Quantitative Real-Time PCR Detection of Inactivated H5 Avian Influenza Virus in Raw Milk Samples by Miniaturized Instruments Designed for On-Site Testing

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ABSTRACT

Highly pathogenic avian influenza virus (HPAIV) of H5 and H7 subtypes has emerged as one of the most important zoonotic pathogens in the 21st century with significant economic consequences. The recent outbreak of H5N1 avian influenza (AI) in dairy cattle highlighted the importance of early detection in managing and mitigating HPAIV outbreaks. A successful high-speed diagnostic response requires rapid site and specimen access, minimal time for test protocols, and prompt communication of the diagnostic results to government officials. A new diagnostic paradigm that consists of miniaturized extractor and qPCR instruments (EZextractor and EZcycler MiniQ), designed for mobile, on-site testing has been compared with a platform of benchtop instruments (QIAGEN RNeasy and QuantStudio™ 5) for detecting inactivated H5 avian influenza virus (AIV) spiked in raw milk samples. Two sets of experiments were performed: 1) 15 raw milk samples, obtained from 15 different farms, diluted with phosphate-buffered saline and spiked with the virus to reach approximately 10 copies/µL virus concentration, and 2) raw milk samples from two farms, each spiked with the inactivated AIV H5 followed by 5 series of dilution to reach AIV concentrations of 1000, 100, 10, 1 and 0.1 copies/µL. Results show that despite the inhibitors in raw milk, AIV in all samples can be detected by both platforms. The MT platform showed higher sensitivity than the benchtop platform: the Ct values from the MT were ~2 units lower than the benchtop Ct values. Our findings demonstrate the robustness of the MT platform for diagnosing AIV H5 in raw milk samples and support its use as an on-site diagnostic for rapid surveillance and response.

Keywords: Avian influenza, H5 avian influenza virus, molecular testing, qPCR, pathogen

detection, avian influenza virus in raw milk, on-site testing

INTRODUCTION

Avian influenza virus (AIV) has been a significant threat to the poultry industries worldwide, with global outbreaks caused primarily by the AIV of H5 and H7 subtypes since the mid-20th century [1]. The current wave of avian influenza started in 2020 and is dominated by the highly pathogenic avian influenza virus (HPAIV) H5N1 strain of the clade 2.3.4.4b, which originated in Europe and first spread to North America in 2021 with frequent spillover events to mammals reported [1, 2]. In the United States, the potential spread of the HPAIV H5N1 strain beyond poultry had a significant upturn when HPAIV H5N1 was isolated from milk in dairy cattle in Texas in February 2024 with cattle showing symptoms such as reduced milk production and decreased feed intake [3]. Zoonotic infection of HPAIV H5N1 of dairy farm workers has also been reported, although the symptoms were milder compared to bird-transmitted HPAIV H5N1 infection [4, 5].

The implications in human public health have been further highlighted by the fact that 70 confirmed zoonotic HPAIV H5 human cases have been reported in the United States as of May 2025 [6,7]. A new genotype D1 typically carried by wild birds was found to be responsible for the first severe HPAIV H5N1 case in Canada in November 2024, and the first severe case in the United States in Louisiana in December 2024 that has resulted in death [8,9].

Since its first discovery in cattle milk, the HPAIV H5N1 has since spread in the United States and as of May 2025, at least 1,052 dairy herds across 17 states have been affected, prompting urgent governmental action to address the growing crisis [6]. California, the largest milk-producing state in the United States bears the brunt of this outbreak accounting for 73% of cases reported in the United States [6]. In response, the state government of California has declared a state of emergency to mobilize resources to combat the virus's rapid spread and uphold consumer confidence in dairy products [10]. Nationwide, to address the importance of early detection and containment to mitigate the spread of H5N1 within dairy herds and protect public health, the United States Department of Agriculture (USDA) announced a new Federal Order to implement a National Milk Testing Strategy on December 6, 2024 [11]. Per the Order, milk samples were to be tested by molecular testing using quantitative reverse transcription polymerase chain reaction (qRT-PCR), which is considered the standard used in the detection of AIV according to the USDA’s Animal and Plant Health Inspection Service [12].

