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Setting a diagnostic benchmark for tumor *BRCA* testing: detection of *BRCA1* and *BRCA2* large genomic rearrangements in FFPE tissue – a pilot study

Nadejda Valtcheva[1]*, Bich Doan Nguyen-Sträuli[2]*, Ulrich Wagner[1], Sandra N. Freiberger[1,3], Zsuzsanna Varga[1], Christian Britschgi[4], Konstantin J. Dedes[2]* and Markus P. Rechsteiner[1]*

[1]Department of Pathology and Molecular Pathology, University Hospital Zurich, 8091 Zurich, Switzerland

[2]Department of Gynecology, Comprehensive Cancer Center Zurich, University Hospital Zurich, 8091 Zurich, Switzerland

[3]University of Zurich, 8091 Zurich, Switzerland

[4]Department of Medical Oncology and Hematology, Comprehensive Cancer Center Zurich, University Hospital Zurich, 8091 Zurich, Switzerland

*These authors contributed equally

Short title: tumor *BRCA* testing for gene or exon deletions

Corresponding author: Nadejda Valtcheva, PhD, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, Tel. +41 (0)44 255 1951, Fax +41 (0)44 255 4416, nadejda.valtcheva@usz.ch

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Abstract

PARP inhibitors are used for treatment of tumors lacking function of the double-strand DNA break repair proteins BRCA1 or BRCA2 and are already approved for several cancer types. Thus, it is clinically crucial to determine germline as well as somatic *BRCA1/2* mutations in those patients. The amplicon-based Oncomine *BRCA1* and *BRCA2* Assay is a test routinely used in diagnostics with FFPE specimens. The assay is validated for the detection of mutations, however, data on its performance in detecting large genomic rearrangements in FFPE tissue, is scarce.

We cross-validated Oncomine *BRCA1* and *BRCA2* Assay in blood samples and/or FFPE tissue with multiplex ligation-dependent probe amplification (MLPA) for exon deletions and with OncoScan and an in-house hybridization-based target capture assay (MelArray) with a customized pipeline for the detection of loss of heterozygosity (LOH) and heterozygous versus complete gene loss.

The Oncomine *BRCA1* and *BRCA2* Assay could detect both exon deletion and mono- and bi-allelic losses of the *BRCA1/2* genes.

We show that the therapeutically relevant large genomic rearrangements are reliably detected with the amplicon-based Oncomine *BRCA1* and *BRCA2* Assay in FFPE tumor tissue. Based on our data, we suggest tumor *BRCA* testing as standard diagnostic prescreening prior to germline *BRCA* testing.

Introduction

With the growing understanding of the molecular characteristics of cancer, novel treatment options specifically targeting tumor vulnerabilities have emerged (Mateo et al., 2019). A paradigm drug affecting only the cancer cells due to tumor specific alterations is the family of Poly(adenosine diphosphate-ribose) polymerase inhibitors (PARPi) in tumors lacking function of BRCA1 or BRCA2. The effect of the PARPi is known as "synthetic lethality" (Kaelin, 2005): the loss of function of BRCA1 or BRCA2 tumor suppressor proteins involved in double-strand DNA break (DSB) repair has no impact on cell survival, rather it contributes to malignant transformation by provoking genomic instability. However, the additional blocking of the single-strand DNA break (SSB) repair pathway by PARP1 leads to cancer cell death due to catastrophic DNA damage, while largely sparing normal cells. Based on two studies with *BRCA1/2* deficient cancer cell lines (Bryant et al., 2005; Farmer et al., 2005), PARPi have been rapidly established as a new standard in targeted cancer therapy. In the last 10 years PARPi showed antitumor activity in breast cancer (Litton et al., 2018), ovarian cancer (Coleman et al., 2017; Mirza et al., 2016; Moore et al., 2018; Pujade-Lauraine et al., 2017), prostate cancer (De Bono et al., 2020; Hussain et al., 2020) and pancreatic cancer (Golan et al., 2019) and have already been approved for several indications. There have been multiple studies reporting the presence of somatic *BRCA1/2* mutations in tumor tissue. Whereas 11 to 15% of cases harbor germline mutations, somatic *BRCA1/2* mutations in ovarian cancer patients occur in approximately 5-7% of the cases (Bell et al., 2011; Cunningham et al., 2014; Hennessy et al., 2010; Pennington et al., 2014; Yates et al., 2014). While the clinical relevance of germline BRCA mutations is established, the full significance of somatic mutations remains unclear and it seems to be dependent on the cancer type. In ovarian and prostate cancer, it appears that patients with somatic BRCA mutations are likely to equally benefit from PARPi treatment as patients with germline mutations (Ledermann et al., 2014, 2012; Mohyuddin et al., 2020). Therefore, in order to detect both somatic and

germline BRCA variants and capture all patients who may benefit from PARPi treatment, a rapid, affordable and reliable *BRCA1/2* mutational test including detection of large genomic rearrangements, which can be applied in formalin-fixed paraffin-embedded (FFPE) tumor tissue is needed.

Several commercial *BRCA1/2* next-generation-sequencing (NGS) tests are meanwhile broadly used in routine diagnostics. Single nucleotide variants (SNV) and small insertions and deletions (indels), ranging from one nucleotide to small nucleotide stretches, are reliably detected with a comparable performance by both amplicon-based and hybridization-capture based assays. However, another class of alterations referred to as large genomic rearrangements (LGR) (Judkins et al., 2012), are challenging for testing in FFPE tissue and particularly for amplicon-based NGS assays. The LGRs include two types of alterations of the genes *BRCA1* or *BRCA2:* 1.) Loss or duplication of one or more exons and 2.) Loss of one or both alleles (copy number alterations). The latter is probably the result of genomic rearrangements during tumorigenesis in line with the Knudson two-hit hypothesis (Knudson, 1971). In contrast, the loss or duplication of one or more exons is considered similar to the pathogenic SNVs and indels in *BRCA1/2*, and they are often germline alterations (Cao et al., 2019). Thus, in clinics a patient tested negative for *BRCA1/2* mutations in the FFPE tissue would often be referred to a geneticist in order to exclude a germline exon deletion by using high quality leukocyte DNA from blood and the gold standard diagnostic test for LGRs, being the multiplex ligation-dependent probe amplification (MLPA). Considering the substantial number of patients tested with *BRCA1/2* wild-type on tumor tissue, this increases the costs and particularly the time needed to reach a clinical decision on the therapeutic strategy.

In our accredited diagnostic laboratory we established and validated the Oncomine *BRCA1* and *BRCA2* Assay on the Ion Torrent platform in 2016 and used the test routinely for testing *BRCA1/2* SNVs and indels. Meanwhile, the bioinformatic pipeline used for analysis was further developed by Thermo Fisher in order to detect LGRs as well. However, although

tested by the company (Thermo Fisher Scientific, White Paper 2019 Evaluation of the Oncomine *BRCA* Research Assay for variant detection by next-generation sequencing) and by Germani et al. (Germani et al., 2018) for diagnostics in blood samples, no detailed information on the performance of this part of the test in FFPE tissue could be obtained, neither from the company nor from peer-reviewed publications by other diagnostic laboratories. Moreover, no threshold is set for the parameters calculated by the software (Ion Reporter) for distinguishing positive and negative results.

In order to test the performance and develop guidelines for the interpretation of the LGR results based on the Oncomine *BRCA1* and *BRCA2* Assay in routine diagnostics, we analyzed a collective of 20 samples and cross-validated the results with different assays: 1) 5 samples from patients with known germline exon deletions revealed in the course of genetic counselling and 2) 15 samples from routine diagnostics were picked based on the altered copy number status in the Oncomine assay (being no CNV, *BRCA1* deletion or *BRCA2* deletion) and were all cross-validated by OncoScan array. In the cases of discordance or equivocal results, an in-house hybridization based target capture assay ("MelArray") was performed as an additional test. Here, we show that after adjusting the filter criteria of the software, the commercially available Oncomine *BRCA1* and *BRCA2* Assay detects reliably all LGRs in FFPE tissue. We summarize the adjusted criteria and the gained experience, including samples difficult to interpret, in user-friendly guidelines.

Methods

Patient cohort

Patient data were collected after informed consent had been obtained in cases, in which it was necessary, and permission to collect data had been given by the Ethical Committee of the Canton Zurich (KEK-2012 553). The patient collective consisted of 20 patients. 5 patients with known germline exon deletions (detected by MLPA testing on DNA from blood

leukocytes in the context of a genetic counseling) were tested in FFPE tissue. Further 15 patients who were tested in routine diagnostics were picked for cross-validation with OncoScan and MelArray. The samples comprised n=5 presumed negatives (copy number 2), n=5 presumed with *BRCA1* deletion (copy number 1) and n=5 presumed with *BRCA2* deletion (copy number 1). Patient history, family history, and gynecological follow-up data were obtained either by the hospital data bank or by contacting primary care providers and gynecologists by telephone. The results from the MLPA *BRCA1/2* test on blood leukocyte DNA were obtained from the medical genetics report through the hospital database.

