Electronic Supplementary Material (ESI) for Analyst.

Ultrasensitive and quantitative detection of *EGFR* mutations in plasma samples from patients with non-small-cell lung cancer using a dual PNA clamping-mediated LNA-PNA PCR clamp †

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List of Electronic Supplementary Information (ESI)

Table S1. Sequences of the PCR primers and probes used for the LNA-dPNA PCR clamp.

Primer name	Sequence (5'-3')	Tm (°C)	Length of amplicon
*EGFR*858-IP-F	CAGCATGTCAAGATCACAGATT	61.0	87 bp
*EGFR*858-IP-R	CCTTACTTTGCCTCCTTCTG	60.0	
*EGFR*858-OP-F	CTACTTGGAGGACCGTCGCTTGGTGC	69.5	200 bp
*EGFR*858-OP-R	CCTGGTCCCTGGTGTCAGGAAAATGCT	69.5	
*EGFR*858-W-PNA	TGGGCTGGCCA	~ 63.9	/
*EGFR*858-M-LNA	6-FAM-TTGGGC*G*GGCCAAA-TAMRA	65.8	
*EGFR*858-UP	HEX-TCTTTCTCTTCCGCACCCAGC-BHQ1	67.3	/
*EGFR*19-2237-IF	ATCCCAGAAGGTGAGAAAGT	60.7	121 bp
*EGFR*19-2237-IR	GGGCCTGAGGTTCAGAG	60.8	
*EGFR*19-2237-OF	TGGCACCATCTCACAATTGCCAGTT	66.9	207 bp
*EGFR*19-2237-OR	GCAGCTGCCAGACATGAGAAAAGGT	66.8	
*EGFR*19-W-PNA	AGGAATTAAGAGAAGCAACATCT	~ 64.5	/
*EGFR*19-2237-LNA	6-FAM-CAAG*GCC*G*A*AAGCC-IABkFQ	70.5	/
*EGFR*19-2240-UP	HEX-ACAGCAAAGCAGAAACTCACATCGA-BHQ1	67.7	
EGFR-T790M-IF	ATCTGCCTCACCTCCAC	60.3	96 bp
EGFR-T790M-IR	GGAGCCAATATTGTCTTTGTGT	61.2	
EGFR-T790M-OF	AGCCACACTGACGTGCCTCTCC	67.9	211 bp
EGFR-T790M-OR	ATCTGCACACACCAGTTGAGCAGGT	68.1	
EGFR-T790M-PNA	GCTCATCACGCAGCTCAT	~ 66.3	/
EGFR-T790M-LNA	6-FAM-TCA*TC*A*TG*C*AG*C-IABkFQ	65.4	/
EGFR-T790M-UP	HEX-TCCCGGACATAGTCCAGGAG-BHQ1	66.3	/
*EGFR*797-2390-IF	ATCTGCCTCACCTCCAC	60.3	115 bp
*EGFR*797-2390-IR	ACCAGTTGAGCAGGTACT	60.1	
*EGFR*797-2390-OF	CTCTCCCTCCCTCCAGGAAGCCTA	67.3	213 bp
*EGFR*797-2390-OR	TCCCTGATTACCTTTGCGATCTGCACAC	67.9	
*EGFR*797-2390-PNA	CTTCGGCTGCCTCCTGG	~ 69.7	/
*EGFR*797-2390-LNA	6-FAM-TTCGGC*TCC*CTCCT-IABkFQ	71.1	/
*EGFR*797-2390-UP	HEX-AGCTGCGTGATGAGCTGC-BHQ1	66.0	/

Note: The Tm values of the primers and probes were calculated by OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc., Iowa, USA). The LNA bases were marked in italic. The Tm values of the PNA probes were 2-13 °C higher than the values calculated by OligoAnalyzer 3.1. Because a base mismatch occurs between the W-PNA and the mutant sequence, the Tm of W-PNA shifts 2-13 °C.

Table S2. Clinical characteristics of patients with NSCLC.

	Patients with NSCLC (N=212)
Age, median (range)	63 (32-83)
Sex (n, %)	
Male	112 (52.8%)
Female	100 (47.2%)
Smoking history (n, %)	
Never smoked	149 (70.3%)
Smoker	63 (29.7%)
Histological type	
Adenocarcinoma	159(75.0%)
Squamous cell carcinoma	38(17.9%)
Others	15(7.1%)
Stage	
Stage I	102 (48.1%)
Stage II	48 (22.7%)
Stage III	49 (23.1%)
Stage IV	13 (6.1%)

