A Robust and Standardized Workflow to Analyze *C. albicans* Differential Protein Expression using Tandem Mass Tag (TMT)

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Abstract

Yeast samples are difficult to process for protein extraction. They are resistant to chemical lysis and exhibit phenotypic appearances which make them even harder to lyse, especially when it comes to the filamentous subtypes. Mechanical treatments can circumvent the use of multiple heating and cooling steps, but they must ensure an efficient and reproducible lysis.

This application note delineates a robust method for reproducible extraction and analysis of almost 3,000 proteins from *C. albicans* in both its yeast and hyphal forms. The process combines Adaptive Focused Acoustics® (AFA®) for the lysis, Single Pot Solid Phase Sample preparation (SP3) for clean-up and digestion, and Tandem Mass Tag (TMT) labeling for Liquid Chromatography-Mass Spectrometry multiplexed analysis. It can be easily adapted for automated, higher-throughput processing as already demonstrated for mammalian cells and tissues in 8-well strips [1] and 96-well plates [2].

Introduction

Candida albicans is the most common human pathogenic yeast and a member of the human gut flora [3]. It can reside harmlessly on the skin, in the oral cavity, and in the urogenital and gastrointestinal tracts causing cutaneous and mucocutaneous candidiasis [4]. It is a polymorphic organism, being able to grow in yeast form, or in hyphal and pseudohyphal types, which form filamentous structures [3]. *C. albicans* can cause life-threatening systemic infections: the hyphal form (which can be induced in vitro using BSA [5]) has an important role in causing disease by invading epithelial cells and causing tissue damage [6].

Studying the difference in protein expression between the two forms has led to discovering the key role for kinases in the cell biology of hyphal growth. The significant mechanical strength of the yeast cell wall, and especially the filamentous form, makes the recovery of proteins, including biologically active proteins, particularly challenging. The techniques often utilized to improve cell lysis employ rigorous mechanical agitation and/or harsh chemicals that can result in both protein denaturation and the loss of protein activity. Alternatively, the use of enzymes (such as lysozyme) to hydrolyze the cell wall may result in the loss of proteins and the elimination of post-translational protein modifications such as phosphorylation or glycosylation.

Mechanical approaches like bead beating or probe sonication can efficiently lyse such cells, but the so-released content can be damaged because of the heat generated during the process [7,8]. In a significant contrast to uncontrolled heating of both bead beating and probe sonication, the precise control of both mechanical and thermal energy of a Covaris AFA-based extraction protocol enables highly reproducible lysis and extraction from yeast [9]. The efficient non-contact isothermal mechanical disruption of cells by AFA leads to a high degree of extraction reproducibility while eliminating temperature fluctuations that can modify or damage proteins and respective PTMs [10].

This application note presents in a combined workflow the use of Adaptive Focused Acoustics for lysis, Single Pot Solid Phase Sample preparation (SP3) [11] for clean-up and digestion, and Tandem Mass Tag (TMT) labeling for multiplexed mass spectrometry analysis, opening new avenues to high-throughput sample preparation for Yeast species.

Materials & Methods

Required Equipment

Covaris

- Covaris M220 Focused-ultrasonicator (PN 500295)
- microtube-130 AFA Fiber Screw-Cap (PN 520216)
- M220 Holder XTU (PN 500414)
- M220 Holder XTU insert microTUBE 130 µL (PN 500489)

Other Supplies

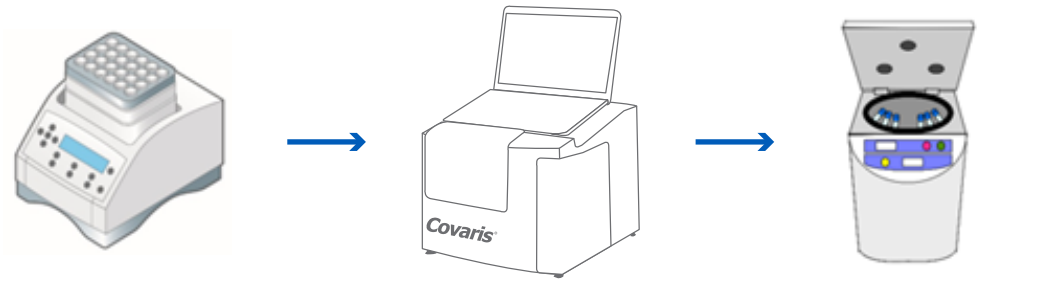
- Centrifuge
- Thermoshaker
- Magnetic Beads
- Magnetic Rack

Culture Conditions

After initial pre-culture of *C. albicans* isolate SC5314, 10 mL culture in YPD medium (named "Yeast") were incubated overnight at 37 °C with moderate shaking. In parallel, 10 mL culture in YPD medium + 10% BSA (named "Hyphae") were incubated overnight at 37 °C with moderate shaking.

