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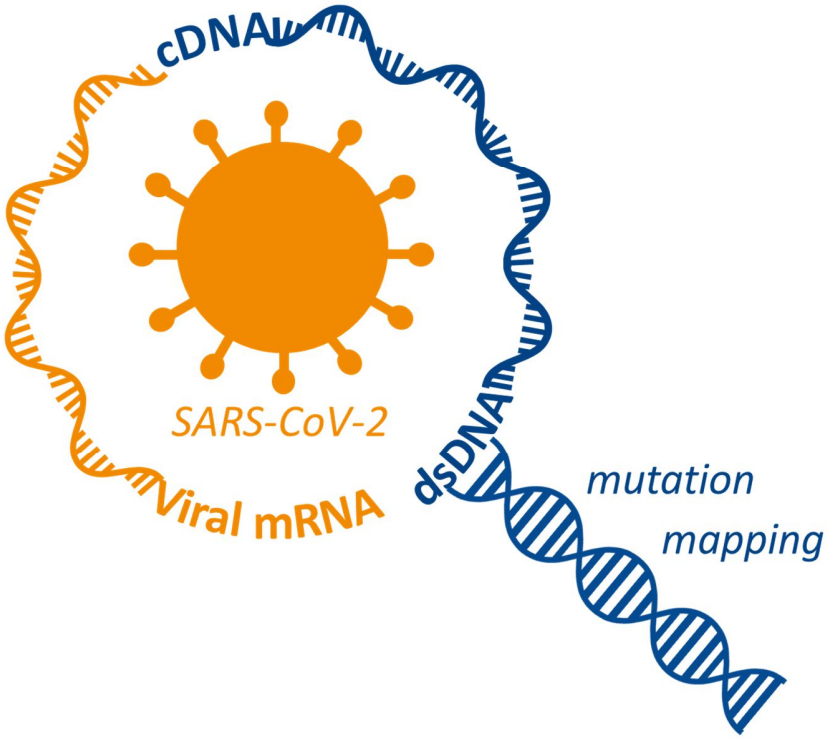
DNA & RNA EXPERTS

# User Manual

# ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2




Version 1.0

Ordering information

(for detailed kit content see **Table 1**)

**ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2:**

| Product Number | Product Name | Application |
|---|---|---|
| **BCK-COV-MM** | ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2 | Discrete mutation detection in new viral strains. |

For References, FAQs and ordering please see online or contact us:

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# ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2

## Introduction and product description:

The ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2 is a research tool that enables rapid, qualitative detection of and differentiation of novel and existing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants from extracted RNA samples by long-read deep sequencing — the practice of sequencing the same genomic region thousands of times to attain a very high degree of precision. The kit contains a specially-designed primer system for full length reverse transcription of extracted SARS-CoV-2 RNA to generate a complete 1:1 copy of the entire viral genome as cDNA. Together with baseclick's specially optimized long-read polymerase blend, additional 5'-phosphorylated primer pairs allow PCR amplifications of three overlapping dsDNA fragments spanning the entire structurally encoding 8kB S-E-M-N region of the genome. Long-read next generation sequencing technologies such as those of PacBio® can then be applied for discrete detection of novel and existing mutations located within a specific, single strain. Thus, with our kit, it is possible to detect both the number of mutations in a specific region, as well as which mutations belong to a given strain. Using newly developed deep sequencing methods, these mutations can be detected with a high degree of certainty. These technologies further facilitate the generation of a comprehensive structural picture of this section of the genome, whereas short read technologies may lead to ambiguous assemblies and limit biological interpretation. The ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2 thus allows users to unambiguously elucidate novel coronavirus mutations both vertically and horizontally, even for example, when multiple variants are present within the same clinical sample.

**For Preparation of Long-Read NGS Libraries**

The ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2 contains reagents to perform 10 full length reverse transcriptions and library preparations from extracted viral RNA.

