A Novel System Control for Quality Control of Diagnostic Tests Based on Next-Generation Sequencing

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Background: We describe a novel system control (SC) implemented in an automated AmpliSeq™-based next-generation sequencing (NGS)2 run that simultaneously acts as (a) an external positive/sensitivity control, (b) a spike-in QC for DNA extraction, and (c) a nontemplate control to detect exogenous DNA contamination.

Methods: Plasmids carrying wild-type tobacco mosaic virus sequence and a sequence with three designed mutations were synthesized and mixed, such that the mutations are present at 5% variant frequency in the mixture designated as SC. SC was used as a stand-alone sample and spiked into each sample in each run. A cell line–derived reference material, in both a formalin-fixed paraffin-embedded (FFPE) sample and genomic DNA (gDNA), was sequenced in the same runs.

Results: By interpolation, 100 fg SC spiked in FFPE sample produced sequencing coverage equivalent to approximately 3 fg in the gDNA. In the SC-only sample, all three designed mutations were recovered around 5% as expected, while no significant reads of human genome were present. In samples with a common PCR inhibitor, coverage for both SC and target amplicons were eliminated. An inverse relationship between the coverage of SC and DNA input was observed. In clinical samples, the ratio of SC to the median coverage of sample can be used to indicate insufficient DNA input.

Conclusions: The SC is an elegant and comprehensive QC concept for NGS-based diagnostic tests.

IMPACT STATEMENT

The methodology presented here is one of the first quality control approaches developed specifically for NGS assays. It provides an important solution for advancing NGS assays into diagnostic tests for clinical use.

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Nonstandard abbreviations: NGS, next-generation sequencing; qPCR, quantitative PCR; NTC, nontemplate control; gDNA, genomic DNA; VF, variant frequency; SC, system control; TMV, tobacco mosaic virus; FFPE, formalin-fixed paraffin-embedded; SNV, single nucleotide variant, indels, insertions and deletions; MNV, multiple nucleotide variant; RM, reference material.
Next-generation sequencing (NGS) is being quickly adopted in the clinical laboratory, especially with respect to targeted resequencing of tumors for finding well-known mutations to stratify treatment (1, 2). While NGS offers genetic analysis at efficiency and throughput unmatchable by single-gene sequencing or quantitative PCR (qPCR) tests, it is also highly complex and multivariate, warranting new quality assurance considerations not encountered in single-gene tests.

Quality control (QC) is defined as those essential measures that must be included in each assay to validate a test and assure the quality of the test results. Although qPCR kits approved by the Food and Drug Administration generally incorporate QCs as part of results interpretation (3, 4), there are no examples of similar controls for amplification-based NGS kits. Single-gene qPCR tests conventionally include exogenous controls in every run, such as a nontemplate control (NTC) for monitoring contamination, and a positive control to verify the reagents. There may also be a sample adequacy (endogenous) control, targeting human DNA in general, to verify the presence of sample material. In addition, a spike-in control, which usually carries sample unrelated sequences such as those derived from phages or plants, may be used to verify the extraction and detection process. For example, in Cepheid (Sweden) tests, genomic DNA (gDNA) of Bacillus globigii is included in each cartridge and processed together with the sample, to verify extraction process and detect sample-associated PCR inhibition of the assay. Results will only be valid if the sample-processing control is positive. In another automated system FilmArray® (Biofire Diagnostics), a transcript from yeast Schizosaccharomyces pombe is included as a process control in a freeze-dried form in the FilmArray pouch. The control material becomes rehydrated when a sample is loaded and is carried throughout the entire process, including lysis, nucleic acid purification, reverse transcription, two PCR stages, and DNA melting. A positive control result indicates that all steps carried out on the sample are successful.

