Abstract No 5110

Development and validation of a liquid biopsy assay with cerebrospinal fluid derived cell free DNA using droplet digital PCR for clinical applications

Dennis O'Rourke, BS[1*], Danyi Wang PhD[1], Zheng Feng PhD, MD[1#]

1. Global Clinical Biomarkers and Companion Diagnostics, Translational Medicine, Global Development, EMD Serono, a business of Merck KGaA, Darmstadt, Germany

*First author, #Corresponding Author

INTRODUCTION

Cerebrospinal fluid (CSF) has been recognized as an important biofluid for biomarker analysis of central nervous system (CNS) tumors due to the proximity to the disease/tumor site as compared to serum or plasma. CNS tumors have significantly enriched tumor-derived cfDNA / circulating tumor DNA (ctDNA) in CSF, however, the absolute quantities are low. In many CNS cases, tissue resection is dangerous or not available for biomarker analysis. Therefore, highly sensitive, liquid biopsy based, targeted methods such as next generation sequencing or digital droplet PCR (ddPCR) are required to efficiently detect clinically actionable mutations in the CSF of patients with CNS tumors.

Recent studies have suggested the feasibility of detecting *H3K27M* mutations in CSF and the potential of these mutations as biomarkers in CNS malignancies. Specifically, mutations in *H3F3A* and *HIST1H3B* have been reported in midline and high-grade gliomas[1]. Furthermore, *H3K27M* mutations in pediatric gliomas can contribute valuable information about the risk stratification of a patient [2].

Here we report the establishment of a protocol for CSF collection, cfDNA extraction and proof of concept study for the analysis of *H3K27* mutations as potential clinical biomarkers for CNS tumors.

METHODS

CSF was collected from healthy donors (n=10) via lumbar puncture. Approximately 2-3mL was obtained per donor. Within 2 hours of the CSF draw the CSF was centrifuged at 850g for 15minutes and if the supernatant was clear collected and stored at -80C until use. If the supernatant was not clear an additional centrifugation step was performed to eliminate any red blood cells.

cfDNA was isolated from approximately 2-3mL CSF from each donor using the Qiagen Circulating Nucleic Acid kit and quantified using the Qubit High Sensitivity DNA kit. Five normal donors were first tested for cfDNA recovery from CSF and then the remaining five were pooled and tested via a spike-in recovery experiment of *KRAS* G12D mutation with a ddPCR assay.

To specifically target use in CNS tumors, ddPCR assays for *H3F3A* p.K27M mutations were designed, and assay characteristics were captured.

RESULTS

Table 1: Patient information for 10 healthy donors of cerebrospinal fluid

Sample ID	Age	Sex	Race
USCBD_CSF_0001	74	Female	Caucasian
USCBD_CSF_0002	70	Female	Caucasian
USCBD_CSF_0003	61	Female	Caucasian
USCBD_CSF_0004	65	Female	Caucasian
USCBD_CSF_0005	62	Female	Caucasian
USCBD_CSF_0006	73	Female	Caucasian
USCBD_CSF_0007	48	Male	Caucasian
USCBD_CSF_0008	35	Male	Caucasian
USCBD_CSF_0009	70	Male	Caucasian
USCBD_CSF_0010	53	Female	Caucasian

