بسم الله الرحمن الرحيم

Topic

CRISPR system and It's application in COVID-19 diagnosis

By:

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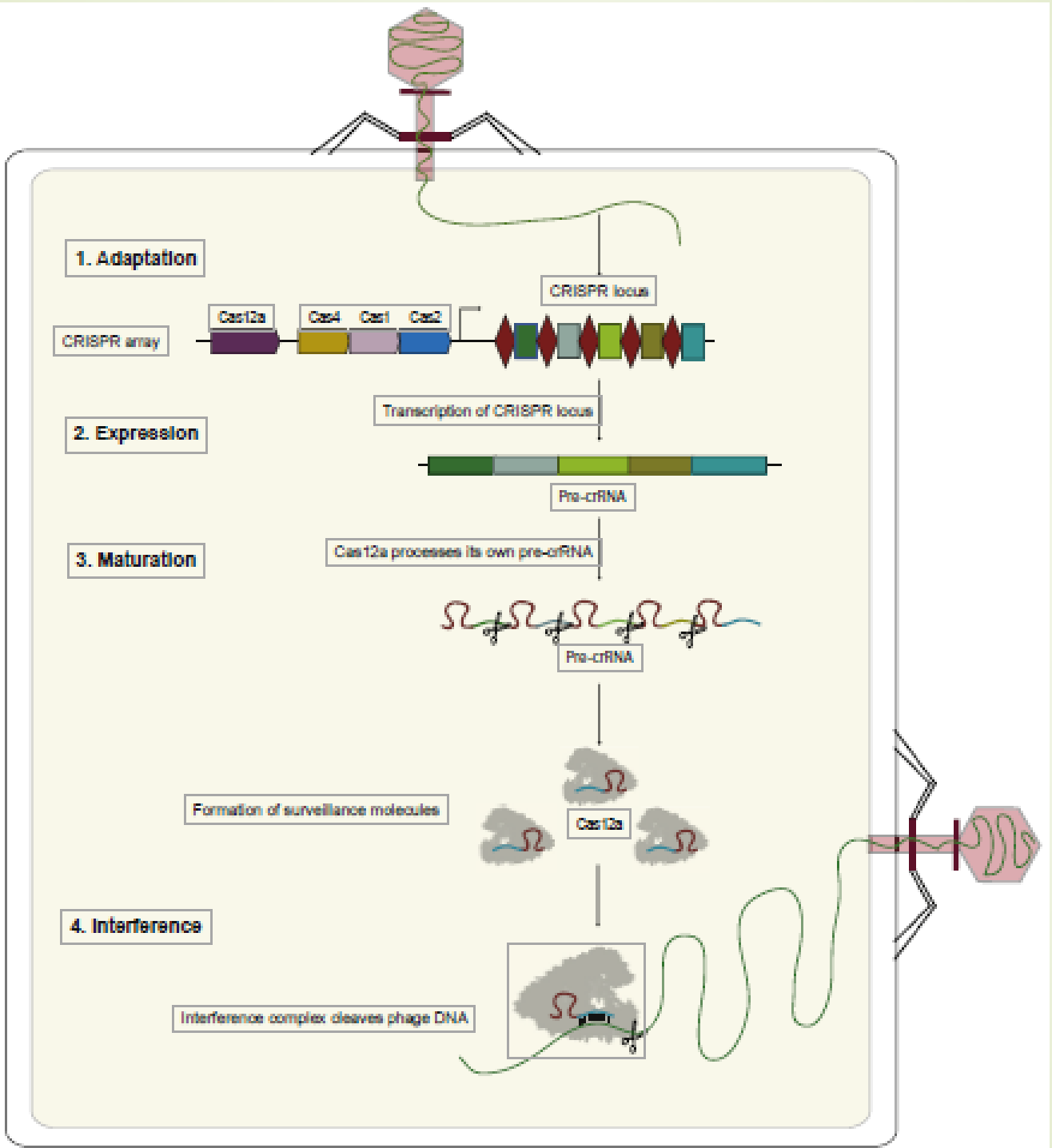
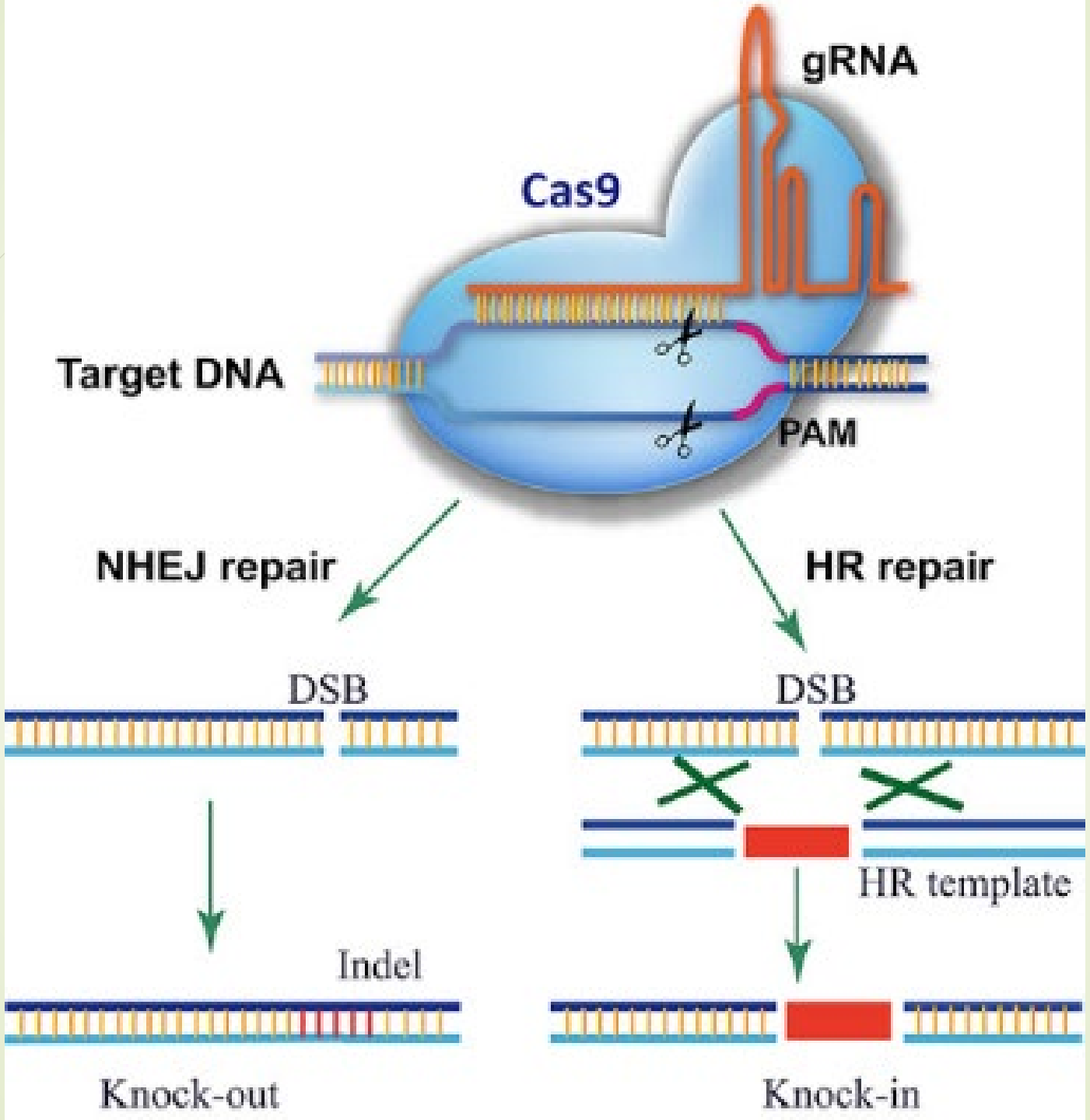
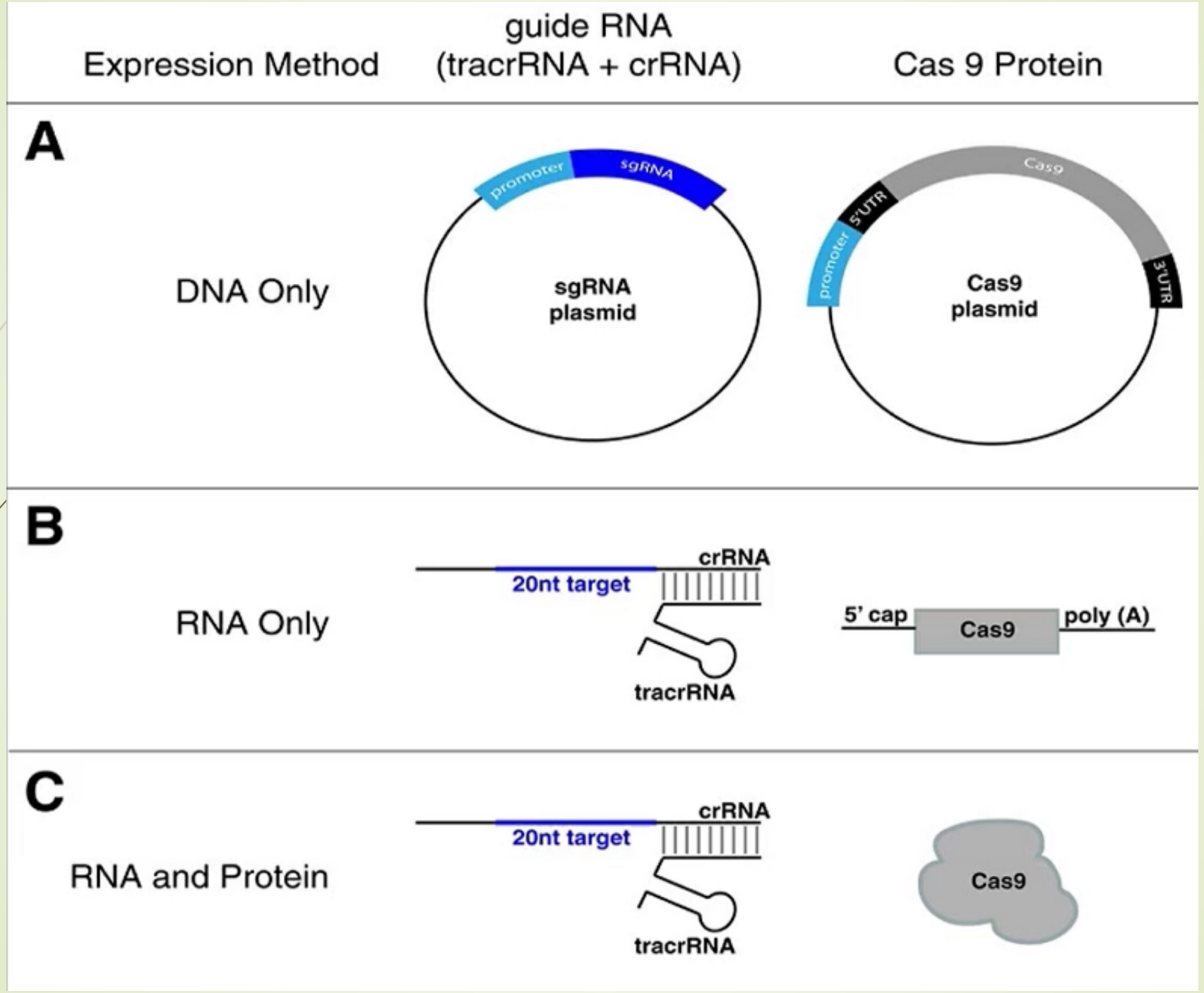
Ph.D of Molecular Medicine

CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments of bacteriophages that had previously infected the prokaryote. They are used to detect and destroy DNA from similar bacteriophages during subsequent infections. Hence these sequences play a key role in the antiviral (i.e. anti-phage) defense system of prokaryotes.


1. Adaptation
CRISPR locus
CRISPR array
Cas12a
Cas4
Cas1
Cas2
Transcription of CRISPR locus
2. Expression
Pre-crRNA
3. Maturation
Cas12a processes its own pre-crRNA
Pre-crRNA
Formation of surveillance molecules
Cas12a
4. Interference
Interference complex cleaves phage DNA

Cas9 (or "CRISPR-associated protein 9") is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence. Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR-Cas9 that can be used to edit genes within organisms. This editing process has a wide variety of applications including basic biological research, development of biotechnology products, and treatment of diseases.


gRNA
Cas9
Target DNA
PAM
NHEJ repair
HR repair
DSB
DSB
HR template
Indel
Knock-out
Knock-in

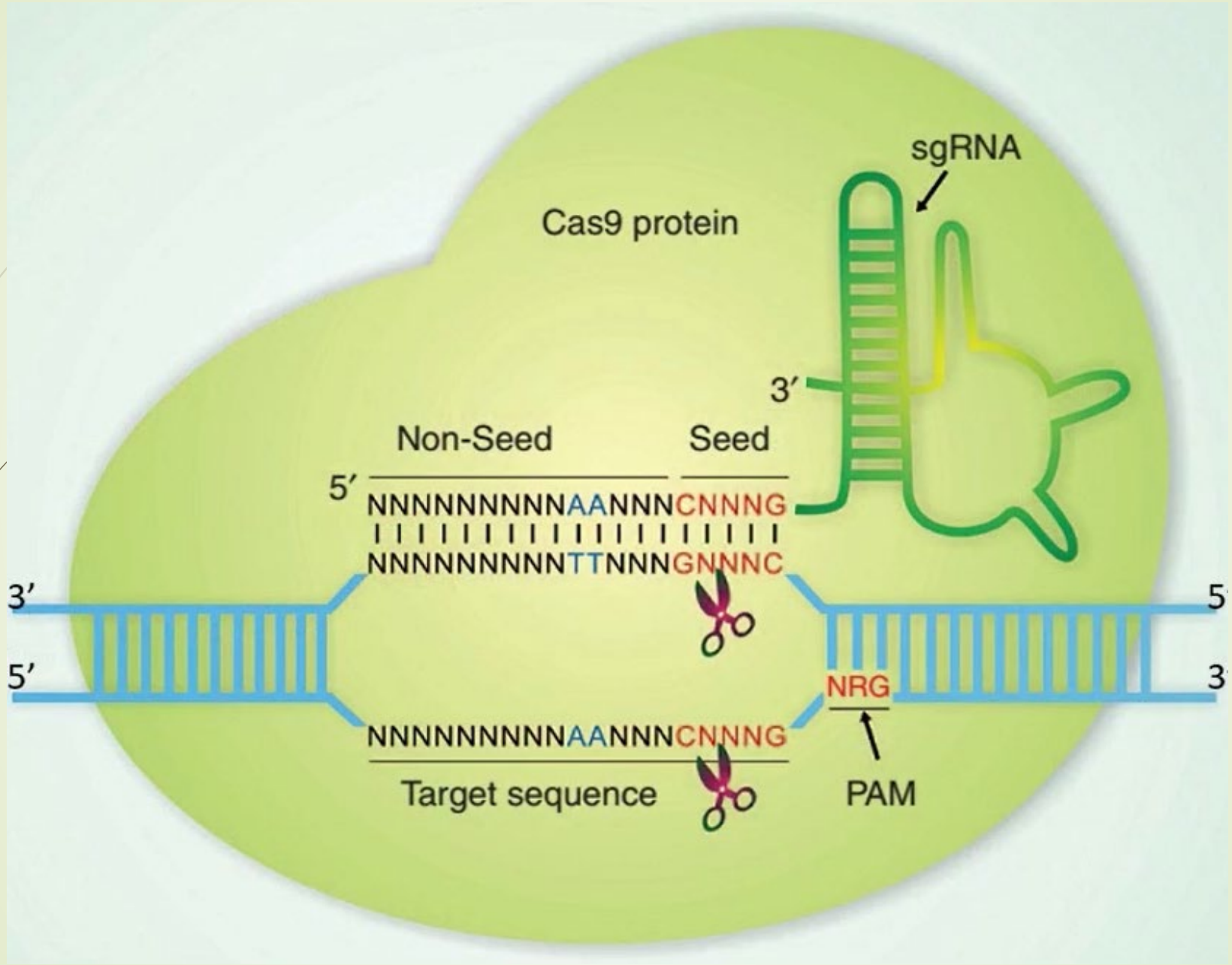

Expression Method
guide RNA (tracrRNA + crRNA)
Cas 9 Protein
A
DNA Only
promoter
sgRNA
sgRNA plasmid
Cas9
5'UTR
promoter
3'UTR
Cas9 plasmid
B
RNA Only
crRNA
20nt target
tracrRNA
5' cap
Cas9
poly (A)
C
RNA and Protein
crRNA
20nt target
tracrRNA
Cas9

The CRISPR-Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity. RNA harboring the spacer sequence helps Cas (CRISPR-associated) proteins recognize and cut foreign pathogenic DNA. CRISPR are found in approximately 50% of sequenced bacterial genomes and nearly 90% of sequenced archaea.

