The Yin-Yang of Sample Preparation: Reagent optimization for maximal yield, activity, and preservation of biomarkers using Adaptive Focused Acoustics™ (AFA)

William Skea
Gary Smejkal
Michael Kaiser
John-David Herlihy
David Green
James Laugharn

Covaris
the pre-analytical advantage™

Covaris, Inc. • 14 Gill Street, Unit H,
Woburn, Massachusetts 01801-1721 USA
Tel: +1 781-932-3959 • Fax: +1 781-932-8705
• Email: info@covarisinc.com • Web: www.covarisinc.com

1. Abstract

The Yin-Yang of sample preparation refers to the divergent downstream applications that dictate the terms and conditions of sample preparation. For example, maximizing the total protein yield from cells and tissues with minimal bias requires the stringency of chaotropic agents, detergents, and reducing agents that are strongly denaturing and frequently destructive to biologically active epitopes. This frequently renders the isolate incompatible with applications requiring antibody recognition such as ELISA and affinity enrichment. (For the same reason, some antibodies that perform well in ELISA will not work for Western blotting.) Hence, reagent selection is based on the intended downstream analysis. Covaris has developed a series of proteomic reagents with performance properties that are enhanced by Adaptive Focused Acoustics (AFA). The non-contact, isothermal, mechanical properties of AFA has been shown to be highly effective for the disruption of cells and tissues with improved compatibility of downstream analytical methods by lessening the dependence on interfering detergents and ionic components. The Covaris Protein Extraction Reagent DF is a detergent-free reagent that efficiently extracts proteins in a milieu compatible with downstream LC/MS analysis. Protein Extraction Reagent TP extracts more total protein at lower detergent concentrations than comparable reagents when used in conjunction with AFA. Covaris Native Protein Extraction Reagents N and SuperB are designed for native applications where the preservation of protein conformation and biological activity is required and is directly compatible with LC/MS, immunoassay, and affinity enrichment. Optimized AFA conditions are described that enhance the performance of each of these new reagents.

3. Materials and Methods

t-PREP cryofracture and AFA extraction

Frozen cardiac muscle samples (12.6 ± 1.7 mg, n = 106) were weighed and then placed in a Covaris t-PREP devices pre-chilled to -80 ℃ (**Figure 1A**). Dry cryofracture of the frozen tissues was performed with a Covaris t-PREP Impactor. Covaris Protein Extraction Buffers N, DF, SuperB, or TP (Woburn, MA), containing protease inhibitors were added to the t-PREP chamber following cryofracture. The t-PREPs were processed in a S220 Focused-ultrasonicator (Covaris, Wobrn, MA, USA) at 35, 70, 140, or 280W peak incident power (PIP) for 60, 120, 240, or 480 seconds. Duty factor and cycles per burst (CPB) were held constant at 10% and 200, respectively. Immediately following AFA, the homogenates were centrifuged at 10,000 RCF for 10 minutes and the supernatants were reserved for analysis.

tissueTube cryofracture and AFA extraction

Larger frozen muscle tissues (51.1 ± 3.8 mg, n = 68) were weighed, placed into TT05 tissueTUBEs (**Figure 1B**), and then cryofractured using an automated cryoPREP Impactor with a 12x12mm roundbottom glass tube (TC12) Protein extraction buffer was added to the tissueTUBE containing cryofractured tissue and the suspension was transferred into the glass tube by inversion. The tissueTUBE was removed from the glass tube, capped, and processed in a S220 Focused-ultrasonicator. Immediately following AFA, the homogenates were cleared by centrifugation at 10,000 RCF for 10 minutes and the supernatants were reserved for analysis.

Total protein and phosphatase activity

Protein concentration was determined by Quick Start Bradford Reagent (Bio-Rad, Hercules, CA, USA). Phosphatase activity was measured by Total Phosphatase Assay Kit from G Biosciences (St. Louis, MO, USA) following the addition of 10 mM $MgCl_2$ to each sample.

SDS-PAGE

SDS-PAGE was performed on 8-16% Tris-HCl Criterion gels (Bio-Rad, Hercules, CA, USA). Gels were stained with colloidal Coomassie stain. High molecular weight proteins (e.g. myosin) were measured by densitometry and expressed as band density as a percentage of the sum of all band densities for each sample.

Heat maps

Heat maps plotting experimental matrices testing sample processing conditions in the t-PREP and the 12x12mm roundbottom glass tube (TC 12) for each of the four reagents were generated using MatLab software (MathWorks, Natick, MA, USA). Circular heat maps consolidating multiple data sets were generated manually.

