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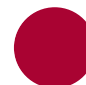

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TECHNICAL ADVANCE

Next-Generation Sequencing—Based Detection of Germline Copy Number Variations in *BRCA1/BRCA2*

Validation of a One-Step Diagnostic Workflow

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Genetic testing of *BRCA1/2* includes screening for single nucleotide variants and small insertions/deletions and for larger copy number variations (CNVs), primarily by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). With the advent of next-generation sequencing (NGS), it has become feasible to provide CNV information and sequence data using a single platform. We report the use of NGS gene panel sequencing on the Illumina MiSeq platform and JSI SeqPilot SeqNext software to call germline CNVs in *BRCA1* and *BRCA2*. For validation 18 different *BRCA1/BRCA2* CNVs previously identified by MLPA in 48 Danish breast and/or ovarian cancer families were analyzed. Moreover, 120 patient samples previously determined as negative for *BRCA1/BRCA2* CNVs by MLPA were included in the analysis. Comparison of the NGS data with the data from MLPA revealed that the sensitivity was 100%, whereas the specificity was 95%. Taken together, this study validates a one-step bioinformatics workflow to call germline *BRCA1/2* CNVs using data obtained by NGS of a breast cancer gene panel. The workflow represents a robust and easy-to-use method for full *BRCA1/2* screening, which can be easily implemented in routine diagnostic testing and adapted to genes other than *BRCA1/2*. *(J Mol Diagn 2017, 19: 809—816; http://dx.doi.org/10.1016/j.jmoldx.2017.07.003)*

Loss-of-function mutations in tumor suppressor genes *BRCA1* (MIM#113705) and *BRCA2* (MIM#600185) predispose to breast and ovarian cancer.[1—3] Genetic testing includes detection of single nucleotide changes, small insertions/deletions, and copy number variations (CNVs) defined as gain or loss of DNA fragments >1 kb. Recently, a total number of 81 and 17 CNVs have been reported for *BRCA1* and *BRCA2*, respectively.[4] The prevalence of *BRCA1/2* CNVs varies greatly among different populations, and numerous *BRCA1* founder events have been identified, for instance in the Dutch[5,6] and Danish[7] populations. The highest contribution of *BRCA1* CNVs is observed in the Dutch population in which 27% to 36% of all germline *BRCA1* mutations are CNVs.[5,6,8] In Denmark, CNVs account for 12.5% of all disease-causing *BRCA1* mutations identified in high-risk breast and ovarian cancer families,[7] a number resembling that of the French,[9] Spanish,[10] and German[11] populations. The proportion of CNVs in the *BRCA2* gene is generally much smaller, ranging from 0% to 8%.[7,12—17] The large difference in the number of genomic rearrangements between the two *BRCA* genes has been explained by the higher number of Alu repeats in *BRCA1*, which are known to increase the occurrence of rearrangements.[18]

For more than a decade, the golden standard for mutational screening of *BRCA1/2* has been Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) for small nucleotide variations and larger genetic rearrangements, respectively, but these techniques are being superseded by

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next-generation sequencing (NGS). Massive parallel sequencing of genomic regions using NGS allows molecular diagnostic laboratories to increase sample throughput, reduce the turn-around time, and analyze more disease-related genes simultaneously. As a result, many diagnostic laboratories have tested and implemented NGS-based platforms as their main technology for clinical testing.[19–25] The main challenge of implementing NGS in diagnostics is the development of a simple and robust bioinformatics pipeline, fulfilling the requirement of quality control for diagnosis.[26] Here, we validate an NGS-based method to identify *BRCA1*/*2* CNVs. Target DNA sequences were captured by hybridization, and the gene panels were sequenced on the Illumina MiSeq platform. Mapping of sequencing reads followed by variant calling, including CNV, was performed using the SeqPilot SeqNext software version 4.0 (JSI Medical Systems, Ettenheim, Germany). The validation set-up included a total number of 18 different *BRCA1*/*2* CNVs previously identified by MLPA and 120 patient samples determined as negative for *BRCA1/BRCA2* CNVs by MLPA.

