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TITLE:

Preparation of cell extracts by cryogrinding in an automated freezer mill

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KEYWORDS:

Freezer mill, cryogrinding, cryogenic grinding, freezer grinding, freezer milling, cryomilling, protein purification, whole cell extract, cell lysate, yeast extract, yeast popcorn, liquid nitrogen (LN_2), immunoprecipitation, forensic, remains, fossil, extinct, lifeform, permafrost, archeological.

SUMMARY:

We describe a reliable method for the preparation of whole cell extracts from yeast or other cells using a cryogenic freezer mill that minimizes degradation and denaturation of proteins. The cell extracts are suitable for purification of functional protein complexes, proteomic analyses, co-immunoprecipitation studies and detection of labile protein modifications.

ABSTRACT:

The ease of genetic manipulation and the strong evolutionary conservation of eukaryotic cellular machinery in the budding yeast *Saccharomyces cerevisiae* has made it a pre-eminent genetic model organism. However, since efficient protein isolation depends upon optimal disruption of cells, the use of yeast for biochemical analysis of cellular proteins is hampered by its cell wall which is expensive to digest enzymatically (using lyticase or zymolyase), and difficult to disrupt mechanically (using a traditional bead beater, a French press or a coffee grinder) without causing heating of samples, which in turn causes protein denaturation and degradation. Although manual grinding of yeast cells under liquid nitrogen (LN_2) using a mortar and pestle avoids overheating of samples, it is labor intensive and subject to variability in cell lysis between operators. For many years, we have been successfully preparing high quality yeast extracts using cryogrinding of cells in an automated freezer mill. The temperature of -196 °C achieved with the use of LN_2 protects the biological material from degradation by proteases and nucleases, allowing the retrieval of intact proteins, nucleic acids and other macromolecules. Here we describe this technique in detail for budding yeast cells which involves first freezing a suspension of cells in a lysis buffer through its dropwise addition into LN_2 to generate frozen droplets of cells known as “popcorn”. This popcorn is then pulverized under LN_2 in a freezer mill to generate a frozen “powdered” extract which is thawed slowly and clarified by centrifugation to remove insoluble debris. The

resulting extracts are ready for downstream applications, such as protein or nucleic acid purification, proteomic analyses, or co-immunoprecipitation studies. This technique is widely applicable for cell extract preparation from a variety of microorganisms, plant and animal tissues, marine specimens including corals, as well as isolating DNA/RNA from forensic and permafrost fossil specimens.

INTRODUCTION:

Yeast is a popular model organism for protein studies, as it is a simple eukaryotic organism with an abundance of genetic and biochemical tools available for researchers[1]. Because of their sturdy cell wall, one challenge that researchers face is in efficiently lysing the cells without damaging the cellular contents. Different methods are available for obtaining protein extracts through disruption of yeast cells which include enzymatic lysis (zymolyase)[2,3], chemical lysis[4], physical lysis by freeze-thaw[5], pressure-based (French press)[6,7], mechanical (glass beads, coffee grinder)[8,9], sonication-based[10] and cryogenic[2,11]. The efficiency of cell lysis and the protein yield can vary considerably depending on the technique employed, thus affecting the end result or suitability for the desired downstream application for the lysate. When studying proteins that are unstable, have fleeting posttranslational modifications, or are temperature sensitive, it is particularly important to use a method that will minimize sample loss or degradation during preparation.

[Place Table 1 here]

Cryogrinding (aka cryogenic grinding/cryogenic milling) is commonly employed to retrieve nucleic acids, proteins or chemicals from temperature sensitive samples in a reliable manner for quantitative or qualitative analyses. It has been used successfully for diverse fields of application including biotechnology, toxicology, forensic science[12,13], environmental science, plant biology[14] and food science. Isolation of intact biological macromolecules is usually critically dependent on the temperature. Extremely low temperatures ensure that the proteases and nucleases stay inactive, resulting in a reliable isolation of intact proteins, nucleic acids and other macromolecules for subsequent analyses. Indeed, a freezer mill typically maintains a sample temperature of -196 °C (the boiling point of LN_2), thus minimizing DNA/RNA or protein denaturation and degradation.

The freezer mill employs an electromagnetic grinding chamber that rapidly moves a solid metal bar or cylinder back and forth within a vial containing the sample to be pulverized between stainless steel end plugs. The instrument creates and rapidly reverses a magnetic field within the grinding chamber. As the magnetic field shifts back and forth, the magnet crushes the sample against the plugs thus achieving the 'cryogrinding' and the pulverization of the popcorn. The freezer mill replaces the mortar and pestle and allows the sequential processing of multiple samples (or up to 4 smaller samples simultaneously) with high reproducibility and avoids the user to user variability associated with manual grinding. Once the samples are processed, the cell extracts can be used for a variety of downstream applications.

PROTOCOL:

1. Preparation of Yeast Popcorn

1.1. Grow yeast cells in 0.5 L of YPD media to a density of 1 x 10^7 cells/mL. Count cells using a Coulter Counter or any other means.

1.2. Centrifuge cells for 10 min at 2,400 x g and 4 °C.

1.3. Wash each sample once with 500 mL of ice-cold deionized 18 mega Ohm Milli-Q water.

1.4. Resuspend pellets in 15 mL of ice-cold Lysis Buffer (20 mM HEPES-KOH [pH 7.5], 110 mM KCl, 0.1% Tween, 10% glycerol, with freshly added reducing agent 10 mM β-mercaptoethanol, protease inhibitor cocktail), 10 μM proteasome inhibitor MG-132, 1 mM deacetylase inhibitor sodium butyrate,) and phosphatase inhibitors (1 mM sodium vanadate, 50 mM sodium fluoride, 50 mM sodium β-glycerophosphate). See the **Table of Materials** for additional details. Keep resuspended cells on ice until the samples are ready for the next step.

NOTE: A wide variety of lysis buffers containing a number of non-ionic detergents can be used with the samples and specific inhibitors included in it based on the downstream application. Inclusion of protease and proteasomal inhibitors are crucial for preventing protein degradation, especially once the extracts are thawed.

1.5. Make snap frozen yeast popcorn by slowly adding the cell suspension one drop at a time using a pre-chilled serological pipette into one or more 50 mL centrifuge tubes kept on dry ice and filled with LN_2 until just below the rim. Top up liquid nitrogen in the tube frequently to compensate for its loss due to rapid evaporation. Work in a well-ventilated area to avoid the hazards associated with nitrogen asphyxiation.

1.5.1. Instead of a 50 mL tube, use multiple 50 mL tubes tied together (or any container with a large mouth, such as a large plastic centrifuge bottle that can tolerate cryogenic temperatures) for pop-corn preparation **(Figure 1A)**. The larger the container and its opening, the easier is the popcorn preparation as this prevents clumping of the popcorn to form large aggregates. A size range of 0.3-0.5 cm for the popcorn is ideal for achieving proper grinding/pulverization using the freezer mill (**Figure 1B**).

1.5.2. Ensure that the LN_2 has evaporated completely from the tubes containing the popcorn prior to storing them at -80 °C. It is possible to stop after preparing the yeast popcorn as they are stable for several years when maintained at -80 °C.

[Place Figure 1 here]

2. Cryogrinding

2.1. To start, ensure that there is enough LN_2 available to chill the freezer mill, grinding vials, supplies and to operate the machine for all samples. For less than five samples, 30-35 L of LN_2

should suffice. Fill the freezer mill chamber with LN_2 up to the fill line.

WARNING: All steps involving LN_2 should be performed in a well-ventilated area and the freezer mill itself should be located in such an area to avoid risk of asphyxiation. Wear personal protective equipment including proper footwear, lab coat, safety glasses, a base layer of nitrile gloves, then thermal gloves, followed by another pair of nitrile gloves. Use extreme caution when handling LN_2.

2.2. Close the freezer mill lid slowly, avoiding the splash of LN_2 and allow a few min for the machine to cool down. It may be necessary to refill the chamber with LN_2 to compensate for the loss due to evaporation before proceeding to the next step.

NOTE: Refill LN_2 as needed only up to the fill line. Do not overfill the chamber as this can be both dangerous and detrimental to the machine. Automated refill systems are available for freezer mill that can replenish the evaporating LN2 as needed directly from a storage tank connected to the freezer mill.

2.3. Pre-chill the large grinding vials and the magnetic impactor bar by dunking them in LN_2 kept in a separate small dewar. Decant all the LN_2 when the LN_2 stops bubbling. Then add the sample/yeast popcorn to the grinding vial and seal it tightly with the two stainless steel end plugs.

