Sequence Artifacts in DNA from Formalin-Fixed Tissues: Causes and Strategies for Minimization

Hongdo Do1,2,3* and Alexander Dobrovic1,2,3*

BACKGROUND: Precision medicine is dependent on identifying actionable mutations in tumors. Accurate detection of mutations is often problematic in formalin-fixed paraffin-embedded (FFPE) tissues. DNA extracted from formalin-fixed tissues is fragmented and also contains DNA lesions that are the sources of sequence artifacts. Sequence artifacts can be difficult to distinguish from true mutations, especially in the context of tumor heterogeneity, and are an increasing interpretive problem in this era of massively parallel sequencing. Understanding of the sources of sequence artifacts in FFPE tissues and implementation of preventative strategies are critical to improve the accurate detection of actionable mutations.

CONTENT: This mini-review focuses on DNA template lesions in FFPE tissues as the source of sequence artifacts in molecular analysis. In particular, fragmentation, base modification (including uracil and thymine deriving from cytosine deamination), and abasic sites are discussed as indirect or direct sources of sequence artifacts. We discuss strategies that can be implemented to minimize sequence artifacts and to distinguish true mutations from sequence artifacts. These strategies are applicable for the detection of actionable mutations in both single amplicon and massively parallel amplicon sequencing approaches.

SUMMARY: Because FFPE tissues are usually the only available material for DNA analysis, it is important to maximize the accurate informational content from FFPE DNA. Careful consideration of each step in the workflow is needed to minimize sequence artifacts. In addition, validation of actionable mutations either by appropriate experimental design or by orthogonal methods should be considered.

Recent advances in molecularly targeted therapies have greatly increased the clinical demand for the detection of actionable mutations in cancer patients. Mutational analysis is key for the stratification of cancer patients for appropriate molecularly targeted therapies. Currently, solid tumors that are selectively treated with small molecule inhibitors on the basis of mutational analysis include epidermal growth factor receptor (EGFR)-mutant lung cancer, v-raf murine sarcoma viral oncogene homolog B (BRAF)-mutant melanoma, and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT)-mutant gastrointestinal stromal tumor cancer. In other cancers, such as colorectal cancer, Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations indicate intrinsic or emerging nonresponsiveness to EGFR-directed antibodies. Thus, accurate detection of these and other clinically actionable mutations is a crucial part of precision medicine.

Formalin-fixed paraffin-embedded (FFPE) tissues are usually the primary material for detection of actionable mutations in solid tumors. Fixation of cancer tissues in buffered formalin (4% formaldehyde) is a standard procedure because formalin fixation preserves tissue and cellular morphology for assessment by anatomical pathologists. It also enables the fixed tissues to be stored at ambient conditions.

However, molecular testing with FFPE DNA is often problematic. In particular, extensive fragmentation significantly reduces the amount of amplifiable templates available for PCR amplification. A second major problem related to FFPE DNA is the occurrence of sequence artifacts, i.e., apparent sequence changes that are not present.

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in the original sample (Table 1). Several studies have demonstrated that the number of sequence variants seen in formalin-fixed tissues is higher than that in matched frozen tissues (3, 4).

It is often difficult to distinguish true sequence changes from artificial sequence changes, thus increasing the risk of false-positive mutation calls (5, 6). In some cases, sequence artifacts can be falsely interpreted as clinically important mutations. Tsao and colleagues reported multiple novel EGFR mutations in FFPE DNA (7) that have never been found in over 2000 fresh-frozen samples of non–small cell lung cancer (8). Other studies have confirmed that multiple artificial sequence alterations in the EGFR gene can arise in FFPE lung tissues (9, 10). A systemic review on the 3381 somatic EGFR mutations detected in 12244 patients with non–small cell lung cancer found that 71% of the EGFR mutations were seen in only a single case (11), suggesting that many of the reported EGFR mutations may be sequence artifacts.

