Electronic Supplementary Material (ESI) for Lab on a Chip.

An Ultrasensitive Test for Profiling Circulating Tumor DNA using Integrated Comprehensive Droplet Digital Detection

Supplementary Tables

Supplementary Table 1. List of primers and probes used in this paper

Oligo Name	Sequence	Vendor	Used in Figure(s)
Multiplex droplet PCR, IC 3D ddPCR detection of KRAS G12D mutant in Jurkat gDNA and LS174T-Spiked WB			
KRAS_forward primer	5'-GGCCTGCTGAAAATGACTGAA-3'	LGC	Figures 5,6,7
KRAS_reverse primer	5'-GCTGTATCGTCAAGGCACTCTT-3'	LGC	Figures 5,6,7
KRAS Wild-type Probe	5'CAL560/pdUGGAGpdCpdUGGpdUGGpdCGpdU/3'BHQ-plus	LGC	Figures 5,6,7
KRAS Mutant Probe	5'FAM/pdUGGAGpdCpdUGApdUGGpdCGpdUA/3'BHQ-plus	LGC	Figures 5,6,7
BRAF_forward primer	5'-ACCTCAGATATATTTCTTCATG-3'	LGC	Figure 3
BRAF_reverse primer	5'- CCAGACAACTGTTCAAAC-3'	LGC	Figure 3
BRAF Wild-type Probe	5'CAL560/pdUpdCGAGApdUpdUpdUpdCApdCpdUGpdUAGpdC/3'BHQ-plus	LGC	Figure 3
BRAF Mutant Probe	5'FAM/pdUpdCGAGApdUpdUpdUpdCpdUpdCpdUGpdUAGpdC/3'BHQ-plus	LGC	Figure 3
Analysis of KRAS G12D in CRC patient samples			
KRAS_forward primer	5'- ATTATAAGGCCTGCTGAAAATGAC-3'	IDT	Figure 4
KRAS_reverse primer	5'- TGTATCGTCAAGGCACTCT-3'	IDT	Figure 4
KRAS Mutant Probe	5'ALEXA647/ TTGGAGCTGATGGCGCCGAC/3'BHQ-2	IDT	Figure 4

*pdC: propynyl-dC; pdU: propynyl-dU

Supplementary Table 2. Summary of synthetic KRAS G12D and BRAF V600E gBlocks® gene fragments

Synthetic KRAS G12D gBlocks® gene fragment	
Molecular Weight	335304.6
fmoles/ng	2.98
Sequence Length (bp)	543
Sequence content (5' -> 3'):	
TGACATACTCCCAAGGAAAGTAAAGTTCCCATATTAATGGTTACATATAACTTGAAACCCAAGGTACATTTCAGATAACTTAACTTTCAGCA TAATTATCTTGTAATAAGTACTCATGAAAATGGTCAGAGAAACCTTTATCTGTATCAAAGAATGGTCCTGCACCAGTAATATGCATATTAAA ACAAGATTTACCTCTATTGTTGGATCATATTCGTCCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCATCAGC TCCAACTACCACAAGTTTATATTCAGTCATTTTCAGCAGGCCTTATAATAAAAATAATGAAAATGTGACTATATTAGAACATGTCACACATA AGGTTAATACACTATCAAATACTCCACCAGTACCTTTTAATACAAACTCACCTTTATATGAAAAATTATTTCAAAATACCTTACAAAATTCAA TCATGAAAATTCCAGTTGACTGCAGACGTGTATCGTAATGAACTGTACTTCATTTACAAACTCCTCCATCGACGCTTAAGAA	

Synthetic BRAF V600E gBlocks® gene fragment	
Molecular Weight	131462.3
fmoles/ng	7.61
Sequence Length (bp)	213
Sequence content (5' -> 3'):	
AGGGCCAAAAATTTAATCAGTGGAAAAATAGCCTCAATTCTTACCATCCACAAAATGGATCCAGACAACTGTTCAAACTGATGGGACCCAC TCCATCGAGATTTCTCTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAA GGAAAACAGTAGATCTCATTTTCCTATCAGA	

