

## 1 Background and Introduction

Cytomegalovirus is prevalent and usually benign in healthy populations. Permanent health problems can arise when transmission occurs prenatally, resulting in congenital cytomegalovirus (cCMV). Screening for cCMV is currently not universal but reactionary to symptoms. Because of this, molecular methods using saliva, urine, or blood freshly collected from symptomatic patients are inadequate as they may no longer be infected or become infected postnatally. Dried Blood Spot (DBS) cards may be a prime sample input for detection of cCMV and may in the future enable retrospective testing of archived samples.

DBS cards facilitate easy collection and transportation of samples. We have developed a two-plex, real-time PCR assay to detect cCMV loci using DNA isolated from a single 3.2 mm punch of a dried blood spot (DBS) using the NeoMDx™ DNA Extraction Kit. The amplification of the house-keeping gene, RPP30, is included in the assay as a positive control of DNA purification and can be used as an internal control to determine relative copy number. The qPCR assay is compatible with the existing NeoMDx™ DNA Extraction Kit, utilizing a simple, alkaline-based DNA extraction, and 2-part qPCR setup. The assay can be fully automated in the same fashion as NeoMDx™ DNA Extraction Kit plus the NeoMDx™ PCR Reagent Kit.

The NeoMDx™ cCMV Real-Time PCR Assay performance was demonstrated on putative normal samples extracted from one 3.2 mm punch of putative normal DBS and DNA from several characterized reference samples and controls. The DBS were extracted using the NeoMDx™ DNA Extraction Kit, and the eluate was sent for two real-time PCR assays, NeoMDx™ PCR Reagent kit testing for TREC, SMN1 and RPP30 as well as the NeoMDx™ cCMV Real-Time PCR Assay. This process shows that a single 3.2 mm punch is enough to run two molecular assays from the same eluate, saving time, dried blood spot punching, and reagents.

## 2 Methodology

712 putative normal DBS were punched into 96-well plates along with one set of NeoMDx™ PCR Reagent Kit and NeoMDx™ cCMV controls per plate. The plates were extracted using the NeoMDx™ Extraction Kit protocol in groups of 4 on the Janus G3 Automated Liquid Handler system, then consolidated for qPCR in two assays. The first consolidation of 3µL per sample was consolidated into a PCR plate containing the NeoMDx™ PCR Reagent Kit, and then a second consolidation of 10µL of each sample was pipetted into a PCR plate containing the NeoMDx™ cCMV PCR Reagents. The qPCR was then run on an Analytik Jena qTower<sup>3</sup>84G instrument.

