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RESEARCH PAPER

Supporting molecular detection of A(H5N1) through development and characterization of RNA reference materials

Eileen A. Moran[1] · Monique E. Hunter[2] · Jason G. Kralj[2] · Kristine Gierz[3] · Julia C. Frederick[4] · Jimma Liddell[4] · Lisa M. Keong[4] · Bo Shu[4] · John R. Barnes[5] · Jasmine Chaitram[6] · Marie K. Kirby[4] · C. Todd Davis[4] · Stephanie L. Servetas[2] · Scott Jackson[2] · Peter M. Vallone[1] · Megan H. Cleveland[1]

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Abstract

In 2024, the US Centers for Disease Control and Prevention (CDC) and National Institute of Standards and Technology (NIST) initiated an interagency collaboration to bolster preparedness in the event of a pandemic scenario due to high pathogenicity avian influenza A(H5N1) clade 2.3.4.4b viruses that had been circulating in North America since 2021. Multiple spillover events into mammalian species, including an outbreak among dairy cattle and a surge in human cases, spurred the development of RNA reference materials to assist with new diagnostics development and validation. With input from CDC on sequences, NIST generated three synthetic RNA materials of A(H5N1) hemagglutinin, neuraminidase, and matrix proteins 1 and 2 gene segments. Here, we describe the development and characterization of these reference materials utilizing droplet digital reverse transcription polymerase chain reaction. Through this collaboration, we were able to rapidly provide quantified, homogeneous, stable, and epidemiologically relevant RNA reference materials to the public to aid new assay development and validation.

Keywords A(H5N1) · Avian influenza · Reference material · Influenza A virus · Bird flu

Introduction

High pathogenicity avian influenza (HPAI) viruses have caused mass wild bird and poultry outbreaks and die-offs globally for decades. HPAI viruses of A/goose/Guangdong/1/1996 lineage have been circulating globally since the earliest detection of these A(H5N1) viruses in 1996 [1, 2]. In late 2020, a new clade 2.3.4.4b A(H5N1) reassortant virus emerged. It rapidly spread across Asia, Europe, and Africa, became the most predominant subtype globally, and arrived in North America by late 2021 [1, 3]. These clade 2.3.4.4b A(H5N1) viruses have demonstrated an expanded host range, infecting and causing outbreaks not only in hundreds of bird species but also many mammalian species, including both marine and terrestrial mammals, such as sea lions, elephant seals, raccoons, rodents, domestic cats, and farmed animals, including minks and dairy cattle [1, 4].

In March 2024, the US Department of Agriculture National Veterinary Services Laboratory confirmed HPAI A(H5N1) in dairy cattle and cats from dairy farms in Texas [5]. Shortly after, a case of HPAI A(H5N1) was reported in a dairy farm worker in Texas, and specimens taken from the

✉ Eileen A. Moran
eileen.moran@nist.gov

Megan H. Cleveland
megan.cleveland@nist.gov

1 Biomolecular Measurement Division, National Institute of Standards and Technology, Gaithersburg, MD, USA

2 Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD, USA

3 Statistical Engineering Division, National Institute of Standards and Technology, Gaithersburg, MD, USA

4 Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA, USA

5 Division of Infectious Disease Readiness and Innovation, Centers for Disease Control and Prevention, Atlanta, GA, USA

6 Office of Readiness and Response, Centers for Disease Control and Prevention, Atlanta, GA, USA

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worker confirmed the virus belonged to clade 2.3.4.4b [6]. HPAI A(H5N1) clade 2.3.4.4b viruses were soon detected in 26 dairy cattle facilities in 8 states and 6 poultry facilities in 3 states [7]. As of early December 2025, these viruses have infected at least 1083 dairy cattle in 18 states, and new cases continue to be reported on a weekly to monthly basis [8]. Outbreaks in dairy cattle have facilitated transmission across species, with the majority of human cases in the USA (57.7%) associated with exposures to dairy herds. Poultry farm or culling operations exposures (33.8%), other unknown animal exposures (4.2%), and unknown exposures (4.2%) constitute the sources of the remaining cases in humans. To date, there have been 71 confirmed cases of clade 2.3.4.4b A(H5) in the USA in humans with 2 resulting in death [9].

HPAIs are prone to mutation as they are influenza A viruses (IAVs) which contain a negative-sense RNA genome and lack a proofreading mechanism, allowing introduction of mutations during viral replication. Furthermore, their genome structure, containing eight segmented RNAs, provides the opportunity for IAVs to incur major genetic changes via reassortment, which occurs when a host cell becomes infected with two or more genetically distinct IAVs, allowing viruses to exchange gene segments, resulting in a novel virus that may have acquired new antigenic properties (antigenic shift). Major changes in human population immunity arising from antigenic shift can pave the way for emergence of pandemic or zoonotic influenza as has occurred in the past, for example, with H3N2 in 1968 and H1N1 in 2009 [1, 10].

The viruses responsible for much of the US dairy cattle and poultry outbreaks and a proportion of the US cases of human infection are novel reassortant viruses, designated as genotypes B3.13, D1.1, and D1.3. B3.13 viruses are A1 reassortant viruses and contain hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 and 2 (MP), and polymerase acidic (PA) gene segments of Eurasian wild bird lineages (ea1 or genotype A1). The remaining segments, nucleoprotein (NP), polymerase basic 2 (PB2), non-structural (NS), and polymerase basic 1 (PB1), are from American wild bird lineages (am8, am2.2, am1.1, and am4, respectively) [1, 11]. Viruses of genotype D1.1 are derived from genotype A3 (Eurasian lineage). In contrast with B3.13, D1.1 viruses acquired NA, PA, PB2, and NP from North American wild bird lineages, which is notable given that viruses circulating in the USA previously had NA segments of mostly Eurasian lineage [2, 12, 13]. D1.3 viruses, similarly to the D1.1 genotype, are reassortants of the A3 genotype, but their NA segments, along with HA, MP, PB1, and NS segments, are of Eurasian lineage [14]. There was also a recent human case of A(H5N5) that was genotype A6 [15]. The simultaneous circulation of multiple genetically distinct HPAIs across multiple species highlights reassortment as a key driver of IAV diversity with direct consequences for emergence and spread of viruses of pandemic concern.

Because of the pandemic potential posed by these novel HPAI A(H5N1) clade 2.3.4.4b viruses, in 2024 the US Centers for Disease Control and Prevention (CDC) issued a call for development of additional A(H5) subtyping diagnostics. At the time, A(H5) diagnostic tests in the USA were limited to CDC and state and local public health laboratories with access to the CDC influenza A(H5) subtyping assay. Therefore, a large outbreak or pandemic scenario could potentially overwhelm national testing capacity, leading to delays in detection, treatment, and case investigations to contain further spread of the virus [16]. Additional diagnostics that could be widely distributed and accessible across healthcare networks would mitigate these issues. However, a lack of relevant control materials for these emerging viruses was a limiting factor for test development. To address this gap, CDC and the National Institute of Standards and Technology (NIST) partnered to develop viral RNA reference materials for A(H5N1).

With guidance on sequences from CDC, NIST produced three synthetic RNAs containing the coding sequences of HA, NA, and MP proteins of influenza A(H5N1) virus. Each RNA material was prepared and bottled separately at NIST, and within 90 days of the start of the collaboration, a portion of the lot of each RNA material was made available to the public as a research grade test material (RGTM), RGTM 10263 [17]. RGTMs are materials provided as exploratory materials to the community without full quantitative characterization and extensive stability testing. This strategy allowed NIST to release RGTM 10263 rapidly, after an initial homogeneity test, to address the critical need for A(H5N1)-specific reference materials that did not require the same biosafety and biosecurity considerations as wild type viruses. While the RGTM was publicly available, characterization, including homogeneity and 26-week stability testing, quantitative concentration value assignment, and compatibility with CDC's influenza A(H5) subtyping diagnostic assay, continued on the same lot of materials which were released eventually as a fully characterized reference material (RM), RM 8038 (see graphical abstract) [18]. Here, we describe the rapid development of influenza A(H5N1) RNA reference materials to aid development and validation of A(H5) subtyping diagnostics.

