Central Laboratory Manual

FOCUS4 – Molecular selection of therapy in colorectal cancer: a molecularly-stratified randomised controlled trial programme

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Author(s) Cardiff and Leeds Laboratory staff and MRC CTU at UCL staff

Position(s) Central Laboratories and Trial Co-ordinating Centre

Date 08th September 2021

Approved by Cardiff Lab: Prof Richard Adams and Rhian White
Position: Biomarker Co-Investigators

Signature **08 Sep 2021**

Signature **08 Sep 2021**

Approved by Leeds Lab: Dr Susan Richman
Position: Senior Research Fellow

Signature **08 Sep 2021**

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FOCUS4 CONTACTS

FOCUS4 CO-ORDINATING CENTRE - MRC CTU AT UCL

FOCUS4 Co-ordinating Centre - MRC CTU at UCL		
MRC Clinical Trials Unit at UCL Institute of Clinical Trials & Methodology 90 High Holborn, 2nd Floor London WC1V 6LJ	Email: mrcctu.focus4@ucl.ac.uk Website: www.focus4trial.org	
Project Lead	Dr Louise Brown	
Clinical Project Manager	Marta Campos	
Trial Manager	Dipa Noor	
Data Manager	Sierra Santana	
First Point of Contact		
Trial Manager	Dipa Noor	

FOCUS4 Central Anayltical Laboratories For Biomarker TESTING

CARDIFF TEAM – Central Laboratory	
Dr Meleri Morgan Consultant Histopathologist Department of Cellular Pathology, University Hospital of Wales, Heath Park, Cardiff, CF14 4XW	Rhian White Principal Clinical Scientist All Wales Molecular Genomics Laboratory Institute of Molecular Genetics University Hospital of Wales Cardiff CF14 4XW
LEEDS TEAM – Central Laboratory	
Professor Phil Quirke Consultant Pathologist Section of Pathology and Data Analytics Leeds Institute of Medical Research St James's University Hospital Leeds LS9 7TF	Dr Susan Richman Senior Research Fellow Section of Pathology and Data Analytics Leeds Institute of Medical Research St James's University Hospital Leeds LS9 7TF

INTRODUCTION AND OVERVIEW

This manual details the processes relating to sample handling and processing for each sample type collected as part of the FOCUS4 Trial Programme: FFPE (Formalin Fixed Paraffin Embedded) blocks (including biopsies and whole tissues), blood samples and plasma samples.

SAMPLE RECEIPT

Blocks are requested by the trials nurses after registration into the FOCUS4 trial where the patient will be assigned an unique FOCUS4 trial number. Samples will have been pre-assigned for analysis at either Cardiff or Leeds. The pathologist will send the appropriate block to the analysing laboratory and send a fax confirming block release to MRC CTU at UCL. For request form see appendix 4.

Block Sample Receipt Leeds

Blocks will be sent directly to the trials laboratory on Level 4 of the Wellcome Trust, Brenner Building. Upon receipt, blocks will be booked in electronically and assigned a unique 'PTB' number. Samples need to be addressed to:

Dr Susan Richman,
FOCUS4 Trial Laboratory,
Level 4,Wellcome Trust,
Brenner Building,
St James University Hospital,
Leeds,
LS9 7TF

BLock Sample Receipt CARDIFF

Blocks are received by the Cardiff & Vale NHS Trust Histopathology laboratory and given a unique "E" number. Following block assessment (see 2; section 5 Sample preparation), a sample will be forwarded to the Molecular Genetics laboratory for DNA analysis. Samples need to be addressed to:

FOCUS4 Trial Programme,
Department of Cellular Pathology,
University Hospital of Wales,
Heath Park,
Cardiff,
CF14 4XW

From 2018 onwards, the laboratory procedures were amended as follows;
All samples received in Cardiff, were forwarded to Leeds for sectioning and H&E. All samples received directly in Leeds were sectioned and H&E'd as before. Unstained sections plus an annotated H&E slides, for all samples, was then sent from Leeds to Cardiiff, to undergo DNA extraction and NGS. All IHC was carried out in Leeds.

Blood and Plasma Sample Receipt Cardiff

All blood/plasma samples should be sent to:

FOCUS4 Trial Programme,
All Wales Medical Genomics Laboratory,
Institute of Medical Genetics,
University Hospital of Wales,
Heath Park,
Cardiff,
CF14 4XW

SAMPLE PREPARATION AND ASSESSMENT

The prioritising of block sectioning will be as follows;

1. The top 5-micron section will be cut for H&E.

2. Six 5-micron sections will be taken for DNA extraction.

If the block is a resection sample, cores will be taken and added to the TMA(s) and then TMA sections should be cut for the IHC. If the block is a biopsy, proceed with the cutting for IHC, as detailed below.

3. Seven 5-micron sections will be taken for MMR and pTEN IHC, providing two spare sections, in case of staining failure.

These sections will all be taken at the same time, to reduce the amount of trimming in and section-waste.

For each block, the following will be documented; whether the block is a resection or a biopsy, and an estimate of tumour content (%) will be made and documented from the H&E slide.

MRC CTU at UCL will inform the relevant laboratory of the need for an expedited assessment (minimum lead time from receipt of block is 4 weeks). Such samples may be processed as whole sections with retrospective entry into TMA.

TMAs will contain 4 cores plus one normal “tumour associated” adjacent mucosa if possible. QA use and IHC efficiency when numbers of registering patients becomes sufficient and a ‘master TMA’ will also be made. This will contain more cases, and will be used to swap samples between the two labs. Each TMA will have a clear orientation core set within it

Section assessment: Leeds

A 5um section will be cut from the block(s) and an H&E stain performed. The slide will then be reviewed by a Histopathologist to confirm the presence of tumour tissue, and mark up and score percent tumour present in the tumour-rich area(s).

As of 2018, all FFPE blocks were sent directly to the laboratory in Leeds, or forwarded to Leeds form Cardiff.
All blocks suitable for processing were macrodissected, and an H&E sections stained. The tumour area was outlined in the glass slide, and the tumour percentage was written on the slide with a marker pen. The unstained sections plus the marked up H&E were sent to the Cardiff laboratory for DNA extraction and NGS analysis.

PTEN (IMMUNOHISTOCHEMISTRY (IHC))

Clinical Relevance/Purpose Of Procedure

To demonstrate antigenic sites in formalin fixed paraffin embedded tissue.

Principle Of Procedure

To perform immunohistochemistry staining using the protocol for Dako pTEN (code: M3627) on the DAKO Autostainer Link 48™ automated stainer.

Personnel / Training Requirements

Training on Autostainer required prior to commencing protocol.

Specimen Requirements

5um paraffin sections (TMA section or whole section if biopsy).
Sections should be picked up on Superfrost Plus slides or DAKO Flex slides (K8020).
Dry Slides overnight in an incubator at 37°C (DO NOT USE A HOT PLATE).

Equipment

DAKO Autostainer Link 48™ automated stainer.
Oven at 37°C.
Standard histology equipment.

Health and safety

Each laboratory will adhere to strict COSHH guidelines and store on-line records of COSHH training. These will be made available upon request.

Autostainer reagents

High pH DAKO target retrieval buffer.
DAKO wash buffer.
DAKO antibody diluent (K8006).
Primary antibody DAKO PTEN (M3627) to be applied at1/100 TMAsand whole sections.
Detection kit: DAKO Flex Envison High pH (K8002).
DAKO Haematoxylin for Autostainer Link™ (this reagent is not included in detection kit).

Quality control

When no internal control exists or there is uncertainty,cores from pTEN +/- cell-lines may be added to each TMA to serve as an internal control. Where biopsy sections are run, a separate slide with cores from these cell-lines may be added to each staining run. Batch controls are not used.

METHODOLOGY

Dewaxing and Antigen retrieval

(i) Load slides into Dako Autostainer racks and scan barcodes into the PT link software.

(ii) Place racks into PT chamber containing High pH Dako Target retrieval buffer***** at working dilution (see Manufacturers data sheet).
(iii) Switch on PT link.
(iv) Programme 97°C 20mins, cool to 65°C.

Remove the slides from the PT link, rinse briefly with water and place into Dako Wash buffer* station for a minimum of 5 minutes. Slides to remain in wash buffer station for a MAXIMUM of five minutes. The kit user guide specifically states 1-5 minutes in buffer at this point.

Autostainer

The Autostainer should be programmed as follows:

1. **Blocking:** Peroxidase block for **5** minutes
2. **Antibody step:** pTEN at 1:100 dilution, 20 minute incubation
3. **Amplification:** 15 minutes mouse linker
4. **Secondary:** 20 minutes HRP-incubation
5. **DAB:** 2 x 5 minutes
6. **Hematoxylin:** 5 minutes

* These reagents are included in Detection kit K8002
Rinses etc should be included as some have longer durations than others. Also the polymer stage has been missed out completely.

Dehydrating and mounting

Slides are dehydrated through alcohol then xylene and finally mounted using DPX mountant.

SCORING OF TISSUE SLIDES

The whole tissue, TMA or biopsy sections are stained at 1:100. The TMA cores should be surveyed and the presence and intensity grade of cytoplasmic staining in the tumor component be noted (0 = absent, 1 = weak; 2 = moderate; 3 = strong).
For tumor core samples where no pTEN staining or weak tissue staining is noted, the corresponding whole section will be stained according to the protocol as above, at the optimal antibody concentration 1:100. This will also apply for all biopsies.

QUALITY ASSURANCE

Stromal cells, if present, should demonstrate a minimum of 50% staining to ensure adequate quality of tissue.
Inter-laboratory reproducibility should be monitored by the swapping of samples between the 2 laboratories (Leeds and Cardiff) at regular, pre-determined intervals.

The dates of swapping samples will be discussed the the regular Biomarker teleconferences between the labs.

MISMATCH REPAIR (MMR) (IHC)

MLH1

Clinical relevance/purpose of procedure

To demonstrate antigenic sites in formalin fixed paraffin embedded tissue.

Principle of procedure

To perform immunohistochemistry staining using the protocol for Dako hMLH1 (Mouse monoclonal Anti-Human MutL Protein Homolog 1. Clone ES05, Ready-to-use, Ref IR079) on the DAKO Autostainer Link 48™ automated stainer.

Personnel / training requirements

Training on Autostainer required prior to commencing protocol.

Specimen requirements

5um paraffin sections.
Sections should be picked up on Superfrost Plus slides or DAKO Flex slides (K8020).
Dry Slides for overnight in an incubator at 37^{o}C (DO NOT USE A HOT PLATE).

Equipment

DAKO Autostainer Link 48™ automated stainer.
Oven at 37^{o}C
Standard histology equipment.

Health and safety

See section 'PTEN IHC' for details.

AUTOSTAINER reagents

High pH DAKO target retrieval buffer.
DAKO wash buffer.
Primary antibody (ready to use).
Detection kit: DAKO Flex Envison High pH (K8002).
DAKO Haematoxylin for Autostainer Link™ (this reagent is not included in detection kit).

Quality control

Staining of tumour lymphocytes will serve as an internal control for acceptable staining (normal colon from a known pMMR sample may also be used).

METHODOLOGY

Dewaxing and Antigen retrieval

(i) Load slides into Dako Autostainer racks and scan barcodes into the PT link software.

(ii) Place racks into PT chamber containing High pH Dako Target retrieval buffer***** at working dilution (see Manufacturers data sheet).
(iii) Switch on PT link.
(iv) Programme 97°C 20mins, cool to 65°C.
(v) Remove the slides from the PT link, rinse briefly with water and place into Dako Wash buffer* station for a minimum of 5 minutes.

* These reagents are included in Detection kit K8002

Autostainer

The Autostainer should be programmed as follows:

Blocking: Peroxidase block for **5** minutes
Antibody step: MLH1 20 minute incubation
Amplification: 15 minutes mouse linker
Secondary: 20 minutes HRP-incubation
DAB: 2 x 5 minutes
Hematoxylin: 5 minutes

Dehydrating and mounting

Slides are dehydrated through alcohol (1x15 secs, 1x1 min, and 2x3 mins) then xylene (3x3 mins) and finally mounted using DPX mountant.

SCORING OF TISSUE SLIDES

The TMA sections or biopsy sections are stained to act as an initial screen. The TMA cores should be surveyed and the presence of nuclear staining in the tumor component be noted (0=absent, 1=present).
For tumor core samples where there is no hMLH1 staining, the corresponding whole section will be stained according to the protocol above.

QUALITY ASSURANCE

Inter-laboratory reproducibility should be monitored by the swapping of samples between the 2 laboratories (Leeds and Cardiff) at regular, pre-determined intervals.

MSH2

Clinical relevance/purpose of procedure

To demonstrate antigenic sites in formalin fixed paraffin embedded tissue.

Principle of procedure

To perform immunohistochemistry staining using the protocol for Dako hMSH2 (Mouse monoclonal Anti-Human MutS Protein Homolog 2. Clone FE11, Ready-to-use, Ref IR085)on the DAKO Autostainer Link 48™ automated stainer.

Personnel / training requirements

Training on Autostainer required prior to commencing protocol.

Specimen requirements

5um paraffin sections.
Sections should be picked up on Superfrost Plus slides or DAKO Flex slides (K8020).
Dry Slides for overnight in an incubator at 37°C (DO NOT USE A HOT PLATE).

Equipment

DAKO Autostainer Link 48™ automated stainer.
Oven at 37°C.
Standard histology equipment.

Health and safety

See section 'PTEN IHC' for details.

AUTOSTAINER reagents

High pH DAKO target retrieval buffer.
DAKO wash buffer.
DAKO antibody diluent (K8006).
Primary antibody.
Detection kit: DAKO Flex Envison High pH (K8002).
DAKO Haematoxylin for Autostainer Link™ (this reagent is not included in detection kit).

Quality control

Staining of tumour lymphocytes will serve as an internal control for acceptable staining.

METHODOLOGY

Dewaxing and Antigen retrieval

1. Load slides into Dako Autostainer racks and scan barcodes into the PT link software.
2. Place racks into PT chamber containing High pH Dako Target retrieval buffer***** at working dilution (see Manufacturers data sheet).
3. Switch on PT link.
4. Programme 97°C 20mins, cool to 65°C.

5. Remove the slides from the PT link, rinse briefly with water and place into Dako Wash buffer* station for a minimum of 5 minutes.

Autostainer

The Autostainer should be programmed as follows:

Blocking: Peroxidase block for **5** minutes
Antibody step: MSH2 20 minute incubation
Amplification: 15 minutes mouse linker
Secondary: 20 minutes HRP-incubation
DAB: 2 x 5 minutes
Hematoxylin: 5 minutes

Dehydrating and mounting

Slides are dehydrated through alcohol (1x15secs, 1x1min, and 2x3mins) then xylene (3x3mins) and finally mounted using DPX mountant.

SCORING OF TISSUE SLIDES

The whole tissue, TMA or biopsy sections are stained, to act as an initial screen. The TMA cores should be surveyed and the presence of nuclear staining in the tumor component be noted (0 = absent, 1 = present).
For tumor core samples where there is no hMSH2 staining, the corresponding whole section will be stained according to the protocol above.

QUALITY ASSURANCE

Inter-laboratory reproducibility should be monitored by the swapping of samples between the 2 laboratories (Leeds and Cardiff) at regular, pre- determined intervals.

MSH6

Clinical relevance/purpose of procedure

To demonstrate antigenic sites in formalin fixed paraffin embedded tissue.

Principle of procedure

To perform immunohistochemistry staining using the protocol for Dako hMSH6 (Mouse monoclonal Anti-Human MutS Protein Homolog 6. Clone EP49, Ready-to-use, Ref IR086) on the DAKO Autostainer Link 48™ automated stainer.

Personnel / training requirements

Training on autostainer required prior to commencing protocol.

Specimen requirements

5um paraffin sections.
Sections should be picked up on Superfrost Plus slides or DAKO Flex slides (K8020).
Dry Slides for overnight in an incubator at 37°C (DO NOT USE A HOT PLATE).

Equipment

DAKO Autostainer Link 48™ automated stainer.
Oven at 37°C.
Standard histology equipment.

Health and safety

See section 'PTEN IHC' for details.

AUTOSTAINER reagents

High pH DAKO target retrieval buffer
DAKO wash buffer.
DAKO antibody diluent (K8006).
Primary antibody.
Detection kit: DAKO Flex Envison High pH (K8002).
DAKO Haematoxylin for Autostainer Link™ (this reagent is not included in detection kit).

Quality control

Staining of tumour lymphocytes will serve as an internal control for acceptable staining.

METHODOLOGY

De-waxing and Antigen retrieval

1. Load slides into Dako Autostainer racks and scan barcodes into the PT link software.
2. Place racks into PT chamber containing High pH Dako Target retrieval buffer***** at working dilution (see Manufacturers data sheet).
3. Switch on PT link.
4. Programme 97°C 20mins, cool to 65°C.

5. Remove the slides from the PT link, rinse briefly with water and place into Dako Wash buffer* station for a minimum of 5 minutes.

Autostainer

The Autostainer should be programmed as follows:

Blocking: Peroxidase block for **5** minutes
Antibody step: MSH6 20 minute incubation
Secondary: 20 minutes HRP-incubation
DAB: 2 x 5 minutes
Hematoxylin: 5 minutes

Dehydrating and mounting

Slides are dehydrated through alcohol (1x15 secs, 1x1 min, and 2x3 mins) then xylene (3x3 mins) and finally mounted using DPX mountant.

SCORING OF TISSUE SLIDES

The whole tissue, TMA or biopsy sections are stained to act as an initial screen. The TMA cores should be surveyed and the presence of nuclear staining in the tumor component be noted (0=absent, 1=present).
For tumor core samples where there is no hMSH6 staining, the corresponding whole section will be stained according to the protocol above.

