

Transitioning from custom amplicon-based parallel sequencing to hybrid capture-based sequencing in molecular pathology routine diagnostics

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INTRODUCTION

In recent years parallel sequencing technologies, also known as “Next-Generation Sequencing (NGS)”, have revolutionized the field of molecular diagnostic testing and have become increasingly integrated into daily clinical practice. Already, many institutions use amplicon-based parallel sequencing approaches for the analysis of multiple targetable genes [1]. By this method, therapeutic genes and gene segments can be amplified, enriched and subsequently sequenced by means of multiplex-PCR for the detection of point mutations, small deletions, insertions or duplications. This method can also be used on fragmented and chemically modified DNA from formalin-fixed, paraffin-embedded (FFPE) tissue, as well as with very little DNA from small biopsies [2, 3]. The disadvantage of amplicon-based parallel sequencing is that this method does not routinely detect chromosomal aberrations such as gene fusions and translocations, or copy number changes on a DNA level. Until now, these aberrations are still widely analysed by fluorescence in situ hybridisation (FISH) and immunohistochemistry (IHC) [4, 5]. The main disadvantage of FISH is that a specific assay has to be performed for each individual chromosomal aberration. Additionally, FISH is both time-consuming and cost-intensive [6].

Thus, the development of new technologies to detect all therapeutically relevant genomic alterations in a single assay is an ongoing process. On the DNA level, hybrid capture-based parallel sequencing enables the simultaneous detection of somatic gene mutations, gene fusions and copy number alterations. With this technology target regions are enriched with target-specific probes [7, 8].

In this project, a diagnostic custom hybrid capture-based assay was tested on FFPE material to enable the simultaneous detection of all genetic alterations and to replace FISH and amplicon-based parallel sequencing with one assay only. The aim was to translate the new assay into clinical practice to advance the establishment of targeted therapies. This will allow rapid, comprehensive and cost-effective testing for routine molecular pathological diagnostics.

MATERIAL AND METHODS

DNA extraction

Tumour areas were marked on H&E stained tissue slides by a pathologist and DNA was extracted from corresponding unstained 10 µm thick slides by manual macrodissection. After proteinase K treatment, the DNA was automatically purified using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (Promega, Madison, USA) on the Maxwell 16 Instrument (Promega) following the manufacturers’ protocol.

Covaris mechanical shearing for DNA fragmentation

After DNA extraction the DNA was quantified using the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and prepared for shearing according to the SureSelect^{XT} Target Enrichment System Manual (Agilent, Santa Clara, CA, USA). 200 ng of DNA was sheared on the Covaris E220 Focused-ultrasonicator (Woburn, MA, USA) to a fragment size of 150 bp following the manufacturer’s instructions. The treatment time was optimised for FFPE material and the Covaris settings are listed in table 1.

Table 1: Settings for using an 8 microTUBE–50 Strip AFA Fiber V2 with the E220 Focused-ultrasonicator

Target (Peak)	150 bp
Peak Incident Power (W)	175
Duty Factor	10%
Cycles per Burst	200
Treatment Time (s)	200
Temperature (°C)	7
Water Level	6
Sample volume (µl)	50
Intensifier (PN 500141)	Yes

Hybrid capture-based sequencing

Custom capture probes were designed using SureDesign (Agilent) for the target regions of 92 genes covering fusion breakpoints, exons and introns of interest. For library preparation SureSelect^{XT} Target Enrichment System (Agilent) was used following manufacturer's instructions. In brief, pre-enriched adapter-ligated libraries were prepared. Subsequently, custom capture probes were hybridised to target sequences to allow for sequence enrichment using streptavidin beads. Post-enriched libraries were quantified, pooled and sequenced on a NextSeq 500 (Illumina, San Diego, CA, USA).

RESULTS

DATA ANALYSIS

Quality of the NextSeq 500 (Illumina) sequencing runs were assessed with the Illumina Sequencing Analysis Viewer (Illumina). FASTQ files were generated using BaseSpace Onsite (Illumina). Sequencing data was analysed with in-house bioinformatics pipelines as well as commercially available software by alignment of FASTQ files against the human reference genome hg19. BAM files were used for visualisation of the sequenced data with the 'Integrated Genomics Viewer' (IGV, Broad Institute).