The implementation of the Federal Order for milk testing will challenge the current capacity of qRT-PCR diagnostic testing in many state diagnostic laboratories. Further, the current laboratory diagnostic routine from taking samples to transportation to a laboratory to results takes time that is critical for tracing the original source of infection. Here we present a new mode of qRT-PCR diagnosis using miniaturized extraction and PCR instruments that can be housed in a mobile laboratory for conducting the diagnosis on-site. The mobile testing (MT) platform for detecting H5N1 in milk samples offers a critical solution for rapid surveillance and early intervention. By enabling on-site and timely detection, MT platform reduces the lag between sample collection and diagnosis, enhancing the ability to contain outbreaks before they escalate.

This study aimed to evaluate the effectiveness of the MT platform for detecting the AIV H5 in raw milk samples and its adaptability to different operational settings by comparing different PCR reagents and systems.

MATERIALS AND METHODS

Virus. Three samples of lysis buffer-inactivated AIV H5 (clade 2.3.4.4b) taken from a goose autopsy tissue/organ sample in Pingtung County, Taiwan, were obtained as part of a research grant awarded to Ya-Mei Chen from the Animal and Plant Health Inspection Agency, Taiwan Ministry of Agriculture (research grant: Research and Analysis of Key Avian Disease Surveillance and Control, ID: 113AS-5.5.5-VP-01; In Chinese: 家禽重要疾病監測及防控研析, ID: 113 農科-5.5.5-檢-01). The concentrations of inactivated AIV H5 suspended in the lysis buffer of the three samples are estimated based on quantitative PCR measurements using the positive control of the ThermoFisher AIV kit with a known RNA concentration of 1,000 copies/µL. The estimated concentrations of samples 1, 2, and 3 are 1,000, 10,000, and 1,000 copies/µL, respectively. All experiments were conducted in the laboratories of the Schweitzer Biotech Company in Taipei, Taiwan.

Milk Samples. Fifteen fresh raw milk samples were collected from different dairy farms in Taiwan. All milk samples were diluted with phosphate-buffered saline (PBS) at a ratio of 1:3 by volume to reduce inhibitory effects of components in the milk. The fifteen raw milk samples were used for evaluating the performance of MT platform (EZextractor + MiniQ) and the USDA-recommended benchtop platform (RNeasy + QS5) in detecting AIV H5. Each of the 15 raw milk samples was spiked with either of

the three inactivated AIV H5 samples (Samples 1, 2, or 3) and diluted to reach a concentration of 10 copies/µL. The spiking and dilution process is outlined in Table 1.

	Sample 1	**Sample 2**	**Sample 3**
Stock concentration	1,000 copies/µL	10,000 copies/µL	1,000 copies/µL
Diluent	Milk-PBS mixture (1:3)	Milk-PBS mixture (1:3)	Milk-PBS mixture (1:3)
Dilution method*	Two ten-fold dilutions	Three ten-fold dilutions	Two ten-fold dilutions
Target concentration	10 copies/µL	10 copies/µL	10 copies/µL

Table 1. Inactivated AIV H5 in milk samples used in this study.
*First dilution was made by adding 40 µL of inactivated virus sample to 100 µL of milk and 260 µL PBS to achieve a concentration of 25% milk by volume. Subsequent ten-fold dilutions were made by adding 100 µL of the prior sample solution to 900 µL of diluent.

Polymerase Chain Reaction. The EZextractor Viral DNA/RNA Extraction Kit (Ref: ATXZ008, Schweitzer Biotech Company, Taipei, Taiwan) was used to isolate viral RNA using the EZextractor Nucleic Acid Extraction System (Model M32, Schweitzer Biotech Company, Taipei, Taiwan) following manufacturer's built-in program V9. The EZextractor system is a fully automated magnetic bead-based platform designed to streamline nucleic acid extraction for laboratories handling medium or large sample sizes. It operates within a compact footprint and features a touchscreen interface for user control. The system supports flexible batch processing with capacities of up to 32 samples per run with an approximate runtime of 30 minutes. As a comparison in this study, QIAGEN RNeasy Mini kit (Cat. #74106 250, QIAGEN N.V., Venlo, The Netherlands) was used to extract AIV H5 RNA spiked into raw milk samples following the Protocol: Purification of Total RNA from Animal Cells Using Spin Technology according to the kit handbook.

