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Directed alteration of *Saccharomyces cerevisiae* mitochondrial DNA by biolistic transformation and homologous recombination

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Abstract

Saccharomyces cerevisiae is currently the only species in which genetic transformation of mitochondria can be used to generate a wide variety of defined alterations in mtDNA. DNA sequences can be delivered into yeast mitochondria by microprojectile bombardment (biolistic transformation) and subsequently incorporated into mtDNA by the highly active homologous recombination machinery present in the organelle. While transformation frequencies are relatively low, the availability of strong mitochondrial selectable markers for the yeast system, both natural and synthetic, makes the isolation of transformants routine. The strategies and procedures reviewed here allow the researcher to insert defined mutations into endogenous mitochondrial genes, and to insert new genes into mtDNA. These methods provide powerful *in vivo* tools for the study of mitochondrial biology.

Keywords

Saccharomyces cerevisiae; mitochondria transformation; biolistic method; gene replacement; homologous recombination

1. Introduction

A key feature of the yeast nuclear genetic system that has made it a preeminent tool for genetic and cell biological research is the fact that DNA transformed into the nuclear chromosomes of *Saccharomyces cerevisiae* is incorporated into the genome only *via* homologous recombination. This fact allows the researcher to add, subtract, and alter genetic information in a highly controlled fashion, easily and cheaply, and essentially rewrite the yeast genome at will. In *S. cerevisiae*, and to date only in that species, similar manipulations based on homologous recombination can be carried out on the mitochondrial genome, using the biolistic transformation method to deliver DNA into the organelle.

1. Summary of the strategies used to create strains with a modified mtDNA

Two properties of *S. cerevisiae* facilitate mitochondrial transformation. First, *S. cerevisiae* cells can survive when they lack part (*rho*[−]) or all (*rho*⁰) of the mitochondrial DNA (mtDNA). Second, *rho*[−] mtDNAs replicate independently of protein synthesis and show no clear requirement for a specific replication origin sequence. These two features are advantageous in

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creating mitochondrial transformants containing defined mtDNAs, since *rho*⁰ yeast strains, entirely lacking mtDNA, can be transformed with bacterial plasmid DNAs that subsequently propagate as 'synthetic' *rho*⁻ molecules (1). The plasmids used for the transformation typically contain a mutant version of a mitochondrial gene, or a foreign piece of DNA flanked by mtDNA sequences. These synthetic *rho*⁻ strains are then used as donors of the new or modified sequence that can be integrated into a *rho*⁺ mtDNA by homologous recombination to yield the desired new strain. Alternatively, when a mutated DNA sequence provides a function that can be selected phenotypically, direct transformation of *rho*⁺ strains bearing deletions in the region of interest can be used to integrate mutations into mtDNA (2,3). Using this strategy, *rho*⁺ recombinants may be obtained more quickly, although the efficiency of transformation is lower than for *rho*⁰ cells (Fig. 1).

2. Bombardment

The standard device for microprojectile bombardment, that functions reproducibly for transformation of *S. cerevisiae* mitochondria, is the PDS-1000/He system. This instrument uses a helium shock wave in an evacuated chamber to accelerate microscopic metal particles coated with DNA towards a lawn of cells on a Petri plate. The shockwave is generated by rupture of a membrane at high pressure, and accelerates a second membrane (the macrocarrier or flying disk), carrying the metal particles, towards the plate. Some cells on the plate are penetrated by particles and survive. DNA precipitated on the particles is thus introduced into cells and is readily taken up by the nucleus. In addition, the mitochondria of a small fraction of such transformants also take up DNA.

