Published in final edited form as:

Methods Mol Biol. 2008 ; 447: 381–394. doi:10.1007/978-1-59745-242-7_25.

Alcoholic Liver Disease and the Mitochondrial Ribosome:

Methods of Analysis

Alan Cahill and Peter Sykora

Summary

Chronic alcohol consumption has been shown to severely compromise mitochondrial protein synthesis. Hepatic mitochondria isolated from alcoholic animals contain decreased levels of respiratory complexes and display depressed respiration rates when compared to pair-fed controls. One underlying mechanism for this involves ethanol-elicited alterations in the structural and functional integrity of the mitochondrial ribosome. Ethanol feeding results in ribosomal changes that include decreased sedimentation rates, larger hydrodynamic volumes, increased levels of unassociated subunits and changes in the levels of specific ribosomal proteins. The methods presented in this chapter detail how to isolate mitochondrial ribosomes, determine ribosomal activity, separate ribosomes into nucleic acid and protein, and perform two-dimensional nonequilibrium pH gradient electrophoretic polyacrylamide gel electrophoresis to separate and subsequently identify mitochondrial ribosomal proteins.

¹A high level of magnesium ions (15–20 mM) is necessary to ensure that mitochondrial ribosomal subunits remain associated during the isolation procedure.

²To dissolve digitonin, place it in a suitable amount of buffer in a glass beaker and heat it up in a microwave. Watch for the buffer to just begin to boil then stop the microwave and remove the beaker.

³Digitonin is a detergent and is being added in a precise amount to the mitochondria to remove the outer mitochondrial membrane and in doing so eliminate the possibility of contamination by cytoplasmic ribosomes. If it is left in contact with the mitochondria for too long it will begin to permeabilize the inner mitochondrial membrane.

⁴Solubilization of the mitoplasts occurs immediately and is accompanied by a darkening of the mitoplast suspension as matrix contents are released.

⁵Puromycin is an antibiotic compound that binds to the ribosome as an analogue of aminoacyl-tRNA and arrests the formation of nascent polypeptide chains. The polypeptides chains are then liberated from the ribosome. If using puromycin be warned that the compound also has an absorbance at 260 nm and this will mask the presence of the 28S subunit (*see* Fig. 1). If the 28S subunit is required we suggest removing the puromycin by pelleting the crude ribosomes through a second sucrose cushion before layering them onto the sucrose gradients.

⁶We have found that complete dissociation of rat liver hepatic mitochondrial ribosomes is achieved by incubating them in a buffer containing no Mg²⁺ rather than low levels of Mg²⁺ as is suggested in some publications. The inclusion of 1 mM EDTA into the Mg²⁺-deficient buffer B ensures a complete absence of Mg²⁺.

⁷On the basis of the molecular weight of the 55S ribosome 1 A_{260 nm} unit approximates to 40 pmol (14).

⁸It is necessary to remove nucleic acid from protein samples that are to be used in isoelectric focusing or NEPHGE analyses because it can bind to the proteins and cause artifactual migration and streaking.

⁹Air bubbles can inhibit gel polymerization.

¹⁰Make sure that no air bubbles are present at the bottom of the tubes as they sit in the lower reservoir solution. If there are, they can easily be removed by blowing reservoir solution across the bottom of the tubes using a glass pipet that has been heated gently and curved at its narrow end.

¹¹It is very important to pipette the agarose solution gently to avoid the formation of bubbles. These can impede electrophoresis and cause distortions of the protein patterns. If air bubbles form, suck them out with a glass pipette. Also make sure that the marker plugs have no air bubbles present.

Keywords

Ethanol, liver; mitochondrial protein synthesis; mitochondrial ribosomes; ribosomal proteins; NEPHGE

1 Introduction

Mitochondrial ribosomes (mitoribosomes) are responsible for the translation of 13 proteins coded for by the mitochondrial genome. These proteins (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, Cyt b, COI, COII, COIII, ATPase6, and ATPase8) are required for the successful assembly of the respiratory complexes NADH dehydrogenase, ubiquinol: cytochrome c oxidoreductase, cytochrome c oxidase, and ATP synthase, respectively. Accurate assembly and subsequent activity of mitoribosomes is therefore essential for the maintenance and correct functioning of the electron transport chain. The rat liver mitochondrial ribosome has a molecular mass of approximately 3.57 MDa, dimensions of 26.2 nm × 23.6 nm, and a sedimentation coefficient of 55S (1). It has a chemical composition of 75% protein and 25% RNA and comprises a large 39S subunit (LSU) and a small 28S subunit (SSU). Subunits are assembled inside the mitochondrial matrix from 12S (SSU) and 16S (LSU) rRNA, encoded for by the mitochondrial genome, and ribosomal proteins, encoded for by the nucleus and imported into the mitochondrion.

