APPENDIX

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APPENDIX A

Cardiac mitochondrial function

1. Solutions

a. Mitochondrial isolation buffer (MIB)

Sucrose	300	mM
EGTA	0.2	mM
TES	5	mM
pH 7.2		

b. Respiration buffer for mitochondrial membrane potential changes (RB)

KCl	150	mM
HEPES	5	mM
$K_2HPO_4.3H_2O$	2	mM
$C_5H_8NNaO_4.xH_2O$	5	mM
$CH_3COCOONa$	5	mM
pH 7.2		

c. Respiration buffer for mitochondrial swelling or ROS production (RH)

KCl	100	mM
Sucrose	50	mM
HEFES	10	mM
KH_2PO_4	5	mM
pH 7.4		

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2. Cardiac mitochondria isolation

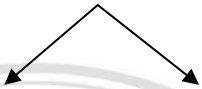
Ventricular tissue in 8 ml cold isolated buffer in homogenate tube

↓

Centrifuge it at 800 g at 4℃ for 5 min

↓

Keep the supernatant and separated tube for RH or RB solution



RH tube RB tube

↓ ↓

Centrifuge it at 8,800 g at 4℃ for 5 min

↓ ↓

Keep pellet and added 2ml isolated buffer

↓ ↓

Centrifuge it at 8,800 g at 4℃ for 5 min

↓ ↓

Keep pellet and added 1 ml of each RES buffer

↓ ↓

50 µl of each tube added 1 ml BCA reagent

↓

Incubated at 60℃ for 30 min in water bath

↓

Measured 562 nm by spectrophotometer

↓

Calculated protein concentration and added each RES buffer for final concentration 0.4 mg/ml

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3. Cardiac mitochondrial ROS production

Well plate	Blank	M
Sample	-	150 µl
RES buffer (RH)	150 µl	-
DI	30 µl	30 µl
DCFH-DA dye	20 µl	20 µl

Incubated at room temperature for 20 min

↓

Measured at 485/ 530 nm by microplate reader

4. Cardiac mitochondrial membrane potential

Well plate	Blank	M
Sample	-	150 µl
RES buffer (RB)	150 µl	-
DI	30 µl	30 µl
JC-1 dye	20 µl	20 µl

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Cover and Incubated at 37℃ for 20 min

↓

Measured at 485/530 and 485/590 nm by microplate reader

5. Cardiac mitochondrial swelling

Well plate	Blank	M
Sample	-	150 µl
RES buffer (RH)	150 µl	-
DI	50 µl	50 µl

Measured at 540 nm by microplate reader

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APPENDIX B

Western blot technique

1. Solutions

1.1 Extraction buffer (stock solution)

1.1.1 Tris (1M; pH 6.8)

Tris	15.76	g
ddH_2O	<100	ml

Add ddH_2O until reach 100 ml and adjust pH to 6.8.

1.1.2 NaF (1M)

NaF	41.98	g
ddH_2O	100	ml

1.1.3 Na_3VO_4 (100 mM)

Na_3VO_4	1.8391	g
ddH_2O	<100	ml

Add H_2O until reach 100 ml and adjust pH to 9.0.

1.2 Extraction buffer (working solution; prepare from stock solution)

Tris (20 mM)	200	μl
NaF (5mM)	50	μl
Na_3VO_4 (100 mM)	1	ml
ddH_2O	17.5	ml
Protease inhibitor tables	1	tab/10ml

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1.3 2X SDS Sample buffer

Glycerol	2	ml
SDS	6	g
Tris	1.4	g

Make up to 100 ml with ddH_2O.

Before use, add 100 μl of mercaptoethanol (10%), 900 μl of 2X sample buffer and 5 μl of bromophenol blue (8% in Ethnol).

1.4 SDS-PAGE gel solutions

1.4.1 Polyacrylamide gel solution

1) Resolving gel (1.5 M Tris; 0.4% SDS; pH to 8.8 with HCl)

SDS	2.0	g
Tris	90.9	g
ddH_2O	300	ml

Add ddH_2O until 500 ml and adjust pH to 8.8

2) Stacking gel (0.5 M Tris; 0.4% SDS; pH to 6.8 with HCl)

SDS	2.0	G
Tris	30.25	g
ddH_2O	300	ml

Add ddH_2O until reach 500 ml and adjust pH to 6.8

3) 10% Ammonium persulfate

Ammonium persulfate	1	g
ddH_2O	10	ml

Polyacrylamide gels were made depending on the concentration according to table.

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Reagent	10%	15%	4% Stacking gel
MW of trget protein	>80	<30	-
ddH_2O (ml)	5	3	3.5
30% Acrylamide (ml)	4	6	1
1.5 M tris-HCl (pH 8.8) (ml)	3	3	-
0.5 M tris-HCl (pH 6.8) (ml)	-	-	1.5
10% Ammonium persulfate (μl)	60	50	50
TEMED (μl)	15	15	5

1.5 Ponceau S Staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid)

This is a reversible staining method to locate protein bands on Western blots.

Ponceau S	1	g
Glacial acetic acid (100%)	50	ml

Add ddH2O until reach 1000 ml.

1.6 Running buffer (10X)

Tris	30.3	g
Glycine	144.2	g
SDS	10	g

Add ddH_2O until reach 1000 ml.

To make 1X Running buffer; add 100 ml of 10X Running buffer and 900 ml of ddH_2O.

1.7 Transfer buffer (10X)

Tris	30.3	g
Glycine	144.2	g

Add ddH_2O until reach 1000 ml.

