Systematic Evaluation of a Novel Formalin- and Methanol-Free FNA Fixative for the Study of Lung Cancer

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Introduction

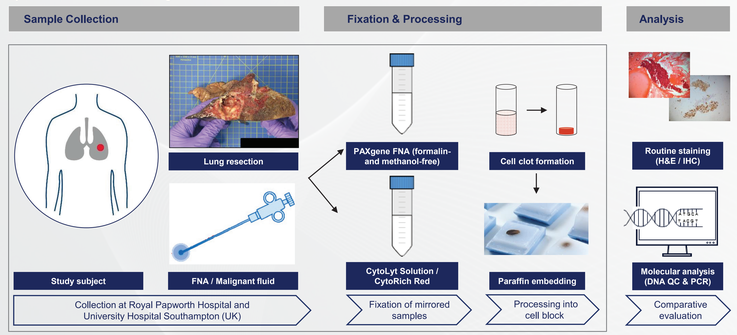
Lung cancer remains a leading cause of cancer mortality worldwide. Identification of actionable mutations is a key focus in cancer research and is highly dependent on nucleic acid quality. Currently, formalin- and methanol-based fixation methods for pathology samples are a rate-limiting step for tumor molecular analysis. STRATFix is an Innovate UK-funded collaborative project between QIAGEN® and NHS pathology teams across the UK. A major aim of the project is to compare a Research Use Only PAXgene FNA fixative* (a novel formalin- and methanol-free fixative) to traditional fixation approaches and evaluate performance in preserving cellular morphology, DNA and RNA, which are now crucial for the molecular study of lung cancer.

Methods

Paired fine needle aspirates (FNAs) of lung tumor resections were collected from up to 16 subjects undergoing surgery for non-small cell lung carcinoma and either fixed in CytoLyt® Solution or PAXgene FNA, a novel fixative specially formulated for cell-rich body fluids. Additionally, 32 paired CytoRich™ Red and PAXgene FNA-fixed FNAs and malignant fluids (ascitic and pleural fluid) were collected at a second clinical site. All samples were fixed for >1 hour, centrifuged, encapsulated in a cell clot and processed into a paraffin block. PAXgene FNA fixation and processing was formalin-free, while samples fixed with CytoLyt Solution and CytoRich Red underwent routine workflow for formalin fixation and paraffin embedding. Routine hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining were performed and sections were blind-scored by pathologists. DNA was extracted using QIAGEN kits and assessed for quantity with the Qubit® 2.0 Fluorometer and for fragmentation with the Agilent® 2200 TapeStation System. PCR performance for DNA was tested using the QIAseq® DNA QuantiMIZE Kit. DNA was further tested for suitability for molecular methods using the *therascreen*® EGFR RGQ PCR Kit.*

Study Design – Sample collection, processing & analysis

- Endobronchial ultrasound-guided FNA mimic and collection of malignant fluid from lung cancer.
- Processing of mirrored samples fixed with PAXgene FNA (formalin- and methanol-free) and CytoLyt Solution or CytoRich Red.
- Analysis with routine histological and molecular methods.

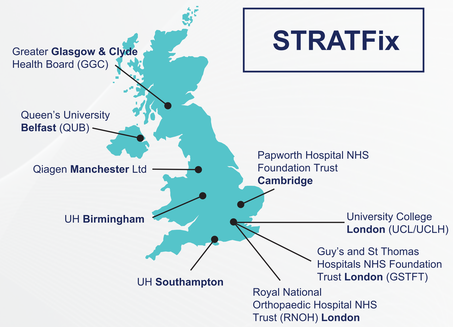


About STRATFix

STRATFix: Enabling stratified medicine with novel fixatives for improved pre-analytical pathology workflows

- In this project, prototypes of integrated systems for sample collection, stabilization and nucleic acid purification were developed for implementation in routine pathology.
- Formaldehyde-free systems were provided for solid tissue, FNAs and circulating cell-free DNA in blood to enable genetic follow-up characterization.
- STRATFix is a joint effort of industry and academia involving cross-sector collaboration and knowledge transfer. All members can build upon excellent and unique expertise in their fields.