Extracted AIV RNA was then amplified by VetMAX™ Gold AIV Detection Kit (Cat# 4485261, ThermoFisher Scientific, Waltham, MA) using Influenza Virus Primer Probe Mix included in the kit (FAM reporter dye). The following control samples were also included in the kit: Influenza Virus-Xeno™ RNA Control Mix for positive control; Xeno™ RNA Control for internal positive control (VIC reporter dye).

The qRT-PCR process was performed and analyzed using two types of qPCR thermocyclers: the benchtop QuantStudio™ 5 (QS5) Real-Time PCR System (Cat#

A28133, ThermoFisher Scientific, Waltham, MA) and the portable EZcycler Mini real-time PCR (MiniQ) System (Schweitzer Biotech Company, Taipei, Taiwan). The MiniQ PCR System is a compact, space-efficient system for precise quantitative analysis suitable for MT. Equipped with a built-in touchscreen, it operates independently of a computer and has a sample capacity of 16 and three detection channels. Two independent qPCR runs were performed for each of the milk samples.

The thermal cycling conditions in reverse transcription followed by qPCR amplification for both thermocyclers were as follows:

Stage	Cycles.	Temperature	Time
Reverse transcription	1	48°C	10 minutes
RT inactivation/initial denaturation	1	95°C	10 minutes
Amplification: Denaturation Amplification: Annealing and extension	40	95°C 60°C	15 seconds 45 seconds*

Table 2. Reverse transcription and qPCR thermocycling conditions.

*: Fluorescence signal is collected at this step.

Sensitivity of AIV H5 Detection. Serial ten-fold dilutions of the inactivated virus sample 2 stock solution (10,000 copies/µL) using raw milk-PBS mixture (raw milk from farm 8 or farm 9) were performed to determine the limits of detection and the efficiency of the MT platform.

Raw Milk Inhibitors. To assess if the potential inhibitors in raw milk influence the detection of AIV, PBS solutions were spiked with inactivated AIV H5. Two raw milk samples were randomly chosen from the stocks and diluted with PBS at 1:3 ratio. Each sample, either raw milk in PBS or PBS only, was spiked using the same source with an unspecified quantity of inactivated viruses. Internal positive control was also used to spike the PBS only as well as the raw milk diluted with PBS. In this comparison, one set of experiments was performed using the combination of Ezextractor and the benchtop QS5, and the other Ezextractor and the miniaturized MiniQ.

RESULTS

Both platforms successfully detected AIV H5 in all 15 spiked raw milk samples

at 10 copies/μL viral concentration (Table 3 and Figure 1). Spearman's correlation of these two assays is 0.96, which shows good consistency between the MT platform and the benchtop platform. The paired t test shows that there is a significant difference in the Ct values between these two assays: The MT assay's Ct values are 2 units smaller than that of the USDA benchtop assay. These results validate the performance of the MT platform for detecting AIV in raw milk.

Ct values of two PCR assays				
Farm ID +AIV Sample ID (10 copies/uL)	Ezextractor + MiniQ (MT)		RNeasy + QS 5 (USDA benchtop)	
Farm 1 + Sample 1	34.8	35.8	35.4	37.0
Farm 2 + Sample 1	30.7	30.7	34.7	35.3
Farm 3 + Sample 1	29.8	29.8	32.3	31.6
Farm 4 + Sample 1	31.3	31.2	33.2	33.0
Farm 5 + Sample 1	30.2	30.2	34.6	36.1
Farm 6 + Sample 2	30.7	31.3	33.0	32.5
Farm 7 + Sample 2	31.0	30.7	32.8	32.8
Farm 8 + Sample 2	30.9	31.1	31.8	32.2
Farm 9 + Sample 2	32.0	31.4	32.5	30.8
Farm 10 + Sample 2	31.3	31.4	32.4	32.5
Farm 11 + Sample 3	31.4	31.5	35.1	35.7
Farm 12 + Sample 3	31.2	31.4	33.4	33.0
Farm 13 + Sample 3	32.2	32.2	35.8	36.6
Farm 14 + Sample 3	31.6	31.7	31.0	31.3
Farm 15 + Sample 3	31.4	30.8	35.4	35.5
PC	24.9	25.1	24.5	23.7
NTC	ND	ND	ND	ND

