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OPTIMIZATION OF REAL-TIME QPCR FOR MEASURING INFLAMMATION INDEX IN DIABETIC FOOT ULCER WOUNDS IN MODEL TISSUE SAMPLES

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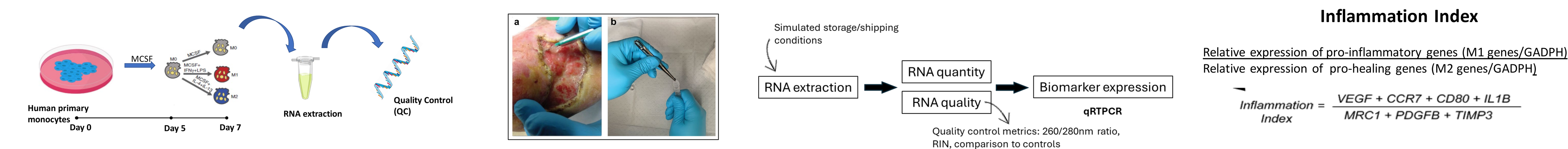
NIH DFC Diabetic Foot Consortium

BACKGROUND

Inflammation and wound healing are complex, linked processes that are dysregulated in nonhealing diabetic foot ulcers (DFU). Our research has shown that while initial pro-inflammatory activation of immune cells is critical for the initiation of wound healing processes, prolonged activation directly impairs it. After recognizing that transition from the early inflammatory to the late resolution phase is required for successful healing, we developed a composite biomarker using the ratio of 4 early-stage pro-inflammatory gene markers to 3 late-stage inflammation-resolution biomarkers, referred to as the Inflammation Index[1]. The Inflammation Index is an indirect measurement of the wound's healing stage. Our previous studies measured the Inflammation Index via qRT-PCR using RNA extracted from debrided wound tissue, suggesting that this score might have the potential to identify those wounds that are more likely to respond to conservative treatment versus those that may benefit from a more aggressive approach. To evaluate the expression of biomarkers that comprise the Inflammation Index, quality RNA is essential. The chronic wound environment is particularly damaging for RNA because of its high levels of enzymes and cellular debris containing RNases. Therefore, **our goal in this project was to optimize biomarker detection and determine the minimum sample quality and quantity in which the Inflammation Index can be reliably detected using RT-qPCR.**

[1]Sina Nassiri, et al., Journal of Investigative Dermatology advance, 2015

METHODOLOGY



RESULTS

Inflammation Index measured in debrided wound tissue

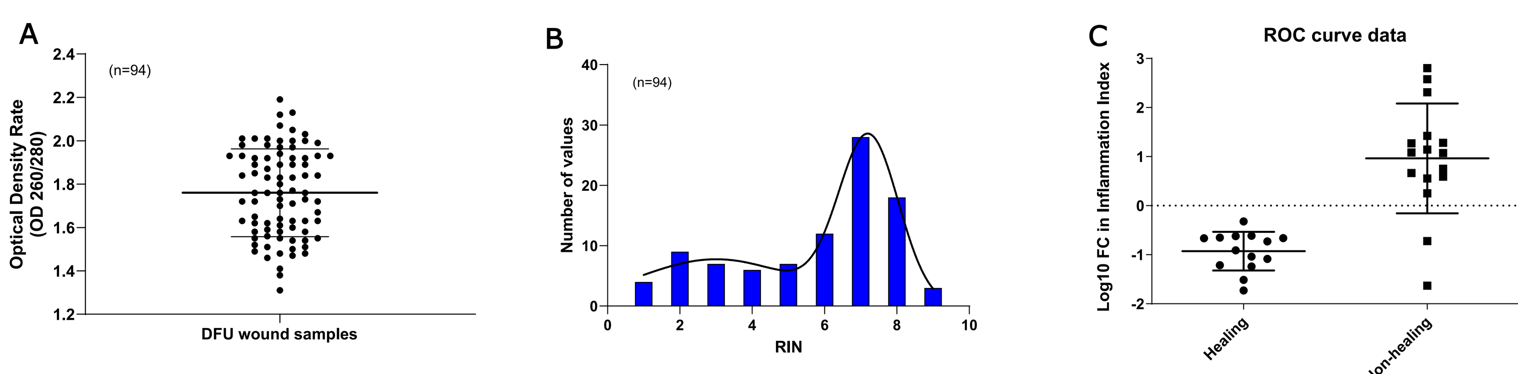


Figure 1. (A) OD 260/280 ratio by nanodrop. (B) Integrity (RIN) by bioanalyzer. (C) Inflammation Index by qRT-PCR

