CENTER FOR PRODUCE SAFETY

CPS 2024 RFP – FINAL PROJECT REPORT

Project Title:
Development of a screening assay for hepatitis A virus which correlates to infectivity

Project Period:
January 1, 2025 – December 31, 2025 (extended to January 31, 2026)

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Objectives:

1. Methods Optimization: Improve the performance of candidate molecular-based infectivity discrimination methods (RNase/proteinase; and PMAxx, or PtCl4 pre-treatments, in conjunction with RT-qPCR) for detection of HAV and compare their performance to infectivity assay.

2. Screening: Perform direct comparison between optimized methods and infectivity assay as applied to fully infectious HAV, and that partially and fully inactivated by exposure to heat and sodium hypochlorite.

3. Matrix Performance: Evaluate the efficacy of the top-performing method(s) in a frozen berry matrix seeded with HAV (fully infectious, partially inactivated, and fully inactivated) processed for detection using the ISO 15216 protocol.

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Summary of Findings and Recommendations

In this study, we optimized and compared three methods to exclude detection of non-infectious hepatitis A virus (HAV) when using molecular amplification detection methods such as those described in the ISO 15216 international standard. This comprehensive study included (i) multiple infectivity discrimination methods and virus having different infectivity ratios (fully, partially, and non-infectious); (ii) direct comparison between cell culture-based infectivity assay and the modified molecular methods; and (iii) evaluation of performance in a soft fruit matrix. Optimization included production of an alternative RT-qPCR method having equivalent performance to the ISO 15216 method but better applicability to the infectivity-discriminating molecular methods. The enzyme-based method we evaluated was unable to effectively discriminate virus infectivity, despite its widespread use. The two nucleic acid intercalation-based methods had greater but not perfect correlation to infectivity when applied to virus partially inactivated by heat. Importantly, though, these two methods were able to completely prevent detection of fully inactivated HAV, with excellent performance in the berry matrix. These simple and inexpensive methods are fully compatible with the internationally accepted standard methods for HAV detection in berries and can be run in parallel with traditional RT-qPCR to discriminate infectivity status. The ability to exclude the detection of non-infectious virus will aid in providing results that are more consistent with actual public health risk when testing is used as part of a comprehensive food safety plan.

Abstract

Hepatitis A virus (HAV) outbreaks have been attributed to contaminated soft fruits, particularly berries. Unlike bacterial pathogens, wild-type HAV cannot be cultivated *in vitro*, hence reliance on viral genome detection methods, primarily RT-qPCR. While highly sensitive, RT-qPCR cannot discriminate between infectious or non-infectious virus, hence the lack of direct correlation between a positive test result and public health risk. Preceding RT-qPCR with (i) enzymatic treatments or (ii) nucleic acid crosslinking can make these assays more discriminatory, but these approaches need optimization and comparative study. The purpose of this project was to develop a prototype screening assay to detect infectious HAV to the exclusion of non-infectious virus. Its design capitalized on the use of three candidate pre-treatment methods, i.e., (i) ProteinaseK/RNase (enzymatic); (ii) propidium monoazide (PMAxx) and (iii) platinum chloride ($PtCl_4$) (both nucleic acid crosslinking). Firstly, a long-amplicon RT-qPCR assay was developed that had comparable performance to the international standard method (ISO 15216) but was better suited for use with enzymatic or crosslinking pre-treatments. Using the cultivable HAV strain HM-175, these methods were optimized and their performance compared to the "gold standard" culture method in buffer and the berry matrix. The Proteinase K/RNase method was unable to discriminate infectivity status. Both nucleic acid crosslinking methods showed incremental reductions in RT-qPCR detection signal for HAV partially inactivated by heat, but still overestimated infectivity. They did not discriminate infectivity for HAV partially inactivated by sodium hypochlorite. However, both PMAxx and $PtCl_4$ completely prevented RT-qPCR detection when viruses were completely inactivated. Similar results were observed in buffer and in a berry matrix. While crosslinking methods fall short of accurately estimating infectious virus concentrations under mild inactivation conditions, they can discriminate well when virus is completely non-infectious, providing valuable context for interpreting positive RT-qPCR when run in parallel to the ISO 15216 standard.

