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Optimization of RNA extraction from FFPE tissues for expression profiling in the DASL assay

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Formalin-fixed paraffin-embedded (FFPE) breast tumor tissues are readily available and represent a largely untapped, vast resource for molecular profiling of clinical samples with long-term follow-up data. We have optimized the conditions and parameters that result in the preparation of total RNA that is of the necessary quality for use in the DASL (cDNA-mediated annealing, selection, extension, and ligation) assay in which expression of 502 genes are analyzed simultaneously using as little as 100 ng of input RNA.

Formalin-fixed paraffin-embedded (FFPE) tissue samples make up a vast archive of pathologically well-characterized clinical samples from randomized trials and are an immense virtually untapped resource that can be used for conducting biomarker investigations. Even though the degradation of RNA that occurs due to the formalin fixation process results in RNA species with an average size of ~200 nt (1), it is feasible to extract and purify RNA from such FFPE tissue and to perform real-time reverse transcription-polymerase chain reaction (RT-PCR)-based gene expression profiling. Some studies have addressed improvements to the process of isolating high quality FFPE RNA suitable for RT-PCR or high-throughput genome-wide gene expression profiling (2-4).

The DASL (cDNA-mediated annealing, selection, extension and ligation) assay is based upon massively multiplex RT-PCR applied in a microarray format that allows for the determination of expression of up to 512 genes (502 genes in the cancer panel used in this study), using RNA isolated from 96 FFPE tumor tissue samples in a high-throughput format (5,6). The DASL assay has been used recently to identify a 16-gene set that correlates with prostate cancer relapse (7).

Here we have compared four FFPE RNA preparation methodologies resulting in an optimized protocol for performance in the DASL assay. Although Illumina Inc. (San Diego, CA, USA) recommends using the High Pure RNA kit (Roche, Mannheim, Germany) based on earlier studies (5,6), several new kits (from Ambion, Austin, TX, USA; Qiagen, Valencia, CA, USA; and SuperArray, Frederick, MD, USA) have not been previously tested for utility in downstream DASL assays. The four commercially available kits for RNA extraction from FFPE tissues that were used in this study are: Ambion’s RecoverAll kit, Roche’s High Pure kit, Qiagen’s RNeasy FFPE kit, and SuperArray’s ArrayGrade FFPE RNA Isolation kit. In our experiments, FFPE blocks from six different breast cancer patients who were estrogen receptor positive (ER+) and epidermal growth factor receptor 2-positive (HER2+) (as determined by immunohistochemistry [IHC]), and were sectioned to enable direct comparison of the same tissues with six individual samples. All samples were then run in the DASL assay as two technical replicates. Here we report our findings on performance of these FFPE RNA extraction methods as well as the finalized optimal protocol in the DASL assay.

Total cellular RNA was extracted with overall yields ranging from 0 to 9 μg with concentrations from 0 to over 300 ng/μL. For each kit, longer digestion with Proteinase K yielded more RNA (Figure 1A), consistent with previous studies (2). Often limited information is available on how the tissue was processed prior to fixation and embedding, but processing likely strongly influences the quantity and quality of nucleic acids recovered from the sample.

Most kits tested in this study produced RNA with an A260/A280 ratio close to 2 (Figure 1B). The most variability in the A260/A280 ratio was seen when using the SuperArray ArrayGrade FFPE RNA Isolation kit. When the A260/A280 ratio decreases, there is a tendency for the RPL13a TaqMan cycle threshold (Ct) values to increase and the DASL replicate reproducibility to decrease. Agilent 2100 bioanalysis (Asilent Technologies, Santa Clara, CA, USA) determined that the median RNA size was approximately 100–200 bp (Figure 1E), with profiles typical for FFPE RNA (8), but the RNA integrity number (RIN) values were not predictive of the utility of RNA samples.