Importantly, sequence artifacts can display the same base changes as recurrent canonical mutations. For example, KRAS mutations and EGFR T790M mutations are predictive markers for resistance to anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors, respectively (12, 13). Sequence artifacts corresponding to KRAS and EGFR T790M mutations have been reported in DNA from formalin-fixed colorectal and non–small cell lung cancers (14, 15). Lamy and colleagues reported that artificial codon 12 and 13 KRAS variants were present in 53 of 993 (5%) formalin-fixed colorectal cancers (14). Similarly, Ye and colleagues found a high rate of EGFR T790M mutations in formalin-fixed lung tumors (41.7%, 15 of 36 cases) and adjacent normal tissues (48.5%, 16 of 33 cases), but in only 1 matching fresh-frozen lung tumor (15), indicating that there were false positives in the tested formalin-fixed tissues.

Sequence artifacts can arise from various sources, including damaged templates preexisting in FFPE DNA (3, 4), oxidative DNA damage during sample preparation (16), DNA polymerase error (17), pseudogene amplification (18), adaptor sequences and adaptor chimeras (19), sequencing chemistry (20), sequence alignment (21), and spontaneous deamination of nucleotides during thermocycling (22, 23). Understanding these issues is important for accurate detection of actionable mutations and thus for implementation of precision medicine into the clinic. In this mini-review we specifically focus on preexisting damage to template DNA as a major source of sequence artifacts in FFPE DNA and discuss the strategies for minimization of sequence artifacts generated from damaged FFPE DNA.

### Types of DNA Damage in Formalin-Fixed Tissues

Several types of DNA damage have been identified in formalin-fixed tissues as sources of sequence artifacts (Fig. 1) and this section addresses these DNA damage types in more detail.

#### FORMALDEHYDE-INDUCED CROSSLINKS

Formaldehyde, the main component of formalin, is a reactive electrophilic chemical that creates various cross-links between intracellular macromolecules such as protein and DNA (24). The formaldehyde-induced crosslinks include protein–protein, protein–DNA, and DNA–formaldehyde adducts and interstrand DNA crosslinks. The interaction of formaldehyde with the functional groups of amino acids (e.g., primary amines and thiols) forms methylol adducts that can further cross-link with other amino acids through methylene bridge formation (25).

Formaldehyde also crosslinks DNA by reacting with the imino groups of DNA bases (26). Because the atoms in the imino groups are involved in hydrogen bonds mediating base pairing, formaldehyde-induced DNA adducts weaken the bonding strength of double-stranded DNA by reducing the number of hydrogen bonds in the

<table>
<thead>
<tr>
<th>Study</th>
<th>Artifactual base changes</th>
<th>Gene</th>
<th>Method</th>
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<tbody>
<tr>
<td>Do and Dobrovic (9)</td>
<td>C&gt;G&gt;T:A 60% C&gt;G&gt;A:T 35% C&gt;G&gt;G:C 0% A&gt;T&gt;G:C 5% Others 0%</td>
<td>EGFR</td>
<td>Sanger sequencing</td>
</tr>
<tr>
<td>Lamy et al. (14)</td>
<td>C&gt;G&gt;T:A 52% C&gt;G&gt;A:T 36% C&gt;G&gt;G:C 11% A&gt;T&gt;G:C 0% Others 1%</td>
<td>KRAS</td>
<td>SNaPshot multiplex PCR assay</td>
</tr>
<tr>
<td>Akabari et al. (53)</td>
<td>C&gt;G&gt;T:A 100% C&gt;G&gt;A:T 0% C&gt;G&gt;G:C 0% A&gt;T&gt;G:C 0% Others 0%</td>
<td>UNG+</td>
<td>Sanger sequencing</td>
</tr>
<tr>
<td>Wong et al. (62)</td>
<td>C&gt;G&gt;T:A 42% C&gt;G&gt;A:T 13% C&gt;G&gt;G:C 0% A&gt;T&gt;G:C 35% Others 10%</td>
<td>BRCA1</td>
<td>Sanger sequencing</td>
</tr>
<tr>
<td>Do et al. (41)</td>
<td>C&gt;G&gt;T:A 80% C&gt;G&gt;A:T 3% C&gt;G&gt;G:C 2% A&gt;T&gt;G:C 6% Others 9%</td>
<td>Gene</td>
<td>Targeted amplicon sequencing</td>
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* UNG, uracil-DNA glycosylase; BRCA, breast cancer 1, early onset.
DNA double helix (27). Furthermore, crosslinking of DNA bases with nearby histones, a dominant form of DNA damage in formaldehyde-exposed cells, results in a conformational change of DNA (28). Thus, formaldehyde-induced crosslinks of DNA reduce the stability of double-stranded DNA, resulting in a partial denaturation of DNA (27).

