

Scalable Library Prep Automation of AmpliSeq™ for Illumina® Kits on the epMotion®

Abstract

Due to the growing number of relevant genes and variants, next generation sequencing (NGS) has become a standard technique in the field of cancer research and molecular pathology. The AmpliSeq for Illumina library preparation panels are used to sequence DNA or RNA isolated from a broad range of specimen, including blood, cell culture, fresh-frozen tissue and challenging samples such as formalin-fixed paraffin-embedded (FFPE) tissue.

With the AmpliSeq Focus panel workflow we here illustrate Eppendorf's scalable solution to easily and reliably automate the construction of NGS libraries and match these to the respective sequencer throughputs. This allows the automated processing of up to 48 samples on the epMotion 5075t NGS solution and 16 samples on the epMotion 5073m NGS solution with the AmpliSeq Focus panel.

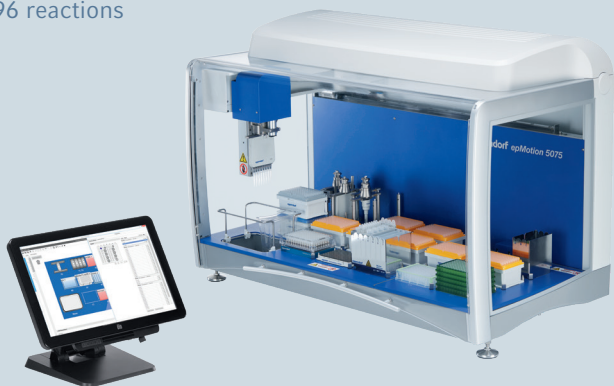
Introduction

AmpliSeq Focus for Illumina is a targeted resequencing solution for a wide-range of sample types, including FFPE tissue and low sample input. Standardization of these workflows is important to deliver accurate data. To address the growing demand for standardization also for lower throughput experiments, we here present a scalable automation solution. Automation of library preparation not only minimizes the loss of samples, wasted reagents and sequencing delays, but also reduces inter-operator

variability as well as errors in sample tracking. Here we combined NGS library prep automation with Illumina's sequencing platforms and cloud-based analysis to deliver a sample-to-answer cancer research workflow. To establish a solution using both DNA and RNA input, as the two common analytes from tumor tissue in the lab, we here demonstrate this workflow using the AmpliSeq Focus Panel which copes equally well with RNA and DNA from degraded FFPE samples.

