Validation of digitalMLPA on the GeneMind FASTASeq 300 and SURFSeq 5000 Platforms

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 GeneMind FASTASeq 300 and SURFSeq 5000 sequencing platforms 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 two digitalMLPA™ NXtec™ assays and one digitalMLPA™ NXtec-MS™ assay (in the pipeline) 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 GeneMind FASTASeq 300 and SURFSeq 5000 platforms are compatible with digitalMLPA technology and deliver 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).

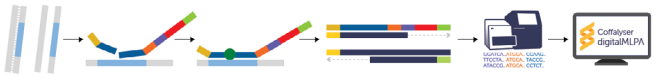
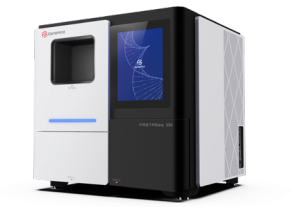


Figure 1. Overview of the digitalMLPA workflow. Genomic DNA is denatured and subjected to probe hybridization and ligation. Ligated probes are amplified by PCR and quantified by NGS. Generated sequencing data are analysed using Coffalyser digitalMLPA to determine copy number and other targeted molecular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

In this study, the performance of digitalMLPA was evaluated on the FASTASeq 300 and SURFSeq 5000 sequencing platforms. Both instruments utilise sequencing-by-synthesis (SBS) chemistry and support a wide range of sequencing applications, including targeted sequencing workflows.



The FASTASeq 300 is a mid-to-low throughput desktop sequencer, optimized for targeted sequencing and low-pass WGS. It supports multiple flow cell formats, flexible loading modes (automated or manual split-lane), and customisable data output. With ready-to-use consumables, RFID recognition, and automated cleaning, it enables a streamlined workflow with rapid turnaround—SE50 in ~4 hrs and PE150 in ~13 hrs, with Q30 > 90%.



The SURFSeq 5000 is a high-throughput desktop platform with output exceeding 2 Tb per run. Integrating ultra-high-density flow cells, rapid chemistry, and AI-based basecall algorithms, it delivers uniform genome coverage and Q50 level data quality. SURFSeq 5000 supports diverse applications including WGS, targeted sequencing, methylomics, and transcriptomics, fully covering DNA and RNA clinical needs.

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 both the FASTASeq 300 and SURFSeq 5000 platforms. Barcode performance was evaluated by comparing each GeneMind platform 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 the manufacturer's instructions. Sequencing was performed on the FASTASeq 300, SURFSeq 5000, and Illumina NextSeq 1000 platforms. Libraries were sequenced in duplicate and loaded at concentrations of 80 pM on the FASTASeq 300, 150 pM on the SURFSeq 5000, and 671 pM on the NextSeq 1000.

The FASTASeq 300 was operated using the FASTASeq 300 Standard V3.0 Kit and an FCP 150-cycle sequencing set, whereas the SURFSeq 5000 was operated using SURFSeq 5000 Standard V2.0 Chemistry and an FCM 200-cycle sequencing set. Prior to loading, the digitalMLPA libraries were diluted to a defined working concentration and chemically denatured. Both GeneMind platforms were operated in flexible mode using SE110 configuration, in which each flowcell lane is loaded manually; the dilution scheme therefore differs between the two instruments. The final pooled library consisted of 70% digitalMLPA libraries and 30% GM Balancer.

The following precautions applied to both platforms. The 0.2 M NaOH denaturing solution was prepared fresh from nuclease-free water immediately before use and verified to pH > 13, since partially neutralised NaOH is the most common cause of reduced sequencing output. The library dilution buffer (FASTASeq 300) or library diluent (SURFSeq 5000) and the library neutralising solution were pre-cooled on ice. Denaturation was started approximately 30 minutes before loading, once the instrument, the sequencing reagents and the flow cell had been fully prepared. The filtered pipette tips were used throughout. Sequencing reagents were thawed as specified in the respective Quick User Guide and used within 24 hours; flowcells were equilibrated at room temperature for at least 30 minutes and used within 12 hours.

FASTASeq 300
Dilution and denaturation (flexible mode, 80 pM)

1. Dilute the digitalMLPA library stock to **1 nM** with pre-cooled library dilution buffer.
2. Combine **40 µL of 0.2 M NaOH** with **40 µL of the 1 nM library**, mix, and incubate for 5 minutes at room temperature.
3. Place the tube on ice and add **40 µL of library neutralising solution** to stop the denaturation reaction.
4. Add **380 µL of pre-cooled hybridisation buffer V5**, giving **500 µL of denatured library at 80 pM**. Vortex, spin briefly and keep on ice.
5. Load **30 µL per flow channel** using the portable loader; dispense at a constant speed over **5–10 seconds** and leave the tip in place for **5 seconds** before withdrawing it.
6. Confirm that the channel is completely filled, remove the loading block vertically, and start the run **within 30 minutes**.

