APPLICATION NOTE

Mitigate index hopping and spread of signal on patterned flow cells.

Key features

- Unique dual index adapters for multiplexing up to 1,536 Illumina® sequencing libraries
- Mitigate index hopping or spread of signal that can occur during sequencing
- Decreases level of mis-assigned reads in sequencing data
- Compatible with both paired-end and single-read Illumina® sequencing

Introduction

The NEXTFLEX® unique dual index barcodes are barcoded adapters for sequencing on Illumina® platforms that provide unprecedented data security in sequencing applications. Increased mis-assignment of indexes has been shown to occur on Illumina® sequencing instruments that feature a patterned flow cell and exclusion amplification technology [1]. The NEXTFLEX® unique dual index barcodes are designed to specifically mitigate the index hopping or spread of signal phenomenon associated with Illumina® platforms that utilize a patterned flow cell [2]. Index mis-assignment can lead to increased false positive rates, which are especially detrimental to sensitive applications. Multiplexing with NEXTFLEX® unique dual index barcodes drastically increases processing capacity while reducing costs by allowing the user to pool multiple libraries in a single flow cell lane.

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Methods

A set of libraries was prepared using the NEXTFLEX® rapid DNA library prep kit with unique, distinct DNA amplicons as starting material. NEXTFLEX® unique dual index barcodes were added during ligation and libraries were sequenced on the Illumina® MiSeq® and HiSeq® 4000 sequencing platforms.

An additional set of libraries was prepared in the same method as the previous experiment. Half of each library was pooled before amplification, while the other half of each library was amplified prior to pooling. Each pool was sequenced on a separate lane of a HiSeq® 4000 platform, and on two separate runs using a MiSeq® sequencing platform.

After sequencing, demultiplexing was performed using both I5 & I7 indexes, and then using only the I7 index. Purity rates from both demultiplexing types and sequencing chemistries were compared to demonstrate the effectiveness of the NEXTFLEX® unique dual index barcodes in preventing read mis-assignment on both sequencing platforms.

Samples Demultiplexed via Unique 8nt i7 Index

	1	2	3	4	5	6	7	8
1	99.7	0	0	0.01	0	0	0	0
2	0	100	0.01	0	0	0	0	0
3	0.01	0	99.97	0	0	0	0	0
4	0.01	0	0.01	99.98	0	0	0	0
5	0	0	0	0	99.99	0	0.01	0
6	0	0	0.01	0.01	0.01	100	0.02	0
7	0.01	0	0	0	0	0	99.97	0.01
8	0	0	0	0	0	0	0	99.99

Samples Demultiplexed via Unique 8nt i7 Index and Unique 8nt i5 Index

	1	2	3	4	5	6	7	8
1	100	0	0	0	0	0	0	0
2	0	100	0	0	0	0	0	0
3	0	0	100	0	0	0	0	0
4	0	0	0	100	0	0	0	0
5	0	0	0	0	100	0	0	0
6	0	0	0	0	0	100	0	0
7	0	0	0	0	0	0	100	0
8	0	0	0	0	0	0	0	100

Figure 1: **NEXTFLEX® unique dual index barcodes decrease index mis-assignment on the Illumina® MiSeq® sequencing platform.** A set of libraries prepared NEXTFLEX® unique dual index barcodes was sequenced on the MiSeq® sequencing platform. The numbers indicate percentage of correct insert reads assigned to index sequences. The resultant data were demultiplexed twice: first by taking only the unique I7 index into account (left panel), and second by taking both unique I7 and I5 indexes into account (right panel). By assessing both the unique I7 and I5 indexes, there was no detectable sequence mis-assignment.

Samples Demultiplexed via Unique 8nt i7 Index

	1	2	3	4	5	6	7	8
1	99.84	0.02	0.02	0.04	0.02	0.02	0.07	0.08
2	0.03	99.82	0.02	0.06	0.03	0.02	0.21	0.09
3	0.03	0.03	99.86	0.07	0.02	0.03	0.11	0.1
4	0.02	0.03	0.02	99.58	0.02	0.02	0.09	0.09
5	0.03	0.03	0.03	0.09	99.85	0.04	0.13	0.09
6	0.02	0.02	0.01	0.05	0.02	99.83	0.08	0.06
7	0.01	0.02	0.01	0.02	0.01	0.01	99.15	0.04
8	0.03	0.04	0.02	0.09	0.03	0.03	0.16	99.46

Samples Demultiplexed via Unique 8nt i7 Index and Unique 8nt i5 Index

	1	2	3	4	5	6	7	8
1	100	0	0	0	0	0	0	0
2	0	99.9	0	0	0	0	0	0
3	0	0.01	100	0	0	0.01	0	0
4	0	0	0	100	0	0	0	0
5	0	0	0	0	100	0	0	0
6	0	0	0	0	0	99.9	0	0
7	0	0	0	0	0	0	100	0
8	0	0	0	0	0	0	0	100

Figure 2: **NEXTFLEX® unique dual index Barcodes decrease index mis-assignment on the Illumina® HiSeq® 4000 platform.** A set of libraries prepared using NEXTFLEX® unique dual index barcodes was sequenced on the HiSeq® 4000 sequencing platform. The numbers indicate percentage of correct insert reads assigned to index sequences. The resultant data was demultiplexed twice: first by taking only the unique I7 index into account (left panel), and second by taking both unique I7 and I5 indexes into account (right panel). By assessing both the unique I7 and I5 indexes, sequence mis-assignment was drastically decreased.