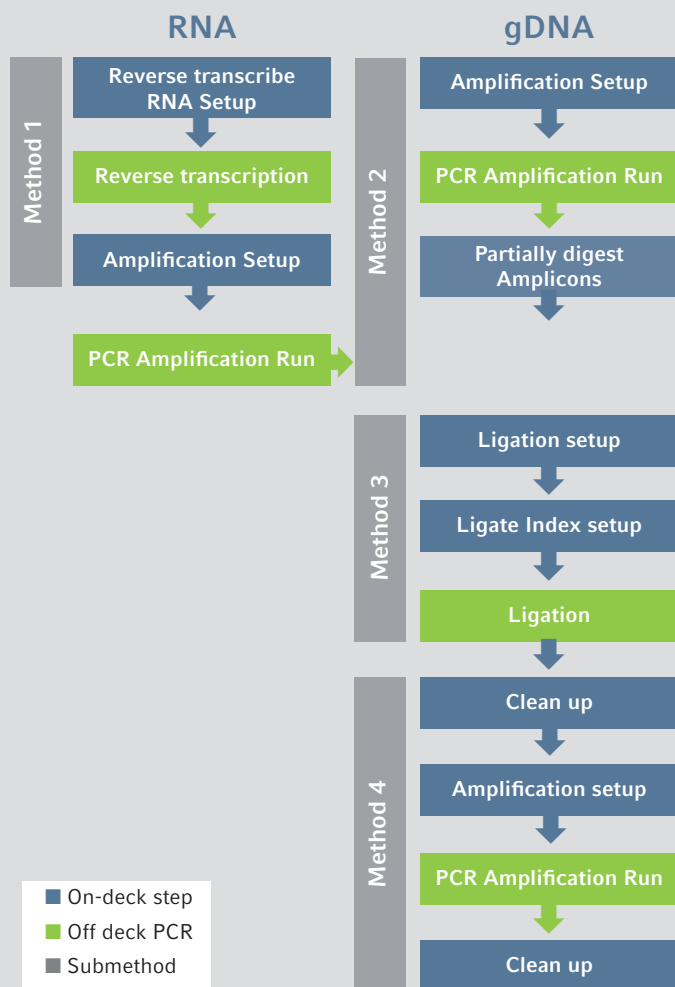
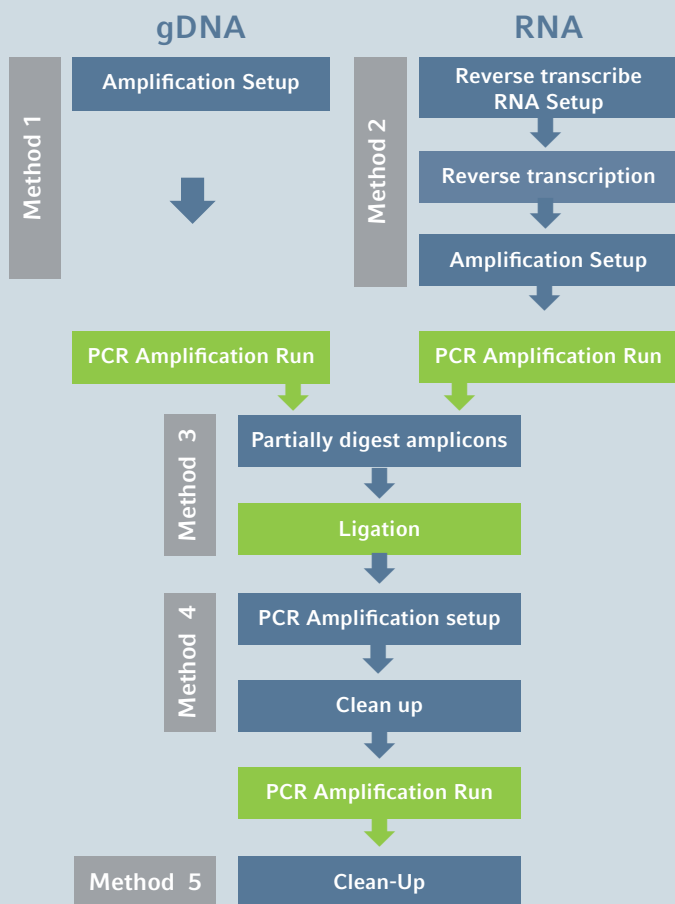
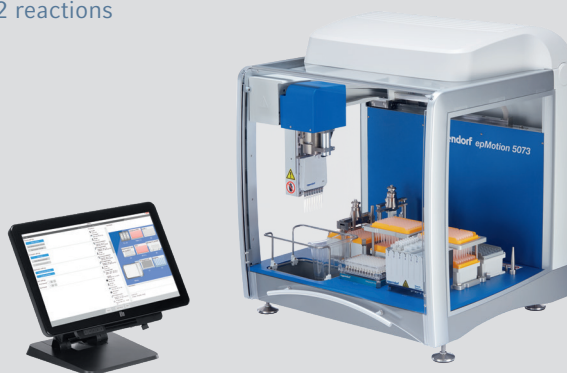
5075t NGS Solution

Up to 48 samples
(48 DNA and 48 RNA libraries)
= 96 reactions



5073m NGS Solution

Up to 16 samples
(16 DNA and 16 RNA libraries)
= 32 reactions



■ On-deck step
■ Off deck PCR
■ Submethod

Figure 1: Overview of the automated workflow for the AmpliSeq for Illumina Focus Panel on the epMotion. The grey boxes show submethods processed on the epMotion. Steps highlighted in green are performed off-deck. PCR amplifications were performed using the Eppendorf Mastercycler® X50. In alignment with the protocol, workflows are compartmented into logical units. Detailed run times and consumable usage are shown in table 1.

