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TITLE:

Labelling and Visualization of Mitochondrial Genome Expression Products in Baker's Yeast *Saccharomyces Cerevisiae*

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KEYWORDS:

Baker's yeast, mitochondria, translation, protein, biosynthesis, labeling, visualization, assay, imaging

SUMMARY:

Baker's yeast mitochondrial genome encodes eight polypeptides. The goal of the current protocol is to label all of them and subsequently visualize them as separate bands.

ABSTRACT:

Mitochondria are essential organelles of eukaryotic cells capable of aerobic respiration. They contain circular genome and gene expression apparatus. A mitochondrial genome of baker's yeast encodes eight proteins: three subunits of the cytochrome c oxidase (Cox1p, Cox2p, and Cox3p), three subunits of the ATP synthase (Atp6p, Atp8p, and Atp9p), a subunit of the ubiquinol-cytochrome c oxidoreductase enzyme, cytochrome b (Cytb), and mitochondrial ribosomal protein Var1p. The purpose of the method described here is to specifically label these proteins with ^{35}S methionine, separate them by electrophoresis and visualize the signals as discrete bands on the screen. The procedure involves several steps. First, yeast cells are cultured in a galactose-containing medium until they reach the late logarithmic growth stage. Next, cycloheximide treatment blocks cytoplasmic translation and allows ^{35}S methionine

incorporation only in mitochondrial translation products. Then, all proteins are extracted from yeast cells and separated by polyacrylamide gel electrophoresis. Finally, the gel is dried and incubated with the storage phosphor screen. The screen is scanned on a phosphorimager revealing the bands. The method can be applied to compare the biosynthesis rate of a single polypeptide in the mitochondria of a mutant yeast strain versus the wild type, which is useful for studying mitochondrial gene expression defects. This protocol gives valuable information about the translation rate of all yeast mitochondrial mRNAs. However, it requires several controls and additional experiments to make proper conclusions.

INTRODUCTION:

Mitochondria are the organelles deeply involved in the metabolism of a eukaryotic cell. Their electron transfer chain supplies the cell with ATP, the main energetic currency used in multiple biochemical pathways. Besides, they are involved in apoptosis, fatty acid and heme synthesis, and other processes. Dysfunction of mitochondria is a well-known source of human disease[1]. It can result from mutations in nuclear or mitochondrial genes encoding structural or regulatory components of the organelles[2]. Baker's yeast *Saccharomyces cerevisiae* is an excellent model organism for studying mitochondrial gene expression due to several reasons. First, their genome is completely sequenced[3], well-annotated, and a big sum of data is already available in literature thanks to the long history of investigations carried out with this organism. Second, the manipulations with their nuclear genome are relatively fast and easy because of their fast growth rate and highly efficient homologous recombination system. Third, baker's yeast *S. cerevisiae* is one of the few organisms for which the manipulations with mitochondrial genomes are developed. Finally, baker's yeast is an aerobe-anaerobe facultative organism, which allows isolation and study of respiratory defective mutants, since they can grow in media containing fermentable carbon sources.

We describe the method to study mitochondrial gene expression of baker's yeast *S. cerevisiae* at the translational level[4]. Its main principle comes from several observations. First, the yeast mitochondrial genome encodes only eight proteins: three subunits of the cytochrome c oxidase (Cox1p, Cox2p, and Cox3p), three subunits of the ATP synthase (Atp6p, Atp8p, and Atp9p), a subunit of the ubiquinol-cytochrome c oxidoreductase enzyme, cytochrome b (Cytb), and mitochondrial ribosomal protein Var1p[5]. This number is small, and all of them can be separated by electrophoresis on a single gel in the appropriate conditions. Second, mitochondrial ribosomes belong to the prokaryotic class rather than eukaryotic[6], and therefore, the sensitivity to antibiotics is different for yeast cytoplasmic and mitochondrial ribosomes. It allows the inhibition of cytoplasmic translation with cycloheximide, providing the conditions when the labeled amino acid (^{35}S-methionine) is incorporated only in mitochondrial translation products. As a result, the experiment gives information about the rate of amino acid incorporation in mitochondrial proteins synthesized de novo, reflecting the overall efficiency of mitochondrial translation for each of the eight products.

PROTOCOL:

1. Yeast culture preparation

1.1. Streak yeast from the frozen stock cultures on fresh plates with the appropriate medium. Put the plates in a culture incubator at 30 °C for 24–48 h.

NOTE: Let the temperature-sensitive mutants grow at the permissive temperature.

