

## **Evaluation of Droplet Digital PCR for the Detection of *BRAF* V600E in Fine-Needle Aspiration Specimens of Thyroid Nodules**

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**Supplemental Data Table S1.** Sequences of the primers and probes used in the *BRAF* V600E ddPCR assay

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<b>Primer/probe</b>	<b>Sequence (5' – 3')</b>
Forward primer	TTCATGAAGACCTCACAGTAAA
Reverse primer	CCACAAAATGGATCCAGACA
Wild-type probe (VIC dye)	CTAGCTACAGTGAAATC
Mutant probe (FAM dye)	TAGCTACAGAGAAATC

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Abbreviation: ddPCR, droplet digital PCR.

**Supplemental Data Table S2.** Precision analysis

<b>Variant allele frequency (%)</b>	<b>Inter-batch precision (SD)</b>	<b>CV (%)</b>
1	0.07	5.8
0.5	0.07	11.4
0.1	0.04	20.5
0.05	0.02	30.3
0.01	0.01	58.8

Abbreviations: SD, standard deviation; CV, coefficient of variation.

**Supplemental Data Table S3.** Discordant results among the molecular assays for *BRAF* V600E detection

<i>BRAF</i> V600E molecular assay results						Clinicopathological features							
No. case	ddPCR	qPCR	MEMO sequencing	Cytological diagnosis	Histological diagnosis	Size of dominant tumor (cm)	Lymphatic invasion	Blood vessel invasion	Extra-thyroidal extension	Surgical margins	Lymph node metastasis	Chronic lymphocytic thyroiditis	Nodular hyperplasia
BRAF-011	D	N	D	AUS/FLUS	PTC	0.7×0.5×0.7	Absent	Absent	Absent	Absent	Present	Absent	Absent
BRAF-064	D	N	N	Malignant	PTC	0.4×0.4×0.3	Present	Absent	Absent	Absent	Present	Present	Present
BRAF-082	D	N	D	Malignant	PTC	2.8×1.5×2.5	Present	Absent	Present	Absent	Present	Absent	Present
BRAF-084	D	N	D	Malignant	PTC	1.2×1×1	Absent	Absent	Absent	Absent	Could not be assessed	Absent	Present
BRAF-088	D	N	D	Malignant	NP								
BRAF-091	D	N	D	Malignant	NP								
BRAF-092	D	N	D	Malignant	PTC	0.8×0.7×0.6	Absent	Absent	Absent	Absent	Present	Present	Absent
BRAF-093	D	N	D	Benign	PTC	0.5×0.4×0.4	Absent	Absent	Absent	Absent	Absent	Absent	Present
BRAF-223	D	N	N	Benign	NP								
BRAF-239	D	N	N	Benign	NP								
BRAF-242	D	N	N	AUS/FLUS	PTC	1.2×1.0×0.8	Absent	Absent	Absent	Absent	Absent	Absent	Absent
BRAF-003	D	Inconclusive	D	Malignant	NP								
BRAF-017	D	Inconclusive	D	AUS/FLUS	PTC	0.8×0.7×0.6	Present	Absent	Absent	Absent	Absent	Present	Absent

BRAF-160	D	Inconclusive	D	Malignant	PTC	0.3×0.2×0.2	Present	Absent	Absent	Absent	Present	Present	Absent
BRAF-164	D	Inconclusive	D	Malignant	NP								
BRAF-165	D	Inconclusive	D	SMC	NP								
BRAF-212	D	Inconclusive	D	Malignant	PTC	0.7×0.6×0.5	Absent	Absent	Absent	Absent	Could not be assessed	Absent	Absent
BRAF-219	D	Inconclusive	D	Malignant	PTC	1.0×1.0	Absent	Absent	Absent	Absent	Could not be assessed	Absent	Absent
BRAF-246	D	Inconclusive	D	AUS/FLUS	PTC	3.2×2×1.5	Present	Absent	Absent	Absent	Present	Present	Absent
BRAF-283	D	Inconclusive	D	SMC	NP								
BRAF-261	N	D	D (K601E)	AUS/FLUS	NP								
BRAF-069	N	Inconclusive	N	Benign	NP								
BRAF-077	N	Inconclusive	N	AUS/FLUS	PTC	0.6×0.5×0.5	Absent	Absent	Absent	Absent	Absent	Present	Absent

