Stabilised Pseudovirions for use as Positive Control in SARS-CoV-2 RNA Detection Kits

A versatile RT-PCR test kit positive control in the form of RNA nanoparticles

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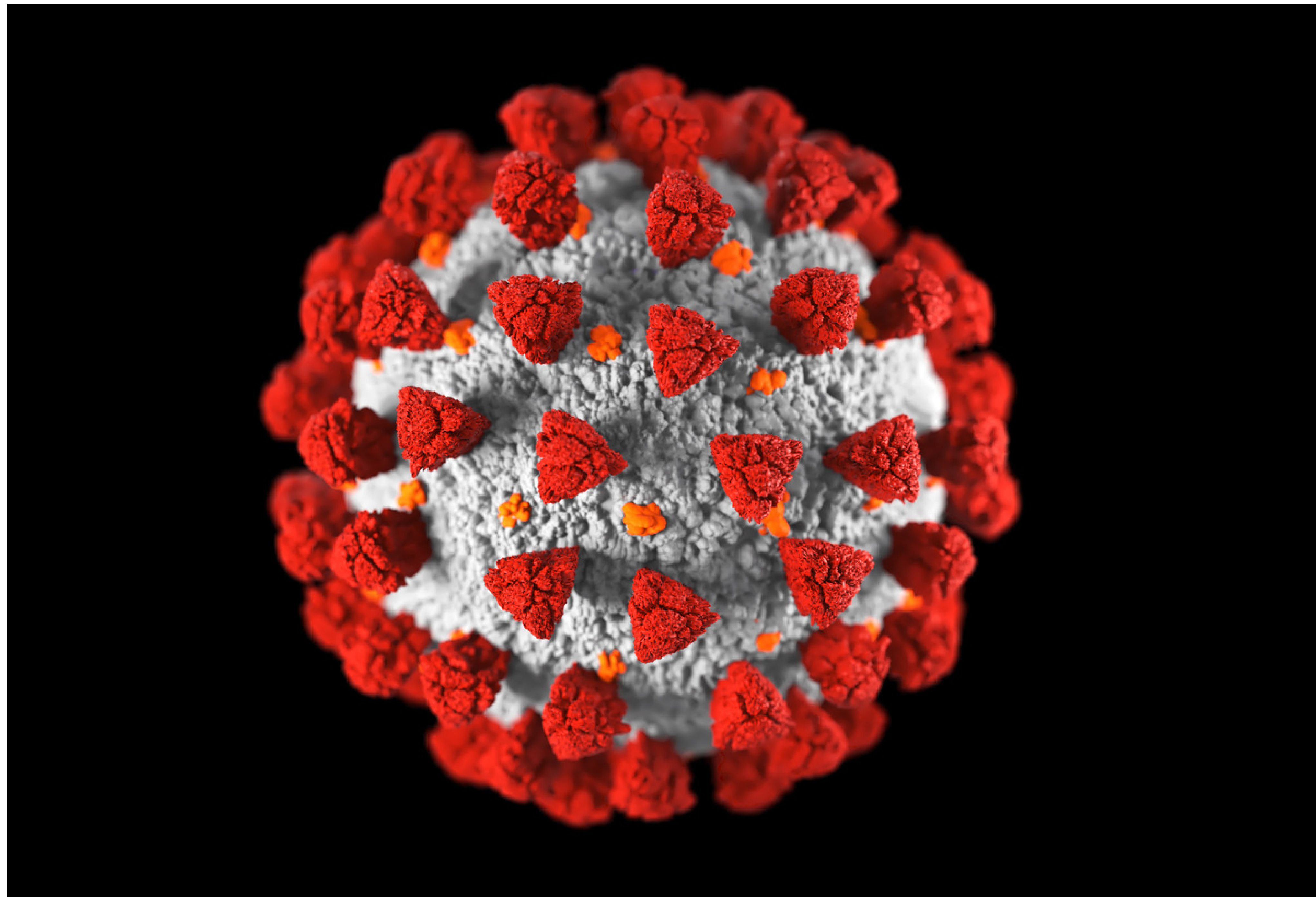

Leone_v, stock.adobe.com

Background

The project involves the production of RNA nanoparticles in plants for use as positive controls in SARS-CoV-2 RNA RT-PCR detection kits. One of the most important tests carried out on patient samples for diagnosis of SARS-CoV-2 infection is reverse transcriptase polymerase chain reaction (RT-PCR), which is designed to amplify any potential viral RNA in the sample, and results in quantitation of viral RNA levels and subsequent assessment of whether a patient is infected or not. All SARS-CoV-2 RT-PCR kits which are used to quantitate the viral load in patient samples need to have positive controls for verification that amplification has occurred i.e., an indication that the reagents in the reaction have performed as required to amplify the correct target/s.