Cell Lysis

1.5 mL of yeast cultures (OD_{600} = 1.2) were transferred into a 2 mL Eppendorf Tube and centrifuged at 5,000 g, 4 °C for 10 minutes. The supernatants were discarded, cell pellets were washed by resuspension in 1 mL of PBS and the process was repeated two additional times before snap freezing cells in liquid nitrogen and storing at -80 °C.



130 µL Lysis Buffer
(4% SDS, 50 mM Tris.HCl pH 7.5)
Boiling a 95 °C for 10 min

Ultrasonication for 180 sec at 75 W,
20% Duty Factor, 200 Cycles per Burst

Centrifugation for 10 min at
10,000 g to remove cell debris

Figure 1. Protein extraction process.

Frozen cell pellets were thawed by the addition of 130 µL lysis buffer consisting of 4% SDS, 50 mM Tris.HCl (pH 7.5). Once pellets were thawed and resuspended, the yeast samples were subjected to boiling at 95 °C on a thermoshaker for 10 minutes (***Figure 1***). After the sample returned to room temperature, the total volume was transferred to a Covaris 130 µL microTUBE and subjected to focused-ultrasonication on a M220 Focused-ultrasonicator for 180 seconds at 75 W, 20% Duty Factor, and 200 Cycles per Burst. Samples were transferred to new 1.5 mL Eppendorf tubes and then centrifuged at 10,000 g for 10 minutes at 20 °C. Protein amount was determined using a Pierce Rapid Gold BCA Protein Assay Kit (cat #A53226) according to manufacturer's instructions, and proteins were analyzed by SDS-PAGE.

SP3-based Protein Digestion, TMT-labeling, and High-pH Reversed-phase Fractionation

Samples (50 µg) were reduced with 10 mM TCEP for 20 min and alkylated with 20 mM iodoacetamide for 20 min in the dark, both at room temperature. The sample buffer was immediately removed using the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) method (***Figure 2***) as described previously [2,11]. Briefly, 500 µg of each bead type were added to samples (10:1 beads-to-protein ratio), then acetonitrile (ACN) was added to a final concentration of 75%. The beads were agitated on a thermoshaker for 10 min at 1,000 rpm at room temperature followed by 2 min incubation on a magnetic rack. The supernatant was removed, and beads were washed two times with 200 µL of 80% Ethanol (EtOH) and one time with 200 µL of ACN. Beads were resuspended in 100 µL of 100 mM TEAB containing 500 ng of Trypsin/Lys-C Protease Mix (1:100 enzyme-to-protein ratio). After a gentle centrifugation step, enzymatic digestion was carried out overnight on a thermoshaker at 37 °C with agitation (1,000 rpm). Following digestion, the samples were centrifuged for 1 min and incubated for 2 min on the magnetic rack.

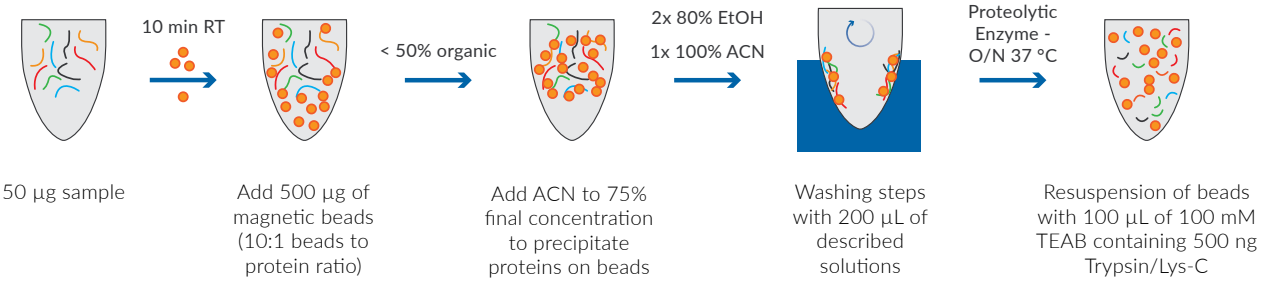


Figure 2. Protein capture, clean-up, and digestion using Single-Pot Solid- Phase Sample Preparation (SP3) (adapted from references 3 and 4).