Inflammation Index measured in "gold standard" lab-prepared samples

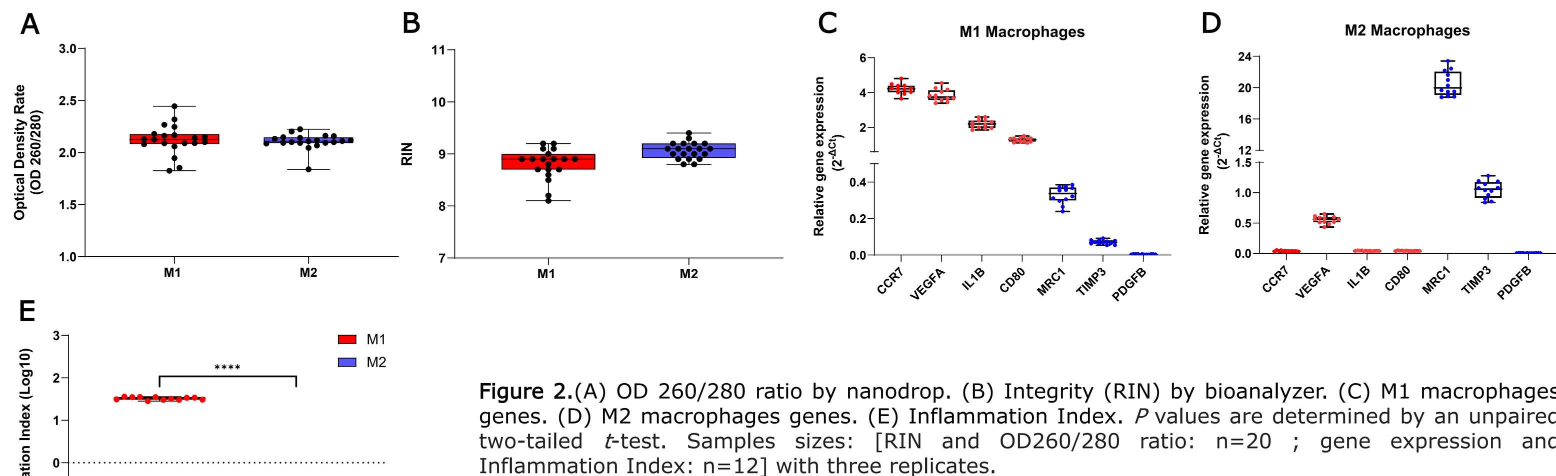


Figure 2.(A) OD 260/280 ratio by nanodrop. (B) Integrity (RIN) by bioanalyzer. (C) M1 macrophages genes. (D) M2 macrophages genes. (E) Inflammation Index. *P* values are determined by an unpaired two-tailed *t*-test. Samples sizes: [RIN and OD260/280 ratio: n=20 ; gene expression and Inflammation Index: n=12] with three replicates.

RNA from "gold standard" samples exhibit a high purity and low grade of degradation. M1 macrophages exhibit a high Inflammation Index while M2 macrophages exhibit a low Inflammation Index.

Reproducibility by level of training (%CV) of Inflammation Index measurements in laboratory-prepared samples

