## Molecular Pathology

### **Histomolecular Mutational Analysis**

The laboratory provides a solid tumour mutation testing service using next generation sequencing (NGS).

The AmpliSeq for Illumina Focus NGS Panel is a targeted resequencing assay for biomarker analysis of 52 genes with known relevance to solid tumours (Table 1). The Focus Panel can simultaneously analyse both DNA and RNA extracted from the same specimen. The Focus Panel is part of a workflow that includes AmpliSeq for Illumina PCR-based library preparation, Illumina sequencing by synthesis (SBS) next-generation sequencing technology and automated analysis.

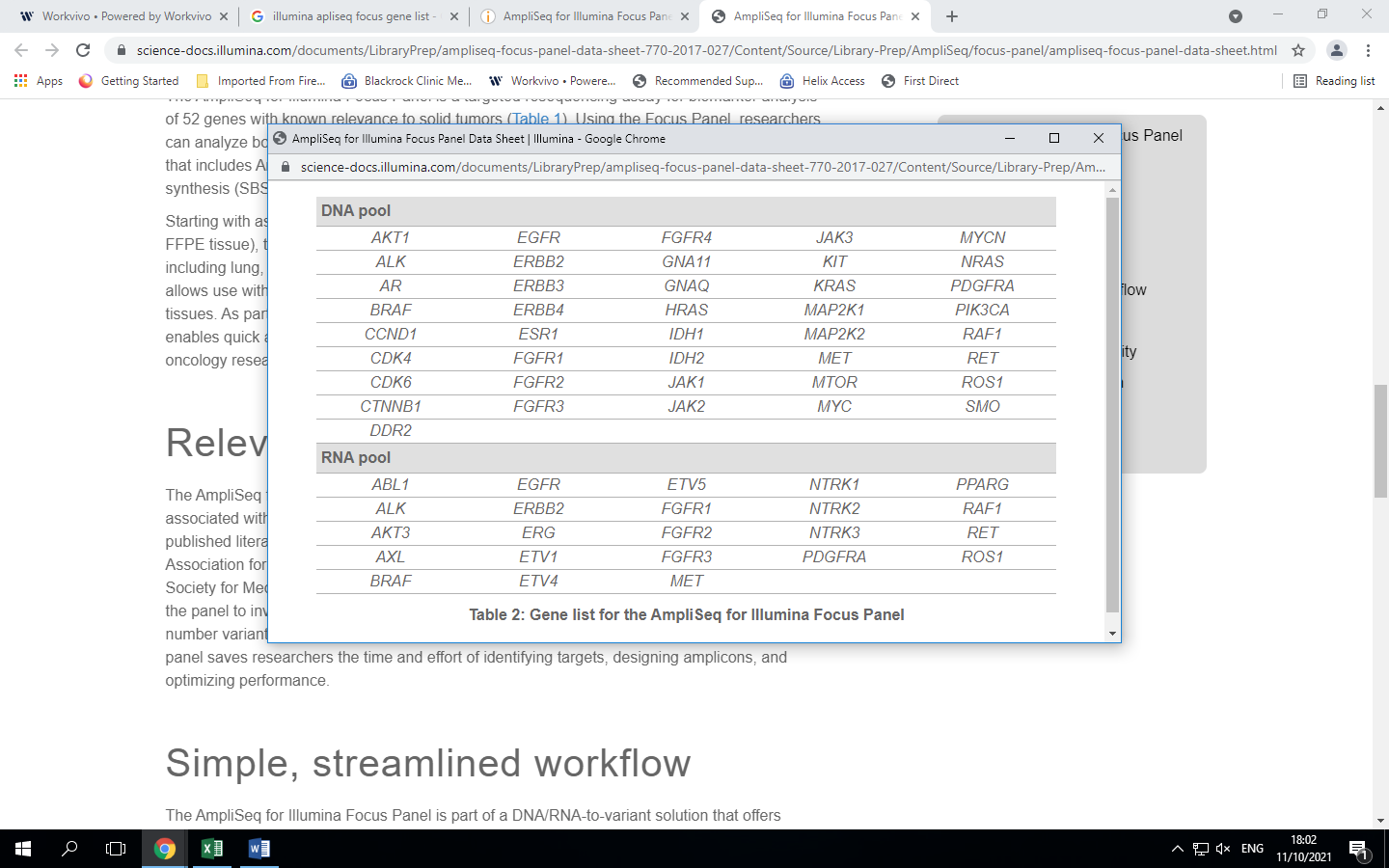
Starting with 10 ng of DNA and RNA, the panel enables the analysis of genes associated with multiple cancer types, including lung, colon, breast, and melanoma. The low-input requirement allows use with various sample types, including formalin-fixed, paraffin-embedded (FFPE) tissues. As part of the AmpliSeq for Illumina targeted resequencing solution, the Focus Panel enables quick and accurate assessment of genomic variation. The reference genome against which NGS is assessed is Ch37. A similar approach is used for BRCA1&2 germline and somatic analysis. Currently germline only analysis is performed for HER2 negative locally advanced or metastatic breast cancer patients.

