

Data by Cycle: % > Q30

Intensity drops and/or lower Q30 scores are the most common ways overclustering can be detected.

Overclustering can affect either Read 1 or Read 2, but Read 2 is commonly more severely affected. This is because during paired-end (PE) chemistry, cluster sizes increase slightly due to extra cycles of amplification, which can lead to an increase in the number of overlapping clusters. With overclustered flow cells, this can affect run image registration and lead to poor Q30 scores and possible run failures (Figure 2).

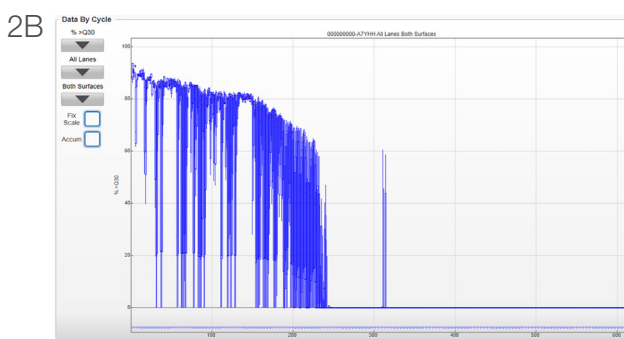
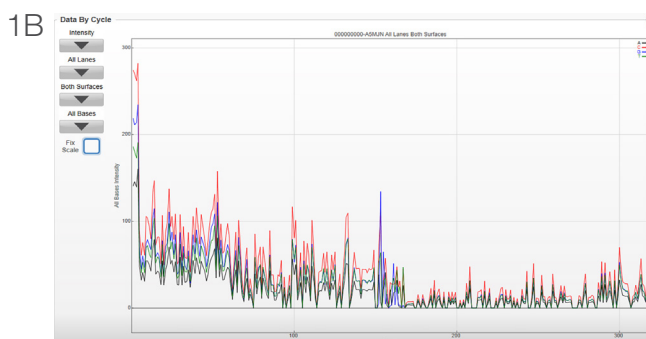
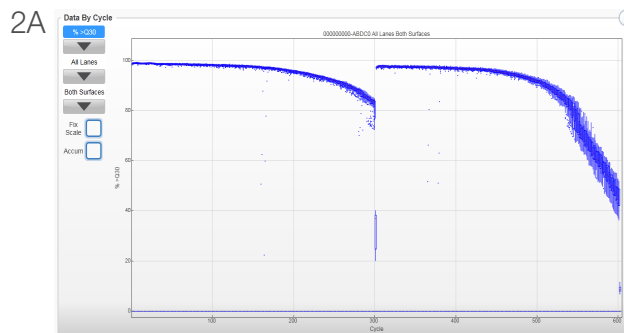
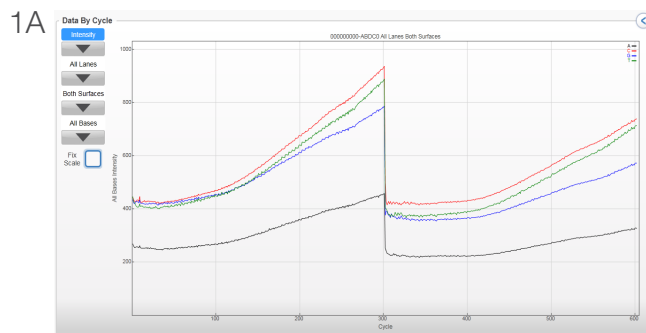


Figure 1: Data by Cycle: Intensity. A) Intensity profile from a normally clustered flow cell. B) Intensity plot shows midrun failure due to an overclustered flow cell.

Figure 2: Data by Cycle: % > Q30. A) % > Q30 profile from a normally clustered flow cell. B) % > Q30 profile shows large standard deviations leading to a run failure due to an overclustered flow cell.

