Supplementary Data. Preliminary study with PCR and Sanger sequencing analysis of blood samples from long-COVID syndrome patients.

Supplementary Methods:

Sample Collection:

Blood samples were collected from a cohort of 81 patients experiencing long-COVID symptoms. The samples were obtained following informed consent and ethical guidelines. Genomic DNA was extracted from the blood samples using a commercially available DNA extraction kit according to the manufacturer's instructions (Blood DNA kit E.N.Z.A., Omega Bio-tek, Inc., Doraville, GA, USA or Exgene Clinic SV mini, GeneAll Biotechnology, Seoul, South Korea).

PCR Analysis:

PCR (Polymerase Chain Reaction) was performed using specific primers designed to target the spike protein sequence derived from the BNT162b2 vaccine (BioNTech/Pfizer mRNA Vaccine) (1). The forward primer (CGAGGTGGCCAAGAATCTGA) and reverse primer (TCTGGAACTAGCAGAGGTGG) were used. PCR amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The PCR products were visualized by agarose gel electrophoresis.

Nested PCR:

Due to the presence of nonspecific amplification in the initial PCR analysis, two rounds of Nested PCR were performed. The same primers and PCR conditions were used for both rounds. DNA extracted from the gel bands was purified using a gel/PCR extraction kit.

Sanger Sequencing:

The PCR products obtained from the Nested PCR were subjected to Sanger sequencing to determine the nucleotide sequence of the amplified fragment. Sequencing was performed in both the forward and reverse directions using fluorescently labeled dideoxynucleotides and a DNA sequencer. The obtained chromatograms were analyzed to confirm the presence of the BNT162b2 vaccine spike protein sequence in the genomic DNA of long-COVID syndome patients.

Supplementary Results:

PCR Analysis:

PCR amplification using the specific primers targeting the BNT162b2 vaccine spike protein sequence resulted in nonspecific amplification in 38 out of the 80 samples (Figure S1). No amplification was observed in 42 samples. A band of approximately 440 base pairs (bp) was

detected, which corresponded to the expected size of the fragment amplified by the BNT162b2 primers.

Nested PCR:

To obtain specific and reliable results, two rounds of Nested PCR were performed on 12 selected samples that showed quality bands after the initial PCR (Figure S2). The second round of Nested PCR successfully amplified a single band of approximately 440 bp, closely matching the expected size (Figure S3).

Sanger Sequencing:

Sanger sequencing of the PCR products obtained from the selected samples possibly suggested the presence of spike protein sequences. The obtained chromatograms were aligned with the wild-type and BioNTech/Pfizer mRNA vaccine BNT162b2 Spike Protein sequences to assess integration. The chromatograms showed significant similarities between the detected spike protein fragments and the reference sequences of the SARS-CoV-2 spike protein (Figure S4).

Supplementary Discussion:

The PCR and Sanger sequencing analysis of genomic DNA from long-COVID patients revealed the presence of a sequence similar to the BNT162b2 vaccine spike protein sequence, possibly indicating potential integration (Figure S2). Alignment of chromatograms with reference sequences suggested the existence of viral spike protein fragments, aligning with intracellular reverse transcription of the Pfizer BioNTech COVID-19 mRNA vaccine (1). Additionally, Lehrer et al. discovered a 117-base pair sequence from the SARS-CoV-2 orf1b gene within the NTNG1 gene on human chromosome 1, raising intriguing questions about the relationship between SARS-CoV-2 genetic material and schizophrenia-related genes (2). The study by Jeong et al., focusing on SARS-CoV-2 spike-encoding mRNA sequences in BNT-162b2 and mRNA-1273 vaccines, directly relates to our investigation of viral and vaccine spike protein fragments in post-COVID-19 syndrome patients (3).

However, it is essential to acknowledge the limitations of this study, including the short length of aligned sequences and primer-related constraints, which necessitate further investigation to confirm vaccine integration and exclude potential cross-reactivity or contamination (1). Validation through alternative techniques like next-generation sequencing and improved primer design in targeted PCR assays is warranted (4). Longitudinal studies with larger sample sizes and control groups are necessary to assess the specificity and prevalence of vaccine spike protein integration in long-COVID patients.

