MOLECULAR DETECTION OF SARS-COV-2 RNA IN NASOPHARYNGEAL/OROPHARYNGEAL SWAB OF PATIENT WITHOUT RNA EXTRACTION

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This is to certify that the research work entitled "**MOLECULAR DETECTION OF SARS-COV-2 RNA IN NASOPHARYNGEAL/OROPHARYNGEAL SWAB OF PATIENT WITHOUT RNA EXTRACTION**" has been carried out by **Ms. Suruchi Karna** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

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I hereby declare that the thesis entitled "**MOLECULAR DETECTION OF SARS-COV-2 RNA IN NASOPHARYNGEAL/OROPHARYNGEAL SWAB OF PATIENT WITHOUT RNA EXTRACTION**" submitted to Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu for partial fulfillment of the requirement for the degree of M.Sc. in Biotechnology is a genuine work performed by me, **Suruchi Karna** (T.U. Registration No: 5-2-37-1839-2014) under the guidance and supervision of **Prof. Dr. Krishna Das Manandhar**. No copies of this work have been published or presented previously anywhere or in any form.

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ACRONYMS

ACE2:	Angiotensin Converting Enzyme
BALF:	Broncho Alveolar Lavage Fluid
CDC:	Centre for Disease Control and Prevention
CoVs:	Coronaviruses
Ct:	Cycle threshold
DNTPs:	Deoxynucleotide Triphosphates
E gene:	Envelope protein Gene
ELISA:	Enzyme Linked Immunosorbent Assay
EUA:	Emergency Use Authorization
FDA:	Food and Drug Administration
IC:	Internal Control
LAMP:	Loop Mediated Isothermal Amplification
LOA:	Limit of Agreement
LOD:	Limit of Detection
MERS:	Middle-East Respiratory Syndrome
MoHP:	Ministry of Health and Population
N gene:	Nucleocapsid Glycoprotein Gene
NPS:	Nasopharyngeal Swab
NSP:	Non-Structural Protein
NTC:	No Template Control
OPS:	Oropharyngeal Swab
ORF:	Open Reading Frame
PC:	Positive Control
RBD:	Receptor Binding Domain

RdRp:	RNA Dependent RNA Polymerase
RDT:	Rapid Diagnostic Test
RNA:	Ribonucleic Acid
RT-qPCR:	Reverse Transcription- quantitative Polymerase Chain Reaction
SARS:	Severe Acute Respiratory Syndrome
SARS-CoV-2:	Severe Acute Respiratory Syndrome Corona Virus 2
SDS:	Sodium Dodecyl Sulfate
TAT:	Turn Around Time
T_m :	Melting Temperature
TMPRSS:	TM protease serine 2
VLP:	Viral Like Particles
VTM:	Viral Transport Media
WHO:	World Health Organization

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ABSTRACT

RT-PCR is the gold standard method used till date for covid19 detection. Owing to the limited supply of SARS-CoV-2 RNA extraction kits in different health care facilities of Nepal, it results in enormous pressure to optimize reagent use, thereby affecting the overall diagnostic quality. This proposed research aimed to detect SARS-CoV-2 RNA from NPS through direct RT-qPCR technique omitting entire RNA extraction process. For this, 184 clinical NPS samples were obtained from Covid19 suspected patients who visited the Kirtipur Municipality-TU Biotech Corona Laboratory, and all subsequent steps were carried out there. These corresponding sample was subjected to RNA extraction followed by RT-qPCR as well as heat inactivated- RT-qPCR for validation. Eventually, their Ct values were compared wherein, the impact of heating temperatures and sample volume on assay sensitivity was also studied. The overall efficacy of these techniques was comparatively analyzed based on their Ct values. Heating NPS samples (n=184) for 20 min at 70 °C yielded a sensitivity, specificity, and accuracy of 93.3%, 96.7%, and 91.3% respectively. According to our paired T-test analysis, the mean Ct values of the N1 and RNase P genes were statistically significant at 95% CI ($p<0.001$), whereas the N2 genes were not ($p>0.001$). The results thus obtained was also compared with that of conventional RT-qPCR technique. Thus, a strong agreement using Cohen's Kappa ($\kappa=0.803$) was found between two methods indicating reliability of heat inactivation assay. Therefore, direct RT-PCR might be a useful method for quickly identifying COVID-19 suspects. This research offers a quick fix for the RNA extraction supply crunch. Furthermore, this emerging concept could even drastically lower costs and accelerate assay TAT by omitting the RNA extraction step.

Keywords: Covid19, direct RT-qPCR, heat-shock, RNA extraction, SARS-CoV-2, RT-qPCR validation, Ct value

Chapter I

INTRODUCTION

1.1 Background

SARS-COV-2, a severe acute respiratory syndrome coronavirus, has caused destruction and claimed many lives. It was initially recognized in December 2019 in Wuhan, China. These are associated with MERS-CoV and SARS-CoV, two extremely dangerous coronaviruses that caused a substantial number of deaths in 2003 and 2012, respectively. (Snijder et al., 2016, and Wu et al., 2022). The most disastrous impacts of all are being and continuing to be caused by SARS-CoV 2. SARS-CoV and MERS-CoV, beta corona viruses that have been found in bats and are related to human transmission either directly or indirectly through intermediate hosts like civet cats for SARS-CoV (Song et al., 2005) and dromedary camels for MERS-CoV (Reusken et al., 2013, Snijder et al., 2016). The four genera of coronaviruses (CoVs) are α-, β-, γ-, and δ-coronaviruses among which α- and β-CoVs infect mammals, γ-CoVs infect avian species, and δ-CoVs infect both mammals and aves (Naqvi et al., 2020). Covid19 symptoms include fever, dry cough, pneumonia, and, in the case of severe complications. Along with the respiratory tract, other organs and cell types that SARS-CoV can infect include the intestinal mucosa, renal tubular epithelial cells, neurons, and cells of the lymphoid and reticuloendothelial system (Gu & Korteweghe, 2007; Hui & Zumla, 2019).

The classification of CoVs has been expanded to include α-, β-, γ-, and δ-coronaviruses of which α- and β- CoVs infect mammals, γ-coronaviruses infect birds, and δ-coronaviruses infect both mammals and aves (Naqvi et al., 2020). In addition, δ variant has been the variant of concern resulting ginormous fatalities and hospitalization rates all around the globe.

1.2. Genome Structure and Organization

Positive sense coronaviruses are enveloped viruses with single-stranded RNA genomes and phospholipid envelopes that are derived from the host's membrane and spike proteins that give them a crown-like appearance are classified as coronaviridae family (Lu et al., 2020). The viral genome codes for 29 proteins, including four structural proteins and 25 putative non-structural and accessory proteins (Wu et al, 2022). With genome sizes

ranging from 26 to 32 kilobases (kb) in length, the corona virus genome encodes four major structural proteins namely, the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a complete viral particle (Mortola, 2004; Wang, 2017; Schoeman & Fielding, 2019). The single stranded RNA genome, associated with nucleocapsid protein, encodes two large genes namely, ORF1a and ORF1b which encodes 16 nonstructural proteins (nsp1-nsp16) (Boopathi et al., 2021).

The S-protein comprises of S1 and S2 subunits which in turn encompasses signal peptide (SP), receptor-binding domain (RBD), fusion peptide (FP), heptad repeat (HR), transmembrane domain (TM), and cytoplasm domain (CP) shown as in figure 2 (Boopathi et al., 2021). CoVs genome contains a 5'-cap structure and a 3'-poly-A tail (Wu et al., 2022). The S-protein plays significant role in pathogenesis due to the presence of RBD which binds to the host cell and initiates the cascade of infections (Naqvi et al., 2020). The conformational change of S-protein during interaction process plays pivotal role in drug/vaccine development. Furthermore, mutations in Spike causes altered antigenicity (Naqvi et al.,2020).

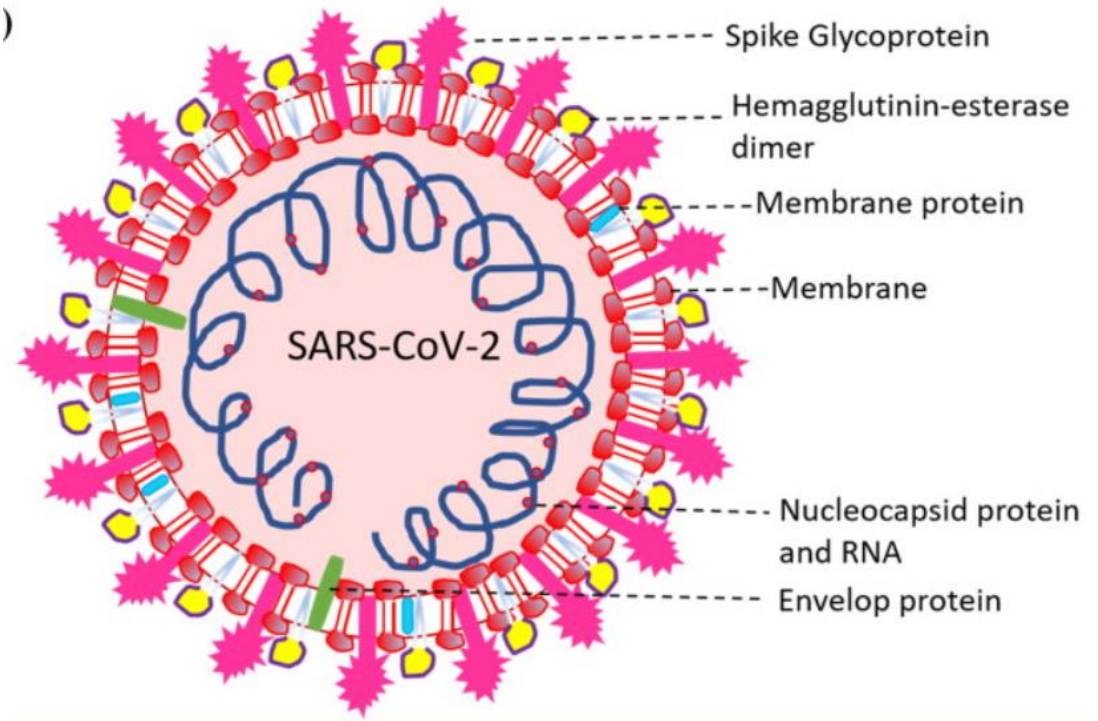


Figure 1: Coronavirus structure (Boopathi et al., 2021)

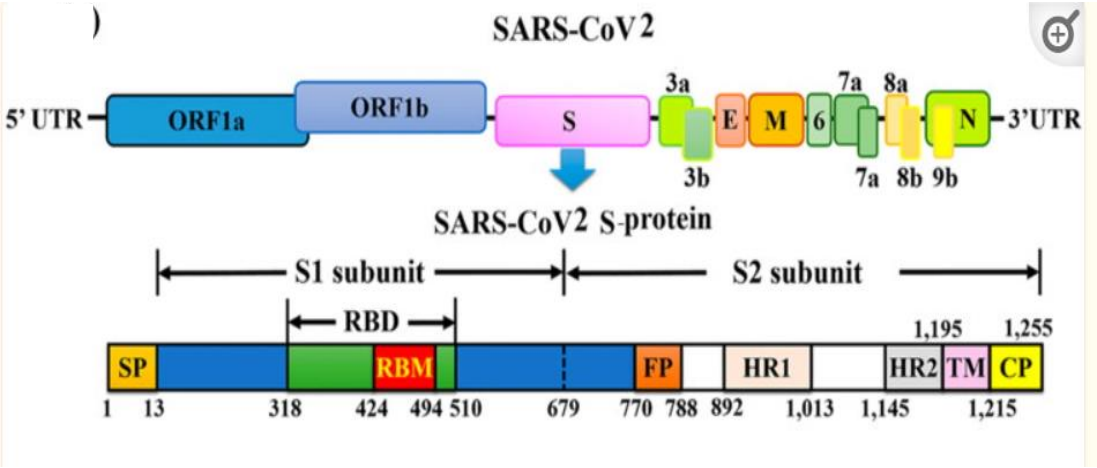
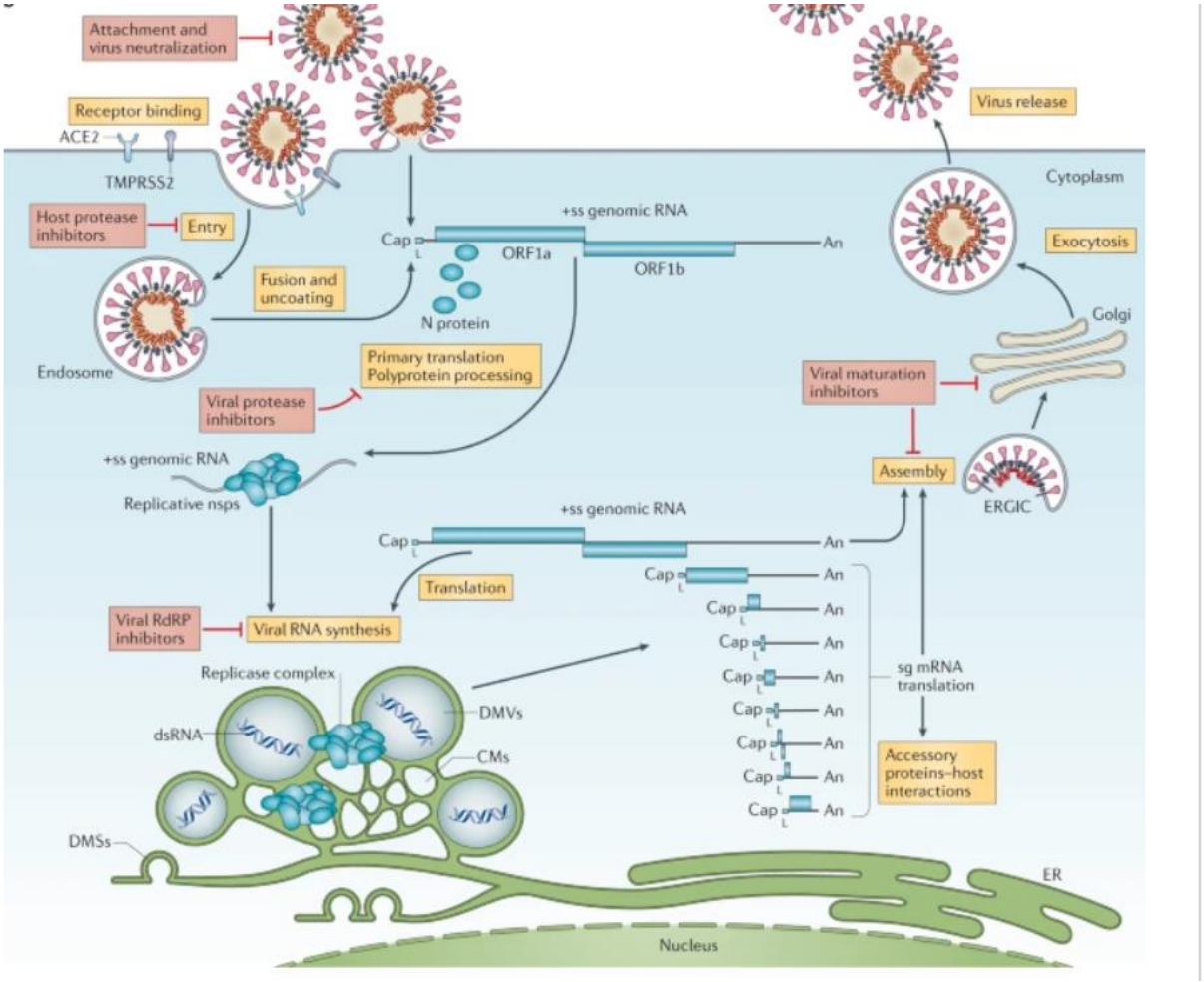


Figure 2: Genome organization and functional domain of S protein (Boopathi et al., 2021)

According to Schoeman et al., 2019 and McBride et al., 2014, the N-protein helps in binding to the CoV RNA genome, in its replication cycle and host cellular response to the infection. M-protein is the most abundant protein present on the viral surface and is responsible for assembly of viral particles in the host's cell. Similarly, Spike proteins or S-proteins protrude from the surface of the viral envelope. These proteins bind onto host cell surface receptors and allows the fusion between the viral and host cell membranes, thereby facilitating entry and invasion into the host cell (Boopathi et al., 2021 and Kirchdoerfer et al., 2016). The E-protein is the smallest of all major structural proteins which are composed of ~76 to 109 amino-acids. It is the minor component of the virus particle, that plays an important role in virus assembly, membrane permeability of the host cell and virus-host cell interaction (Boopathi et al., 2021 and Gupta et al., 2020). Both M and E proteins are pivotal for the production and release of viral like particles (VLPs).

1.3. Pathogenesis



(V'kovski et al., 2021)

Figure 3: Lifecycle of coronavirus Viral entry

The S-proteins are the fusion glycoproteins which are divided into S1 and S2 subunits as stated above. This protein is primarily responsible for the fusion of viral and host cell membrane through RBD of surface exposed S1 subunit (V'kovski et al., 2021). It latches onto ACE2 (Angiotensin Converting Enzyme) receptor on human cells, including those in lungs, facilitating the viral entry. Besides lungs, ACE2 are also highly expressed in oral, esophageal, ileal epithelial cells, myocardial cells, proximal tubule cells of the kidneys as well as urothelial cells of the bladder (Zou X et al., 2020 and Kordzadeh-Kermani et al., 2020). The host proteases aid in the proteolytic cleavage of S-protein at two sites located at the boundary between S1/S2 site as highlighted by dotted lines in figure 2. According to V'kovski et al., 2021, the cell-surface serine protease TMPRSS2, expressed in human respiratory tract has been reported to aid in priming and entry of virus and thus, contributes to both spread of SARS-CoV-2 infection and pathogenesis. Eventually, the

cleavage of S2 domain causes the release of fusion peptide, triggering the activation of membrane fusion mechanism at low pH.

- Viral replication

Once entering into the cytoplasm through a process called endocytosis, the virus causes conformational changes in Spike (S) glycoprotein followed by cathepsin L proteolysis through intracellular proteases and further activation of membrane fusion mechanism within endosomes (Simmons et al., 2005 and Boopathi et al., 2021). This causes the release of virus to the cytoplasm marks the cascade of viral gene expression and additionally, uncoating of nucleocapsid starts via proteasomes. Finally, single stranded RNA is fully released into the cytoplasm, followed by replication and transcription processes mediated by nsp called, RTC (Replication/Transcription Complex).

