

## Application Note

# A Pyrosequencing® workflow for the simple and rapid detection of SARS-CoV-2 variants that can be quickly adapted to new mutations

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## Introduction

To keep pace with SARS-CoV-2 viral mutation, the prevalence of variants of concern and variants of interest in the population must be rapidly detected and reported. Variants are typically detected using two methods: next-generation sequencing (NGS) of the viral genome in whole or in part and RT-qPCR assays. Genotyping SARS-CoV-2 from patient samples using NGS is the most thorough method and is able to identify new lineages, but incurs high costs and requires technical expertise. Further, the long turnaround time required for NGS makes this method an unrealistic option for genotyping all samples in a population to support real-time surveillance. RT-qPCR assays have become a useful proxy for NGS and can differentiate known variants by screening a combination of defined loci [1]. This enables front-line testing labs to participate in surveillance, reducing turnaround times and eliminating the need for additional equipment and resources.

As new viral lineages and mutations are identified with NGS, new RT-qPCR protocols for screening the relevant loci must be rapidly developed and implemented, and a delay could result in unmonitored growth of these

lineages in the population. However, labs actively performing SARS-CoV-2 variant screening have limited time and resources to develop new protocols. In addition, RT-qPCR assay design is time-consuming and challenging, typically requiring optimization of spurious amplification and low-sensitivity results due to primer and probe design.

In contrast, Pyrosequencing represents a complementary screening approach that combines simple assay design with the quick turnaround time and low capital investment and running costs of RT-qPCR. Pyrosequencing assays can be created quickly: regions containing loci of interest are amplified using RT-PCR, then an additional primer dictates which part of the amplicon is sequenced to genotype relevant mutation(s). Unlike genotyping by RT-qPCR, primers can be placed in conserved sites, enabling an easy design process. As a result, following the identification of a positive virus sample from a RT-qPCR screen, Pyrosequencing could be used to genotype the sample on the same day, thereby supporting surveillance activities. We sought to test this concept by sequencing 20 virus-positive patient samples, representing different known SARS-CoV-2 ▶

lineages, using a standard Pyrosequencing protocol on the PyroMark® Q48 Autoprep Instrument. Here, we show the ease of designing successful, rapid Pyrosequencing assays to screen for SARS-CoV-2 variants. We have additionally begun development of even faster Pyrosequencing protocols.

## Materials and methods

20 patient samples that had previously been confirmed as containing the “wildtype”, B.1.1.7 or B.1.351 SARS-CoV-2 strain were collected from various European labs. When screened for SARS-CoV-2 using the QIAprep& Viral RNA UM Kit with the SARS-CoV-2 N1+N2 Assay Kit, these samples exhibited Ct values ranging from 20 to 34. We note that as this was a proof of concept study, we had a limited sample size that lacked examples of the P.1, B.1.617.2 and B.1.617.1 SARS-CoV-2 lineages.

As shown in Table 1, specific detection of the B.1.1.7, B.1.351, P.1, B.1.617.2 or B.1.617.1 strains was performed

by identifying combinations of the mutations N501Y, E484K, E484Q, K417T, K417N, P681H and P681R. Pyrosequencing primers were designed using PyroMark Assay Design Software 2.0 and manufactured by Eurofins. RT-PCR was performed using the PyroMark OneStep RT-PCR Kit and QIAprep& Viral RNA UM Kit. Pyrosequencing was performed on a PyroMark Q48 AutoPrep Instrument and data was analyzed using the PyroMark Q48 AutoPrep Instrument Software Version 2.4.2.