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Abbreviations: D, detected; N, not detected; AUS/FLUS, atypia of undermined significance; ddPCR: droplet digital PCR; PTC: papillary thyroid carcinoma; SMC: suspicious for malignant cells/follicular lesion of undetermined significance; SMC, suspicious for malignant cells; PTC, papillary thyroid carcinoma; NP, not performed.

**Supplemental Data Table S4.** Clinicopathological parameters and *BRAF* V600E status determined by ddPCR

<b>Variable</b>	<b><i>BRAF</i> V600E mutation + (N=84)</b>	<b><i>BRAF</i> V600E mutation – (N=18)</b>	<b><i>P</i></b>
Tumor size (cm)	1.16 (0.7–1.2)	1.33 (0.7–1.35)	0.509 <sup>*</sup>
Tumor size			0.142 <sup>†</sup>
<1 cm	44 (88.0)	6 (12.0)	
≥1 cm	40 (76.9)	12 (23.1)	
Lymphatic invasion	53 (85.5)	9 (14.5)	0.302 <sup>†</sup>
Blood vessel invasion	1 (50)	1 (50)	0.323 <sup>†</sup>
Extra-thyroid extension	8 (88.9)	1 (11.1)	1.000 <sup>†</sup>
Lymph-node metastasis	45 (83.3)	9 (16.7)	0.782 <sup>†</sup>
Tumor, node, metastasis stage			0.421 <sup>‡</sup>
I	61 (72.6)	15 (83.3)	
II	14 (16.7)	1 (5.56)	
III	1 (1.19)	0 (0.00)	
IV	8 (9.52)	2 (11.11)	

