Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information for

Mito-magneto: A Tool for Nanoparticle Mediated Mitochondria Isolation

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Materials. All chemicals were used as received without further purification unless otherwise noted. Oleic acid, 1,2 hexadecanediol, oleyl amine, 6-bromohexanoic acid, triphenylphosphine, benzyl ether, and MTT were purchased from Sigma Aldrich. Ultra-pure LPS was purchased from Invivogen, CA, USA. Glutamine, penicillin/streptomycin, trypsin-ethylenediamminetetraacetic acid (EDTA) solution, (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (1 M), and sodium pyruvate were procured from Sigma Life Sciences. Qdot 705 ITK amino PEG quantum dots (QDs) was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Gibco Life Technologies. IL-6, and TNF-α cytokines were tested using BD OptEIA mouse ELISA kits. Iron (II, III) oxide magnetic NP solution (5 mg/mL, in toluene) for cytotoxicity and MRI measurements was purchased from Sigma Aldrich (Cat. # 700320). Citrate synthase assay kit (Cat. # K318-100) and Cytochrome C oxidase (COX) activity kit (Cat. # K287-100) were purchased from Biovision Inc. MitoTox™ Complex V OXPHOS Activity Microplate assay kit (Cat. # ab109907) was purchased from Abcam. ATP production by the isolated mitochondria was assessed by a CellTiter-Glo® Luminescent Cell Viability Assay kit (Cat. # G7570) procured from Promega kit. Pierce® Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific. Reagents for mitochondria isolation was from a mitochondria isolation kit (Cat. # 89874) procured from ThermoFiser scientific. TOM22 magnetic beads based Mitochondria MidiMACS starting kit (Cat. # 130-094-872) was procured from MACS Miltenyi Biotec. Tween 20 was purchased from Fisher Bio-reagent. The primary antibodies against TFAM (Cat. # ab131607), VDAC1/porin (Cat. # ab14734), Lamin-A (Cat. # ab26300), and calnexin (Cat. # ab10286) for western blot were purchased from

Abcam. Secondary goat anti-rabbit IgG H&L (HRP) preadsorbed (Cat. # ab97080) and goat anti-mouse IgG H&L (HRP) preadsorbed (Cat. # ab ab6789) antibodies were also purchased from Abcam. $CDCl_3$ and DMSO-d_6 were purchased from Cambridge Isotope Laboratories Inc. Regenerative cellulose membrane Amicon ultra centrifugal 100 kDa filters were purchased from Merck Millipore Ltd. Oligomycin, rotenone, antimycin-A, and FCCP were purchased from Sigma Aldrich. XF24-well cell culture microplates were purchased from Seahorse Biosciences. Ammonium persulfate (Cat. # 161-0180), tris/glycine/SDS buffer (Cat. # 161-0732), SDS-PAGE gel preparation kit TGX stain-free TM fast cast TM acrylamine 10% (Cat. # 161-0182), and Clarity™ western ECL substrate (Cat. # 170-5060) were purchased from Bio-Rad Inc. Nitrocellulose membrane (Cat. # 88018) was purchased from Thermo Scientific. JC-1 dye (Cat. # T3168) was purchased from Life Technologies.

Instrumentations. ^{31}P spectra were recorded on a 500 MHz Varian NMR spectrometer. Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 MΩ) containing a 0.22 μm filter. Transmission electron microscopy (TEM) images were acquired using a Philips/FEI Tecnai 20 microscope. Cells were counted using Countess® automated cell counter procured from Invitrogen life technology. Plate reader analyses were performed on a Bio-Tek Synergy HT microplate reader. Dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS system. MRI (Magnetic Resonance Imaging) images were obtained using an Agilent (7 Tesla, 200 mm) horizontal bore magnet based MRI instrument. Mitochondrial bioenergetics assay was performed on a Seahorse XF24 (Seahorse Biosciences, North Billerica, MA, USA) analyzer. ICP-MS

studies on the subcellular fractions were done on a VG SerumQuad 3 ICP mass spectrometer. ICP-OES studies were performed on Perkin Elmer Optima 8300 ICP-OES spectrometer**.** Western blots were imaged on a FluorChem HD2 system from Alpha Innotech (Protein Simple, Santa Clara, CA, USA). Magnetic isolation of mitochondrial fraction was done on a EasySep^{TM} magnet (Cat. # 18000) from Stemcell technologies. Probe sonicator for cell lysis was procured from Misonix Sonicators (Newtown, CT, USA). TEM studies of isolated mitochondria were performed using a JEOL JEM 1011 Transmission Electron Microscope.

Methods.

Synthesis of 1-Carboxyhexyl-6-triphenylphosphonium bromide (TPP-hexanoic acid). TPP-hexanoic acid synthesis and characterization were performed as reported by us in previous publications.[1-3]

Synthesis of PLGA-b-PEG-QD, PLGA-b-PEG-OH, PLGA-b-PEG-TPP and Targeted and Non-targeted QD loaded nanoparticles. All the syntheses were performed as previously described.[1, 3]

Cell Viability Studies on H9C2 Cells

Cytotoxicity of the NT-IONP or Mito-magneto was studied in H9C2 cardiomyocytes using MTT assay. H9C2 cardiomyocytes (4000 cells/well) were seeded on a 96-well plate and allowed to grow 24 h at 37 °C in 5% CO_2. Mito-magneto or NT-IONP were added to the cells in fresh DMEM containing 1% DMSO (5% for NT-IONP) and incubated for 12 h. Media was aspirated from the wells and fresh media was added and the cells were incubated for another 60 h following which, MTT (4 mg/mL, 25 μL/well) was added to the wells. The plates were then incubated for another 5 h for the

conversion of MTT to formazan by cellular reductase enzymes. The media was removed and cells were lysed using 100 μL of DMSO and homogenized with gentle shaking at room temperature. The absorbance of the resultant solution in each well was read at 550 nm with a background reading at 800 nm. Cytotoxicity was expressed as mean percentage increase relative to the untreated control ± standard deviation. Control values were set at 0% cytotoxicity or 100% cell viability. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a three parameters logistic model used to calculate the inhibitory concentration-50 (IC_{50}) that is the concentration of test article under investigation showing 50% inhibition in comparison to untreated controls. These analyses were performed with GraphPad Prism (San Diego, U.S.A).