One of the earliest observations seen in the liver during chronic ethanol feeding is decreased mitochondrial protein synthesis (2,3). Although a number of potential molecular mechanisms may explain this decrease, one specific mechanism involves the impaired assembly of mitoribosomes. Investigations conducted by the Cunningham lab have revealed that mitoribosomes isolated from young male rats (200 g) fed ethanol (Lieber-DiCarli diet) (4) for 31 d exhibit decreased sedimentation rates through sucrose density gradients when compared with their paired controls (5,6). Further, sedimentation velocity experiments reveal a significant decrease in the average sedimentation coefficient for the intact ethanol mitoribosome (52.2, ethanol-fed; 53.7, control) (6) as well as for the small ribosomal subunit (27.0, ethanol-fed; 28.3, control) (6). In addition, mitoribosomes isolated from ethanol-fed animals exhibit significant decreases in their translational diffusion coefficients ($1.02 \times 10^{-7} \text{cm}^2 \text{s}^{-1}$, ethanol-fed; 1.10, control) (6), and larger hydrodynamic diameters (42.1 nm, ethanol-fed; 39.1, control) (6). These ethanol-elicited alterations in the physicochemical properties of the hepatic mitoribosome are accompanied by significant amounts of disassociation of the intact 55S monosome (8–14%) into its constituent subunits (5,6). Further, in vitro studies on the translation capacity of mitoribosomes have revealed that while total translation activity is depressed by 29% in the ethanol-fed animals relative to the controls, no difference is detected between the two treatment groups in the activity of the intact monosomes (6), which suggests that the decrease in translation activity observed in ethanol-fed animals is caused by either increased disassociation or decreased association of functional ribosomes rather than impaired activity of intact monosomes. Whether this means that ethanol feeding induces increased disassociation or decreased association of mitoribosomes in vivo or whether it causes a more relaxed and loosely assembled monosome which becomes disassociated upon isolation has yet to be determined.

The sedimentation properties of mitochondrial ribosomes are affected not only by the size of the ribosomes but also by their shape. Any ethanol-elicited alterations in the ratios of constitutive mitochondrial ribosomal proteins (MRPs) may lead to a change in the shape of the ribosome and consequently a decrease in sedimentation rates. Additionally, altered MRP levels may lead to formation of inaccurately assembled ribosomes that are easily dissociated into subunits or unable to bind to functionally important docking sites on the inner mitochondrial

membrane (IMM). Further, it is unclear as to whether any of the MRPs are susceptible to the kinds of protein damage commonly incurred during chronic ethanol feeding, that is oxidative modification, nitrosative damage and formation of adducts such as acetaldehyde (AA), malondialdehyde-acetaldehyde (MAA), and the hydroxyethyl radical (HER). Ethanol-elicited modifications of MRPs may result in an alteration in their binding to other MRPs, rRNA or IMM components, as well as alter their rates of degradation. In addition to altering the overall structure of the mitochondrial ribosome, ethanol feeding has been shown to significantly decrease the levels of a specific population of MRPs (5,7). The reasons for the ethanol-elicited decreases in a specific population of MRPs as opposed to a global down-regulation are unclear; however, a recent proteomics study by Chevallet et al. (8) has demonstrated the selective depression of nine MRPs upon depletion of mitochondrial DNA (mtDNA) in 143B osteosarcoma cells. Our laboratory has shown that mtDNA depletion is a common occurrence during chronic ethanol feeding (9-11).

In this chapter, we detail methodologies for the isolation of mitochondrial ribosomes from hepatic mitochondria, determination of their activities, extraction of MRPs from the ribosomes and two-dimensional nonequilibrium pH gradient electrophoretic (NEPHGE) analysis of MRPs. Separated proteins can then be excised from the gels and subjected to mass spectrometry or transferred to nitrocellulose membranes and probed for oxidative and nitrosative modifications by western blotting.