Materials and Methods

Experimental design on the epMotion® 5075t NGS solution

Libraries were generated using an automated version of the previously established Illumina protocol [1]. All steps off-deck were performed on the Mastercycler® X50 as shown in figure 1. To test the DNA route of the AmpliSeq protocol 6 replicates of gDNA samples (each 1 ng) of Quantitative Multiplex Reference Standard Dx HD701 from Horizon Discovery and 6 replicates of gDNA samples of Quantitative Multiplex Formalin Compromised (Mildly degraded) Dx HD798 from Horizon Discovery (with 10 ng DNA input each), and 4 replicates of Quantitative Multiplex Formalin Compromised (Moderately degraded) Dx HD799 from Horizon Discovery with 10 ng DNA input each were tested. For the RNA workflow we tested 5 replicates of Seraseq Fusion RNA Mix v2 from Seracare with 10 ng RNA input each and 3 replicates of Universal Human Reference RNA (UHR) from Agilent with 1 ng RNA input each. QC was performed with the DNA 1000 kit on the Agilent® 2100 BioAnalyzer®. Finished libraries were sequenced on a MiSeq® (2x15-0 bp). Data was analyzed using Illumina's BaseSpace® DNA Amplicon and RNA Amplicon App. Table 1 summarizes the used consumables and runtimes for this setup for the library

preparation for comparison with reference guide where the manual procedures are stated with 135 min for the corresponding steps. For incubation steps on the device Vapor Lock® was used as a vapor barrier.

Experimental design on the epMotion® 5073m NGS solution

For labs with lower throughputs we sought to establish an automated workflow on the epMotion 5073m NGS that can accommodate up to 16 samples. To this end all off-deck steps were performed on the Eppendorf Mastercycler X50 as shown in figure 1. The DNA workflow was tested using 12 replicates HD701 from Horizon Discovery with 10 ng DNA input each. For the RNA workflow we tested 6 replicates of Seraseq Fusion RNA Mix v2 from Seracare with 10 ng RNA input each and 6 replicates of Universal Human Reference RNA from Agilent with 10ng RNA input each. QC was performed with the DNA 1000 kit on the Bioanalyzer 2100. Table 1 summarizes the used consumables and time for this setup.

Table 1: Exemplified consumption of epT.I.P.S.® Motion and twin.tec® 96 PCR plates to process 12 samples (=24 libraries) per run on the epMotion 5073m NGS solution and 48 samples (=96 libraries) on the epMotion 5075t NGS solution. The automated methods are capable of processing variable numbers of samples as shown in Figure 1. The run times do not include the short manual setups and off-deck steps on the Mastercycler X50.

Step	Focus Panel					
	48 samples on epMotion 5075t (48 DNA and 48 RNA libraries)			12 samples on epMotion 5073m (12 DNA and 12 RNA libraries)		
	Tip consumption	Plate consumption	epMotion run time (min)	Tip consumption	Plate consumption	epMotion run time (min)
Method 1	50 µl: 48 300 µl: 0	1	24	50 µl: 24 300 µl: 0	1	25
Method 2	50 µl: 97 300 µl: 0	1	92	50 µl: 50 300 µl: 0	1	85
Method 3	50 µl: 152 300 µl: 0	0	58	50 µl: 72 300 µl: 0	0	39
Method 4	50 µl: 320 300 µl: 384	0	121	50 µl: 0 300 µl: 354	1	111
Method 5	50 µl: 192 300 µl: 488	2	89	-		
Total	50 µl: 809 300 µl: 872	4	384	50 µl: 146 300 µl: 354	3	190

Results & Discussion

Establishing a scalable automated solution for the Ampliseq for Illumina Focus Panel

To demonstrate the benefits of the automated AmpliSeq for Illumina Focus Panel solution we prepared sequencing libraries using different quality and quantity inputs of high-quality DNA (HD701) and moderately degraded DNA (HD798 and HD799) as well as Universal Human Reference RNA (UHR; Agilent) and Seraseq Fusion RNA

Mix v2 from Seracare®. As shown in figure 2, the Bioanalyzer traces of those libraries are as expected in terms of yield and library size [2] on the epMotion 5075t NGS. Even weaker replicates of UHR and Horizon DNA 799 yielded sufficient material and were therefore sequenced and aligned.