Western Blot Method

Protein content in each of the subcellular fractions was measured using BCA assay and stock solutions containing 1 mg/mL protein were made for western blot. The subcellular fractions were suspended in mitochondria assay solution comprised of 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1 mM ethyleneglycoltetraacetic acid (EGTA) and 0.2% (w.v) fatty acid-free bovine serum albumin (BSA). Proteins were resolved on a 10% SDS-PAGE and transferred onto nitrocellulose membranes (Biorad). Membranes were blocked using 5% milk in TBST (Tris-buffered saline, 0.1% Tween 20) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and incubated with primary antibodies overnight (~16 h) at 4 °C. Membranes were then washed (3X) in TBST buffer and incubated with the secondary antibody for 2 h and developed by chemiluminescence and the image acquired using FluorChem HD2 system.

Enzyme-linked Immunosorbent assay (ELISA) for Immunogenic Effect of Mito-magneto on RAW 264.7 Cells

The levels of pro-inflammatory cytokines IL-6 and TNF-α in RAW 264.7 macrophages upon incubation with Mito-magneto for 24 h was measured using ELISA kits for the respective cytokines following manufacturer's protocol. RAW 264.7 macrophage cells were plated in 96-well plates at a cell density of 10,000 cells/well and allowed to grow for 24 h. Mito-magneto (50 μg/mL,) was then added to the cells in 1% DMSO containing DMEM and incubated for 24 h. To the capture antibody pre-coated strips, the assay diluent (20 μL) was added. To this, supernatant DMEM (80 μL) from the RAW 264.7 cells treated with Mito-magneto, were added. 100 μL of standard (for the respective cytokine) was also added to the respective wells in the plate. The plate was then sealed with the adhesive strip provided and incubated for 2 h at room temperature on an orbital shaker. The wells were then aspirated and washed 5 times with the wash buffer (~300 μL each wash). The freshly washed plates were then incubated with either IL-6, or TNF-α detection antibodies (100 μL). Once again, they were sealed with the provided adhesive strips for 2 h at room temperature on an orbital shaker. The wells were then washed using wash buffer for 5 times and incubated with streptavidin-HRP conjugate (100 μL, diluted with assay diluent) and incubated for 30 min. The solutions were aspirated and washed 5 times with the wash buffer (~300 μL for each wash). Then, the wells were incubated with the substrate solution (100 μL) for 30 min at room temperature in the dark. After 30 min, the reaction was stopped with the stop solution (100 μL) with gentle mixing to ensure a uniform solution. The plates were then read for absorbance using a plate reader at 450 nm.

TEM of Isolated Mitochondria

The mitochondrial fraction was pelleted down *via* centrifugation at 12,000 g for 15 min and the pellet (~1 mg) was treated with 2% glutaraldehyde in 1X mitochondria isolation buffer for overnight. The fixed mitochondria were then rinsed three times with mitochondria isolation buffer (1X) for 15 min each. The mitochondria were then post fixed with 1% OsO_4 in mitochondria isolation buffer for an hour and then rinsed with deionized water (3X) for 10 min each. The samples were dehydrated in ethanol (30, 50, 75, 95, and 100%) for 15 min in each step. The last step with 100% ethanol was repeated twice. The samples were then rinsed with 100% propylene oxide twice for 15 min each. The samples were then infiltrated 50% and 75% Spurr in propylene oxide for 120 min each. The samples were further treated with 1:1 Araldi:Spurr mixture twice for 30 min each. The samples were then allowed to polymerize at 70-80 °C inside the oven for overnight. The samples were then sliced, mounted on copper grids, and imaged using a JEOL JEM 1011 Transmission Electron Microscope.

MitoStress Test

XF sensor cartridges were hydrated in XF calibrant (1 mL) overnight prior to the test at 37 °C in a CO_2 free incubator. H9C2, J3TBG or A2780 cells were cultured in XF24-well cell culture microplates (Seahorse Bioscience) at a density of 40,000 cells/well (0.32 cm^2) in 200 μL growth medium and then incubated for 24 h at 37 °C under 5% CO_2 atmosphere. Mito-magneto (20, 40, 60 and 100 μg/mL) was then added to the cells and the cells were incubated for 24 h at 37 °C under 5% CO_2 atmosphere. After 24 h, all but 50 μL of the culture medium was removed from each well and the cells were rinsed three times with 500 μL of XF stress test glycolysis optimization medium

supplemented with sodium pyruvate and D-glucose and pre-warmed to 37 °C, and finally 500 μL of the optimization medium was added to each well and the plate was incubated at 37 °C without CO_2 for 1 h prior to assay. The oxygen consumption rate (OCR) was measured simultaneously for 16 min to establish a baseline rate. Different parameters of respiration, basal respiration, coupling efficiency, and spare respiratory capacity, were investigated by using a Seahorse XF-24 cell Mito Stress Test Kit. The different parameters of respiration were calculated by subtracting the average respiration rates before and after the addition of the electron transport inhibitors oligomycin (2.0 μM) and FCCP (2.0 μM), an ionophore that is a mobile ion carrier, and a mixture of antimycin-A (1.0 μM) (which is a complex III inhibitor) and rotenone (1.0 μM), a mitochondrial inhibitor that prevents the transfer of electrons from the Fe–S center in complex I to ubiquinone. Test articles on each well had four replicates.

Table S1. Cell Viability During Mito-magneto Based Isolation		
	Untreated Cells	Mito-magneto Treated Cells
A2780		
Live Cells	$5.25X10^6$	$4.95X10^6$
Dead Cells	$9.0X10^4$	$6.0X10^4$
Total Cells	$5.35X10^6$	$5.0X10^6$
Percent Viability	98.5	98.5
J3TBG		
Live Cells	$3.15X10^6$	$3.5X10^6$
Dead Cells	$1.4X10^4$	$1.0X10^5$
Total Cells	$3.25X10^6$	$3.6X10^6$
Percent Viability	96	97
H9C2		
Live Cells	$1.12X10^6$	$1.1X10^6$
Dead Cells	$5.0X10^5$	$4.0X10^4$
Total Cells	$1.19X10^6$	$1.2X10^6$
Percent Viability	96	97