2.3.1. Do not fill more than one-third of the grinding vial with the sample as that can reduce the efficiency of grinding. Instead, larger samples can be processed by dividing them into two or more vials and grinding them sequentially. Smaller grinding vials can be used for smaller samples (up to 3 mL).

2.4. Place the grinding vial in the freezer mill chamber and lock it in place. Close the lid.

2.5. For budding yeast, grind the samples for a total of three cycles for 2 min/cycle (with 2 min break for cooling between cycles) at a crushing rate of 14. When the machine stops after completing the cycles, open the freezer mill lid slowly and carefully unlock the vial with the powdered frozen cell lysate and remove it from the freezer mill. If multiple small vials are used, work quickly to remove one vial at a time and place them in dry ice.

NOTE: There is usually no need to adjust the grinding parameters when using vials of different sizes with the same type of sample. However, the grinding parameters such as the number of cycles and the grinding rate need to be empirically determined for different sample types, depending on the ease with which they lyse. Most mammalian cells and soft tissues will lyse in 1-2 cycles at a crushing rate of 10. Harder to lyse samples such as bacteria, yeast, fly larvae and adult fruit flies require 3-6 cycles at the maximum rate, while hard tissues such as bone, teeth, etc. may require up to 10 cycles. A small aliquot of the thawed sample can be viewed under the microscope before and after grinding to count the number of intact, unlysed cells to determine the lysis efficiency. An excellent analysis of the impact of cryogrinding parameters on the release of proteins and DNA from budding yeast has been published previously[15].

2.6. Working quickly so as not to allow the sample to thaw out, carefully unscrew one of the end pieces (pick the one that appears to have become somewhat loose during the grinding) using the opening tool. Then, use a pair of long forceps (that are pre-chilled in a bucket with dry ice) to remove the impactor bar. Collect the pulverized sample by inverting and tapping the grinding vial onto a polystyrene weighing dish pre-chilled with LN_2 and kept on dry ice.

2.7. Once all of the powdered frozen cell lysate has been recovered from the grinding vial, pour the powdered lysate from the weighing dish back into the 50 mL tube and proceed immediately to the slow thawing step described next for best results. Alternatively, store the frozen powdered lysate overnight at -80 °C, although this may result in some degradation.

2.8. Prepare an ice bucket with a 50% slurry of ice and water and place it on a stir plate with a magnetic stirrer. Submerge a wire rack or another suitable rack in the ice slurry to hold the samples.

2.8.1. Then, slowly thaw the samples on an ice slurry bath with constant agitation of the slurry using a magnetic stirrer. Add more ice to replace the melting ice (some water may need to be discarded to prevent the ice bucket from overflowing). Since several inhibitors have short half lives in aqueous buffers, add additional protease inhibitor cocktail (see **Table of Materials**) and 10 μM proteasome inhibitor MG-132 to the lysate once the samples start thawing (after approximately 30 min).

2.8.2. Remove ice formed on the outside of the tubes every 5 min to expedite the thawing process. Note that rapid thawing of the samples at room temperature or higher can lead to significant degradation.

2.9. After the samples have thawed completely (it may take well over an hour depending on the amount of sample), centrifuge the lysate at ~3,220 x g for 20 min in a refrigerated tabletop centrifuge at 4 °C to remove the bulk of the cell debris from the lysate.

2.10. Transfer the supernatant to 50 mL polycarbonate centrifuge tubes that have been pre-chilled on ice and centrifuge the samples at 16,000 x g for 20 min at 4 °C (see **Table of Materials**).

2.11. Transfer only the clear supernatant consisting of the clarified whole cell extract from the center of the liquid column in the tube very slowly and carefully, without disturbing the cloudy lipid layer (**Figure 2**), into a chilled 15 mL tube using pre-chilled serological pipettes. Avoid removing all of the lysate in the centrifuge tube to prevent carryover of lipids and debris. The leftover lysate in the centrifuge tube can be centrifuged multiple times to recover small amounts of extract after each spin.

NOTE: Lipids along with any denatured proteins would appear cloudy/milky and are located at the top of the liquid/air interface, and occasionally above the pellet itself that consists of insoluble cellular debris (**Figure 2**).

[Place Figure 2 here]

2.12. Centrifuge any remaining lysate for 5 min to recover more of the extract. Repeat this 5 min centrifugation multiple times if needed to recover as much of the clear extract as possible. Discard the cloudy lysate containing lipids close to the bottom of the tube near the pellet.

2.13. Use these extracts for downstream applications such as protein complex purification, immunoprecipitation and proteomic analysis.

REPRESENTATIVE RESULTS:

We compared two different methods for yeast cell lysis, namely glass bead milling at 4 °C and an automated cryogrinding method at -196 °C, to assess the relative recovery proteins in the cell extracts prepared with both methods. For this study, we chose to use a budding yeast strain YAG 1177 (MAT a lys2–810 leu2–3,-112 ura3–52 his3-Δ200 trp1–1[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 [pUB39] [pUB221])[16] carrying a high copy plasmid expressing a tandem HIS-MYC tagged Ubiquitin (HIS-MYC-Ub) so we can assess the efficiency of recovery of tagged ubiquitinated proteins from extracts following affinity purification.

We tested a quick protocol that uses glass bead milling to lyse yeast cells[9] at 4 °C and the cryogrinding method using frozen yeast cells (yeast popcorn) and grinding them in LN_2 in a freezer mill. We report a clear difference with the two methods of extract preparation.

In **Figure 3A** we demonstrate that we can achieve a decidedly higher total protein yield from whole cell extracts (WCE) prepared using freezer grinding as determined by Ponceau S staining after loading equivalent amounts of WCE prepared from the same number of cells using the two different extract preparation methods. Further, **Figure 3B** shows higher recovery of HIS-MYC tagged ubiquitylated proteins following pull down using Talon beads from the WCE prepared by cryogrinding, when compared to the WCE prepared using the bead beater, despite using extracts prepared from the same number of cells in both cases.

[Place Figure 3 here]

Overall, the representative results show that cryogrinding of yeast popcorn leads to higher yields and superior protein isolation based on the stronger Ponceau stained protein bands in the WCE prepared by cryogrinding (**Figure 3A**). This is likely due to more efficient lysis and limited protein degradation in the extract prepared by cryogrinding, as indicated by the presence of stronger bands in the Western blot lane showing HIS-tagged proteins pulled down (IP) from the cryogenic extract (**Figure 3B**). While the glass bead milling protocol is faster and less labor intensive, it may not be suitable for proteins that are temperature sensitive or highly unstable. We also find that the isolation of posttranslational modifications such as polyubiquitinated proteins is more efficient with the cryogrinding protocol.[16-18]

FIGURE AND TABLE LEGENDS:

Table 1: Comparison of methods available for the preparation of yeast extracts.

Figure 1: Yeast popcorn preparation. (A) Yeast "popcorn" is made by the dropwise freezing of the cell suspension in LN_2. We use one to three 50 mL tubes held together with a rubber band and placed in an ice bucket filled with dry ice. The tubes are filled with LN_2 until just below their rims and are topped up with liquid nitrogen frequently to keep them nearly full until all the cell suspension had been made into popcorn (**B)** The size of the popcorn is an important determinant of optimal grinding efficiency. The size range of the popcorn should be between 0.3 and 0.5 cm in diameter.

Figure 2: Extract layers in a centrifuge tube. The major visible features of the whole cell extract in a tube following centrifugation at 16,000 g for 20 min are indicated. The relative abundance of each feature depends on the sample type, the growth phase of the cells (exponential versus stationary), the amount of lysis buffer used to resuspend cells and the lysis efficiency.

Figure 3: Cryogrinding leads to higher total protein yields in cell extracts and better recovery of tagged proteins following pull down of proteins from these extracts. (A) Whole cell extracts (WCE) were prepared as described in this protocol for the cryogrinding or as previously described for the bead milling method.[9] 500 mL of the yeast strain carrying HIS-MYC-Ub was grown to density of 10^7 cells/mL. Then 250 mL of the culture was used in parallel to prepare extracts by each method. Equivalent amounts of WCE prepared from the same number of cells using the two different methods, as well as HIS tagged proteins pulled down using Talon beads (IP) from the same amount of each extract were compared by resolving on a denaturing polyacrylamide gel and transferring to a nitrocellulose membrane followed by staining with Ponceau S. Notice the stronger staining throughout the lane containing WCE prepared using the freezer grinding method. **(B)** The Ponceau S stained nitrocellulose membrane shown in (A) was then processed for Western blotting using HIS tag antibodies to compare the amounts of HIS-Ub tagged proteins pulled down from equal amounts of extract prepared by the two methods. Several bands were clearly overrepresented in the pulldown using the WCE prepared by freezer grinding (indicated by the red arrows), while the corresponding bands in the pulldown using WCE prepared by bead beating were either considerably weaker in intensity, or undetectable.