1.2. Inoculate yeast cultures in 2 mL of YPGal medium (2% peptone, 1% yeast extract, 2% galactose) from the fresh streak in 15 mL tubes and incubate them overnight agitating at 200 rpm at 30 °C.

1.3. Measure the optical density of the culture at a wavelength of 600 nm (OD_{600}).

1.4. Take the volume corresponding to 0.2 absorbance units in sterile tubes, pellet yeast cells at 9,000 x *g* for 30 s at room temperature, and discard the supernatant.

1.5. Wash cells with 0.5 mL of sterile water by vortexing for 5 s. Pellet yeast cells at 9,000 x *g* for 30 s at room temperature and discard the supernatant. Dilute cells in 2 mL of fresh YPGal medium.

1.6. Incubate agitating at 200 rpm and 30 °C until OD_{600} reaches 1.5–1.9 values.

NOTE: Yeast growth rates vary, so be prepared to wait. It is reasonable to make steps 1.3–1.6 early in the morning. It usually takes 4–5 h, but can take longer.

2. Radioactive isotope incorporation

2.1. Transfer the culture volume equivalent to one optical unit in a microcentrifuge tube. Spin the tubes at 3,000 x *g* for 1 min and discard the supernatant. Wash with 0.5 mL of sterile water by vortexing for 5 s.

2.1.1. Pellet yeast cells at 9,000 x *g* for 30 s at room temperature and discard the supernatant. Resuspend yeast cells in 0.5 mL of sterile translation buffer. Place the suspension in a 15 mL tube.

NOTE: Translation buffer is a solution containing 2% galactose (w/v) and 50 mM potassium phosphate with pH 6.0.

2.2. Add cycloheximide to cell suspension up to a final concentration of 0.2 mg/mL. Incubate for 5 min agitating at 200 rpm and 30 °C to inhibit cytosolic translation.

NOTE: Cycloheximide solution (20 mg/mL, in ethanol) should be prepared fresh before the experiment. Chloramphenicol can be successfully substituted with anisomycin in the same concentration[7].

2.3. Add 25–30 µCi of ^{35}S-methionine to the cell suspension and incubate for 30 min agitating at 200 rpm and 30 °C.

NOTE: The mixture of ^{35}S-methionine and ^{35}S-cysteine can also be used. Pure ^{35}S-methionine results in the strongest signal and the best signal-to-noise ratio. Generally, methionine is more effective than cysteine because the content of this amino acid in mitochondrial proteins is higher. However, the EasyTag mixture of ^{35}S-methionine and cysteine is less expensive and gives comparable results[7].

CAUTION: ^{35}S-methionine is radioactive. Follow the usual safety practices for handling radioactive materials.

NOTE: It is well known that the incorporation of radioactivity reaches a limit due to which the total signals level off over time. Once this limit is reached, it is almost impossible to determine the rates by which the different translation products are synthesized. To analyze the rate of mitochondrial translation, it is imperative to be in a condition in which the signal intensity increases with time of incubation with ^{35}S-methionine in a linear manner. For common laboratory wild-type strains, the signal is already saturated for incubation times far shorter than the 30 min. Translational mutants can behave very differently. A time-course should be performed to establish the kinetics of radioactivity incorporation and thus the rate of translation, at least when working with a new strain. For this, we suggest taking samples with an interval of several minutes (e.g., 2.5, 5.0, 7,5, 10, and 20 min).

2.4. Add unlabeled "cold" methionine (final concentration should be 20 mM) and puromycine (final concentration should be 1–10 µg/mL) to stop the labeling. Incubate for 10 min agitating at 200 rpm and 30 °C.

NOTE: This step is critical to give ribosomes time to finish translating the peptides. Otherwise, all polypeptides shorter than full-length will be detected at a different size. A time-course experiment (pulse-chase) can be done at this step to determine the stability of mitochondrial translation products. For this, continue the incubation taking samples with an interval of 30 min (e.g., 30, 60, 90, and 120 min).

3. Yeast cell lysis and extraction of proteins

3.1. Collect yeast cells by centrifugation at 9,000 x *g* for 30 s. Wash the cells with 0.5 mL of sterile water by vortexing for 5 s. Pellet yeast cells at 9,000 x *g* for 30 s at room temperature. Discard the supernatant.

NOTE. The protocol can be paused here. Store the samples at -20 °C.