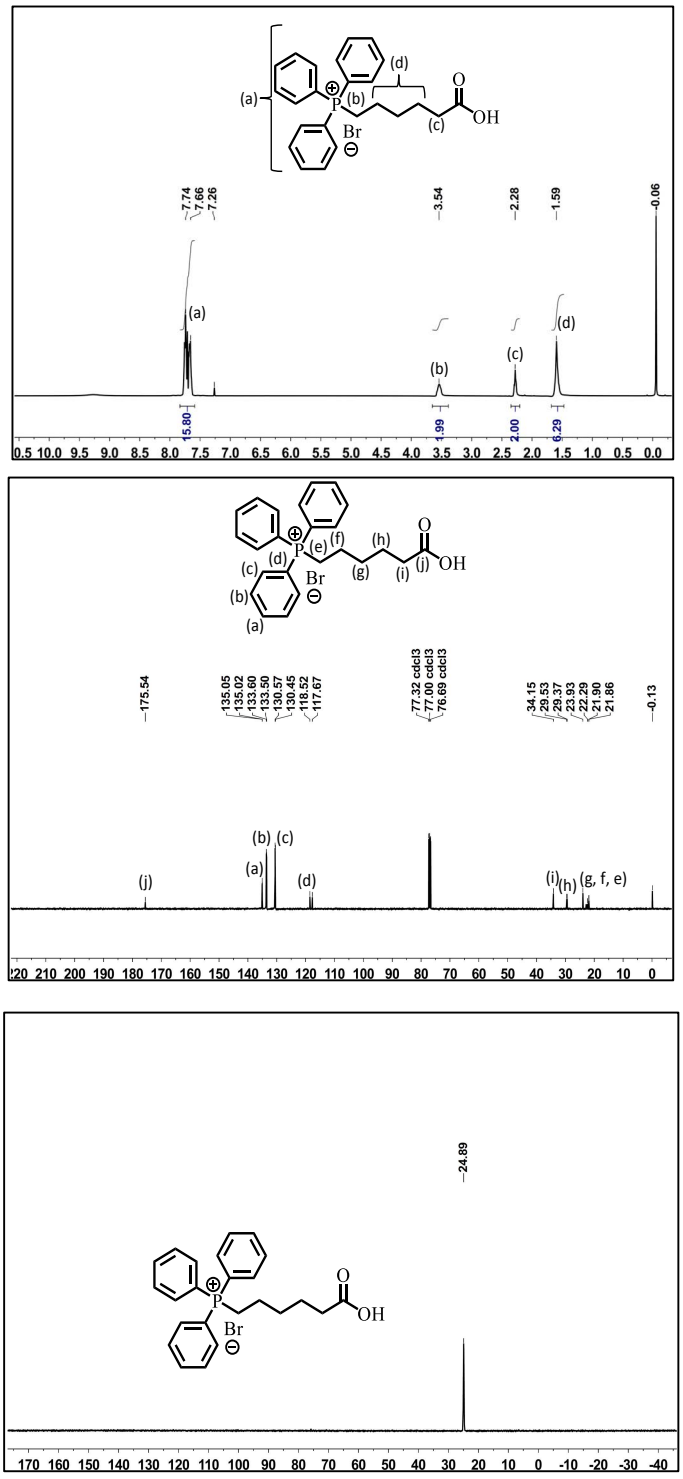


Figure S1. ^{1}H proton (top), ^{13}C (middle), and ^{31}P (bottom) spectra of TPP-$(CH_2)_5$-COOH.

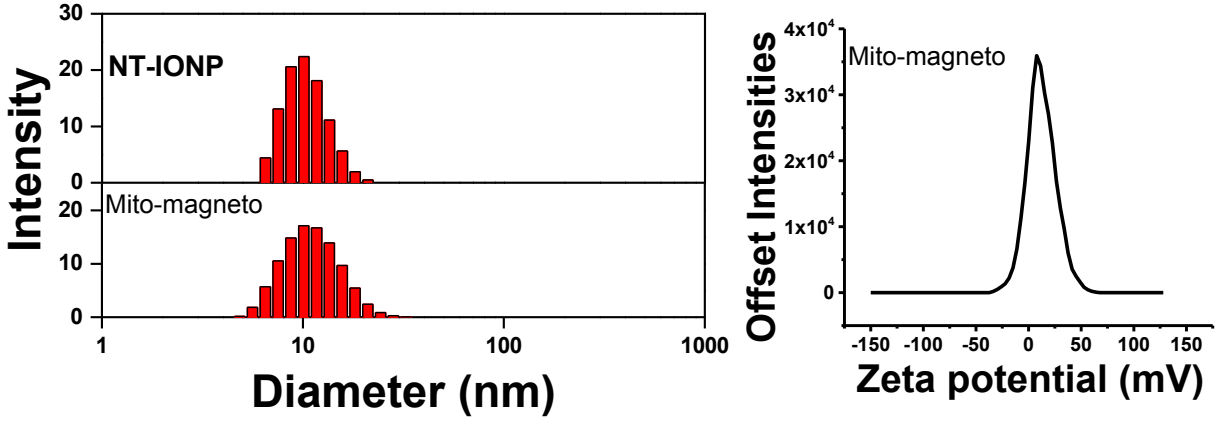


Figure S2. DLS plots for hydrodynamic diameter and zeta potential of Mito-magneto.

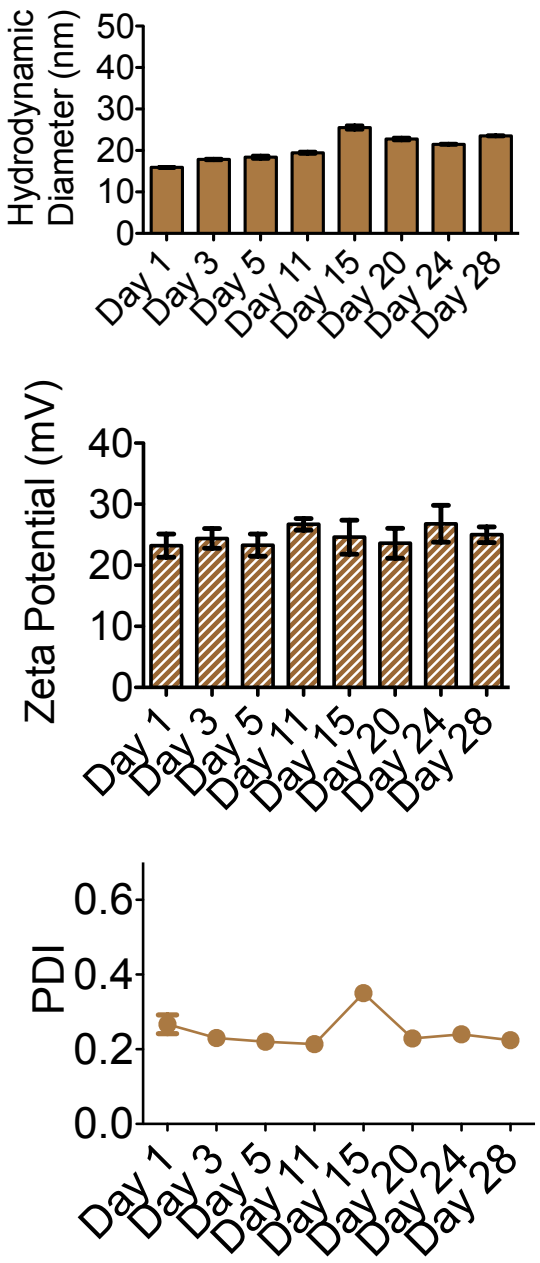
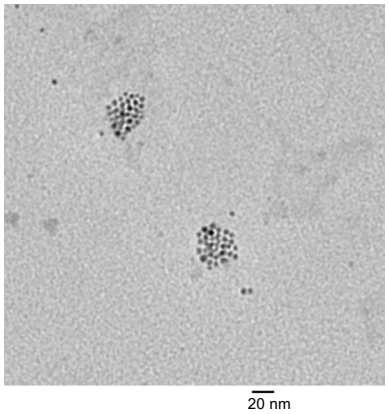
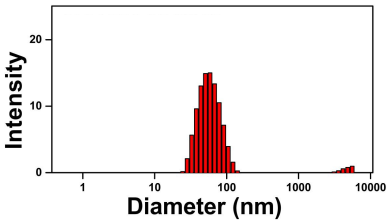