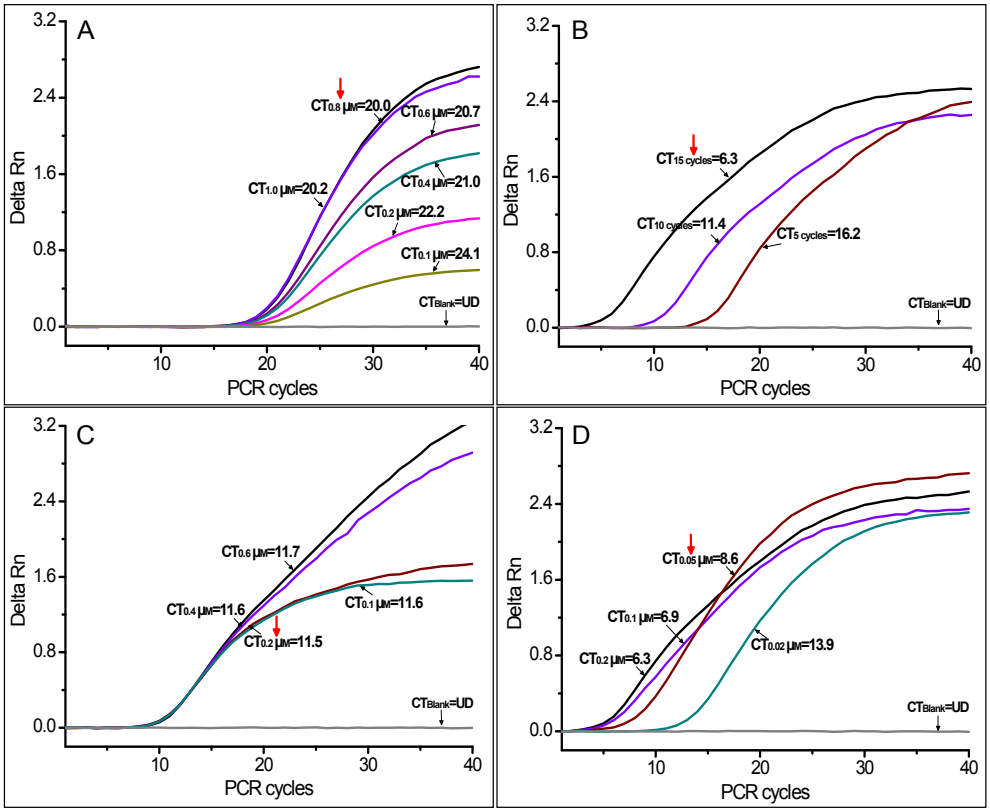

Figure S1. Efficiency of the LNA-dPNA PCR clamp at various concentrations of M-LNA probe (A), cycle numbers (B), concentrations of IPs (C), and concentrations of OPs (D). All experiments were performed at the same concentration of template (10^6 mutant plasmid), and each assessment was performed twice. The selected conditions are marked with red arrows.

Note: The efficiency of the LNA-dPNA PCR clamp was affected by the concentrations of the M-LNA probe, IPs, and OPs as well as the cycle numbers. Therefore, we optimized these key factors and found that a high efficiency was achieved when performing the LNA-dPNA PCR clamp with 0.8 μM M-LNA probe (Figure S1A), 0.2 μM IPs (Figure S1B), and 15 cycles of pre-amplification (Figure S1C). We also found that a higher efficiency was achieved at a higher concentration of OPs (0.2 μM), but a smoother S-curve was achieved at a lower concentration of OPs (0.05 μM). Therefore, we selected 0.05 μM as the optimized concentration (Figure S1D).

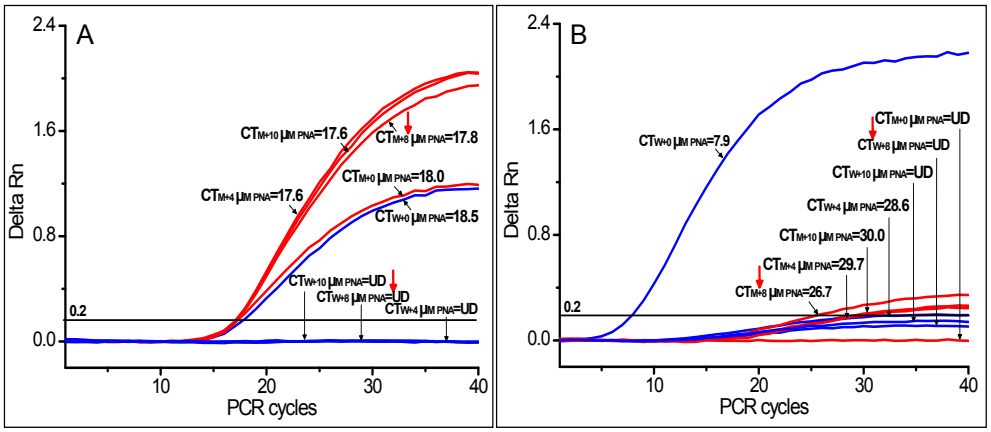

Figure S2. Mutation enrichment detected by the LNA-dPNA PCR clamp at various concentrations of the W-PNA probe using 10^3 M:10^3 W (A) and 10^3 M:10^6 W (B) as templates. "M" means mutant DNA; "W" means wild-type DNA; "UD" means "Undetected". Each assessment was performed twice, and the selected conditions are marked with red arrows.

