Supplementary Information for

STAMP-Based Digital CRISPR-Cas13a (STAMP-dCRISPR) for amplification-Free Quantification of HIV-1 Plasma Viral Load

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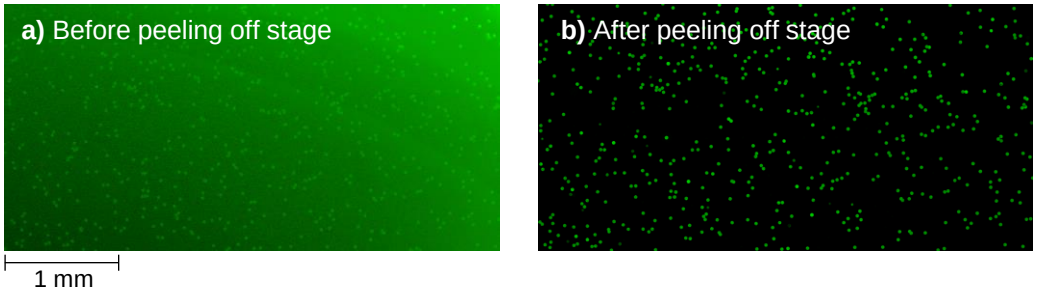


Figure S1. Effect of peeling-off stage on the excess sample removal process. Fluorescent images of the membrane a) before and b) after the peeling-off stage of the stamping process. These images confirmed the excess sample removal process in the peeling-off stage.

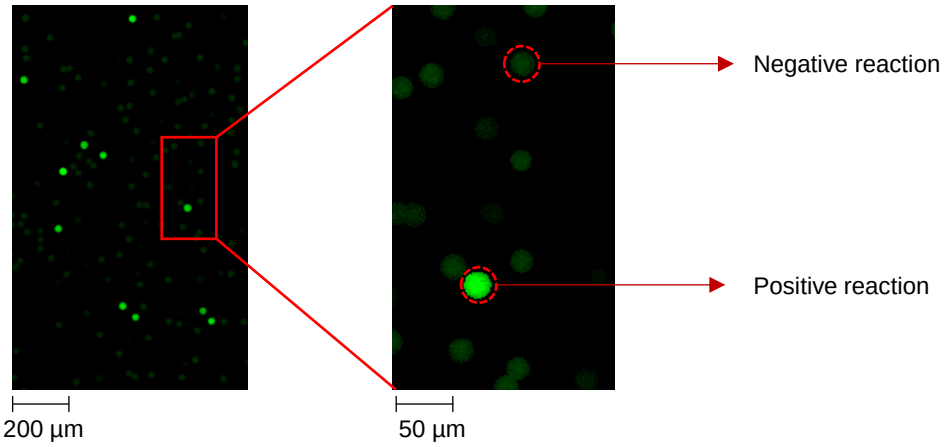


Figure S2. Typical fluorescent signals from a negative and positive reaction. Fluorescent image of part of a membrane demonstrating typical fluorescent signals from a negative and positive reaction. The negative reaction containing the unquenched FAM reporters also shows a weak fluorescence signal in our fluorescent images.