Background

Wild-type HAV (the strains that cause illness) cannot be cultivated in the laboratory, so unlike many bacterial pathogens, cultural enrichment is not possible. HAV detection methods used for food and environmental samples therefore rely on the sequential steps of virus concentration and purification, followed by detection using molecular amplification targeting a fragment of the viral genome (in this case, single stranded RNA), typically using reverse transcription–quantitative PCR (RT-qPCR). Unfortunately, this method does not unequivocally correlate to virus infectivity, so positive detection can overestimate risk to human health. Scientific consensus is that there is a need to develop methods specifically designed to detect infectious virus, but that will not be possible any time soon using culture methods.

RT-qPCR is itself a form of non-cultural enrichment, in that one can "enrich" a part of the pathogen's nucleic acid up to a million-fold. While it clearly cannot replace cultural enrichment, it provides a way to get many more copies of the viral nucleic acid, effectively improving assay detection limits. So, it remains a better detection platform for the low levels of contamination expected in foods and the environment. To address the so-called infectivity dilemma, two general approaches for viability discrimination have been investigated, one being enzyme-based and the second involving the use of nucleic acid crosslinking agents. Both are applied just prior to RNA extraction. They effectively degrade free RNA, or otherwise prevent RNA surrounded by damaged or degraded capsid protein, from being amplified by RT-qPCR. Theoretically, the only RNA that will be amplified should be that associated with full-integrity capsids, i.e., presumably infectious virus.

Enzyme-based methods rely on the use of RNase to degrade free or partially encapsidated viral nucleic acid, while proteinase degrades damaged virus capsid proteins to expose the protected RNA genome. When used together, this treatment essentially prevents RT-qPCR amplification of the RNA not associated with an intact capsid. Nucleic acid crosslinking methods rely on the ability of chemicals like propidium monoazide (PMA/PMAxx) and platinum (IV) chloride ($PtCl_4$) to covalently bind to free or partially encapsidated viral RNA, making it impossible to be amplified by RT-qPCR. These compounds cannot penetrate intact virus capsids, but can penetrate those that are damaged or destroyed. $PtCl_4$ is particularly appealing because it is inexpensive and has the least complicated protocol. Both enzyme and nucleic acid crosslinking approaches are applied as pre-treatments before RNA extraction, so upstream sample processing steps (virus concentration and purification) remain unchanged and there is simply an extra step or two added to existing detection protocols, the most important of which is the ISO 15216 (https://www.iso.org/standard/74263.html), considered the international "gold standard." While both methodological categories have been investigated by others, their performance has been spotty and key usage variables have not been considered and/or optimized. Further, few studies have compared methodological performance to the "gold standard" of infectivity assay using a cultivable HAV strain. Finally, most previous studies have evaluated human norovirus, not HAV, and heat is the only inactivation method usually evaluated, even though chemical disinfection is an important control strategy. Rarely is the method applied to real-world samples such as foods.

Capitalizing on enzyme-based and nucleic acid crosslinking-based methods, the purpose of this project was to develop a prototype screening (rapid) assay that could detect infectious HAV to the exclusion of non-infectious virus, one which could be readily incorporated into the ISO 15216 method for detecting enteric viruses in soft fruits.

Research Methods and Results

Methodological Overview: Three research objectives were identified for this project, roughly outlined as (i) infectivity discrimination methods optimization and development of a long-amplicon RT-qPCR assay; (ii) direct comparison of performance of optimized methods to mammalian cell culture infectivity assay; and (iii) evaluation of performance in a candidate frozen berry matrix. The methods optimization is described briefly below. To evaluate performance, stock solutions having varying degrees of HAV inactivation were generated using either heat or sodium hypochlorite treatments, with infectivity validated/quantified using a standard tissue culture infectious dose 50 ($TCID_{50}$) infectivity assay. To assay performance of the optimized infectivity discrimination methods (i.e., Proteinase K/RNase, PMAxx, and $PtCl_4$), phosphate buffered saline (PBS) or an ISO 15216-concentrated strawberry matrix was spiked with the various virus stock solutions and further processed using the pre-treatments followed by RNA extraction and RT-qPCR, the latter using standard ISO 15216 primers and long-amplicon primers developed in this study. This overall experimental design is shown in **Figure 1**.

