Issue III **2015**

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in this issue

NEW ENGLAND BioLabs® Inc.

The Quantitation Question: How does accurate library quantitation influence sequencing?

The determination of the number of sequencing-ready molecules present after library preparation is an important step in the next generation sequencing (NGS) workflow and has a strong influence on the success of both a sequencing run and a sequencing-based experiment. Before selecting the quantitation method you'll use, it can be helpful to understand what happens to a library during sequencing, and exactly what quantitation does and does not tell you.
A typical NGS workflow starts with library preparation and ends with sequencing and data analysis; each of these steps is of critical importance to the quality and reproducibility of the sequencing data. However, between library preparation and sequencing is, perhaps, one of the less-discussed steps in the NGS workflow: library quantitation.

WHY DO I NEED TO QUANTITATE MY LIBRARY?

There are two primary reasons that libraries must be quantitated.

1. The chemistries that underlie Illumina sequencing require an optimal amount of adaptor-ligated DNA fragments to be loaded into the cluster generation step, for example 6-10 pM for the MiSeq® instrument (v3 chemistry).
2. If multiple libraries are sequenced in one run, it is desirable for the sequence coverage to be equal for each library, and therefore an equal amount of each library should be moved into the cluster generation step.

What happens to your library during sequencing?

To fully understand the importance of accurate library quantitation before sequencing, it is first necessary to understand sequencing chemistries and their interactions with the samples you'll be sequencing.

For the purposes of this article, we'll focus on the chemistries that underlie the popular (and market leading) Illumina sequencers, although library quantitation is an important step for sequencing on any platform.

Building bridges & counting clusters

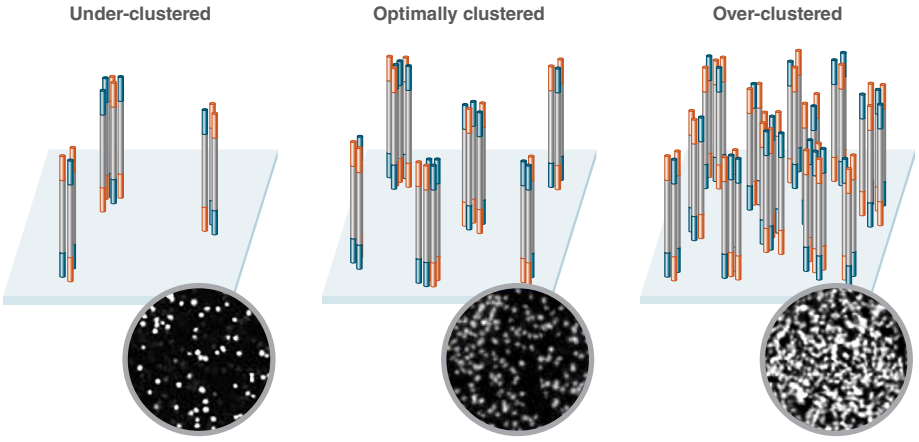
Core components of Illumina's sequencing technology are its flow cells and their cluster-generating capabilities. Illumina's sequencers are based on optical detection of DNA clusters that form on the glass flow cell, a phenomenon enabled by a dense lawn of primers pre-immobilized to the flow cell channel. As you add your library to the flow cell, the single-stranded, adaptor-ligated fragments hybridize to the immobilized primers studded across the flow cell. This step is where the accuracy of your library quantitation is put to the test.

Cluster generation then occurs: each hybridized molecule undergoes multiple rounds of amplification to produce up to 1,000 copies of the same molecule in the same location on the flow cell: a "cluster", whose diameter is 1 micron or less. For more details on cluster generation, visit Illumina.com.

The amount of DNA initially loaded onto the flow cell directly influences the density of the clusters that form. Too little DNA and the clusters are likely to sparsely populate the flow cell. Too much DNA and the clusters will be too close together, making it difficult to interpret the sequencing data due to poor resolution, and resequencing of libraries will be required (Figure 1). Illumina's recommended input ranges, which differ depending on the specific Illumina instrument, help to ensure that the clusters forming on the flow cell have sufficient resolution, without wasting valuable flow cell space.

FIGURE 1:
Optimal cluster density enables efficient & accurate sequencing

The density of library clusters as they form on the flow cell prior to sequencing is a key factor in the success of a sequencing run. Low concentration libraries (Left) fail to make optimal use of the space, while high concentration libraries (Right) lead to densely packed clusters that are difficult to call. Optimal cluster density (Center) makes the best use of flow cell real estate, without over crowding. Representative optical data generated during sequencing depicts variation in cluster densities as shown in the insets.



A deeper dive into equivalent representation

When you pool libraries, you increase the value of each sequencing run by increasing the number of samples that can be sequenced in a single run. However, if libraries are combined in unequal concentrations, this leads to biased representation of certain libraries over others. In cases where libraries are significantly under-represented, these libraries will need to be resequenced, costing time and money. Over-representation of libraries can result in generation of more sequence data than required, and the subsequent discarding of sequence reads, wasting sequence capacity.

Figure 2 (next page) is an example of uneven library pooling resulting in uneven sequence coverage and the need to resequence. With 16 libraries in this pool, each library should theoretically have 6.25% of the sequence reads. However, this is not the case, and some of the libraries, such as libraries 5 and 15, would need to be resequenced.

Why do my library fragments need to be adaptor-ligated?

Sequences required downstream of library preparation, such as those for cluster generation and sequencing, must be added to the DNA fragments to be sequenced, and this is the primary goal of library preparation. In PCR-free library preparation workflows, all of the required sequences must be included in the adaptor sequence. In workflows including amplification, some of the sequences, including the sequences required for cluster generation (indicated by P5 and P7 in Figure 3, next page), can be added during PCR instead.

Only fragments that have a P5 sequence at one end and a P7 sequence at the other are capable of participating successfully in cluster generation. Therefore, ideally, only fragments to which both of these sequences have been attached should be counted during a library quantitation step.

FIGURE 2: **Uneven pooling of libraries yields uneven sequence coverage**

Inadequate or uneven pooling of libraries can result in suboptimal data, and even lead to the need for library resequencing, as seen with library #5.

