Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Quantitative Isothermal Amplification on Paper Membranes using Amplification Nucleation Site Analysis

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This Supplementary Information document describes:

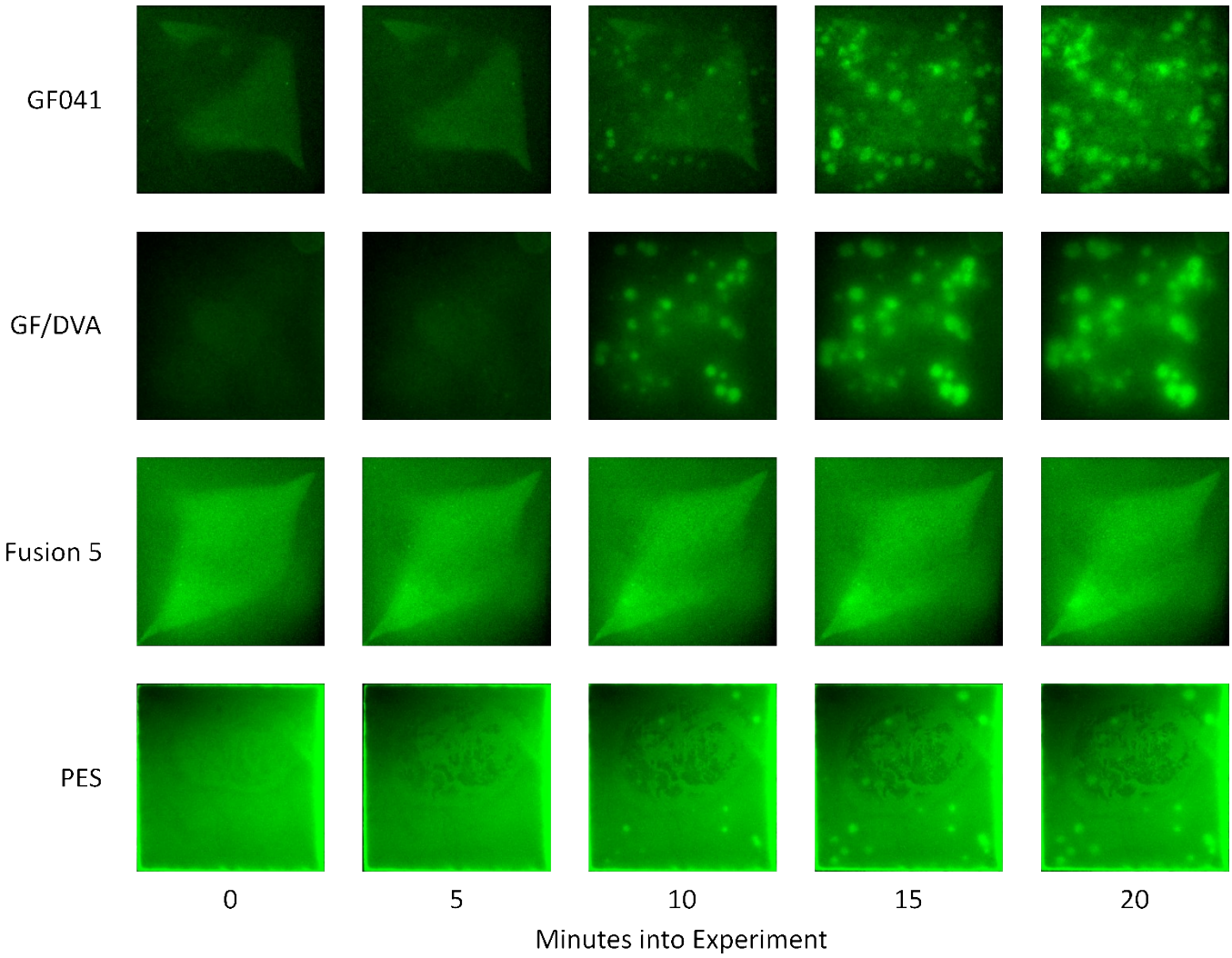

Figure S1: Representative amplification reactions on various membranes. From top to bottom: Millipore GF041, Whatman GF/DVA, Whatman Fusion 5, Millipore PES GPWP04700. All experiments are at 1,000 cps DNA per rxn. Due to the low water absorbency (~ 14 µL/cm^2) of the polyethersulfone (PES) membrane, a pad to hold the full 50 µL RPA mastermix volume would be too large for the imaging set up. Consequently, only 25 µL mastermix was used for the PES experiments. Strong amplification is seen in both the Millipore GF041 and the Whatman GF/DVA, with many distinct amplification nucleation sites visible. However, the Millipore GF041 exhibits brighter background fluorescence, reducing the contrast of the amplification nucleation sites compared to the Whatman GF/DVA membrane. Amplification within the Whatman Fusion 5 is relatively poor, with only one nucleation site appearing after 20 minutes and high background signal. The Millipore PES membrane supports amplification, though air bubbles (visible in the center of the pad) would consistently form, obscuring amplification nucleation sites.

