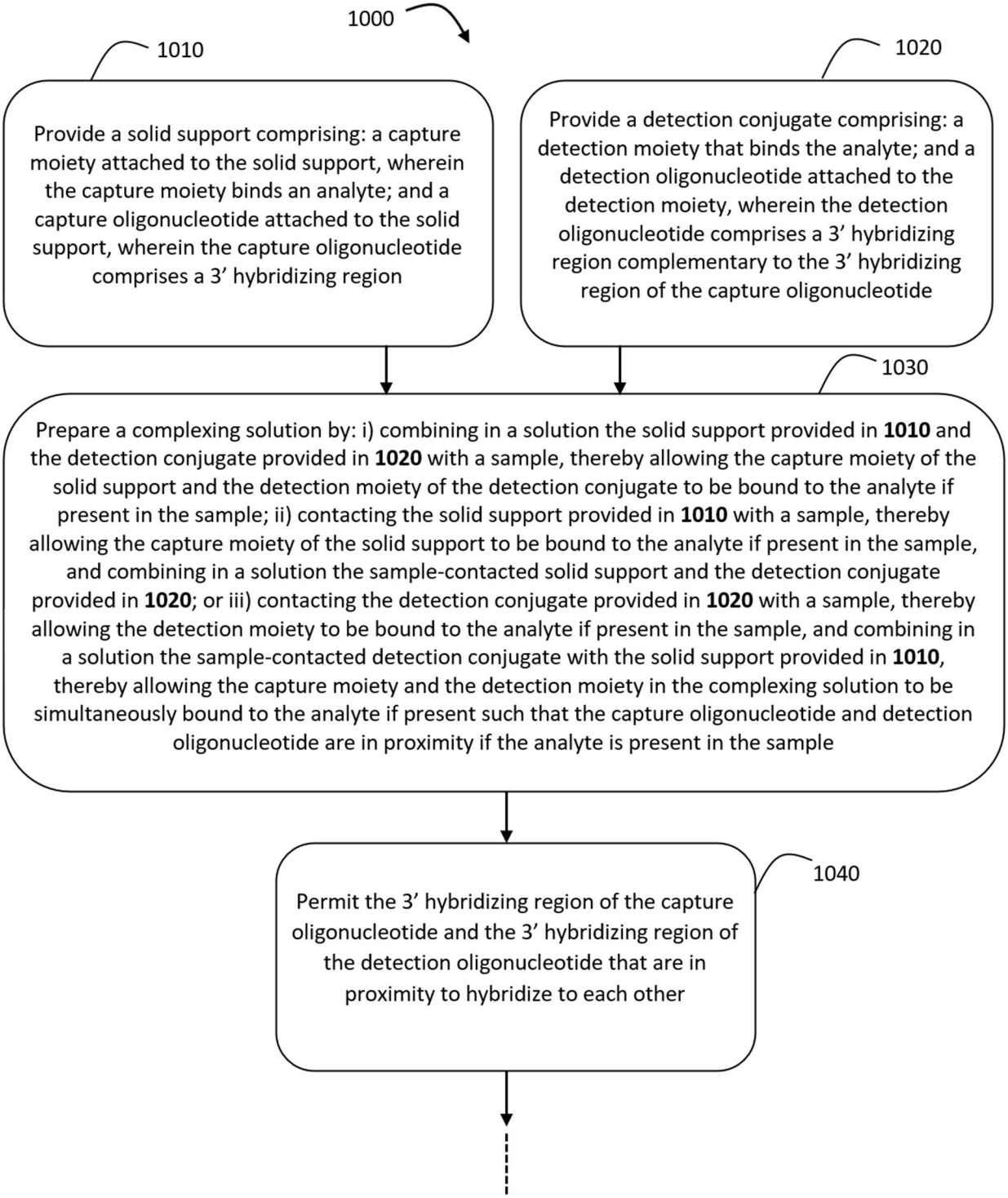

1000
1010
Provide a solid support comprising: a capture moiety attached to the solid support, wherein the capture moiety binds an analyte; and a capture oligonucleotide attached to the solid support, wherein the capture oligonucleotide comprises a 3' hybridizing region
1020
Provide a detection conjugate comprising: a detection moiety that binds the analyte; and a detection oligonucleotide attached to the detection moiety, wherein the detection oligonucleotide comprises a 3' hybridizing region complementary to the 3' hybridizing region of the capture oligonucleotide
1030
Prepare a complexing solution by: i) combining in a solution the solid support provided in **1010** and the detection conjugate provided in **1020** with a sample, thereby allowing the capture moiety of the solid support and the detection moiety of the detection conjugate to be bound to the analyte if present in the sample; ii) contacting the solid support provided in **1010** with a sample, thereby allowing the capture moiety of the solid support to be bound to the analyte if present in the sample, and combining in a solution the sample-contacted solid support and the detection conjugate provided in **1020**; or iii) contacting the detection conjugate provided in **1020** with a sample, thereby allowing the detection moiety to be bound to the analyte if present in the sample, and combining in a solution the sample-contacted detection conjugate with the solid support provided in **1010**, thereby allowing the capture moiety and the detection moiety in the complexing solution to be simultaneously bound to the analyte if present such that the capture oligonucleotide and detection oligonucleotide are in proximity if the analyte is present in the sample
1040
Permit the 3' hybridizing region of the capture oligonucleotide and the 3' hybridizing region of the detection oligonucleotide that are in proximity to hybridize to each other

FIG. 1

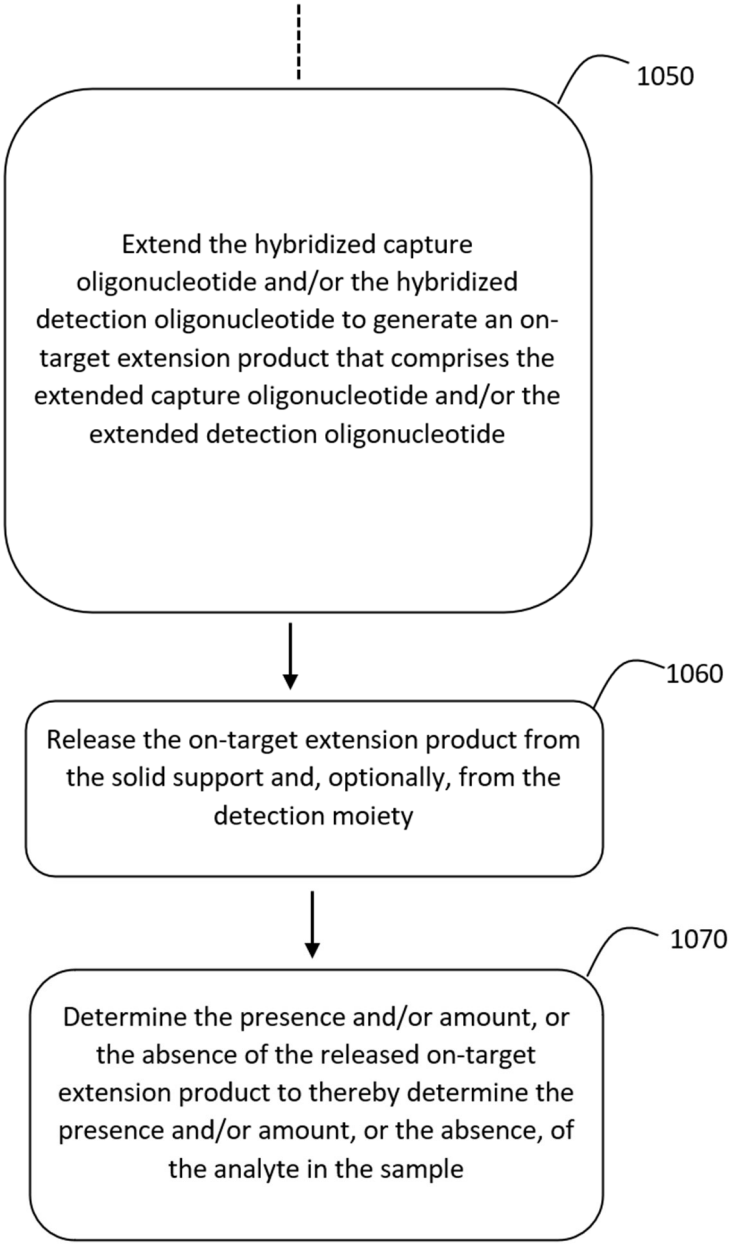


FIG. 1

CONTINUED

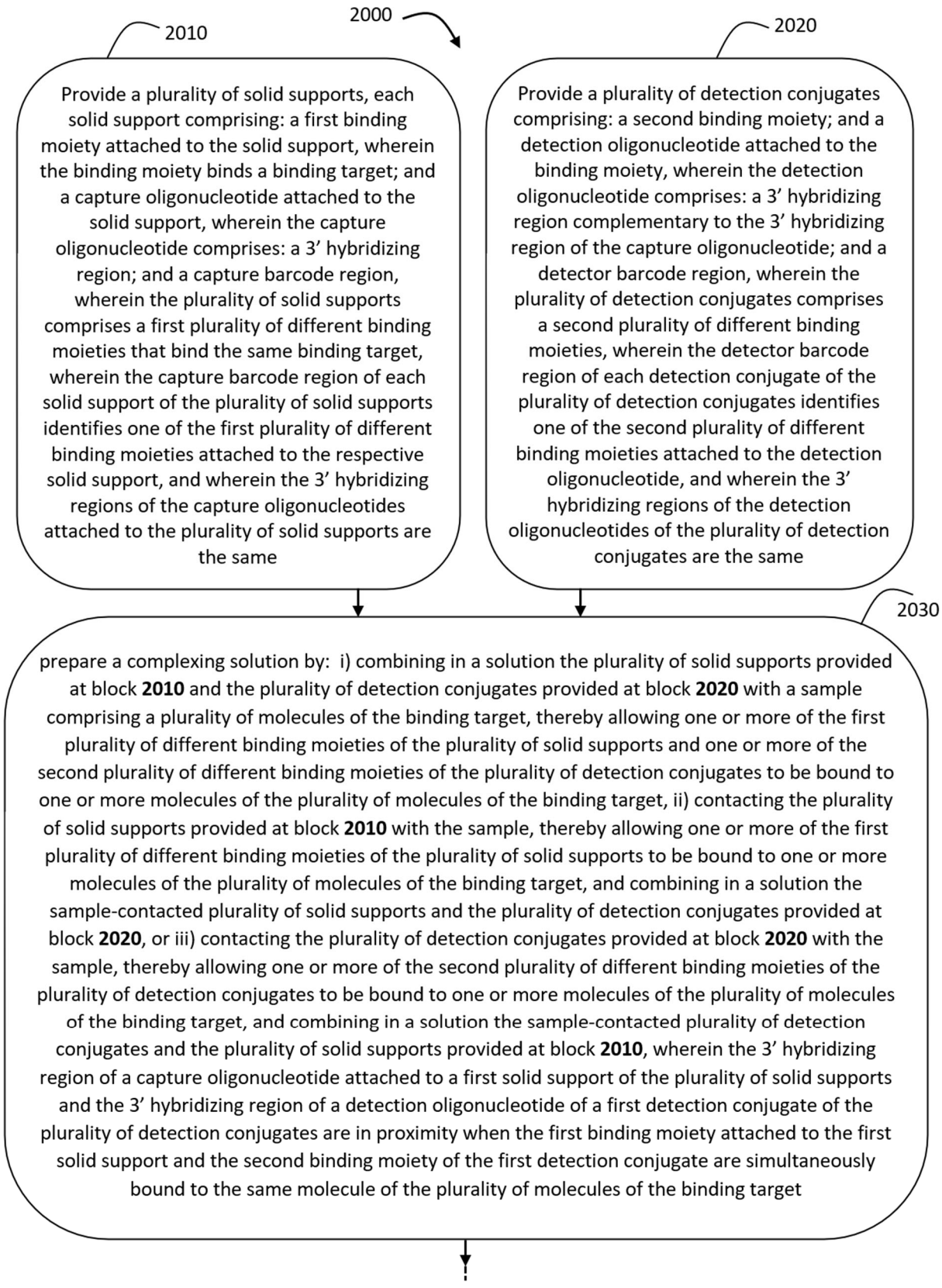

2000
2010
Provide a plurality of solid supports, each solid support comprising: a first binding moiety attached to the solid support, wherein the binding moiety binds a binding target; and a capture oligonucleotide attached to the solid support, wherein the capture oligonucleotide comprises: a 3' hybridizing region; and a capture barcode region, wherein the plurality of solid supports comprises a first plurality of different binding moieties that bind the same binding target, wherein the capture barcode region of each solid support of the plurality of solid supports identifies one of the first plurality of different binding moieties attached to the respective solid support, and wherein the 3' hybridizing regions of the capture oligonucleotides attached to the plurality of solid supports are the same
2020
Provide a plurality of detection conjugates comprising: a second binding moiety; and a detection oligonucleotide attached to the binding moiety, wherein the detection oligonucleotide comprises: a 3' hybridizing region complementary to the 3' hybridizing region of the capture oligonucleotide; and a detector barcode region, wherein the plurality of detection conjugates comprises a second plurality of different binding moieties, wherein the detector barcode region of each detection conjugate of the plurality of detection conjugates identifies one of the second plurality of different binding moieties attached to the detection oligonucleotide, and wherein the 3' hybridizing regions of the detection oligonucleotides of the plurality of detection conjugates are the same
2030
prepare a complexing solution by: i) combining in a solution the plurality of solid supports provided at block **2010** and the plurality of detection conjugates provided at block **2020** with a sample comprising a plurality of molecules of the binding target, thereby allowing one or more of the first plurality of different binding moieties of the plurality of solid supports and one or more of the second plurality of different binding moieties of the plurality of detection conjugates to be bound to one or more molecules of the plurality of molecules of the binding target, ii) contacting the plurality of solid supports provided at block **2010** with the sample, thereby allowing one or more of the first plurality of different binding moieties of the plurality of solid supports to be bound to one or more molecules of the plurality of molecules of the binding target, and combining in a solution the sample-contacted plurality of solid supports and the plurality of detection conjugates provided at block **2020**, or iii) contacting the plurality of detection conjugates provided at block **2020** with the sample, thereby allowing one or more of the second plurality of different binding moieties of the plurality of detection conjugates to be bound to one or more molecules of the plurality of molecules of the binding target, and combining in a solution the sample-contacted plurality of detection conjugates and the plurality of solid supports provided at block **2010**, wherein the 3' hybridizing region of a capture oligonucleotide attached to a first solid support of the plurality of solid supports and the 3' hybridizing region of a detection oligonucleotide of a first detection conjugate of the plurality of detection conjugates are in proximity when the first binding moiety attached to the first solid support and the second binding moiety of the first detection conjugate are simultaneously bound to the same molecule of the plurality of molecules of the binding target

FIG. 2

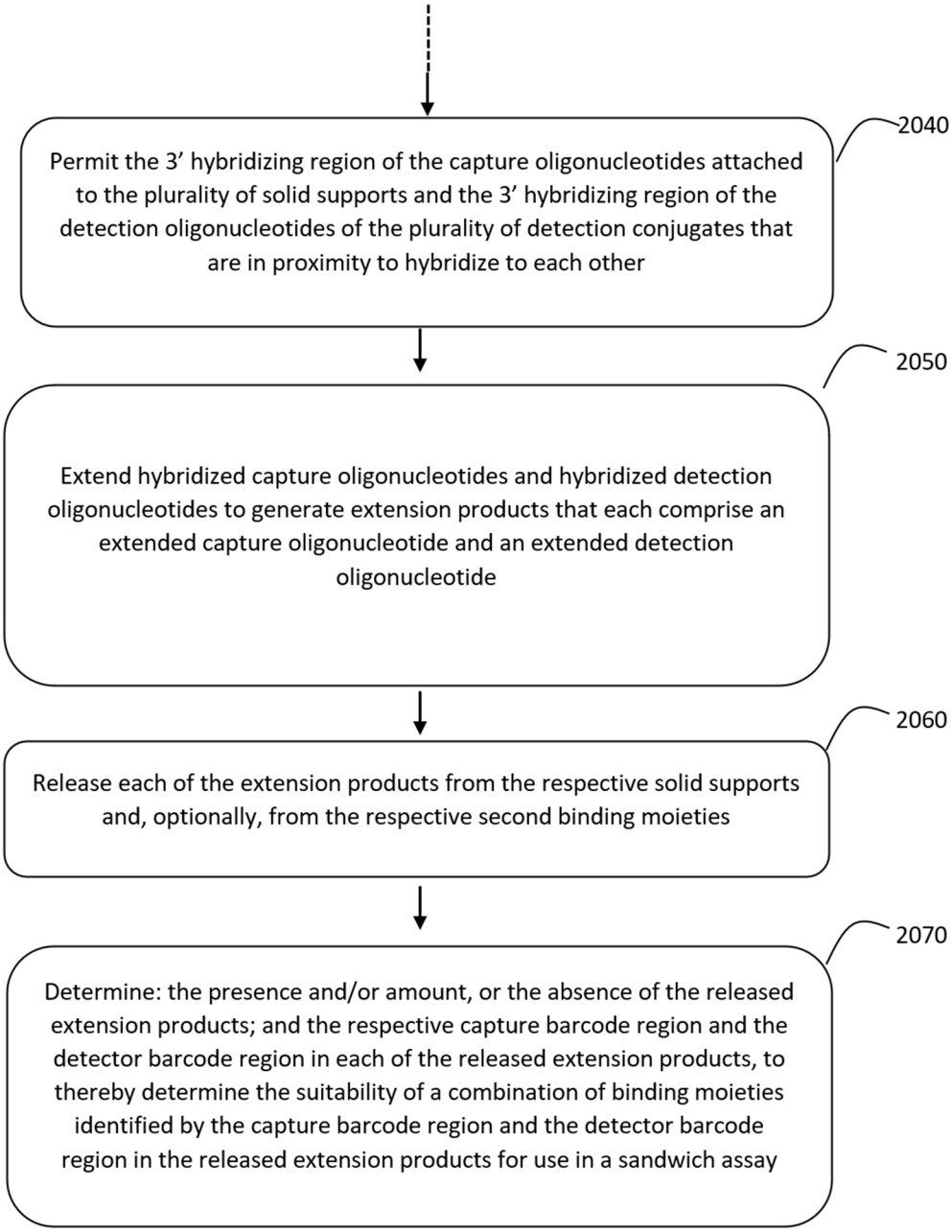


FIG. 2

CONTINUED



FIG. 3A



FIG. 3B

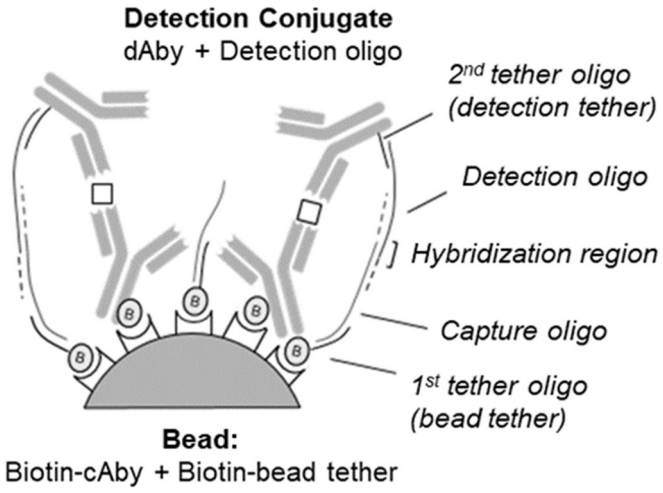


FIG. 3C

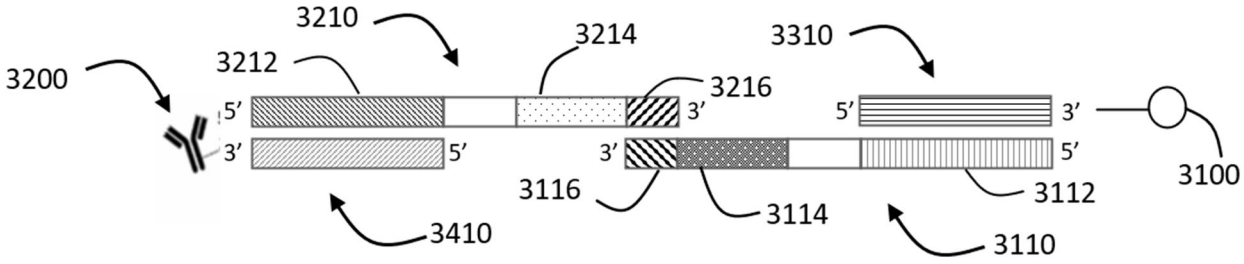


FIG. 3D

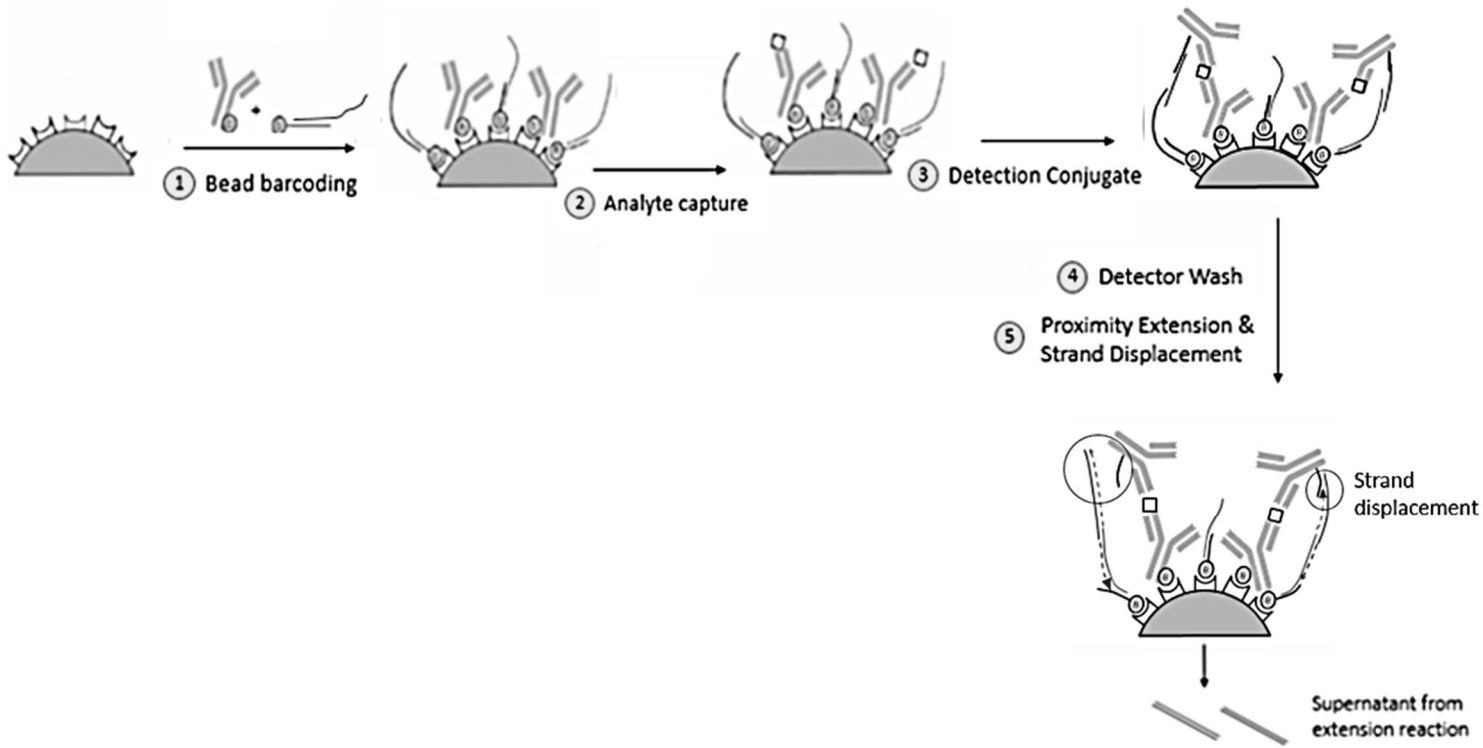

1 Bead barcoding
2 Analyte capture
3 Detection Conjugate
4 Detector Wash
5 Proximity Extension & Strand Displacement
Strand displacement
Supernatant from extension reaction

FIG. 4



FIG. 5A

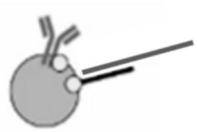


FIG. 5B

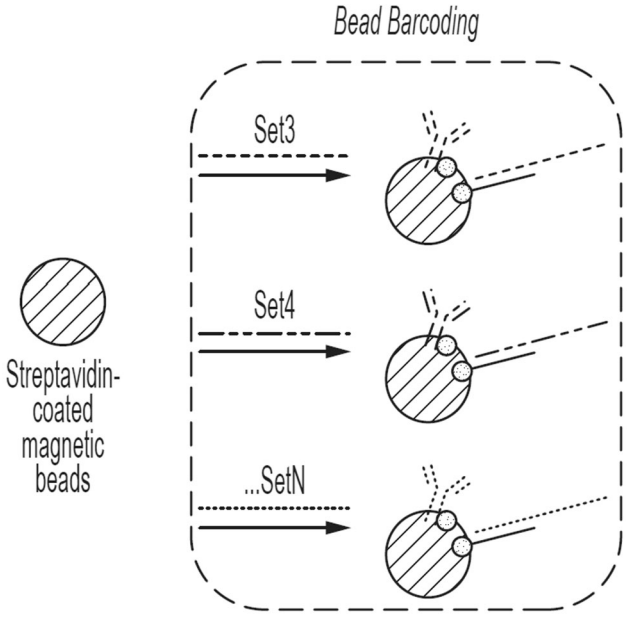


FIG. 5C



FIG. 5D

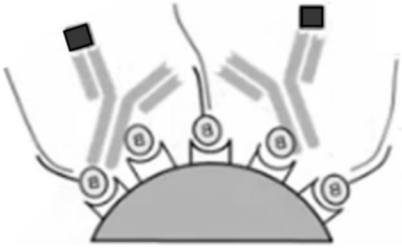


FIG. 5E

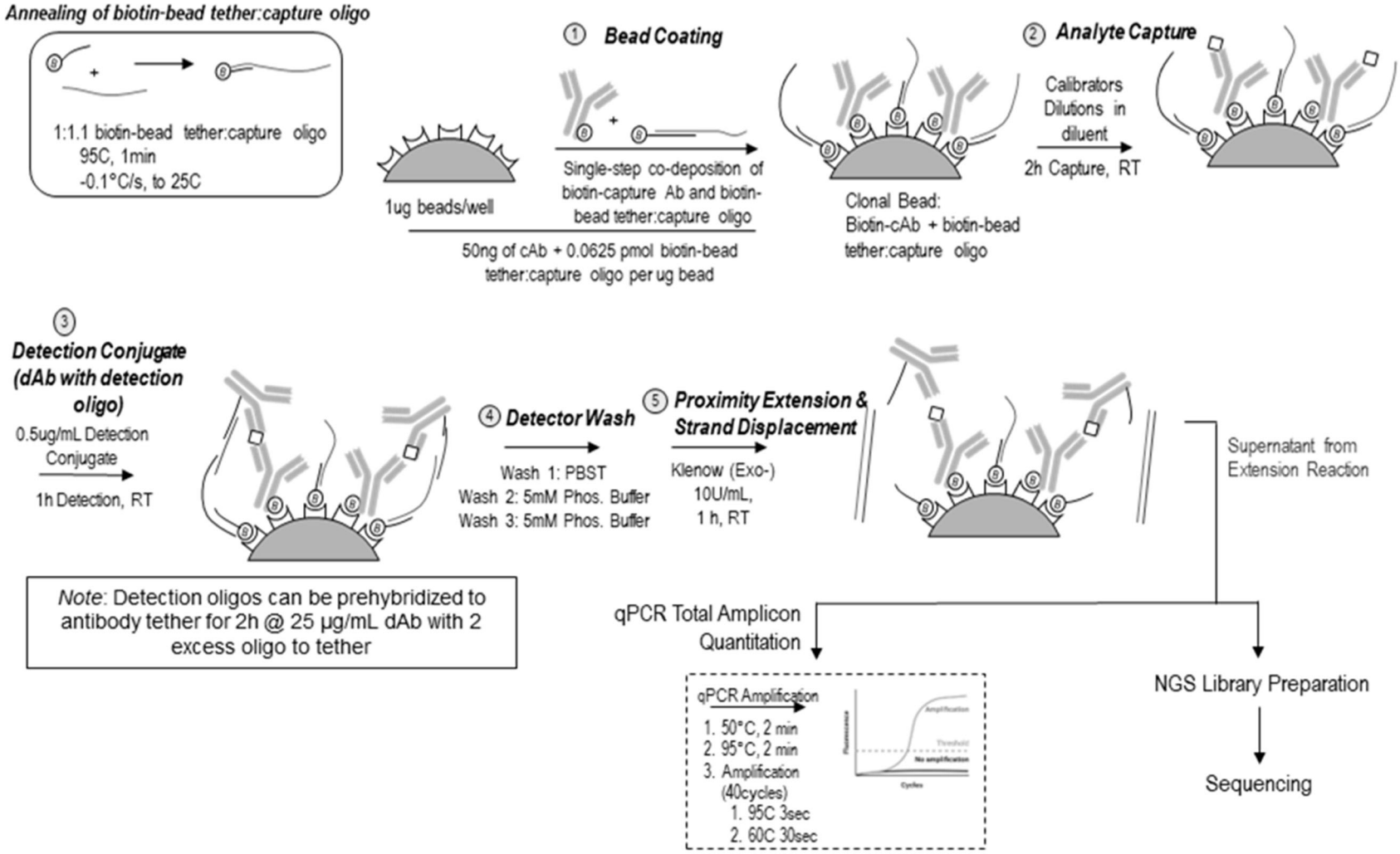

Annealing of biotin-bead tether:capture oligo
1:1.1 biotin-bead tether:capture oligo
95C, 1min
-0.1°C/s, to 25C
1ug beads/well
① Bead Coating
Single-step co-deposition of biotin-capture Ab and biotin-bead tether:capture oligo
50ng of cAb + 0.0625 pmol biotin-bead tether:capture oligo per ug bead
Clonal Bead:
Biotin-cAb + biotin-bead tether:capture oligo
② Analyte Capture
Calibrators
Dilutions in diluent
2h Capture, RT
③
Detection Conjugate (dAb with detection oligo)
0.5ug/mL Detection Conjugate
1h Detection, RT
④ Detector Wash
Wash 1: PBST
Wash 2: 5mM Phos. Buffer
Wash 3: 5mM Phos. Buffer
⑤ Proximity Extension & Strand Displacement
Klenow (Exo-)
10U/mL,
1 h, RT
Supernatant from Extension Reaction
Note: Detection oligos can be prehybridized to antibody tether for 2h @ 25 µg/mL dAb with 2 excess oligo to tether
qPCR Total Amplicon Quantitation
qPCR Amplification
1. 50°C, 2 min
2. 95°C, 2 min
3. Amplification (40cycles)
1. 95C 3sec
2. 60C 30sec
NGS Library Preparation
Sequencing

