SUPPORTING INFORMATION TO:

“Next generation digital PCR: high dynamic range single molecule DNA counting via ultra-partitioning”

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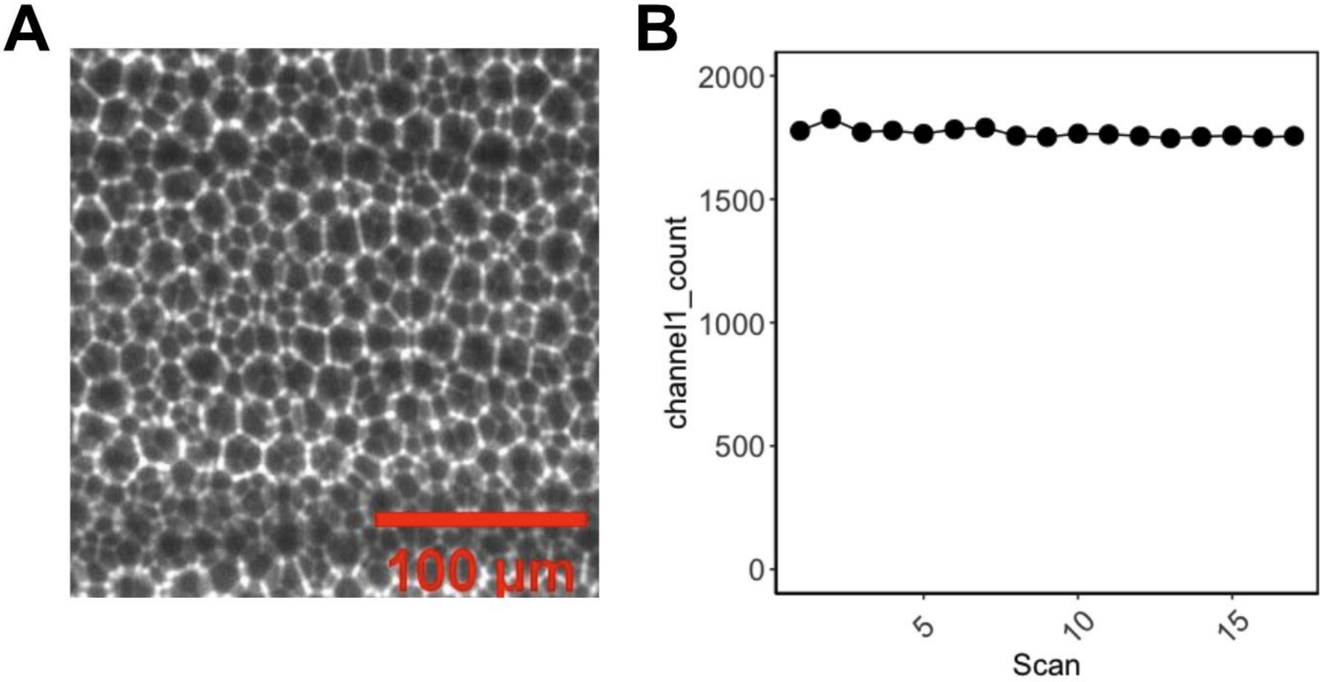

Figure S1. Ultra-partitions have high packing efficiency and are immobilized.

(A) Image from lightsheet microscopy of ultra-partitions where Nile Red oil stain is spiked into the emulsion oil to visualize droplet shells via fluorescence. The centrifugation step during ultra-partitioning enables droplets to be densely packed, showcased by this Nile Red image. The resulting ultra-partitions forms a viscous gel that immobilizes the emulsion, allowing for stable and repeatable 3D imaging of ultra-partitions.

(B) UltraPCR emulsion stability test. A sample with *prfA* template from *L. monocytogenes* was measured using UltraPCR repeatedly scanned 17 times over the course of 7 months. Using a *prfA*-FAM conjugated TaqMan probe, droplet fluorescence intensity decreases after each scanning event due to photobleaching (data not shown), but can still be counted using the droplet counting algorithm.