DISCUSSION:

A limitation of studying native proteins from yeast is the efficient lysis of yeast cells due to the integrity of their cell wall. Although several methods have been developed, the most consistent and efficient method in our hands is the cryogrinding of yeast cells flash frozen as popcorn. This method allows the reliable preparation of high-quality whole cell extracts from budding yeast compared to other lysis methods. The representative results demonstrated that cryogrinding is superior to a popular mechanical method for yeast lysis that employs bead milling at 4 °C (**Figure 3A and 3B**). Cryogrinding efficiently lyses yeast cells while the samples are processed at a temperature of -196 °C in a LN_2 bath, therefore maintaining protein integrity by greatly limiting heat induced protein denaturation and degradation that occurs with many other methods of extract preparation. This allows for the analysis of highly labile macromolecules in the samples and permits the purification of functional protein complexes. Further, the use of the freezer mill

also replaces the highly labor-intensive traditional manual grinding of yeast popcorn with a mortar, allowing for faster and efficient sample processing. Finally, the achievement of yeast cell lysis without the use of any expensive enzymes provides for significant savings in the long run.

The major downside of a freezer mill is the upfront cost involved in purchasing the equipment, as well as the minimal maintenance and running costs (i.e., large quantities of liquid nitrogen) involved. It also requires careful handling of potentially dangerous LN_2 for both the preparation of yeast popcorn and the cryogrinding steps. Larger samples need to be processed individually, which may become time consuming if multiple samples need to be processed. In addition, when processing multiple samples, the grinding vials, caps and impactor bars must be properly washed and wiped clean before re-use (alternatively, several sets of spare accessories must be maintained).

The protocol demonstrated here is valuable for the preparation of yeast protein samples for protein affinity purification, immunoprecipitation studies, sample preparation for Western blotting for labile proteins or their modifications, or for proteomic analyses[16,19]. In addition, cryogrinding of the yeast popcorn can also be utilized for sheared genomic DNA[13,20,21] or RNA[22,23] isolation while minimizing nuclease activity, thus ensuring sample preservation[24]. This versatile method can be used as is for most microorganisms such as bacteria[21,22] and yeasts[15], and can be quickly adapted for mammalian cells, both plant[25] and animal[26] tissues, food products[27], marine specimens[21,28] including corals[23], forensic samples[20] including hair[29] and even fossils[30] as well as the remains of extinct lifeforms frozen in permafrost[31].

In conclusion, cryogrinding in a freezer mill is an efficient method for protein and other macromolecule isolation from a variety of samples derived from diverse sources for multiple downstream biochemical applications.

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DISCLOSURES:

The authors have nothing to disclose.

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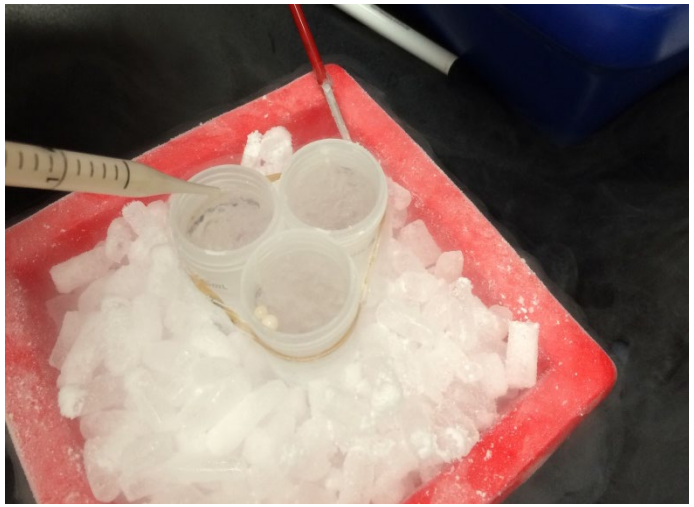
Figure 1

A



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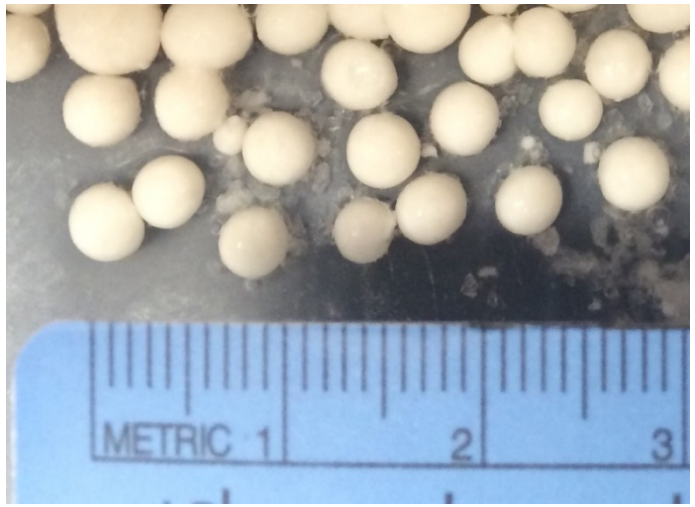


Figure 2

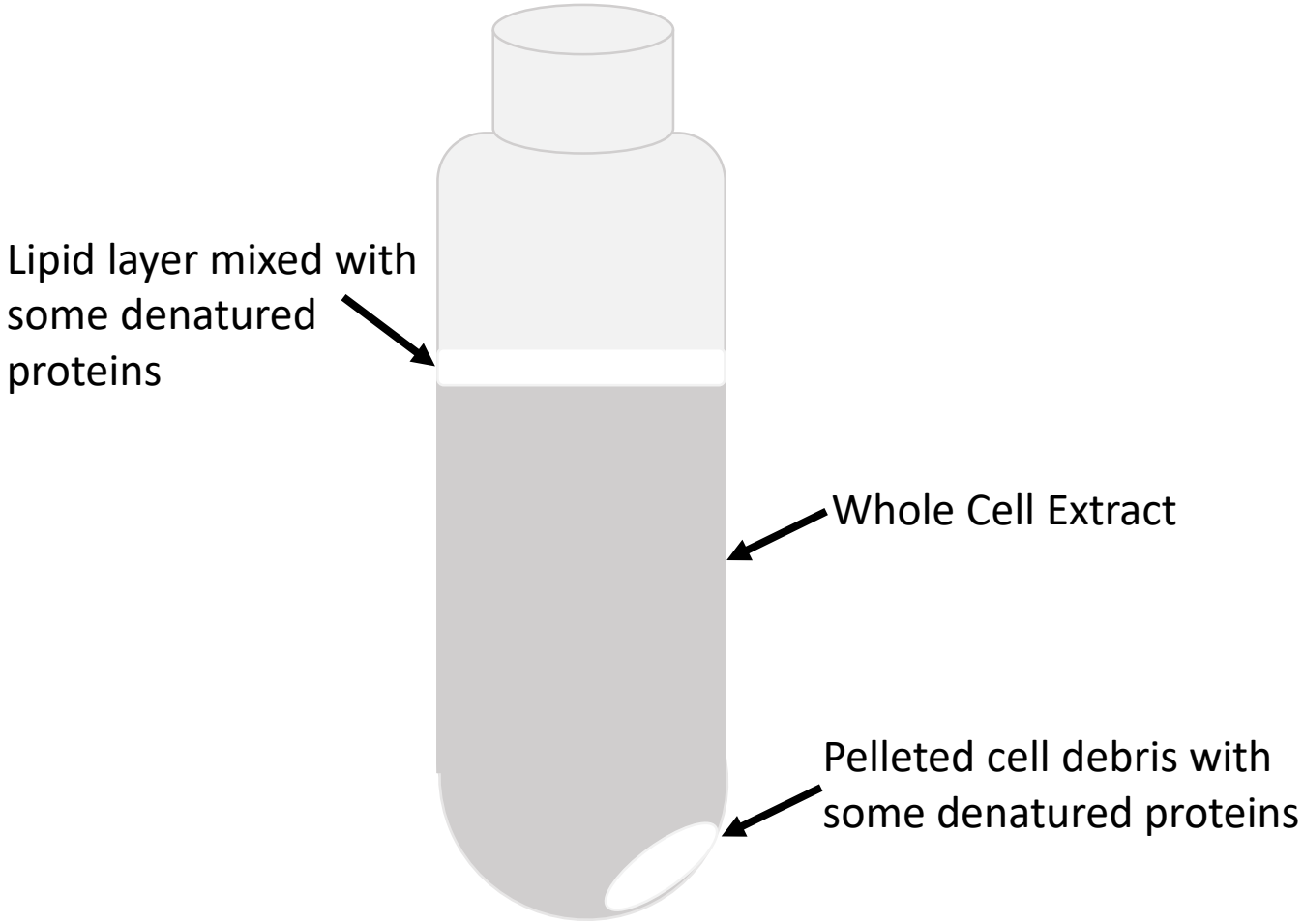
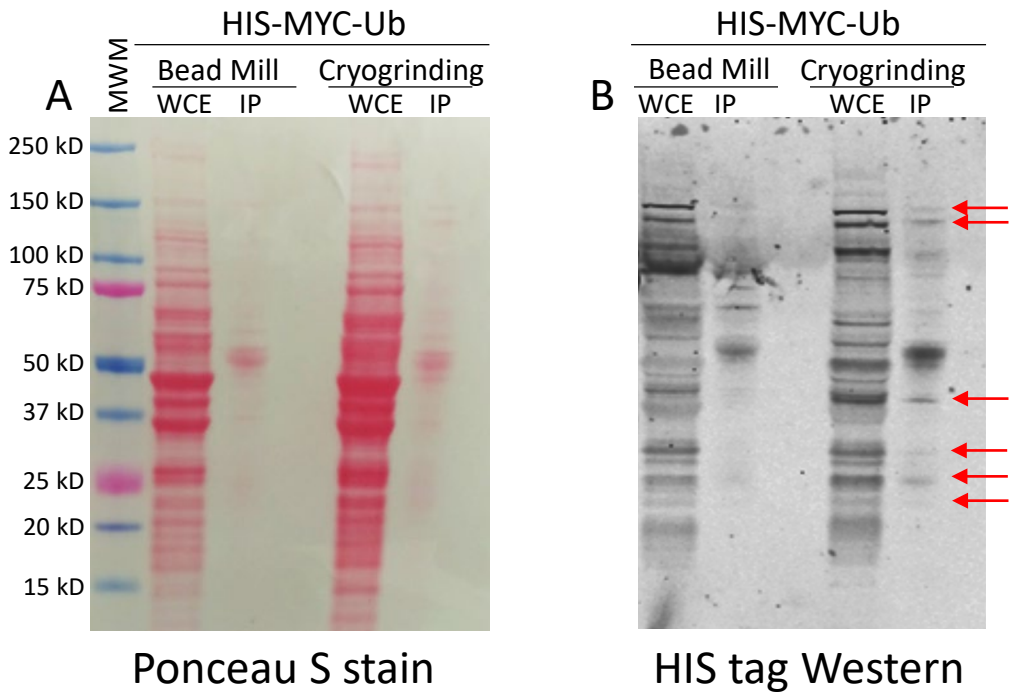