Materials and methods

Design and synthesis of RNAs for RGTM/RM

Sequences from A1 genotype A/American Wigeon/South Carolina/22/2021 virus isolate were used to generate HA (GenBank: OR051630.1), NA (GenBank: OR051629.1),

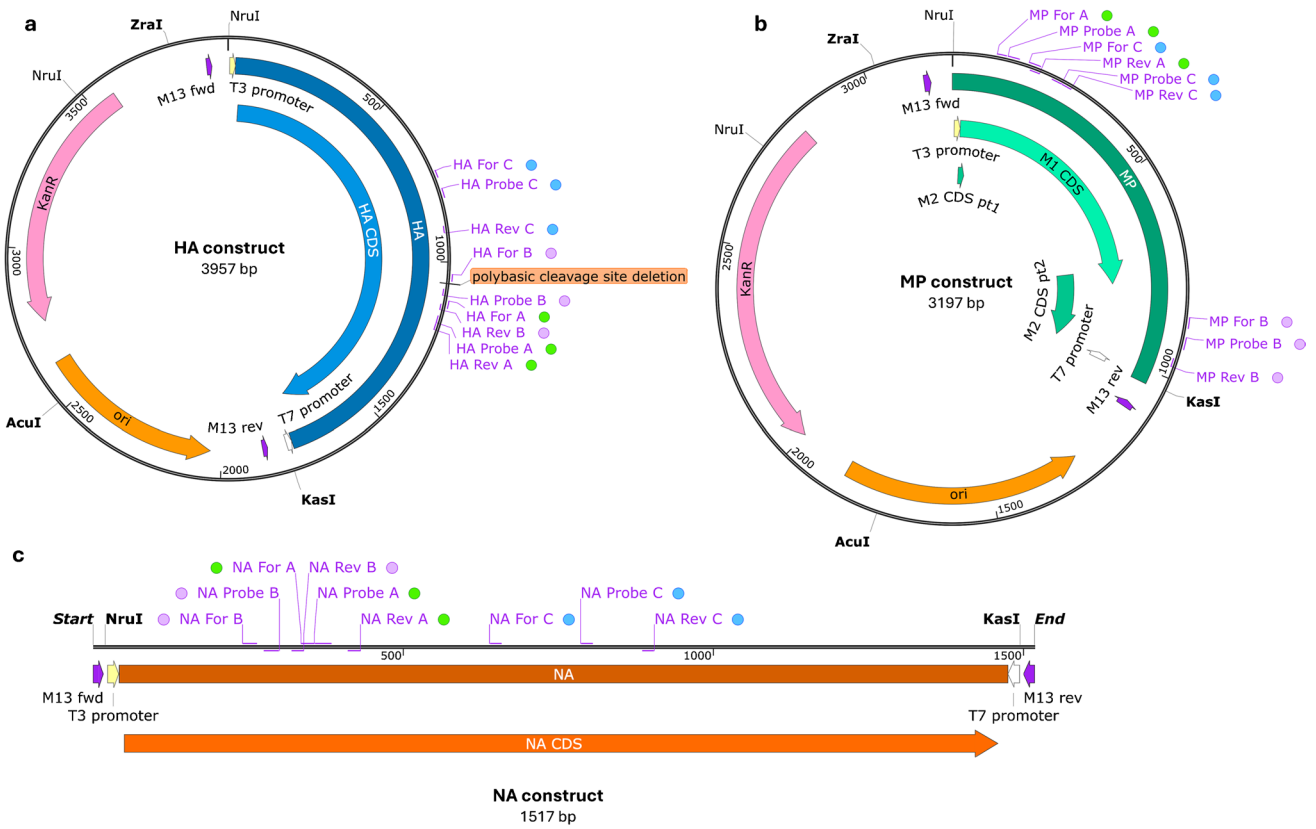

Fig. 1 Plasmid or linear DNA fragments containing the coding sequences of **a** HA, **b** MP, and **c** NA were synthesized. Plasmids were linearized by ZraI and AcuI restriction digestion. HA, MP, and NA fragments were PCR-amplified using the M13 priming sites (purple arrows). After amplification, M13 priming sites were removed by KasI and NruI restriction digest, and negative-sense RNA was generated by in vitro transcription using the T7 promoter sites (white arrows). RT-ddPCR assays for assigning concentration values (Assays A and B) and for homogeneity and stability assessment (Assays C) are displayed in purple text for each construct

and MP (GenBank: OQ958047.1) RNA. For HA, a nine-nucleotide deletion was made to remove the polybasic cleavage site.

To generate HA and MP RNAs, plasmids (pTwist Kan High Copy) containing the coding sequence of each target were purchased from Twist Biosciences (Fig. 1a, b). The fragments of interest, flanked by M13 priming sites, were excised by restriction enzyme digest with ZraI and AcuI (NEB, catalog # R0659S and R0641S). After confirming the digested DNA for correct size and linearization by DNA FlashGel (Lonza, catalog # 57023), the fragments of interest were amplified by polymerase chain reaction (PCR) using the M13 priming sites and Phusion High-Fidelity DNA Polymerase (NEB, catalog # M0530S). PCR products were confirmed by gel again and purified with Monarch PCR and DNA Cleanup Kit (NEB, catalog # T1030S). For generation of NA RNA, the NA coding sequence was synthesized by Twist Biosciences as a linear DNA fragment with flanking M13 priming sites (Fig. 1c).

To remove the M13 priming sites from all 3 DNA fragments, DNA was double digested with KasI and NruI-HF (NEB catalog # R0544S and R3192S) (Fig. 1a–c). The digested products were confirmed again by DNA FlashGel and purified by Monarch PCR and DNA Cleanup Kit.

Negative-sense RNAs were transcribed by MEGAscript T7 Transcription Kit (Invitrogen catalog # AM1334) utilizing the T7 promoter inserted in the DNA constructs immediately downstream of the A(H5N1) gene sequences. RNAs were purified with the RNeasy Mini Kit (QIAGEN catalog # 74104). RNAs were confirmed on an RNA FlashGel (Lonza catalog # 57027), and concentrations were determined using a Nanodrop UV-Vis spectrophotometer (Thermo Fisher Scientific, MA).

Plasmid constructs were visualized using SnapGene Version 8.0.3.

Sequencing confirmation of RNAs for RGTM/RM

Prior to bottling, HA, NA, and MP RNAs were amplified for sequencing using SuperScript III One-Step RT-PCR with Platinum Taq High Fidelity (Invitrogen) and multiple primer pairs to target the HA, NA, or MP segments. The

full HA was amplified using forward H5_HA_F23M(5′-GTC AAA ATG GAG AAM ATA GTR CTW CT-3′) and reverse H5_HA_R1771 (5′-AGG GTG TTT TTA ACT ACA ATC TGA GC-3′), the full NA was amplified using two different primer pairs (1) forward NIST_NA_F(5′-GGA GTT CAA AAT GAA TCC AAA TCA AA-3′) and reverse H5N1NA_R1435 (5′-ACA AAC TAC TTG TCA ATG GTG AAT GG-3′) and (2) forward NIST_NA_F(5′-GGA GTT CAA AAT GAA TCC AAA TCA AA-3′) and reverse H5N1NA_R1444 (5′-GTT TTT TGR ACA AAC TAC TTG TCA ATG GT-3′), and the full MP was amplified using forward InfA_M_F25m (5′-GAT GAG TCT TCT AAC MGA GGT CGA AAC-3′) and reverse un_M_R972 (5′-TCG TCA RCA TCC ACA GCA YTC TG-3′). The full HA and NA segments were amplified using the cycling conditions of 50 °C for 30 min; 94 °C for 2 min; 45 cycles [94 °C for 15 s, 55 °C for 30 s, 68 °C for 2 min]; 68 °C for 7 min; hold at 4 °C. For the MP segment, the extension time was reduced to 1 min for the cycling conditions of 50 °C for 30 min; 94 °C for 2 min; 45 cycles [94°C for 15 s, 55 °C for 30 s, 68 °C for 1 min]; 68 °C for 7 min; hold at 4°C. Illumina libraries were then generated from the amplicons using quarter-volume reactions of the Illumina DNA Prep Kit. Libraries were individually barcoded and pooled to 2 nmol/L final concentration based on average library size, and the concentration was quantified on a Qubit using the 1 × dsHigh Sensitivity Kit (Invitrogen). The final pool was loaded on an Illumina MiSeq using a 300-cycle v2 kit with the run set for 150 base paired-end sequencing. Sequencing data were demultiplexed using bcl2fastq v2.20.0 and consensus assemblies generated using the FLU-utr module in IRMA v1.2.0 [19]. Data are available as an NCBI BioProject PRJNA1456179 (https://www.ncbi.nlm.nih.gov/bioproject/1456179).

