Challenge
NGS library prep complexity affects efficiency, resource usage, and requires precise quantification for ideal sequencing

Solution
Analytik Jena's Biometra TAdvanced and qTOWERiris systems ensure precise, reproducible library prep, maximizing sequencing data yield and minimizing technical biases for optimal results

Intended audience
Research and contract laboratories with strong demand of reproducible and high-quality DNA amplicon sequencing library construction for NGS applications

DNA Amplicon Library Preparation for Illumina® Sequencing

Introduction

NGS is a massively parallel sequencing technology and enables a variety of applications that allows the detailed investigation of whole genomes or specific target regions.[1]
Targeted sequencing allows to focus time, cost, and data analysis on specific areas of interest of a genome and enables sequencing with a much higher level of coverage. Targeted sequencing involves the enrichment of a gene fragment or panel of genes from a genome. The enrichment required for targeted sequencing is performed particularly quickly and cost-effectively by polymerase chain reaction (PCR). In particular, amplicon sequencing is an affordable, robust and well-established NGS approach that offers high throughput capacity in a broad range of application areas, including oncology, evolutionary biology and microbiome research. In all NGS applications, the quality of DNA sequencing libraries has a major impact on the preparation steps and thus on the final result.[2] For this reason, it is important to perform DNA library preparation with the highest precision and reproducibility. DNA library preparation for amplicon sequencing involves a series of steps with enzymatic incubations, adapter ligation, barcoding with unique index sequences during library enrichment and quality control measurements,[3] for which thermal cyclers are used.
For excellent library quality, the features of the thermal cycler are critical. The precision of temperature control, efficient heating and cooling rates, and excellent temperature homogeneity across the entire block leads to the reproducible generation of preparation products and amplicons in all sample wells, without non-specific side reactions and PCR bias.[4]
The Biometra TAdvanced thermal cycler (Analytik Jena GmbH+Co. KG) ideally meets the requirements of NGS library preparation and is therefore popular among scientists for this purpose.
In this application note, we exemplified the preparation of DNA amplicon sequencing libraries for NGS using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs GmbH) on the Biometra TAdvanced 96 SG and performed library quantification using the real-time thermal cycler qTOWERiris (Analytik Jena GmbH+Co. KG).

