

Introduction

Recent genomic technologies have made possible the accurate and rapid assessment of specific pathogens directly at the site of patient care without the time delays associated with diagnostic laboratories. One of the major advantages of recent Point-of-Care (POC) instrumentation is their ease of use, making diagnostic tests accessible to personnel without specialized laboratory training. Nucleic acid-based techniques enable the accurate assessment of specific pathogens directly at the site of patient care. Essential to these DNA/RNA amplification-based assays is the reliable collection and storage of clinical samples in media that protects nucleic acids. The goal of this study was to assess the ability of a MK inactivation buffer solution to preserve viral RNA for genetic testing following storage in temperatures of 4° and 30°C for up to 30 days. Detection of viral RSV-A RNA was assessed using quantitative real-time PCR (QPCR) and Loop-Mediated Isothermal Amplification (LAMP) assays.



The goal of this project is

- To evaluate Puritan MK inactivation buffer solution's ability to preserve known concentrations of viral RSV-A RNA and allow recovery and quantitation of RNA for genetic testing using two nucleic acid-based detection protocols employed by current POC instruments.
- To assess the effect of storage RSV-A from 1 to 30 days at both 4° and 30° C on the RNA concentrations as measured by QPCR and for 1 day for LAMP assays.

Methods

RSV-A (ATCC strain VR-26) was propagated on monolayers of Hep-2 Cells. Virus stocks were enumerated using the Reed-Muench method and reported as 50% tissue culture infectious dose per milliliter (TCID₅₀/mL). Serial dilutions were then made resulting in aliquots with concentrations ranging from 1x10⁶ to 1x10¹ TCID₅₀/mL from which swabs were used to transfer ~100 µL of RSV-A added to 1 mL of MK buffered solution. Samples were stored at 4° and 30° C for 0, 1, 7 and 30 days. Following storage total RNA isolated from 300 µL of media. Three replicates were quantified using Quantitative real-time PCR using Promega GoTaq® Probe 1-Step RT-qPCR System. To establish that MK buffered solution is compatible with LAMP protocols the same RSV-A RNA samples were reverse transcribed and cDNA's were assayed in a 25 µL reaction mixture per manufacturers protocol using the WarmStart® LAMP Kit (New England BioLabs).

QPCR Assay

ProMega GoTaq Probe 1-step RT-qPCR	
	one rxn
GoTaq qPCR Master Mix	10.0 ul
GoScript RT Mix	0.4 ul
Combined Primer/Probe Mix	1.5 ul
Nuclease-free water	3.1 ul
	15.0 ul/sample + 5 ul RNA
Thermocycle Program	
Reverse Transcriptase	45 °C 15 min
RT Inactivation	95 °C 2 min
Denaturation	95 °C 15 sec 45 X
Annealing/Extension *	60 °C 1 min * Detect FAM

QPCR fluorescence readings were measured by a BioRad CFX 96 Real-Time PCR System. The threshold for determining Ct value for each sample is based on the baseline threshold – above background and within the exponential phase of amplification curve.

Methods (continued)

Loop-mediated Isothermal Amplification (LAMP)

Six primer sequences for the detection of the RSV-A matrix gene were used (Mahony et al. 2013). The LAMP assay was carried out in a 25 µL reaction mixture per manufacturers protocol using the WarmStart® LAMP Kit (New England BioLabs). 5 µL of the diluted samples were used for target detection using SYBR Green® and an BioRad CFX 96Real-Time PCR System.