Positive controls for currently commercially available SARS-CoV-2 RT PCR kits are varied (see Table 1 at end of document) and many manufacturers have not divulged their nature. Of those that we have been able to discern, some use RNA encoding various SARS-CoV-2 targets (presumably stabilised somehow), while others use non-infectious plasmid DNA encoding SARS-CoV2 sequences, non-infectious RNA virus containing SARS-CoV-2 RNA sequences, as well as non-infectious and replication deficient viral material of unknown source.

This research project addresses the absolute requirement for positive controls for SARS-CoV-2 RT PCR diagnostic assays. The positive control is usually included in RT-PCR test kits which are being developed for commercialisation. However, in some cases the assays require the additional use of external controls for calibration of the diagnostic assays.

Technology Overview

This project has used biopharming technology to develop a versatile RT-PCR test kit positive control in the form of RNA nanoparticles called pseudovirions (PsVs) (RNA encapsidated with plant virus coat protein), which can be used for the detection of any desired SARS-CoV-2 targets. The plant (Nicotiana benthamiana) production platform for this positive control provides a reliable, scalable and safe method for its production. In addition, the method ensures that the control is stable and can be adapted at low cost and most importantly, very rapidly, to cater for changes that may be requisite in SARS-CoV-2 targets with the evolution of variant viral strains. Ultimately this product will fit in with any SARS-CoV-2 RT PCR diagnostic kits that are being designed in South Africa, and in this case, targeting of specific SARS-CoV-2 variants will be most applicable.

The positive control PsVs are made in plants, and once purified, can be used immediately with commercially available test kits/RT-PCR reagents. The RNA was made by designing and synthesising an artificial DNA sequence containing a concatenate of 13 SARS-CoV-2-specific primer/probe sequence sets (approx. 1300 nucleotides in length) that encoded the most commonly used targets (Table 1) for amplification in SARS-CoV-2 RT-PCR tests reported in scientific literature at the time. The construct also contained the RNAse P internal control primer/probe sequence set and was designed to have some flexibility by incorporating various restriction enzyme sites whereby certain of the primer/probe sequence sets could be removed if not required at any stage, or new ones inserted, if required. This would occur for example, if target sequences were required to detect new SARS-CoV-2 variants or even the spike (S) protein (not included in our original design), making it very adaptable. The synthesised DNA sequence was cloned into a construct capable of allowing for the production of the PsVs in plants.

The PsVs were expressed in plants and purified from leaves using conventional plant virus purification methods involving sequencing polyethylene glycol precipitation and centrifugation. After 3 sequential PEG precipitations, the PsV pellet was resuspended in ½ volume buffer and samples were analysed by SDS-PAGE followed by Coomassie blue staining as well as western blotting to verify the presence of plant virus coat protein. The PsVs were also imaged using the electron microscope to confirm their presence and morphology.

To demonstrate the presence of the SARS-CoV-2 concatenate mRNA within the PsVs, RT-PCR was conducted on PsVs purified from the leaf extract. cDNA was synthesized by reverse transcription of putative ssRNA contained within the PsVs, followed by PCR amplification using the appropriate primers. The presence of the SARS-Cov-2 mRNA isolated from the PsVs was confirmed by visualising the correct DNA band size from the PCR on an agarose gel.

The PsVs were then tested for functionality in routine SARS-CoV-2 diagnostic assays by researchers at the National Health Laboratory Services facility in Cape Town and the National Health Laboratory Services facility at WITS university in Johannesburg.

Cape Town researchers tested the PsVs using the following SARS-CoV-2 RT-PCR kit:

- Allplex™ 2019-nCov Assay (Seegene)

Real Time qPCR of five different PsV samples successfully amplified all 3 targets tested – the RdRp, N and E genes (Table 1). Routine extraction methods were used whereby 50ul TMV PsV sample aliquots were incubated at 98 oC for 5 min followed by 4 oC for 2 minutes before adding a 2ul sample 5 to the 28ul reaction ie. only 2ul samples were required per test. Samples were tested as undiluted, 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 dilutions. All samples appeared similar in concentration, demonstrating similar Ct (cycle threshold = no of cycles required for signal to exceed background level) values with samples that were tested at 10-5 dilutions demonstrating Ct values closest to what is observed for typical diagnostic samples. It would therefore appear that only 2 x 10-5 ul of undiluted sample is required per reaction, indicating the suitability of the PsVs for use as a positive control in the Seegene RT PCR kit.

The researchers tested the PsVs using the following SARV-CoV-2 RT-PCR assays:

- BD BioGX SARS-CoV-2 for MD Max™ system (BD)
- Xpert® Xpress SARS-CoV-2 for GeneXpert system (Cepheid Innovation)
- TaqPath Covid19 CE IVD RT-PCR kit (Thermo Fischer)

Similar results were obtained in these three assays (Table 1): in the BD BioGX test, RT qPCR of the two different PsV samples sent to WITS successfully amplified the N1 and N2 regions of the N gene; in the Xpert Xpress assay, the E and N genes were amplified, and in the TaqPath assay, the N gene was amplified. The ORF1ab target was not amplified, however it is possible that a different part of the sequence was included in the TaqPath assay – the specific sequence in the assay has not been disclosed.

