Title: FnCas9 based low-cost CRISPR diagnostics for rapid detection of monogenic and infectious diseases.

Introduction:

CRISPR/Cas systems with their ability to precisely target nucleotide sequences have reformed the field of therapeutic genome editing. Recently, we have shown that the orthogonal Ca9 protein from *Francisella novicida* (FnCas9) can effectively discriminate sequences with single nucleotide variations, allowing it to be used for disease diagnosis (Azhar, Phutela, Kumar, Ansari et al. *Biosensors and Bioelectronics* 2021). Our previous reports highlighted the very low binding affinity of FnCas9 to mismatched substrates which can be adapted to readouts that are useful for developing diagnostic assays for identifying nucleotide variants. We call this strategy FnCas9 Editor Linked Uniform Detection Assay (FELUDA) and had previously reported a web-server JATAYU, which accepts a nucleotide sequence from the user and returns its FELUDA design parameters. Further, FELUDA repurposed Rapid variant AssaY (RAY) diagnosis is highly robust and provides a visual readout on a dipstick for the detection of monogenic and infectious diseases like SARS-CoV-2 and its variants.

Objective

Development of FnCas9 based, a rapid, robust, and inexpensive diagnostic tool that provides a visual read out on a dipstick for the detection of monogenic and infectious diseases.

Material and Methods

Experimental design: The idea was to develop an easy, rapid and robust protocol for single-step RT-PCR (Reverse transcription-polymerase chain reaction) rapid nucleic acid visual detection, which can be performed under general laboratory conditions, without requiring many optimizations. To accomplish this, we first optimized protocols for rapid nucleic acid amplification and reverse transcription separately. We then tried combining both by putting together reverse transcriptase and DNA Taq polymerase enzymes in a single reaction and checked for the robustness of this protocol by changing reagents. This method can be really useful for direct amplification of shorter amplicons (≤ 400bp) from RNA specimens, where rapid amplification can help in predicting the presence or absence of target RNA. Using FnCas9, shown with single nucleotide mismatch sensitivity [1], and combining with a lateral flow assay (LFA) paper strip chemistry, this assay can provide visual readouts differentiating RNA/DNA sequences with single nucleotide changes within an hour [2,3].

Equipment and reagents

- Micropipettes
- Plastic ware (sterile filter tips, microcentrifuge and PCR tubes).
- PCR thermal cycler
- DNA agarose gel electrophoresis apparatus
- UV GelDoc system
- Single step RT-PCR Buffer (10X):
  200 mM Tris-Cl (pH 8.4)
  500 mM KCl
- Reverse transcriptase enzyme
- Taq polymerase enzyme
- 1X TAE Buffer
- 5X DNA Loading Buffer
- 2.5 mM dNTP
- Agarose powder
- Nuclease Free water
- Dimethyl sulfoxide (DMSO)
- Milenia HybriDetect Lateral flow assay (LFA) paper strip

FnCas9 based SNP detection:

i) via in-vitro cleavage (IVC) assay, Reverse transcribed, and PCR amplified amplicons were treated with 100 nM Cas9 RNP complex in a tube containing reaction buffer (20 mM HEPES (pH7.5), 150mM KCl, 10% glycerol, 1 mM DTT, 10 mM $MgCl_2$) at 37°C for 10 min, and the products were looked on a 2% agarose gel for the quantification.

ii) via lateral flow assay (LFA), 5' biotin-labeled amplicons were mixed with reconstituted Cas9 RNP complex for 10 min at 37°C. Cas9 RNP is prepared by equimolar addition of 3' FAM labelled-Chimeric gRNA and catalytically inactive or dead-Cas9 in a buffer solution made of 20 mM HEPES (pH7.5), 150 mM KCl, 10% glycerol, 1 mM DTT, 10 mM $MgCl_2$ and incubated for 10 min at room temperature. Along with the addition of 80 µl Dipstick buffer solution to the reaction tube, a Milenia HybriDetect one lateral flow strip was inserted to observe for test and control band intensities at room temperature. In addition, the background-corrected band intensity values were calculated through a smartphone application (TOPSE) [2].

Results

Optimizing PCR conditions for rapid amplification

We first worked towards establishing rapid and reproducible PCR cycling conditions for getting detectable amount of amplification from a DNA template. We initially used 3 ng of DNA plasmid containing target sequence and tried amplifying a 228bp amplicon with various PCR cycling conditions.

