Supplementary Information

Simple, low-cost production of DNA MS2 virus-like particles as viral diagnostic controls

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Supplementary Tables

Supplementary Table 1. Oligonucleotides required for exogenous DNA amplification

Process	Forward Sequence	Reverse Sequence
Exogenous DNA [T7 Exonuclease]	A*A*C*A*T*GAGGATTACCCATGTATGGCTGCTAGGCTGTACTGC	TTAAATGTATACCCAGAGACAAAAGAAAATTG
	ATGGCTGCTAGGCTGTACTGC	T*T*A*A*A*TGTATACCCAGAGACAAAAGAAAATTG
Exogenous DNA [Lambda Exonuclease]	AACATGAGGATTACCCATGTATGGCTGCTAGGCTGTACTGC	/5Phos/TTAAATGTATACCCAGAGACAAAAGAAAATTG
	/5Phos/ATGGCTGCTAGGCTGTACTGC	TTAAATGTATACCCAGAGACAAAAGAAAATTG

Supplementary Table 2. Oligonucleotides required for qPCR and ddPCR quantification (derived from a previously published assay[1]).

Set	Forward Sequence	Reverse Sequence	Probe Sequence
A	GTCCTCCAATTTGTCCTGG	TGAGGCATAGCAGCAGGAT	/56-FAM/CTGGATGTG/ZEN/TCTGCGGCGTTTTATCAT/3IABkFQ/
B	CACCTGTATTCCCATCCCATC	AGCCCTACGAACCACTGAACA	/5HEX/AAACGGACT/ZEN/GAGGCCCACTCCCA/3IABkFQ/

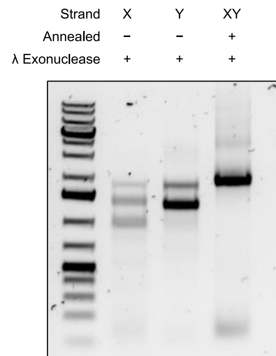
Supplementary Table 3. Cost comparison between the method described by Zhang et al. and the method described in this study for the production of a single DNA VLP. Costs of common consumables like pipette tips and chemicals used to make buffers have been omitted. Pricing is current as of August 2022, but could be subject to change.

					Zhang et al.[2]	This Study
Stage	**Item**	**Manufacturer**	**Catalog Number**	**List Price**	**Per VLP**	**Per VLP**
Exogenous DNA Production	DNA Synthesis (1000 bp)	Twist Bioscience		£50	£50	£50
	Oligo Synthesis	IDT			£100	£45
	Q5® High-Fidelity 2X Master Mix	NEB	M0492S	£134	£21	£43
	Amicon® Ultra 0.5mL 3kDa	Merck Millipore	UFC500308	£46.60	£5.83	
	Amicon® Ultra 0.5mL 50kDa	Merck Millipore	UFC505008	£46.60	£11.65	
	Zeba™ Spin Desalting Columns, 40K MWCO, 0.5 mL	ThermoFisher Scientific	87766	£154.00	£6.16	
	T7 Exonuclease	NEB	M0263S	£57		£18
	AMPure XP	Beckman Coulter	A63880	£230.90		£3.23
	Amicon® Ultra 0.5mL 30kDa	Merck Millipore	UFC503008	£46.60		£5.83
	TE Buffer	ThermoFisher Scientific	12090015	£47	£0.47	£0.47
				Total DNA production	£195.54	£166
Protein Purification	TURBO™ DNase	ThermoFisher Scientific	AM2238	£125	£18.75	£18.75
	RNase A	Qiagen	19101	£225	£9.00	£9.00
	Basemuncher	Abcam	ab270049	£155	£15.50	£15.50
	HiPrep 16/60 Sephacryl S-200 HR	Cytiva	17116601	£585	£585	
	HiLoad 16/600 Superdex 75 pg	Cytiva	28989333	£1,904	£1,904	
	SnakeSkin™ Dialysis Tubing, 10K MWCO, 22 mm	ThermoFisher Scientific	68100	£184	£5	
	Minisart® Syringe Filter, SFCA, Pore Size 5 mm	Sartorius	17594	£848		£1.70
	HiTrap TALON crude	Cytiva	28953767	£510		£102
	Amicon® Ultra-15 10 kDa (2)	Merck Millipore	UFC901008	£97.90		£24.48
	Amicon® Ultra-15 30 kDa	Merck Millipore	UFC903008	£97.90		£12.24
				Total Protein Purification	£2,537.36	£183.71
				Total Cost	**£2,732.90**	**£349.36**