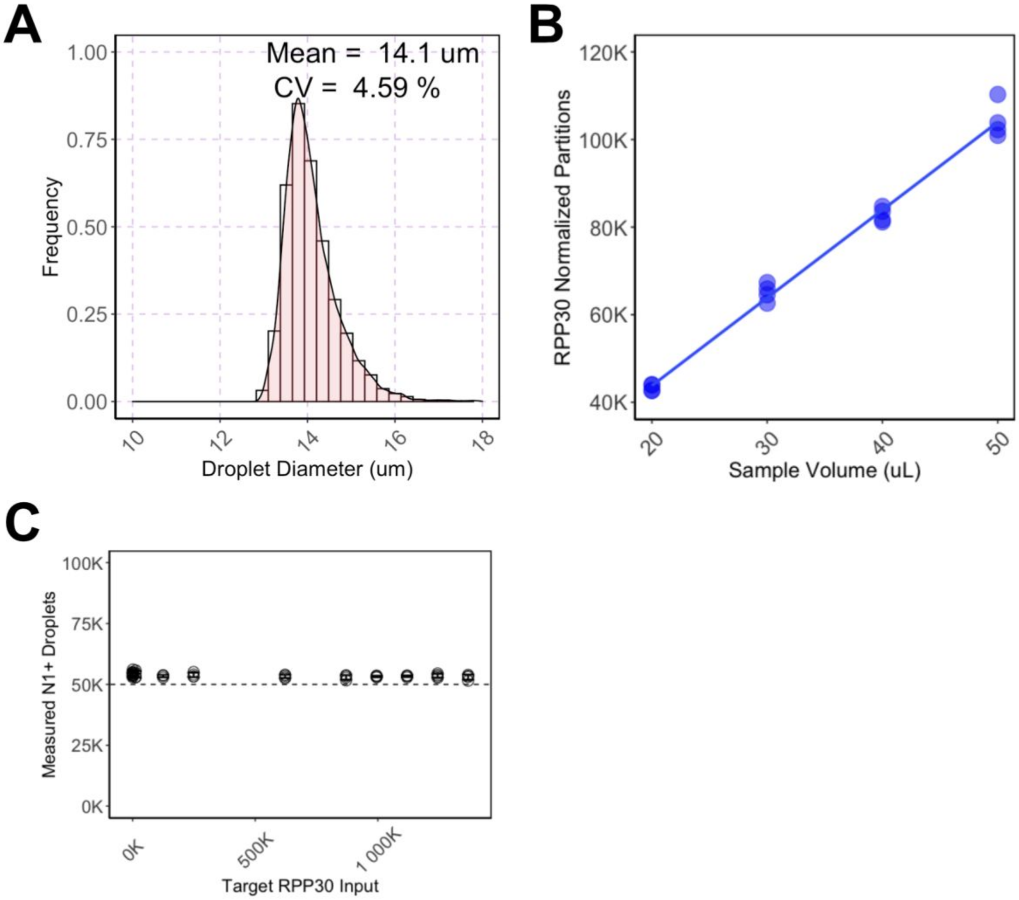

Figure S2. UltraPCR emulsion and dynamic range characterization.

(A) Droplet diameter of ultra-partitions, analyzed via brightfield microscopy and MATLAB. Due to the dense packing of ultra-partitions, more accurate droplet size measurement is achieved through dilution of the emulsion with Emulsion Oil and counted on a hemocytometer chip.

(B) Volume titration using the same PCR mix to amplify human *RPP30* amplicon. Each volume has 4 technical replicates with a linear regression fitted (Adjusted R2 = 0.9621).

(C) In the 6-log *RPP30* titration experiment shown Figure 4, each replicate was also multiplexed with *SARS-CoV-2 N1* assay with a target of ~50K copies of target DNA. Even when adding *RPP30* at higher DNA copies through a 6-log range, the accuracy and precision of multiplexing with *N1* remains consistent.