Bottling of RGTM/RM RNA

Each RNA component of the RGTM/RM (HA, NA, or MP) was bottled separately into screw cap microcentrifuge tubes (USA Scientific catalog # 1405-9710) on separate days on a Scinomix VXL instrument (Earth City, MO). Each RNA component was added to a solution of 5 ng/µL Jurkat RNA (BioChain catalog # R1255815-50) in THE RNA Storage Solution (Invitrogen catalog # AM7001) (RSS) to reach a concentration of approximately 10^5 copies/µL to 10^6 copies/µL. The targeted concentration range (in copies/µL) for each RNA was initially estimated by the length of the RNA, the corresponding mass concentration (in ng/µL) determined by Nanodrop, and the average molecular weight per nucleotide for single-stranded RNA. For each RNA component, on each day of bottling, approximately 250 tubes labeled as RGTM 10263 were filled, followed by 750 tubes labeled as RM 8038. When all tubes were filled, they were transferred to 11 freezer storage boxes (3 for RGTM 10263 tubes and 8 for RM 8038 tubes) in the order in which they were bottled and placed at −80 °C for storage. A single unit of RGTM 10263 or RM 8038 consists of one tube each of HA, NA, and MP RNA.

Sequence comparison of reference materials and clinical isolates

A search for clade 2.3.4.4b influenza A(H5N1) viral sequences from human isolates collected in the USA during 2024 and 2025 was performed in GISAID, returning a total of 60 virus isolates [20]. Twenty-nine isolates were excluded from analysis because they did not have an assigned Geno-FLU genotype in GISAID [13]. The metadata associated with the 31 sequences used in this study up to February 20, 2026, are available on GISAID and accessible at https://doi.org/10.55876/gis8.260408gv. One isolate was assigned genotype D1.3. HA, NA, and MP segment nucleotide sequence alignments were generated in GISAID from 22 B3.13 and 8 D1.1 sequences. The consensus sequences generated from these alignments were aligned with the sequences used to generate the HA, NA, and MP RNA RGTM/RM at NIST using Jalview (Version 2.11.5.1). The single D1.3 sequence was added to these alignments. Microsoft Excel was used to generate the figure of the alignments and to count mismatched nucleotides using the RGTM/RM sequence as the reference sequence for each alignment. Unresolved base calls in the consensus sequences resulting from an even number of sequences with conflicting nucleotides (HA B3.13 = 1; NA D1.1 = 3; MP D1.1 = 1) and the nine-nucleotide gap created by the polybasic cleavage site deletion in the HA component of the RGTM/RM were ignored in the mismatch counts.

Reverse transcription droplet digital PCR

The concentrations of the RGTM/RM components were measured by RT-ddPCR using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, catalog #1864022). HA, NA, and MP RNAs were diluted (1:200) separately into THE RNA Storage solution (Invitrogen, catalog # AM7001) (RSS) to be within the dynamic range of the ddPCR instrument. RT-ddPCR mixtures were prepared according to the manufacturer's protocol with a final volume of 22 µL. A volume of 2 µL diluted RNA was added to each reaction which were prepared in technical triplicates. RT-ddPCR cycling conditions and primer and probe concentrations are listed in appropriate sections below.

Droplets were generated on an Automated Droplet Generator (Bio-Rad, catalog # 1864101) or on a QX200 Droplet Generator (Bio-Rad, catalog # 1864002), and RT-ddPCR was performed on a ProFlex PCR System (Applied Biosystems, catalog # 4484075). Droplets were read on a QX200 Droplet Reader (Bio-Rad, catalog # 1864003) and analyzed

Table 1 Primers and probes sequences for RT-ddPCR assays (5′–3′)

NA	
NA Forward Primer A	GGGTGGGCAATATACAGTAAGG
NA Reverse Primer A	CAAGTGGGAGCATGAGATGAA
NA Probe A	FAM/ACAACGGTATAAGAATTGGGTCCAAGGG/MGB-NFQ
NA Forward Primer B	GACGTATGTCAACATCAGCAATAC
NA Reverse Primer B	CACCCACTAATAGGGCAAAGA
NA Probe B	FAM/TTACCGAAGTAACAGCCTGCTCAGC/MGB-NFQ
NA Forward Primer C	GTCCAGACAATGGAGCTGTG
NA Reverse Primer C	CGCATCAGGATAACAGGAGC
NA Probe C	FAM/CAAGCAATGGGCAGGCCTCA/BHQ-1
HA	
HA Forward Primer A	AGGGAATGGTTGATGGTTGG
HA Reverse Primer A	TGCCTTTTGGGTGGATTCTT
HA Probe A	FAM/TGTCCGCAGCGTACCCACTCCCC/MGB-NFQ
HA Forward Primer B	GGCTCAGAAATAGTCCTC
HA Reverse Primer B	GTACCCATACCAACCATC
HA Probe B	FAM/TATAAACCCTGCTATCGCCC/MGB-NFQ
HA Forward Primer C	ATCCCAAGTAAACGGGCAAC
HA Reverse Primer C	TGTTGCAGTGGCCATATTCC
HA Probe C	FAM/ACCAGATGATGCAATCCATTTCGAGA/BHQ-1
MP	
MP Forward Primer A	GCGCAGAGACTTGAAGATG
MP Reverse Primer A	CTTAGTCAGAGGTGACAGGATTG
MP Probe A	FAM/TGCAGGGAAGAACACCGATCTTGA/MGB-NFQ
MP Forward Primer B	GCGTTTATCGTCGCCTTAAATAC
MP Reverse Primer B	GTCAACATCCACAGCACTCT
MP Probe B	FAM/ACGGAAGGAGTACCTGAGTCCATGA/MGB-NFQ
MP Forward Primer C	CAAGACCAATCYTGTCACCTCTGAC
MP Reverse Primer C	GCATTYTGGACAAAVCGTCTACG
MP Probe C	FAM/TGCAGTCCTCGCTCACTGGGCACG/BHQ-1

by QX Manager Software 2.2 Standard Edition (Bio-Rad, QX-MANAGER-V.2.2-STD). Thresholds separating positive and negative droplets were manually set by inspection of 1D plots. Data were exported to Microsoft Excel for further analysis.

Primers and probes

The RT-ddPCR primer and probe sequences for each assay used for homogeneity, stability, and concentration value assignment are shown in Table 1. Two assays each for HA, NA, and MP RM 8038 components (Assays A and B) were selected for concentration value assignment. Primers for Assays A and B for HA, NA, and MP RNAs were purchased from Eurofins Genomics, and corresponding probes were purchased from Thermo Fisher Scientific (TaqMan MGB Probe, catalog # 4316034). A single assay each for HA, NA, and MP (Assays C) was selected for RGTM/RM homogeneity and stability. Because of the immediate need for and rapid turnaround of RGTM 10263, only minimal RT-ddPCR assay and PCR optimization was performed before characterization and release of RGTM 10263. After RGTM 10263 was available publicly, it allowed additional time for methodical comparative evaluation and optimization of RT-ddPCR assays and PCR conditions to provide the most accurate quantification and concentration value assignment (HA, NA, and MP Assays A and B). However, because the initial homogeneity and stability assessments were performed with Assays C, those assays continued to be used for the additional RM 8038 homogeneity and stability analysis for consistency and comparability to the original data. Assays C for HA, NA, and MP RNAs were purchased from Biosearch Technologies. All probes for Assays A, B, and C contained a 5′ FAM reporter. Probes for Assays A and B contained a 3′ nonfluorescent quencher (NFQ), and probes for Assays C included a 3′ Black Hole Quencher.

Protocols and assay sequences for the CDC Human Influenza Virus Real-time RT-PCR Diagnostic Panel: Influenza A/B Typing Kit (VER 2), Influenza A Subtyping Kit (VER 4), Influenza B Lineage Genotyping Kit (VER 1.1 and VER

2), Influenza A/H5 Subtyping Kit (VER 4) (2025) have been previously published [21, 22].

Concentration value assignment for RM 8038

The copy number concentration in copies/µL of each RNA component of RM 8038 was determined by RT-ddPCR using Assays A and B. Two assays were used to account for assay-specific RT-ddPCR performance differences and ensure concordance in the observed concentration values. A single tube from each of the eight RM freezer storage boxes for each RNA was tested. Tubes from even-numbered storage boxes were tested on day one, and tubes from odd-numbered storage boxes were tested on day two. Diluted samples were heated at 65 °C for 5 min and then placed on ice before adding to chilled PCR mixtures with a final concentration of primers and probe at 900 nmol/L and 250 nmol/L, respectively. RT-ddPCR cycling conditions were as follows: 1 h at 50 °C, 10 min at 95 °C, 30 s at 94 °C followed by 1 min at 58 °C for 60 cycles, and a final hold for 10 min at 98 °C.

