Started Dec 12

Establishment of Human Cell Lines Lacking Mitochondrial DNA

Mitochondrial DNA pp 383-391 | Cite as

- Kazunari Hashiguchi (1)
- Qiu-Mei Zhang-Akiyama (1)

1. Laboratory of Radiation Biology, Graduate School of Science, Kyoto University, , Kyoto, Japan

Protocol

Part of the Methods in Molecular Biology™ book series (MIMB, volume 554)

554 Bio 16 Fc6

5, 5.1.2

Abstract

Mitochondria have their own genome, and mitochondrial DNA (mtDNA) encodes 2 ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides that function in oxidative phosphorylation (OXPHOS). mtDNA mutations lead to dysfunction of OXPHOS, resulting in cell death and/or compromised cellular activity. Cell lines lacking mtDNA (termed ρ^0 cells) are very effective tools for studying the consequences of mtDNA mutations. ρ^0cell lines have been used widely to investigate relationships between mtDNA mutation, mitochondrial function, and a variety of cellular processes. In this chapter, we summarize the yeast and animal ρ^0 cell lines that have been studied. We provide simple protocols for the generation of human ρ^0 cells by exposure to ethidium bromide and PCR verification of their ρ^0 status.

Key words

Mitochondria, mitochondrial DNA (mtDNA), ρ^0 cells, oxidative phosphorylation (OXPHOS), uridine pyruvate, ethidium bromide, PCR

1 Introduction

Since mitochondrial DNA (mtDNA) encodes genes related to oxidative phosphorylation (OXPHOS), the stability of mtDNA is crucial for the survival of cells in vivo (1, 2, 3, 4). However, cells depleted of mtDNA (ρ^0 cells) are viable in culture, provided appropriate conditions are met (5, 6, 7). These ρ^0 cells are important tools for investigating the pathogenesis of specific mtDNA mutations, and for developing a better understanding of interactions between nuclear and mitochondrial genomes in mitochondrial disease (8). The ρ^0 cells also have been used to study mtDNA replication and repair processes, since many of the proteins involved in mtDNA transactions continue to localize to mitochondria even in the absence of mtDNA (9, 10, 11). Cybrid cells have been produced by fusing ρ^0 cells with enucleated cells (6), and using this technique, mitochondrial disorders have been investigated in human (12 , 13) and mouse cells (14, 15, 16).

As summarized in **Table** 23.1, ρ^0 cell lines have now been established from various tissues and species. Human cells lacking mtDNA (ρ^0 cells) were originally obtained from the human cell line 143B.TK$^-$ (6) by chronic exposure to a DNA intercalating dye ethidium bromide (EtBr). This same approach has been used to establish an avian ρ^0cell line (17). In mouse cells, EtBr is not effective in producing ρ^0 cells for unknown reasons. Inoue and co-workers, however, successfully isolated ρ^0 mouse cell lines by exposure to the antitumor bis-intercalating agent ditercalinium (DC) (14).

Table 23.1

and seven *Eco*RI-sensitive sites, respectively (accession number of human, mouse, and rat mtDNA reference sequence; AC 000021, NC 005089, and AC 000022), it is thought that *Eco*RI cleaves mtDNA, producing several short linear segments that are not effectively replicated. The comparison of the human ρ^0 line generated using the restriction approach (143B.TK$^-$K7) with 143B.TK$^-$$\rho^0$ cells generated by EtBr treatment revealed very similar proliferation rates, glucose consumption rates, lactate production rates, and mitochondrial morphology and reticular structure.

Here, we describe a method for establishing human ρ^0 cells and propagating these cell lines in culture. A convenient PCR analysis for verifying the ρ^0 state is also described. Modifications to the culture medium required to support the viability of ρ^0 cells are provided and explained. While the method is outlined for a human cell line, it is broadly applicable, with suitable modifications (*see* **Table 23.1**) for use with other cells.

2 Materials

2.1 Cell Culture

1. 1.