**For research use only.**

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**Literature Citation:** When describing a procedure for publication using this product, please refer to it as the *ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2.*

## Coronaviruses and SARS-CoV-2

Coronaviruses belong to a category of highly variable RNA viruses.[1] Most strains of coronavirus trigger typical winter cold symptoms, with an estimated number of Germans infected of about 170,000 per year. As with influenza viruses, however, there exists a danger that animal strains can be passed to humans and cause severe health problems.[2] This has been the case with the two previous and locally limited epidemics of SARS and MERS. With the emergence of the SARS-CoV-2 virus, an entirely new and unprecedented coronavirus pandemic has arisen. Although the SARS-CoV-2 virus does not have as high a mortality rate as the SARS and MERS inducing coronaviruses, new potentially harmful mutations are occurring rapidly.

Coronaviruses such as SARS-CoV-2, which possess the largest genomes of all known RNA viruses (~30 kb), consist of a viral envelope containing a positive-sense, single-stranded RNA genome.[1] Each viral transcript resembles a fully functional mRNA, complete with a 5'-Cap structure and a 3'-Poly(A)tail.[3] Negative-sense RNA intermediates generated by RNA-dependent RNA polymerases, are then used as templates for the synthesis of up to nine sub genomic RNAs (**Figure 1**). The high mutation rate and variability in the proteins Spike, Envelope, Membrane and Nucleocapsid ("S-E-M-N") can lead to rapid generation of novel Corona viruses that evade the host immune system even after first infection, thus limiting the success of former vaccine development programs. One such mutation of the 614th amino acid residue, for example, gave rise to SARS-CoV-2 virus particles that can more easily penetrate cells.[4] This modification, known as the D614G mutation, has increased in frequency since February at an alarming rate, and rapidly became the dominant SARS-CoV-2 lineage in Europe, Canada, Australia, and the United States. Studying such mutations in detail may be critical for controlling the pandemic and designing effective vaccines and treatments. This kit provides the tools needed to detect critical mutations in the genomic S-E-M-N region coding for those proteins.

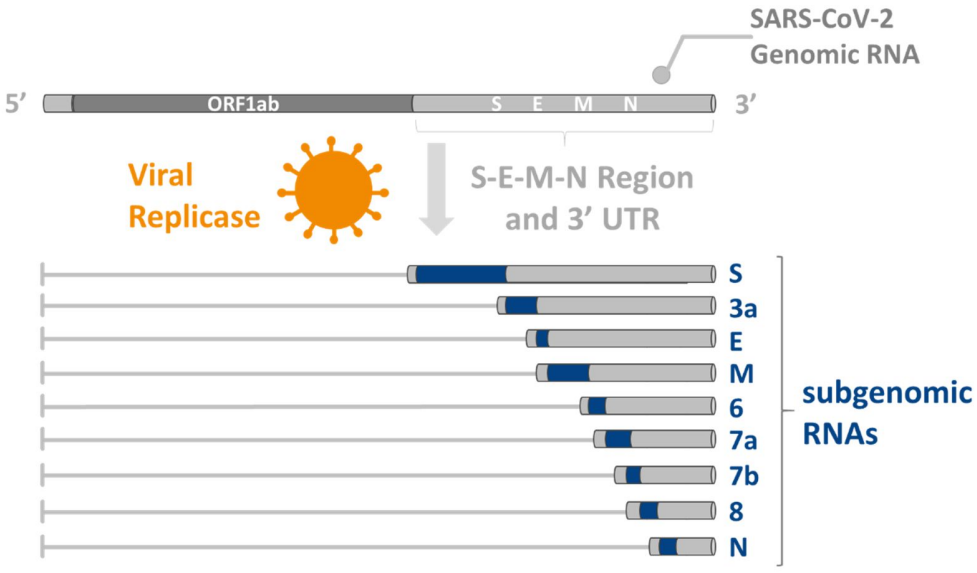


*Figure 1: The transcriptomic architecture of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[3] Full length genomic RNA serves as mRNA, whose open reading frames 1a and 1b (ORF1ab) are translated directly within cells. As well as the genomic RNA, nine major sub genomic RNAs are also generated by the virus's replicase machinery. Blue segments representing genes encoding for small accessory proteins are depicted larger than their actual size for better visualization. The grey segment labelled S-E-M-N represents the genomic region encoding for the structurally significant spike, envelope, membrane and nucleocapsid proteins.*