**DNA Fragmentation**

Fragmentation, often extensive, is the common form of DNA damage found in formalin-fixed tissues (5). Fragmentation of DNA in formalin-fixed tissues was shown to be increased with longer storage time and lower pH of formalin used in tissue fixation (29). Compared to DNA from fresh formalin-fixed tissues, the PCR success rate of DNA from older formalin-fixed tissues was shown to be decreased (29), indicating that DNA fragmentation may continuously occur during storage. Fragmentation damage in FFPE DNA directly influences the amount of templates available for PCR amplification (30). Thus, the same quantity of FFPE DNA from different samples may contain significantly different amounts of amplifiable templates, depending on the degree of fragmentation damage (6).

**ABasic Sites**

Formaldehyde is readily oxidized to formic acid in the reaction with atmospheric oxygen. The formation of formic acid reduces the pH of formalin. Formalin is thus usually buffered to maintain a neutral pH level. The $N$-glycosidic bonds of the purine bases to the sugar backbone are susceptible to hydrolysis at low pH (31), generating abasic sites in the DNA. Thus, fixation of tissues in unbuffered formalin will significantly lower the amount of amplifiable DNA templates (32).

The depurination rate of single-stranded DNA is 4 times higher than that of double-stranded DNA (33). Purine bases at the terminals of DNA strands are more readily depurinated than those located at internal positions (31). The aldehyde residue of abasic sites can generate an interstrand crosslink by reacting with the exocyclic amino group of a guanine base (34). Furthermore, abasic sites in DNA strongly destabilize the double helix (35), leading to local denaturation of the DNA. Because the rate of DNA damage in single-stranded DNA is higher than in double-stranded DNA, the DNA denaturation, induced by formaldehyde, may promote further DNA damage.

Abasic sites cause problems in sequence analysis. DNA polymerases have generally low bypass efficiencies at abasic sites (36), preventing amplification of DNA templates with abasic sites (37). However, when DNA polymerases read through abasic sites, sequence artifacts can be generated. Adenines are preferentially incorporated opposite to abasic sites by many DNA polymerases, but guanines or short deletions (1 to 3 bases) are also incorporated to a lesser extent (36). As a result, various types of artifactual single nucleotide variants (SNVs) and deletions can arise from abasic sites. In addition, abasic sites can undergo spontaneous cleavage through the $\beta$-elimination reaction leading to breakage of DNA strands (38).

**Deamination of Cytosine Bases**

Hydrolitic deamination of cytosine bases to uracil takes place at an estimated rate of 70–200 events/day in a living cell (39). In living cells, uracil lesions in DNA are removed by uracil-DNA glycosylase (UDG). In the resulting abasic site, the cytosine is then correctly restored by base excision repair due to the guanine in the com-
Pathological review is necessary to identify tumor-rich areas for macrodissection or coring of tumor tissues and to estimate the tumor purity within the sampled area. Tumor purity information is important for interpretation of the results because mutations will be present at lower frequency if there is a predominant amount of normal tissue. The analytic sensitivity of detection methods used for molecular testing varies substantially, and the minimum required levels of tumor purity differ depending on the detection method used. For Sanger sequencing, a minimum purity of 20% is desirable but deep sequencing by massively parallel sequencing (MPS) allows mutations to be detectable with lower tumor purities. However, at these lower tumor purities, it becomes more difficult to distinguish sequence artifacts from true mutations because both will be present at similar frequencies.