Primer Name: RSV A matrix gene

Ref: Mahony et al. J Clin Microbiol. 2013 51:2696

FIP: TCTGCTGGCATGGATGATTGGAGACGATGATCCTGCATCA

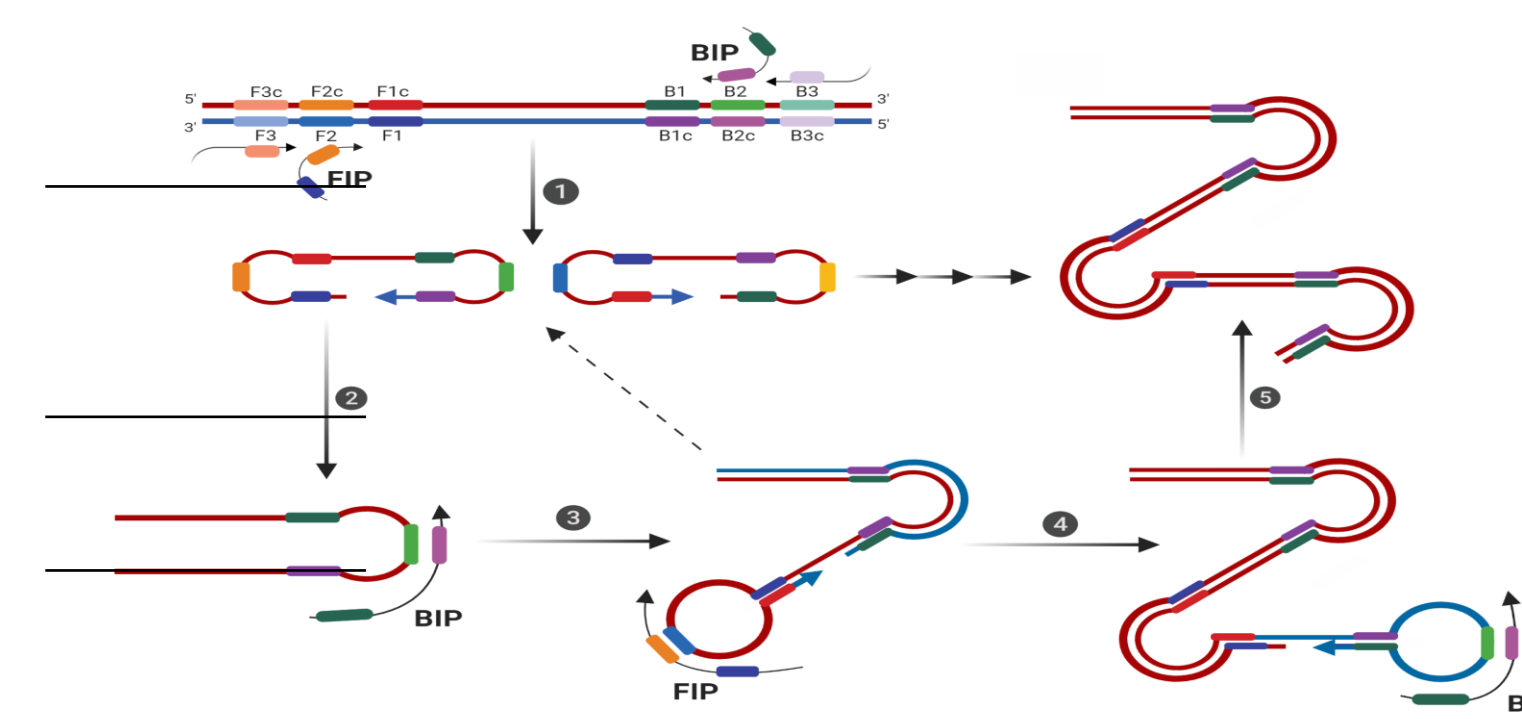
BIP: CTAGTGAACAAATATCCACACCCAGCACTGCACCTTCTTGAGTT

LF: ACATGGGCACCCATATTGTAAG

LB: AGGGACCTTCATTAAGAGTCATGAT

F3: GCTGTTCAATACAATGTCCTAGA

