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

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EDITION CONGENITAL CMV
FROM PREGNANCY TO INFANCY: LET'S FACE IT
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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 in most areas, but instead is reactionary to symptoms. By waiting for symptom onset, the viral infection can diminish resulting in false negatives, or occur postnatally resulting in false positives. This process also skips patients with delayed symptom onset and misses their window of detectable prenatal viral infection.

- Typical reactionary tests use molecular methods that utilize saliva, urine, and plasma as the sample input for the detection of CMV. These sample inputs are limited to testing within a certain testing window to catch the cCMV infection. Universal screening of newborns has the appeal of detecting cCMV in symptomatic, and asymptomatic patients as well as those that might experience delayed symptom onset.

- Currently, newborn screening (NBS) uses dried blood spot (DBS) cards that are collected neonatally for other screening tests. DBS are an archivable snapshot of the neonate's health. This makes DBS a prime sample input for universal screening of cCMV among other reasons, such as:
  - DBS collection is routine, no additional training of medical personnel
  - Newborn screening labs are familiar with this sample type
  - Automatable and compatible with high-throughput screening
  - Mitigates issues of viral infection onset ambiguity due to proximity of sample collection to birth
  - Catches asymptomatic and those that will have delayed symptom onset
  - Catches infection when actionable healthcare is possible
  - Retrospective testing possible using archived DBS samples for older patient testing

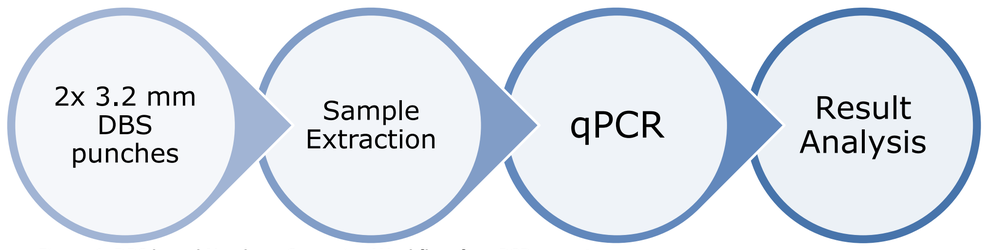

Figure 1: DBS based Newborn Screening Workflow for qPCR assay types

- 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 2x 3.2 mm DBS punches.

