Variant of Concern Screening with PACE-RT™

(**P**CR **A**llelic **C**ompetitive **E**xtension- **R**everse **T**ranscription)

This document contains:

PACE-RT™ is a reverse transcriptase version of the classic 'PACE' chemistry (**P**CR **A**llelic **C**ompetitive **E**xtension - **R**everse **T**ranscription) that may be used to detect SARS-CoV-2 variants [1]. An RNA sample is reverse transcribed to DNA and then probed using PACE-specific DNA probes. It is an end point reaction rather than real-time with the data analysed at the end of a PCR reaction. The method requires a thermal cycler and a plate scanner capable of reading FAM, HEX (or VIC) and ROX fluorescent dyes. While real-time PCR equipment may be used to perform the PCR and plate reading steps, it should be noted that the chemistry works as an end-point reaction with a room temperature sample and that results cannot be obtained in a real-time sense. Please consult the manual for the specific equipment to perform the reaction as an end-point protocol.

For all Sample Size Formats

Starting RNA Material

Sample quality plays a part in the accuracy of this assay. We have had good results with RNA extracted using the Kingfisher method, but poor results with samples harvested for bacterial studies that were subsequently used for viral RNA. In these instances, the results will appear as low and non-specific amplification. Try to limit freeze-thaw prior to genotyping.

An ideal starting concentration of RNA is 1.5-2.5ng μL^{-1} or a cT (cycle-threshold) value < 30 in RNase free water.

Samples from an RNA extraction will work considerably better than a total nucleic acid extraction. As any RNA measurement will include human as well as viral RNA, this starting amount may vary. If total nucleic acid is used, a considerably higher starting concentration is suggested as the viral RNA will only represent a small amount of the measured concentration. The Nanodrop, which is fast and easy to use, can also have a slight over-estimation of RNA concentration.

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For clinical samples, an RNA concentration may be difficult to obtain, a cT score from initial testing will suffice. We obtain good results with cT<30 after which the calling becomes more difficult and low or non-specific amplification occurs.

Create Primer Set

Primer sets can be updated as new variants occur [1], the complete pipeline along with links to example input data files is available from https://github.com/pr0kary0te/SARSmarkers.

Design oligos compatible with One Step PACE-RT™ chemistry. For each of the markers in the panel, two allele-specific forward primers and one common reverse primer were designed with a PACE-specific tail (Supplementary Table 1). Aside from this, the primers require no further modification. 3CR Bioscience offer a free-of-charge primer design and Primers can be ordered from any preferred oligo manufacturer (e.g. Merck UK, Eurofins etc).

Each SNP marker is probed by a primer triplet which require combining prior to use:

	Volume
Forward Primer 1 (100µM)	12µL
Forward Primer 2 (100µM)	12µL
Reverse Primer (100µM)	30µL

Table 1: Marker master mix. This combination is required for each marker so a panel of 10 targets will require 30 primers to be ordered and combined into 10 marker master mix as described here.

PACE-RT™ with Manual 96-well Capability

Create the RNA Sample Plate

Distribute samples into a 96 well plate. 1-2 negative controls (water) and 1-2 positive controls are recommended alongside the sample panel.

For manual testing, a recommended layout is to repeat the same sample across the row of the plate. In this example A1-A12 are multiple aliquots of sample 1, B1-B12 are multiple aliquots of sample 2, C1-C12 are multiple aliquots of sample 3 and so forth. A row of negative control (water) is recommended.

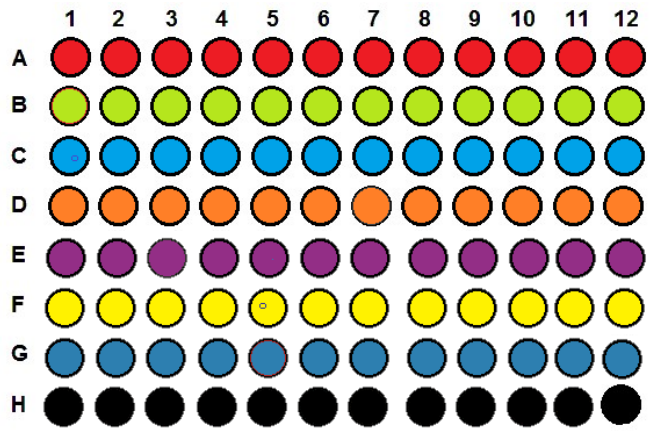

Figure 1: RNA distribution into sample plate. Each sample replicated 12 times. 5 µL RNA in water in each well.

This layout allows 8 samples to be screened with 12 markers, for different sample/marker combinations different layouts or multiple plates can be used but please adapt the volumes of master-mix solutions accordingly.

