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Exogenous RNA virus-like particles (VLPs) for precise molecular diagnostics: A pathway to enhanced detection and analysis for real-time PCR and NGS applications

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Introduction

Why use an exogenous RNA VLP control in molecular assays? An exogenous control is crucial in molecular assays as it eliminates false negatives and false positives, ensures accurate detection, validates extraction and amplification, enables quantification and normalization, and promotes standardization in inter-laboratory comparisons. This is particularly important in workflows involving RNA due to the presence of ubiquitous ribonucleases (RNases). Unreliable RNA controls create data interpretation challenges and add significant time, effort, and expense during validation and execution. Therefore, a dependable exogenous RNA VLP (virus-like particle) control that can go through the entire molecular workflow is crucial to mitigate these issues.

Enhanced VLP RNA for diverse applications: we introduce a single plasmid with a dual-expression mechanism for encapsulating any RNA sequence using the MS2 phage viral coat protein to get VLPs. This methodology yields exceptionally stable but noncontagious VLPs of RNA. These capsules work well as calibration standards or internal assay controls since they resist RNase degradation. By using common extraction methods, encapsulated VLP RNA can be easily extracted for quantification or size standards. This technology exhibits significant potential across a range of applications, encompassing infectious disease detection, NGS workflows, and beyond. It provides a high-quality RNA encapsulation method that enhances experimental precision and control.

Materials and Methods

To develop a versatile VLP serving as a universal process control, we designed a unique 1-kilobase (kb) exogenous sequence. This VLP exhibits no sequence similarity to known human pathogens, including viruses, bacteria and fungi. The generation of this unique sequence involved creating a shortest common supersequence (SCS) from exclusive 14-mers that are absent in the reference genomes of human pathogens and the human and mouse genome databases.

Our design occurs in living cells (in vivo), enabling proper protein translation and folding. To ensure its quality and specificity, our design was meticulously validated through in silico analysis, confirming its uniqueness and lack of similarity to known sequences of common human pathogens. Laboratory spike-in experiments with raw samples like saliva, as well as stability tests, ensure the VLP's reliability. RNA purity and quantity were verified through qPCR and dPCR, and stability was assessed through freeze-thaw and real-time equivalence stability tests, followed by RT-qPCR analysis. **Importantly, linearity, spike-in, and stability results apply to all our VLPs, as validation has been performed across multiple Takara Bio VLPs.**

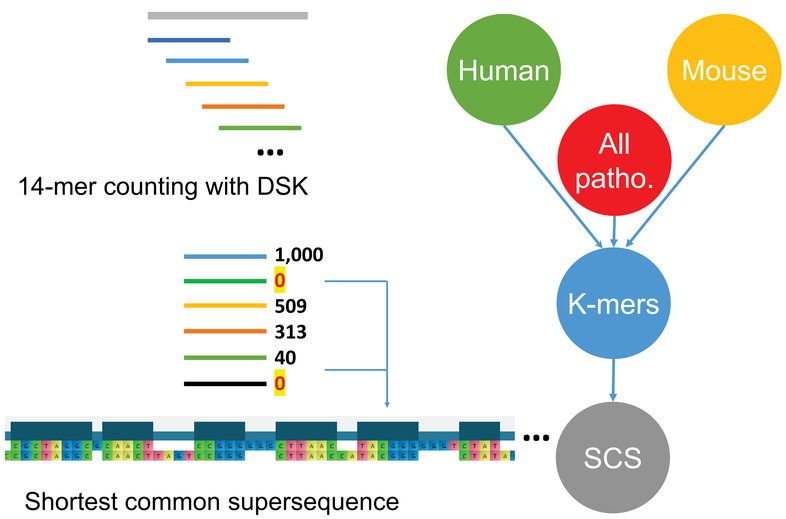

Figure 1. Creation of the exogenous 1-kb sequence, a nonhomologous sequence that does not match any genome. The exogenous sequence was extensively tested in silico to not match any of the genomes so far represented in the databases of human pathogens (incl. bacteria, viruses, fungi, and protozoa) or nonpathogens (incl. common host genomes, human and mouse).