3. Generation of a synthetic *rho*⁻ transformant

In a typical mitochondrial transformation experiment, a large number of *rho*⁰ cells are randomly bombarded by a large number of particles. In the first step, cells that have been hit and that survived are allowed to make colonies on the Petri plates by selecting for a nuclear genetic marker that is included in the DNA precipitated on the particles (Fig. 1, top plates). Mitochondrial transformants are identified among these colonies by genetic tests for the presence of new genetic information in the mitochondrial genome (Fig. 1, bottom plates). This new information is typically a portion of wild-type mitochondrial DNA (mtDNA) sequence that can rescue a known mitochondrial marker mutation by recombination, after the transformants are mated to an appropriate *rho*⁺ tester strain, resulting in recombinants with a detectable growth phenotype. The new wild-type sequence may be an unaltered region of the gene of interest, or it may be another piece of wild-type mtDNA incorporated into a vector. Such marker rescue can work with as little as 50 bp of homologous sequence flanking the site of the mutation in the tester mtDNA. The mitochondrial transformants are then purified by series of re-streaking and crosses to the tester before obtaining the final synthetic *rho*⁻ strains.

4. Generation of a *rho*⁺ strain with a modified mtDNA

In a second step, synthetic *rho*⁻ are mated with a wild-type *rho*⁺ recipient strain. As a result of this second mating, mitochondria from the two strains fuse and recombination between the two mtDNAs produces recombinant *rho*⁺ strains in which the new mtDNA sequence is integrated by double cross over events. Pure recombinant strains are generated by subsequent mitotic segregation. Since mitochondrial DNA recombination and segregation is so frequent, this simple procedure typically yields the desired integrants at frequencies between 1% and 50% of clones derived from zygotes. Using a *rho*⁺ recipient that carries a deletion in the region present in the synthetic *rho*⁻ can greatly facilitate the detection of homologous recombinants, if one of the strains in such a cross carries the karyogamy defective mutation *kar1-1* (4) (Fig. 2). In this case, haploid mitochondrial mutant cytoductants can be identified after such a mating by their acquired ability to rescue a defined marker within the deleted region.

5. Transformation of a ρ^+ strains carrying a deletion in the region of interest

Selection for transformation by DNA fragments restoring wild-type or near wild-type function to the ρ^+ recipient is straight forward, by selecting first for nuclear transformants, as described above in section 1.2, and then screening for the mitochondrial phenotype by replica plating (Fig. 3, right) (5). Respiring mitochondrial transformants can also be selected in a single step, by directly bombarding lawns of mutant ρ^+ cells spread on non-fermentable plates supplemented with 0.1% glucose, to allow a brief period of outgrowth, and 1M sorbitol (Fig. 3, left). We have also been able to select for ρ^+ transformants expressing the recoded gene *ARG8^m* (6) by directly bombarding lawns spread on appropriate minimal glucose medium supplemented with sorbitol (2,3). In addition, transformation can also be performed with linear DNA molecules obtained either from plasmid clones or PCR amplification (7). Linear DNA fragments having as little as 260bp of homologous sequence flanking each side of a deletion mutation in a ρ^+ recipient were able to yield respiring transformants at frequencies similar to those obtained with circular plasmids. The ability to use PCR-generated fragments to transform defined mtDNA deletion recipients can substantially accelerate strain construction.

The detailed protocols used to conduct all the different steps of mitochondria transformation and integration of directed mutations or new genes in mtDNA by homologous recombination are described in §3. For a review of the important features of *S. cerevisiae* mitochondrial genetics that underly the methods presented here, refer to (7).

2. Materials

1. Preparation of cells

1. ρ^0 or ρ^+ recipient strain, preferably of DBY947 genetic background (Note 1) and *kar1-1*.
2. YPR: Yeast extract 1%, Bacto-peptone 2%, Raffinose 2%, Glucose 0.1%, Adenine 40µg/ml (Note 2).
3. YPD: Yeast extract 1%, Bacto-peptone 2%, Glucose 2%, Adenine 40µg/ml.
4. SD-Sorbitol: Yeast nitrogen base 0.67%, Glucose 5%, Sorbitol 1M, Adenine 100µg/ml, Agar 3%, other supplements as required 40µg/µl except for the selection marker.
5. YPEG-Sorbitol is used for direct selection of respiring transformants into ρ^+ cells: Yeast extract 1%, Bacto-peptone 2%, Glycerol 3%, Ethanol 3%, Glucose 0.1%, Agar 3%.