> Tip: To prevent contamination during plate filling with PACE-RT™ mix, the RNA may be dried down within the plate. Spin for 10 seconds then put the plate in an oven/incubator at 48°C for 40 minutes. Cool the plate to room temperature before applying the PACE-RT™ ready mix.
>
> This step is not essential for the 96-well protocol and may be omitted if required.

Apply PACE-RT™ Mix to the RNA Sample Plate

Create a master mix of PACE-RT™ mix and RT-enzyme sufficient for 12 markers. Make immediately prior to use and store on ice. Protect from light.

	Volume
PACE-RT™ mix	870 µL
Reverse Transcription-Enzyme	8.7 µL

Table 2: PACE-RT™ master mix for 12 markers. Scale up or down as required. PACE-RT™ may be ordered from 3CR with order number 103-0002

Now combine the PACE-RT™ master mix with other reagents.

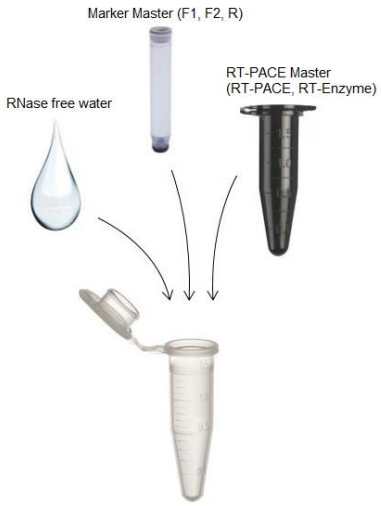

Figure 4: PACE-RT™ Ready mix. Mix carefully to avoid bubbles and use immediately.

	Volume	Perform this 12 times for each marker option.
PACE-RT™ master mix from Table 2	66 µL	
RNase-free Water**	-	
Marker master mix from Table 1	5.9 µL	

Table 3: PACE-RT™ Ready mix. Mix carefully and use immediately. Note that the marker master mix components may separate in storage so ensure it is mixed well before adding to Ready Mix.

** If the RNA was dried in the plate before use, change this to 64 µL and dispense 10 µL per well rather than 5 µL.

Apply 5 µL of the ready mix solution (Table 3) for each marker to the 96 well plate with aliquots of the same marker in columns. In this example A1-H1 is multiple aliquots of the ready mix with the marker master mix for marker 1, A2-H2 is multiple aliquots of the ready mix with marker master mix for marker 2, and so forth.

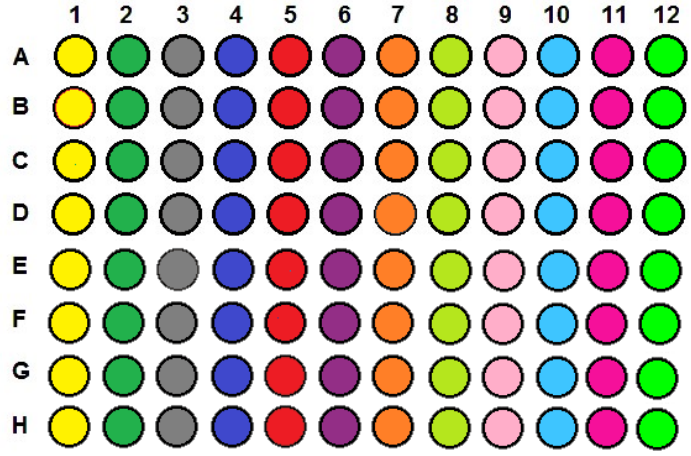

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Figure 4: Marker ready mix distribution into sample plate. Each marker replicated 12 times. 5 µL ready mix added to each well.

Seal carefully using an optically clear seal, laser seal if possible and centrifuge for 10 seconds before PCR. If you are preparing multiple plates before running, store in the fridge protected from light for up to 30 minutes.

End-point PCR Procedure

Transfer to thermal cycler, preheated where possible. If plates are not able to be laser sealed, consider hotlid options to reduce volume loss by evaporation. This is an end-point reaction, so do not scan the plate as it is being cycled.

Temperature	Time	Cycling	
50°C	10 minutes		
94°C	15 minutes		
94°C	20 seconds	10 cycles	(Dropping 0.8°C per cycle)
65-57°C	60 seconds		
94°C	20 seconds	35-40 cycles	
67°C	60 seconds		

Table 4: PCR parameters. This procedure was optimised using a water bath thermal cycler to reduce ramping time. Complete PCR steps prior to scanning.

When cycling is complete it is possible to scan the plate.

Remove the plate and allow to cool to room temperature (this should only take a few seconds). If laser sealed, spin the plate upside-down for 10 seconds to capture condensation (only perform this step with laser sealed plates!). With the plate right side-up, centrifuge for 10 seconds. If condensation persists, allow another 30 seconds of cooling and re-centrifuge.

