MicroSEC: Sequence error filtering pipeline for formalin-fixed and paraffin-embedded samples

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Brief Communication

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Brief Communication

MicroSEC: Sequence error filtering pipeline for formalin-fixed and paraffin-embedded samples

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37 **Abstract**

38 The clinical sequencing of tumors is usually performed on formalin-fixed, paraffin-embedded
39 (FFPE) samples and results in many sequencing errors. Most of these errors are detected in
40 chimeric reads caused by single-strand DNA molecules with microhomology. Our filtering
41 pipeline, MicroSEC, focuses on the uneven distribution of mutations in each read and
42 removes the sequencing errors in FFPE samples without eliminating the true mutations that
43 are also detected in fresh frozen samples.

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Cancer gene panel testing using next-generation sequencing has been applied in routine practice to identify the somatic as well as germline mutations and to determine the appropriate treatment strategy for cancer patients¹. Somatic mutations often only occur in a small subset of cells and are present in a small fraction of DNA molecules from tumor samples. Somatic mutations can be detected in fresh or fresh frozen (FF) samples with the next-generation sequencers, but those materials are not always available for clinical sequencing.

As a result, the nucleic acids extracted from formalin-fixed and paraffin-embedded (FFPE) tumor tissues collected in surgeries or biopsies are more commonly used²⁻⁴. Formalin fixation and prolonged storage cause various changes on nucleic acids, such as the cross-linking between nucleic acids and proteins, denaturation, cytosine deamination, and chemical modification⁵. As a result, the DNA extracted from FFPE tissues is usually fragmented and contains single-stranded DNA (ssDNA)⁶. Low DNA quality causes significant noise in the sequencing reaction. Therefore, it is highly challenging to detect the mutations that occur at low variant allele fractions (VAF) in FFPE samples⁷⁻⁹. However, the development of filtering pipelines for FFPE artifacts has not progressed significantly beyond the well-known CG-to-TG mutation caused by cytosine deamination¹⁰⁻¹⁴.

Here, we propose a major mechanism for FFPE sequencing errors, microhomology-induced chimeric read (MICR) formation in the capture-based target sequencing. MICRs are

ssDNA-derived artifacts and classified into two types induced by different mechanisms. The first type of MICRs result from the hairpin structure formed by two palindromic sequences in the same ssDNA molecule. The second type of MICRs is formed from the mis-annealing of two ssDNA molecules derived from different homologous regions (Fig. 1a-c and Supplementary Fig. 1).

MICRs are formed during the end-repair step in the library preparation for clinical sequencing, when a considerable amount of extracted DNA is denatured to ssDNA and behaves as site-directed mutagenesis polymerase chain reaction (PCR) primers¹⁵. In the case of a true mutation, the altered bases are expected to be evenly distributed across the reads. Conversely, in case of an MICR error, the altered bases are distributed preferentially near the effective ends of the read, with severely limited lengths that match the reference sequence upstream or downstream of the mutations. VarScan2 mutation caller employs it as a filtering metric¹⁶. However, such bias is vaguely known. As MICRs form before the adapter ligation step in the library preparation, it cannot be ruled out in principle using template tagmentation techniques such as molecular identifier (ID) barcoding¹. Therefore, there is a significant need for specifically designed filtering pipelines.

Based on our theory that artifacts are derived from ssDNA-annealing, we have developed a MICR-originating Sequence Error Cleaning pipeline (MicroSEC), a post hoc filtering pipeline to predict if a given mutation is an MICR-derived error, utilizing BAM files

and lists of mutation-supporting read IDs. MicroSEC is based on three criteria (Fig. 1d and Online Methods). First, the local palindromic sequences causing hairpin-induced errors are detected (Filter 2). Second, the distribution of mutation positions in sequencing reads is interrogated if it is too uneven to occur probabilistically (Filter 1 and 3). Third, distant regions in the genome are searched, of which sequences are exactly matched to the query FFPE reads (Filter 4).

During the step of detecting hairpin-induced errors, 15-base sequences containing the mutation are extracted from each read. We consider the mutation an artifact if more than half of the sequences existed on the opposite strand within 150 bases of the neighboring sequence. During the step of detecting mutated base position bias, the number of bases from the mutated base to the farthest mapped base in each read is defined as the 3' or 5'-supporting lengths. The probability of the mutation distribution of the supporting lengths is calculated based on the multinomial distribution; mutations with $p < 10^{-6}$ are considered artifacts. During the step of detecting distant homologous regions, 40-base sequences including the mutation are extracted from each read. The mutation is considered an artifact if more than 15% of the sequences match another region in the genome.

Then, we examined the sensitivity of MicroSEC in distinguishing true mutations from FFPE artifacts. We analyzed the target sequencing data of 53 FF and 190 FFPE normal breast tissue samples with a high mean coverage of 400 or more. Our initial somatic mutation

analysis pipeline identified an average of 0.3 and 11.8 mutations per sample in FF and FFPE samples, respectively (Table 1). With the MicroSEC pipeline, 0 (0%) and 10.1 (85.8%) mutations per sample were filtered out from the data of the FF and FFPE samples, respectively. In the FFPE samples, possible artifacts such as the CG-to-TG mutations or mutations in or adjacent to a homopolymer equal to or longer than 10-base, accounted for half of the mutations that had passed through the filter (Table 1). The mutations passing through the filter had similar VAFs to the filtered-out mutations (Supplementary Fig. 2a).