SURFSeq 5000
Dilution and denaturation (flexible mode, 150 pM)

1. Dilute the digitalMLPA library stock to **0.75 nM** with pre-cooled library diluent.
2. For each flowcell lane, prepare **14 µL of 0.75 nM undenatured library** in a 1.5 mL tube.
3. Add **2 µL of 0.2 M NaOH**, mix, and incubate for **5 minutes at room temperature**.
4. Place the tube on ice and add **2 µL of library neutralising solution**, giving **18 µL of denatured library**.
5. Pre-mix the isothermal clustering reagents **in the stated order: 8.1 µL of reagent 4** followed by **17.9 µL of reagent 5** (26 µL total). Pipette slowly, invert eight times, spin for **3–5 seconds only**, and keep on ice.
6. Add **9 µL of the denatured library** to the clustering mix, giving **35 µL of clustering reaction solution**. Invert eight times, spin for 3–5 seconds and keep on ice. **Do not vortex**, and use within 30 minutes.
7. Load **30 µL per flowcell lane**: insert the tip vertically into the loading port, release the tip and allow the solution to enter by gravity (approximately 35 seconds), then remove the tip without passing over the other ports.
8. Place the loaded flow cell on the instrument **within 10 minutes** of loading.

RECOMMENDED SETTINGS AND CONSUMABLES
On-machine concentration used for digitalMLPA in flexible mode: **80 pM** on the FASTASeq 300 (FCP flow cell) and **150 pM** on the SURFSeq 5000 (FCM flow cell).
Recommended tips for manual loading on the SURFSeq 5000: **QSP TF140-200-Q**; other tips may cause loading failure.
A slight white turbidity on mixing the isothermal clustering reagents is normal, as are small bubbles in the flow channel after loading.

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 both the FASTASeq 300 and SURFSeq 5000 platforms 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 Plates 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).

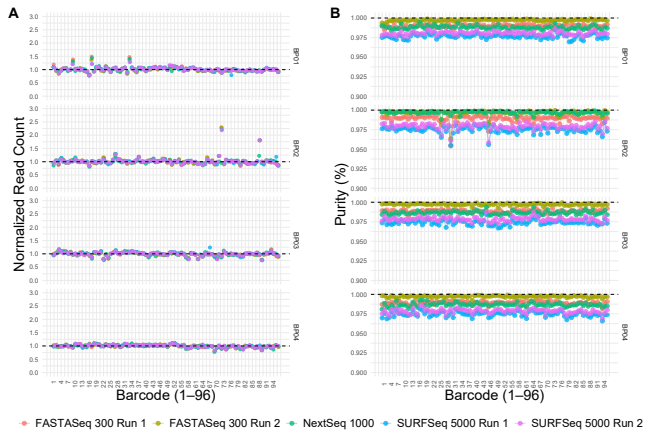


Figure 2. Barcode Performance on the FASTASeq 300 and SURFSeq 5000 platforms. (A) Normalised read count across all NXtec Barcode Plates (BP01-IL, BP02-IL, BP03-IL, BP04-IL). Read counts were normalised to the median read count of all barcodes within each barcode plate. (B) Barcode purity reflects the accuracy of barcode assignment. A value of 1.00 indicates perfect barcode assignment and purity.

Barcode purity values were consistently high across all evaluated barcode plates (Figure 2B). FASTASeq 300 and NextSeq 1000 demonstrated barcode purity values above 98% across the majority of evaluated barcodes, while SURFSeq 5000 barcode purity values generally ranged between 97% and 98%. Despite these small differences, barcode assignment remained highly accurate and no impact on digitalMLPA results was observed. These results demonstrate accurate barcode assignment across both GeneMind platforms.

Concordance of digitalMLPA Results

Inter ratios 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). Probe measurements from all digitalMLPA products included in the study showed highly comparable inter ratio distributions. The majority of probe measurements resulted in 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 and discordant classifications observed.