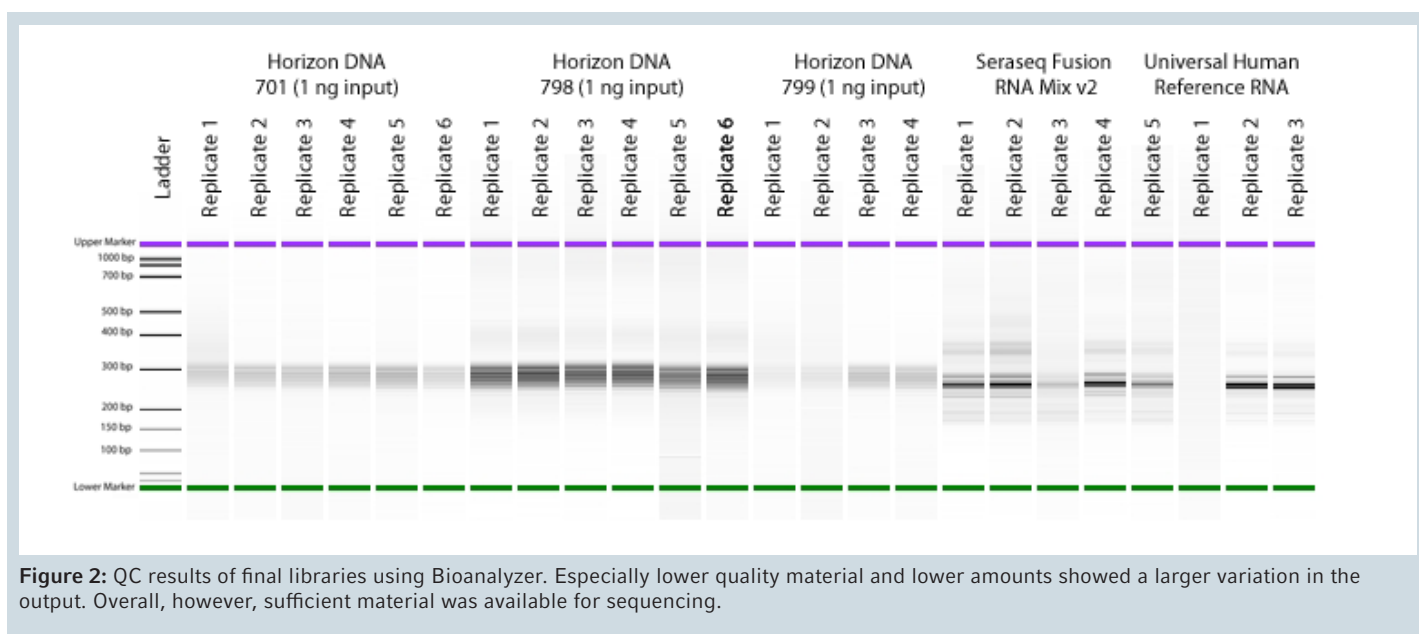


Figure 2: QC results of final libraries using Bioanalyzer. Especially lower quality material and lower amounts showed a larger variation in the output. Overall, however, sufficient material was available for sequencing.

Next, we examined, whether the coverage and reads passing filter would be different between the different replicates and workflows. We therefore analysed and aligned data. As shown in figure 3, panel A, the coverage of DNA samples was highly uniform with consistent amounts of data passing filter. Although the RNA samples showed lesser uniformity, overall all samples passed far above quality thresholds

(figure 3). This translated to the detection of fusion transcripts in the Seraseq pool, but not the UHR samples as expected. Fusion transcript detection was highly consistent in the Seraseq v2 samples (not shown), with the exception of 1 transcript in 3 replicates all transcripts were identified. As a result, these findings qualified the automated method.