Data by Lane: Density

Data by Lane: Density box plots compare the raw cluster density to the %PF cluster density (Figure 3). With optimal density, the raw cluster density and %PF box plots appear close to one another (Figure 3A). As the cluster density increases beyond optimal density, the %PF decreases and the box plots appear further apart (Figure 3B). Also, clusters will not be identified correctly, which can result in underestimation of the raw cluster density. With severe overclustering, no clusters passed filter and the %PF plot is displayed as a green line at 0 density (Figure 3C).

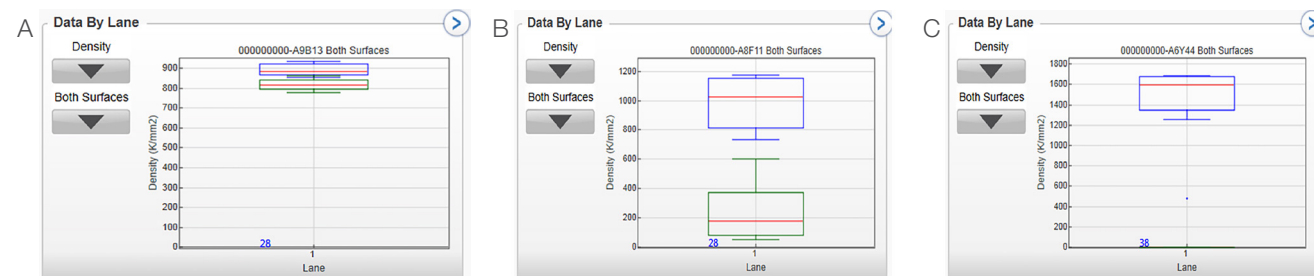
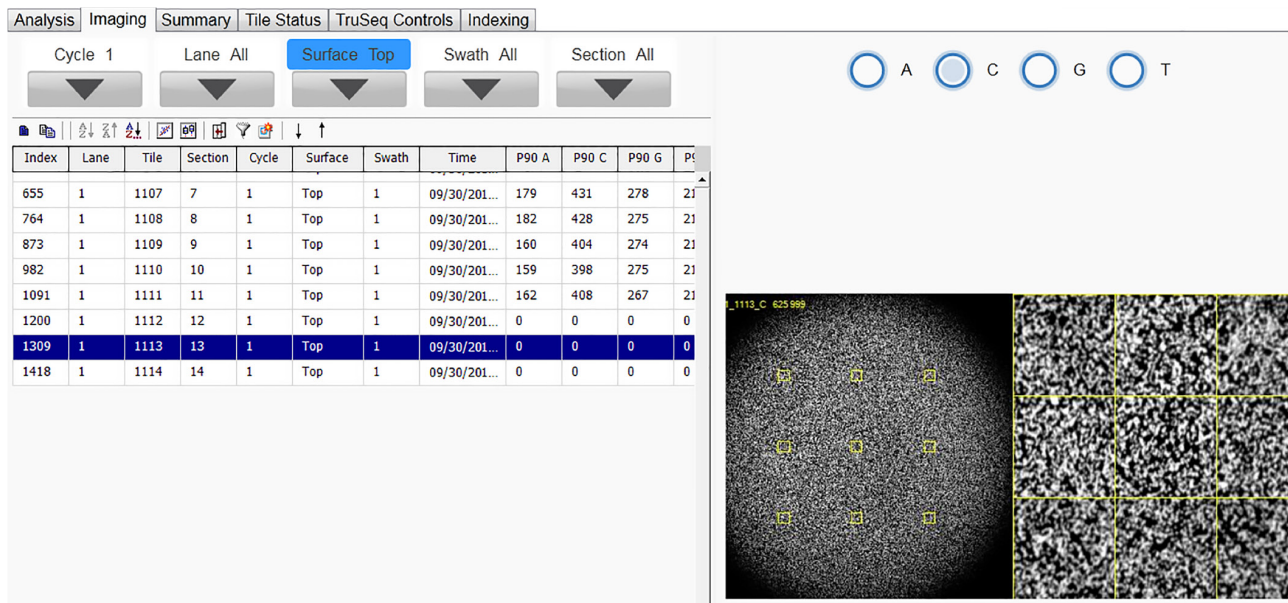


Figure 3: Data by Lane: Density. The blue boxes illustrate the raw cluster density range, the green boxes illustrate the %PF cluster density range, and the red lines indicate the median cluster density values. A) Optimal density. B) Overclustered. C) Severely overclustered.

