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Supporting Information

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RNA-Directed Packaging of Enzymes within Virus-like Particles**

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Supporting Information

A. Materials and Methods

1. Cloning

Peptidase E (AP_004522, NCBI) was first coded into a fusion with the Qβ coat protein in an attempt to create hybrid particles with enzymes expressed on the outer capsid surface. The PepE gene was amplified by PCR directly from One Shot Top10 (Invitrogen) *E. coli* with the primers pepE-F1 and pepE-R1 (Table S1). Overlap extension PCR with PepE gene PCR product and Qβ coat protein (CP) amplified with CP-F1 and CP-R1 resulted in CP fused to PepE through a 24 bp linker sequence, corresponding to amino acids GGASESGG. The fusion product was digested with NcoI and Xho, gel purified, and ligated into similarly digested pCDF-1b (Novagen) to make plasmid pCDF-CP-pepE. Dual expression of this plasmid and the corresponding plasmid coding for the coat protein alone gave rise to hybrid particles as previously described for other fusion domains.[1] However, the hybrid VLPs produced in this case proved to be difficult to purify due to their unstable nature and were not pursued.

Table S1. PepE and Qb coat protein primers used for production of a fused coat protein construct as well as encapsidated proteins. Overlap sequences are noted in italics; NcoI/XhoI sites are underlined.

primer name	primer sequence
pepE-F1	5'-*cgcgagcgaaagcggcggt*atggaactgcttttattgagtaa-3'
pepE-R1	5'-aagctggtcaccgtttttaactcgagcgg-3'
CP-F1	5'-catgccatggcaaaattagagactgttact-3'
CP-R1	5'-*cgctttcgctcgcgccacc*atacgctgggttcagct-3'
pepE-F2	5'-catgccatggaactgcttttattgagtaa-3'
pepE-his-F1	5'-catgccatgg*cacatcaccaccaccatcac* atggaactgcttttattgagtaac-3'
Luc-F2	5'-catgccatggaagacgccaaaaac-3'
Luc-R1	5'-gcggaaagtccaaattgtaactcgagcgg-3'
Luc-E354K-F1	5'-ctattctgattacacccaaaggggatgataaac-3'
Luc-E354K-R1	5'-gtttatcatccccttgggtgtaatcagaatag-3'

The Rev-pepE fusion was prepared as follows. The pepE gene was amplified by PCR from the pCDF-CP-pepE coding plasmid with primers pepE-F2 and pepE-R1, digested with NcoI and XhoI, gel purified and ligated into a similarly digested pCDF vector coding for the synthetic Rev-peptide in-frame and directly upstream from the NcoI site. For free PepE, amplification by PCR from the CP-pepE coding plasmid was performed with forward primer pepE-his-F1 (Table S1, sequence in bold corresponds to the hexahistidine motif) and pepE-R1. The resulting fragment was again digested and ligated into a similarly digested pCDF-1b vector, creating pCDF-pepE. Rev-pepE S120A was created by using site-selective mutagenesis with primers pepE-S120A-F1 and pepE-S120A-R1 to replace the active-site serine with alanine. This was fused to the Rev-peptide in the same manner as above. For Rev-luciferase, firefly luciferase was amplified by PCR from pRevTRE-Luc (Clontech) with primers Luc-F2 and Luc-R1 (Table S1). The resulting fragment was fused to the plasmid-encoded Rev peptide in the same manner as for Rev-pepE. The thermal-stable luciferase was generated by site-selective mutagenesis PCR using primers Luc-E354K-F1 and Luc-E354K-R1 to replace the glutamate at position 354 with a lysine. This was amplified and fused to Rev in the same manner as WT

luciferase.

All sequences were verified by direct sequencing of forward and reverse strands using unique primers at either ends (Retrogen). Plasmids were propagated in DH5α cells (BioPioneer) or One Shot Top10 (Invitrogen) and grown in SOB (Difco).

