Digital CRISPR-based quantification of HIV-1

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***Abstract*—Here, we demonstrate a membrane-based digital CRISPR-Cas13a system for amplification-free absolute quantification of viral particles of HIV-1. We established a stamping technique to digitalize the Cas13 assay inside a commercial track-etched polycarbonate (PCTE) membrane. We evaluated the performance of our system by quantifying the synthetic HIV-1 RNA, where a limit of detection of 100 aM was achieved in 30 min reaction. Absolute quantification of the viral particles HIV-1 in plasma background using our system confirmed that our system can quantify spiked samples.**

Keywords—HIV-1; Digital assay; Cas13a; CRISPR; Nucleic acid testing

I. Introduction

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) infection, a notorious fatal epidemic, has led to millions of deaths worldwide since its origin [1]. Early awareness of infection does enable not only timely treatment for exposed patients but also prevents further transmission [2]. Therefore, nucleic acid tests (NAT) such as real-time PCR (RT-PCR) that have been demonstrated to shorten the window period of AIDS than antigen/antibody tests hold tremendous promise in AIDS diagnosis [3].

While RT-PCR has been the gold standard for viral load quantification of HIV-1 RNA due to accessibility, external references are needed for quantification. Digital assays concepts have many potential advantages over real-time PCR, including obtaining absolute quantification without external references and robustness to variations in assay efficiency [4]. In addition, the recent discovery of the collateral cleavage in other Cas proteins like Cas12 and Cas13 made it possible to translate the sequence-specific targeting to other detectable signals, which has led to the increasing emergence of CRISPR-mediated biosensors [5, 6]. Recently, few groups have developed digital CRISPR assays where an amplification-free absolute quantification was obtained [7-9].

In this study, a membrane-based digital CRISPR-Cas13a system for amplification-free absolute quantification of viral particles HIV-1. We established a stamping technique to digitalize the Cas13 assay inside a commercial track-etched polycarbonate (PCTE) membrane. To optimize the Cas13 crRNA design, we initially designed five crRNAs along the HIV-1 genome and compared their cleavage speed (fluorescent signal formation speed). The quantitative performance of our system was evaluated by quantifying the synthetic HIV-1 RNA at different concentrations from 10 aM to 1 pM. Finally, we tested our system performance for quantifying viral particles of HIV-1 in the plasma background.

II. Working Principle

Fig. 1a depicts the working steps of the digital CRISPR-based system for HIV-1 RNA quantification. Prior to the digitalization of the assay, all components of the Cas13a reaction, including HIV-1 RNA, Cas13a and crRNA complex, fluorophore quencher (FQ)-labeled single-stranded RNA reporters, CRISPR buffer, RNase inhibitor, and pure water would be mixed. Subsequently, we utilized the track-etched membranes as a platform to digitize the assay. Decreasing the reaction volume from microliter-scale to picolitre-scale of membrane pores would increase the product (cleaved reporter) concentration by around 6 orders of magnitude. Therefore, we expect to reach a lower detection limit and improve the system's performance in a fixed reaction time.

Upon the specific RNA-guided target binding, the activated Cas13a proteins would perform trans-cleavage on the surrounding non-target reporters. The positive wells would be distinguished from the negative wells using the fluorescent intensity of the wells. In the last step, Poisson statistics would be utilized to quantify the number of HIV-1 RNA targets without external references.

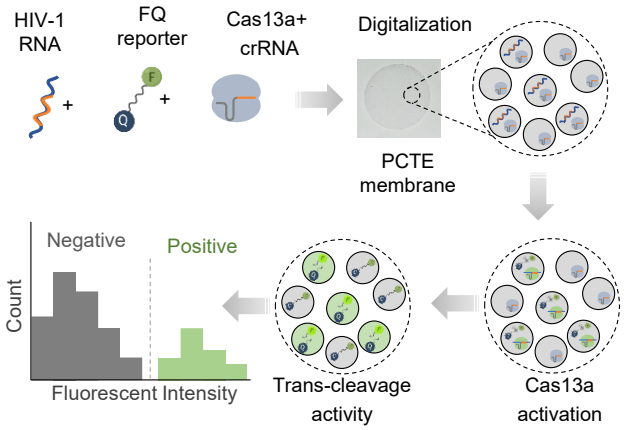

Fig.1. The overall workflow of the CRISPR-Cas13a-assisted HIV-1 quantification system.

