A Novel Real Time PCR for Highly Sensitive and Accurate Detection of KRAS Mutations and SARS-CoV-2 Variants

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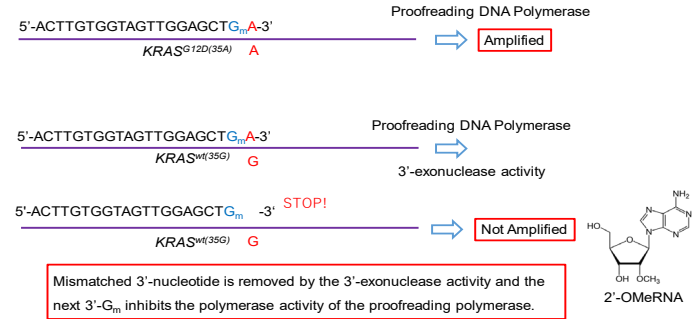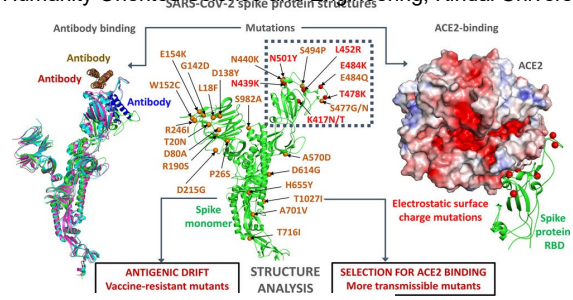
Abstract.

In the present study, quantitative analysis of KRAS gene discriminating single nucleotide mutation by allele specific real time PCR using chemically modified primers is described. Taq DNA polymerases which do not have 3'-exonuclease activity and HiFi taq DNA polymerase which has 3'-exonuclease activity, namely proof-reading activity were investigated. As a result, PCR using regular phosphate or phosphorothioate primers was not specific enough for a quantitative analysis discriminating single base mutation. On the other hand, real time PCR method using some chemically modified primers and proofreading polymerases or non-proofreading polymerases was highly specific for a quantitative analysis discriminating single base mutation between $KRAS^{wt}$ and $KRAS^{G12D}$, $KRAS^{G12A}$ and $KARS^{G12V}$. Only 10 copies of the template in one sample could be detected in a concentration dependent manner and 0.01 % of $KRAS^{G12D}$, $KRAS^{G12A}$ or $KARS^{G12V}$ template mixed with $KRAS^{wt}$ template could be detected without any interference by the $KRAS^{wt}$ template.

SARS-CoV-2 Spike protein structures

5'-ACTTGTGGTAGTTGGAGCTG_mA-3' → Proofreading DNA Polymerase → Amplified
$KRAS^{G12D(35A)}$ A

5'-ACTTGTGGTAGTTGGAGCTG_mA-3' → Proofreading DNA Polymerase → 3'-exonuclease activity
$KRAS^{wt(35G)}$ G

5'-ACTTGTGGTAGTTGGAGCTG_m -3' STOP! → Not Amplified
$KRAS^{wt(35G)}$ G

Mismatched 3'-nucleotide is removed by the 3'-exonuclease activity and the next 3'-G_m inhibits the polymerase activity of the proofreading polymerase.

2'-OMeRNA

Tx: KRAS(35x)
5'-acttgtggtagttggagctg(g/a/c/t)tggcgtaggcaagagtgccttgacgatacagctaattcagaatcattttgtggacgaatatgatccaacaataga-3'
Reverse Primer; 5'-tctattgttggatcatattcgt-3'
Forward Primers;
POM2c; 5'-acttgtggtagttggagct(G)c-3'
POM2a; 5'-acttgtggtagttggagct(G^m)a-3'
POM2g; 5'-acttgtggtagttggagct(G^m)g-3'
POM2t; 5'-acttgtggtagttggagct(G ^{n})t-3'
PSM2c; 5'-acttgtggtagttggagct(G)^c-3'
PSM2a; 5'-acttgtggtagttggagct(G^m)^a-3'
PSM2g; 5'-acttgtggtagttggagct(G^m)^g-3'
PSM2t; 5'-acttgtggtagttggagct(G ^{n})^t-3'