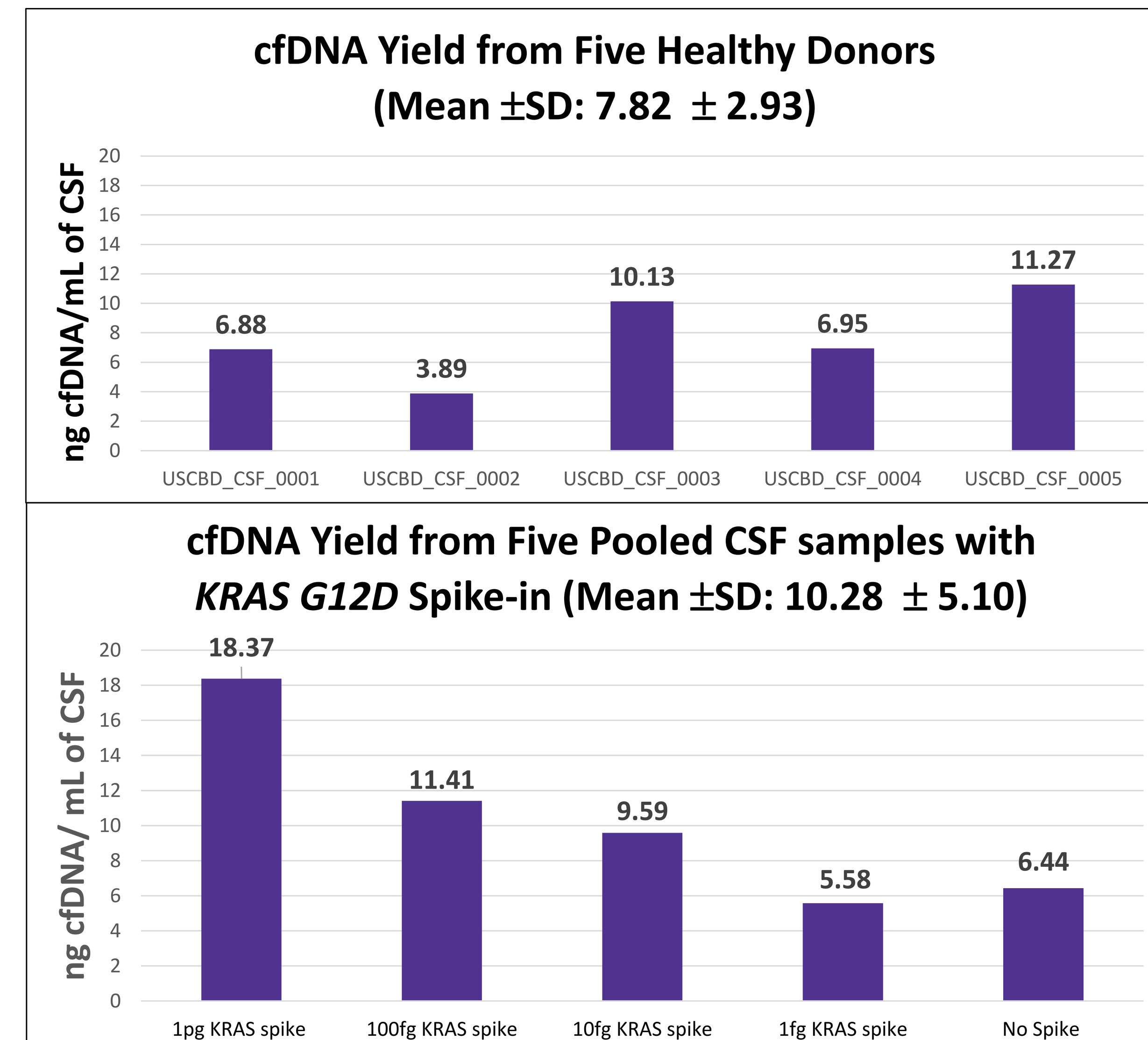

Figure 1: cfDNA yield per mL of CSF consumed. cfDNA was extracted using the Qiagen Circulating Nucleic Acid Kit and quantified using the Qubit dsDNA High Sensitivity Kit. Top: Five healthy CSF samples tested individually. Bottom: Five different CSF samples pooled with *KRAS* G12D DNA spiked-in

