Nucleic acid extraction

Heat inactivation of respiratory viruses in raw saliva for nucleic acid extraction

Introduction

Saliva specimens contain high-quality DNA for ancestry and genetic carrier research as well as pharmacogenomic research. More recently, saliva has been emerging as a suitable specimen for detection of respiratory viruses. Collecting saliva using the Thermo Scientific™ SpeciMAX™ Saliva Collection Kit is noninvasive and comfortable for the subject. The kit reduces the risk of infection to staff members because individuals of all ages and backgrounds can administer the collection themselves with minimal supervision. The 6 mL specimen collection tube in the kit is designed to be compatible with most liquid handlers and automated sample transfer systems, allowing low- to ultrahigh-throughput sample processing, including viral heat inactivation. This study provides a method to inactivate human respiratory viral pathogens in saliva specimens for safer downstream sample processing such as nucleic acid extraction. Raw saliva may be referred to as plain, neat, or raw saliva; in this study we will refer to it as raw saliva.

Background

Heat and UV inactivation are common methods to inactivate respiratory viruses, including SARS-CoV-2. Heat is understood to denature the capsid and envelope proteins, thus destroying infectivity while preserving downstream functionality of the genomic material [1]. This study proposes using a 10-minute incubation at 95°C to rapidly denature common respiratory viral proteins to destroy infectivity while maintaining genomic integrity. Figure 1 details a possible workflow for labs that perform low- to ultrahigh-throughput sample processing and wish to conduct heat inactivation on raw saliva specimens before extracting the RNA or DNA. Upon receipt, specimens collected with the SpeciMAX Saliva Collection Kit can be put into a 95°C (± 5°C) incubator for 10 minutes to inactivate respiratory viral pathogens. Thermo Fisher Scientific offers ovens that can hold small to very large quantities of SpeciMAX collection tubes. The SpeciMAX tube is versatile enough to fit in common tube racks that fit within general oven incubators such as the Thermo Scientific™ Heratherm™ oven. If nucleic acid extraction is required, the samples can be cooled to ambient temperatures and immediately placed directly into an extraction workflow. Automated or semi-automated workflows utilize a magnetic sample processing instrument, such as one of the Thermo Scientific™ KingFisher™ Purification Systems, with a magnetic bead–based extraction kit such as the Applied Biosystems™ MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit. After extraction, viral RNA or DNA may be detected using Applied Biosystems™ TaqMan™ Assays, or an equivalent, along with a real-time PCR instrument such as the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System.

Figure 1. Schematic of a possible sample analysis workflow that incorporates heat inactivation.

Experimental procedures

To confirm that heat inactivation of saliva specimens does not impact detection of viral nucleic acids after magnetic bead–based extraction, SpeciMAX Saliva Collection Kits were used to collect saliva from 18 different donors. Contrived samples were created by spiking the saliva samples with RP Multimarker Controls (ZeptoMetrix Corporation) containing the viral targets in Table 1. After thorough mixing, 1 mL of spiked saliva from each donor was aliquoted into a 96 deep-well plate and set aside until extraction. The collection tubes, containing the remaining 1 mL of saliva, were re-capped and placed into a 95°C (± 5°C) oven and incubated for 10 minutes. After incubation, the samples were cooled on ice and brought to ambient temperature. The 18 donors' samples (heat-inactivated and non–heat-inactivated) were processed on the KingFisher Flex Purification System using the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (RUO)* with the saliva protocol. Following nucleic acid extraction, TaqMan Assays were used with Applied Biosystems™ TaqMan™ Fast Virus 1-Step Master Mix to detect HCoV-229E, RSV-A, and AdV-3 nucleic acids on the QuantStudio 5 Real-Time PCR System. C_t values of heat-inactivated samples were compared with C_t values of non–heat-inactivated samples to determine if viral nucleic acids remained intact for detection.

* Research use only (RUO) version of the kit.

