

Supplementary Information

Evaluation of potential reference genes in the biting midge *Culicoides sonorensis* for real-time quantitative PCR analyses

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Supplementary Table S1

Reported Ct values of six candidate reference genes for seven experiments in this study.

Three biological replicates (each the average of two technical replicates) are reported for each experimental condition. Reference genes are *actin*, beta-tubulin (*β-tubulin*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S18 (*RPS18*), vacuolar-type ATPase subunit A (*VhaA*), and elongation factor 1-beta (*EF1b*).

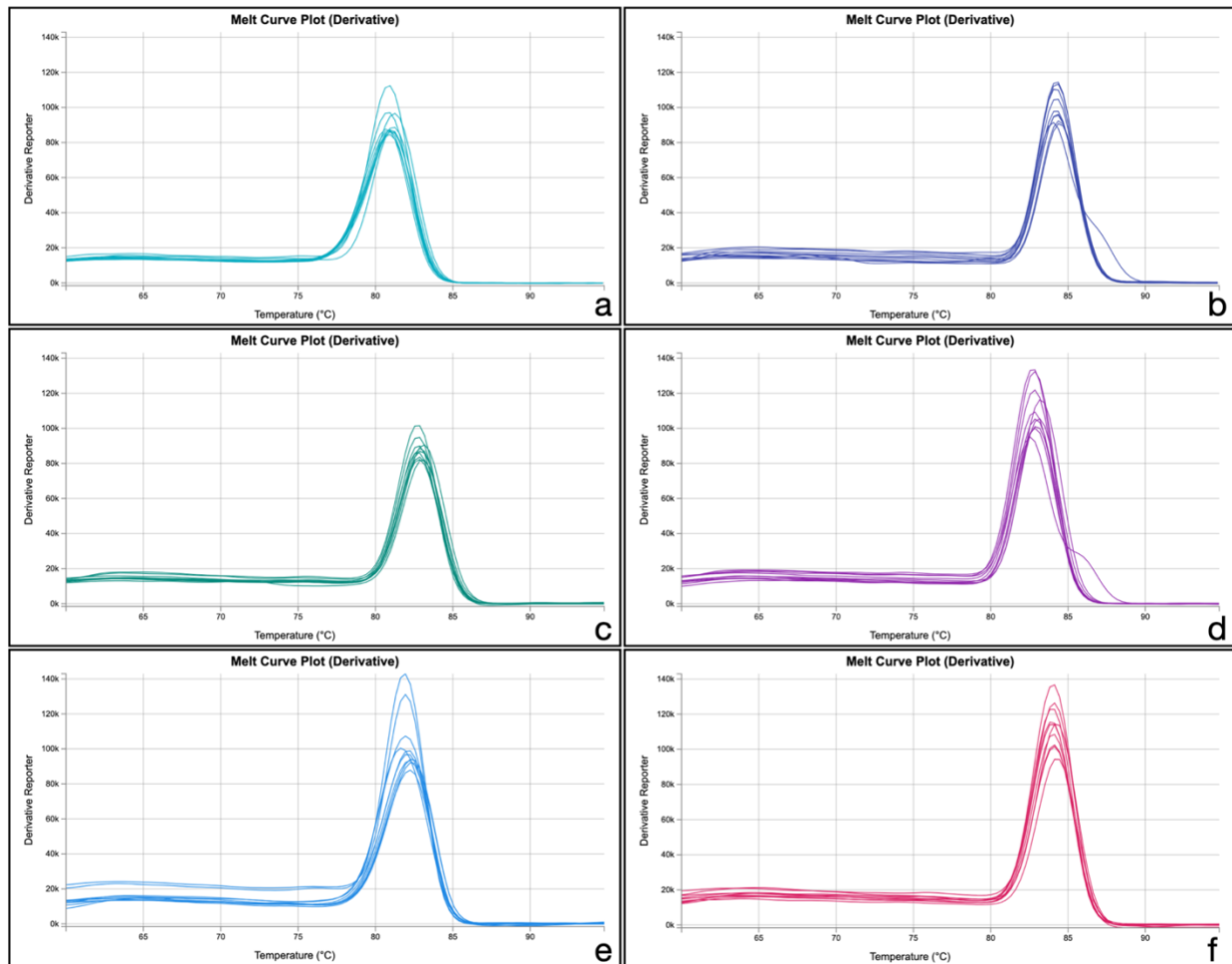
	<i>Actin</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>VhaA</i>
Cells treated with water or <i>dseGFP</i>						
Water	15.78	15.79	13.16	19.18	18.16	16.92
	16.48	16.59	13.88	19.96	18.85	17.69
	15.87	15.74	13.20	19.20	18.10	16.85
1 µg/mL <i>dseGFP</i>	16.29	16.26	13.59	19.65	18.48	17.39
	15.95	16.15	13.57	19.56	18.50	17.24
	15.91	16.05	13.43	19.43	18.42	17.01
100 ng/mL <i>dseGFP</i>	15.88	15.91	13.32	19.38	18.23	16.99
	15.76	15.78	13.19	19.21	18.12	16.86
	15.47	15.38	12.88	18.89	17.77	16.53
10 ng/mL <i>dseGFP</i>	15.47	15.58	13.07	18.91	18.05	16.74
	15.59	15.69	13.24	19.01	18.16	16.91
	15.39	15.44	13.00	18.84	17.84	16.69
Cells treated with <i>dseGFP</i> or <i>dsVhaA</i>						
1 µg/mL <i>dseGFP</i>	15.99	15.93	13.45	19.21	18.88	17.18
	17.62	18.90	14.51	19.97	19.81	19.57
	15.69	15.49	13.30	18.88	18.50	16.87
10 µg/mL <i>dsVhaA</i>	15.76	15.69	13.52	18.82	18.82	23.01
	15.73	15.63	13.26	18.69	18.65	23.23
	15.52	15.38	13.25	18.63	18.57	23.22
1 µg/mL <i>dsVhaA</i>	15.63	15.43	13.22	18.73	18.48	20.18
	15.57	15.60	13.06	18.73	18.41	20.41

