Theoretical Design and Analysis of Multivolume Digital Assays with Wide Dynamic Range Validated Experimentally with Microfluidic Digital PCR

Jason E. Kreutz[a], *Todd Munson*[b], *Toan Huynh*[a], *Feng Shen*[a†], *Wenbin Du*[a‡], *and Rustem F. Ismagilov*[a&]*

a Department of Chemistry and Institute for Biophysical Dynamics, The University of Chicago, 929 East. 57th St., Chicago, Illinois 60637

b University of Chicago, Computation Institute, Chicago, IL 60637 USA and Argonne Natl Lab, Argonne, IL 60439 USA

[†]Current Address: SlipChip, LLC, 2201 Campbell Park Drive, Chicago, IL, 60612

[‡]Current Address: Department of Chemistry, Renmin University of China, Beijing, China, 100872

[&]Current Address: Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 E. California Blvd, Pasadena, CA 91125.

Computer programs: (Programs with bold names are initiated by the user, other programs are initiated by the programs with bold names)

MVdPCR_DataInput.m- This program is used to analyze experimental data, which has been entered into Datainput.xls. It reads Datainput.xls to obtain parameters and experimental results, then calculates λ and σ using MVdPCR_MLE.m. For experimental results being tested for statistical significance, the Z-score (Eq. 14) and confidence level are also determined.

MVdPCR_MLE.m- This program uses a globalized Newton method to calculate the MPN, by solving for λ in Eq. 8, for the parameters input from MVdPCR_DataInput.m or MVdPCR_Simulate.m. It also calculates σ using Eq. 12.

MVdPCR_Simulate.m- This program uses the input parameters from MVdPCR_RunSim, MVdPCR_Plot_points, or MVdPCR_Find_device_lower.m to generate simulated results. These results are then analyzed through MVdPCR_MLE.m, and the power level for the set of results are determined.

MVdPCR_RunSim.m- This program provides the input parameters for MVdPCR_Simulate.m for specific input concentrations and resolution levels.

MVdPCR_Find_device_param.m- This is the first of a set of programs used to find the minimum concentration at which a desired resolution level can be achieved. This program provides the device parameters and specified resolution levels that will be input into MVdPCR_Find_device_resol.m

MVdPCR_Find_device_resol.m- This program uses the input values from MVdPCR_Find_device_param.m and for each resolution level it performs stages of optimization using MVdPCR_Find_device_lower.m at increasing levels of precision.

MVdPCR_Find_device_lower.m- This program runs the actual optimization protocol that tests concentrations to find where the desired power level is achieved for the specified input parameters.

MVdPCR_Find_approx1.m- A fitting program used within MVdPCR_Find_lower.m to help find the target concentration.

MVdPCR_RunPlot.m- The goal of MVdPCR_RunPlot is to provide a graphical summary of the resolution ability of the design specified by the parameters in the program. It provides the initial parameters for the design, the resolution conditions, and the precision that the resolution will be measured at. These parameters are input into MVdPCR_Plot _points.m, which uniformly spaces the points on a log scale across the entire dynamic range. Then, for the points defined in MVdPCR_Plot_points.m the actual power level is determined. The program then plots the power level with respect to the lower of the two concentrations being compared at each point defined by the

parameters. While the computer processing time of this program is much faster than MVdPCR_Find_device_param.m, the latter gives more precise determination of the lowest concentration where the resolution is achieved. An example output from MVdPCR_RunPlot.m is shown for 100 sets of concentrations at each resolution level whose power level was determined from 10,000 simulations (see Results and Discussion).

MVdPCR_Plot_points.m- This program uses the input parameters from MVdPCR_Runplot.m to generate a set of points that are uniformly distributed on a log scale, and then inputs those conditions into MVdPCR_Simulate.m to generate the power level. The results are then exported back to MVdPCR_Runplot.m to be plotted.