Mutations in Spike Protein of SARS-CoV-2 Variants

wt	440N:**1320 T**	452L:1355T	484E:1450G	496G:1486G	501N:1501A
δ	440N:1320 T	L452R:1355T>G	484E:1450G	496G:1486G	501N:1501A
o-BA1	440N:1320 T	452L:1355T	E484A:1451A>C	G496S:1486G>A	N501Y:1501A>T
o-BA2	N440K:1320 T>G	452L:1355T	E484A:1451A>C	496G:1486G	N501Y:1501A>T
oBA2.12.1	N440K:1320 T>G	L452Q:1355T>A	E484A:1451A>C	496G:1486G	N501Y:1501A>T
oBA4	N440K:1320 T>G	L452R:1355T>G	E484A:1451A>C	496G:1486G	N501Y:1501A>T
oBA5	N440K:1320 T>G	L452R:1355T>G	E484A:1451A>C	496G:1486G	N501Y:1501A>T

Detection of SARS-CoV-2 Variants

δ — F1355 δ : 5'- ta aggttggtgg taattataat tacCg-3' (δ)
R1486w/BA2: 3'-cCaaaggttggggtgatttaccacaac-5' (wt, δ, BA2)

o-BA.1 — F1355w/o: 5'-ta aggttggtgg taattataat tacCt-3' (wt, BA1, BA2)
R1486BA1: 3'-tCaaaggttggggtgaataccacaac-5' (BA1)

o-BA.2 — F1355w/o: 5'-ta aggttggtgg taattataat tacCt-3' (wt, BA1, BA2)
R1486w/BA2: 3'-cCaaaggttggggtgaataccacaac-5' (wt, δ, BA2)

Table 2. Mutations in Surface Glycoprotein of SARS-CoV-2 Variants

	1355	1486	1451
Wuhan-Hu-1	Ct-3'	3'-cC	5'-gaa
δ	Cg-3'	3'-cC	5'-gaa
o-BA.1	Ct-3'	3'-tC	5'-gaa
o-BA.2	Ct-3'	3'-cC	5'-gca

Detection of SARS-CoV-2 δ variant

(F1355 δ/R1486w/BA2) δ   (F1355 δ/R1486w/BA2) o BA.1   (F1355 δ/R1486w/BA2) o BA.2

Detection of SARS-CoV-2 o BA.1 variant

(F1355 δ/R1486w/BA2) o BA.1   (F1355 δ/R1486w/BA2) δ   (F1355 δ/R1486w/BA2) o BA.2

Detection of SARS-CoV-2 o BA.2 variant

(F1355 δ/R1486w/BA2) o BA.2   (F1355 δ/R1486w/BA2) δ   (F1355 δ/R1486w/BA2) o BA.1

Table 3. Detection of SARS-CoV-2 Variants

Primers	SARS-CoV-2 δ (ΔCt)	SARS-CoV-2 o BA.1(ΔCt)	SARS-CoV-2 o BA.2(ΔCt)
F1355 δ R1486w/BA2	20.57 ± 0.07	NA	NA
F1355w/o R1486BA1	NA	25.32 ± 0.35	NA
F1355w/o R1486w/BA2	NA	NA	26.48 ± 0.19

POM2g-Tg: $KRAS^{wt(35G)}$ POM2g-Ta: $KRAS^{G12D(35A)}$ POM2a-Ta: $KRAS^{G12D(35A)}$