Plate Scanning

PACE-RT™ (reverse transcription PACE) is an end-point reaction. While it can be processed using the thermal cycling and scanning capability of an RT-PCR (Real time PCR) machine, it is very **important to not use real-time scanning**. The results will be meaningless as the plate must be cooled to room temperature before scanning.

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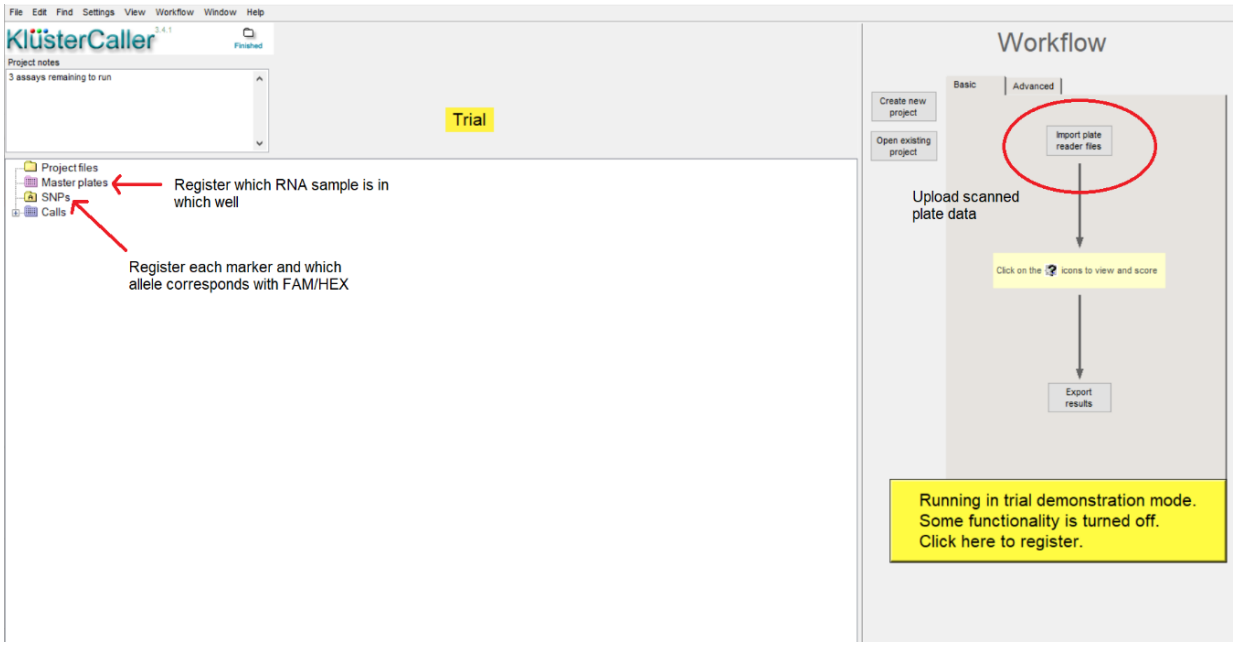
Use a scanner fitted with Fl 485/520, Fl 520/560 and Fl 570/610 optic modules. The method of scanning will vary greatly with manufacturer. The aim is to obtain readings for FAM, HEX (same parameters as VIC) and ROX dyes with ROX used for normalisation.

> It is possible to scan the plate, then return to the thermal cycler for additional cycles if the initial amplification was not sufficient. This is not usually required but may be useful if starting sample concentration is low. Allow the plate to cool before re-scanning. Be careful to save both files not just overwrite.

Data Analysis

Where qPCR is used for scanning, software already exists to create cluster plots and this may be the best choice for lab workflow.

Alternatively use Kraken (may already be owned by DHSC) or KlusterCaller. A free version of Klustercaller exists alongside instruction manual here: https://www.biosearchtech.com/support/tools/genotyping-software/klustercaller