To begin with PCRs were set up using following composition:

Sl. No.	Reagent (Stock Conc.)	Volume (µl)	Final Concentration
1	Forward Primer (10 µM)	0.5	500 nM
2	Reverse Primer (10 µM)	0.5	500 nM
3	dNTPs (10 mM)	0.1	200 µM
4	10X Reaction Buffer (200 mM Tris-Cl pH 8.4, 500 mM KCl)	1.0	1X
5	$MgCl_2$ (50 mM)	0.3	1.5 mM
6	Taq DNA polymerase (5 U/µl)	0.1	1 U
7	DNA template		3 ng
8	Total Volume (with nuclease free water)	Upto 10 µl	

Table 1: Showing list of different reagents with their working amounts used to set-up PCR reaction.

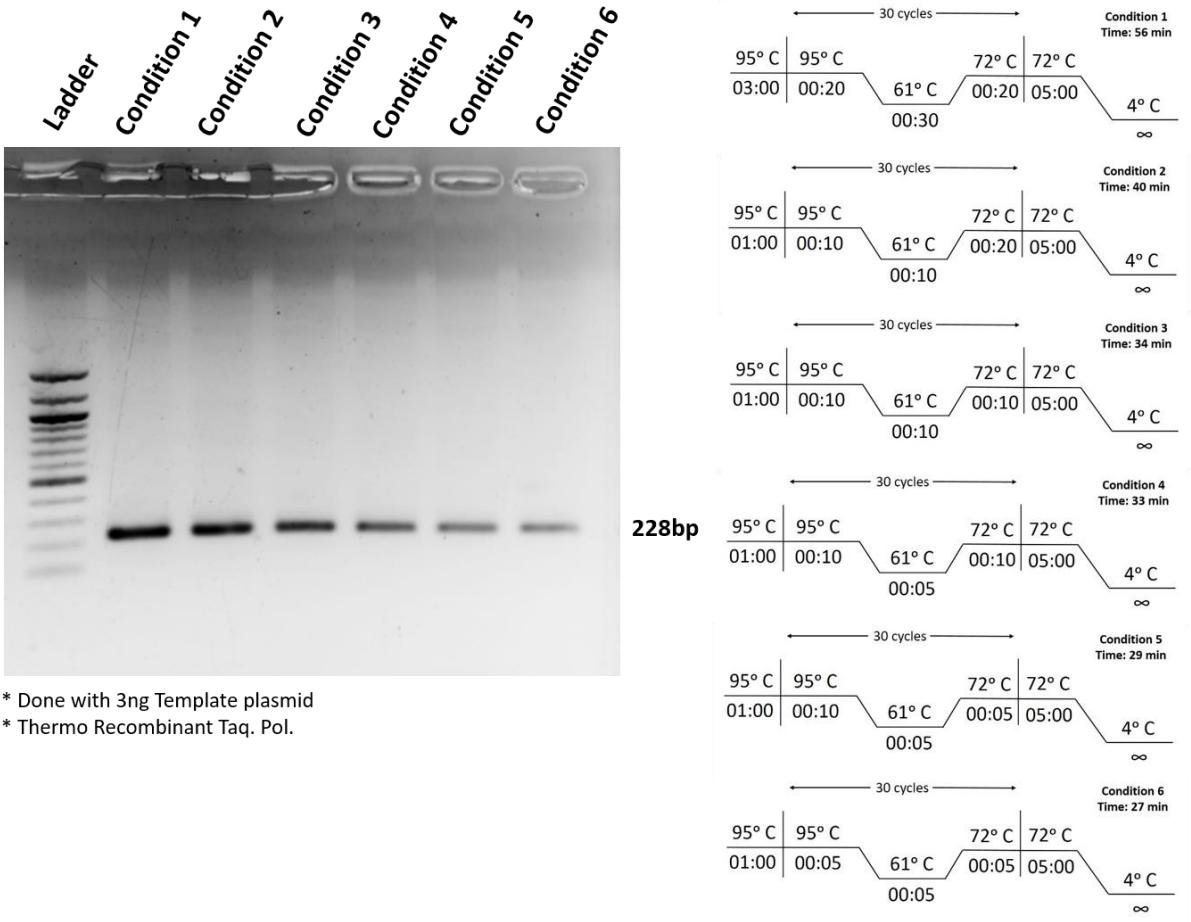
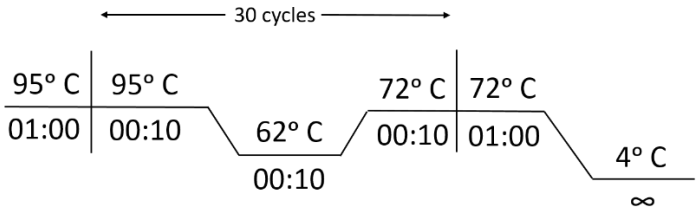