Lipid layer mixed with some denatured proteins
Whole Cell Extract
Pelleted cell debris with some denatured proteins

Figure 3



Ponceau S stain

HIS tag Western

Table 1: Comparison of methods available for the preparation of yeast extracts

Extract preparation techniques	Characteristics	Advantages	Disadvantages	Downstream analysis	Reference
French press: High-pressure homogenizer (aka Microfluidizer) with enzymatic pretreatment using Zymolyase	Zymolyase-20T, a Microfluidizer high-pressure homogenizer (model MllOT with extra heavy duty pump; Microfluidics Corp., Newton, MA., U.S.). The disruptor consists of an air-driven, high-pressure pump (ratio 1:250; required air pressure 0.6-l MPa) and a special disruption chamber with an additional back pressure unit. A minimum sample size of 20 mL is required for processing.	Final total disruption obtained using the combined protocol approached 100 % with 4 passes at a pressure of 95 MPa, as compared to only 32 % disruption with 4 passes at 95 MPa using only homogenization without the Zymolyase.	Not appropriate for small scale applications. The enzymes can get expensive for large scale preparations.	Protein purification	6
Bead beater: Zymolyase treated cells lysed with glass beads in a	Roughly equal volume of cold, dry, acid-washed 0.5 mm glass beads are added to a given volume of cell pellet in lysis buffer and the cells are disrupted by vigorous manual agitation.	It is particularly useful when making extracts from many different small yeast cultures for assaying purposes rather than for protein purification.	During the glass bead procedure, proteins are treated harshly causing extensive foaming leading to protein denaturation. The amount of cell breakage varies, while proteolysis as well as modification of the proteins may result from heating of the extract above 4°C during the mechanical breakage.	Mostly DNA & RNA analyses, but also protein analysis by denturing gel electropheoresis, either with or without Western blotting.	8

Table 1: Comparison of methods available for the preparation of yeast extracts

Zymolyase treatment followed by lysis using a combination of osmotic shock and Dounce homogenization	After enzymatic digestion of cell walls, spheroplasts are lysed with 15 to 20 strokes of a tight-fitting pestle (clearance 1 to 3 µm) in a Dounce homogenizer.	Advantageous to use protease-deficient strains such as BJ926 or EJ101. This is the most gentle way to break yeast cells and hence it is most suitable for preparing extracts that can carry out complex enzymatic functions (e.g., translation, transcription, DNA replication) and in which the integrity of macromolecular structures (e.g., ribosomes, splicesomes) has to be maintained. It is also useful for isolating intact nuclei that can be used for chromatin studies (Bloom and Carbon, 1982) or for nuclear protein extracts (Lue and Kornberg, 1987).	The major disadvantages of the spheroplast lysis procedure are that it is relatively tedious and expensive, especially for large-scale preparations (>10 liters), and the long incubation periods can lead to proteolysis or protein modification. For chromatin preparations, they seem to be of varying or lower quality than those produced by the differential centrifugation (based on nucleosome ladder integrity).	Isolating intact nuclei for chromatin studies, extracts that can carry out complex enzymatic functions, extracts requiring the integrity of macromolecular structures, nuclear protein extracts.	2

Table 1: Comparison of methods available for the preparation of yeast extracts

Cell Disruption of flash frozen cells by grinding in Liquid Nitrogen using a mortar/pestle or a blender	Cells are frozen immediately in liquid nitrogen and then lysed by grinding manually in a mortar using a pestle, or using a Waring blender in the presence of liquid nitrogen.	The protocol is quick and easy. It can accommodate varying amounts of yeast cells including very large cultures. Its main advantage is that cells are taken immediately from the actively growing state into liquid nitrogen (−196°C), decreasing degradative enzyme activities such as proteases and nucleases as well as activities that modify proteins (e.g., phosphatases and kinases). It is particularly suited for making whole-cell extracts from a single yeast culture for large-scale protein purification.	A bit messy and potentially dangerous to the careless investigator. Small samples (i.e., 10- to 100-ml yeast cultures) are not easily processed because there is not enough mass of frozen cell clumps to fracture effectively in the blender. It is time-consuming to process individual samples and to clean the equipment between uses.	Whole-cell extracts from a single yeast culture for large-scale protein purification.	2

Table 1: Comparison of methods available for the preparation of yeast extracts

Autolysis, Bead mill	pH 5.0, 50 °C, 24 h, 200 rpm / Ø 0.5 mm, 5 × 3 min/3 min	Quick and efficient lysis, especially for small scale extract preparation	Heat generation leads to denaturation and degradation of macromolecules. Bead beating equipment required.	Small scale analyses.	10
Autolysis, Sonication	pH 5.0, 50 °C, 24 h, 200 rpm, 4 × 5 min/2 min, pulser 80%, power 80%	Sonication equipment is usally available in most institutions.	Heat generation leads to denaturation and degradation of macromolecules. Sonication equipment required. Slow lysis can take more than 24 hours.	Yeast cell wall preparations.	
Boiling and freeze-thaw process	No specilized equipment needed other than a standard freezer and a heating block or hot water bath.	Efficient, reproducible, simple and inexpensive.	Heat generation leads to denaturation and degradation of macromolecules.	DNA analyses by PCR.	5

