Vigilant: An engineered VirD2-Cas9 complex for lateral flow assay-based detection of SARS-CoV2

Tin Marsic¹, Zahir Ali¹, Muhammad Tehseen², Ahmed Mahas¹, Samir Hamdan², and Magdy Mahfouz¹, *

¹Laboratory for Genome Engineering and Synthetic Biology, Division of Biological Sciences, 4700 King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia.
²Laboratory of DNA Replication and Recombination, Biological and Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia.

*Correspondence: Magdy M. Mahfouz (magdy.mahfouz@kaust.edu.sa)

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MATERIALS AND METHODS

Nucleic acid preparation

a) Plasmids, ssDNA probe, and oligos

VirD2-SpCas9 and SpCas9-VirD2 clones were used to purify the fusion proteins. Mutant VirD2-SpdCas9 (dead SpCas9-VirD2), SpdCas9-VirD2 (VirD2-dead SpCas9), and SpdCas9-dVirD2 (dead SpCas9-dead VirD2) clones for expression of the respective fusion proteins were custom synthesized by GenScript. Guide RNAs for SpCas9 experiments were designed using SnapGene and ordered as gBlocks from Integrated DNA Technologies under the T7 promoter for in vitro transcription. The FAM-labeled ssDNA probe and biotin-labeled oligos were ordered from Integrated DNA Technologies. Sequences of the plasmids and oligos are listed in the supplementary file.

b) In vitro transcription of sgRNA

In vitro transcription was performed using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) following the manufacturer’s instructions. Briefly, 10 µL of 5X TranscriptAid Reaction Buffer, 20 µL NTP mix, 10 µL of the DNA template (annealed sgRNA gBlock and T7 promoter oligo), 0.5 µL of RNase Out, 5 µL of TranscriptAid Enzyme Mix, and 4.5 µL of DEPC-treated water were incubated at 37°C for 8 hours. In vitro transcribed RNA was purified using Direct-zol RNA MiniPrep Kit (Zymo Research). Production of the proper size sgRNA fragments was confirmed on a 2% agarose gel run in Tris-Borate-EDTA buffer.

Protein purification

Protein purification was performed as previously described [13]. Briefly, a single colony of BL21(DE3) was grown in 2X-YT media and induced at 0.6 OD_{600} with 0.3 mM IPTG and incubated at 18°C for 15 h at 180 rpm. Proteins were isolated using affinity purification column and further purified by size fractionation using the ÄKTA pure system (Cytiva).

Functional characterization of in-house produced enzymes
a) SpCas9 nuclease activity assay of the fusion proteins

Target fragment (SARS-CoV-2, N-gene fragment) was amplified by PCR for SpCas9-based cleavage assays. Ribo-nucleoprotein particles of VirD2-SpCas9, SpCas9-VirD2, and catalytically dead mutants VirD2-SpdCas9, SpdCas9-VirD2 and SpdCas9-dVirD2 were assembled at 37°C for 10 minutes in 16.3 µL reaction consisting of 250 nM of the respective protein, 250 nM sgRNA in the cleavage buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM MgCl₂). Following the incubation, 3.7 µL containing 150 ng of the target was added into the tube and the reaction was incubated for 1 hour at 37°C. The protein was denatured at 95°C for 5 minutes, the reaction was cooled on ice for 3 minutes, and the DNA products were separated on a 2% agarose gel.

b) VirD2 and probe covalent binding assay of the fusion proteins

Biotin-labeled probe (ssDNA harboring the T-DNA right border sequence) was mixed with the fusion proteins (VirD2-SpCas9, SpCas9-VirD2, and catalytically dead mutants VirD2-SpdCas9, SpdCas9-VirD2 and SpdCas9-dVirD2) at the ratio 1:1 (250 nM each) V2 buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM MgCl₂). The reaction mix was incubated at 37°C for 60 minutes. Protein loading dye was added to the reaction and heated for 95°C for 3 minutes. The complex was resolved on 10% NuPAGE (Invitrogen) for 3 h at 4°C and transferred to nitrocellulose membrane for 3 h at 4°C. The membrane was immuno-blotted with anti-biotin mouse primary antibody 1:1000 (Cat. No. SC-53179, Santa Cruz) and anti-mouse secondary antibody 1:2000 (Cat. No. A3688, Sigma) and detected with chemiluminescent ECL solution (BioRad). Alternatively, for direct gel mobility shift, probe (64 bp ssDNA harboring the T-DNA right border sequence) was mixed with the fusion proteins (VirD2-SpCas9, SpCas9-VirD2, and catalytically dead mutants VirD2-SpdCas9, SpdCas9-VirD2 and SpdCas9-dVirD2) at the ratio 1:1 (250 nM each) V2 buffer (10 mM Tris-HCl pH=8.0, 50 mM NaCl, 10 mM MgCl₂). The reaction mix was incubated at 37°C for 60 minutes. Protein loading dye was added to the reaction and heated for 95°C for 3 minutes. The complex was resolved on 10% NuPAGE (Invitrogen) for 3 h at 4°C. Gel was stained with Coomassie brilliant blue stain (MBP). Gel photos were taken using Gel Doc XR (BioRad).
RT-RPA reactions with synthetic targets