Fig. 1, Optimization of PCR conditions for rapid amplification. Different cycling conditions with changing duration of Denaturation: Annealing: Extension steps were tried, to obtain detectable amount of amplification using agarose gel electrophoresis stained with ethidium bromide.

As observed, there was a linear reduction in amount of amplification as the time for Annealing: Extension steps was reduced (**Fig. 1)**. Analysing and reproducing cycling conditions multiple times, we choose following cycling conditions, where we could every time observe a detectable amount of amplification:



Optimizing RT conditions for rapid amplification

After selecting a best PCR cycling conditions we then looked to reduce time for reverse transcription by taking an *in vitro* transcribed template RNA. cDNA preparation reaction mix was assembled as per the manufacturer's protocol (QuantiTect Reverse Transcription Kit, Qiagen) but rather keeping the reaction for recommended time of 15min, we tried changing reaction time and reducing overall duration, **(Fig. 2)** .

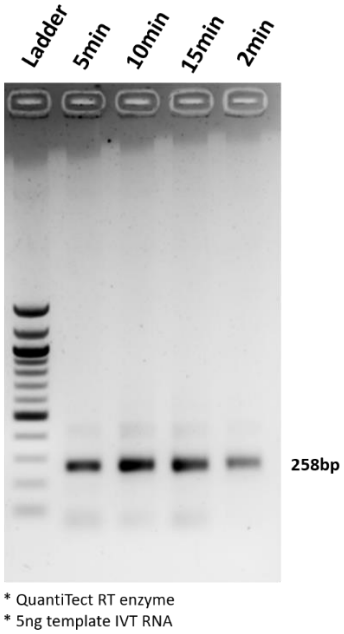
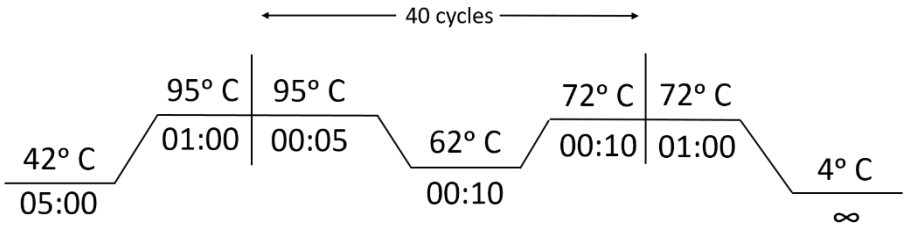


Fig. 2, Reverse transcription for different duration followed by semi-quant PCR (two step). Time shown is for reverse transcription step, further converted cDNA was amplified by previously optimized PCR condition.

Set-up single step rapid RT-PCR reaction

After having optimized both PCR as well as reverse transcription conditions separately, we tried combining them in a single reaction trying different buffer conditions. To previously optimized PCR condition and additional initial step of 5 min was added for RT reaction to occur in combination. Thus, making it single step rapid RT-PCR set up.

Thermal cycling condition optimized for single-step rapid RT-PCR:



