CPS 2024 RFP – FINAL PROJECT REPORT

Project Title:
Two validated first-level screening assays for infectious hepatitis A virus by detection of an intact capsid on frozen berries

Project Period:
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Objectives:

1. Develop and quantify the sensitivity of two first-level detection methods for infectious HAV on frozen strawberries and irrigation water by specific detection of viral capsid-protected RNA.

2. Replicate these two methods in separate laboratories and validate these two methods comparing their performance against infectious HAV, inactivated HAV and free HAV RNA.

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Summary of Findings and Recommendations

A CRISPR-Cas13a detection assay was developed to identify hepatitis A virus (HAV) RNA. This assay was coupled with an RNase pretreatment and was capable of specifically detecting HAV RNA, presumably derived from intact capsids.

Two methods were developed, based on ISO-15216 (parts 1 and 2) and validated on frozen strawberries and irrigation water. For method 1, prior to RT-qPCR assay, a step with RNase pretreatment was added and for method 2, RNase pretreatment was also included, and RT-qPCR was substituted by CRISPR-Cas13a.

Abstract

Hepatitis A virus (HAV) contamination in fresh and frozen berries causes significant food safety challenges and economic losses worldwide. Current ISO 15216-based detection methods are expensive and time-consuming. Furthermore, the industry lacks rapid tools to distinguish between infectious and non-infectious HAV, which is essential for accurate risk assessment. We validated a novel CRISPR-Cas13a-based method, coupled with RNase pretreatment, for detecting intact HAV capsids, as a proxy for viral infectivity, in frozen strawberries and artificial ground water (AGW). Two genomic targets were developed using a two-step isothermal RT-RPA/T7-CRISPR-Cas13a system. An RNase ONE pretreatment (20 U; 37°C/90 min) was optimized to assess capsid integrity. The method was validated per ISO 15216-2:2019 using frozen strawberries (25 g, 80°C overnight) and AGW (500 mL) inoculated from 10^2 to 10^6 genome copies (gc)/sample. Results were compared against standard RT-qPCR. The CRISPR-Cas13a assay demonstrated high specificity, detecting targets within 36 min. RNase pretreatment degraded free RNA (5.76 to 0.33 $\log_{10}$ reduction), while intact HAV remained unaffected ($p > 0.05$), confirming its reliability as a structural proxy, complete viral inactivation was confirmed by TCID50 assays. In matrix trials, no significant difference was identified between CRISPR-Cas13a and RT-qPCR ($p > 0.05$). At 10^2 gc/sample, CRISPR-Cas13a was able to detect 1/3 positives in AGW (RT-qPCR: 0/3) and 3/3 in strawberries (RT-qPCR: 2/3). This study demonstrates CRISPR-Cas13a as a robust, cost-effective alternative to RT-qPCR for enteric virus detection. Integrating RNase pretreatment provides the produce industry with a rapid tool to assess HAV infectivity proxies, potentially reducing unnecessary recalls and improving public health decision-making in agricultural settings.

Background

Consumption of frozen produce contaminated with infectious HAV poses a recognized food safety risk, as illustrated by the 2022–2023 multistate outbreak in the United States linked to frozen strawberries (1). Although the outbreak source was investigated, the event undermined consumer confidence in frozen berries and highlighted persistent challenges faced by regulators and industry when viral contamination is suspected. In particular, the absence of validated methods to determine whether HAV detected on frozen produce is infectious limited the ability to clear implicated products, contributing to unnecessary product condemnation, food waste, and economic losses.

In response to multiple outbreaks of norovirus and HAV associated with frozen berries, the U.S. Food and Drug Administration (FDA) initiated surveillance activities targeting viral RNA detection in frozen

strawberries, raspberries, and blackberries (2). While these efforts provided valuable data on the presence of viral RNA, they also underscored a critical limitation of current standard methods. RT-qPCR-based assays, including ISO 15216-2:2019 and FDA BAM Chapter 26, detect viral RNA but do not distinguish between infectious virus and non-infectious or environmentally persistent RNA. As a result, positive molecular signals cannot be directly interpreted as an indicator of public health risk.

The persistence of viral RNA in environmental and food matrices, even after viral infectivity has been lost, is well documented. This phenomenon, often referred to as "relic RNA," has been observed for several viruses, including norovirus and SARS-CoV-2, and can lead to prolonged RNA detection in the absence of infectious virus (3,4). Studies have shown that viral RNA may remain detectable for weeks to months in water, food, and built environments, even when infectivity assays indicate virus inactivation. These findings demonstrate that RNA detection alone may overestimate risk and may not be an appropriate sole basis for regulatory actions such as recalls or market withdrawals.