Note: The mutation enrichment detected by the LNA-dPNA PCR clamp was affected by the W-PNA probe concentration and annealing temperature. Therefore, we first optimized the PNA concentration and found that mutant DNA amplification was unaffected, and wild-type DNA amplification was completely blocked when the concentration of W-PNA was changed from 4 μM to 10 μM at a lower concentration of wild-type template (10^3 copies) (Figure S2A). We selected 8 μM W-PNA as the optimal concentration because more efficient blockade of wild-type DNA (UD vs. 29.7) and more efficient mutant DNA amplification (26.7 vs. UD) were obtained when the W-PNA concentration was 8 μM vs. 4 μM at a higher concentration of wild-type DNA (10^6 copies) (Figure S2B).

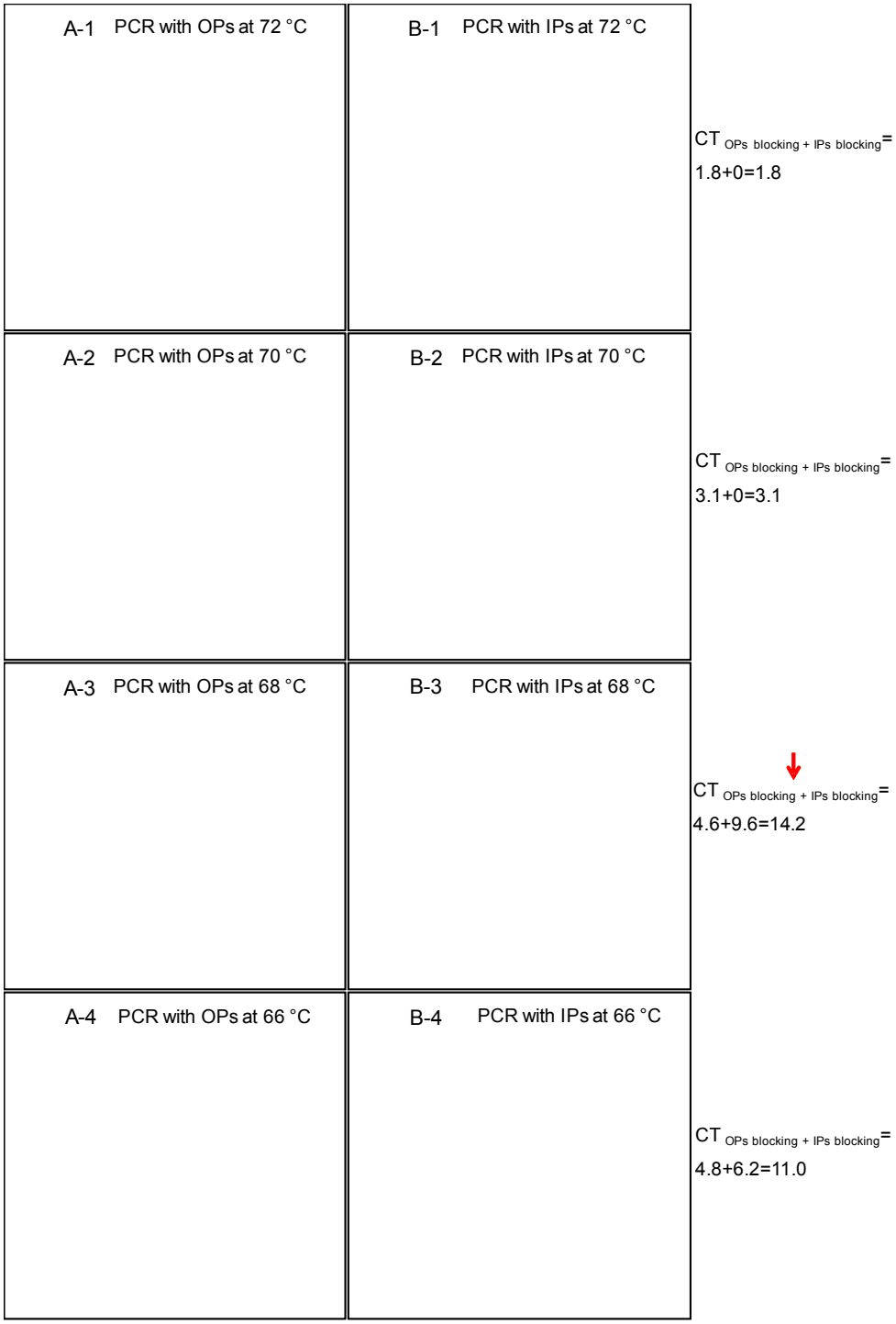

Figure S3. Mutation enrichment detected by the LNA-dPNA PCR clamp at annealing temperatures of 72 °C (A-1, B-1), 70 °C (A-2, B-2), 68 °C (A-3, B-3), and 66 °C (A-4, B-4) using OPs and IPs. “M” means mutant DNA; “W” means wild-type DNA; “PNA” means W-PNA probe; “UD” means “Undetected”. Each assessment was performed twice, and the selected conditions are marked with red arrows.