2. VLP Production

E. coli BL21 (DE3) (Invitrogen) cells harboring the appropriate plasmids were grown in either SOB (Difco or Amresco) or MEM[2] supplemented with carbenicilin, kanaymycin, or spectinomycin at 50, 100, and 100 μg/mL, respectively. Starter cultures were grown overnight at 37 °C, and were used to inoculate larger cultures. Induction was performed with 1 mM IPTG at an OD_{600} of 1.0 in SOB or 2.0 in MEM for 4 hours at 37 °C for all PepE constructs or 16 hours at 30 °C for luciferase constructs. Cells were harvested by centrifugation in a JA-17 rotor at 10K RPM. and were either processed immediately or stored as a pellet at -80 °C. The cell lysate was prepared by resuspending the cell pellet with 5 mL Qβ buffer (20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$) or TBS and sonicating at 30W for 3 min with 5-second bursts and 5-second intervals. Cell debris was pelleted in a JA-17 rotor at 14K RPM and 2M ammonium sulfate was added to the supernatant to precipitate the VLPs. These were pelleted and resuspended in 0.5 mL of Qβ buffer or TBS. Lipids and membrane proteins were then extracted from particles with 1:1 n-butanol:chloroform; VLPs remain in the aqueous layer. Crude VLPs were further purified by sucrose density ultra-centrifugation (10-40% w:v). Particles were either precipitated from the sucrose solution with 10% w:v PEG8000 or pelleted out by ultracentrifugation in a 70.1 Ti rotor (Beckman) at 70K RPM for at least 2 hours. After assessment of purity as described below, additional sucrose gradients were used to further purify VLPs to >95% if necessary.

The catalytic activity of Qβ@$(RevPepE)_n$ particles was unaffected by organic extraction extraction, but the catalytic activity of Qβ@$(RevLuc)_n$ particles was sensitive to such treatment. Therefore, the organic extraction step was omitted for the luciferase particles. No change in the number of proteins packaged per VLP was observed between samples that were extracted and those that were instead subjected to many rounds of sucrose gradient ultracentrifugation.

3. VLP Purification and Characterization

a. Purity and quantitation of encapsidated proteins. The purity of assembled VLPs was assessed by isocratic size-exclusion chromatography with a Superose 6 column on an Akta Explorer FPLC instrument. Non-aggregated Qβ particles elute approximately 3 mL after the void volume-associated peaks.

The protein content of each sample was analyzed with a Bioanalyzer 2100 Protein 80 microfluidics chip. The average number of encapsidated proteins was determined by normalizing the area integration of coat protein and cargo protein peaks to the calculated molecular weight of the proteins they signified, determining the molar ratio of coat protein to cargo protein and multiplying by 180 to obtain the number of cargo proteins loaded per VLP. Overall protein concentration was determined with Coomassie Plus Protein Reagant (Pierce) according to the manufacturer's instructions.

b. Electron microscopy. TEM images were acquired with a HP CM100 electron microscope (HP) with 80kV, 1s exposure and Kodak SO163 film on carbon formavor grids stained with 2% uranyl acetate.

c. Dynamic light scattering. Purified particles were analyzed on a light-scattering plate reader (Wyatt Dynapro), with the results shown below in Table S3.

d. Analytical ultracentrifugation (AUC). Sedimentation velocity experiments were performed on a Beckman XL-I analytical ultracentrifuge, using both absorbance (260 nm) and interference optics, giving the data shown in Figure S2. Experiments were run at 15,000 rpm at 25 °C, after a one-hour equilibration period. Data were fit to a "continuous species model" with Sedfit.[3] Estimated molecular weight of each VLP was obtained by assuming WT VLPs package the same amount of RNA as the

infectious virion (4200 nucleotides ssRNA). VLPs packaging an enzyme were estimated to package ~80% the amount of RNA of WT based on spectroscopic measurement at 260 nm of equal amounts of protein. Estimated molecular weight: WT(empty) and Qβ@$(RevLuc)_4$ = 3.8 MDa; Qβ@$(RevPepE)_{18}$ = 4.0 MDa. This corresponds to the differences of peak density constants obtained from AUC: WT(empty) = 76 S; Qβ@$(RevLuc)_4$ =79 S; and Qβ@$(RevPepE)_{18}$ = 86 S. Infectious virions, which package the RNA genome and infection-related proteins were calculated to have a density constant of 84 S *(16).* This suggests that the calculated densities are in the correct range of values and that we are able to significantly increase this density with our RNA-directed protein packaging system.

