PERIÓDICO TCHÊ QUÍMICA
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SEQUÊNCIAMENTO DE COBERTURA ULTRA BAIXO É O MÉTODO DE QUANTIFICAÇÃO DE BIBLIOTECA MAIS PRECISA ANTES DO SEQUÊNCIAMENTO DE EXOMA

ULTRALOW COVERAGE SEQUENCING IS THE MOST ACCURATE LIBRARY QUANTIFICATION METHOD PRIOR TO EXOME SEQUENCING

СЕКВЕНИРОВАНИЕ СО СВЕРХНИЗКИМ ПОКРЫТИЕМ ЯВЛЯЕТСЯ НАИБОЛЕЕ ТОЧНЫМ МЕТОДОМ КВАНТИФИКАЦИИ БИБЛИОТЕК ДЛЯ СЕКВЕНИРОВАНИЯ ЭКЗОМА

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RESUMO

A quantificação precisa da biblioteca de DNA é muito importante no pós-agrupamento do seqüenciamento do exoma capturado. Bibliotecas sub-representadas precisarão de sequenciamento adicional, o que demanda mais tempo e dinheiro, enquanto bibliotecas de DNA superexpressas podem levar à geração de mais dados do que o necessário, o que leva ao desperdício de capacidade de sequência e um número reduzido de amostras por lote. Existe uma ampla quantidade de métodos disponíveis para quantificar as bibliotecas de DNA antes dos sequenciamentos, tais como absorção de UV, uso de corantes intercalantes, eletroforese capilar, sondas de hidrólise 5´ (TaqMan ©) acopladas com PCR quantitativo (qPCR) ou PCR digital por gota. Mas não há o padrão ouro para a quantificação de bibliotecas de DNA. Este estudo compara métodos comuns de quantificação de bibliotecas, incluindo LabChip (PerkinElmer Inc., MA, EUA), Qubit 3.0 (Thermo Fisher Scientific, MA, EUA), várias abordagens qPCR e sequenciamento de cobertura ultralow na Illumina MiSeqplatform (com e sem correção de tamanho de inserção). As bibliotecas de ADN foram preparadas utilizando o Kit NEBNext Ultra DNA Library Prep para Illumina (New England Biolabs, MA, EUA). Para comparar as abordagens acima mencionadas, o custo, o tempo e a precisão da quantificação foram avaliados em nosso estudo. Os métodos de quantificação envolvendo o uso de Qubit e MiSeq foram melhores do que as abordagens qPCR e LabChip na previsão da concentração final da biblioteca. Também foi revelado que o MiSeq com correção do tamanho da inserção foi o método mais preciso para quantificação de bibliotecas antes do sequenciamento do exoma. Este método permite mudanças de correção na razão devido ao enriquecimento. O sequenciamento de cobertura Ultralow na plataforma Illumina MiSeq é o método mais preciso de quantificação de bibliotecas antes do *pooling* e *post-pooling* do enriquecimento do exoma.

Palavras-chave: *quantificação de bibliotecas, sequenciamento de exoma, enriquecimento do exoma*.

ABSTRACT

Accurate DNA library quantification is very important in post-pooling captured exome sequencing. Underrepresented libraries will need additional sequencing, which takes extra time and money, whereas overexpressed DNA libraries can lead to the generation of more data than required, which leads to the waste of

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sequence capacity and a reduced number of samples per batch. There is a number of methods available to quantify DNA libraries prior to sequencings, such as UV absorption, use of intercalating dyes, capillary electrophoresis, 5'-hydrolysis probes (TaqMan©) coupled with quantitative PCR (qPCR) or droplet digital PCR. But there is no gold standard for the quantification of DNA libraries. This study compares common library quantification methods, including LabChip (PerkinElmer Inc., MA, USA), Qubit 3.0 (Thermo Fisher Scientific, MA, USA), several qPCR approaches, and ultralow coverage sequencing on Illumina MiSeq platform (with and without insert size correction). DNA libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA). To compare the above-mentioned approaches, cost, time, and quantification accuracy were assessed in our study. Quantification methods involving the use of Qubit and MiSeq were found to be better than qPCR and LabChip approaches at predicting the final library concentration. It was also revealed that MiSeq with insert size correction was the most accurate method for library quantification prior to exome sequencing. This method allows for correction shifts in the ratio due to enrichment. Ultralow coverage sequencing on the Illumina MiSeq platform is the most accurate method of library quantification prior to pooling and post-pooling exome enrichment.

