Validation of digitalMLPA on the Saluseq Nimbo platform

Abstract

digitalMLPA is a multiplexed NGS-based technique that enables simultaneous analysis of up to 1600 targets in a single reaction. This study evaluates the compatibility of the Saluseq Nimbo sequencing platform with digitalMLPA, using the Illumina NextSeq 1000 as a reference platform. Performance was evaluated through assessment of barcode compatibility, normalised probe signal generation, classification agreement across probe types, repeatability and reproducibility using three digitalMLPA products across two independent sequencing runs.

The results demonstrated high concordance of normalised probe signals across sequencing runs and platforms. Assessment of four NXtec Barcode Plates that are currently on the market demonstrated uniform read distribution across the majority of barcodes and high barcode purity. Repeatability and reproducibility analyses demonstrated strong agreement across runs and sequencing platforms, and classification agreement was high for copy number, methylation-specific, and mutation-specific probe types.

Overall, the results demonstrate that the Saluseq Nimbo platform generates sequencing data suitable for digitalMLPA analysis and delivers performance comparable to the Illumina NextSeq 1000 reference platform.

Introduction

Copy number variations play a role in a wide range of genetic disorders and cancers. digitalMLPA is a semi-quantitative targeted technique that combines the gold-standard MLPA technology with NGS, enabling simultaneous analysis of hundreds to thousands of genomic targets within a single reaction[1,2]. Following DNA denaturation, probe hybridisation and ligation are performed, after which ligated probes are amplified by PCR. Individual probe amplicons are subsequently quantified by NGS and analysed using the free digitalMLPA analysis software Coffalyser digitalMLPA (Figure 1).

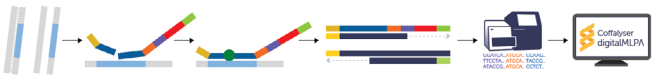
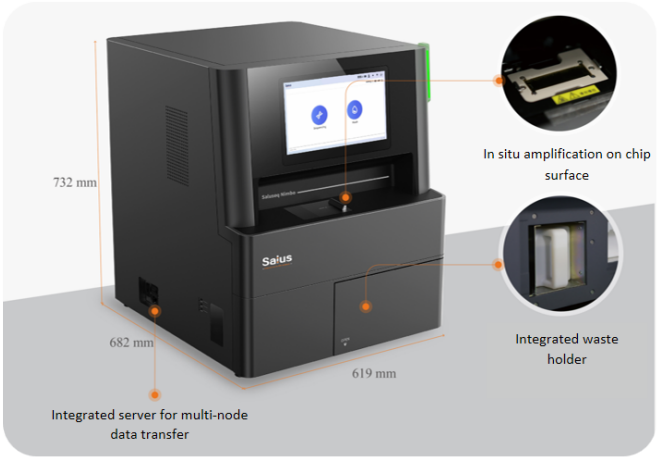


Figure 1. Overview of the digitalMLPA workflow. Genomic DNA is denatured and subjected to probe hybridisation and ligation. Ligated probes are amplified by PCR and quantified by NGS. Generated sequencing data are analysed using Coffalyser digitalMLPA to determine copy number and other targeted molecular alterations.

In this study, the performance of digitalMLPA was evaluated on the Saluseq Nimbo sequencing platform. The Saluseq Nimbo is a low-throughput benchtop NGS platform that utilizes sequencing-by-synthesis (SBS) chemistry and supports a wide range of sequencing applications, including targeted sequencing workflows. With run times ranging from approximately 2.2 to 25 hours, the platform is designed to support routine sequencing workflows in both research and diagnostic laboratories. Its throughput, flexibility, and streamlined workflow make the Saluseq Nimbo well suited for targeted applications such as digitalMLPA. Saluseq Nimbo provides three different types of flow cell (25M/60M/100M) for different application scenarios and is easy to operate.





Study Design and Methods

digitalMLPA Libraries

digitalMLPA experiments were performed according to manufacturer's instructions. Two NXtec and one NXtec-MS (in the pipeline) assays representing different application types were included in the validation study (Table 1).

Table 1. Overview of Study Components and Objectives.

Study Component	Objective
Barcode library	Evaluation of barcode compatibility and barcode purity
NXtec D002-A1 Hereditary Cancer Panel 2 library	Evaluation of copy number and mutation-specific probe performance
NXtec D024-A1 KaryoProfiler library	Evaluation of copy number probe performance
NXtec-MS DM025-X1 Congenital Anomalies library	Evaluation of copy number and methylation-specific probe performance
Independent sequencing runs	Assessment of repeatability
Comparison with NextSeq 1000	Assessment of reproducibility

Sequencing Design

Two independent sequencing runs were performed on the Saluseq Nimbo platform. Barcode performance was evaluated by comparing the data from the Saluseq Nimbo sequencing runs with a single NextSeq 1000 reference dataset. Repeatability was assessed through comparisons between independent sequencing runs performed on the same platform, while reproducibility was assessed through comparisons with the Illumina NextSeq 1000 reference platform.

