eP487: Universal Newborn Screening of Congenital Cytomegalovirus using Dried Blood Spots and qPCR

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1 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 are inadequate as symptomatic patients may no longer be infected or become infected postnatally.

Newborn Screening (NBS) currently uses dried blood spot (DBS) cards that are collected neonatally for other screening. This makes DBS a prime sample input for universal screening of cCMV, as well as retrospective testing using archived samples.

Historically, issues with DBS for NBS of cCMV were due to sensitivity, scalability, and input needs. To address these concerns, we have developed a relatively sensitive, high-throughput compatible, simple workflow, sample extraction to qPCR assay kit using only one or two 3.2 mm DBS punches.

Assay Key Features

- DBS-based sample input for NBS compatibility
- Simple workflow that is user friendly and has short turn around times
- Scalable from a partial 96-well plate to a full 384-well plate
- Manual and Automation compatible

2 Methods

Two different manual DBS extraction methods were tested and compared, the simplified NeoMDx™ alkaline based extraction and Thermal Shock. Both methods used 2x 3.2 mm DBS punches and a 65 µL elution volume for compatible comparison.

In addition, three automated DBS extraction methods were evaluated:

- **Extraction Workflow 1:** simplified NeoMDx™™ alkaline extraction with 1x 3.2 mm DBS punch and 50 µL elution
- **Extraction Workflow 2:** simplified NeoMDx™ alkaline extraction with 2x 3.2 mm DBS punches and 65 µL elution
- **Extraction Workflow 3:** standard NeoMDx™ alkaline extraction with 1x 3.2 mm DBS punch and 80 µL elution used with SCID/SMA assay

The workflow for each is shown in Figure 1. Each scheme's eluents were used as direct input into a 15 µL PCR reaction using the NeoMDx™ cCMV Kit reagents via workflow shown in Figure 2.

The assay quantifies a CMV gene marker in FAM, and a human housekeeping gene, RPP30, in Cy5, as well as a background baseline reading in ROX. This design is compatible with all commercially available real-time PCR instruments without the need of additional instrument color compensation.

For each test, the assay uses DBS controls that monitor the overall workflow from sample extraction to real-time PCR detection. Due to the lack of access to cCMV confirmed newborn DBS, contrived DBS samples were used for development.

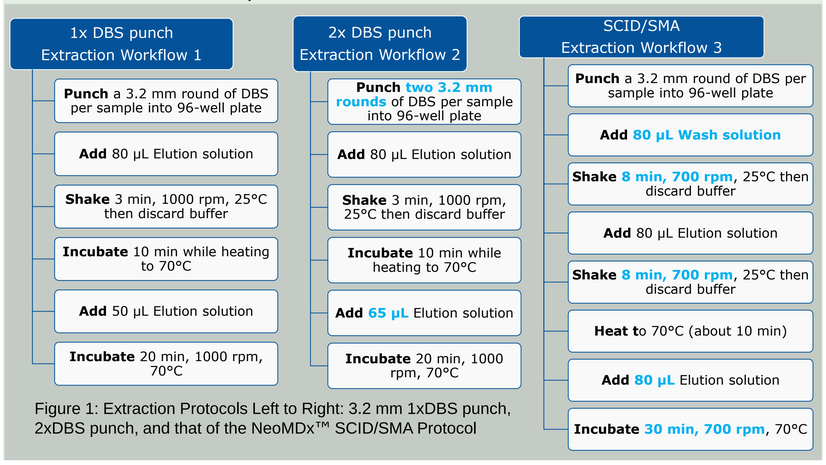

Figure 1: Extraction Protocols Left to Right: 3.2 mm 1xDBS punch, 2xDBS punch, and that of the NeoMDx™ SCID/SMA Protocol

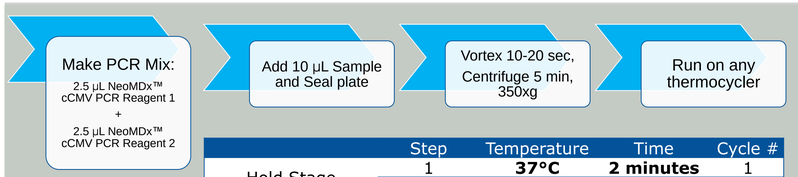

Figure 2: qPCR Assay procedure

Table 1: Thermocycling protocol

	Step	Temperature	Time	Cycle #
Hold Stage	1	**37°C**	**2 minutes**	1
	2	**94°C**	**10 minutes**	1
PCR Stage	3	**93°C**	**10 seconds**	40
		60°C*	**30 seconds**	
		69°C	**40 seconds**	

* Collect fluorescence signal at this step.