2 Materials

2.1 Isolation of Mitochondrial Ribosomes From Mitochondria

1. Isolation buffer: 0.25 M sucrose and 2 mM HEPES, pH 7.4 (pH with 6 M KOH). Store at 4°C.
2. RC DC Protein Assay Reagents (BioRad, cat. no. 500-0120).
3. Digitonin (solid, free of water-insoluble constituents, Sigma cat. no. D-141).
4. Buffer A: 0.26 M sucrose, 40 mM KCl, 15 mM MgCl₂, 14 mM Tris-HCl, pH 7.5, and 0.8 mM ethylene diamine tetraacetic acid (EDTA; *see Note 1*). Store at 4°C. Just before use add β-mercaptoethanol to a final concentration of 5 mM.
5. Buffer B: 100 mM KCl, 20 mM MgCl₂, and 20 mM triethanolamine pH 7.5. Store at 4°C. Just before use, add β-mercaptoethanol to a final concentration of 5 mM.
6. Puromycin dihydrochloride (solid, Sigma, cat. no. P 7255).
7. Magnesium-deficient buffer B: buffer B without the MgCl₂ but including 1 mM EDTA.

2.2 Ribosome Activity Assay

1. Ribosome activity buffer: 50 mM KCl, 50 mM Tris-HCl, pH 7.8, and 20 mM MgCl₂. Store at 4°C.
2. Transfer RNA (from baker's yeast, Type, X., Sigma cat. no. R9001).
3. Pyruvate kinase (from rabbit muscle, Type VII, cat. no. P7768).
4. Phosphoenolpyruvate (Sigma, cat. no. P 7127).
5. ATP (Sigma, cat. no. A 7699) and GTP (Sigma, cat. no. G 5884).
6. L-[4-³H]Phenylalanine (Amersham, cat. no. TRK204-250UC).
7. Econo-Safe (RPI, cat. no. 111175).

2.3 Preparation of Soluble Mitochondrial Translation Factors

1. Hypotonic buffer: 20 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 40 mM KCl. Store at 4°C. Just before, use add β-mercaptoethanol to a final concentration of 5 mM.
2. 2 M KCl.
3. Sephadex G-25 (Amersham, cat. no. 17-0031-02).

2.4 Extraction of Ribosomal Proteins From Pelleted Mitochondrial Ribosomes

Both MRP extraction procedures produce similar 2DE profiles.

2.4.1 Lithium Chloride-Urea Procedure—This procedure is used in the lab when purified ribosomes or subunits are first pelleted; starting material usually two livers.

1. Ribosomal protein solubilization solution: 6 M urea, 3 M LiCl, 50 mM KCl, and 5 mM β-mercaptoethanol, pH 3.5–5.5, with 3 N HCl. Can be stored at -20°C in 500 μL aliquots.
2. 3 N HCl.
3. 20% (w/v) trichloroacetic acid (TCA).
4. Ethanol/ether (1:1 vol/vol).

2.4.2 Acetic Acid-Acetone Procedure—This procedure is used when MRPs are extracted directly from sucrose density gradient fractions without prior sedimentation of purified ribosomes or subunits; starting material can be one liver.

1. Glacial acetic acid.
2. 1 M magnesium chloride.
3. Acetone.

2.5 2D-Electrophoretic Analyses Of Mitochondrial Ribosomal Proteins (NEPHGE)

1. 40% Biolyte 3/10 ampholyte (BioRad, cat. no. 163-1113) and 40% Biolyte 5/7 ampholyte (BioRad, cat. no. 163-1153).
2. Ribosomal protein lysis buffer: 9.5 M urea (ultrapure™ urea, Invitrogen, cat #. 15505-035), 2% (w/v) Triton X-100, 2% ampholytes (1% Biolyte 3/10 ampholyte, 1% Biolyte 5/7 ampholyte). Store at -20°C as 500 μL aliquots.
3. 30% acrylamide / bis solution, 29, 1 ratio (BioRad, cat. no. 161-0156). Store at 4°C.
4. 10% (w/v) Triton X-100.
5. 10% (w/v) ammonium persulfate (Sigma, cat #. A3678). Make up fresh every 2 wk.
6. TEMED (BioRad, cat. no. 161-0801).
7. Equilibration buffer: 10% (w/v) glycerol, 2.3% (w/v) sodium dodecyl sulfate (SDS), 0.002% bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8.
8. 0.5% (w/v) agarose (ultrapure™ agarose, Invitrogen, cat. no. 15110-019) in equilibration buffer.
9. Dithiothreitol (DTT), solid (BioRad, cat. no. 161-0610).

10. Possible staining procedures: SYPRO® Ruby protein gel stain (Invitrogen, cat. no. S-12000), Brilliant Blue R staining solution (Sigma, cat. no. B6529) and SilverQuest™ Silver Staining kit (Invitrogen, cat. no. LC6070).
11. Destaining solution (for Brilliant Blue R staining only): 30% (v/v) ethanol and 10% (v/v) acetic acid.