Sample preparation

Tumor area was marked on the HE slide and relative tumor cell content was determined by a trained pathologist (Supplemetary table 2). Cores (1-3 cylinders, 0.6 mm diameter) from the tumor area of FFPE blocks or 300 µl of peripheral blood collected in BD Vacutainer K2 (EDTA 18.0 mg) were used for DNA isolation using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (Promega, #AS1130), and the Maxwell 16 LEV Blood DNA kit (Promega, #AS1290) respectively, according to the manufacturer's recommendations. The double-strand DNA concentration (dsDNA) was determined using the fluorescence-based Qubit dsDNA HS Assay Kit.

NGS

Oncomine *BRCA1* and *BRCA2* Assay

Library preparation with 20 ng DNA input was performed with the Oncomine *BRCA1* and *BRCA2* Assay following the manufacturer's instructions. Adaptor/barcode ligation, purification and equilibration was automated with Tecan Liquid Handler (EVO-100).

NGS libraries were templated using Ion Chef and sequenced on a S5 (Thermo Fisher Scientific Schweiz AG, Reinach, Switzerland), and the data were analyzed using Ion Reporter Software 5.12 with default settings (*BRCA* Oncomine 5.12, Thermo Fisher Scientific).

Briefly, the software uses the coverage of each amplicon compared to the coverage of all amplicons in order to detect large deletions.

MelArray

The KAPA HyperPlus Kit was used for DNA fragmentation and library preparation. Hybrid capture was performed using a customized probe set (Freiberger et al., 2019) by Roche NimbleGen (Basel, Switzerland). Libraries were sequenced paired-end (100 bp) on a NextSeq550 Illumina machine (Illumina, San Diego, CA, USA).

A customized pipeline with open source software was used to analyze the data as described previously (Freiberger S.N., Cheng P., Pornputtapong N., Turko P., Kong Y., Irmisch A., Khan M., Halaban R., Dummer R., n.d.; Freiberger et al., 2019). Briefly, heterozygous SNPs are equally spaced across the genome to detect reliably copy number alterations.

OncoScan

The samples were processed by IMGM Laboratories GmbH (Martinsried, Germany) for CNV determination according to the Affymetrix OncoScan FFPE Array recommended protocol.

The assay uses locus-specific molecular inversion probes (MIPs) optimized for highly degraded FFPE samples with a 40-bp probe interrogation site. Probes were hybridized to separate OncoScan microarrays for A/T and G/C signals/sample. After hybridization, microarrays were scanned with a 3000-7G GeneChip scanner (Affymetrix) and image files processed to yield signal intensity (.cel-) files. Cel files were then converted to .OSCHP files using OncoScan Console 1.3 (Affymetrix, Inc. St. Clara, CA, USA), yielding normalized log intensity ratios (sample/reference), B-allele frequencies and a set of metrics for quality control, including a median of absolute pair-wise difference (MAPD), normal diploid SNP QC (ndSNPQC) or normal diploid waviness standard deviation (ndWavinessSD). After quality checking, samples were categorized as high, normal or low CN based on the log intensity ratio using the TuScan algorithm as implemented in Nexus Express Software for OncoScan 3.0.1 (Biodiscovery, Inc. 2014, El Segundo, CA, USA).

Results

The bioinformatic algorithm behind the calculation of LGRs in the Thermo Fisher companion software Ion Reporter consists of two parts reflecting the two kinds of rearrangements. One algorithm is used for the calculation of exon deletions and duplications and a second one for the detection of copy number alterations of the entire *BRCA1* and *BRCA2* genes.

Performance of the Oncomine *BRCA1* and *BRCA2* Assay for exon deletions

First, we tested the performance of the Oncomine *BRCA1* and *BRCA2* assay in detecting exon deletions and duplications by sequencing three patients with known germline exon deletions (detected by MLPA testing on DNA from blood leukocytes in the context of a genetic counseling). We analyzed high-quality DNA from blood samples and DNA from the FFPE biopsies and compared the results. The parameters calculated by the Ion Reporter LGR algorithm include the Oncomine variant class ("exon deletion"), the copy number ("1") at the locus called missing and the "CNV confidence" being the estimated probability for the copy number (Fig. 1A). The assay includes an internal control, the so-called sample ID, that consists of amplicons distributed over the genome and covering genes others than *BRCA1/2*. All exons of *BRCA1* and *BRCA2* together with the sample ID used for calibration can be visualized in the software (Fig. 1B). All three exon losses (Exon19, Exon16 and Exon20-21) could be detected and are visible in both the result table and the graphic representation. Interestingly, in all three patients, the CNV confidence was similar in blood and FFPE but between the cases, the confidence for exon deletion differed (Fig. 1A). In these three validation cases, the exon deletions were visible and concordant between the CNV confidence number calculated and the visualization plot. In summary, these three cases showed a clear result and straightforward interpretation, in both blood and FFPE tissue.

In contrast, two further cases – ExDel#4 and ExDel#5, for which only FFPE was tested, showed results controversial between the result table and the visualization plot (Fig. 2A and B). For the case ExDel#4 the algorithm called a duplication of exons 2-8 with a CNV confidence of 100 (Fig. 2A). The visualization plot clearly shows (also in comparison to the *BRCA2* exons and the sample ID) that in fact the sample carries a deletion of exons 9-23 rather than a duplication of exons 2-8. The second case difficult to interpret displays a deletion of exons 7 and 8 that is considerable in the visualization graph (Fig. 2B) but has a rather low CNV confidence of 18 (Fig. 2A). Both samples could be interpreted correctly when including the visualization plot in the decision-making.

Performance of the Oncomine *BRCA1* and *BRCA2* assay for CNV detection compared to OncoScan

In the second part of the validation study, we aimed at testing the performance of the Oncomine *BRCA1* and *BRCA2* assay on detecting whole gene mono- or bi-allelic losses in comparison to OncoScan array (probe distribution shown in Suppl. Figure 13) and its performance to distinguish real homozygous from heterozygous deletions with an additional in-house hybridization based target capture assay (MelArray). The usual data reported by the software for three different cases – Copy number CN 2 (REF), loss of *BRCA1* (GeneCNV, BRCA1DEL) and loss of *BRCA2* (GeneCNV, BRCA2DEL) are depicted in Fig. 3A. All three constellations were called with a confidence of 100. For each case, the visualization by IR 5.12 (Fig. 3B), the genomic view, and the zoom-in on the *BRCA1* and *BRCA2* loci from the OncoScan array (Fig. 3C) are represented. Interestingly, in contrast to the exon deletion calling, the visualization plot of the CNV reporting is not always suggestive of the result reported in the table. GeneDel sample #2 has a very slight decrease of *BRCA2* as compared to *BRCA1* and the sample ID in the plot and still has a CNV confidence of 100. In total, we tested 15 samples for copy numbers of *BRCA1* and *BRCA2* with both the Oncomine *BRCA1*

and *BRCA2* assay and the OncoScan array (Table 1, Supplementary Figures). We found discordant results between the two assays in four of the 15 cases. Three cases were not concordant, since Ion Reporter calculated 2 copies of *BRCA1* and *BRCA2* and the OncoScan array detected deletions and one gain (amplification) (Table 1: GeneDel#4, GeneDel#7 and GeneDel#11). In one case, the Oncomine *BRCA1* and *BRCA2* assay and the OncoScan array found both a *BRCA1* deletion but the OncoScan array reported also a *BRCA2* deletion (GeneDel#12).

Cross-Validation of Oncomine *BRCA1* and *BRCA2* Assay with MelArray assay

In order to clarify the reasons for this discordance, we added the complementary MelArray that takes into account the percentage tumor cells for the calculation of CNVs and has a numeric cut-off for the amplifications, hetero- and homozygous deletions for each sample. In all four discordant samples, the MelArray confirmed the result obtained by the Oncomine *BRCA1* and *BRCA2* assay. The additional deletions and gains observed in the OncoScan data were also detected by the MelArray but were beyond the numeric threshold for deletion/amplification (s. Suppl. table 1). We further elucidated if a result "*BRCA1* or *BRCA2* deletion" can be subdivided into heterozygous deletion or homozygous deletion by the Oncomine *BRCA1* and *BRCA2* assay. In one sample (GeneDel #15), a homozygous deletion was suspected due to two calls from the two different algorithms. GeneCNV CN 1 and an additional Exon Deletion of exons 5-27 (Fig. 4A and B). This was also reflected by the tremendous drop of the exons of *BRCA2* when compared to *BRCA1* and the sample ID in the visualization plot. This sample was compared to another sample (GeneDel#13) with only a *BRCA2* deletion detected, suggesting a heterozygous deletion (Fig. 4C and D). Both samples were sequenced additionally with the MelArray and by the corresponding CNV calculation pipeline a homozygous deletion in *BRCA2* was detected for GeneDel#15 and a heterozygous deletion for GeneDel#13 (s. Suppl. table 1). Whereas most of the samples sequenced

according to the diagnostic quality criteria show a good correlation between the calculated values and CNV confidence and the visualization plot, some samples are difficult to interpret. In sample GeneDel#1, according to the table with copy numbers, the algorithm evaluates some of the exons as "NOCALL" but with generally low CNV confidence (Fig. 5A). The only alteration with CNV confidence of 100 is the *BRCA2* deletion GeneCNV that can be unambiguously recognized on the visualization plot. In general, this sample was with relative high median of absolute pair-wise difference - MAPD (0.318), the value being a measurement of the variation in read coverage. High variation resulting in high MAPD values impairs the calculation of CNVs and thus, these results should be handled with care.

