Library Prep Method Melds DNA Quantification and Size Selection into Single Step with Digital PCR

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Premium

A Seattle team has come up with a scheme that harnesses droplet digital PCR technology during next-generation sequencing library preparation, making it possible to measure DNA quantity and amplicon size in a single step.

The researchers outlined the rationale behind this approach, which they called QuantiSize, in the August issue of *Biotechniques*. There, they demonstrated the potential of using QuantiSize — done with the help of Bio-Rad's QX100 ddPCR system — to simultaneously see DNA quantity and insert sizes while preparing libraries for Illumina MiSeq sequencing.

"We're very happy with it currently," the Fred Hutchinson Cancer Research Center's Jason Bielas, senior author on the study, told *In Sequence*, noting that he and his colleagues rarely have failed sequencing runs due to DNA over- or under-loading since they began using QuantiSize.

The approach relies on a relationship between amplicon size and fluorescence amplitude displayed by ddPCR droplets, allowing precise DNA quantification and amplicon size information during library preparation in one rather than two steps.

"By exploiting a correlation we have discovered between droplet fluorescence and amplicon size, we achieve the joint quantification and size determination of target DNA with a single ddPCR assay," Bielas and his colleagues wrote.

This past April, Bio-Rad introduced a ddPCR-based library quantification kit for use with Illumina's TruSeq library preparation protocol. Along with DNA quantification, that kit uses fluorescence data as a means of gauging both amplicon size and quality, Bio-Rad representatives noted.

The ability to perform an optimal sequencing run on most high-throughput sequencing platforms hinges on the ability to control DNA quantity in a given sequencing library, Bielas and his co-authors noted in *Biotechniques*. And that, in turn, requires methods for quantifying DNA overall and selecting appropriate insert sizes.

"Performing a sequencing run with either too many or too few library molecules results in compromised data yields or completely failed sequencing runs that waste sample, expensive

reagents, user time, and instrument time," they wrote. "Similarly, if library molecules are not the appropriate length to fully utilize the capabilities of the sequencing platform, fewer bases can be sequenced in [a next-generation sequencing] run and the throughput is wasted."

But upon embarking on next-generation sequencing experiments, the team discovered that these properties are usually determined in independent steps that are not always especially accurate.

Methods such as real-time qPCR and gel or capillary electrophoresis have made their way into standard library preparation protocols for quantifying DNA and selecting appropriate DNA sizes, respectively, the researchers noted. Even so, they argued that each approach has potential drawbacks on the efficiency or accuracy side.

In the case of qPCR, for instance, DNA may be amplified differently depending on the size of the template on hand and its sequence composition. Alternative approaches for characterizing library DNA such as UV spectrometry, the Quant-iT PicoGreen assay from Invitrogen, or Agilent's BioAnalyzer assay are useful but may have limited accuracy, the authors pointed out, since they don't distinguish between amplified DNA and non-amplified molecules.

In an attempt to rein in such potential sources of inaccuracy while performing DNA quantification and size assessments in a single step, the researchers decided to try using ddPCR during the next-gen sequencing library preparation process.

This application of the ddPCR technology, which eventually became the QuantiSize assay, stemmed from the realization that the fluorescence amplitude of droplets formed during ddPCR increased as the length of the amplicons decreased and vice versa.

The group got the first hints of this relationship while performing experiments for another study published recently in *Aging Cell* that used ddPCR as part of an assay for finding DNA deletions.

As they began preparing next-generation sequencing libraries for other experiments, the researchers suspected that the relationship between droplet fluorescence and amplicon size might be useful for streamlining sample prep steps, Bielas noted.

"We thought we could apply this correlation that we discovered between the size of the amplicon that had been amplified and the fluorescence to determine the relative size of our libraries for next-generation sequencing," he said. "And given that digital PCR is more accurate and sensitive than quantitative PCR, we thought we could do everything in one single step — not only to determine how many molecules were to be sequenced, but also the relative size of them."