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Results

1 Exogenous sequence does not cross-react with pathogen detection assays

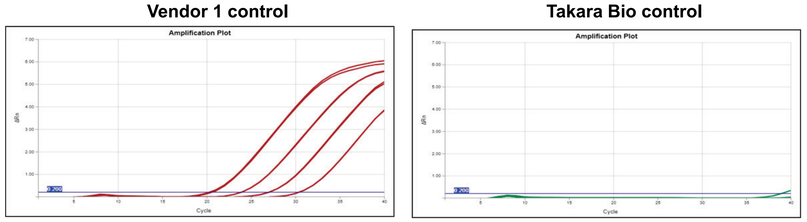
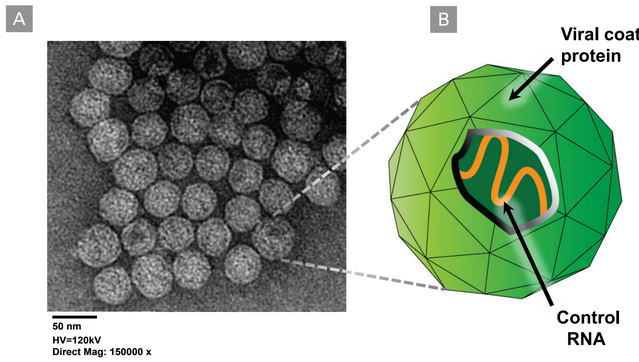


Figure 2. Exogenous sequence gives clean result with antibiotic resistance detection assays. In a qPCR assay for the beta-lactamase resistance gene, Vendor 1's control exhibited cross-reactivity while our exogenous-sequence control did not. The exogenous-sequence control's design was based on the latest sequence data from current databases that minimizes cross-reactivity with pathogens and their associated genes. Serial dilution from 9 x 10^5 to 9 x 10^2 copies per reaction was used in the comparison.

2 Takara Bio VLPs are highly stable



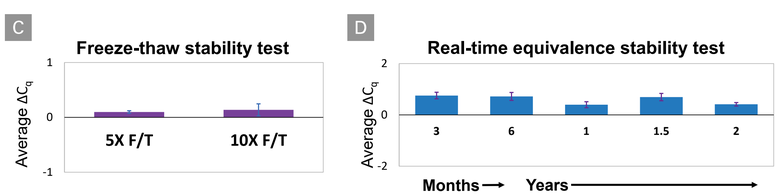


Figure 3. VLP RNA particles are stable, noninfectious positive controls for a variety of applications. Confirmed by transmission electron microscopy, Takara Bio's VLP RNA particles **(Panel A)** were intact in the final storage buffer. The calculated average size of particles was very close to the size of the natural virus (~27 nm), and most of the individual capsules had icosahedral symmetry **(Panel B).** VLPs subjected to 5 or 10 freeze-thaw cycles were compared to a control that experienced one freeze-thaw cycle **(Panel C)**. By this test, ΔC_q was <1, indicating high stability. By real-time equivalence accelerated stability test **(Panel D),** the ΔC_q of timepoints up to 2 years was also <1, relative to the zero timepoint.

Conclusions

- Our VLP controls demonstrate exceptional accuracy and stability throughout the RNA processing workflow.
- They maintain consistent performance across diverse sample types, unaffected by sample volume or extraction method variations.
- Moreover, our VLP controls outperform standard controls in RT-qPCR, providing enhanced sensitivity for pathogen detection.
- Thus, they serve as a unique and optimal choice for various applications, including molecular diagnostics workflows, infectious disease detection, gene expression experiments, and biomolecular studies.