Guidelines for NGS-based diagnostics have emerged in the past few years (5, 6). However, unlike conventional single-gene tests where there are clear regulatory requirements for positive and negative controls (7–9), requirements regarding controls for NGS diagnostics remain somewhat uncertain. Moreover, the multiplex nature of NGS makes it impractical to have contextual positive controls for every targeted gene in one run. The Guidelines for Somatic Genetic Variant Detection issued by the US Department of Health (http://web.archive.org/web/20150906150119/http://www.wadsworth.org/labcert/TestApproval/forms/NextGenSeq_ONCO_Guidelines.pdf) recommend that there must be an NTC and positive/sensitivity control in every run. Furthermore, this positive/sensitivity control should be an individually bar-coded, low positive DNA sample containing multiple known variants to be detected near the sensitivity of the assay. Yet, in the American College of Medical Genetics and Genomics Standards and Guidelines (5), positive controls do not need to be tested concurrently with routine clinical tests. Instead, measures to control for possible contamination before, during, and at the end of the sequencing run must be present. Furthermore, guidelines published by a CDC-established national workgroup of experts (6) only require an external control for each run, while a variety of controls can be run over a reasonable timeframe.

Recently, the Food and Drug Administration cleared Illumina’s cystic fibrosis carrier screening assay and cystic fibrosis diagnosis assay using the MiSeqDx™ platform. According to the manufacturer’s guidelines, positive and negative controls are required for the assays but are neither provided nor specified. Genomes containing published variants, and known positives from genetic disorders, are available, such as DNA from the National Human Genome Research Institute (NHGRI) Sample Repository for Human Genetic Research, or the Human Varia-
tion Subcollection maintained at the Coriell Institute. However, rare variants may not be presented, or variant frequencies (VFs) (defined as the proportion of mutant allele among all alleles) may not be low enough for limit of detection studies. Notably, the CDC’s guidance only applies to clinical detection of constitutional germ line variants at 50% or 100% frequencies, as in Illumina’s cystic fibrosis assays. It may not be applicable to other applications, including oncology, since somatic mutations in tumors often present at much lower frequencies because of the heterogeneous nature of the samples. At such low frequencies, false negatives arising from system error and false positives resulting from contamination must be tightly controlled and distinguished from the real signals. Therefore, control design in such an assay is much more challenging but indeed is essential.

We aimed to derive a comprehensive system of controls for NGS that would include an exogenous positive control, NTC to monitor contamination, and spike-in controls to monitor DNA extraction and to distinguish between suboptimal DNA inputs and presence of inhibitory substances in the event of amplification failure. Herein, we proposed a novel system control (SC) that included a plasmid carrying a wild-type tobacco mosaic virus (TMV) sequence and one carrying a perturbed TMV sequence with three artificially designed mutations. TMV is a plant RNA virus for which the sequence is least likely to be presented in the DNA extracted from human samples. In this study, we implemented the SC into an automated workflow commercialized by Vela Diagnostics (Singapore) for QC use in the detection of somatic mutations in formalin-fixed paraffin-embedded (FFPE) samples.

**MATERIALS AND METHODS**

**Platform and workflow**

The standard workflow is a 1.5-day automated process that included FFPE-DNA extraction followed by library preparation and equalization (Fig. 1). After an off-board overnight incubation of 10 μm FFPE section per sample, the workflow begins by adding 1 pg SC (50 μL of 20 fg/μL) to the SC-only sample and spiking 100 fg SC (5 μL) into 7 FFPE samples. The FFPE-DNA is extracted by a proprietary magnetic bead-based purification method. Uracil-DNA Glycosylase (Enzymatics) is added after the DNA extraction to prevent amplification of carry-over amplicons. AmpliSeq™ library construction and equalization are automated based on the original manual protocols (Thermo Fisher Scientific) with some modifications. For the purpose of controlled experimentation, a modified workflow starting from DNA is also used. On completion of sequencing, read alignment and variant call analyses were done with Torrent Server tools. Variant call filtering and reporting were done with SQ Reporter, software developed by Vela Research. This software reports the QC status of the run and the samples as well as variants detected in the valid samples.