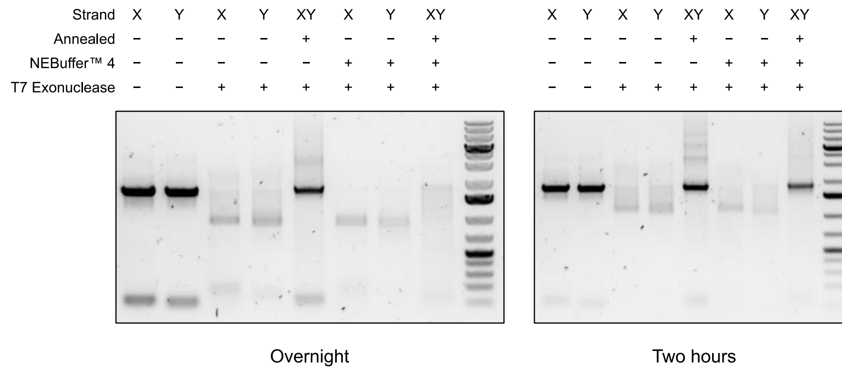
Supplementary Table 4. Raw Roche® cobas 6800 results for the diluted International Standard and VLP samples using the Roche cobas® HBV assay.

Sample	Target 1 Ct	Measured IU/mL	QS Ct	QS Result	Valid
95500	21.92	107000	32.43	Valid	Yes
95500	22.08	98200	32.46	Valid	Yes
95500	22.09	85100	32.26	Valid	Yes
9550	25.28	15100	32.93	Valid	Yes
9550	25.45	8390	32.24	Valid	Yes
9550	25.4	9140	32.32	Valid	Yes
995	28.73	940	32.33	Valid	Yes
995	28.7	1010	32.41	Valid	Yes
995	28.85	1030	32.58	Valid	Yes
99.5	32.09	98	32.4	Valid	Yes
99.5	31.86	115	32.4	Valid	Yes
99.5	31.88	147	32.77	Valid	Yes
VLP	28.81	932	32.4	Valid	Yes
VLP	28.8	835	32.23	Valid	Yes
VLP	29.13	908	32.68	Valid	Yes
VLP	28.96	756	32.25	Valid	Yes
Blank	-	Target Not Detected	32.41	Valid	Yes

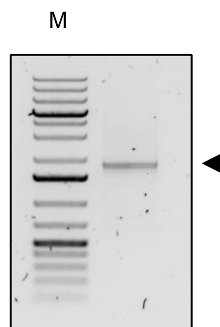
Supplementary Figures



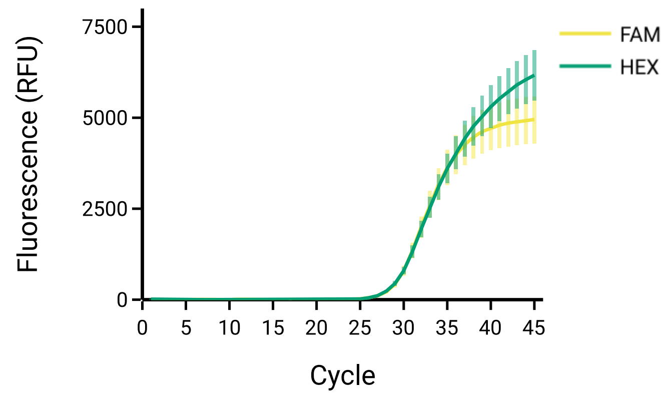
Supplementary Figure 1. Agarose Electrophoresis showing digestion with λ Exonuclease. λ Exonuclease was added directly to the completed PCR reactions without reaction buffer.



