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# Use of the Ct difference between the Nucleocapside (N) and the Spike (S) or RNA-dependent RNA polymerase (RdRP) genes as a preliminary screening for SARS-CoV-2 variants with the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay

## Searching the N in Variants

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### ABSTRACT

**Purpose:** With the rise of the different Variants of Concern (VOC) and Variants of Interest (VOI) in order to control the SARS-CoV-2 pandemic, strategies for accurately tracking these different variants have been developed. While most of these strategies rely heavily on specific PCRs targeting the characteristic mutations of some lineages, several approaches using the alterations at the cycle threshold (Ct) of different commercial PCR diagnostic tests have been described.

The objective of this study is to analyse the use of the Ct difference at the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay (Seegene, Korea) between the Nucleocapside (N) and the Spike (S) or RNA-dependent RNA polymerase (RdRP) genes as a preliminary screening for variant tracking.

**Methods:** The samples analysed with the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay from 1<sup>st</sup> of March 2021 to 26<sup>th</sup> of December 2021 were selected. The Ct values for N, S, RdRP were collected, and the differences between N and S ( $\Delta S$ ) and N and RdRP ( $\Delta RdRP$ ) were calculated. Using  $\Delta S$  and  $\Delta RdRP$  a diagnostic test was designed and these results were compared to the routine Variant assessment.

**Results:** The mean  $\Delta S$  and  $\Delta RdRP$  were characteristic for Alpha and Delta. This difference was statistically significant. For Every analysed Variant the diagnostic test achieved a higher than 90% sensitivity with a noteworthy performance with the Omicron variant (97% sensitivity and 90% specificity).

**Conclusions:** The analysis of the Ct alterations at the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay may be a suitable method for an early approach to SARS-CoV-2 variant assessment.

### 1. Introduction

The pandemic caused by SARS-CoV-2 has severely impacted all human activity since early 2020. For the most part of 2020 Clinical Microbiology laboratories have been struggling trying to find the best solutions to maximize the diagnostic capacities following WHO recommendations. Moreover 2021 supposed a new challenge with the appearance of the Variants of Concern (VOC) and the Variants of Interest (VOI). In Europe since it's firsts descriptions at the end of 2020 (Virological, 2020) the Alpha variant acquired a noticeably predominant

situation amongst the other variants such as Beta or Gamma representing in many reports up to 90% of the cases (Funk et al., 2021; Davies et al., 2021). During the second half of 2021 the Alpha variant is being progressively replaced by the Delta variant in many European countries (EU-EEA, 2021). For Alpha several descriptions of PCR primer compromises have being described, most noticeably the S gene dropout at the Thermofisher assay (Bal et al., 2021) (Kidd et al., 2021), which has also been described for the Omicron variant (SARS-CoV-2, 2021), and the N gene late amplification at the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay (Seegene, Korea) assay

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(Sánchez-Calvo et al., 2021; Wollschläger et al., 2021). This has not been the case for Delta or any other VOC or VOI.

The objective of this study is to analyse the use of the cycle threshold (Ct) difference at the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay (Seegene, Korea) between the Nucleocapsid (N) and the Spike (S) or RNA-dependent RNA polymerase (RdRP) genes as a preliminary screening for variant tracking.

## 2. Materials and Methods

### 2.1. Sample selection

We selected every positive sample analysed with the Allplex™ SARS-CoV-2/FluA/FluB/RSV Assay (Seegene, Korea) from 1<sup>st</sup> of March 2021 to 26<sup>th</sup> of December 2021 retrieving the Ct values for the N, RdRP and S genes.

