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Application Note

NEOonsite assays automated on the Hamilton NGS STAR™

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Key project features

- High reproducibility of Hybrid Capture-based NGS library preparation
- Flexible protocol, allows user to start and stop at various steps in the protocol, incl. optional QC step
- 3–4 hours of true walk-away time for lab operator
- Parallel processing of different NEOonsite assays

Abstract

In this application note, we discuss the results and findings from a customer project to automate NEOonsite assays using Hamilton's NGS STAR™ workstation, aiming for a reduction of hands-on time and number of user interventions, while keeping a high level of reproducibility.

Based on an automation protocol developed in the course of the project, the NGS STAR™ workstation allowed for nearly full automation of the NEOonsite wet lab workflow including on-deck PCR cycling and hybridization, bead-based purifications and stringent washing at elevated temperatures post hybridization, resulting in efficient target enrichment. The protocol was programmed aiming for high user flexibility, being able to start and end the process at various protocol steps. Thus, sample numbers can be adjusted in one sample increment and different NEOonsite assays can be processed in parallel. The need for user interventions could be limited to optimize walk-away time for laboratory staff. The automation protocol produced sequence-ready libraries. It is available from Hamilton as a method qualified by NEO New Oncology[1].

NEOonsite assays enrich for selected genomic regions using Hybrid Capture technology and cover from 39 genes up to 340 genes[2] to simultaneously detect genomic alterations such as single nucleotide variants (SNVs), small insertions and deletions (InDels), copy number alterations, gene fusions, microsatellite instability[3] (MSI) and tumor mutational burden[3] (TMB). NEOonsite assays are designed to detect genetic alterations in solid tumors and hematological malignancies[4].

The automated process uses fragmented genomic DNA from various sources (e.g. FFPE or fresh-frozen tissue, blood) utilizing the same reagents as the manual workflow. In the project, the performance of the automated workflow was compared to the manual workflow across different NEOonsite assays with routine pathology samples. It could be demonstrated that performance is consistent, as discussed in this application note.

Method overview

NEOonsite assays use fragmented genomic DNA that can be extracted from various sources such as FFPE, fresh-frozen tissue, blood and other typical materials. After library preparation, including DNA end-repair, A-tailing and sequencing adapter ligation, samples are hybridized against complementary RNA baits to enrich for selected genes. Hybridization is followed by a stringent wash protocol at elevated temperatures and subsequent PCR amplification. A second round of hybridization and washing allows for efficient enrichment of targeted genes. Most protocol steps were automated, and the user only needed to supply consumables, reagent master mixes and samples to the instrument. User intervention was needed at the beginning of the workflow for instrument set-up after which the instrument runs independently for 3–4 hours resulting in extended walk-away time for laboratory staff. Short user intervention (approx. 15–30 minutes) was required after this time to supply freshly prepared hybridization reagents, before the automated process continued independently running the overnight hybridization *(Fig. 1)*.

Introduction to experiments

Assessment of reproducibility of the protocol was performed using NEOselect v1 assay and sheared genomic cell line DNA from a HapMap normal sample as well as FFPE samples. Experiments were performed in four independent runs using cell line DNA with 24, 10, 3 and 6 replicates or using 5 FFPE samples. All samples were sequenced using a NextSeq® 500 sequencing instrument in an overnight sequencing run (paired-end; e.g. NextSeq® 500/550 Mid-Output v2.5 Kit for 150 cycles). Data was analyzed using NEO New Oncology's proprietary bioinformatic data analysis and all samples were in-silico down sampled to 5 M read pairs (mrp) to allow for comparison of sequencing parameters. After establishing the robustness of the automated protocol, further tests using the NEOplus v2 RUO assay were performed employing cell-line-derived genomic DNA and FFPE DNA with known various levels of tumor mutational burden (TMB).

Furthermore, we checked for cross-contamination between samples and compared the performance of the automated protocol against the performance of manual library preparation by two users using FFPE samples.