Summary of results

In summary, we cross-validated the results of LGRs gained by sequencing 20 samples with the Oncomine *BRCA1* and *BRCA2* assay and after integrating the numeric results from the IR 5.12 CNV algorithm and the visual representation we could detect correctly all 20 LGRs (Table 2). Based on our data, we suggest a decision tree (Fig. 6) that will help using all assay parameters and increase the sensitivity and positive predictive value of the assay in the diagnostic routine. However, the interpretation should be performed with caution since the detection of CNVs/exon deletions by the Oncomine *BRCA1* and *BRCA2* Assay is tremendously influenced by the quality of the library in terms of MAPD and uniformity.

Discussion

BRCA1/2 mutation status and genomic instability testing are an important part of treatment decisions in different tumor entities like ovarian, breast, prostate and pancreatic cancer (Coleman et al., 2017; De Bono et al., 2020; Golan et al., 2019; Hussain et al., 2020; Litton et al., 2018; Mirza et al., 2016; Moore et al., 2018; Pujade-Lauraine et al., 2017)

The Oncomine *BRCA1* and *BRCA2* Assay by Thermo Fisher is an assay commonly used on FFPE tissue for the detection of SNVs and small Indels in the genes *BRCA1* and *BRCA2*. However, an add-on of the assay enables the detection of larger rearrangements, the LGRs, including loss of exons and loss of the whole gene. Although the assay has been validated with high quality DNA from blood leukocytes and FFPE samples by the provider (Thermo Fisher Scientific, White Paper 2019 Evaluation of the Oncomine *BRCA* Research Assay for variant detection by next-generation sequencing), there is no study so far that systematically validated the performance of the assay in terms of detecting the LGRs in FFPE tumor tissue in the diagnostic setting. Moreover, according to the manufacturer no official thresholds for the detection of LGRs are available. Additionally, unlike the detection of SNVs and indels, the quality of the results for the detection of CNVs/exon deletions are tremendously influenced by the quality of the library in terms of low median absolute pair-wise difference MAPD/good uniformity. Thus, some diagnostic laboratories still do not report CNVs/exon deletions considering this part of the assay difficult to interpret (personal communication). Consequently, a wild-type status of the assay might miss this infrequent type of *BRCA1/2* aberrations and for these patients an additional test for germline mutations on blood is often performed by the medical geneticists. This increases costs and particularly turnaround times. Moreover, approximately 5 % of *BRCA1/2* mutations in ovarian cancer are somatic and remain undetected in germline testing (Bell et al., 2011; Cunningham et al., 2014; Hennessy et al., 2010; Pennington et al., 2014; Yates et al., 2014).

In order to use the Oncomine *BRCA1* and *BRCA2* Assay also for the detection of exon deletions and CNVs in the diagnostic routine, we performed a cross-validation of the assay using in total 20 FFPE samples. MLPA is routinely applied with good quality blood DNA and thus was used for cross-validation of the germline exon deletions. However, MLPA is not "gold standard" for degraded FFPE DNA; therefore, we subjected the test samples to OncoScan and MelArray for the detection of whole gene CNVs.

Starting with three samples with known germline exon losses, we showed that the detection of the exon losses was with a similar confidence in both good quality blood DNA and degraded FFPE DNA but was unique for the specific deletion of each patient. We analyzed two more patients only in FFPE tissue and found a tendency for higher CNV confidence in the cases with loss of two or more exons, probably due to the higher number of amplicons covering the area and thus increasing the certainty (Fig 2). Of note, a threshold is important to be set, since samples with high variation in the amplicon coverage can produce low CNV confidence calls for exon deletions or duplications (Fig. 5). The CNV confidence of 18 in sample ExDel#5 is rather low and although the visual representation is suggestive of a true positive result, confirmation with an additional assay would be recommended in the diagnostic report. This value was set as the lowest possible found in true positive samples.

The second algorithm is set to detect larger losses like a mono- or bi-allelic loss of the entire *BRCA1* or *BRCA2* gene. All samples were analyzed also with other versions of IR 5.6 and 5.10 (data not shown). Of note, the algorithm of IR 5.10 cannot detect whole gene losses of *BRCA1*.

In general, true positive whole gene losses are accompanied by a CNV confidence of 100, whereas small numbers are a sign of high standard deviation (MAPD high). We compared the data from the Oncomine *BRCA1* and *BRCA2* Assay with the OncoScan array data and set a threshold of 0.3 for the MAPD for the calling of exon losses and mono- or bi-allelic loss of *BRCA1* and *BRCA2* by IR5.12, since higher numbers reduce the reliability of the test. However, it is important to evaluate the visual representation of the data: the bi-allelic loss of *BRCA2* failed the QC for calling exon and gene losses probably due to the almost complete absence of amplicons covering *BRCA2* (MAPD 0.644, CNV QC- failed by Ion Reporter). The calling of *BRCA2* gene loss and the parallel loss of *BRCA2* exons 5-27 and considering the clear drop of all exons in *BRCA2* in the visual representation, however, lead to the correct result of biallelic loss of *BRCA2*. Of note is that the clinical significance of a mono-allelic loss

of *BRCA1* or *BRCA2* is questionable (Maxwell et al., 2017) and some assays do not report it (for example Foundation One CDx). Interestingly, we analyzed the three samples with known germline exon losses with another broadly used Oncomine assay – the Oncomine Comprehensive Assay (data not shown) and the corresponding Ion Reporter Workflow and this assay was not able to detect the exon deletions. This result underlines the need to use the specific Oncomine *BRCA1* and *BRCA2* Assay although the Oncomine Comprehensive Assay covers the genes *BRCA1* and *BRCA2*.

In summary, we performed an orthogonal validation of the Oncomine *BRCA1* and *BRCA2* Assay and show that it reliably detects known therapeutically relevant aberrations in the genes *BRCA1* and *BRCA2*, including large genomic rearrangements. Our data is in agreement with recently published reports that prove NGS assays capable of detecting LGRs in *BRCA1/2* (Germani et al., 2018; Han et al., 2020). The thorough validation of the Oncomine *BRCA1* and *BRCA2* test enables screening for targetable alterations directly in FFPE tumor tissue. Thus, a rapid, reliable and affordable tumor *BRCA1/2* testing could be used in the future as standard analysis after diagnosis with ovarian, breast, pancreatic and prostate cancer in routine diagnostics. This will immensely shorten the time for treatment decision, especially for patients without *BRCA1/2* alterations since only patients with tumor B*RCA1/2* mutations will be referred to the more time consuming genetic counselling and germline (*gBRCA1/2*) testing. However, we would like to stress that in samples of lesser library quality or with borderline MAPD of the assay, MLPA in blood remains the gold standard for both, confirming true positives and avoiding false positives.

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References

Bell, D., Berchuck, A., Birrer, M., Chien, J., Cramer, D.W., Dao, F., Dhir, R., Disaia, P., Gabra, H., Glenn, P., Godwin, A.K., Gross, J., Hartmann, L., Huang, M., Huntsman, D.G., Iacocca, M., Imielinski, M., Kalloger, S., Karlan, B.Y., Levine, D.A., Mills, G.B., Morrison, C., Mutch, D., Olvera, N., Orsulic, S., Park, K., Petrelli, N., Rabeno, B., Rader, J.S., Sikic, B.I., Smith-Mccune, K., Sood, A.K., Bowtell, D., Penny, R., Testa, J.R., Chang, K., Dinh, H.H., Drummond, J.A., Fowler, G., Gunaratne, P., Hawes, A.C., Kovar, C.L., Lewis, L.R., Morgan, M.B., Newsham, I.F., Santibanez, J., Reid, J.G., Trevino, L.R., Wu, Y.Q., Wang, M., Muzny, D.M., Wheeler, D.A., Gibbs, R.A., Getz, G., Lawrence, M.S., Cibulskis, K., Sivachenko, A.Y., Sougnez, C., Voet, D., Wilkinson, J., Bloom, T., Ardlie, K., Fennell, T., Baldwin, J., Gabriel, S., Lander, E.S., Ding, L., Fulton, R.S., Koboldt, D.C., McLellan, M.D., Wylie, T., Walker, J., O'Laughlin, M., Dooling, D.J., Fulton, L., Abbott, K., Dees, N.D., Zhang, Q., Kandoth, C., Wendl, M., Schierding, W., Shen, D., Harris, C.C., Schmidt, H., Kalicki, J., Delehaunty, K.D., Fronick, C.C., Demeter, R., Cook, L., Wallis, J.W., Lin, L., Magrini, V.J., Hodges, J.S., Eldred, J.M., Smith, S.M., Pohl, C.S., Vandin, F., Raphael, B.J., Weinstock, G.M., Mardis, E.R., Wilson, R.K., Meyerson, M., Winckler, W., Verhaak, R.G.W., Carter, S.L., Mermel, C.H., Saksena, G., Nguyen, H., Onofrio, R.C., Hubbard, D., Gupta, S., Crenshaw, A., Ramos, A.H., Chin, L., Protopopov, A., Zhang, J., Kim, T.M., Perna, I., Xiao, Y., Zhang, H., Ren, G., Sathiamoorthy, N., Park, R.W., Lee, E., Park, P.J., Kucherlapati, R., Absher, D.M., Waite, L., Sherlock, G., Brooks, J.D., Li, J.Z., Xu, J., Myers, R.M., Laird, P.W., Cope, L., Herman, J.G., Shen, H., Weisenberger, D.J., Noushmehr, H., Pan, F., Triche, T., Berman, B.P., Van Den Berg, D.J., Buckley, J., Baylin, S.B., Spellman, P.T., Purdom, E., Neuvial, P., Bengtsson, H., Jakkula, L.R.,