A separate experiment was conducted to confirm heat inactivation of a panel of respiratory viruses (Table 2). HSV-1, while not considered a respiratory virus, was included as a herpesvirus family surrogate for Epstein-Barr virus (EBV). Undiluted viral stocks in various cell culture media were tested along with contrived samples of virus prepared in human saliva or saliva-like matrices (PBS, or PBS with 3% BSA). Undiluted viral stocks and contrived samples were incubated for 10 minutes at 95°C (± 5°C) followed by standard titering via plaque assay. Each sample was also serially passaged three times to test for the presence of virus below the plaque assay's limit of detection, with the exception of virus diluted in saliva. Blind passages showed bacterial contamination in the saliva matrix after heating, so serial passaging could not be performed. Blind passages were visually monitored daily for the presence of viral effects on the cellular monolayer, and scored at three time points in hours post-infection (hpi) as appropriate for each virus: RSV and AdV-5 at 24, 120, and 168 hpi; MHV and HSV-1 at 24, 48, and 72 hpi; and HCoV-229E at 24, 96, and 120 hpi. Cell lines and viruses used in this experiment are summarized in Table 3. Each cell line was maintained as recommended by the manufacturer in the Gibco™ medium noted, and a standard plaque assay was performed for each virus utilizing either agarose, agar, or carboxymethylcellulose overlays in 12-well or 6-well plates as applicable [2-6]. The plaque assay is quantitative, and results are reported in mean pfu/mL from triplicate conditions.

Table 1. Viral targets spiked into 18 whole-saliva specimens collected with the SpeciMAX Saliva Collection Kit.

Target	Nucleic acid type	TaqMan Assay ID
Human coronavirus 229E (HCoV-229E)	Positive-sense single-stranded RNA (+ssRNA)	Vi06439671_s1
RSV type A (RSV-A)	Negative-sense single-stranded RNA (–ssRNA)	Vi99990014_po
Adenovirus type 3 (AdV-3)	Double-stranded DNA (dsDNA)	Vi99990001_po

Table 2. Virus panel used for confirmation of heat inactivation.

Material	Genome type	Average genome size (kb)	Segmented genome	Viral envelope	Average virion size (nm)
Respiratory syncytial virus type A strain 2 (RSV-A2)	–ssRNA	15.2	Yes	Yes	150
Human coronavirus strain 229E (HCoV-229E)	+ssRNA	32	No	Yes	115
Murine hepatitis virus strain A59 (MHV-A59)	+ssRNA	29.9	No	Yes	85
Adenovirus type 5 (AdV-5) strain adenoid-75	dsDNA	36	No	No	80
HSV type 1 (HSV-1) strain KOS	dsDNA	152	No	Yes	200

Table 3. Virus and cell materials used for plaque assays and serial passaging.

Material	Supplier	Cat. No.
Virus materials		
Respiratory syncytial virus type A strain 2 (RSV-A2)	BEI Resources	NR-12149
Human coronavirus strain 229E (HCoV-229E)	ATCC	VR-740
Murine hepatitis virus strain A59 (MHV-A59)	ATCC	VR-764
Adenovirus type 5 (AdV-5) strain adenoid-75	ATCC	VR-5
HSV type 1 (HSV-1) strain KOS	ATCC	VR-1493
Cell materials*		
HEp-2 (DMEM/F-12, Cat. No. 11320)	ATCC	CCL-23
MRC-5 (MEM, Cat. No. 11095)	ATCC	CCL-171
A549 (DMEM, Cat. No. 11995)	ATCC	CCL-185
L929 (Medium 199, Cat. No. 11150)	Sigma	85103115-1VL
Vero (DMEM, Cat. No. 11995)	ATCC	CRL-1586

* Gibco medium used for maintenance of each cell line is shown in parentheses.