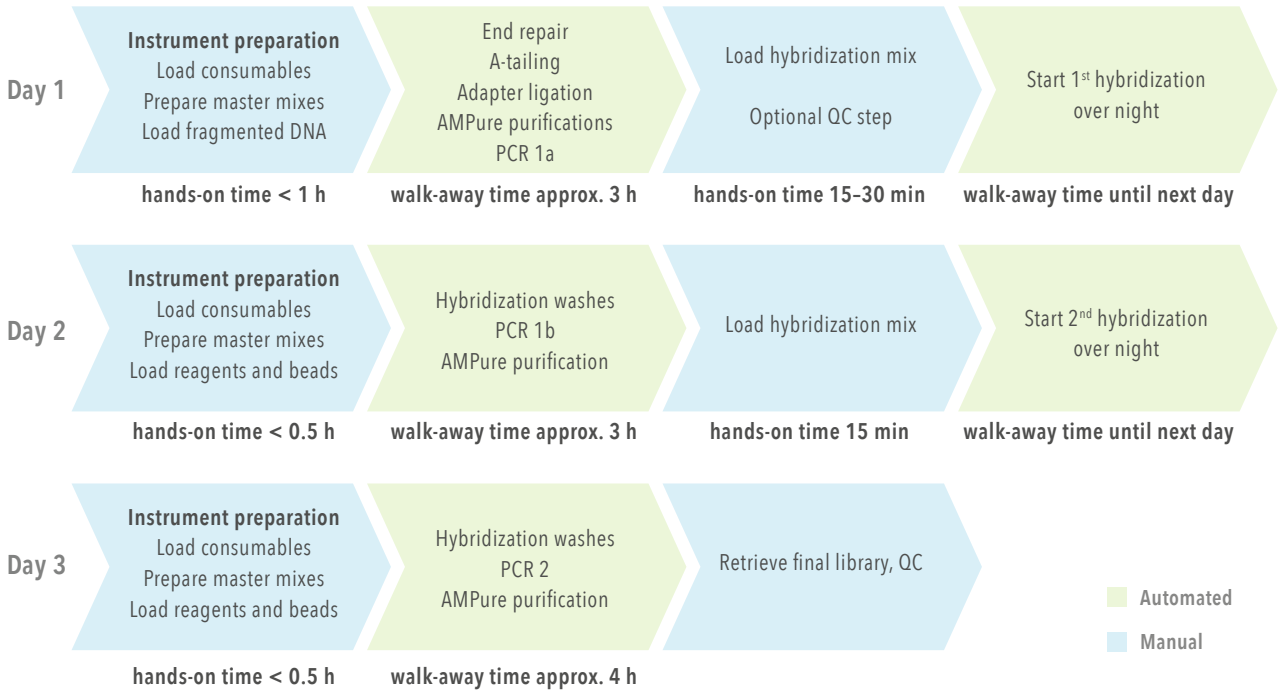


Fig. 1: Workflow overview

Experimental results

Reproducibility

NEOselect v1 enriches for 39 target genes and was used to establish the automation protocol on Hamilton's NGS STAR™ workstation. Four independent runs were performed using HapMap normal cell line DNA with 200 ng of input amount, with varying numbers of replicates *(Fig. 2)*. We analyzed the *percent reads on target,* a measure that allows to judge the efficacy of the stringent post-hybridization washes. As can be seen in *Fig. 2A* reads on target rates were stable at an average of 82% with a coefficient of variation (CV) of 2.2% across all cell line DNA runs. Highly similar results were obtained when using DNA extracted from FFPE samples with an average of 82% *reads on target* across all five samples and a CV of 2.1%, *Fig. 2B*.