Table of Materials

Click here to access/download;Table of Materials;JoVE Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50 mL polycarbonate tubes with screw caps	Beckman	357002	Centrifuge tubes
BD Bacto Peptone	BD Biosiences	211677	Yeast YPD media component
BD Bacto Yeast Extract	BD Biosiences	212750	Yeast YPD media component
Beckman Avanti centrifuge	Beckman	B38624	High speed centrifuge
Beckman JLA-9.1000	Beckman	366754	Rotor
D-(+)-Dextrose Anhydrous	MP Biomedicals	901521	YeastYPD media component
Eppendorf A-4-44	Eppendorf	22637461	Swinging bucket rotor
Eppendorf refrigerated centrifuge 5810 R	Eppendorf	22625101	Refrigerated centrifuge
Glycerol	SIGMA-ALDRICH	G5150-1GA	Volume excluder and cryoprotectant
HEPES	FisherBiotech	BP310-100	Buffer
HIS6 antibody	Novagen	70796	Antibody for HIS tag
KCl	SIGMA-ALDRICH	P9541-1KG	Salt for maintaining ionic strength
MG-132	CALBIOCHEM	474790	Proteasome Inhibitor
Phosphatase inhibitor cocktail	ThermoFisher Scientific	A32957	Phosphatase inhibitor cocktail
Ponceau S	SIGMA	P7170-1L	Protein Stain
Protease inhibitor cocktail	ThermoFisher Scientific	A32963	Protease inhibitor cocktail
Rotor JLA 25.500	Beckman	JLA 25.500	Rotor
Sodium Butyrate	EM Science	BX2165-1	Histone Deacetylase Inhibitor
Sodium Fluoride	Sigma-Aldrich	S6521	Phosphatase Inhibitor
Sodium Vanadate	MP Biomedicals	159664	Phosphatase Inhibitor
Sodium β-glycerophosphate	Alfa Aesar	13408-09-8	Phosphatase Inhibitor
Spex Certiprep 6850 freezer mill	SPEX Sample Prep	6850	Freezer Mill
TALON Metal Affinity Resin	BD Biosiences	635502	For pulling down HIS tagged proteins
Tween 20	VWR International	VW1521-07	Non-ionic etergent
β-Mercaptoethanol	AMRESCO	M131-250ML	Reducing agent

REBUTTAL

Dear editors and reviewers,

Thank you for your valuable feedback on our manuscript. We found the detailed feedback to be very useful in revising our manuscript. We have addressed all the concerns/comments and made all the requested changes in the text of the manuscript as well as the video as detailed below in blue font.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have done this to the best of our ability.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) All your steps need rewriting. For example, “Yeast cells are grown in 0.5 liters of YPD media…” should be “ Grow yeast cells in 0.5 liters of YPD media…”

We have made changes throughout the manuscript to fix this issue.

• **Protocol Detail:** Please ensure that all specific details from the video are mentioned in the manuscript (the manuscript should be able to serve as a standalone article). Please ensure homogeneity between the video and the manuscript.
1) 1.1: mention centrifuge speeds in g.

This has been fixed now.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have revised the discussion section to adhere to the format suggested above.

• **References:**
1) Please move weblinks (line 73, 118, 175) to the reference list and use superscripted citations.
2) Please spell out journal names.

We have fixed both these issues.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your

manuscript are Beckman Avanti, JLA 9.1000, Spex Certiprep 6850, Beckman polycarbonate tubes (Ref#357002), JLA 25.500, SPEX CertiPrep 6850 freezer mill etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have removed all brand names and trademarks from the manuscript.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures are original.

Video Comments:

Audio / Visual Quality:
• Please reduce the overall volume by 3 dB
• 03:40 There is a single frame at the beginning of the dissolve from the incoming clip that is flashing as the CU of the "popcorn" fades out.

We have corrected this.

Video Format:
• 12:22 Conclusion - this section contains the Representative Results, which should be its own separate section apart from the Conclusion. Please give it its own standalone chapter title card and move the Conclusion title card to a point where you think you've moved past talking about the Results and more generally about the procedure to conclude the video.

We have fixed this issue.

VO Coverage & Performance:
• 00:00 Consider giving the speaker a lower third (a name caption that appears near the bottom of the frame) and also, if you can, you may want to do another take of this. While this performance is very good, there are a couple of spots that have a jumbled performance. Ensure that this is the best take and consider some editing if you have a better performance. Use a light touch, as this is pretty good as-is.

This has been fixed now.

"• 02:35 Misspoke: "heat generization" should be "heat generation" and "regarding" should probably be "regardless" (if you mean that the method is scalable without regard to the number of cells lysed)

This is the line in question:
"It is essential to use as easily scalable lysis method that will minimize heat generation while allowing for efficient lysis regardless of the quantity of cells."

We have fixed this issue.

• 03:10 a bit of a jumbled performance here. "Premafrost" should be "permafrost". Please re-record this line:
"This method can be reliably adapted for the use of any imaginable type of species or biological sample, including: bacteria, yeast, worms, fruit flies, and mammalian tissues; as well as forensics or even ancient samples recovered from archeological sites or found frozen in *perma*frost."

This has been fixed now.

• You may want to consider re-working the narration content from 02:43 to 03:55 as you are a bit redundant when you say @03:51 "and produces functional macromolecules that can be used for a variety of downstream purposes". Being more concise is a good thing for these videos, so if you catch the narration repeating itself, go ahead and chop it out and compress this section a little bit so we can get to the meat of the video- the protocol.

We have rephrased the narration for this part.

• 09:53 a bit of a jumbled performance here. Consider re-recording this line. "Recover as much of the frozen extract as possible by banging the vial on the wooden breadboard"

The line has been re-recorded.

• 12:41 "For this study" There are people heard speaking in the background. This line should be re-recorded in a quiet place.

This has been fixed now.

• Please upload the revised video here:
https://www.dropbox.com/request/3bAewut05HoUphqJN3PN?oref=e

New video has been uploaded now.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
Review of Giovinazzi et al.: "Preparation of cell extracts by cryogenic grinding in a freezer mill" (JoVE61164)

This paper describes a good protocol for the preparation of whole cell extracts from budding yeast that

is particularly reliable for obtaining intact proteins, a main goal for those working with these molecules. The method is based on the cryogenic grinding of yeast cells, which helps to prevent the degradation of proteins, and uses an automated freezer mill to provide high reproducibility. This method can be applied to different studies such as protein complex purification, proteomic analyses or co-immunoprecipitation, among others, and is therefore very useful and of interest to many labs.
The protocol is nicely described in the manuscript and also in the video, which is accurate, clear and easy to follow. Both the manuscript and the video give enough detail for the experiments, and in my opinion the method can be reproduced without difficulty by following the instructions of the authors. Taking all into consideration, I clearly recommend the publication of this paper in JoVE. I would only suggest a few minor corrections to the manuscript as indicated below:

Minor Concerns:
- I would write "min" instead of "minutes" and "L" instead of "liters". These appear in different places of the text. I think it is better to use international units in a scientific text.

These have been corrected.

- 1.2. Is the water used ddH2O or Milli-Q? If so, it should be indicated.

The water used was deionized 18 mega Ohm Milli-Q and this has been corrected.

- 1.3. The authors write "1X Lysis Buffer" when describe it. It is unnecessary to mention "1X" , as a buffer is "1X" unless otherwise indicated.

The redundant 1X have been deleted.

- 1.3 and 2.8. They use "1X protease inhibitor cocktail". When they write "1X", do they mean one tablet? What type of inhibitor cocktail do they use?

The Pierce protease inhibitor cocktail was used at a final concentration of 1X, after diluting form a freshly made 10X stock solution.

- 1.4. The authors write: "The popcorn samples are stable for several years when stored properly at -80 ºC". What do they mean by "properly"? The reader could understand a needed action or a particular way of preserving the samples in addition to keep them at -80 ºC.

We have clarified this by replacing “properly” with “maintained” at 80 ºC. As long as the samples are maintained frozen at 80 ºC and not allowed to thaw out as a result of the freezer door being left open for too long or during the process of defrosting the freezer, the samples are fine for years.

- I would leave an space between numbers and units when appropriate. For instance, I would write "0.5 cm" instead of "0.5cm", or "50 mL" instead of "50mL".

We have corrected this now.

Reviewer #2:
Manuscript Summary:
As stated by the authors, the manuscript and its associated video describe a reliable method for the

preparation of whole cell extracts from yeast using a cryogenic freezer mill. The method minimizes degradation and denaturation of proteins. The cell extracts are particularly suitable for purification of biologically active macromolecules that can be affected by other mechanical extraction methods which do not use liquid nitrogen. Extracts generated should be enriched in large proteins, intact proteins, proteins of less abundance or carrying labile post-translational modifications as well as functional protein complexes. This makes extract used this way suitable for enzymatic assays, proteomic analyses, co-immunoprecipitation studies and detection of labile protein modifications. Additional advantages of using a freezer mill to manual grinding in liquid nitrogen is the rapidity and reproducibility of extracts preparation.