QUALITY ASSURANCE

Inter-laboratory reproducibility should be monitored by the swapping of samples between the 2 laboratories (Leeds and Cardiff) at regular, pre- determined intervals.

PMS2

Clinical relevance/purpose of procedure

To demonstrate antigenic sites in formalin fixed paraffin embedded tissue.

Principle of procedure

To perform immunohistochemistry staining using the protocol for DAKO PMS2 (Rabbit monoclonal Anti-Human Postmeiotic Segregation Increased 2. Clone EP51, (Concentrate) M3647 on the DAKO Autostainer Link 48™ automated stainer.

Personnel / training requirements

Training on Autostainer required prior to commencing protocol.

Specimen requirements

5um paraffin sections.
Sections should be picked up on Superfrost Plus slides or DAKO Flex slides (K8020).
Dry Slides overnight in an incubator at 37°C (DO NOT USE A HOT PLATE).

Equipment

DAKO Autostainer Link 48™ automated stainer.
Oven at 37°C.
Standard histology equipment.

Health and safety

See section 'PTEN IHC' for details.

AUTOSTAINER reagents

High pH DAKO target retrieval buffer
DAKO wash buffer
Primary antibody (M3647)
Antibody Diluent (S0809)
Detection kit: DAKO Flex Envison High pH (K8002)
DAKO Haematoxylin for Autostainer Link™ (this reagent is not included in detection kit).

Quality control

Staining of tumour lymphocytes will serve as an internal control for acceptable staining.

METHODOLOGY

Dewaxing and Antigen retrieval

1. Load slides into Dako Autostainer racks and scan barcodes into the PT link software.
2. Place racks into PT chamber containing High pH Dako Target retrieval buffer***** at working dilution (see Manufacturers data sheet).
3. Switch on PT link:
4. Programme 97°C 20mins, cool to 65°C.

5. Remove the slides from the PT link, rinse briefly with water and place into Dako Wash buffer* station for a minimum of 5 minutes.

Autostainer

The Autostainer should be programmed as follows:

Blocking: Peroxidase block for **5** minutes
Antibody step: PMS2 @ 1:40 dilution, 1 hour incubation
Amplification: 15 minutes rabbit linker
Secondary: 30 minutes HRP-incubation
DAB: 2 x 5 minutes
Hematoxylin: 5 minutes

Dehydrating and mounting

Slides are dehydrated through alcohol (1x15secs, 1x1min, and 2x3mins) then xylene (3x3mins) and finally mounted using DPX mountant.

SCORING OF TISSUE SLIDES

(i) The whole tissue, TMA or biopsy sections are stained to act as an initial screen. The TMA cores should be surveyed and the presence of nuclear staining in the tumor component be noted (0 = absent, 1 = present).

(ii) For tumour core samples where there is no PMS2 staining, the corresponding whole section will be stained according to the protocol above.

QUALITY ASSURANCE

(i) Inter-laboratory reproducibility should be monitored by the swapping of samples between the 2 laboratories (Leeds and Cardiff) at regular, pre- determined intervals.

(ii) Under specific circumstances where uncertainty continues after assessment by both labs, Ki-67 IHC may be utilized to clarify the proliferation of the relevant tumour sample. Those samples that are “positive” for Ki-67 staining and negative for MMR IHC are more indicative of a “true” MMR deficient sample.

DNA EXTRACTION

For the extraction of DNA from FFPE blocks, each laboratory will adopt identical preparative and quantitative protocols, but will carry out different extraction methodologies. Extensive cross validation, particularly of KRAS and BRAF mutation status has been carried out between laboratories. With a 100% concordance rate to prove that the actual extraction methodologies, although different, will generate DNA of almost identical quality.

Preparing samples for macrodissection

1. Blocks to be cut for sections should be placed face down on ice for at least 10 minutes prior to cutting sections. This helps the sections to come off evenly.
2. The microtome should be set to 5 microns.
3. Wipe a new microtome blade with a dry tissue, then with Xylene and again with a dry tissue. This will remove the protective film on the blade.
4. Insert the blade into the blade holder.
5. Mount the block in the microtome block holder. It is preferable to mount all blocks in the same plane. However, if not possible then orientate the block so that the face of the block is square with the blade.
6. Gently trim the face of the block. Remove enough sections to expose the full face of the block using forceps to draw the sections away from the block. Try not to take more than 1-2 minutes to do this because the block will warm up. If this occurs, transfer the block face down to the ice for a couple of minutes and then replace in the holder.
7. Using a forceps which have been cooled on ice begin cutting sections. It should be possible to generate a ribbon of sections 3 or 4 in length.
8. Sections are then carefully transferred to a 37°C water bath so that the sections float on the surface of the water.
9. The block is once more returned to the ice.
10. Sections floating on the water are then separated using a curved forceps and guided on a Superfrost slide.
11. Repeat until the number of slides required have been made.
12. Ensure slides are appropriately labelled.
13. Slides are air dried overnight or put into the oven at 55°C for one hour to ensure adherence to the slides.

DNA Extraction-Leeds

From 2018 onwards, no dna extraction was carried out in leeds. All extractions were carried out in the cardiff lab, using a marked-up H&E slide, provided by the Leeds laboratory.

DNA Extraction-Cardiff

MACRODISSECT THE FFPE SAMPLE:

1. Place the labelled screw top tube next to the corresponding slide tray.
2. Place the unstained slides under the marked stained slide and align. With a marker pen, trace the marked area on to the back of the unstained slide.

3. An appropriate trained member of staff must carry out the slides check:

4. QC step: Checker must:
   a. Check patient demographics and sample received
   b. Check the referral form and slides match (this includes checking the slide number in addition to the laboratory number)
   c. Check the order of slides match the extraction paperwork
   d. Check that the tumour area has been correctly marked on the unstained slides
   e. Check that the lab number on the slides and tubes matches

5. Scrape the marked area off the slide, using a new scalpel blade per sample, into the correctly labelled eppendorf tube.

DNA Extraction using the Maxwell® 16 FFPE Plus

1. Spin for 2 minutes at 13000 rpm to collect material at the bottom of the tube.

2. Add 180µl of the supplied incubation buffer into the incubation tube, containing the FFPE material. It is important to ensure that the FFPE material is completely covered by the liquid.

3. Add 20µl of Proteinase K (PK) solution to each incubation tube.

4. Spin the incubation tube again for 2 minutes at 13000rpm to collect the liquid at the bottom of the tube.

5. Incubate the incubation tubes in the heating block (set to 70°C) for a minimum of 2 hours at 1300rpm. (Better yields can be obtained by prolonged incubation of 8 hours or overnight).

6. After incubation, add 400µl of the supplied lysis buffer to each incubation tube containing the sample.

7. Vortex the solution briefly.

8. Once incubation is complete, label the prefilled reagent cartridges with sample label and collect the rack from the Maxwell.

9. Prepare the labelled elution tubes by adding 70µl of Nuclease Free Water

10. Prepare the Maxwell and remove the rack from the machine, take to the hood. The door can be opened manually at any point once the machine is switched on. The Maxwell has a touchscreen or the buttons can be used to navigate:

11. Note: To proceed to the next step, once correct option selected click Run/Stop

12. A check is required for the next step, this is referred to as Transfer Check 2:

13. **QC step:** Following incubation, the checker ensures that the label on the incubation tube, Maxwell tube and cartridge are all correct and check the sample order on the worksheet.
    a. For each sample hand the incubation, Maxwell and cartridge to the person carrying out the extraction.
    b. Observe that the cartridge is placed in the correct position in the Maxwell rack, and that the incubated sample is transferred into position one of the cartridge.
    c. Observe that the Maxwell elution tube is filled and placed in the corresponding position on the Maxwell rack.

14. In the hood, remove the seal from position 1 of the cartridge and place it into the Maxwell rack. Transfer the incubated/ lysed sample/ aqueous (blue) phase into position 1 (see figure below) of the Maxwell cartridge

15. Note for FFPE samples: Any paraffin visible in the sample tube should not be transferred into the Maxwell cartridge

16. Take the rack containing the cartridges and elution tubes to the Maxwell

17. Add the plunger in the last position of the cartridge position 8 (see diagram above).
18. *Plunger must be aligned, so sides face outwards (otherwise plunger will not be picked up)

19. Place the Maxwell rack into the Maxwell, and ensure elution tubes are open.

20. Move to the final sample and the screen will show the items to check before running:

    - Cartridges (present in rack)
    - Elution tubes (present and open)
    - Plungers (present with sides facing outwards)
    - Start the run, using the green run/stop button.

21. Once the run is complete screen shows “Data Transfer – Yes/No” select No. (press green run/stop button)

22. Screen asks to “Open door”. Do so and press green button.

23. Take the eluted samples out, check the colour and close lid

24. If the eluted sample is not clear, inform Lead Genetic Technologist and fill in the fail samples log: LF-GEN-ExtractFailRecord (sample will need re-extraction if more material/ sample is available)

25. "Remove cartridges". Discard the cartridges into the sharps bin. Click green button.

26. "Close door" and switch off

Once the run is complete samples must be transferred (check required), quantified using the Qubit and banked.

Genomic DNA extraction from blood samples-Cardiff

Automated extraction of nucleic acids allows for greater accuracy through reduced user error and standardisation of the technique. It also leads to a higher throughput of samples as well as time and cost efficiencies.

The **Chemagic Star** is a stand-alone nucleic acid extraction system that combines Perkin Elmer's leading nucleic acid purification chemistry, Chemagen, with Hamilton's superior automated liquid handling robotics.

Arrange all request forms in worksheet order.

1. Re-confirm all blood tubes have at least 2ml of blood. Samples with less than 2ml of blood cannot be processed on the current Chemagic Star protocol.
2. Check that the tube type received is a 4ml vacuette with no gel or barrier at the bottom. Samples received in any other tube type have to be transferred into the 4ml test tubes provided during the second checking process. Labelling of these test tubes with a 1D code should be performed at this stage.
3. Once all samples have been retrieved they should be lined up in worksheet order .
4. All samples that need to be transferred into a transfer test tube need to have both the blood tube and the test tube together ready for the transfer check.
5. The Chemagic Star blood tube carriers should then be lined up next to the rack in preparation for tubes to be transferred into them by the checker. Place this in the same direction as the blood tubes; so numbers should go from left to right.
6. Finally, the elution tubes should be prepared, using a label printed using the Zebra printer. It is very important that 8100HT White High Tack Polyester Labels are used as these are suitable for fridge and freezer use.
    a. These are Fluid X tubes they should be arranged at the bottom of the lockable Fluid X rack .
    b. If you have a mixture of internal and external samples in one run then where possible use eppendorfs for all samples. This prevents wastage of the more expensive FluidX tubes. If this is carried out then a manual transfer of banked samples into FluidX tubes will be necessary post Chemagic run.
7. When all items are arranged an extraction checker must be called to perform a second check of all request forms and samples against the worksheet for that run.
8. All blood samples should be opened within a class 1 cabinet. This should be prepared as follows:

   a. Ensure the bottom of the class 1 cabinet is lined with blue tissue to absorb spillages
   b. Ensure there is an appropriate flow of air through the cabinet.
   c. Ensure there is a sharp safe placed within the class 1 cabinet for disposal of contaminated sharps waste – orange lidded sharp.
   d. Check that all tip boxes, pipettes and reagents are lined up along the back and sides of the cabinet to allow for an adequate work area.
   e. Ensure there is a plastic sample bag in the cabinet labelled with today's date for temporary storage of used and spare blood tubes in the freezer.
9. While the check is being performed the initiation and preparation of the deck can be Started.

Chemagic Star Checking Procedure

1. The Chemagic Star performs the extraction and elutes directly into the FluidX tube that is banked, so the checking procedure is vital for quality purposes.
2. Check samples one at a time.
3. Compare demographic data on the request form against the DNA label, blood tube, FluidX tube and the worksheet
4. Place the 4ml blood vacuette into the blood tube carrier Starting at the first slot numbered one.
5. Any blood tubes that require a transfer step (labelled 4ml transfer tube) will be passed to the extraction Medical Technical Officer (MTO1) for transfer before moving onto the next sample.
   a. Any sample handling during sample transfer will take place within a class 1 cabinet.
   b. When transferring blood samples, 1ml filter tips must be used.
6. The MTO1 transfers a maximum of 3mls and a minimum of 2mls of blood from Vacuette blood tube to 4ml labelled test tube and covered with parafilm one at a time. At this point the transfer tube is re-checked and placed in rack request form and worksheet (for position).
7. The checker must place the transfer tube into the correct position, before checking the next sample. This is vital to ensure that no sample switches occur on the run.
8. The tip will be disposed of in the sharp safe and the blood tube should be re-sealed and placed in the plastic storage bag (with today's date).
9. FluidX tubes will simultaneously be checked against the worksheet for demographics and tube order. These will be found lined up at the bottom of the FluidX tube
10. As they are checked they will be moved up to rows A and B (set 1) and C and D (set 2)
11. The following blood tube, checked as in point 2, will be placed in the next available slot in the blood tube carrier. If there are more than 12 samples then two or more blood tube carriers will be used. The checker will Start with the carrier labelled 'set 1' (for samples 1 to 12) before placing the next set of 12 samples in the carrier labelled 'set 2' (for samples 13 to 24), and then 'set 3' (for samples 25-36) and 'set 4' (for samples 37-48)

12. Once all samples have been checked and blood tubes placed in the blood tube carrier, the carrier will be transferred to the Chemagic Star and placed in the appropriate. Set 1 tubes are placed in the slot for 'Input tube set 1' and set 2 is placed in the slot for 'Input tube set 2'.
13. If using FluidX tubes, these are placed on the FluidX rack slot
14. If eppendorf tubes are used as the output tube then these are put into the slots to the left of the input tube carriers. This would occur if more service samples are being extracted than diagnostic.
15. At this point a deck check is performed by the checker. Here the checker will check that the blood tube carrier/s has been placed at the correct position. This would be carried out by checking the two tubes in each of the sets, at random, against the corresponding output tubes and the worksheet.
16. Once check is complete, invert all of the bloods with lids and remove lids. Lids should be numbered so that they can go back on after the run, in case any samples need to be repeated.

Chemagic Star initiation

1. Instructions for loading the deck will be given by the instrument at the beginning of the run. This protocol details every step involved from sample preparation to DNA output, and should be used in conjunction with the onscreen instructions.
2. At the beginning of the day, prior to initiating any runs, complete the daily maintenance
3. To initiate the machine, switch on the two green switches at the side of the Chemagen unit and the Star unit. The door to the Star unit has an interlocking system whereas the Chemagic unit is not interlocked. To access the Chemagic unit unlock using the key at the bottom of the glass door.
4. Turn on the computer and log into the system by clicking on the IMG Cardiff icon.
5. Once logged in, double click the Chemagic Star icon shown below to open the control program
6. The following screen should appear. Select the correct protocols and lab ware specific for the blood kit and output tubes used.
7. The screen will always default to the selections shown and the last method used.
8. The fields on the screen above give the following selections:
9. Select the 'Medium Volume' head from the 'Configuration' field
10. The 'Input Labware' shows '4ml Vacuette' as the only option. This is a description of the 4ml blood tube.
11. The 'Output Labware' should be selected as:
12. 'Fluix075ml' for output into a 2D Fluidx tube to be banked in the onsite banking facility.
13. The 'Sample Type' defaults to 'Blood', which is the only protocol we currently use, but the following are also available:
    - 'Buffy Coat'
    - 'Plasma'
    - 'Saliva'
    - 'Cells'

14. The 'Kit' field will always default to the last kit used and is specific to the sample volume and sample type. The kit currently being used in the Cardiff lab is '3-4ml Blood (Medium volume)'. The 'Sample mixing' option is always set at '2 times'.
15. The 'Mix Ratio' field is set to '50% of maximum volume'. Please note that this only functions if the 'Sample Mixing' field indicates a mix number
16. The 'Editable values' details quantity and ranges for the following:
17. The 'Sample Set' which is the number of samples per set. Each set accommodating a minimum of 1 sample and a maximum of 24 samples. As previously mentioned 2 sets (referred to as 'Sample Set 1' and 'Sample Set 2') can be loaded per run. The Chemagic should be run in batches of 24 only to minimise plastic wastage
18. The 'Sample Volume' specifies the minimum volume loaded. The range is determined by the kit type. For the blood protocol the sample volume is 3000µl.
19. The 'Elution Volume' is currently optimised to 300µl
20. 'Sample transfer with 5ml tips' should always be selected. This allows for transfer of the blood samples from the blood tube to the initial DWP.
21. The 'Skip sample transfer' option should only be selected if the samples have already been transferred from the incoming blood tubes to the DWP. This step should be avoided unless absolutely necessary, for example, a software error that requires an instrument re-Start.
22. The 'Reset all tip counters' option resets the instrument's tip tracker to zero so that it assumes that there are no tips on deck. The Chemagic Star keeps track of the number of tips left on the deck at the end of each run. Please do not use this option unless absolutely necessary.
23. If the tip counter is not reset, the instrument will calculate the number of tips required to process the number of samples chosen. If there are not enough tips on the deck the instrument will prompt you to load new racks of tips.

Preparing the Instrument

1. Once the Chemagic Star has been initiated, maintenance completed and the correct fields selected for the specific protocol the instrument will have to be loaded with the correct reagents, plastics, input tubes and output tubes.
2. The instructions for loading the deck will be given by the instrument at the beginning of the run. This SOP gives additional information and explanations and should be used as an aid to running the instrument but NOT as a substitute to following the instructions supplied by the instrument.

Tip Loading

1. The instrument keeps tracks of the tips left on the deck at the end of each run. You will only be asked to load tips if there aren't enough tips on the deck. If you are running 24 samples, you will need full sets of the 5ml tips.
2. All tips should be loaded with the barcodes on the right and should be clicked firmly into the carriers. To do this, hold the tip rack in the centre, pressing the two clips inwards at the top and place it in the carrier then release the clips and check that it is firmly pressed down by gently tugging it.