Keywords*: library quantification, exome sequencing, enrichment.*

АННОТАЦИЯ

Точная подсчет концентрации ДНК-библиотек после пулирования крайне важен при секвенировании экзома. Библиотеки, представленные в пулах в более низкой концентрации в сравнении с исходной, необходимо секвенировать повторно, что требует дополнительных затрат и времени, тогда как библиотеки с избыточной экспрессией могут привести к созданию большего количества данных, чем требуется, что снижает эффективность секвенирования и приводит к уменьшению количества исследуемых образцов за один анализ. Существует ряд методов для количественной оценки библиотек перед секвенированием, но нет единого стандарта для их квантификации. В этом исследовании сравниваются широко используемые методы количественной оценки библиотек (Labchip, Qubit 3.0, qPCR с тремя наборами праймеров) с секвенированием сверхнизкого покрытия (MiSeq с коррекцией размера вставки и без нее). Однако в настоящее время нет единого стандарта для количественной оценки качества ДНК-библиотек для секвенирования. В настоящей работе нами проведен сравнительный анализ стоимости, времени и точности количественного анализа методом секвенирования со сверхнизким покрытием с другими методами анализа библиотек. Библиотеки фрагментов ДНК готовили с использованием наборов NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, MA, США). Методы количественной оценки на основе Qubit и MiSeq, оказались лучше в подсчете конечной концентрации библиотек, в сравнении с подходами на основе qPCR и LabChip. Также было обнаружено, что MiSeq с коррекцией размера вставки был наиболее точным методом анализа ДНК-библиотек. Данный метод позволяет корректировать изменения представленности ДНК-библиотек образцов в составе пулов, возникающих в процессе обогащения. Секвенирование методом сверхнизкого покрытия на платформе Illumina MiSeq является наиболее точным методом количественного анализа библиотек.

Ключевые слова*: квантификация библиотек, экзомное секвенирование, обогащение.*

1. INTRODUCTION

Accurate equimolar pooling is important to the equal distribution of reads among samples in a single batch (Sham *et al.,* 2002). The unequal combination of libraries leads to the biased representation of certain libraries over others. Underrepresented libraries will need resequencing, which takes time and money. Overrepresentation of libraries can result from the generation of more sequence data than required, which leads to the waste of sequence capacity and a reduced number of samples per batch. Considering a fixed price per sequencing run, it is economically sound to pool more samples in each exome sequencing run with perfectly equal concentrations.

Post-pooling exome enrichment is more cost-effective than pre-pooling enrichment, but it can cause unpredictable shifts in the ratios among samples in the same enrichment batch. Bacterial contamination of initial samples (e.g., extracted from saliva samples), differences in the library insert length distribution, and many other factors cannot be considered simultaneously by common library quantification methods.