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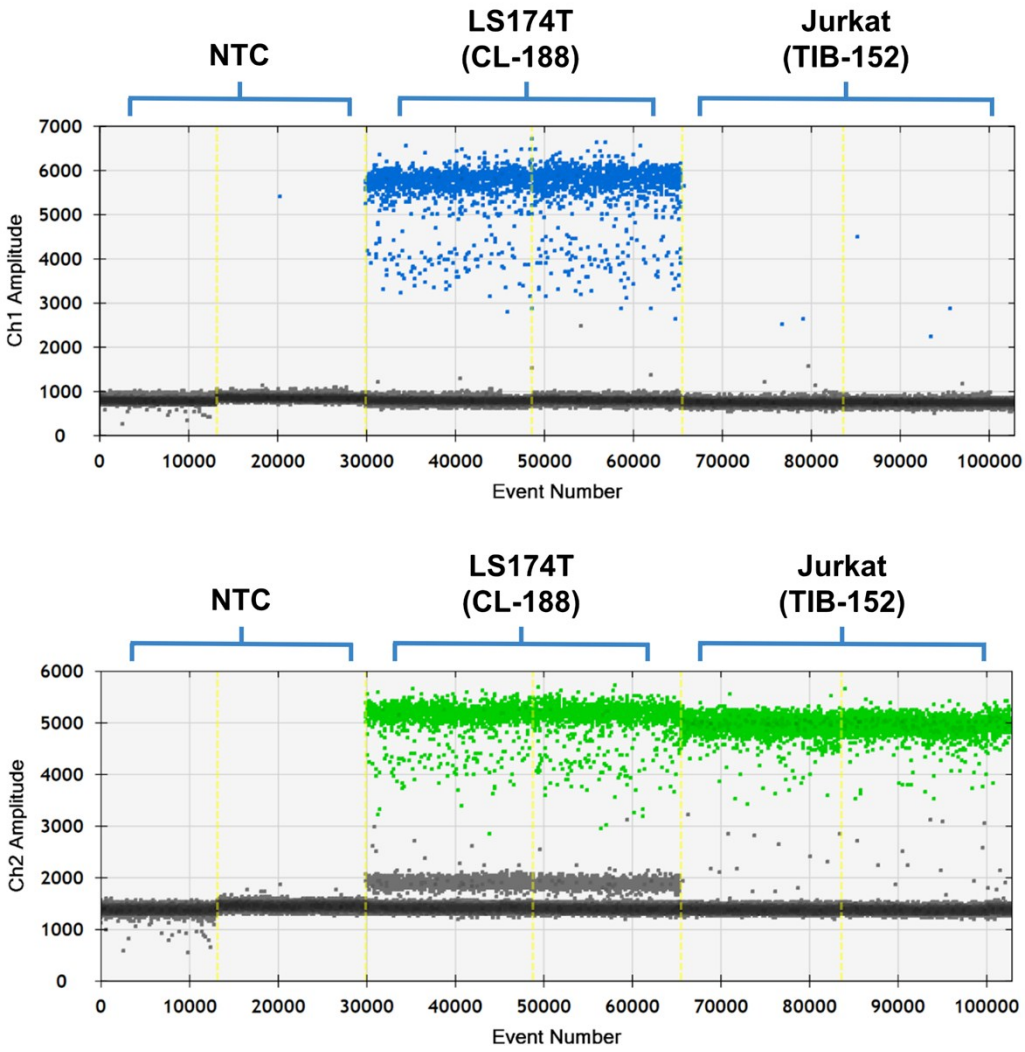
Supplementary Figure 1. Determining KRASG12D mutant allele frequency of LS174T via Bio-Rad QX-200 ddPCR