In conjunction with germline analysis, FFPE material is required for somatic analysis for prostate cancer patients. Homologous Recombination deficiency (HRD) testing is now performed on patients who have ovarian, fallopian tube or primary peritoneal cancer. FFPE tissue is required for the test while it is advised that a blood sample is also sent for MLPA testing. Sequencing of germline and somatic testing for these tumour types is performed in accordance with National testing guidelines.

#### Relevant gene content

The AmpliSeq for Illumina Focus Panel targets hundreds of mutations across 52 key genes associated with solid tumours (Table 2). Gene content for this panel was selected based on published literature, current guidelines (National Comprehensive Cancer Network [NCCN], Association for Molecular Pathology [AMP], College of American Pathologists [CAP], European Society for Medical Oncology [ESMO], etc.), and relevant clinical trials. A positive control sample is included on each run and the expected variant allele frequency (VAF) is compared to what is detected per run. For each sample the limit of detection is the following: VAF is set to detect a 2% variant allelic frequency (VAF) with at least 1000 reads.

Like most similar panels, this is a hotspot panel and does not cover all exons for all genes. Please contact the laboratory if you have a specific variant to analyse.



#### Colorectal Cancer (CRC) Mutation Panel:

#### KRAS & NRAS

*KRAS* & *NRAS* mutation status are critical when evaluating patients with a view to placing them on *EGFR*-targeted monoclonal antibody therapy. The presence of an activating *KRAS* or *NRAS* mutation is generally associated with a lack of response to anti-*EGFR* therapy.

#### BRAF

Mutations in position p.V600 in *BRAF* have been associated with poor prognosis, especially in patients with metastatic disease. Currently there is insufficient evidence to recommend *BRAF* V600 mutational status as a predictive molecular biomarker for response to anti-*EGFR* therapy but this is a rapidly evolving field.

#### **Microsatellite instability (MSI)/Mismatch repair deficiency (dMMR)**

MSI/dMMR CRC have been shown to have increased sensitivity to immune-oncological (IO) agents such as PD-L1 inhibitors. In addition, while the majority of these tumours are sporadic, MSI/dMMR tumours are more likely to be associated with Lynch syndrome than MSS/MMR intact tumours. MSI and/or immunohistochemistry (IHC) testing is performed on tumour tissue samples to predict likely response to IO agents. In addition, the results of this testing allow for risk stratification in relation to Lynch syndrome (in certain circumstances this will be the primary indication for this testing).

MSI testing is performed initially and, if required, samples will be reflexed for MMR IHC. IHC uses a panel of 4 mismatch protein IHC markers (MLH1, PMS2, MSH2 & MSH6). MSI testing is performed using a multiplex PCR approach for thirteen different microsatellite loci followed by DNA fragment analysis using the SeqStudio™ Genetic Analyser. PCR is carried out using the Applied Biosystems TrueMark™ MSI Assay which can identify microsatellite instability in FFPE samples from multiple tumour tissue types.