Surprisingly, two unique mutations with VAF of >50% in FFPE samples were eliminated by Filter 1 or 2 (Supplementary Table 1). Thus, a high VAF does not necessarily indicate the mutation as a true one. The adequate threshold of mutation depth was also examined using FFPE samples from normal breast tissues, which were expected to have few or no mutations. When there were 10 or more reads supporting mutations, more than 90% of the mutations were consistently filtered out (Supplementary Fig. 3).

We then tested the specificity of MicroSEC by analyzing the target sequencing data of 23 FF and 33 FFPE breast cancer samples with a high mean coverage, including eight sets of matched FF and FFPE samples from the same patients. Our pipeline identified 4.0 and 7.5 mutations per sample in the FF and FFPE samples, respectively (Table 1). All the mutations in the FF samples passed through the filter, while 3.2 mutations per sample (30.1%) were filtered out in the FFPE samples. Mutations passing through the filter tended to have a higher

122 VAF than the filtered mutations (Supplementary Fig. 2b). Notably, MicroSEC did not
123 remove any mutations detected in matched FF samples (Supplementary Fig. 4). We revealed
124 the different distribution of the rates among low-quality bases, soft-clipped reads, artifacts
125 from homologous regions, and mutations derived from hairpin structures in the FF and FFPE
126 samples. We also confirmed that the thresholds used for filtering were appropriate
127 (Supplementary Fig. 5).

128 The MicroSEC analysis results were validated with amplicon-based sequencing that
129 enriches target genomic regions by PCR. Thirty mutations including germline mutations and
130 low VAF ones, found in the breast tissue samples, were examined. In theory, MICR-
131 originated artifacts cannot occur in amplification-based sequencing because PCR does not
132 amplify small structures formed by microhomology. Consistent with this prediction, 11
133 mutations that passed through the MicroSEC filter were all detected by amplicon-based
134 sequencing with a similar level of VAF, whereas 14 filtered mutations were not detected by
135 amplicon-based sequencing (Fig. 1e and Supplementary Table 2). A CG-to-TG mutation with
136 5.5% of VAF was not amplified in amplicon-based sequencing because this artifact arose
137 from deaminated cytosine, and the Q5 DNA polymerase could not amplify such degenerated
138 template DNA.

139 We further examined the utility of MicroSEC in the clinical setting by re-analyzing
140 the sequencing data of 54 clinical FFPE samples with a high mean coverage (Table 1)². At

first, 21.7 somatic mutations per sample were detected by the conventional mutation caller pipeline. MicroSEC detected 3.7 artifacts per sample (16.9%), including five unique pathogenic mutations (Supplementary Table 3). Since annotating artifacts as pathogenic can compromise the reliability of the clinical sequencing, the post hoc filtering with MicroSEC is of great value.

Most other existing error detection algorithms utilize strand bias, k-mer frequency, suffix trie, multiple sequence alignment, and statistical error models^{12, 13}. The application of these algorithms is limited because they are not highly accurate. These methods disregard detailed genomic information such as the local palindromic sequences and the position of mutations within reads. A few pipelines are constructed to remove the chimeric reads generated by PCR, only during the sequencing of about 1.5 kilobase pairs of the 16S ribosomal RNA^{14, 17, 18}.

In contrast, MicroSEC utilizes such overlooked information. MicroSEC's focus on the distribution of mutations in each read enables it to remove only FFPE artifacts without eliminating the true mutations. The effectiveness of MicroSEC is similar to the experimental reduction of FFPE artifacts by degrading ssDNA with S1 nuclease⁶. MicroSEC is useful in applications in target deep sequencing or whole-exome sequencing data with very high coverage. A caveat of MicroSEC lies in its basis on probability calculations; therefore, it requires a high number of reads supporting each mutation. Overall, our pipeline will increase

the reliability of the studies that use FFPE samples, thus advancing cancer research significantly. Our algorithm can also help to convey the correct information to cancer patients who underwent clinical sequencing, resulting in the improvement of quality and precision of their treatments. The source code of MicroSEC is freely available at <https://github.com/MANO-B/MicroSEC>.

ONLINE METHODS

Specimens

The study cohort comprised 26 patients with breast cancer who underwent tumor resection or prophylactic surgery because of hereditary breast and ovarian cancer syndrome at St. Luke's International Hospital between 2010 and 2020. A pathologist (N.K.) specializing in breast cancer reviewed the histological features and location of the tumors. Fifty-six FF samples of normal breast tissue were obtained from 14 patients, and 211 FFPE samples were obtained from 26 patients. Twenty-three FF breast cancer samples were obtained from nine patients, and 37 FFPE samples were obtained from 11 patients, including matching FF and FFPE samples from eight patients. The FFPE specimens were fixed in 10% neutral-buffered formalin for 15–72 hours and then embedded in paraffin blocks. All FFPE specimens had been stored at room temperature for at least six months before DNA extraction. Blood samples were obtained from all the patients as a source of matching normal DNA.

The study protocol was approved by the Ethics Committees of St Luke's International Hospital and the National Cancer Center Research Institute. Written informed consent was obtained from all the participants.