Experimental Section

Materials: All compounds purchased from commercial sources were used as received unless otherwise stated. Bovine serum albumin (BSA) solution was ordered from Roche Diagnostics (Indianapolis, IN). Tetradecane, chloroform, acetone, ethanol, and DEPC-treated and nuclease free water were obtained from Fisher Scientific (Hanover Park, IL). Dichlorodimethylsilane was purchased from Sigma-Aldrich (St. Louis, MO). All PCR primers were purchased from Integrated DNA Technologies (Coralville, IA). SsoFast EvaGreen Supermix (2X) was obtained from Bio-Rad Laboratories (Hercules, CA). Microposit^{TM} MF^{TM}-CD-26 developer was purchased from Rohm and Hass Electronic Materials LLC (Marlborough, MA). Soda−lime glass plates coated with photoresist and chromium were ordered from Telic Company (Valencia, CA). Photomasks were designed using AutoCAD (San Rafael, CA) and ordered from CAD/Art Services, Inc. (Bandon, OR). Amorphous diamond coated drill bits were purchased from Harvey Tool (0.030 inch cutter diameter, Rowley, MA). MinElute PCR purification kit was purchased from Qiagen Inc (Valencia, CA). PCR tubes and barrier pipette tips were purchased from Molecular BioProducts (San Diego, CA). PCR Mastercycler and *in situ* adapter were purchased from Eppendorf (Hamburg, Germany).

DNA template preparation: The control template DNA, originating from the LITMUS 28iMal Control Plasmid, used to validate the MV digital PCR design was generated using standard PCR methods using the primers designated MalF197 and MalR526. The product, 631 bp in length, was purified using the MinElute PCR purification kit, and the stock solution of template DNA was quantified using UV-Vis absorption. The sequence of the control template was confirmed by DNA sequencing.

DNA Template and primer sequences: The control template had the following sequence as confirmed by DNA sequencing.

5'-

*ctgcaagaac cgtacttcac ctg*gccgctg attgctgctg acgggggtta tgcgttcaag tatgaaaacg gcaagtacga cattaaagac gtgggcgt**gg ataacgctgg cgcgaaagcg** ggtctgacct tcctggttga cctgattaaa aacaaacaca tgaatgcaga caccgattac tccatcgcag aagctgcctt taataaaggc gaaacagcga tgaccatcaa cggcccgtgg gcatggtcca acatcgacac cagcaaagtg aattatggtg taacggtact gccgaccttc aagggtcaac catccaaacc gttcgttggc gtgctgagcg caggtattaa cgccgccagt ccgaacaaag agctggcaaa agagttcctc gaaaactatc tgct**gactga tgaaggtctg gaagcgg**tta ataaagacaa accgctgggt gccgtagcgc tgaagtctta cgaggaagag ttggcgaaag atccacgtat tgccgccact atggaaaacg cccagaaagg tgaaatcatg ccgaacatcc cgcagatgtc cgctttctgg tatgccgtgc gtactgcggt gatcaacgcc gccagcggt*c gtcagactgt cgatgaagcc c*-3'

Primers used for template preparation:

5'- *ctgcaagaaccgtacttcacctg* -3'

5'-*gggcttcatcgacagtctgac* g-3'

Primers used for multivolume digital PCR experiments:

MalF197: 5'-**ggataacgctggcgcgaaagcg**-3'

MalR526: 5'-**ccgcttccagaccttcatcagtc**-3'

MV digital PCR experiments: Details of fabrication and preparation of chips for experiments is provided in **Fabrication of SlipChip for Multivolume digital PCR** below. For use in digital PCR experiments the stock solution was diluted into Tris buffer/BSA (10 mM Tris pH 8.0, 75 mM NaCl, 50 μM EDTA, 2 mg/mL BSA) and all template solutions were stored at 4 °C. A typical digital PCR reaction solution contained 50 μL of SsoFast EvaGreen Supermix, 10 μL each of 2 μM primer in Tris buffer (10 mM Tris pH 8.0, 75 mM NaCl, 50 μM EDTA; final primer concentration was 200 nM of each primer), 10 μL of 20 mg/mL BSA, 10 μL Tris buffer and 10 μL of template DNA. This was enough for three separate SlipChip experiments. 30 μL of the reaction mixture was placed over the inlet hole of the assembled chip (if necessary, 1-2 μL of tetradecane was added to the inlet to prevent trapping of air bubbles) and loaded using dead-end filling by applying a positive pressure of ~ 0.02 atm.[1] The chip was placed on the in situ adapter (a layer of tetradecane was used to ensure uniform thermal contact) for the PCR Mastercycler and the following protocol was run: 5 minutes at 95 °C; then 35 cycles of 45 seconds at 95 °C, 45 seconds at 55 °C, and 60 seconds at 72 °C; followed by 2 minutes at 72 °C; and finally cooled to room temperature. Chips were imaged on a Leica fluorescent microscope and analyzed using Metamorph software.