3 Results

Thermal Shock versus NeoMDx™ Alkaline-based Extraction
Thermal Shock based extraction had a lower sensitivity than a NeoMDx™ in an initial comparison using a proposed 1x 3.2 mm DBS punch 80 µL Elution Volume extraction schema for NeoMDx™ using input volumes for a 15 µL qPCR reaction as shown in Table 2. However, when more concentrated NeoMDx™ qPCR reagents were used with an increased qPCR sample volume was used the Thermal Shock scheme could not detect any CMV in the samples.

On the other hand, NeoMDx™ was able to detect down to 50 IU/mL with the increased sample input volume with the same sample scheme of two DBS punches input with 65µL elution volume with NeoMDx™ alkaline based elution solution.

This shows that the NeoMDx™ Extraction method can outperform the DBS gold standard extraction method for CMV via its robust detection abilities in addition to being a simpler protocol and automation compatible, which Thermal Shock is not.

Extraction Method	Sample Scheme	Input Volume	Lowest CMVAQP Verification Panel CMV concentration detected (IU/mL)
Thermal Shock	2x 3.2 mm DBS punches 65 µL Elution Volume	3 µL	15000
		6 µL	5000
		10 µL	-
NeoMDx™ Extraction	Extraction Workflow 1 with 80 µL Elution Volume	3 µL	-
		6 µL	50000
	Extraction Workflow 2	10 µL	50

Table 2: Thermal Shock versus NeoMDx™ Alkaline Extractions

Automated Extraction Workflow Performance
The automation study shown in Figure 3 and Table 3 compares the three NeoMDx™ alkaline extraction schemes and shows that all three are compatible to detect CMV. The three extraction workflows have Ct differences as seen with the Extraction Workflow 2 due to the increased DBS input and more concentrated final elution volume comparatively. The reduced Ct of Extraction Workflow 2 shows benefit to having 2 punches as the extraction sample. While Extraction Workflow 3 shows that the NeoMDx™ CMV assay can be run with the same elution material as the SCID/SMA assay. On the other hand, while Extraction Workflow 1 has higher Cts for each type, it is shown to be competitive to the other types and a good choice when additional DBS punches cannot be used.

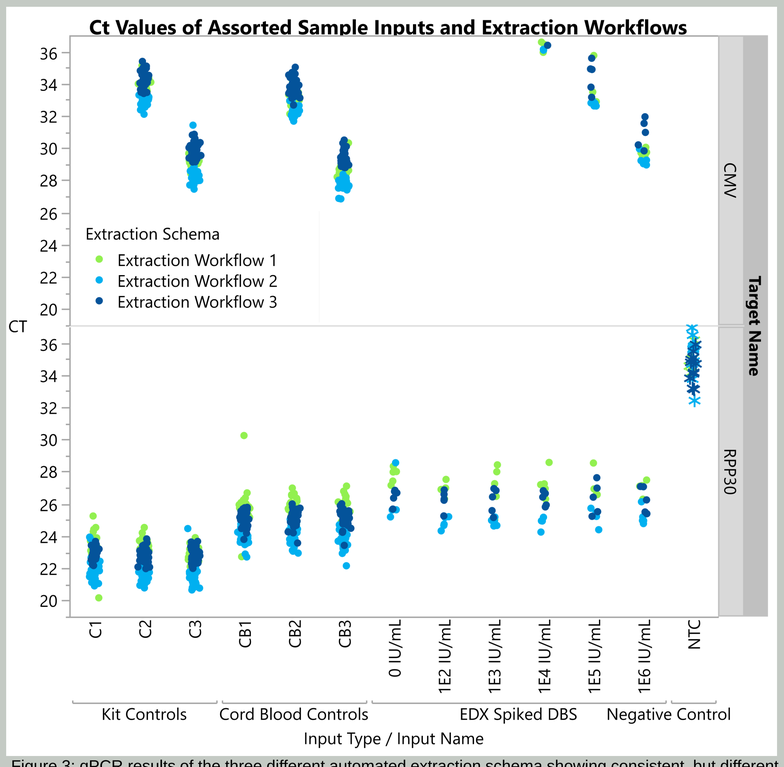

Figure 3: qPCR results of the three different automated extraction schema showing consistent, but different Ct values of the different types.

