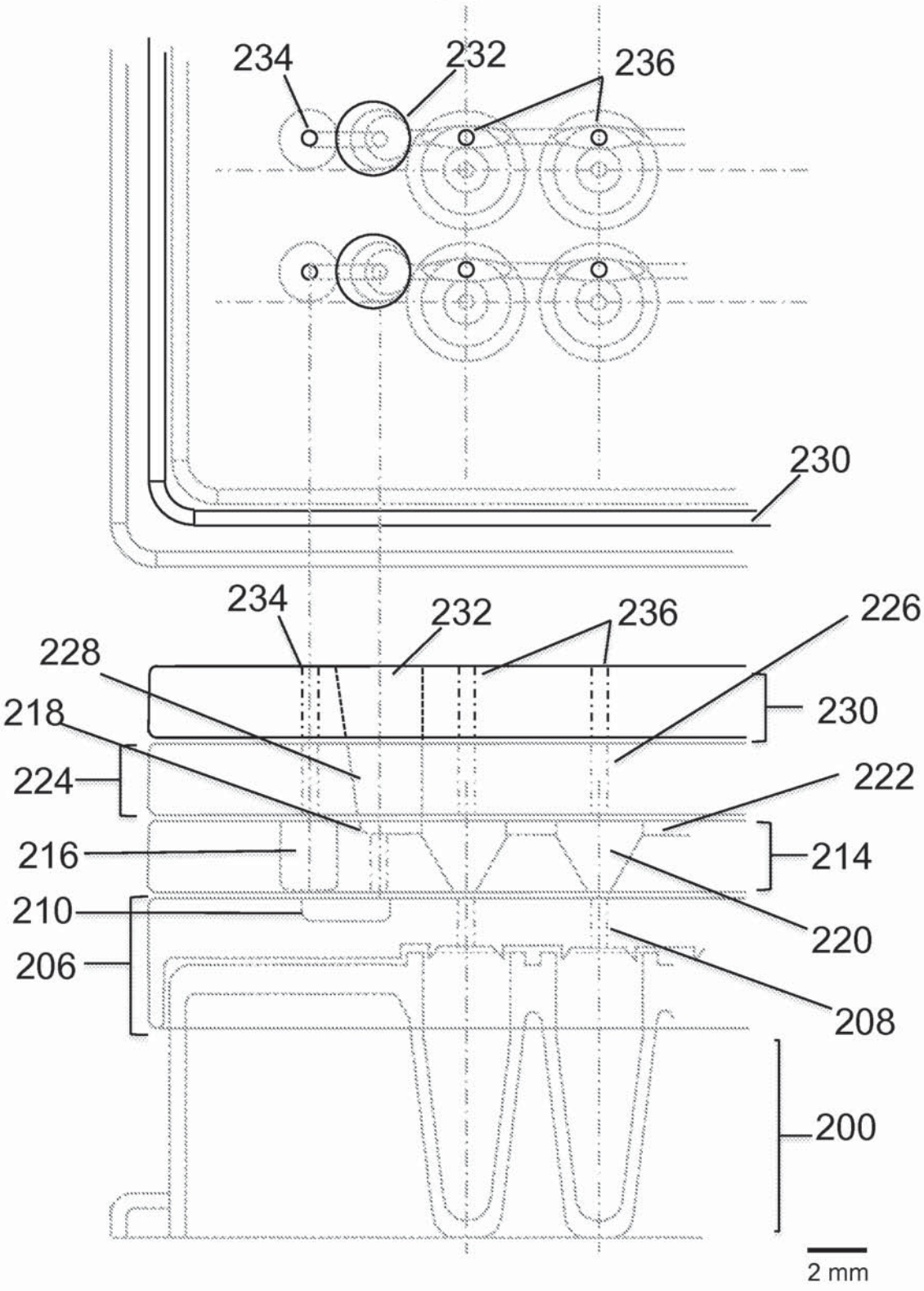
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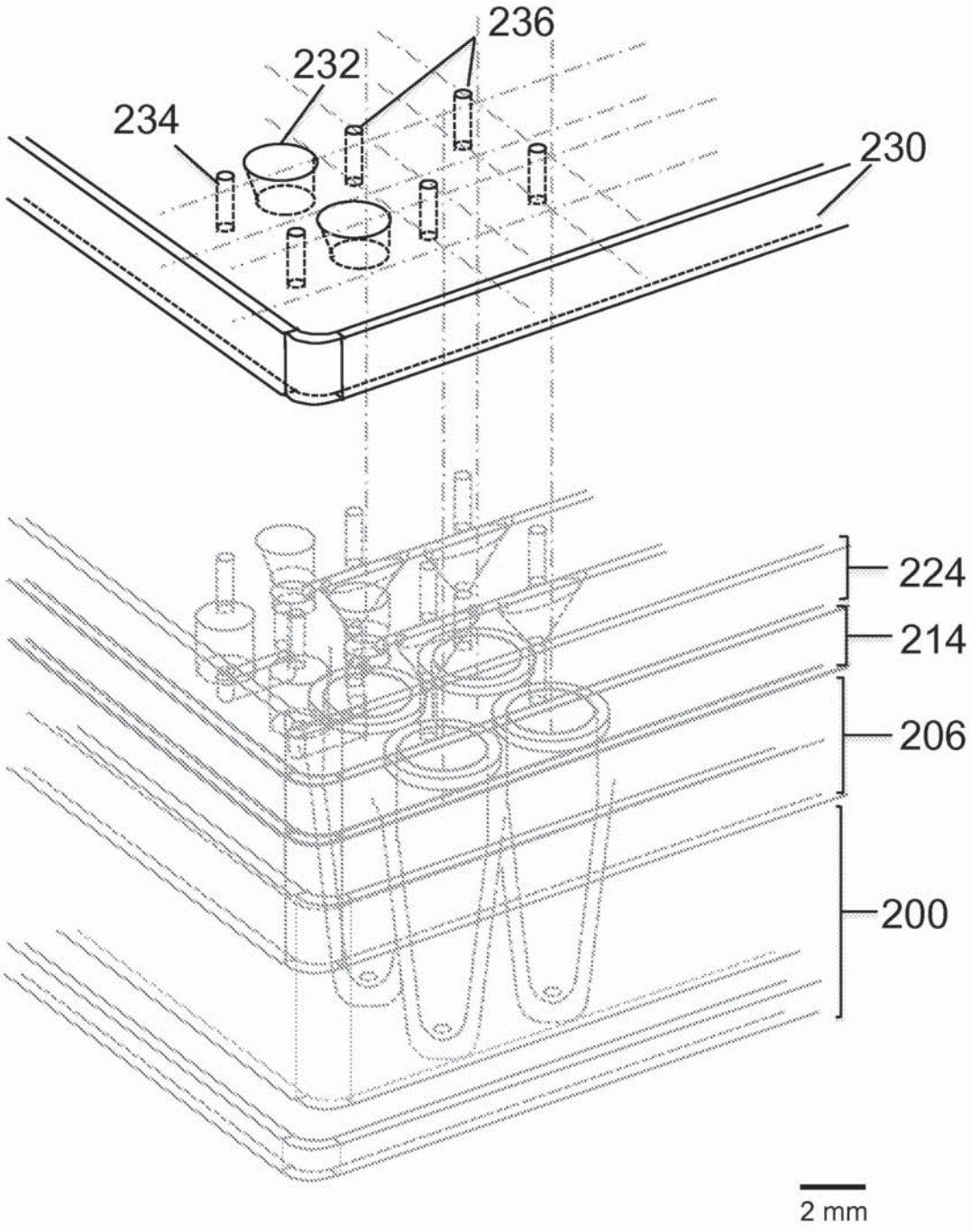
Figure 117



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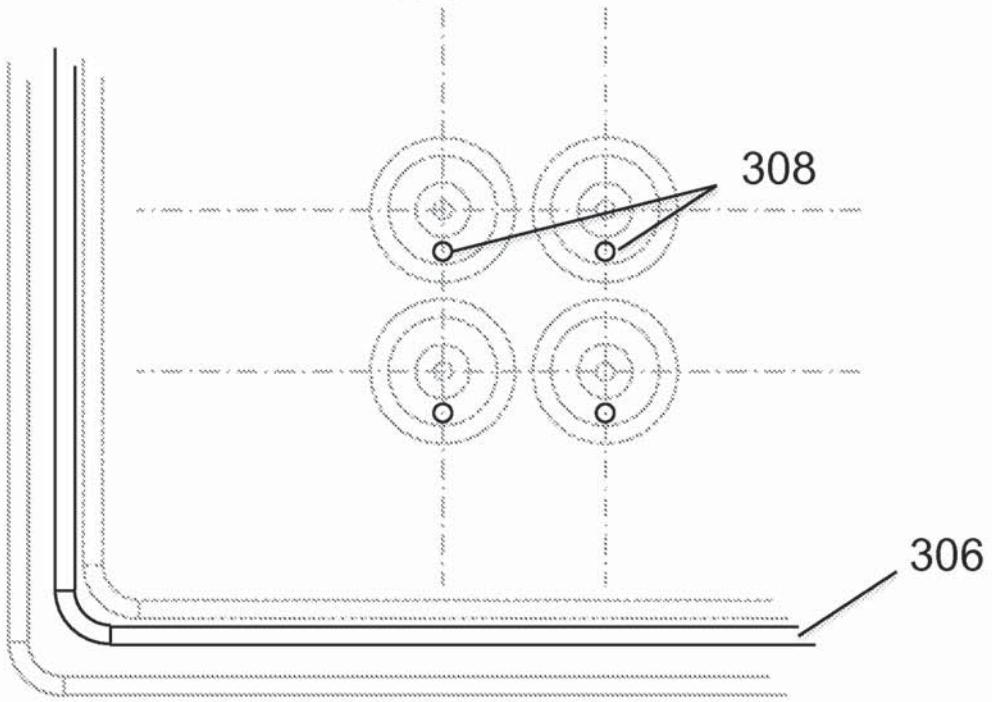

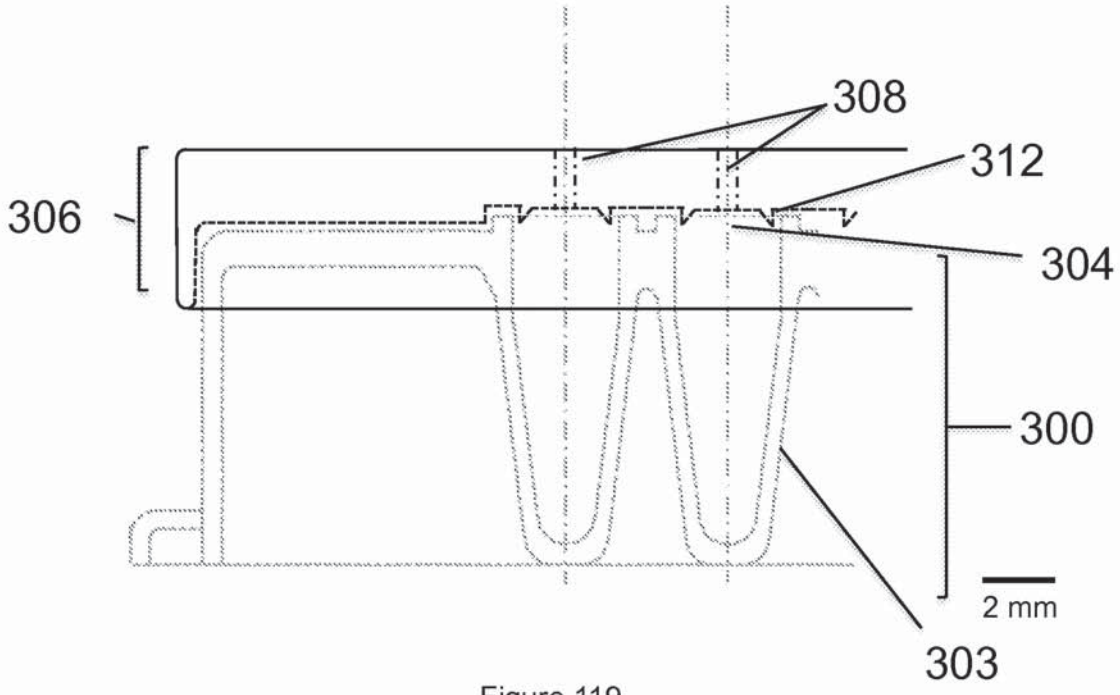

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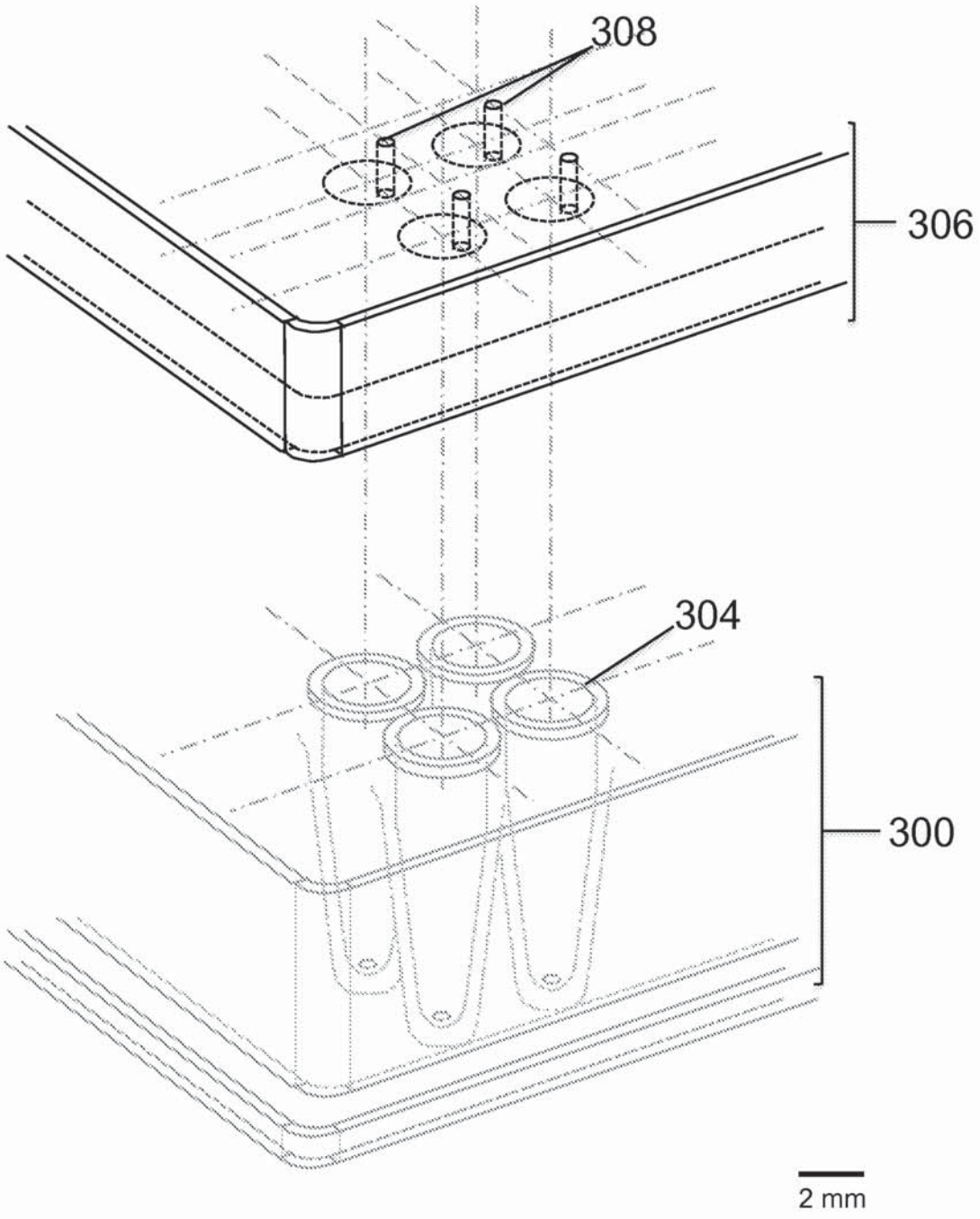

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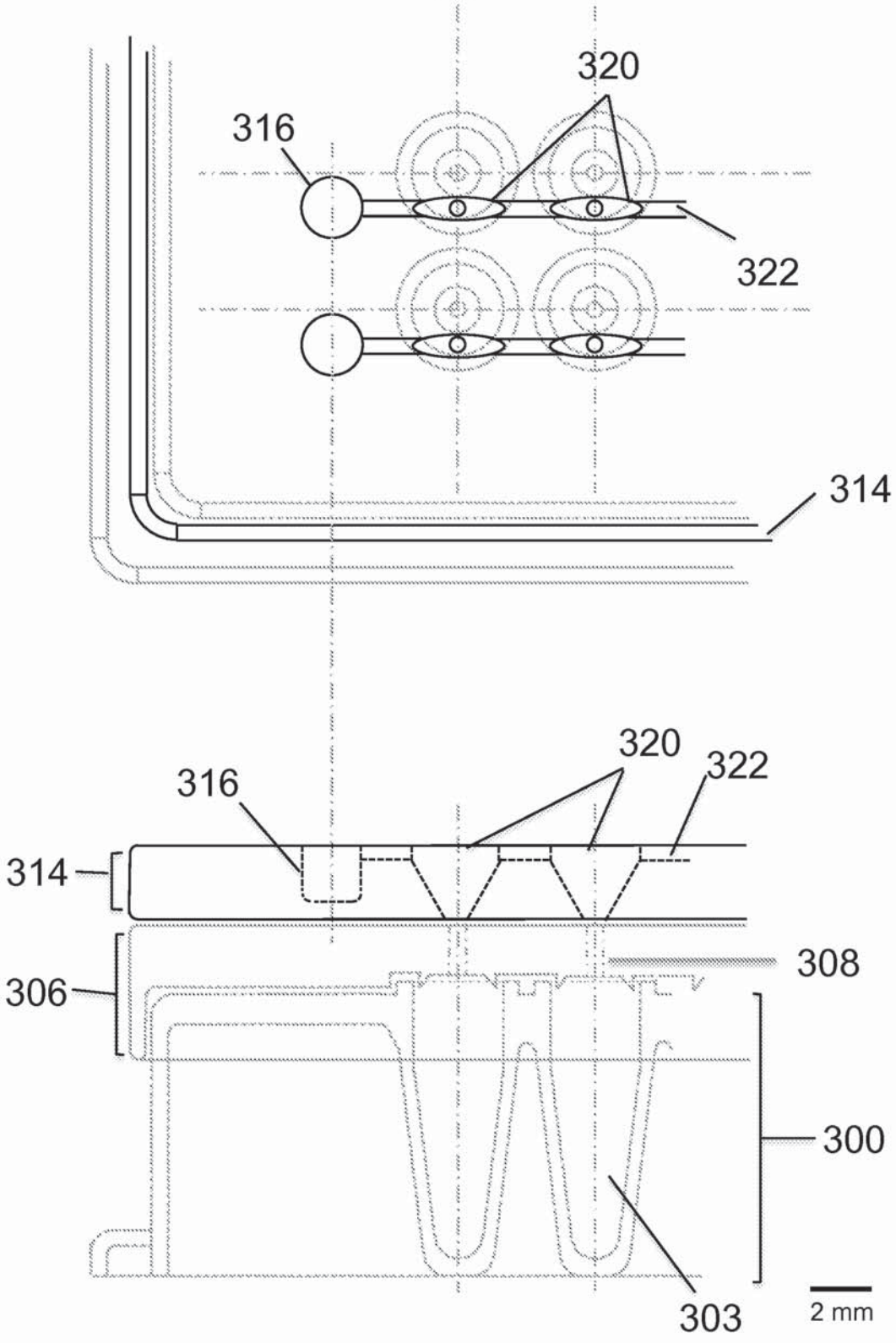

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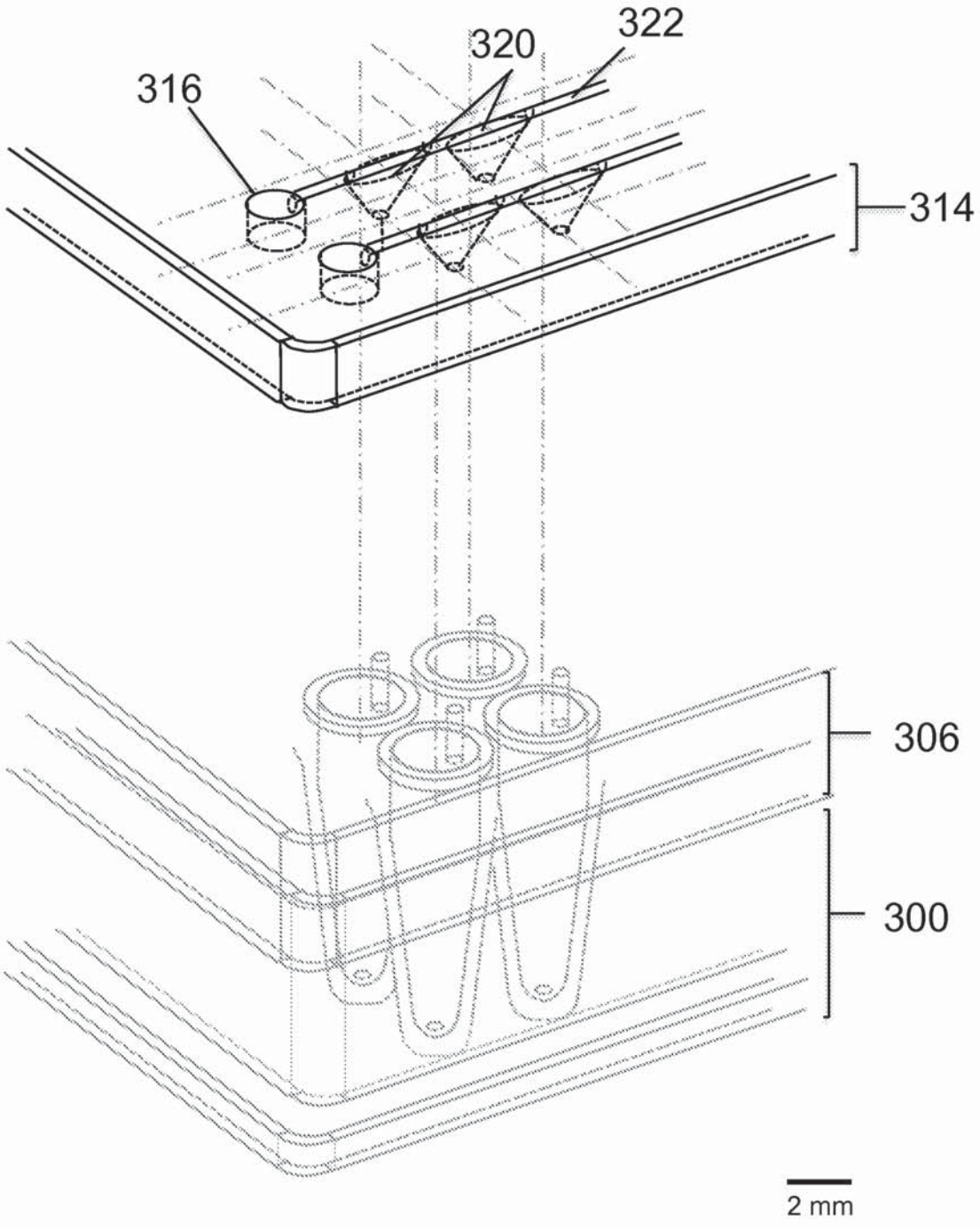

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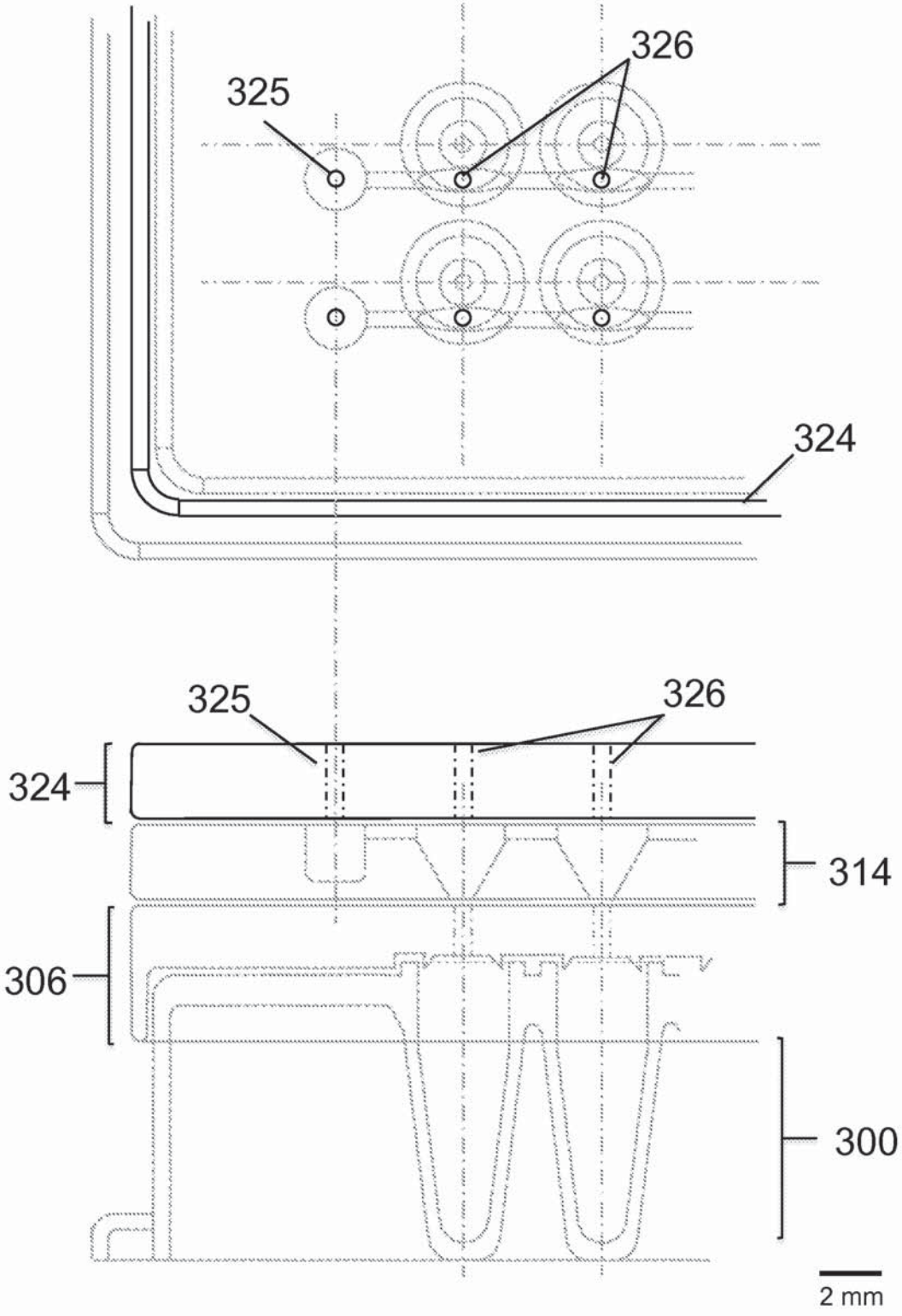

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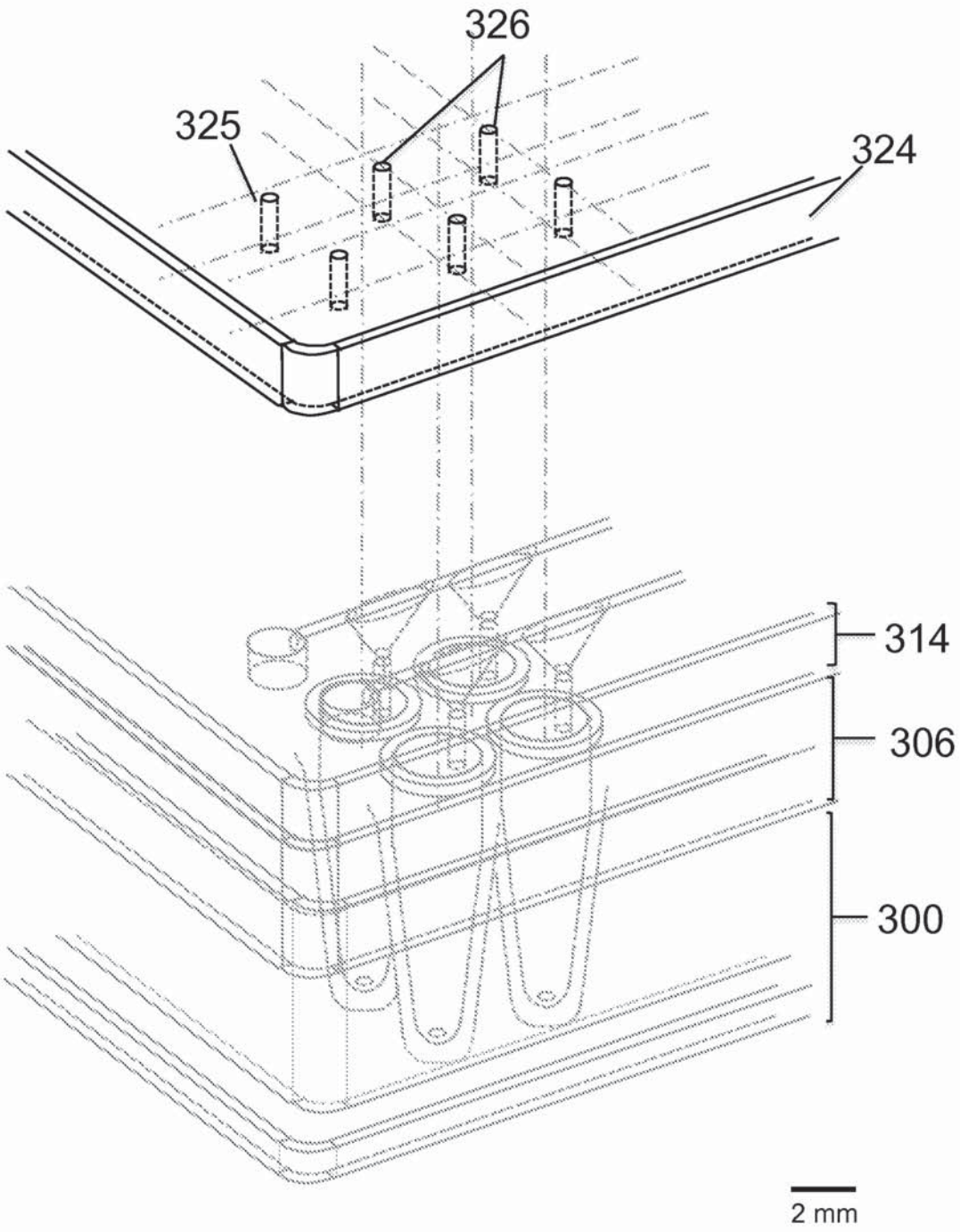