Fabrication of SlipChip for Multivolume digital PCR: The procedure for fabrication of SlipChip from soda lime glass was based on the methods developed previously.[2,3] In order to fabricate SlipChip with wells of different depths, the following procedures were used: 1) The glass plate coated with chromium and photoresist was aligned with a photomask containing the design for the deepest wells and channels (125 and 25 nL wells, 100 μm desired depth) by using a mask aligner, then the photoresist layer was exposed to UV light using standard exposure protocols. 2) After exposure, the glass plate was immersed in MF-CD-26 developer to immediately remove the photoresist from exposed areas. 3) The exposed underlying chromium layer was removed by using a chromium etchant (a solution of 0.6:0.365 mol/L $HClO_4$ / $(NH_4)_2Ce(NO_3)_6$). 4) The glass plate was then thoroughly rinsed with Millipore water

and dried with nitrogen gas. 5) The glass plate was aligned with a second photomask containing the design for the shallower wells and channels (5 and 1 nL wells, 40 μm desired depth and was exposed to UV light by using the standard exposure protocols. 6) After protecting the back side of the glass plate with tape the glass plate was then immersed in a glass etching solution (1:0.5:0.75 mol/L $HF/NH_4F/HNO_3$) to etch the glass surface where photoresist layer and chromium coating were removed in step 3, and the glass was etched to a depth of 60 μm. 7) The glass plate was thoroughly washed with Millipore water and dried with nitrogen gas. 8) Steps 2-4 were then repeated to remove the photoresist and chromium from the second exposure. 9) Then steps 6-7 were repeated (glass was etched a further 40 μm). 10) The protective tape was removed and was aligned with a third photomask containing a design for an array of 100 μm x 100 μm posts (to serve as a "nano pattern" that assists in oil drainage) and was exposed to UV light by using the standard exposure protocols.[4] 11) Steps 2-4 and 6-7 were then repeated to etch the glass 1.5 μm. Finally, the remaining photoresist was removed by using acetone, and the underlying chromium layer was removed by using the chromium etchant. The etched depth was controlled by the etching time and speed, which was controlled by the etching temperature. Through-holes for sample loading and oil drainage were drilled into the top plate.

The glass plate was oxidized in a plasma cleaner (Structure Probe, Inc., West Chester, PA) for 10 minutes and then immediately transferred into a desiccator (Fisher Scientific, Hanover Park, IL). Dichlorodimethylsilane (150 μL) was injected into the desiccator and then a vacuum was applied to perform gas-phase silanization for one hour. The silanized glass plate was thoroughly cleaned with chloroform, acetone, and ethanol, and then dried with nitrogen gas. The silanized glass plate was stored under nitrogen and used for Multivolume digital PCR within one day.

The glass plate was reused by performing the following cleaning procedure to avoid contamination of previously amplified DNA product:

1) The assembled chip was taken to an isolated room for initial cleaning.
2) The wax used to hold the device in place during thermocycling was removed with a razor blade.

3) The assembled chip was opened and rinsed with water.
4) The chip was rinsed with chloroform, hexane and acetone. (Ethanol was avoided to prevent precipitation of DNA.)
5) The chip was then sonicated in hexane for 20-40 minutes.
6) The chip was rinsed again with water, chloroform, hexane and acetone and then dried with air, and placed on large cleanroom wipe (rinse well to avoid leaving waxy residue).
7) The chip was treated with DNAZap (1-1.5 squirts of each solution per chip) then covered with a second cleanroom wipe (to help DNAZap wick across entire chip surface) and let sit for at least 3 minutes.
8) The chip was then rinsed and placed in telfon holder for 2" chip and cleaned with piranha solution (3:1 sulfuric acid : hydrogen peroxide) for at least 1.5 hours.:
9) The chip was thoroughly rinsed with water and then with acetone and soaked in hexane for 40-60 minutes
10) Steps 6-8 were repeated. Any residual wax can resist piranha and store DNA providing a source for contamination, so this second cleaning sequence was necessary. The chip was then silanized as described above for the initial fabrication.
11) Cleaned and silanized chips were stored in a desiccator under N_2.

