# CHAPTER III

# RESEARCH METHODOLOGY

## Preparation of BM extract

Brahmi or BM was collected from Phetburi province, Thailand and identified by Associate Professor Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The specimen (Phrompittayarat001) was kept at the PBM Herbarium, Mahidol University, Thailand. BM was cut into small pieces and dried in a hot air oven at 50 °C for 12 hours. The dried plant material was coarsely powdered and soaked in water. After 24 hours, the water was mechanically squeezed out of the plant material. The plant material were percolated with circulating 95% ethanol for 8 hours. The residue was extracted again twice using the same procedure. The combined extract was filtrated and dried under reduced pressure (Kamonwannasit, et al., 2008; Phrompittayarat, et al., 2008). The extract contained 5% (w/w) of total saponins, the mixture of bacoside $A_3$, bacoside II, bacopasaponin X, bacopasaponin C and bacopaside I. The total saponin content was determine using HPLC as previously reported (Phrompittayarat, et al., 2007). The extract was stored at -20 °C in a dark bottle until used. Brahmi extract was generously provided by Associate Professor Dr. Kornkanok Ingkaninan, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand.

For preparing the stock BM solution, paste ethanolic BM extract were firstly dissolved in 1:1 ratio of DMSO: absolute 95% Ethanol, aliquoted and stored at -20 °C in a dark tube. This stock BM solution were further diluted with sterile D-PBSA to obtaine proper concentrations of working solution, freshly immediately before the experiment. The working solution contains both DMSO and ethanol of lesser than 0.03% for excluding from the cytotoxicity of these substances (Limpeanchob, et al., 2008)

**Cell culture**

Normally, neuronal PC12 and neuroblastoma SH-SY5Y cells were brought up in 75 $cm^2$ flask containing 10 mL DMEM/F12 medium (Gibco, CA, USA) with 10% fetal bovine serum and incubated at an atmospheric 37 °C. The cells were refreshed with new medium every 2-3 days and subcultured when cell density reached between 2-4x$10^6$ viable cells/ mL.

**Cell subculturing**

The old culture medium was removed from each of 75 $cm^2$ culture flask. Fresh sterilized D-PBSA (10 ml) was added into each flask. Each flask was brought back to incubate at an atmospheric 37 °C for 5-10 min. The cells were detached from each flask and each cell suspension was transfered to each sterilized 15 mL centrifuge tube. The cell suspension was centrifuged at 180-225 xg for 10-15 min at 4 °C before decanting the supernatant and collecting the cell pellet. The pellet was resuspended in an appropriated volume of fresh DMEM/F12 medium (about one tenth of the original volume). The cell suspension was gently aspirated 15-20 times and then an appropriate aliquot of the cell suspension was added to new 75 $cm^2$ flask carrying 10 mL fresh DMEM/F12 medium. The 5x$10^5$ to 1x$10^6$ viable cells/mL or subcultivation ratio of 1:2 to 1:4 was seeded into each flask. The culture flasks were brought back to incubate at an atmospheric 37 °C.

**Freezing cells**

The cells were harvested as usual, washed once with an appropriate volume of DMEM/F12 medium, resuspended and counted for determining the cell viability. After that the cell suspension was centrifuged at 180-225 xg for 10-15 min at 4 °C before decanting the supernatant and collecting the cell pellet. The $10^6$ to $10^7$ viable cells/ mL was added into freshly prepared freezing medium containing 85% fetal bovine serum, 10% sterilize DMSO, and 5% DMEM/F12 medium. One mL aliquots were transferred to a fresh sterilized freezering vial and the put in a container, containing sufficient isopropanol, at room temperature. The container was kept in -50 °C refrigerator overnight and then all culture vials were transferred to preserve in liquid nitrogen on the next day.

