Methodology for assessing quality of RNA from FFPE samples for the Oncomine Immune Response Research Assay

Introduction

We describe an RT-qPCR–based method for determining RNA concentration and integrity of samples prior to use in the Ion Torrent™ Oncomine™ Immune Response Research Assay. RNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples is fragmented to varying degrees. While the Invitrogen™ Qubit™ 3 Fluorometer is typically used to quantify RNA after extraction, its output does not consistently predict successful library preparation and results in next-generation sequencing (NGS) assays. Electrophoretic approaches using the Agilent™ Bioanalyzer™ system are also not predictive of FFPE RNA performance in downstream analyses. The assay described here, the functional RNA quantitation (FRQ) assay, was designed as a rapid and inexpensive test to ascertain RNA quality and determine amplifiable RNA quantity from FFPE samples prior to running samples in the Oncomine Immune Response Research Assay.

Description of the FRQ assay

FRQ is a rapid test that uses off-the-shelf reagents to perform qPCR, delivering results in less than 2 hours. The test is performed in a simple qPCR workflow and requires total RNA that can be extracted from FFPE sections. A commercially available standard of known concentration is serially diluted to generate a standard curve. After the run, the test sample is compared to the standard curve to obtain the functional RNA quantity. The test output helps the researcher determine whether to proceed with a downstream NGS workflow, as shown in Figure 1. Details of the protocol and required reagents are available in Appendix B (Supplemental information) of the Oncomine Immune Response Research Assay User Guide (**Pub. No. MAN0015867**).

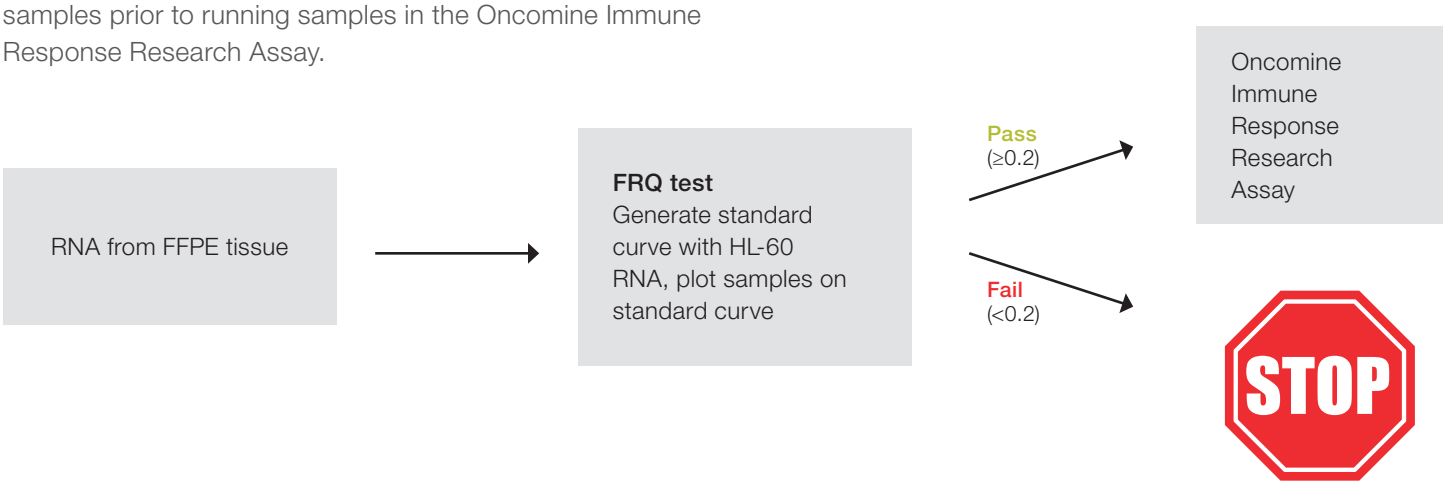

Figure 1. Overview of the FRQ assay.

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Correlation of test output and Oncomine assay results

Results are presented for 17 RNA research samples extracted from FFPE tissue of 6 different tumor types: breast, colorectal, head and neck, melanoma, non-small cell lung carcinoma, and renal cell carcinoma, as well as H838 and H727 human lung cell lines (ATCC Cat. No. CRL-5844 and CRL-5815, respectively). The FRQ assay was performed with 10 ng of input RNA, quantified using the Qubit 3 Fluorometer. The FRQ output indicating the amplifiable RNA concentration varied from 0.01 to 5.93 ng/µL (median 0.25 ng/µL), shown in Figure 2.

While the FRQ concentration correlated well overall with the percentage of valid mapped reads (correlation coefficient of 0.9), samples with low FRQ values showed considerable variability in mapped reads. Samples with FRQ of ≥0.2 ng/µL (–0.7 on the x-axis of Figure 2, indicated by the red dashed line) consistently yielded mapped reads above a 200K threshold with at least 70% valid mapped reads. In the FRQ concentration range of 0.2–5.93 ng/µL, 70–95% valid mapped reads were observed. Based on these results, 0.2 ng/µL is recommended as a cutoff, where samples with an FRQ concentration of ≥0.2 ng/µL are suitable for use in the Oncomine Immune Response Research Assay.

