Figure S1

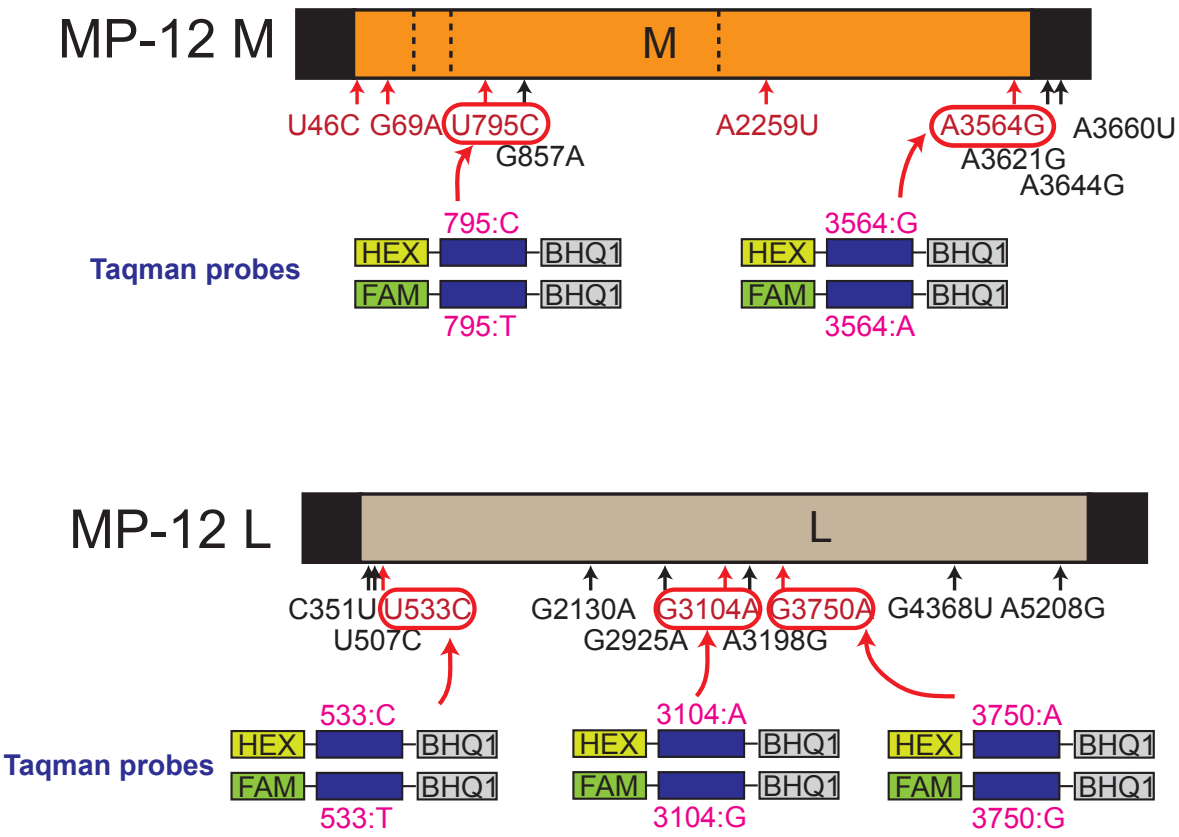

Fig. S1. Probe design for M-795, M-3564, L-533, L-3104, and L-3750. We designed two Taqman DNA probes at the M-795, M-3564, L-533, L-3104, and L-3750 sites. One probe was 5' conjugated with either hexachlorofluorescein (HEX; Absorbance max: 535 nm; Emission max: 556 nm) to detect parental MP-12 genotype, or 6-carboxyfluorescein (6-FAM; Absorbance max: 494 nm; Emission max: 518 nm) to detect mutant genotype reverted to pathogenic genotype. The other probe was 3' conjugated with Black Hole Quencher-1 (BHQ-1). Sequences of probes and primers are shown in Table S2.