	15.58	15.44	12.99	18.72	18.46	20.56
100 ng/mL ds <i>VhaA</i>	15.82	15.84	13.49	18.85	18.63	19.20
	15.60	15.48	13.33	18.63	18.44	18.81
	15.64	15.65	13.38	18.71	18.51	18.97
L₃ and L₄ larvae treated with <i>dseGFP</i> or <i>dsIAP1</i>						
L ₃ : 10 µg/mL <i>dseGFP</i>	19.62	18.61	13.77	21.44	17.29	17.20
	19.62	18.66	13.59	21.50	17.12	17.21
	18.95	18.21	13.21	20.77	16.68	16.78
L ₄ : 10 µg/mL <i>dseGFP</i>	18.79	18.04	13.18	20.84	16.64	16.55
	20.14	18.50	13.46	21.14	17.02	17.34
	19.40	18.45	13.49	20.96	16.90	16.99
L ₃ : 10 µg/mL <i>dsIAP1</i>	19.25	18.38	13.31	20.80	16.70	16.75
	19.03	18.21	13.30	20.83	16.67	16.87
	19.34	18.28	13.97	20.70	16.83	16.86
L ₄ : 10 µg/mL <i>dsIAP1</i>	19.41	18.46	13.68	21.03	16.60	17.00
	19.06	18.64	13.44	20.72	16.64	16.92
	19.40	18.35	13.64	20.79	16.84	17.26
Developmental Stages						
L ₃ Larvae	20.72	19.72	13.73	23.66	18.24	19.12
	20.76	18.99	14.01	23.64	18.28	18.93
	21.00	19.78	14.25	23.81	18.45	20.00
L ₄ Larvae	21.12	19.73	13.56	24.21	18.94	19.65
	20.89	19.60	13.44	23.98	18.81	19.61
	20.96	19.36	13.55	23.83	18.78	19.64
Male Pupae	20.52	18.58	16.11	21.99	19.03	18.35
	20.93	19.18	16.99	22.44	19.45	18.75
	20.84	18.91	15.97	22.36	19.80	18.62
Female Pupae	20.38	17.99	16.53	21.84	18.63	18.14
	20.51	18.79	16.62	22.19	18.97	18.16
	20.21	18.05	15.89	21.76	18.91	18.00
Males	21.60	20.95	14.97	23.52	20.26	19.32