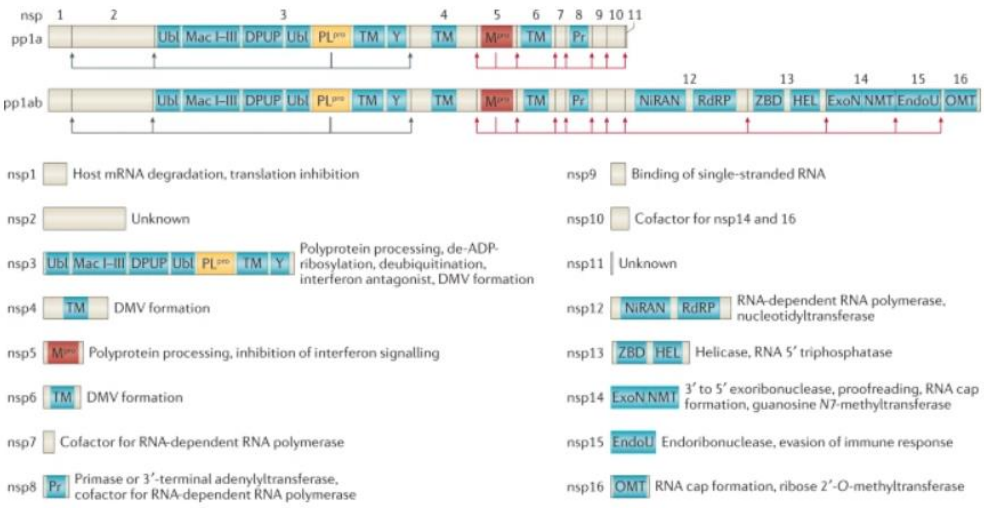


Figure 4: polyprotein and non-structural protein domains of corona virus and their roles in viral gene expression and RNA synthesis (V'kovski et al., 2021)

The translation of ORF1a and ORF1b results in the synthesis of polyprotein pp1a and pp1ab respectively. Moreover, sixteen non-structural proteins are co-translationally and post-translationally released from pp1a (nsp1–11) and pp1ab (nsp1–10, nsp12–16) upon proteolytic cleavage by two cysteine proteases that are located within nsp3 (papain-like protease; PLpro) and nsp5 (chymotrypsin-like protease). Proteolytic release of nsp1 which is known to occur rapidly helps hijack the host cell machinery whereas, nsp2-16 help determine the course of replication cycle. Besides, Nsp2–11 are believed to provide the necessary supporting functions to accommodate the viral RTC, such as modulating intracellular membranes, host immune evasion and providing cofactors for replication,

whereas nsp12–16 contain the core enzymatic functions involved in RNA synthesis, RNA proofreading and RNA modification (V'kovski et al., 2021). To be more specific, RNA synthesis is performed by nsp12 RNA dependent RNA polymerase (RdRp) whereas for RNA proofreading function, nsp14 provides a 3'–5' exonuclease activity (Eckerle et al., 2007 and V'kovski et al., 2021)

- Assembly and release

Then positive RNA genome is translated to generate replicase proteins from open reading frame 1a/b (ORF 1a/b) which are used to generate negative sense RNAs. Concordantly, the synthesis of M, S and E protein in the cytoplasm take place, whereby gets inserted into ER (Endoplasmic Reticulum) and eventually gets transferred to ER-Golgi intermediate compartment (ERGIC) (Masters, 2006; Song et al., 2004 and Boopathi et al., 2021). This whole process results in self-assembly into new virions which get transported to cell membrane in smooth-walled vesicles and secreted via exocytosis (Boopathi et al., 2021).

Additionally, it has been shown that the levels of cytokines including TNF-, GCSF, IP-10, MCP-1, MIP-1A, and interleukins (IL-2, IL-6, IL-7, and IL-10) are positively connected with the severity of covid19 (Kordzadeh-Kermani et al., 2020). Additionally, certain test results showed D-dimer presence, a high C reactive protein level, and a low leukocyte count (leukopenia) (Rothan et al., 2020). As stated in by Kordzadeh-Kermani et al., 2020, even the flow cytometry analysis demonstrated reduction in T lymphocyte cells ($CD4^+$ and $CD8^+$) and Natural Killer (NK) cells in covid19 patients.

1.4. Laboratory based diagnostic techniques for covid19

There is various lab based diagnostic tools opted for SARS-CoV-2 detection till date. Notably, nucleic acid based technique like RT-qPCR is considered as the gold standard tool for SARS-CoV-2 diagnosis. Besides serological tests such as antigen and antibody tests also aid in its diagnosis and control.

1.4.1. Real-time quantification RT-PCR

CDC and WHO have recommended to follow the standardized protocol which includes RNA extraction followed by RT-qPCR for efficacious SARS-COV-2 RNA detection (Bruce et.al and Barza et al., 2020). The most commonly targeted gene for the detection of virus

includes ORF1ab, N and E genes and in some countries RdRp gene is also targeted as a human Internal Control (IC).

Table 1: Primer/Probe sequence of SARS-CoV-2 gene targeted for RT-qPCR (Li et al., 2020)

Gene target	Probe (5′-3′)	Former primer (5′-3′)	Reverse primer (5′-3′)
ORF1ab gene	FAM-CCGTCTGCGGTATGT GGA AAGGTTATGG-BHQ1	CCCTGTGGGTTTTACACTTAA	ACGATTGTGCATCAGCTGA
N gene	FAM-TTGCTGCTGCTTGACAGA TT-TAMRA	GGGGAACTTCTCCTGCTAGAAT	CAGACATTTTGCTCTCAAGCTG
N1 target	FAM-ACCCCGCATTAC GTT TGGTGGACC-BHQ1	GAC CCC AAA ATC AGC GAA AT	TCT GGT TAC TGC CAG TTG AAT CTG
N2 target	FAM-ACAATTTGCCCCCAGCGC TTCAG-BHQ1	TTA CAA ACA TTG GCC GCA AA	GCG CGA CAT TCC GAA GAA'
N3 target	FAM-AYCACATTGGCACCCGCA ATCCTG-BHQ1	GGG AGC CTT GAA TAC ACC AAA A	TGT AGC ACG ATT GCA GCA TTG
RdRP1 target	HEX-AGATGTCTTGTGCTGCCG GTA-BHQ1	ATGAGCTTAGTCCTGTTG	CTCCCTTTGTTGTGTTGT
RdRP2 target	FAM-TCATACAAACCACGCCAG G-BHQ1	GGTAACTGGTATGATTTCG	CTGGTCAAGGTTAATATAGG
N gene	FAM-ATGTCGCGCATTGGCATG GA-BHQ	AAATTTTGGGGACCAGGAAC	TGGCAGCTGTGTAGGTCAAC
RdRP gene	FAM-CAGGTGGAACCTCATCAG GAGATGC-BBQ	GTGARATGGTCATGTGTGGCGG	CARATGTTAAASACACTATTAGCATA
E gene	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA
N gene	FAM-CAACTGGCAGTAACCA-BQH1	CGTTTGGTGGACCCTCAGAT-	CCCCACTGCGTTCTCCATT
ORF1b-nsp14 gene	FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA	TGGGGYTTTACRGGTAACCT	AACRCGCTTAACAAAGCACTC
N gene	FAM-GCAAATTGTGCAATTTGCGG-TAMRA	TAATCAGACAAGGAACTGATTA	CGAAGGTGTGACTTCCATG

The most repeatedly used clinical specimen used for SARS-CoV-2 diagnosis include nasopharyngeal/oropharyngeal swabs (Chan et al., 2020 and Zhang et al., 2020). Besides, throat swabs, sputum, Broncho alveolar lavage fluid (BALF), whole blood, serum, stool, urine, saliva, rectal swabs and conjunctival swabs also detected the presence of SARS-CoV-2 RNA (Li et al., 2020). Moreover, upper/lower respiratory tract specimens, stool specimens, whole blood specimens, and serum specimens, and the respiratory secretions are the acceptable specimens used as laboratory diagnosis (Li et al., 2020).

1.4.2. Loop-mediated isothermal amplification (LAMP)

LAMP is also rapid and sensitive detection technique merged with reverse transcription (RT-LAMP) based on amplification of the genetic material of the virus at a constant temperature. Consequently, the reaction product can be analyzed using agarose gel electrophoresis, UV illuminance or RT-qPCR. Alternatively, visual colorimetric RT-LAMP has also been successfully used for reliable detection of SARS-CoV-2 in NP fluids from COVID-19 patient (Amaral et al., 2021).

1.4.3. Serological tests

These tests are based on antibodies that bind to SARS-CoV-2 antigen in the virus, which are present if the person had past exposure to virus. The most commonly used serological tests are ELISA (Enzyme Linked Immunosorbent Assay) and RDT (Rapid Diagnostic Test).

The former is the lab based test and typically utilizes blood, plasma or serum samples from patients. The ELISA plate is coated with specific viral antigen which binds to the antibody (IgG, IgM, IgA) of covid19 patients if present in his/her blood. Subsequently, the secondary antibody is allowed to react with the so formed antigen-antibody complex and eventually, any visible change in color formed by a substrate is detected and the infection is confirmed on that basis (Ghaffari et al., 2020). The latter test is based on lateral flow immunoassay (LFIA) technology which makes use of a drop blood to detect the presence of IgG, IgM or IgA antibodies in the patient's blood formed against a specific SARS-CoV-2 antigen (Ghaffari et al., 2020).

1.4.4. Imaging technologies

The person with covid19 typically manifests ground-glass opacities as characteristic feature during chest CT (Computed Tomography) scan (Shi et al.,2020 and Wiersinga et al., 2020). However, CT scan of chest is regarded as non-specific for covid19 due to its overlapping results with other lung infections (Wiersinga et al., 2020). Besides CT scan, conventional X-rays and ultrasound have also been used as diagnostic tool, howbeit, these have very low sensitivity and specificity and affected by number of factors such as disease severity, patient weight and operator skills (Pascarella et al., 2020).

1.5. Treatment Options

SARS-CoV-2 had undergone various mutation since 2019 and the most lethal variant was delta variant (B.1.617.2) with higher transmissibility, higher mortality, and hospitalization rates (Rashedi et al., 2022). During the beginning of the pandemic, when no drugs or vaccines were available, quarantine was the most efficacious step for clinical management of covid19 cases. Anyone who had been manifesting symptoms like fever, sore throat or cough were advised to isolate themselves at home while maintaining sufficient hydration and nutrition (Pascarella et al., 2020). Moreover, self-quarantine has been proved much effective in minimizing the contagion rate (Pascarella et al., 2020). As of now, the vaccines have been the most promising contrivance to curb worldwide covid19 inflation rates. Pfizer/BioNtech was the first vaccine to receive approval for emergency use in UK in December 2nd 2020. Since then various other companies have developed vaccines with the approval from WHO. Here are the following lists of some commonly administered vaccine worldwide.

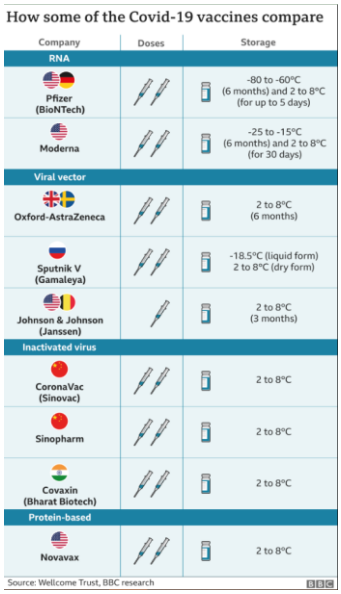


Figure 5: List of some WHO approved covid19 vaccines

Despite the administration of various doses of vaccines and protection measures, there still remain few populations which are still infected with covid19. The probable cause might be the low immunization coverage especially in developing countries as well as the adaptive mutations that SARS-CoV-2 virus undergoes periodically that eventually causes changes in the pathogenicity, virulence and infectivity (Rashedi et al., 2022). Nevertheless, self-quarantine, restricted mass gatherings, social distancing and proper sanitation can be the key to circumvent future public health crisis.

According to MoHP (Ministry of Health and Population), currently there are 999575 total cases of covid19 in Nepal of which 986335 have already been recovered, including 12017 deaths. The data as of 25th September 2022 is given below.

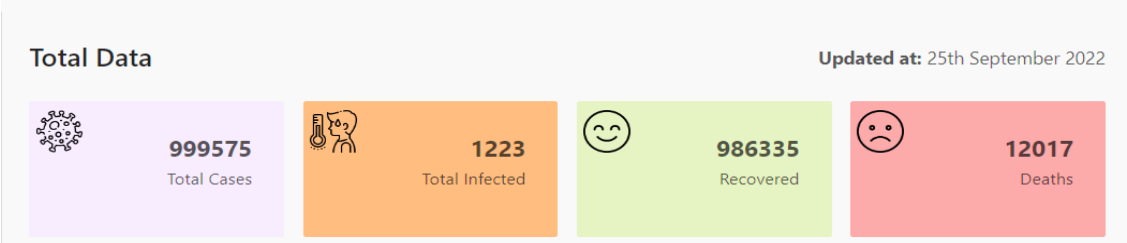


Figure 6: Total active cases of covid19 in Nepal according to MoHP

1.6. Problem Statement

During early pandemic, there were shortage of diagnostic kits across the world and in Nepal too, we had limited stocks as we are completely dependent on imports. Nepal being a developing country, was having a hard time managing the surge and rampant inflation

of SARS-COV-2 virus amidst sparsity. Many alternatives have so far been introduced in the market and an increasing demand of molecular tests to ensure equitable and quality health care facilities. Pertaining to increasing requirement for testing worldwide, an easy and rapid method can help monitor the COVID cases in developing countries like ours. Therefore, this study seeks to address these problems by rapid detection of SARS-COV-2 RNA through extraction free RT-qPCR approach. This study is also supposed to provide an insight on how rapid and easy detection can be accomplished, concomitantly maintaining high sensitivity and specificity. Conforming to a recent paper by Visseaux et al, 2021, a good sensitivity of extraction free SARS-COV-2 RT-PCR assays compared to a standard reference RT-PCR assay for samples with Ct above 30 was reported. Thus approach like heat inactivation, extraction-free reagents can provide a valuable option, cheaper, easier and less reagent consuming for SARS-CoV-2 diagnostic, especially in laboratory with lower experience and equipment for molecular assays (Visseaux et al., 2021). Therefore, it is important to understand the importance of extraction-free approaches in the middle-income countries to boost the laboratory based surveillance system.

1.7. Rationale

RNA extraction kits have been on high demand for SARS-COV-2 diagnosis. However, long turnaround time is the limitation of diagnostic techniques used during early pandemic. RNA extraction of exceedingly large number of sample is indeed an arduous task. Because of potential transmission to healthcare professionals, the COVID-19 has been of significant public health importance, and its specimen needs to be treated carefully. Prior to RT-qPCR, heat inactivation of the sample might enable secure testing regime. Therefore, it's crucial to evaluate how heat inactivation affects SARS-CoV-2 RT-PCR detection in circumstances with restricted resources. Besides, eliminating RNA extraction process will thus save time and culminate human error. It may prove to be cost effective and feasible alternative with low technical requirement for any diagnostic lab. Besides, heat shock NPS are non-invasive and easy to work with. It lessens the risk of viral transmission to health workers. In addition, it reduces the sample handling time and contamination across many samples during processing. As a result, these approaches would provide a promising solution for laboratories with limited biomedical resources. However, at present, such studies are very limited in Nepal. Thus, this study is believed to provide new dimension and refinement to the existing protocols and at the same time, an insight on heat shock

RT-PCR strategies to be considered as new diagnostic testing solutions and to be implemented for diagnosis in different labs of Nepal.

1.9. Hypothesis

- Null Hypothesis (Ho): heat inactivation/thermal shock, without RNA extraction approach cannot be an alternative technique
- Alternative hypothesis (H1): heat inactivation/thermal shock, without RNA extraction can be an alternative to standard RT-PCR technique

Research questions

- Is sensitivity and specificity of heat shock RT-PCR strategies higher or comparable to that of standard RT-qPCR method?
- Does heat shock RT-PCR strategies have diagnostic values?
- Is simple heat-RNA release method reasonable alternative to automated RNA extraction system and help overcome the cost and availability issues of RNA extraction reagents?

1.10. Objectives

1.10.1. General Objective

- To assess the performance of heat-RNA release technique in the detection of SARS-CoV-2 without nucleic acid extraction.

1.10.2. Specific Objectives

1. To determine the effective thermal temperature and exposure time for SARS-CoV-2 inactivation
2. To determine the sensitivity and specificity of extraction free methods with that of standard RT-qPCR method by comparing their Ct values.
3. To determine if heat inactivation step is alternative to conventional standard RNA extraction technique
4. To determine if this strategy could serve for diagnostic purpose.

CHAPTER II

Literature Review

There seems a need to expand more stringent rapid diagnostic techniques to control the alleviating public health concern, especially in developing countries like Nepal, where the shortage of RNA extraction kits is a prime challenge. CDC and WHO have recommended to follow the standardized protocol which includes RNA extraction followed by RT-qPCR for efficacious SARS-COV-2 RNA detection (Bruce et.al and Barza et al., 2020). However, it becomes a major challenge to purchase expensive RNA kits frequently to procure test reagents to address growing need of tests for our population. To combat this financial predicament, heat inactivation as an alternative to tedious RNA extraction, prior to PCR would be a potential solution.

2.1. Different RNA Extraction methods

There are several methods used for RNA extraction during early pandemic when no commercial kits were available. Following basic techniques were used to isolate RNA from covid samples.

2.1.1. Trizol Reagent

A monophasic solution of guanidinium isothiocyanate and phenol called Trizol, simultaneously solubilizes biological material and denatures protein (Rio et al., 2010). It helps in solubilizing cells and tissues for RNA extraction. The addition of trizol followed by chloroform results in phase separation causing DNA to reside at interface and RNA in the clear aqueous phase (Rio et al., 2010). However, the disadvantages of employing Trizol in RNA extraction includes the possible DNA contamination and high salt concentration during phase separation.

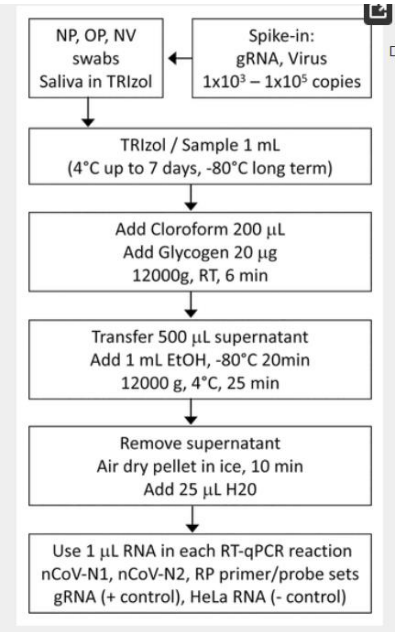


Figure 7: A simplified Trizol protocol for the extraction of SARS-CoV-2 RNA (Paz et al., 2020)

2.1.2. Guanidine Cesium Chloride

Application of Guanidine Cesium Chloride for RNA extraction is based on the buoyant density among protein, RNA, and DNA during centrifugation. 2'-hydroxyl group of RNA is significantly denser than DNA and protein which causes RNA to traverse CsCl cushion, forming pellets at the bottom of the tube (Nilsen et al., 2013). Here the density of CsCl is so adjusted which prevents DNA from entering into the cushion (Nilsen et al., 2013).

2.1.3. SDS/Proteinase K/Phenol: Chloroform

RNA extraction requiring SDS solubilization followed by proteinase K digestion and phase separation by phenol: chloroform is widely used wherein SDS aids in disrupting protein-nucleic acid interaction (Rio et al., 2010). Additionally, in combination with phenol-chloroform, it also denatures the protein, thereby making it insoluble and hence available for the separation in the aqueous solution (Rio et al., 2010).