FIG. 6

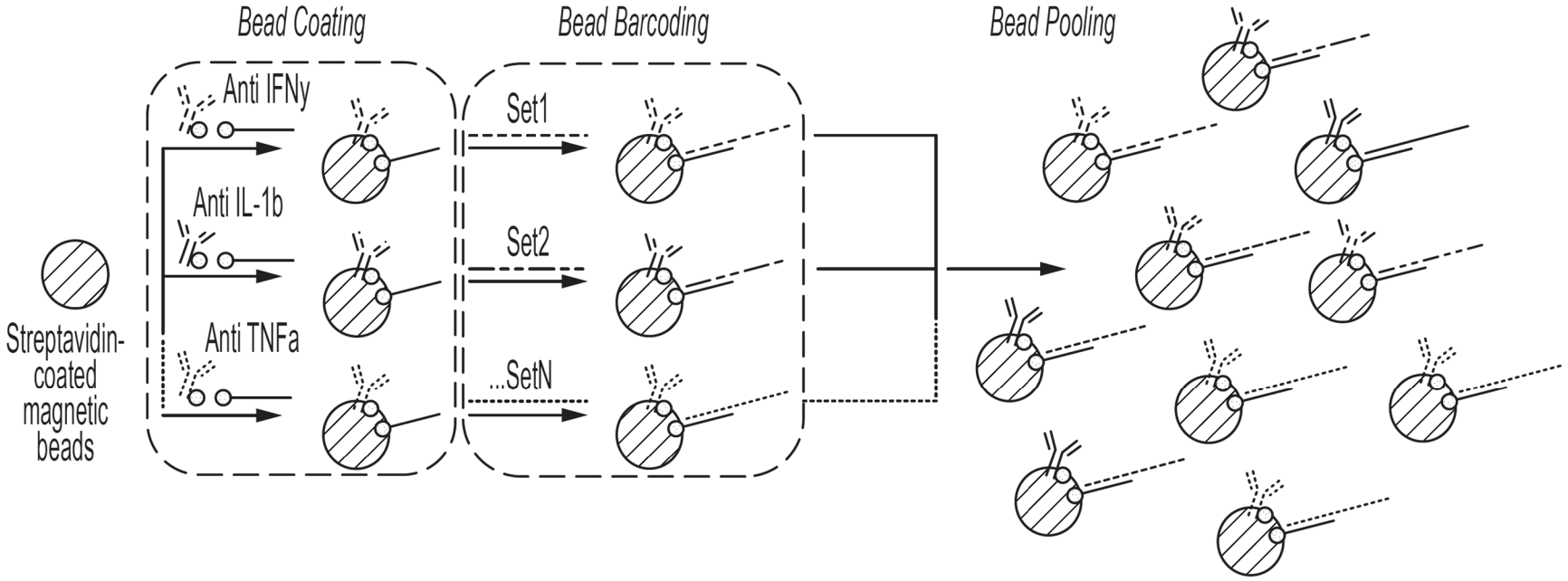

Bead Coating
Bead Barcoding
Bead Pooling
Anti IFNy
Anti IL-1b
Anti TNFa
Set1
Set2
...SetN
Streptavidin-coated magnetic beads

FIG. 7A

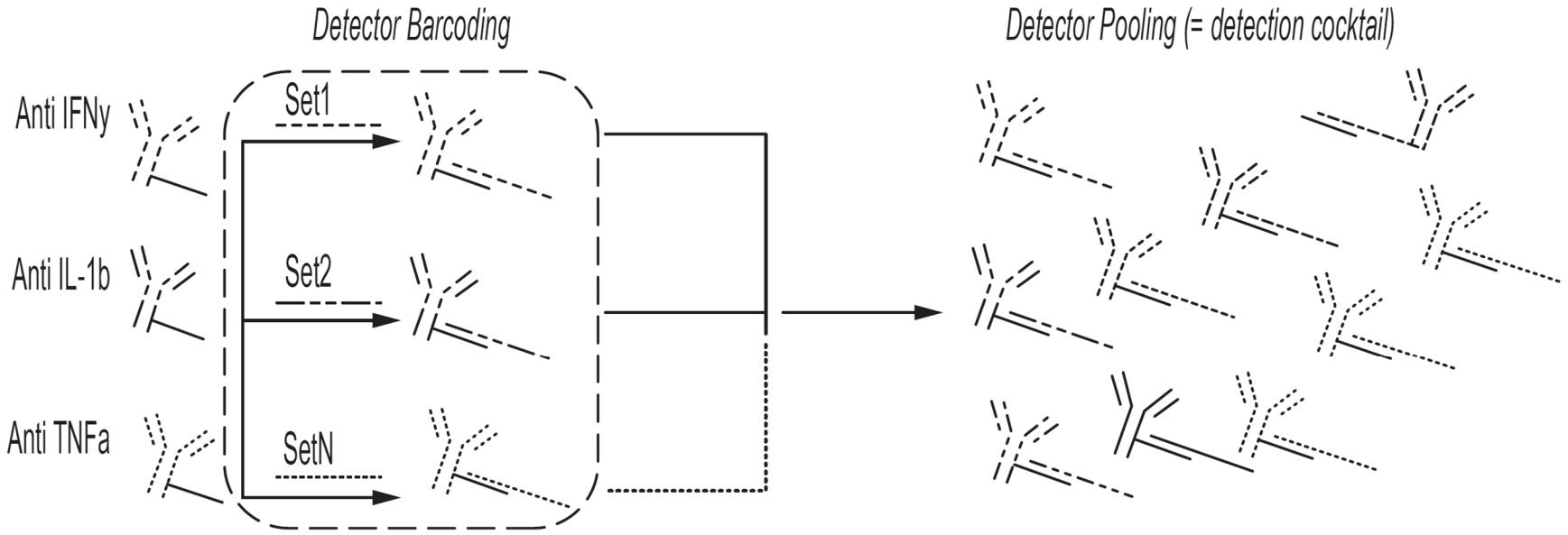

Detector Barcoding
Detector Pooling (= detection cocktail)
Anti IFNy
Anti IL-1b
Anti TNFa
Set1
Set2
SetN

FIG. 7B

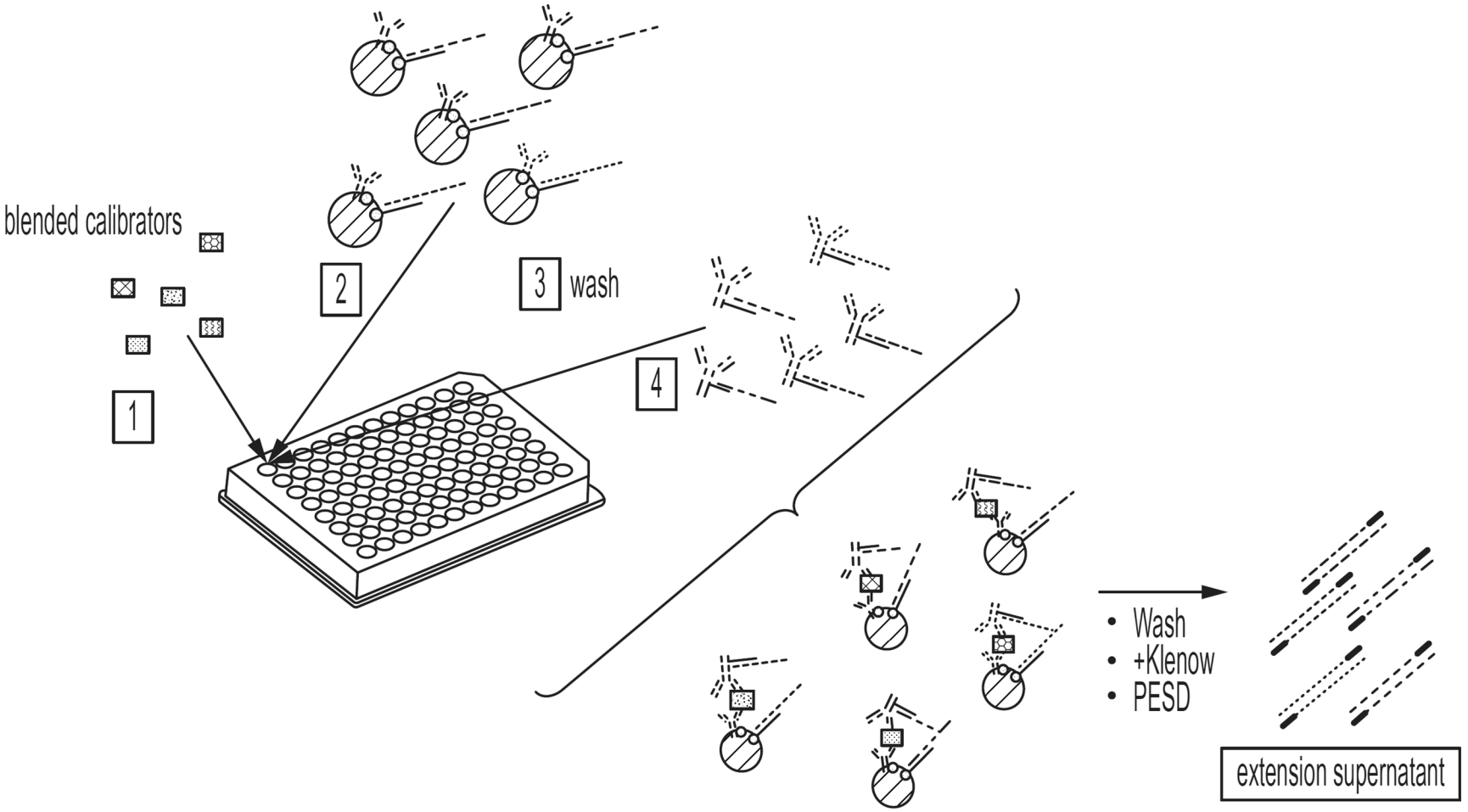

blended calibrators
1
2
3 wash
4
• Wash
• +Klenow
• PESD
extension supernatant

FIG. 8

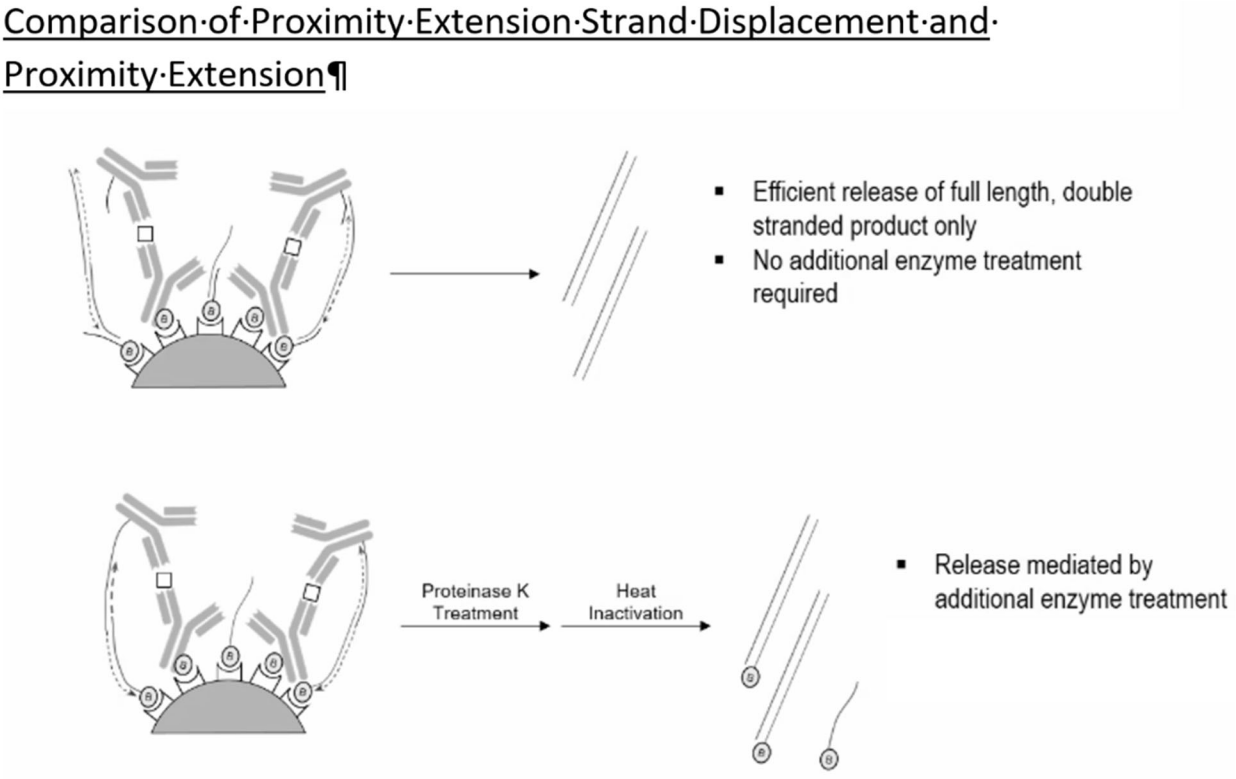

Comparison·of·Proximity·Extension·Strand·Displacement·and·
Proximity·Extension¶
Efficient release of full length, double stranded product only
No additional enzyme treatment required
Proteinase K Treatment
Heat Inactivation
Release mediated by additional enzyme treatment

FIG. 9

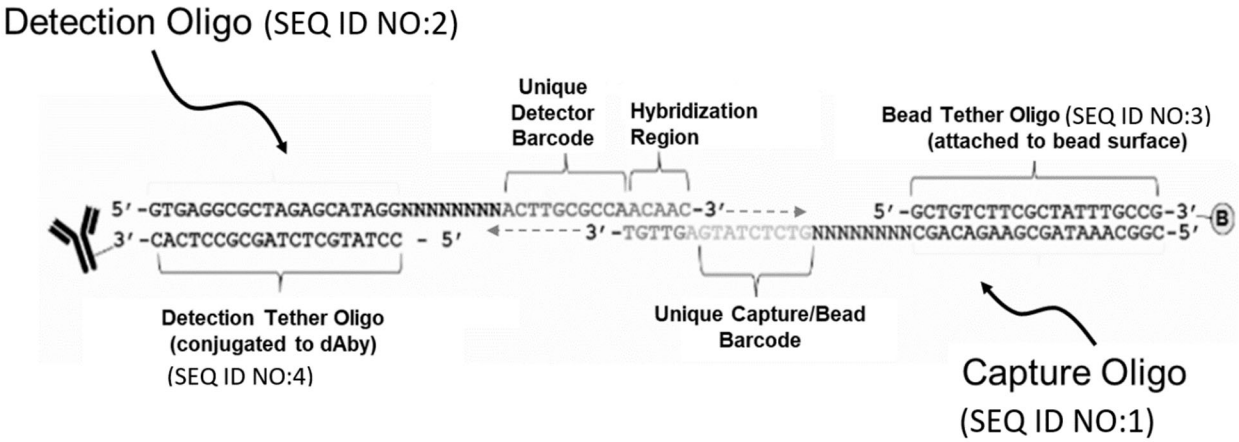

Detection Oligo (SEQ ID NO:2)
Unique Detector Barcode
Hybridization Region
Bead Tether Oligo (SEQ ID NO:3) (attached to bead surface)
5′-GTGAGGCGCTAGAGCATAGGNNNNNNNNACTTGCGCCAACAAC-3′
3′-CACTCCGCGATCTCGTATCC - 5′
3′-TGTTGAGTATCTCTGNNNNNNNNCGACAGAAGCGATAAACGGC-5′
5′-GCTGTCTTCGCTATTTGCCG-3′
B
Detection Tether Oligo (conjugated to dAby)
(SEQ ID NO:4)
Unique Capture/Bead Barcode
Capture Oligo
(SEQ ID NO:1)

FIG. 10

IL-13 PESD Assay

[IL-13] (pg/mL)	1	2
482	11.49	11.25
120.5	12.91	12.54
30.125	14.98	14.46
7.531	17.43	16.90
1.883	18.42	18.39
0.471	20.77	20.40
0.118	22.17	22.04
0	23.90	23.80

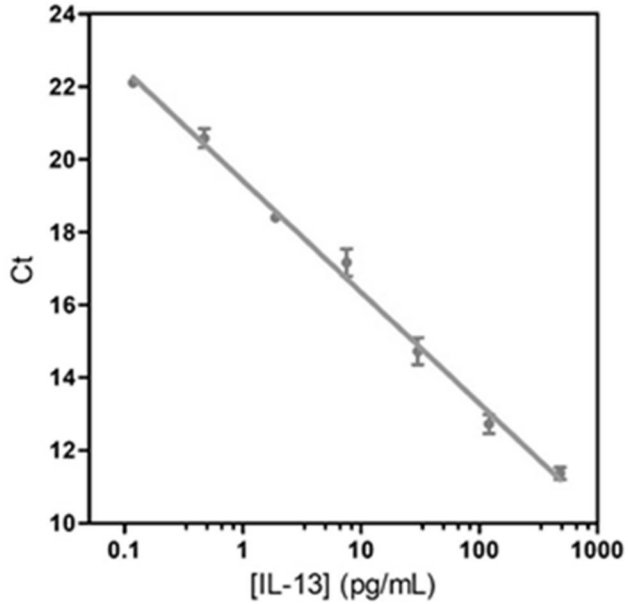


FIG. 11

	IFNy		IL-1b		IL-2		IL-4		IL-6		IL-8		IL-10		IL-12p70		IL-13		TNFa	
Calibrator #	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	5363	2718	13218	7098	19211	10815	64429	32872	229754	124449	6424	3812	12811	6829	13524	7762	134866	76384	712	402
2	1541	1509	3416	3342	5224	5006	17964	16763	73188	71921	1678	1588	3470	3517	4090	4250	34883	32760	171	169
3	434	306	894	717	1312	1005	4794	3491	21228	16055	426	282	974	738	1116	900	5915	4390	47	31
4	229	195	311	213	466	328	1690	1222	7178	5365	143	127	301	232	373	305	1376	983	27	22
5	156	87	107	63	161	99	626	342	2319	1418	57	58	105	66	119	83	350	234	14	5
6	71	68	18	22	28	42	86	138	332	418	16	38	20	15	23	27	46	59	15	6
7	75	69	6	3	20	20	71	46	103	79	20	21	10	5	12	21	22	15	5	4
8	28	229	3	9	7	60	37	200	7	120	10	64	4	19	4	25	2	23	3	11

FIG. 12A

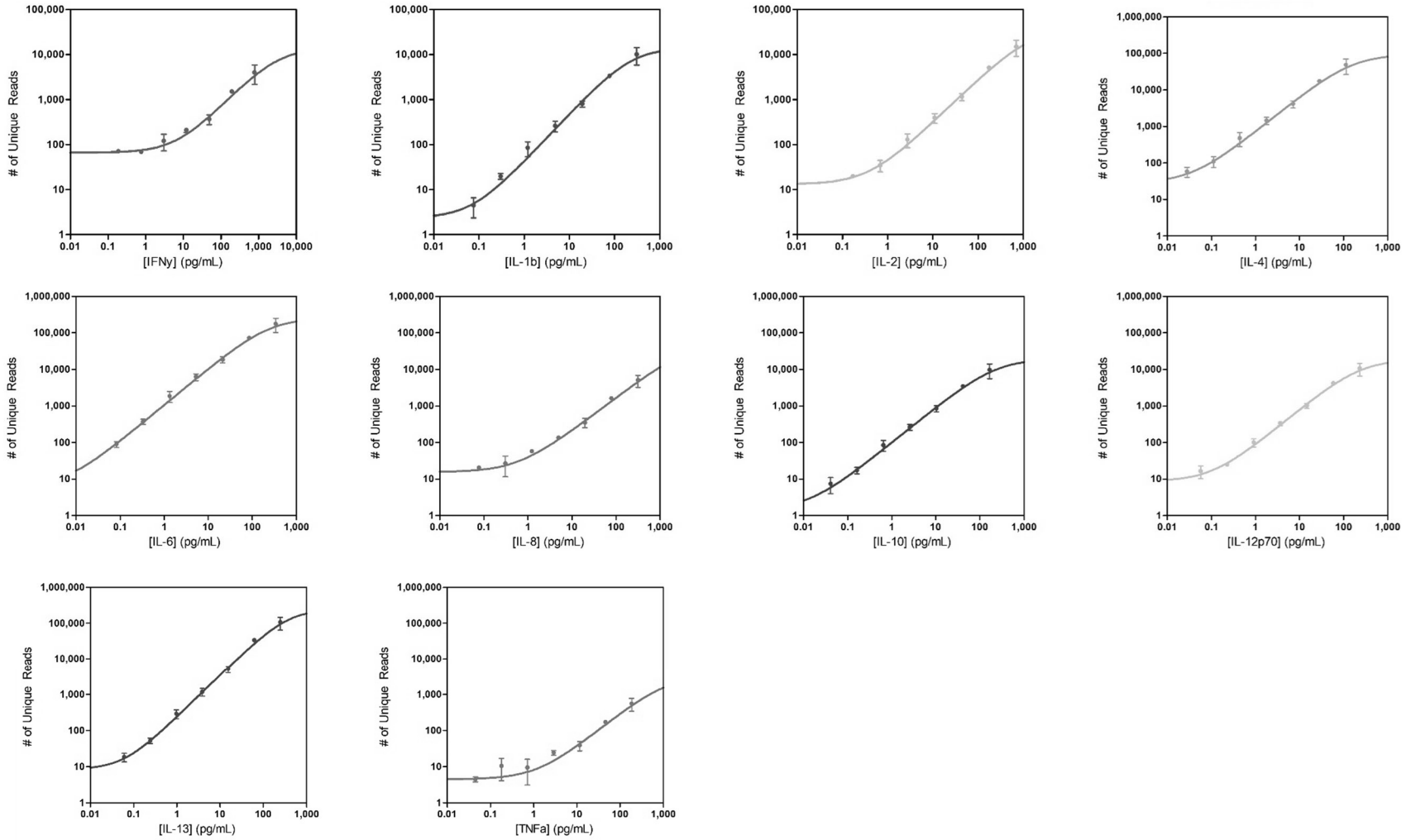

# of Unique Reads
[IFNy] (pg/mL)
[IL-1b] (pg/mL)
[IL-2] (pg/mL)
[IL-4] (pg/mL)
[IL-6] (pg/mL)
[IL-8] (pg/mL)
[IL-10] (pg/mL)
[IL-12p70] (pg/mL)
[IL-13] (pg/mL)
[TNFa] (pg/mL)

FIG. 12B

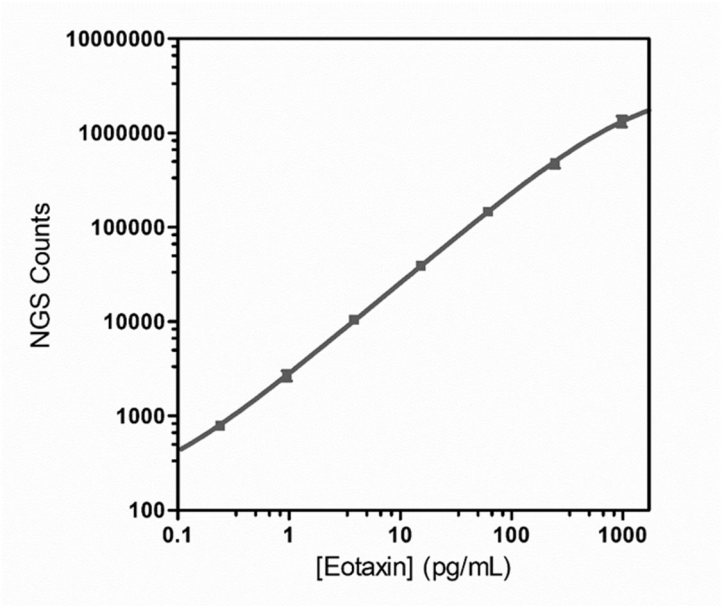

10000000
1000000
100000
10000
1000
100
NGS Counts
0.1
1
10
100
1000
[Eotaxin] (pg/mL)

FIG. 13

Assay \ Calibrator	IL-27	Eotaxin	IL-22	IL-31	MIP-3a	Granzyme A	MIP-1a	MIP-1b	MCP-1	IL-18	IL-6	FLT-1	IL-8	PlGF	VEGF-D	IL-10	GM-CSF	MCP-4	IL-4	MDC	TARC	TNF-b	IL-2Ra	IL-5	IL-15	IL-7	Eotaxin-3	Tie-2	IL-29	IL-21	IL-1b	IL-17a	IP-10	IL-2
IL-27	100%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%
Eotaxin	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
IL-22	0.01%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
IL-31	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.04%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
MIP-3a	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
Granzyme A	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%
MIP-1a	0.22%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.43%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.56%
MIP-1b	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
MCP-1	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	100%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
IL-18	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
IL-6	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
FLT-1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%
IL-8	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
PlGF	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.00%	0.02%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
VEGF-D	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
IL-10	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	100%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
GM-CSF	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
MCP-4	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.07%	1.80%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%
IL-4	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
MDC	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%
TARC	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%
TNF-b	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
IL-2Ra	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
IL-5	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.03%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%
IL-15	0.08%	0.02%	0.00%	0.01%	0.00%	0.00%	0.01%	0.08%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	100%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
IL-7	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%
Eotaxin-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%
Tie-2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	100%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%
IL-29	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	100%	0.00%	0.00%	0.00%	0.01%
IL-21	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%	0.01%
IL-1b	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.00%	0.00%
IL-17a	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	100%	0.02%
IP-10	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%
IL-2	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100%