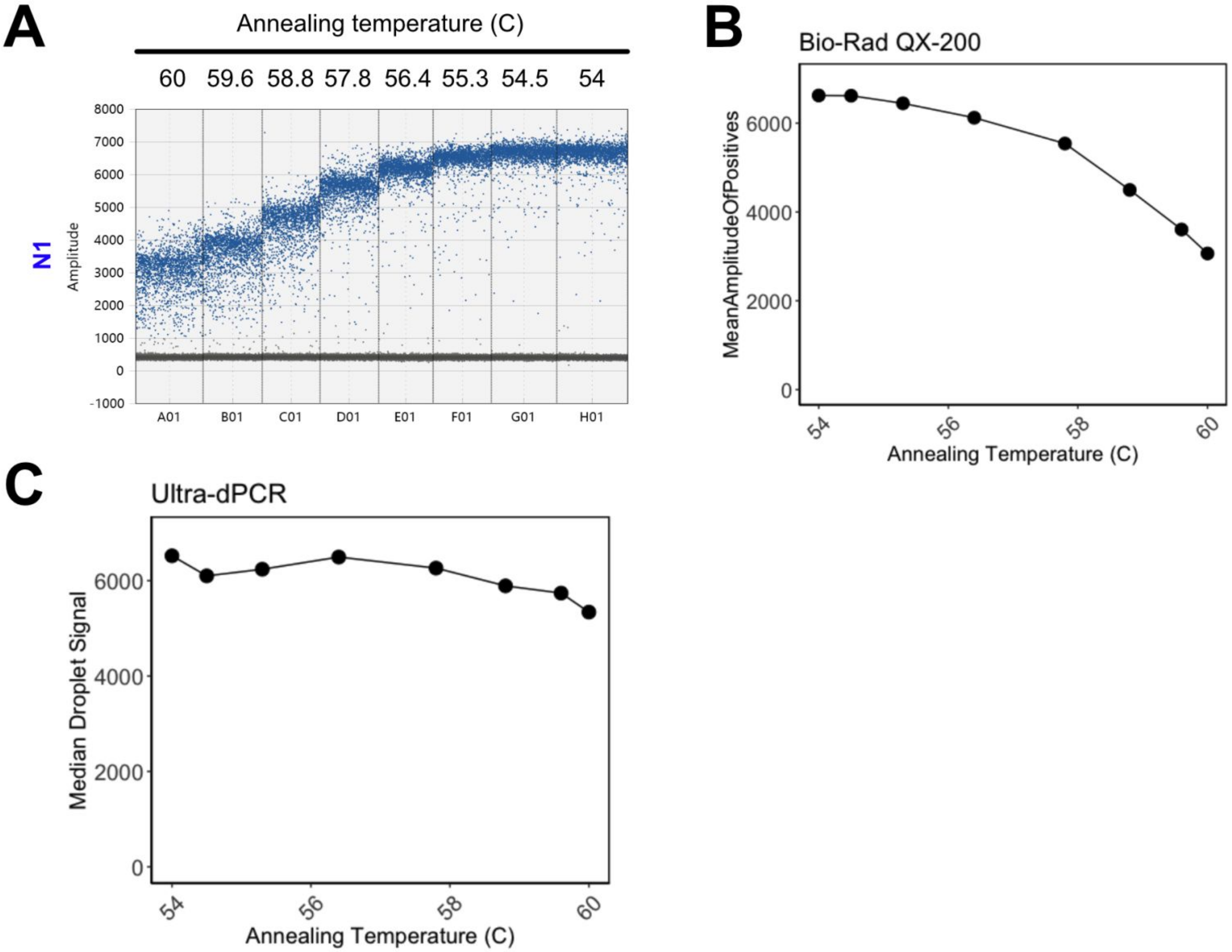

Figure S3. Assay optimization for orthogonal testing.

(A) 1D amplitude plot representing data from annealing temperature gradient optimization on Bio-Rad QX-200 of SARS-CoV-2 *N1* amplicon to establish an amplification protocol for orthogonal comparison. This assay is particularly sensitive in Bio-Rad QX-200 system. To maximize signal-to-noise separation, 54°C was chosen as the optimized annealing temperature on this system.

(B) Mean amplitude of positive droplets versus annealing temperature as provided by the QuantaSoft software to further showcase that this *N1* assay performance is highly variable on QX-200, as amplitude of signal of positive partitions drops by ~50% from annealing temperature of 54°C to 60°C.

(C) Median amplitude of *RPP30*-positive droplets measured by UltraPCR. Compared to Bio-Rad QX-200, *N1* assay performance on UltraPCR is less susceptible to annealing temperature change, exemplified by only a mild drop in median positive-partition signal. Due to the nature of UltraPCR having low DNA occupancy relative to the ~30 million partition capacity, standard 1D amplitude plots are not representative methods to visualize positive droplet metrics.