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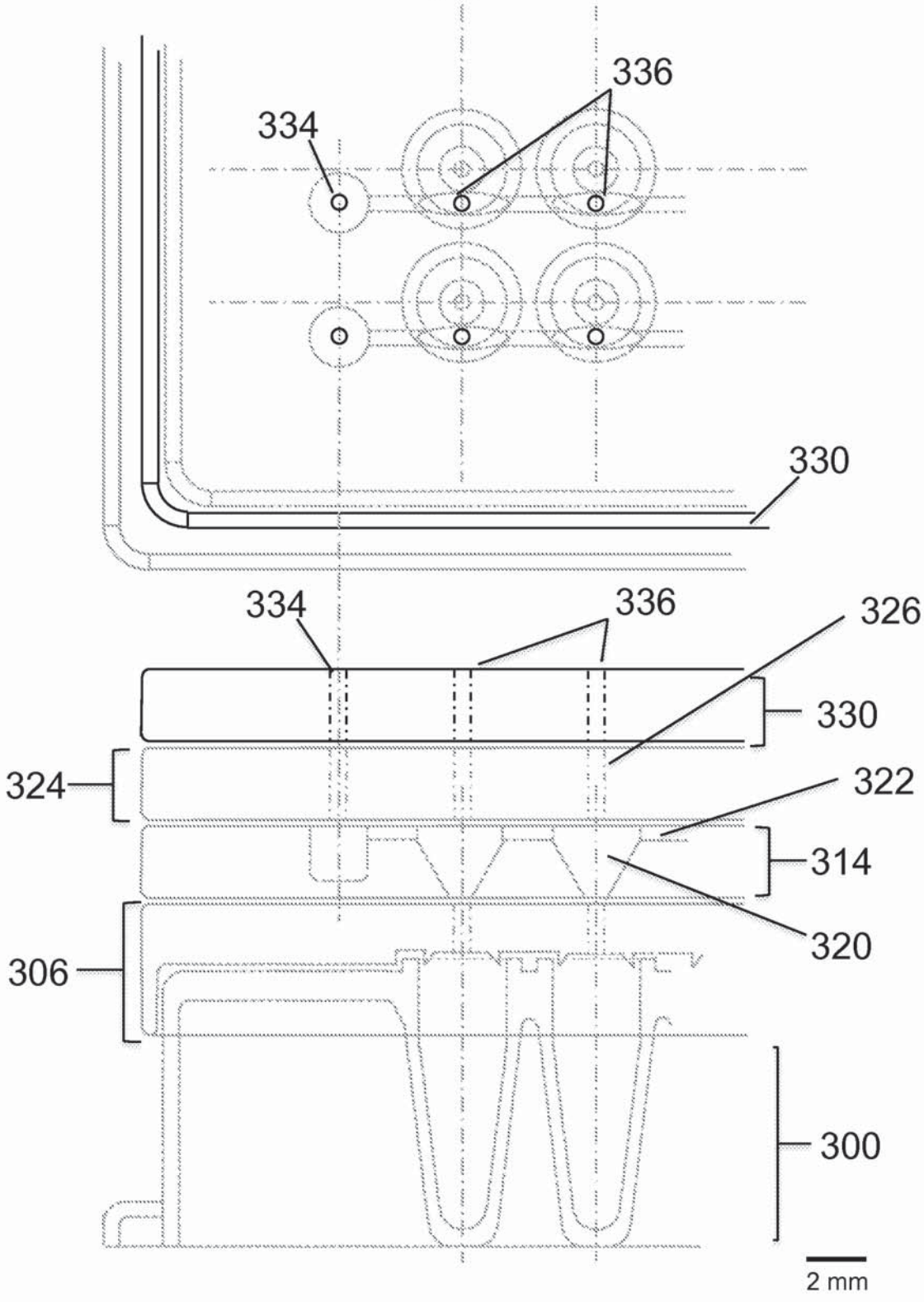

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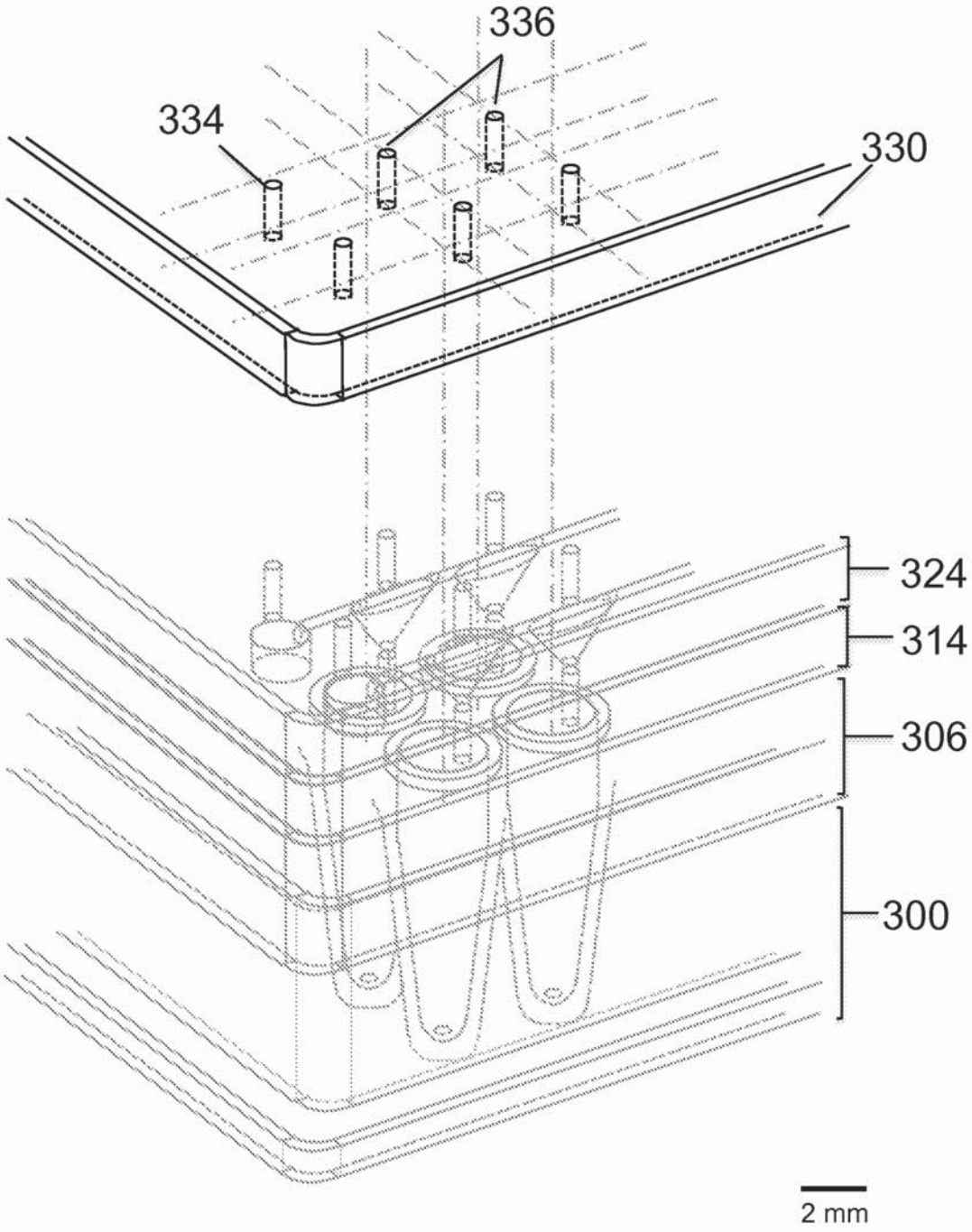

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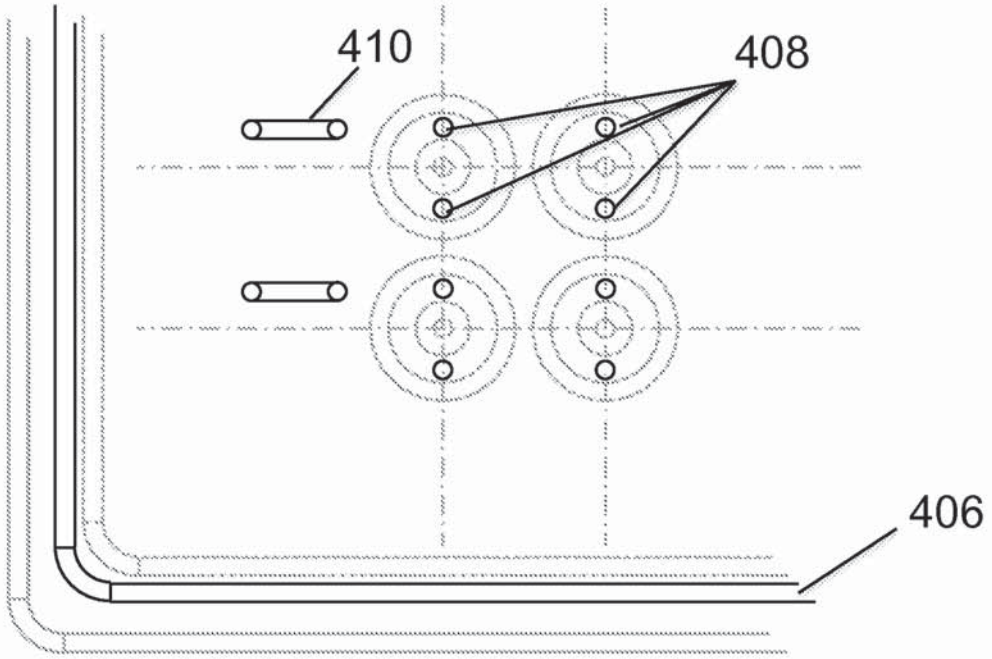

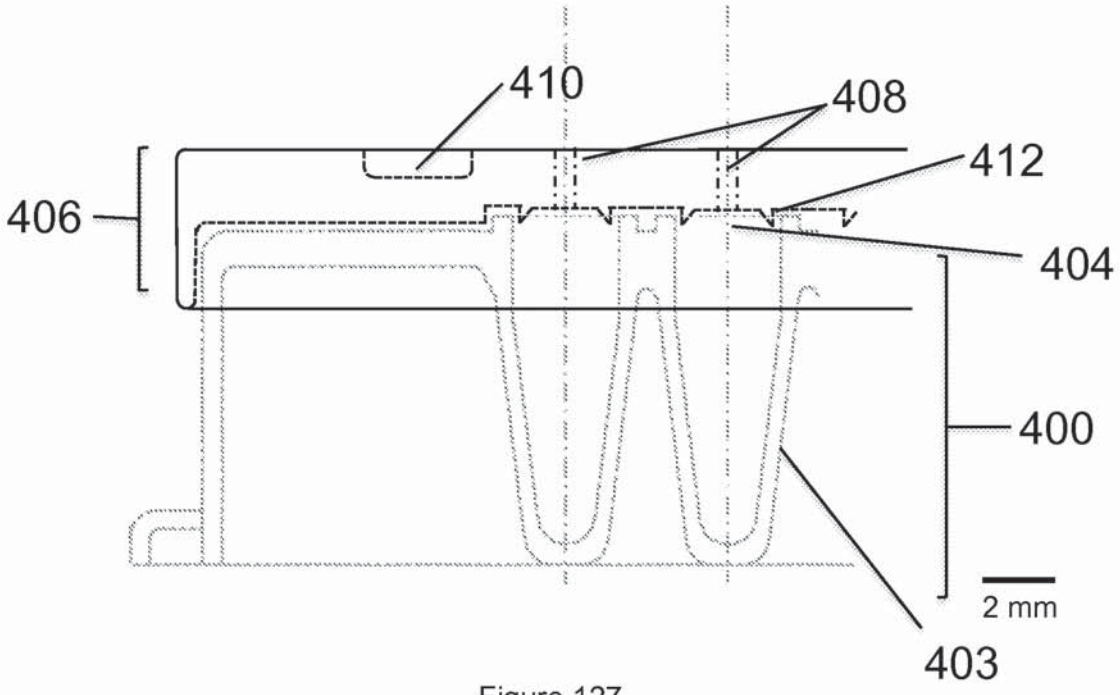

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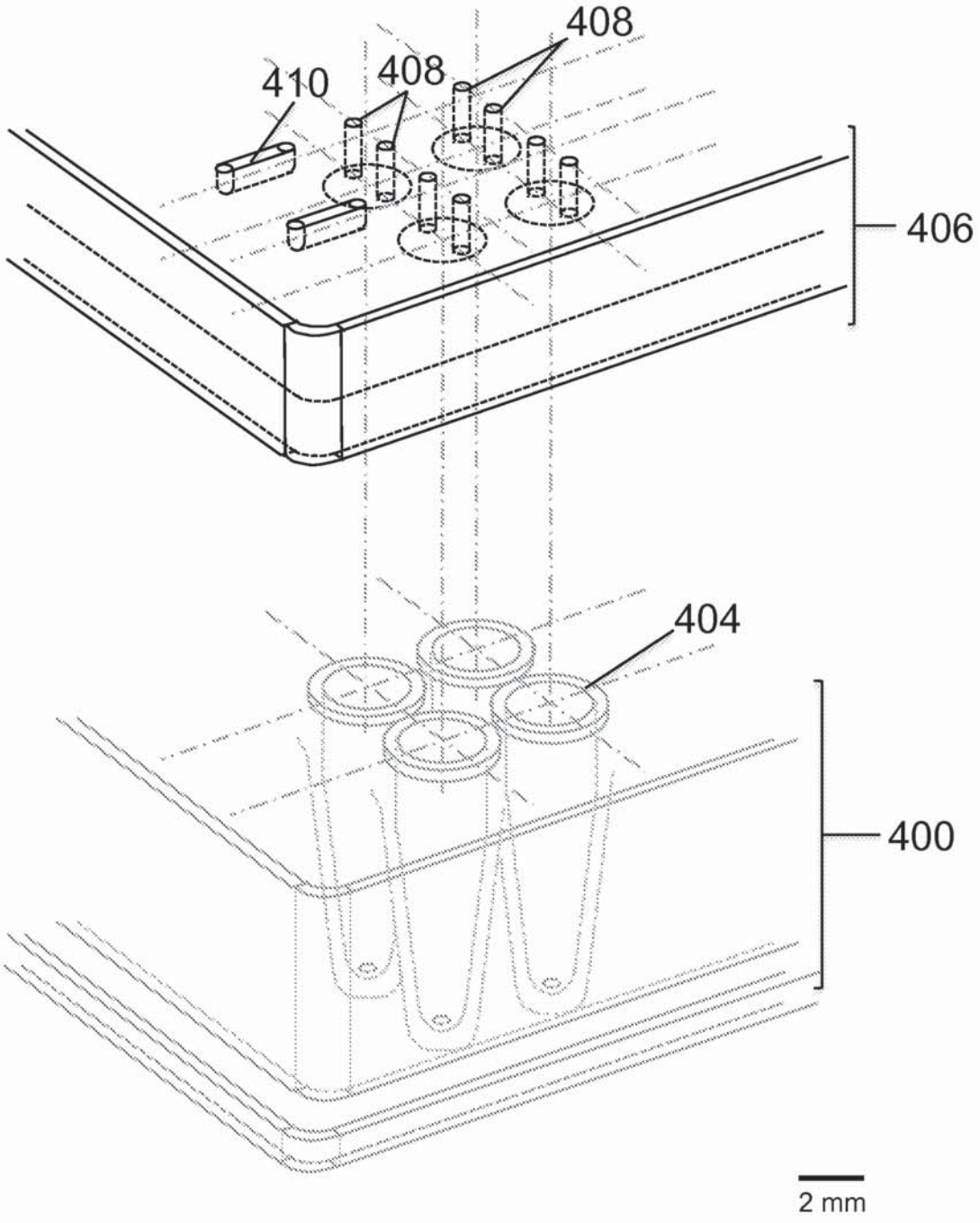

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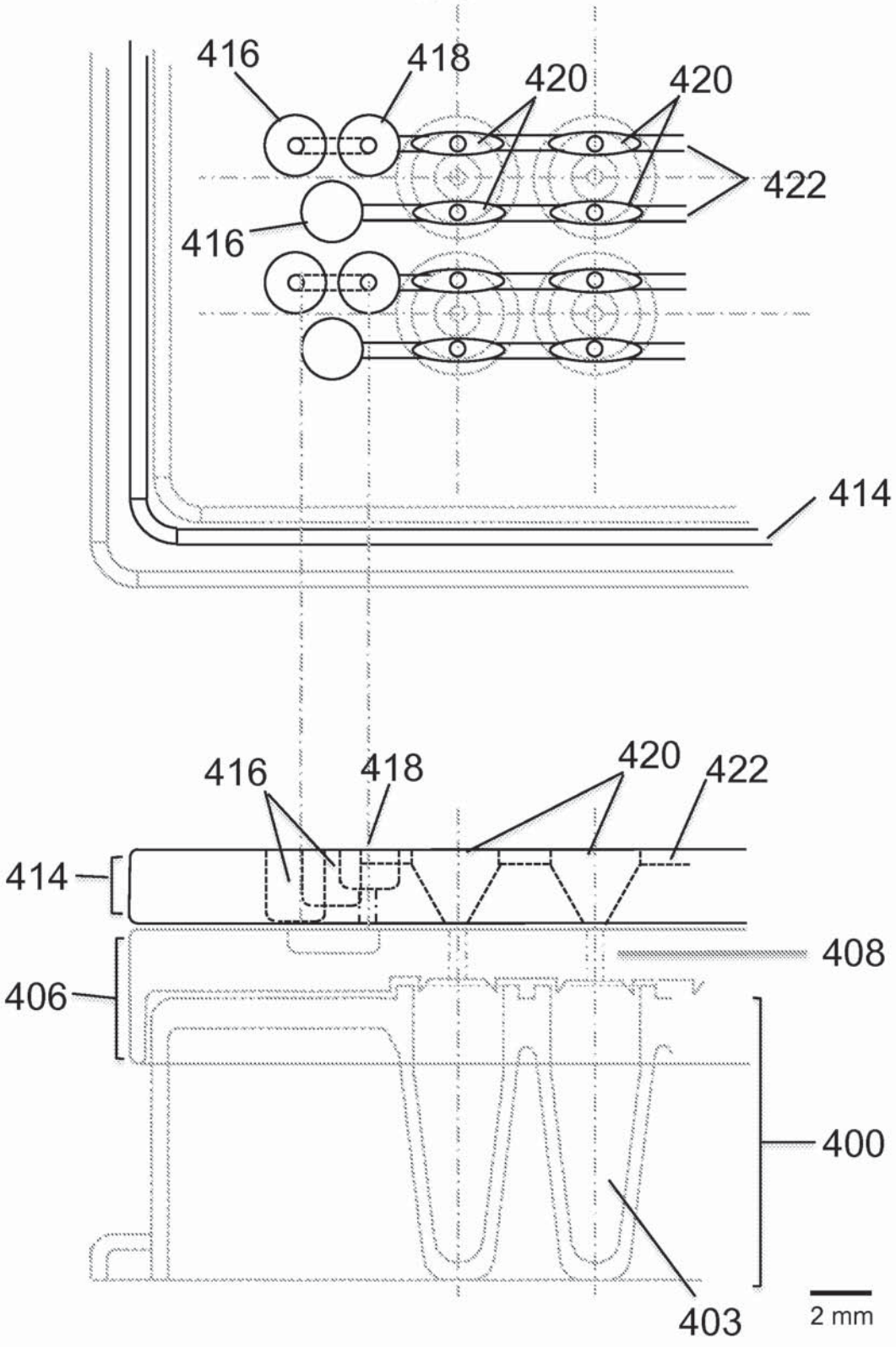
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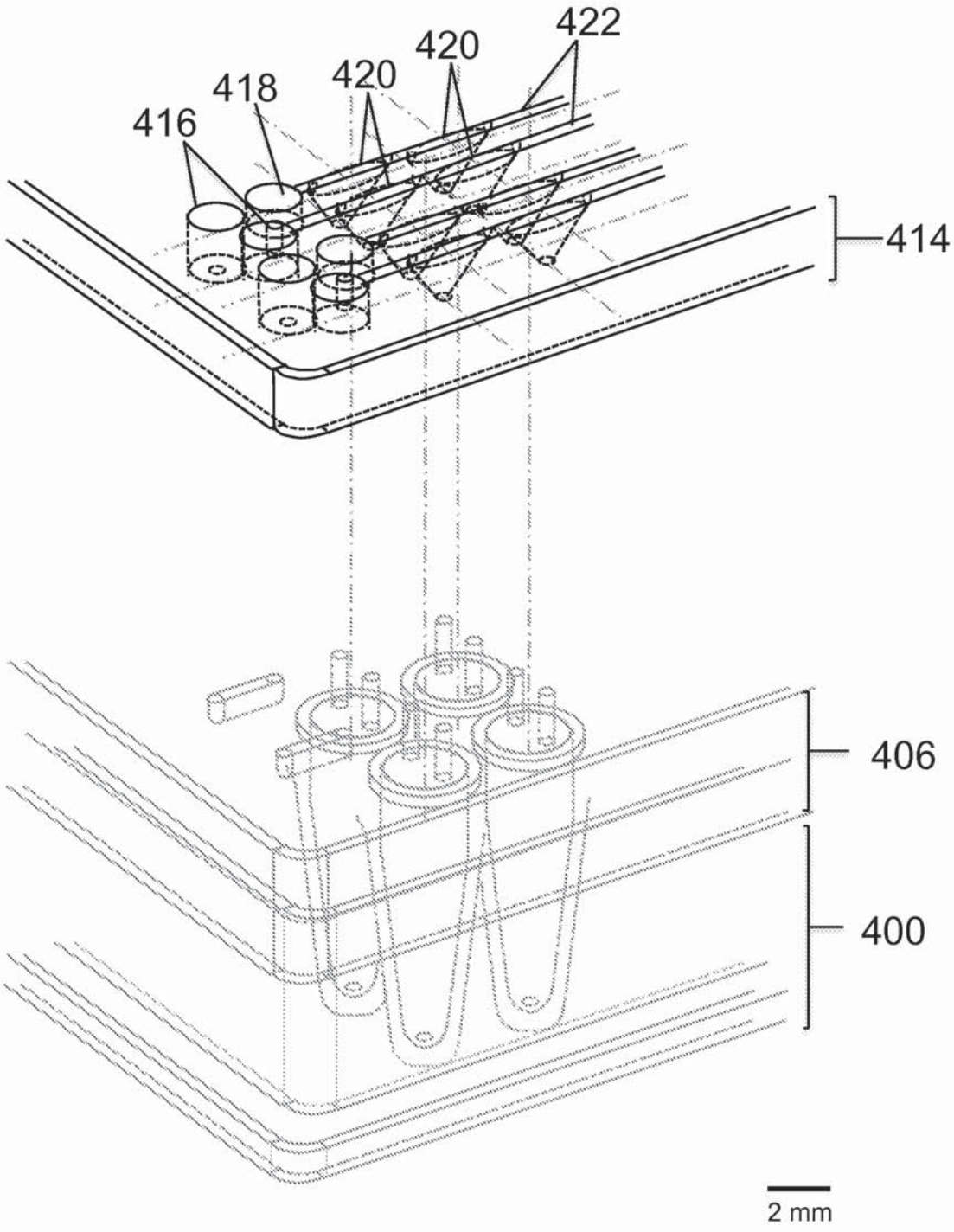

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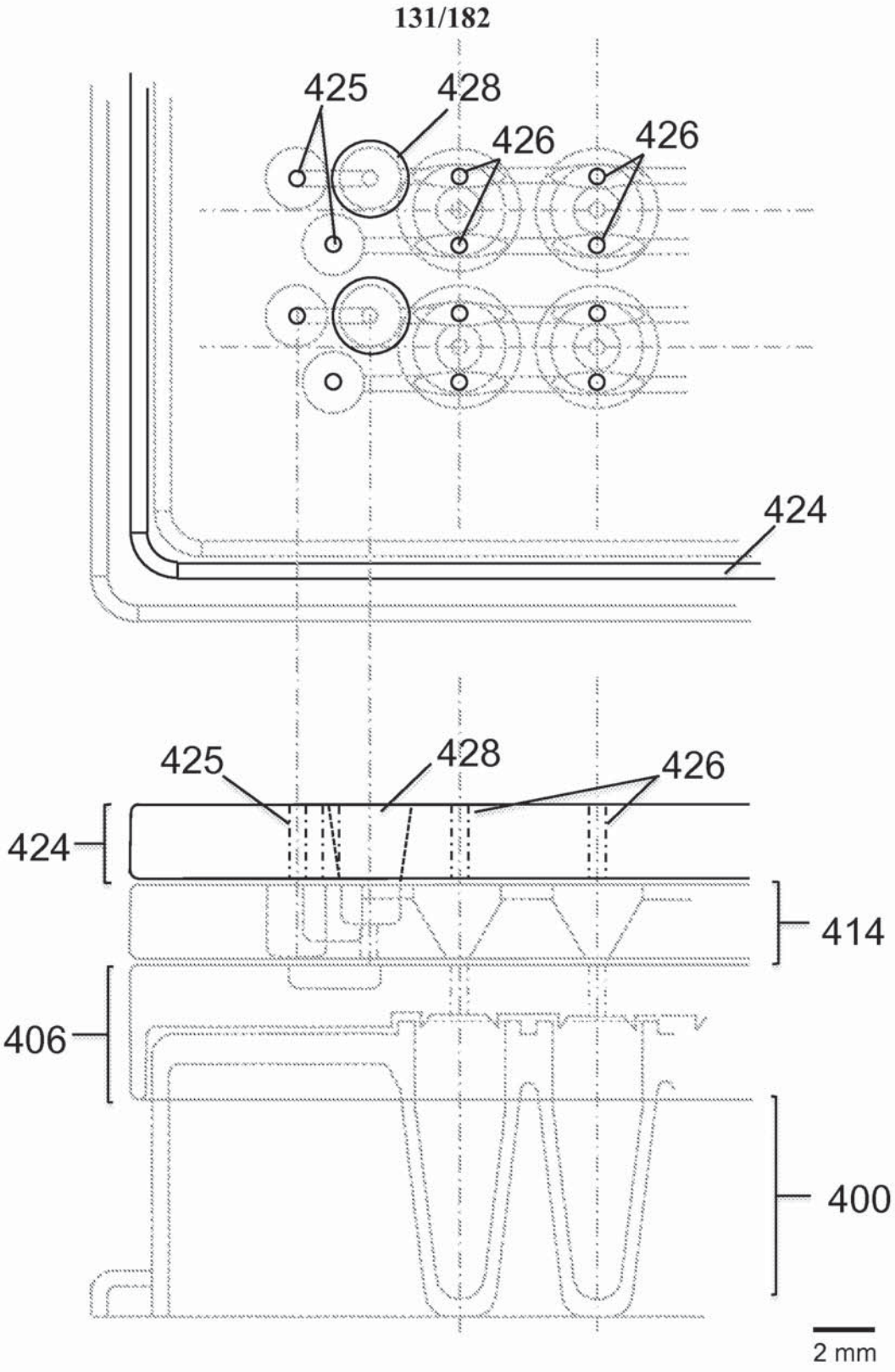

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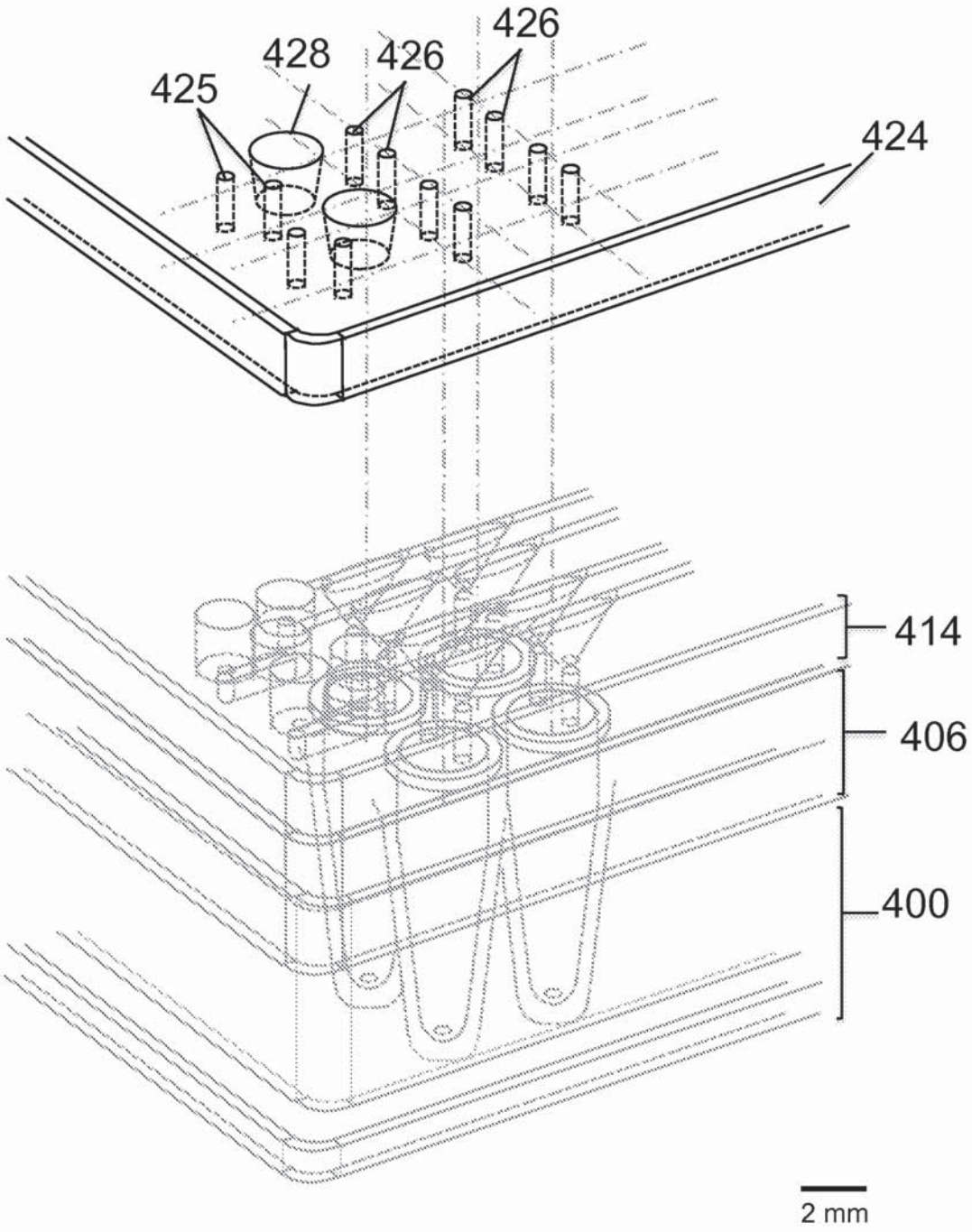

