**Supplemental data**

**Generic Validation / Verification Form**

This form is intended as a guide to aid laboratories in developing a suitable validation / verification procedure. A suggested methodology for using the form has been given below.

The terms ‘investigating scientist’ and ‘senior scientist’ have been used for illustration of an authorization procedure – organisation of authorization systems is a matter for individual laboratories.

1. Section 1 should be completed as far as possible to establish the goals and general format of the validation / verification.

* Sections 1.1 “**Intended use or application**” and 1.2 “**Requirements**” *must* be completed at the start of the procedure. The assessment of the validation/verification depends formally on confirmation, through the provision of objective evidence, that these requirements have been fulfilled[[1]](#footnote-1).
* If mentioned (1.2), the “Expected Performance” should be distinguished from the “Requirements”, which must be shown to have been fulfilled.

***E.g.*** *The statement “should detect all known point mutations of hemophilia A” could be included as a guide in the Expected performance; if stated as a requirement, however, it would need to be proved.*

1. Section 2 covers the validation of utility which should be carried out for all validations and verifications. In the majority of cases this section can be completed on objective evidence from developmental work, design procedures (e.g. SNP checking primers) or by the use of limitations or controls in the on-going test. Where this is not the case, work plans for relevant parameters should be prepared as in 3 below.
2. Appropriate parameters for experimental investigation should be identified with the aid of the table appendix A – a checklist is also provided at the top of section 3. For each parameter required, the investigating scientist develops a work plan based on section 3 (these are referenced 3.1, 3.2 to 3.*n*) by completing copies of sections 3.*n*.1 (‘Aims’, ‘Samples’ and ‘Methodology’). It is suggested that these be maintained in a single document.

***Note****: several parameters may be tested in a single experiment, for example sensitivity and specificity.*

1. The work plan[s] should be agreed and authorized by the investigating and the senior scientist by signing and dating in the boxes provided.
2. The experimental work is performed and analysed by the investigating scientist who should then complete the ‘experimental results’ and ‘interpretation’ sections 3.*n*.2.
3. The ‘outcome and limitations’ should be agreed between the investigating and senior scientists by signing and dating in the boxes provided.
4. Points 3 to 6 should be repeated for each parameter to be tested.
5. If there is any non compliance between the experimental results and the required performance specification detailed in section 1.2 the parameter in question should to be re-examined to determine if the methodology can be changed or new limitations introduced to rectify the non-compliance. Any further work should be recorded in a new section 3 work plan. Alternatively the implementation can be abandoned.
6. Once all the parameters have been satisfactorily investigated the investigating and senior scientist can agree and sign off the final conclusions in section 4.
7. Assuming the validation / verification has been completed satisfactorily an implementation plan can be drawn up. Appendix B provides a basis for an administrative checklist for the implementation.
8. **Validation Details**

|  |  |  |  |
| --- | --- | --- | --- |
| **Test name** | Validation of Copy number variation for ABC-Bio panel | **Reference** | VAL27A |

