Sensitive genetic analysis of enriched cell populations from FFPE carcinoma samples by NGS

Sensitive genetic analysis of enriched cell populations from FFPE carcinoma samples by next-generation sequencing

Stefan Küffer[1], Carsten Poggel[2], René Hilgers[2], and Philipp Ströbel[1]

[1] Institute of Pathology, University Medical Center Göttingen (UMG), Göttingen, Germany

[2] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Background

Formalin-fixed paraffin-embedded (FFPE) tissue samples are the worldwide standard in routine pathology procedures for the investigation of human diseases including cancer. Huge archives of FFPE samples represent an invaluable and easily accessible resource of biological specimens for retrospective large-cohort studies on the identification of neoantigens or biomarkers by next-generation sequencing (NGS).

Although handling and storage of FFPE samples is easy and effortless, there are also quite a few drawbacks. Besides cancer cells, samples of solid tumors contain various non-cancer cells, including tumor-infiltrating leukocytes (TILs) as well as nerves, blood vessels, and fibroblasts. Amounts and proportions of these cells can vary depending on the type of tumor, disease stage, and prior tumor treatment. These cells can influence molecular analysis of cancer cells considerably by diluting the ratio between non-neoplastic and neoplastic cells in a sample. Pancreatic cancer is a prototypical example of this problem. Pancreatic carcinomas characteristically show few tumor cells in a background of a dense fibrotic stroma. In such tumors, enrichment of tumor cells prior to their analysis is of critical importance. The current methods for separating tumor cells from bulk tumor for subsequent molecular analysis include macrodissection, expression-based microdissection (xMD)[1], and laser capture microdissection. Each of these techniques has its own advantages and disadvantages.

In this application note, we describe a complete workflow for the efficient isolation of tumor cells from FFPE samples using reagents and instruments from Miltenyi Biotec.

The workflow starts with automated tissue dissociation based on a combination of enzymatic and mechanical treatment with the FFPE Tissue Dissociation Kit and the gentleMACS™ Octo Dissociator with Heaters to obtain single-cell suspensions with preserved epitopes. Tumor cells and non-tumor cells are subsequently isolated by flow cytometry–based cell sorting using MACS® Antibodies. Isolated tumor cells are suitable for genetic analyses by NGS or flow cytometry, including determination of ploidy status, somatic mutations, and variation of mutation copy numbers. Isolated non-tumor cells can be used, for example, as a germline reference in NGS instead of a matched normal sample, such as patient blood.

Materials and methods

Tissue dissociation

FFPE sections (50 µm) of human pancreas carcinomas were dissociated into single-cell suspensions using the FFPE Tissue Dissociation Kit and the gentleMACS Octo Dissociator with Heaters. Dissociation was performed according to the protocol provided with the kit. The protocol includes deparaffinization and rehydration of the FFPE samples, heat-induced antigen retrieval to reverse formalin-induced modifications, and the actual enzymatic and mechanical dissociation process. Heat-induced antigen retrieval has been described to enhance immunohistochemical staining of FFPE samples[2], and mild enzymatic treatment has been used previously in the context of FFPE tissue dissociation for flow cytometry[3]. Tissue dissociation by the FFPE Tissue Dissociation Kit and the gentleMACS Octo Dissociator with Heaters is gentle and ensures preservation of epitopes, including the cytoskeletal markers cytokeratin and vimentin. Hence, this process enables subsequent cell separation based on these markers. Besides cytokeratin, carcinoma cells sometimes express variable levels of vimentin, depending on the epithelial-mesenchymal transition status[4]. In contrast, stromal cells and TILs express only the cytoskeletal marker vimentin.

Flow cytometry of dissociated carcinoma tissue

After dissociation of carcinoma tissue into single-cell suspensions, cells were labeled with Anti-Cytokeratin-FITC

(REA831) and Anti-Vimentin-APC (REA409) antibodies in addition to DAPI for flow cytometry–based cell sorting and analysis.

DNA extraction

DNA was extracted from bulk tumor or isolated tumor cells using the QIAamp® DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions except that the deparaffinization step was omitted. Instead, the extraction procedure was started from the proteinase K digest on. DNA was quantified on a Qubit™ fluorometer using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and fragmentation was determined on an Agilent® 2100 Bioanalyzer®.

Next-generation sequencing

Next-generation sequencing was performed by GATC Biotech AG (Konstanz, Germany) based on the INVIEW Oncopanel all-in-one.