Durinck, S., Han, J., Dorton, S., Marr, H., Choi, Y.G., Wang, V., Wang, N.J., Ngai, J., Conboy, J.G., Parvin, B., Feiler, H.S., Speed, T.P., Gray, J.W., Socci, N.D., Liang, Y., Taylor, B.S., Schultz, N., Borsu, L., Lash, A.E., Brennan, C., Viale, A., Sander, C., Ladanyi, M., Hoadley, K.A., Meng, S., Du, Y., Shi, Y., Li, L., Turman, Y.J., Zang, D., Helms, E.B., Balu, S., Zhou, X., Wu, J., Topal, M.D., Hayes, D.N., Perou, C.M., Wu, C.J., Shukla, S., Sivachenko, A., Jing, R., Liu, Y., Noble, M., Carter, H., Kim, D., Karchin, R., Korkola, J.E., Heiser, L.M., Cho, R.J., Hu, Z., Cerami, E., Olshen, A., Reva, B., Antipin, Y., Shen, R., Mankoo, P., Sheridan, R., Ciriello, G., Chang, W.K., Bernanke, J.A., Haussler, D., Benz, C.C., Stuart, J.M., Benz, S.C., Sanborn, J.Z., Vaske, C.J., Zhu, J., Szeto, C., Scott, G.K., Yau, C., Wilkerson, M.D., Zhang, N., Akbani, R., Baggerly, K.A., Yung, W.K., Weinstein, J.N., Sheinson, T., Grimm, D., Hatfield, M., Morris, S., Yena, P., Rhodes, P., Sherman, M., Paulauskis, J., Millis, S., Kahn, A., Greene, J.M., Sfeir, R., Jensen, M.A., Chen, J., Whitmore, J., Alonso, S., Jordan, J., Chu, A., Barker, A., Compton, C., Eley, G., Ferguson, M., Fielding, P., Gerhard, D.S., Myles, R., Schaefer, C., Mills Shaw, K.R., Vaught, J., Vockley, J.B., Good, P.J., Guyer, M.S., Ozenberger, B., Peterson, J., Thomson, E., 2011. Integrated genomic analyses of ovarian carcinoma. Nature. doi:10.1038/nature10166

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., Helleday, T., 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. doi:10.1038/nature03443

Cao, W.M., Zheng, Y.B., Gao, Y., Ding, X.W., Sun, Y., Huang, Y., Lou, C.J., Pan, Z.W., Peng, G., Wang, X.J., 2019. Comprehensive mutation detection of BRCA1/2 genes reveals large genomic rearrangements contribute to hereditary breast and ovarian cancer in Chinese women. BMC Cancer. doi:10.1186/s12885-019-5765-3

Coleman, R.L., Oza, A.M., Lorusso, D., Aghajanian, C., Oaknin, A., Dean, Andrew,

Colombo, N., Weberpals, J.I., Clamp, A., Scambia, G., Leary, A., Holloway, R.W., Gancedo, M.A., Fong, P.C., Goh, J.C., O'Malley, D.M., Armstrong, D.K., Garcia-Donas, J., Swisher, E.M., Floquet, A., Konecny, G.E., McNeish, I.A., Scott, C.L., Cameron, T., Maloney, L., Isaacson, J., Goble, S., Grace, C., Harding, T.C., Raponi, M., Sun, J., Lin, K.K., Giordano, H., Ledermann, Jonathan A., Buck, M., Dean, A., Friedlander, M.L., Harnett, P., Kichenadasse, G., Denys, H., Dirix, L., Vergote, I., Elit, L., Ghatage, P., Plante, M., Provencher, D., Welch, S., Gladieff, L., Joly, F., Lortholary, A., Lotz, J., Medioni, J., Tredan, O., You, B., El-Balat, A., Harle, C., Krabisch, P., Neunhöffer, T., Pölcher, M., Wimberger, P., Amit, A., Kovel, S., Leviov, M., Safra, T., Shapira-Frommer, R., Stemmer, S., Bologna, A., Pignata, S., Sabbatini, R.F., Tamberi, S., Zamagni, C., O'Donnell, A., Herraez, A.C., Guerra, E.M., Palacio, I., Romero, I., Sanchez, A., Banerjee, S.N., Drew, Y., Gabra, H.G., Jackson, D., Ledermann, J. A., Parkinson, C., Powell, M., Birrer, M.J., Buss, M.K., Chambers, S.K., Chen, L.M., Ma, L., Morgan, M.A., Morris, R.T., Mutch, D.G., Slomovitz, B.M., Vanderkwaak, T., Vulfovich, M., 2017. Rucaparib maintenance treatment for recurrent ovarian carcinoma after response to platinum therapy (ARIEL3): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. doi:10.1016/S0140-6736(17)32440-6

Cunningham, J.M., Cicek, M.S., Larson, N.B., Davila, J., Wang, C., Larson, M.C., Song, H., Dicks, E.M., Harrington, P., Wick, M., Winterhoff, B.J., Hamidi, H., Konecny, G.E., Chien, J., Bibikova, M., Fan, J.B., Kalli, K.R., Lindor, N.M., Fridley, B.L., Pharoah, P.P.D., Goode, E.L., 2014. Clinical characteristics of ovarian cancer classified by BRCA1, BRCA2, and RAD51C status. Sci. Rep. doi:10.1038/srep04026

De Bono, J., Mateo, J., Fizazi, K., Saad, F., Shore, N., Sandhu, S., Chi, K.N., Sartor, O., Agarwal, N., Olmos, D., Thiery-Vuillemin, A., Twardowski, P., Mehra, N., Goessl, C., Kang, J., Burgents, J., Wu, W., Kohlmann, A., Adelman, C.A., Hussain, M., 2020. Olaparib for metastatic castration-resistant prostate cancer. N. Engl. J. Med.

doi:10.1056/NEJMoa1911440

Farmer, H., McCabe, H., Lord, C.J., Tutt, A.H.J., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., Martin, N.M.B., Jackson, S.P., Smith, G.C.M., Ashworth, A., 2005. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. doi:10.1038/nature03445

Freiberger S.N., Cheng P., Pornputtapong N., Turko P., Kong Y., Irmisch A., Khan M., Halaban R., Dummer R., W.P.J., n.d. MelArray: An integrated targeted sequencing panel to assess mutations, copy number, structural variants, and immunological features in melanoma for clinical decision support. maniscript Prep.

Freiberger, S.N., Morand, G.B., Turko, P., Wager, U., Dummer, R., Hüllner, M., Holzmann, D., Rupp, N.J., Levesque, M.P., 2019. Morpho-molecular assessment indicates new prognostic aspects and personalized therapeutic options in sinonasal melanoma. Cancers (Basel). doi:10.3390/cancers11091329

Germani, A., Libi, F., Maggi, S., Stanzani, G., Lombardi, A., Pellegrini, P., Mattei, M., De Marchis, L., Amanti, C., Pizzuti, A., Torrisi, M.R., Piane, M., 2018. Rapid detection of copy number variations and point mutations in BRCA1/2 genes using a single workflow by ion semiconductor sequencing pipeline. Oncotarget 9, 33648–33655. doi:10.18632/oncotarget.26000

Golan, T., Hammel, P., Reni, M., Van Cutsem, E., Macarulla, T., Hall, M.J., Park, J.O., Hochhauser, D., Arnold, D., Oh, D.Y., Reinacher-Schick, A., Tortora, G., Algül, H., O'Reilly, E.M., McGuinness, D., Cui, K.Y., Schlienger, K., Locker, G.Y., Kindler, H.L., 2019. Maintenance olaparib for germline BRCA-mutated metastatic pancreatic cancer. N. Engl. J. Med. doi:10.1056/NEJMoa1903387

Han, E., Yoo, J., Chae, H., Lee, S., Kim, D.-H., Kim, K.J., Kim, Y., Kim, M., 2020. Detection of BRCA1/2 large genomic rearrangement including BRCA1 promoter-region deletions using next-generation sequencing. Clin. Chim. Acta. 505, 49–54.

doi:10.1016/j.cca.2020.02.023

Hennessy, B.T.J., Timms, K.M., Carey, M.S., Gutin, A., Meyer, L.A., Flake, D.D., Abkevich, V., Potter, J., Pruss, D., Glenn, P., Li, Y., Li, J., Gonzalez-Angulo, A.M., McCune, K.S., Markman, M., Broaddus, R.R., Lanchbury, J.S., Lu, K.H., Mills, G.B., 2010. Somatic mutations in BRCA1 and BRCA2 could expand the number of patients that benefit from poly (ADP ribose) polymerase inhibitors in ovarian cancer. J. Clin. Oncol. doi:10.1200/JCO.2009.27.2997

Hussain, M., Mateo, J., Fizazi, K., Saad, F., Shore, N., Sandhu, S., Chi, K.N., Sartor, O., Agarwal, N., Olmos, D., Thiery-Vuillemin, A., Twardowski, P., Roubaud, G., Özgüroğlu, M., Kang, J., Burgents, J., Gresty, C., Corcoran, C., Adelman, C.A., de Bono, J., 2020. Survival with Olaparib in Metastatic Castration-Resistant Prostate Cancer. N. Engl. J. Med. 383, 2345–2357. doi:10.1056/nejmoa2022485

