

# A rapid and easy protocol for quantification of Illumina -NGS libraries

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## Introduction

The exact quantification of an NGS library by concentration is a crucial step in obtaining good NGS data on Illumina platforms. Indeed, the amount of DNA loaded onto the flow cell can affect the sequencing yield and quality directly. Introducing too much DNA can result in poor quality data, as individual clusters may not be resolved sufficiently. However, loading too little DNA can generate fewer clusters and result in a low sequencing yield. Different approaches for DNA quantification are available currently and the most commonly used are based on the analysis of the DNA physical properties, through spectrophotometry or fluorometry. Although these methods are usually technically easy, they do not allow the exact quantification of the amount of adaptor ligated molecules, i.e. the amount of the functional library. This exact quantification can only be achieved with real-time PCR (qPCR). qPCR has been recognized as a very sensitive method for DNA quantification [1] specifically in the case of NGS library preparation [2]. To achieve this level of performance the qPCR reaction needs to be specific, efficient and reliable.

The combination of a fast qPCR mix and a magnetic induction thermocycler allows exact and rapid quantification of NGS libraries as demonstrated by the workflow (Figure 1a).

## Methods

### Library preparation:

An Illumina NGS library was prepared using a JetSeq™ DNA Library Preparation Kit (Bioline Reagents Ltd) and human genomic DNA was mechanically fragmented (approximately 200 bp) following the manufacturers recommended protocol (Covaris, Inc. USA)

### Library quantification by qPCR:

The library quantification was performed on a Mic thermocycler (Bioline Reagents Ltd, UK/BMS) using SYBR® detection technology. The cycling conditions were 95°C for 2 minutes; 35 cycles of 95°C for 5 seconds and lastly 60°C for 45 seconds. A melt curve was then prepared using the preset protocol.

The primers used were the universal P5/P7 amplification primers (Illumina, Inc., USA) (Figure 1b)

**-Q-primer 1 F: 5'-AAT GAT ACG GCG ACC ACC GA-3'**

**-Q-primer 2 R: 5'-CAA GCA GAA GAC GGC ATA CGA-3'**

Six DNA standards ranging from 0.0001 pM up to 10 pM (5 orders of magnitude) were used to build a standard curve and compared to three dilutions of the sample library (1:1000, 1:10000 and 1:100000). The library concentration was calculated using the formula:

$$\text{library concentration (pM)} = 10^{\left[\left(\frac{Ct-b}{a}\right) \times \left(\frac{340}{\text{average fragment length}}\right) \times \text{dilution factor}\right]}$$

Where a and b are the slope and intercept of the standard curve generated with the DNA standards.

### Library quantification by fluorometry:

Library samples were also quantified using a Qubit (dsDNA HS Assay kit, ThermoFisher USA) and a 2100 Bioanalyzer (High Sensitivity DNA analysis kit, Agilent Technologies USA) accordingly to the manufacturers' recommended protocols.