2. Preparation of microprojectiles and precipitation of DNA

1. Tungsten powder <1µm 99.95% (metals basis), Alfa Aesar/Johnson Matthey, item 44210, CAS# 7440-33-7 (Note 3).
2. Ethanol 70% and 100%, room temperature.

¹The strain genetic background is an important factor affecting the efficiency of transformation (Fig. 1 and Table 1). We have obtained the best results with ρ^0 strains in the S288c background, in particular those derived from DBY947 (11), like MCC109rho0 (12), (American Type Culture Collection # 201440), MCC123rho0, which is the identical strain with *MATa* (ATCC# 201442), and DFS160, which is recommended for *ARG8^m* constructs (6). Strains derived from W303 (13), (ATCC# 200060) give lower but satisfactory efficiencies (see W303-1B/A/50, Fig. 1), while strains in the D273-10B (ATCC# 24657) background are very difficult to transform. In our hands, mitochondrial transformation is 10 to 20 times more efficient during bombardment of a ρ^0 strain than of an isogenic ρ^+ strain containing a small deletion in mtDNA (Fig. 1). This effect could be due either to physiological differences between the strains such as the properties of the mitochondrial inner membrane, or to an advantage in establishing an incoming DNA molecule in the absence of endogenous mtDNA. Effects consistent with the latter notion have been observed in comparisons of mtDNA behavior after ρ^+ x ρ^+ matings as opposed to ρ^+ x ρ^0 matings (14).

²The 0.1% glucose supplement accelerates growth, and adenine seems to increase the transformation efficiency, even for Ade⁺ prototrophs. Raffinose can be replaced by galactose (which is less expensive) with no or very limited decrease in transformation efficiency.

3. Sterile water.
4. Glycerol 50%, sterile, -20°C .
5. DNA for nuclear transformation, carrying the nuclear marker and a nuclear replication origin, *e.g.* for the *LEU2* marker Yep351 (8) or pRS315 (9). Qiagen-based midi-or maxi-preps are recommended, at $2\mu\text{g}/\mu\text{l}$ or more.
6. DNA for mitochondria transformation, Qiagen-preps, at least $2\mu\text{g}/\mu\text{l}$.
7. Spermidine free-base 1M (Sigma)
8. Calcium chloride 2.5 M, -20°C , filter-sterilized
9. 100% ethanol, -20°C

3. Bombardment

1. Macrocarrier holders, sterilized in a Pasteur oven.
2. Macrocarriers (Biorad).
3. Rupture disks, 1100 psi (Note 4).
4. PDS-1000He biolistic gun (Biorad) with helium bottle and vacuum pump.

4. Identification of mitochondrial transformants

1. $\rho^{+} \text{mit}^{-}$ tester strain, carrying a mitochondrial mutation in the region covered by the DNA for mitochondrial transformation, and mating type opposite to the recipient ρ^{0} strain.
2. SD plates: 0.67% Yeast nitrogen base, Glucose 2%, Agar 2%, supplements as required $40\mu\text{g}/\mu\text{l}$.
3. YPD plates: like liquid YPD but with 2% agar.
4. YPEG plates: Yeast extract 1%, Bacto-peptone 2%, Glycerol 3%, Ethanol 3%, Agar 2%.

5. Mating and isolation of recombinant cytoductants

1. Recipient wild-type ρ^{+} strain or $\text{mit}^{-} \rho^{+}$ strain. To recover haploid recombinants, this strain must carry the *kar1-1* mutation unless the ρ^{0} strain used for the transformation (section 2.1.1) is *kar1-1*.