The quantity of DNA can be measured by spectrophotometry or fluorometry. Importantly, the same measured quantities of DNA from different FFPE samples can contain widely different amounts of amplifiable templates, depending on the degree of fragmentation (6). Both spectrophotometry and fluorometry tend to overestimate, often seriously, the actual amount of amplifiable templates in FFPE DNA (18). For this reason, PCR-based methods such as quantitative real-time PCR (qPCR) and digital PCR are preferable to quantify the amount of amplifiable templates in FFPE DNA. The amplicon size used in estimating targets should reflect the mean amplicon size used in the sequencing protocol (18, 46).

Information on the number of amplifiable templates will enable determination as to how reliable the accurate detection of variants might be. In low amplifiable template situations, the allele frequency of true variants cannot be reliably measured because of stochastic variation in allelic representation. Thus, the fewer the templates used in mutation analysis, the higher the risk of false negatives, especially in the case of low tumor purity, in which even true mutations are present at reduced frequencies. In addition, artifactual sequence variants arising from DNA damage will be more frequently detected because of stochastic enrichment in the low copy number context, increasing the risk of false positives (9, 18).

REMOVAL OF CROSSLINKS BY HEAT TREATMENT
Formaldehyde-induced DNA–DNA and DNA–protein crosslinks adversely affect the isolation of DNA from formalin-fixed tissues and the amount of amplifiable DNA templates by PCR. Formaldehyde-induced crosslinks are reversible by heat treatment (47). The reversal rate of formaldehyde crosslinks is closely dependent on the temperature and pH of the buffer solution (48, 49). The half-life of formaldehyde crosslinks is inversely correlated with temperature (48). High-
temperature heating methods, usually at >90 °C, have been shown to be effective not only for the yield of DNA (50), but also for the yield of amplifiable templates from FFPE tissues (49, 51).

IN VITRO REMOVAL OF MODIFIED BASES USING DNA-GLYCOSYLASES

Transitional C:G>T:A SNVs are the most frequent sequence artifacts arising from deamination of cytosine in FFPE DNA (8, 40). In vitro removal of uracil bases from FFPE DNA using UDG before PCR amplification markedly reduces the artificial C:G>T:A SNVs (40). This can be as high as 60%–80% in some FFPE DNAs (41).

UDG removes uracil bases from U:G mismatches in double-stranded DNA, generating abasic sites. The resulting abasic sites significantly hinder the amplification of templates by diminishing the DNA polymerase extension rate and causing thermal cleavage of templates under PCR cycling conditions (36). Thus, UDG pretreatment of FFPE DNA before PCR amplification can selectively prevent the enrichment of artifactual sequence reads from uracil lesions when a polymerase is used that does not read through abasic sites.

After UDG treatment, a certain number of artifactual C>T changes can still be observed. Many of these are at CpG sites which are presumably methylated (40, 41). Thymine lesions generated by deamination of 5-mC are theoretically removable from double-stranded DNA using either of the base excision repair enzymes MBD4 and thymine-DNA glycosylase (52). Up to now, this strategy has not, to our knowledge, been used in the context of FFPE DNA.

USING SHORT AMPICONS

There is a significant relationship between the amount of amplifiable DNA used in mutational analysis and the frequency of sequence artifacts (9, 18). Sequence artifacts in FFPE DNA are observed more frequently as the number of input DNA templates decreases (53). The fewer amplifiable templates that are used in molecular analysis, the more chance that DNA templates with lesions leading to subsequent sequencing errors will be de-