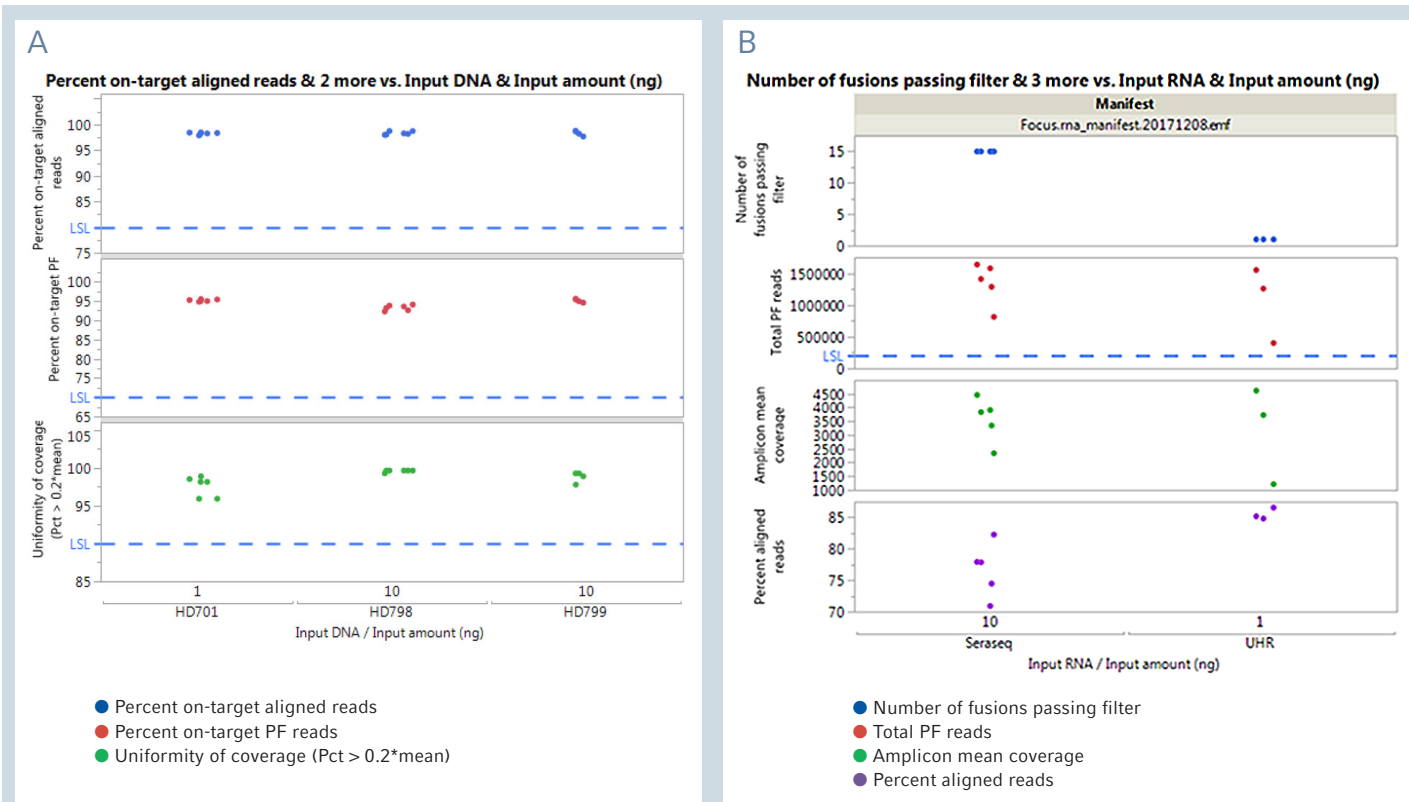


Figure 3: Consistency of sequencing results along panels with varying quality using the Basespace Amplicon Sequencing Analysis app quality metrics. Panel A shows the heterogeneity of samples with different inputs of degraded material. From top to bottom the target aligned reads, the percent on target reads passing filter and the uniformity of coverage were highly reproducible between replicates and between different inputs. Panel B shows similar statistics comparing Seraseq pool and UHR RNA. While the spread of total passing filter (PF), the average amplicon coverage and the percent aligned reads do show greater variability between the RNA samples, overall the number of fusions called is consistent.