Table 3. Ct values of qPCR run of 15 raw milk samples spiked with inactivated AIV H5 using the MT and the USDA benchtop platforms. Two independent PCR runs were performed for each sample using each platform. PC: positive control. NTC: no template control. ND: not detected.

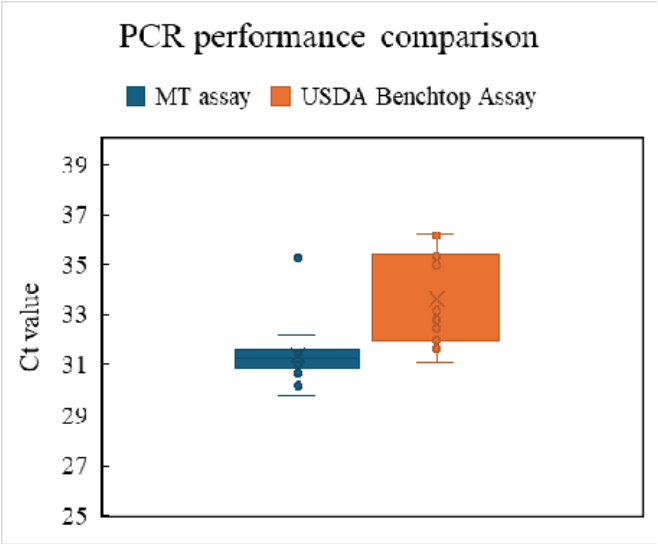

Figure 1. Comparison of performance of the MT vs. the benchtop PCR testing platforms. The Ct values listed in Table 3 from the two platforms respectively are analyzed. The Spearman's correlation is calculated to be 0.96 and a paired Student's t test is calculated to be 0.0002. The difference of the mean Ct values of the MT assay and the USDA-benchtop assay is calculated to be -2.3.

Sensitivity of the MT platform

Raw milk samples from Farm 8 and Farm 9, undergone serial dilutions, were diagnosed using the MT platform to determine its detection sensitivity and efficiency. The Ct values are listed in Table 4. The Ct values of two independent runs of each sample at the five different dilution levels with estimated virus density of 1000, 100, 10, 1.0, and 0.10 copies/µL are consistent with each other and show the expected trend according to the virus density. The Ct values as a function of dilution level, the PCR standard curves in Figure 2, show that the amplification efficiencies are 93.6% and 102.5% for Farms 8 and 9 respectively. The R^2 values (0.987 and 0.989) of both dilution series show good PCR performance of the MT platform.

Farm ID/Dilution factor	Ct values of the dilution series	
Farm $8/10^1$	24.1	23.9
Farm $8/10^2$	26.1	26.1
Farm $8/10^3$	31.1	31.1
Farm $8/10^4$	33.7	34.0
Farm $8/10^5$	38.0	37.1

Farm 9/10^1	23.7	23.6
Farm 9/10^2	27.4	27.3
Farm 9/10^3	31.3	31.1
Farm 9/10^4	34.0	34.2
Farm 9/10^5	36.1	37.0
PC	25.1	25.1
NTC	ND	ND

Table 4. Ct values of qPCR run using the MT platform of raw milk samples from two farms spiked with inactivated AIV H5 following serial ten-fold dilutions. Two independent PCR runs were performed for each sample. PC: positive control. NTC: no template control. ND: not detected. Note: the first (10^1) dilution was made by adding 40 µL of inactivated virus sample to 100 µL of milk and 260 µL PBS to achieve a virus density of 1000 copies/µL. Subsequent ten-fold dilutions were made by adding 100 µL of prior dilution to 900 µL of diluent described in Table 2.