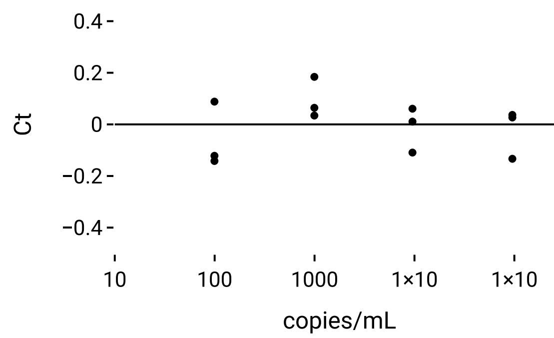
Supplementary Figure 2. Agarose Electrophoresis showing optimisation of digestion conditions with T7 Exonuclease. Reactions were tested with and without buffer, individually and after adding F and R together and with a two hour digestion or overnight at 25 °C.



Supplementary Figure 3. Agarose Electrophoresis with DNA Ladder (M) and magnetic bead purified product after digestion and annealing (indicated with the arrow).



Supplementary Figure 4. Raw qPCR curves of DNA MS2 VLPs detected with the duplex assay[1]. Error bars represent the SD of n = 5 technical replicates.



Supplementary Figure 5. Residuals obtained after fitting the standard curve using linear regression (SEq 1).

Supplementary Methods

Production of partially single stranded DNA for packaging using λ Exonuclease

Two sets of PCR reactions were setup to create the exogenous DNA for packaging. The X PCR was performed using a phosphorylated reverse primer and unmodified forward primer and the Y PCR was performed with a phosphorylated forward primer and unmodified reverse primer. PCR reactions were performed with a final primer concentration of 1 µM using Q5® High-Fidelity 2X Master Mix (NEB). Equal volumes of X and Y PCR reactions were then combined, supplemented with 0.2 U/µL of λ Exonuclease (ThermoFisher Scientific) and incubated at 37 °C for 30 minutes before being heated to 95 °C and slowly annealed (-0.1 °C/sec) in a thermocycler.

Concentration and error calculation for working standard calibration

The working standard calibration was performed using linear regression and the error was calculated using a stastistical tool[3] based on Fieller's theorem[4]. For completeness the equations below are included as found in Pizzamiglio et al[3].

The data from the standard curve was initially fit by linear regression to SEq 1. The residuals obtained using the fitted equation can be visualized in Supplementary Figure 5.

$$y_{ij} = \beta_0 + \beta_1 x_i \qquad \textbf{SEq 1}$$

where y_{ij} specifies the value of the Ct values determined for the j-th replicate (j = 1,2,...,Ji) at log x_i different (i = 1,2,...,I) WHO international standard dilutions.

PCR efficiency could then be calculated using SEq 2:

$$\text{Efficiency} = 10^{(-1/\beta_1)} - 1 \qquad \textbf{SEq 2}$$

The estimate of the unknown log starting concentration ($\hat{x}_0$) of the unknown VLP concentration (x_0) is then calculated by substituting the mean Ct of the replicates ($\bar{y}$) of the VLP working standard into SEq 3.

$$\hat{x}_0 = \frac{\bar{y} - \beta_0}{\beta_1} \qquad \textbf{SEq 3}$$

The variance for the standard was then estimated using SEq 4.

$$s_p^2 = \frac{\sum_{i=1}^{I} \sum_{j=1}^{J_i} (y_{ij} - \bar{y}_i)^2}{\sum_{i=1}^{I} (J_i - 1)} \qquad \textbf{SEq 4}$$

where $\bar{y}_i$ is the mean of the Ct values at the i-th standard dilution

We can then define the deviance (s_{xx}) of the x_i values as SEq 5:

$$s_{xx} = \sum_{i=1}^{I} J_i (x_i - \bar{\bar{x}})^2 \qquad \textbf{SEq 5}$$

And the mean ($\bar{\bar{x}}$) of the x_i values as SEq 6:

$$\bar{\bar{x}} = \frac{\sum_{i=1}^{I} J_i x_i}{\sum_{i=1}^{I} J_i} \qquad \textbf{SEq 6}$$

$t_{f;1-\alpha/2}$ is the value which corresponds to a critical value where the significance is α and there are f degrees of freedom.