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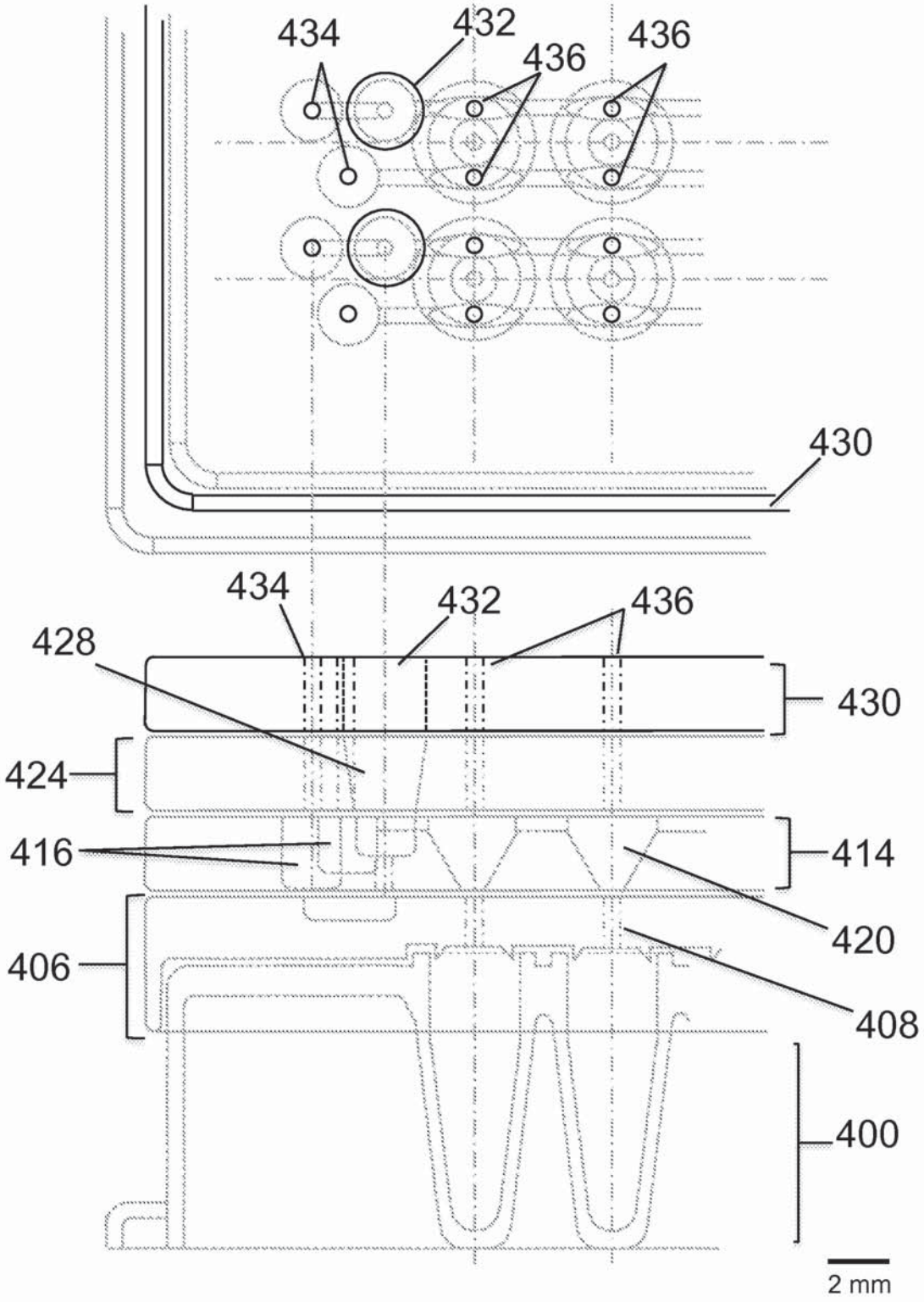
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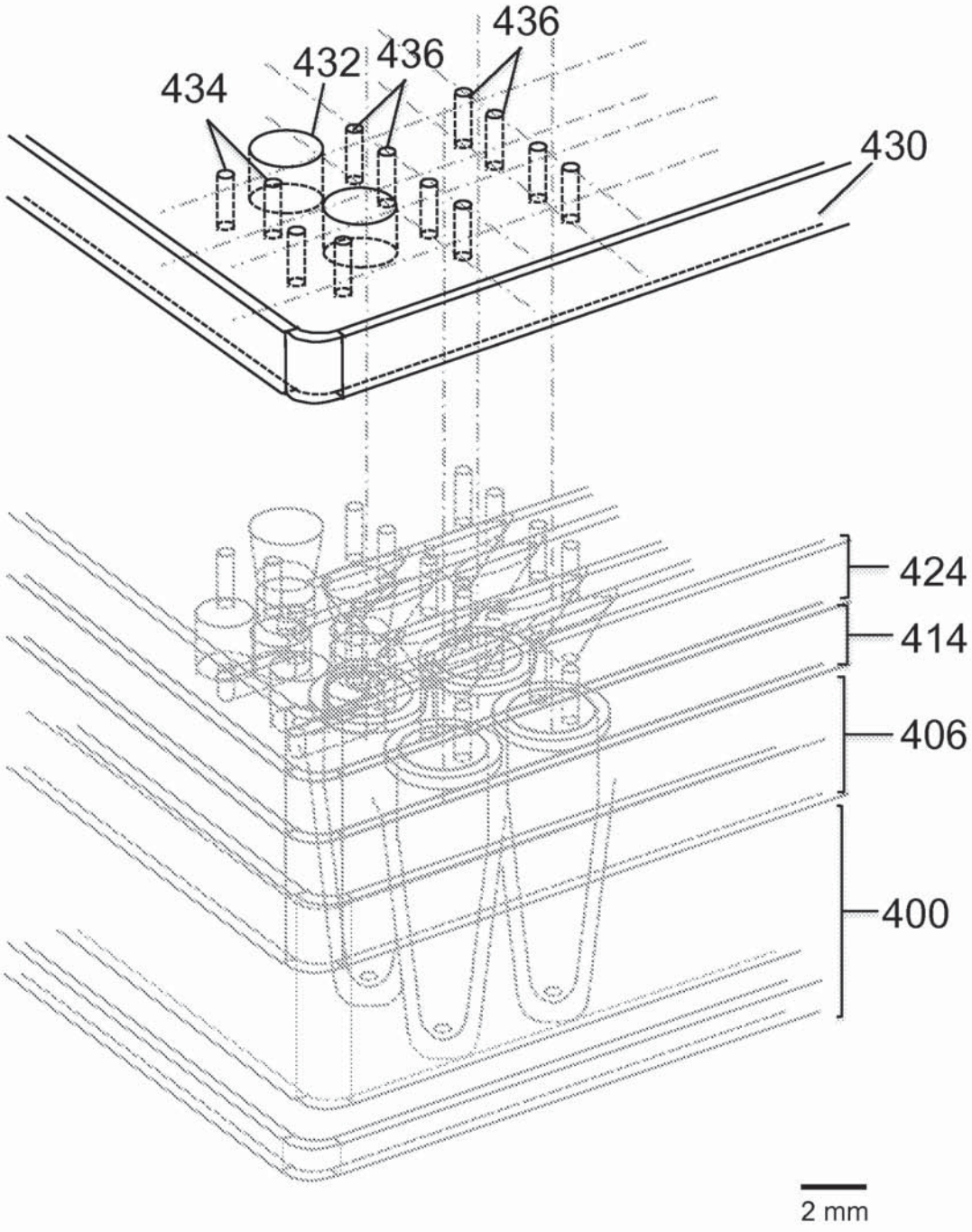
Figure 133



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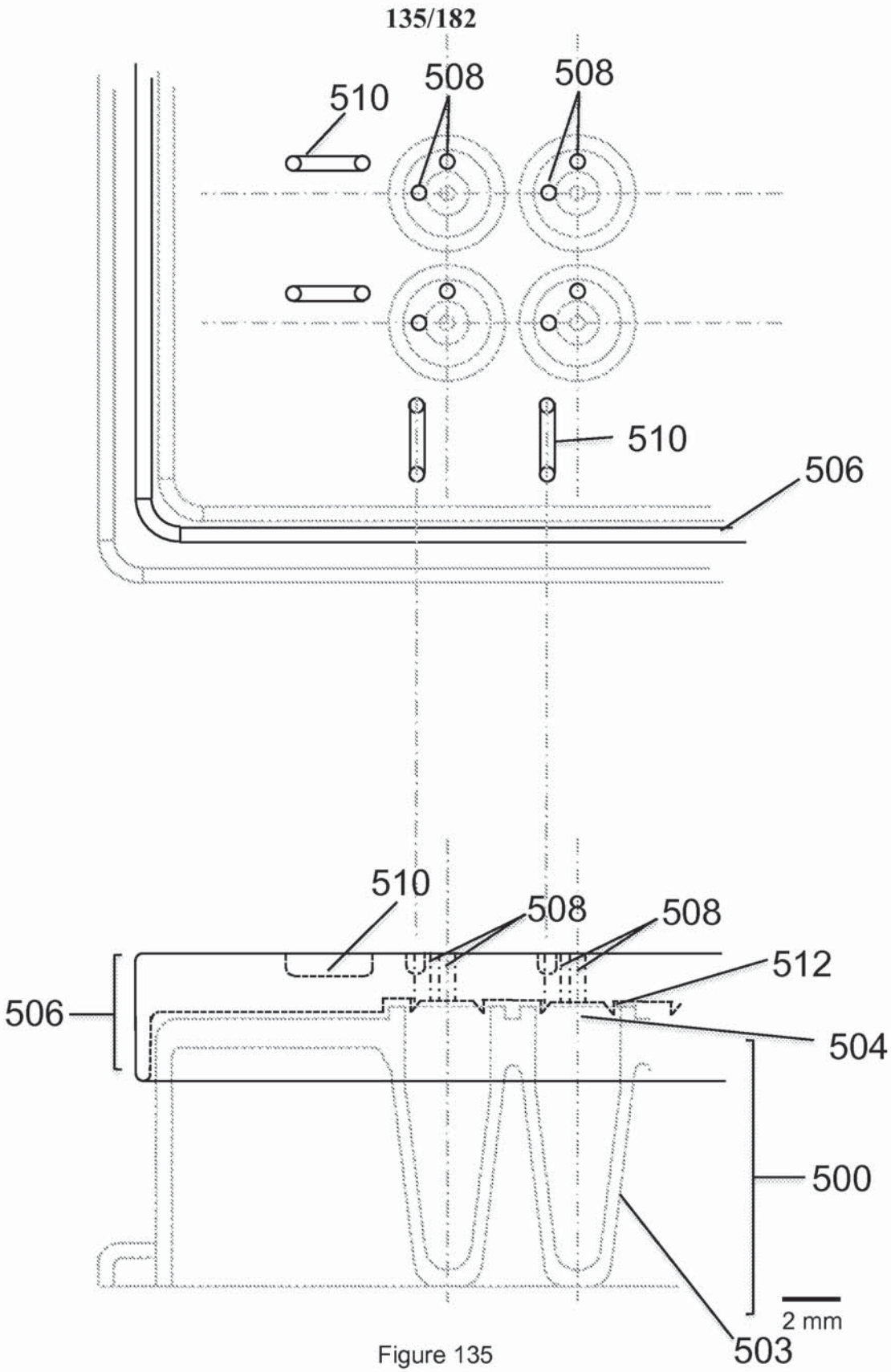

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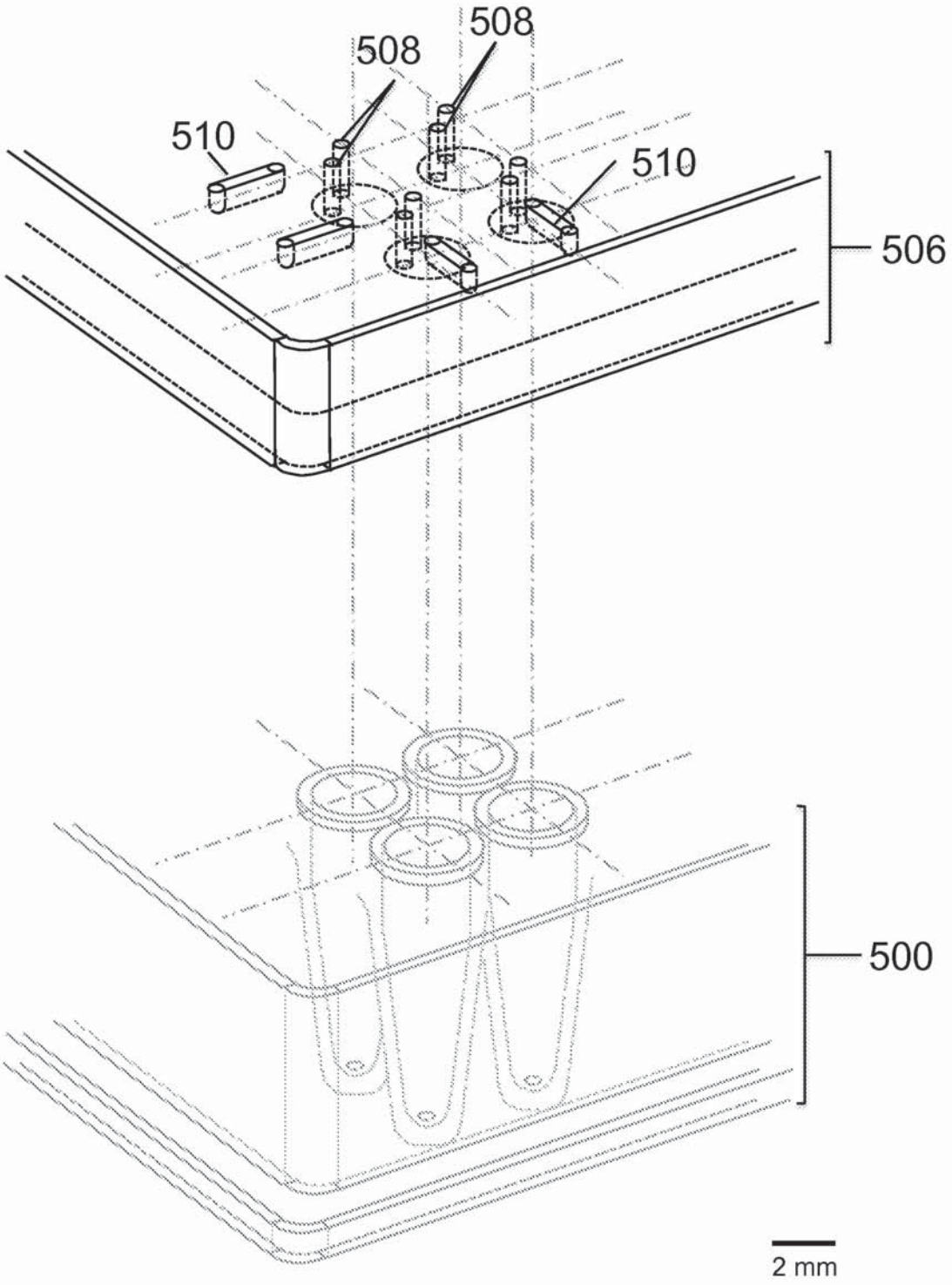

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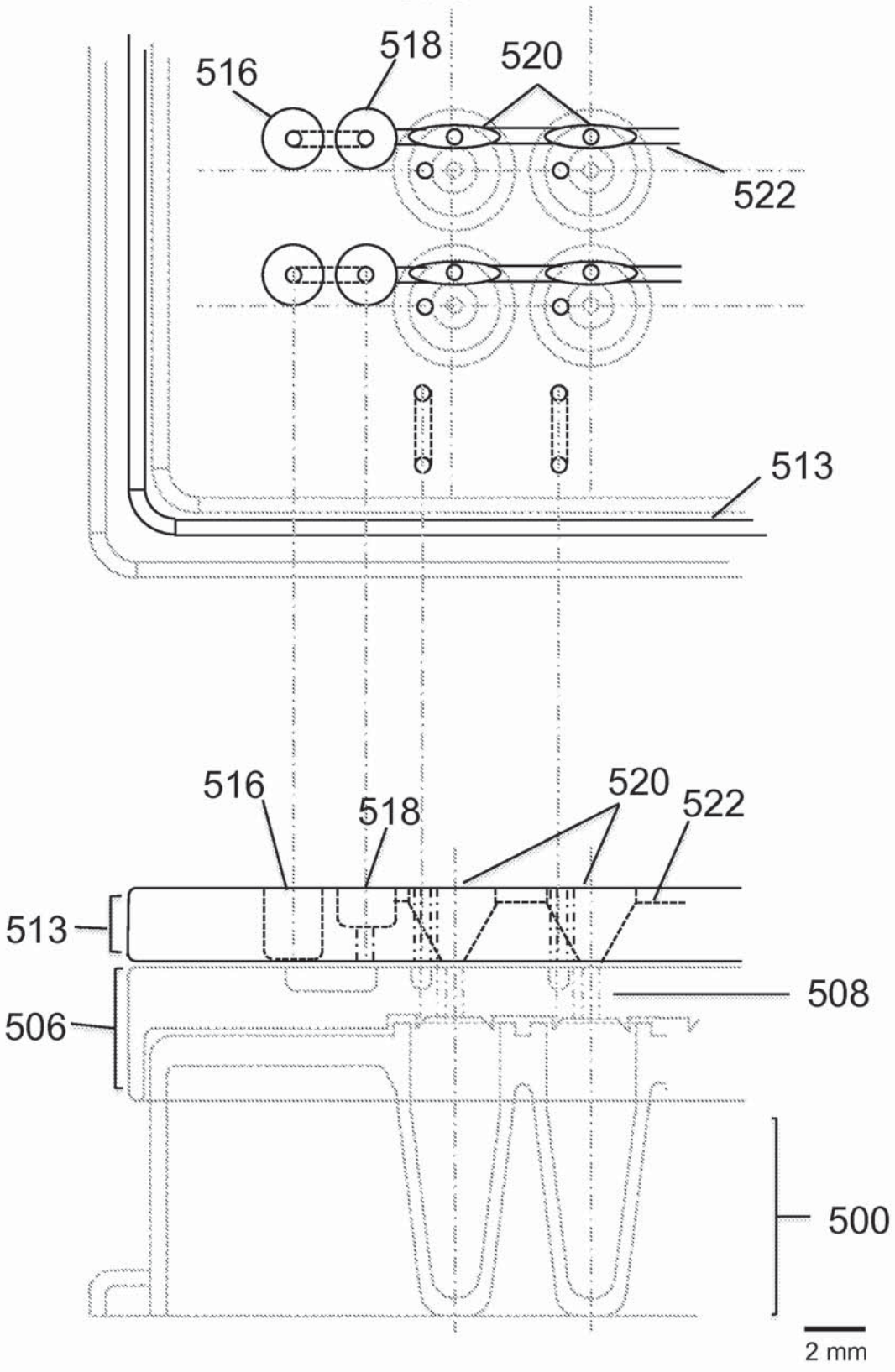

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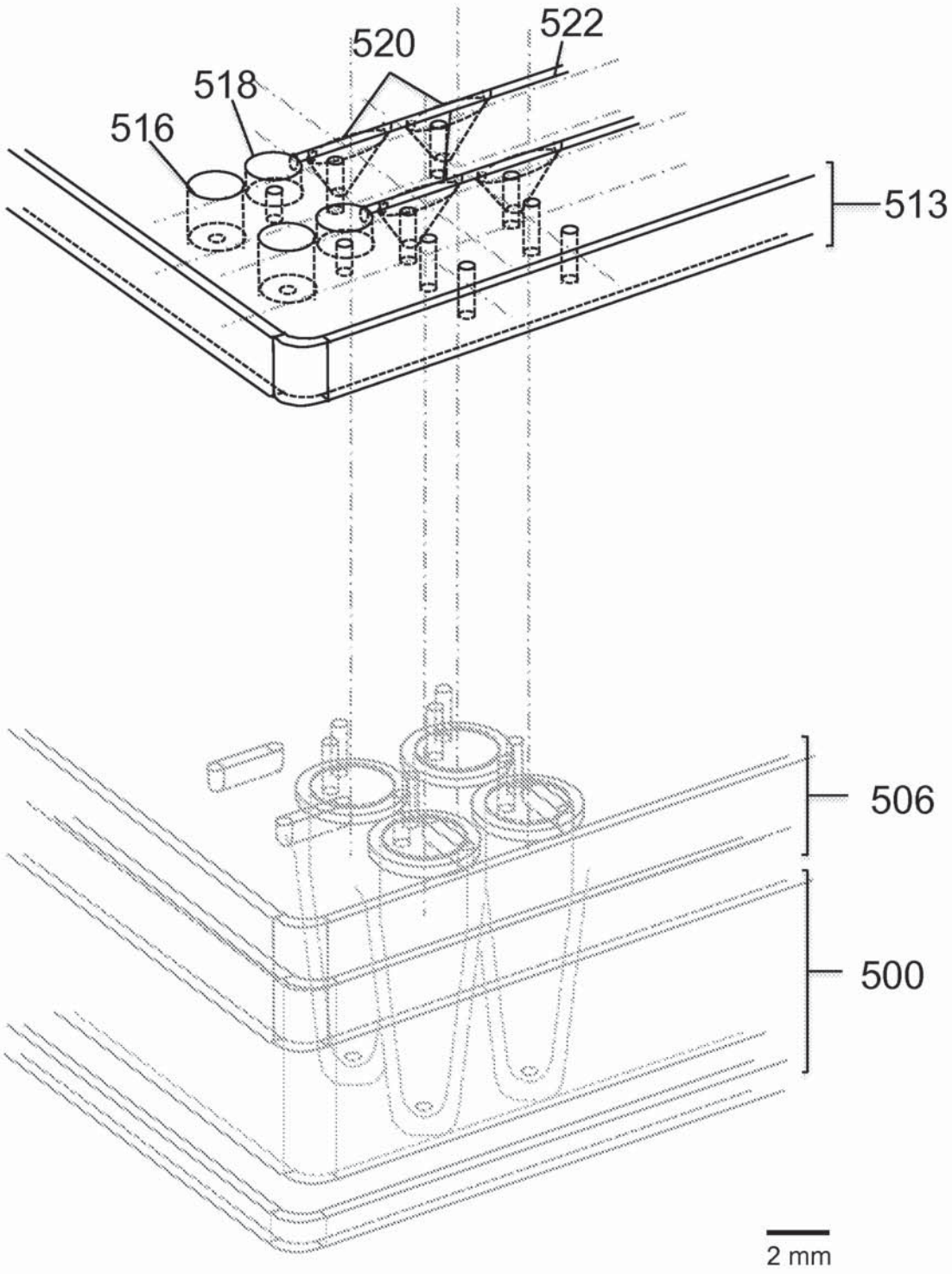

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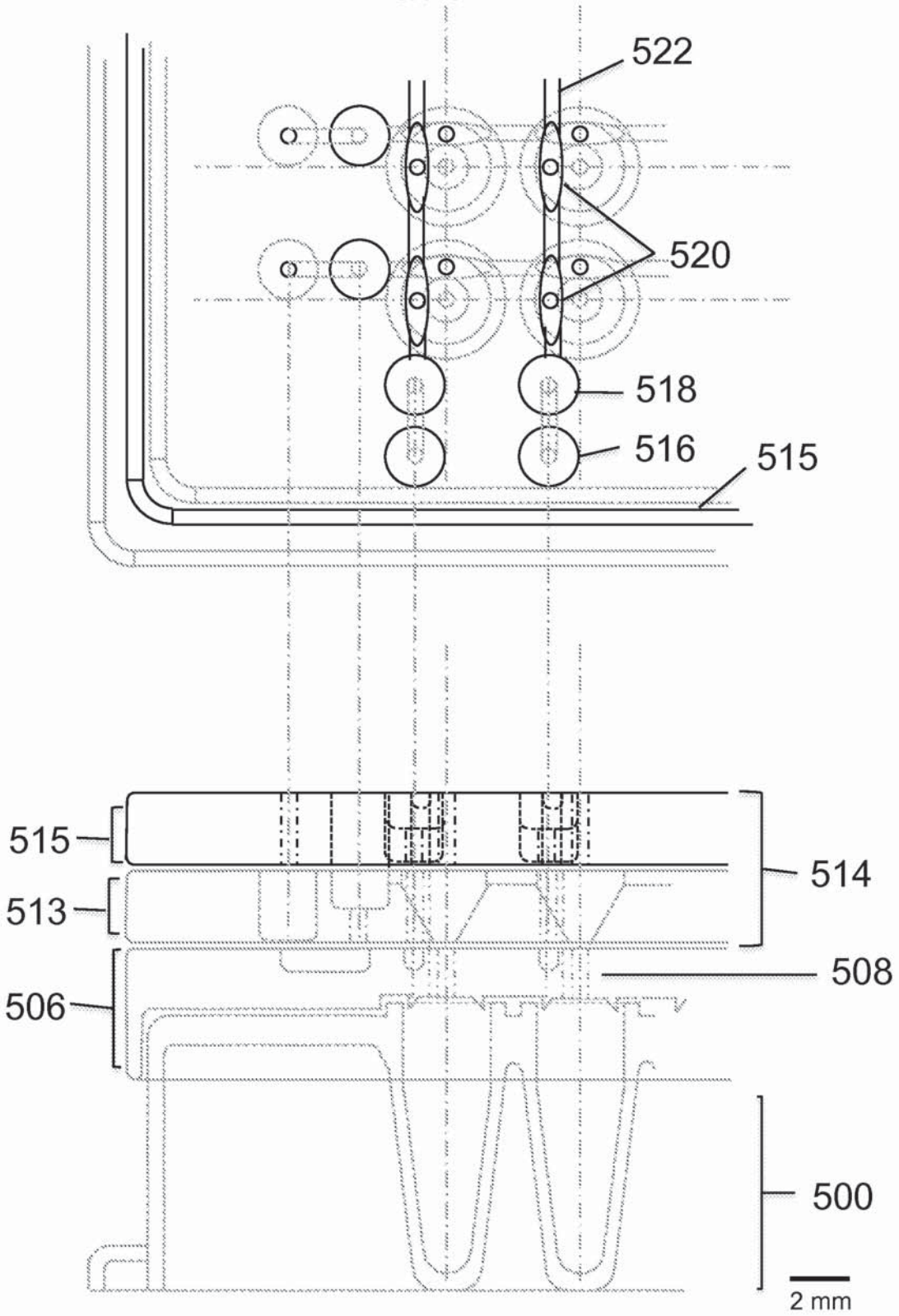

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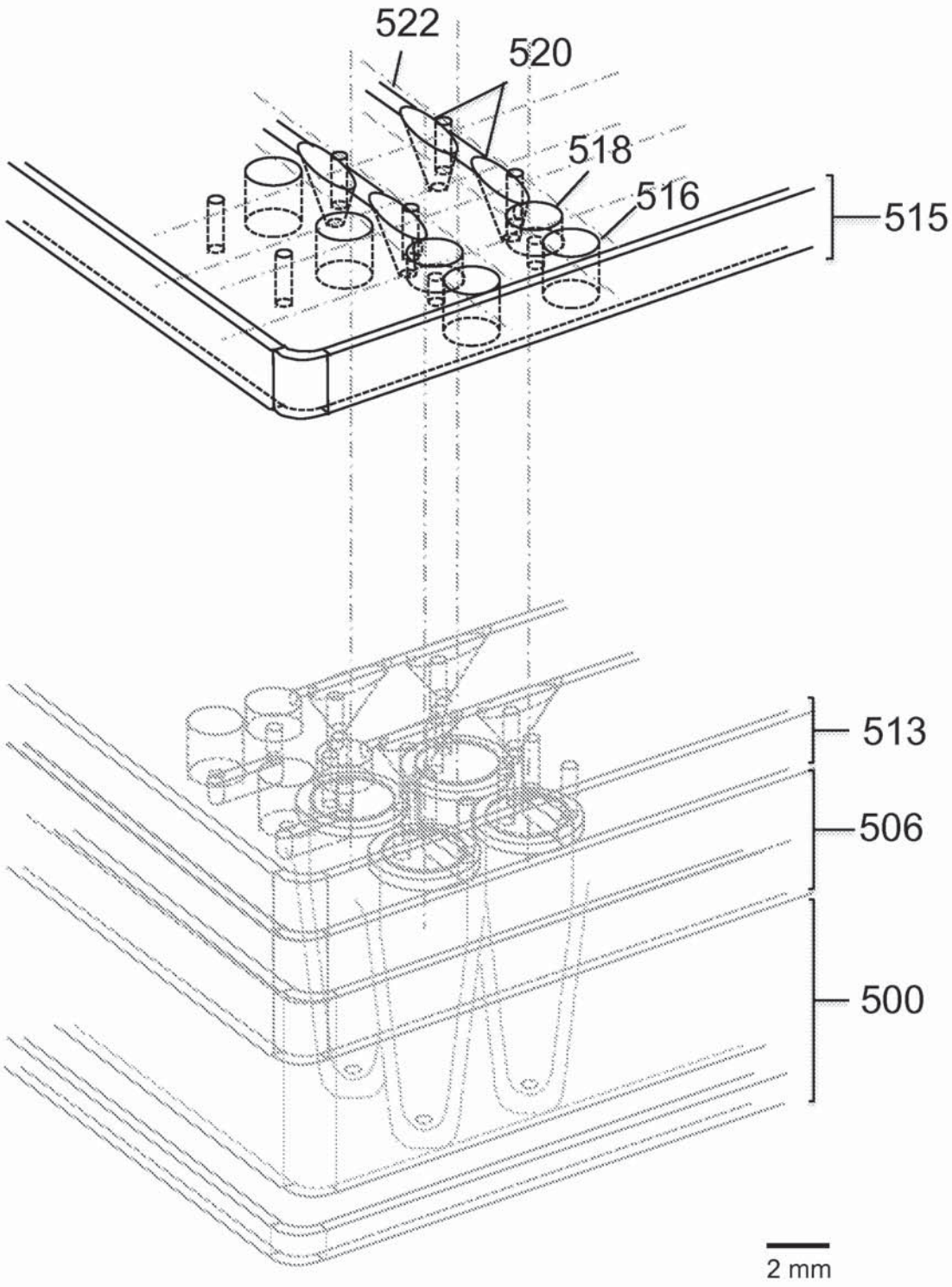

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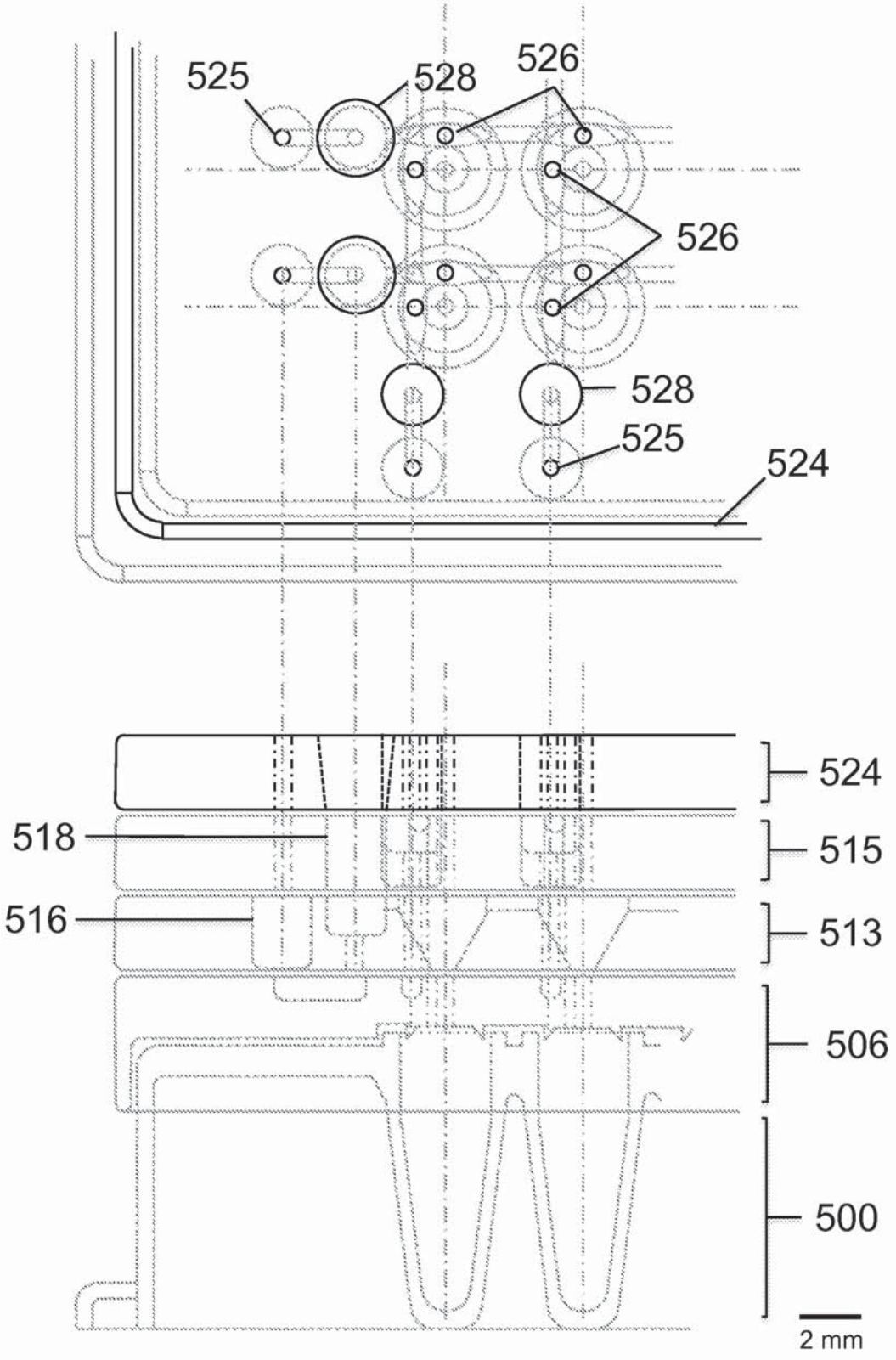

