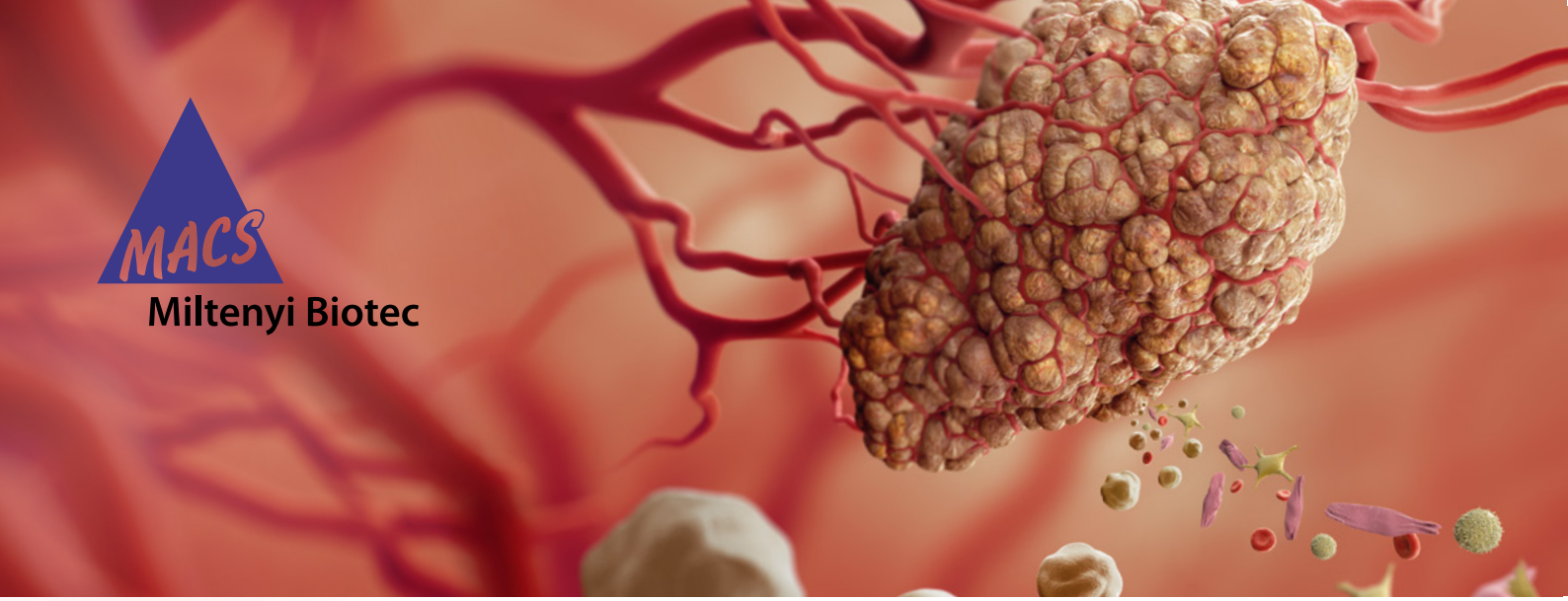

Enrichment of tumor cells from FFPE lung adenocarcinoma resection specimens increases sensitivity of mutation analysis

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Background

Formalin-fixed paraffin-embedded tissue (FFPE) represents the standard preservation procedure for diagnostic surgical pathology. The use of FFPE tissues for molecular analysis is increasing significantly in the last decade due to advances in new sequencing technologies[1]. Next generation sequencing (NGS) of tumor tissue enables personalized therapeutic approaches and is a widely used technique in routine diagnostics. Several guidelines require upfront testing of every non-small cell lung cancer (NSCLC) for PDL1, EGFR, ALK, ROS1, and BRAF within 10 working days, in order to decide between different therapy options. Clinical trials targeting further aberrations are also underway; thus, panel testing by parallel sequencing is widely recommended.

Samples with low tumor content represent a challenge for quality-assured molecular analysis. The smaller the proportion of tumor cells in a given sample, the higher the probability of false-negative results. For the reliable detection of a specific mutation it is usually required that the allelic fraction is 5% or higher; meaning that tumor tissue with a tumor cell content of less than 10% is not examined by NGS. In current routine diagnostics, up to 10% of lung cancer samples requested for NGS testing are below 10% and cannot be analyzed. Since additional tumor material of a patient is limited or unavailable, the enrichment of tumor cells in a sample below 10% tumor cell content could improve and simplify molecular analysis in any given sample.

In this application note, we describe the complete workflow to enrich tumor cells from bulk FFPE NSCLC sections to improve NGS-based cancer diagnostics. Molecular analysis of FFPE NSCLC resection specimens was performed on enriched tumor cells and compared to standard macrodissection. FFPE NSCLS sections were dissociated using the gentleMACS™ Octo Dissociator with Heaters in combination with the FFPE Tissue Dissociation Kit. Subsequent magnetic cell enrichment of tumor cells based on cytokeratin selection was carried out by using the new Anti-Cytokeratin MicroBeads, human and autoMACS® Pro Separator.