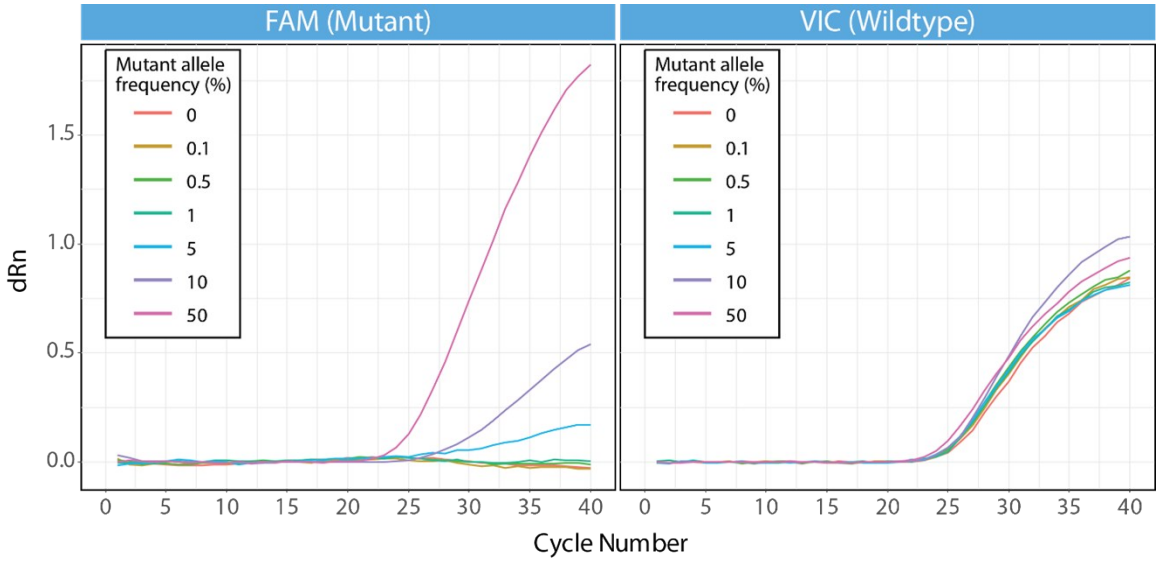
Sample Type	gDNA Loaded per well (ng)	KRASG12D Mutant (CH1 - FAM)			Wildtype (CH2 - VIC)			Measured Mutant Fraction (%)	Measured WT Fraction (%)
		Positive Droplets	Negative Droplets	Copies Detected	Positive Droplets	Negative Droplets	Copies Detected		
Blank	0	1	30,022	1.4	0	30,023	0	0	0
LS174T	12	2,505	33,042	3,446	5,184	32,163	3,352	50.69	49.31
Jurkat	12	7	37,340	9	6,768	7,426	7,040	0.12	99.88

*n = 2 for all sample types. Blank = no template control (nuclease-free water).

Supplementary Table 3. Blood spiking conditions with LS174T cells and theoretical mutant and wild-type copies calculations

# of LS174T Cells Spiked In 1 ml WB	# of Leukocytes in 1 mL WB	Theoretical # of KRASG12D copies	Theoretical # of Wild-Type copies	Theoretical KRASG12D AF (%)
409,600	3.95×10^6	415,252	8,309,522	4.76
102,400	3.95×10^6	103,813	8,002,380	1.28
25,600	3.95×10^6	25,953	7,925,595	0.327
6,400	3.95×10^6	6,488	7,906,398	0.082
1,600	3.95×10^6	1,622	7,901,600	0.021
400	3.95×10^6	405	7,900,399	0.005
100	3.95×10^6	101	7,900,100	0.00125
0 (WT)	3.95×10^6	0	7,900,000	0

* An expected background of 3.95×10^6 leukocytes cells per ml were determined with a hemocytometer. 50.69% mutant zygosity was used to calculate the theoretical # of KRASG12D copies contribution from LS174T.



Supplementary Figure 2. Amplification plots for qPCR data (parallel to synthetic KRAS G12D detection experiment)