Capture-based panel sequencing

Genomic DNA was isolated from FF or blood samples using QIAamp DNA Mini Kits (Qiagen, Germany) or FFPE samples using GeneRead DNA FFPE Kits (Qiagen, Germany). Then, uracil DNA glycosylase treatment was performed for FFPE samples to remove C-to-T artifacts. Lastly, 500 ng of genomic DNA was subjected to target fragment enrichment using SureSelectXT Custom kits (Agilent Technologies, USA) or Twist Custom Panels (Twist Bioscience, USA). Custom-made probes were designed to hybridize and capture the target genes listed in the Todai OncoPanel (TOP) as previously described². Double-stranded linear DNA libraries for Illumina sequencer were converted to single-stranded circular DNA libraries for MGI sequencer with the MGIEasy Universal Library Conversion Kit (MGI Technologies, China). A library's quantity and quality were assessed with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and the Agilent 2200 TapeStation System (Agilent Technologies, USA). The massive parallel sequencing of the isolated fragments with 124–151 base pair read lengths was performed using the HiSeq 2500 System, NovaSeq 6000 System (Illumina, USA), or DNBSEQ-G400 sequencer (MGI Technologies, China). Samples with an insufficient mean coverage of less than 400 were excluded (n = 28, 8.6%).

197 **Amplicon-based sequencing**

198 The genomic regions of 106–156 bases around a mutation were amplified by PCR using the
199 NEB Q5 Hot Start HiFi PCR Master Mix (New England BioLabs, USA) and appropriate
200 primer sets (Supplementary Table 3). PCR products were purified using Agencourt AMPure
201 XP beads (Beckman Coulter, USA) and subjected to library preparation with the NEBNext
202 Ultra II DNA Library Prep Kit for Illumina (New England BioLabs, USA). The libraries were
203 sequenced to generate 150 base paired-end reads using the Miseq system (Illumina, USA).

204 **Somatic mutation calls**

205 Paired-end reads were independently aligned to the human reference genome (University of
206 California, Santa Cruz Genome Browser assembly ID: hg38) using Burrows-Wheeler Aligner
207 (v0.7.17)¹⁹ and Bowtie2 (v2.1.0)²⁰. Potential PCR duplicates were removed with SAMtools
208 (v1.9)²¹. Somatic mutations were identified using MuTect2 (GATK v4.1.3.0)²², VarScan2
209 (v2.4.3)¹⁶, and our in-house pipeline.

210 Mutations were discarded if the read depth was shorter than 100 base pairs or the
211 number of mutation-supporting reads was fewer than 10 or VAF was less than 5%. Mutations
212 were also eliminated if the reads were supported by only one strand of the chromosome,
213 observed in normal human genomes in the 1000 Genomes Project dataset²³ with a frequency
214 greater than 1% or in our in-house database, located outside the 3.4 megabase TOP target
215 region, or located at simple repeat sequences downloaded from the Tandem Repeats Database

216 (<https://tandem.bu.edu/cgi-bin/trdb/trdb.exe>)^{24, 25}. In addition, the mutations were removed if
217 a homopolymer consisting of more than 14 bases was present within 20 bases around the
218 mutation; or more than 10% of the bases within the mutation supporting reads had a Phred
219 quality score of less than Q18. The mapped reads and called mutations were visualized and
220 manually checked with the Integrative Genomics Viewer (v2.4.10)²⁶.

221 **MicroSEC pipeline**

222 MicroSEC (v1.1.18) is a filtering pipeline written in R language designed to discover MICR-
223 derived sequencing errors in FFPE samples. It detects local palindromic sequences, the
224 uneven distribution of mutated bases within each read, and the pseudo-mutations introduced
225 by distant homologous regions.

226 The workflow of MicroSEC in this study was composed of several steps. First, the
227 positions of the mutated bases were detected with the matchPattern function from the
228 Biostrings package (v2.54.0). Second, the adapter sequence at the 3' end of each read was
229 trimmed with the trimLRPatterns function from the Biostrings package with the maximum
230 mismatch rate of 10%. Then, the distance from the altered base to the read's effective ends
231 was calculated based on the Compact Idiosyncratic Gapped Alignment Report (CIGAR)
232 strings of the alignment results (see "Supporting length analysis"). Next, the hairpin structure-
233 induced chimeric reads were detected (see "Hairpin structure detection"). Afterward, the
234 entire genome was searched for regions that were homologous to the sequence around the

235 mutation using the countPDict function from the Biostrings package (see "Homologous
236 region detection"). Lastly, the above results were combined to construct the MicroSEC
237 filtering pipeline. The results of MicroSEC's filtering of the somatic mutations in breast
238 tissue samples and clinical sequencing samples were provided as Supplementary Data.

239 **Supporting length analysis**

240 It is essential to identify the uneven distribution of the mutations in the mutation-supporting
241 reads for discriminating between the MICR-derived errors and true mutations. Here, we have
242 introduced the concept of support lengths, which are the base lengths from the mutated bases
243 (excluding the mutated bases) to the furthest mapped bases in each read, based on the CIGAR
244 strings in BAM files. When a mutation is a substitution of a number (N) of nucleotides, and
245 the read length is another number (L) of nucleotides, the 3'- or 5'-supporting lengths are
246 distributed between zero and $L - N$. When a mutation is an N-nucleotide deletion or
247 insertion, the possibility of the mutation being the part of a repeated sequence should be
248 considered, because the mutation-supporting reads must contain all the repetitive sequence to
249 determine the presence or absence of small insertions/deletions (indels).