2.6 Equipment

1. Nitrocellulose filter disks (Millipore); used in Subheading 3.2.
2. Gel extrusion needles (BioRad, cat. no. 165–1944); used in Subheading 3.5.
3. Appropriate ultracentrifuge, rotors and centrifuge tubes.
4. Microcentrifuge.
5. UV plate reader or spectrophotometer; used in Subheading 3.1.
6. Scintillation counter; used in Subheading 3.2.
7. Gradient fractionator (e.g. Labconco, Auto-Densiflow), 3.1.
8. Liquid nitrogen; used in Subheading 3.3.
9. Probe sonicator (e.g., Fisher Sonic Dismembrator 300, with Microtip); used in Subheading 3.3.
10. Lyophilizer; used in Subheading 3.3.
11. Glass tubes for NEPHGE (e.g., 7 mm OD, 5 mm ID, 15 cm length); used in Subheading 3.5.
12. Electrophoresis unit (e.g., Hoefer Scientific Instruments, GT1); used in Subheading 3.5.
13. Image analysis system and suitable software; used in Subheading 3.6.
14. Microwave (optional); used in Subheading 3.1.

3 Methods

3.1 Isolation of Mitochondrial Ribosomes From Mitochondria

This procedure provides sufficient yields of mitochondrial ribosomes for sedimentation profile analyses and, when used in conjunction with silver staining, 2D/SDS-PAGE mini-gel analyses of ribosomal proteins. If 55S ribosomal activity is to be determined or 2D electrophoretic analysis of ribosomal proteins using larger gels is intended we suggest using two rat livers as the starting tissue.

1. Isolate hepatic mitochondria using standard procedures (12).
2. Perform a protein assay on the isolated mitochondria. We use the Lowry protein assay but any detergent compatible assay will work just as well. Digitonin needs to be added to the mitochondria at a ratio of 0.11 mg of digitonin per milligram of mitochondrial protein. Dissolve a suitable amount of digitonin (*see Note 2*) in mitochondrial isolation buffer such that when the digitonin solution is added to the mitochondria it not only gives the required detergent to protein ratio but that it also dilutes the mitochondrial protein concentration down to 20 mg/mL.
3. Let the digitonin solution cool down before adding it to the mitochondria on ice. Stir the mitochondrial suspension gently for exactly 15 min (*see Note 3*).

4. Dilute the suspension down 10-fold with ice-cold isolation buffer, allow to continue stirring for a further 10 s, then centrifuge immediately at 12,000 *g* for 10 min.
5. Collect the pelleted mitoplasts and resuspend them in isolation buffer to wash them. Re-pellet the mitoplasts and resuspend them in buffer A. Mitoplasts can be stored overnight at -70°C.
6. Perform a protein assay on the mitoplasts and dilute them down with buffer A to a concentration of 20 mg/mL. Lyse them (*see Note 4*) with Triton X-100 (2% w/v final concentration).
7. Clarify the suspension at 12,000 *g* for 45 min. Remove the supernatant and layer it onto 15 ml cushions of 1 M sucrose and 1% (w/v) Triton X-100 dissolved in buffer B. Centrifuge at 200,000 *g* for 4 h (a 50.2Ti is a good choice of rotor). The pellets consist of mitochondrial ribosomes and some mitoplast membrane fragments.
8. Decant the supernatant, resuspend the crude mitochondrial ribosomal pellets in 1 ml of buffer B and layer them on top of a 10–30% linear sucrose gradient (30 mL) in buffer B and centrifuge at 53,000 *g* for 14 h (SW27 rotor). Fractionate the gradients and monitor the $A_{260\text{ nm}}$ of the fractions for ribosomal RNA. We detect the $A_{260\text{ nm}}$ of 50- μL aliquots of our gradient fractions using a 96-well fluorescent plate reader that is also capable of reading U. V. Alternatively, fractions can be monitored spectrophotometrically. Absorbance peaks of free ribosomal particles corresponding to the small (28S) and large (39S) ribosomal subunits and the intact 55S monosome should be detected (*see Fig. 1*). A small peak is also detected at the bottom of the gradient. This corresponds to a population of ribosomes that may be attached to inner mitochondrial membrane fragments via unique attachments, for example, nascent polypeptide chains being inserted into the inner mitochondrial membrane.
9. If the aim is to isolate mitochondrial ribosomal proteins without any contaminating nascent polypeptides or, alternatively, if it is required to strip from the IMM those ribosomes attached via newly synthesized polypeptides, then incubate the crude ribosomal pellet in 1 ml buffer B containing 1 mM puromycin (*see Note 5*) for 15 min at 37°C before layering on top of the sucrose gradients.
10. Fractions corresponding to the intact ribosomes or the ribosomal subunits can be centrifuged at 230,000 *g* for 5 h (60Ti rotor) and stored as a pellet at -70°C. Alternatively, the proteins associated with the intact 55S ribosome or the respective subunits can be subjected directly to electrophoresis and detected by silver staining.
11. If it is desired to separate 55S mitochondrial ribosomes into their 28S and 39S, small and large ribosomal subunits, respectively, resuspend the pelleted 55S monosomes or crude ribosomal pellets in magnesium-deficient buffer B and sediment them through 10–30% sucrose gradients made up in the same buffer (*see Note 6*).