FIG. 14

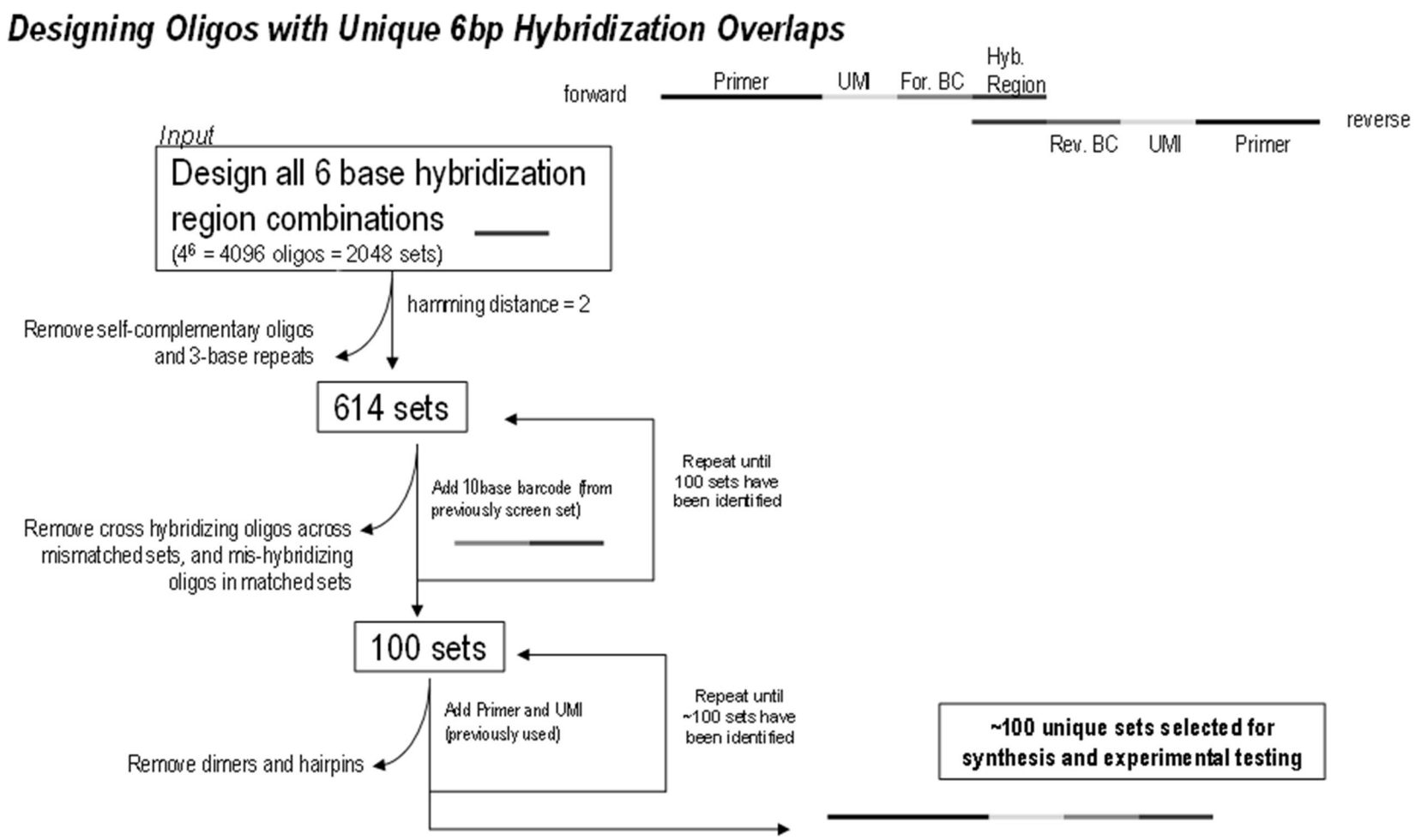

Designing Oligos with Unique 6bp Hybridization Overlaps
forward
Primer
UMI
For. BC
Hyb. Region
reverse
Rev. BC
UMI
Primer
Input
Design all 6 base hybridization region combinations
(4^6 = 4096 oligos = 2048 sets)
harmming distance = 2
Remove self-complementary oligos and 3-base repeats
614 sets
Add 10base barcode (from previously screen set)
Repeat until 100 sets have been identified
Remove cross hybridizing oligos across mismatched sets, and mis-hybridizing oligos in matched sets
100 sets
Add Primer and UMI (previously used)
Repeat until ~100 sets have been identified
Remove dimers and hairpins
~100 unique sets selected for synthesis and experimental testing

FIG. 15

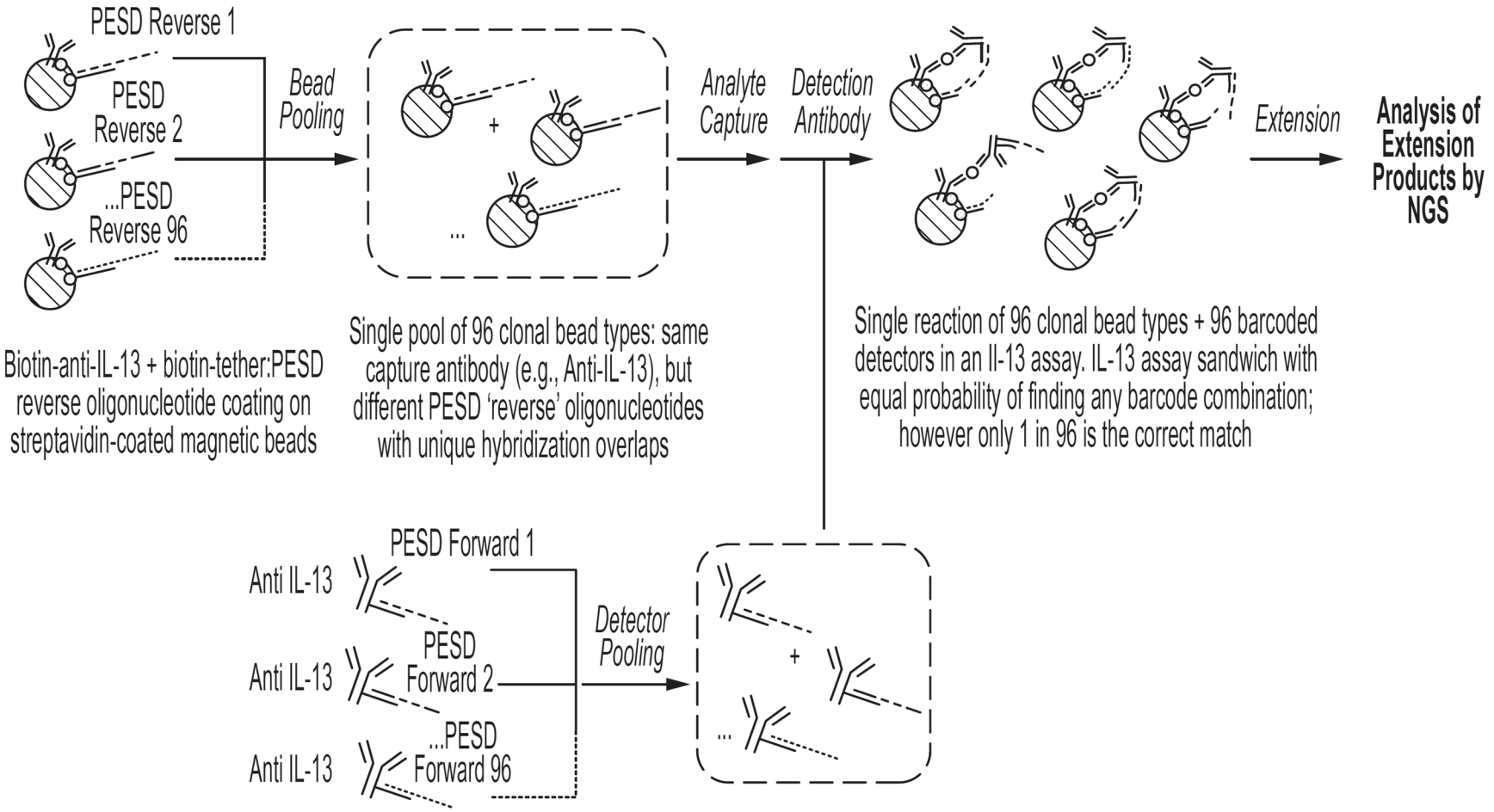
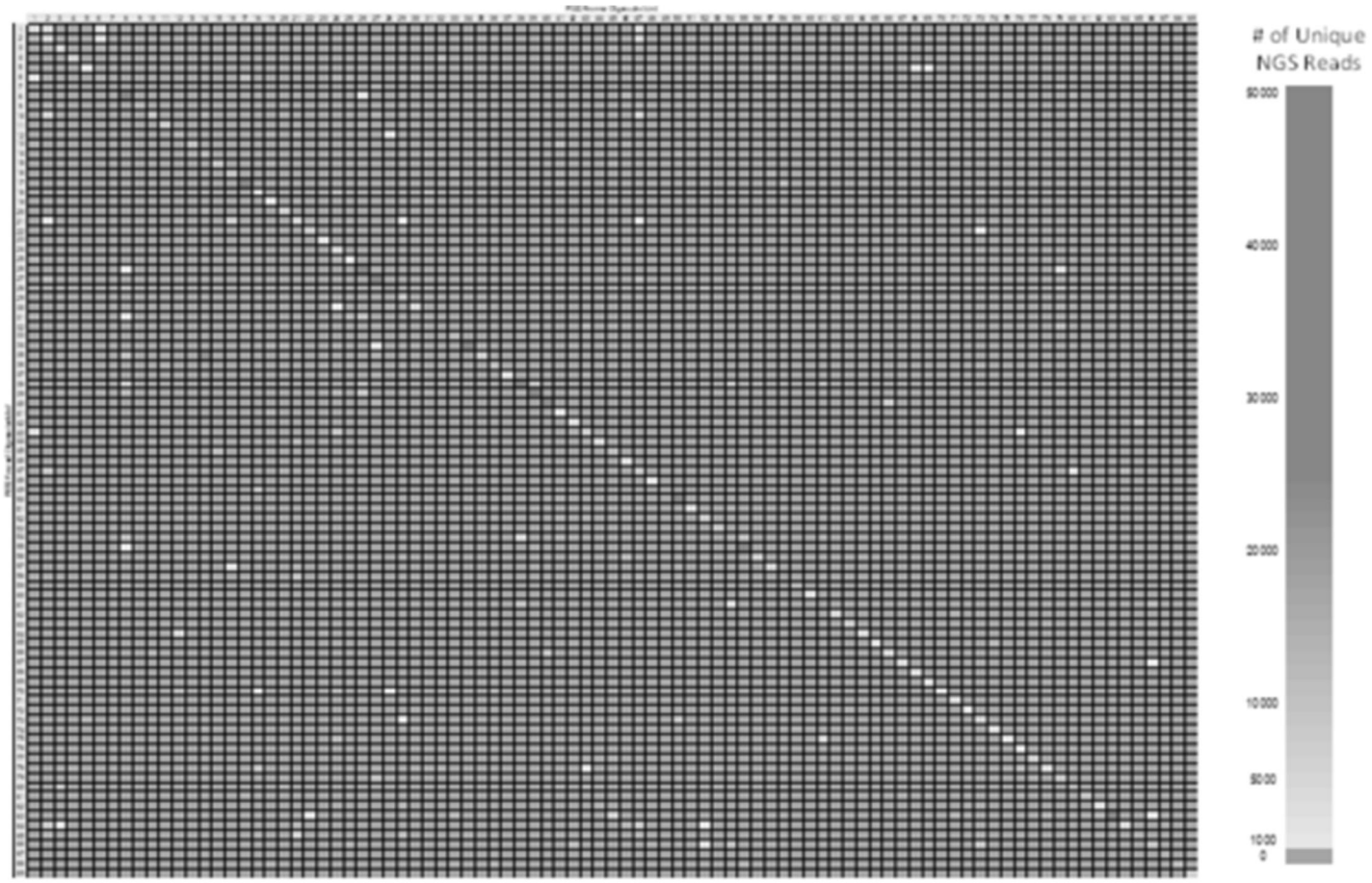

PESD Reverse 1
PESD Reverse 2
...PESD Reverse 96
Bead Pooling
Biotin-anti-IL-13 + biotin-tether:PESD reverse oligonucleotide coating on streptavidin-coated magnetic beads
Single pool of 96 clonal bead types: same capture antibody (e.g., Anti-IL-13), but different PESD 'reverse' oligonucleotides with unique hybridization overlaps
Analyte Capture
Detection Antibody
Single reaction of 96 clonal bead types + 96 barcoded detectors in an Il-13 assay. IL-13 assay sandwich with equal probability of finding any barcode combination; however only 1 in 96 is the correct match
Extension
Analysis of Extension Products by NGS
PESD Forward 1
Anti IL-13
PESD Forward 2
Anti IL-13
...PESD Forward 96
Anti IL-13
Detector Pooling

FIG. 16



The number of unique NGS reads identified for amplicons containing 2 barcodes during screening for 89 sets of PESD oligos with unique hybridization overlaps. Note that each forward oligo and reverse oligo are paired (by-design) with complementary sequences in their hybridization overlap. For visualization of on- and off-target interactions, those paired oligonucleotides were sequentially ordered in this matrix such that each complement is found at the diagonal intersection of the table from top-left to bottom-right.

FIG. 17

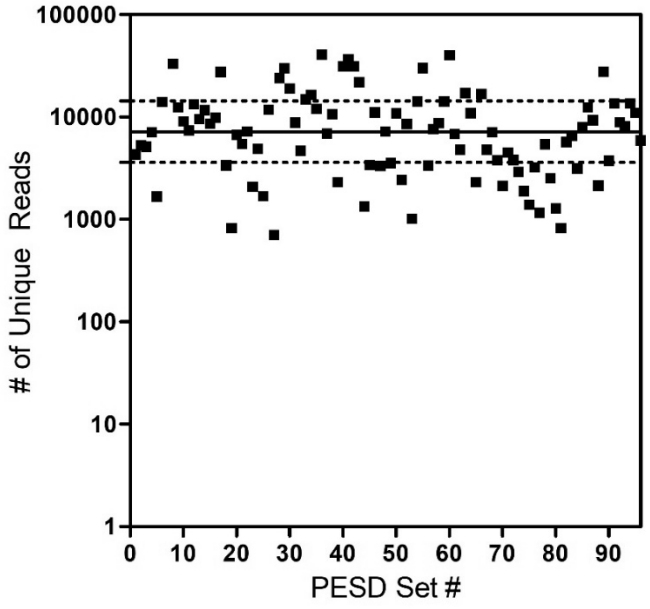
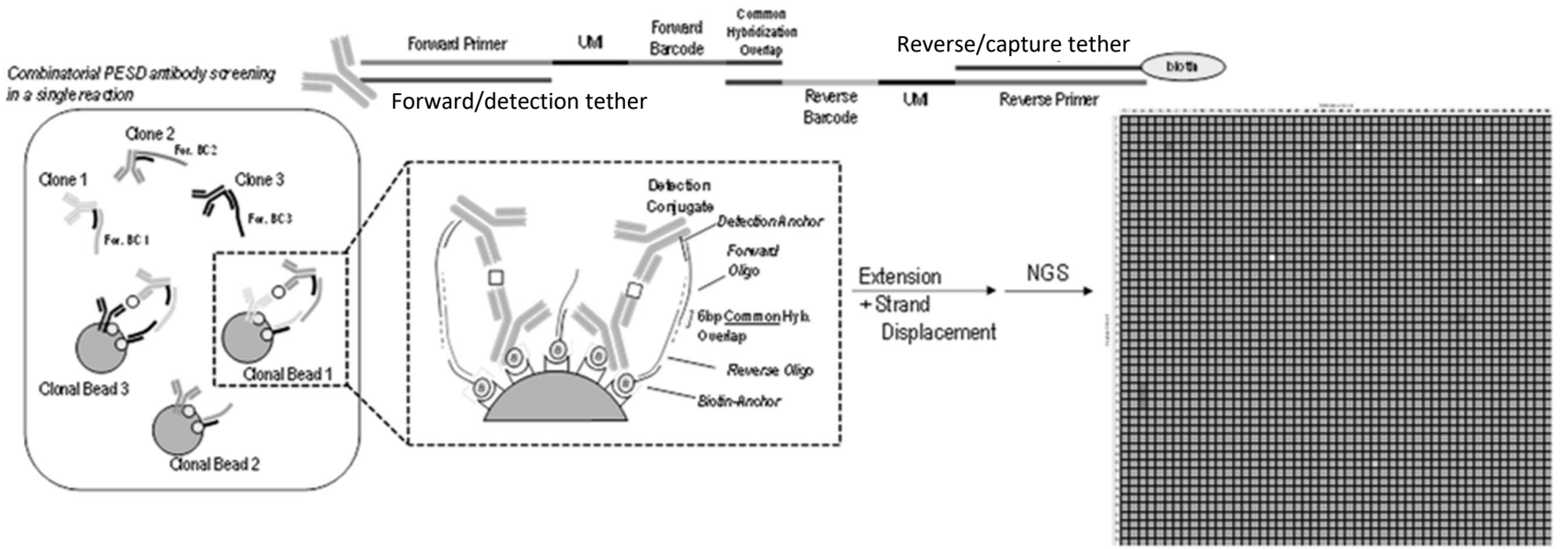
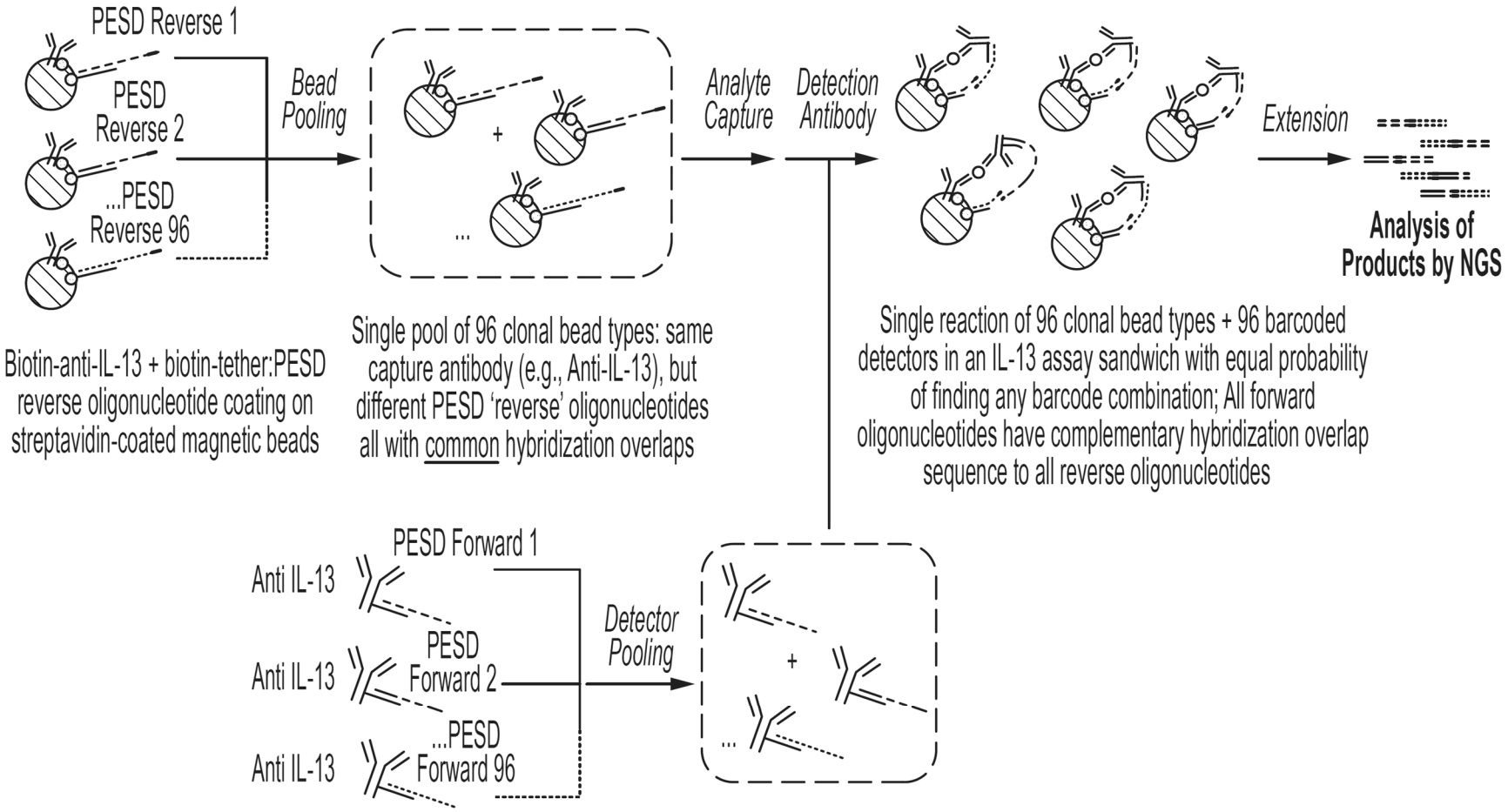

100000
10000
1000
100
10
1
# of Unique Reads
0
10
20
30
40
50
60
70
80
90
PESD Set #

FIG. 18



Example data matrix representing the number of doubly-barcoded NGS reads corresponding to each capture and detection antibody clone combination uncovered by PESD in a theoretical 50-antibody combinatorial screen; select combinations that generate a significant number of reads above background (shown as yellow and red squares) may be selected for additional antibody testing.

FIG. 19



Scheme for the experimental evaluation of 96 different forward/detector and reverse/capture PESD oligonucleotides, read out by NGS.

FIG. 20

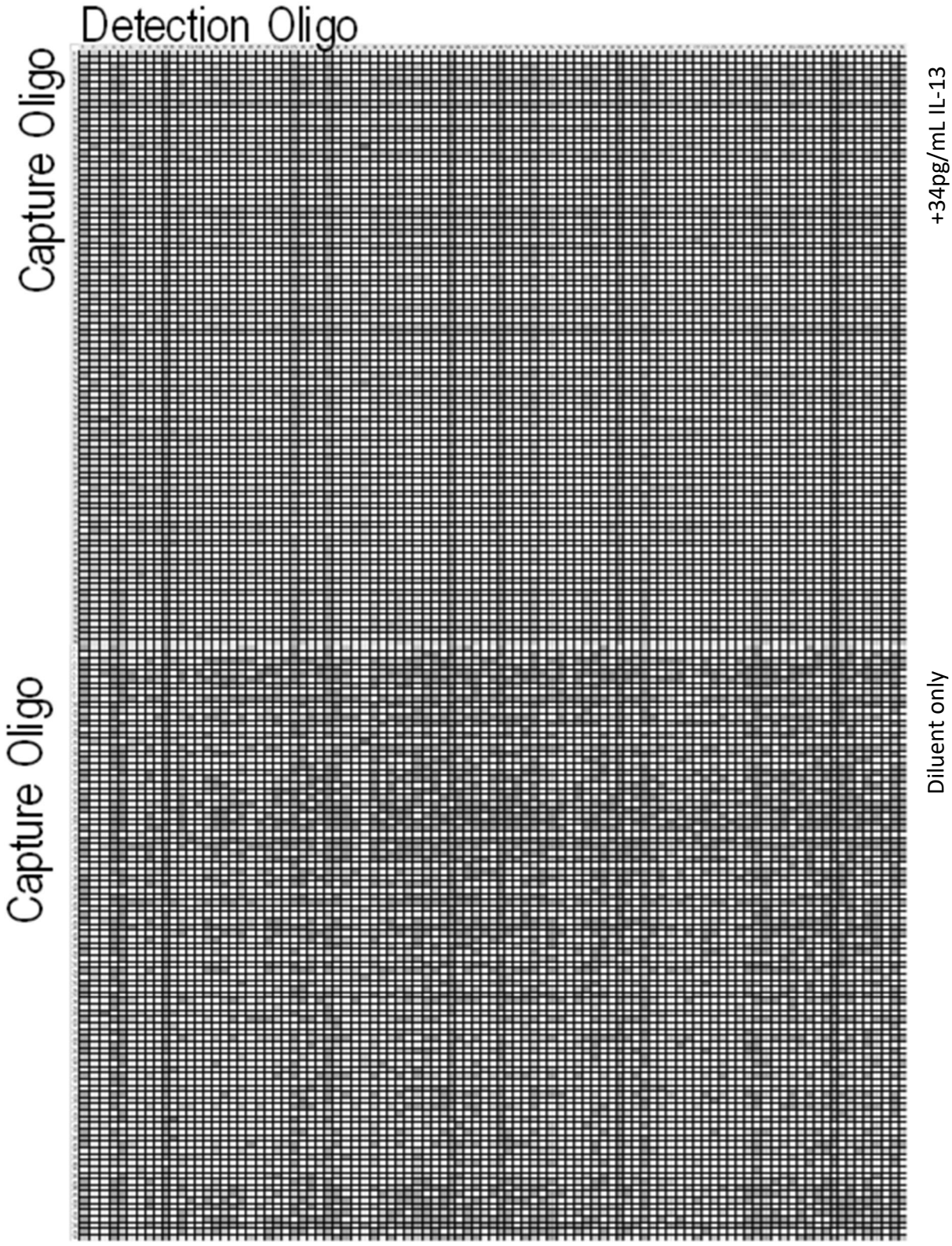

Detection Oligo
Capture Oligo
Capture Oligo
+34pg/mL IL-13
Diluent only

FIG. 21


Set3
IFNγ
5'
(SEQ ID NO:5)
TGGTCCTACGAGACATCGGTNNNNNNNNGTATGGCGTCACAAC
3'
(SEQ ID NO:11)
3'TGTTGAAGAGTGGCTNNNNNNNNTAGGACGGCCACTATCGTAC5'—Ⓑ
Set4
IL4
(SEQ ID NO:6)
GTAGCGCCTTGCATGTTAGCNNNNNNNNCAGGTCGGAAACAAC
(SEQ ID NO:12)
TGTTGTCTGCGTGTGNNNNNNNNCACGGGACCAAGTCAATCGA—Ⓑ
Set5
IL6
(SEQ ID NO:7)
GCTGCCGGCATCCTATAGTTNNNNNNNNGGACTGCGATACAAC
(SEQ ID NO:13)
TGTTGGTTGCGTAGTNNNNNNNNCTTAACAGCGTACTGCTGCC—Ⓑ
Set6
IL10
(SEQ ID NO:8)
CAGGTATACCATCGCCGCTTNNNNNNNNGCGACTTCAGACAAC
(SEQ ID NO:14)
TGTTGAACAACGCGGNNNNNNNNATAAATTACGTGCCGTCGCG—Ⓑ
Set7
IL12
(SEQ ID NO:9)
CCGACCAGTAAACACGTGGANNNNNNNNGTCAGGTGAAACAAC
(SEQ ID NO:15)
TGTTGCCTGTTCATGNNNNNNNNATTCGCAACAATGGCGTGGA—Ⓑ
Set8
IL13
(SEQ ID NO:2)
GTGAGGCGCTAGAGCATAGGNNNNNNNNACTTGCGCCAACAAC
(SEQ ID NO:1)
TGTTGAGTATCTCTGNNNNNNNNCGACAGAAGCGATAAACGGC—Ⓑ
Set9
TNFa
(SEQ ID NO:10)
CGCTAGAGCTGGGTGATCTGNNNNNNNNTGTATGGTACACAAC
(SEQ ID NO:16)
TGTTGACGATGTTCTNNNNNNNNATGGTTACCTGAGCGGTTGG—Ⓑ