250 Furthermore, we need to consider the penalties by Burrows-Wheeler Aligner for
251 mapping with gaps. Since the penalty due to an N-base gap is $N + 6$ and the soft-clipping
252 penalty is 5, a sequence supporting the indel would be soft-clipped if there are no more than
253 $N + 1$ matching sequences outside the gap. Given the 5' and 3' repetitive sequences around

254 the mutation of lengths $R_{5'}$ and $R_{3'}$, the supporting lengths can be distributed between $\max(R_{3'}$
 255 $\text{or } 5', N + 1)$ and $L - \max(R_{5' \text{ or } 3'}, N + 1)$ for deletions, or between $\max(R_{3' \text{ or } 5'}, N + 1)$ and $L -$
 256 $N - \max(R_{5' \text{ or } 3'}, N + 1)$ for insertions, with $\max(X, Y)$ denoting the larger value of X and Y .

257 We also have introduced the concept of the shorter support length, which is the
 258 shorter of the 3'- and 5'-supporting lengths in each read. The theoretical distributions are
 259 between zero and $[(L - N) / 2]$, between $\min(\max(R_{3'}, N + 1), \max(R_{5'}, N + 1))$ and $[L / 2]$,
 260 and between $\min(\max(R_{3'}, N + 1), \max(R_{5'}, N + 1))$ and $[(L - N) / 2]$, for substitutions,
 261 deletions, and insertions, respectively. $[X]$ represents the greatest integer less than or equal to
 262 X , with $\min(X, Y)$ denoting the smaller value of X and Y .

263 Suppose the actual supporting length distribution is between A and B , the theoretical
 264 distribution is between C and D , and the coverage depth of the mutation-supporting reads is
 265 E . In that case, the probability p is calculated based on the multinomial distribution by the
 266 following equations:

$$267 \quad p = \left\{ \sum_{m=A}^B f(m) / \sum_{n=C}^D f(n) \right\}^E$$

268 $f(x) = [\text{The number of reads of which the supporting length is } x.]$

269 We regard mutations with $p < 10^{-6}$ as errors in principle. During deep sequencing, however,
 270 p becomes too small with large E , leading to the overfiltering of the mutations. Thus, the

271 filter is not applied to the mutations if $B - A + 1$ is greater than 75% of $D - C + 1$, or

272 $\sum_{m=A}^{m=B} f(m)$ is greater than 75% of $\sum_{n=C}^{n=D} f(n)$.

273 When multiple mutations are close to each other, the minimum required supporting
274 length will increase because of the cumulative mismatch penalties of the mutations.
275 Overfiltering can be avoided by fixing the minimum supporting length to zero if there are
276 more than three mismatched bases in the 10 bases surrounding the mutation.

277 **Hairpin structure detection**

278 A sequence of at least 15 consecutive bases, with the mutated bases at the center, was
279 extracted from each read. If the sequence was present on the opposite strand and within 150
280 bases of the neighboring sequence, the mutation was suspected to be derived from a hairpin
281 structure. If more than 50% of the reads had such sequences, the mutation would be
282 considered an error.

283 **Homologous region detection**

284 We extracted a sequence comprising the mutated bases and the surrounding repetitive
285 sequences if available from each read. Four upstream or downstream bases were added to that
286 sequence, and a sequence further from the sequence with the mutation was added on the
287 opposite side to create two sequences with a total of 40 bases. We searched the entire genome
288 for regions matching exactly to one of the two sequences. If more than 15% of the reads had
289 such homologous sequences, the mutation would be considered an artifact.

290 **Data availability**

291 We have deposited the raw sequencing data of the breast tissue samples examined in this
292 study under the accession number JGASxxxxxxxxxx (accession codes will be available
293 before publication) in the Japanese Genotype-Phenotype Archive
294 (<http://trace.ddbj.nig.ac.jp/jga>) hosted by the DNA Data Bank of Japan. The target
295 sequencing data of 54 cancer specimens are available for download at the Japanese
296 Genotype-Phenotype Archive under the accession number JGAS00000000164.

297 **Code availability**

298 All the source code written in R for MISEC is publicly available at
299 <https://github.com/MANO-B/MicroSEC> and [https://cran.r-](https://cran.r-project.org/web/packages/MicroSEC)
300 [project.org/web/packages/MicroSEC](https://cran.r-project.org/web/packages/MicroSEC).

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310 **Author contributions**

311 M.I. conceived the project and developed the algorithm. N.K. and H.Y. collected breast tissue
312 samples. M.I., S.K., T.H., and T.U. designed and analyzed the experiments. H.K. and S.T.

supervised the projects. M.I., S.K., T.H., T.U., and H.M. wrote the manuscript with input from all authors. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Figure 1. An example of microhomology-induced chimeric read (MICR)-originated sequencing error.

a. The genomic sequence visualized by Integrative Genomics Viewer exhibits a T-to-C artifact in the *FGFR4* gene. In all mutation-supporting reads, only six bases downstream of the mutation were mapped, and the rest is soft-clipped.

b. A representative read supporting the mutation. The upstream sequence was mapped to the forward strand (blue arrow), and the downstream sequence was mapped to the reverse strand (green arrow). The red box represented the T-to-C artifact. Most of the downstream bases were soft-clipped.