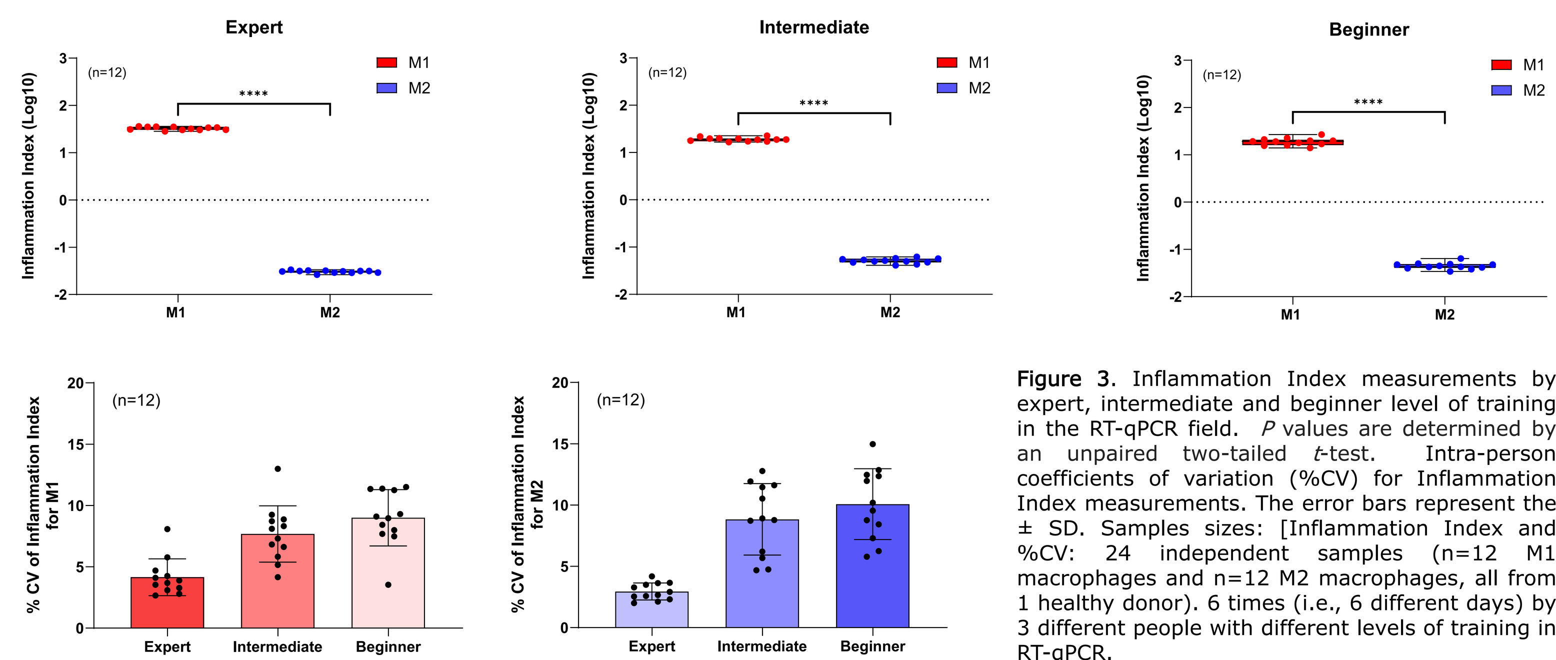


Figure 3. Inflammation Index measurements by expert, intermediate and beginner level of training in the RT-qPCR field. *P* values are determined by an unpaired two-tailed *t*-test. Intra-person coefficients of variation (%CV) for Inflammation Index measurements. The error bars represent the ± SD. Samples sizes: [Inflammation Index and %CV: 24 independent samples (n=12 M1 macrophages and n=12 M2 macrophages, all from 1 healthy donor). 6 times (i.e., 6 different days) by 3 different people with different levels of training in RT-qPCR.

We optimized measurement of the Inflammation Index and demonstrated reproducibility using lab-prepared "gold standard" samples.

Validation sample stability at simulated storage and shipping conditions compared to freshly collected lab-prepared samples

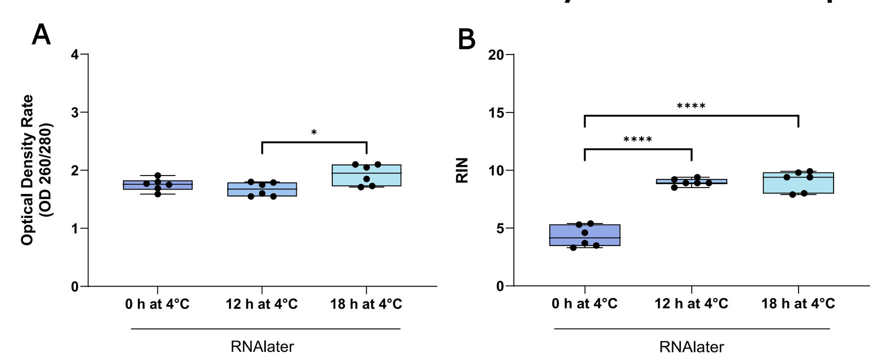


Figure 4. Impact of RNAlater pre-treatment on RNA stabilization. Effect of 0, 12, 18 h-RNAlater pre-treatment before freezing -80°C on Optical density rate (A) and RNA Integrity Numbers (RIN) (B). The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [RIN and OD260/280 ratio: n=6].