Quality metrics on the 5073m NGS solution are consistent with the results obtained on the 5075t NGS solution

In order to validate the protocol for the 5073m NGS, we tested this protocol using samples similar to aforementioned experiment. We analysed the replicates of these libraries on the Bioanalyzer (Figure 4). In doing so we found that DNA as well as RNA samples yielded consistent profiles, which match those of Figure 2. In general, profiles seem to be more consistent, which is most likely related to the higher quality and amount of the input material. This is also evident with the very tight mean sizes of the DNA libraries (282 bp

with a CV of 1.2%) and RNA libraries (274 bp with a CV of 1.0% for the Seraseq Fusion and 284 bp with a CV of 0.7% for UHR RNA, respectively). Also, yields showed very little variation for DNA library replicates (mean 26 nM with a CV of 5.8%) and RNA library replicates (152 nM with a CV of 5.8% for the Seraseq Fusion and 171 nM with a CV of 3.0% for UHR RNA, respectively). Note the higher reproducibility of replicates is most likely linked to the higher inputs as described in the Materials and Methods section.

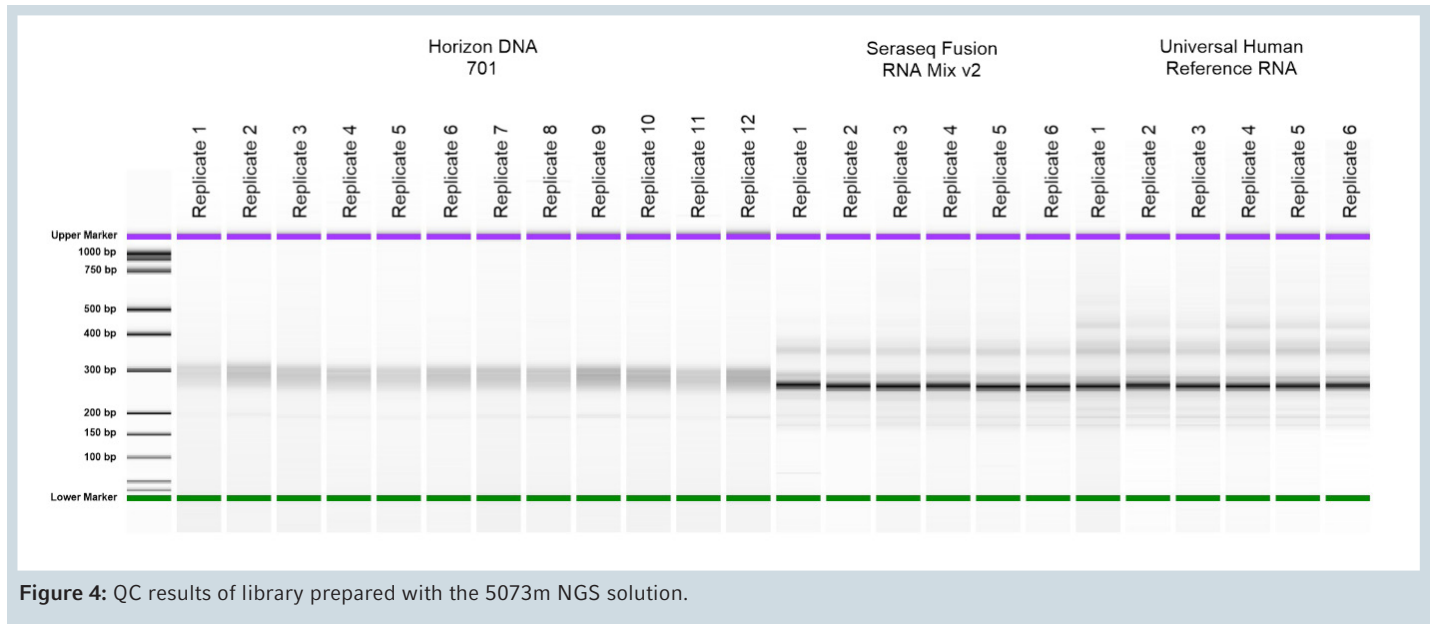


Figure 4: QC results of library prepared with the 5073m NGS solution.

Conclusion

Here we demonstrate the successful automation of the AmpliSeq for Illumina Focus Panel on two types of *epMotions*. This panel stands exemplary for further AmpliSeq workflows that feature either the DNA or RNA route of sample processing. Given, that the automated library prep solution can be scaled to the throughput, with the *epMotion* NGS solution, Eppendorf

provides a reliable and attractive companion for different sequencers. Table 2 exemplifies how different *epMotion* systems can provide the required flexibility to produce the matching number of libraries for different bench top sequencers.

