DEVELOPMENT AND QUALIFICATION OF IDENTITY TEST FOR CORONAVIRUS VACCINES

A dissertation thesis submitted to Nirma University in partial fulfillment for the Degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

Submitted by

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Under guidance of

Dr. Rajeev Mehla

Dr. Amee Nair

INSTITUTE OF SCIENCE

NIRMA UNIVERSITY

AHMEDABAD

MAY 2022

NIRMA UNIVERSITY

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CERTIFICATE

This is to certify that the thesis entitled **"Development and Qualification of Identity Test for Coronavirus Vaccines"** submitted to Institute of Science, Nirma University in partial fulfilment of the requirements for the award of the degree of **M.Sc. Biotechnology**, is a record research work carried out by **Ms. Gupta Prachi Manmohanbhai (20MBT014)** under the guidance of **Dr. Amee K. Nair**. No part of the thesis has been submitted for any other degree or diploma.

Dr. Amee K. Nair
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Director, ISNU

Declaration

The above dissertation was carried out by **Ms. Gupta Prachi Manmohanbhai (20MBT014)** under my guidance.

Dr. Amee K. Nair
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CERTIFICATE

This is to certify that **Ms. Gupta Prachi Manmohan bhai**, student of M.Sc Biotechnology from Institute of Science, Nirma University, Ahmedabad, Gujarat has carried out and accomplished her dissertation work on **"Development and Qualification of Identity test for Coronavirus vaccines."** successfully in the department of R&D Dengue & COVID vaccine of Serum Institute of India Pvt. Ltd., Pune under our guidance and to our satisfaction. This work is in partial fulfillment for award a degree in Master of Science Biotechnology. This report is now ready for examination.

Pune, 26.04.2022.

Place & Date

Dr. Rajeev Mehla
Research guide,
Deputy Manager. Dengue & COVID R&D QC,
Serum Institute of India Pvt. Ltd., Hadapsar, Pune.

DECLARATION

I, hereby solemnly declare that the project work entitled “Development and Qualification of identity test for Coronavirus vaccines” submitted to the Institute of Science Nirma University, Ahmedabad, towards partial fulfillment of Master of Science in Biotechnology, is the result of research work carried out by me under the guidance of Dr. Rajeev Mehla, Deputy Manager Dengue & COVID R&D QC, Serum Institute of India Pvt. Ltd., Hadapsar, Pune.

I further declare that the work reported in this project has not been previously for the award of any degree, diploma, or any other work of a similar title.

Place & Date: Prachi Gupta

STUDENT UNDERTAKING

I, Ms. Gupta Prachi Manmohanbhai student of M.Sc. Biotechnology Semester IV, Nirma University, Ahmedabad willingly undersign that, I will not publish/communicate any data of the project or any other data of Serum Institute of India Pvt. Ltd., Hadapsar, Pune during the academic year 2021-2022, without prior permission of the respective project guide. I shall be held responsible for self-indiscipline behavior/act during the project tenure. Further, I assure you that I shall abide by the rules and regulations of the institute I shall be working at.

Name: Prachi Gupta
Signature of student:
Name of the project guide: Dr. Rajeev Mehla
Project tenure: December 2021 to April 2022

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Dr. Amee K. Nair — Dissertation Guide

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At last, I will thank Almighty God who enables me to continue my journey in the field of Biotechnology and by his grace, I shall be able to touch a peak.

Place:

Prachi Gupta

Date:

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List of symbols, abbreviations & nomenclature

Sr No.	Abbreviation	Meaning
1	API	Active pharmaceutical ingredient
2	BSA	Bovine serum albumin
3	BIP	Backward inner primer
4	B3	Backward outer primer
5	CDC	Centre for Disease Control and Prevention
6	cDNA	Complementary Deoxyribonucleic acid
7	COVID-19	Corona Virus Disease 2019
8	CoV	Corona Virus
9	DNA	Deoxyribonucleic acid
10	DP	Drug Product
11	DS	Drug Substance
12	EDC	Ethyl(dimethylaminopropyl) carbodiimide
13	ELISA	Enzyme-linked immunosorbent assay
14	Fc	Fragment crystallizable
15	FIP	Forward inner primer
16	F3	Forward outer primer
17	fM	Femto Molar
18	gblock	Gene fragments
19	HOT	Hands-on time
20	HR	Heptad repeat
21	IgG	Immunoglobulin G
22	kDa	Kilo Dalton
23	LAMP	Loop-mediated isothermal amplification
24	LBP	Latex bead particles
25	LB	Loop backward primer
26	LF	Loop forward primer
27	MeV	Measles Virus
28	ml	Millilitre
29	NHS	N-Hydroxyl succinimide
30	NSP	Non-Structural Protein
31	ORF	Open reading frame
32	PCR	Polymerase chain reaction
33	PFU	Plaque forming unit
34	pM	Pico molar
35	RNA	Ribonucleic Acid
36	Rpm	Rotation per minute
37	RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
38	RuV	Rubella virus
39	S1	Subunit 1
40	S2	Subunit 2
41	SARS-CoV-2	Saviour Acute respiratory syndrome – Coronavirus – 2

42	+ssRNA	Positive single-stranded Ribonucleic Acid
43	TAT	Turnaround time
44	VOC	Variants of Concern
45	WHO	World Health Organization
46	μM	Micro molar
47	μL	Microlitre

ABSTRACT

The main purpose of the identity test is to ensure that each component shall be tested for conformation for purity, strength, and quality of the drug product. Molecular techniques like Loop-mediated isothermal amplification, Real-time RT-PCR, and Latex agglutination assays are the method for reliable quantification of low-abundance antigen in fill finished drug products. These methods are comparatively quick, specific, sensitive, robust, accurate, and precise to another method. The live-attenuated vaccine contains a live virus that provides a long-lasting immune response. In identity tests, methods are used to check for parameters like suitability, specificity, sensitivity, detection range, accuracy, and robustness.

Hypothesis

1. Nucleotides are very specific; they should be able to detect and therefore can be used in identity tests.

2. We're having monoclonal and polyclonal antibodies which bind to the antigen; these antibodies are specific to spike protein and they should be able to identify Coronavirus.

Objective

1. Development of a loop-mediated isothermal amplification method to detection of Coronavirus in Coronavirus vaccine strain.

2. Development of a one-step real-time RT-PCR for detection of Measles, Rubella, and Coronavirus in a combination vaccine.

3. Development of latex bead agglutination assay to detect COVID-19 vaccine strain.

Chapter I
Introduction

1.1 SARS-CoV-2

Coronavirus disease 2019 (COVID-19), a highly contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported in Wuhan, China in late 2019. It is a highly contagious disease and is transmitted directly or indirectly, primarily through the respiratory droplets and aerosols of an infected person. SARS-CoV-2 has had a terrible effect on the global demographic, killing over 6.1 million people and becoming one of the world's most critical health disasters.

1.2 Etiology

Coronaviruses are a broad family of viruses that cause infection in humans in some cases, while others cause disease in animals such as bats, camels, and civets. SARS-CoV-2 is a new strain of coronavirus that has not yet been identified in humans. SARS-CoV-2 belongs to the subfamily *Orthocoronavirinae* of the *Coronaviridae* family (order - *Nidovirales*) classifies into four genera of CoVs:

- Alpha Coronavirus
- Beta Coronavirus
- Delta Coronavirus
- Gamma Coronavirus

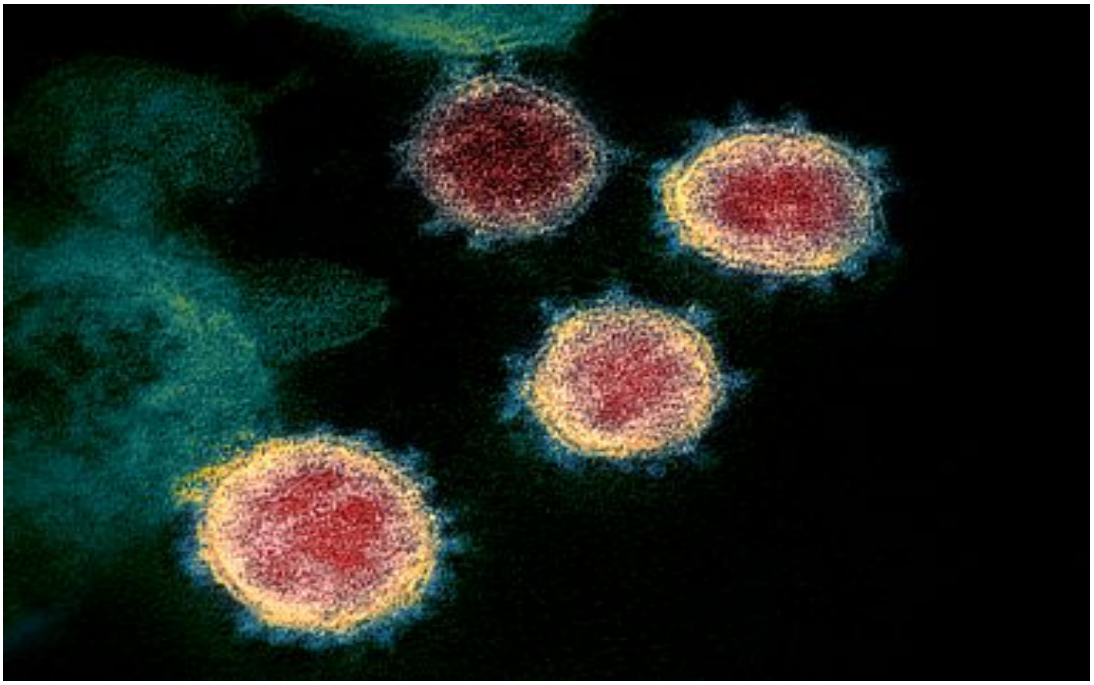
Attributed to the prevalence of spike proteins on the envelope, SARS-CoV-2 is a unique beta CoV and positive-stranded RNA(+ssRNA) virus with a crown-like appearance on cryo-electron tomography (Fig.1). The single-stranded RNA of the SARS-CoV-2 genome has 29891 nucleotides, which code for 9860 amino acids. SARS-CoV-2 is susceptible to evolutionary processes, resulting in several mutants with different features than the original strain. Frequent genomic sequencing of viral samples is critical, especially in a pandemic situation, because it aids in the detection of new SARS-CoV-2 genetic variations. The CDC and WHO have established their sification model to classify emerging SARS-CoV-2 mutations as Variant of Concern. (Cascella et al., 2022).

SARS-CoV-2 Variants of Concern (VOCs)

- Alpha (B.1.1.7 lineage) – First reported in the UK
- Beta (B.1.351 lineage) - First reported in Africa

- Gamma (P.1 lineage) - First reported in Brazil
- Delta (B.1.617.2 lineage) - First reported in India
- Omicron (B.1.1.529 lineage) - First reported in South Africa

Structure of SARS-CoV-2:



https://www.nih.gov/news-events/nih-research-matters/novel-coronavirus-structure-reveals-targets-vaccines-treatments

Figure 1. Transmission electron microscope image shows SARS-CoV-2, the virus that causes COVID-19, isolated from a patient in the U.S. Virus particles are emerging from the surface of cells cultured in the lab. The spikes on the outer edge of the virus particles give coronaviruses their name, crown-like. *NIAID-RML*

Structural Protein of SARS-CoV-2

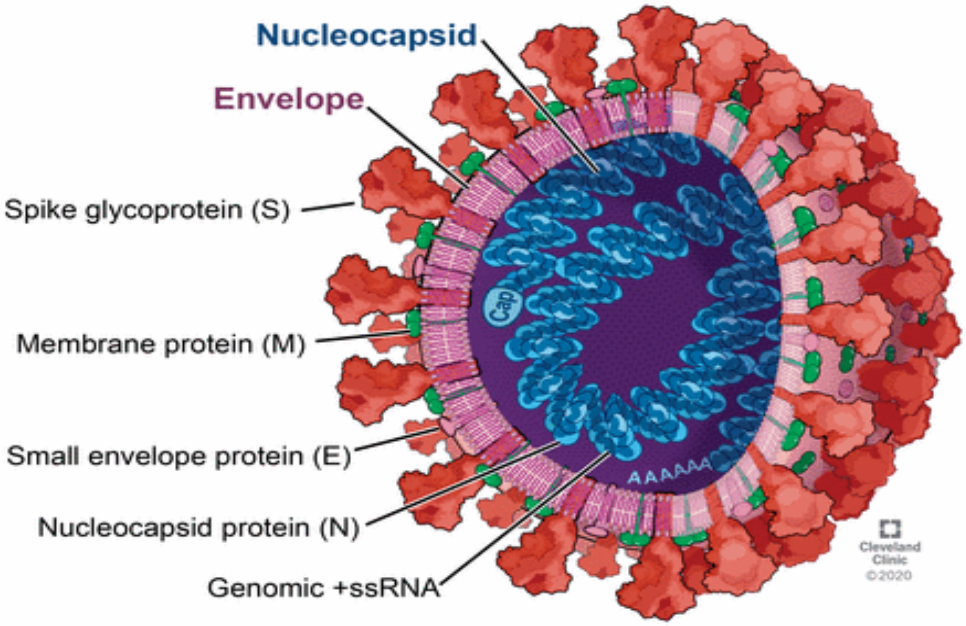


Figure 2. Structure of structural protein of SARS-CoV-2 (Bergman et al., 2020)

Coronaviruses are enveloped viruses with a single strand of positive-sense RNA that is between 26 and 32 kb in length. Around 70% of viral RNA mainly situated in the first open reading frame (ORF 1a/b) encodes 16 non-structural proteins (Table 1) (Yadav R et al, 2021) the remaining part of the virus genome encodes four essential structural proteins, including spike protein (S), a small envelope protein (E), membrane protein (M), and nucleocapsid protein (N) (Fig. 2).