Figure S2

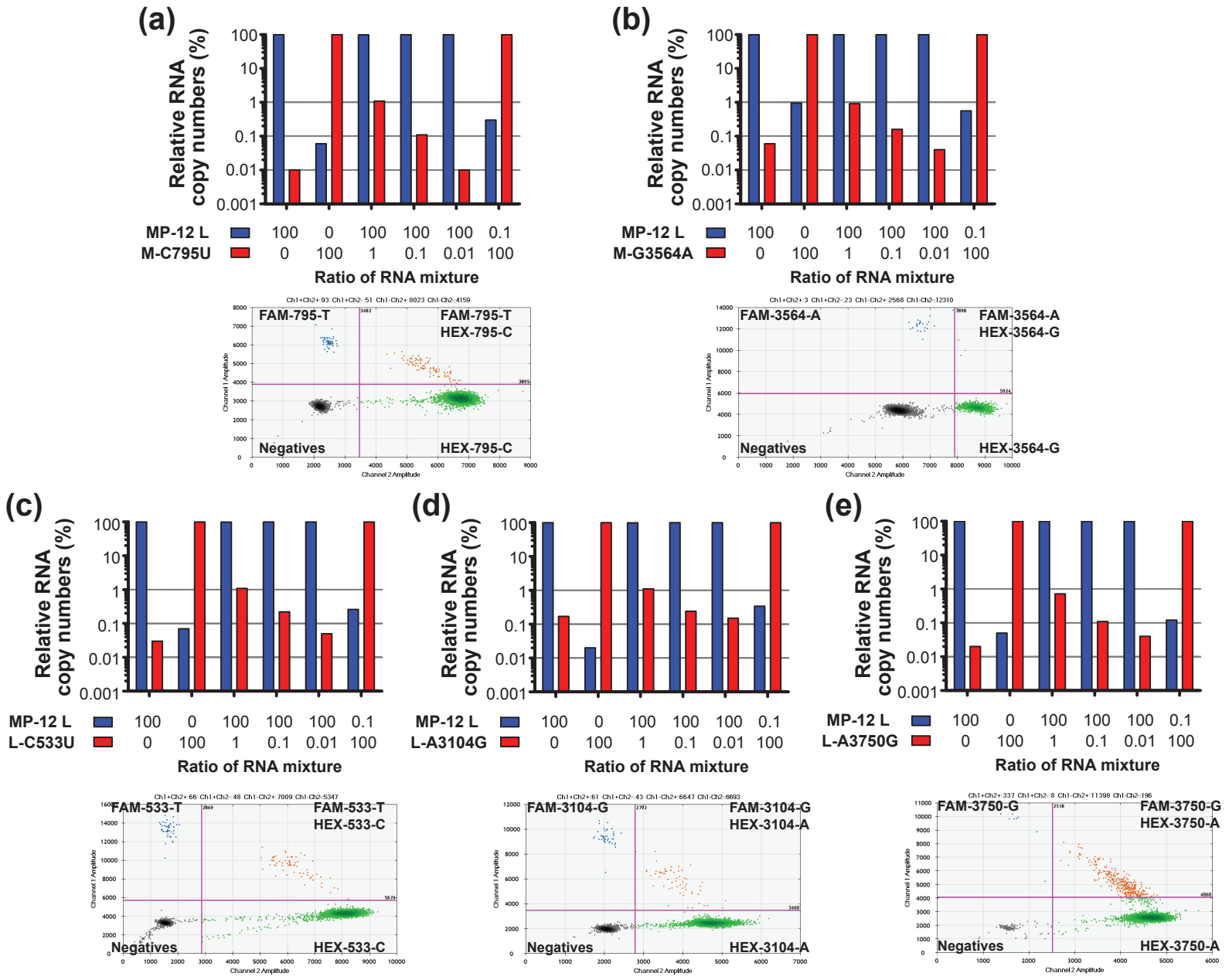

Fig. S2. Validation of ddPCR using Taqman probes for M-795, M-3564, L-533, L-3104, and L-3750. The accuracy of ddPCR assays were validated using five different Taqman probe sets. Full-length MP-12 M- or L-segment RNAs were in vitro synthesized by MEGAscript T7 Transcription kit (Thermofisher), using linearized pProT7-vM(+) or pProT7-vL(+) plasmids, respectively. In vitro synthesized full-length M- or L-segment RNAs encoding M-C795U, M-G3564A, L-C533U, L-A3104G, or L-A3750G (mutant RNA) were prepared. Synthesized parental MP-12 RNA and mutant RNA were mixed at ratios of 100:0, 0:100, 100:1, 100:0.1, 100:0.01, or 0.1:100. First-stranded cDNA was then synthesized and ddPCR was performed, as described in Material and Methods. The ddPCR results for M-795 (a), M-3564 (b), L-533 (c), L-3104 (d), and L-3750 (e) are shown. The graph represents the relative percentage of parental and mutant genotype RNA copy numbers. Bottom panels represent the raw images of QX100 Droplet Reader output [parental (HEX): mutant (FAM) = 100:1].