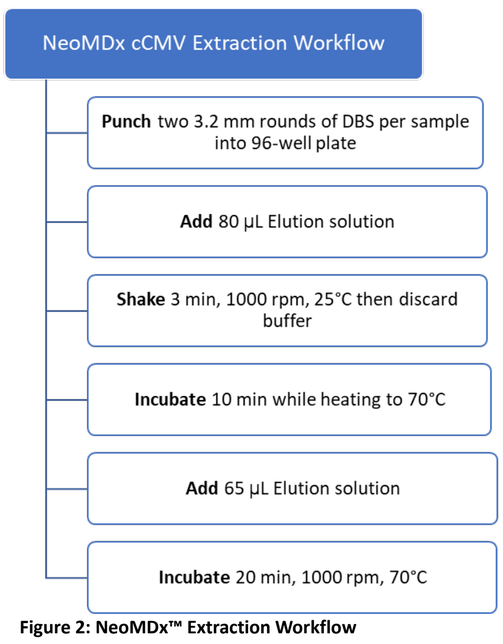

Figure 2: NeoMDx™ Extraction Workflow

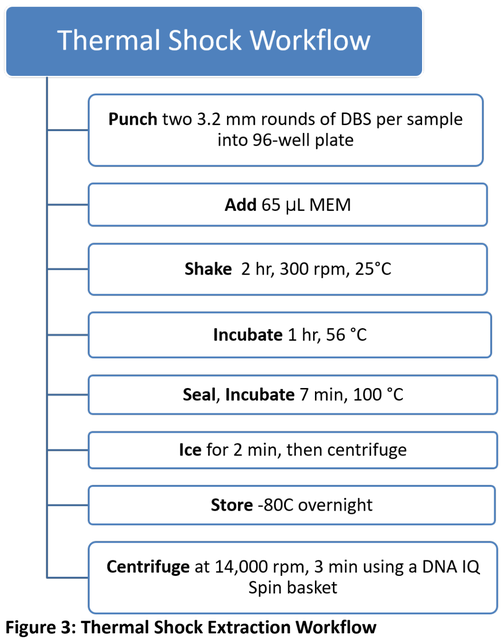

Figure 3: Thermal Shock Extraction Workflow

METHODOLOGY

- Two 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 a compatible comparison. In addition, both eluents were used as direct input into a 15 µL PCR reaction using the NeoMDx™ cCMV Kit reagents.

- The qPCR 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 cCMV confirmed newborn DBS, commercial analytical and proficiency panels, contrived samples, and cCMV negative newborn DBS were used for the tests.

- Evaluation samples: Kit Controls, Randox CMVAQP Verification Panel, QAV064127 2020 QCMD Proficiency Panel

RESULTS

- The simplified NeoMDx™ extraction takes around 30 minutes manually for 96-wells, with only two buffer exchanges and two incubation temperatures. The Thermal Shock method had a larger sample input prior to extraction, and a lower limit of detection (LoD) as seen in Table 1. Table 2 shows comparable performance with the same sample scheme for NeoMDx™. Yet, when used in the assay as a 10 µL input for the qPCR reaction, Thermal Shock had no amplification of the targets (Table 3).

- NeoMDx™-based runs went on to reach over 95% analytical sensitivity (n=84) with the commercial proficiency panel and 100% analytical specificity with known cCMV negative DBS (n=192) as shown in Table 4.

- The LoD of the qPCR assay is 3.3 international units (IU) per reaction as shown in Table 5. While the LoD of the full extraction to qPCR workflow is 10 IU/µL based on contrived DBS as shown in Table 6.

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

Table 1: Thermal Shock with an increased sample scheme of 2x 3.2 mm DBS punches with 65 µL Elution Volume had better sensitivity than NeoMDx™ Extraction with a 1x 3.2 mm DBS, 80 µL Elution Volume scheme with either input volumes.

Sample Type	CMV Call	Sample ID	Viral Loads (IU/mL)	CMV (FAM)			RPP30 (Cy5)		
				Mean	Std Dev	N	Mean	Std Dev	N
Control	Control	Kit control C1	0	-	-	0	20.24	0.969	12
		Kit control C2	low control	33.25	0.375	12	20.19	1.054	12
		Kit control C3	high control	28.45	0.379	12	19.72	1.052	12
		NTC	0	-	-	0	-	-	0
DBS Sample	Negative	DBS Sample	Negative	-	-	0	23.00	1.222	180
Proficiency Panel	Negative	CMVDBS20S-03	CMV Negative	-	-	0	22.97	0.928	12
	Positive	CMVDBS20S-01	CMV (AD169)	33.72	0.468	12	22.84	0.794	12
		CMVDBS20S-02	CMV clinical	35.75	3.132	12	23.13	0.909	12
		CMVDBS20S-04	CMV (AD169)	35.95	0.676	10	23.11	0.997	12
		CMVDBS20S-05	CMV clinical	37.28	0.498	10	23.12	1.093	12
		CMVDBS20S-06	CMV (AD169)	36.51	0.778	12	22.96	0.839	12
		CMVDBS20S-07	CMV clinical	36.68	1.132	12	22.97	0.978	12
		CMVDBS20S-08	CMV (AD169)	34.93	0.582	12	22.96	0.843	12
Verification Panel	Negative	CMVAQP02-S09	0	-	-	0	23.89	1.856	12
	Positive	CMVAQP02-S01	50000	31.65	0.399	12	24.57	1.168	12
		CMVAQP02-S02	15000	33.19	0.666	12	24.26	1.116	12
		CMVAQP02-S03	5000	35.20	0.770	12	24.02	1.612	12
		CMVAQP02-S04	1500	34.92	0.803	6	23.95	1.637	12
		CMVAQP02-S05	500	35.84	0.057	2	24.11	1.597	12
		CMVAQP02-S06	150	-	-	0	24.64	1.729	12
		CMVAQP02-S07	50	-	-	0	23.71	1.293	12
		CMVAQP02-S08	15	-	-	0	24.35	1.737	12