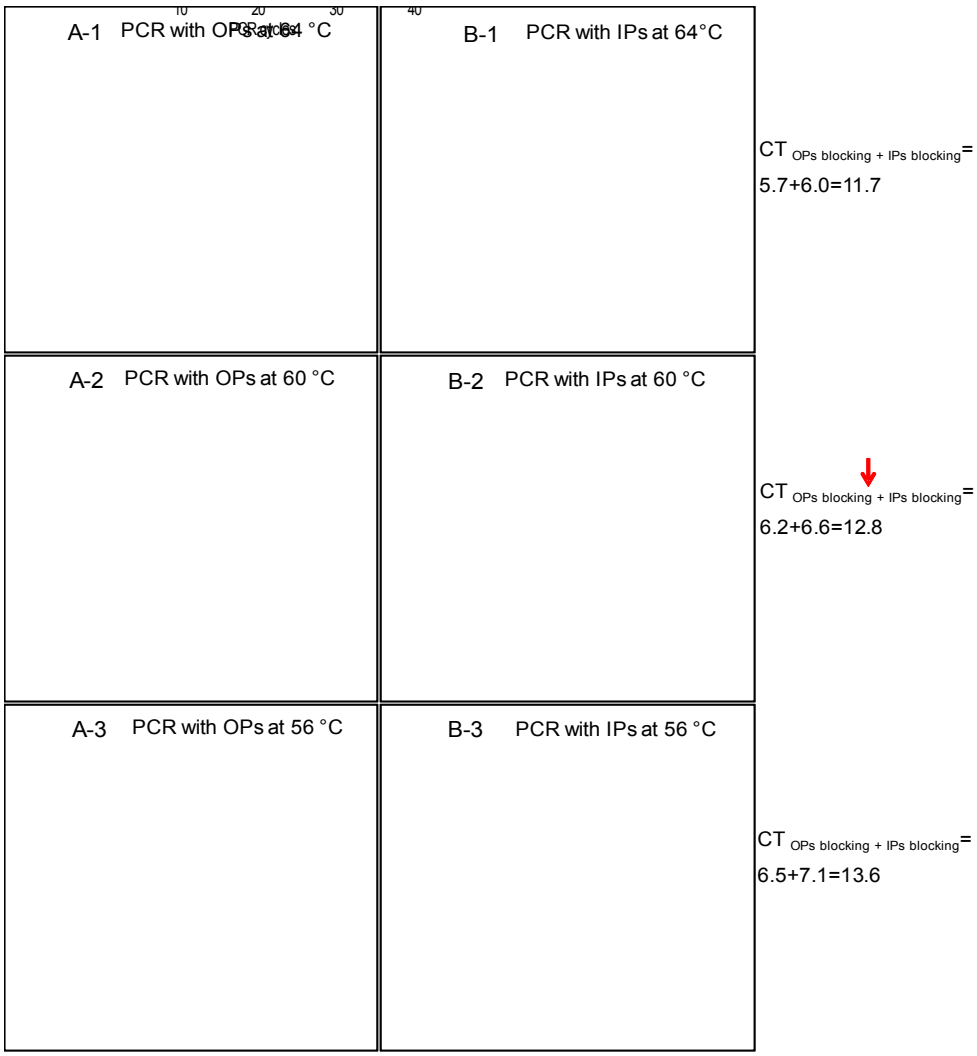

Figure S4. Mutation enrichment detected by the LNA-dPNA PCR clamp at annealing temperatures of 64 °C (A-1, B-1), 60 °C (A-2, B-2), and 56 °C (A-3, B-3) using OPs or IPs. "M" means mutant DNA; "W" means wild-type DNA; "PNA" means W-PNA probe; "UD" means "Undetected". Each assessment was performed twice, and the selected conditions are marked with red arrows.

Note: We optimized the annealing temperatures, and 68 °C for the OPs and 56 °C for the IPs were selected because we found that more efficient blockade was achieved when the OPs were amplified at 68 °C (14.2) rather than 72 °C (1.8), 70 °C (3.2), or 66 °C (11.0) (Figure S3). We further observed that more efficient blockade was achieved when the IPs were amplified at 56 °C (13.6) rather than 60 °C (12.8) or 62 °C (11.7) (Figure S4). A better specificity was achieved when the IPs were amplified at 60 °C rather than 56 °C when the clinical samples were amplified (data not shown).