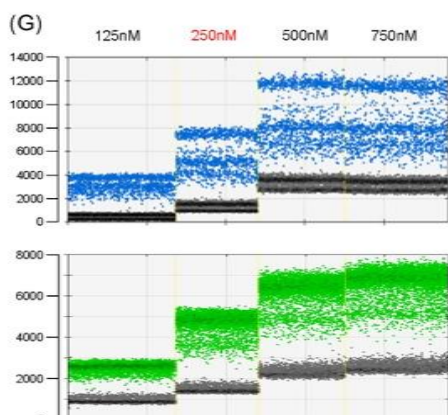
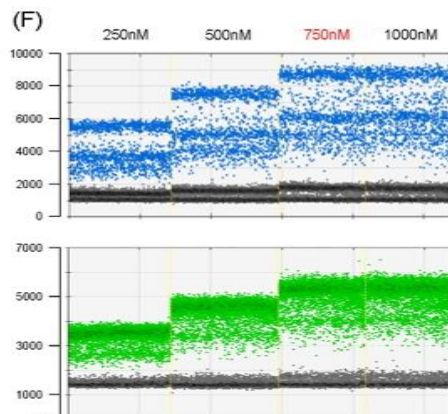
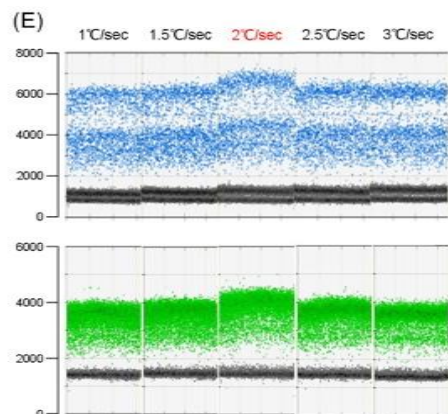
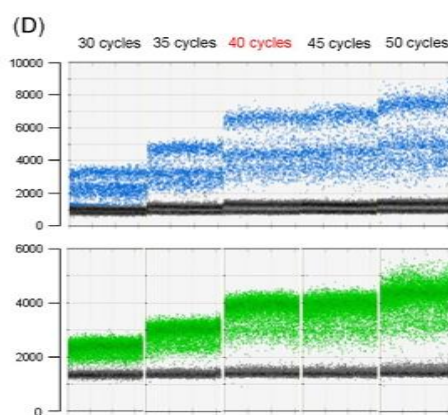
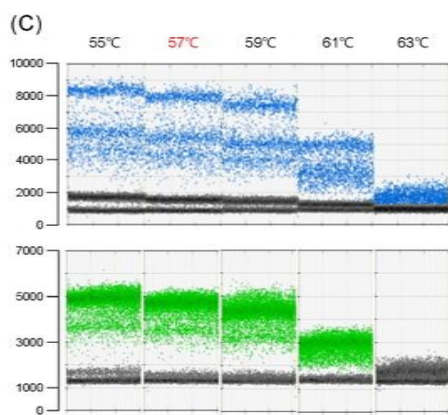
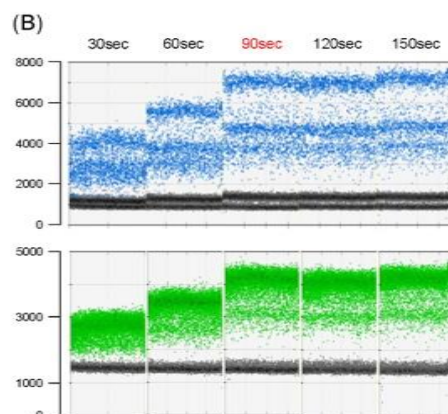
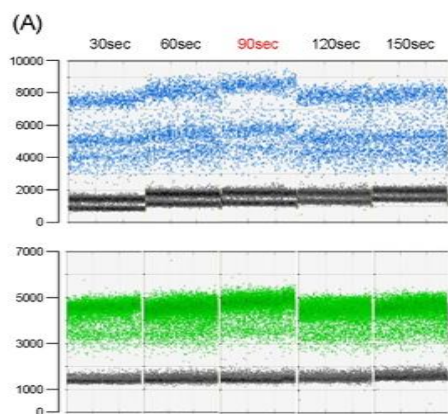
Statistical analysis was performed using SPSS Statistics v20.0.0 (SPSS Inc., Chicago, IL, USA) and R v4.2.0. The chi-squared test, Fisher's exact test, and Cochran–Armitage trend test were used to compare categorical variables. The Mann–Whitney test for two groups was used to compare continuous variables. Data are expressed as number (%) or median (interquartile range).

<sup>\*</sup>Mann–Whitney test.

<sup>†</sup>Pearson's chi-squared test and Fisher's exact test.

<sup>‡</sup>Cochran–Armitage trend test.

