All-in-one dual CRISPR-Cas12a (AIOD-CRISPR) assay protocol for SARS-CoV-2 detection

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Method Article

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Abstract

This protocol presents the all-in-one dual CRISPR-Cas12a (AIOD-CRISPR) assay to ultra-sensitively and visually detect SARS-CoV-2. The procedure of AIOD-CRISPR assay typically consists of three parts including sample preparation, AIOD-CRISPR reaction, and fluorescence detection. Sample preparation involves the synthetic RNA preparation and the nucleic acid extraction from SARS-CoV-2 samples. The prepared nucleic acids were then added into the AIOD-CRISPR reaction systems as templates, followed by incubation at 37°C for 20-40 min. After incubation, visual detection was immediately conducted by placing the tubes in a portable LED blue transilluminator (Maestrogen UltraSlim) or the ChemiDoc™ MP Imaging System (Bio-Rad) with its built-in UV channel. In addition to endpoint visual detection, real-time fluorescence detection was also available for AIOD-CRISPR assay. This protocol is helpful for applying AIOD-CRISPR assay to rapid, sensitive, one-pot point-of-care SARS-CoV-2 detection.

Introduction

Reagents

- QIAamp DSP Viral RNA Mini Kit Print (Qiagen, cat. no. 61904)
- TwistAmp® Liquid Basic Kit (TwistDx, cat. no. TALQBAS01)
- PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, cat. no. K210012)
- TURBO DNA-free™ Kit (Thermo Fisher Scientific, cat. no. AM1907)
- RNeasy® MinElute TM Cleanup Kit (Qiagen, cat. no. 74204)
- RiboMAX™ Large Scale RNA Production Systems-T7 (Sigma-Aldrich, cat. no. P1300)
- SsoAdvanced™ Universal SYBR® Green PCR Supermix (Bio-Rad Laboratories, cat. no. 1725270)
- EnGen® Lba Cas12a (Cpf1) (100 µM) (New England BioLabs, cat. no. M0653T)
- Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (10,000 units/mL) (New England BioLabs, cat. no. M0277L)
- dNTP Set (100 mM) (ThermoFisher Scientific, cat. no. 10297018)
- Nuclease-free water (New England BioLabs, cat. no. B1500L)
- Single-stranded fluorescent reporter (ssDNA-FQ): /56-FAM/TTATT/3IABkFQ/ (Integrated DNA Technologies)
- The pUCIDT (Amp) plasmid with 316 bp N gene from SARS-CoV-2 (Integrated DNA Technologies)

- Forward primer (FP) targeting N gene: 5'-AGGCAGCAGTAGGGGAACTTCTCCTGCTAGAAT-3' (HPLC grade, Integrated DNA Technologies)
- Reverse primer (RP) targeting N gene: 5'-TTGGCCTTTACCAGACATTTTGCTCTCAAGCTG-3' (HPLC grade, Integrated DNA Technologies)
- CRISPR RNA 1 (crRNA1) targeting N gene: 5'-UAAUUUCUACUAAGUGUAGAUCAUCACCGCCAUUGCCAGCC-3' (Integrated DNA Technologies)
- CRISPR RNA 2 (crRNA2) targeting N gene: 5'-UAAUUUCUACUAAGUGUAGAUUUGCUGCUGCUUGACAGAUU-3' (Integrated DNA Technologies)
- T7 promotor-tagged PCR forward primer (T7-PCR-F) targeting SARS-CoV-2 N gene: TATACGACTCACTATAGGGACTCAAGGAACAACATTGCCA (HPLC grade, Integrated DNA Technologies)
- PCR reverse primer (PCR-R) targeting SARS-CoV-2 N gene: GCTTTAGTGGCAGTACGTT (HPLC grade, Integrated DNA Technologies)

Equipment

- MaestroGen UltraSlim LED Transilluminator (Fisher Scientific)
- ChemiDoc™ MP Imaging System (Bio-Rad Laboratories)
- CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories)
- NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometry (Thermo Fisher Scientific)

Procedure

CRITICAL: Thaw all frozen reagents thoroughly before use.

Sample preparation

Preparation of synthetic RNA

1. Run PCR to amplify 316 bp SARS-CoV-2 N gene sequence in the pUCIDT plasmid. The 25-μL PCR system contains 1× SsoAdvanced™ Universal SYBR® Green PCR Supermix, 0.4 μM of T7-PCR-F, 0.4 μM of PCR-R, and 1.0 μL of 1.3× 10⁵ copies/μL SARS-CoV-2 N plasmid solution. The thermal cycling is 2.5 min at 98°C for initial denaturation, 35 cycles of 15 s at 95°C for denaturation and 30 s at 60°C for annealing and extension.