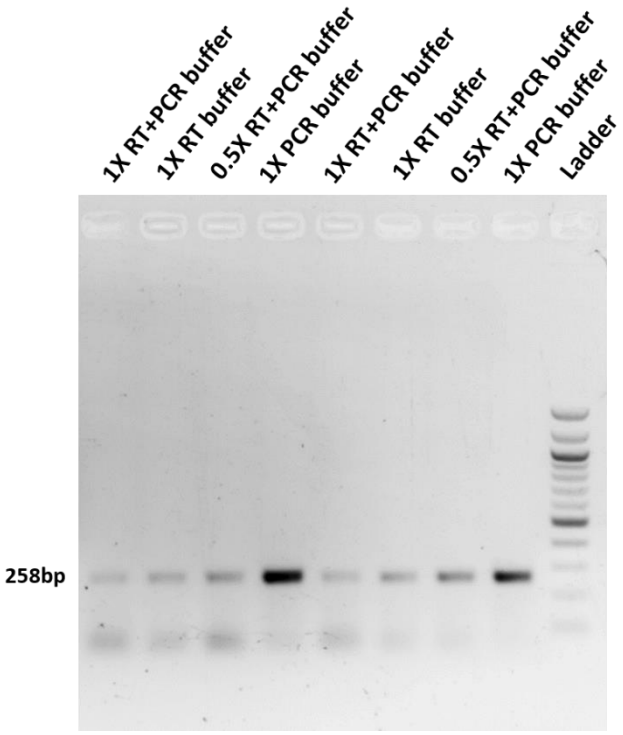


Fig. 3, Single step RT-PCR performed different buffer combination by mixing RT buffer provided along with the kit having unknown composition and lab prepared minimal PCR buffer constituting 200mM Tris-Cl pH 8.4, 500mM KCl and separately.

As can be observed, the lab prepared and filtered PCR buffer having 200mM Tris-Cl pH 8.4, 500mM KCl) is good enough to perform single step RT-PCR, and could be reproduced efficiently, **(Fig. 3)**. This shows that both Reverse transcriptase and Taq polymerase enzymes can work together in a single buffer without requiring any specialized reagents.

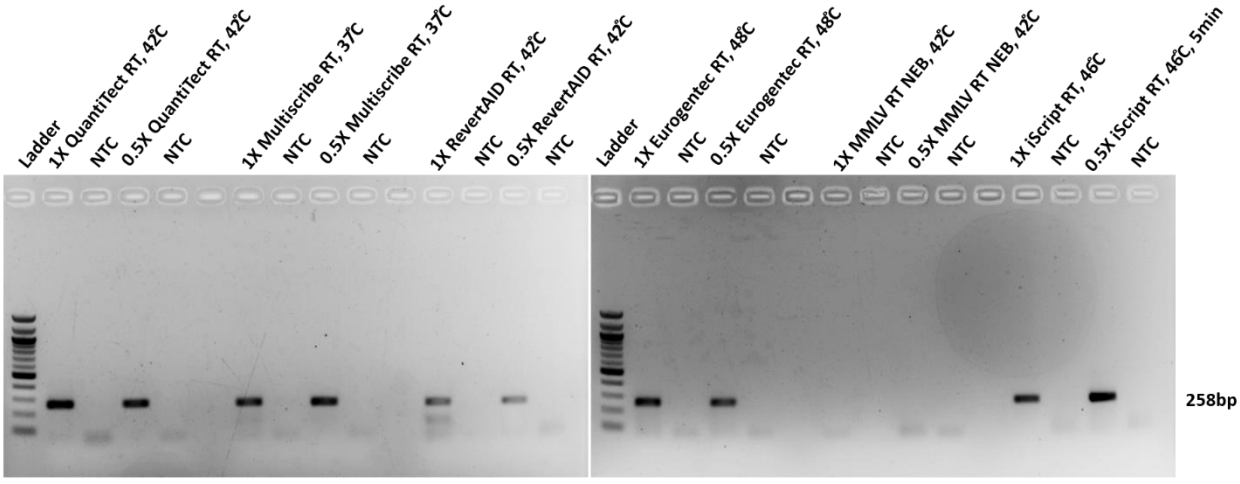
Different reverse transcriptase (RTase) and Taq polymerase enzymes tested for Single-step RT-PCR using in minimal PCR buffer

After multiple iterations a final composition of reaction was formulated, as given in **Table 2**. To check whether this protocol can be used with different RTase and Taq polymerase enzymes, we tried putting reaction with different RTase enzymes by keeping Taq polymerase and *vice versa.*