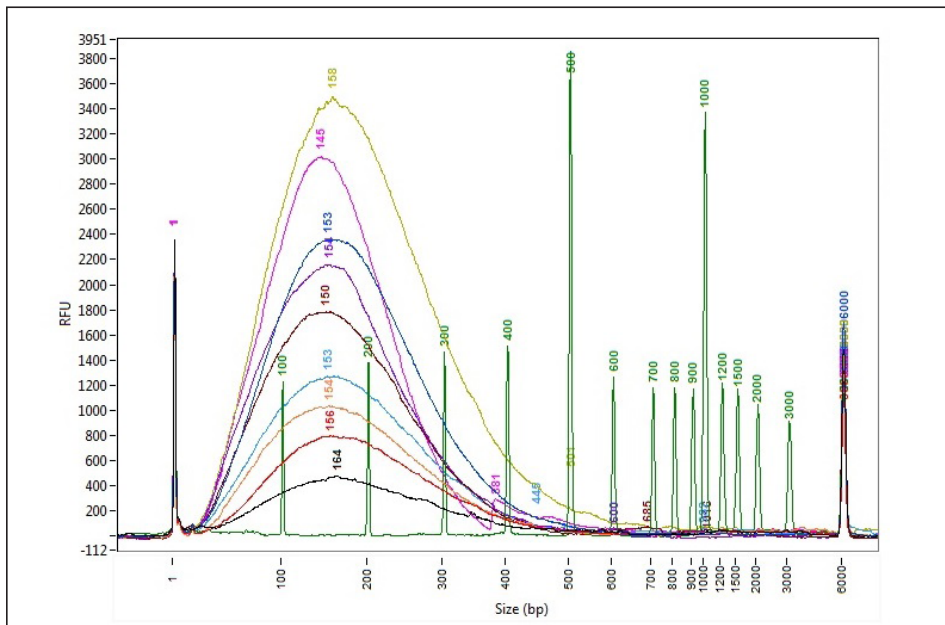


FIGURE 1: DNA shearing using different FFPE DNA concentrations and inputs on the Covaris E220. DNA derived from FFPE samples of different concentrations (50 – 415 ng) and levels of degradation were sheared with the optimised shearing conditions listed in table 1 and were analysed with the Fragment Analyzer (Advanced Analytical, Ankeny, IA, USA). The FFPE samples were from different tumour types including lung cancer, gastrointestinal cancer, ovarian cancer and melanoma and were collected in the years 2013 – 2015. Green peaks correspond to the ladder of the High Sensitivity NGS Fragment Analysis Kit (Advanced Analytical). The mean median bp size was always around ± 150 bps, indicating a consistent and robust shearing of different FFPE samples and concentrations.

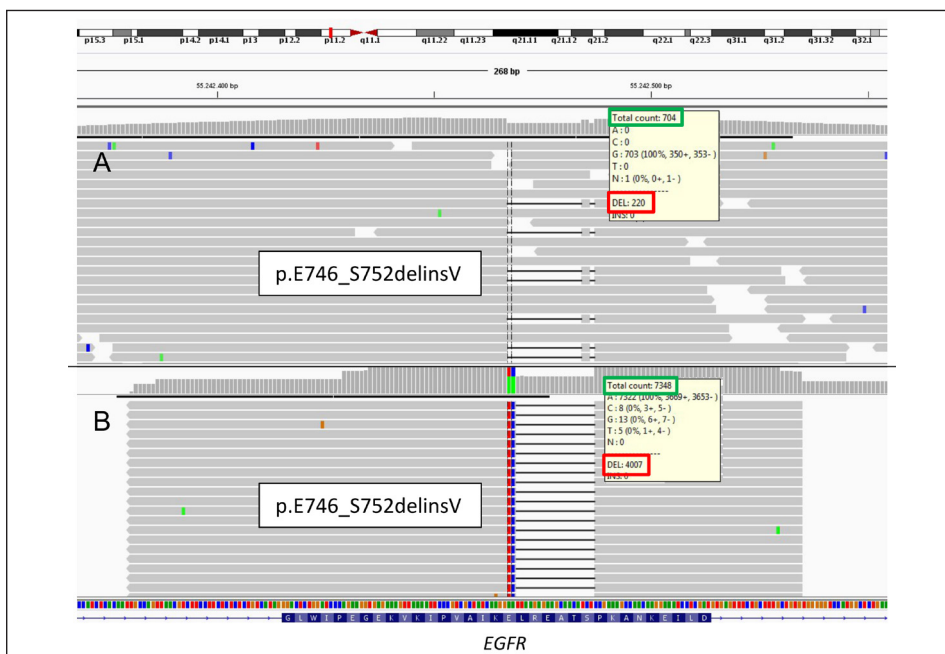


FIGURE 2: Illustration of hybrid capture-based (A) versus amplicon-based (B) parallel sequencing data and a genomic alteration in a lung cancer sample. Sequence reads were mapped to the target region of EGFR Exon 19. EGFR p.E746_S752delinsV deletion is visualised on chromosome 7 by the IGV (Integrative Genomics Viewer) in the hybrid capture-based (A) and amplicon-based (B) sequencing reads. The number of reads for the reference allele and the variant allele are shown for each enrichment method. Wild type reads are highlighted in green and mutated reads are highlighted in red. Differences in the aligned sequence reads for EGFR Exon 19 are clearly shown between the two enrichment technologies.