### NeoMDx™ Extraction Workflow

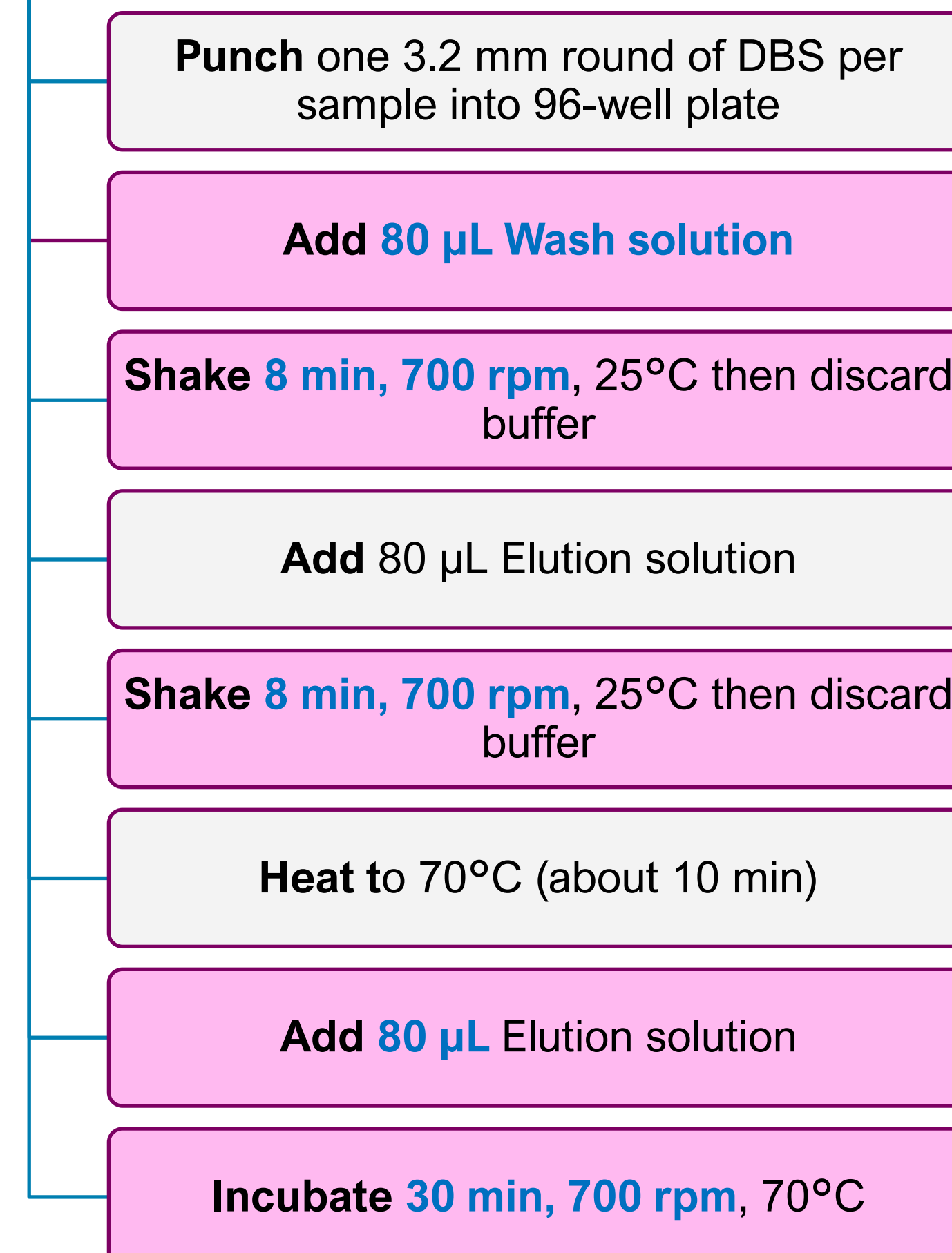


Figure 1: Revvity Panthera DBS puncher.

