Chapter 18

Genome-Wide Analysis of RNA Extracted from Isolated Mitochondria

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Abstract

Isolating mitochondria by subcellular fractionation is a well-established method for retrieving intact and functional mitochondria. This procedure has been used to identify proteins of the mitochondria and to explore import mechanisms. Using the same method, it was shown that mitochondria can be purified along with cytoplasmic ribosomes and nuclear-encoded mRNAs attached to the outer membrane. Combining this procedure with DNA microarray analysis allows for global identification of the mRNAs associated with mitochondria, and hence a better understanding of the underlying molecular mechanisms. In this chapter, we will describe a procedure for the isolation of mitochondria from yeast and RNA purification. We will then describe the process of labeling and hybridization to DNA microarrays, and comment on a few aspects of the data analysis.

Key words: DNA microarrays, Mitochondria, RNA localization

1. Introduction

A key feature of eukaryotic cells is their organization into distinct compartments having specific functions. Each cellular compartment contains a unique set of proteins that are essential for its activity. As protein synthesis occurs throughout the cytoplasm, these proteins need to be targeted to their proper compartment. This targeting may occur through the utilization of various chaperones and import receptors that bind the fully synthesized protein and escort it to the correct site (1–3). It is well established, however, that many organellar proteins are synthesized near their site of activity and not at distant sites in the cytoplasm. ER resident proteins, for example, are translated directly into the ER membrane or lumen (4). Such a localized protein synthesis minimizes the chances of function at inappropriate sites and may

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increase the efficiency of protein targeting. Consistent with this process, many mRNAs were found to localize near the site of action of their encoded protein. Moreover, in many cases, mRNA localization occurs prior to the initiation of protein synthesis, and mRNA mislocalization may be associated with mislocalization of its encoded protein (5).

The mitochondrion, the cell respiratory organelle, is enclosed with a double membrane. It contains one circular chromosome that contains a small number of genes with roles mainly in protein synthesis. However, the majority of mitochondrial proteins is encoded in the nucleus and must be transported into the mitochondria (6). It was suggested more than 30 years ago that many of these proteins are synthesized near the mitochondria and transported co-translationally. This was based on the observation that ribosomes associated with mRNAs encoding mitochondrial proteins accumulate on the surface of yeast mitochondria (7–10). More recent in vivo studies, in which either the import of fully synthesized proteins into mitochondria (11) or the targeting of ribosomes to the mitochondria surface was inhibited (12), further supported the notion that many mitochondrial proteins are inserted into the mitochondria while being translated. The predominance of this co-translational process became apparent following genome-wide studies in *Saccharomyces cerevisiae*, which identified the mRNA population associated with mitochondria (13–15). Whether these mRNAs approach the mitochondria prior to translation initiation or while being translated is unclear; some models suggest that targeting occurs while the protein is being synthesized, and the translated mitochondrial targeting sequence (MTS) assists in the association of the translation complex (mRNA-ribosome and nascent peptide) with the mitochondria (16). In agreement with this model, we have recently shown that either changing just two amino acids in an MTS or deleting a mitochondria import receptor leads to a profound decrease in the mitochondrial association of mRNAs (15). On the other hand, noncoding domains (most significantly 3′ UTRs) were also shown to be involved in mRNA association to the mitochondria (17, 18). The association through these domains appears to necessitate in some cases an RNA-binding protein from the PUM family (Puf3) (19). Since many PUM family proteins are known to be involved in translation inhibition (20), it is possible that the role of Puf3 is to exclude translation of its target mRNA while en route to the mitochondria. Future studies may reveal the exact mechanisms and dynamics of mRNA targeting to the mitochondria.