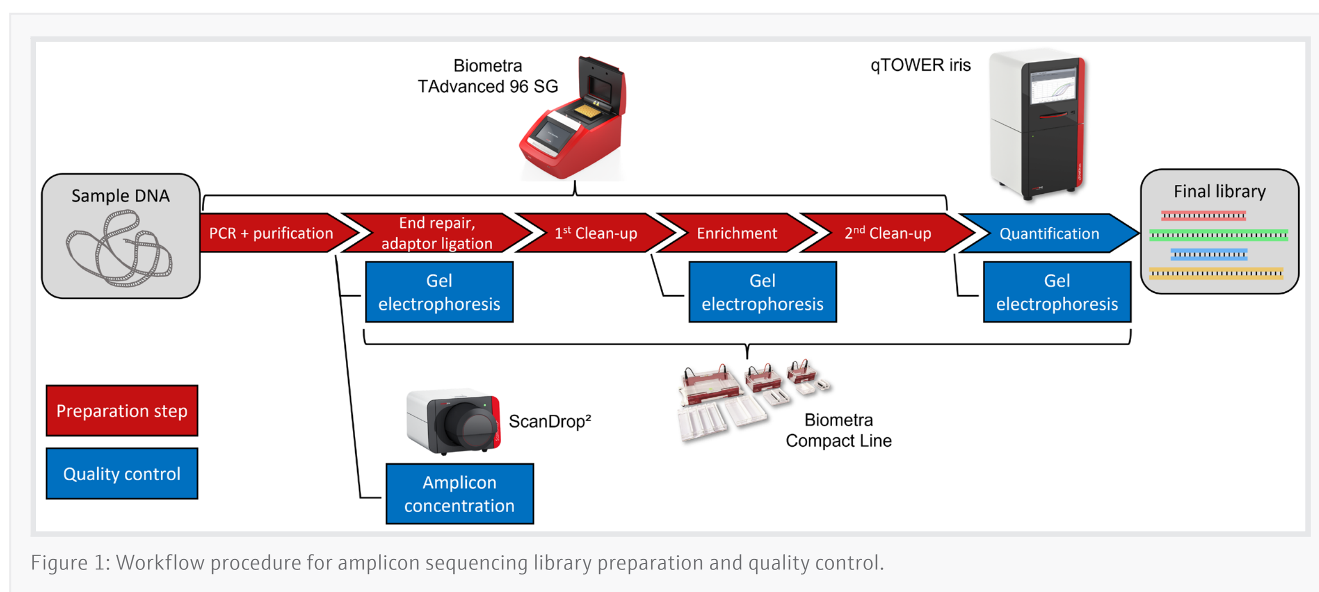


Figure 1: Workflow procedure for amplicon sequencing library preparation and quality control.

Materials and Methods

Samples and reagents

- Human Genomic DNA – Promega Cooperation, cat: G3041
- Commercial PCR master mix
- Commercial PCR purification kit
- NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® with sample purification beads – New England Biolabs Inc., cat: #E7103
- NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) – New England Biolabs Inc., cat: #E7335
- NEBNext® Library Quant Kit for Illumina® – New England Biolabs Inc., cat: #E7630

Sample preparation

Prior to the library preparation procedure, PCR was performed targeting four genes from human genomic DNA (Promega Cooperation) (Table 1), using a commercial master mix in 50 µL volume and six replicate reactions for each gene. Subsequently, a PCR clean-up was conducted using a commercial PCR purification kit. Product integrity and fragment size was checked by agarose gel electrophoresis using Biometra Compact M (Figure 2A). The DNA concentrations of PCR amplicons were determined with the ScanDrop² microvolume spectrophotometer (Analytik Jena GmbH+Co KG).

Table 1: Four gene targets for amplicon library preparation were chosen derived from human genomic DNA (Promega Cooperation, G3041) representing varying amplicon lengths and GC content.

Gene targets	Amplicon length (bp)	GC content (%)	Concentration (ng/µL)
ACTB	250	61.2	11.09
MYC	480	36.0	16.84
B2M short	203	41.4	16.37
B2M long	473	43.3	18.95

Instrumentation

- Biometra TAdvanced 96 SG thermal cycler – (Analytik Jena GmbH+Co. KG), cat: 846-2-070-241
- Biometra Compact Line gel electrophoresis system – (Analytik Jena GmbH+Co. KG), cat: 846-025-xxx*
- ScanDrop² microvolume spectrophotometer – (Analytik Jena GmbH+Co. KG), cat: 844-00203-x*
- qTOWERiris real-time PCR cycler – (Analytik Jena GmbH+Co. KG), cat: 844-0085x-x*
- Standard vortex mixer
- Standard benchtop centrifuge for 2 mL tubes
- Standard centrifuge for PCR plates

*Multiple device configurations available

Library preparation workflow

Purified PCR products between 300 ng to 1 µg were used as input for library preparation and all steps were performed according to the manual of the NEB Next Ultra™ II DNA Library Prep Kit for Illumina®. Samples were distributed across the block of the Biometra TAdvanced thermal cycler (Figure 2B) in a way that tested the uniformity of sample temperature control for library preparation protocols. The NEBNext® adaptors were ligated without any dilution followed by a first library clean-up without size selection. Afterwards, the libraries were checked by gel electrophoresis (Figure 2C). Adaptor-ligated DNA libraries were barcoded using the Index Primers Set1 of the NEBNext® Multiplex Oligos for Illumina® and were enriched during four PCR cycles. The final library integrity was checked by gel electrophoresis (Figure 2D).