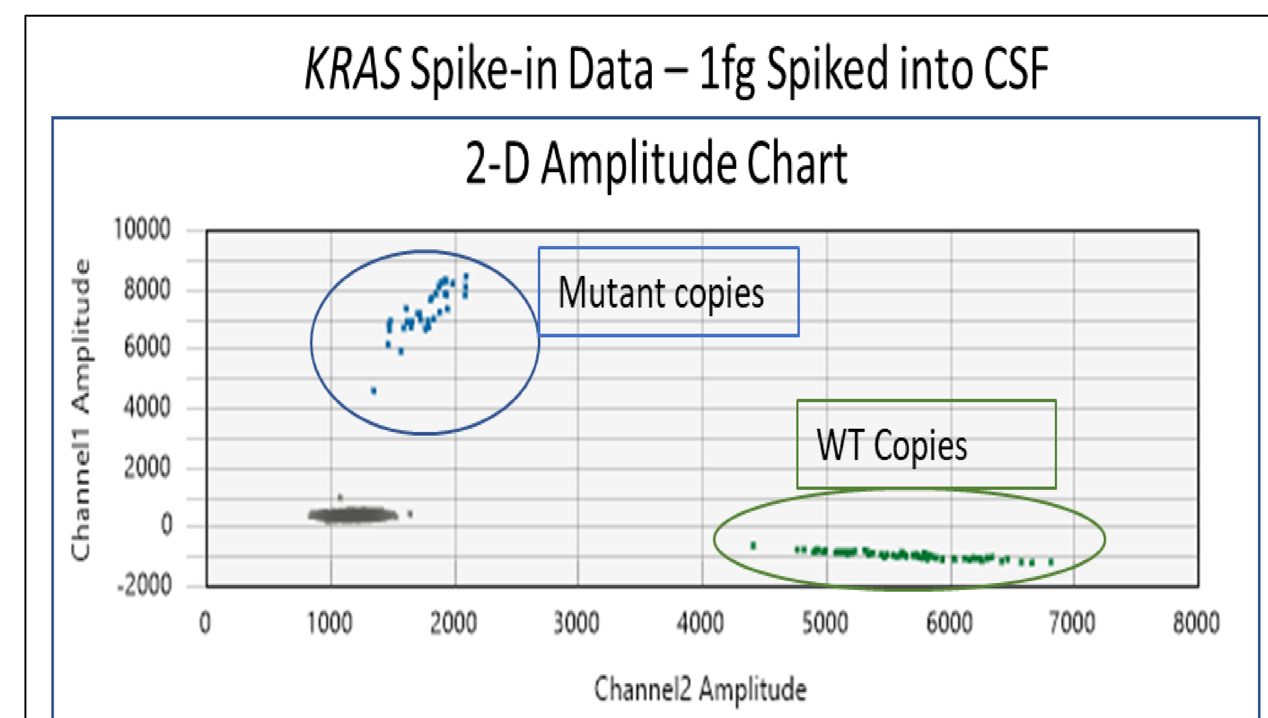

Figure 2: *KRAS G12D* synthetic DNA was spiked into pooled healthy patient CSF extracted with the cfDNA and measured. As low as approximately 40 copies of *KRAS G12D* DNA were spiked into the CSF. In those samples an average of 27 copies was recovered (67.5%) which corresponded to approximately 11 positive droplets. This demonstrated the ability to recover low levels of target mutations from CSF material using ddPCR.