Data visualizations were created in Microsoft Excel.

Homogeneity analysis of RGTM 10263 and RM 8038

Two homogeneity assessments were conducted. For both assessments, the HA, NA, and MP Assays C were used (Table 1). The first homogeneity assessment was conducted in October 2024, prior to the release of RGTM 10263. An additional homogeneity analysis was conducted for RM 8038 in March 2025. These data correspond to week 20 of the stability assessment (described below). For the initial homogeneity test, concentration measurements for each RNA component were carried out on 1 tube from each of the 8 RM and 3 RGTM freezer storage boxes (11 tubes total). From these data, the mean across all RGTM and RM samples for each RNA component was taken as the concentration estimate for RGTM 10263 and provided to RGTM customers. Subsequent homogeneity and stability analyses (as described in the following section), were conducted only on eight tubes from each of the RM freezer storage boxes for each RNA component. For both homogeneity analyses, the RT-ddPCR cycling conditions were as follows: 1 h at 50 °C, 10 min at 95 °C, 30 s at 94 °C followed by 1 min at 60 °C for 60 cycles, and a final hold for 10 min at 98 °C.

Graphs displaying data from concentration measurements were created in Microsoft Excel.

Stability analysis of RM 8038

The HA, NA, and MP Assays C were used for stability analysis of RM 8038 (Table 1). For each RNA component, one tube from each of the eight RM freezer storage boxes was tested. Measurements from the 8 RM freezer boxes taken during the initial homogeneity assessment (made within 2 weeks after bottling was completed in October 2024) were used as the starting time point for the stability analysis. Fresh tubes from the same 8 freezer storage boxes were thawed approximately 20 weeks later on 3/14/2025. The same tubes were tested again at 22 weeks (3/28/2025) and 26 weeks (4/24/2025) after the initial time point. After testing, the original, undiluted tubes were returned to −80 °C storage until the next time point, undergoing a total of at least three freeze/thaw cycles (one per time point 3/14/2025 to 4/24/2025). Repeat testing was performed on 3 NA tubes during week 22 testing and thus underwent a total of 5 freeze/thaw cycles. The 26-week stability study was designed to assess whether the material exhibited measurable degradation over the study period under the intended storage conditions and was not intended to serve as a predictive model for the full assigned shelf life provided to customers [18].

At each time point, diluted RNA materials were added to PCR mixtures at room temperature with a final concentration of primers and probe at 375 nmol/L and 250 nmol/L, respectively. RT-ddPCR cycling conditions were the same as described for the homogeneity analyses.

Real-time reverse transcription PCR

Before bottling, the HA, NA, and MP RNA was evaluated by the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel Influenza A/H5 Subtyping Kit assay and a CDC A(H5N1) laboratory-developed test (LDT) assay targeting the NA gene segment. The A/H5 Subtyping Kit consists of four oligonucleotide primers and probes that identify a pan influenza A target (InfA) on the MP gene segment, two avian influenza differentiation targets H5a and H5b on the HA gene segment, and a human RNase P. The A(H5N1) LDT assay consists of oligonucleotide primers and a probe that identify the influenza A(H5N1) NA gene. The single-plex real-time reverse transcription polymerase chain reactions (rRT-PCR) were performed using Invitrogen SuperScript III Platinum One-Step quantitative RT-PCR (Life Technologies, 11732088) on the Applied Biosystems (AB) 7500 Fast Dx Real-Time PCR instrument. All rRT-PCR reactions performed consisted of 5 µL of RNA template and a total reaction volume of 25 µL with primer and probe reaction concentrations of 0.8 µmol/L and 0.2 µmol/L, respectively. HA, NA, and MP RNA templates were prepared as ten, five-fold serial dilutions against the InfA, H5a, H5b, and A(H5N1) assays. HA, NA, and MP RNA templates were subsequently tested undiluted (neat) against the human RNase P assay. Thermocycling rRT-PCR conditions were as follows: 50 °C for 30 min, Taq activation at 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and 55 °C for 30 s.

Statistical analysis

Hierarchical Bayesian mixed-effects models were used to assess homogeneity as well as assigned concentration values and associated uncertainties for RM 8038 (see Electronic Supplementary Material 1 (ESM_1) Figs. S1 and S2). The models accounted for variability due to box differences, amplicon differences, droplet volume, and repeatability. Priors were chosen to be weakly informative and anchored in the observed data to ensure that model parameters are expressed on a meaningful scale without being overly restrictive [23–26].

The stability of RM 8038 was specifically characterized by using a weighted linear regression model of the RT-ddPCR concentration measurements, where the weights are the inverse of the variance at each date (Fig. S3). In this way, we incorporate replicate uncertainty and can naturally handle differences in the number of replicates or variability across the time points.

All figures generated from the statistical analysis were created in the R programming language using ggplot2 [27, 28]. Modeling was performed in the R programming language using Stan [29].

Survey

Feedback requests in the form of a Google Forms survey were sent by email to RGTM 10263 customers who placed orders between 12/12/2024 and 8/11/2025. During this period, there were 47 different customers, and a total of 20 customers completed surveys. The survey consisted of seven questions with the goal of understanding how RGTM 10263 was being used and seeking feedback to improve the quality of future NIST RGTM/RM offerings. The survey was conducted in accordance with the Paperwork Reduction Act of 1995 (codified at 44 U.S.C. Chapter 35) and implemented according to the Code of Federal Regulations 5 C.F.R Part 1320 (OMB Control Number 0690-0030).

Data visualizations were created in Microsoft Excel.

Results

Production and confirmation of RGTM/RM RNA

We sought to produce RNA materials that could be established as reference materials and demonstrate their fitness for purpose for molecular assay development, validation, and harmonization. First, we developed three RNA materials that contained A(H5N1) gene sequences similar to those of viruses circulating during the outbreaks at the time in 2024. All three RNAs were successfully generated by in vitro transcription and size-verified by electrophoresis. The 3 in vitro transcription reactions yielded between 60 µg and 110 µg RNA. Before bottling, aliquots of HA, NA, and MP RNA were sent to CDC for sequencing confirmation (BioProject PRJNA1456179).

The viral sequences used to generate the RGTM/RM were compared to clade 2.3.4.4b viruses that had infected humans. HA, NA, and MP sequences from human isolates of clade 2.3.4.4b influenza A(H5N1) collected in the USA during 2024 and 2025 were compiled in GISAID. Nucleotide B3.13 and D1.1 consensus sequences generated from clinical isolates were aligned with the viral gene segment sequences used to generate the RGTM/RM along with a single D1.3 isolate sequence. Analysis of these alignments revealed 31 mismatches for B3.13, 30 mismatches for D1.1, and 34 mismatches for D1.3 when compared to the 1695-nucleotide coding sequence used for the HA RGTM/RM (Fig. S4). For MP (982-nucleotide coding sequence), there were 10 mismatches for B3.13 and 9 mismatches each for D1.1 and D1.3 sequences. For NA (1410-nucleotide coding sequence), there were 16 mismatches for B3.13, 198 mismatches for D1.1, and 39 mismatches for D1.3 sequences when compared to the sequence of the RGTM/RM (Fig. S4). Overall, the RGTM/RM sequences, derived from an A1 genotype virus, were highly similar to circulating viruses. In particular, B3.13 and D1.3 genotypes exhibited minimal mismatches with the RGTM/RM. B3.13 viruses are A1 reassortants with their HA, NA, and MP segments derived from A1 genotypes, which are fully Eurasian lineage viruses. D1.3 viruses are A3 reassortants with their HA, NA, and MP segments from Eurasian lineage viruses. While D1.1 genotypes are also A3 reassortants, their NA segments exhibited a higher number of mismatches with the A1 RGTM/RM sequence. D1.1 genotypes acquired a North American lineage NA segment while their HA and MP segments are from Eurasian lineages.

RM 8038 RNA concentration value assignment

NIST RMs are provided with property values that are suitable for the materials' intended use [30]. In order to be useful for method development, validation, and harmonization, we determined the concentration values and uncertainty intervals of each RNA component of RM 8038. The concentration of each RM 8038 RNA component was determined by RT-ddPCR over two days, using two different assays targeting different amplicons for each RNA. Eight tubes per RNA component (HA, NA, MP) were tested in total, one from each RM freezer storage box.