To make 1X Transfer Buffer; add 100 ml of 10X Transfer Buffer to 200 ml of methanol and 700 ml of ddH_2O

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1.8 TBS buffer (10X)

Tris	24.2	g
NaCl	80	g

To make 1X TBST; add 100 ml of 10X TBS to 900 ml of ddH2O and 1 ml of Tween-20.

1.9 Blocking buffer

1X TBST	100	ml
Skimmed Milk powder	5	g

1.10 Antibody dilution buffer

1X TBST	100	ml
Skimmed Milk powder	1	g

2. Sample preparation

Frozen heart samples were homogenized with extraction buffer
(Add 1 ml of extraction buffer/ 100 mg sample)
↓
Centrifuged at 13,000 rpm for 10 minutes at 4℃
↓
Collect supernatant and add 2X SDS Sample buffer (1:1)
↓
Boil 95℃, 10 min

3. SDS-Acrylamide gel preparation

Clean loading gel glass with 70% Ethanol
↓
Load the 10% or 15% separating gel, fill the space above the gel with isopropanol, and leave it for 30 min
↓
After gel is set, discard isopropanol, wash with ddH_2O
↓
Add 4% stacking gel, place comb, and leave it for 15 min
↓

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After gel is set, move gels into electrophoresis chamber, and add 1X running buffer

4. Immunolotting

Add 10μl of Protein ladder and 20 μl of protein sample/well

↓

Run gel at constant voltage of 90 Volts for initial 10 min and increase the voltage to 120 Volts for approximately 2 h until the protein touch the end of the gel

↓

Transfer gel to mitocellulose membrane at 100 Volts, 1 h (sponge-blotting paper-gel-membrane-blotting paper-sponge)

↓

Check transfer by straining membrane with Ponceau S for 5 min, wash with ddH2O follow by 1X TBST until red band disappear

↓

Block membrane with 5% milk in 1X TBST for 1 hour on an orbital shaker

Discard the blocking solution, add primary antibody 1:1000 with 1% milk in 1X TBST, and incubate overnight at 4℃

Wash membrane with 1X TBST 5 min, 4 times

Add anti-rabit IgG conjugate HRP in TBST for 1 hour on an orbital shaker

Wash membrane with 1X TBST 5 min, 6 times

5. ECL exposure

Immerse the membrane in ECL reagent mixed with 1:1 for 1 min at room temperature

↓

Adjust exposure time according to the signal strength and specificity

↓

Protein was exposed by ChemiDoc^{TM} Touch Imaging System

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APPENDIX C

Determination of cardiac MDA level

Reagents

Phosphate buffer

$NaH_2PO_42H_20$	712	mg
H_2PO_4	68	l
ddH_2O	1000	ml
Phosphoric acid		
H_3PO_4	30	ml
ddH_2O	970	ml

10%TCA in 50 ppm BHT

TCA	100	g
BHT	50	mg
ddH2O	1000	ml

50 ppm BHT in methanol

BHT	50	mg
Methanol	1000	ml

0.6% TBA in ddH_2O

TBA	6	g
ddH_2O	1000	ml

MDA standard

MDA stock solution	100	ml
ddH_2O	9900	ml

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Mobile phase for MDA determination

1 M $K_2HPO_4.3H_20$

$K_2HPO_4.3H_20$	780	g
ddH_20	5000	ml

1 M KH_2PO_4

KH_2PO_4	1141.15	g
ddH_20	5000	ml

100 mM KPB

KH_2PO_4	192.5	ml
$K_2HPO_4.3H20$	307.5	ml
ddH_20	4500	ml

50 mM KPB

KPB	3250	ml
Methanol	1750	ml

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Peer reviewed abstract

Shinlapawittayatorn K, **Nuntaphum W**, Tanajak P, Thummasorn S, Khamseekaew J, Wongjaikam S, Chattipakorn S and Chattipakorn N., Vagus Nerve Stimulation Requires both Ipsilateral and Contralateral Efferent Vagal Activity to Fully Provide its Cardioprotection Against I/R Injury. *J Am Coll Cardiol* 2017;69(11):50 Suppl. (Impact Factor = 17.759)

Peer-review Articles

Nuntaphum W, Pongkan W, Wolovengjaikam S, Thummasorn S, Tanajak P, Khamseekaew J, Chattipakorn S, Intachai N, Chattipakorn N and Shinlapawittayatorn K. Vagus Nerve Stimulation Protects the Heart Against Ischemia /Reperfusion Injury Predominantly Through its Efferent Vagal Fibers. *Basic Res Cardiol*. 2018 May 9;113(4):22. (Impact Factor = 5.306)

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Conference abstract

Nuntaphum W, Tanajak P, Thummasorn S, Khamseekaew J, Wongjaikam S, Chattipakorn C, Chattipakorn N and Shinlapawittayatorn K., Vagus Nerve Stimulation Protects the Heart Against Ischemia /Reperfusion Injury Predominantly Through its Efferent Vagal Fibers. *International Graduate Research Conference (iGRC)* 2016

Scientific abstract participation at international meeting

February 2017 The International Graduate Research Conference (iGRC 2016), Empress Hotel Chiang Mai, Thailand

Scientific abstract participation at national meeting

December 2016 The Physiological society of Thailand conference 2016 (PSTC2016), Empress Hotel Chiang Mai, Thailand

May 2017 Research Day, Department of Physiology, Faculty of Medicine, Chiang Mai University Chiang Mai, Thailand

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