From the 1<sup>st</sup> of March to the 3<sup>rd</sup> of May lineage-specific analysis was conducted with the Allplex™ SARS-CoV-2 Variants I Assay (Seegene, Korea) targeting H69/V70-, E484 K and N501Y mutations. Samples presenting H69/V70- and N501Y mutations were assigned to Alpha. Those specimens with the E484 K and N501Y mutations were further examined for K417 N and V1176 F with the VirSNIPs K417 N and V1176 F (TIBMOlBiol, Germany). The samples with compatible melting temperatures to the K417 T and V1176 F were assigned to Gamma and those with the K417 N and V1176 T to Beta. In this phase, triple negatives by the Variants I Assay were assigned as no clinically relevant variants. However, from the 3<sup>rd</sup> of May to the 15<sup>th</sup> of July two additional mutations were searched to these samples with VirSNIP assays L452R and P681R. Those samples positive to both assays were assigned as Delta and those assays with only L452R were assigned as Epsilon. Lastly from the 15<sup>th</sup> of July 2021 all samples were studied with both Allplex™ SARS-CoV-2 Variants I and Allplex™ SARS-CoV-2 Variants II (targeting W152C, K417 N, K417 T and L452R) assays simultaneously and interpreted as follows: Alpha (H69/V70- and N501Y), Beta (K417 N, E484 K and N501Y), Gamma (K417 T, E484 K and N501Y), Delta (L452R), Epsilon (W152C and L452R), Eta (H69/V70- and N501Y), Mu (E484 K and N501Y) and Omicron (H69/V70-, K417 N and N501Y).

Samples with other mutation profiles or with a suspicion of a clinically relevant variant were sequenced.

### 2.2. Statistical analysis

#### 2.2.1. N-dropout

The samples testing negative for the N gene while being positive for both other genes (S and RdRP) were analysed. Mean Ct values ( $\bar{x}$ ) and standard deviations ( $\sigma$ ) of the different Variants were calculated. Statistical significance was considered when the two-tailed p value at the T-test was less than 0.05.

#### 2.2.2. N-late amplification

The mean difference between Ct values of N and S ( $\Delta S$ ) or N and RdRP ( $\Delta RdRP$ ) were calculated for every VOC or Variants of Interest (VOI) individually and for non VOC-VOI variants collectively. Statistical significance was considered when the two-tailed p value at the T-test was less than 0.05.

#### 2.2.3. Variant SeNSoR

For the most prevalent VOCs in our media (Alpha, Gamma and Delta) a diagnostic test with  $\Delta S$  and  $\Delta RdRP$  was developed and named Variant SeNSoR. If  $\Delta S$  or  $\Delta RdRP$  was inside 95% confidence interval ( $\bar{x}_{\Delta S} \pm 1,96\sigma_{\Delta S}$  or  $\bar{x}_{\Delta RdRP} \pm 1,96\sigma_{\Delta RdRP}$ ) the test was considered positive for that variant. Susceptibility and Specificity values were calculated for these tests.

### 2.3. Sequencing and bioinformatic analysis

For Whole Genome Sequencing, a targeted approach was employed using the Ion AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific) (Alessandrini et al., 2020). The libraries were prepared following the manufacturer's instructions and loaded on a 540 chip and run on the Ion GeneStudio™ S5 System (Thermo Fisher Scientific). Genome assembly was obtained with the IRMAreport plugin (Shepard et al., 2016) and the consistency of the nucleotide calls was checked with Integrative Genomics Viewer (IGV) (Nature Biotechnology, 2021).

The N gene of the studied variants was aligned with the ClustalO-mega pipeline at the Ugene (Unipro) software suite (Unipro UGENE, 2021). The mutations found on each variant were checked with other sequences of the same variant at the GISAID database (Khare et al., 2021).

## 3. Results

For this study, 10538 samples were selected. After the corresponding classification with specific PCRs, 3046 (28.9%) were classified as Alpha, 11 (0.1%) as Beta, 74 (0.69%) as Gamma, 6703 (63.6%) as Delta, 3 (0.03%) as Eta, 5 (0.04%) as Epsilon, 9 (0.08%) as Mu, 502 (4.76%) as Omicron and 185 (1.74%) as other non-VOC-VOI variants.

### 3.1. N-Dropout

From the total of 10538 samples, 695 (6.6%) tested negative for the N gene while being positive for both other genes (S and RdRP). The N-Dropout was most frequently found with the Alpha variant and up to 470 (15.43%) of the samples classified as Alpha presented the N-Dropout, while only 225 (3.35%) of the Delta variant failed to amplify the N gene.

Analysing the Ct values for the S and RdRP genes in both Alpha and Delta variants we found that the Ct values were much higher in the group with the N-Dropout (Table 1). This difference was statistically significant ( $p < 0.001$ ).