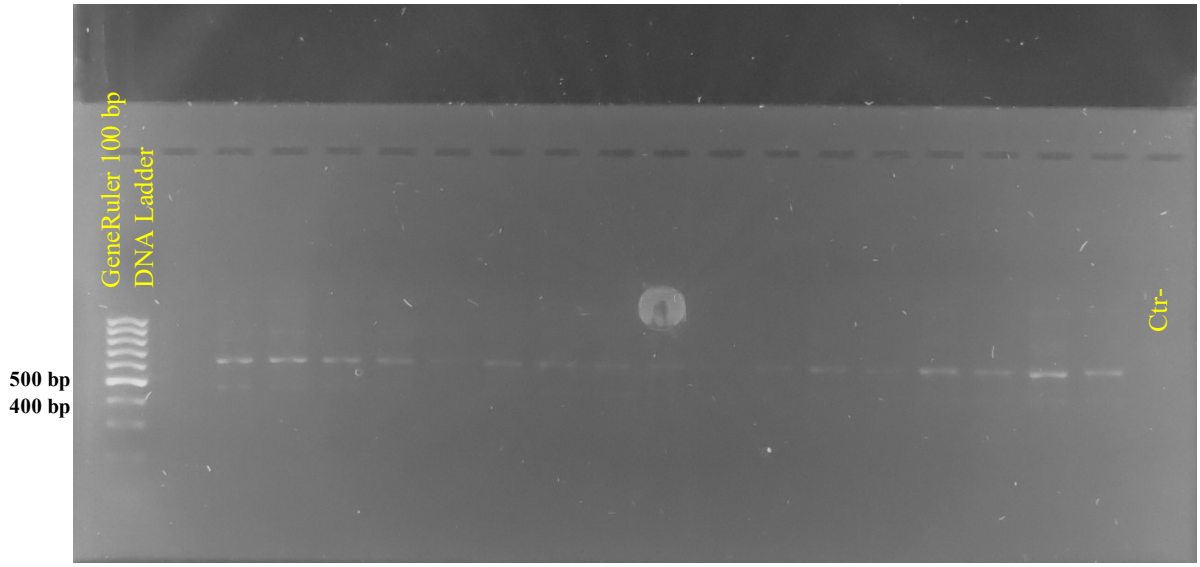

Figure S1. Example of PCR products on 1.4% agarose gel.

