APPLICATION NOTE No. 375 I December 2016

Eppendorf twin.tec® PCR Plates 96 LoBind Increase Yield of Transcript Species and Number of Reads of NGS Libraries

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Abstract

Sequencing is an increasingly important technique in life sciences. Multiple sequencing strategies exist, but most of them require high sample quality and, often, a large amount of starting material. Currently the most sophisticated method is Next Generation Sequencing (NGS).Due to its complexity, many factors may interfere with the procedure and impair the quality as well as quantity of the final library. Amongst the multitude of these factors, consumable selection is often overlooked. We compared Eppendorf twin.tec PCR plate LoBind 96 (Eppendorf LoBind®) with low binding characteristics and Eppendorf twin.tec PCR plate 96 (standard) for their sequencing read depth and number of transcript species. To demonstrate the advantage of Eppendorf LoBind consumables, two NGS libraries (10 and 50 ng total RNA input) were prepared in both plate types, sequenced, and the data were compared. It was shown that samples processed in Eppendorf LoBind plates produced a higher average number of reads, as well as broader variation of transcript species. NGS libraries of low quality and low starting material should therefore benefit even more from using the Eppendorf LoBind PCR plates.

Introduction

Nowadays, sequencing is one of the most important technologies in almost every research field. A number of approaches exist and the classic Sanger chain termination method invented in 1977 was further developed over the past decades. Amongst these methods, NGS is the most sophisticated, which offers the possibility to sequence up to 1.8 terabases in one sequencing run [1]. Preparing a library for NGS can be a long, multi-step process. Typically, four major steps are involved. First, the DNA sample is fragmented. Secondly, the broken DNA ends are enzymatically repaired for further modification. The third step includes attaching oligonucleotides to both ends of the fragments to allow sample distinction and universal amplification. The fourth step is the enrichment PCR to increase the amount of the library. Quality of a library can be assessed by many metrics after a sequencing run. A commonly-used indice is the number of reads, which should be as high as possible (with controlled cluster density) to increase the chance of capturing all variants. Another indice is the number of various transcript species (in the case of RNA sequencing), which can be used to gauge the faithfulness of sequencing data.

Another important element for successful sequencing is sample quality. Ideally samples are supplied at high purity and quantity. But in reality, samples derived from ending sources such as FFPE or forensic samples can be very limited and poor in quality. One of the methods to treat these samples is Massive Analysis of cDNA Ends (MACE) [2]. In MACE, only one read per transcript molecule is sequenced. So short and rare transcripts can be identified at 10-20 times lower sequencing depth when compared to full length RNA-Seq. Moreover, no length-based normalization is required [3]. Therefore, this technique can be used to process challenging samples for NGS sequencing. The analysis method TrueQuant offers the possibility to clean sequencing data from sequencing artifacts and low quality reads. When it comes to sensitive samples with low input, high-quality cluster generation and good sequencing depth and coverage are essential to data fidelity. So improving the quality of critical aspects, including purity, number of reads, and preservation of transcript variants can all lead to better sequencing results.

One way to ensure high sample quality of a NGS library is using special consumables that protect the DNA. Multiple studies have found that standard laboratory polypropylene consumables may lead to DNA denaturation, multimerization, as well as increased binding of short DNA fragments to the plastic surface [4, 5]. Additionally, substances leached from the plastics can interfere with the sample and inhibit binding of receptors, increase photometric readings, and alter the activity of enzymes [6]. Using plastics that do not affect DNA, such as polyallomers, for the production of laboratory consumables is one approach to answer those challenges [4]. To demonstrate the effectiveness of the low-binding feature for NGS workflows, we used Eppendorf twin.tec PCR plate 96 LoBind (Eppendorf LoBind) with low binding characteristics and Eppendorf twin.tec PCR plate 96 (standard) for the preparation of NGS libraries. Then we compared their performance by number of reads from sequencing, and variation of transcript species.