S Protein: Surface S Protein is a ~600 kDa trimeric glycoprotein present in all human coronaviruses. The trimer of the S protein positioned on the surface of the viral envelope is the basic unit by which the S protein binds to the receptor. S Protein divides into two domains: the S1 subunit contains the RBD, which is responsible for the binding of the virus to the receptor, while the S2 subunit mainly contains the HR (Heptad repeats) domain, including HR1 and HR2, which is closely related to virus fusion.

M protein: M protein is the most abundant protein on the surface and is responsible for determining the shape of an envelope. It is also responsible for the transmembrane transport of nutrients, the bud release.

E Protein: E Protein is the smallest of the structural proteins on the viable membrane, and it appears to play several roles in transforming host cell machinery into workshops where the virus and human cells collaborate to create new viral particles.

N Protein: Capsid is a protein shell that encloses the genetic material of the virus called nucleocapsid or N Protein. This protein binds to the virus' single strand of RNA which is where its all-domestic information is held to allow itself to replicate. The N Protein seems to be multifunctional. In addition, it basically constrains a lot of the host cell defense mechanism and assists the viral RNA in replicating itself.

Table 1. Non-structural protein and its functions

Non-structural protein	Functions
NSP1	Leader protein acts as a host translation inhibitor and also degrades host mRNAs
NSP2	Binds to prohibitin 1 and prohibitin 2 (PHB1 and PHB2)
NSP3	NSP1, NSP2, and NSP3 are released from the N-terminal region of pp1a and pp1ab.
NSP4	Viral replication-transcription complex and helps modify ER Membranes
NSP5	Produces mature and intermediate non-structural proteins by cleaving at numerous different locations.
NSP6	Induces the formation of ER-derived autophagosomes as well as induces double-membrane vesicles
NSP7	NSP8's RNA polymerase activity is achieved by forming a complex with NSP8 and NSP12.
NSP8	Makes heterodimer with NSP8 and 12
NSP9	May bind to helicase
NSP10	Yet to be deciphered
NSP11	Unknown
NSP12	Replication and methylation
NSP13	The core domain of a helicase that binds ATP. The zinc-binding domain is crucial in the replication and transcription activities.
NSP14	N7-guanine methyltransferase activity and exoribonuclease activity working in a 3′-5′ direction
NSP15	Mn2+ dependent endoribonuclease activity
NSP16	mRNA cap 2′-O-ribose methylation of the 5′-cap structure of viral mRNAs is mediated by a methyltransferase.

Chapter II
Literature review

2.1 Vaccines

Vaccines are immune preparations for the prevention of infectious diseases made by pathogenic microorganisms and their metabolites through artificial detoxification, inactivation, or genetic engineering. The vaccine plays an increasingly significant role in disease prevention.

Types of vaccines:

- Live-ed vaccines - MMR combined vaccine, Rotavirus, Chickenpox
- Inactivated vaccines - Hepatitis A, Polio
- Subunit, recombinant, and polysaccharide vaccines - Hemophilus influenza type-b, Hepatitis B, Pneumococcal disease, Meningococcal disease, Human papillomavirus
- Toxoid vaccines - Diphtheria and tetanus
- Viral vector vaccines - Ebola virus and COVID-19
- mRNA vaccines - COVID-19

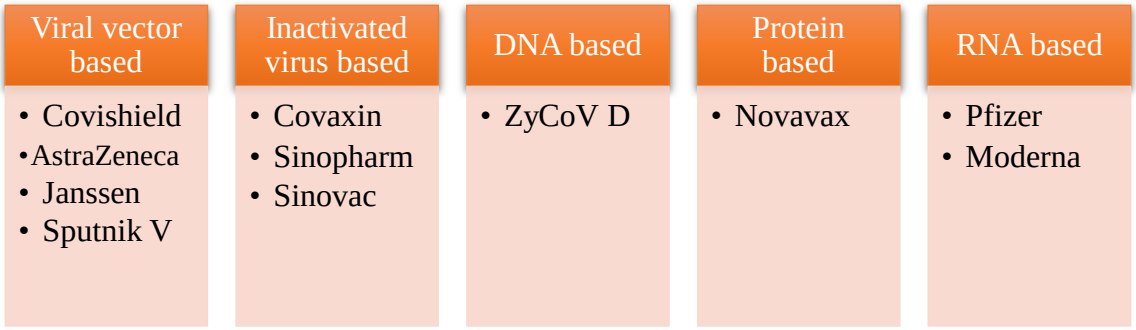


Figure 3. COVID-19 available vaccines

A viral pandemic necessitates billions of vaccine doses across the globe which leads rapid manufacture of effective treatments and vaccines. These days, different types of vaccines are available on the market (Fig 3). Live-attenuated vaccines use a disease-causing virus that has been weakened (or attenuated). These vaccines are quite similar to natural infections, but they do not cause any major disease under normal circumstances. As a result, the live-attenuated vaccine triggers cellular immune responses dominated by type 1 helper T cells and cytotoxic T cells. Moreover, a live-attenuated vaccine contains a large number of antigen molecules that are available to stimulate e strong immune response. Live attenuated vaccine provides contact immunity in unimmunized individuals who are associated with immunized people. In-short live-attenuated vaccines provide a strong and long-lasting immunresponsesse.

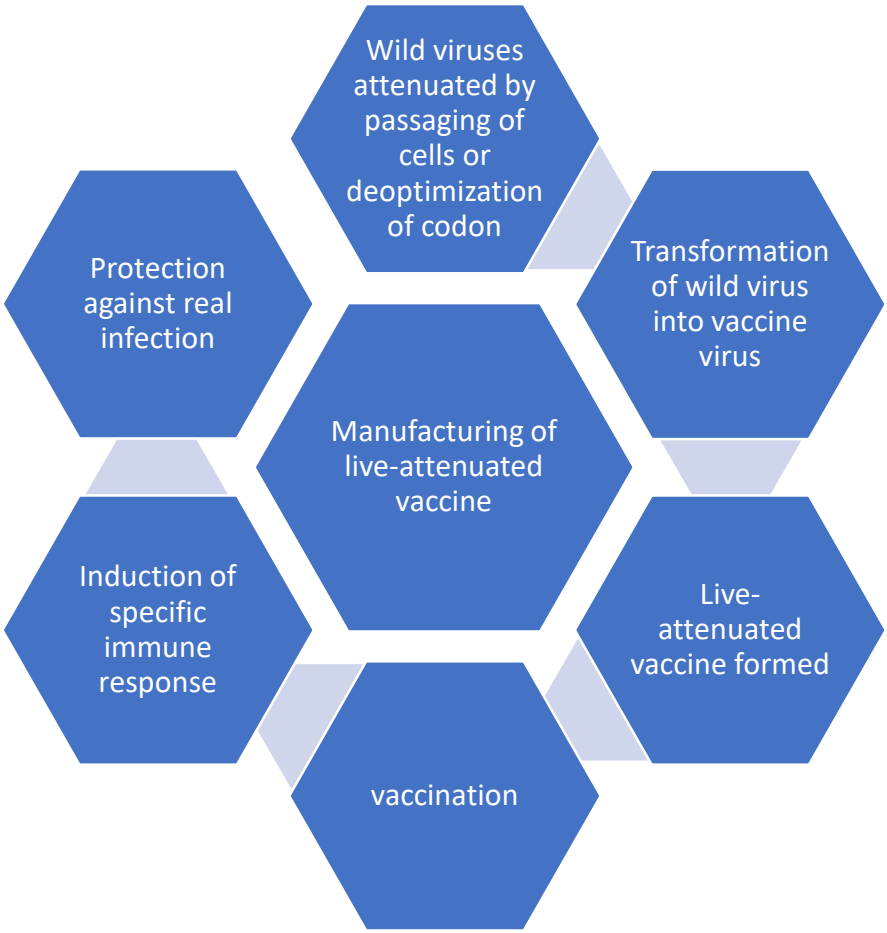


Figure 4. The manufacturing process of live-attenuated vaccines

Among all vaccines, live-attenuated vaccines provide a strong and long-lasting immune response. Since vaccines contain live-attenuated viruses, the safety of these vaccines remains a matter of concern. It needs to be ensured that every lot of the vaccine in goes out on the market for commercial use underwent proper testing for maintaining consistently high standards of quality. The purpose of identification testing is to verify the identity of the active pharmaceutical ingredient (API) or drug substance in fill finished drug product. The identification test can distinguish between chemicals with virtually identical structures that are most likely present.

Traditional and revolutionary vaccine identity tests are created by combining related technology from diverse disciplines. Until date, no single technology has been capable of completing the entire development of identification checks. These identity tests should be high throughput, less time taking, cost-effective, and highly robust in the manner of specificity and sensitivity. There are cell-culture-based (Neutralization assay) and molecular biology-based (Nucleic acid-based & Protein-based) techniques available for identity.

The neutralization test is relatively cumbersome and time-intensive (a few days) relative to a molecular diagnostic test that gives quick results (usually several minutes to a few hours). To fill finished DP it is required to have a faster and more reliable method for quick confirmation of the presence of drug substance. So molecular diagnostic techniques have been found useful for the Identity of the Coronavirus vaccine strain.

2.2 Molecular diagnostic techniques

Molecular diagnostics is a rapidly evolving topic within the economically significant field of biotechnology. In biological, agricultural, veterinary, medicinal, and forensic research, it has found a home. In most biomedical science laboratories, nucleic acid-based techniques & protein-based techniques have been established.

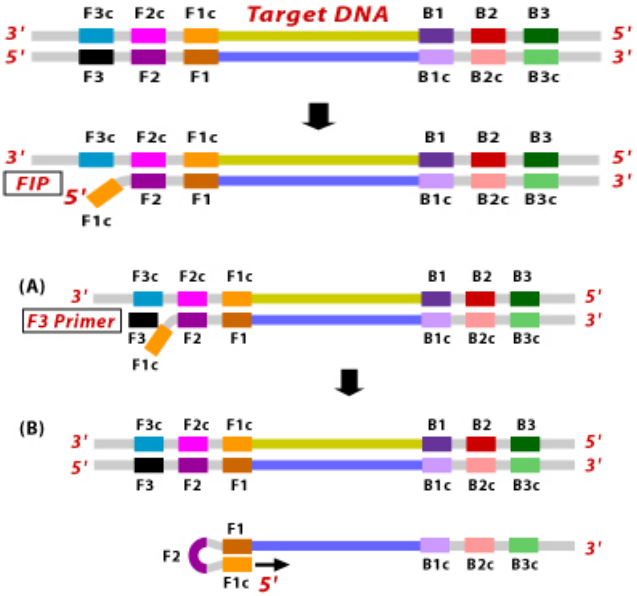
2.2.1 Nucleic acid-based techniques

The identification of viral genomes is made possible by a wide range of customized PCR applications. PCR is a molecular biology technique for amplifying a specific sequence of DNA in vitro by cycling the DNA into two strands and incubating it with oligonucleotides, primers, and DNA polymerase. Denaturation, annealing, and elongation stages are usually performed at different temperatures in PCR. The number of cycles is determined by how much target is present. The most common nucleic acid-based detection systems include conventional PCR, Real-time RT-PCR, and LAMP.

(A) LAMP

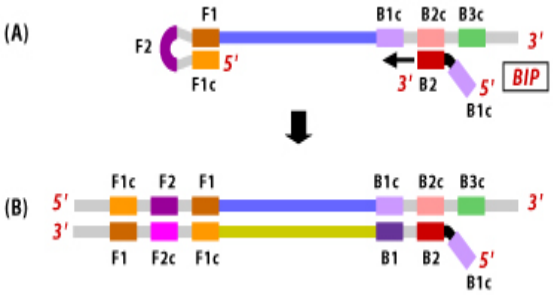
"LAMP" which stands for Loop-mediated isothermal amplification is a simple, quick, accurate, and cost-effective nucleic acid amplification method. It is characterized by the use of 4-6 different primers and the reaction process proceeds at a constant temperature using strand displacement activity. LAMP method has the characteristics of no special reagent required, no sophisticated temperature control device required, the template can be simply detected through the presence of the amplified product. Amplification and detection of genes can be accomplished in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity, and substrates at a constant temperature (65°C) resulting in high amplification efficiency. Because of its high specificity, the presence of amplified products can designate the presence of the target gene. (Francois et al., 2011). The LAMP technique uses four to six different primers (FIP,

BIP, F3. B3, LF, and LB) and strand displacement activity by DNA polymerase. The mechanism of the LAMP method is described below (Fig 5).