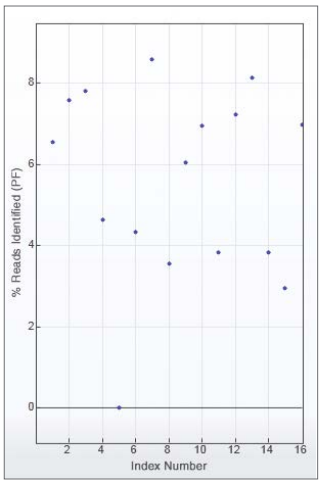
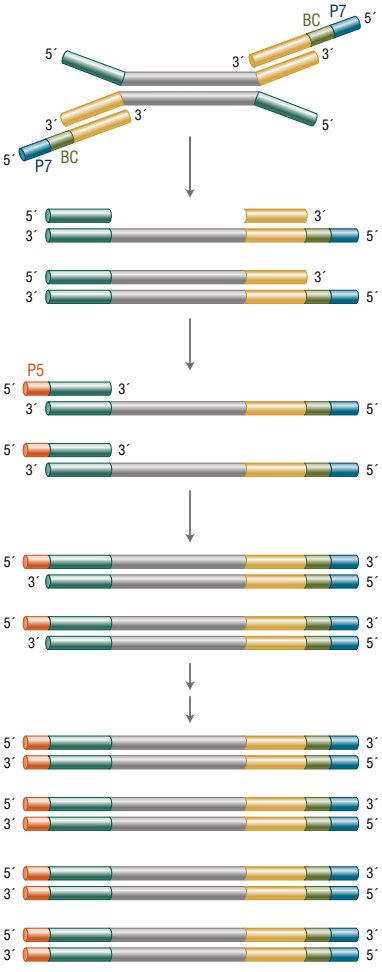


FIGURE 3: **Adaptor ligation workflow**

The stepwise addition of the sequences P5 and P7 and the barcode (BC) can be achieved during PCR amplification of the library.



BC = Barcode

However, in addition to the desired fragments with an adaptor at both ends, libraries may also contain fragments that have no adaptors, one adaptor or adaptor-dimers. Fragments with no adaptors or one adaptor ligated will not form clusters. Adaptor-dimers will efficiently cluster, but contain no DNA of interest (Figure 4, page 4).

HOW SHOULD I QUANTITATE MY DNA LIBRARY?

Simply put, library quantitation refers to a variety of methods for determining the number of nucleic acid molecules present in a specific volume of your library. Unlike other molecular biology techniques, where the recommended input range is broad and forgiving, the basic chemistry of NGS requires that a narrow input range of library fragments be further prepared for sequencing. Therefore, quantitation must be precise. It's also important to consider whether you're quantitating productive library molecules – ones that will be (clustered and) sequenced – or if you're simply quantitating total DNA or even total nucleic acid. Accurate quantitation of a library is essential for optimal sequencing outcomes, so choosing the right quantitation method may mean the difference between a successful run and a sub-optimal, or even failed, run, meaning the library will then need to be adjusted and resequenced – an expensive and time-consuming proposition.

When choosing a quantitation method, there are many important considerations, including accuracy, throughput and cost. Several common methods are compared (Table 1) and discussed below.

WHAT ABOUT SPECTROPHOTOMETRIC RATIOS AND FLUOROMETRIC QUANTITATION?

Due to their utility in multiple molecular biology applications, many labs already have spectrophotometers and fluorometers, and these enable relatively low cost quantitation.

For quantitating nucleic acid, spectrophotometers assess the amount of UV light absorbed by the sample at two wavelengths, 260 nm and 280 nm. A ratio of the absorbance values can then be used to determine whether or not the sample has contaminating proteins. The 260/280 ratio of a purified DNA sample should be between 1.7 and 1.9. Spectrophotometers are

continued on page 4...

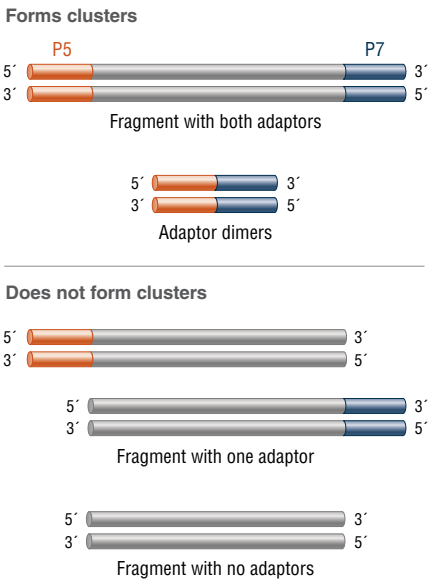
TABLE 1: **Comparison of common library quantitation methods**

When choosing a quantitation method there are many important considerations, including cost, throughput and accuracy.

METHOD	EXAMPLE	BRIEF DESCRIPTION	BENEFITS/LIMITATIONS
Spectrophotometry (260/280)	NanoDrop™	This method detects the absorption of UV light by the macromolecules in the sample.	✓ Low cost, as most laboratories already have access to UV/vis spectrophotometers ✗ Not specific for DNA ✗ Results can be skewed by RNA or protein contamination ✗ Cannot determine fragment sizes
Fluorimetry	Qubit®	This method measures the enhanced fluorescence of a dye upon binding to DNA/ macromolecules.	✓ Low cost, as most laboratories already have access to fluorimeters ✓ Can quantitate specifically dsDNA, ssDNA, RNA or protein ✗ Quantitates all nucleic acid present in sample, not just molecules to be sequenced ✗ Cannot determine fragment sizes
Electrophoretic	Bioanalyzer®, TapeStation®, Fragment Analyzer™	This method relies on capillary electrophoresis of DNA fragments for size estimation, as well as intercalating dyes for quantity determination.	✓ Accurate determination of fragment size distribution ✗ Less reliable quantitation ✗ Requires expensive equipment
Quantitative PCR (qPCR)	NEBNext	This method measures fluorescence of a dye bound to dsDNA at each PCR cycle, quantitating relative to included standards.	✓ Most accurate quantitation method ✗ More expensive ✗ Cannot determine fragment sizes

FIGURE 4:
Adaptors are the hallmark of productive molecules

Only library fragments containing both a P5 and a P7 adaptor will result in a flow-cell cluster. Other molecules are insufficient for cluster formation or contain no DNA of interest, so efforts should be made to exclude them from quantitation.



great at estimating the total amount and relative purity of nucleic acid in solution, but they can't provide information about fragment size, and they can be confounded by an abundance of either RNA or protein in the sample.

Fluorometers, unlike UV-Vis spectrophotometers, rely on nucleic acid-specific dyes to assess the amount of nucleic acids in the sample. In this way, they avoid the pitfalls of spectrophotometry, and can specifically quantitate dsDNA, ssDNA, RNA, or protein, depending on the dye used. However, they too are limited to gathering data about the entire complement of dsDNA or ssDNA in the sample, and not just molecules that will be sequenced.

It is generally recommended not to use only spectrophotometry, fluorometery, or even a combination of the two as your sole quantitation method before sample loading.

What can electrophoretic methods/ instruments tell me?