Major Concerns:
I have no concerns that could prevent the publication of the work presented here. This work is an helpful guide on to preparing freezer mill yeast extracts of quality. It is clearly presented, of scientific accuracy, useful, compliant with research standards, of technical quality and efficiency, and will be helpful to researchers who work with yeasts as a model system. I only have minor concerns to be addressed and added to the current manuscript/videos to clarify some specific points.

Minor Concerns:
- Comment on the importance of the lysis buffer used and its composition - it is also an important factor allowing the detection of intact proteins carrying post-translational modifications.

We have now included text to emphasize specific buffer components that are especially crucial.

- The most serious health risk of handling LN2 is asphyxiation due to displacement of Oxygen by Nitrogen gas expansion. This should be stated in the manuscript and the protocol should instruct to manipulating the large amount of LN2 needed to operate the freezer mill in a well ventilated room, possibly equipped of an O2 level monitor.

We have included wording to stress the importance of performing all procedures involving liquid nitrogen in a well-ventilated area.

- Adding a table covering characteristics, advantages and disadvantages of the different extracts preparation techniques that can be use to prepare yeast extracts would be beneficial to the article/video. Both cost involved and the type of investigations that technics allow (because they are leading to different extracts quality) could be included in this table.

A table with the requested information has been included in the revised manuscript.

- Regarding the setting used for freezer mill in step 2.5, it is unclear if the protocol needs to be optimised by researchers for their own purpose/proteins. Is there any value of playing with parameters (time, cycle nb…) to optimise the quality of the extra? Is there a risk of overgrinding? It is also unclear if the setting would have to be changed if vials of different size were used. It would be helpful if this information is known and added to the manuscript. In addition pointers at how to check the quality of the extracts would be of help.

We have added text to clarify the issue of optimizing parameters for different types of samples. Changing vial size for the same sample does not require a change in grinding parameters. Pointers to follow the degree of cell lysis have also been included. An excellent analysis of the impact of

cryogrinding parameters on the release of proteins and DNA from budding yeast has been published previously in Singh, Roy & Bellare et al., 2009.

- The description of the data presented in Figure 2, that compares the quality of the extracts generated with the Freezer mill or the glass bead milling is a very important part of the validation of the techniques. This descritpion should be more specific and its discussion should be expanded. Some points to address are:

We have expanded the description and discussion on this topic in the revised manuscript.

o If Figure 2A demonstrates that the freezer mill produces a higher total protein yield, the authors should clarify that the same volume of WCE (as oppose to same amount of total proteins) has been loaded in this gel. The reader should be looking at the increased signal intensity of the ponceau stain freezer mill extract versus glass bead milling. Could the author provide an estimation of the increase yield? This could be estimated by loading of several volumes of same extract in 3 or 4 lanes.

Indeed, **equivalent amounts WCE prepared from the same number of cells** (determined by counting on a Coulter Counter) using the two different methods were loaded on the gel. Although the yields vary somewhat from experiment to experiment, we typically always see a 1.25 to 2-fold improvement in total protein yields using the freezer mill over other methods. However, the use of Ponceau staining to measure total proteins is not the best way to infer the real quality of the WCE, as the Ponceau signal does not allow us to distinguish intact proteins from partially degraded proteins, or modified proteins from proteins that have lost their posttranslational modifications. Western blotting is needed to get a better idea of the quality of the extracts.

We are unable to perform additional experiments to better quantitate the quality of the extracts since our lab has been mostly closed due to the Covid-19 pandemic since early March. We re-opened with 25% personnel in June, but soon an outbreak in our lab (as well as the periodic worsening of the pandemic in the state of Florida) resulted in a shutdown of lab work again and we do not have a clear idea regarding when we will be allowed back in the lab at full strength.

o Similarly detailed provided to explain Figure 2B, and its discussion are not sufficient for the reader to understand how the authors reached their conclusion. For the HIS IP to assess the lesser level of protein degradation with the freezer mill technique- the same amount of proteins, assessed in the WCEs lanes, should be used in both IPs (as opposed to the same volume of WCEs). Therefore the volumes of WCE lanes should be different Figure 2A versus 2B - and different in the 2 WCE lanes in figure 2B. This does not seem to be the case considering the presentation of the 2 panels and the figure description. This needs to be clarified and better explained.

Figure 2A and 2B correspond to Figure 3A and 3B and the revised manuscript. The two images correspond to the same gel which was transferred to a nitrocellulose membrane for Ponceau staining (Figure 3A) prior to Western blotting with a HIS antibody (Figure 3B). As explained above, equivalent amounts of WCE prepared from the same number of cells using the two different methods were loaded on the gel and used for affinity purification. Doing so may be the only way to compare not only the efficiency of lysis based on the total amounts of proteins extracted from the same number of cells by each method, but also the integrity of proteins in the WCE based on Western blotting for labile proteins and their posttranslational modifications. On the other hand, loading equal amounts of protein obtained by the two methods in this experiment will not be as informative, since it will simply show equal loading

of protein by Ponceau staining, while the Western blotting results would still report any difference in the abundance of specific labile proteins or their posttranslational modifications between the two samples. Consistent with this notion, in the representative experiment shown here, we observe only a slight difference in the total abundance of proteins by Ponceau staining in Figure 3A, while the Western blot shows a bigger difference in the abundance of specific HIS-ubiquitin tagged proteins in Figure 2B, especially following the IP. We have clarified this issue greater detail in the text of the revised manuscript.

o Please add the size of the MW markers use in Figure 2A and 2B. Note that there is a cloudy spot in the WCE/IP lane of Bead mill lanes in panel B that might make the comparison with cryogrinding difficult to make for the reader.

The MW marker sizes have been added. The cloudy spot on the Western is indeed unfortunate, but it only occludes a small part of the lane. The data in the rest of the lane is clearly visible and adequate to highlight the differences that we typically observed between the two methods. We have also converted the Western blot from a colored to a grayscale image in Figure 3B which allows for better visualization of the bands.

- Could authors refer the reader to specific examples (highly labile macromolecules, post-translational modifications or purification of functional protein complexes…), citing references if possible, where the techniques could be used?

Certainly. These references (Singh et al 2009, Singh et al, 2012, Liang et al, 2012) have now been cited in the text. Our reference list has nearly tripled since the initial submission.

- Comments on the video:

o Understanding of the commentary would be easier if the video was subtitled

Adding subtitles would be a considerable undertaking for the audiovisual resources at our institution that are already stretched due to the Covid-19 pandemic. However, we have requested them to do this and are currently in queue for the subtitles to be added. We will upload the sub-titled version of the video when it is ready. In the meantime, we feel that the text of the manuscript accompanying the video provides sufficient details of every step and should easily clarify any issues with the voiceover. We also slightly concerned that the subtitles may detract from the visual details of the process being described via voiceover.

o A couple of formulations are in the commentary are incorrect: "Denaturisation" used in places instead of denaturation, "Celsius degree" instead of degree Celsius.

These have been fixed now.

o There could be a better used of illustrations/ in particular when comparing the different extraction preparation techniques - the slides are a bit static.

We have added a table in the manuscript to better illustrate the differences between the different methods.

o The asphyxiation risk when using LN2 or dry ice should be mentioned

We have added text to emphasize this.

o The use of a specific sample buffer should be commented on

Most lysis buffer will work just fine for lysis using the freezer mill. To facilitate WCE with intact and functional protein complexes, we generally use the mild lysis buffer listed here. It is important to include protease and proteasomal inhibitors to block protein degradation after thawing the lysate, as well as phosphatase inhibitors if studying phosphorylated proteins. Additional inhibitors should be added for the study of other posttranslational modifications.

o Popcorn size maintenance by rotating movement to release drops and the use of small with forceps: Movement is not obvious. Would the use of a container with a wider opening than a 50mL tube make this step easier?

Yes, the use of a wide mouth container would indeed make this step easier and we do use such containers for the pop-corn preparation, especially when larger number of cells are involved. We have added this to the revised manuscript.

Reviewer #3:
The authors provide a valuable step-by-step protocol to generate protein lysates from the budding yeast Saccharomyces cerevisiae. In addition to this widely used model organism, the technique described by the authors will prove valuable to prepare native protein extracts from other model organisms (e.g. the fission yeast Schizosaccharomyces pombe) and fungal pathogens such as Candida and Aspergillus species, among many others. I have a few concerns that need taking care before this manuscript is suitable for publication.