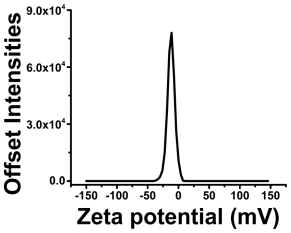
Figure S3. Stability of Mito-magneto by storing these particles in DMF at a concentration of 5 mg/mL at 4 °C for 28 days.

(A)



(B)





Diameter ($Z_{average}$)/nm	Zeta Potential (mV)	PDI
56.3±1.3	-11.5±0.1	0.189±0.012

Figure S4. (A) TEM image of Mito-magneto encapsulated PLGA-*b*-PEG-NPs demonstrating efficient encapsulation of Mito-magneto in the hydrophobic core of other nanomaterials. (B) Diameter, zeta potential, and polydispersity index (PDI) of Mito-magneto encapsulated PLGA-*b*-PEG-NPs as determined by dynamic light scattering method.

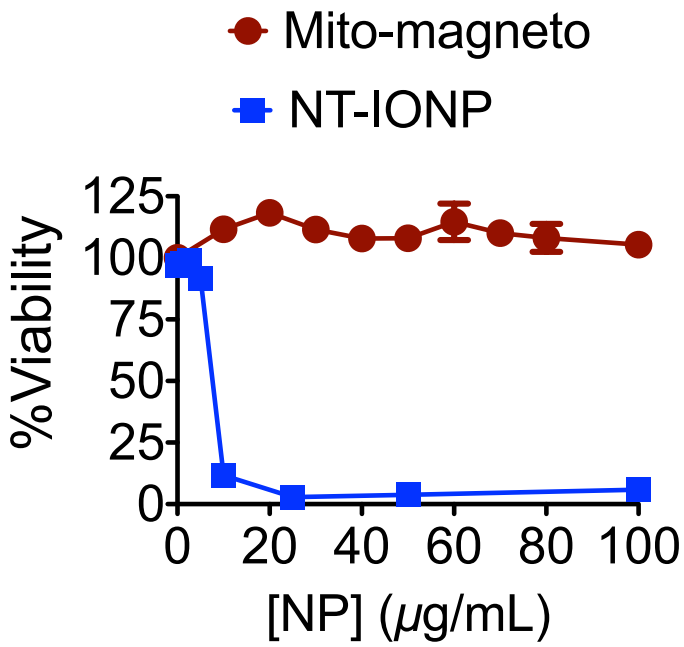


Figure S5. Comparison of cytotoxicity of Mito-magneto with commercially available IO-NPs on H9C2 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

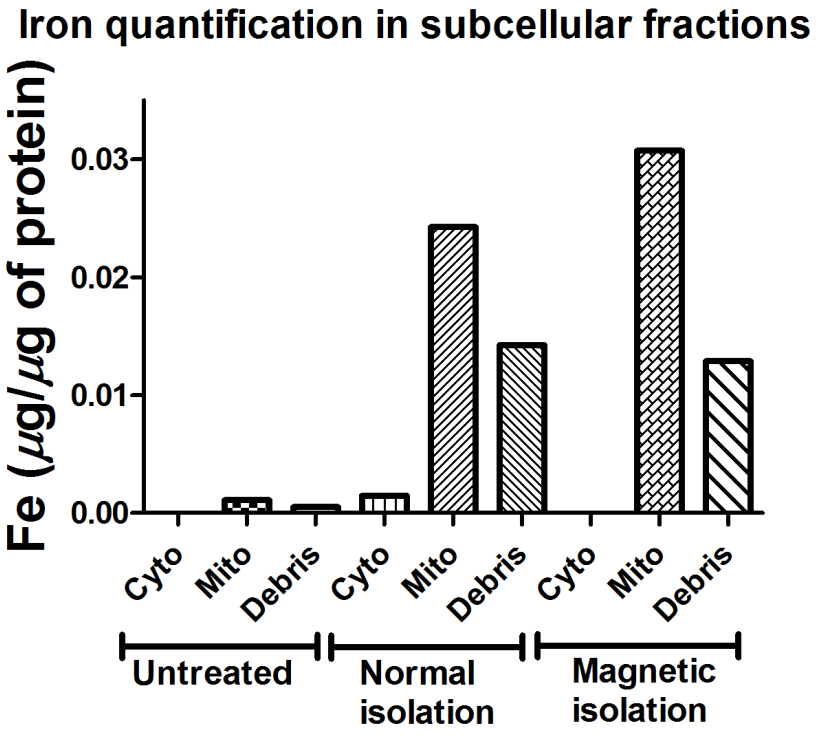


Figure S6. Quantification of Mito-magneto in different cellular compartments from H9C2 cells by iron ICP-OES.