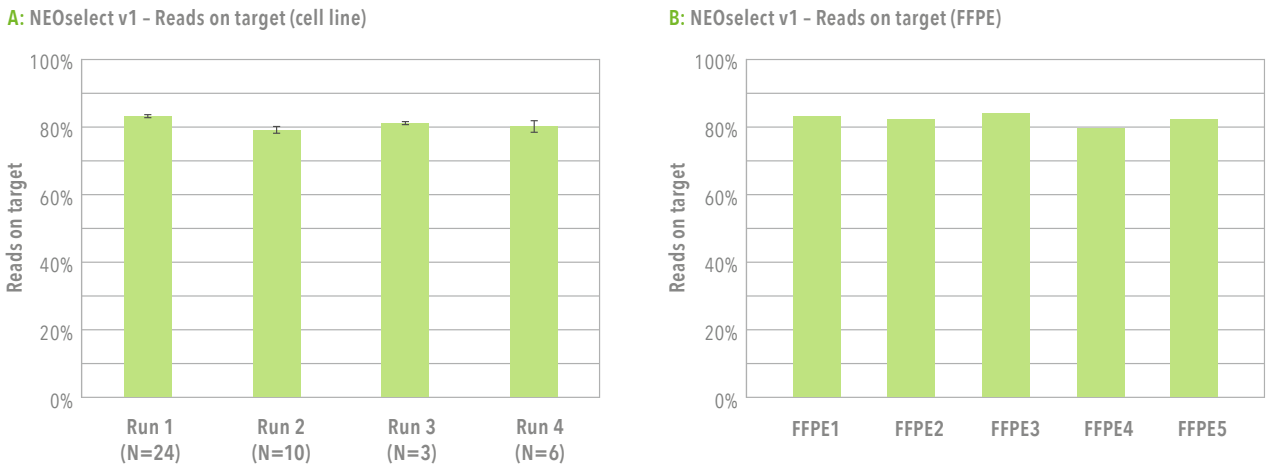


Fig. 2A and 2B: Reads on target of NEOselect v1 on cell line and FFPE DNA

Using NEOplus v2 RUO, an enrichment assay designed to cover a large number of genes (up to 340 genes) and to assess the TMB values of a research sample, we were able to demonstrate a high level of *percent reads on target* as measured before using NEOselect v1 in the automated setting. We used NEOplus v2 RUO with 100 ng of HapMap cell line DNA in triplicate and also five different FFPE samples *(Fig. 3)*. Overall reads on target rates are slightly lower compared to NEOselect v1, but still high around an average of 79% for cell line DNA samples with a CV of 2.2% and at an average of 76% and a CV of 2.5% across five FFPE samples.

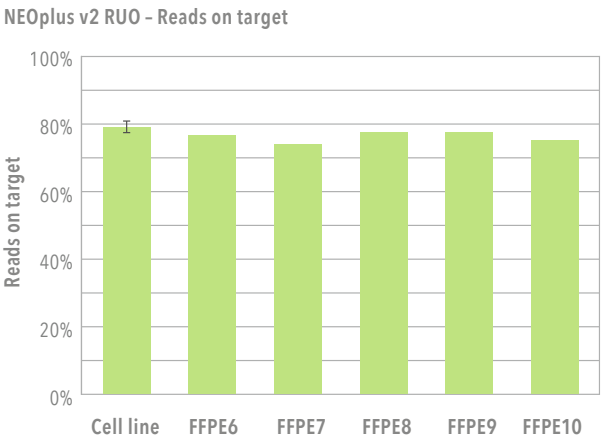
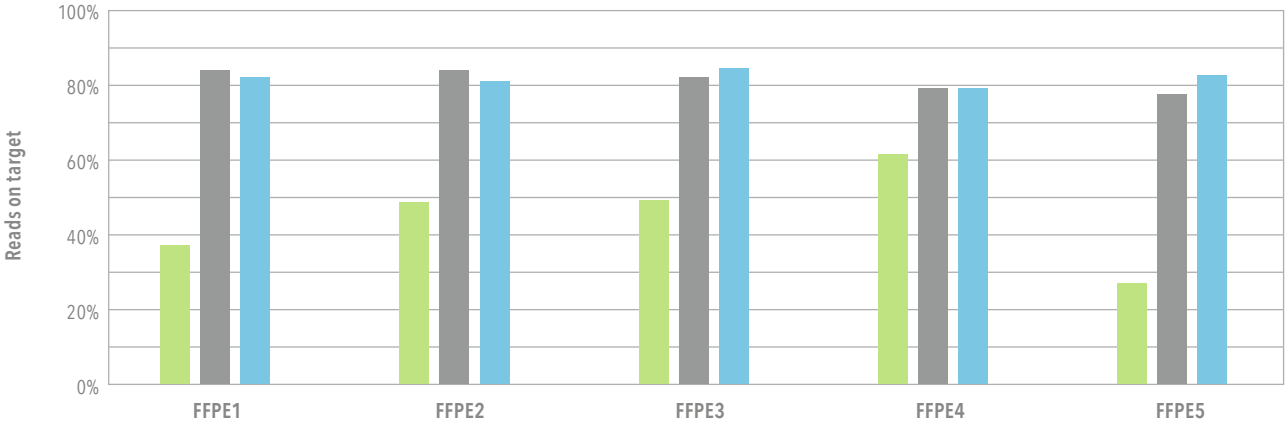