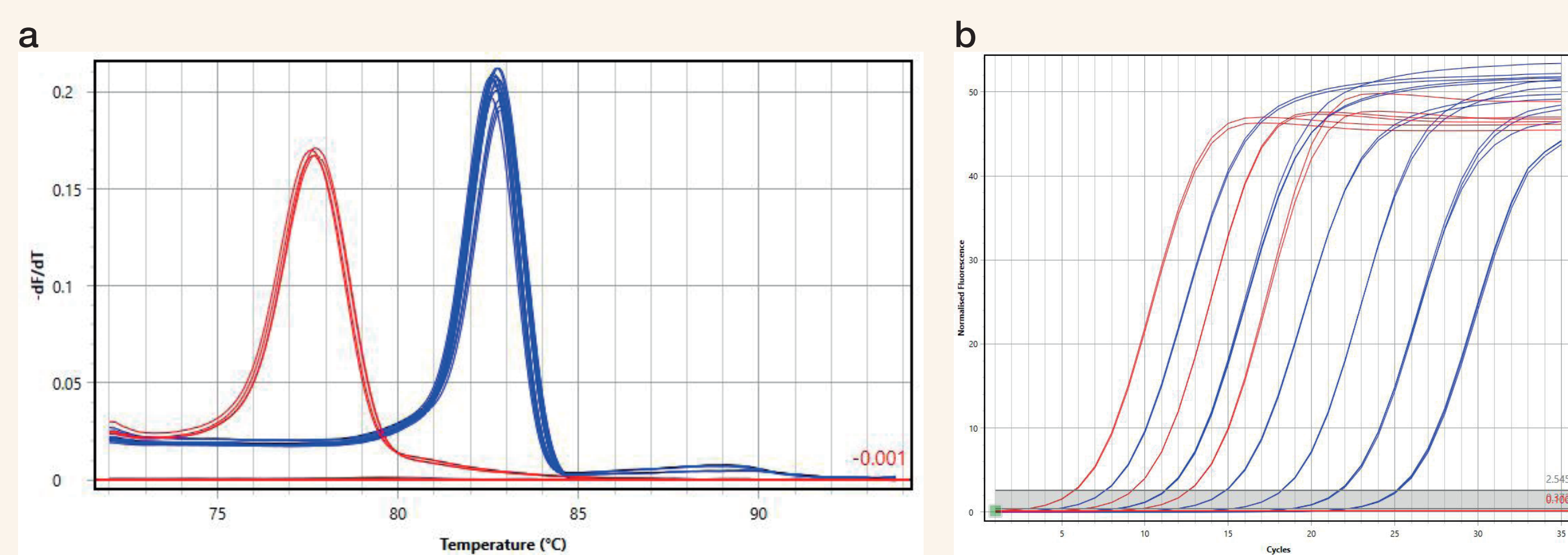
## An Efficient Reaction for a Specific Quantification

A unique melting peak was observed for the six DNA standards as well as for the sample library dilutions even at the lowest concentrations (Figure 2a). The amplification plots confirm the sensitivity of this assay as the limit of detection was 0.0001 pM (Figure 2b).

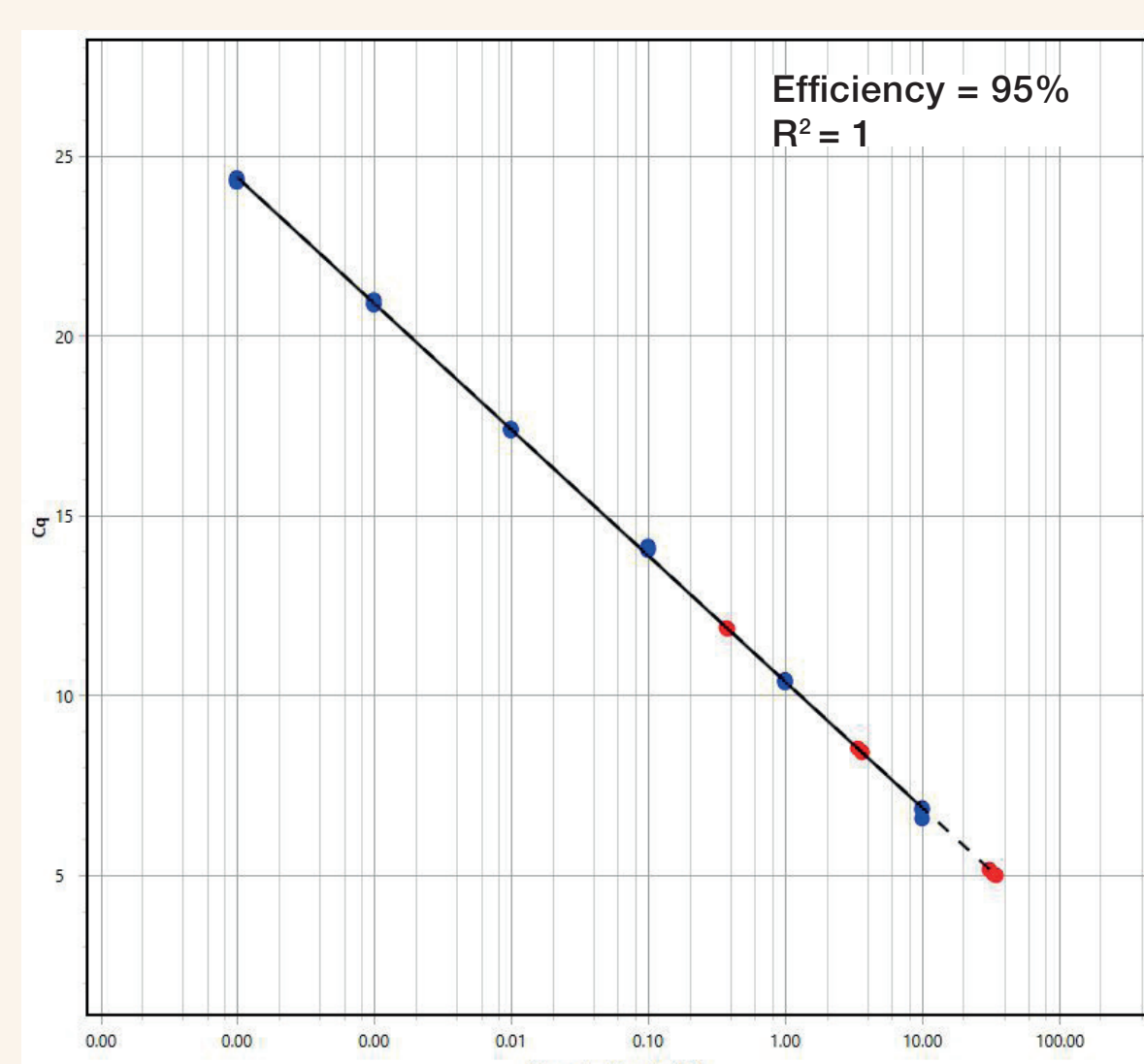
The efficiency of the reaction and R<sup>2</sup> coefficient were calculated and showed values of 90-110 % and 1 respectively (Figure 3).