c. Two palindromic sequences in a single-stranded DNA (ssDNA) formed a hairpin structure. After nicking and partial denaturation, the double-stranded DNA was regenerated during the end-repair step of library preparation. The mismatched base between two palindromic sequences was defined as a mutation.

d. The MicroSEC algorithm is based on three criteria. Filter 1, 3: the distance from the mutation position to the most distant mapped base is distributed over a limited range for any reads. Filter 2: MICR-originated sequencing errors are generated when two palindromic sequences are in the same DNA fragment. Filter 4: The mis-annealing of ssDNA derived from two distant homologous regions also creates an artifact.

e. Mutations identified by capture-based sequencing were validated by amplicon-based

402 sequencing. The mutations that passed through the MicroSEC filter were detected with a
403 similar level of variant allele frequency (VAF) by both methods (blue). A potential CG-to-TG
404 artifact with a VAF of 5.5% was not amplified by amplicon-based sequencing (red). Filtered-
405 out mutations were not detected by amplicon-based sequencing (green).
406

407 **Table Legend**

	Normal Breast Tissue		Breast cancer		Clinical sequencing
	Fresh frozen (N = 53)	FFPE (N = 190)	Fresh frozen (N = 23)	FFPE (N = 33)	FFPE (N = 54)
Total reads (in millions)	61.3 (36.6–120.4)	83.5 (37.3–154.8)	60.8 (43.3–114.2)	67.5 (20.0–127.6)	87.8 (38.0–216.0)
Mapped reads (%)	92.2 (89.7–93.7)	93.4 (89.4–96.8)	91.9 (88.7–93.6)	92.3 (89.0–94.3)	90.5 (85.9–92.9)
Unique reads (%)	77.1 (55.9–87.9)	51.1 (32.6–79.8)	76.5 (51.9–84.9)	55.3 (38.6–86.6)	41.1 (17.8–77.2)
Mean coverage	928 (417–1,584)	919 (438–1,544)	944 (550–1,880)	767 (403–1,525)	817 (404–1589)
Median insert size (base)	217 (163–312)	158 (117–196)	202 (158–293)	142 (119–173)	135 (104–177)
Somatic mutations	0.3 (0–9)	11.8 (0–117)	4.0 (0–13)	10.8 (2–34)	21.7 (1–135)
removed by					
Filter 1	0 (0–0)	4.3 (0–88)	0 (0–0)	1.0 (0–7)	0.5 (0–12)
Filter 2	0 (0–0)	2.3 (0–29)	0 (0–0)	0.7 (0–4)	0.1 (0–1)
Filter 3	0 (0–0)	3.6 (0–76)	0 (0–0)	0.8 (0–6)	0.7 (0–17)
Filter 4	0 (0–0)	5.4 (0–40)	0 (0–0)	1.8 (0–9)	3.2 (0–89)
Any of Filter 1–4	0 (0–0)	10.1 (0–111)	0 (0–0)	3.2 (0–15)	3.7 (0–94)
Mutations passing the filter	0.3 (0–9)	1.7 (0–50)	4.0 (0–13)	7.5 (0–32)	18.0 (0–135)
Filtered rate (%)	0	85.8	0	30.1	16.9
CG-to-TG potential artifacts	NA	0.1 (0–12)	NA	0.2 (0–1)	2.6 (0–37)
Intra ≥10-base homopolymer	0.0 (0–1)	0.7 (0–14)	0.0 (0–0)	0.2 (0–2)	0.1 (0–1)
Remaining mutations	0.3 (0–9)	0.8 (0–38)	4.0 (0–13)	7.2 (0–32)	15.3 (0–128)

Data are shown as mean (range).
NA, not applicable; FFPE, formalin-fixed and paraffin-embedded.

408

409 **Table 1. MicroSEC filtering summary.**

410

Supplementary Figure Legends

Supplementary Figure 1. Two proposed mechanisms of microhomology-induced chimeric read (MICR) formation.

a. In the region with two proximal palindromic sequences, single-stranded DNA (ssDNA) is

416 formed after degradation and denaturation. The annealing between the two palindromic
417 sequences forms a hairpin structure. The chimeric double-stranded DNA is formed during the
418 shearing and end-repair steps of library preparation. Adapter and index sequences are added to
419 the original sequence. The ligation product is amplified by polymerase chain reaction (PCR),
420 sequenced, and mapped to the reference genome. In a chimeric read of two sequences, only
421 one is correctly mapped, and the other is clipped by the mapping software.

422 **b.** MICR is also formed by the homologous sequences in two distant regions in the genome.
423 Chimeric reads are generated by mis-annealing of ssDNA derived from the distant homologous
424 regions.

425

427

428 **Supplementary Figure 2. The distribution of the variant allele frequencies of the breast**

429 **tissue samples.**

430 **a.** FFPE samples of normal breast tissues (n = 190) with total mutations (upper), mutations
431 filtered out by MicroSEC filter (middle), and mutations passing through the filter (lower).
432 **b.** FFPE samples of breast tumor tissues (n = 33) with total mutations (upper), mutations
433 filtered out by MicroSEC filter (middle), and mutations passed through the filter (lower).
434 FFPE, formalin-fixed and paraffin-embedded.
435

Supplementary Figure 3. The relationship between the mutation depth and filtering efficiency in the 190 FFPE normal breast tissue samples.