3. Protocols

Optima for the parameters of sections 3.1 to 3.3 are summarized in the Table 1.

1. Preparation of cells

1. Grow the ρ^{0} (or ρ^{+}) strain to be bombarded in a few ml of liquid YPR for one to two nights at 30°C with agitation.

³We routinely use Alfa Aesar tungsten powder $<1\mu\text{m}$, which is inexpensive and very effective, but tungsten powder 0.4 to $0.7\mu\text{m}$ is also available from Bio-Rad (catalog numbers 165–2265 or 165–2266 respectively), as is gold powder $0.6\mu\text{m}$ (Biorad 165–2262). In our hands, gold powder gave a similar yield to the Aesar tungsten, whereas tungsten powder from Bio-Rad was three times less efficient.

⁴Rupture disks of 1350 psi can also be used for efficient transformation of yeast, but in our hands 1100 psi disks tend to give better results.

2. Use this fresh culture to inoculate at 1/100 a larger volume of YPR, and grow for two or three days (stationary phase) at 30°C with agitation (Note 2 and ⁵). On average 30 ml to 50 ml of cultured cells are used for 6 shots.
3. Harvest cells and concentrate 40 to 100 times in liquid YPD medium to reach a cell density of 1 to 5×10^9 cells/ml.
4. Spread 0.1 ml of cells onto the appropriate SD-Sorbitol supplemented to provide the appropriated prototrophic selection (Note 6 and ⁷).

2. Preparation of microprojectiles and precipitation of DNA

1. Pre-cool a microcentrifuge for step 3.2.4.
2. Weigh and sterilize in a microfuge tube 10 to 50 mg of tungsten particles by vigorous suspension in 1.5 ml of 70% ethanol (100% ethanol for gold particles, (10)), and incubation at room temperature for 10 minutes. Centrifuge 15 minutes at room temperature at maximum speed in a microcentrifuge and carefully remove the supernatant. Wash the particles with 1.5 ml of sterile water, resuspend at 60 mg/ml in freezer 50% glycerol and keep on ice (Note 8).
3. Precipitation of DNA onto particles is conducted on ice with ice cold or freezer-stored reagents. For 6 shots, mix in a microfuge tube (Note 9), 5 µg of plasmid for the nuclear selection (Yep351) with 15 to 30 µg of plasmid carrying the mitochondrial DNA of interest in a total volume of 15–20 µl (Note 10). Add 100 µl of tungsten particles (Note 11), 4 µl of spermidine 1M and 100µl of 2.5 M CaCl₂ from the freezer, in that order, vortexing immediately after each addition. Incubate 10 to 15 minutes with occasional vortexing.
4. Spin briefly in the pre-cooled microcentrifuge, remove the supernatant and add 200 µl of freezer stock 100% ethanol. Take extreme care to scrape off the side of the tube and to fragment aggregates of particles using the pipette tip, then resuspend the particles thoroughly. Repeat spinning and resuspension, at least once, until the particles resuspend easily (Note 12).
5. Spin briefly, remove the supernatant, and add 60 µl of 100% ethanol. Distribute the resulting suspension evenly at the center of 6 macrocarriers (flying disks) previously placed in their holders. Allow the ethanol to evaporate (Note 13).

⁵For *rho*^o cells in the DBY947 background, a three day culture is optimum, but this could differ for other backgrounds.

⁶Cells can be used between 1 up to 3 hours after plating. Plates that are still wet can be bombarded without drastic changes in the efficiency. We usually plate cells with a glass rod spreader rather than beads, since the heterogeneity of plating will provide both some zones of optimum cell density for the transformation, and a pattern of transformants that plays the role of useful identification marks to compare the plates when picking the mitochondrial transformants at step 3.4.5.

⁷YPEG-Sorbitol can be used instead of SD-Sorbitol when transforming a respiratory deficient *rho*⁺ strain and selecting directly for respiratory proficient transformants. SD-Sorbitol lacking arginine is used for selection of transformants from *rho*⁺ *arg8* cells transformed with a construct allowing the production of functional Arg8p from the *ARG8^m* reporter.