III. Result and Discussion

A. Membrane Based Digitalization

In this work, a commercial track-etched polycarbonate (PCTE) membrane was utilized for digitalization. Fig. 2a shows a photograph of the commercial PCTE membrane (1.3 cm diameter), which is transparent and flexible. The membrane has a smooth surface and contains a high density of cylindrical pores with an average pore size of 25 ± 1 µm and pore density of 10^4 pores/cm^2, as confirmed in Fig. 2b and c. For some commercial PCTE membranes with polyvinylpyrrolidone (PVP) coating, this hydrophilic

coating needs to be removed since it affects the removal of the excess assay from the membrane.

In order to fill the membrane reliably, we developed a stamping technique (Fig. 2d). The membrane was sandwiched between a polymethyl methacrylate (PMMA) holder and double-sided tape (stamp system). Afterward, the assay solution was dropped on top of a glass surface, and the stamp was placed on top of it. After 60 seconds, we sealed the top surface of the membrane by adding mineral oil and removing the stamp from the glass to remove the excess liquid at the bottom. Afterward, the stamp system was placed on top of the system base, which consisted of a glass substrate and a double-sided tape filled with mineral oil to seal the bottom side of the membrane.

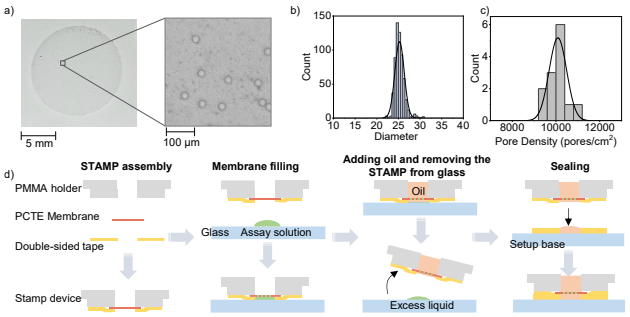

Fig.2. a) Images of the commercial PCTE membranes. b) Pore size distribution. c) Pore density distribution. d) Filling process using a stamping technique.

B. *HIV-1 CAS13 crRNA design and optimization*

To optimize the Cas13 crRNA design, we initially designed five crRNAs along the HIV-1 genome (Fig. 3a). Also, we synthesized five 100 nucleotides target to cover each designed crRNA. Fig. 3b shows the fluorescent intensity over 60 minutes of Cas13 reactions. To validate the specificity of the assay, we cross-react the crRNAs and target RNAs in 25 samples. In addition, we tested each crRNA without any target in the assay (Negative control cases) in 5 samples. Fig. 3b shows the fluorescent intensity over 60 minutes of Cas13 reactions. Only in cases where targets and crRNAs were matched (positive cases) an increase in fluorescent intensity was detected, confirming our assay's specificity. In addition, crRNA1 and 4 showed the highest trans-cleavage activity among the cases where the higher fluorescent intensity was observed from the 60 minutes reaction. Therefore, we selected crRNA1 and 4 for the following steps of our study. In the next phase, the sensitivity of the bulk Cas13 assay using crRNA1 and 4 was measured by changing the target concentration from 1 to 100 pM. In each case, three no-target control (NTC) cases were tested to determine the background fluorescent intensity as $\mu_{NTC}+2\sigma_{NTC}$, where μ_{NTC} and σ_{NTC} are the averages and standard deviation of the NTC cases, respectively. Cas 13 assay using crRNA1 showed higher sensitivity where the limit of detection of 20 pM was measured compared to 100 pM in the crRNA4 assay (Fig. 3c). It is noteworthy that this result is comparable with the amplification-free Sherlock method, where a limit of detection of ~50 pM was achieved [5]. To further compare the performance of the Cas13a assay using crRNA 1 and 4, we performed a Michaelis-Menten kinetic study on the system using crRNA1, and 4. Fig. 3d presents the cleavage speed of the reaction at different FQ reporter concentrations to obtain K_M (Michaelis constant.) k_{cat} (catalytic rate). For the reaction using crRNA 1 and 4, the catalytic rate of 29.49 and 60.32 1/s were measured, respectively. Therefore, crRNA1 was chosen for our digital assay since it showed a higher trans cleavage rate.