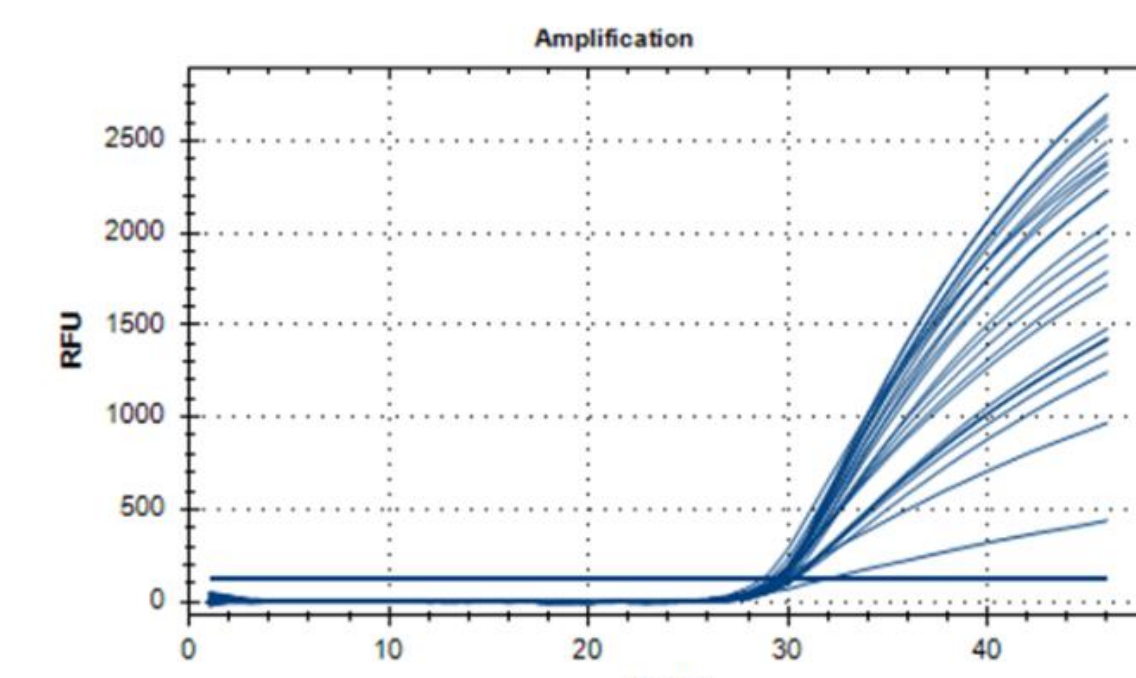
B3: GGTAATTTGCTGGGCATT



For LAMP	one rxn	Lamp Cycle
WarmStart Master mix (2x)	12.5µL	65 °C 10 sec 50 X
Fluorescent dye - FAM (50x)	0.5µL	65 °C 1 min * Detect FAM
Lamp Primers (10x)	2.5µL	
Sterile H ₂ O	4.5µL	
	20.0µL	20 ul/sample + 5 ul cDNA or RNA