Table S1: Properties and dimensions of paper membranes used for amplification pads.

Membrane Type	Water Absorbency [µL/cm^2]	Dimensions of Amplification Pad
Millipore GF041	40	11.2 mm x 11.2 mm
Whatman GF/DVA	75	8.2 mm x 8.2 mm
Whatman Fusion 5	31	12.7 mm x 12.7 mm
Millipore PES GPWP04700	14	13.4 mm x 13.4 mm*

*Experiments using the Millipore PES GPWP04700 utilized a 25 µL reaction volume.

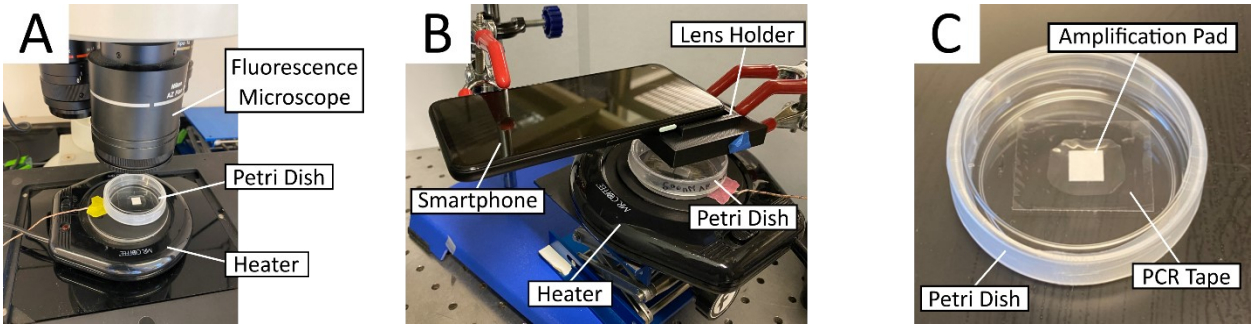

Figure S2. Images of experimental setup. (A) Microscope setup (B) Smartphone setup (C) Detailed view of the amplification pad

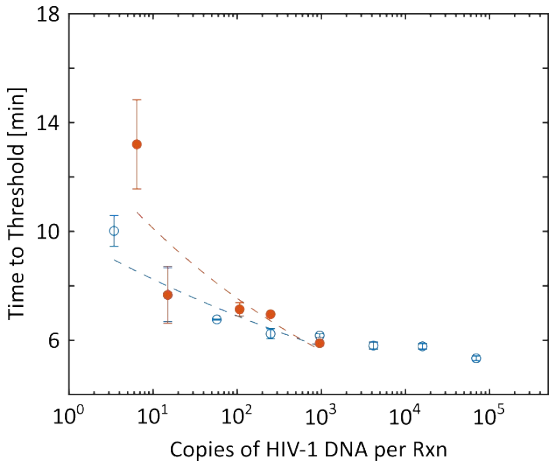

Figure S3: Time-to-threshold analysis of tube-based RPA experiments using the same primers and probe as used in paper-based amplification nucleation site analysis, for both HIV DNA (blue open circles) and HIV RNA (red closed circles). Circles represent mean values with standard deviations shown (n=3). We observe a relationship between time-to-threshold and copy number, with higher copy numbers taking less time to reach the fluorescent threshold though there is often significant overlap when comparing various copy numbers.

