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The NovaSeq™ Xp workflow provides flexibility and control without sacrificing data quality or yield

Without compromising on data quality or yield, the NovaSeq Xp workflow delivers the flexibility of independent lane loading and higher library multiplexing per flow cell.

Introduction

The NovaSeq Xp workflow adds a level of flexibility and control to experimental planning and flow cell configuration on the NovaSeq 6000 System. By enabling individual lane loading, the Xp workflow supports the ability to run incompatible library types on the same flow cell (eg, long and short insert libraries in separate lanes). The Xp workflow also supports a reduction in the amount of library required and higher multiplexing per flow cell (96-plex library pools with an S4 flow cell amounts to 384 libraries per run) compared to the standard workflow.

While the Xp workflow provides significant advantages, there are important operational differences between the standard and Xp workflows (Figure 1). With the Xp workflow, preparation of the ExAmp master mix and combining of the master mix with denatured libraries are performed manually. Furthermore, the ExAmp–library mixtures are manually loaded onto the flow cell, lane by lane. This contrasts with the standard workflow, where denatured libraries are loaded into the NovaSeq reagent cartridge and delivery of the ExAmp–library mixture to the flow cell is automated onboard the NovaSeq System. As a result, with the standard workflow, ExAmp staging times are minimized and hard-coded into the instrument workflow. Conversely, with the Xp workflow, unenforced staging times can vary by user due to manual execution of the process.

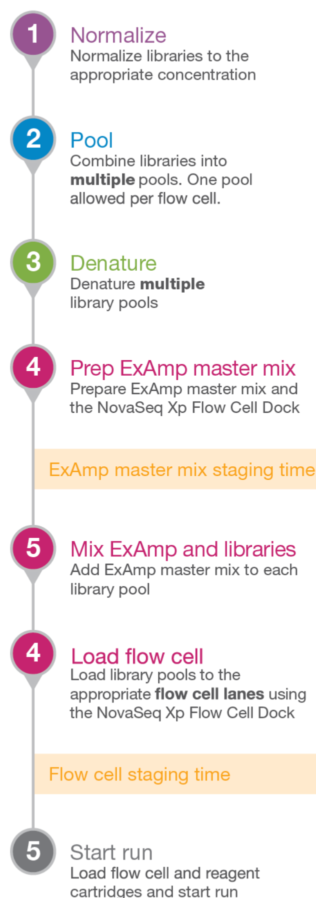
Staging time: The time between the completion of one step and starting the next step. For example, flow cell staging time refers to the time a flow cell sits loaded with the ExAmp mixture before starting the sequencing run.

In addition to these operational differences, the standard and Xp workflows require different flow cell loading concentrations and display slight differences in certain sequencing metrics. To provide insight into the differences between the two workflows, libraries were prepared from the same DNA sample using the standard and Xp workflows. This technical note demonstrates how the standard and Xp workflows deliver equivalent data yield and quality and highlights a series of best practices designed to optimize the performance of the Xp workflow.

Methods

Library preparation and sequencing

Libraries were prepared from Coriell NA12878 genomic DNA using the TruSeq™ DNA PCR-Free Library Preparation Kit (Illumina, Catalog No. FC-121-3001) and the TruSeq Nano DNA Library Preparation Kit (Illumina, Catalog No. 20015964). After library quantitation, libraries were prepared for loading according to the NovaSeq Xp workflow with the NovaSeq 6000 S4 Reagent Kit (Illumina, Catalog No. 20012866) or the standard workflow with the NovaSeq 6000 S2 Reagent Kit (Illumina, Catalog No. 20012860). All