**System control**

The SC is a plasmid mixture composed of two pUC57-based plasmids: one carrying a wild-type TMV sequence and the other carrying the TMV sequence with three designed mutations (c.8G>A, c.28_30delAAA, and c.44_45CA>AG) representing the single nucleotide variant (SNV), insertions and deletions (indels), and the multiple nucleotide variant (MNV). Synthesized plasmids at 1 μg/μL (GeneScript) were diluted at 1:10⁶ in Lo-Bind tubes (Eppendorf) with diluent containing 1× TE buffer, pH 8.0 (BST Scientific), 0.001% Triton X-100 (Sigma), and carrier RNA (1 ng/μL) (Qiagen). Fluorescent probe–based droplet digital PCR (ddPCR) was designed to quantify the copy number of both plasmids. The primers used were 5′-ACGTGCGAAATTCCTCAGGGA and 5′-CCAGTATGCAACATCACCCT; probes used were /5HEX/AAGGATAACGTGTTAACCCTTGATT/3BHQ_1/ (for wild-type TMV sequence) and /56-FAM/CGAAGGATAACGTGTTAACCCTTGATT/3BHQ_1/ (for TMV sequence with mutations) (Integrated DNA Technologies). Digital
PCR was performed using QX200™ Droplet Digital™ PCR System (Bio-Rad) according to the manufacturer’s instructions. Plasmids were then mixed at the desired molar ratio to carry mutants at 5% VF at a final concentration of 20 fg/μL. The mutation VFs within SC were confirmed using droplet digital PCR with both probes combined in one assay.

**Assay, reference material, and clinical samples**

The onco-panel assay consists of 28 primer pairs (Integrated DNA Technologies) designed by AmpliSeq designer to amplify mutation hotspot regions of 11 actionable cancer genes and one pair to amplify the TMV sequence in the SC. The SC amplicon is 100 bp, while the 28 target amplicons range between 74 and 137 bp, with 98 bp in average and 2710 bp in total. The FFPE reference material (RM) is an FFPE-treated proprietary cell line mixture (Horizon Diagnostics), which carries 9 known mutations at different VFs detectable by the Onco-panel. Genomic DNA extracted from the cell line mixture of the same batch before FFPE treatment, named gDNA-RM, was also used for comparison. FFPE clinical samples were procured from Proteogenix (colorectal tumors) and Cybridi (lung tumors). Individual scrolls of 10 μm were obtained and stored at 4 °C.

**Equivalency test**

A standard run was performed to evaluate the recovery of SC coverage. Extracted FFPE-DNA was quantitated after the run using the Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific) according to manufacturer’s instructions. To estimate the equivalent amount of SC to be used in the modified workflow starting with gDNA, 3 FFPE-RM–derived DNA samples containing SC were selected to be individually processed together with 6 gDNA-RM samples containing a various amount of SC (0.1–10 fg) in 3 runs. The DNA input of the gDNA-RM and the FFPE-RM was matched in each run.
Tests for suboptimum DNA and melanin interference

To characterize the relationship between SC coverage and DNA input, SC was kept constant at 5 fg per sample, with the gDNA-RM input varied from 0 to 185 ng in a modified workflow. Five replicate runs were performed. Melanin, a well-known strong inhibitor of PCR, was used for interference substances study. Melanin (Sigma-Aldrich) was dissolved in 1 mol/L ammonium hydroxide (Fluka) to 100 ng/μL and then diluted with 10 mmol/L ammonium hydroxide and added into samples at various concentrations. This test was performed with fixed DNA input of 5 fg SC and 5 ng gDNA-RM.

Intellectual property rights

The designs of the automation, assay, and SQ Reporter are proprietary commercialized products of Vela Research. The patent of SC is pending.