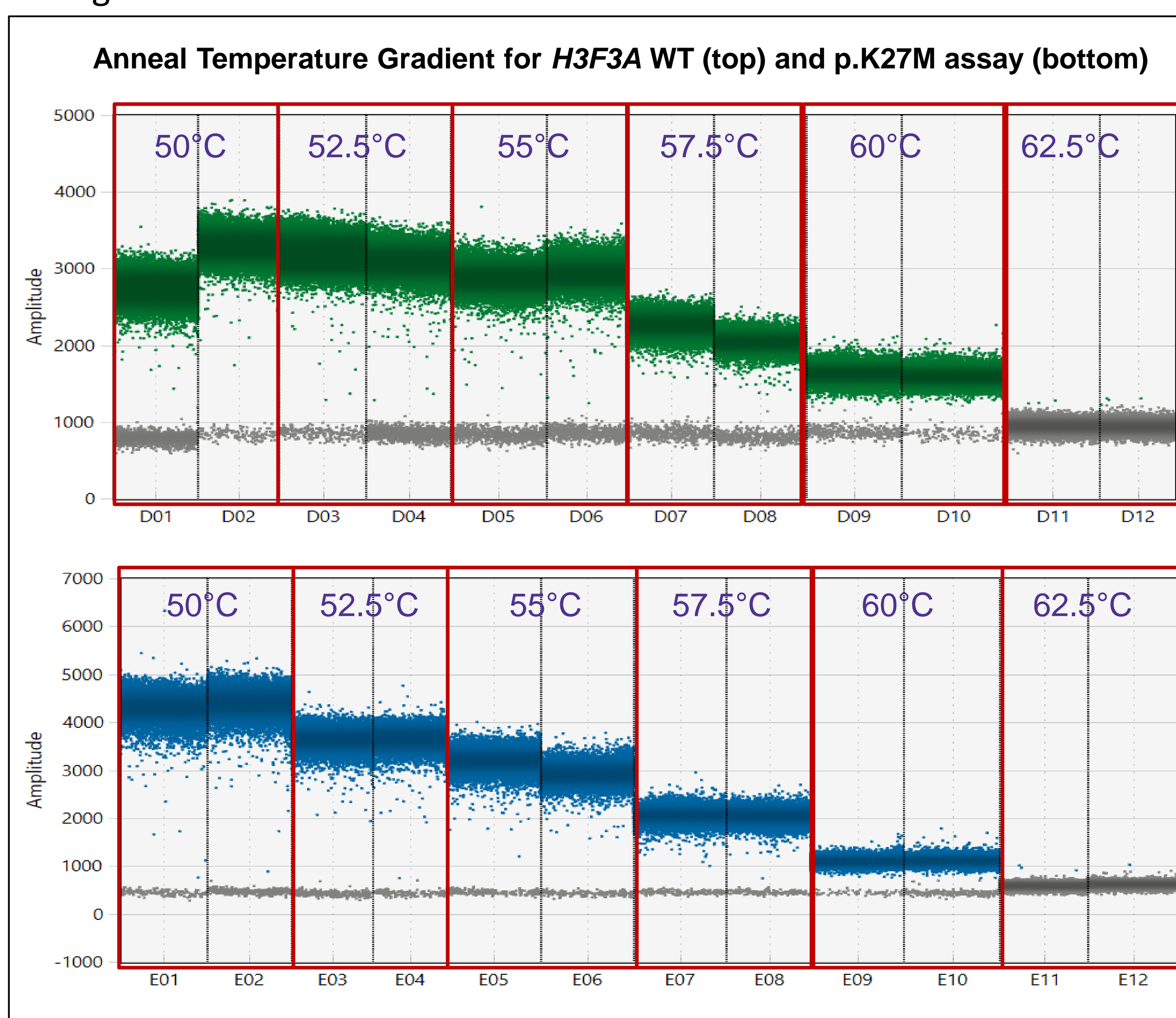

Figure 3: An anneal temperature gradient from 50°C to 62.5 °C was run to determine the optimal anneal temperature for the assay. There was clear decrease in separation between positive and negative droplet population above 55°C. Moving forward 52.5°C was used as the optimal annealing temperature.