* 1. **Test details**

|  |  |
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| **Intended use or application** | This is verification for a new NGS capture panel specifically for breast cancer samples. This uses the same sample prep and analysis method detailed in CCMD\_15 (Nimblegen gastrointestinal capture panel) and CCMD\_22 (Enzymatic fragmentation verification). This panel will be used initially as part of the ABC-BIO trial to identify somatic mutations in FFPE samples from breast cancer patients. |
| **Locus / Gene / Marker** | Panel has previously been validated for SNVs and indels (VAL27). This validation is for copy number variation of 15 genes. In first instance validation includes amplification of the gene alone, not small gains or deletions.  Gene amplification is an increase in the number of copies of a gene without a proportional increase in other genes.  Unlike constitutive copy number variations (CNVs), focal CNAs are the result of many selection events during the evolution of cancer genomes. Therefore, it is possible that a single gene in a focal CNA gives the tumour a selective growth advantage. The term ‘focal CNAs’ has been used without a strict consensus. Many laboratories, have defined an exact upper cutoff size value, of either 1-3 Mb. Others have used ‘smaller than a chromosome-arm’ to define a focal CNA. Alternative operational cutoffs for focal CNAs could be: ‘CNAs that contain maximally one annotated element’, and we will use that definition here.  Amplification of *CDKN1A, ESR1, EGFR, FGFR1, CDKN2A, CDKN2B, PTEN, FGFR2, BRCA2, RB1, CDH1, TP53, MAP2K4, ERBB2* and *BRCA1* can be detected with the panel.  Detection of *ESR1, MDM2, MYCC* and *ERBB2* amplification are used for validation. |
| **Reference Sequence** | Hg19 |
| **Outline methodology** | This is a process for enrichment of individual or multiplexed genomic DNA (gDNA) sample libraries using SeqCap EZ Libraries, and the amplification of these sample libraries by ligation-mediated PCR (LM-PCR). Specifically, this is a protocol for the preparation of gDNA sample libraries using KAPA Biosystems Hyper Prep Kit with NimbleGen SeqCap Adapter Kit A and B, and the capture of these sample libraries using SeqCap EZ Choice Libraries, SeqCap EZ Accessory Kit v2, and SeqCap HE-Oligo Kit A and B. The output of this protocol consists of enriched gDNA fragments that can be quantified by qPCR before sequenced directly using an Illumina Miseq sequencing instrument. Tumour sample may be analysed along with a non-tumour sample from the same patient to allow for the analysis of only acquired somatic variants.  Capture panel results were compared with FISH results for ESR1, and IHC results for ERBB2, as well as ddPCR (plasmaMATCH assay).  FISH and IHC are gold standard techniques for copy number detection in FFPE tissue sections.  FISH scores on ESR1 were done by Vera Martins from Breast Cancer Now, Chelsea. The average of the 20 cells/sample score values were used for comparison.  ddPCR provides high precision, absolute quantification by encapsulation of nucleic acid molecules into discrete water in oil droplets prior to PCR amplification. The PCR product is detected by TaqMan hydrolysis probes using FAM and VIC as the reporter fluorophores. PCR reactions are partitioned in ≤20, 000 droplets using Bio-Rad’s Automated Droplet Generator and placed on a thermal cycler for PCR amplification. The droplets are then streamed and read single-file on Bio-Rad’s QX200 Droplet Reader which counts the number of positive and negative droplets prior to application of a Poisson algorithm and calculation of DNA concentration.  The QuantSoft software compares the concentration of ERBB2 to EFTUD2 and a ratio is calculated. A ratio >2 indicates amplification of ERBB2.  The QuantSoft software compares the concentration of MDM2 to average of reference genes (RNaseP, GAPDH, NAGK) and a ratio is calculated. A ratio ≥1.6 indicates amplification of MDM2, borderline range being 1.3-1.5.  The QuantSoft software compares the concentration of MYC genes (C, L and N) to 3 reference genes on corresponding chromosomes (EIF2C1, NAGK and RNaseP) and a ratio is calculated. A ratio >2 indicates amplification of MYC. Only MYCC is covered by ABC-Bio panel. |
| **SOP** | SMD74 Targeted Capture of DNA Using KAPA HyperPlus Prep Kit and Nimblegen Baits  SMD91 Somatic Mutation Report Using Molecular Diagnostics Information Management System  SMD115\_Multiplex\_ddPCR\_for\_plasmaMATCH  SMD164 MDM2 amplification testing by ddPCR  SMD161\_MYC amplification ddPCR assay |
| **References** | Kapa Biosystems Hyper Prep Kit Protocol: http://www.kapabiosystems.com/document/kapa-hyper-prep-kit-tds/  Roche Nimblegen SeqCap Ez: <http://www.nimblegen.com/products/seqcap/ez-system/index.html> |

**1.2 Validation details**

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| --- | --- |
| **Overall Aims** | Aim is to show ESR1, MDM2, MYCC and ERBB2 amplification detected by ABC-Bio Panel is consistent when compared with FISH/ICH/droplet digital PCR (ddPCR). |
| **Requirements** | Samples where amplification was detected are expected to be found amplified with alternative methods. Samples detected to be negative by ABC-Bio panel are expected to be found negative in the validation. |
| **Validation / Verification** | Summary of ongoing validation, as indicated in VAL27. |
| **Type** | This test is a type C Categorical test |
| **Scope / limitations** | This test may be performed on DNA extracted from a wide variety of sources including FFPE, cell pellet or fresh frozen material. However, it is known that DNA extracted by Cobas does not generate good libraries. |
| **Turn around time** | 21 working days |
| **Other considerations** | Degradation of DNA can seriously affect the performance of the assay. Samples should be run on a gDNA Tapestation prior to starting to determine the level of degradation. This is most applicable to FFPE samples. |