Materials and Methods

CNV Nomenclature

The *BRCA1*/*2* CNVs are named according to the Human Genome Variation Society (*http://www.HGVS.org/varnomen*, last accessed January 3, 2017) guidelines for CNVs with characterized and uncharacterized breakpoints. For CNVs with uncharacterized breakpoints the nomenclature indicates that the breakpoints are located somewhere in the intronic sequences flanking the deleted or duplicated exons.

DNA Purification

Genomic DNA was purified from whole blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or the ReliaPrep Large Volume HT gDNA Isolation Kit (Promega, Madison, WI) using a Tecan Freedom EVO HSM2.0 Workstation according to the manufacturer's instructions.

MLPA Analysis

MLPA analysis was performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands) using the SALSA P002 and P087 *BRCA1* and SALSA P045 and P077 *BRCA2* MLPA Kits as recently described.[7]

Gene Panel Sequencing

A gene panel comprising six breast and ovarian cancer-predisposing genes (*BRCA1*, *BRCA2*, *RAD51C*, *CDH1*, *PTEN*, and *TP53*) was examined by NGS. Target DNA sequences were captured using biotinylated oligos provided through Roche NimbleGen (Roche, Basel, Switzerland). The oligos were designed to capture all exons, including 100 bp of flanking intronic sequence, from the six genes transcribed to the following transcripts: NM_007294 (*BRCA1*), NM_000059 (*BRCA2*), NM_058216 (*RAD51C*), NM_004360 (*CDH1*), NM_000314 (*PTEN*), and NM_000546 (*TP53*), respectively. Library was constructed using 500 ng of genomic DNA. The DNA was fragmented into an average size of 200 bp using a Covaris S2 AFA ultrasonicator, and adaptors from Illumina (Illumina, San Diego, CA) or Roche NimbleGen were ligated to the fragments. Illumina adaptors included in the TruSeq DNA LT Sample Preparation Kit were attached using an SPRI-works System I for Illumina Genome Analyzer (Beckman Coulter, Brea, CA). Adaptor sequences provided by Roche NimbleGen (SeqCap Adaptor Kit A/B) were ligated using the KAPA HTP Library Preparation Kit Illumina on a Sciclone G3 NGS Workstation (Perkin Elmer, Waltham, MA). Sequence capture was performed using the double capture protocol as described by Roche NimbleGen, where 6 to 12 samples are multiplexed before hybridization. Importantly, these 6 to 12 samples must follow the same type of library construction to successfully call CNVs. Finally, 2 × 76-bp paired-end sequencing was performed on the Illumina MiSeq platform to an average depth of 5 to 600× with a minimum coverage of at least 50×.

CNV Detection

CNVs were identified from the Illumina sequencing data by loading FASTQ files into the SeqPilot SeqNext software. This software detects CNVs based on the coverage in specific target regions of interest (ROIs). The ROIs are defined as exons ± 50-bp intronic sequence in *BRCA1*/*2* (and *RAD51C*, *CDH1*, *PTEN*, and *TP53*) and 24 control fragments localized on several different chromosomes. A minimum of six samples in a batch were simultaneously analyzed to obtain at least five control samples for each sample, assuming they did not contain common CNVs. Therefore, including family members in a batch should be avoided. To normalize the absolute amounts of *BRCA1*/*BRCA2* ROIs, the average coverage for the 24 control fragments is calculated for each sample. Then the relative coverage was calculated for each *BRCA1/BRCA2* ROI against the average coverage of the control fragments. These calculations are applied to all control samples and the analyzed sample. The percentage of the relative coverages of every specific *BRCA1/BRCA2* ROI in the analyzed sample is then compared with the average relative coverage of the control samples for the same ROI. If the ratio between the patient and control samples exceeds the lower (70%) or upper (135%) limit, it is regarded as a deletion or duplication, and the finding is verified by MLPA analysis. In cases when NGS and MLPA results are conflicting, new blood sample is requested and the MLPA analysis is repeated.