⁸Tungsten particles can be kept frozen in 50% glycerol for several months to several years without loss of transformation efficiency. We have not tried gold.

⁹Precipitations can be up-scaled by a factor of 2; a siliconized tube can be used.

¹⁰Try to keep the DNA volume to a minimum to optimize the precipitation.

¹¹Vortex vigorously the tungsten suspension before pipetting, because the particules tend to settle extremely rapidly.

¹²This step is crucial to obtain a very finely dispersed precipitate that will efficiently transform the cells.

¹³We usually work under a laminar flow hood, using sterile forceps to insert the macrocarrier disks into the holders. Holders can be conveniently kept in sterile Petri dishes. There is no need to pre-wash the macrocarriers nor to desiccate them after coating, the ethanol will quickly evaporate.

3. Bombardment

1. Thoroughly wash the chamber and the removable parts by soaking with 70% ethanol. Wipe dry the parts and the chamber meticulously, since remaining ethanol will prevent efficient evacuation of the chamber (Note 14).
2. Carefully follow the manufacturer's instructions for use of the PDS-1000He apparatus (it employs gas under high pressure). Place the rupture disk in its retaining cap and tighten using the torque wrench (Note 15).
3. Load the macrocarrier in its holder into the assembly system. Do not assemble the stopping screen (Note 16).
4. Place the open Petri plate carrying the lawn of cells at 5 cm from the macrocarrier assembly (Note 17).
5. Evacuate the vacuum chamber to reach a reading of 29 to 29.5 inches Hg (the higher the better) on the PDS-1000's gauge (Note 18).
6. Fire.
7. Remove any fragments of the macrocarrier disk with sterile forceps.
8. Incubate the plate at 30°C for 4 to 5 days until colonies appear. Expect between 1000 and 10000 nuclear transformants per plate for S288c related strains transformed to prototrophy, or from 0 up to 20 transformants per plate for direct selection of ρ^+ mitochondrial transformants from a ρ^+ recipient. For transformation of ρ^0 recipients, proceed with the following section 3.4 to identify ρ^- transformants. In the case of ρ^+ transformation, nuclear prototrophic transformants are printed to selective medium to isolate mitochondrial transformants, or mitochondrial transformants can be picked directly if the bombardment was made on selective medium. Analyze them genetically as in Fig. 2B.

4. Identification of mitochondrial ρ^- transformants after bombardment of a ρ^0 recipient strain

1. During the incubation of the bombarded plates, set up a liquid YPD culture of an appropriate ρ^+ mutant (mit^-) tester strain.
2. Replica-plate the transformants onto SD medium with the appropriate supplements (to keep the transformants), and onto a lawn of the tester strain freshly spread on a YPD plate. Mark the plates precisely to facilitate the subsequent step 3.4.5.
3. Incubate at 30°C for two days to allow mating and recombination.

¹⁴For a more efficient sterilization, the chamber and parts can be soaked with 70% ethanol before starting the cell preparation and DNA precipitation, allowed to sit, and wiped dry just before the bombardment. The chamber is washed in a similar way after the bombardment.

¹⁵Be careful not to use two rupture disks stacked together.

¹⁶Interestingly, we have found that simply allowing the carrier disk to fly to the surface of the Petri plate, by not assembling the stopping screen, yields more transformants than are obtained if the stopping screen is employed. However in this case it is important to use sorbitol plates containing 3% agar, as advised in the materials section, to prevent severe damage to the agar surface.

¹⁷Shorter distances result in very high colony densities in the center of the plate with few colonies at the periphery, while longer distances decrease the transformation efficiency.