2.1.4. Column Chromatography

The commercially available kits utilize column chromatography technique where cells or tissues are dissolved in guanidine isothiocyanate followed by adsorption in silica gel column. Here, RNA adheres to the silica, while DNA and protein are removed by washing with polar buffers. Eventually, Rna gets eluted with the use of RNAse free water (Rio et al., 2010). Moreover, the extraction kits are accompanied with buffers that contain

chaotropic salts, solvents and detergents to efficiently lyse virus (Thom et al., 2021). Chaotropic salts include Guanidinium salts, such as guanidinium isothiocyanate (GITC), which helps in efficient inactivation of different viruses such as, alphaviruses, flaviviruses, filoviruses, and a bunyavirus (Blow et al., 2004; Ngo et al., 2017 and Thom et al., 2021). Therefore, RNA extraction with commercially available kits are hassle free and easy to work with unlike other chemicals above mentioned.

2.1.5. Thermal Inactivation Method

It has been found that heat treatment fosters virus inactivation by denaturing its protein and causing disassembly of its protein structure (Loveday et al., 2021). In this same article by Loveday et al., 2021, he found that heat inactivation even for a longer duration did not have any effect on integrity of virus. However, it did result in deformation and reduced antigenicity of virus.

The heat inactivation method causes the release of viral RNA by destabilizing the physical integrity of virus, thereby aiding in detection through RT-PCR provided that, the heating temperatures are carefully monitored. This is because the virus particle disintegration takes place at different temperatures according to the type of virus and physicochemical conditions (Pastorino et al., 2005). According to Hessling et al., 2020, SARS-CoV-2 can be inactivated at 60°C, 80°C, and 100°C for approximately 32.5, 3.7, and 0.5 minutes, respectively, without genetic material being compromised. Besides, these samples at different heating temperatures do not interfere with the amplification by RT-PCR (Ñique et al., 2021).

Few reports have already described a potential solution to overcome RNA extraction kits dependency by using a simple heat inactivation and extraction step as an alternative to automated RNA extraction kit-based systems, which are also more expensive and time and labor demanding (Barza et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020). An article by Alaifan, 2020, states that the advantages of extraction free protocols encompass decreases the diagnostic test time from 2.5 hr. to 1.5 hr., help cope up during limited supply periods, minimizes labor force and plausible contamination. In the article by Kampf et al., 2020, heating SARS-COV-2 and MERS-COV at 60º, 65º and 80º for 30min, 15min and 1 min respectively, virus infectivity can be reduced by at least 4 log10. Various results have been generated in various studies

by varying the degree of temperature and time exposure implying, the effect of temperature help in reduction of viral infectivity. In one of the findings by Burton et al, 2021, heating to 80 °C for 30 min or more led to an increase in Ct value and therefore a reduction in RT-PCR sensitivity.

2.2. RT-qPCR assay

RT-qPCR is a technique used for amplification and quantitative detection of genes wherein the region of interest is amplified using specific oligonucleotide primers and DNA polymerase enzyme (Steward, 2022). As PCR is extremely sensitive, only minute amount of sample will suffice. Inside the machine, the target region undergoes multiple cycles of amplification which increases exponentially generation large number of copies, which can be tracked using an intercalating dye or sequence-specific probes producing fluorescence (Steward, 2022). Eventually, the computer then uses software to generate a graph in the form of Ct values. Ultimately, the Ct values help determine if the person is infected with SARS-CoV-2 and requires self-quarantine or needs to be treated for COVID19 infection (SARS-COV-2 RT-PCR controls, 2020).

The advancement in PCR (Polymerase Chain Reaction) gave rise to RT-PCR which is the combination of both Reverse Transcription and PCR. The RT-qPCR technique requires the information on virus genomic sequence along with the sequence information of specific primers and probes. The SARS-COV-2 genomic sequence was first made available on January 10th 2020, and the first primers and probes targeting the virus were described on January 13th (Bustin et al., 2021). As fluorescent is required for the quantitative detection purpose, the technique is named as RT-qPCR. Therefore, RT-qPCR, not only detects SARS-COV-2 gene but also quantifies the amount of genetic information present in the sample. The unprecedented spike in covid19 cases and death toll fueled stringent RT-qPCR protocols for large mass scale testing. It is now considered as a gold standard technique with high sensitivity and specificity.

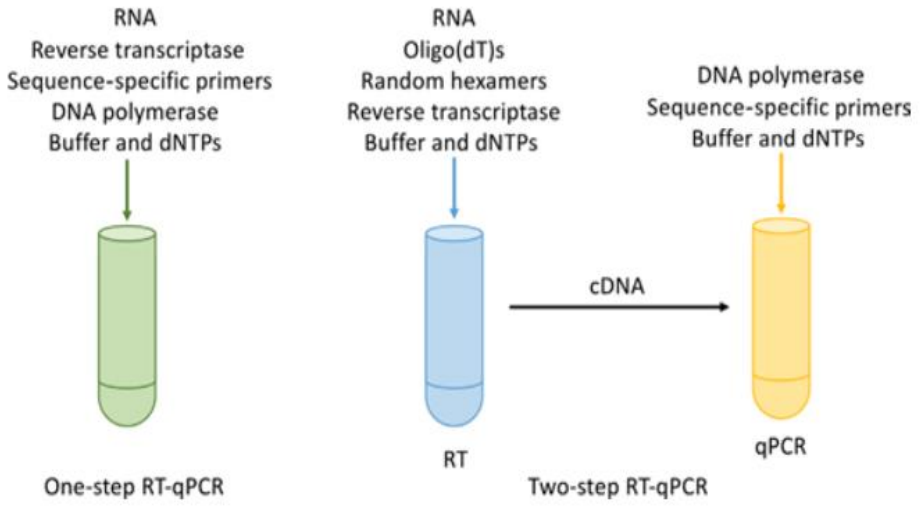
2.2.1. Steps in RT-qPCR

2.2.1.1. Sample Preparation

It is the most crucial step requiring precision during extraction of RNA or DNA of sample of interest. Any contamination would lead to the inhibition of PCR resulting in inaccurate results. Currently there are myriad of commercial RNA extraction kits available in the

market which are of high quality and easy to use, having high RNA yield. Unlike manual RNA extraction, which is labor demanding, cumbersome, with probability of contamination and low RNA yield. Since SARS-CoV-2 has RNA as its genetic element, and RNA backbone being more sensitive to breakage than DNA due to the presence of 2' -hydroxyl group attached to the pentose ring, proper care should be taken to prevent RNA degradation (Nilsen et al., 2013). The 2' -hydroxyl group adjacent to phosphodiester linkages can act as an intramolecular nucleophile, and may attack the adjacent phosphodiester bond, creating a 2', 3' cyclic phosphate thereby, cleaving the RNA backbone (Nilsen et al., 2013). Therefore, the major challenge here is to retain RNA intact throughout the extraction as well as for downstream processes.

After the RNA being extracted, the final step would be to generate cDNA. This step may either be a part of qPCR (single- step RT-qPCR) or may be generated separately using oligo (dT) primers or random hexamers (two-step RT-qPCR) (Adams, 2020). These oligo (dT) primers anneal to the polyA tail of RNA, whereas random hexamers anneal at multiple points along the RNA transcript (Adams, 2020).



(Adams, 2020)

Figure 8: Single- step RT-qPCR involving generation of cDNA via reverse transcription and qPCR in same step. Two-step RT-qPCR, involving reverse transcription and qPCR in two different steps.

2.2.1.2. PCR amplification and amplicon detection

PCR cycles encompasses,

- Initial Denaturation: denaturation is the process in which secondary structure of dsDNA separates to ssDNA. The reaction temperature rises to 95ºC with incubation period of 2-5 min or 10 min depending on enzyme characteristics or template complexity (Sigma-Aldrich® Solutions). The duration should be long enough to allow separation of all the strands of DNA, without causing any damage to DNA, thereby making it available for the priming (Sigma-Aldrich® Solutions).
- Cycling
    - Denaturation: the optimum temperature for denaturation is considered as 95ºC which is enough to break the hydrogen bonds between two strands of DNA. This step usually lasts for about 1min.
    - Annealing: it is the process in which primers gets attached to the template DNA. Usually, the temperature is lowered to 5ºC below the melting temperature (T_m) of the primers. Temperature typically between (45-60) ºC promotes primer binding to template and lasts from 30sec to 1min (Sigma-Aldrich® Solutions). Here the primer designing plays a pivotal role in determining its specificity to the template.
    - Extension: it is the process in which there is the addition of nucleotides to the annealed primer by Taq polymerase. Here the temperature is increased to 72ºC for about 20 secs to 1min which is considered as an optimum temperature for Taq polymerase activity.
- Repeat: it is required for exponential amplification of the amplicons (Sigma-Aldrich® Solutions). Here, denaturation, annealing and extension take place in a cyclic manner.

The number of cycles may vary depending upon the desired yield of PCR product. At the final stage, in case of conventional PCR, the PCR products can be analyzed using gel electrophoresis and UV-illuminance. However, qPCR does not require post amplification analysis as the results can be analyzed in real-time displayed in monitor.

Amplicons are detected either using DNA binding dyes or fluorescent probes inside the PCR machine. SYBR® Green 1 is an example of DNA intercalating dyes which fluoresces

only when intercalated to dsDNA (figure 9). However, nonspecific PCR products and primer-dimer formation are the major cons associated with using SYBER Green as it binds to all dsDNAs formed during reaction (Arya et al., 2005). Unlike, fluorescent probes for instance, TaqMan probes which are more specific and binds to the minor groove of DNA (figure 10). With this probe, specific hybridization between probe and template is necessary for fluorescence emission which averts the possibility of non-specific priming and primer-dimer formation (Arya et al., 2005).

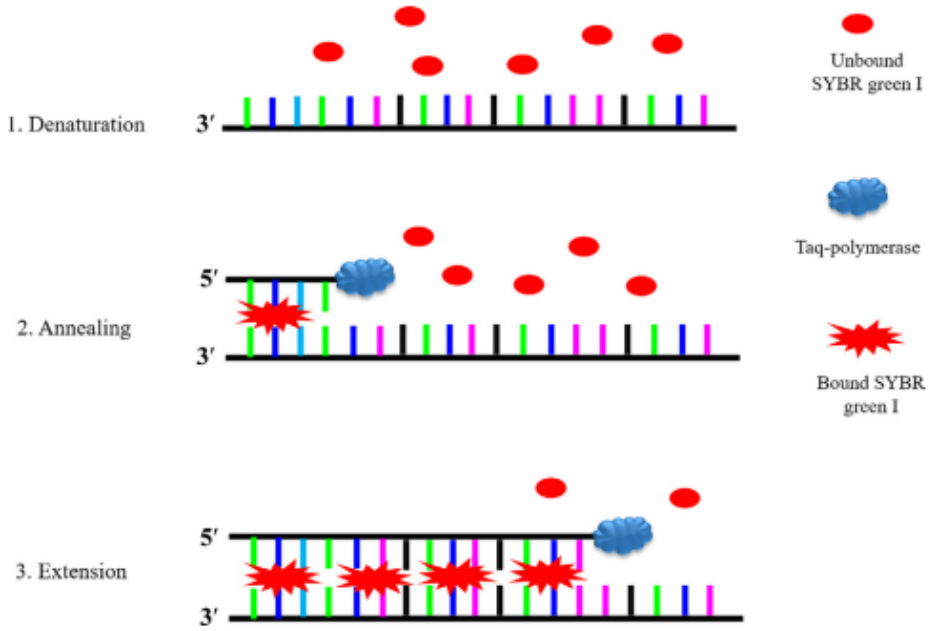
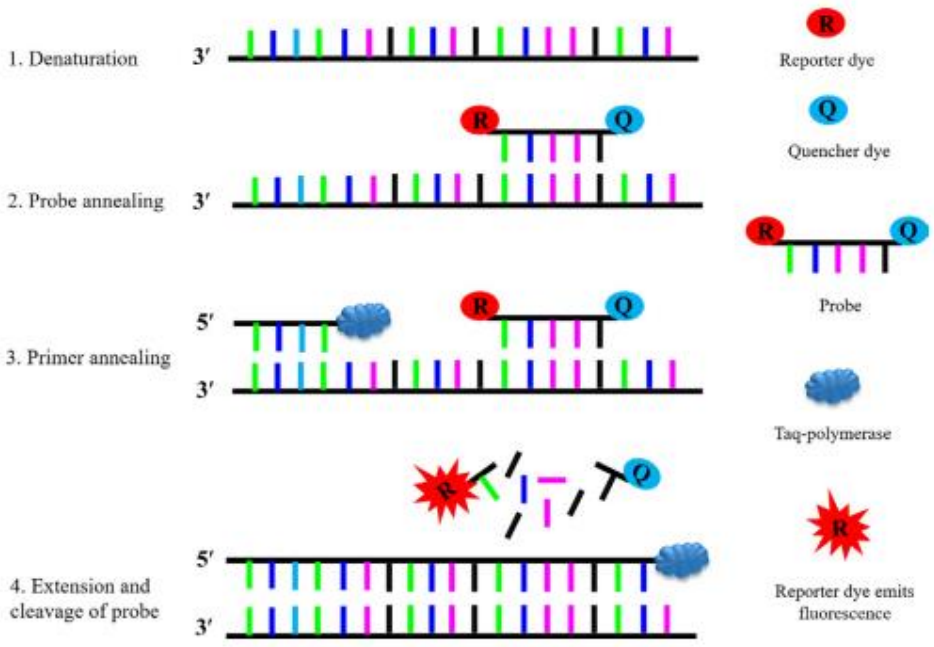


Figure 9: RT-qPCR reaction involving detection by SYBER green probe

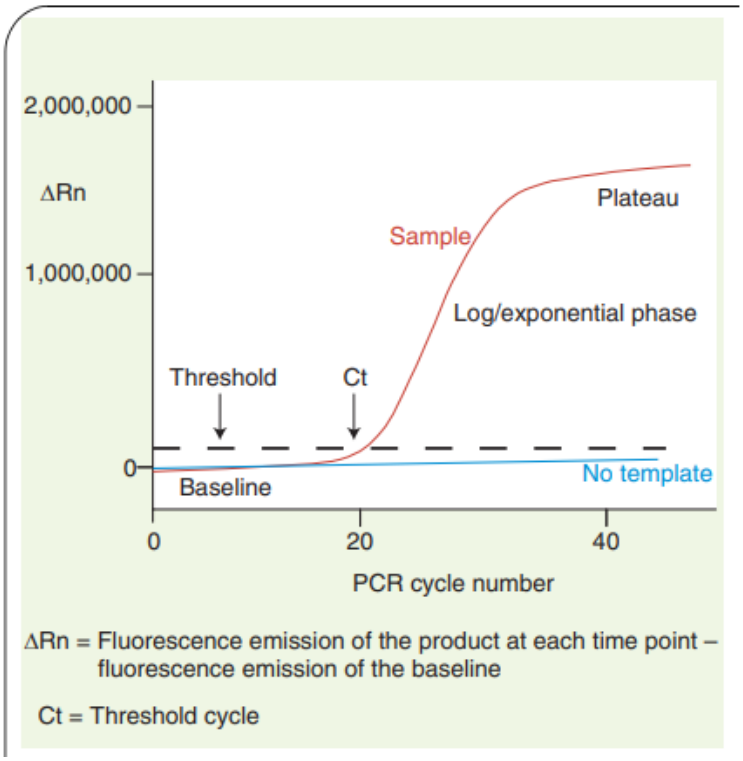


(Roy et al., 2019)

Figure 10: RT-qPCR reaction involving detection by TaqMan probe

2.2.1.3. Amplification Curve analysis

The RT-qPCR amplification curve has mainly four phases namely, the linear ground phase, early exponential phase, log (also known as exponential) phase, and plateau phase (Wong & Medrano, 2005). The linear ground phase is the initial phase implying to first 10-15 cycles where no fluorescence emission has risen above the background. Here the emitted fluorescence cannot be distinguished from the baseline. As the cycle progresses, and when the PCR product begins to amplify, the stage is referred to as log phase (Cycle 16-25) where the amount of product doubles at each cycle (Wong & Medrano, 2005). During the final phase (Cycle 26-38/40) where reaction components get used up, the amplification cycle reaches the plateau phase and thus no increase in product is detected. The fluorescence emitted data are required to generate amplification curves by a computer software which are important for calculating background signal, cycle threshold (Ct), and amplification efficiency (Wong & Medrano, 2005; Arya et al., 2005). Below is the figure representing amplification curve after the completion of cycle. Some of the terminologies associated with this curve are described below:



(Arya et al., 2005)

Figure 11: Phases of RT-qPCR amplification curve

- Baseline: the baseline of the amplification curve refers to the fluorescent signal that appears during the beginning of the cycle, usually from cycles 3-15 and beneath the limits of detection of the instrument (Arya et al., 2005).
- Threshold: it is the minimal detection level calculated as 10 times of standard deviation of average signal between cycles 3-15 (Arya et al., 2005). Computers actually selects the arbitrary threshold based on the baseline data generated (Arya et al., 2005). Any fluorescent signal detected above this threshold is considered real signal that pertains to the Ct value or Cycle threshold (Arya et al., 2005).

- Ct value: Ct value is defined as the number of cycles required by reporter fluorescent signal to cross the threshold or minimal detection value (Arya et al., 2005). During the exponential phase, none of the reaction components is limiting and therefore Ct values are very reproducible for replicate reactions and reliable measure of starting copy number (Arya et al., 2005).
- Viral load: viral load is known to be the predictor of covid19 severity (Silva et al., 2022). It is a measure of active viral replication and provides information on progression of infection and response to treatment (Zheng et al., 2020). It is measured as viral

copies/ml per swab (Challenger et al., 2022). The viral load data can be measured using patients NPS/OPS and obtained through analyzing the Ct value after RT-qPCR reaction (Dadras et al., 2021). The Ct value and viral load is inversely proportional to each other, meaning, higher the Ct value, lower the viral load and vice-versa.

In one of the studies by Zou et al., 2020, he depicted that there is no difference between viral loads in upper respiratory specimen of symptomatic and asymptomatic patients infected from covid19, i.e. it was seen equally high among both type of patients (Zheng et al., 2020). Similarly, in a study of viral dynamics in mild and severe cases of covid19 by Liu et al., 2020, had found that the viral load in case of severe cases were 60 times higher than that in mild cases in NPS during the first 12 days of infection (Silva et al., 2022).

- ΔRn: it is the change in fluorescence signal which is calculated as the difference in Rn values of the sample and Rn value of the baseline signal. It represents magnitude of signal generated during PCR (Wong & Medrano, 2005).

  Rn= Rnf- Rnb

  Where,

  Rnf= the fluorescence emission of the product at each time point and

  Rnb= the fluorescence emission of the baseline

  (Heid et al., 1996; Gibson et al., 1996; Arya et al., 2005)

- Types of Real-time Quantification

A. Absolute Quantitation

It gives us information about exactly how many copies of virus are present in any sample. Absolute quantification requires standard curve which can be generated by serial dilution of standard samples of known concentration and its amplification (Wong & Medrano, 2005). Then the quantities of unknown samples can be extrapolated from the standard curve. The standard curve gives the linear relationship between the Ct value and initial concentration of SARS-CoV-2 RNA (Wong & Medrano, 2005). The known concentration of samples here means either plasmid containing gene of interest or synthetic single stranded sense oligonucleotides (Arya et al., 2005) or dsDNA (Wong & Medrano, 2005). The quantity of standard samples can be known by spectrophotometer and the number

of copies of a template in an unknown sample can be known with great precision (Arya et al., 2005).

B. Relative quantitation

It requires mathematical expressions to calculate relative gene expression in sample relative to a reference control or calibrator. The source of calibrator may be untreated sample or RNA from normal tissue (Arya et al., 2005).