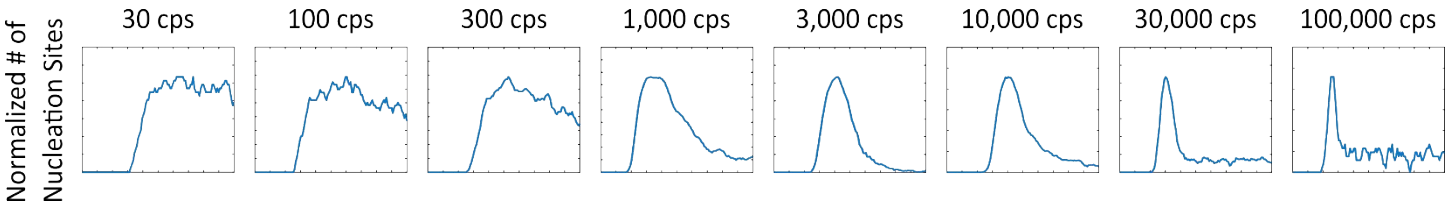

Figure S4: Representative normalized number of nucleation sites over the course of 20-minute experiments using HIV-1 DNA copy numbers 30 – 100,000 cps/rxn, captured via the microscope setup. Number of nucleation sites is normalized by the maximum number of nucleation sites. At very low copy numbers, the nucleation sites are spatially separated such that there is little site merging, and consequently, we do not observe any distinguishable peak of number of quantified nucleation sites. As the copy number increases, we observe gradually more pronounced peaks, as the increase in number of sites leads to more site merging. At very high copy numbers, the number of quantified nucleation sites increases rapidly, then decreases very quickly as site merging dominates very soon after the nucleation sites become visible.

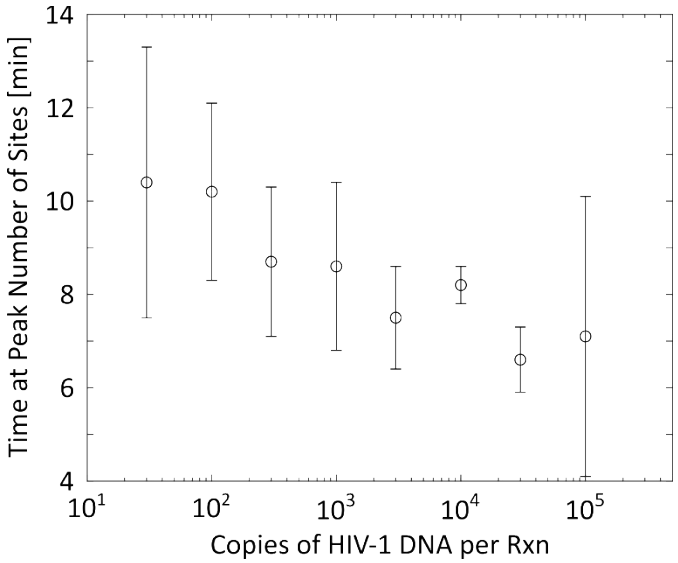

Figure S5: Time at peak number of nucleation sites for HIV-1 DNA experiments. As copy number increases, we generally observe the peak number of nucleation sites occur earlier in the experiment, as site merging becomes dominant very quickly at high copy numbers.

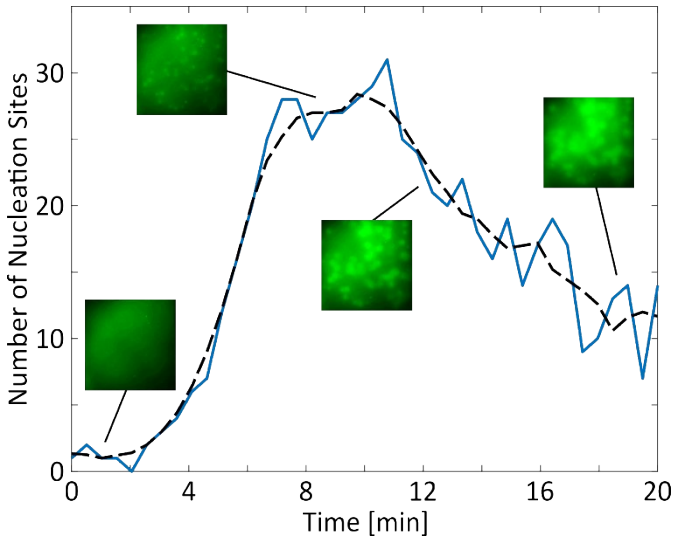

Figure S6: Representative experiment of 1,000 cps/rxn DNA on Whatman GF/DVA membrane captured by the mobile phone imaging system, showing the number of amplification nucleation sites as a function of experiment time. We observe very similar trends in the mobile phone-recorded experiments (in terms of site merging events) compared to microscope-recorded experiments.