Table 2: Matching automation platform for different sequencers. Depending on the coverage, panels of different sizes can be accommodated on different sequencers. For this a coarse division between different platforms can be made [3]. As this is proportional to the number of primer pools per panel (DNA and RNA) this definition can be further divided for the *epMotion* 5073m and 5075t NGS platforms to match that number of samples per sequencer, where the limiting variable is the number of PCR reactions (for each primer pool) that can be prepared on the liquid handler [1].

	iSeq® 100	MiniSeq®	MiSeq®	NextSeq®
epMotion 5073 (for up to 32 reactions*)	<ul style="list-style-type: none"> > Focus Panel (8 samples) > BRCA Somatic (12 samples) > Cancer Hotspot (16 samples) 	<ul style="list-style-type: none"> > Immune Response (24 samples) > Immune Repertoire (24 samples) 	<ul style="list-style-type: none"> > Immune Response (24 samples) > Immune Repertoire (24 samples) 	<ul style="list-style-type: none"> > No kits specified
epMotion 5075 (for up to 96 reactions*)	<ul style="list-style-type: none"> > BRCA Germline (96 samples) 	<ul style="list-style-type: none"> > Focus Panel (48 samples) > BRCA Somatic (80 samples) > Cancer Hotspot (96 samples) > BRCA Germline (96 samples) > Germline (96 samples) > Myeloid Panel (24 samples) 	<ul style="list-style-type: none"> > Focus Panel (48 samples) > BRCA Somatic (80 samples) > Cancer Hotspot (96 samples) > BRCA Germline (96 samples) > Germline (96 samples) > Myeloid Panel (24 samples) 	<ul style="list-style-type: none"> > Comprehensive Cancer (12 samples) > Exome (8 samples) > Transcriptome (40 samples) > Comprehensive v3 (48 samples)

*This is roughly illustrated in the table below based on 96 total PCR reactions divided on the different pools per kit below for the *epMotion* 5075 NGS solution and 32 total PCR reactions for the 5073m NGS solution. For example the focus panel features 1 DNA and 1 RNA primer pool, that thus allows the preparation of up to 16 samples on the *epMotion* 5073m and 48 samples on the *epMotion* 5075t, respectively matching iSeq and MiSeq throughputs.

References

- [1] Flexible Library Preparation for Your Cancer Research Lab – Automate AmpliSeq® for Illumina® Kits on the *epMotion*®. Eppendorf Flyer; www.eppendorf.com
- [2] AmpliSeq for Illumina Focus Panel. Reference Guide. Version: January 2018; www.illumina.com
- [3] AmpliSeq for Illumina. Sequencing amplified. Available at www.illumina.com.

Ordering information

Description	Order no. International	Order no. North America
epMotion® 5073m NGS Solution (w/o CleanCap and with EasyCon)	5073000930	–
epMotion® 5073m NGS Solution (w/o CleanCap and with Multicon)	5073000949	–
epMotion® 5073m NGS Solution (w CleanCap and with EasyCon)	5073000957	–
epMotion® 5073m NGS Solution (w CleanCap and with Multicon)	5073000965	–
or		
epMotion® 5075t NGS Solution (w/o CleanCap)	5075000962	5075000965
epMotion® 5075t NGS Solution (w CleanCap)	5075000963	5075000966
Thermoadapter PCR 96 (1x)	5075787008	960002199
Mastercycler® X50s	6311000010	6311000010
AmpliSeq® Library PLUS for Illumina®	(96 rxns): Illumina®, #200190102 (24 rxns): Illumina® #200190101	–
AmpliSeq® for Illumina® Focus Panel	Illumina® #20019164	–
AmpliSeq® CD indexes set A for Illumina® (96 indexes, 96 samples)	Illumina® #20019105	–
Agencourt® AMPure XP bead	Fisher Scientific® / NC9959336 or A63881	–
Vapor Lock®	Qiagen® / 981611	–

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