Sl. No.	Reagent	Volume (µl)	Final Concentration
1	Forward Primer (10 µM)	0.2	200 nM
2	Reverse Primer (10 µM)	0.2	200 nM
3	dNTPs (10 mM)	0.1	100 µM
4	10X Reaction Buffer (200 mM Tris-Cl pH 8.4, 500 mM KCl)	1.0	1X
5	$MgCl_2$ (50 mM)	0.3	1.5 mM
6	DMSO (100 %)	0.3	3 %
7	Taq DNA polymerase (5 U/µl)	0.05	0.5 U
8	Reverse Transcriptase (200 U/µl)	0.25	5 U/µl
9	RNase inhibitor (Optional, in case not present with RTase enzyme) 20U/µl	0.2	0.4 U/µl
10	RNA sample	As per sample	
11	Total Volume (with nuclease free water)	Upto 10 µl	

Table 2: Showing optimized working amounts of different reagents used to set-up single step rapid RT-PCR reaction.

a)



* Recombinant Taq Pol., ThermoFisher
* Temperature is of reverse transcription step.

b)

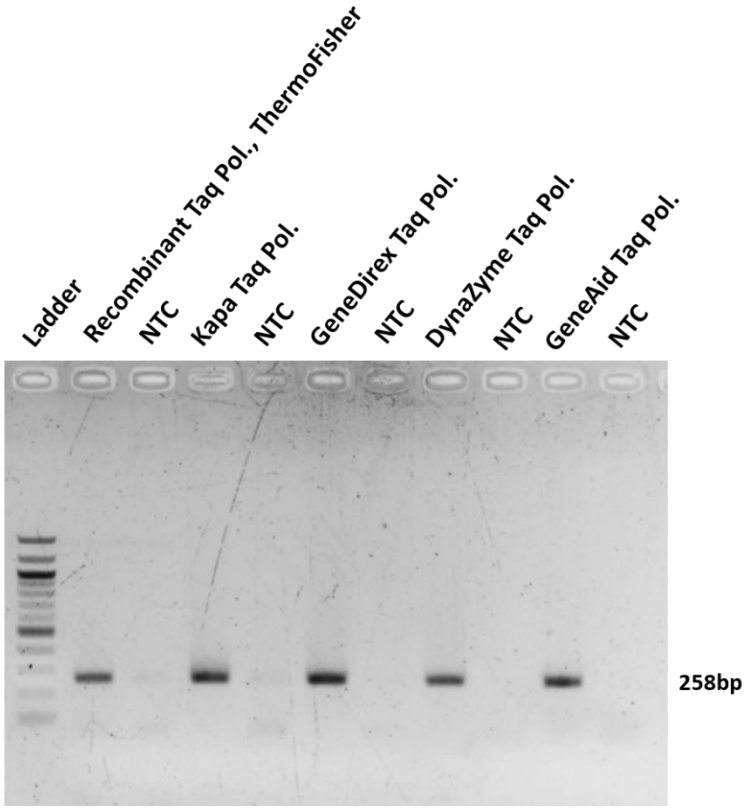


Fig. 4, a) Different RTase enzymes tested shown compatible with single step RT-PCR using in minimal PCR buffer. **b)** Different Taq polymerase enzymes tested shown compatible with Single-step RT-PCR using in minimal PCR buffer. For a set of reactions one non-template control (NTC) was included, to check for non-specific amplification. Enzymes were chose based on their availability and keeping as variable as possible.

Most of the RTase worked well with our single step RT-PCR protocol except MMLV RTase from NEB (New England Biolabs), hinting that this particular enzyme might require some special reagents or conditions different from the ones we used here, **(Fig. 4, a)**. Moreover, the established protocol worked well with all the Taq polymerase enzymes those are tested, (**Fig. 4, b)**. Taken together such single step protocols could be really helpful to design nucleic acid detection platforms, where RNA to cDNA conversion, and further amplification for detection is required.

Finally, to test the limit of detection of optimized single step RT-PCR protocol, we serially diluted 5 ng of template IVT RNA and looked for the signal till highest degree of dilution. Through agarose gel electrophoresis along with ethidium bromide stain amplification from as low as 20-200 molecules could be visually detected (**Fig. 5)**.