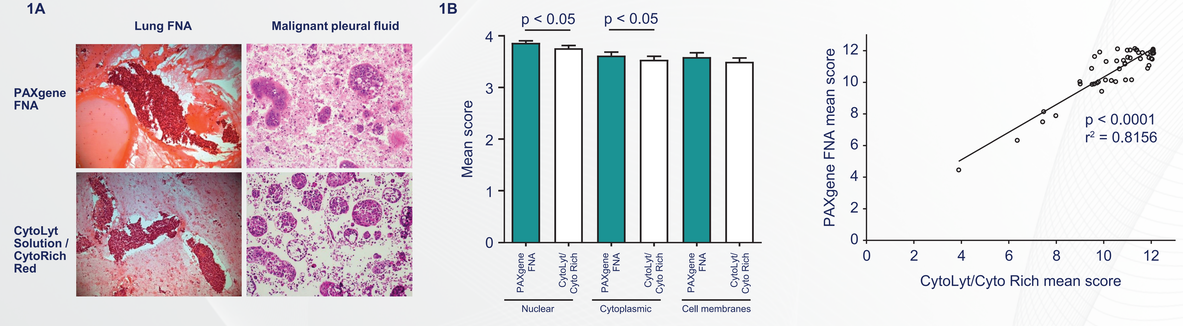
www.spidia.eu/stratfix-project/



Results

H&E staining

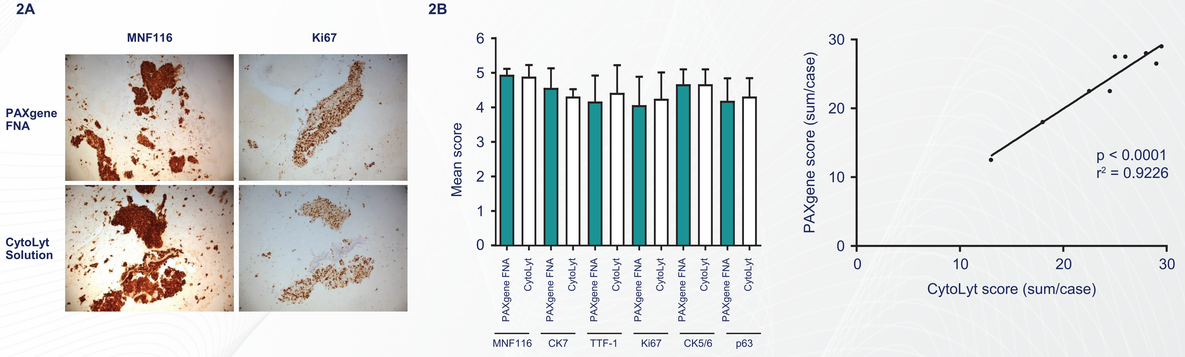
- Despite some eosinophilia of PAXgene FNA-fixed samples, adequate and comparable H&E staining was observed.



H&E staining. Lung EBUS FNA and malignant fluids were stained with hematoxylin and eosin (H&E) and sections were blind-scored by pathologists. **1A:** Representative stains of samples fixed with PAXgene FNA and with CytoLyt Solution and CytoRich Red. **1B:** Quality of H&E sections was evaluated using a published and accepted scoring system *(Craft et al. 2014)* whereby a score of 0–4 was assigned to nuclear, cytoplasmic and cell membrane features (n = 51).

Immunohistochemical staining

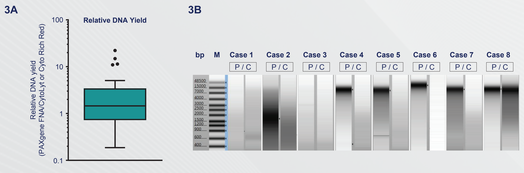
- IHC was successful and comparable to CytoLyt Solution in all samples scored.