FIG. 22

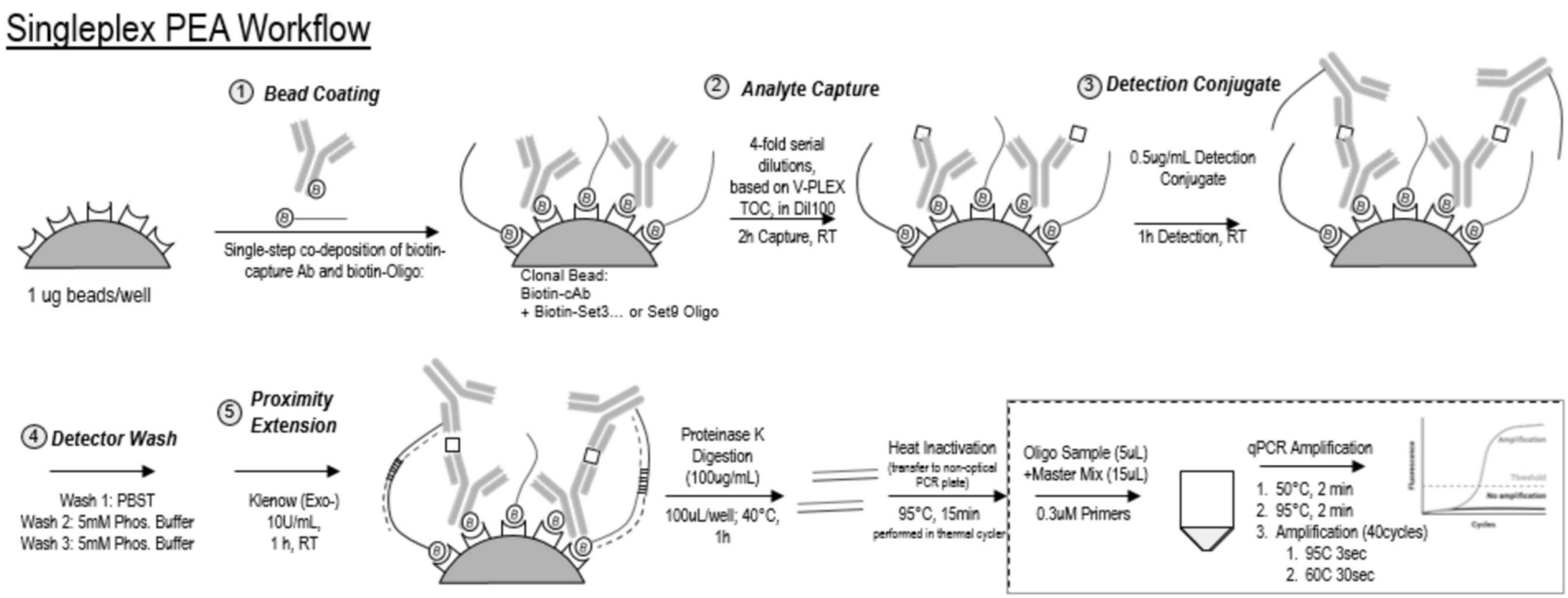

Singleplex PEA Workflow
① Bead Coating
1 ug beads/well
Single-step co-deposition of biotin-capture Ab and biotin-Oligo:
Clonal Bead:
Biotin-cAb
+ Biotin-Set3... or Set9 Oligo
② Analyte Capture
4-fold serial dilutions, based on V-PLEX TOC, in Dil100
2h Capture, RT
③ Detection Conjugate
0.5ug/mL Detection Conjugate
1h Detection, RT
④ Detector Wash
Wash 1: PBST
Wash 2: 5mM Phos. Buffer
Wash 3: 5mM Phos. Buffer
⑤ Proximity Extension
Klenow (Exo-)
10U/mL,
1 h, RT
Proteinase K Digestion (100ug/mL)
100uL/well; 40°C, 1h
Heat Inactivation
(transfer to non-optical PCR plate)
95°C, 15min
performed in thermal cycler
Oligo Sample (5uL)
+Master Mix (15uL)
0.3uM Primers
qPCR Amplification
1. 50°C, 2 min
2. 95°C, 2 min
3. Amplification (40cycles)
1. 95C 3sec
2. 60C 30sec

FIG. 23

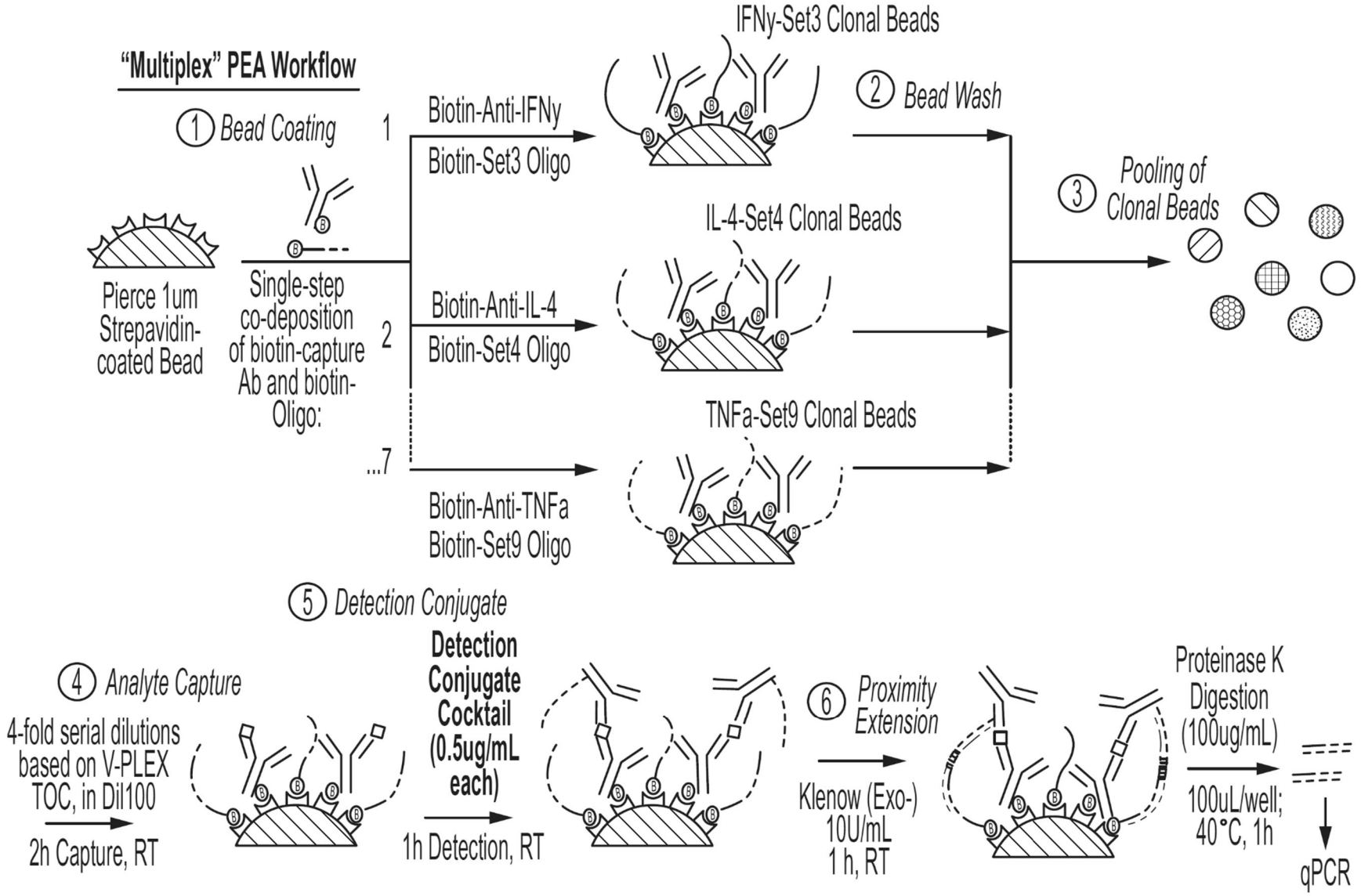

"Multiplex" PEA Workflow
1 Bead Coating
Pierce 1um Streptavidin-coated Bead
Single-step co-deposition of biotin-capture Ab and biotin-Oligo:
1
Biotin-Anti-IFNy
Biotin-Set3 Oligo
IFNy-Set3 Clonal Beads
2
Biotin-Anti-IL-4
Biotin-Set4 Oligo
IL-4-Set4 Clonal Beads
...7
Biotin-Anti-TNFa
Biotin-Set9 Oligo
TNFa-Set9 Clonal Beads
2 Bead Wash
3 Pooling of Clonal Beads
4 Analyte Capture
4-fold serial dilutions based on V-PLEX TOC, in Dil100
2h Capture, RT
5 Detection Conjugate
Detection Conjugate Cocktail (0.5ug/mL each)
1h Detection, RT
6 Proximity Extension
Klenow (Exo-) 10U/mL
1 h, RT
Proteinase K Digestion (100ug/mL)
100uL/well; 40°C, 1h
qPCR

FIG. 24

IFNγ

Antibody version	v1		v2		v2	
Assay Format	Singleplex		Singleplex		Multiplex	
Experiment Date	11-Jan-2022		18-Mar-2022		23-Mar-2022	
[IFNγ] (pg/mL)	1	2	1	2	1	2
1460			17.60		17.20	
365.0			19.46		18.88	
91.25			21.39		21.09	
22.81			23.21		23.19	
5.703			24.58		24.76	
1.426			26.36		26.13	
0.356			27.46		26.93	
0.000			28.38		27.65	

IL-4

Antibody version	v1		v2		v2	
Assay Format	Singleplex		Singleplex		Multiplex	
Experiment Date	11-Jan-2022		18-Mar-2022		23-Mar-2022	
[IL-4] (pg/mL)	1	2	1	2	1	2
263	16.66	16.99	15.81		15.38	
65.8	18.90	18.55	17.31		17.29	
16.44	20.69	20.24	19.19		18.96	
4.11	22.34	22.35	21.48		21.24	
1.027	24.12	24.18	23.08		23.21	
0.257	26.28	26.30	25.34		25.13	
0.064	28.73	27.48	27.21		26.67	
0.000	30.12	28.75	28.91		28.22	

IL-6

Antibody version	v1		v2		v2	
Assay Format	Singleplex		Singleplex		Multiplex	
Experiment Date	11-Jan-2022		18-Mar-2022		23-Mar-2022	
[IL-6] (pg/mL)	1	2	1	2	1	2
732	17.54	17.17	14.41		13.88	
183.0	19.78	19.12	15.61		15.77	
45.75	21.40	21.34	17.63		17.57	
11.44	23.40	23.63	19.44		19.70	
2.859	25.31	25.23	21.61		21.60	
0.715	27.38	27.27	23.43		22.94	
0.179	28.60	28.15	24.95		23.49	
0.000	28.64	29.90	26.72		23.74	

IL-10

Antibody version	v1		v2		v2	
Assay Format	Singleplex		Singleplex		Multiplex	
Experiment Date	11-Jan-2022		18-Mar-2022		23-Mar-2022	
[IL-10] (pg/mL)	1	2	1	2	1	2
383	16.06	16.55	17.96		17.16	
95.8	18.24	18.32	19.75		19.00	
23.94	20.24	20.51	22.09		21.19	
5.98	22.16	22.14	23.69		22.75	
1.496	24.18	24.46	25.38		24.45	
0.374	25.47	25.68	26.96		24.99	
0.094	26.96	27.10	27.19		25.08	
0.000	27.49	27.31	27.56		25.29	

IL-12p70

Antibody version	v1		v2			v2	
Assay Format	Singleplex		Singleplex			Multiplex	
Experiment Date	11-Jan-2022		21-Mar-2022			23-Mar-2022	
[IL-12p70] (pg/mL)	1	2	1	2	3	1	2
383	16.06	16.55	18.35	18.57	18.49	17.16	
95.8	18.24	18.32	20.30	20.45	20.28	19.00	
23.94	20.24	20.51	22.06	22.34	22.25	21.19	
5.98	22.16	22.14	24.32	24.25	23.98	22.75	
1.496	24.18	24.46	26.06	26.21	26.17	24.45	
0.374	25.47	25.68	27.59	27.56	27.65	24.99	
0.094	26.96	27.10	28.99	28.85	28.62	25.08	
0.000	27.49	27.31	29.60	29.38	29.59	25.29	

IL-13

Antibody version	v1		v2		v2	
Assay Format	Singleplex		Singleplex		Multiplex	
Experiment Date	11-Jan-2022		18-Mar-2022		23-Mar-2022	
[IL-13] (pg/mL)	1	2	1	2	1	2
482			15.77		15.73	
121.0			17.67		17.71	
30.00			19.89		20.23	
7.53			21.89		22.31	
1.883			23.69		24.24	
0.471			26.79		26.24	
0.118			28.17		28.13	
0.000			31.37		31.74	

TNFa

Antibody version	v1		v2			v2	
Assay Format	Singleplex		Singleplex			Multiplex	
Experiment Date	11-Jan-2022		21-Mar-2022			23-Mar-2022	
[TNFa] (pg/mL)	1	2	1	2	3	1	2
362	20.58	20.63	19.18	18.12	18.90	16.95	
90.5	22.05	21.93	21.49	20.92	21.45	19.01	
22.63	24.60	24.48	22.76	22.85	22.87	20.84	
5.66	26.88	26.48	24.07	24.48	24.18	21.93	
1.414	28.12	28.24	24.22	24.53	24.67	22.54	
0.354	30.13	29.92	25.29	24.78	25.28	22.42	
0.088	30.31	30.60	24.94	25.00	25.23	22.35	
0.010	30.05	31.50	23.96	25.12	25.06	22.74	

FIG. 25

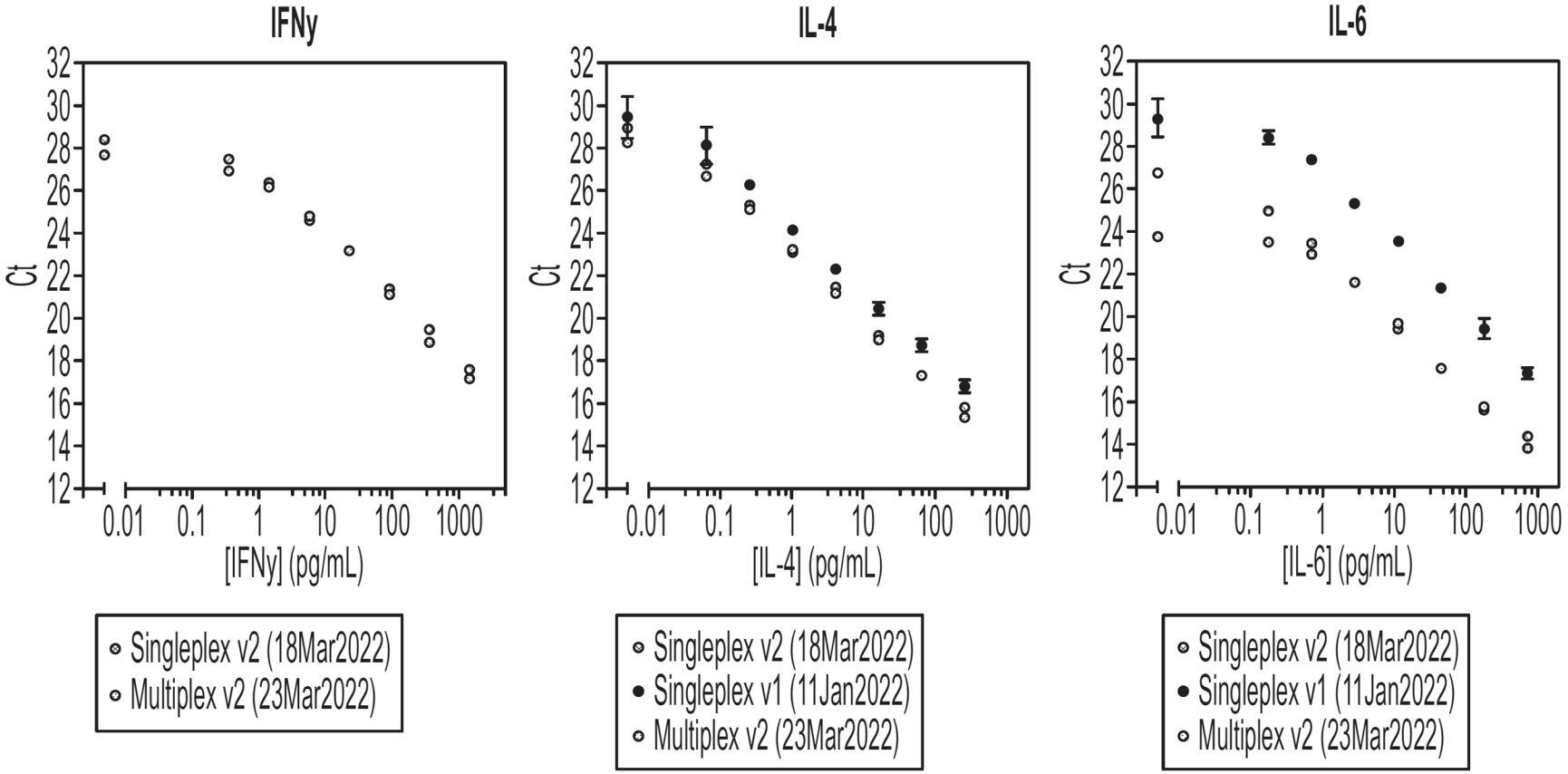

IFNy
IL-4
IL-6
Ct
32
30
28
26
24
22
20
18
16
14
12
0.01
0.1
1
10
100
1000
[IFNy] (pg/mL)
[IL-4] (pg/mL)
[IL-6] (pg/mL)
Singleplex v2 (18Mar2022)
Multiplex v2 (23Mar2022)
Singleplex v2 (18Mar2022)
Singleplex v1 (11Jan2022)
Multiplex v2 (23Mar2022)
Singleplex v2 (18Mar2022)
Singleplex v1 (11Jan2022)
Multiplex v2 (23Mar2022)

FIG. 26

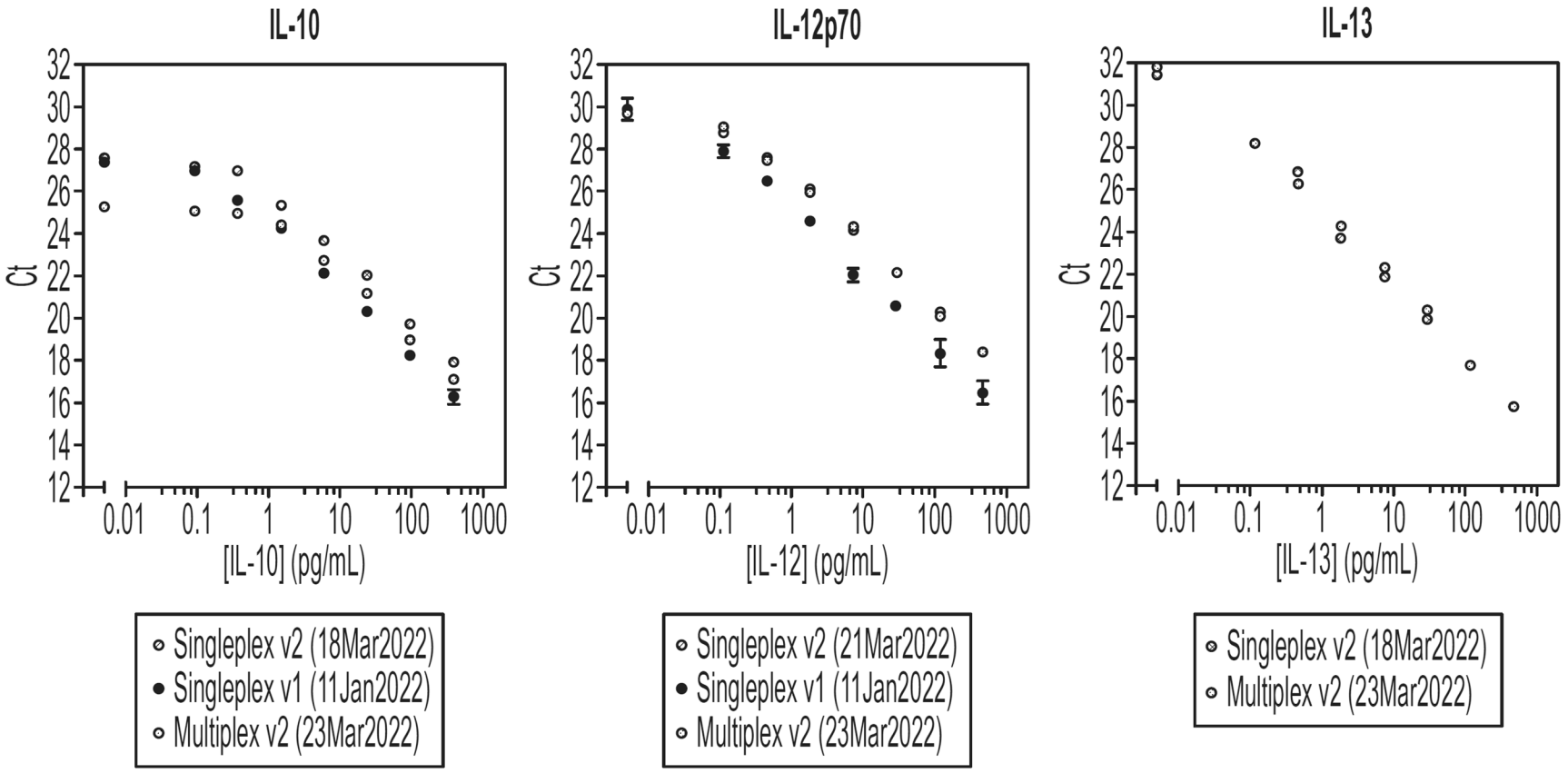


FIG. 26

CONTINUED

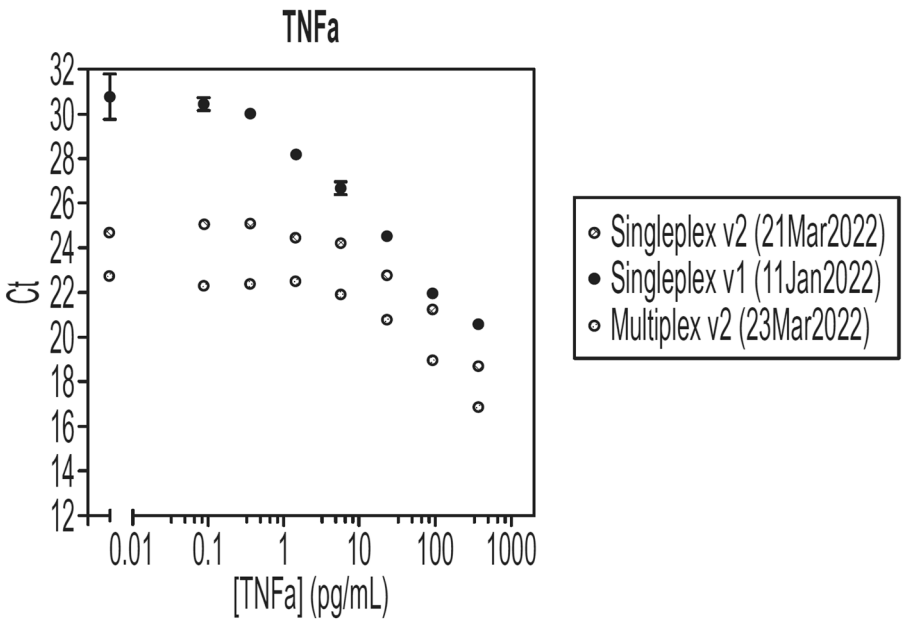


FIG. 26

CONTINUED

	Eotaxin-RRC	Eotaxin3-RRC	FLT1-RRC	GMCSF-RRC	GranzymeA-RRC	IFN?-RRC	IL1?-RRC	IL10-RRC	IL12p70-RRC	IL13-RRC	IL15-RRC	IL17a-RRC	IL18-RRC	IL1a-RRC	IL2-RRC	IL21-RRC	IL22-RRC	IL27-RRC	IL29-RRC	IL2Ra-RRC	IL31-RRC	IL33-RRC	IL4-RRC	IL5-RRC	IL6-RRC	IL7-RRC	IL8-RRC	IP10-RRC	MCP1-RRC	MCP4-RRC	MDC-RRC	MIP1a-RRC	MIP1b-RRC	MIP3a-RRC	PlGF-RRC	TARC-RRC	Tie2-RRC	TNF?-RRC	TNFb-RRC	VEGFA-RRC	VEGFD-RRC
Eotaxin-FW	1E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA
Eotaxin3-F	NA	1E+06	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FLT1-FW	NA	NA	2E+05	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA
GMCSF-FW	NA	NA	NA	9E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Granzyme/	1	NA	1	NA	7E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IFN?-FW	NA	NA	NA	NA	NA	9E+04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL1?-FW	NA	NA	NA	11	NA	NA	7E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL10-FW	NA	NA	NA	NA	NA	NA	NA	3E+05	NA	NA	4	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL12p70-FV	NA	NA	NA	NA	1	NA	NA	NA	203	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL13-FW	NA	NA	NA	NA	NA	NA	2	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
IL15-FW	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL17a-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+06	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	11	NA	NA	NA	NA	NA	NA	NA
IL18-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4E+05	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL1a-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL2-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4E+05	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL21-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL22-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL27-FW	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	1	NA	NA	NA	NA	NA	5E+05	NA	16	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	4
IL29-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	6E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL2Ra-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	8E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL31-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL33-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL4-FW	NA	NA	NA	NA	NA	1	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL5-FW	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	6E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA
IL6-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06	NA	NA	NA	2	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL7-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IL8-FW	NA	NA	NA	NA	4	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	9E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
IP10-FW	NA	NA	NA	NA	NA	NA	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MCP1-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	9E+05	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	1	NA	NA
MCP4-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MDC-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+05	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA
MIP1a-FW	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MIP1b-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+06	NA	NA	NA	NA	NA	NA	NA	NA	NA
MIP3a-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	NA	NA
PlGF-FW	NA	NA	1	NA	NA	NA	1	NA	NA	NA	NA	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06	NA	NA	NA	NA	NA	NA	NA
TARC-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	2	NA	NA	NA	NA	5E+05	NA	NA	NA	NA	NA	NA	NA
Tie2-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2E+05	NA	NA	NA	NA	NA
TNF?-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	NA	NA	NA	NA	5E+05	NA	NA	2
TNFb-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	9E+05	NA	NA
VEGFA-FW	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	4E+05	NA
VEGFD-FW	NA	NA	NA	1	NA	NA	NA	1	NA	NA	NA	6	NA	NA	NA	NA	2	NA	NA	NA	NA	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1E+06

FIG. 27

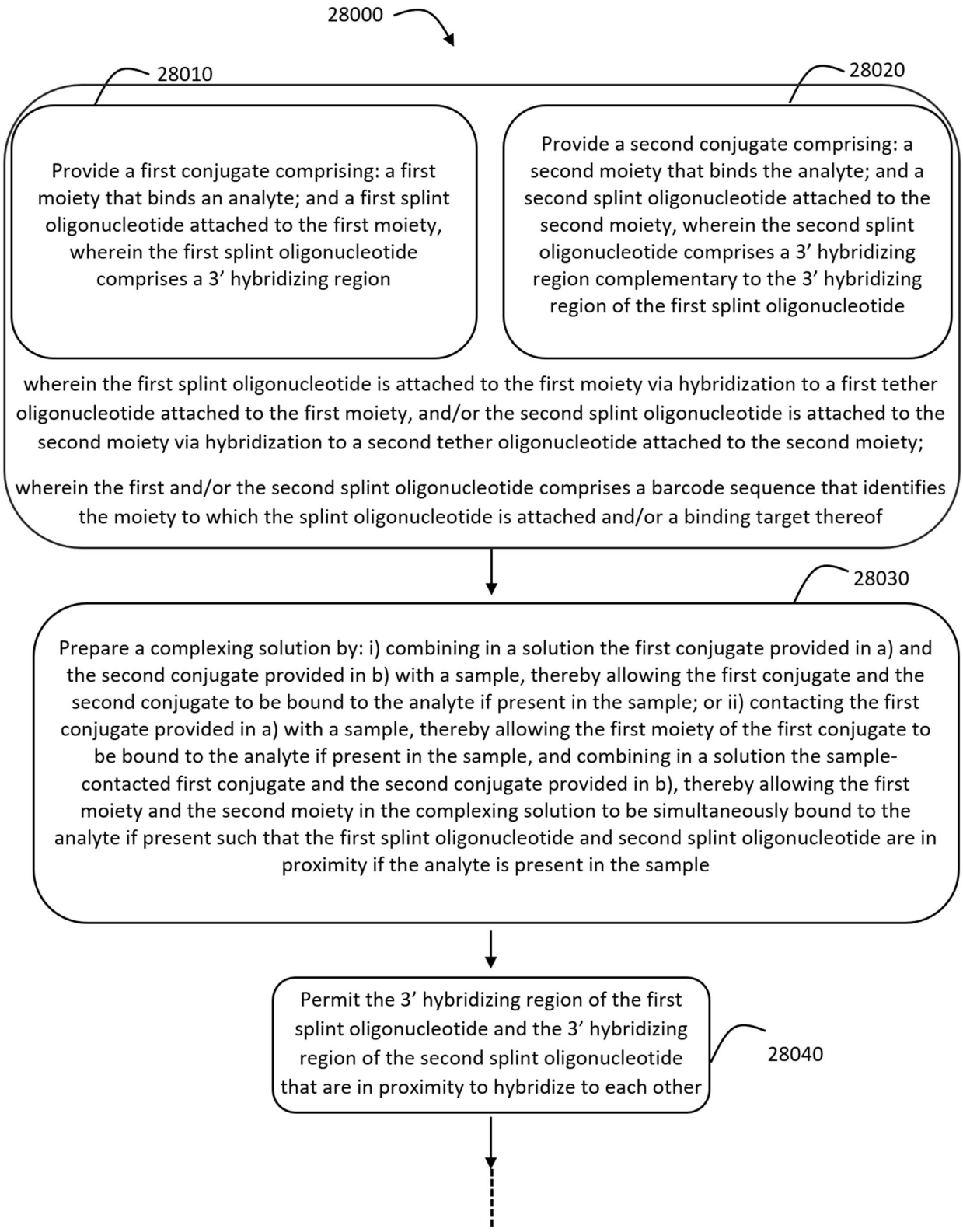

28000
28010
28020
Provide a first conjugate comprising: a first moiety that binds an analyte; and a first splint oligonucleotide attached to the first moiety, wherein the first splint oligonucleotide comprises a 3' hybridizing region
Provide a second conjugate comprising: a second moiety that binds the analyte; and a second splint oligonucleotide attached to the second moiety, wherein the second splint oligonucleotide comprises a 3' hybridizing region complementary to the 3' hybridizing region of the first splint oligonucleotide
wherein the first splint oligonucleotide is attached to the first moiety via hybridization to a first tether oligonucleotide attached to the first moiety, and/or the second splint oligonucleotide is attached to the second moiety via hybridization to a second tether oligonucleotide attached to the second moiety;
wherein the first and/or the second splint oligonucleotide comprises a barcode sequence that identifies the moiety to which the splint oligonucleotide is attached and/or a binding target thereof
28030
Prepare a complexing solution by: i) combining in a solution the first conjugate provided in a) and the second conjugate provided in b) with a sample, thereby allowing the first conjugate and the second conjugate to be bound to the analyte if present in the sample; or ii) contacting the first conjugate provided in a) with a sample, thereby allowing the first moiety of the first conjugate to be bound to the analyte if present in the sample, and combining in a solution the sample-contacted first conjugate and the second conjugate provided in b), thereby allowing the first moiety and the second moiety in the complexing solution to be simultaneously bound to the analyte if present such that the first splint oligonucleotide and second splint oligonucleotide are in proximity if the analyte is present in the sample
Permit the 3' hybridizing region of the first splint oligonucleotide and the 3' hybridizing region of the second splint oligonucleotide that are in proximity to hybridize to each other
28040