	21.18	20.68	13.83	22.85	19.85	18.92
	21.28	20.80	13.64	22.97	20.16	19.04
Females	20.36	19.83	15.36	21.86	17.39	17.99
	20.22	19.49	14.85	21.93	17.72	17.67
	20.55	20.29	14.30	22.20	17.82	18.00
Female Body Parts and Tissue Groups						
Midgut	21.63	24.82	19.77	26.06	20.50	21.66
	21.02	23.77	19.04	25.08	20.05	21.04
	21.70	25.25	20.00	26.54	20.69	21.73
Remaining Gut	19.56	20.97	16.24	20.69	17.08	19.70
	20.54	21.37	17.30	21.77	18.92	19.43
	21.83	23.03	18.34	23.32	20.11	20.99
Remaining Body	20.82	25.51	17.59	23.95	19.12	20.15
	21.25	25.01	17.65	23.33	18.77	20.96
	21.06	25.19	17.71	23.43	19.30	20.68
Head	23.68	29.00	22.43	31.65	22.54	22.71
	22.83	28.27	21.58	32.87	21.36	21.36
	22.85	28.13	21.80	35.46	21.79	21.80
VSV-Injected Females						
Media Injected	19.78	19.75	16.59	21.91	17.25	19.04
	20.67	21.22	17.08	22.89	18.41	20.34
	21.78	22.22	18.22	23.95	19.39	21.32
VSV Injected	19.77	20.05	17.06	22.01	17.54	19.11
	19.63	19.97	16.68	21.99	17.31	18.97
	19.52	19.89	17.01	21.84	17.38	19.05
BTV-Injected Females						
Media Injected	20.01	20.19	17.03	22.06	17.70	18.97
	19.97	20.13	16.94	21.75	17.63	18.95
	20.03	20.21	17.01	22.02	17.82	19.17
BTV Injected	20.10	20.54	17.19	22.07	17.99	19.16
	20.12	20.32	17.62	22.05	17.94	19.23

	19.98	20.13	16.92	22.24	17.92	19.82
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Supplementary Figure S1. Melt curve analyses of six *Culicoides sonorensis* candidate reference genes. Analysis was performed on a QuantStudio 7 Pro (Thermo Fisher Scientific). Outputs are from the Design & Analysis Software (Release Version: 2.6.0, Thermo Fisher Scientific). The figure shows the melt curves for *EF1b* (a), β -*tubulin* (b), *VhaA* (c), *actin* (d), *RPS18* (e), and *GAPDH* (f).



Supplementary Table S2. Primers for dsRNA synthesis for *C. sonorensis* experiments.

Target	Accession/Source	Primer Sequence (5' ->3')	Product Size (bp)
ds <i>IAP1</i> ¹	GAWM01009039.1	F: [T7]-AGTTGAAGAACAACCTTGAGATGG	344
		R: [T7]-GCCAATCTTCATACGACACC	
ds <i>VhaA</i>	GAWM01018658.1	F: [T7]-CAACTGCAATACCAGGAGCG	409
		R: [T7]-CGTGACCTAAATATGCGGG	
dse <i>GFP</i> ²	Ac5-STABLE1-neo (Plasmid #32425)	F: [T7]-TGACCACCCTGACCTAC	304
		R: [T7]-TTGATGCCGTTCTTCTGC	

Abbreviations: bp, base pair; ds, double-stranded; *IAP1*, inhibitor of apoptosis 1; *VhaA*, vacuolar-type ATPase subunit A; *eGFP*, enhanced green fluorescent protein; [T7], T7 Promoter Sequence (TAATACGACTCACTATAGGG). Primer references are superscripted. Ac5-STABLE1-neo was a gift from Rosa Barrio & James Sutherland (Addgene plasmid # 32425 ; <http://n2t.net/addgene:32425> ; RRID:Addgene_32425).