Sequencing Workflow and Parameters

Libraries were loaded and sequenced according to manufacturer's instructions. Sequencing was performed on both the Saluseq Nimbo and Illumina NextSeq 1000 platforms. Saluseq Nimbo sequencing was performed using single-end 110-cycle (SE110) configuration on a 100M flow cell, with run configured per manufacturer guidelines and a target raw data quality of Q30 >95%. A 10% PhiX control spike-in was included in each run. Libraries were sequenced in duplicate and loaded at concentrations ranging from 3.2 to 6.0 pM.

The NextSeq 1000 was operated using either the NextSeq 1000/2000 P1 Reagents (v3) kit or the NextSeq 1000/2000 P1 Reagents (XLEAP-SBS) kit, depending on the sequencing run. The libraries were loaded at a final concentration of 671 pM.

Saluseq Nimbo

Library Dilution and Denaturation

1. A 10% PhiX control library spike-in was added and the library was diluted to a concentration of 4 nM.
2. Libraries were denatured by combining 5 µL of library with 5 µL of 0.2 M denaturation buffer.
3. After 5 min, denatured libraries were neutralised by the addition of 5 µL Neutralization Buffer.
4. Neutralised libraries were diluted with 485 µL Hybridization Buffer 1, resulting in an intermediate library concentration of approximately 40 pM.
5. The diluted library was further diluted in Hybridization Buffer 1 to the desired loading concentration (3.2-6.0 pM), and the final library preparation was loaded onto the Saluseq Nimbo instrument.

Data Analysis

FASTQ files generated on all sequencing platforms were analysed using Coffalyser digitalMLPA v2.5 software according to the standard digitalMLPA analysis workflow.

Results

Barcode Compatibility

Barcode performance was assessed using the four commercially available NXtec Barcode Plates (BP01-IL, BP02-IL, BP03-IL, BP04-IL). Two independent sequencing runs were performed on the Saluseq Nimbo platform and compared with a single NextSeq 1000 reference dataset. Normalised read counts were generally comparable across barcode plates and sequencing platforms (Figure 2A). NXtec Barcode Plates 3 and 4 showed the most uniform performance, with normalised read count values consistently close to 1.0. A small number of barcodes within NXtec Barcode Plate 1 and 2 demonstrated slightly elevated normalised read counts relative to the plate median. These differences were limited in magnitude and did not affect barcode purity (Figure 2B).

Barcode purity values were consistently high across all evaluated barcode plates (Figure 2B). Saluseq Nimbo runs demonstrated barcode purity values exceeding 99% for the majority of barcodes, while the NextSeq 1000 reference run showed slightly lower purity values for NXtec Barcode Plates 1, 3 and 4. NXtec Barcode plate 2 showed comparable purity between platforms. However, three individual barcodes within NXtec Barcode Plate 2 demonstrated slightly reduced purity values; although all remained above 97% purity. Overall, the results demonstrate uniform read distribution and accurate barcode assignment on the Saluseq Nimbo platform.

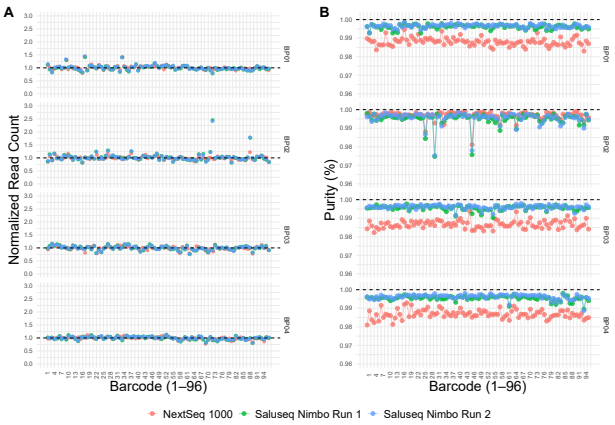


Figure 2. Barcode Performance on the Saluseq Nimbo Platform. (A) Normalised read count across NXtec Barcode Plates (BP01-IL, BP02-IL, BP03-IL, BP04-IL). Read counts were normalised to the median read count of all barcodes within each barcode plate. (B) Barcode purity reflects the accuracy of barcode assignment. A value of 1.00 indicates perfect barcode assignment and purity.

Concordance of digitalMLPA Results

Inter ratios, which allow determination of copy numbers, represent normalised probe signals after normalisation using reference probes and comparison with a reference sample set. Comparison of inter ratios between sequencing runs and platforms enables assessment of agreement at the level of individual probe measurements.

Inter ratio comparisons demonstrated strong agreement between sequencing runs and platforms (Figure 3). The displayed measurements represent probe-level data from all products included in the study. The majority of probe measurements showed concordant classifications, i.e. equal copy number, mutation status and/or methylation status determination as obtained with the NextSeq 1000, with only a small number of ambiguous classifications and at most two discordant classifications observed per comparison.