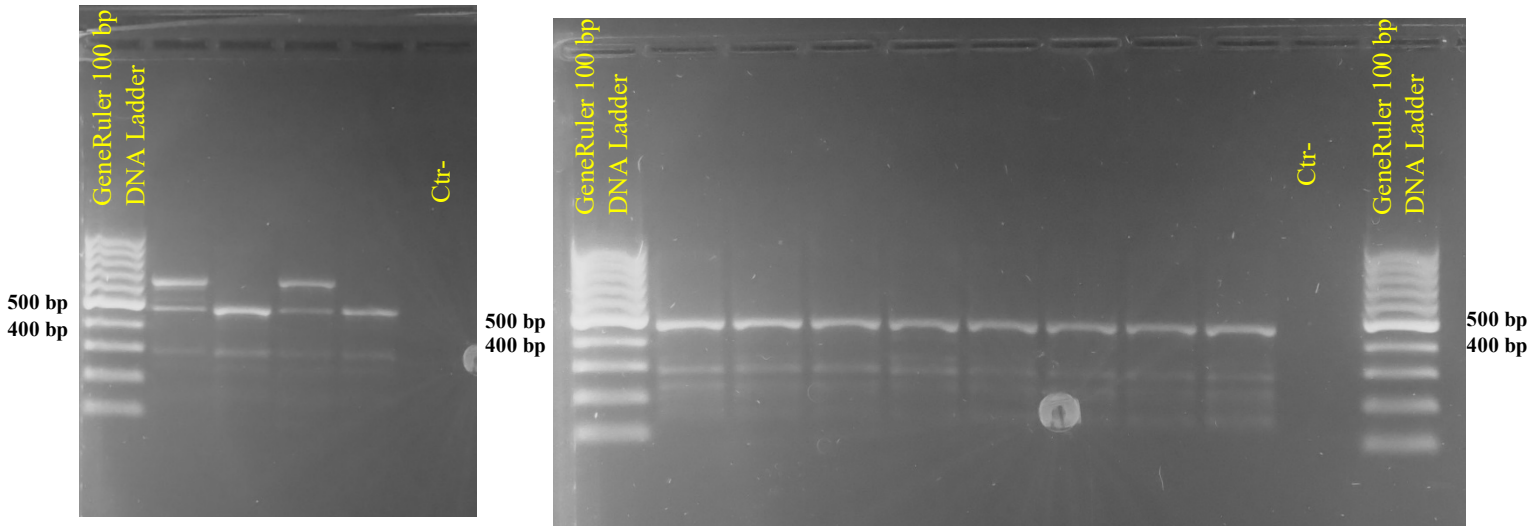

Figure S2. First Nested PCR products on 1.4% agarose gel.

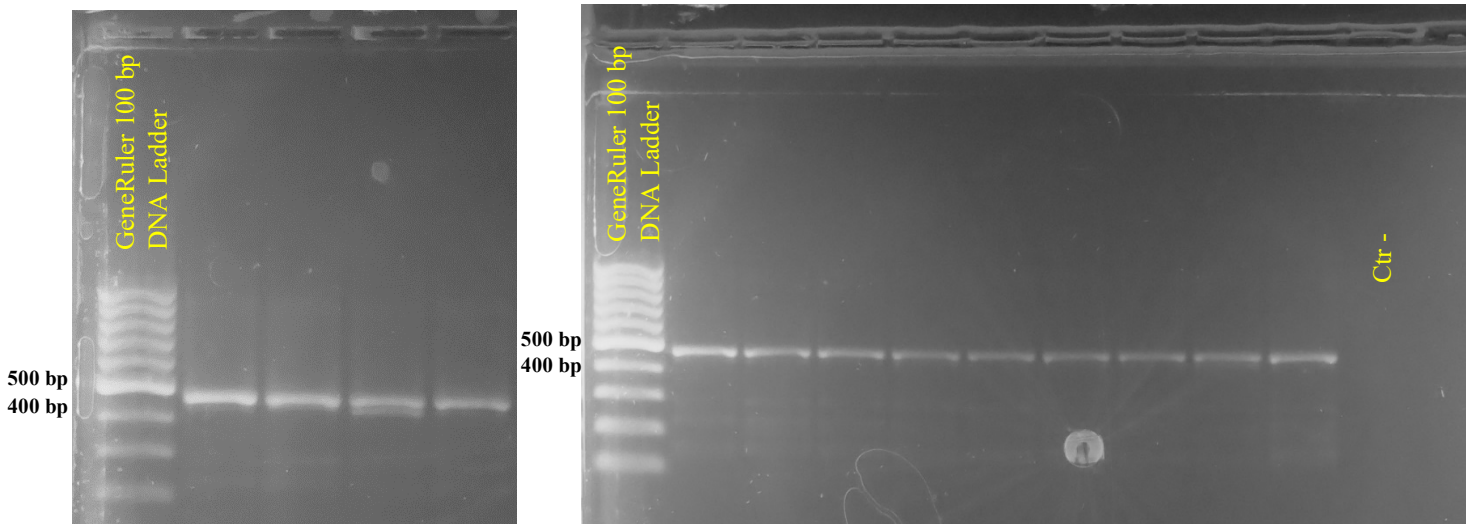

Figure S3. Second Nested PCR on 1.4% agarose gel.