Imaging Tab Metrics Table

Issues with cluster registration can also be diagnosed with the **Imaging Tab Metrics Table**. For each tile, SAV generates a report of run metrics. The P90 A, C, G, and T metrics show the intensity values extracted from each cluster. With optimal clustering, they will reflect numeric intensity values. With overclustering, 0s and/or NaNs (not a number) can be reported in these fields even though clusters are visible in the thumbnail images. This is an indication that image extraction failed due to overclustering. Example run metrics tables for the MiSeq (Figure 6A) and the HiSeq 2500 (Figure 6B) are shown.

A



B

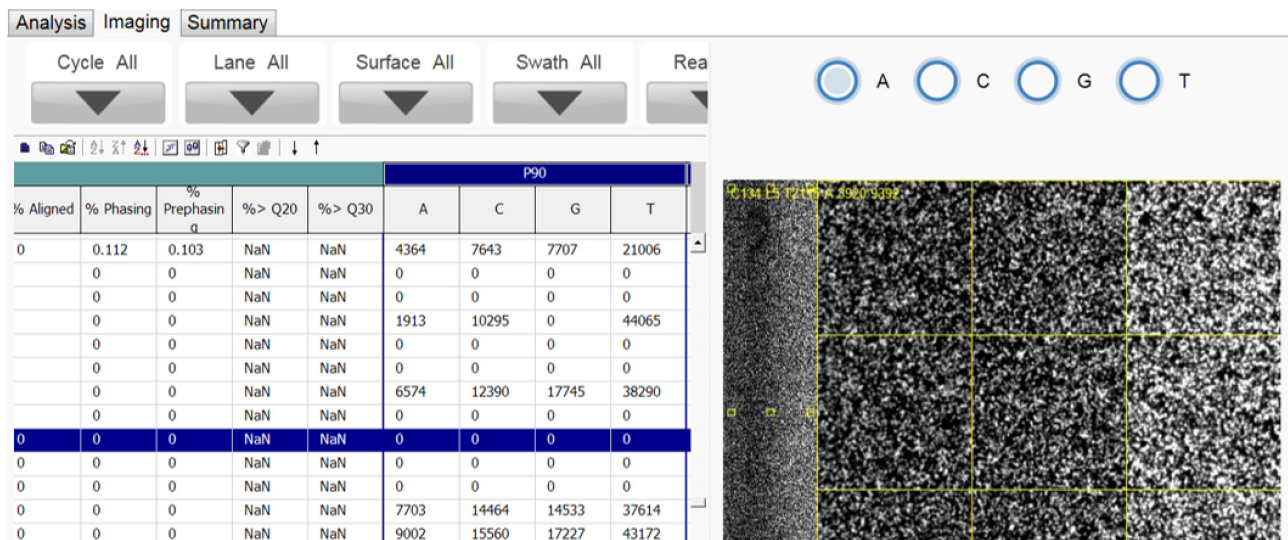


Figure 6: Run Metrics. (A) An example run metrics table for the MiSeq is shown. (B) An example run metrics table for the HiSeq 2500 is shown. "0" and "NaN" values indicate that the software was unable to extract intensity values.

c. The Summary Tab

After cycle 25 of Read 1, the following run metrics in the **Summary** tab can be used to check if the run is overclustered:

- **Clusters PF**—Reported percentages are below 80%. This value is not an official specification for this metric from Illumina; however, it can be used as a general guideline.
- **Raw Density**—Reported raw density value exceeds cluster density range recommended by Illumina.* Alternatively, reported raw density can be an underestimation of the true density due to saturation.
- **% Aligned**—If the reported percentage of reads aligning to PhiX is significantly lower compared to what was spiked-in, it is possible that the starting concentration of the libraries were underestimated, leading to overclustering.