Although HAV infectivity can be assessed using cell culture-based methods, these assays are poorly suited for routine monitoring of foods. Assays are often inhibited by complex food matrices, and sensitivity is insufficient to detect low levels of virus relevant to food contamination events (5). As an alternative, several studies have explored the use of infectivity proxies, such as capsid integrity assays, in which RNase pretreatment or viability dyes are applied prior to RNA amplification to remove RNA from damaged or non-intact virus particles (6,7). However, only a limited number of studies have applied these approaches to produce or irrigation water, and none have been fully validated for use on frozen berries.

The lack of validated methods to discriminate infectious HAV from non-infectious viral RNA represents a critical gap for the frozen produce industry and for regulatory decision-making. This gap contributes to uncertainty during outbreak investigations and surveillance activities and increases the likelihood of precautionary but unnecessary recalls.

The purpose of this project was to address this need by developing and validating two complementary methods capable of detecting infectious HAV on frozen strawberries and in irrigation water while discriminating non-infectious virus, including free viral RNA. Both methods integrate an RNase pretreatment step to assess capsid integrity prior to nucleic acid amplification. Method 1 builds upon the existing ISO 15216-2:2019 framework for HAV detection in soft fruits and water. Method 2 follows the same workflow but replaces the RT-qPCR amplification step with a CRISPR-Cas–based RNA detection assay designed to improve sensitivity, reduce assay time, and lower per-sample cost.

CRISPR-Cas–based diagnostics have demonstrated performance comparable to RT-qPCR while requiring less specialized equipment and offering improved tolerance to inhibition from complex matrices (8). Although CRISPR-based assays have been reported for several foodborne bacterial pathogens, their application to foodborne viruses, and HAV in particular, has remained largely unexplored. Importantly, because CRISPR assays also rely on RNA detection, the integration of an RNase pretreatment step was essential to enable discrimination between infectious and non-infectious virus in both methods evaluated in this project.

Together, these approaches were designed to generate practical, validated tools that support risk-based decision-making for the frozen produce industry. By distinguishing infectious HAV from non-infectious viral RNA on frozen strawberries and in irrigation water, the methods developed in this project aim to improve consumer protection, enhance confidence in surveillance data, support targeted recall decisions, and reduce unnecessary food waste associated with precautionary product withdrawal.

Research Methods and Results

Methods

Frozen strawberry and irrigation water samples were processed following the ISO 15216-2:2019 standard method for the detection of HAV in soft fruits and water, with the integration of an RNase pretreatment step to assess viral capsid integrity. Briefly, viral particles were eluted, concentrated, and purified according to the ISO protocol prior to molecular detection.

To discriminate infectious HAV from non-infectious virus and free viral RNA, samples were treated with RNase prior to nucleic acid amplification. RNase treatment was performed by adding 2 µL of RNase (20 U) to 138 µL of processed sample and incubating under conditions optimized to degrade exposed RNA while preserving RNA protected within intact viral capsids. Following RNase treatment, samples were immediately subjected to RNA extraction using the ISO-recommended procedures.

Extracted RNA was analyzed using two amplification approaches. For Method 1, HAV RNA was detected using RT-qPCR as specified in ISO 15216-2:2019. For Method 2, the RT-qPCR amplification step was replaced with a CRISPR-Cas13a-based RNA detection assay. This assay employed a two-step workflow consisting of reverse transcription and recombinase polymerase amplification (RT-RPA), followed by in vitro transcription and Cas13a-mediated detection using a sequence-specific guide RNA targeting HAV. Fluorescence generated by Cas13a collateral cleavage of an RNA reporter was measured as the analytical readout.

Both methods were applied to untreated samples and RNase-treated samples to evaluate their ability to discriminate between infectious HAV, inactivated virus, and free viral RNA. Method performance was assessed in terms of sensitivity, resistance to matrix-associated inhibition, and practical applicability to frozen produce and irrigation water matrices.

Results

Detection limit of HAV

The RT-qPCR assay for HAV detection demonstrated a robust linear range across seven orders of magnitude. The operational limit of detection (LOD) was determined to be 1.90 $\log_{10}$ genome copies/reaction (gc/rx) (equivalent to $10^{4.24}$ gc/ml). Concentrations at or below 0.90 $\log_{10}$ gc/rx fell below the threshold of detection, yielding 'Not Detected' (ND) results (**Table 1**).