2. Pipette 20 μL of the PCR products for 2% agarose gel electrophoresis analysis (120 V and 45 min). Then cut the band at the size of about 336 bp, followed by gel extraction using PureLink™ Quick Gel

Extraction Kit. Further confirm the sequence by shipping the purified PCR products to Genewiz® for Sanger sequencing.

3. Incubate the solution containing 8 µL of 5× T7 Transcription Buffer, 3 µL each of 100 mM rNTPs, 4 µL of the Enzyme Mix with T7 RNA polymerase, 16 µL of the gel-extracted PCR products at 37°C for 4 h. The reagents are from the RiboMAX™ Large Scale RNA Production Systems-T7 Kit.
4. Treat the transcription products using the DNase (from the TURBO DNA-free™ Kit) to degrading the DNA.
5. Purify the resulting RNA using the RNeasy® MinElute™ Cleanup Kit.
6. Determined the purity and concentration of the collected nucleic acid using NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometry.
7. Keep the synthetic RNA at -80°C before use. **TIP:** Do not thaw-freeze the RNA too many times in case of degradation.

Extraction of RNA from SARS-CoV-2 samples

· This procedure strictly follows the handbook of QIAamp DSP Viral RNA Mini Kit. The handbook can be downloaded at this link, <https://www.qiagen.com/us/resources/resourcedetail?id=46638e95-df58-4874-9015-732e75587524&lang=en>

AIOD-CRISPR reaction

1. Prepare Component A by mixing 12.5 µL of 2× Reaction Buffer from the TwistAmp® Liquid Basic Kit, 2.5 µL of 10× Basic E-mix from the TwistAmp® Liquid Basic Kit, 1.25 µL of 280 mM MgOAc from the TwistAmp® Liquid Basic Kit, 0.08 µL of 100 µM FP, 0.08 µL of 100 µM RP, 0.2 µL of 100 mM dATP, 0.2 µL of 100 mM dGTP, 0.2 µL of 100 mM dTTP, 0.2 µL of 100 mM dCTP, and 0.8 µL of 10 U/µL AMV reverse transcriptase. **TIP:** Prepare the mixture enough for at least 8 reactions for easy and accurate pipetting.
2. Prepare Component B by mixing 2 µL of ssDNA-FQ and 1.25 µL of 20× Core Reaction Buffer from the TwistAmp® Liquid Basic Kit. **TIP:** Thoroughly vortex the Core Reaction Buffer before pipetting.
3. Pipette 2.5 µL of the synthetic RNA or the extracted nucleic acid solutions into the prepared Component A, followed by adding 3.25 of the prepared Component B. The assembled mixture is left at room temperature during the preparation of Component C. **TIP:** Thoroughly vortex the assembled mixture and invert the tube 3 times prior to slight centrifuging.

4. Prepare Component C by mixing 0.32 μ L of 50 μ M crRNA1, 0.32 μ L of 50 μ M crRNA2, 0.32 μ L of 100 μ M EnGen® Lba Cas12a (Cpf1), and 0.28 μ L nuclease-free water. **TIP:** It is preferable to leave the mixture for about 10 min at room temperature for fully forming the crRNA-Cas12a complex. Prepare the mixture enough for at least 2 reactions for easy and accurate pipetting. For better performance, the Component C is recommended to be fresh prepared when testing.
5. Pipette 1.24 μ L of the Component C into the assembled mixture in Step 3 to form the final reaction system. **TIP:** Thoroughly vortex the reaction system and invert the tube 3 times prior to slight centrifuging.
6. Incubate the final reaction system at 37°C for 20-40 min prior to fluorescence detection.

Fluorescence detection

- Take out the tubes after the AIOD-CRISPR reaction and place them in the LED blue transilluminator or the Imaging System for visual fluorescence detection.
- Real-time fluorescence detection can be achieved by incubating the AIOD-CRISPR reaction systems in the CFX96 Touch™ Real-Time PCR Detection System.

Time Taken

For real sample testing

- Extract RNA from samples: **at least 20 min.** The actual time depends on sample size, manual or fully automated operation (e.g. the QIAcube).
- Prepare the AIOD-CRISPR reaction system: **at least 5 min.** Component A, B, and C can be prepared as stock solutions and kept at -20°C until use. The actual time also depends on sample size.
- Incubate the AIOD-CRISPR solution: **20-40 min.**
- Run fluorescence detection and visually judge the test results: **1-2 min.**

Totally, an AIOD-CRISPR assay can be finished as short as 46 min from sample preparation to reporting results.