Fig. 3: Performance of NEOplus v2 RUO on cell line and FFPE DNA

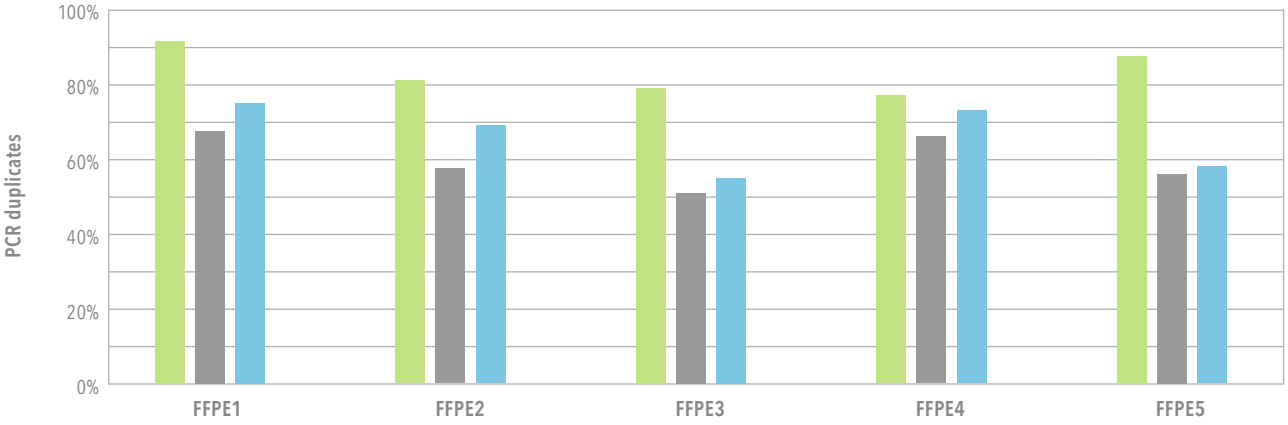
Assessing influence of user performance

After demonstrating high reproducibility of the automated protocol for two different NEOonsite assays, we aimed to assess the effect of independent manual operators on results of complex NGS testing methods. Therefore, sequencing libraries of five FFPE samples were prepared using NEOselect v1 by two different users and compared to libraries produced using the automated protocol. User 1 was new to the manual workflow, whereas user 2 was experienced in NGS sample handling. Libraries were prepared from the same DNA extract for all three settings and within a limited timespan to avoid influences of using different