1. Detection Range and Linearity

The data demonstrates a clear correlation across a serial dilution ranging from a high concentration of 7.90 $\log_{10}$ gc/rx down to 0.90 $\log_{10}$ gc/rx.

- Precision: The assay shows high reproducibility, evidenced by the low Standard Deviation (SD) values. Most SD values remain below 0.10, indicating minimal variance between replicates.
- Scaling: There is a consistent mathematical relationship between the copies per reaction (gc/rx) and the concentration per milliliter (gc/ml).

2. Identification of the LOD

The most significant finding occurs at the lower end of the dilution series:

- Quantification Limit: The assay effectively quantifies the virus down to 1.90 $\log_{10}$ gc/rx, which corresponds to an environmental/sample concentration of 4.24 ± 0.13 $\log_{10}$ gc/ml.
- Failure to Detect (ND): Once concentration drops to 0.90 $\log_{10}$ gc/rx, the result is marked as ND.
- Conclusion: The functional sensitivity of this RT-qPCR protocol is established at the 1.90 $\log_{10}$ gc/rx threshold.

The CRISPR-Cas13a assay demonstrates rapid kinetics, with detectable fluorescence emerging within the first 15 minutes of the reaction (**Fig. 1A**). Visual endpoint detection (**Fig. 1B**) confirms the presence of the HAV genome across the entire dilution range, including the lowest concentration of 0.90 $\log_{10}$ gc/rx, while the No Template Control (NTC) remains consistently negative.

Figure 1A: Kinetic fluorescence curves – This figure displays the real-time increase in Relative Fluorescence Units (RFUs) over a 60-minute period.

- Positive Signal Progression: All samples containing the viral genome (from 7.90 down to 0.90 $\log_{10}$ gc/rx) show a progressive increase in fluorescence starting shortly after the 5-minute mark.
- Signal Intensity: Interestingly, the samples with lower concentrations (e.g., 2.90 and 1.90 $\log_{10}$ gc/rx) show higher RFU peaks compared to some higher concentrations, which may be attributed to local reaction kinetics or saturation in the specific assay conditions.
- Negative Control: The NTC (No Template Control) remains completely flat at the baseline (0 RFUs), confirming the absence of contamination or non-specific amplification.
- Consistency: The variation between replicates, showed higher stability in the lower-concentration samples toward the end of the 60 minutes.

Figure 1B: Endpoint visual detection – This figure shows the reaction tubes under UV light or a blue light transilluminator at the end of the assay.

- Visual Confirmation: A clear fluorescent glow is visible in all tubes from 7.90 $\log_{10}$ gc/rx to 0.90 $\log_{10}$ gc/rx.
- Sensitivity: Note that while the previous table (**Table 1**) suggested a detection limit of 1.90 $\log_{10}$ gc/rx for qPCR quantification, this visual assay (**Figure 1B**) shows a detectable signal even at 0.90 $\log_{10}$ gc/rx, suggesting high qualitative sensitivity.
- NTC Comparison: The NTC tube is clearly dark/non-fluorescent, providing a sharp contrast against the positive samples.

Detection of HAV on strawberries (RT-qPCR vs CRISPR)

Data suggest that while both methods are highly effective for detecting HAV in strawberries, RT-qPCR appears slightly more sensitive overall, as indicated by its lower LOD_{50} value. However, the overlapping confidence intervals and the strong performance of CRISPR at the 2.69 $\log_{10}$ gc/sample level suggest that both are viable options for food safety testing in frozen fruit matrices.

RNase treatment significantly reduced detectable RNA, likely by degrading free viral RNA not protected by capsids. Under total RNA conditions (No RNase), RT-qPCR exhibited a higher value (LOD_{50} = 12.6 gc/g) compared to CRISPR (LOD_{50} = 47.5 gc/g). However, following RNase treatment, the limit of detection for both methods decreased substantially, 148 gc/g for RT-qPCR and 185.1 gc/g for CRISPR (**Table 2**).