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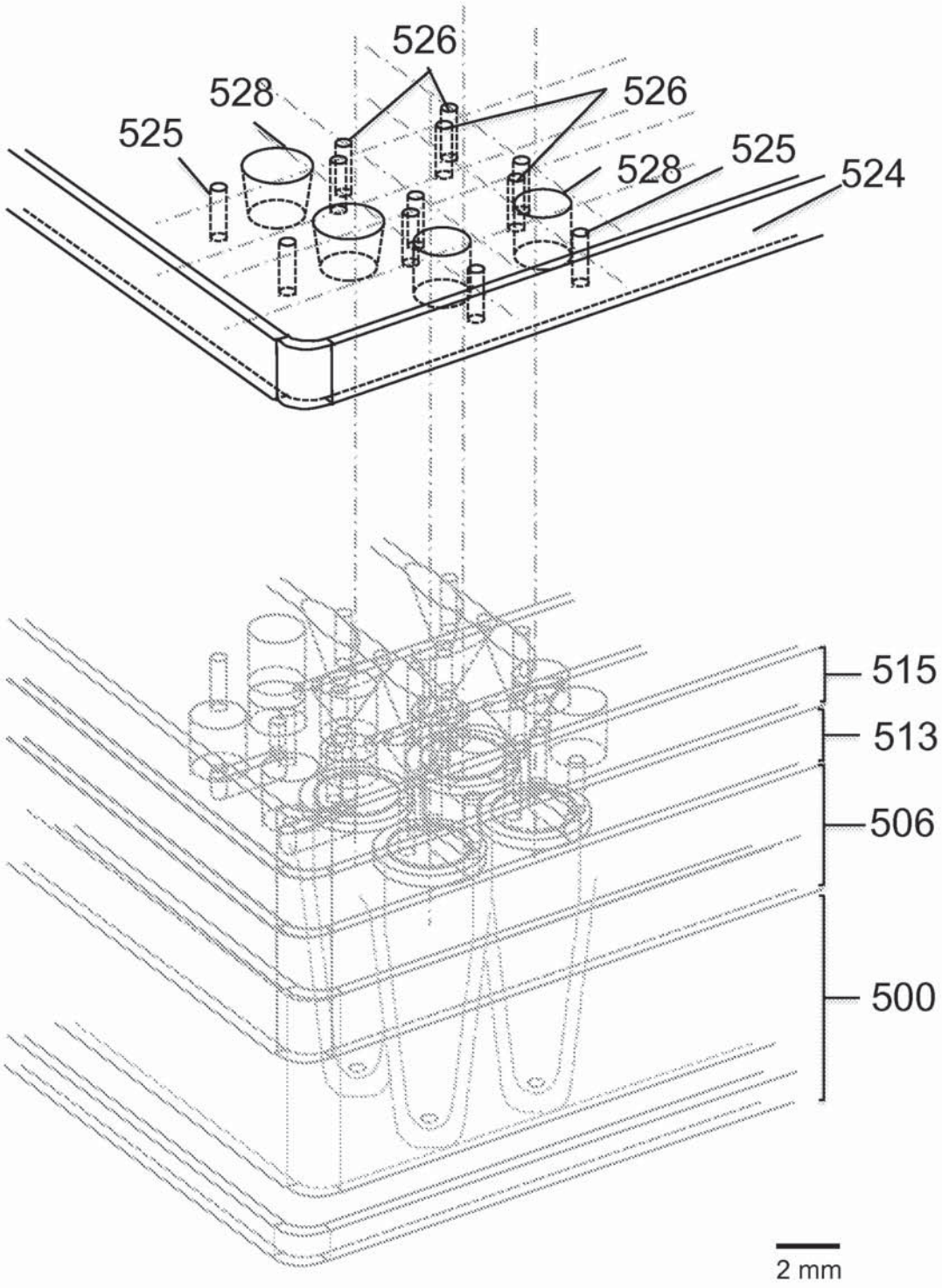

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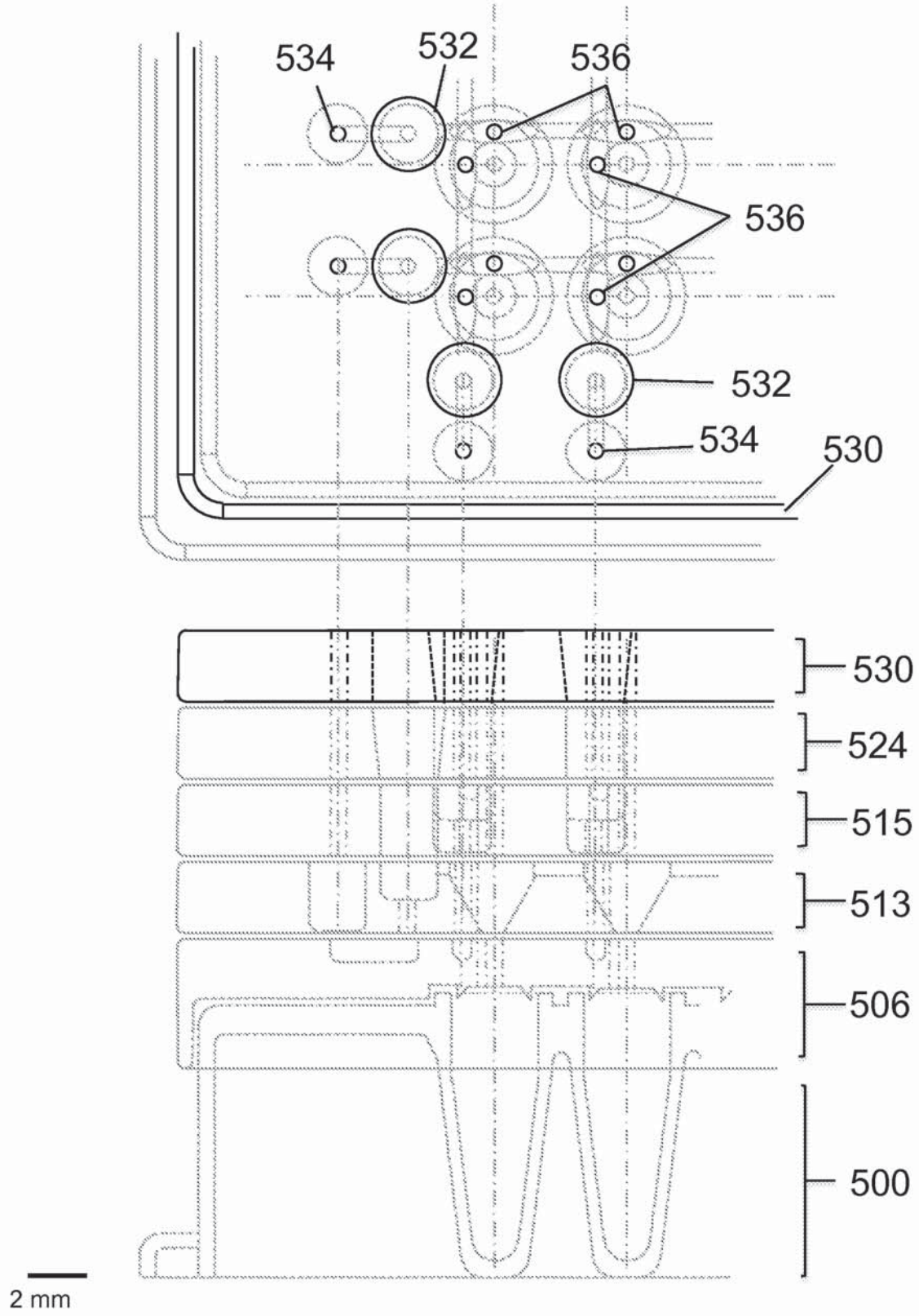

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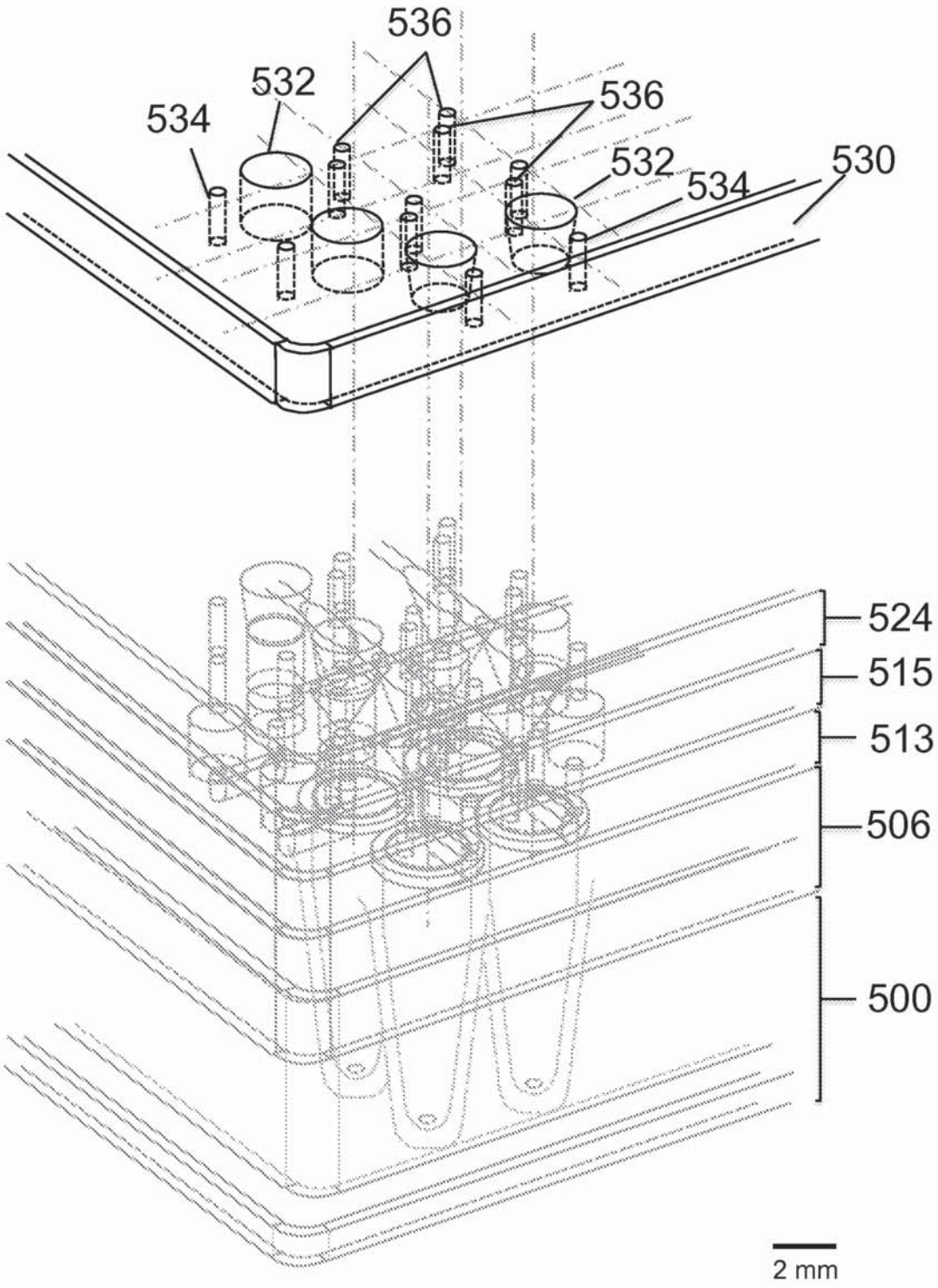


Figure 144

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention. Upstream locus primers contain mutation-specific ribose bases (mr), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Primers with Wt sequence remain blocked. Preferentially amplify mutation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Ci') for subsequent PCR amplification, while wild-type probe (Wt) does not. 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. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

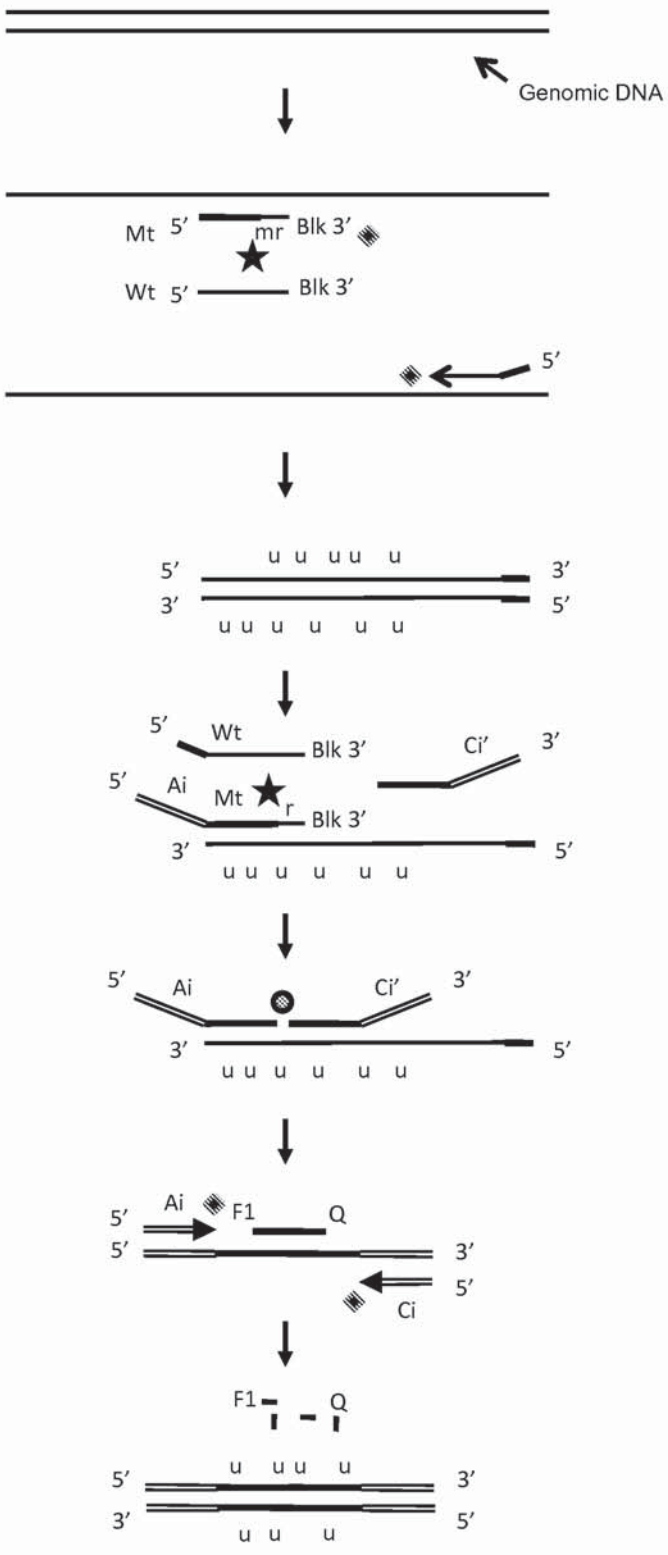


Figure 145

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention. Upstream locus primers contain mutation-specific ribose bases (mr), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Primers with Wt sequence remain blocked. Preferentially amplify mutation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Bi', Ci') for subsequent PCR amplification, while wild-type probe (Wt) does not. 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. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

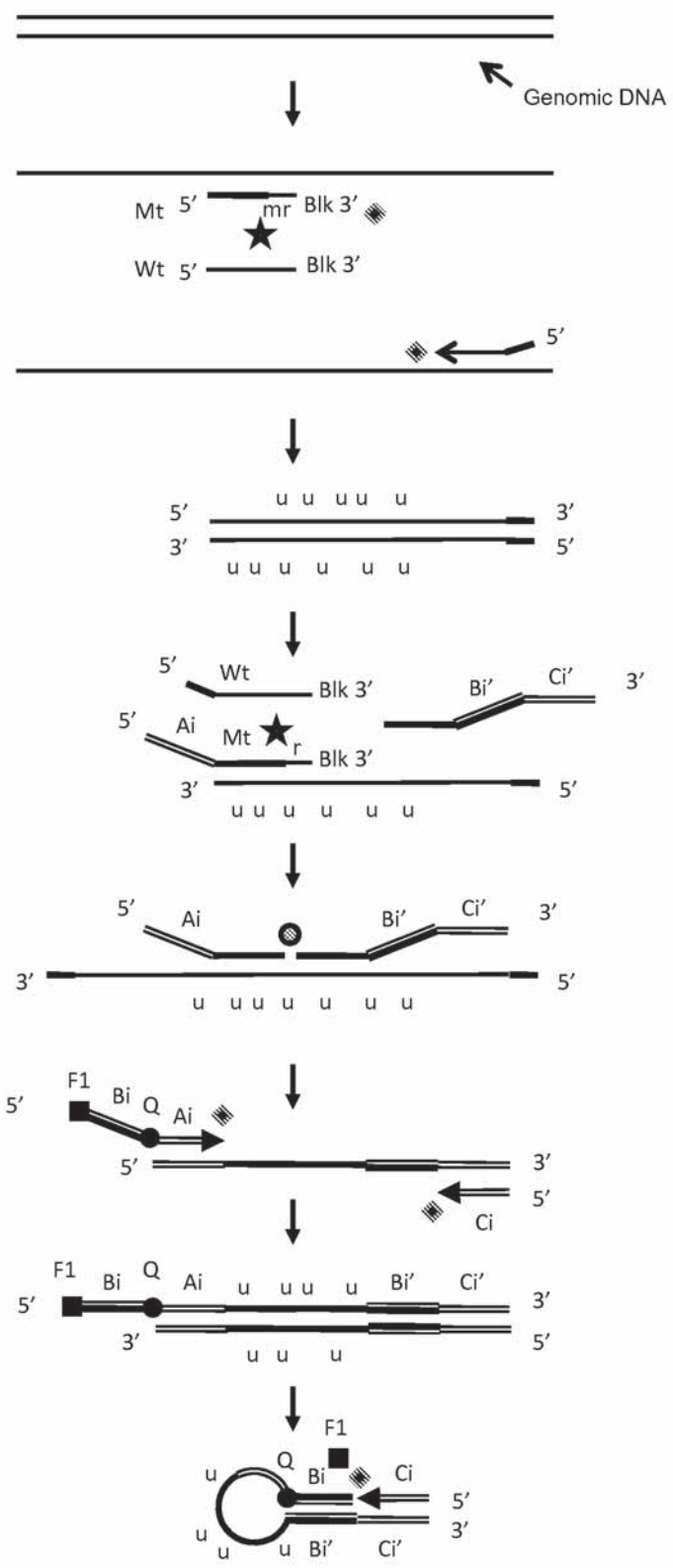


Figure 146

A. PCR-qLDR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention. Upstream locus primers contain mutation-specific ribose bases (mr), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Primers with Wt sequence remain blocked. Preferentially amplify mutation containing regions using PCR (and dUTP). Downstream primers contain universal tails to enable universal primer amplification to append a 5′ biotin.

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Mutation-specific ligation oligonucleotides (Mt) contain tails for subsequent FRET detection, while wild-type probe (Wt) does not. 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 upstream and phosphorylated downstream oligonucleotides together.

F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

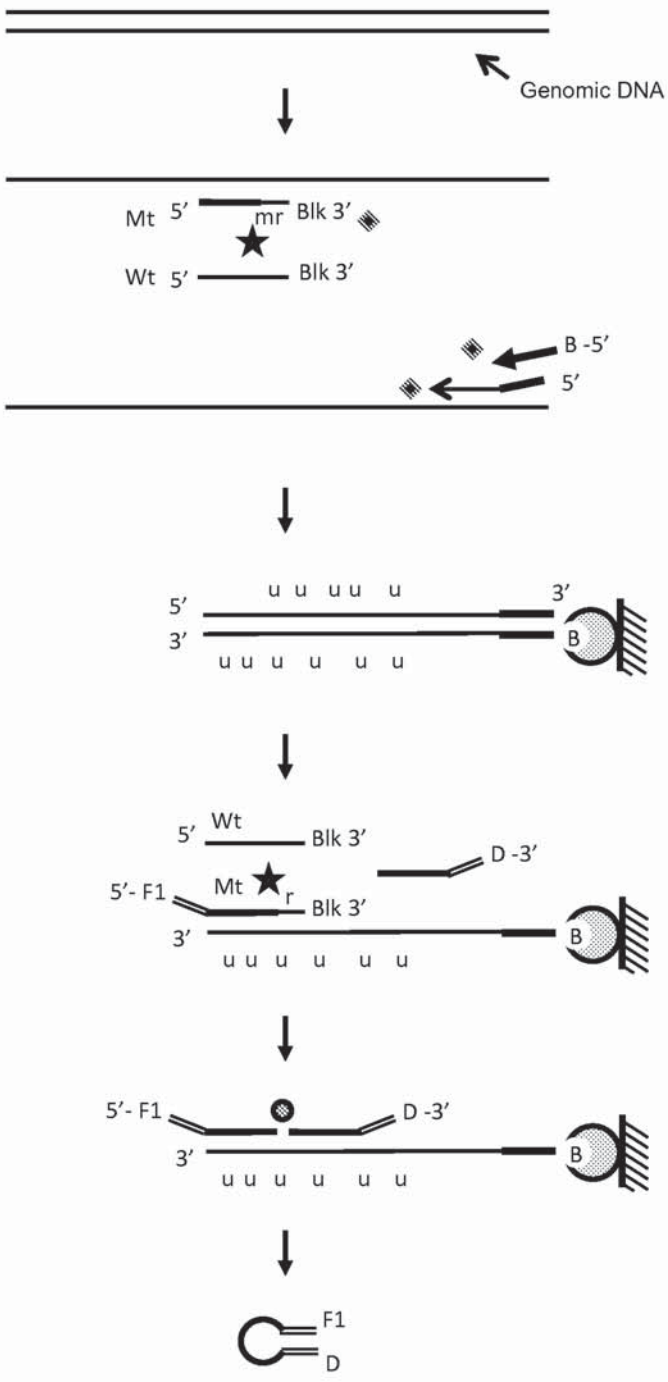


Figure 147

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. 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. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

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. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

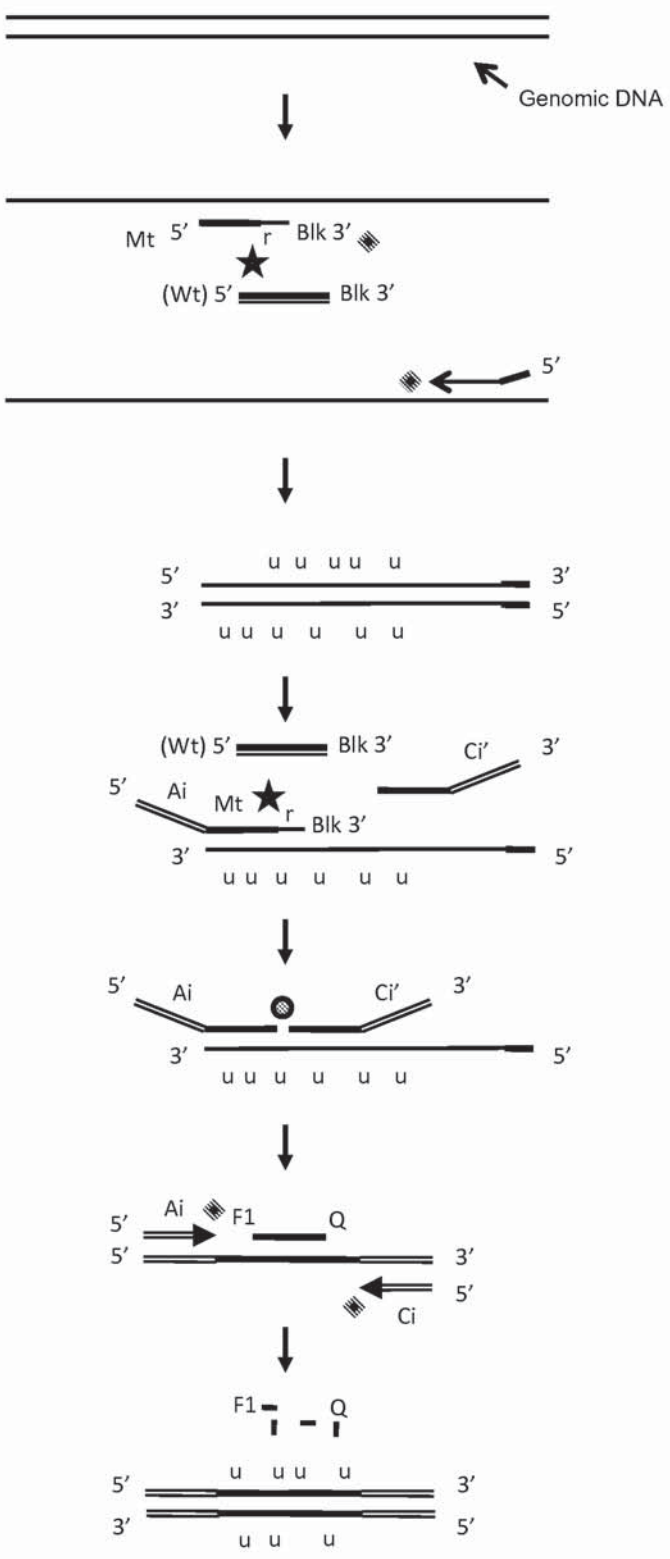


Figure 148

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. 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. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

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. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

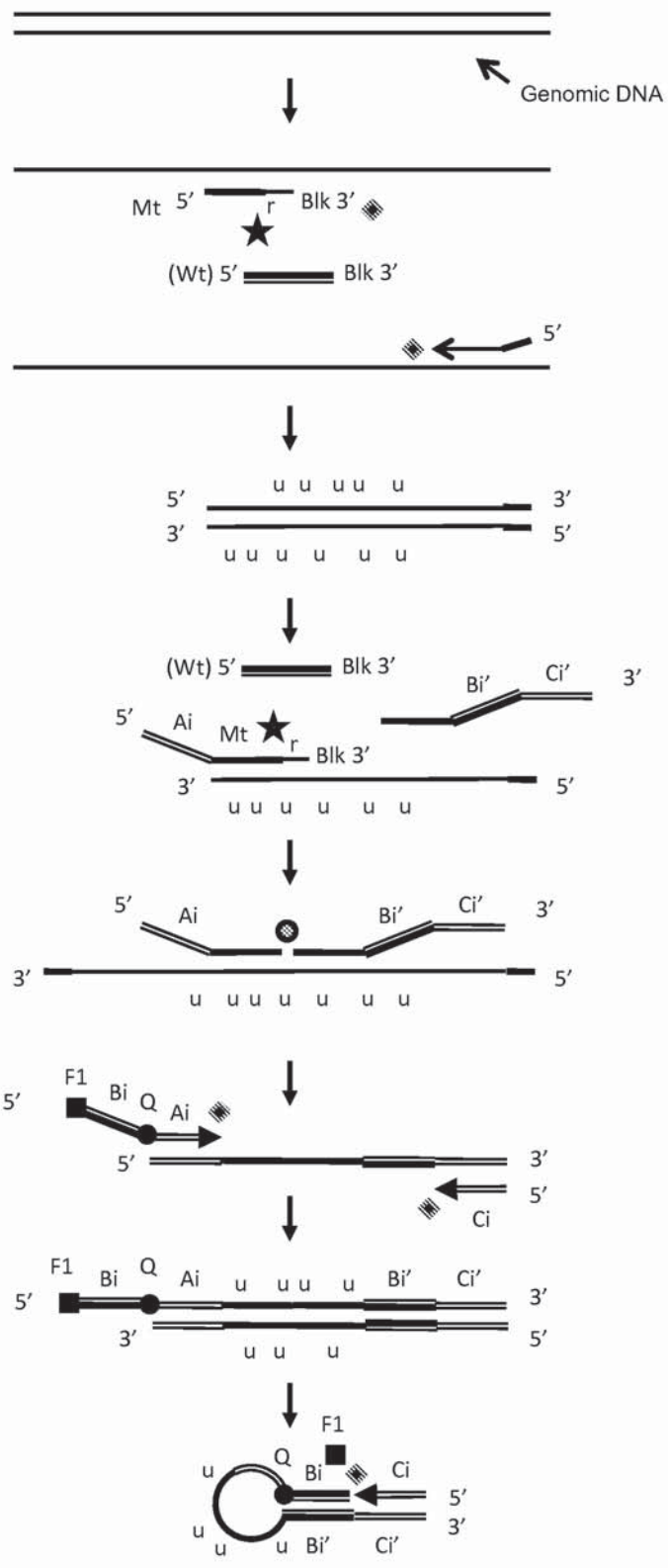


Figure 149

A. PCR-qLDR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. 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 universal tails to enable universal primer amplification to append a 5′ biotin. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Mutation-specific ligation oligonucleotides (Mt) contain tails for subsequent FRET detection. 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 upstream and phosphorylated downstream oligonucleotides together.