A: NEOselect v1 – Reads on target



B: NEOselect v1 – PCR duplicates



C: NEOselect v1 – Percent target base coverage 100x

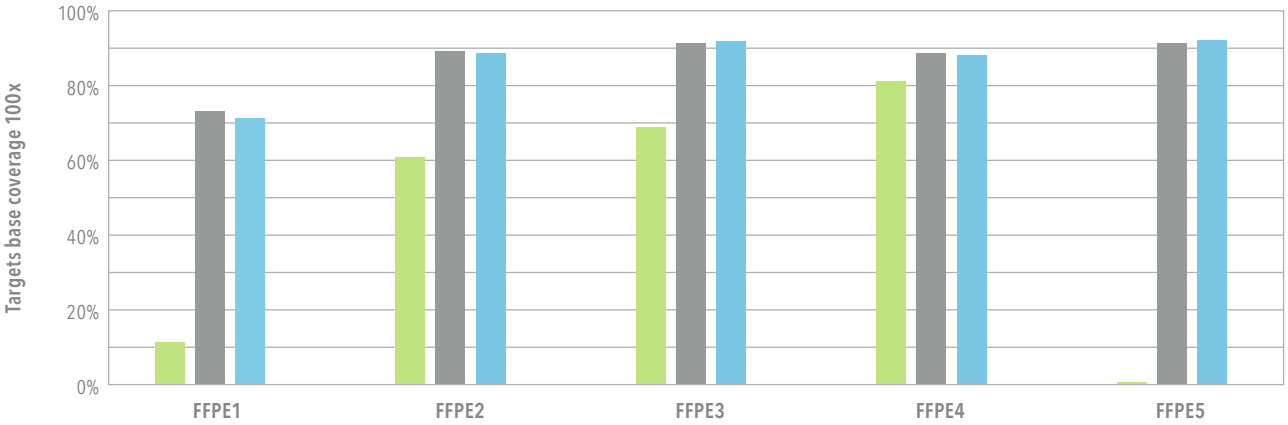


Fig. 4A, 4B and 4C: User-introduced variability and effects on *percentage reads on target, PCR duplication rates* and *target base 100x coverage* in a manual and automated setting

batches of DNA extractions or of DNA storage effects. As shown in *Fig. 4A* results from samples prepared by user 1 and user 2 are different for *percent reads on target* ranging from 30–60% for user 1, but rather consistent for user 2 with rates around 81%, similar to the automated protocol.

The *percentage of PCR duplicates* is an indicator for library complexity and can be used as a measure for sample quality and for the loss of sample throughout the library preparation process. A loss of DNA leads to a lower number of diverse molecules in the final library and subsequent sequencing results in re-sequencing of PCR duplicates. The same holds true for low quality DNA samples which cannot be turned over into a sequenceable library, because the necessary DNA modifying enzymes during library preparation are inhibited by DNA damages or modifications resulting from fixation procedures and sample handling.

Fig. 4B shows *PCR duplication percentages* between 77% and up to 92% for user 1, whereas results from user 2 show 51% to 68% PCR duplicates, indicating a putative loss of sample during library preparation by user 1. Similar values to user 2 were obtained when using the automated protocol.

Percent target base coverage was assessed as a result for efficient Hybrid Capture washes and ability to maintain a high library diversity throughout the library preparation workflow. *Fig. 4C* shows a high percentage of target base coverage 100x for the results achieved by user 2 and the automated workflow of approx. 90%. Nevertheless, the effect of low quality FFPE DNA becomes apparent in FFPE sample 1, where a maximum of target base 100x percentage of only 73% could be reached for user 2 and the automated workflow. Sample 5 showed fewest reads on target and high PCR duplicates for user 1, leading to a low overall coverage and as such a loss of the sample.

These results showed that the performance of the automated workflow can be almost identical to experienced users. Overall, one must acknowledge the challenges of handling routine FFPE specimen regarding sample quantity and quality. Therefore, a robust automated protocol taking variation in human performance out of the equation is beneficial when analyzing precious and scarce samples.

Cross-contamination

Testing for cross-contamination is important as samples and reagents are being handled in open vessels in the instrument and the robotic arm moves in x- and y-axis across the entire deck. Testing was achieved by using three different cell line DNA samples and following the respective inherent mutations of those cell lines. We used a HapMap normal cell line DNA and cancer cell line DNA HCC-827 (EGFR ex. 19 del) and HCT-15 (KRAS p.G13D). The samples were arranged in a pattern across three columns of a 96-well plate as shown in *Fig. 5* and NEOselect v1 testing of all samples was performed in parallel. After automated library preparation, sequencing and bioinformatic data analysis we sought to identify mutations of each cell line in the other cell line pool but were not able to identify cross-contaminating sequencing reads. We therefore assess the risk of cross-contamination using the automated protocol as low.