2.2.2. PCR assay components

- Template: templates are obtained through extraction of either RNA or DNA according to the gene of interest. Template contains the target sequence required to be amplified by PCR machine.
- Primers: these are the short oligonucleotide sequence of DNA specifically designed complementary to the target sequence. The length of the primers is crucial for the PCR experiment. It is because, if the primers are too short, hybridization at non-specific sites might occur resulting in undesirable products (Brown, 2016). Similarly, longer primers cause reduction in PCR efficiency as hybridization take place at slower rate (Brown, 2016). Therefore, oligonucleotides between 18-24 bases and with GC content 40%-60% (Jalali et al., 2017) are more commonly used with melting temperatures set within a few degrees of the primer (Dieffenbach et al., 1993). Melting temperature or T_m is the temperature at which primer-template hybrid dissociates (Brown, 2016). It can be calculated by following formula:

$$T_m = (4 \times [G + C]) + (2 \times [A + T])\ ºC \text{ (Brown, 2016)}$$

A temperature 1-2ºC below this is chosen to allow the formation of stable primer-template hybrid (Brown, 2016).

Table 2: The primer and probe sequences of SARS-CoV-2 (Bruce et al., 2020)

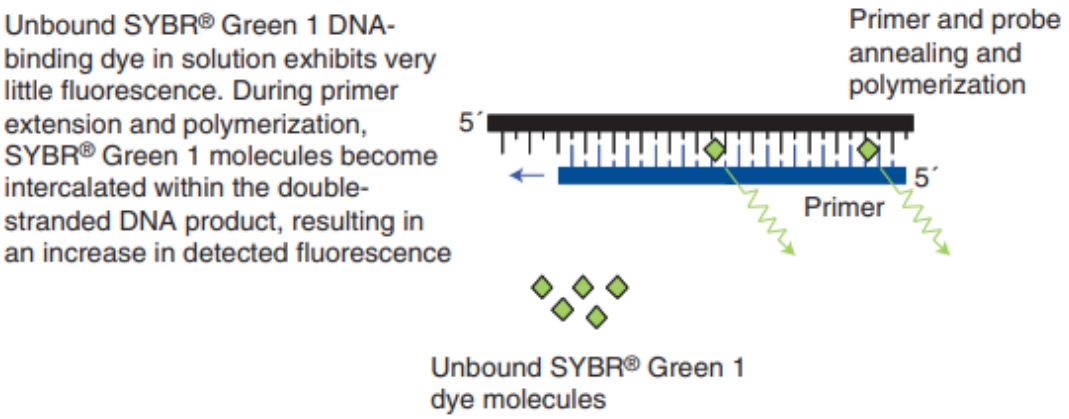
Name	Description	Oligonucleotide Sequence (5'>3')
2019-nCoV_N 1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
2019-nCoV_N 1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'
2019-nCoV_N2-P	2019-nCoV_N2 Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'
RP-F	RNase P Forward Primer	5'-AGA TTT GGA CCT GCG AGC G-3'
RP-R	RNase P Reverse Primer	5'-GAG CGG CTG TCT CCA CAA GT-3'
RP-P	RNase P Probe	5'-FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1-3'

- DNA polymerase: it is the most important enzyme required in PCR that helps in addition of new dNTPs for the synthesis of new strands, complementary to DNA or RNA template (Brown, 2016). The most frequently used DNA polymerase is Taq Polymerase which is isolated from the bacterium living in the hot springs, *Thermus aquaticus*. As this bacterium can withstand high temperature, it is thermostable, meaning they are resistant to denaturation (Brown, 2016). However, it can get inactivated during denaturation step when the temperature inside the PCR rises to 95º C.

- $MgCl_2$: Mg^{++} acts as a cofactor for Taq DNA polymerase activity (Jalali et al., 2017). It aids in DNA amplification by enhancing the catalytic activity of DNA polymerase. An optimal concentration of 1.5–2.0mM (Jalali et al., 2017) is generally used during PCR. It is because too much or too little concentration of magnesium ions may perhaps lead to unspecific binding or weak amplification. Therefore, its concentration needs to be optimized prior to PCR.

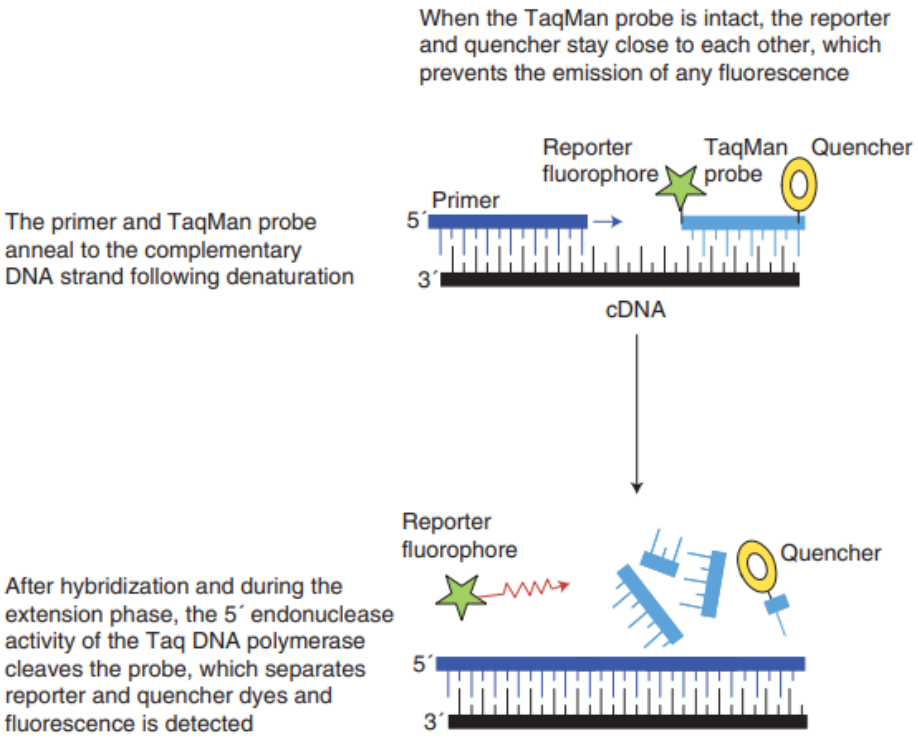
- Fluorescent dyes/probes: the application of fluorescent dyes/probes in qPCR eliminated the post PCR processing. The most commonly used DNA intercalating dye is SYBER green. It binds to the dsDNA and emits fluorescence (figure 11) whose intensity is proportional to the concentration of PCR product (Jalali et al., 2017). It is relatively cheaper than TaqMan but the pitfall is it being non-sequence specific which implies that the presence of any dsDNA (e.g. primer dimer) generates fluorescence (Arya et al., 2005 and Jalali et al., 2017). Thus, the specificity is highly compromised. Contrastingly, dual-labeled oligonucleotide fluorogenic probes has reporter

fluorophore at 5’ end and quencher at 3’ end as shown in figure 12 (Arya et al., 2005). Initially, the quencher and reporter dye are in close proximity but as soon as it is encountered by Taq polymerase, the 5´ nuclease activity of enzyme cleaves the probe, resulting in the separation of both the dyes during extension phase (Arya et al., 2005). This engenders phenomenon called FRET (Fluorescence Resonance Energy Transfer) in which, the emission of the reporter dye is absorbed by the quenching dye (Arya et al., 2005). Moreover, cleavage of probe removes it from the target stand, allowing primer extension till the end of the template strand (Arya et al., 2005).



(Arya et al., 2005)

Figure 12: An example of action of DNA intercalating Dye, SYBER green



(Arya et al., 2005)

Figure 13: An example of action of hydrolysis probe i.e., TaqMan probe

2.2.3. Controls used in RT-qPCR

2.2.3.1. Negative Control

Negative control lacks the template DNA/RNA so also known as No Template Control (NTC). It contains PCR reaction mix and nuclease free water/molecular grade water instead of DNA/RNA template in the separate well (Czurda et al., 2016; Moldovan & Moldovan, 2020). The use of negative control gives us information regarding presence/absence of contamination (i.e. foreign DNA/Viral DNA) in the reaction mix, and formation of primer dimers (Moldovan & Moldovan, 2020). The formation of primer dimers may pertain to the stage during the designing of primers or using high concentration of primers in the master mix, leading to primer amplification despite the absence of template.

2.2.3.2. Positive Control

The positive control is used in a separate well which when undergoes PCR reaction, exhibit positive amplification curve of specific target regions. This helps us confirm that there are no PCR inhibitors present in the PCR reaction mix and that the preparation of PCR mix is

also contamination free. Besides, it helps verify that all reagents are working and all the PCR cycle set up, i.e., primer annealing temperature, and extension temperature are accurate and error-free (Moldovan & Moldovan, 2020). In nutshell, it helps prevent false negative results. There are various examples of positive control used in the PCR reaction mix. The study of Pavšič et al., 2017 illustrated plasmid DNA as a positive control for the study of human cytomegalovirus. Similarly, in the study of leukemia-associated fusion gene transcript by Lion, 2001, control RNA, commonly derived from cell lines is used as positive control.

2.2.3.3. Internal Control

Another control which is widely used in PCR is internal control (IC) to prevent false negative results. The term "housekeeping genes" is also used more commonly for IC. It is used to minimize errors during PCR which may arise due minor differences in the starting amount of RNA, nucleic acid purification or differences in efficiency of cDNA synthesis and PCR amplification (Arya et al., 2005). IC gets amplified together with the target sequence during same PCR reaction but does not compete with target region of interest due to different fluorophore marking (Moldovan & Moldovan, 2020). A study by Wagner, 2013 mentioned that IC and target genes should have difference of 12 amplification cycle within same PCR reaction (Moldovan & Moldovan, 2020). There are basically 2 types of internal controls namely, exogenous IC and endogenous IC.

- Exogenous IC: exogenous control is added directly into the sample before extraction. It helps to confirm that the RNA is successfully extracted and also transcribed. If the control is present and properly amplified, it implies that the negative sample is purely negative. Contrastingly, in the absence of control amplification, the sample cannot be confirmed negative and should be subjected to retesting ("SARS-COV-2 RT-PCR controls", 2020). The TaqPath COVID-19 CE-IVD RT-PCR Kit (Applied Biosystems, USA), used for covid19 diagnosis utilizes Bacteriophage MS2 as an exogenous control (Moldovan & Moldovan, 2020).
- Endogenous IC: endogenous controls are the housekeeping genes which are already present in the sample. These genes are commonly used for normalization (Arya et al., 2005), i.e., study of gene expression profiles (Moldovan & Moldovan, 2020). β-actin, glceraldehyde-3-phosphate dehydrogenase (GAPDH), and

ribosomal RNA (rRNA) are some of the examples of housekeeping genes most commonly used as endogenous control in PCR experiment (Arya et al., 2005).

2.2.4. Direct method for SARS-CoV-2 detection

Because of potential transmission to healthcare professionals, COVID-19 has been of significant public health importance, and its specimen needs to be treated carefully. Prior to nucleic acid isolation, heat inactivation of the material can enable secure testing procedures. Therefore, it's crucial to evaluate how heat inactivation affects SARS-CoV-2 RT-PCR detection in settings with restricted resources (Woldesemayat et al., 2022)

In the current stage of the pandemic, direct PCR amplification from samples is a technique that may be helpful. Through the elimination of the extraction step in the samples handling workflow, direct amplification can drastically shorten (TAT) time and lower testing expenses. Additionally, this type of approach cuts down on sample handling time, which lowers the risk of sample contamination. In bacterial culture, human cells, and plants, this technique has previously produced promising results. The use of direct RT-PCR is an intriguing approach for labs with constrained supplies and reagents in light of the current pandemic (Alaifan et al., 2021).

The SARS-CoV-2 virus can be inactivated using a variety of techniques, including chemical inactivation using a 0.5% solution of Povidone-Iodine oral antiseptic and/or 70% alcohol. Without the use of additional tools, the lysis buffers included in RNA extraction kits are likewise effective at inactivating SARS-CoV-2 (Woldesemayat et al., 2022). However, some detergents that are used to treat samples can stifle PCR processes. Without RNA degradation, some viruses cannot be effectively inactivated. With a similar temperature range, many virus strains may become inactive (Abraham et al., 2020). It has been demonstrated that the well-known approach of inactivating SARS-CoV-2 at 56 °C for 30 min prior to extraction processes causes a noticeable decline in viral infectivity (> 5 Log10 reduction) (Wang et al., 2020, Woldesemayat et al., 2022). The viral infectivity in a clinical specimen may also be greatly reduced by the other SARS-CoV-2 inactivation at 60 °C for 60 min, 92 °C for 15 min, 80 °C for 5 min, and 100 °C for one minute. Prior to molecular testing, sample preparation with heat may, however, degrade viral RNA and result in false-negative findings (Chen et al., 2020; Woldesemayat et al., 2022).

Chapter III

MATERIALS AND METHODOLOGY

3.1. Study Area

The overall study was a cross-sectional research which was carried out in Kirtipur Municipality-TU Biotech Corona Laboratory located at Kirtipur. The study began from 20th February, 2022 using the NPS/OPS clinical samples that were routinely received in TU Biotech Corona Laboratory. Both positive and negative clinical samples were stored at -20º C in VTM (Viral Transport Media) post-confirmation via RT-qPCR in the lab. A total of 184 samples were used for research purposes. Ethical approval was sought from IRC (Institutional Review Committee) for the utilization of the sample for research purposes. Informed consent was taken from all the patients involved in this study.

3.2. Sample Collection

For nasopharyngeal swab collection, patient head was slightly tilted (CDC recommends tilting patient head back to 70 degrees). Then cotton swab was inserted through the nostril parallel to the palate, only few centimeters inside, followed by gentle rolling. The swab was eventually removed slowly, placed into 3ml VTM tube, clearly labelled and preserved in ice box until further downstream processing. Similar process was carried out for oropharyngeal swab collection except for the swab was collected rotating around the tonsillar area. Since, each VTM came in combination with two types of swabs, one for oral and another for nasal, both naso- and oropharyngeal swabs were collected from single patient.

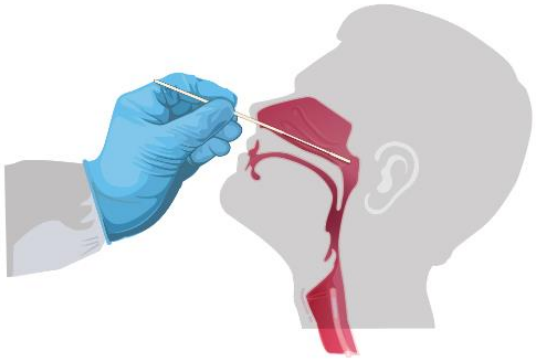


Figure 14: Nasal Swab collection from upper respiratory tract (Source: CDC, 2019)

Standard precaution guidelines as per CDC recommendation were inculcated during handling, processing and disposal of Covid19 infectious specimens. This includes the use of PPE (Personal Protective Equipment), such as laboratory gowns and coats, gloves, face shields, disposable masks, shoe cover, and hair cover during handling and processing of sample.

3.3. Sample size

The total COVID19 cases as of 20th February, 2022 was around 970000 according to MoHP website. Since the study population is infinitely large, 95% confidence interval and 6% margin of error was considered. The prevalence rate was calculated by dividing total no. of covid19 cases by total PCR tests done, which gave a sample size of 150. However, total of 184 samples were used for this study, out of which 154 were COVID positive and the rest were negative.

Sample size (n) = $\frac{Z_{\alpha/2}^2 \times P \times (1-P)}{d^2}$

Where,

Z = Z Statistic for a level of significance

α = Level of significance

P = Expected proportion/ Prevalence rate

d = Absolute Precision

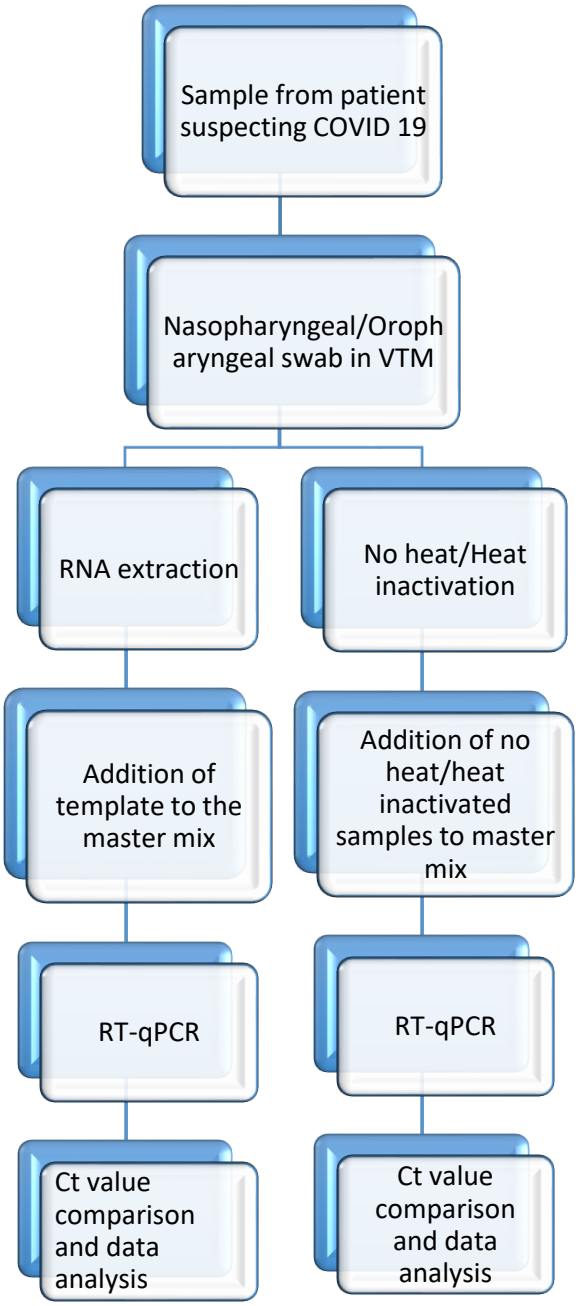
Prevalence Rate = $\frac{970000}{5700000}$

= 0.17

Sample size (n) = $\frac{1.96^2 \times 0.17\ (1-0.17)}{0.06^2}$

≈ 150

3.4. Outline of Research methodology



3.5. Sample Processing

The clinical samples during culmination of Omicron variant were selected for this study, i.e. between Dec 2021 to Feb 2022, stored at -20ºC. The same samples which were subjected to RNA extraction followed by quantitative PCR, were also subjected to direct RT-qPCR approach for its validation.