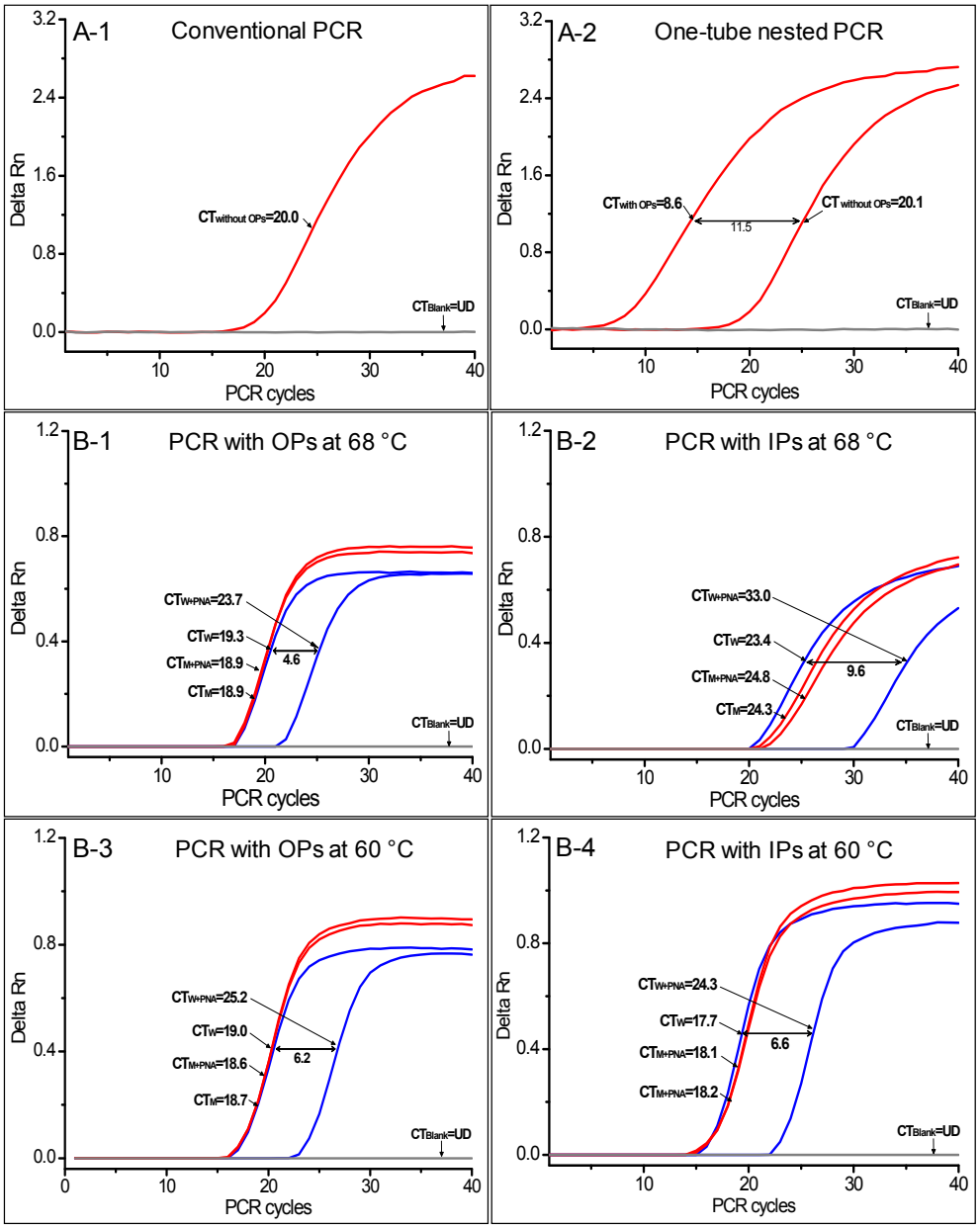

Figure S5. Evaluation of the effects of one-tubed nested PCR (A) and dual PNA clamp (B) on the LNA-dPNA PCR clamp. “M” means mutant DNA; “W” means wild-type DNA; “PNA” means W-PNA probe. Each assessment was performed twice.