2. Materials

1. Galactose Growth Medium – autoclaved 1% yeast extract, 2% Bacto peptone medium supplemented with 2% filtered galactose.
2. Dithiothreitol (DTT) Buffer – freshly prepared 0.1 M Tris–HCl at pH 9.4 supplemented with 10 mM DTT.
3. Sorbitol Buffer – 1.2 M sorbitol in Tris–HCl at pH 7.4 (use filtered buffer).
4. Zymolyase – Zymolyase 20T (Seikagaku America, INC). Should be weighed and dissolved in Sorbitol buffer just before use.
5. Recovery Medium – Galactose growth medium supplemented with 1 M sorbitol.
6. Lysis Buffer – 0.6 M mannitol, 30 mM Tris–HCl at pH 7.6, 5 mM MgAc, 100 mM KCl and freshly added 0.1 mg/ml cycloheximide (CHX), 0.5 mg/ml Heparin, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Filter and use ice-cold. Can be stored at 4°C for at least a month.
7. Phenol:Chloroform (5:1) at pH 4.7 (Sigma P1944).
8. ImProm-II reverse transcription system (Promega, A3802).
9. 25× Amino-allyl mix – 12.5 mM of each dATP, dGTP, dCTP, 5 mM dTTP, and 7.5 mM amino-allyl dUTP (Ambion #8439).
10. DNA clean and concentrator kit (Zymo Research, D4004).
11. Slides Blocking Buffer – 0.5% BSA (A7906 sigma), 5× saline-sodium citrate buffer (SSC), and 1% SDS.
12. Hybridization Buffer – 2× MWG buffer (Ocimum Biosolutions 1180-000010).
13. PolyA (Sigma, P9403) dissolved to 10 µg/µl with water. Keep at –20°C.

3. Methods

The following protocol is designed for global determination of mRNA association with the mitochondria of yeast *S. cerevisiae*. It entails enzymatic degradation of the cell wall and gentle breakage of the plasma membrane by moderate mechanical force, without the use of detergent. Heavy complexes, which include mitochondria and other large compartments, are precipitated at 10,000 × *g* and RNA is extracted from this crude mitochondrial pellet. The

RNA is then labeled with red fluorescent dye and hybridized to DNA microarrays together with green-labeled reference RNA. The resulting fluorescent signals represent the relative association of mRNAs to the mitochondria. Herein we provide a detailed protocol for this procedure, which could be modified easily for the analyses of cells grown under different conditions or of deleted genes that may be involved in the targeting process.

3.1. Mitochondrial Fractionation

This part describes a procedure for the isolation of mitochondria that results in a crude mitochondrial fraction (i.e., that may include other cellular compartments) (Fig. 1). Further purification using a sucrose density gradient (e.g., according to (21)) is possible, yet it may lead to loss of mitochondria-associated mRNAs and therefore reduce the quality of the results. In some experimental settings (such as comparative analyses), the presence of other cellular parts (e.g., plasma membrane and ER) may not pose a limitation as they will be present at similar levels in all samples.

1. Grow 500 ml of cells to OD 0.8 at A_{600} in 30°C on Galactose Growth Medium (see Note 1).
2. Centrifuge cells at 3,000 × *g* for 4 min at room temperature, resuspend cells in double distilled water, centrifuge again, and discard the supernatant.
3. Resuspend the pellet in 20 ml of DTT Buffer. Incubate at 30°C for 10 min with gentle shaking (see Note 2).
4. Centrifuge the sample at 3,000 × *g* for 4 min at room temperature, discard the supernatant, and resuspend the pellet in 10 ml Sorbitol Buffer. Measure the OD, at A_{600}, of a 20 µl aliquot diluted with 980 µl of water.

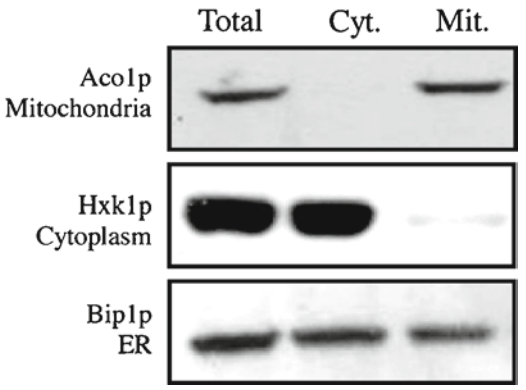

Fig. 1. Western analysis of fractionation quality. Equal fractions from the unfractionated extract (Total), cytosolic extract (Cyt), and mitochondria (Mit) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and tested with antibodies recognizing the indicated marker proteins.