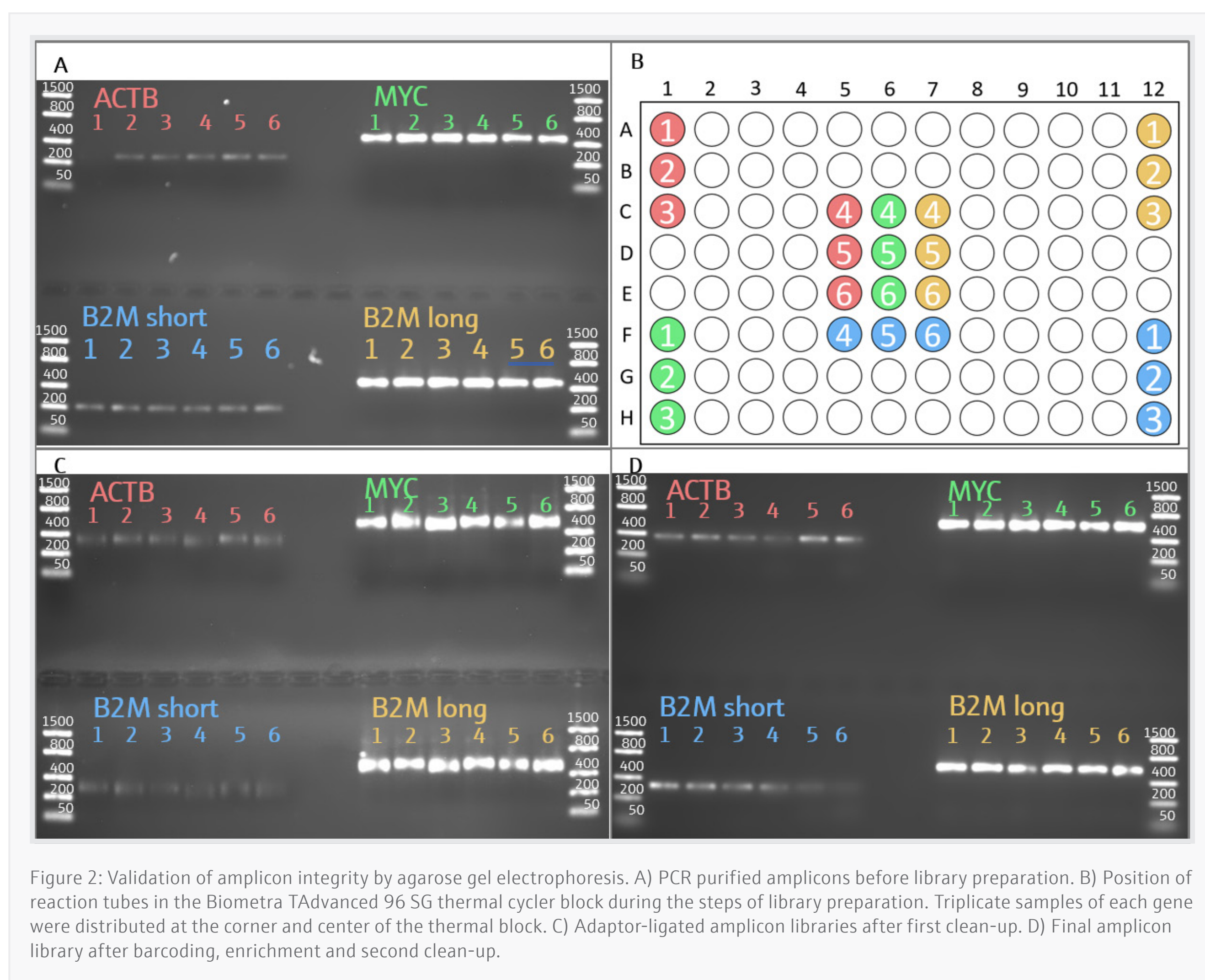


Figure 2: Validation of amplicon integrity by agarose gel electrophoresis. A) PCR purified amplicons before library preparation. B) Position of reaction tubes in the Biometra TAdvanced 96 SG thermal cycler block during the steps of library preparation. Triplicate samples of each gene were distributed at the corner and center of the thermal block. C) Adaptor-ligated amplicon libraries after first clean-up. D) Final amplicon library after barcoding, enrichment and second clean-up.