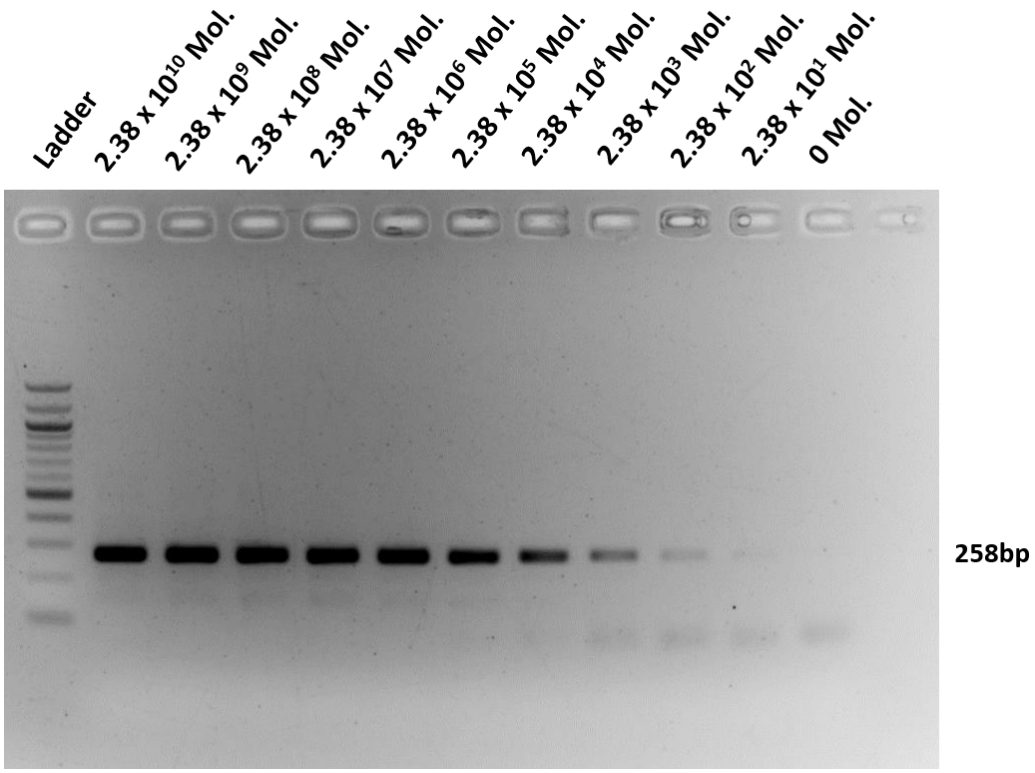


Fig. 5, LOD with IVT synthesized and purified target RNA (372bases). An *in vitro* transcribed synthetic target RNA was 1:10 serially diluted for ten times from $2.38x10^{10}$ copies/µl to perform single step RT-PCR. The amplified products where then visualized on EtBr stained 2% agarose gel.

Lateral flow paper strip based visual detection of SARS-CoV-2 and its variants

Combining an optimized single-step Reverse Transcription-PCR protocol, FnCas9 complexes, and lateral flow paper strip, FnCas9 Editor Linked Uniform Detection Assay (FELUDA) uses a direct binding based enzymatic readout for nucleotide sequences identification. For a point-of-care (POC) suited visual detection the commercially available LFA paper strips could capture FAM-labeled chimeric gRNA-Cas9 (RNP) bound biotinylated DNA substrate on a well-defined test band of the paper strip. As shown in **Fig. 6**, FELUDA can detect SARS-CoV-2 sequences from RNA samples within an hour. Further, to harness the single nucleotide specificity of FnCas9 in order to develop a point of care (POC) diagnosis for rapidly mutating SARS-CoV2 variants, we have repurposed another paper-strip based platform, Rapid variant AssaY (RAY). Which uses FnCas9 along with fine-tuned gRNA design and lateral flow paper strip, to provide visual readouts confirming if a sample contains a variant lineage within an hour. Currently, RAY can successfully detect both positive SARS-CoV2 infection in addition to distinguishing it with a recently evolved and highly infective N501Y variant containing CoV-2 lineage in a patient sample, thus acting as a rapid and robust alternative for labor-intensive and expensive sequencing-based diagnostics. Through a fine-tuned gRNA design shown in our previous study [2,3], RAY can be utilized to

detect single nucleotide variants within any RNA/DNA targets fitting with the gRNA designing criteria.