#### Lung Cancer Mutation Panel:

#### EGFR

*EGFR* mutation status is critical when evaluating patients with a view to placing them on anti-*EGFR* Tyrosine Kinase Inhibitors (TKIs). The presence of a sensitising mutation is associated with a favourable response to treatment with *EGFR* TKIs. The presence of resistance mutations need to be interpreted in the context of any previous treatment regimes.

#### ERBB2 (HER2) and KRAS G12C

Clinical trials are currently enrolling patients with EBRB2 exon 20 insertions and KRAS variants. The results of these trials will determine whether these treatments become part of standard care for patients with these mutations.

#### BRAF

*BRAF* mutation status is critical when evaluating patients with a view to placing them on *BRAF* targeted therapies. The presence of a mutation in codon 600 of *BRAF* is required for treatment with *BRAF* targeted therapies.

#### **ALK**

ALK translocation has been associated with response to anti-ALK targeted therapies such as crizotinib. ALK translocations can be assessed by a number of different methodologies. Any of immunohistochemistry, in-situ hybridisation or next-generation sequencing is acceptable methodologies for assessing the presence of translocations.

#### **ROS1**

ROS1 translocation has been associated with response to targeted therapies, including crizotinib. ROS1 translocations can be assessed by a number of different methodologies. Any of in-situ hybridisation or next-generation sequencing is acceptable methodologies for assessing the presence of translocations. While antibodies exist for ROS1 immunohistochemistry it is not currently an accepted method for assessing ROS1 translocations.

Note: *ALK* and ROS1 FISH testing is routinely performed on all lung cases

#### Melanoma Mutation Panel:

#### BRAF

*BRAF* mutation status is critical when evaluating patients with a view to placing them on *BRAF* targeted therapies. The presence of a mutation in codon 600 of *BRAF* is required for treatment with *BRAF* targeted therapies.

#### KIT

KIT gene analysis enables the selection of those melanoma patients with KIT variants that will benefit from TKIs.

#### Breast Cancer Mutation Panel:

#### PIK3CA

PIK3CA mutation status provides information to guide treatment with PIK3CA inhibitors. It may also have a role in predicting response to chemotherapy.

#### BRCA 1 & 2 analysis and Multi Ligation-dependant Probe Amplification (MLPA)

This assay detects variants in BRCA1 or BRCA2, helping to identify patients that may benefit from treatment with PARP inhibitor. MLPA analysis also looks for largescale alterations in the BRCA 1 and 2 genes.

There is also an important role in BRCA testing for identifying germline variants that may be responsible for Hereditary Breast and Ovarian Cancer (HBOC). The results of these tests can then be used by clinicians to guide family testing as required. MLPA is performed on patients samples referred for germline testing.

Currently, we offer germline only analysis to HER2 negative locally advanced or metastatic breast cancer patients.

We offer somatic analysis and MLPA for patients with prostate cancer. Germline testing is performed reflexively if a significant variant is detected. Ideally, both a germline (EDTA blood) and a tumour sample (FFPE) will be provided on the same patient.

A separate request from for BRCA analysis is available on the Beaumont internet and intranet.

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| **Service** | **Specimen type** | **Specimen Requirements** |
| IHC & ISH | FFPE Blocks/slides | Ensure that sections are mounted on adhesive slides. |
| Mutational Analysis | FFPE Blocks | A representative block of tumour (resection, biopsy or cytology preparation) should be provided. The analysis can only be performed on specimens where there is adequate tumour material. |
| MSI | FFPE Blocks | Send representative blocks of tumour and normal tissue and if these are not available then contact us to discuss alternatives. The normal tissue sample need not be from the same specimen. If no normal tissue is available the analysis will be performed using a generic normal control. However, this can affect the interpretation of results. |
| Germline BRCA1 & 2 (including MLPA) | EDTA blood | Germline only analysis is currently performed for HER2 negative locally advanced or metastatic breast cancer patients.  FFPE material will be required is somatic analysis is required for prostate, ovarian, fallopian tube or primary peritoneal cancer. |