RT-RPA was performed using the TwistAmp Basic kit following the manufacturer’s instructions. Briefly, a well-mixed 47.5-µL sample (1 µl RNA template, 2.4 µL of 10 µM biotin-labeled forward and 2.4 µL of unlabeled reverse primers, 29.5 µL of Rehydration buffer, 0.5 µL of SuperScript IV reverse transcriptase, 1 µL of RNase H, 0.5 µL of RNase Out, 10.2 µL H2O) was added to the lyophilized RPA reaction components (TwistAmp Basic) and homogenized by pipetting. Magnesium acetate (2.5 µL of 280 mM) was added to each tube and mixed. The isothermal amplification was performed at 42°C for 25 minutes. To confirm the DNA isothermal amplification, 10 µL of the reactions were purified using QIAquick PCR Purification Kit and separated on a 1.5 % agarose gel.

VirD2-SpdCas9 reporter complex assembly

The reporter complex (final concentration of 250 nM each VirD2-SpdCas9, sgRNA and FAM-labeled reporter oligo) was prepared by combining 1.25 µL of 5 µM VirD2-SpCas9 secondary stock, 1.25 µL of 5 µM sgRNA, 1.25 µL of 5 µM FAM-labeled reporter oligo, 5 µL of 5X RPA reaction buffer (50 mM Tris-HCl pH 8.0, 500 mM KCl, 250 mM NaCl, 5 mM DTT, 50 mM MgCl₂, 250 mM L-arginine, and 250 mM L-glutamic acid) or 5 µL 5X PCR reaction buffer (100 mM HEPES pH 8.0, 500 mM KCl, 250 mM NaCl, 5 mM DTT, 25 mM MgCl₂), 0.5 µL of RNase Out and ultra-pure water to 20 µL. The reaction was incubated at 37°C for 60 minutes. Two reactions, 20 µL each, were combined in a single tube to make 40 µL preassembled reporter reaction.

VirD2-SpdCas9 detection assay and lateral flow assay

RT-RPA product (5 µL) was mixed with 40 µL of preassembled reporter. The 45-µL reaction mix was incubated at 37°C for 10 minutes followed by 1 minute at 60°C. Following the incubation, 55 µL of the running buffer (44.5 µL of the HybriDetect Assay Buffer with 10.5 µL of 10% BSA) was added directly into the reaction and mixed. Room temperature adjusted HybriDetect Dipsticks were placed into the tube containing the reaction mixture. Lateral flow strips were removed from the tube as soon as the control band appeared and the result was called
within 10 minutes. Images of the strips were taken within 25 minutes after the beginning of the LFA.

Limit of detection assay

SARS-CoV-2 RNA (synthetic RNA from IDT) was diluted to final concentrations corresponding to 1, 2.5, 7.5, 10, 50, and 100 copies/µL or 50, 125, 375, 500, 2500, and 5000 copies/reaction. The respective volume of RNA sample was added to the RT-RPA reactions. Nuclease-free water was used as the negative control. Following the RT-RPA reaction, 5 µL of the product was transferred into the reaction containing the preassembled reporter complex and the samples were detected as previously described. The detection limit was considered as the concentration that could be successfully detected within 10 minutes of the LFA assay in all three replicates.

Validation of the developed protocol with SARS-CoV-2 clinical sample

RNA samples from SARS-CoV-2 RT-PCR positive (26 clinical samples) and negative (4 clinical samples) were used for evaluation of our Vigilant protocol. RNA (4 µL) was added to the RT-RPA reaction and 5 µL of the amplified product was used for detection in the next step.
List of Supplementary Figures

Supplementary Figure 1. VirD2 alone is capable of cleaving ssDNA containing the specific RB recognition sequence.

Supplementary Figure 2. Covalent binding of VirD2 and ssDNA probe.

Supplementary Figure 3. Covalent binding of VirD2 and ssDNA.

Supplementary Figure 4. Confirmation of VirD2-SpCas9 and SpCas9-VirD2 nuclease activity.

Supplementary Figure 5. Selection of the optimal RT-RPA primer set.

Supplementary Figure 6. Selection of the optimal reaction buffer.

Supplementary Figure 7. Evaluation of the Vigilante at 42°C.

Supplementary Figure 8. Comparison of VirD2-SpCas9 and VirD2-SpdCas9.