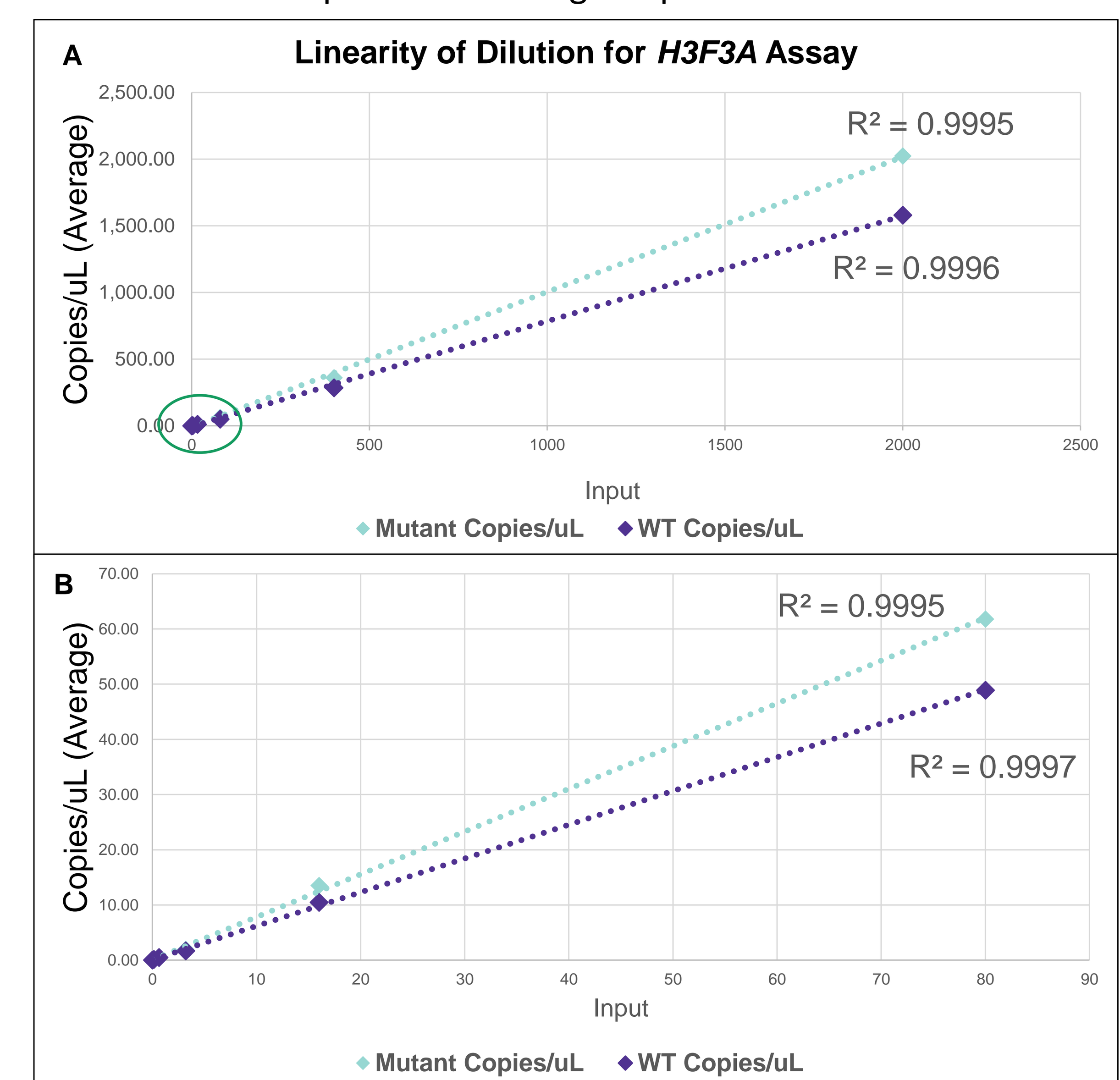

Figure 4: Linearity of our *H3F3A* p.K27M mutation detection ddPCR assay. A seven point, 1:5 titration of a 50:50 mix of WT and Mutant DNA was run in triplicate. **A:** All seven points including a No Template Control. **B:** A zoomed in view of lower 5 points (circled in Figure 4A) of the dilution curve.

Table 2: Assay performance using synthetic DNA. Further assessment is needed using biological samples to validate the assay performance characteristics displayed here.

Lower limit of Detection	~2 copies/uL
CV at Lower limit of Detection	22%
Upper limit of detection	>2,000 copies/uL
CV at upper limit of detection	1.9%
Limit of Blank	0.02 copies/uL

