B49 Optimization of a Droplet Digital™ Polymerase Chain Reaction (ddPCR™) Assay for the Quantitative and Qualitative Analysis of Illumina® MiSeq® Massively Parallel Sequencing (MPS) Libraries

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After attending this presentation, attendees will better understand ddPCR™, a cutting-edge technique used to quantify nucleic acids. Focus will be placed on the use of ddPCR™ to accurately quantify prepared MPS libraries. Additionally, the use of ddPCR™ as a way to determine the average length of fragments in an MPS library will be discussed.

This presentation will impact the forensic science community by describing a newly optimized method that enables laboratories to fully assess prepared MPS libraries. This level of library assessment results in the generation of large amounts of high-quality data without sample or sequencing run failures. Furthermore, the assay described has been optimized to reduce the potential for contamination.

MPS methods are quickly being adopted by the forensic community for analysis of precious evidentiary samples. These methods are capable of generating an unprecedented amount of data, particularly when analysis is performed using commercially available highly multiplexed panels designed to target hundreds of loci per amplification. Accurate qualitative and quantitative assessment of prepared MPS libraries is of paramount importance for obtaining maximum yield of high-quality data from a sequencing run. Many vendor-recommended protocols suggest assessment of the final library using fluorometric methods, agarose gel or chip-based electrophoresis, or quantitative PCR (qPCR); however, these methods can be problematic because they often: (1) result in over- or underestimation of library DNA concentrations; (2) do not enable estimation of the size of DNA fragments in the library, which can lead to incompatible kit selection; and, (3) are not typically specific for those fragments that are MPS-ready.

A ddPCR™ is similar to qPCR in that target-specific primers and 5' nuclease probes are utilized for detection following an end-point PCR reaction; however, due to the nature of the method, no standard curve is needed for estimation of DNA concentration. With ddPCR™, a 20µL aqueous PCR reaction is emulsified into 1nL uniformly sized droplets. Each droplet is then counted as fluorescence positive or negative and a Poisson correction is applied to estimate the starting copy number of DNA fragments in the sample. In addition, previous literature has shown that droplet florescence intensity is dependent upon the average length of the fragments being assessed.[1] Longer fragments tend to result in lower droplet fluorescence intensities than shorter fragments due to the kinetics and stoichiometry of the PCR reaction. As a result of this observed fluorescence intensity:fragment-size correlation, Laurie et al. have developed a ddPCR™ assay that allows for simultaneous quantitative and qualitative assessment of MPS libraries.[1] The assay specifically targets MPS platform-specific library modifications (i.e., flow cell adapter sequences) to enable quantification of only those fragments that are sequenceable. The assay also includes the use of a series of size standards to facilitate estimation of average length of fragments in the prepared library; however, the standards are derived from a commercially available agarose gel electrophoresis ladder. The reported preparation is time-consuming, labor intensive, and may give rise to low-level contamination evident in MPS data.

This presentation reports an optimization of the aforementioned assay using synthetically prepared size standards. Initially, a series of oligonucleotides with known sizes ranging from 25bp to 700bp was designed to consist of PhiX

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DNA with Illumina® MiSeq® sequencing primer flanking regions. The oligonucleotides were designed using PhiX to reduce possible run contamination from exogenous sources. PhiX is supplied for use as an MPS control, and data generated from any part of the PhiX genome is easily identified and bioinformatically filtered from raw data. The sequencing primer region serves as a primer binding site to allow for additional incorporation of barcoding indices and flow cell adapters into the synthetic oligonucleotide during the limited-cycle PCR step. Final products are then normalized for reaction input of 10,000 copies to avoid fluorescence intensity variability due to copy number and not length. Average observed droplet fluorescence intensities range from 13,157RFU (+/- 203.3) for the 25bp standard to 3,860.4RFU (+/-352.3) for the 700bp standard. The standard series appears to be efficient at predicting the average size of Illumina® MiSeq® libraries while avoiding quantification of adapter dimers and other artifacts often generated during library preparation. This increases first-pass Illumina® MiSeq® run success.

Reference(s):

1. Laurie M.T., Bertour J.A., Taylor S.D., Burton J.N., Shendure J.A., Bielas J.H. Simultaneous digital quatification and fluorescence-based size characterization of massively parallel sequencing libraries. *Biotechniques* 2013; 55:61-67.

Quantitative PCR, Droplet Digital™ PCR, Massively Parallel Sequencing

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