Electrophoretic instruments, such as the Agilent Bioanalyzer, TapeStation, and AATI Fragment Analyzer, provide valuable data in a variety of forms. The output of these instruments is a visualization based upon laser excitation of an intercalating dye during the sample's passage through a chip matrix, and measurement of the time taken to travel through the matrix. The data can be formatted to look like the familiar banding pattern of gel electrophoresis or as a graph (a "trace"). On-chip electrophoresis enables faster, more standardized quantitation of nucleic acid samples than standard slab gel electrophoresis, with much smaller sample amounts.

Overall, electrophoretic instruments are exceptionally useful tools for library quantitation, and they are a part of many laboratories' NGS workflows. Electrophoretic methods are able to determine both the average library size and the size distribution of the library (important as a tight size range is generally more desirable than a broad size range). Still, electrophoresis-based quantitation of NGS libraries is not as accurate or consistent as qPCR-based methods, and is not specific for adaptor-ligated fragments. Additionally, electrophoresis-based methods are not sufficient for quantitating PCR-free library construction, as there is no PCR enrichment of adaptor-ligated molecules and they cannot discern between adaptor-ligated DNA molecules and unligated molecules.

qPCR: What's in a name?

As the name implies, qPCR (or quantitative PCR) can provide an additional level of information about your library. Beyond simply reporting the total amount of DNA in your sample, qPCR-based library quantitation uses specific primers that hybridize to the adaptor sequences and, therefore, measures only molecules with adaptor sequences at both ends. This added specificity ensures that the fraction of the library loaded onto the sequencer contains the expected number of adaptor-ligated molecules. As described above, exact titration of adaptor-ligated DNA molecules is important for NGS as only molecules with an adaptor on each end can be successfully processed through the sequencing workflow. The quantity of the library is determined by comparing to a standard curve generated from DNA standards (known concentrations of DNA of a known size), followed by a simple calculation to account for any difference in size between the library being measured and the DNA standards. Methods such as electrophoretic analysis, described above, are useful for library size determination.

qPCR-based methods, which quantitate DNA sequences that are attached to adaptors, will also quantitate adaptor-dimers (e.g., two ligated adaptors without any intervening library sequence). The presence of excessive adaptor-dimers in your library can skew your quantitation, but if this situation is suspected, running the sample on a Bioanalyzer or similar instrument will be informative. Frequently, qPCR-based quantitation methods and electrophoretic methods are used in parallel, to determine both the quantity and quality of your library.

Specific details on the use of qPCR-based library quantitation are available in the product manual for the NEBNext Library Quant Kit for Illumina which can be downloaded at www.neb.com/E7630. More information can also be found in our application note on page 11.

THE BIG PICTURE

So, which method for library quantitation is right for you? Your answer will depend on a number of factors that are specific to your situation, including your laboratory's preferred DNA quantitation method, the tools you have available, your source material, and the size and scope of your experiment. No matter which platform you'll be sequencing on, it is important to accurately determine the amount of sequence-ready DNA present. As we've described, accurate quantitation makes a meaningful difference in the quality of the data you'll create and the overall value of your experiment, by ensuring generation of optimal cluster densities and the equivalent representation of multiplexed libraries when pooling.

Using a qPCR-based approach, as we've just reviewed, ensures the most accurate quantitation, providing optimal conditions for Illumina's sequencing chemistries. To make NGS library quantitation more accurate and reproducible, New England Biolabs® (NEB®) offers the NEBNext Library Quant Kit for Illumina. This qPCR-based kit is compatible with a broad range of library insert sizes and GC content, and has a user-friendly workflow.

ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
NEBNext Library Quant Kit for Illumina	E7630S/L	100/500 reactions

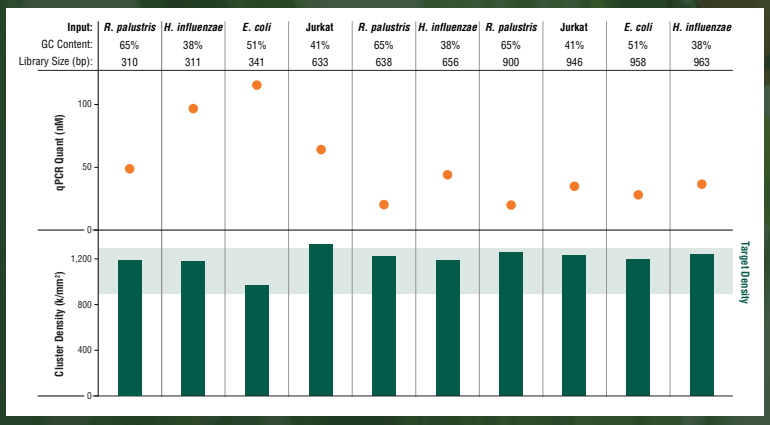
Count on it.

Introducing the NEBNext Library Quant Kit for Illumina

Accurate quantitation of next generation sequencing libraries is essential for maximizing sequencing data output and quality. The NEBNext Library Quant Kit for Illumina is a qPCR-based method that delivers higher consistency and reproducibility of quantitation than other currently available methods. With optimized kit components and a more convenient protocol, you can count on your quantitation values, every time.

To learn more and request a sample, visit **www.neb.com/E7630**

With the NEBNext Library Quant Kit, optimal cluster density is achieved from quantitated libraries with a broad range of library size and GC content.



Libraries of 310–963 bp from the indicated sources were quantitated using the NEBNext Library Quant Kit, then diluted to 8 pM and loaded onto a MiSeq® (v2 chemistry; MCS v2.4.1.3). Library concentrations ranged from 7–120 nM, and resulting raw cluster density for all libraries was 965–1300 k/mm2 (avg. =1199). Optimal cluster density was achieved using concentrations determined by the NEBNext Library Quant Kit for all library sizes.

Being conscious of the need for a "greener" laboratory

Environmental stewardship is one of the founding principles of NEB. By promoting sound ecological practices and environmental sustainability, NEB helps to ensure the protection and preservation of natural resources, both locally and globally. We continuously strive to improve our business processes in order to minimize and, wherever possible, mitigate our impact on the environment. Examples of these initiatives include:

- Establishment of a **LEED®-certified** (Leadership in Energy and Environmental Design) laboratory and production facility
- Implementation of a state-of-the-art **Solar Aquatics System™** that utilizes and accelerates the process found in streams and wetlands to treat the campus' wastewater, making it clean enough for groundwater recharge
- Pioneering the first **shipping box recycling program** over 30 years ago, effectively diverting unnecessary packaging from landfills
- Utilization of **recycled paper** and **soy-based inks** for all NEB publications and marketing materials. In fact, the NEB Catalog is the first **CarbonNeutral® certified catalog** produced in the United States.
- Availability of **cafeteria composting** and extensive **recycling programs** for NEB employees.

labconscious

In line with these company principles, NEB scientists are always mindful of the fact that daily work in the laboratory can generate significant amounts of waste. Simple changes in our lab practices can reduce our impact on the environment. It is in this spirit that NEB has sponsored Labconscious, an open community for researchers to share ideas, protocols and best practices that help reduce the environmental footprint of bench science. It is our hope that Labconscious will become an educational platform and resource repository that will connect companies and brands with end users, and be used to identify greener processes and products. Together, we can try to make a better world with better labs.