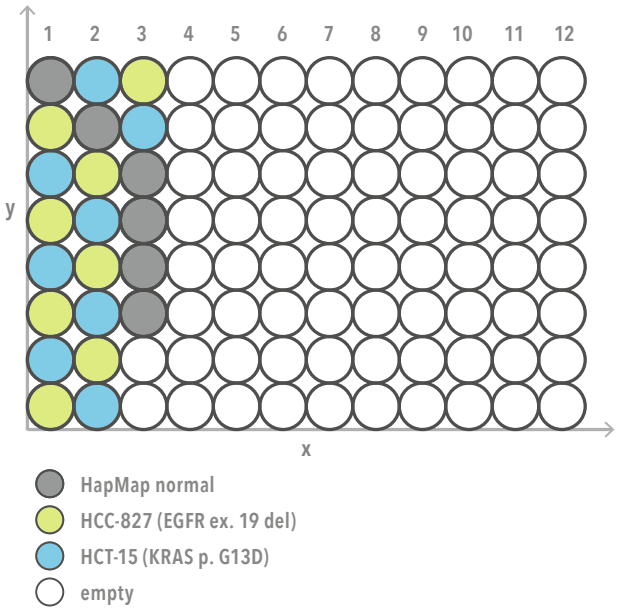


Fig. 5: Cross-contamination assessment (motion of robotic arm in x- and y-axis across sample plate)

Mutation calling and TMB results of manual and automated processing

NEOselect v1 was used to assess FFPE samples which had been tested before using an orthogonal amplicon-based NGS method. NEOselect v1 was run using the automated protocol and was then compared to the manual protocol processing. Results after bioinformatic analysis and data evaluation are shown in *Table 1*. All mutations that could be identified by the orthogonal method could be verified using NEOselect v1, in both the manual and automated library preparations with nearly identical allelic frequencies. In addition, TP53 mutations and CDK4 and MDM2 amplifications were identified in three out of five samples only when using NEOselect v1 compared to the amplicon-based method.

Table 1: NEOselect v1 identified mutations in manual and automated library preparations (VAF: variant allelic frequency; n/a: not applicable; n.d.: not detected; + fold change)

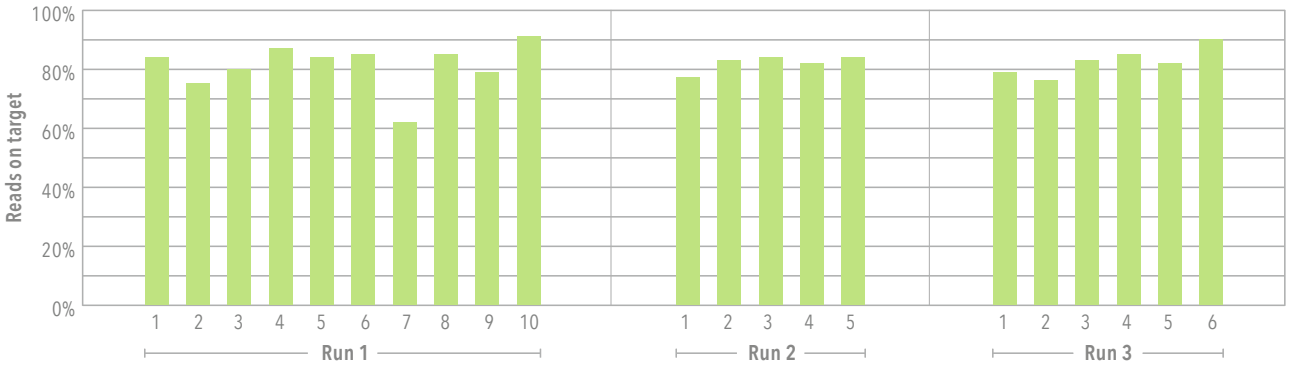
Sample	Mutation DNA	Mutation protein	Amplicon-based testing (VAF)	NEOselect v1 manual (VAF)	NEOselect v1 automated (VAF)
FFPE 1	KRAS c.35G>C	KRAS p.G12A	22%	21%	27%
	TP53 c.386_427dup	TP53 p.A129_P142dup	n.d.	17%	15%
FFPE 2	KRAS, NRAS, BRAF negative	n/a	n/a	n/a	n/a
	TP53 c.254del	TP53 p.P85Lfs*38	n.d.	45%	44%
FFPE 3	EGFR c.2235_2249del	EGFR p.E746_A750del (EGFR ex. 19del)	36%	25%	26%
	CDK4 amplification		n.d.	6.9+	7.3+
	MDM2 amplification		n.d.	7.4+	8.1+
FFPE 4	TP53 c.370del	TP53 p.C124Afs*46	44%	44%	47%
FFPE 5	MET c.3082G>A	MET p.D1028N	32%	38%	38%
	TP53 c.734G>A	TP53 p.G245D	19%	19%	21%