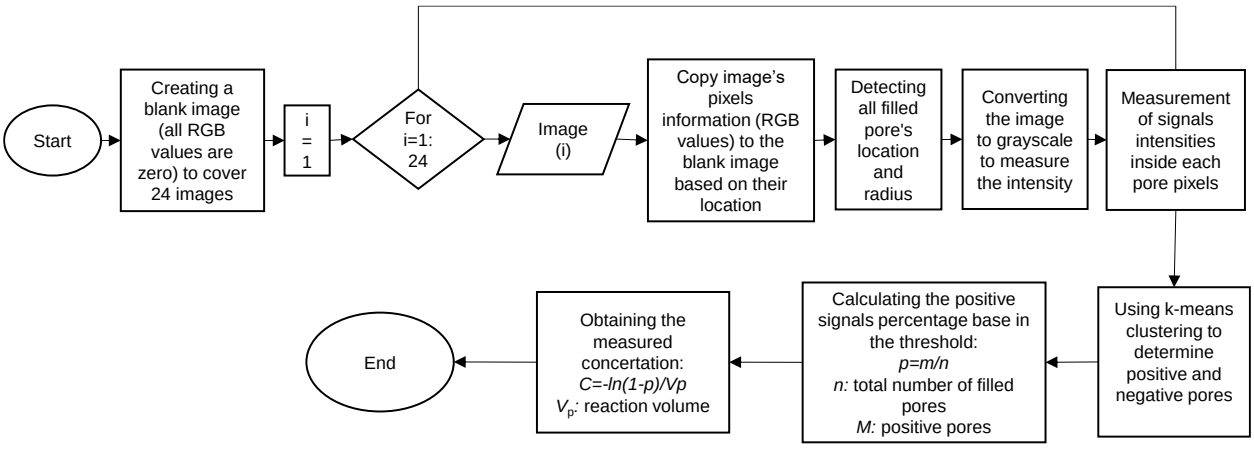


Figure S3. Algorithm for acquiring the fluorescent intensity and stitching of the images. The algorithm runs through 24 images and identifies the pixels inside the filled pores in each case. Afterward, the fluorescent intensity of each pore is calculated by combining the intensity of the pixels inside the pores. After examining all 24 images, a *k*-means algorithm would classify the positive and negative pores based on the fluorescent intensity. k-means clustering is a method that aims to partition n observations (n pores in our case) into k clusters (2 clusters in our case, positive and negative). Our k-means algorithm uses an iterative refinement technique.

Given an initial set of *k* means $m_1^{(1)},\dots m_k^{(1)}$ (In our particular case, we know there are two populations after each reaction, therefore k=2), the algorithm proceeds by alternating between two steps:

Step 1-Assignment: Assign each observation to the cluster with the nearest mean. Mathematically, this means partitioning the observations to *k* cluster.

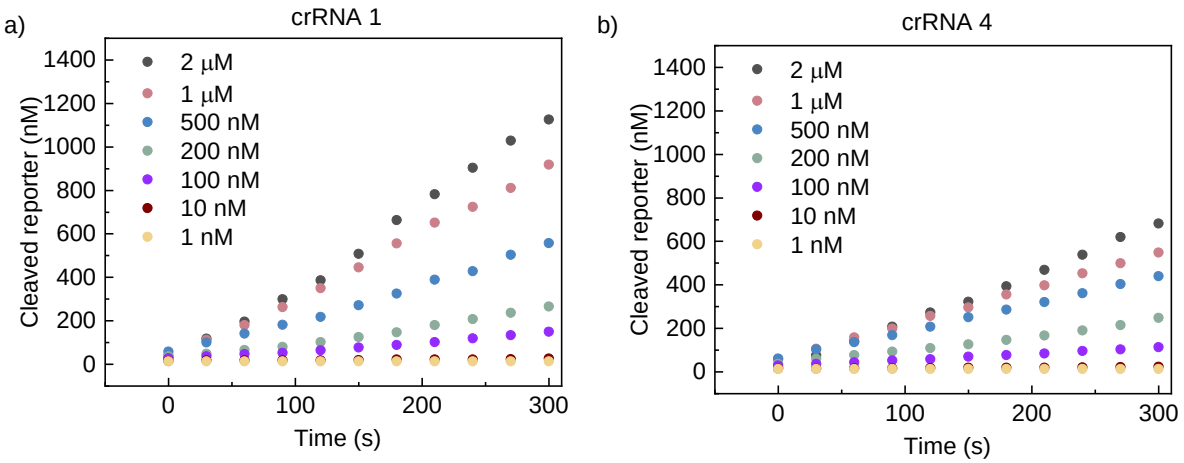
$$S_i^{(t)} = \left\{x_p: \left\|x_p - m_i^{(t)}\right\|^2 \le \left\|x_p - m_j^{(t)}\right\|^2 \forall j, 1 \le j \le k\right\} \qquad (1)$$

Step 2-Update: Recalculate means (centroids) for observations assigned to each cluster.

$$m_i^{(t+1)} = \frac{1}{\left|S_i^{(t)}\right|} \sum_{x_j \in S_i^{(t)}} x_j$$

The algorithm converged when the assignments no longer changed (thus the calculation is finished).