4. Free pepE Production and Purification

The conditions used for expression of free PepE were the same as used for the VLPs. To isolate the desired material, the cleared cell lysate was passed through a cobalt-NTA Talon resin column (0.5 mL bed volume). The column was washed with 3 column volumes of T buffer (20mM Tris-HCl pH 7.5), 3 volumes of T + 20 mM imidazole, 2 volumes of T + 100 mM imidazole and eluted with T + 300 mM imadazole. Fractions containing PepE were pooled and dialyzed against two changes of 2L of T and concentrated with an Amicon Ultra centrifugal filtration unit (10 kDa MWCO, Millipore). Purity was assayed by chip-based electrophoresis as above.

5. Enzymatic Activity

All experiments were run in triplicate and all runs with encapsidated enzyme were performed in parallel with purified free enzyme for comparison. All assays were performed with respect to the overall enzyme concentration, not the total protein concentration.

Peptidase E activity and kinetics were analyzed with the fluorescent substrate aspartate-4-amino-7-methyl-coumarin (Asp-AMC) (Bachem), using a Thermo Varioskan Flash plate reader (excitation 352 nm, emission 438 nm, 5 nm slit, 100 ms read time). For determinations of kinetic parameters, 95 µL of 0-0.8 mM substrate in PBS buffer was added to 5 µL of a 20x enzyme solution of His_6-PepE or Qβ@(Rev-PepE) and read immediately. VLPs that packaged an active-site knockout mutant (S120A) of pepE displayed no cleavage of the substrate.

For thermal protection studies, 60 µL of a 4.0 µg/mL solution (PepE concentration, in PBS) was incubated at the indicated temperature for 30 minutes. The solutions were then allowed to equilibrate to room temperature for 30 minutes before 50 µl was added to 50 µl of the substrate (0.6 mM final concentration). Initial velocities for every incubation temperature were normalized to the initial velocity of the free or packaged pepE incubated at 4 °C. To determine the thermal half-life of the enzymes, the assay was the same as above, except the temperature was maintained at either 45 °C or 50 °C. At the time point specified, 60 µL were taken and maintained at 4 °C until the end of the experiment. All samples were equilibrated to room temperature for 30 min and activity was assayed as described above. Initial velocities were normalized to activity at the t=0 time point for either 45 °C or 50 °C temperature. Activity measurements were plotted vs. time and an exponential decay non-linear fit was used to obtain half life values.

For protease protection studies, 0.2 mg of Proteinase K (Invitrogen, >20U/mg) was added to 150 µL of a 0.04 mg/mL His_6-pepE or Qβ@$(RevPepE)_9$ (in PBS) and incubated at room temperature. At the time points indicated, 5 µL aliquots were taken and 95 µL of substrate (0.76 mM final concentration Asp-AMC) was added and initial rates were measured. All data points were normalized to control treatments where proteinase K was not added. WT Qβ VLPs were added to the His_6-pepE samples to make the total protein concentrations in both samples equal.

Luciferase activity and kinetics were assayed by measuring the intensity of luminescence induced with D-luciferin (Anaspec, Inc.) in the plate reader. Purified luciferase (US Biological) was reconstituted as recommended by the manufacturer and aliquots were stored at -80° C and thawed immediately before use. K_m values for D-luciferin and ATP were identified using a range of concentrations of each substrate (0-2 mM and 0-3 mM, respectively) in 30 mM HEPES pH 7.5 with 15

mM $MgSO_4$, 0.16 nM enzyme (final concentrations). Activity was initiated by injecting 50 µL of a 2x enzyme solution into 50 µL luciferin or ATP of varying concentrations with all other components. Luminescence was measured immediately for 10 s. This emission intensity was plotted vs substrate concentration of the varied reagent. A Michaelis-Menten non-linear fit was used to obtain $K_{m,app}$ and V_{max} values. For time course measurements, luminescence was measured for 1 s every 2 min.