The CRISPR-Cas systems are classified into two classes (Classes 1 and 2) that are subdivided into six types (types I through VI). Class 1 (types I, III and IV) systems use multiple Cas proteins in their CRISPR ribonucleoprotein effector nucleases and Class 2 systems (types II, V and VI) use a single Cas protein.

Class 1 CRISPR-Cas systems are most commonly found in bacteria and archaea, and comprise ~90% of all identified CRISPR-Cas loci. The Class 2 CRISPR-Cas systems, comprising the remaining ~10%, exists almost exclusively in bacteria, and assemble a ribonucleoprotein complex, consisting of a CRISPR-RNA (crRNA) and a Cas protein.

The crRNA contains information to target a specific DNA sequence. These multi-domain effector proteins achieve interference by complementarity between the crRNA and the target sequence after recognition of the PAM (Protospacer Adjacent Motif) sequence, which is adjacent to the target DNA.


sgRNA
Cas9 protein
3′
Non-Seed
Seed
5′
NNNNNNNNNAANNNCNNNG
NNNNNNNNNTTNNNGNNNC
3′
5′
5
3
NRG
NNNNNNNNNAANNNCNNNG
Target sequence
PAM

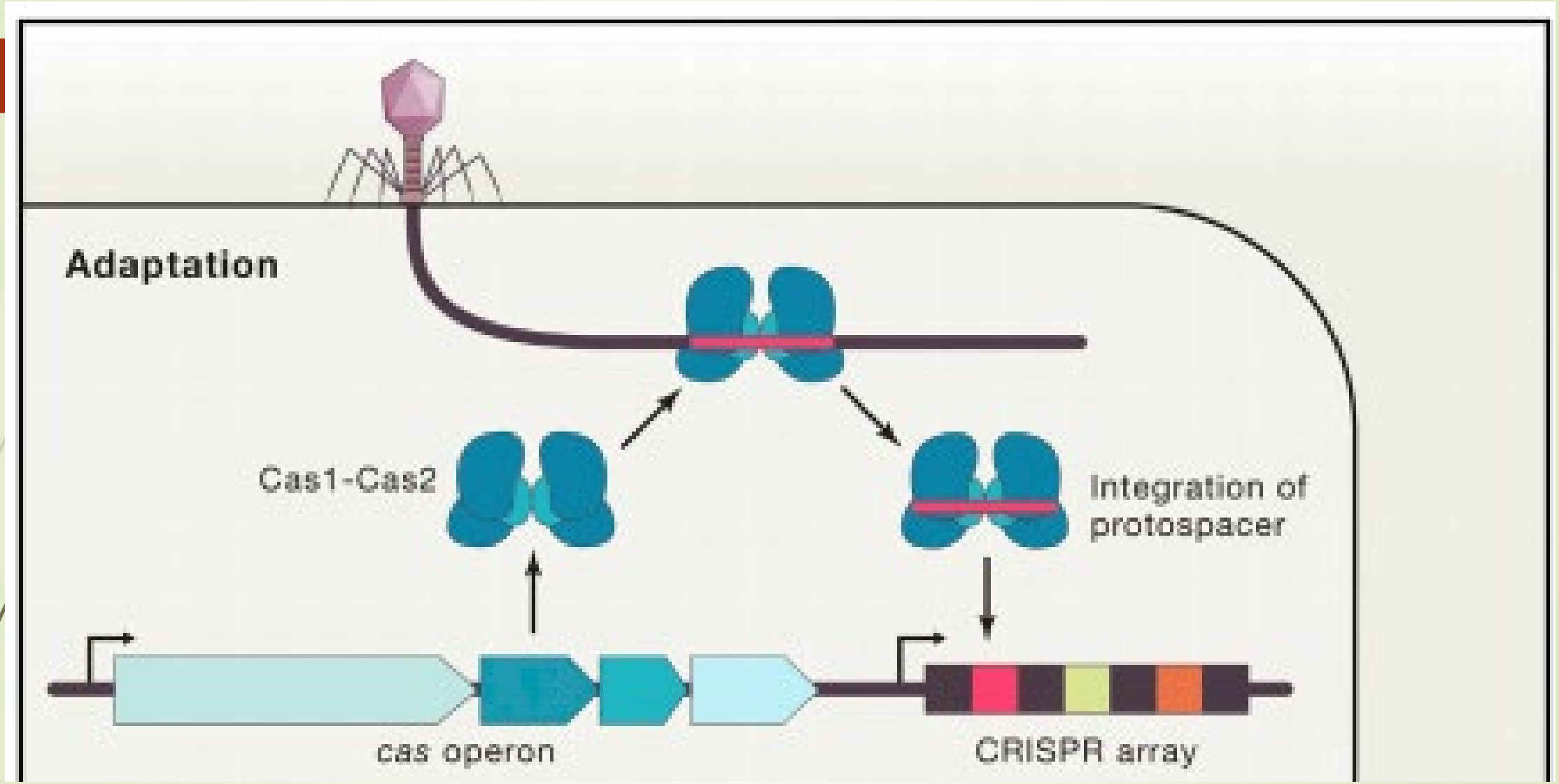
These ribonucleoprotein complexes have been redesigned for precise genome editing by providing a crRNA with a redesigned guide sequence, which is complementary to the sequence of the targeted DNA. The most widely characterized CRISPR-Cas system is the type II subtype II-A that is found in Streptococcus pyogenes (Sp), which uses the protein SpCas9, Cas9 was the first Cas-protein engineered for use in gene editing. Class 2 type V is further classified into 4 subtypes (V-A, V-B, V-C, V-U).

At present, V-C and V-U remain widely uncharacterised and no structural information on these systems is available. V-A encodes the protein Cas12a (also known as Cpf1) and recently several high resolution structures of Cas12a have provided an insight into its working mechanism.

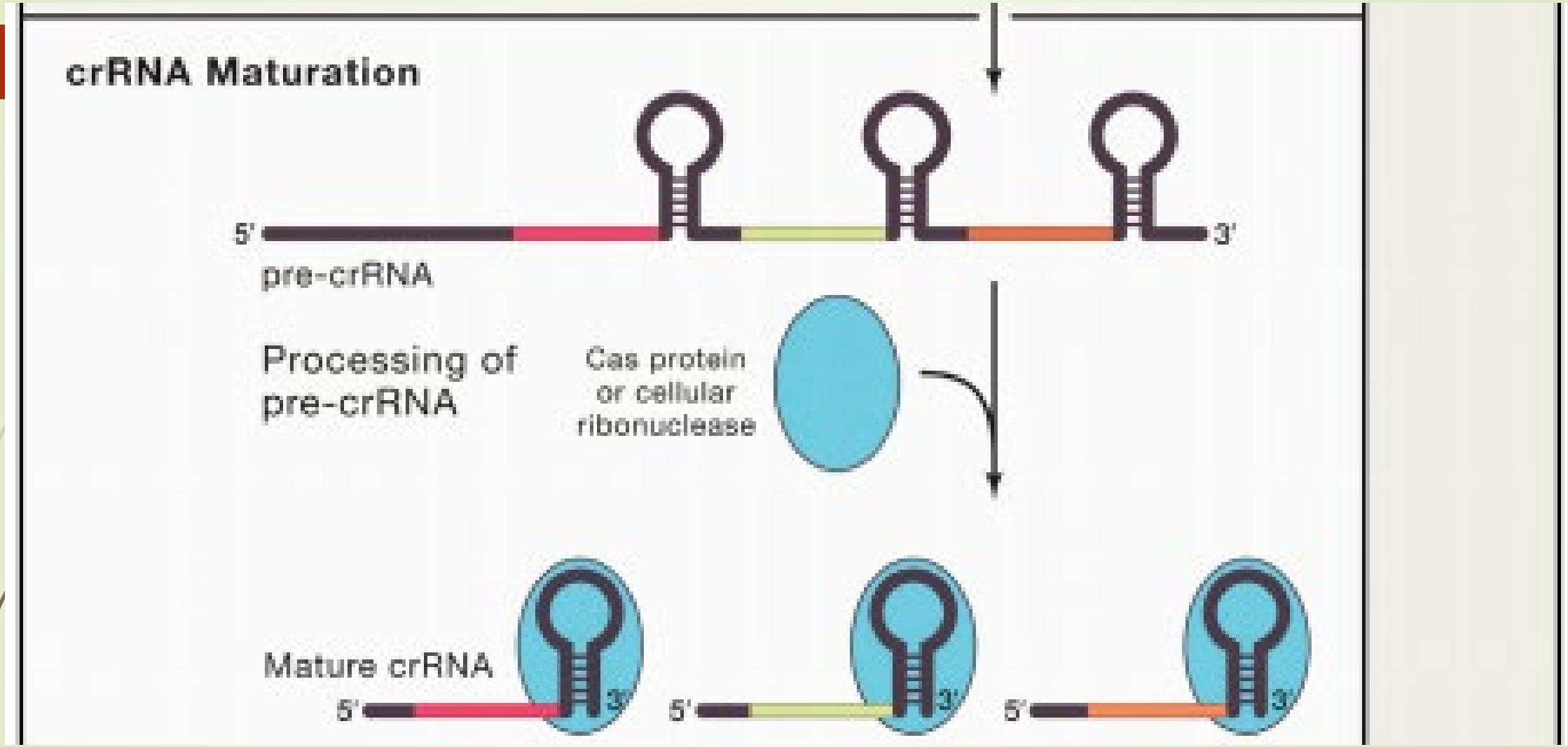
This system, involving RNA-guided interference, has been harnessed into a versatile biotechnological tool for genome editing, whereby a simple exchange of the RNA guide sequence can be employed to re-engineer nuclease specificity, leading to a revolution in the life sciences.

CRISPR-Cas immunity involves three major sequential steps: adaptation, expression/maturation and interference, each step needs specific Cas proteins encoded by the cas genes near the CRISPR array, together with other accessory proteins.

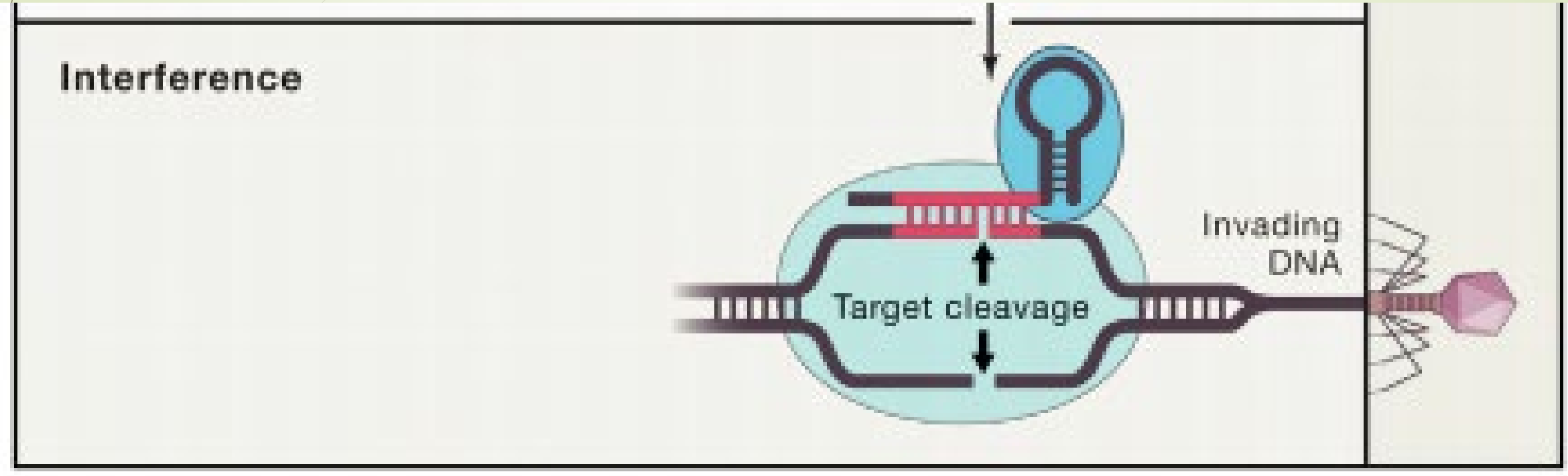
The CRISPR-Cas adaptation stage involves the identification and extraction of the protospacer from the invading DNA/RNA and its subsequent incorporation into the CRISPR array. Both these functions are performed by the versatile Cas1-Cas2 adaptation complex.