Assembling the SlipChip: The SlipChip was assembled under filtered, degassed tetradecane. The bottom plate was first immersed into tetradecane in a Petri dish, with the patterned wells facing up. The top plate was then immersed into tetradecane and placed on top of the bottom plate with the patterned side facing down. The two plates were aligned under a stereoscope (Leica, Germany) and stabilized using binder clips.

The filling chamber used to apply positive pressure for filling consisted of a PDMS gasket and glass cover slide. The glass slide had a hole drilled in it, where tubing was inserted and sealed. The tubing was connected to a valve and could be pressurized with nitrogen.

Verifying the validity of the Z-test using permutation tests.

For the Z-test, which assumes a normal distribution, to be useful, it is important to verify that it can be used under a wide range of conditions, including at low concentrations when there are few positive wells, or when the design consists of very few wells. While in the limit of many wells the binomial distribution can be approximated by a normal distribution, it is not obvious that a normal approximation is appropriate for the test statistic generated from the MPN-combined binomial distributions. To validate the use of the Z-test under these conditions, the confidence level measured from the Z-test was compared to the confidence level measured based on permutation tests. The permutation test[5-7] is an exact method for determining the confidence level based on the actual statistical distribution of two results being compared. It was performed here by first determining the concentration that would correspond to the null hypothesis for the two sets of results. The null hypothesis concentration was used to generate simulated sets of results (10,000 simulations), and the confidence level was calculated based on where the original two results fell within the simulated distribution.

Permutation tests for eight pairs of results generated using the design of the device characterized in this paper were used to validate that use of the Z-test is appropriate. In addition permutation tests were performed for designs with fewer well numbers (down to eight at each volume) (Table S1). The results from the Z-test and permutation test are in very good agreement even for systems with much fewer well numbers than 160 (Table S1 and as used in the accompanying paper[8]).

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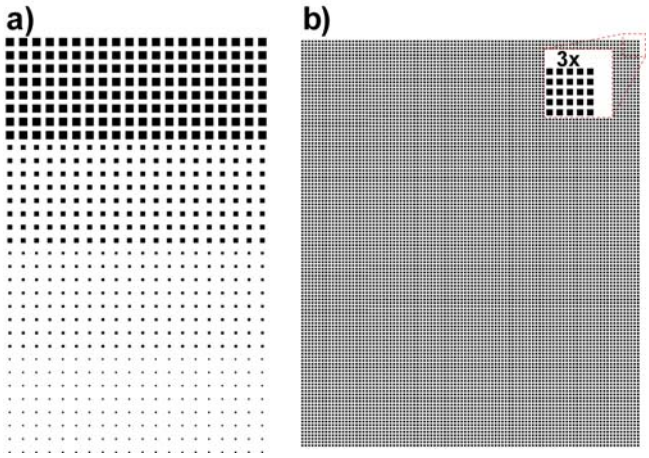

Figure S1: Two hypothetical devices with identical dynamic range and similar footprint, to demonstrate visual readout ability. a) The MV design (160 wells each at 125, 25, 5 and 1 nL) are spaced far enough apart for easy visualization, while b) the single volume design (12,000 wells at 2.08 nL) are too tightly packed for easy resolution. Note: Well sizes are based on assumption of cubic well dimensions. Spacing was based off of a distance between wells that was 60% of the well dimension for the largest wells.

Table S1a: Comparison of Z-tests and permutation tests for eight simulated pairs of results generated using the design of the device characterized in this paper

	Result 1 (# of negative wells)					Result 2 (# of negative wells)								
Trial	125 nL	25 nL	5 nL	1 nL	MPN	125 nL	25 nL	5 nL	1 nL	MPN	Z	Z-test conf. (1-α)	Permutation test confidence	Z-test value as percent of perm. test value
1	156	159	160	160	202	152	158	160	160	409	1.2853	0.801	0.812	98.74%
2	156	159	160	160	202	148	158	160	160	579	2.0176	0.956	0.967	98.94%
3	156	159	160	160	202	141	156	159	160	1013	3.2748	0.999	1.000	99.91%
4	150	158	160	160	494	145	157	159	160	793	1.2842	0.801	0.803	99.69%
5	150	158	160	160	494	140	156	159	160	1058	2.1704	0.970	0.973	99.70%
6	150	158	160	160	494	132	154	159	160	1517	3.3552	0.999	1.000	99.93%
7	13	97	145	157	20002	7	86	141	156	24982	2.0376	0.958	0.957	100.16%
8	13	97	145	157	20002	4	76	138	155	29717	3.6029	1.000	1.000	100.00%
													Average	99.63%

Table S1b: Comparison of Z-tests and permutation tests for 7 simulated pairs of results generated using 32 wells at each of the volumes used in this paper.