RESULTS

Spike-in SC as extraction control

As illustrated in Fig. 1, the automated workflow used in the study includes steps of DNA extraction, amplification-based NGS library construction, library equalization, and pooling. Pooling of molecularly barcoded NGS libraries for one sequencing run is a commonly used approach to increase sample throughput per run while reducing cost per sample. The equalization step is to normalize the 8 barcoded libraries before pooling so that each library achieves similar sequencing output, approximately 12.5% (1/8) of the total reads/bases. A standard run starts with 1 pg of the SC-only sample and 7 FFPE-RM samples with 100 fg of spike-in SC. Sequencing results from the automated workflow showed that although the SC-only sample had a much lower DNA input, it can be recovered with similar percentage of total reads (e.g., 10.28% in Supplemental Table 1, which appears in the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol1/issue1) to the other FFPE samples in the run, ranging from 7.31% to 15.50%. Notably, the equalization is only approximate with some coverage variation among samples.

To demonstrate that SC spiked into the sample before extraction can be used as a control for this process, 6 gDNA-RM samples containing various amounts of SC (0.1–10 fg) were run together with an FFPE-RM–derived, SC-containing DNA sample that remained from the previous standard run at a matched DNA concentration in 3 replicate runs. After the analysis of each run, a mean coverage was reported for every amplicon, which is the average of the coverage of every base in the amplicon. The sample median coverage is also reported as the middle value in the list of all 29 mean amplicon coverages, including the SC amplicon. The mean SC coverage spiked in each gDNA-RM sample was normalized with median coverage of that sample and plotted against the amount of SC added into the sample (Fig. 2A). By interpolation, the FFPE-RM sample produced an SC coverage equivalent to 2.78 fg (95% CI, 1.46–4.10 fg) added to gDNA-RM, comparable to the expected recovery of spike-in SC from extraction. Assuming 100% extraction recovery, the DNA eluted in 100 μL after extraction would contain SC at 1 fg/μL, of which 5 μL (5 fg) was used for the following AmpliSeq reaction. These data support the fact that SC, when spiked into FFPE sample, can be the control of the extraction process. For the subsequent experiments, 5 fg SC was added to DNA samples, for the sake of simplicity and providing a safety margin.

Furthermore, a chart of sample median coverage against internal SC amount used in gDNA-RM (Fig. 2B) in these 3 runs showed that there was a slight decrease in the overall target coverage at 5 and 10 fg, but no significant difference was observed with increasing SC at the levels tested. Coverage variation could also be resulted from the
variation of the process including equalization. Overall, these data suggest that there was no significant PCR competition with target amplification from the SC at these amounts.

**Spike-in SC as indicator for suboptimal DNA input and presence of inhibitors**

To demonstrate SC functions as an internal control for DNA quantity and quality, we next characterized the relationship between SC coverage and DNA input. In five replicate runs, SC was kept constant at 5 fg per sample, with the DNA varied from 0–185 ng. The resulting mean SC coverage within each sample was plotted against the respective DNA input. Interestingly, SC coverage varied inversely with DNA input (Fig. 3A), suggesting that SC coverage may be used as an indication for DNA input. Such function was confirmed by a large cohort of 186 procured clinical colorectal and 186 lung FFPE samples using the standard workflow. A total of 57 runs (28 runs of colorectal and 29 runs of lung FFPE samples) were performed, with some samples run in replications. Excess extracted DNA was quantitated after the run using a Qubit ds-DNA high-sensitivity assay. Input DNA concentrations were then plotted against the ratios of mean SC coverage vs median sample coverage (Fig. 3C). Indeed, a higher ratio is often correlated with lower DNA input leading to failed sequencing results (colored samples), suggesting that the SC can be used to infer the DNA concentration extracted from FFPE samples in an automated workflow. Notably, the sample median coverage, which is the middle value in the list of 29 amplicon coverage, was reported instead of mean coverage. In the case of low starting DNA, sample median coverage will still be low even though SC amplicon will have much higher coverage than the other target amplicons. A value of 55 ($\approx 10^{-5}$) was chosen as the threshold for the ratio of SC vs sample median coverage for warning, above which samples may have a high probability of sequencing QC failure due to insufficient input DNA.