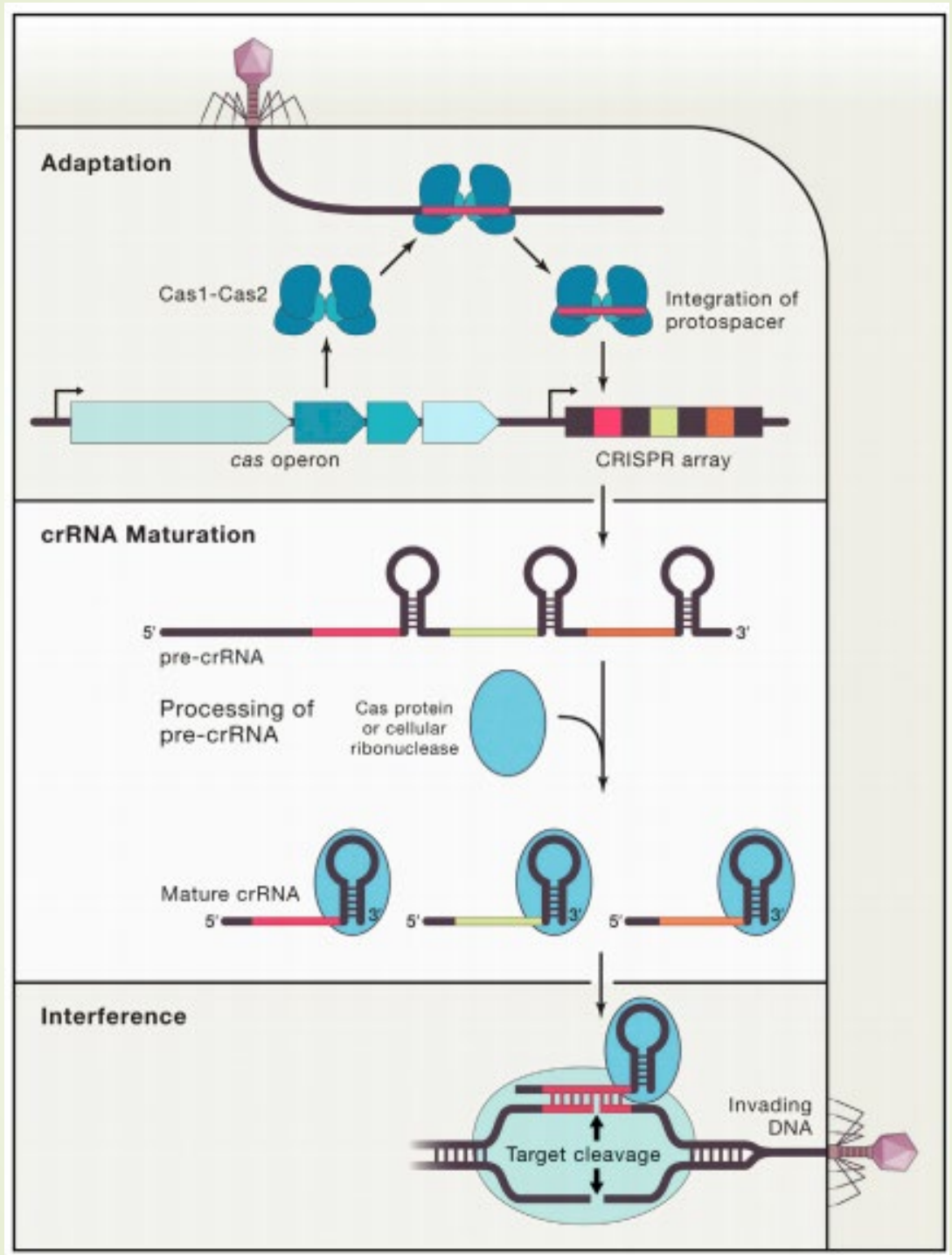

Adaptation
Cas1-Cas2
Integration of protospacer
cas operon
CRISPR array

During the expression/maturation stage, the CRISPR array is transcribed into a long pre-CRISPR RNA (pre-crRNA) molecule. The pre-crRNA is processed into shorter crRNA molecules each containing a spacer and a part of the repeat sequence.


crRNA Maturation
5'
3'
pre-crRNA
Processing of pre-crRNA
Cas protein or cellular ribonuclease
Mature crRNA
5'
3'
5'
3'
5'
3'

Finally, interference can occur, after the crRNA forms a complex with the effector protein, forming a functional RNA guided endonuclease. This endonuclease is guided by the crRNA, which after PAM recognition hybridizes with the target DNA through its spacer sequence, and eventually, cuts the target DNA sequence.

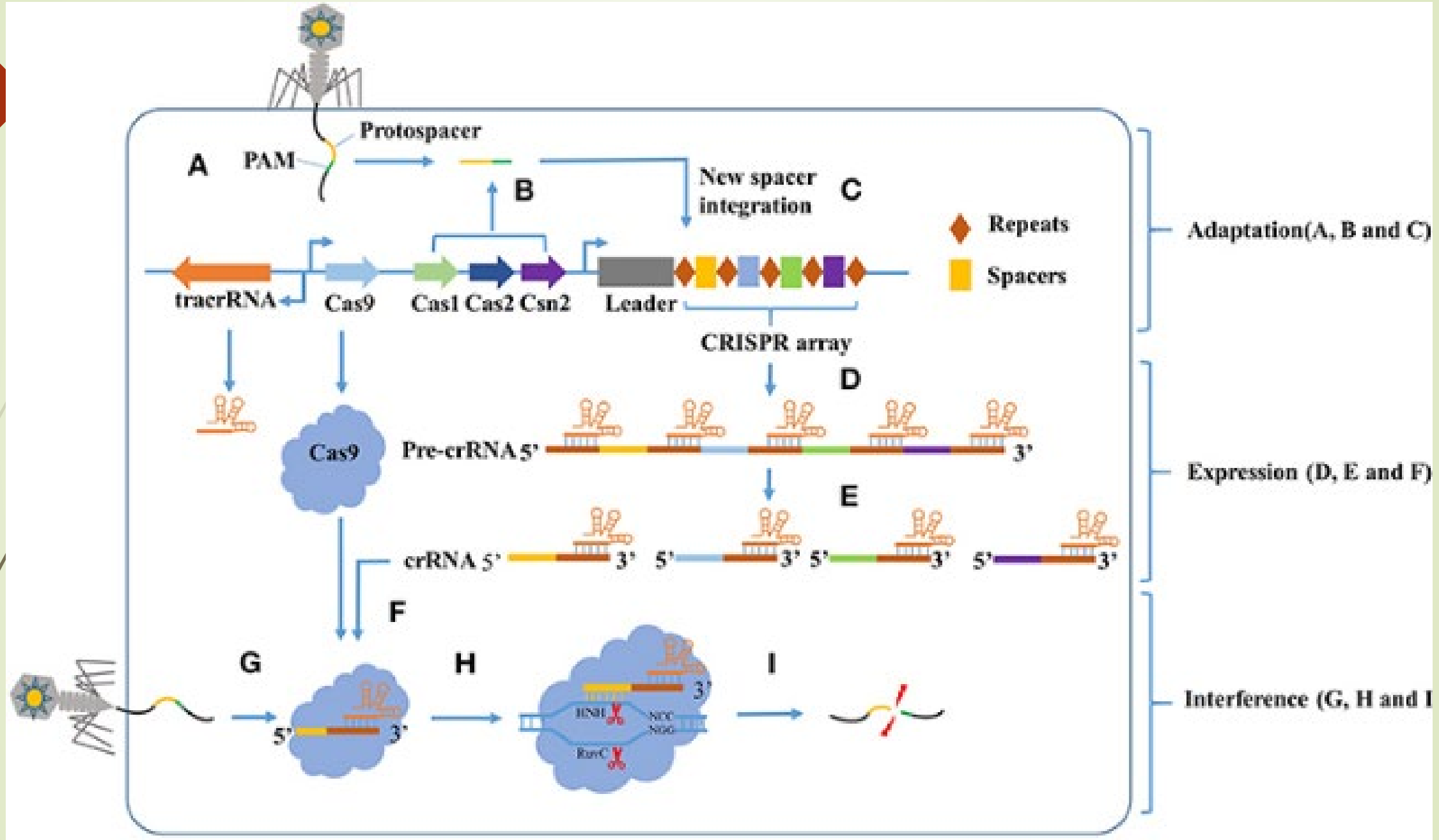

Interference
Target cleavage
Invading
DNA


Adaptation
Cas1-Cas2
Integration of protospacer
cas operon
CRISPR array
crRNA Maturation
5'
3'
pre-crRNA
Processing of pre-crRNA
Cas protein or cellular ribonuclease
Mature crRNA
Interference
Invading DNA
Target cleavage

Cas12a vs Cas9

Cas12a and Cas9 have striking functional similarities despite having evolved through independent pathways, with similar sizes (1368 amino acids for SpCas9; 1307 for FnCas12a).

- ✓ Cas9 requires two RNA molecules: tracrRNA and a crRNA, whereas Cas12a requires only a single RNA molecule, the crRNA.


Protospacer
A
PAM
B
New spacer integration
C
Repeats
Spacers
tracrRNA
Cas9
Cas1 Cas2 Csn2
Leader
CRISPR array
Adaptation(A, B and C)
D
Cas9
Pre-crRNA 5'
3'
Expression (D, E and F)
E
crRNA 5'
3'
F
G
H
I
5'
3'
Interference (G, H and I

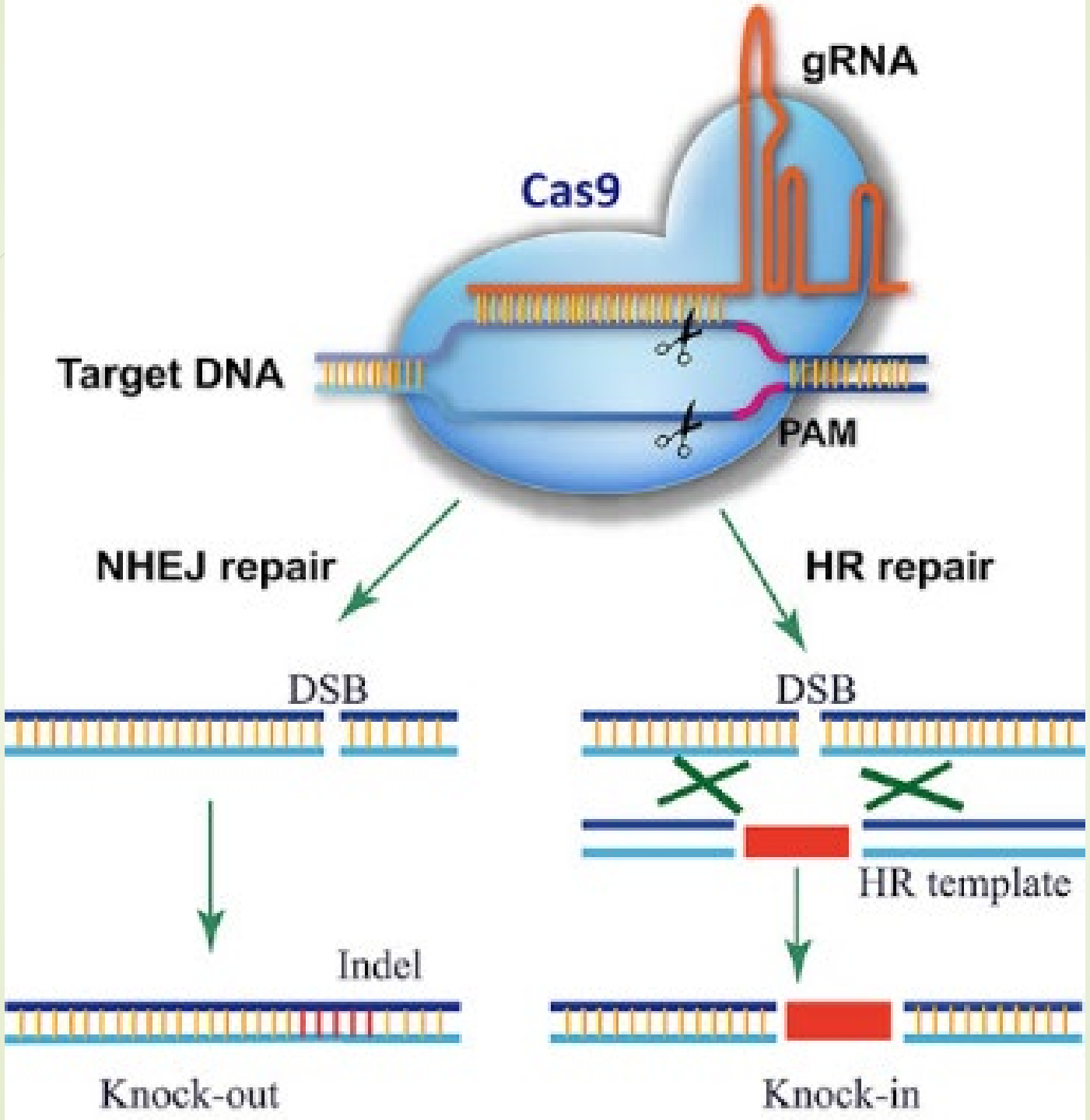
- ✓ Cas9 possesses two nuclease sites HNH and RuvC domains, while Cas12a possesses only one nuclease site in the RuvC domain.
- ✓ Cas12a also possesses an RNA processing site.
- ✓ There are distinct differences in the mechanisms employed by the two proteins when it comes to RNA processing, PAM recognition, target DNA binding and eventually catalysis.

- ✓ For Cas9 targeted DNA sequences, the PAM is situated downstream of the spacer sequence on the non-template strand, and is recognized by the PI domain, which is primed for identifying a 5'-NGG-3' PAM.
- ✓ In contrast, Cas12a recognizes A-T rich sequences, with the PAM, located upstream of the spacer. Upon PAM recognition, the target DNA is unzipped and hybridization of the RNA/DNA takes place.
- ✓ For both enzymes exists a crucial seed sequence next to the PAM to determine the specificity of target DNA binding. The seed sequence for Cas9 is about ~10 nt whereas for Cas12a it is about ~5-6 nt.

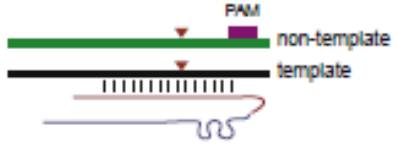
- ✓ When the hybridization of the DNA with the RNA is complete, Cas9 cleaves the template strand and the non-template strands in the catalytic sites located in the HNH and the RuvC domains respectively, producing a blunt DSB, with the cleavage site being 3 base pairs upstream from the PAM sequence.
- ✓ However, in the case of Cas12a due to the presence of a single nuclease site, the strands of the DNA are cut in the same nuclease site. Since it has been shown that the two strands follow different pathways to reach the catalytic site, explaining the staggered DSB produced by Cas12a.

A recent comparison of different Cas12a and Cas9 from different species using nuclease digestion and deep sequencing in vitro, revealed that both enzymes share similar types of specificities and tolerate similar mismatches, in contrast to in vivo reports that show the lower off-target effects of Cas12a.

Application of CRISPR-Cas systems as molecular tools for genome editing exploits their ability to produce a double strand break (DSB) at a specific genomic locus, and depends entirely on the host cell DNA repair machinery to fix the lesion produced by these systems. The repair mechanisms can be either of the following processes: homology-directed repair (HDR) or non-homologous end joining (NHEJ). HDR utilizes a template DNA that is homologous to the break site (an unbroken sister chromatid or a homologous chromosome) to repair the DSB, whereas NHEJ is based on direct joining of broken ends of the DSB, making NHEJ the more error prone mechanism of the two.


gRNA
Cas9
Target DNA
PAM
NHEJ repair
HR repair
DSB
DSB
HR template
Indel
Knock-out
Knock-in

At the center of CRISPR mediated genome engineering today is Cas9, with applications including, but not limited to, gene knockout and precise genome editing. Despite the rapid advances in genome editing by Cas9, it still presents challenges owing to the possibility of off-target effects and difficulty of delivering the ribonucleoprotein particle. Cas12a, owing to its substantial differences with Cas9, presents an alternate molecular genome editing tool.