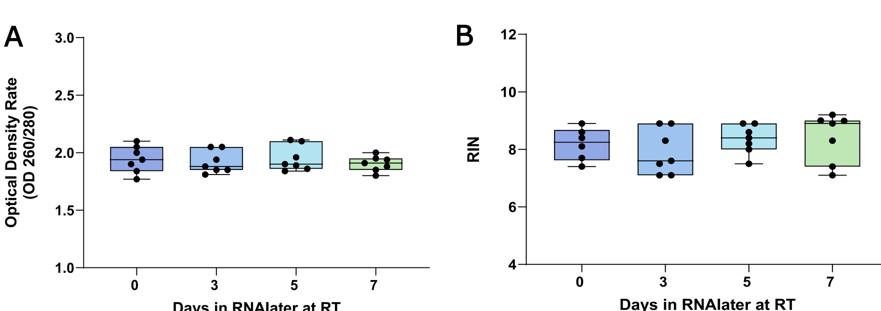


Figure 5. Impact of storing RNA samples in RNAlater at RT storage before freezing -80°C on RNA quality. Effect of 0, 3, 5, and 7 days at RT RNAlater following initial incubation on Optical density rate (OD 260/280) (A), and RNA Integrity Numbers (RIN) (B). The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [RIN and OD260/280 ratio: n=7].

Samples can be collected and stored overnight at 4°C in the clinic and then shipped at regular shipping speeds and without cold storage to a testing laboratory for analysis.

RNA quality in samples subjected to controlled degradation

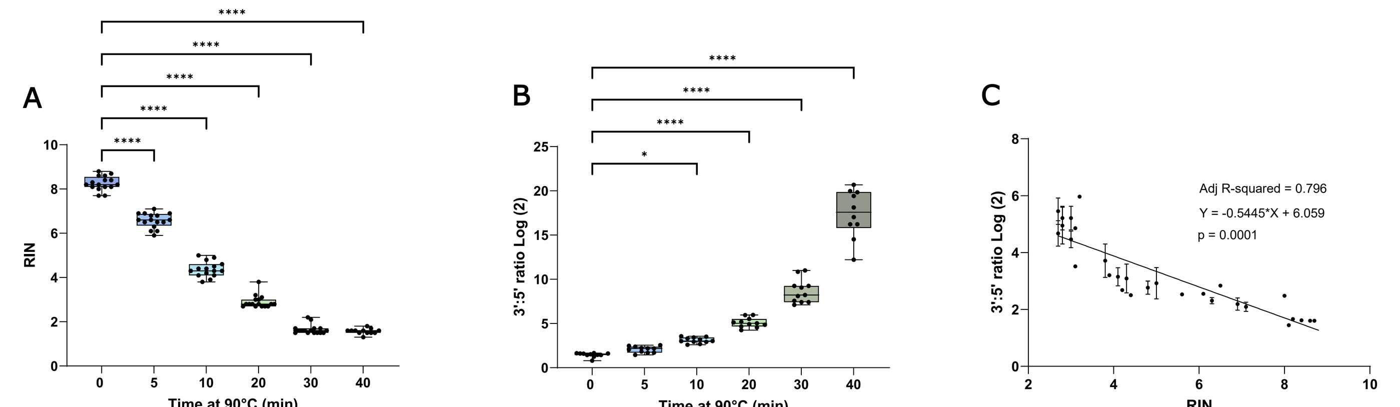


Figure 6. Increasing time of heat degradation decreases mRNA integrity as measured by the RIN (A) and 3':5' assay (B). Correlation between RIN and 3':5' assay (C). The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [RIN: n=16 ; 3':5' assay: n=11].

Increasing time at 90°C increases the degradation of RNA. RIN is a good indicator of mRNA integrity, and we found a good correlation between decreasing RIN values and decreasing 3':5' ratios, suggesting that the 3':5' ratio may be helpful for measuring other genes that comprise the Inflammation Index.