The concentration of the sample would be calculated using Poisson statistics. In the last step to visualize the whole image, a MATLAB code was implemented, which utilizes the coordinates of the motorized stage for each image to stitch all 24 images together.



c)

Reporter concentration (nM)	Cleavage Velocity using crRNA 1 (nM/s)	Cleavage Velocity using crRNA 4 (nM/s)
2000	3.657189	2.208361
1000	2.917791	1.634262
500	1.664232	1.261519
200	0.762344	0.709897
100	0.410205	0.283772
10	0.035589	0.028096

Figure S4. Cleavage velocity measurements using crRNA1 and 4. Measurements of cleaved reporters associated with the trans-cleavage activity of the CRISPR Cas13 proteins using a) crRNA 1 and b) crRNA 4. In each case, cleaved reporters were measured for 420 s to extract the cleavage speed (V (nM/s)). c) Details of measured cleavage speed of CRISPR Cas13 proteins using crRNA 1 and crRNA 4 at different reporter concentrations.

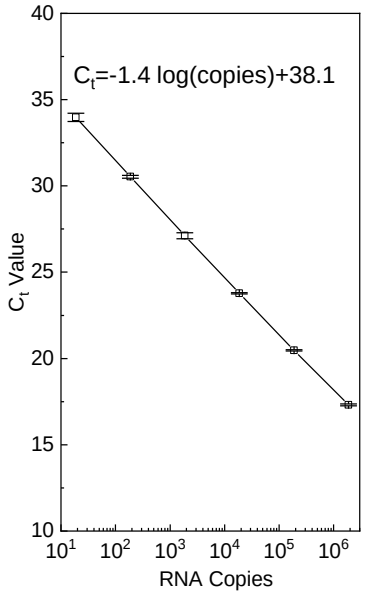


Figure S5. The C_t values obtained from the RT-PCR versus the target RNA copies. C_t values showed a logarithmic relationship with the sample concentration and verified our assay.

Supplementary Tables

Table S1. The total number of filled and positive pores and the measured positive pore ratio for 4 negative control cases. The average and standard deviation of the positive pore ratio in negative control cases were measured as 0.00033 and 0.0002, respectively. Therefore, our threshold for negative samples was set as $\mu_{NTC}+3\sigma_{NTC}=0.00093$.

Case Id	Total filled number of wells	Number of positive wells	PPR
1	8652	2	0.000231163
2	9321	3	0.000321854
3	8923	1	0.000112072
4	9145	6	0.000656096

Table S2. Detailed sequences of crRNAs

Description	Sequence
crRNA 1	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACGGCUAUACAUUCUUACUAUUU UAUUUAA
crRNA 2	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACAAACCUCCAAUUCCCCCUA UCAUUUUUG
crRNA 3	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACGCUUUUAUUUUUUCUUCUG UCAAUGGCC
crRNA 4	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACCUAAUUUAUCUACUUGTTC AUUUCCUCC
crRNA 5	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACUCCCUGUAAUAAACCCGAA AAUUUUGAA

Table S3. Detailed sequences of target RNAs

Description	Sequence
Target 1 fragment	UAGGAGAAAUUUAUAAAAGAUGGAUAAUCCUGGGAUUAAAUAAAAUAGUAAGAAUC UAUAGCCCUACCAGCAUUCUGGACAUAAGACAAGGACCAAAG
Target 2 fragment	AUUAGAAGAAAUGAGUUUGCCAGGAAGAUGGAAACCAAAAAUGAUAGGGGGAAU UGGAGGUUUUAUCAAAGUAAGACAGUAUGAUCAGAUACUCAUAG
Target 3 fragment	AAAGCCAGGAAUGGAUGGCCCAAAAGUUAAACAAUGGCCAUUGACAGAAGAAAAA AUAAAAGCAUUAGUAGAAAUUUGUACAGAGAUGGAAAAGGAAG
Target 4 fragment	UCUAUCUGGCAUGGGUACCAGCACACAAAGGAAUUGGAGGAAAUGAACAAGUAGA UAAAUUAGUCAGUGCUGGAAUCAGGAAAGUACUAUUUUUAGAU
Target 5 fragment	ACAAACUAAAGAAUUACAAAAACAAAUUACAAAAAUUCAAAAUUUUCGGGUUUAU UACAGGGACAGCAGAAAUCCACUUUGGAAAGGACCAGCAAAGC

Table S4. Detailed information for testing assay reaction times.