### Table 2. Strategies for minimization of sequence artifacts from FFPE DNA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Strategy</th>
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</thead>
<tbody>
<tr>
<td>DNA extraction</td>
<td>Assessment of tumor purity and identification of tumor-enriched areas by a pathologist</td>
</tr>
<tr>
<td></td>
<td>Macrodissection or coring of the tumor-enriched areas</td>
</tr>
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<td></td>
<td>Use of sufficient tissue, whenever possible, to ensure that a sufficient quantity of DNA is isolated for subsequent molecular testing</td>
</tr>
<tr>
<td></td>
<td>Heat treatment to remove formaldehyde-induced crosslinks and to facilitate subsequent tissue digestion with protease</td>
</tr>
<tr>
<td></td>
<td>Extended protease K treatment to digest tissue and to remove proteins cross-linked to DNA</td>
</tr>
<tr>
<td>DNA assessment</td>
<td>Assessment of double-stranded DNA quantity using fluorometry</td>
</tr>
<tr>
<td></td>
<td>Quantification of amplifiable templates using qPCR or digital PCR, especially for massively parallel sequencing. Use amplicons sizes that correspond to the mean amplicon size of the sequencing assay</td>
</tr>
<tr>
<td>Sample library preparation</td>
<td>In vitro removal of uracil prior to PCR amplification of FFPE DNA</td>
</tr>
<tr>
<td></td>
<td>Using assays generating short amplicons to increase the number of templates for PCR</td>
</tr>
<tr>
<td></td>
<td>Capture-based target enrichment allowing the recognition of the initial templates in sequence reads using their unique start and end sites</td>
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<tr>
<td></td>
<td>Using primers specific for each strand of the DNA template in amplicon-based target enrichment approach</td>
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<tr>
<td></td>
<td>Molecularly tagging DNA templates for identification of sequence artifacts</td>
</tr>
<tr>
<td>PCR amplification</td>
<td>Use of specific DNA polymerases (e.g. Pfu and KAPA) that have low bypass efficiency over DNA lesions such as uracil and abasic sites</td>
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<tr>
<td></td>
<td>Use a high-fidelity DNA polymerase to reduce polymerase errors</td>
</tr>
<tr>
<td>Validation of sequence variants from amplicon-based MPS</td>
<td>Running each test in duplicate so that separate pools of templates are used</td>
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<tr>
<td></td>
<td>Using orthogonal methods for clinically actionable mutations</td>
</tr>
</tbody>
</table>
Minimization of Sequence Artifacts

Fragmentation of FFPE DNA directly influences the amount of amplifiable templates available for PCR (30). The PCR success rate of FFPE DNA is strongly correlated with the size of the amplicon (49, 54), confirming the benefit of designing shorter amplicons. Thus, the use of short amplicon (e.g., 120 bp or less) should be considered to maximize the number of templates to be used for PCR.

REduced Amplification of Damaged Templates By High Fidelity Polymerases

DNA polymerases with low bypass efficiencies over various DNA lesions can be used for reduction of sequence artifacts. Many DNA polymerases incorporate adenine opposite to uracil during extension. However, other DNA polymerases, especially from the family B DNA polymerases (e.g., Pfu and KAPA), have a read-ahead function to recognize uracil lesions and terminate extension before misincorporation of adenine (55). Pfu polymerase has been shown to terminate the extension over uracil lesions in 70%–99% of templates (36). The bypass efficiency of DNA polymerases also differs markedly at abasic sites (36). Thus, the use of specific DNA polymerases with low bypass efficiencies over various DNA lesions would be a simple but effective way to minimize sequence artifacts generated from the DNA lesions.

Sequencing Both Strands of DNA

Accuracy in variant calling can be improved by protocols that have the capacity to sequence the sense and antisense strands of target sequences independently, e.g., molecular inversion probes and other extension-ligation techniques. Because each template lesion will be present in only 1 of the DNA strands, this approach can distinguish these and other sequence artifacts arising from DNA lesions from true mutations.

Molecular Tagging of DNA Templates

Tagging of DNA templates with unique sequences is a powerful approach which effectively reduces sequence artifacts. Recently, 2 methods, Safe-Sequencing System (Safe-SeqS) and duplex sequencing, were reported to enable more sensitive and accurate rare variant detection by stringently eliminating sequence errors (17, 56).

In the Safe-SeqS method, individual single-stranded DNAs are tagged with a unique identifier (UID) of 14-bp degenerate sequences to allow the tracking of the initiating templates in the sequence reads (17). The traceability of templates in their sequence reads enables the allelic frequency of each sequence variant to be readily counted. True mutations are present in all daughter molecules, whereas any errors introduced during the various experimental steps are present only in a lower proportion of a UID family. By eliminating variants present at less than <95% in the sequence reads with the same UID, this approach has been shown to reduce the error rate by approximately 20-fold (17).