Judkins, T., Rosenthal, E., Arnell, C., Burbidge, L.A., Geary, W., Barrus, T., Schoenberger, J., Trost, J., Wenstrup, R.J., Roa, B.B., 2012. Clinical significance of large rearrangements in BRCA1 and BRCA2. Cancer 118, 5210–5216. doi:10.1002/cncr.27556

Kaelin, W.G., 2005. The concept of synthetic lethality in the context of anticancer therapy. Nat. Rev. Cancer. doi:10.1038/nrc1691

Knudson, A.G., 1971. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.68.4.820

Ledermann, J., Harter, P., Gourley, C., Friedlander, M., Vergote, I., Rustin, G., Scott, C., Meier, W., Shapira-Frommer, R., Safra, T., Matei, D., Macpherson, E., Watkins, C., Carmichael, J., Matulonis, U., 2012. Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N. Engl. J. Med. doi:10.1056/NEJMoa1105535

Ledermann, J., Harter, P., Gourley, C., Friedlander, M., Vergote, I., Rustin, G., Scott, C.L., Meier, W., Shapira-Frommer, R., Safra, T., Matei, D., Fielding, A., Spencer, S.,

Dougherty, B., Orr, M., Hodgson, D., Barrett, J.C., Matulonis, U., 2014. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: A preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol. doi:10.1016/S1470-2045(14)70228-1

Litton, J.K., Rugo, H.S., Ettl, J., Hurvitz, S.A., Gonçalves, A., Lee, K.H., Fehrenbacher, L., Yerushalmi, R., Mina, L.A., Martin, M., Roché, H., Im, Y.H., Quek, R.G.W., Markova, D., Tudor, I.C., Hannah, A.L., Eiermann, W., Blum, J.L., 2018. Talazoparib in patients with advanced breast cancer and a germline BRCA mutation. N. Engl. J. Med. doi:10.1056/NEJMoa1802905

Mateo, J., Lord, C.J., Serra, V., Tutt, A., Balmaña, J., Castroviejo-Bermejo, M., Cruz, C., Oaknin, A., Kaye, S.B., de Bono, J.S., 2019. A decade of clinical development of PARP inhibitors in perspective. Ann. Oncol. 30, 1437–1447. doi:10.1093/annonc/mdz192

Maxwell, K.N., Wubbenhorst, B., Wenz, B.M., De Sloover, D., Pluta, J., Emery, L., Barrett, A., Kraya, A.A., Anastopoulos, I.N., Yu, S., Jiang, Y., Chen, H., Zhang, N.R., Hackman, N., D'Andrea, K., Daber, R., Morrissette, J.J.D., Mitra, N., Feldman, M., Domchek, S.M., Nathanson, K.L., 2017. BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. Nat. Commun. doi:10.1038/s41467-017-00388-9

Mirza, M.R., Monk, B.J., Herrstedt, J., Oza, A.M., Mahner, S., Redondo, A., Fabbro, M., Ledermann, J.A., Lorusso, D., Vergote, I., Ben-Baruch, N.E., Marth, C., Madry, R., Christensen, R.D., Berek, J.S., Dørum, A., Tinker, A. V., Du Bois, A., González-Martín, A., Follana, P., Benigno, B., Rosenberg, P., Gilbert, L., Rimel, B.J., Buscema, J., Balser, J.P., Agarwal, S., Matulonis, U.A., 2016. Niraparib maintenance therapy in platinum-sensitive, recurrent ovarian cancer. N. Engl. J. Med. doi:10.1056/NEJMoa1611310

Mohyuddin, G.R., Aziz, M., Britt, A., Wade, L., Sun, W., Baranda, J., Al-Rajabi, R., Saeed, A., Kasi, A., 2020. Similar response rates and survival with PARP inhibitors for patients with solid tumors harboring somatic versus Germline BRCA mutations: A Meta-analysis

and systematic review. BMC Cancer 20, 1–9. doi:10.1186/s12885-020-06948-5

Moore, K., Colombo, N., Scambia, G., Kim, B.-G., Oaknin, A., Friedlander, M., Lisyanskaya, A., Floquet, A., Leary, A., Sonke, G.S., Gourley, C., Banerjee, S., Oza, A., González-Martín, A., Aghajanian, C., Bradley, W., Mathews, C., Liu, J., Lowe, E.S., Bloomfield, R., DiSilvestro, P., 2018. Maintenance Olaparib in Patients with Newly Diagnosed Advanced Ovarian Cancer. N. Engl. J. Med. 379, 2495–2505. doi:10.1056/NEJMoa1810858

Pennington, K.P., Walsh, T., Harrell, M.I., Lee, M.K., Pennil, C.C., Rendi, M.H., Thornton, A., Norquist, B.M., Casadei, S., Nord, A.S., Agnew, K.J., Pritchard, C.C., Scroggins, S., Garcia, R.L., King, M.C., Swisher, E.M., 2014. Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. Clin. Cancer Res. doi:10.1158/1078-0432.CCR-13-2287

Pujade-Lauraine, E., Ledermann, J.A., Selle, F., Gebski, V., Penson, R.T., Oza, A.M., Korach, J., Huzarski, T., Poveda, A., Pignata, S., Friedlander, M., Colombo, N., Harter, P., Fujiwara, K., Ray-Coquard, I., Banerjee, S., Liu, J., Lowe, E.S., Bloomfield, R., Pautier, P., Korach, J., Huzarski, T., Byrski, T., Pautier, P., Harter, P., Colombo, N., Scambia, G., Nicoletto, M., Nussey, F., Clamp, A., Penson, R., Oza, A., Poveda Velasco, A., Rodrigues, M., Lotz, J.P., Selle, F., Ray-Coquard, I., Provencher, D., Prat Aparicio, A., Vidal Boixader, L., Scott, C., Tamura, K., Yunokawa, M., Lisyanskaya, A., Medioni, J., Pécuchet, N., Dubot, C., de la Motte Rouge, T., Kaminsky, M.C., Weber, B., Lortholary, A., Parkinson, C., Ledermann, J., Williams, S., Banerjee, S., Cosin, J., Hoffman, J., Penson, R., Plante, M., Covens, A., Sonke, G., Joly, F., Floquet, A., Banerjee, S., Hirte, H., Amit, A., Park-Simon, T.W., Matsumoto, K., Tjulandin, S., Kim, J.H., Gladieff, L., Sabbatini, R., O'Malley, D., Timmins, P., Kredentser, D., Laínez Milagro, N., Barretina Ginesta, M.P., Tibau Martorell, A., Gómez de Liaño Lista, A.,

Ojeda González, B., Mileshkin, L., Mandai, M., Boere, I., Ottevanger, P., Nam, J.H., Filho, E., Hamizi, S., Cognetti, F., Warshal, D., Dickson-Michelson, E., Kamelle, S., McKenzie, N., Rodriguez, G., Armstrong, D., Chalas, E., Celano, P., Behbakht, K., Davidson, S., Welch, S., Helpman, L., Fishman, A., Bruchim, I., Sikorska, M., Słowińska, A., Rogowski, W., Bidziński, M., Śpiewankiewicz, B., Casado Herraez, A., Mendiola Fernández, C., Gropp-Meier, M., Saito, T., Takehara, K., Enomoto, T., Watari, H., Choi, C.H., Kim, B.G., Kim, J.W., Hegg, R., Vergote, I., 2017. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. Lancet Oncol. 18, 1274–1284. doi:10.1016/S1470-2045(17)30469-2

Yates, M., Timms, K., Daniels, M., Batte, B., Ring, K., Neff, C., Potter, J., Chau, S., Chen, J., Williams, D., Perry, M., Morris, B., Guan, A., Amin, Y., Munsell, M., Schmeler, K., Lanchbury, J., Lu, K., 2014. Next Generation Sequencing of Brca1/2 in High Grade Ovarian Tumors Expands Brca Defects Beyond Germline Mutations. Ann. Oncol. doi:10.1093/annonc/mdu238.10

Figure legends

Fig. 1. Validation of the detection of exon deletions by Oncomine *BRCA1* and *BRCA2* panel. A. Summary of the test samples and the parameters reported by Ion Reporter 5.12. B, C Visualization of the exons of *BRCA1* (orange) and *BRCA2* (green) based on the coverage and compared to the sample ID (yellow box). The deleted exons (arrows) display lower coverage compared to the baseline. The validation was performed with high quality DNA from blood

leukocytes (B) and consequently with DNA from the FFPE biopsies of the same patients (C). The CNV confidence is listed in A for both, blood and FFPE sample.

Fig. 2. Examples for FFPE samples with exon deletions difficult for interpretation. A. Summary of the parameters reported by Ion Reporter 5.12. B. Visualization of the exons of *BRCA1* (orange) and *BRCA2* (green) based on the coverage and compared to the sample ID (yellow box). The deleted exons (arrows) display lower coverage compared to the baseline (dotted line).