3 Takara Bio VLPs give accurate quantification

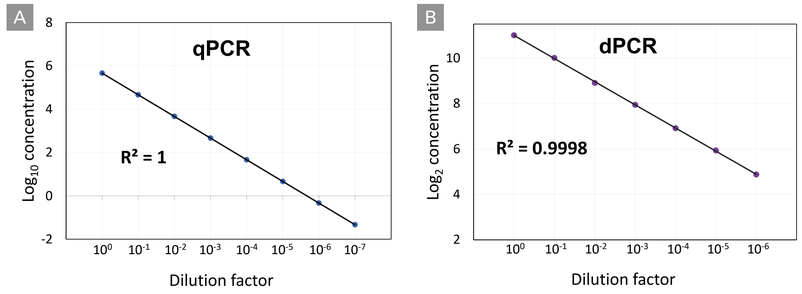


Figure 4. Serial dilution of Takara Bio VLPs shows excellent correlation with concentration (copies/µl) detected by qPCR and digital PCR. Both qPCR **(Panel A)** and dPCR **(Panel B)** consistently produced highly reproducible results across a wide range of VLP concentrations. This underscores the confidence with which the exogenous VLP can be employed in constructing standard curves, serving as a reliable reference for quantifying the detected pathogen. Furthermore, it is worth highlighting that the concentrations determined by qPCR and dPCR exhibit a strong positive correlation.

4 Takara Bio VLPs deliver more accurate quantity of RNA

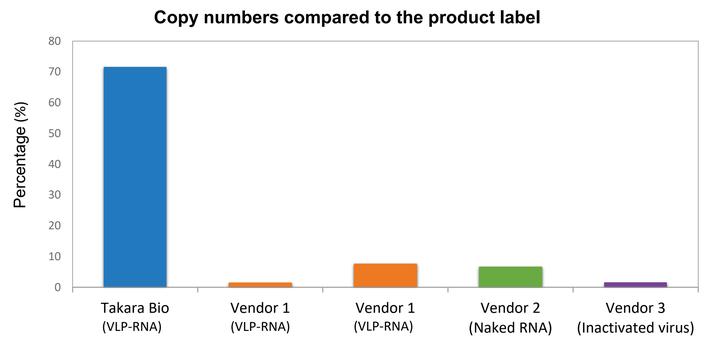


Figure 5. A more accurate quantity of RNA template was present in Takara Bio VLPs than those from different vendors. By digital PCR (or qPCR, data not shown), the RNA in Takara Bio VLPs most closely matched the copy number on the product label. All products were stored at the recommended temperature.

5 Takara Bio VLPs tolerate saliva and nuclease

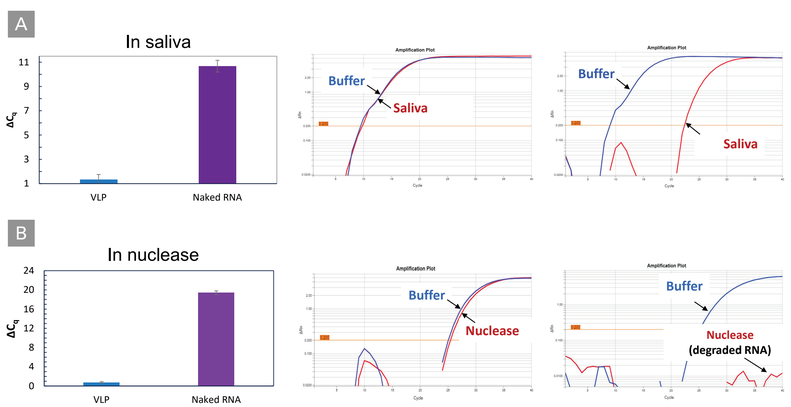


Figure 6. VLP controls are more resistant to degradation than naked RNA. The qPCR amplification curves demonstrate the stability of VLPs in saliva **(Panel A)** and nuclease **(Panel B)** (red curves) as compared to the same in buffer (blue curves).

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