Materials and Methods

Comparison of qPCR efficacy in Eppendorf LoBind and standard PCR plates

A general comparison of qPCR efficiency between Eppendorf twin.tec PCR plate 96 LoBind, semi-skirted (Eppendorf LoBind; Lot. No: E161876M; Eppendorf AG, Hamburg, Germany) and Eppendorf twin.tec PCR plate 96, semi-skirted (standard; Eppendorf AG, Hamburg, Germany) was done to ensure that the LoBind material does not interfere with the PCR reaction. HOT MOLPol EvaGreen® qPCR Mix Plus (projodis GmbH, Butzbach, Germany) was used in 20 µL reaction volume. The template used was 1 µL of a pooled NGS library (0.1 ng/uL) produced and purified via SPRIselect® SPRI bead-based size selection (Beckman Coulter, California, USA). The reactions were prepared in both the Eppendorf LoBind and standard plates, then consolidated to a MicroAmp® Fast Optical 48-well reaction plate (REF: 4375816, Applied Biosystems®, California, USA) for qPCR analysis. The cycler used was Applied Bio-systems StepOne™ Real-Time PCR System (Applied Bio-systems, California, USA).

Comparison of sequencing efficiency of NGS library of primary cells in Eppendorf LoBind and standard PCR plates

10 and 50 ng total RNA from primary HUVEC cells was used to prepare a NGS library according to the MACE protocol [2] by GenXpro GmbH, Frankfurt a. M., Germany. cDNA synthesis and all following steps were accomplished in Eppendorf LoBind and standard plates. Both plate types were treated in parallel with 3 replicates each. Ten PCR cycles were applied for both groups. Sequencing of all products was done on the same Illumina® NextSeq® 500 lane (Illumina, Inc., California, USA). The adapter sequences had a length of 75 bps. All sequencing data was cleared from artifacts and low quality reads with the TrueQuant method (GenXpro GmbH, Frankfurt a. M., Germany). All MACE sequencing data was annotated on the human genome hg19 using the GenXpro annotation pipeline with novoalign (http://novocraft.com/). All annotation coordinates were aligned with Refseq-tracks to receive the genetic information. Normalization of the data was accomplished by calculating the number of each transcript over the number of all sequenced molecules followed by multiplication of 106 (TPM = tags per million).

Results and Discussion

Comparison of qPCR efficacy in Eppendorf LoBind and standard PCR plates

To demonstrate that the Eppendorf LoBind property does not affect the PCR performance, amplification rate in the Eppendorf LoBind and standard PCR plates must be comparable. A qPCR experiment in both plate types was done using the same NGS library as template. Figure 1 shows that the amplification rates of a target in the Eppendorf LoBind and standard plates are similar, at C_t values of 8.83 and 8.81, respectively.

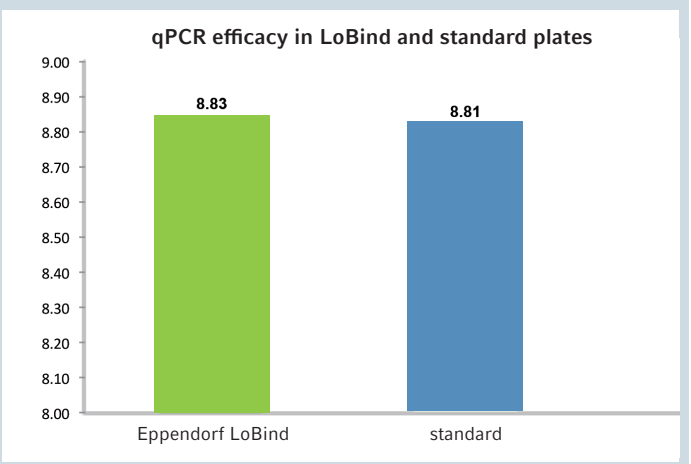

Figure 1: Comparison of amplification rates and PCR efficacy in Eppendorf LoBind and standard plates (n=3).

