Validation of PAXgene Tissue Fixation for Whole Genome Sequencing

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1 Document History and Control

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1.1 Version History

Version	Date	Description
0.1	09/01/2018	First draft
0.2	10/01/2018	Louise's edits
0.3	15/01/2018	Sandi's edits
0.4	29/01/2018	Incorporating Sandi's edits from FNA doc
0.5	5/2/18	New title and checklist

2 Purpose & Scope

Preliminary experiments using PAXgene as an alternative tissue fixative have demonstrated reduced detrimental effects on the quality of the DNA and subsequent whole genome sequencing (WGS) in comparisonto formalin fixed tissue. The purpose of this protocol is to validate this method of sample handling in a bigger cohort.

NHS GMCs can opt to take part in a pilot of this technique and the requirements for this are outlined in this document.

Any solid tumour can be fixed with PAXgene. Where there is a requirement for immunohistochemical testing of the tissue for diagnosis then PAXgene fixation should be avoided until completion of this validation.

3 Roles & Responsibilities

Role	***Responsibility***
Clinical Lead for validation cohort	Nominated NHS GMC individual responsible for ensuring protocol is adhered to; samples are tracked and data is collected and submitted correctly.
Radiologist or other sample taker	The radiologist is responsible for ensuring there is adequate material for conventional processing in formalin for diagnosis with the genomic biopsy being placed into PAXgene.
Biomedical Scientist, Clinical Scientist or Pathologist	Ensuring the PAXgene samples are processed in a formalin free environment; preparation for histological interpretation; tumour content assessment and DNA extraction.

4 Background to validation cohort

The chemical bonds formed by formaldehyde include protein-DNA, inter strand DNA, and protein-protein crosslinking and DNA-formaldehyde adducts[i]. Formaldehyde destabilises the DNA double helix leading to local structural changes and causes DNA fragmentation. False positive single nucleotide variants are also found as formaldehyde causes hydrolytic deamination of cytosine bases to uracil (or to thyamine if methylated). In combination, exposure of tissue to formaldehyde results in false positive mutations and an increased risk of missing structural changes which can result in false negative reports.

Proof of principle experiments have shown that tissue fixed in PAXgene shows WGS results comparable to fresh frozen tissue. A validation project to analyse a pilot cohort has now been initiated and NHS GMCs are invited to participate.

Validation of this sampling method will require approximately 50 cases. The number required may increase during the course of the pilot if initial data reveals unexpected findings that require further work to fully understand.

It is hoped that PAXgene fixation will increase access to genomic testing for patients where tumours are not macroscopically identifiable or all tissue needs histological assessment e.g. where only limited biopsy material is available or melanoma samples. Samples will be

submitted as part of the main programme with a Whole Genome Analysis result returned to the GMC and these samples will count towards the recruitment trajectory and be eligible for funding.

5 General Information

When a sample is submitted as part of this validation cohort then how the tissue is fixed and sampled for DNA extraction will be affected as well as how data is collected and submitted. There is a requirement to track these cases through the Genomics England service desk as outlined below.

For all other aspects the usual guidance applies to how patients should be approached for consenting and how sampling should be carried out. The current sample handling guidance can be found here: https://www.genomicsengland.co.uk/information-for-gmc-staff/sample-handling-guidance/ and guidance on consenting can be found as part of the Genomics Education Programme here: https://www.genomicseducation.hee.nhs.uk/courses/courses/consent-ethics/

5.1 Consenting

This work is considered service development work. The samples are therefore diagnostic samples. Once a PAXgene sample has been examined histologically and is found to be eligible then a decision on which area of the tumour to sample must be taken. Sampling for DNA can take place in the patient's best interests prior to specific consent for participation in the 100,000 Genomes Project in accordance with the Consensus Statement which can be accessed here: https://www.genomicsengland.co.uk/information-for-gmc-staff/cancer-programme/cancer-mdt-engagement-pack/

5.2 Germline DNA sampling

A germline peripheral blood sample must be taken in an EDTA tube and the extracted germline DNA must be submitted with the tumour DNA sample to UKB for plating as set out in the current Sample Handling Guidance.