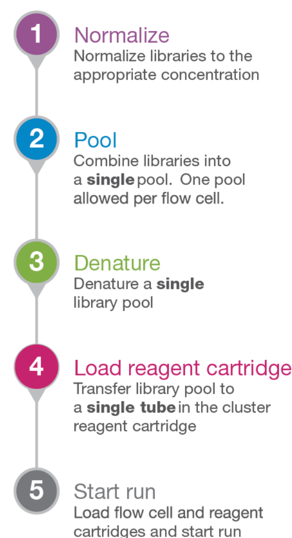


Figure 1: Comparison of NovaSeq Xp and standard workflows—In comparison to the standard workflow, the Xp workflow supports individual lane loading and includes several additional manual touchpoints. These additional steps create staging times (ExAmp master mix staging time and flow cell staging time) that do not exist in the standard workflow and may vary according to automation protocols or user handling.

runs were performed on a NovaSeq 6000 System with a run configuration of 2 × 151 bp.

Data analysis

Data files generated by the NovaSeq 6000 System were aligned against the human reference genome GRCh38.[1] Primary data analysis metrics, including percentage of reads passing filter (%PF Reads), percentage of duplicate reads passing filter (%Duplicate Reads), percentage of reads that do not pass filter (%Non-PF Reads), and percent nanowell occupancy (%Occupancy) were calculated with Real-Time Analysis Software[2] and Sequencing Analysis Viewer.[3]

Results

NovaSeq standard and Xp workflows produce equivalent yield of usable reads

Users may observe elevated duplicate read levels when running the Xp workflow relative to the standard workflow. However, when library loading concentration is optimal, an increase in the %Duplicate Reads is typically compensated for by a higher %PF Reads in the Xp workflow (Figure 2). In contrast, the standard workflow typically generates a higher %Non-PF Reads and a lower %Duplicate Reads. Therefore, in sum, the standard and Xp workflows produce equivalent levels of usable reads passing filter (%Usable Reads) when the percentages of Non-PF Reads and Duplicate Reads are subtracted from the total read set.

%Usable Reads: The percentage of reads passing filter after Non-PF Reads and Duplicate Reads have been subtracted.

NovaSeq standard and Xp workflows produce equivalent high-quality data

To assess data quality resulting from the NovaSeq standard and Xp workflows, libraries prepared from each workflow were sequenced on the NovaSeq System. Four lanes of sequencing data from each library type were analyzed to calculate quality scores (Q-scores).

Q-score: A prediction of the probability of an error in base calling. It serves as a compact way to communicate small error probabilities.

A Q-score of 30 (Q30) corresponds to a 0.1% error rate in base calling, and is widely considered a benchmark for high-quality data.[4] A comparison of Q30 metrics show that the standard and Xp workflows generate equivalent percentages of high-quality sequencing data (Figure 3).

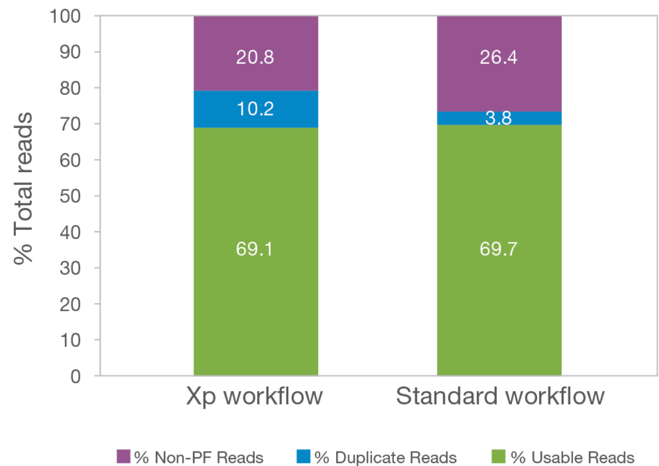


Figure 2: Comparison of NovaSeq standard and Xp workflow sequencing metrics—The Xp workflow data exhibits higher %Duplicate Reads than observed in the standard workflow, while the standard workflow produces higher %Non-PF Reads. Despite these differences, the fraction of Usable Reads remains equivalent between the two workflows.