Fig. 3. Validation of the detection of gene CNVs by Oncomine *BRCA1* and *BRCA2* panel (extraction of the validation cohort). A. Summary of the test samples (examples depicted) and the parameters reported by Ion Reporter 5.12. B. Visualization of the exons of *BRCA1* (orange) and *BRCA2* (green) based on the coverage and compared to the sample ID (yellow box). The deletions display lower coverage compared to the baseline. C: Samples were cross-validated with OncoScan Array. Normalized log2-ratios of tumor to reference signal intensities at different SNP-positions across the entire genome of a human sample (upper panel). Chromosomal segments of copy number gains and losses are shown by blue and red boxes, resp., with the heights of the boxes indicating the average log2-ratio within each box. The yellow arrows indicate the location of BRCA1- (chromosome 17) and BRCA2-gene (chromosome 13). The middle panels show tumor to reference log2-ratio signal intensities of SNPs within (red dots) and in close vicinity (black dots) of BRCA1 and BRCA2 gene bodies, resp. The gene bodies are marked by blue boxes. The lower panels show tumor to reference log2-ratio signal intensities of SNPs within exons (blue boxes) and introns (white) of BRCA1 and BRCA2 gene bodies. The orientation of transcription is indicated by black arrows.

Fig. 4. Examples homozygous (A, B) vs. heterozygous loss (C, D) of *BRCA2*. A. and C. Parameters reported by Ion Reporter 5.12. B. and D. Visualization of the exons of *BRCA1* (orange) and *BRCA2* (green) based on the coverage and compared to the sample ID (yellow box).

Fig. 5. Examples difficult for interpretation. A. Summary of the parameters reported by Ion Reporter 5.12. B. Visualization of the exons of *BRCA1* (orange) and *BRCA2* (green) based on the coverage and compared to the sample ID (yellow box).

Fig. 6. Guidelines for reporting LGR the results obtained with Oncomine *BRCA1* and *BRCA2* Assay.

Supplementary Figure

S1 – S12. Samples were cross-validated with OncoScan Array. Normalized log2-ratios of tumor to reference signal intensities at different SNP-positions across the entire genome of a human sample (upper panel). Chromosomal segments of copy number gains and losses are shown by blue and red boxes, resp., with the heights of the boxes indicating the average log2-ratio within each box. The yellow arrows indicate the location of BRCA1- (chromosome 17) and BRCA2-gene (chromosome 13). The middle panels show tumor to reference log2-ratio signal intensities of SNPs within (red dots) and in close vicinity (black dots) of BRCA1 and BRCA2 gene bodies, resp. The gene bodies are marked by blue boxes. The lower panels show tumor to reference log2-ratio signal intensities of SNPs within exons (blue boxes) and introns (white) of BRCA1 and BRCA2 gene bodies. The orientation of transcription is indicated by black arrows.

S13. Distribution of the OncoScan probes across the *BRCA1* and *BRCA2* loci. *BRCA1* is covered by 65 in intronic regions and 15 probes in the exonic regions. *BRCA2* is covered by 60 intronic and 20 exonic probes. The locations of intronic probes are marked by red triangles , the locations of exonic probes are marked by white triangles.

Table 1. Concordance between *BRCA1* and *BRCA2* Oncomine assay with OncoScan and MelArray.

Sample ID	Oncomine w5.12		OncoScan	MelArray
	Call	Confidence		
GeneDel#1	*BRCA2* Deletion	100	*BRCA2* Deletion	-
GeneDel#2	*BRCA2* Deletion	100	*BRCA2* Deletion	-
GeneDel#3	*BRCA1* Deletion	100	*BRCA1* Deletion	-
GeneDel#4	**No CNV (*BRCA1/BRCA2)***	**-**	***BRCA2* Gain**	**No CNV (*BRCA1/BRCA2)***
GeneDel#5	No CNV (*BRCA1/BRCA2)*	-	No CNV (*BRCA1/BRCA2)*	-
GeneDel#6	*BRCA1* Deletion	100	*BRCA1* Deletion	-
GeneDel#7	**No CNV (*BRCA1/BRCA2)***	**100**	***BRCA1, BRCA2* Deletion**	**No CNV (*BRCA1/BRCA2)***
GeneDel#8	*BRCA2* Deletion	100	*BRCA2* Deletion	-
GeneDel#9	*BRCA1* Deletion	100	*BRCA1* Deletion	-
GeneDel#10	*BRCA1* Deletion	100	*BRCA1* Deletion	-
GeneDel#11	**No CNV (*BRCA1/BRCA2)***	**-**	***BRCA1* Deletion**	**No CNV (*BRCA1/BRCA2)***
GeneDel#12	***BRCA1* Deletion**	**100**	***BRCA1, BRCA2* Deletion**	***BRCA1* Deletion**
GeneDel#13	*BRCA2* Deletion	100	*BRCA2* Deletion	-
GeneDel#14	No CNV (*BRCA1/BRCA2)*	-	No CNV (*BRCA1/BRCA2)*	-
GeneDel#15	*BRCA2* Deletion	100	*BRCA2* Deletion	-

Table 2. Performance of the Oncomine *BRCA1* and *BRCA2* panel. Overlap of the NGS results (Oncomine *BRCA1* and *BRCA2* panel) with the results of other methods. No positive percentage agreement (PPA) and positive predictive value (PPV) are calculated due to the small number of cases analyzed. The asterix shows the numbers if the borderline case (ExDel#5) is considered true positive, in brackets the numbers if considered false negative.

NGS Result	Other test positive	Other test negative	Total
Positive	15* (14)	0	15
Negative	0* (1)	5	5
Total	15	5	20

Universitätsspital Zürich, PATH D 75, 8091 Zurich, Switzerland

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230 Park Avenue, Suite 800
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Nadejda Valtcheva, Dr. rer. nat.

University Hospital Zurich
Schmelzbergstrasse 12
8091 Zürich
Switzerland

Direct +41 44 255 19 51
nadejda.valtcheva@usz.ch

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Dear Dr. Lewis, dear Reviewers,

Thank you very much for considering our manuscript for publication. We appreciate the careful proof-reading by the reviewers and have corrected the errors.

According to the comment of Reviewer #2 we have changed the title to:

"Setting a diagnostic benchmark for tumor BRCA testing: detection of BRCA1 and BRCA2 large genomic rearrangements in FFPE tissue – a pilot study".

We hope, you find the manuscript in his current version suitable for publication.

Please find our detailed point-to-point reply in the rebuttal letter.

Kind regards,

Nadejda Valtcheva

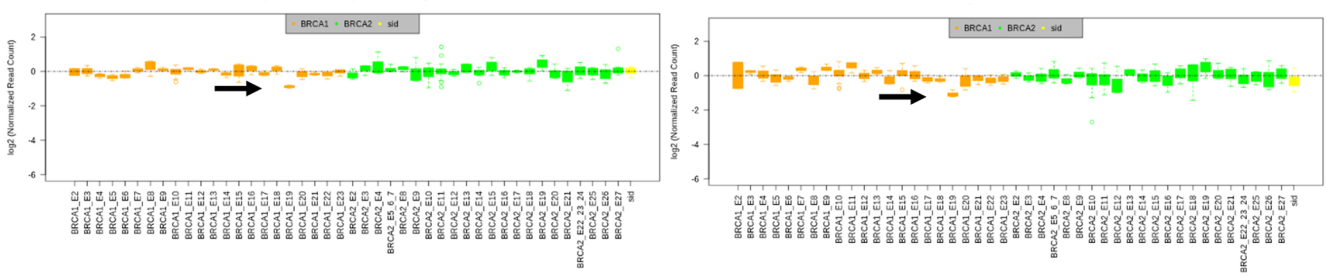
A.

Sample	Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence (Blood/FFPE)	Confirmed by other test?
ExDel#1	*BRCA1*	1	ExonDeletion	BigDel	EXON19	57/56	MLPA (blood)
ExDel#2	*BRCA1*	1	ExonDeletion	BigDel	EXON16	73/67	MLPA (blood)
ExDel#3	*BRCA1*	1	ExonDeletion	BigDel	EXON20-21	100/100	MLPA (blood)