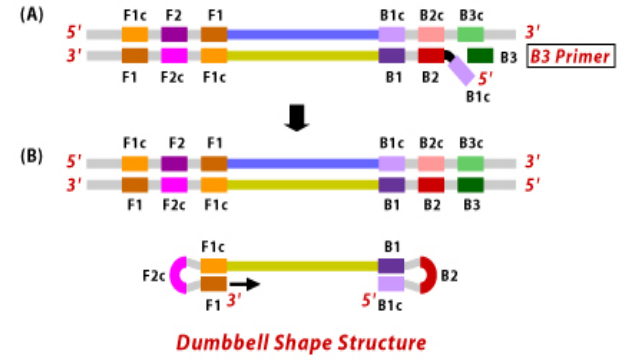


1. Starting from the 3' end of the F2 region of the FIP, a DNA strand complementary to the target DNA is produced using DNA polymerase with strand displacement activity.

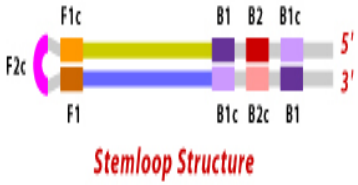
2. The F3 Primer binds to the target DNA's F3c region outside of FIP and triggers strand displacement DNA synthesis, releasing the FIP-linked complementary strand and the loop will form at the 5’ end of displaces strand.



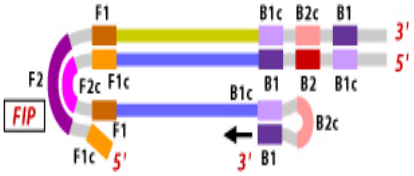
3. This single-stranded DNA with a loop at the 5' end processes as a template for BIP. B2 binds to the template DNA's B2c region. The synthesis of DNA has now begun, leading to the formation of a complementary strand by the opening of the 5' end loop.



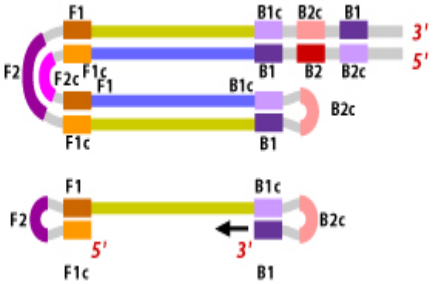
4. The outer primer B3 now intertwines with the target DNA's B3c region and extends by displacing the BIP - associated complementary strand. A dumbbell-shaped structure is developed as a result of this.



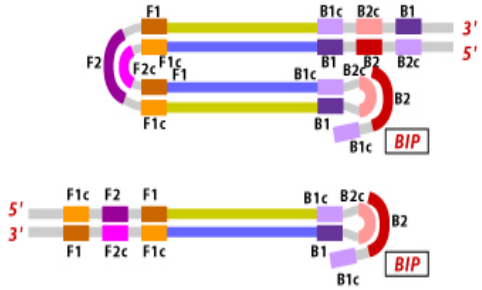
5. DNA polymerase adds the nucleotides at the 3' end of the F1 region, extending and opening up the loop at the 5' end. A stem-loop framework has been developed from the dumbbell-shaped structure of DNA. For LAMP cycling, this stem-loop structure acts as an initiator structure.



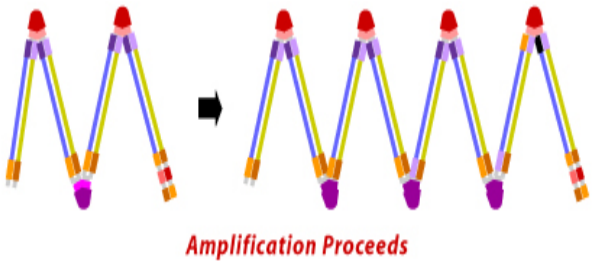
6. The FIP binds to the loop of the stem-loop DNA structure to induce LAMP cycling. The strand synthesis process has begun here. The F1 strand is displaced and forms a loop at the 3' end as the FIP binds to the loop.



7. Nucleotides are added to the 3' end of B1. The FIP strand is displaced to allow for the extension. This strand is displaced again, forming a dumbbell-shaped DNA structure. Self-primed strand displacement after that One complementary structure of the original stem-loop structure DNA and one gap repaired stem-loop structure DNA are produced during DNA synthesis.



8. In subsequent cycles, both of these products function as templates for a BIP strand displacement reaction. As a result, a LAMP target sequence is amplified excessively.



9. The outcome is a mix of stem-loop DNA with varying stem lengths and diverse cauliflower-like forms with multiple loops. Annealing between alternately inverted copies of the target sequence in the same strand produces the structures.

Figure 5. Schematic representation of LAMP method principle

(B) Real-time RT-PCR

In 1987, the technique of reverse transcription-polymerase chain reaction (RT-PCR) was first described. The enzymatic steps in RT-PCR are the synthesis of a single-strand complementary DNA copy (cDNA) from reverse transcription of RNA which is used as a template, and an amplification of the cDNA produced by reverse transcription. (Emery et al., 2004) It can be done in two separate tubes with two different enzymes (two-tube/two-enzyme) or in one tube with one or two enzymes (one tube or two-enzyme).

Real-time PCR is based on the observation of a fluorescent signal generated during the amplification process. Post-PCR processing, such as observing post-PCR products on gel electrophoresis, is eliminated with real-time PCR. This increases throughput while lowering the risk of contaminant carryover.

Detection Chemistries

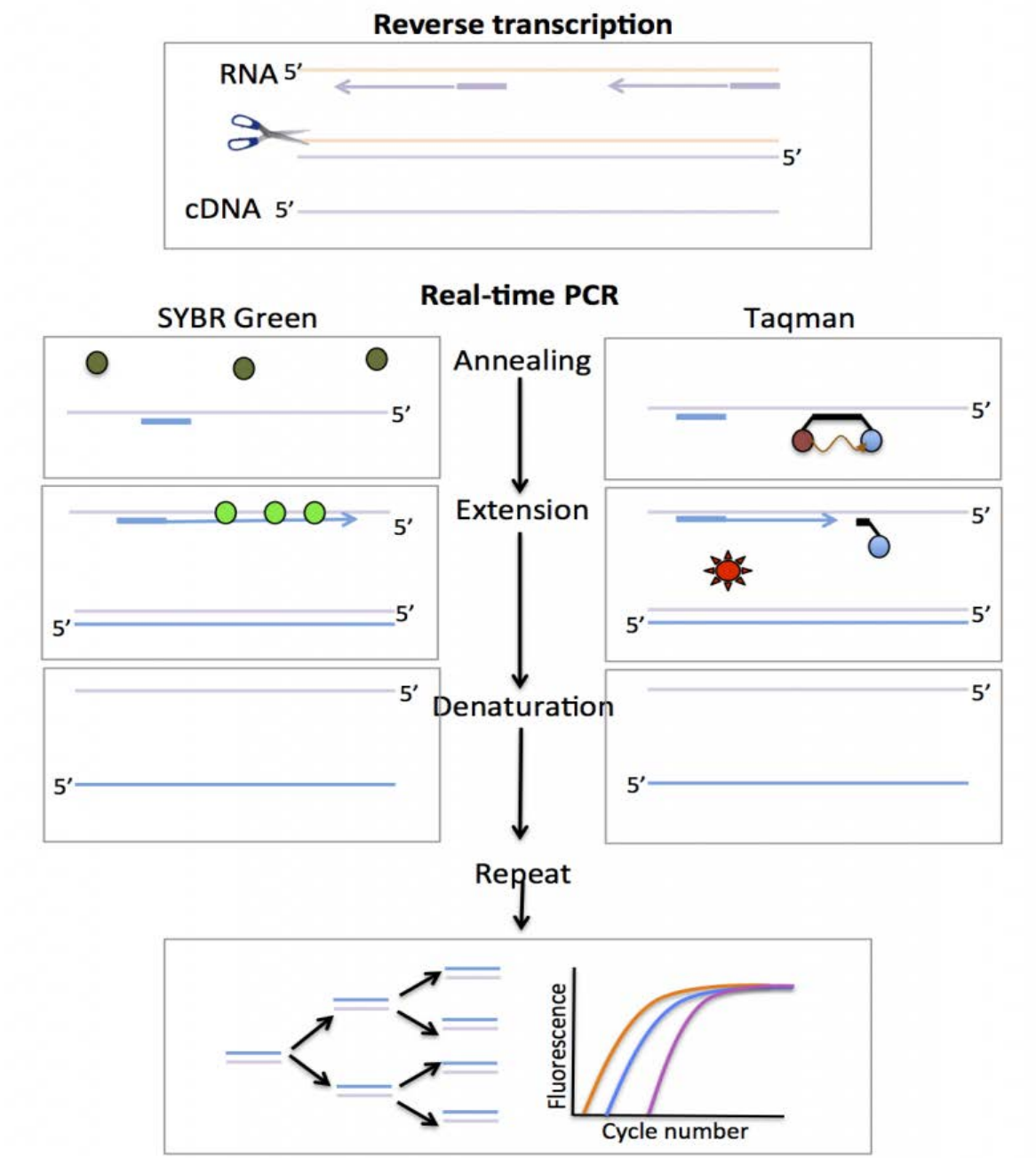
There are two detection chemistries (Navarro et al., 2015) mentioned here:

(I) SYBR® Green

(II) TaqMan®

(I) SYBR® Green

SYBR Green is a fluorescent dye used in Molecular Biology to stain nucleic acids, particularly double-stranded DNA. During real-time PCR, the SYBR Green technique is utilized to quantify PCR products. The resulting DNA-dye combination absorbs blue light and emits strong green light once it attaches to DNA. It happens because the dye molecule undergoes a structural shift when it binds to double-stranded DNA. More dye molecules bond to DNA as PCR generates greater DNA, resulting in more fluorescence.



https://www.ebi.ac.uk/training/online/courses/functional-genomics-ii-common-technologies-and-data-analysis-methods/real-time-pcr/

Figure 6. Detection chemistries for real-time RT-PCR

As a result, as the PCR product accumulates, the fluorescence increases. As a result, the SYBR Green fluorescence detection can be used to quantify the amount of PCR product.

(II) TaqMan®

Taqman® is a real-time PCR monitoring system that replaces SYBR Green. The 5'–3' exonuclease activity of the Taq polymerase enzyme is being used to degrade the probes during the extension of the new strand and fluorophore released in this method. This

method uses dual-labeled probes and is based on probe hydrolysis. Probes are fluorescently labeled DNA oligonucleotides with a fluorescent reporter molecule (fluorophore) at the 5' end and a quencher molecule at the 3' end. Taq polymerase extends the new strand towards the dual-labeled probes by adding nucleotides to the primer. Exonuclease activity of the Taq polymerase activates and degrades the probe. The probe is subjected to complete degradation and releases of the fluorophore when Taq polymerase completes the synthesis of the new strand. Fluorescence is generated when a fluorophore is released. The quencher molecule effectively quenches the emitted fluorescence and produces the output for PCR product quantification. The amount of fluorophores emitted and the amount of PCR products produced are proportional. As a result, the Taqman® probe chemistry makes quantification simple.

Table 2. Comparison of LAMP & Real-time RT-PCR

Sr No.	Parameter	LAMP	Real-time RT-PCR
1	Thermal conditions	Isothermal and continuous amplification at a single temperature.	Thermal cycling steps like multiple heating and cooling cycle; hence, bulky and cumbersome.
2	Reagents / Primers	4–6 primers are used, and 6–8 unique locations of the target gene are recognized.	Two primers are used, and two regions of the target gene are recognized
3	Detection	The use of bioluminescence allows for easy detection.	The use of probes increases specificity and offers easier detection.
4	Maintenance	Simpler, smaller equipment is used in this method, which has no moving components, and hence is easier to maintain.	This method involves more complicated, bulkier equipment - requires periodic calibration, moving parts, and a light source, filters, and detectors for fluorescence.
5	TAT	Turnaround time is about 4-5 hr.	Turnaround time is about 8-9 hr.
6	HOT	Hands-on time is about 3 - 3.5 hr.	Hands-on time is about 5-6 hr.
7	Ease of performance	This approach adheres to streamlined protocols, which are the same for all targets and require minimal	Multiple protocols have to be followed. Hence requires a skilled technician.

		steps. As a result, it is simpler to carry out.	
8	Result observation	Results are visually observed so no post-reaction analysis is required.	Post reaction analysis can be done with gel electrophoresis.
9	Establishment	Applications using LAMP assays are being explored.	RT-qPCR is an established technique.