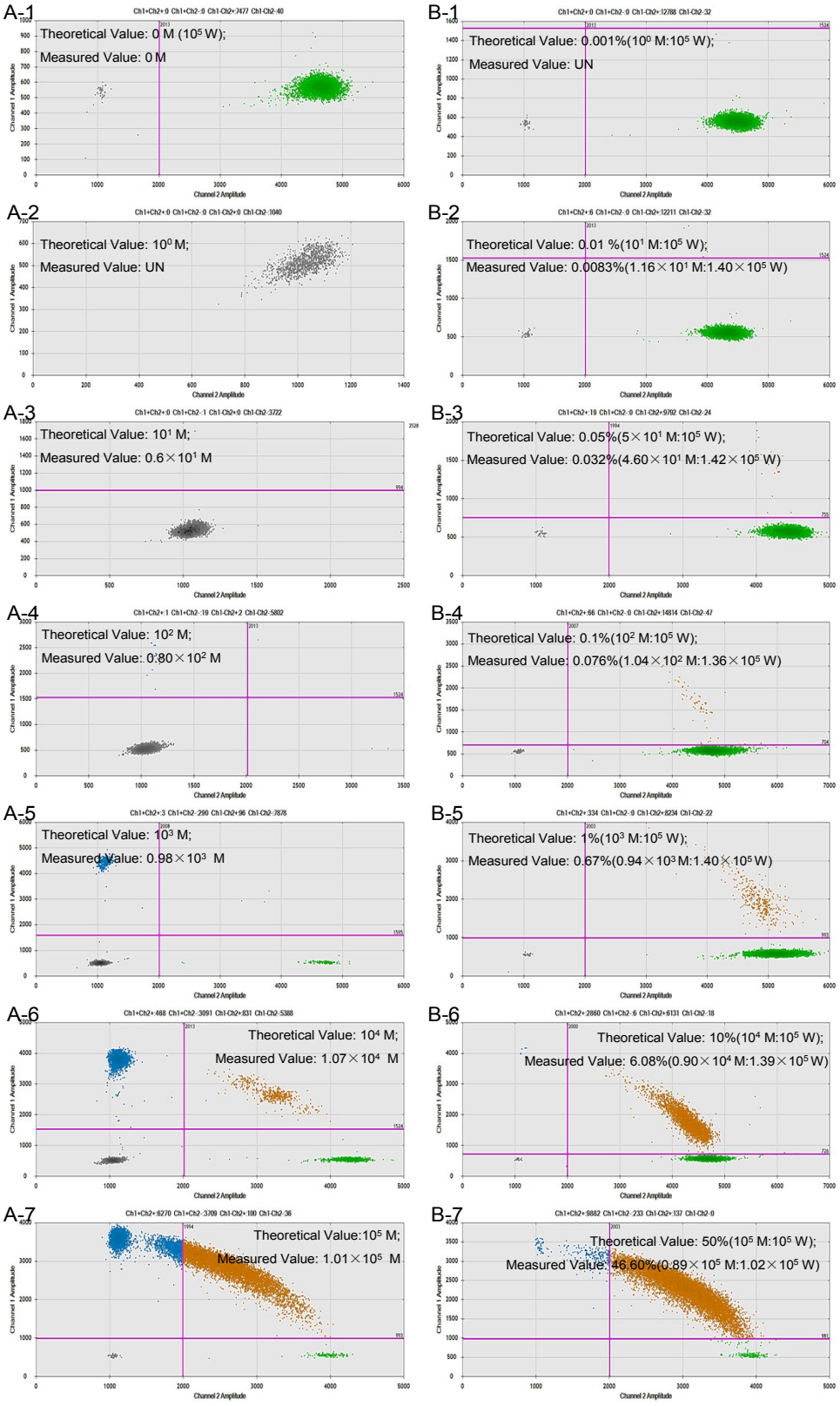

Figure S6. Evaluation of the sensitivity (A), specificity (B), and linearity (A) using ddPCR for *EGFR* L858R detection. “M” means mutant DNA; “W” means wild-type DNA; “UN” means undetermined. Each assessment was performed twice.