   Human cultured cell lines (HeLa, 143B.TK$^-$, and so on) can be obtained from ATCC (http://www.atcc.org (http://www.atcc.org)).

2. 2.

   Culture medium: Dulbecco's Modified Eagle's Medium (high glucose, cat# 10938-025, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 μg/ml sodium pyruvate (Invitrogen), and 50 μg/ml uridine (Sigma). For culture of 143B.TK$^-$ cells, 100 μg/ml bromodeoxyuridine (BrdU, Sigma) is also required (*see* **Note 1**). A stock solution of 10 mg/ml uridine and 10 mg/ml BrdU is dissolved in distilled water, sterilized by filtration, and stored at −20°C. BrdU is light-sensitive, so this should be shielded from light.

3. 3.

   EtBr stock solution: 10 mg/ml; prepared in distilled water, filter-sterilized, and stored at 4°C in dark (*see* **Note 2**). Before use, dilute this stock solution with sterilized water to 5 μg/ml.

4. 4.

   Sterilized PBS (Ca, Mg-free).

5. 5.

   Trypsin–EDTA solution (Invitrogen).

6. 6.

   CO_2 incubator.

7. 7.

   Centrifuge with swinging rotor.

8.

Centrifuge with 1.5-mL minifuge tube rotor.

2.2 DNA Extraction, PCR, and Polyacrylamide Gel Electrophoresis

1. 1.

PCR primer sequences for amplification of mtDNA encoded tRNA-Leu gene and the nuclear encoded poly gene (as positive control) are as follows (*see* **Note 3**).

For mtDNA encoded tRNA-Leu gene:

5′-GATGGCAGAGCCCGGTAATCGC-3′

5′-TAAGCATTAGGAATGCCATTGCG-3′

For nuclear poly gene:

5′-AGCGACGGGCAGCGGCGGCGGCA-3′

5′-CCCTCCGAGGATAGCACTTGCGGC-3′

2. 2.

Cell scraper (can be obtained from BD Falcon, Corning, and Greiner Bio-one).

3. 3.

Total DNA extraction kit. Any commercial DNA extraction kit for use with cultured cells is acceptable.

4. 4.

Conventional recombinant Taq polymerase is useful as PCR enzyme (Promega, Takara, Roche, NEB, Stratagene). High-fidelity enzyme is not required. PCR buffer and dNTPs are generally supplemented with PCR enzyme.

5. 5.

Radioactive [γ-^{32}P]ATP and [α-^{32}P]dCTP (3,000 Ci/mmol) can be purchased from ICN (*see* **Note 4**).

6. 6.

Molecular weight marker (50 Base-Pair Ladder, GE Healthcare).

7. 7.

T4 polynucleotide kinase (New England BioLabs).

8. 8.

Electrophoresis buffer: 1X TBE buffer (89 mM Tris–borate and 2 mM EDTA). Store at room temperature.

9. 9.

8% Polyacrylamide gel prepared from 30% acrylamide/bis-acrylamide (29:1) stock solution and contains 1X TBE.

10. 10.

10% Ammonium persulfate (APS) solution prepared in distilled water. Store aliquot at −20°C, and should be used in 6 months.

11. 11.

N,N,N′,N′-Tetramethylethylenediamine (TEMED) can be purchased from Bio-Rad.

12. 12.

Appropriate electrophoresis system for polyacrylamide gel.

13. 13.

Phosphorimager (e.g., Storm 860; Molecular Dynamics).

14. 14.

Transilluminator (if not using phosphorimager).

15. 15.

UV–Vis spectrophotometer for qualitative analysis of isolated DNA purity.

