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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 al[2] 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 study[1], 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 al[2] aliquoted CSF and extracted

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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 bias[3] and introduction of false-positives[4]. Notably, Kim et al[2] 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 cfDNA[1], highlighting the clinical utility of testing CSF for somatic variants.

To verify that false-positive artefacts arise from pre-amplification, we tested our *LIS1* K64X ddPCR assay[1] 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-fee 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