	Cas12a	Cas9
Size of protein	~1300 amino acids	~1000-1600 amino acids
RNA	Single RNA molecule	Two RNA molecules
Nuclease sites	Single nuclease site RuvC-Nuc	2 nuclease domains HNH and RuvC
Type of cut	Staggered ends	 Blunt ends
PAM requirements	Recognises 5' T-rich PAM sequences of 3-4 nt	Recognises 3' G-rich PAM sequences of 3-5 nt
precrRNA processing	possesses intrinsic RNase activity to process precr-RNA	requires host RNase III and tracrRNA

Cas12a vs Cas9

All-in-One Dual CRISPR-Cas12a (AIOD-CRISPR) Assay: A Case for Rapid, Ultrasensitive and Visual Detection of Novel Coronavirus SARS-CoV-2 and HIV virus

A recent outbreak of novel coronavirus (SARS-CoV-2), the causative agent of COVID-19, has spread rapidly all over the world. Human immunodeficiency virus (HIV) is another deadly virus and causes acquired immunodeficiency syndrome (AIDS). Rapid and early detection of these viruses will facilitate early intervention and reduce disease transmission risk. Here, we present an **A**ll-**I**n-**O**ne **D**ual **CRISPR**-Cas12a (termed "AIOD-CRISPR") assay method for simple, rapid, ultrasensitive, one-pot, and visual detection of coronavirus SARS-CoV-2 and HIV virus.

In our AIOD-CRISPR assay, a pair of crRNAs was introduced to initiate dual CRISPR-Cas12a detection and improve detection sensitivity. The AIOD-CRISPR assay system was successfully utilized to detect nucleic acids (DNA and RNA) of SARS-CoV-2 and HIV with a sensitivity of few copies. Also, it was evaluated by detecting HIV-1 RNA extracted from human plasma samples, achieving a comparable sensitivity with real-time RT-PCR method. Thus, our method has a great potential for developing next-generation point-of-care molecular diagnostics.

Polymerase chain reaction (PCR) method is the most commonly used technology for pathogen nucleic acid detection and has been considered as a “gold standard” for disease diagnostics due to high sensitivity and specificity. However, it typically relies on expensive equipment and well-trained personnel, all of which is not suitable for point of care diagnostic application.

In recent decades, several isothermal amplification methods, such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), have been developed as attractive alternatives to conventional PCR method because of their simplicity, rapidity and low cost. However, there is still a challenge to apply it to develop a reliable diagnostics for clinical applications due to non-specific signals (e.g., false-positive).

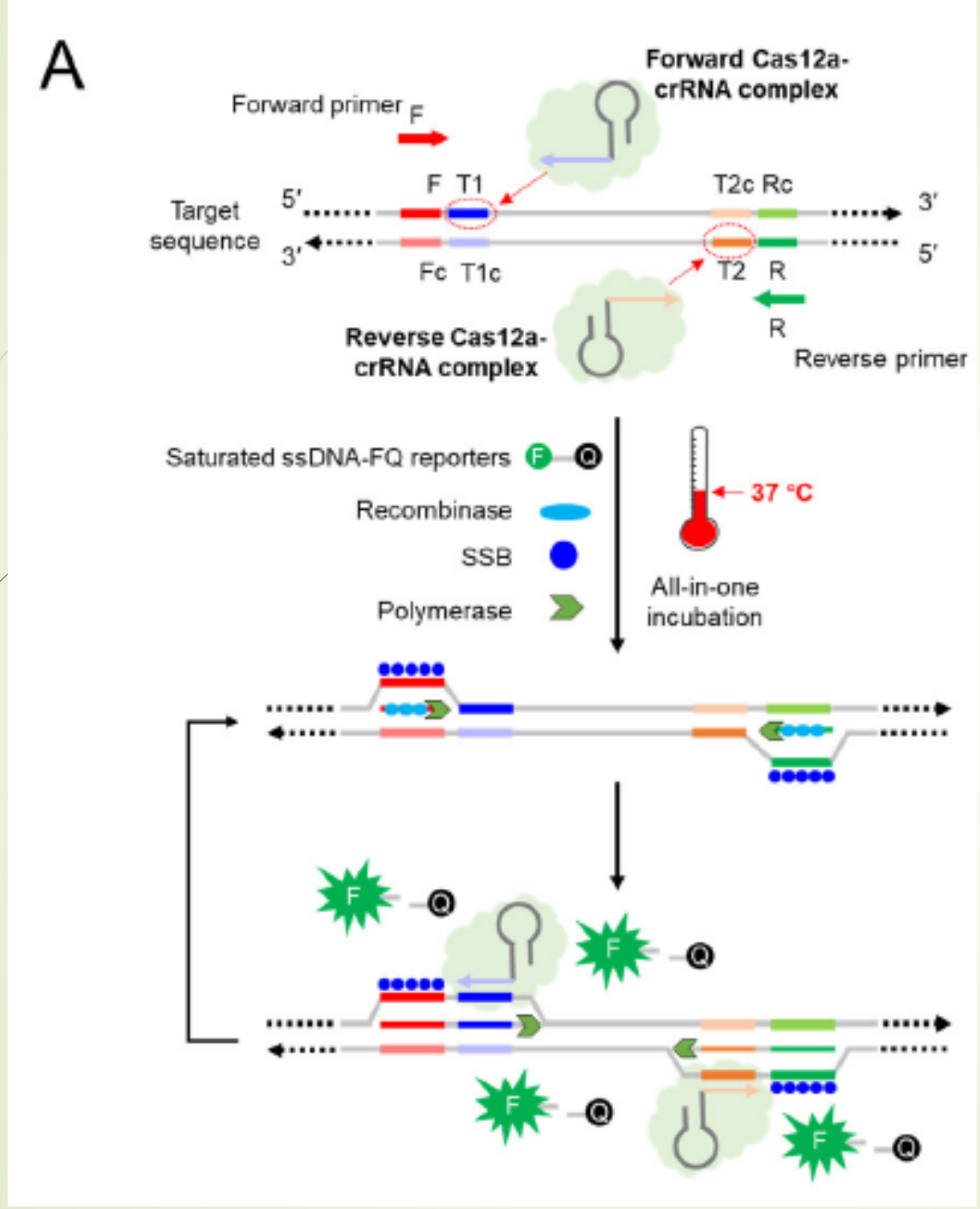
Recently, RNA-guided CRISPR/Cas nuclease-based nucleic acid detection has shown great promise for the development of next-generation molecular diagnostics technology due to its high sensitivity, specificity and reliability. For example, some Cas nucleases (e.g., Cas12a, Cas12b and Cas13a) perform strong collateral cleavage activities in which a crRNA-target binding activated Cas can indiscriminately cleave surrounding non-target single-stranded nucleic acids.

In this study, reported an **A**ll-**I**n-**O**ne **D**ual **CRISPR**-Cas12a (termed "AIOD-CRISPR") assay for rapid, ultrasensitive, specific and visual detection of nucleic acid. Dual crRNAs are introduced to initiate highly efficient CRISPR-based nucleic acid detection. In AIOD-CRISPR assay, all components for nucleic acid amplification and CRISPR detection are thoroughly mixed in a single, one-pot reaction system and incubated at a single temperature (e.g., 37 °C), eliminating the need for separate pre-amplification and amplified product transferring.

As application examples, the AIOD-CRISPR assay was engineered to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)19 and human immunodeficiency virus type 1 (HIV-1). Since SARS-CoV-2 and HIV-1 are retrovirus, evaluated the performance of AIOD-CRISPR assay by detecting both of their DNA and RNA. Especially, the test results of AIOD-CRISPR assay can be directly visualized by naked eye. Therefore, we anticipate that the AIOD-CRISPR assay will facilitate CRISPR based next-generation molecular diagnostics towards point-of-care applications.

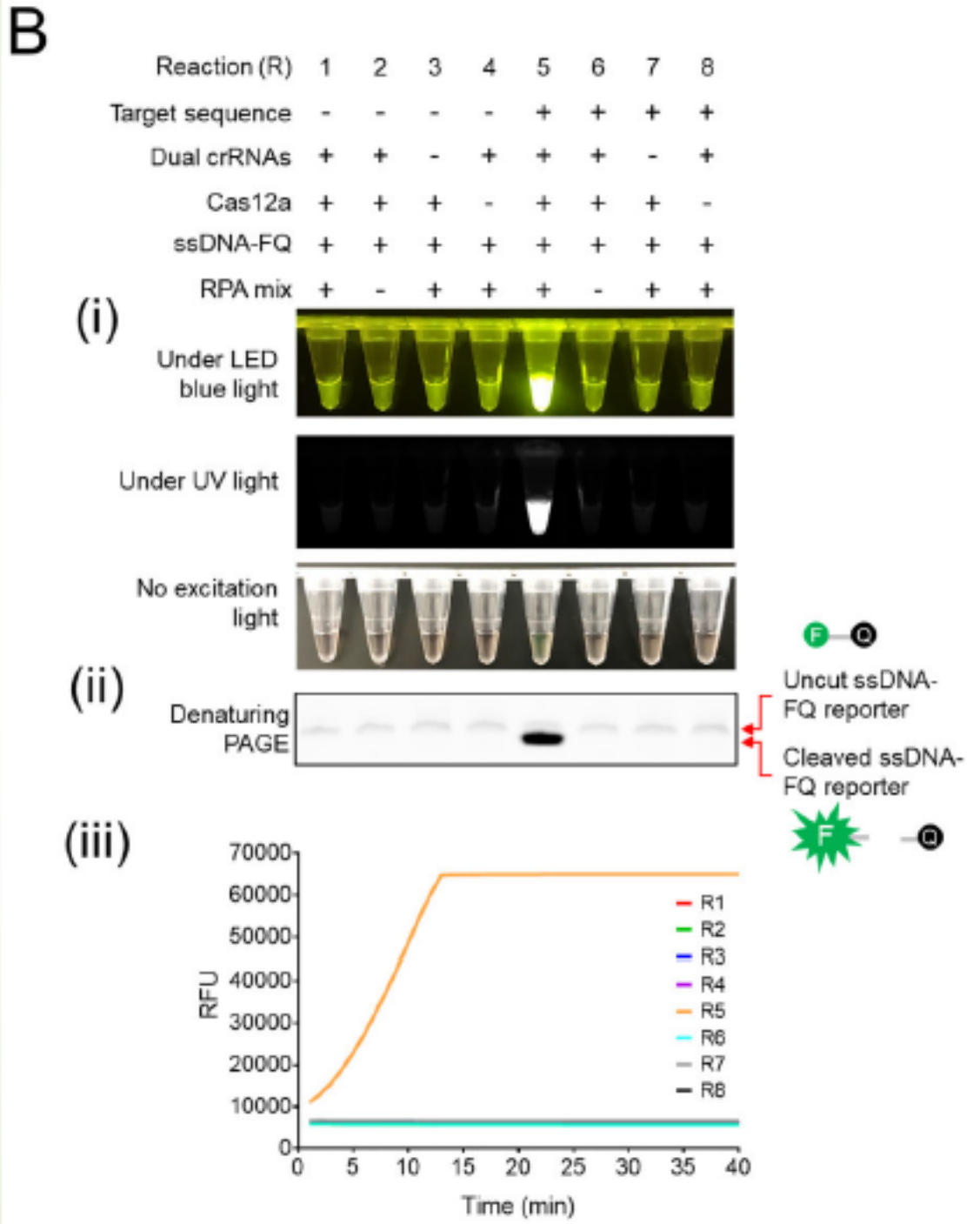
AIOD-CRISPR assay system. As shown in **Figure 1A**, the AIOD-CRISPR assay system used a pair of Cas12a-crRNA complexes generated by two individual crRNAs to bind two corresponding sites which are close to the recognition sites of primers in the target sequence. The Cas12a-crRNA complexes were separately prepared prior to being loaded into the solution containing two RPA primers, ssDNA-FQ reporters, recombinase, single-stranded DNA binding protein (SSB), strand-displacement DNA polymerase, and target sequences.

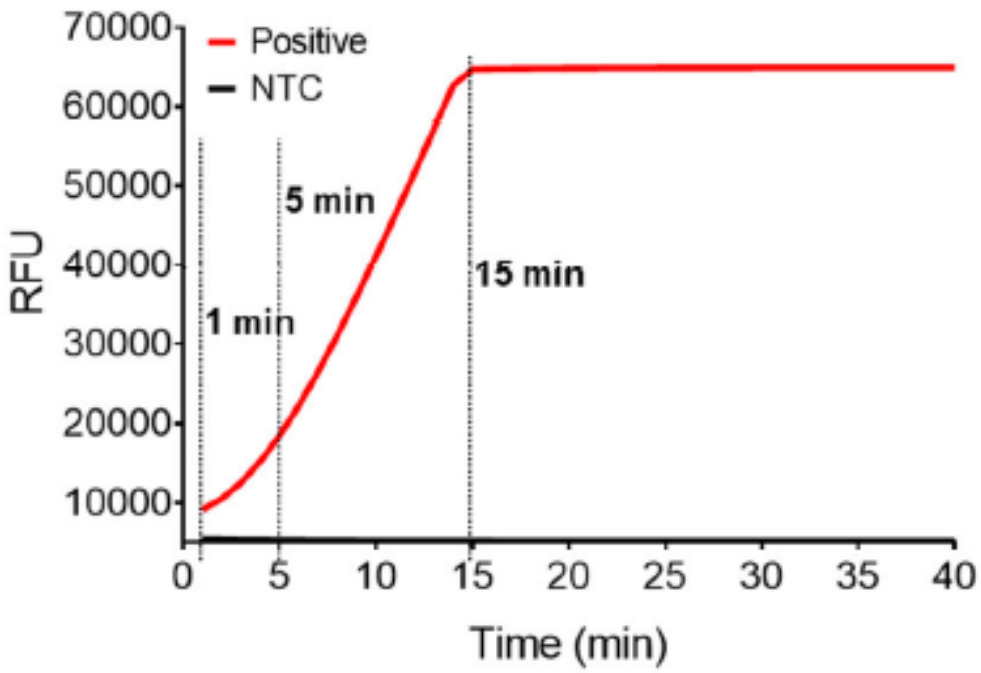
When incubating the AIOD-CRISPR system in one pot at 37°C, RPA amplification is first initiated and exposes the binding sites of the Cas12a-crRNA complexes due to the strand displacement. Once the Cas12a-crRNA complexes bind the target sites, Cas12a endonuclease is activated and cleaves the nearby ssDNA-FQ reporters to produce fluorescence. Similarly, RPA amplified products are also the substrates to undertake the same process and continuously trigger CRISPR-Cas12a-based collateral cleavage activity.