Virus, Cell Lines, and Infectivity Assay: Fetal rhesus monkey kidney (FRhK-4) cells (University of North Carolina, Lineberger Cancer Center, Chapel Hill, NC) were used to cultivate HAV HM-175/18f (VR-1402, ATCC, Manassas, VA). Infectious virus titers were determined using the $TCID_{50}$ method performed in 96-well plates, with 12 wells per dilution. Virus dilutions were prepared in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4mM $CaCl_2$ and 2% Fetal Bovine Serum (FBS), and 25µl of sample or sample dilution were added to each well. After 1h incubation to allow for virus binding, 175µl of DMEM with 2% FBS was added to each well and plates were incubated in 5% CO_2 at 37°C. After 10 days, wells were visually examined microscopically for cytopathic effects (CPE) and $TCID_{50}$ for infectious virus was determined using the Reed-Muench method.

Partially and Fully Inactivated Virus Stocks: Heat and sodium hypochlorite treatments, representing physical and chemical methods of damage, respectively, were used to generate virus stocks with a range of infectious:non-infectious virus ratios. For heat treatment, HAV was incubated at 20, 62, 66, and 90°C in a thermocycler for 3 min, then rapidly cooled to 4°C. Chlorine treatment was performed using a modified ASTM E1052 method (ASTM International, 2020), with volumes changed to minimize virus dilution. Chlorine treatments were performed at 0ppm, 550ppm, 625ppm, and 1000ppm. The infectivity data for these treatments are provided in **Tables 1** and **2**. Aliquots of partially and fully inactivated HAV stocks were stored in Protein Lo-bind microcentrifuge tubes at -80°C until use. The final virus stock concentration used for each experiment was generated by diluting each virus stock 1:10 in the respective matrix for a total sample volume of 200µl. Initial screening was performed using PBS as the matrix, with additional testing using a berry matrix that was prepared by processing berries through the ISO 15216 method with virus inoculation after pre-analytical sample processing but before RNA extraction.

RNA Extraction and RT-qPCR: RNA was extracted using the NucliSense EasyMAG (Biomerieux, St. Louis, MO) platform as per manufacturer instructions, with elution to a final sample volume of 40µl for experiments with PBS, and 80µl with the strawberry extract. RT-qPCR was performed using the Superscript III One-Step kit (Invitrogen, Waltham, MA) in a 25µl reaction volume. Parallel assays were performed using the ISO 15216 primers and the long-amplicon primers developed in this project, using 400nM forward primer, 800mM reverse primer, and 200nM probe. Reverse transcription was done for 30 min at 55°C, followed by 45 amplification cycles of 95°C for 15 sec and 60°C for 60 sec. Ct values were converted to log_{10} RT-qPCR-amplifiable units using standard curves prepared using a 1:10 dilution series of extracted viral RNA. Limit of detection for each RT-qPCR assay design was determined based on the highest consistently detected dilution from a 1:2 dilution series starting at a Ct value of 35.

Long-Amplicon PCR Assay Design: A long amplicon RT-qPCR method was designed to take into account the need for minimal secondary structure in amplified regions as a criterion for efficient RNase activity. A longer amplicon region could also help reduce detection of shorter genome fragments in cases for which the infectivity discrimination method fails to prevent amplification. To keep consistent with the ISO 15216 method, we retained the forward primer and probe from that method but selected an alternative reverse primer that was located further downstream. To determine the optimal primer location, all full-length HAV genomes from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) were downloaded (total of 341), of which 336 were used after quality control checks. Sequences were genotyped using the RIVM Hepatitis A Genotyping Tool (National Institute for Public Health and the Environment, http://www.rivm.nl/mpf/hav/typingtool), and grouped by genotype (IA, IB, IIA, IIB, IIIA, IIIB). Sequence alignment was performed for each genogroup individually, then the consensus sequence for each genogroup was used for an overall alignment (**Figure 2**). To further guide identification of optimal reverse primer location for amplifications involving the use of RNase and PMAxx, RNA secondary structure analysis was also performed using RNAfold (ViennaRNA Package 2.0, http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Aligned sequences were manually examined to determine the primer candidate locations based on secondary structure and sequence conservation.

Multiple candidate primers were identified, but the one having best performance extended the amplicon length to 344 bases (compared to 173 for the ISO 15216 method). Comparatively, this primer produced assay sensitivity similar to that of the ISO method primers (data not shown). Additionally, this alternative reverse primer demonstrated near perfect conservation across all HAV genotypes, an improvement over the ISO primer set (**Figure 2**).

Infectivity Discrimination using Enzymatic Treatment with Proteinase K and RNase: Initial optimization of the RNase method focused on improving activity in the presence of a high degree of genomic RNA secondary structure, which can be presented to the enzyme as “double stranded” in nature; RNA in this configuration is relatively resistant to RNase activity. Two different RNase formulations, i.e., RNase ONE (Promega, Madison, WI) and RNase I_f (New England Biolabs, Ipswich, MA), were compared, each using their original buffer or NEBuffer 3 (New England Biolabs, Ipswich, MA) supplemented with 4mM $CaCl_2$; $CaCl_2$ has been shown to induce double-stranded RNase activity which is typically lacking for RNase I (Grunburg et al., 2021). The best combination was RNase ONE using NEBuffer 3 with 4mM $CaCl_2$, however the improvement was marginal with only a 0.7 Ct increase compared to the standard buffer (data not shown). An alternative attempt to improve performance included the addition of 6U Proteinase K (Thermo Scientific, Waltham, MA) treatment, followed by the RNase ONE with $CaCl_2$, again with limited success (data not shown). A combined Proteinase K/RNase protocol was used in comparative studies.

Infectivity Discrimination using Platinum Chloride: Platinum chloride ($PtCl_4$) has shown promise, but the literature is clear that its performance is significantly inhibited by high organic load (Cuevas-Ferrando et al., 2021) which would be expected in soft fruit or other food matrices. To overcome this challenge, we initially optimized (increased) $PtCl_4$ concentration to identify the maximum concentration that could be used without continued activity during RNA extraction or negative downstream detection interference. Concentrations of 0mM, 2.5mM, 5mM, and 10mM $PtCl_4$ dissolved in DMSO were tested, and no continued activity or negative effects were observed. The 10 mM concentration was chosen for use in the final assay, with a treatment time of 10 min at room temperature (RT).

Infectivity Discrimination using PMAxx: For PMAxx, the protocol was adapted from Randazzo et al. (2018) using 100µM PMAxx (Biotium, Fremont, CA) and 0.5% Triton X-100 (Biotium, Fremont, CA). A

potential shortcoming with using PMA-based methods in food matrices is that the resulting changes in sample opacity and color from the food matrix can negatively affect light penetration during the light activation treatments, a necessary step for crosslinking (Moreno et al., 2015). To promote light penetration and achieve a more uniform light exposure, light activation steps were performed in combination with orbital shaking. In the final protocol, done at RT, samples were subjected to orbital shaking (200rpm for 30 min) in the dark, followed by exposure to blue light using a PMA-Lite LED Photolysis Device (Biotium, Fremont, CA) placed on top of an orbital shaker for an additional 15 min. The samples were exposed to an additional dark cycle for 15 min, and a final light cycle for 15 min. This was the final protocol used in comparative studies.

Screening of Infectivity Discrimination Methods Compared to Cell Culture Infectivity in PBS: Fully and partially inactivated virus stocks were spiked at a 1:10 dilution into PBS. Proteinase K/RNase, PMAxx, and $PtCl_4$ pre-treatments were performed as described above, in parallel with untreated control samples. Following pre-treatments, samples were subjected to RNA extraction, followed by RT-qPCR using both the ISO 15216 primers and the long-amplicon primers developed here.

Results for experiments performed in PBS are shown in **Table 1**. Under control conditions (no heat or hypochlorite inactivation), the RT-qPCR assays overestimated virus infectivity predicted by $TCID_{50}$ by ~1 log_{10}. This is consistent with the naturally occurring infectious:non-infectious virus ratio for cultured HAV. Without any pre-treatments, RT-qPCR signals remained stable for all heat treatments except the highest (90°C), which was associated with full inactivation of infectious virus. This is demonstrative of the infectivity dilemma and was not ameliorated by the Proteinase K/RNase enzymatic treatment. When stocks of various infectious virus concentration derived from heat treatment were pre-treated using PMAxx or $PtCl_4$, both methods performed similarly and demonstrated incremental reductions in detection signal but fell short of reaching complete parity with cell culture infectivity results.
For partially inactivated virus stocks prepared with sodium hypochlorite, all three methods failed to produce any demonstrable infectivity discrimination. Only in the most extreme case (the 1000ppm treatment that completely abolished infectious virus) did PMAxx and $PtCl_4$ prevent detection, but these results did not differ from the sample receiving no crosslinking agent pre-treatment. This is likely due to genomic damage that occurs upon exposure to high chlorine concentrations.
Collectively, for PMAxx and $PtCl_4$, the long-amplicon assay was better at infectivity discrimination than was the standard ISO 15216 RT-qPCR protocol. This was particularly notable for fully inactivated virus, for which the long-amplicon assay in combination with either PMAxx and $PtCl_4$ was able to completely prevent detection (abolish RT-qPCR signal).

Screening of Infectivity Discrimination Methods in the Berry Matrix: Strawberries were processed using the sample concentration protocol described in the ISO 15216 and then spiked with a 1:10 dilution of the fully and partially inactivated virus stocks. Proteinase K/RNase, PMAxx, and $PtCl_4$ pre-treatments were performed as described above, in parallel with untreated control samples. Following pre-treatments, samples were subjected to RNA extraction, followed by RT-qPCR using both the ISO 15216 primers and the long-primers developed here. Results for the berry matrix were similar to those for PBS, with complete failure of the enzymatic method to discriminate infectivity, and partial success with the PMAxx and $PtCl_4$, which was most notable when the virus was fully inactivated by heat (**Table 2**).

Outcomes and Accomplishments

Outcomes:

- To facilitate effective performance of the molecular-based infectivity discrimination assays, a long-amplicon RT-qPCR method was developed that considered secondary RNA structure. Its design necessitated replacement of only one ISO 15216 primer with performance (sensitivity) was nearly identical that of the ISO 15216 standard RT-qPCR assay.
- Attempts were made to optimize enzyme-based methods for infectivity discrimination, with minimal success, although joint use of Proteinase and RNase provided modest improvements.
- Optimization of the nucleic acid crosslinking methods ($PtCl_4$ and PMAxx) resulted in improved performance for application to complex sample matrices (i.e., high organic load) as would be expected for soft fruits.
- The enzymatic method (Proteinase/RNase) alone failed to produce incremental infectivity discrimination by RT-qPCR.
- When stocks of various partially infectious virus concentrations derived from heat treatment (i.e., 62°C and 66°C) were pre-processed using PMAxx or $PtCl_4$ both methods performed similarly and demonstrated incremental reductions in detection signal but fell short of reaching complete parity with cell culture infectivity results (overestimated infectivity). This was consistent regardless of sample matrix (buffer or berry concentrate).
- When stocks of various partially infectious virus concentration derived from treatment with sodium hypochlorite (i.e., 550ppm and 625ppm) were pre-processed using PMAxx or $PtCl_4$, no incremental reductions in detection signal could be observed, regardless of sample matrix (buffer or berry concentrate).
- Both PMAxx and $PtCl_4$ were successful in preventing RT-qPCR amplification of completely inactivated virus (i.e., 90°C and 1000ppm sodium hypochlorite).

Accomplishments:

In this study, we optimized and then compared the performance of one enzymatic method and two nucleic acid crosslinking methods, at discriminating infectivity as applied to fully infectious, partially infectious and fully inactivated HAV. This study was unique in its: (i) focus on methods optimization; (ii) design to be incorporated into the ISO 15216 standard method for detection of viruses in soft fruits; (iii) inclusion of virus with different infectious:non-infectious ratios; (iv) consideration of virus inactivation by a physical (heat) and a chemical (chlorine) treatment; (v) head-to-head comparison of multiple infectivity discrimination methods; (vi) inclusion of direct comparison between infectivity (cell culture) and molecular (RT-qPCR) methods; and (vii) evaluation of performance in a both pristine and relevant sample matrices (soft fruit). Key accomplishments include the following:

- Production of a long-range RT-qPCR method that has equivalent analytical sensitivity to the ISO 15216 RT-qPCR method, broader reactivity (i.e., genogroups IIIA and IIIB), and may be more amenable to use with infectivity-discriminating crosslinking agents.
- Further proof that the widely used Proteinase K/RNase pre-treatment has little efficacy in HAV infectivity discrimination.
- Promising data to support the use of the optimized PMAxx or $PtCl_4$ methods, perhaps not to estimate infectious virus titer but certainly to indicate the presence of non-infectious virus were parallel assays (with and without infectivity discrimination pre-treatments) to be done simultaneously.

References

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APPENDICES

Publications and Presentations

Poster was presented at the Center for Produce Safety Research Symposium, June 2025.

Budget Summary

This project was awarded $57,646 in research funds. All funds were spent by project end (with extension to January 31, 2026).

Figures 1–2 and Tables 1–2 (see below)

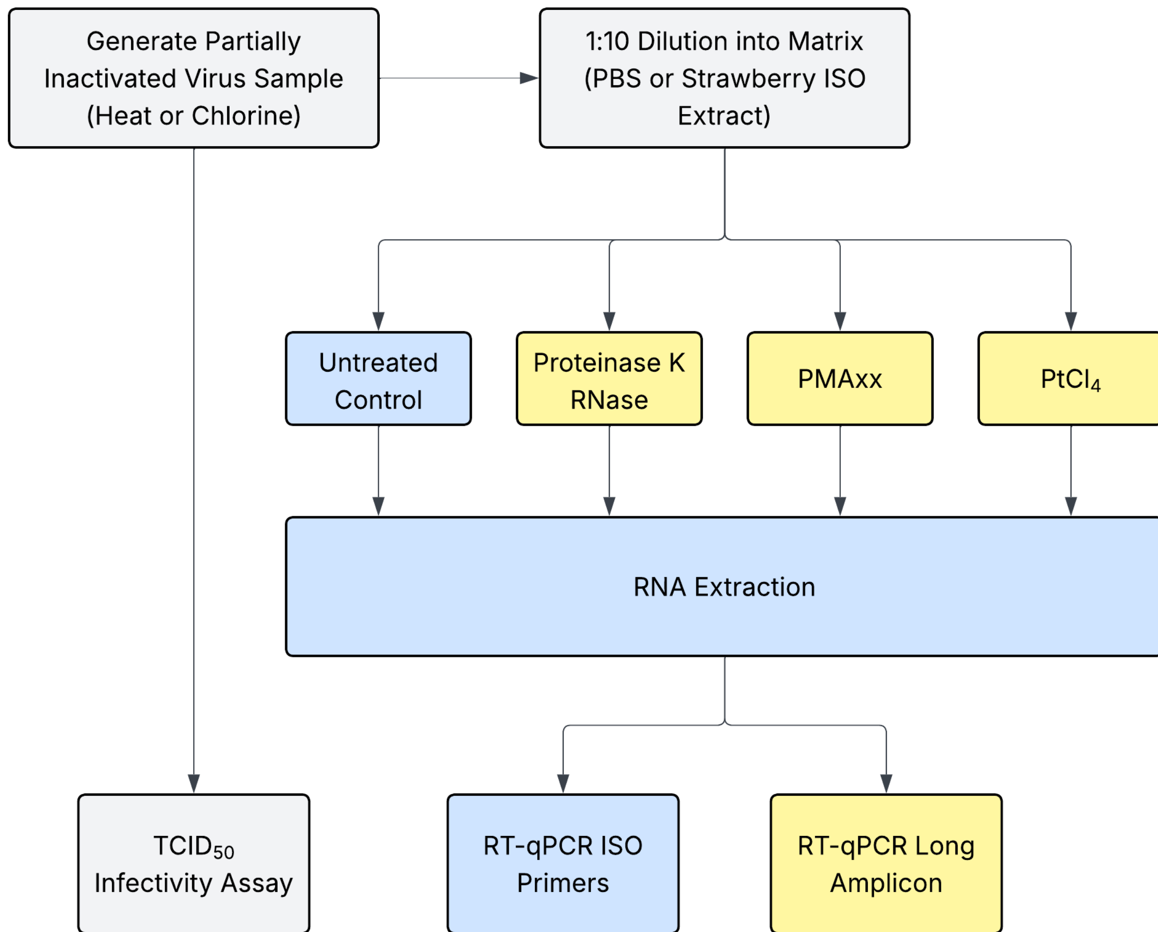

Figure 1. Experimental overview, with the standard ISO detection method shown in blue, and the tested modifications for improving infectivity discrimination shown in yellow.

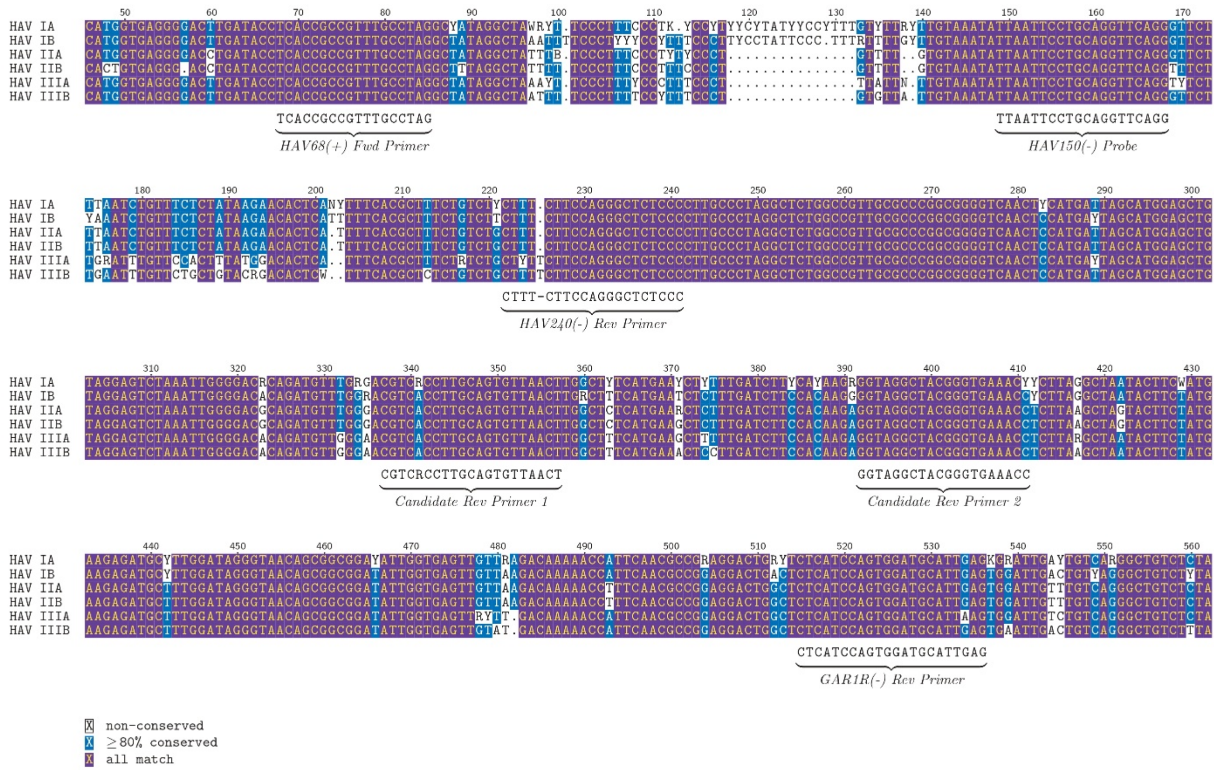

Figure 2. Final sequence alignment showing the positions of the ISO primers and probe, along with the two candidate primer locations and the reverse primer from the U.S. FDA Bacteriological Analytical Manual (BAM) method for detection of HAV in soft fruits.

Table 1. Results for experiments performed in PBS. Values in parentheses are for the long-amplicon version of the RT-qPCR assay.

	Infectivity	RT-qPCR (log_{10} PCRU/20µl)			
Heat	**($TCID_{50}$/20µl)**	**Control**	**ProtK/RNase**	**PMAxx**	**PtCl4**
20°C	5.65	6.69 (6.58)	6.59 (6.47)	6.71 (6.56)	6.49 (6.21)
62°C	2.21	6.55 (6.38)	6.48 (6.32)	4.89 (4.70)	4.67 (4.29)
66°C	0.36	6.54 (6.40)	6.44 (6.34)	4.21 (3.99)	4.22 (3.57)
90°C	<0.20	5.20 (4.83)	5.09 (4.64)	1.85 (<1.20)	2.77 (<1.20)
Hypochlorite					
0ppm	4.88	6.10 (5.94)	6.03 (5.85)	6.08 (5.88)	5.98 (5.75)
550ppm	3.41	6.05 (5.83)	6.04 (5.82)	6.02 (5.78)	5.74 (5.54)
625ppm	0.90	6.01 (5.77)	5.92 (5.73)	5.89 (5.74)	5.63 (5.41)
1000ppm	<0.20	2.37 (1.86)	2.25 (1.93)	1.27 (<1.20)	<1.20 (<1.20)

Table 2. Results for experiments performed in the representative strawberry matrix. Values in parentheses are for the long-amplicon version of the RT-qPCR assay.

	Infectivity	RT-qPCR (log_{10} PCRU/20µl)			
Heat	**($TCID_{50}$/20µl)**	**Control**	**ProtK/RNase**	**PMAxx**	**PtCl4**
20°C	5.65	6.43 (6.14)	6.31 (5.97)	6.66 (6.36)	6.39 (6.04)
62°C	2.21	6.12 (5.84)	6.01 (5.73)	4.78 (4.47)	4.61 (4.13)
66°C	0.36	6.07 (5.79)	5.66 (5.42)	4.00 (3.72)	4.12 (3.35)
90°C	<0.20	4.43 (3.97)	4.00 (3.57)	<1.51 (<1.51)	2.87 (<1.51)
Hypochlorite					
0ppm	4.88	5.67 (5.48)	5.49 (5.34)	5.86 (5.64)	5.67 (5.51)
550ppm	3.41	5.58 (5.41)	5.46 (5.28)	5.77 (5.55)	5.47 (5.26)
625ppm	0.90	5.51 (5.33)	5.34 (5.18)	5.71 (5.49)	5.33 (5.16)
1000ppm	<0.20	1.88 (<1.51)	1.90 (1.62)	<1.51 (<1.51)	<1.51 (<1.51)