For luciferase, absolute k_{cat} values are difficult to determine because the conversion between light output and number of catalytic turnovers is not clearly quantified. However, values of k_{cat} could be calculated by assuming that the output light intensity at saturation represents V_{max} of free luciferase in all experiments. Saturating relative light units can then be converted to turnovers by converting the specific activity of the enzyme into molecules of pyrophosphate released.

B. Supplemental Data

1. Aptamer-Guided Packaging

We sought to control the number of encapsulated enzymes per VLP by removing components of the Rev–αRev RNA aptamer interaction, by changing the expression media, or by changing the Mg^{2+} concentration. The results are given in Table S2. The enzymes were found to differ in their media-dependent packaging efficiency. In SOB, the omission of either the Rev peptide or the bifunctional RNA gave rise to substantially less efficient packaging (entry 1 *vs.* 5 and 7). In MEM media, the Rev-tag was more important than the bifunctional RNA (entries 3 *vs.* 6,8).

Table S2. Encapsulated enzyme molecules per VLP as a function of plasmid and expression conditions.

Entry	Bifunctional RNA	Rev tag	Cargo	Exp. media	[$MgSO_4$] (mM)	# per VLP [a]	Yield [b]	N
1	+	+	pepE	SOB(D)	20	18 ± 2	76	4
2	+	+	pepE	SOB(A)	20	12.7 ± 0.5	47	3
3	+	+	pepE	MEM	2	8 ± 1	45	12
4	+	+	pepE	MEM	20	5.1 ± 0.8	23	9
5	–	+	pepE	SOB(D)	20	8.5 ± 0.6	41	3
6	–	+	pepE	MEM	2 or 20	6.9 ± 0.1	58	2
7	+	–	pepE	SOB(D)	20	1.6 ± 0.6	98	3
8	+	–	pepE	MEM	2	0	150	1
9	+	+	Luc	SOB(D)	20	4	140	1
10	+	+	Luc	MEM	2	0	100	3
11	+	+	Luc	MEM	20	5	150	1
12	–	+	Luc	MEM	20	8 ± 1.8	140	3
13	+	+	tsLuc	SOB(D)	20	2.7 ± 0.5	100	4
14	+	+	tsLuc	MEM	20	10 ± 1	77	2
15	–	+	tsLuc	MEM	20	7.4	58	1

(a) Average number of enzymes packaged within each VLP, determined as explained above. The reported error is the standard deviation among "N" independently expressed and purified samples. (b) Average amount of purified VLPs (mg) obtained from 1 liter of bacterial culture as estimated by Bradford Assay. MEM: Minimal Expression Media, SOB(D): SOB from Difco, SOB(A): SOB from Amresco.

It therefore appears that the positively-charged Rev tag promotes pepE packaging by itself, but more effectively when paired with the αRev aptamer. RevLuc and RevtsLuc VLPs were less extensively explored, but it appears that a higher concentration of Mg^{2+} ions is required to package either enzyme when MEM is the expression medium. As noted in the text, luciferase encapsidation was less variable, probably because its much larger surface area offers more opportunity for

noncovalent interaction with the coat protein and/or packaged RNA. Interestingly, the maximum amount of packaged enzyme in the two cases was approximately the same in terms of mass (approx. 600 KDa), composed either of approximately 20 PepE or 10 for Luc molecules. This suggests 600 KDa as an overall limit for this methodology. Although it is enticing to attempt to package the largest amount of cargo possible, more may not always be better for enzymatic catalytic activity (Table 1).