A
Forward Cas12a-crRNA complex
Forward primer F
F T1
T2c Rc
Target sequence
5′
3′
3′
5′
Fc T1c
T2 R
R
Reverse Cas12a-crRNA complex
Reverse primer
Saturated ssDNA-FQ reporters
Recombinase
SSB
Polymerase
37 °C
All-in-one incubation
F
Q

Previous study has demonstrated that this collateral cleavage activity is irrelevant to target strand cleavage in CRISPR-Cas12a system. Therefore, target sequences for the AIOD CRISPR assay are not limited by the Cas12a's protospacer adjacent motif (PAM).

To evaluate and develop AIOD-CRISPR assay, (**Figure 1B (i)**): prepared and tested eight reaction systems (Reactions # 1-8) with various components. **Figure 1B (ii)**: The assay products were verified by denaturing polyacrylamide gel electrophoresis (PAGE). (**Figure 1B(iii)**): in real-time fluorescence detection, only the reaction # 5 showed an elevated fluorescence signals that saturated within 13 min.





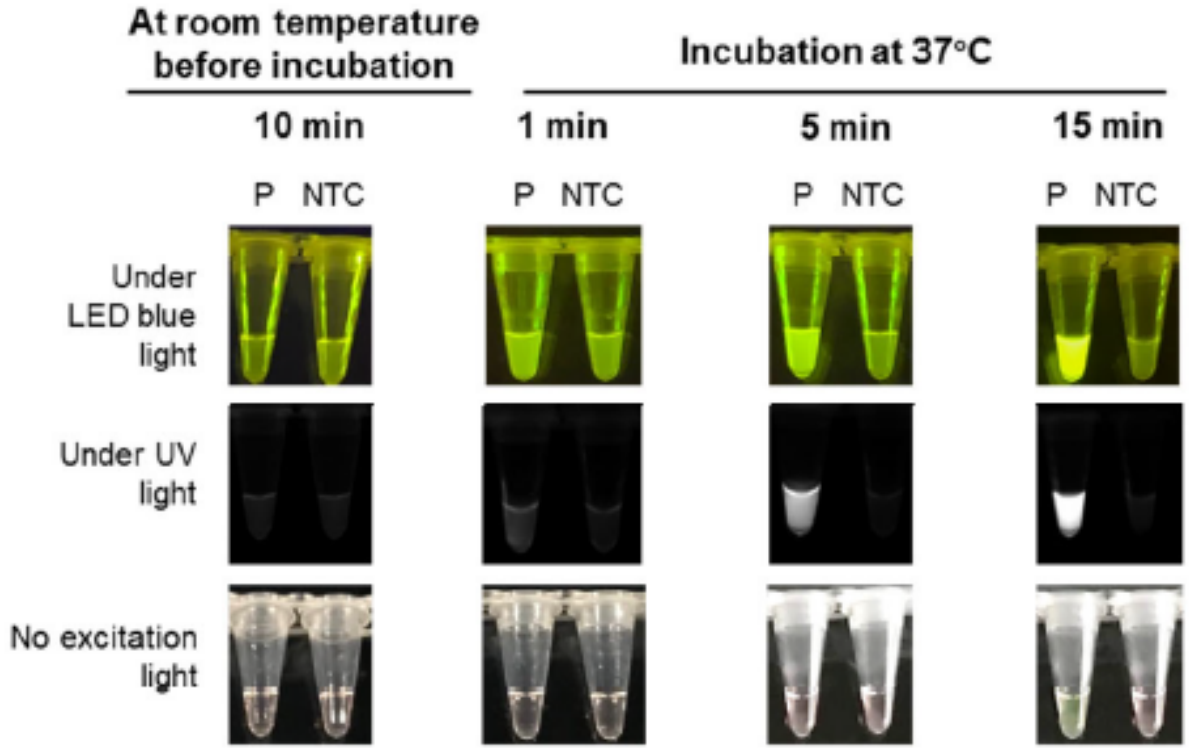


Figure S1. The AIOD-CRISPR assay at room temperature for 10 min before incubation and incubated at 37°C for 1, 5, and 15 min. Positive (P), the reaction with 1.2× 105 copies of HIV-1 p24 plasmids. NTC, non-target control reaction.

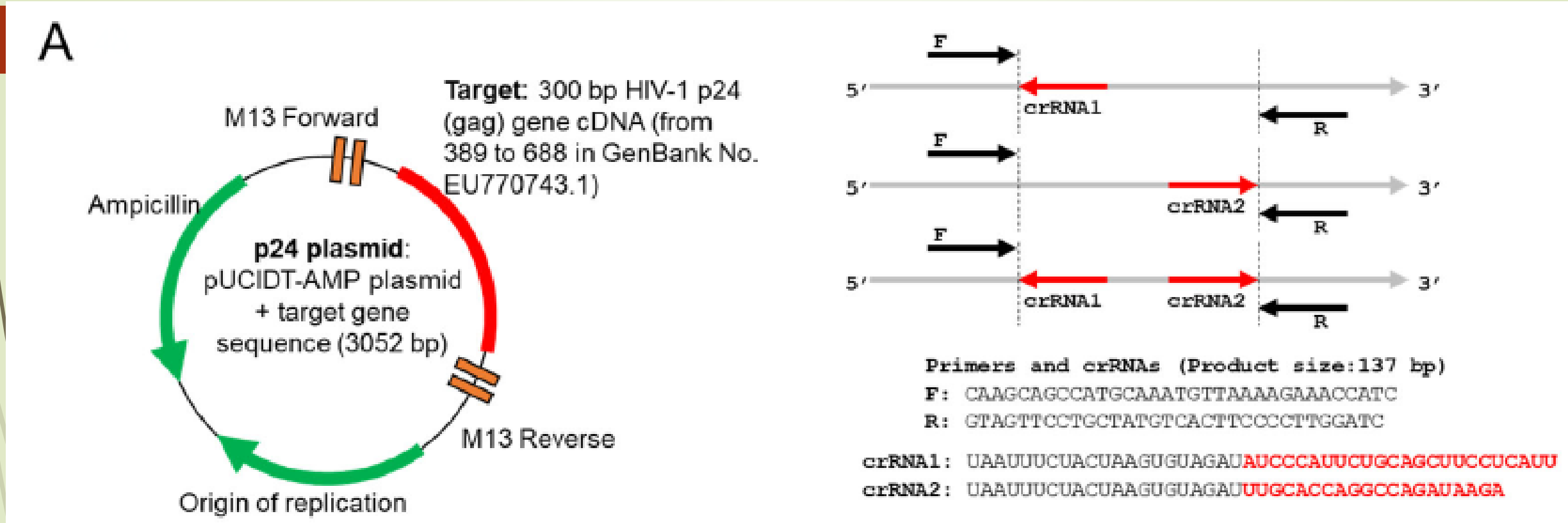


Figure 2. Comparison of the all-in-one CRISPR-Cas12a assay using dual crRNAs or single crRNA. (A) The pUCIDT-AMP plasmid containing 300 bp HIV-1 p24 gene cDNA (p24 plasmid) and the sequences of its primers and crRNAs.

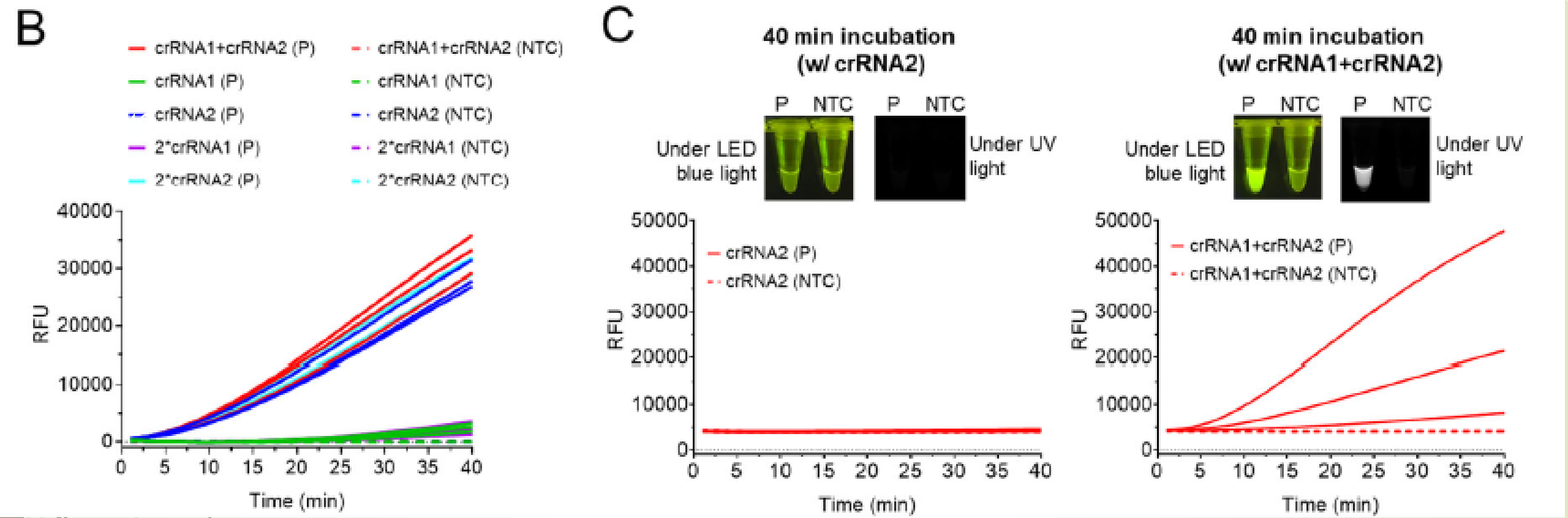


Figure 2. (B) Real-time fluorescence detection of the all-in-one CRISPR-Cas12a assay using dual crRNAs (crRNA1+crRNA2) or single crRNA (crRNA1/crRNA2). 2*crRNA1/2*crRNA2 means doubling its amount. P, the positive reaction with 1.2× 103 copies of HIV-1 p24 plasmids. (C) The CRISPR-Cas12a assays with dual crRNAs (crRNA1+crRNA2) or crRNA2 for the detection of 1.2 copies of HIV-1 p24 plasmids (P). Three replicates ran for each reaction with the plasmid. NTC, non-template control reaction. Each reaction contained 2 μM ssDNA-FQ. Fluorescence images were taken after 40 min incubation.

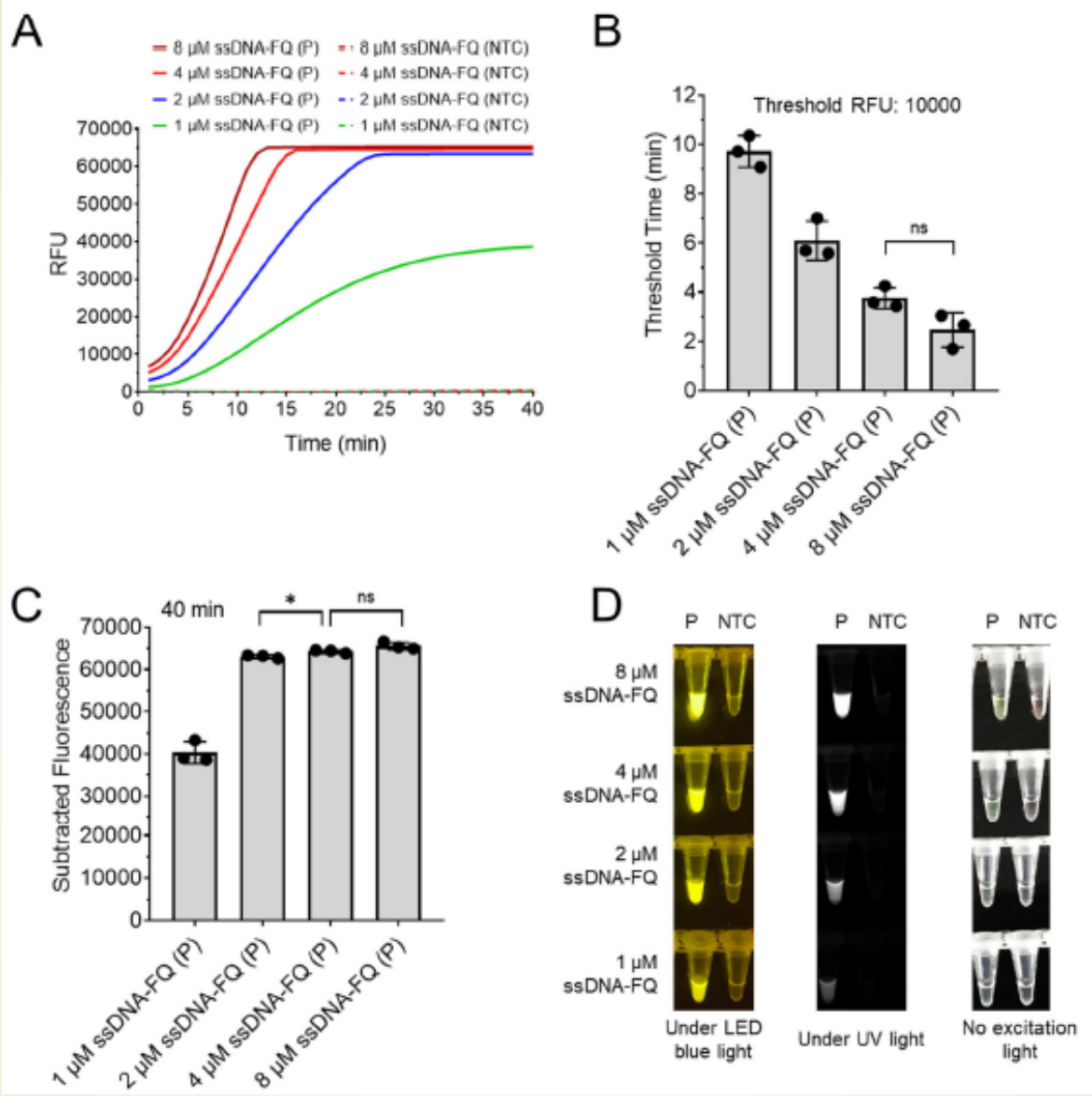
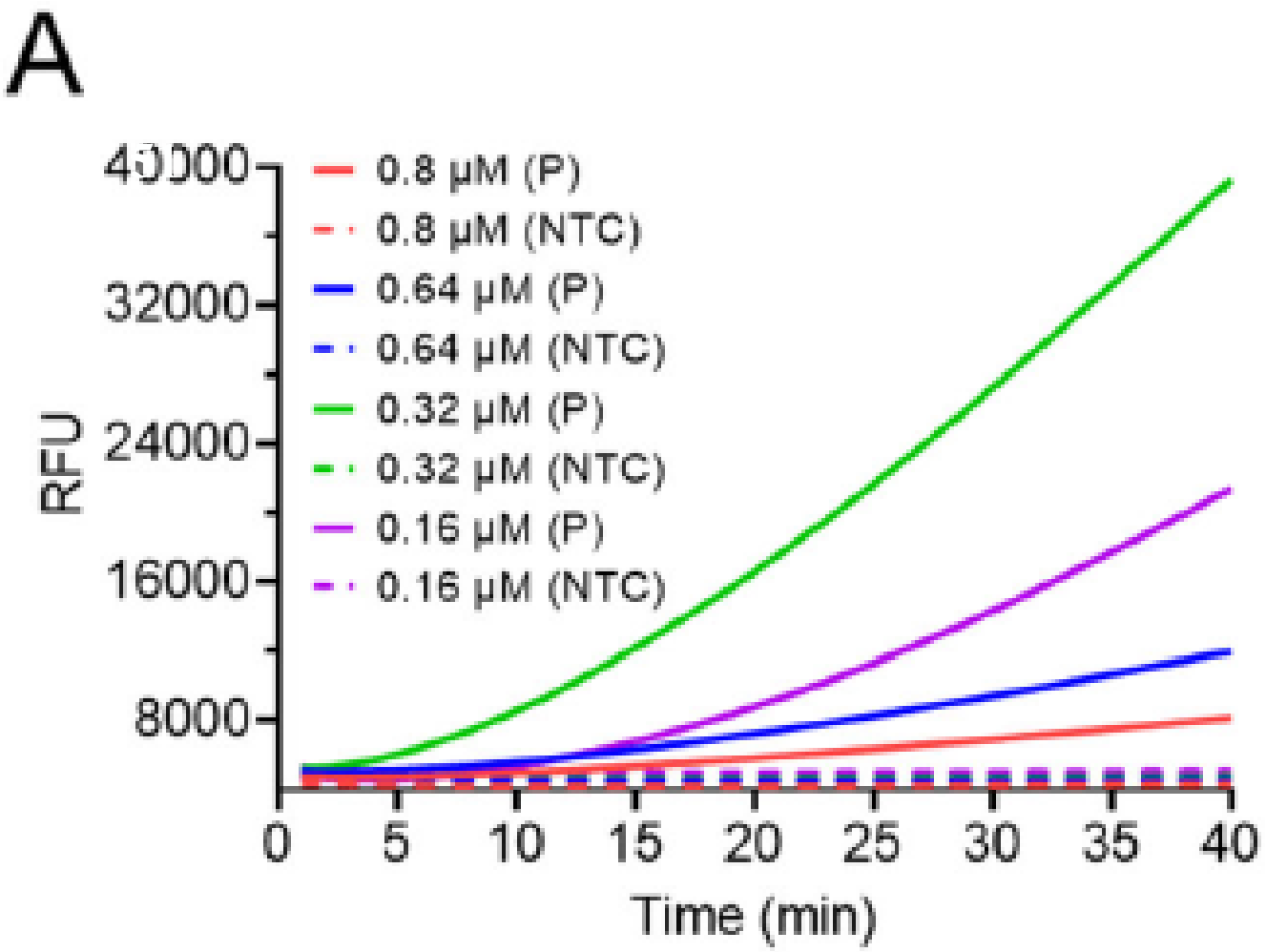