5. To break the cell wall and convert cells to spheroplasts, add 36 mg of Zymolyase. Incubate cells with gentle shaking for 15 min at 30°C. Completion of cell wall hydrolysis is verified by mixing 20 µl from the sample with 980 µl of water and measuring the OD at A_{600}; a tenfold decrease in the OD, at A_{600}, compared to the previous step is expected because spheroplasts are quickly lysed in water. If not, continue the incubation until the required decrease is reached (see Note 3).
6. Centrifuge the sample at 2,000 × *g* for 4 min at room temperature. Wash once with 20 ml of Sorbitol Buffer, discard the supernatant, and resuspend the pellet in 100 ml Recovery Medium. Incubate the spheroplasts for 2 h at 30°C while shaking (see Note 4).
7. Add 0.1 mg/ml CHX and immediately spin the samples at 2,000 × *g* for 4 min at 4°C (see Note 5).
8. Wash the sample twice with cold 5 ml Lysis Buffer.
9. Resuspend the sample with 5 ml Lysis Buffer and gently break the cells with 15–20 strokes with a tight-fitting Dounce homogenizer. Move the sample to 13-ml tubes (e.g., Sarstedt D-51588).
10. Centrifuge the sample at 450 × *g* for 6 min at 4°C and carefully place the supernatant in a new tube. Avoid touching the pellet, which contains unbroken cells and nuclei. Set aside 25% of the supernatant, which can be used to extract RNA (by the "hot phenol" method (22)) or proteins (by adding a protein loading buffer). This will serve as an unfractionated ("Total") control.
11. Centrifuge the supernatant at 10,000 × *g* for 10 min at 4°C. Place the supernatant in another tube. RNA or proteins can be extracted from this sample and serve as a cytosolic control.
12. Resuspend the pellet, which includes the mitochondria, with 5 ml of cold Lysis Buffer, centrifuge at 10,000 × *g* for 10 min at 4°C, and remove residual cytosolic components.
13. Resuspend the pellet with 3 ml of cold Lysis Buffer. This is the crude mitochondrial fraction.
14. The quality of the fractionation procedure can be evaluated by Western analysis. Aliquots of the unfractionated (Total) sample (Subheading 3.1, step 10), cytosolic sample (Subheading 3.1, step 11), and mitochondrial sample (Subheading 3.1, step 13) are resolved by SDS-PAGE and probed with antibodies to various cellular markers. Figure 1 presents representative results of such an analysis. Two aspects should be evaluated: (1) losses during fractionation that will be apparent if the sum of signals in the Mit and Cyt fractions

is not equal to the signal in the Total sample; and (2) purity of the fractions – the mitochondrial sample should be devoid of cytosolic markers and the cytosolic fraction devoid from mitochondria markers. It should be noted that markers of some cellular organelles (most significantly the ER) usually appear in the mitochondria sample. This presence may be due to their similar mass or to their established physical connections (23–25).

3.2. RNA Extraction

1. Add one volume of 8 M *Guanidinium-HCl* to the mitochondrial fraction, mix gently, add two volumes of 100% ethanol and mix gently by inverting the tube. Incubate in –20°C for at least 2 h. (The same extraction procedure can be done for the cytosol fraction or the unfractionated sample if RNA extraction is desired).
2. Centrifuge the samples at 11,000×*g* for 20 min at 4°C and discard the supernatant. Wash the pellet with 5 ml of ice-cold 80% ethanol (do not resuspend) and centrifuge again at 11,000×*g* for 20 min at 4°C. The pellet is visible and stable at this stage due to the presence of heparin.
3. Remove the supernatant and resuspend the pellet with 400 µl RNAse-free water. Transfer the sample to a microtube, and precipitate the RNA by adding 0.1 volume of 3 M sodium acetate at pH 5.2 and two volumes of 100% ethanol. Incubate for at least 2 h at –20°C and spin at maximal speed for 20 min at 4°C. Wash with ice-cold 80% ethanol and spin again for 20 min at 4°C.
4. Resuspend the pellet with 650 µl of RNase-free water. Add an equal volume of Phenol:Chloroform (5:1) (pH 4.7) and vortex vigorously. Spin at top speed for 5 min at room temperature. Take 500 µl of the aqueous phase (upper layer) into a new microtube (see Note 6).
5. Add 350 µl of RNase-free water and 150 µl 10 M LiCl (final concentration of 1.5 M), and incubate overnight at –20°C. Thaw the sample on ice and centrifuge at top speed for 20 min at 4°C. Wash the pellet carefully (as the pellet is transparent and unstable) with 200 µl of cold 80% ethanol and resuspend in 150 µl of RNase-free water (see Note 7).
6. Precipitate the RNA with sodium acetate as in Subheading 3.2, step 3, wash with 80% ethanol, and air dry. Resuspend the pellet in RNase-free water. The sample can be stored at –80°C.

3.3. Fluorescent Labeling

The protocol below is a general one that can be used for labeling RNA obtained by various methods (e.g., "hot phenol" extraction, polysomal mRNA preparations, or co-immunoprecipitated mRNAs).