**2. Validation of Utility**

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| **Test name** | Validation of Copy number variation for ABC-Bio panel | **Reference** | VAL27A |

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| **Applicability of measurements** | FFPE samples analysed with ABC-Bio panel reanalysed by droplet digital PCR and FISH/IHC. |
| **Selectivity** | None identified. |
| **Interferences** | None identified. |
| **Cross-reactivity** | None identified. |

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| **Authorization** | Name | Signature | Date |
| Investigating scientist | Paula Proszek |  | 29/9/17 |
| Senior Scientist (Authorization) | Michael Hubank |  |  |

**3 Validation for Copy number Detection**

A copy of this section should be filled in for all parameters to be tested.

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| **Test name** | Validation of Copy number variation for ABC-Bio panel | **Reference** | VAL27A |

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| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Sensitivity |  | Trueness |  | Reproducibility |  | Limit of quantification |
|  |  |  |  |  |  |  |  |
|  | Specificity |  | Repeatability |  | Robustness |  | Linearity |
|  |  |  |  |  |  |  |  |
| **X** | Accuracy |  | Intermediate precision |  | Limit of detection |  | Measurement uncertainty |

**3.1 Work plan**

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| **Section aims** | The aim is to determine how fit for purpose CNV detection in the panel is by analysing samples found positive, negative and borderline by ABC-Bio panel with FISH or ddPCR to obtain result. The purpose of this experiment is not to find samples amplified/ negative to diagnostic level, but to prove that ABC-Bio panel functions as reliable method to evaluate copy number variation to same level as other methods. |
| **Samples** | There were 92 ABC-Bio samples identified with amplifications in 21 genes, including *AKT1, BRAF, CCND1, CCNE1, CDK4, CDKN2A, CDKN2B, EGFR, ERBB2, ESR1, FGFR1, GATA3, IGF1R, KIT, KRAS, MAP2K4, MCL1, MDM2, MYC, PDGFRA* and *PIK3CA*.  Data was available from orthogonal methods for the following: *ERBB2* by DDISH (dual-color dual-hapten brightfield in situ hybridization), FISH, Immunostaining (IHC) or ddPCR (for 25 cases), *ESR1* amp assessment by FISH (5 cases), MYCC (12 cases) and MDM2 (17 cases) by ddPCR.  A total of 59 cases were used in this verification. |
| **Methodology** | The ddPCR CNV assays will be run as describe in SOP: SMD115 from FFPE samples. Preferably with 5 ng, or max. 10 µL (if required amount of DNA cannot be used sample will be run with the remaining DNA, or Hyperplus material from this tumour). Samples are loaded into a 25 µL PCR reaction containing TaqMan assays for ERBB2 (reference gene EFTUD2), FGFR2 (reference gene HOGA1), KRAS (reference gene TERT). The reaction will then be partitioned on the Automated Droplet Generator (BioRad), followed by PCR and then run on the QX200 Droplet Reader (BioRad). Comparison of the concentrations of gene of interest to their reference genes will be used to calculate a ratio of target to reference gene.  For FISH scoring, the following ratio rules were applied:  ESR1/CEP6:  < 1.5 Non-amplified  ≥ 1.5 Gain  ≥ 2.0 Amplified  For sequencing data, the amplification threshold was set as >2.4, with at least 80% ROI in the gene above it. The maximum value was used in the table.  The low level amplification (gain) was called when <80% ROI in a given gene were above 2.4 threshold.  Some samples were also included in this comparison, when the normalised regions across the panel were very low and some gene probes were noticeably higher, to check the limits of copy number detection by the current method.  For ddPCR ERBB2 amplification status, a ratio ≥2 is regarded as positive and borderline as 1.80-2.  For ddPCR MDM2 amplification status, a ratio ≥1.6 is regarded as positive, and borderline as 1.3-1.5.  The QuantSoft software compares the concentration of MYC genes (C, L and N) to 3 reference genes on corresponding chromosomes (EIF2C1, NAGK and RNaseP) and a ratio is calculated. A ratio >2 indicates amplification of MYC. Only MYCC is covered by ABC-Bio panel. |

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| **Authorization** | Name | Signature | Date |
| Investigating scientist | Paula Proszek |  |  |
| Senior Scientist (Authorization) | Michael Hubank |  |  |