Library quantification

The final libraries were quantified on the real-time thermal cycler qTOWERiris according to the following sample layout (Figure 3) and PCR program (Figure 4) according to the manual from the NEBNext® Library Quant Kit for Illumina®. Sample libraries were diluted 1:100,000 and measured in triplicate reactions using the color channel SYBR Green/ FAM.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1 100 pM	Standard 1 100 pM	Standard 1 100 pM	ACTB_1	ACTB_1	ACTB_1	MYC_3	MYC_3	MYC_3	B2M short_5	B2M short_5	B2M short_5
B	Standard 2 10 pM	Standard 2 10 pM	Standard 2 10 pM	ACTB_2	ACTB_2	ACTB_2	MYC_4	MYC_4	MYC_4	B2M short_6	B2M short_6	B2M short_6
C	Standard 3 1 pM	Standard 3 1 pM	Standard 3 1 pM	ACTB_3	ACTB_3	ACTB_3	MYC_5	MYC_5	MYC_5	B2M long_1	B2M long_1	B2M long_1
D	Standard 4 0.1 pM	Standard 4 0.1 pM	Standard 4 0.1 pM	ACTB_4	ACTB_4	ACTB_4	MYC_6	MYC_6	MYC_6	B2M long_2	B2M long_2	B2M long_2
E	Standard 5 0.01 pM	Standard 5 0.01 pM	Standard 5 0.01 pM	ACTB_5	ACTB_5	ACTB_5	B2M short_1	B2M short_1	B2M short_1	B2M long_3	B2M long_3	B2M long_3
F	Standard 6 0.001 pM	Standard 6 0.001 pM	Standard 6 0.001 pM	ACTB_6	ACTB_6	ACTB_6	B2M short_2	B2M short_2	B2M short_2	B2M long_4	B2M long_4	B2M long_4
G	NTC	NTC	NTC	MYC_1	MYC_1	MYC_1	B2M short_3	B2M short_3	B2M short_3	B2M long_5	B2M long_5	B2M long_5
H				MYC_2	MYC_2	MYC_2	B2M short_4	B2M short_4	B2M short_4	B2M long_6	B2M long_6	B2M long_6

Figure 3: Plate layout for the quantification of amplicon libraries using the NEBNext® Library Quant Kit for Illumina® in a 20 µL qPCR reaction volume. The quantification assay consisted of six provided standards, the 24 gene libraries and no-template controls (NTC) in triplicate reactions.