## 1. Materials provided with the Kit and Storage Conditions

*Table 1: Contents of the Kit and storage conditions.*

| Vial Lid | Quantity | Component | Storage | Thaw Before Use |
|---|---|---|---|---|
| colorless | 15 µL | dNTP Mix (10 mM) | –20 °C | 2 - 8 °C |
| blue | 15 µL | CTL Primer RT (100 µM) | –20 °C | 2 - 8 °C |
| blue | 45 µL | 5x Reverse Transcription Buffer | –20 °C | 2 - 8 °C |
| blue | 15 µL | DTT (100 mM) | –20 °C | 2 - 8 °C |
| blue | 12 µL | Reverse Transcriptase (200 U/µL) | –20 °C | n.r. |
| yellow | 35 µL | 10x RNase H Buffer | –20 °C | 2 - 8 °C |
| yellow | 45 µL | Nucleotide Away (1 U/µL) | –20 °C | n.r. |
| yellow | 16 µL | RNase H (5 U/µL) | –20 °C | n.r. |
| yellow | 15 µL | RNase A (10 mg/mL) | –20 °C | n.r. |
| purple | 400 µL | 2x baseclick PCR Master Mix | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer S Forward (10 µM) | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer S Reverse (10 µM) | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer EM Forward (10 µM) | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer EM Reverse (10 µM) | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer MN Forward (10 µM) | –20 °C | 2 - 8 °C |
| purple | 15 µL | Primer MN Reverse (10 µM) | –20 °C | 2 - 8 °C |

## 2. Required Material and Equipment not included in this Kit

- Extracted RNA or total nucleic acid from SARS-CoV-2 infected patients or viral culture
- RNase free water (e.g. DEPC-treated water)
- RNase free microcentrifuge tubes and pipette tips
- RNase AWAY or similar to clean surfaces
- Thermal cycler
- Agarose gel electrophoresis
- Appropriate spin column-based nucleic acid purification system

**IMPORTANT:** The quality of extracted RNA or total nucleic acid template is critical to the performance of the entire kit system. For optimal performance, high-concentration extracted and purified RNA is recommended.