One hundred (100) µL of the supernatant was transferred to a new 1.5 mL Eppendorf tube, then 200 µg of TMT label dissolved in 41 µL ACN was added for labeling reaction at room temperature for one hour (***Figure 3***).

The reaction was quenched by an addition of 8 µL of 5% hydroxylamine, followed by a 15 min incubation at room temperature. Samples were pooled and dried using a SpeedVac for one hour at 60 °C. Approximately 200 µg were fractionated with a 32 min basic pH reverse-phase HPLC separation on a Waters UPLC CSH C18 Peptide (2.1 x 150 mm, 1.7 µm). Ninety-six fractions were collected and combined down to 24 fractions prior to subsequent LC-MS/MS processing.

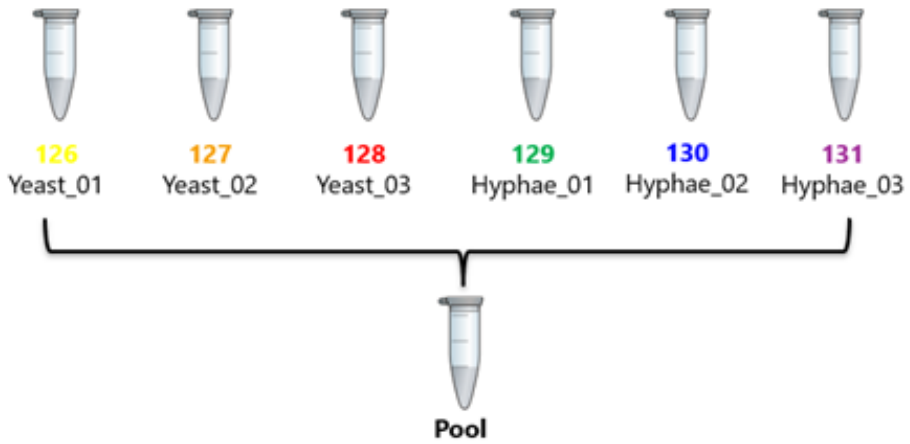


Figure 3. TMT labeling.

Mass Spectrometry Acquisition

Reverse phase separation of peptides was performed on an Ultimate 3000 RSLC Nano system using a 25 cm column (75 µm internal diameter, Reprosil-Pur C18-AQ 1.9 µm phase, PepSep) at a flow rate of 500 nl/min and maintained at 60 °C. Solvent A was 0.2% formic acid in LC-MS grade water and solvent B 0.2% formic acid in 80% ACN. Gradient consisted of a first increase of solvent B from 10 to 30% over 83 minutes then from 30 to 45% over 17 minutes. Total run time including column wash and re-equilibration was 120 minutes.

Tandem mass spectrometry analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with an Easy-Spray nanosource. A full scan was acquired between 375 and 1,400 Th at a resolution of 70,000 (AGC target of 3e6 or 60 ms maximal injection time). The top fifteen precursors were then selected for MS2 analysis at a resolution of 35,000 (AGC target of 2e5 or 110 ms maximal injection time, NCE 35%) with an isolation window of 1.6 Th and a fixed first mass of 120 m/z.

Mass Spectrometry Data Analysis

All raw files that used DDA LC–MS/MS were analyzed by MaxQuant v1.6.0.1 software using the integrated Andromeda Search engine and searched against the *Candida albicans* (strain SC5314 / ATCC MYA-2876) UniProt Proteome (July 2020 release with 6 040 protein sequences). Trypsin was specified as the enzyme, cleaving after all lysine and arginine residues and allowing up to two missed cleavages. Carbamidomethylation of cysteine was specified as fixed modification and protein N-terminal acetylation, oxidation of methionine, and pyro-glutamate formation from glutamine were considered variable modifications. TMT data was set to the first parameter group that further specified TMT6-plex as label with a reporter ion mass accuracy of 0.003 Da.

Results and Discussion

The differences in expression patterns between the three "Yeast" and the three "Hyphae" strains were first analyzed by SDS-PAGE. Several bands seemed differentially expressed between the two phenotypes, and the main observation came from a 60 kDa band clearly overexpressed in the Hyphae samples.