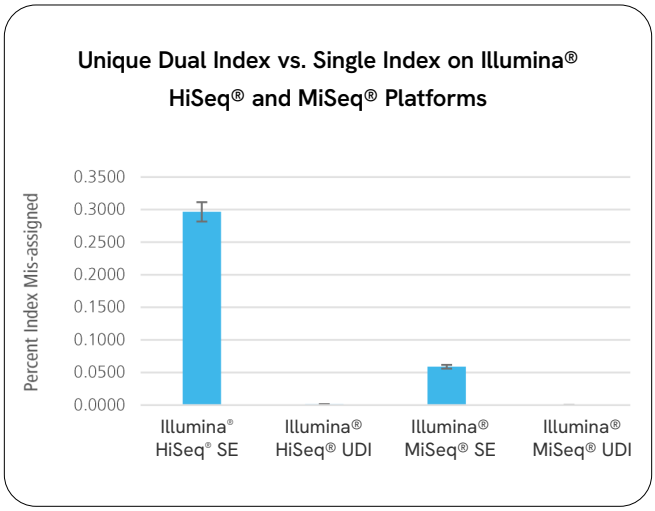

Figure 3: **NEXTFLEX® Unique Dual Index Barcodes increase confidence in sequencing data.** The data generated on the HiSeq® 4000 platform showed a dramatic reduction in the percentage of mis-assigned reads, and the data generated on the MiSeq® platform also showed a reduction. The results indicate that the NEXTFLEX® unique dual index barcodes greatly reduce the amount of mis-assigned reads in data sets, and that this occurs at a higher rate on the instruments that use exclusion amplification chemistry and patterned flow cells.

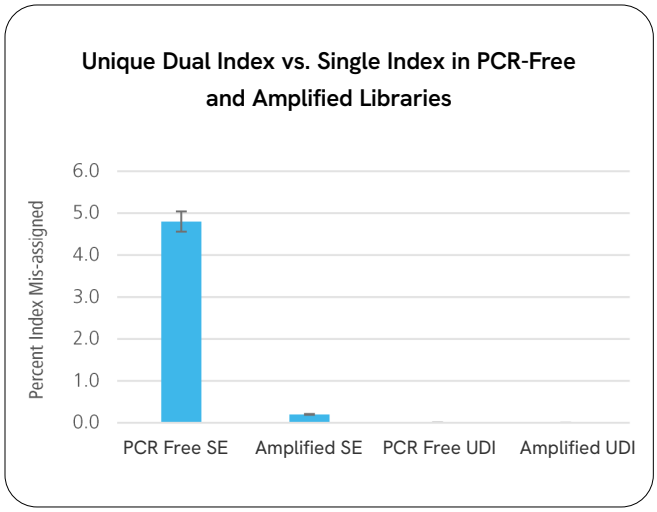

Figure 4: **PCR-free workflows benefit from NEXTFLEX® Unique Dual Index Barcodes.** A set of libraries were prepared from unique, distinct amplicons using NEXTFLEX® Unique Dual Index Barcodes. Prior to PCR amplification, each library was quantified and divided into equal halves. One half of each of the libraries was reserved in the un-amplified state and sequenced on a HiSeq® 4000 instrument. The remaining half was PCR-amplified and sequenced on a separate lane of a HiSeq® 4000 instrument. This entire data set was demultiplexed twice: once with only taking a single index into account (SI), then demultiplexed again when taking both indexes into account (UDI).

Conclusion

Illumina® sequencing instruments that utilize a patterned flow cell and exclusion amplification technology is known to suffer from increased levels of sample mis-assignment. The use of NEXTFLEX® unique dual index barcodes prevents such mis-assigned reads from appearing in final data sets allowing for the highest assurance of data integrity.

References

1. Sinha, R. et al. (2017) Index Switching Causes "Spreading-Of-Signal" Among Multiplexed Samples In Illumina HiSeq 4000 DNA Sequencing. Cold Spring Harbor Laboratory. bioRxiv 125724; doi: https://doi.org/10.1101/125724

2. Effects of Index Misassignment on Multiplexing and Downstream Analysis. https://www.illumina.com/content/dam/illumina-marketing/documents/products/whitepapers/index-hopping-white-paper-770-2017-004.pdf

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