Mitochondrial DNA Purification Kit

(Catalog # BN00647; 25 purifications; Store at Multiple Temperatures)

I. Introduction:

Mitochondria are essential double-membrane bound organelles that support aerobic respiration and most of the cellular ATP production in all nucleated mammalian cells. To date, hundreds of pathogenic mitochondrial DNA mutations have been identified to result in disease phenotypes such as cancer, ataxia, and diabetes. As a result, isolation of high quality mitochondrial DNA from mammalian cells and tissues in sufficient quantities is important for many basic research and clinical applications. Assay Genie's Mitochondrial DNA Isolation Kit facilitates purification of quality mitochondrial DNA from mammalian cultured cells or tissue. This kit utilizes a density separation step to isolate mitochondria from the rest of the cellular organelles, followed by an enzymatic reaction to release DNA from the mitochondria. Upon release, mitochondrial DNA is further purified from nuclear DNA contamination and adsorbed onto a silica spin-column under chaotropic conditions, eliminating the use of toxic organic compounds or solvents. Mitochondrial DNA purified by this kit is suitable for various downstream molecular biology applications such as PCR, cloning, DNA hybridization, Southern Blotting, sequencing, genotyping, enzymatic analysis, and more.

II. Applications:

- PCR, cloning, DNA hybridization, southern blotting, genotyping, restriction enzyme digest analysis, sequencing, etc.

III. Sample Type:

- Mammalian cultured cells or tissue

IV. Kit Contents:

Components	BN00647	Cap Code	Part Number	Storage (°C)
Buffer H [Homogenization Buffer]	90 ml	WM/Clear	BN00647-1	RT
Buffer R [Re-suspension Buffer]	9 ml	NM/Clear	BN00647-2	RT
Enzyme Mix	380 µl	Red	BN00647-3	-20°C
RNAse A	380 µl	Blue	BN00647-4	-20°C
Buffer L [Lysis Buffer]	9 ml	NM/Brown	BN00647-5	RT
Buffer N [Neutralization Buffer]	9 ml	NM/Clear	BN00647-6	RT
Buffer W [Wash Buffer]	8 ml	WM	BN00647-7	RT
Buffer E [Elution Buffer]	4 ml	NM	BN00647-8	RT
Spin Columns/Collection Tubes	25 tubes	-	BN00647-9	RT

V. User Supplied Reagents and Equipment:

- DNAse-free aerosol tips and micro-centrifuge tubes, 100% Ethanol, PBS, Heating Block, Refrigerated Centrifuge

VI. Storage Conditions and Reagent Preparation:

Refer to section IV (Kit contents) for proper storage. Protect from light. Briefly centrifuge small vials prior opening. Read entire protocol before performing the assay.

- **Buffer H, Buffer R, Buffer L, Buffer N, and Buffer E:** Ready to use. Store at room temperature.
- **Buffer W:** Add 34 ml of 100% Ethanol, molecular biology grade. Mix well and store at room temperature.
- **Enzyme Mix and RNAse A:** Ready to use. Store at -20°C. Keep on ice at all times while in use.
- **Spin Columns/Collection Tubes:** Ready to use. Store at room temperature in dry conditions.

VII. Mitochondrial DNA Isolation Protocol:

1. Sample Preparation:

a. Start with 20 x 10^6 – 50 x 10^6 cells or 100 – 300 mg of fresh perfused tissue per isolation. If using cultured cells, proceed with steps b-d. For fresh perfused tissue samples, proceed directly to step e.

b. Transfer the cells from tissue culture plate to a fresh tube and centrifuge cells at 2000 x g for 5 minutes at 4°C. Discard the supernatant.

c. Thoroughly re-suspend the cell pellet in 1 ml of PBS to wash and centrifuge at 2000 x g for 5 minutes at 4°C.

d. Discard the supernatant and move immediately to the next step. Alternatively cells may be flash frozen and stored at -80°C for later use.

2. Isolation of Mitochondria:

e. Re-suspend cells or tissue in 1 ml of Buffer H [Homogenization Buffer] and transfer the sample to a tight-fitting Dounce Tissue Homogenizer.

f. Homogenize the samples by performing 50-100 passes, or what is recommended for a particular sample type. Do not over-homogenize the samples to avoid rupturing the mitochondria.

g. Transfer the homogenate to a 1.5 ml microcentrifuge tube and centrifuge at ***1,000 x g*** for 10 minutes at 4°C. *The pellet contains nuclei and cell debris, while the supernatant contains mitochondria at this point.*

h. Transfer the supernatant to a fresh tube being careful not to touch the pellet and centrifuge at ***10,000 x g*** for 30 minutes at 4°C. The pellet resulting from this centrifugation step contains mitochondria.

i. Discard the supernatant and thoroughly re-suspend the pellet in 0.5 ml of Buffer H [Homogenization Buffer] to wash the mitochondria.

j. Centrifuge at 10,000 x *g* for 30 minutes at 4°C, discard the supernatant, and move immediately to "Mitochondrial DNA Release" procedure.