As the qPCR assay used primers targeting the P5/P7 regions of the Illumina adaptors, only fragments containing these adaptors are amplified and consequently quantified. This ensured that only functional fragments of the library were quantified.

These results demonstrated not only the specificity of the assay, but also its high sensitivity. The sensitivity was superior to the other fluorimetric method available



**Figure 2. Specific reactions.** Melting curves (A) and amplification plots (B) of the six standards (blue) and the sample library dilutions (red) are shown.

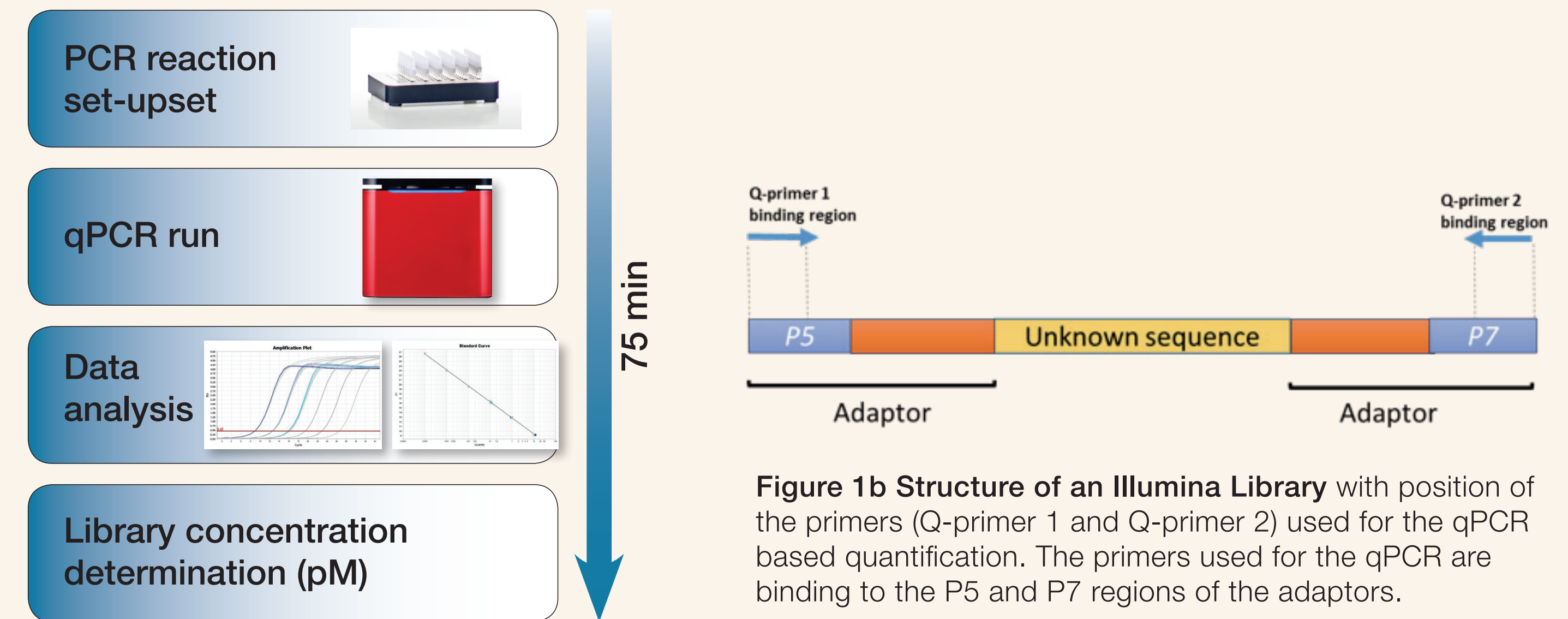