The histograms of mutation depth for total mutations (gray) and mutation passing the MicroSEC filter (blue) were shown. Most of the called mutations are considered to be artifacts, because normal breast tissues have little or no mutations. The mutation filtering rate (black line) was not high at low mutation depth, and more than 90% of mutations were filtered by the MicroSEC pipeline when the mutation coverage was 10 or larger (dotted red line). FFPE, formalin-fixed and paraffin-embedded.

Supplementary Figure 4. The mutations detected in matching FF and FFPE samples.

The mutations detected in the matching sets of frozen (blue) and FFPE breast cancer (peach) samples from eight patients. Eleven mutations found in only FFPE samples were filtered by MicroSEC (green). All mutations found in fresh frozen samples passed through the MicroSEC filter. FFPE, formalin-fixed and paraffin-embedded.

453

454 **Supplementary Figure 5. The distribution of the mutations in breast tissue samples.**

455 a. The rate of low-quality bases in mutation-supporting reads in 190 FFPE normal breast tissue
456 samples (left) and 23 frozen breast tumor samples (right).

457 b. The rate of soft-clipped reads in FFPE samples of normal breast tissue (left) and frozen
458 samples of breast tumor (right).

459 c. The rate of reads derived from other homologous regions in FFPE samples of normal breast
460 tissue (left) and frozen samples of breast tumor (right).

461 d. The rate of reads derived from the hairpin structure in FFPE samples of normal breast tissue
462 (left) and frozen samples of breast tumor (right).

463 Dotted red lines represent the thresholds.

464

465 **Supplementary Table Legends**

Sample	Chr	Position	Ref	Alt	VAF (%)	Mutation depth	Soft-clipped read	Hairpin- structure	Read length	Supporting length			Probability		
										5'	3'	Shorter	5'	3'	Shorter
SL_0001_L_FFPE_4	12	106255381	G	GTGAC	55.4	652	645 (98.9%)	632 (96.9%)	150	145	125	10	NA	NA	0
SL_1003_R_FFPE_66	12	106255382	A	T	70.2	536	474 (88.4%)	523 (97.6%)	150	148	136	36	NA	NA	3.0×10^{-25}
SL_1008_L_FFPE_13	12	106255382	A	T	74.3	916	807 (88.1%)	899 (98.1%)	150	149	136	37	NA	NA	1.4×10^{-34}
SL_1008_R_FFPE_32	12	106255382	A	T	53.4	378	335 (88.6%)	368 (97.3%)	150	147	139	38	NA	NA	1.4×10^{-24}
SL_1008_R_FFPE_50	12	106255382	A	T	75.9	950	880 (92.6%)	935 (98.4%)	150	149	136	37	NA	NA	3.9×10^{-34}
SL_1015_R_69_FFPE	12	106255382	A	T	65.4	461	407 (88.2%)	447 (97.0%)	124	123	110	59	NA	NA	NA

Chr, chromosome; Ref, reference sequence; Alt, altered sequence; VAF, variant allele frequency; NA, not assessed.

Probability is calculated only if the supporting length is < 80% of the read length.

466

467 **Supplementary Table 1. Filtered mutations with high variant allele frequency in FFPE**
468 **samples.**