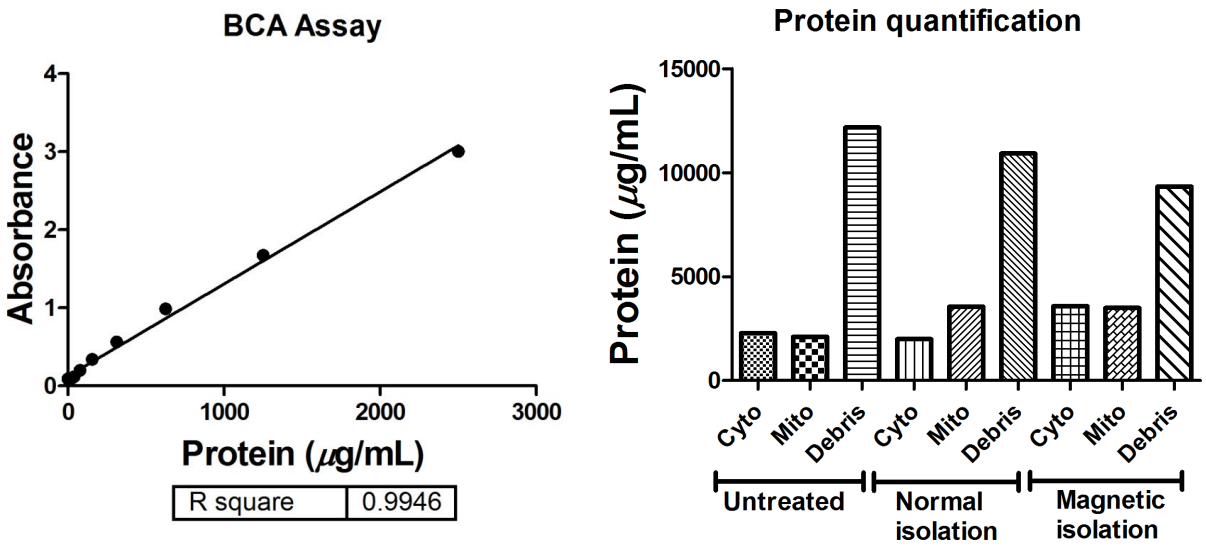
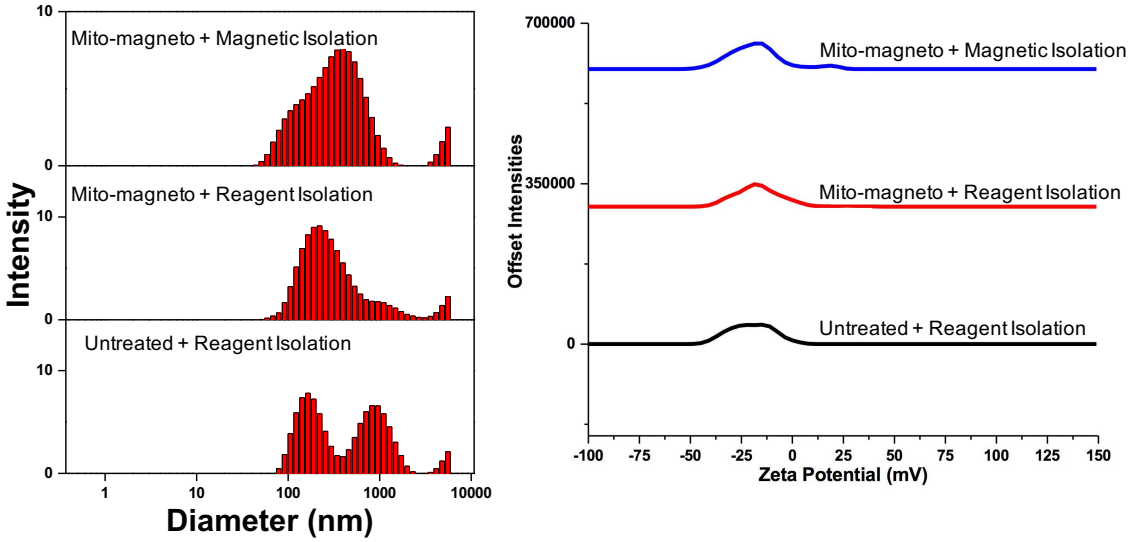


Figure S7. Protein quantification of cellular fractions by BCA assay.

(A) Mitochondria from H9C2 Cells



(B) Mitochondria from A2780 Cells

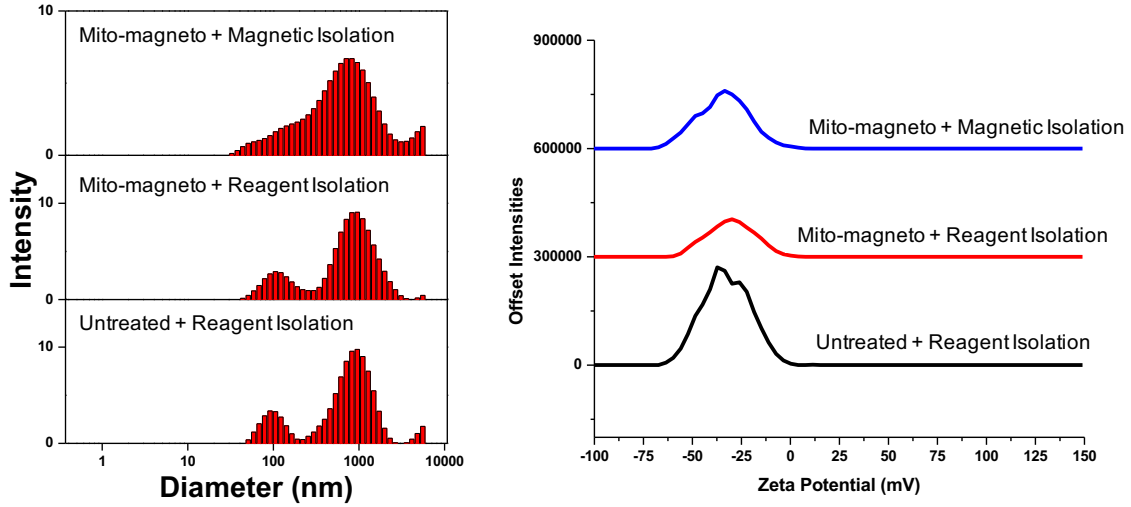


Figure S8. Diameter and zeta potential of isolated mitochondria from H9C2 and A2780 cells by different methods using DLS.