3 Methods

A high concentration of glucose, as well as addition of uridine and pyruvate to the culture medium (6) is required for the viability of cells lacking mtDNA. The requirement for glucose is due to the complete reliance of ρ^0 cells on glucose fermentation for ATP synthesis. The requirement for uridine is due to a deficiency of pyrimidine biosynthesis in ρ^0 cells. Addition of uridine and pyruvate into the culture medium and at least 1 month of culture in the presence of EtBr or DC are commonly used for generating ρ^0 vertebrate cell lines. Once mtDNA is completely lost, there is no further requirement for EtBr in the culture medium. The method described below for human cells can be applied with minor modifications to a wide variety of eukaryotic cells (*see* **Note 5**).

3.1 Isolation of a Human ρ^0 Cell Line

1. 1.

Maintain human cell lines in culture medium and passage by trypsinization twice a week or as required. Do not allow cells to reach confluency.

2. 2.

Twenty-four hours after plating cells to a 100-mm dish (~1 × 10^5 cells), add 50 ng/ml EtBr to culture medium.

3. 3.

Approximately 1 week after the addition of EtBr, dead cells will begin to peel off from the dish. Remove these cells by washing the plate once with PBS, then add fresh medium supplemented with 50 ng/ml EtBr.

4. 4.

Monitor the pH of the culture medium closely, and change medium as required (*see* **Note 6**), continuing to supplement with 50 ng/ml EtBr.

5. 5.

Approximately 1 month following the initial addition of EtBr, colonies will be visible (these might be ρ^0 cells). Isolate single colonies by penicillin cup, and transfer cell suspension to 24-well.

6. 6.

Continue to grow cells, changing medium every 2 days or sooner if medium begins to appear yellow.

7. 7.

Expand cells to isolate total DNA (*see* **Section 3.2** below).

8. 8.

Isolated cell lines can be stored in liquid nitrogen using a general cell freezing medium.

3.2 Total DNA Extraction and PCR

1. 1.

Propagate candidate ρ^0 cell lines generated above in 100-mm culture dishes to near confluency (this will be approx. 5×10^{6}~1×10^{7} cells).

2. 2.

Remove the culture medium by aspiration and wash cell surface with 5 ml of PBS.

3. 3.

Add fresh 1 ml of PBS and scrape cells by cell scraper (*see* **Note 7**).

4. 4.

Transfer cell suspension to a minifuge tube.

5. 5.

Centrifuge at 300 × g for 3 min.

6. 6.

Aspirate supernatant. At this point, the cell pellet can be stored at −80°C until use.

7. 7.

Extract total DNA from the cell pellet using any commercial kit, according to the instructions provided.

8. 8.

Measure DNA concentration and purity using a spectrophotometer.

9. 9.

Prepare 50 µl reactions in PCR tubes according to the manufacturer of your PCR enzyme. Generally add 1× PCR buffer, $MgCl_2$ (if not included in PCR buffer). Include 200 µM dNTPs and 10 µCi [α-^{32}P]dCTP (3,000 Ci/mmol) to the reaction (*see* **Note 4**). Add 0.1 µM each primer, 20 ng of total DNA, and 0.5 U Taq DNA polymerase.

- used normal fusion protocol w/ T_m = 72°c phusion 2

10. 10.

Incubate reaction mixtures at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min in a PCR machine. The reaction is completed by a final incubation at 72°C for 5 min, and the reaction products are stored at 4°C until use (*see* **Note 8**).

- Separated on 2.5% Agarose (overnight)

3.3 Polyacrylamide Gel Electrophoresis

1. 1.

Before electrophoresis, mix 50 pmol of molecular weight marker with 50 pmol of [γ-^{32}P]ATP and 20 units of T4 polynucleotide kinase in 1×T4 polynucleotide kinase buffer and incubate at 37°C for 30 min. To inactivate kinase, incubate reaction at 65°C for 20 min. This reaction can be loaded directly onto gel and should be stored at −20°C.

2. 2.

Clean the glass plates, comb, and spacers. It is better to wipe with ethanol before use.

3. 3.

Assemble the glass plates.

4. 4.

Mix 3.2 ml of 30% acrylamide stock with 7.6 ml of distilled water, 1.2 ml of 10× TBE, and 10 µl of TEMED (*see* **Note 9**).