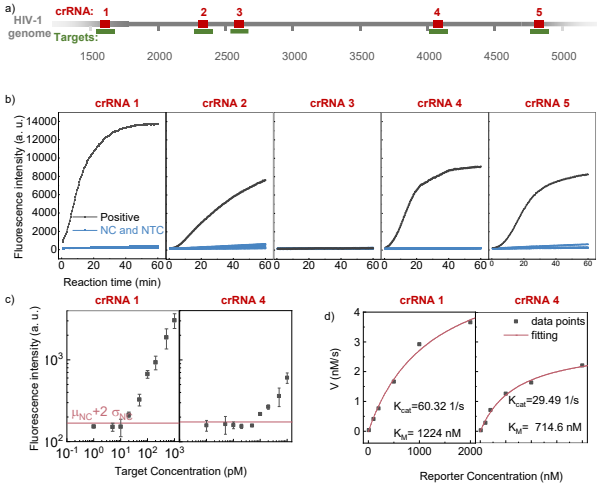

Fig.3. a) Schematic of the HIV-1 genome and the location of each crRNA spacer and the target region. b) Fluorescence intensity values over 60 minutes for 5 different crRNA and their corresponding targets (positive), no-target (NTC), and negative control (NC) samples. c) Sensitivity test of CRISPR assay using crRNA 1 and crRNA 4. d) Michaelis-Menten kinetic study of the Cas13a assay using crRNA1 and 4.

C. *Reaction Time effect on the measurement*

As reaction time increases, more reporters would be degraded in the positive wells, and consequently, the higher fluorescent intensity would be detected. Therefore, reaction times would affect the detectable positive samples. To obtain the optimal reaction time for our system, we measured the positive wells ratio (positive well/total number of wells) at different reaction times for a Cas13a assay containing 5 fM HIV-1 synthetic RNA. Fig. 4a presents the fluorescence images of part of the membrane at different reaction times. As time increased to 30 minutes, more positive wells were detected. However, after 30 minutes, the ratio of the positive wells becomes stable. Therefore, we chose 30 minutes reaction in our experiments to detect the positive wells more accurately and efficiently.

D. *Absolute quantification validation using synthesized HIV-1 RNA*

To test the quantitative performance of our system, series of synthetic HIV-1 RNA dilutions from 10 aM to 1pM were quantified. In each case, the positive wells ratio was measured at 30 minutes reaction (Fig. 5a). We also measured 4 no-target control cases to obtain the background noise of our system. Fig. 5a also demonstrates the limitations of absolute quantification at high (more than 1 pM) and low (less than 50 aM) concentrations. In cases where the concertation is higher than 1 pM, the positive well ratio would saturate at 1, and absolute quantification is unfeasible. To solve this problem, the high concentrated samples could be diluted to the dynamic range of the system (50 aM to 1 pM). For a lower concentration than 50

aM, the background noise of the system would interfere with the quantification, and accurate measurement could not be achieved.

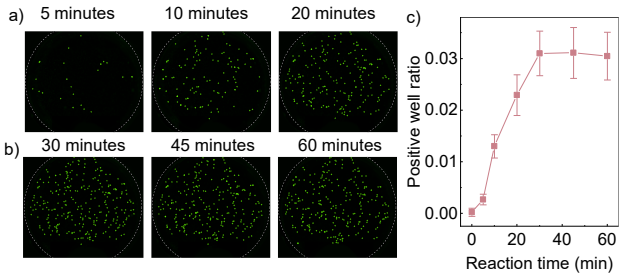

Fig.4. a) Floursent images illustrating the positive and negative wells at different reaction times from 0 to 60 minutes. b) The proportion of positive wells at different reaction times.

In the next step, Poisson statistics were utilized to quantify the target concentrations as $C = -ln\,(1-p)/V_d$ Where p is the positive well ratio, and V_d is the average volume of each well (13 pL). Fig. 5b presents the measured concentrations for each case. In the dynamic range (50 aM to 1pM), the measured concentrations correspond very well to the expected concentrations (R^2= 0.998), confirming our system's quantitative performance.