#### Homologous Recombination Deficiency

This assay gives a Genomic Instability Score (GIS) A score of ≥ 48 is positive. BRCA1 or BRCA2 are also assessed in the same assay and a positive GIS and/or positive for a pathogenic/likely pathogenic BRCA1/2 variant will identify patients that may benefit from treatment with PARP inhibitor. MLPA analysis also looks for largescale alterations in the BRCA 1 and 2 genes This is performed on the blood sample requested to accompany the FFPE block for HRD testing. The request form is available on the internet and intranet. Detailed on the request form is the sampel type required, the FFPE block and an EDTA Blood sample.

### Neuromolecular Pathology Tests and Requirements:

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| **Molecular Test performed** | **Requirement:** |
| 1p19q Array CGH | 10x5micron sections of requested block on unbaked glass slides. |
| MGMT methylation analysis | 10x5micron sections of requested block on unbaked glass slides. |
| BRAF Fusion qPCR | 5x5micron sections of requested block on unbaked glass slides. |
| IDH 1&2 sequencing analysis | 10x5micron sections of requested block on unbaked glass slides. |
| DNA Methylation profiling | 10x5micron sections of requested block on unbaked glass slides. |
| FusionPlex NGS | 10x5micron sections of requested block on unbaked glass slides. |
| DNA/RNA NGS CNS Tumours  (External Referral to SIHMDS-AG) | 10x5micron sections of requested block in two separate labelled sterile 1.5mL tubes. |

From an external referral centre the samples must arrive with adequate documentation and request form, outlining patient details as detailed in 10 below.

#### Array CGH

Microarray - Comparative Genomic Hybridization (array-CGH) is a molecular cytogenetic method for analysing Copy Number Variations (CNVs) relative to ploidy level in the DNA of a test sample (eg. tumour) compared to a reference control sample. Test (eg. tumour DNA) is labelled in one fluorescent dye (eg. Cy3) while reference (sample with a normal complement of chromosomes) is labelled in another fluorescent dye (eg. Cy5). Fluorescent intensities from both dyes are then scanned and compared to each other for every locus that is represented on the microarray. The final output is a genome-wide graph of copy number gains (gain or amplification) or deletions.

#### MGMT methylation analysis

*MGMT* in gliomas is a useful predictor of the responsiveness of tumours to alkylating agents. The protein O6-methylguanine-DNA methyltransferase (MGMT) functions to repair alkylated guanine in DNA by transferring the alkyl group at the O-6 position to a cysteine residue in the enzyme. This activity confers a certain chemoresistance to tumour cells and the silencing of *MGMT* through promoter methylation results in a better response to alkylating chemotherapy. In 2005 Hegi *et al*., reported that patients with methylated *MGMT* demonstrated a significant survival advantage with temozolomide treatment in a prospective phase III trial. The assessment of the methylation status of the *MGMT* promoter has therefore become an important genetic marker which is associated with response to alkylating chemotherapy and subsequent increased overall and progression free survival in GBM patients.

##### Assay Principle

The MGMT assay is based on the pyrosequencing of 8 CpG sites within the MGMT gene modified from Dunn J et al. 2009. The average methylation across the 8 CpG sites is calculated automatically by the PyroMark software. Methylated samples are defined as having an average methylation of > 9% methylation in accordance with the clinically significant thresholds reported by Dunn *et al*.

#### BRAF Fusion

Molecular detection of the *BRAF-KIAA1549* fusion gene on chromosome 7q32 has been identified in up to 70% of PAs and is therefore of diagnostic value in these tumours (JONES, D. T. et al, Cancer Research, 2008). A qPCR based method is employed which based on the amplification of the 3 most common fusion partners in pilocytic astrocytoma. Primers specific for each of the exons above are used to amplify the fusion product. Fluorescent probes specific for the fusion junctions are used to detect the amplified product. A positive control (*GAPDH)* is included in each analysis to ensure the quality of tumour RNA. The assay is based on the publication by Tian *et al*. Journal of Molecular Diagnostics, 2011.