3.2 Ribosome Activity Assay

1. Incubate 2–5 pmol of 55S mitochondrial ribosomes (*see Note 7*) in ribosome activity buffer supplemented with (final concentrations) 500 mg/mL tRNA, 100 mg/mL pyruvate kinase, 2 mM phosphoenolpyruvate, 5 mM ATP, 0.3 mM GTP, 0.2 mg/mL translation factors (*see Subheading 3.3*) and 10 μCi [^3H]phenylalanine in a final volume of 50 μL .
2. Remove 5 μL of the reaction mixture and spot onto a nitrocellulose filter disk. This represents time zero. Start the reaction by adding poly(U) to a final concentration of 1 mg/mL, remove 5 μL aliquots at suitable time points up to 1 h (we find that radiolabel

incorporation is linear for this time period) and spot them onto nitrocellulose filter disks (1 cm²).

3. Incubate the filter disks in ice-cold 10% TCA (w/v, 2 mL per 1 cm² filter) for 30 min with gentle swirling to stop the reaction and to precipitate the protein onto the filter.
4. Wash the disks in fresh ice-cold 10% TCA for 10 min with gentle swirling.
5. Place the disks in 5% TCA and heat to 90°C for 15 min.
6. Quantify the radioactivity present on the filters by adding 5 mL of a scintillation cocktail of choice (Econo-Safe works well for us). Express the phenylalanine polymerization as pmol [³H]phenylalanine / mg RNA / pmol mitochondrial ribosomes.

3.3 Preparation of Soluble Mitochondrial Translation Factors

1. Resuspend mitochondria in hypotonic buffer at a concentration of 5 mg/mL and incubate on ice for 15 min to allow the mitochondria to swell.
2. Subject the mitochondria to three cycles of freeze-thawing using liquid nitrogen then sonicate (we use 4 × 30 s pulses at 50%; *see* Subheading 2.5.) to disrupt the mitochondria.
3. Clarify the mitochondrial suspension at 16,000 g for 20 min at 4°C. Remove the supernatant and adjust it to 500 mM KCl.
4. Centrifuge at 200,000 g for 4 h at 4°C to pellet mitochondrial ribosomes.
5. Pass the supernatant over a Sephadex G-25 column (to remove endogenous amino acids) and collect the void volume. This contains the mitochondrial translation factors. Lyophilize the translation factors and store at -70°C.

3.4 Extraction of MRPs From Pelleted Mitochondrial Ribosomes

3.4.1 Lithium Chloride-Urea Procedure—

1. Resuspend 55S mitochondrial ribosomes or mitoribosomal subunits in 150 µL of ribosomal protein solubilization solution in a 1.5 mL microcentrifuge tube.
2. Place a micro stir bar in the centrifuge tube (the stir bar will stand on its end) and stir the ribosomes overnight at 4°C (make sure the sample is stirring and it is not the tube that is spinning).
3. Pellet the RNA at 200,000 g (we use a TLA-100 rotor with 200 µL capacity tubes) for 1 h. Remove the supernatant and reserve. Re-extract the RNA pellet by adding another 150 µL of ribosomal protein solubilizing solution. Stir for 6 h and pellet the RNA at 200,000 g for 1 h (*see* **Note 7**). Remove the supernatant and combine it with the supernatant from the first extraction.
4. The RNA pellet should contain rRNA and any mRNA that remains associated with the ribosome.
5. Precipitate the ribosomal proteins overnight at 4°C by adding 2 volumes of 20% TCA. Centrifuge at 10,000 g in a microcentrifuge for 15 min. Remove the supernatant and wash the pellet in 1:1 (v/v) ethanol:ether.
6. Allow to air-dry for 5–10 min before resuspending the ribosomal proteins in an electrophoresis buffer of choice. We suggest using ribosomal protein lysis buffer supplemented with DTT to a final concentration of 1% (w/v) just before use.