¹⁸We have found that failure to draw the greatest vacuum possible dramatically reduces the transformation efficiency. Teflon sealing tape can be used to reduce air leakage in the connections between the chamber and the pump. Cell viability is not significantly affected by a prolonged stay under these vacuum conditions.

4. Print to YPEG medium to detect respiring diploids (or another appropriate selection medium if scoring another phenotype) (Note 19). Incubate two to three days (Note 20).
5. Pick colonies off the bombarded plate (or its direct replica) that correspond to the position of respiring recombinants. Streak these colonies on YPD and repeat the marker rescue with the tester strain, as above. Such subcloning and retesting must usually be done three times before pure stable synthetic *rho*⁻ clones are obtained. Cells usually lose the nuclear marker plasmid during these subcloning steps if no selection is applied for its maintenance.

5. Mating and isolation of recombinant cytoductants

1. Grow cultures of the subcloned synthetic *rho*⁻ strain and the recipient wild-type *rho*⁺ strain overnight in liquid YPD. At least one of these two strains (usually the synthetic *rho*⁻) must carry the *kar1-1* mutation (Note 21).
2. Mix 0.5 ml of each parent (alternatively 1 ml of synthetic *rho*⁻ and 0.2 ml of wild-type *rho*⁺, see Note 22) in a microfuge tube, spin, remove the supernatant, resuspend in residual liquid and spread the mixture onto a YPD plate.
3. Incubate at 30°C for 4 to 5 hours. Check zygote formation microscopically. Scrape the mating cells from the plate and use them to inoculate fresh YPD liquid medium. Incubate at 30°C with agitation for a few hours to overnight.
4. Dilute the culture and plate to obtain single colonies on minimal medium selecting for the recipient nuclear genotype and against the donor nuclear genotype, if possible. Alternatively, plate on YPD medium. Densities of 50 to 200 colonies per plates should be obtained.
5. Replica plate the colonies obtained to medium that will reveal the altered phenotype of the recipient strain as a result of integration of the mutant donor sequences into its mtDNA. For example, print to YPEG to identify clones that have acquired a mutation preventing respiratory growth.
6. Mate nonrespiring candidate clones to a known *rho*⁻ strain that covers the region you are seeking to mutate. The desired *rho*⁺ recombinant cytoductants will produce respiring diploids after mating to this known *rho*⁻ strain. This step eliminates cytoductants that simply acquired the transformed synthetic *rho*⁻ mtDNA by cytoduction.

¹⁹In cases where a high number of nuclear transformants are present, it may be useful to also replicate the mated cells on medium that selects for the diploids, since these diploid plates may by comparison facilitate the identification of the mitochondrial transformants on the original bombarded plate.

²⁰Respiratory proficient colonies typically appear earlier if the transformed DNA allows complementation of the *mit*⁻ mutation in *trans*.

²¹The introduction of a mutation causing respiratory deficiency in an otherwise *rho*⁺ genome can be facilitated by using as *rho*⁺ recipient a strain that carries a deletion mutation in the region of interest, that can recombine with the synthetic *rho*⁻ sequences. Some of the colonies obtained in step 3.5.4 will be respiratory deficient *rho*⁺ haploid recombinants that have the deleted region restored but containing the desired mutation (Fig. 2A). Find them by mating to freshly spread lawns of two different testers: a *rho*⁺ tester strain that has another mutation within the recipient's deleted region distinct from the new mutation to be introduced (Fig. 2B), and a *rho*⁻ strain carrying wild-type information in the region deleted in the original recipient strain. The desired colonies will yield respiratory proficient diploids after two days incubation at 30° and print to YPEG. Identify the corresponding haploid *rho*⁺ cytoductants colonies on the plates from step 3.5.4, restreak and retest.

²²If the synthetic *rho*⁻ donor and the *rho*⁺ recipient strains share nuclear markers, and therefore cannot be distinguished selectively on glucose medium, mating mixtures should contain equal numbers of cells of both strains. If nuclear auxotrophic or drug resistance markers allow selection against the synthetic *rho*⁻ donor strain, then the mating mixture should contain a five-fold excess of donor cells.

