Rapid intra-surgical real-time Glioma Characterization using a defined Marker Panel

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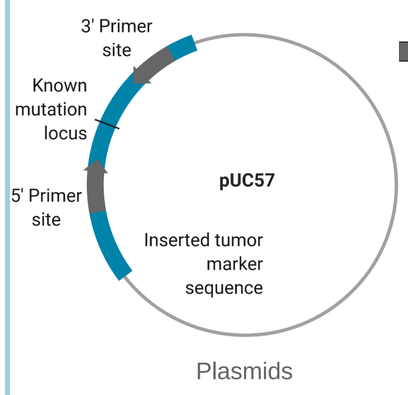
1. Abstract

Divergent to classical histology diagnostics of brain tumors, we developed an amplification-based **workflow (4)** to investigate molecular tumor markers during surgery. We **designed (2)** amplification systems for defined marker regions containing known mutations (*IDH1, IDH2, pTERT, H3F3A, Hist1H3B, BRAF*). Those amplicons were used for library preparation using either Ligation or Rapid Sequencing Kits. Resulting sequences were mapped and variants called in real-time using **custom scripts (3)**. As proof of concept, a **clinical demonstrator (5)** was performed alongside a tumor resection surgery (4 markers: *IDH1, IDH2, pTERT, H3F3A*). The resulting intra-surgical classification of the tumor was confirmed by classical histological methods after surgery.

2. Design



Plasmids

Amplicons

Sequencing

1 2 3 4 5 6
ACT — GCT
ACTGAGCT

Mapping

Amplicon sizes:

IDH1	-	962 bp
IDH2	-	990 bp
pTERT	-	489 bp
H3F3A	-	914 bp
Hist1H3B	-	916 bp
BRAF	-	1098 bp