Figure S2. The AIOD-CRISPR assay with various concentrations of ssDNA-FQ reporters. (A) Realtime fluorescence detection. (B) Threshold time comparison. (C) Background-subtracted fluorescence comparison after 40 min incubation. (D) Visual detection comparison after 40 min incubation.



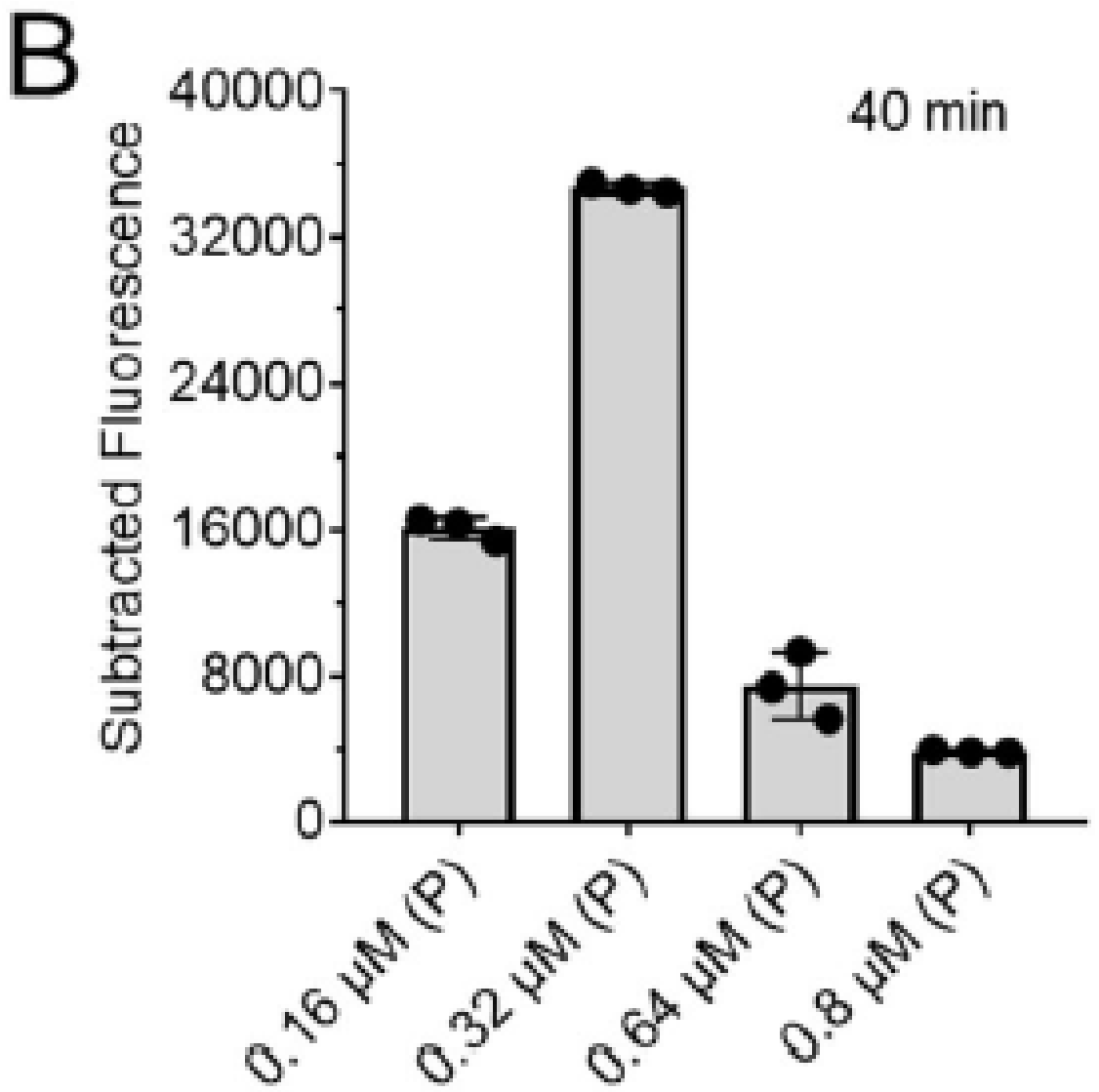


Figure S3. The AIOD-CRISPR assay with various concentrations of primers. (A) Real-time fluorescence detection. (C) Background-subtracted fluorescence comparison after 40 min incubation. P, the positive reaction with 1.2× 103 copies of HIV-1 p24 plasmids. Each reaction contained 2 μM ssDNA-FQ.

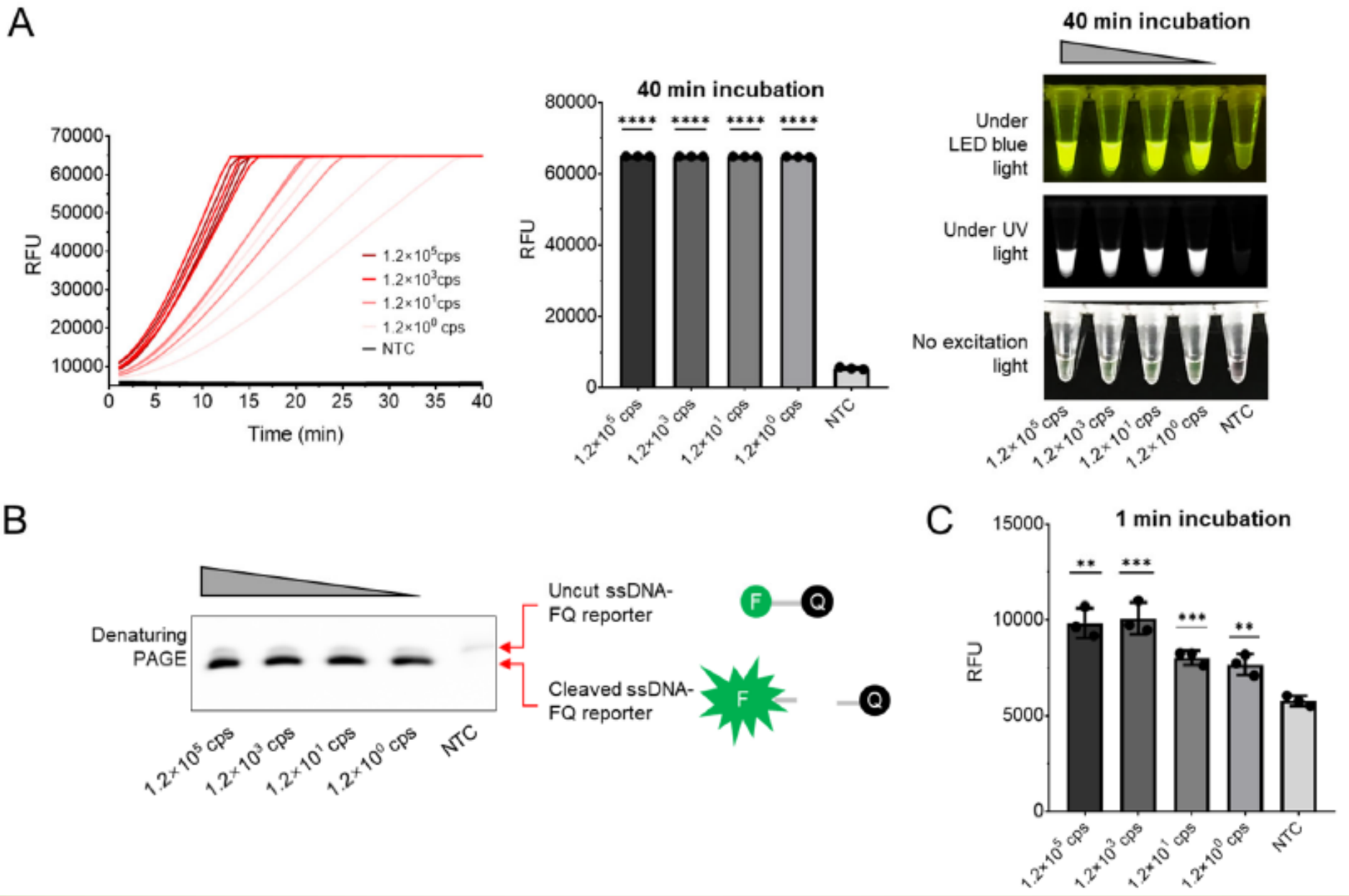


Figure 3. HIV-1 p24 plasmid (DNA) detection by the AIOD-CRISPR assay. (A) Real-time detection and endpoint fluorescence/visual detection. (B) Denaturing PAGE analysis of the AIOD-CRISPR products. (C) Endpoint fluorescence comparison after 1 min incubation at 37 °C.