Reaction time (min)	Total number of filled pores	Number of positive pores	Positive pore ratio (PPR)
0	9421	2	0.000212292
5	9235	24	0.002598809
10	9230	110	0.01191766
20	8901	202	0.022694079
30	8872	275	0.030996393
45	8710	271	0.031113662
60	8431	257	0.030482742

Table S5. Summary of the synthetic RNA test results. The synthetic RNA used in each test is 2 µL in a total of 20 µL Cas 13a reaction mixture. Each STAMP device takes ~100 nL of this 20 µL for analysis.

Expected Target Concentration (fM)	Total number of filled pores	Number of positive pores	Positive pore ratio	Measured Concentration (fM)
First batch				
0.1	8625	4	0.000463768	0.6
0.2	7835	4	0.00051053	0.7
0.5	7545	7	0.000927767	1.2
1	9231	10	0.001083306	1.4
10	8729	57	0.006529958	8.6
50	7342	280	0.038136747	29.9
100	6686	400	0.059826503	78.8
200	5451	912	0.167308751	233.2
1000	6925	4136	0.597256318	1161.7
10000	8765	8722	0.995094124	7977.4
20000	6950	6941	0.998705036	8493.4
50000	8574	8571	0.999650105	10163.4
Second batch				
0.1	9053	5	0.000552303	0.7
0.2	7432	6	0.00080732	1.0
0.5	8312	7	0.000842156	1.1
1	8653	12	0.001386802	1.8
10	8981	81	0.00901904	11.5
50	9232	503	0.054484402	71.3
100	9367	766	0.081776449	119.6
200	8869	1271	0.143308152	197.5
1000	8882	4150	0.467237109	804.3
10000	9080	9069	0.998788546	8578.5
20000	9456	9453	0.999682741	10291.7
50000	8938	8931	0.999216827	9562.1
Third batch				
0.1	8322	1	0.000120163	0.16
0.2	7325	5	0.000682594	0.9
0.5	7880	6	0.000761421	1.0
1	9321	11	0.001180131	1.5
10	10755	80	0.007438401	9.5
50	9242	390	0.042198658	55.3
100	7329	623	0.085004776	113.4
200	6408	826	0.128901373	176.2
1000	8713	5007	0.574658556	1093.2
10000	8425	8235	0.977448071	4843.6
20000	8133	8125	0.999016353	9212.1
50000	7123	7111	0.998315317	8151.2

Table S6. Summary of the contrived plasma sample test results. The volume of the plasma sample used in each test is 140 µL. The whole volume of the 10 µL eluted RNAs were used to form a total of 20 µL Cas 13a reaction mixture. Each STAMP device then takes ~ 100 nL of this 20 µL CRISPR mixture for quantification analysis.

# spiked into 140 µL plasma	Plasma viral load (copy/ml)	Total number of filled pores	Number of positive pores	Positive Pore ratio	Measured targets copies in 140 µL plasma	Measured Viral load (copy/ml plasma)
First batch						
0	0	8845	2	0.000226116	347	2479
1000	7143	8423	6	0.000712335	1096	7829
1500	10714	7510	9	0.001198402	1844	13171
2000	14286	8585	11	0.001281305	1972	14086
2500	17857	8428	14	0.00166113	2557	18264
3000	21429	9041	16	0.001769716	2725	19464
Second batch						
0	0	6752	1	0.000148104	227	1621
1000	7143	9115	8	0.000877674	1350	9643
1500	10714	8252	9	0.001090645	1678	11986
2000	14286	8765	13	0.001483172	2285	16321
2500	17857	7921	14	0.001767454	2721	19436
3000	21429	8321	17	0.002043024	3146	22471
Third batch						
0	0	8501	2	0.000235266	361	2579
1000	7143	8346	7	0.000838725	1290	9214
1500	10714	8895	9	0.001011804	1557	11121
2000	14286	8871	11	0.001239995	1908	13629
2500	17857	6521	11	0.001686858	2597	18550
3000	21429	8124	16	0.001969473	2876	20543

Table S7. Summary of clinical sample testing. The volume of the plasma sample used in each test is 140 µL. The whole volume of the 10 µL eluted RNAs were used to form a total of 20 µL Cas 13a reaction mixture. Each STAMP device takes ~ 100 nL of this 20 µL CRISPR mixture for quantification analysis. The RT-PCR test sued all 10 µL eluted RNAs.