3.5.1. Manual RNA Extraction

The NPS and OPS sample from the previously tested and confirmed patient sorted according to their high, intermediate and low Ct values. For RNA extraction, the kits from CWBIO were used as per manufacturer's instructions. It consisted of lysis buffer, washing buffer 1, washing buffer 2, RNase-Free Water, adsorption column and collection tubes. Prior to commencing general RNA purification, isopropanol was added to Washing Buffer 1 and 100% ethanol to Washing Buffer 2 according to the label of the reagent bottle. They were then mixed by gentle inversion to ensure the solution is homogenized. The BSL-2 hood was first and foremost disinfected with 70% ethanol and UV exposure prior to working inside with samples and reagents. The NPS samples collected in VTM tubes were drawn out from a -20ºC refrigerator and kept at 4ºC for thawing. Once thawed, the samples were vortexed, the adsorption column were laid out onto micro-centrifuge tube racks. About 200µl of lysis buffer was pipetted out followed by 300µl of isopropanol and 200µl of sample into each column. This solution mixture was then vortexed for 5 secs and incubated at room temperature for 5 min. All the columns were then centrifuged at 12000rpm for 1 min. So obtained liquid was discarded carefully to avoid any contamination. To these columns, 500µl of Wash Buffer 1 was added and centrifuged at 12000rpm for 1min. Again, the liquid obtained was discarded and 500µl of Wash Buffer 2 was added to the same column. It was then centrifuged at 12000rpm for 1min and the liquid was discarded. The adsorption columns were put back into the same collection tubes and centrifuged at 12000 rpm for 2min. The adsorption columns were transferred to the new micro-centrifuge tubes to which 40µl of RNase-Free Water was added to the middle part of the adsorption column membrane, followed by incubation at RT for 1min and centrifugation for 30secs at 12000rpm. The so collected RNA in the micro-centrifuge tube were stored at -20ºC to avoid degradation until PCR mixture was prepared.

3.5.2. Automated RNA extraction

Some of the samples were also subjected to automated RNA extractor machine, Liferiver EX3600 Shanghai ZJ Bio-Tech Co. Ltd. This instrument utilizes Liferiver Viral RNA Isolation Kit (Preloaded for Auto-Extraction) for SARS-COV-2 detection. It utilizes magnetic particle technology for isolation and purification of pathogen's nucleic acids from biological specimens. Each kit consisted of 5 Preloaded plates, 5 magnetic caps, 1.3 ml of Proteinase K, 1 tube of Carrier RNA and 600µl Carrier RNA Buffer for 60 preps. The workbench was

clean and disinfected before starting the experiment. The automated extraction machine incorporates UV function for sterilization purpose, pre and post extraction. 500µl of carrier RNA buffer was added to lyophilized carrier RNA. 300µl of NPS sample, 2µl of IC, 20µl of Proteinase K and 6µl of carrier RNA was added to each well of preloaded plate labelled from A1 to 12. The preloaded plate was placed on the transport platform carefully and magnetic cap was inserted. The "RNA Isolation 2" program was selected according to the user manual of Automated Nucleic Acid Extraction Instrument. Then, the START button was tapped to run the instrument. The completion of extraction was indicated by the beep sound. The magnetic cap was then discarded and the elute from preloaded plate (A1-A12) was transferred into well labelled DNase/RNase free micro-centrifuge tubes. All the tubes were immediately stored at -20ºC.

3.5.3. Master mix preparation

For master mix preparation, IDT (Integrated DNA Technology) kit from La Jolla Institute of Immunology, California, USA was used to financially facilitate this study. Prior to master mix preparation, the virus specific forward and reverse primers N1, N2 and RNase P and their respective probes were diluted using TE buffer. RNase P was used as an internal control (IC) for the detection of human RP gene. The 200µl PCR tubes were labelled according to different primers and probes required for dilution. For 200µl of a primer diluted mixture, 40µl of each primer was added to 160µl of TE. Similarly, 10µl of individual probes were added to 190µl of TE buffer. The following table 3 shows the calculated amount of components needed for the preparation of master mix. However, the saliva sample was replaced with clinical NPS/OPS and heat inactivated samples.

Table 3: The calculated amount of components needed for the preparation of master mix.

Master Mix N1, N2, RP				
# Samples	48			
Rxn Vol	20			
# of Replicates/Sample	2			
Overage	15%			
Component	ul x1	ul batch	[Stock] X	[Final] X
Premix N1	1.28	**140.8**	40	0.85
Premix N2	1.50	**165.6**	40	1
Premix RP	1.28	**140.8**	40	0.85
Saliva + TX	10	**1104.0**		
TaqPath	5	**552.0**		
H2O	0.95	**104.9**		
Total	**20.00**	**2208.0**		

The master mix was prepared in clean DNase/RNase free 2ml Eppendorf tube. The diluted primer/probe premixes(40X) and TaqPath (4X) were thawed on wet ice. With reference to the calculations above, according to the no. of samples per batch, the components were added in the Eppendorf tube and mixed properly. All these process were performed on ice plate/cooler. Once the master mix was ready, about 3µl of extracted RNA was added into each well of PCR tubes and sealed. 2019-Cov Plasmid containing complete nucleocapsid gene from 2019-nCoV was used as a positive control and RNase free water was used as negative template control (NTC). It was then vortexed for few seconds to ensure proper mixing. Finally, the PCR tubes were inserted into the RT-qPCR cycler machine following the programs of cyclic parameters. BioRad CFX program was chosen as shown in table 4. All the cycling parameters and dyes/targets were properly defined, and ensured prior to starting thermocycler. The run program was saved choosing the data export location on the computer. Once the qPCR program was completed, the curves were inspected and thresholds were adjusted for each target according to software protocols to include amplifications and disregard noise such as background fluorescence. In the final stage, the data was exported according to Azure Cielo Manager 1.0.0.287 software version protocol and data analysis was performed. The positive value was indicated by a Ct value less than 40, and a negative value was indicated by a Ct value over 40 i.e., NaN (Not A Number). All the templates were maintained at -20º C in between RNA extraction step and qPCR to prevent the degradation of template.

Table 4: PCR cycling parameters

FDA CDC Instructions
Standard

Step	Deg C	Time	Purpose
1	25	2 min	UNG Incubation
2	50	15 min	RT Incubation
3	95	2 min	Enz Activation
4	95	5 sec	Amplification
5	56	30 sec	
7	Go to step 4 45 times total		

BioRad CFX

Step	Deg C	Time	Purpose
1	25	2 min	UNG Incubation
2	50	15 min	RT Incubation
3	95	2 min	Enz Activation
4	95	5 sec	Amplification
5	56	15 sec	
6	56	15 sec	Amplification /Read
7	Go to step 4 45 times total		

Table 5: Selection of dyes according to different probes using Azure Cielo Manager software for thermocycler

Peak Channel (Quantstudio 3 and 5)	Color	Filter Wavelength (nm)[1]		Reporter Dye	Probe Name
		Excitation	Emission		
x1-m1	Blue	470 ± 15	520 ± 15	FAM	N1
x2-m2	Green	520 ± 10	558 ± 12	VIC	N2
x5-m5	Red	640 ± 10	682 ± 14	Cy5	RP

3.5.4. Heat inactivation

Different temperatures and sample loading volumes were tested in order to validate the direct RT-qPCR protocol. 250µl of samples from VTM were transferred in Eppendorf tubes and labelled accordingly. All the samples were heated in batch using heat block at varying temperatures, i.e. 95 and 70 degrees centigrade for 10 and 20 minutes respectively to validate the direct RT-qPCR approach. Subsequently, the Eppendorf tubes were placed into calibrated heat block, pre-heated to temperature (i) 95º C and (ii) 70 º C to inactivate SARS-CoV-2 virus. The lysis plate was then prepared using 10% Triton-X. about 50µl of Triton-X was dispensed into each well of PCR tubes according to the no. of samples per batch. Since each sample required each well, about 50µl of sample was transferred into a well containing 50µl of the Triton-X. It was then pipetted and mixed slowly to avoid the formation of excess bubbles or foams followed by incubation for 10mins at room

temperature to complete lysis reaction. The master mix was prepared by the above method explained. To validate this method, different volumes of samples were tested. About 1µl, 3µl, 5µl and 10µl of samples were added into master mix and loaded in the thermocycler to determine the impact of temperature and sample loading volume on the detection/assay sensitivity. All the samples were processed in duplicate as shown in the plate layout below. Additionally, 1µl, 3µl, 5µl and 10µl of swab was directly added to the RT-qPCR reaction mix, without preheating and loaded in the thermocycler (Program: BioRad CFX) to determine the impact of heatless treatment on assay sensitivity.

50ul

Triton -X Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39						
B	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40						
C	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41						
D	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42						
E	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43						
F	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44						
G	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45						
H	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46						
	Triton-X 10%						Empty					

MM + Sample Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	NTC	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
B	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Plasmid	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
C	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41
D	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42
E	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43
F	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44
G	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45
H	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46
	Replicate 1						Replicate 2					

Figure 15: PCR plate layout

3.5.5. Data Analysis

Data were analyzed using both Microsoft Excel and SPSS version 29.0.0.0. Bland-Altman plot was created using excel to study the agreement and differences between two different methods/strategies. SPSS was used to study other all tests including Cohen's Kappa inter rater reliability, paired T-test, Pearson's correlation coefficient and McNemar's test. All these statistical tools were applied to determine and study the quantitative and qualitative changes existing between the two different methods.

CHAPTER IV

RESULTS

4.1. Validation of direct RT-qPCR method

For validation of direct RT-qPCR method, 11 out of 184 clinical NPS samples were used for validation purpose. These samples which were collected and stored in VTMs at -20º C were subjected directly to RT-PCR mix at different volumes, i.e. 10µl, 5µl, 3µl and 1µl. Table 6 provides the information on the qPCR results of N1(FAM), N2(HEX) and RP (Cy5) genes generated by the software. No fluorescence was detected when raw sample were directly subjected to 10µl of IDT master mix in each well. Therefore, no Ct values were recorded.

Table 6: Validation of direct RT-qPCR (without RNA extraction and heat inactivation), i.e., directly loaded 11 clinical swab samples into RT-qPCR mix.

Direct RT-qPCR (Without RNA extraction and without heat inactivation)												
Sample Vol.	**10µl**			**5µl**			**3µl**			**1µl**		
Sample IDs	**Ct values**			**Ct values**			**Ct values**			**Ct values**		
	N1	**N2**	**RP**	**N1**	**N2**	**RP**	**N1**	**N2**	**RP**	**N1**	**N2**	**RP**
NP1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

***ND: NOT DETECTED**

For the heat inactivation, about 250µl of samples from VTMs were transferred into Eppendorf tubes and short centrifuged prior to heating. Then, all the 11 samples were placed into the heat block, preset to 95º C. The lid was closed and the samples in the Eppendorf tubes were heated for 20 min. Table 6 shows the qPCR results in terms of Ct values for three different genes and figure below shows erratic curve obtained post PCR.

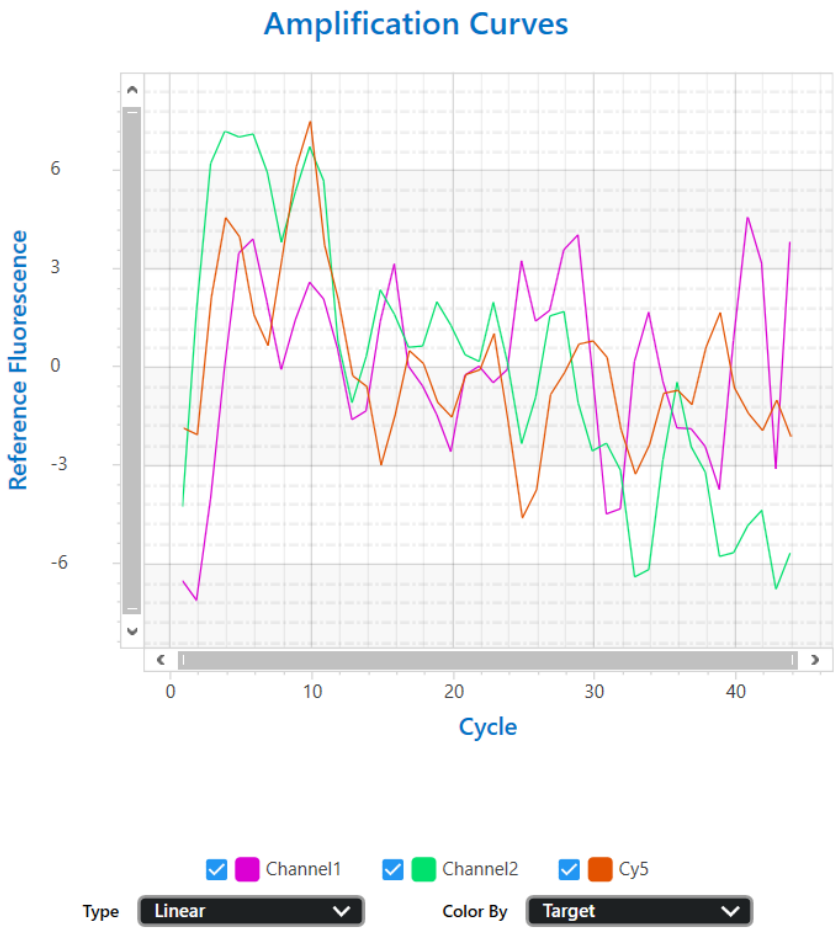


Figure 16: Amplification curves obtained after validation with unheated sample

Table 7: Validation of direct RT-qPCR i.e., without RNA extraction and heat inactivation at 95ºC for 10 min.

Sample Vol.	5µl			3µl			1µl		
Sample IDs	Ct values			Ct values			Ct values		
	N1	N2	RP	N1	N2	RP	N1	N2	RP
NP1	ND	ND	ND	27.48	32.74	32.31	27.56	29.84	24.36
NP2	ND	ND	ND	38.36	33.75	22.79	28.06	30.91	29.96

NP3	29.41	ND	ND	38.75	35.65	33	36.08	ND	23.35
NP4	ND	ND	39	32.17	38.36	33.17	ND	36.95	24.11
NP5	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP6	26.55	38.91	6.02	30.53	22.53	38.97	22.01	38.80	27
NP7	29.77	35.46	38.53	31.50	30.02	34.24	22	29.27	ND
NP8	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP9	28.74	3.09	ND	ND	ND	ND	ND	ND	32.07
NP10	ND	ND	ND	ND	ND	ND	ND	ND	ND
NP11	ND	ND	ND	37.25	ND	ND	ND	ND	ND

6 out of 11 previously confirmed positive samples from Kirtipur Municipality-TU Biotech Corona Laboratory, TU were also positive for heat inactivation at 95º C. Since the no. of positive samples detected at this temperature was not significant for validation, therefore, for more reliable result assessment, we next sought for heat inactivation at 70º C for 20 min to analyze the effect of different temperatures on covid19 positive sample through the change in Ct values.

***T*able 8: Validation of direct RT-qPCR i.e., without RNA extraction and heat inactivation at 70ºC for 20 min**.

swab Sample Vol.	**5µl**			**3µl**			**1µl**		
Sample IDs	**Ct values**			**Ct values**			**Ct values**		
	N1	**N2**	**RP**	**N1**	**N2**	**RP**	**N1**	**N2**	**RP**
NP1	ND	32.81	ND	24.41	31.49	23.04	28.59	29.28	16.18
NP2	ND	ND	14.91	29.73	32.11	38.48	ND	ND	14.03
NP3	ND	ND	18.63	24.16	33.87	30.33	35.47	30.05	21.86
NP4	35.13	20.12	22.37	38.6	22.02	39.79	ND	36.95	19.64
NP5	ND	ND	ND	17.19	26.69	26.08	ND	ND	ND
NP6	27.43	ND	35.66	32.02	38.03	37.39	22.74	38.80	27
NP7	ND	29.81	ND	27.63	27.61	29.41	26.24	29.27	30.98
NP8	3.87	ND	18.54	ND	ND	ND	ND	ND	24.02

NP9	28.74	ND	ND	30.89	19.29	33.78	30.49	29.46	26.27
NP10	ND	ND	ND	21.73	22.45	24.74	ND	ND	ND
NP11	ND	ND	ND	ND	ND	ND	ND	ND	ND

9 out of 11 samples were also positive for heat inactivation method, i.e. without RNA extraction when 3µl of sample were mixed with IDT master mix. SARS-CoV-2 failed to be detected in 2 of the samples, i.e. no fluorescence was detected for any genes. Additionally, the amplification curves generated were comparatively erratic and didn't have clear demarcation of Ct values so the 2 samples were assigned ND (Not Detected). PC was detected with Ct values of 33.28, 34.03 and 24.75 for N1, N2, and RP genes respectively. NTC was also run to check for any contamination during the process. No contamination was detected in NTC well. Besides, sample volume of 5µl and 1µl did not provide consistent results and also, no clear demarcation of Ct values, so these volumes were aborted for further study in this research. The figure 17 below illustrates the fluorescent signals of 3 different genes namely N1, N2, and RP associated with FAM (Pink), HEX (Green), and Cy5 (Orange) fluorophore respectively.

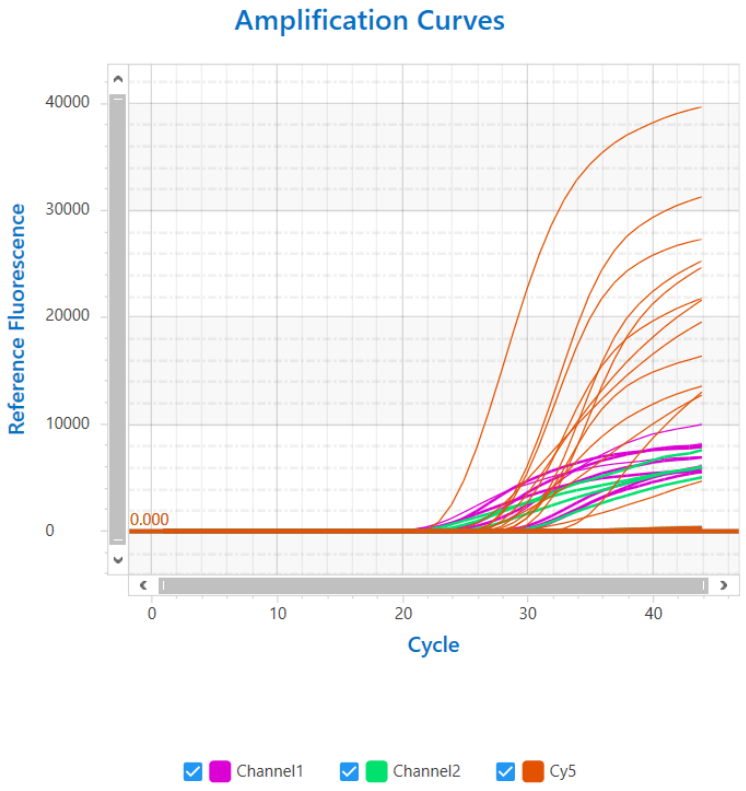


Figure 17: Amplification curves generated from NPS samples of 11 different NPS samples of covid19 patients for validation of heat inactivation method (heated at 70ºC for 20 min).

4.2. Demographic data for COVID19 with reference to gender

A total of 184 samples were tested for covid19, and as a result, demographic information based on the sex and gender of potential patients was generated. Positive and negative proportion of RNA extracted RT-qPCR was 154 (84%) and 30 (16%) respectively. Thermal inactivation at 70 °C for 20 min was also applied to 154 samples that were examined and found to be positive using standard qPCR.