3.4.2 Acetic Acid-Acetone Procedure—We have used this procedure on as little as 0.1 A_{260 nm} units of mitochondrial ribosomes.

1. Collect fractions from sucrose density gradients (*see* Fig. 1) corresponding to intact 55S monosomes or ribosomal subunits.
2. Add magnesium chloride to a final concentration of 100 mM.
3. Add 2 volumes of glacial acetic acid. Invert a couple of times. Leave for 45 min on ice.
4. Pellet precipitated RNA at 5000 g for 10 min.
5. Add 5 volumes of ice-cold acetone. Invert a couple of times. Leave at -20°C overnight.
6. Pellet precipitated protein at 5000 g for 10 min.
7. Wash pellets with ice-cold acetone. Re-pellet protein at 5000 g for 10 min.
8. Remove supernatant and allow pellet to air-dry (approximately 10 min).
9. Resuspend pellet in ribosomal protein lysis buffer supplemented with DTT to a final concentration of 1% (w/v) just before use (*see* Subheading 2.4.).

3.5 2D-Electrophoretic Analyses Of Mitochondrial Ribosomal Proteins (NEPHGE)

Early isoelectric focusing methodologies in which ampholytes were used to form pH gradients suffered from gradient drift. The development in recent years of immobilines (buffering acrylamide derivatives that contain either a free carboxylic acid group or a tertiary amino group) has allowed the formation of stable pH gradients in the range of pH 3–12. These immobilized pH gradients (IPGs) are reproducible and insensitive to disturbances from sample components. We have used the ZOOM® IPGRunner™ system from Invitrogen in conjunction with silver staining to analyze the protein content of the large ribosomal subunit from control and ethanol mitochondrial ribosomes. Figure 1 shows a sucrose density gradient profile of mitochondrial ribosomes isolated from control and ethanol-fed animals. Ethanol-fed animals show decreased levels of intact 55S ribosomes but an increase in the level of 39S ribosomal subunits. This is suggestive of an ethanol-elicited increase in ribosome dissociation or a decrease in ribosome association. The 39S peaks from Fig. 1 were extracted using the acetic acid-acetone procedure (*see* Subheading 2.3.2.) and separated in the first dimension by isoelectric focusing using IPGs (ZOOM strips, Invitrogen) of range pH 6–10 followed by second dimension SDS-polyacrylamide gel electrophoresis (PAGE; Fig. 2). A significantly greater amount of ribosomal protein was extracted from the 39S peak in the ethanol-fed animals when compared with the paired control. This greater yield of ribosomal protein reflects the increase in 39S subunit seen in Fig. 1 and suggests that the increase in 39S fraction as measured by A_{260 nm} is the result of increased levels of intact 39S subunits and not due to increased levels of mRNA and rRNA associated with the 39S fraction.

The methodologies involved in using Invitrogen's ZOOM® IPGRunner™ system are described in detail in the chapter "Proteomic Approaches to Identify and Characterize Alterations to the Mitochondrial Proteome in Alcoholic Liver Disease" by Bailey et al. in this volume. We will therefore focus here on an alternative and excellent method of separating ribosomal proteins, that is, NEPHGE. Ribosomal proteins are, for the most part, extremely basic in charge. The 39S ribosomal subunit for example is believed to comprise 48 proteins with pIs ranging from 6.6 to 12.3 with an average of 9.6 (13). Even if these proteins were to be focused using IPGs, it would be difficult to include them all on the same 2D/IEF/ SDS-PAGE gel. During NEPHGE proteins are not necessarily focused to their pIs but instead migrate at different rates across the gel due to their charge. At the end of the run, some proteins will have focused to their pIs while the others will still be migrating. The protein pattern

produced on the gel is dependent on the accumulated volt hours so to ensure reproducibility this must remain constant for a specific population of proteins. The method described below is intended for use with the larger 2D system, that is, 15-cm tube gels and 16 cm × 16 cm SDS-PAGE slab gels which we find to be excellent for resolving the complete population of ribosomal proteins. Mini-systems such as the Mini-PROTEAN® Tube Cell Module from Bio-Rad can be used but we find the larger set-up easier to manipulate (Fig. 3).