469

Sample	Chr	Pos	Ref	Alt	Gene	HGVS.p	Capture-based sequencing				Amplicon-based sequencing		
							Total read	Mutation depth	VAF (%)	MicroSEC	Total read	Mutation depth	VAF (%)
SL_1006_L_FFPE_16	1	36469724	G	T	CSF3R	p.Pro468Thr	668	36	5.4	Filter 1, 3	21,677	3	0.01
SL_1006_L_FFPE_22	1	36469724	G	T	CSF3R	p.Pro468Thr	555	61	11	Filter 1, 3	33,998	6	0.02
SL_0001_R_FFPE_58	1	43360798	T	A	CDC20	p.Leu305Gln	104	22	21.2	Filter 1–3	37,241	4	0.01
SL_0006_RN_4	3	52403601	TT	GG	BAP1	p.Asn515Pro	1,825	29	1.6	Filter 3	156,752	0	0.00
SL_0007_R_FFPE_48	3	179199690	G	A	PIK3CA	p.Gly118Asp	1,046	19	1.8	Passed	55,234	709	1.62
SL_1010-N4	3	179218294	G	A	PIK3CA	p.Glu542Lys	599	172	28.7	Passed	132,576	36,391	27.45
SL_1010-N5	3	179218294	G	A	PIK3CA	p.Glu542Lys	780	215	27.6	Passed	155,872	42,841	27.48
SL_0007_LT_9	3	179234297	A	G	PIK3CA	p.His1047Arg	897	12	1.3	Passed	48,955	312	0.64
SL_0008_LT_8	3	179234297	A	G	PIK3CA	p.His1047Arg	958	101	10.5	Passed	47,909	5,492	11.46
SL_1007_L_FFPE_26	3	179234297	A	G	PIK3CA	p.His1047Arg	901	46	5.1	Passed	49,568	4,452	8.98
SL_1004_R_49_FFPE	3	179234297	A	G	PIK3CA	p.His1047Arg	641	268	41.8	Passed	115,256	42,213	36.63
SL0001_L_FFPE_10	3	179234297	A	G	PIK3CA	p.His1047Arg	554	10	1.8	Passed	50,261	1,638	3.26
SL0001_R_FFPE_58	3	179234297	A	G	PIK3CA	p.His1047Arg	491	22	4.5	Passed	48,777	2,216	4.54
SL_0001_L_FFPE_3	6	137881182	G	A	TNFAIP3	p.Gly746Arg	553	63	11.4	Filter 1, 2	16,044	8	0.05
SL_1007_T2	6	137881182	G	A	TNFAIP3	p.Gly746Arg	384	23	6	Filter 2	8,099	8	0.10
SL_1010-N2	6	152011697	G	C	ESR1	p.Glu380Gln	1,332	182	13.7	Filter 3	83,395	32	0.04
SL_1004_R_FFPE_61	6	152011739	C	T	ESR1	p.Arg394Cys	785	43	5.5	Passed (Cg>Tg)	146,849	248	0.17
SL_0001_L_FFPE_10	12	68815634	A	G	MDM2	p.Ser55Gly	189	49	25.9	Filter 4	3,604	1	0.03
SL_0012_RT_5	12	12745786	A	G	APOLD1	-	1,065	20	1.9	Filter 1	138,907	179	0.13
SL_1010_L_FFPE_6	13	32356550	C	T	BRCA2	p.Arg2520*	540	12	2.2	Passed (Cg>Tg)	36,182	134	0.37
SL_1010_L_FFPE_9	13	32356550	C	T	BRCA2	p.Arg2520*	651	7	1.1	Passed (Cg>Tg)	40,544	328	0.81
SL_1007-T2	13	48360023	TATTAC	T	RB1	p.Leu206fs	347	104	30	Passed	213,320	69,215	32.45
SL_0001_R_FFPE_52	14	36519170	G	A	NKX2-1	p.Ala63Val	741	42	5.7	Filter 4	42,628	3	0.01
SL_0001_L_FFPE_29	14	68594559	C	T	RAD51B	p.Gln371*	538	101	19.1	Filter 4	74,485	7	0.01
SL_1010-T1	17	7670685	G	A	TP53	p.Arg342*	707	85	12	Passed (Cg>Tg)	85,333	9,867	11.56
SL_1004_L_36_FFPE	17	7673803	G	A	TP53	p.Arg273Cys	729	72	9.9	Passed (Cg>Tg)	197,963	24,528	12.39
SL_0001_R_FFPE_56	17	7674385	A	G	TP53	-	1466	82	5.6	Filter 4	8,742	1	0.01
SL_1006_L_FFPE_22	19	6828091	G	A	VAV1	p.Ala315Thr	298	15	5	Filter 1, 3	17,710	14	0.08
SL_0007_L_FFPE_19	19	46919618	C	T	ARHGAP35	p.Gln315*	1,130	67	5.9	Passed	28,581	1,911	6.69
SL_1007-T2	X	15801329	CA	AG	ZRSR2	p.His87Ser	197	41	20.8	Filter 4	2,329	0	0.00

Chr, chromosome; Ref, reference sequence; Alt, altered sequence; HGVS, Human Genome Variation Society; VAF, variant allele frequency.

Supplementary Table 2. Comparison between the results of capture-based sequencing and amplicon-based sequencing.

Sample	Gene	HGVS.c	HGVS.p	VAF (%)	Mutation depth	Soft-clipped read	Read length	Supporting length			Reads from distant region	MicroSEC
								5'	3'	Shorter		
H12-13366-21	RAD51B	c.1111C>T	p.Gln371*	12.2	24	13 (54.2%)	150	143	39	39	10 (41.7%)	Filter 4
H17-09165-20	PPP2R1A	c.108delT	p.Leu36fs	13.4	17	5(29.4%)	150	46	141	46	9 (52.9%)	Filter 4
H17-06211-11	FAM175A	c.229C>T	p.Arg77*	11.8	16	1 (6.3%)	151	141	68	68	11 (68.8%)	Filter 4
H17-06211-11	RAD51B	c.1111C>T	p.Gln371*	10.9	57	24 (42.1%)	151	150	50	50	22 (38.6%)	Filter 4
H17-06211-11	PPP2R1A	c.108delT	p.Leu36fs	21.1	23	6 (26.1%)	151	63	145	63	15 (65.2%)	Filter 4
H17-09880-1	TP53	c.1021T>G	p.Phe341Val	8.8	62	4 (6.5%)	151	102	150	75	0 (0%)	Filter 3
H17-10196-1	PPP2R1A	c.108delT	p.Leu36fs	9.3	23	6 (26.1%)	151	52	144	52	13 (56.5%)	Filter 4
H17-10196-1	ZRSR2	c.283C>T	p.Arg95*	6.0	10	5 (50%)	151	149	115	55	6 (60%)	Filter 4

Chr, chromosome; Ref, reference sequence; Alt, altered sequence; NA, not assessed.

Probability is calculated only if the supporting length is < 80% of the read length.