F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

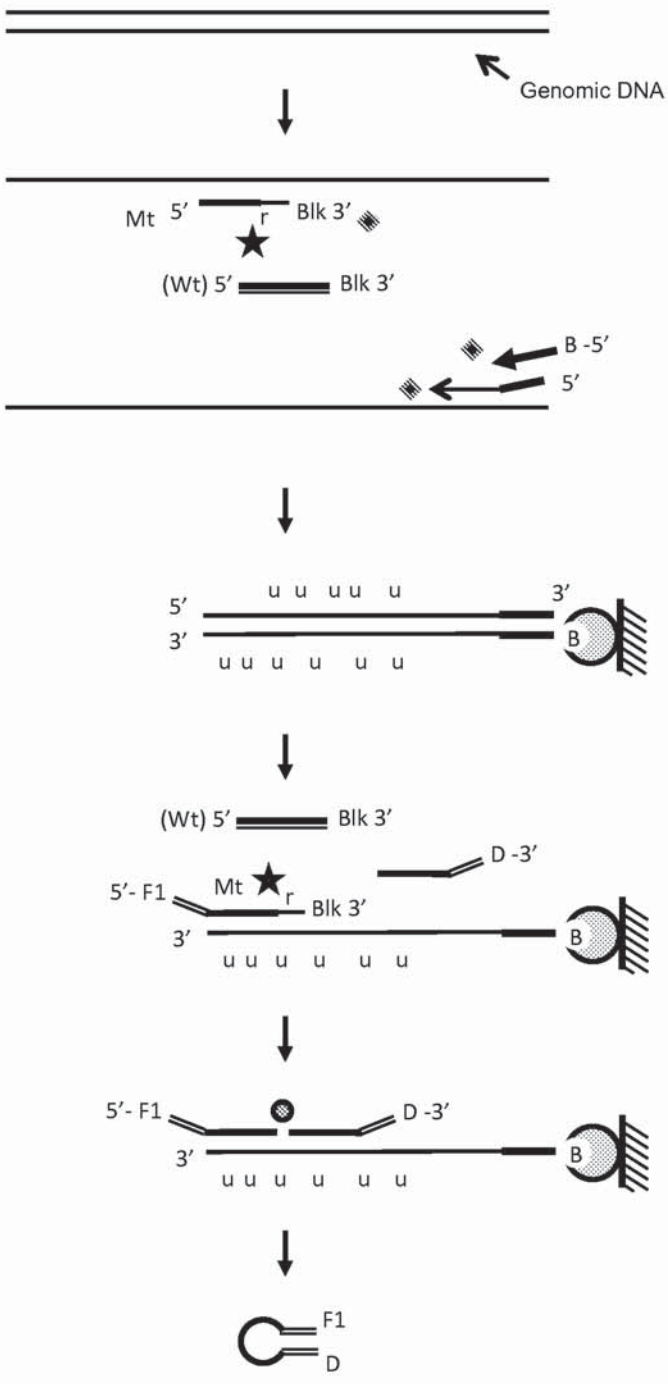


Figure 150

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest un-methylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Blocking LNA or PNA probes may be used to limit amplification of originally un-methylated DNA using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Methyl region-specific ligation oligonucleotides (m) contain tags (Ai, Ci') for subsequent PCR amplification. Blocking LNA or PNA probes suppress ligation to originally un-methylated DNA 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 methyl region-specific upstream and phosphorylated downstream oligonucleotides together. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

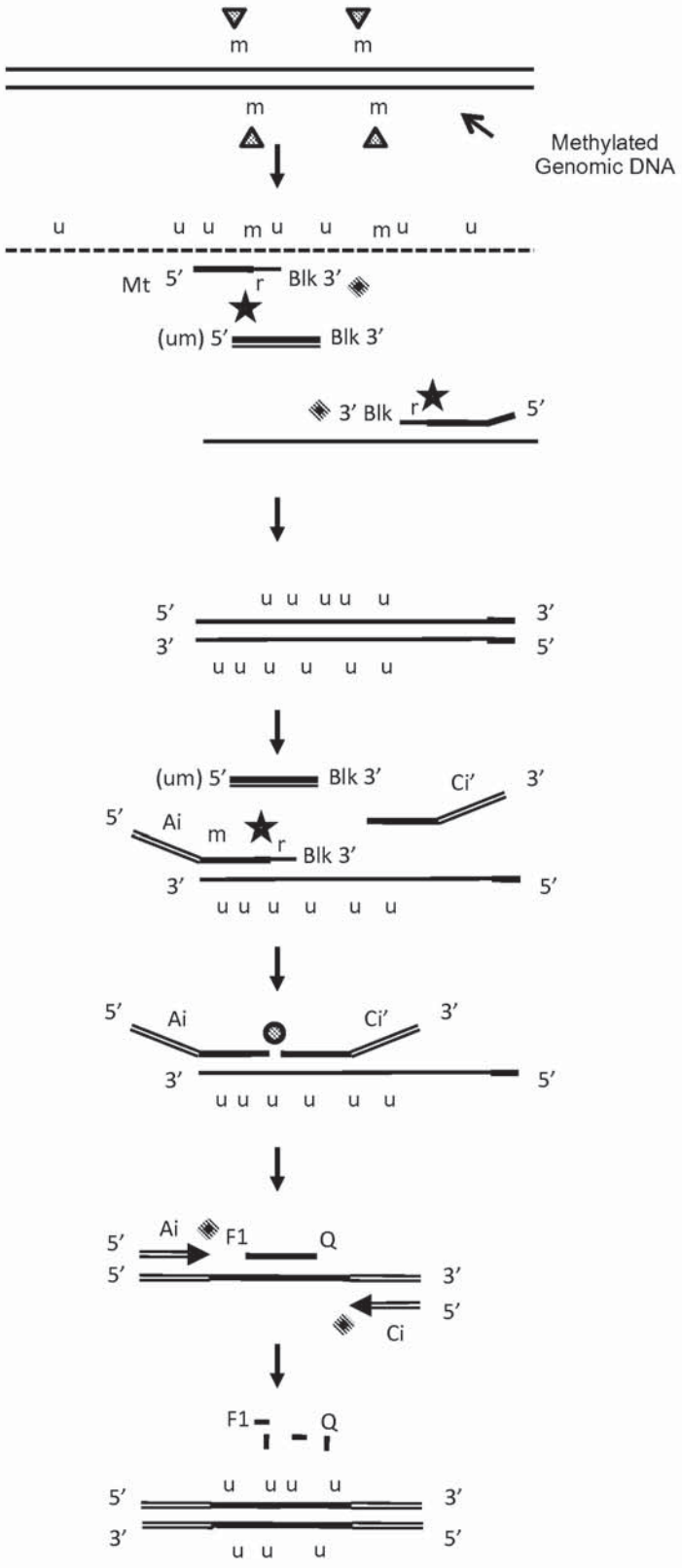


Figure 151

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest un-methylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Blocking LNA or PNA probes may be used to limit amplification of originally un-methylated DNA using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention.

D. Methyl region-specific ligation oligonucleotides (m) contain tags (Ai, Bi', Ci') for subsequent PCR amplification. Blocking LNA or PNA probes suppress ligation to originally un-methylated DNA 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 methyl region-specific upstream and phosphorylated downstream oligonucleotides together. Aliquot into separate wells for detection.

F. Treat with UDG for carryover prevention, which also destroys original target amplicons. 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.)

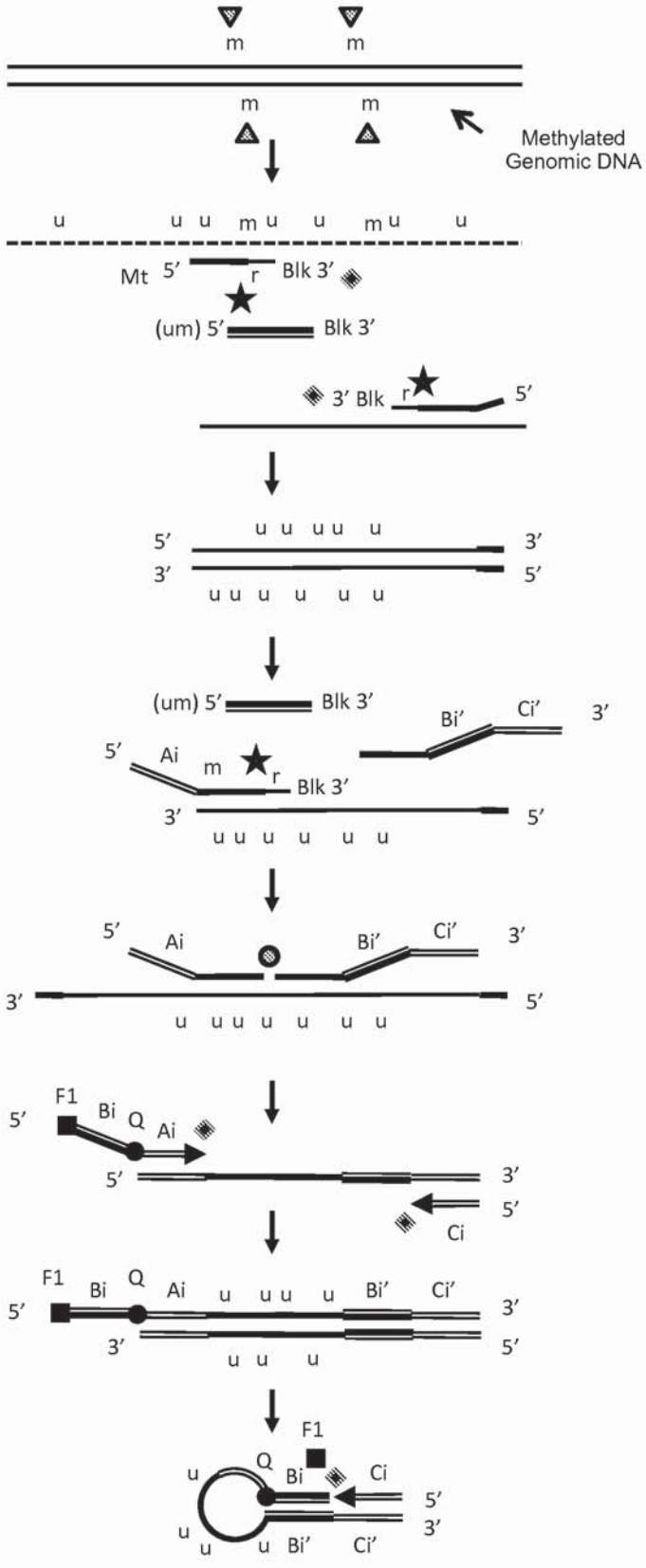


Figure 152

A. PCR-LDR-qPCR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UNG (37oC, 30-60 minutes) to completely digest un-methylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Locus primers contain ribose bases (r), which are removed with RNaseH2 only when bound to target, thus unblocking primer. Blocking LNA or PNA probes may be used to limit amplification of originally un-methylated DNA using PCR (and dUTP). Downstream primers contain universal tails to enable universal primer amplification to append a 5′ biotin. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support.

D. Methyl region-specific ligation oligonucleotides (m) contain tails for subsequent FRET detection. Blocking LNA or PNA probes suppress ligation to originally un-methylated DNA 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 upstream and phosphorylated downstream oligonucleotides together.

F. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

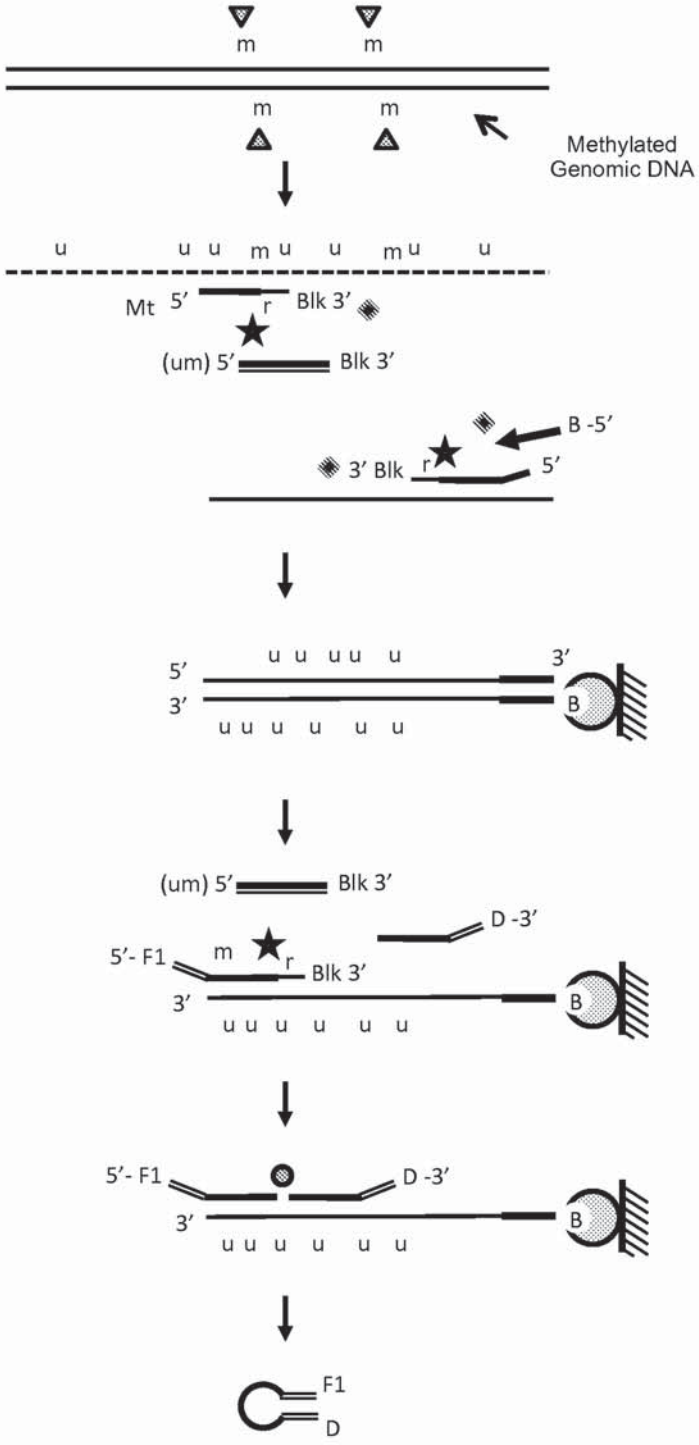


Figure 153

A. Loop-PCR-LDR-qPCR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention. Upstream locus-specific primers also contain 5′ sequences complementary to Wt sequence of top strand, and are unblocked with RNaseH2 only when bound to target. Amplify mutation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. (PCR products incorporate dU, allowing for carryover prevention.)

D. In detail: PCR is performed with a polymerase lacking 5′ nuclease, 3′ nuclease, and strand-displacement activity. (i) Denaturation of Wt bottom strand results in hairpin with perfect match at 3′ end, which is extended by polymerase. (ii) Denaturation of Mt bottom strand results in hairpin with mismatch at 3′ end, which generally is not extended by polymerase. (iii) Denaturation of top strand results in hairpin on 5′ side, which denatures during the extend step of PCR (72oC).

E. (i) After extension of hairpin on wild-type DNA, extended hairpin sequence does not denature at 72oC and prevents upstream primer from generating full-length top strand. (ii) Hairpin sequence of mutant DNA does not extend, and thus denatures at 72oC, enabling upstream primer to generate full-length top strand. (iii) Likewise, top strand product denatures at 72oC, allowing polymerase to generate full-length bottom strand. The difference in hairpin extension preference of upstream primers with (i) wild-type and (ii) mutant template results in preferential amplification of mutant DNA.

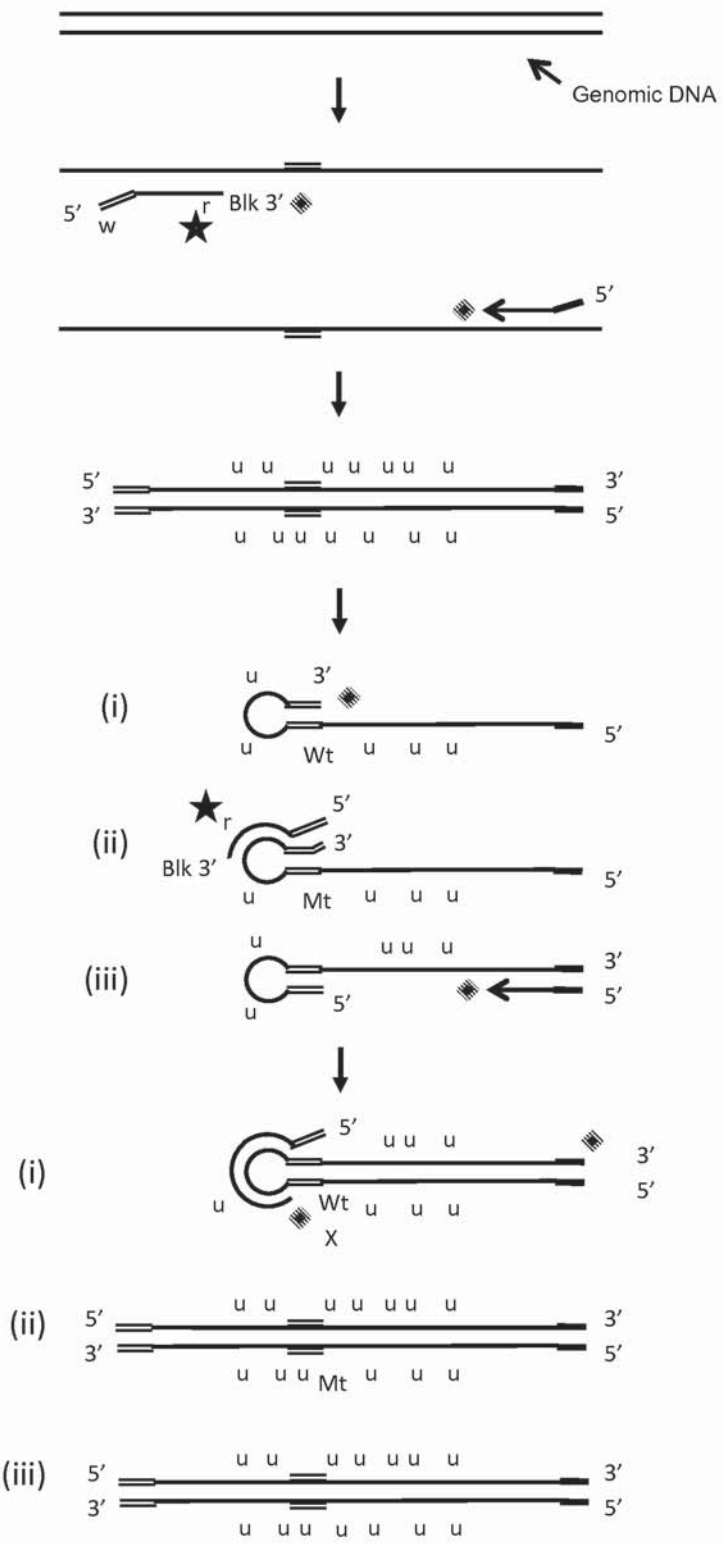


Figure 154

F. (PCR products incorporate dU, allowing for carryover prevention. Optional: wild-type sequence on end may be clipped off with restriction endonuclease.)

G. Mutation-specific ligation oligonucleotides (Mt) contain tags (Ai, Ci') for subsequent PCR amplification, while (optional) wild-type probe (Wt) does not. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target. (Optional: wild-type sequence on 3' end may be clipped off with exonuclease III)

H. Ligase covalently seals the unblocked upstream and phosphorylated downstream oligonucleotides together. Aliquot into separate wells for detection.

I. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci'). Each Taqman probe spans the ligation junction.

J. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

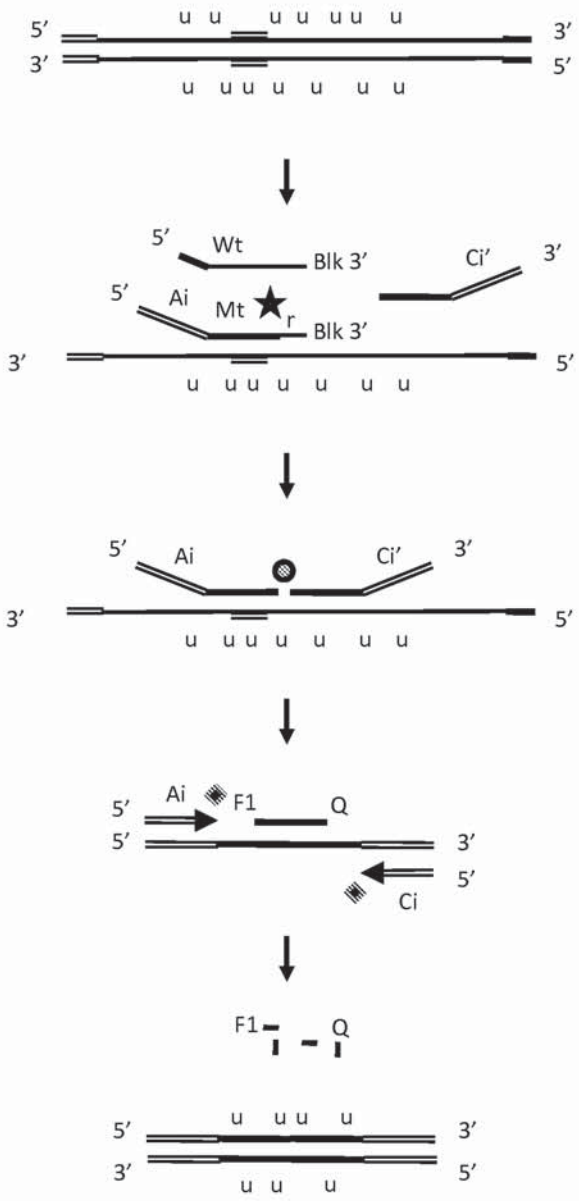


Figure 154 (Cont.)

A. Loop-PCR-qLDR carryover prevention reaction to detect low-level mutation. Isolate genomic or cfDNA.

B. Treat with UDG for carryover prevention. Upstream locus-specific primers also contain 5′ sequences complementary to Wt sequence of top strand, and are unblocked with RNaseH2 only when bound to target. Amplify mutation containing regions using PCR (and dUTP). Downstream primers contain universal tails to enable universal primer amplification to append a 5′ biotin. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. (PCR products incorporate dU, allowing for carryover prevention.)

D. In detail: PCR is performed with a polymerase lacking 5′ nuclease, 3′ nuclease, and strand-displacement activity. (i) Denaturation of Wt bottom strand results in hairpin with perfect match at 3′ end, which is extended by polymerase. (ii) Denaturation of Mt bottom strand results in hairpin with mismatch at 3′ end, which generally is not extended by polymerase. (iii) Denaturation of top strand results in hairpin on 5′ side, which denatures during the extend step of PCR (72oC).

E. (i) After extension of hairpin on wild-type DNA, extended hairpin sequence does not denature at 72oC and prevents upstream primer from generating full-length top strand. (ii) Hairpin sequence of mutant DNA does not extend, and thus denatures at 72oC, enabling upstream primer to generate full-length top strand. (iii) Likewise, top strand product denatures at 72oC, allowing polymerase to generate full-length bottom strand. The difference in hairpin extension preference of upstream primers with (i) wild-type and (ii) mutant template results in preferential amplification of mutant DNA.

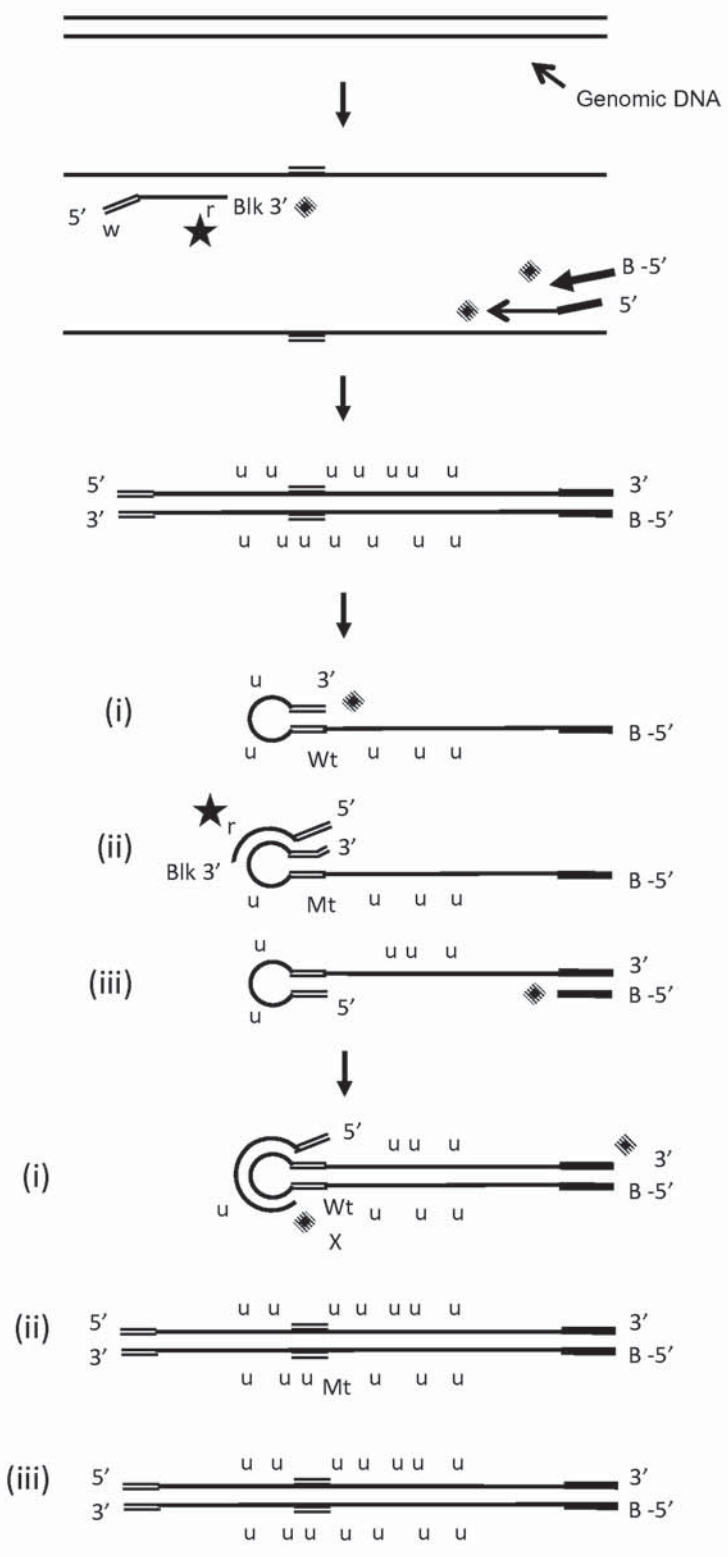


Figure 155

F. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support. Optional: wild-type sequence on end may be clipped off with restriction endonuclease.

G. Mutation-specific ligation oligonucleotides (Mt) contain tails for subsequent FRET detection, while (optional) wild-type probe (Wt) does not. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target. (Optional: wild-type sequence on 3′ end may be clipped off with exonuclease III)

H. Ligase covalently seals the unblocked upstream and phosphorylated downstream oligonucleotides together.

I. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

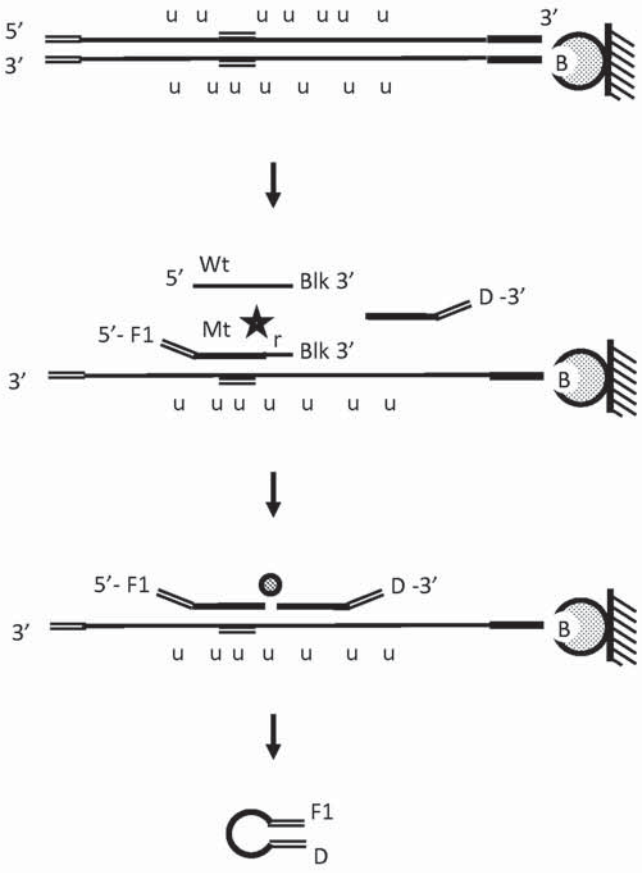


Figure 155 (Cont.)

A. Loop-PCR-LDR-qPCR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UDG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Upstream locus-specific primers also contain 5′ sequences complementary to bisulfite-treated unmethylated sequence of top strand, and are unblocked with RNaseH2 only when bound to target. Amplify methylation containing regions using PCR (and dUTP). Downstream primers contain identical 8-11 base tails to prevent primer dimers. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. (PCR products incorporate dU, allowing for carryover prevention.)

D. In detail: PCR is performed with a polymerase lacking 5′ nuclease, 3′ nuclease, and strand-displacement activity. (i) Denaturation of unmethylated bottom strand results in hairpin with perfect match at 3′ end, which is extended by polymerase. (ii) Denaturation of methylated bottom strand results in hairpin with mismatches, which generally is not extended by polymerase. (iii) Denaturation of top strand results in hairpin on 5′ side, which denatures during the extend step of PCR (72oC).

E. (i) After extension of hairpin on unmethylated DNA, extended hairpin sequence does not denature at 72oC and prevents upstream primer from generating full-length top strand. (ii) Hairpin sequence of methylated DNA does not extend, and thus denatures at 72oC, enabling upstream primer to generate full-length top strand. (iii) Likewise, top strand product denatures at 72oC, allowing polymerase to generate full-length bottom strand. The difference in hairpin extension preference of upstream primers with (i) unmethylated and (ii) methylated template results in preferential amplification of methylated DNA.

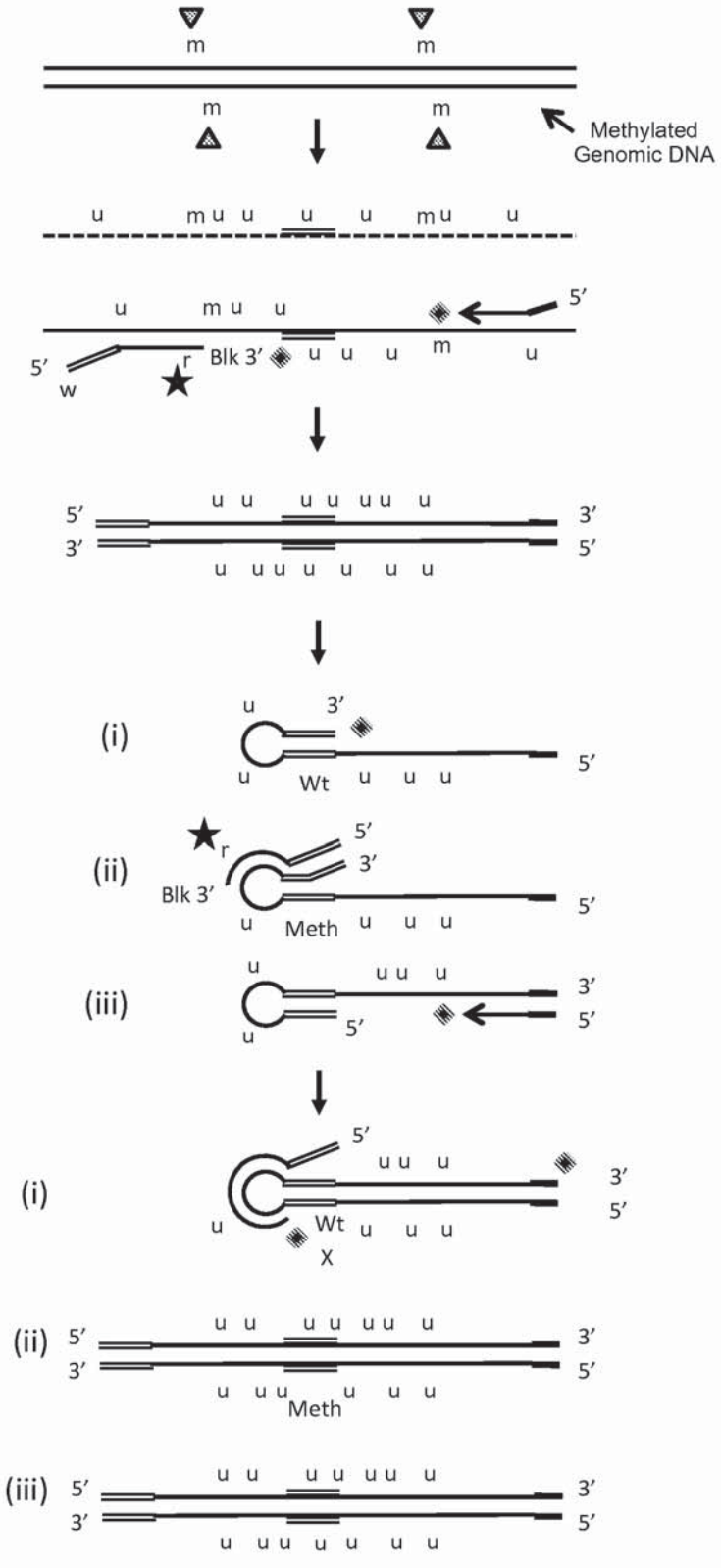


Figure 156

F. PCR products incorporate dU, allowing for carryover prevention. Optional: sequence on end complementary to unmethylated region may be clipped off with restriction endonuclease.