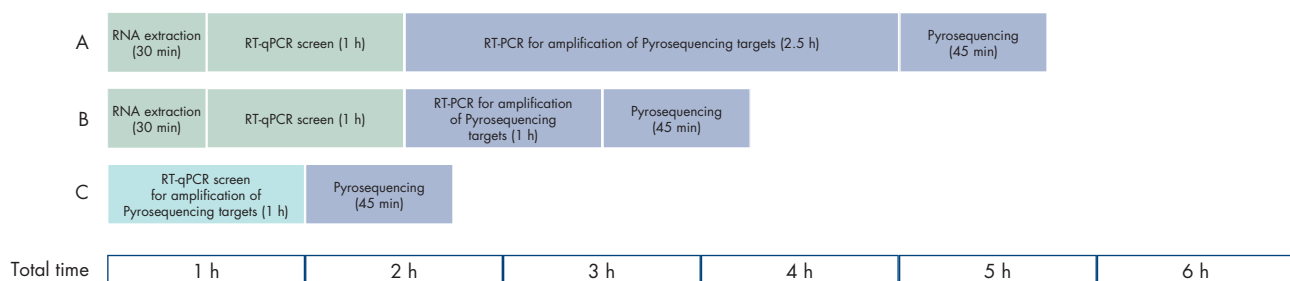
**Table 1. Mutations associated with various SARS-CoV-2 lineages were assessed and used as markers as reported by outbreak.info [2]**

	B.1.1.7	B.1.351	P.1	B.1.617.2	B.1.617.1
N501Y	x	x	x	–	–
E484K	–	x	x	–	–
E484Q	–	–	–	–	x
K417T	–	–	x	–	–
K417N	–	x	–	–	–
P681H	x	–	–	–	–
P681R	–	–	–	x	x

## Results and discussion

The *de novo* design of Pyrosequencing primers for the seven mutations shown in Table 1 was straightforward

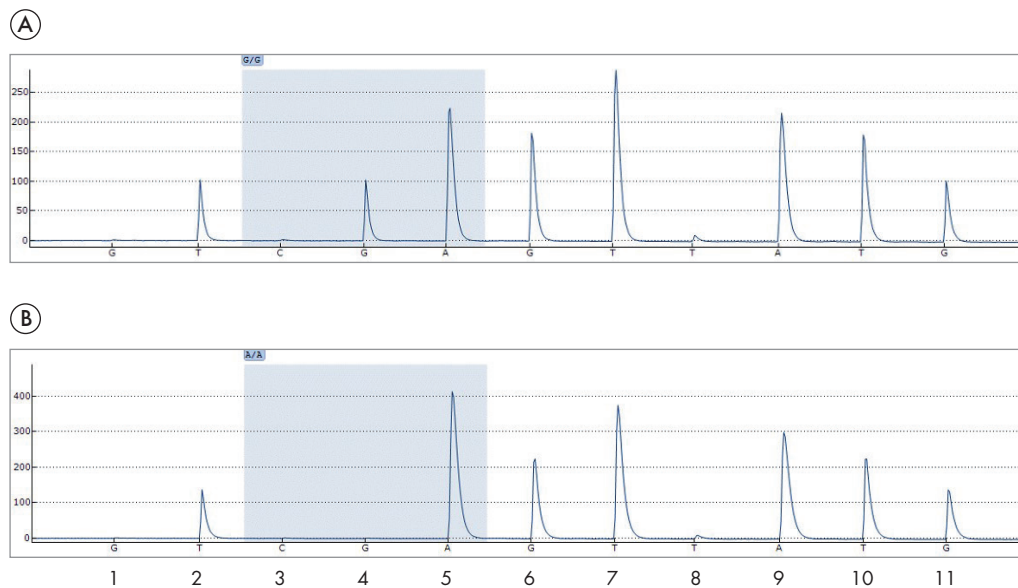
and could be completed in one hour. Three potential variant identification workflows incorporating these



**Figure 1. Estimated time required for workflows combining RT-qPCR screening (green) and Pyrosequencing (blue) of samples for variant detection.** A Workflow using a traditional Pyrosequencing RT-PCR with the PyroMark OneStep RT-PCR Kit. The estimated total time of this workflow is 4 h 45 min. This workflow was implemented for sample analysis in this study. B Workflow where the Pyrosequencing RT-PCR step is substituted with QIAGEN's QIAprep& Viral RNA UM Kit to reduce RT-PCR time. The estimated total time of this workflow is 3 h 15 min. C Workflow where both the Pyrosequencing RT-PCR and RT-qPCR screening steps are replaced by the QIAprep& Viral RNA UM Kit. In this case, the mastermix would also contain primers required for generating the Pyrosequencing amplicons. This would reduce the time needed for the RT-qPCR screening step, as well as the time required to perform a Pyrosequencing reaction. The estimated total time of this workflow is 1 h 45 min.