Ct values of genes that comprise the Inflammation Index vs. RNA fragmentation

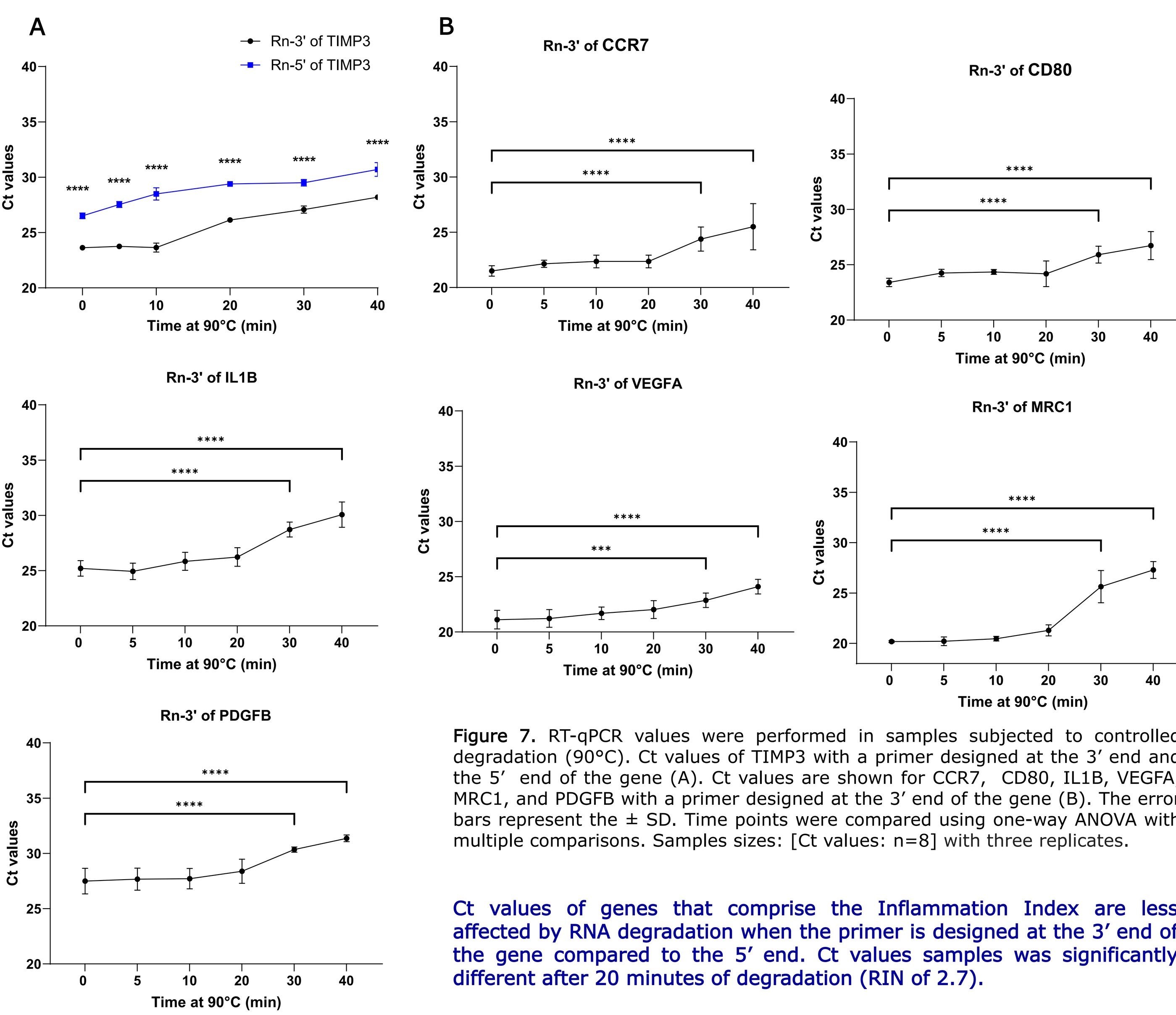
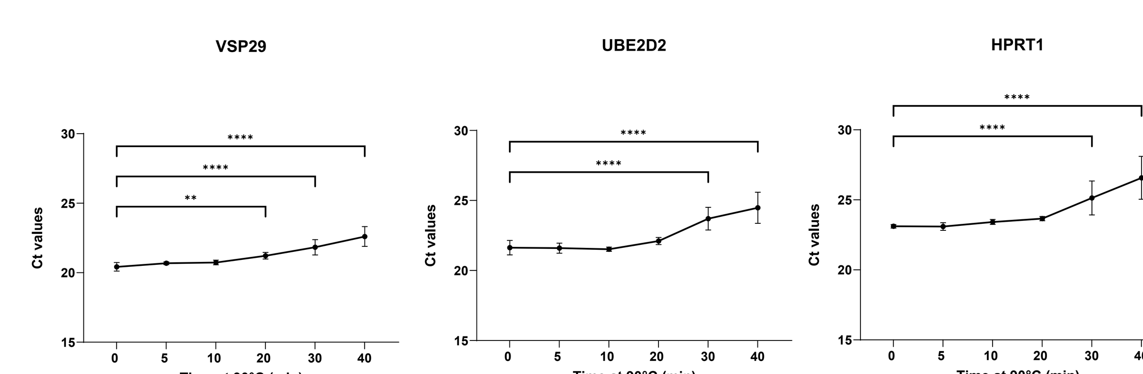