**3.1.2 Partial results and conclusions**

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| **Experimental results** | Table comparing NGS based results with dPCR (for HER2 amplification), FISH (scored on the same pathology number blocks for ESR1), and EPR data when available for ERBB2 status, from the same block/timepoint (DDISH, IHC, FISH).  Also in-house ddPCR amplification assays data was used for MYCC and MDM2 genes copy number status.  This was done for 59 tumour samples from ABC-Bio trial (for details see Appendix 1 at the end).  **ESR1**  All ESR1 amplification samples were concordant with FISH findings. I.e L092 neg in primary and amplified in metastatic sample, see below:    The identified samples with discrepant results will be described below:  **ERBB2**  In sample 16/11243 L131 sequencing identified an ERBB2 gain. The sample was marked as amplified on EPR. By NGS had 57% of gene over the threshold of 2.4.  It was tested by ddPCR for ERBB2 amplification and showed a ratio of 6.2. This sample will be counted as true positive.      In sample 17/02497 S013, NGS sequencing did not detect ERBB2 amplification. This sample had a record on EPR as ambiguous, with DDISH result positive (ratio 2.12), and immunostaining for Her2 was negative (0) [BLOCK 8].  This sample was tested by ddPCR, and gave a borderline ratio of 1.86. This result will be counted as true negative.    In sample 17/03979 S104, NGS sequencing did not detect ERBB2 amplification. This block was marked as amplified on EPR by immunostaining for Her2 positive (3+).  This sample was amplified by ddPCR (ratio 2.2).  This result will be counted as false negative.    **MYCC**  In sample 17/00442\_L122, less than half of the probes were amplified in NGS so perhaps not detected by the dPCR MYCC assay (which is in intron 2/exon 3) or the NGS is a false-positive result. NGS ROI>2.40 = 8, %gene>2.40 = 47, max 14.16. This result will be counted as false positive.    **MDM2**  In sample 18/01712\_L195, 71% of the probes were amplified in NGS.  ROI>2.40 = 15, %gene>2.40 =71, max 5.51 (Pool 757).  This sample was amplified by ddPCR MDM2 assay, with ratio 2.2.  This result will be counted as true positive.    Sample 16/05707\_L089 was selected for ddPCR testing, as the CNV plot and igv showed higher coverage in MDM2 region. It did not reach the amplification threshold by NGS.  ROI>2.40 = 0, %gene>2.40 = 0, max 2.3 (Pool 388)  This sample was amplified by ddPCR (ratio 2.3). This result will be counted as true positive.  This same sample was then also tested with MYC assay by ddPCR, as it also did not cross amplification threshold of 2.4, but the plot quality was good and suggesting amplification event. The ddPCR MYCC assay ratio was 2.8 (amplified).    Sample 17/06421\_S111 was selected for ddPCR testing, as the CNV plot and igv showed higher coverage in MDM2 region. It did not reach the amplification threshold by NGS.  ROI>2.40 = 0, %gene>2.40 = 0, max 2.27 (Pool 643)  This sample was non-amplified by ddPCR (ratio 1.0).  This result will be counted as true negative.    Sample 15/8787\_S026 was selected for ddPCR testing, as the CNV plot and igv showed higher coverage in MDM2 region. It did not reach the amplification threshold by NGS.  ROI>2.40 = 1, %gene>2.40 = 4, max 2.45(Pool 267)  This sample was non-amplified by ddPCR (ratio 1.3).  This result will be counted as true negative.    Sample 18/00241\_L187 was selected for ddPCR testing, as the CNV plot and igv showed higher coverage in MDM2 region. It did not reach the amplification treshold by NGS. ROI>2.40 = 0, %gene>2.40 = 0, max 2.21(Pool 676)  This sample was amplified by ddPCR (ratio 2.7).  This result will be counted as false negative. |
| **Interpretation** | Copy number analysis by ABC-Bio panel proved to be suitable for strong focal amplification of genes (here ERBB2 and ESR1). Within 59 samples tested, 55 were concordant in calls (93.2%).  All discordant or interesting cases will be discussed below.  Overall, the concordance between NGS and FISH/IHC calls for ERBB2 was very good. ddPCR ratio differences most possibly result from poor quality, degraded FFPE DNA being more likely to amplify better by ddPCR, as well as technological differences between compared techniques (i.e. probe size).  There were a lot of cases were, when NGS called gain (low level) instead of amplification. In one case the ERBB2 amplification was called for 57% of the gene (L131), or not called it (S104), and the samples were amplified by ddPCR. There was one discordant case - an ambiguous sample, possibly highly heterogeneous, with conflicting results with 4 techniques used: IHC was negative, DDISH positive (ratio 2.12), ddPCR borderline (ratio 1.86).  There were many samples reported as gain in NGS, while being either borderline or positive by ddPCR. Those results were counted as true positives.  To calculate specificity, sensitivity and accuracy of NGS CNV calling we had to classify findings into 4 categories:  TP [true positive] is when the NGS called amplification or gain, and one of the orthogonal techniques agreed with the finding (amplified by FISH, amplified and borderline ratio by ddPCR),  TN [true negative] is when the NGS called no amplification, and one of the orthogonal techniques agreed with the finding (Negative IHC, normal ratio by ddPCR),  FP [false positive] is when the NGS called amplification or gain, and at least one of the orthogonal techniques did not agree with the finding (normal by FISH, IHC or ddPCR ratio).  FN [false negative] is when the NGS called neither amplification nor gain, but one of the orthogonal techniques did not agree with the finding (amplified by FISH, amplified and borderline ratio by ddPCR). Details in Appendix 1.   |  |  | | --- | --- | | FN | 2 | | FP | 1 | | TN | 15 | | TP | 41 |   All discrepant cases were discussed for possible reasons in experimental results section.  Due to small number of samples tested in this validation, to calculate sensitivity, specificity and accuracy we used a method described in [3, 4]:  Sensitivity = 95.3% (95%CI: 0.842-0.994)  Specificity = 93.8% (95%CI: 0.698 – 0.998)  Accuracy = 94.92% (95%CI: 0.858-0.989) |
| **Outcome / limitations** | * Validation requirements are met as stated in section 1.2. * The ongoing monitoring will be done for all ABC-Bio cases for ERBB2 status and any discrepancies followed up when possible by ddPCR. * The nature of capture and sequencing of FFPE samples of variable quality makes it difficult to apply a precise threshold for gain and amplification that applies in every case, especially because genes differ in AT/GC ratios and the control is derived from fresh material (blood). * Techniques taken to compare NGS CNVs calls differ in terms of copy number assessment, i.e. FISH has probes much longer than ddPCR, NGS and ddPCR normalising the results, so calling lower values on heterogeneous samples (i.e. with strong amplification in small number of cells). |