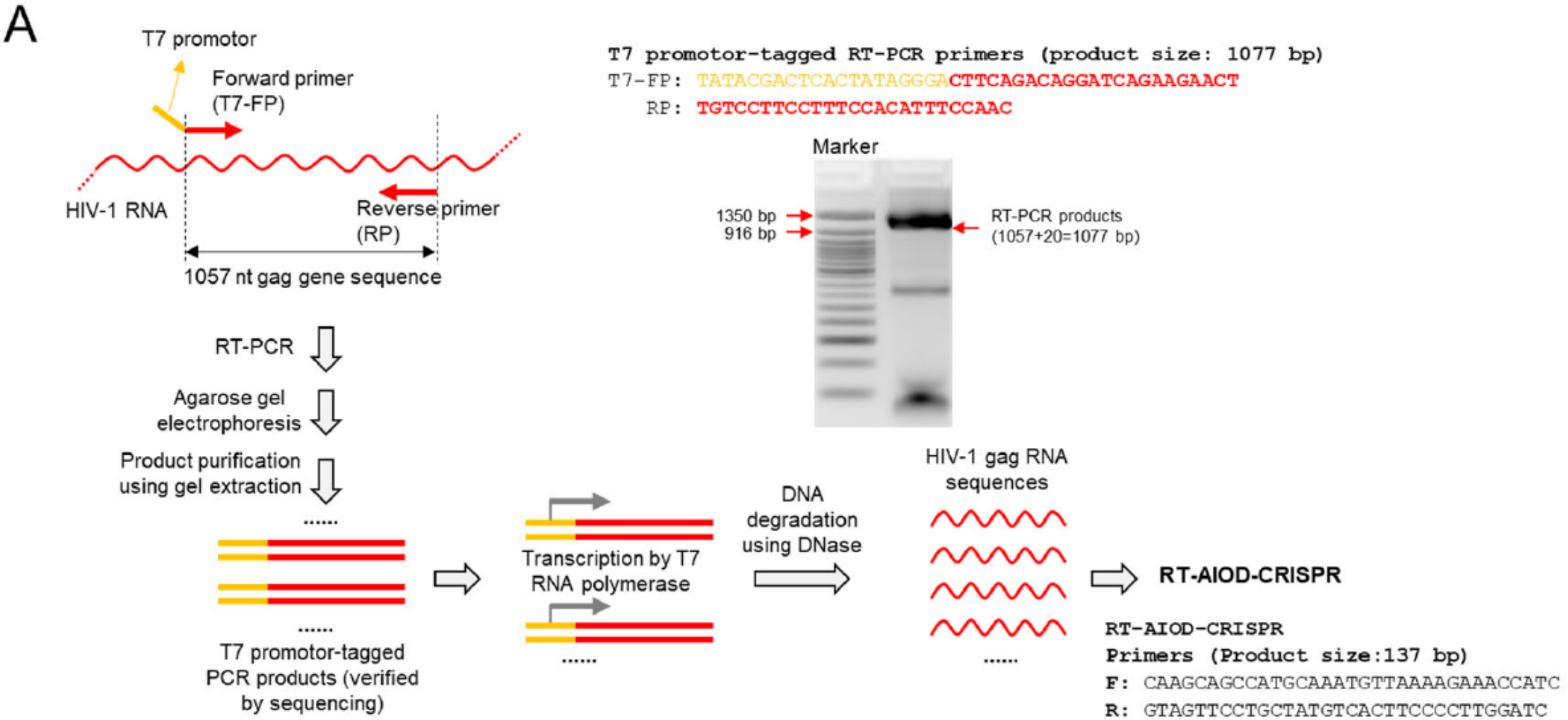
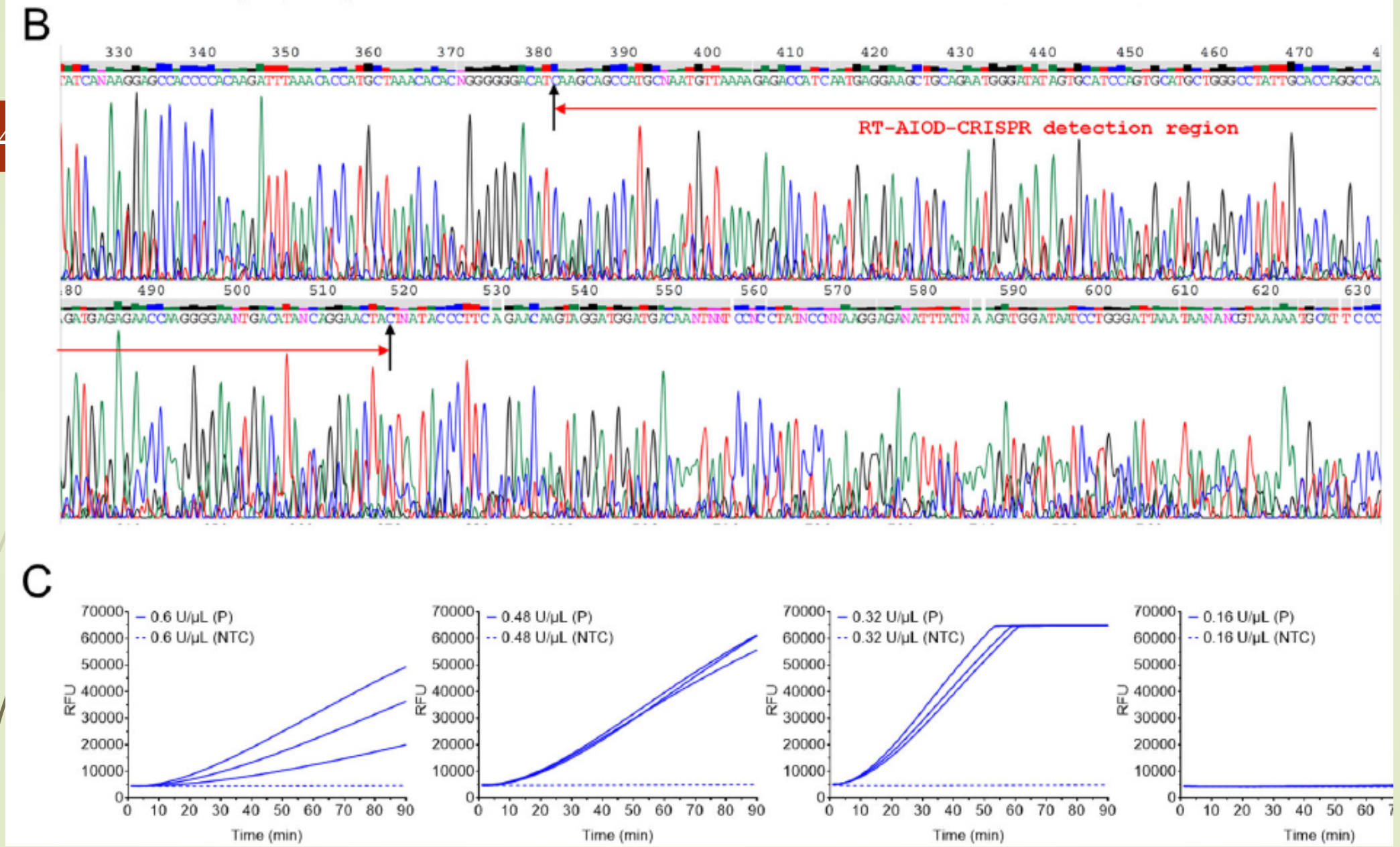


Figure S4. Application of AIOD-CRISPR assay to detect HIV-1 RNA sequence by adding Avian Myeloblastosis Virus (AMV) Reverse Transcriptase, namely reverse transcription AIOD-CRISPR (RT-AIOD-CRISPR). (A) Protocol and RT-PCR primers for preparing HIV-1 gag RNA sequences.



(B) Sanger sequencing of RT-AIOD-Cas12aR-based detection region in the prepared HIV-1 gag RNA. (C) The RT-AIOD-CRISPR assay using various concentrations of AMV reverse transcriptase.

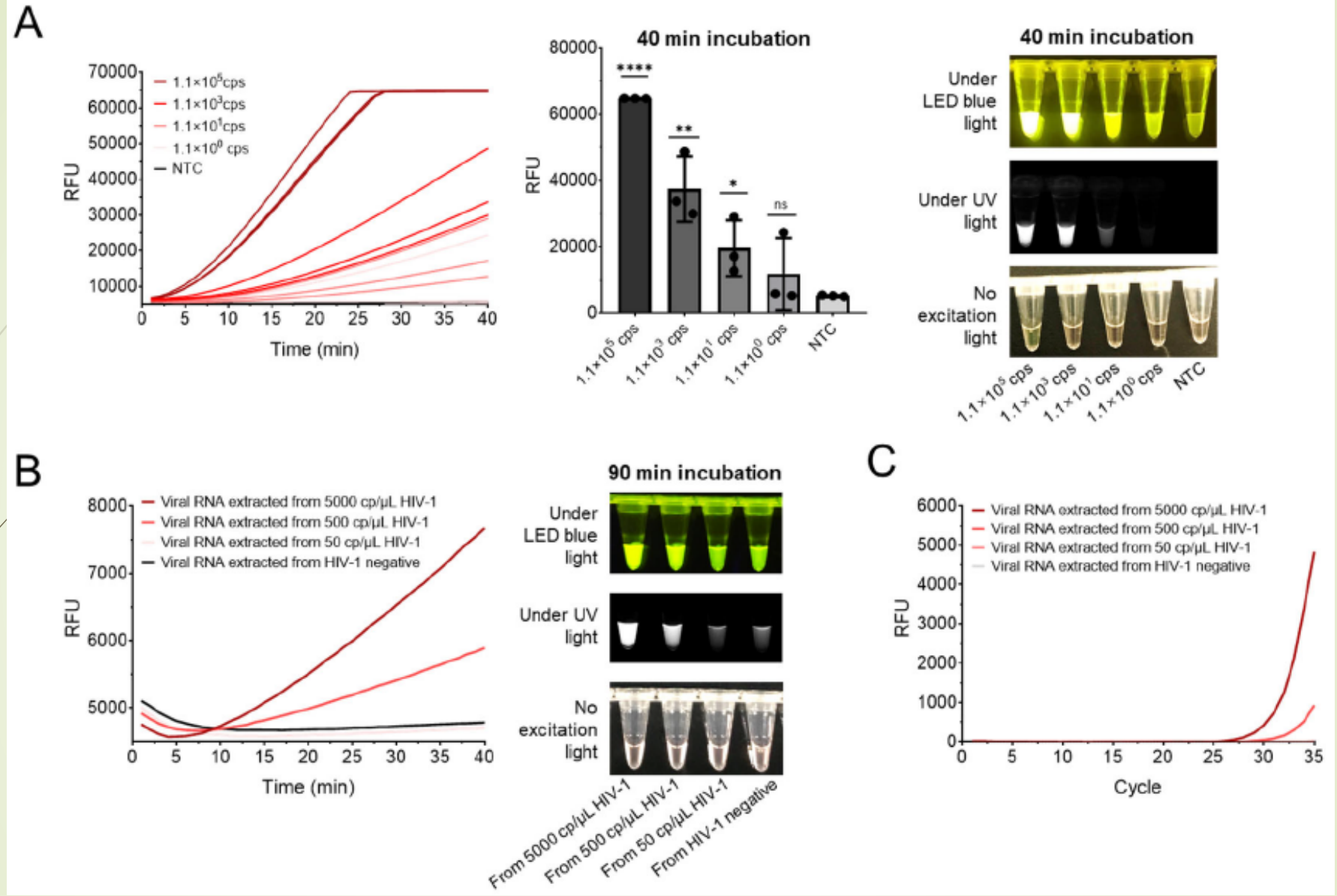


Figure 4. HIV-1 RNA detection by the RT-AIOD-CRISPR assay. (A) Sensitivity of real-time and endpoint fluorescence/visual detections. (B) Real-time and visual AIOD-CRISPR assays for the detection of HIV-1 RNA extracted from human plasma samples. (C) Real-time RT-PCR assay to detect HIV-1 RNA extracted from human plasma samples.

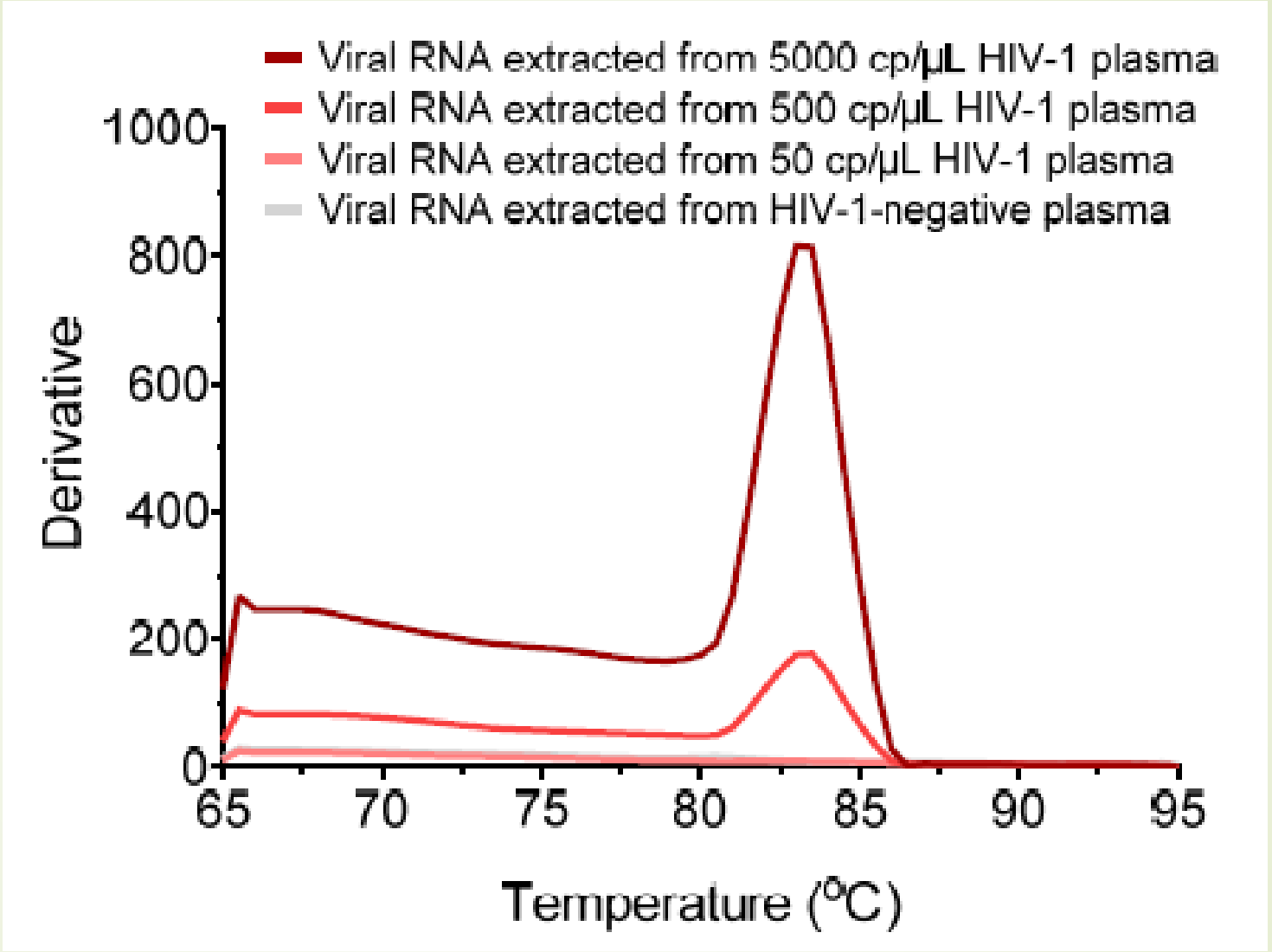


Figure S6. The melting curves for the OneStep RT-PCR products on detecting viral RNA extracted from various copies per microliter of HIV-1 plasma. The melting temperature of the products was about 83.5 °C.

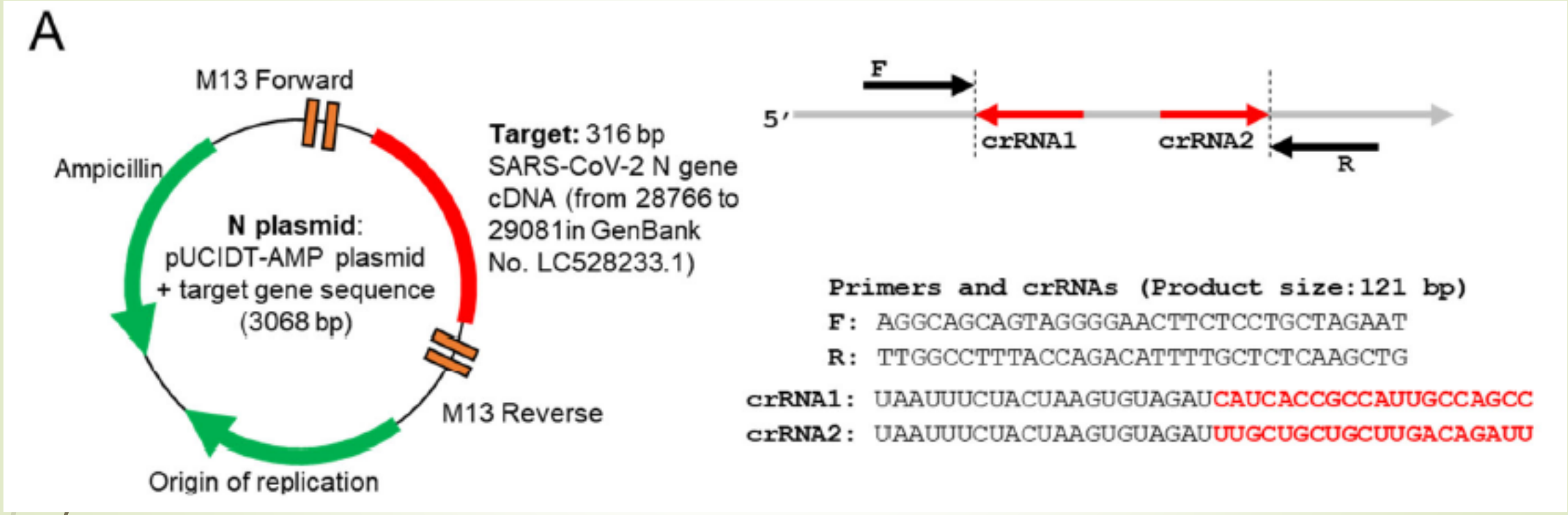


Figure 5. SARS-CoV-2 N DNA detection by the AIOD-CRISPR assay. (A) The pUCIDT-AMP plasmid containing 316 bp SARS-CoV-2 N gene cDNA (N plasmid) and the primers and crRNAs.