3. After loading the requested tips slide the carrier into the in position.
4. Filter and non-filter tips look similar. From above, you will be able to see the white filter in the tips (non-filter tips are completely black inside). The tip type is also written on the barcode label and the outside of the tip packaging.
5. NB 5ml filter tips are referred to as 4ml filter tips and the 5ml non-filter tips are referred to as 5ml tips.
6. You will see specific messages for each tip type – the picture below is an example. It shows the item to load and its position. The numbers at the bottom indicate the lane position on the machine.

Loading of Source (Input) Blood tubes

1. You would have already selected your tube type prior to Starting the run. You should always ensure that the tubes loaded are of the correct type. For current blood extraction protocols only the 4ml vacuettes can be used in a single run. These have a diameter of 13mm.
2. Label the lids of the tubes with position number of the worksheet. Please ensure all lids are removed prior to starting a run.
3. As there are 2 sample sets of 24 per run, there are four source tube loading bays, holding 12 each, and these are labelled as 'Input tube Set 1', 'Input tube set 2', 'Input tube set 3' and 'Input tube set 4' from right to left..
4. Never use blood vacuettes of a different diameter, that are longer (e.g. 8ml vacuettes) or that contain gel at the bottom of the tube.
5. Any samples that arrive in unsuitable blood containers should be transferred into a 4ml vacuette size test tube during the checking procedure.
6. The first sample is at the back of the instrument with sample 12 at the front.
7. Please ensure that you place the tubes in the flashing positions (in blue) indicated on the computer screen during loading. A typical loading instruction is shown below. Here the input tubes are loaded in the set 1 position .

Destination (output) tube loading

1. These would also have been selected prior to Starting the run. Please make sure that the labware loaded is the right type and do not mix tube types within a run.
2. Internal samples; samples to be banked in house, will be output into 2D FluidX tubes. These are racked in a 96 position rack and are filled in from the back right in rows of 12 (position A1 is the top right corner, so that the rows of bloods and elution tubes line up the same.). Please ensure all lids are removed prior to Starting a run.
3. The output carriers for these are situated at the left of the input tube carriers for each set as shown below.

Loading Plastics

1. Sleeves and elution tubes should be added to the deck.
2. All plasticware is provided with the kits. For the 13ml elution tube, use the racks with holes at the bottom, to ensure they fit the machine correctly.

3. Important Note: The DWP fits onto the carrier in a specific manner. It should not be raised on the carrier at all, but should fit snugly inside, with a small amount of wiggle room around. Check the fit of the DWP before proceeding, as any misalignment will result in a machinery error.

Reagent Loading

1. Reagents are added up to the fill lines marked on the trough. Elution buffer and magnetic beads must be filled as close to the top of the trough as possible.
2. The troughs should be washed once a week with water and should be dried prior to re-filling them. This should always be performed at the end of the week as it will allow them to dry over the weekend and will remind staff to clean down the Chemagic at the end of the week.
3. Any that appear to have cracks or any other visible damage should be replaced immediately.
4. Filling of all reagent troughs should be carried out off the instrument. They should never be filled on the instrument as any spillages could leak into the body of the instrument and affect the wiring or corrode the machinery.
5. They should also be kept in the carriers to avoid them toppling over as they are not stable on their own.
6. Do not shake the reagents vigorously prior to filling the troughs as this will result in bubbles forming; rather rotate the bottle gently. Any bubbles forming will interfere with the instrument's liquid level detection.
7. If you do end up with a lot of bubbles forming then use a pipette to transfer liquid from beneath the bubbles to the reagent reservoir. The trough should never contain more than a few bubbles.
8. The other reagents can be filled to the marked line, indicated on the sides of the trough. Though overfilling the troughs for the other reagents will not affect the quality of the extraction, it will result in wastage from possible spillage.
    a. Lysis buffer must be changed every day to avoid crystallisation.
    b. Only ¾ of the Binding Buffer 2 is used, therefore ensure positions x and y are swapped daily to avoid stagnant buffers being used for extraction.
9. At the end of the week, decant any remaining solutions into the reagent bottles. These can be re-used the following week.
10. Wash all troughs with de-ionised water and leave to dry ready for re-use the following week.
11. The protease for each kit comes with volumes of re-suspension indicated on the protease tube, and once resuspended must be transferred to eppendorf tube. This eppendorf tube must be labelled with "protease" and the lot number and date.
12. Once re-suspended, the protease needs to be stored in the fridge. All other reagents can be kept at room temperature. Please ensure you always return this to the fridge at the end of each WORKING DAY.
13. The magnetic beads should always be loaded onto the instrument last. This is to avoid them sedimenting at the bottom of the trough which would clog the tips thereby hindering mixing and pipetting.

14. The magnetic bead solution must be completely suspended prior to pouring it into the trough. Shake well to ensure this occurs. The trough should never be re-filled with beads without first emptying it of all beads and washing it. If this is not carried out properly then the density of the beads will increase overtime which will again cause clogging of the tips and mis-pipetting.
15. Note: As beads are used, operator must ensure there is at least 2inch of liquid left in the bottle for resuspension. Do not let them get really low, otherwise they get too thick and this affects the quality of the run. The same lot numbers can be mixed up to use up the 'leftover' beads. When different lot numbers are available, the beads can be mixed up but they will require an additional batch testing process. Please refer to Extraction Lead for further instructions.
16. Once all reagent troughs are filled, return the carriers to the instrument ensuring they are slotted in to the correct lanes.. The carriers should not be pushed in; rather they should be left in the out position. They will be drawn into their lanes by the instrument once the run has been initiated. This avoids spillage of reagents onto the deck.
17. If any reagents are spilled on the instrument wipe them off immediately and clean the area with solution.

Starting a Run

1. Prior to initialising the run carry out the following checks:
2. Ensure that there are no items on the deck that should not be on deck
3. Check that the items on the deck are enough for the number of samples to be processed and that they are within the correct carriers
4. Check that all the carriers are slotted into the right lane and that all solution carriers and input tube carriers are in the out position.
5. The instrument will prompt you to check this.
6. At this point the user should have all the blood vacuettes lined up as per worksheet order in the input tube carriers.
7. All output tubes, whether FluidX or 2ml should also be lined up as per worksheet order in the correct carriers.
8. A deck check should have been carried out in order to ensure input and output tubes for each set are correct.
9. Once all carriers, plastics, input tubes and output tubes are loaded on the machine the run can be started:
10. To initiate a run click the green tick on the control program
11. Both the Chemagic robot and the Star will initialise at this point.
12. If all the channels are functioning properly the machine will initiate the extraction protocol.
13. If not then a warning message will appear. The operator has to immediately call through to customer services asking for a call out to be put through to the service engineer. The user will then be able to continue with this run and one subsequent run prior to the engineer visit (maximum of two runs).

14. Once the extraction protocol has been initiated you will be prompted to carry out a deck check of all the plastics as follows:
15. Check tip loading. Remember: Do not reset the tip counter unless asked to do so. The tip count should carry on from the previous run.
16. If this is correct press ok.
17. You will then be prompted to load the input blood tubes
18. Their position will be indicated on the deck view below by flashing blue lights (top four positions).
19. Load output tubes, FluidX tubes for in house samples and 2ml tubes for external samples (referred to as Anachem 2ml).
20. Check loading of plastics for Set 1 (You will be prompted to check Set 2 as well if you have more than 24 samples).
21. Check reagent loading
22. Always add the beads at this point – these are left till the end to reduce the amount of sedimentation. Even though the machine mixes them before aliquoting them out into the deep well plate, this will reduce the likelihood of the tip clogging up.
23. You will now be asked to select the users Nadex number or users name
24. Finally, you will be asked to enter the lot numbers for all reagents as shown below:
25. All this information will now be displayed on the screen for confirmation
26. This information can also be extracted from the runs log file. A shortcut to this can be found on the desktop or it can be accessed through C:\ Program files\ Hamilton\ log files.
27. Message: “Run is about to Start”, will appear on the screen. Check the door is closed before clicking ok
28. The machine will now scan the barcodes.
29. The current lab barcodes are too compact to be scanned by the screen so an error message will flash up
30. To enter barcodes manually select the position you want to name, select ‘Repeat’ and then Barcode.
31. The sample identifiers have to be entered in manually. This is only numbers 1-24, as the laboratory barcodes are not recognised by the machine. Once this has been entered for a position the position will be flagged green as shown below This must be repeated for set 2.
32. You can now select ‘execute’.
33. You will now be prompted to enter barcodes for your output racks as in the last two diagrams if the final output tubes are eppendorfs.
34. Once all barcodes are entered the run will be initiated.
35. As the run progresses, the different steps can be followed on the ‘Trace View’ window
36. The machines progress on the Chemagen arm of the instrument can also be followed on the Chemagen screen

DNA quantification-Cardiff

The Qubit is a benchtop fluorometer for the quantification of DNA, RNA and protein. It uses fluorescent dyes that are specific to the target of interest (either DNA, RNA or protein as selected). The dyes only emit fluorescence when bound to the target molecules, even at low concentrations. As a general rule the high sensitivity kit should be used to measure FFPE DNA and broad range kit to measure bloods. The Qubit does not give an indication of fragmentation of the sample or presence of contaminants. Therefore, it does not give an indication of quality.

Qubit Protocol

1. Take Standards (there are 2 per kit) AND SAMPLES out of the fridge and allow to warm to room temperature for 20-30 minutes.
    - If samples are frozen, take plate out of freezer and allow to warm to room temperature for 20-30 minutes.
2. Prepare assay tubes. You will need one assay tube for each patient sample, an assay tube for each of the standards and one assay tube for the control. For example, if quantifying 10 patient samples, 13 assay tubes will be required.
3. Prepare the Qubit Working Solution, as below, for the assay you require (broad range or high sensitivity – there are different kits please check you have the correct one):

	x1 tube	**x10 tubes**	**X20 tubes**
Qubit Buffer	199µl	1990µl	3980µl
Qubit Reagent	1µl	10µl	20µl

N.B. Qubit Reagent is light sensitive, keep the working solution in the dark where possible.

4. Standards and control should be made and tested prior to samples, in case standards fail and working solution needs to be prepared again.
5. Make standards:
    a. Each kit contains 2 standard bottles, see below for conc.
    b. Add 190µl of the working solution to each assay tube and 10µl of each standard.

	Standard #1	Standard #2
Broad Range	0ng/µl	100ng/µl
High Sensitivity	0ng/µl	10ng/µl

The Qubit readings should be within the recommended ranges shown below:

	Standard #1	Standard #2
BR	150 – 180	14,000 – 18,000
HS	35 - 65	18,000 – 23,000

6. If the readings are outside these values, the standards should be re-made, but if the readings are still outside these ranges, a new Working Solution will need to be made.
7. Make control a. Add 198µl * of the working solution to the assay tube and 2µl * of standard #2 (standard specific to kit you are using). The standard is a known concentration (see above) and is therefore good control.

Note: BE SURE NOT TO INTRODUCE BUBBLES INTO SAMPLES

*Note: these values can be changed, 199µl of working solution and 1µl of standard 2 may be used. The volume of standard used to make the control should be the same volume used to make up the samples.

8. Vortex samples for 2-3 seconds
9. Incubate the tubes for 2 minutes at room temperature.
10. Switch on the Qubit:
    a. select assay: DNA
    b. select assay: DNA dsBR or dsHS
    c. Once selected the screen below appears - Read new standards, click 'yes'
    d. Insert S1 tube – click read
11. Note: Do not excessively hold the assay tube in your hand before performing the measurement (as this can affect the temperature of the tube).
12. Note: If you are performing multiple readings of a single tube, remove the tube from the instrument and let it equilibrate to room temperature for 30 seconds before taking another reading (as per manufacturer's instructions).
    a. Insert S2 tube – click read
    b. Once, the standards have been assessed, the following screen appears - Insert assay tube (control) – click read
    c. Click 'calculate stock concentration', screen below appears, and scroll through the volumes, select '2'
    d. Click on the units (usually ug/ml) and change to 'ng/µl'
13. **Important: Control must be checked each time a standard curve is generated to ensure accuracy of quantification. If the control does not match the indicated concentration +/-10% repeat standard step (for example if standard concentration is 10ng/ul control conc should be between 9.0 and 11ng/µl)**
14. Note: for the Qubit dDNA BR and HS, the readings given by standard 2 should be at least 10x higher than that of standard 1 (as per manufacturer's booklet).
15. For frozen samples: after 20-30 minutes warming to room temperature, use a plate shaker (1800rpm for 1 minute) to ensure that the plate is thoroughly mixed. Centrifuge the plate briefly and continue as below.
16. Prepare patient samples in the same way as the control*
    a. Add 198µl of the working solution to the assay tube and 2µl of sample.

Note: BE SURE NOT TO INTRODUCE BUBBLES INTO SAMPLES

*Note: these values can be changed, 199µl of working solution and µl of sample may be used. When preparing samples with low volume e.g. FFPE samples it is advisable to use 1µl of the sample rather than 2µls.

17. Vortex for 2-3 seconds
18. Incubate for 2 minutes
19. Continue with Qubit. As standards are already done, insert assay tube, then click 'read next sample'. Click calculate stock concentration to show the same parameters selected as control tube – this can be altered as necessary (volume and units)
20. Note: Do not excessively hold the assay tube in your hand before performing the
21. measurement (as this can affect the temperature of the tube).
22. Note: If you are performing multiple readings of a single tube, remove the tube from the instrument and let it equilibrate to room temperature for 30 seconds before taking another reading (as per manufacturer's instructions).
23. Note: The fluorescence signal of samples is stable for 3 hours at room temperature (following initial 2 minutes incubation period).

Banking of plasma samples

Frozen blood plasma samples must remain frozen at -80°C. These samples are currently for storage only. Plasma samples are checked by a Duty Scientist and logged in on Shire before being added to an extraction worksheet with details of the freezer storage location. This information is added to Shire by the technical section.

NEXT GENERATION SEQUENCING (NGS) – CARDIFF AWMGS

Since 2017, NGS has been used in AWMGS for the detection of mutations in the KRAS, NRAS, BRAF, PIK3CA, and TP53 genes. All samples will be analysed for mutations at specified codons/exons within the following genes; BRAF codons 599, 600 and 601 (exon 15), KRAS codons 12 & 13 (exon 2), 61 (exon 3), 117 and 146 (exon 4), NRAS codons 12 & 13 (exon 2),61 (exon 3), 117 and 146 (exon 4), PIK3CA codons 542, 545, 546 (exon 10*), 1047 and 1049 (exon 21*) and TP53.

*Note: PIK3CA numbering corrected from previous SOP versions in line with ref seq NM_006218.2 (previously referred to as exons 9 and 20).

Any changes in these specific codons will be reported, as long as they are not considered to be polymorphisms.

Introduction: GeneRead Clinically Relevant Mutation panel

The GeneRead Clinically Relevant Mutation panel used in the Cardiff lab interrogates the following genes:

Reference sequences

Gene	Refseq
AKT1	NM_001014432.1
ALK	NM_004304.4
AR	NM_000044.3
BRAF	NM_004333.4
CTNNB1	NM_001098209.1
DDR2	NM_001014796.1
EGFR	NM_005228.3
ERBB2	NM_001289936.1
FGFR3	NM_000142.4
GNA11	NM_002067.4
GNAQ	NM_002072.4
IDH1	NM_001282386.1
IDH2	NM_002168.3
KIT	NM_000222.2
KRAS	NM_004985.3
MAP2K1	NM_002755.3
MET	NM_001127500.1
NRAS	NM_002524.4*
PDGFRA	NM_006206.4
PIK3CA	NM_006218.2
PTEN	NM_000314.6
RET	NM_020975.4
STK11	NM_000455.4
TP53	NM_000546.4

GeneReadDNAseq Targeted Panels V2 uses multiplex PCR-based targeted enrichment technology, in combination with a sophisticated primer design algorithm; which enables amplification and enrichment of any gene or targeted region in the human genome in order to detect genetic variation using NGS. Adjacent and potentially interacting primer pairs are separated into different pools for optimal performance. GeneReadDNAseq Targeted Panels V2 are designed to analyze a panel of genes and/or regions related to a disease state and can be used with any major NGS platform.

GeneReadDNAseq Targeted Panels V2 are provided as sets of 1, 2, 3 or 4 pools, each containing primer mixes, with up to 9600 primer pairs per 4-pool set. The number of pools included is determined by the region covered by amplicons. Most panels cover full coding regions of genes, so one 4-pool set is provided. For special panels, like the Tumour Actionable Mutations Panel covering tumor mutation hotspots, one pool is provided. GeneReadDNAseq Targeted Panels V2 can enrich selected genes and/or regions using as little as 40 or 20 ng genomic DNA in 3 hours for a 4- or 1-pool panel, respectively (Figure 2). Briefly, genomic DNA samples are combined with primer mix and PCR reagent and PCR is performed in a standard thermocycler. After the PCR is complete, the reactions for each sample are pooled and the enriched DNA is purified. The purified DNA then is ready for NGS library construction and sequencing using the NGS platform of your choice. Since amplicons from each sample are pooled before library construction, each sample results in one library only.