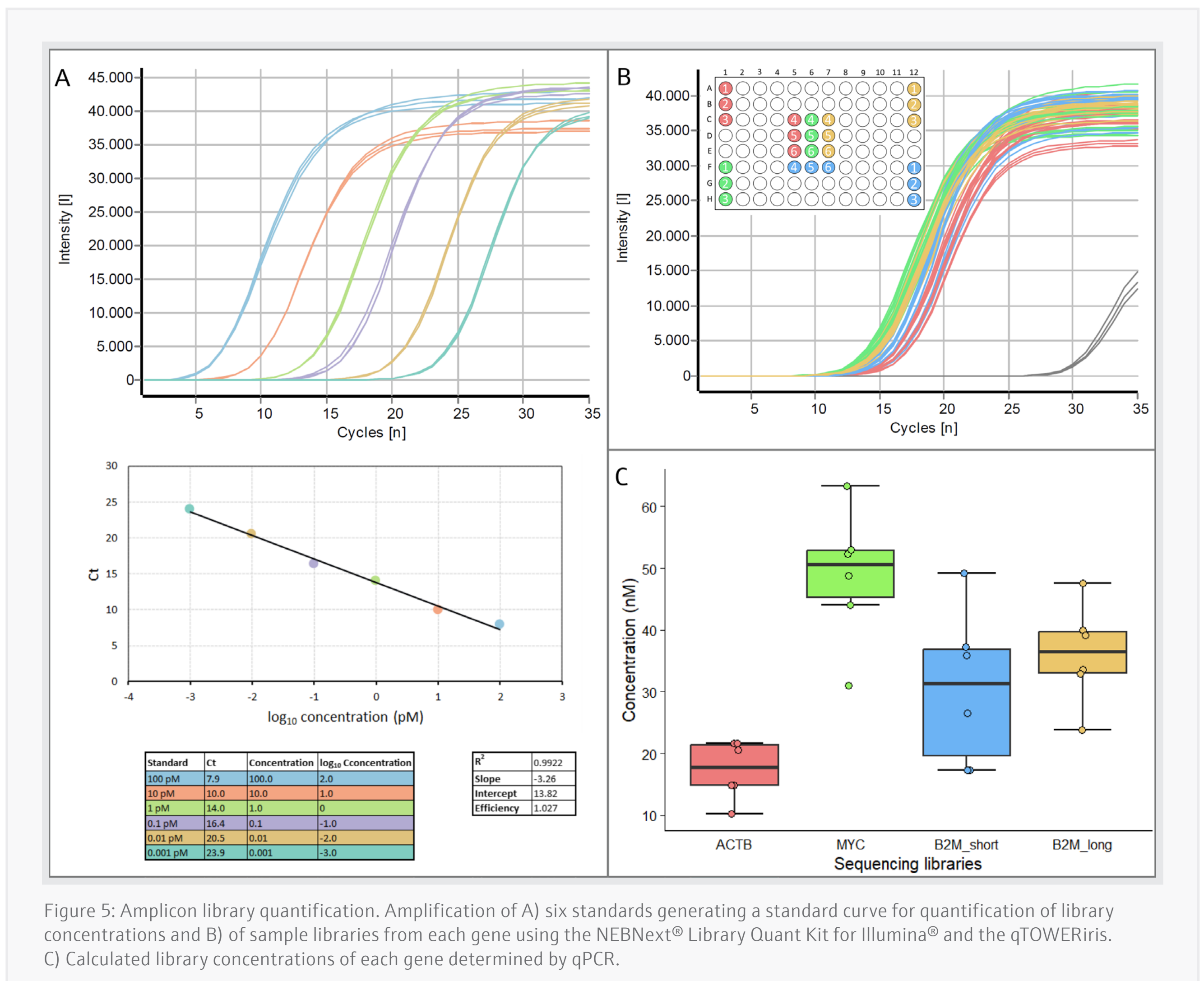
Preheated lid: ✔ Lid temp. °C: 100

Step	Scan	Temp. (°C)	Time (m:s)	Go to	Loops	Ramp (°C/s)
1		95.0	01:00	0	0	8.0
2		95.0	00:15	0	0	8.0
3	✔	63.0	00:45	2	34	5.5

Figure 4: PCR program for the library quantification, screenshot taken from the qPCRsoft 5.0.2.0 software.