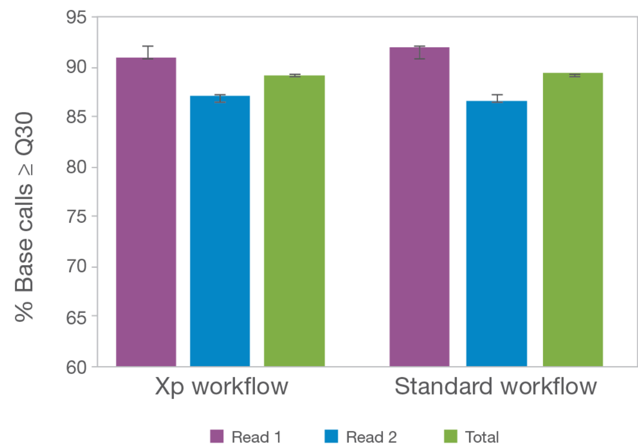


Figure 3: Comparison of NovaSeq standard and Xp workflow data quality results—The graph illustrates the percentage of base calls in the data set with a Q-score of 30 or higher. The results show that both Read 1 and Read 2 data for the standard and Xp workflows produce equivalent levels of high-quality sequencing data.

Optimization of the NovaSeq Xp workflow

While the NovaSeq standard and Xp workflows produce equivalent yield and high-quality data, it is important to understand the operational differences between the two workflows. Because the Xp workflow includes several manual staging steps, it is helpful to be aware of best practices for minimizing staging times and improving consistency. Here we highlight best practices for optimization of the Xp workflow.

Optimization of ExAmp master mix staging time

The manual preparation of ExAmp master mix creates a potential staging point for the Xp workflow that does not exist under the standard workflow. After the ExAmp master mix reagents are combined and mixed, the workflow recommends proceeding directly to addition of denatured library and to flow cell filling. However, certain considerations such as automation steps or reagent handling time may prevent this. If staging of the ExAmp master mix is required, the preferred method is to place the master mix on ice. ExAmp master

mix stored on ice is stable for up to 60 minutes without an appreciable decrease in performance (Figure 4). Storage at room temperature should not exceed 30 minutes. Room temperature storage longer than 30 minutes can have a negative impact on data quality and yield.

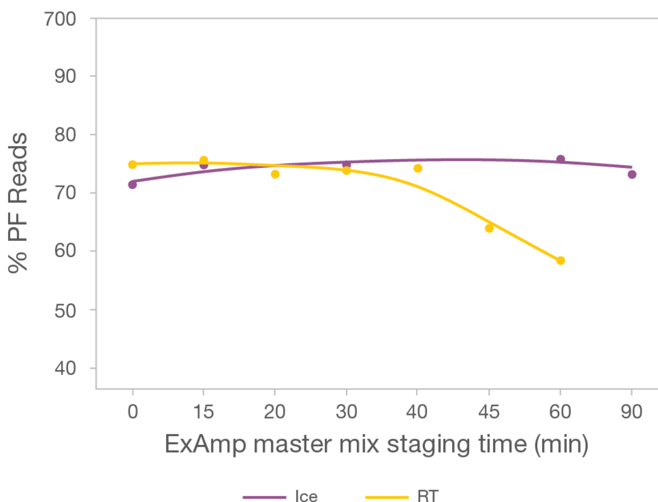


Figure 4: Impact of ExAmp master mix storage temperature and staging time—ExAmp master mix is stable for up to 60 minutes when stored on ice and stable up to 30 minutes when stored at room temperature.

Optimization of flow cell staging time

After pooled libraries and ExAmp master mix are combined, they must be manually loaded onto the appropriate lane of the flow cell. After the flow cell is loaded, Illumina recommends starting the run as soon as possible to minimize flow cell staging time. Increased flow cell staging times can result in decreased yield, a lower %PF Reads, and higher %Occupancy. Therefore, as a general rule, the maximum recommended flow cell staging time is 30 minutes.