3.2. Add 75 µL of lysis buffer to the pellet and vortex for 5–10 s.

NOTE: Lysis buffer is a solution of 1.8 M NaOH, 1 M β-mercaptoethanol, and 1 mM PMSF in

water. Avoid excessive incubation with lysis buffer leading to alkaline hydrolysis of proteins. Immediately proceed to step 3.3.

3.3. Add 500 µL of 0.5 M Tris-HCl buffer with pH 6.8. Vortex briefly.

4. Precipitation of proteins

CAUTION: Methanol and chloroform are organic solvents. Follow the usual safety practices for handling organic substances.

4.1. Add 600 µL of methanol to the sample. Vortex for 5 s.

4.2. Add 150 µL of chloroform to the sample. Vortex for 5 s.

4.3. Centrifuge the samples for 2 min at 12,000 x *g*. Carefully discard the upper phase with a sampler.

4.4. Add 600 µL of methanol to the sample. Mix carefully by inverting the tube several times.

4.5. Centrifuge the samples for 2 min at 12,000 x *g*. Discard the supernatant.

4.6. Air-dry the pellet for 2 min at 80 °C.

NOTE: All of the liquid should evaporate. There is a risk to have an aberrant separation of proteins in the gel if the pellet was dried insufficiently. However, it is not possible to over-dry the pellet, so it can even be stored overnight at 4 °C.

4.7. Dissolve precipitated proteins in 60 µL of 1x Laemmli sample buffer.

4.8. Heat for 10 min at 40 °C.

NOTE: Avoid boiling the samples at 95 °C, because this causes aggregation. If the aggregates still form, Spin the samples at 12,000 x *g* for 2 min and collect the supernatant. Samples can be stored at -20 °C. The protocol can be paused here.

5. SDS-PAGE

5.1. Cast the 17.5% Laemmli SDS-polyacrylamide gel.

NOTE: 6 M urea can be added to the gel to resolve better atp8 and atp9. Gradient 15%–20% gels can also give better resolution.

5.2. Load 15 µL (40–50 µg) of each sample in the pockets.

NOTE: It is not necessary to measure protein concentration if OD_{600} values were close in step 1.6. If not, measure protein concentrations using the assay with detergent-compatible reagents.

5.3. Run the gel in a cold room until the blue dye reaches approximately 65% of the gel length.

NOTE: For the system used (e.g., Protean II xi cell), we use either of the two modes: 16–17 h at 5 V/cm or 5 h at 15 V/cm. No proteins run faster than the bromophenol blue dye, but long runs can result in blurring of atp8 and atp9 signals.

5.4. Stain the gel with Coomassie brilliant blue and make a scan or photo, which is required as a loading control.

NOTE: An alternative way to make a loading control is immunoblotting with an antibody to mitochondrial "house-keeping" gene, e. g., porin 1.

6. Autoradiography

6.1. Dry the gel in a gel-dryer. Keep it in the cassette with a storage phosphor screen for 3–5 days.

NOTE: An alternative to screening the dried gel is the transfer of proteins to a nitrocellulose membrane by electro-blotting and screening it thereafter. It results in stronger signals and sharper bands.

6.2. Scan the screen on a phosphorimager.

NOTE: As an alternative to phosphor imaging, X-ray film can also be used to reveal the signals.

REPRESENTATIVE RESULTS:

Following the protocol described above, we assigned mitochondrial translation products from two *S. cerevisiae* strains: the wild type (*WT*) and a mutant bearing deletion of the *AIM23* gene *(AIM23Δ*), encoding mitochondrial translation initiation factor 3 (**Table 1**)[8]*.* Mitochondrial translation products were radioactively labeled and separated in SDS-PAAG[9]. The samples were collected every 2.5 min before saturation to build a time course (**Figure 1A**). The gel was stained, dried, and screened after the 5-day exposition (**Figure 1A**).

In the case of a successful experiment, the picture demonstrates eight bands assigned according to the standard pattern[4]. However, the intensities of individual bands can be highly variable depending on the strain and experimental conditions. Each band corresponds to one translation product. The data (**Figure 1A**) suggest that the *AIM23Δ* strain is capable of mitochondrial protein synthesis because all products appearing in the WT are visible in this mutant. However, the intensities of the bands are different from the WT, meaning that the deletion of *AIM23* affects mitochondrial gene expression[8]. Coomassie Brilliant Blue staining

serves as a loading control.

The resulting data can be quantified (**Figure 1B**) to identify differences between strains or experimental conditions using ImageJ[10] or ImageQuant software. For this, the ratios of the signal corresponding to every product to the total signal are calculated. Mean values and standard deviations are calculated in at least three independent experiments.