The following SARS-CoV-2 RT PCR assays are still under investigation:

- Abbot RealTime SARS-CoV-2 (Abbot)
- cobas® SARS-CoV-2 for cobas system (Roche)

Overall, proof of concept of our idea has largely been shown in that the PsVs have functioned as positive controls for the 4 tests carried out to date, with the SARS-CoV-2 targets that should be detected by the specific primers in the assays being amplified from the RNA in the PsVs.

Overall goal

The overall goal of this project is to provide a plant-produced product which can be incorporated into RT-PCR assays which are apparently being developed in South Africa and elsewhere, as well as to provide a reagent which can be used on its own as an external control for diagnostic assays and as a reagent in research laboratories. It would be most beneficial if this product could be manufactured in South Africa.

Novelty

The idea is novel in respect that a plant virus coat protein is used to stabilize single-stranded RNA that can be used as a positive control in nucleic acid-based detection systems. The purification process is highly scalable and very cost effective; the end product – PsVs – can be stored at 4 oC in liquid form indefinitely. Most importantly, additional targets can be added to the pre-existing RNA without having to remove any existing targets, as the PsV has capacity for RNA up to at least 6400 nucleotides and the current concatenate is approximately only 1300 nucleotides in length.

Stage of Development

Probably TRL 5/6 - the technology has been validated in the relevant environment and its functionality has been confirmed in the situation in which it would be used; however it has not been incorporated into a SARS-CoV-2 RT-PCR kit which is essentially the main goal. For the purposes of using it as an external control or in the laboratory as a reagent, it could be used as is, although a certificate of analysis would have to be generated to provide information on stability data (this is currently being collected) etc.

Proof of concept has been shown and the product has been validated using 4 different commercially available methods/assays by laboratories who are licensed to carry out such assays and who do them routinely.

Some further work could be done on:

- additional optimization to determine the concentration at which to use the product.
- investigation of formulation to incorporate the product into specific test kits which seem to vary in design/protocol to a certain extent eg freeze drying, attachment to beads etc.
- assessment of regulatory requirements for addressing manufacture eg specific ISO standards of manufacture etc.
- most importantly, additional laboratory work to include the SARS-Cov-2 S protein and other targets into the PsV mRNA.

Benefits

- Most importantly, easy manipulation allows inclusion of additional or different targets that are required for screening in the assay eg if targets change in the event of differences in sequences of SARS-CoV-2 variants, the relevant sequences can be easily synthesized, cloned and expressed for encapsidation.
- Easier to scale up – sterility requirements and therefore costs are not as high compared to controls which involve plasmid DNA or RNA preparation, or the production of controls which are made in cell culture.
- The TMV PsVs are potentially more stable and less prone to degradation than some of the current controls which are included in the commercially available assays.

Applications

- SARS-CoV-2 diagnostic kit manufacturers for internal assay control incorporation
- Diagnostic reagent suppliers for external control kits required for routine calibration of assays
- Research laboratories working on SARS-CoV-2

Opportunity

- Collaboration with experts in formulation of the product for incorporation into diagnostic kits
- Assistance in assessment of regulatory requirements for scaled up manufacturing
- Funding for further laboratory work to include S and other targets into the control RNA sequence

Patents

- Priority Application: United Kingdom Patent Application No. 2108392.8 (filed 11 June 2021)

IP Status

- Patent application submitted

Seeking

- Development partner
- Licensing
- Seeking investment

TMV PsV targets in encapsidated RNA	-	+ (RNAse P)	+(WHO RdRP IP2, WHO RdRP IP4, RdRP Charite)	+(WHO E Sarbecco)	+(CDC N1, CDC N2, CDC N3, WHO/CHINA CDC N, Charite German N, NIID Japan N, HKU N)	+	-
BioGX	Not known	√			√ CDC N1, CDC N2		
XPert Xpress	No info but freeze-dried attached to beads; also refs Accuplex (see Seegene)	√		√	√ CDC N2		
TaqMan TaqPath	TaqPath Covid19 control (1 x [illegible] copies) - RNA control containing targets specific to the SARS-CoV-2 genomic regions	MS2 phage			√	x	
Cobas Roche	Non-infectious plasmid DNA (microbial) encoding SARS-CoV-2 sequences						
Abbot	Non-infectious Sindbis virus containing SARS-Cov-2 RNA sequences						
Seegene	Non-infectious and replication deficient control fully extractable	√	√	√	√		
	with a real viral protein coat (5000 copies/ml). Also see Accuplex SARS-CoV-2 ref material Seracare Life						