## 3. Workflow

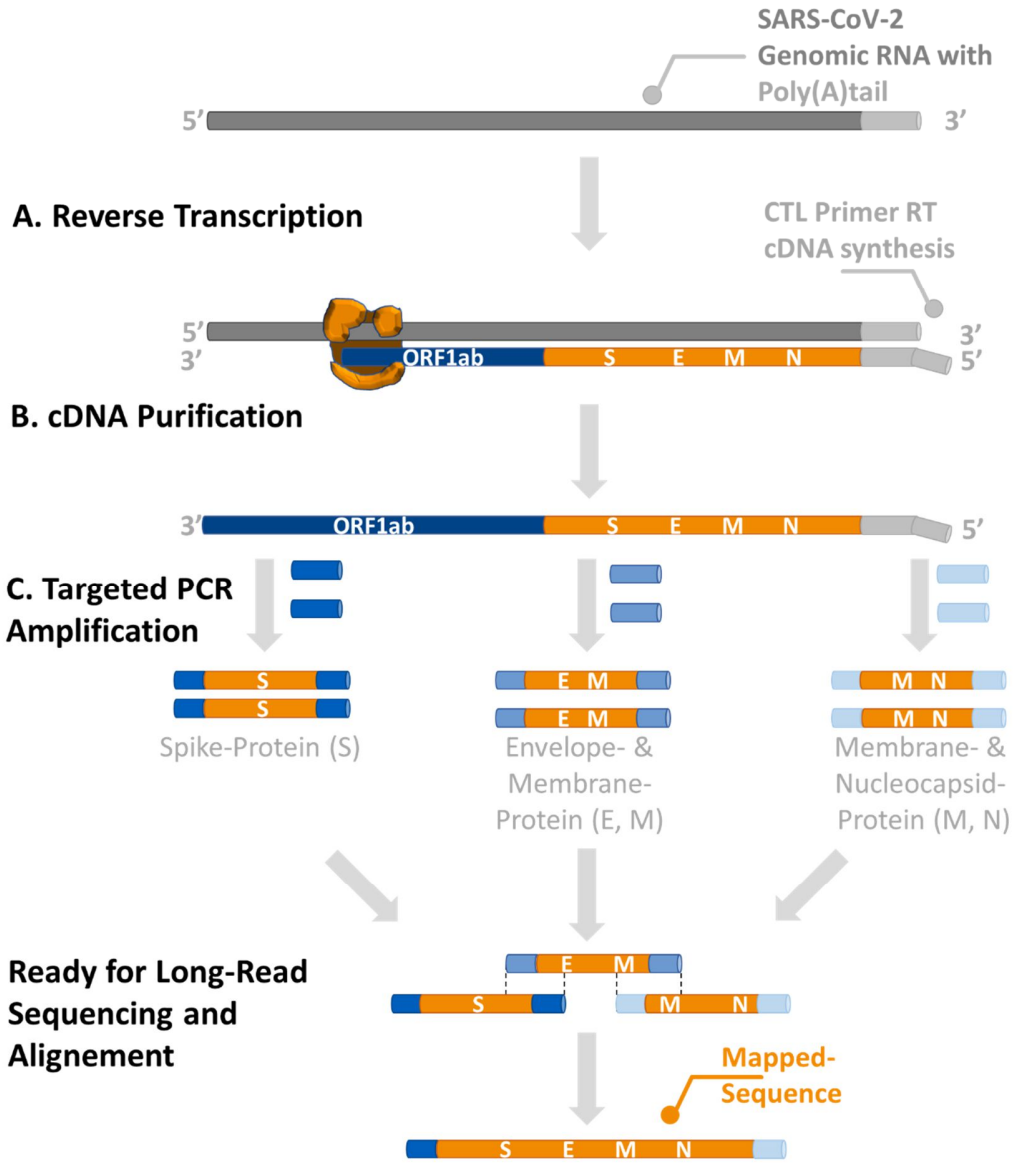


*Figure 2: General workflow of ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2.*

## 4. Reverse Transcription and cDNA Preparation

In general, 2 µg of extracted and purified RNA or total nucleic acid template is suitable in the following protocol. To prevent RNase contamination, always wear gloves and use dedicated solutions e.g. RNase AWAY to clean pipettes and maintain a clean working area. For a proper workflow please take care that all specified solutions are thawed before usage.

### 4.1 Hybridization of CTL Primer RT (100 µM)

- Thaw the **dNTP Mix** on ice and spin down briefly.
- Thaw **CTL Primer RT (100 µM)** on ice and spin down briefly.
- Prepare the following reaction mixture at room temperature:

*Table 2: Reagents and quantities for hybridization.*

| Color Code | Quantity | Component |
|---|---|---|
| ***Not Provided*** | 2 µg | RNA Template |
| **colorless** | 1 µL | dNTP Mix (10 mM) |
| **blue** | 1 µL | **CTL Primer RT (100 µM)** |
| ***Not Provided*** | to 13 µL total volume | RNase-free $H_2O$ |

- Mix by pipetting, spin down briefly.
- For hybridization heat the mixture for 5 min at 65 °C and cool down for 3 min at 0 °C.
- Promptly proceed with step **4.2**, Reverse Transcription.