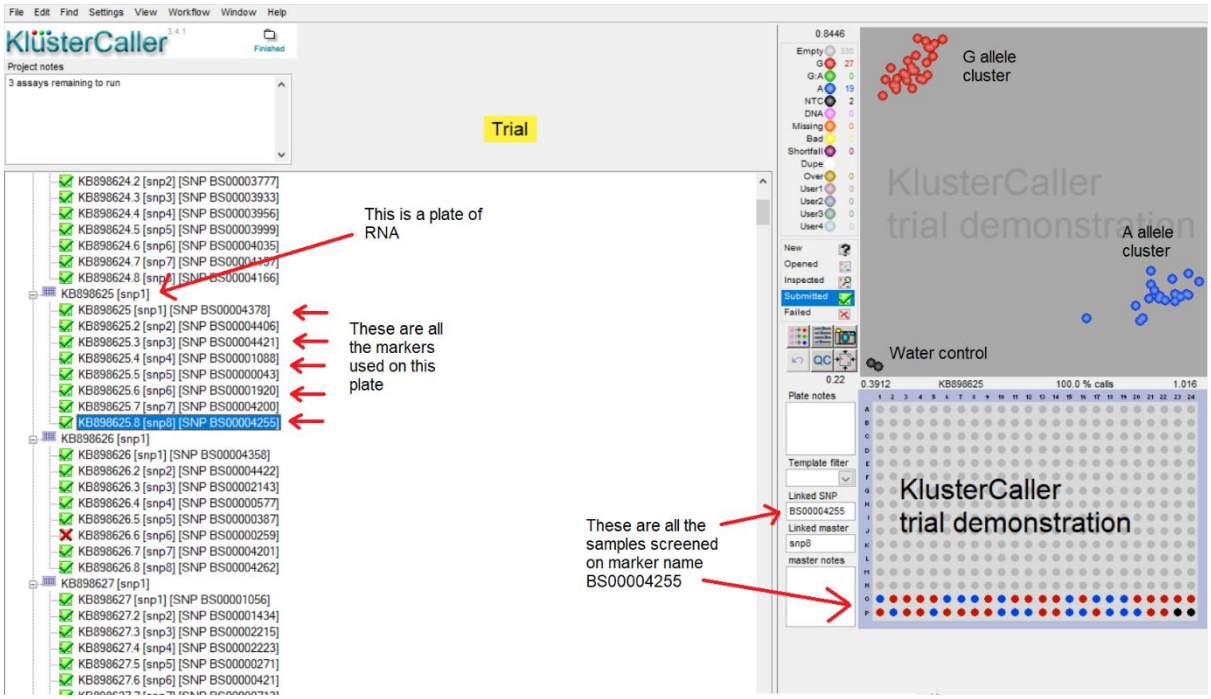


Figure 5: KlusterCaller software screenshot.

Using the Klustercaller software, import all the scanned plates, these will automatically appear in the project and will be listed by plate and separated by marker. For each marker, check the cluster plot as an indicator or genotype. In each case the FAM signal will represent one allele and the HEX signal will represent the other allele.

The auto-calling function is very weak, so manually circle and assign samples to clusters if they cluster well but are not coloured by the software.

> Be aware that Kraken will adjust the grid axis depending on the samples present, so if you only have one allele present in your sample plate, Kraken will auto-scale to make the plate appear to have both alleles (green) in all samples. Compare axis across the project before submitting for export to ensure that this has not occurred.

Export as “Submitted results” will generate an Excel spreadsheet with each sample compared to each marker.

	A	B	C	D	E	F	G	H	I	J	K
1	DNA \ Assay	BS00001108	BS00011451	BS00012880	BS00021950	BS00021957	BS00021982	BS00021991	BS00022016	BS00022188	BS00022195
2	Sample 1	G:C	G:G	T:T	G:G	A:A	A:A	A:A	T:G	C:C	C:C
3	Sample 2	G:G	A:G	?	G:G	A:A	A:A	A:A	T:G	C:C	C:C
4	Sample 3	G:C	G:G	T:T	G:G	A:A	A:A	A:A	T:G	C:C	C:C
5	Sample 4	G:G	A:G	T:T	G:G	A:A	A:A	A:A	T:G	C:C	C:C
6	Sample 5	G:C	G:G	T:T	G:G	A:A	A:A	A:A	T:G	C:C	C:C
7	Sample 6	G:G	A:G	T:T	G:G	A:A	A:A	A:A	T:G	T:C	C:C
8	Sample 7	G:G	?	T:T	G:G	A:A	A:A	A:A	G:G	T:C	C:C
9	Sample 8	G:G	A:G	T:T	G:G	A:A	A:A	A:A	T:G	T:C	C:C

Figure 6: Example of data output from KlusterCaller software.

Primer	PACE-specific tail	Example sequence
Forward Primer 1	GAAGGTGACCAAGTTCATGCT	GAAGGTGACCAAGTTCATGCTGTCTCTAGTCAGTGTGTTAATCTTACAAA
Forward Primer 2	GAAGGTCGGAGTCAACGGATT	GAAGGTCGGAGTCAACGGATTCTCTAGTCAGTGTGTTAATCTTACAAC
Common Reverse	-none-	AATTAGTGTATGCAGGGGGTAATTGAGTT

Supplementary Table 1: PACE-specific tails for oligonucleotide probes. Each SNP is targeted by two forward primers with a PACE-specific tail and a reverse primer common to both. In this example, the primers are targeting an A/C SNP.

Reference

[1] Harper H, Burridge A, Winfield M, Finn A, Davidson A, Matthews D, et al. (2021) Detecting SARS-CoV-2 variants with SNP genotyping. PLoS ONE 16(2): e0243185. https://doi.org/10.1371/journal.pone.0243185