1) My main concern is Figure 2. Based on a blot probed with an antibody against the His tag, the authors claim that there are more His-MYC-ubiquitylated proteins (HMU proteins) when the IP is performed from a whole-cell extract (WCE) prepared by cryogrinding, rather than a WCE generated with a bead mill (commonly known as a bead beater). If I squint the right way, I can see a few bands that are detectable in the IP from cyogrinding cells, but "seemingly absent" in the IP from cells broken with a bead beater (the bands are already very weak in the IP from cryoground cells; they may well be just below the detection threshold). Conversely, I see at least two bands that are present in the IP derived from a WCE prepared from a bead beater, but those bands are below the threshold for detection in the IP from the WCE obtained cryoground cells. Perhaps the exposure of the blot that I have is not optimal, but Figure 2B is far from convincing that cryogrinding is better than bead beating in that specific application. I would imagine that the authors use the freezer mill routinely and, therefore, should be able to provide more convincing evidence that cryogrinding is more effective than bead beating.

The reviewer does have a valid point. However, there is likely a very trivial explanation for the presence of a couple of bands in the IP of WCE prepared by bead beating, but not in the IP of the WCE prepared using the freezer mill. These bands are likely the partial breakdown products of full length ubiquitylated proteins in the WCE prepared by bead beating, whereas these bands are “missing” from the WCE prepared using the freezer mill as they are still intact as full length (or at least near full length) proteins in the latter. More importantly, as clearly pointed out by the reviewer, there are many more bands in

the IP from WCE prepared using the freezer mill that are absent from the IP of the WCE prepared using the bead beater, which is fully consistent with our view that the freezer mill is superior to bead beating for preparing extracts. We have converted the Western blot from a colored to a grayscale image in Figure 3B which allows for better visualization of the bands. Because the freezer mill produces better quality extracts, we routinely use only the freezer mill and have rarely used the bead beater over the past 2 decades. We have a volume of published work spanning a decade using the freezer mill which are cited here (Gunjan & Verreault, 2003; Singh et al 2009, Singh et al, 2012, Liang et al, 2012) and that offer many additional examples of the detection of labile proteins as well as their fleeting posttranslational modifications using extracts prepared with the freezer mill, which are impossible to detect using extracts prepared by other means including the bead beater. Please also see our response to Reviewer 2 above regarding the original figure 2.

I am not an expert in this at all, but I would like to make a few suggestions that "might" help illustrate more clearly the difference between IPs prepared from WCE obtained by cryogrinding versus IPs prepared from WCE obtained from a bead beater. The first is that there may be many deubiquitylating enzymes (DUBs) present in yeast WCEs. Lines 97-100: There is no N-ethylmaleimide (NEM), a generic inhibitor of DUBs. Lines 97-100: MG-132 is a potent inhibitor of ubiquitylated protein degradation by the proteasomes. However, it does not inhibit lysosomal proteases that are independent of target ubiquitylation of proteasoms. At least some of the lysosomal proteases can be inhibited by cathepsins.

We have indeed used NEM and other inhibitors of DUBs in our extracts in the past depending on the questions that we were interested in. Further, our protease inhibitor cocktail includes two inhibitors that block lysosomal proteases such as cathepsin B and L. In general, unlike MG132, we did not find a detectable difference between the absence or the presence of the DUB inhibitors in the extracts in blocking the degradation of proteins in bulk (the Ponceau staining pattern was essentially the same), although they are clearly important in the context of specific proteins.

Antibodies against the His tag are not very sensitive. Unless there is a technical against this, would it be whort proping the IP lanes with anti-MYC antibody (e.g. 9E10), rather than anti-His.

Even in our experience, most HIS antibodies are not very good. However, we have found an excellent monoclonal antibody for the HIS6 tag that was developed by Novagen and we have been using this for well over a decade, including the experiment shown in Figure 3. In our experience, the 9E10 monoclonal MYC antibody gives rise to much higher background signal compared to the HIS antibody (presumably due to the presence of a single MYC epitope) and hence we probed the blot first with the HIS antibody. The plan was to re-probe the blot with a MYC antibody, but unfortunately the blot was destroyed accidentally during the re-probing. As detailed above in our response to reviewer 2, we are currently unable to perform additional experiments in the lab due to the Covid-19 pandemic.

2) Legend to Figure 2: "Cryogenic grinding leads to higher total protein yields in cell extracts". To back this up, the authors indicate that equal numbers of cells were lysed with either a bead beater or a freezer and, because ther Ponceau S of the WCE prepared by cryogrinding is more intense (perhaps slightly more than 2-fold) than the WCE obtained from bead beating, the freezer mill is far better than the bead beater at generating protein extracts. First of all, on order to properly assess this claim, I would need to know that the volumes of extracts prepared by both instruments are the same or nearly the same. Rather than having to eyeball how much more protein is present in the extract derived by cryogrinding, why not perform a Bradford or BCA on the two extracts. This would provide an actual number. It would be be necesary to remove Tween and any other substances that interfere with

Bradford or BCA from the lysis buffer but, in my opinion, it would give rise to a more accurate estimate of protein concentrations than Ponceau S.

As detailed above in our response to Reviewer 2, equivalent amounts of WCE prepared from the same number of cells using the two different methods were loaded on the gel. Due to the reasons cited by the reviewer, we do not perform the Bradford assay on these extracts (nevertheless, results from such a Bradford assay performed on budding yeast freezer mill extracts are available in Singh, Roy & Bellare et al., 2009). However, in other qualitative experiments in the past, we have precipitated the proteins using TCA prior to measuring total protein levels and the freezer mill consistently gives us higher protein yields. The higher protein yield from the freezer mill is also seen on the ponceau stain in Figure 3A.

3) There has to be a less confusing manner to write the legend to Figure 2. I am specifically referring to this sentence: "Small but equal aliquots of each WCE" (these refer to panel A if I'm not mistaken, and panel A was not probed to generate panel, as one might intuitively led to believe, because panel B from pull dows of HMU proteins derived from the same WCEs). Ant this deeply misleading sentence goes on: "... as well as HIS tagged proteins pulled down using Talon beads (IP) from each extract were compared by resolving on a denaturing gel and transferring to a nitrocellulose membrane followed by staining with Ponceau S"
Why not just say at the top that two types of experiments were conducted from the same WCEs, and then describe (A) and (B).

We have reworded much of the legend for the original figure 2, which is figure 3 in the revised version. Hopefully, this will clarify things.

4) The article often claims that cryogrinding under liquid nitrogen is an effective way to retrieve nucleic acids. Their statement seems largely based on references 7 and 8 (two papers in the Journal of Forensic Science), where tooth DNA is retrieved by cryogrinding. I was curious about the sizes of DNA that were retrieved from ground teeth, and necessary for forensic studies but, unfortunately, I was not able to gain access to references 7 and 8.

Indeed, the DNA present in the extracts prepared using the freezer mill is sheared, but more than adequate for the identification of forensic samples using DNA fingerprinting, as demonstrated by its use in the identification of the remains of the Romanov family members related to the former Russian Tsar (Gill et al, 1994; Nature Genetics 6: 130–5). More recently, one of our colleagues was able to extract DNA using our freezer mill from a piece of a dinosaur femur found frozen in permafrost and is on the verge of obtaining libraries for whole genome sequencing! In our budding yeast extracts, the DNA ranges from ~150bp to nearly 50Kb (it extends all the way into the wells of the agarose gels; also see Singh, Roy & Bellare et al., 2009). This is better than what has been reported by Smith et al, 1993, although we should expect much more degradation in the forensic samples.

In our experience, under conditions where lysis of S. cerevisiae and S. pombe is efficient, chromosomal DNA is sheared into a size range that spans from approximately 100 bp up to >3Kb. This deserves to be mentioned. Otherwise it is misleading the reader to state that "cryogrinding preserves the integrity of nucleic acids".

We have included the word “sheared” to clarify that intact, full length chromosomal DNA is neither expected nor obtained.

Reviewer #4:

Manuscript Summary:

In this manuscript, Giovinazzi and colleagues describes an important method used in molecular biology experiments using the budding yeast Saccharomyces cerevisiae. As the authors mentioned, this method has been extremely useful to prepare whole cell extracts and subsequently purify protein complexes, proteomic analyses and co-immunoprecipitation studies and detection of labile protein modifications. There are a large number of molecular details found using this way to prepare cell extracts. The method is very well written and details are easy to be followed by readers and viewers.

Major Concerns:

1) How do authors make sure that the amount of powdered extracts between different samples is the same in order to be able to compare them experimentally? Do authors weigh the popcorn at any step of the experiment? I guess depending on the cell line used there will be differences in the weight of cells assuming that authors always collect precise number of cells in each experiment. All these points should be addressed. I recommend that author explain the use of smaller vial that are very useful to prepare different samples at the same time from a time course for instance. Reader should understand other applications that can be achieved using other vials.