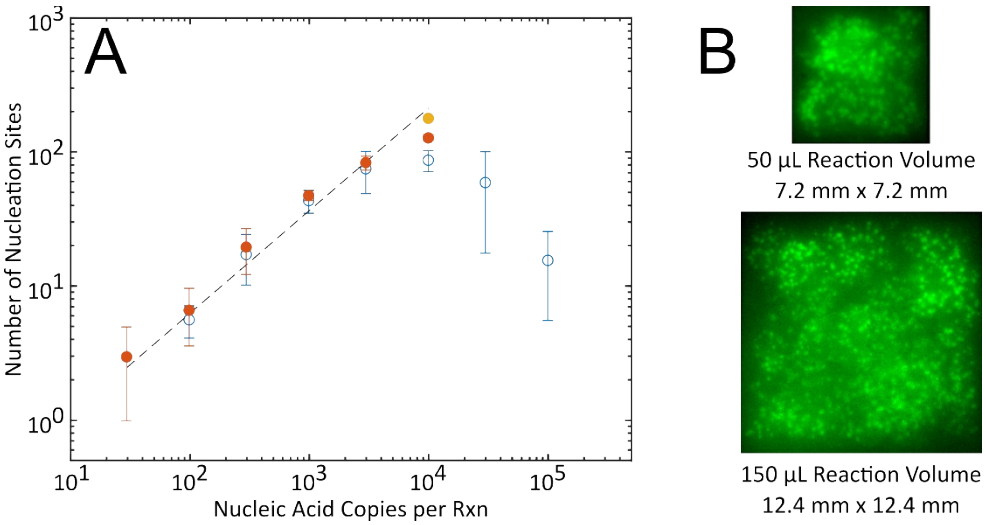

Figure S7: (A) Log-log plots of quantification of amplification nucleation sites for HIV DNA, using the CHT algorithm (blue) and a manual count (red). Also included is preliminary data using an increased amplification pad size accommodating a 150 µL reaction volume (yellow). The effects of the algorithmic undercount (relative to the manual count) are seen at 10,000 copies, suggesting that a more robust algorithm could further extend the dynamic range of amplification nucleation site analysis. Manual counts past 10,000 cps/rxn prove to be extremely difficult to perform, as there is very little separating the point in time when the nucleation sites are sufficiently bright to identify and when they begin merging. (B) Comparison of a typically sized amplification pad (50 µL reaction volume) to an increased amplification pad (150 µL reaction volume). Both images show 10,000 copies HIV DNA per reaction, at the peak number of nucleation sites. The nucleation sites in the 150 µL reaction volume amplification pad are less dense, reducing the effects of site merging. Consequently, this preliminary experiment has a nucleation site count of 179 (in good agreement with the calculated calibration curve for HIV DNA, shown in dotted black) whereas the typical 50 µL reaction volume experiment shown has a nucleation site count of 105. This suggests that increasing the amplification pad size/area is a viable strategy to increase the dynamic range of this method, though at the expense of increased reagent consumption.

Concentration prediction comparison between amplification nucleation site analysis and tube-based time-to-threshold analysis

Calibration curve fit for amplification nucleation site analysis (DNA: 100-3,000 cps/rxn):

$$\#\ of\ Amplification\ Nucleation\ Sites = 0.184 * (DNA\ cps\ per\ rxn)^{0.766}$$

Calibration curve fit for tube-based time-to-threshold analysis (DNA:100-3,000 cps/rxn)

$$TimeToThreshold[s] = 742.16 * (DNA\ cps\ per\ rxn)^{-0.095}$$

Average predicted concentrations and standard deviations are calculated from triplicates (n=3). All units are in log(cps/rxn) unless otherwise noted.

Copies per Rxn	Log(Copies per Rxn)	Amplification Nucleation Site Analysis				Tube-Based Time-to-Threshold Analysis			
		Average Predicted Concentration	Std. Dev.	Average Error	Average Absolute Error	Average Predicted Concentration	Std. Dev.	Error	Average Absolute Error
3,000	3.48	3.39	0.16	-0.09	0.17	3.18	0.05	-0.30	0.30
1,000	3.00	3.09	0.10	0.09	0.12	3.13	0.14	0.13	0.17
300	2.48	2.55	0.19	0.07	0.17	2.76	0.01	0.28	0.28
100	2.00	1.93	0.13	-0.07	0.11	2.22	0.58	0.22	0.58
		Average Absolute Error			0.14 (±0.09)	Average Absolute Error			0.33 (±0.19)

Calibration curve fit for amplification nucleation site analysis (RNA: 100-1,000 cps/rxn):

$$\#\ of\ Amplification\ Nucleation\ Sites = 1.047 * (cps\ per\ rxn)^{0.551}$$

Calibration curve fit for tube-based time-to-threshold analysis (RNA:100-1,000 cps/rxn)

$$TimeToThreshold[s] = 1182.1 * (cps\ per\ rxn)^{-0.156}$$

Average predicted concentrations and standard deviations are calculated from triplicates (n=3). All units are in log(cps/rxn) unless otherwise noted.

