# Quantitative PCR Completely Discriminating Point Mutation of *KRAS*

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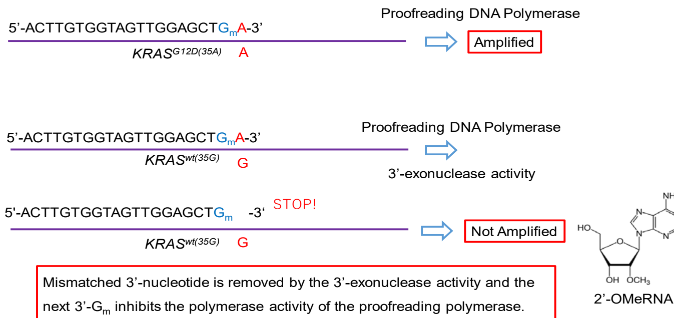
Abstract.

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.

## Amplification of $KRAS^{wt}$ and $KRAS^{G12D}$ by $KRAS^{wt}$ Primer (POM2g)




Tx: KRAS(35x)
Acttgtggtagttggagctg(g/a/c/t)tggcgtaggcaagagtgccttgacgatacagctaattcagaatcattttgtggacgaa tatgatccaacaataga-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 $^m$)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 $^m$)^t-3'

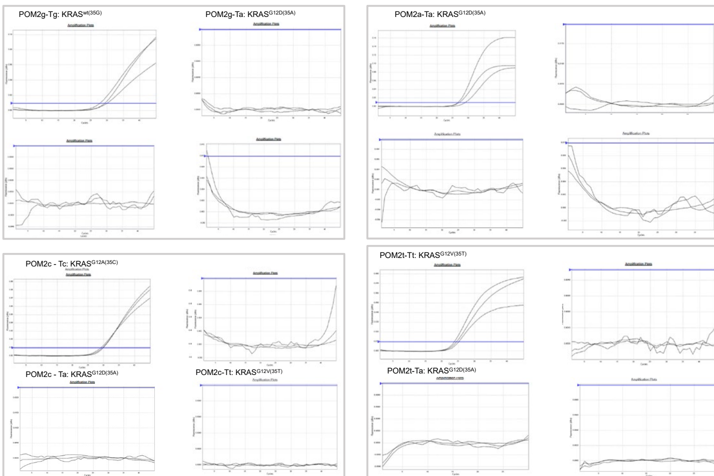
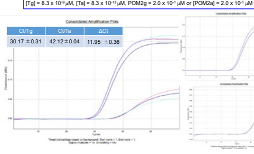



Table 1. qPCR by TaKaRa Ex Taq Polymerase and 2'-OMeRNA Modified Primers

| 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 | ∞ |

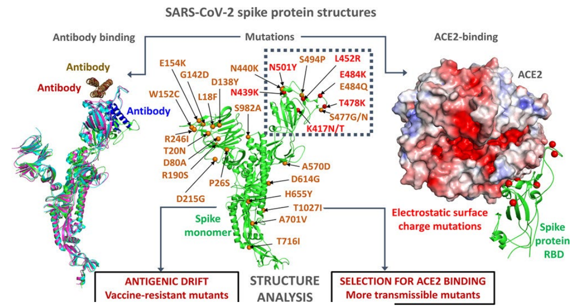
Detection of 0.01% $KRAS^{G12D}$(Ta) under the background of $KRAS^{wt}$(Tg): Tg/Ta (1/0.0001)
[Tg] = 8.3 x $10^{-9}$ μM, [Ta] = 8.3 x $10^{-13}$ μM, POM2g = 2.0 x $10^{-1}$ μM or [POM2a] = 2.0 x $10^{-1}$ μM



| Ct/Tg | Ct/Ta | ΔCt |
|---|---|---|
| 30.17 ± 0.31 | 42.12 ± 0.04 | 11.95 ± 0.36 |

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 |




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'-cCaaaggttgggtgattaccacaac-5' (wt, δ, BA2)

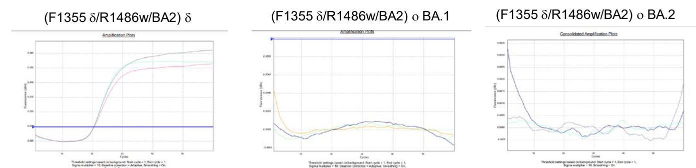
o-BA.1
F1355w/o: 5'-ta aggttggtgg taattataat tacCt-3' (wt, BA1, BA2)
R1486BA1: 3'-tCaaaggttgggtgaataccacaac-5' (BA1)

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

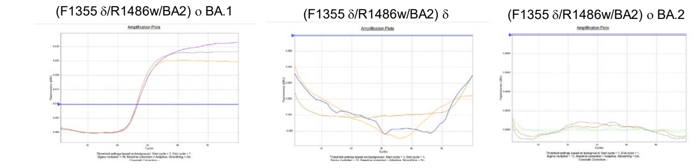
Table 2. Mutations in Surface Glycoprotein of SARS-CoV-2 Variants

| | 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




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




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

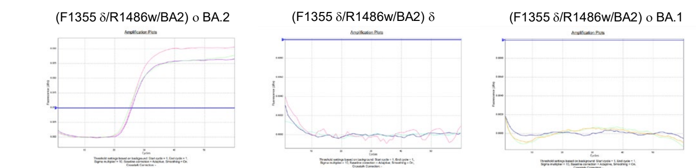



Table 3. Detection of SARS-CoV-2 Variants

| 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 |

## 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.

COI: We have no conflict of interest to disclose for this presentation.