**Figure 3. High efficiency reaction.** Standard curve obtained with the six standards (blue points), library dilutions (red points) are shown.

## References:

- (1) Meyer, M. et al. From micrograms to picograms: quantitative PCR reduces the material demands of high-throughput sequencing. *Nucleic Acids Research*, 36 (1): e5. (2008)
- (2) Robin, J. D. et al. Comparison of DNA quantification methods for Next Generation Sequencing. *Nature, Scientific report* 6: 24067. (2016)

The library to be quantified is compared to DNA standards of known concentrations through Ct values determination



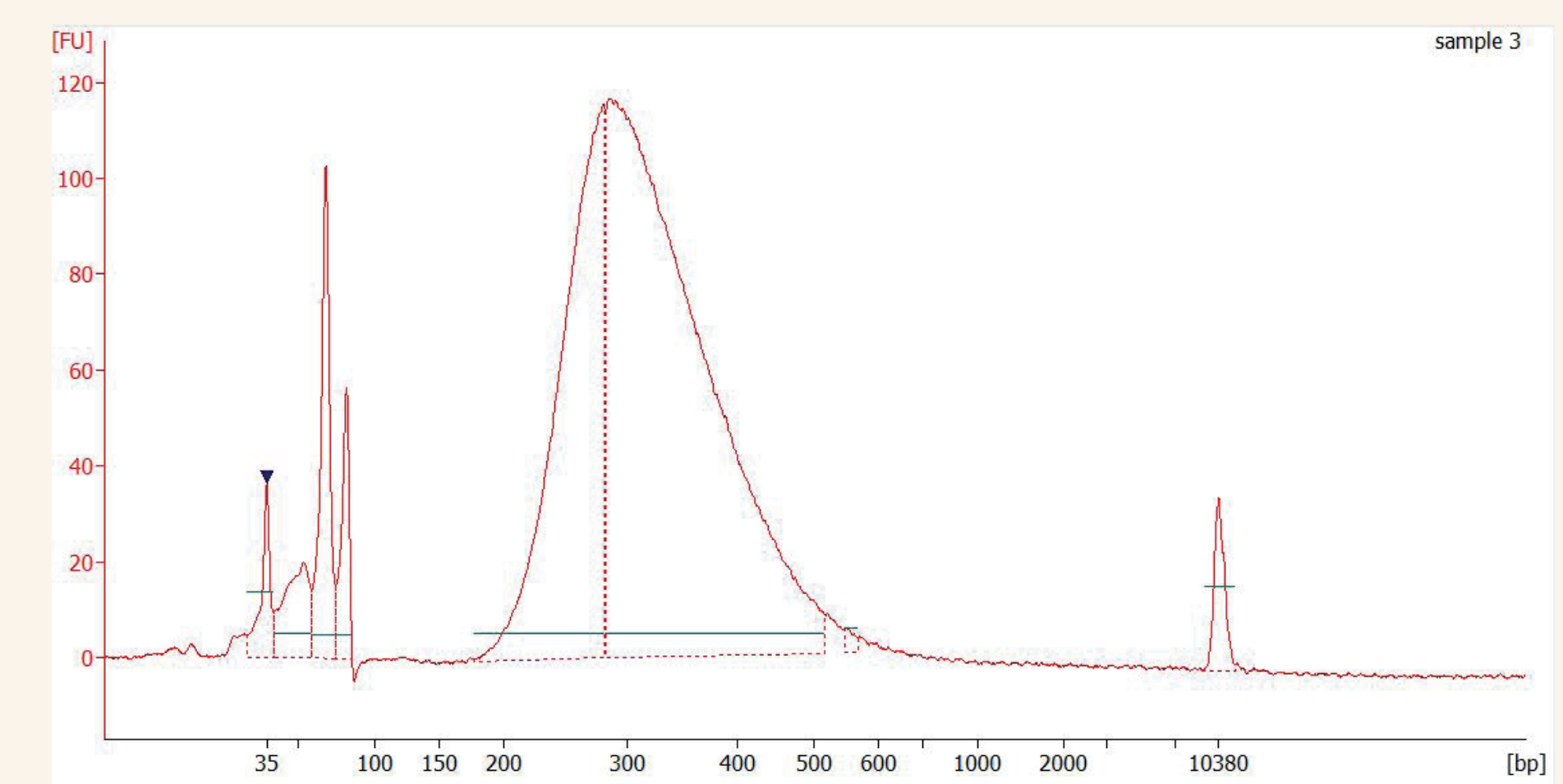
**Figure 1a Library quantification Workflow in 75 min**