Start the discussion today!

labconscious.com

NEW PRODUCT

NEBNext Ultra II

Even more from less.

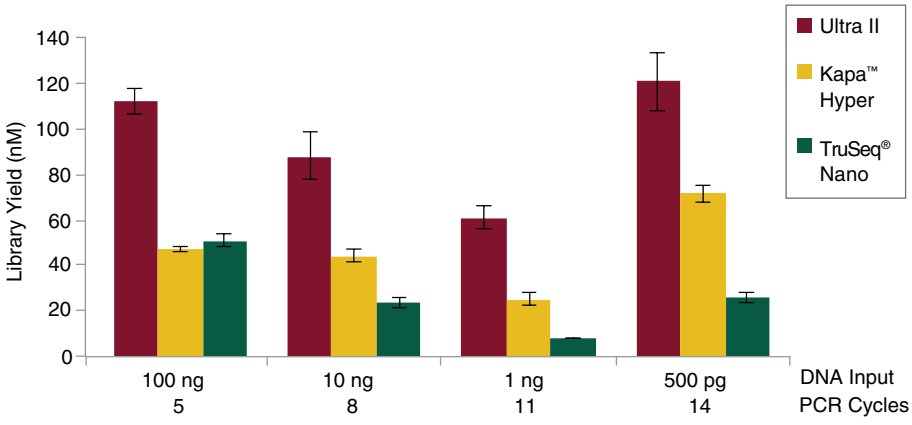
NEW NEBNext® Ultra™ II DNA Library Prep

Are you challenged with trying to get higher library yields using ever-decreasing input amounts? Each component in the NEBNext Ultra II DNA Library Prep Kit from NEB has been reformulated, resulting in a several-fold increase in library yield with as little as 500 picograms of input DNA. These advances deliver unprecedented performance, while enabling lower inputs and fewer PCR cycles. Get even more from less with NEBNext Ultra II.

An important measure of the success of library preparation is the yield of the final library. Optimization of each reagent in the library prep workflow enables substantially higher yields from the NEBNext Ultra II kit as compared to other commercially available kits. Achieving yields for high quality cluster generation and sequencing from very low input amounts can be challenging, a fact compounded by the preference to amplify the library using as few PCR cycles as possible. NEBNext Ultra II overcomes this challenge, and users can now obtain higher library yields with lower inputs, as shown below.

FIGURE 1: **The NEBNext Ultra II DNA Library Prep Kit produces the highest yield libraries from a broad range of input amounts**

Libraries were prepared from Human NA19240 genomic DNA using the input amounts and number of PCR cycles shown. Manufacturers' recommended protocols were followed, with the exception that size selection was omitted.



advantages

- Get more of what you need, with the **highest library yields**
- Use to generate high quality libraries, even when you have only limited amounts of DNA, with **inputs as low as 500 pg**
- Improved library complexity with **fewer PCR cycles**
- Prepare libraries from all of your samples, including **GC-rich** and **FFPE samples**
- Save time with **streamlined workflows, reduced hands-on time, and automation compatibility**
- Enjoy the **flexibility** of **kit or module format** products

Here's what customers are saying about NEBNext Ultra II:

The new NEBNext DNA Ultra II kit has provided us with a critical opportunity to process challenging low-input genomic samples. These samples otherwise didn't yield libraries of adequate complexity required for exploring their genomes comprehensively. In libraries prepared with a different kit, the proportion of duplicated reads, which don't provide new information, is too high. NEBNext DNA Ultra II has alleviated this problem and enabled us to achieve high quality, high content sequencing data that are relevant for our users.

– Vladimir Benes, Ph.D., Head, GeneCore Facility, EMBL Heidelberg, Germany

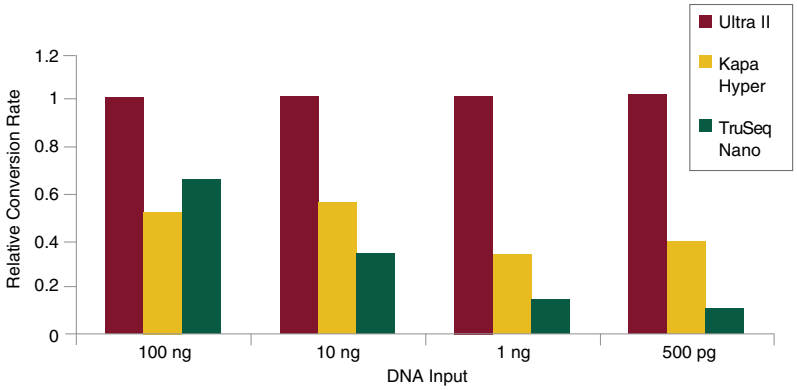
The efficiency of the End Repair, dA-Tailing and Adaptor Ligation steps during library construction can be measured separately from the PCR step by performing qPCR quantitation of adaptor-ligated fragments prior to library amplification. This enables determination of the rate of conversion of input DNA to adaptor-ligated fragments, or sequenceable molecules. Therefore, measuring conversion rates is another way to assess the efficiency of library construction and also provide information on the diversity of the library. Again, NEBNext Ultra II enables substantially higher rates of conversion as compared to other commercially available kits.

Interested in learning more?

Visit **NEBNextUltraII.com** to learn more about how NEBNext Ultra II addresses low input amounts and challenging sample types. While you are there, you can also request a sample and **download our technical note**.

FIGURE 2: **NEBNext Ultra II produces the highest rates of conversion to adaptor-ligated molecules from a broad range of input amounts.**

Libraries were prepared from Human NA19240 genomic DNA using the input amounts and library prep kits shown without an amplification step, and following manufacturers' recommendations. qPCR was used to quantitate adaptor-ligated molecules, and quantitation values were then normalized to the conversion rate for Ultra II. The Ultra II kit produces the highest rate of conversion to adaptor-ligated molecules, for a broad range of input amounts.