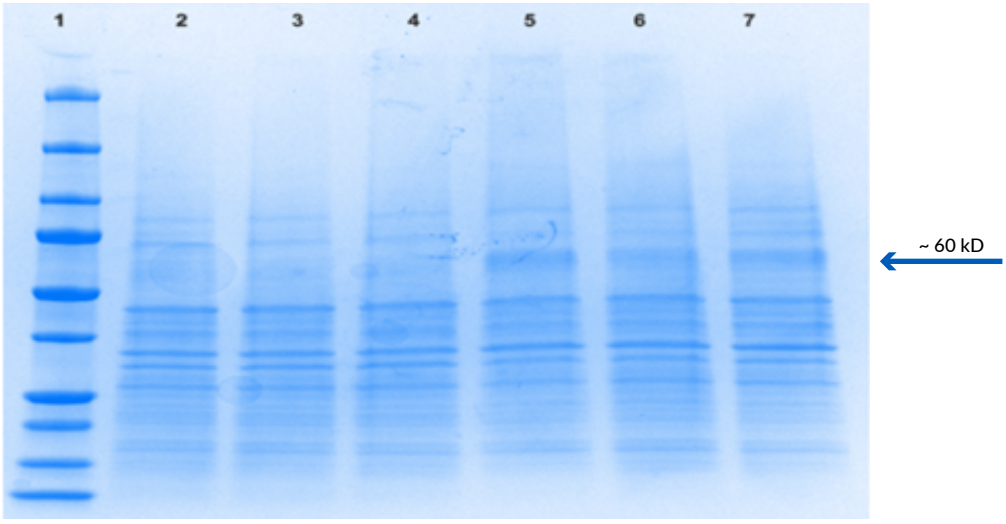


Figure 4. 1D SDS PAGE analysis. Thirty (30) µg of protein from each sample were analyzed on a 12% acrylamide SDS-PAGE gel (Biorad) under reducing conditions followed by Coomasie Brillant Blue staining. Lane 1: MW marker, Lanes 2 to 4: Yeast samples, and Lanes 5 to 7: Hyphae samples.

Peptides and proteins were analyzed (***Table 1***): 2933 proteins in total were identified, corresponding to 29,148 unique peptides (29,648 total). A Principal Component Analysis (PCA) was used to summarize protein expression into a reduced dimension based on a Euclidian distance. This is used to illustrate the samples in two dimensions based on the 1st and 2nd principal components. This PCA was run on the same 6 samples to evaluate the reproducibility of extraction. As expected, we observe a separation between the two phenotypes with the 3 Yeast samples clustered together as well as the 3 Hyphae samples (***Figure 5***).

Proteins[a]	Unique Peptides	Total Peptides	Sig. Dif. Proteins Up	Sig. Dif. Proteins Down[b]
2,993	29,148	29,648	65	130

Table 1. Protein and peptide quantification. a - Proteins are quantified across 6 channels with ≥ 2 unique peptides and CV < 30% for each conditions. b - Significantly differentiated proteins are defined as those with Benjamini-Hochberg adjusted *p*-value < 0.01 and a $\log_2$ ratio exceeding ± 1.

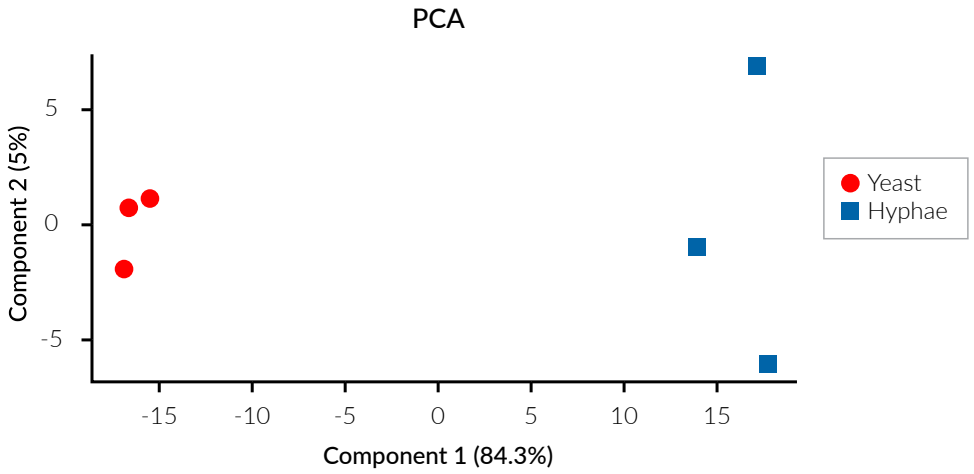


Figure 5. Principal Component Analysis of the two different phenotypes. The PCA based on the first and the second component, coloured by strains shows a discrimination between Yeast and Hyphae on the first component with a variance of 84.3%.