Results

DNA fragmentation analysis

Comprehensive genetic analysis requires DNA samples of high integrity. To test whether the tissue dissociation procedure has an effect on DNA quality, we compared the fragmentation patterns of DNA that was prepared directly from FFPE samples and DNA prepared from the dissociated FFPE tissue. Figure 1 shows that the fragmentation patterns were similar between the two DNA samples.

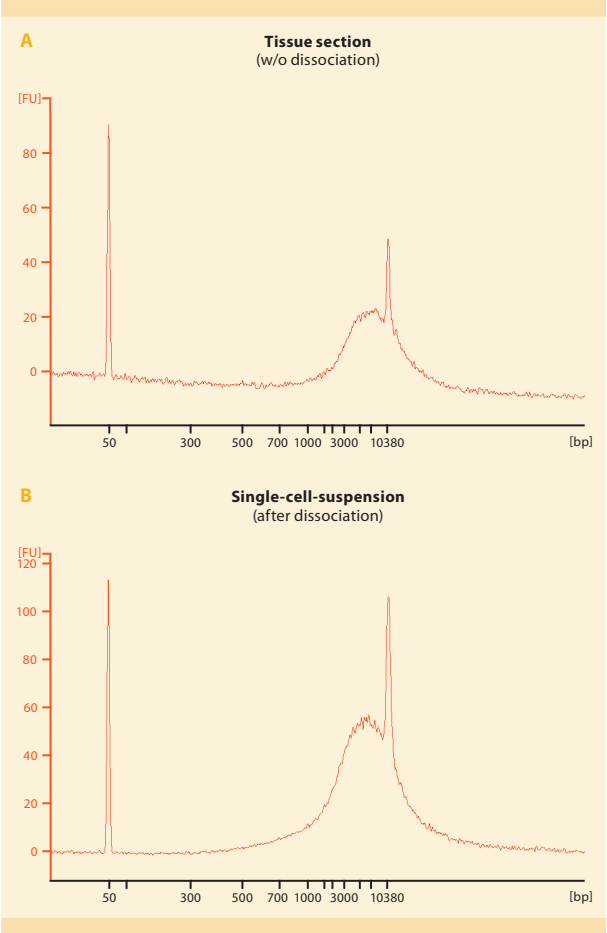

Figure 1: Fragmentation analysis of DNA prepared directly from an FFPE tissue section (A) or after dissociation of the tissue section into a single-cell suspension (B).

Flow cytometry analysis

Carcinoma cells can be distinguished from stromal cells and TILs by the cytoskeletal markers cytokeratin and vimentin. Cytokeratin is expressed in normal and neoplastic epithelial cells (i.e. carcinoma cells), whereas stromal cells and TILs express only vimentin. Both markers can be easily detected by flow cytometry in a single-cell suspension prepared by the FFPE Tissue Dissociation Kit (fig. 2A). This demonstrates that the dissociation procedure preserves these epitopes and thus enables subsequent cell sorting based on these markers. Indeed, cytokeratin-positive carcinoma cells could be separated effectively from vimentin-positive, cytokeratin-negative non-cancer cells, resulting in two pure and distinct cell populations (fig. 2B). The number of isolated cells yielded sufficient DNA for subsequent analysis (table 1).

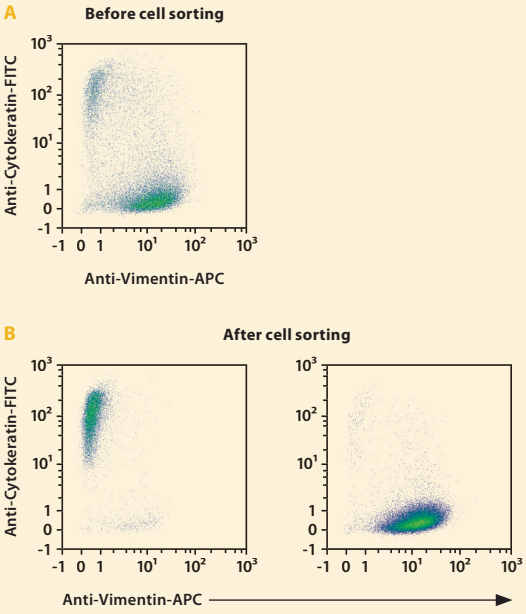

Figure 2: Flow cytometry analysis of cytokeratin-positive and vimentin-positive cells. Cells were labeled with fluorochrome-conjugated antibodies as indicated and analyzed before (A) and after sorting (B). Sorting resulted in two populations of cytokeratin-positive cells and vimentin-positive cells (B). Cells were analyzed using the MACSQuant® Analyzer 10.