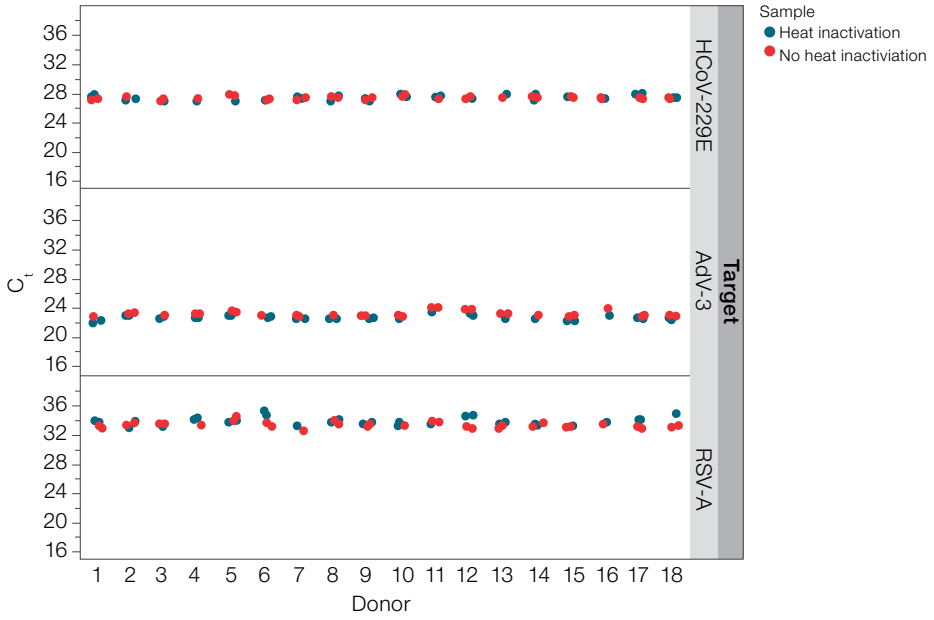

Figure 2. Detection of viral targets across 18 different donors after heat inactivation. Samples were processed with the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (RUO) and detected with TaqMan Assays on the QuantStudio 5 Real-Time PCR System.

Results

Results from real-time PCR of nucleic acids from heated compared to non-heated raw saliva are shown in Figure 2. Minimal variation between heated and non-heated samples across 18 donors for HCoV-229E, RSV-A, and AdV-3 targets indicates that viral nucleic acid degradation is not observed after heat inactivation.

All respiratory viruses in culture media, tested at the titer levels indicated, are inactivated by heating at 95°C for 10 minutes (Figure 3). All contrived viral samples in PBS, PBS with BSA, or saliva contained inactivated virus after incubation at 95°C for 10 minutes.

Virus	Stock virus (input control)				95°C, stock virus				95°C, stock virus diluted in PBS				95°C, stock virus diluted in PBS with BSA				95°C, stock virus in saliva	Result
	Plaque assay (pfu/mL)	Passage positive control			Plaque assay	Serial passages			Plaque assay	Serial passages			Plaque assay	Serial passages			Plaque assay	
		P1	P2	P3		P1	P2	P3		P1	P2	P3		P1	P2	P3		
RSV-A2	1.2×10^7	+	+	+	<	<	<	<	<	<	<	<	<	<	<	<	<	Inactivated
HCoV-229E	2.0×10^6	+	+	+	<	<	<	<	<	<	<	<	<	<	<	<	<	Inactivated
MHV-A59	9.0×10^5	+	+	+	<	<	<	<	<	<	<	<	<	<	<	<	<	Inactivated
HSV-1	4.0×10^7	+	+	+	<	<	<	<	ND	ND	ND	ND	ND	ND	ND	ND	ND	Inactivated
AdV-5	1.4×10^8	+	+	+	<	<	<	<	<	<	<	<	<	<	<	<	<	Inactivated

Figure 3. Plaque and serial passage assay results across respiratory and enteric viruses. "+" indicates the presence of cytopathic effect on the monolayer, "<" indicates no observable titer or cytopathic effect, and "ND" indicates not done.

Conclusions

Thermo Fisher offers a comprehensive way to collect saliva specimens, inactivate the samples for respiratory viral targets, extract viral nucleic acids from the samples with a semi-automated or fully automated workflow, and analyze the results for research purposes. Given the results in this study, heat inactivation did not degrade the selected viral targets within the samples.

References

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3. Lundin A, Dijkman R, Bergström T et al. (2014) Targeting membrane-bound viral RNA synthesis reveals potent inhibition of diverse coronaviruses including the Middle East respiratory syndrome virus. *PLoS Pathog* 10(5):e1004166.
4. Hirose S, Tormanen K, Kato M et al. (2021) Protocol for a mouse CNS demyelination model induced by a combination of HSV-1 and IL-2. *STAR Protoc* 2(1):100287.
5. McClain ME, Spendlove RS, Lennette EH (1967) Infectivity assay of Reoviruses: comparison of immunofluorescent cell count and plaque methods. *J Immunol* 98(6):1301–1308.
6. Leibowitz J, Kaufman G, Liu P (2011) Coronaviruses: propagation, quantification, storage, and construction of recombinant mouse hepatitis virus. *Curr Protoc Microbiol* Chapter 15(1):Unit 15E.1.

Ordering information

Description	Cat. No.
SpeciMAX Saliva Collection Kit	A50696
Heratherm Advanced Protocol Oven	51028152
MagMAX DNA Multi-Sample Ultra 2.0 Kit	A36570
MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (RUO)	A48383R
MagMAX *mir*Vana Total RNA Isolation Kit	A27828
KingFisher Flex Purification System*	5400640

* **For Laboratory Use.**

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