5. 5.

Add 200 µl of 10% APS to the gel mixture, and immediately pour into gel plates and set the comb into the top of the gel (*see* **Note 10**).

6. 6.

Allow at least 60 min for gel polymerization (*see* **Note 11**).

7. 7.

Set the gel plate into the electrophoresis apparatus and pour the 1× TBE running buffer.

8. 8.

Mix PCR product with appropriate gel loading dye and load sample onto the gel.

9. 9.

Run the gel at 1–8 V/cm constant voltage. Stop gel running at appropriate point.

10. 10.

When radioactive materials have been used in PCR, visualize products using a phosphorimager. If not using radioactive materials, soak the gel in 1:20,000 diluted 10 mg/ml EtBr in 1 × TBE and visualize using a transilluminator.

4 Notes

1. 1.

Normally, Dulbecco's Modified Eagle Medium contains pyruvate. You may choose pyruvate pre-mixed medium. Important materials in medium are high concentrations of glucose (4,500 mg/l), pyruvate, l-glutamine, and uridine. The reason for the addition of BrdU is that this cell line is deficient in thymidine kinase and is resistant to BrdU.

2. 2.

Filtration of lower than 10 mg/ml EtBr causes significant reduction of EtBr concentration, because of binding to the filter.

The sizes of PCR products from mitochondrial tRNA-Leu and nuclear polymerase γ genes are 139 and 92 bp, respectively.

4. 4.

This protocol is a highly sensitive detection method because of the use of radioactive materials. If use of radioactivity is a complication in your lab, this can be omitted, and conventional PCR without radioactive materials can be done.

5. 5.

Gamen et al. (20) used 5 ng/ml EtBr for isolation of human U937 ρ^0 cells. Miller et al. (21) used 5 μg/ml EtBr for isolation of human 64/5 ρ^0 cells. Compared with EtBr concentration used for 143B206 cells (50 ng/ml), it is obvious that the optimal concentration of EtBr differs in different cell lines. For isolation of murine ρ^0 cell lines, concentrations of DC used include 1.5 μg/ml for C2, B82cap, and NIH3T3 cell lines and 56 ng/ml for MIN6 cell line (14 , 22). For the isolation of the avian LSCC-32H ρ^0cell line, a final concentration of 400 ng/ml EtBr is used (17). Thus, when generating ρ^0cells using a unique cell line, optimization of EtBr/DC concentration is required. For generation of ρ^0cells in the budding yeast *Saccharomyces cerevisiae*, the parental line is cultivated for 40 generations in the presence of 20 μg/ml EtBr in YPD (2% Bacto-pepton, 1% Bacto-yeast extract, and 2% glucose) medium. Cells are plated onto YPD plate, and single colonies that appeared are replicated onto both fresh YPD and YPG [2% Bacto-pepton, 1 % Bacto-yeast extract, and 3% (v/v) glycerol] plates. Since yeast ρ^0 line cannot use glycerol as a carbon source, the ρ^0 state of candidates that cannot replicate on YPG can be used as a preliminary check of mtDNA status that is verified by PCR.

6. 6.

Due to their reliance on glucose fermentation, ρ^0 cells produce large amounts of lactic acid that can rapidly acidify the culture medium and interfere with cell growth and viability. Therefore, close attention to media pH is necessary.

7. 7.

This step can be replaced by trypsinization.

8. 8.

These reaction times are not critical. Obey your instruction manual attached to PCR enzyme.

9. 9.

The total volume of gel is 12 ml. You may change this volume according to your gel size. Acrylamide before polymerization is neurotoxic. You need to wear gloves when you handle with unpolymerized acrylamide.

10. 10.

After addition of APS, polymerization is started. Any bubble in the gel has to be omitted.

11. 11.

Before use ensure that the gel is completely polymerized. With lower room temperature, acrylamide becomes difficult to polymerize. Keep around 25°C as room temperature.