FIG. 28

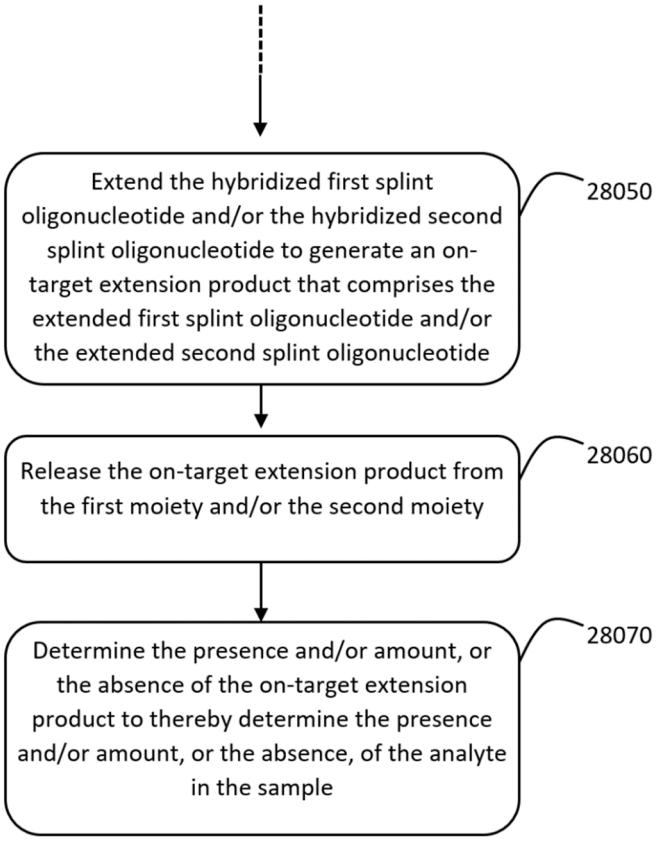


FIG. 28

CONTINUED

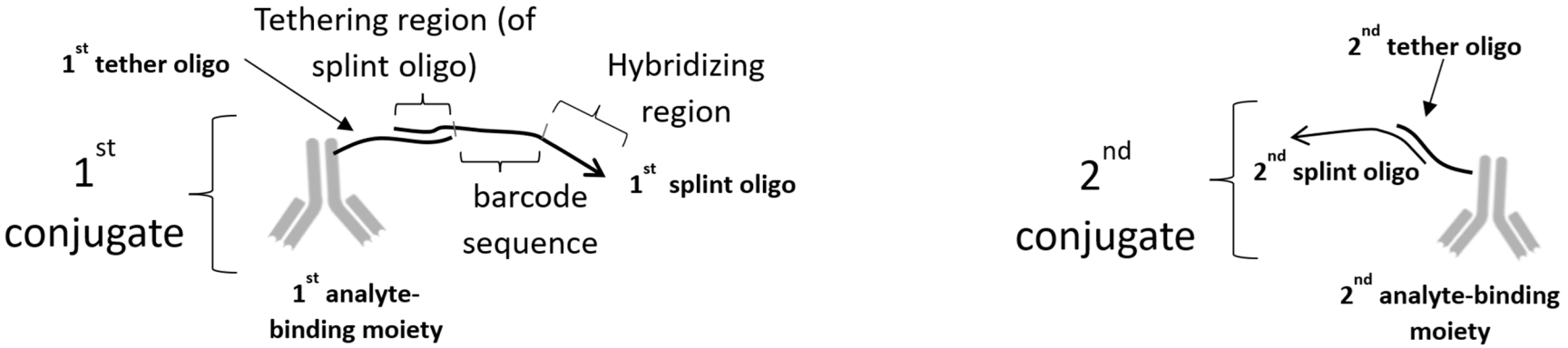


FIG. 29A

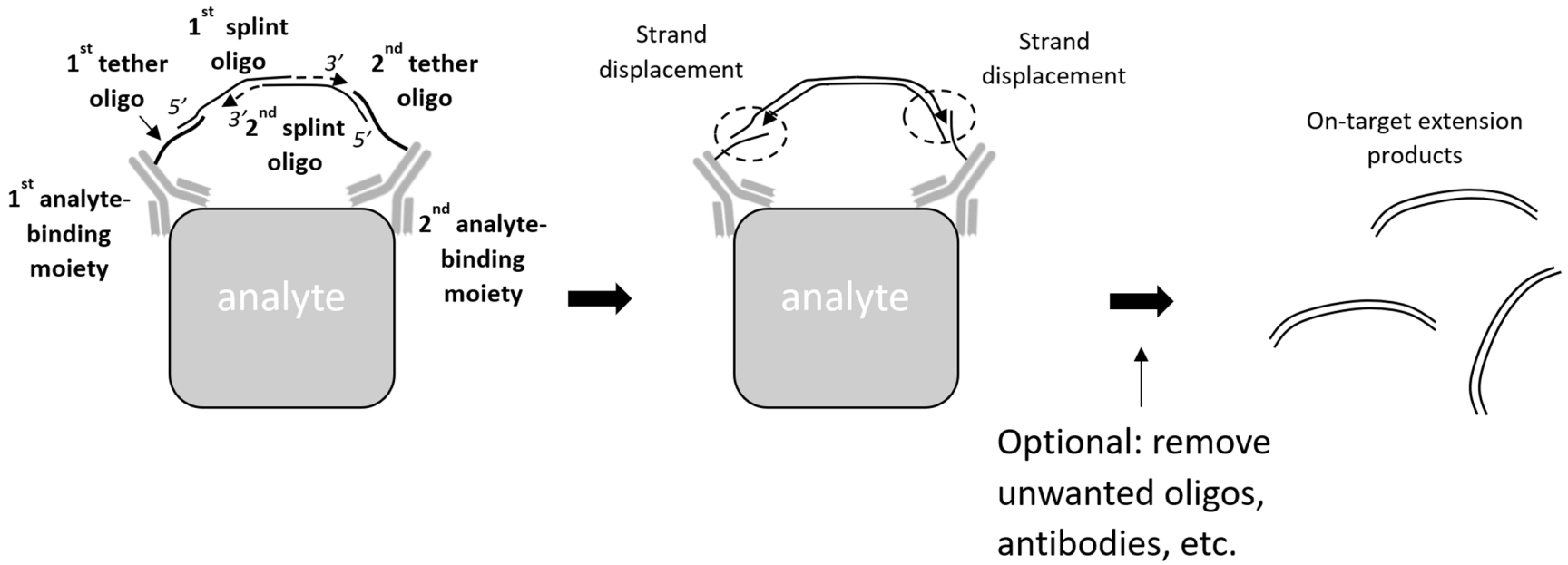


FIG. 29B

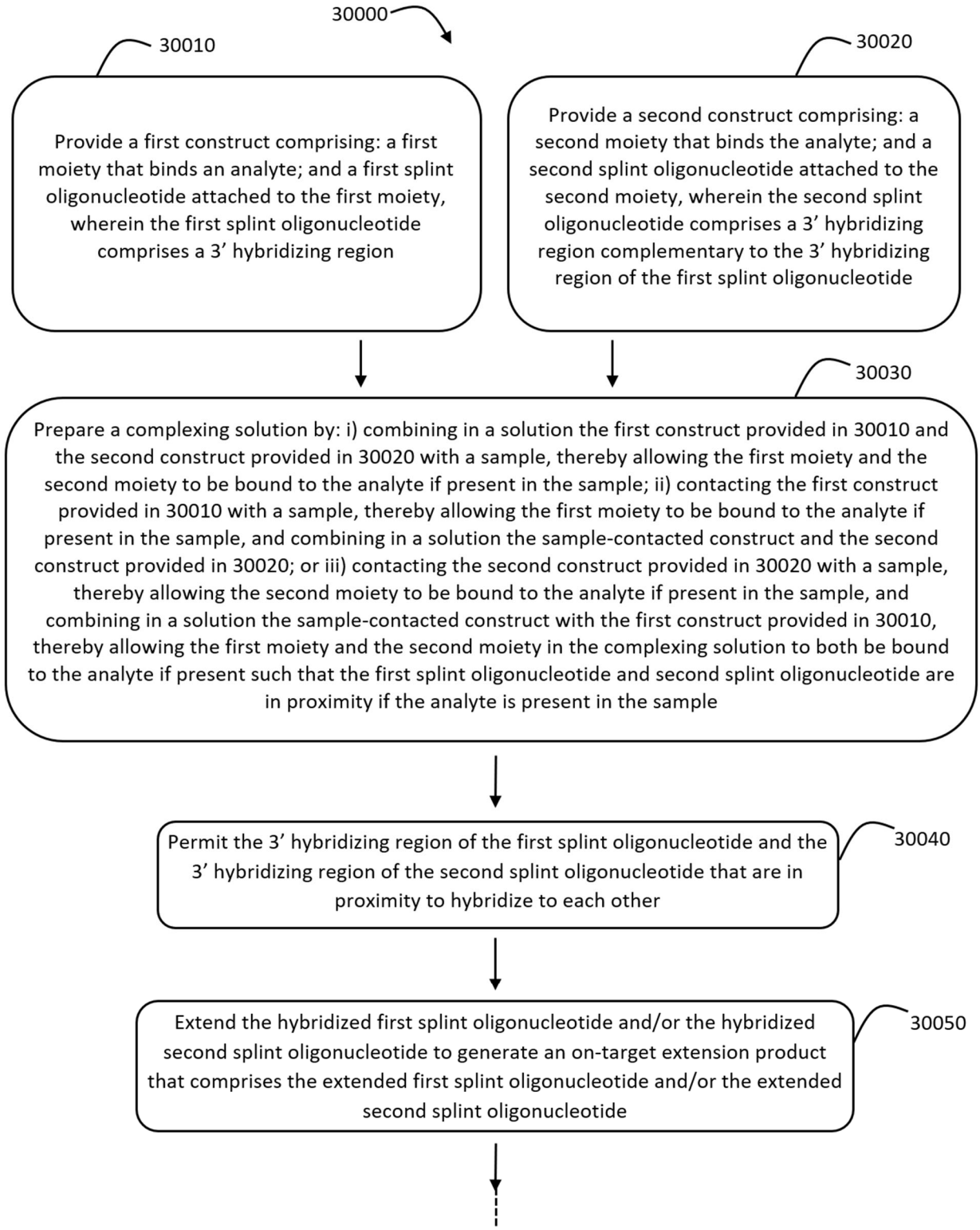

30000
30010
Provide a first construct comprising: a first moiety that binds an analyte; and a first splint oligonucleotide attached to the first moiety, wherein the first splint oligonucleotide comprises a 3' hybridizing region
30020
Provide a second construct comprising: a second moiety that binds the analyte; and a second splint oligonucleotide attached to the second moiety, wherein the second splint oligonucleotide comprises a 3' hybridizing region complementary to the 3' hybridizing region of the first splint oligonucleotide
30030
Prepare a complexing solution by: i) combining in a solution the first construct provided in 30010 and the second construct provided in 30020 with a sample, thereby allowing the first moiety and the second moiety to be bound to the analyte if present in the sample; ii) contacting the first construct provided in 30010 with a sample, thereby allowing the first moiety to be bound to the analyte if present in the sample, and combining in a solution the sample-contacted construct and the second construct provided in 30020; or iii) contacting the second construct provided in 30020 with a sample, thereby allowing the second moiety to be bound to the analyte if present in the sample, and combining in a solution the sample-contacted construct with the first construct provided in 30010, thereby allowing the first moiety and the second moiety in the complexing solution to both be bound to the analyte if present such that the first splint oligonucleotide and second splint oligonucleotide are in proximity if the analyte is present in the sample
Permit the 3' hybridizing region of the first splint oligonucleotide and the 3' hybridizing region of the second splint oligonucleotide that are in proximity to hybridize to each other
30040
Extend the hybridized first splint oligonucleotide and/or the hybridized second splint oligonucleotide to generate an on-target extension product that comprises the extended first splint oligonucleotide and/or the extended second splint oligonucleotide
30050

FIG. 30A

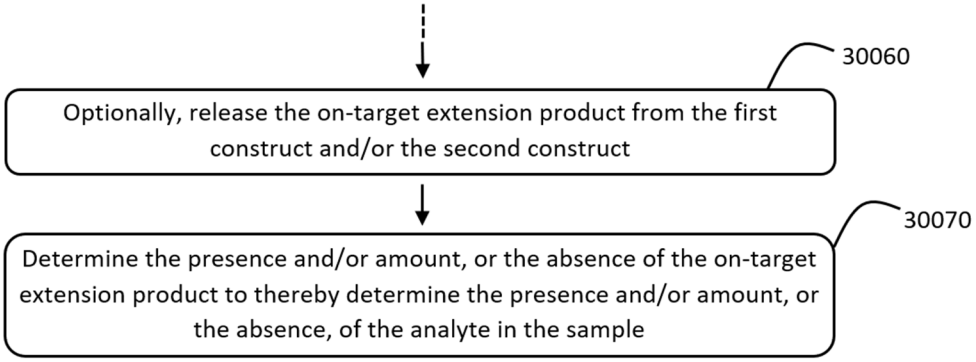


FIG. 30A

CONTINUED

30000

(I) The complexing solution comprises one or more blocker oligonucleotides, wherein each blocker oligonucleotide hybridizes to a portion of the first splint oligonucleotide and/or of the second splint oligonucleotide.

(II) Analyze the sample for a first analyte and a second analyte, wherein preparing the complexing solution in 30030 comprises: preparing the complexing solution in a plurality of subpools comprising a first subpool and second subpool, wherein the first moiety and second moiety in the prepared complexing solution of the first subpool bind the first analyte, and the first moiety and second moiety in the prepared complexing solution of the second subpool bind the second analyte; and combining the plurality of subpools before determining the presence and/or amount, or the absence of the on-target extension product in 30070.

(III) Provide a plurality of paired combinations of the first and second constructs, wherein the 3' hybridizing region of the splint oligonucleotides of one or more of the plurality of paired combinations is 1, 2, 3 or more nucleotides shorter than the 3' hybridizing region of the splint oligonucleotides of at least another one of the plurality of paired combinations, wherein the 3' hybridizing regions of the splint oligonucleotides of the at least another one of the plurality of paired combinations is different from and is not complementary to any contiguous stretch of the 3' hybridizing region of the splint oligonucleotides of the one or more of the plurality of paired combinations.

and/or

(IV) Attenuate an amount of amplification products by reducing or interfering with a binding interaction between the analyte and the first moiety or the second moiety and/or suppressing on-target interactions between the conjugate splint oligonucleotide and the first splint oligonucleotide when the first moiety and the second moiety are both bound to the analyte.

FIG. 30B

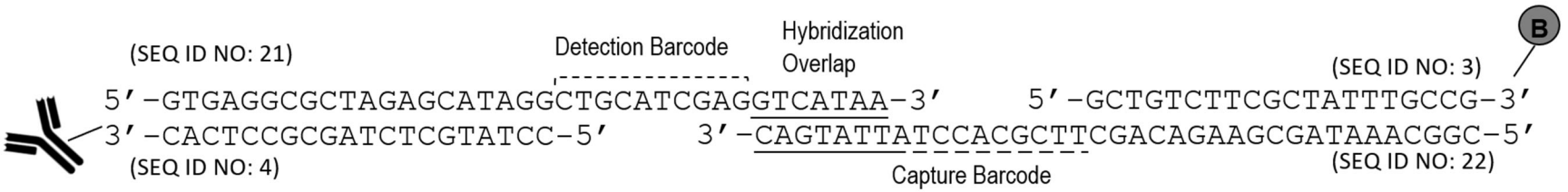
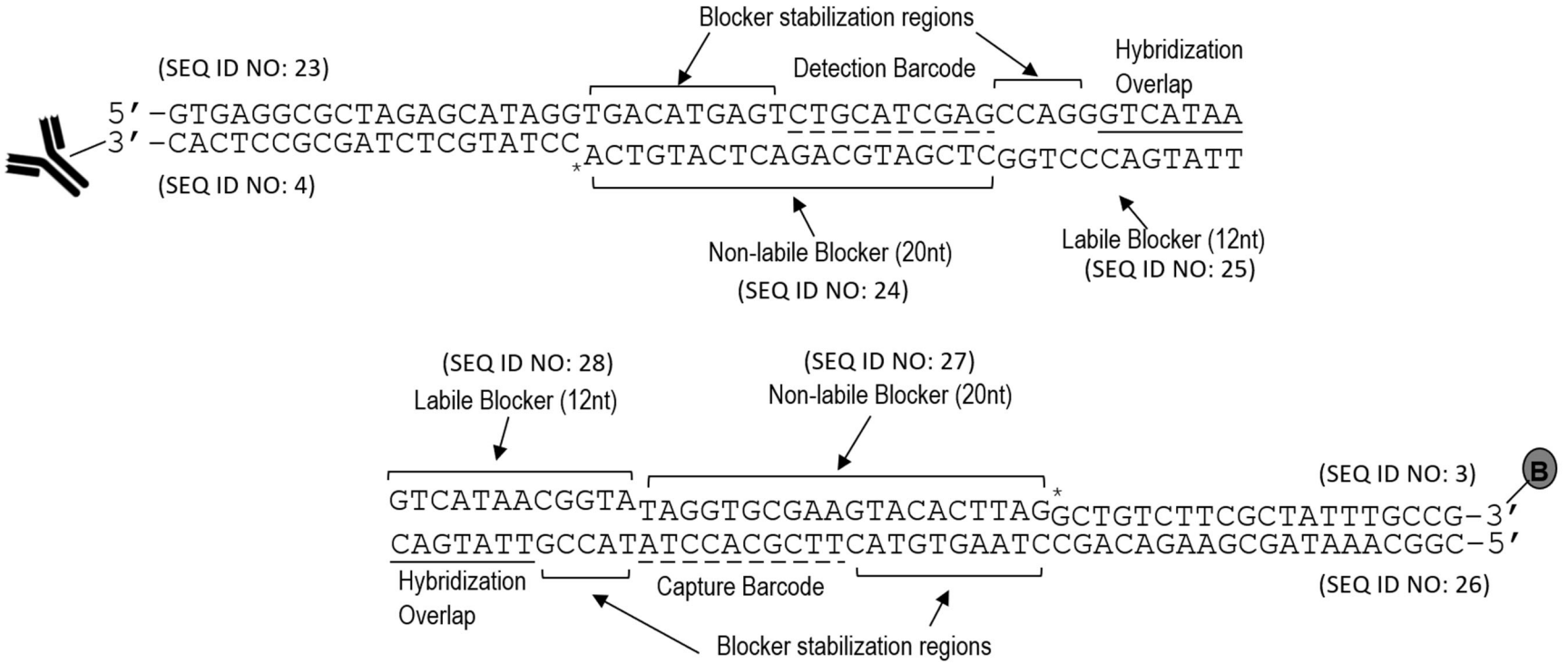


FIG. 31A



* Indicates a 3' terminator, or modified base, to inhibit 5'->3' extension from this site

FIG. 31B

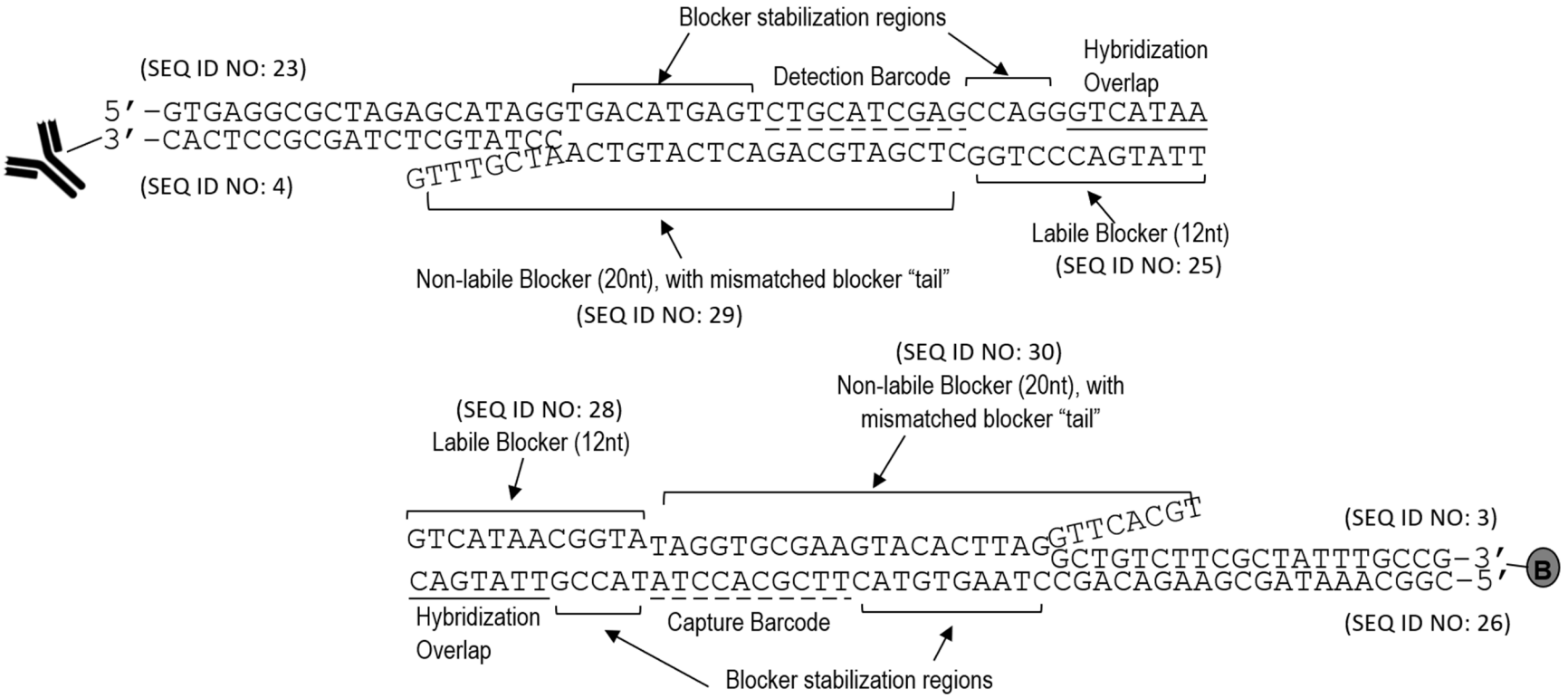


FIG. 31C

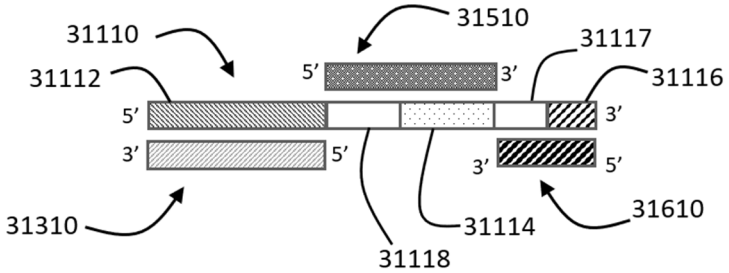


FIG. 31D

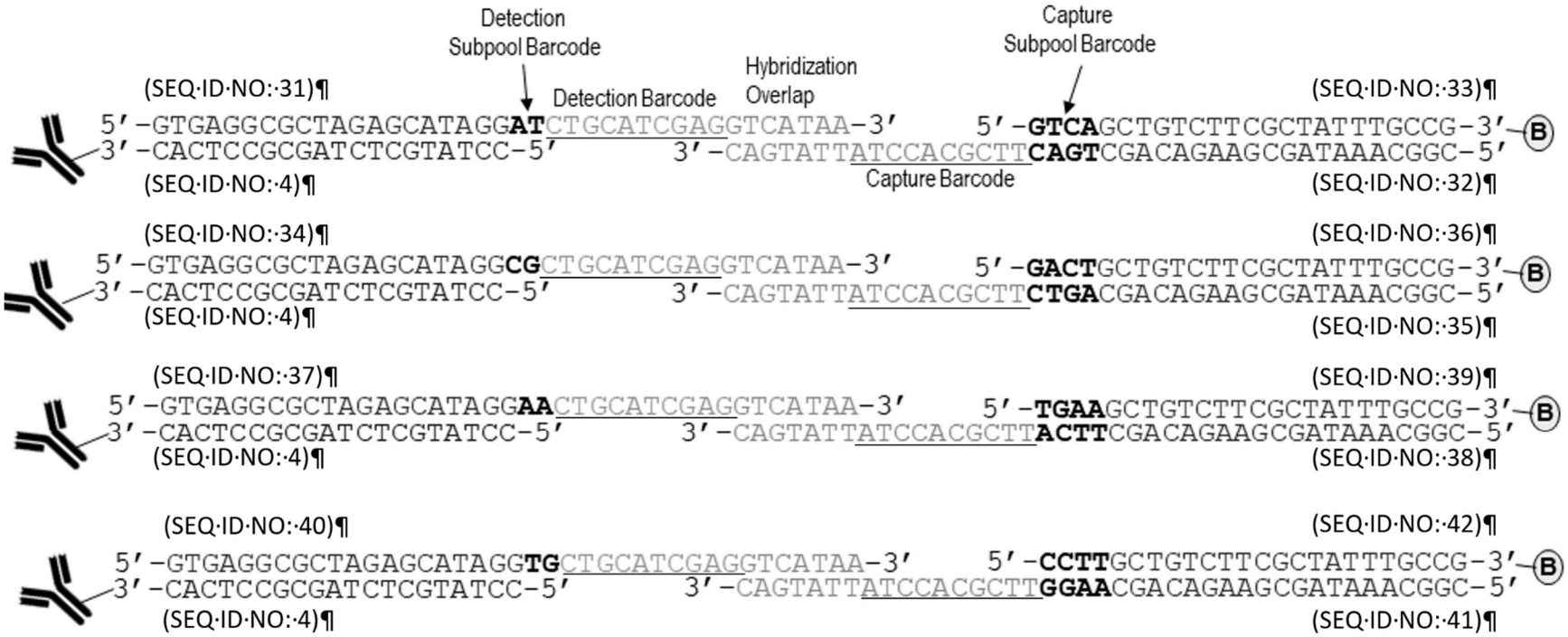

Detection
Subpool Barcode
Detection Barcode
Hybridization
Overlap
Capture
Subpool Barcode
Capture Barcode
(SEQ·ID·NO:·31)¶
5′–GTGAGGCGCTAGAGCATAGGATCTGCATCGAGGTCATAA–3′
3′–CACTCCGCGATCTCGTATCC–5′
(SEQ·ID·NO:·4)¶
3′–CAGTATTATCCACGCTTCAGTCGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·32)¶
5′–GTCAGCTGTCTTCGCTATTTGCCG–3′
(SEQ·ID·NO:·33)¶
B
(SEQ·ID·NO:·34)¶
5′–GTGAGGCGCTAGAGCATAGGCGCTGCATCGAGGTCATAA–3′
3′–CACTCCGCGATCTCGTATCC–5′
(SEQ·ID·NO:·4)¶
3′–CAGTATTATCCACGCTTCTGACGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·35)¶
5′–GACTGCTGTCTTCGCTATTTGCCG–3′
(SEQ·ID·NO:·36)¶
B
(SEQ·ID·NO:·37)¶
5′–GTGAGGCGCTAGAGCATAGGAACTGCATCGAGGTCATAA–3′
3′–CACTCCGCGATCTCGTATCC–5′
(SEQ·ID·NO:·4)¶
3′–CAGTATTATCCACGCTTACTTCGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·38)¶
5′–TGAAGCTGTCTTCGCTATTTGCCG–3′
(SEQ·ID·NO:·39)¶
B
(SEQ·ID·NO:·40)¶
5′–GTGAGGCGCTAGAGCATAGGTGCTGCATCGAGGTCATAA–3′
3′–CACTCCGCGATCTCGTATCC–5′
(SEQ·ID·NO:·4)¶
3′–CAGTATTATCCACGCTTGGAACGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·41)¶
5′–CCTTGCTGTCTTCGCTATTTGCCG–3′
(SEQ·ID·NO:·42)¶
B