5.3 Tumour sampling

The tumours must fulfil the eligibility criteria detailed here: https://www.genomicsengland.co.uk/information-for-gmc-staff/cancer-programme/eligibility/

5.4 Data entry

The patient must have a completed Registration file including consent information and a completed sample metadata file and Clinical Sample Test csv file (for tumour content assessment) prior to sample submission to UKB.

5.5 Sample submission

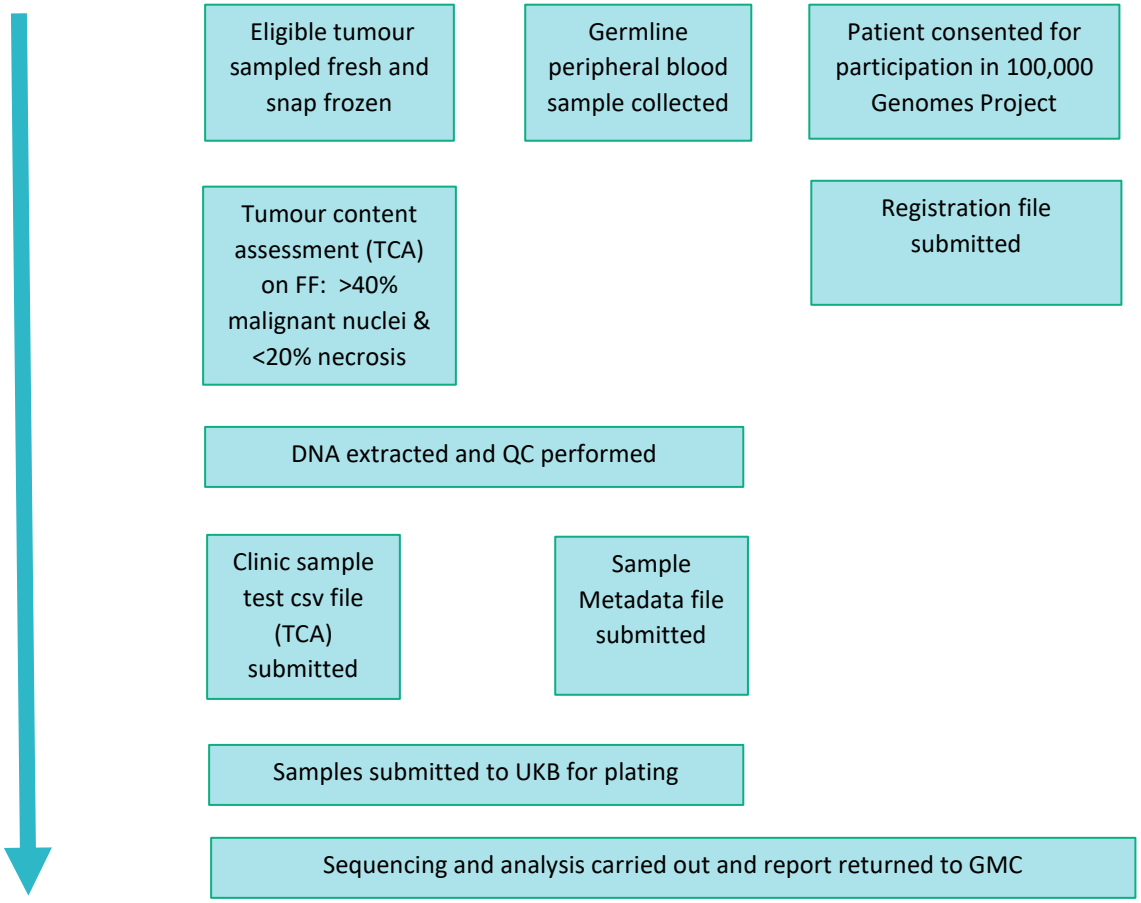
After DNA extraction, samples must be submitted to UKB following the recommendations of the current Sample Handling Guidance. Samples from this pilot should not be a clinical priority for fast or medium track slots, however, if they meet the volume and concentration requirements for fast track cases then they can be included on a fast track plate. Where possible samples submitted as part of the pilot should fulfil the fast track criteria in order to expedite the validation work.