Materials and methods

Macrodissection
Estimations of tumor cell content of FFPE tumor sections resected from NSCLC patients were made by a trained pathologist (table 1). For macrodissection, a pathologist marked tumor areas on hematoxylin and eosin (H&E) stained tissue slides. The corresponding areas were scraped from three to six serial FFPE sections of 10 µm thickness.

Tissue dissociation
Two FFPE serial sections of 50 µm thickness were dissociated into single-cell suspensions from each resection specimen using the FFPE Tissue Dissociation Kit and gentleMACS Octo Dissociator with Heaters. Briefly, sections were deparaffinized and rehydrated, followed by a heat-induced antigen retrieval step prior to automated enzymatic and mechanic dissociation. All steps were performed according to the protocol provided with the FFPE Tissue Dissociation Kit **data sheet** (The data sheet is available for direct download on the product page).

Product	Order No.
FFPE Tissue Dissociation Kit	130-118-052
gentleMACS Octo Dissociator with Heaters	130-096-427
gentleMACS C Tubes (25 tubes, sterile-packed)	130-093-237
Pre-Separation Filters (20 µm)	130-101-812
autoMACS Pro Separator Starter Kit	130-092-545
Anti-Cytokeratin MicroBeads, human	130-123-094

Specimen ID	H&E staining	Estimated tumor cell content
Specimen 1		70% tumor cells
Specimen 2		10% tumor cells
Specimen 3		< 5% tumor cells

Table 1: Resected FFPE tumor specimen sections used for the study. For each specimen hematoxylin and eosin (H&E) stained slides are shown, as well as corresponding estimated tumor cell contents.

Tumor cell isolation
After dissociation, automated enrichment of carcinoma tumor cells was performed based on cytokeratin expression using Anti-Cytokeratin Microbeads, human and the autoMACS® Pro Separator.

DNA extraction
DNA was extracted from enriched tumor cells and from tumor areas after macrodissection. DNA extraction after macrodissection was performed using the Maxwell® 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega) in a final volume of 70 µL. DNA extraction from enriched tumor cells was performed using a combination of the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit and the RSC Blood DNA Kit (AS 1400) (Promega) in a final volume of 50 µL according to the manufacturer's recommendation.

Next-Generation Sequencing
Extracted DNA was quantified using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific). Target-enriched DNA libraries were prepared according to the manufacturer's recommendation (TruSight Tumor 170, Illumina).
DNA quality and fragment sizes were verified using the Agilent 4200 TapeStation system. Normalized libraries were pooled for sequencing with the NextSeq 500 (Illumina) using the NextSeq® 500/550 High Output Kit (Illumina) and analyzed using BaseSpace™ Sequence Hub (Illumina).

Results

NGS-based mutation analysis of specific driver mutations was conducted on FFPE NSCLC patient resection specimens, after macrodissection of specific tumor areas, and compared to enrichment of carcinoma tumor cells.

In resection specimens with high estimated tumor cell content (70%), identification of the driver mutation was comparable when performing macrodissection to enrichment of tumor cells. However, the sensitivity of the molecular analysis of specimens with low estimated tumor cell content (10% or below) was increased by the enrichment of tumor cells from the bulk sample (table 2). On average, the allele frequency of the identified driver mutation increased by 4.5-fold after enrichment of tumor cells compared to macrodissection.

Conclusions

- The FFPE Tissue Dissociation Kit, in combination with the gentleMACS™ Octo Dissociator with Heaters, allowed the efficient dissociation of FFPE NSCLC resection specimens for the subsequent isolation of carcinoma cells using Anti-Cytokeratin MicroBeads, human and the autoMACS Pro Separator.
- Isolation of carinoma cells from FFPE NSCLC resection specimens with low tumor cell content, enabled the increase in sensitivity of the NGS-based mutation analysis.
- This methodology has potential to introduce NGS-based multiplex analysis of FFPE tumor specimens with low tumor cell content into routine workflows, and ultimately allow physicians to make more reliable and rational therapy decisions for their patients.

References

1. Kokkat, T.J. *et al.* (2013) Archived formalin-fixed paraffin-embedded (FFPE) blocks: a valuable underexploited resource for extraction of DNA, RNA, and protein. Biopreserv Biobank., 11 (no 2): 101–106.

Specimen ID	Pre-processing prior DNA extraction	Concentration of extracted DNA (ng/µL)	DNA input for NGS	Driver mutation identified	Coverage	Allele frequency
Specimen 1 (70% tumor cells)	Macrodissection	48.80	40	EGFR: c.2236_2250del; p.E746_A750del	1471	52.3%
	Enriched tumor cells	38.00	40	EGFR: c.2236_2250del; p.E746_A750del	2522	59.9%
Specimen 2 (10% tumor cells)	Macrodissection	4.78	20	KRAS: c.34G>T; p.G12C	388	11%
	Enriched tumor cells	0.46	20	KRAS: c.34G>T; p.G12C	623	28%
Specimen 3 (< 5% tumor cells)	Macrodissection	5.64	40	KRAS: c.34G>T; p.G12C	405	1%
	Enriched tumor cells	2.12	40	KRAS: c.34G>T; p.G12C	951	7%

Table 2: Sequencing results from DNA extracted after macrodissection or from enriched tumor cells.

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