**Cell treatment with BM extract**

**NGF-deprived PC12 cells**

PC12 cells were brought up in normal-serum (NR) RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 2.05 mM L-Glutamine, and 1x penicillin/streptomycin (PAA) (applied from Greene and Tischler (1976))for 7 days. The culture medium was refreshed every 2-3 days. The cultures were shifted to low-serum (LW) RPMI 1640 medium containing 3% heat-inactivated horse serum, 2% heat-inactivated fetal bovine serum, 2.05 mM L-Glutamine, and 1x penicillin/streptomycin (PAA) in the second week. A set of the cells was also maintained in the previous NR-serum RPMI 1640. The cells were harvested as usual, washed once with an appropriate volume of DMEM/F12 medium, resuspended and counted for determining cell viability. After that the cells were reflasked onto fresh poly-D-lysine-coated flasks. The first set of the cells were maintained in low-serum (LW) RPMI 1640 medium while the second one was brought up in apoptotic-induced serum-free (SF) media (RPMI 1640 media containing 1X Insulin-transferrin-sodium selenite (Sigma), 0.2% bovine albumin fraction V (Calbiochem), 2.05 mM L-Glutamine, 1x penicillin/streptomycin) for another 7 days applied from Shelton and Johnson (2001)). Again, another set of the cells was reflasked onto fresh poly- D-lysine-coated flasks containing NR-serum medium.

**1. Cell culturing for MTT assay**

The cells were harvested as usual, washed once with an appropriate volume of DMEM/F12 medium, resuspended and counted for determining cell count viability. About 0.5 – $10x10^3$ cells were plated onto each well of round-bottomed 96 well plate coated with poly-D lysine carrying NR-, LW-, or SF-serum RPMI1640 medium and incubated in a humidified atmosphere at 37 $^{o}$C for 1-3 days so that the cells were in exponential phase of growth at the time the drug was added. After that the cells were exposed to BM extract at final concentration of 0 (control), 50, 100, 150, 200, 250, and 300 μg/mL for 1-3 days.

**2. Cell culturing for immunoblotting**

Each set of the cells was refreshed with either NR-, LW-, or SF-serum RPMI 1640 and then exposed to BM extract at final concentration of 0 (control), 50, and 100μg/mL for 1-3 days.

**Differentiated human SH-SY5Y neuroblastoma cells**

SH-SY5Y cells were brought up in normal-serum (NR) RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 2.05 mM L-Glutamine, and 1x penicillin/streptomycin (PAA) (applied from Greene and Tischler (1976)) for 7 days. The cultures were refreshed the medium every 2-3 days. The culturing medium was then changed to be low-serum (LW) RPMI 1640 medium containing 3% heat-inactivated horse serum, 2% heat-inactivated fetal bovine serum, 20 μM retinoic acid, 2.05 mM L-Glutamine, and 1x penicillin/streptomycin (PAA) in the second week.

1. **Cell culturing for MTT assay**

The cells were harvested as usual, washed once with an appropriate volume of DMEM/F12 medium, resuspended and counted for determining the cell viability. About 0.5 – $10x10^3$ cells were plated onto each well of round-bottomed 96 well plate and incubated in a humidified atmosphere at 37 °C for 1-3 days such that the cells were in exponential phase of growth at the time the drug is added. After that the control cells were exposed to the medium without any other reagents and the treated cells were exposed to absolute 0.025% DMSO (vehicle), absolute 5 μM camptothecin, and BM extract at the final concentration range from 50-300 μg/mL for 12, 24, and 48 hours.

2. **Cell culturing for immunoblotting and for immunocytochemistry**

The culture was shifted to grow in LW –serum RPMI1640 medium (without retinoic acid). Then one set of the cells was exposed to the medium without any other reagents. The other treated cells were exposed to absolute 0.025% DMSO (vehicle), absolute 5 μM camptothecin, and BM extract at the final concentration of 50 and 100 μg/mL for 12, 24, and 48 hours.