2.2.2 Protein-based techniques

In addition to ongoing nucleic acid-based testing for COVID-19 detection, Protein-based detection can be an excellent supplement for better mass screening for early measurements in a point-of-care context. Antigens and antibodies are two of the most common protein-based entities that have long been employed to identify a variety of disorders. The most common protein-based detection systems include ELISA, lateral flow-based detection, and the Latex agglutination test.

Latex Agglutination Test

latex agglutination assays have been used to detect antibodies against a variety of different viruses. A latex fixation test is used clinically in the identification and typing of many important pathogens. Agglutination tests, specific to a variety of pathogens, can be designed and manufactured for clinicians by coating microbeads of latex with pathogen-specific antigens or antibodies.

This method works on the principle of ELISA. In this method antigens or antibodies are coated on the latex bead particles and then introduce to the sample, if the sample has antigens or antibodies then agglutination is found and one can identify a particular antigen or antibody.

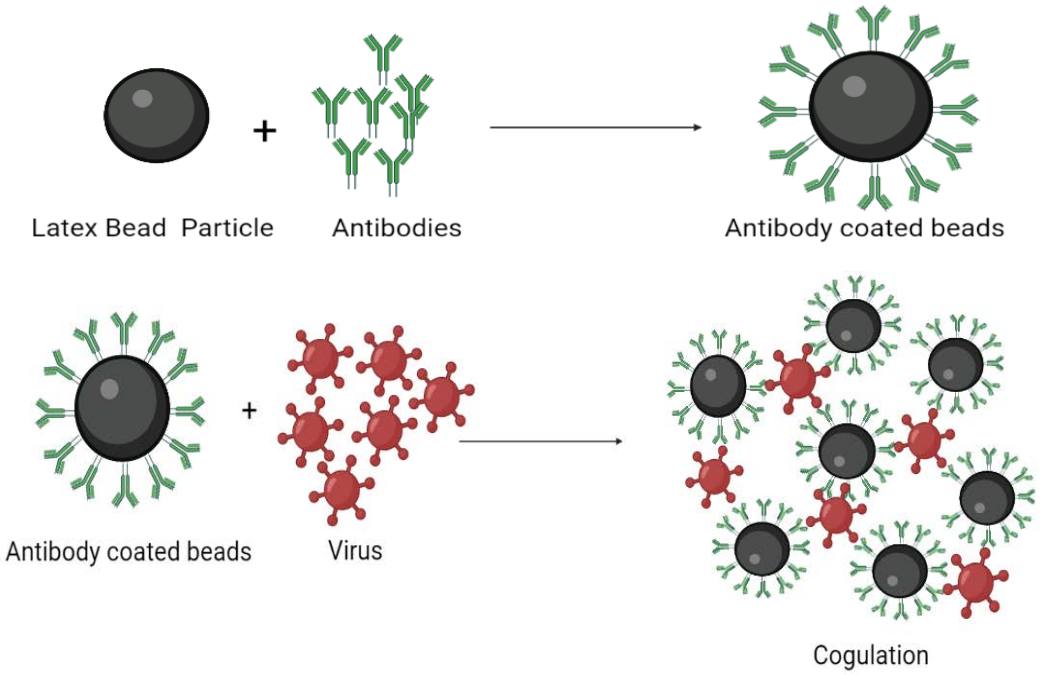


Figure 7. Representative image of latex agglutination assay

Types of binding antibodies to the latex bead particles:

1. Passive adsorption

In this type of method, monoclonal antibodies were directly bound to the latex bead particles and do not need any linker molecule on the surface of the bead.

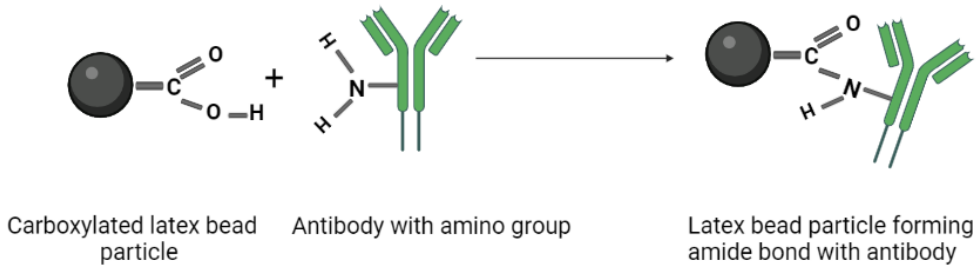


Figure 8. Passive adsorption on latex bead particle

2. Covalent adsorption

Immunoglobulins can easily be immobilized on plain polystyrene, to prevent this activity co-valent adsorption method was used. This method contains an adaptor molecule as NHS-EDC. EDC is a water-soluble cross-linker agent, which forms amide bonds between the carboxyl group of polystyrene beads and the amine group of antibodies. These antibodies bind to the beads, which were later used as an important reagent in further experiments.

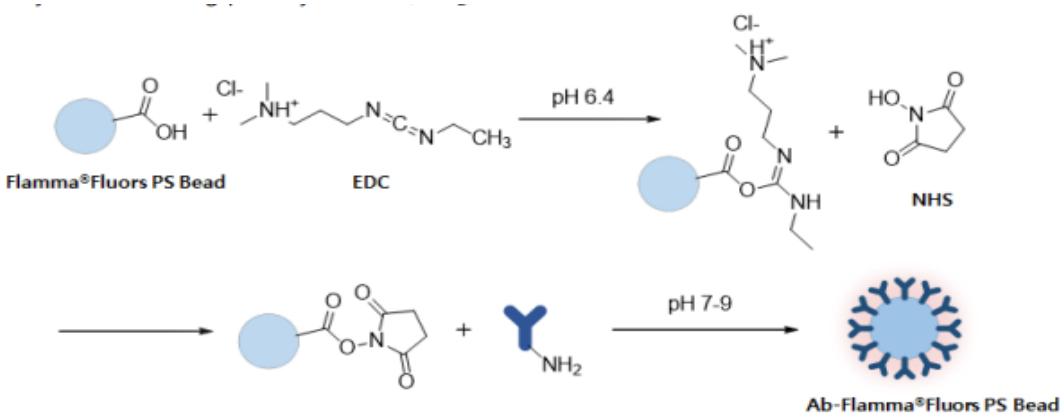


Figure 9. Co-valent adsorption on latex bead particle

2.3 Method scouting

Here is a comparative analysis of different parameters of developed techniques shown in Table 3.

Table 3. Comparative analysis of developed techniques

Parameters	LAMP	Real-time RT-PCR	Latex agglutination assay
Specificity	High	High	High
Robustness	High	High	High
Speed of obtaining results	Fast	Slow compared to LAMP	Very Fast
Reliability / accuracy / precision	Very good	Excellent	Good

Chapter III
Material & Method

3.1 Materials

Materials were used for the development of the identity test mentioned below:

Table 4. Materials used for identity test development

Sr No.	Equipment/ Instrument	Make
1.	Pipettes of different range (1-10µL, 1-20µL, 20-200µL, 100-1000µL)	Eppendorf
2.	Biosafety Cabinet	Esco Life science
3.	Freezer (-20°C)	DAIRE
4.	Laminar airflow	Microfilt
5.	Microcentrifuge	Minispin plus
6.	Refrigerated microcentrifuge	Thermo Scientific
7.	PCR Machine	Applied biosystem 7500
8.	Vortex	SPINIX
9.	0.2 ml PCR tube	Eppendorf
10.	Thermal cycler	Applied biosystem
11.	Water bath	Labycare
12.	1.5 ml microcentrifuge tube	Eppendorf
13.	Nucleo-clean spray	Merck
14.	PCR reaction plate	Applied biosystem
15.	PCR Cooler	Eppendorf
16.	Electrophoresis unit	Biorad
17.	Benchtop cooler	Tarson
18.	Support base	Applied biosystem
19.	Optical adhesive film	Applied biosystem
20.	Aluminium foil	Superwrap
21.	QIAamp Viral RNA Kits	QIAGEN
22.	Ethanol	Pharmaco
23.	Gel electrophoresis unit	Biorad
24.	Spectrophotometer	Tecan

3.2 Methods

Different methods used during the development of the identity tests are mentioned here.

3.2.1 RNA extraction for LAMP / Real-time RT-PCR assay:

RNA extraction was done using QIAamp Viral RNA Kits using the standard protocol provided by the manufacturer given below:

1. Transfer 560 µl Buffer AVL to a 1.5 ml microcentrifuge tube.
2. In the same microcentrifuge tube, add 140 µl of sample to the Buffer AVL followed by 15 seconds of pulse-vortexing
3. After a 10-minute incubation period at room temperature, centrifuge the tube briefly.
4. Pour 560 µl of ethanol (96–100%) into the sample and pulse vortex for 15 seconds. After mixing, centrifuge the tube briefly.
5. Carefully pipette 630 µl of the step 4 solution into the spin column (in a 2 ml collection tube) and centrifuge for 1 minute at 8000 rpm. Place the spin column in a clean 2 ml collection tube and discard the filtrate-filled tube.
6. Open the spin column, and repeat step 5.
7. Add 500 µl Buffer AW1 to the spin column and centrifuge for 1 minute at 8000 rpm. Place the spin column in a clean 2 mL collection tube and discard the filtrate-filled tube.
8. Carefully open the spin column and add 500 µl AW2 Buffer. Centrifuge the column at 14,000 rpm for 3 minutes.
9. Transfer the spin column to a fresh 2 ml collection tube and discard the filtrate in the previous collection tube. Centrifuge the tube at 14000 rpm for 1 minute.
10. Place the spin column in a clean 1.5 ml microcentrifuge tube. Discard the collection tube containing the filtrate. Add 60 µl Buffer AVE and incubate at room temperature for 1 minute.
11. Centrifuge at 8000 rpm for 1 minute. Discard spin column and store extracted RNA at -80°C.

3.2.2 LAMP reaction method

Primer mix preparation

1X Primer mix containing all 6 LAMP primers. FIP & BIP primer contains 1.6 µM, F3 & B3 primers contains 0.2 µM and LF & LB primer contains 0.4 µM concentration. (Zhang et al., 2020)

LAMP reaction RNA was extracted by following section 3.2.1. The reaction was incubated at 65°C for different time intervals using a water bath pr thermal cycler. The reaction mixture composition for this method is shown in (Table 5).

Table 5. Reaction mixture composition for the LAMP method

Sr. No.	Reagent	Conc.	Reaction volume (µL)	
			25	15
1	WarmStart® Colorimetric LAMP 2X Master Mix	2X	12.5	7.5
2	Primer mix	1X	2.5	1
3	NFW	-	9	5.5
4	Tested sample	-	1	1

3.2.3 Reaction mixture composition

For master mix preparation add the given component as per the required no. of reaction. The reaction mixture composition for SYBR Green chemistry and TaqMan chemistry is shown below in Table 6 and Table 7 respectively.

Table 6. Reaction mixture composition for SYBR Green chemistry

Sr. No.	Component	Conc.	Reaction volume (µL)		Final Conc.
			15	20	
1.	Power SYBR® Green RT-PCR Mix	2X	7.5	10	1X
2.	RT Enzyme Mix	125X	0.12	0.16	1X
3.	Nuclease free water	-	Makeup to 15 µL	Makeup to 20 µL	-
4.	Primer (Forward & Reverse)	2.5 µM	0.9	1.2	0.15 µm
5.	Template	-	1.5	2	-

Table 7. Reaction mixture composition for TaqMan chemistry

Sr. No.	Component	Conc.	Reaction volume (µL)		Final Conc.
			15	20	
1.	TaqMan Fast Virus 1-Step Master Mix	4X	3.75	5	1X
2.	Nuclease free water	-	Makeup to 15 µL	Makeup to 20 µL	-
3.	Primer (Forward & Reverse)	2.5 µM	0.9	1.2	0.15 µM
4.	Probe	2.5 µM	0.9	1.2	0.15 µM
5.	Template	-	1.5	2	-

3.2.4 Run Method for real-time RT-PCR reaction

Real-time RT-PCR has the same procedure as conventional PCR but in RT-PCR additionally, firstly RNA converts into cDNA followed by denaturation, annealing, and extension. Run programs for SYBR® Green and TaqMan® chemistry are mentioned in (Table 8). The threshold is set to 0.2, and the baseline is automatic.

Table 8. Run program for SYBR® Green and TaqMan® chemistry

Chemistry	cDNA synthe-sis	RT inacti-vation	Denaturation & Extension		Melt curve		
SYBR® Green	50°C / 30 min	95°C / 5 min	95°C / 15 sec	60°C / 30 sec*	95°C / 15 sec	60°C / 30 sec	95°C / 30 sec
			40 cycles		1 cycle*		
TaqMan®	50°C / 30 min	95°C / 5 min	95°C / 15 sec	60°C / 30* sec			
			40 cycles				

*Fluorescence captured at this stage

3.2.5 Method for latex bead agglutination test

100µL LBP (Sigma-Aldrich, US) were spin at 12000 rpm for 3 min at 4°C to remove extra surfactants. LBP were washed three times with washing buffer followed by spin at 12000 rpm for 3 min at 4°C. For passive adsorption of monoclonal antibody against anti-spike S1 protein-coated to LBP by incubating it for 2 hrs / 24 hrs. After completion of incubation, LBP were washed with washing buffer 3 times to remove excessive unbound antibodies following a spin at 12000 rpm for 3 min at 4°C. The supernatant of

every wash had been stored to check the amount of protein-bound to LBP (Bradford method). For covalent adsorption 20µL of each NHS & EDC (Sigma-Aldrich, US).