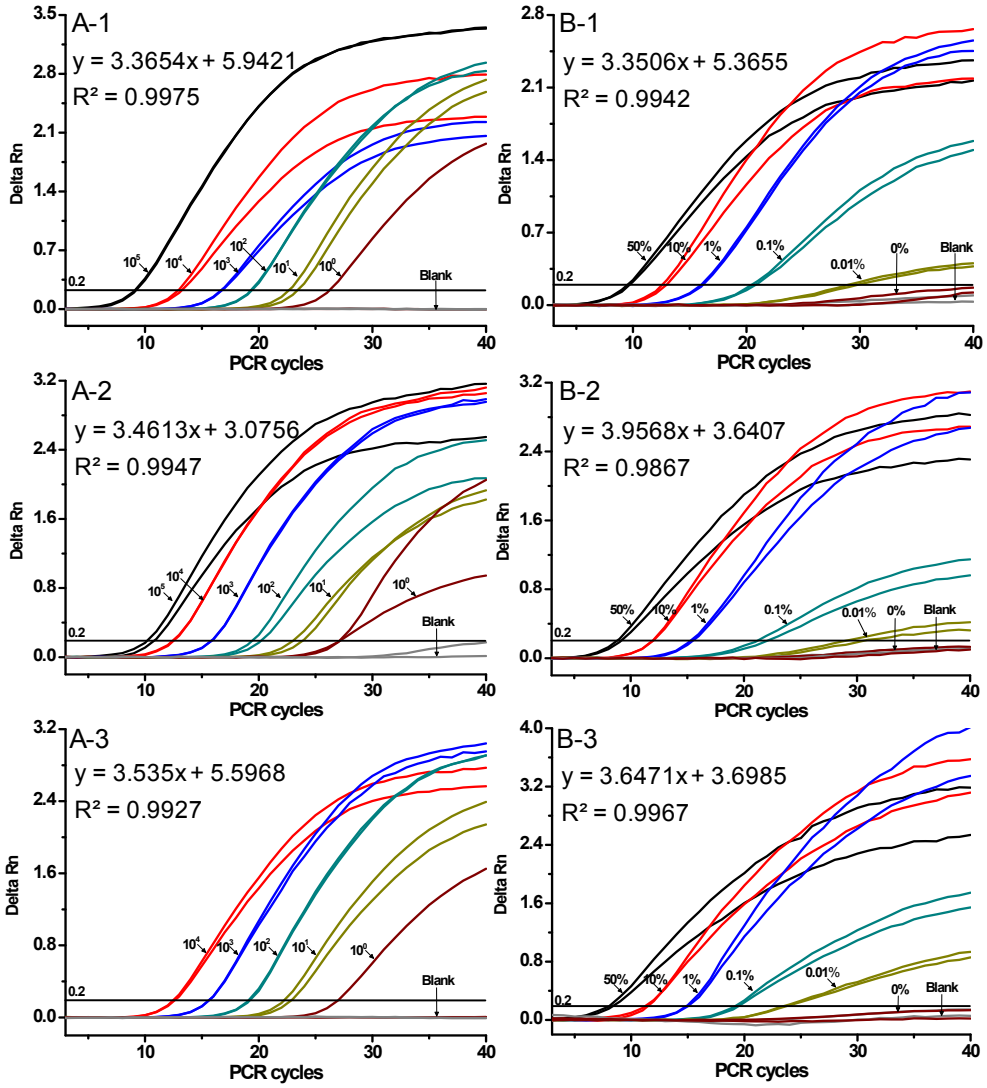

Figure S7. Evaluation of the reproducibility of LNA-dPNA PCR clamp in detection of *EGFR* L858R. The amplification sensitivity (A) and limit of detection (LOD) (B) were repeatedly detected in triplicate from sample dilution to real-time PCR. A-1: The amplification sensitivity was tested first; A-2: the amplification sensitivity was tested second; A-3: the amplification sensitivity was tested third. B-1: the LOD was tested first; B-2: the LOD was tested second; B-3: the LOD was tested third. Each assessment was performed twice.

Note: 0.01% (10^1M:10^5W) was repeatedly detected in the six parallel tests. A 10^0 copy/reaction was detected four times in six parallel tests due to its extremely low content.