### 4.2 Reverse Transcription

- Keep the **Reverse Transcriptase (200 U/µL)** in an enzyme cooler (–20 °C) or on ice.
- Thaw the **Reverse Transcription Buffer (5x)** on ice and spin down briefly.
- Thaw the **DTT (100 mM)** on ice and spin down briefly.
- Add the following volumes to the reaction prepared before in step **4.1** (**Table 3**).

*Table 3: Reagents and quantities for Reverse Transcription.*

| Color Code | Quantity | Component |
|---|---|---|
| **see step 4.1** | 13 µL | Reaction mixture from step **4.1** |
| **blue** | 4 µL | **5x Reverse Transcription Buffer** |
| **blue** | 1 µL | **DTT (100 mM)** |
| **blue** | 1 µL | **Reverse Transcriptase (200 U/µL)** |
| ***Not Provided*** | 1 µL | RNase-free $H_2O$ |
| **n.r.** | total volume 20 µL | n.r. |

- Mix by pipetting, spin down briefly.
- For cDNA synthesis heat the mixture for 20 min at 50 °C, 10 min at 80 °C and cool down for 3 min at 4 °C.

## 5. cDNA Purification

After cDNA synthesis, RNA digestion and spin-column purification are necessary to remove RNA, excess dNTPs, and all enzymes.

### 5.1 RNA Digestion

- Thaw the **10x RNase H Buffer** on ice and spin down briefly.
- Keep the **RNase H (5 U/µL)** in an enzyme cooler (–20 °C) or on ice.
- Keep the **RNase A (10 mg/mL)** in an enzyme cooler (–20 °C) or on ice.
- Keep the **Nucleotide Away (1 U/µL)** in an enzyme cooler (–20 °C) or on ice.
- Prepare the reaction mixture at room temperature:

*Table 4: Reagents and quantities for RNA Digestion.*

| Color Code | Quantity | Component |
|---|---|---|
| **see step 4.2** | 20 µL | Reaction mixture from step **4.2** |
| **yellow** | 3 µL | 10x RNase H Buffer |
| **yellow** | 4 µL | Nucleotide Away (1 U/µL) |
| **yellow** | 1 µL | RNase A (10 mg/mL) |
| **yellow** | 1.4 µL | RNase H (5 U/µL) |
| ***Not Provided*** | 0.6 µL | $H_2O$ |
| **n.r.** | total volume 30 µL | n.r. |

- Mix by pipetting and spin down briefly.
- Heat the mixture for 25 min at 37 °C, 15 min at 65 °C and cool down for 3 min at 0 °C.
- Proceed with step **5.2** Spin Column Purification.

### 5.2 Spin Column Purification

After RNA digestion, the mixture obtained must be purified using an appropriate spin column-based nucleic acid purification system (**Not Provided**), to obtain a final volume of 17 µL using ca. 19 µL of Elution Buffer.

At this point the purified cDNA can be maintained at –20 °C for long-term storage.

# 6. PCR Amplification

At this point, proceeding with the instructions below will allow users to amplify a given cDNA pool using the specific targeted Primers provided with this kit, to obtain three overlapping fragments corresponding to the S-E-M-N region of the SARS-CoV-2 genome (see **Figure 1**).

**Notice:** If desired, other **sequence-specific amplifications** are also possible, and can be achieved by replacing provided forward and reverse primers with independently designed DNA primers (not included in the kit). To this end, existing sequencing data for SARS-CoV-2 should be used as a reference.

## 6.1 Spike-Protein Targeted PCR Amplification

In a 200 µL DNase and RNase free tube, add all the reaction components as listed in **Table 5**.

*Table 5: Reagents and quantities for Spike-Protein-Targeted PCR Amplification.*

| Color Code | Quantity | Final Concentration | Component |
|---|---|---|---|
| **see step 5.2** | 10 ng - 500 ng | n.r. | Purified cDNA step **5.2** |
| purple | 1 µL | 0.4 µM | Primer S Forward (10 µM) |
| purple | 1 µL | 0.4 µM | Primer S Reverse (10 µM) |
| purple | 12.5 µL | 1x | 2x baseclick PCR Master Mix |
| ***Not Provided*** | 25 µL total volume | n.r. | $H_2O$ |

- Mix by pipetting, spin down briefly.
- Transfer the mixture to the thermal cycler block with the lid temperature set to 100°C, and begin thermocycling using the program shown in **Table 6**.