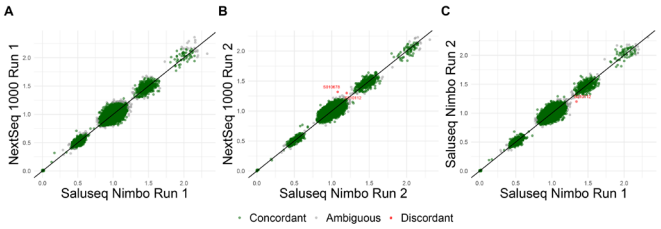


Figure 3. Concordance of digitalMLPA inter ratios between sequencing runs and platforms. (A) Saluseq Nimbo Run 1 compared with NextSeq 1000 Run 1. (B) Saluseq Nimbo Run 2 compared with NextSeq 1000 Run 2. (C) Saluseq Nimbo Run 1 compared with Saluseq Nimbo Run 2. Each data point represents an individual probe measurement, with probe measurements from all digitalMLPA products included in the study shown. Green points indicate concordant classification, grey points indicate ambiguous classification, and red points indicate discordant classification.

To quantify classification agreement, overall percentage agreement was calculated for copy number, methylation-specific, and mutation-specific probes across all sequencing run comparisons (Table 2, Page 4). Ambiguous and discordant classifications were considered non-concordant.

Classification agreement was consistently high across all evaluated comparisons (Table 2). Copy number probe agreement ranged from 99.70 to 99.86%, while methylation-specific probe agreement ranged from 97.21 to 98.84%. Mutation-specific probe agreement was 100% across all comparisons. No more than two discordant copy number calls were observed in any comparison, whereas no discordant calls for methylation-specific or mutation-specific probes were identified. These results demonstrate highly comparable classification outcomes between the Saluseq Nimbo and NextSeq 1000 platforms.

Precision

Precision analysis demonstrated strong agreement both within and between sequencing platforms (Table 3). The Pearson correlation coefficient observed between independent Saluseq Nimbo sequencing runs was identical to that observed between independent NextSeq 1000 sequencing runs. Comparison between Saluseq Nimbo and NextSeq 1000 platforms yielded Pearson correlation coefficients ranging from 0.90 to 0.93, demonstrating highly comparable inter ratio measurements across platforms. Collectively, these findings support the repeatability and reproducibility of digitalMLPA analysis on the Saluseq Nimbo sequencing platform.

Table 3. Precision assessment based on inter ratio correlation. Repeatability was assessed through comparisons of independent sequencing runs performed on the same platform, while reproducibility was assessed through comparison between Saluseq Nimbo and Illumina NextSeq 1000 platforms.

Comparison	Pearson correlation coefficient (r)
NextSeq 1000 Run 1 vs Run 2	0.92
Saluseq Nimbo Run 1 vs Run 2	0.92
Saluseq Nimbo Run 1 vs NextSeq 1000 Run 1	0.90
Saluseq Nimbo Run 1 vs NextSeq 1000 Run 2	0.92
Saluseq Nimbo Run 2 vs NextSeq 1000 Run 1	0.91
Saluseq Nimbo Run 2 vs NextSeq 1000 Run 2	0.93

Conclusion

Overall, the results of this study demonstrate that the Saluseq Nimbo platform generates sequencing data suitable for digitalMLPA analysis and delivers performance comparable to the Illumina NextSeq 1000 reference platform. The platform showed robust barcode performance, high classification agreement, and reproducible inter ratio measurements, supporting its use for digitalMLPA sequencing applications.

References

1. Benard-Slagter, Anne, et al. "Digital multiplex ligation-dependent probe amplification for detection of key copy number alterations in T-and B-cell lymphoblastic leukemia." *The Journal of Molecular Diagnostics* 19.5 (2017): 659-672.

2. Schouten, Jan P., et al. "Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification." *Nucleic Acids Research* 30.12 (2002): e57-e57.

Table 2. Comparison of inter ratio classifications between sequencing runs and platforms. Overall Percentage Agreement (OPA) was calculated as the percentage of concordant calls relative to the total number of calls for each probe type. Ambiguous and discordant calls were considered non-concordant. Probe types included copy number (CN), methylation-specific (MET), and mutation-specific (MUT). Results represent classifications from all digitalMLPA products used in the study.

Comparison	Probe Type	Total Calls	Discordant	Ambiguous	OPA (%)
NextSeq 1000 Run 1 vs Run 2	CN	35457	1	85	99.76
	MET	430	0	12	97.21
	MUT	90	0	0	100
Saluseq Nimbo Run 1 vs Run 2	CN	35457	1	74	99.79
	MET	430	0	5	98.84
	MUT	90	0	0	100
Saluseq Nimbo Run 1 vs NextSeq 1000 Run 1	CN	35457	0	107	99.7
	MET	430	0	12	97.21
	MUT	90	0	0	100
Saluseq Nimbo Run 1 vs NextSeq 1000 Run 2	CN	35457	1	66	99.81
	MET	430	0	6	98.6
	MUT	90	0	0	100
Saluseq Nimbo Run 2 vs NextSeq 1000 Run 1	CN	35457	1	86	99.75
	MET	430	0	12	97.21
	MUT	90	0	0	100
Saluseq Nimbo Run 2 vs NextSeq 1000 Run 2	CN	35457	2	49	99.86
	MET	430	0	6	98.6
	MUT	90	0	0	100

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