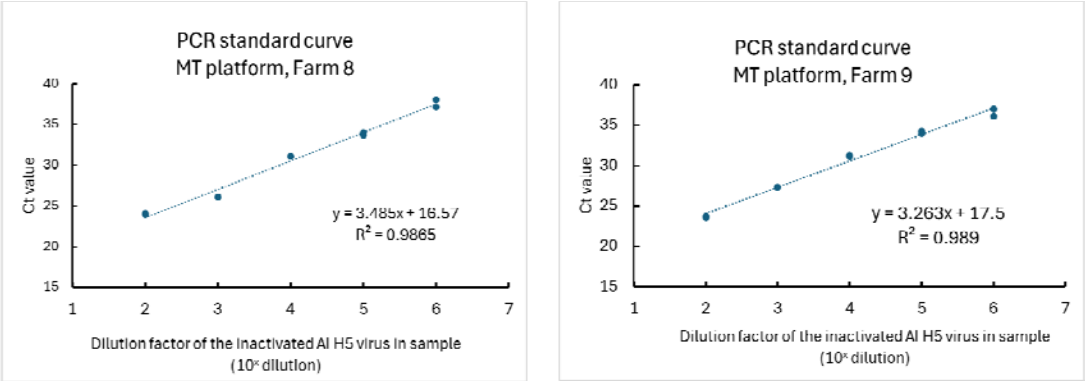

Figure 2. PCR standard curves, Ct values as a function of dilution level, from the data in Table 4. The amplification efficiency is calculated to be 93.6% for Farm 8 samples and 102.5% for Farm 9 samples.

Effect of Raw Milk Inhibitors on PCR Diagnosis

The data obtained using both platforms (Table 3) clearly show their effectiveness in detecting inactivated AIV H5 in raw milk. Table 5 reports comparison of diagnoses performed on raw milk vs. PBS samples spiked with the same virus density. All runs on the internal positive control (Xeno™ RNA Control) spiked samples, whether they contain raw milk or not, showed similar Ct values. In contrast, the runs on inactivated viruses showed notable differences in Ct values: The runs on samples containing raw milk showed consistently 1.5 higher Ct values than the pure PBS samples. Further, it appears that whether the combination of Ezextractor+QS5 or Ezextractor + MiniQ, the Ct values for both virus- and internal positive control- spiked samples appear the same.

Ezextractor + QS5	Ct values	
	AIV H5	**Internal positive control**
PBS	32.8, 32.6	30.7, 30.5
Raw milk + PBS	34.0, 34.0	30.0, 29.8
PC	28.1, 28.1	27.9, 27.9
NTC	ND	ND
Ezextractor + MiniQ	**Ct values**	
	AIV H5	**Internal positive control**
PBS	32.5, 32.5	29.5, 29.3
Raw milk + PBS	34.3, 34.0	30.1, 29.3
PC	27.8, 27.8	27.0, 26.8
NTC	ND	ND

Table 5. Ct values of qPCR run on two raw milk samples in comparison with PBS solutions. In both comparisons, the raw milk sample and the corresponding PBS solution were spiked with the same unknown quantity of inactivated AIV H5. PC: positive control. NTC: no template control. ND: not detected.

DISCUSSION

Milk is a complex mixture containing fat globules, proteins, sugar, and metal ions. [13] Components in milk that may interfere with the AIV detection process include calcium ions, collagen, and myoglobin. These ions/molecules can inhibit DNA polymerase or reverse transcriptase, and *Taq* polymerase-degrading plasmin [14]. To mitigate the inhibitory effect of raw milk, we first diluted milk samples in PBS, and we were able to demonstrate the feasibility of detecting AIV H5 in diluted raw milk samples using both platforms. Both qPCR systems (MiniQ and QS5) exhibited consistent performance in detecting AIV H5 in raw milk samples. Virus detection sensitivity remained robust despite the presence of PCR inhibitory substances in raw milk. The results also illustrated the impact of milk from different sources on AIV H5 detection. Table 3 shows that the Ct values from the MT platform range from 30 to 32 for all samples except the raw milk from Farm 1 which has Ct values higher than 35. The Ct values from the benchtop platform show a larger variation from 31 to 37, again with the Farm 1 sample displaying the highest Ct values.