The kinetics of synthesized protein turnover is studied in a pulse-chase experiment (**Figure 1C**). Samples are collected at the indicated time points after the labeling reaction is stopped by cold methionine and puromycine in step 2.4. This control is necessary to estimate the stability of the products because the intensity of the signal is a result of two opposite processes: synthesis of new chains and protein degradation. Immunostaining with anti-porin 1 antibodies is a loading control.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative radioactive labeling of yeast mitochondrial translation products. (**A**) Time course of ^{35}S-methionine incorporation in mitochondrially synthesized proteins in live yeast cells of *WT* and *AIM23Δ* strains. Coomassie Brilliant Blue staining is a loading control. (**B**) Levels of mitochondrially-encoded proteins after 5 min labeling with ^{35}S-methionine. The relative expression is normalized to the total expression of mitochondrially encoded protein genes. Error bars indicate the standard deviation of the mean of at least three independent experiments. (**C**) Turnover of mitochondrially synthesized proteins in wild type and *Aim23Δ* strains. The labeling was stopped and the samples were collected at the indicated time points. Immunostaining with anti-porin 1 antibody is a loading control. (**D**) Sub-optimal experiment with old 35S-methionine, radioautography. **Figures 1A,B,C** are adapted from[8] with minor modifications.

Table 1. Genotypes of *S. cerevisiae* strains.

DISCUSSION:

Investigations of gene expression occupy a central part in modern life sciences. Numerous methods providing insights into this complex process have been developed. Here, we described the method allowing to access protein biosynthesis in baker's yeast *S. cerevisiae* mitochondria. It is usually applied to compare translation efficiencies of the mRNAs in mitochondria of mutant yeast strain versus wild type to access the consequences of the studied mutation. This is one of the basic experiments the researchers conduct when they study the mitochondrial function of yeast cells bearing the mutation suggested to influence mitochondria[8,11–13]. It is often combined with the measurements of oxygen consumption rate and mitochondrial membrane potential. However, the information it provides is not sufficient to distinguish what stage of gene expression is affected. A set of additional experiments is required to find it out. First, northern blot or RT-qPCR evaluation of mitochondrial mRNAs is necessary to assess the transcriptional step. Second, a Western blot of total protein extracts with specific antibodies should be done to assess the protein level. Third, the pulse of labeled ^{35}S-methionine (hot) should be continued

with the addition of unlabeled (cold) methionine (chase) and several time points should be collected and analyzed on the gel to investigate the stability of the proteins.

Accurate analysis of mitochondrial gene expression using ^{35}S pulse labeling requires control reactions, especially when the researcher lacks the experience handling it or works with a new yeast strain or a mutant. In these cases, good negative control is a *rho*^{0} strain devoid of mitochondrial DNA. It shows efficient cycloheximide inhibition of cytosolic translation and confirms that the banding pattern is mitochondrial translation specific. If the *rho*^{0} strain is not available, then we suggest including chloramphenicol along with cycloheximide to inhibit all protein synthesis to confirm cycloheximide efficiency and specificity of the banding pattern.

The closest modification of the protocol is the pulse-chase when the culture is incubated in the shaker (step 2.4) longer than suggested in the pulse experiment (**Figure 1C**). It is used to study the turnover and stability of mitochondrial translation products. There is another modification of the method when the radioactive labeling is done in organello, not in vivo[4]. It suggests the isolation of mitochondria from yeast cells. This modification is faster if frozen mitochondria were previously stocked in aliquots. Another advantage is the absence of cycloheximide treatment, which affects different aspects of cellular metabolism. However, isolation of mitochondria and freeze-thawing them can perturb the translation complexes in the organelles providing an artificial picture. Another important modification of the protocol can be done after the separation of mitochondrial translation products in polyacrylamide gel (step 5). Instead of Coomassie Brilliant Blue staining and drying the gel, the protein can be transferred to the nitrocellulose membrane by electro-blotting. This results in stronger and sharper signals. The main reason is that ^{35}S decays by emission of beta particles with very short penetration, so the signal is easily screened in this approach. Electrophoretic conditions can also be modified to provide better resolution. One point is to add 6M urea in the gel, which improves the separation of atp8 and atp9[14]. Another way is using gradient 15%–20% gels.