474

475 **Supplementary Table 3. Pathogenic mutations in clinical FFPE samples filtered out by**

476 **MicroESC.**

477

Chr	Pos	Target region		Gene	HGVS.p	Insert (bases)	Forward primer	Reverse primer
		Ref	Alt					
1	36469724	G	T	CSF3R	p.Pro468Thr	114	TTCAGCAGAAACCCCGTGGC	TCCATGGCCTCAGGGCTATG
1	43360798	T	A	CDC20	p.Leu305Gln	128	TGGTTCACGTTCTGGCCACATC	GACCAAGTTATCATTACCACCACTG
3	52403601	TT	GG	BAP1	p.Asn515Pro	136	GCTGTCATCCTCTCCAAAAGCAC	TACAGACACGGCCTCTGAGATC
3	179199690	G	A	PIK3CA	p.Gly118Asp	137	GTTTGCAAAAAAACATGTTTCATGCTGTG	GGAAGTCTGTACTTCTGGATC
3	179218294	G	A	PIK3CA	p.Glu542Lys	127	CTAGAGACAATGAATTAAGGAAAATGAC	CATTTTAGCACTTACCTGTGACTCC
3	179234297	A	G	PIK3CA	p.His1047Arg	136	ACATTGAAAGACCCTAGCCTTAG	ATGCTGTTTAATTGTGTGGAAGATCC
6	137881182	G	A	TNFAIP3	p.Gly746Arg	156	CAAGAACATCCTGGCCTGCC	GCATTGCCAAAATGATCACAGGC
6	152011697	G	C	ESR1	p.Glu380Gln	119	TGTTTTCAGGCTTTGTGGATTGAC	TAGCTTCCCTGGGTGCTCCATG
6	152011739	C	T	ESR1	p.Arg394Cys	122	CTCCATGATCAGGTCCACCTTC	CTGTCCAAGAGCAAGTTAGGAG
12	12745786	A	G	APOLD1	-	106	AGGATGACTCAGAATAGCACAAATAG	GAAAGATCTGCTGTAGGCAAGACC
12	68815634	A	G	MDM2	p.Ser55Gly	149	AGTGCTGCAGTAGCTAGGACTAC	ACTTTAGGAGGCCGAGGTAAGCAG
13	32356550	C	T	BRCA2	p.Arg2520*	153	GAAGAAACAAAGGCAACGCGTC	CAGTATTGTAGACAAACATACCTG
13	48360023	TATTAC	T	RB1	p.Leu206fs	127	TTTCTCTACATAAAGATCTGAATCTC	AGTCAAGGACACATAGCATTAAGT
14	36519170	G	A	NKX2-1	p.Ala63Val	125	CAGGTTGCCGTTGCAGTAGC	CAGCGGCCATGCAGCAGCAC
14	68594559	C	T	RAD51B	p.Gln371*	126	CCAAGCTGAAGTGAAGTGGGCTC	ACATAATGAGACCCACCTCTAC
17	7670685	G	A	TP53	p.Arg342*	148	CTGAGGTCACCTGAGTAGGAG	ACTTCTCCCCCTCTCTGTTGCTG
17	7673803	G	A	TP53	p.Arg273Cys	113	GGCTCCCTTTCTTGCGGAGATTC	ATCCTGAGTAGTGGTAATCTACTG
17	7674385	A	G	TP53	-	124	GGGGAGACCTGTGGCAAGCAG	ACCTGGGCGGTGGAGCTTGC
19	6828091	G	A	VAV1	p.Ala315Thr	151	CAACTGGCTGTTTCTGGGACC	TGGAGAAGGAGGTGATATTTGAGAAC
19	46919618	C	T	ARHGAP35	p.Gln315*	148	GGCTGAGTGTGAGCCGAAAG	GGTACAGCTTTCTCCTACGCTC
X	15801329	CA	AG	ZRSR2	p.His87Ser	147	TGTTTTGTTGAGACGGACTCTCG	TGGCGGTGCGTGCTGTAATC

Chr, chromosome; Ref, reference sequence; Alt, altered sequence; HGVS, Human Genome Variation Society.

Supplementary Table 4. PCR primer sets used in this study.

Figures

Figure 1

An example of microhomology-induced chimeric 383 read (MICR)-originated sequencing error. a. The genomic sequence visualized by Integrative Genomics Viewer exhibits a T-to-C artifact in the FGFR4 gene. In all mutation-supporting reads, only six bases downstream of the mutation were mapped, and the rest is

soft-clipped. b. A representative read supporting the mutation. The upstream sequence was mapped to the forward strand (blue arrow), and the downstream sequence was mapped to the reverse strand (green arrow). The red box represented the T-to-C artifact. Most of the downstream bases were soft-clipped. c. Two palindromic sequences in a single-stranded DNA (ssDNA) formed a hairpin structure. After nicking and partial denaturation, the double-stranded DNA was regenerated during the end-repair step of library preparation. The mismatched base between two palindromic sequences was defined as a mutation. d. The MicroSEC algorithm is based on three criteria. Filter 1, 3: the distance from the mutation position to the most distant mapped base is distributed over a limited range for any reads. Filter 2: MICR-originated sequencing errors are generated when two palindromic sequences are in the same DNA fragment. Filter 4: The mis-annealing of ssDNA derived from two distant homologous regions also creates an artifact. e. Mutations identified by capture-based sequencing were validated by amplicon-based sequencing. The mutations that passed through the MicroSEC filter were detected with a similar level of variant allele frequency (VAF) by both methods (blue). A potential CG-to-TG artifact with a VAF of 5.5% was not amplified by amplicon-based sequencing (red). Filtered out mutations were not detected by amplicon-based sequencing (green).

Supplementary Files

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