## A Reliable Library Quantification Method

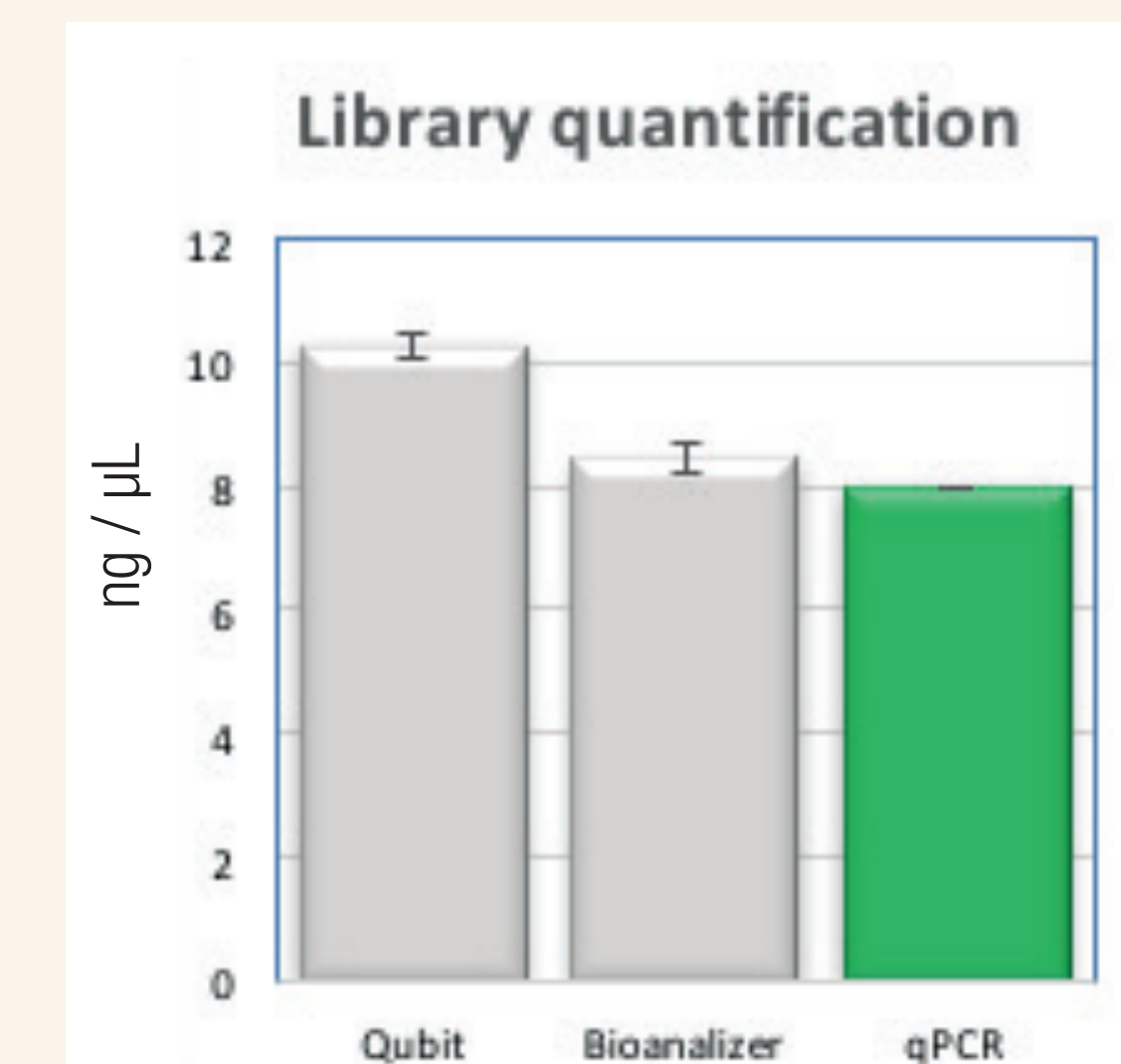
The ability of the qPCR method to quantify NGS library was compared with commonly used fluorometric methods.