The solution is used as an adaptor molecule to increase the surface area of LBP and provide more space for antibodies to binding. Antibodies were coated as mentioned above. 50µL of BSA (Sigma-Aldrich, US) was added for blocking empty spaces on LBP for 1 hr of incubation to antibody-coated LBP followed by 3 washes with the same spinning method and store the supernatants. Add 100µL of Coronavirus (1:5 dilution) as an antigen on antibody-coated LBP and incubated it for 10 min to check agglutination.

3.2.6 Bradford Method

Bradford method used for protein estimation, steps to perform this assay are described below: Prepare BSA standards (as shown in Table 9) and sample dilutions in PBS. Add 50 µl sample/standards as per the plate template given below. Add 150 µl Bradford reagent per well. Incubate 96-well plate at room temperature for 30 min. After incubation is over take a reading at 595 nm.

Table 9. Preparation of BSA standards

Standard	Final BSA conc. (µg/mL)	Vol of diluent (PBS) µl	Volume (µl) and source of BSA
Std 1	1000	200	200 of 2 mg/ml stock
Std 2	500.0		200 of Std 1
Std 3	250.0		200 of Std 2
Std 4	125.0		200 of Std 3
Std 5	62.5		200 of Std 4
Std 6	31.3		200 of Std 5
Std 7	15.6		200 of Std 6
Std 8	7.8		200 of Std 7

Chapter IV
Result & Discussion

4.1 Results of LAMP

Experiments were done during the development of the identity test are mentioned below:

4.1.1 Primer suitability

Two sets of primers (Sigma-Aldrich, US), primer set I and set II were checked for their suitability with SuperScript™ IV RT-LAMP Master Mix (ThermoFisher, US). The reaction was performed in duplicate and the volume considered was 25µL for each reaction (Table 5). Samples tested were NTC, MR-COVID19 vaccine and gB_CoV_NC. RNA was extracted by following section 3.2.1. Tests were run in a PCR machine at 65°C for 30 min followed by 95°C for 30 sec for 60 cycles.

NTC, test sample found amplified while positive control was not amplified using primer set I. While NTC, positive control was not amplified and the test sample was amplified with primer set II. Positive control does not show amplification because of some short stretch of nucleotides found to be missing. Hence, primer set II was found suitable to reagent SuperScript™ IV RT-LAMP Master Mix. To ensure the amplification process, LAMP PCR products were gel-electrophoresed on 2.5% agarose gel. Amplified products are shown in the image mentioned in Fig 10.

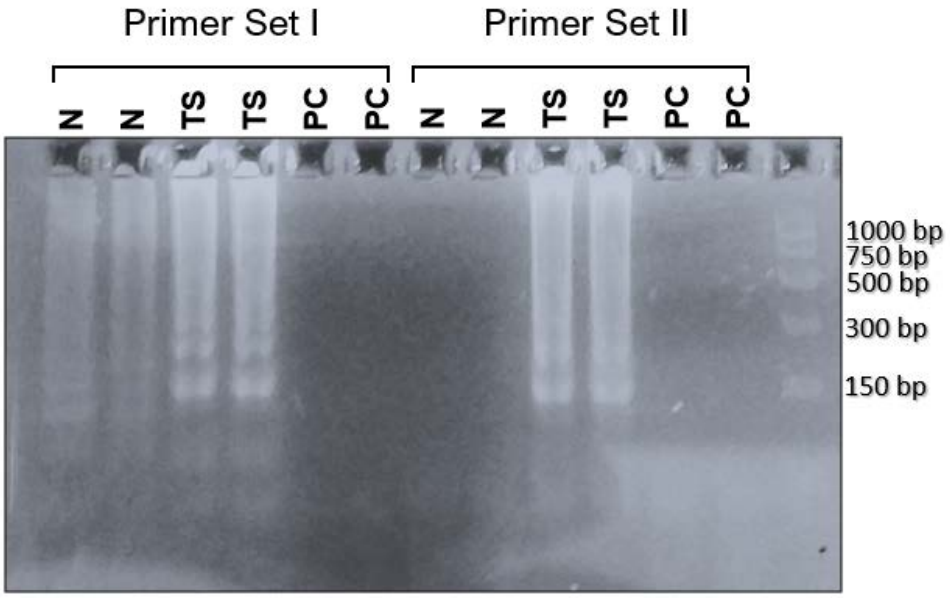


Figure 10. LAMP PCR product gel electrophoresed on 2.5% gel
N: No template control; TS: Test sample; PC: Positive control

4.1.2 Reagent suitability and establishment of positive control:

WarmStart® Colorimetric LAMP 2X Master Mix (NEB, US) is a colorimetric reagent and does not require any dye to detect the result so reagent suitability needs to be observed with primers. In this test WarmStart® Colorimetric LAMP 2X Master Mix was used with primer set II (Table 5).

In the primer suitability test positive control was found missing a short segment of nucleotides hence another positive control PCR product Amplicon no.36 synthesized with help of whole-genome sequencing diluted to 10^5 in TE buffer was used. Reaction incubated at 65°C for 30 min. Color changes from pink to yellow show amplification of the target gene.

NTC remained pink in color indicating no amplification process occurred, test sample and positive control showed a color change from pink to yellow which indicates amplification of the target gene at 65°C for 30 min. (Table 10, Fig 11)

Table 10. Observation of color change for reagent suitability and establishment of positive control

Sr. No.	Sample	Sample type	65°C/30 min
1	NFW	Negative control	-
2	CoV RNA	Test sample	+
3	PCR product – Amplicon no. 36 (10^5 dilution in TE buffer)	Positive control	+

Figure 11. Visual observations of LAMP assay reactions for the establishment of positive control: Reaction tubes incubated at 65°C for 30 min. NTC: No template control; CoV: Coronavirus RNA; PC: Positive control.

4.1.3 Assessment of detection range and incubation time

The assay was assessed w.r.t parameters like detection range and incubation time.

Detection range - Intranasal vaccine is filled at a set titer of 7.0 $\log_{10}$ PFU / 0.5 ml. While the injectable vaccine for coronavirus vaccine strain component is not less than 3.0 $\log_{10}$ PFU / 0.5 ml. Therefore, the working range of the 3.0 – 7.0 $\log_{10}$ PFU / 0.5 ml was evaluated in the assay. Dilution of half the concentration of lower vaccine titer (2.7 $\log_{10}$ PFU/0.5 mL) was also tested (sample dilutions details given in Table 11) and RNA was extracted by following section 3.2.1

Table 11. Dilution of samples

Sr No.	Intended titer (log10 PFU/0.5 mL)	Stock volume (µL)	Diluent (µL)
1	7.0	427 of neat	573
2	6.0	100 of 1	900
3	5.0	100 of 2	900
4	4.0	100 of 3	900
5	3.0	100 of 4	900
6	2.7	500 of 5	500

Incubation time: The kit manufacturer has recommended the reaction incubation time of 30 min at 65 °C and observed color change at time intervals from 15 min to 30 min.

Table 12. Observations of detection range

Sample Tested	Sample type	Incubation time		
		15 min	20min	30 min
NFW	No template control	-	-	-
Bulk diluent	Negative extraction control	-	-	-
CoV (Titer: 7.0)	Test sample	+	+	+
CoV (Titer: 4.0)		-	+	+
CoV (Titer: 3.0)		-	+	+
CoV (Titer: 2.7)		-	+	+
PCR Product (Amplicon no. 36)	Positive control	+	+	+

+: Yellow color, –: Pink color,

The samples prepared from COVID-19 vaccine containing titer ranging between 2.7 to 7.0 log_{10} PFU/0.5 mL were tested positive (Table 12). No template control and negative extraction control were negative that indicating no external factor interfere with the reaction and positive control was amplified and the color changed from pink to yellow. (Fig. 12)

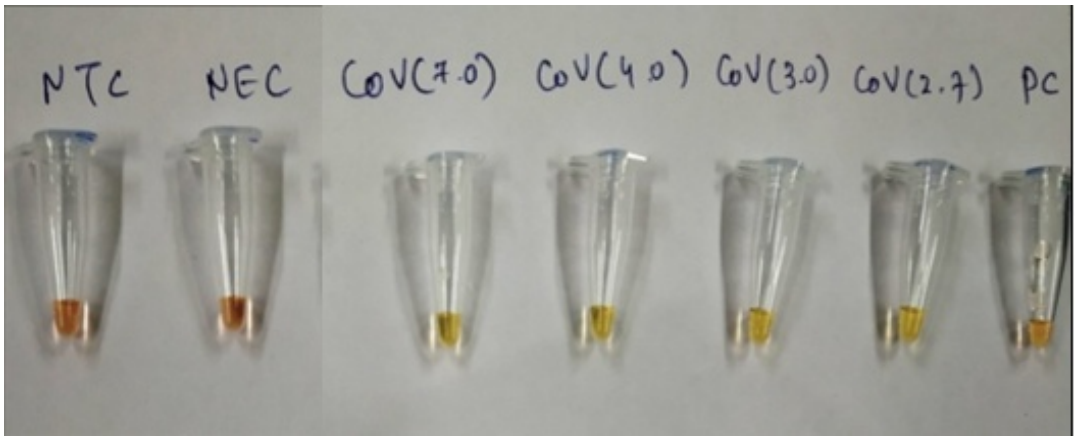


Figure 12. Visual observations of LAMP assay reactions for detection range: Detection range of LAMP assay reaction tested with Coronavirus titer – 2.7 to 7.0 log_{10}PFU / 0.5 ml titer. NTC: No template control; NEC: Negative extraction control; CoV (7.0): Coronavirus RNA (7 log_{10} PFU / 0.5 ml); CoV (4.0): Corona virus RNA (4 log_{10} PFU / 0.5 ml); CoV (3.0): Corona virus RNA (3 log_{10} PFU / 0.5 ml); CoV (2.7), Coronavirus RNA (2.7 log_{10} PFU / 0.5 ml); PC: Positive control (PCR product Amplicon no. 36 -5 log dilution).

4.1.4 Assay specificity and robustness

Specificity - To check the specificity of primers used in the assay, samples of other virus samples (Measles and Rubella virus) were included to test false-positive amplification.

The assay robustness was tested w.r.t. following:

1. **Primer concentration** - Primer concentration was tested at a half concentration (0.5X) of the original reaction.
2. **Incubation time** - The reactions were incubated at 65 °C by extending the incubation time to 40 min.

Negative control, negative extraction control, and specificity control of measles and rubella were tested negative. The test sample and positive control sample reactions containing 1X primer concentration were tested positive (Table 13). The reaction containing primer 0.5X and coronavirus RNA (titer 2.7 $\log_{10}$ PFU / 0.5 ml) was negative till 20 min but later tested positive after 40 min. The color change was stable up to 24h (Fig. 13).