	STAMP-dCRIPSR Result					RT-PCR Result		
Sample #	Total number of filled pores	Number of positive pores	Positive pore ratio	Measured RNA Conc in 20 µl of reaction mix (aM)	Measured viral load in 140 µl plasma (copies/ml)	Ct Value (mean)	Ct Value (STD)	Measured viral load in 140 µl plasma (copies/ml)
1	8651	2	0.000231187	29.534	2540	34.53	0.22	255
2	7515	3	0.000399202	51.002	4386	35.39	0.14	141
3	7234	235	0.032485485	4206.6	361768	23.86	0.09	402719
4	8723	4	0.000458558	58.58	5038	31.48	0.23	2095
5	9316	1	0.000107342	13.712	1179	ND	ND	ND
6	6153	2	0.000325045	41.526	3571	36.08	0.05	88
7	8424	94	0.011158594	1433	123238	25.3	0.2	149075
8	6928	3	0.000433025	55.324	4758	ND	ND	ND
9	10127	3	0.000296238	37.845	3255	33.28	0.18	605
10	10870	39	0.003587856	459.1	39483	27.51	0.16	32436
11	8324	2	0.000240269	30.69	2639	ND	ND	ND
12	8412	3	0.000356633	45.562	3918	ND	ND	ND
13	8021	2	0.000249345	31.854	2739	ND	ND	ND
14	8740	1	0.000114416	14.616	1257	ND	ND	ND
15	8801	55	0.00624929	800.75	68865	26.87	0.11	50448
16	9201	1	0.000108684	13.883	1194	ND	ND	ND
17	7338	1	0.000136277	17.408	1497	ND	ND	ND
18	9021	9	0.000997672	127.5	10965	29.48	0.11	8329
19	7668	3	0.000391236	49.984	4299	ND	ND	ND
20	7123	1	0.00014039	17.934	1542	ND	ND	ND

Table S8. Summary of current digital CRISPR works that are using Cas13a for RNA detection.

Pathogen	Target	Amplification	Effector Nuclease	Partitioning Method	Volume of Partition (pL)	Number of Partitions	Detection Readout	LoD (aM)	Dynamic Range (fold)	Refs
SARS-CoV-2	RNA	None	LwaCas13a	microchamber	0.003	1.00E+06	Fluorescence	9300	4	Shinoda et. al.
SARS-CoV-2	RNA and microRNA	None	LbuCas13a	droplet emulsion	1500	1.00E+05	Fluorescence	10	6	Tian et. al
SARS-CoV-2	RNA	None	LbuCas13a	droplet emulsion	10	4.78E+02	Fluorescence	8.3	4	Son et. al.
SARS-CoV-2	RNA	None	LtrCas13a	microchamber	0.03	5.00E+08	Fluorescence	2.4	6	Shinoda et. al.
SARS-CoV-2	RNA	None	LbuCas13a	droplet emulsion	10	1.60E+05	Fluorescence	1.6	4	Son et. al.
SARS-CoV-2	RNA	None	LwaCas13a	microchamber	0.003	1.00E+06	Fluorescence	5700	4	Shinoda et. al.
SARS-Cov-2	RNA	None	LbuCas13a	vortex emulsion	4.19-524	4.50E+03	Fluorescence	10	4-5	Xue et. al.
SARS-CoV-2	RNA	None	LwaCas13a	microchamber	0.003	1.00E+06	Fluorescence	89	3	Minagawa et. al
HPV	RNA	RT-RPA	LwaCas13a	droplet emulsion	14.1	1.40E+06	Fluorescence	5	5	Liu et. al.

Supplementary Videos

Supplementary Video S1. (Duration: 90 s) Video demonstrating the workflow of the STAMP device and the data acquisition.