Pyrosequencing primers, including initial sample screening for the presence of SARS-CoV-2, are shown in Figure 1. The workflow shown in Figure 1A was implemented in this study. As shown in Figure 2, the PyroMark Q48 AutoPrep Instrument generates Pyrograms

for the amplicons produced during Pyrosequencing. These Pyrograms are first assessed to check the quality of the runs, and then analyzed further to determine the genotype of the viral sample. The results of the 20 samples used in this study are summarized in Table 2.



**Figure 2. Pyrosequencing results are illustrated as Pyrograms.** **A** The direct detection of the sequence for sample 7 around the E484 locus of the SARS-CoV-2 S gene is shown. Highlighted in grey is codon 484, GAA, representing the wildtype codon. Peaks outside the highlighted region represent reference signals for automatic quality assessment. **B** The Pyrogram for sample 1 at the same locus shows an absence of the G peak at dispensation 4 and an increased signal for the A peak at dispensation 5, representing the sequence AAA for codon 484. Thus, this sample harbors the E484K mutation. The absence of a G peak at dispensation 5 and occurrence of a C peak at dispensation 3 would alternatively indicate a sequence of CAA for codon 484, and thus an E484Q mutation.

In all cases, the Pyrosequencing reactions were successful upon the first run. The Pyrosequencing results for all 20 samples correlated well with the expected genotypes for known SARS-CoV-2 lineages, even when the RT-qPCR Ct values were high (e.g. >30). In addition, we found that mutations in the same loci could be analyzed on one amplicon with separate sequencing primers (e.g. N501Y and E484K/Q) or even with one sequencing primer (e.g. K417N and K417T or E484K and E484Q). Together, these results indicate that this method can successfully be used to quickly design new screening assays for genomic loci of interest.

Additional optimization of this Pyrosequencing protocol can be performed to further reduce the total turnaround time from screening to variant detection. For example, the QIAprep&amp; Viral RNA UM Kit can be implemented to directly perform RT-PCR on primary samples, thereby omitting the RNA extraction step (Figure 1B). In addition, Pyrosequencing primers can be included in the initial RT-qPCR screening reactions so that positive samples can be immediately Pyrosequenced (Figure 1C).

**Table 2. Pyrosequencing results for the 20 samples analyzed in this study. The Ct levels from the initial screening RT-qPCR are shown, as well as the mutations detected in the samples and the corresponding reference lineages (as indicated in Table 1). The correlation between the detected mutations and known lineages is also shown.**

Sample	Ct	N501Y	E484K	E484Q	K417T	K417N	P681H	P681R	Reference result	Correlation
1	23.1	x	x	–	–	x	–	–	B.1.351	Yes
2	20.7	–	x	–	–	–	–	–	Unknown lineage with E484K mutation	Yes
3	20.6	x	x	–	–	x	–	–	B.1.351	Yes
4	21.3	x	x	–	–	x	–	–	B.1.351	Yes
5	19.6	–	–	–	–	–	–	–	Wildtype	Yes
6	34.0	x	x	–	–	x	–	–	B.1.351	Yes
7	30.7	x	–	–	–	–	x	–	B.1.1.7	Yes
8	27.0	x	–	–	–	–	x	–	B.1.1.7	Yes
9	25.1	–	–	–	–	–	–	–	Wildtype	Yes
10	28.1	–	–	–	–	–	–	–	Wildtype	Yes
11	28.7	–	–	–	–	–	–	–	Wildtype	Yes
12	27.7	x	–	–	–	–	x	–	B.1.1.7	Yes
13	26.5	x	–	–	–	–	x	–	B.1.1.7	Yes
14	25.1	x	–	–	–	–	x	–	B.1.1.7	Yes
15	32.1	–	–	–	–	–	–	–	Wildtype	Yes
16	27.8	–	–	–	–	–	–	–	Wildtype	Yes
17	30.3	x	–	–	–	–	x	–	B.1.1.7	Yes
18	22.2	x	–	–	–	–	x	–	B.1.1.7	Yes
19	25.1	x	–	–	–	–	x	–	B.1.1.7	Yes
20	32.7	–	–	–	–	–	–	–	Wildtype	Yes