Table 13. Observation of samples at different incubation times and temperature

Sample Tested	Sample type	Primer Conc.	Incubation time / temperature			
			0 min/ 25 ±3 ºC	20min/ 65ºC	40 min/ 65ºC	24 hr/ 25 ±3 ºC
NFW	No template control	1X	-	-	-	-
		0.5X	-	-	-	-
Bulk diluent	Negative extraction control	1X	-	-	-	-
		0.5X	-	-	-	-
Me (CVP)	Negative control for specificity	1X	-	-	-	-
		0.5X	-	-	-	-
Ru (CVP)		1X	-	-	-	-
		0.5X	-	-	-	-
CoV (Titer:2.7)	Test sample	1X	-	+	+	+
		0.5X	-	-	+	+
MRCoV		1X	-	+	+	+
		0.5X	-	+	+	+
PCR Product (Amplicon no. 36)	Positive control	1X	-	+	+	+
		0.5X	-	+	+	+

+ : Yellow color; – : Pink color

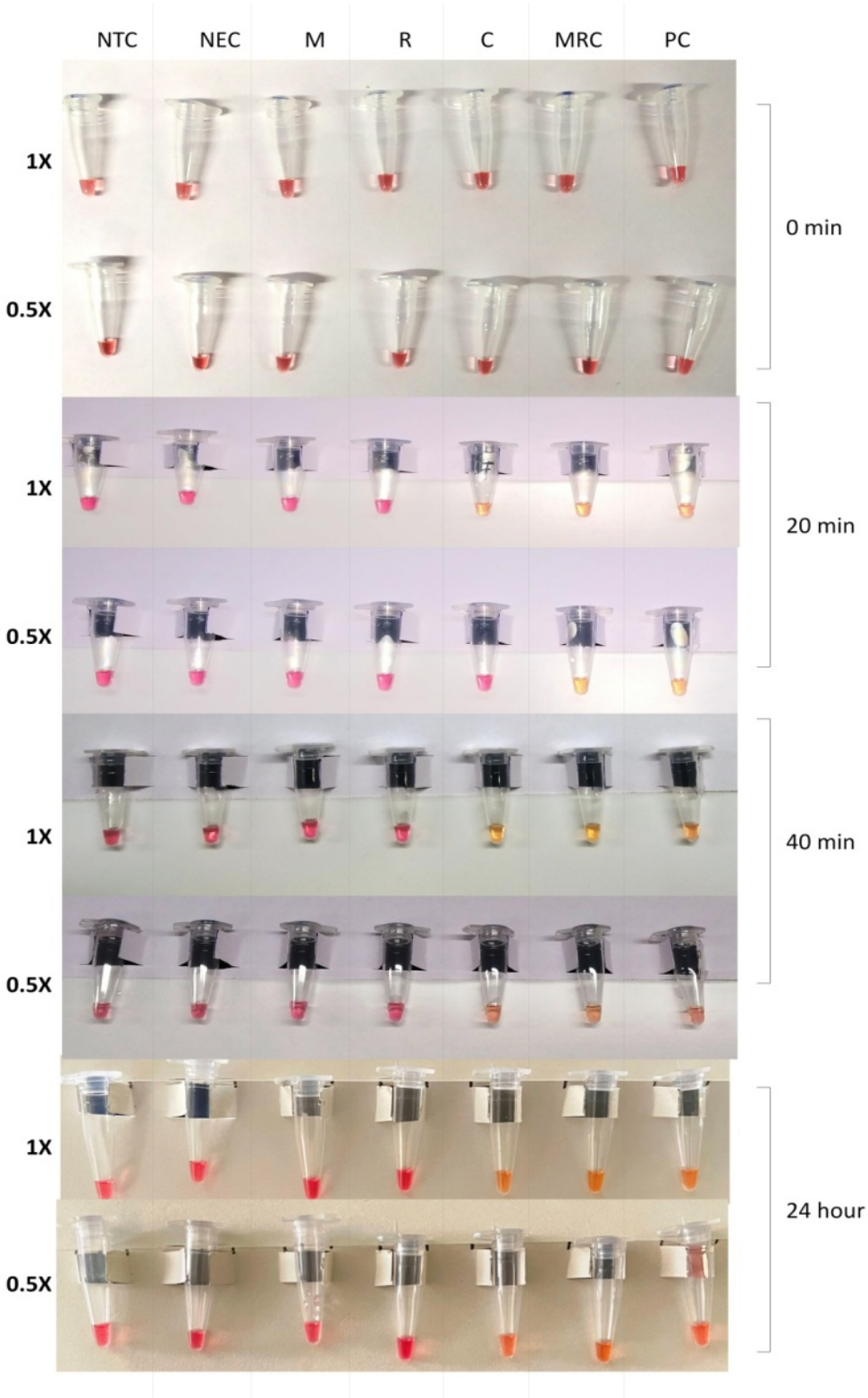


Figure 13. Visual observations of evaluation for primer concentration & incubation time: The tubes were incubated at 65 °C for 40 min. After incubation tubes were held at 25 ± 3 °C and photographed. NTC: No template control; NEC: negative extraction control; M: Measles RNA; R: Rubella RNA; C: Coronavirus RNA; MRC: MR-COVID19 Vaccine RNA; PC: positive control (Amplicon no. 36, -5 log dilution)

4.1.5 Spectrum reading:

Absorbance maxima of the positive and negative samples were determined by taking spectral absorbance on pooled negative and positive PCR products in a spectrophotometer. For clarity spectral readings from 350 to 680 nm are shown below (Fig 14). A clear shift in spectral absorption maxima from 560 nm (pink) to 430 nm (yellow) was observed in the positive PCR products and was visibly distinguishable.

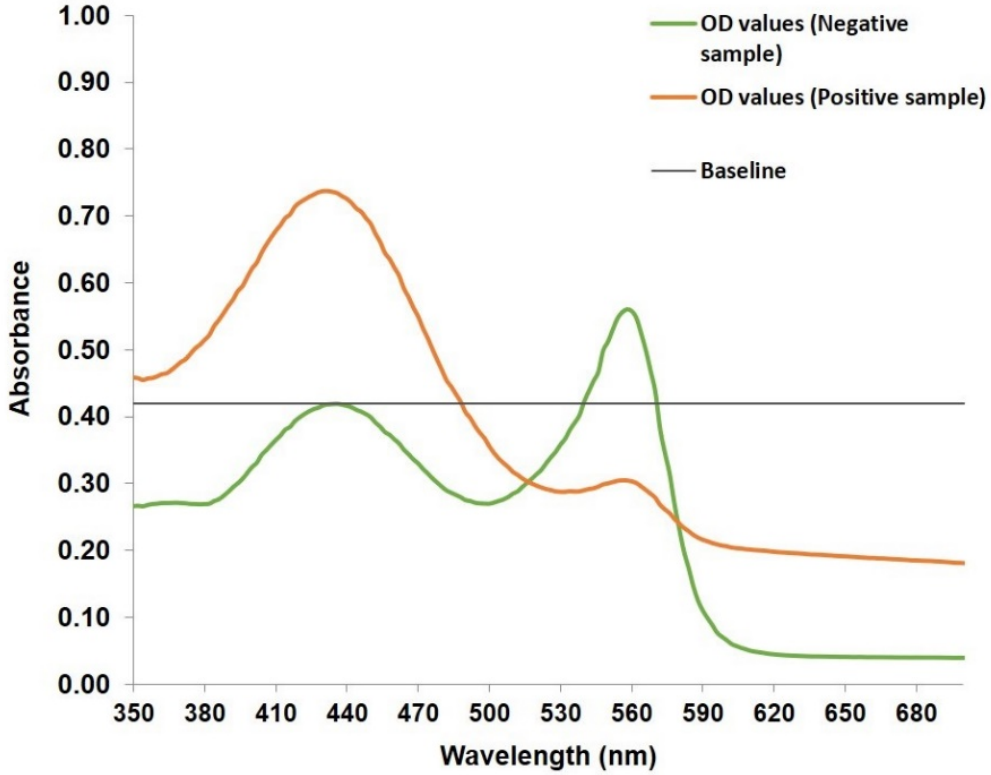


Figure 14. Absorption spectrum graph of LAMP products.

4.2 Results – Real-time RT-PCR

Experiments were done during the development of the identity test are mentioned below:

4.2.1 Specificity of primers using SYBR chemistry

Different sets of primers were checked for their specificity against the test sample using SYBR Green chemistry for 20µL reaction volume. The reaction composition and run program are mentioned in (Tables 6 & 8) respectively. Samples tested were Measles virus, Rubella virus, Coronavirus, and MR-COVID19 vaccine corresponding to their primers. A different set of primer details is given in Table 14.

Table 14. Primer used for specificity using SYBR® chemistry

Forward Primer	Me2	Ru1	COV
Reverse Primer	Me2	Ru1	COV
Final Conc. (µM)	0.3	0.9	0.6

Primer mix (forward primer and reverse primer) and template added along with master mix (reagent) are shown in Table 14.1

Table 14.1. Primer mix & template addition layout

</>	1	3	5	7	9
A	Me2+ NFW	Me2+ MeV	Me2+ RuV	Me2+ CoV	Me2+ MRCoV
B					
C					
D	Ru1 + NFW	Ru1 + MeV	Ru1 + RuV	Ru1 + CoV	Ru1 + MRCoV
E					
F					
G	COV+ NFW	COV+ MeV	COV+ RuV	COV+ CoV	COV+ MRCoV
H					

RuV: Rubella virus RNA, CoV: Coronavirus RNA, MRCoV: MR-COVID19 vaccine RNA

All the primer sets give specific binding to a target gene. Ct value for each primer set mentioned in Table 14.2. The melt curve of each primer showed a single peak (Fig.15).

Table 14.2 Ct Value

</>	1	3	5	7	9
A	UD	26.72	UD	UD	26.56
B	UD	26.15	UD	UD	26.87
C					
D	UD	UD	31.78	UD	31.44
E	UD	UD	31.15	UD	31.35
F					
G	UD	UD	UD	24.28	22.42
H	UD	UD	UD	23.73	22.13

UD: Undetermined

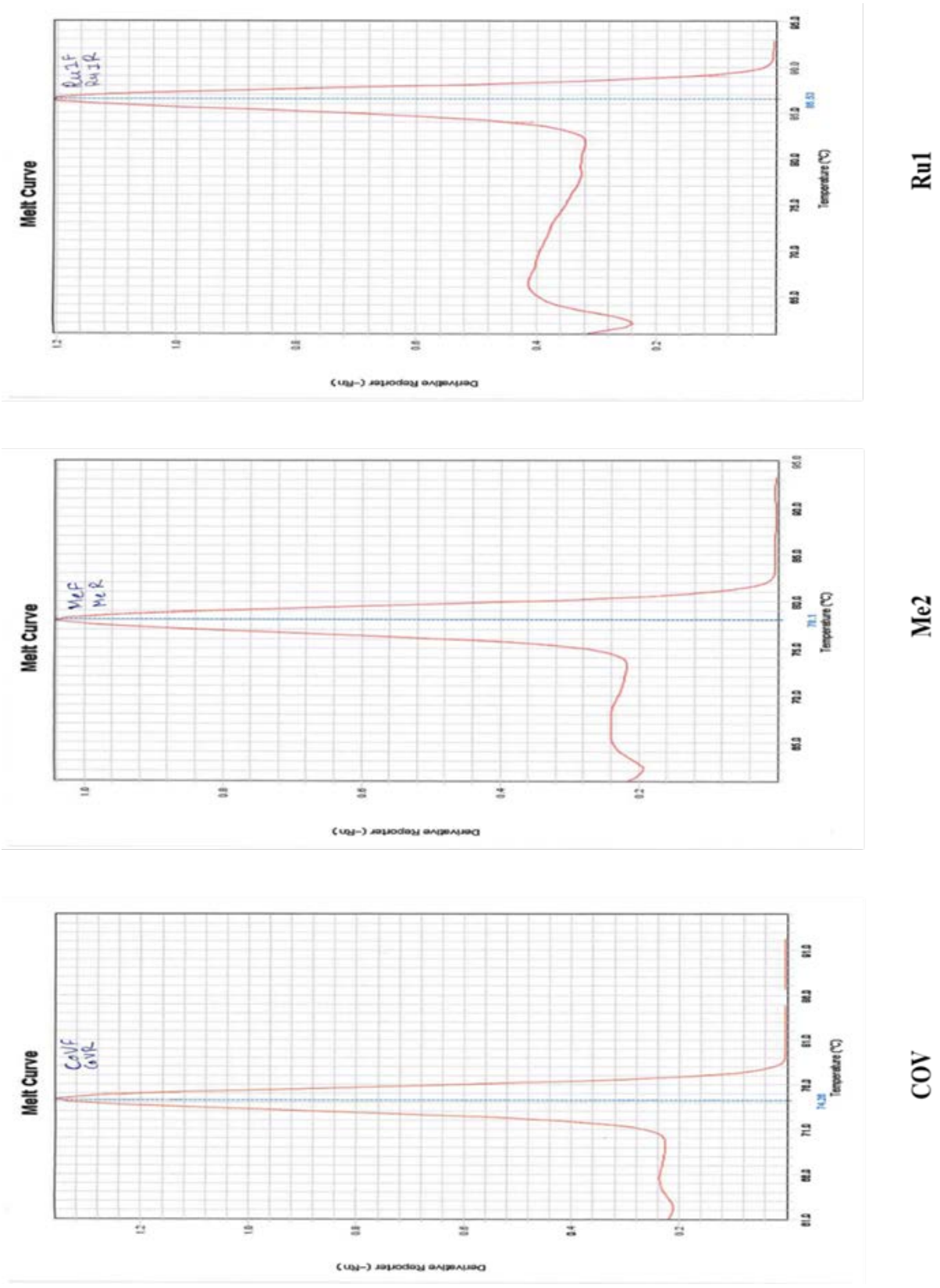


Figure 15. Melting curve of primers used for specificity with SYBR® Green chemistry

4.2.2 Specificity of primers using Taqman® chemistry

A different set of primers and probes were checked for their specificity against the test sample using Taqman® chemistry for 20µL reaction volume. The reaction composition and run program are mentioned in (Tables 7 & 8) respectively. Samples tested were Measles virus, Rubella virus, Coronavirus, and MR-COVID19 vaccine corresponding to their primers. A different set of primer details is given in Table 15.