For the proof-of-principle study, the researchers focused on the potential of using QuantiSize during Illumina sequencing library preparation with the firm's Nextera and Nextera XT kits, starting with libraries prepared with snippets of DNA with known sizes ranging from a couple dozen bases to 1,000 bases. For the Nextera XT kit, they noted, QuantiSize replaced an optional bead normalization step included in the sample prep protocol.

Along with QuantiSize libraries prepared with known DNA size standards, they also took a crack at using the approach for DNA quantification and amplicon size selection during library preparation using genomic DNA from a cell line generated from human colon cancer.

In addition to confirming the linear relationship between increasing amplicon size and diminishing mean fluorescence amplitude in the ddPCR droplets, the researchers examined how the slope of

the line linking these properties shifted depending on the ddPCR cycling conditions — in particular, the elongation time.

In general, the team found that the PCR cycling conditions contributed to how well the approach can resolve amplicons of different sizes, while the ability to count DNA molecules is more or less independent of those conditions.

Once the relationship is worked out between fluorescence and amplicon size for given conditions, it's possible to generalize this relationship to determine the size of any target amplified under the same conditions, Bielas said.

Indeed, with the help of QuantiSize data, he and his colleagues demonstrated that they could accurately predict the number of reads that can be generated for a given MiSeq library. The size range of amplicons predicted by QuantiSize more or less mirrored that determined by direct sequencing, they reported, albeit with a slightly broader distribution.

The approach presented in *Biotechniques* highlights the digital data and precision provided by ddPCR, as well as the analog information that can be gleaned from it — in this case the amplicon size estimates made using fluorescence amplitude profiles, George Karlin-Neumann, director of scientific affairs for Bio-Rad's Digital Biology Center, told *IS*.

The kit that Bio-Rad released this spring for doing DNA quantification, quality, and size assessments with ddPCR during Illumina TruSeq sample preparation protocol also relies on the relationship between fluorescence amplitude and amplicon size, Karlin-Neumann said. But he noted that there are slight differences in the company's approach to doing DNA quantification and size assessments during next-gen library preparation.

In particular, the Bio-Rad method for Illumina TruSeq libraries includes two colored probes, each targeting an opposite end of the library insert-containing cassette. Those probes make it possible to look at whether an insert is flanked by both adaptor types, providing information on a library's quality as well as DNA quantity and insert size.

"You can also get a quality measure as to whether you actually have malformed products in your library that aren't going to sequence well," Karlin-Neumann noted.

"A well-constructed library molecule should have [both adaptors]," he explained. "If it doesn't, it's not going to form clusters on the sequencing cell, so it's good to know about that."

The Bio-Rad kit is designed to be used with the company's QX100 and QX200 ddPCR systems and contains probes that target sequences found specifically in adaptors used for Illumina TruSeq sample prep methods.

The firm is in the process of developing a similar kit that's compatible with the adaptors currently used to prepare Ion Torrent sequencing libraries and is considering the possibility of developing kits that are compatible with library prep protocols for other instruments and chemistries in the future.

The QuantiSize assay described by Bielas and colleagues in the *Biotechniques* paper is expected to be applicable to sample prep steps used for other sequencing instruments too, though that is not something the team has tried so far.

"All the methodology would be the same, you would just need a different primer set," Bielas said.

"The primers would have to anneal to the sequencing adaptor and you could use a TaqMan probe as well within that region."

Though the researchers used DNA in these droplets for subsequent steps of the experiments done for the *Aging Cell* study, the QuantiSize method does not currently involve taking the ddPCR droplets forward for later stages of the library preparation.

"We only used the data for counts to optimize the reaction — we didn't break them and sequence anything in the droplets — just to get an idea of how many templates to put down on the sequencing machine," Bielas said.

"This is just a way to characterize your library," he explained. "So the idea is that you make multiple libraries and choose the one that's appropriate, in terms of length and such."

His lab is not currently collaborating with Bio-Rad to commercialize any methods related to QuantiSize applications of the company's ddPCR system, though he noted that the Fred Hutchinson Cancer Research Center holds intellectual property related to the QuantiSize assay.

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