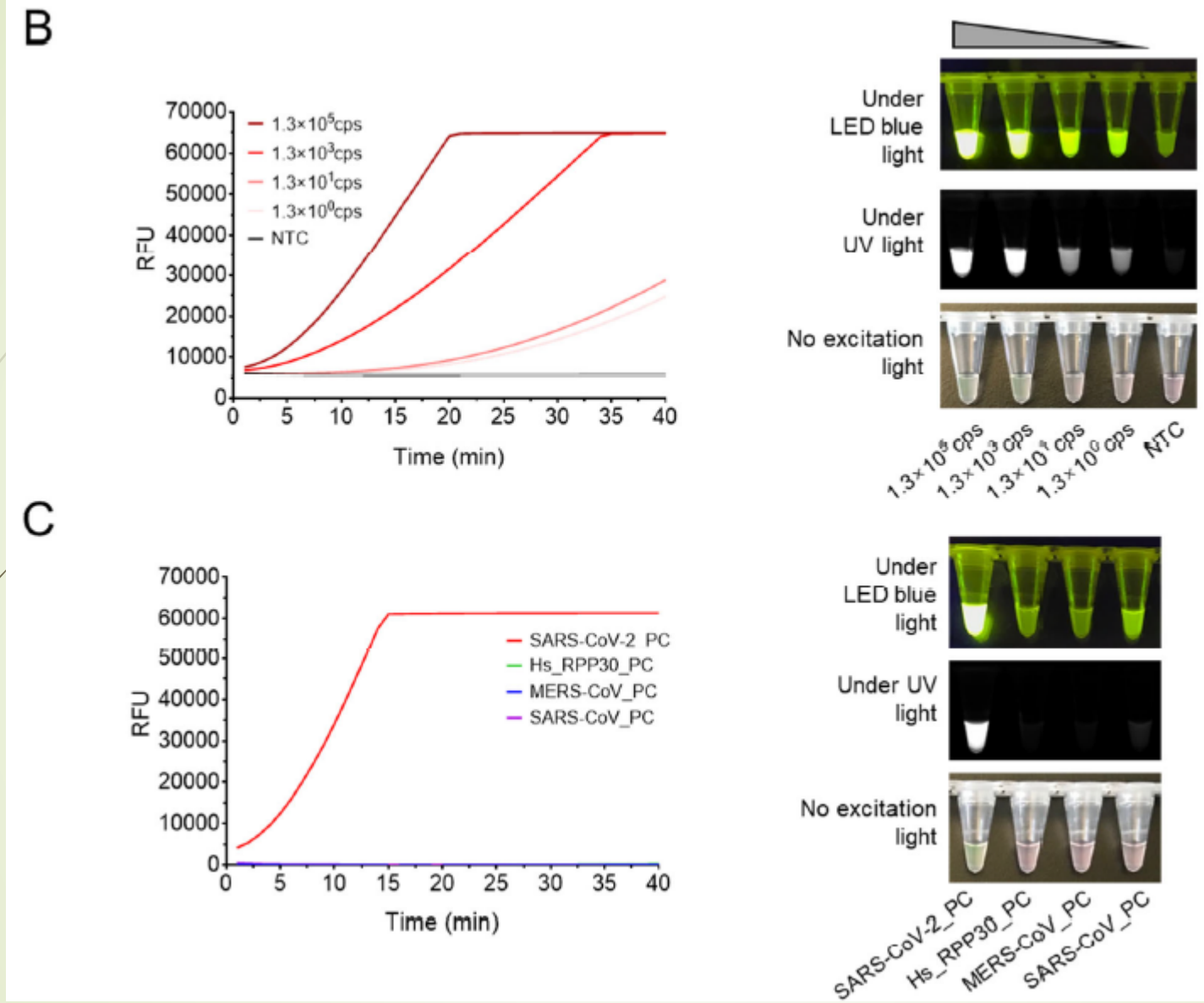


Figure 5. SARS-CoV-2 N DNA detection by the AIOD-CRISPR assay. (B) Real-time AIOD-CRISPR detection with various copies of SARS-CoV-2 N DNA. (C) Specificity assay of the AIOD-CRISPR assay on SARS-CoV-2 N detection. Tube images were taken after 40 min incubation.

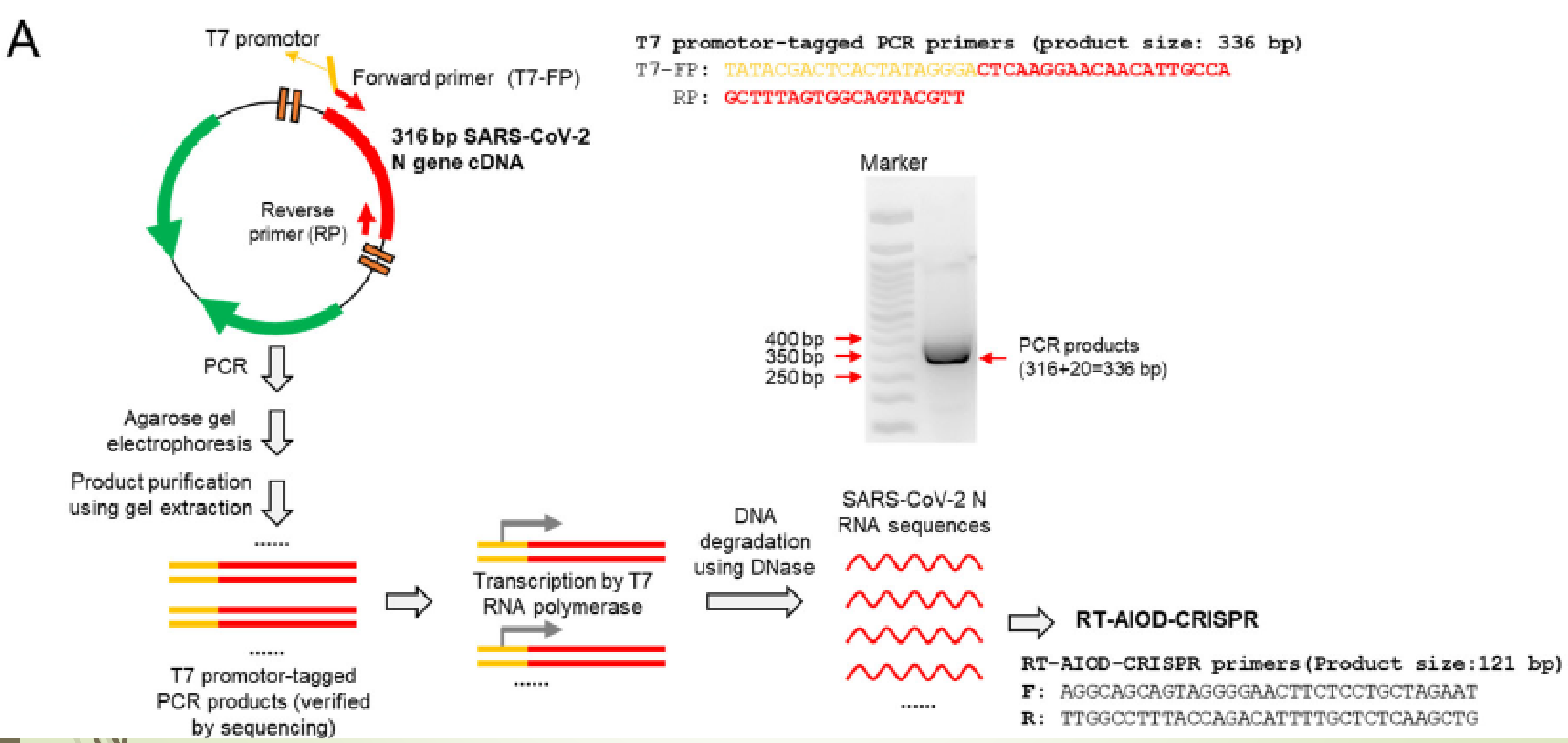


Figure 6. use of T7 promotor-tagged PCR and T7 RNA polymerase to prepare SARS-CoV-2 N gene RNA sequences to develop the RT-AIOD-CRISPR assay. (A) Protocol and PCR primers for preparing the SARS-CoV-2 N RNA sequences to detection by RT-AIOD-CRISPR assay.

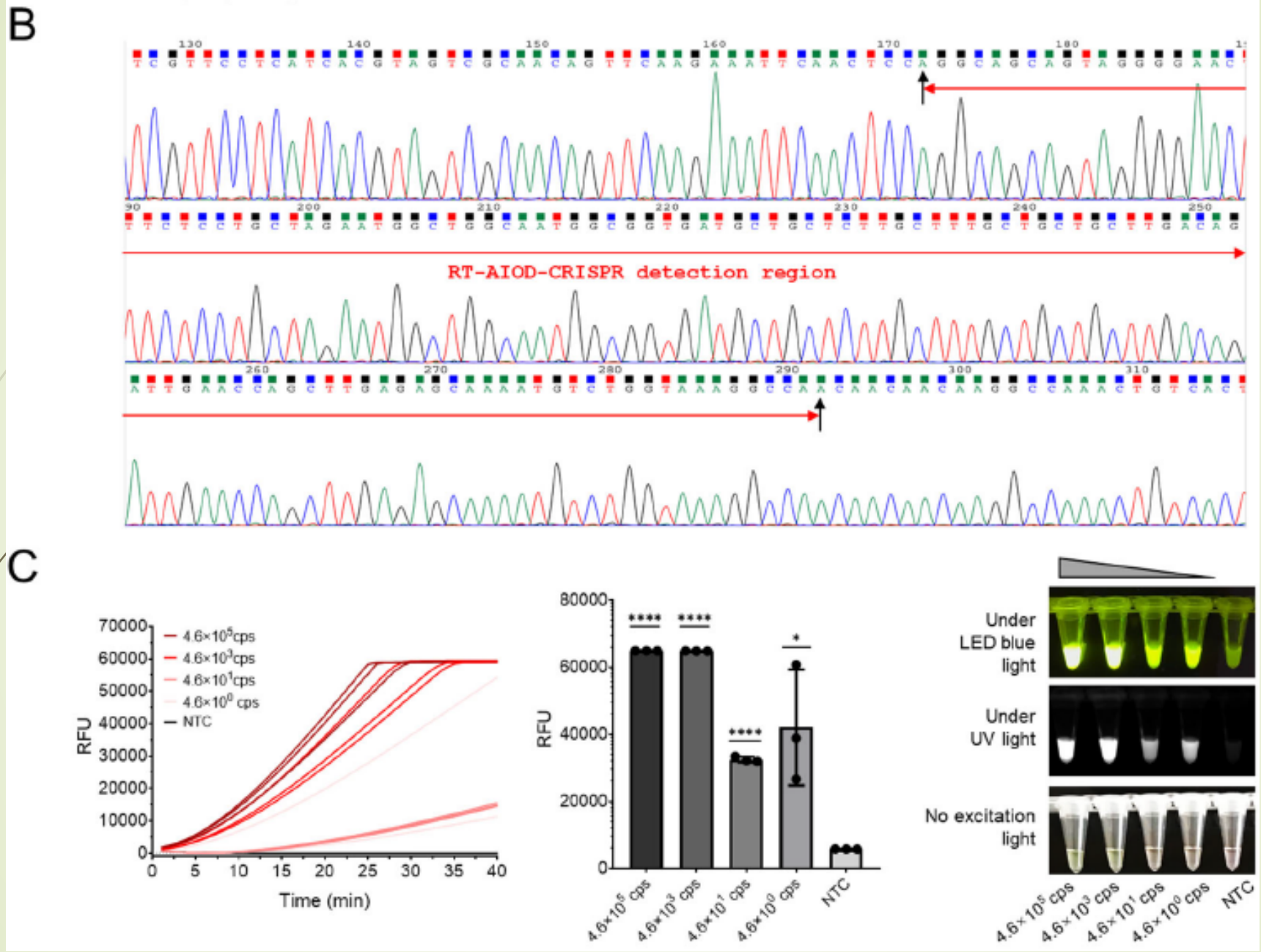


Figure 6. (B) Sanger sequencing of the AIOD-CRISPR detection region in the prepared SARS-CoV-2 N RNA. (C) Sensitivity of real-time and endpoint fluorescence/visual AIOD-CRISPR detections.

Discussion

In this study, we describe a new CRISPR-Cas12a reaction, AIOD-CRISPR in which all components are incubated in a single reaction system to rapidly, highly sensitively and specifically detect nucleic acids without separate pre-amplification steps. Upon the design of AIOD-CRISPR, dual crRNAs are introduced to improve the sensitivity and high-concentration ssDNA-FQ reporters are added to strengthen the detection signals.

In addition to real-time detection, AIOD-CRISPR assay is also beneficial for point-of-care detection because its detection results can be visually judged based on the fluorescence and color change of reaction solutions. As proof-of-concept assays, AIOD-CRISPR has been successfully developed for SARS-CoV-2 and HIV-1 detection.

Compared to previously reported CRISPR-based nucleic acid detection, our versatile and robust AIOD-CRISPR has some distinctive advantages. First, AIOD-CRISPR system is a true single reaction system. Second, AIOD-CRISPR is a true isothermal nucleic acid detection method. AIOD-CRISPR is conducted in one-step and one-pot format at one temperature, eliminating the need of expensive thermocycler in PCR methods.

Third, AIOD-CRISPR-based detection is very fast, robust, highly specific, and nearly single-molecule sensitive. In the application examples of HIV-1 and SARS-CoV-2 detections, our engineered AIOD-CRISPR without pre-amplification is able to detect as low as 1.2 copies DNA targets and 4.6 copies RNA targets in 40 min incubation.

For emerging SARS-CoV-2, in addition to high sensitivity, the AIOD-CRISPR also performs high detection specificity. Fourth, the AIOD-CRISPR enables one-step CRISPR-Cas12a-based RNA detection. By supplementing AMV reverse transcriptase, AIOD-CRISPR can be easily developed as one-step RT-AIOD-CRISPR to detect RNA targets such as HIV-1 and SARSCoV-2 RNAs, which facilitates the CRISPR-Cas12a-based RNA detection without the preparation of cDNA.

In summary, AIOD-CRISPR using a true single reaction system is a rapid, robust, highly specific, and nearly single-molecule sensitive isothermal nucleic acid detection method. This straightforward CRISPR-Cas12a nucleic acid sensing system has great potential in enabling CRISPR-based next-generation molecular diagnostics towards point-of-care, quantitation, and digital analysis.