Translational profiling[15] is the method used to dive deeper into the alterations of mitochondrial translation. As compared to radioactive labeling, it allows establishing positions of mitochondrial ribosomes on the mRNA, which makes it possible to find the exact step (initiation, elongation, or termination) being affected. However, profiling is much more expensive, complicated, and time-consuming. Rationally it can be done after the label incorporation experiment, not vice versa. Recently, a novel approach to monitoring yeast mitochondrial translation has been developed[16]. It avoids treatment with cycloheximide, which is advantageous because such treatment affects cellular metabolism and signaling pathways. Instead of radioactively labeled amino acid incorporation, it utilizes the insertion of a recoded gene for super-folded GFP (sfGFP) in yeast mitochondrial genome, which allows direct measurement of mitochondrial translation using flow cytometry. However, the application of this approach requires the special yeast strain with modified mitochondrial DNA containing sfGFP coding sequence placed between 5'- and 3'- flanking sequences of a certain mitochondrial gene.

The label incorporation experiment includes several critical steps, which cannot be

compromised in a successful experiment. First, fresh yeast cultures should be used (step 1.1). Keeping yeasts on the plates longer than 1 month is not recommend; otherwise, they can behave unpredictably in this assay. Second, the cycloheximide solution should be prepared fresh before the experiment and stored frozen no longer than 1 week (step 2.1). The old solution loses its ability to inhibit cytoplasmic translation resulting in a completely aberrant band pattern in the radioautography. Third, ^{35}S-methionine should be fresh and active (step 2.2), otherwise, the intensities of the bands will be weak (**Figure 1D**). Using the reagent that passed four half-lives (4 x 87.4 days) is not recommended. Avoid boiling the protein samples at 95 °C as standard sample preparation guides suggest (step 4.8) because mitochondrial proteins are highly hydrophobic and prone to aggregation.

There are several common issues one can experience dealing with this method. The first one is the weak intensity of the bands on the radioautography. To fix it make sure, that fresh ^{35}S-methionine is used, a sufficient amount of yeast cells is taken, and the proteins do not aggregate in the pockets on the gel, which can be controlled by Coomassie staining. Keep the dried gel with the screen for no less than 3 days. The second issue is the incorrect band pattern. If it is encountered, make sure that fresh yeast plates and freshly prepared cycloheximide are used. Keep in mind that relative intensities of the bands can vastly vary in different yeast strains and electrophoresis conditions. It makes little sense to load the protein molecular weight ladder on the gel since mitochondrial translation products are never separated according to their molecular masses in this procedure because they are highly hydrophobic. Thus, Cox I (58 kDa) migrates faster than Var 1 (47 kDa). Depending on the buffer conditions, the proteins can even switch positions with one of the neighbors. The third common issue is the blurred picture with no sharp bands observed after Coomassie staining. It indicates the mistakes in the casting of the gel, incorrect buffer composition, or degradation of proteins in the samples. It is recommended to prepare new gel buffers and running buffer carefully checking the composition and the pH values.

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DISCLOSURES:

The authors have nothing to disclose

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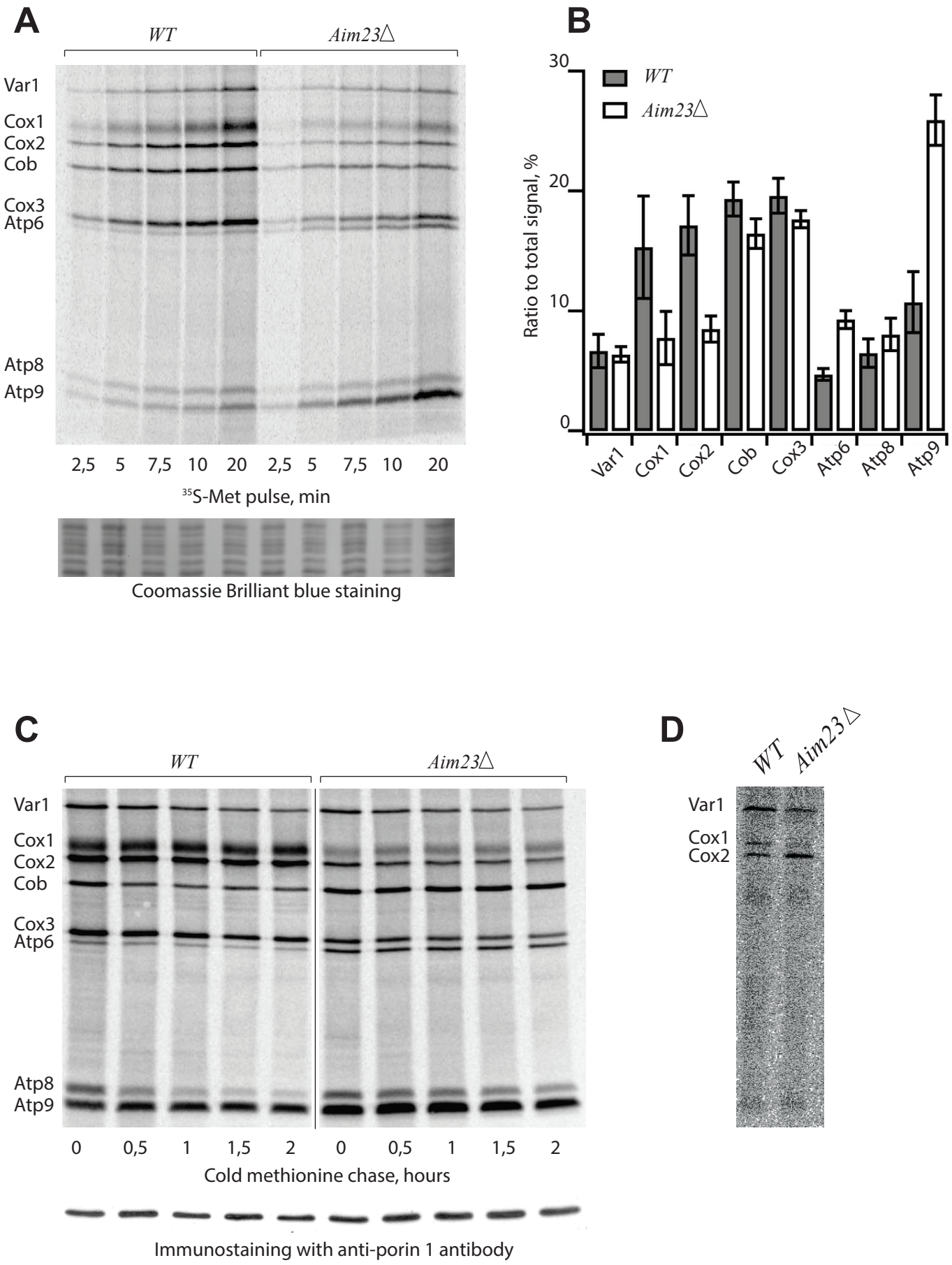

Table

strain	genotype
WT	*MATα mal*
AIM23Δ	*MATα mal, AIM23::KanMX4*

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-Mercaptoethanol	Sigma-Aldrich	M3148	
Acrylamide	Sigma-Aldrich	A9099	
Ammonium persulfate	Sigma-Aldrich	A3678	
Bacteriological agar	Sigma-Aldrich	A5306	
Biowave Cell Density Meter CO8000	BIOCHROM US BE	80-3000-45	
BRAND standard disposable cuvettes	Sigma-Aldrich	Z330361	
chloroform	Sigma-Aldrich	288306	
cycloheximide	Sigma-Aldrich	C1988	
D-(+)-Galactose	Sigma-Aldrich	G5388	
D-(+)-Glucose	Sigma-Aldrich	G7021	
digital block heater	Thermo Scientific	88870001	
EasyTag L-[35S]-Methionine, 500µCi (18.5MBq), Stabilized Aqueous Solution	Perkin Elmer	NEG709A500UC	
Eppendorf Centrifuge 5425	Thermo Scientific	13-864-457	
GE Storage Phosphor Screens	Sigma-Aldrich	GE29-0171-33	
L-methionine	Sigma-Aldrich	M9625	
methanol	Sigma-Aldrich	34860	
N,N,N′,N′-Tetramethylethylenediamine	Sigma-Aldrich	T9281	
N,N′-Methylenebisacrylamide	Sigma-Aldrich	M7279	
New Brunswick Innova 44/44R Shaker Incubator	New Brunswick Scientific		
Peptone from meat, bacteriological	Millipore	91249	

Phenylmethanesulfonyl fluoride	Sigma-Aldrich	P7626
Pierce 660nm Protein Assay Kit	Thermo Scientific	22662
PowerPac Basic Power Supply	Bio-Rad	1645050
Protean II xi cell	Bio-Rad	1651802
Puromycin dihydrochloride from Streptomyces alboniger	Sigma-Aldrich	P8833
Sodium hydroxide	Sigma-Aldrich	221465
Storm 865 phosphor imager	GE Healthcare	
Trizma base	Sigma-Aldrich	93352
Vacuum Heated Gel Dryer	Cleaver Scientific	CSL-GDVH
Yeast extract	Sigma-Aldrich	Y1625

REBUTTAL LETTER

Editorial and production comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- We have done the proofreading using the Grammarly application. We corrected many grammar and spelling issues.