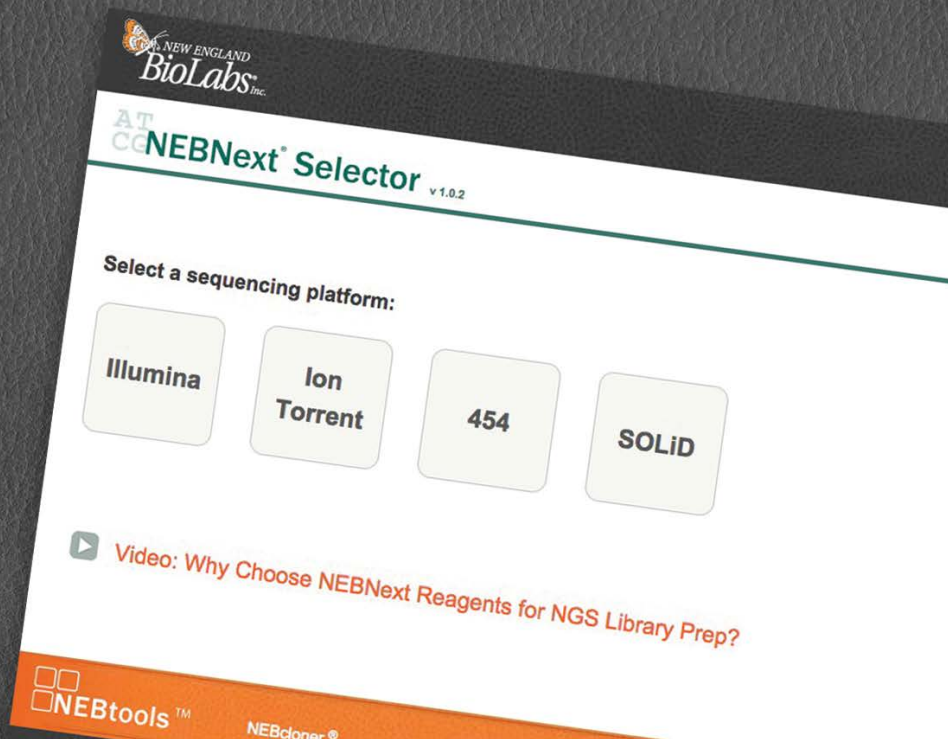
ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
NEBNext Ultra II DNA Library Prep Kit for Illumina	E7645S/L	24/96 reactions
NEBNext Ultra II End Repair/dA-tailing Module	E7546S/L	24/96 reactions
NEBNext Ultra II Ligation Module	E7595S/L	24/96 reactions
NEBNext Ultra II Q5® Master Mix	M0544S/L	50/250 units

FEATURED TOOL

NEBNext® Selector v1.0

- Find recommended NGS sample prep products easily for your **sample type** and **platform**
- **Easily identify** which **step** in the library preparation workflow reagents are suitable for
- Quickly find **additional resources** to help with successful library preparation
- Easy access to **neb.com** for ordering

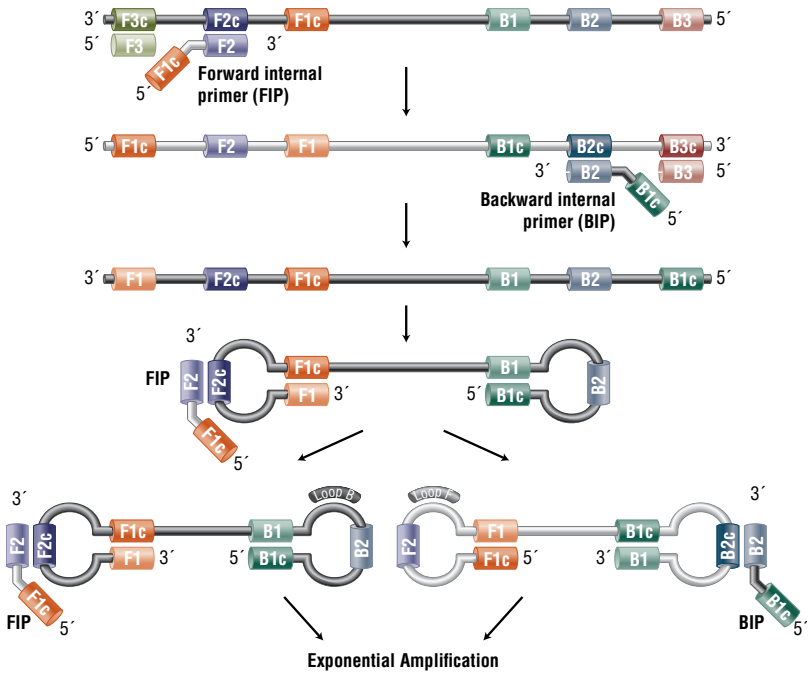


LOOP-MEDIATED Isothermal Amplification

LAMP (loop-mediated isothermal amplification) is designed to detect a target nucleic acid without sophisticated equipment. LAMP uses 4-6 primers recognizing 6-8 distinct regions of the target DNA. A strand-displacing DNA polymerase initiates synthesis and two of the primers form loop structures to facilitate subsequent rounds of amplification. LAMP provides high sensitivity (fg levels or <10 copies of target), and reactions can be performed in as little as 5–10 minutes. Additionally, reactions can be performed with limited resources (e.g., using a water bath for incubation, and detection of results by eye), or with real-time measurement and high-throughput instruments.

Detection of RNA targets is accomplished by simple addition of a reverse transcriptase to the LAMP reaction (e.g., WarmStart® RTx Reverse Transcriptase, NEB #M0380), or by use of a DNA polymerase with RT activity (e.g., *Bst* 3.0 DNA Polymerase), with RT-LAMP performed as a true one-step, isothermal workflow.