For the HA component, the mean concentration for the 8 samples tested was 1.72×10^6 copies/µL for Assay A and 1.71×10^6 copies/µL for Assay B (Fig. 2). For the NA component, the mean concentrations for NA Assays A and B were 2.38×10^6 copies/µL and 2.87×10^6 copies/µL, respectively. For

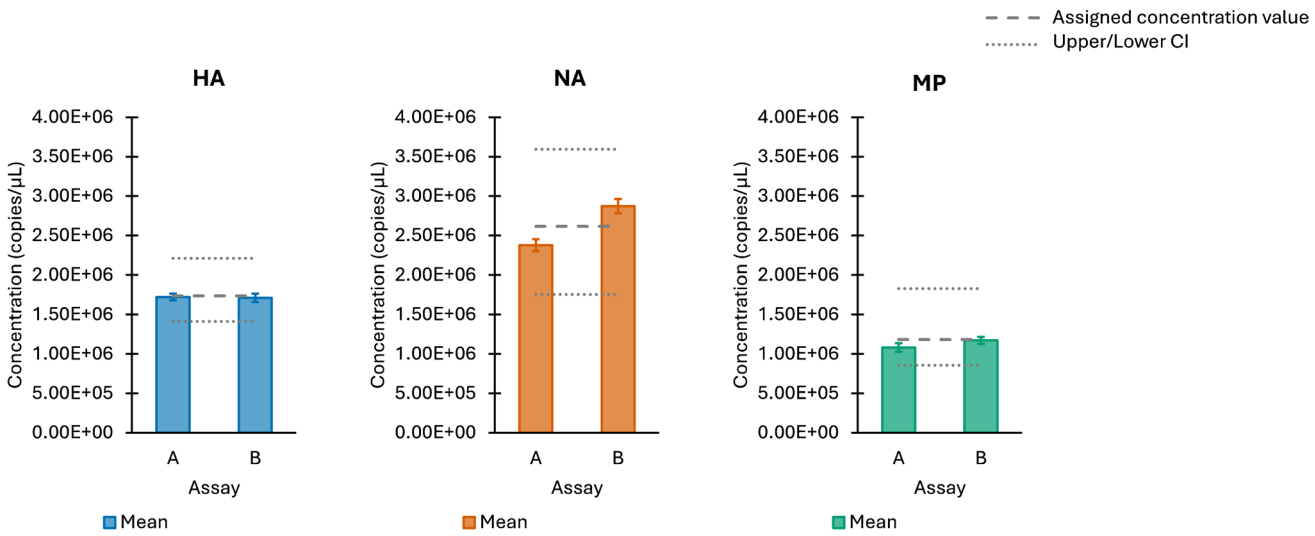

Fig. 2 Concentration values for each RM 8038 RNA component were determined by RT-ddPCR measurements. RT-ddPCR was performed over two days for two amplicons (Assays A and B) per RM 8038 component. Shaded bars indicate the mean of all replicate measurements on 8 RNA samples per RNA component with error bars indicating ± standard deviation. The dashed lines indicate the assigned concentration values for each RNA component based on concentration measurements taken together from both assays, and dotted lines indicate the 95% upper and lower credible intervals (CI) for the respective assigned concentration values

Table 2 Assigned concentration values for RM 8038 with uncertainty

RNA	Concentration (copies/µL)	Upper credible interval (copies/µL)	Lower credible interval (copies/µL)	Standard uncertainty (copies/µL)
HA	1.738×10^6	2.210×10^6	1.414×10^6	1.866×10^5
NA	2.615×10^6	3.595×10^6	1.755×10^6	4.387×10^5
MP	1.182×10^6	1.830×10^6	8.562×10^5	2.452×10^5

the MP component, the mean concentration was 1.08×10^6 copies/µL for MP Assay A and 1.17×10^6 copies/µL for MP Assay B.

To assign a single concentration value to each RM 8038 RNA component, a hierarchical statistical model was used, accounting for uncertainty due to box differences, amplicon differences, droplet volume, and repeatability (Fig. S1). The final assigned concentration values for HA, NA, and MP RM 8038 RNA components are 1.738×10^6 copies/µL, 2.615×10^6 copies/µL, and 1.182×10^6 copies/µL, respectively (Table 2). The 95% credible intervals and standard uncertainty determined for each RM 8038 component are displayed in Table 2. While the concentration measurements for each of the two amplicons for HA and for MP are quite similar, the two amplicons for NA exhibit a larger difference. As such, the standard uncertainty for NA is substantially larger than that for HA or MP (Table 2).

RM 8038 homogeneity

Next, we demonstrated that the RM 8038 RNA components are sufficiently homogeneous for their intended use by assessing concentration measurements from RM 8038 components sampled across the lot materials. To test for any sample concentration variation that may have occurred during bottling of the RNA such as from evaporation or insufficient mixing, a single tube per RNA component was tested from each RM 8038 freezer storage box to account for loading the tubes into storage boxes in the order in which they were bottled.

For each of the 3 RNAs, variability in concentration measurements across the 8 RM storage boxes was low with % CV values calculated from the mean and standard deviation of replicates ranging from 3.29% to 4.67% (Fig. 3).

To assess whether the RM 8038 RNA components were homogeneous, a hierarchical Bayesian mixed-effects model was used to estimate the magnitude and uncertainty associated with between-box variability using concentration measurements from one randomly selected tube from each of the eight storage boxes (Fig. S2). Since tubes were selected randomly from each storage box, the sampled tubes were treated as representative of the overall

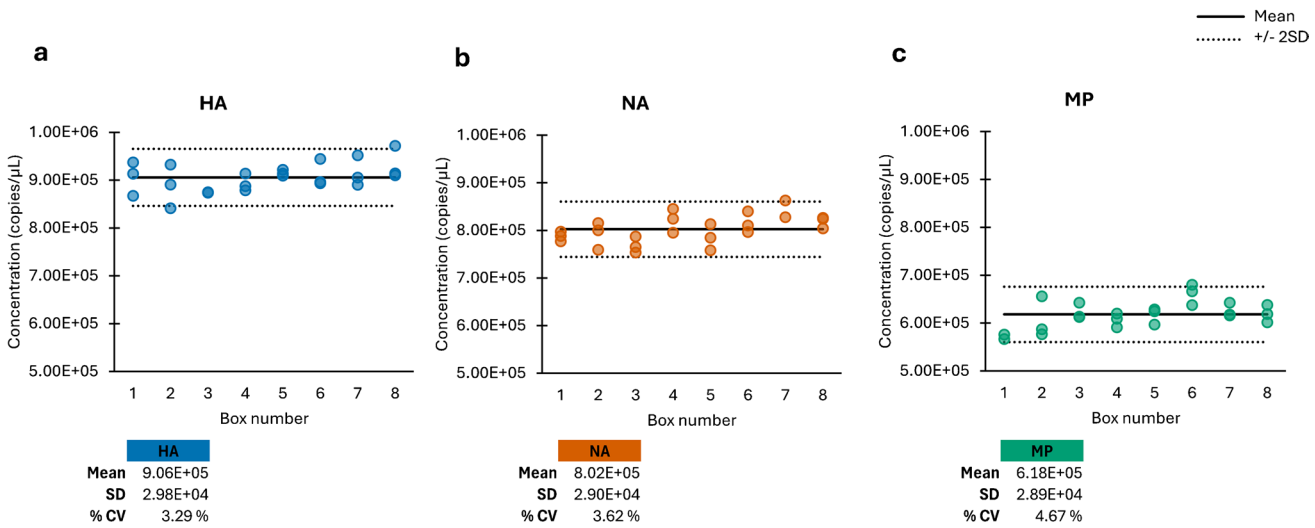

Fig. 3 Concentration measurements by RT-ddPCR were taken for **a** HA, **b** NA, and **c** MP RNA from each of 8 RM freezer storage boxes per RM 8038 RNA component to assess homogeneity. Data points shown for each box are technical triplicate RT-ddPCR measurements taken from a single sample. The solid black line indicates the mean of replicates across all boxes, and the dotted black line indicates ± two standard deviations. The values for the mean, standard deviation (SD), and coefficient of variation expressed as a percentage (% CV) are also displayed for each RM 8038 component. Statistical analysis (Figs. S2 and S5) determined that the RM 8038 RNA components are sufficiently homogeneous

production lot. Consequently, the analysis is primarily sensitive to large-scale bottling or storage effects but does not directly assess tube-to-tube variability within individual boxes.

For all 3 RNA RM components, each box deviation from the overall mean had 95% credible intervals which include zero, indicating that the boxes do not differ significantly from the overall mean for each RNA component (Fig. S5). Thus, the RM 8038 RNA components are sufficiently homogeneous and fit for purpose.