### 3.2. N-Late Amplification

In this subanalysis we included 9836 samples.  $\bar{x}_{\Delta S}$  and  $\bar{x}_{\Delta RdRP}$  were 4.66 and 4.5 respectively when analysing the whole sample, with a broad interval due to having a disperse population, with relatively high standard deviations. The biggest difference in both  $\Delta S$  (8.64) and  $\Delta RdRP$  (8.11) was observed in the Alpha variant, being this difference statistically significant ( $p < 0.001$ ). Even if the number of processed Eta variants is not great, the observations are consistent, and a décalage of the N gene is also noteworthy. For the Delta Variant  $\Delta S$  is 3.65 and  $\Delta RdRP$  3.6 being this difference also statistically significant ( $p < 0.001$ ).

For the Gamma variant the N gene has lower Ct values than both the S or RdRP genes and this difference is also statistically significant. For Omicron variant there are no significant differences between the Ct values of the different genes. (Table 2)

### 3.3. Variant SeNSoR

For every analysed Variant the Variant SeNSoR test achieved a

**Table 1**  
Mean Ct Values ( $\bar{x}$ ) and standard deviation ( $\sigma$ ) for S and RdRP genes in both N-Dropout Alpha and Delta and non N-Dropout Alpha and Delta.

		n	$\bar{x}_S$	$\sigma_S$	$\bar{x}_{RdRP}$	$\sigma_{RdRP}$
Alpha	Dropout	470	31,52	2,66	32,76	2,63
	No Dropout	2576	19,81	4,57	20,34	4,74
Delta	Dropout	225	31,28	2,15	32,1	2,16
	No Dropout	6478	19,99	4,89	19,97	5,2

**Table 2**

Mean difference between Ct Values of N and S( $\Delta S$ ) or RdRP ( $\Delta RdrP$ ) in all the detected VOC or VOI.

Variant	N	$\bar{x}_{\Delta S}$	$\sigma_{\Delta S}$	$\bar{x}_{\Delta RdrP}$	$\sigma_{\Delta RdrP}$
Alpha	3046	8,65	1,57	8,11	1,49
Beta	11	-1,61	1,43	-0,84	1,61
Gamma	74	-0,88	0,99	-1,21	0,92
Delta	6703	3,65	1,25	3,67	1,43
Eta	3	7,17	0,93	6,53	0,93
Epsilon	5	2,89	2,77	2,27	2,55
Mu	9	-0,89	0,72	0,22	0,56
Omicron	502	0,01	0,98	-0,33	0,80
Non VOC	185	-0,16	1,76	-0,97	1,71
Total	10538	6,10	3,80	5,69	3,50

higher than 90% sensitivity, however the specificity varies greatly between variants (Table 3). While for Alpha SeNSoR performs better with  $\bar{x}_{\Delta S}$  than with  $\bar{x}_{\Delta RdrP}$  with both better sensitivity and specificity, for Delta is the other way round and  $\bar{x}_{\Delta RdrP}$  is more accurate to classify this variant.

For omicron SeNSoR is an accurate tool achieving in both with  $\bar{x}_{\Delta S}$  and with  $\bar{x}_{\Delta RdrP}$  sensitivities above 97% while still retaining a 90% specificity in case of  $\bar{x}_{\Delta RdrP}$ .

### 3.4. Genomic analysis of the N gene

The analysed sequences of the Beta, Gamma, Epsilon and of a B.1 lineage variant were identical to the original Wuhan sequence at the studied region of the N gene. Alpha, Delta and Omicron, all three, have the 28271 deletion (position 2 on Fig. 1). In addition to this, Alpha also presents G28280C, A28281 T and T28282A (position 11 to 13 on Image 1). Meanwhile, Delta Variant harboured the mutation A28299 T (Corresponding to the position 30 on the Image 1) and Omicron C28311 T (position 42). Eta Variant presents the 28278-28280 deletion (position 9 to 11) and the C28308 G (position 39) mutation. Finally, Mu acquires the A28272 T (position 3) mutation.

## 4. Discussion

As stated by other authors previously the appearance of either Dropouts or late amplifications at validated commercial assays should serve as a hint on the search for potential new variants (Kidd et al., 2021; Sánchez-Calvo et al., 2021). Especially so when the affected regions such as the N gene are known to be more conserved than others (Wollschläger et al., 2021; Grifoni et al., 2020).

The results of this study agree with those of the previously described for the Alpha Variant, which described a N-Dropout or N-late amplification by the Allplex™ SARS-CoV-2/FluA/FluB/RSV assay. In addition to that, despite the small number of Eta Variants on this study, the results were cohesive and close to what was expected and suggested in previous studies. Nevertheless, the difference between Alpha and Eta was narrow and therefore this kind of study with a different local ecology may vary greatly and using only this system to detect variants

**Table 3**

Diagnostic yield of the proposed Variant SeNSoR test.