Results

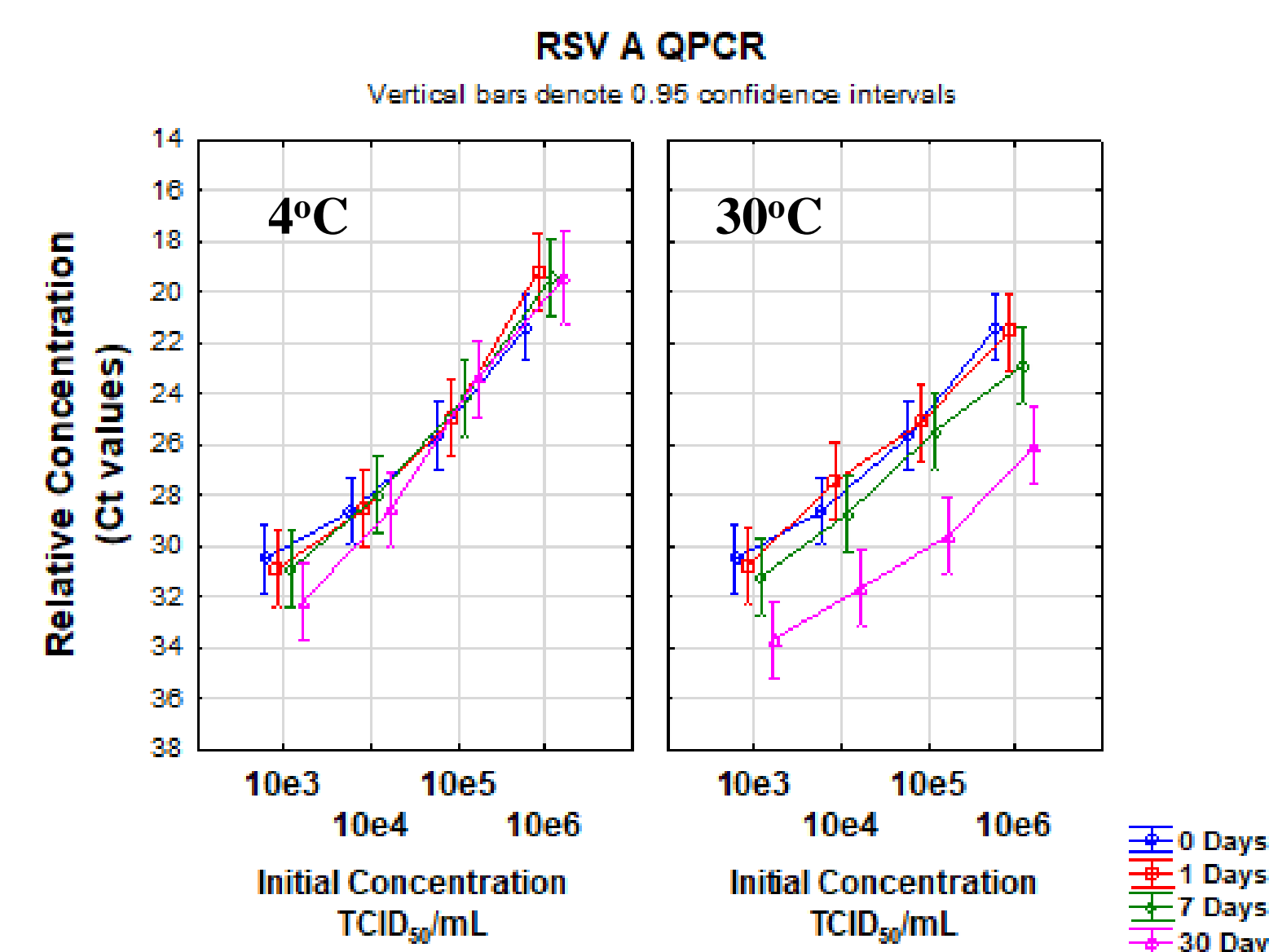
QPCR Assay



QPCR curves for 25 replicates of RSV-A at a concentration of 10³ TCID₅₀/mL.

RSV-A RNA stability at 4° and 30°C

	Concentration 10 ³ TCID ₅₀ /ml						
	Stability at 4°C			Stability at 30°C			
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	30.5	30.9	30.9	32.1	30.8	31.2	33.7
Std Dev Ct	0.67	0.26	0.27	0.36	1.62	1.75	1.67
	Concentration 10 ⁴ TCID ₅₀ /ml						
	Stability at 4°C			Stability at 30°C			
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	28.6	28.5	28.0	28.6	27.4	28.7	31.6
Std Dev Ct	1.42	1.31	0.97	1.03	0.28	0.30	0.61
	Concentration 10 ⁵ TCID ₅₀ /ml						
	Stability at 4°C			Stability at 30°C			
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	25.7	25.0	24.1	23.4	25.1	25.5	29.6
Std Dev Ct	2.27	2.31	1.00	0.43	0.55	1.51	1.31
	Concentration 10 ⁶ TCID ₅₀ /ml						
	Stability at 4°C			Stability at 30°C			
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	21.4	19.2	19.4	19.4	21.5	22.9	26.1
Std Dev Ct	1.79	0.41	0.52	0.57	0.30	1.85	0.91



For samples stored at 4°C, there was no significant difference in Ct values versus time (ANOVA, F=1.43, P=0.25). There was a significant difference in Ct values among the samples stored at 30°C due to degradation among the day 30 samples (ANOVA, F=22.36, P<0.001) though the relationship with starting concentration was maintained.

Results (continued)