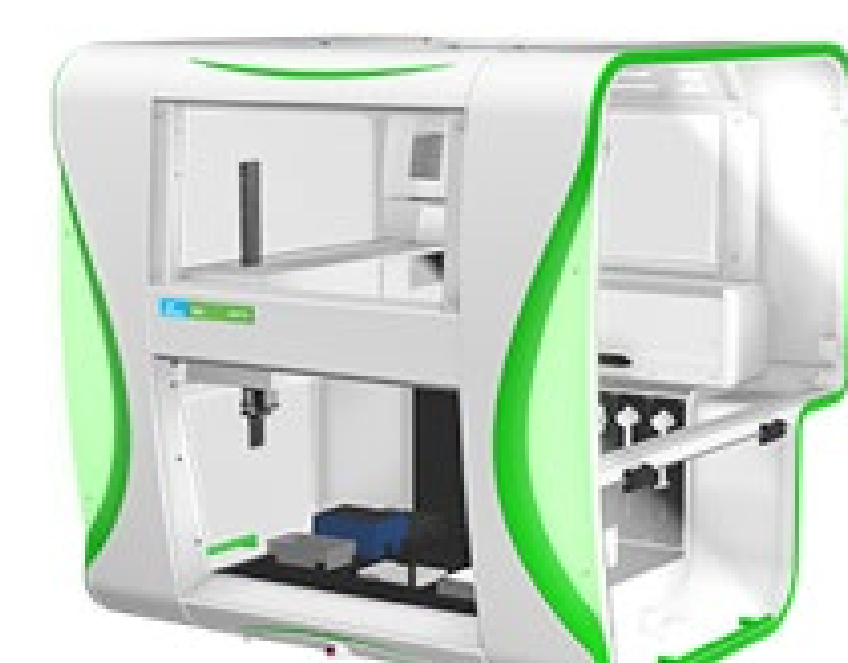


Figure 2: Revvity Janus G3 Automated Liquid Handler



Figure 3: Analytik Jena qTower<sup>3</sup>84G Instrument

## 3 Results

Valid Results (CMV)			DBS SAMPLE INTERPRETATION		
Input	CT*		FAM (CMV)	Cy5 (RPP30)	Result interpretation*
	CMV (FAM)	RNase P (Cy5)			
NTC	No Ct or > 40	> 35	Y	Y/N	CMV detected
C1	No Ct or > 40	Detected < 30	N	Y	CMV not detected
C2	Detected < 36	Detected < 30	N	N	Invalid. Sample needs to be re-tested by repeat PCR if there is sufficient extracted DNA. Otherwise, sample needs to be re-tested by re-extraction or re-collection.
C3	Detected < 32	Detected < 30			
DBS Sample	≤ 40	≤ 35			

\*Based on QuantStudio 7, 1x DBS, 50 µL Elution Buffer. It is subject to change for different real-time PCR instruments.

\*With valid C1, C2, C3

Valid Results (TREC/SMN1)			DBS SAMPLE INTERPRETATION				
Input	CT*			FAM (TREC)	Cy5 (SMN1)	Cy5.5 (RPP30)	Result interpretation*
	TREC (FAM)	SMN1 (Cy5)	RPP30 (Cy5.5)				
NTC	No Ct or > 40	No Ct or > 40	> 35	Y	Y	Y/N	TREC and/or SMN1 detected
C1	No Ct or > 40	No Ct or > 40	Detected < 30	N	N	Y	TREC and/or SMN1 not detected
C2	Detected < 36	Detected < 30	Detected < 30	N	N	N	Invalid. Sample needs to be re-tested by repeat PCR if there is sufficient extracted DNA. Otherwise, sample needs to be re-tested by re-extraction or re-collection.
C3	Detected < 32	Detected < 30	Detected < 30				
DBS Sample	≤ 40	≤ 40	≤ 35				

\*Based on QuantStudio Dx, 1x DBS, 80 µL Elution Buffer. It is subject to change for different real-time PCR instruments.

\*With valid C1, C2, C3

n=8	CMV	std dev	RPP30	std dev
C1			24.6	0.62
C2	34.4	0.21	24.5	0.38
C3	30.1	0.34	24.2	0.46

n=8	TREC	std dev	SMN1	std dev	RPP30	std dev
C1					22.6	0.35
C2	33.2	0.39	21.3	0.36	22.0	0.52
C3	28.7	0.26	21.0	0.25	22.0	0.28

## 4 Discussion

The data shows that using 1x 3.2 mm DBS is sufficient for running two molecular assays. The extractions were done by two operators on different days, and the qPCR was set up to run one assay following the other to utilize the same qPCR instrument.

The controls for each assay are valid and within the QC limits to pass.

Of the 712 samples run, 7 were QNS (0.98%) and had similar results in both tests. There were 6 samples with a blood spot loss (0.84%) during extraction, and again, yielded similar results with both PCR assays. RPP30 Ct values for the runs ranged from a low in the CMV assay of 22.13 to a high of 33.16. In the NeoMDx PCR assay, RPP30 Ct values ranged from 21.16 to 31.2.

There is a loss of 3 sample spots in the extraction due to the need for both sets of controls to be punched, but it saves up to 2 punches from the sample dried blood spot card using this method.

## 6 Conclusion

We have demonstrated that the NeoMDx™ Extraction Kit yields enough high-quality DNA to run both the NeoMDx™ PCR Reagent Kit and the NeoMDx™ cCMV PCR Reagents.

This method of using a single 3.2 mm punch is to run two molecular assays from the same eluate, saves time, dried blood spot availability, time punching, and reagents.

*This testing service has not been cleared or approved by the U.S. Food and Drug Administration. Testing services may not be licensed in accordance with the laws in all countries. The content of this pamphlet is provided for informational purposes only, not as medical advice. It is not intended to substitute for the consultation, diagnosis, and/or treatment provided by a qualified licensed physician or other medical professional.*