Figure 1: Below is a summary of the overall workflow:

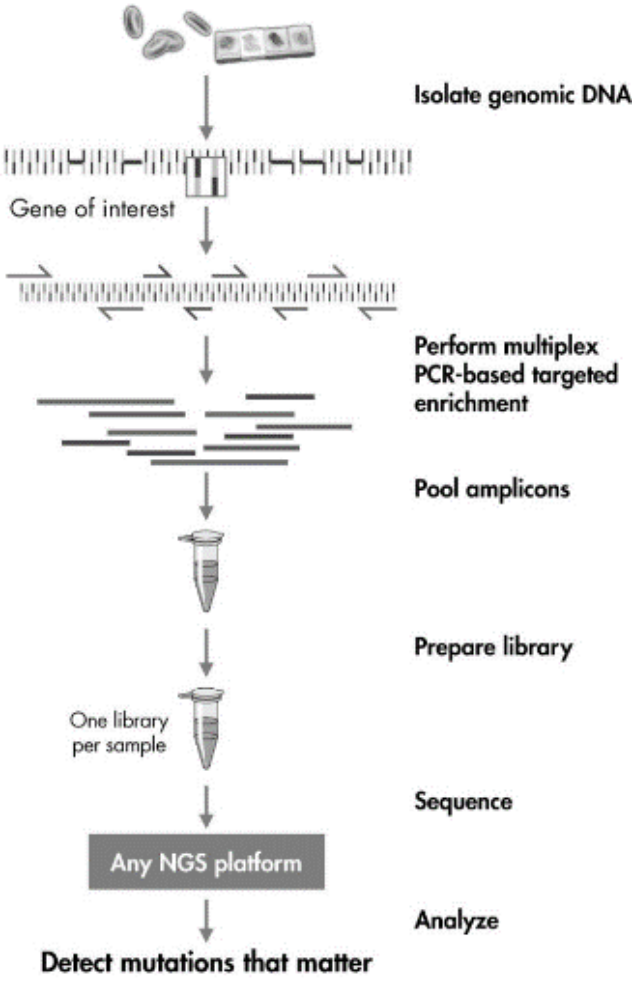


Figure 2. The GeneReadDNAseq Targeted Panel V2 procedure.

Day	Stage of Process, Reagents used and Plate name.	Information
1	**PCR Set Up** Reagents Stock DNA PCR Buffer (5x) Primer Mixes (Oligo Panel) HotStarTaq DNase-free Water Plate Name: PCR 1	**Prepare DNA** • Dilute to 2.5 ng/µl (vol of at least 16µl). **Multiplex PCR** • 1-4 PCR Primer Sets are carried out in separate reactions to target and amplify regions of interest. • This prevents competition between primers that are targeting similar regions. PCR set up takes up to 90 minutes PCR takes 3 hrs. **SAFE STOPPING POINT**: After PCR Plate can be stored in the freezer.
	Sample Pooling, Purificationand Quantification Reagents: AMpure XP beads 80% Ethanol Nuclease Free water Broad Range Qubit Standard Broad Range Qubit Reagent Broad Range Qubit Buffer Plate name: PCR 1/ PCR Trans/ DNA Amp 1/DNA Amp 2	**Sample Pooling:** • Combine up to 4 primer reactions into the 1 well per sample. All targets will be in one pool per sample. **Sample Purification:** • 2 rounds of sample purification to ensure the correct sized fragments are selected to proceed into the next stage of library preparation. Pooling takes up to 30 minutes Purification takes between 90 - 120 minutes Quantification: takes up to 60 minutes **SAFE STOPPING POINT**: Once library has been cleaned, you can store in the freezer.
2	**End Repair of DNA** Reagents: PCR-Enriched DNA from previous step End-Repair Buffer, 10x End-Repair Enzyme Mix Plate Name: End Repair	**End Repair:** • DNA fragments are repaired so that all strands are blunt ended. Procedure takes 40 minutes Incubation takes 50 minutes **SAFE STOPPINGPOINT**: Samples can be stored in the freezer after incubation.
	A-addition Reagents: End Repaired DNA from previous step	**A-addition:** • An A-tail is ligated to the 3` end of each end repaired DNA strand. Procedure takes 30 minutes

	A-addition Buffer Klenow Fragment (3'-> 5'exo-) Plate Name: End Repair	Incubation takes <u>40 minutes</u> **<u>SAFE STOPPING POINT</u>**: Samples can be stored in the freezer after incubation.
	Adapter Ligation Reagents: DNA from End Repair step Ligation Buffer 2x Adapters (Sets A and B) T5 DNA Ligase DNAse-Free water Ampure XP Beads 80% Ethanol PCR Plate: End Repair, AMP ER	**Adapter Ligation:** • Adapters are ligated to both ends of the DNA fragments. • These allow for further amplification and sequencing. • Within in the adapters lays a unique identifying sequence which allows for multiplexing of samples. **Sample Purification:** Adapter Ligation set up takes <u>60 minutes</u> Adapter Ligation Incubation: <u>15 minutes</u> Sample purification takes <u>60-90 minutes</u> **<u>SAFE STOPPING POINT:</u>** After Adapter Ligation and Sample Purification. Samples can be stored in the freezer.
3	**PCR Amplification of Purified Library** <u>Reagents:</u> Barcoded DNA from Previous Step Hifi PCR Master Mix 2x Primer Mix (10uM each) RNase-free water AMPure XP beads 80% Beads High Sensitivity Qubit Standard Broad Range Reagent Broad Range Buffer High Sensitivity Bioanalyser Reagents Plates: AMP ER, AMP T1 and FINAL	**PCR Amplification:** • Amplification of only the DNA fragments that have successfully bound to adapters. **Sample Purification:** • 2 rounds of sample purification to ensure the correct sized fragments are selected to proceed into the next stage of library preparation. **<u>Quantification:</u>** • Final quantification using the Qubit. • Bioanalyser run with only pooled samples ensures library is of the right shape and at the expected fragment size. PCR setup takes <u>40 minutes</u> PCR takes <u>25 minutes</u> Sample purification takes <u>90-120 minutes</u> Final Quantification takes <u>60-90 minutes</u>

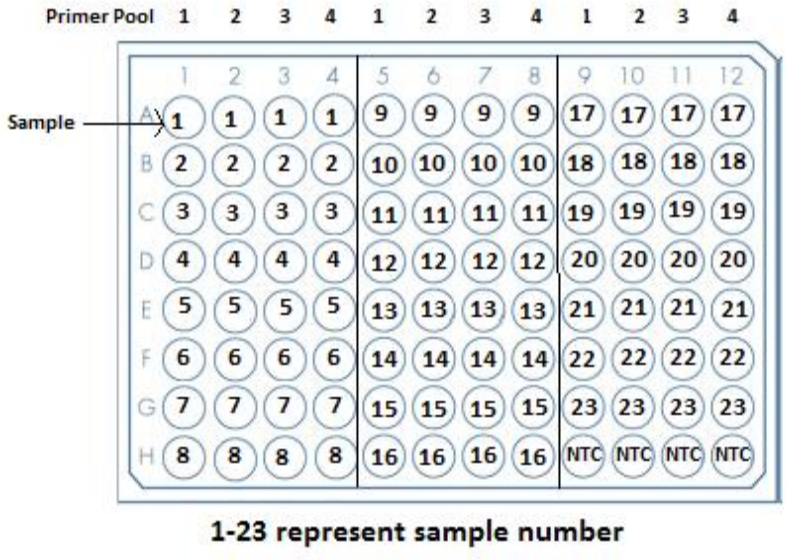
Definitions

PCR	Polymerase Chain Reaction
NTC	No template control
WCB	Wales Cancer Bank
IMG	Institute of Medical Genetics
NGS	Next Generation Sequencing
GRPCR1	GeneRead PCR 1
AMPT1	Ampure Transfer 1
AMPT2	Ampure Transfer 2
ENDR	End Repair
CLN2	Clean 2
AMPT3	Ampure Transfer 3
FIN	Final

Protocol: GeneRead Clinically Relevant Mutation panel

PCR Set-up

1. Prepare a worksheet with no more than 23 samples and 1 NTC. If you are running 46 samples (2
   MiSeq runs) then you need to prepare two separate worksheets.
2. Measure stock DNA concentration using Qubit HS reagents and Diluteto 2.5ng/µl (+/- 0.5ng/µl) with DNase-free water, in a LoBind tube. Make a minimum of 16µl.
3. Label a 96 well PCR plate with the worksheet number, date and also “PCR”.
4. Ensure that each primer pool location is clearly marked out on the PCR plate, according to the DNA layout below.
    - The first pool will be aliquoted into columns 1, 5 and 9. Therefore mark above the columns with the number 1.
    - The second pool will be aliquoted into columns 2, 6 and 10. Mark above these columns with the number 2.
    - The third pool will be aliquoted into columns 3, 7 and 11. Mark above these columns with the number 3.
    - The fourth pool with be aliquoted into columns 4, 8 and 12. Mark above these columns with the number 4.



1-23 represent sample number

Check required: Checker must visually confirm the sample tube order matches sample order on the worksheet and that the plate is labelled with plate name, worksheet number and pool numbers. Plate numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

5. Aliquot 4µl of each sample into each of the assigned 4 wells (see diagram above).

Note:Transferring from tubes to plates without using a multichannel pipette is a challenging process; always take extra care and concentrate.

6. Prepare a PCR master mix using the table below. Make the master mix with at least an additional
   2 samples to allow for pipetting error i.e. if you have 46 samples then make a master mix for 48 samples.

A check is required; Check requires visual confirmation that the correct Primer Panel is being used. Use the table in 4.4 if you are unsure which panel is which.

Component	x4 Pool Panel Per Sample	X 4 Pool Panel x 26 samples	X 4 Pool Panel x 50 samples
GeneRead DNAsea Panel PCR Buffer	17.6	457.6	880
GeneRead Hotstart Taq	6	156	300
DNAse Free Water	2.8	72.8	140
Total	26.4	686.4	1320

7. Separate the master mix into four separate 1.7 ml Eppendorf tubes (using the table below). Label tubes 1-4.

A check is required: Checker must visually confirm that the correct Primer Panel is being used and Pool tubes are labelled appropriately.

8. Add each primer pool to the corresponding Eppendorf tubes using the table below. The volumes are representative of one pool only.

Component	Per Sample	x 26 samples	x 50 samples
PCR master Mix (Made Previously)	6.6	171.6	330
Primer Mix Pool (A1-A4)	11	286	550
Total	17.6	457.6	844.8

9. Aliquot 16µl of each PCR pool mix into the appropriate wells of the PCR plate. Refer to DNA layout on point 2. Mix gently by pipetting up and down x5 using a p20 multichannel pipette.

10. Seal the plate. Pulse centrifuge for 20 seconds. Using a thermocycler to carry out the PCR set according to the parameters below. If not already saved, save protocol as GRPCR1

Cycle	Temperature	Time
1	95	15 min
26	95	15s
	60	4 min
1	72	10 min
1	4	∞

11. When the reaction is complete, either proceed to sample pooling and purification or **store plate at -20^{0}C for up to 3 days. Or the plate can be left overnight on the PCR block.**

Sample Pooling and Purification

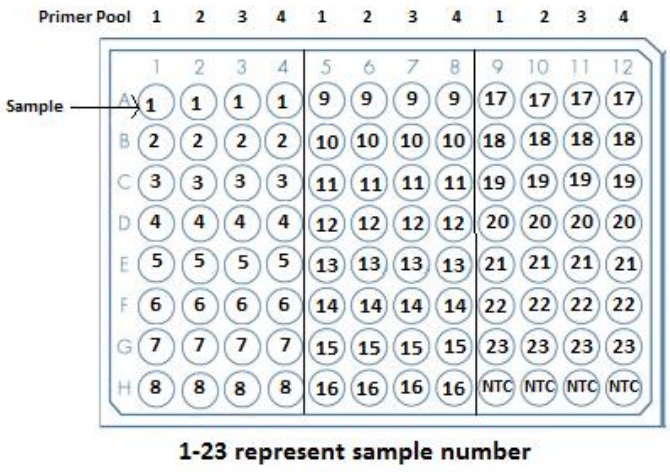
1. Take out Ampure XP beads from the fridge to equilibrate to room temperature for at least 30 minutes prior to purification.
2. Prepare 80% ethanol by mixing 100% EtOH and nuclease free water. Use the table below for the volumes to make.

Component	24 Samples	48 Samples
100% Ethanol	10 mL	20 mL
Nuclease Free Water	2.5 mL	5 mL

A check is required: Checker must visually confirm that the correct samples are being combined as indicated in the diagram below and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run. *The checker must watch the entire combining process.*

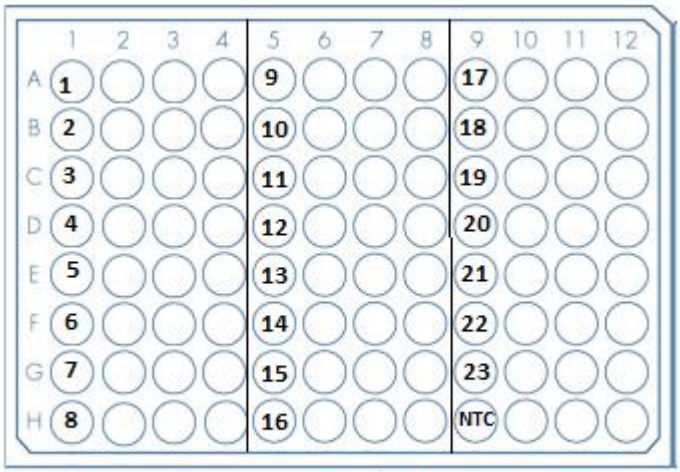
3. Once the PCR plate has been removed from the thermocycler, pulse centrifuge for 20 seconds.
4. Combine all 4 PCR reactions for 1 sample into a single well of the 96 well PCR plate. The total volume in the well should be ~80µl. See the diagram below for combining layout.

Original layout



1-23 represent sample number

Combined layout

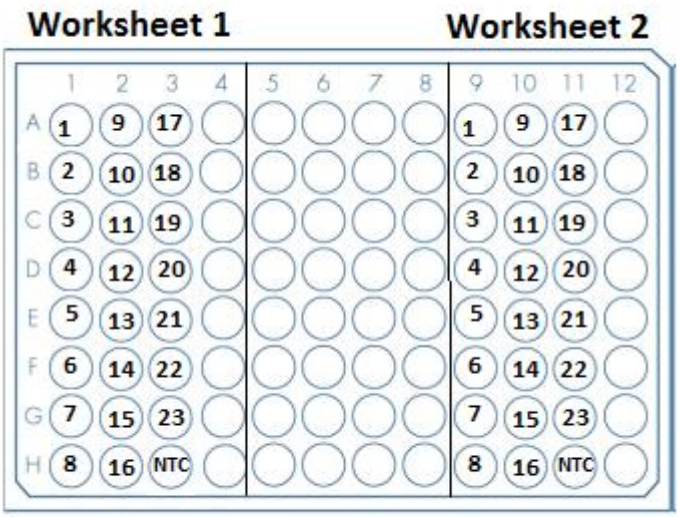


1-23 represent sample number

5. Mix by pipetting up and down at least 10 times.
6. Label a new 96 well plate NPCR and worksheet number.

A Check is required; Checker must visually confirm PCR and NPCR plate orientations are correct (see combined layout diagram above for PCR and below for NPCR) and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

7. Transfer 40µl of each combined pooled sample to NPCR plate. Store the remaining 40µl at -20°C. See diagram below for new plate layout. If running more than one worksheet (23 samples) a second worksheet can be added to the same plate.



1-23 represent sample number

8. Using a multichannel pipette, add 36 µl (0.9x volume) AMPure XP beads to 40 µl PCR product. Pipette mix up and down 10 times. Check that the beads are mixed evenly. If

not, repeat mixing process until the beads are mixed evenly. Seal the plate. If using a trough to dispense the beads, aliquot a small amount into the trough and discard the remaining beads once added.

9. Incubate the mixture for 5 minutes at room temperature.
10. During incubation. Prepare 20ml of 80% ethanol solution and label a new PCR plate AMPT1 with appropriate worksheet numbers.
11. Pulse centrifuge for 20 seconds.
12. Place the plate on a magnetic rack to separate the beads from the supernatant. Leave on the magnetic rack until solution is clear.

A Check is required: Checker must visually confirm NPCR and AMPT1 plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

13. Once solution is clear, transfer 70µl of the supernatant to AMPT1 plate. This will leave behind 6µl of supernatant which prevents bead carry over.

DO NOT DISCARD the supernatant. Store in fridge for until library prep has been successful. This plate can be used for troubleshooting (see section 5).

14. Using a multichannel pipette, add 64µl ofAMPure XP beads to the samples on plate AMPT1. Pipette mix up and down 10 times. Check that the beads are mixed evenly. If not, mix up and down a further 10 times. Repeat mixing process until the beads are mixed evenly. Seal the plate.
15. Incubate the mixture for 5 minutes at room temperature.
16. Pulse centrifuge for 20 seconds.
17. Place the plate on a magnetic stand to separate the beads from solution. Wait until solution is clear.
18. Carefully remove and discard the supernatant.

DO NOT DISCARD the beads

19. Using a multichannel add 200µl of fresh 80% ethanol to the beads whilst on the magnetic plate. Incubate at room temperature for 30 seconds
20. Carefully remove and discard the supernatant.
21. Repeat steps 14-15 for a total of two washes.
22. Completely remove residual ethanol using a multi/single channel p20.
23. Leaving plate on the magnetic rack, air dry beads for at least 10 minutes. Cracks in the beads should be visible before moving onto the next step.
24. Whilst the beads are air drying. Label a new PCR plate with AMPT2 and appropriate worksheet numbers.
25. Remove plate from the magnetic rack. Add 28µl of nuclease free water. Seal the plate.
26. Mix up and down at least 10 times until beads are dispersed equally in solution.
27. Pulse centrifuge for 20 seconds.
28. Place plate back onto the magnetic rack for at least 5 minutes. Make sure solution is completely clear.
29. **A Check is required:** Checker must visually confirm AMPT1and AMPT2 plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

30. Using a P20 set to 12.5µl, transfer a total of 25µl of the supernatant to AMPT2 plate.
31. Quantify each sample library using the qubit broad range kit. Expected yield is 10-200ng of PCR-enriched DNA.