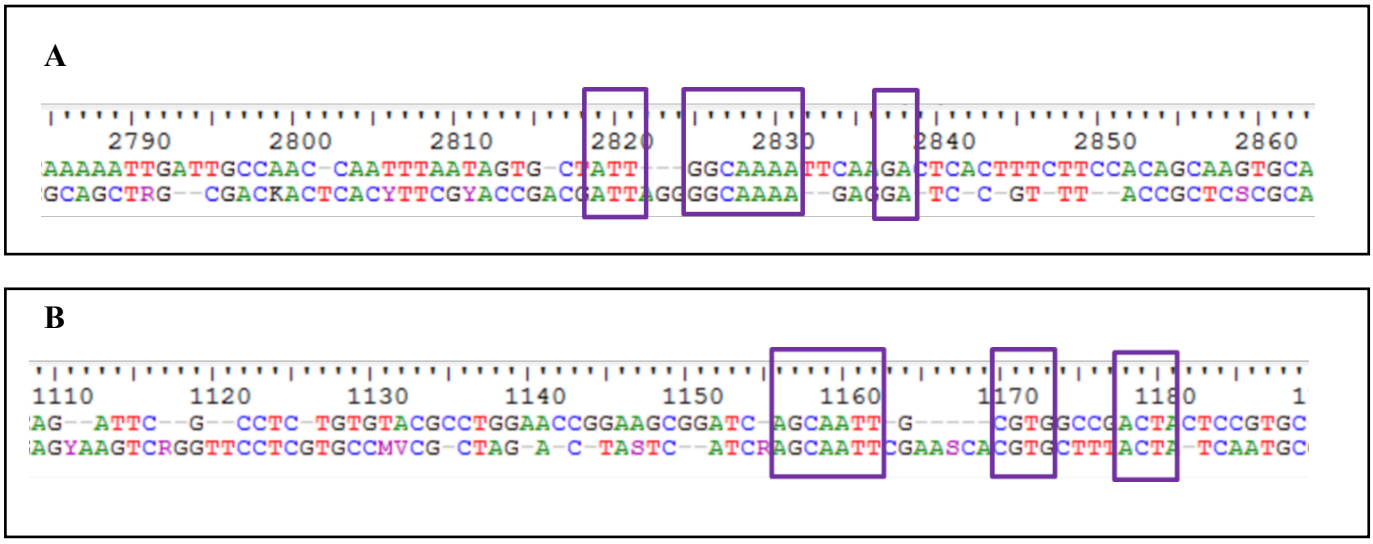

Figure S4. Example of sequences alignment with BioEdit Sequence Alignment Editor tool results. **A**, Wild-type Spike Protein sequence alignment with patient genomic sequence; (**B**), Vaccine BNT162b2 sequence alignment with patient genomic sequence.

Side by Side Alignment

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00403 gtgtcctattcac 00415
>>>>> ||||||||||||| >>>>>
28001 gtgtcctattcac 28013
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Figure S5. Example of BLAT alignment of genomic DNA from long-COVID patient (upper sequences) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (lower sequences.

acccccctaat	gactggcgtt	ccatctgagc	caattttccc	accgaccggt	400
ggGTGTCCTA	TTCACggtgg	gttcagggtc	cctactacga	tggttgcggc	450

tgtagtttac	agtcatgtac	tcaacatcaa	ccatatgtag	ttgatgaccc	28000
GTGTCCTATT	CACttctatt	ctaaatggta	tattagagta	ggagctagaa	28050

Supplementary Table I. Alignment of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome and long-COVID patient genome. Matching bases in cDNA and genomic sequences are colored blue and capitalized. Light blue bases mark the boundaries of gaps in either sequence (often splice sites).

References

1. Aldén M, Olofsson Falla F, Yang D, Barghouth M, Luan C, Rasmussen M, De Marinis Y. Intracellular Reverse Transcription of Pfizer BioNTech COVID-19 mRNA Vaccine BNT162b2 In Vitro in Human Liver Cell Line. Curr Issues Mol Biol 2022; 44: 1115-1126.

2. Lehrer S, Rheinstein PH. SARS-CoV-2 *orf1b* Gene Sequence in the *NTNG1* Gene on Human Chromosome 1. In Vivo 2020; 34: 1629-1632.

3. Jeong DE, McCoy M, Artiles K, Ilbay O, Fire A, Nadeau K, Park H, Betts B, Boyd S, Hoh R, Shoura M. Assemblies-of-putative-SARS-CoV2-spike-encoding-mRNA-sequences-for-vaccines-BNT-162b2-and-mRNA-1273. Available at: https://virological.org/t/assemblies-of-putative-sars-cov2-spike-encoding-mrna-sequences-for-vaccines-bnt-162b2-and-mrna-1273/663.

4. Beaudoin CA, Bartas M, Volná A, Pečinka P, Blundell TL. Are there hidden genes in DNA/RNA vaccines? Front Immunol 2022; 13: 801915.