Comparison of sequencing efficiency of a NGS library of primary cells in Eppendorf LoBind and standard PCR plates

To compare the effectiveness of sequencing in Eppendorf LoBind and standard plates, NGS libraries were prepared from 10 and 50 ng total RNA. Then both libraries were sequenced on the same lane of a flow cell. All sequences were cleaned-up by the TrueQuant method. This method excludes sequencing artifacts as well as low quality reads, resulting in an average of 549,165 reads from the library prepared in the Eppendorf LoBind plate and 442,699 reads from the standard plate with 10 ng total RNA input. The data shows a lead of 20 % in reads using the LoBind plates versus the standard ones, which is repeated with 50 ng group (~23 % more reads from the Eppendorf LoBind plate). The average number of reads in LoBind plates was 2,836,281 while in standard plates 2,184,589 reads could be detected. This indicates that the usage of Eppendorf LoBind plates for the preparation of NGS libraries leads to more raw data output, leaving room for evaluation and a higher chance of catching all desired sequences in the library.

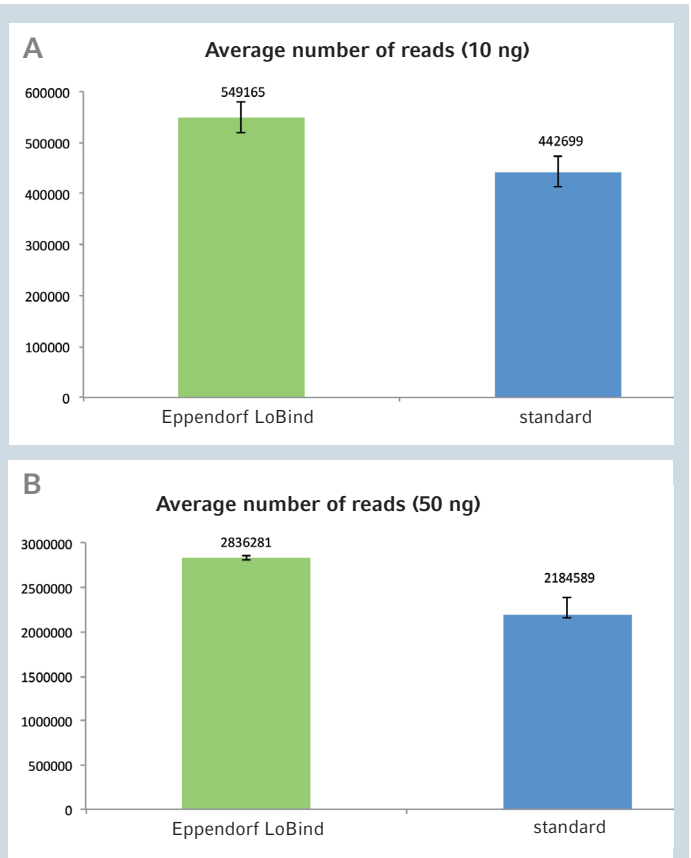

Figure 2: The average number of reads of a 10 ng (2 A) and 50 ng (2 B) NGS library prepared from total RNA of primary HUVEC cells in Eppendorf LoBind and standard plates. The average number of reads is up to 23 % higher in Eppendorf LoBind than in standard plates (n=3).

Not only the average read depth is a quality measure of a sequencing run, but also the number of different transcript species detected. The more different species identified, the more comprehensive the sequencing data is, and with this the evaluation possibilities increase. It was found that using Eppendorf LoBind plates for the preparation of NGS libraries led to an increase in the average number of transcript species. 13,800 different transcript species in LoBind plates and 12,800 in standard plates from 10 ng total RNA for the library set up shows that Eppendorf LoBind plates helped to preserve the transcript variability (figure 3 A). This enhancement was more robust as the starting material increased (50 ng), an average number of 18,000 in Eppendorf LoBind plates and 12,000 in standard plates or 30 % more transcript species from the Eppendorf LoBind plates were reported (figure 3 B).