	Result 1 (# of negative wells)					Result 2 (# of negative wells)								
Trial	125 nL	25 nL	5 nL	1 nL	MPN	125 nL	25 nL	5 nL	1 nL	MPN	Z	Z-test conf. (1-α)	Permutation test confidence	Z-test value as percent of perm. test value
1	28	31	32	32	1058	25	30	32	32	1995	1.1355	0.7438	0.7533	98.75%
2	28	31	32	32	1058	22	30	32	32	2787	1.8188	0.9311	0.9412	98.93%
3	28	31	32	32	1058	17	28	31	32	5126	3.1458	0.9983	0.9992	99.91%
4	22	30	32	32	2787	18	29	31	32	4506	1.2847	0.8011	0.7987	100.30%
5	22	30	32	32	2787	15	28	31	32	5871	2.0660	0.9612	0.9639	99.72%
6	22	30	32	32	2787	10	26	31	32	8817	3.3254	0.9991	0.9995	99.96%
7	2	18	28	31	23166	0	13	27	31	37468	1.9352	0.9470	0.9425	100.48%
													Average	99.72%

Table S1c: Comparison of Z-tests and permutation tests for 7 simulated pairs of results generated using20 wells at each of the volumes used in this paper.

	Result 1 (# of negative wells)					Result 2 (# of negative wells)								
Trial	125 nL	25 nL	5 nL	1 nL	MPN	125 nL	25 nL	5 nL	1 nL	MPN	Z	Z-test conf. (1-α)	Permutation test confidence	Z-test value as percent of perm. test value
1	16	19	20	20	1755	12	18	20	20	3915	1.4619	0.8562	0.8649	99.00%
2	16	19	20	20	1755	9	17	19	20	6524	2.5263	0.9885	0.9926	99.59%
3	16	19	20	20	1755	6	16	19	20	9391	3.3076	0.9991	0.9997	99.94%
4	11	18	20	20	4435	8	17	19	20	7215	1.2277	0.7804	0.7828	99.70%
5	11	18	20	20	4435	6	16	19	20	9391	1.9478	0.9486	0.9507	99.78%
6	11	18	20	20	4435	3	14	19	20	14338	3.1313	0.9983	0.9995	99.88%
7	2	13	18	20	17911	1	10	17	19	27163	1.3443	0.8212	0.8062	101.85%
													Average	99.96%

Table S1d: Comparison of Z-tests and permutation tests for 7 simulated pairs of results generated using 8 wells at each of the volumes used in this paper.

	Result 1 (# of negative wells)					Result 2 (# of negative wells)								
Trial	125 nL	25 nL	5 nL	1 nL	MPN	125 nL	25 nL	5 nL	1 nL	MPN	Z	Z-test conf. (1-α)	Permutation test confidence	Z-test value as percent of perm. test value
1	4	7	8	8	5218	2	6	8	8	10420	1.1888	0.7655	0.7673	99.76%
2	4	7	8	8	5218	1	5	7	8	17820	2.1663	0.9697	0.9700	99.98%
3	4	7	8	8	5218	0	4	7	8	30180	3.0699	0.9979	0.9977	100.02%
4	1	5	7	8	17820	0	4	7	8	30180	1.0706	0.7157	0.7119	100.52%
5	1	5	7	8	17820	0	3	7	8	36750	1.4594	0.8555	0.8276	103.37%
6	1	5	7	8	17820	0	2	6	8	53218	2.2047	0.9725	0.9470	102.69%
7	4	7	8	8	5218	2	6	8	8	10420	1.1888	0.7655	0.7673	99.76%
													Average	101.06%

Table S2a: Average etch depth and well volume for full SlipChips prepared for experiments