Supplementary Table S3. Rankings from 1 (best) to 6 (worst) of six candidate C.

sonorensis reference genes under seven experimental conditions as determined by four analysis tools (RefFinder, BestKeeper, NormFinder, and geNorm). Comprehensive rankings are reported from RefFinder and this study's analyses.

Experiment	Algorithm	Ranking					
		1	2	3	4	5	6
Cells (dseGFP)	RF ^A : Delta Ct	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Normfinder	<i>β-tubulin</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>
	RF: geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>
	RF: Comprehensive	<i>GAPDH</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>
	BestKeeper	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>
	NormFinder	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>EF1b</i>	<i>Actin</i>
	geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>
	Comprehensive	<i>GAPDH</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>
Cells (dsVhaA)	RF: Delta Ct	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: Normfinder	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: Comprehensive	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	NormFinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>VhaA</i>
	geNorm	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	Comprehensive	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
Larvae (dsIAP1)	RF: Delta Ct	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>

	RF: Normfinder	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: geNorm	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	BestKeeper	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>
	NormFinder	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	geNorm	<i>VhaA</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>Actin</i>
	Comprehensive	<i>VhaA</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>Actin</i>
Life Stages	RF: Delta Ct	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>
	RF: Normfinder	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: geNorm	<i>RPS18</i>	<i>VhaA</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	BestKeeper	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>
	NormFinder	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	geNorm	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>GAPDH</i>
	Comprehensive	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
Female Tissues	RF: Delta Ct	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: BestKeeper	<i>VhaA</i>	<i>Actin</i>	<i>EF1b</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Normfinder	<i>GAPDH</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>
	RF: geNorm	<i>Actin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
	BestKeeper	<i>VhaA</i>	<i>Actin</i>	<i>EF1b</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	NormFinder	<i>GAPDH</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>	<i>RPS18</i>
	geNorm	<i>Actin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
VSV Injected	RF: Delta Ct	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>
	RF: Normfinder	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>

	RF: geNorm	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>
	BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>
	NormFinder	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	geNorm	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	Comprehensive	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>
BTV Injected	RF: Delta Ct	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: BestKeeper	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: Normfinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: geNorm	<i>Actin</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	BestKeeper	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	NormFinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	geNorm	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>
	Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>

^ARefFinder (RF) reports rankings for each of the other analysis tools (BestKeeper, NormFinder, geNorm) and includes the delta Ct ranking when creating a comprehensive rank. Bolded text call attention to comprehensive ranks.

Supplementary Table S4. Pairwise V score values for each of the experiments in this study as determined by the geNorm analysis. A value of 0.15 or greater suggests a significant contribution of adding another reference gene. The experiments were cells treated with water or varying concentrations of *dseGFP* (Cells (G)), cells treated with *dseGFP* or varying concentrations of *dsVhaA* (Cells (V)), larvae treated with *dseGFP* or *dsIAP1* (Larvae (I)), midge developmental stages (Stages), midge body parts or tissue groups (Body), and female midges injected with vesicular stomatitis virus (VSV) or bluetongue virus (BTV).

	Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV
V2/V3	0.00213	0.00562	0.00482	0.01246	0.01660	0.00222	0.00273
V3/V4	0.00180	0.00487	0.00329	0.01410	0.01861	0.00304	0.00249
V4/V5	0.00132	0.00932	0.00328	0.01223	0.01615	0.00291	0.00376
V5/V6	0.00147	0.02785	0.00366	0.02411	0.02514	0.00489	0.00391

References for Supplementary Materials

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<https://doi.org/10.1111/imb.12139>
- 2 Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K. & Whitfield, A. E. Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLoS One*. **8**, e70243 (2013). <https://doi.org/10.1371/journal.pone.0070243>