Results

By MLPA analysis, a total of 48 families positive for a *BRCA1*/*2* CNV were previously identified. Forty-five

Table 1 Large CNVs in *BRCA1* and *BRCA2* Identified in Danish Breast and/or Ovarian Cancer Families by MLPA and NGS Analysis

Families, *n*	Gene	MLPA	Genomic alteration	CNV calling from NGS data*	Reference
1	*BRCA1*	Del exon 1–3	c.(?_-232)_(134+1_135-1)del	E1 52%; E2 52%; E3 48%	27,28
1	*BRCA1*	Del exon 1–7	c.(?_-232)_(441+1_442-1)del	E1 66%; E2 48%; E3 56%; E5 48%; E6 52%; E7 54%	29–31
1	*BRCA1*	Del exon 1–14	c.(?_-232)_(4484+1_4485-1)del	E1 49%; E2 47%; E3 50%; E5 58%; E6 51%; E7 49%; E8 57%; E9 50%; E10 52%; E11 54%; E12 58%; E13 56%; E14 55%	32
2	*BRCA1*	Del exon 3	c.(80+1_81-1)_(134+1_135-1)del	E3 62%	16,28,33
24	*BRCA1*	Del exon 3–16	c.81-1018_4986+716del46586	E3 52%; E5 54%; E6 58%; E7 56%; E8 51%; E9 53%; E10 53%; E11 60%; E12 52%; E13 54%; E14 51%; E15 54%; E16 52%	7,34
2	*BRCA1*	Del exon 5–7	c.136-623_441+1958del4994	E5 56%; E6 52%; E7 52%	7,35–37
2	*BRCA1*	Dup exon 13	c.(4185+1_4186-1)_(4357+1_4358-1) dup	E13 172%	5,7,28,38–47
4	*BRCA1*	Del exon 13–15	c.(4185+1_4186-1)_(4675+1_4676-1) del	E13 50%; E14 51%; E15 48%	30,34,43,48,49
1	*BRCA1*	Del exon 17	c.(4986+1_4987-1)_(5074+1_5075-1) del	E17 54%	11,28,30,35,37,50–52
1	*BRCA1*	Del exon 17–18	c.4986+498_5152+171del6727	E17 50%; E18 54%	7
2	*BRCA1*	Del exon 17–19	c.(4986+1_4987-1)_(5193+1_5194-1) del	E17 52%; E18 54%; E19 58%	7,46,53
1	*BRCA1*	Del exon 18–19	c.(5074+1_5075-1)_(5193+1_5194-1) del	E18 49%; E19 47%	30,54–56
1	*BRCA1*	Del exon 19	c.(5152+1_5153-1)_(5193+1_5194-1) del	E19 50%	7
1	*BRCA1*	Del Exon 22	c.(5332+1_5333-1)_(5406+1_5407-1) del	E22 63%	6,28,30,39,40,57,58
1	*BRCA1*	Del exon 23–24	c.(5406+1_5407-1)_(1383_?)del	E23 51%; E24 56%	4,10,30,35,59
1	*BRCA2*	Del exon 12–16	c.(6841+1_6842-1)_(7805+1_7806-1) del	E12 54%; E13 48%; E14 46%; E15 51%; E16 51%	60
1	*BRCA2*	Dup exon 17–20	c.(7805+1_7806-1)_(8632+1_8633-1) dup	E17 190%; E18 165%; E19 183%; E20 176%	61
1	*BRCA2*	Del exon 20	c.8531_8632+1054del	E20 51%	7,15,56

*Coverage ≤70%: deletion; coverage ≥130%: duplication.
CNV, copy number variation; Del, deletion; Dup, duplication; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing.

families carried a *BRCA1* mutation, whereas only three families had a *BRCA2* mutation. Eighteen unique CNVs were identified—15 in *BRCA1* and three in *BRCA2*—including 16 gross deletions and two large duplications (Table 1).