Supplementary Figure 9. Repeats 2 and 3 of LOD determination.

Supplementary Figure 10. Copy number determination by RT-qPCR for Ct value relevance in clinical samples.

Supplementary Figure 11. Reporter complex stability.

Supplementary Figure 12. Additional evaluated clinical samples and their original scan.

Supplementary table 1. Designation and the Ct value of the clinical samples used in this study. RT-qPCR was performed on Trizol based isolated RNA.
Supplementary Figure 1. VirD2 alone is capable of cleaving ssDNA containing the specific RB recognition sequence. Following the cleavage of the oligonucleotide, VirD2 remains covalently bound to Tyr29 moiety, leaving three nucleotides of the original sequence. This property can be exploited to attach labels at the 3’ end of oligonucleotide sequence bound to VirD2.
Supplementary Figure 2. A, B), Covalent binding of VirD2 and ssDNA probe. Biotin labeled probe harboring the T-DNA right border sequence was incubated with fusion proteins in the presence of Mg\(^{2+}\). VirD2-Cas9, Cas9-VirD2, VirD2-dCas9, dCas9-VirD2 bound to biotin labeled probe were detected by western blot. Biotin labeled probe, unlabeled probe, and dCas9-dVirD2 (no binding to RB sequence containing probe) were used as experimental control. Red arrow head indicated the immuno-detection of the biotin labeled probe bounded to fusion proteins.
**Supplementary Figure 3.** Covalent binding of VirD2 and ssDNA. ssDNA strand (64 bp), harboring the T-DNA right border sequence was incubated with fusion proteins in the presence of Mg^{2+}. VirD2-Cas9, Cas9-VirD2, VirD2-dCas9, dCas9-VirD2 bound to biotin labeled probe were detected by Coomassie staining. RB containing probe did not bind dCas9-dVirD2. Red arrow head indicated the ssDNA - fusion protein complex.
Supplementary Figure 4. Confirmation of VirD2-Cas9 and Cas9-VirD2 nuclease activity. N-gene target DNA was incubated with fusion proteins in the presence sgRNA and Mg$^{2+}$. Samples without sgRNA were used as controls. Arrow heads, indicate the respective DNA fragments.
**Supplementary Figure 5.** Selection of the optimal RT-RPA primer set. N-gene target DNA was amplified by RT-RPA kit using manufacturer's instructions. Black arrow heads indicate the expected amplicons. Red arrow head, indicates the selected primer set. Primer set was selected based on the absence of non-specific amplicons and primer dimers.
Supplementary Figure 6. A. Selection of the optimal reaction buffer. 1, specific target sample. 2, non-specific target sample. 3, non-specific sgRNA. B. Selection of the optimal reporter complex concentration. Protein:sgRNA:FAM-probe were mixed in 1:1:1 ratio in 50, 100, 250, 500 nM concentration in a 25 µL reaction volume. C. Selection of the optimal RT-RPA product volume for detection. Different volumes (0, 1, 2, 3, 4, 5 µL) of the RT-RPA product were added to the preassembled reporter complex. No template and no sgRNA were used as negative controls. D. Selection of the optimal duration for detection. RT-RPA product was incubated with preassembled reporter complex for different time periods. Incubation at 37 °C for 10 minutes followed by 1 minute at 60 °C results in robust, rapid and consistent appearance of the bands at the test line.
**Supplementary Figure 7.** Evaluation of the Vigilante at 42 °C. All steps, the RT-RPA reaction, reporter complex assembly and detection reaction were carried out at 42 °C. N-gene specific target and specific sgRNA were used as positive and no template samples was used as negative control.
**Supplementary Figure 8.** Comparison of VirD2-Cas9 and VirD2-dCas9. SARS-CoV-2 RT-RPA amplified N-gene product was detected with the Vigilante platform. N-gene target 1, N-gene specific target; N-gene target 2, N-gene non-specific target; N-gene sgRNA 1, specific sgRNA; N-gene sgRNA 2, non-specific sgRNA.
Replicates of the LoD experiment conducted in Figure 3A

Replicate 2

| Copies per reaction | Copies per microliter |
|---------------------|-----------------------|
| 0 0 50 125 375 500 2500 5000 | 0 0 1 2.5 7.5 10 50 100 |