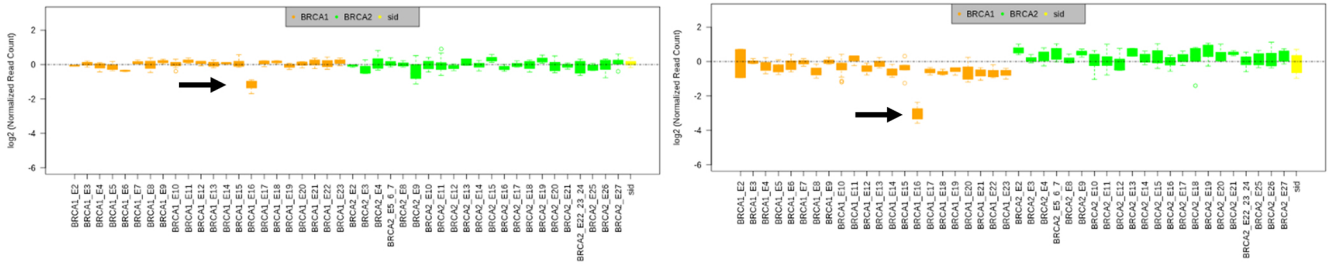
B. **Blood**

C. **FFPE**

ExDel#1



ExDel#2



ExDel#3

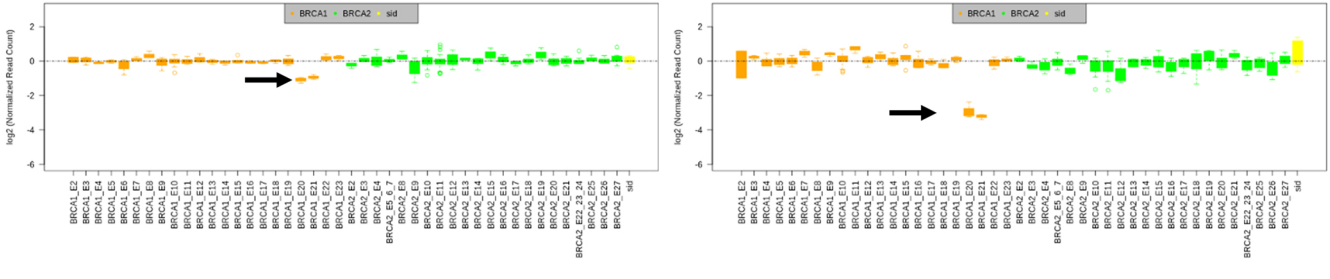


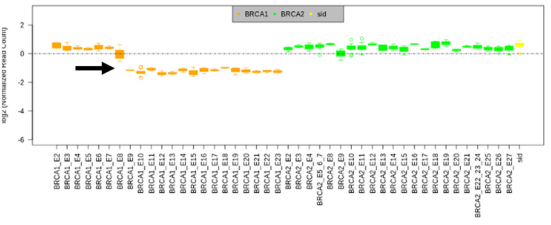
Figure 1

A.

Sample	Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence (FFPE)	Confirmed by other test?
ExDel#4	*BRCA1*	3	ExonDeletion	**BigDup**	EXON2-8	100	Yes, MLPA in blood (big del)
ExDel#5	*BRCA1*	1	ExonDeletion	BigDel	EXON7-8	**18**	Yes, MLPA in blood

B.

ExDel#4



ExDel#5

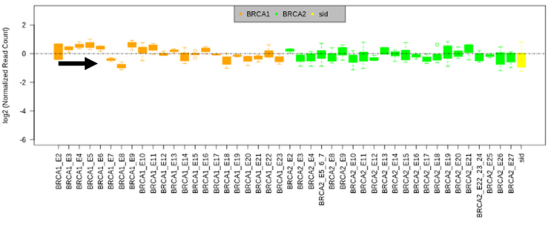


Figure 2

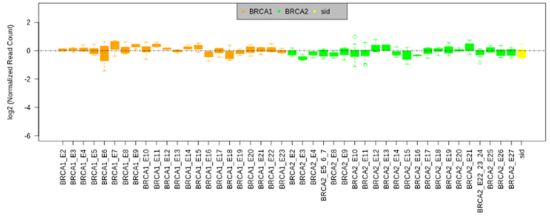
A.

Sample	Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence
GeneDel#5	*BRCA1*	2	-	REF	BRCA1 EXON2-23	100
	BRCA2	2	-	REF	BRCA2 EXON2-12	100
		2.63	-	NOCALL	BRCA2 EXON3	10.23
		2	-	REF	BRCA2 EXON14-27	100
GeneDel#3	*BRCA1*	1	Deletion	GeneCNV	BRCA1DEL	100
GeneDel#2	*BRCA2*	1	Deletion	GeneCNV	BRCA2DEL	100

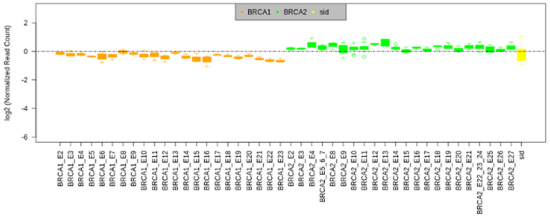
B.

IR 5.12

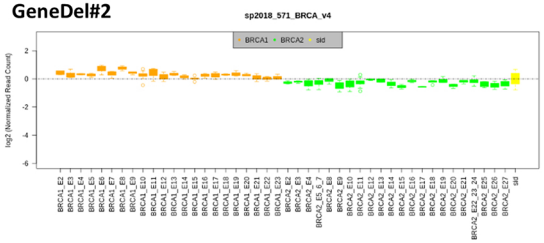
GeneDel#5



GeneDel#3



GeneDel#2



C.

OncoScan Array

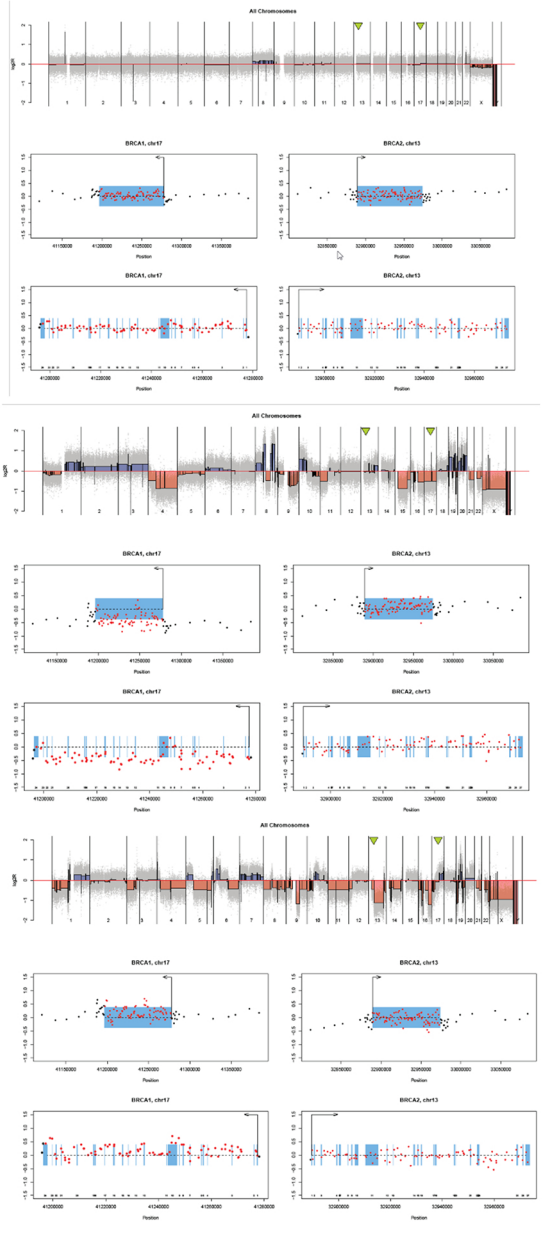
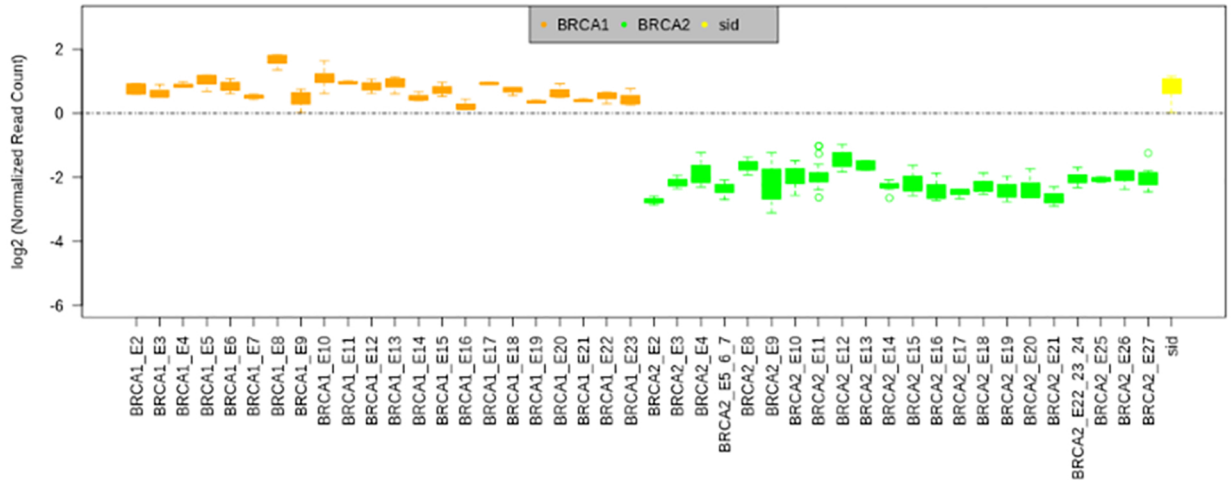


Figure 3

A. **GeneDel#15**

Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence
BRCA2	1	Deletion	GeneCNV	BRCA2DEL	100
BRCA2	0.53		NOCALL	EXON2-4	3.33
BRCA2	1	ExonDeletion	BigDel	EXON5-27	70.6
BRCA1	2.18		NOCALL	EXON3-23	1.26
BRCA1	3		BigDup	EXON2	11.44

B.



C. **GeneDel#13**

Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence
BRCA2	1	Deletion	GeneCNV	BRCA2DEL	100
BRCA2	2		REF	EXON2-27	100
BRCA1	2		REF	EXON3-23	100
BRCA1	3		BigDup	EXON2	14.45

D.