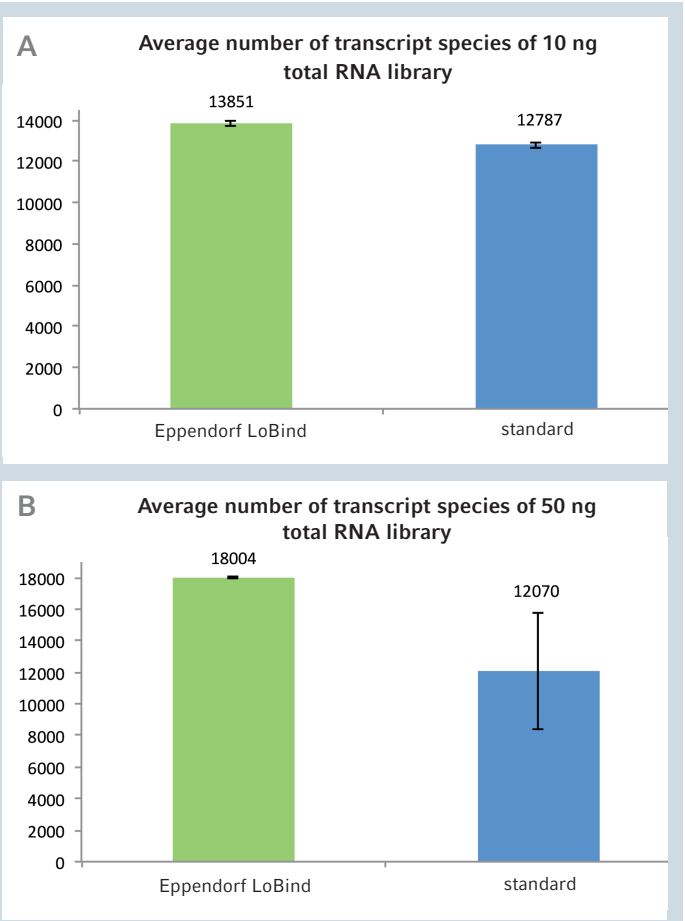

Figure 3: The average number of transcript species of NGS libraries prepared from 10 ng (3 A) and 50 ng (3 B) total RNA of primary HUVEC cells in Eppendorf LoBind and standard plates. The average number of transcript species is up to 30 % higher in Eppendorf LoBind than in standard plates (n=3).

Conclusion

Preparing NGS libraries in Eppendorf LoBind plates instead of standard plates showed multiple advantages. While the PCR efficiency in both plate types is identical, the quality and quantity of the resulting sequencing data is improved. Moreover, especially NGS libraries of lower quality may benefit from using Eppendorf LoBind plates. More reads and transcript species were identified by sequencing the same library prepared in the LoBind plates as compared to preparation in the standard plates. So a low quality NGS library prepared in a Eppendorf LoBind plate can have comparable results to a high quality library prepared in a standard plate. Using Eppendorf LoBind plates means less work and fewer repetitions have to be done during library preparation. This is especially advantageous when low quality or low starting material has to be used. An increased amount of raw sequencing data by preparing a library in Eppendorf LoBind plates adds to more assurance at detecting rare species from challenging samples.

Acknowledgement

We want to thank Dr. Björn Rotter and his team at GenXpro GmbH for their enthusiasm in the project and the scientific guidance.

Literature

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Ordering information

Description	Order no. international	Order no. North America
Eppendorf twin.tec® PCR plates 96 Eppendorf LoBind®, semi-skirted	0030 129.504	0030129504
Eppendorf twin.tec® PCR plates 96 Eppendorf LoBind®, skirted	0030 129.512	0030129512

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