Table 15. Primer – probe used for specificity using TaqMan® chemistry

Forward Primer	Me	Ru1	COVNC
Reverse Primer	Me	Ru1	COVNC
Probe	Me	Ru1	COVNC
Final Conc. (µM)	0.15	0.15	0.15

Primer (Forward and reverse primer) – probe mix and template added along with master mix shown in Table 15.1

Table 15.1. Primer-probe mix and template addition layout

</>	1	3	5	7	9
A	Me.Ru1.COVNC+ NFW	Me.Ru1.COVNC+ MeV	Me.Ru1.COVNC+ RuV	Me.Ru1.COVNC+ CoV	Me.Ru1.COVNC+ MRCOV
B					

RuV: Rubella virus RNA, CoV: Coronavirus RNA, MRCoV: MR-COVID19 vaccine RNA

All the primer sets give specific binding to the target gene. Ct value for each primer set mentioned in Table 15.2 and amplification plot shown in Fig. 16.

For Measles, Rubella, and Coronavirus the Fluorophore molecules are FAM, CY5, and JOE respectively.

Table 15.2. Ct value

</>		1	3	5	7	9
A	Target- FAM	ND	16.41	ND	ND	25.36
	Target – CY5	ND	ND	21.52	ND	29.65
	Target - JOE	ND	ND	ND	11.32	22.73

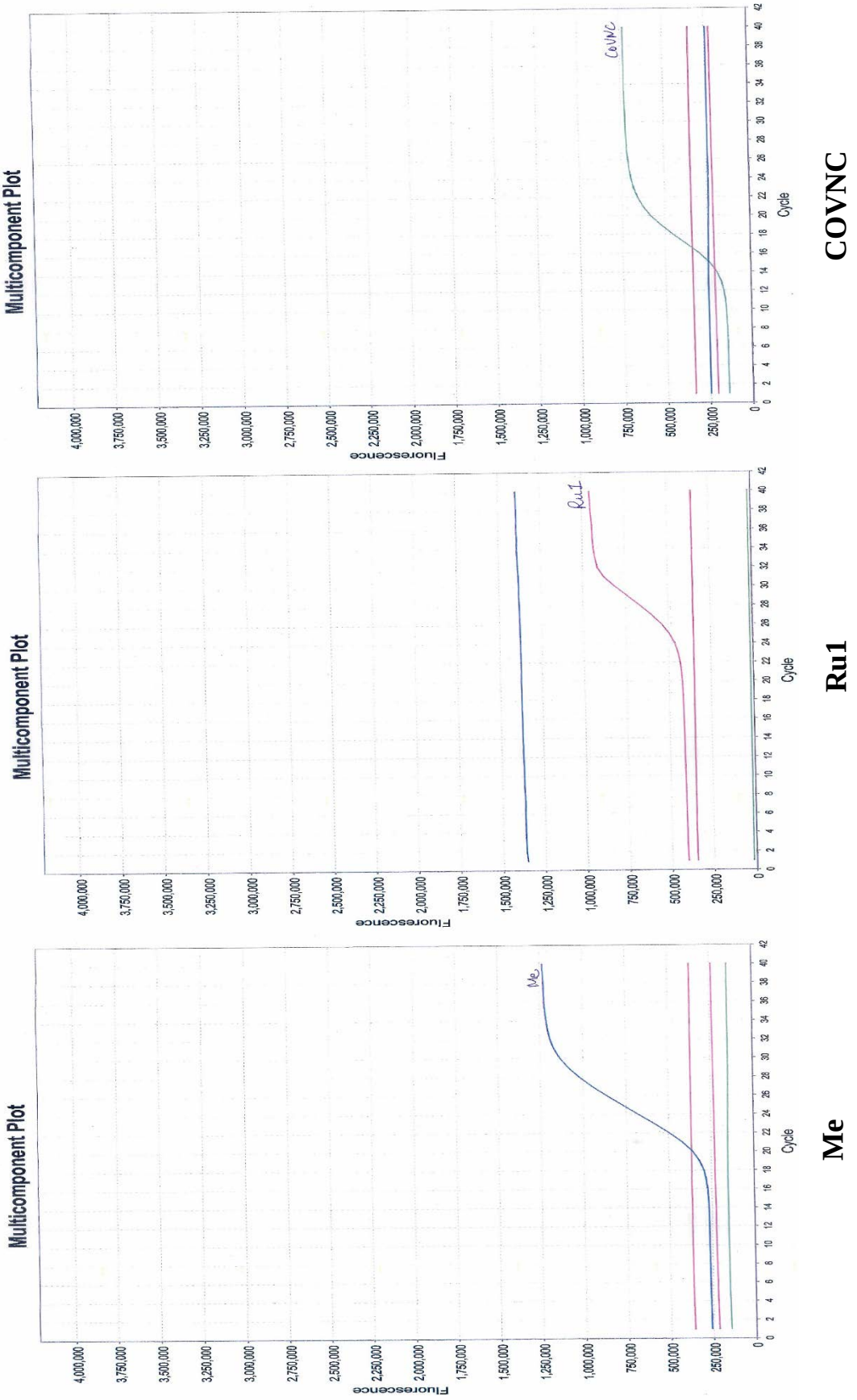


Figure 16. Amplification of primers used for specificity with TaqMan® chemistry

4.2.3 Reagent suitability

Primers were tested with another manufacturer's reagent (SOLIS BIODYNE) and compared with an established reagent (Thermo Fisher). Test sample MRCoV was tested against primers of Measles, Rubella, and Coronavirus in singleplex and multiplex with both of the reagents.

RNA of the test sample was isolated as mentioned in section 3.2.1. For each reaction, 10% overages were added in reaction composition (Table 6) and run method as mentioned in (Table 8).

Table 16. Primer used to check reagent suitability

Forward Primer	Me2	Ru1	COV
Reverse Primer	Me2	Ru1	COV
Final Conc. (μM)	0.3	0.9	0.6

Primer mix (Forward and reverse primer) and template added along with master mix shown in Table 16.1

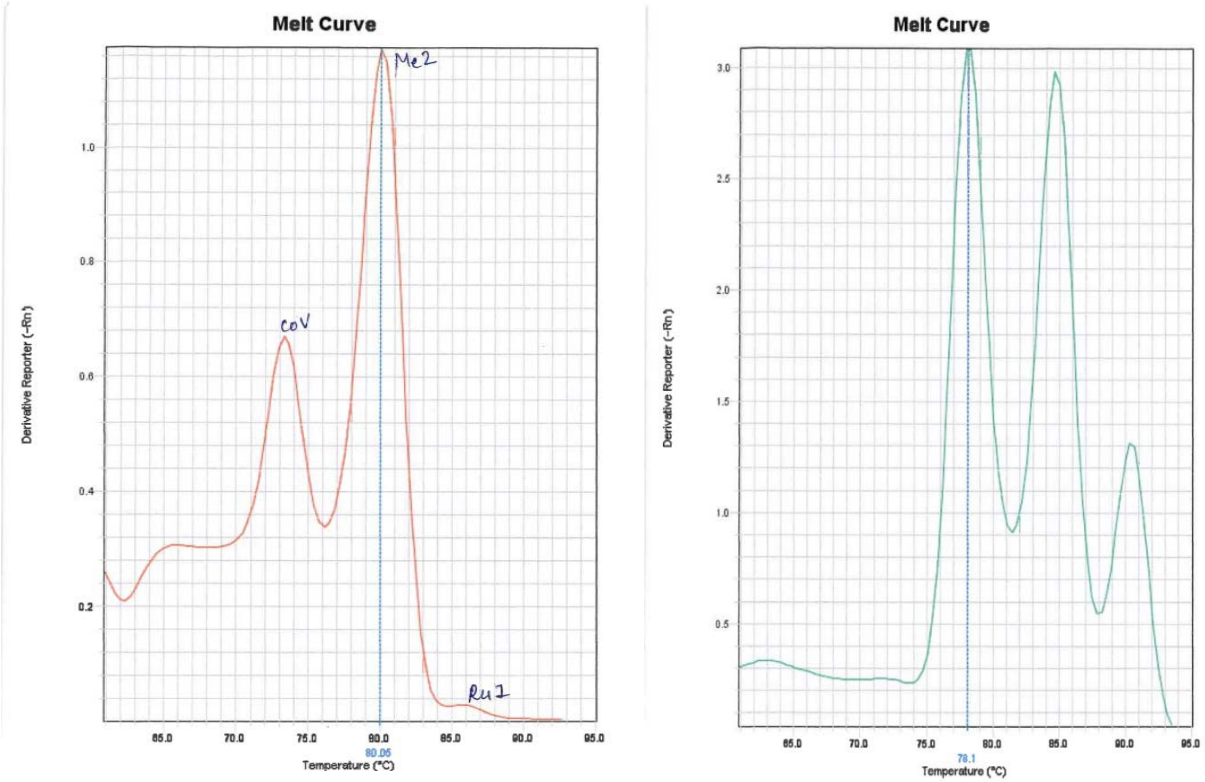
Table 16.1. Primer mix and template addition layout

</>	1	3	5	7	9
A	Me2.Ru1. COV +NFW	Ru1 + MRCoV	Me2 + MRCoV	COV + MRCoV	Me2.Ru1. COV + MRCoV
B					
C					
D	Solis Mix + Me2.Ru1.COV + NFW	Solis Mix + Ru1 + MRCoV	Solis Mix + Me2 + MRCoV	Solis Mix + COV + MRCoV	Solis Mix + Me2.Ru1.COV + MRCoV
E					

Table 16.2. Ct value

</>	1	3	5	7	9
A	UD	32.91	26.28	23.03	23.06
B	UD	32.85	26.08	22.88	22.92
C					
D	UD	28.26	25.73	20.58	28.17
E	UD	27.97	25.77	20.5	19.66

In the melting curve Rubella primers show a small peak in a multiplex while, using SOLIS BIODYNE reagent all of the primers in multiplex, gave a sharp peak in the melting curve Fig 17.



(A) ThermoFisher **(B) SOLIS BIODYNE**

Figure 17. Melting curve of multiplex reaction using (A) ThermoFisher (B) SOLIS BIODYNE

4.2.4 Establishment of positive control and dose-response

Dose-response of MR-COVID19 vaccine evaluated using positive control – gBlocks for all the three virus components. gBlocks having 10nM concentration was serially diluted to achieve concentration of 1nM, 100pM, 10pM, 1pM, 100fM, 10fM in TE buffer. Each reaction volume contains 20µL and is performed in a singleplex considering 10% overages, using TaqMan® chemistry. The reaction composition mentioned in Table 7 and the run method were followed as described in Table 8. Primer-probe mix prepared as shown in Table 17.

Table 17. Primer-probe used for dose-response

Forward Primer	Me	Ru1	COVNC
Reverse Primer	Me	Ru1	COVNC
Probe	Me	Ru1	COVNC
Final Conc. (µM)	0.15	0.15	0.15

Primer mix (Forward and reverse primer) and template added along with master mix shown in Table 17.1

Table 17.1. Plate layout of Mastermix and template addition

</>	**1**	**3**	**5**	**7**	**9**	**11**	**12**
	NTC	**1nM**	**100pM**	**10pM**	**1pM**	**100fM**	**10fM**
A	Me + NFW	Me + gB_MeFP	Me + gB_MeFP	Me + gB_MeFP	Me + gB_MeFP	Me + gB_MeFP	Me + gB_MeFP
B							
C							
D	Ru1 + NFW	Ru1 + gB_RuSPP1	Ru1 + gB_RuSPP1	Ru1 + gB_RuSPP1	Ru1 + gB_RuSPP1	Ru1 + gB_RuSPP1	Ru1 + gB_RuSPP1
E							
F							
G	COVNC + NFW	COVNC + gB_COVNC	COVNC + gB_COVNC	COVNC + gB_COVNC	COVNC + gB_COVNC	COVNC + gB_COVNC	COVNC + gB_COVNC
H							

gB: gBlock

From the Ct value finalized dose-response concentration for Measles, Rubella and Coronavirus is 1pM, 100pM, 100pM respectively. The multicomponent curve showed that the concentration of gblock is proportional to the Ct value. As concentration decreases Ct value decreases (Fig 18).