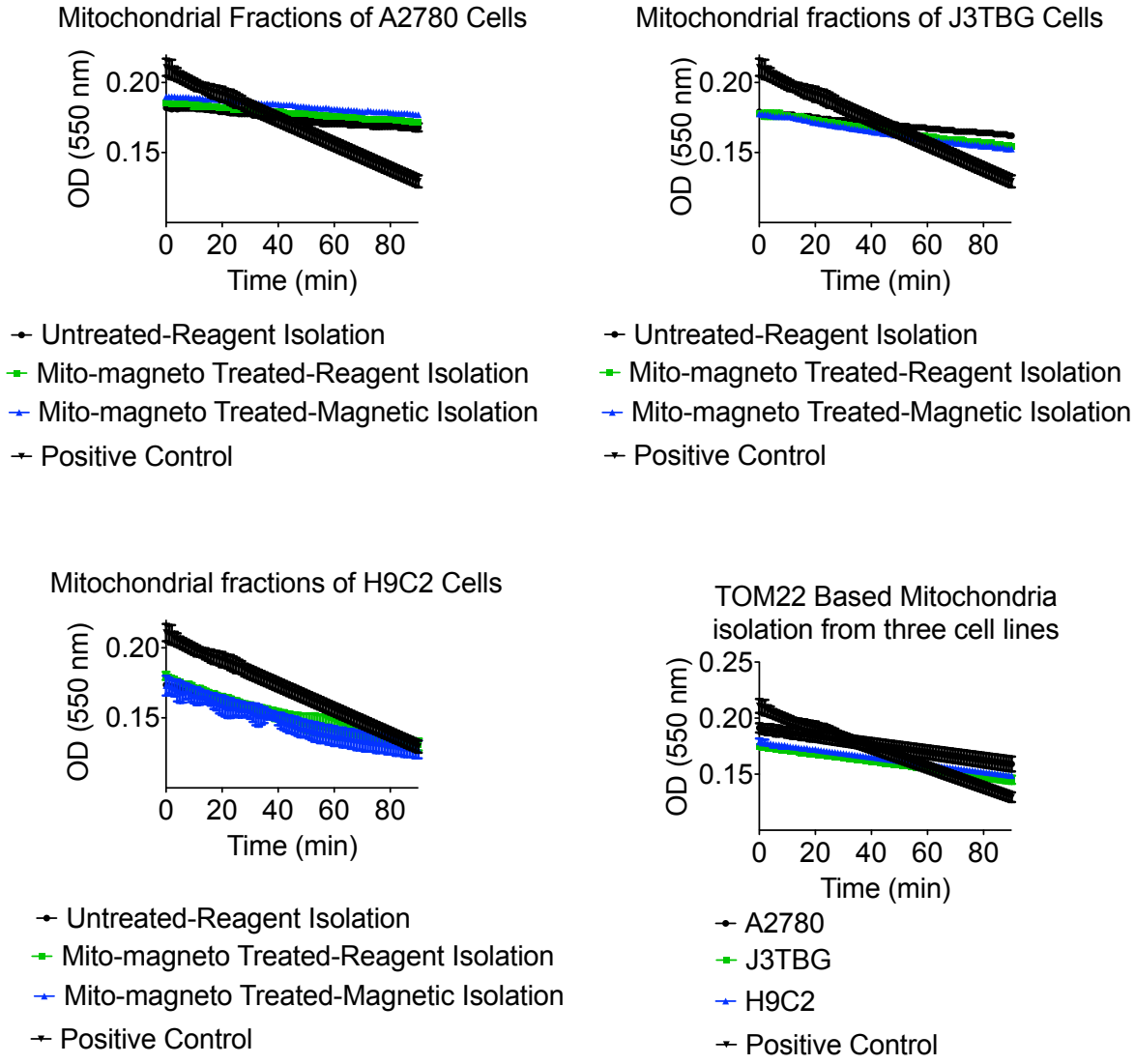


Figure S9. COX activity of isolated mitochondria from different cell lines by various methods.