	Average etch depth (μm) of full chips*				Calculated avg. volumes (nL) of full chips*			
SlipChip Label	"125" nL	"25" nL	"5" nL	"1" nL	"125" nL	"25" nL	"5" nL	"1" nL
4A	100.26	100.67	41.14	40.36	127.10	27.43	5.03	1.12
4B	100.33	100.65	41.19	40.23	127.22	27.42	5.04	1.12
4C	100.47	100.89	41.25	40.36	127.45	27.53	5.05	1.12
4D	100.59	101.00	41.30	40.30	127.65	27.57	5.06	1.12
4E	100.50	100.89	40.99	40.18	127.50	27.53	5.00	1.11
4F	100.39	100.87	40.97	40.04	127.31	27.52	5.00	1.11
4G	100.62	100.96	41.02	40.13	127.69	27.56	5.01	1.11
4H	100.68	100.95	41.08	40.21	127.78	27.55	5.02	1.12
4I	100.50	100.81	41.15	40.16	127.50	27.49	5.03	1.11
Summary of overall chip characteristics								
	"125" nL	"25" nL	"5" nL	"1" nL	"125" nL	"25" nL	"5" nL	"1" nL
Average	100.48	100.86	41.12	40.22	127.47	27.51	5.03	1.12
St. Dev	0.14	0.12	0.12	0.11	0.22	0.05	0.021	0.005
CV%					0.18%	0.20%	0.42%	0.47%

Table S2b: Variations from the overall average for half chip averages and individual wells and for replacement chips

	Etch depth (μm)				Calculated volumes (nL)			
	"125" nL	"25" nL	"5" nL	"1" nL	"125" nL	"25" nL	"5" nL	"1" nL
Half chip min.*	99.97	100.51	40.83	39.89	126.64	27.36	4.97	1.10
Half chip max.*	100.74	101.19	41.39	40.44	127.88	27.66	5.07	1.13
St. dev.	0.20	0.20	0.18	0.14	0.33	0.09	0.032	0.007
CV%					0.26%	0.31%	0.63%	0.58%
Individual well min.*	98.67	99.9	40.47	39.51	124.54	27.09	4.91	1.09
Individual well max.*	101.62	101.82	41.62	41.00	129.3	27.93	5.12	1.15
St. dev.	0.52	0.36	0.23	0.25	0.85	0.16	0.041	0.012
CV%					0.66%	0.58%	0.82%	1.07%
Replacement Chip 4E2 average**	99.96	100.14	41.26	40.32	126.62	27.20	5.05	1.12
Replacement Chip 4Ib average**	100.47	100.78	40.57	39.54	127.45	27.48	4.93	1.08

* For each half chip, the etch depth of approximately 12 wells at each nominal volume was measured and the corresponding actual well volume was calculated. The depth of the nanopattern was factored in to account for the gap in the chip. The average for each half chip was determined this way, and the full chip averages were determined by averaging the two halves.

** Several chips were broken during the course of the experiments and replacements were needed to complete the experiments. 4E2 and 4Ib correspond to 4E and 4I with the top half of the original chip replaced.

Table S3: Experimental results testing 3-fold resolution (500 molecules/mL vs. 1500 molecules/mL, 40 experiments total)

	# of positive wells				500 molec./mL		# of positive wells				1500 molec./mL			
Pair	125 nL	25 nL	5 nL	1 nL	MPN	σ	125 nL	25 nL	5 nL	1 nL	MPN	σ	Z	Z-test conf. (1-α)
1	15	2	0	0	686	0.242	27	8	1	0	1507	0.167	2.675	0.992
2	11	3	0	0	559	0.267	30	3	0	0	1388	0.173	2.853	0.995
3	13	1	0	0	562	0.267	22	9	2	0	1363	0.175	2.780	0.994
4	10	3	1	0	558	0.268	20	6	0	0	1066	0.196	1.950	0.945
5	10	3	2	0	598	0.259	26	6	0	0	1334	0.177	2.560	0.989
6	8	4	0	0	476	0.289	18	6	2	0	1060	0.197	2.291	0.978
7	7	4	0	0	435	0.302	32	9	1	0	1784	0.155	4.159	0.9999
8	7	3	1	1	475	0.290	25	5	1	0	1288	0.180	2.930	0.996
9	9	1	0	0	397	0.316	27	7	2	0	1506	0.167	3.731	0.9995
10	9	5	0	0	557	0.268	33	1	2	0	1526	0.166	3.198	0.998
11	20	1	0	0	858	0.217	33	5	1	0	1657	0.160	2.438	0.985
12	14	1	0	0	603	0.258	26	5	1	0	1333	0.177	2.537	0.988
13	10	1	1	0	478	0.289	25	5	2	0	1330	0.177	3.024	0.997
14	7	2	1	0	395	0.317	33	10	0	0	1833	0.153	4.365	0.9999
15	12	3	0	0	601	0.258	23	9	1	1	1408	0.172	2.744	0.993
16	5	1	1	0	275	0.378	28	6	1	0	1467	0.169	4.040	0.9999
17	9	1	2	0	477	0.289	29	8	0	0	1557	0.165	3.561	0.9995
18	15	1	1	0	686	0.242	28	2	1	0	1296	0.179	2.114	0.965
19	9	2	1	0	477	0.289	21	9	1	0	1277	0.180	2.893	0.996
20	12	0	2	0	560	0.267	26	4	0	0	1249	0.182	2.480	0.986
Avg	10.6	2.1	0.7	0.1	535	0.273	26.6	6.2	1.0	0.1	1409	0.172	3.002	0.997