Table 2: TMB values from five FFPE samples using NEOplus v2 RUO (TMB as mutations/Mb with LoD 5%)

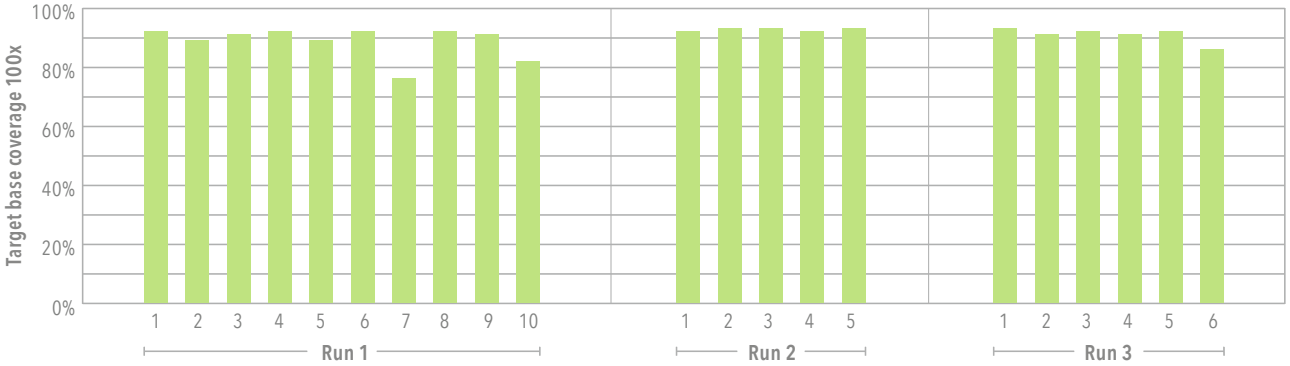
Sample	TMB value manual	TMB value automated
FFPE 6	3.5	2.6
FFPE 7	20.1	20.9
FFPE 8	29.7	28.8
FFPE 9	9.6	9.6
FFPE 10	7.0	7.0

Furthermore, five FFPE samples with different TMB values were examined with NEOplus v2 RUO. TMB values ranged from 2.6 muts/Mb up to 29.7 muts/Mb. Values for manual and automated sample processing showed a high concordance *(Table 2)*.

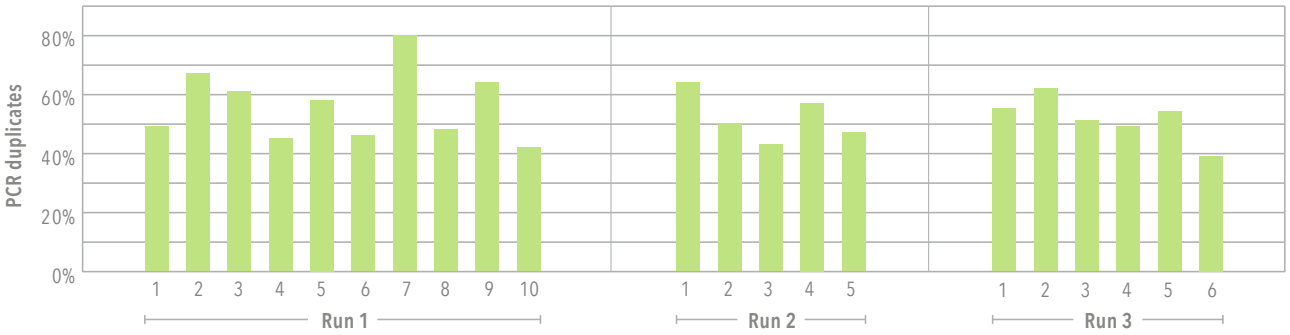
A: NEOselect v1 – Reads on target (routine use)



B: NEOselect v1 – Target base coverage 100x (routine use)



C: NEOselect v1 – PCR duplicates (routine use)



D: NEOselect v1 – Mean coverage (routine use)