FIG. 32A

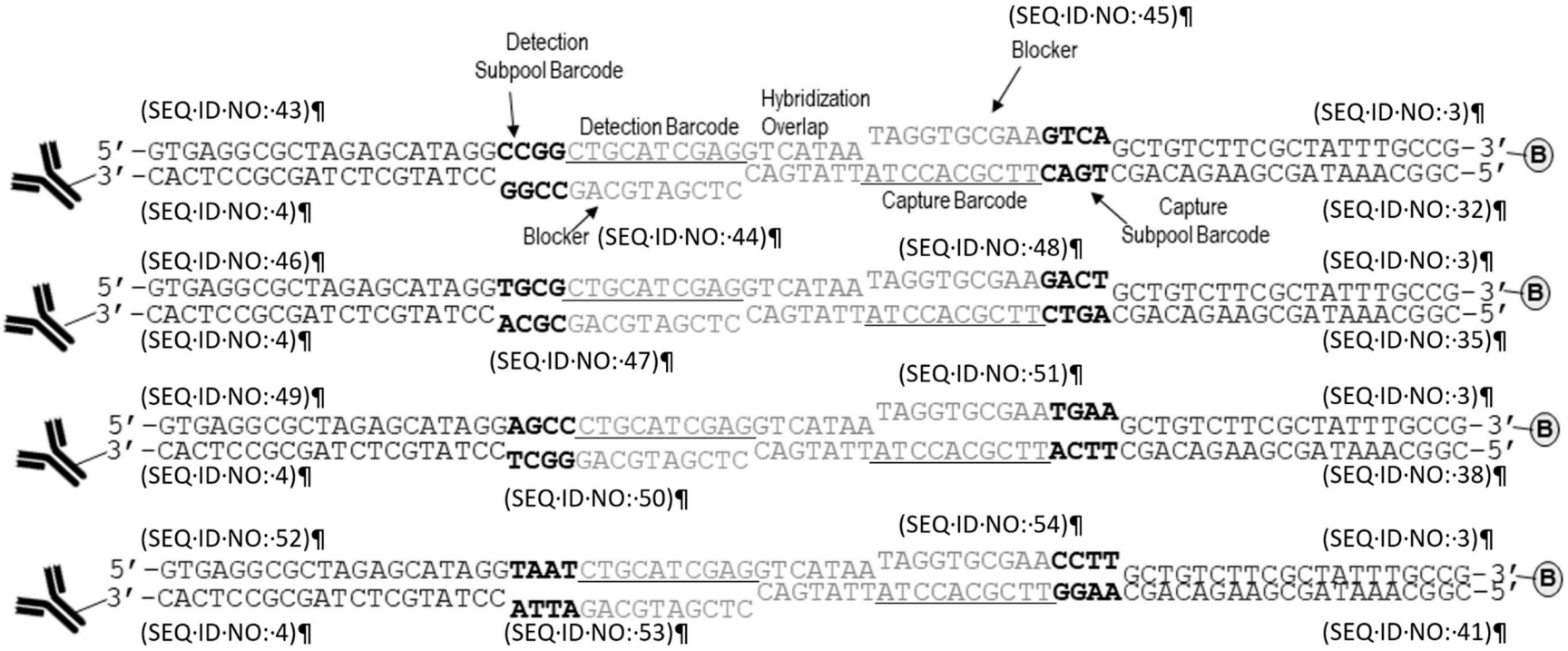

(SEQ·ID·NO:·45)¶
Blocker
Detection
Subpool Barcode
Hybridization
Overlap
(SEQ·ID·NO:·43)¶
Detection Barcode
(SEQ·ID·NO:·3)¶
5′–GTGAGGCGCTAGAGCATAGGCCGGCTGCATCGAGGTCATAA TAGGTGCGAAGTCA GCTGTCTTCGCTATTTGCCG–3′
3′–CACTCCGCGATCTCGTATCC GGCCGACGTAGCTC CAGTATTATCCACGCTTCAGTCGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·4)¶
Capture Barcode
Capture
Subpool Barcode
(SEQ·ID·NO:·32)¶
Blocker (SEQ·ID·NO:·44)¶
(SEQ·ID·NO:·48)¶
(SEQ·ID·NO:·46)¶
(SEQ·ID·NO:·3)¶
5′–GTGAGGCGCTAGAGCATAGGTGCGCTGCATCGAGGTCATAA TAGGTGCGAAGACT GCTGTCTTCGCTATTTGCCG–3′
3′–CACTCCGCGATCTCGTATCC ACGCGACGTAGCTC CAGTATTATCCACGCTTCTGACGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·4)¶
(SEQ·ID·NO:·35)¶
(SEQ·ID·NO:·47)¶
(SEQ·ID·NO:·51)¶
(SEQ·ID·NO:·49)¶
(SEQ·ID·NO:·3)¶
5′–GTGAGGCGCTAGAGCATAGGAGCCCTGCATCGAGGTCATAA TAGGTGCGAATGAA GCTGTCTTCGCTATTTGCCG–3′
3′–CACTCCGCGATCTCGTATCC TCGGGACGTAGCTC CAGTATTATCCACGCTTACTTCGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·4)¶
(SEQ·ID·NO:·38)¶
(SEQ·ID·NO:·50)¶
(SEQ·ID·NO:·54)¶
(SEQ·ID·NO:·52)¶
(SEQ·ID·NO:·3)¶
5′–GTGAGGCGCTAGAGCATAGGTAATCTGCATCGAGGTCATAA TAGGTGCGAACCTT GCTGTCTTCGCTATTTGCCG–3′
3′–CACTCCGCGATCTCGTATCC ATTAGACGTAGCTC CAGTATTATCCACGCTTGGAACGACAGAAGCGATAAACGGC–5′
(SEQ·ID·NO:·4)¶
(SEQ·ID·NO:·53)¶
(SEQ·ID·NO:·41)¶

FIG. 32B

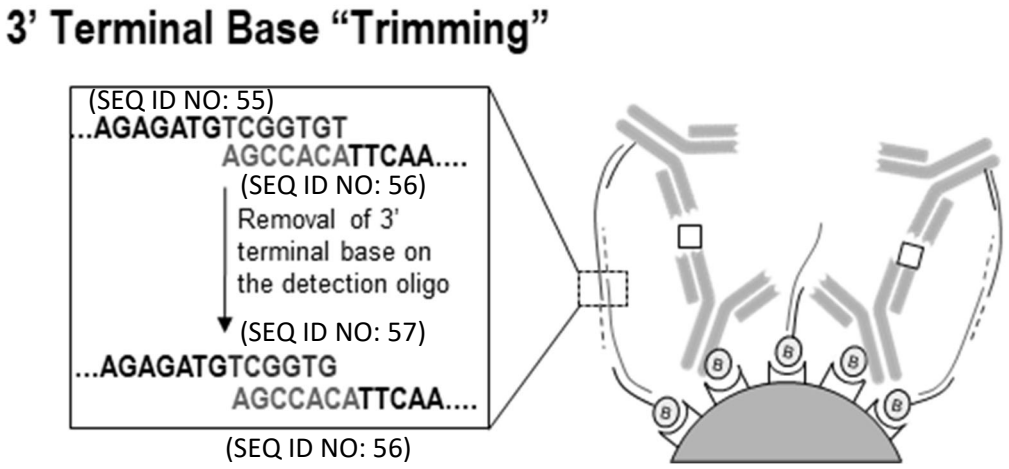


FIG. 33

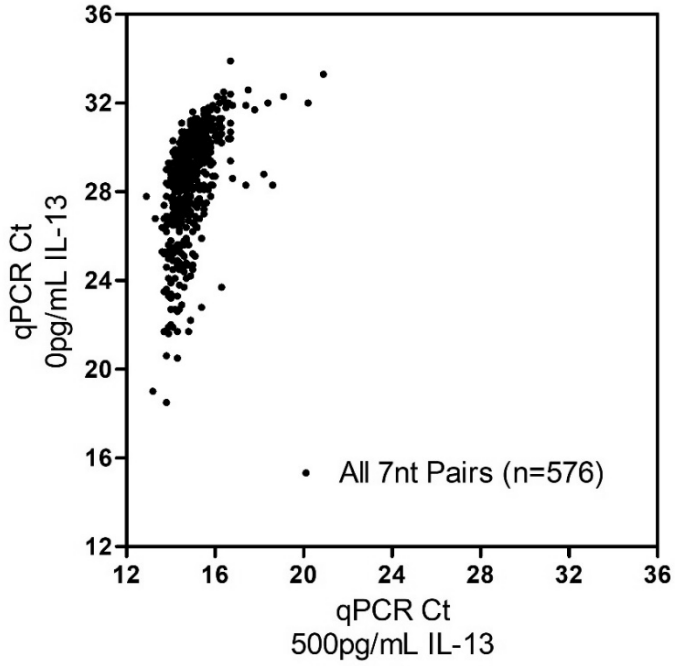


FIG. 34A

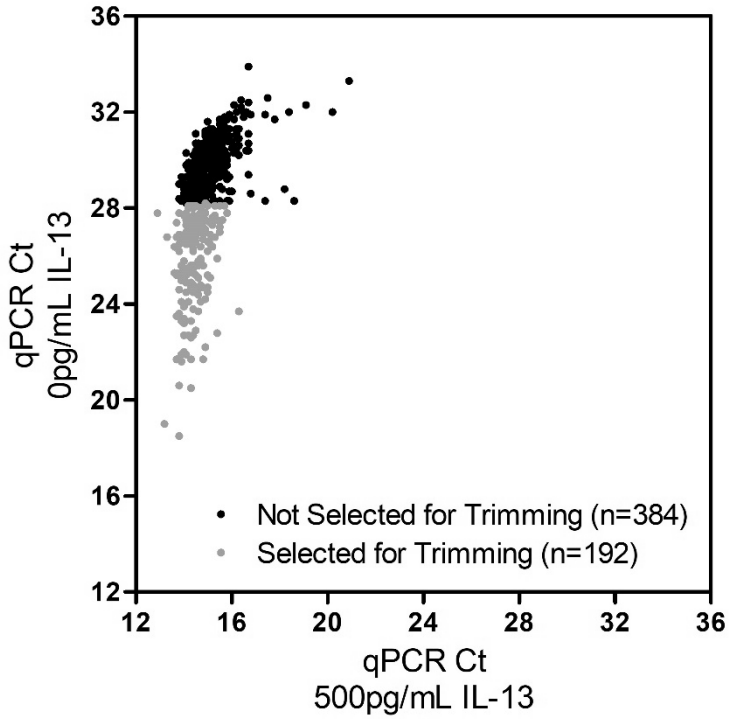


FIG. 34B

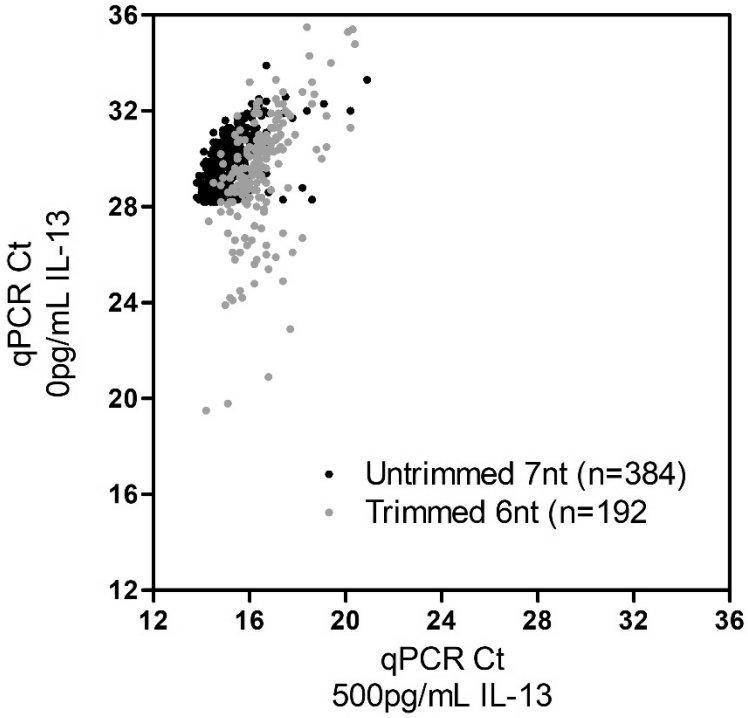


FIG. 34C

Ct Value	TOC (384 pg/mL IL-13)						NSB (Diluent 2)						Amount of Bt-Anchor Oligo (pmol Oligo/µg bead)	
	1	2	3	4	5	6	7	8	9	10	11	12		
A	9.7	9.7	10.1	11.9	13.3	15.4	13.6	13.6	14.4	19.0	22.1	24.9	0.563	9x
B	11.1	9.6	10.1	11.8	13.3	15.4	16.1	16.3	17.8	22.6	25.4	28.1	0.1875	3x
C	10.7	10.5	10.9	12.3	14.0	15.9	22.5	22.4	23.6	25.9	28.3	31.0	0.0625	1x
D	11.8	11.7	11.9	13.2	14.9	17.2	26.5	26.4	26.8	28.2	30.2	32.7	0.0208	1x/3
E	13.4	13.2	13.4	14.6	16.2	18.2	28.3	28.4	28.7	29.8	31.4	28.6	0.00694	1x/9
F	15.0	14.7	15.0	15.9	17.7	19.6	29.8	30.1	29.8	31.4	32.9	33.8	0.00231	1x/27
G	16.6	16.4	16.3	17.4	19.2	21.1	31.8	32.0	31.9	32.8	36.1	34.6	0.000772	1x/81
H	18.0	18.0	18.3	19.8	21.3	23.5	32.8	35.3	33.9	35.1	41.0	41.0	0.000257	1x/243
	8.00	2.00	0.500	0.125	0.0313	0.00781	8.00	2.00	0.500	0.125	0.0313	0.00781		
	4x	1x	1/4x	1/16x	1/64x	1/256x	4x	1x	1/4x	1/16x	1/64x	1/256x		

Delta Ct	Delta Ct Value between TOC (384 pg/mL IL-13) and NSB (Diluent 2)												Amount of Bt-Anchor Oligo (pmol Oligo/µg bead)	
	1	2	3	4	5	6	7	8	9	10	11	12		
A	4.0	3.9	4.4	7.2	8.8	9.5							0.563	9x
B	5.0	6.6	7.7	10.9	12.1	12.7							0.1875	3x
C	11.7	11.9	12.7	13.6	14.3	15.1							0.0625	1x
D	14.7	14.7	15.0	15.0	15.3	15.5							0.0208	1x/3
E	15.0	15.2	15.3	15.2	15.2	10.4							0.00694	1x/9
F	14.8	15.4	14.9	15.5	15.2	14.2							0.00231	1x/27
G	15.2	15.5	15.5	15.4	16.9	13.5							0.000772	1x/81
H	14.8	17.3	15.6	15.3	19.7	17.5							0.000257	1x/243
	8.00	2.00	0.500	0.125	0.0313	0.00781								
	4x	1x	1/4x	1/16x	1/64x	1/256x								

FIG. 35

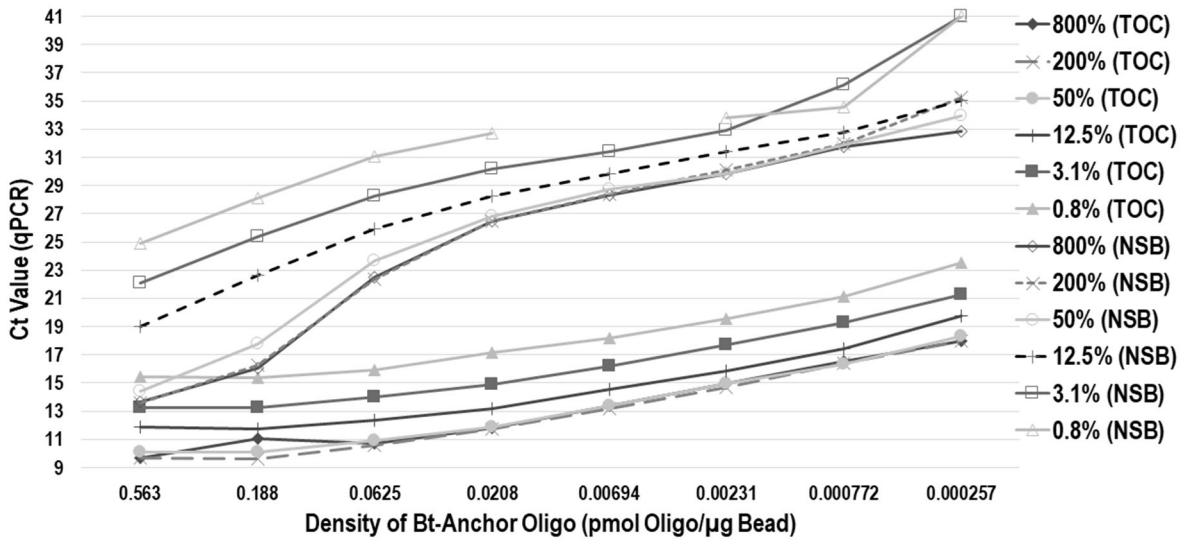


FIG. 36A

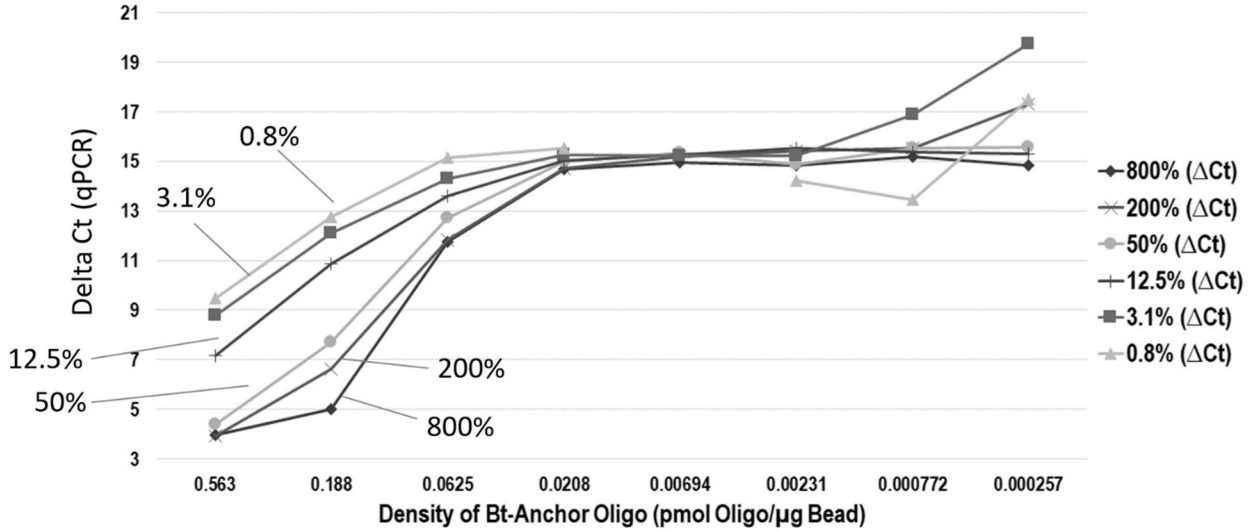


FIG. 36B

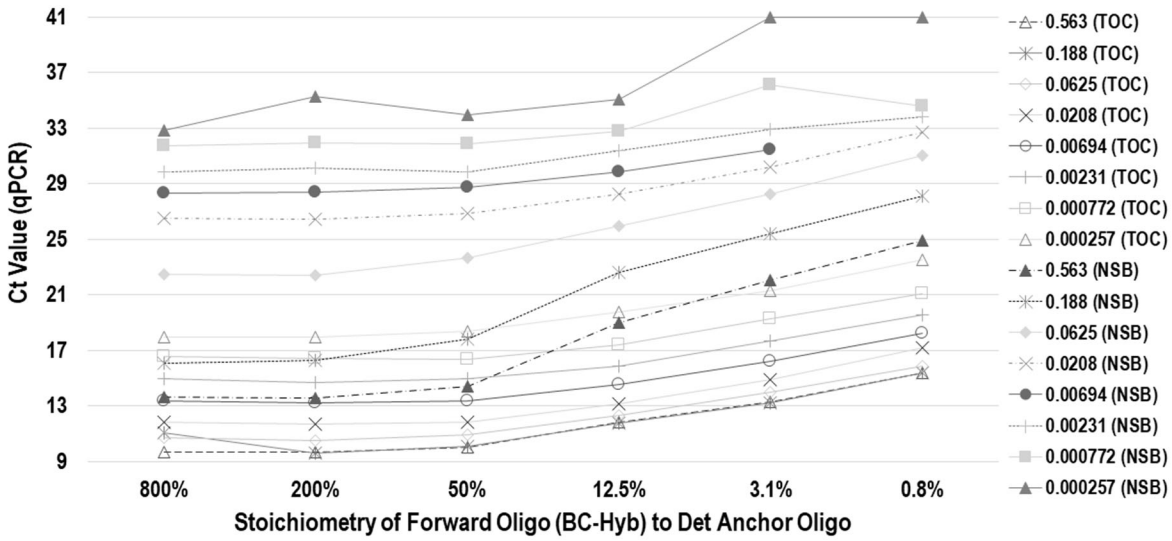


FIG. 37A

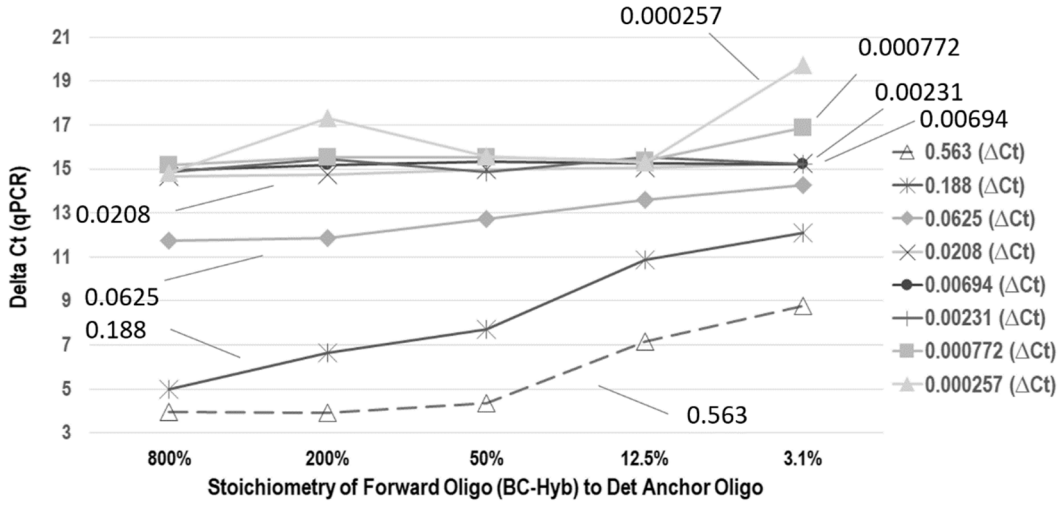


FIG. 37B

	None				None				25	None	nM Conc in Dil2
	3,125	625	125	25	3,125	625	125	25	25	0	nM Conc in Dil3
	Forward Hyb Competitor (9NT covers 7NT Hyb + 2NT BC)				**Reverse Hyb Competitor** (9NT covers 7NT Hyb + 2NT BC)				**Reverse Hyb Competitor** (9NT covers 7NT Hyb + 2NT BC)		**Competitor Type**

Ct Value	1	2	3	4	5	6	7	8	9	10	11	12	Calibrator (IL-13) Conc. in pg/mL
A	11.2	10.8	10.8	10.7	11.4	11.2	11.0	10.9	10.9	10.7	10.6	11.1	384
B	13.1	12.7	12.5	12.5	13.2	13.1	12.9	12.8	12.3	12.2	12.4	12.9	96.0
C	15.8	14.8	14.6	14.5	16.2	15.7	15.5	14.6	14.4	13.9	14.4	15.1	24.0
D	17.7	16.8	16.5	16.5	18.5	18.3	17.5	16.9	16.3	15.9	16.3	16.8	6.00
E	19.9	18.7	18.4	18.4	20.5	20.3	19.5	18.9	18.5	17.8	18.2	18.6	1.50
F	21.9	20.7	20.4	20.3	22.6	22.2	21.6	20.7	20.4	19.6	20.4	20.9	0.375
G	25.1	23.7	22.3	22.0	26.4	26.1	25.6	24.4	23.2	21.0	21.8	22.6	0
H	25.7	23.9	22.5	22.3	26.4	26.5	25.8	24.4	23.4	20.9	22.3	22.6	0

Delta Ct	1	2	3	4	5	6	7	8	9	10	11	12	Calibrator (IL-13) Conc. in pg/mL
A	14.2	13.0	11.6	11.4	15.0	15.1	14.7	13.5	12.4	10.3	11.5	11.5	384
B	12.3	11.1	9.9	9.6	13.2	13.2	12.8	11.7	11.0	8.8	9.7	9.7	96.0
C	9.7	9.0	7.8	7.6	10.2	10.6	10.3	9.8	8.9	7.1	7.7	7.5	24.0
D	7.7	7.0	5.9	5.7	7.9	8.0	8.2	7.5	7.0	5.1	5.8	5.7	6.00
E	5.6	5.1	3.9	3.7	5.9	6.0	6.2	5.6	4.8	3.2	3.9	4.0	1.50
F	3.5	3.1	2.0	1.9	3.8	4.1	4.1	3.7	2.9	1.4	1.6	1.7	0.375
G	0.3	0.1	0.1	0.1	0.0	0.2	0.1	0.0	0.1	0.0	0.3	0.0	0
H	-0.3	-0.1	-0.1	-0.1	0.0	-0.2	-0.1	0.0	-0.1	0.0	-0.3	0.0	0

	None				None				25	None	nM Conc in Dil2
	3,125	625	125	25	3,125	625	125	25	25	0	nM Conc in Dil3
	Forward Hyb Competitor (9NT covers 7NT Hyb + 2NT BC)				**Reverse Hyb Competitor** (9NT covers 7NT Hyb + 2NT BC)				**Reverse Hyb Competitor** (9NT covers 7NT Hyb + 2NT BC)		**Competitor Type**