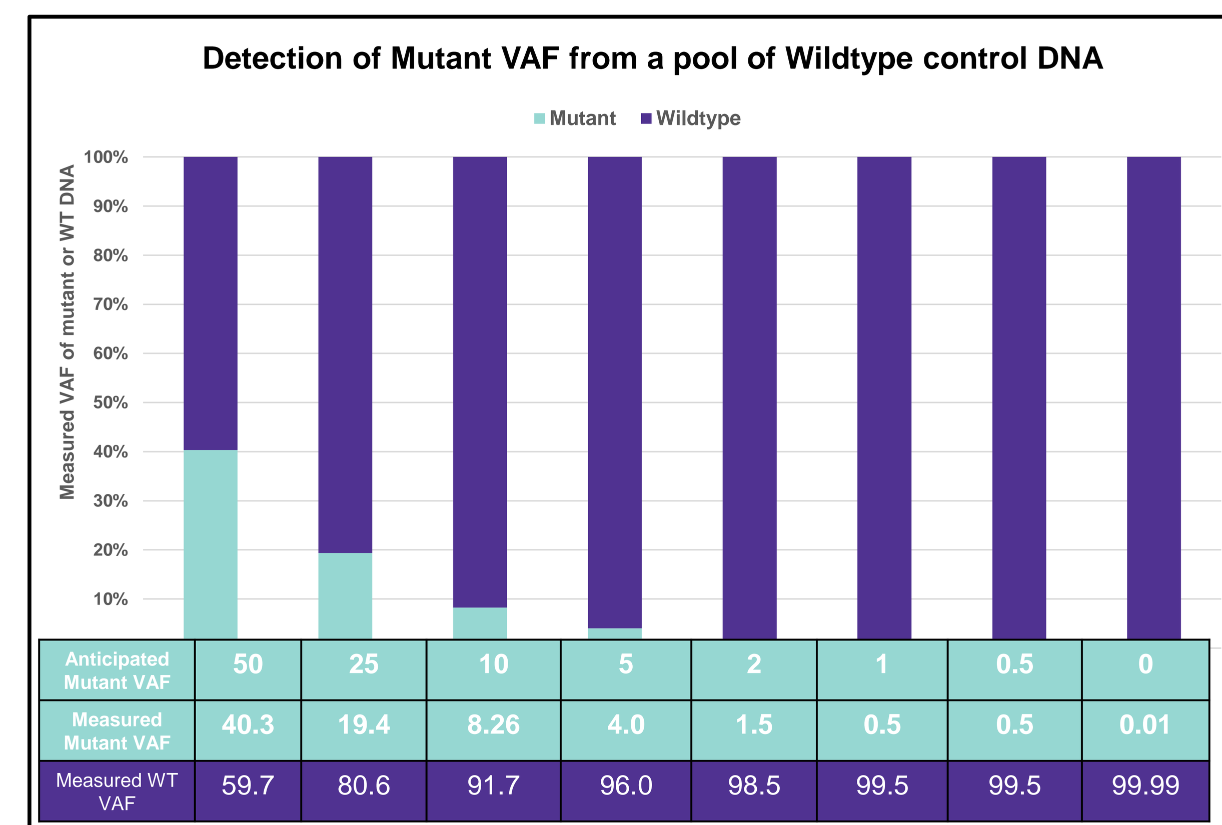

Figure 5: A range of *H3F3A* p.K27M control DNA were mixed with a pool of wildtype *H3F3A* DNA. Mutant VAFs were measured. Results indicate that our assay is sensitive to detect as low as 2% VAF.

CONCLUSIONS

- Circulating free DNA was successfully isolated from CSF samples collected from healthy volunteers. The low concentration of cfDNA from extraction emphasizes the need for high-sensitive testing methods using CSF.
- ddPCR offers a sensitive method of mutation detection, gene expression, epigenetic analysis etc., which could be applicable for a targeted analysis of biomarkers in CSF in a clinical setting
- We developed a ddPCR assay for detection of *H3F3A p.K27M* in CSF material.
- While the assay was characterized using synthetic DNA sequences, biological samples still need to be tested to assess the assay in real world scenarios, which is planned.

REFERENCES

1. Gielen GH, Gessi M, Hammes J, Kramm CM, Waha A, Pietsch T (2013) H3F3A K27M mutation in pediatric CNS tumors: a marker for diffuse high-grade astrocytomas. Am J Clin Pathol 139:345–349
2. Mosaab, A., El-Ayadi, M., Khorshed, E.N. *et al.* Histone H3K27M Mutation Overrides Histological Grading in Pediatric Gliomas. *Sci Rep* **10**, 8368 (2020). https://doi.org/10.1038/s41598-020-65272-x

DISCLOSURES

This study was funded fully by EMD Serono Inc., a business of Merck KGaA, Darmstadt Germany.

The authors are full time employees of EMD Serono Inc., a business of Merck KGaA, Darmstadt Germany.