The feasibility of using SC to monitor PCR inhibition was also investigated. Melanin was used as a surrogate for a well-known strong PCR inhibitor.
often present in highly vascularized tumor samples such as melanoma. Known concentrations of melanin were added into a 5-ng gDNA-RM sample with 5 fg spike-in SC in the modified workflow. Resulting internal SC coverage and median non-SC target coverage were plotted against melanin input (Fig. 3B). Both SC and target were reduced or diminished because of the PCR inhibition with in-
creasing melanin concentration. Hence, in the event of negative or lower target human amplicon generation in a sample, indicated by lower median sample coverage, the presence of high SC or low SC coverage can be used to differentiate between sample failure due to suboptimal DNA inputs or PCR inhibitors, respectively.

SC-only sample as external positive control and NTC

Throughout the entire process, 1 pg SC was used as a stand-alone SC-only sample. In 18 replicated runs starting with 5 ng gDNA-RM, SC-only samples were all sequenced successfully (see Supplemental Table 2 in the online Data Supplement). Moreover, 3 mutations in SC representing SNV, MNV, and indels were consistently recovered at approximately 5%, confirming the sensitivity of every run (Table 1). This result suggests that the SC-only sample serves as an external positive control for the extraction and library construction reagents as well as a sequencing quality and analysis pipeline to achieve 5% detection sensitivity. Additionally, the SC-only sample also functioned as NTC for human gDNA or amplicon contamination. The coverages of all the amplicons in the SC-only sample were recorded for each run (see Supplemental Table 2 in the online Data Supplement). In Fig. 4, the sum of coverages from 18 runs was shown for the SC as well as the 28 target amplicons. On average, the median coverage of sample containing only SC being 282 905 (95% CI, 235 011–330 800) is in 5 order of magnitude of the maximum coverage of non-SC amplicons being 3 (95% CI, 2–4) in this sample (Table 1). Such coverage difference suggests that there is minimal contamination in the system and an SC-only sample can serve as NTC for contamination.

QC matrix

The extensive data support that SC functioned as a comprehensive control as intended. The status of the SC-only sample, spiked-in SC, and target amplicons in the FFPE sample can be tabulated in a logic table (Table 2) for validating a successful run/sample or troubleshooting a run/sample that failed for various reasons, including DNA extraction, suboptimal DNA input, PCR inhibition or contamination. Notably, because the FFPE samples are of different quantities and qualities, it is possible that an individual FFPE sample could fail the sample criteria in a successful run. The combination of SC-only and spike-in SC is designed to indicate such a situation.

DISCUSSION

The current paradigm of “one-drug/one-gene Dx” appears increasingly unsustainable, due to its severe limitations in specimen amount and turnaround time and because more molecular information is needed with the advancement of