DAY 1 Stopping Point. AMPT2 can be stored at -20°C for up to three days before proceeding onto **7.2.3 End repair.**

End Repair

1. Using the Qubit concentration results, calculate the average library concentration by adding each library concentration together and dividing by the total number of sample libraries (normally 23). Do not include the NTC in this calculation.
2. Using the average library concentration calculate the volume of each library to transfer to the next plate using the calculation below; Aim transfer at least 200ng DNA and a volume of least 12.5ul.

For example:
Aiming to transfer at least 200ng in total of each sample library using calculation below;

Calculation: 200/Average = Volume of Sample to transfer
E.g. if average concentration is 33
200/33=6.1µl of each sample library to be transferred. This is under the 12.5ul threshold, thus transfer 12.5ul. There will be more than 200ng/ul of PCR product going through to end repair.

3. If many of your libraries (over 50% of your samples) are on the low side (5-10ng/ul), you can transfer 20.5 of your PCR product. The next steps will work as long as you have a concentration of at least 10ng of PCR product. However, the Qubit concentration includes all the background primer noise and large unwanted fragments. Thus, you have less of the desired library than you anticipate. If your library is across the board very low (<5ng/ul), then it is likely your library has failed.
4. ***You may be asked to transfer exactly the same amount of each sample at this stage. This will be confirmed by your line manager. If so, use the same concentration calculation, but instead of dividing by the average concentration you divide by the individual sample concentration.***

Calculation: Concentration Required (200ng)/ Individual sample concentration = Volume of sample transfer.

5. Label a new 96 well PCR plate ENDR and worksheet number.

A Check is required: Checker must visually confirm AMPT2 and ENDR plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

6. Transfer the appropriate volume of each library to the ENDR plate as calculated in step 4.
7. Top up the volume of each library to 20.5µl using nuclease free water. For example if transferring 12.5 µl of library from AMPT2 to ENDR then add 8 µl of water.

8. Prepare a reaction mix for end-repair according to the table below (30 sample volume should give you enough for 24 samples. Due to the viscosity of the mix more needs to be made up).

Component	Volume (µl) x 1 sample	Volume x 30 samples	Volume x 60 samples
End-Repair Buffer	2.5	75	150
End-Repair Enzyme Mix	2.0	60	120
Total	4.5	126	225

9. Pipette 4.5µl of the end repair master mix into each sample well.
10. Pulse centrifuge for 20 seconds.
11. Incubate in a thermocycler for 30 min at 25°C followed by 20 mins at 75°C. If not already saved, save protocolas GENENDR.
12. Once the programme has finished, pulse centrifuge plate for 20 seconds.

Note: Safe stopping point. Plate can be left on the thermocycler overnight or at -20˚C for 3 days.

A-Addition

1. Prepare a reaction mix for the A-addition using the table below. Pipette mix solution.

Component	Volume (µl) x 1 Sample	Volume (µl) x 26 samples	Volume (µl) x 48 samples
A-addition buffer 10x	3.0	84	150
Klenow Fragment	3.0	84	150
Total	6.0	168	300

2. Add 6µl of the mix to each sample well. Mix by pipetting up and down at least 5 times.
3. Incubate in a thermal cycler for 30 min at 37˚C, followed by 10 minutes at 75˚C. If not already saved, save protocol as GENAAD.
4. Once finished pulse centrifuge plate for 20 seconds.

Note: Safe stopping point. Plate can be left on the thermocycler overnight or at-20˚C for 3 days.

Adapter Ligation

1. Prepare a reaction mix for adapter ligation according to the table below.

Component	Volume (µl) x 1 sample	Volume (µl) x 26 samples	Volume (µl) x 48 samples
Ligation Buffer	45	1170	2160
T4 DNA Ligase	4	104	192
DNAse free water	9	234	432
Total	58	1508	2784

2. Add 58µl of the mix to each sample. Mix by pipetting up and down at least 5 times.

A check is required. Checker must visually confirm that the correct Barcode PCR plate has been removed from the freezer and the plate in the correct orientation with the well A1 being at the top left corner.

3. Add 1µl of Adapters from the Generead Barcode pcr plate. The orientation is all ready in order from barcodes Bc1-Bc27 (total of 24 adapters). If the plate is empty or some barcodes are missing, then a new plate of barcodes needs to be set up. This is outlined in the appendix of this protocol. See **10.1.2J** for how to generate a new barcode plate.
4. Pulse centrifuge for 20 seconds.
5. Incubate on a thermocycler at 25°C for 10 minutes. **Ensure you leave the lid open.** If not already saved, save protocol as GENADL.

Purification of adapter-ligated DNA with AMPure XP bead

1. Add 108µl (1.2x Volume) AMPure XP beads to the 90µl DNA solution. Mix well by pipetting up and down at least 5 times. If using a trough to dispense the beads, aliquot a small amount into the trough and discard the remaining beads once added.
2. Incubate for 5 minutes at room temperature.
3. Place the 96 well plate on a magnetic rack to separate beads from supernatant. Leave on the rack for 15 minutes.
4. Whilst the plate is on the magnetic plate. Prepare 80% ethanol by mixing 100% EtOH and nuclease free water. Use the table below for the volumes to make.

Component	**24 Samples**	**48 Samples**
100% Ethanol	10 mL	20 mL
Nuclease Free Water	2.5 mL	5 mL

5. Carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain the DNA target.
6. Whilst the plate is on the magnetic rack, add 200µl of fresh 80% ethanol to each of the wells. Wait 30 seconds, then carefully remove and discard the supernatant.
7. Repeat step 5 for a total of 2 ethanol washes.
8. Using a P20 multichannel or single channel pipette completely remove ethanol and dry beads for 10 minutes while the plate is on the rack. If the beads haven't form cracks by the end of 10 minutes, leave to dry on the magnetic rack until you see cracks appear in the beads.
9. Remove plate from the magnetic rack. Elute the DNA from the beads in 19µl nuclease free water. Mix well by pipetting up and down, until the beads are homogenous in the solution.
10. Pulse centrifuge for 20 seconds.
11. Label a new PCR plate and the worksheet number.

A Check is required: Checker must visually confirm ENDR and CLN2 plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

12. Place the plate back onto the magnetic rack for 5 minutes until solution clears. Transfer 17µl of the supernatant to a new 96 PCR plate labelled CLN2.

PCR amplification of purified Library

13. Prepare a PCR master mix using the table below.

Component	Volume (µl) x 1 sample	Volume x 26 samples	Volume x 48 samples
HiFi PCR Master Mix, 2x	25	650	1200
Primer Mix (10uM Each)	1.5	39	72
RNase-free water	6.5	169	312
Total	33	858	1584

14. Add 33µl of the master mix to each well, to give a total volume of 50µl.
15. Pulse centrifuge for 20 seconds.
16. Amplify products using a thermal cycler according to the parameters below. If not already saved, save the protocol as GENAMP.

Cycle	Temperature	Time
1	98	2min
5	98	20sec
	60	30sec
	72	30sec
1	72	1min
1	4	∞

17. After the reaction is complete proceed with the AMPure XP clean up or store plate at-20°C. Samples are stable for 3 days.

Purification of amplified library with AMPure XP beads

1. Add 40µl (0.8x volume) AMPure XP beads to 50µl PCR solution. Mix well by pipetting up and down at least 5 times, until the solution is homogenous. If using a trough to dispense the beads, aliquot a small amount into the trough and discard the remaining beads once added.
2. Whilst the plate is on the magnetic plate. Prepare 80% ethanol by mixing 100% EtOH and nuclease free water. Use the table below for the volumes to make.

Component	24 Samples	48 Samples
100% Ethanol	10 mL	20 mL
Nuclease Free Water	2.5 mL	5 mL

3. Incubate for 5 minutes at room temperature.
4. Pulse centrifuge for 20 seconds.
5. Place the PCR plate on a magnetic rack to separate the beads.
6. Incubate at room temperature for at least 5 minutes until the solution clears.
7. Label a new PCR plate AMPT3 and worksheet number.

A Check is required: Checker must visually confirm CLN2 and AMPT3 plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

8. Carefully transfer 86µl of the supernatant to AMPT3, without disturbing the beads.

Discard the beads, which contain unwanted large DNA fragments

9. Add 20µl (0.4x the original volume of PCR product, which was 50µl) AMPure XP beads to the supernatant, mix well by pipetting and incubate at room temperature for 5 minutes.
10. Pulse centrifuge for 20 seconds.
11. Place the PCR plate on a magnetic rack and wait at least 5 minutes until the solution is clear.
12. Carefully remove and discard supernatant. **Be careful not to disturb the beads**, which contain the DNA target.
13. Add 200µl of fresh 80% ethanol to each well containing the DNA target. Wait 30 seconds, then carefully remove and discard supernatant.
14. Repeat step 12 for a total of 2 washes.
15. Completely remove ethanol and dry beads for 10 min with the plate still on the rack.
16. Add 30µl nuclease-free water to each well to elute DNA from the beads. Mix well by pipetting up and down, until solution is homogenous.
17. Pulse centrifuge for 20 seconds.
18. Label a new PCR plate FIN and the worksheet number.

A Check is required: Checker must visually confirm AMPT3 and FIN plate orientations match and the plates are labelled with correct plate name and worksheet number. Numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

19. Place the plate on the magnetic rack until solution is clear. Transfer 28µl supernatant to FIN.
20. Quantify the libraries using high sensitivity Qubit reagents. Above 5ng/ul is considered really good. 2-5ng/ul is considered adequate. Anything below 2ng/ul will likely be poor and have a high primer dimer peak or probably dropped out completely.

Libraries can be stored at-20°C and are stable for 1 month at this temperature.

Prepping and pooling the libraries for MiSeq loading

1. Remove a 300 cycle V2 cartridge from the freezer and defrost in warm water.
   Note: Ensure a V2 flow cell is used.
2. In the L drive, open the Generead Final Pool Calculator. Enter your final Qubit concentrations into the A column. Full instructions are in the document as to which columns represent nM conversion and the equimolar volume for each sample to pool into the final pool. If the calculator is not available then continue to point 3 and 4 for manual calculations.
3. From the Qubit concentrations calculate the nano molar concentration and make a note on the sample sheet, using the following calculation.

$$\frac{\text{(concentration in ng/µl)}}{\text{(660g/mol * average library size)}} \times 10^6 = \text{Concentration in nM}$$

The average library size is 280bp.
For example:
Final library Qubit value is 8ng/µl

$$\frac{8\text{ng/µl}}{184{,}800} \times 10^6 = 43.3\text{nM}$$

4. Using the nM concentrations, calculate the volume of each sample needed to create an equimolar final library pool.

To do this, divide the sample with the highest nM concentration with each sample in the run concentration. This gives the volume required to pool the each sample.

For example:

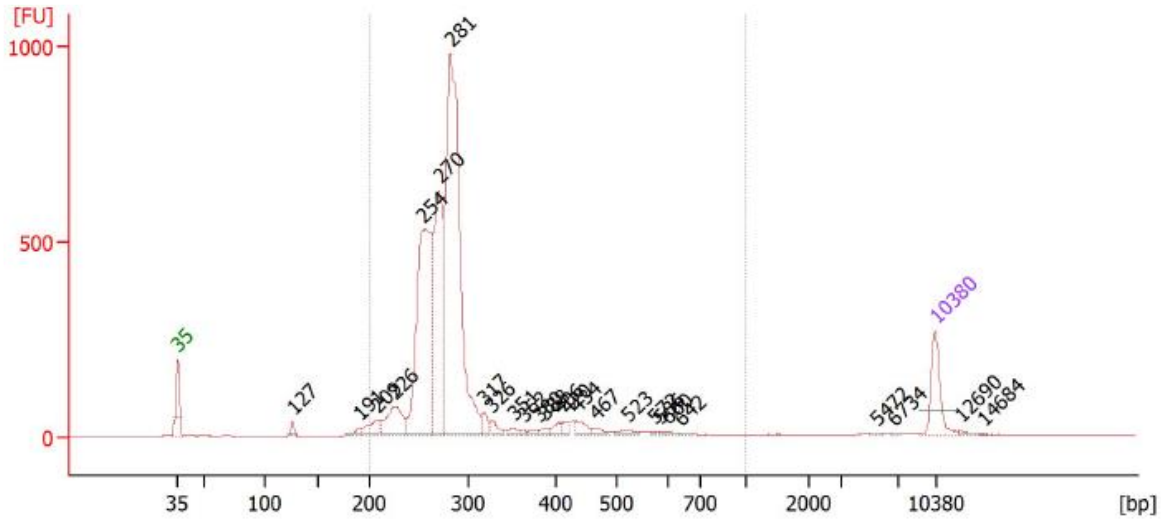
Sample No	nM concentration	Equimolar Calculation
1	10	20/10 = 2µl
2	4	20/4 = 5µl
3	13	20/13 = 1.5µl
4	20	20/20 = 1µl

5. Label 5 tubes 1-5 with the K numbers of the worksheets on the lids and sides.

- Tube 1 needs to be labelled Final Pool
- Tube 2 needs to be labelled 4nM
- Tube 3 needs to be labelled 2nM
- Tube 4 needs to be labelled 20pM
- Tube 5 needs to be labelled 12pM

A check is required: Checker must visually confirm that FIN plate and tubes 1-4 are labelled with correct plate/tube name and worksheet number. Plate numbering 1-12 and A-G should be in the correct orientation with sample wells outlined and A1 clearly marked to indicate the start of the run.

6. Pool all the samples using the volumes generated from the equimolar calculation into tube 1, labelled final pool.
7. Run the final pool on the bioanalyser using the HS reagents. Run the pooled library in duplicate plus run the NTC of each worksheet as a singlet. This ensures no contamination is present in the run. The image below illustrates what is expected to be seen.
   Note: CRM and BRCA will have a different profile. The example shows a CRM library.



Example of a good CRM Bioanalyser trace. Note the very small primer peak at 127 bp

8. If you notice a big primer dimer peak as illustrated below, seek advice from your GT lead. You will need to increase the loading concentration onto the MiSeq to counteract the primer dimer. The lead GT will make this decision. See the troubleshooting section for more information.
9. Qubit the final pool using HS reagents. **Don't forget to convert the final qubit concentration to nM using the calculation displayed in point 3.** From this make a 20ul 4nM stock solution of your library in tube 2, labelled 4nM.
10. Qubit the 4nM dilution.
11. From this make a 20ul 2nM stock solution of your library in tube 3, labelled 2nM.
12. Transfer10µl of the 2nM stock solution to tube 4 (labelled 20pM) and add 10µl of 0.1N NaOH (made fresh from 1N stock).
13. Vortex thoroughly, pulse centrifuge and incubate at room temperature for 5 minutes.
14. Add 980µl of ice cold HT1 and vortex thoroughly.
15. Make a 12pM loading aliquot for the MiSeq into tube 4 labelled 12pM.

Vol of 20pM stock to add	**Vol of ice cold HT1 to add**
360µl	240 µl

16. The 12pM library is now ready to load on the MiSeq. Store at 2-8^{o}C until you are ready to load onto the MiSeq. See LP-GEN-MiSeqOp for details on loading the MiSeq.

A check is required: Checker must visually confirm that 11pM pool tube is labelled with correct tube name and worksheet number and that the correct type of MiSeq cartridge is being used. MiSeq sample sheet should be checked to confirm worksheet number, sample numbers and indexes are correct.

Index Sequences

Setting up a Generead Barcode (indices) PCR plate. Take out from the freezer Barcode sets A and B. Set A will contain Barcodes 1-12 and Set B will contain Barcodes 13-27. Note 17, 24 and 26 are not included in the barcode sets. **See Barcode lists below**.