1. Rinse out the glass tubes with water followed by acetone. Allow to air dry.
2. Stopper one end of the tubes with parafilm.
3. To make 10 mL of gel mix, place 5.5 g of urea, 1.33 mL of 30% acrylamide mix, 2 mL of 10% Triton X-100, 1.97 mL of dH₂O, 0.25 mL of Biolyte 3/10 ampholyte (40%), and 0.25 mL of Biolyte 5/7 ampholyte (40%) into a 125-mL side-arm flask.
4. Heat slightly to dissolve urea.
5. Add 30 µL of AMPS.
6. De-gas for 1 min (*see Note 9*).
7. Add 21 µL of TEMED.
8. Pour tube gels using a suitable syringe to approx 1 cm from the top. Overlay with dH₂O. Leave to polymerize for 1–2 h. To test whether the gel mix has polymerized successfully we suggest sucking up any remaining gel mix, after the tubes have been poured, into a plastic pipet. Once it has polymerized inside the pipet it is usually safe to assume that it has also polymerized inside the tubes.
9. Fill the lower reservoir of the electrophoresis apparatus with 0.02 N NaOH. Remove the parafilm from the bottom of the tubes. Place the tubes inside the tube holder according to apparatus instructions (*see Note 10*).
10. Load ribosomal protein sample solubilized in ribosomal protein lysis buffer (50 µL is a good volume) onto polymerized gel. For Coomassie blue staining of gels, we suggest 200 µg per tube gel, and for silver staining of gels we suggest 50 µg. We suggest using the RC DC Protein Assay (BioRad, cat. no. 500–0120) to quantify sample protein levels. This assay is compatible with a number of components of the ribosomal protein lysis buffer, for example, ampholytes and thiol reducing agents that would normally interfere with protein determination.
11. Overlay with ribosomal protein lysis buffer diluted 1 to 4 leaving a small space at the top.
12. Fill the tube to the top with upper reservoir solution, that is, 0.01 M phosphoric acid.
13. Run electrophoresis with the polarity reversed i.e. red lead from apparatus into black port on power pack, black into red. Run at 400 V for 1 h and 30 min depending upon the desired separation profile. This corresponds to 600 Vh.
14. Extrude gel from glass tube using a syringe with a long thin gel extrusion needle. Fill the syringe with dH₂O and insert it gently inside the glass tube down the side of the gel. The needles recommended can easily be slid inside the tubes to just over half way. Pipet water gently while sliding the needle around the outside of the gel. Then withdraw the needle, turn the glass tube around and reinsert the needle into the other side of the tube. Gently pipet water as before. On withdrawing the needle from the tube this time, the gel inside should slide easily out. If not, determine which end of the gel is sticking and reinsert the needle into that side and pipette. We suggest placing a sheet of parafilm or aluminum foil on the lab bench to catch the tube gel should it slide out unexpectedly during extrusion.

15. Place tube gel into 15 mL conical tube and add 5 mL of equilibration buffer supplemented with DTT (0.5% w/v) just before use. Incubate with gentle rocking for 30 min.
16. Pour an SDS slab gel (8–20% resolving gel works well, 5% stacking gel) according to standard procedures. Assemble the electrophoresis apparatus according to manufacturer's instructions.
17. Add a small amount of 1% agarose dissolved in equilibration buffer containing 0.5% (w/v) DTT to the top of the gel to serve as a platform for the tube gel to sit on. Once the agarose has set, lay the tube gel on top. Allow some room at one end to add molecular weight markers. We normally embed a small volume of markers in about 20 μ L of 1% agarose in equilibration buffer containing 0.5% DTT in the bottom of a 1.5 mL centrifuge tube. After the agarose has set pop out the markers plug and sit it next to the tube gel on the agarose platform.
18. Pipet more of the agarose/equilibration buffer solution over the top of the tube gel and markers plug to hold them in place (*see Note 11*).
19. Run the gel at 25 mA for 7 h or until the Bromophenol blue has migrated to the bottom of the gel.
20. Detect ribosomal proteins using a suitable staining procedure. We have successfully used Coomassie Brilliant Blue R250, SYPRO® Ruby protein gel stain and silver staining procedures. All these staining systems are compatible with the excision of protein spots from the gels, tryptic digestion of the proteins and analysis by mass spectrometry.
21. Alternatively, the proteins can be transferred from the gel to nitrocellulose membranes and analyzed for specific protein modifications by western blotting.

3.6 Analysis of Proteins Separated by 2DG

A number of current systems and software products are available for protein analyses in 2D gels. Our laboratory possesses a Kodak ImageStation 440CF with Kodak Digital Science 1D & 2D Software. This is convenient for protein staining via Coomassie Brilliant Blue, SYPRO® Ruby and silver.

Acknowledgments

This work was supported by grant AA14151 from the National Institute of Alcohol and Alcohol Abuse.