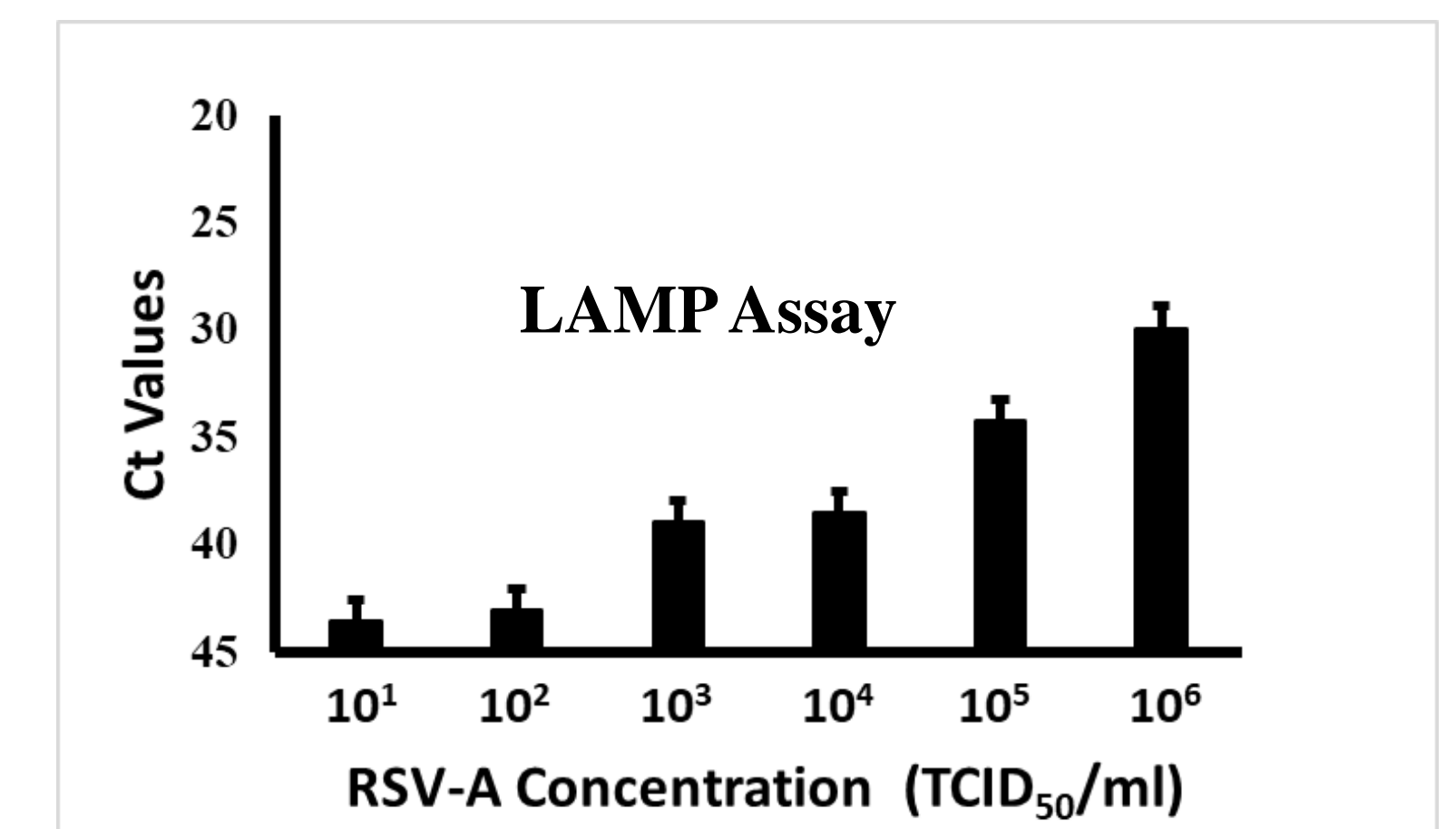
LAMP Assay

To establish that MK inactivation buffer solution is compatible with protocols employing isothermal amplification rather than QPCR. RSV-A RNA samples were tested using Loop-mediated Isothermal Amplification (LAMP). There were no differences in Ct values with time for samples stored at either 4°C or 30°C