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| **Authorization** | Name | Signature | Date |
| Investigating scientist | Paula Proszek |  |  |
| Senior Scientist (Authorization) | Michael Hubank |  |  |

**4. Validation Final Conclusions**

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| **Test name** | Validation of Copy number variation for ABC-Bio panel | **Reference** | VAL27 |

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| **Overall Conclusion** | Requirements of the validation as described in section 1.2 have been met. |
| **Estimates of accuracy and measures of uncertainty** | **ABC-Bio panel is thought to be fit for purpose to detect samples with focal gene amplifications (>80% gene ROI >2.4 ratio).**  **In this validation, all samples, apart from three (1 false positive, 2 false negatives), were found comparable to alternative methods calls.**  **Amplifications were correctly identified by NGS with:**  **Sensitivity = 95.3% (95%CI: 0.842-0.994)**  **Specificity = 93.8% (95%CI: 0.698 – 0.998)**  **Accuracy = 94.92% (95%CI: 0.858-0.989)** |
| **Limitations and/or predictable interferences** | **The ongoing monitoring will be done for all ERBB2 amplified cases, and any discrepancies followed up when possible.**  **HER2 CNV event is expected to be rare. Changes in HER2 status between primary tumour and recurrence have been documented in 5-10% patients.** |
| **Internal QC** | **Analysing amplified samples with alternative methods when necessary; low amplification samples, borderline samples etc.** |
| **External QA** | **No external quality schemes exist at the moment that ABC-Bio panel could be applied to.** |

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| **Authorization** | Name | Signature | Date |
| Investigating scientist | Paula Proszek |  |  |
| Senior Scientist (Authorization) | Michael Hubank |  |  |

**Appendix A: Types of test.**  (Refer to main paper for full descriptions of test types)

*NB. In addition to the parameters detailed below appropriate* ***robustness*** *testing should be carried out for all types of test.*