2. Characterization Data

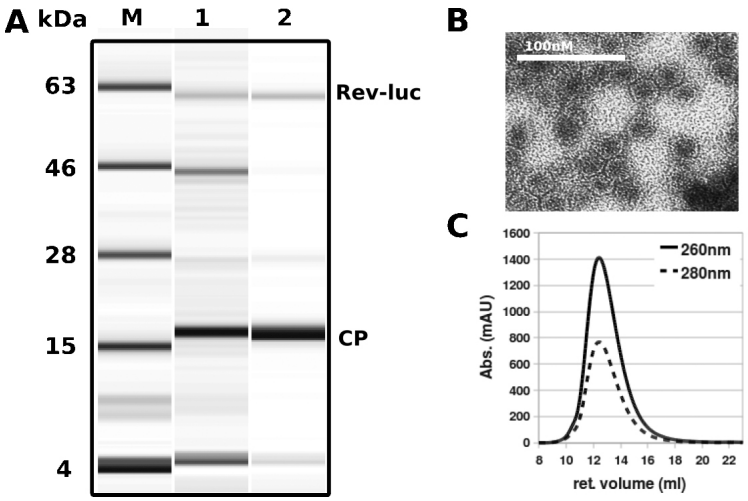


Figure S1. Characterization of Qβ@(RevLuc)$_8$. (A) Transmission electron micrograph with uranyl acetate staining; images are indistinguishable from those of WT Qβ VLPs. (B) Graphical representation of microfluidic chip electropherogram (Agilent Bioanalyzer Protein-80 analysis chip) M: protein ladder marker, 1: WT Qβ VLPs, 2: *E. coli* cells 16 h after induction with IPTG, 3: purified Qβ@(RevLuc)$_4$ VLPs showing CP and RevLuc band (C) Size-exclusion FPLC (Superose 6).

Table S3. Radii of Qβ VLPs encapsidating enzymes by dynamic light scattering. The packaging of different proteins in different amounts had no significant effect on the size of the VLPs. No aggregation was observed in measurements at different concentrations.

Name	Radius (nm)	St. Dev
WT Qβ VLP	14.2	0.5
Qβ@(RevPepE)$_{18}$	14.3	0.6
Qβ@(RevPepE)$_9$	15.6	0.7
Qβ@(RevLuc)$_{10}$	14.6	1.5
Qβ@(RevLuc)$_4$	16.6	0.5
Qβ@(RevtsLuc)$_9$	13.9	0.3
Qβ@(RevtsLuc)$_2$	13.7	0.4

Analytical Ultracentrifugation (AUC, Figure S2). A minor component of lower density and unknown structure was evident in each case, even when the particles were purified over multiple sucrose gradients with no contaminating protein evident by denaturing gel electrophoresis or size-exclusion FPLC. We therefore suspect that this is an experimental artifact.

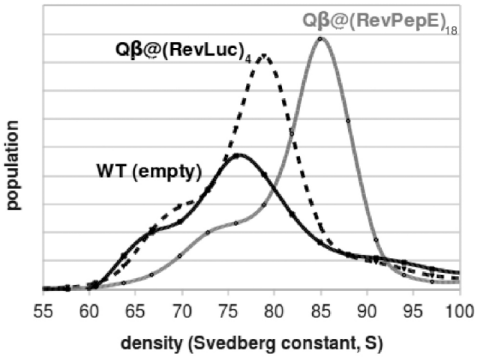


Figure S2. Analytical ultracentrifugation of WT and enzyme-loaded VLPs. Results are typical of three independent runs. The distribution maxima reported in the text are for the major peak in each case; all samples showed a proteinaceous component of lower density.

Protection from Denaturation and Adsorption (Figures S3–S5)

Figure S3. Determination of enzymatic half-life at elevated temperature. Samples of Qβ@(RevPepE)$_9$ and His$_6$-pepE were incubated at 45 °C and 50 °C for the indicated time, cooled to room temperature and then assayed for activity with Asp-AMC; the activity before incubation (t = 0) was set to 100%. Data were fit to a exponential decay curve (line) to determine half-life.