RM 8038 stability

We also demonstrated that the RM 8038 RNA components are sufficiently stable for their intended use by assessing concentration measurements from RM 8038 components sampled across the lot materials at 4 time points over the course of 26 weeks of storage at −80 °C. A single tube from each of the 8 RM freezer storage boxes per RNA component was initially tested in late October 2024, shortly after bottling. Testing was repeated on a new set of 8 tubes from each RM storage box (per RNA) approximately 20, 22, and 26 weeks later in March and April 2025, incorporating at least 3 freeze-thaw cycles into the stability study.

For all 3 RM 8038 RNA components, variability in concentration measurements across all time points was low with % CV values calculated from the mean and standard deviation of all replicate concentration measurements across time points ranging from 4.74% to 5.26% (Fig. 4). The measured concentration of each RNA component at the 26-week time point ranged from 92 to 95% of the concentration at the initial time point (Fig. 4).

Stability data were assessed using a weighted linear regression model incorporating data from all time points (Fig. S3). All regression slopes were non-significant with 95% confidence, indicating that the variations across time for each RM 8038 RNA component do not follow a specific trend and that all components are sufficiently stable (HA $p = 0.25$; NA $p = 0.12$; MP $p = 0.31$) (Fig. 4).

CDC subtyping assay compatibility

Before the materials were bottled, we tested and determined that the HA, NA, and MP RNAs were compatible with and accurately detected by the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, Influenza A/H5 Subtyping Kit. The subtyping kit includes two assays that detect A(H5) influenza viruses (H5a and H5b) and one assay that detects pan-influenza A viruses (InfA). An A(H5N1) LDT assay to detect N1 was also included.

The HA, NA, and MP RNAs were detected by the CDC A/H5 Subtyping Kit with high accuracy (Table 3). For each dilution series, the RNA target was amplified exclusively by its respective assay(s). No cross-reactivity with non-targeting assays was observed across the tested dilution range, as indicated by "Undetermined" results for those assays. The highly diluted samples (dilutions beyond a dilution factor of 5^{-7} or 5^{-8}) also produced an "Undetermined" result with their respective assays, likely because of low target

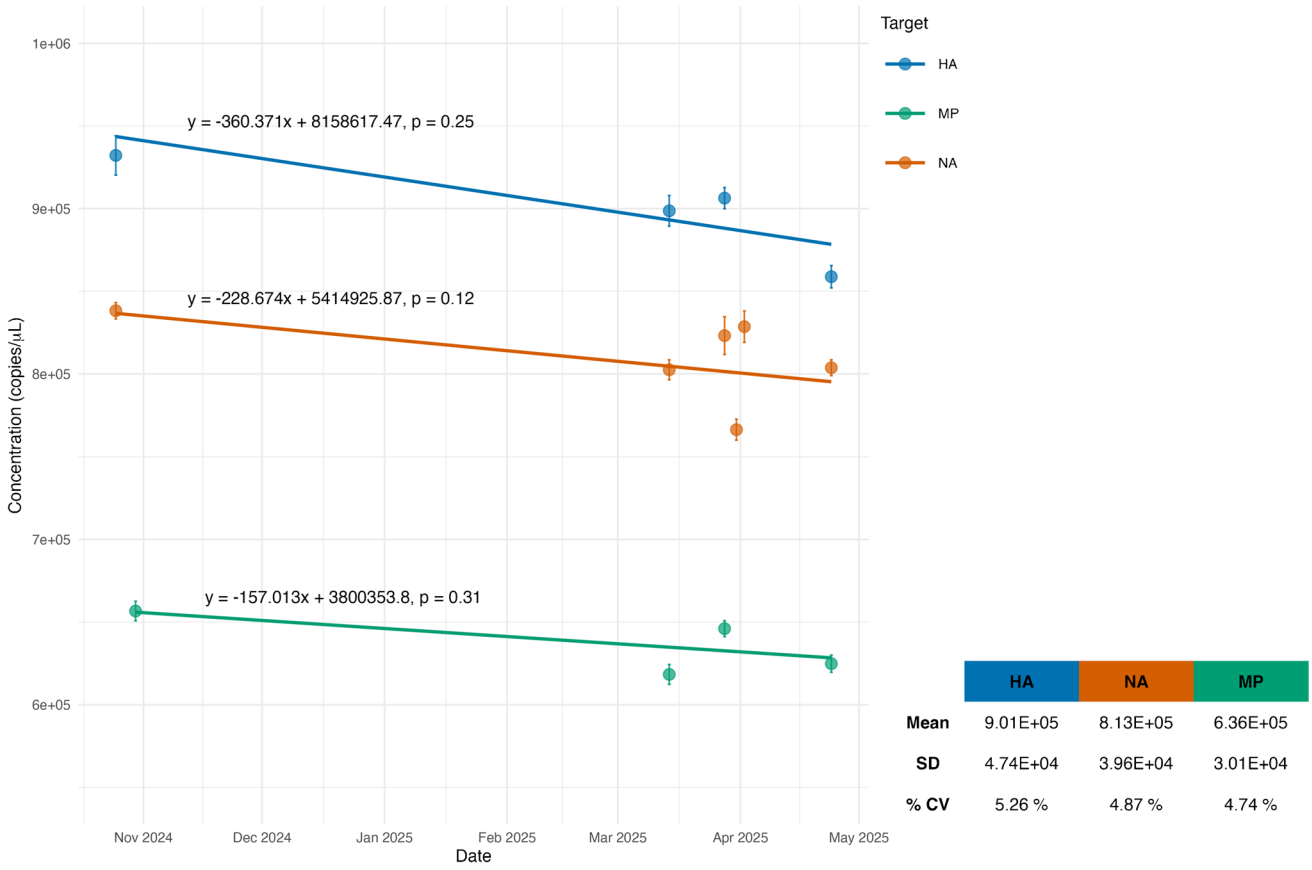

Fig. 4 The RM 8038 RNA components are sufficiently stable. Mean concentration measurements of 8 samples per RM 8038 RNA component are indicated by circular data points with the vertical lines at each point indicating the standard error. Concentration measurements were taken after materials were initially bottled (October 2024) and at approximately 20 (3/14/25), 22 (3/28/25), and 26 (4/24/25) weeks after bottling. Weighted linear regressions with equations and *p*-values are displayed for each RM 8038 RNA component. All slopes are not significant at a 95% confidence level, indicating sufficient stability

availability as late-cycle amplification was observed in the preceding dilution samples.

All three RNAs were tested with the human RNase P assay. Approximately equal C_T values for RNase P were observed among all three RNAs (Table S1).

RGTM 10263 survey

Finally, we also investigated the usefulness of RGTM 10263 to the community and found that most customers used it for new assay development and validation of user-defined assays. As part of NIST's RGTM program, RGTMs are provided at low to no cost to customers before extensive stability and quantitative characterization is complete. Because of the urgent need for influenza A(H5N1) control materials and the protracted process of full characterization of a reference material, NIST released a portion of the lot of influenza A(H5N1) materials to the public as an RGTM (RGTM 10263) within 90 days of initiating work on the materials and after an initial homogeneity test was complete (Fig. S6a–c). This allowed expedited access to the materials, which were provided with an initial concentration estimate, derived from the initial homogeneity measurements (Fig. S6d). The initial homogeneity measurements could then serve as the first time point in the 6-month stability study conducted for RM 8038 while we also developed and optimized new RT-ddPCR assays with improved sensitivity for the concentration value assignment (Figs. 2 and 4). Characterization and public release of RM 8038 took 14 months; however, RGTM 10263 was available to the scientific community during this time.