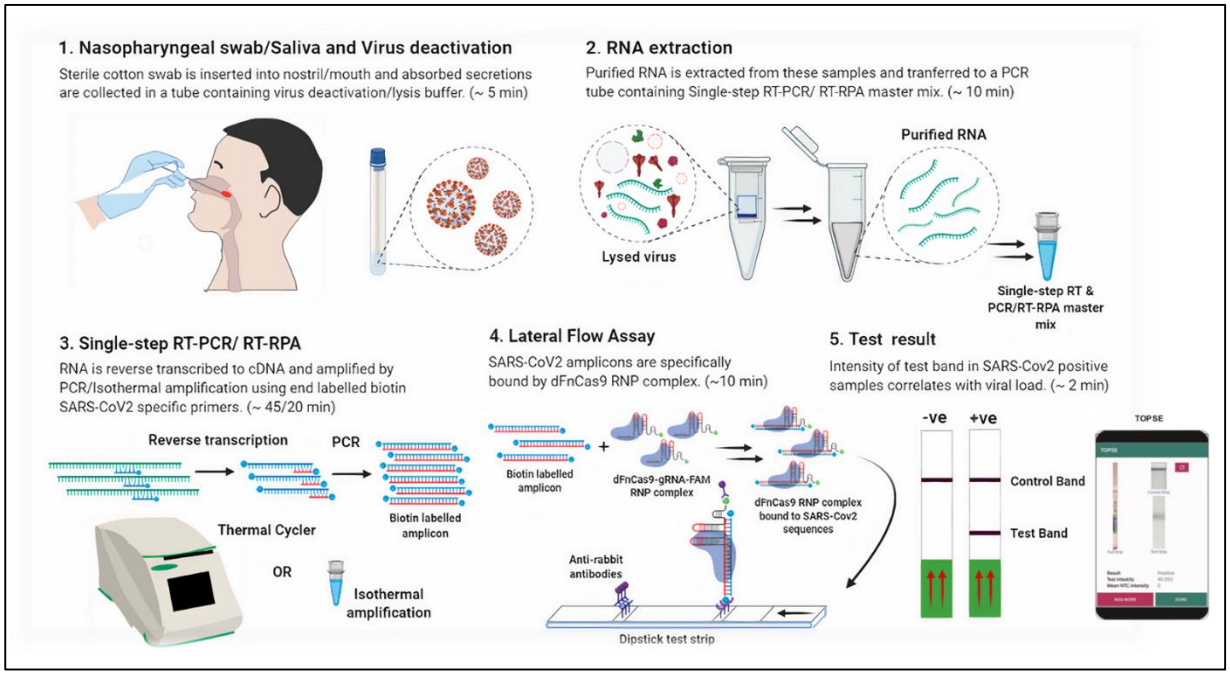


Fig. 6, Visual schematic showing different steps involved in the FnCas9 based nucleic acid detection, FnCas9 Linked Uniform Detection Assay (FELUDA). Starting from collection of the sample to RNA extraction, reverse transcription, and pre-amplification followed by detection and quantification on a lateral flow paper strip through a smartphone application.