1. Detection Sensitivity by Inoculation Level

The table shows the number of positive detections out of three replicates for various viral concentrations:

- High Concentrations (6.69 and 4.69 $\log_{10}$ gc/sample): Both RT-qPCR and CRISPR methods achieved perfect detection (3/3 positive replicates).
- Mid-to-Low Concentrations (3.69 and 2.69 $\log_{10}$ gc/sample): At 3.69 $\log_{10}$ gc/sample, RT-qPCR maintained 100% detection (3/3), while CRISPR detected 2 out of 3 replicates. At 2.69 $\log_{10}$ gc/sample, the trend reversed; CRISPR detected 3 out of 3 replicates, whereas RT-qPCR only detected 2 out of 3.
- Negative Control (0): Both methods correctly yielded zero detections, confirming the specificity of the assays.

2. Impact of RNase Treatment on Detection

The addition of RNase treatment significantly reduced the detection of HAV RNA in both methods (RT-qPCR and CRISPR), as seen by the lower number of positive replicates at lower inoculation levels.

- High Concentrations (6.69 and 4.69 log_{10} gc/sample): Both methods maintained perfect 3/3 detection regardless of RNase treatment.
- At 3.69 log_{10} gc/sample inoculation: Without RNase, RT-qPCR detected 3/3 replicates, but this dropped to 2/3 with RNase treatment. CRISPR dropped from 2/3 to 1/3.
- At 2.69 log_{10} gc/sample inoculation: Detection became much more difficult. RT-qPCR went from 2/3 to 0/3 (no detection), and CRISPR dropped from 3/3 to 1/3.

HAV detection reduction: RNase treatment increased the LOD_{50} by more than 10-fold for RT-qPCR (from 12.6 to 148.6 gc/g) and approximately 4-fold for CRISPR (from 47.5 to 185.1 gc/g).

Method comparison: For frozen strawberries, RT-qPCR was slightly more sensitive (lower LOD_{50}) than CRISPR. The CRISPR, compared to RT-qPCR, assay, combined with RNase, detected a higher percentage of replicates at the lowest seeding dose (2.69 log_{10} gc/sample), with 1/3 detection where RT-qPCR did not detect any replicates (**Table 3**).

Detection of HAV on AGW (RT-qPCR vs CRISPR)

In agricultural water samples, the CRISPR-based method demonstrated an apparent superior sensitivity compared to RT-qPCR, as evidenced by its lower quantification (33.9 vs 94.2 gc/ml), however, the overlapping confidence intervals is seen between both LOD_{50} (CRISPR and RT-qPCR). CRISPR, without RNase, provided a positive signal at (2.69 log_{10} gc/sample) but RT-qPCR did not, suggesting it may be more robust against the inhibitors or conditions present in large-volume water samples (**Table 4**).

- High Inoculation (6.69 log_{10} gc/sample): Both methods achieved a perfect detection rate of 3 out of 3 replicates.
- Intermediate Inoculation (4.69 and 3.69 log_{10} gc/sample): At 4.69 log_{10} gc/sample, CRISPR outperformed RT-qPCR by detecting 2 out of 3 replicates, compared to only 1 out of 3 for RT-qPCR. At 3.69 log_{10} gc/sample, both methods showed limited sensitivity, each detecting only 1 out of 3 replicates.
- Low Inoculation (2.69 log_{10} gc/sample): CRISPR successfully detected the virus in 1 out of 3 replicates, while RT-qPCR failed to detect any virus (0/3).
- Negative Control (0): As expected, both methods yielded 0 detections.

Sensitivity and LOD_{50}

The LOD_{50} was reported in genome copies per milliliter (gc/ml) with a 95% Confidence Interval (CI):

- CRISPR: Demonstrated higher sensitivity in water with an LOD_{50} of 33.9 gc/ml.
- RT-qPCR: Showed a significantly higher LOD_{50} of 94.2 gc/ml, indicating lower sensitivity in water samples compared to the CRISPR method.

In conclusion, in AGW and frozen strawberry samples without RNase pretreatment and strawberry samples with RNase pretreatment, recovery of HAV was affected by virus concentration ($P \leq 0.0005$, Chi-Square test); however, no significant difference in HAV amplification was seen between the RT-qPCR and CRISPR-Cas13a methods ($P > 0.05$).

Outcomes and Accomplishments

The primary outcome of this project was the successful development and validation of two first-level screening assays capable of distinguishing potentially infectious HAV from non-infectious free RNA. We optimized an RNase ONE pretreatment protocol (37°C for 90 min) that effectively degraded free viral RNA, achieving a reduction from 5.76 to 0.33 $\log_{10}$. This accomplishment addresses a critical industry gap by providing a structural proxy for infectivity, which current ISO 15216 standards lack.

Technical milestones included the validation of a novel CRISPR-Cas13a-based assay that demonstrated rapid detection kinetics, with visible results in under 40 minutes. In comparative matrix trials, both RT-qPCR and CRISPR proved highly effective. In frozen strawberry samples (25 g), RT-qPCR maintained equivalent sensitivity, with an LOD_{50} of 12.6 gc/g compared to 47.5 gc/g for CRISPR. Likewise, in large-volume agricultural water samples (500 mL), the CRISPR method performed comparably to RT-qPCR. It demonstrated an LOD_{50} of 33.9 gc/ml vs. 94.2 gc/ml, further optimization may be required to enhance its robustness against matrix-associated inhibitors.

These validated methods offer the produce industry a rapid, cost-effective tool to improve risk-based decision-making. By accurately identifying intact capsids and reducing false-positive signals from "relic" RNA, these workflows can prevent unnecessary product recalls and minimize economic losses for berry growers and stakeholders. Finally, the project's impact is being extended through the preparation of three scientific manuscripts and two abstracts for IAFP 2026, ensuring that these validated diagnostic tools are available for broader regulatory and commercial adoption.

APPENDICES

Publications and Presentations

Publications: There have not been any articles published to date. The team is currently working on three manuscripts.

Article 1.
"CRISPR-Cas13a coupled with RNase pretreatment for the detection of potentially infectious hepatitis A virus"
Results:

- Basic detection limit.
- Comparison with RT-qPCR (with and without RNase).
- Comparison with infectivity assay.
- Proof of concept: it is effective in reducing false positives.

Article 2.
"Application of a CRISPR-Cas13a RNase-based assay for hepatitis A virus detection in frozen strawberries and irrigation water"
Results:

- Recovery in frozen strawberries and water.
- Sensitivity and specificity.
- Comparison with ISO/RT-qPCR methods.
- Robustness in the presence of inhibitors.

Article 3.
"Interlaboratory validation of a CRISPR-Cas13a RNase-based assay for hepatitis A virus detection in frozen strawberries and irrigation water"
Results:

- Comparative testing in multiple laboratories.
- Correlation with RT-qPCR and control samples.
- Sensitivity/specificity analysis under various conditions.

Presentations: Two abstracts were submitted to be presented at IAFP 2026

1. Hepatitis A Virus Detection Using a Novel CRISPR-Cas13a and RNase-Pretreatment Based Method
2. Novel CRISPR-Cas13a-based RNA detection assay for hepatitis A virus: Validation in agricultural water and frozen strawberries

Budget Summary

This project was awarded a total of $104,750 in research funds. The majority of funds have been spent as planned.

Tables and Figure

Table 1. Detection limit of HAV by RT-qPCR

log_{10} (gc/rx)	log_{10} gc/ml (SD)	log_{10} gc/rx (SD)
7.90	10.78 ± 0.03	7.56 ± 0.03
6.90	10.02 ± 0.05	6.8 ± 0.05
5.90	8.57 ± 0.01	5.35 ± 0.01
4.90	7.00 ± 0.06	3.47 ± 0.06
3.90	6.22 ± 0.02	2.69 ± 0.02
2.90	5.03 ± 0.18	1.51 ± 0.18
1.90	4.24 ± 0.13	0.72 ± 0.13
0.90	ND	ND

ND, Not Detected. Represents samples where the target nucleic acid was below the assay's limit of detection (LOD). For statistical purposes, these values are treated as non-quantifiable on the log_{10} scale.

Table 2. Detection of hepatitis A virus from frozen strawberry samples (25g)

Inoculation level (log_{10} gc/sample)	No RNase		RNase	
	RT-qPCR	CRISPR	RT-qPCR	CRISPR
6.69	3	3	3	3
4.69	3	3	3	3
3.69	3	2	2	1
2.69	2	3	0	1
ND	0	0	0	0
LOD_{50} genome copies/g (95% CI)	12.6 (2.7 – 55.6)	47.5 (12.4 – 182.3)	148.6 (35.9 – 614.9)	185.1 (42.0 – 815.3)

ND, Not Detected. Represents samples where the target nucleic acid was below the assay's limit of detection (LOD). For statistical purposes, these values are treated as non-quantifiable on the log_{10} scale.