As part of the RGTM 10263 release, NIST requested feedback on the RGTM from customers via a survey to better understand the performance of the materials for customers' individual needs and improve the quality of future offerings. Of the 20 survey respondents, the majority were affiliated with clinical testing laboratories (6 of 20, or 30%), followed by academic and other commercial laboratories (4 of 20 respondents each, or 20%) (Fig. 5a). Other respondents were affiliated with US state governments, non-profits, and a

Table 3 rRT-PCR results (C_T values) for the CDC A/H5 subtyping assays (InfA, H5a, H5b) for the MP and HA segments, and a CDC-designed assay, A(H5N1), for the NA gene segment

Sample	InfA	H5a	H5b	A(H5N1)
HA Dilution 1	Undetermined	20.39	19.25	Undetermined
HA Dilution 2	Undetermined	22.68	23.2	Undetermined
HA Dilution 3	Undetermined	25.1	26.11	Undetermined
HA Dilution 4	Undetermined	27.91	30.6	Undetermined
HA Dilution 5	Undetermined	30.47	34.48	Undetermined
HA Dilution 6	Undetermined	33.31	36.82	Undetermined
HA Dilution 7	Undetermined	35.41	39.24	Undetermined
HA Dilution 8	Undetermined	Undetermined	40.18	Undetermined
HA Dilution 9	Undetermined	Undetermined	Undetermined	Undetermined
HA Dilution 10	Undetermined	Undetermined	Undetermined	Undetermined
NA Dilution 1	Undetermined	Undetermined	Undetermined	18.51
NA Dilution 2	Undetermined	Undetermined	Undetermined	21.06
NA Dilution 3	Undetermined	Undetermined	Undetermined	23.93
NA Dilution 4	Undetermined	Undetermined	Undetermined	26.72
NA Dilution 5	Undetermined	Undetermined	Undetermined	29.48
NA Dilution 6	Undetermined	Undetermined	Undetermined	32.71
NA Dilution 7	Undetermined	Undetermined	Undetermined	34.76
NA Dilution 8	Undetermined	Undetermined	Undetermined	38.62
NA Dilution 9	Undetermined	Undetermined	Undetermined	Undetermined
NA Dilution 10	Undetermined	Undetermined	Undetermined	Undetermined
MP Dilution 1	16.74	Undetermined	Undetermined	Undetermined
MP Dilution 2	19.28	Undetermined	Undetermined	Undetermined
MP Dilution 3	22.84	Undetermined	Undetermined	Undetermined
MP Dilution 4	26.1	Undetermined	Undetermined	Undetermined
MP Dilution 5	28.72	Undetermined	Undetermined	Undetermined
MP Dilution 6	33.73	Undetermined	Undetermined	Undetermined
MP Dilution 7	36.04	Undetermined	Undetermined	Undetermined
MP Dilution 8	Undetermined	Undetermined	Undetermined	Undetermined
MP Dilution 9	Undetermined	Undetermined	Undetermined	Undetermined
MP Dilution 10	Undetermined	Undetermined	Undetermined	Undetermined

diagnostic company. Survey responses showed that RGTM 10263 was predominately used to support development and validation of customers' own independent assays by digital PCR and quantitative PCR techniques, demonstrating the utility of the materials for the scientific community (Fig. 5b, c).

Discussion

Here we described the rapid production and characterization of influenza A(H5N1) RNA reference materials with the goal of addressing diagnostic testing gaps for pandemic preparedness. With the surge in influenza A(H5N1) human infections facilitated by dairy cattle and poultry outbreaks across the USA, there was a concern that testing capacities of laboratories may be overwhelmed in the event that the outbreaks progress to a pandemic. Development of additional diagnostic assays to alleviate this potential burden was hindered because of a lack of influenza A(H5N1)-specific reference materials that could be handled outside of Biosafety Level 3 enhanced (BSL-3E) containment. In collaboration, NIST and CDC addressed this deficiency by producing stable RNA research grade test materials containing the sequences of HA, NA, and MP influenza A(H5N1) gene segments within 90 days of establishing our collaboration. During this 90-day window, we also began the extended characterization required to transition the same lot of materials to NIST reference materials. The research grade test materials have already been used for their intended purpose in a variety of laboratory settings, including clinical and academic laboratories, facilitating customers' new assay development and validation efforts.

In addition to their utility as tools for assay development and validation, these RNA materials offer several additional advantages. First, because they are non-infectious, they can be handled safely at the most basic level of containment, in a Biosafety Level 1 facility. In contrast, it is required that

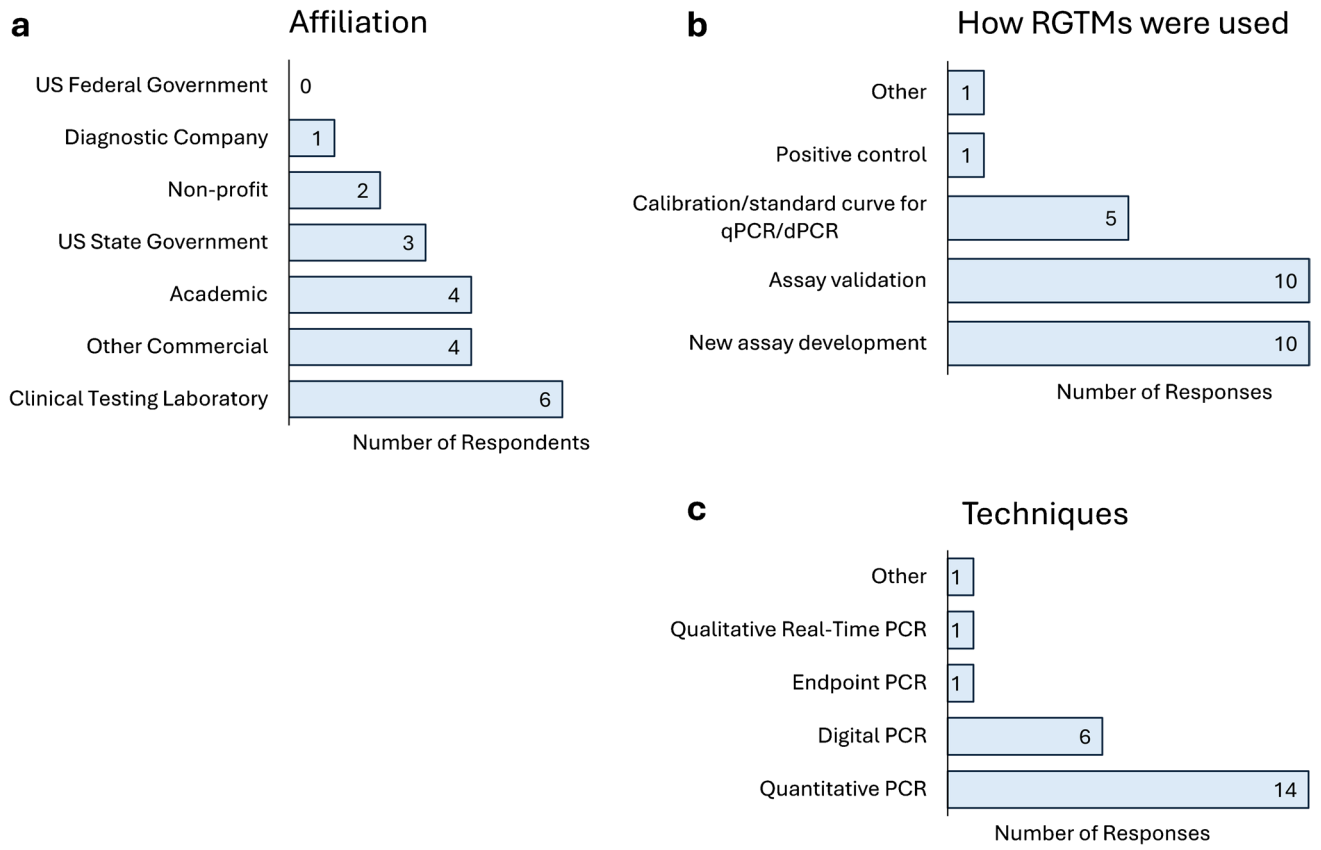

Fig. 5 Research grade test materials were useful for customers. Google survey results showing **a** respondents' affiliations, **b** how RGTM 10263 was used, and **c** the measurement methods used with RGTM 10263

infectious HPAI influenza A(H5N1) viruses be handled at BSL-3E containment, which may be a limiting factor for some researchers [31]. Furthermore, until the June 6, 2024 temporary exemption of influenza A(H5) HPAI viruses as a Select Agent, possession, transfer, or use of infectious HPAI A(H5) viruses required registration of facilities and staff with the Federal Select Agent Program [32]. In the future, this may limit or delay access to biologically relevant materials to laboratories or individuals who are not registered to work with HPAI A(H5) viruses. Because of the potential for severe disease in humans and poultry caused by influenza A(H5N1) viruses and the barriers to working safely with them, inactivated viruses or nucleic acid reference materials are preferred.

Second, the RNA materials (RM 8038) we have developed are quantitative, with assigned concentration values in copies per microliter. Providing rigorously tested quantitative concentration values ensures that the materials can be easily implemented for a variety of molecular assays and applications, including validating and establishing performance characteristics, such as limit of detection and limit of quantitation. Furthermore, they can be used directly as standard curves for quantitative rRT-PCR and allow for harmonization of measurements across laboratories. Additionally, we have demonstrated that these RNA RMs are stable up to 26 weeks when stored frozen at −80 °C and can be frozen and thawed at least 3 times without affecting the concentration of the material. The stability evidence, particularly freeze-thaw stability, confirms their suitability as a stable control for applications such as multi-day validation protocols where repeated handling is required. Ongoing studies will continue to monitor longer term stability of the RNAs at different storage temperatures and after many freeze-thaw cycles to demonstrate the stability and consistent performance of synthetic RNA across repeated use cycles to extend usability for customers.