3. Mitochondrial DNA Release:

k. Re-suspend mitochondrial pellet in 250 µl Buffer R [Re-suspension Buffer] thoroughly without leaving clumps.

l. *Optional: Add 15 µl of RNAse A at this point if you wish to obtain RNA-free DNA product.*

m. Add 15 µl of Enzyme Mix and mix by inverting the tube 5 times. Incubate for 30 minutes at 55°C.

4. Mitochondrial Lysis and DNA Binding:

n. Add 250 µl of Buffer L [Lysis Buffer] and mix by inverting 5-6 times. Incubate on ice for 5 minutes.

o. Add 350 µl of Buffer N [Neutralization Buffer] and mix by inverting 5-6 times.

p. Centrifuge the tubes at 12,000 x g for 10 minutes at 4°C.

q. Place the spin column into the 2 ml collection tube and apply the entire supernatant from step "p" onto the top of the spin column. *Avoid pipetting the precipitate.*

r. Centrifuge at 12,000 x g for 1 minute at 4°C and discard the flow through.

5. Washing:

s. Add 750 µl of Buffer W [Wash Buffer] onto the top of the spin column and centrifuge at 12,000 x *g* for 1 minute at 4°C. Discard the flow through.

t. Repeat step "s" one more time.

u. Centrifuge the spin column at 12,000 x g for 2 minutes at 4°C to dry.

6. Elution:

v. Transfer the spin column to a clean, *DNAse-free* 1.5 ml tube.

w. Add 50 µl of Buffer E [Elution Buffer] to the top of the spin column and incubate for 1-2 minutes at RT.

x. Centrifuge at 12,000 x g for 1 minute at 4°C. The flow through contains purified DNA.

y. Store DNA at -20°C or immediately use the sample in a downstream application of your choice.
Note: *Good quality mitochondrial DNA will have A260/280 of 1.7- 1.99 and exhibit a clear band at 16.5 kb molecular weight on 1% agarose gel. You may also see a high molecular weight band resulting from mitochondrial plasmid concatenation. See troubleshooting guide in Section IX for help.*

VIII. Trouble Shooting

Issue	Possible Reason	Recommendations
Low yield	The tissue is not fresh / the cells are not viable	Use fresh tissue / ensure that the cultured cells are viable prior to their lysis.
	Incomplete DNA release	Increase the number of passages during homogenization by Dounce homogenizer. Alternatively, increase incubation with Enzyme Mix up to 45 min – 1hr.
Low A260/280 (<1.7)	Protein Contamination	Increase incubation time with Enzyme Mix up to 45min – 1hr.
High A260/280 (>2.0)	RNA Contamination	Add RNAse A during DNA release reaction step "L".
No DNA band/smear	DNAse contamination	Use DNAse free aerosol tips, DNAse-free tubes, and practice good sterile technique.
	Over homogenization of cells or tissue	Reduce the number of passages during homogenization by Dounce homogenizer.
Several high molecular DNA Bands observed on gel	Concatenated and/or supercoiled mitochondrial plasmid topology	Mitochondrial plasmid can be concatenated, supercoiled, or relaxed after purification. For human plasmid, linearize mitochondrial plasmid by digesting it with BamHI enzyme. In the case of pure mitochondrial plasmid, multiple bands will merge into one 16.5 kb band after digestion. If multiple bands are still observed after digestion, then nuclear DNA contamination is more likely. Warning: different mammalian species have different endonuclease digestion sites, therefore we recommend analyzing the DNA sequence for unique cut sites prior to enzymatic digestion of non-human mitochondrial plasmid.
	Genomic DNA Contamination	Avoid touching the pellet when transferring the supernatant to a fresh tube in step "h". If you suspect residual pellet transfer at this step, repeat the 1000 x g centrifugation step prior to performing high speed centrifugation step.
Clogged Column	Incomplete disruption of tissue / contaminants present	Use a smaller amount of cells or tissue free of fat and other contaminants.

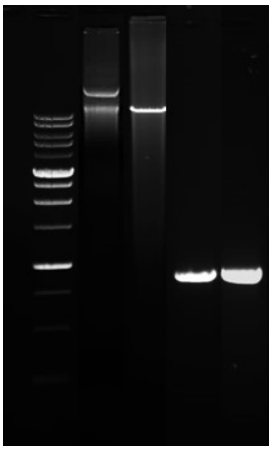

Figure 1: Mitochondrial DNA detection by electrophoresis:

Lane 1 – 1 kb DNA Ladder; Lane 2 – undigested human mtDNA purified by this kit; Lane 3 – human mtDNA digested with BamHI; Lane 4 – rat mtDNA amplified by PCR using rat-specific mtDNA primers; Lane 5 – human mtDNA amplified by PCR using human-specific mtDNA primers.

FOR RESEARCH USE ONLY! Not to be used on humans