## MTT assay

The old culture medium was removed from each well of round-bottomed 96 well plates, and the 100 μL of MTT (1 mg/ mL in appropriate media) was then added daily to each well and incubated further in dark for 2 hours. At the end of the MTT treatment, the medium was removed, and 100 μL DMSO (aMRESCO) was added to each well to dissolve the purple formazan crystal. The color was quantified using

microplate reader (iEMS Reader MF, Labsystem) at 540 nm. The viability percentages were analyzed by statistic one-way ANOVA with $\alpha = 0.05$.

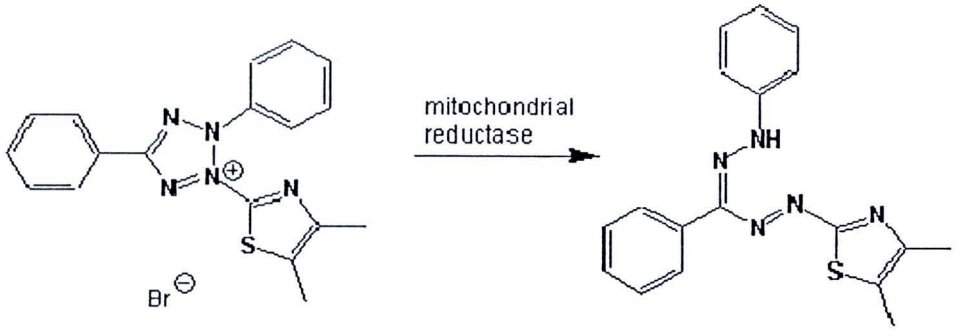



**Figure 8 The reduction of MTT to formazan**

**Source:** http://en.wikipedia.org/wiki/MTT_assay

**Antibodies**

Mouse anti-Tau monoclonal antibody (Clone name Tau-5, MAB361, Millipore) is phosphorylation-independent antibody specific detecting total tau protein while mouse anti-Tau-1 monoclonal antibody (MAB3420, Millipore) is a dephosphorylation-dependent antibody specific detecting dephosphorylated epitope at amino acid 189-207 (Binder, et al., 1985; Szendrei, et al., 1993). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (MAB374, Millipore) is an antibody specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a ubiquitous glycolytic enzyme present in reasonable high levels in almost all tissues. These all are primary antibodies applicable for immunoblotting technique. By the same method, goat anti-mouse IgG (H&L) peroxidase conjugated affinity purified antibody (AP124P, Millipore) is used as a secondary antibody for following the detection.

**Immunoblotting**

The cells from each flask were detached and the cell suspension was transferred to sterilized 15 mL centrifuge tube. The cell suspension was centrifuged at 180-225 xg for 10-15 min at 4 °C. The supernatant was decanted and the cell pellet

was collected. The pellet was lysed with ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) by at least 5x106 cells/ 1 mL RIPA buffer, and was incubated on ice for 30 min. The cell suspension was centrifuged with 14,000 xg at 4 °C for 15 min and was collected for protein-part supernatant. The total protein concentrations were determined by using quick startTM Bradford reagent (BIO-RAD) by measuring the absorbance at 620 nm. After that an equal amount of total protein (50-125 μg of total protein) was resolved on 10-12% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. This membrane was incubated in a blocking buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST) plus 5% dry milk (wt/v) overnight at 4 °C. Then the primary antibodies: anti-Tau (clone Tau-5) (1:500), anti-Tau-1 (1:500), or anti-GAPDH (1:200) was incubated for 3 hours at room temperature. The membranes were washed three times in TBST (5 min/ each). The membranes were further incubated in goat anti-mouse IgG (H&L) peroxidase conjugated secondary antibody (1:5000) for 1 hour at room temperature. The Western blots were analyzed using chemiluminescent HRP-substrate and quantified the antigen expression basing on density measurements of protein bands using Scion Image program or Image J software (http://rsb.info.inh.gov/ij/).