1. Please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol so that users can use the written manuscript and the video concurrently.

- We revised the narration and now it is a word for word reading of the protocol

2. Title card: Please italicize Saccharomyces cerevisiae

- Fixed.

3. Consider holding another moment on the title card

- We made it longer. Now it is approximately 5 seconds.

4. 05:42 Chapter title card: "6. Autoradigraphy" typo, should be spelled Autoradiography

- Fixed.

5. Please reduce the overall audio volume by 3 dB

- It has been done.

RESPONSE TO THE REVIEWERS

Reviewer 1

It is well known that the incorporation of radioactivity reaches a limit due to which the total signals level off over time. Once this limit is reached, it is basically impossible to determine the rates by which the different translation products are synthesized. To analyze the rate of mitochondrial translation, it is imperative to be in a condition in which the signal intensity increases with time of incubation with S35-methionine in a linear manner. For common laboratory wild-type strains, the signal is saturated already for incubation times far shorter than the 30-45 minutes suggested in this protocol. Translational mutants can behave very differently. A time course should be performed, at least when working with a new strain, in order to establish the kinetics of radioactivity incorporation/ rate of translation.

- We agree with your suggestion. We added a text note in point 2.3 (lines 140-149). Also, we reworked the figures. Now figure 1A shows the time-course of label incorporation. We used this figure from our previous work published in SciRep (2016) because the format of the publication allows us to do it.

In the abstract and introduction, the expression "protein part of cytochrome b" should be corrected. Cytochrome b (Cytb) is a subunit of the ubiquinol-cytochrome c oxidoreductase enzyme or CIII.

- The expression has been corrected, see lines 37-38 and 77-78.

In the introduction amongst the qualities that made S. cerevisiae an excellent model system for the study of mitochondria, it could be included the fact that baker's yeast is an aerobe-anaerobe facultative organism and respiratory defective mutants can be easily isolated and study since they can grow in media containing fermentable carbon sources.

- The information was added, see lines 70-72.

The transfer of proteins to nitrocellulose membrane by electro-blotting represents a very advantageous alternative to drying the gel. When we have tested the two approaches in parallel, we have obtained stronger and sharper signals after transferring the proteins to a membrane. The main reason is that S35 decays by emission of beta particles with very short penetration. The S35 signal is easily screened and the dried gel material is sufficient to screen some of the signal. The electro-blotting should be mentioned at least as an alternative approach.

- We did not try it personally, but we respect your experience and indicate electrotransfer as an alternative approach. See lines 325-330 and 238-240.

Variations in the electrophoretic conditions have been optimized to resolve the Atp8 and Atp9 proteins and could also be mention in the discussion.

- We have found that adding 6M urea in the gel can provide a better resolution of atp8 and atp9 (PMID: 22916027). Also, gradient 15-20% gels can help to resolve them better. We made a note about it in step 5.1 (lines 212-213) and mentioned it in the discussion (lines 331-332).

In the example presented in figure 1, Cox1 and Atp6 are undetectable.

- Indeed, the quality of the figures was not the best one. We tried to generate them specifically for this publication, but they did not look nice. Now we remodeled the figures and used our gels and blots from the previous publication in SciRep (2016). We believe they are more convincing. See figure 1.

Reviewer 2:

1. While the procedure is outlined clearly and as such can definitely be followed up, the manuscript needs some English language editing.

- We are not native speakers and the problem with the English language follows us in every publication. Now we worked with the text and corrected multiple wrong articles, prepositions, and spelling mistakes. We hope, now the text has become better.

2. If the authors find it possible, they could analyze a larger set of commonly used "wt" yeast strains to show the differences in mitochondrial translation products.

- We work with the D273-10b strain, which was specifically constructed for mitochondrial studies. We are mitochondrial biologists and work exclusively with this strain. We do not have other commonly used strains at our disposal.

Reviewer 3:

1. Recommend removing Figure 1D from the manuscript and the associated text (lines 225-226 and 287-291). It is unclear the precise error that gave rise to these results, but it was not likely directly related to the main protocol being described. Including the protein identity of the bands in Figure 1C is recommended, if known.

- Figure 1D and the associated text have been removed. We remodeled the figures, so now figure 1C from the previous iteration has become figure 1D. The identity of the bands was included where possible.