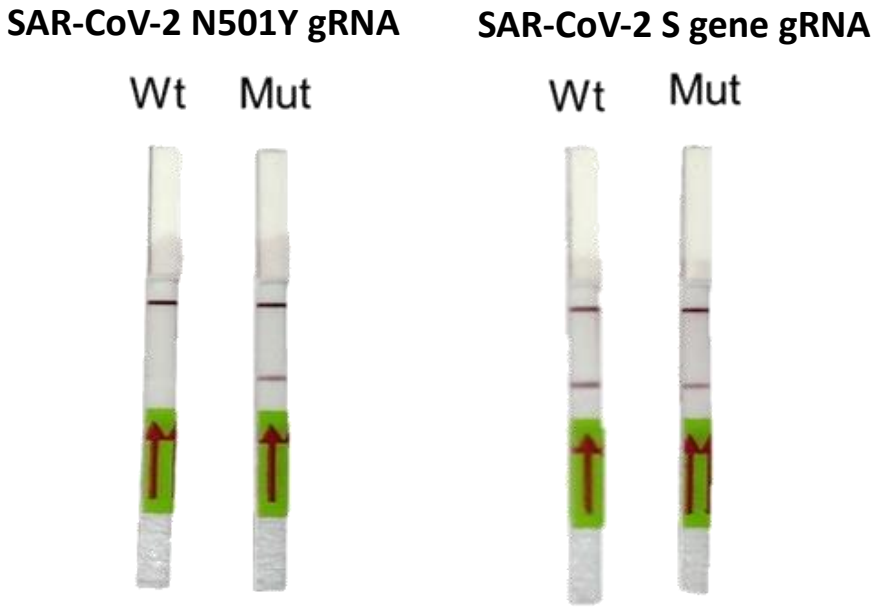


Fig. 7, RAY (Rapid variant AssaY), for successful discrimination of Wild-type (WT) and N501Y SARS-CoV-2 (Mut.) infected samples. Right Panel, visual test bands with S gene gRNA is confirming the infection of SARS-CoV-2 in both WT and Mut. infected SARS-CoV-2 samples. Left

panel, sample infected with N501Y SARS-CoV-2 but not the parent lineage will give visual test band with N501Y gRNA, indicating infection with N501Y SARS-CoV-2 (alpha) variant.

Discussion

Unexpected health emergencies like the recent COVID-19 pandemic necessitated the development of rapid and robust nucleic acid detection methods for widespread use. qRT-PCR being a gold standard for the nucleic acid diagnostic is laborious, expensive, and requires a special instrument. As shown by our previous reports, FELUDA being semi-quantitative can be adapted to multiple signal readouts. When commissioned with a lateral flow readout, FELUDA could determine a range of viral loads in clinical samples within a 1hr [2]. In combination with isothermal pre-amplification using RT-RPA and background-corrected signal intensities through a smartphone application True Outcome Predicted via Strip Evaluation (TOPSE), we also showed a working prototype for FELUDA based CoV-2 detection for home settings [2] and could utilize it for ingenious applications such as molecular diagnosis during infectious disease like COVID-19.

The COVID-19 pandemic was worsen with the SARS-CoV-2 virus undergoing mutations, several of which have also been shown to be associated with the higher transmission, immune escape, and disease severity. Tracking these variants is of immense help to understand how the virus is propagating, responding to vaccines, and thereby help shape strategies to counter its spread. At present, the sequencing of viral genomes is the best we have to understand the evolution of viral mutations and risk stratification. However, sequencing is tough to implement as a general diagnostic test since it is expensive, requires specialized infrastructure and trained personnel, and has a long turnaround time. Using this simple strategy of FELUDA improvised RAY, we could target many of the other CDC-listed major VOCs/VOIs reported in different SARS-CoV-2 emerging lineages [3]. Which makes RAY a very useful assay to be exercised for the primary surveillance to isolate cases infected with evolved lineages and follow appropriate COVID management protocols.

Statistical Analysis

The methods suggested in this thesis are well replicated and part of it is previously published as Azhar, Phutela, Kumar, Ansari et al. *Biosensors and Bioelectronics* 2021; Kumar, Gulati et al. *eLife* 2021. In addition, TataMD CHECK, a CRISPR based commercialized nucleic acid diagnostics test for COVID-19, is developed based on FELUDA.

Impact of the research in the advancement of knowledge or benefit to mankind

During our initial research to characterize FnCas9 for its highly specific DNA cleavage events, we found that FnCas9 is specific to different DNA sequences with single nucleotide changes. Because of its specificity, we could repurpose FnCas9 based FELUDA for nucleic acid diagnostics test for COVID-19. TataMD CHECK, the commercial FELUDA kit, is deployed at several diagnostic centers

and airports. Also now available are mobile vans with a robotic platform that can take it to the difficult-to-reach places where RT-PCR test is inaccessible.

A web-based server JATAYU 2.0 (Junction for Analysis and TArget design for Your felUda) to aid users with no prior experience of performing a FELUDA experiment to design and validate an assay for SNV discrimination. By providing very minimal information like rs ID or flanking sequence for any human SNV, the user can quickly design and implement a nucleic acid screening assay for that SNV across samples. JATAYU 2.0 returns sequences for both the FnCas9 sgRNA and flanking amplification primers for the provided SNV.

Shown a working prototype of FnCas9 based, a rapid, robust, and inexpensive diagnostic tool that provides a visual readout on a dipstick for the detection of monogenic and infectious diseases, e.g. sickle cell anemia, SARS-CoV-2 and its variants etc. Which in the future can be useful for early detection or primary surveillance of diseases caused by single nucleotide changes both within humans and through other bodily infections.

References:

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