The current methods for DNA library quantification use a variety of techniques including UV absorption (e.g., Nanodrop, Thermo Fisher Scientific, USA) (Ponti *et al.*, 2018; McGown, 2000), intercalating dyes (e.g., Qubit, Invitrogen, USA) (Ahn *et al.,* 1996; Vitzthum *et al.,* 1999), capillary electrophoresis (Agilent Bioanalyzer 2100, Agilent Technologies Inc,

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USA) (Panaro *et al.*, 2000), 5'-hydrolysis probes (e.g., TaqMan© probes) coupled with quantitative PCR (e.g., qPCR assays by Roche) (Bunce *et al*., 2012; Mardis and McCombie, 2017) or droplet digital emulsion PCR (ddPCR, Bio-Rad Inc, USA) (Aigrain *et al.,* 2016). These common methods have several limitations and may provide inaccurate results (Haque *et al.,* 2003). For example, UV spectrophotometers detect not only DNA but also UV-absorbing materials such as RNA, protein and phenol and are not sensitive enough to detect small amounts of DNA (Nielsen *et al.*, 2008). Fluorometric methods that only detect double-stranded DNA, such as Qubit, potentially overinflate the actual library concentration due to the binding of the dyes with partially ligated double-stranded libraries and adapter dimers. PicoGreen also binds with dsDNA, but this method is not specific to human DNA; any animal, bacterial or fungal DNA co-purified with the human DNA of interest will contribute to the final reading and could give a falsely high DNA quantification. Several studies indicate that qPCR is the most effective method for library quantification (Meyer *et al.*, 2008; Buehler *et al*., 2010; Hussing *et al*., 2018; Robin *et al*., 2016; Dang *et al.,* 2016).

Because the economic outcome of post-pooling capture exome sequencing experiments depends on the library quantification accuracy, it is crucial to choose the most accurate, reliable, and reproducible method. In this study, several library quantification methods were compared by accuracy and cost to finally select the best method for library quantification prior to pooling before exome capture and Illumina sequencing.

2. MATERIALS AND METHODS

The DNA extraction was performed from both blood and saliva samples of patients using the QIAmp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. All the samples were obtained with informed consent. DNA libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA). The study design was a comparison of several techniques used for the quantification of libraries prior to pooling and exome sequencing, including LabChip (PerkinElmer Inc., MA, USA), Qubit 3.0 (Thermo Fisher Scientific, MA, USA), several qPCR approaches, and Illumina MiSeq (with and without insert size correction according to our study (Krasnenko *et al.*, 2018).

2.1. Qubit 3.0

Quantification using Qubit 3.0 was performed according to the manufacturer's recommended protocol.

2.2. Labchip

Quantification using Labchip was performed according to the manufacturer's recommended protocol. Library concentrations with fragment sizes ranging from 200 to 1000 bp were estimated. This allowed us to exclude too short and too long fragments. Fragments that are too short drop out in enrichment, while fragments that are too long do not participate in sequencing due to the peculiarities of cluster generation during Illumina sequencing.

2.3. qPCR quantification

The library quantification was performed using the StepOnePlus real-time PCR System (Thermo Fisher Scientific, MA, USA) with SYBRGreen I. The cycling conditions were 95°C for 5 minutes followed by 45 cycles of 95°C for 20 seconds, 62°C for 20 seconds and 72°C for 55 seconds. The following amplification primers were used:

1. P5/P7. This primer set was used to detect Illumina-compatible libraries irrespective of their insert size and sequence. This is the most common principle for quantifying sequencing libraries, such as in the QIAseq™ Library Quant Assay Kit, NEBNext Library Quant Kit for Illumina, KAPA Library Quantification Kit Illumina platforms, PerfeCTa NGS Library Quantification Kit for Illumina and other commercially available kits.

P5 AATGATACGGCGACCACCGA
P7 CAAGCAGAAGACGGCATACGAGAT

2. GHRf/GHRr. Both primers anneal to the human GHR gene; thus, we detected the amount of human DNA irrespective of the presence of Illumina sequencing adapters.

GHRf CCCCTCTAAGGAGTGTAGCA
GHRr CTTTTGGTGCCTGGTAAGTT

3. P5/GHRf. The P5 primer anneals to the Illumina adapter, and GHRf anneals to the human GHR gene. This allowed us to detect Illumina-compatible library fragments containing GHR gene fragments.