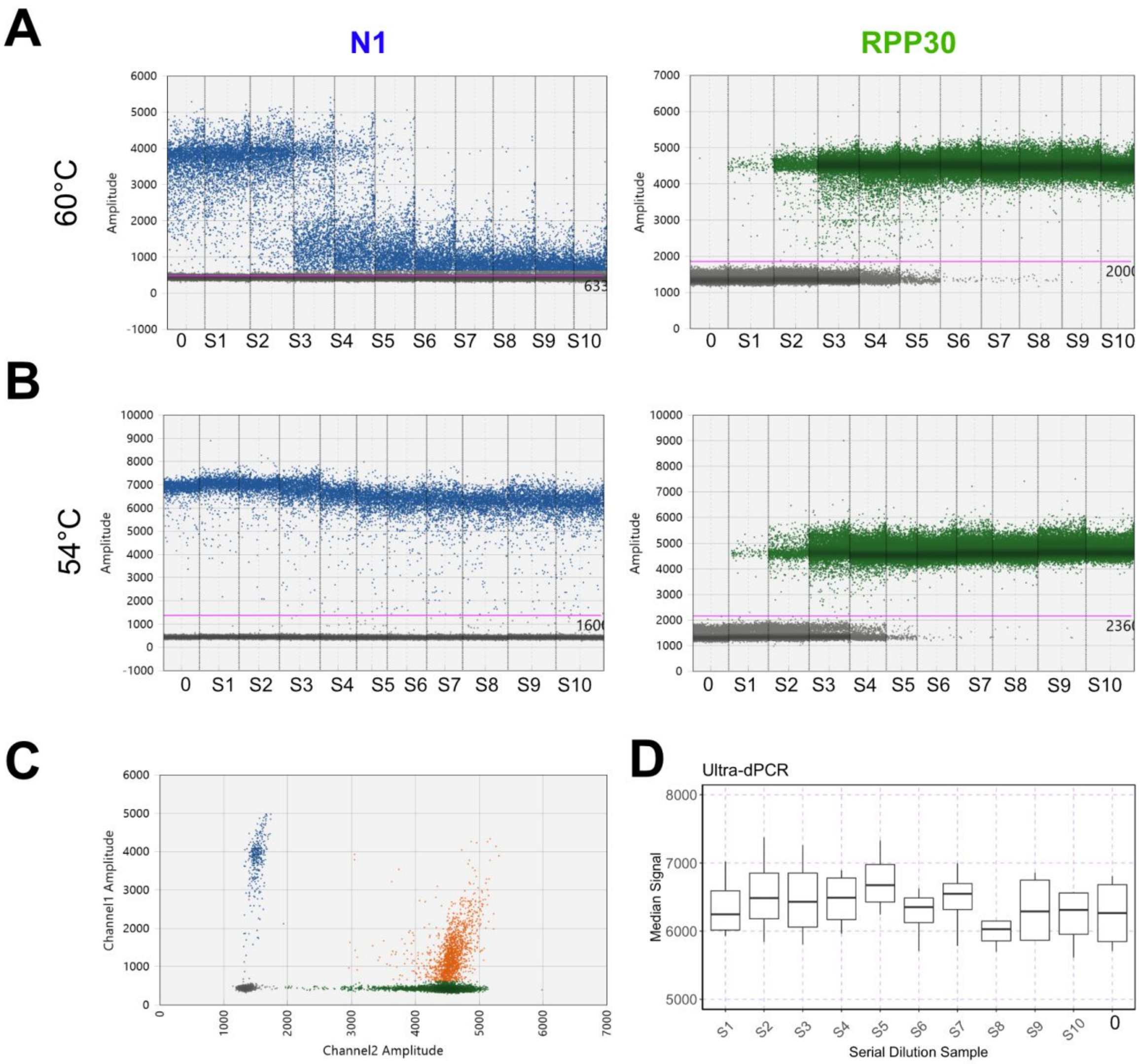

Figure S4. UltraPCR is immune to partition-specific competition (PSC).

(A) 1D amplitude of SARS-CoV-2 *N1* (left, channel1) and *RPP30* (right, channel2) from QX-200 with different *RPP30* dilutions at 60°C annealing temperature. At this temperature, PSC effects are apparent as *RPP30* input increases, *N1* amplitude becomes noticeably lower and, in many partitions, the *N1* counts are lost. Note that "0" is defined as 0 expected *RPP30* input from serial dilution but contains *N1* input.

(B) 1D amplitude of SARS-CoV-2 *N1* (left, channel1) and *RPP30* (right, channel2) from QX-200 with different *RPP30* dilutions at 54°C annealing temperature. Note that "0" is defined as 0 expected *RPP30* input from serial dilution but contains *N1* input.

(C) 2D amplitude of a sample in (A) where orange points denote droplets that are channel1+ and channel2+, blue points denote channel1+ only, green points denote channel2+ only, and grey points denote double negative droplets. This is another depiction of the effect of PSCs, as multi-target occupied droplets in QX-200 have significantly lower amplitude in *N1* channel.

(D) Median signal of positive droplets of SARS-CoV-2 *N1* in UltraPCR displayed as a boxplot for 4 technical replicates per serial dilution. This plot shows that in a realm where DNA targets are partitioned at low occupancy, PSC is not an issue, as exemplified by consistent signal amplitude of *N1* across all *RPP30* loading conditions. This phenomenon is observed in all annealing temperatures tested. Note that "0" is defined as 0 expected *RPP30* input from serial dilution but contains *N1* input.