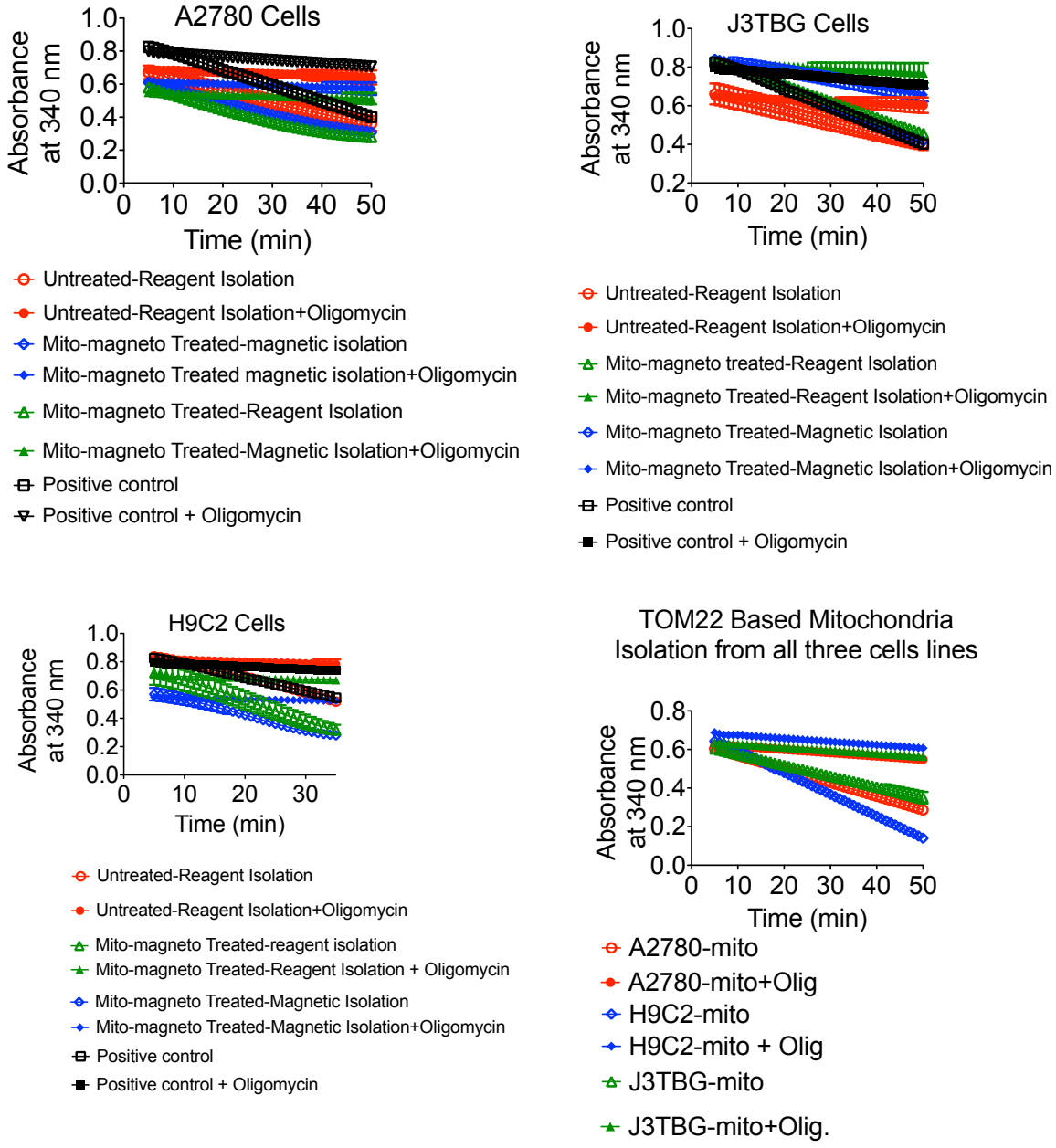


Figure S10. ATP synthase activity in the presence or absence of ATP synthase inhibitor oligomycin of isolated mitochondria from different cell lines by various methods.

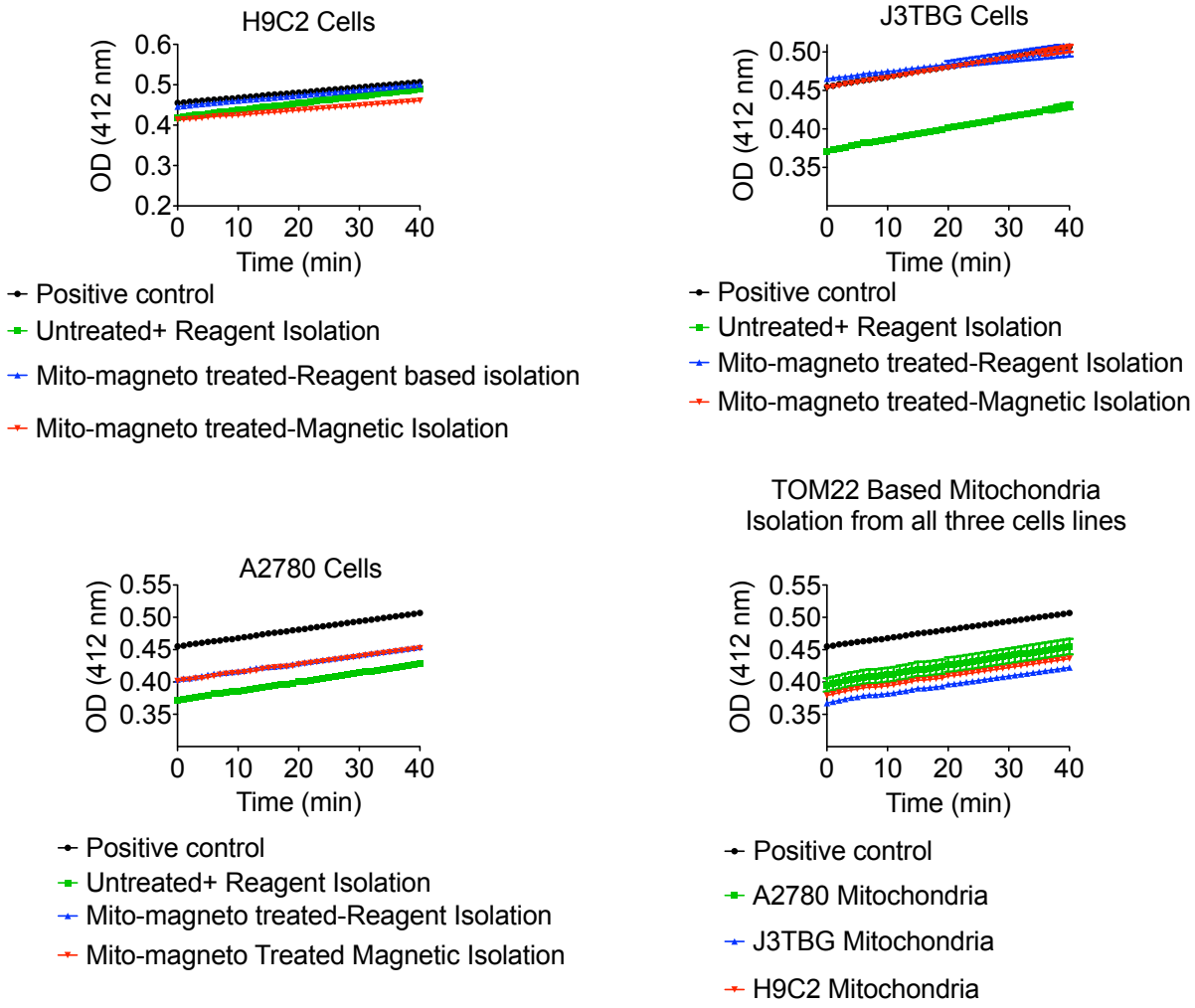


Figure S11. Citrate Synthase activity of isolated mitochondria from different cell lines by various methods.