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**Table 1. Performance of SC-only sample in 18 replicate runs.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>18 runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median coverage of SC-only sample</td>
<td>282 905</td>
</tr>
<tr>
<td>Maximum coverage of non-SC amplicon</td>
<td>3</td>
</tr>
<tr>
<td>SNV: c.8G&gt;A, %</td>
<td>4.83</td>
</tr>
<tr>
<td>indels: c.28_30delAAA, %</td>
<td>4.70</td>
</tr>
<tr>
<td>MNV: c.44_45CA&gt;AG, %</td>
<td>4.45</td>
</tr>
<tr>
<td>Total bases</td>
<td>218 050 623</td>
</tr>
<tr>
<td>Total reads</td>
<td>2 181 508</td>
</tr>
<tr>
<td>Sample median coverage</td>
<td>8167</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>18 runs</th>
<th>Average</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median coverage of SC-only sample</td>
<td>235 011</td>
<td>330 800</td>
<td></td>
</tr>
<tr>
<td>Maximum coverage of non-SC amplicon</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SNV: c.8G&gt;A, %</td>
<td>4.59</td>
<td>5.07</td>
<td></td>
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<tr>
<td>indels: c.28_30delAAA, %</td>
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<td>4.92</td>
<td></td>
</tr>
<tr>
<td>MNV: c.44_45CA&gt;AG, %</td>
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<td>4.71</td>
<td></td>
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<tr>
<td>Total bases</td>
<td>183 527 211</td>
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<tr>
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<tr>
<td>Sample median coverage</td>
<td>6743</td>
<td>9591</td>
<td></td>
</tr>
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</table>
precision medicine (10). While NGS will provide potential clinically important data for each patient at unprecedented throughput and speed, it is rather expensive and complicated. Many factors, such as poor input DNA quality, contamination with human DNA or amplicons, or sample mix-up, can result in a waste of samples, raised costs, and increased risk of false positives and false negatives. Some of these risks can be minimized in clinical implementation, through developing robotic processes such as the automated workflow presented in this study. However, controls are still crucial for monitoring the occurrences of these risks. Clearly, it is not feasible to have controls for all the targeted amplicons to assure the accuracy for NGS runs. Moreover, conventional qPCR assays detect...
the presence or absence of a mutation, while in NGS, the mutation is detected in terms of VF (%). Further, for the workflow combining DNA extraction and library construction, it would be necessary to include a control to examine the DNA quality and input amount. Hence, the SC is designed to include control for the extraction process and also includes 3 “surrogate” mutations representing common alterations (SNV, MNV, and indels) to confirm the variant detection sensitivity of 5% VF at sufficient coverage (>1000 for 5% VF) in a successful run. For reason of economy, in the current design, there would be no NTC for the SC itself, since the TMV sequences used are unlikely to interfere mutation detection in human genome sequences.

The amplicon-based AmpliSeq method was chosen for FFPE samples because of its low DNA input requirements and proven efficacy for FFPE samples (11). There may also be an upper limit at which amplification or equalization will fail, but it is not likely to be reached by FFPE samples (see Supplemental Fig. 1 in the online Data Supplement).

The observation that SC coverage is inversely related to DNA input is particularly useful because the automated workflow does not have a DNA quantification step such as NanoDrop and Qubit in concert (12) or library quantification kits (Thermo Fisher Scientific). Suboptimal DNA inputs may lead to false positives from high noise background and false negatives from true mutations with low frequencies. The calling of large numbers of mutations at very low VFs may be indicative of suboptimal DNA inputs (13); however, it is not definitive. High SC and low sample median coverage can infer low DNA input and alert compromised sequencing results. However, a large cohort of clinical samples are required to further refine this QC parameter. The Onco-panel is applicable to many solid tumors, including colorectal, lung and thyroid tumors, and melanoma. In the PCR inhibitor test, melanin was used as a representative. It was found that the suppression of both SC and sample coverage could be an indication of PCR inhibition, whereas high SC and low sample coverage could suggest a suboptimal DNA input.

Novel control concepts are critical in addressing basic QC requirements for NGS as a diagnostic test, as well as for the parameters specific to this new technology, e.g., mutation detection at low VF (5%) in oncology samples. In this study, a novel SC concept was developed and tested. This SC can be easily generated by mixing two synthetic plasmids containing TMV-derived sequences at a fixed molar ratio (e.g., 5%). By using the SC as a stand-alone sample as well as spiking into FFPE samples at the step of DNA purification, one SC can provide comprehensive control functions as follow: (a) the SC-only sample is processed throughout the whole workflow as a positive control for a valid run; (b) the detection of the known mutations at 5% VF in the SC serves as a positive control for the quality of the library construction, sequencing, and variant call to achieve the required sensitivity; (c) the minimal presence of human DNA sequence in the SC-only sample functions as an NTC for the potential crossover and carry-over contaminations; and (d) the spike-in SC serves as an extraction control to surrogate the DNA quantity and quality, especially the presence of PCR inhibitors. All the control functions have been demonstrated for one automated NGS workflow in this study. However, it can be applied to other automated workflows as well as manual NGS library preparation workflows not limited to oncology application. In conclusion, the SC is a simple yet comprehensive control that supplements the sequencing QC to assure the sensitivity and specificity of a NGS diagnostic test.
Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.


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REFERENCES