Of greater predictive power for assessment of RNA performance in the DASL assay is TaqMan real-time PCR analysis of RPL13a (6). TaqMan analysis suggested that most samples with a Ct value of ≤29 had sufficient quality RNA to give reproducible results in the DASL assay (Figure 1C). As the Ct value increased, the replicate reproducibility value decreased. The lowest Ct values, and thus the most usable RNA, were obtained for RNA prepared with the Ambion and Roche kits paired with overnight Proteinase K digestion and an RNA concentration of ≥20 ng/μL. The RNA prepared with the Qiagen kit achieved higher Ct values in this assay at both the 15 min and 3 h Proteinase K digestion time points compared with Ambion or Roche at the
Figure 1. Quality control analysis of total RNA samples prepared using Ambion’s RecoverAll kit, Roche’s RNA High Pure kit, Qiagen’s RNeasy FFPE kit, and SuperArray’s ArrayGrade FFPE RNA Isolation kit. (A) Concentration of samples varied from sample to sample and from kit to kit, but it was clearly tissue-dependent. The overnight incubation with Proteinase K increased the yield of total RNA across the methods tested. (B) The A_{260}/A_{280} ratio was close to the ideal value of 2, with the exception of the SuperArray kit. (C) The lowest C_{T} values were obtained using the Ambion and Roche kits, while the SuperArray kit provided the least amount of usable RNA. (D) Replicate reproducibility was highest in samples that were incubated overnight, with the best results obtained using the Ambion and Roche kits. (E) Representative Agilent 2100 bioanalysis of the same patient (sample 4) using the Roche kit with overnight and 3 h Proteinase K digestion is shown. Median RNA size is approximately 100–200 nt.
In order to determine which RNA isolation method yielded the highest quality RNA for the DASL assay (Figure 1D), we analyzed A$_{260}$/A$_{280}$ ratios, RPL13a C$_T$ values, and DASL assay replicate data. The results for all analyses are summarized in Table 1. We found that by using at least 100 ng (preferably 200 ng) of input RNA (at 20 ng/μL), an A$_{260}$/A$_{280}$ ratio ≥ 1.5, and RPL13a TaqMan assay C$_T$ values ≤ 29, we could achieve a Log R$^2$ value of >0.9 for 92% of the replicate samples (n = 26) in the DASL assay. Therefore, these were the minimum RNA quality parameters required for obtaining an acceptable replicate reproducibility score in the DASL assay (see Table 2 for optimized protocol).

Although many manufacturers recommend a shorter Proteinase K digestion in order to save time, the additional incubation time significantly increases RNA yield and overall quality. Upon examination of replicate reproducibility between two samples from the same patient (Figure 2), longer Proteinase K digestion also produced tighter scatter plots with higher Log R$^2$ values in the DASL assay for all kits tested. The best replicate reproducibility was achieved with the Roche kit using an overnight Proteinase K digestion, but the Ambion kit also gave comparable reproducibility with an overnight digestion (Figure 1D and Figure 2).

We were not able to compare the performance of RNA from fresh frozen samples with RNA from FFPE samples because frozen specimens were not available from this cohort of patients. However, previous studies have found that the DASL assay generates data that is highly correlated between frozen and fixed tissues from autopsies (8). Our technical replicate reproducibility was generally higher than those studies using similar RNA preparation methods, which may be due to the fact that autopsy specimens may have slightly greater RNA degradation than surgical specimens.

In the DASL assay, the number of detected genes (P < 0.01) and reproducibility of technical replicates (Log R$^2$ values) is largely determined by the 3 h or overnight Proteinase K digestion time points.