To validate the hybridization-based gene panel set-up for CNV analysis, all of the *BRCA1/2* CNVs described above were examined. Coding regions plus 100 bp of flanking intronic sequence of six genes were captured by hybridization and sequenced on the Illumina MiSeq platform. The samples were hybridized in batches with at least six bar-coded diagnostic samples to obtain a minimum of five control samples, assuming they do not contain common CNVs. Sequence data were processed and analyzed using the SeqPilot SeqNext software, and CNVs were called based on differences in read depth. After normalization, a relative coverage was calculated for each *BRCA1/BRCA2* ROI in the patient sample of interest and control samples. All 18 CNVs tested were identified in the NGS data. The CNVs consisted of the following aberrations: *BRCA1* exon 1 to 3 deletion, *BRCA1* exon 1 to 7 deletion, *BRCA1* exon 1 to 14 deletion, *BRCA1* exon 3 deletion, *BRCA1* exon 3 to 16 deletion, *BRCA1* exon 5 to 7 deletion, *BRCA1* exon 13 duplication, *BRCA1* exon 13 to 15 deletion, *BRCA1* exon 17 deletion, *BRCA1* exon 17 to 18 deletion, *BRCA1* exon 17 to 19 deletion, *BRCA1* exon 18 to 19 deletion, *BRCA1* exon 19 deletion, *BRCA1* exon 22 deletion, *BRCA1* exon 23 to 24 deletion, *BRCA2* exon 12 to 16 deletion, *BRCA2* exon 17 to 20 duplication, and *BRCA2* exon 20 deletion (Table 1, Figure 1, and Supplemental Figures S1–S5). The average relative coverage compared with controls was 52.9% (range, 47% to 66%) for an exon deletion and 177.2% (range, 165% to 190%) for an exon duplication. Thus, there was a 100% concordance between the CNVs identified by MLPA and the CNVs identified by NGS analysis. To examine the specificity of the analysis 120 samples previously reported

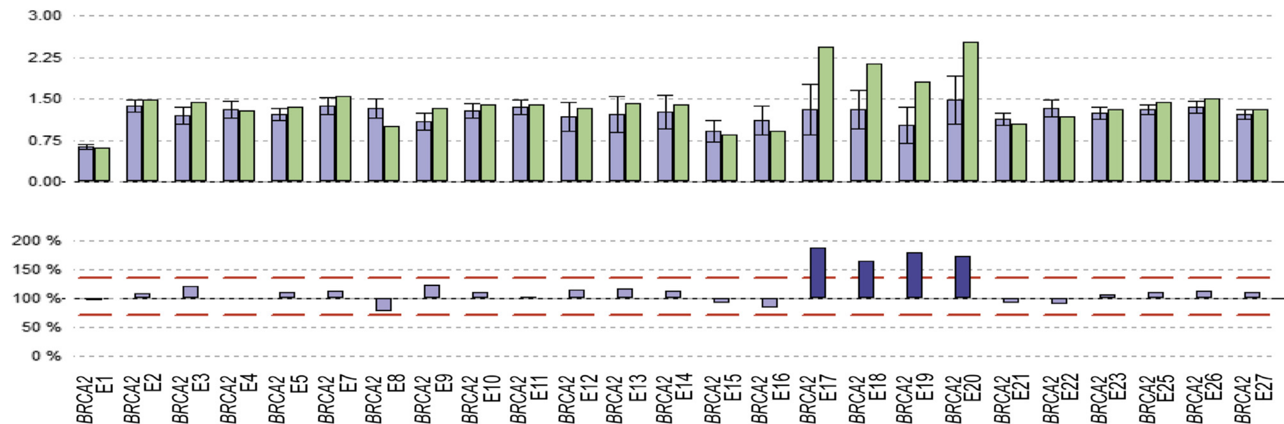

Figure 1 Visualization of *BRCA2* germline exon 17 to 20 duplication in JSI SeqPilot SeqNext software. The upper histogram shows the relative coverage of every target region of interest (ROI) of the patient sample in green and the average relative target coverage of control samples in blue. The lower histogram shows the ratio of the relative coverage of target ROIs calculated from patient versus controls. If the ratio exceeds the defined limits indicated by red lines, the bars change from light blue to dark blue, indicating a genomic change. A lower limit (**lower red dotted line**) of 70% (deletion) and an upper limit (**upper red dotted line**) of 135% (duplication) was used. Data are expressed as means ± SD of control samples.

as CNV negative by MLPA analysis were analyzed. Four samples had between one and four exon deletions and/or duplications in the *BRCA1* and/or *BRCA2* genes with an average relative coverage of 60.3% (range, 52% to 70%) for exon deletions and 138.8% (range, 137% to 143%) for exon duplications. In three of these four samples the deletions/duplications were separated by exons with normal average relative coverage. Moreover, two samples revealed multiple (>20) deletions and duplications. Overall, the results showed a 95% concordance between the MLPA and NGS results.