Workflow from FFPE tissue to Oncomine assay

The FRQ assay, performed in a simple 1-step RT-qPCR process, requires 3.5 µL per sample of total RNA isolated from FFPE sections. A test sample may be diluted 1:4 if its volume is limiting. A commercially available standard of known concentration, such as Invitrogen™ Promyelocytic (HL-60) Total RNA, is serially diluted to generate a standard curve for a range of 0.05–50 ng per reaction. The test sample is compared to the standard curve to obtain the functional RNA concentration. If an FRQ of ≥0.2 ng/µL is obtained, the sample is suitable for library preparation, sequencing, and downstream analyses, as depicted in Figure 3.

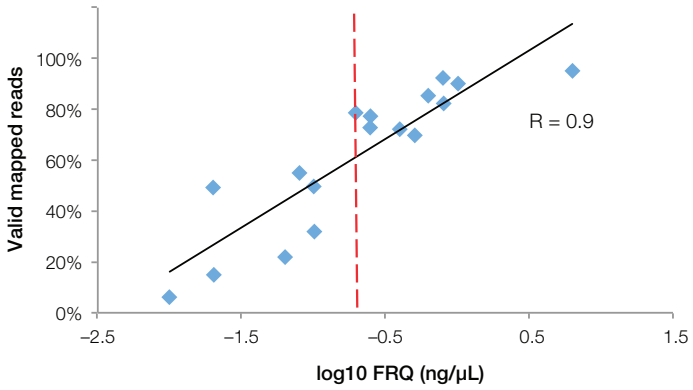

Figure 2. FRQ concentration vs. valid mapped reads. The dashed red line corresponds to an FRQ concentration of 0.2 ng/µL.

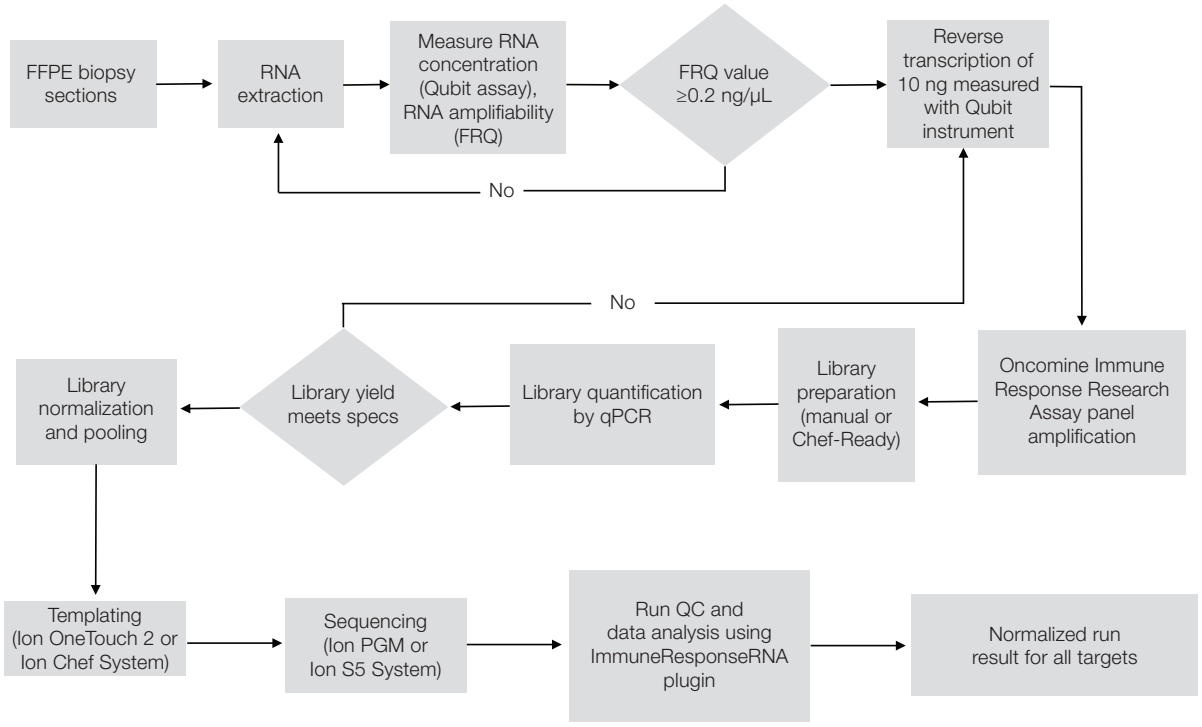

Figure 3. Detailed workflow from FFPE tissue to Oncomine Immune Response Research Assay.

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Conclusions and recommendations

Here, we show that assessing RNA amplifiability using the FRQ assay can be used as a quality check prior to performing the Oncomine Immune Response Research Assay. While the FRQ assay is not a required part of the workflow, it is recommended. This quality check may be particularly beneficial when the target markers are typically expressed at low levels and therefore the input RNA's quality has a greater effect on the assay. Samples with FRQ concentrations of <0.2 ng/µL are likely of poor quality and will not reliably produce good sequencing results. Using RNA with an FRQ concentration of ≥0.2 ng/µL for the Oncomine Immune Response Research Assay increases the likelihood of good sequencing results.

Ordering information

Product	Cat. No.
Promyelocytic Leukemia (HL-60) Total RNA	AM7836
TaqMan Fast Virus 1-Step Master Mix	4444434
Nuclease-free water (not DEPC-treated)	AM9938
20X TaqMan Gene Expression assay, *GUSB*	Hs00939627
Nonstick RNase-free 0.5 mL microfuge tubes	AM12350
Nonstick RNase-free 1.5 mL microfuge tubes	AM12450
MicroAmp Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp Optical Adhesive Film	4311971

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