A volcano plot analysis confirmed the differential expression and the protein preservation during the extraction: 65 proteins are up-regulated and 130 are down regulated in the Hyphae type (***Figure 6***).

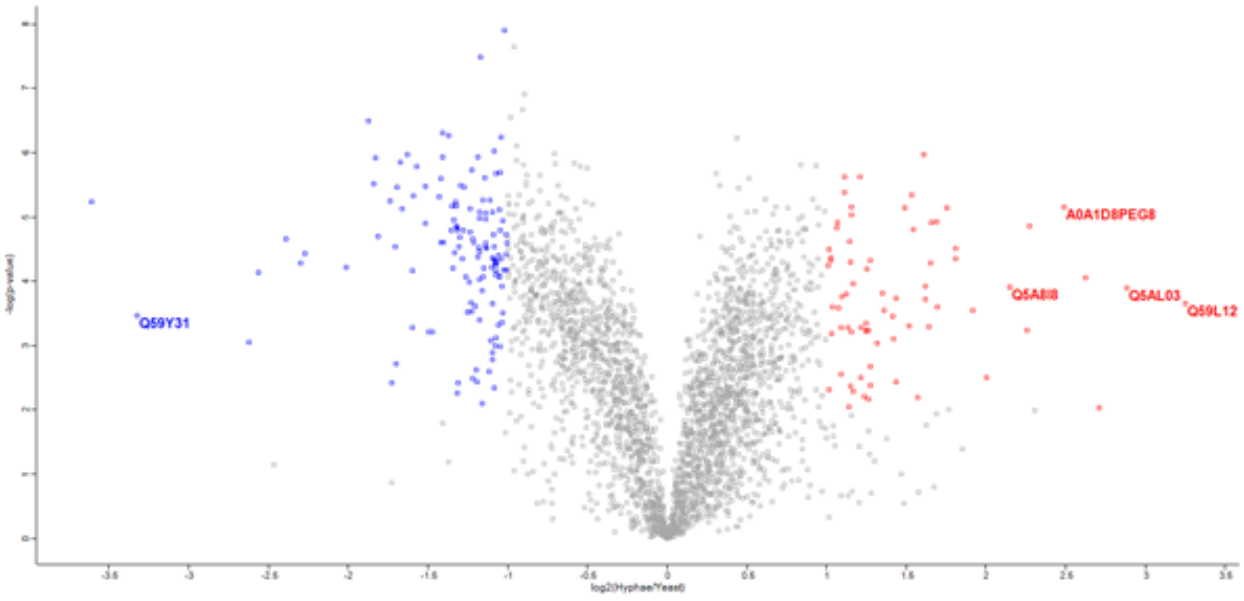


Figure 6. Differential expression between Yeast and Hyphae subtypes. Significantly differentiated proteins are defined as those with a Benjamini-Hochberg adjusted p-value < 0.01 and a log2 ratio exceeding ± 1. In Hyphae, proteins in red are up-regulated and proteins in blue are down-regulated.

Those results are in line with previous observations. For example, Agglutinin-like protein 3 (ALS3, Q59L12), Hyphally regulated cell wall protein 1 (Q5AL03), and Induced during Hyphae Development Protein 1 (Q5A8I8) are known to be induced during Hyphae formation. Of particular interest, ALS3 is a key protein in the adherence of *C. albicans* hyphae to epithelial cells [12]. On the other hand, Yeast-form wall Protein 1 (Q59Y31) undergoes a downregulation under filamentation [13].

Conclusion

As the analytical methods of proteomic analysis have become more sensitive, it has opened the field for the utilization of limited and difficult sample types. The proposed workflow combines some of the most advanced preparation techniques for proteomics. The precise energy and thermal control of Covaris AFA allows for an isothermal and reproducible protein extraction from challenging samples like yeast cells making it an ideal mechanical extraction technology for applications where reproducible pre-analytical sample preparation is critical. It is fully compatible with single pot, automation-oriented clean-up approaches like SP3, and with TMT-based multiplexing. Covaris Focused-ultrasonicators are proven to be an extremely robust and flexible solution for protein processing for any throughput and format, regardless of buffers and clean-up methods.

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