*Table 6: Thermocycling conditions for S-Targeted PCR Amplification.*

| Step | Temperature | Time |
|---|---|---|
| **Initial Denaturation** | 94 °C | 30 seconds |
| **35 Cycles** | 94 °C<br>57 °C<br>65 °C | 20 seconds<br>30 seconds<br>5 minutes |
| **Final Extension** | 65 °C | 10 minutes |
| **Hold** | 4 °C | ∞ |

## 6.2 Envelope-Protein to Membrane-Protein Targeted PCR Amplification

In a 200 µL DNase and RNase free tube, add all the reaction components as listed in **Table 7**.

*Table 7: Reagents and quantities for OEM-Targeted PCR Amplification.*

| Color Code | Quantity | Final Concentration | Component |
|---|---|---|---|
| **see step 5.2** | 10 ng - 500 ng | n.r. | Purified cDNA step **5.2** |
| purple | 1 µL | 0.4 µM | Primer EM Forward (10 µM) |
| purple | 1 µL | 0.4 µM | Primer EM Reverse (10 µM) |
| purple | 12.5 µL | 1x | 2x baseclick PCR Master Mix |
| ***Not Provided*** | 25 µL total volume | n.r. | $H_2O$ |

- Mix by pipetting, spin down briefly.
- Transfer the mixture to the thermal cycler block with the lid temperature set to 100°C, and begin thermocycling using the program shown in **Table 6**.

*Table 8: Thermocycling conditions for PCR Amplification.*

| Step | Temperature | Time |
|---|---|---|
| **Initial Denaturation** | 94 °C | 30 seconds |
| **35 Cycles** | 94 °C<br>55 °C<br>65 °C | 20 seconds<br>30 seconds<br>5 minutes |
| **Final Extension** | 65 °C | 10 minutes |
| **Hold** | 4 °C | ∞ |

### 6.3 M-Protein → N-Protein -Targeted PCR Amplification

In a 200 µL DNase and RNase free tube, add all the reaction components as listed in **Table 9**.

*Table 9: Reagents and quantities for OEM-Targeted PCR Amplification.*

| Color Code | Quantity | Final Concentration | Component |
|---|---|---|---|
| **see step 5.2** | 10 ng - 500 ng | n.r. | Purified cDNA step **5.2** |
| **purple** | 1 µL | 0.4 µM | **Primer MN Forward (10 µM)** |
| **purple** | 1 µL | 0.4 µM | **Primer MN Reverse (10 µM)** |
| **purple** | 12.5 µL | 1x | **2x baseclick PCR Master Mix** |
| ***Not Provided*** | 25 µL total volume | n.r. | Nuclease-free $H_2O$ |

- Mix by pipetting, spin down briefly.
- Transfer the mixture to the thermal cycler block with the lid temperature set to 100°C, and begin thermocycling using the program shown in **Table 10**.

*Table 10: Thermocycling conditions for PCR Amplification.*

| Step | Temperature | Time |
|---|---|---|
| **Initial Denaturation** | 94 °C | 30 seconds |
| **35 Cycles** | 94 °C<br>57 °C<br>65 °C | 20 seconds<br>30 seconds<br>5 minutes |
| **Final Extension** | 65 °C | 10 minutes |
| **Hold** | 4 °C | ∞ |

### 6.4 Spin Column Purification

The mixtures obtained in steps **6.1**, **6.2** and **6.3** should once more be purified using an appropriate spin column-based nucleic acid purification system (**Not Provided**).

At this point the purified cDNA can be maintained at –20 °C for long-term storage.