Table S1. Genome sequences of plaque clones from passage 25 samples in Vero cells

Virus	Segment	Gene	Location	nt. Mutation[1]	aa. Mutation[2]	Plaque clone #
MP-12 Vero P25 Exp-1	S	N	183	G to A	D to N	2,3
		N	761	C to U	-	1,2,3,4
		NSs	908	A to G	M to T	1,2,3,4
		NSs	1244	C to U	R to K	3
		NSs	1409	G to A	A to V	2,4
	M	78kD/NSm	171	G to A	E to K	1,2,3,4
		78kD/NSm	385	A to G	D to G	1,3
		Gn	587	C to U	-	1,3,4
		Gn	829	A to G	K to R	2
		Gn	1149	C to U	H to Y	2,3,4
		Gn	1204	A to G	K to R	2,3,4
		Gn	1876	A to G	N to S	2,3,4
		Gc	2646	G to A	G to R	1
		5' UTR	3648	G to A	-	3
	L	L	876	U to C	-	1
		L	2553	G to U	-	1,2
		L	5005	U to C	Y to H	1,2,3,4
		L	6066	U to C	-	1,2,3,4
rMP12-ΔNSs16/198 Vero P25 Exp-1	S	NSs	891	U to A	S to C	3
	M	78kD	99	G to A	E to K	2
		78kD/NSm	361	U to A	I to K	3
		Gn	877	A to U	Q to L	2,3,4
		Gn	1165	A to G	K to R	1,3
		Gn	1852	A to G	K to G	1,3
		Gn	1861	C to A	P to Q	2,4
		Gc	2907	U to C	-	1,2,3,4
		Gc	2909	G to C	L to F	1,2,3,4
	L	L	231	G to A	-	3
		L	3022	A to G	T to A	4
		L	3750[3]	A to G	I to M	1,2,3,4
		L	4584	G to A	-	2
		L	4602	G to A	-	2
		L	4971	U to C	-	1

[1]nt., nucleotide; [2]aa., amino acid. [3]Reversion mutation to parental ZH548 strain.

Table S2. Primers and probes for droplet digital PCR analysis

Mutation site	Name[2,3]	Sequence[1]
M-795	HEX-MP-M795-BHQ	5'-HEX-AGT CAG CTC ATC ACC TCA ACA-BHQ-3'
	FAM-ZH-M795-BHQ	5'-FAM-AGT CAG CTC ATT ACC TCA ACA-BHQ-3'
	Taq-M795F	5'-ACA CAC TGT CCA AAT GAC TAC C-3'
	Taq-M795R	5'-TAG GAG GGC ACT TGA CTG AA-3'
M-3564	HEX-MP-M3564-BHQ	5'-HEX-ATA TAT CTT GGA GGA ACA GGC CT-BHQ-3'
	FAM-ZH-M3564-BHQ	5'-FAM-ATA TAT CTT GGA AGA ACA GGC CT-BHQ-3'
	Taq-M3564F	5'-TTG GGC TCT TTT TCC TCC TT-3'
	Taq-M3564R	5'-CCT TCT TAG TGG CAG CAA GC-3'
L-533	HEX-MP-L533-BHQ	5'-HEX-CAT GGT GCA TGG TCT AAT CTG G-BHQ-3'
	FAM-ZH-L533-BHQ	5'-FAM-CAT GGT GTA TGG TCT AAT CTG G-BHQ-3'
	Taq-L533F	5'-GCA GGA CTG TTG TTC TTT ACG-3'
	Taq-L533R	5'-ACC TAT AAA CCA TCT CCT CTG CT-3'
L-3104	HEX-MP-L3104-BHQ	5'-HEX-TGC TCA ATG TTT ACC AAG AAA AGG A -BHQ-3'
	FAM-ZH-L3104-BHQ	5'-FAM-TGC TCA ATG TTT ACC AGG AAA AGG A-BHQ-3'
	Taq-L3104F	5'-GTG GCC GCT GAT CAT TAG G-3'
	Taq-L3104R	5'-ATC AAG CTC CCG ATG ACC AT-3'
L-3750	HEX-MP-L3750-BHQ	5'-HEX-CTC CTT AGC TGC AAT AAT TCA G-BHQ-3'
	FAM-ZH-L3750-BHQ	5'-FAM-CTC CTT AGC TGC AAT GAT TCA G-BHQ-3'
	Taq-L3750F	5'-GAA GTG GAA ACA CTA GTA GC-3'
	Taq-L3750R	5'-TGT AAT GGA GAG TAC ACT GA-3'

[1]A single nucleotide difference between two probes is underlined.

[2]HEX, hexachlorofluorescein; FAM, 6-carboxyfluorescein; BHQ, Black Hole Quencher-1.

[3]HEX and FAM probes specifically bind to MP-12 and ZH548 sequences, respectively.