P5 ATGATACGGCGACCACCGA
GHRf CCCCTCTAAGGAGTGTAGCA

2.4. Ultralow coverage Illumina sequencing

The libraries were sequenced using MiSeq (Liu *et al.,* 2012) with 150 bp PE reads on average. The reads were considered if they mapped to the human genome. Then the relative concentration of the samples in the pool was calculated.

2.5. Ultralow coverage Illumina sequencing with insert size correction

Fragments with different insert lengths are enriched with different efficiencies (Krasnenko *et al.*, 2018; Head *et al.,* 2014). Therefore, the number of reads obtained for each sample was corrected by MiSeq sequencing using coefficients reflecting the enrichment efficiency of fragments with specific lengths.

2.6. Postcapture pooling and exome sequencing

After the quantification libraries were pooled, enrichment was performed with SureSelectXT2 Focused Exome (Agilent Technologies, CA, USA). Exome sequencing was performed using a HiSeq 2500 (Illumina, CA, USA) (Liu *et al.,* 2012). Reads were filtered and mapped to the human genome. The final distribution of reads was considered standard, as the purpose of this work was to determine the most accurate prediction of the data output from exome sequencing.

2.7. Statistical analysis

Log-transformation was used to reduce skewness. The Shapiro-Wilk test was applied to ensure that the data had a normal distribution upon the outlier removal. The Student's t-test was applied to check for bias. To estimate the accuracy, the quantification results obtained by the studied methods were compared with the HiSeq results. The associations between the relative HiSeq concentration and the quantification methods were evaluated by Pearson correlation and linear regression.

3. RESULTS AND DISCUSSION

In this study, several methods for library quantification were compared, including Labchip, Qubit 3.0, qPCR with three primer sets, Illumina MiSeq, and Illumina MiSeq with insert size correction. For each method, we analyzed the accuracy (Fig. 1), cost per sample, and time (Table 1).

We used the library concentration determined by HiSeq as the reference library concentration. All the methods were compared by their ability to predict this concentration. A correlation analysis revealed that for 4 quantification methods (GHR qPCR, Qubit, MiSeq and MiSeq with insert size correction) the p-value is below 0.05, which can be interpreted as an association (Fig. 1). Generally, Qubit and MiSeq were better than qPCR and LabChip at predicting the final concentration. Thus, these methods were chosen for further comparison.

In the additional investigation, the data from Qubit and MiSeq were analyzed by linear regression. The best correlation with HiSeq was revealed for MiSeq with insert size correction (R^2=85,63%, P<0.001). There was a strong correlation between HiSeq and MiSeq data without insert size correction (R^2 = 80,48%, P < 0.001) and Qubit (R^2 = 81,12%, P < 0.001).

By comparing the accuracy of the different quantification methods, we revealed that MiSeq with insert size correction was the most accurate method for library quantification prior to post-pooling capture exome sequencing.

The various instruments for library quantification vary in accuracy, reproducibility, and sensitivity, as well as in labor intensity, speed, and cost. A reliable and accurate quantification strategy will permit investigators to fully utilize the sequencer capacity, reducing the costs of sequencing even further. Therefore, the basic chemistry of NGS requires that a narrow input range of library fragments be prepared for sequencing.

Many studies have previously compared different NGS library quantification methods and shown contradictory results (Brzobohatá *et al*., 2017; Hussing *et al.*, 2015; Katsuoka *et al.*, 2014; Laurie *et al.,* 2013; Nakayama *et al.,* 2016; Robin *et al.*, 2016; White *et al.,* 2009). Hussing with colleagues quantified dsDNA oligos and revealed that BioAnalyzer, TapeStation, and Qubit instruments give concentrations closest to the expected (Hussing *et al.*, 2015). Katsuoka with colleagues have shown that MiSeq is an effective quantification method, but authors have not compared it with other methods (Katsuoka *et al.*, 2014). There is no comparative analysis of methods for library quantification prior to pooling before exome capture and Illumina sequencing.