Copies per Rxn	Log(Copies per Rxn)	Amplification Nucleation Site Analysis				Tube-Based Time-to-Threshold Analysis			
		Average Predicted Concentration	Std. Dev.	Error	Average Absolute Error	Average Predicted Concentration	Std. Dev.	Error	Average Absolute Error
3,000	3.48	3.35	0.20	-0.13	0.22	3.36	0.03	-0.11	0.11
1,000	3.00	3.03	0.07	0.03	0.07	2.89	0.03	-0.11	0.11
300	2.48	2.72	0.07	0.02	0.06	2.83	0.08	0.13	0.13
100	2.00	1.95	0.32	-0.05	0.28	2.64	0.30	0.64	0.64
		Average Absolute Error			0.16 (±0.13)	Average Absolute Error			0.25 (±0.28)

Circular Hough Transform (CHT) Method Algorithm – MATLAB Based

```
clear all; close all; clc;

%% User Inputs
namefile = uigetfile('*tif');

%% Crop Image
figure(1)
preview=imread(namefile,1200);
imshow(preview,[0 max(max(preview))])
draw = drawrectangle('Color','b','FaceAlpha',0.01); % Select region of interest
rect = customWait(draw);
xmin = double(rect(1));
ymin = double(rect(2));
height = double(rect(4));
L = double(rect(3));

%% Compile Image Data

k = length(imfinfo(namefile)); %number of frames in data stack
for j=1:k
 Image=imread(namefile,j); % read in multiple image tiff file
 imcell=imcrop(Image,[xmin ymin L height]);

 %Resample to higher pixel density
 sz = size(imcell);
 xg = 1:sz(1);
 yg = 1:sz(2);
 F = griddedInterpolant({xg,yg},double(imcell));
 xq = (0:1/3:sz(1))';
 yq = (0:1/3:sz(2))';
 vq = F({xq,yq});

 X(:,j) = vertcat(vq(:));
end

%% Smooth Data - Average Every N Frames
[m1,n1] = size(X);
aveint = 10;
X_aveint = reshape(X, m1, aveint, n1/aveint);
X_smooth = squeeze(mean(X_aveint,2));

[m2,n2] = size(vq);
[m3,n3] = size(X_smooth);

%% Subtract Background - ATB Method

fr = 0.1*n3; %Frame that is 0.1 way through image stack
X_back = mean(X_smooth(:,1:fr),2);

X_new = bsxfun(@minus,X_smooth,X_back);
[m2,n2] = size(vq);
se = strel('disk',15);

for i = 1:k/aveint
 new = reshape(X_new(:,i),m2,n2);
 new = mat2gray(new);
 new_all(:,:,i)=new;
 background = imopen(new,se);
 newback = (new - background);

J1 = newback;
 J1_all(:,:,i) = J1;
 [centers, radii, metric] = imfindcircles(J1,...
 [8 20],'objectpolarity','bright');... % Alter for performance boost!
 radii_all{i} = radii;
 metric_all{i} = metric;
 centers_all{i} = centers;
 dots(i) = length(radii);
 % Visualize dot size increase over time
 metricbest = metric(metric>0.2);
 averad(i) = mean(radii(1:length(metricbest)));
 averad(isnan(averad))=0;


 figure(2)
 imshow(J1)

 if isempty(centers) == 0
 figure(3)
 plot(centers(:,1),-centers(:,2),'k.','MarkerSize',15)
 hold on
 end
clc
end
```

```
%% Visualize Nucleation Sites
figure(2)
imshow(new_all(:,:,k/aveint/2))
for i = 1:k/aveint
 viscircles(centers_all{i},radii_all{i},'EdgeColor','b');
end
```

Threshold Analyze Particles (TAP) Method Algorithm – ImageJ Based

```
run("Grouped Z Project...", "projection=[Average Intensity] group=10");

run("Subtract Background...", "rolling=10 stack");

setSlice(48)

run("Make Binary", "method=Otsu background=Dark calculate");

run("Median...", "radius=2 stack");

run("Watershed", "stack");

run("Analyze Particles...", "size=20-Infinity exclude summarize stack");
```