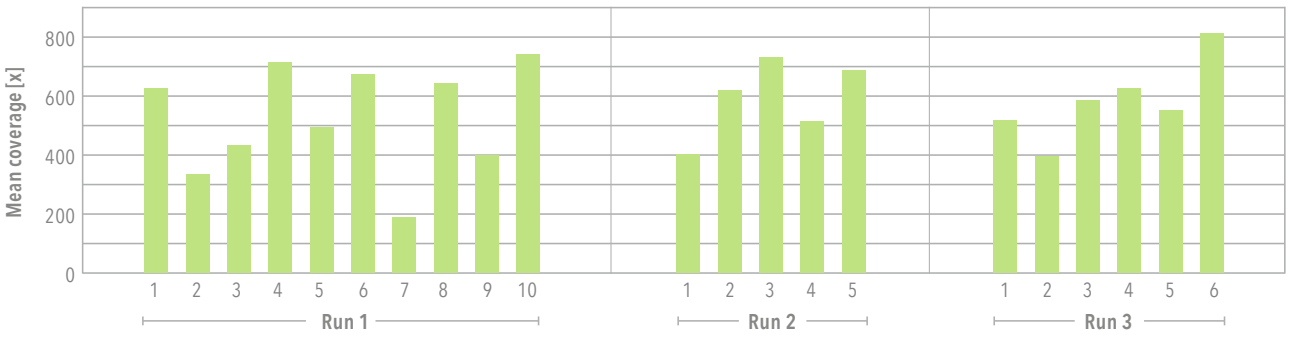


Fig. 6A–D: Monitoring of performance in routine clinical setting

Performance of automated library preparation in a routine clinical setting

To determine the performance of the automated protocol in a routine clinical setting we monitored several library preparation runs after installation of the protocol at a customer site. All runs were performed using clinical grade FFPE samples. The high level of reproducibility allowed the user to perform an efficient target enrichment for all samples throughout several runs as shown by high *reads on target* and *target base coverage 100x* rates *(Fig. 6A and 6B).* The strong influence of DNA quality becomes apparent when monitoring percent *PCR duplicates* since large variations were observed *(Fig. 6C).* As mentioned previously, PCR duplicates can be used as a measure for DNA quality because low-quality samples produce fewer unique molecules in library preparations and thus more duplicate molecules are being sequenced. One sample showed very poor quality with 80% *PCR duplicates (Fig. 6C; Run 1, sample 7)* but was kept in the data evaluation to demonstrate clinical routine conditions. Nevertheless, due to efficient target enrichment high sample *mean coverages* between 200x and 800x were achieved *(Fig. 6D),* showing the usefulness of automation in a routine clinical setting.

Conclusion

The automated NEOonsite protocol was tested with two different NEOonsite assays, NEOselect v1 and NEOplus v2 RUO, targeting 39 and up to 340 genes, respectively. Although very different in target size, both assays showed good results with high reproducibility. High performance of the protocol was not only demonstrated using high-quality cell line DNA, but also using clinical-grade FFPE samples. Use of the automated protocol in a routine clinical setting with FFPE samples demonstrated high performance in respect to enrichment efficiency and sequencing library quality. High sample coverages were reached in samples of superior DNA quality. Lack of measurable cross-contamination and demonstration of robust performance compared to human-introduced variability in sample handling made this protocol a great choice for the customers' routine applications, where high reproducibility is needed. The overall hands-on time could be kept short and the user gained several hours of hands-off time after initial set-up of the instrument. This automation project using Hamilton's NGS STAR™ workstation in combination with NEOonsite assays enabled the user with true flexibility, high performance NGS assays and autonomy for the lab operator.

[1] While the project was carried out in the specific customer laboratory, the automated workflow for Hamilton's NGS STAR platform is, in principle, suitable to provide performance comparable to manual preparation of sequencing libraries. However, individual implementation of the automation solution requires specific validation according to the applicable quality assurance requirements.

[2] Detailed information on the coverage of the respective genes for the specific assays can be found in the NEOonsite instructions for use.

[3] Tumor mutational burden and microsatellite instability detection with NEOplus v2 RUO. For research use only. Not for use in diagnostic procedures.

[4] Analysis of genes associated with myeloid malignancies with NEOmyeloid v1 RUO. For research use only. Not for use in diagnostic procedures.

The products/features (here mentioned) are not commercially available in all countries. Due to regulatory reasons their future availability cannot be guaranteed. Please contact NEO New Oncology GmbH for further details.

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