**Deprobing western blot**

The appropriate volume of stripping buffer was warmed to 50 °C in a tight lid container. The PVDF membrane was added into that stripping buffer and incubated at 50 °C for up to 30 min with some agitation at 80 rpm. Then the β-mercaptoethanol-based stripping buffer was decanted. The membrane was rinsed with TBST in a fresh container at 40 rpm room temperature 5 min twice. Then the membrane was transferred to a new container and was rinsed with fresh TBST three times again (for avoiding the remnant of β-mercaptoethanol which can impede a new probe to bind to the target protein.). The membrane was ready to be blocked and further processed immunodetection again.

**Reverse-transcription PCR**

The cells were detached from each flask and cell suspension was transferred to fresh sterilized 15 mL centrifuge tube. The cell suspension was centrifuged at 180-

225 xg for 10-15 min at 4 °C. The supernatant was decanted and the cell pellet was collected. The cultured cells were lysed directly in a culture dish by adding 1 ml of TRIzol reagent to 3.5 $cm^2$ diameter dish or 10 $cm^2$ dish area (380 µl of TRIzol reagent for each well of 12 well culture plate, dish area is 3.8 $cm^2$), and the cell lysate was aspirated several times through a pipette. An insufficient amount of TRIzol reagent may result in contamination of the isolated RNA with DNA. The homogenized samples were incubated for 5 minutes at 15-30°C to permit the complete separation of nucleoprotein complexes and then 200 µl of chloroform was added to the samples. The sample tubes were capped, shook vigorously by hand for 15 seconds and incubated for 2-3 minutes at 15-30°C. The samples were centrifuged at no more than 12,000xg for 15 minutes at 2-8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol reagent used for homogenization. The aqueous phase was transferred to a fresh tube and then 500 µl of isopropyl alcohol was added to the isolate, which 1 ml of TRIzol reagent was used for the initial homogenization, to precipitate the RNA from the aqueous. The samples were incubated at 15-30°C for 10 minutes and centrifuged at no more than 7,500xg for 10 minutes at 2-8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The supernatant was decanted and then the RNA pellet was washed once with 75% ethanol, and then at least 1 ml of 75% ethanol was added per 1 ml of TRIzol reagent used for the initial homogenization. The sample was mixed by vortexing and centrifuged at no more than 7,500xg for 5 minutes at 2-8°C. The RNA pellet was briefly dried (air-dry for 5-10 minutes). After that the RNA samples were dissolved in DEPC-treated water. The RNA concentration was determined by measuring the absorbance at 260 and 280 nm. High quality RNA should have an A260/280 ratio of at least 1.8. For cDNA synthesis, appropriated amount of mRNA template was mixed with oligo-dT primers and incubated at 65 °C for 5 min. The reverse transcription PCR was performed at 42 °C for 60 min and stop the reaction by further incubated at 70 °C for 5 min by using RevertAid ® First Strand cDNA Synthesis Kit (Fermentas, Leon-Rot, Germany). The

cDNA concentration was determined by measuring the absorbance of the cDNA products at 260 nm and then calculated for their concentration by the following formula:

cDNA concentration (μg/ mL) = OD260 nm X dilution factor X 50


**Measurement of gene expression**

1. **Determination of human Glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) gene expression**

One hundred ng of each cDNA sample was undergone 15 μL PCR reaction containing 1X PCR buffer, 2 mM $Mg_2Cl$, 0.4 mM dNTPs, 300 pmole of each primer, and 0.06 U of Taq DNA polymerase (Invitrogen, NY, USA). The sequence of GAPDH forward primer is 5'AGGTCGGAGTCAACGGATTTG 3' and the sequence of GAPDH reverse primer is 5' TGGTGTCAGGTACGGTAGTG 3'. The cDNA samples were initially denatured at 95 °C for 5 min, then 35 cycles of amplification were performed with Gene Amp ® PCR Systems 9700 (Applied Biosystems, CA, USA) with a step program (95 °C 30 sec, 56 °C 30 sec, and 72 °C 40 sec) and followed by a final extension at 72 °C for 7 min. The PCR products were resolved on 1.5% agarose gel and, soaked in ethidium bromide and then visualized under UV light. The gene expression was quantified based on density measurements of PCR product bands using Scion Image program or Image J software (**http://rsb.info.inh.gov/ij/**).