Table 17.2. Ct value

</>	**1**	**3**	**5**	**7**	**9**	**11**	**12**
	NTC	**1nM**	**100pM**	**10pM**	**1pM**	**100fM**	**10fM**
A	ND	6.97	10.91	14.02	17.44	20.51	24.55
B	ND	6.83	10.94	13.79	17.29	20.43	24.53
C							
D	ND	UD	17.4	19.05	21.97	25.46	28.73
E	ND	UD	17.41	18.98	21.99	25.57	28.7
F							
G	ND	14.3	17.48	19.09	26.27	26.54	30.83
H	ND	13.45	17.56	18.95	25.97	26.58	29.81

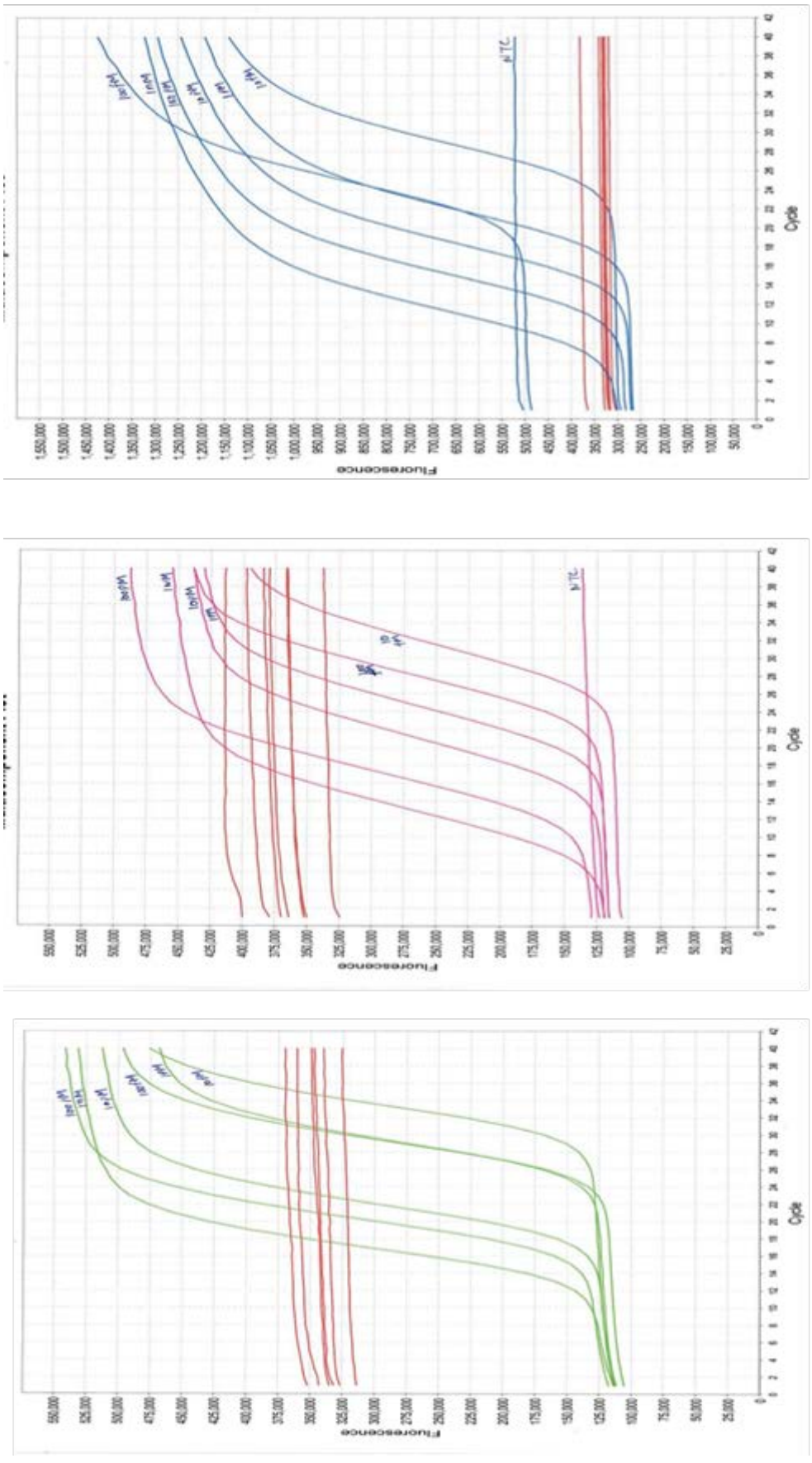


Figure. 18 Graph of gblock of Measles, Rubella, and Coronavirus having different concentrations.

4.2.5 Optimization of sample detection range

The detection range of the sample was optimized to check the sensitivity of the assay w.r.t a small amount of RNA could be identified or not. To optimize the detection range of the sample, the dilution of the MR-COVID19 vaccine was diluted in 1:2, 1:4 & 1:8, and RNA was isolated as mentioned in section 3.2.1. As a positive control concoction of the gBlock (MeFP + Ru1SPP1 + COVNC) was used. Bulk diluent was used as a negative extraction control and Coronavirus vaccine was spiked to bulk diluent used as a test sample. Primer-probe mix details have been mentioned in the table given below. (Table 18). Three different probes were used and all probes have their fluorophore molecule. For Measles, Rubella, and Coronavirus probe reporter dye is FAM, CY5, and JOE respectively. Reaction mixture preparation (Table 7) and run program mentioned in (Table 8) was followed.

Table 18. Primer-probe used for a detection range of sample

Forward Primer	Me	Ru1	COVNC
Reverse Primer	Me	Ru1	COVNC
Probe	Me	Ru1	COVNC
Final Conc. (μM)	0.15		

Table 18.1. Master mix & template addition plate layout

</>	1	3	5	7	9	12
A	Me.Ru1. COVNC + NFW	Me.Ru1. COVNC + Neat sample	Me.Ru1. COVNC + 1: 2 sample	Me.Ru1. COVNC + 1:4 sample	Me.Ru1. COVNC + 1:8 sample	Me.Ru1. COVNC Gblock (MeFP + RuSPP1 + COVNC)
B						

Table 18.2. Ct value

</>	1	3	5	7	9	12
A	UD	20.01	22.94	24.95	25.73	15.96
	UD	21.77	24.38	25.95	26.95	19.15
	UD	27.99	30.74	32.63	35.31	17.25
B	UD	20.06	22.85	24.44	25.5	15.98
	UD	21.88	24.39	25.96	26.79	19.35
	UD	28.35	30.9	33.03	35.37	17.24

JOE – CoV FAM- Measles CY5-Rubella

UD: undetermined

The result showed Ct value of the neat sample, 1:2 diluted sample, 1:4 diluted sample, and 1:8 diluted sample of MR-COVID19 vaccine gradually increase (Table 18.2) which indicates the sensitivity of an assay that less amount of RNA sample can also be detected. Fig19 shows a comparative analysis of the test sample with a neat sample and positive control.

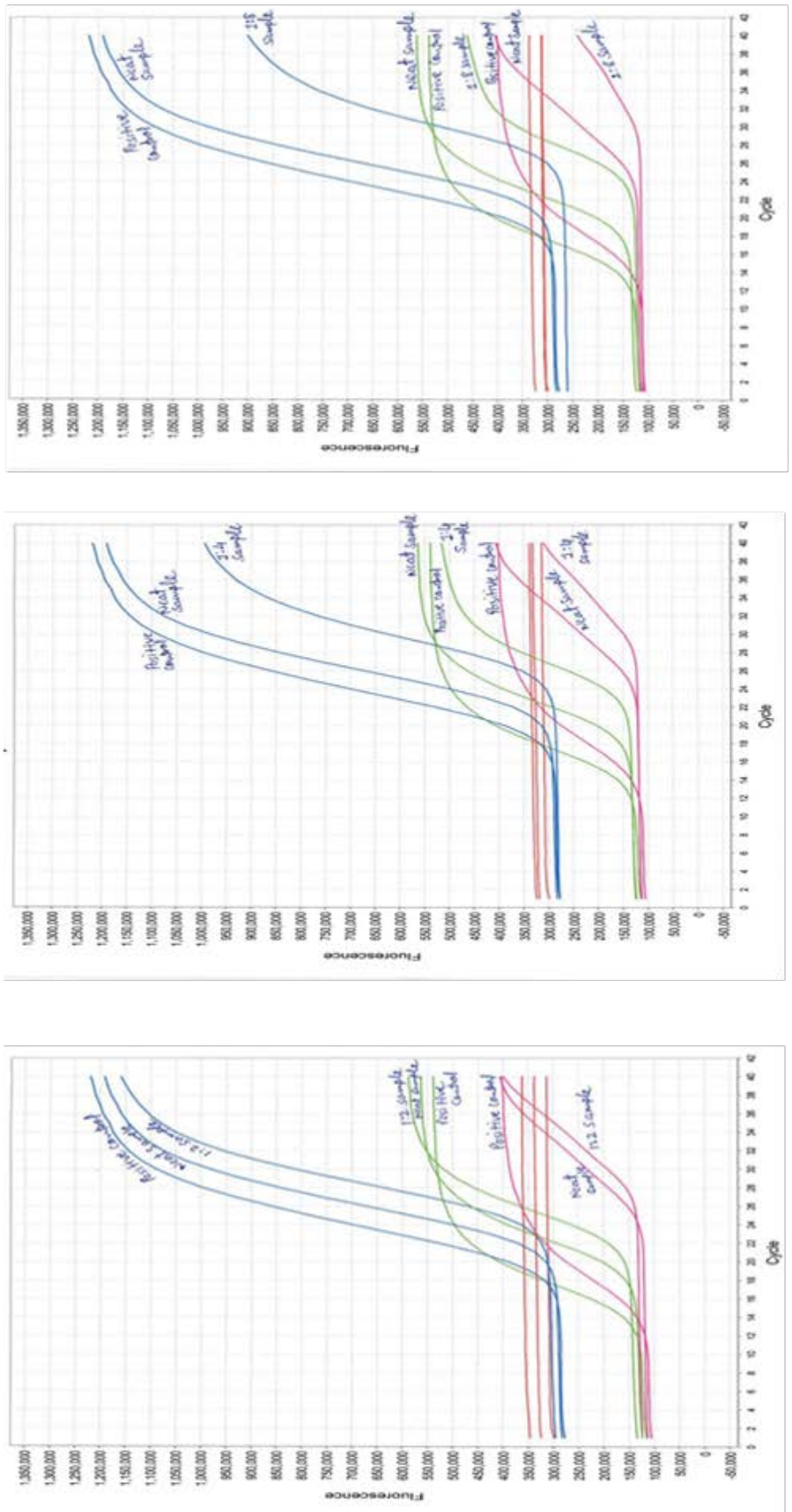


Figure 19. Graph showing samples tested for detection range

4.3 Results – Latex agglutination assay

Experiments were done during the development of the identity test are mentioned below:

4.3.1 Passive adsorption of monoclonal antibodies to a latex bead

LBP were washed with washing buffer and Mab was used to coat the beads with passive adsorption by following the method as described in section 3.2.5. Several beads were counted manually by 1:1000 dilution of beads under an inverted microscope.

Table 19. Details of beads used in passive adsorption

Exp No.	Bead size	Surface area/ml	No. Of beads	Washing buffer	Amount of antibody use
1	0.8 µ	2.7×10^{11}	18×10^{7} beads / ml	PBS 1X (pH-7.2),	50 µg
2	1.1 µ	1.98×10^{11}		PBST	2 mg

The observation made during the test showed the clumping of beads in smaller-sized LBP. After the addition of antigen LBP were incubated for 10 min and overnight, no agglutination was seen.

4.3.2 Covalent adsorption of antibodies on beads

LBP were washed with washing buffer and different types of antibodies like Monoclonal antibodies against S1 spike protein, Sera of rabbit and hamster which contain polyclonal antibodies. To coat the beads with covalent adsorption by following the same method as described in section 3.2.5. To measure protein estimation with the Bradford method by following section 3.2.6, the supernatant was used as a sample, and protein concentration was measured (Table 20).

The formula used to find % of binding capacity:

$$\% \textbf{ of binding capacity } = \frac{\textbf{IPC} - \textbf{ FPC x 1000}}{\textbf{IPC}}$$

IPC: Initial protein conc., FPC: Final protein conc.

Table 20. Details of beads used in co-valent adsorption

Exp No.	Bead size	Surface area/ml	Washing buffer	Amount of antibody use	% of binding ab on LBP
3	0.75µ	2.9×10^{11}	MES (pH-6.1)	Mab (1mg)	69
4				Pab of rabbit & hamster (500µg)	224.54 & 196.17
5				Mab + Pab of rabbit (150µg)	25.19%

Reason for non-agglutination conditions:

1. An Insufficient number of antibodies binding on LBP.
2. Orientation of antibody binding of LBP may wrong.
3. Probability of antigen could not bind with antibodies.

Chapter V
Conclusion

LAMP method used for identity test of Coronavirus vaccine can detect the lower titer of 2.7 $\log_{10}$ PFU /0.5 ml in the sample at a minimum concentration of primer. The method is specific for the detection of the Coronavirus vaccine.

Real-time RT-PCR method used for identity test of MR-COVID19 vaccine which is specific to detect samples of Measles, Rubella, and Coronavirus. The established concentration of gblock as a positive control for Measles, Rubella, and Coronavirus are 10 pM, 100 pM, and 100 pM respectively. The lower titer of antigen presented in a sample (diluted to 1:8) can also be detected.

Latex agglutination assay needs optimization for agglutination using Protein A and other parameters. Protein A-coated latex beads bind specific IgGs at the Fc portion of antibodies, resulting in increased activity due to proper orientation. Other parameters considered as pH of the buffer, No. of washes of beads, and Size of LBP need to be evaluated.

The developed Loop-mediated isothermal amplification and real-time RT-PCR method were evaluated specific, selective, sensitive, accurate, precise, and ready for qualification and validation.

Chapter VI
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