FIG. 38

	SEQ ID NO:		
Set 1			
FwPESD412-12	59	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC TAT CT	
FwPESD412-11	60	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC TAT C	
FwPESD412-10	61	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC TAT	
FwPESD412-09	62	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC TA	
FwPESD412-08	63	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC T	
FwPESD412-07	64	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GAC	
FwPESD412-06	65	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC GA	
FwPESD412-05	66	GTG AGG CGC TAG AGC ATA GGN NNN NNN NAA CAC GAG GAG TTC G	
	58	C AAG CTG ATA GAG GCG NNN NNN NNC GAC AGA AGC GAT AAA CGG C	Rv-PESD512
Set 2			
FwPESD427-12	68	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC CTT CG	
FwPESD427-11	69	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC CTT C	
FwPESD427-10	70	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC CTT	
FwPESD427-09	71	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC CT	
FwPESD427-08	72	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC C	
FwPESD427-07	73	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AGC	
FwPESD427-06	74	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA AG	
FwPESD427-05	75	GTG AGG CGC TAG AGC ATA GGN NNN NNN NGC CAA GAA GGG CGA A	
	67	C GCT TCG GAA GCT AAT NNN NNN NNC GAC AGA AGC GAT AAA CGG C	Rv-PESD527
Set 3			
FwPESD451-12	77	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC TTG TT	
FwPESD451-11	78	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC TTG T	
FwPESD451-10	79	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC TTG	
FwPESD451-09	80	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC TT	
FwPESD451-08	81	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC T	
FwPESD451-07	82	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA ACC	
FwPESD451-06	83	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA AC	
FwPESD451-05	84	GTG AGG CGC TAG AGC ATA GGN NNN NNN NCT CAA TCT GAA GGA A	
	76	T CCT TGG AAC AAG CAA NNN NNN NNC GAC AGA AGC GAT AAA CGG C	Rv-PESD551

FIG. 39A

Hybridization Length (bp)	Set 1		Set 2		Set 3	
	497 pg/mL IL-13	0 pg/mL IL-13	497 pg/mL IL-13	0 pg/mL IL-13	497 pg/mL IL-13	0 pg/mL IL-13
12	7.5	8.4	9.6	17.5	8.2	11.3
11	7.3	9.7	9.7	17.9	8.1	11.9
10	8.2	13.6	9.8	19	8.5	15.4
9	8.1	14.7	10.1	21.4	8.6	17.4
8	8.4	14.6	10.3	24.3	9.4	19.8
7	9.2	15.4	10.2	24.4	9.4	23
6	9.9	21.7	10.6	24.8	10.6	25.6
5	10.9	23.6	13.3	26.7	12.6	27.9

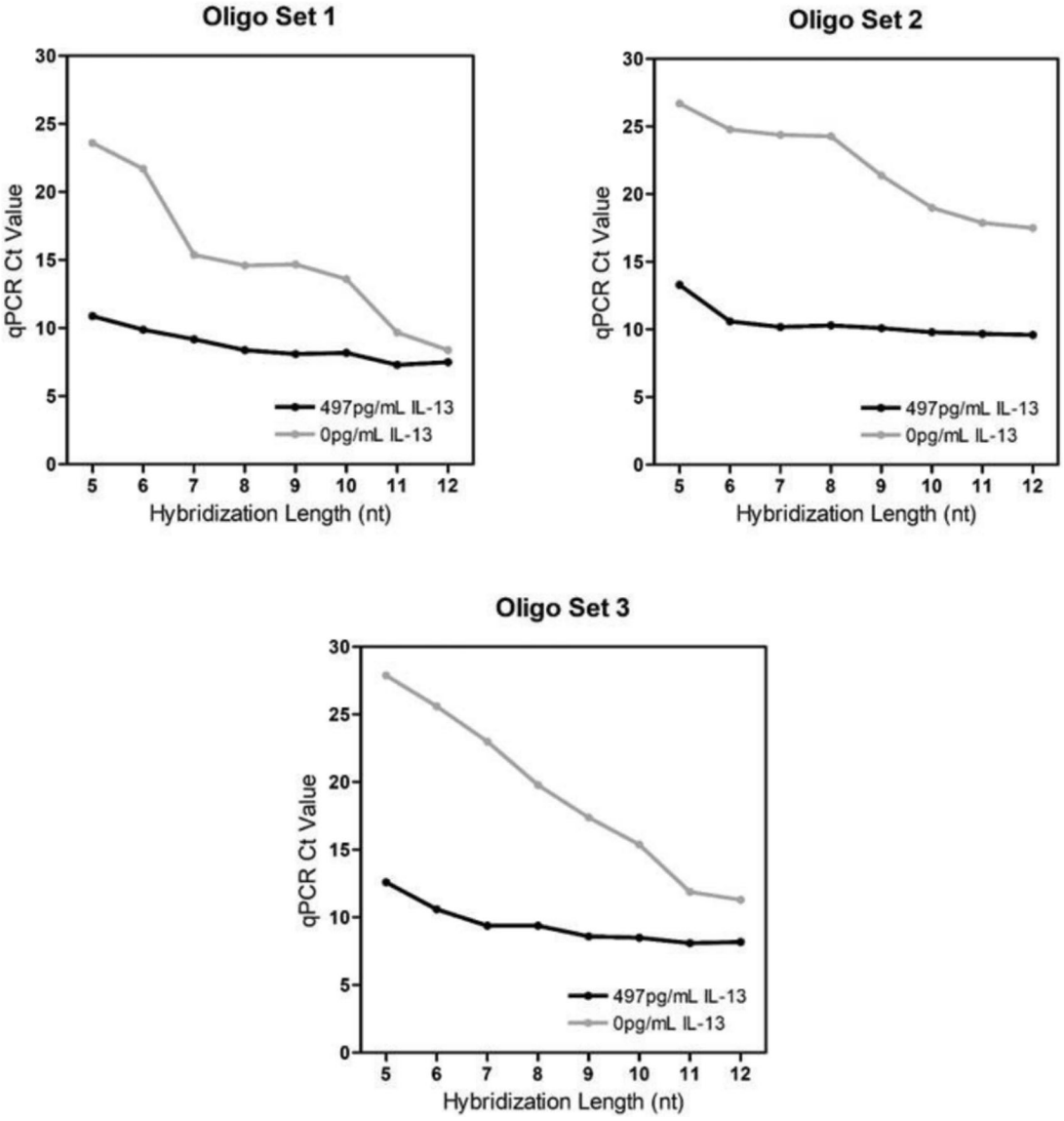


FIG. 39B

	deltaCt			Dynamic Range (fold-change)		
Hybridization Length (bp)	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
12	0.9	7.9	3.1	2	239	9
11	2.4	8.2	3.8	5	294	14
10	5.4	9.2	6.9	42	588	119
9	6.6	11.3	8.8	97	2521	446
8	6.2	14.0	10.4	74	16384	1351
7	6.2	14.2	13.6	74	18820	12417
6	11.8	14.2	15.0	3566	18820	32768
5	12.7	13.4	15.3	6654	10809	40342

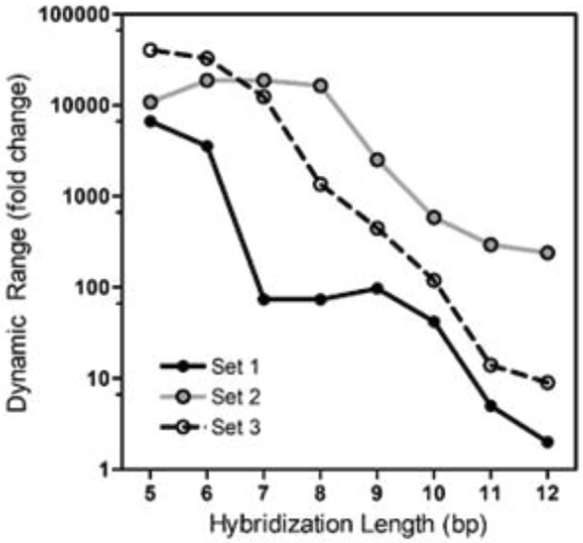


FIG. 39C

Cold cAb (ng)	0	150	450	4950
[IL-13] (pg/mL)	1X Calibration Curve			
1000	13.6	16.2	18.1	21.4
250	14.1	17.9	19.6	22.7
62.5	15.2	19.7	21.6	24.6
15.625	17.5	22.0	23.7	26.5
3.906	19.3	24.2	25.6	27.8
0.977	21.3	25.8	27.2	28.9
0.244	23.3	27.6	28.8	29.1
0	29.2	29.6	29.4	29.8
Dynamic Range	15.5	13.5	11.4	8.4

FIG. 40A

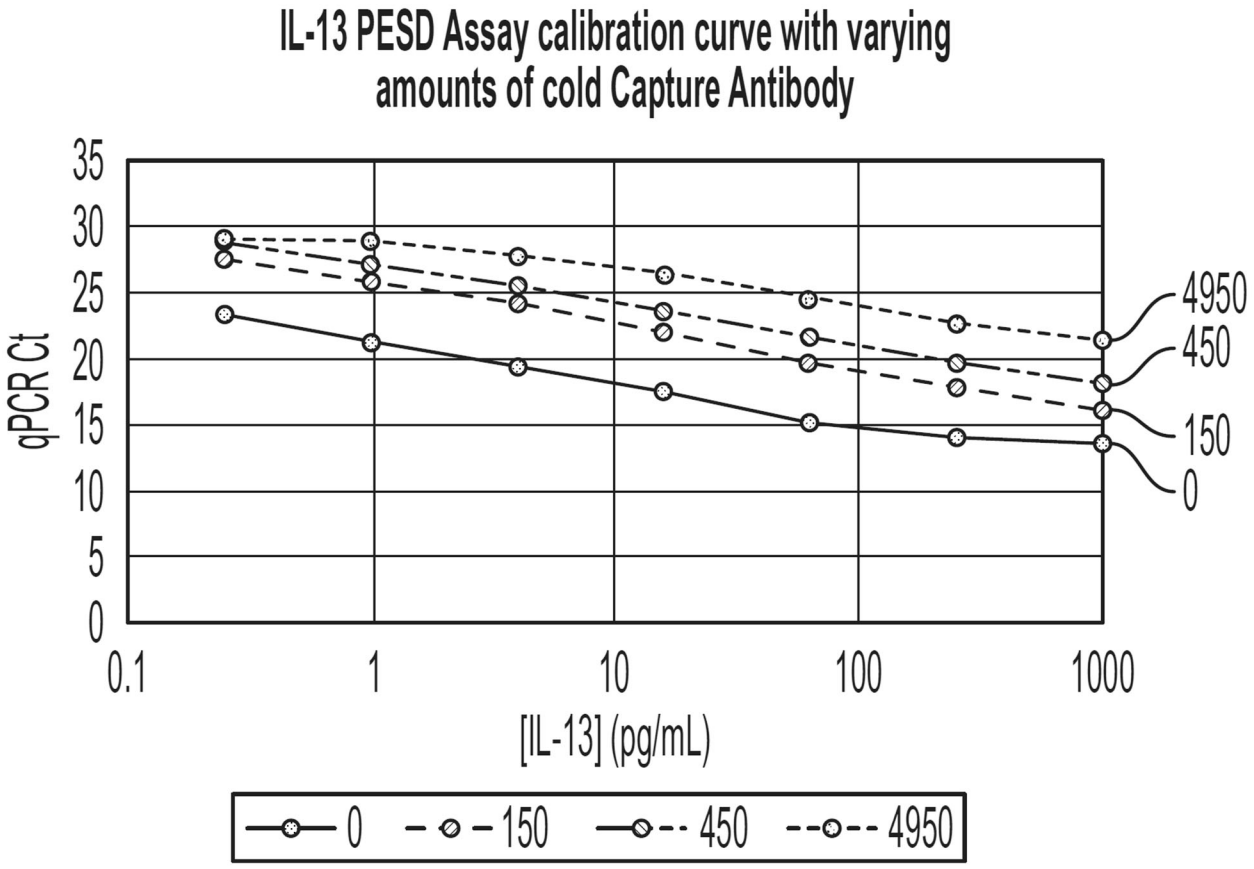

IL-13 PESD Assay calibration curve with varying amounts of cold Capture Antibody
qPCR Ct
35
30
25
20
15
10
5
0
0.1
1
10
100
1000
[IL-13] (pg/mL)
4950
450
150
0
0
150
450
4950

FIG. 40B

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal 1	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Cal 1	Sample 41	Sample 49	Sample 57	Sample 65	Sample 73
B	Cal 2	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Cal 2	Sample 42	Sample 50	Sample 58	Sample 66	Sample 74
C	Cal 3	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Cal 3	Sample 43	Sample 51	Sample 59	Sample 67	Sample 75
D	Cal 4	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Cal 4	Sample 44	Sample 52	Sample 60	Sample 68	Sample 76
E	Cal 5	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Cal 5	Sample 45	Sample 53	Sample 61	Sample 69	Sample 77
F	Cal 6	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Cal 6	Sample 46	Sample 54	Sample 62	Sample 70	Sample 78
G	Cal 7	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Cal 7	Sample 47	Sample 55	Sample 63	Sample 71	Sample 79
H	Cal 8	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Cal 8	Sample 48	Sample 56	Sample 64	Sample 72	Sample 80

96-well plate:

- 16 wells reserved for Calibrators 1-8 (duplicates)
- 80 wells reserved for patient samples (singlicate)

[Total PESD Product[(pM)

	1	2	3	4	5	6	7	8	9	10	11	12
A	100,000	1,000	1,000	1,000	1,000	1,000	100,000	1,000	1,000	1,000	1,000	1,000
B	25,000	1,000	1,000	1,000	1,000	1,000	25,000	1,000	1,000	1,000	1,000	1,000
C	6,250	1,000	1,000	1,000	1,000	1,000	6,250	1,000	1,000	1,000	1,000	1,000
D	1,563	1,000	1,000	1,000	1,000	1,000	1,563	1,000	1,000	1,000	1,000	1,000
E	391	1,000	1,000	1,000	1,000	1,000	391	1,000	1,000	1,000	1,000	1,000
F	98	1,000	1,000	1,000	1,000	1,000	98	1,000	1,000	1,000	1,000	1,000
G	24	1,000	1,000	1,000	1,000	1,000	24	1,000	1,000	1,000	1,000	1,000
H	6	1,000	1,000	1,000	1,000	1,000	6	1,000	1,000	1,000	1,000	1,000

8 PCR Cycles, [Primers] = 5µM
No primer depletion

[Post Adapter PCR Product] (pM)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,560,000	25,600	25,600	25,600	25,600	25,600	2,560,000	25,600	25,600	25,600	25,600	25,600
B	640,000	25,600	25,600	25,600	25,600	25,600	640,000	25,600	25,600	25,600	25,600	25,600
C	160,000	25,600	25,600	25,600	25,600	25,600	160,000	25,600	25,600	25,600	25,600	25,600
D	40,000	25,600	25,600	25,600	25,600	25,600	40,000	25,600	25,600	25,600	25,600	25,600
E	10,000	25,600	25,600	25,600	25,600	25,600	10,000	25,600	25,600	25,600	25,600	25,600
F	2,500	25,600	25,600	25,600	25,600	25,600	2,500	25,600	25,600	25,600	25,600	25,600
G	625	25,600	25,600	25,600	25,600	25,600	625	25,600	25,600	25,600	25,600	25,600
H	156	25,600	25,600	25,600	25,600	25,600	156	25,600	25,600	25,600	25,600	25,600

Fraction of molecules (and thus NGS reads) allocated to standards: 76.9%

8 PCR Cycles, [Primers] = 0.5µM

[Post Adapter PCR Product] (pM)

	1	2	3	4	5	6	7	8	9	10	11	12
A	500,000	25,600	25,600	25,600	25,600	25,600	500,000	25,600	25,600	25,600	25,600	25,600
B	500,000	25,600	25,600	25,600	25,600	25,600	500,000	25,600	25,600	25,600	25,600	25,600
C	160,000	25,600	25,600	25,600	25,600	25,600	160,000	25,600	25,600	25,600	25,600	25,600
D	40,000	25,600	25,600	25,600	25,600	25,600	40,000	25,600	25,600	25,600	25,600	25,600
E	10,000	25,600	25,600	25,600	25,600	25,600	10,000	25,600	25,600	25,600	25,600	25,600
F	2,500	25,600	25,600	25,600	25,600	25,600	2,500	25,600	25,600	25,600	25,600	25,600
G	625	25,600	25,600	25,600	25,600	25,600	625	25,600	25,600	25,600	25,600	25,600
H	156	25,600	25,600	25,600	25,600	25,600	156	25,600	25,600	25,600	25,600	25,600

Fraction of molecules (and thus NGS reads) allocated to standards: 54.2%

8 PCR Cycles, [Primers] = 0.05µM

[Post Adapter PCR Product] (pM)

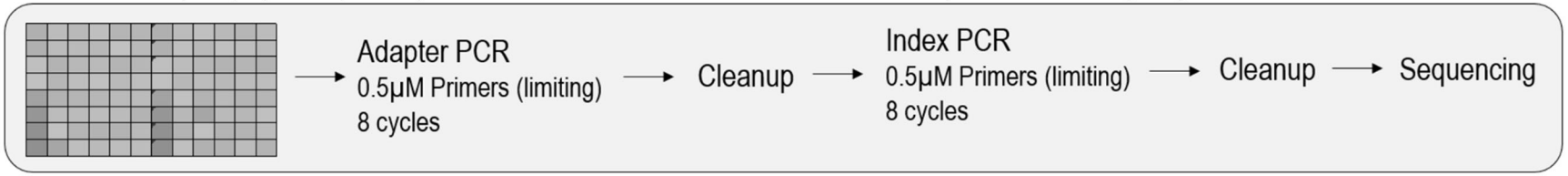
	1	2	3	4	5	6	7	8	9	10	11	12
A	50,000	25,600	25,600	25,600	25,600	25,600	50,000	25,600	25,600	25,600	25,600	25,600
B	50,000	25,600	25,600	25,600	25,600	25,600	50,000	25,600	25,600	25,600	25,600	25,600
C	50,000	25,600	25,600	25,600	25,600	25,600	50,000	25,600	25,600	25,600	25,600	25,600
D	40,000	25,600	25,600	25,600	25,600	25,600	40,000	25,600	25,600	25,600	25,600	25,600
E	10,000	25,600	25,600	25,600	25,600	25,600	10,000	25,600	25,600	25,600	25,600	25,600
F	2,500	25,600	25,600	25,600	25,600	25,600	2,500	25,600	25,600	25,600	25,600	25,600
G	625	25,600	25,600	25,600	25,600	25,600	625	25,600	25,600	25,600	25,600	25,600
H	156	25,600	25,600	25,600	25,600	25,600	156	25,600	25,600	25,600	25,600	25,600

Fraction of molecules (and thus NGS reads) allocated to standards: 16.6%

FIG. 41A

96-well plate:

- 16 wells reserved for Calibrators 1-8 (duplicates)
- 40 mock samples (duplicates) – samples contrived with random concentrations of PESD product within 1log range



	Actual [PESD Product] (pM)		Post Adapter PCR Product (pM)		# of NGS Reads	
Calibrator	1	2	1	2	1	2
1	597,523	640,513	6,221,356	6,885,041	2,716,834	2,593,010
2	154,404	194,320	5,932,921	6,778,813	3,873,512	2,719,794
3	45,365	35,960	5,976,459	5,626,744	3,182,911	2,164,672
4	12,815	9,356	4,018,016	3,166,562	3,565,562	81,2704
5	2,520	2,890	2,427,755	1,956,901	388,068	221,144
6	671	753	939,638	762,605	100,961	54,153
7	144	222	337,269	238,396	28,529	16,098
8	0	0	2	2	2	10

Collapsing of calibrator range observed at the top of the cal curve due to primer depletion

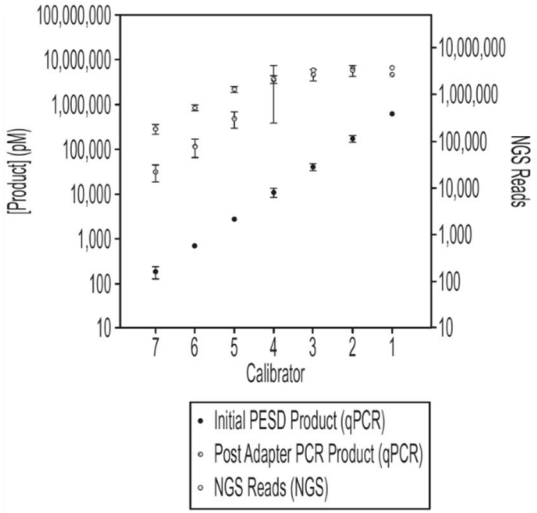
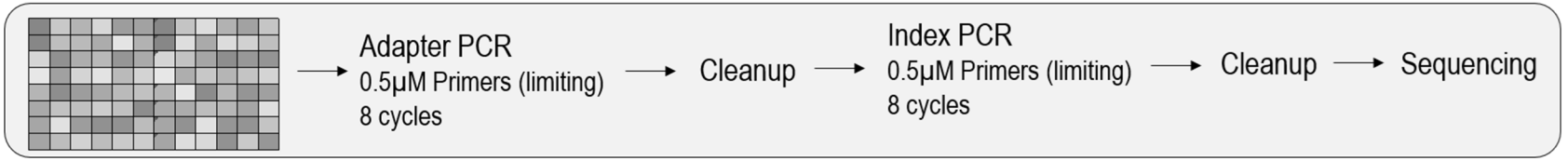


FIG. 41B

96-well plate:

- 16 wells reserved for Calibrators 1-8 (duplicates)
- 40 mock samples (duplicates) – samples contrived with random concentrations of PESD product within 1log range



	Actual [PESD Product] (pM)		Post Adapter PCR Product (pM)		# of NGS Reads	
Sample #	1	2	1	2	1	2
1	22,910	23,290	5,725,854	4,961,139	1,895,241	2,056,097
2	23,919	21,932	175,688	5,531,937	14,165	1,641,071
3	20,047	18,748	4,824,211	3,801,101	1,581,145	1,513,304
4	7,635	7,524	3,639,896	3,304,046	512,526	510,660
5	31,005	27,615	164,528	4,650,935	9,362	2,090,859
6	13,831	11,047	4,761,847	3,581,862	948,790	829,445
7	6,316	7,053	3,364,131	3,249,779	484,234	384,086
8	18,347	18,190	4,907,221	4,920,821	2,008,328	1,548,464
9	36,891	42,557	6,283,490	6,674,116	2,861,910	2,790,236
10	33,623	39,417	5,069,037	6,255,317	2,108,890	2,137,797
11	39,423	41,519	112,869	6,537,612	5,506	2,187,236
12	24,579	24,159	3,923,167	5,845,461	596,474	1,667,109
13	51,206	56,808	5,962,108	5,927,412	3,077,795	2,767,411
14	40,578	40,817	6,827,381	4,978,384	2,824,813	2,203,355
15	26,750	10,221	3,277,400	2,742,931	596,175	514,438
16	6,042	5,691	2,853,021	2,230,348	492,392	425,536
17	16,151	16,463	5,209,531	4,739,308	1,773,954	1,594,566
18	8,602	10,648	4,357,322	3,704,791	780,002	644,073
19	10,893	11,735	344,369	5,158,642	21,317	625,268
20	49,656	52,505	5,357,922	6,132,720	2,580,426	2,432,348

	Actual [PESD Product] (pM)		Post Adapter PCR Product (pM)		# of NGS Reads	
Sample #	1	2	1	2	1	2
21	3,158	8,094	2,614,560	3,428,464	507,789	507,375
22	7,807	8,462	3,002,391	3,600,136	593,198	436,644
23	32,744	35,267	6,394,232	5,802,432	2,695,597	1,876,395
24	17,350	17,244	4,348,093	4,960,646	1,507,451	1,398,700
25	23,686	19,298	5,622,260	5,096,529	1,323,473	1,885,384
26	6,851	6,440	3,260,807	4,042,450	418,854	535,039
27	68,937	60,594	5,981,642	6,331,675	2,487,932	2,706,966
28	25,399	27,851	4,788,317	6,950,571	1,856,535	1,721,834
29	28,424	33,180	5,253,426	5,965,070	1,654,230	2,045,866
30	40,754	45,635	5,322,428	5,986,471	2,624,550	2,695,698
31	30,838	37,367	5,680,413	6,111,545	2,124,733	2,329,368
32	7,985	7,929	3,547,116	4,037,647	701,968	604,503
33	13,005	11,627	6,466,827	5,082,075	1,073,764	1,324,280
34	35,360	22,134	6,428,568	7,062,604	2,347,907	2,782,061
35	8,781	8,882	3,289,135	5,215,218	590,189	691,574
36	16,632	14,090	4,623,559	3,764,616	940,230	1,232,774
37	7,623	8,233	3,112,798	4,032,629	529,604	576,936
38	23,396	25,715	4,772,390	5,215,428	1,802,043	2,073,075
39	36,637	34,760	4,920,623	6,796,599	2,165,202	2,510,228
40	28,932	28,255	4,368,409	6,097,551	2,378,179	2,651,293

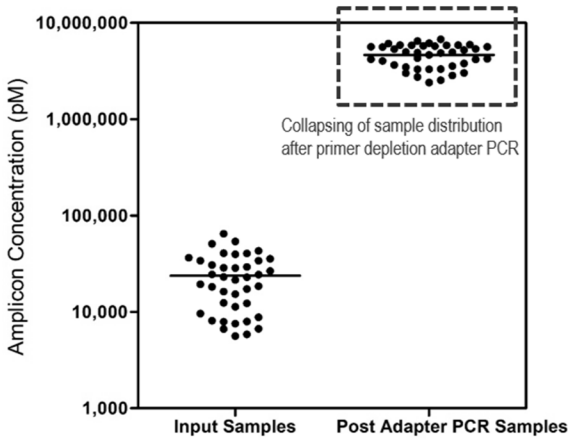


FIG. 41C

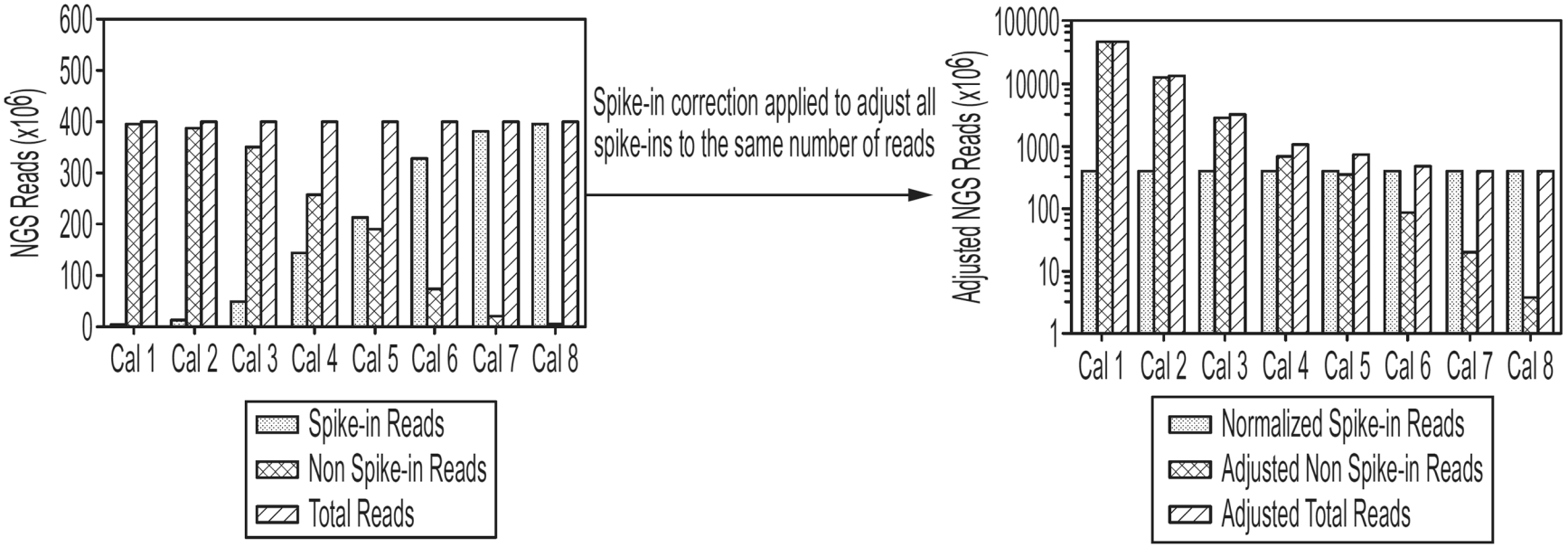
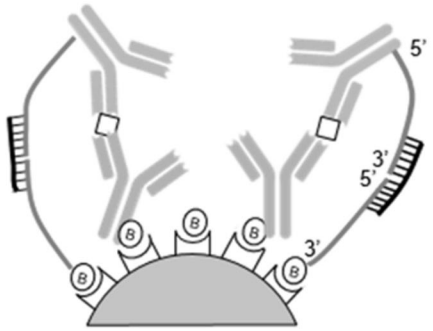