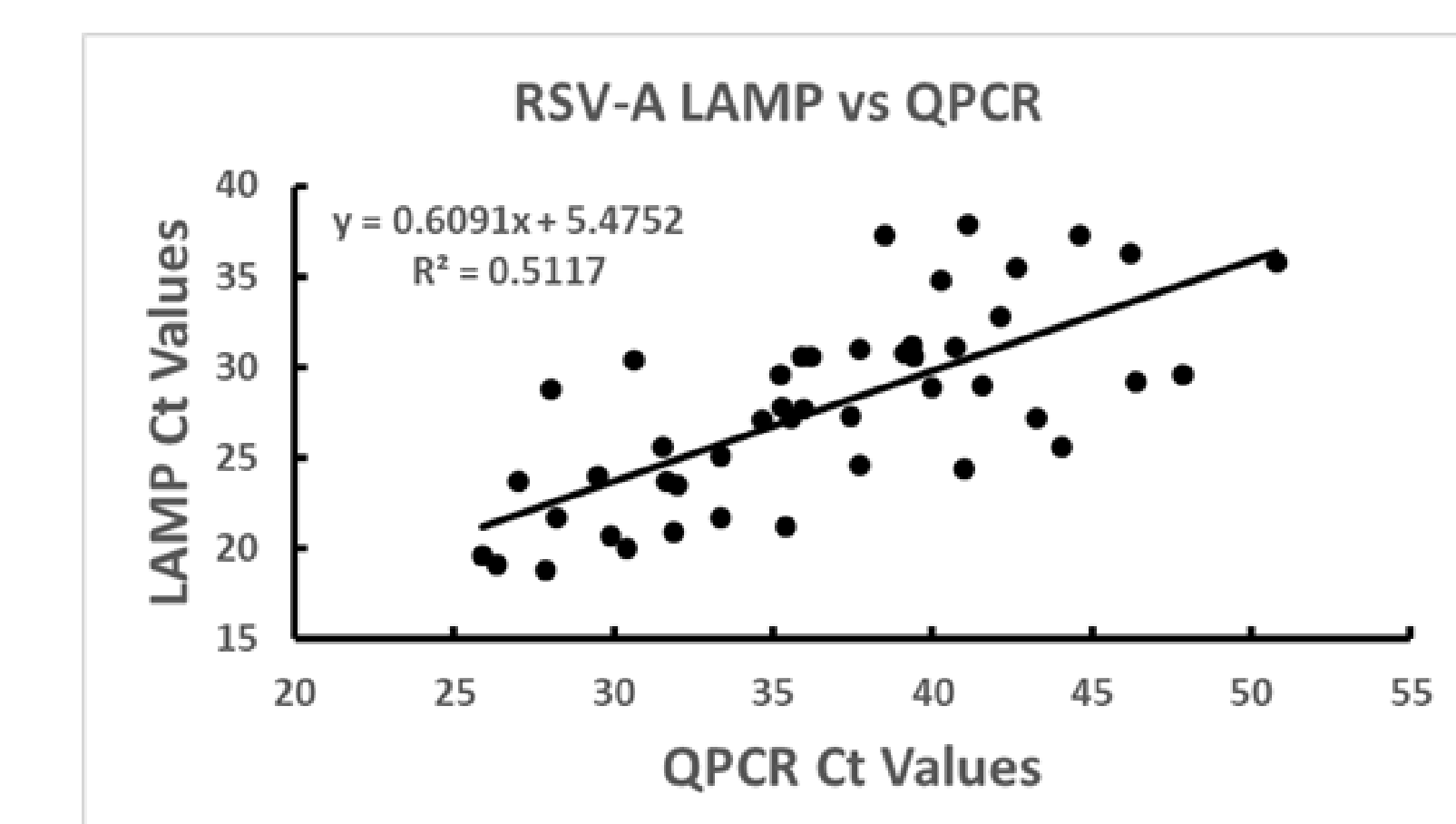
Concentration	N	Mean	Std Dev
10 ¹ TCID ₅₀ /mL	4	43.5	2.3
10 ² TCID ₅₀ /mL	4	43.1	5.4
10 ³ TCID ₅₀ /mL	9	38.9	3.4
10 ⁴ TCID ₅₀ /mL	9	38.5	5.2
10 ⁵ TCID ₅₀ /mL	10	34.2	5.4
10 ⁶ TCID ₅₀ /mL	10	29.9	3.0
All	46	36.6	6.2

Ct values for the RSV-A samples at concentrations ranging from 10¹ to 10⁶ TCID₅₀/mL.

Mean and Std Dev Ct values for the RSV-A



Relationship between the QPCR and LAMP Ct values.



Conclusions

These results indicate that for RSV-A the MK inactivation buffer solution allows for the rapid detection and quantification of viral (RSV-A) RNA following storage for up to 30 days at temperatures up to 30°C using either QPCR or LAMP assay-based methods

Acknowledgements

This project was supported by funding from Puritan Medical Products, Guilford ME.