2. **Determination of human Tau gene expression**

One hundred ng of each cDNA sample was undergone 15 μL PCR reaction containing 1X PCR buffer, 2 mM $Mg_2Cl$, 0.4 mM dNTPs, 300 pmole of each primer, and 0.06 U of Taq DNA polymerase (Invitrogen, NY, USA). The sequence of GAPDH forward primer is 5' GCCAACGCCACCAGGATTCCAG 3', corresponding to nucleotide 1769-1790 of the longest isoform of Tau mRNA and the sequence of GAPDH reverse primer is 5' GGAGTACGGACCACTGCCACCT 3', corresponding to the inverse complement of nucleotide 2001-2022 of the longest isoform of Tau mRNA . The cDNA samples were initially denatured at 95 °C for 5 min, then 35 cycles of amplification were performed with Gene Amp ® PCR Systems 9700

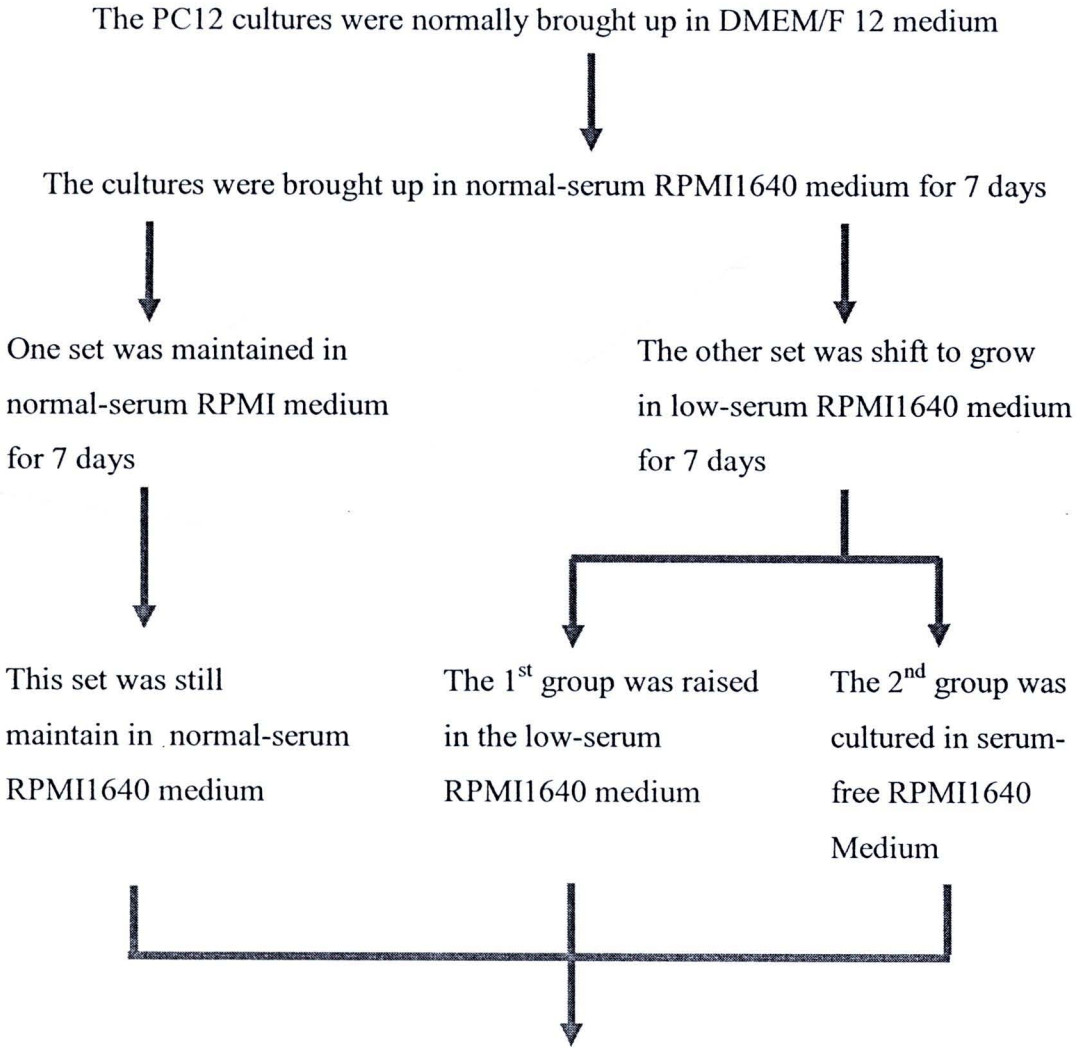
(Applied Biosystem, CA, USA) with a step program (95 °C 30 sec, 57 °C 30 sec, and 72 °C 40 sec) and followed by a final extension at 72 °C for 7 min.