Figure 7. RT-qPCR values were performed in samples subjected to controlled degradation (90°C). Ct values of TIMP3 with a primer designed at the 3' end and the 5' end of the gene (A). Ct values are shown for CCR7, CD80, IL1B, VEGFA, MRC1, and PDGFB with a primer designed at the 3' end of the gene (B). The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [Ct values: n=8] with three replicates.

Ct values of genes that comprise the Inflammation Index are less affected by RNA degradation when the primer is designed at the 3' end of the gene compared to the 5' end. Ct values samples was significantly different after 20 minutes of degradation (RIN of 2.7).

Ct values of housekeeping genes vs. RNA fragmentation



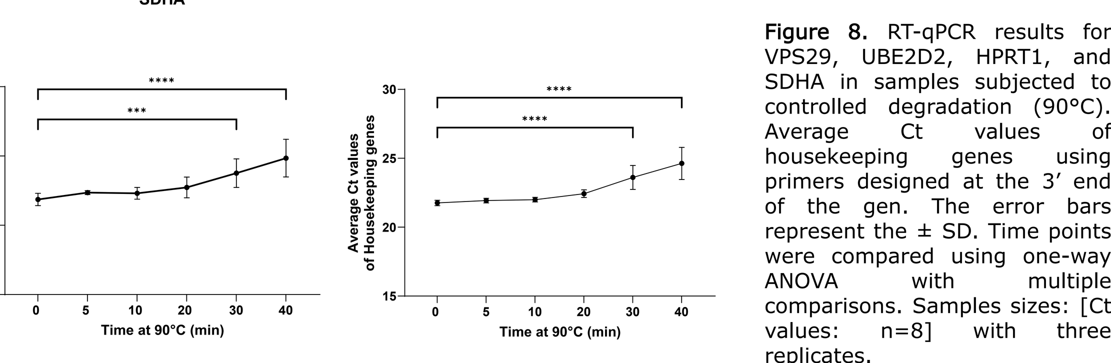


Figure 8. RT-qPCR results for VPS29, UBE2D2, HPRT1, and SDHA in samples subjected to controlled degradation (90°C). Average Ct values of housekeeping genes using primers designed at the 3' end of the gen. The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [Ct values: n=8] with three replicates.

Average of Ct values of housekeeping genes using primers designed at 3' end eliminates the effects of RNA degradation on individual housekeeping gene expression, even for very highly degraded samples. Ct values samples was significantly different after 20 minutes of degradation (RIN of 2.7).

Relative expression levels of the genes that comprise the Inflammation Index vs. RNA fragmentation

Figure 9. Relative expression levels of genes were performed in samples subjected to controlled degradation (90°C). Expression values of each gene was normalized to the average of the housekeeping genes using the 2^-delta Ct method and using primers designed near the 3' end. The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [Relative RNA expression: n=8] with three replicates.

Normalizing biomarker expression to the housekeeping genes' mean, and using primers designed near the 3' end, largely abrogates the effects of RNA degradation on expression of each gene. Relative expression levels of genes was significantly different after 20 minutes of degradation (RIN of 2.7).