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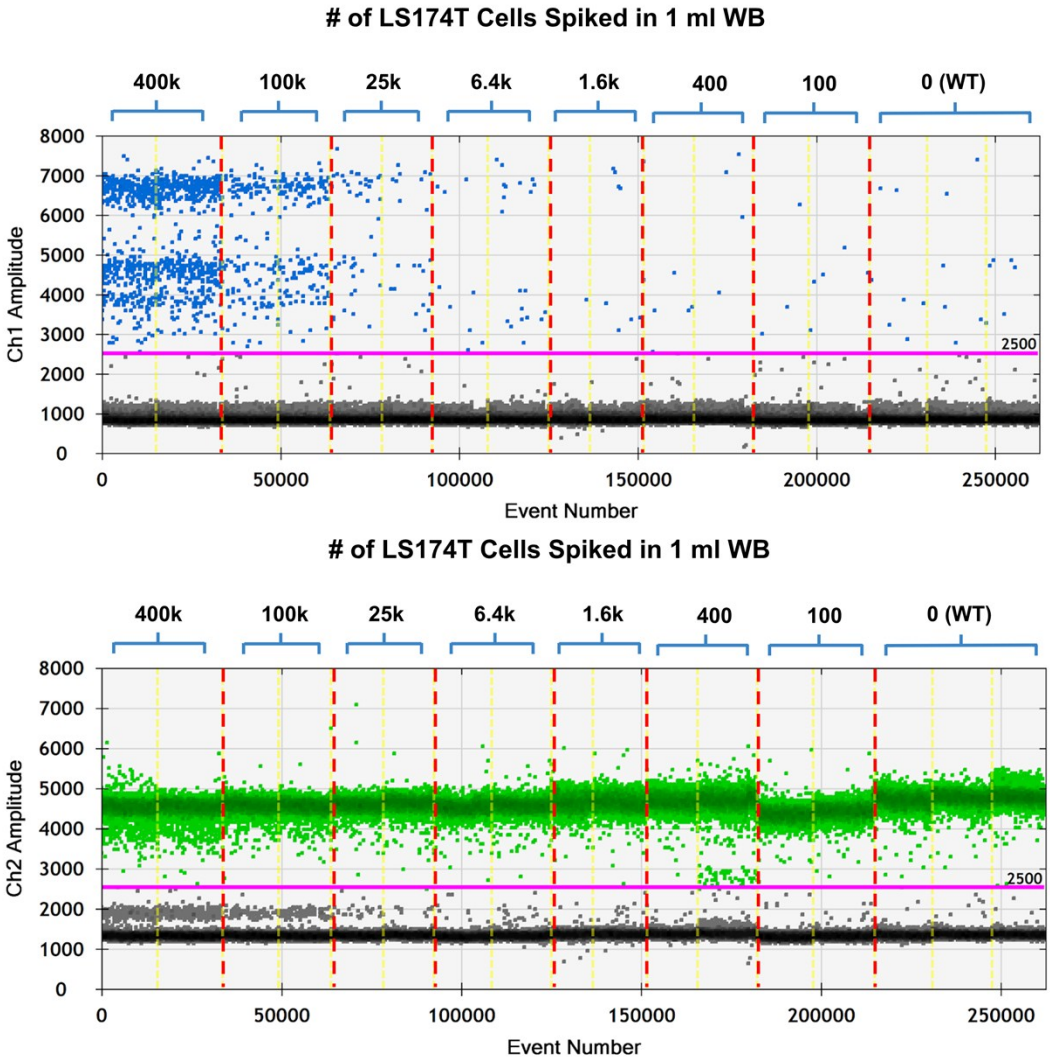
Supplementary Table 4. IC3D hit counts for synthetic KRAS G12D experiment

Replicate	AF %	Hits / 120s
A	BLANK	0
A	0.00000%	0
A	0.01000%	1
A	0.10000%	9
A	0.50000%	82
A	1.00000%	257
A	5.00000%	427
B	0.00000%	0
B	0.01000%	3
B	0.10000%	13
B	0.50000%	27
B	1.00000%	146
B	5.00000%	536
C	0.00000%	0
C	0.01000%	4
C	0.10000%	9
C	0.50000%	104
C	1.00000%	139
C	5.00000%	668

Supplementary Table 5. IC3D hit counts for LS174T cell-spiking experiment

Replicate	# Spiked Cells/ ml whole blood	AF %	Hits / 120s
A	0	0.00000%	0
A	100	0.00125%	3
A	400	0.00500%	1
A	1600	0.02100%	9
A	6400	0.08200%	48
A	25600	0.32700%	67
B	0	0.00000%	0
B	100	0.00125%	1
B	400	0.00500%	3
B	1600	0.02100%	14
B	6400	0.08200%	66
B	25600	0.32700%	123
C	0	0.00000%	0
C	100	0.00125%	0
C	400	0.00500%	16
C	1600	0.02100%	13
C	6400	0.08200%	70
C	25600	0.32700%	114