FIGURE 1. (A) Covaris t-PREP and (B) tissueTube with 12 x 12 mm glass tube with cap (TC12).
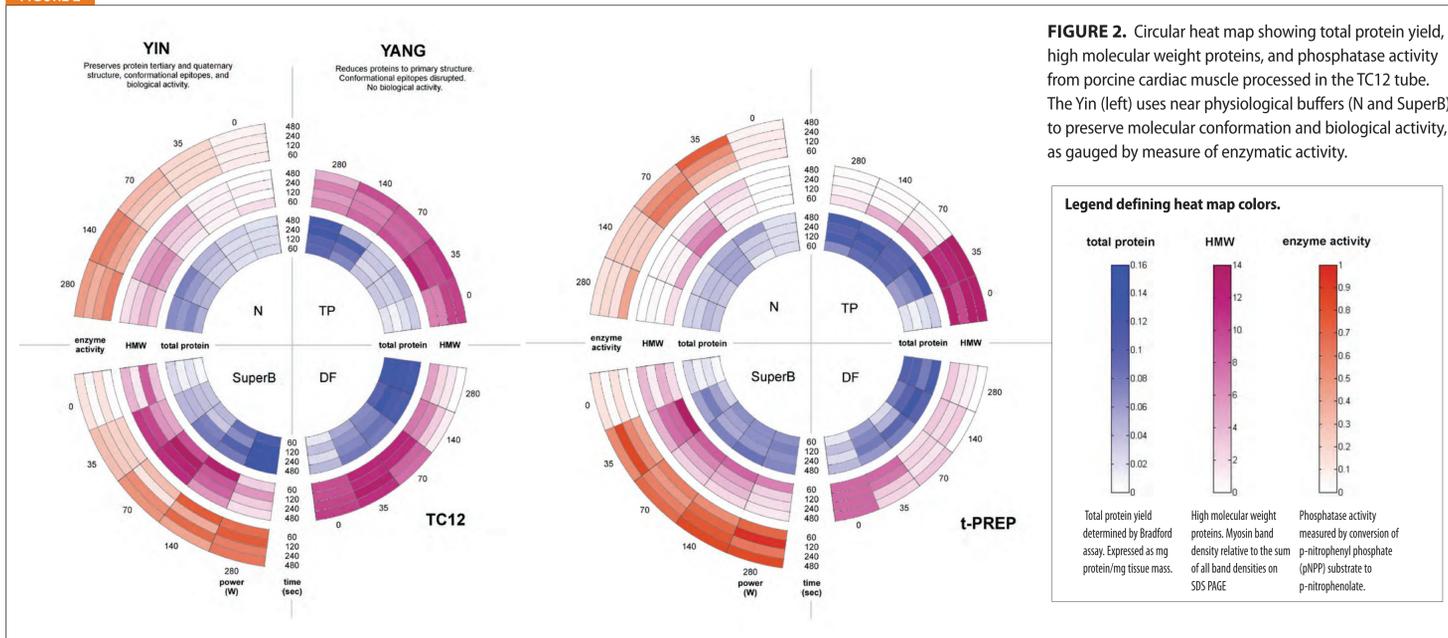

FIGURE 2. Circular heat map showing total protein yield, high molecular weight proteins, and phosphatase activity from porcine cardiac muscle processed in the TC12 tube. The Yin (left) uses near physiological buffers (N and SuperB) to preserve molecular conformation and biological activity, as gauged by measure of enzymatic activity.
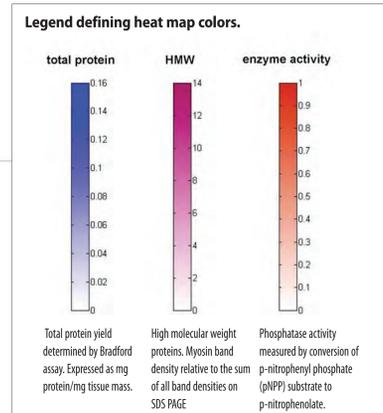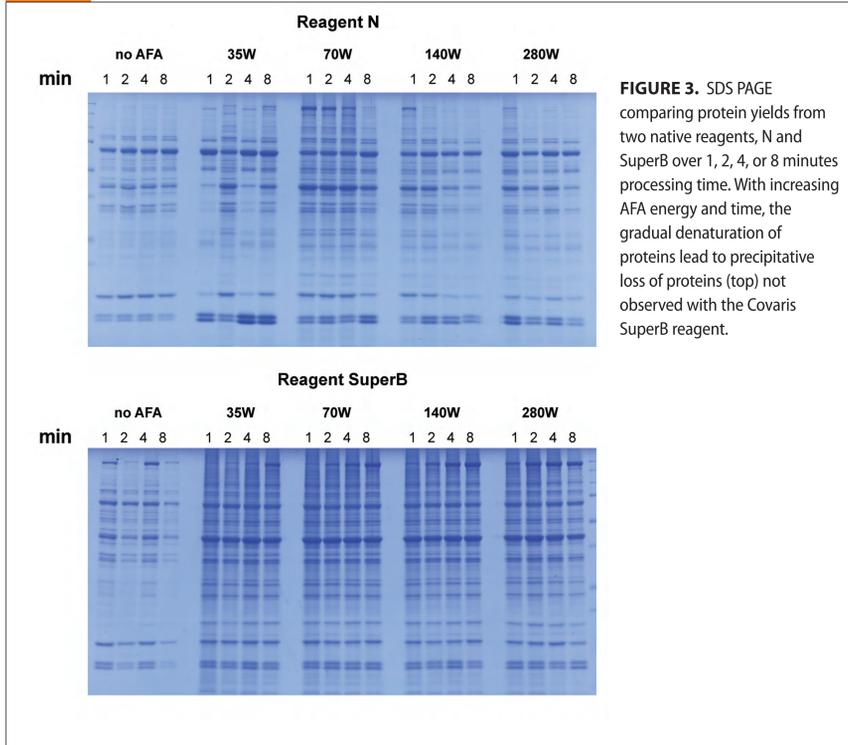