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## Conclusion

Pyrosequencing can be quickly implemented for new loci of interest for SARS-CoV-2 variant detection due to its fast assay design process and high first-time success rate. Costs are similar to RT-qPCR, but unlike RT-qPCR the technique provides a sequence readout to directly interrogate multiple mutation sites in an amplicon. Using this approach, we were able to identify viral mutations and lineages that correlated well with previously determined results for the samples tested. Even samples with high Ct values (e.g. >30) for the SARS-CoV-2 screening RT-qPCR could easily be analyzed, indicating the high sensitivity of this method.

As Pyrosequencing has a short turnaround time, samples can be genotyped on the same day a positive result is confirmed. In addition, the time required for this workflow can be further reduced by implementing the QIAprep& Viral RNA UM Kit. Using this protocol, the RT-qPCR step for Pyrosequencing can be shortened to reduce the overall time by 1.5 h. In addition, the RT-qPCR screening step can be shortened and combined with Pyrosequencing to reduce the overall time by a further 1.5 h.

## Summary

Pyrosequencing with the PyroMark OneStep RT-PCR Kit on the PyroMark Q48 AutoPrep Instrument can help labs respond more quickly to the emergence of new SARS-CoV-2 viral mutations in a population by allowing them to rapidly begin surveilling new viral genomic loci of interest.

## References

1. Vogels, C. B. F. et al. (2021) PCR assay to enhance global surveillance for SARS-CoV-2 variants of concern. medRxiv <https://doi.org/10.1101/2021.01.28.21250486>
2. Mullen, J. L. et al. (2020) Outbreak.info. Scripps Research. <https://outbreak.info/>

## Ordering Information

Product	Contents	Cat. no.
PyroMark Q48 Autoprep Instrument	PyroMark Q48 Instrument, multistep pipet, software and documentation	9002471
PyroMark Q48 AutoPrep Starter Kit	PyroMark Q48 Magnetic Beads (300), PyroMark Q48 Advanced CpG Reagents (4 x 48), PyroMark Control Oligo, PyroMark Q48 Discs (50) and PyroMark Q48 Absorber Strips (100)	974230
PyroMark OneStep RT-PCR Kit (200)*	For 200 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, optimized QIAGEN OneStep RT-PCR Buffer, 10x CoralLoad Concentrate, 5x Q-Solution, dNTP Mix, and RNase-Free Water	978803
PyroMark Q48 Software License (1)	One additional license for PyroMark Q48 Software. Only valid together with PyroMark Q48 Autoprep.	9024325
SARS-CoV-2 N1+N2 Assay Kit (600)*	For 600 x 20 µl reactions: 1x 600 µl SARS-CoV-2 N1+N2 assay, 20x concentrate	222015
QIAprep& Viral RNA UM Kit (600)*	For 600 x 20 µl reactions: 1.2 ml Viral RNA UM Prep Buffer, 2x 1.5 ml Viral RNA Master Mix, 4x, 1.2 ml RNA IC Template + Assay, 0.6 ml Human Sampling IC Assay, 1 ml QN ROX, 2x 1.9 ml RNase-Free Water	221415

\*Additional kit sizes are available on [www.qiagen.com](http://www.qiagen.com)

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