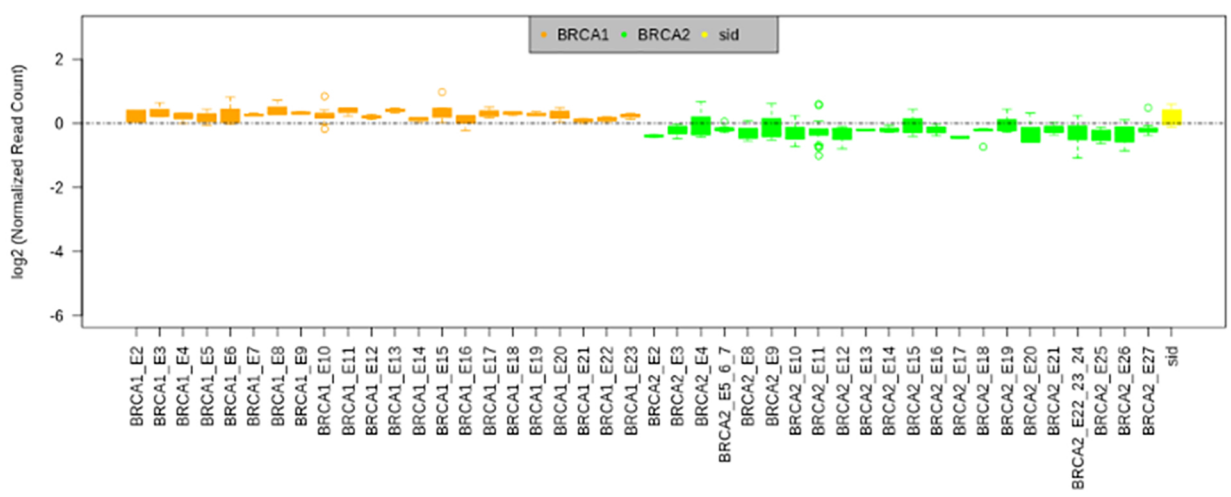


Figure 4

A. **GeneDel#1**

Gene	Copy number	Oncomine Variant Class	CNV Subtype	Call	CNV Confidence
BRCA2	1	Deletion	GeneCNV	BRCA2DEL	100
BRCA2	1.36		NOCALL	EXON2	7.18
BRCA2	2		REF	EXON3	15.16
BRCA2	1.63		NOCALL	EXON4	9.85
BRCA2	2		REF	EXON5-7	12.66
BRCA2	2.5		NOCALL	EXON8	7.81
BRCA2	1	ExonDeletion	BigDel	EXON9	12.31
BRCA2	2		REF	EXON10-14	58.28
BRCA2	1.41		NOCALL	EXON15-16	5.89
BRCA2	2		REF	EXON17	11.88
BRCA2	1.56		NOCALL	EXON18-20	4.16
BRCA2	1	ExonDeletion	BigDel	EXON21	24.11
BRCA2	2		REF	EXON22-24	10.31
BRCA2	1.49		NOCALL	EXON25	4.99
BRCA2	2		REF	EXON26	14.38
BRCA2	1.32		NOCALL	EXON27	15.15
BRCA1	2		REF	EXON23	12.5
BRCA1	1.48		NOCALL	EXON21-22	4.36
BRCA1	2		REF	EXON20	12.82
BRCA1	1.3		NOCALL	EXON18-19	8.99
BRCA1	2		REF	EXON15-17	38.44
BRCA1	1.53		NOCALL	EXON14	8.28
BRCA1	2		REF	EXON5-13	100
BRCA1	2.83		NOCALL	EXON4	9.61
BRCA1	2		REF	EXON3	13.75
BRCA1	3		BigDup	EXON2	20.92

B.

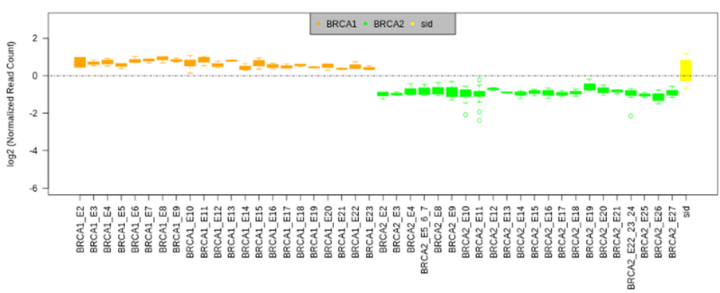


Figure 5

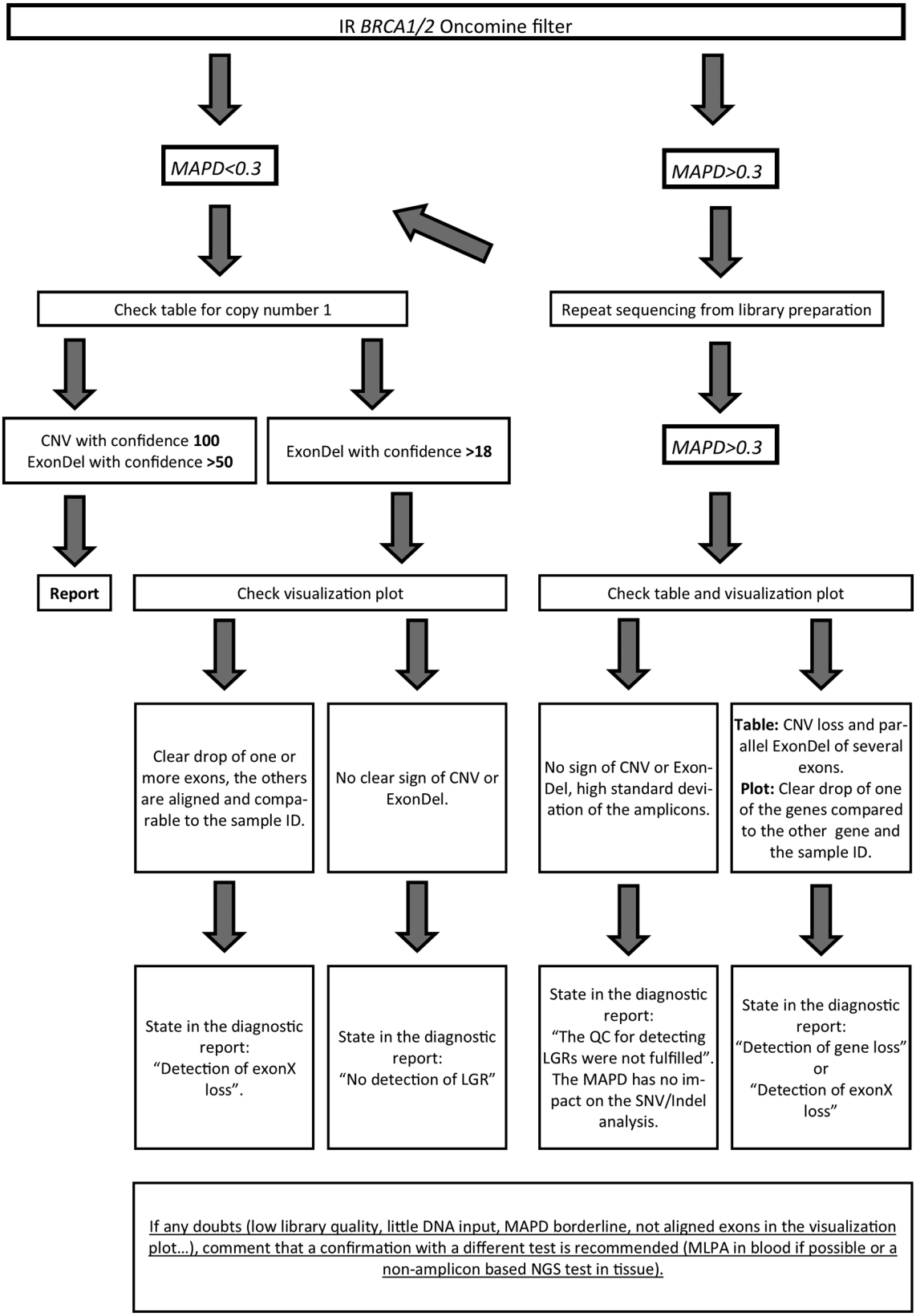

IR *BRCA1/2* Oncomine filter
MAPD<0.3
MAPD>0.3
Check table for copy number 1
Repeat sequencing from library preparation
CNV with confidence **100**
ExonDel with confidence **>50**
ExonDel with confidence **>18**
MAPD>0.3
Report
Check visualization plot
Check table and visualization plot
Clear drop of one or more exons, the others are aligned and comparable to the sample ID.
No clear sign of CNV or ExonDel.
No sign of CNV or ExonDel, high standard deviation of the amplicons.
Table: CNV loss and parallel ExonDel of several exons.
Plot: Clear drop of one of the genes compared to the other gene and the sample ID.
State in the diagnostic report:
"Detection of exonX loss".
State in the diagnostic report:
"No detection of LGR"
State in the diagnostic report:
"The QC for detecting LGRs were not fulfilled". The MAPD has no impact on the SNV/Indel analysis.
State in the diagnostic report:
"Detection of gene loss" or
"Detection of exonX loss"
If any doubts (low library quality, little DNA input, MAPD borderline, not aligned exons in the visualization plot…), comment that a confirmation with a different test is recommended (MLPA in blood if possible or a non-amplicon based NGS test in tissue).

Figure 6