FIGURE 3. SDS PAGE comparing protein yields from two native reagents, N and SuperB over 1, 2, 4, or 8 minutes processing time. With increasing AFA energy and time, the gradual denaturation of proteins lead to precipitation loss of proteins (top) not observed with the Covaris SuperB reagent.
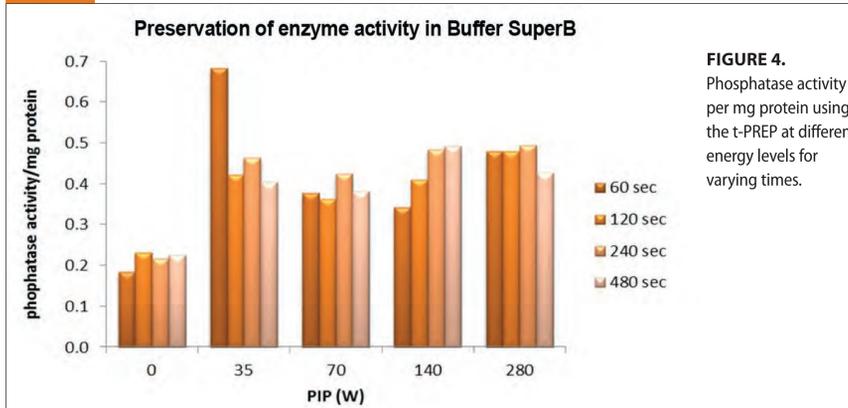

FIGURE 4. Phosphatase activity per mg protein using the t-PREP at different energy levels for varying times.

4. Results and Discussion

Circular heat maps provide multivariate analysis to visually guide the user towards optimal sample processing parameters (**Figures 2**). The Yin refers to extraction conditions and buffers that preserve protein integrity in applications such as the recovery of enzymatically active proteins, immunoprecipitation, ELISA, and biomarker research where the preservation of conformational epitopes is essential. A recent survey of 327 validated antibodies showed that 17% of ELISA compatible antibodies were not compatible with Western blotting, indicating that conformational epitopes needed to be preserved for antibody recognition. The proprietary Covaris SuperB Reagent was formulated to enhance protein recoveries, while retaining enzymatic activity under native conditions (**Figure 3 and 4**). However, the mild conditions required to preserve conformation frequently bias toward cytoplasmic and more hydrophilic proteins. The Yang refers to the more stringent conditions and reagents (denaturing) required to gain a more comprehensive view of proteomes, including membrane proteins.

Precise control over sample processing conditions attained by AFA and AFA-optimized reagents enables highly reproducibe results. Furthermore, the introduction of the t-PREP for processing very small (less than 10 mg tissue), and frequently very rare tissue samples, offers added flexibility to the Biomarker Extraction System.

5. Conclusion

- The Yin and Yang charts provide guidelines for optimizing AFA conditions after using the t-PREP or tissueTUBE for tissue cryofracture.

- Buffer selection using AFA is based on the intended downstream analysis.

- "Slow and Gentle" AFA energy provides better extraction of whole protein than traditional extraction methods.

- Covaris Buffer SuperB provides high enzymatic recovery using native conditions.

- Covaris Buffer DF is compatible with mass spectrometry workflows.