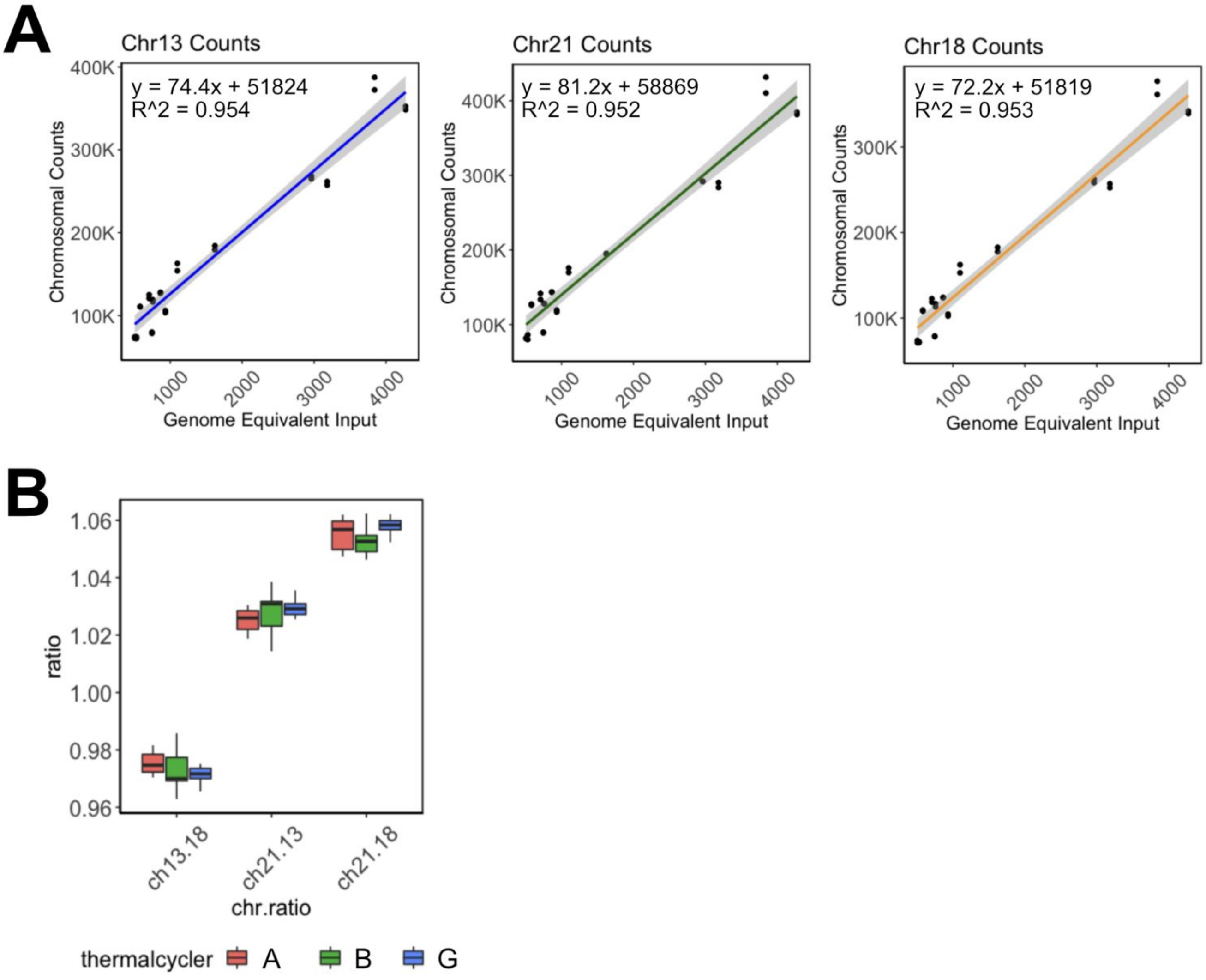

Figure S5. UltraPCR NIPT assay characterization.

(A) cfDNA samples from healthy donors measured using UltraPCR NIPT assay versus TapeStation cfDNA ScreenTape (ng of cfDNA converted to genome equivalent (GE), based on a 6.6 pg/GE conversion factor). Two 10 mL blood tubes were obtained per donor and for each blood tube, extracted cfDNA was divided into 2 UltraPCR reactions. For the UltraPCR NIPT assay, Chr13, Chr18, and Chr21 counts were measured and compared independently to TapeStation data, fitted with a linear regression. Slope of chromosomal counts to GE is between 72.2-81.2, which is close to the expected increase of 74-plex per chromosome of the assay, suggesting high efficiency in assay performance.

(B) UltraPCR NIPT assay is optimized to have low variability between thermal cyclers, as shown in this thermal cycler reproducibility study measuring the differences in chromosomal ratios.