A library sample (Figure 4) was quantified using the qPCR method described here as well as the Qubit and 2100 Bioanalyzer methods. Measurements were carried out in triplicate and the results are displayed in Figure 5. The Qubit gave the highest estimations while qPCR correlated with the Bioanalyzer results.

The overestimation of the Qubit can be explained by the nature of the assay, which measures any dsDNA, including primer or adaptor dimers, as well as any non-adaptor ligated DNA (Figure 4).



**Figure 4. Prepared library electropherogram (331/bp) before clean-up and containing short unspecific DNA fragments.**



**Figure 5. Comparison of library concentrations obtained with Qubit, Bioanalyzer and qPCR methods.**

## A Rapid Library Quantification Method

In order to speed up the library quantification workflow, our qPCR chemistry was combined with a fast thermocycler, Mic (Bioline Reagent Ltd/BMS). This qPCR machine uses magnetic induction to control temperatures during cycling. These allow fast cycling, demonstrated by the completion of a protocol including melt curve in 75 min.

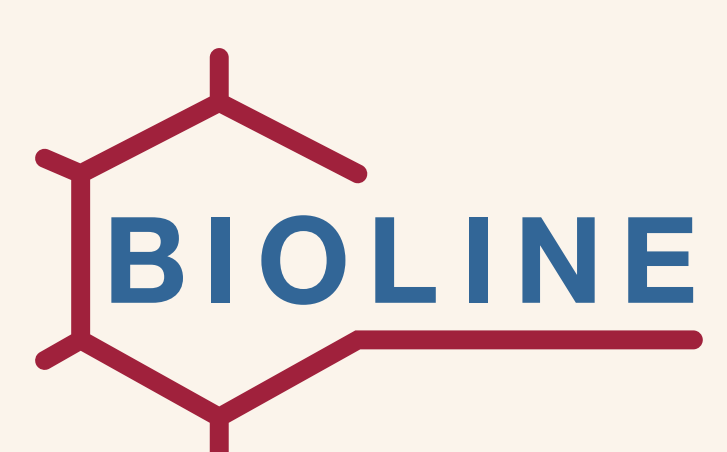
The time required to carry out the quantification with our protocol was compared to other market leading NGS library quantification kits. Our protocol showed the shortest time in comparison with five different suppliers (Table 1).

Supplier	Protocol Time
B	2h 20 min
C	2h 05 min
D	2h 00 min
E	1h 45 min
F	1h 30 min
G	1h 15 min

**Table 1. Time comparison between Bioline and others manufacturers' protocols.**

## Conclusion

This work demonstrates a fast and reliable method to quantify functional Illumina libraries. In addition, we have shown that the complete library quantification workflow can be performed in 75 min by using a magnetic induction thermocycler, making this protocol one of the fastest on the market.



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