To examine the most accurate and suitable library quantification methods prior to exome sequencing, four quantification methods were compared that involved using LabChip, Qubit, quantitative PCR (qPCR) with three primer sets and Illumina MiSeq. Quantification using

Miseq was performed using 2 methods, with and without insert size correction. Seven different approaches were applied to estimate the number of reads and these estimates compared with the HiSeq data. It was revealed that MiSeq data correlated most strongly with those obtained by HiSeq. This was confirmed by the linear regression analysis. MiSeq and insert size correction combined led to improved correlations with HiSeq data.

In addition to the actual library quantification, low-depth MiSeq sequencing allows us to determine the library insert size distribution with high details; we have previously shown that this affects the library enrichment efficiency, and, therefore, the relative library representation in the resulting enriched pool (Krasnenko *et al.*, 2018). The enrichment efficiency differences caused by the insert length distribution allowed us to further improve the prediction accuracy of the library concentration in the final pool.

When comparing the cost and time required for the different methods, it appears that MiSeq is costlier and more time consuming than the other quantification methods. However, more hands-on time and a higher price for more accurate quantification may be preferable compared to a higher risk of large variations in library coverage, especially in clinical and forensic genetic laboratories.

When used to quantify NGS libraries, MiSeq decreases overall sequencing costs by ensuring an accurate quantification upfront, which minimizes the need to re-run or repeat sequencing of samples. Nevertheless, our work also reported comparable quality results from the Qubit assay, suggesting that this method can be used when one has a clean and homogenous library with no primer dimer problems.

4. CONCLUSIONS

In summary, this work presents a comparative analysis of cost efficiency and accuracy of DNA library quantification methods for the subsequent exome sequencing.

When predicting the final library concentration, the use of Qubit and MiSeq platform were found to be better than compared to qPCR and LabChip approaches.

The method using ultralow coverage sequencing on Illumina MiSeq platform with insert size correction was the most accurate for DNA library quantification prior to pooling and post-pooling exome enrichment. At the same time, as compared to other methods, the use of MiSeq for quantification is more expensive and requires more time to prepare DNA libraries,

Finally, the study reveals that sequencing on the MiSeq platform is the most accurate, reliable, and reproducible method for library quantification prior to post-pooling capture exome sequencing.

5. LIST OF ABBREVIATIONS

ddPCR – droplet digital emulsion Polymerase Chain Reaction.
NGS – Next Generation Sequencing.
qPCR – a quantitative Polymerase Chain Reaction.

6. DECLARATIONS

6.1. Ethics approval and consent to participate

All the research was approved by the ethics committee of Genotek Ltd (08/2018). The patients or patient's parents provided written informed consent.

6.2. Consent for publication

The patients or patient's parents gave written informed consent to studies and publication of sequencing data.

6.3. Availability of data and materials

Not applicable as the data are included in the results, figure, and table.

6.4. Competing interests

AYu, IF, AS, VV are employees of the commercial organization – Genotek Ltd. The authors declare that they have no other competing interests.

6.5. Funding

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6.6. Authors' contribution

AYu, IF, AS, AV, VV met the International Committee of Medical Journal Editors (ICMJE) criteria for authorship. AYu, IF and AS contributed to the data collection and the first draft of the manuscript. AS also carried out the statistical analysis. VV was mentor. AC has reviewed and edited the text of the manuscript,

corrected the manuscript and prepared the final version. All authors read and approved the final manuscript.

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Table 1. *Comparison of cost and time for the studied library quantification methods*

Quantification method	Hands-on time, hours	Total time, hours	Cost per sample, USD
Labchip	<1	2.5	2.08
Qubit	<1	1	0.86
qPCR p5/GHRf	1.5	3	0.8
qPCR GHRf/GHRr	1.5	3	0.8
qPCR p5/p7	1.5	3	0.8
Ultralow sequencing	2	24	4.95
Ultralow sequencing with insert size correction	2	24	4.95