#### IDH 1&2 Sequencing

*IDH1* mutations have been reported in 60-80% of WHO grade II and III gliomas, and secondary glioblastomas, whilst 2-5% of these tumours have *IDH2* mutations. Approximately 5% of primary GBM harbour *IDH* mutations. *IDH1* mutations have been associated with better clinical outcome; they are suitable predictive markers for adult glioma patients. In terms of diagnosis the presence of an *IDH* mutation can help to distinguish oligodendrogliomas from other tumours such as clear cell ependymomas and dysembryonic neuroepithelial tumours, as well as helping to differentiate between ganglioliomas and diffuse gliomas2-5. Mutations affecting *IDH1* and *IDH2* have been shown to be limited to the binding site of the proteins –cDNA positions 394 and 395 in *IDH1* and 514, 515 and 516 in *IDH2*, with mutations thought to be mutually exclusive.

#### DNA Methylation profiling

DNA methylation plays an important and dynamic role in regulating gene expression. It allows cells to become specialized and stably maintain those unique characteristics throughout the life of the organism, suppresses the deleterious expression of viral genes and other non-host DNA elements, and provides a mechanism for response to environmental stimuli. Aberrant DNA methylation (hyper or hypomethylation) and its impact on gene expression have been implicated in many disease processes, including cancer. By providing quantitative methylation measurement at the single-CpG–site level for normal and formalin-fixed paraffin-embedded (FFPE) samples, this assay offers powerful resolution for understanding epigenetic changes.

Following bisulfite conversion of DNA samples, DNA restoration is carried out using the Infinium HD FFPE Restoration Kit to optimise the processing of DNA previously extracted from FFPE tissue. The Illumina EPIC array Kit is then used to amplify, fragment and hybridise DNA to a beadchip which can be analysed on the Illumina iScan instrument to determine the methylation profile of the sample DNA.

#### FusionPlex NGS

Archer FusionPlex NGS is an RNA-based next generation sequencing panel for the identification of fusions and variants from FFPE tissue including NTRK fusions. The panel consists of 57 genes (shown below) which are assessed under the direction of the reporting neuropathologist. Any of these genes can be assessed upon request by contacting the reporting neuropathologist.

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| *AKT1* | *DDR2* | *FGFR2* | *IDH1* | *MAP3K8* | *NTRK3* | *PRKCA* | *TRIM11* |
| *ALK* | *DNAJB1* | *FGFR3* | *IDH2* | *MET* | *NUTM1* | *PRKCB* |  |
| *AXL* | *EGFR* | *GNA11* | *KEAP1* | *MYB* | *PAX8* | *RAF1* |  |
| *BRAF* | *ERBB2* | *GNAQ* | *KIT* | *MYBL1* | *PDGFRA* | *RET* |  |
| *BRD3* | *ERBB4* | *GNAS* | *KRAS* | *NRAS* | *PIK3CA* | *ROS1* |  |
| *BRD4* | *ERG* | *H3F3A* | *LTK* | *NRG1* | *POLD1* | *STK11* |  |
| *CTNNB1* | *ESR1* | *HIST1H3B* | *MAP2K1* | *NTRK1* | *POLE* | *TMPRSS2* |  |
| *CYSLTR2* | *FGFR1* | *HRAS* | *MAP3K3* | *NTRK2* | *PPARG* | *TP53* |  |

#### DNA/RNA NGS for CNS Tumours

CNS tumours are referred to the external laboratory listed below for the purpose of DNA and RNA next generation sequencing. The purpose of this testing is to identify variants and fusions clinically relevant to CNS tumours as either diagnostic markers or indications for treatment.