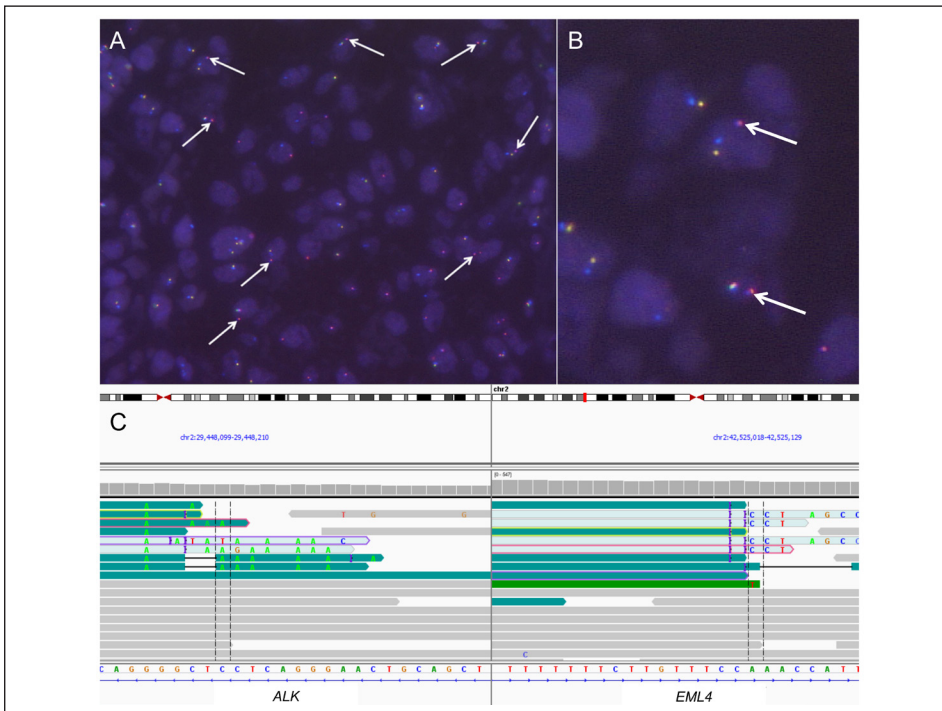


FIGURE 3: Detection of *EML4-ALK* rearrangement by FISH (A, B) and hybrid capture-based parallel sequencing (C). (A, B) The *EML4-ALK* rearrangement is detected with the ZytoLight® SPEC ALK/*EML4* TriCheck™Probe (ZytoVision, Bremerhaven, Germany): Orange and green fluorescent signals for the *ALK* break-apart probes and blue fluorescent signal for the *EML4* probe. The arrows point to *EML4-ALK* rearranged nuclei. (B) A magnification of two *EML4-ALK* rearrangements is shown. (C) *EML4-ALK* rearrangement as visualised on chromosome 2 by the IGV (Integrative Genomics Viewer). Highlighted are normal *ALK* and *EML4* reads (grey), as well as *EML4-ALK* fusion reads identified by discordant and split reads. Bioinformatics detected 29 split and 14 discordant reads for this *EML4-ALK* rearrangement. Paired-reads are highlighted with the same coloured border.

DISCUSSION

Hybrid capture-based parallel sequencing enables the simultaneous detection of chromosomal aberrations and somatic gene mutations in a single assay, and offers the potential to replace the traditional combination of FISH and amplicon-based parallel sequencing.

The covered target region is typically a few megabases in size, whereas amplicon-based parallel sequencing panels cover only a few kilobases. Thus, only one panel is needed for all different tumour entities. Additionally, fixation artefacts are avoided or minimised and PCR-duplicates can be filtered out. Disadvantages of this method are the complex custom panel design, higher sample input requirements, and the need for larger sequencers. While we have evaluated our hybrid-based sequencing workflow for input as low 50 ng, it still necessitates concurrent use of amplicon-based methods for some lung cancer samples with lower DNA yield. Another challenge is the bioinformatics for hybrid-capture based sequencing data, especially the detection of gene fusions and copy number changes. The highly complex data analysis requires custom data analysis pipelines and the field is in need of improvements in commercially available software.

Mechanical DNA shearing with the Covaris E220 Focused-ultrasonicator is a reliable tool for fragmentation of FFPE DNA as part of hybrid capture-based parallel sequencing approaches. For this workflow, DNA shearing is highly robust and enables consistent performances across a wide range of FFPE DNA of various concentrations or levels of degradation, thus enabling routine use in

molecular pathology diagnostics laboratories.

In summary, new parallel sequencing-based approaches have shown the potential to replace FISH as the gold standard in molecular pathology routine diagnostics for the detection of gene fusions and copy number alterations. However, FISH may always be used as a confirmatory test. In the following years, the time and the amount of tissue for diagnostic tests will become increasingly limiting, while at the same time an increase in sensitivity will be needed. Ongoing development of diagnostic tests, as well as the adaption to recent publications in cancer research, will become more and more important.

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