5.6 Return of Results

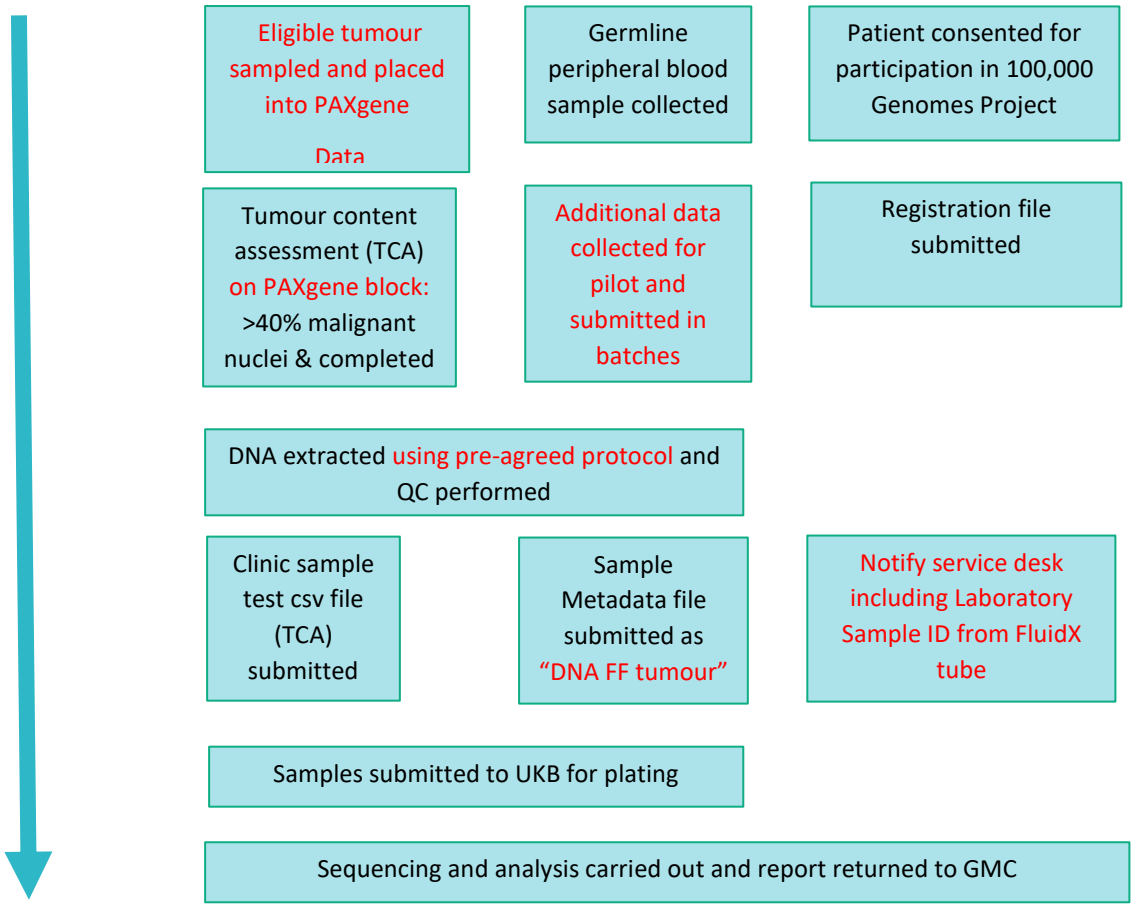
Samples submitted as part of the validation cohort will be processed through the main programme and results will be returned to NHS GMCs via the usual route (cancer interpretation portal). As with all 100,000 Genomes Project cancer cases results should be interpreted and reported in accordance with the NHSE Cancer Validation and Reporting working group guidance document (in preparation).