Results and Discussion

The sample libraries were reproducible processed across all library preparation steps, regardless of the position across the thermal block of the Biometra TAdvanced 96 SG, as documented by gel electrophoresis after adaptor ligation (Figure 2C) and PCR enrichment using Index Primers Set1 (Figure 2D). No PCR bias or cross-contamination was observed between the libraries. Libraries with varying length and GC content were successfully created which demonstrates the suitable performance of instruments and reagents for the whole preparation process.
For normalization of the sequencing libraries, concentrations were assessed following the NEB protocol for library quantification. The amplification of libraries (Figure 5B) had an acceptable PCR efficiency of 103 % determined by linear regression of the standard curve ($R^2 > 0.99$) (Figure 5A). Ct values of all replicates had a standard deviation of < 0.3 depicting an excellent homogenous measurement of the system. Quantification of the libraries yielded in concentrations between 10 to 63 nM (Figure 5C), which corresponded to the range of recommended library concentrations for Illumina® sequencing.
In conclusion, the depicted workflow using PCR and real-time PCR thermal cyclers from Analytik Jena and a library preparation kit from NEB demonstrated a reliable and efficient solution for NGS DNA library preparation for amplicon sequencing regardless of gene target, amplicon length and GC content.



Standard	Ct	Concentration	log_{10} Concentration
100 pM	7.9	100.0	2.0
10 pM	10.0	10.0	1.0
1 pM	14.0	1.0	0
0.1 pM	16.4	0.1	-1.0
0.01 pM	20.5	0.01	-2.0
0.001 pM	23.9	0.001	-3.0

R^2	0.9922
Slope	-3.26
Intercept	13.82
Efficiency	1.027

Figure 5: Amplicon library quantification. Amplification of A) six standards generating a standard curve for quantification of library concentrations and B) of sample libraries from each gene using the NEBNext® Library Quant Kit for Illumina® and the qTOWERiris. C) Calculated library concentrations of each gene determined by qPCR.

Summary

This application note highlights the importance of generating high-quality sequencing libraries in Next Generation Sequencing (NGS) applications, specifically focusing on amplicon sequencing of genes from human genomic DNA. The depicted library preparation workflow included targeted PCR, end repair, adaptor ligation, barcoding, library enrichment and purification steps as well as fragment integrity verification by agarose gel electrophoresis. In particular, the Biometra TAdvanced thermal cycler (Figure 6) demonstrated excellent performance in combination with the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® kit to create reliable and reproducible DNA amplicon libraries. The resulting libraries exhibited varying lengths and GC content, proving the suitability of the instruments and reagents for this workflow. The library quantification for subsequent normalizing showed accurate results using the qTOWERiris real-time PCR thermal cycler. The library amplification had an optimal PCR efficiency and quantification yielded concentrations within the recommended range for Illumina® sequencing.

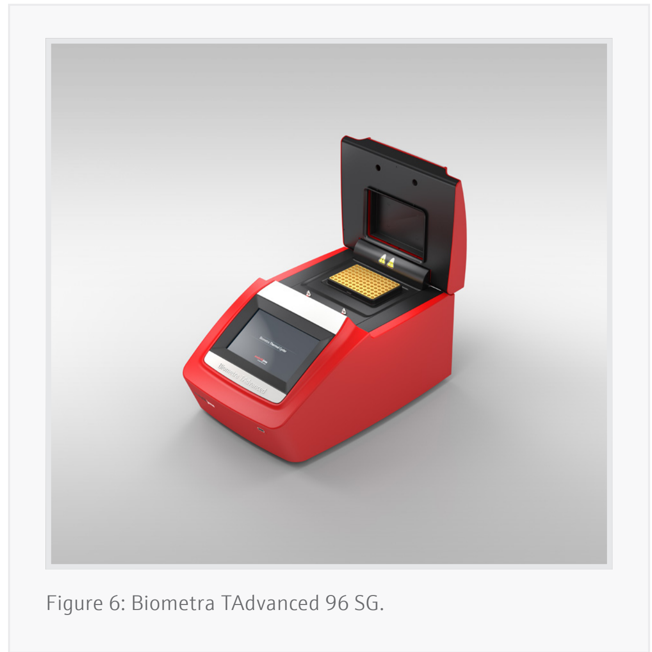


Figure 6: Biometra TAdvanced 96 SG.

References

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