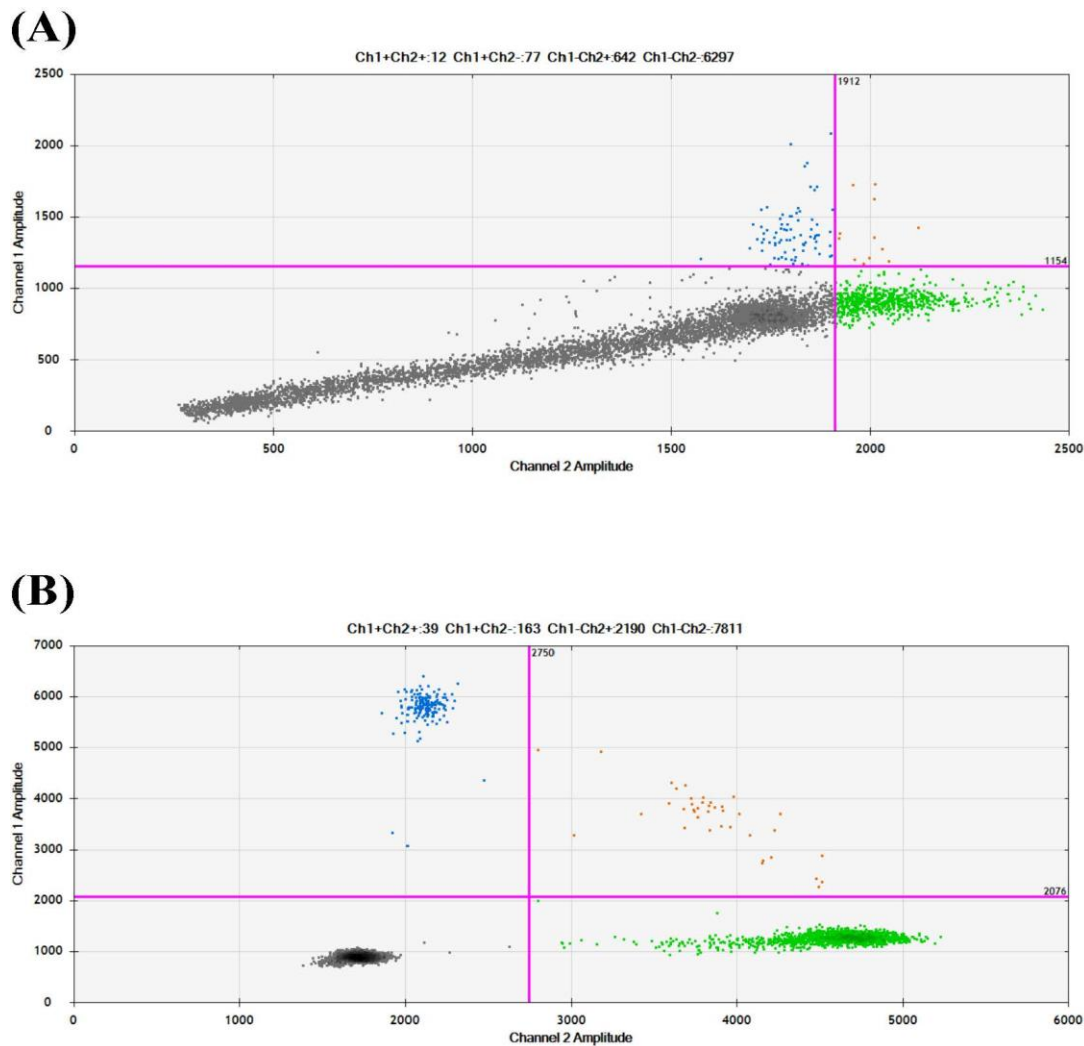
Abbreviation: ddPCR, droplet digital PCR.



**Supplemental Data Fig. S1.** Optimization results of ddPCR parameters. The one-dimensional ddPCR plot shows the influence of each of the following parameters: (A) denaturation time, (B) annealing time, (C) annealing temperature, (D) number of PCR cycles, (E) ramp rate, (F) primer concentration, and (G) probe concentration. The conditions selected for the optimized ddPCR assay are highlighted in red font. Blue dots represent *BRAF* V600E, and green dots represent wild-type *BRAF* V600E.