Table 4 shows the sensitivity of qPCR detection of inactivated AIV H5 in raw milk samples diluted to different virus densities. The five levels of dilution correspond

to approximately 1,000, 100, 10, 1, 0.1 copies/μL. Even the lowest density AIV can be detected by the MT platform with good PCR efficiency. The standard curves quantitatively illustrate the precision of the MT platform diagnosis.

From the comparison of Ct values listed in Table 5, it seems that the inhibitors do have effect on detection efficiency. The runs on samples containing raw milk show consistently 1.5 higher Ct values than the pure PBS samples. Furthermore, by comparing the experiments performed by using the same Ezextractor but different qPCR (QS5 vs MiniQ), it appears that the difference in detection efficiency between the bench top vs miniaturized instruments lies primarily with the extraction method/instrument.

Examination of the Ct values in Table 3 also shows that the MT platform displays higher sensitivity than the benchtop platform in this study. Ct values for all 15 raw milk samples from the MT platform are on average ~2 units lower than the ones from the benchtop platform. This difference may be attributed to the different extraction methods used in these two platforms.

CONCLUSION

In this study, we have shown that, even though the presence of the inhibitors in raw milk challenges the operation of extraction and PCR detection, both the MT and the benchtop platforms can detect inactivated AIV H5 in raw milk samples from 15 different farms. Further, the MT platform performs well and consistently for inactivated virus samples diluted over a 5-order magnitude. The experiments performed so far illustrate that AIV H5 can be detected with high sensitivity even though there is discernible interference from milk components. The MT platform performance in all samples tested in this work is more sensitive than the benchtop platform used here.

The MT platform utilizing miniaturized extraction and PCR instruments for inactivated AIV H5 detection in raw milk samples represents a highly sensitive, versatile, and portable approach for managing AIV outbreaks. Successful containment and mitigation of outbreaks at the earliest instance call for early detection and surveillance of the virus. Given that viral loads in milk may vary depending on infection stage and severity, the ability to detect the virus at low concentrations ensures that even minimally infected herds can be identified and managed promptly. This capability is crucial for preventing the spread of infection within and between

herds. Putting into perspective the recent outbreak of H5N1 in the United States dairy cattle and the associated zoonotic risks, the implementation of the MT platform offers a practical solution for minimizing the public health and economic impacts. The portability of these systems could facilitate rapid on-site testing, reducing the delay between sample collection and results and communication with government officials. This is particularly important in high-risk areas in many states which have experienced significant outbreaks.

Data Availability. All data generated or analyzed in this study are included in this article. PCR amplification curves of data shown in Table 3 and 4 are shown in Figure 3 and 4 respectively at the end of this article.

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Competing Interests. Authors declare no competing interests.