G. Methylation-specific ligation oligonucleotides (Meth) contain tags (Ai, Ci') for subsequent PCR amplification, while (optional) unmethylated probe (Umet) does not. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target. (Optional: wild-type sequence on 3' end may be clipped off with exonuclease III)

H. Ligase covalently seals the unblocked upstream and phosphorylated downstream oligonucleotides together. Aliquot into separate wells for detection.

I. Treat with UDG for carryover prevention, which also destroys original target amplicons. Only authentic LDR products will amplify, when using PCR (and dUTP) with tag-specific primers (Ai, Ci'). Each Taqman probe spans the ligation junction.

J. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

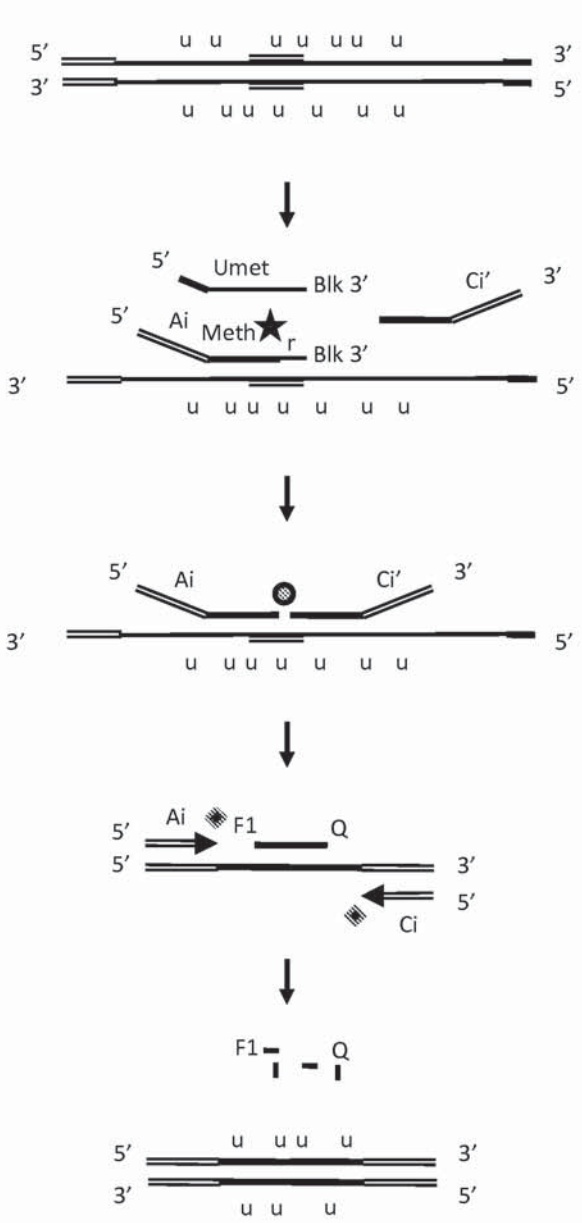


Figure 156 (Cont.)

A. Loop-PCR-qLDR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Treat with Bsh1236I (CG^CG) and UDG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover. Treat with bisulfite, which renders the strands non-complementary.

B. Upstream locus-specific primers also contain 5′ sequences complementary to bisulfite-treated unmethylated sequence of bottom strand, and are unblocked with RNaseH2 only when bound to target. Amplify methylation containing regions using PCR (and dUTP). Downstream primers contain universal tails to enable universal primer amplification to append a 5′ biotin. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. (PCR products incorporate dU, allowing for carryover prevention.)

D. In detail: PCR is performed with a polymerase lacking 5′ nuclease, 3′ nuclease, and strand-displacement activity. (i) Denaturation of unmethylated bottom strand results in hairpin with perfect match at 3′ end, which is extended by polymerase. (ii) Denaturation of methylated bottom strand results in hairpin with mismatches, which generally is not extended by polymerase. (iii) Denaturation of top strand results in hairpin on 5′ side, which denatures during the extend step of PCR (72oC).

E. (i) After extension of hairpin on unmethylated DNA, extended hairpin sequence does not denature at 72oC and prevents upstream primer from generating full-length top strand. (ii) Hairpin sequence of methylated DNA does not extend, and thus denatures at 72oC, enabling upstream primer to generate full-length top strand. (iii) Likewise, top strand product denatures at 72oC, allowing polymerase to generate full-length bottom strand. The difference in hairpin extension preference of upstream primers with (i) unmethylated and (ii) methylated template results in preferential amplification of methylated DNA.

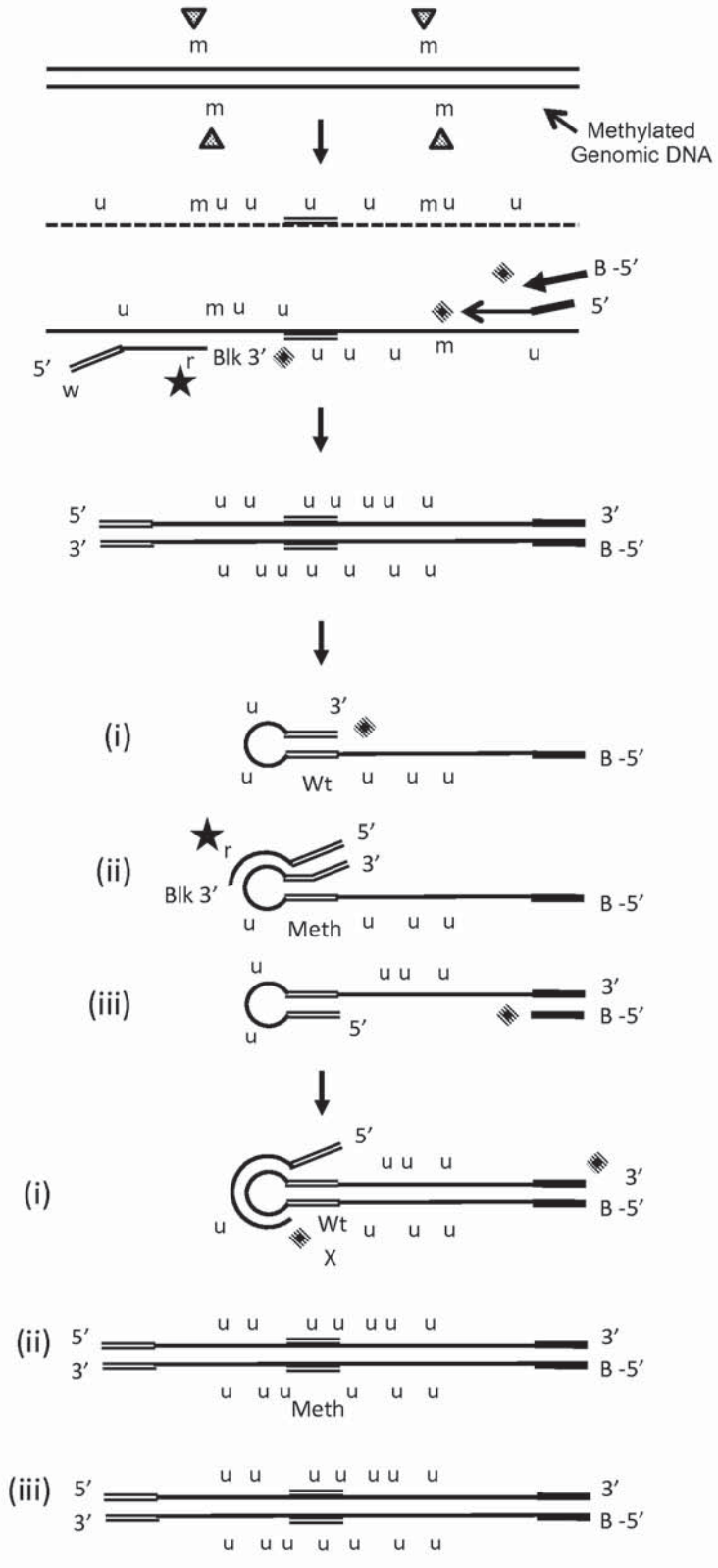


Figure 157

F. PCR products incorporate dU, allowing for carryover prevention. Separate product from primers and capture biotinylated product on solid support. Optional: sequence on end complementary to unmethylated region may be clipped off with restriction endonuclease.

G. Methytion-specific ligation oligonucleotides (Meth) contain tails for subsequent FRET detection, while (optional) unmethylated probe (Umet) does not. Upstream ligation primer contains block, which may be removed by cleaving ribose base with RNaseH2 only when hybridized to target. (Optional: wild-type sequence on 3′ end may be clipped off with exonuclease III)

H. Ligase covalently seals the unblocked upstream and phosphorylated downstream oligonucleotides together.

I. Wash away unligated primers. Denature product from target, then detect, and enumerate FRET signal.

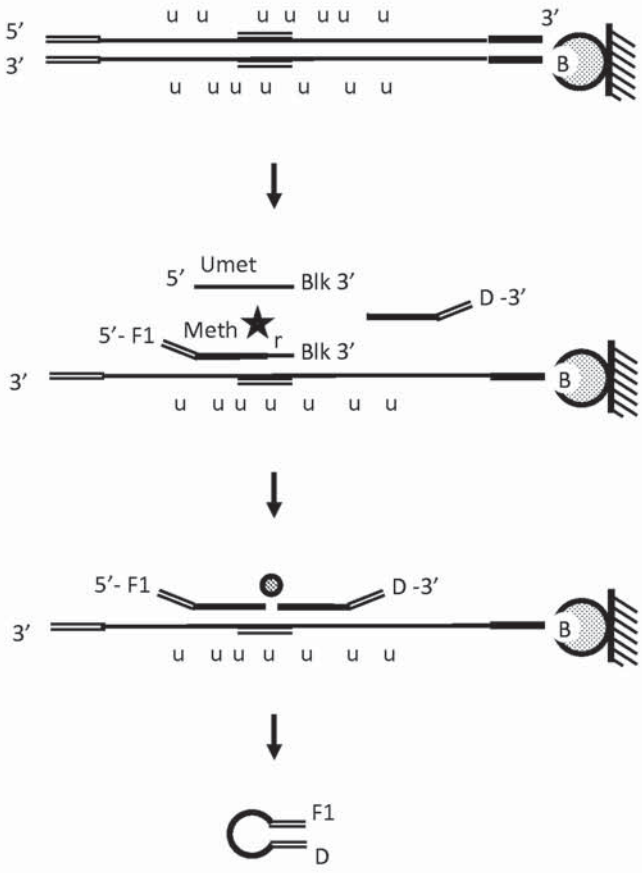


Figure 157 (Cont.)

A. PCR-qPCR carryover prevention reaction to detect low-level methylation. Isolate genomic or cfDNA. Optional: enrich for methylated DNA using antibodies. Treat with methyl-sensitive restriction endonucleases Bsh1236I (CG^CG) and HinP1I (G^CGC), and UNG (37oC, 30-60 minutes) to completely digest unmethylated DNA and prevent carryover.

B. Amplify methyl containing regions using PCR (and dUTP) with locus-specific primers. Perform limited cycle amplification (12-20) to maintain relative ratios of different amplicons. (Optional: aliquot into 12, 24, 48, or 96 wells prior to PCR.)

C. PCR products incorporate dU, allowing for carryover prevention. (Products lack methyl groups, providing additional protection.)

D. Optional: Aliquot into separate wells for detection. Amplify methyl containing regions using PCR (and dUTP) with nested locus-specific primers and internal Taqman probes.

E. The 5'->3' nuclease activity of Taq polymerase liberates the fluorescent group to generate signal. (PCR products incorporate dU, allowing for carryover prevention.)

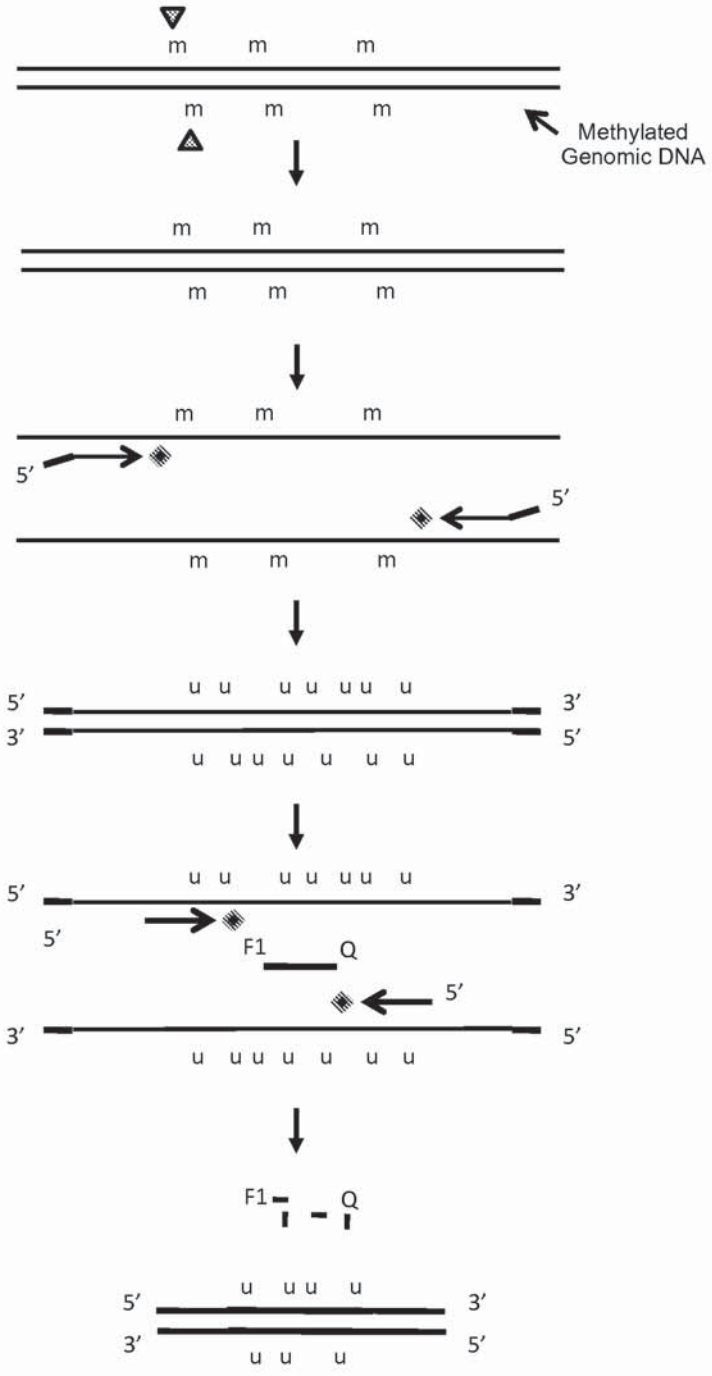


Figure 158

Detection of BRAF V600E by PCR-LDR-qPCR

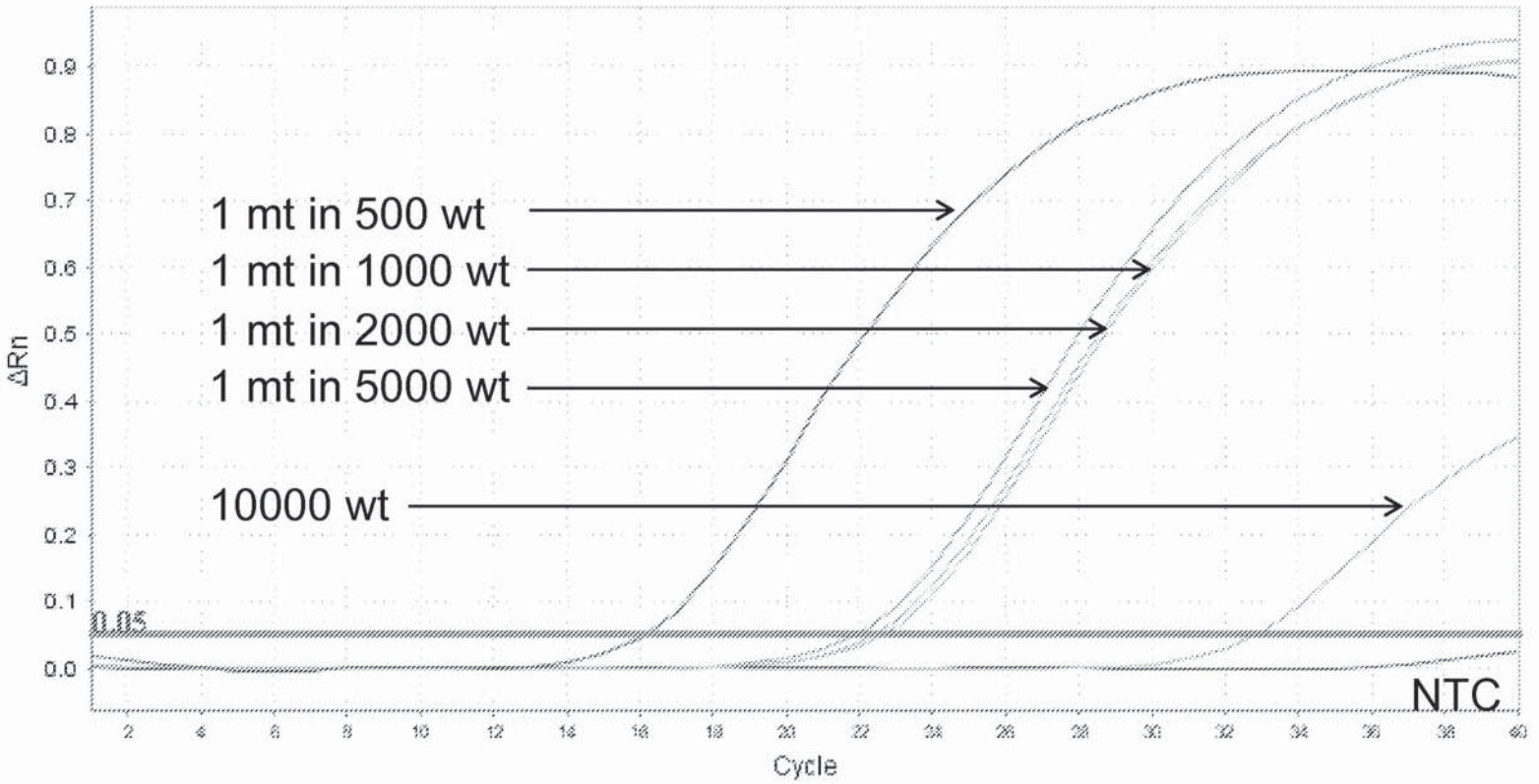
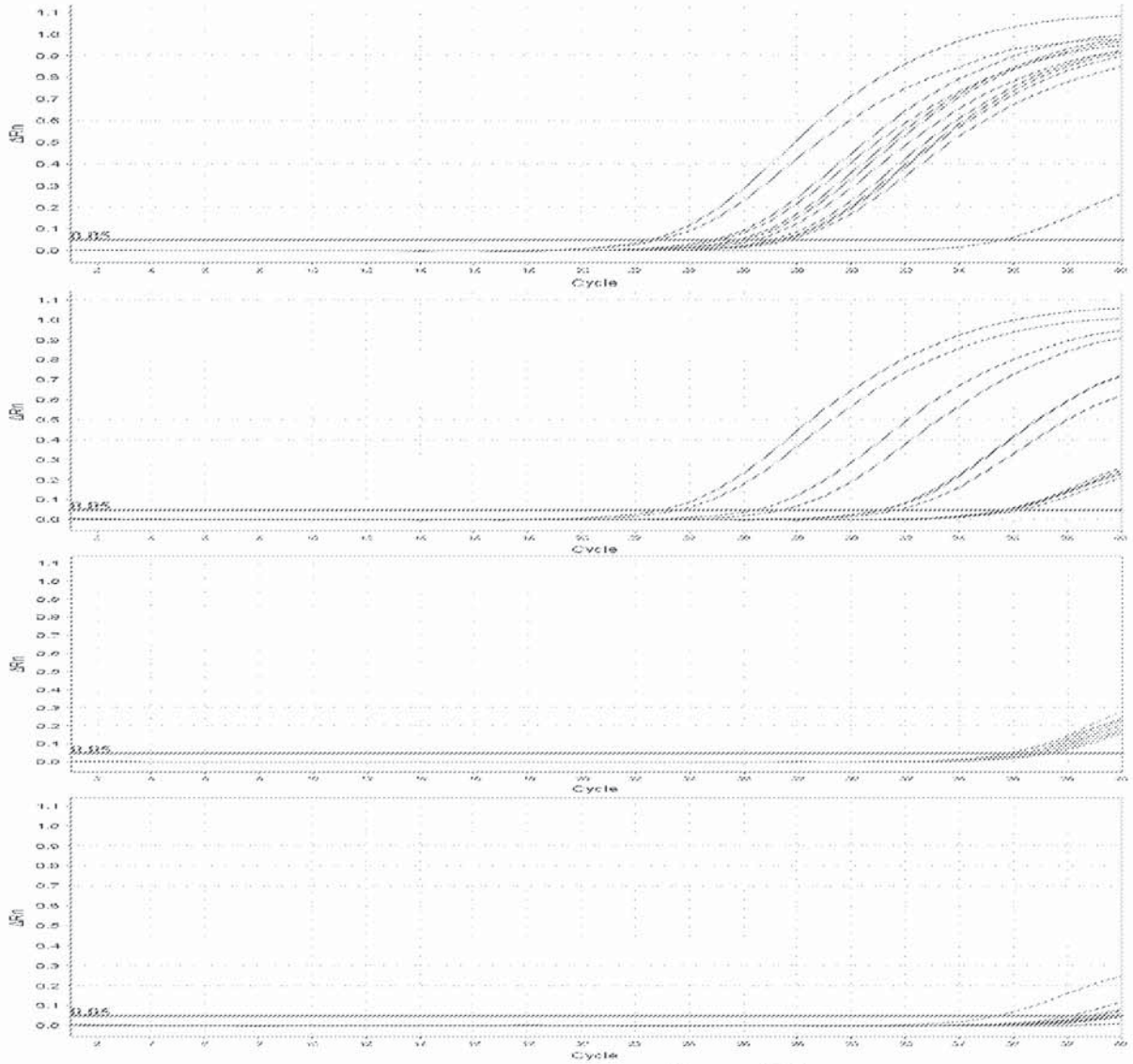


Figure 159

Single molecule detection of BRAF V600E by PCR-LDR-qPCR



10 mt molecules + 2500 wt molecules divided in 12 tubes
11 Amplifications

5 mt molecules + 2500 wt molecules divided in 12 tubes
7 Amplifications

2500 wt molecules divided in 12 tubes
0 Amplification

0 molecules
0 Amplifications

Figure 160

Single molecule detection of BRAF V600E by PCR-LDR-qPCR in Plasma

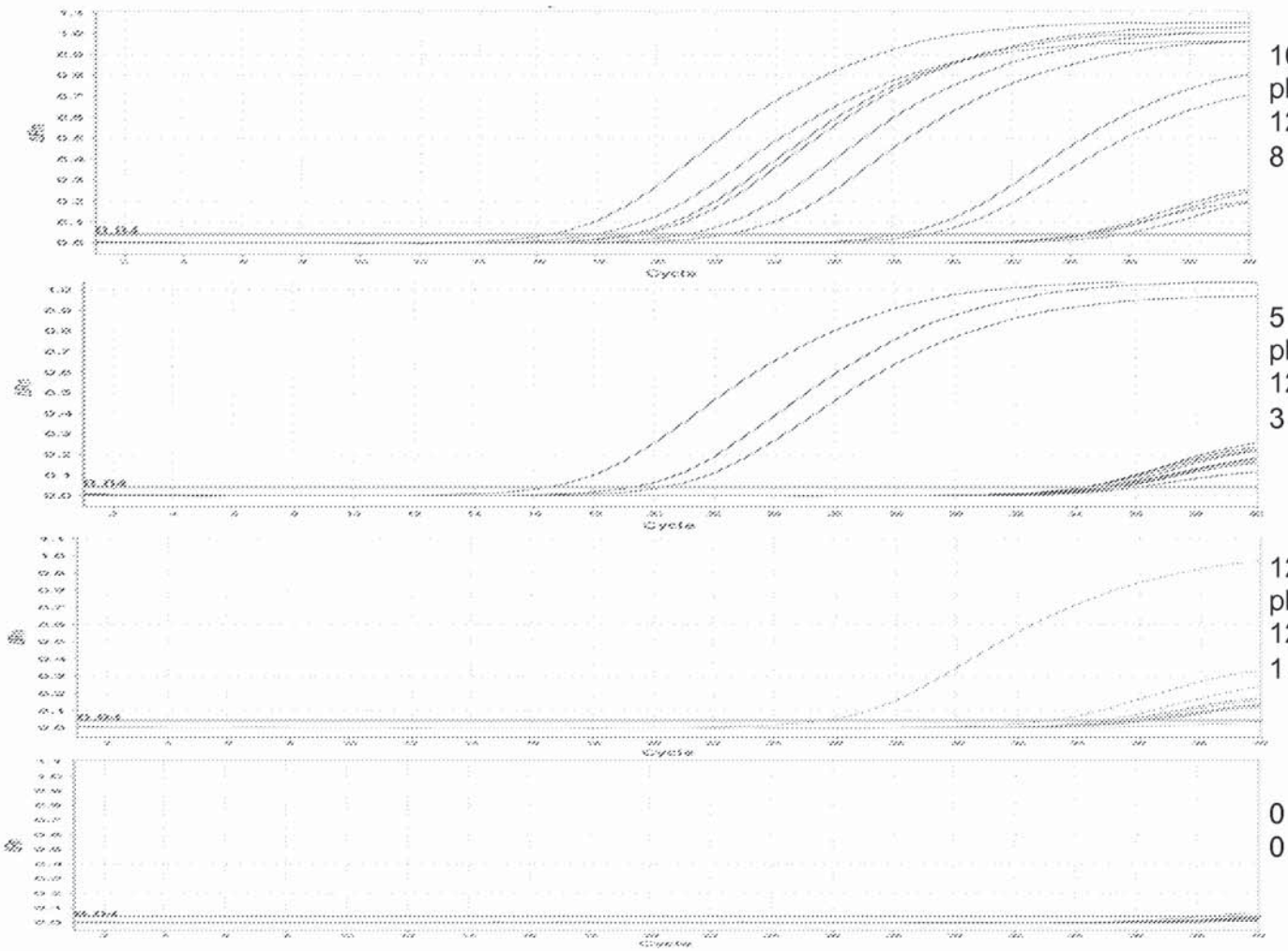


Figure 161

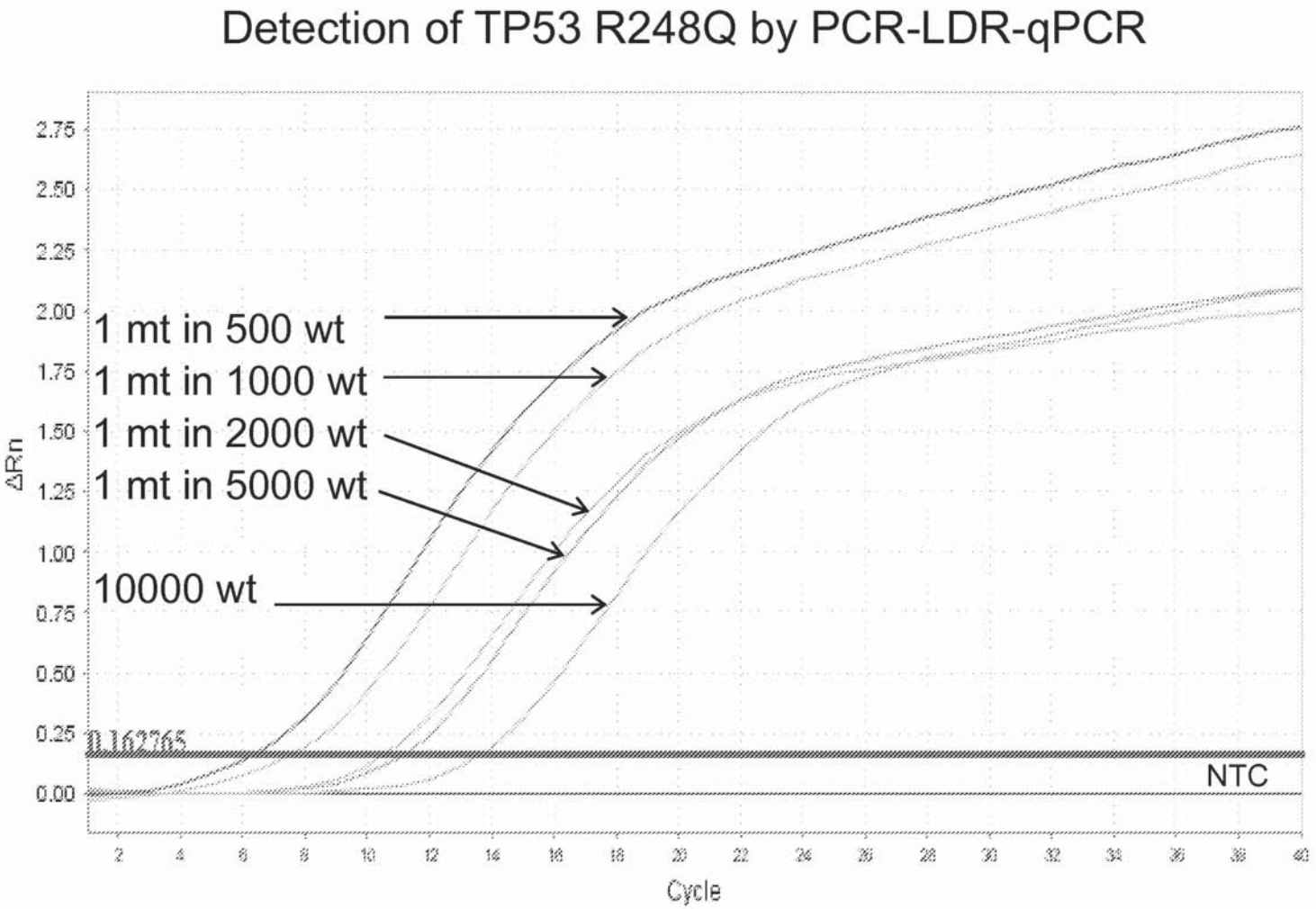

Detection of TP53 R248Q by PCR-LDR-qPCR
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
1 mt in 5000 wt
10000 wt
NTC
0.162765
ΔRn
Cycle

