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Title:

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Date:

2021-10-01

Citation:

Ye, Z., Scheffer, I. E., Berkovic, S. F. & Hildebrand, M. S. (2021). Improving Specificity of Cerebrospinal Fluid Liquid Biopsy for Genetic Testing. *Annals of Neurology*, 90 (4), pp.693-694. <https://doi.org/10.1002/ana.26191>.

Persistent Link:

<https://hdl.handle.net/11343/298850>

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Improving Specificity of CSF Liquid Biopsy for Genetic Testing

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To the Editor:

We recently published the first study of CSF liquid biopsy for epilepsy.¹ We were delighted to see this approach replicated and independently validated with detection of brain somatic mutations in CSF cell-free DNA (cfDNA) from patients with drug-resistant focal epilepsy by Kim et al.² While the results were similar, there were some key differences in the protocols used which influence the specificity of this approach essential for clinical implementation of CSF liquid biopsy for etiological diagnosis.

Kim et al² collected 1-6 mL CSF from patients with epilepsy or controls. Samples were divided into 1mL aliquots and cfDNA extracted from each aliquot. Qubit assays were used to determine cfDNA concentration. Due to the limited cfDNA obtained from aliquots, pre-amplification was performed using 13 PCR cycles prior to detection of variants in 3/12 patients using droplet digital PCR (ddPCR).

In our study¹, 0.25-10.5 mL CSF was collected from patients with epilepsy or controls, and absolute cfDNA concentration was measured by ddPCR. We tested 10 µL of neat CSF cfDNA directly in our ddPCR assays without pre-amplification, detecting variants in 3/3 patients.

Despite the different quantitation methods, total CSF cfDNA concentration was comparable between the two studies. The main difference was that Kim et al² aliquoted CSF and extracted

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ana.26191

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cfDNA from each aliquot, meaning pre-amplification was required. While pre-amplification increases the DNA input for ddPCR, it can also lead to allele bias³ and introduction of false-positives⁴. Notably, Kim et al² detected false-positives in almost all negative controls. Indeed, for the *BRAF* V600E assay, the negative controls had significantly higher variant allele frequency (VAF) due to artefact, than their patient KR-6.² In contrast, our patient with a ganglioglioma had not had prior genetic testing; we detected the pathogenic *BRAF* V600E variant in CSF cfDNA¹, highlighting the clinical utility of testing CSF for somatic variants.

To verify that false-positive artefacts arise from pre-amplification, we tested our *LISI* K64X ddPCR assay¹ on 10 control CSF cfDNA samples with and without pre-amplification (Table 1). We confirmed that pre-amplification introduced false-positive artefacts. A clean-up step after pre-amplification reduced, but did not eliminate, false-positives.

To reduce false-positive results and maximize specificity for diagnostic testing pre-amplification should be avoided. With low cfDNA sample input, results need to be interpreted with extreme caution. (372/400 words)

Potential Conflicts of Interest

The authors declare no conflicts of interest.

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Table 1. Comparison of Different ddPCR Protocols for Mosaic Variant Detection in CSF cfDNA

Sample ID	Standard ddPCR [#]		Pre-Amplified ddPCR [*]		Modified Pre-Amplified ddPCR ^{**}	
	VAF	MUT copies/WT copies	VAF	MUT copies/WT copies	VAF	MUT copies/WT copies
Control-1	0	0/6.61	0.0961%	9.67/10047	0	0/10526
Control-2	0	0/43.7	0.00595%	6.55/110201	0.00323%	1.32/40796
Control-3	0	0/2.07	0	0/11431	0	0/2381
Control-4	0	0/12.9	0.00503%	1.53/30446	0	0/16216
Control-5	0	0/4.57	0	0/5578	0	0/10951
Control-6	0	0/5.61	0	0/6382	0	0/8160
Control-7	0	0/7.25	0.0112%	2.51/22433	0	0/10892
Control-8	0	0/4.49	0.0258%	1.47/5692	0	0/5751
Control-9	0	0/16.8	0	0/10852	0	0/15560
Control-10	0	0/2.67	0	0/7297	0	0/6130
Patient 1[^]	9.95%	35.7/324	-	-	6.9%	3936/53077
Patient 1[^]	8.84%	36.4/375	-	-	6.87%	3967/53762

[#]**Standard ddPCR:** 10µl CSF cell-free DNA directly input for ddPCR, no pre-amplification

^{*}**Pre-Amplified ddPCR:** 15-cycle pre-amplification using ddPCR primers, then 10µl product input for ddPCR

^{**}**Modified Pre-Amplified ddPCR:** 15-cycle pre-amplification using ddPCR primers followed by a clean-up step using Zymo Research DNA Clean & Concentrator-5 (CA USA) with 25ul elution, then 10µl of the elution used as input for ddPCR

[^]**Patient 1** with *LIS1* p.K64X variant from our previous study (Ye et al. Brain Commun. 2021), same sample tested in duplicate

VAF: variant allele frequency; **MUT copies:** mutant copies/20µl ddPCR reaction; **WT copies:** Wild-type copies/20µl ddPCR reaction