## 7. Example Results

This section depicts agarose gel imaging results that can typically be obtained through normal use of the ClickTech Single Strain Mutation Mapping Kit for SARS-CoV-2.

After completing **step 5 cDNA Purification**, independent PCR amplifications spanning small regions throughout the ~30 kB SARS-CoV-2 genome are able to confirm complete reverse transcription of the viral mRNA (full 3' to 5' coverage), as illustrated in **Figure 3**.

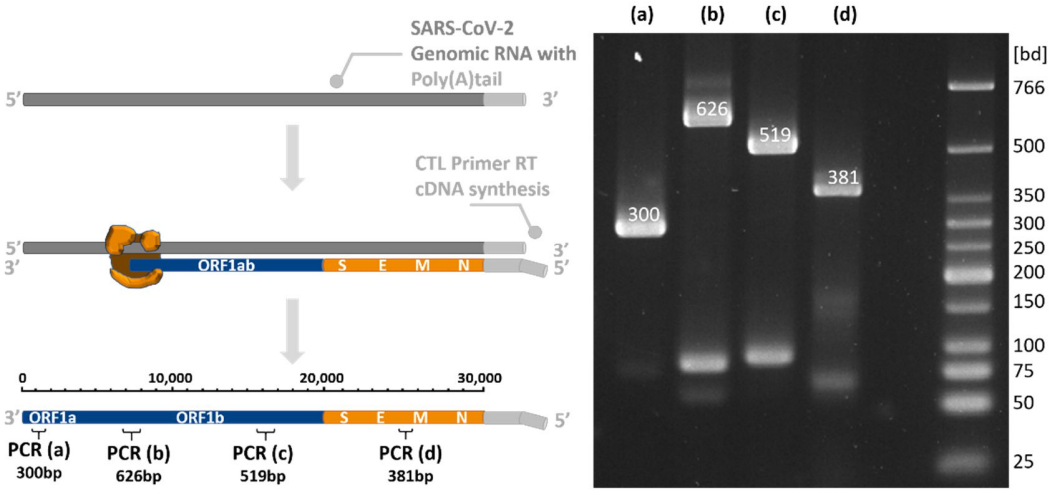


*Figure 3 Agarose gel electrophoresis showing control PCR fragments (a, b, c & d) that were generated from reverse transcribed SARS-CoV-2 mRNA using this kit. All PCR products have the desired fragment size and show that the complete RNA sequence from the very 5'End (PCR (a)) to the 3'End (PCR (d)) was successfully reverse transcribed. This kit thus ensures complete reverse transcription of the entire 30,000 nt SARS-CoV-2 genome. Lower bands are also observed for (b), (c) and (d) due to non-specific amplifications.*

Carrying out **step 6 PCR Amplification** followed by visualization *via* gel electrophoresis will afford imaging results such as those shown in **Figure 4**, indicating successful generation of the desired overlapping "S", "EM" and "MN"-targeted dsDNA fragments.