Table S1. Primers and probes used for UltraPCR assays in this study.

Assay	Product length (bp)	Forward Primer	Reverse Primer	Taqman Probe	Final primer concentration (nM)	Final probe concentration (nM)
prfA	280	CCGCAAATAGAGCCAAGCTT	GTGAGAACGGGACCATCATG	AGCCAACCGATGTTTCTGTATCAA	200	200
RPP30	65	AGATTTGGACCTGCGAGCG	GAGCGGCTGTCTCCACAAGT	TTCTGACCTGAAGGCTCTGCGCG	200	200
nCoV_N1	71	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	ACCCCGCATTACGTTTGGTGGACC	200	200
nCoV_N2	67	TTACAAACATTGGCCGCAAA	GCGCGACATTCCGAAGAA	ACAATTTGCCCCCAGCGCTTCAG	200	200

Table S2. Thermocycling conditions used for UltraPCR characterization testing.

Assay	Stage 1	Stage 2			Stage 3
prfA RPP30 N1 N2	95℃	95℃	55℃	58℃	12℃
	2 min	20 s	30 s	30 s	∞
	1x (Hold)	39x (Cycles)			1x (Hold)
NIPT	94℃	94℃	62℃		8℃
	2 min	20 s	1 min 30 s		∞
	1x (Hold)	39x (Cycles)			1x (Hold)

Note: all protocols have ramp rate set at 2℃/s

Table S3. Orthogonal testing data on Bio-Rad QX-200

Well	Dilution	Target	Conc(copies/µL)	Copies/20µLWell	PoissonConfMax	PoissonConfMin	Accepted Droplets	Positives	Negatives	Ch1+Ch2+	Ch1+Ch2-	Ch1-Ch2+
A01	NTC	N1-FAM	127	2545	134	121	15276	1566	13710	0	1566	0
B01	NTC	N1-FAM	124	2489	131	118	12930	1298	11632	0	1298	0
C01	NTC	N1-FAM	119	2377	125	113	16944	1628	15316	0	1628	0
D01	NTC	N1-FAM	121	2430	127	116	16809	1649	15160	0	1649	2
A01	NTC	RPP30-HEX	0	0	0.231	0	15276	0	15276	0	1566	0
B01	NTC	RPP30-HEX	0	0	0.273	0	12930	0	12930	0	1298	0
C01	NTC	RPP30-HEX	0	0	0.208	0	16944	0	16944	0	1628	0
D01	NTC	RPP30-HEX	0.14	2.8	0.448	0.0212	16809	2	16807	0	1649	2
A02	S10	N1-FAM	128	2553	134	121	14100	1450	12650	6	1444	87
B02	S10	N1-FAM	122	2448	129	116	14402	1423	12979	4	1419	88
C02	S10	N1-FAM	116	2311	121	110	15619	1461	14158	12	1449	101
D02	S10	N1-FAM	123	2467	130	117	15620	1555	14065	17	1538	88
A02	S10	RPP30-HEX	7.79	156	9.49	6.3	14100	93	14007	6	1444	87
B02	S10	RPP30-HEX	7.54	151	9.19	6.1	14402	92	14310	4	1419	88
C02	S10	RPP30-HEX	8.54	171	10.1	6.97	15619	113	15506	12	1449	101
D02	S10	RPP30-HEX	7.94	159	9.45	6.42	15620	105	15515	17	1538	88
A03	S9	N1-FAM	122	2444	129	116	14598	1440	13158	122	1318	1104
B03	S9	N1-FAM	123	2458	129	117	14790	1467	13323	126	1341	1104
C03	S9	N1-FAM	122	2442	129	116	14020	1382	12638	104	1278	983
D03	S9	N1-FAM	123	2450	129	116	15865	1569	14296	153	1416	1171
A03	S9	RPP30-HEX	103	2064	109	97.4	14598	1226	13372	122	1318	1104
B03	S9	RPP30-HEX	102	2043	108	96.5	14790	1230	13560	126	1341	1104
C03	S9	RPP30-HEX	94.9	1899	101	89.3	14020	1087	12933	104	1278	983