IHC staining. Lung EBUS FNA samples were stained with antibodies against MNF116, CK7, TTF-1, Ki67, CK5/6 and p63. **2A:** Representative stains of samples fixed with PAXgene FNA and CytoLyt Solution. **2B:** Quality of IHC sections was evaluated by two independent observers whereby a score of 0–5 was assigned: 1 or 2 = Unacceptable, 3 = Borderline Acceptable and 4 or 5 = Acceptable (n = 9).

DNA quality control

- Higher DNA yield was recovered from PAXgene FNA-fixed vs. CytoLyt Solution/ CytoRich-fixed mirrored samples.
- Less DNA fragmentation was observed in PAXgene FNA-fixed samples.

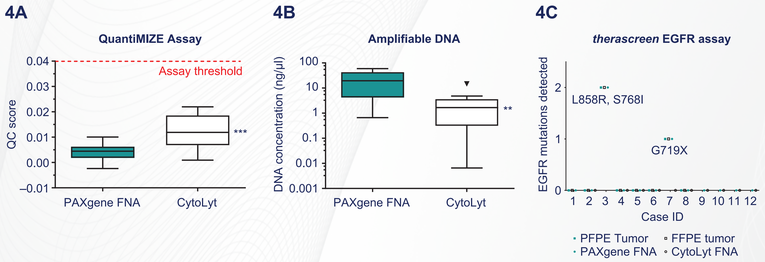


DNA yield and integrity. DNA was extracted from samples fixed with PAXgene FNA or CytoLyt Solution/CytoRich Red using commercial kits and following the manufacturer's protocol. Sections of a paraffin block served as input for each DNA extraction. **3A:** DNA yield was assessed by Qubit dsDNA Broad Range fluorimetry and High Sensitivity Kit (n = 32). **3B:** DNA integrity was assessed by Agilent 2200 TapeStation and Genomic Screen Tape assay: 5/8 cases show one high-molecular weight band, while corresponding formalin samples lack this distinct band. In 3/8 cases both PAXgene and formalin samples show poor DNA integrity. (bp = base pairs, M = marker, P = PAXgene FNA, C = CytoLyt Solution).

PCR assays and mutation analysis

- DNA from PAXgene FNA fixation resulted in superior performance in a PCR-based assay. A low quality control score indicates similar amplification of smaller and larger PCR fragments (Figure 4A).
- Similar EGFR mutations were detected in 2/12 subjects, in both PAXgene FNA- and CytoLyt Solution-fixed samples (Figure 4C).

PCR-based DNA analysis. To evaluate DNA performance in downstream PCR applications, a commercial assay from QIAGEN (QIAseq DNA QuantiMIZE Kit) was used to determine DNA quality **(4A)** and quantity **(4B)** according to the manufacturer's instructions. DNA (1 µl) was amplified in two qPCR assays that interrogate 20 genomic loci to generate 100 and 200 bp amplicons, respectively. C_T values of control and case samples from assays for each of the amplicons (100 and 200) were used for DNA quality scoring and quantification. Statistically significant differences were observed for both DNA quality (***p = 0.0005 < 0.05) and quantity (**p = 0.0027 < 0.05). **(4C)** The *therascreen* EGFR RGQ PCR Kit, which detects exon 19 deletions, L858R, L861Q, G719X, S768I, exon 20 insertions, and the resistance mutation T790M in the EGFR gene, was used for qPCR-based mutation analysis. The minimum required DNA volume is determined in a control reaction prior to a mutation reaction run. The ΔC_T value from the mutation run (C_T[MUT] – C_T[CTRL]) is the basis for mutation positive or mutation negative calls.



Conclusions

- The majority of lung cancer patients present with advanced-stage and inoperable disease resulting in limited availability of tissue for subsequent molecular analyses.
- PAXgene FNA-fixed samples show comparable morphology to formalin and methanol fixation, are adequate for histopathology and IHC, and are more suitable for molecular studies, which is important for low-yield samples such as FNA and malignant fluids.
- Formalin- and methanol-free PAXgene FNA fixation is adequate for the lung cancer molecular research.

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Handbooks and user manuals are available at **www.qiagen.com** and **www.preanalytix.com** or can be requested from QIAGEN Technical Services or your local distributor.

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