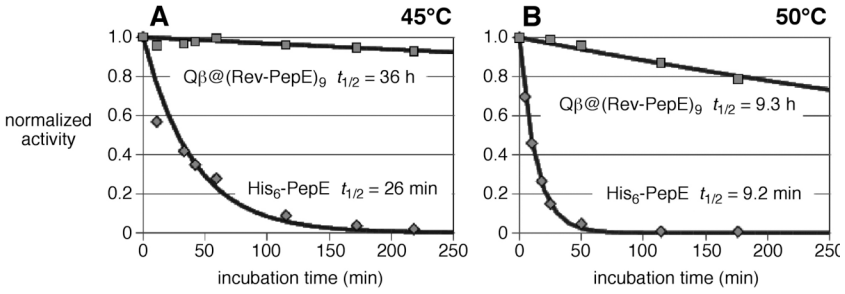


Figure S4. Size-exclusion FPLC of Qβ@(RevPepE)$_9$ before and after incubation at 55 °C for 30 min. The observed elution volumes and A_{260}/A_{280} ratios are consistent with intact particles in both cases, showing that the VLP is not degraded under these conditions (solid lines = absorbance at 260 nm; dashed lines = absorbance at 280 nm).

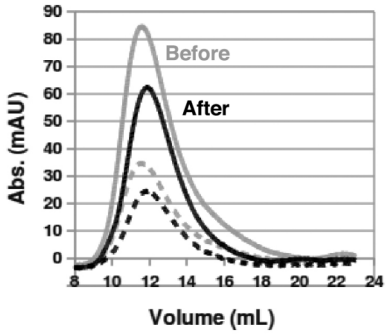
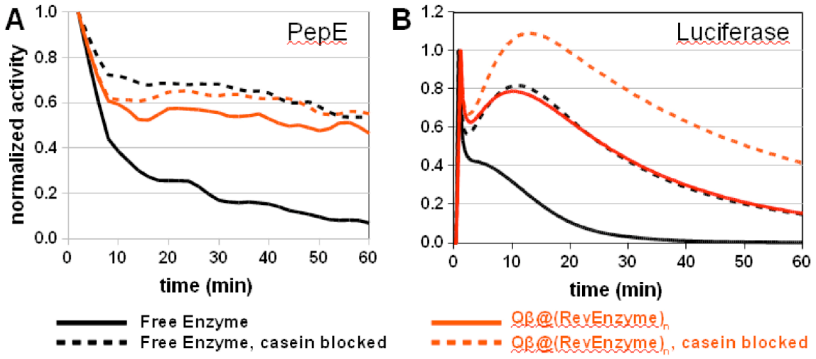


Figure S5. Activities of free vs. encapsulated enzymes in polystyrene microtiter plates, with and without blocking of the wells with purified casein (1 mg/mL).
(A) Qβ@(RevPepE)$_{18}$ vs. His$_6$-PepE. Data normalized to activity at t=0
(B) Qβ@(RevLuc)$_4$ vs. free luciferase. Data normalized to the initial light output at the "burst" time point.



Representative Kinetics Measurements (Figure S6)