D03	S9	RPP30-HEX	103	2050	108	97	15865	1324	14541	153	1416	1171
A04	S8	N1-FAM	133	2655	139	126	15569	1661	13908	1053	608	8759
B04	S8	N1-FAM	124	2474	130	117	14746	1472	13274	899	573	8116
C04	S8	N1-FAM	123	2470	130	117	15888	1583	14305	1004	579	8921
D04	S8	N1-FAM	122	2445	128	116	15815	1561	14254	938	623	8708
A04	S8	RPP30-HEX	1170	23409	1195	1147	15569	9812	5757	1053	608	8759
B04	S8	RPP30-HEX	1112	22237	1136	1088	14746	9015	5731	899	573	8116
C04	S8	RPP30-HEX	1153	23059	1177	1130	15888	9925	5963	1004	579	8921
D04	S8	RPP30-HEX	1108	22151	1131	1085	15815	9646	6169	938	623	8708
A05	S7	N1-FAM	121	2415	127	115	16628	1622	15006	1430	192	13201
B05	S7	N1-FAM	116	2330	123	110	13281	1252	12029	1090	162	10543
C05	S7	N1-FAM	118	2367	124	112	15841	1516	14325	1315	201	12472
D05	S7	N1-FAM	116	2321	122	110	16647	1564	15083	1369	195	13252
A05	S7	RPP30-HEX	2493	49869	2543	2446	16628	14631	1997	1430	192	13201
B05	S7	RPP30-HEX	2455	49101	2509	2403	13281	11633	1648	1090	162	10543
C05	S7	RPP30-HEX	2403	48066	2452	2357	15841	13787	2054	1315	201	12472
D05	S7	RPP30-HEX	2478	49557	2527	2431	16647	14621	2026	1369	195	13252
A06	S6	N1-FAM	125	2499	131	119	16325	1645	14680	1627	18	14494
B06	S6	N1-FAM	116	2317	123	109	11133	1044	10089	1029	15	9970
C06	S6	N1-FAM	116	2313	122	110	15008	1405	13603	1384	21	13443
D06	S6	N1-FAM	118	2354	124	112	15461	1472	13989	1465	7	13820
A06	S6	RPP30-HEX	5156	103114	5328	5005	16325	16121	204	1627	18	14494
B06	S6	RPP30-HEX	5200	103996	5417	5017	11133	10999	134	1029	15	9970
C06	S6	RPP30-HEX	5197	103949	5381	5038	15008	14827	181	1384	21	13443
D06	S6	RPP30-HEX	5265	105308	5452	5104	15461	15285	176	1465	7	13820
A07	S5	N1-FAM	121	2422	127	115	15241	1491	13750	1488	3	13726

B07	S5	N1-FAM	120	2409	127	114	14357	1397	12960	1396	1	12946
C07	S5	N1-FAM	122	2432	128	115	15315	1504	13811	1498	6	13790
D07	S5	N1-FAM	118	2362	124	112	14387	1374	13013	1372	2	12998
A07	S5	RPP30-HEX	7454	149080	7929	7035	15241	15214	27	1488	3	13726
B07	S5	RPP30-HEX	8075	161505	8729	7523	14357	14342	15	1396	1	12946
C07	S5	RPP30-HEX	7460	149194	7935	7041	15315	15288	27	1498	6	13790
D07	S5	RPP30-HEX	7930	158609	8541	7410	14387	14370	17	1372	2	12998
A08	S4	N1-FAM	117	2337	123	111	16213	1533	14680	1530	3	14664
B08	S4	N1-FAM	121	2421	128	114	13069	1278	11791	1277	1	11786
C08	S4	N1-FAM	121	2413	127	115	16435	1602	14833	1602	0	14817
D08	S4	N1-FAM	129	2579	135	123	15288	1587	13701	1584	3	13691
A08	S4	RPP30-HEX	7940	158803	8515	7446	16213	16194	19	1530	3	14664
B08	S4	RPP30-HEX	9043	180853	10145	8202	13069	13063	6	1277	1	11786
C08	S4	RPP30-HEX	8158	163167	8790	7623	16435	16419	16	1602	0	14817
D08	S4	RPP30-HEX	8317	166350	9026	7728	15288	15275	13	1584	3	13691
A09	S3	N1-FAM	112	2233	117	106	16048	1453	14595	1452	1	14591
B09	S3	N1-FAM	117	2331	122	111	16473	1554	14919	1551	3	14916
C09	S3	N1-FAM	125	2496	131	119	15319	1542	13777	1540	2	13769
D09	S3	N1-FAM	129	2571	135	122	16078	1664	14414	1664	0	14409
A09	S3	RPP30-HEX	9499	189974	10728	8586	16048	16043	5	1452	1	14591
B09	S3	RPP30-HEX	9315	186299	10418	8474	16473	16467	6	1551	3	14916
C09	S3	RPP30-HEX	8629	172571	9449	7963	15319	15309	10	1540	2	13769
D09	S3	RPP30-HEX	9501	190018	10731	8588	16078	16073	5	1664	0	14409
A10	S2	N1-FAM	126	2517	132	120	16315	1655	14660	1655	0	14658
B10	S2	N1-FAM	124	2476	130	118	17460	1744	15716	1743	1	15714
C10	S2	N1-FAM	125	2493	130	119	17594	1769	15825	1769	0	15821