References

1. Patel VB, Cunningham CC, Hantgan RR. Physiochemical properties of rat liver mitochondrial ribosomes. *J. Biol. Chem* 2001;276:6739–6746. [PubMed: 11106644]
2. Coleman WB, Cunningham CC. Effects of chronic ethanol consumption on the synthesis of polypeptides encoded by the hepatic mitochondrial genome. *Biochim. Biophys. Acta* 1990;1019:142–150. [PubMed: 2169877]
3. Coleman WB, Cunningham CC. Effect of chronic ethanol consumption on hepatic mitochondrial transcription and translation. *Biochim. Biophys. Acta* 1991;1058:178–186. [PubMed: 1710928]
4. Lieber CS, DeCarli LM. The feeding of alcohol in liquid diets: Two decades of applications and 1982 update. *Alcohol. Clin. Exp. Res* 1982;6:523–531. [PubMed: 6758624]
5. Cahill A, Baio DL, Ivester P, Cunningham CC. Differential effects of chronic ethanol consumption on hepatic mitochondrial and cytoplasmic ribosomes. *Alcohol. Clin. Exp. Res* 1996;20:1362. [PubMed: 8947311]

6. Patel VB, Cunningham CC. Altered hepatic mitochondrial ribosome structure following chronic ethanol consumption. *Arch. Biochem. Biophys* 2002;398:41–50. [PubMed: 11811947]
7. Cahill A, Cunningham CC. Effects of chronic ethanol feeding on the protein composition of mitochondrial ribosomes. *Electrophoresis* 2000;21:3420–3426. [PubMed: 11079562]
8. Chevallet M, Lescuyer P, Diemer H, van Dorsselaer A, Leize-Wagner E, Rabilloud T. Alterations of the mitochondrial proteome caused by the absence of mitochondrial DNA: A proteomic view. *Electrophoresis* 2006;27:1574–1583. [PubMed: 16548050]
9. Cahill A, Wang X, Hoek JB. Increased oxidative damage to mitochondrial DNA following chronic ethanol consumption. *Biochem. Biophys. Res. Commun* 1997;235:286–290. [PubMed: 9199183]
10. Cahill A, Stabley GJ, Wang X, Hoek JB. Chronic ethanol consumption causes alterations in the structural integrity of mitochondrial DNA in aged rats. *Hepatology* 1999;30:881–888. [PubMed: 10498638]
11. Cahill A, Hershman S, Davies A, Sykora P. Ethanol feeding enhances age-related deterioration of the rat hepatic mitochondrion. *Am. J. Physiol. Gastrointest. Liver Physiol* 2005;289:G1115–G1123. [PubMed: 16020655]
12. Cohen NS, Kyan FS, Kyan SS, Cheung CW, Rajman L. The apparent K_m of ammonia for carbamoyl phosphate synthetase (ammonia) in situ. *Biochem. J* 1985;229:205–211. [PubMed: 4038255]
13. Koc EC, Burkhardt W, Blackburn K, Moyer MB, Schlatzer DM, Moseley A, Spremulli LL. The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. *J. Biol. Chem* 2001;276:43958–4369. [PubMed: 11551941]
14. Ulbrich B, Czempel W, Bass R. Mammalian mitochondrial ribosomes. (1980) Studies on the exchangeability of polypeptide chain elongation factors from bacterial and mitochondrial systems. *Eur. J. Biochem* 108:337–343. [PubMed: 6157531]

Fig. 1. Effects of chronic ethanol feeding (12 mo) upon the sucrose density gradient profiles of mitochondrial ribosomes and their subunits isolated from a single control and alcoholic liver. Sucrose cushion purified hepatic mitochondrial ribosomes were separated through 10–30% sucrose gradients into monosomes and subunits. The A_{260 nm} of the 50-μL fraction aliquots was monitored using a 96-well fluorescent plate-reader (in UV mode)

Fig. 2. Two-dimensional (IEF/SDS-PAGE) proteomic analysis of the hepatic mitochondrial LSU. Proteins were extracted from the LSU 39S peaks shown in Fig. 1 using acetic acid and acetone (see Subheading 3.4.2.), separated in the first dimension on pH 6–10 ZOOM IPG strips (see chapter by Bailey et al. in this volume) and in the second dimension by 12% SDS-PAGE. Proteins were stained with silver. (A) Proteins were extracted from control 39S peak and (B) from ethanol 39S peak

Fig. 3. Two-dimensional (NEPHGE/SDS-PAGE) proteomic analysis of mitochondrial ribosomal proteins extracted from intact 55S monosomes (modified from ⁷). Proteins were extracted from the 55S monosome using lithium chloride and urea (*see Subheading 3.4.1.*), separated in the first dimension by NEPHGE (pH 3–10) and in the second dimension by 12% SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue