# Facilitating Purification and Detection of Viral Nucleic Acids from Milk

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## INTRODUCTION

Outbreaks of Highly Pathogenic Avian Influenza (HPAI) A (H5N1) have been reported worldwide in wild birds and poultry since 2022 with incidences of sporadic spillover into mammalian species (1-5). On March 25th, 2024, a multi-state outbreak of HPAI A (H5N1) bird flu in dairy cows was first reported in the United States with 46 herds in 9 states reported infected as of May 14, 2024 (6,7). The spread of H5N1 in cattle poses a health risk to the animals as well as our agricultural workforce that has occupational exposure to livestock. The CDC, FDA and USDA have provided situation updates and measures to limit the impact of H5N1 spread including implementation of surveillance systems, guidance for H5N1 testing in cattle, and safety assessment of milk and dairy products available for public consumption (8).

In the Testing Guidance for Influenza A in Livestock (Version.2024.05.01), the USDA recommends using milk and nasal swabs for H5N1 testing in cows (9). Additionally, initial reports by the FDA on April 25th, 2024, indicated that 1 in 5 retail milk samples tested positive for HPAI viral fragments (10). In this evolving scenario, broader testing of milk samples and convenient research tools are needed to better understand this public health challenge.

Here we demonstrate use of milk as a sample type. Viral nucleic acid was extracted from milk containing inactivated Influenza A and SARS-CoV-2 viruses using NEB's Monarch® Mag Viral DNA/RNA Extraction Kit (NEB #T4010) and subsequently amplified using RT-qPCR or RT-LAMP. Additionally, we demonstrate the tolerance of milk with NEB's Luna® RT-qPCR and RT-LAMP reagents to support direct detection assays.

Considering the current supply challenges of H5N1 test material, this app note focuses on detecting Influenza A and SARS-CoV-2 as a representative proxy input.

## RESULTS

### Milk is a compatible sample type for Monarch Mag Viral DNA/RNA Extraction Kit (NEB #T4010)

The Monarch Mag Viral DNA/RNA Extraction Kit provides a simple, magnetic bead-based workflow for extracting viral nucleic acids. The procedure employs an optimized buffer chemistry in a lyse-bind-wash-elute workflow which has been previously demonstrated to be compatible with swabs, saliva and wastewater samples. Here we report that the Monarch Mag Viral DNA/RNA Extraction Kit protocol can be used to extract viral nucleic acids from milk using manual and automated workflows.

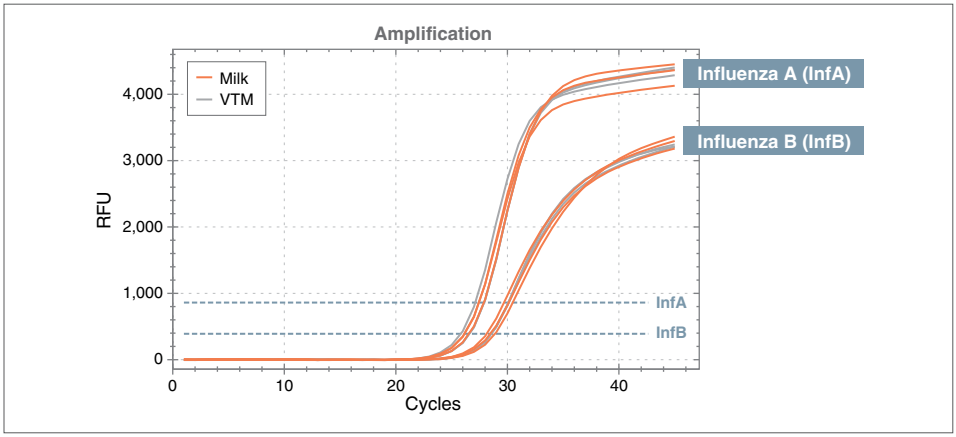
To simulate infected milk, mock samples were created by spiking store-bought pasteurized whole milk with known inactivated viruses and used as the starting point for extraction. Given the complex composition of milk in terms of high protein and fat content, different points of optimization were explored, including phase separation, additional Proteinase K treatment and other workflow modifications. Here we present an optimized manual and automated protocol for extracting viral nucleic acids from milk.

### Manual protocol for extraction of viral nucleic acids from milk

Our recommendation for manual extraction for viral nucleic acids from milk follows the current kit protocol provided on www.neb.com: Protocol for Manual Isolation of Viral DNA/RNA in Microfuge Tubes (1.5 or 2.0 ml).

We evaluated this protocol using virus-spiked milk and Viral Transport Medium (Hardy Diagnostics®) samples spiked with swabs containing inactivated Influenza A, Influenza B, Respiratory Syncytial Virus and SARS-CoV-2 (Microbiologics®, Cat 8246). After extraction, the eluates were subjected to RT-qPCR using NEB's Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019) with primers/probes targeting Influenza A and Influenza B, as described in CDC's influenza SARS-CoV-2 (Flu SC2) multiplex assay granted an Emergency Use Authorization (EUA) in 2020 (11). Results from the RT-qPCR indicate that the Monarch Mag Viral DNA/RNA Extraction Kit enables successful extraction of viral nucleic acids from milk, with no $C_q$ delay in the mock samples compared to the VTM controls (Figure 1).

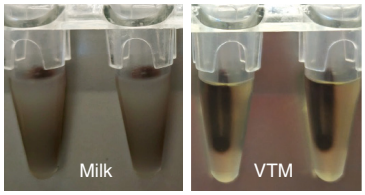
FIGURE 1: **Milk matrix is not inhibitory to extraction of viral nucleic acids**



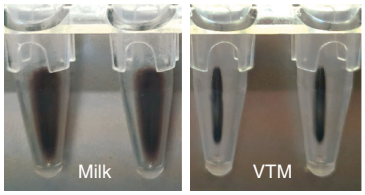

RT-qPCR amplification curves showing detection of InfA and InfB from viral RNA extracted using Monarch Mag Viral DNA/RNA Extraction Kit from virus-spiked milk (orange) and virus-spiked VTM (gray) samples. Milk and VTM were spiked with swabs containing inactivated Influenza A, Influenza B, Respiratory Syncytial Virus and SARS-CoV-2 and RT-qPCR was performed using Luna Probe One-Step RT-qPCR 4X Mix with UDG in a 20 µL reaction with CDC InfA (FAM) and InfB (HEX) primers/probes on a Bio-Rad® CFX-96 qPCR instrument. Dotted light blue lines represent the instrument-defined threshold for InfB (HEX) and InfA (FAM).

FIGURE 2: **Practical observations when processing milk samples with the Monarch Mag Viral DNA/RNA Extraction Kit**

**A. Turbidity**

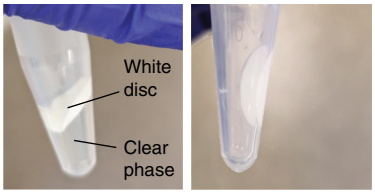



Before supernatant removal




After supernatant removal

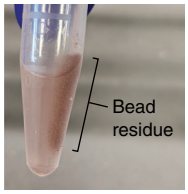
**B. Disc formation**




White disc formed after centrifugation

Disc pushed aside after clear phase has been drawn out

**C. Bead residue**




## Useful guidance for milk extraction

Below we summarize some observations specific to milk as a sample type and helpful tips to achieve optimal results.

1. The sample will appear in two phases when the lysis buffer bead mix is added. At this point, vortex the sample thoroughly to ensure the even dispersion of reagents within the sample.

2. During the steps on the magnet, the milk samples may retain a level of turbidity. This does not impact the magnetic separation and bead-free supernatant can still be successfully removed after the magnetic separation is complete (Figure 2A).

3. In our testing, centrifugation of milk did not improve end results and is therefore not recommended. Milk samples are directly compatible with our extraction kit. If, however, centrifugation of milk is strongly desired, we suggest 16000 x g for 2 minutes at room temperature, and proceeding with the clear phase in the next steps, after carefully pushing the top layer (disc) aside (Figure 2B).

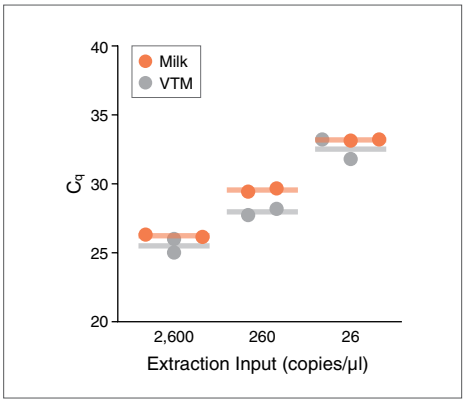
4. Milk sample tubes may appear to have a bead residue along the tube wall, even at stages away from the magnet (Figure 2C). Vortex vigorously to resuspend the beads as much as possible. Vortexing at the elution step removes the bead residue and does not have an impact on performance.

5. Based on our testing, additional Proteinase K or higher incubation temperatures for protein digestion were not helpful. Therefore, we recommend performing the Proteinase K incubation step at room temperature with 5 µl of Proteinase K per sample as outlined in the protocol.

## KingFisher® workflow for extraction of viral nucleic acids from milk

Our recommendation for automated extraction for viral nucleic acids from milk using the KingFisher platform (Thermo Scientific) follows the current kit protocol provided on www.neb.com: Protocol for KingFisher Flex Automated Isolation of Viral DNA/RNA. We evaluated this protocol using milk and Viral Transport Medium (Hardy Diagnostics) samples spiked with a 10-fold serial load of heat-inactivated SARS-CoV-2 (ATCC VR-1086HK). After extraction, the eluates were subjected to RT-qPCR using the Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (NEB #E3019). Results from the RT-qPCR assay demonstrate that the Monarch Mag Viral DNA/RNA Extraction Kit enables successful extraction of viral nucleic acids from milk using the KingFisher platform. A $C_q$ delay of one cycle was observed in milk samples compared to VTM samples (Figure 3) but a linear response was observed in both cases.

FIGURE 3: **Quantitative recovery of viral RNA extracted using Monarch Mag Viral DNA/RNA Extraction Kit on KingFisher platform**




RT-qPCR detection of SARS-CoV-2 on RNA extracted using Monarch Mag Viral DNA/RNA Extraction Kit from virus-spiked milk (orange) and VTM (gray) samples. Milk and VTM were spiked with a dilution series of heat inactivated SARS-CoV-2 (ATCC) at 2600 copies/ µl, 260 copies/µl and 26 copies/µl. RT-qPCR was performed using 10 µl of input in the Luna SARS-CoV-2 RT-qPCR Multiplex Assay Kit (NEB #E3019) in duplicate as outlined in the protocol (20 µl reactions) on a Bio-Rad CFX-96 qPCR instrument. Data shown is for SARS-CoV-2 N1 detection (HEX) and the lines denote the average $C_q$ value.

## Whole milk is tolerated in direct RT- qPCR assays

Currently, raw milk and commercial dairy products are sample types for H5N1 surveillance to monitor spread among dairy cows and mitigate any risks to agricultural workers. Purification of viral RNA from milk is typically recommended prior to amplification-based detection strategies. However, understanding if milk samples could be interrogated directly without up-front RNA purification steps would reduce assay turnaround times and the cost per sample. Therefore, we evaluated how pasteurized store-bought whole milk is tolerated in amplification assays using the Luna Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019).

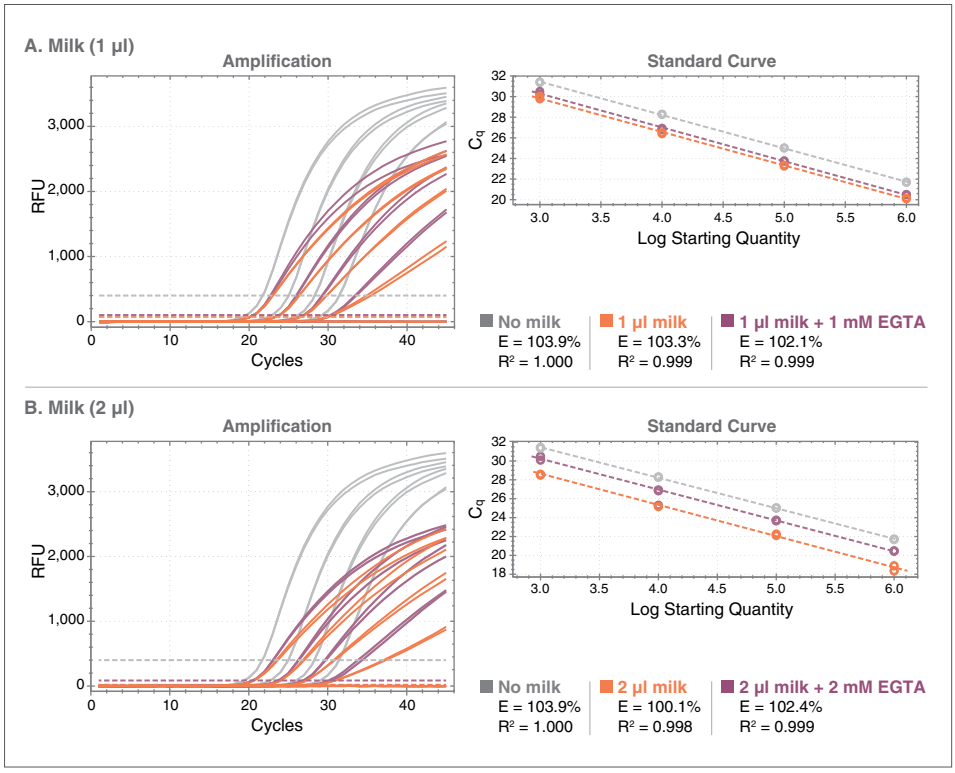
We investigated direct addition of whole milk up to 10 % (v/v) of the total reaction volume in SARS-CoV-2 assays. To mitigate any potential inhibitory effects due to high concentrations of calcium in milk, we evaluated the impact of ethylene glycol-bis (2-aminoethyl ether)-N,N,N´,N´-tetraacetic acid (EGTA), a known calcium chelator, on the amplification reactions.

For RT-qPCR, up to 2 µl of milk was tolerated in a 20 µl reaction (10% v/v) for detection of SARS-CoV-2 N1 (HEX) over a 4-log range of synthetic SARS-CoV-2 RNA input (100,000 cps to 100 cps) using the SARS-CoV-2 Primer/Probe Mix from NEB #E3019 (Figure 4). Addition of milk slightly inhibited detection by approximately 2 cycles and reduced the endpoint fluorescence signal of HEX compared to a no milk control, but linearity and sensitivity were unaffected. Including EGTA improved the robustness of amplification by increasing the endpoint fluorescence signal in milk containing reactions, particularly at low input. However, no signal was observed for SARS-CoV-2 N2 (FAM) in the presence of 10% milk (v/v), even with the addition of 2 mM EGTA (data not shown). The lack of amplification of N2 may be due to milk's impact on primer/probe annealing, which was previously observed for this target in direct assays with VTM (13).

## Whole milk is tolerated in direct RT-LAMP assays with minor adjustments

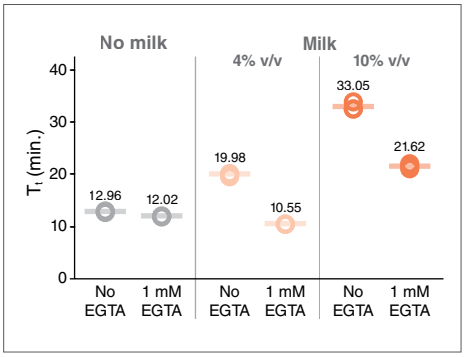
Loop-Mediated Isothermal Amplification (LAMP) is a commonly used method for rapid and simplified point-of-care diagnostics. Given the advantages that it can offer over RT-qPCR, we also evaluated the tolerance of milk for viral RNA detection using NEB #E1708, WarmStart Fluorescent LAMP/RT-LAMP Kit (with UDG). This kit is a simple and one-step solution for fluorescence detection of DNA (LAMP) or RNA (RT-LAMP) targets. Compared to the pH-based colorimetric LAMP approach described below, the NEB #M1708 master mix in this kit is fully buffered and offers robust detection of different sample types while being compatible with various detection strategies. In this study, we monitored SARS-CoV-2 detection in real time using our SARS-CoV-2 LAMP Primer Mix (N/E) from NEB #E2019. Following successful detection of SARS-CoV-2 from RNA isolated with the Monarch Mag Viral DNA/RNA Extraction Kit from virus-spiked milk samples (data not shown), the impact of whole milk on RT-LAMP was investigated. Addition of milk up to 10% (v/v) in a 25 µl RT-LAMP reaction delayed detection of 5000 cps of synthetic SARS-CoV-2 RNA up to 8 minutes (Figure 5). Reducing the amount of milk to 4% (v/v) improved time to detection but the reaction still failed to match the performance of a no milk control. Including 1 mM EGTA significantly improved assay performance, and the 4% (v/v) milk reaction gave results substantially equivalent to the no milk control.

FIGURE 4: **Tolerance of Luna Probe RT-qPCR 4X Mix with UDG to whole milk**




Luna Probe One-Step RT-qPCR 4X Mix with UDG was used to detect Twist synthetic SARS-CoV-2 RNA starting from 100,000 cps to 100 cps in the presence of up to 10% (v/v) whole milk in a 20 µl reaction. Data was collected on a Bio-Rad CFX-96 qPCR instrument using the instrument-defined threshold for each condition. Addition of milk results in minor inhibition ($C_q$ delay) and dampens the overall fluorescence signal. Inclusion of EGTA improved robustness of the amplification signal.

FIGURE 5: **Tolerance of WarmStart® RT-LAMP 2X Master Mix with UDG to whole milk**




Fluorescent RT-LAMP detection of Twist synthetic SARS-CoV-2 RNA at 5000 cps in the presence of whole milk up to 10% (v/v) using WarmStart Fluorescent LAMP/RT-LAMP Kit (with UDG). Triplicate reactions (25 µl) containing 0.5X LAMP Fluorescent dye and 1X SARS-CoV-2 LAMP Primer Mix (N/E) were incubated at 65°C for 40 minutes and fluorescence was monitored in the FAM/SYBR channel of a Bio-Rad CFX-96 qPCR instrument. Each dot represents the time at which the fluorescence signal for a single reaction crosses the instrument-defined threshold. All three replicates were detected for each condition and the average time to detection is denoted by the line and numerical values (note that dots frequently overlap given similar detection time for the replicates). Whole milk inhibited fluorescent RT-LAMP reactions significantly but including 1 mM EGTA greatly improved detection times.

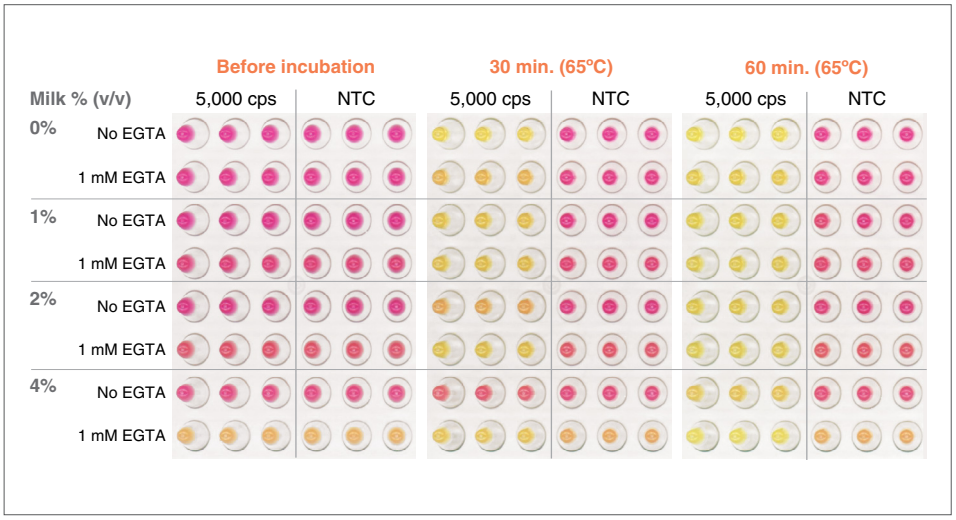
LAMP also enables simple, visual detection of amplification, which has been used previously to detect multiple influenza viruses including H5N1 (14) The WarmStart Colorimetric LAMP 2X Master Mix with UDG (NEB #M1804) utilizes a rapid pink to yellow color change to indicate amplification. However, this color change is pH-dependent, utilizing proton production during DNA amplification, and factors such as sample pH and buffering must be considered when developing a colorimetric LAMP workflow. To evaluate the effect of milk in pH-based colorimetric LAMP, we tested amplification of 5000 cps of Twist synthetic SARS-CoV-2 RNA in the presence of whole milk and EGTA at 65°C. We also evaluated whether the turbid nature of milk would mask the color change. Up to 2% (v/v) milk in 25 µl reaction was tolerated well, resulting in robust color change within 30 minutes (Figure 6). A volume of 4% (v/v) milk also resulted in successful amplification but required additional incubation time (60 min) to detect a color change. In this colorimetric experiment, adding EGTA did not improve detection and in the case of the 4% (v/v) milk reactions, resulted in a yellow color prior to incubation, suggesting volumes of milk greater than or equal to 4% (v/v) in the presence of EGTA are incompatible with pH-based colorimetric LAMP.

## Nucleic acids from viral particles can be detected directly in milk without isolation

Detecting viral RNA in milk without viral isolation can streamline testing workflows and shorten assay turnaround time. To determine if direct detection assays using milk can be developed, we spiked milk with inactivated viruses to simulate a real-world sample. An inactivated swab containing Influenza A, Influenza B, Respiratory Syncytial Virus, and SARS-CoV-2 viral particles (Microbiologics, Cat 8246) was swirled in 500 µl whole milk. This milk sample was tested for Influenza A (FAM, primers/probes from CDC Flu SC2 multiplex assay) and SARS-CoV-2 (HEX, primers/probe from E3019) using Luna Probe One-Step RT- qPCR 4X Mix with UDG.

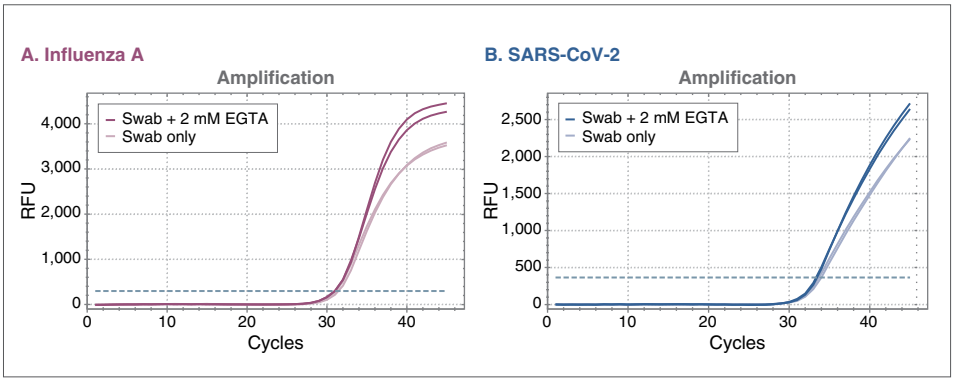
In the RT-qPCR assay, 2 µl of the virus-spiked milk sample was added directly to the reaction in the presence and absence of 2 mM EGTA. Both SARS-CoV-2 (HEX) and InfA (FAM) RNA were evaluated in single-plex reactions, and resulted in $C_q$ values of 33 and 31, respectively (Figure 7), which corresponds to less than 120 copies based on the standard curves of synthetic viral RNA that were included in the run. Addition of 2 mM EGTA in the RT-qPCR assay again improved the endpoint fluorescence signal. This data shows that viral detection directly from milk is possible, but additional optimization to ensure robust amplification and sensitive detection for direct assay workflows may be required.

FIGURE 6: **Tolerance of WarmStart Colorimetric LAMP 2X Master Mix with UDG to whole milk.**




Detection of 5000 copies of Twist synthetic SARS-CoV-2 RNA using NEB's WarmStart Colorimetric LAMP 2X Master Mix (with UDG). Reactions were incubated at 65°C for either 30 or 60 minutes in the presence of 1X SARS-CoV-2 LAMP Primer Mix (N/E). Whole milk was added up to 4% (v/v) in a 25 µl reaction in the presence or absence of 1 mM EGTA. Up to 2% (v/v) milk resulted in robust detection within 30 minutes while inclusion of 1 mM EGTA at milk volumes of 4% (v/v) resulted in an undesired color change prior to incubation.

FIGURE 7: **Detecting viral RNA from whole milk without extraction and purification**



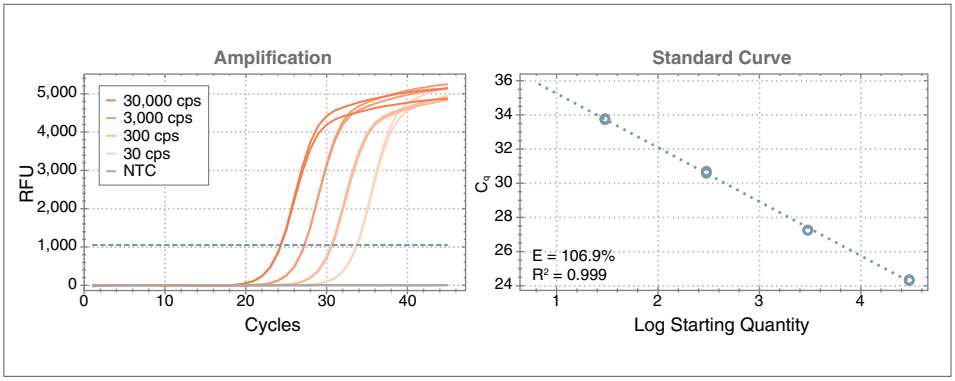

Testing a virus-spiked milk sample prepared from an inactivated swab containing Influenza A and SARS-CoV-2 using Luna Probe One-Step RT-qPCR 4X Mix with UDG. A sample of milk (2 µl) was directly added to the RT-qPCR assay without any pretreatment and tested in the presence and absence of 2 mM EGTA. Influenza A (FAM) and SARS-CoV-2 (HEX) were successfully detected in both independent reactions.

## H5N1 viral RNA can be detected using CDC's InfA primers and probe in RT- qPCR

The CDC Flu SC2 multiplex assay mentioned above utilizes two forward and two reverse primers with a FAM-labeled fluorescent probe designed to detect InfA (11). We previously demonstrated that these primer sets could detect three Influenza A strains (H1N1/1934, H1N1/2009, H3N2/2009) consistently over a 5-log range of viral RNA input using Luna Probe One-Step RT-qPCR 4X Mix with UDG (12).

For testing applicability for H5N1 detection, the InfA primers and probe were used in reactions containing AMPLIRUN Influenza the H5 RNA Control from Vircell® (30,000 cps to 30 cps). Sensitive detection with good linearity and efficiency was observed for H5 RNA Control (Figure 8, page 5), highlighting that these primers also detect the H5N1 subtype of influenza A.

FIGURE 8: **Testing Influenza H5 synthetic RNA control using InfA primers and probe from the CDC**




Influenza H5 RNA Control (AMPLIRUN) was tested over 4-log template input starting from 30,000 cps to 30 cps using Luna Probe One-Step RT-qPCR 4X Mix with UDG in a 20 µl reaction in the presence of CDC InfA primer sets and probe on a Bio-rad CFX-96 qPCR instrument.

## CONCLUSION

The data presented here are intended to serve as a starting point, which we hope will enable further studies utilizing milk as a sample type to research H5N1. In this study, we used inactivated swabs that contained Influenza A, Influenza B and SARS-CoV-2 viral particles as a proxy for H5N1 due to challenges in obtaining sufficient quantities of commercially available H5N1 viral RNA. We demonstrated that the Monarch Mag Viral DNA/RNA Extraction Kit is compatible with milk and supports purification of viral RNA from mock samples, which can be subsequently amplified using either RT-qPCR or RT-LAMP. Additionally, both RT-qPCR and RT-LAMP reactions tolerate the presence of milk directly and we were able to observe detection of both Influenza A and SARS-CoV-2 when a virus-spiked milk sample was used as template in RT-qPCR. Additional questions remain in detecting viral nucleic acid directly from milk using RT-qPCR or RT-LAMP and further studies are needed to determine the overall robustness and sensitivity of such a workflow, or if additional pretreatment would be beneficial.

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