5.7 Where pilot cases differ from normal practice

Conventional process



Validation pilot process

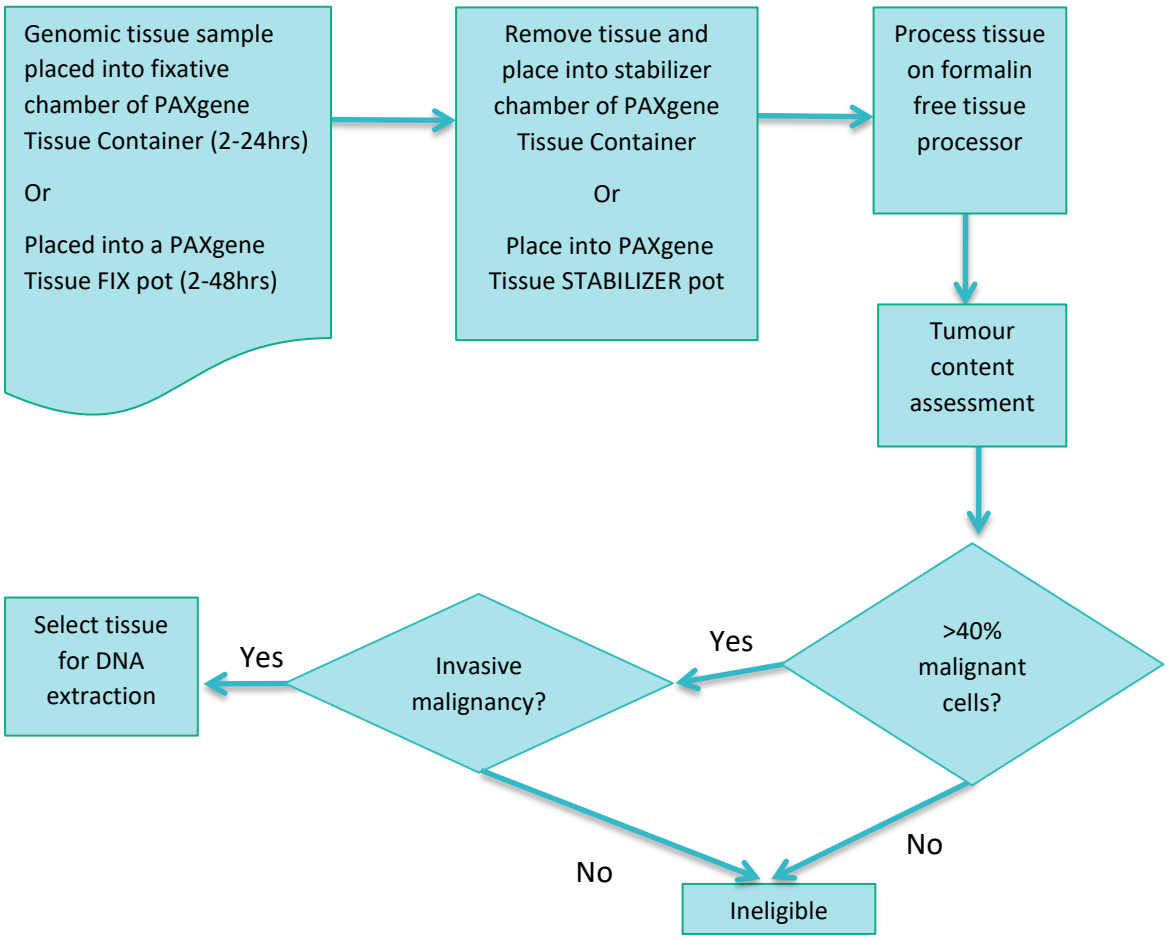


6 Procedure

6.1 Introduction

Tissue samples may include both biopsy specimens and sections from surgical resection samples that were collected fresh but where tumour could not be identified macroscopically. Melanoma samples where the entire sample must be examined histologically for diagnosis, can be collected fresh and bisected with half fixed in PAXgene and half in formalin. This will enable validation of any impact on morphology and immunohistochemistry.