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Description** | **Examples** | **Sensitivitya** | **Specificityb** | **Accuracyc** | **Trueness** | **Precisiond** | **Limits of detection** | **Probabilitye** |
| **A** | Q**uantitative** tests.  The result can have any value between two limits (including decimals). | Determination of methylation load (%); characterization of a mosaic mutation; heteroplasmy of mitochondrial variants. | v |  |  | **++** | **++** | **++** |  |
| **B** | **Categorical** tests where the quantitative signal is placed into an ordinal series to give the final result. | Sizing a PCR product; determination of triplet repeat size (FRAXA, Huntington disease, etc.) |  |  | **+** | **++** | **++** | **++** | **+** |
| **C** | **Categorical** tests where the quantitative signal is placed into one of a limited series of predefined categories to give the final result. | Determination of copy number using PCR or MLPA.: exon deletion / duplication in *BRCA1*; *PMP22* gene dosage in CMT and HNPP; |  |  | **+** | To establish correction factors and/or cut-offs | |  | **++** |
| **D** | **Qualitative** tests where the true quantitative signal can have one of many possible values, but the required result can only have one of two possible values. | Mutation scanning for unknown mutations e.g. by sequencing or high resolution melt. | **++** | **++** | **+** | To establish correction factors and/or cut-offs | | **++**f |  |
| **E** | **Qualitative [binary]** tests where the true quantitative signal can only have one of two possible values | Genotyping for a specific mutation e.g. *CFTR* Phe508del in cystic fibrosis or *HFE* Cys282Tyr in hemochromatosis. | **++** | **++** | **+** | To establish correction factors and/or cut-offs | | **++** f | **+** |

**Legend Notes**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Metric used for implementation validation | a. | Sensitivity = True Positive / (True Positive + False Negative) |
|  | Metric used for implementation or ongoing validation | b. | Specificity = True Negative / (True Negative + False Positive) |
|  | Metric used for ongoing validation | c. | Accuracy = True Result / (True Result + False Result) |
| **++** | Recommended parameter | d. | Precision should be measured in terms of repeatability and intermediate precision (as well as reproducibility for inter-laboratory validations) |
| **+** | Applicable parameter (less used) | e. | The term ‘probability’ is used to describe situations where a probability that the result is correct can be assigned – primarily in ongoing validation (e.g. competitive hypothesis testing) |
|  |  | f. | Should be used in tests where genotyping of low level variations is required for example mitochondrial DNA |

**Appendix B: Administrative checklist**

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|  |  | Date | Initials |
|  | Validation completed and approved |  |  |
|  |  |  |  |
|  | Complete SOP |  |  |
|  |  |  |  |
|  | Order reagents |  |  |
|  |  |  |  |
|  | Health and safety aspects (personal, reagents) |  |  |
|  |  |  |  |
|  | Equipment (electrical testing, maintenance) |  |  |
|  |  |  |  |
|  | Subscribe to EQA |  |  |
|  |  |  |  |
|  | Update request forms |  |  |
|  |  |  |  |
|  | Update website and any directory listings |  |  |
|  |  |  |  |
|  | Billing procedure |  |  |
|  |  |  |  |
|  | Training |  |  |
|  |  |  |  |
|  | LIMS functionality |  |  |
|  |  |  |  |
|  | Worksheets |  |  |
|  |  |  |  |
|  | Inform clients |  |  |
|  |  |  |  |
|  | Report template |  |  |