Table 4: Out of the 276 Proficiency Panel Samples and DBS Samples, there were 80 true positive, 0 false positive, 192 true negative, and 4 false negative. This results in an accuracy of 98.6%, positive predictive value of 100%, analytical specificity of 100% and analytical sensitivity of 95.2%.

	Lowest CMVAQP Verification Panel CMV concentration detected (IU/mL)	Percent of QAV064127 2020 QCMD Proficiency Panel Samples Correct
1x 3.2 mm DBS punch 50 µL Elution Volume	15000	63%
2x 3.2 mm DBS punches 65 µL Elution Volume	5000	100%

Table 2: 2x 3.2 mm DBS input with 65 µL Elution Volume have a better sensitivity and detection rate than the 1x 3.2 mm DBS input with 50 µL Elution Volume.

Sample Scheme	Input Volume	Extraction Method	CMVAQP Verification Panel CMV concentrations detected (IU/mL)
2x 3.2 mm DBS punches 65 µL Elution Volume	10 µL	Thermal Shock	None
		NeoMDx™ Extraction	50000, 15000, 5000, 500

Table 3: At 10 µL with a 2x 3.2 mm DBS, 65 µL Elution Volume Scheme, Thermal Shock was not able to detect CMV due to inhibitors persisting from extraction method. The NeoMDx™ Extraction Method not only detected CMV but had an increase in detection rate compared to smaller input volumes of same type.

Concentration of CMV in EDX Plasma used as direct input (IU/rxn)	CMV			RPP30		
	Mean	Std Dev	N	Mean	Std Dev	N
100	33.53	0.229	5	39.10	-	1
10	35.76	0.666	5	-	-	0
1	37.77	0.340	2	-	-	0
NTC	-	-	0	-	-	0
Follow-up						
10	34.85	0.428	20	38.88	-	1
3.3	37.97	0.866	19	-	-	0
NTC	-	-	0	-	-	0

Table 5: For the qPCR aspect of the kit, the limit of detection is 3.3 IU/rxn based on direct addition of EDX plasma to the qPCR reaction as the sample input.

Concentration of CMV in DBS from EDX Plasma spike (IU/µL)	CMV			RPP30		
	Mean	Std Dev	N	Mean	Std Dev	N
1000	30.18	0.235	3	25.79	0.055	3
100	33.47	0.367	3	25.35	0.224	3
10	36.73	0.647	3	25.14	0.151	3
1	-	-	0	24.99	0.121	3
0.1	-	-	0	26.62	0.237	3
0	-	-	0	25.43	0.421	3
Follow-up						
10	36.81	0.628	20	25.22	0.281	20

Table 6: For the whole workflow of DBS extraction to qPCR, the limit of detection is 10 IU/µL based on the contrived samples of EDX plasma and human blood for DBS.

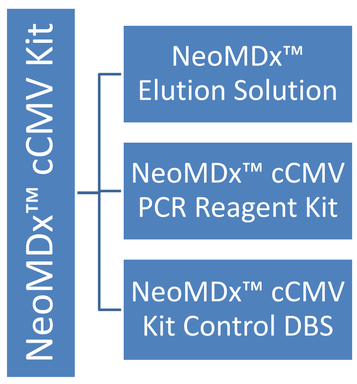

Figure 4: NeoMDx™ cCMV Kit Components

DISCUSSION

- The NeoMDx™ cCMV kit can be used for different throughput labs due to its scalable extraction protocol and 96-well and 384-well compatibility for qPCR. Due to hospitals and testing sites already collecting and testing DBS, it is the easiest sample type to implement for universal screening. Having a scalable 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 test the robustness.

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.

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