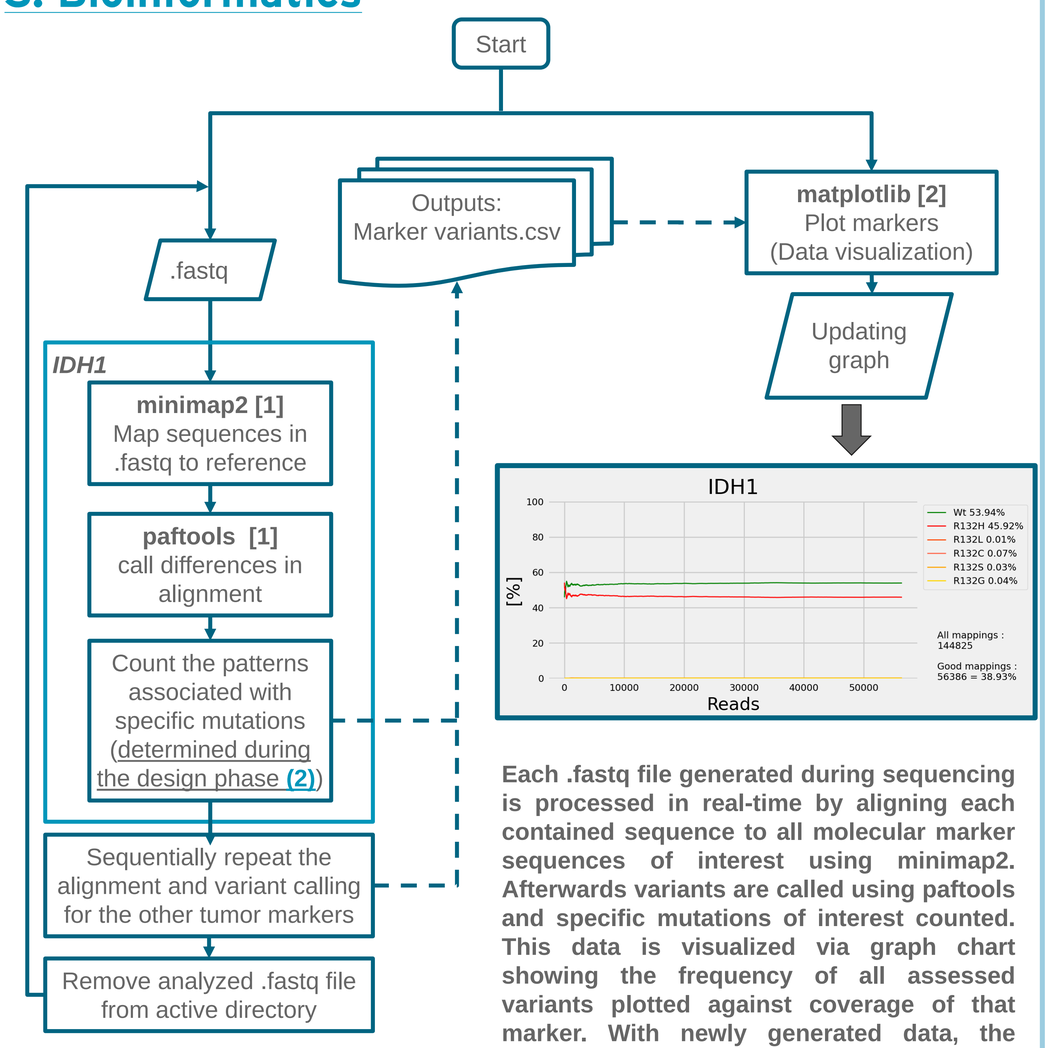
Start	End	Reference	Sequence
4	4	-	GA

Listing the sequencing variations corresponding to each marker variant

Scripts to automate analysis **(3)**

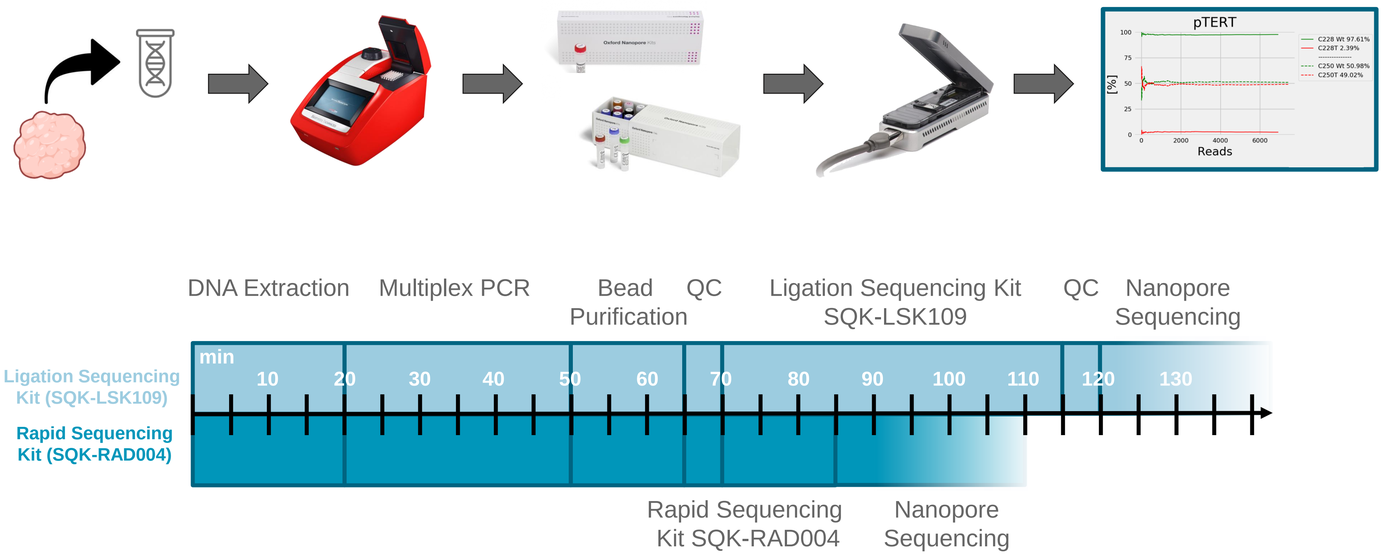
pUC57 plasmids containing tumor marker sequences with defined mutations were amplified. The resulting amplicons were used for library preparation and sequencing. Those sequences were mapped to references that had the bases of interest deleted. Variant calling of the alignments revealed a list of unique ‚insertions' corresponding to either wildtype or mutant sequences. This data was used to write several python scripts (3) that have the potential to help automatically detect tumor marker variants in amplicons from uncharacterized gDNA.

3. Bioinformatics



Each .fastq file generated during sequencing is processed in real-time by aligning each contained sequence to all molecular marker sequences of interest using minimap2. Afterwards variants are called using paftools and specific mutations of interest counted. This data is visualized via graph chart showing the frequency of all assessed variants plotted against coverage of that marker. With newly generated data, the graph extends in x-axis direction.

4. Workflow & Timeline



Tumor tissue (10 – 20 mg) is lysed via bead mill before Proteinase K and RNase A treatment. DNA is extracted via spin column and its concentration subsequently measured using a Nanodrop spectrophotometer. Between 4 and 40 ng gDNA are used as template for a multiplex PCR (35 cycles, 5 s @ 95 °C, 10 s @ 59 °C, 20 s @ 72 °C) containing amplification systems for the tumor marker genes of interest. Phusion HSII polymerase is used for high replication fidelity. After bead purification using AMPure XP beads DNA concentration is measured again via Nanodrop. Up to 400 ng amplicon DNA are then used for library preparation according to Ligation or Rapid Sequencing protocols. Nanopore sequencing using MinION R9.4.1 Flowcells and live analysis of generated reads are started. Results may be assessed after the graphs indicating the variant frequencies stabilize, typically after ~100 – 1000x coverage / 15 – 30 min of sequencing.

5. Clinical Demonstrator

Using the workflow described above (4) we assessed clinically relevant variations of *IDH1*, *IDH2*, *pTERT* and *H3F3A*. The data suggests heterozygous mutations in *IDH1* (R132H) and *pTERT* (C250T), and wildtype *IDH2* and *H3F3A*. Histological analysis confirmed the assessments of *IDH1* and *IDH2*. The status of the molecular markers could be assessed ~2h after receipt of the tumor tissue.

The chosen molecular markers hold significance in the latest WHO Classification of Tumors of the Central Nervous System [3]. Intra-surgical detection of relevant tumor markers may permit an adjustment of the surgical strategy, application of targeted local therapeutic modalities and deliver the prognosis to patients right after surgery.

6. References

[1] H. Li, Minimap2: pairwise alignment for nucleotide sequences, *Bioinformatics*, Volume 34, Issue 18, 15 September 2018, Pages 3094–3100, https://doi.org/10.1093/bioinformatics/bty191
[2] J. D. Hunter, Matplotlib: A 2D Graphics Environment, Computing in Science & Engineering, vol. 9, no. 3, pp. 90-95, 2007
[3] D. N. Louis et. al., The 2021 WHO Classification of Tumors of the Central Nervous System: a summary, *Neuro-Oncology*, Volume 23, Issue 8, August 2021, Pages 1231–1251, https://doi.org/10.1093/neuonc/noab106

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