The RNA is converted to cDNA that contains an amino-allyl modified nucleotide (usually deoxyuridine) by reverse transcription reaction. This nucleotide is then coupled with a fluorescent dye. We usually label the mitochondria fraction with a Cy5 fluorescent dye and hybridize it to a DNA microarray together with a reference RNA that is labeled with Cy3 dye. The reference sample allows correcting problems in hybridization and variations in spot quality (see Note 8 for selection of reference sample). Thus, for every gene, the microarray results are best interpreted as the ratio of fluorescence signals between the sample and the reference.

1. Mix 15–50 µg RNA with 5 µg Oligo dT (T20VN (V = any nucleotide except T)). Adjust to 15.5 µl with nuclease-free water (see Note 9).
2. Denature RNA secondary structures by incubating the mixture for 10 min at 70°C and then transfer to ice for 10 min.
3. We utilize Promega's ImProm-II reverse transcription system for cDNA synthesis. Mix 6 µl 5× reaction buffer, 4 µl 25 mM $MgCl_2$, 1.2 µl 25× amino-allyl mix, 3.0 µl reverse transcriptase, and 0.3 µl nuclease-free water (total volume of 14.5 µl) and incubate at 42°C for 2 h.
4. Degrade RNA by adding 10 µl of 1 N NaOH and 10 µl of 0.5 M EDTA, and incubate at 65°C for 15 min.
5. Neutralize by adding 25 µl of 1 M HEPES (pH 7.0) and 25 µl of nuclease-free water.
6. Purify the cDNA using a DNA clean and concentrator kit (Zymo Research, D4004), and resuspend in 9 µl of nuclease-free water. The amino-allyl labeled cDNAs can be stored at –80°C for at least a month.
7. Add 1 µl of sodium bicarbonate 1 M (pH 9.0) to the cDNA and 1 µl of a fluorescent dye (see Note 10).
8. Incubate at room temperature protected from light for 1 h to allow coupling of the dye to the amino-allyl groups.
9. Purify the Cy-labeled cDNA using a DNA clean and concentrator kit (Zymo Research, D4004). Elute with nuclease-free water in a final volume of 5 µl.

3.4. Microarray Hybridization

The following hybridization procedure is for aminosilane-coated glass slides that are spotted with long (70 mer) oligonucleotides (Operon AROS for yeast).The oligos were designed to yield minimal cross-hybridization between genes. Slides are stored in the dark under desiccation and are handled with powder-free gloves. As the spotted DNA will not be visible during the process, the array boundaries should be marked with a diamond pen on the back of the slide prior to their use.

1. Place the slides at 42°C for 1 h in a coupling jar containing preheated and filtered slide-blocking buffer.
2. Carefully remove the slides from the blocking solution and wash three times with 0.1× SSC for 5 min each. Wash the slide in double distilled *water* for 30 s and spin at 500 rpm for 5 min at room temperature to dry. Slides are ready for hybridization.
3. Prepare the hybridization mixture by combining 10 µl labeled cDNA (5 µl of the mitochondria sample and 5 µl of the reference sample) with 12.5 µl of hybridization buffer and 1 µl of PolyA that was preheated at 95°C for 3 min.
4. Set the microarray slide in a hybridization chamber. Carefully pipette the hybridization mixture on the spotted area and cover with a cover slip. Incubate in a water bath at 42°C overnight in the dark.
5. Wash the slides at the end of the hybridization by three consecutive washes, each for 12 min with gentle shaking: first wash with 2× SSC and 0.2% SDS that were prewarmed to 42°C, then with 2× SSC, and finally with 0.2× SSC (see Note 11).
6. Spin-dry the slide by centrifugation at 50×*g* for 5 min at room temperature. Store the dried slides in a dark box until scanning.
7. Scan with a laser scanner (e.g., GenePix 4000B) with settings that yield less than 10% saturated signals. If necessary, scan at two laser intensities. Spots with signals that are too close to their local background include too few pixels or those having an irregular structure are flagged out (we use "GenePix" software from Molecular Devices to assist with the flagging procedure and the data retrieval).