POM2t-Tc: $KRAS^{G12A(35C)}$ POM2t-Tt: $KRAS^{G12V(35T)}$

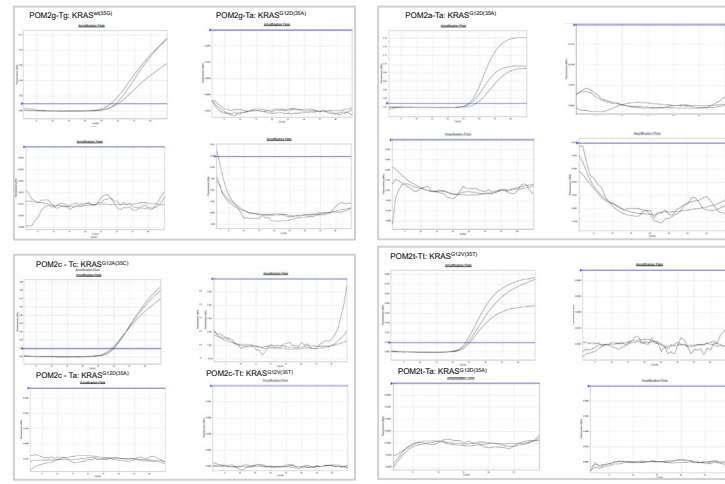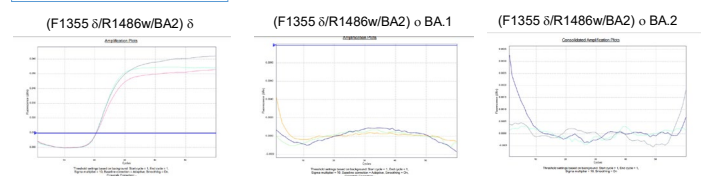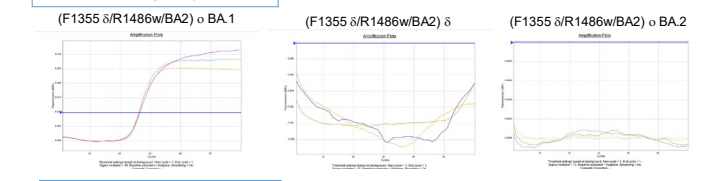POM2t-Ta: $KRAS^{G12D(35A)}$ POM2c-Tt: $KRAS^{G12V(35T)}$ POM2t-Ta: $KRAS^{G12D(35A)}$

Table 1. qPCR by TaKaRa Ex Taq Polymerase and 2'-OMeRNA Modified Primers

Primers	Ct/Tg (wt)	Ct/Ta (G12D)	Ct/Tc(G12A)	Ct/Tt(G12V)	ΔCt
POM2t	NA	NA	NA	24.77 ± 0.81	∞
POM2c	NA	NA	25.95 ± 0.20	NA	∞
POM2a	NA	22.67 ± 0.92	NA	NA	∞
POM2g	25.41 ± 0.84	NA(G-T)	NA	NA	∞
PSM2t	NA/	NA	NA	24.69 ± 0.15	∞
PSM2c	NA	NA	25.31 ± 0.30	NA	∞
PSM2a	NA	23.63 ± 0.19	NA	NA	∞
PSM2g	24.02 ± 0.85	NA(G-T)	NA	NA	∞

Table 2. Detection of 0.1%~0.01% Mutant $KRAS^{G12D}$, $KRAS^{G12A}$ and $KRAS^{G12V}$ under the background of $KRAS^{wt}$

[Tg] (μM)	[Ta] (G12D)(μM)	Ct (Tg)	Ct (Ta)	ΔCt
8.3 x 10⁻⁹	8.3 x 10⁻¹²	30.17 ±0.31	39.78 ±0.29	9.61 ±0.41
8.3 x 10⁻⁹	8.3 x 10⁻¹³	30.17 ±0.31	42.12 ±0.04	11.95 ±0.36

[Tg] (μM)	[Tc](G12A) (μM)	Ct (Tg)	Ct (Tc)	ΔCt
8.3 x 10⁻⁹	8.3 x 10⁻¹²	30.17 ±0.31	37.52 ±0.37	7.35 ±0.44
8.3 x 10⁻⁹	8.3 x 10⁻¹³	30.17 ±0.31	41.96 ±0.64	11.87 ±0.57

[Tg] (μM)	[Tt] (G12V)(μM)	Ct (Tg)	Ct (Tt)	ΔCt
8.3 x 10⁻⁹	8.3 x 10⁻¹²	30.17 ±0.31	37.68 ±0.13	7.51 ±0.41
8.3 x 10⁻⁹	8.3 x 10⁻¹³	30.17 ±0.31	41.17 ±0.71	11.00 ±0.78

Conclusion
We achieved highly specific and quantitative PCR analysis discriminating single base mutation between $KRAS^{wt}$, $KRAS^{G12D}$, $KRAS^{G12A}$ and $KRAS^{G12V}$ genes using chemically modified primers and proofreading polymerases. We also demonstrated highly sensitive and rapid detection of SARS-CoV-2 δ, o-BA1 and o-BA2 variants by the PCR method. The forward primer was designed to discriminate the mutation at 1355 position of spike glycoprotein and the reverse primer was designed to discriminate the mutation at 1486 position. We could show that PCR using these sets of primers clearly discriminated these three variants of SARS-CoV-2 with high sensitivity and accuracy.