Table 3. Comparison of the Limit of Detection (LOD_{50}) of hepatitis A virus from frozen strawberry samples (25g)

Condition	Method	LOD_{50} (gc/g)	95% Confidence Interval
No RNase	RT-qPCR	12.6	2.7 – 55.6
	CRISPR	47.5	12.4 – 182.3
With RNase	RT-qPCR	148.6	35.9 – 614.9
	CRISPR	185.1	42.0 – 815.3

Table 4. Detection of hepatitis A virus from agricultural water samples (500 mL)

Inoculation level (log_{10} gc/sample)	No RNase	
	RT-qPCR	CRISPR
6.69	3	3
4.69	1	2
3.69	1	1
2.69	0	1
ND	0	0
LOD_{50} genome copies/ml (95% CI)	94.2 (20.6 – 431.3)	33.9 (9.5 – 120.6)

ND, Not Detected. Represents samples where the target nucleic acid was below the assay's limit of detection (LOD). For statistical purposes, these values are treated as non-quantifiable on the log_{10} scale.

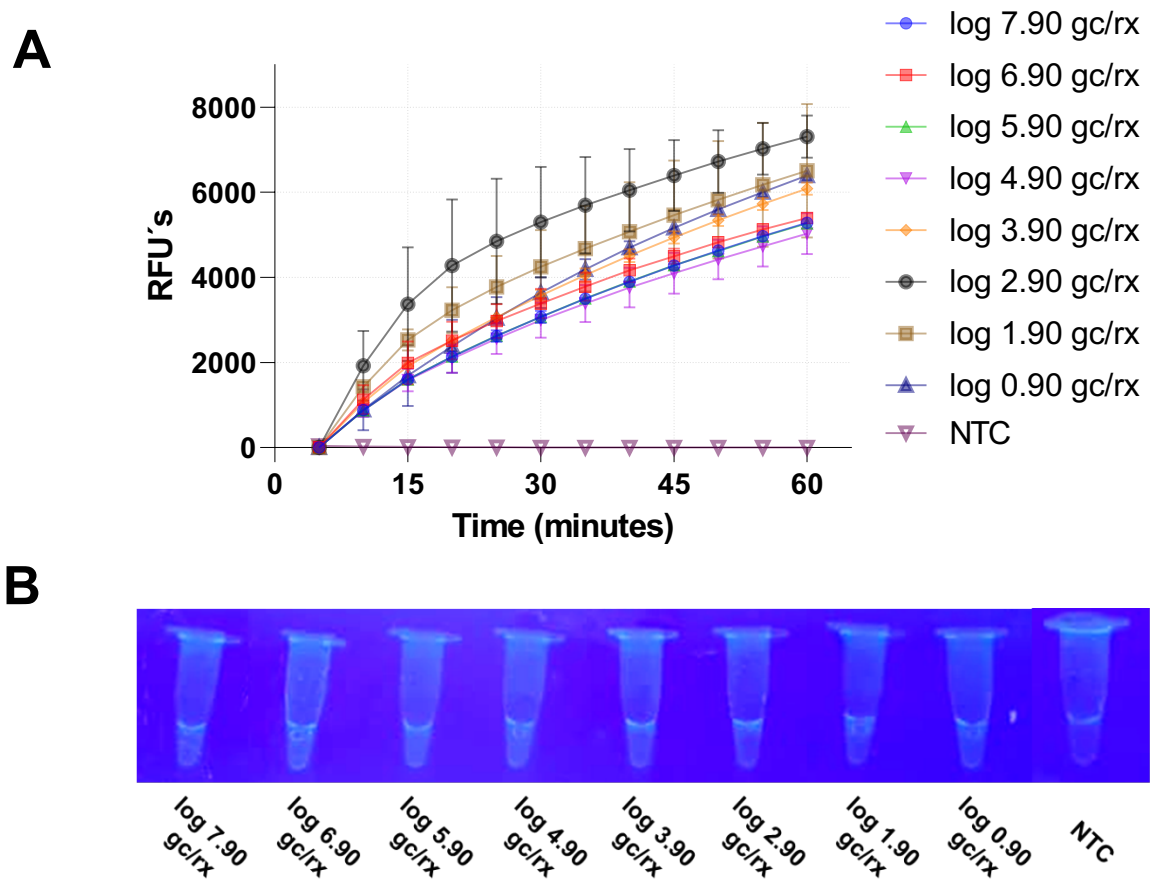

Figure 1. CRISPR-Cas13a HAV detection LOD. The CRISPR-Cas13a assay demonstrates rapid kinetics, with detectable fluorescence emerging within the first 15 minutes of the reaction (A); visual endpoint detection (B).

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