Learn More

For more information, consult the “Sequencing Analysis Viewer (SAV) Software Guide.”¹

IV. Common Causes of Under- and Overclustering and Strategies for Prevention

a. Library Quality

Insufficient library clean-up steps can lead to the presence of library preparation contaminants such as adapter dimers, primer dimers, or partial library constructs that can greatly affect library quantification and subsequent clustering efficiency. Verification of all libraries for quality and purity using the Bioanalyzer or similar technology will check for library integrity, average insert size, and the presence of contamination. Detailed instructions regarding quality assessment with the Bioanalyzer are included in the Illumina library preparation user guides.

Sample Denaturation Considerations:

Preparing DNA samples for cluster generation requires denaturation with sodium hydroxide (NaOH). Special consideration should be given, as 2 factors can adversely affect cluster generation and density:

- NaOH used for sample denaturation must be freshly diluted with a pH > 12.5. NaOH that is not freshly diluted can acidify; the resulting decrease in pH will impair DNA denaturation and result in lower cluster density.
- Excess NaOH concentrations (> 1 mM) in diluted samples inhibits the formation of clusters.

For optimal cluster density, denature samples with freshly diluted NaOH (pH > 12.5) and make sure that the final concentration of NaOH in diluted samples is < 1 mM. If the diluted library must have a final concentration of NaOH > 1 mM, use Tris-HCl to neutralize the pH, as indicated in system specific denature and dilution guid^{2, 2-5}

b. Library Quantification

The most common cause of under- and overclustering is inaccurate library quantification. While this section provides several recommendations for accurate library quantification, some library preparation protocols include a final bead-based normalization step and do not require additional quantification steps. The choice of library quantification methods will depend on the type of Illumina library preparation kit used and access to quantification resources.

Recommended Quantification Methods:

- **qPCR**—qPCR is the most effective method of library quantification when paired with a standard of similar size range. This technique is powerful as it measures only functional library fragments rather than all DNA species within a sample (eg primer dimers, free nucleotides, library fragments). However, this method may not be appropriate for quantifying libraries that have a broad insert size. For more information on library quantification with qPCR, see the “Sequencing Library qPCR Quantification Guide and Nextera® Library Validation and Cluster Density Optimization Technical Note.”^{6,7}

* Cluster density recommendations are listed on page 8.

Sequencing a New or Unknown Library

If a library is new or has unknown nucleotide diversity, choose a more conservative loading concentration (ie target the lower end of the recommended cluster density range). Spiking in a system-specific amount of PhiX is also good practice with libraries of unknown nucleotide diversity.

