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Quality Materials for Quality Assurance in the Analysis of Liquid Biopsy Samples

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The analysis of circulating cell-free DNA for the detection of cancer-associated genetic and genomic changes, frequently called liquid biopsy, has become an important tool for the management of cancer patients. For example, the analysis of epidermal growth factor receptor (*EGFR*) mutations in the plasma of cancer patients suffering from non-small cell lung cancers has been rapidly adopted as a routine clinical service for guiding the use of EGFR tyrosine kinase inhibitors in many countries *(1)*. The noninvasive nature of liquid biopsy, as well as its rapid turnaround time, makes it a favorable choice for patients over tissue biopsy. In some patients, the *EGFR* mutation status can be determined by circulating tumoral DNA analysis before imaging-guided tumor biopsies are performed. Recently, whole-exome and targeted sequencing of cancer-associated genes from plasma DNA have also been performed in patients with advanced cancer to search for cancer-associated alterations that are potential therapeutic targets and to detect disease recurrence *(2, 3)*. With the increasing utilization of liquid biopsies, there is a growing demand for quality control materials for circulating tumoral DNA analysis for platform comparison, assay development, internal quality control, and proficiency testing. However, the production of good-quality control materials that biologically resemble authentic circulating DNA in cancer patients is challenging and was the subject of a study by Zhang and colleagues reported in this issue of *Clinical Chemistry (4)*.

Circulating cell-free DNA exhibits a characteristic nucleosomal size profile predominantly comprising DNA fragments approximately 166 bp in length *(5)*; tumor-derived cell-free DNA fragments are shorter than those derived from nontumoral-derived cell-free DNA *(6)*. The fragmentation pattern of cell-free DNA is nonrandom and is related to nucleosome positioning *(7, 8)*. This property has important implications on the performance of different analytical platforms in detecting cell-free tumoral DNA. For example, locus-specific PCR assays would favor the amplification of longer DNA fragments over shorter ones and would be affected by the availability of primer annealing sites on the DNA fragments, whereas adaptor ligation-based methods commonly used in next-generation sequencing platforms would have preference toward shorter fragments. Therefore, quality control materials with similar fragment size distributions as the circulating DNA, in particular for tumor-derived DNA, would be required to reflect the analytical characteristics of different detection approaches. Good-quality control materials should also cover a wide spectrum of clinically relevant genetic alterations ranging from single-nucleotide alterations, small indels, and large copy number alterations of deletion and amplification to structural changes like chromosomal translocation. The genetic background of the cell-free DNA also should be completely defined and isogenic to the mutant DNA. These characteristics would be important in the assessment of variant identification performance of the bioinformatics pipeline in whole-exome sequencing-based assays. Crucially, the total cell-free DNA amount and the allelic fraction of the mutations in the quality control material should be quantitatively defined and representative of different disease stages. For a comprehensive quality assessment of the whole laboratory workflow, differences between sample matrix composition in quality control materials and authentic patient plasma should also be minimized. The idea of cell-free tumoral DNA circulating as a simple mixture of duplex noninteracting DNA molecules is oversimplified. These species can associate with nucleosome proteins and immunoglobulins and exhibit marked differences in stability and configuration in different bodily fluid compartments *(9)*. Preanalytical processing can significantly impact the performance of the analytical system *(10)*. Although plasma samples collected from cancer patients would be the ideal source of quality control materials in terms of biochemical properties, it is impractical to obtain a sufficient volume of plasma from one or a few cancer patients for quality assurance programs involving a large number of laboratories. Furthermore, the absolute or fractional concentrations of mutations in the plasma samples cannot be easily adjusted to test the performance of the

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assays at clinically critical levels, for example, at the limit of detection.

In the study by Zhang and colleagues in the current issue, the authors developed a set of synthetic cell-free DNA quality control materials that mimicked the plasma DNA of cancer patients *(4)*. Using site-directed mutagenesis and the CRISPR-associated protein 9 (CRISPR/Cas9) system, they engineered DNA fragments carrying a variety of clinically important alterations, including the *EGFR* T790M, L858R and exon 19 deletion, *KRAS* G12D mutation, and more complex *EML4-ALK* rearrangements and mixed them with micrococcal nuclease-digested HEK293 cell-line DNA to obtain DNA samples with varying allelic fractions of mutant DNA. Interestingly, the size profile of the micrococcal nuclease-digested genomic DNA was found to be shorter than that of authentic plasma DNA from normal and early breast cancer patients, but it resembled the core nucleosomic pattern previously reported in tumor-derived cell-free DNA *(6, 11)*.

Zhang and colleagues distributed these synthetic DNA samples containing cancer-associated alterations at different fractional concentrations to 11 laboratories for proficiency testing, covering massively parallel sequencing (MPS)-based, digital PCR, and amplification refractory mutation system (ARMS)-based methods. No 2 laboratories had identical workflows and informatics pipelines. This study demonstrated that most laboratories were able to accurately detect oncogenic alterations present at around 1% allele frequency in the synthetic quality control materials, regardless of methodology. Through the analysis of these quality control samples, 1 laboratory was identified to have a high proportion of false-positive and false-negative results. The incorrect results were reported by methods based on both NGS and ARMS, suggesting that the assays used by this laboratory would need further optimization and validation before being provided as a clinical service.

The synthetic DNA quality control materials developed by Zhang and colleagues cover a wide range of oncogenic alterations ranging from single-nucleotide changes to large rearrangements *(4)*. The use of genetically defined cell lines also allows scalable manufacturing of the quality control materials. With the increasing accessibility of genomic engineering technology, it can be envisioned that more complex alterations can be generated to cope with future clinical needs and to fully mimic the diverse genomic aberrations in cancer. The use of micrococcal nuclease digestion in DNA fragmentation may be more biologically relevant than ultrasonic shearing because plasma DNA exhibits nonrandom fragmentation patterns related to the positioning of nucleosomes *(7, 8)*. The additional benefits such as minimization of platform-specific bias and suitability in nonsequence composition-based cell-free tumoral DNA analysis require further studies. Nevertheless, the work of Zhang and colleagues will add to the ongoing efforts to develop robust and commutable quality control materials by commercial and quality assurance program providers. Such materials will be critical for ensuring laboratory quality and patient safety.

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