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

Methods

Two different manual DBS extraction procedures were tested and compared, the alkaline-based extraction and Thermal Shock. Both methods used 2x 3.2 mm DBS punches and a 65 µL elution volume for comparable 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/SCMA 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 for 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.

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 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/SCMA 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.

**Automated Extraction Workflow Performance**

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**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 used 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.

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. With a high-throughput compatible and sensitive DBS based assay 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.