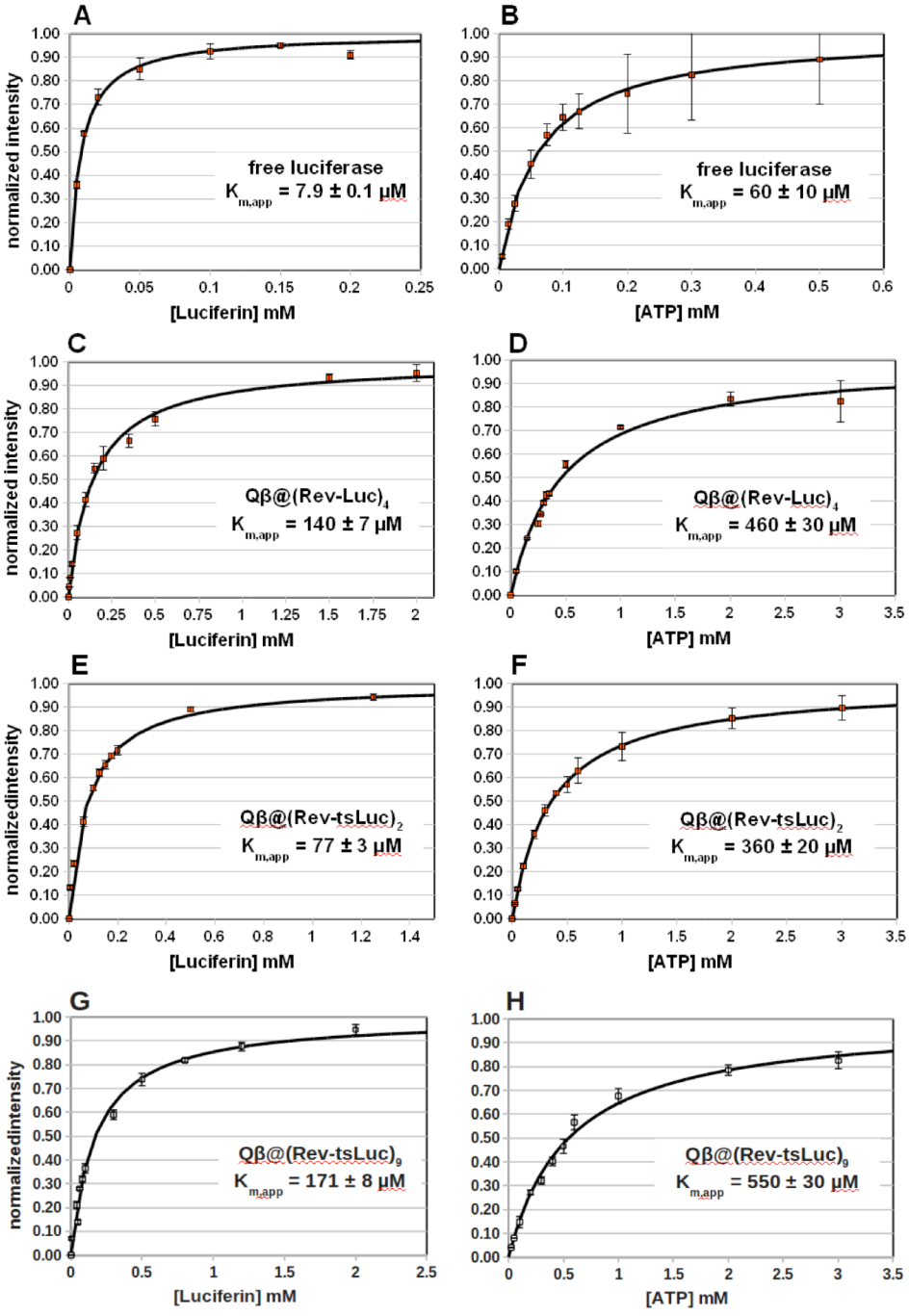


Figure S6. Kinetics of free luciferase and VLPs encapsidating RevLuc and RevtsLuc enzymes. Reactions were initiated by injection of enzyme into the well and the first ten seconds of light output were integrated and plotted for different concentrations of luciferin (A, C, E, G; holding ATP constant at 3 mM) and ATP (B, D, F, H; holding luciferin constant at 2 mM). Error bars are the standard deviations of triplicate measurements. Each line is the Michaelis-Menton fit to the data with the kinetic value indicated. All data were normalized to the calculated V_{max}. Assay conditions: 30 mM HEPES buffer, pH 7.8, 15 mM $MgSO_4$, 0.16 nM enzyme

References

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2. Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **41**, 207-234 (2005).
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