-19 out of 20 (95%) experimental pairs show at least 95% confidence as expected.

- If experiments are randomly paired then 91.8% reach the desired confidence (based on 50 sets of randomly scrambling the 20 pairs).

Table S4: Complete experimental results testing 1.5-fold resolution (20,000 molecules/mL vs. 30,000 molecules/mL, 20 experiments total)

	# of positive wells				20K molec./mL		# of positive wells				30K molec./mL			
Pair	125 nL	25 nL	5 nL	1 nL	MPN	σ	125 nL	25 nL	5 nL	1 nL	MPN	σ	Z	Z-test conf. (1-α)
1	145	65	12	4	18,528	0.076	156	88	19	8	28,995	0.078	4.120	0.9999
2	145	66	17	3	19,126	0.076	158	80	15	5	27,027	0.077	3.192	0.9980
3	149	54	8	1	17,354	0.076	152	81	26	6	25,954	0.077	3.717	0.9995
4	144	56	11	5	17,077	0.076	157	89	22	5	29,976	0.078	5.163	1.0000
5	136	55	13	3	15,183	0.077	156	93	24	3	30,357	0.078	6.330	1.0000
6	137	65	11	5	16,518	0.076	153	79	24	3	25,279	0.077	3.929	0.9999
7	145	59	16	2	17,948	0.076	157	109	26	10	38,315	0.079	6.898	1.0000
8	146	73	14	6	20,442	0.076	159	96	32	7	36,545	0.079	5.294	1.0000
9	146	60	11	4	18,013	0.076	158	98	22	4	33,205	0.079	5.592	1.0000
10	153	77	19	2	23,967	0.077	158	92	22	6	31,779	0.078	2.577	0.9900
Avg.	144.6	63	13.2	3.5	18,226	0.076	156.4	90.5	23.2	5.7	30,316	0.078	4.672	1.0000

Table S5: Five designs at constant total volume and dynamic range that demonstrate the impact of changing VS on the total number of wells and resolution

Design parameters					
Design	1	2	3	4	5
Well # per volume	36	58	160	1100	12,000
Well Volumes (nL)	625, 62.5, 6.25, 0.625	378.9, 47.36, 5.92, 0.74	125, 25, 5, 1	12, 6, 3, 1.5	2.08
VS	10	8	5	2	1
Total Volume (μL)	25.00	25.18	24.96	24.75	24.96
LDL (molec./mL)	120	119.5	120	121	120
ULQ (molec./mL)	$3.976x10^6$	$4.002x10^6$	$3.976x10^6$	$3.942x10^6$	$3.987x10^6$
Log dynamic range (Log_{10}(ULQ/LDL)	4.52	4.52	4.52	4.51	4.52
Total # of wells	144	232	640	4,400	12,000
Resolution level	Minimum concentration (in molecules/mL) at which 95% power is achieved for a given resolution level (LLQ-X).* ULQ is approximately $4.0x10^6$ for all designs.				
5-fold	210	205	195	190	180
3-fold	730	600	530	505	495
2-fold	-	-	1765	1560	1525
1.5-fold	-	-	10,120	5320	5160
1.3-fold	-	-	-	14,470	13,480

Complete References

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