Supporting Information

Screening of additive formulations enables off-chip drop reverse transcription quantitative PCR of single influenza A virus genomes

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Table S1. Supplemental data table for Fig. 1B and 1C.

Additive	$(D-D_0)/D_0$ mean	$(D-D_0)/D_0$ standard deviation	$(D-D_0)/D_0$ median	D CV	$(I-I_B)$ mean	$(I-I_B)$ standard deviation	$(I-I_B)$ median	$(I-I_B)$ CV	N_{drops}
No Additives	0.31	0.43	0.16	33%	33.6	10.4	34.0	30.9%	405
BSA	0.12	0.28	0.01	25%	83.6	20.1	85.7	24.0%	915
PEG-6K	0.25	0.40	0.09	32%	79.2	15.0	80.0	19.0%	622
Betaine	0.46	0.60	0.29	41%	72.6	10.9	73.8	15.1%	270
Tween-20	-0.10	0.08	-0.12	9%	97.0	17.9	99.6	18.5%	1948
Tween-20 / PEG-6K	-0.05	0.14	-0.09	15%	63.9	16.9	65.3	26.4%	1534
Tween-20 / BSA	-0.07	0.11	-0.12	12%	76.2	23.5	80.5	30.8%	1460
Tween-20 / BSA / Betaine	-0.01	0.12	-0.04	12%	88.9	14.1	89.7	15.9%	1155

Table S2. Supplemental data table for endpoint analysis Fig. 3E and 5D.

M gene copies/drop (cpd)	N_+/N_{total} (measured) (M gene standards)	N_+/N_{total} (Poisson) (M gene standards)	Supernatant dilution factor	N_+/N_{total} (measured) (infected supernatant)	N_+/N_{total} (Poisson) (infected supernatant)
1.7×10^4	1 (N_{total} = 2051)	1.00	10^0	1.00 (N_{total} = 138)	1.00
1.7×10^3	1 (N_{total} = 2100)	1.00	10^{-1}	0.968 (N_{total} = 95)	1.00
1.7×10^2	1 (N_{total} = 1889)	1.00	10^{-2}	0.771 (N_{total} = 280)	0.826
1.7×10^1	0.997 (N_{total} = 2038)	1.00	10^{-3}	0.274 (N_{total} = 208)	0.161
1.7×10^0	0.606 (N_{total} = 2076)	0.819	10^{-4}	0.071 (N_{total} = 141)	0.017
1.7×10^{-1}	0.107 (N_{total} = 2298)	0.157	10^{-5}	0.123 (N_{total} = 155)	0.002
Negative Control	0.000 (N_{total} = 1774)	N/A	Negative Control	0.082 (N_{total} = 208)	N/A

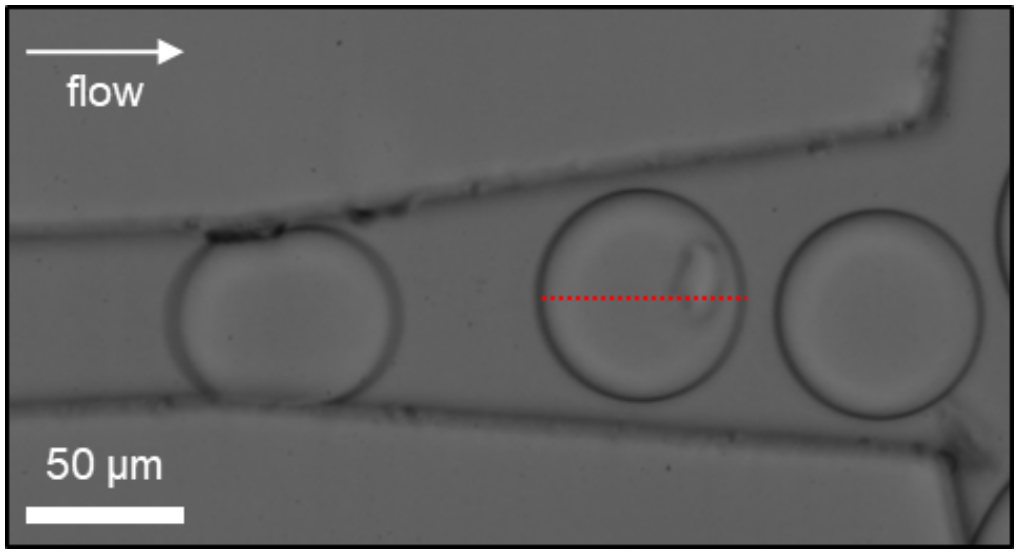

Figure S1. Drops at the exit of a drop making device. Initial drop diameter (D_0) is measured as indicated by the dotted red line.

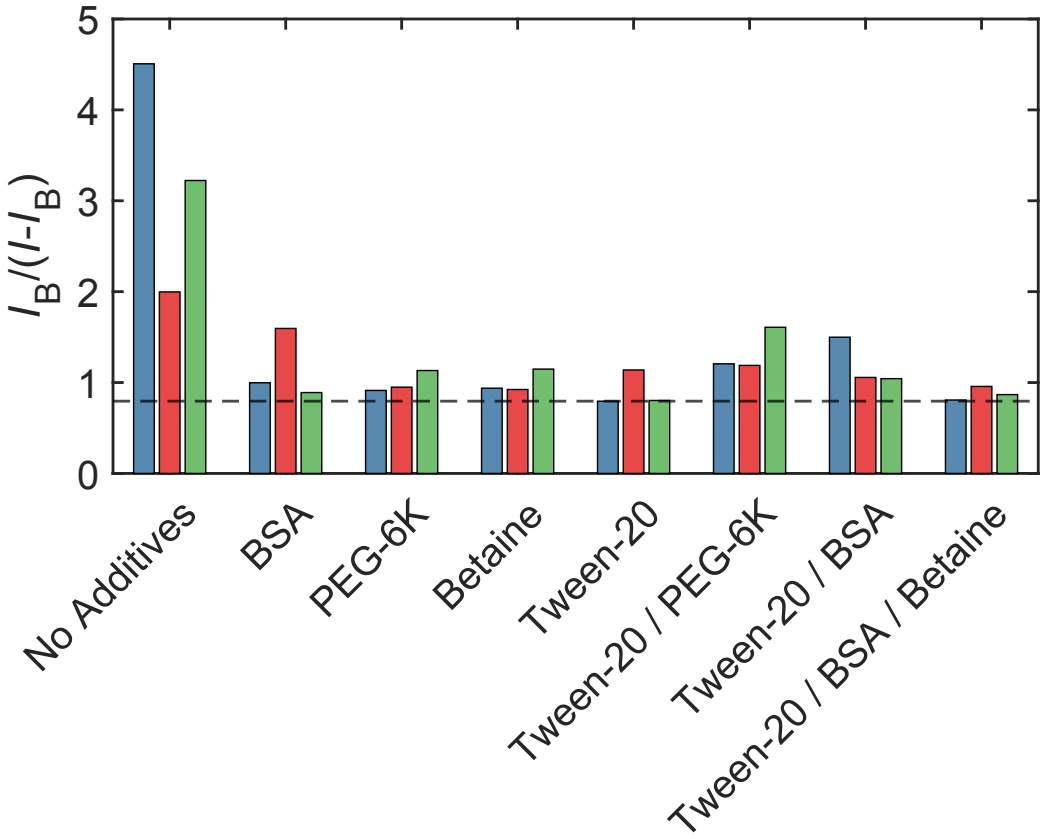

Figure S2. Mean normalized background fluorescence intensity $\frac{I_B}{I-I_B}$ for each PCR additive condition. Colored bars for each additive represent pooled image data from each qPCR tube sampled. The dashed line indicates that the Tween-20 alone and the Tween-20 / BSA / betaine combination had the lowest normalized background, indicating the best retention of ROX dye in the drops during thermocycling.

Expanded experimental details

Microfluidic device fabrication. Microfluidic devices for making 50 μm and 100 μm diameter drops were fabricated using standard soft lithographic methods. Photoresist (Microchem SU-8 3050) was patterned on silicon wafers (University Wafer, ID# 447, test grade) using photolithography Polydimethylsiloxane (PDMS) (Sylgard 184) at 10:1 mass ratio of polymer to cross-linking agent and was poured onto the patterned device master molds. Air was purged from the uncured PDMS by placing the filled mold in a vacuum chamber for at least 1 h. The PDMS was cured in an oven at 55 °C for 24 h and then removed from the mold with a scalpel. Ports were punched into the PDMS slab with a 0.75 mm diameter biopsy punch (EMS Rapid-Core, Electron Microscopy Sciences). The PDMS slab was bonded to a 2-in by 3-in glass slide (VWR micro

slides, cat. #48382-179) after plasma treatment (Harrick Plasma PDC-001) for 60 s at high power (30 W) and 700 mTorr oxygen pressure. The drop making devices were made hydrophobic by flowing Aquapel (Pittsburgh Glass Works) through the channels, followed by blowing the channels with air filtered through a GVS ABLUO 25 mm 0.2-µm filter (Fisher Scientific) before baking the devices in an oven at 55 °C for 1 h.