3.5. Data Analysis

Hybridization procedures in which two differently labeled samples are hybridized to the same DNA microarray result in two values per gene: a value that represents its transcript levels in one sample and a second value that represent the amount of transcripts in the second sample. In the specific example described herein, one value represents the mRNAs that are associated with the mitochondria, and the other value represents presence in the cytosolic fraction. Thus, the ratio between these values is the relative association of the transcripts of a gene with the mitochondria. Changes in this ratio upon altering of growth conditions or utilizing mutants of various genes provide important information regarding the mechanisms of targeting to the mitochondria. These studies necessitate multiple microarray hybridizations that are used to obtain relative associations to the mitochondria for thousands of genes. The simplest way to identify changes in mRNAs association is by dividing the ratios obtained in one

experiment by those obtained in another experiment. This will give the fold-change in mRNAs association to the mitochondria. Genes, in which the association of their mRNAs to the mitochondria was most affected (increased or decreased), can be selected for further analysis by defining a cut-off (e.g., differences having twofold changes from the mean change of all genes). An alternative and more robust way to identify affected targets is to generate a scatter plot (e.g., Fig. 2a) in which the ratios obtained from one sample are plotted against those from another sample. This plot identifies global changes occurring throughout the genome, which will be apparent from the slope of the best-fit trend line and from the distribution of spots around that line. It is possible to set standard deviation lines around the trend line and extract genes that deviate beyond these lines.

The extended experimental procedure and the fact that many mRNAs are not associated with mitochondria usually result in a low number of genes that pass the filtration criteria. This relatively low number limits statistical analyses because many genes may not pass the filtration criteria in all/most experimental

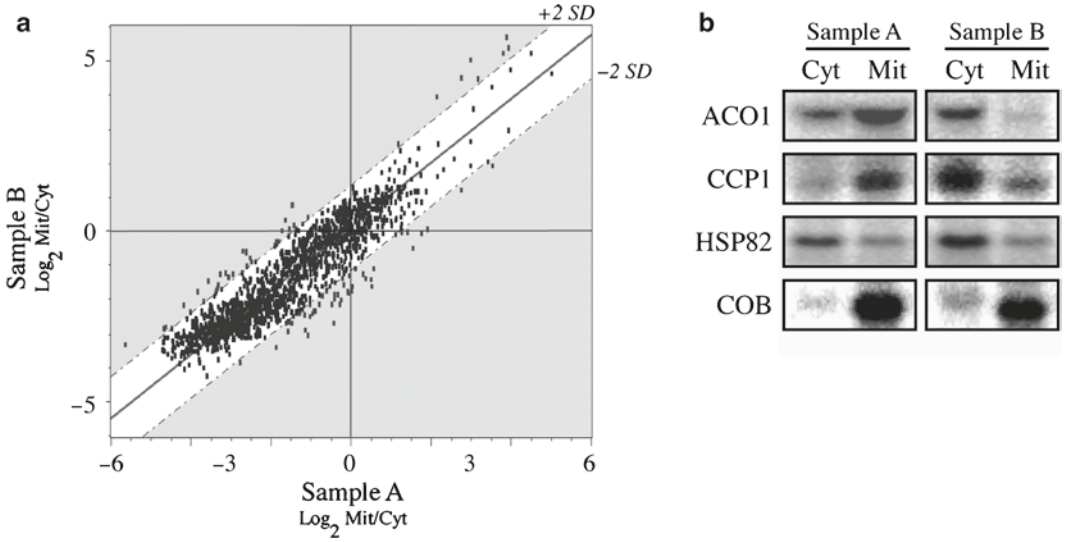

Fig. 2. Representative results of microarray analysis of two fractionated samples. Two yeast strains were subjected to the fractionation procedure. The Mit and Cyt fractions from each strain were labeled differentially and cohybridized to a DNA microarray. This resulted in a Mit/Cyt ratio for transcripts of thousands of genes from either Strain A or Strain B. (**a**) Scatter plot representation of microarray results from Strain A (*X*-axis) versus Strain B (*Y*-axis). Each *dot* indicates the Log_2 of the Mit/Cyt ratio of transcripts of a particular gene. The best-fit linear trend line is indicated and the ±2 standard deviation (SD) lines (*dashed lines*). Spots below the −2 SD or above the +2 SD lines (*gray areas*) indicate mRNAs that are relatively less associated or more associated with mitochondria in Strain B compared to Strain A, respectively. (**b**) Northern analysis for representative genes. Equal amounts of RNA from the Cyt or Mit fractions of Strain A or Strain B were subjected to Northern analyses using probes recognizing the indicated mRNAs. *ACO1* and *CCP1* mRNAs present significant change in their Cyt:Mit distribution and are likely to appear in the *gray area* in the scatter plot. The distribution of *HSP82* and *COB* does not change and is therefore likely to be within the two SD lines (*white area*).