D10	S2	N1-FAM	122	2442	128	116	18256	1800	16456	1800	0	16453
A10	S2	RPP30-HEX	10596	211922	12816	9227	16315	16313	2	1655	0	14658
B10	S2	RPP30-HEX	10199	203978	11891	9052	17460	17457	3	1743	1	15714
C10	S2	RPP30-HEX	9869	197389	11279	8860	17594	17590	4	1769	0	15821
D10	S2	RPP30-HEX	10251	205027	11943	9105	18256	18253	3	1800	0	16453
A11	S1	N1-FAM	124	2477	130	118	15101	1509	13592	1509	0	13592
B11	S1	N1-FAM	120	2396	126	114	17505	1695	15810	1695	0	15809
C11	S1	N1-FAM	120	2402	126	114	16733	1624	15109	1624	0	15109
D11	S1	N1-FAM	123	2461	129	117	16773	1666	15107	1666	0	15106
A11	S1	RPP30-HEX	1000000	20000000	1000000	10030	15101	15101	0	1509	0	13592
B11	S1	RPP30-HEX	11494	229888	15224	9655	17505	17504	1	1695	0	15809
C11	S1	RPP30-HEX	1000000	20000000	1000000	10150	16733	16733	0	1624	0	15109
D11	S1	RPP30-HEX	11444	228883	15174	9605	16773	16772	1	1666	0	15106

Table S4. Orthogonal testing data on UltraPCR

Dilution	Sample_vol_ul	channel1_count	channel2_count	median_signal_channel1	median_noise_channel1	median_signal_channel2	median_noise_channel2
NTC	52.9956	1822	3	3067	368	3067	563
NTC	55.5795	1684	4	6810	370	3200.5	584
NTC	53.7933	1910	6	5711	371	4209	606
NTC	54.3236	1948	7	5892	369	7664	631
S10	52.077	1918	174	6572	373	8240	550
S10	54.3553	1950	170	6555	375	7876.5	570
S10	52.7465	1939	149	5613	370	7355.5	602
S10	54.3407	1959	143	6067	372	7667	612
S9	50.4001	1863	2186	6711.5	368	8172	542
S9	53.515	1968	2370	6858.5	374	8505	564
S9	52.1148	1872	2203	5865.5	374	7689	590
S9	53.9695	1973	2277	5865	376	7824.5	598
S8	46.9913	1655	21732	6151	358	7536	537
S8	53.5456	2007	25659	5910	361	7629	577
S8	52.5247	1895	24678	5697	373	7794.5	652
S8	53.7508	1876	25702	6146.5	366	8069	611
S7	53.4778	1899	52492	6994	390	8686.5	600
S7	54.5839	2108	54689	6599	394	8372	636.5
S7	52.1288	2065	53101	5783	389	8046	643
S7	54.2903	1980	54221	6495	392	8517.5	648
S6	52.4052	1859	118844	6626	375	8574	639

S6	55.6687	1941	126902	6440	387	8551	690
S6	53.8211	1861	122789	6264	374	8437	687
S6	53.488	1711	122521	5708	376	7971	684
S5	50.9371	1774	152460	6858.5	383	8613	651
S5	55.5952	2048	165562	6489	384	8627	688
S5	52.6996	1965	160076	7330	384	10260	722
S5	53.508	1930	161063	6241	385	8498	708
S4	52.3932	1661	164251	6891	381	9290	692
S4	54.9414	1860	171007	6741	382	9184	701
S4	52.5204	1851	167586	6238	383	8420	719
S4	53.9262	1939	170839	5963.5	388	8243	719
S3	54.6043	1913	189074	6715.5	386	8692	694
S3	52.3681	1945	181751	7261	401	9216	710
S3	51.0249	1822	182480	5802	390	7932	721
S3	54.4086	1881	188421	6144.5	383	8566	739
S2	53.279	1900	192130	6676	392	8697	699
S2	53.3843	1810	191515	7375	394	9574	736
S2	50.931	1875	185897	5842	403	7899	750
S2	53.5131	1975	191108	6293	393	8881	764
S1	50.7397	1967	235454	6448	396	8256	710
S1	53.458	1858	244005	7020	397	9363	755
S1	50.1706	1829	237441	6044	407	8429	822
S1	52.7064	1784	241517	5930	401	8143	773