OVERVIEW OF LAMP



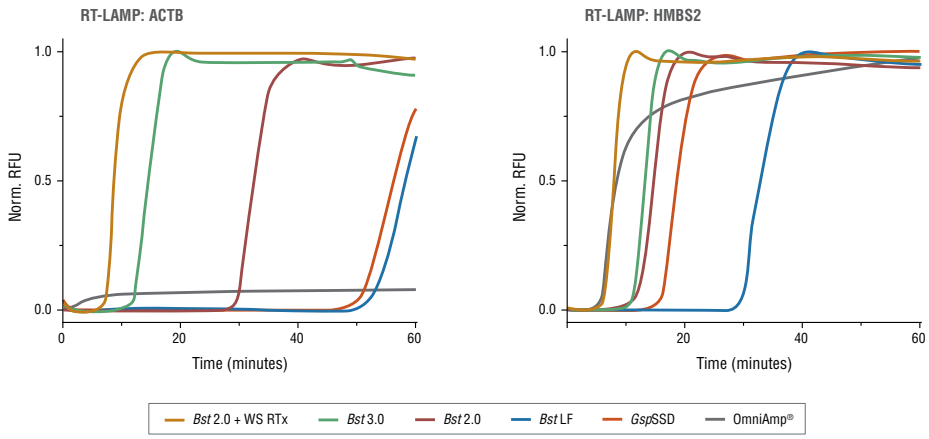
Validated for LAMP

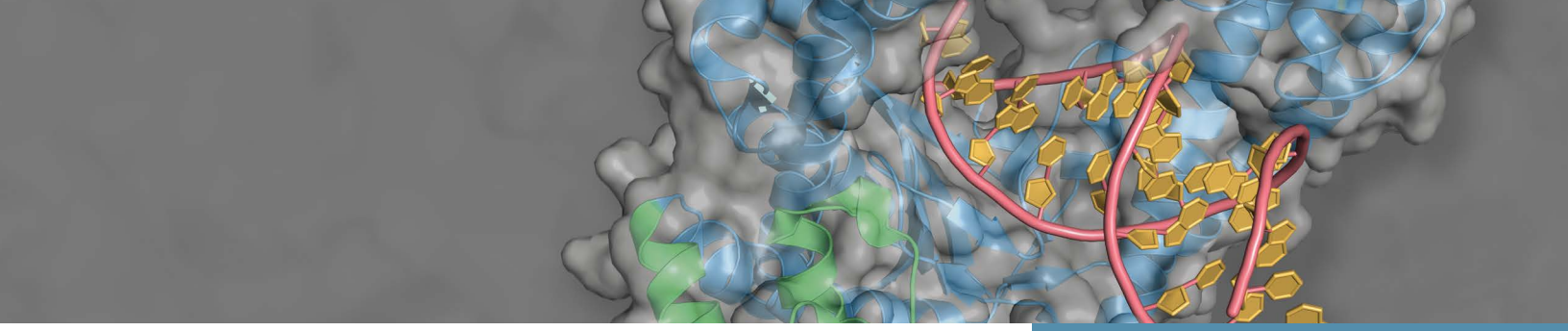
Bst 3.0 DNA Polymerase

Bst 3.0 DNA Polymerase (NEB #M0374) is an *in silico*-designed homolog of *Bacillus stearothermophilus* DNA Polymerase I, Large Fragment (NEB #M0275), engineered for improved isothermal amplification performance and increased reverse transcription activity. *Bst* 3.0 contains 5´→ 3´ DNA polymerase activity with either DNA or RNA templates and strong strand displacement activity, but lacks 5´→ 3´ and 3´ → 5´ exonuclease activity. *Bst* 3.0 demonstrates robust performance, even in high concentrations of amplification inhibitors, and features significantly increased reverse transcriptase activity compared to *Bst* DNA Polymerase.

FIGURE 1: **Fast, single-enzyme RT-LAMP can be performed using *Bst* 3.0 without additional reverse transcriptase**

RT-LAMP (reverse-transcriptase LAMP) was performed using indicated DNA polymerase and Jurkat total RNA and primers for two genes (ACTB, left; HMBS2, right). Fastest results were observed with a 2-enzyme system, *Bst* 2.0 DNA Polymerase and WarmStart RTx Reverse Transcriptase (WS RTx), but robust amplification was also observed using *Bst* 3.0 without additional RT. *Bst* DNA Polymerase, Large Fragment (*Bst* LF), *Bst* 2.0 DNA Polymerase and competitor enzymes showed highly variable performance, with slow threshold times or reaction failure on one of the two targets.





Validated for RT-LAMP

WarmStart RTx Reverse Transcriptase

WarmStart RTx Reverse Transcriptase (NEB #M0380) is a unique *in silico*-designed, RNA-directed DNA polymerase coupled with a reversibly-bound aptamer that inhibits RTx activity below 40°C. This enzyme can synthesize a complementary DNA strand initiating from a primer using RNA (cDNA synthesis) or single-stranded DNA as a template. RTx is a robust enzyme for RNA detection in amplification reactions and is particularly well-suited for use in loop-mediated isothermal amplification. The WarmStart property enables high throughput applications, room temperature setup, and increases the consistency and specificity of amplification reactions. RTx contains intact RNase H activity.

FIGURE 2: **WarmStart control of WarmStart RTx**

cDNA synthesis was performed for 10 minutes, followed by qPCR analysis. Resulting Cts were normalized to a "no RT" control for 0% activity and fastest Ct for 100% activity. WarmStart RTx is inhibited by a reversibly bound aptamer at temperatures below 40°C, and is fully active at temperatures 42°C and higher.

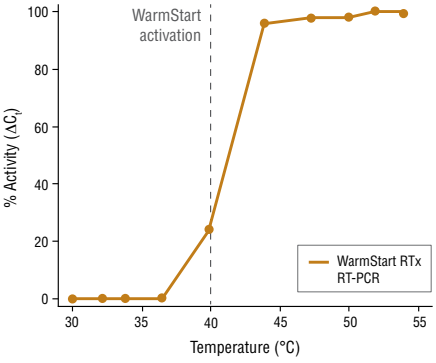
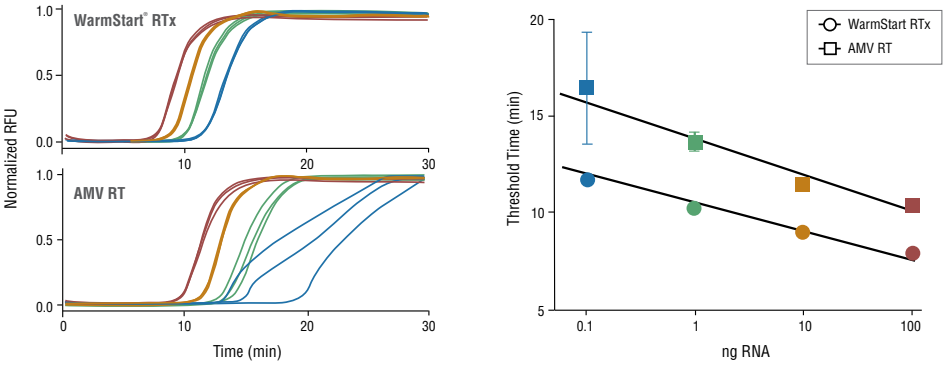


FIGURE 1:
WarmStart improves speed and sensitivity in RT-LAMP

RT-LAMP reactions with *Bst* 2.0 WarmStart DNA Polymerase and the indicated reverse transcriptase were incubated at 65°C with 1 pg – 100 ng of Jurkat total RNA. Reactions were monitored with real-time fluorescence, and resulting curves are shown (left), with corresponding threshold times (right). WarmStart RTx provides faster reaction threshold times for improved consistency and sensitivity with lower input RNA amounts. RT-LAMP reactions performed with AMV Reverse Transcriptase resulted in inconsistent detection, as indicated by wide variation at lower RNA input concentrations (blue curves).



ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
Bst 3.0 DNA Polymerase	M0374S/L/M	1,600/8,000/8,000 units
Bst 2.0 WarmStart DNA Polymerase	M0538S/M/L	1,600/8,000 units
Bst 2.0 DNA Polymerase	M0537S/M/L	1,600/8,000 units
Bst DNA Polymerase, Large Fragment	M0275S/M/L	1,600/8,000 units
Bst DNA Polymerase, Full Length	M0328S/L	500/2,500 units
WarmStart RTx Reverse Transcriptase	M0380S/L	50/250 rxns

Interested in learning more?

Interested in learning how NEB scientists are using isothermal amplification in their research?

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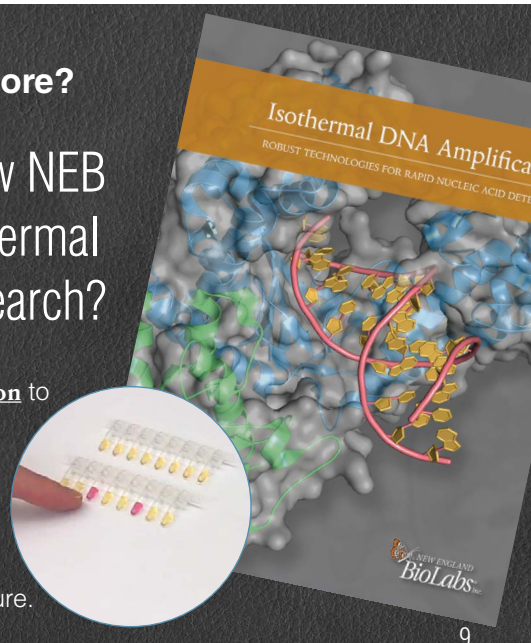


TECHNICAL TIPS

Easy Tips for Success with the NEB PCR Cloning Kit

Improve the success of your PCR cloning experiments by following these simple tips:

1 Be sure to follow the protocol

The protocol has been optimized to have a low background; if you have inadvertently deviated from the optimized protocol (e.g., extended ligation incubation, overly-concentrated outgrowth), compensate by plating less outgrowth medium (< 50 µl).

2 Use with blunt or single-base overhangs:

You can use the NEB PCR Cloning Kit to clone any fragment that has a blunt end or a single-base overhang.

3 Use an insert:vector ratio of 3:1

A higher insert:vector ratio can actually result in fewer colonies because inserts may ligate to both ends of the vector, preventing cloning.

4 Add the cloning mixes 1 and 2 to the reaction last

Some people try to save time by preparing a mix of water, cloning mixes and pMiniT, aliquoting this to tubes, then adding the inserts. This will lead to vector backbone depletion since the pMiniT would be recircularized by the ligase before the inserts were present, thus lowering your cloning efficiency. For convenience, you can mix the two cloning mixes together for your day's ligations.

5 Did you know that freezers not cold enough to actually freeze ligation samples can be a problem?

If ligation reactions remain liquid, low level end trimming/vector backbone recircularization can continue even at these cold temperatures, raising the background. To easily avoid this, if you wish to do your transformations at a later time, quick-freeze your ligation reactions with a dry ice/alcohol bath before transferring to a -20°C freezer.

6 Plate 50 µl or less of the 1 ml outgrowth

Plating too much of the outgrowth can increase background, and cause problems with colony PCR. If you need more colonies, spread 50 µl of outgrowth onto each of multiple plates.

7 Follow the transformation protocol carefully

The number of colonies will decrease significantly if you incubate the ligated DNA with the competent cells for less than 20 minutes, or if the outgrowth with SOC is less than 60 minutes.

8 Check compatibility with other strains

You can use other strains of high-efficiency competent *E. coli*, as long as they grow quickly. NEB Stable Competent *E. coli* (NEB #C3040) is an excellent choice for cloning direct and indirect repeats, and works well with the NEB PCR Cloning Kit. NEB Turbo Competent *E. coli* (NEB #C2984) and NEB Express Competent *E. coli* (NEB #C2523) are also good choices. NEB 5-alpha Competent *E. coli* (NEB #C2987) grows slowly, so the number of background colonies is higher than with NEB 10-beta Competent *E. coli* (NEB #C3019).

9 Do not incubate the transformation plates at room temperature

The slow growth rate of the cells at room temperature will increase the number of background colonies.

FEATURED PRODUCT

NEB PCR Cloning Kit

With or without competent cells

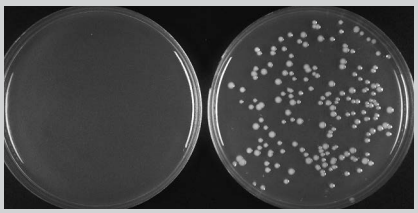
The NEB PCR Cloning Kit allows quick and simple cloning of all your PCR amplicons, regardless of the PCR polymerase used. Now available with or without competent cells, this kit utilizes a novel mechanism for background colony suppression – a toxic mini gene is generated when the vector closes upon itself – and allows for direct cloning from your reaction with no purification step. Enjoy faster cloning with more flexible conditions.

advantages

- Works with both **blunt** and **TA ends**
- Clone faster, with **low/no background**
- Get the colonies you need, with **high transformation efficiency**
- **No** need for **end-modification steps**
- **No blue/white screening** required
- Now available at a **lower price**

FIGURE 1: **PCR cloning with low/no background**

A 500 bp PCR product incubated with the linearized vector in a 3:1 ratio according to recommended protocol. 2 µl of reaction was transformed into the provided NEB 10-beta Competent *E. coli* and 1/20th of the outgrowth was plated. The left plate serves as the control, with vector backbone only. The right plate shows colonies containing the PCR insert.



ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
NEB PCR Cloning Kit	E1202S	20 rxns
NEB PCR Cloning Kit (without competent cells)	E1203S	20 rxns

APPLICATION NOTE

Improved library quantitation for a broad range of library types using the NEBNext Library Quant Kit for Illumina

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INTRODUCTION

Accurate quantitation of a next generation sequencing (NGS) library is essential for maximizing data output and quality from each sequencing run. qPCR is widely accepted as the most effective method for library quantitation, as it measures only sequenceable library fragments, with a high level of accuracy and consistency. The NEBNext Library Quant Kit for Illumina offers a simple, robust, qPCR-based method for the quantitation of libraries to be sequenced on the Illumina platform.