Table 1. RNA QC Values for Each Sample Preparation

<table>
<thead>
<tr>
<th>Sample Preparation</th>
<th>DASL R$^2$</th>
<th>RIN</th>
<th>Rpl13A Ct</th>
<th>[DNA] ng/μL</th>
<th>A$<em>{260}$/A$</em>{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambion O/N</td>
<td>0.95</td>
<td>1.2</td>
<td>25.8</td>
<td>58.8</td>
<td>2.0</td>
</tr>
<tr>
<td>(0.91–0.98)</td>
<td>(0–2.3)</td>
<td>(24.4–26.3)</td>
<td>(8.4–173.6)</td>
<td>(1.9–2.1)</td>
<td></td>
</tr>
<tr>
<td>Ambion 3 h</td>
<td>0.90</td>
<td>1.0</td>
<td>26.7</td>
<td>33.0</td>
<td>2.0</td>
</tr>
<tr>
<td>(0.69–0.98)</td>
<td>(0–1.7)</td>
<td>(24–30)</td>
<td>(21–54.2)</td>
<td>(1.9–2.1)</td>
<td></td>
</tr>
<tr>
<td>Roche O/N</td>
<td>0.97</td>
<td>1.5</td>
<td>25.9</td>
<td>67.3</td>
<td>2.0</td>
</tr>
<tr>
<td>(0.94–0.98)</td>
<td>(1.1–2.3)</td>
<td>(24.3–27.8)</td>
<td>(31.5–167.9)</td>
<td>(1.8–2.1)</td>
<td></td>
</tr>
<tr>
<td>Roche 3 h</td>
<td>0.94</td>
<td>1.8</td>
<td>26.4</td>
<td>50.8</td>
<td>1.9</td>
</tr>
<tr>
<td>(0.89–0.97)</td>
<td>(1.0–3.0)</td>
<td>(24.2–28.9)</td>
<td>(14.5–101.6)</td>
<td>(1.8–2.1)</td>
<td></td>
</tr>
<tr>
<td>Qiagen 3h</td>
<td>0.81</td>
<td>2.0</td>
<td>27.8</td>
<td>97.2</td>
<td>2.0</td>
</tr>
<tr>
<td>(0.57–0.98)</td>
<td>(1.1–2.4)</td>
<td>(26.2–30.1)</td>
<td>(16.2–314.3)</td>
<td>(1.9–2.1)</td>
<td></td>
</tr>
<tr>
<td>Qiagen 15 min</td>
<td>0.86</td>
<td>1.7</td>
<td>28.5</td>
<td>47.6</td>
<td>1.9</td>
</tr>
<tr>
<td>(0.67–0.97)</td>
<td>(0.9–2.4)</td>
<td>(26.8–30.9)</td>
<td>(10.3–124.5)</td>
<td>(1.7–2.0)</td>
<td></td>
</tr>
<tr>
<td>SuperArray O/N</td>
<td>0.85</td>
<td>0.9</td>
<td>27.7</td>
<td>53.9</td>
<td>1.9</td>
</tr>
<tr>
<td>(0.55–0.97)</td>
<td>(0.1–1.7)</td>
<td>(25.2–30.8)</td>
<td>(1.6–163)</td>
<td>(1.2–2.1)</td>
<td></td>
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<tr>
<td>SuperArray 3 h</td>
<td>0.73</td>
<td>0.3</td>
<td>31.2</td>
<td>9.6</td>
<td>1.2</td>
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<tr>
<td>(0.34–0.9)</td>
<td>(0–1.0)</td>
<td>(28.9–36.4)</td>
<td>(8.3–10.3)</td>
<td>(0–2.6)</td>
<td></td>
</tr>
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</table>

RNA QC values for each sample preparation. The average and range are given for the R$^2$ correlation for technical replicates, the RNA integrity number (RIN) from the Agilent Bioanalyzer, the cycle threshold (C$_T$) for the TaqMan Rpl13a assay, the DNA concentration, and the A$_{260}$/A$_{280}$ ratios. Roche=RNA High Pure kit, Ambion=RecoverAll kit, Qiagen=RNeasy FFPE kit, SuperArray=ArrayGrade FFPE RNA Isolation kit.
Signal-to-noise ratio. We found that while the expression patterns of RNA prepared using different kits clustered together for each patient, regardless of the RNA extraction method (Figure 3A), certain kits performed better. In fact, the DASL replicate reproducibility of samples prepared from the same patient using different RNA isolation methods was dependent upon the kits used, indicating that RNA preparation methods had important effects on the overall gene expression pattern (Figure 3B). There were significant differences in gene expression between patients, as evidenced by both our clustering and scatterplot comparisons (Figure 3, A and B, respectively). A heat map generated for all 502 genes for the 6 ER and HER2 positive samples (Figure 3C) showed that there was a substantial number of differentially expressed genes, pointing to significant and potentially important differences in the gene expression patterns between patient samples from the same major breast cancer subtype. These data suggest that the DASL assay is robust, and while quite tolerant to differences in RNA extraction methods, optimization and standardization of the RNA extraction protocol will enable the undertaking of future large studies.

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**Competing Interests Statement**

The authors declare no competing interests.
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