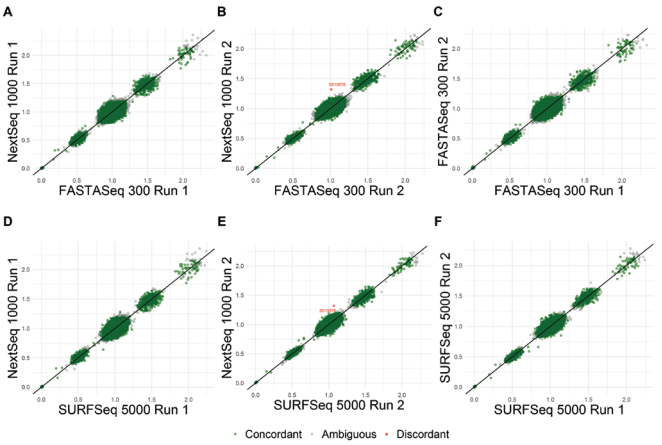


Figure 3. Concordance of digitalMLPA inter ratios between sequencing runs and platforms. (A) FASTASeq 300 Run 1 compared with NextSeq 1000 Run 1. (B) FASTASeq 300 Run 2 compared with NextSeq 1000 Run 2. (C) FASTASeq 300 Run 1 compared with FASTASeq 300 Run 2. (D) SURFSeq 5000 Run 1 compared with NextSeq 1000 Run 1. (E) SURFSeq 5000 Run 2 compared with NextSeq 1000 Run 2. (F) SURFSeq 5000 Run 1 compared with SURFSeq 5000 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 5). Ambiguous and discordant classifications were considered non-concordant.

Classification agreement was consistently high across all evaluated comparisons (Table 2, Page 5). Copy number probe agreement ranged from 99.63% to 99.86%, while methylation-specific probe agreement ranged from 96.74% to 98.84%. Mutation-specific probe agreement was 100% across all comparisons. Discordant classifications were rare, with no more than a single discordant copy number classification

observed in any comparison. No discordant methylation-specific or mutation-specific classifications were identified. These results demonstrate highly comparable classification outcomes between the FASTASeq 300, SURFSeq 5000, and NextSeq 1000 sequencing platforms.

Precision

Precision analyses demonstrated strong agreement both within and between sequencing platforms (Table 3). Repeatability correlation coefficients were 0.89 for FASTASeq 300 and 0.93 for SURFSeq 5000, which were comparable to the repeatability observed for the NextSeq 1000 reference platform (r = 0.92). Reproducibility analyses demonstrated strong agreement between the GeneMind platforms and the NextSeq 1000 reference platform, with Pearson correlation coefficients ranging from 0.89 to 0.91 for FASTASeq 300 and from 0.91 to 0.94 for SURFSeq 5000. These results support the repeatability and reproducibility of digitalMLPA analysis on both GeneMind sequencing platforms.

Table 3. Precision assessment based on inter ratio correlation. Repeatability was assessed through comparison of independent sequencing runs performed on the same platform. Reproducibility was assessed through comparison of inter ratio measurements generated on FASTASeq 300 and SURFSeq 5000 relative to the NextSeq 1000 reference platform. For reproducibility, Pearson correlation coefficients are reported as the range observed across both comparisons with the NextSeq 1000 reference runs.

Platform	Repeatability Pearson correlation coefficient (r)	Reproducibility Pearson correlation coefficient (r)
NextSeq 1000	0.92	Reference
FASTASeq 300	0.89	0.89-0.91
SURFSeq 5000	0.93	0.91-0.94

Conclusion

Overall, the results of this study demonstrate that the FASTASeq 300 and SURFSeq 5000 platforms deliver performance comparable to the Illumina NextSeq 1000 reference platform. Both platforms demonstrated robust barcode performance, high classification agreement, and reproducible inter ratio measurements, supporting their 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
FASTASeq 300 Run 1 vs Run 2	CN	35457	0	118	99.67
	MET	430	0	9	97.91
	MUT	90	0	0	100
FASTASeq 300 Run 1 vs NextSeq 1000 Run 1	CN	35457	0	117	99.67
	MET	430	0	12	97.21
	MUT	90	0	0	100
FASTASeq 300 Run 1 vs NextSeq 1000 Run 2	CN	35457	1	78	99.78
	MET	430	0	6	98.6
	MUT	90	0	0	100
FASTASeq 300 Run 2 vs NextSeq 1000 Run 1	CN	35457	0	130	99.63
	MET	430	0	14	96.74
	MUT	90	0	0	100
FASTASeq 300 Run 2 vs NextSeq 1000 Run 2	CN	35457	1	87	99.75
	MET	430	0	8	98.14
	MUT	90	0	0	100
SURFSeq 5000 Run 1 vs Run 2	CN	35457	0	57	99.84
	MET	430	0	5	98.84
	MUT	90	0	0	100
SURFSeq 5000 Run 1 vs NextSeq 1000 Run 1	CN	35457	0	88	99.75
	MET	430	0	11	97.44
	MUT	90	0	0	100
SURFSeq 5000 Run 1 vs NextSeq 1000 Run 2	CN	35457	1	50	99.86
	MET	430	0	5	98.84
	MUT	90	0	0	100
SURFSeq 5000 Run 2 vs NextSeq 1000 Run 1	CN	35457	0	85	99.76
	MET	430	0	12	97.21
	MUT	90	0	0	100
SURFSeq 5000 Run 2 vs NextSeq 1000 Run 2	CN	35457	1	47	99.86
	MET	430	0	6	98.6
	MUT	90	0	0	100

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