Here we demonstrate the effectiveness of the NEBNext Library Quant Kit for a broad range of library types and sizes, while also highlighting the advantages offered by qPCR quantitation for obtaining optimal cluster density and performance consistency.

RESULTS

For a method or kit to be a trusted way to quantitate libraries, the values obtained must not only be accurate but also consistent, both between libraries and between users.

qPCR accuracy

Electrophoretic quantitation methods such as the Agilent Bioanalyzer® instrument can provide information on library size, but for quantitation this method can be less accurate and consistent. Also, electrophoretic methods quantitate all DNA molecules present in a library, in contrast to qPCR which quantitates only molecules with an adaptor ligated to each end.

In this experiment, quantitation values were obtained for multiple replicates of libraries of different size and GC content (Figure 1). Concentrations of four libraries were determined by the NEBNext Library Quant Kit (orange) and compared to values measured by the Bioanalyzer (blue). Compared to NEBNext qPCR, Bioanalyzer concentrations displayed a greater level of variation, demonstrating the benefits of qPCR for library quantitation.

In addition to the method itself, consistency of the reagents involved and ease-of-use of the protocol both minimize variability. Replicates of 340–400 bp libraries from *E. coli*, *H. influenzae* and human (IMR-90) genomic DNA were quantitated by four different users with the NEBNext or Kapa Library Quant Kit (Universal). While both kits were able to successfully quantitate the various sample types, a marked improvement in quantitation consistency was observed using the NEBNext Library Quant Kit (see full application note for data).

Correlation with cluster density

The most relevant measure of accuracy of library quantitation is the density of clusters achieved after loading the recommended amount of library. If the quantitation value is too low, more library will be loaded, and over-clustering will result. In contrast, if the quantitation value is too high, less library than desired will be loaded, producing under-clustered samples. In this experiment, seven different libraries at a range of concentrations were quantitated using the NEBNext Library Quant Kit, then diluted to 8 pM and loaded into cluster generation was observed. A raw cluster density average of 1160 k/mm² for libraries prepared with the NEBNext Kit. This falls directly in the optimal range of 900–1300 k/mm² (see full application note for data).

Quantitation of a broad range of libraries

The consistent and reliable performance of a method or kit with a variety of libraries, in terms of GC content and insert size, is critical for practical utility. The ability of the NEBNext Library Quant Kit to accurately quantitate a wide range of library types was tested by using libraries from 10 different sources, including human and microbial DNA, of high GC and high AT content, and a broad range of library sizes (150 – 963 bp) (Figure 2). In all cases, optimal cluster density was achieved using concentrations determined by the NEBNext Library Quant Kit.

Furthermore, the NEBNext Library Quant Kit has been used to successfully quantitate libraries from 20–70% GC, with a broad range of sizes, made with several library prep kits, including NEBNext, Illumina TruSeq® Nano and Kapa Hyper library prep kits (see full application note for data).

CONCLUSION

The NEBNext Library Quant Kit provides accurate and reliable qPCR-based library quantitation of Illumina libraries, as shown by the production of optimal cluster densities. The NEBNext kit demonstrates improved reproducibility and consistency when compared to alternative methods and kits. Furthermore, this kit can successfully quantitate samples from a wide variety of sample types, as well as a broad range of sizes and GC-content.

FIGURE 1: qPCR provides more consistent library quantitation results than Bioanalyzer analysis

Concentrations of four libraries were determined by the NEBNext Library Quant Kit (orange) and compared to values measured using the Aglient Bioanalyzer (blue).

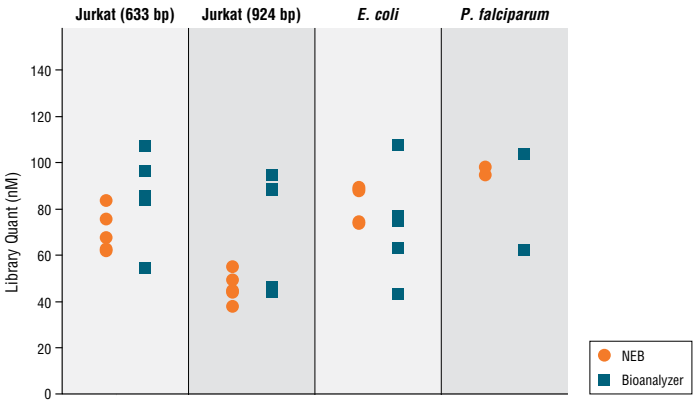
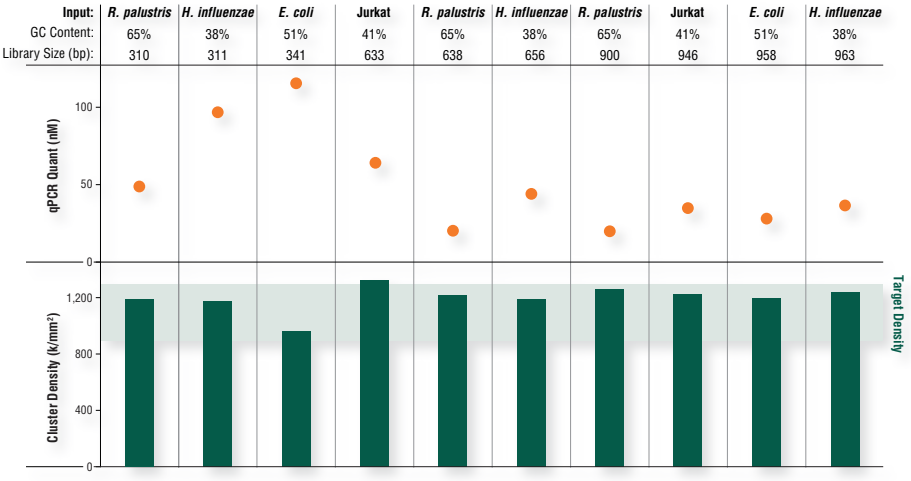


FIGURE 2: NEBNext Library Quant Kit delivers accurate quantitation for a variety of sample types and sizes

Libraries of 310–963 bp from the indicated sources were quantitated using the NEBNext Library Quant Kit, then diluted to 8 pM and loaded onto a MiSeq (v2 chemistry; MCS v2.4.1.3). Library concentrations ranged from 7–120 nM, and resulting raw cluster density for all libraries was 965–1300 k/mm2 (ave. =1199). Optimal cluster density was achieved using concentrations determined by the NEBNext Library Quant Kit for all library sizes.



Interested in learning more?

Visit **www.neb.com/E7630** to **download the full application note**, which includes figures not shown.

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