Appendix 1

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **MDx** | **Trial ID** | **Tumour %**  **Peak size** | **Gene** | **NGS Result** | **NGS max** | **Comment FISH - EPR** | **FISH DDISH score** | **IHC** | **dPCR ratio** | **Comment** |
| 16/01408 | S029 | 70 high  553 bp | ERBB2 | No amp |  | No amp |  | negative (0) |  | TN |
| 16/05702 | L060 | 70 int  1390 bp | ERBB2 | No amp |  | No amp |  | negative (0) |  | TN |
| 17/08168 | S041 | 80 int  900 bp | ERBB2 | No amp |  | No amp |  | negative (0) |  | TN |
| 17/08171 | S112 | 80 low  900 bp | ERBB2 | No amp |  | No amp | 1.51 | equivocal (2+) |  | TN |
| 18/03806 | L196 | 40 low-i  20473 bp | ERBB2 | No amp |  | No amp |  | negative (0) |  | TN |
| 17/02510 | S102 | 50 low-i  2023bp | ERBB2 | No amp |  | No amp | 1.58 | No amp (1+) | 1 | TN |
| 15/3483 | L002 | 30 int  1453 bp | ERBB2 | Amp | 7.29 | Amp |  | Amp (3+) |  | TP |
| 15/8981 | L022 | 70 low-i  1964 bp | ERBB2 | Amp | 4.79 | Amp |  | Amp (3+) |  | TP |
| 16/01480 | S008 | 60 low-i  0 | ERBB2 | Amp | 15.07 | Amp |  | Amp (3+) |  | TP |
| 16/04585 | L083 | 90 low  1978 bp | ERBB2 | Amp | 10.48 | Amp |  | Amp (3+) |  | TP |
| 16/05705 | L087 | 30 low  16180 bp | ERBB2 | Amp | 12.89 | Amp |  | Amp (3+) |  | TP |
| 16/05710 | S042 | 60 high  2565 bp | ERBB2 | Amp | 13.65 | Amp |  |  |  | TP |
| 16/07577 | S049 | 70 low  3147 bp | ERBB2 | Amp | 18.01 | Amp |  |  |  | TP |
| 16/07583 | L097 | 50 int  2357 bp | ERBB2 | Amp | 4.94 | Amp | 5.7 | equivocal (2+) |  | TP |
| 16/08954 | L064 | 70 low-i  0 | ERBB2 | Amp | 7.37 | Amp |  | Amp (3+) |  | TP |
| 16/08963 | S058 | 60 int  2513 bp | ERBB2 | Amp | 17.58 | Amp |  | Amp (2+) 2009. neg 2010 |  | TP |
| 16/09039 | S042 | 50 low-i  0 | ERBB2 | Amp | 30.25 | Amp |  |  |  | TP |
| 17/00444 | L144 | 50 int  747 bp | ERBB2 | Amp | 7.76 | Amp |  |  |  | TP |
| 17/00445 | L145 | 50 low  509 bp | ERBB2 | Amp | 18.28 | Amp |  |  |  | TP |
| 17/02498 | S027 | 60 low  0 | ERBB2 | Amp | 14.87 | Amp |  | Amp (3+) |  | TP |
| 17/09391 | S104 | 60 int  1850 bp | ERBB2 | Amp | 6.46 | Amp |  | Amp (3+) |  | TP |
| 18/00240 | L186 | 60 int  13503 bp | ERBB2 | Amp | 17.29 | Amp |  | Amp (3+) |  | TP |
| 16/11243 | L131 | 70 low  3382 bp | ERBB2 | Gain | 6.72 | Amp |  |  | 6.2 | TP |
| 17/03979 | S104 | 60 low-i  2424bp | ERBB2 | **No amp** | 2.25 | Amp |  | **Amp (3+)** | 2.2 | **FN** |
| 17/02497 | S013 | 40 low-i  1233 bp | ERBB2 | No amp | 1.77 | **Amp** | 2.12 | negative (0) | 1.86 | TN |
| 17/05099 | L092 prim | 60 low-i  0 | ESR1 | No amp | No amp | No amp |  |  |  | TN |
| 16/01408 | S029 | 70 high  553 bp | ESR1 | Amp | 8.37 | Amp 10115/15 | 2.30 |  |  | TP |
| 17/01237 | L092 met | 50 low-i  0 | ESR1 | Amp | 4.05 | Amp | 3.18 |  |  | TP |
| 17/03981 | S106 | 40 low  14757 | ESR1 | Amp | 6.06 | Amp | 4.45 |  |  | TP |
| 17/06418 | L137 met | 60 int  3000bp | ESR1 | Amp | 17.66 | Amp | 9.1 |  |  | TP |
| 15/8981 | L022 | 70 low-i  1964 bp | MYCC | Amp | 11.5 |  |  |  | 5.52 | TP |
| 16/01800 | L035 | 90 int  2247bp | MYCC | Amp | 21.26 |  |  |  | 6.26 | TP |
| 15/8787 | S026 | 80 low  2324bp | MYCC | gain 65% | 4.46 |  |  |  | 1.81 | TP |
| 16/11252 | S087 | 60 low  0 | MYCC | gain 76% | 6.67 |  |  |  | 1.72 | TP |
| 17/00444 | L144 | 50 int  747bp | MYCC | gain with control region 12% | 3.68 |  |  |  | 1.73 | TP |
| 17/00448 | L151 | 70 low  15812 | MYCC | gain 6% | 2.61 |  |  |  | 1.53 | TP |
| 16/03819 | L075 | 50 int  12478 | MYCC | gain 41% | 3.05 |  |  |  | 2.69 | TP |
| 16/08973 | S073 | 50 low-i  3324bp | MYCC | gain 36% | 4.26 |  |  |  | 2.39 | TP |
| 17/02501 | S086 | 60 low  4000bp | MYCC | gain 88% | 5.71 |  |  |  | 2.29 | TP |
| 17/03966 | L152 | 60i-high  1829bp | MYCC | 8q gain, aneup 94% | 4.92 |  |  |  | 3.48 | TP |
| 17/03981 | S106 | 40 low  14757 | MYCC | gain 82% | 5.16 |  |  |  | 2.46 | TP |
| 17/00442 | L122 | 50int  2224bp | MYCC | **gain 47%** | 14.16 |  |  |  | **1.17** | **FP** |
| 16/03818 | L074 | 50 int  0 | MDM2 | No amp |  |  |  |  | 0.8 | TN |
| 16/04585 | L083 | 90low  1978bp | MDM2 | No amp |  |  |  |  | 0.9 | TN |
| 16/04586 | L086 | 80low  2780bp | MDM2 | No amp |  |  |  |  | 0.8 | TN |
| 16/05705 | L087 | 30 low  16180 | MDM2 | No amp |  |  |  |  | 0.9 | TN |
| 16/06476 | L093 | 70low | MDM2 | No amp |  |  |  |  | 1.2 | TN |
| 15/8148 | L030 | 80 high  2177bp | MDM2 | Amp | 7.58 |  |  |  | 2.6 | TP |
| 16/05701 | L011 | 50low-i  17859 | MDM2 | Amp | 13.85 |  |  |  | 12.6 | TP |
| 16/05703 | L077 | 60int  16689 | MDM2 | Amp | 8.54 |  |  |  | 7.8 | TP |
| 16/08956 | L109 | 50low-i  2314bp | MDM2 | Amp | 10.46 |  |  |  | 5.0 | TP |
| 16/11252 | S087 | 60low  0 | MDM2 | Amp | 8.5 |  |  |  | 2.7 | TP |
| 17/08165 | L181 | 40low-i  1043bp | MDM2 | Amp | 8.51 |  |  |  | 8.0 | TP |
| 17/11014 | L111 | 50int  2672bp | MDM2 | Amp | 7.54 |  |  |  | 3.1 | TP |
| 18/01712 | L195 | 40low-i  1007bp | MDM2 | gain 71% | 5.51 |  |  |  | 2.2 | TP |
| 16/05707 | L089 | 90low  16778 | MDM2 | gain 0 | 2.3 |  |  |  | 2.3 | TP |
| 17/06421 | S111 | 70low-i  2441bp | MDM2 | small gain 0 | 2.27 |  |  |  | 1.0 | TN |
| 18/00241 | L187 | 30int  2326bp | MDM2 | **gain 0** | 2.21 |  |  |  | 2.7 | **FN** |
| 15/8787 | S026 | 80low  2324bp | MDM2 | gain 4% | 2.45 |  |  |  | 1.3 | TN |

Citations:

[1] Focal chromosomal copy number aberrations in cancer—Needles in a genome haystack. OscarKrijgsman, BeatrizCarvalho, Gerrit A.Meijer, Renske D.M.Steenbergen, BaukeY lstra

[2] A standardized framework for the validation and verification of clinical molecular genetic tests. Christopher J Mattocks, Michael A Morris, Gert Matthijs, Elfriede Swinnen, Anniek Corveleyn, Els Dequeker, Clemens R Mu¨ller, Victoria Pratt and Andrew Wallace, for the EuroGentest Validation Group.

[3] For calculating Specificity and sensitivity, the following method was used:

NIST/SEMATECH e-Handbook of Statistical Methods <https://www.itl.nist.gov/div898/handbook/prc/section2/prc241.htm>

[4] medcalc: <https://www.medcalc.org/calc/diagnostic_test.php>

[5] Buckley, N. E. *et al.* Quantification of HER2 heterogeneity in breast cancer – implications for identification of sub-dominant clones for personalised treatment. *Sci. Rep.* **6**, 23383; doi: 10.1038/srep23383 (2016).

1. (ISO 9000:2005 3.8.4, 3.8.5) [↑](#footnote-ref-1)