Moreover, we demonstrated that the RNA materials were compatible with existing CDC diagnostic assays and demonstrated the impact of our interagency collaboration. Combining our distinct and complementary areas of expertise—CDC's leadership in genomic evaluation of viral pathogens of public health concern and assay development and validation and NIST's strengths in measurement science and reference materials production—ensured that the resulting RMs reflected the predominant, currently circulating viral genotypes and were compatible with the only

influenza A(H5)-specific, FDA 510(k)-cleared, molecular in vitro diagnostic [33]. Integrating our unique skillsets in this collaboration accelerated the availability of critical tools needed to support molecular diagnostics and pandemic preparedness.

The establishment of this collaboration facilitates the rapid production of future RGTMs or RMs. As influenza A(H5N1) and other influenza viruses with pandemic potential continue to spread and evolve, the need may arise for additional RGTMs or RMs to reflect the emergence of novel viral reassortants or genotypes. An advantage of packaging the viral RGTM/RM as three separate components is that as novel HPAI A(H5) viruses are detected in humans, as has occurred in November 2025 with the first ever human case of influenza A(H5N5) in the USA and globally, additional RM components can be added to reflect the major sequence changes observed [34]. For example, the substantial sequence differences between N1 and N5 NA segments can be accommodated with the addition of an N5 NA RM component. Similarly, as we observed with the differences in the NA sequences between D1.1 and the B3.13, D1.3, or A1 genotypes, a D1.1-specific NA RM component may be a valuable tool to help develop assays to distinguish genotypes when both D1.1 and B3.13 viruses are co-circulating (Fig. S4).

Finally, these RNA materials have applications beyond development and validation of clinical diagnostic tests for humans. They may be useful as control materials or standards for animal diagnostic testing and surveillance, including for applications such as milk testing for dairy cattle or community-based surveillance such as wastewater testing. As avian influenza virus outbreaks have affected a range of animal species and industries for decades, these materials can be useful in continually improving surveillance measures to reduce the number of affected individuals, animals, and industries.

While these materials have demonstrated utility, they do have some limitations. First, because they are not whole viruses and do not mimic a real clinical sample, they are limited in their application for diagnostic testing. Because they lack a viral envelope and surface protein antigens, they are not suitable as control materials in applications which rely on the presence of viral antigens, such as antigen-based lateral flow assays or hemagglutination or hemagglutination inhibition assays. Furthermore, because these materials are not whole viruses and do not need to undergo a nucleic acid extraction step, they cannot be used as full process controls or in full process validations to verify a successful extraction step or assess nucleic acid extraction efficiency.

Another potential limitation is that during in vitro transcription, byproducts such as prematurely terminated or extended transcripts may form [35, 36]. As these byproducts can affect the accurate quantification of full-length transcripts, we took several steps to minimize potential transcriptional byproducts and ensure accurate quantification.

For example, to minimize byproducts, we utilized restriction enzymes which generate blunt ends or 5′ overhangs and incorporated two separate double digest steps to ensure potential for double-stranded RNAs or extended transcripts was minimal [35, 37]. Transcriptional by-products were not detectable by electrophoresis, and each of the RNA materials were checked by sequencing to verify integrity.

Furthermore, to ensure accurate quantification during RM 8038 characterization, we first designed 5 to 8 RT-ddPCR assays spanning the length of each RNA component to confirm consensus of the concentration values across each fragment (data not shown). After adding a template pre-heating step to minimize reverse transcription inefficiencies due to RNA secondary structures, there was less than a twofold difference between the highest and lowest values measured along each RNA component. These measurement differences could be further minimized by optimizing the template matrix and increasing primer concentrations of the assays that generated the lowest concentration measurements for each RNA component (data not shown), suggesting a combination of RNA secondary structure and reagent availability as the major contributors to minor differences in concentration values. To accommodate remaining minor measurement differences after assay optimization, two RT-ddPCR assays generating distinct amplicons were used to determine the assigned concentration values and uncertainty intervals for each RM 8038 RNA component. As we observed for HA and MP, the values generated from the two assays used to assign values to each RNA were very similar, whereas there was a slightly larger difference in values generated by the two assays used to assign values to the NA RNA. While NA assay optimization, particularly the pre-heating step combined with Assay A, reduced the measurement differences between assays, it is possible that additional secondary structure-related optimization could have reduced the differences even further. Despite the difference in values from the two assays, they were selected to generate the assigned value for the NA RNA component as they were the best performing assays in the range of conditions tested, and the variability observed between the assays results in a wider uncertainty interval.

Together the optimized RT-ddPCR assays and conditions resulted in RM 8038 assigned concentration values that were two- to threefold higher than the estimated values that were reported for RGTM 10263 in Fig. S6 and provided to customers in the RGTM 10263 Guidance Document, highlighting important distinctions between NIST RGTMs and NIST RMs [17]. RGTM 10263 was released relatively quickly with limited characterization and was provided with concentration estimates in a "Guidance Document" whereas

the more deeply characterized RM 8038 took longer to release and came with concentration values and uncertainty that was assigned based on optimized conditions and assays. The RM 8038 concentration values are provided to customers in a "Reference Material Information Sheet" and are fit for the material's intended uses such as method development and harmonization but do not provide metrological traceability to the International System of Units (SI), and as such, the values are not considered "certified values" [18]. While utilizing whole virus reference materials is viewed as the "gold standard" for full process validation, they do not escape the issues of uncertainty due to possible molecular heterogeneity and RT-PCR inefficiencies, and they add the biological complexity of intact virions, introducing new sources of measurement uncertainty such as particle to infectivity ratios and nucleic acid extraction efficiencies, which are difficult to standardize.

Conclusion

In conclusion, to address gaps in diagnostic test capacity and accessibility for the sake of pandemic preparedness, NIST and CDC developed synthetic RNA reference materials to assist molecular diagnostics development for emerging influenza A(H5N1) clade 2.3.4.4b viruses. We have demonstrated rapid production and dissemination of these stable and homogeneous materials for assay development and validation in clinical, academic, and commercial laboratories. With this established development and distribution pipeline and interagency collaboration, we will be well-positioned for rapid responses to future outbreaks.

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Author contribution Conceptualization: John R. Barnes, Jasmine Chaitram, Marie K. Kirby, C. Todd Davis, Stephanie L. Servetas, Scott Jackson, Peter M. Vallone, Megan H. Cleveland.

Formal analysis: Eileen A. Moran, Kristine Gierz, Megan H. Cleveland.

Investigation: Eileen A. Moran, Monique E. Hunter, Kristine Gierz, Julia C. Frederick, Jimma Liddell, Lisa M. Keong, Bo Shu, Megan H. Cleveland.

Methodology: Eileen A. Moran, Kristine Gierz, Jason G. Kralj, Megan H. Cleveland.

Project Administration: Eileen A. Moran, Stephanie L. Servetas, Scott Jackson, Peter M. Vallone, Megan H. Cleveland.

Supervision: John R. Barnes, C. Todd Davis, Marie K. Kirby, Stephanie L. Servetas, Scott Jackson, Peter M. Vallone.

Visualization: Eileen A. Moran, Kristine Gierz.

Writing—original draft preparation: Eileen A. Moran.

Writing—review and editing: all authors.

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Data availability All data supporting the findings of this study are available within the paper and its supplementary information (ESM_1 and ESM_2), or they are publicly accessible at the following locations: Sequencing data can be found here: BioProject PRJNA1456179, https://www.ncbi.nlm.nih.gov/bioproject/1456179; Clinical isolate sequences are available here: https://doi.org/10.55876/gis8.260408gv.

Declarations

Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Commerce, the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry. Certain commercial software, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by NIST, nor does it imply that any of the materials, instruments, or equipment identified are necessarily the best available for the purpose. All work has been reviewed and approved by the U. S. National Institute of Standards and Technology Research Protections Office. This study was determined to be "not human subjects research" (often referred to as research not involving human subjects) as defined in U. S. Department of Commerce Regulations, 15 CFR 27, also known as the Common Rule (45 CFR 46, Subpart A), for the Protection of Human Subjects by the NIST Human Research Protections Office and therefore not subject to oversight by the NIST Institutional Review Board.
The authors have no relevant financial or non-financial interests to disclose.

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