%Occupancy: The percentage of flow cell nanowells that contain a cluster (or clusters). %Occupancy differs from %PF Reads in that it signifies the total percentage of amplified nanowells, not whether a nanowell passes filtering. Occupied wells can fail filtering if multiple DNA strands produce multiple clusters within the same well.

It is important to note that sensitivity to flow cell staging time varies by library type. For example, PCR-amplified libraries (prepared with the TruSeq Nano DNA Library Prep Kit) are quite robust for up to 60 minutes of flow cell staging time, while PCR-free libraries (prepared with the TruSeq DNA PCR-Free Library Prep Kit) exhibit a significant sensitivity to flow cell staging time (Figure 5). Although both library types produced a decrease in %PF Reads and an increase in %Occupancy with increasing flow cell time, the PCR-free library had a more dramatic response.

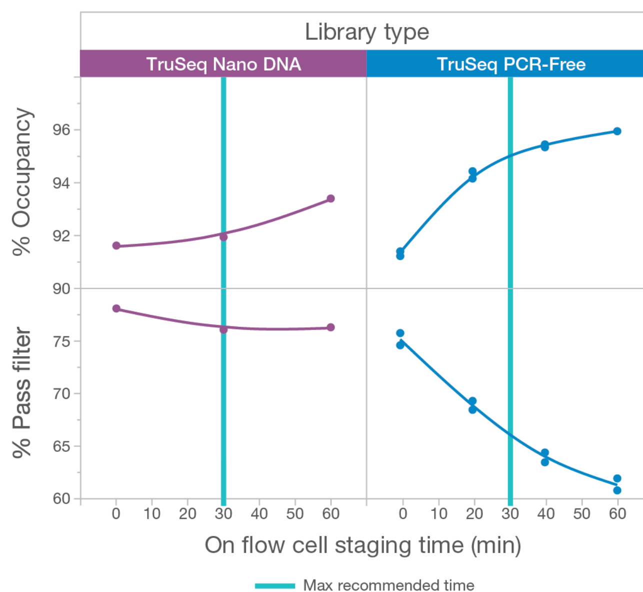


Figure 5: Increased flow cell staging time and library types—The graph shows the relationship between increasing levels of flow cell staging time and two sequencing metrics: %PF Reads and %Occupancy. PCR-amplified libraries remain robust to extended staging while PCR-free libraries exhibit heightened sensitivity. The light blue vertical lines represents the maximum recommended staging times. Different library types may result in varied sensitivity relative to those shown here.

Optimization of library loading concentration

Different library types as well as different workflows may result in different responses to library loading concentration (Figure 6). The Xp workflow generally requires a lower DNA input than observed for the standard workflow. Despite these differences, the Usable PF between the standard and Xp workflows is equivalent when input amount is optimized. Careful and proper optimization of library loading concentration is critical for avoiding depressed %PF Reads or elevated %Duplicate Reads. For more on workflow specific loading instructions, see the NovaSeq 6000 Sequencing System Guide.

Additionally, Illumina recommends performing library concentration titration and optimization experiments in a setting that is consistent with the final intended workflow. This specifically applies if enforced staging times are inherent to the workflow (eg, due to automation or transport requirements). Maintaining consistency in experimental workflows will maximize the performance of the NovaSeq 6000 System and the sequencing results in Xp mode.