The interbatch reproducibility of the method was tested by repeating the library construction and targeted sequencing of 10 samples without *BRCA1/BRCA2* CNVs and 10 samples with *BRCA1* CNVs, including exon 1 to 3 deletion, exon 1 to 7 deletion (three different samples), exon 1 to 14 deletion, exon 3 to 16 deletion, exon 5 to 7 deletion, exon 13 to 15 deletion, exon 17 deletion, and exon 19 deletion. For all samples the result resembled the previous result. No deletions or duplication were called by repeated analysis of the 10 samples without *BRCA1/BRCA2* CNVs (Table 2).

In addition, 23 family members found to be *BRCA1* CNV positive by MLPA analysis were examined for *BRCA1* CNVs by NGS analysis, including seven cases with exon 3 deletion, seven cases with exon 3 to 16 deletion, three cases with exon 17 to 19 deletion, two cases of exon 1 to 7 deletion, one case of exon 1 to 14 deletion, one case of exon 5 to 7 deletion, one case of exon 13 to 15 deletion, and one case of exon 19 deletion. In all cases the deletion was identified by NGS analysis with an average relative coverage of 50.7% (range, 43% to 60%). Moreover, the intrabatch reproducibility of the method was tested by repeating the library construction and targeted sequencing of two samples (*BRCA1* deletion of exon 17 and *BRCA1* deletion of exons 3 to 16) six times each in one batch containing 12 samples, respectively. The data revealed an average relative coverage of 48.2% (range, 43% to 53%) for the exon 17 deletion and 51.5% (range, 42% to 68%) for exon 3 to 16 deletion.

Finally, the influence of the number of identical duplications in a single batch on the CNV calling were examined. Data containing one, two, or three identical duplications (*BRCA1* exon 13 duplication) in a batch of six samples (Supplemental Figure S6) were analyzed. The data revealed that including one sample in a batch resulted in a *BRCA1* exon 13 CNV call of 147%, including two samples resulted in a *BRCA1* exon 13 CNV call of 135%, whereas including three samples resulted in a *BRCA1* exon 13 CNV call of 127%, which does not exceed the limit of 135%. Inclusion of three identical exon 13 duplications however did not

Table 2 Average Relative Coverage of Two Independent Sequencing Runs of *BRCA1* CNVs

CNVs	RUN 1 Average relative coverage, % (range)	RUN 2 Average relative coverage, % (range)
BRCA1 exon 1–3 deletion	52 (49–58)	47 (43–49)
BRCA1 exon 1–7 deletion (sample 1)	53 (51–56)	49 (47–51)
BRCA1 exon 1–7 deletion (sample 2)	52 (48–60)	50 (47–56)
BRCA1 exon 1–7 deletion (sample 3)	49 (45–59)	48 (42–68)
BRCA1 exon 1–14 deletion	51 (47–56)	50 (45–54)
BRCA1 exon 3–16 deletion	50 (47–53)	51 (48–56)
BRCA1 exon 5–7 deletion	47 (43–50)	46 (43–49)
BRCA1 exon 13–15 deletion	50 (48–52)	49 (49–50)
BRCA1 exon 17 deletion	52	52
BRCA1 exon 19 deletion	56	54

CNV, copy number variation.

influence the CNV call of samples without rearrangement (data not shown). The analysis was also performed with identical deletions (*BRCA1* exon 3 to 16 deletion) in a single batch of six samples. In this case it was possible to include up to three samples with deletion and still call the CNV (Supplemental Figure S7). However, this in contrast influenced the CNV call of samples without rearrangement and led to the identification of a false-positive duplication call (Supplemental Figure S8).

Discussion

In recent years, NGS has paved its way into the molecular diagnostic set-up and made it possible to screen more genes and patients simultaneously. According to data from the European Molecular Genetics Quality Network, the number of laboratories using NGS for *BRCA1/2* screening has increased. In 2012/2013 only 6% of the laboratories involved in external quality assessment of *BRCA1* and *BRCA2* mutations used NGS, but by 2014 and 2015 this number had increased to 19% and 32%, respectively. These figures reflect the general trend toward using NGS-based approaches for genetic screening and consequently the need for validation of simple and accurate bioinformatics pipelines.