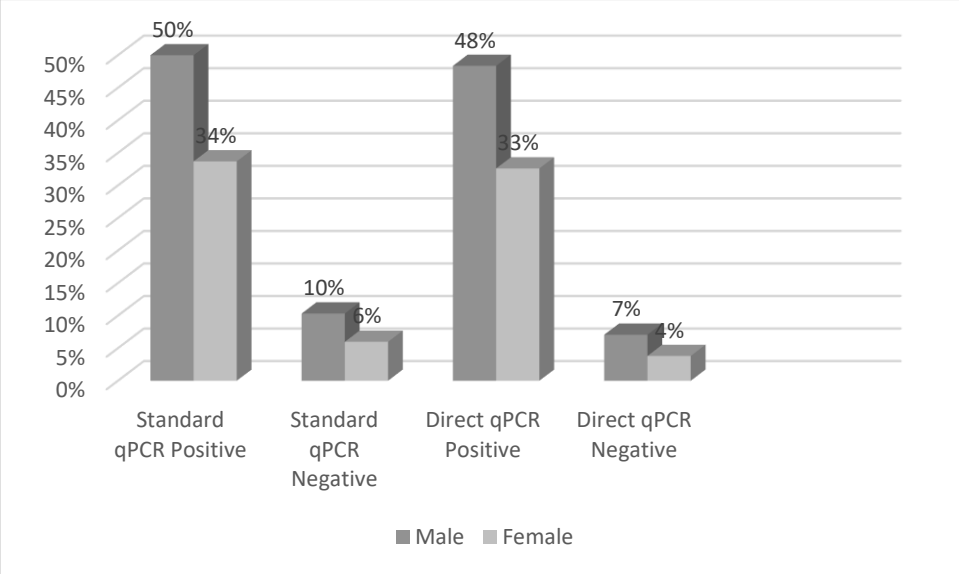


Figure 18: Bar graph illustrating covid19 positive cases according to sex/gender

By using a thermal inactivation method, 149 (81%) out of 184 samples were found to be positive. Of them, 89 patients were male and 60 were female, or 48% and 33% respectively. Similar to this, a total of 30 negative samples that were verified using standard qPCR were also put through the thermal inactivation process. However, only 20 (11%) were found to be negative, with 7 females and 13 males, or 4% and 7% respectively. Additionally, the heat inactivation method left 5 (2.71%) samples undetected even after retest, hence they were not included in this bar graph.

4.3. Demographic data for covid19 with respect to different age groups

Table 9: Positive and negative covid19 cases according to different age groups with different test methods

	Heat Inactivated SARS-Cov-2 RT-qPCR		RNA Extracted SARS-CoV-2 RT-qPCR	
Age Group	**Positive %**	**Negative %**	**Positive %**	**Negative %**
>20	1.086957	0	1.086957	0
20-30	34.23913	5.978261	35.32609	4.347826
31-40	20.1087	3.804348	18.47826	4.891304
41-50	18.47826	2.173913	16.84783	3.26087
51-60	4.347826	1.086957	4.347826	0.543478
61-70	3.804348	2.173913	3.26087	2.717391
71-80	0.543478	1.086957	1.630435	0

Age groups between 15 to 80 are included in this study for suspected cases. The highly infectious group, which visited the lab between January and March, was between the ages of 20 and 30, whereas the least infectious group, which visited between January and March, was above the age of 70. A total of 37 out of the total sample, or around 34.23% of those between the ages of 20 and 30, tested positive utilizing the heat inactivation method. In a similar vein, the usual standard technique revealed 35.32% of people of a similar age to be positive.

4.4. Performance of direct heat inactivation approach

Table 10: Effect of direct heat inactivated RT-qPCR approach for the detection of SARS-CoV-2

Total samples	**True positive**	**True negative**	**False positive**	**False negative**	**Undetected**
184 (+ve=154 -ve=30)	139/154 (90.25%)	29/30 (96.66%)	1/30 (3.33%)	10/154 (6.49%)	5/184 (2.71%)

154 out of a total of 184 samples had positive results using an RNA extracted RT-qPCR method. These samples were thought to be authentically positive samples. In a similar vein, 30 samples were determined to be truly negative for SARS-CoV-2 using a similar

approach. 1 false positive and 10 false negative results were obtained from RT-qPCR using samples that had been heat inactivated. The accuracy of this technique can simply be calculated by the sum of true positive and negative results divided by total no. of samples. This can be calculated as,

$$\text{Accuracy} = \frac{139+29}{184}$$

$$= 0.913$$

$$= 91.30\%$$

Table 11: Clinical effectiveness of the heat shock approach for SARS-CoV-2 detection

Methods	RT-qPCR Qualitative Results		
	Positive N1 gene %	**Positive N2 gene %**	**RNase P gene %**
Heat Inactivated RT-qPCR	153 (99.35%)	149 (96.75%)	183 (99.35%)
RNA Extracted RT-qPCR	154	154	184

154 samples with RNA extracted for RT-qPCR were positive for both the N1 and N2 genes. A total of 153 (99.35%) samples were N1 gene positive and 149 (86.75%) samples were N2 gene positive after being heat-inactivated at 70 °C for 20 min. 184 of the total samples tested positive for the presence of the RNase P gene using RNA extracted for RT-qPCR, whereas 183 (99.35%) tested positive using heat inactivation. Three samples (3/184 = 1.63%) were continuously double target gene positive even after re-testing, but two samples (1.08%) using the heat inactivation technique was single gene positive even after re-testing.

4.5. Comparative analysis of Ct values between two methods

Comparison of the CT values obtained from COVID-19 patient NP swabs after heat inactivation and RT-qPCR with conventional standard RT-qPCR was carried out using the following graph.

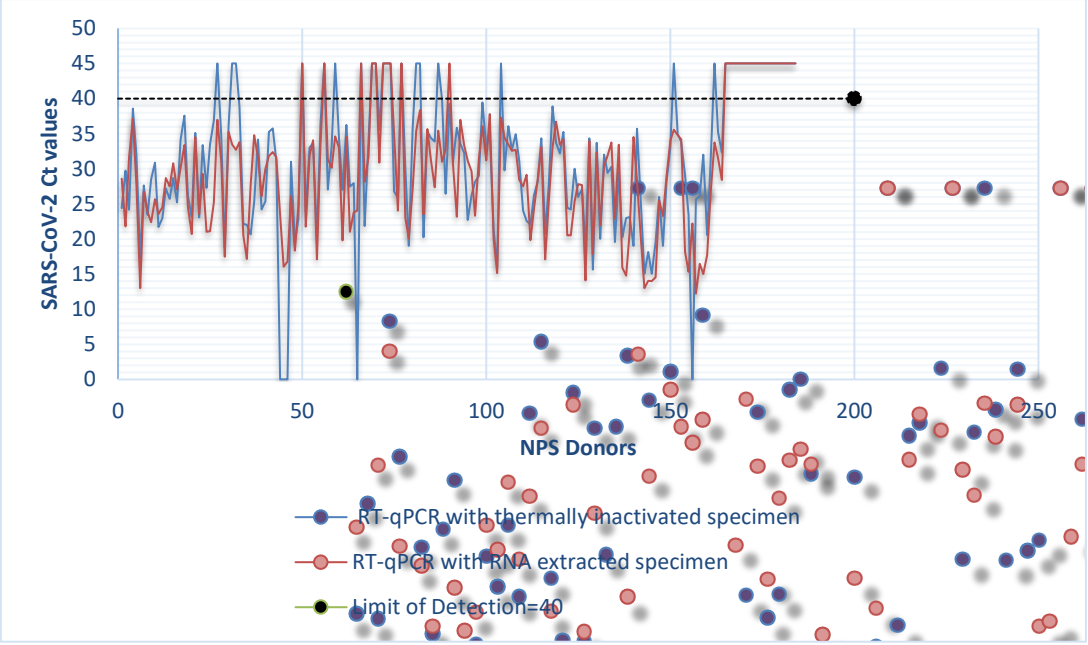


Figure 19: Distribution of Ct values of the RT-qPCR with and without RNA extraction approaches

Figure compares the Ct values from the original clinical RT-qPCR performed on the equivalent of 3µl of NP swab with that of heat inactivated sample of same volume. The 5 samples (blue dots adjoining X-axis) that remained undetected by heating approach came from donors having high Ct values, i.e. with low viral RNA content. The samples which were above the limit of detection (LOD) line (denoted with black dashed line) are considered negative for SARS-CoV-2 RNA. In contrary, the samples below the LOD are considered to be positive for SARS-CoV-2 RNA. The above scatterplot was created using RNA from 3µl of the same samples used in RT-qPCR (pink circles), allowing for a side-by-side comparison of direct RT-qPCR (blue circles) on the same amount of NP swab.

Table 12: Agreement statistics of heat inactivated samples in relation to different categories of Ct values

Viral RNA load	RT-qPCR with heat inactivated samples	RT-qPCR with RNA extracted samples	Detection sensitivity of heat inactivated method
High (Ct < 20)	14	25	14/25 (56%)
Intermediate (Ct 20 - 30)	42	62	42/62 (67.74%)

Low (Ct > 30)	59	67	59/67 (88.05%)

154 NP swabs from COVID-19 patients representing high (Ct less than 20), intermediate (Ct between 20 - 30), or low (Ct of more than 30) viral RNA loads were tested by direct RT-qPCR to obtain an accurate picture of how omission of the RNA extraction would perform in a real-world clinical setting. In case of standard detection technique, 25 samples were detected to be having Ct values lower than 20. Similarly, 62 and 67 samples were detected to have Ct values between 20 to 30 and greater than 30. These figures were compared to those obtained from a heat inactivation method, which detected only 14 samples with Ct values less than 20, yielding an agreement of 56%. 42 out of 62 SARS-CoV-2 intermediate positive samples for RNA extraction were also positive for the heat inactivation method, resulting in an agreement of 67.74%. Likewise, agreement between samples greater than 30 was 88.05%. Therefore, samples with high Ct values had the highest agreement between two methods, while samples with low Ct values had the lowest.

Table 13: Qualitative analysis results of direct and indirect qPCR estimation indicating sensitivity and specificity

HEAT INACTIVATED qPCR * STANDARD qPCR Cross tabulation

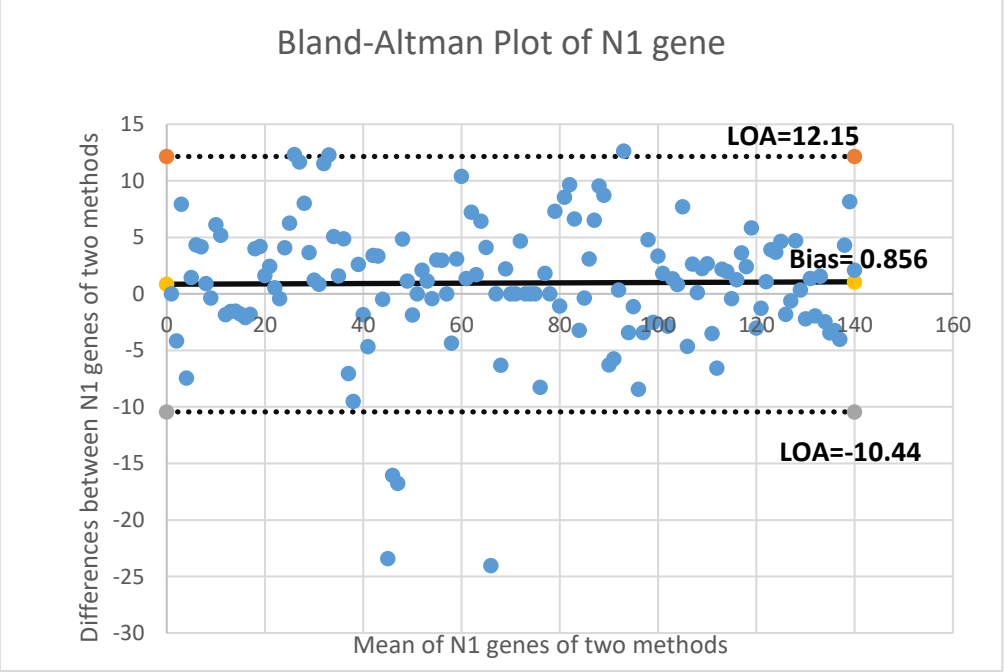
			STANDARD qPCR		
			0	1	Total
HEAT INACTIVATED qPCR	0	Count	29	10	39
		% within HEAT INACTIVATED qPCR	74.4%	25.6%	100.0%
		% within STANDARD qPCR	96.7%	6.7%	21.8%
	1	Count	1	139	140
		% within HEAT INACTIVATED qPCR	0.7%	99.3%	100.0%
		% within STANDARD qPCR	3.3%	93.3%	78.2%

Total	Count	30	30	149
	% within HEAT INACTIVATED qPCR	16.8%	16.8%	83.2%
	% within STANDARD qPCR	100.0%	100.0%	100.0%

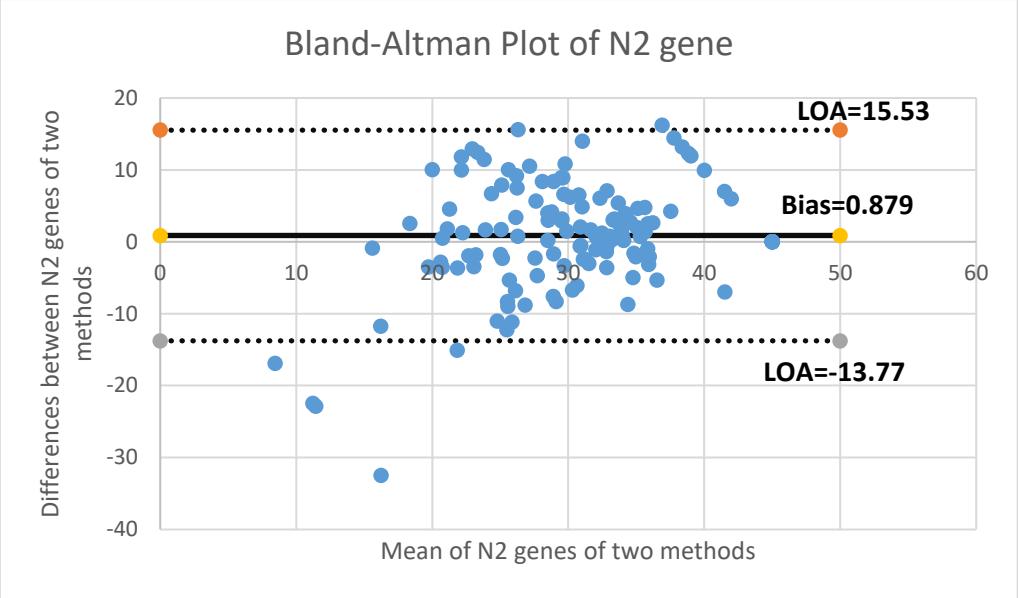
Ninety-five percent confidence intervals (CI) for sensitivity, and specificity were calculated by the inter- rater reliability with McNemar's test in SPSS. The sensitivity and specificity of heat inactivation method was cross tabulated as above and found to be 93.3% and 96.7% respectively.

4.6. Agreement and correlation between standard RT-qPCR and direct method

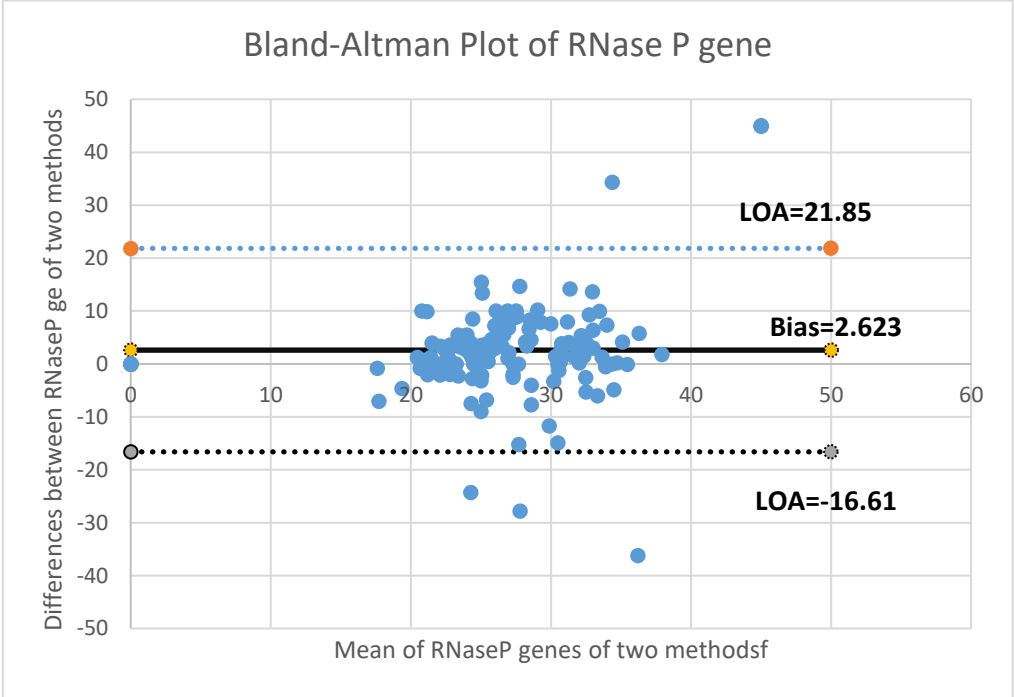
In order to compare and appraise the differences between this new heating technique with that of the standard existing technique, Bland-Altman graph was plotted between different Ct values of two measurements against the mean of two measurements.



(A)



(B)



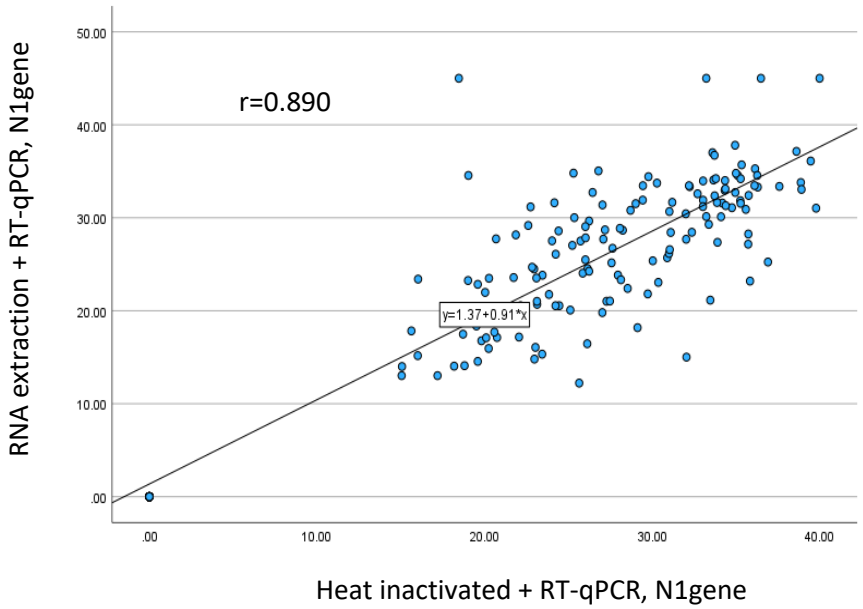
(C)

Figure 20: The Bland-Altman Comparison of the Ct values for the SARS CoV-2 virus's (A) N1, (B) N2, and (C) RNase P genes using two different techniques

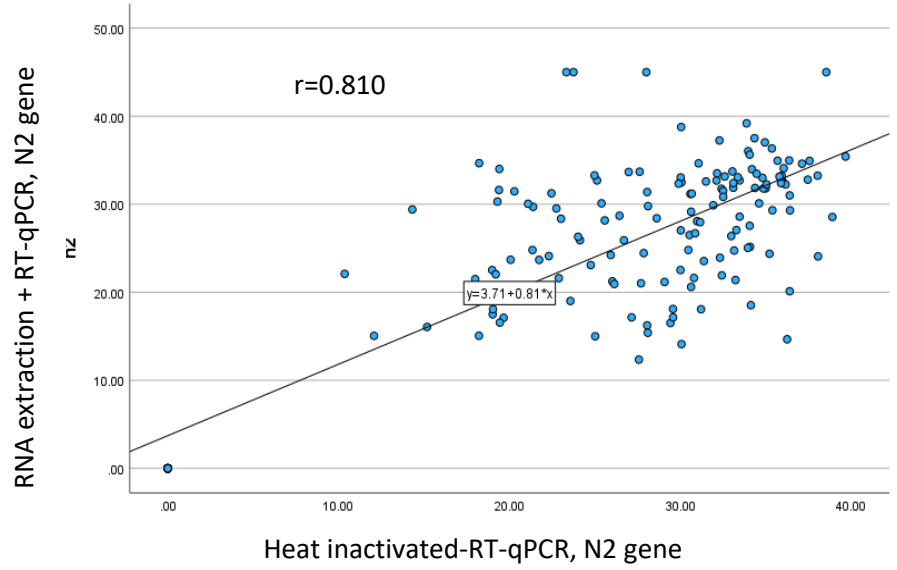
Bland-Altman graphs were constructed to examine agreement between two separate techniques, and they revealed good agreement between the Ct values of all three genes obtained using both methods, with only few samples exceeding the LOA (Limit of Agreement). The Bland Altman plot revealed inconsistent Ct values both below and above

the mean difference. The Bland Altman comparison also showed that, for N1 gene, the lower and upper LOA between heat treated and RNA extracted samples were -10.44 and 12.15 at 95% CI respectively. Similarly, for N2 genes, the higher and lower LOA were 15.53 and -13.77 respectively. Finally, for RNase P gene the higher and lower LOA were 21.85 and -16.61 respectively. 95% of the differences in Ct values between the two instruments are expected to fall in these ranges of upper and lower limits. The chart's horizontal line in the center, which represents the average measurement difference between the two techniques, is termed as "bias". For N1, N2 and RNase P, the biases are 0.856, 0.879 and 2.623 respectively. The average discrepancy in measurements between the equipment increases as this number deviates from zero. Therefore, on average Ct values measured via heat inactivation method is slightly higher than that measured via RNA extraction by 0.856, 0.879 and 2.623 for N1, N2, and RNase P genes respectively.