2. To be consistent with the text, the protein names in Figure 1B should be presented as Cox1, Cox2, and Cox3 rather than CoxI, CoxII, and CoxIII.

- It has been done.

3. Suggest mentioning within the text the possibility of including some additional control reactions: a) Using a rho⁰ strain. This strain is devoid of mitochondrial DNA and therefore would be a good control to show efficient cycloheximide inhibition of cytosolic translation thereby confirming banding pattern is mitochondrial translation specific, or b) The possibility of including chloramphenicol along with cycloheximide to inhibit all protein synthesis to confirm cycloheximide efficiency for situations where a rho⁰ strain is not available.

- These are good controls, for sure. We added the information in Discussion lines 307-313.

4. Can the resulting data be quantified to identify differences between strains or experimental conditions? If so, this may be information (along with approaches used to normalize the data) that could be considered to be included in the protocol.

- Yes, they can. We use ImageJ or ImageQuant software for this. We have written about it in Results (lines 263-266) and added a figure (1B). However, we did not put it in the protocol; otherwise, we ought to provide step-by-step instruction and describe it in detail by showing what buttons we click and how we build the boxes. The paper dedicated to Northern blot published in the same journal (PMID: 25177861) does not include the quantification guide, but it also contains diagrams. We do normalization relative to the total signal.

5. Line 212-216: "… deletion of aim23 affects mitochondrial gene expression" should have citation #7 indicated as this has been shown previously.

- Fixed, see line 262.

6. Line 125-126; Protocol Step 2.3: Could ^{35}S Cysteine-Methionine be substituted here in place of ^{35}S-Methionine? If so, are there any 'adjustments' to the protocol or the results that should be noted?

- Yes, it can. We found a paper where people used a mixture of cysteine and methionine (PMID: 22215550). We added this information in step 2.3 note 1 lines 132-136. As far as we know, there are no adjustments to the protocol. The mixture is less expensive and gives comparable results.

7. Line 131; Protocol Step 2.4: It may be helpful if a working range for puromycin is given rather than just a maximum amount.

- We agree, 1 mg/ml is too much. The working range is 1-10 µg/ml. This point has been corrected (step 2.4, lines 149-150).

8. Line 142; Protocol Step 3.2: Is 1M β-mercaptoethanol correct? Please check.

- Yes, it is. We checked it. In the classical paper (PMID: 11381608) people use 7,5% beta- mercaptoethanol in the lysis buffer. Pure stock is approximately 14M, so 7,5% solution is 14 x 7,5 / 100 = 1,05 M. It is close to what we write.

9. Line 166; Protocol Step 4.6: How important is it for all of the liquid solution to be evaporated? Are there complications if any methanol solution remains or if the pellet is 'overdried'? If so, maybe a statement in the form of a "NOTE" is warranted for this step.

- If the pellet is dried insufficiently, you risk having an aberrant separation of proteins in the gel. However, it is not possible to over-dry the pellet, so it can be even stored overnight at +4°C. We added the note about it in 4.6 (lines 193-195).

10. Line 178; Protocol Step 5.1 and Line 186; Protocol 5.3: A specific size gel is given (20x20cm) along with time for the run (16-17hrs; 5hrs). Are these invariable and essential

for protocol success? The apparatus for PAGE is likely to vary from lab to lab. Essential information may include information on: % acrylamide, the acrylamide:bisacrylamide ratio used, electrophoresis temperature, voltage/cm. If this is a standard Laemmli SDS-PAGE, no proteins run in front of the dye front, so maybe include a statement as to why is it important to stop the electrophoresis when the dye has traveled only 2/3 of the gel length.

- It's a standard denaturing Laemmli gel for the separation of proteins. The parameters are variables and should be adjusted for the instruments in the lab. They are just what we use in the Protean II xi cell. The information about casting the gel and electrophoresis conditions was reworked following your recommendations in steps 5.1 (line 207) and 5.3 (lines 218-223). It is important to stop the electrophoresis when the dye has traveled only 2/3 of the gel length because according to our experience longer runs can result in blurring of atp8 and atp9 signals.

11. Line 194 and 196; Protocol Step 6.1 and 6.2: Is the use of a phosphor imager essential? If the dried gel can be exposed to autoradiography film for a period of time, then maybe include a statement to the effect.

- X-ray film can also be used to reveal the signals as an alternative to phosphor imaging. We added the note about it in step 6.2 (line 241).