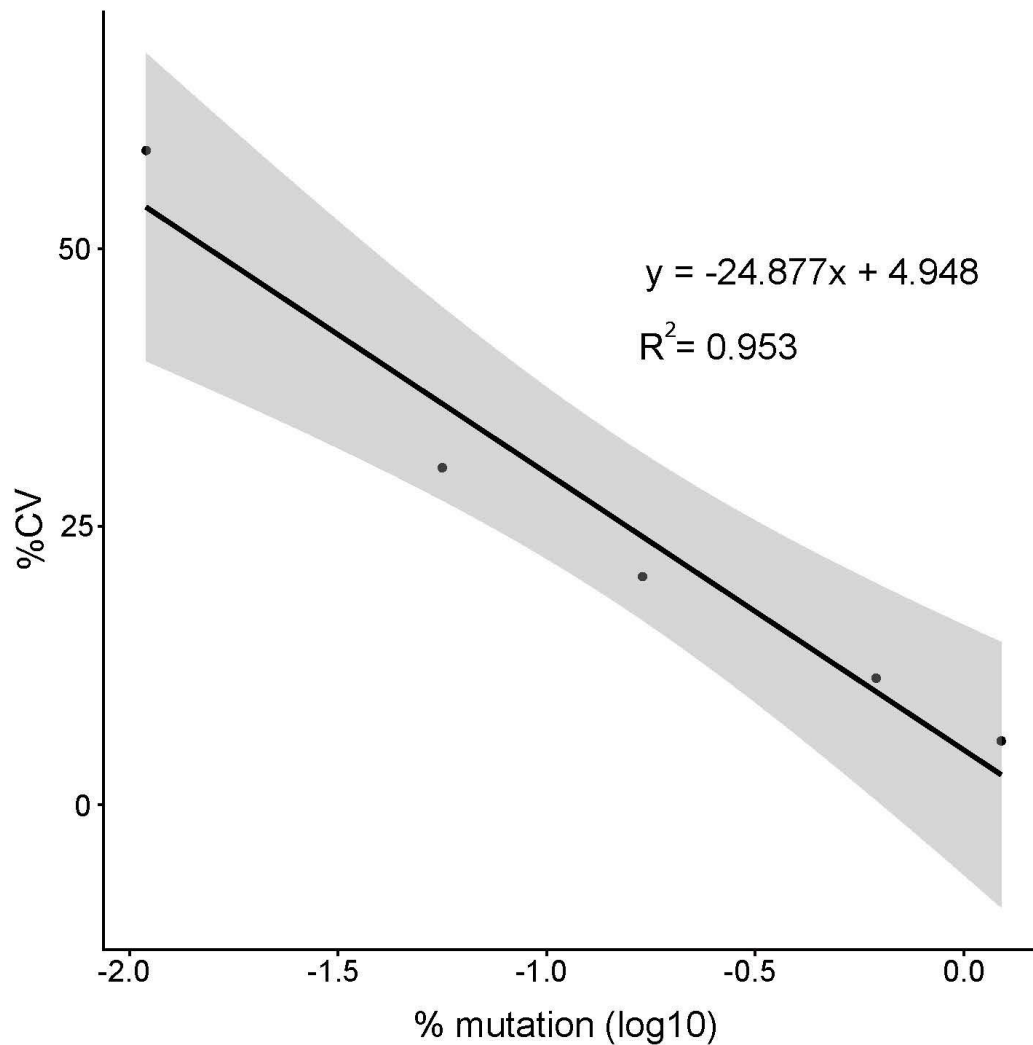
Abbreviation: ddPCR, droplet digital PCR.





**Supplemental Data Fig. S2.** Comparison of the standard and optimized protocols. The standard and optimized protocols using FNA clinical specimens were compared to assess whether there were significant differences in the detection rate. However, it was challenging to set the threshold to differentiate positive and negative droplets in the first experiment using the standard protocol. The clinical specimens comprised 32 positive and 16 negative specimens. The VAF of positive specimens varied from 0.04% to 42.5% with the optimized protocol. Representative 2D graphs for a clinical specimen with a VAF of 7.5% assessed using the standard protocol (A) and optimized protocol (B) are shown.

Abbreviations: FNA, fine-needle aspiration; VAF, variant allele frequency.



**Supplemental Data Fig. S3.** Precision of the optimized ddPCR assay for *BRAF* V600E detection. The CV (%) was evaluated on the basis of the percentage of mutation ( $\log_{10}$  transformation) using linear regression. The gray-shaded area indicates the 95% confidence interval.

Abbreviation: ddPCR, droplet digital PCR.