Inflammation Index vs. RNA fragmentation

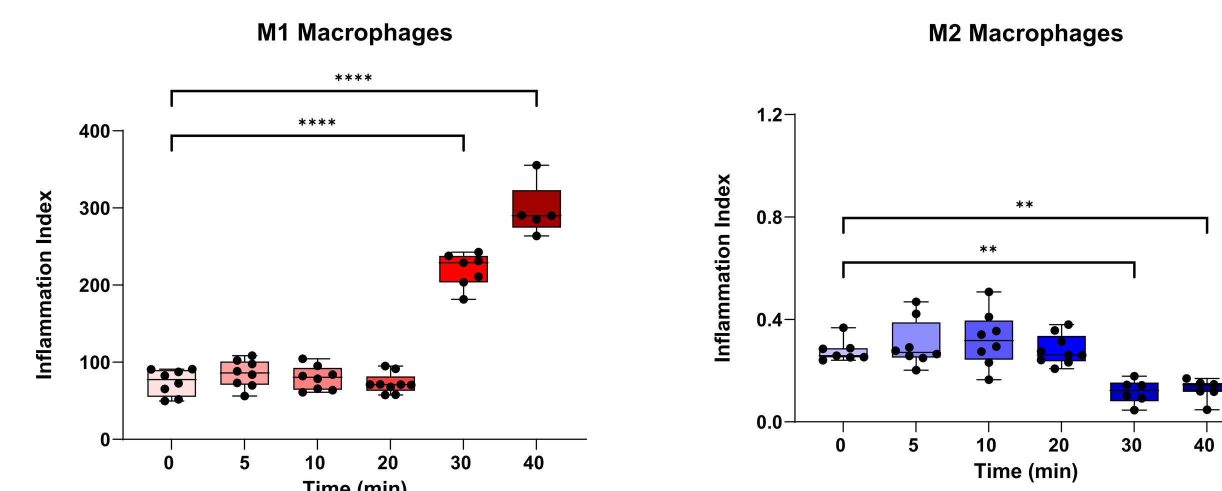


Figure 10. Time-dependent degradation of the Inflammation Index in M1 and M2 macrophage samples subjected to controlled heat degradation (90°C). The error bars represent the ± SD. Time points were compared using two-way ANOVA with multiple comparisons. Samples sizes: [Inflammation Index: n=8] with three replicates.

Inflammation index is affected significantly by RNA degradation after 20 minutes of degradation (RIN of 2.7).

Inflammation Index vs. RNA purity

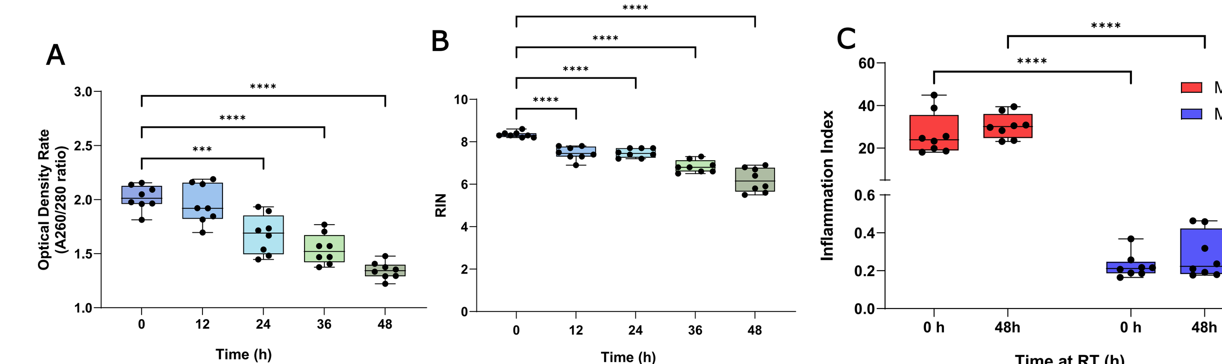


Figure 11. RNA purity (A) and quality (B) from samples incubated at different times in PBS (controlled degradation). Results of RT-qPCR for Inflammatory Index in samples stored under normal conditions or 48 h in PBS at RT (C). The error bars represent the ± SD. Time points were compared using one-way ANOVA with multiple comparisons. Samples sizes: [RIN and OD260/280 ratio: n=8; Inflammation Index: n=18] with three replicates.

Increasing incubation time of cells in PBS results in RNA samples decreasing purity and quality. RNA samples with decreasing purity do not significantly impact Inflammation index measurement. (at least to samples with 260/280nm ratio of 1.39).

The minimum QC metrics for RNA degradation and purity are RIN of 2.7 and 260/280nm ratio of 1.39, respectively, in order to accept samples for analysis.