Generate a K worksheet number and list the description as Generead Barcode set up. Print a generic K worksheet from Q-Pulse and label it with the K worksheet number just generated. The list for K numbers is located in Pre PCR of the main IMG building. Set up the barcodes in order of 1 – 27 on an 8 by 12

PCR rack. Label a PCR plate with the K worksheet number. A check is required to make sure that the barcodes are labelled ordered correctly and the PCR plate is labelled correctly and in the correct orientation with A1 being in the top left hand corner. Once completed, transfer the full volume of the barcodes one at a time from the tube in the plate. This pate is now your Generead Barcode plate and should last you for about 30 library preparations

Set A

Adapter name	Barcode
Adapter Bc1 Illumina	ATCACG
Adapter Bc2 Illumina	CGATGT
Adapter Bc3 Illumina	TTAGGC
Adapter Bc4 Illumina	TGACCA
Adapter Bc5 Illumina	ACAGTG
Adapter Bc6 Illumina	GCCAAT
Adapter Bc7 Illumina	CAGATC
Adapter Bc8 Illumina	ACTTGA
Adapter Bc9 Illumina	GATCAG
Adapter Bc10 Illumina	TAGCTT
Adapter Bc11 Illumina	GGCTAC
Adapter Bc12 Illumina	CTTGTA

Set B

Adapter name	Barcode
Adapter Bc13 Illumina	AGTCAA
Adapter Bc14 Illumina	AGTTCC
Adapter Bc15 Illumina	ATGTCA
Adapter Bc16 Illumina	CCGTCC
Adapter Bc18 Illumina	GTCCGC
Adapter Bc19 Illumina	GTGAAA
Adapter Bc20 Illumina	GTGGCC
Adapter Bc21 Illumina	GTTTCG
Adapter Bc22 Illumina	CGTACG
Adapter Bc23 Illumina	GAGTGG
Adapter Bc25 Illumina	ACTGAT
Adapter Bc27 Illumina	ATTCCT

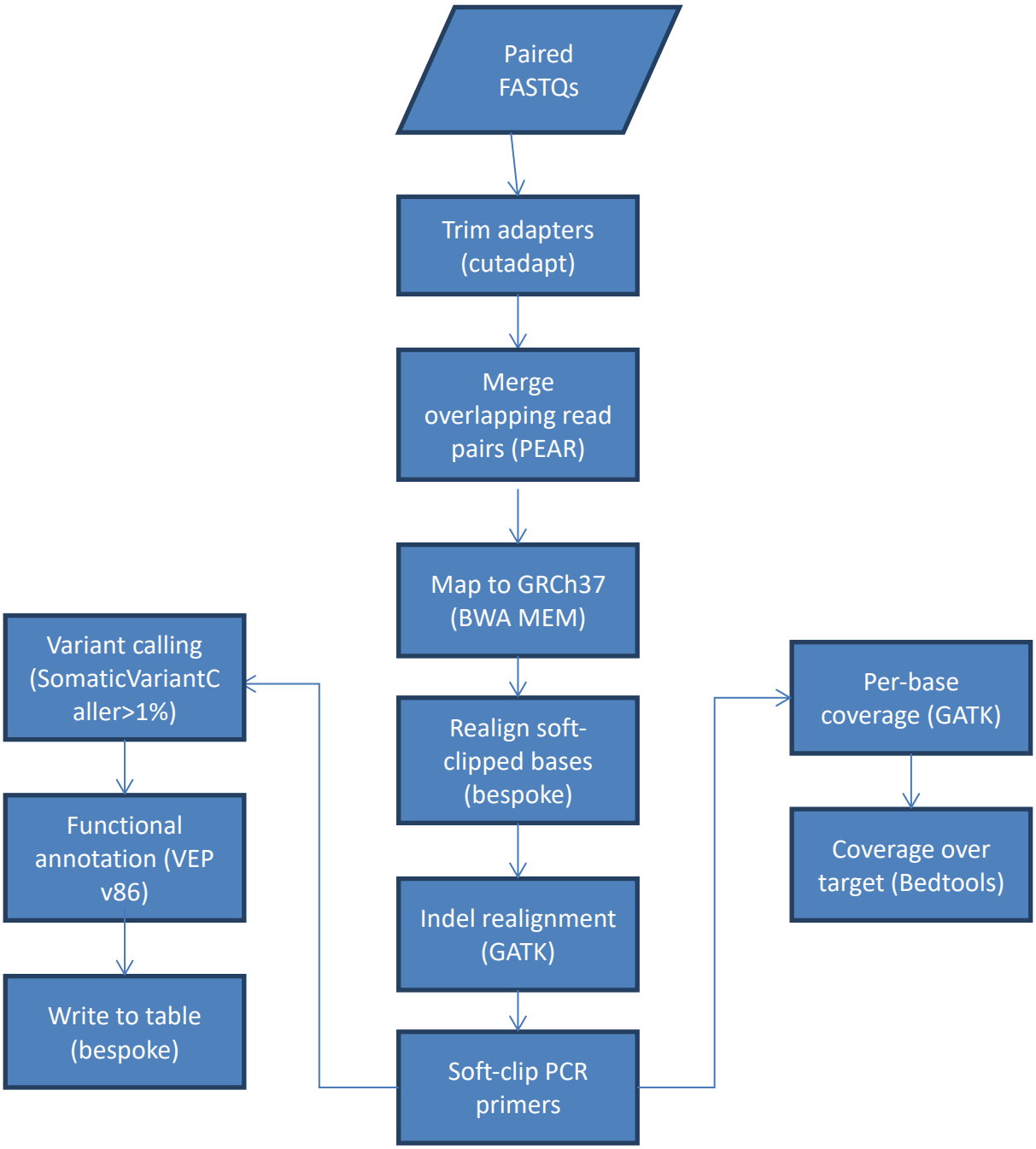
NGS Bioinformatic Analysis

Somatic Amplicon bioinformatics pipeline for detecting low level variation in amplicon datasets.

Introduction

The somatic amplicon pipeline is designed to detect low level variation (>1%) from amplicon next-generation sequencing library preparations. The pipeline accepts paired-end FASTQs files and requires a minimum of 10bp overlap between the first and second read. Therefore the maximum insert size must be less than the total cycle number minus ten.

Pipeline flowchart



Method

1. Adapters are trimmed from the reads (Cutadapt).
2. Overlapping read pairs are merged into one contiguous sequence. Non-overlapping reads are discarded.
    a. Most amplicons in the GeneRead kit are short enough to be covered in both directions using 2 x 151bp sequencing. This information can be used to eliminate sequencing error seen in one direction.
    b. Once merged, the reads must start and end with PCR primer which is useful for downstream processes (i.e. steps 5 and 6).
3. Whole genome alignment using BWA MEM.
4. Realign soft-clipped bases.
    a. Following paired-end read merge (step 2) all sequences must start and end with PCR primer and should align correctly to the reference genome. Evidence of soft clipping can suggest the presence of a large indel.
    b. Reads overlapping the ROI showing soft-clipping are realigned to the expected amplicon sequence using global alignment (Needleman–Wunsch).
5. Indel realignment using GATK.
6. Soft-clip PCR primers.
    a. PCR primers contain false-positive variation due to errors in synthesis.
    b. The sequence may also mask a true-positive in an overlapping amplicon.
7. Variant calling using SomaticVariantCaller (Illumina).

NGS analysis

1. Obtain the NGS worksheet from the NGS technical team
2. Open the FOCUS4 database and complete cells G to M of each patient using data from the NGS worksheet (S:\MedGen\SHARED\MOLEC\DISEASES\Pharmacogenetics\FOCUS4\Databases\FOCUS 4 database.xls)
3. Navigate to L:\NGS ANALYSIS\CRM\FOCUS 4\Results analysis and create a new folder named with the worksheet number. Within this folder create three new subfolders called: '1st Checking', '2nd Checking' and 'Completed'.
4. Open the NGS results drive - W:\ or Z:\ to retrieve the data from your NGS run (e.g. 180305_M02641_0058_000000000-BL4YN) using the flow cell ID reference (BL4YN in this example; the flow cell ID is handwritten on the NGS worksheet. Select the relevant Excel analysis files and copy them across to the new folder in L:\NGS ANALYSIS\CRM\FOCUS 4\Results analysis.

Check results data for individual patients

1. Open the appropriate patient analysis worksheet (from your new folder in L:\NGS ANALYSIS\CRM\FOCUS 4\Results analysis)
2. Complete the 'Patients demographics' tab using data from the FOCUS4 database and the NGS worksheet (i.e. find patient in the FOCUS4 database and copy and paste cells A-M only into the 'Patients demographics' tab of the FOCUS 4 analysis worksheet).
3. In the NTC Subpanel check tab determine that any reads in the NTC cannot influence the calls made in your patient. The depth of coverage achieved for the patient and NTC within the regions of interest are auto-populated. The % NTC column NTC allows identification of regions that are covered in the NTC at greater than 10% when compared to the patient's

coverage data. Check the values in the % NTC column for any that are greater than 10% (displayed as a decimal so 0.1). Examine any regions with >10% NTC reads in IGV (patient vs patient) and exclude them from downstream analyses if you are confident of the presence of NTC contamination

4. Enter either 'OK' (if no contamination or <10%) or FAIL (if you suspect there to be NTC contamination' i.e. if >10%) – in the box adjacent to 'NTC check1'.

5. Open the 'Variant Calls' tab and initial and date in the box adjacent to '1st checker'.

6. Data within the cells and columns of this tab is automatically populated by the bioinformatics pipeline.

    a. If any patient calls were also detected in the NTC, the % level of NTC contamination will be determined. Examine any variants present at >10% in the NTC when compared to the patient to determine whether that is true contamination (there could be more than one change being called at that location in the NTC and your call might have a lower allele frequency than the one given by the bio-informatics pipeline. The pipeline does not always assign the correct AF to a variant when there are more than 2 calls at a location).

    b. Any variants that have previously been classified as benign polymorphisms or sequence artefacts will be highlighted in the '1st and 2nd checker' columns within the 'Variant Calls' tab, as 'Known polymorphisms' and 'Known artefacts', respectively. These variants need not be checked again.

    c. Variants detected at <5% AF should not be reported as these are below the limit of sensitivity for the assay. These do not need to be checked in IGV, type 'below sensitivity of assay' into the conclusion box.

7. Verify that any other variants called (i.e. not known SNPs or artefacts) as genuine calls using the Integrated Genomics Viewer (IGV).

8. Open IGV (Navigate to L:\NGS ANALYSIS\IGV\IGV_2.3.88 and click the IGV.bat icon)

    a. Ensure that you are using Hg19 as the genome reference (top left window should show – gatk_bundle2.8_hg19_14042015b - If not – select it from the drop-down list).

    b. If you've already got IGV open click on 'new session' to ensure that the previous patient's data (.bam file) is removed.
        i. This is to minimize the chance of detecting incidental findings.

    c. Copy the genomic coordinates for the variant you want to visualise from the 'variant' column (column K) of the 'Variant-Calls' tab in the worksheet analysis template.

9. Paste these genomic coordinates into the IGV search window.

    a. Press – Enter or Click 'Go' [IGV will centre in on your variant (black vertical dashed lines)]

b. Load your patient BAM files (Click -'File'; select – 'Load from File'; Navigate to the NGS results drive [see 8.3.1.1 (d) and (e)] and select – e.g. V-BCPBB_DNA#.bam).

c. Sorting the sample reads so that those containing the mutant base filter to the top

d. With your chosen genomic region/mutation within the centre line (two dashed vertical lines), right click anywhere within the IGV read window and from the popup window, select >sort alignments by >base

10. Determine whether the change appears genuine
    a. Please note that these are some things to consider when determining whether a change is genuine. It is not a definitive list and there are no hard and fast rules

    b. Hovering over the bam Coverage plot directly above the variant gives you the depth of coverage at that location and the allele frequency with which the variant.

    c. Is the variant covered by more than one amplicon (note: some regions are only covered by one amplicon so this is not always possible)? If so does it appear in all?

    d. Is the variant covered in both directions by each amplicon? Note: Amplicons greater than 200bp long will not be fully sequenced in both directions – Hence a change may still be genuine and yet only be covered in one direction.

    e. Due to errors in sequencing insertion and/deletion mutations are frequently called at homopolymer repeats. Loading BAM files from other patients on the same run is useful at highlighting such artefacts.

    f. How clean does the sequence generally look in that region? There's generally more noise at the 3' ends of reads so care should be taken when determining whether calls in these reads are genuine

    g. Take care when calling indels as they may appear as noise especially the ends of the reads (i.e. the variant calling software may call them as individual calls including single nucleotide variants, deletions and insertions. That region might be classed as being of low quality and the call ignored.

    h. Is it a C>T or G>A change that could be a deamination artefact?

    i. What is the quality (QC) score – A change is more likely to be an artefact if it has a QC score of less than 90

11. Score the variant with your conclusion: A variant is either scored as genuine or as an artefact – based on its presentation in IGV as discussed above (i.e. either type – 'Genuine' or 'Artefact' in the 'Conclusion 1st checker' column (L) of the 'Variant-Calls' tab in the analysis worksheet.

12. TP53 variants called are checked against an internal database of previously detected variants. If the called variant has been seen previously column Q in the variant calls tab of the analysis worksheet will display the classification. Check if a classification is present for the variant(s) in the sample:
    a. Variants classed as 'mutation' have been investigated previously and are actionable for the trial. Nonsense or PTC variants and variants occurring in canonical splice sites are always actionable for the trial. Type 'Genuine – Report' in the conclusion box.
    b. Variants previously classed as 'benign' are not reported for any sample. Type 'Genuine – Benign' in the conclusion box

13. Variant investigation may be required to determine whether a variant is actionable and if it should be reported to the trial. TP53 variants determine patient stratification in the trial when a RAS variant is also detected. Variants classed as VUS or not previously classified which are detected in a sample that also has an actionable variant in KRAS or NRAS are investigated using the in-house Somatic Variant Analysis procedure (see SOP LP-GEN-SomVarAna). If more than one unclassified TP53 variant occurs in a sample with a KRAS or NRAS variant these variants should also be investigated.
    a. Samples with KRAS/NRAS variant: If no variant classification is available, or a classification of VUS is given for a TP53 variant detected variant investigation is required. Indicate this when you score the variant by typing 'Genuine – VUS required'.
    b. Samples without a KRAS/NRAS variant If no variant classification is available, or a classification of VUS is given for a TP53 variant detected indicate this by typing 'Genuine – not assessed'.

14. Copy cells 'A to L' of the rows containing all 'Genuine' calls and 'Known Polymorphisms' into the 'Mutations & SNPs' tab of the analysis worksheet.

    a. If no mutations/variants are detected in a patient (and therefore there are no mutations to be copied to the 'Mutations & SNPs' tab; List the genes for which analysis has been requested in column B (Gene) of the 'Mutations & SNPs' tab and type 'No variants' in the corresponding results cell in the 1st checker column (column L).

15. Finally determine the regions of interest that have not been covered to sufficient depth (i.e. the 'gaps' in coverage). Open the 'Hotspots.gaps' tab to see the identified gaps. These auto-populate cells in the final 'Report' tab of the analysis worksheet.

    a. if there are no gaps for that panel of genes, type 'No gaps' in the comments box on the 'Report' tab.

16. If there are gaps determine how the sample should be reported:

    i. Gap(s) in BRAF:

Mutations at c.1798 account for <1% of BRAF mutations
Mutations at c.1799 account for 94% of BRAF mutations
Mutations at c.1800 account for <1% of BRAF mutations
BRAF c.1799 must pass for BRAF to be called WT. If c.1799 does not have sufficient coverage (and no BRAF variants are identified) then BRAF gene testing has failed.

- Type 'c.1799 covered' or 'fail' into the comments box on the 'Report Tab'

ii. Gap(s) in KRAS:

Codon 12 accounts for 76% of KRAS mutations
Codon 13 accounts for 20% of KRAS mutations
Codon 61 accounts for 1% of KRAS mutations
Codon 117 accounts for 0.1% of KRAS mutations
Codon 146 accounts for 0.8% of KRAS mutations

When no KRAS mutation is identified, codon 12 must pass for the KRAS gene to be called WT, given the high % of mutations found within this codon. If codon 12 fails, then the gene should be classed as a fail. If one of the other codon/s fails, the gene can be reported based on the codon 12 results including a comment in the report (Macro and Shire) to say for example: SUBOPTIMAL: KRAS codon 61 failed.

- Type 'suboptimal' or 'fail' into the comments box on the 'Report Tab'

iii. Gap(s) in NRAS:

When no NRAS mutation is identified, NRAS codon 61 must pass for NRAS to be called WT, given the high % of mutations found within this codon. If codon 61 fails, then the gene should be classed as a fail. If one of the other codon/s fails, the gene can be reported based on the codon 61 results including a comment in the report (Macro and Shire) to say for example: SUBOPTIMAL: NRAS codon 117 failed.

- Type 'suboptimal' or 'fail' into the comments box on the 'Report Tab'

iv. Gap(s) in PIK3CA:

Exon 9 accounts for 47% of PIK3CA mutations
Exon 20 accounts for 25% of PIK3CA mutations

When no PIK3CA mutation is identified, exon 9 must pass for the PIK3CA gene to be called WT, given the high % of mutations found within this exon. If exon 9 fails, then the gene should be classed as a fail. If exon 20 fails, the gene can be reported based on the exon 9 results including a comment in the report (Macro and Shire) to say: SUBOPTIMAL: PIK3CA exon 20 failed.

- Type 'suboptimal' or 'fail' into the comments box on the 'Report Tab'

v. Gap(s) in TP53:

If a pathogenic variant is identified in TP53 then the overall coverage value of the TP53 gene does not need to be inspected. However, if TP53 is WT (no variants identified or only benign/polys found) then the gene can only be reported as WT if over 45% (rounded up to 50%) of TP53 has achieved 500x coverage; if <45% TP53 achieves coverage then TP53 should be reported as a fail.

- Type 'fail' into the comments box on the 'Report Tab' if appropriate

17. Save the analysis worksheet and move it to the '2nd Checking' folder created earlier. The sheet is now ready to be 2nd checked.

2nd checking of the NGS results

1. Open the saved analysis worksheet from the '2nd Checking' folder. Check that the correct patient demographics have been added from the FOCUS4 database.

2. Go to the 'Subpanel NTC check' tab of the analysis spreadsheet and check that the conclusions derived by the 1st checker regarding NTC checks are correct; i.e. the values in

column E, which highlight regions within the NTC that are covered at greater than 10% when compared to the patient's coverage data are not over 10%.

3. Go to the 'Variant Calls' tab of the analysis spreadsheet and add your initials and date to the box underneath '2nd checker name and date'.

4. Verify that the variants called are genuine calls using the Integrated Genomics Viewer (IGV)

5. Add your conclusion to what you've determined the variants to be – i.e. genuine/SNPs/artefacts – into the 'Mutations & SNPs' tab of the patient's analysis spreadsheet – see above. Check the classification of any TP53 variants and whether somatic variant investigation is required.

6. Check the gaps identified in the 'hotspots.gaps' tab and whether somatic variant interpretation is required for TP53 and add your initials and date to the comment added in the 'Report' tab.

7. Print out the summary sheet – to be scanned in with the patient's referral documentation and report

    - Note: The 2nd checker has to physically sign the printed summary sheet.

8. If somatic variant analysis is required these procedures should be activated by the 2nd/checker or FOCUS4 GT at the time the analysis summary sheet is printed out.

9. Once analysis is complete, the relevant worksheet/s & copy of the request form updated with the results, should be passed to the Section Lead/Authorising Scientist so that the results can be checked.