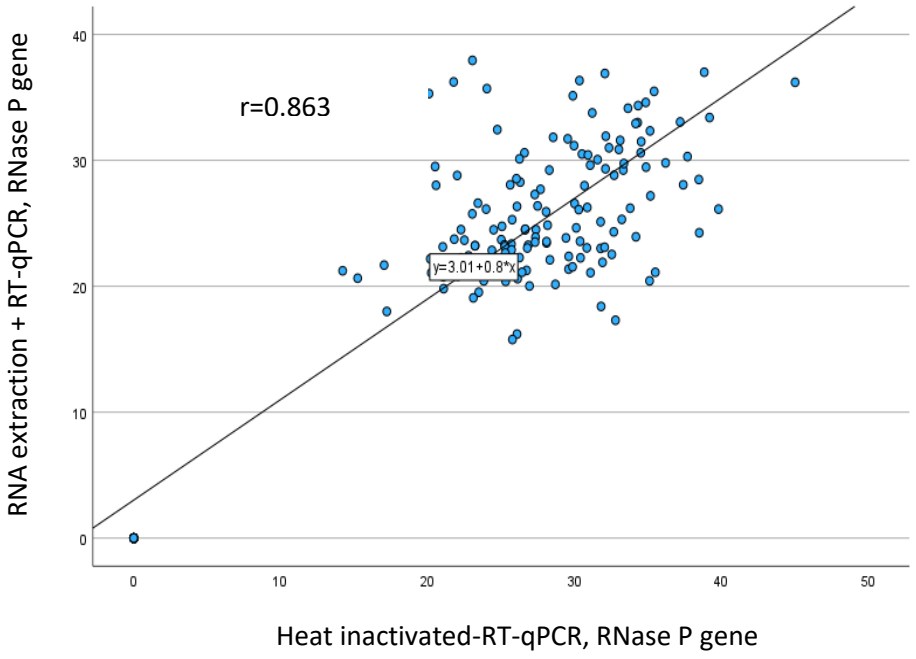
Since this plot gives no information about the correlation between two different methods, Pearson's correlation coefficient (r) was calculated for each gene namely, N1, N2 and RNase P and was found to be 0.89, 0.81 and 0.86 respectively with $P < 0.001$. This suggests that there is a strong positive correlation between the two approaches, indicating that the relation is significant.



(A)



(B)



(C)

Figure 21: Correlation of heat inactivated- and RNA extracted-RT-qPCR Ct values of (A) N1- gene (B) N2-gene and (C) RNase P-gene obtained by Azure Biosystems

The results for paired T-Test revealed that the mean difference between both methods of N1 gene Ct was significantly different ($p < 0.001$) for the heat inactivation method (Ct =

24.62) compared to the RNA extraction method (Ct = 23.33). However, the mean difference between both methods of the N2 gene Ct was not significantly different (p = 0.02) for the heat inactivation method (Ct = 25.55) compared to the RNA extraction method (Ct = 24). The mean difference between both methods of the RNase P gene Ct was significantly different (p < 0.001) for the heat inactivation method (Ct = 24.68) compared to the RNA extraction method (Ct = 22.80). Overall, the average differences in Ct values of with and without RNA extraction was 1.28 for N1 gene, 1.546 for N2 gene and 1.877 for RNase P gene. It means that after heat inactivation the N1, N2 and RNase P gene was increased by 1.28 and 1.546 and 1.877 averagely on each sample respectively.

For qualitative assessment of data obtained from both the methods, McNemar test and Cohen's Kappa inter rater reliability test were applied. Consequently, McNemar test indicated that, the qualitative detection of SARS-CoV-2 (negativity or positivity) between heat-inactivated and RNA extracted group results had statistical significant difference (p=0.012) at 95% CI (p<0.05). Similarly, the Cohen's Kappa measures the agreement between two methods. Consequently, the kappa value was found to be 0.803 (95% CI). Thus, substantial agreement between two methods can be inferred from this value obtained.

CHAPTER V

DISCUSSION

Eliminating RNA extraction step for the detection of SARS-COV-2 RNA is completely a novel approach in the context of Nepal and also in many developed countries. Few papers have been published so far regarding this strategy. However, this unprecedented approach is not known to many. Thus, this study carries paradigm shifting implications for how simplifying the pre-PCR step, i.e., simple heat-RNA release step can be an alternative to the traditional RNA extraction approach. It is sought to provide a valuable option, cheaper, easier and less reagent consuming for SARS-CoV-2 diagnostic, especially in low to middle-income countries with laboratories having lower experience and equipment for molecular assays.

Molecular testing for SARS-CoV-2 is required for a more precise diagnosis because clinical symptoms for COVID-19 might occasionally be non-specific (cough, moderate fever, sore throat, weariness), comparable to other respiratory infections, or even absent despite infection (Fomsgaard et al. 2020). Our Kirtipur Municipality- TU Biotech Corona laboratory typically uses the CW Bio Viral DNA/RNA kit followed by Azure Biosystems SARS-CoV-2 RT-qPCR assay to diagnose COVID-19. To circumvent the cumbersome RNA extraction process, an alternative technique of heating for 20 min at 70 °C that yielded a sensitivity, specificity, and accuracy of 93.3%, 96.7%, and 91.3% respectively can be utilized. These findings suggest that in situations where the extraction step is not possible, direct RT-PCR can be a dependable technique. Moreover, in a related study by Fomsgaard et al. 2020, showed 97.4% sensitivity, 100% specificity and 98.3% accuracy (98º C for 5mins). Similar techniques were employed in a subsequent study by Hasan et al. in 2020. Their assay was able to identify 95% of the positive samples and had 99% specificity. Compared to our study, both papers' indirect technique sensitivity results were higher. Moreover, they employed, more sensitive amplification kit, which contributes to the high sensitivity percentage.

We experimented with several sample volumes and temperatures to validate our approach. Initially, temperature of 95º C for 10 min was applied on 11 NPS specimen resulting in only 6 samples being detected by PCR machine. To achieve sufficiently good

validation, we did opt for another temperature variation at 70º C for 20 min followed by 10% Triton-X treatment for 10 min. additionally with this, different volumes of swab samples were tested including 10µl, 5µl, 3µl and 1µl. However, 3µl swab loading volume was observed to be optimal since it detected all three genes in 9 out of 11 samples. Contrarily, using high heat (95 °C for a 10 minutes) to perform direct RT-PCR instead of RNA extraction frequently led to reduced RT-PCR sensitivity, which is probably triggered by the breakdown of phosphodiester bonds in the targeted sequence (Barza et al., 2020). Furthermore, we failed to get ideal results with larger volume of 10µl and 5µl. this can be explained by the study of Alaifan et al., 2021 who discussed that as with direct RT-PCR, where the greater the VTM volume, the greater the risk of introducing inhibitors, as was also discovered in another study by Smyrlaki et al., 2020, higher volume inputs did not enhance sensitivity.

RT-qPCR testing success is influenced by a variety of parameters. Because RNA extraction concentrates and purifies the RNA targets while excluding PCR inhibiting chemicals, it is preferable to using direct specimens (Hasan et al., 2020). When untreated/unheated sample were mixed with IDT master mix and subjected to RT-qPCR, aberrant curves were detected. This can be explained by the work of Hasan et al., 2020, which shows that it is challenging to use pre-treated or untreated material directly in RT-qPCR due to the presence of inhibitors and/or RNases. In addition to this, the five samples that went undetected after thermal exposure to 70º C for 20 min may have been missed due to the RNA loss by heating. As a result, this technique correctly detected 159 samples out of 184, yielding an accuracy of 91.3%. Besides, in this direct heating technique, the VTM components were added directly to the master mix, which may have interfered with or partially inhibited RT-PCR and delayed Ct results (Alaifan et al., 2021), especially in samples with low viral loads (Ct > 30), which has impacted the sensitivity of our assay.

The results so far provided supports the heat inactivation at 70º C for 20 min followed by RT-qPCR procedure's validity as a comparable alternative to the column based manual extraction protocol typically used for SARS-CoV-2 detection. Consequently, it is anticipated that there will be low or modest clinical impact if the new direct RT-qPCR methodology replaces the standard protocol currently used in our laboratory since 1/154 (3.33%) false positive and 10/154 (6.49%) false negative results have been estimated. The

reason with single false positive even after retesting may be due to cross contamination with positive samples in the VTM containing sample during storage or former processing.

In samples with high Ct values, the test revealed substantial false-negative rates (6.49%). Also, low viral load samples failed to be detected (2.71%). Those 10 samples with false negative results had mean Ct value of 35.35. Numerous articles have written about the problems of false-negative RT-PCR results. In this regard, Kong et al., 2022, have mentioned that false negative results might be brought on by variations in the sampling site or sample condition, incorrect sample storage, long processing times, or low reagent sensitivity. Also, according to Pan et al., 2020, false negative results in some weak positive samples may be because of prolonged storage or preservation at ambient temperature. Additionally, Pan et al.,2020 discovered that the effects of thermal inactivation varied amongst various sample types, indicating that the composition of studied samples may affect the viral thermal-stability. Such variations in thermal stability have also been observed in earlier investigations, which discovered that the effects of fetal calf serum (20%) or antithrombin III on SARS-CoV thermal inactivation might be reduced. During pandemic, the only way to slow the COVID-19 infection's rapid spread is through early identification and detection, prevention, and early control (Woldesemayat et al., 2022). On some measures, nevertheless, it might have a detrimental effect since false-negative individuals in the population may be transferred to a wide spectrum of people, even while heat inactivation is quite safer and non-invasive than RNA extraction.

By adopting the heat inactivation technique, discernible shift toward higher Ct values for the N1, N2, and RNase P genes were noticeable despite the use of the same volume of sample for the RT-qPCR. Furthermore, the N1 gene sensitivity (83.15%) by heat inactivation was found to be lower than the N1 of standard RT-qPCR (83.69%). This might be attributed to experimental variability (Bruce et al., 2021) which encompasses NPS collected in different VTMs or instrumentation or sample preparation). Besides, for Ct range below 20, a considerable reduction of sensitivity was seen. Because centrifugation and elution on a smaller volume result in less effective RNA concentration than using ionic binding columns, such as those found in RNA extraction kits, the lack of sensitivity seen is likely caused by this (Bruno et al., 2020). However, we discovered a sensitivity of 93.3 % for our heat inactivation approach overall. This table, which is utilized to compare our

findings, was taken from a study by Bruno et al.,2021, in which they compared several research papers with their own.

Table 14: Comparison of published studies' clinical results for SARS-CoV-2 detection without RNA extraction

sample treatment	sample size (SARS-CoV-2 +)	sensitivity (%)	reference
65°C/20min	86	94.0	(Fomsgaard & Rosenstierne, 2020)
98°C/5min - no treatment	40	58.0 - 56.0	(Freire-Paspuel et al., 2020)
98°C/5min	39	97.4	(Freire-Paspuel et al., 2020)
99°C/5min	91	81.3	(Freire-Paspuel et al., 2020)
65°C/10min	19	95.0	(Freire-Paspuel et al., 2020)
95°C/10min - no treatment	150	92.0 - 84.0	(Lu et al.. 2020)
99°C/5min	543	77.5	Bruno et al, 2021

Previous studies addressing heat shock methods without RNA extraction for SARS-CoV-2 detection made it abundantly clear that high Ct values in the range of 32 to 40 were associated with failure to detect SARS-CoV-2 positive samples (Barza et al. 2020; Bruce et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; and Bruce et al., 2021). Our study's findings are also consistent with those from these studies mentioned since 5 samples with Ct values between 30-40 went undetected.

According to our paired T-test analysis, the mean Ct values of the N1 and RNase P genes were statistically significant at 95% CI ($p<0.001$), whereas the N2 genes were not ($p>0.001$). This contradicts with the studies done at Charite University in Berlin, Germany, Renmin Hospital of Wuhan University in Huazhong, China, Republic of South Korea, and Zhejiang University School of Medicine in China, which mentioned that there was no statistically significant difference between the Ct values of the non-inactivated group and that had been heat-inactivated at 56 °C for 30 min. These discrepancies can be the result of sample size, sample type, or a different strain circulating in the community, all of which demand for more research (Woldesemayat et al., 2022). Besides our paired T-test result also revealed that after heat inactivation, the N1, N2 and RNase P genes were increased by 1.28 and 1.546 and 1.877 averagely on each sample respectively. Heat inactivation prior to extraction can dramatically increase Ct values compared to the original sample

according to research by two Chinese, Pan et al. 2022and Chen et al., 2020. In this research correlation between heating technique and standard technique was also examined. Pearson's correlation coefficient (r) for N1, N2 and RNase P genes was found to be 0.89, 0.81 and 0.86 respectively with $P < 0.001$. Therefore, excellent correlation between Ct values of both techniques can be deciphered.

Considering the limited sample size in our study, a larger number of specimens could have led us to a more significant result. It's better to emphasize that all RT-qPCR assays utilized in conjunction with the heat-processing workflow should be verified prior to being employed in clinical diagnostics due to variations between RT-qPCR assays. We further highlight that oropharyngeal swabs heated for 20 minutes at 70 °C before a SARS-CoV-2 RT-qPCR reaction are not as precise or sensitive as standardized RT-qPCR experiments conducted on purified/RNA extracted samples. Furthermore, we cannot completely rule out the possibility of RNA degradation during heating, even though kappa value resulted in good reliability between the Ct values for the heat-processed samples and the RNA extracted samples via Kohen's Kappa statistical test. However, when necessary, the adoption of this type of technique can be beneficial for TAT and emergency situations like a paucity of supplies and the pandemic's high testing demand (Alaifan et al., 2021). This statement can be supported by our results obtained through inter-rater reliability Cohen's Kappa test which correctly measured an agreement of 0.803 between two methods. On that account, we fail to reject null hypothesis (H_o).

Direct RT-PCR has demonstrated good outcomes in the detection of DNA viruses in hepatitis B and Mycoplasma organisms (Baaity et al., 2019; Cheng et al., 2007, Alaifan et al., 2021). According to the study by Wiedbrauk et al., 1995, the most common reason for inadequate testing sensitivity in various fluids is PCR inhibition, some of which are found to be heat-resistant and require the extraction stage with sample dilution to remove the inhibition.

In a nutshell, our novel method showed good specificity and sensitivity in the detection of SARS-CoV-2 RNA. This emerging concept is believed to drastically lower costs and accelerate assay TAT by omitting the RNA extraction step. Inadequate and delayed laboratory testing can seriously impair pandemic control efforts. Our findings will be useful to numerous ill-equipped laboratories that are battling a reagent shortage and

need to continue testing for SARS-CoV-2. Similar case was observed in Ecuador where more than 100,000 samples were tested by this same method to cope up with extraction kit demands and control the infection rates in the nation. Therefore, the enhanced direct approach we presented may help resource-constrained countries increase their capacity for RT-qPCR testing because it is significantly less expensive. Moreover, the key benefits of adopting this assay include cutting the diagnostic test time in half, boosting cost effectiveness, reducing the amount of manual work required, which reduces the risk of contamination, and overcoming extraction reagent supply issues (Esona et al., 2013). The significance of our study increases in times of scarcity, but the direct assay can be enhanced by adjusting the lysis reagent and scrutinizing the various kinds of VTMs, which are crucial for incorporating PCR inhibitors. Even though this approach enables physicians to significantly shorten processing times, we think its application should be limited to clinical laboratories where the absence of RNA extraction reagents poses a challenge to meeting diagnostic requirements since, it's crucial to guarantee the accuracy of the analysis while making a patient diagnosis.

Under this scenario, this study expects to contribute in the development of more standardized and revised protocols to be implemented in any labs in Nepal. It is anticipated to more effectively and minimally invasively manage infection rates among populations.

CHAPTER VI

CONCLUSION

RT-qPCR has undoubtedly been a gold standard technique till date with highest sensitivity and specificity for detection of SARS-CoV-2 RNA. However, this method involves RNA extraction which is quite cumbersome and labor intensive. Besides, repeat testing is one of the commonest problems encountered due to contamination during RNA extraction or storage causing inconclusive results by PCR thereby resulting in long turnaround time (TAT). Data from our validation provides an insight about the heating method as an alternative for standard approach with minimal clinical impact especially for those labs dealing with supply shortage. For the qualitative detection, i.e. positive and negative, we found no statistical significance between two methods ($p=0.012$). However, statistical significance of N1 and RNase P genes Ct values between both the methods were observed ($p<0.001$). Therefore, slight differences between Ct values measured by both the methods is evident. Additionally, Ct value increment post heat treatment at 70º C for 20 min was noted with small percentage of false negative which adds to the limitation of this protocol. During the SARS-CoV-2 pandemic crisis, we were able to conduct and assess a direct and easy RT-PCR test that does not require extraction. The direct RT-PCR method did produce adequate results that were well-concordant with the gold standard test. Direct RT-PCR might be a useful method for quickly identifying COVID-19 suspects. Our research offers a quick fix for the RNA extraction supply crunch. Thus, this heating approach, replacing RNA extraction can be a cutting edge strategy for considerable population provided that repeat test using RNA extraction be still reserved for those suspecting infection but a negative initial results.