Inflammation Index measured in real-world samples collected from the Diabetic Foot Consortium (DFC)

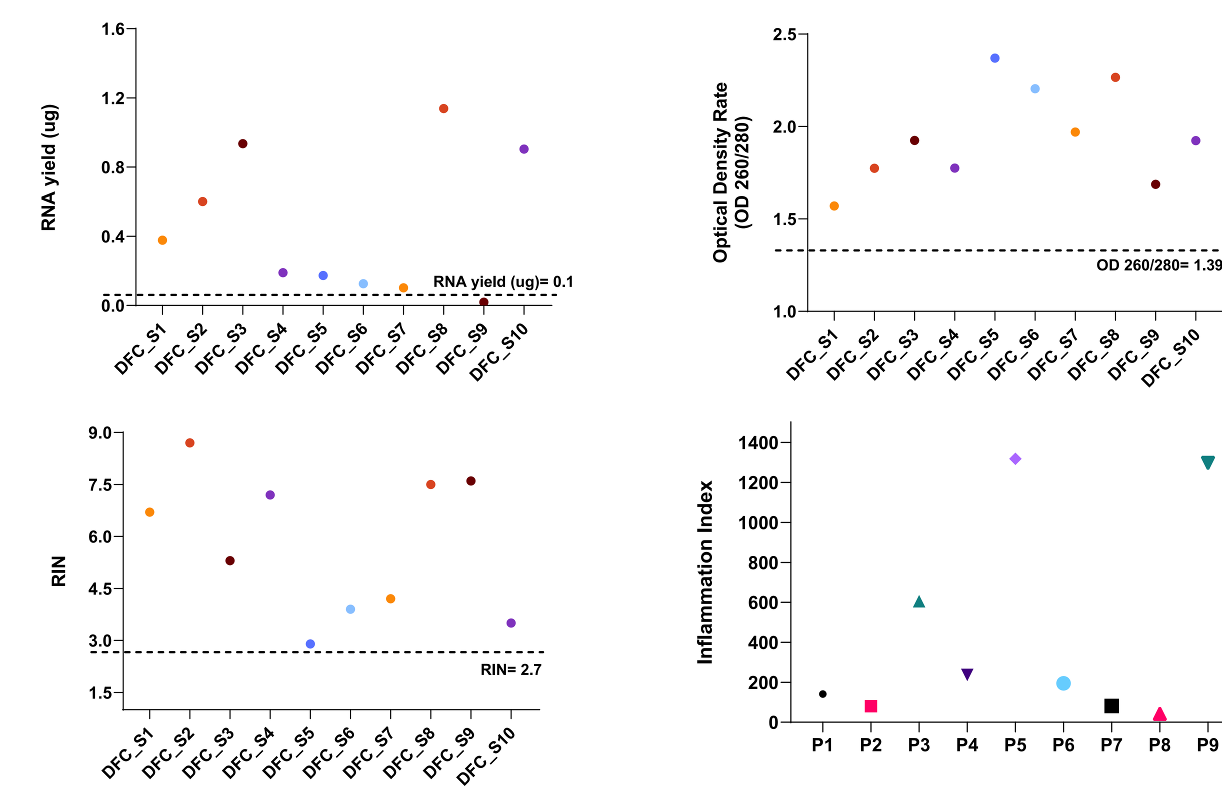


Figure 12. Analysis of RNA yield, purity (260/280nm ratio) and quality (RIN) (C) in 10 samples received from DFC. Inflammation Index measured in 9 samples received from the DFC that passed QC metrics.

9 of 10 samples from DFC met the minimal QC threshold (one sample yielded too little RNA to measure all 7 genes that comprise the Inflammation Index). We successfully measured expression of all 7 genes for the 9 samples received from the DFC that passed QC metrics .

CONCLUSIONS

Based on these results, we conclude that by using controllably degraded cell samples in vitro to model damaged tissue, the measurement of the Inflammation Index in DFU samples was appropriately optimized. We show that the Inflammation Index can be reliably detected in even highly degraded samples and in those contaminated with nucleic acid and/or proteins. From a translational perspective, we determined the minimum QC metrics that are satisfied for the biomarkers can be reliably measured in real-world samples collected from the Diabetic Foot Consortium (DFC). Acknowledgments: This work was funded by NIH R61 DK131917