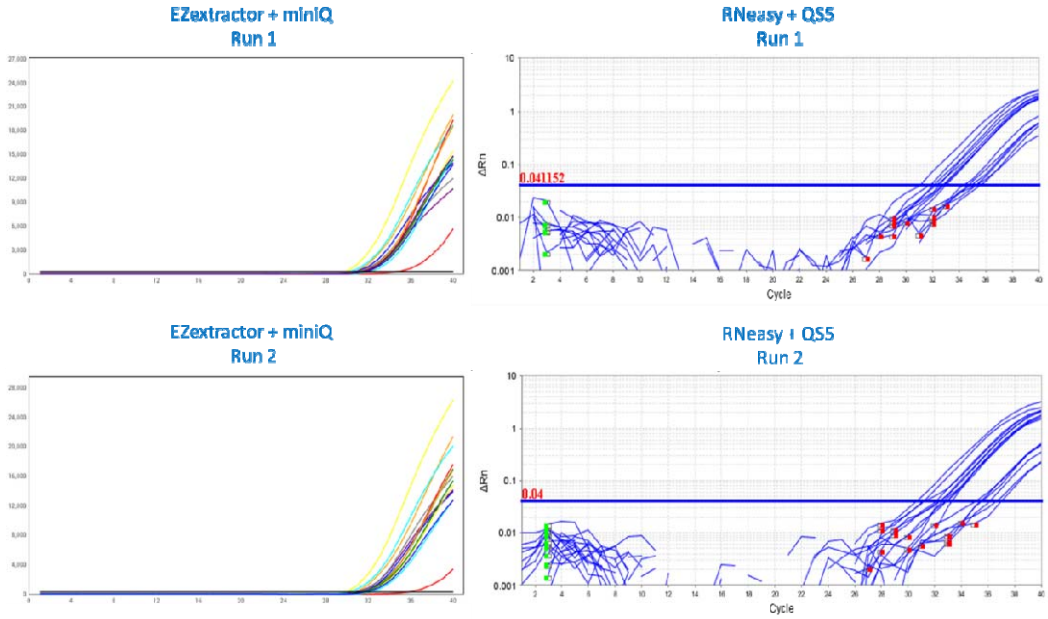

Figure 3. Amplification plots of two qPCR runs for the detection of inactivated AIV H5 spiked into raw milk samples from 15 farms. The qPCR runs were performed using the MT platform (EZextractor for RNA extraction and MiniQ thermocycler; right panels) or the benchtop platform (RNeasy for RNA extraction and QS5 thermocycler; left panels).

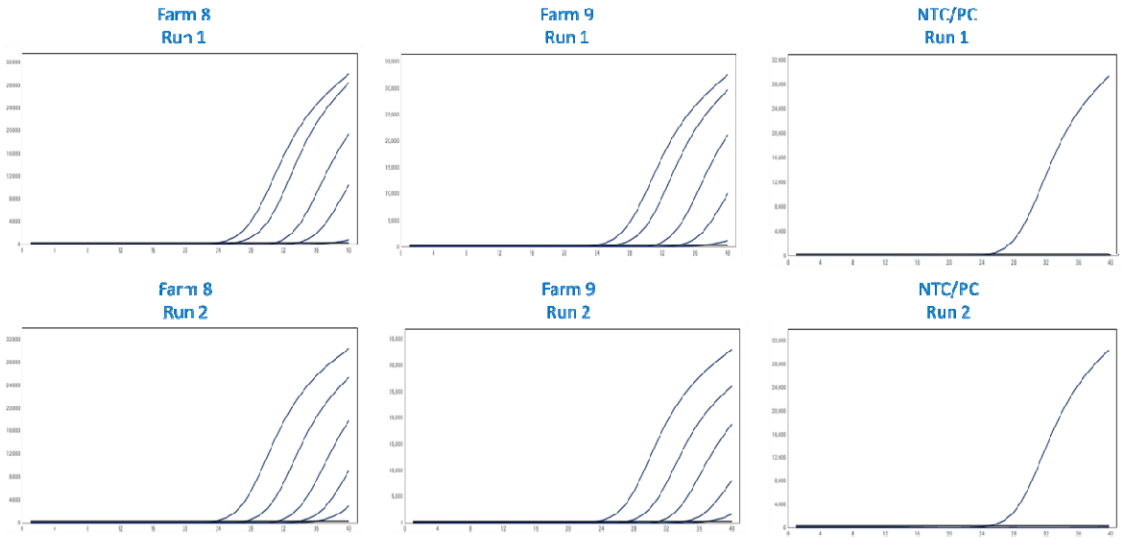

Figure 4. Amplification plots of two qPCR runs for the detection of AIV H5 in serial ten-fold dilutions of inactivated AIV H5 spiked into raw milk samples from two farms. The qPCR runs were performed using the MT platform (EZextractor for RNA extraction and MiniQ thermocycler).

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