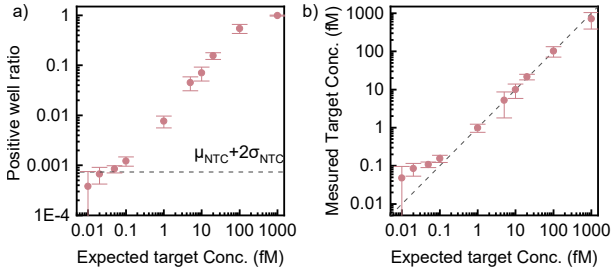

Fig.5. a) The measured positive well ratios at different HIV-1 RNA concentrations from 10 aM to 1 PM. The dashed line represents the background noise b) Comparison of measured RNA concentrations to the expected concentrations.

E. Test with spiked plasma samples

In the next step, we evaluated the system performance for quantifying HIV RNAs in a plasma background. Spiked plasma samples were prepared by adding serially diluted synthesized HIV-1 RNA (3000, 2000, and 1000 copies) into the 1 ml of healthy plasma samples. Fig. 6a illustrates the steps for quantifying the spiked samples. A column-based extraction process (QIAMP viral RNA Mini Kit) was performed prior to the digital assay. Afterward, the extracted HIV-1 RNAs were quantified using our membrane-based digital assay. We prepared two batches for each concentration, followed by one no-target control sample (7 samples in total). Fig. 6b shows the measured concentration for the 7 cases. We observed two points. First, the average relative error for 1000, 2000, and 3000 (copies/ml of plasma) quantification was measured as 0.22, 0.06, and 0.02, respectively, which confirms the capability of our system for absolute quantification of spiked samples. Second, this result confirmed that our system could differentiate between spiked samples with a resolution of 1000 (copies/ml of plasma) where the average measured concentration for 1000, 2000, and 3000 (copies/ml of plasma) were 1223, 2128, and 2935.5 (copies/ml of plasma).

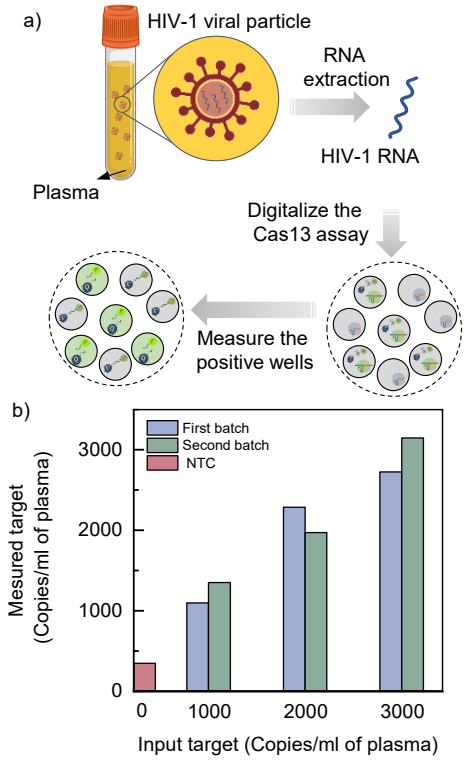

Fig.6. a) Workflow for direct HIV-1 testing in spiked plasma samples. b) Measured concentration for serially diluted spiked HIV-1 RNA samples and one no-target control sample.

IV. CONCLUSION

In conclusion, we presented a membrane-based digital CRISPR-Cas13a system for amplification-free absolute quantification of viral particles HIV-1. To fill the PCTE membranes readily, we developed a stamp device by sandwiching the membrane with double-sided tape and a PMMA handle. Five crRNA were designed to target the HIV-1 RNA, and crRNA1, which showed the highest trans cleavage rate, was chosen for the digital assay. The result of the reaction time effect on the digital assay showed that 30 minutes reaction is the optimal reaction time to obtain the fluorescent images. The quantitative performance of our system was confirmed when a series of synthetic HIV-1 RNA was quantified accurately from 50 aM to 1 pM. Finally, a test with spiked samples showed that our system is capable of quantifying HIV-1 RNAs with the resolution of 1000 copies per 1 ml of plasma.

V. ACKNOWLEDGMENT

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