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Fig. 1. Nuclear transformants and mitochondrial co-transformants obtained by bombardment of different yeast strains

The nuclear *LEU2* plasmid Yep351 (8) and the *COX2* plasmid pNB69 (2) were precipitated together onto tungsten particles and bombarded on lawns of the *rho*^o strains W303-1B/A/50 (*MATa*, *ade2-1*, *ura3-1*, *his3-11,15*, *trp1-1*, *leu2-3,112*, *can1-100* [*rho*^o]) (a *rho*^o derivative of W303-1B, (13)) and DFS160 (*MATa ade2-101*, *leu2D*, *ura3-52*, *arg8D::URA3*, *kar1-1*, [*rho*^o]) (6), or on lawns of the *rho*⁺ strain NB104. NB104 *rho*⁺ mtDNA carries a 129 bp deletion, *cox2-60*, located around *COX2* first codon (2), and is isonuclear to DFS160. The top plates correspond to minimal medium supplemented with sorbitol and lacking leucine. Typical plates showing about 3000 nuclear transformants for each strain have been presented. Nuclear transformants were crossed by replica plating to the nonrespiring tester strain (NB160), carrying a mutation of *COX2* initiation codon (2), and mitochondrial transformants (bottom plates) were detected by replica-plating the mated cells onto non-fermentable medium. Reprinted from (7) with permission.

Fig. 2. Schematic diagram of recombination events that allow identification of nonrespiring recombinant cytoductants by marker rescue

Thick lines represent mtDNA sequences, thin lines represent vector DNA. The box represents a gene under study. (A) A karyogamy defective (*kar1-1*) synthetic ρ^- donor containing an experimentally induced mutation “e” is mated to a ρ^+ recipient strain with a deletion in the region of interest. Among the cells present in the mixture after mating, are the desired ρ^+ recombinant cytoductants. (B) To distinguish the desired ρ^+ recombinant cytoductants from the unaltered recipient cells and other cell types present, clones derived from the mating mixture are mated to a ρ^+ tester strain bearing the marker mutation “m.” The desired ρ^+ recombinant cytoductants can yield respiring recombinants when mated to this tester by a crossover between

“e” and “m” (and a second resolving crossover anywhere else). The ability to produce respiring recombinants identifies the desired cytoductant clones. Unaltered recipient clones, and other cells types present, cannot yield such respiring recombinants. Reprinted from (7) with permission.

Fig. 3. Selection of *rho*⁺ mitochondrial transformants directly after bombardment

The nuclear shuttle vector Yep351 (*LEU2*) and plasmid pNB69 (*COX2*) were bombarded together onto lawns of the *rho*⁺ *cox2-60* strain NB104 (see legend of Fig. 1). The bombarded lawns had been spread either on minimal medium supplemented with sorbitol but lacking leucine (top right), or on non-fermentable YPEG medium supplemented with sorbitol and 0.1% glucose (top left). Leu⁺ transformants were replica plated to non-fermentable medium (bottom plate) to select for mitochondrial transformants. Reprinted from (7) with permission.

Table 1
Summary of the factors influencing mitochondrial transformation efficiency, and their known optima.

Recipient strain		DNA precipitation		Biolistic parameters	
Parameter	Optimum	Parameter	Optimum	Parameter	Optimum
Genetic background : -nuclear	DBY947	DNA: -size -purity -concentration -volume -quantity	5–6 kb Qiagen >2 µg/µl <15–20 µl 5µg (nuclear) 20–30 µg (mito) Aesar 44210 Tungsten powder, <1 micron ice cold finely dispersed	Rupture disks Stopping screens	1100 psi none
-mitochondrial Carbon source	<i>rho</i> ^o Raffinose	Particles Temperature Precipitate		Plate distance Vacuum	5 cm 29–29.5