We have found that the best way to ensure that the same amount of extracts is obtained from each sample by counting the cells using a Coulter Counter and using the same number of cells resuspended in the same volume of the lysis buffer for preparing extracts under identical conditions and settings on the freezer mill. We do not weigh the popcorn at any point as there is no need to do so, since the majority of their weight will be contributed by the volume of the lysis buffer used. The smaller vials can accommodate 4 smaller samples at a time and can be used without changing the freezer mill settings as long as the sample type is the same. We do not see any detectable difference between processing four samples simultaneously or sequentially using the 4 smaller vials, as long as the samples and settings remain the same. We have included additional details on the use of the smaller vials in the revised manuscript.

2) In point 2.7 authors state that powdered extracts can be stored overnight. Is this step recommended? In the video is mentioned that just overnight, but not in the text.

As long as the frozen extracts are not thawed out following their recovery from the freezer mill and stored immediately in the -80C freezer, we have not seen any appreciable increase in protein degradation. However, we would not recommend this step, since ideally the extracts should be used right away to minimize protein degradation, especially for labile proteins and their modifications. We have clarified this in the text.

3) I am confused about point 2.8 on how authors thaw the samples being kept on ice with constant stirring. It is shown in video but without watching the video it is difficult to imagine. More detailed description is needed in the main text.

We have provided additional details in the text for the extract thawing procedure.

4) In Figure 2A I see clear difference between the two methods in amount of protein in WCE but we are missing loading control. Something that would clearly show the same amount of starting material between the two to be able to judge the difference (like TCA extract from the sample before the

cryogrinding).

As pointed out above in response to Reviewers 2 and 3, equivalent amounts WCE prepared from the same number of cells using the two different methods were loaded on the gel. Because we use the same number of cells and the same volume of lysis buffer, loading the same amount of extract allows us to directly compare the amounts of protein present in each extract using Ponceau staining as shown in Figure 2A. We have compared this type of analysis to TCA precipitated proteins in the past and the results were very similar between the extracts run directly on the gel, or following TCA precipitation. In our experience, we have not found any method superior to starting out with accurate cell counts and using equal number of cells for extract preparation to ensure equal protein content.

5) I am convinced that cryogrinding is an outstanding method, but I reckon that the example shown if Figure 2 does not completely show the benefit of this method. I was wondering if authors could show a more striking example. Maybe the use of His antibodies would help.

The Western shown in Figure 3B is using HIS antibodies. As pointed out above in response to Reviewer 3, we have a volume of published work spanning a decade using the freezer mill which are cited here (Gunjan & Verreault, 2003; Singh et al 2009, Singh et al, 2012, Liang et al, 2012) and that offer many additional examples of the detection of labile proteins as well as their fleeting posttranslational modifications using extracts prepared with the freezer mill.

6) There are already some published cryogrinding methods for budding yeast. This manuscript perfectly complements those. I would recommend to include those references for readers to get as much information to adapt to their own work.

Of course! As pointed out above, we have published a significant amount of budding yeast work performed using the freezer mill and these references have been included already. Additional references (15) from other labs using the freezer mill for preparing budding yeast extracts are also listed in the revised manuscript.

Minor Concerns:
1) I would consider adding budding yeast Saccharomyces cerevisiae to the title as the paper describes precisely cryogenic grinding using budding yeast extracts.

We have refrained from being too specific in the title as the method described here can be used pretty much as is for a variety of samples from other species, ranging from fossils to forensic samples. We felt that by not making the title too specific, it will reach the widest possible audience.

2) The summary authors includes "describe a reliable method for the preparation of whole cell extracts from yeast and other samples". I would recommend describing here what they mean by other "samples".

We have replaced the word “samples” with “cells”. As described in the manuscript, cryogrinding using a freezer mill is very versatile and it is possible to process just about any imaginable type of cells, tissues, forensic or permafrost specimens to obtain biological macromolecules from them. We have elaborated some more on this.

3) Line 46 should state "budding" yeast.

This has been fixed now.

4) Line 50 missing coma after applications.

The comma has been inserted now.

5) Line 86 model of the freezer mill could be mentioned as there are models that would fit 4 samples and there are models that would fit only one sample.

We have to follow the publishing guidelines which do not allow us to include brand names and model number in the main body of the manuscript, but we can and have included this information in the table with all the materials listed.

6) Figure 1A and Figure 1B should be mentioned next to where authors refer to their content

We have fixed this oversight.

7) Line 122 "Close the freezer mill slowly, avoiding the splash of LN2" could be added to warn readers it is a hazardous action.

We have included this now.

8) Line 132 should give the full protocol followed (time for grinding, time for breaks and rate). I would suggest to include a Figure showing the freezer mill display.

We have included all the details now.

9) Line 133 I would recommend to include "carefully open the lid" after "cycles".

This has been included now.

10) Line 135 I would recommend to include "work with vials quickly removing one vial at the time".

We have included this now.

11) Line 137 I suggest that authors could include how they keep chilled forceps

The forceps are kept chilled by simply sticking them in the dry ice bucket and we have included this information in the revised manuscript.

12) Authors need to unify the centrifuge speed writing either 3220g or 3220xg.

We have fixed this and removed the unnecessary x.

13) Point 2.11 lipid layer is mentioned and it would need a bit more explanation. Maybe even a

schematic representation or even a picture with what reader will find when following the protocol.

We have added Figure 2 with a schematic representation and have provided additional details regarding this step which should clarify things.

14) Line 156 the description "top half of the sample" is quite confusing. It should be explained better for reader to understand it.

We have reworded this and added additional explanation for this step.

15) Point 2.12 is confusing and needs to be reorganized. There was no mention of any division of the extracts earlier so "remaining extract" use is confusing. Also what do authors mean by "centrifuging for 5 minutes between collections". This fragment has to be rewritten in more precise way.

This passage has been reworded to clarify the procedure.

16) Authors use cryogenic grinding, cryogrinding or cryogenic milling terms. I recommend to be unified.

We have replaced all these terms with cryogrinding.

17) Line 168-169 Authors need to include budding yeast cell line and strain name.

We have now included the budding yeast strain details and a reference for the same.

18) Line 197 Authors could mention that LN2 needs to be topped up as it gets evaporated.

We have included this information now.

19) In the figure they show three 50ml tubes held with a rubber band, while in the video there is only one. Author should clarify why the use 3 50ml tubes or update the figure with just one 50ml tube as in the video.

This step can be performed with different variations depending on the size and number of samples. We have included additional text to clarify this. Please also see our response to Reviewer 2 regarding this step.

20) Lines 241-242 References for the use of the freezer mill with other organisms should be included.

We have added additional references (in fact, our references have nearly tripled in number compared to the initial submission!).

21) Figure 2 is missing the molecular weight markers.

We have added them now.

22) Do authors ensure careful removal of the ground frozen sample from the vial e.g. by scrapping the leftovers of the frozen powder from the walls of the vial or they simply invert the vial in order to remove

the powder.

To maintain the powdered extract in frozen form and prevent their rapid thawing out, we work quickly and hence do not scrape the frozen extract from inside the vials. We have found that by inverting and tapping the vial we recover ~95% of the frozen extract in the vial.

23) Authors mention the use of this method in forensic studies. It is a bit surprising the use for those studies as one could imagine that the size sample is not abundant. I was wondering if authors could mention something about that or even include a reference.

References 12 and 13 in the revised manuscript refer to the use of such grinding for obtaining DNA from forensic samples. In fact, one of the primary uses of freezer mills is to obtain DNA from forensic samples to aid their identification by DNA fingerprinting. Additional references (20) have been included now and many more are easily found in databases. Please see our response above to Reviewer 3 as well.

24) Authors could consider to include that, as large amount of LN2 are used, a LN2 could be installed in the room where freezer mill will be used.

This is indeed possible and the latest models of freezer mills are designed to work with such automated liquid nitrogen supply systems. However, due to the high evaporation rate of liquid nitrogen and the asphyxiation risk associated with its vapors in enclosed places, doing this would be advantageous only if the freezer mill was used nearly every day. We have included this possibility in the manuscript.

Video comments
1) Cells were spun at 2392g as it says in the method or 2400g (or 4000rpm) as it says in the video?

Cells were centrifuged at 4000rpm which corresponds to 2392g (or approximately 2400g) for the rotor. We have clarified this now.

2) I guess some of the popcorn beads are bigger that 0,5 cm in the video attached.

Yes, the 0.5cm refers to the largest size of the popcorn desired for the experiment (range 0.3-0.5cm), but individual droplets vary in size depending on the person performing the procedure. We use a pair of chilled forceps the breakup popcorn kernels that are much larger than 0.5cm before freezing them.