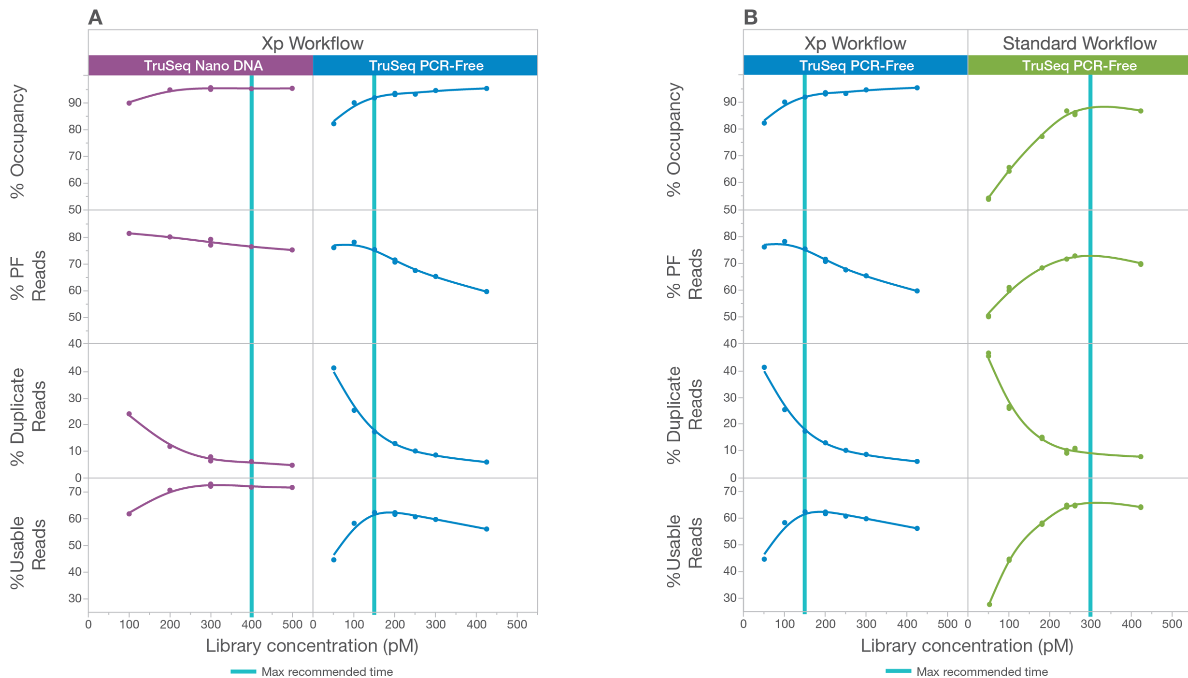


Figure 6: DNA titrations of TruSeq Nano and PCR-Free libraries with the standard and Xp workflows—(A) Response to increased library input differs according to library type. %Occupancy rises for both as library concentration increases. The TruSeq PCR-Free library is more sensitive to overloading the flow cell, as shown by the %PF Reads and %Usable Reads curves. (B) Response to increased library input also differs between the standard and Xp workflow. Xp loaded flow cells generally require a lower library input concentration to maximize performance compared to the standard workflow. Although %PF Reads and %Duplicate Reads may differ between workflows, %Usable Reads remains equivalent when library loading concentration is optimized.

Summary

Users can confidently choose between the NovaSeq standard and Xp workflow without compromising on data quality or quantity. The Xp workflow was designed to enable individual lane loading for a variety of library pool combinations on the NovaSeq 6000 System. Although the standard and Xp workflows show slight differences in sequencing metrics such as %Non-PF Reads and %Duplicate Reads, these differences do not impact sequencing data quality or data yield. By following the best practices described in this technical note, users can optimize instrument run performance and maintain repeatability between runs to advance their research goals.

References

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3. SAV. support.illumina.com/sequencing/sequencing_software/sequencing_analysis_viewer_sav.html. Accessed June 18, 2018.
4. Illumina (2011). Quality Scores for Next-Generation Sequencing. Accessed June 18, 2018.

Xp workflow best practices

- Immediately after preparing ExAmp master mix, add library pools. If staging is required, ExAmp master mix stored on ice is stable for up to 60 minutes. Storage at RT should not exceed 30 minutes.
- Start run immediately after loading libraries onto the flow cell, or within 30 minutes.
- Optimize DNA loading concentration according to workflow type (standard vs. Xp), library type, and insert size.
- Perform optimization experiments under conditions consistent with the final intended workflow (eg, robotic wait times, sample transport times).

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