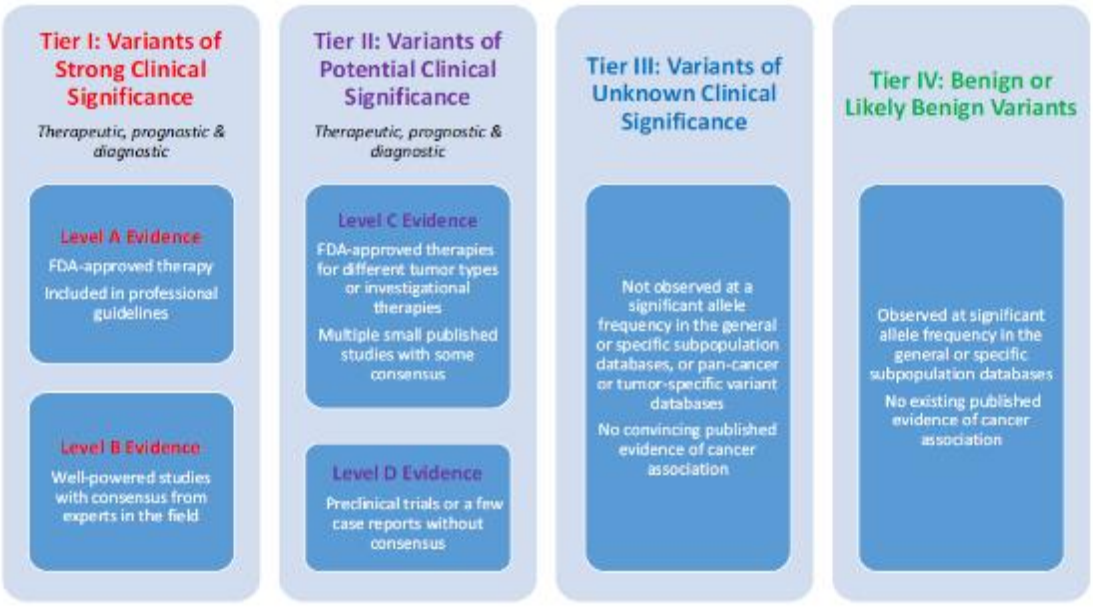
10. The worksheets will be put in to be scanned and the authorised request form will be passed on to the FOCUS 4 GT so, once all results are gathered, the lab FOCUS4 excel database should be updated Shire reports and clinical report can be written.

SOMATIC VARIANT ANALYSIS

Introduction

Due to the implementation of Next Generation Sequencing panels for somatic cancer analysis across both solid tumours and haematological cancers the laboratory must have effective guidance on variant analysis. In 2018 the laboratory adopted the American College of Medical Genetics and Genomics (ACMG) guidance for the investigation of germline sequencing variants. The introduction of these guidelines for germline disorders has highlighted the needed to adopt guidance to standardise the interpretation of somatic sequencing variants. Somatic variants can be used to guide treatment and provide prognostic, diagnostic information or inclusion for a clinical trial. In 2015 the Association of Molecular Pathology (AMP) in American established a working group to produce recommendations for the interpretation and reporting of sequencing variants in cancer.

The AMP guidelines focus on establishing if a sequencing variant can be used as a biomarker i.e. can it be used to impact on the patient's clinical care, rather than the pathogenicity of a variant. A variant can be considered a biomarker and therefore actionable if it predicts treatment response to a specific therapy, affects the patient's prognosis or diagnosis or indicates that the patient requires surveillance for the early detection of cancer. The AMP recommends that currently available evidence should be used to classify the variant into one of four classes. Tier I, variants of strong clinical significance, tier II, variants of potential clinical significance, tier III, variants of unknown significance and tier IV, benign or likely benign. Tier I & tier II are considered clinically actionable therefore the evidence for these tiers is split into four levels to aid classification. The levels can be seen in the figure below.



This document details the procedure for the investigation of sequencing variants in cancer and incorporates the guidelines outlined by the AMP.

Definitions

Tier I: variants of strong clinical significance – made up of variants with level A & B evidence.

Tier II: Variants of potential clinical significance – made up of variants with level C & D evidence.

Tier III: Variants of unknown significance.

Tier IV: Benign or likely benign.

Biomarker – a variant that has been found to be actionable i.e. has been shown to be tier I or II.

Fill the form in completing the tabs in order. The somatic variant interpretation form can be found here S:\MedGen\SHARED\Lab Genetics\Laboratory Genetics Group Meetings\Cancer & Const Mtgs upto 2018\Cancer Section Meeting Minutes\somatic variant interpretation\Training.

Getting started

1. In the summary tab please fill out the case ID (i.e. lab number), reason the gene is being investigated and the location that the form is being saved to.
2. Copy and paste the variant details from the 'Mutations & SNPS' tab of the analysis spreadsheet into A31 of the 'summary' tab of the somatic VUS form. The variant details will auto populate into the summary table.
3. Once the variant details have been completed, copy and paste the Alamut variant summary report (section 4.2 – in appendix) and a screen shot of the splice site tool with the differences highlighted (section 4.3 – in appendix) into the 'paste here summary' tab.

Step 1

1. This step is to check if the variant requires a full investigation.
2. If the variant has been seen in the lab before and classed as actionable (tier 1 or 2) in the **same** cancer type being investigated or has been classed as benign (tier IV) then it can reported accordingly. Previous instances of the variant can be seen in the occurrences tab of the variant summary in Alamut.
3. If the variant hasn't been seen previously or was classed as a VUS (tier III) then continue.
4. If the variant is present in gnomAD then it should have auto populated from the Alamut summary. If the variant is present in the population at or above 1% then the variant can

be considered benign and therefore non-actionable. For variants that lie between 0.5% and 1% pay close attention to the individual populations in gnomAD as it could be that the exceed the 1% threshold in a certain population or that homozygotes have been reported as healthy in some populations (which would increase the likelihood that it is benign).

5. If variant has been classified as benign stop and report if not continue.
6. Check the in house list of guidelines to ensure that the variant is not on them if it is then report according to the guidance, if not then continue to complete to investigation.

Databases

7. Please note that **all data recorded at in this tab** should be **limited to the specific variant being investigated**.
8. If the variant is found in any of the databases listed please record the details and ensure that the link is recorded and/or the paper is saved under the relevant references folder of the gene being investigated; especially for papers where journal access is not freely available. If the variant is present in ClinVar it should auto populate from the Alamut summary however, ClinVar should always be check either by linking from Alamut or checking directly. The details of any ClinVar classifications should then be recorded.
9. Databases searches should not be limited to those listed in the form. If any other databases (e.g. Jackson Laboratory or IARC) including disease specific databases are used the details should be recorded below the databases listed. Both the databases listed and

further databases can be found through the Alamut google search, which can be accessed by clicking the google button in the Alamut variant annotation window.

10. The databases with also provide links to literature that might be useful in the next step.
11. Once the details of all databases used and the links have been recorded proceed to the literature search.
12. To check each database separately follow the directions below.
    - **MyCancerGenome** can be found here https://www.mycancergenome.org/ if the variant isn't in the drop down list google search the variant followed by MyCancerGenome.
    - **ClinVar** can be found here https://preview.ncbi.nlm.nih.gov/clinvar/ enter the variant into the search box.
    - The Catalogue of Somatic Mutation In Cancer (**COSMIC**) can be found here https://cancer.sanger.ac.uk/cosmic
    - HGMD can be found here http://www.hgmd.cf.ac.uk/ac/index.php
    - HGMD professional can be found here https://portal.biobase-international.com/cgi-bin/portal/login.cgi more information on HGMD can be found in appendix 4.4.1.

Literature search

1. The literature search should focus on the **specific variant** (nucleotide change) being investigated. If information not specific to the variant is found it should be recorded in the final tab; you may decide to come back to it if required following the literature search. Please copy and paste the Alamut google search terms into box provided. The literature search should include any references from databases. The following details should be recorded on any literature found:
    - The reference
    - The details on the variant described by the paper.
        - How they have classed the variant.
        - How big the sample size is.
    - Any comments on the paper.
        - Any reason why the paper is not useful for classification
    - The type of evidence.
    - A link to the paper
2. Once the relevant literature has been recorded and classified assess the evidence to establish if it possible to classify the variant. Variants should be classified according the level of evidence, the levels of evidence are seen below;

Tier 1A – Present in guidelines

Tier 1B – Large studies with outcome data

Tier 2C – Multiple small studies with consensus

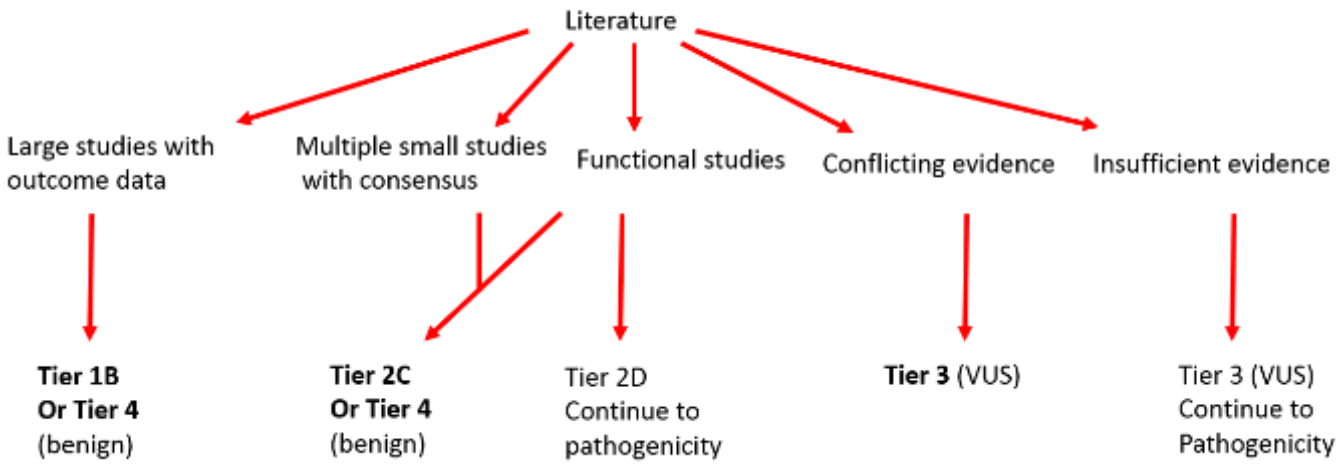
Tier 2D – Preclinical evidence

Tier 3 – Conflicting evidence - VUS

Tier 4 – Evidence suggests benign

3. If during the literature search the variant is found to be in guidelines, the literature search can be stopped and the variant reported as recommended by the guidance. If the guidance recommends that the variant is actionable it can be classed as a **tier 1A**. The guidance should also be added to the in house database so that it can be easily found in the future.
4. The following relies on the databases and literature showing a consensus on the actionability of a variant. If the databases and literature is conflicting make a note of any piece of evidence that can be discarded and the reason it is not considered reliable/useful. If you do not think that any of the evidence can be discarded please continue the investigation of the variant.

Tier IB	**Tier 2C**	**Tier 2D**	**Tier 3 (VUS)**	**Tier 4**
Large studies with outcome data	**Mutiple** (3-4) **small studies** with	Mutiple **Functional**	Literature is **conflicting** on	**Large studies** class variant as

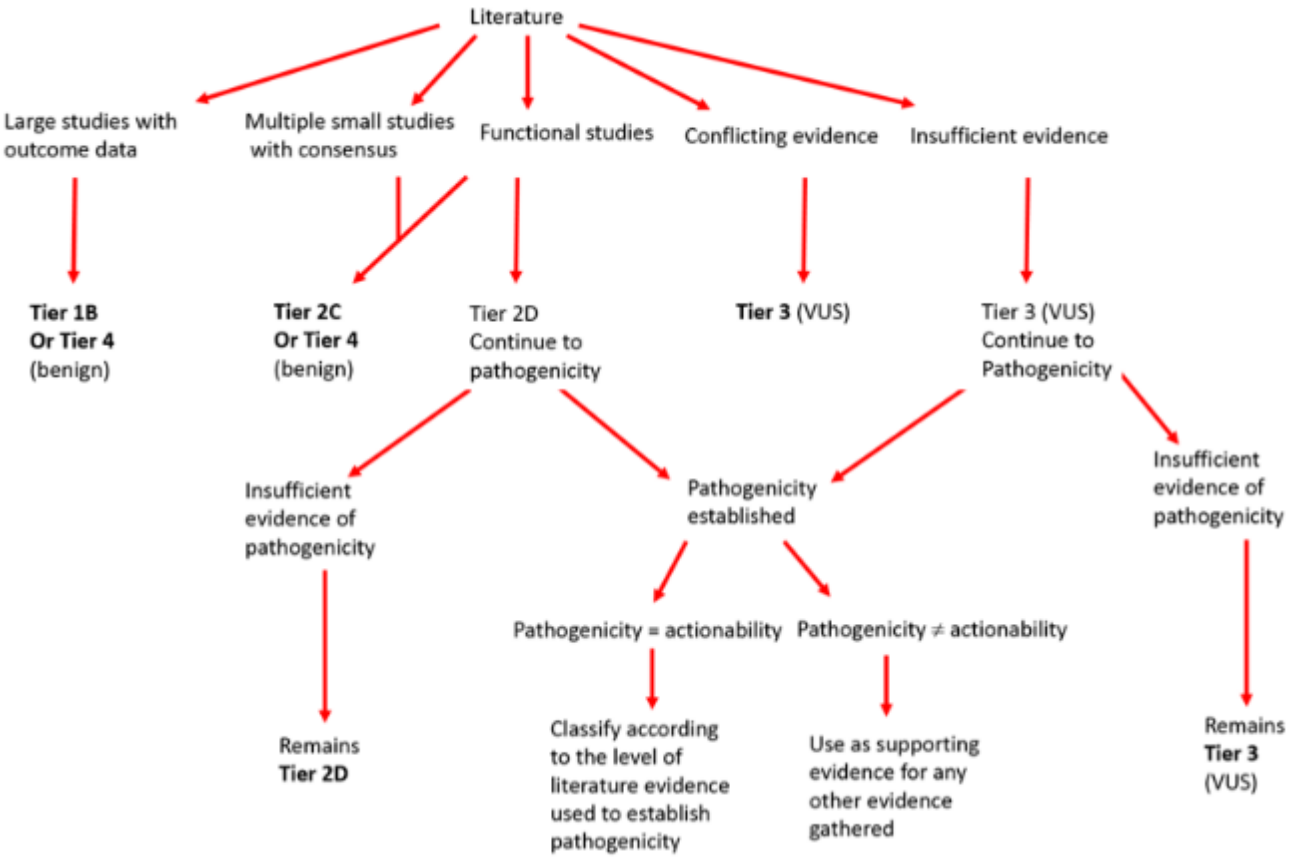


5. If the literature evidence is insufficient or only allows the variant to be classified as tier 2D please continue the investigation by moving onto the pathogenicity analysis.

Pathogenicity Analysis

1. This step is only required if the specific variant cannot be classified from the literature, if the variant already meets the criteria for classification do not complete this step.
2. The pathogenicity analysis is used to establish if there is evidence that the variant is having an effect on the protein. This can be used as supporting evidence that a variant is actionable. The level at which this evidence can be used is dependent on the gene being investigated. If pathogenicity = actionable then it might be possible to classify the variant on this evidence alone.
3. This is the only place where evidence not relating to the specific variant should be recorded. You may decide to record information found during the literature search on this tab and come back to it if required. Any literature used for this analysis next to the questions in the same way as the literature step.
4. Answer **all** the Yes/No questions in the pathogenicity analysis tab and record the associated evidence. The literature evidence for pathogenicity should be applied at the same levels as the literature search above. Therefore, the questions should only be answered 'Yes' if the literature associated with the question can be classed as tier 2 or above. The questions are as follows.
    i. Shown to be actionable in a different cancer type? Need to ensure the level of evidence found would make this variant reportable on its own.
    ii. Has the same amino acid change been established as an actionable variant due to a different nucleotide change? Need to ensure the level of evidence found would make this variant reportable on its own.
    iii. Missense change at an amino acid where a different missense change has previously been established as actionable.
    iv. Variant located in a mutational hotspot or functional domain?
    v. Mutation type consistent with the disease mechanism? Be aware that loss of function variants at the 3' end of the protein may not be pathogenic. Check previously reported variants downstream.
    vi. Is there an effect on splice sites? (Specify number of tools) See screen shot in the 'paste here' tab
    vii. Multiple lines of computational evidence support a deleterious effect on the gene or gene product. See Alamut data, PolyPhen will need to be access via Alamut

5. Pathogenicity evidence can be used for the variant if sufficient evidence to answer 'Yes' to one of the following combinations of questions.
   - 1
   - 2
   - 3 + 7 + 5
   - 4+5+7
   - 4 + 6
   - 5+ 6
6. If the above criteria has been met then pathogenicity has been demonstrated.
7. If pathogenicity equals actionability then classification is based on gene in question and evidence used to derive pathogenicity. Genes with known germline associations e.g. TP53.
8. If pathogenicity does not equal actionability then pathogenicity can be considered (as one piece of evidence) in conjunction with other evidence gathered prior to this to classify the variant and knowledge of the gene and disease being investigated.



Summary

1. Once analysis of the variant has been completed return to the summary tab and write a short (1 or 2 sentences) explanation of the classification given. Use the drop boxes to fill out the AMP Classification, Evidence used and Actionability.
2. Initial and date in the 'form completed by:' box, next to this please record the time spent investigating this variant.
3. Once the form has been completed the variant should be recorded in Alamut in occurrences. This is found by clicking on the occurrence tab at the top of the summary. Then select new occurrence and enter the case details:
   - The case ID – to allow the variant interpretation form to be located.
   - The cancer type the variant has been seen in.
   - Under comment the classification and the short explanation of the classification.