	DAPI⁺cytokeratin⁺ cells	DAPI⁺vimentin⁺ cells
Cell number	2.1×10^5	2.5×10^5
Extracted DNA (µg)	1.7	2.7

Table 1: Cell yield after sorting of cytokeratin-positive and vimentin-positive cells from dissociated FFPE tissue and yield of DNA extracted from the sorted cell populations.

Gene	Location	AA change	Codon change	Mutation frequency				Mutation type
				Non-dissociated	Unsorted	Vimentin+	Cytokeratin+	
TP53	chr17:7579472	p.P72R	c.215C>G	100.0%	99.5%	99.6%	99.4%	germline
ABCB1	chr7:87160618	p.S893A	c.2677T>G	99.8%	100.0%	99.8%	99.5%	germline
TAS2R38	chr7:141673345	p.A49P	c.145G>C	99.8%	99.8%	100.0%	100.0%	germline
TAS2R38	chr7:141672604	p.I296V	c.886A>G	99.6%	99.7%	99.7%	99.5%	germline
CYP2B6	chr19:41512841	p.Q172H	c.516G>T	53.6%	57.2%	48.5%	87.0%	germline
FGFR4	chr5:176520243	p.G388R	c.1162G>A	43.8%	45.0%	46.8%	55.8%	germline
CYP2B6	chr19:41515263	p.K262R	c.785A>G	39.3%	36.0%	29.2%	81.8%	germline
KRAS	chr12:25398284	p.G12V	c.35G>T	13.9%	14.7%	<1.0%	52.3%	somatic
TP53	chr17:7577120	p.R273H	c.818G>A	11.9%	13.8%	<1.0%	80.5%	somatic
BAX	chr19:49458970	p.E41fs	c.121insG	1.3%	<1.0%	<1.0%	1.5%	noise

Table 2: NGS analysis of various oncogenes in carcinoma and non-tumor cells. DNA was extracted from non-dissociated FFPE tissue samples, unsorted cells from dissociated tissue, and sorted vimentin-positive or cytokeratin-positive cells.

Molecular analysis of carcinoma versus non-tumor cells

To test whether the tissue dissociation procedure has an effect on DNA mutation frequencies, we compared the mutation frequencies from DNA that was prepared directly from whole FFPE samples (blue in table 2) and DNA prepared from the dissociated FFPE tissue before cell sorting (green in table 2). The mutation frequencies were similar between the two DNA samples.

Flow cytometry–based cell sorting yielded a cytokeratin-positive carcinoma cell population and a vimentin-positive non-tumor cell population. High purities of the isolated populations enabled sensitive analysis of cancer-associated genetic alterations. Several mutations were significantly enriched in isolated cytokeratin+ cells, e.g., CYP2B6 (chr19:41512841). Moreover, as DNA from carcinoma cells was analyzed without interference by DNA from non-tumor cells, certain alterations, e.g. KRAS (chr12:25398284) and TP53 (chr17:7577120), could be specifically attributed to the tumor cells, which indicates the presence of somatic mutations. Alterations that were also detectable in DNA from non-tumor cells indicated germline mutations.

Conclusions

- FFPE Tissue Dissociation Kit in combination with the gentleMACS Octo Dissociator with Heaters enables fully automated FFPE tissue dissociation of multiple samples at the same time. The dissociation process resulted in single-cell suspensions with preserved markers for flow cytometry of FFPE carcinoma samples.
- Isolation of tumor cells from bulk tumor enhances the sensitivity of genetic analysis. Tumor-specific signals are enriched and can be easily analyzed and distinguished from background signals, e.g., by NGS.
- No additional healthy tissue or blood sample is required as a control because isolated vimentin-positive, cytokeratin-negative cells provide an ideal reference to distinguish somatic from germline mutations.
- Preservation of cytokeratin and vimentin may allow for the separation of tumor cells of different epithelial-mesenchymal transition statuses to obtain information on tumor heterogeneity.

MACS Product	Order no.
FFPE Tissue Dissociation Kit	130-118-052
gentleMACS Octo Dissociator with Heaters	130-096-427
MACSQuant Analyzer 10	130-096-343
DAPI Staining Solution	130-111-570
Anti-Cytokeratin-FITC, human (clone REA831)*	
Anti-Vimentin-APC, human (clone REA409)*	

* These antibodies are available conjugated to a wide range of other fluorochromes. For more information on MACS Antibodies visit **www.miltenyibiotec.com/antibodies**

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Miltenyi Biotec GmbH | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macs@miltenyibiotec.de | **www.miltenyibiotec.com**

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