repeats. Thus, the validity of the results for mRNAs of interest should be tested using alternative methods, such as Northern analysis (Fig. 2b), qRT-PCR, or Fluorescence In Situ Hybridization (FISH) (14). Yet, the genome-wide analysis of mitochondrial association provides important information beyond identification of a gene with altered association. It allows selection of a group of mRNAs with altered association and testing for features that are common to this group. These features may be their intracellular localization (e.g., regions of the mitochondria such as inner or outer membrane), or their molecular function (e.g., oxidative phosphorylation and mitochondria translation). Hints regarding their mechanisms of targeting can be obtained from examining physical properties of the encoded protein (e.g., the presence of a signal peptide and its charge) or mRNA features such binding motifs for RNA-binding proteins. Several tools are available for these analyses. The *Saccharomyces* genome database (SGD) Gene Ontology Term Finder, and Slim Mapper tools (http://www.yeastgenome.org/GOContents.shtml) allow rapid and reliable identification of enrichment of certain intracellular functions, locations, or molecular processes among a group of genes. Some physical properties of mitochondria targeting signals can be obtained from the Signal P server (http://www.cbs.dtu.dk/services/SignalP/) and from a recent publication (26). For identifying mRNA motifs, we usually utilize the MEME algorithm (27).

4. Notes

1. It is preferable to use galactose or a non-fermentable carbon source for mitochondrial enrichment. Based on our experience, growth in selection media rather than rich media yields similar amounts of RNA.
2. Treatment with DTT enhances cell wall hydrolysis by breaking the disulfide bonds within the cell wall proteins, thereby easing later access of the 1,3-glucanase to the glucan linkages.
3. Cells that retain their cell wall will not be broken properly in later steps, hence the efficiency of the Zymolyase step is critical. Since spheroplasts are very sensitive to hypo-osmotic conditions, they must be maintained in an isotonic solution during and after Zymolyase treatment to prevent premature lysis. Here, the non-metabolized sugar alcohol sorbitol provides the osmotic support.
4. The stress imposed by the Zymolyase treatment or by the hyper-osmotic buffer may disrupt the normal mRNA localization, and hence the recovery step is necessary.

5. Cycloheximide inhibits ribosomal translocation yet it does not lead to the disassembly of ribosomes from the mRNA. As an outcome, mRNAs that are associated with mitochondria through their ribosomes remain associated throughout the procedure. It should be noted, however, that CHX was shown to induce a differential effect on mRNA association with the mitochondria (19).
6. This step removes any residual DNA and proteins. Avoid taking any of the phenol (lower) layer as it will inhibit later enzymatic steps.
7. The LiCl precipitation is necessary to remove residual Heparin, which inhibits the fluorescent labeling reaction.
8. To analyze enrichment to the mitochondria, it is possible to use two types of references: the first is unfractionated RNA, i.e., an RNA sample that was collected just prior to the spin down of the mitochondria from the cytosolic fraction (Subheading 3.1, step 10). Since the cell lysis procedure induces a significant change in the transcriptome, this sample will represent the amount of each gene's transcripts prior to fractionation. Thus, the resulting ratio between the mitochondria and the unfractionated RNA signal will represent the relative enrichment of RNAs in the mitochondria, with high ratios indicating a relatively high enrichment. A second possible reference is RNA that was extracted from the cytosolic fraction (Subheading 3.1, step 11). This RNA sample is the complement of the mitochondrial fraction, and the resulting hybridization ratio reports directly on the distribution of mRNAs between these two populations. High ratios represent a relatively high association with the mitochondria, and low ratios represent a relatively high cytosolic association. Such a direct comparison appears to be refractory to changes in total RNA levels, yet is problematic for genes that are highly associated with one of the fractions.
9. The amounts of RNA removed from each fraction may vary without affecting the data interpretation. This is because the signal per mRNA is relative to all other mRNAs and this relativity (or ranking) will be maintained regardless of the amount of RNA taken.
10. Cy dyes are dissolved in DMSO (12 μl) and divided into aliquots of 1 μl each. Unused dyes are dried and stored at 4°C in a desiccator for later use. It is important to minimize the exposure of the dyes or labeled samples to light.
11. The cover slip should be gently removed from the slides during the first wash (using a needle can ease the process; do not let the cover slip smear on the printed area). Avoid the slides' drying during the procedure.

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