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Collaborative Study to evaluate the proposed WHO 1st International Standards for Cancer Genomes

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **27 September 2019** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

With an ever-increasing number of clinically actionable genomic variants in cancer, there is an urgent need for the provision of reference standards to aid the definition of assay limit-of-detection and for the harmonization of variant measurement in response to treatment. By using tumor cell lines carrying multiple rather than single actionable variants, the development of cancer genomic DNA (gDNA) standards may be accelerated. Furthermore, as additional variants within the cancer gDNA standards become clinically relevant, the data on such variants may be added to pre-existing WHO International Standards, thus enhancing their clinical utility. It is proposed that the candidate cancer gDNA standards presented here be the first in a series of cancer gDNA standards and that they will together act as calibrants for many clinically-relevant variants. The background of non-clinically relevant variants in these candidate cancer gDNAs standards may also have value in the validation of next-generation sequencing (NGS) assays.
An international collaborative study assessed the suitability of three genomic gDNA materials as proposed WHO 1st International Standards for two cell line cancer genomes carrying a total of five clinically relevant markers, and a wild-type cell line genome. The three gDNA materials (18/118, 18/130, and 18/164) were derived from the following cell lines, respectively: HCT 15, a colon adenocarcinoma cell line (putatively carrying the *PIK3CA* p.E545 variant); MOLT-4, an acute T lymphoblastic leukemia cell line (putatively carrying the *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9, and *MAP2K1/MEK1* p.D67N variants); ATDB102, a wild-type cell line intended for use both as a common reference and to dilute the variant-positive standards.
The study encompassed both NGS and digital polymerase chain reaction (dPCR) methods. In addition to characterizing the five clinically-relevant variants, the study also captured the presence of other variants in the three materials which may be useful in the broader validation of NGS pipelines, although not for calibration or diagnostic purposes.
Participants evaluated the materials using their routine established methods and against in-house controls (previously characterized patient samples and cell line-derived gDNA), or commercial materials. Participants evaluated both the crude (undiluted) materials, and each material at a range of dilutions, in order to both determine their suitability for use at a range of variant levels for assay or secondary standard calibration, and to enable the derivation of the cancer gene copy numbers by mathematical modelling. Participants were asked to report data for the five putative variants, along with any additional sequence data for the three materials. With one exception, results were reported quantitatively in order to assign consensus values to each of the materials. Thirty nine laboratories in twenty two countries took part in the study, and results were returned by thirty five; thirty eight datasets were returned as three laboratories assessed the materials each with two different approaches.

Conclusions from this study indicate that all three materials are suitable for use as International Standards for the calibration of the five variants, with verified performance in NGS and dPCR. The proposed consensus percentage for each of the five clinically relevant variants is derived from the median values of NGS and dPCR methods as: 52.1% *PIK3CA* p.E545K (18/118), 31.8% *TP53* p.R306*, 24.7% *NRAS* p.G12C, 100.0% *PTEN* p.K267fs*9, and 25.3% *MAP2K1/MEK1* p.D67N (18/130), along with common wild-type material (18/164). Additionally, as per the 1st International WHO Reference Panel for genomic *KRAS* codons 12 and 13 mutations (NIBSC panel 16/250), the collaborative study also analyzed the response of the candidate cancer gDNA standards to dilution (with putative wild-type material 18/164).

These dilution data were used to calculate the *PIK3CA*, *TP53*, *NRAS*, *PTEN*, and *MAP2K1/MEK1* variant and total gene copy numbers, also to be formally associated with each proposed standard. These copy number data can be applied to a mathematical formula, with which the end-user calculates how to prepare further standards at lower variant percentages for each specific variant (by dilution with wild-type material 18/164, or another wild-type gDNA aligned to 18/164). Thus, these materials and their dilutions will enable the calibration of assays, kits, and secondary standards for each of the five clinically relevant variants at any range of percentage variant lower than the crude material variant percentage. Furthermore, additional (qualitative) data on other verified variants present in the materials are available for the broader validation of NGS pipelines but are not intended for calibration or diagnostic purposes. Accelerated degradation studies have indicated the retained stability of the materials at elevated temperatures (7 months at +56°C).
The collaborative study participants agreed with the proposed genotype, consensus variant percentage, and gene copy number for each material, and agreed that the three materials be submitted for WHO ECBS approval as the WHO 1st International Standard for HCT 15 Cancer Genome, the WHO 1st International Standard for MOLT-4 Cancer Genome, and the WHO 1st International Standard for ATDB102 Reference Genome (18/118, 18/130, and 18/164 respectively).

Introduction

The development of precision molecular technologies, for example NGS and dPCR, along with advanced computational data analysis approaches has allowed the shift from traditional single biomarker analysis towards the development of multiplexed diagnostic panels (Offit, 2016) and the use of genomic-based assays for the selection of the most appropriate targeted therapies and the study of acquired resistance (Hayes *et al.*, 2015).

Good examples of the employment of multiplex diagnostics assay to improve patient care are the Oncomine Dx Target Test (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the FoundationOne CDx (Foundation Medicine, Cambridge, Massachusetts, USA). The Oncomine Dx Target Test received US Food and Drug Administration (FDA) pre-market approval in 2017; this test detects single-nucleotide variants (SNVs) and deletions in 23 genes from DNA and fusions in *ROS1* from RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples from patients with non-small cell lung cancer (NSCLC) using the Ion PGM Dx System and serves as a companion diagnostic for multiple therapies, yielding results useful to predict treatment response. The FoundationOne CDx, also FDA approved in 2017, detects variants in 324 genes and 2 genomic signatures in any solid tumor; the test is a companion diagnostic for several solid tumor therapies to identify patients with certain genomic variants who may benefit from specific treatments for non-small cell lung cancer, melanoma, breast, colorectal, and ovarian cancers.

Multiplex target analysis, and NGS-based tests, in particular, allow the investigation of different aspects of the human genome including presence of SNVs, small insertions and deletions, copy number alterations, structural rearrangements, and loss of heterozygosity in tumor DNA samples as well as characterization of transcriptomes and epigenomes; thus the clinical utility of NGS-based cancer genomics is rapidly expanding. Moreover, due to the rapid uptake of this

technology for tumor profiling, there is a continual development of different strategies involving a variety of technologies and computational analysis pipelines for the detection and reporting of clinically-relevant somatic variants (Berger *et al.* 2018). Overall, all NGS methods include four major components: sample preparation, library preparation, sequencing, and data analysis; with each of these components there is a potential source of error impacting the final diagnostic outcome. Therefore, despite the progress made in this rapidly growing field, there are still several challenges for the successful clinical implementation of multiplex targets assays in cancer genomics, including the calibration of these somatic analysis tools for cancer genomics being hampered by a lack of well-characterized multiplex target tumor/normal reference standards (Craig *et al.* 2016).

In order to support clinical diagnostics in improving the identification of multiple somatic variants in cancer and the calibration of assays identifying these alterations, in 2017 the WHO ECBS (Expert Committee on Biological Standardization) endorsed the National Institute for Biological Standards and Control (NIBSC) proposal to develop the WHO 1st International Reference Panel for Cancer Genomes. However, because of the diversity and rapidly evolving field of clinically relevant variants, in 2018 NIBSC successfully proposed rather to develop individual WHO International Standards, such that an ongoing programme using a common approach to efficiently develop standards containing multiplex targets could be established. Additionally, it was proposed that each standard could be further formally characterized as and when new clinical variants are identified, and new data added to the original WHO International Standards.

In this first series of WHO International Standards for cancer genomes, NIBSC developed two candidate cancer gDNA standards that would allow the global harmonization of genomic diagnostics for the detection and quantification of five broadly clinically-relevant variants (*PIK3CA* p.E545, *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9, and *MAP2K1/MEK1* p.D67N) using NGS and dPCR, along with a wild-type material (18/164). The presence of other (non-clinical) variants in these materials will allow for the broader validation of NGS pipelines. The availability of primary standards for these five variants should improve the quality of cancer genomic diagnostics by enabling the calibration of assays and kits, the derivation of secondary standards for routine diagnostic use in determining testing accuracy and sensitivity, and the validation of NGS pipelines, thus providing inter-laboratory comparison towards the harmonization of variant measurement.
The three gDNAs (18/118, 18/130, and 18/164) were derived respectively from HCT 15 colon adenocarcinoma cell line (putatively carrying the *PIK3CA* p.E545 variant), MOLT-4 acute T lymphoblastic leukemia cell line (putatively carrying the *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9, and *MAP2K1/MEK1* p.D67N variants), and a putative wild-type lymphoblastoid cell line, ATDB102.

A collaborative study was conducted to confirm genotype of these five variants, derive consensus variant percentages and determine variant and total gene copy numbers. This information will be provided to the end-users along with the formula to derive dilution responses for each clinically-relevant variant so that assay calibration across a wide variant percentage range is possible. Additional qualitative genotyping data for other verified variants present in the

materials are available for the validation of sequencing pipelines but are not intended for calibration or diagnostic purposes.
A total of 5000 ampoules per each material are available from NIBSC. These standards are intended for use in *in vitro* diagnostics and relate to BS EN ISO 17511:2003 Section 5.5.

Aims of the Collaborative Study

The study evaluated three freeze-dried gDNA materials carrying five putative clinically-relevant markers (*PIK3CA* p.E545K, *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9, and *MAP2K1/MEK1* p.D67N), plus additional (unknown) variants, in an international collaborative study involving laboratories using a variety of established genotyping approaches, thereby assessing their suitability as WHO Cancer Genome International Standards for use as primary standards in the calibration of secondary standards, kits, and assays targeting the five clinically-relevant markers, and for the (non-diagnostic) validation of NGS pipelines based on additional sequencing data. The data were used to establish the genotypes and variant percentages for each of the materials. The materials were also evaluated at several dilutions (each material diluted in the nominal wild-type material 18/164). These data were used to derive consensus variant and total gene copy numbers for each of the clinically-relevant markers in each of the candidate cancer gDNA standards, and to establish a formula which determines how a dilution should be performed (with the wild-type ATDB102 Reference Genome material 18/164, or another wild-type gDNA aligned to 18/164) to generate further standards at any specified lower variant percentage for the clinically-relevant markers.

Candidate Materials

Three materials were evaluated as the proposed WHO 1st International Standard for HCT 15 Cancer Genome, WHO 1st International Standard for MOLT-4 Cancer Genome and WHO 1st International Standard for ATDB102 Reference Genome (18/118, 18/130, and 18/164 respectively). Putative genotypes for each of the two candidate cancer gDNA standards were indicated by the COSMIC Cell lines Project (COSMIC, https://cancer.sanger.ac.uk/cosmic) and the Cancer Cell Line Encyclopedia (Broad Institute, https://portals.broadinstitute.org/ccle/about) databases and verified in-house with droplet digital PCR (ddPCR; BioRad, Hercules, CA, USA), whole genome sequencing (WGS) with NextSeq 550 System (Illumina, San Diego, CA, USA), Sanger sequencing (Eurofins Genomics Germany GmbH, Ebersberg, Germany), and INVIEW Oncopanel All-in-one (tumor-specific target panel; Eurofins, Germany GmbH, Ebersberg, Germany), data not shown. Droplet digital PCR was also used to confirm the presence of two copies of each of the five wild-type alleles in the ATDB102 gDNA (material 18/164), which when used in the dilution of the candidate cancer gDNAs standards enabled the calculation of the allelic ratio (variant: wild-type) and total gene copy number (variant plus wild-type) in each of the variant materials (see Copy Numbers: Establishment of a Dilution Formula, below).

All materials were of freeze-dried, purified gDNA extracted from three cell lines of either cancer or wild-type genotypes. Materials were freeze-dried in glass ampoules as an established format for ensuring long-term stability of gDNAs. Ideally, the formulation for reference materials should be as close as possible to the usual patient analyte, cover the entire analytical process, and

be applicable to methods in use throughout the world. Nevertheless, materials generated using gDNA derived from cell culture offers a close mimic of cancer patient samples by containing the variants of interest in the context of the complete genome, thereby making gDNA one of the most commutable materials for calibration and validation, especially for increasingly-used NGS approaches. Additionally, it is essential that the formulation be stable for many years, and that it is practically possible to produce batches of sufficient size to satisfy demand over a similar period of time. Furthermore, it should ideally be possible to generate replacement standards from the same source material to ensure consistency in formulation and to minimize value drift. It would be impossible to obtain sufficient primary patient material to produce materials for each of the five clinically-relevant markers at both high quality and sufficient quantity. Also, since the process of DNA extraction from cultured cells is different from that of solid tissue (typically formalin-fixed paraffin-embedded (FFPE) sections) or blood (if the analyte is circulating tumor DNA (ctDNA) in liquid biopsy, or blood cells for leukemia), providing the materials as cultured cells would not provide standardization for this step of the process in the most optimal way. Materials were provided as high molecular weight gDNA rather than the fragmented DNA often obtained from FFPE sections, or present as ctDNA, due to stability concerns and the intent to provide materials applicable to potentially any substrate used for the detection of the five clinically-relevant markers.

The nominal wild-type ATDB102 lymphoblastoid human cell line was established at NIBSC following Epstein-Barr virus (EBV)-transformation of isolated monocytes from a whole blood sample provided by a consenting healthy donor, and was confirmed as having a diploid genomic content by karyotyping (data not shown). The human colon adenocarcinoma HCT 15 and human acute T lymphoblastic leukemia MOLT-4 human cancer cell lines were derived from patient tumor tissue and obtained from the European Collection of Cell Cultures (ECACC; Public Health England, Salisbury, UK; Table 1).

Table 1. Source cell lines of materials 18/118, 18/130, and 18/164. Genotypes were indicated by COSMIC Cell lines Project and Cancer Cell Line Encyclopedia and/or in-house ddPCR, WGS, Sanger sequencing and INVIEW Oncopanel All-in-one sequencing. Na, not applicable.

NIBSC material code	Source cell line	Human tissue source	Gene	CDS	aa	COSMIC ID	Genomic coordinates (hg 19)
18/118	HCT 15	Colon adenocarcinoma	*PIK3CA*	c.1633G>A	p.E545K	COSM125370	3:178936091-178936091
18/130	MOLT-4	Acute T lymphoblastic leukemia	*TP53*	c.916C>T	p.R306*	COSM10663	17:7577022-7577022
			NRAS	c.34G>T	p.G12C	COSM562	1:115258748-115258748
			PTEN	c.795delA	p.K267fs*9	COSM30622	10:89717769-89717769
			MAP2K1/MEK1	c.199G>A	p.D67N	COSM1678546	15:66727483-66727483
18/164	ATDB102	EBV-transformed lymphoblastoids	Wild-type for all the above				na

All cell lines were tested and found negative for HIV1, HTLV1, Hepatitis B, Hepatitis C, and Mycoplasma by PCR; master and working cell banks were produced in-house to ensure a continual future cell supply. Large-scale cell culture for the wild-type and cancer cell lines was carried out, and frozen cell pellets of 5×10^7 to 1×10^8 cells prepared. Genomic DNA was extracted from the cell pellets using Gentra Puregene chemistry with a Gentra Autopure LS robot (Qiagen, Hilden, Germany). The DNA extraction process involved RNAse treatment, protein denaturation, protein removal, and 70% ethanol washing. The use of 70% ethanol is an established method for viral inactivation (Roberts *et al.*, 2007). Additionally, gDNA extracted in-house using the same purification procedure from other EBV-transformed cell lines did not show EBV infectivity (Hawkins *et al*., 2010). However, these materials should be handled with care, and according to local laboratory safety precautions for biological materials.
Many tumors exhibit high levels of genomic instability, including variant mosaicism and variability in gene copy number, zygosity, and overall ploidy. Cell lines derived from tumors are believed to provide a snapshot of the tumor at the time of biopsy (Lansford *et al*., 1999), with evidence to support this including data from histopathology, molecular genetics, receptor expression, gene expression, and drug sensitivity (Masters, 2000). However, it is unclear as to what extent variability continues to occur within the cell line over time. Overall it is expected that the materials used in this study are a useful mimic for the *in vivo* genomic complexity and variability of a tumor sample, and thus some commutability is achieved. Furthermore, since these materials are each prepared as a large batch, they are a long-term source of an unchanging genomic content.

Each of the gDNA materials was prepared at approximately 10 µg/ml gDNA concentration in 2.0 mM Tris, 0.2 mM EDTA, with 5 mg/ml D-(+)-trehalose dehydrate (Sigma-Aldrich, St. Louis, MO, USA; Table 2). Aliquots of 0.5 ml were dispensed into 2.5 ml autoclaved DIN glass ampoules (Schott, Pont-sur-Yonne, France) using an automated AFV5090 ampoule filling line (Bausch & Strobel, Ilfshofen, Germany) with the bulk continually stirred at a slow rate using a magnetic stirrer whilst at ambient temperature. The homogeneity of the fill was determined by on-line check-weighing of the wet weight of triplicate ampoules for every 90 ampoules filled, with any ampoule outside the defined specification (0.5000 g to 0.5300 g). The ampoules were partially stoppered with 13 mm Igloo stoppers (West, St Austell, UK) before the materials were freeze-dried in a CS15 (Serail, Argenteuil, France) to ensure long-term stability: the ampoules were frozen to -50°C, with primary drying at -35°C, 50 µbar, for 30 hours, followed by secondary drying at +30°C, 30 µbar, for 40 hours. The vacuum was then released and the ampoules back-filled using boil-off gas from high purity liquid nitrogen (99.99%), before stoppering *in situ* in the dryer and flame sealing of the ampoules.

Measurement of the mean oxygen head space after sealing served as a measure of ampoule integrity. This was measured non-invasively by frequency modulated spectroscopy (FMS 760, Lighthouse Instruments, Charlottesville, VA, USA), based upon the Near Infra-Red absorbance by oxygen at 760 nm when excited using a laser. Controls of 0% and 20% oxygen were tested before samples were analyzed to verify the method. Twelve ampoules were tested at random from each material; oxygen should be less than 1.14%. Residual moisture content was measured for the same 12 ampoules per material using the coulometric Karl Fischer method in a dry box environment (Mitsubishi CA100, A1 Environsciences, Cramlington, UK) with total moisture expressed as a percentage of the mean dry weight of the ampoule contents. Individual ampoules

were opened in the dry box and reconstituted with approximately 1-3 ml Karl Fischer anolyte reagent which was then injected back into the Karl Fischer reaction cell and the water present in the sample determined coulometrically. Dry weight was determined for six ampoules per material weighed before and after drying, with the measured water expressed as a percentage of the dry weight. Residual moisture levels of less than 1% was obtained for material 18/164 and greater than 1% for materials 18/118 and 18/130; residual moisture levels of less than 1% are typically expected, but where the dry weight is low (as here) the moisture level can be higher, with the materials still expected to demonstrate long-term stability (as seen for the similarly prepared WHO 1st International Genetic Reference Panel for Prader Willi & Angelman Syndromes, NIBSC panel code 09/140, which continues to demonstrate high stability ten years post-manufacture). Ongoing stability for these materials will be confirmed by accelerated degradation studies.

Upon reconstitution with 100 µl nuclease-free water, the DNA concentration was approximately 50 µg/ml in 10 mM Tris, 1 mM EDTA (1x TE buffer) with 25 mg/ml D-(+)-trehalose dehydrate. Homogeneity of each fill was determined by analysis of ampoules from the beginning, middle, and end of the filling process with quality and quantity of the freeze-dried gDNAs confirmed by 260/280 nm absorbance (Nanodrop, Thermo Fisher Scientific), Qubit fluorometric DNA quantification (Thermo Fisher Scientific), TapeStation electrophoresis (Agilent, Santa Clara, CA, USA), and ddPCR, which also acted as a pilot study to determine the performance of the materials in this increasingly-used diagnostic technique (Table 2). All three materials were of acceptable gDNA quality, as determined by the high DNA integrity numbers (DIN). Microbiological results were negative for all three materials. The ampoules are stored at -20°C at NIBSC under continuous temperature monitoring for the lifetime of the product. Shipping will typically be at ambient temperature, as studies have indicated the retained stability of the materials at elevated temperatures (7 months at +56°C, see Degradation Studies, below).

Table 2. Production and testing summary of the three materials of the proposed WHO 1st International Standard for HCT 15 Cancer Genome, WHO 1st International Standard for MOLT-4 Cancer Genome, and WHO 1st International Standard for ATDB102 Reference Genome. N/C, not calculated as all values were zero.

NIBSC material code	18/118	18/130				18/164
Nominal variant	*PIK3CA* c.1633G>A (E545K)	*TP53* c.916C>T (R306*)	*NRAS* c.34G>T (G12C)	*PTEN* c.795delA (K267fs*9)	*MAP2K1/MEK1* c.199G>A (D67N)	Wild-type
Date filled	18/05/2018	14/06/2018				03/10/2018
Mean DNA concentration (Qubit HS) upon filling (µg/ml; n=18 to 21)	12.47 (~5µg total; 18)	11.13 (~5µg total; 18)				11.12 (~5µg total; 21)
Mean fill mass (g; n=180 to 258)	0.5151 (258)	0.5159 (249)				0.5149 (180)
Mean pH upon filling (n= 12-14)	7.0 (12)	7.0 (12)				7.0 (14)
Coefficient of variation of fill mass (%; n= 180 to 258)	0.14 (258)	0.20 (249)				0.14 (180)
Mean dry weight (g; n= 6)	0.002	0.002				0.002
Coefficient of variation of dry mass (%; n= 6)	3.07	3.13				6.72
Mean residual moisture after lyophilisation (%; n= 12)	1.44124	1.71176				0.40374
Coefficient of variation of residual moisture (%; n= 12)	17.20	20.94				22.63
Mean residual oxygen (%; n= 12)	0.35	0.40				0.68
Coefficient of variation of residual oxygen (%; n= 12)	41.49	37.78				28.54
Mean DNA concentration (Qubit BR) upon reconstitution (µg/ml; n= 3-9)	48.60 (3)	48.70 (3)				48.99 (9)

Mean OD ratio ($A_{260/280}$ nm; n= 3-9)	1.84 (3)	1.84 (3)				1.91 (9)
Mean TapeStation DIN (n= 1-3)	9.10 (1)	9.20 (1)				8.73 (3)
Mean variant % (ddPCR; n= 3-9)	53.03 (3)	33.87 (3)	25.33 (3)	99.99 (3)	25.30 (3)	0.00 for all variants (9)
Coefficient of variation of variant % (%; n=3-9)	0.76 (3)	0.95 (3)	1.78 (3)	0.02 (3)	2.20 (3)	N/C
Number of ampoules available	5000	5000				5000
Presentation	Sealed, glass DIN ampoules, 2.5 ml					
Excipient	Trehalose, 5 mg/ml in 2.0 mM Tris, 0.2 mM EDTA buffer					
Address of facility where material was processed	NIBSC, South Mimms, Hertfordshire, UK					
Present custodian	NIBSC, South Mimms, Hertfordshire, UK					
Storage temperature	-20ºC					

Participants

Participants were recruited according their ability to test (and possibly quantify) the five clinically relevant variants.

A total of thirty nine participants were recruited to the collaborative study, through membership of the European Molecular Genetics Quality Network (EMQN; Manchester, UK) and UK National External Quality Assessment Service (UK NEQAS) for Molecular Genetics (Edinburgh, UK), publications on genomic diagnostics for the five clinically-relevant markers using NGS and dPCR, an open call on social media, and personal contacts. Four participants were unable to proceed with the study, either due to import constraints (n=1), costs associated with the import of the materials (n=1), or limited laboratory resources (n=2); all remaining thirty five participants took part in the study (Appendix I) and returned data. Twenty two countries were represented by the participants returning results, encompassing Europe, Asia, North America, South America, and Australia. Each participant was assigned a code number (1 to 39) which does not reflect the order of listing in Appendix I. Where participants submitted data from more than one method, each method is referred to by an alphabetical suffix, for example 05a and 05b. Data from a total of 38 methods were returned.

Study Design

Triplicate coded samples of the three gDNA materials (n= 9) were sent to each laboratory with instructions for reconstitution and storage. Overall the materials were each to be tested at five different dilutions (crude, 1:1.4, 1:2, 1:4, and 1:10), by dilution with the nominal wild-type material 18/164. However, since it was not reasonable to request each laboratory to analyze such a high number of samples, the materials and their dilutions were distributed amongst the participants based upon their method and reported assay sensitivity. Diluted samples were assigned to laboratories according to the limit of detection (LOD) declared during the recruiting phase; details of the collaborative study design are provided in Appendices II and III. Participants were asked to perform their routine testing method(s) for the investigation of the five variants by testing the nine coded materials over three separate days, such that on each day all three materials are tested at a single dilution, plus two of the materials were each tested at an additional dilution (n=15 tests total). One participant (Laboratory 05) performed only nine tests due to their costly WGS approach; one participant (Laboratory 07) also performed only nine tests due to the delayed receipt of the materials.

Participants were requested to use different batches of reagents and/or different operators if possible, alongside in-house patient samples (or other control materials) if typically used. Laboratories were asked to report quantitative results where possible (including sequencing depth for NGS analysis and copies/µl for dPCR analysis), together with the clinical interpretation, in a Microsoft Excel template provided by NIBSC. A second Excel template was given for the logging of full details of the techniques used, any reference samples used, and reasons for failure of any of the samples tested. The two Excel templates, along with raw data for each sample tested were to returned by uploading to a dedicated (secure and encrypted) ShareFile Web Page hosted by NIBSC (https://nibsc.sharefile.com/r-r8365f8e6d164eb2b).

Collaborative Study Results

Two main principal quantitative technologies were used by the thirty five participants of the collaborative study: Illumina sequencing (Illumina), Ion Torrent: Proton /PGM sequencing (Thermo Fisher Scientific), along with one laboratory using GeneReader NGS System (Qiagen), and three laboratories using dPCR (Figure 1 A).

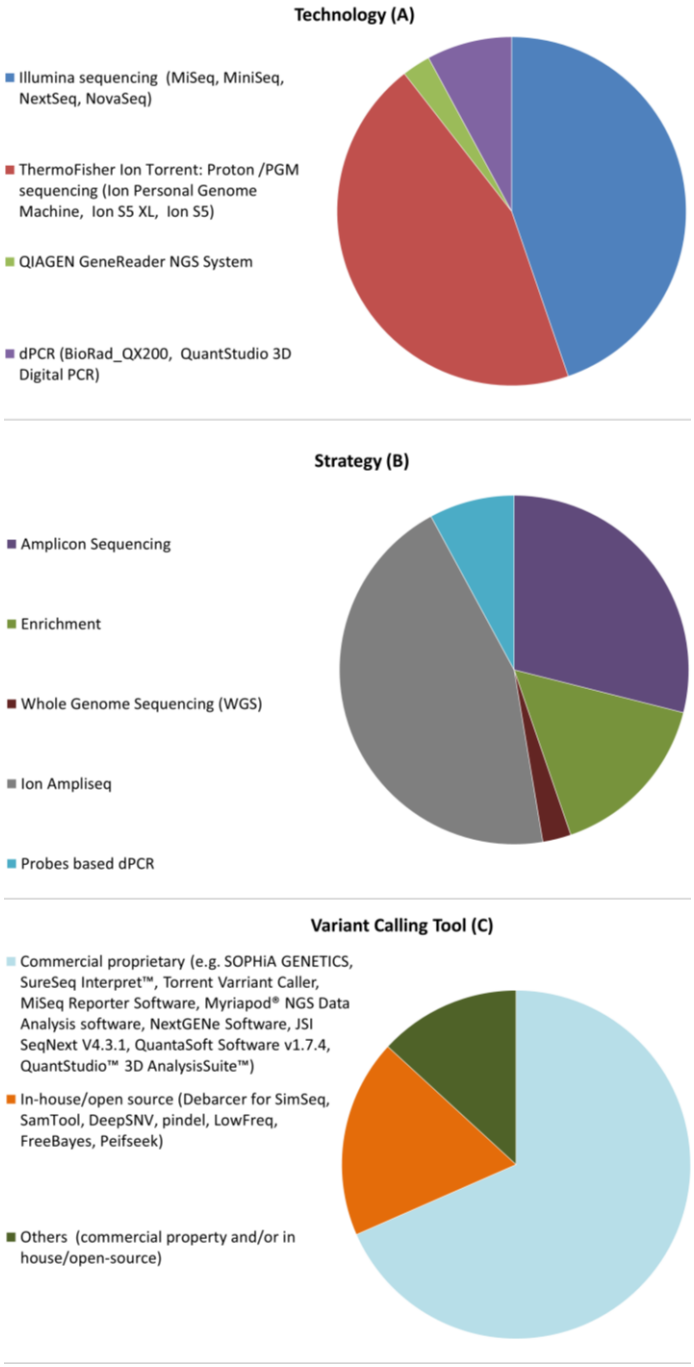

Figure 1: **Technologies, strategies and variant calling tools used by collaborative study participants.** (A) n=17 for Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq), n=17 for ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5), n=1 for Qiagen (GeneReader Platform), n=3 for dPCR (BioRad_QX200, QuantStudio 3D Digital PCR). (B) n=11 for Amplicon Sequencing, n=6 for Enrichment, n=17 for Ion Ampliseq, n=3 for Probe-based ddPCR, n=1 for WGS. (C) n= 26 for Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite™), n=7 for in-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek), n= 5 for others (e.g. commercial property and/or in house/open-source). Sanger sequencing analysis carried out by participant 17 for the targeting of the *PTEN* p.K267fs*9 variant is not captured here.

Illumina sequencing technology was used in combination with amplicon sequencing strategy by ten participants (laboratories 02, 03, 12, 14, 15, 17, 19, 23, 34, and 13); participant 13 also used the GeneReader NGS System. Illumina sequencing technology was also used in conjunction with an enrichment strategy by six participants (laboratories 01, 04, 05, 09, 11, and 16); participant 05 used the Illumina sequencing technology with both enrichment and WGS strategy.
All seventeen participants using the Ion Torrent: Proton / PGM sequencing technology (laboratories 07, 10, 18, 20, 21, 22, 24, 25, 26, 28, 29, 31, 32, 33, 35, 37, and 38) also followed the Ion Ampliseq strategy. In addition to the Ion Torrent: Proton / PGM sequencing technology in conjunction with Ion Ampliseq strategy, participant 31 also used the QuantStudio 3D Digital PCR (Thermo Fisher Scientific) for the analysis of *PIK3CA* p.E545K, *TP53* p.R306*, *NRAS* p.G12C, and *MAP2K1/MEK1* p.D67N variants whilst participants 30 and 39 used QX200 ddPCR (BioRad) for the analysis of all five variants. Participant 17 also carried out Sanger sequencing for the *PTEN* p.K267fs*9 variant to produce a qualitative-only result.
Several variant calling tools were used by the participants, herein simplified as three main subgroups (Figure 1 C):

1) Commercial proprietary, including participants using SOPHiA DDM, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, and QuantStudio™ 3D AnalysisSuite™ software;
2) In-house/open source software, including participants using Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, and Peifseek software;
3) Other, including participants using a mix of commercial property and/or in house/open-source software.

Details of the technology, strategy, and variant calling tool (along with the procedures and reagents) used by each participant are provided in Appendix IV.

Expected Genotypes

The nine blinded materials comprised triplicate samples of each of the three candidate materials; 18/118 (with the putative *PIK3CA* p.E545K variant), 18/130 (with the putative *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9, and *MAP2K1/MEK1* p.D67N variants), and 18/164 (putative wild-type), Table 3. The nominal variant genotypes were indicated by COSMIC Cell lines Project and Cancer Cell Line Encyclopedia and/or in-house ddPCR (data not shown). Additionally, according to COSMIC Cell lines Project and Cancer Cell Line Encyclopedia databases and in-house WGS with NextSeq 500 Illumina System (Illumina) and INVIEW Oncopanel All-in-one (tumor-specific target panel), the two candidate cancer gDNA standards were reported to contain additional variants, not yet determined to be clinically-relevant (data not shown).

Each material was tested as crude and at four dilutions (1:1.4, 1:2, 1:4 and 1:10), by dilution with the nominal wild-type material 18/164 on three separate days (in total, n=15 tests). The expected percentages for each clinically-relevant variant or the other variants present in each material are not shown as these were to be determined by the collaborative study.

Table 3. Collaborative study expected genotypes. Participants tested 9 blinded samples which comprised triplicates of the 3 materials of the different variant genotypes. Each of these materials was tested as crude, 1:1.4, 1:2, 1:4, or 1:10 diluted with the nominal wild-type material (18/164); expected variant percentages are not shown as they were to be established by the collaborative study.

NIBSC material code	18/118	18/130				18/164
Nominal variant	*PIK3CA* c.1633G>A (E545K)	*TP53* c.916C>T (R306*)	*NRAS* c.34G>T (G12C)	*PTEN* c.795delA (K267fs*9)	*MAP2K1/MEK1* c.199G>A (D67N)	Wild-type
Sample 1	✓					
Sample 2		✓	✓	✓	✓	
Sample 3						✓
Sample 4	✓					
Sample 5		✓	✓	✓	✓	
Sample 6						✓
Sample 7	✓					
Sample 8		✓	✓	✓	✓	
Sample 9						✓

Quantitative Genotyping Data

Quantitative data were reported for thirty eight datasets. An initial assessment of the within-laboratory variability showed a small number of triplicate datasets (seven cases) with high variability (difference between the minimum and maximum values >10). In these cases, individual replicate results were excluded if they differed from any of the other replicates by >5% (see Appendix V for further information; this rule resulted in all replicates being excluded in some cases). The participant's mean variant percentage for the triplicate tested samples was calculated and used for further analysis. The final overall consensus variant percentages for each gene and dilution were calculated as both the median and Huber's robust mean values of the participants' mean variant percentages using the R package 'WRS2' (Mair *et al*., 2017; R Core Team, 2018). As there were insufficient data to confirm the assumption of a normal distribution of results in all cases, and to avoid the influence of any outliers, median values are proposed as appropriate final consensus values for each variant (and dilution). For all genes, the robust mean obtained for the crude sample did not differ from the median by more than 1%.

A summary of final consensus percentage for each variant and dilution is shown in Table 4, with laboratory mean percentage variant values shown in Figure 2.
It is recognized that the NGS and dPCR data are herein considered as a single dataset for ease of analysis and timeliness. A preliminary statistical analysis of the different subgroups has been performed and indicates that the different technologies are broadly in agreement (Appendix VI).

Further analyses of the sub-grouped data, including sequencing depth, and copies/µl will be carried out at a later stage to maximize the value of such a large dataset.
Therefore, consensus variant percentages are presented as the overall median value for each material and dilution according to all quantitative methods, i.e. NGS and dPCR (Table 4)

Table 4. Summary data of final percentage for each variant and dilution tested in the collaborative study.

NIBSC material code	Nominal variant	Dilution	Number of participants	Minimum participant's mean	Maximum participant's mean	Overall mean	Standard Deviation	Median	Inter-Quartile Range	Robust Mean	Difference from Median
18/118	*PIK3CA* c.1633G>A (E545K)	Crude	20	25.9	55.3	50.7	6.4	52.1	2.9	51.9	-0.1%
		1:1.4	17	16.2	43.5	35.6	5.8	37.1	4.7	36.8	-0.8%
		1:2	13	14.7	29.3	24.9	4.0	26.2	2.0	26.1	-0.6%
		1:4	15	7.0	18.9	12.2	3.1	12.7	2.8	12.2	-3.9%
		1:10	10	3.0	6.5	5.1	1.0	5.2	1.3	5.2	-1.1%
18/130	*TP53* c.916C>T (R306*)	Crude	17	28.0	35.3	31.7	1.9	31.8	2.0	31.8	0.1%
		1:1.4	16	14.6	26.3	20.4	2.5	20.6	1.9	20.5	-0.6%
		1:2	15	12.0	18.0	13.9	1.4	13.8	1.2	13.8	-0.2%
		1:4	16	5.0	7.2	6.3	0.7	6.5	1.0	6.4	-1.4%
		1:10	10	0.0	2.7	2.1	0.8	2.3	0.6	2.2	-3.3%
18/130	*NRAS* c.34G>T (G12C)	Crude	21	22.7	30.0	24.8	1.6	24.7	2.0	24.6	-0.5%
		1:1.4	19	12.4	23.3	17.5	2.2	17.2	1.9	17.4	0.9%
		1:2	16	10.2	16.3	12.0	1.4	12.0	1.0	11.8	-1.2%
		1:4	16	4.4	7.3	5.8	0.7	5.7	0.6	5.8	0.8%
		1:10	9	0.0	3.0	2.1	0.9	2.3	0.2	2.3	0.5%
18/130	*PTEN* c.795delA (K267fs*9)	Crude	13	83.6	100.0	96.9	5.7	100.0	3.7	99.9	-0.1%
		1:1.4	12	50.2	81.2	70.2	7.4	70.6	4.9	70.9	0.4%
		1:2	11	30.2	59.8	46.2	7.3	46.7	2.6	47.2	-1.2%
		1:4	11	19.3	26.0	21.7	1.7	21.5	2.4	22.2	3.3%
		1:10	7	2.9	12.3	8.5	3.0	8.3	1.4	8.6	3.8%
18/130	*MAP2K1/ MEK1* c.199G>A (D67N)	Crude	15	22.4	28.8	25.1	1.7	25.3	1.6	25.0	-0.9%
		1:1.4	12	14.7	19.1	17.5	1.2	17.4	1.4	17.6	1.1%
		1:2	11	11.4	13.8	12.2	0.9	11.6	1.1	11.9	2.6%
		1:4	15	4.4	7.2	5.9	0.8	5.9	0.8	5.9	0.3%
		1:10	10	0.0	3.1	2.0	1.1	2.3	0.3	2.3	-0.4%

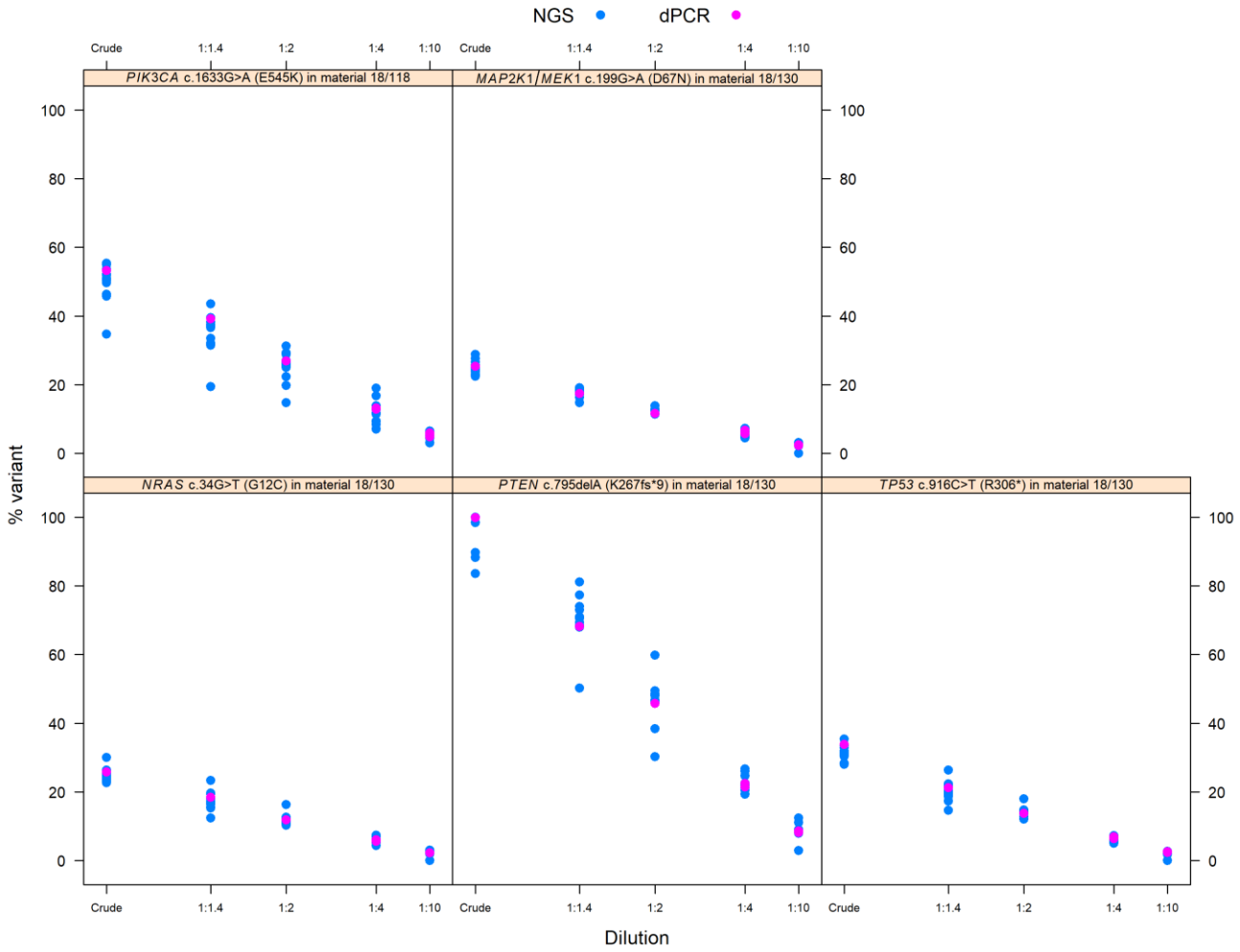

Figure 2. Participants' mean percentages for each variant and dilution. Data are shown the mean of triplicate samples for each material at crude, 1:1.4, 1:2, 1: 4 and 1:10 dilutions for each quantitative method.

Particular observations were:

a. Not all laboratories were able to test all five clinically relevant variants due to the coverage of their targeted NGS panels or the laboratory's focus;
b. Participant 04 reported 37.0% *PTEN* p.K267fs*9 variant in sample 2 of material 18/130 (1:4 dilution), compared with 21.0% for sample 5 and 22.0% for sample 8, so samples 2 was considered an outlier and excluded from further analysis. The participant also noted that not all samples achieved their in-house requirement for minimum coverage (1000x) and uniformity of coverage (99% of bases at >20% of the mean). As such some variants would not usually be reported after the application of somatic filters;
c. Participant 05 performed only five tests with method b due to the costly WGS approach;
d. Participant 07 was unable to test sample 8 of material 18/130 due to the ampoule being damaged during transit;
e. Participant 13 performed only three tests using method b;
f. Participant 16 reported 44.0% *PIK3CA* p.E545K variant in sample 1 of material 18/118 (crude), compared with 51.0% for sample 4, and 56.0% for sample 7, so sample 1 was considered an outlier and excluded from further analysis;
g. Participant 18 noted that reporting limits setting of the kit used are equal to or greater than 5% variant, therefore results below this are reported as negative. The laboratory also reported that accurate reporting of *PTEN* p.K267fs*9 variant was difficult for all samples due to the homopolymer regions;
h. Participant 23 failed in testing sample 7 at 1:1.4 dilution;
i. Participant 26 reported *PIK3CA* p.E545K variant as below LOD in samples 1 and 4 of material 18/118 at dilutions 1:2 and 1:4 and therefore was excluded from further analysis;
j. Participant 28 reported 18.3% *PTEN* p.K267fs*9 variant in sample 5 of material 18/130 at 1:2 dilution, compared with 47.8% for sample 2 and 45.4% for sample 8, so sample 5 was considered an outlier and excluded from further analysis. Additionally, this laboratory reported a wide range of percentage *PTEN* p.K267fs*9 variant in the three triplicate samples (2, 5, and 8) at 1:4 dilution, i.e. 5.3%, 0.0%, and 21.6%. Due to large difference observed in these triplicate results, they were all excluded from further analysis;
k. Participant 30 reported results as the mean of two replicates, except for *PIK3CA* p.E545K variant in sample 1 (dilution 1:4) which was a single replicate;
l. Participant 33 reported 33.4% *PTEN* p.K267fs*9 variant in sample 5 of material 18/130 at 1:4 dilution, compared with 20.1% for sample 2, and 20.5% for sample 8, so sample 5 was considered an outlier and excluded from further analysis. Additionally, this laboratory reported a wide range of percentage *PTEN* p.K267fs*9 variant in the three triplicate samples (2, 5, and 8) at 1:10 dilution, i.e. 0.0%, 19.2%, and 7.9%. Due to large difference observed in these triplicate results, they were all excluded from further analysis;
m. Participant 38 reported 39.7% *PIK3CA* p.E545K variant in sample 1 of material 18/118 (1:2 dilution), compared with 25.9% for sample 4 and 28.2% for sample 7, so sample 1 was considered an outlier and excluded from further analysis.

Variant Copy Numbers: Establishment of a Dilution Formula

In addition to percentage variant analysis, analysis of copy number variation (CNV), or the number of variant gene copies, is becoming increasingly relevant to clinical oncology assessment. Therefore, in this study the CNV of *PIK3CA, TP53, NRAS, PTEN, MAP2K1/MEK1* genes was analyzed.

As per the assigment of gene copy number in the WHO 1st International Reference Panel for genomic *KRAS* codons 12 and 13 mutations (Sanzone *et al*., 2017), each crude material of the proposed WHO Cancer Genome International Standards was tested crude and at four dilutions (1:1.4, 1:2, 1:4 and 1:10, by combination with the nominal wild-type material 18/164). The presence of two copies of the wild-type alleles for the above five genes in material 18/164 was determined by ddPCR by reference firstly to MRC-5, a primary diploid cell line derived from normal lung tissue of a 14 week-old male foetus (Jacobs *et al*., 1970) and commonly used in vaccine development, and for *in vitro* cytotoxicity testing, and secondly to a commercial human gDNA derived from multiple anonymous donors (catalogue number G3041, Promega, Madison, WI, USA). The use of this wild-type material (18/164) of assumed average diploid genome mass (~6.6pg) as indicated by the 2n=46 karyology (data not shown), and containing verified two wild-type copies of each of the five genes of interest in the dilution of the variant-containing materials enabled the calculation of the variant allelic ratio and total gene copy numbers (variant plus wild-type) in materials 18/118 and 18/130.

Since the dilutions were performed based on gDNA mass, for example a 1:2 dilution used 20ng cancer gDNA plus 20ng wild-type gDNA, the derived gene copy numbers for the cancer gDNA materials are per mass equivalent of a normal diploid human genome.
A model-fitting was performed using Python 2.7 SciPy (scipy.optimize.curve_fit function). The chosen model is given by the following equation:

$$y = \frac{x}{(ax + b)} \quad (1)$$

where x and y are the dilution performed and the output percentage variant used to fit the model, respectively, and a and b are the fitting parameters.

Table 5 summarizes the optimal fitting values for each material obtained after the fitting algorithm has converged.

Table 5. Derivation of the *a* and *b* coefficients for the five genes dilution curves. Coefficients, their lower and upper 95% confidence intervals (CI), and standard error (SE) were derived from a model-fitting algorithm using Python 2.7 SciPy.

Gene	coefficient *a*				coefficient *b*			
	value	lower 95% CI	upper 95% CI	SE	value	lower 95% CI	upper 95% CI	SE
PIK3CA	-0.00006	-0.00057	0.00045	0.00026	0.01923	0.01879	0.01966	0.00022
TP53	-0.00992	-0.01094	-0.00890	0.00052	0.04138	0.04048	0.04229	0.00046
NRAS	-0.00308	-0.00424	-0.00192	0.00059	0.04352	0.04252	0.04452	0.00051
PTEN	-0.00120	-0.00238	-0.00032	0.00053	0.01120	0.01039	0.01218	0.00046
MAP2K1/ MEK1	-0.00546	-0.00754	-0.00338	0.00106	0.04497	0.04316	0.04678	0.00093

In contrast to the dilution responses obtained for the WHO 1st International Reference Panel for genomic *KRAS* codons 12 and 13 mutations which were clearly non-linear, in this case the data appear to have a more linear trend (Figure 3).

Therefore, if the parameter *a* in equation (1) is set to zero, the fitting model becomes linear and the formula becomes:

$$x = \frac{1}{(b * x)} \quad (2)$$

Thus, when the best fit to the data is approximately a straight line, parameter *a* tends to zero as shown in Table 5 (e.g. -0.00006 for *PIK3CA*).

For completeness, the fitting has been performed using both non-linear and linear fitting models (Figure 3). This confirmed that the non-linear fitting function best fits the data:

1) when all data points lay approximately in a straight line, where the two models are overlapping (e.g. green and orange line in *PIK3CA* plot in Figure 3);
2) when non-linear behavior is observed (e.g. orange lines in *TP53* and *PTEN* plots in Figure 3).

Therefore, it is proposed to continue to use the non-linear approach as it would allow the use of a model that works in both scenarios and that can be applied to any gene/dilution response for these and future cancer gDNAs standards.

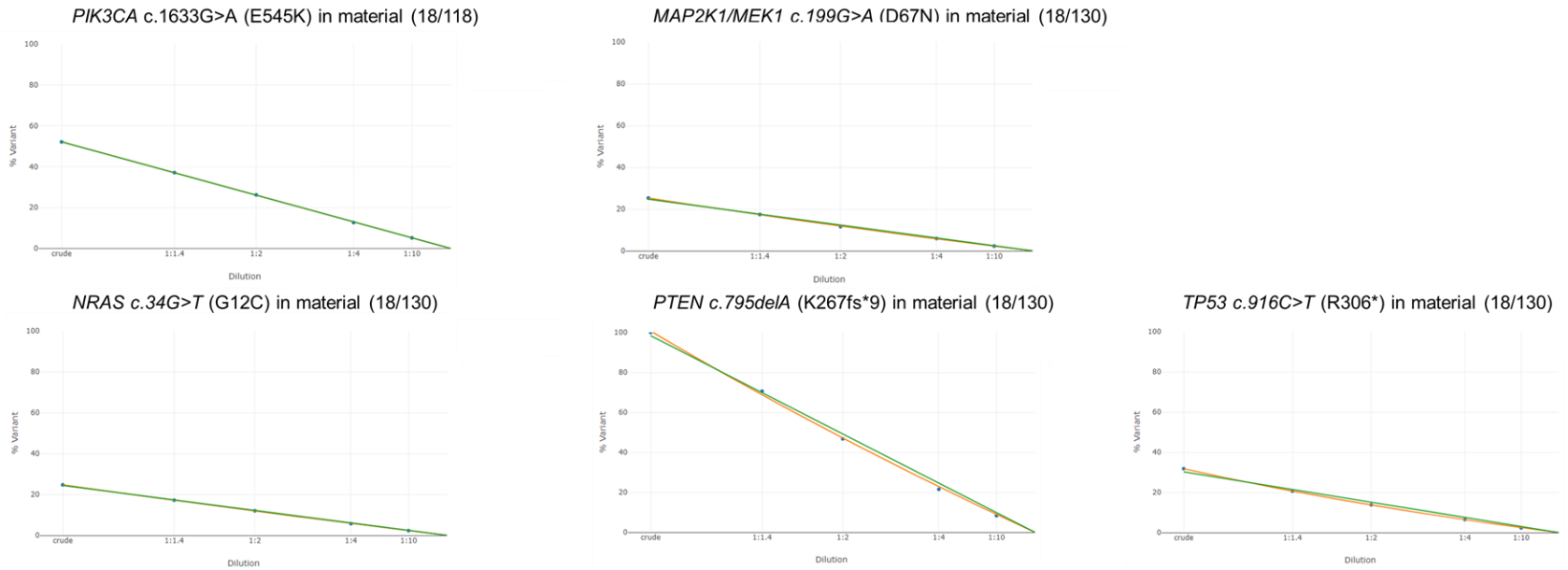

Figure 3: Assessment of linear and non-linear fitting of variant percentage dilution response. Data shown are the consensus variant percentages for each of materials 18/118 and 18/130 and their dilutions (crude, 1:1.4, 1:2, 1:4, and 1:10). The green lines represent the linear fitting model; the orange lines represent the non-linear fitting; the blue dots represent the collaborative study median values for each dilution.

The above model was used to derive zygosity and consensus copy number for the five clinically-relevant genes in each candidate cancer gDNA standard, whereby

$$variant\ copy\ number = \frac{2}{(b * 100)} \quad (3)$$

$$wild - type\ copy\ number = \frac{2 * (a + b)}{b} - \frac{2}{(b * 100)} \quad (4)$$

$$total\ copy\ number = \frac{2 * (a + b)}{b} \quad (5)$$

All gene copy numbers are noted to be <2, likely attributable to sequence or gene deletions, or even aneuploidy. Furthermore, the model was used to determine how the end-user may dilute each variant material (with wild-type 18/164, or an in-house wild-type gDNA aligned to 18/164) to achieve standards at any desired lower variant percentage. This was done by derivation of the coefficients for the allelic ratio (variant: wild-type) for the genes containing the clinically-relevant variants, and the total gene copy number for each material as shown in Table 6.

In the proposed use of these materials as calibrants and in determining assay limit of detection, the end-user may produce a further standard at any desired lower consensus percentage by dilution of the crude cancer gDNA standard with the wild-type standard 18/164 (or a wild-type gDNA calibrated to 18/164) by using the formula:

$$x = \left(\frac{variant\ copy\ number}{y} * 100 - total\ copy\ number \right) * \frac{1}{2} + 1 \quad (6)$$

where *x* is the dilution to be performed and *y* is the wanted variant percentage.
NIBSC will supply online interactive plots if end-users do not wish to perform their own calculations. The Instructions for Use will also contain dilution examples for the generation of a range of typical consensus variant percentages (Appendix VII). Thus, the proposed consensus variant percentages for the crude materials and the dilution formula can be used to prepare a range of standards at multiple variant percentages from which assay calibration can be achieved. It should be emphasized however, that these data derived from the consensus values are not necessarily empirical, but the materials and their dilutions achieve standardization since all participants will be deriving the same values. Furthermore it is acknowledged that these measurements are derived from targeted sub-gene regions, and therefore the extrapolation to whole gene copy number is inferred but considered to be appropriate due to the well-known phenomenon of cancer gene copy number variation in tumors, although this will be further verified.

Table 6. Proposed consensus variant and wild-type gene copy numbers from mathematical modelling in each of the crude candidate cancer gDNA standards. *reported as 0.00001 for completeness but likely to be zero.

NIBSC material code	Gene	Consensus variant copy number per diploid human genome mass	Consensus wild-type copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass
18/118	*PIK3CA*	1.04014	0.95373	1.99387
18/130	*TP53*	0.48327	1.03723	1.52050
	NRAS	0.45954	1.39909	1.85863
	PTEN	1.78544	0.00001*	1.78545
	MAP2K1/MEK1	0.44476	1.31226	1.75702

Whilst these variant and wild-type copy number data are derived by a model fitting established from the collaborative study dilution response data, it is recognized that some laboratories were able to perform CNV analysis in the collaborative study. However, no laboratories reported any CNV for the five clinically actionable variants, possibly because the threshold set for CNV detection was higher than CNV levels seen here, which are only marginally (although significantly) < 2 copies.

Particular observations from participants performing CNV analysis were:

a) Participant 13 reported no CNV detection using the QCI Analyze for GeneReader 1.5.0 software with AIT UMI FFPE 1.0 analysis workflow in the genes covered by their panel;
b) Participant 16 reported that their bioinformatic pipeline was still under development and thus it was not possible to provide reliable CNV (and fusion gene data);
c) Participant 18 reported no CNVs ≥7 copies using Thermo Fisher Scientific Torrent Suite ™ Software 5.2.2 integrated with Ion Reporter version 5.2 workflow in the genes covered by their panel;
d) Participant 29 reported no CNV detection using MAPD-0.5 in the genes covered by their panel;
e) Participant 32 reported no CNV detection using Thermo Fisher Scientific Torrent Suite ™ Software 5.2.2 integrated with Ion ReporterTM version 5.10 in the genes covered by their panel;
f) Participant 37 reported a deletion in *CDKN2A* gene (chr9:21,968,187-21,975,146) using an in house CNV tool in samples 2, 5 and 8 (crude and at dilution 1:1.4).

Consensus Value Assignment

Using the median variant percentage for each of five clinically-relevant variants, derived from the mean quantitative value of triplicate samples tested by NGS and dPCR methods (excluding statistical outliers), the genotype and consensus variant percentage for each of the three materials proposed as the WHO 1st International Standards for MOLT-4 Cancer Genome, HCT 15 Cancer

Genome and ATDB102 Reference Genome (18/118, 18/130, and 18/164) is shown (Table 7). These data will be reported in the Instructions for Use (Appendix VII).

End-users will be able to further dilute the variant materials (with wild-type 18/164, or another wild-type gDNA calibrated to material 18/164) using a dilution formula, to achieve further standards at a range of lower consensus variant percentages from which assay calibration is achieved.

The proposed dilution formula to be used is as follows:

$$dilution\ response\ = \left(\frac{variant\ copy\ number}{percentage\ of\ variant} * 100 - total\ copy\ number \right) * \frac{1}{2} + 1 \qquad (7)$$

where the consensus variant copy number and total copy number are specific to each gene (Table 7).
End-users will be referred to the WHO report (via the Instructions for Use) for further details of this complex data analysis.

Table 7. Proposed consensus values for materials 18/118, 18/130, and 18/164. Genotype, consensus variant percentage, and consensus copy numbers for use in calculating how each variant material may be diluted to prepare further standards at lower variant levels, are shown. As in Table 6, the wild-type gene copy number is calculated as 0.00001% but likely to be zero, therefore the consensus variant percentage is shown as 100.0%.

NIBSC material code	Nominal Variant	Consensus variant percentage (%)	Consensus variant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass
18/118	*PIK3CA* c.1633G>A (E545K)	52.1	1.04014	1.99387
18/130	*TP53* c.916C>T (R306*)	31.8	0.48327	1.52050
	NRAS c.34G>T (G12C)	24.7	0.45954	1.85863
	PTEN c.795delA (K267fs*9)	100.0*	1.78544	1.78545
	MAP2K1/MEK1 c.199G>A (D67N)	25.3	0.44476	1.75702
18/164	Wild-type			

Qualitative Genotyping Data

Participant 17 carried out qualitative testing (Sanger sequencing) for the *PTEN* p.K267fs*9 variant in samples 2, 5, and 8 for material 18/130 (crude and 1:2 dilution). These data could not be used in the derivation of the consensus values. However, they valuably demonstrate validity of using Sanger sequencing for the identification of the *PTEN* p.K267fs*9 variant in material 18/130.

In addition to the five clinical variants, thirty two participants also reported other variants in the three materials. Despite the data being reported quantitatively, meaningful quantitative

conclusions have not yet been derived because of the small dataset for each variant, primarily due to the select coverage of the targeted NGS panels used by the laboratories or the laboratory's focus. Nevertheless, the 30 most frequently reported variants for each material is given as additional information (Appendix VIII). It must be noted that variants are ranked according to the number of participants that have reported them, with no accounting for the variant percentage. These additional variants may be used for the general validation of NGS pipelines and will be included in the final Instructions for Use provided with the products as supplementary information. NIBSC will endeavor to further analyze the data towards publication with participants' agreement. Additionally, upon participants' agreement, full details of these additional variants, for example variant percentage and sequencing depth, will be available on request.

Material 18/164 (nominal wild-type)

Participants analyzing material 18/164 used similar terminology in reporting, broadly 'not present', '0%', and '-', or leaving an empty cell when the variant was not analyzed (for full data see Appendix IX), with the following exceptions:

a) Participant 02 reported 0.75%, 0.79%, and 0.70% for *PIK3CA* c.1633G>A in the three samples;
b) Participant 30 reported 0.29%, 1.07%, and 0.03% for *PIK3CA* c.1633G>A in the three samples; 0.04%, 0.04%, and 0.04% for *TP53* c.916C>T in the three samples; 0.06%, 0.05%, and 0.05% for *PTEN* c.795delA in the three samples; 0.02%, 0.02%, and 0.04% for *MAP2K1/MEK1*c.199G>A in the three samples;
c) Participant 31 method b reported 0.03%, 0.03%, and 0.4% for *PIK3CA* c.1633G>A in the three samples; 0.05%, 0.05%, and 0.05% for *TP53* c.916C>T in the three samples; 3.64%, 2.86%, and 3.60% for *PTEN* c.795delA in the three samples; 0.03%, 0.06%, and 0.03% for *MAP2K1/MEK1* c.199G>A in the three samples;
d) Participant 39 reported 0.02% for *PIK3CA* c.1633G>A, 0.06% for *TP53* c.916C>T, 0.10% for *PTEN* c.795delA, and 0.03% for *MAP2K1/MEK1* c.199G>A, all in sample 3.
e)

Given that >83% of results (Appendix IX) are negative, and the median result is zero in each case, the use of 'wild-type' for each of the five clinically-relevant variants in material 18/164 is supported.

Additionally, other currently non-clinically relevant variants were reported in the collaborative study assessment of this material and may also be of use in the validation of NGS pipelines (Appendix VIII).

Testing carried out by Participants

More than one batch of consumables (for example, reagents, sequencing cartridges, or flow cells), or different instruments (for example, sequencing instruments), or bioinformatics tools, were reported as used in all thirty eight datasets. Testing was noted as carried out by more than one operator by eighteen laboratories.

Reference Samples used in the Study

The following participants used positive and/or negative controls in their testing:

a) Participant 2 used gDNA from breast cancer cell lines as an internal control for quality of library preparation;
b) Participant 3 used two control samples (a gDNA source from an external supplier and an on-board control provided with the TruSeq Amplicon Cancer Panel kit, ACD1) for the library preparation and on-sequencing acceptance;
c) Participant 9 used a SeraCare Reference material (Seraseq™ Circulating Tumor DNA-I Variant Mix Kit (AF5-WT), Reference number 0710-0018) as internal control for the library preparation, sequencing run, and bioinformatic pipeline;
d) Participant 13 method b and participant 25 both used a Horizon Discovery reference material;
e) Participant 14 used a Diatech NGS positive control for the library preparation reaction and pre-PCR TE buffer as a negative control, resulting in libraries below the 2nM threshold;
f) Participant 15 used *KRAS*, *NRAS*, and *BRAF* positive FFPE samples;
g) Participant 19 used NA24385 from the Corriell Institute and a no template control;
h) Participant 21 used a buffy coat from a healthy person;
i) Participants 22 and 30 both used a no template control;
j) Participant 23 used a no template control to set up each library and 5% Phix spiked in each run;
k) Participant 28 used a positive control (Horizon Discovery Tru-Q Control HD732), a no template control, and an extraction blank;
l) Participant 31 method b used a positive and negative control in each preparation;
m) Participant 33 used a sample negative for all the clinically-relevant variants indicated;
n) Participant 35 used 20ng of a positive control (Horizon Discovery Tru-Q 3 5%);
o) Participant 37 used a control sample included in their regular diagnostics runs;
p) Participant 39 used a no template control as negative sample and Promega genomic DNA (Cat. No. G3041) as a positive control;
q) Ten participants did not use control samples;
r) Eight participants did not indicate whether or not control samples were used.

Regarding the human reference genome sequence used for the alignment of NGS data, participants 05 and 12 used GRCh38, whilst all others used GRCh37.

Comments from the Participants

Few participants provided additional comments. One participant commented that this was a great initiative and noted that the collaborative study instructions were clear, the study was well organized and the dedicated ShareFile Web Page was easy to use; the same participant also recognized the importance of analyzing cancer genome samples in their complexity and therefore the need to consider every structural variant (single nucleotide, indels, copy number variation, etc.) in the diagnostic environment.

Two participants noted the complexity of data coming from the collaborative study and recognized the scope in expanding the characterization of these materials and how valuable that would be for the genomic field.

One participant commented that glass ampoules were less-preferable than plastic tubes. This issue is recognized, and NIBSC is accruing long-term stability data for gDNA stored in plastic tubes as a possible alternative format. However, freeze-drying in glass ampoules which can be completely sealed is the currently preferred method for ensuring the long-term stability of WHO International Standards.

One participant commented on the comparability of these materials with FFPE patient samples and therefore would prefer the use of DNA extracted from the FFPE samples; our reasons for using gDNA are practical, and the reduced commutability with FFPE-extracted DNA is recognized, but as a standardization effort, the batch size, stability, homogeneity, replaceability, and usability in as many diagnostic approaches as possible are importantly addressed. Once standardization begins, by all laboratories aligning to the same reference standards, any differences between methods are revealed, adjustments can be made, and harmonization is achieved.

One participant commented that clinical interpretation would be easier if the DNA source (e.g. cancer type) would be revealed; this is acknowledged however for the assignment of the final consensus values it was important to withhold information that could be potential source of bias. One participant expressed some concerns about the standard deviation of *PIK3CA* c.1633G>A variant in material 18/118 and *PTEN* c.795delA variant in material 18/130 being higher than for other variants; this is acknowledged, however, the WHO international standards are indeed developed to highlight inter-laboratory variability which is reduced by their use as common standard for assay calibration. The same participant also expressed some concerns about the stability of the materials due to their humidity being near the threshold; this is also acknowledged, however in NIBSC's experience materials behaving in this way are still expected to demonstrate long-term stability (as seen for the similarly prepared WHO 1st International Genetic Reference Panel for Prader Willi & Angelman Syndromes, NIBSC panel code 09/140, which continues to demonstrate high stability ten years post-manufacture). Preliminary accelerated degradation stability monitoring at seven months' showed absence of degradation at elevated temperature. Ongoing stability monitoring for these materials will be confirmed by (annual) accelerated degradation studies.

All participants noted that would be happy to receive full details about other variants and to share their own findings about these additional variants with other participants.

Degradation Studies

Accelerated Degradation Studies

Multiple samples were reserved for in-house long-term accelerated degradation studies by storage at elevated temperatures (+37°C, +45°C, and +56°C), as well as for real-time stability monitoring at -20°C. Preliminary samples were assessed after seven months' storage and

demonstrated no apparent degradation at +56°C, -20°C, or the baseline temperature of -150°C, as measured by electrophoresis (TapeStation; Figure 4), 260/280 nm absorbance (Nanodrop), DNA quantification (Qubit BR), and ddPCR (Table 8), with data comparable to that seen at the time of manufacture (Table 2). The absence of degradation at elevated temperature resulted in the inability to predict loss of real-time stability. However, assurance that the materials are suitable for shipping at ambient temperatures was provided. Samples will continue to be assessed on a regular basis (typically annually) to ensure ongoing long-term stability for the lifetime of the panel. Previous experience with similar gDNA reference panels has demonstrated ongoing real-time stability at least twelve years post-manufacture.

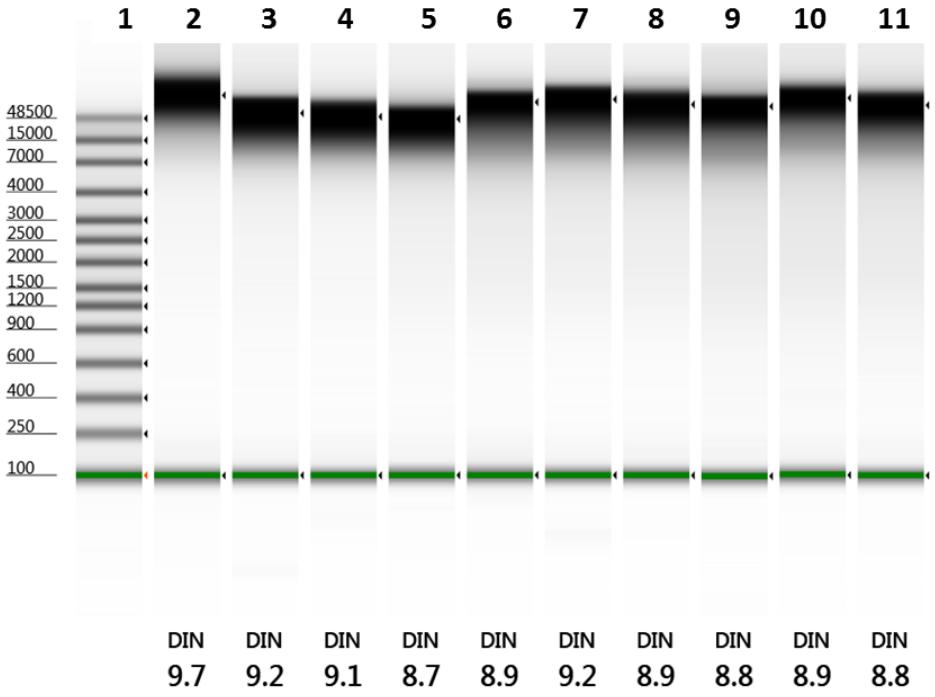

Figure 4. TapeStation analysis of materials 18/118, 18/130, and 18/164 following 7 months' storage at various temperatures. High quality gDNA as indicated by a high molecular weight band and the absence of lower molecular weight fragmented gDNA, and as quantified by a high DIN, was apparent for all materials, including at elevated temperature, indicating the absence of degradation. Lane 1, DNA ladder (catalogue number 5190-6292, Agilent); lane 2, positive control (catalogue number G3041, Promega); lanes 3-11, ampoules stored for seven months at -150°C, -20°C, or +56°C for each material as follows: 18/118, 18/130, and 18/164.

Table 8. Nanodrop, Qubit, and ddPCR analyses of materials 18/118, 18/130, and 18/164 following seven months' storage at various temperatures. gDNA of high purity and quality as indicated by expected 260/280 nm absorbances, approximately consistent DNA concentrations, and reproducible variant percentages, as measured by ddPCR, including at elevated temperature, indicated the absence of degradation in all materials. N/C, not calculated as all values were zero.

NIBSC material code	Temperature	Nominal variant	Mean OD, $A_{260/280}$ (n= 3)	Mean DNA concentration (QuBit BR, µg/ml; n= 3)	Mean variant % (ddPCR; n= 3)	Coefficient of variation of variant % (% ; n=3)
18/118	-150C	*PIK3CA* c.1633G>A (E545K)	1.89	51.93	52.67	0.90
	-20C		1.90	50.25	52.77	2.96
	+56C		1.86	49.10	53.17	2.85
18/130	-150	*TP53* c.916C>T (R306*)	1.94	45.90	33.07	4.06
		NRAS c.34G>T (G12C)			26.40	6.33
		PTEN c.795delA (K267fs*9)			99.97	0.06
		MAP2K1/MEK1 c.199G>A (D67N)			25.47	0.99
	-20C	*TP53* c.916C>T (R306*)	1.91	45.60	32.90	1.22
		NRAS c.34G>T (G12C)			25.07	1.61
		PTEN c.795delA (K267fs*9)			99.97	0.06
		MAP2K1/MEK1 c.199G>A (D67N)			24.60	6.91
	+56C	*TP53* c.916C>T (R306*)	1.91	45.20	33.80	3.36
		NRAS c.34G>T (G12C)			25.73	3.59
		PTEN c.795delA (K267fs*9)			99.97	0.06
		MAP2K1/MEK1 c.199G>A (D67N)			24.37	2.73
18/164	-150C	PIK3CA c.1633G>A (E545K)	1.90	44.10	0.01	173.21
		TP53 c.916C>T (R306*)			0.07	57.66
		NRAS c.34G>T (G12C)			0.00	173.21
		PTEN c.795delA (K267fs*9)			0.00	N/C
		MAP2K1/MEK1 c.199G>A (D67N)			0.09	51.73
	-20C	PIK3CA c.1633G>A (E545K)	1.92	41.80	0.00	N/C
		TP53 c.916C>T (R306*)			0.00	N/C
		NRAS c.34G>T (G12C)			0.00	N/C
		PTEN c.795delA (K267fs*9)			0.00	N/C
		MAP2K1/MEK1 c.199G>A (p.D67N)			0.05	88.63
	+56C	PIK3CA c.1633G>A (E545K)	1.87	42.40	0.02	173.21
		TP53 c.916C>T (R306*)			0.09	49.05
		NRAS c.34G>T (G12C)			0.01	173.21
		PTEN c.795delA (K267fs*9)			0.00	N/C
		MAP2K1/MEK1 c.199G>A (D67N)			0.06	116.38

Post-Reconstitution Stability Studies

End-users are recommended to use the materials on the day of reconstitution. However, in-house analysis determined reconstituted freeze-dried gDNA to be stable for at least four days at +4°C, or two months at -20°C (Figure 5 and Table 9), with data comparable to that seen at the time of manufacture (Table 2).

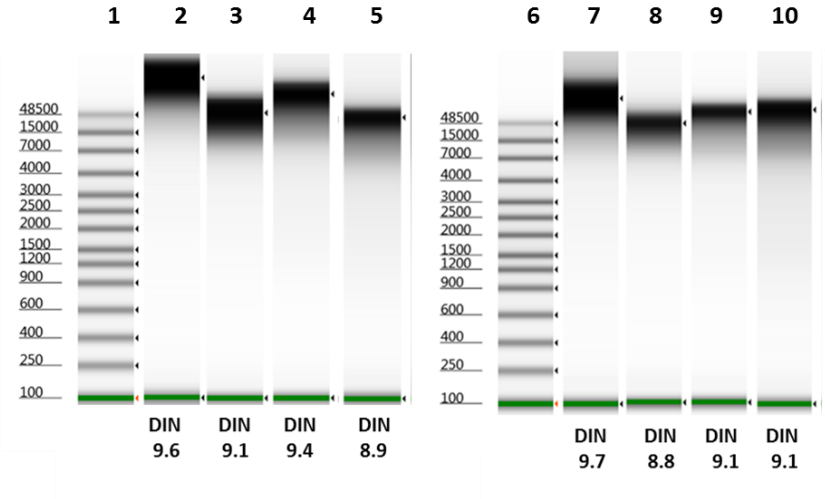

Figure 5. Post-reconstitution analysis of materials 18/118, 18/130, and 18/164 by TapeStation. High quality gDNA as indicated by a high molecular weight band and the absence of lower molecular weight fragmented gDNA, and as quantified by a high DIN, was apparent for all materials, indicating post-reconstitution stability following 4 days at +4°C or 3 months at -20°C. Lanes 1 and 6, DNA ladder (Agilent); lanes 2 and 7, positive control (Promega); lanes 3 to 5, reconstituted materials stored for four days at +4°C, and lanes 8 to 10 for two months at -20°C as follows: 18/118, 18/130 and 18/164*. *Material 18/164 was assessed at three months due to time constraints.

Table 9. Post-reconstitution analyses of materials 18/118, 18/130, and 18/164* by Nanodrop, Qubit, and ddPCR. gDNA of high purity and quality as indicated by expected 260/280 nm absorbances, approximately consistent DNA concentrations, and reproducible variant percentages, as measured by ddPCR, indicating post-reconstitution stability following four days at +4°C or two months at -20°C. N/C, not calculated as all values were zero. Material 18/164 was assessed at three months due to time constraints.

NIBSC material code	Temperature	Nominal variant	Mean OD, $A_{260/280}$ (n= 3)	Mean DNA concentration (QuBit BR, µg/ml; n= 3)	Mean variant % (ddPCR; n= 4-9)	Coefficient of variation of variant % (% ; n=4-9)
18/118	+4C/4d	*PIK3CA* c.1633G>A (E545K)	1.74	56.77	53.40	3.06
	-20C/2m		1.98	52.67	52.78	1.02
18/130	+4C/4d	*TP53* c.916C>T (R306*)	1.71	49.80	34.10	0.93
		NRAS c.34G>T (G12C)			25.88	2.70
		PTEN c.795delA (K267fs*9)			100.00	0.00
		MAP2K1/MEK1 c.199G>A (D67N)			24.73	3.87
	-20C/2m	*TP53* c.916C>T (R306*)	1.93	54.70	33.18	4.27
		NRAS c.34G>T (G12C)			25.70	2.46
		PTEN c.795delA (K267fs*9)			99.99	0.02
		MAP2K1/MEK1 c.199G>A (D67N)			25.00	3.44
18/164	-150C	PIK3CA c.1633G>A (E545K)	1.89	46.26	0.00	N/C
		TP53 c.916C>T (R306*)			0.00	N/C
		NRAS c.34G>T (G12C)			0.00	300.00
		PTEN c.795delA (K267fs*9)			0.00	300.00
		MAP2K1/MEK1 c.199G>A (D67N)			0.00	N/C
	-20C	PIK3CA c.1633G>A (E545K)	1.90	54.37	0.00	N/C
		TP53 c.916C>T (R306*)			0.00	N/C
		NRAS c.34G>T (G12C)			0.00	208.33
		PTEN c.795delA (K267fs*9)			0.00	N/C
		MAP2K1/MEK1 c.199G>A (D67N)			0.00	300.00

Discussion

The first International Genomic Reference Material was approved by the WHO ECBS in November 2004 and comprised a panel of three materials for the genomic diagnosis of Factor V Leiden. The same approach has been used at NIBSC for the subsequent preparation of a range of other International Reference Panels including for Prothrombin Variant G20210A, Factor VIII intron 22 inversion, Fragile X, Prader Willi & Angelman Syndromes, *JAK2* V617F and *KRAS* codon 12 and 13 variants. In the current study, a similar approach was adopted with the establishment of in-house cell line banks so that a continual future supply of the same gDNA materials could be assured, along with large-scale cell culture and gDNA extraction.
Unlike previous programmes whereby International Reference Panels were produced, here individual International Standards are proposed, i.e. the proposed WHO 1st International Standards for HCT 15 Cancer Genome, MOLT-4 Cancer Genome, and ATDB102 Reference Genome (18/118, 18/130, and 18/164). Also, unlike previously, these materials were characterized for multiple, rather than single actionable variants. The drivers of these choices were:

a) to begin an ongoing programme for the establishment of International Standards which provides increasing coverage of clinically-relevant cancer genomic variants;
b) to respond in a timely manner to the need for harmonized cancer variant diagnostics and measurement in response to treatment when multiplex targets technologies are used;
b) to accelerate the development of cancer gDNA standards by adding data for new variants in pre-existing standards as and when they become clinically relevant;
c) to generate a wild-type gDNA standard to serve as common reference genome and diluent for these and future cancer gDNA standards.

Additionally, in this study, supplementary currently non-clinically relevant variant data are provided to aid the broader validation of NGS pipelines, although these are not intended for calibration or diagnostic purposes. These data are also expected to be further refined following additional verification of the data generated in this study.

Thirty five laboratories participated in the international collaborative study to evaluate the three materials as the proposed WHO 1st International Standards for HCT 15 Cancer Genome, MOLT-4 Cancer Genome, and ATDB102 Reference Genome (18/118, 18/130, and 18/164). The study was designed to determine the performance of the three materials using a variety of established methods. In order to assess the consistency of the materials' performance, participants were requested to perform the study on three separate days and where possible with different operators and batches of reagents where possible.

All materials and their replicates performed well in the study, across the range of established methods used. Whilst the materials were tested primarily using NGS approaches and therefore are expected to be most suitable for this technology, they may also be used for single-analyte methodologies such as dPCR, considered by some as the 'gold standard' method for precision medicine and therefore ultrasensitive detection and absolute quantification (Alcaide *et al.*, 2018). The collaborative study has reflected this and provided a small dataset for cross-methodology comparisons with data from NGS and dPCR methods showing good concordance.

The collaborative study also showed that whilst most laboratories were using NGS, different approaches for sample quantification and preparation, library preparation (e.g. amplicon and hybridization capture), sequencing (e.g. Illumina, Ion Torrent: Proton /PGM and GeneReader NGS System sequencing) and tools for dry data analysis (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek) were used according the laboratory's focus and expertise. It is therefore recognized that each of these components can be the source of bias in the final diagnostic test outcome. A preliminary statistical analysis assessing the impact of different technologies and library preparation approaches showed that overall results are broadly in agreement despite the different approaches. Therefore, for ease of analysis and timeliness, data have been considered as a single dataset, whilst further in-depth studies, possibly with a larger dataset, are planned to maximize the value of this study.

Consensus percentages for each variant were therefore assigned to each of the materials as the median values of all NGS and dPCR methods, but further analysis of different subgroups to take into account other features (e.g. sequencing depth, copies/µl) will be carried out at later stages. Additionally, other currently non-clinically relevant variants were reported in the collaborative study assessment of the three materials, thus suggesting the value of these materials in mimicking the *in vivo* genomic complexity and variability of a tumor sample (18/118 and 18/130) and true genomic variability in the genome of healthy individuals (18/164). This information will be included in the final Instructions for Use provided with the products as supplementary information (Appendix VII). NIBSC will endeavor to further analyze these data towards publication with participants' agreement. Additionally, upon participants' agreement, the full details of these additional variants, for example percentage variant, sequencing depth, will be available on request.

It was also noted that reporting only variant percentage would not be fully representative of the genomic complexity of cancer samples, therefore as per the assignment of gene copy numbers in the WHO 1st International Reference Panel for genomic *KRAS* codons 12 and 13 mutations (NIBSC panel 16/250), it is proposed that the consensus variant, wild-type and total copy numbers of *PIK3CA* gene in material 18/118 and *TP53, NRAS, PTEN,* and *MAPK1/MEK1* genes in material 18/130 is also provided. Such information was derived from the response of the materials to dilution with the diploid (two wild-type gene copies) material (18/164) and can be applied to a dilution formula specific to each of the cancer gDNA materials in the preparation of additional standards at any further lower variant percentage. In contrast to the dilution responses obtained for the WHO 1st International Reference Panel for genomic *KRAS* codons 12 and 13 mutations which were clearly non-linear, in this case the data appeared to have a more linear trend therefore for completeness a comparison between a linear and non-linear fitting was carried out; this showed the non-linear fitting to be a more generically-applicable model able to work in all cases, including when reduced copy numbers were observed as for all five variants here. Whilst these variant and wild-type copy number data are derived by a model fitting established from the collaborative study dilution response data, it is recognized that some laboratories were able to perform CNV analysis in the collaborative study. However, no laboratories reported any CNV for the five clinically actionable variants, possibly because the threshold settled for CNV detection was higher than CNV levels seen here, which are only marginally (although significantly) < 2 copies or the genes not being covered by the analysis performed by the participants. It should be emphasized however, that the calculations derive the copy number

variation for each of the five genes, and make no inference on the rest of the genome; additionally the gene copy number derived from the variant percentage consensus values are derived from targeted sub-gene regions, and therefore the extrapolation to whole gene copy number is inferred and needs to be verified experimentally in future studies; moreover the data derived from the consensus values are not necessarily empirical but the materials and their dilutions achieve standardization since all participants will be deriving the same values.

Furthermore, it is noted that the wild-type proposed WHO 1st International Standard for ATDB102 Reference Genome could serve as common wild-type reference genome as well as diluent for the variant-positive standards; if insufficient wild-type material 18/164 were available for the preparation for dilutions, an in-house wild-type gDNA could be aligned to material 18/164 and then used as the diluent.

Finally, the provision of the materials as high quality gDNAs, albeit dissimilar to the fragmented DNA sometimes analyzed in patient samples, enables the harmonization of assays, kits and secondary standards targeting each of the five clinically relevant variants in any patient sample. Whilst the collaborative study was not able to assess the materials with other NGS technologies, such as Oxford Nanopore and PacBio, the most commonly used multiplex technologies in the cancer diagnostics environment were included, and it is expected that these standards will also perform in these other technologies. Future studies of these standards will also endeavor to further characterize other genomic alterations not typically captured by the methodologies used in this collaborative study, such as insertions, deletions, and translocations.

This effort to develop the first in a series of WHO International Standards for Cancer Genomes is considered to be an important first step towards the standardization of the rapidly expanding field of multiplex target analysis, and NGS- based cancer diagnostics. These candidate WHO International Standards will allow the derivation of secondary standards for routine diagnostic use in determining testing accuracy and sensitivity for *PIK3CA*, p.E545, *TP53* p.R306*, *NRAS* p.G12C, *PTEN* p.K267fs*9 and *MAP2K1/MEK1* p.D67N variants, thus providing inter-laboratory comparison towards the harmonization of variant measurement. The additional qualitative genotyping information allows the broader validation of NGS pipelines.

Conclusions and Proposal

The results of this international multi-centre study demonstrated that the three materials are suitable for use as WHO International Standards in laboratories carrying out cancer genotyping, with the proposed consensus variant percentages derived from the median values of NGS and dPCR methods as: 52.1% *PIK3CA* p.E545K (18/118), 31.8% *TP53* p.R306*, 24.7% *NRAS* p.G12C, 100.0% *PTEN* p.K267fs*9, and 25.3% *MAP2K1/MEK1* p.D67N (18/130), and associated gene copy numbers (Table 7), along with reference wild-type material (18/164). The materials may also be used for the broader validation of NGS pipelines.

These materials may be diluted (with wild-type material 18/164 or another wild-type gDNA aligned to 18/164) by application of a calculation specific to each material (based on its consensus variant and total copy number), to produce standards at a range of consensus variant percentages which enable the calibration of quantitative assays.

NIBSC would like to propose that the three materials be established as the WHO 1st International Standard for HCT 15 Cancer Genome, WHO 1st International Standard for MOLT-4 Cancer Genome, and WHO 1st International Standard for ATDB102 Reference Genome (18/118, 18/130, and 18/164 respectively).

These standards are proposed as the first in a series of standards for cancer genomics that will act as calibrants for an increasing number of clinically-relevant variants. Data may be added to pre-existing standards as further variants within those materials become significant.

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Appendix I. Collaborative Study Participants

Country	Participant(s)	Institution
Argentina	Javier Sfalcin Guadalupe Méjico Nadia Cambados	HERITAS, Instituto de Agrobiotecnología, Ocampo
Australia	Michael Christie Kym Pham	University of Melbourne Centre for Cancer Research, Victorian Comprehensive Cancer Centre, Melbourne
Austria	Leonhard Müllauer Felicitas Oberndofer	Department of Pathology, Medical University Vienna, Vienna
Austria	Christian Oberkanins Anne Berndt Kevin Gesson	ViennaLab Diagnostics GmbH, Vienna
Belgium	Pascal Vannuffel Celine DeRop	Institut de Pathologie et de Génétique, Department de Biologie Moleculaire-Oncohemato, Gosselies
Brazil	Rui Reis Flávia Escremim de Paula Escremim Gustavo Noriz Berardinelli	Molecular Diagnosis Center Barretos Cancer Hospital- PioXII Fundation, Barretos , São Paulo
Czech Republic	Jiri Drabek Rastislav Slavkovsky	IMTM, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc
Germany	Christian Thiede	AgenDix GmbH, Dresden
Germany	Joachim Arnemann Stefanie Stepanow	Laboratory Dr Wisplinghoff, Molecular Genetics/ 3. OG, Cologne
Germany	Andreas Jung	Pathologisches Institut, Medizinische Fakultät, LMU, München
Germany	Carina Heydt Janna Siemanowsky Sabine Merkelbach-Bruse	University Hospital Cologne, Institute of Pathology, Molecular Pathology Diagnostic, Cologne
India	Rashmi Khadapkar	SRL LIMITED, Mumbai
India	Atul Thatai	Dr Lal PathLabs Ltd., New Dehli,
India	Dadasaheb B Akolkar	Datar Cancer Genetics Limited, Nashik, Maharashtra
Ireland	Paul Kennedy	Beaumont Hospital, Haematology Department, Dublin
Italy	Gianmarco Musciano Edoardo Petrini	Diatech Pharmacogenetics, Jesi, Ancona
Italy	Alessandro Maria Vannucchi Paola Guglielmelli	CRIMM, Center Reserach and Innovation of Myeloproliferative Neoplasms, University of Florence, Florence
Italy	Massimo Negrini Laura Lupini Cristian Bassi	Dept of Morphology, surgery and experimental Medicine, University of Ferrara, Ferrara
Japan	Hiroki Beppu	SRL. Inc., Genetic & Chromosome Analysis Department, Tokyo
Luxembourg	Wim Ammerlaan Brian De Witt	IBBL (Integrated BioBank of Luxembourg), Dudelange
Malaysia	Low Chui Thean Tan Lu Ping	Institute for Medical Research, Molecular Pathology Unit, Cancer Research Centre, Kuala Lumpur
Netherlands	Tom van Wezel Nienke Solleveld	Leiden University Medical Center, Department of Pathology, Leiden
Portugal	Ana Carla Sousa	GenoMed - Diagnósticos de Medicina Molecular, Instituto de Medicina Molecular, Faculdade de Medicina Universidade de Lisboa, Lisbon
Singapore	Tan Min-Han	Lucence Diagnostics Pte Ltd, Singapore
Spain	Michele Biscuola	Laboratorio Patologia Molecular- Departamento de Anatomia Patologica- Hospital Virgen del Rocio, Seville
Sweden	Anders Ståhlberg Stefan Filges	Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg

	Gustav Johansson Junrui Li	
Switzerland	Adrian Willig Ray Marin	SOPHiA GENETICS, Geneva, Lausanne
Turkey	Serdar Ceylaner Haldun Dogan	Intergen Genetic Diagnosis and Research Centre, Ankara
UK	Jacqueline Chan Lyudmila Georgieva	Oxford Gene Technology (Operations) Ltd. (OGT), Begbroke Science Park, Begbroke, Oxfordshire, UK
UK	Susie Cooke	Wolfson Wohl Cancer Research Centre, Glasgow
UK	Kevin Balbi	Sarah Cannon Molecular Diagnostics (Part of HCA Healthcare UK), London
UK	A. Pia Sanzone	National Institute for Biological Standards and Control, South Mimms
USA	Adam Corner Diana Maar	Digital Biology Group, Bio-Rad Laboratories, Pleasanton
USA	Navid Sadri	UH Translational Laboratory, Cleveland
USA	Marco Tsui	Si Paradigm, Pine Brook

Appendix II. Collaborative Study Design

Each material was provided as triplicate coded ampoules. The materials were tested crude and at four dilutions (1:1.4, 1:2, 1:4 and 1:10).

Protocol	A	B	C	D	E & EdPCR
Participant	5, 7, 39	3, 13, 15, 16, 18, 21, 22, 35, 37, 39	1, 10, 12, 14, 17, 23, 25, 38, 39	19, 20, 24, 26, 28, 32, 34, 39	2, 4, 9, 11, 29, 30, 31, 33, 39
Dilutions/ Samples/ Materials	[crude] samples 1, 4, 7 (material 18/118)	[crude] samples 1, 4, 7 (material 18/118)	[crude] samples 1, 4, 7 (material 18/118)	[crude] samples 2, 5, 8 (material 18/130)	[crude] samples 3, 6, 9 (material 18/164)
	[crude] samples 2, 5, 8 (material 18/130)	[crude] samples 2, 5, 8 (material 18/130)			
	[crude] samples 3, 6, 9 (material 18/164)	[crude] samples 3, 6, 9 (material 18/164)			
		[dilution a], 1:1.4 samples 1, 4, 7 (material 18/118)	[dilution a], 1:1.4 samples 1, 4, 7 (material 18/118)		
		[dilution a], 1:1.4 samples 2, 5, 8 (material 18/130)	[dilution a] 1:1.4 samples 2, 5, 8 (material 18/130)		
			[dilution b], 1:2 samples 1, 4, 7 (material 18/118)	[dilution b], 1:2 samples 1, 4, 7 (material 18/118)	
			[dilution b], 1:2 samples 2, 5, 8 (material 18/130)	[dilution b], 1:2 samples 2, 5, 8 (material 18/130)	
				[dilution c], 1:4 samples 1, 4, 7 (material 18/118)	[dilution c], 1:4 samples 1, 4, 7 (material 18/118)
				[dilution c], 1:4 samples 2, 5, 8 (material 18/130)	[dilution c], 1:4 samples 2, 5, 8 (material 18/130)
					[dilution d], 1:10 samples 1, 4, 7 (material 18/118)
					[dilution d], 1:10 samples 2, 5, 8 (material 18/130)

Appendix III. Example of Collaborative Study Protocol and Results Forms

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The proposed WHO 1st International Standards for Cancer Genomes (CS626) Protocol B

Thank you for participating in the collaborative study to evaluate the proposed WHO 1st International Standards for Cancer Genomes.

Aim of the Study

The purpose of the study is to evaluate a panel of three freeze-dried genomic DNA materials extracted from cell lines expected to collectively represent variants in *PIK3CA*, *TP53*, *NRAS*, *PTEN*, and *MAP2K1/MEK1*. The performance of the materials will be assessed across a range of methods, resulting in the assignment of consensus values for clinically-relevant variants in these genes, and a large dataset for additional genes and variants, on which your agreement will be sought. A report will be submitted to the World Health Organization, proposing the establishment of the materials as International Standards for the calibration of secondary standards, kits, and assays for the clinically-relevant variants, and as reference materials to validate assay performance with multiple other genome-wide variants in these pan-cancer materials.

Table 1 lists the clinically-relevant variants expected to be represented in the three materials, for which quantitative reporting is requested:

Gene	COSMIC ID	AA Mutation	CDS Mutation	Mutation genome position (GRCh37) Assembly*	Mutation genome position (GRCh38) Assembly*
PIK3CA	COSM125370	c.1633G>A	p.E545K	3:178936091 -178936091	3:179218303-179218303
TP53	COSM10663	c.916C>T	p.R306*	17:7577022-7577022	17:7673704-7673704
NRAS	COSM562	c.34G>T	p.G12C	1:115258748 -115258748	1:114716127-114716127
PTEN	COSM30622	c.795delA	p.K267fs*9	10:89717769 -89717769	10:87958012-87958012
MAP2K1 / MEK1	COSM1678546	c.199G>A	p.D67N	15:66727483 -66727483	15:66435145-66435145

* indel positions recorded as in vcf format

Additional information (quantitative or qualitative) is requested for any other variant noted in your testing.

Please read the protocol completely before starting and contact Pia Sanzone (Pia.Sanzone@nibsc.org; telephone +44 (0)1707 641000) if anything is unclear.

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Samples

All samples are genomic DNAs freeze-dried in TE buffer and presented in sealed glass ampoules. Each participant will receive 9 'blinded' ampoules comprised of triplicate samples of the 3 materials. The amount of genomic DNA in each ampoule is approximately 5µg and the concentration after reconstitution with water will be approximately 50ng/µl in 1x TE (as determined by Qubit quantification).

Sample Testing Overview

Participants are requested to:

- Perform their routine testing method(s) for the analysis of the variants listed in Table 1. If more than one method is routinely used, please test the samples with all methods and complete multiple copies of the results forms (see "Reporting of Data", below).
- Report data for any additional variant noted in their testing (quantitative or qualitative), using the results forms (see "Reporting of Data", below).
- Where possible, provide raw data (VCF files, BAM files, .qlp files; see "Reporting of Data", below).
- Test the 9 samples once each but spread over 3 separate days using different batches of reagents and/or operators if possible i.e. test samples 1-3 on Day 1, samples 4-6 on Day 2, and samples 7-9 on Day 3.
- Additionally, test two samples per day at a pre-defined dilution (dilution a). i.e. test diluted samples 1 and 2 on Day 1, diluted samples 4 and 5 on Day 2, diluted samples 7 and 8 on Day 3.

Sample Handling and Testing Protocol

The samples should be handled as follows:

1. Store all unopened ampoules of the freeze-dried materials at -20°C or below. **Please note that because of the inherent stability of freeze-dried material, NIBSC may ship these materials at ambient temperature.**
2. DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. **Tap the ampoule gently to collect the material at the bottom (labelled) end.** Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. **Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar. Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule.** Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.
3. It is highly recommended that the material is used on the day it is reconstituted and is not stored. However, in-house analysis determined reconstituted freeze-dried genomic DNA to be stable for up to 3 days at +4°C (or 1 month at -20°C). Care should be taken to avoid cross-contamination with other samples.

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Protocol B, Day 1

- **Samples to be analysed:**
  - 'crude' sample 1;
  - 'crude' sample 2;
  - 'crude' sample 3;
  - 'dilution a' sample 1;
  - 'dilution a' sample 2;

4. Follow step 2 to open ampoules 1-3 and rehydrate the contents of each at room temperature with 100µl nuclease-free water; transfer each sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.
5. Allow the materials to reconstitute for 1 hour at room temperature and pipette well to mix.
6. The DNA concentration for all samples will now be approximately 50ng/µl in 1x TE.
7. Test all 3 samples (as 'crude' samples) by adding the required amount to your assay. You may dilute the samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
8. Additionally, test sample 1 following a dilution (dilution a) with sample 3 as follows; combine 25µl sample 1 with 10µl sample 3 in a new nuclease-free tube, and pipette well to mix. The DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required amount to your assay. As for step 7, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
9. Additionally, test sample 2 following a dilution (dilution a) with sample 3 as follows; combine 25µl sample 2 with 10µl sample 3 in a new nuclease-free tube, and pipette well to mix. The DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required amount to your assay. As for step 7, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.

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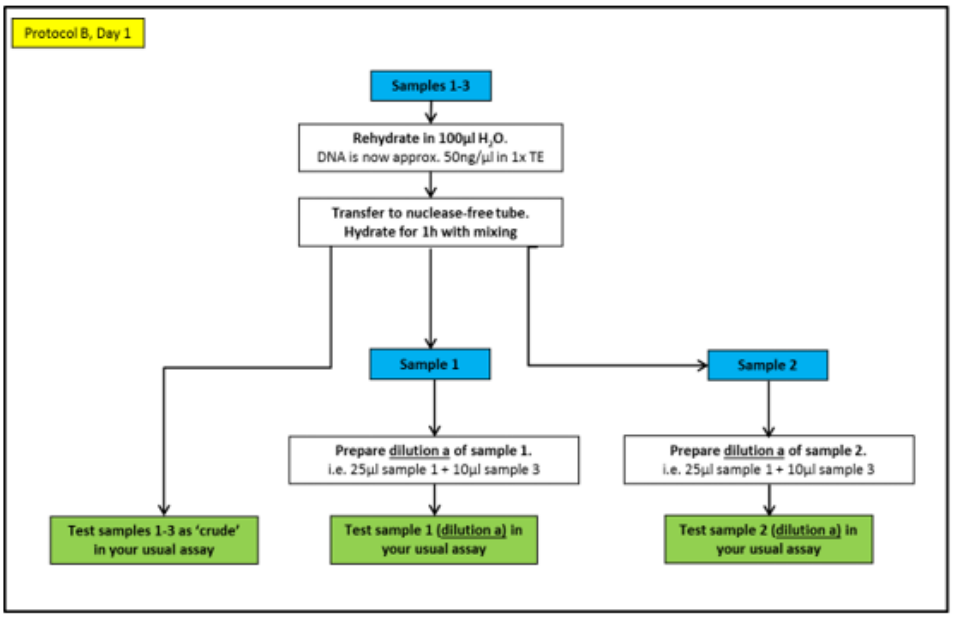

Protocol B, Day 2

- **Samples to be analysed:**
  - 'crude' sample 4;
  - 'crude' sample 5;
  - 'crude' sample 6;
  - 'dilution a' sample 4;
  - 'dilution a' sample 5;

10. Follow step 2 to open ampoules 4-6 and rehydrate the contents of each at room temperature with 100µl nuclease-free water; transfer each sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.
11. Allow the materials to reconstitute for 1 hour at room temperature and pipette well to mix.
12. The DNA concentration for all samples will now be approximately 50ng/µl in 1x TE.
13. Test all 3 samples (as 'crude' samples) by adding the required amount to your assay. You may dilute the samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
14. Additionally, test sample 4 following a dilution (dilution a) with sample 6 as follows; combine 25µl sample 4 with 10µl sample 6 in a new nuclease-free tube, and pipette well to mix. The DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required amount to your assay. As for step 13, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
15. Additionally, test sample 5 following a dilution (dilution a) with sample 6 as follows; combine 25µl sample 5 with 10µl sample 6 in a new nuclease-free tube, and pipette well to mix. The

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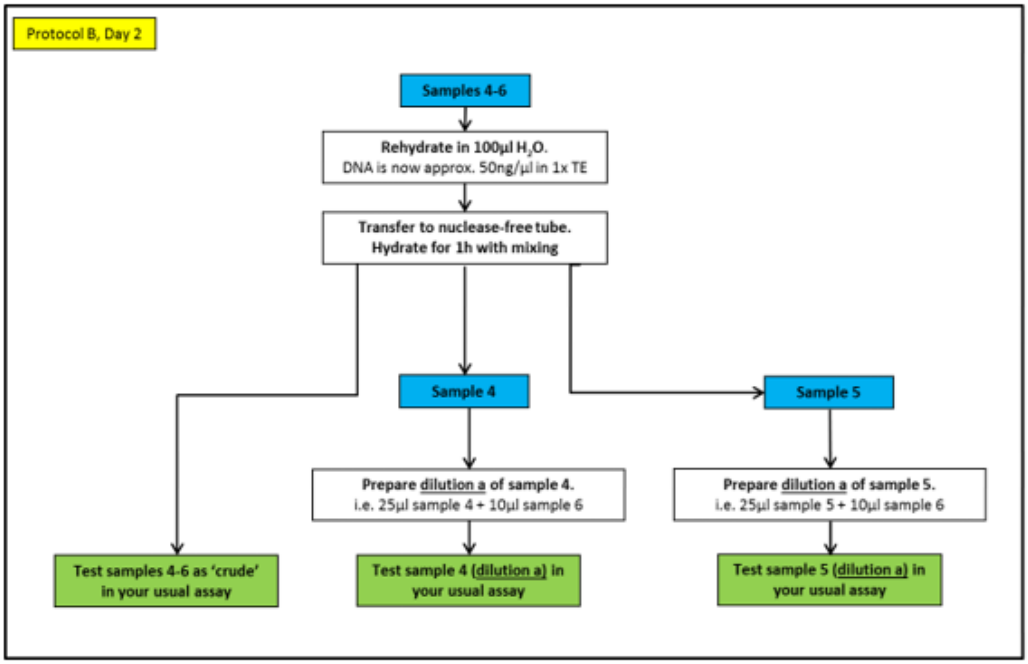
DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required amount to your assay. As for step 13, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.



Protocol B, Day 3

- **Samples to be analysed:**
  - 'crude' sample 7;
  - 'crude' sample 8;
  - 'crude' sample 9;
  - 'dilution a' sample 7;
  - 'dilution a' sample 8;

16. Follow step 2 to open ampoules 7-9 and rehydrate the contents of each at room temperature with 100µl nuclease-free water; transfer each sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.
17. Allow the materials to reconstitute for 1 hour at room temperature and pipette well to mix.
18. The DNA concentration for all samples will now be approximately 50ng/µl in 1x TE.
19. Test all 3 samples (as 'crude' samples) by adding the required amount to your assay. You may dilute the samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
20. Additionally, test sample 7 following a dilution (dilution a) with sample 9 as follows; combine 25µl sample 7 with 10µl sample 9 in a new nuclease-free tube, and pipette well to mix. The DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required

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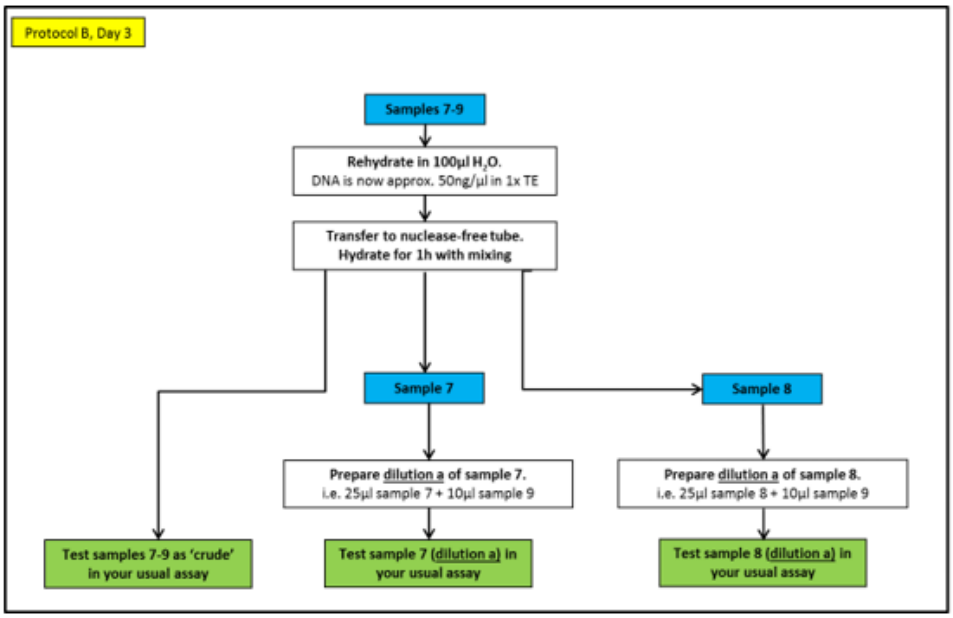
amount to your assay. As for step 19, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.

21. Additionally, test sample 8 following a dilution (dilution a) with sample 9 as follows; combine 25µl sample 8 with 10µl sample 9 in a new nuclease-free tube, and pipette well to mix. The DNA concentration for this sample will be approximately 50 ng/µl in 1x TE. Add the required amount to your assay. As for step 19, you may dilute these samples further (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.



Reporting of Data

- Overall findings (for the clinically-relevant variants listed in Table 1, and any additional variants) should be recorded in the Excel Results Forms provided with this protocol;

1. File name: PROTOCOL B_SNV&Indels_LabXX.xlsx:
    a. Record the % mutation detected for the clinically-relevant variants listed in Table 1, and any additional SNV or indel information (quantitative or qualitative data is acceptable).
    b. Save and re-name the file as PROTOCOL B_ SNV&Indels_LabXX.xlsx (where XX is the number assigned to your lab). Please check the file is saved as .xlsx
2. File names: PROTOCOL B_CNV_LabXX.xlsx, and PROTOCOL B_Fusion Genes_LabXX.xlsx:
    a. Record any additional variants (for example, CNV, fusions genes) in the appropriate file (quantitative or qualitative data is acceptable).
    b. Save and re-name the files as PROTOCOL B_CNV_LabXX.xlsx (where XX is the number assigned to your lab), and/or PROTOCOL B_Fusion Genes_LabXX.xlsx (where XX is the number assigned to your lab). Please check the files are saved as .xlsx
3. File name: COVERsheet.xlsx:

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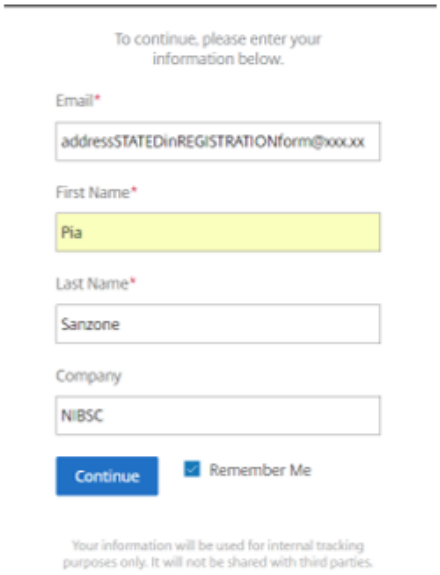
a. Provide details of the method(s) used by giving full details of all techniques, any in-house or commercial controls, and reasons for any failures (if known).
b. Save and re-name the file as COVERsheet_LabXX.xlsx (where XX is the number assigned to your lab). Please check the file is saved as .xlsx

4. Please also return copies of any raw data (VCF files, BAM files, .qlp files), or protocols used for the calculation of results e.g. digital PCR counts, formulae. Please append "_Lab XX" to each file name (where XX is the number assigned to your lab).

- All Excel and raw data files should be returned to NIBSC by uploading them to the dedicated (secure and encrypted) ShareFile Web Page as follows:
  - Click on the following link: https://nibsc.sharefile.com/r-r8365f8e6d164eb2b
  - Complete your details on the registration page as in the example below:



  - Click on "Continue" to see the following page:

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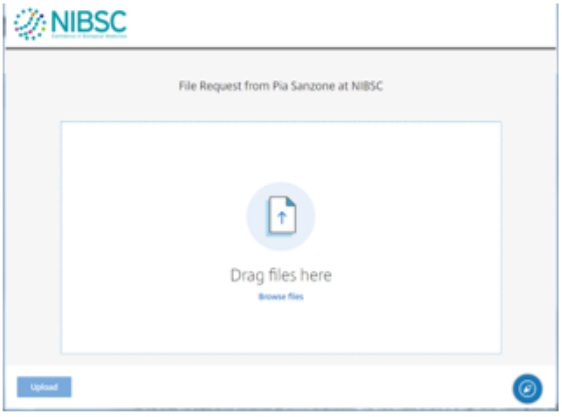
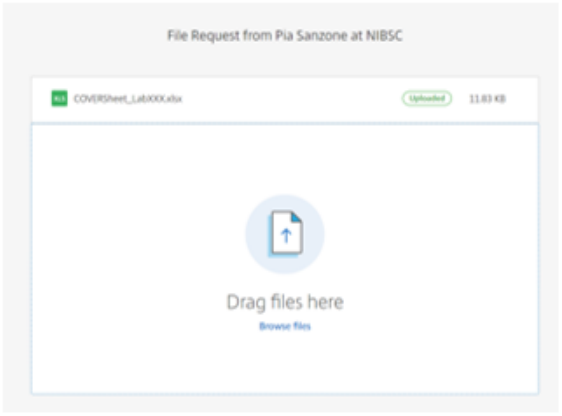


- Click on "Browse files", select the file(s) you wish to upload and click on "Upload".
- Once the upload has finished the following will appear on your screen:



Note:

- While you can upload files up to 100GB, **any upload that takes longer than 18 hours to complete will fail. Please contact us if you have any issues.**
- Uploaded data will only be available to NIBSC and will only be used for the above purposes of the collaborative study CS626 unless further written consent is obtained. All public communications, including WHO reports and publications will anonymise the participants. Data will be stored indefinitely but may be destroyed at any time upon written request from the submitting laboratory.

Please electronically complete and return the results forms online by Friday 21st December 2018.

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Sequencing Method	
Sequencing platform	e.g. Illumina NextSeq 500
Enrichment method (if any)	e.g. Agilent SureSelect Target Enrichment
Sequencing strategy	e.g. Whole-Genome Sequencing
Library construction protocol	e.g. Illumina Nextera DNA Flex
Run Metrics	
Read length	e.g. 250 nt
Read type	e.g. Paired-End
Total # of reads	e.g. 400 M reads
Total # of bases after trimming / filtering	e.g. 48 Gb
Average coverage against targeted region	e.g. 20.5 x coverage across whole genome per sample
Bioinformatic Analysis	
Reference Genome	e.g. GRCh37
Trimming / filtering tool(s) and settings	e.g. Trimmomatic v. 0.38: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36
Alignment tool(s) and settings	e.g. BWA v. 0.7.12-r1039, bwa mem -k 16 -M
Variant calling tool(s) and settings	e.g. GATK somatic short variant discovery pipeline (version updated 18/03/06)
CNV	e.g. CNV Kit, copy number events with minimum 100x coverage reported
Fusion gene calling tool(s) and settings	e.g. Delly v. 0.7.7, Ensembl release 92 annotation
Additional Information	
1. Please give full details of the method used, for example probe and primer sequences (and supplier(s), purification method(s)), PCR programme, PCR machine, software, purchased kits. Please indicate how any quantitative mutations results were generated (e.g. by reference to a standard curve, or by using another calculation). If a published method was used, please reference the publication. If the DNA was quantified prior to use, please indicate method:	
2. If you are unable to report results for any samples, please give details:	
3. Please indicate if different operators or batches of reagents were used:	
4. Please give details of any control samples used:	
5. Please make any other comments about the samples or the study organisation:	

Sample 8 (dilution a)

	SNV & Indels							
	Gene	CDS Mutation	AA Mutation	Mutation Frequency (%)	COSMIC ID or rs (optional)	Genomic coordinates (hg	Depth	ClinicalVar ID (optional)	Clinical Interpretation
(predicted) clinically-relevant variants	PIK3CA	c.1633G>A	p.E545K		COSM12530	3:178936091-178936091			
	TP53	c.992C>T	p.P308*		COSM10663	17:7577022-7577022			
	NRAS	c.34G>T	p.G12C		COSM562	1:115258748-115258748			
	PTEN	c.795delA	p.K267fs*9		COSM30622	10:89717769-89717769			
	MAP2K1/MEK1	c.199G>A	p.D67N		COSM1678546	15:66727483-66727483			
other genes & variants									

Sample 7 (crude) | Sample 8 (crude) | Sample 9 (crude) | Sample 7 (dilution a) | **Sample 8 (dilution a)**

Sample 8 (dilution a)

Fusion Gene Discovery (translocation and inversion)		
fusion pair 5'-3'	5' gene junction Genomic coordinates	3' gene junction Genomic coordinates

Sample 7 (crude) | Sample 8 (crude) | Sample 9 (crude) | Sample 7 (dilution a) | **Sample 8 (dilution a)**

Sample 8 (dilution a)

Copy Number Analysis			
Gene	CN	Depth	Genomic coordinates

Sample 7 (crude) | Sample 8 (crude) | Sample 9 (crude) | Sample 7 (dilution a) | **Sample 8 (dilution a)**

Appendix IV. Details of the Methods used in the Collaborative Study

		Subgroups		
Participant/ Method	**Procedure/Reagents**	**Platform**	**Strategy**	**Variant calling TOOL(S)**
Lab01	Library preparation using the Solid Tumor Solution by SOPHiA GENETICS. Amplification using Biometra thermocycler (Analytik Jena). Sequencing performed on Illumina MiSeq. Data analyzed using SOPHiA DDM.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab02	SiMSen-Seq (Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing) used. Sequencing perfomed on Illumina MiSeq. Data analyzed using Debacer v. 0.3.1. Wet analysis performed by three operators. Different batches used only for some reagents.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)
Lab03	Library preparation using Illumina TruSeq Amplicon Cancer Panel kit. Amplification using C-1000 thermocycler (Bio-Rad). Sequencing performed on Illumina MiSeq. Data analyzed using VCFtool v. 0.1.13, bcftool v. 1.1, htslib v. 1.1 Wet analysis performed by only one operator.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)

Lab04	DNA quantified by fluorometric quantitation using Qubit Fluorimeter with Qubit DNA dsDNA BR Assay kit from Invitrogen™ prior to library preparation. Samples processed with Oxford Gene Technology's (OGT) library preparation kit and hybridised with a custom panel created for this study, which enriches for all coding exons of PIK3CA, TP53, NRAS, PTEN, and MAP2K1, and an early access version of OGT's Hyb and Wash buffer. Sequencing on Illumina MiSeq using v2 2 x 150 bp cartridge. Data analysed using OGT SureSeq Interpret™ software v. 3.0.89 set to report on VAF >1% in the following targets: PIK3CA exon 10, NRAS exon 2, PTEN exon7, MAP2K1 exon 2, and TP53 exon 8. Wet analysis performed by three operators. One batch of reagents used.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab05_a	Library preparation using Agilent SureSelect Target Enrichment & SureSelectXT. Sequencing performed Illumina NextSeq 500. Data analyzed using deepSNV v. 1.22 and pindel v. 0.2.5b8 Alignment to human reference genome hg38. Wet analysis performed by three operators.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)
Lab05_b	Library preparation using Illumina TruSeq. Sequencing performed on Illumina NovaSeq. Data analyzed using deepSNV v. 1.22 and pindel v. 0.2.5b8. Alignment to human reference genome hg38. Wet analysis performed by three operators.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Whole genome sequencing (WGS)	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)
Lab07	Library preparation using Thermofisher Oncomine Solid Tumor DNA kit. Sequencing performed on Ion Personal Genome Machine. Data analyzed using Ion Reporter Software. Wet analysis performed by only one	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant

	operator. One batch of reagents used.			Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab09	DNA quantified using a flurometric method Quant-iT PicoGreen dsDNA Assay Kit - Thermo Fisher Scientific. Library preparation using the Archer Reveal ctDNA™ 28 panel (target enrichment panel) with Archer Variant Plex reagents and 50ng of input DNA. Amplification using BioRad T100 Thermal Cycler. Sequencing performed on Illumina MiniSeq. Data analyzed using Archer Analysis software v. 6.0.3.2. Wet analysis performed by two operators (operator A on day 1 and 2; operator B on day 3). Different bacthes of reagents used (Day 1: Archer® Reveal ctDNA™ 28 Kit REF SK0095/ LOT ADX0031101 + Archer Variant Plex REF: SK00117/ LOT ADX003510; Day 2: Operator A /Reagents: Archer® Reveal ctDNA™ 28 Kit REF SK0095/ LOT ADX003650 + Archer Variant Plex REF: SK00117/ LOT ADX003704; Day 3: Archer® Reveal ctDNA™ 28 Kit REF SK0095/ LOT ADX0031101 + Archer Variant Plex REF: SK00117/ LOT ADX003510).	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	Others (e.g. commercial property and/or in house/open-source)

Lab10	DNA quantified by fluorometric quantitation using Qubit™ 3.0 Fluorimeter with Qubit DNA dsDNA HS Assay kit from Invitrogen™ and diluted to the ~5ng/mL. Libraries prepared using 10ng of DNA of each sample accordingly to manufacturer's protocol for Oncomine™ Solid Tumor DNA Kit (Life Technologies™) and pooled. Amplification using Veriti™ 96-Well Thermal Cycler (Life Technologies™). The pool of libraries was sent to an external laboratory to proceed with next generation sequencing, where template preparation was performed with Ion One Touch™ System using the Ion PGM Template Kit. Sequencing performed on Ion Personal Genome Machine. Data analyzed using Ion Torrent Suite™ Browser version 5.8.0 and Ion Reporter™ version 5.10, using the workflow AmpliSeq Colon and Lung Cancer v2 single sample that detects and annotates low frequency variants (SNPs, InDels) from targeted DNA libraries. Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab11	DNA quantified by fluorometric quantitation using Qubit Fluorimeter. The overall method used for capture of targets is a lab-developed method which is broadly described as multiplex PCR followed by sequencing. Target-specific primer pairs are used to capture tagets in a multiplex PCR reaction, the products of which undergo a final universal amplification to prepare library suitable for sequencing on the Illumina platform. Sequencing reads are aligned to reference genome, variants are identified and variant allele frequency is calculated. Sequencing libaries were prepared on different days. Sequencing performed on Illumina MiniSeq. Data analyzed using in house variant caller, lofreq and Mutect. Wet analysis performed by only one operator. One batch of reagents used.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)

Lab12	Regions of interests are amplified by using in-house designed primers and standart PCR methods. PCR products checked by using 2% agarose gel electrophoresis, and repeated if needed. Pool of PCR products per each of the samples; when combining the PCRs, amplification efficiencies are taken into consideration. Purification of PCR pools using a vacuum filtering method and quantification using micro volume spectrophotometer. NexteraXT is used as the sample prep kit and V2-300 kit. Sequencing performed on Illumina MiSeq. Wet analysis performed by only one operator.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Others (e.g. commercial property and/or in house/open-source)
Lab13_a	DNA quantified using NanoDrop™ 8000 and by fluorometric quantitation using Qubit Fluorimeter with Qubit DNA dsDNA HS Assay kit from Invitrogen™. Libraries prepared using Illumina AmpliSeq Focus Panel. Sequencing performed on Illumina MiSeq. Data Analyzed using Illumina BaseSpace. Amplification using Applied Biosystems Veriti Thermal Cycler and Agilent 2200 TapeStation System. Wet analysis performed by three operators.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab13_b	DNA quantified using NanoDrop™ 8000 and and by fluorometric quantitation using Qubit Fluorimeter with Qubit DNA dsDNA HS Assay kit from Invitrogen™. Libraries prepared using QIAact AIT DNA UMI Panel. Sequencing performed on GeneReader. Data Analyzed using QCI Analyze for GeneReader v. 1.5.0 with AIT UMI FFPE v. 1.0 workflow. Amplification using Applied Biosystems Veriti Thermal Cycler and Qiagen QIAxcel Advanced system were used during library preparation. Wet analysis performed by only one operator. Only 1 set of samples (first 5 samples).	Qiagen (GeneReader Platform)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab14	Myriapod NGS-IL 56G Onco Panel used. Sequencing performed on Illumina MiSeq. Data analyzed using in house variant caller. Wet analysis performed by only one operator.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab15	DNA was not quantified. Proprietary Multiplex PCR amplification and parallel indexing of samples for further demultiplexing. Clinicaly relevant KRAS, NRAS (both genes exons 2, 3, and 4) and BRAF (exon 16) regions are targets. Amplification on Biorad CFX 96 thermocycler with realtime detection. Sequencing performed on Illumina MiSeq. Data analyzed using Illumina Somatic Variant Caller v. 3.5.2.1. Different batches of reagents used.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab16	DNA quantified by fluorometric quantitation using Qubit Fluorimeter. quantified DNA (250 – 500 ng) and subjected to enzymatic fragmentation and subsequent end repair, A-tailing and adapter ligation in a single tube. After adapter ligation fragments were dual-indexed via PCR, followed by 16h of hybridization with target-specific probes. Following target capture, samples were amplified for a second time. Libraries quantified by qPCR. Sequencing performed on MiSeq using a 300 cycle V2 cartridge. Amplification on an Eppendorf X50s using KAPA Hifi mix according to standard protocol and PCR programme. For purification steps JetSeq Clean beads from Bioline were used. Purified libraries from intermediate steps were quality controlled by running the samples on a Fragment Analyzer. Primer and probe sequences are proprietary and cannot be disclosed. Bioinformatic pipeline is under development, unable to provide reliable CNV and fusion gene data, and deletion detection not optimised. A deletion in the	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Enrichment	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)

	APC gene (chr5:g.112175539delC) in samples 1, 4 and 7 (crude) was observed and hence also in sample 1, 4 and 7 (dilution a), did not end up in the filtered SNV/InDel calls of samples 4 and 7 (crude). This is due to a cut-off setting of a minimum coverage of 35 for SNV/InDel calling. Obviously, a homozygous deletion would ideally have 0 reads at this position and thus 0 coverage. Because of this cut-off, chr5:g.112175539delC was filtered out in samples 4 and 7 (crude) with a local coverage of 30 and 34, respectively. The deletion was however called and is present in the respective vcf files. Thus, it cannot be ruled out that this might have happened for other deletions as well. Pre-filtered pipeline output for SNVs/InDels was further filtered for panel target genes. Sequencing performed on Illumina MiSeq. Data analyzed usingFreeBayes and lofreq. One batch of reagents used.			
Lab17	Illumina TruSight Tumor 15 was used according to manufacturer's instructions. Different opperators performed the analyses. Sequencing performed on Illumina MiSeq. Data analyzed using Sophia Genetics pipeline.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab18	OCAv3 (Oncomine Comprehensive Assay v3: Thermo Fisher Scientific Inc.) used. DNA amplified by multiplex PCR. Amplicon used for library construction. The reporting limits setting of the OCAv3 kit are equal to or greater than 5% for SNVs and INDELs, 7 copies for CNVs, and 40 reads for Fusion analysis. ・ Negative Results: The results are below reporting limits mentioned above. ・ Indeterminate Results: Accurate determination of PTEN deletions were difficult for all sample due to the homopolymer regions. ・ CNVs more than 7 copies are undetected for all samples. ・ Fusion detection is unable to analyze for all samples, as RNA samples are required fusions identification using this OCAv3 system. Sequencing perfomed on Ion S5 XL. Data analyzed using Ion Torrent Suite Software v. 5.2.2. Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab19	In-house IVD targeted panel (University of Melbourne IP) used. Sequenicng perfromed on Illumina MiSeq. Data analyzed using Illumina MiSeq reporter v. 2.6.3, GATK and Varscan 2. Wet analysis performed by only one operator. One batch of reagents used.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Others (e.g. commercial property and/or in house/open-source)

Lab20	DNA quatified by fluorometric quantitation using Qubit Fluorimeter and used to prepare genomic DNA libraries using Ion AmpliSeq Library Kit 2.0 (Life Technologies) and Ion AmpliSeq Custom Panel (Life Technologies) that had been designed using the AmpliSeq Designer Tool 2.2.1 (Life Technologies). Libraries purified using Agentcourt AMPure XP (Beckman Coulter, Brea, CA) and quantified with Ion Library Quantitation Kit (Life Technologies) on StepOne Plus system (Applied Biosystems). Customized primers designed to cover all coding sequence of NRAS, DNMT3A, IKZF1, EZH2, KRAS, TP53, and partial portion of genes SF3B1 (exons 13-16), NFE2 (exon 3), LNK (exons 2-4), U2AF1 (exons 2, 6, 8). Sequencing performed on Ion GeneStudio S5. Data analyzed using NextGENe software (version 2.4.1; SoftGenetics, State College, PA). Alignment to human reference genome hg38. Functionally annotated variants filtered based on the information retrieved from public databases (Single Nucleotide Polymorphism database [dbSNP], ExAc, Catalogue of Somatic Mutations in Cancer [COSMIC]). The potential pathogenetic role of filtered variants was assessed using available tools (SIFT, Polyphen,MutationTester, FATHMM, GERP++). All samples displayed a target coverage higher than 95% except for dilution B sample 1 (target coverage of 67.6%), but we were able to detect all the variants found in the other samples. For this reason and to avoid any other technical variables influencing the analysis, we decided to not run again the sample. Sequencing perfomed on Ion S5. Data analyzed using NextGENe v. 2.4.1, US patent 8271206. Wet analysis performed by two operators (operator A on Day 1 and 2; operator B on Day 3). Two different batches of reagents used (batch 1 on Day 1 and batch 2 on Day 2 and 3).	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab21	DNA was not quantified. Method outlined by Stasik et al. 2017 used. AmpliSeq ColonLungV2.20140523 and selfdesign (supplier: TibMolBiol Berlin) used for primers. AMpureXP sizeselection protocol (Agencourt® AMPure® XP Reagent (Beckman Coulter) used for purification. PCR programme: 98°C for 30 sec, followed by 35 cycles at 98°C for 5 s, XX°C* for 10 sec, and 72°C for 20 sec, with final extension at 72°C for 2 min, hold at 4°C. Amplification on Thermofisher Proflex Thermocycler. Sequenincing performed on Ion S5 XL. Data analyzed using Ion Torrent Suite Software 5.8.0, ION 510/520/530 KIT-CHEF 2R/I 1 KIT (catalog no. A34461, Thermofisher). Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab22	DNA quantified by fluorometric quantitation using Qubit Fluorimeter. Library preparation performed according to the Ion AmpliSeq Library Kit 2.0 protocol (Thermo Fisher). Amplification using Bio-Rad T100 thermalcycler. Two 19-cycle multiplex amplification reactions of the regions of interest performed by using AmpliSeq custom oligos, starting with 10 ng of genomic DNA per reaction. One-hundred and twenty-five different amplicons generated, overall, across 18.4 kb of target regions, covering the following genes: ATM, BIRC3, KIT, KRAS, MYD88, NOTCH1, NRAS, PIK3CA, PTEN, TP53. The following primers were used to amplify the four target regions: PIK3CA (CTGTAAATCATCTGTGAATCCAGAGG; AGCACTTACCTGTGACTCCATAGAA); TP53 (GCACCCTTGGTCTCCTCCAC; GATTTCCTTACTGCCTCTTGCTTCT); NRAS (CGACAAGTGAGAGACAGGATCAG; TGTAGATGTGGCTCGCCAATTAA);	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

	PTEN (TTAACCATGCAGATCCTCAGTTTG T; CTGTCCTTATTTTGGATATTTCTCC CAA). Ion Xpress Barcode Adapters Kit (Thermo Fisher) was to add Ion Torrent specific motifs to amplicons. Purification using Agencourt AMPure XP reagent (Beckman Coulter). Final libraries quantification using Bioanalyzer instrument with the High Sensitivity DNA Kit (Agilent), dilution and pooling in equimolar amounts. Twenty-five microliters of a 26 pM pool of all libraries were mixed with Ion Sphere Particles and clonally amplified in an emulsion PCR, performed in accordance with the Ion PGM Hi-Q View OT2 Kit protocol and using the Ion OneTouch 2 instrument (Thermo Fisher). Enrichment-System and Ion PGM Enrichment Beads (Thermo Fisher) used to enrich template-positive Ion Sphere Particles. Enriched sample loaded onto Ion 318. Sequencing performed on Ion Torrent Personal Genome Machine (PGM) following the Ion PGM Hi-Q View Sequencing Kit protocol (Thermo Fisher). Data analyzed using Torrent Suite software v. 5.10.0 (Thermo Fisher). Low-quality reads were removed, adapter sequences trimmed and alignment against a reference genome (hg19) performed by using the Torrent Mapping Alignment Program. The Variant Caller plugin was used to identify variations from the reference sequence and to quantify them. Intronic and synonymous alterations found were not annotated in the final result table. Wet analysis performed by three operators.			
Lab23	DNA quantified by fluorometric quantitation using Qubit Fluorimeter. Illumina Myeloid Trusight Panel used. Amplification using Verdi Thermocycler and analysed on a Miseq. Sequencing performed on Illumina MiSeq. Data analyzed using JSI SeqNext v. 4.3.1. Wet analysis performed by two operators.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter

				Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab24	Colon-Lung Panel (Thermofisher) used. Sequencing performed on Ion Torrent S5. Data analyzed using Torrent Server and Ion Reporter.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab25	DNA quantified by fluorometric quantitation using Qubit Fluorimeter. Ion Ampliseq Cancer Hotspot Panel V2 used. Sequencing performed using Ion Proton. Data Analyzed using Ion Reporter v. 5.0. Different batches of reagents used.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data

				Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab26	Primer sequences were selected via the AmpliSeq Designer Software and supplied by Thermo. All amplifications and purifications steps were performed according to theThermofisher's instructions. DNA was on a Qiacube, according to the Qiagen's protocol and quantified and quantified by fluorometric quantitation using Qubit Fluorimeter. Librairies were quantified by quantified by fluorometric quantitation using Qubit Fluorimeter and loaded on 138 chip on a 100 pM concentration each. Sequencing performed on Ion Personal Genome Machine. Data Analyzed using NextGene and NextGene viewer v. 5. 8.0.19. Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab28	Life Technologies Ampliseq and the Oncomine Solid Tumor panel used for library preparation. The library was prepared for sequencing using the Life Technologies High-Q View sequencing kit. Prior to analysis DNA quantified using an Agilent Tapestation 4200. Each set of samples ([1,2,3], [4,5,6], and [7,8,9]) were reconstituted, and the dilutions prepared by, a different oprators. The second and third set were sequenced by the same operator. All three sets were sequenced on separate days. The third set was sequenced using a	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Others (e.g. commercial property and/or in house/open-source)

	sequencing kit with a different lot number. Sequencing performed on Ion Personal Genome Machine. Data Analyzed using Torrent Variant Caller v. 5.0.4.0 with custom analysis parameters.			
Lab29	Library preparation was carried out using the Oncomine Comprehensive Assay ™V3 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturers instructions, using a total of 10 ng input DNA per sample. Amplification using GeneAmp® PCR System 9700, ABI, USA and SureCycler 8800, Agilent Technologies, USA); amplification with two primer pools included in the panel (Oncomine Comprehensive Panel V3). The amplicon mixes were combined and treated with FuPa reagent (Thermo Fisher Scientific, Waltham, MA, USA) to partially digest primer sequences and phosphorylate the amplicons, which were then ligated to multiplexing barcodes (Thermo Fisher Scientific, Waltham, MA, USA). Purification using Beckman Coulter (Nyon, Switzerland) AMPure XP or by JetSeq™ Clean (Bioline, USA) magnetic beads and amplified using Amplification master mix (Thermofisher Scientific). Prepared Library underwent quality control (QC) using an E-GelTM Agarose Gel 2 % (Thermofisher Scientific). Purified library was quantified by Real Time PCR Machin (Quant studio 12 k Flex, Thermofisher, USA) using Ion library TaqMan quantification kit (Thermofisher Scientific, USA) as per manufacturers instructions. Following the manufacturer's instructions, the volume of each of the prepared libraries was adjusted to add equimolar amounts of each library into the emulsion PCR for a final total molarity 100 pMoles. The emulsion PCRs were carried out using Ion PI™ Hi-Q™ OT2 200 Kit. (Thermofisher scientific, Waltham, MA, USA, cat no- A26434) and loaded in the	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

	Ion OneTouch™ 2 System, after which non-templated Ion Sphere Particles (ISP) beads were removed by magnetic bead purification (included in the Ion PI™ Hi-Q™ OT2 200 Kit). After ISP bead enrichment, each library was sequenced using the Ion PI™ Hi-Q™ Sequencing 200 Kit (Thermofisher scientific, Waltham, MA, USA); cat. no. A 26433. The enriched sequencing microreactors from each emulsion PCR were loaded in an Ion PI V3 Chip (Thermo Fisher Scientific; cat no- A 26770). Sequencing performed on Ion Personal Genome Machine. Data Analyzed using Torrent Variant Caller v. 5.10, Ion Reporte v. 5.10.			
Lab30	ddPCR QX200 from BioRad was used. No DNA quantification carried out and assumed that reconstituted DNA was at 50 ng/ µl; 60 ng of DNA added to each well. Assays from BioRad catalogue. Data analyzed using QuantaSoft v. 1.7.4. Amplification on Biorad C1000 Thermocycler. Only one well was excluded from analysis due to failed droplet formation. This was well 1D on Day 1 data collection. This was a replicate of two wells used so the sample was counted. 3 unique lots of all reagents and consumables were used for each day. Wet analysis performed by only one operator.	dPCR (BioRad_QX200, QuantStudio 3D Digital PCR)	Probes based dPCR	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab31_a	AmpliSeq design from ThermoFisher Scientific: Patho-HotSpot (IAD166800_231, bed-file provided) used. Libraries prepared using the AmpliSeq library kit 2.0 (4480441, ThermoFisher Scientific) following the manufacturers protocol MAN0006735. Multiplex PCR performed using 10 ng DNA input and 20 cycles. Input DNA was quantified using Qubit 4 fluorometer and the Qubit dsDNA HS assay kit (Q32851, Invitrogen). Quality of the NGS libraries controlled by qPCR kit Ion library TaqMan quantification kit (4468802, ThermoFisher Scientific). Template preparation performed on IonChef. Sequencing performed on Ion Gene Studio S5 with the 510 & Ion 520 & Ion 530 Kit -Chef (A34019, ThermoFisher Scientific) following manufacturer's protocol MAN0016854. Additional to the above mentioned bioinformatic analysis using the TorrentSuite software workflow, data analysed with NextGene v2.4.2.2 (Softgenetics) using standard settings for targeted resequencing. Allele frequency for variant call was 1 %. The results (bam/vcf) of both bioinformatic analyses (TorrentSuite & NextGene) are then combined using the Varvis software v1.8.0 (Limbus Medical Technologies). Sequencing performed on Ion S5. Data Analyzed using Variant Caller Plugin for Torrent Suite software v. 5.10. Wet analysis performed by two operators (operator A on Day 1; operator B on Day 2 and 3). One batch of reagents used.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab31_b	DNA quantified by fluorometric quantitation using Qubit Fluorimeter with Qubit DNA dsDNA HS Assay kit from Invitrogen™ and diluted it to 7.5 ng/µl or 10 ng/µL depending on TaqMan probes. dPCR performed using QuantStudio™ 3D Digital PCR Master Mix v2 (A26358, ThermoFisher Scientific), the QuantStudio™ 3D Digital PCR 30K Chip Kit v2 (A26316, ThermoFisher Scientific) and the TaqMan probes: PIK3CA: c_150852487_10, 10 ng/µL DNA input conc.; TP53:c__2403511_20, 7,5 ng/µL DNA input conc. and	dPCR (BioRad_QX200, QuantStudio 3D Digital PCR)	Probes based dPCR	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis

	MAP2K1/MEK1: c_335004619_10, 7,5 ng/µL DNA input conc. All probes ordered from ThermoFisher Scientific using cat.no 4351379 and the assay no. (see above). The preparation followed manufacturers protocol MAN0007720 (ThermoFisher Scientific). For all probes 60°C annealing temperature was used. Wet analysis performed by two operators (operator A on Day 1 and 2; operator B on Day 3). Analysis and interpretation were done by safe.			software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab32	Lung colorectal cancer assay used. Sequencing performed on Ion Personal Genome Machine. Data Analyzed using Ion Reporter v. 5.10, Varsome, IGV.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Others (e.g. commercial property and/or in house/open-source)
Lab33	Oncomine Solid Tumor Kit used for DNA libraries preparation. Amplification using Verity PCR instrument, 7900 HT Fast Real Time PCR system for libraries quantification. Template prepared using Ion Chef. Sequencing performed on GeneStudio S5. Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab34	Method outlined by Quaas A, Heydt C, Gebauer F, et al Genomic Characterization of TP53-Wild-Type Esophageal Carcinoma. Transl Oncol. 2018;12(1):154-161.; Gültekin SE, Aziz R, Heydt C, et al. The landscape of genetic alterations in ameloblastomas relates to clinical features. Virchows Arch. 2018;472(5):807-814. Alidousty C, Baar T, Martelotto LG, et al. Genetic instability and recurrent MYC amplification in ALK-translocated NSCLC: a central role of TP53 mutations. J Pathol. 2018;246(1):67-76 used. Only an updated primer set for lung cancer samples was used covering hotspots of the following genes: ALK, BRAF, CTNNB1, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, FGFR4, IDH1, IDH2, KRAS, MAP2K1, MET, NRAS, PIK3CA, PTEN, ROS1, TP53. Sequencing performed on Illumina MiSeq. Data Analyzed using peifseek in house variant caller. Wet analysis performed three operators. One batch of reagents used.	Illumina (MiSeq, MiniSeq, NextSeq, NovaSeq)	Amplicon Sequencing	In-house/open source (e.g. Debarcer for SimSeq, SamTool, DeepSNV, pindel, LowFreq, FreeBayes, Peifseek)
Lab35	Sample diluted from an assumed 50ng/uL concentration and set up at 15ng input for the initial PCR reaction. Amplification using SimpliAmp Thermal Cyclers from Applied Biosystmes/ Thermo Fisher Scientific. Library Preparation using the Ion Ampliseq Library Kit 2.0 and Ion Torrent Equilizer Kits. Custom ampliseq primer set used. Purifications using AMPure XP Beads from Beckman Coulter. Instrumentation used for templating and sequencing were the Personal Genome Machine (PGM), Gene Studio (S5), and Ion Chef System all from Ion Torrent. Senquencing performed on Ion Personal Genome Machine and Ion S5. Data analyzed using Ion Torrent Suite 5.8. Wet analysis performed by two operators. One batch of reagents used.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab37	Customized Ampliseq Cancer Hotspot Panel composed of 371 amplicons covering hotspot regions in 73 tumor associated genes, including PIK3CA, KRAS, NRAS, TP53, PTEN, and MAP2K1 used. Amplification using BioRad C1000 Thermal Cycler. Clonal amplification and library enrichment by Ion Chef System. Sequencing performed on Ion Torrent S5. Data analyzed using in Torrent Variant Caller. Wet analysis performed by three operators.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)
Lab38	Ion AmpliSeq Colon and Lung panel used. Sequencing performed on Ion S5 and Ion S5XL. Data Analyzed using Ion Reporter.	ThermoFisher (Ion Personal Genome Machine, Ion S5 XL, Ion S5)	Ion Ampliseq	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Lab39	ddPCR QX200 from BioRad was used. DNA quantified by fluorometric quantitation using Qubit Fluorimeter with Qubit DNA dsDNA BR Assay kit from Invitrogen™; 20 ng of DNA added to each well. Assays from BioRad catalogue. Droplets generated using QX200 AutoDG Droplet Digital PCR System. Data analyzed using QuantaSoft v. 1.7.4. Amplification on BioRad C1000 Thermocycler. Wet analysis performed by only one operator. Different batches of reagents were used.	dPCR (BioRad_QX200, QuantStudio 3D Digital PCR)	Probes based dPCR	Commercial proprietary (e.g. SOPHiA GENETICS, SureSeq Interpret™, Torrent Varriant Caller, MiSeq Reporter Software, Myriapod® NGS Data Analysis software, NextGENe Software, JSI SeqNext V4.3.1, QuantaSoft Software v1.7.4, QuantStudio™ 3D AnalysisSuite ™)

Appendix V. Collaborative Study Results for Quantitative Genotyping Data

Data in yellow were excluded from further analysis. See section Quantitative Genotyping Data for details.

	***PIK3CA* c.1633G>A (E545K), (Material 18/118)**														
	Sample 1	Sample 4 [crude]	Sample 7	Sample 1	Sample 4 [dilution a], 1:1.4	Sample 7	Sample 1	Sample 4 [dilution b], 1:2	Sample 7	Sample 1	Sample 4 [dilution c], 1:4	Sample 7	Sample 1	Sample 4 [dilution d], 1:10	Sample 7
Lab01	52.1	51.5	52.2	35.7	36.3	39.9	25.5	25	25.3						
Lab02										10.5	9.9	6.9	5.6	4.6	3
Lab03	52.4	49.6	49.5	32	33.6	34.7									
Lab04										16	12	10	5	5	3
Lab05_a	54	47	48												
Lab05_b	55														
Lab07	54.8	55	55.1												
Lab09										14.9	8.1	11.1	5.4	7.2	5
Lab10	54.6	53.1	53	37.4	36.6	35.7	26.6	28	24.1						
Lab11										17.6	18.1	21.2	6.9	6.3	6.3
Lab12	50	50	53	36	37	39	25	27	27						
Lab13_a	53.5	52	54.6	35.4	36	39									
Lab13_b	52			43.5											
Lab14	46.5	44.3	46.6	29.7	31.4	33.2	22.3	22.3	22.2						
Lab16	44	51	56	34	32	30									
Lab17	53.9	52.5	53.9	35.2	39.1	40.4	28.4	28.6	29.4						
Lab18	50.4	51.8	53	39	39.3	36.3									
Lab19							14	15	15	7	7	7			
Lab21	25.5	25.5	26.6	16.5	17.2	15									
Lab22	48.2	47.2	43.5	33.5	33.9	33.1									
Lab23						failed									
Lab24							29.7	29.8	28.3	18.6	16.8	14.9			
Lab25	51.3	52.5	55.7	39.9	37.4	41.2	29.2	26.6	25.3						
Lab26							< LOD	< LOD	3	< LOD	< LOD	< LOD			
Lab28							27.2	23.4	24.4	12.4	10.6	12.1			
Lab29										14.4	13.6	13.6	4.4	6.4	6.3
Lab30										12.5	14.2	12.8	5.5	6.7	5.7
Lab31a										11.4	11	15.3	5.5	5	4.7
Lab31b										11.8	12.6	14.2	4.2	4.6	5.5
Lab32							25.6	25.4	26.5	12.4	12.4	13.3			
Lab33										7.4	9.6	8	9	0	0
Lab34							20.6	19.5	19.2	10	9.4	8.7			
Lab35	52.8	53.5	50.1	35.3	38.8	37.3									
Lab37	49.4	49.4	53.1	36.6	35.8	38									
Lab38	53.4	52.2	60.3	38.1	39.9	34.3	39.7	25.9	28.2						
Lab39	53.3			39.1			26.9			13.2			5.4		

	***TP53* c.916C>T (R306*), (Material 18/130)**														
	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8
		[crude]			[dilution a], 1:1.4			[dilution b], 1:2			[dilution c], 1:4			[dilution d], 1:10	
Lab01				19.9	21.3	25.7	15.6	13.3	15.3						
Lab02										7.8	5.4	6.2	2	2.2	2.3
Lab03	28.5	27.8	28.9	17	18.8	16									
Lab04										9	6	6	2	4	2
Lab05_a	31	35	29.4							7.9	5.4	6.7	2	2.3	2.6
Lab05_b	28														
Lab07	30.2	31.4	damaged ampoule												
Lab10				22.7	21.3	19	13.4	13.8	14.1						
Lab11										5.7	5.6	5.6	1.8	1.9	1.7
Lab12				26	27	26	17	18	19						
Lab14				20.6	20.3	19.9	12	13.1	13.1						
Lab16	29	31	31	18	18	20									
Lab17				18.9	17.7	22.3	10.9	11.4	13.7						
Lab18	32.8	29.1	33.3	22.6	21.7	21.2									
Lab19	31	32	32				13	11	12	5	5	5			
Lab20	30.3	33.6	32				14.2	14.3	14.4	4.4	6.4	5.6			
Lab21	33.6	34.9	32.1	13.8	15.3	14.8									
Lab22	28.5	31.9	31.4	21.3	21.1	18.4									
Lab23				23	20	22	15	16	13						
Lab24	31.4	33.4	29.6				11.3	10.7	16.2	8.9	5.7	7.1			
Lab25				21.4	20.8	21.1	15.1	12.9	13.5						
Lab28	31.5	33	34				11.1	15	14.6	3.8	7.8	6.5			
Lab29										5.1	5.8	4.9	0	0	0
Lab30										6.2	6.3	6.6	2.2	2.6	2.2
Lab31_a										6.7	6.1	7.9	2.7	2.4	2.7
Lab31_b										6.6	7.2	7	2.7	2.5	2.8
Lab32	35.4	33	32.4				14.2	13.2	13.2	7.5	6.5	7			
Lab33										5.5	8.9	5	0	5.5	0
Lab34	31.4	32.2	32.3				14.1	14	14.8	7.1	6.2				
Lab35	35.6	32.1	38.3	19.8	20.7	23.7									
Lab37	31.9	28.8	32	20.6	17.9	19.2									
Lab38				19.3	20.4	20.4	14.1	14.5	14						
Lab39	33.8			21.2			13.7			6.3			2.3		

	***NRAS* c.34G>T (G12C), (Material 18/130)**														
	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8
		[crude]			[dilution a], 1:1.4			[dilution b], 1:2			[dilution c], 1:4			[dilution d], 1:10	
Lab01				19.1	18.7	21.1	13.1	11.7	12.2						
Lab02										8.1	5.3	5.4	2	2.5	2.2
Lab03	25.9	26.4	24.9	16.9	16.4	15.7									
Lab04										8	5	9	2	5	2
Lab05_a	23	23	23.1												
Lab05_b	30														
Lab07	24.6	24.9	damaged ampoule												
Lab09										4.5	5.6	5.4	2.4	2.5	2.3
Lab10				18.7	18.3	17.8	11.7	13.8	12						
Lab11										5.3	5.8	5.1	2.5	2.1	2.2
Lab12				21	25	24	16	17	16						
Lab13_a	24.7	26.2	28	19.9	17.8	16.7									
Lab13_b	23.5			19.3											
Lab14				18.3	17.2	16.3	12.1	12.2	11.3						
Lab15	24.7	24	26	16.2	16.9	16.2									
Lab16	24	21	23	14	16	16									
Lab17				18	18.1	17.7	13.6	12.3	11.8						
Lab18	25.4	25.4	26	18.6	16.1	20.3									
Lab19	24	24	25				11	10	11	6	5	5			
Lab20	24.7	25.4	26.4				13.1	11.6	11.6	4.9	5.6	6			
Lab21	26.4	23.9	20.4	12.1	12.1	13.1									
Lab22	24.9	19.6	24.3	18.5	18.1	18.1									
Lab 23				16	17	16	12	13	12						
Lab24	23.7	28.6	24.6				10.7	10.5	11.2	6	5.8	5.1			
Lab25				17	15.3	17.6	10.9	10.1	12.3						
Lab26	24.6	21.8	24.4				11.8	13.1	12.2	6.2	5.9	5.7			
Lab28	24	22	26.4				10.8	10.2	9.7	6.1	6	5.8			
Lab29										7.5	5.9	7.5	0	0	0
Lab30										5.4	5.5	5.8	2.2	2.4	2.2
Lab31_a										5.9	5.8	6.9	2.9	2.7	2.7
Lab31_b															
Lab32	25.5	24.7	22.5				13.3	11.2	11.9	5.3	5.9	6.2			
Lab33										5.6	7.5	0	0	5.7	0
Lab34	24.6	24.3	26.1				11.5	11.9	11	5.6	5.6	5.8			
Lab35	26.1	23.2	25.9	15.1	16.4	15.1									
Lab37	24.3	23.1	25.7	16.9	17.3	17.2									
Lab38				17.1	18.2	15.5	10.1	12.1	11.9						
Lab39	25.8			18.4			11.9			6.1			2.3		

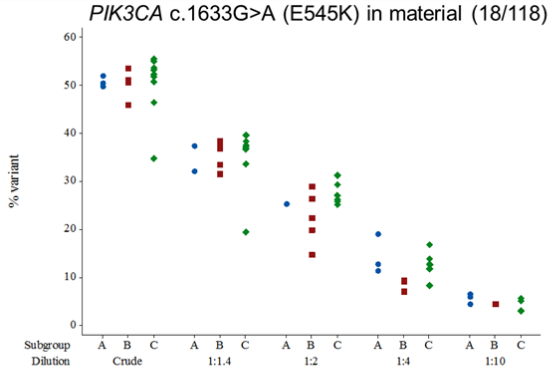
	***PTEN* c.795delA (K267fs*9), (Material 18/130)**														
	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8
		[crude]			[dilution a], 1:1.4			[dilution b], 1:2			[dilution c], 1:4			[dilution d], 1:10	
Lab02										22.3	21.6	22.2	8.1	8	8.7
Lab03	98.6	98.5	98.3	73.8	76.4	71.9									
Lab04										37	21	22	10	16	7
Lab05_a	87	90	88												
Lab05_b	100														
Lab07	100	100	damaged ampoule												
Lab10				71.7	69.8	70.6	48.4	47.9	49						
Lab11										21	21.4	20.9	7.1	8.2	8.4
Lab12				71	70	72	47	48	49						
Lab14				47.9	52.1	50.6	32.7	31	26.9						
Lab21	100	99.8	99.9	67.8	68.7	69									
Lab22	100	100	100	72.4	72.5	74.2									
Lab23				66	69	69	46	48	46						
Lab24	88.9	88.1	92.3				47	44	48	21	21.3	22.9			
Lab25				80.8	81.3	81.4	58.8	61.5	59.2						
Lab28	100	100	100				47.8	18.3	45.4	5.3	0	21.6			
Lab29										19.6	22.7	19.2	3.2	1.5	4.1
Lab30										22	22.5	22.8	8.3	9	8.5
Lab31_a										25.7	24.4	27.8	12.9	10.6	13.5
Lab31_b															
Lab32	100	100	100				51.4	47.5	46.8	24.1	22.2	21.5			
Lab33										20.1	33.4	20.5	0	19.2	7.9
Lab34	81.1	86.9	82.9				37.9	42.3	34.9	19.2	18.1	20.6			
Lab35	100	100	100	69.2	74.5	67.9									
Lab37	100	100	100	80	71	81									
Lab38				70	70.9	66.9	52.1	48.4	48						
Lab39	99.9			68.2			45.8			21.4			8.1		

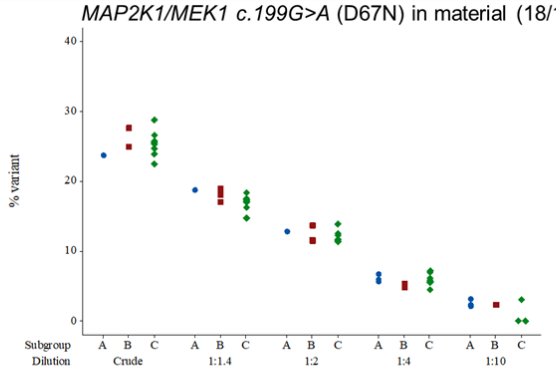
	***MAP2K1/MEK1* c.199G>A (D67N), (Material 18/130)**														
	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8	Sample 2	Sample 5	Sample 8
		[crude]			[dilution a], 1:1.4			[dilution b], 1:2			[dilution c], 1:4			[dilution d], 1:10	
Lab01				18.6	16.4	21	12.8	12.8	12.7						
Lab02										4.8	4.9	4.6	2.1	2.3	2.4
Lab04										9	5	3	2	4	1
Lab05_a	24	26	21												
Lab05_b	23														
Lab07	26.6	24.3	damaged ampoule												
Lab09										5.6	8.1	6.5	3.7	3.7	2.1
Lab10				18.1	18.5	18.6	13.9	11.7	11.9						
Lab11										5.3	6.4	6	2.6	1.7	2
Lab12				19	17	18	14	14	13						
Lab13_a	28.7	26.8	27.2	18.6	17.3	20.8									
Lab13_b	24.1			19.1											
Lab14				17.4	17.2	16.5	11.4	12.1	11						
Lab18	23.3	24.8	27.9	18.1	18	16.4									
Lab21	23.4	25.6	24.8	13	15.2	15.9									
Lab24	28.8	27.4	30.1				13.1	12.6	15.7	7.5	6.5	7.6			
Lab26	22.9	21.3	23				13	11.4	10.2	4.8	5.6	6.3			
Lab28	23.3	27.3	25.2				11.3	12.5	12.9	7.2	6.6	4.5			
Lab29										5.9	5.7	5.2	0	0	0
Lab30										5.6	5.9	5.7	2.2	2.3	2.2
Lab31_a										7.3	7.5	6.2	2.5	3.3	3.3
Lab31_b										6.6	6.8	6.7	2.3	2.4	2.6
Lab32	25.1	25.9	26.1				11.7	11.7	10.6	6.2	5.5	5.9			
Lab33										0	7.3	6	0	0	0
Lab34	24.7	24.4	25.7				11.1	11.7	11.9	5.2	5.3	5.5			
Lab35	24	24.8	22.9	17.8	16.9	16.4									
Lab37	27.1	26.9	25.7	15.2	18.6	14.9									
Lab38				18.3	17.7	15.8	10.3	13.6	11						
Lab39	25.3			17.4			11.6			5.9			2.3		

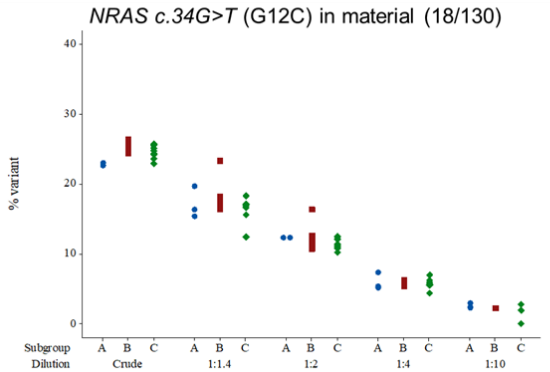
Appendix VI. Preliminary next-generation sequencing subgrouping analysis

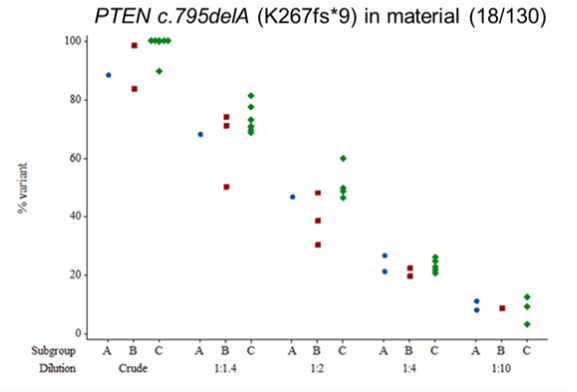
Preliminary subgroups analysis of all NGS data suggests broad agreement of the data, excepting the *PTEN* p.K267fs*9 variant where Ion Torrent: Proton/PGM sequencing technology /IonAmpliseq strategy appears to give higher results than Illumina sequencing technology/Amplicon Sequencing strategy.

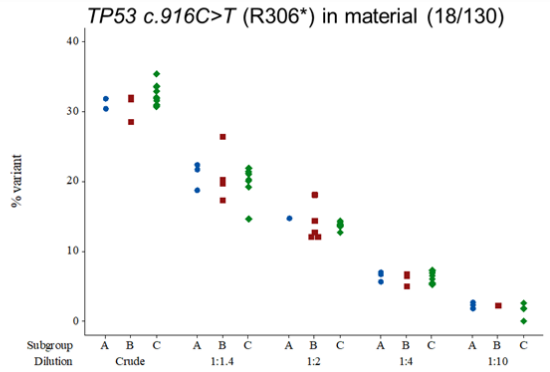
Subgroup A (in blue): Illumina sequencing & Enrichment strategy; Subgroup B (in red): Illumina sequencing & Amplicon Sequencing strategy; Subgroup C (in green): ThermoFisher Ion Torrent: Proton /PGM sequencing & Ion Ampliseq strategy.











Appendix VII. Instructions for Use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st International Standard for HCT 15 Cancer Genome
NIBSC code: 18/118
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

Material 18/118 is of freeze-dried, purified genomic DNA (gDNA) extracted from HCT 15 human cell lines. The material has consensus variant percentage for *PIK3CA* c.1633G>A (E545K), and consensus variant and total *PIK3CA* gene copy numbers. The material may be diluted by application of a calculation specific to each material to produce standards at a range of *PIK3CA* c.1633G>A (E545K) consensus mutation percentages. The material is intended for use as primary standards for the calibration of secondary standards, kits, and assays. The material is not intended as run controls. The material was tested by external laboratories and show suitability as standards in next-generation sequencing (NGS) and digital PCR (dPCR). In addition to the *PIK3CA* c.1633G>A (E545K) clinically relevant variant, the material comprises also non-clinically relevant variants in the background that may be used in the clinical validation of NGS assays. The material was established in 2019 by the Expert Committee on Biological Standardization of the World Health Organization (WHO) as the WHO 1st International Standard for HCT-15 Cancer Genome, NIBSC material code 18/118. This material should not be put to any other use. Data analysis must be focussed on *PIK3CA* c.1633G>A (E545K) and *PIK3CA* gene copy numbers.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The cell line used in the preparation of this material tested and found negative for mycoplasma, HIV1, HTLV1, HBV, and HCV by PCR. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The material was tested in an international collaborative study involving 35 laboratories and 38 testing methods. The genotype and consensus mutation percentage was obtained from NGS and dPCR (Table 1). End-users are able to further dilute the material (with wild-type material 18/164, or another wild-type genomic DNA calibrated to material 18/164) using a dilution formula based on the variant and total gene copy numbers, to achieve further standards at a range of lower consensus variation percentages from which assay calibration may be achieved, see section 7 and Appendix I, below.

NIBSC material code	Nominal Variant	Consensus variant percentage (%)	Consensus variant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass
18/118	*PIK3CA* c.1633G>A (E545K)	52.1	1.04014	1.99387

Table 1. Consensus values for the WHO 1st International Standard for HCT 15 Cancer Genome (NIBSC material code 18/118). Genotype, consensus variant percentage, and consensus gene copy numbers for use in calculating how the material may be diluted to prepare further standards at lower variant levels, are shown.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The coded ampoule contains approximately 5µg freeze-dried, purified genomic DNA extracted from human cell lines. The gDNA was extracted using a 'salting out' method, and diluted in Tris-EDTA buffer with 5mg/ml Trehalose before freeze-drying.

5. STORAGE

Store all unopened ampoules of the freeze-dried materials at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
a. Open the ampoule as described in section 6, above.
b. Reconstitute the freeze-dried materials at room temperature with 100µl nuclease-free water.
c. Transfer the sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.
d. Allow the materials to reconstitute for 1 hour at room temperature and pipette well to mix. The DNA concentration will now be approximately 50ng/µl in 1x Tris-EDTA buffer but confirmation with own quantification method is recommended before use. The possible appearance of white flecks in the materials should not be of concern.
e. This variant material may be combined with material 18/164 (wild-type) to produce standards at any chosen mutation percentage; please see details in Appendix I.

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

f. Add the required amount to your assay. Materials may be further diluted (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
g. Primary and secondary standards should be analysed in the same assay to assign values to the secondary standards. If further information is required, please contact jennifer.boyle@nibsc.org.

8. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of the WHO to not assign an expiry date to their international reference materials. They remain valid with the assigned values and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. Accelerated degradation studies have indicated that these materials are suitably stable when stored at -20°C or below, for the assigned values to remain valid until the materials are withdrawn or replaced. These studies have also shown that the materials are suitably stable for shipment at ambient temperature without any effect on the assigned values. It is highly recommended that the material is used on the day it is reconstituted and is not stored. However, in-house analysis determined reconstituted freeze-dried genomic DNA to be stable for up to 4 days at +4°C (or 2 months at -20°C). Care should be taken to avoid cross contamination with other samples. Users who have any data supporting any deterioration in the characteristics of materials are encouraged to contact NIBSC.

9. REFERENCES

1. WHO document XXX (not final)

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the significant contributions of all collaborative study participants.
Particular thanks go to Simon Patton of EMQN (Manchester, UK) for connecting us with the participants. We would also like to extend our gratitude to Paul Matejtschuk, Sara Jane Holmes, James Condron and the Standardization Science group at NIBSC, along with the Standards Processing Division for their development, and processing of the materials; Dahud Kahan for helping us to set up the dedicated (secure and encrypted) ShareFile Web Page and Sophie McLachlan from the MHRA communications team. This project is funded by UK Department of Health and Social Care

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

hysical and Chemical properties			
Physical appearance: white crystalline solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	contains material of human origin		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		
Effects of ingestion:	Not established, avoid ingestion		
Effects of skin absorption:	Not established, avoid contact with skin		
Suggested First Aid			
Inhalation:	Seek medical advice		
Ingestion:	Seek medical advice		
Contact with eyes:	Wash with copious amounts of water. Seek medical advice		
Contact with skin:	Wash thoroughly with water		
Action on Spillage and Method of Disposal			
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.			

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 3.5g per ampoule
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards

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http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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APPENDIX I. DILUTION OF THE MUTANT MATERIALS TO GENERATE ADDITIONAL STANDARDS

Each of the mutant materials may be diluted to produce further standards at lower consensus mutation percentages. The preferable diluent is the wild-type material 18/164. However, if insufficient material 18/164 is available to perform the dilutions, an alternative wild-type DNA may be aligned to material 18/164 and used as the diluent i.e. it should be confirmed as being fully wild-type, diploid, and containing two copies wild-type.

Further details on the dilution response of these materials may be found in the WHO report on the collaborative study to evaluate the proposed WHO 1st International Standards for Cancer Genomes: (not final) http://www.who.int/biologicals/WHO ECBS/en/.

Dilutions of the mutant materials may be established as follows:

1. By use of the formula:

$$dilution\ response = \left(\frac{variant\ copy\ number}{percentage\ of\ variant} * 100 - total\ copy\ number\right) * \frac{1}{2} + 1; \quad (1)$$

where the variant copy number and total copy number can be taken from Table 1, above.

For example, to prepare a standard of consensus variant percentage of 25%, the allelic content figures are used thus:

$$\left(\frac{1.04014}{25} * 100 - 1.99387\right) * \frac{1}{2} + 1 = 2.08 \quad (2)$$

Meaning that a 1 in 2.08 dilution (in blue in example formula 2) of material 18/118 with the wild-type material 18/164 (or another wild-type gDNA aligned to 18/164), will yield a further standard of consensus mutant percentage 25% (in green in example formula 2) *PIK3CA* c.1633G>A (E545K), for example, 2.0 µl material 18/118, plus 2.2µl material 18/164.

N.B. It is important to use the 5 decimal places for copy numbers in the calculation to achieve a maximally accurate answer.

2. By reference to dilution curves available from NIBSC:

Use Google Chrome to open the link for an interactive dilution curve:
(not final) http://www.nibsc.org/science and research/advanced therapies/genomic reference materials.aspx

For each material, hover the "+" cursor over the dilution curve at the mutation percentage required to see the dilution to be performed. For example, to prepare a further standard of consensus mutation percentage 25% for material 18/118 (*PIK3CA* c.1633G>A (E545K), hover the "+" cursor over 25% on the curve to see the dilution "2.08" i. e. 1 in 2.08 dilution will yield a further standard of consensus mutant percentage 25% (in green in example formula 2) *PIK3CA* c.1633G>A (E545K), for example, 2.0 µl material 18/118, plus 2.2µl material 18/164.

N.B. Performance in other browsers cannot be guaranteed.

3. By use of pre-calculated dilutions:

Refer to Table 2 (not final), over, for details on the preparation of further standards for each of the materials at a range of consensus mutation percentages.

NIBSC material code	Nominal Variant	Consensus mutant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass	Wanted variant %	Dilution to be performed	Volume mutant material (µl)	Volume wild-type material (µl)	Total volume (µl)
18/118	*PIK3CA* c.1633G>A (E545K)	1.04014	1.99387	50	1.04	8.0	0.3	8.3
				25	2.08	2.0	2.2	4.2
				10	5.20	1.0	4.2	5.2
				5	10.40	1.0	9.4	10.4
				1	52.01	1.0	51.0	52.0

Table 2. Example dilutions in the preparation of further standards for material 18/118 (*PIK3CA* c.1633G>A (E545K). Dilutions calculated using formula 1.

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WHO International Standard
1 st International Standard for MOLT-4 Cancer Genome
NIBSC code: 18/130
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

Material 18/130 is of freeze-dried, purified genomic DNA (gDNA) extracted from MOLT-4 human cell lines. The material has consensus variant percentage for *TP53* c.916C>T (R306*), *NRAS* c.34G>T (G12C), *PTEN* c.795delA (K267fs*9) and *MAP2K1/MEK1* c.199G>A (D67N) variants and consensus variant and total *TP53, NRAS, PTEN, MAP2K1/MEK1* gene copy numbers. The material may be diluted by application of a calculation specific to each material to produce standards at a range of consensus variant percentages for any of the above variants. The material is intended for use as primary standards for the calibration of secondary standards, kits, and assays. The material is not intended as run controls. The material was tested by external laboratories and show suitability as standards in next-generation sequencing (NGS) and digital PCR (dPCR). In addition to the above clinically relevant variants, the material comprises also non-clinically relevant variants in the background that may be used in the clinical validation of NGS assays. The material was established in 2019 by the Expert Committee on Biological Standardization of the World Health Organization (WHO) as the WHO 1st International Standard for HCT-15 Cancer Genome, NIBSC material code 18/130. This material should not be put to any other use. Data analysis must be focussed on *TP53* c.916C>T (R306*), *NRAS* c.34G>T (G12C), *PTEN* c.795delA (K267fs*9) and *MAP2K1/MEK1* c.199G>A (D67N) and *TP53, NRAS, PTEN, MAP2K1/MEK1* gene copy numbers.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The cell line used in the preparation of this material was tested and found negative for mycoplasma, HIV1, HTLV1, HBV, and HCV by PCR. However, the potential for viable virus to survive cannot be eliminated. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The material was tested in an international collaborative study involving 35 laboratories and 38 testing methods. The genotype and consensus mutation percentage was obtained from NGS and dPCR (Table 1). End-users are able to further dilute the material (with wild-type material 18/164, or another wild-type genomic DNA calibrated to material 18/164) using a dilution formula based on the variant and total gene copy numbers, to achieve further standards at a range of lower consensus mutation percentages from which assay calibration may be achieved, see section 7 and Appendix I, below.

NIBSC material code	Nominal Variant	Consensus variant percentage (%)	Consensus variant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass
18/130	*TP53* c.916C>T (R306*)	31.8	0.48327	1.52050
	NRAS c.34G>T (G12C)	24.7	0.45954	1.85863
	PTEN c.795delA (K267fs*9)	100.0*	1.78544	1.78545
	MAP2K1/MEK1 c.199G>A (D67N)	25.3	0.44476	1.75702

Table 1. Consensus values for the WHO 1st International Standard for MOLT-4 Cancer Genome (NIBSC material code 18/130). Genotype, consensus variant percentage, and consensus gene copy numbers for use in calculating how the material may be diluted to prepare further standards at lower variant levels, are shown. * wild-type calculated as 0.00001% but likely to be 0 therefore consensus variant percentage shown as 100.0%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
The coded ampoule contains approximately 5µg freeze-dried, purified genomic DNA extracted from human cell lines. The gDNA was extracted using a 'salting out' method, and diluted in Tris-EDTA buffer with 5mg/ml Trehalose before freeze-drying.

5. STORAGE

Store all unopened ampoules of the freeze-dried materials at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
a. Open the ampoule as described in section 6, above.
b. Reconstitute the freeze-dried materials at room temperature with 100µl nuclease-free water.

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c. Transfer the sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.
d. Allow the materials to reconstitute for 1 hour at room temperature and pipette well to mix. The DNA concentration will now be approximately 50ng/µl in 1x Tris-EDTA buffer but confirmation with own quantification method is recommended before use. The possible appearance of white flecks in the materials should not be of concern.
e. This variant material may be combined with material 18/164 (wild-type) to produce standards at any chosen mutation percentage; please see details in Appendix I.
f. Add the required amount to your assay. Materials may be further diluted (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
g. Primary and secondary standards should be analysed in the same assay to assign values to the secondary standards. If further information is required, please contact jennifer.boyle@nibsc.org.

8. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of the WHO to not assign an expiry date to their international reference materials. They remain valid with the assigned values and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. Accelerated degradation studies have indicated that these materials are suitably stable when stored at -20°C or below, for the assigned values to remain valid until the materials are withdrawn or replaced. These studies have also shown that the materials are suitably stable for shipment at ambient temperature without any effect on the assigned values. It is highly recommended that the material is used on the day it is reconstituted and is not stored. However, in-house analysis determined reconstituted freeze-dried genomic DNA to be stable for up to 4 days at +4°C (or 2 months at -20°C). Care should be taken to avoid cross contamination with other samples. Users who have any data supporting any deterioration in the characteristics of materials are encouraged to contact NIBSC.

9. REFERENCES

1. WHO document XXX (not final)

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the significant contributions of all collaborative study participants.
Particular thanks go to Simon Patton of EMQN (Manchester, UK) for connecting us with the participants. We would also like to extend our gratitude to Paul Matejtschuk, Sara Jane Holmes, James Condron and the Standardization Science group at NIBSC, along with the Standards Processing Division for their development, and processing of the materials; Dahud Kahan for helping us to set up the dedicated (secure and encrypted) ShareFile Web Page and Sophie McLachlan from the MHRA communications team. This project is funded by UK Department of Health and Social Care

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

hysical and Chemical properties			
Physical appearance: white crystalline solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 3.5g per ampoule
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

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17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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APPENDIX I. DILUTION OF THE MUTANT MATERIALS TO GENERATE ADDITIONAL STANDARDS

Each of the mutant materials may be diluted to produce further standards at lower consensus mutation percentages. The preferable diluent is the wild-type material 18/164. However, if insufficient material 18/164 is available to perform the dilutions, an alternative wild-type DNA may be aligned to material 18/164 and used as the diluent i.e. it should be confirmed as being fully wild-type, diploid, and containing two copies wild-type.

Further details on the dilution response of these materials may be found in the WHO report on the collaborative study to evaluate the proposed WHO 1st International Standards for Cancer Genomes: (not final) http://www.who.int/biologicals/WHO ECBS/en/.

Dilutions of the mutant materials may be established as follows:

1. By use of the formula:

$$dilution\ response = \left(\frac{variant\ copy\ number}{percentage\ of\ variant} * 100 - total\ copy\ number \right) * \frac{1}{2} + 1; \qquad (1)$$

where the variant copy number and total copy number can be taken from Table 1, above.

For example, to prepare a standard of consensus mutation percentage 15% for *TP53* c.916C>T (R306*) variant, the allelic content figures are used thus:

$$\left(\frac{0.48327}{15} * 100 - 1.5205 \right) * \frac{1}{2} + 1 = 1.85 \qquad (2)$$

Meaning that a 1 in 1.85 dilution (in blue in example formula 2) of material 18/130 with the wild-type material 18/164 (or another wild-type gDNA aligned to 18/164), will yield a further standard of consensus mutant percentage 15% (in green in example formula 2) *TP53* c.916C>T (R306*), for example, 1.0 µl material 18/130, plus 0.9µl material 18/164.

N.B. It is important to use the 5 decimal places for copy numbers in the calculation to achieve a maximally accurate answer.

2. By reference to dilution curves available from NIBSC:

Use Google Chrome to open the link for an interactive dilution curve:
(not final) http://www.nibsc.org/science and research/advanced therapies/genomic reference materials.aspx

For each material, hover the "+" cursor over the dilution curve at the mutation percentage required to see the dilution to be performed. For example, to prepare a further standard of consensus mutation percentage 15% for *TP53* c.916C>T (R306*) variant in material 18/130, hover the "+" cursor over 15% on the curve to see the dilution "1.85" i. e. 1 in 1.85 dilution will yield a further standard of consensus mutant percentage 15% (in green in example formula 2) *TP53 c.916C>T (R306*)*, for example, 1.0 µl material 18/130, plus 0.9µl material 18/164.

N.B. Performance in other browsers cannot be guaranteed.

3. By use of pre-calculated dilutions:

Refer to Table 2 (not final), over, for details on the preparation of further standards for each of the materials at a range of consensus mutation percentages.

NIBSC material code	Nominal Variant	Consensus mutant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass	Wanted variant %	Dilution to be performed	Volume mutant material (µl)	Volume wild-type material (µl)	Total volume (µl)
18/130	*TP53* c.916C>T (R306*)	0.48327	1.5205	15	1.85	1.0	0.9	1.9
				10	2.66	1.0	1.7	2.7
				5	5.07	1.0	4.1	5.1
				1	24.40	1.0	23.4	24.4
	NRAS c.34G>T (G12C)	0.45954	1.85863	15	1.60	1.0	0.6	1.6
				10	2.37	1.0	1.4	2.4
				5	4.67	1.0	3.7	4.7
				1	23.05	1.0	22.0	23.0
	PTEN c.795delA (K267fs*9)	1.78544	1.78545	50	1.89	1.0	0.9	1.9
				25	3.68	1.0	2.7	3.7
				10	9.03	1.0	8.0	9.0
				5	17.96	1.0	17.0	18.0
				1	89.38	1.0	88.4	89.4
	MAP2K1/MEK1 c.199G>A (D67N)	0.44476	1.75702	15	1.60	1.0	0.6	1.6
				10	2.35	1.0	1.3	2.3
				5	4.57	1.0	3.6	4.6
				1	22.36	1.0	21.4	22.4

Table 2. Example dilutions in the preparation of further standards for TP53 c.916C>T (R306*), NRAS c.34G>T (G12C), PTEN c.795delA (K267fs*9) and MAP2K1/MEK1 c.199G>A (D67N) variants in material 18/130. Dilutions calculated using formula 1.

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WHO International Standard
1st International Standard for ATDB102 Reference Genome
NIBSC code: 18/164
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

Material 18/164 is of freeze-dried, purified genomic DNA (gDNA) extracted from human cell lines. The material has proven to be wild-type for *PIK3CA* c.1633G>A (E545K), *TP53* c.916C>T (R306*), *NRAS* c.34G>T (G12C), *PTEN* c.795delA (K267fs*9) and *MAP2K1/MEK1* c.199G>A (D67N) variants and diplod for *PIK3CA*, *TP53*, *NRAS*, *PTEN*, *MAP2K1/MEK1* gene copy numbers. The material may be used both as common reference and diluent of for the above variations in the 1st International Standard for HCT 15 and MOLT-4 Cancer Genome (NIBSC material code 18/118 and 18/130). Details on how to use the material as dilutent is provided in material 18/118 and 18/130 Instruction for Use. The material is intended for use as primary standards for the calibration of secondary standards, kits, and assays. The material is not intended as run control. The material was tested by external laboratories and show suitability as standards in next-generation sequencing (NGS) and digital PCR (dPCR). The material comprises non-clinically relevant variants in the background that may be used in the clinical validation of NGS assays. The material was established in 2019 by the Expert Committee on Biological Standardization of the World Health Organization (WHO) as the WHO 1st International Standard for ATDB102 Reference Genome, NIBSC material code 18/164. This material should not be put to any other use. Data analysis must be focussed on *PIK3CA* c.1633G>A (E545K), *TP53* c.916C>T (R306*), *NRAS* c.34G>T (G12C), *PTEN* c.795delA (K267fs*9) and *MAP2K1/MEK1* c.199G>A (D67N) and *PIK3CA*, *TP53*, *NRAS*, *PTEN*, *MAP2K1/MEK1* gene copy numbers. No attempt must be made to identify the source material donor.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The cell line used in the preparation of this material was tested and found negative for mycoplasma, HIV1, HTLV1, HBV, and HCV by PCR. This cell line is an Epstein Barr virus (EBV)-transformed lymphoblastoid cell line. EBV is a category 2 pathogen as classified by the UK Advisory Committee on Dangerous Pathogens. EBV sequences may be present in these materials, but the DNA has been prepared using a protocol in which proteins are denatured and removed, thus likely inactivating the virus. However, the potential for viable virus to survive cannot be eliminated. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The material was tested in an international collaborative study involving 35 laboratories and 38 testing methods. The genotype and consensus mutation percentage was obtained from NGS and dPCR (Table 1).

NIBSC material code	Nominal Variant	Consensus variant percentage (%)	Consensus variant copy number per diploid human genome mass	Consensus total copy number per diploid human genome mass
18/164	*PIK3CA* c.1633G>A (E545K)	Wild-type		
	TP53 c.916C>T (R306*)	Wild-type		
	NRAS c.34G>T (G12C)	Wild-type		
	PTEN c.795delA (K267fs*9)	Wild-type		
	MAP2K1/MEK1 c.199G>A (D67N)	Wild-type		

Table 1. Consensus values for the WHO 1st International Standard for ATDB102 Reference Genome (NIBSC material code 18/164).

4. CONTENTS

Country of origin of biological material: United Kingdom.
The coded ampoule contains approximately 5µg freeze-dried, purified genomic DNA extracted from human cell lines. The gDNA was extracted using a 'salting out' method, and diluted in Tris-EDTA buffer with 5mg/ml Trehalose before freeze-drying.

5. STORAGE

Store all unopened ampoules of the freeze-dried materials at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
a. Open the ampoule as described in section 6, above.
b. Reconstitute the freeze-dried material at room temperature with 100µl nuclease-free water.
c. Transfer the sample to a nuclease-free tube using a pipette, ensuring the maximum available volume is collected.

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d. Allow the material to reconstitute for 1 hour at room temperature and pipette well to mix. The DNA concentration will now be approximately 50ng/µl in 1x Tris-EDTA buffer but confirmation with own quantification method is recommended before use. The possible appearance of white flecks in the materials should not be of concern.
e. This material may be combined with material 18/118 and 18/130 (cancer gDNAs) to produce standards at any chosen mutation percentage; please see relevant Instruction for Use for details on how to use it as diluent.
f. Add the required amount to your assay. Material may be further diluted (with nuclease-free water or suitable buffer) to achieve a DNA concentration appropriate for your assay.
g. Primary and secondary standards should be analysed in the same assay to assign values to the secondary standards. If further information is required, please contact jennifer.boyle@nibsc.org.

8. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of the WHO to not assign an expiry date to their international reference materials. They remain valid with the assigned values and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. Accelerated degradation studies have indicated that these materials are suitably stable when stored at -20°C or below, for the assigned values to remain valid until the materials are withdrawn or replaced. These studies have also shown that the materials are suitably stable for shipment at ambient temperature without any effect on the assigned values. It is highly recommended that the material is used on the day it is reconstituted and is not stored. However, in-house analysis determined reconstituted freeze-dried genomic DNA to be stable for up to 4 days at +4°C (or 2 months at -20°C). Care should be taken to avoid cross contamination with other samples. Users who have any data supporting any deterioration in the characteristics of materials are encouraged to contact NIBSC.

9. REFERENCES

1. WHO document XXX (not final)

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the significant contributions of all collaborative study participants.
Particular thanks go to Simon Patton of EMQN (Manchester, UK) for connecting us with the participants. We would also like to extend our gratitude to Paul Matejtschuk, Sara Jane Holmes, James Condron and the Standardization Science group at NIBSC, along with the Standards Processing Division for their development, and processing of the materials; Dahud Kahan for helping us to set up the dedicated (secure and encrypted) ShareFile Web Page and Sophie McLachlan from the MHRA communications team. This project is funded by UK Department of Health and Social Care

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

hysical and Chemical properties			
Physical appearance: white crystalline solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 3.5g per ampoule
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

Appendix VIII. Collaborative Study Results for Additional Genotyping Data

For each of the three materials, addition variants were reported by laboratories performing NGS. Only the 30 most-frequent variants are listed below, ranked according to the number of laboratories observing them. These data are supplementary to the five clinically-actionable variants, and are intended to aid the broader validation of NGS pipelines but are not intended for calibration or diagnostic purposes.

Material 18/118		
Gene	**CDS**	**Participants reporting the variant**
KRAS	c.38G>A	Lab01; Lab03; Lab05_a; Lab07; Lab10; Lab13_a; Lab13_b; Lab14; Lab15; Lab16; Lab17; Lab18; Lab22; Lab25; Lab35; Lab37; Lab38
PIK3CA	c.1645G>A	Lab01; Lab03; Lab05_a; Lab07; Lab10; Lab12; Lab13_a; Lab13_b; Lab16; Lab17; Lab21; Lab22; Lab25; Lab35; Lab38
TP53	c.722C>T	Lab01; Lab05_a; Lab07; Lab10; Lab12; Lab14; Lab17; Lab18; Lab22; Lab23; Lab35; Lab37; Lab38
DDR2	c.2287A>G	Lab01; Lab05_a; Lab10; Lab13_b; Lab35; Lab38
KIT	c.1594G>A	Lab01; Lab05_a; Lab13_a; Lab14; Lab22; Lab35
NOTCH1	c.4742C>T	Lab05_a; Lab10; Lab13_b; Lab14; Lab22; Lab38
TP53	c.215C>G	Lab01; Lab03; Lab10; Lab16; Lab38
APC	c.4248delC	Lab05_a; Lab13_a; Lab16; Lab35
EGFR	c.2361G>A	Lab01; Lab03; Lab10; Lab13_b
APC	c.4683G>T	Lab03; Lab05_a; Lab16
DDR2	c.1323G>A	Lab10; Lab13_b; Lab38
EGFR	c.1839C>T	Lab03; Lab13_b; Lab16
FGFR3	c.1953G>A	Lab01; Lab10; Lab13_b
FGFR3	c.2370C>A	Lab05_a; Lab10; Lab13_b
PDGFRA	c.1701A>G	Lab01; Lab13_a; Lab13_b
TP53	c.1101-2A>C	Lab05_a; Lab17; Lab23
TP53	c.461G>A	Lab01; Lab35; Lab38
ALK	c.1597A>G	Lab05_a; Lab13_b
ALK	c.3375C>A	Lab01; Lab13_a
ALK	c.4381A>G	Lab13_a; Lab13_b
ALK	c.4587C>G	Lab13_a; Lab13_b
APC	c.2180G>T	Lab05_a; Lab16
APC	c.2979G>T	Lab05_a; Lab16
APC	c.5337A>G	Lab05_a; Lab16
APC	c.6496C>T	Lab05_a; Lab16
APC	c.7648G>C	Lab05_a; Lab16
CDH1	c.1138-1G>A	Lab05_a; Lab37
EGFR	c.1562G>A	Lab13_b; Lab16
EGFR,EGFR-AS1	c.2361G>A	Lab13_a; Lab16
ERBB2	c.3508C>G	Lab13_b; Lab16

Material 18/130		
Gene	**CDS**	**Participants reporting the variant**
NOTCH1	c.4799T>C	Lab03; Lab05_a; Lab07; Lab13_b; Lab18; Lab22; Lab24; Lab28; Lab32; Lab37
ERBB4	c.859G>A	Lab05_a; Lab13_b; Lab24; Lab28; Lab32
FGFR3	c.2135G>A	Lab05_a; Lab13_b; Lab32; Lab34; Lab35
FGFR3	c.2139G>C	Lab05_a; Lab13_b; Lab32; Lab34; Lab35
KIT	c.1621A>C	Lab03; Lab13_a; Lab13_b; Lab22; Lab26
SMAD4	c.787+2T>C	Lab05_a; Lab24; Lab32; Lab37
EGFR	c.1839C>T	Lab03; Lab13_b; Lab16
EGFR	c.2361G>A	Lab03; Lab13_b; Lab26
ERBB2	c.1173G>A	Lab05_a; Lab13_b; Lab16
ERBB2	c.2316C>T	Lab05_a; Lab13_b; Lab16
FGFR3	c.1862G>A	Lab05_a; Lab13_b; Lab34
FGFR3	c.2149G>A	Lab13_b; Lab32; Lab35
GNA11	c.502G>A	Lab05_a; Lab13_b; Lab35
PDGFRA	c.1701A>G	Lab13_a; Lab13_b; Lab26
TP53	c.215C>G	Lab03; Lab16; Lab28
ALK	c.4338C>T	Lab13_a; Lab13_b
ALK	c.4381A>G	Lab13_a; Lab13_b
ALK	c.4587C>G	Lab13_a; Lab13_b
APC	c.3637T>C	Lab05_a; Lab16
DNMT3A	c.2096delG	Lab05_a; Lab20
EGFR	c.1536delC	Lab05_a; Lab16
EGFR	c.1562G>A	Lab13_b; Lab16
EGFR,EGFR-AS1	c.2361G>A	Lab13_a; Lab16
ERBB2	c.3508C>G	Lab13_b; Lab16
ERBB2	c.3694delG	Lab05_a; Lab16
FGFR3	c.2082G>A	Lab05_a; Lab13_b
FGFR3	c.2392C>A	Lab13_b; Lab34
FGFR4	c.1121C>T	Lab05_a; Lab34
FGFR4	c.1397+1G>A	Lab05_a; Lab35
HRAS	c.368G>A	Lab05_a; Lab13_b

Material 18/164		
Gene	**CDS**	**Participants reporting the variant**
ATM	c.2572T>C	Lab03; Lab22; Lab29
ALK	c.4338C>T	Lab13_a; Lab13_b
ALK	c.4381A>G	Lab13_a; Lab13_b
ALK	c.4587C>G	Lab13_a; Lab13_b
APC	c.5009C>T	Lab05_a; Lab16
APC	c.7201C>T	Lab05_a; Lab16
EGFR	c.1562G>A	Lab13_b; Lab16
EGFR	c.2361G>A	Lab03; Lab13_b
EGFR	c.2982C>T	Lab13_b; Lab16
EGFR	c.474C>T	Lab13_b; Lab16
EGFR,EGFR-AS1	c.2361G>A	Lab13_a; Lab16
PDGFRA	c.1701A>G	Lab13_a; Lab13_b
TP53	c.215C>G	Lab03; Lab16
AKT1	c.1329G>A	Lab05_a
ALK	c.2535T>C	Lab13_b
ALK	c.27C>G	Lab13_b
ALK	c.3036G>A	Lab13_b
ALK	c.82C>T	Lab13_b
APC	c.1458T>C	Lab16
APC	c.2507C>G	Lab16
APC	c.2510C>G	Lab16
APC	c.5465T>A	Lab16
BRAF	c.1799T>A	Lab15
BRAF	c.900A>C	Lab16
BRAF	c.949T>C	Lab16
BRAF	c.955T>C	Lab16
BRAF	c.960A>C	Lab16
BRCA1	c.4357+1G>A	Lab29
EGFR	c.1498+22A>C	Lab13_a
EGFR	c.1856_1857delTGinsCA	Lab13_b

Appendix IX. Material 18/164 (nominal wild-type) Data

"-" and empty cells indicate that the participant did not perform the analysis.

	Material 18/164														
	***PIK3CA* c.1633G>A (E545K)**			***TP53* c.916C>T (R306*)**			***NRAS* c.34G>T (G12C)**			***PTEN* c.795delA (K267fs*9)**			***MAP2K1/MEK1* c.199G>A (D67N)**		
	Sample 3	Sample 6	Sample 9	Sample 3	Sample 6	Sample 9	Sample 3	Sample 6	Sample 9	Sample 3	Sample 6	Sample 9	Sample 3	Sample 6	Sample 9
		[crude]			[crude]			[crude]			[crude]			[crude]	
Lab02	0.75	0.79	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						
Lab04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab05a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab05b	0.00			0.00			0.00			-	-	-	-	-	-
Lab07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.00	0.00
Lab11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab13_a	0.00	0.00	0.00				0.00	0.00	0.00				0.00	0.00	0.00
Lab13_b	0.00						0.00						0.00		
Lab15							0.00	0.00	0.00						
Lab16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						
Lab18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.00	0.00
Lab21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Lab29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab30	0.29	1.07	0.03	0.04	0.04	0.04	0.00	0.00	0.00	0.06	0.05	0.05	0.02	0.02	0.04
Lab31_a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				0.00	0.00	0.00
Lab_31b	0.03	0.03	0.04	0.05	0.05	0.05	0.00	0.01	0.00	3.64	2.86	3.60	0.03	0.06	0.03
Lab33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab39	0.02	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.03	0.00	0.00
%estimates zero		84%			88%			97%			83%			87%	
Median		0.00			0.00			0.00			0.00			0.00	