Figure 162

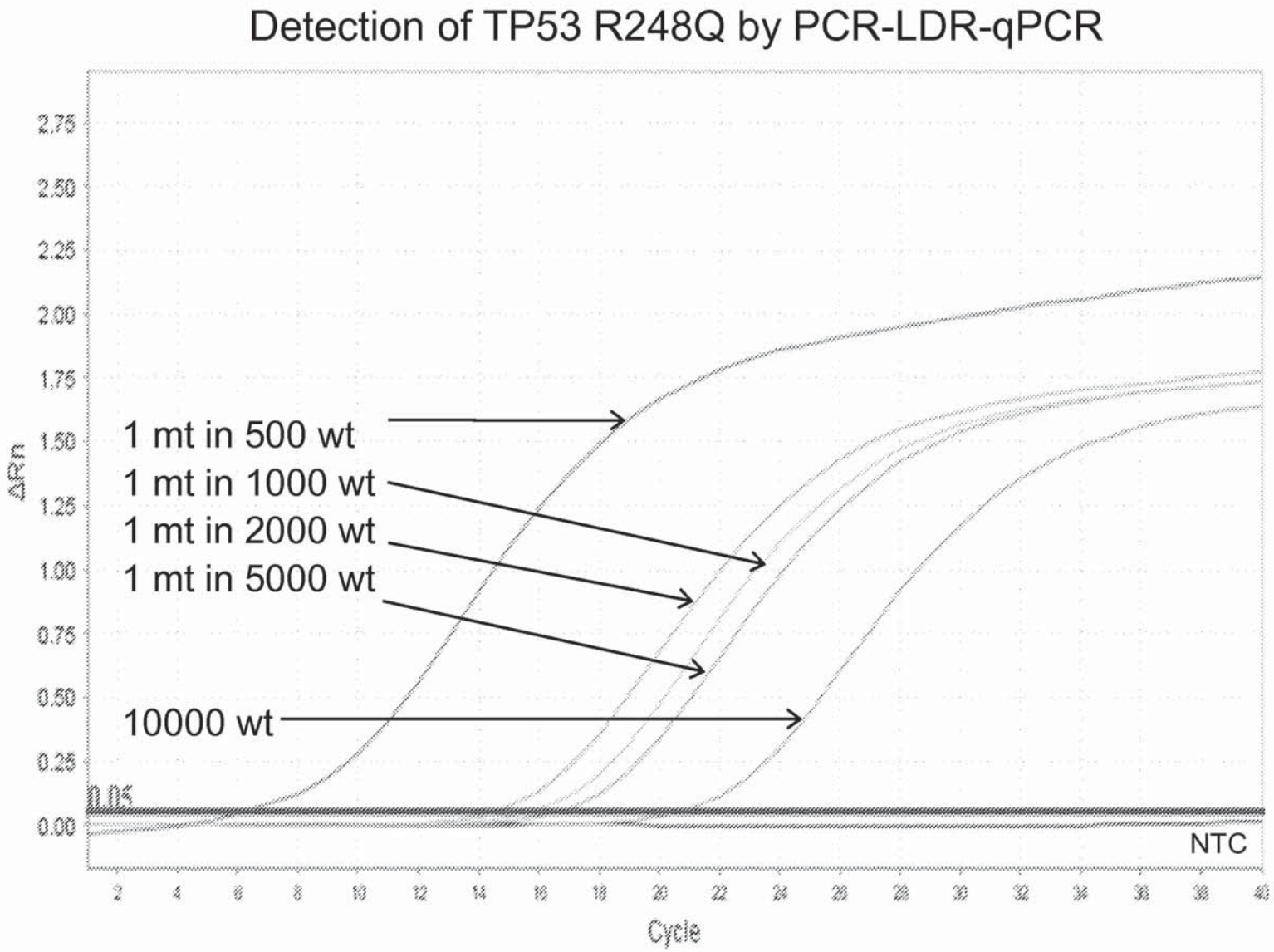
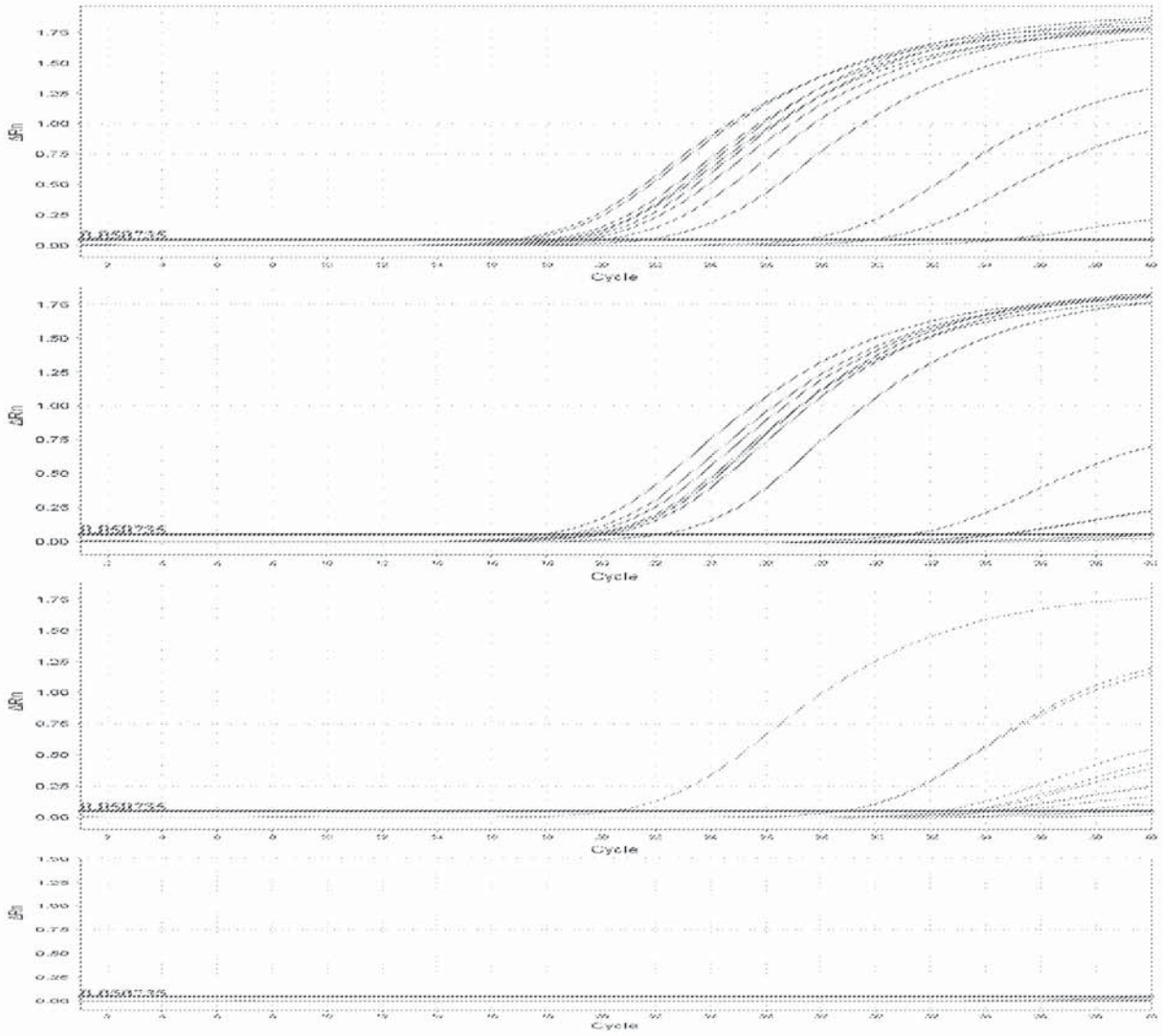

Detection of TP53 R248Q by PCR-LDR-qPCR
ΔRn
Cycle
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
1 mt in 5000 wt
10000 wt
0.05
NTC

Figure 163

Single molecule detection of TP53 R248Q by PCR-LDR-qPCR



10 mt molecules + 2500 wt molecules divided in 12 tubes
9 Amplifications

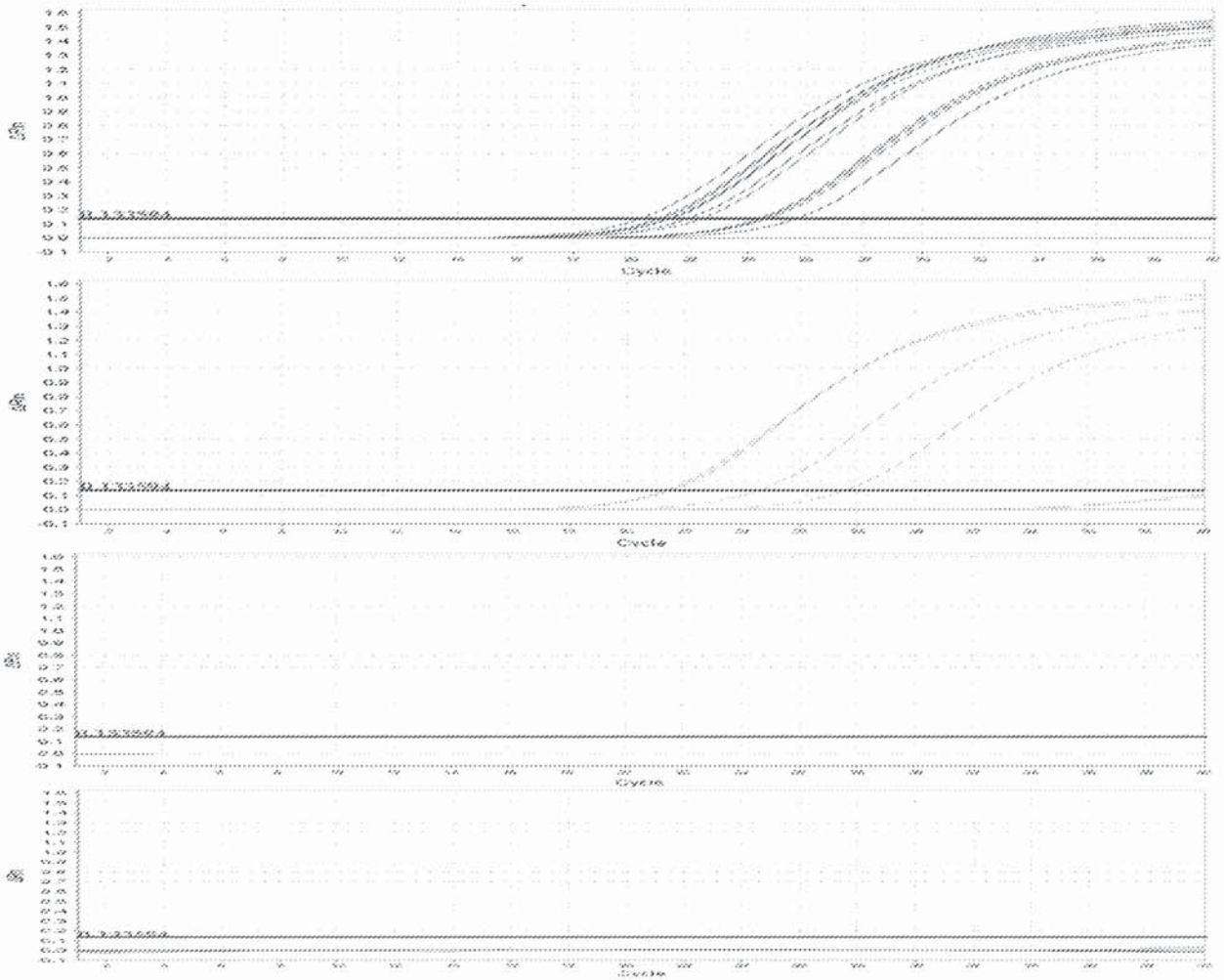
5 mt molecules + 2500 wt molecules divided in 12 tubes
7 Amplifications

2500 wt molecules divided in 12 tubes
1 Amplifications

0 molecules
0 Amplifications

Figure 164

Single molecule detection of TP53 R248Q by PCR-LDR-qPCR in Plasma



10 mt molecules + 1250 wt
plasma molecules divided in
12 tubes
11 Amplifications

5 mt molecules + 1250 wt
plasma molecules divided in
12 tubes
4 Amplifications

1250 wt
plasma molecules divided in
12 tubes
0 Amplifications

0 molecules
0 Amplifications

Figure 165

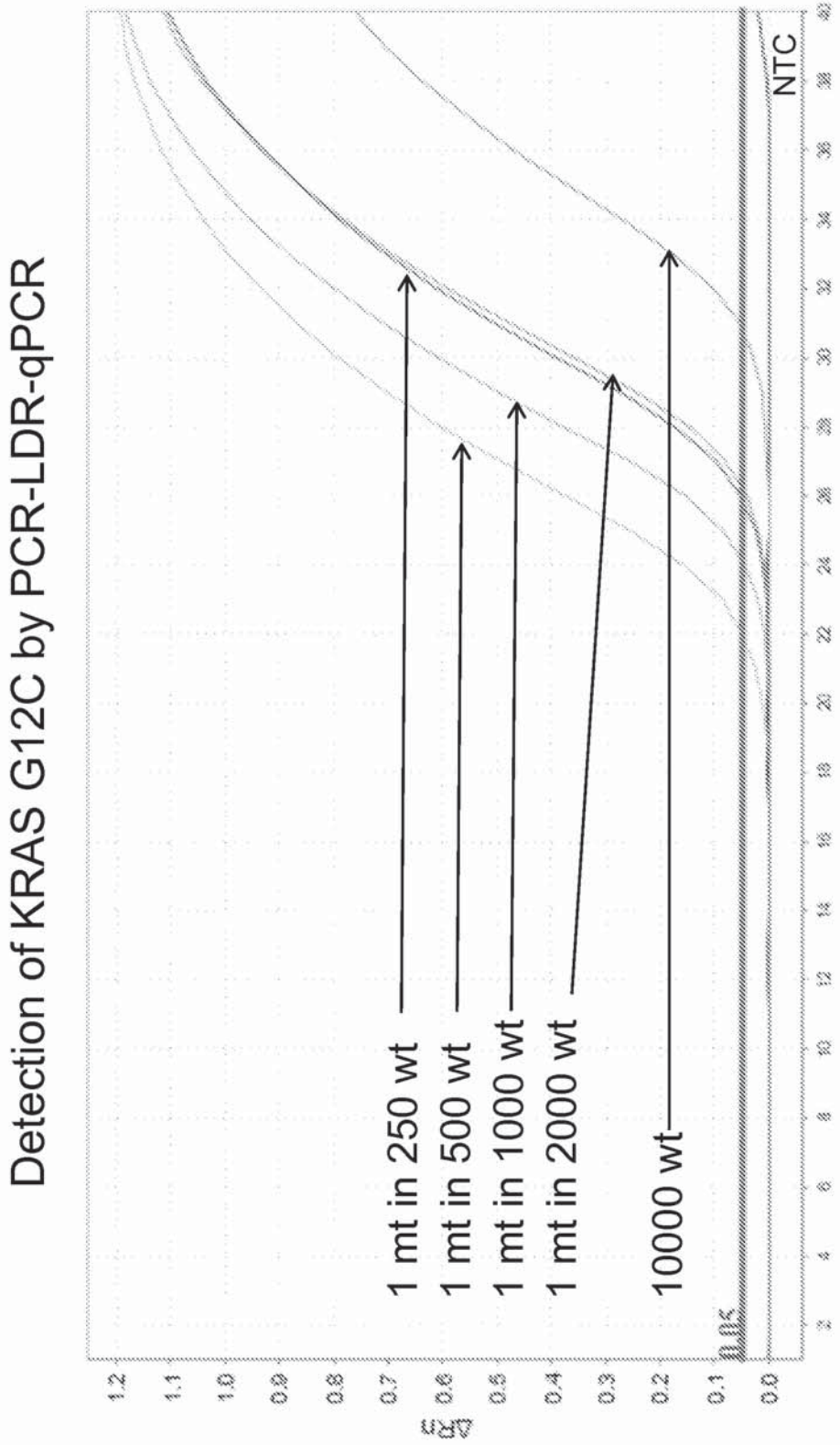

Detection of KRAS G12C by PCR-LDR-qPCR
1 mt in 250 wt
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
10000 wt
NTC
Cycle

Figure 166

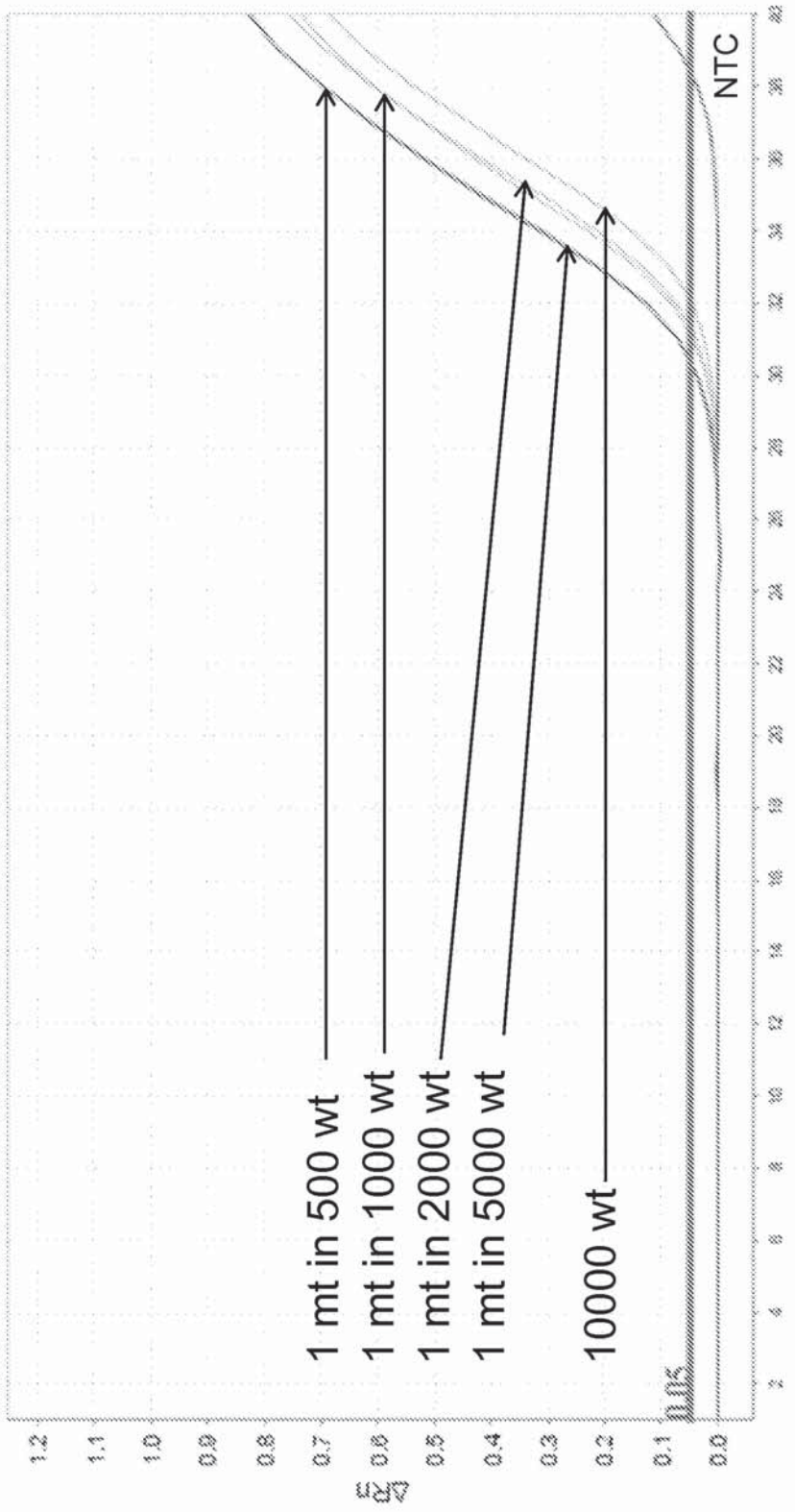
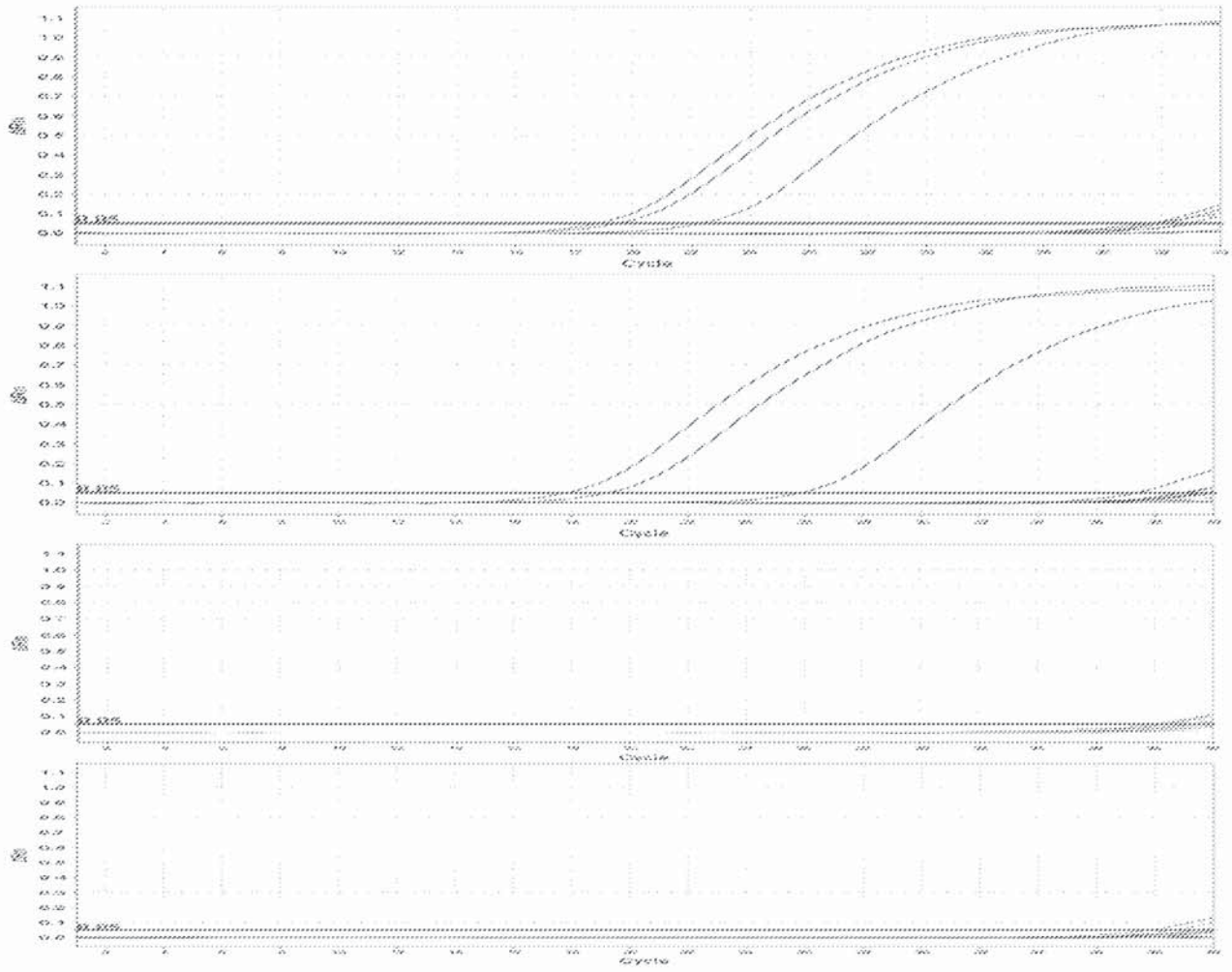

Detection of KRAS G12S by PCR-LDR-qPCR
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
1 mt in 5000 wt
10000 wt
NTC
0.05
Cycle

Figure 167

Single molecule detection of KRAS G12C by PCR-LDR-qPCR in Plasma



10 mt molecules + 1000 wt plasma molecules divided in 12 tubes
3 Amplifications

5 mt molecules + 1000 wt plasma molecules divided in 12 tubes
3 Amplifications

1000 wt plasma molecules divided in 12 tubes
0 Amplifications

0 molecules
0 Amplifications

Figure 168

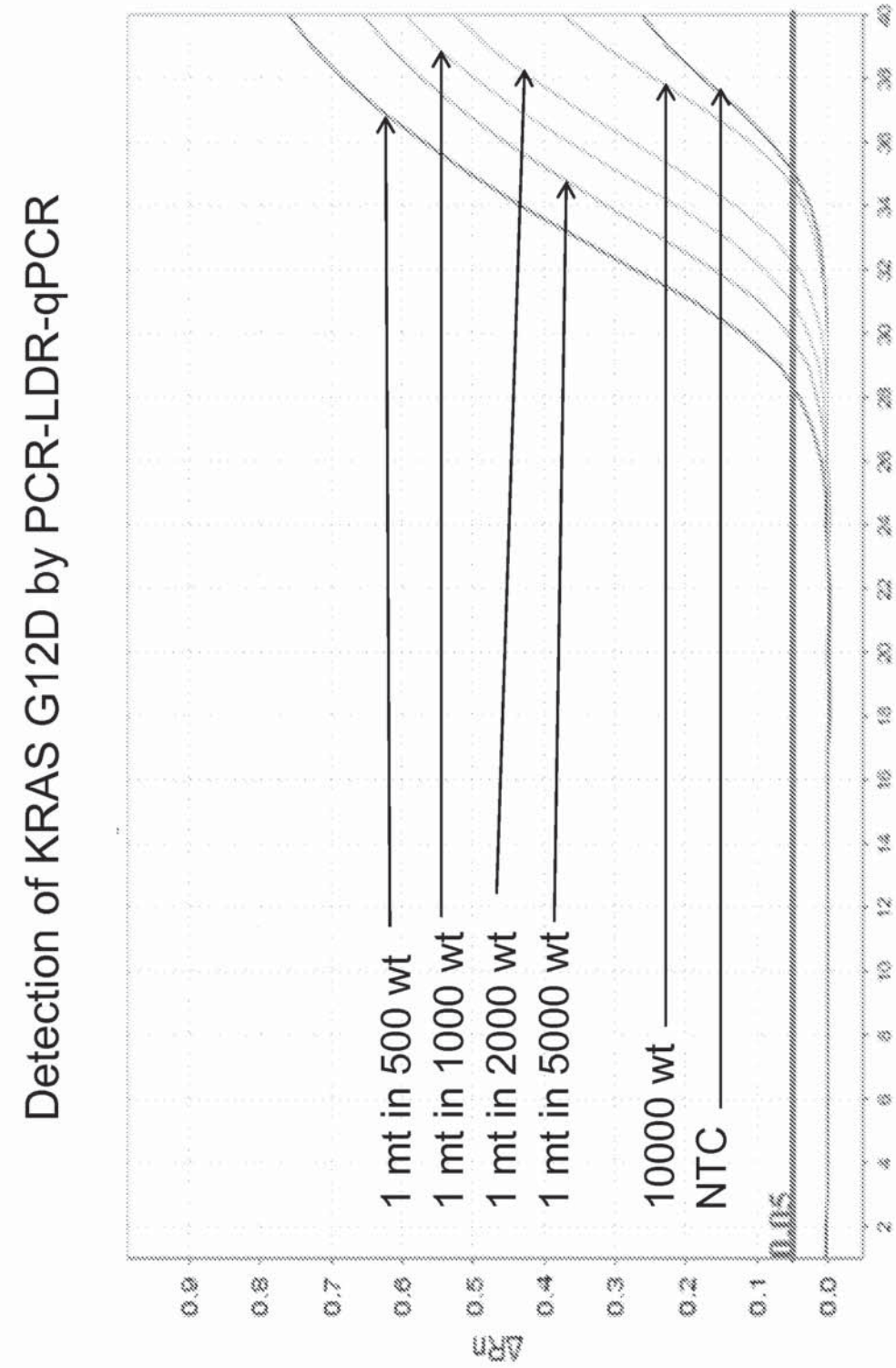

Detection of KRAS G12D by PCR-LDR-qPCR
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
1 mt in 5000 wt
10000 wt
NTC
ΔRn
Cycle

Figure 169

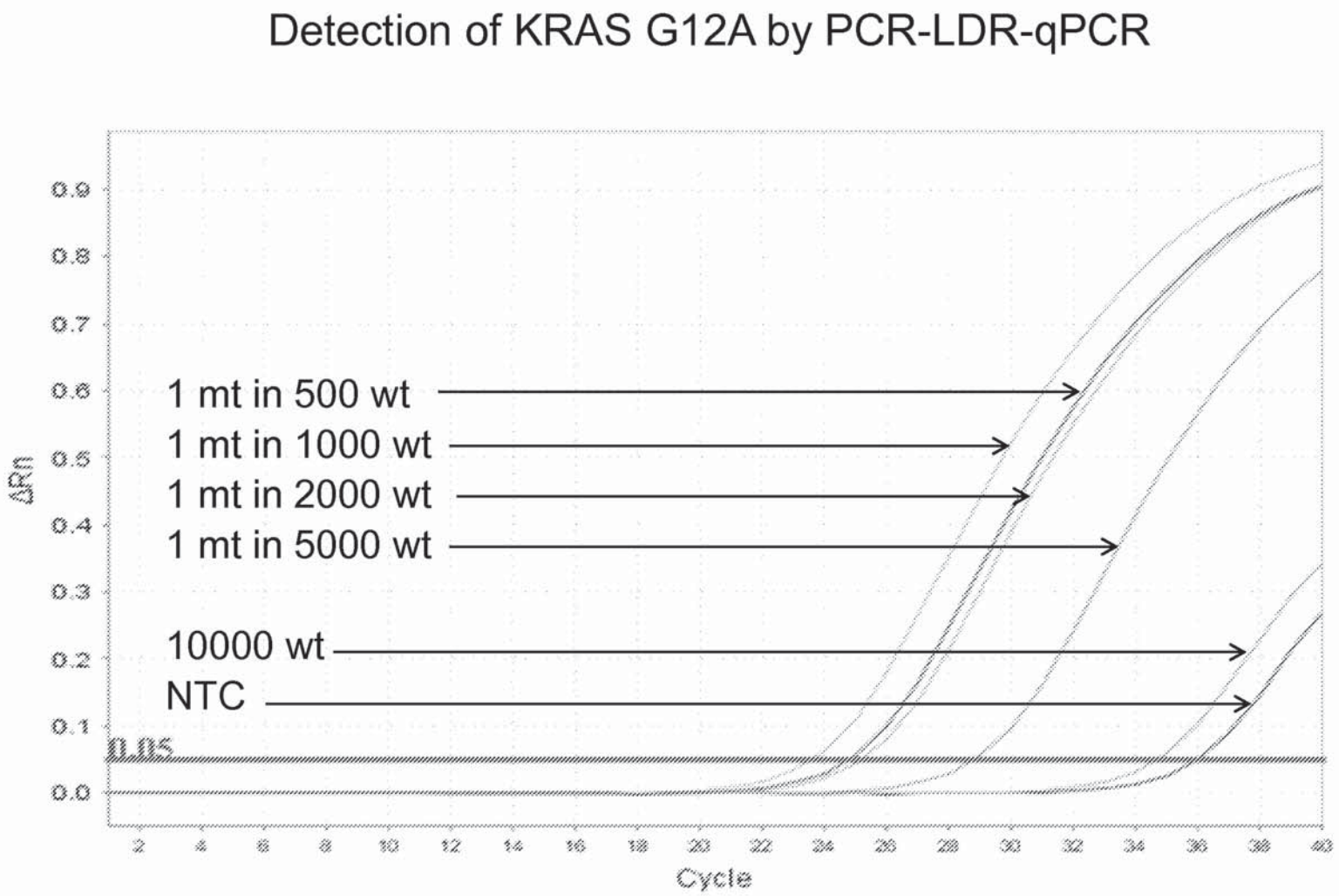

Detection of KRAS G12A by PCR-LDR-qPCR
ΔRn
Cycle
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
1 mt in 5000 wt
10000 wt
NTC
0.05