SIHMDS Acquired Genomics Laboratory,

North Thames Genomic Laboratory Hub,

Level 4 Barclay House,

37 Queen Square,

London

WC1N 3BH

Samples are referred as cut sections in sterile tubes which undergo nucleic acid extraction at the referral site. This material is then sent to the Clinical Genomics laboratory at the Royal Marsden Hospital (also part of the North Thames genomic hub), where it undergoes DNA and RNA NGS using their custom gene/fusion panels. The data output is then returned to SIHMDS-AG for analysis, interpretation and reporting. For more information regarding the NGS panels and genes included, please contact the Molecular Pathology Laboratory, Beaumont Hospital.

### Test Request Forms

Test request forms are available to download via the Beaumont Hospital Molecular department website at:

https://www.beaumont.ie/pages/health-A-Z/laboratorypathology or by contacting the laboratory.

### Delivery of Specimens for Analysis

Courier Services Specimens can be delivered via courier directly to the Molecular Pathology Department care of Pathology Specimen reception in the Laboratory Directorate addressed to the following.

Molecular Pathology Laboratory

c/o Pathology Specimen Reception

Beaumont Hospital

Beaumont Road

P.O. Box 9063

Dublin 9

### Test Result Queries

Despite our best efforts, it is possible that an error can occur. If you have concerns about a report please draw it to our attention without delay, and we will investigate immediately.

### Specimen Referral

When we are unable to provide a request or required follow-on analysis, we will attempt to source a referral laboratory, to which specimens may be sent. We welcome input from interested clinicians in this process. The choice of laboratory is primarily based on quality grounds, with accredited laboratories being chosen preferentially. Other factors such as cost and turnaround times are also considered.

### Details Required for All Specimens

Regardless of the specimen type, the minimum essential information and minimum criteria that must be supplied **legibly** include:

On the specimen block/slide:

Histopathology block number

On the blood sample:

On the request form:

* Name of patient
* Date of Birth
* Requesting Clinician/Pathologist
* Referring Hospital
* Relevant clinical details
* Specimen type

**Note:** Please send the pathology report relating to the sample to be tested and give ***as much clinical information on the form / letter*** as possible, as this will be required by the Pathologist when considering interpretations and advice. Specimens will not be accepted without a minimum of three forms of identification on the request form and will be returned to the source of origin to be completed / labelled correctly. All hazard labels where appropriate must be used for the health and safety of the staff that will be handling the specimen.

### Turnaround Times for Results (TATs)

The turnaround time of specimens will vary depending on the nature of the specimen and the complexity of the investigations required. The following is an outline of estimated turn-around times for different specimen types from time of receipt in the laboratory:

Solid tumour mutation analysis 15 days

*ALK* & *ROS1* FISH testing 15 days

Microsatellite Instability analysis (MSI) 20 days

MLH1 Hypermethylation analysis 20 days

Neuromolecular testing (aCGH, MGMT, BRAF fusion, IDHSeq,DMET, NGS) 42 days

*HRD testing* 48 days

*BRCA* 1&2 testing (including MLPA) 48 days

**Notes**

* TATs refer to working days from receipt of specimen until report has been authorised. Time refers to 90% of referrals.
* All reports are emailed by the laboratory staff to the requesting clinical and referral site. No results are issued over the phone.
* There is no time limit for requesting additional examinations but requests should be made by emailing the laboratory at [molecular@beaumont.ie](mailto:molecular@beaumont.ie) including an updated request form and stating the patient’s name, DOB and original sample number of available.
* Urgent specimens will be “fast tracked” as appropriate.

### Reports

Reports are available through the laboratory.

* External Reports are sent to those listed on the MolecularPathology request form or Consent form
* If a report has not been received, and the test request has exceeded the stated TAT, an enquiry can be made by emailing [molecular@beaumont.ie](mailto:molecular@beaumont.ie) Please do not email unless the TAT has been exceeded.
* Only authorised reports are available from the laboratory.
* If an interim report, clinical advice or result interpretation is required please contact the Consultant Histopathologist/Neuropathologist.