**PCR product detection**

The PCR products were resolved on 1.75% agarose gel and, soaked in ethidium bromide and then visualized under UV light. The gene expression was quantified based on density measurements of PCR product bands using Scion Image program or Image J software (**http://rsb.info.inh.gov/ij/**).

**Methodology Diagrams**

**PC12 cells**



**SH-SY5Y cells**

The PC12 cultures were normally brought up in DMEM/F 12 medium

↓

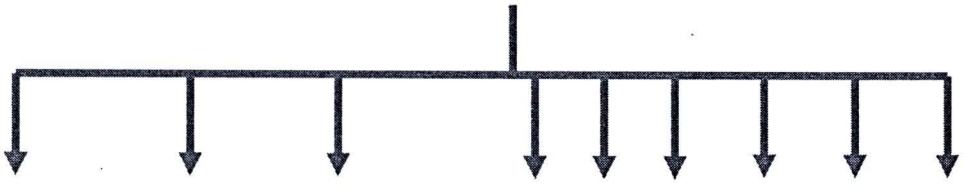
The cultures were brought up in normal-serum RPMI1640 medium for 7 days

↓

The cultures was shift to grow in differentiating low-serum RPMI1640 medium containing 20 μM retinoic acid for another 7 days

↓

The cultures were raised up in the low-serum RPMI1640 medium containing no any retinoic acid



Absolute, RPMI 1640 | Absolute, DMSO | Absolute, Camptothecin | 0, 50, 100, 150, 200, 250 μg/ mL BM extract

for 12, 24, 48, 72 hours (MTT viability assay)

**or**

Absolute, RPMI 1640 | Absolute, DMSO | Absolute, Camptothecin | 0, 50, 100 μg/ mL BM extract

for 12, 24, 48, 72 hours (Immunoblot analysis and Gene expression determination)