Figure 170

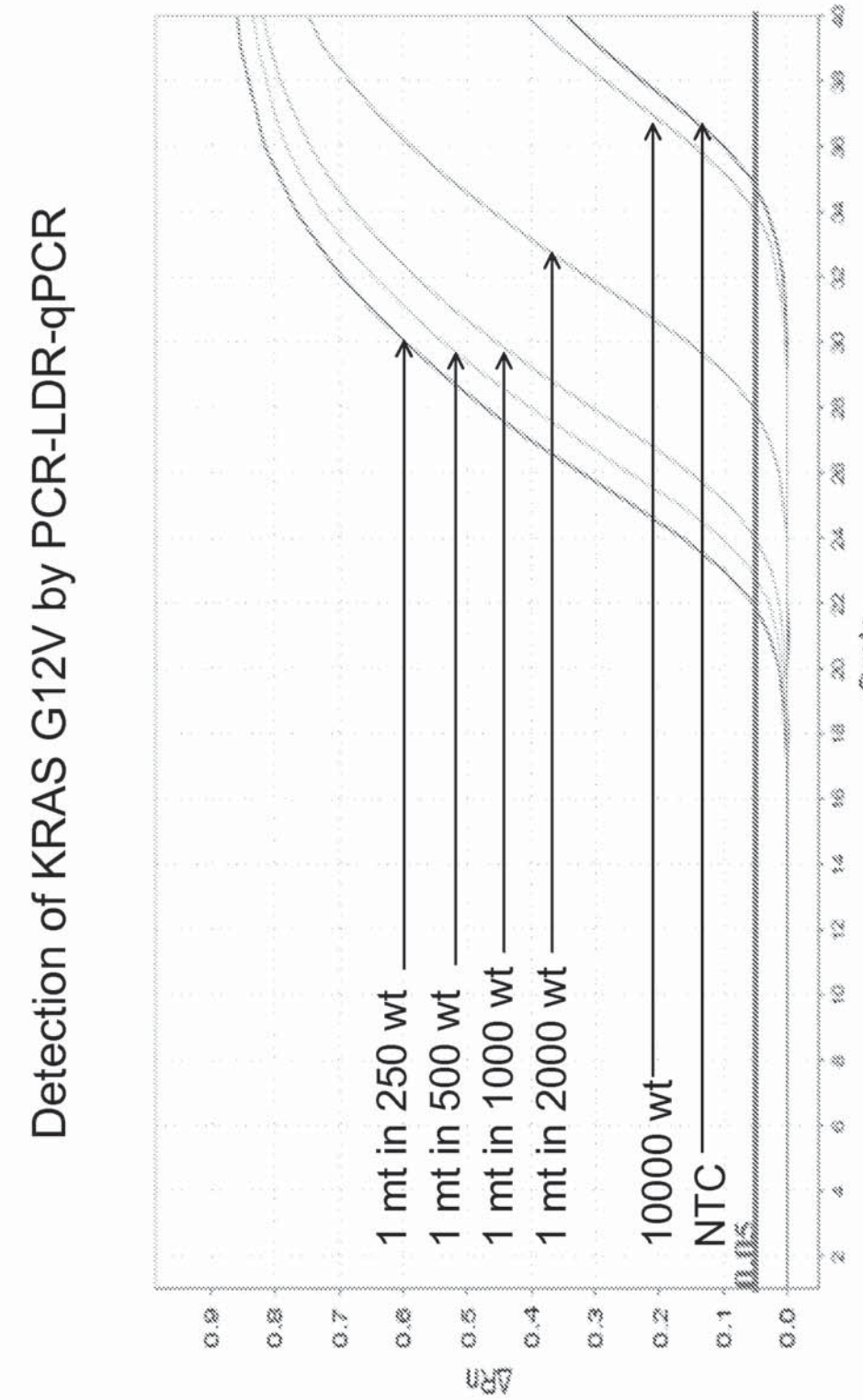

Detection of KRAS G12V by PCR-LDR-qPCR
1 mt in 250 wt
1 mt in 500 wt
1 mt in 1000 wt
1 mt in 2000 wt
10000 wt
NTC
Cycle

Figure 171

Single molecule detection of KRAS G12V by PCR-LDR-qPCR in Plasma

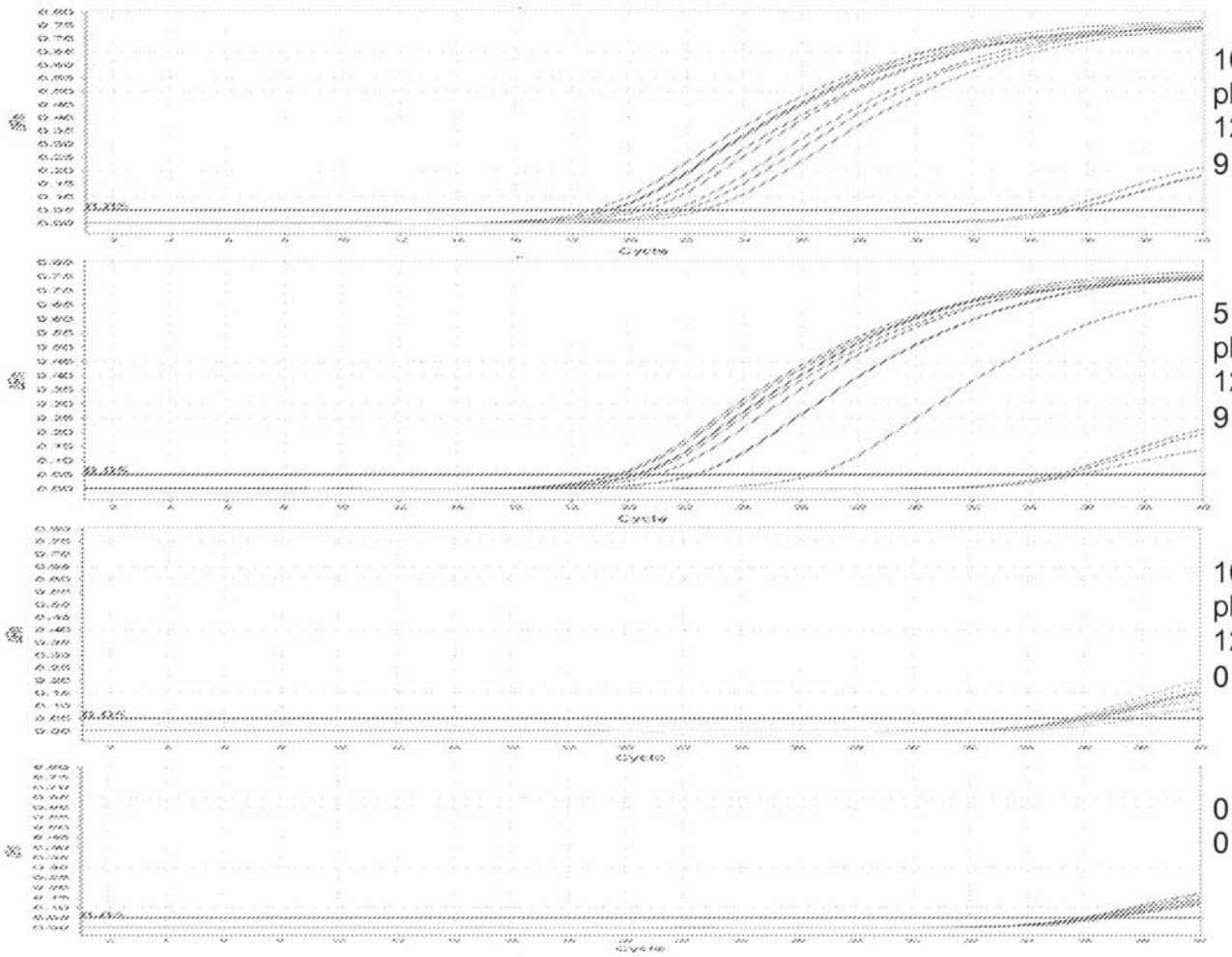


Figure 172

Detection of methylation at VIM-S3 by PCR-LDR-qPCR

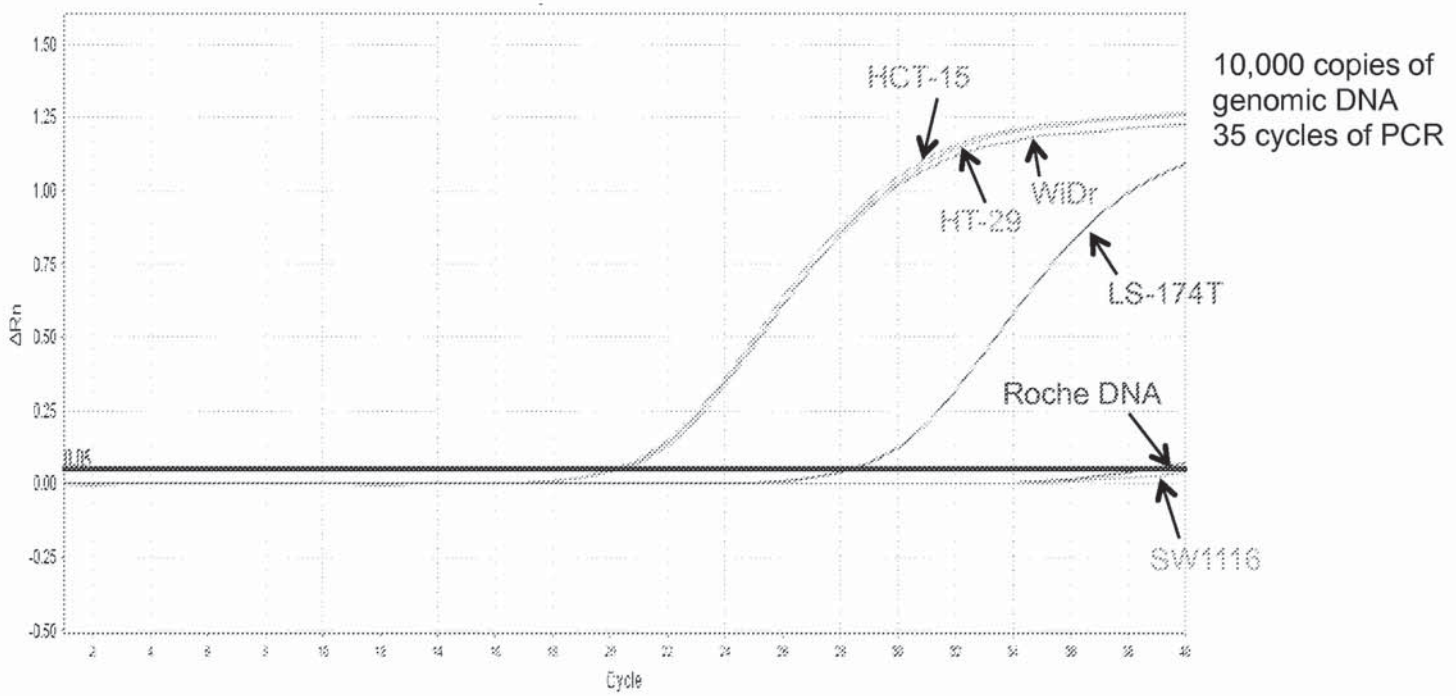


Figure 173

Single molecule detection of methylation of VIM_S3 by PCR-LDR-qPCR

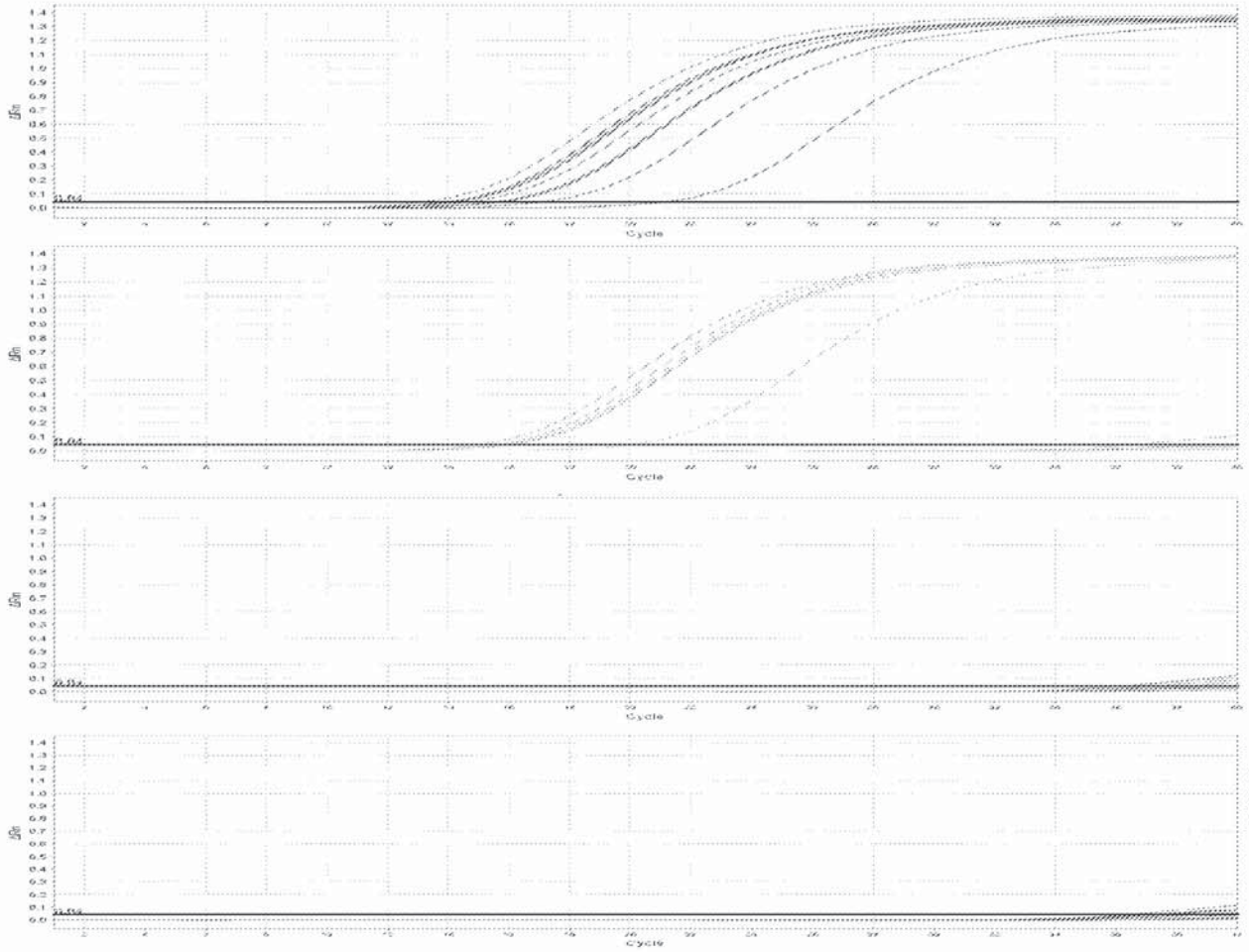


Figure 174

Methylation Detection VIM_S3 Top strand using PCR-LDR-qPCR and Taqman Probe version "A"

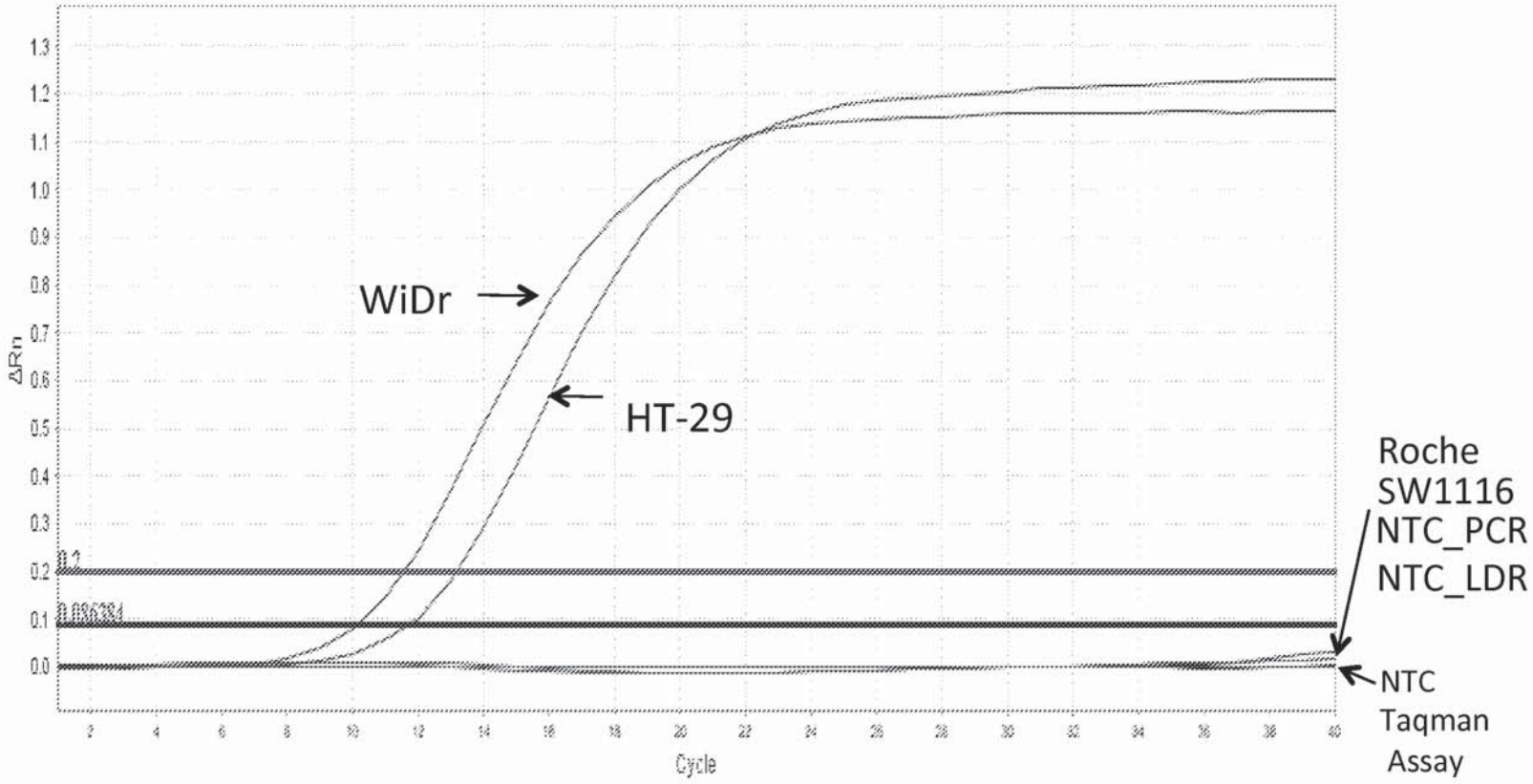


Figure 175

Methylation Detection VIM_S3 Bottom strand using PCR-LDR-qPCR and Taqman Probe version “A”

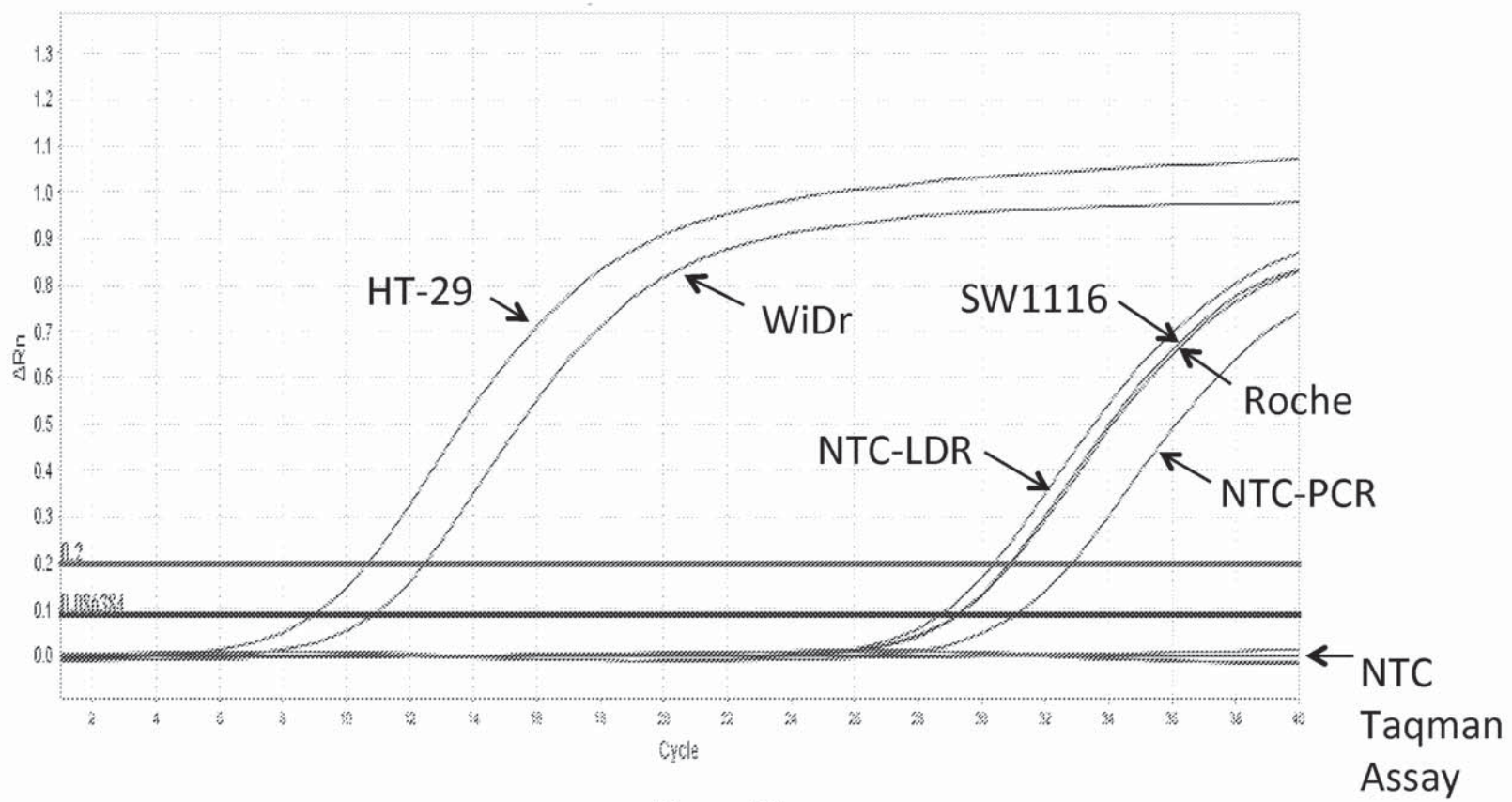


Figure 176

Methylation Detection VIM_S3 Top strand using PCR-LDR-qPCR and Taqman Probe version "B"

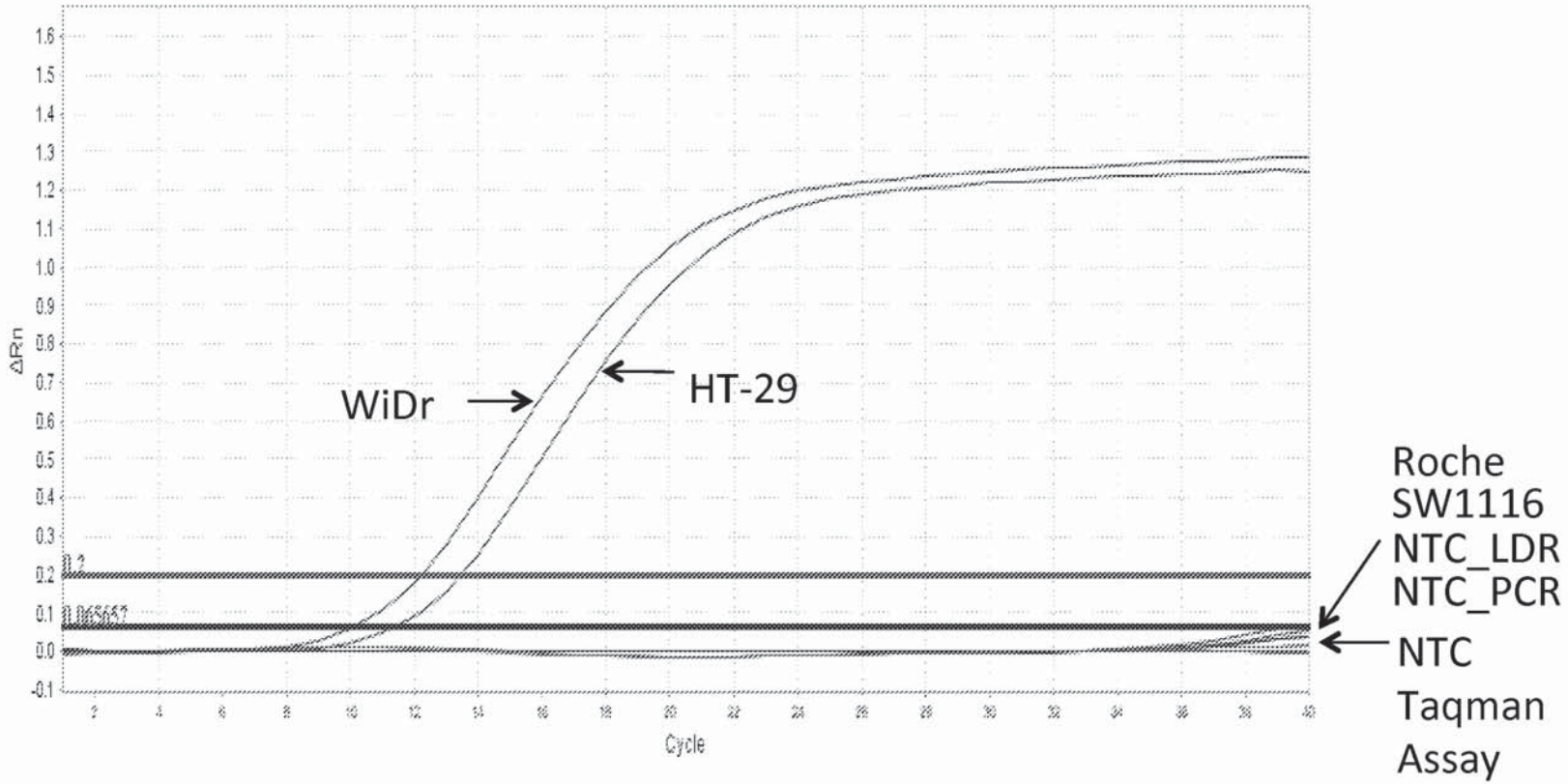


Figure 177

Methylation Detection VIM_S3 Bottom strand using PCR-LDR-qPCR and Taqman Probe version "B"

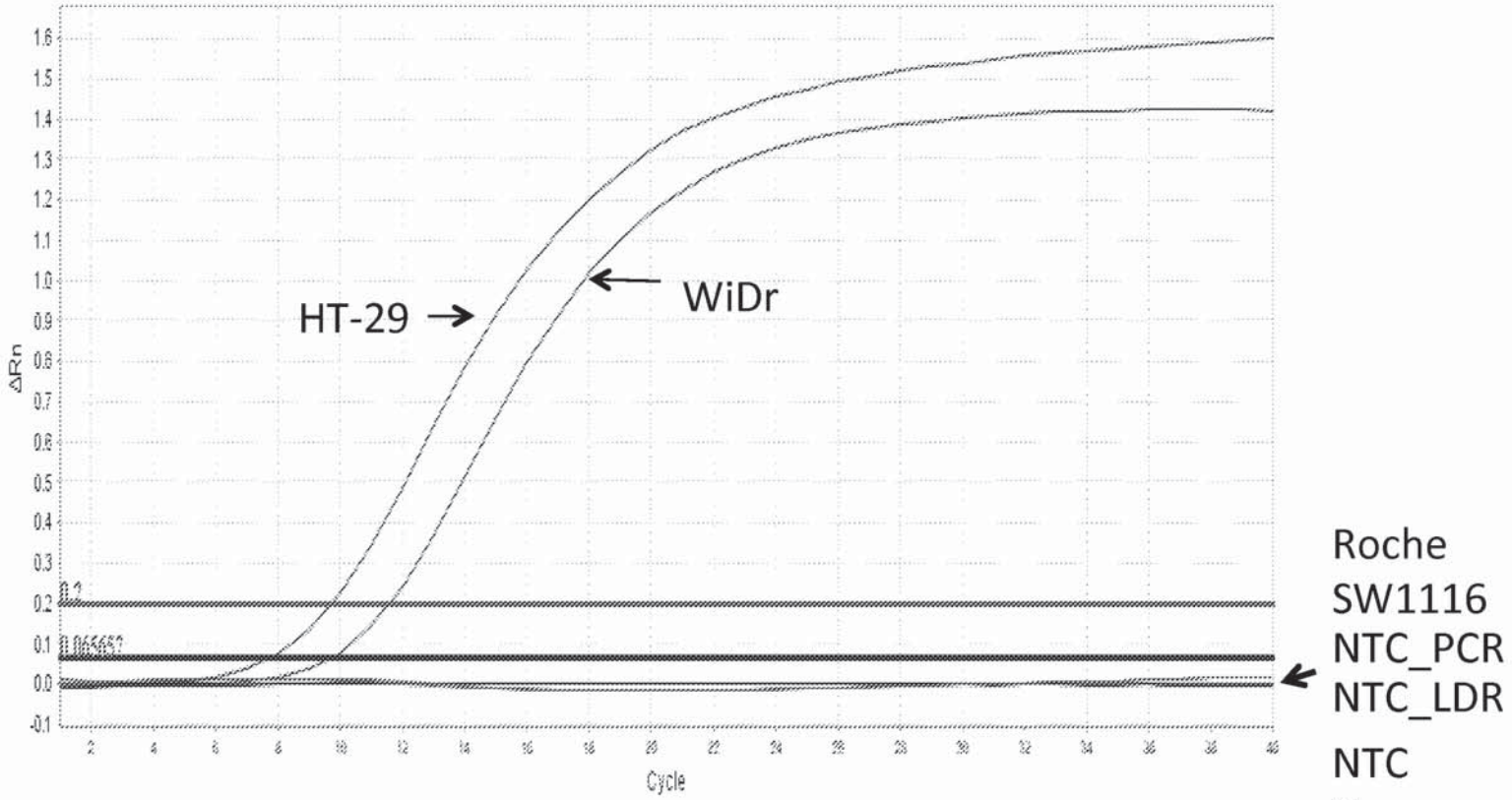


Figure 178