NGS Reads (x10^6)
600
500
400
300
200
100
0
Cal 1
Cal 2
Cal 3
Cal 4
Cal 5
Cal 6
Cal 7
Cal 8
Spike-in Reads
Non Spike-in Reads
Total Reads
Spike-in correction applied to adjust all spike-ins to the same number of reads
Adjusted NGS Reads (x10^6)
100000
10000
1000
100
10
1
Normalized Spike-in Reads
Adjusted Non Spike-in Reads
Adjusted Total Reads

FIG. 42

Proximity Ligation Assay (PLA) Oligo Design
"Asymmetrical" Splints



Antibody-conjugated "Detector-side" Oligo
(same antibody-oligo conjugate used in PEA)

Biotinylated "Capture-side" Oligo on Clonal Bead

5' Thiol — For. Primer Region — UMI — Detector Barcode — Splint Hyb Region — 3' | 5' Phos — Splint Hyb Region — Capture Barcode — UMI — Rev. Primer Region — 3' Biotin

GTA TGC GAT GCC AGG TTT GAN NNN NNN GGG CTA AAT TGT CTT ACA AC | AGA CAA CGG CAG CAT TCT AGN NNN NNN TAT GAC CAG CGT TTC CTT GT
(SEQ ID NO: 85) (SEQ ID NO: 86)

'Asymmetrical' splints for PLA

3' – CA GAA TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 87)
3' – A GAA TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 88)
3' – GAA TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 89)
3' – AA TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 90)
3' – A TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 91)
3' – TGT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 92)
3' – GT TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 93)
3' – T TG	TCT GTT GCC GTC GTA AGA TC - 5' (SEQ ID NO: 94)

- ✓ Variable overhangs (10-3bp) complementary to detection conjugate oligo
- ✓ 20bp region for hybridization to bead-coating oligo

FIG. 43

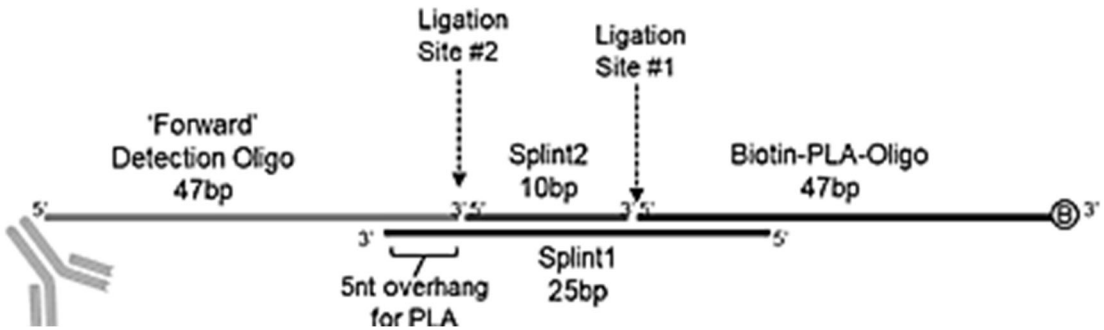

Ligation
Site #2
Ligation
Site #1
'Forward'
Detection Oligo
47bp
Splint2
10bp
Biotin-PLA-Oligo
47bp
5'
3'
5'
3'
5'
3'
B
3'
5'
Splint1
25bp
5nt overhang
for PLA

FIG. 44A

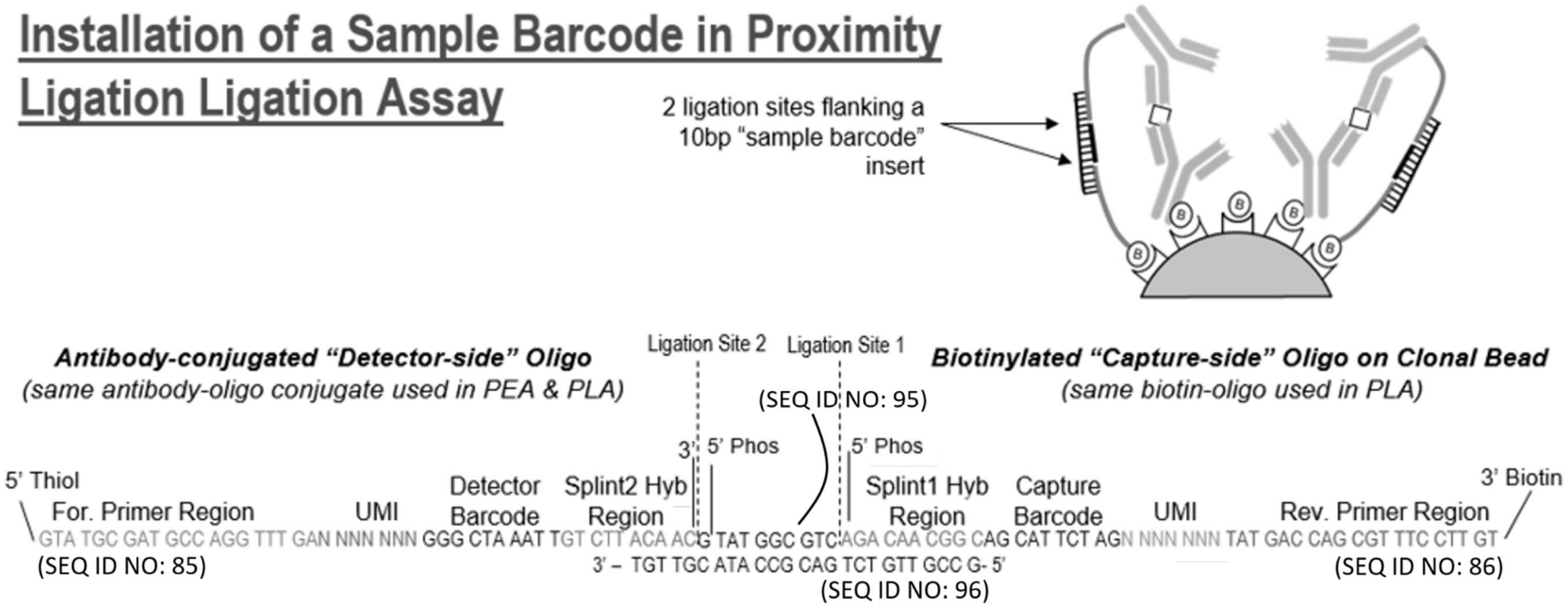

Installation of a Sample Barcode in Proximity Ligation Ligation Assay
2 ligation sites flanking a 10bp "sample barcode" insert
Antibody-conjugated "Detector-side" Oligo
(same antibody-oligo conjugate used in PEA & PLA)
Ligation Site 2
Ligation Site 1
Biotinylated "Capture-side" Oligo on Clonal Bead
(same biotin-oligo used in PLA)
(SEQ ID NO: 95)
3'
5' Phos
5' Phos
5' Thiol
For. Primer Region
UMI
Detector Barcode
Splint2 Hyb Region
Splint1 Hyb Region
Capture Barcode
UMI
Rev. Primer Region
3' Biotin
GTA TGC GAT GCC AGG TTT GAN NNN NNN GGG CTA AAT TGT CTT ACA ACG TAT GGC GTC AGA CAA CGG CAG CAT TCT AGN NNN NNN TAT GAC CAG CGT TTC CTT GT
(SEQ ID NO: 85)
3' – TGT TGC ATA CCG CAG TCT GTT GCC G- 5'
(SEQ ID NO: 96)
(SEQ ID NO: 86)

FIG. 44B

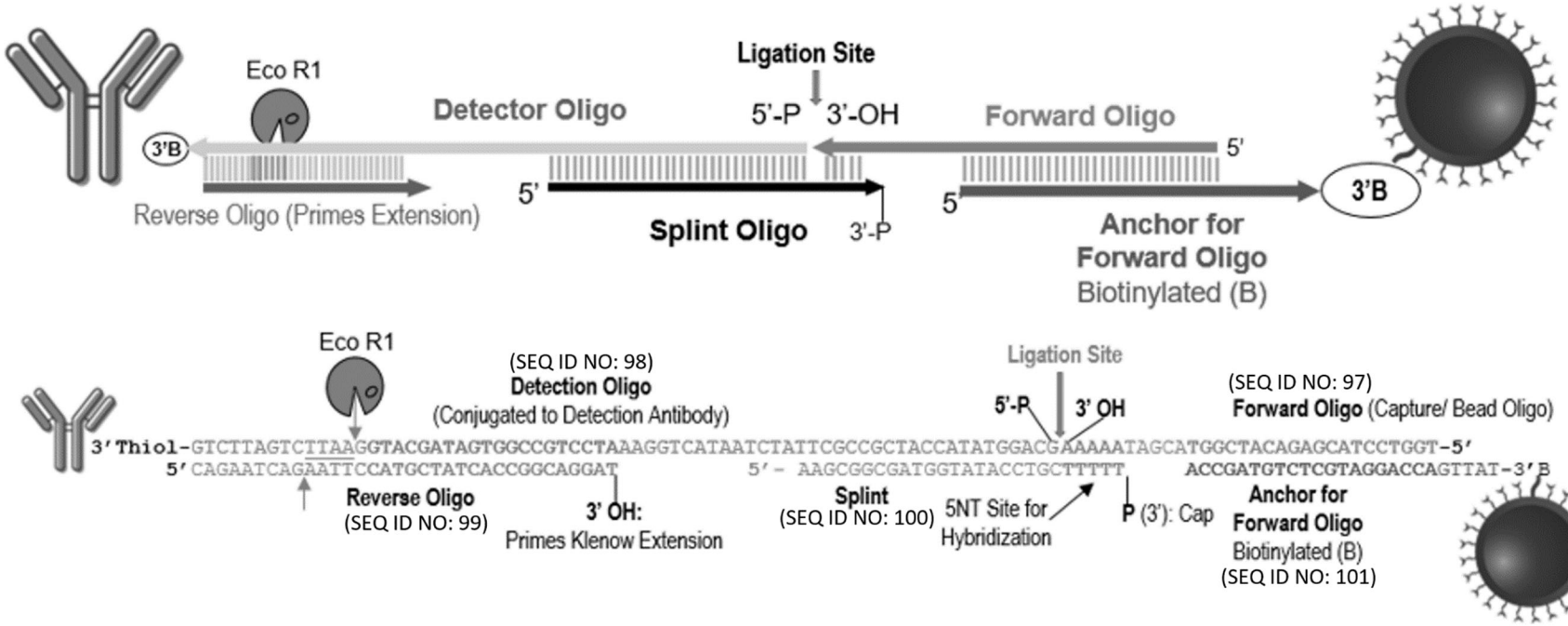

Eco R1
Ligation Site
Detector Oligo
5'-P
3'-OH
Forward Oligo
3'B
5'
Reverse Oligo (Primes Extension)
Splint Oligo
3'-P
Anchor for
Forward Oligo
Biotinylated (B)
(SEQ ID NO: 98)
Detection Oligo
(Conjugated to Detection Antibody)
(SEQ ID NO: 97)
Forward Oligo (Capture/ Bead Oligo)
3'Thiol-GTCTTAGTCTTAAGGTACGATAGTGGCCGTCCTAAAGGTCATAATCTATTCGCCGCTACCATATGGACGAAAAATAGCATGGCTACAGAGCATCCTGGT-5'
5'CAGAATCAGAATTCCATGCTATCACCGGCAGGAT
5'- AAGCGGCGATGGTATACCTGCTTTTT
ACCGATGTCTCGTAGGACCAGTTAT-3'B
Reverse Oligo
(SEQ ID NO: 99)
3' OH:
Primes Klenow Extension
Splint
(SEQ ID NO: 100)
5NT Site for
Hybridization
P (3'): Cap
Anchor for
Forward Oligo
Biotinylated (B)
(SEQ ID NO: 101)
(SEQ ID NO: 102)
qPCR
5'-CATGCTATCACCGGCAGGAT-3' Reverse Primer (PEA Set3)
(SEQ ID NO: 103)
3'-GTCTTAGTCTTAAGGTACGATAGTGGCCGTCCTAAAGGTCATAATCTATTCGCCGCTACCATATGGACGAAAAATAGCATGGCTACAGAGCATCCTGGTCAATA-5'
5'-CAGAATCAGAATTCCATGCTATCACCGGCAGGATTTCCAGTATTAGATAAGCGGCGATGGTATACCTGCTTTTTATCGTACCGATGTCTCGTAGGACCAGTTAT-3'
(SEQ ID NO: 104)
Forward Primer (PEA Set3) 3'-TGGCTACAGAGCATCCTGGT-5'
(SEQ ID NO: 105)
3' GAATTC
5' CTTAAG
Eco R1
Recognition
Site
PLSD_Reverse CAGAATCAGAATTCCATGCTATCACCGGCAGGAT (SEQ ID NO: 99)
PLSD_Splint AAGCGGCGATGGTATACCTGCTTTTT (SEQ ID NO: 100)
PLSD_Detector_3Thiol /5Phos/GCAGGTATACCATCGCCGCTTATCTAATACTGGAAATCCTGCCGGTGATAGCATGGAATTCTGATTCTG/3ThioMC3-D/
PLSD_ForwardV2 TGGTCCTACGAGACATCGGTACGATAAAAA (SEQ ID NO: 97)
(SEQ ID NO: 98)
PLSD_For_AnchorV2 ACCGATGTCTCGTAGGACCAGTTAT/3Bio/ (SEQ ID NO: 101)
PEA Set3 Forward Primer (qPCR) TGGTCCTACGAGACATCGGT (SEQ ID NO: 105)
PEA Set3 Reverse Primer (qPCR) CATGCTATCACCGGCAGGAT (SEQ ID NO: 102)

FIG. 45

[T4 Ligase] (ng/mL)		25				5				1			
[IL-12] (pg/mL)		0		464		0		464		0		464	
		1	2	1	2	1	2	1	2	1	2	1	2
Splint Overhang (nt)	3	34.74	Undetermined	23.77	23.93	36.54	Undetermined	24.87	25.55	34.37	Undetermined	26.67	27.21
	4	34.46	38.48	22.39	22.48	35.64	Undetermined	24.32	24.45	36.57	38.52	26.49	26.73
	5	29.85	29.43	15.82	15.94	30.90	31.10	17.24	17.59	30.80	31.50	18.66	19.18
	6	28.14	27.51	15.11	15.20	28.83	28.67	15.29	15.58	29.78	28.95	16.28	16.63
	7	24.73	23.77	14.72	14.95	25.50	25.85	15.13	15.24	26.26	26.57	15.41	15.74
	8	22.66	24.18	14.75	14.44	23.92	23.60	14.59	15.09	23.66	23.92	15.00	15.14
	9	23.74	21.65	14.55	14.77	24.31	21.88	14.74	14.94	23.46	23.36	14.67	15.38
	10	25.60	22.32	14.74	14.69	25.06	22.49	14.60	14.80	25.68	24.52	14.69	15.33

FIG. 46A

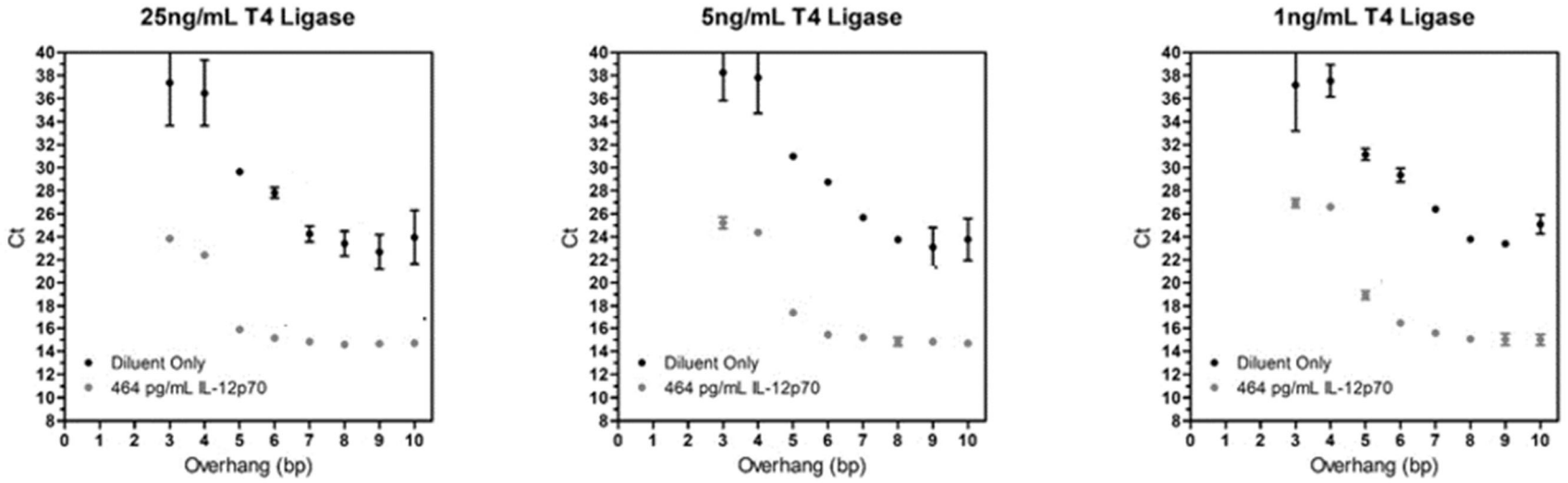


FIG. 46B

	PLLA Singleplex IL-12		No Splint 2		No Splint 1		No Ligase (during Sample BC addition)	
[IL-12] (pg/mL)	1	2	1	2	1	2	1	2
464	16.67	18.51	29.32	29.80	29.41	30.39	18.47	20.53
116	19.44	18.63	30.15	30.82	30.66	31.79	19.63	22.12
29	20.95	20.80	32.63	32.41	32.53	32.05	21.87	21.64
7.25	22.35	23.08	35.72	36.06	34.29	35.74	23.84	23.44
1.81	24.20	24.83	Undetermined	35.65	35.85	35.91	26.05	25.66
0.453	26.59	26.85	35.92	Undetermined	36.97	34.95	28.18	27.67
0.113	28.25	34.74	35.75	35.76	Undetermined	Undetermined	29.33	29.35
0.000	29.06	36.86	35.49	35.98	36.70	36.89	30.56	31.06
NTC							33.58	35.81

FIG. 47

NaCl	50	50	10	50	50	10	50	50	10	50	50	10	NaCl (mM)
$MgCl_2$	10	2	2	10	2	2	10	2	2	10	2	2	$MgCl_2$ (mM)
	#2	#3	#5	#2	#3	#5	#2	#3	#5	#2	#3	#5	In-house Buffer

Ct	1	2	3	4	5	6	7	8	9	10	11	12	Analyte Conc.
A	10.8	9.9	9.9	13.4	12.6	12.7	10.5	9.8	9.8	13.2	12.6	12.5	8×TOC
B	13.6	12.9	12.8	16.4	15.5	15.8	13.3	12.6	12.4	16.3	15.7	15.9	1×TOC
C	17.2	16.4	16.3	19.3	18.5	18.7	17.0	16.3	16.2	19.0	18.6	18.5	1/8×TOC
D	20.2	19.6	19.6	20.0	19.7	21.6	20.4	19.6	19.4	20.6	20.5	20.2	1/64×TOC
E	23.1	22.6	22.4	21.5	20.8	20.9	23.1	22.4	22.3	22.1	22.2	22.3	1/512×TOC
F	24.8	24.8	24.7	20.9	21.0	20.7	25.2	24.1	24.2	22.1	21.1	21.4	1/4096×TOC
G	25.9	25.3	25.3	22.5	21.4	20.6	26.2	25.7	25.4	20.9	19.0	20.3	1/32768×TOC
H	25.9	25.8	25.9	23.2	20.4	22.8	25.9	25.8	25.5	22.7	20.2	21.4	0
	IL-13			TNFa			IL-13			TNFa			Analyte Type

Ct (Avg)	1	2	3	4	5	6	7	8	9	10	11	12	Analyte Conc.
A	10.7	9.9	9.9	13.3	12.6	12.6							8×TOC
B	13.5	12.8	12.6	16.3	15.6	15.9							1×TOC
C	17.1	16.4	16.2	19.2	18.5	18.6							1/8×TOC
D	20.3	19.6	19.5	20.3	20.1	20.9							1/64×TOC
E	23.1	22.5	22.3	21.8	21.5	21.6							1/512×TOC
F	25.0	24.5	24.5	21.5	21.0	21.1							1/4096×TOC
G	26.1	25.5	25.4	21.7	20.2	20.5							1/32768×TOC
H	25.9	25.8	25.7	23.0	20.3	22.1							0
	IL-13			TNFa			IL-13			TNFa			Analyte Type

FIG. 48

Assay Pair (Capture/Detect)	Top	Bottom	Midpoint	Hill Slope	R^2	LLOD (pg/mL)
401-01R/MAM02-008	64823	19.7	4552	1.07	0.78	1.56
401-01R/6162-D63-1	43107	5.6	73951	0.87	0.99	0.30
401-01R/6162-A19-4	49344	7.6	9755	1.00	0.97	0.36
401-01R/6162-D85-4	6357	5.3	6034	1.07	0.88	0.67
6162-B40-3/6162-C57-1	52654	10.6	242169	1.00	0.99	1.87
6162-C69-2t/MAM02-008	93444	35.6	54539	1.05	0.94	0.71
6162-G14-3/6162-F78-2	50382	14.8	211260	1.06	0.97	0.25
6162-G14-3/6162-G76-1	17149	10.4	324621	1.00	0.98	2.04
6162-H85-2t/6162-D11-5	47798	32.9	62130	1.05	0.98	1.59
MAM02008/6162-C69-2t	42579	16.4	26686	0.95	0.99	5.10

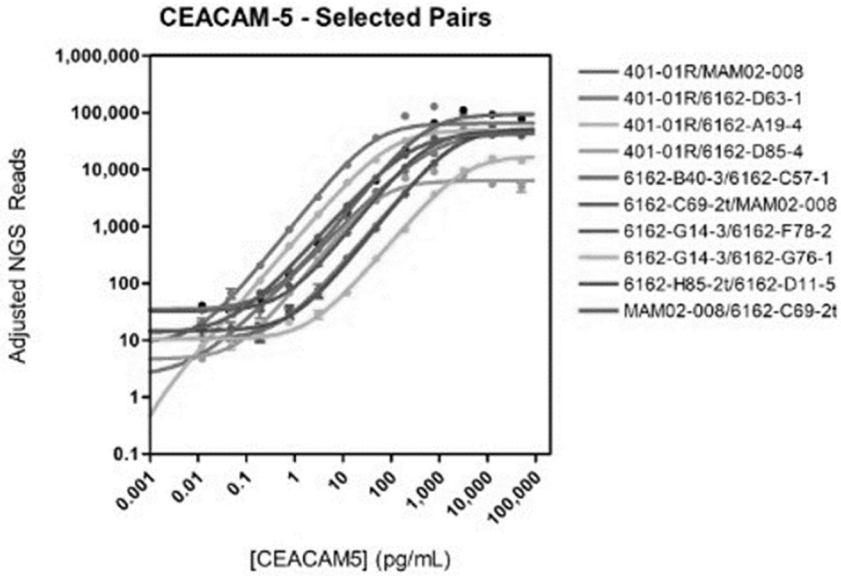
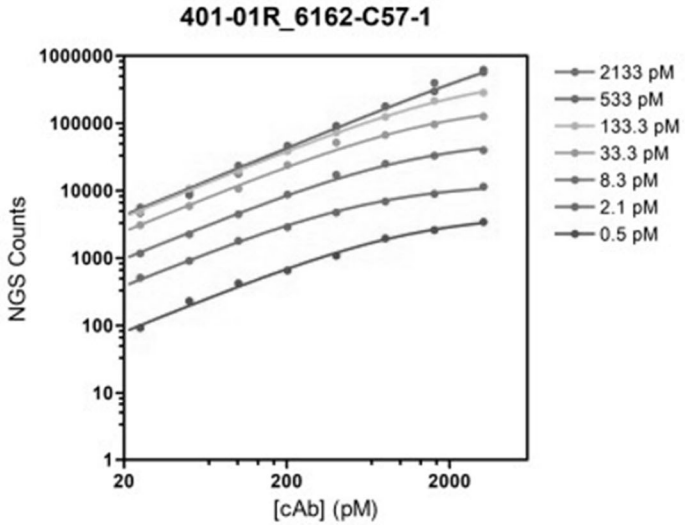


FIG. 49

401-01R_6162-C57-1								
	Cal-1	Cal-2	Cal-3	Cal-4	Cal-5	Cal-6	Cal-7	* Cal-8
[Analyte] (pg/mL)	320000	80000	20000	5000	1250	312.5	78.125	0
[Analyte] (pM)	2133.3	533.3	133.3	33.3	8.3	2.1	0.5	0
[cAb] (pM)	NGS Counts							
3225.8	626008	571215	285369	127803	40079	11487	3455	582
1612.9	398698	298881	215815	96298	33117	9047	2618	392
806.5	179261	176721	125682	67589	25209	6956	1973	155
403.2	92106	87134	73172	51741	17322	4777	1094	85
201.6	44362	46572	38722	23993	8792	2887	658	52
100.8	18013	23590	19426	10745	4467	1816	424	18
50.4	8700	9350	10878	5934	2261	915	231	19
25.2	4708	5645	4923	3096	1180	515	93	5
* 12.6	2078	2639	2281	1244	567	205	64	4
* 6.3	1082	1438	1183	615	334	107	24	1
* 3.2	521	686	807	397	239	74	28	2
* 1.6	257	316	311	206	95	20	5	1

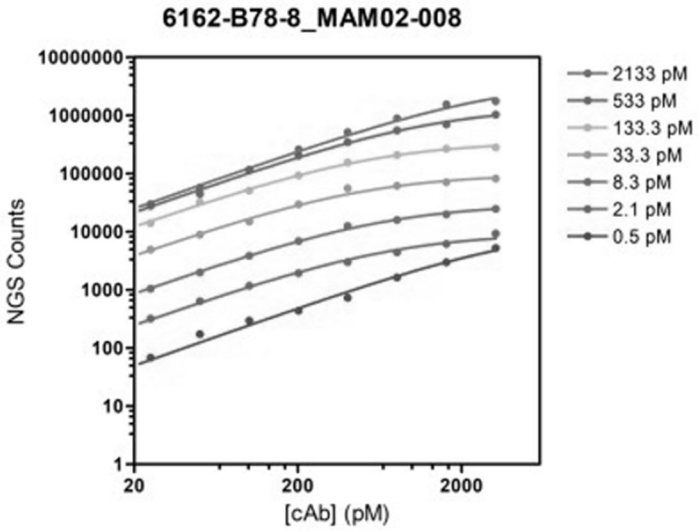


Average Kd: 906 pM

* Excluded from Analysis

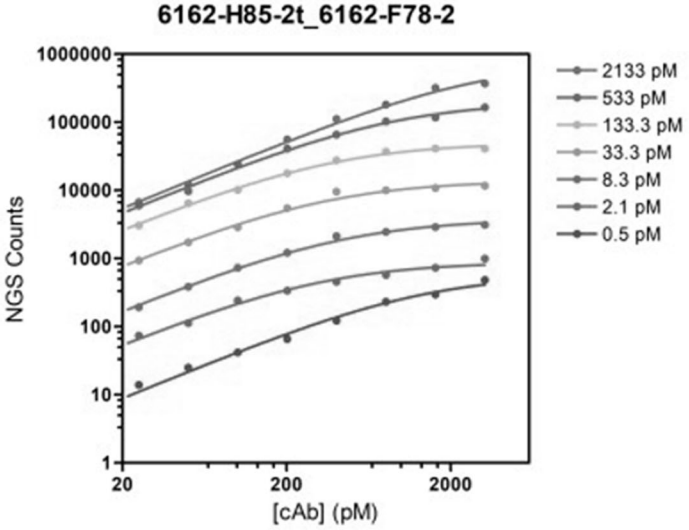
FIG. 50

Antibody with relatively poor affinity



Average Kd: 1944 pM

Antibody with relatively good affinity



Average Kd: 352 pM

* Excluded from Analysis

FIG. 51