**Instrument and Material**

| | |
|---|---|
| All-trans-retinoic acid | (Sigma Aldrich, MO, USA) |
| Amersham Hyperfilm ™ ECL | (GE Health care, Buckinghamshire, UK) |
| Amersham™ ECL Plus Western Blotting Detection System | (GE Healthcare, Buckinghamshire, UK) |
| Ammonium persulfate | (BIO-RAD, CA, USA) |
| Bovine albumin fraction V | (Calbiochem, Darmstadt, Germany) |
| Bromophenol blue | (BIO-RAD, CA, USA) |
| Camptothecin | (Calbiochem, Darmstadt, Germany) |
| Chloroform | (Lab-Scan Analytical Sciences, Bangkok, Thailand) |
| Chromatein Prestained Protein Ladder | (Vivantis, CA, USA) |
| DMEM/F12 medium | (GIBCO, invitrogen, CA, USA) |
| DMSO | (aMRESCO, OH, USA) |
| DMSO | (Invitrogen,CA, USA) |
| DNA ladder | (Invitrogen,CA, USA) |
| 95% Ethanol | (VMR, IL, USA) |
| EDTA, disodium salt | (USB corporation, OH, USA) |
| Fetal bovine serum | (Gibco, Invitrogen, CA, USA) |
| GAPDH forward primer | (Bio Basic Inc., Markham Ontario, Canada) |
| GAPDH reverse primer | (Bio Basic Inc., Markham Ontario, Canada) |
| Gene Amp ® PCR Systems 9700 | (Applied Biosystems, CA, USA) |
| Glacial acetic acid | (Lab-Scan Analytical Sciences, Bangkok, Thailand) |
| L-Glutamine | (Gibco, Invitrogen, CA, USA) |
| Glycine | (USB corporation, OH, USA) |
| 87% Glycerol | (Appli Chem, Darmstadt, Germany) |
| Goat anti-mouse IgG (H&L) peroxidase conjugated affinity purified antibody | (Millipore, MA, USA) |

Horse serum (Gibco, Invitrogen, CA, USA)

Human neuroblastoma SH-SY5Ycells (ATCC, VA, USA)

100X Insulin-transferrin-sodium selenite (Sigma Aldrich, MO, USA)

KCl (VMR, IL, USA)

$KH_2PO_4$ (VMR, IL, USA)

Microplate reader (Labsystems iEMS Reader MF, Helsinki, Finland)

β-Mercaptoethanol (BIO-RAD, CA, USA)

Methanol (ACI Labscan, Bangkok, Thailand)

Mouse anti-glyceraldehyde-3- phosphate dehydrogenase monoclonal antibody (Millipore, MA, USA)

Mouse anti-Tau monoclonal antibody (Tau-5, Millipore, MA, USA)

Mouse anti-Tau-1 monoclonal antibody (Millipore, MA, USA)

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT (BioBasic Inc, Markham Ontario, Canada)

NaCl (MERCK, Darmstadt, Germany),

$Na_2HPO_4$ (VMR, IL, USA)

NP-40 (USB corporation, OH, USA),

PC12 cells (ATCC, VA, USA)

10x Penicillin/Streptomycin (PAA, Australia)

Platimum® Taq polymerase (Invitrogen, NY, USA)

Polyacrylamide (National diagnostic, GE, USA)

Poly-D-lysine hydrobromide (Sigma Aldirch, MO, USA)

Polyvinylidene difluoride membrane (GE Health care, Buckinghamshire, UK)

Protease inhibitor cocktail (Sigma Aldrich, MO, USA)

Quick start™ Bradford reagent (BIO-RAD, CA, USA)

| | |
|---|---|
| RevertAid™ Premiun First Strand cDNA Synthesis Kit | (Fermentas, Thermo Fisher Life Science, Leon-Rot, Germany) |
| RPMI1640 medium | (Gibco, Invitrogen, CA, USA) |
| Sodium dodecyl Sulfate (SDS) | (USB corporation, OH, USA) |
| Sodium deoxycholate | (Bio Basic Inc., Markham Ontario, Canada) |
| Platimum® Taq polymerase | (Invitrogen,CA, USA) |
| Tau forward primer | (Bio Basic Inc., Markham Ontario, Canada) |
| Tau reward primer | (Bio Basic Inc., Markham Ontario, Canada) |
| Tris | (USB corporation, OH, USA) |
| TRIzol | (Invitrogen, CA, USA) |
| Tween 20 | (Bio Basic Inc., Markham Ontario, Canada) |
| Xylene cyanol FF | (BIO-RAD, CA, USA) |