Variant	Variant SeNSoR test		
	[IC]	Sensitivity %	Specificity %
Alpha	$\bar{x}_{\Delta S}$ [5,47-11,82]	92,68	77,71
	$\bar{x}_{\Delta RdrP}$ [5,11-11,11]	91,96	74,6
Delta	$\bar{x}_{\Delta S}$ [-0,91-8,22]	95,04	54,56
	$\bar{x}_{\Delta RdrP}$ [-0,39-7,74]	95,19	59,09
Gamma	$\bar{x}_{\Delta S}$ [-2,81-1,06]	94,59	88,44
	$\bar{x}_{\Delta RdrP}$ [-3,01-0,58]	95,94	91,8
Omicron	$\bar{x}_{\Delta S}$ [0,01-0,98]	97,01	75,78
	$\bar{x}_{\Delta RdrP}$ [-0,32-0,79]	97,81	90,53

may induce to a misclassification. However, this may also occur with other methods as the S gene Dropout at ThermoFisher, since both Alpha, Eta and Omicron share the well-known H69/V70 deletion (Pereira et al., 2021).

While N gene dropouts and late amplifications as diagnostic marker for Alpha variant have been as stated before already reported for the Allplex™ SARS-CoV-2/FluA/FluB/RSV assay, to the best of our knowledge, this is the first report of the same phenomenon being relevant for the Delta Variant. Moreover, being Delta and Omicron the most predominant variants at the end of 2021, the absence of N gene dropouts or late amplifications in Omicron variant may represent a useful hint comparable to the S gene dropout.

All in all, there is a quite strong correlation between the conclusions that could be carried away from the genome analysis and these findings. The greater the genetic diversion from the original SARS-CoV-2 sequence in this region the bigger  $\Delta S$  and  $\Delta RdrP$  values, particularly when does mutations are in the 28280-28300 range.

Since the mean Cts of the samples with N gene dropout, in both Alpha and Delta, were well above 30 this is most probably explained not due to a relevant deletion but due to the late appearance of the N curve which does not have the time to appear at all.

As detecting new Variants is becoming an additional task of increasing importance the availability of using simple methods such searching for dropouts or late amplifications at various genes is proving to be a very helpful tool, even though in our opinion it should rather not substitute the other molecular variant tracking assays but rather to complement them, for example as initial screening at epidemiological peaks.

### Author statement

Mikel Urrutikoetxea-Gutierrez: Conceptualization, Writing, Original draft preparation M<sup>a</sup> Carmen Nieto Toboso: Writing- Reviewing Estibaliz Ugalde Zarraga: Writing- Reviewing Mikele Macho Aizpurua: Writing- Reviewing Jose Luis Díaz de Tuesta del Arco: Reviewing Supervision

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### Declaration of Competing Interest

The authors declare no conflicts of interest or competing interests.

### Code availability

Not applicable.

### Authors' contributions

MUG participated in the conceptualization, data curation and analyzation as well as in the writing of the original draft. All other authors contributed at the writing of the Manuscript. All authors work in the Clinical Microbiology Laboratory led by Jose Luis Diaz de Tuesta del Arco.

### Ethics approval

Not applicable.

### Consent to participate

Not applicable.

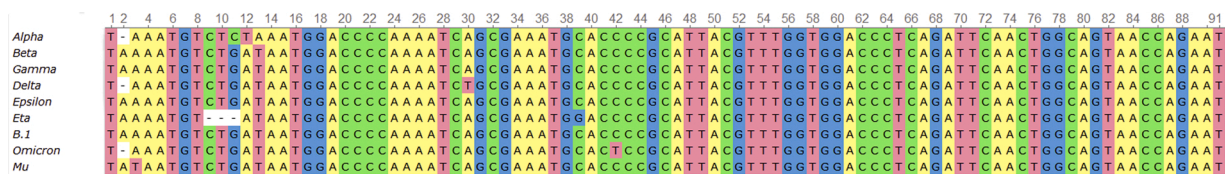


Fig. 1. Alignment of the 28270-28360 region from Alpha, Beta, Gamma, Delta, Epsilon, Eta, Mu and Omicron.

Consent for publication

Not applicable.

Data Availability

Data will be made available on request.

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