The confidence intervals of x_0 are obtained by calculating the roots of the quadratic equation SEq 7.

$$Ax^2 + 2Bx + C = 0 \qquad \textbf{SEq 7}$$

where:

$$A = \beta_1^2 - \frac{s_p^2}{s_{xx}} t_{f;1-\alpha/2}^2 \qquad \textbf{SEq 8}$$

$$B = \beta_0\beta_1 - \bar{y}\beta_1 + \frac{s_p^2 \bar{\bar{x}}}{s_{xx}} t_{f;1-\alpha/2}^2 \qquad \textbf{SEq 9}$$

$$C = \bar{y}^2 + \beta_0^2 - 2\bar{y}\beta_0 - \frac{s_p^2}{K} t_{f;1-\alpha/2}^2 - \left(\frac{s_p^2 \sum_{i=1}^{I} J_i x_i^2}{s_{xx} \sum_{i=1}^{I} J_i} \right) t_{f;1-\alpha/2}^2 \qquad \textbf{SEq 10}$$

finally, by defining g as:

$$g = \frac{s_p^2 t_{f;1-\alpha/2}^2}{s_{xx} \beta_1^2} \qquad \textbf{SEq 11}$$

We can obtain the two roots of SEq 7 and the required confidence limits of x_0 using SEq 12:

$$\left.\begin{matrix} \hat{x}_{upper} \\ \hat{x}_{lower} \end{matrix}\right\} = \hat{x}_0 + \frac{(\hat{x}_0 - \bar{\bar{x}})g \pm (s_p t_{f;1-\alpha/2}/\beta_1)\{[(\hat{x}_0 - \bar{\bar{x}})^2/s_{xx}] + (1-g)(1/K + 1/n)\}^{\frac{1}{2}}}{1-g} \qquad \textbf{SEq 12}$$

The prediction limits of the Ct value of the VLP sample ($\bar{y}$) are then calculated using SEq 13:

$$\left.\begin{matrix} \hat{y}_{upper} \\ \hat{y}_{lower} \end{matrix}\right\} = \bar{y} \pm t_{f;1-\alpha/2} s_p \left(\frac{(x - \bar{\bar{x}})^2}{s_{xx}} + \frac{1}{K} + \frac{1}{n} \right)^{\frac{1}{2}} \qquad \textbf{SEq 13}$$

Variable	Value
β_0	38.66645943863722
β_1	-3.335984211745958
PCR Efficiency	0.9941673919755294
$\bar{y}$	28.925
$\hat{x}_0$	831.9851413821814
s_p^2	0.00981666666666674
s_{xx}	14.787116116160096
$\bar{\bar{x}}$	3.4889132261647355
g	0.00031721429593360836
x_u (95% PI)	757.1503874590653 - 913.763660990488
$\bar{y}_u$ (95% PI)	28.78882848628346 - 29.061171513716534

Supplementary References

(1) Sun, S.; Meng, S.; Zhang, R.; Zhang, K.; Wang, L.; Li, J. Development of a New Duplex Real-Time Polymerase Chain Reaction Assay for Hepatitis B Viral DNA Detection. *Virol J* 2011, *8* (1), 227. https://doi.org/10.1186/1743-422x-8-227.

(2) Zhang, L.; Sun, Y.; Chang, L.; Jia, T.; Wang, G.; Zhang, R.; Zhang, K.; Li, J. A Novel Method to Produce Armored Double-Stranded DNA by Encapsulation of MS2 Viral Capsids. *Appl Microbiol Biot* 2015, *99* (17), 7047–7057. https://doi.org/10.1007/s00253-015-6664-4.

(3) Pizzamiglio, S.; Verderio, P.; Orlando, C.; Marubini, E. Confidence Interval for DNA/MRNA Concentration by Real-Time PCR. *Int J Biological Markers* 2007, *22* (3), 232–236. https://doi.org/10.1177/172460080702200312.

(4) Fieller, E. C. The Biological Standardization of Insulin. *Suppl J Royal Statistical Soc* 2018, *7* (1), 1–54. https://doi.org/10.2307/2983630.