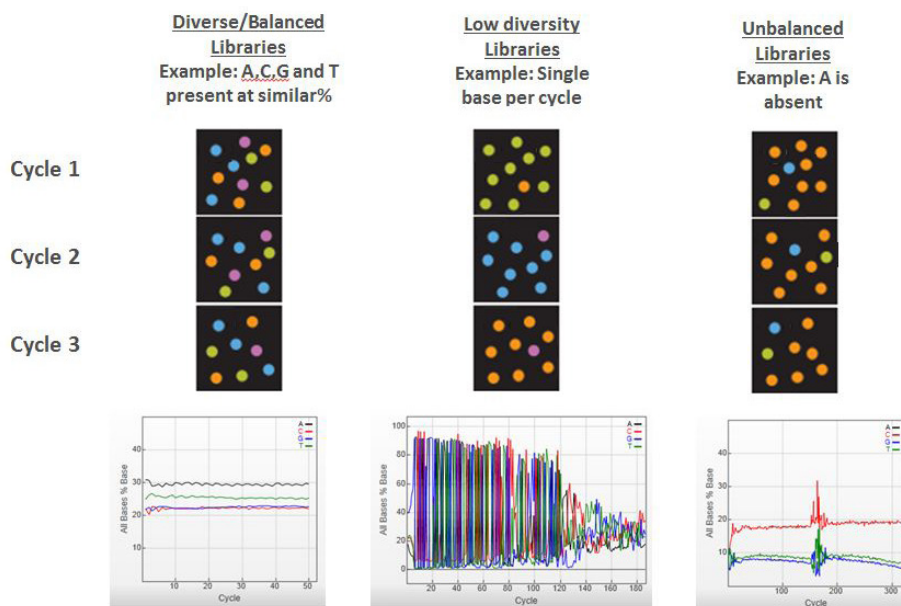


Figure 8: Nucleotide Diversity and Data by Cycle: % Base Plots. A) Diverse/balanced libraries contains equal proportions of A, T, C, and G nucleotides. The Data by Cycle: % Base plot shows even, horizontal curves centered on 25%. B) Low-diversity libraries, such as PCR amplicon, metagenomic, enriched/targeted, or ChIP libraries, have an uneven proportion of nucleotides across the flow cell from one cycle to the next. The Data by Cycle: % Bases plot shows large intensity spikes at each cycle. C) Unbalanced libraries, such as bisulfite converted libraries, have one base at a much lower percentage than the others. This example shows a library with a low percentage of the "A" nucleotide.

V. Summary

Cluster density optimization is a critical step for the generation of high-quality data and high yield runs. Under- and overclustering can be largely avoided by taking precautions early on, such as careful library quantification (using recommended quantification methods), assessing the library quality, and performing mitigations for low diversity libraries. Empirical testing may be needed to determine the best library concentration range to meet the Illumina recommended cluster density range. When the run is in progress, understanding how overclustering affects SAV run metrics, thumbnail images, and summary data allows real-time monitoring of run quality. Diagnosing overclustering early in the run, aborting runs when necessary, or quickly identifying the root cause of run failures will save valuable time and effort, allowing researchers to meet their goals with greater speed and efficiency.

VI. Glossary

Image Registration: Intensity values are extracted during a process called “image registration.” During image registration, fluorescence is converted to a numeric intensity value and assigned to an X, Y-position. This “map” of cluster positions is applied to the entire run.

Passing Filter: During cycles 1–25 of Read 1, the chastity filter removes the least reliable clusters from the image extraction results. Clusters “pass filter” if no more than 1 base call has a chastity value below 0.6 in the first 25 cycles. Chastity is defined as the ratio of the brightest base intensity divided by the sum of the brightest and the second brightest base intensities.⁴

Template Generation: Template generation involves analyzing images from the first 4–5 cycles of a run to map the location of each individual cluster. This is referred to as the “cluster template” or “cluster map.” By detecting clusters from multiple cycles, there is a better chance of resolving overlapped clusters.

VII. References

1. Illumina (2016) Sequence Analysis Viewer v1.11 software guide. (support.illumina.com/sequencing/sequencing_software/sequencing_analysis_viewer_sav/documentation.html)
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3. Illumina (2016) MiSeq system denature and dilute libraries guide. (support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-01.pdf)
4. Illumina (2015) NextSeq system denature and dilute libraries guide. (support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/nextseq/nextseq-denature-dilute-libraries-guide-15048776-02.pdf)
5. Illumina (2016) HiSeq and GA_{MX} systems denature and dilute libraries guide. (support.illumina.com/downloads/hiseq-denature-dilute-libraries-guide-15050107.html)
6. Illumina (2011) Sequencing library qPCR quantification guide. (support.illumina.com/downloads/sequencing_library_qpcr_quantification_guide_11322363.html).
7. Illumina (2013) Nextera library validation and cluster density optimization technical note. (www.illumina.com/documents/products/technotes/technote_nextera_library_validation.pdf).
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