6.2 Process Flow



6.3 Equipment needed

PAXgene Tissue FIX

PAXgene Tissue STABILIZER

Dedicated formalin free tissue processing machine

6.4 Procedural Steps

6.4.1 Fixation

1. For each tissue pathway a plan must be put in place between the radiologist or other sample taker and the pathologist to agree scenarios where a genomic biopsy or PAXgene block should be taken where possible.
2. Biopsy tissue or a section from a surgical resection should be selected for fixation.
3. A dual chamber PAXgene Tissue Container pot can be used which can take a cassette containing a single tissue sample up to 4 x 15 x 15 mm. Alternatively, a PAXgene Tissue FIX container can be used which can take 4 tissue cassettes each containing up to 4 x 15 x 15mm pieces of tissue or a single tissue sample up to 20 x 20 x 20 mm.
4. The tissue should be kept in the fixative for a minimum of 2 hours or until the tissue is fixed. It penetrates at approximately 1mm every 30 minutes.
5. Tissue should not be left in the PAXgene Tissue Container fixative for longer than 24 hours or the PAXgeneTissue FIX solution for longer than 48 hours at room temperature.
6. Once fixation is complete the tissue should be transferred from the PAXgene Tissue Container fixative chamber to the stabiliser chamber. If using PAXgene Tissue FIX the tissue should be transferred into a PAXgene Tissue STABILIZER pot.
7. Tissue can be kept in PAXgene stabilizer solution (either in the dual chamber or separate pot system) for up to 7 days at room temperature (15-25^{o}C) or up to 4 weeks at 2-8^{o}C.

6.4.2 Processing

8. The PAXgene fixed samples must be kept away from formalin and must be processed on a formalin free processor.
9. An example of a suitable formalin-free 12 hour processing program schedule is attached (Appendix A, as used at Wessex GMC as part of the experimental pathway). This schedule follows the PAXgene manufacturer's example tissue processing protocols as described in the PAXgene Tissue System product circular.

6.4.3 Staining and examination of slide

10. Histological sections should be stained using an adjusted protocol to prevent hypereosinophilia and to optimise the chromatin staining and a proposed schedule for this is attached (Appendix B as used at Wessex GMC as part of the experimental pathway).
11. The pathologist must ensure the sample is of an eligible invasive malignancy and contains greater than 40% malignant cells to ensure eligibility.

7 Additional Data

In addition to the submission of sample metadata the following data must be collected on each case within the pilot.

1. Participant ID
2. Disease Type
3. Disease subtype
4. Diagnostic morphology code
5. Tumour content assessment
6. DNA yield
7. Local DNA QC metrics
8. Results of any immunohistochemical or other special testing that has been carried out on the PAXgene sample and any conventionally fixed material.
9. Where there is sufficient residual material this should be noted so that future testing of the effects on immunohistochemistry could be carried out.

Any NHS GMCs participating in the pilot must submit Standard Operating Procedures for the DNA extraction method they propose to use for PAXgene samples prior to commencing the pilot. This method must be used for all cases in the pilot. These methods must be submitted to the Clinical Lead for the validation cohort when requesting permission to participate in the pilot.

This data must be sent to the Clinical Lead for the PAXgene fixation validation cohort at Genomics England, monthly or after every 10 cases have been sampled whichever is soonest. Genomics England will then assess the following outcomes for these samples:

1. DNA yield
2. Tumour purity
3. Coverage
4. Variant calling rate
5. Pick up rate for known mutations

8 Definitions & Abbreviations

Abbreviation / Term	Description
DNA	Deoxyribonucleic Acid
QC	Quality Control
NHS	National Health Service
GMC	Genomic Medicine Centre
UKB	United Kingdom Biobank
WGS	Whole Genome Sequencing
FF	Fresh Frozen

9 Data Model Requirements

There are a number of specific requirements around entering sample metadata on these samples since the current data model does not fully support PAXgene fixation.

Clinic Sample Type should be entered as DNA FF Tumour since there is no DNA Tumour Other enumeration in the current data model. Laboratory Sample ID from the FluidX tube for the DNA sample from the tumour FNA should be documented.

10 DNA Extraction Protocol

The DNA extraction technique is critical to achieving adequate DNA yield and quality. Extraction must be performed using a kit and protocol which has been recommended by the manufacturer as appropriate for PAXgene fixed samples, e.g. Qiagen *PAXgene Tissue DNA kit*. Extraction techniques suitable for formalin fixed tissueshould **not** be used. QC requirements of the resulting DNA are the same as for a fresh frozen tissue sample.

11 Permission to Commence Collection of PAXgene samples for the 100,000 genomes cancer programme

Prior to commencing collection of PAXgene samples NHS GMCs will be required to have submitted a completed checklist (see Appendix C) and to have reviewed procedural aspects

of the PAXgene fixation and the details of the DNA extraction method they plan to use with Genomics England and NHS England (usually via a teleconference).