In duplex sequencing, both the sense and the antisense strands of each DNA template are tagged with a unique double-stranded sequence at each end (56). This strategy enables any sequence variant detected in 1 strand of DNA to be crosschecked using the corresponding sequence reads from the other strand of the same template. Thus, sequence artifacts arising from DNA lesions are readily distinguished from true mutations because artificial sequence variants are detectable in only 1 of the strands but true mutations are present in both strands. Duplex sequencing thus enables sequence artifacts to be readily recognized by their strand specificity, resulting in exquisite sensitivities of 1 mutant molecule in 10000 wild-type molecules (56).

Capture-based Sequencing Approaches

Amplicons generated by PCR do not retain the information on the number of initiating templates of sequence reads, making it difficult to distinguish true mutations from sequence artifacts without adequate validation. For this reason, a capture-based approach is particularly helpful because the varying insert sequences of each captured template enable the differentiation of the templates (5). Because all of the sequence reads from the same template have the same insert sequence, the number of independent templates harboring the same sequence variants can be readily determined using a bioinformatic tool like Picard.

An important advantage of the capture-based approach is that the capture baits are shorter than amplicons and can be overlapped, enabling more templates to be captured. Orthogonal validation is usually not required when a mutation is seen in multiple independent templates. If a sequence variant is found in only 1 template, although detected in multiple sequence reads of the same template, the variant should be interpreted with caution because it may be a sequence artifact. Although the capture-based approach requires more setup time and may require shearing of DNA before library generation, it is amenable to automation (57). It is thus possible that capture-based approaches will become the preferred technology to analyze FFPE DNA for mutations.

Validation of Sequence Variants

Sequence artifacts are often present above the intrinsic background level of sequencing variation of MPS, which is operationally considered to be 1% (23). It can be difficult to distinguish artifacts from true low-level mutations which are present as the result of low tumor purity or tumor heterogeneity. Bioinformatic filtering has been
suggested as a potential strategy for artifact reduction (58, 59), but the bioinformatic removal of sequence variants can also increase the risk of false negatives for clinically important mutations, especially mutation results present at low levels.

When locus-specific singleplex assays are used in mutational analysis, all sequence variants detected can be readily be verified by using independent PCR products. However, validation of every single variant detected in MPS-based approaches is not feasible because of the high number of sequence variants.

It is, however, desirable to validate clinically important (actionable) mutations. The simplest approach is to consider only variants present in 2 independent MPS runs of separate aliquots of each sample (60). Another MPS methodology can also be used for validation (60) but is unlikely to be practical in diagnostic situations. Alternatively, validation can be built into the assay design, e.g., if the design of the assay means that independent templates can be identified, as in capture-based methods or by molecular tagging, or with strand-specific amplification.

Orthogonal methodologies not using MPS can also be used, particularly when only 1 or a few mutations need to be validated. Typically, a singleplex sequencing method such as Sanger sequencing or pyrosequencing is used, although the lower sensitivity can be an issue (61).

Conclusions

Detection of actionable mutations from formalin-fixed tissues is often problematic because of sequence artifacts arising from DNA damage. DNA fragmentation not only reduces the amount of amplifiable templates but also increases the sequence artifact rate due to stochastic enrichment of artificial changes.

A number of measures need to be implemented to reduce the danger of false-positive and false-negative calls in the diagnostic context. First, preanalytical assessment of amplifiable templates in FFPE DNA should be implemented into the workflow for reliable interpretation of mutational results. For conventional amplicon-based approaches, the removal of damaged templates is desirable directly and/or indirectly by the use of enzymes that do not read through modified or abasic bases. Marked reduction of C:G>T:A sequence artifacts by UDG pretreatment of FFPE DNA in combination with an enzyme that does not read through abasic sites demonstrates the validity of this approach.

To avoid false positives arising from sequence artifacts, sequence variants detected in FFPE DNA may need to be validated by 1 of several approaches, such as molecular barcodes to tag individual DNA templates to enable the origin of templates to be traced from the sequence reads or duplicate sequencing reads. It may be possible to simultaneously repair the multiple types of DNA damage seen in formalin-fixed tissues using a mixture of multiple DNA repair enzymes. Implementation of these approaches in mutational analysis will greatly improve accurate detection of clinically important mutations in formalin-fixed tissues.

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