Supplementary Figure 3. Bio-RAD QX-200 ddPCR for LS174T cell-spiking experiment



Expected Mutant Fraction (%)	# of LS174T Cells Spiked In 1 ml WB	KRASG12D (CH1 - FAM)	Wild-Type (CH2 - VIC)	Measured Mutant Fraction (%)	Standard Deviation	*t*-value	*p*-value	Confidence Level (%)
		Copies Detected	Copies Detected					
4.76	409,600	1480	27,840	5.05	0.0044	980	< 0.01	< 99.99
1.28	102,400	458	30,760	1.47	0.0682	19.4	< 0.01	< 99.9
0.33	25,600	114	30,480	0.37	0.1996	1.44	0.031	96.9
0.08	6,400	41.2	30,360	0.14	0.0723	0.869	0.09	91
0.02	1,600	16.2	31,220	0.05	0.0551	0.299	0.295	70.5
0.005	400	17.4	31,980	0.05	0.0297	0.473	0.207	79.3
0.0013	100	11.4	28,560	0.04	0.0108	2.52	0.008	99.2
0	0 (WT)	35.4	51,080	0.07	0.0013	**reference**	**reference**	**reference**
0	0 (NF H2O)	5.8	0	100.00	0	464	< 0.01	100

*n = 3 for WT and NF H20 controls; n = 2 for all other samples

Supplementary Table 6. Droplet throughput comparison of Velox prototype IC3D system to commercial ddPCR systems

		Velox IC3D (3-D) *	RainDance (1-D)	BioRad (1-D)
	Analytical Sensitivity	0.00125 – 0.005% (present study)	0.0022% [a]	0.08 – 0.33% (present study)
Sample	WT concentration limit per reaction	> 400 ng/ 20 µl (20 µg/ml)	1500 ng/ 25 µl (60 µg/ml) [b]	66 ng/ 20 µl [f] (3.3 µg/ml)
Sample	Final dilution volume (clinical sample = 20 µg total)	1 ml	0.33 ml	6.06 ml
Sample	Number of samples	1 x 1 ml	14 x 25 µl	304 x 20 µl
Sample	Total number of droplets (partitions)	15.3 million	70 million [c]	6.1 million [g]
Throughput	Droplet Generation	66 min (1 ml/ 66 min)	60 min 2 x (8-wells/30 min) [d]	180 min 4x (96-wells/45 min) [h]
Throughput	Droplet scanning	2 min (2 min/ sample)	4 hours 2 x (2 hrs/8 wells in "fast mode") [e]	380 min 304 x (120 min/96 wells) [i]
Throughput	Total Time**	1hr, 8 min	5 hours	9hr, 20min

[a] Reported empirical LOD of 0.0022% AF for KRAS G12D (3.5 E7 WT copies) [C. A. Milbury, Q. Zhong, J. Lin, M. Williams, J. Olson, D. R. Link and B. Hutchison, *Biomol Detect Quantif*, 2014, **1**, 8–22.]

[b] Recommended DNA loading up to 10% target occupancy (RainDrop® assay guidelines), which for a 25 µl reaction (5E6 droplets) is approximately 500,000 DNA molecules, which is the equivalent of approximately 1.5 µg gDNA

[c] Approximately 5E6 droplets per 25 µl reaction (RainDrop® assay guidelines)

[d] Approximately 30 minutes to generate droplets from 1 chip (8 wells) (ThunderBolts™ System/RainDance Source Operator's Manual)

[e] Droplet scanning time for 8 wells = 2 hours (fast mode); 4 hours (standard mode) (RainDrop® Sense Operator's manual)

[f] "Recommendations for Optimal Results" (ddPCR™ Supermix for Probes): "The concentration of intact human genomic DNA should be ≤ 66 ng per 20 µl reaction"

[g] Approximately 20,000 droplets per sample ("QX100/QX200 Workflow / Droplet Generation"): "Droplet Digital™ PCR Applications Guide." Bulletin 6407 Ver B. Bio-Rad Laboratories, Inc.)

[h] Approximately 45 minutes to generate droplets for 96 wells ("1.3 Installation and General Operation", pg. 4): "Automated Droplet Generator." Instruction Manual. Catalog #1864101. BioRad © 2018)

[i] Approximately 120 minutes to read/analyze droplets from one 96-well plate [S. H. Te, E. Y. Chen and K. Y.-H. Gin, Appl. Environ. Microbiol., 2015, **81**, 5203.], [E. Mazaika and J. Homsy, *Current protocols in human genetics / editorial board, Jonathan L. Haines ... [et al.]*, 2014, **82**, 7.24.1-7.24.13.]

* IC3D performance and throughput estimates are solely based on this proof-of-concept study and do not represent limitations of the technology.

** Droplet thermocycling and droplet transfer steps not included

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VIDEO: “IC3D for Cancer Liquid Biopsy: Step-by-step workflow for user training and education”

https://vimeo.com/313872068