Second Checking

1. All forms should be second checked by another competent scientist/technologist. The second checker should work through the form in order; checking each piece of evidence recorded. The checker should fill in each of the grey boxes from the drop down box. If the checker has any comments or anything to add it should be recorded in the comments box. Only once all the form has been checked will the second checker be able to initial and date the form. The checker should indicate the time spent checking the form in cell D16.
2. If the first and second checker disagree about the actionability of the variant a third checker should check the form

If for any reason, either laboratory is unable to carry out NGS in their own facility, DNA samples must be sent to the alternative laboratory for testing. DNA will be extracted from the samples in one lab, then posted (Royal Mail recorded delivery) to the other laboratory for sequencing. The laboratory carrying out the DNA extraction will take responsibility for ensuring that the correct area of tissue has been macrodissected. An MTA form must accompany each batch of samples (see below) and the MRC CTU at UCL must be alerted every time a batch of DNAs are transferred.

The NGS results must be reported by the laboratory carrying out the sequencing and likewise, the IHC results must also be reported by the laboratory carrying out the assays.

INTER-LABORATORY AND EXTERNAL QUALITY ASSURANCE

Prior to the trial commencement all newly validated assays (i.e. PTEN and MMR IHC and KRAS/NRAS/BRAF/PI3KCA Pyrosequencing) underwent a sample swap of a total of 97 samples to ensure standardisation of techniques and results in the two laboratories. The full set of samples were be analysed for all assays as per the trial protocol, and published in J Clin Path. (see below)

Original article

OPEN ACCESS

Pre-trial inter-laboratory analytical validation of the FOCUS4 personalised therapy trial

Susan D Richman,[1] Richard Adams,[2] Phil Quirke,[1] Rachel Butler,[3] Gemma Hemmings,[1] Phil Chambers,[1] Helen Roberts,[3] Michelle D James,[4] Sue Wozniak,[4] Riya Bathia,[5] Cheryl Pugh,[5] Timothy Maughan,[6] Bharat Jasani,[7] on behalf of FOCUS4 Trial Management Group

▸ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2015-203097)

For numbered affiliations see end of article.

Correspondence to
Dr Richard Adams, Institute of Cancer & Genetics, Cardiff University School of Medicine, Velindre Hospital, Cardiff CF14 2TL, UK;
Richard.Adams@wales.nhs.uk

SDR and RA are joint first authors.

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ABSTRACT

Introduction Molecular characterisation of tumours is increasing personalisation of cancer therapy, tailored to an individual and their cancer. FOCUS4 is a molecularly stratified clinical trial for patients with advanced colorectal cancer. During an initial 16-week period of standard first-line chemotherapy, tumour tissue will undergo several molecular assays, with the results used for cohort allocation, then randomisation. Laboratories in Leeds and Cardiff will perform the molecular testing. The results of a rigorous pre-trial inter-laboratory analytical validation are presented and discussed.

Methods Wales Cancer Bank supplied FFPE tumour blocks from 97 mCRC patients with consent for use in further research. Both laboratories processed each sample according to an agreed definitive FOCUS4 laboratory protocol, reporting results directly to the MRC Trial Management Group for independent cross-referencing.

Results Pyrosequencing analysis of mutation status at *KRAS* codons12/13/61/146, *NRAS* codons12/13/61, *BRAF* codon600 and *PIK3CA* codons542/545/546/1047, generated highly concordant results. Two samples gave discrepant results; in one a *PIK3CA* mutation was detected only in Leeds, and in the other, a *PIK3CA* mutation was only detected in Cardiff. pTEN and mismatch repair (MMR) protein expression was assessed by immunohistochemistry (IHC) resulting in 6/97 discordant results for pTEN and 5/388 for MMR, resolved upon joint review. Tumour heterogeneity was likely responsible for pyrosequencing discrepancies. The presence of signet-ring cells, necrosis, mucin, edge-effects and over-counterstaining influenced IHC discrepancies.

Conclusions Pre-trial assay analytical validation is essential to ensure appropriate selection of patients for targeted therapies. This is feasible for both mutation testing and immunohistochemical assays and must be built into the workup of such trials.

Trial registration number ISRCTN90061564.

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INTRODUCTION

Molecular characterisation of tumours is leading to increasing personalisation of cancer therapy tailored to an individual and the target cancer. FOCUS4 (figure 1) marks a significant advancement in terms of clinical trial design.[1] It aims to stratify patients to novel targeted agents by a prospective, progressive molecular stratification process. Following patient registration, archival formalin-fixed, paraffin-embedded (FFPE) tumour samples from patients with advanced or metastatic colorectal cancer (CRC) will be sent from collaborating centres to undergo mutation and immunohistochemical testing at one of the two centralised testing laboratories in Leeds and Cardiff, to allow the allocation of patients into molecular cohorts, within which there is a specific randomised comparison.

The current panel of molecular markers selected for the trial is based on biomarkers which have been identified or are hypothesised as having the capacity to predict responses to specific targeted therapies. The trial is designed to allow the panel to progressively change during the life span of the trial, with identification of novel biomarkers and new treatment approaches being incorporated for differing biomarker-defined cohorts.[1]

Mutational analysis of *KRAS* and *NRAS* is already used routinely to determine suitability to receive anti-epidermal growth factor receptor (EGFR) therapy.[2–5] Mutational status of *BRAF* is becoming widely recognised as a prognostic marker, with the presence of an activating mutation in stage IV being associated with very poor prognosis.[2 6–8] Mutational activation of *PIK3CA*[9] or loss of pTEN protein expression[10 11] has been implicated in driving signalling through the AKT pathway, which is a feature in up to 30% of CRC. While not being used initially to randomise patients, mismatch repair (MMR) status is being determined in order to further stratify patients in the post-randomisation phase.

The aim of this study was to carry out a pre-trial analytical validation between the two designated biomarker testing laboratories in Leeds and Cardiff, in order to identify the inter-observer laboratory agreement on 97 samples of stage IV CRC and to ensure that laboratory testing was accurate and fit for purpose in both laboratories.

MATERIALS AND METHODS

Samples

Ninety-seven FFPE tumour resection blocks, from patients previously entered into either the FOCUS3 trial[12] or consented outside clinical trial to the Wales Cancer Bank (WCB), and stored with prior consent for further use in research, were retrieved from the WCB. The matched diagnostic biopsy blocks were also retrieved in 14 cases, to reflect the fact that 20–40% of patients in FOCUS4 will only have biopsy samples available for analysis. From the outset, these biopsies were only intended to be

A continual programme of EQA will be undertaken through sample swaps between the two centres (where appropriate). UKNEQAS currently provides regular schemes for KRAS, NRAS, BRAF, PI3KCA for which both labs participate. Participation scores for these schemes will be returned to the MRC CTU at UCL. The Leeds laboratory also participates in the UKNEQAS IHC & ISH module- Alimentary Tract Pathology (Lynch Syndrome/HNPCC) scheme.

Note: when both Leeds and Cardiff laboratories performed both IHC and genetic profiling, two sample swaps each year were performed, consisting of 10 samples each year; to include wild-type and mutant samples. The results for both labs should be returned to the MRC CTU at UCL within the timeline defined by the CTU, however a minimum period of 4 weeks is required from receipt of the DNA. Any discrepant results will be highlighted by MRC CTU and followed up by both laboratories.

For more details and the dates of sample swaps, please refer to the following document (Ongoing Pyrosequencing and Next Generation sequencing QA) stored at MRC CTU at UCL (S:\Focus4\16. Laboratory\QA). This document will continue to be updated after each sample swap.

REPORTING TO MRC CTU AT UCL AND SITES

Confirming receipt of sample with MRC CTU at UCL

Once per week, the MRCCTU will send each labortatory an 'outstanding results' spreadsheet, which must be filled in and returned to the FOCUS4 team (mrcctu.focus4@ucl.ac.uk) within 3 working days
This spreadsheet will show all registered patients, and the status of their sample within the sample processing pipeline. Any sample issues must be highlighted to the MRCCTU.

Reporting of biomarker panel results

Each report will include the results of:

- KRAS, NRAS, BRAF, PI3KCA and TP53 analyses.

Mutations are reported using HGVS nomenclature; the amino acid change should also be included.
e.g. KRAS c.35G>A [p.Gly12Asp]. Furthermore, the variant allele frequency (VAF) should be reported where appropriate.

OR **Wild-type or Fail or could not be tested.**

- PTEN, and MMR IHC.

Reported as Present/No Loss or Absent/Loss or Inconclusive or could not be tested.

- Any failed sampled samples (for any assays) will be reported as **FAIL.**
- Signature of designated authorising scientists and date reported.

Failed results
The genetic mutation analyses and PTEN IHC are fundamentally driving the different arms with the MMR IHC results adding some subtlety and further refinement.

If the mutations or DNA extraction fails:

1. If DNA extraction fails due to inadequate tumour, inadequate fixation or some other technical issue – the biomarker test has **FAILED** irrespective of any results from IHC.

Some samples will yield DNA but this will not be amplifiable – these should also be treated as a **FAIL.**

2. If DNA is amplifiable then it is most likely that all or most mutations will be successfully tested. If this is the case we can allocate patients simply on the mutation calls:
    a. TP53 and RAS mutant – into FOCUS4-C or FOCUS4-N
    b. Any other mutation combination- into FOCUS4-N (following the closure of FOCUS4-B and FOCUS4-D)

3. Some samples will give partial results (i.e. <100% of the region of interest of a gene will achieve the desirable NGS coverage. Refer to 'NGS section' section for details of reporting fail and suboptimal NGS results.

IHC Fails:
Allocations can happen irrespective of the results of the IHC results. Therefore failed IHC samples are not considered as fails even if the results are impossible to interpret.

Biomarker panel results data will be entered directly into the FOCUS4 Registration MACRO Database by staff at both Leeds and Cardiff. All results should be appropriately authorised prior to entering onto the database. Electronic sign off required on the database is to confirm that the data entered is accurate and has the appropriate authorisation.

As a backup, the paper Biomarker Panel Results CRF (Appendix 1) can be completed on paper and faxed to **0207 670 4653** or scanned and emailed to mrcctu.focus4@ucl.ac.uk

Return of blocks to sites/sending blocks to Wales Cancer Bank (WCB)

At the end of the recruitment phase of FOCUS4, samples will be catalogued and stored for future bowel cancer research.

Blocks for which no additional consent has been received for future research will be returned to the originating centre by the respective Leeds or Cardiff laboratory. The date of sending and confirmation of receipt should be noted and the MRC CTU informed.

MRC CTU will be the first port of call for all centres wishing for the return of tumour blocks, it is to be emphasised that this should only occur under exceptional circumstances (over the first 15 years) or in the case of clinical need. All samples in which additional consent has been given for future bowel cancer research will have been received in good faith to allow such research to take place, in a time appropriate fashion. If the criteria for return are met the MRC CTU will contact WCB who will undertake the direct return of the given sample(s)

APPENDIX 1: BIOMARKER PANEL REQUEST CRFs

Cardiff

MRC Clinical Trials Unit

FOCUS4

Form 2a - Biomarker Panel Request Form
Version 4.0 16 May 2019

FOCUS4

Patient's Initials: ☐☐☐☐ Date of Birth: d d m m y y y y

Hospital:..... Trial Number: ☐☐☐☐

SECTION 1.To be completed by the RESEARCH NURSE

Please complete this section and fax a copy of this form together with a copy of the Registration Consent Form (CF1) to the Histopathology Department and the MRC CTU. The results will be sent to the nurse whose details are given below.

Pathology Hospital: ..... **Pathology Number if known:** .....

Date of Resection/Biopsy: d d m m y y y y

Date tumour sample block requested: d d m m y y y y

Has the patient consented for future bowel cancer research? (Item 9, CF1) *This question is mandatory* Y ☐ N ☐

Name (print): ..... Signature:.....

Date form completed: .....

Email: ..... Telephone: .....

SECTION 2. To be completed by the HISTOPATHOLOGY DEPARTMENT

⇒ Please identify the FFPE block with the maximum quantity of viable tumour ***(no megablocks or slides)***
⇒ Please complete this section and send a copy by first class post together with the anonymised tumour sample and pathology report *(using trial number and date of birth only, do not send the attached Consent Form)* to:

FOCUS4, Department of Cellular Pathology, University Hospital of Wales
Heath Park, Cardiff, CF14 4XW

⇒ Please also fax both this form and the anonymised pathology report to the MRC CTU on 0207 670 4653 to confirm that the sample has been sent

Pathology No *(must be completed)* **:** .....

Sample Type: *(Please tick one)*
☐ Resection
☐ Biopsy

Fixation: *(Please tick one)*
☐ Neutral Buffered formalin
☐ Formol saline
☐ Acidified formalin
☐ Bouin's fixative
☐ Other, please specify.....

Site: *(Please tick one)*
☐ Primary cancer—Right Colon (Appendix, Caecum, Hepatic flexure, Ascending & Transverse colon)
☐ Primary cancer—Left Colon (Splenic flexture, Descending & Sigmoid colon)
☐ Primary cancer—Rectum (Rectosigmoid, Rectum)
☐ Primary cancer—Small Bowel
☐ Metastases—Lung
☐ Metastases—Liver
☐ Metastases—Peritoneum
☐ Metastases—Lymph node
☐ Metastases—Other, please specify.....
☐ Local Recurrence— Colonic
☐ Local Recurrence— Rectal

Date tumour sample block sent to Cardiff Laboratory:
d d m m y y y y

Name (print): .....

Signature:..... Date form completed:.....

Email: ..... Telephone: .....

For MRC CTU use only:
Date Part 1 entered onto database : dd - mm - yyyy Initials of data enterer: ☐☐
Date Part 2 entered onto database : dd - mm - yyyy Initials of data enterer: ☐☐

Leeds

MRC Clinical Trials Unit

FOCUS4

Form 2a - Biomarker Panel Request Form

Version 4.0 16 May 2019

FOCUS4

Patient's Initials: ☐☐☐☐ Date of Birth: d d m m y y y y

Hospital:..... Trial Number: ☐☐☐☐

SECTION 1.To be completed by the RESEARCH NURSE

Please complete this section and fax a copy of this form together with a copy of the Registration Consent Form (CF1) to the Histopathology Department and the MRC CTU. The results will be sent to the nurse whose details are given below.

Pathology Hospital: ..... **Pathology Number if known:** .....

Date of Resection/Biopsy: d d m m y y y y

Date tumour sample block requested: d d m m y y y y

Has the patient consented for future bowel cancer research? (Item 9, CF1) *This question is mandatory* Y ☐ N ☐

Name (print): ..... Signature:.....

Date form completed: .....

Email: ..... Telephone: .....

SECTION 2. To be completed by the HISTOPATHOLOGY DEPARTMENT

⇒ Please identify the FFPE block with the maximum quantity of viable tumour ***(no megablocks or slides)***

⇒ Please complete this section and send a copy by first class post together with the anonymised tumour sample and pathology report *(using trial number and date of birth only, do not send the attached Consent Form)* to:

Dr Susan Richman, FOCUS4 Trial Laboratory, Level 4, Wellcome Trust Brenner Building, St James University Hospital, Leeds, LS9 7TF

⇒ Please also fax both this form and the anonymised pathology report to the MRC CTU on 0207 670 4653 to confirm that the sample has been sent

Pathology No *(must be completed)* **:** .....

Sample Type: *(Please tick one)*
- ☐ Resection
- ☐ Biopsy

Fixation: *(Please tick one)*
- ☐ Neutral Buffered formalin
- ☐ Formol saline
- ☐ Acidified formalin
- ☐ Bouin's fixative
- ☐ Other, please specify.....

Site: *(Please tick one)*
- ☐ Primary cancer—Right Colon (Appendix, Caecum, Hepatic flexure, Ascending & Transverse colon)
- ☐ Primary cancer—Left Colon (Splenic flexture, Descending & Sigmoid colon)
- ☐ Primary cancer—Rectum (Rectosigmoid, Rectum)
- ☐ Primary cancer—Small Bowel
- ☐ Metastases—Lung
- ☐ Metastases—Liver
- ☐ Metastases—Peritoneum
- ☐ Metastases—Lymph node
- ☐ Metastases—Other, please specify.....
- ☐ Local Recurrence— Colonic
- ☐ Local Recurrence— Rectal

Date tumour sample block sent to Leeds Laboratory:

d d m m y y y y

Name (print): .....

Signature:..... Date form completed:.....

Email: ..... Telephone: .....

For MRC CTU use only:

Date Part 1 entered onto database : dd - mm - yyyy Initials of data enterer: ☐☐

Date Part 2 entered onto database : dd - mm - yyyy Initials of data enterer: ☐☐

APPENDIX 2: AMENDMENTS

Version	Date	Revised by	Change	Reason for change
2.2	30-Apr-2020	E. Yates	MRC CTU FOCUS4 staff details updated	Staff changes since v2.0 of lab manual
2.3	08-July-2020	S. Richman	Additional information added to 'Section assessment:Leeds'	All samples being processed initially in Leeds. Details added to demonstrate this
			Removal of details about sectioning for RNA extraction	RNA extraction no longer done
			Removal of H3K36me3 IHC protocol	No longer carried out
			Removal of DNA extraction protocol for Leeds lab	No longer carried out
			Removal of NGS protocol for Leeds Lab	No longer carried out
			Removal of 'PIK3CA cohort and 'KRAS, NRAS-mut cohort'	These randomisations do not exist
			Removal of EREG assay	No longer carried out
			Removal of EREG, AREG, ERBB3 Q-PCR analysis	Assay not carried out, therefore analysis not required
2.4	01-Sep-2020	R Dodds	Cardiff lab contact details updated	Staff changes since v2.0 of lab manual
2.5	25-Sep-2020	R Dodds	Cardiff lab contact details updated	Staff changes since v2.0 of lab manual
2.6	20-Nov-20	E. Yates and S. Richman	Current version of the Biomarker Panel Request Forms added to Appendix 1	Change to CRF version