In this study, we validated a simple Illumina NGS set-up based on the capture of a panel of six genes involved in hereditary breast and ovarian cancer to identify *BRCA1/2* CNVs in routine genetic testing. In total, 18 large rearrangements in *BRCA1/2* were examined, and the analysis showed a 100% concordance between NGS and MLPA results. Moreover, the results were reproducible. The NGS set-up usually included hybridization of 12 barcoded samples in the same reaction. However, it is possible to include a minimum of two samples per batch if they are prepared in the same library construction and do not contain the same CNV in *BRCA1* or *BRCA2*. We recommend a minimum of five controls for each sample in a single batch. This makes it possible to analyze data even if some of the samples have false-positive CNVs or if patients with identical CNVs are included in the batch. Identical CNVs can disturb the CNV calling, a problem that also affects MLPA analysis. Therefore, it is also recommended that family members are not analyzed in the same batch. Our data however suggest that two samples with identical duplications (*BRCA1* exon 13 duplication) or identical deletions (*BRCA1* exon 3 to 16 deletion) of six samples do not affect the CNV calling of a true duplication or deletion or the CNV-negative samples in the batch. In contrast, including three identical deletions results in a false-positive duplication call in samples without rearrangements, whereas inclusion of three identical duplications results in a false-negative call of a sample with a *BRCA1* exon 13 duplication. Given the rarity of *BRCA1* and *BRCA2* CNVs in the Danish population, the risk of including more than two samples with identical CNV, when excluding family members, is considered very low.

The specificity of the NGS analysis was found to be 95%. Six of 120 CNV-negative samples revealed a false-positive CNV result by NGS analysis. In these cases the average relative coverage was 60.3% for exon deletions and 138.8% for exon duplications, which is closer to the threshold values of 70% and 135% than true deletions/duplications. However, the data indicate that all deletions/duplications called by NGS analysis should be verified by MLPA analysis. In cases when NGS and MLPA data are conflicting, a second blood sample is requested from the patient and the MLPA analysis is repeated.

Previous publications have described other streamlined NGS pipelines for diagnostic screening of *BRCA1* and *BRCA2*.[23,25] In these studies, 13 and 6 large *BRCA1/2* CNVs were included, respectively.[23,25] In contrast to these studies that involved more complicated bioinformatics work-flows, our set-up relies on a single software for detection of both CNVs, single nucleotide variants, and small deletions/insertions. The SeqPilot SeqNext software is very simple and user-friendly, and the processing time for full *BRCA1/2* mutational screening only takes a few minutes after upload of data.

The NGS-based sequencing and CNV calling has several advantages compared with the standard diagnostic set-up involving Sanger sequencing and MLPA. The most obvious is that only one method is required to perform full screening of *BRCA1* and *BRCA2*, thus reducing the diagnostic turnaround and costs per sample. Second, NGS requires a lower amount of DNA for multiple analyses. Third, calling CNVs using an NGS-based strategy avoids one group of false-positive results that are a feature of MLPA because of mutations present in the MLPA primer hybridization site. All of these advantages are important in a diagnostic setting; however, the NGS-based method also has limitations, especially for pseudogenes because of the hybridization-based capture approach. We have observed false-positive CNVs in *PTEN* because of the existence of highly homologous pseudogenes.[62] MLPA is also affected by some of these problems, but to a lesser extent, because probes are designed to avoid the pseudogenes. To circumvent the influence of pseudogenes, future set-ups could include an amplicon-based NGS method designed to preclude exon amplification from pseudogenes.

Conclusion

This study describes the validation of a simple one-step bioinformatics pipeline for CNV calling in *BRCA1* and *BRCA2* using Illumina gene panel data and the SeqPilot SeqNext software. Because of the existence of many different CNVs in *BRCA1* and *BRCA2*, these genes constitute a good model for testing the diagnostic NGS set-up.[25] We infer that the set-up may be adapted to other genes involved in hereditary diseases.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2017.07.003*.

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