



Figure S1. Comparison between 1 µL and 5 µL of the extracted RNA and different extraction protocols.

Lane 1 negative control (water-no virus), Lane 2—VEEV 7×10^5 PFU/mL, Lane 3—EEEV/Sinv 1.2×10^5 PFU/mL, Lane 4—VEEV 5×10^7 PFU/mL, Lane 5—VEEV 5×10^4 PFU/mL, Lane 6—VEEV 5×10^3 PFU/mL, Lane 7—EEEV/Sinv 4.5×10^5 PFU/mL, Lane 8—EEEV/Sinv 4.5×10^4 PFU/mL, Lane 9—EEEV/Sinv 4.5×10^3 PFU/mL, Lane 10—EEEV/Sinv 4.5×10^2 PFU/mL.

Table S1. Retro transcription protocol.

Master Mix Reagents	20 µL of Reaction	Cycle	Temperature	Time
Oligo dt-primers 100 µM	6.6 µM (1 µL)	1	50 °C	30 min
dNTP mix 10 µM	0.5 mM (1 µL)	1	95 °C	15 min
water	8 µL			
extracted sample	5 µL			
RT-Buffer 5×	1× (4 µL)			
Maxima H minus RT Enzyme mix 200 U/µL	0.5 U/µL (1 µL)			

Table S2. First phase PCR two steps format of the Grywna, Pfeiffer, and Sanchez protocols.

Grywna PCR Protocol				
Master Mix Reagents	12.5 μL of Reaction	Cycle	Temperature	Time
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
2×				
primer Gry 1 10 μM	0.5 μM	10 cycles touchdown 30 cycles	95 °C	20 s
primer Gry 2 10 μM	0.5 μM		65–56 °C	20 s
Retrotranscribed product	1 μL		72 °C	20 s
			95 °C	20 s
			55 °C	20 s
			72 °C	20 s

		final step	4 °C	infinite
Grywna nested PCR protocol. The first phase.				
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
primer Gry 3	0.2 μM		95 °C	20 s
primer Gry 4	0.1 μM	40 cycles	58 °C	20 s
primers Gry5	0.1 μM		72 °C	20 s
MgCl ₂ 25 mM	1.6 mM	final step	4 °C	infinite
DNA	1 μL			
Pfeffer PCR protocol.				
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
2×				
M2W forward primer 10 μM	1 μM		94 °C	20 s
cM3W reverse primer 10 μM	1 μM	35 cycles	50 °C	30 s
Retrotranscribed product	5 μL		72 °C	60 s
		1 cycle	72 °C	10 min
		final step	4 °C	infinite
Pfeffer-nested PCR.				
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
M2W2 forward primer 10 μM	1 μM		94 °C	20 s
cM3W reverse primer 10 μM	1 μM	30 cycles	55 °C	30 s
DNA	2 μL		72 °C	30 s
		1 cycle	72 °C	5 min
		final step	4 °C	infinite
Sanchez PCR protocol.				
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
2×				
Alpha1+ forward primer 20 μM	0.8 μM		94 °C	30 s
Alpha1- reverse primer 20 μM	0.8 μM	40 cycles	52 °C	60 s
Retrotranscribed product	5 μL		68 °C	30 s
		1 cycle	68 °C	5 min
		final step	4 °C	infinite
Sanchez-nested PCR.				
DreamTaq Hot Start Green Master Mix	1×	1	95 °C	3 min
2×				
Alpha2+ forward primer 20 μM	0.4 μM		94 °C	20 s
Alpha2- reverse primer 20 μM	0.4 μM	30 cycles	52 °C	30 s
DNA	1 μL		72 °C	15 s
		1 cycle	72 °C	5 min
		final step	4 °C	infinite

Table S3. Phase II, RT-PCR one-step format of the Pfeffer, Sanchez, and Torii protocols.

Pfeffer PCR One-Step RT-PCR Protocol.				
Master Mix Reagents	12.5 μL of Reaction	Cycle	Temp	Time
Buffer One step 5×	1×	1	50 °C	30 min
dNTPs 10 mM	0.4 mM	1	95 °C	15 min
M2W forward primer 10 mM	1 μM		94 °C	20 s
cM3W reverse primer 10 mM	1 μM	35 cycles	50 °C	30 s
Rnase inhibitor 4U/μL	0.04U/μL		72 °C	60 s
One-step RT-PCR Enzyme 25×	1×	1 cycle	72 °C	10 min
Retrotranscribed product	5 μL	final step	4 °C	infinite
Pfeffer-nested PCR protocol.				

DreamTaq Hot Start Green Master Mix 2×	1×	1	95 °C	3 min
M2W2 forward primer 10 mM	1 μM	30 cycles	94 °C	20 s
cM3W reverse primer 10 mM	1 μM		55 °C	30 s
Retrotranscribed product	2 μL		72 °C	30 s
		1 cycle	72 °C	5 min
		final step	4 °C	infinite
Sanchez PCR. The second phase.				
Buffer One step 5×	1×	1	50 °C	30 min
dNTPs 10 mM	0.4 mM	1	95 °C	15 min
Alpha1+ forward primer 20 μM	0.8 μM	40 cycles	94 °C	30 s
Alpha1- reverse primer 20 μM	0.8 μM		52 °C	60 s
Rnase inhibitor 4U/μL	0.04U/μL		68 °C	30 s
One-step RT-PCR Enzyme 25×	1×	1 cycle	68 °C	5 min
Retrotranscribed product	5 μL	final step	4 °C	infinite
Sanchez-nested PCR. The second phase.				
DreamTaq Hot Start Green Master Mix 2×	1×	1	95 °C	3 min
Alpha2+ forward primer 20 μM	0.4 μM	30 cycles	94 °C	30 s
Alpha2- reverse primer 20 μM	0.4 μM		52 °C	60 s
DNA	2 μL		72 °C	15 s
		1 cycle	72 °C	5 min
		final step	4 °C	infinite
Torii Protocol One-step RT-PCR.				
Buffer One step 5×	1×	1	50 °C	30 min
dNTPs 10 mM	0.4 mM	1	95 °C	15 min
Alpha1+ forward primer 20 μM	1 μM	43 cycles	94 °C	30 s
Alpha1- reverse primer 20 μM	1 μM		52 °C	30 s
Rnase inhibitor 4U/μL	0.04U/μL		72 °C	30 s
One-step RT-PCR Enzyme 25×	1×	1 cycle	72 °C	5 min
Retrotranscribed product	1 μL	final step	4 °C	infinite