APPENDIX

APPENDIX A

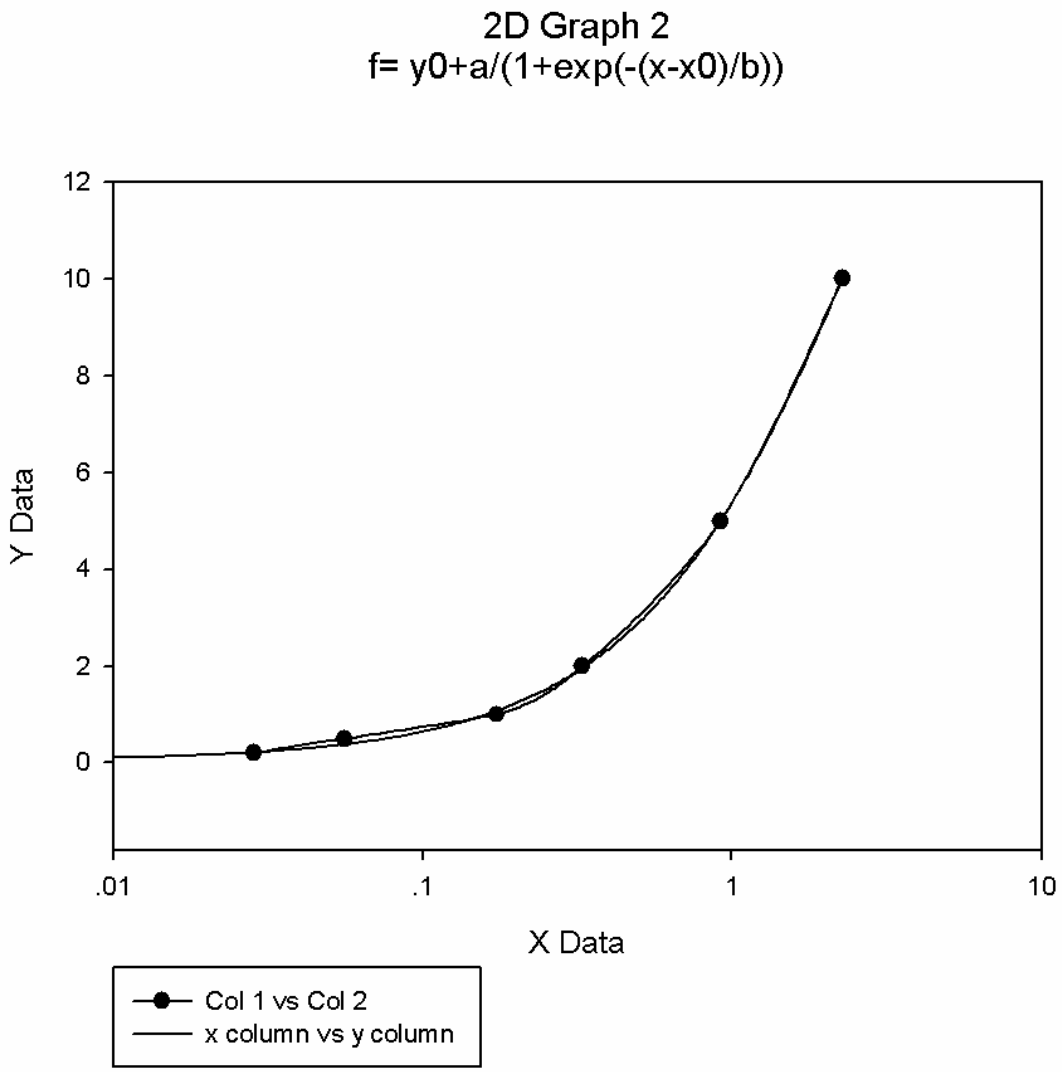

Figure A-1 Standard curve for determination of insulin concentration based on Sandwich ELISA method

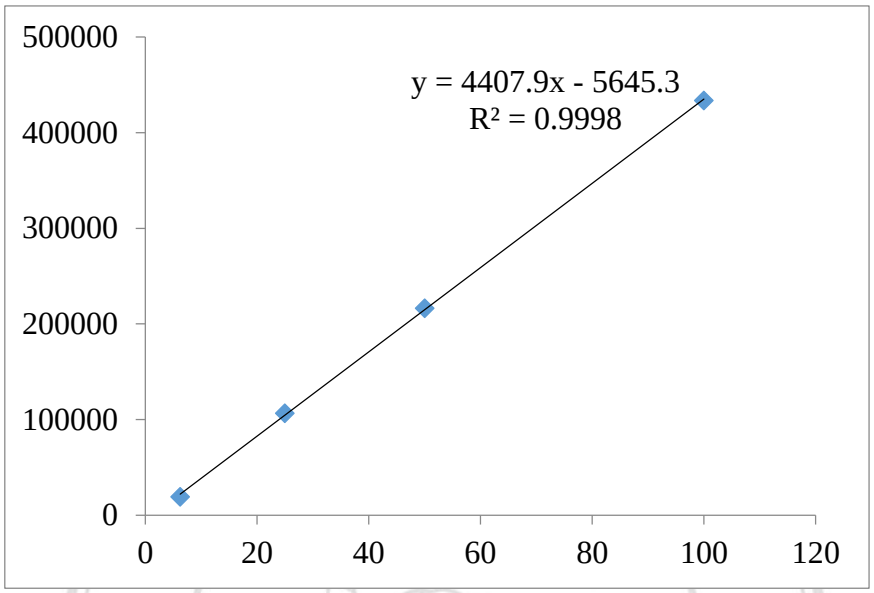

Figure A-2 Standard curve for determination of cardiac MDA level

APPENDIX B

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

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

100mM 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

APPENDIX C

Western blot technique

1. Solutions

1.1 Extraction buffer (stock solution)

1.1.1 Tris HCl (1M; pH 6.8)

Tris HCl	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 HCl (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

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.

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 Coomassie blue staining solution

Protein can be visualized in the gel by exposure to Coomassie blue solution followed by de-stained to remove background staining.

Coomassie brilliant blue R250	1.25	g
Methanol	225	ml
ddH_2O	225	ml
Glacial acetic acid	50	ml

Filter through Whatman #1 Filter paper

1.6 Coomassie blue destain solution

Methanol	500	ml
Glacial acetic acid (100%)	100	ml
H_2O	400	ml

1.7 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.

1.8 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.

1.9 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

1.10 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.

1.11 Blocking buffer

1X TBST	100	ml
Skimmed Milk powder	5	g

1.12 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

↓

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 PVDF membrane at 90 Volts, 2 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 and staining gel with Coomassie Brilliant Blue for 5 min, and destain until gel is clear

↓

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

↓

Place the membrane between the plastic sheets, put into an x-ray film cassette, and close the cassette

↓

Adjust exposure time according to the signal strength and specificity

↓

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

6. Re-use the Membrane for another protein

After membrane exposed to ECL, wash with 1X TBST for 5 min

Incubate the membrane with stripping buffer (10X dilution with ddH_2O) for 30 min, wash with 1X TBST for 5 min

Incubate the membrane in the blocking buffer (5% milk in 1X TBST) for 40 min

Incubate the membrane with another primary antibody

APPENDIX D

Cardiac mitochondrial function

1. Solutions

1.1 Mitochondrial isolation buffer (MIB)

Sucrose	300	mM
EGTA	0.2	mM
TES	5	mM

pH 7.2

1.2 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

1.3 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

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

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

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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