LIMITATIONS OF THE STUDY

- SARS-CoV-2 viral quantification (LOD) was not undertaken. As a result, the viral copy number of these two strategies could not be compared.
- Since fresh samples weren't used, the tests' sensitivity and specificity might have been impacted.
- The use of hot blocks for this study resulted in unequal heating or spikes in temperature.

RECOMMENDATIONS AND FUTURE PERSPECTIVES

- For widespread clinical application, it is advised that more standardized and revised protocols be developed.
- Further research is needed before we can pinpoint the optimal conditions and compositions that will allow the approach to function at its best.
- Assessing VTMs, lysis temperature and buffers would improve sensitivity, specificity and accuracy of this assay
- It’s better to emphasize that all RT-qPCR assays utilized in conjunction with the heat-processing workflow should be verified prior to being employed in clinical diagnostics due to variations between RT-qPCR assays

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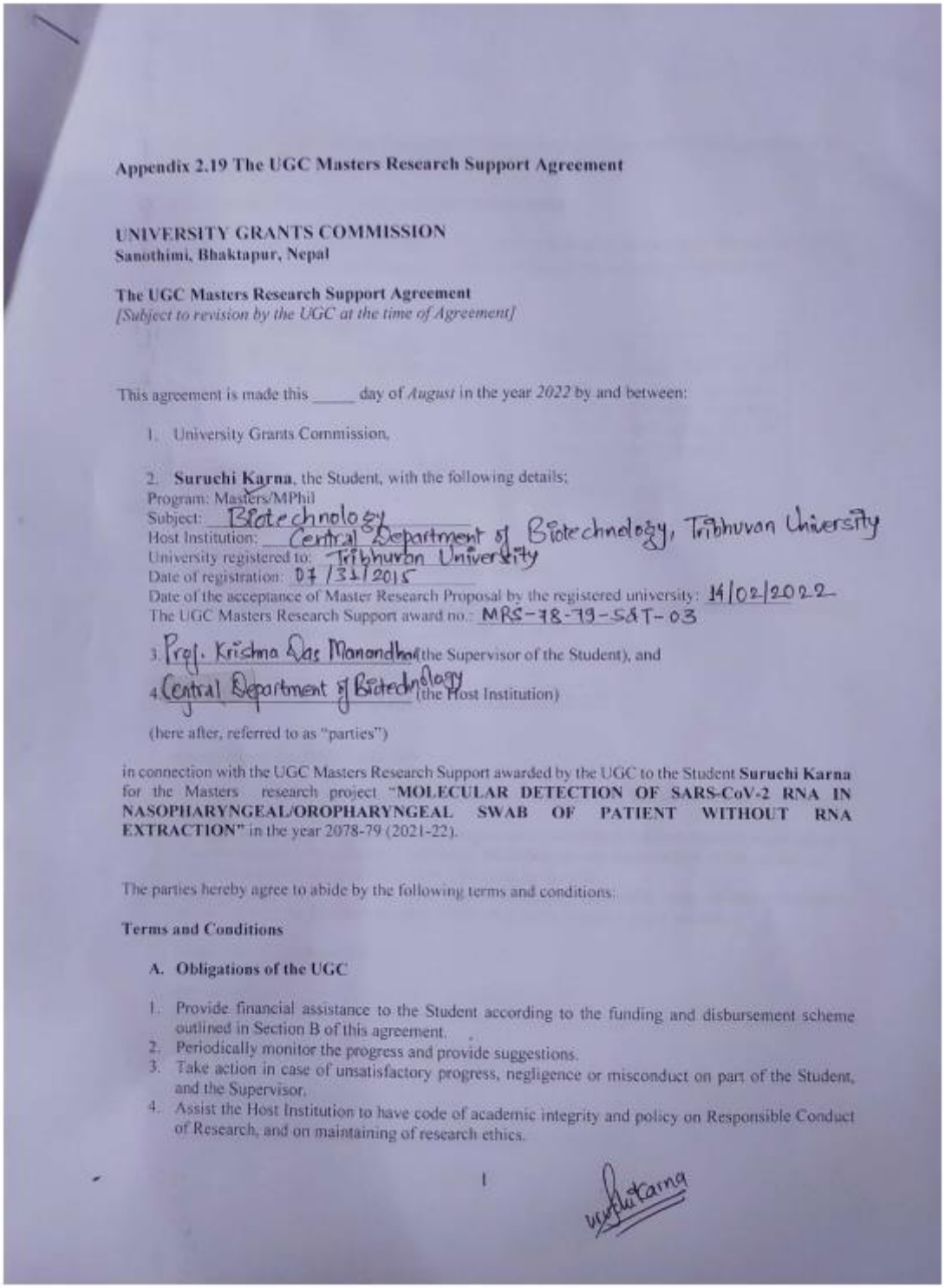
APPENDIX I: UGC Grant Agreement Form



Appendix 2.19 The UGC Masters Research Support Agreement

UNIVERSITY GRANTS COMMISSION
Sanothimi, Bhaktapur, Nepal

The UGC Masters Research Support Agreement
[Subject to revision by the UGC at the time of Agreement]

This agreement is made this _____ day of *August* in the year *2022* by and between:

1. University Grants Commission,

2. **Suruchi Karna**, the Student, with the following details;
Program: Masters/MPhil
Subject: Biotechnology
Host Institution: Central Department of Biotechnology, Tribhuvan University
University registered to: Tribhuvan University
Date of registration: 07/31/2015
Date of the acceptance of Master Research Proposal by the registered university: 14/02/2022
The UGC Masters Research Support award no.: MRS-78-79-SAT-03

3. Prof. Krishna Das Manandhar (the Supervisor of the Student), and

4. Central Department of Biotechnology (the Host Institution)

(here after, referred to as "parties")

in connection with the UGC Masters Research Support awarded by the UGC to the Student **Suruchi Karna** for the Masters research project "**MOLECULAR DETECTION OF SARS-CoV-2 RNA IN NASOPHARYNGEAL/OROPHARYNGEAL SWAB OF PATIENT WITHOUT RNA EXTRACTION**" in the year 2078-79 (2021-22).

The parties hereby agree to abide by the following terms and conditions:

Terms and Conditions

A. Obligations of the UGC

1. Provide financial assistance to the Student according to the funding and disbursement scheme outlined in Section B of this agreement.
2. Periodically monitor the progress and provide suggestions.
3. Take action in case of unsatisfactory progress, negligence or misconduct on part of the Student, and the Supervisor.
4. Assist the Host Institution to have code of academic integrity and policy on Responsible Conduct of Research, and on maintaining of research ethics.

1

Suruchi Karna

APPENDIX II: Informed Consent Form

Detection of SARS-CoV-2 using qRT-PCR in Nasopharyngeal/oropharyngeal swab obtained from asymptomatic or mild COVID-19 patients

सम्बन्धि अध्ययनमा सहभागी मञ्जुरीनामा फाराम

विश्व महामारीको रुपमा फैलिरहेको COVID-19 ,कोरोना भाइरसको नयाँ प्रजाति SARS–COV–2 ले हुने भाईरल संक्रमण हो। यो श्वास प्रश्वास , खोक्दा, हाच्छ्युँ गर्दा निस्कने थुक, खकार तथा सिगाँनका कणहरुका माध्यमबाट सर्दछ । माथि उल्लेखित शिर्षक Detection of SARS-CoV-2 using qRT-PCR in Nasopharyngeal/oropharyngeal swab obtained from asymptomatic or mild COVID-19 patient मा नेपालमा हाल कोरोना भाईरस संक्रमणको प्रभाव र प्रकृति बुझ्नका लागि गरिएको एउटा अनुसन्धान हो । तपाईलाई यस अनुसन्धानमा सहभागी गराउनुको मुख्य उद्धेश्य तपाईमा देखिएको स्वास्थ्य समस्या कोरोना संक्रमणले गर्दा हो वा होईन, हो भने यसले तपाईलाई कस्तो प्रभाव पारिरहेको छ, तपाईमा यसको संक्रमणकाल कति रहेको छ अध्ययन गर्नु हो ।

तपाई यस अनुसन्धानको स्वयंसेवकको रुपमा आफ्नो नाक तथा मुख बाट तरल पदाथ दिनुपर्नेछ । तपाइबाट लिएको जैविकपदार्थलाई Kirtipur Municipality-TU Biotech Corona Laboratoryमा विभिन्न साधन प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र (Questionnaire) प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ । तपाईबाट लिइएको जैविकपदार्थ र यसबाट आएको तथ्याङ्कप्रयोग गरि कुनै किसिमको व्यापारिकरण गरिने छैन ।

तपाईबाट लिइएको जैविकनमुना तथा तथ्याङ्क भण्डार गरेर राखिनेछ र भविष्यमा चाहीएको खण्डमा प्रयोग गर्न सकिनेछ ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनमा हुने परिक्षणमा तपाइको शरिरमा नविन कोरोना भाइरस भए नभएको विषयमा RT-PCR प्रविधि प्रयोग गरि गरिन्छ र यसले रोगको पहिचान गरी संक्रमण कम गर्न मद्दत गर्न सक्छ । तपाईले Nasopharyngeal/oropharyngeal swab दिदा केही जोखिम हुने छैन । तपाईले आफ्नो रिपोर्ट प्राप्त गर्नुहुनेछ र positive भएमा isolation को लागी पनि उचित परामर्श प्राप्त गर्नुहुनेछ । यदि कुनै पनि गम्भीर लक्षण र समस्या देखिएमा अस्पतालमा सम्पर्क गर्नुहोस् ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

यो रोग नेपालमा मात्र नभएर संसारभर नै महामारीको रुपमा फैलिरहेको छ । यसले बेलाबेलामा आफ्नो स्वरुप फेरीरहने र नयां नया भेरीयन्टको रुपमा फैलिने गरेको पनि पाइएको छ । त्यसैले तपाईको शरीरमा भईसको मात्रा कति छ र उक्त भाईरस निस्कृय हुन कति समय लाग्यो भनी अध्ययन गर्नाले नेपालमा यो रोगको अवस्था थाहा पाउन तपाइको सहभागिले ठूलो भूमिका खेल्ने छ ।

जोखिम : स्वाब लिँदा तपाईले हल्का असहज महसुस गर्न सक्नुहुनेछ । nasal bleeding पनि हुन सक्ने सम्भावना छ ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैल आफ्नो इच्छाले बिना जरिवाना ,बिना डरत्रास, अनुसन्धानकर्ता समक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

सहभागीको हस्ताक्षर :

(अथवा सहभागीको हकमा अनुमती प्रदान गर्ने व्यक्तिको हस्ताक्षर)

सहभागिको नाम :

ठेगाना :

सम्पर्क नं :

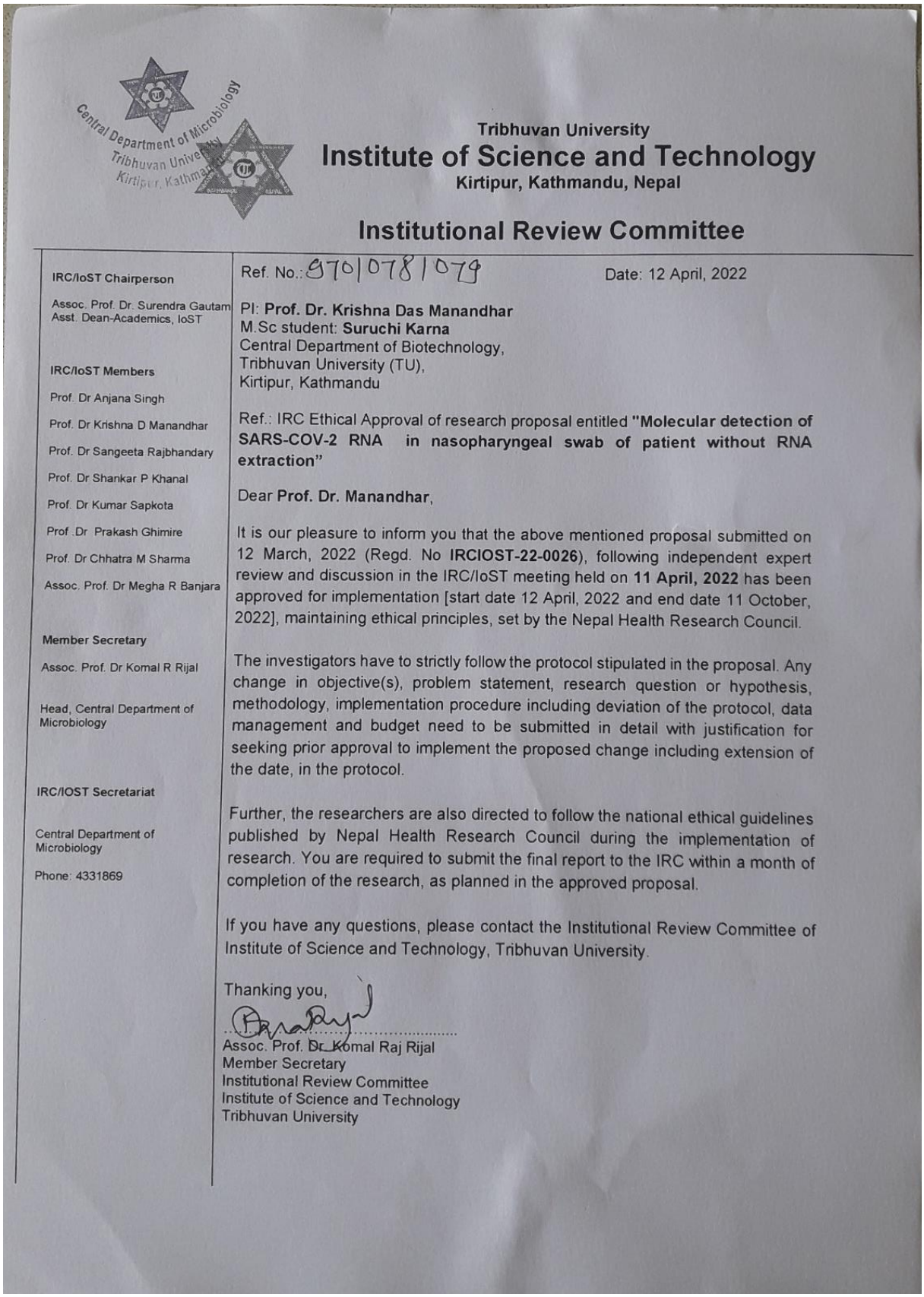
अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर :

मिति :

APPENDIX III: Ethical Approval



Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

Institutional Review Committee

IRC/IoST Chairperson

Assoc. Prof. Dr. Surendra Gautam
Asst. Dean-Academics, IoST

IRC/IoST Members

Prof. Dr Anjana Singh
Prof. Dr Krishna D Manandhar
Prof. Dr Sangeeta Rajbhandary
Prof. Dr Shankar P Khanal
Prof. Dr Kumar Sapkota
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Prof. Dr Chhatra M Sharma
Assoc. Prof. Dr Megha R Banjara

Member Secretary

Assoc. Prof. Dr Komal R Rijal

Head, Central Department of Microbiology

IRC/IOST Secretariat

Central Department of Microbiology

Phone: 4331869

Ref. No.: 970|078|079 Date: 12 April, 2022

PI: **Prof. Dr. Krishna Das Manandhar**
M.Sc student: **Suruchi Karna**
Central Department of Biotechnology,
Tribhuvan University (TU),
Kirtipur, Kathmandu

Ref.: IRC Ethical Approval of research proposal entitled **"Molecular detection of SARS-COV-2 RNA in nasopharyngeal swab of patient without RNA extraction"**

Dear **Prof. Dr. Manandhar**,

It is our pleasure to inform you that the above mentioned proposal submitted on 12 March, 2022 (Regd. No **IRCIOST-22-0026**), following independent expert review and discussion in the IRC/IoST meeting held on **11 April, 2022** has been approved for implementation [start date 12 April, 2022 and end date 11 October, 2022], maintaining ethical principles, set by the Nepal Health Research Council.

The investigators have to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure including deviation of the protocol, data management and budget need to be submitted in detail with justification for seeking prior approval to implement the proposed change including extension of the date, in the protocol.

Further, the researchers are also directed to follow the national ethical guidelines published by Nepal Health Research Council during the implementation of research. You are required to submit the final report to the IRC within a month of completion of the research, as planned in the approved proposal.

If you have any questions, please contact the Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Thanking you,

.....
Assoc. Prof. Dr. Komal Raj Rijal
Member Secretary
Institutional Review Committee
Institute of Science and Technology
Tribhuvan University

APPENDIX IV: IDT (Integrated DNA Technology) MASTER MIX COMPONENTS

S.N	Primers/Probes	Catalogue no.	Lot no.
1.	N1 Forward Primer	10006830	0000591228
2.	N1 Reverse primer	10006831	0000604126
3.	N1 probe	10006832	0000591233
4.	N2 Forward Primer	10006833	0000586232
5.	N2 Reverse Primer	10006834	0000586233
6.	N2 Probe	10007049	0000604708
7.	RNase P Forward Primer	10006836	0000583665
8.	RNase P Reverse Primer	10006837	0000583167
9.	RNase P Probe	10007062	0000593832
10.	Positive Control	10006621	0000568956

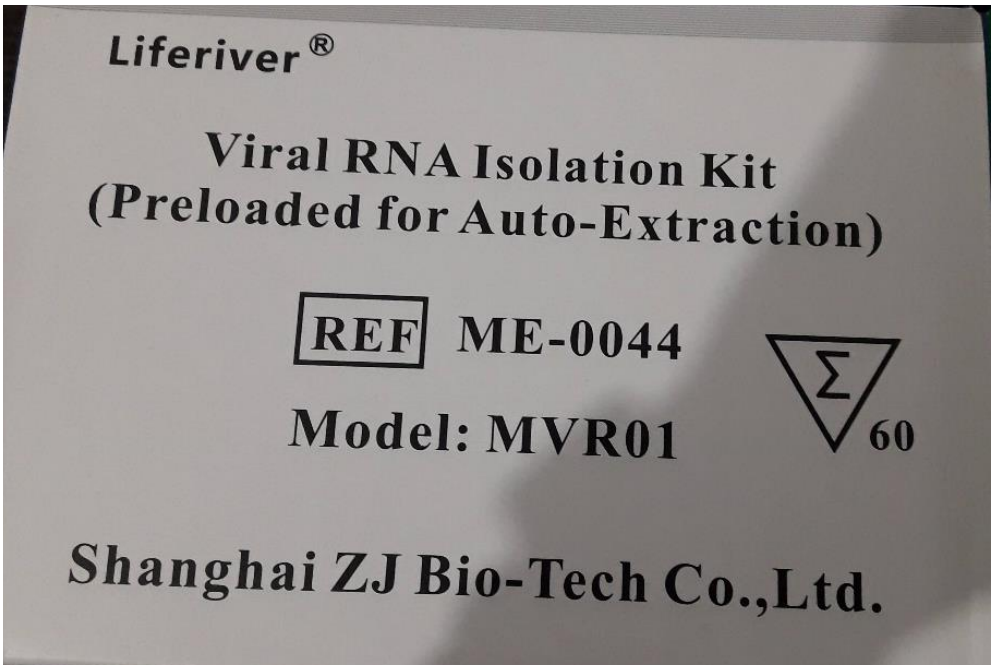
APPENDIX V: Photographs

RNA extraction

Setting up program for PCR

Azure Biosystem RT-PCR machine

Heat Block



RNA extraction kit for automated machine

Automated RNA Extraction Machine