Replicate 3

| Copies per reaction | Copies per microliter |
|---------------------|-----------------------|
| 0 0 50 125 375 500 2500 5000 | 0 0 1 2.5 7.5 10 50 100 |
Supplementary Figure 10. Copy number determination by RT-qPCR for Ct value relevance in clinical samples. Synthetic SARS-CoV-2 RNA template was used to determine the LOD by RT-qPCR using One-step RT-qPCR kit (Invitrogen). Ct value was determined using two independent sets primer sets, N1 and N2. Blank sample (no template) was used as negative control.
Supplementary Figure 11. Reporter complex stability. Protein:sgRNA:FAM-probe were mixed in 1:1:1 ratio in 250 nM concentration and incubated at 37 °C for 60 minutes. The reporter complex was stored at different temperatures for different periods of time. N-gene specific target and specific sgRNA were used as positive and no template samples was used as negative control.
Supplementary Figure 12. Additional evaluated clinical samples and their original scan.
| Sample designation | Ct value (RT-qPCR) |
|--------------------|-------------------|
| 155418             | 21                |
| 155378             | 34                |
| 155381             | 21                |
| 155383             | 21                |
| 155384             | 14                |
| 155385             | 22                |
| 155388             | 22                |
| 155389             | 23                |
| 155369             | 16                |
| 155397             | 18                |
| 155398             | 23                |
| 155902             | 15                |
| 155905             | 20                |
| 155908             | 17                |
| 155916             | 22                |
| 155960             | 15                |
| 155974             | 26                |
| 156852             | 21                |
| 161058             | 17                |
| 161063             | 16.5              |
| 151504             | 30.75             |
| 151611             | 30.77             |
| 151605             | 31.12             |
| 151547             | 31.45             |
| 151606             | 31.52             |
| 151560             | 31.57             |
| NID-1              | >38               |
| NID-2              | >38               |
| NID-3              | >38               |
| NID-4              | >38               |

| Sample | Ct value |
|--------|----------|
| K0250  | 34.64    |
| K0252  | 36.06    |
| K0253  | 35.57    |
| K0367  | 35.16    |
| S-1617 | 30.00    |
| S-1541 | 30.55    |
| S-1504 | 30.75    |
| S-1611 | 30.77    |
| S-1605 | 31.12    |
| S-1547 | 31.45    |
| S-1606 | 31.52    |
| S-1560 | 31.57    |
| S-437  | 32.47    |
| S-578  | 33.25    |
| S-786  | 32.88    |
| S-573  | 33.25    |
| S-270  | 33.80    |
| S-272  | 32.70    |
| S-263  | 33.29    |
| S-766  | 31.99    |

Supplementary table 1. Designation and the Ct value of the clinical samples used in this study. RT-qPCR was performed on Trizol based isolated RNA.
Name | Sequence | Experimental description
--- | --- | ---
virD2 reporter-FAM-S | 5'-gtaaccgcacatatctcttgctcaTTTTT/56-FAM/-3' | Short, FAM-labeled probe for VirD2
virD2 reporter-FAM-L | 5'-gtaaccgcacatatctcttgctcaTTTTT/56-FAM/-3' | Long, FAM-labeled probe for VirD2
virD2 reporter-B-L | 5'-gtaaccgcacatatctcttgctcaTTTTT/56-FAM/-3' | Long, FAM-labeled probe for VirD2

PCR-F1-N3 | 5'/biotin/CCGAAAGCTACCCGACAGACATTC/3' | Forward PCR primer used for proof-of-concept assay
PCR-R2-N3 | 5'/TGACACGATGTCAGGTT/3' | Reverse PCR primer used for proof-of-concept assay

gBlock-N3-sgRNA-1 | 5'/GTCTCACGCCATATATGCTACTATAGGCcagaagctgcttactaaT/3' | gBlock template used for IVT of sgRNA targeting both the PCR and RT-RPA generated amplicons

**Sequences of 3 different mutant proteins**

```plaintext
>3_SpdCas9-dVirD2-Mutated
Ccaccctgaattgctctccgggcgtatcagcatacgctgaaaggttagttgcctcttgccattcgatggttgtccgggatctcgacgctctcccttatagcgactcctgcattaggaagcagcccagtagtaggttgag

5'/acgattgcagcattgttagcaggattgcgg/3'

5'/biotin/aattggctactaccgaagagctaccagacg/3'

5'/biotin/agctaccagacgaattcgtggtggtgacgg/3'

5'/biotin/gtactgccactaaagcatacaatgtgacac/3'

5'/gtttactccgaatatatcctgtca/56-FAM/-3'

5'/GTCTCAGGCATAATACGACTCACTATAGGcagaagctggacttccctaGTTTTAGAGCT/3'
```

**Experimental description**

- blot analysis of VirD2 nuclease- and binding activities
- Selected RT-RPA fwd primer
- Used for primer screening
- Used for primer screening
- Used for primer screening
- Used for primer screening
- Used for primer screening
- Selected RT-RPA rev primer
- Covalent binding confirmation of VirD2.dQ9 fusions

**Additional sequences**

```plaintext
Ccaccctgaattgctctccgggcgtatcagcatacgctgaaaggttagttgcctcttgccattcgatggttgtccgggatctcgacgctctcccttatagcgactcctgcattaggaagcagcccagtagtaggttgag
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