Input Type	CMV Conc. (IU/mL)	CMV Ct Value per Extraction Schema: Extraction Workflow 1 Mean	Std Dev	N	Extraction Workflow 2 Mean	Std Dev	N	Extraction Workflow 3 Mean	Std Dev	N
Kit Controls	C1	-	-	0	-	-	0	-	-	0
	C2	33.95	0.514	36	32.98	0.345	36	34.27	0.520	36
	C3	29.14	0.452	36	28.33	0.634	36	29.91	0.437	36
Cord Blood Controls	CB1	-	-	0	-	-	0	-	-	0
	CB2	33.02	0.406	36	32.26	0.326	36	33.73	0.531	36
	CB3	28.62	0.431	36	27.79	0.319	36	29.33	0.460	36
EDX Spiked DBS	0	-	-	0	-	-	0	-	-	0
	100	-	-	0	-	-	0	-	-	0
	1000	-	-	0	-	-	0	-	-	0
	10000	36.32	0.333	3	36.10	0.035	2	36.38	-	1
	100000	33.69	1.412	4	32.74	0.119	5	34.46	0.974	5
	1000000	29.68	0.246	5	29.28	0.394	5	30.89	0.885	5
Negative Control	NTC	-	-	0	-	-	0	-	-	0

Table 3: CMV Ct values of 13 different sample inputs and the three different automated extraction methods. N = 36 for each Kit Control and Cord Blood Controls types. N = 5 for each EDX set. N = 72 for each Negative Control set.

The C2 control, paired with the CMV negative C1 control and the more concentrated CMV positive C3 control establishes a strong design to catch boundary cases of the kit's detection abilities with confidence. This is seen with the EDX Spiked DBS Samples of similar Ct values to the C2 control which is shown to be near the detection limit of the assay. By including that control level, lower viral load samples will more definitively not be missed due to inadequate extraction or qPCR setup whereas higher viral load samples and controls might still pass.

To further support automated extraction in a lab setting, clinical samples must be run to establish a Ct cutoff value that balances true CMV detection in true newborn samples and background. This is especially critical in the detection of CMV due to its nature of being a viral component of variable viral load rather than a native gene to every nucleated cell.

Extraction Type	Input	Target Name: CMV Mean	CMV Std Dev	CMV N	RPP30 Mean	RPP30 Std Dev	RPP30 N
Automated	C1	-	-	0	23.39	0.796	36
	C2	33.95	0.514	36	23.27	0.402	36
	C3	29.14	0.452	36	22.93	0.444	36
Manual	C1	-	-	0	21.89	0.385	14
	C2	33.72	0.580	14	22.06	0.387	14
	C3	28.89	0.492	14	21.87	0.361	14

Table 4: Comparison of Extraction Workflow 1 both manually and via automation shows comparable Ct values and reproducibility.

Manual versus Automated Extraction Workflows
The qPCR results of kit control samples are compared between a manual and an automated extraction scheme in Table 4. For both manual and automated extractions were run with the Extraction Workflow 1 base scheme. For CMV there is a negligible difference in Ct shows that the automated and manual processes are comparable. This establishes the feasibility of using automation of the NeoMDx™ cCMV kit as a high-throughput compatible method for the detection of CMV in addition to the low-throughput manual method.

4 Conclusion

The NeoMDx™ cCMV kit can be used for different throughput labs, high or low, due to its scalable extraction protocol and 96-well and 384-well compatibility for qPCR. As hospitals and screening labs are already collecting and testing DBS, it is the easiest sample type to implement for NBS. Having a high-throughput compatible and sensitive DBS based assay is instrumental to adding cCMV to NBS as well as retrospective testing of high-risk patients. This makes the NeoMDx™ cCMV kit a steppingstone to universal screening of cCMV though access to relevant/known clinical samples is needed to further vet robustness and finalize Ct cutoffs.

For research use only. Not for use in diagnostic procedures. The product is currently under development. Please check with your local representative for more details.