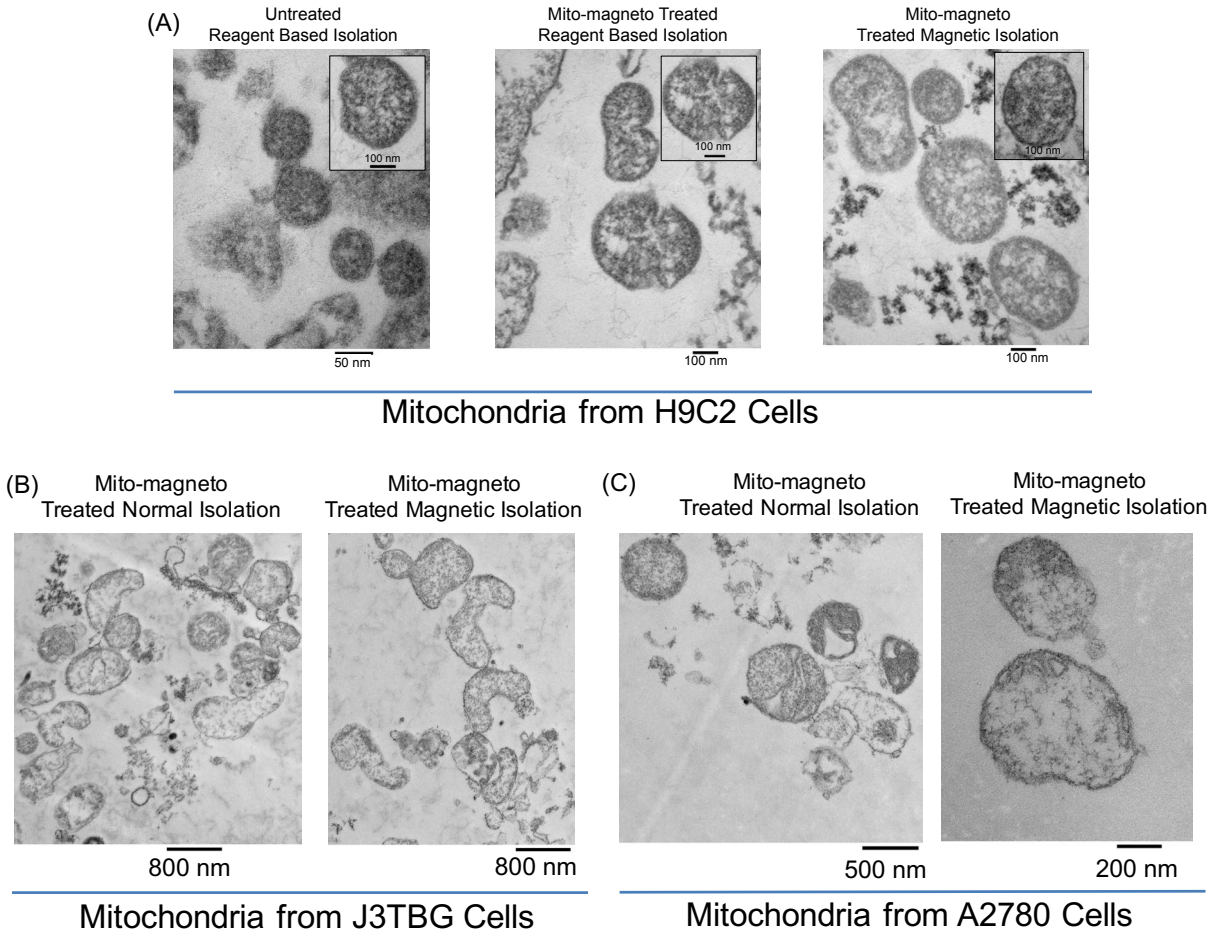


Figure S12. Morphological analyses of isolated mitochondria using Mito-magneto from H9C2, J3TBG, and A2780 cells by TEM analyses.

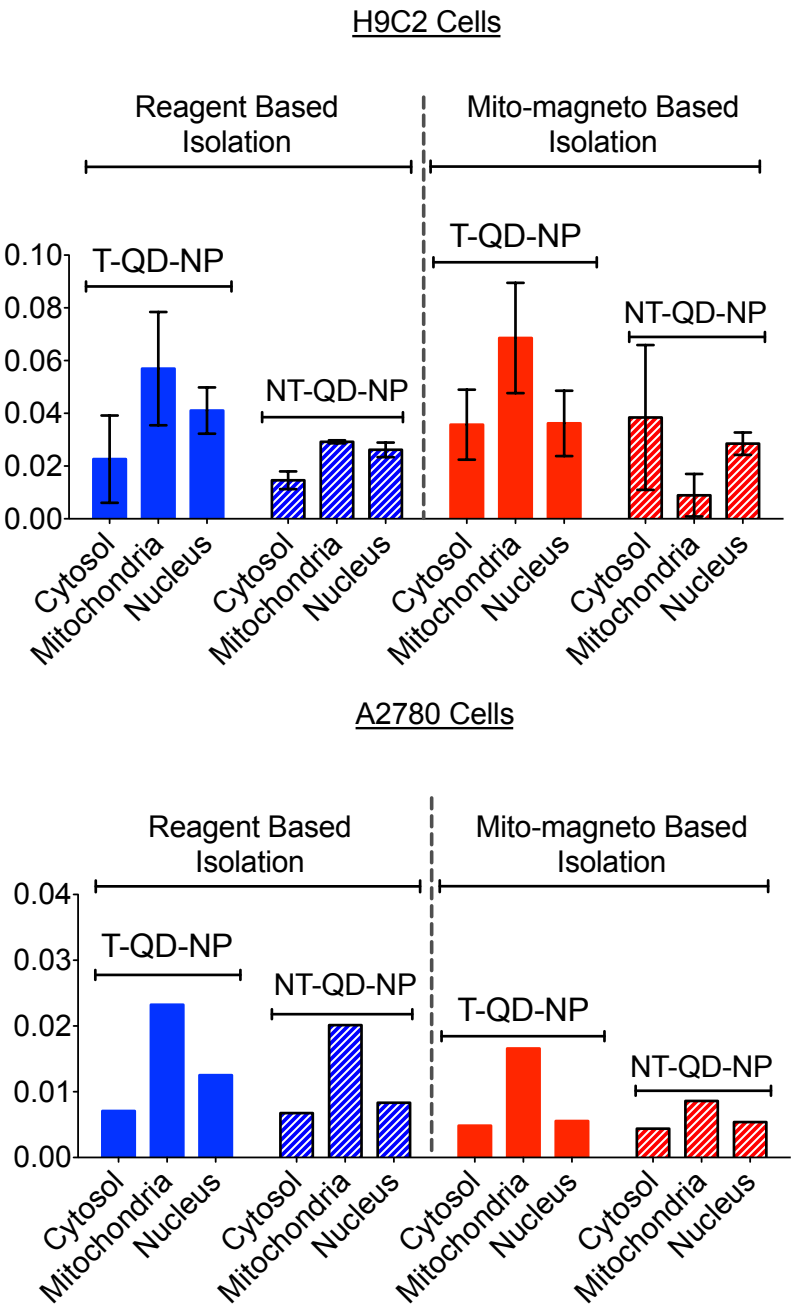


Figure S13. T/NT NP quantification in mitochondrial compartments of H9C2 and A2780 cells by conventional reagent isolation and Mito-magneto driven magnetic isolation.

References:

1. S. Marrache and S. Dhar, *Proc Natl Acad Sci U S A*, 2012, **109**, 16288-16293.
2. R. K. Pathak, S. Marrache, D. A. Harn and S. Dhar, *ACS Chem Biol*, 2014, **9**, 1178-1187.
3. S. Marrache, R. K. Pathak and S. Dhar, *Proc Natl Acad Sci U S A*, 2014, **111**, 10444-10449.