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Supporting Information

Artifact-Free Quantification and Sequencing of Rare Recombinant Viruses by Using Drop-Based Microfluidics

Ye Tao,[a, b] Assaf Rotem,[a] Huidan Zhang,[a, c] Shelley K. Cockrell,[d] Stephan A. Koehler,[a] Connie B. Chang,[a, e] Lloyd W. Ung,[a] Paul G. Cantalupo,[d] Yukun Ren,[b] Jeffrey S. Lin,[f] Andrew B. Feldman,[f, g] Christiane E. Wobus,[h] James M. Pipas,[d] and David A. Weitz*[a, i]

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MATERIAL AND METHODS

Preparation of viral RNA samples

Murine RAW 264.7 macrophage cells are purchased from ATCC and maintained in DMEM supplemented with 10% low-endotoxin fetal calf serum 100 U penicillin/mL, 100 µg/mL streptomycin, 10 mM N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES), and 2 mM L-glutamine.[1] The two parental viral strains are the plaque-purified MNV-1 clone (GV/MNV1/2002/USA) MNV-1.CW3 and the fecal isolate WU20 (GV/WU20/2005/USA) at the passage six.[2] The infectivity titers of the virus-containing cell lysates are 10^8 plaque forming units (PFU)/mL and 5×10^8 PFU/mL for MNV-1 and WU20, respectively. To generate recombinants, 2 mL of $2\text{x}10^6$/mL RAW cells are co-infected with 40 µL of 10^8 PFU/mL MNV-1 and 8 µL of $5\text{x}10^8$ PFU/mL WU20 each with a multiplicity of infection of 2 for 16 hours. The viruses are released using three freeze/thaw cycles, followed by centrifugation to reduce cell debris, and the supernatant is stored at -80 °C. Viral genome RNA is purified using the QIAamp Viral RNA Mini Kit (Qiagen, CA) from 140 µL of viral supernatant and re-suspended in 60 µL of ddH_2O.

Design and Fabrication of microfluidic devices

Polydimethylsiloxane (PDMS) Microfluidic devices for drop encapsulation and sorting are similar in design to those described in our previous work.[3] Although it is tempting to design a single microfluidic chip that performs all functions (drop encapsulation, PCR and sorting) on a single device, in practice, decoupling the different steps and performing them on separate devices provides a modular platform that is more stable, versatile and flexible. Particularly, when performing all steps on a single chip, every change in flow settings of one microfluidic junction affects the function of downstream and requires the re-design of the entire chip. By contrast, in a modular platform, microfluidic junctions are decoupled and can be optimized individually. In addition, preforming PCR off-chip allows easy manipulation of the sample, including its splitting into multiple samples that can be used for experimental controls and for optimization of thermocycling parameters.

We use the standard soft lithographic method to fabricate the microfluidic devices used in this study.[4] To make the channel hydrophobic, we treat the microfluidic channels by flushing with Aquapel (Aquapel Glass Treatment, PA). The electrodes for sorting drops are made by filling channels with Indalloy 19 (Indium Corp., NY).[5]

Overview of experimental flow

To characterize rare recombinants, we encapsulate and amplify single viral recombinants in drops, and then count, isolate and sequence the target amplicons in individual drops. The loading of single viral templates in drops is required to avoid artificial recombination (PCR or RT-PCR chimera) which cannot be distinguished from the true recombinant viral genomes. We assume that viral genomes are suspended homogenously in the purified viral RNA solution, meaning that the loading of suspended viral genomes into drops is solely determined by the concentration of genomes in the suspension: if

the genomes are dilute enough in the suspension, then only a small number of genomes will be co-encapsulated in the same drop and the chance for chimera generation is negligible (our measurements show that this is true for concentrations below one genome per drop, see Figure 3C and main text for more details). Thus, to avoid chimeras and to calculate the relative fraction of recombinant genomes in the sample we must first determine the concentration of viral genomes in our sample. Then we dilute the sample to one genome per drop to avoid chimeras and finally we count, sort and sequence the recombinant bearing drops. A more detailed description of each of the experimental steps follows.

RT-PCR cocktails for amplifying viral genomes

We perform drop-based one-step RT-PCR to amplify viral RNA genomes in an amplification cocktail containing both reverse transcriptase and DNA polymerase. To render the successful amplification in drops for their counting and sorting, we add a fluorescent marker to the cocktail. We use one in-drop RT-PCR protocol to determine the total concentration of viral genomes, and a second protocol to measure the concentration of recombinant viral genomes.

To measure the concentration of all viral genomes in the sample we amplify a loci conserved for all murine norovirus (MNV) strains near the 5' UTR with the following primers: F-5'-CGGGCTGAGCTTCCTGC-3', R-5'-GTGCGCAACACAGAGAAACG-3' (IDT, IA).[6] To mark the drops with successfully amplification we add a Taqman probe (Life Tech, CA): 5'-FAM-CTAGTGTCTCCTTTGGAGCACCTA-3'-MGB. We include 1 µL of OneStep RT-PCR enzyme (Qiagen, CA) and 1 µL of serially diluted viral RNA in a total volume of 25 µL of 1X OneStep RT-PCR buffer (Qiagen, CA) containing 400 µM dNTPs, 0.25 µM forward and reverse primers, 0.24 µM Taqman probe, 0.2 µg/µL BSA and 0.2% Tween 20. The cocktail is thermocycled at: 50 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 58 °C for 50 s, 72 °C for 1 min.

To measure the concentration of recombinant genomes in the sample we target a specific recombinant fragment in the viral genome (nt 4701 – nt 5904) using the following differential primers: F-5'-CAGGGGATTTCCTTTCTGCGTCGC-3' (Targeting WU20 genome at nt 4701) and R-5'-ACCGGAGATTGGGGTGGTACCAAGC-3' (Targeting MNV-1 genome at nt 5904). To mark the drops with successful amplification we use a non-specific intercalating dye, Eva Green (Biotum, CA). We include 0.5 µL of SuperScript™ III RT/Platinum Taq High Fidelity Enzyme (Invitrogen, NY) and 1 µL of purified RNA in a total volume of 25 µL of 1X reaction buffer (Invitrogen, NY) containing 200 µM dNTPs, 0.2 µM forward and reverse primers, 0.08 × Eva Green, 0.2 µg/µL BSA and 0.2% Tween 20. The cocktail is thermocycled at: 55 °C for 30 min 94 °C for 2 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 68 °C for 90 s, and a final extension at 68 °C for 5 min. Since we determine the concentration of total RNA using our drop-based assay, the precise RNA amount used in the first RT-PCR reaction is only known after the reaction is completed. We then use this measured amount to control the exact concentration of parental viruses in the second RT-PCR reaction, which is used to identify recombinants. For a dilution of one parental virus per drop the RNA mass per 1µL is 12.8 pg.

Formation of monodisperse aqueous drops and RT-PCR

We use a microfluidic device that contains a flow-focusing drop maker with a cross section of 15 µm x 25 µm to generate 25 µm monodisperse aqueous drops in fluorinated oil, HFE-7500 (3M, MN) containing 1% (w/w) Krytox-PEG diblock co-polymer surfactant (RAN Biotechnologies, MA). The flow is driven by applying a -0.4 PSI vacuum at the outlet, as shown in Figure S2. The drops are collected in a PCR tube and covered with mineral oil to prevent evaporation during thermocycling. RT-PCR is then performed according to the thermal profiles described in the previous section.

Detection and sorting of successfully amplified drops

The amplified drops are re-injected into our microfluidic reading and sorting device with a cross section of 25 µm × 25 µm.[3] We use syringe pumps (Harvard aparatus, MA) to drive the drop sample at a flow rate of 15 µL/h and the spacing oil at a flow rate of 180 µL/h. The microfluidic device is mounted on a microscope (Olympus, PA) and a laser beam (488 nm excitation, Laserg, Canada) is focused on the channel where drops are flowing so that successfully amplified drops are excited and their fluorescence is collected via the microscope in a photomultiplier tube (PMT) (Hammamatsu, Japan). The fluorescent pulses are acquired by a real-time field-programmable gate array card (National Instruments, TX) and analyzed by a LabView program so that drops above a given pulse height are considered successfully amplified. Positive drops are immediately sorted via an alternating electric field created by the electrodes which exerts a dielectrophoretic force that deflects the drop into the sorting channel (Figure 1C).

Isolation and sequencing of individual recombinant fragments

To separately sequence the contents of each positive drop we isolate each positive drops into a separate well by distributing the sample of positive drops into a large number of micro-titer wells (3 times more than the number of sorted drops) so that the probability that two drops were distributed into the same well is low (<5% according to Poisson distribution). First, drops that are deflected into the sorting channel are collected into a tip containing a large number of empty “carrier drops”. Carrier drops contain only ddH_2O, have the same size as the sample drops and are used to protect the sample drops against evaporation, absorption to channel walls and coalescence. Since the sorted drops are collected into the carrier emulsion, the total volume of sample drops and buffering drops is macroscopic and can be handled with ease, without risking loss of sample drops during manipulation. For example, after collecting 15 positive drops into a total volume of 45 ul of carrier drops, we homogenize the sorted sample by mixing and then dispense the emulsion into 45 wells, 1 µL each.

To sequence the amplicons of sorted drops we perform a second round of PCR for each of the wells containing 1 µL of emulsion. We first add 1 µL of 1H,1H,2H,2H-perfluorooctanol (Alfa Aesar, MA) to each well and vortex and centrifuge the well plate, followed by adding 5 µL of ddH_2O. Consequently, the emulsion is separated into a bottom oil phase and a top aqueous phase, from which we transfer 5 µl to a new well containing 20 µL of a PCR cocktail mix. The final reaction volume of 25 µL includes

0.5 µL of KAPAHiFi DNA Polymerase (KAPA bio, MA), 1X buffer, 200 µM dNTPs, 0.2 µM forward and reverse primers of the conserved fragment (see previous section about RT-PCR cocktail), and 5 µl of the template. PCR is performed by preheating the reaction mixture to 95°C for 3 min, followed by 35 cycles of 98°C for 20 s, 60°C for 15 s, and 72°C for 90 s, and a final extension at 72°C for 5 min. Finally, we analyze the contents of each well using gel electrophoresis, purify the amplicons from wells whose size equals that of the amplified region (1205-bp) using GenElute™ Gel Extraction Kit (Sigma, CA) and Sanger sequence them (Beckman Coulter , MA).

Next generation sequencing of viral genomes

Viral cDNA is generated either from cell lysate that co-infected by both MNV-1 and WU20 or from a negative control (cell-free mix of MNV-1 and WU20 viral RNA genomes). 500 ng of RNA is reverse transcribed with Superscript II RT (Invitrogen, NY) and random hexamer primers. After treatment with RNAse H, 0.5 µl, cDNA is used for each PCR with Accuprime Taq High Fidelity DNA Polymerase (Invitrogen, NY) and the primers listed in Table S2, designed to anneal to both MNV-1 and WU20 sequences so that all parental and recombinant genomes would be amplified. Each forward (F) primer used has the universal tag 5'-GTTTCCCAGTCACGATA*CCTAGA* or GTTTCCCAGTCACGATA*CTCCAA* at its 5' end, where *CCTAGA* and *CTCCAA* are the barcodes for co-infected cell cDNA or the negative control. Each reverse (R) primer has the tag 5'-CAGGAAACAGCTATGAC at its 5' end. The underlined sequences are universal primers used in a second round of amplification (see below). cDNA (0.5 µl), Accuprime Taq High Fidelity DNA Polymerase (Invitrogen), and amplicon primers are used in a total volume of 25 µl with the following conditions: 94°C, 1 min; 30x[94°C, 30 s; 59°C, 30 s; 68°C, 30 s]; 68°C, 1 min. Each PCR product is diluted 1:10 in ddH2O and used as the template for a second round of PCR amplification with the following primers:

5'- **CCATCTCATCCCTGCGTGTCTCCGACTCAG**GTTTCCCAGTCACGATA and

5'- **CCTATCCCCTGTGTGCCTTGGCAGTCTCAG**CAGGAAACAGCTATGAC,

where the bold sequences are the Roche/454 Lib-L Primers A and B and the underlined sequences are the universal primers added in the first round of PCR. The conditions for the second PCR are: 94°C, 1 min; 35x[94°C, 30 s; 50°C, 30 s; 68°C, 30 s]; 68°C, 1 min. The final PCR amplicons ranged from 386-472 bp. The amplicons are purified with a Qiaquick PCR purification kit (Qiagen, CA) and stored in TE buffer. Each amplicon is quantified on an Agilent Bioanalyzer (University of Pittsburgh Genomics and Proteomics Core, PA), and the amplicon stocks are diluted to 10^8 molecules/µl in TE buffer. Equal volumes of each diluted amplicon are pooled to form the sequencing library. The library is amplified with a Roche/454 Lib-L emPCR kit prior to the sequencing run on a GS-FLX instrument (performed at Johns Hopkins University Applied Physics Lab, MD).

Analysis of next generation sequencing data

The universal tag 5'-GTTTCCCAGTCACGATA and 5'-CAGGAAACAGCTATGAC sequences are trimmed from the raw reads. Reads are binned into two groups, co-infected or mixed, based on their barcode (co-infected, 5' CCTAGA; mixed, 5' CTCCAA). After removal of the barcodes, sequences >200bp are aligned to mouse norovirus MNV-1 and WU20 with BLASTN (v2.2.27) using default parameters. The alignment position on the norovirus genome is used to determine the amplicon identity of each sequence (A through L). Unidentified sequences are labeled "Z". Recombinant sequences are identified using UCHIME (version 4.2.40; http://drive5.com/usearch/manual/uchime_algo.html) with default parameters. The number of parental and recombinant fragments for each condition (co-infected and mixed) is plotted along the genome in Figure S1.

TABLES

Table S1. Comparison of available methods for characterization of rare recombinants.[7]

	Drop Based Microfluidics	Clone Sequencing	Next Generation Sequencing	Emulsion PCR	Microfluidic on-chip Digital PCR
Description	Single templates screened in drops and then each drop is Sanger sequenced.	Single templates inserted into plasmids, amplified in bacterial colonies and Sanger sequenced.[8]	Templates amplified in bulk and deep sequenced.[9]	Single templates amplified in drops. Pool of all drops can be retrieved for sequencing.[10]	Single templates amplified and screened in nano-wells. Contents of wells cannot be retrieved for sequencing.[11]
Counting accuracy	Good A single template is associated with each drop counted.	Biased Amplification bias accumulates during library preparation.	Biased Amplification Bias accumulates during library preparation Bias can be avoided when using UMI*.[12]	Biased Amplification bias is generated due to size polydispersity of emulsion.	Good A single template is associated with each nano-well counted.
Chimera artifacts	None if only one template per drop.	Yes, generated during library preparation.	Yes, 0.01 - 30% chimeras generated during library preparation,[13] see also Figure S1.	None if only one template per drop.	None if only one template per nano-well.
Sequencing method	Sanger	Sanger	Deep sequencing	Any method of choice	Unavailable Samples cannot be retrieved for sequencing.
# of templates screened	$\sim 10^6$ $\sim 10^6$ drops can be screened in an hour.	~10 No screening involved. Each sequence requires a monoclonal bacterial colony.	$\sim 10^7$ $\sim 10^{10}$ bp (Illumina) sequenced per run, covering 10^7 fragments that are 1kb long	Not applicable, because emulsion PCR only is a method for chimera-free amplification.	$\sim 10^4$ Current technology is limited to $\sim 10^4$ nano-wells.
Library preparation and sequencing time	Hours ~2 hours of drop manipulation. ~4 hours of amplification cycles	Days Growing monoclonal colonies.	Days sequencing time depends upon sequencer and specified read length.[14]	Hours	Hours
Read length	~1kb	~1kb	500bp (Illumina) 500bp-1kb (454 Life Sciences) +10kb (PacBio)	~1kb	~1kb
Summary	Method of choice for sequencing and counting rare (<0.1%) recombinants.	Very low throughput Appropriate for sequencing and counting abundant templates.	Appropriate for sequencing frequent (>0.1%) recombinants. Appropriate for counting frequent recombinants when using UMI*.	Combined with NGS, can be appropriate for sequencing rare recombinants but inappropriate for counting.	Appropriate for counting frequent (>0.1%) recombinants Inappropriate for sequencing.

*UMI - Universal Molecular Identifiers

Table S2. Sequencing amplicon primers.

Sequencing amplicon	Locus (bp)	Oligo sequences
1	2406-2691	F: 5'-AAGGAGGCCCGCCTCCGCT
		R: 5'-TACTCCTCATCCGTGAGCCCACG
2	2670-3018	F: 5'-CGTGGGCTCACGGATGAGGAGTA
		R: 5'-G**S**ACAACACGGGACCAGATGGA
3	2997-3361	F: 5'-TCCATCTGGTCCCGTGTTGT**S**C
		R: 5'-GAGCCAGTCAGGAGCATGCCAGT
4	3339-3636	F: 5'-ACTGGCATGCTCCTGACTGGCTC
		R: 5'-GCCAGAACATGGTCTTGGTGCTCA
5	3613-3941	F: 5'-TGAGCACCAAGACCATGTTCTGGC
		R: 5'-GTCCAGTCCTTGCTCTTCTG**Y**TTGTG
6	3915-4211	F: 5'-CACAA**R**CAGAAGAGCAAGGACTGGAC
		R: 5'-**R**TTCATCGACATGCCCACTCTGA
7	4188-4516	F: 5'-ATCAGAGTGGGCATGTCGATGAA**Y**
		R: 5'-ACCTCAACCATTGCACA**R**AGGGTC
8	4493-4864	F: 5'-GACCCT**Y**TGTGCAATGGTTGAGGT
		R: 5'-AGTTGGGAGGGTCTCTGAGC**R**TGTC
9	4840-5152	F: 5'-GACA**Y**GCTCAGAGACCCTCCCAACT
		R: 5'-GAATGGGGACGGCCTGTTCAAC
10	5131-5479	F: 5'-GTTGAACAGGCCGTCCCCATTC
		R: 5'-TGATCTG**R**GCAGTGGTGAGTGACCC
11	5455-5773	F: 5'-GGGTCACTCACCACTGC**Y**CAGATCA
		R: 5'-ACAGCCGCGGCTGTA**K**CACG
12	5754-6071	F: 5'-CGTG**M**TACAGCCGCGGCTGT
		R: 5'-G**S**ACCTCGATCTCCAGTTGCCC
13	6052-6392	F: 5'-GGGCAACTGGAGATCGAGGTSC
		R: 5'-CTGCGGCGTCAGCGGTGTC

F, forward primer. R, reverse primer. Bold letters indicate ambiguous nucleotides.

REFERENCES

[1] C. E. Wobus, S. M. Karst, L. B. Thackray, K. O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green, H. W. Virgin, *PLoS biology* **2004**, *2*, e432.

[2] L. B. Thackray, C. E. Wobus, K. A. Chachu, B. Liu, E. R. Alegre, K. S. Henderson, S. T. Kelley, H. W. t. Virgin, *Journal of virology* **2007**, *81*, 10460-10473.

[3] J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J. C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths, D. A. Weitz, *Proceedings of the National Academy of Sciences of the United States of America* **2010**, *107*, 4004-4009.

[4] J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller, G. M. Whitesides, *Electrophoresis* **2000**, *21*, 27-40.

[5] L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths, J. A. Heyman, *Nature protocols* **2013**, *8*, 870-891.

[6] S. Taube, J. W. Perry, K. Yetming, S. P. Patel, H. Auble, L. Shu, H. F. Nawar, C. H. Lee, T. D. Connell, J. A. Shayman, C. E. Wobus, *Journal of virology* **2009**, *83*, 4092-4101.

[7] aM. A. Quail, M. Smith, P. Coupland, T. D. Otto, S. R. Harris, T. R. Connor, A. Bertoni, H. P. Swerdlow, Y. Gu, *BMC genomics* **2012**, *13*, 341; bL. Liu, Y. Li, S. Li, N. Hu, Y. He, R. Pong, D. Lin, L. Lu, M. Law, *Journal of biomedicine & biotechnology* **2012**, *2012*, 251364.

[8] E. Mathijs, B. Muylkens, A. Mauroy, D. Ziant, T. Delwiche, E. Thiry, *The Journal of general virology* **2010**, *91*, 2723-2733.

[9] D. C. Koboldt, K. M. Steinberg, D. E. Larson, R. K. Wilson, E. R. Mardis, *Cell* **2013**, *155*, 27-38.

[10] D. Dressman, H. Yan, G. Traverso, K. W. Kinzler, B. Vogelstein, *Proceedings of the National Academy of Sciences of the United States of America* **2003**, *100*, 8817-8822.

[11] aH. C. Fan, Y. J. Blumenfeld, Y. Y. El-Sayed, J. Chueh, S. R. Quake, *American journal of obstetrics and gynecology* **2009**, *200*, 543 e541-547; bE. A. Ottesen, J. W. Hong, S. R. Quake, J. R. Leadbetter, *Science* **2006**, *314*, 1464-1467.

[12] G. K. Fu, W. Xu, J. Wilhelmy, M. N. Mindrinos, R. W. Davis, W. Xiao, S. P. Fodor, *Proceedings of the National Academy of Sciences of the United States of America* **2014**, *111*, 1891-1896.

[13] aF. Di Giallonardo, O. Zagordi, Y. Duport, C. Leemann, B. Joos, M. Kunzli-Gontarczyk, R. Bruggmann, N. Beerenwinkel, H. F. Gunthard, K. J. Metzner, *PloS one* **2013**, *8*, e74249; bM. Mild, C. Hedskog, J. Jernberg, J. Albert, *PloS one* **2011**, *6*, e22741; cW. Shao, V. F. Boltz, J. E. Spindler, M. F. Kearney, F. Maldarelli, J. W. Mellors, C. Stewart, N. Volfovsky, A. Levitsky, R. M. Stephens, J. M. Coffin, *Retrovirology* **2013**, *10*, 18; dR. P. Smyth, T. E. Schlub, A. Grimm, V. Venturi, A. Chopra, S. Mallal, M. P. Davenport, J. Mak, *Gene* **2010**, *469*, 45-51; eA. Routh, P. Ordoukhanian, J. E. Johnson, *Journal of molecular biology* **2012**, *424*, 257-269.

[14] A. H. van Vliet, *FEMS microbiology letters* **2010**, *302*, 1-7.

FIGURES

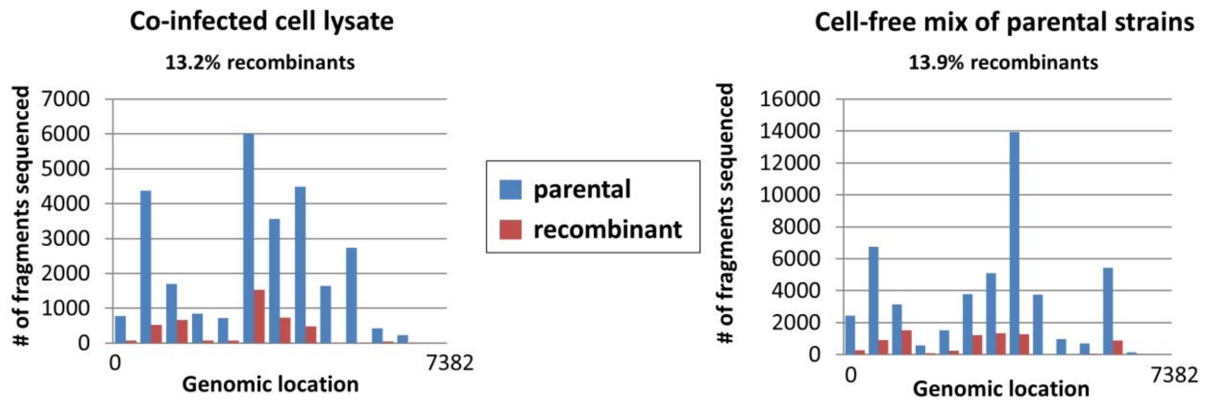

Figure S1. Next generation sequencing of murine norovirus RNA genomes. The number of fragments matching either of the two parental genomes (blue) and the number of recombinant fragments (red) is plotted along the MNV genome. The fraction of recombinant fragments found in lysate of co-infected cells (left) is roughly equal to that found in a cell-free mix of the two parental strains, ~13%. For experimental procedures see Supplementary Information.

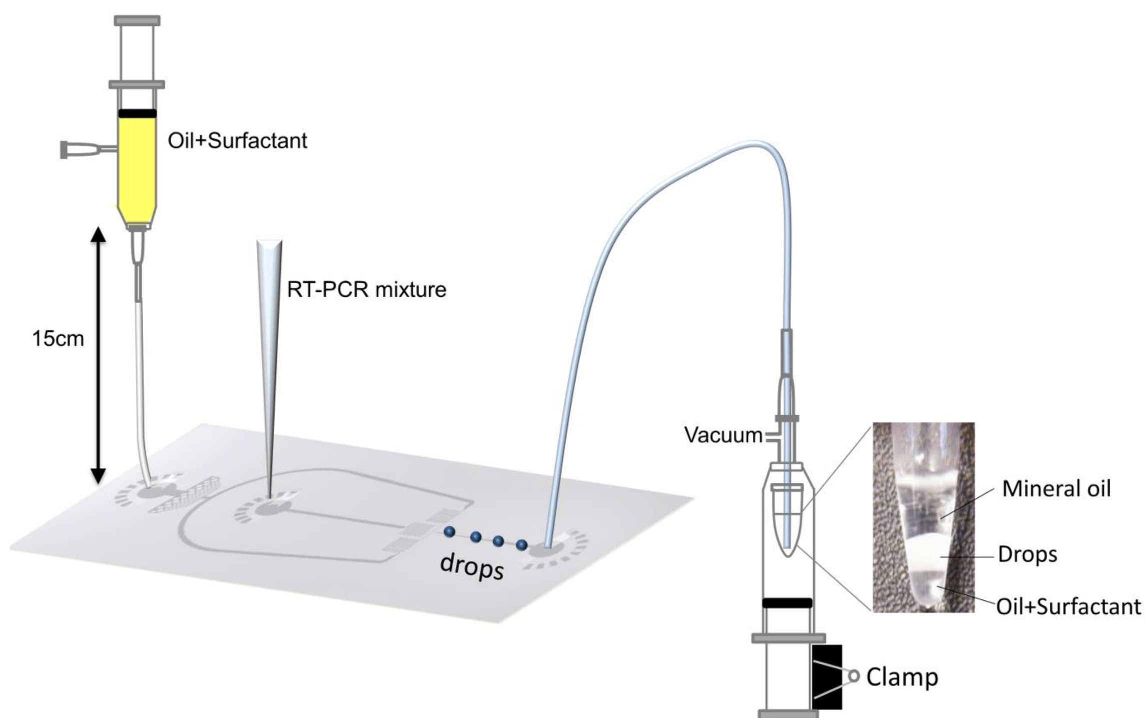

Figure S2. A microfluidic vacuum setup for drop making. The RT-PCR cocktail is first loaded into a gel-loading tip and the tip is inserted into the sample inlet of the microfluidic device. The fluorinated oil HFE-7500 containing 1% (w/w) surfactant is placed in a ventilated 10 mL plastic syringe located 15cm above the microfluidic device and connected into the oil inlet via tubing. We collect the drops via tubing into a PCR tube placed inside a plastic syringe equipped with a T-branch pipe. The syringe is kept at a negative pressure by connecting the T junction to a vacuum source and gluing the tubing sealed to a 18 TW needle fitted onto the T branch pipe. The benefits of a vacuum system compared to traditional syringe pumps are 1) no initial transients in drop size, 2) no dead volume of reagents remaining inside the syringe, tubing and device and 3) simplicity and low cost.

Address (nt)	4929	4950	4968	5092	5101	5106	5126	5155	5200	5239	5266	5287	5455
WU20	G	T	G	C	A	T	A	G	C	A	A	G	A
MNV-1	T	A	A	T	G	G	G	A	T	G	G	A	G
Drop-1	G	T	A	T	G	G	G	A	T	G	G	A	G
Drop-2	G	T	A	T	G	G	G	A	T	G	G	A	G
Drop-3	G	T	G	T	G	G	G	A	T	G	G	A	G
Drop-4	G	T	G	T	G	G	G	A	T	G	G	A	G
Drop-5	G	T	G	T	G	G	G	A	T	G	G	A	G
Drop-6	G	T	G	T	G	G	G	A	T	G	G	A	G
Drop-7	G	T	G	T	G	G	G	A	T	G	G	A	G
Drop-8	G	T	G	C	A	T	G	A	T	G	G	A	G
Drop-9	G	T	G	C	A	T	A	G	C	A	G	A	G

Figure S3. Recombinants obtained by Sanger sequencing of gel-verified drops. Summary of the recombination for the sequences obtained from four different drops, where the nucleotide addresses of 13 mismatches between WU20 and MNV-1 are shown. Each drop is aligned to different parental sequences at these loci. **3.** Recombinants obtained by Sanger sequencing of gel-verified drops. The sequences align with WU20 genome at the 5' end, with MNV-1 genome at the 3' end, and in between is the region of recombination where they align with both parental viruses. We identified four unique regions in the sequenced drops.