12 Notification

Whilst early experimental outcomes for this technique have been very encouraging, this has only been on a limited number of tumours. In order that we may monitor the metadata and sequencing metrics on these samples the Genomics England Helpdesk must be notified (ssd.genomics@hscic.gov.uk) with the subject title 'PAXgene fixation'; the DNAhe extraction technique used and both the Participant ID and Laboratory Sample ID (for the tumour DNA sample) should be included in the text of the notification.

13 Related Documents, References & Procedures

Sampling of tumours with this technique should adhere to the guidance set out in the current Sample Handling Guidance.

14 Requirements for success

There are two tiers to the findings of a successful validation cohort:

1. Whole genome sequencing of a PAXgene fixed sample provides results which are equivalent or an improvement on those obtained from fresh frozen sampling.
2. Whole genome sequencing of a PAXgene fixed sample provides results which are adequate for detecting diagnostic changes but not as sensitive as fresh frozen sampling.

The outcome may vary by tumour type.
Where PAXgene fixation is adequate but not as good as fresh frozen sampling then fresh frozen will remain the gold standard for sampling of large tumours but PAXgene fixation will provide an option for sampling biopsies and macroscopically unidentifiable tumours that otherwise couldn't be sampled.

Results of the validation from each GMC cohort will be shared with the participating GMC once they are available.

Once results are available for the validation cohort as a whole these will be shared with all GMCs.

15 Appendix A

Processing conditions for PAXgene fixed tissue samples

Step	Reagent	Time (hh:mm)	Temperature	Vacuum/ Pressure	Mix
1	80% ethanol	01:00	Ambient	On	Slow
2	90% ethanol	01:00	Ambient	On	Slow
3	100% ethanol	01:00	Ambient	On	Slow
4	100% ethanol	01:00	Ambient	On	Slow
5	100% ethanol	01:00	Ambient	On	Slow
6	Isopropanol	01:00	Ambient	On	Slow
7	Isopropanol	01:00	Ambient	On	Slow
8	Xylene	01:00	Ambient	On	Slow
9	Xylene	01:00	Ambient	On	Slow
10	Paraplast Xtra low melting point (LMP) paraffin	01:00	56ºC	On	Slow
11	Paraplast Xtra LMP paraffin	01:00	56ºC	On	Slow
12	Paraplast Xtra LMP paraffin	01:00	56ºC	On	Slow
Total program time		12:00			

16 Appendix B

Dako Coverstainer Haemotoxylin & Eosin staining protocol

Step	Reagent	Time (hh:mm)
1	60°C oven	12:00
2	Xylene	03:00
3	Xylene	03:00
4	96% Ethanol	01:00
5	96% Ethanol	01:00
6	70% Ethanol	01:00
7	Tap water	01:00
8	Dako Haematoxylin	00:15
9	Deionised water	01:00
10	Dako Bluing buffer	01:00
11	Tap water	01:00
12	96% Ethanol, 1% Acetic acid	01:00
13	Dako Eosin	01:30
14	Dako Eosin 2	01:30
15	99% Ethanol, 1% Acetic acid	01:00
16	100% Ethanol	01:00
17	100% Ethanol	01:00
18	100% Ethanol	01:00
19	Xylene	01:00
20	Mounting	-
21	Drying	10:00
Total program time		44:15

17 Appendix C

Checklist for participation in PAXgene pilot	
Sample Handling	
Proposed tumour types that will be sampled	
Number of cases expected	
Pathologists or Scientists who will be carrying out procedure	
Confirmation of which laboratories will process the samples for (i) tissue processing and (ii) DNA extraction e.g. tissue bank or pathology laboratory	
Storage and transportation requirements prior to DNA extraction	
Details of proposed DNA extraction method and SOP	
Data capture	
Confirmation of how Pathology Department will record cases including data on ineligible cases and those that fail QC **i) Tissue sampling details** **ii) DNA extraction details**	
Designated responsibility for collecting the meta-data	
Sample tracking	
Process for tracking when these patients have been diagnosed, consented to GEL and sample has been retrieved, including responsibility to complete this action	
Confirmed process to notify GEL service desk that a sample has been submitted and associated patient identifier, including responsibility to complete this action	
Teleconferences	
Proposed operators to talk through procedural aspects of tissue fixation and processing	
Clinical Scientist to talk through DNA extraction methods proposed	

[i] Do, H. and A. Dobrovic, *Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization.* Clin Chem, 2015. **61**(1): p. 64-71