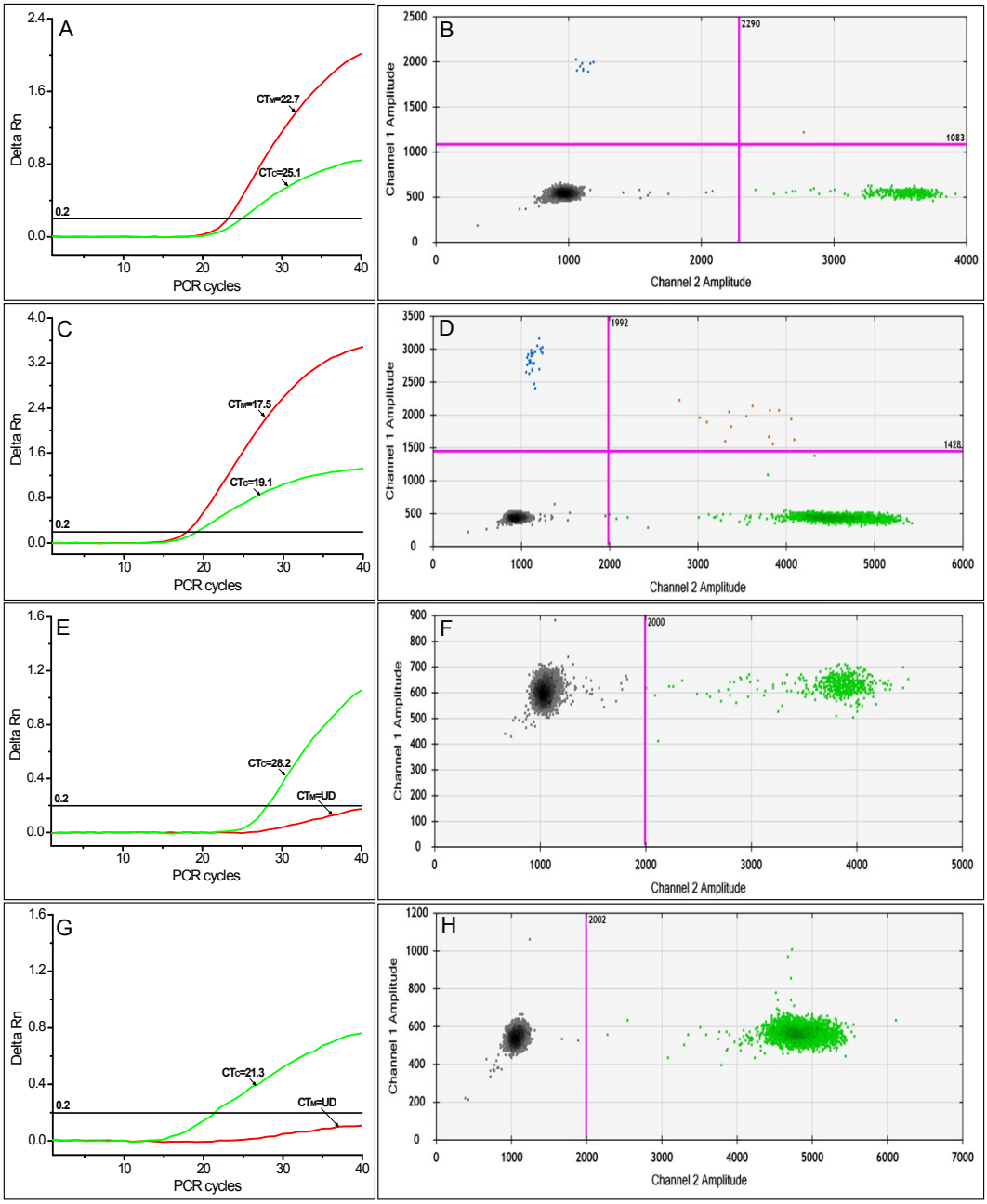

Figure S8. Typical results for *EGFR* L858R analysed using the LNA-dPNA PCR clamp (A, C, E, G) and ddPCR (B, D, F, H) from plasma (A, B, E, F) and tissue (C, D, G, H) samples. "M" means mutant DNA; "C" means universal control; "UD" means "Undetected." Each assessment was performed twice.

Table S3. Comparison of ddPCR and the LNA-dPNA PCR clamp for detecting plasma *EGFR* mutations and tissue *EGFR* mutations.

ddPCR		LNA-dPNA PCR clamp		Total	Sensitivity (%)	Specificity (%)
		Positive	Negative			
Tissues	Positive	46	0	46	100.0	/
	Negative	0	86	86	/	100.0
	Total	46	86	132	/	/
Plasmas	Positive	12	5	17	70.6	/
	Negative	2	113	115	/	98.3
	Total	14	118	132	/	/

Table S4. Comparison of *EGFR* mutations in tumor tissues and matched plasma samples for different tumour stages.

Tumor stages	Tissues	Matched plasma samples		Sensitivity
		Positive	Negative	
I	29	2	27	6.9%
II	11	2	9	18.2%
III	8	1	7	12.5%
IV	3	3	0	100%

Table S5. Comparison of labour and cost between the LNA-dPNA PCR clamp and ddPCR for quantifying plasma mutations.

LNA-dPNA PCR clamp			**ddPCR**		
Steps	Time (h)	Cost ($)	Steps	Time (h)	Cost ($)
Standard curve preparation	~0.5	0.02	Reagent preparation	~0.5	22.04
Reagent preparation	~0.5	39.52	Droplet preparation	~0.5	28.08
PCR	~1.25	0	PCR	~2.0	0
Data analysis	~0.5	0	Droplet reading	~0.5	5.92
			Data analysis	~0.5	0
Total	~2.75	39.68[a]	Total	~4.0	56.04 [b]

[a] Eight reactions are needed to quantify one mutation site with the LNA-dPNA PCR clamp, including 5 reactions for the standard curve, 1 reaction for the negative control, 1 reaction for the blank control, and 1 reaction for the sample. The cost of one reaction is 4.96 $, and the cost for the reaction is as follows: EASY Dilution (for real time PCR): 0.02 $/reaction; PNA probe: 2.49 $/reaction; TaKaRa Premix Ex Taq™ (probe qPCR): 0.69 $/reaction; PCR tube (Axygen): 0.09 $/reaction; LNA Probe: 1.54 $/reaction; universal probe: 0.13 $/reaction.

[b] Four reactions are needed to quantify one mutation site by ddPCR, including 1 reaction for the positive control, 1 reaction for the negative control, 1 reaction for the blank control, and 1 reaction for the sample. The cost of one reaction is 14.01 $, and the cost for the reaction is as follows: PrimePCR™ ddPCR™ Mutation Assay (Bio-Rad): 3.52 $/reaction; probes: 1.83 $/reaction; PCR tube and seal foil: 0.16 $/reaction; droplet generation oil: 0.96 $/reaction; droplet generator: 4.76 $/reaction; DG8 gaskets: 1.3 $/reaction; droplet-reading oil: 1.48 $/reaction.