PCR sequences and reaction concentrations. Primers and probes were ordered from Eurofins Operon and were prepared as 100 µM stocks. The working stocks of the primers were 25 µM with a final reaction concentration of 400 nM. The working stock of the probe was 10 µM with a final reaction concentration of 200 nM. Each reaction mix contained 0.05 µM ROX reference dye, 2.0 mM $MgSO_4$ and 0.32 U/µL SUPERase RNase Inhibitor (Invitrogen AM2694), and 2.5 µL of RNA or supernatant. Tested additives were added to the qRT-PCR reaction mix at the following concentrations: 1.0% w/v Tween-20 (Calbiochem 655204-100mL), 0.8 µg/µL BSA (Fisher BP675-1), 2.5% w/v PEG-6K (Acros Organics 192280010), and 1.0 M betaine (Sigma B0300-1VL).

Imaging of drops containing PCR additives. 20 µL of thermocycled drops were imaged to capture a full field of view (FOV) of drops with a 10× objective. Brightfield and fluorescence images (Texas Red, TXRED ex. 540-580 nm, em. 592-669 nm) of the drops were captured on an inverted epifluorescence microscope (Leica DMi8) with a 10× objective (Leica, NA 0.32). The Leica Application Suite X was used for image acquisition. For each of the eight additive conditions, drops were imaged from three PCR tubes and three FOV per tube (>100 drops per FOV) were captured. The device used for imaging had a channel height of 50 µm, thereby compressing drops larger than 50 µm in diameter and skewing larger drop diameter measurements. Thus, drop diameters greater than this height were adjusted by estimating the compressed drops as oblate spheroids and then using the oblate spheroid volume to calculate the equivalent sphere diameter using the equation $D_S = \sqrt[3]{D_E^2 h}$ where D_S is the diameter of the equivalent sphere, D_E is the measured diameter of a compressed drop, and h is the channel height of 50 µm.

Analysis of endpoint epifluorescence images of drops containing PCR additives. Drops imaged on the epifluorescence microscope were processed with a custom MATLAB (R2019a) script to measure drop diameter and ROX fluorescence intensity. Diameters D from at least 270 drops per condition were made. ROX fluorescence intensities I were normalized by subtracting the background fluorescence I_B, set as the average pixel value measured from a 100 px^2 background region using Fiji.[1] Normalization of measured D from an initial drop diameter D_0 was performed using the expression $(D\text{-}D_0)/D_0$. The initial D_0 is the average drop diameter measured from drops without PCR additives upon exit from a drop making device (*SI Appendix*, Figure S1).

Imaging of drops during OCD-qPCR thermocycling. The qPCR machine was paused and one PCR tube was removed to sample drops. Drops were imaged using epifluorescence microscopy to quantify fluorescence from the FAM (6-carboxyfluorescein) dye-labeled TaqMan probe and ROX (6-carboxy-X-rhodamine) reference dye. Five FOV were captured of drops from each tube at each cycle on the epifluorescence microscope for an average of 650 drops after pooling from all FOV.

Imaging of multiplexed OCD-qPCR drops. M gene (2.62×10^5 copies/μL) and β-actin (6.98×10^4 copies/μL) were encapsulated with qPCR mix in 50 μm diameter drops and thermocycled. Drops were imaged using epifluorescence microscopy to quantify fluorescence from the FAM (6-carboxyfluorescein) dye-labeled TaqMan probe for M gene, Cy5 (Cyanine5 NHS ester, ex. 649 nm, 666 em.) dye-labeled TaqMan probe for β-actin, and ROX (6-carboxy-X-rhodamine) reference dye. Five FOV were captured of drops on the epifluorescence microscope for a total of 1127 drops after pooling from all FOV.

SI References

1 Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol. Reprod. Dev.* **82**, 518-529, doi:10.1002/mrd.22489 (2015).