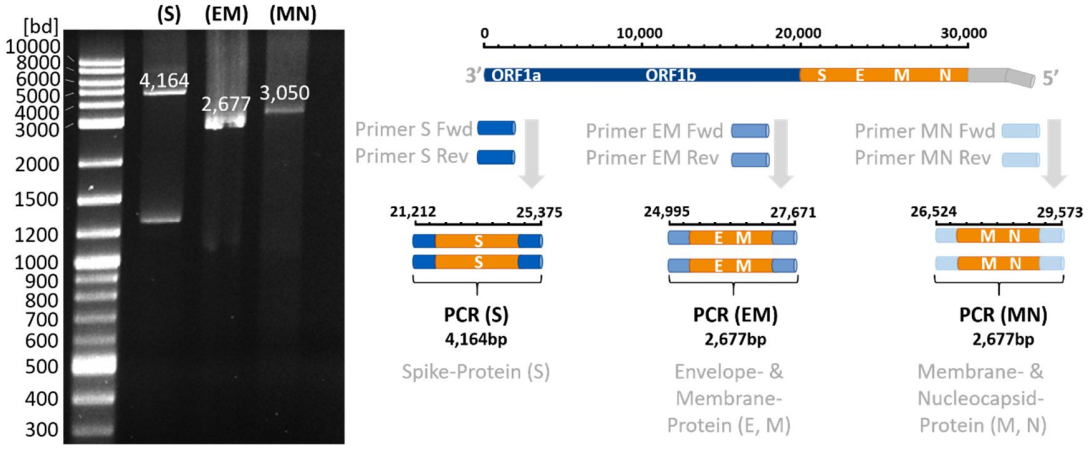


*Figure 4 shows agarose gel electrophoresis of three lengthy PCR products (4164 bp, 2677 bp and 3050 bp) derived from cDNA from SARS-CoV-2. The three primer pairs used here cover the entire structurally-encoding protein sequence (S-,E-,M-,N-proteins), known for having high mutation rates. Additionally, the three amplicons that are generated have overlapping segments and ensure exact determination of mutations in these regions for individual co-detected strains SARS-CoV-2. Also see Figure 2 for reference. A lower band is also observed for (s) due to non-specific amplification (does not detrimentally affect the quality of sequencing data).*

## 8. Trouble Shooting

*Table 11: Trouble Shooting solutions.*

| Problem | Possible Reason | Solution |
|---|---|---|
| **Low cDNA synthesis yield** | Contamination of RNase during or prior to cDNA synthesis. | Wear gloves and use dedicated solutions e.g. RNase AWAY to clean pipettes and tips and maintain a clean working area. Ensure that all reagents are RNase free. |
| | Low quality RNA template. | Review your given RNA extraction procedure and if necessary, follow an appropriate RNA purification protocol. Use nuclease-free $H_2O$ to dilute the RNA sample and potential inhibitors, if needed. |
| **Column purification: Low or no recovery of cDNA** | Too much cDNA product applied to the spin column. | Check the manufacturer's specifications to ensure the maximum amount of material is not being exceeded. |
| | Incomplete elution of cDNA. | Make sure that the elution solution has been completely absorbed by the silica column membrane before centrifugation. |
| | The size of cDNA too large for the given spin column. | Check the manufacturer's specifications to ensure the maximum length of the cDNA is not being exceeded. |
| **Poor performance in downstream applications.** | Salt residue remains in eluted cDNA. | Wash the silica column twice with Wash Buffer. |
| | Ethanol residue remains in eluted DNA. | Discard the flow-through after washing with Wash Buffer and centrifuge for an additional 3 min. |
| **PCR amplicons don't have the expected size.** | Extension time of the PCR cycling is too short. | For amplification of specific sequences using independently designed DNA primers, please calculate the extension time using 1kb/1min. |
| | Shorter length amplicons are occasionally obtained, depending on the quality of the cDNA template (see **Figure 4**). | The quality of extracted RNA or total nucleic acid template is critical to the performance of the entire kit system. For optimal performance, high-concentration extracted and purified RNA is recommended. Use electrophoresis or another means to check the quality of input mRNA. |
| **Low, or no yield of PCR product.** | Insufficient template was used. | Increase the amount of cDNA template. |
| | Number of PCR cycles was not sufficient. | Try increasing the number of PCR cycles by five. If low or no PCR product yield is still obtained, try using 1 µL of crude PCR as the template for further reactions. |

## 9. References


1. Rajčáni, J. Coronaviruses. *Acta Virol.* **64**, 264–267 (2020).
2. Salajegheh Tazerji, S. *et al.* Transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to animals: An updated review. *Journal of Translational Medicine* **18**, (2020).
3. Kim, D. *et al.* The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914-921.e10 (2020).
4. Li, Q. *et al.* The Impact of Mutations in SARS-CoV-2 Spike on Viral Infectivity and Antigenicity. *Cell* **182**, 1284-1294.e9 (2020).