ADVANCED BIOTECHNOLOGY
MASTER DEGREE

EPIGENETIC MECHANISMS IN PATHOLOGICAL AND PHYSIOLOGICAL PROCESSES
-PRACTICAL EXERCISES IN LABORATORY-

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In this laboratory experience, students will approach different techniques of cellular and molecular biology:

- Grow cells in culture
- Nucleic acids isolation and purification
- RNA Reverse Transcription (cDNA synthesis)
- Real-Time Quantitative Reverse Transcription PCR (Real Time qRT-PCR)
- DNA bisulfite conversion
- Pyrosequencing
- Chromatin Immunoprecipitation (ChiP) Assay

DAY 1: CELLS CULTURE PROTOCOL FOR THE SH-SY5Y NEUROBLASTOMA CELL LINE.

Before You Get Started: Important Terminology

Aseptic Techniques	Procedures to prevent contaminations and maintain sterility
Basal Media	Growth medium containing salts, aminoacids, sugars and other factors
Buffered Saline Solutions	Maintains required ph (7.2-7.6) for cell cultures; uses CO_2
Cell Type	Refers to all cells with a common phenotype
Confluency	The amount of the growth surface covered with cells expressed as a percentage
Conicals	Centrifuge tubes for volumes ranging between 15 ml to 50 ml
Continuous Cell Lines	Cells that can propagate in vitro for an indefinite time period
Cryopreservation	Process to store cells for an indefinite time period
Cryoprotectant solution	Solution containing components to help protect cells from damage during cryopreservation
Dewars Vessel	Vessel containing liquid nitrogen (-156°C gas phase, -196°C liquid phase) for cryopreservation
DMSO (Dimethyl sulfoxide)	Used in cryopreservation to minimize ice crystals from puncturing cell membranes (preventing membrane permeability)
FBS (Fetal Bovine Serum)	Plasma from fetal calf; contains growth factors
Heat-Inactivated FBS	Heated for 30 minutes at 56°C with mixing to inactivate complement proteins that are part of the immune response
Incubator	Maintains optimum environment for cell cultures (5% CO2, 37°C, humidity)—unless otherwise noted
In Vitro	Taking place outside a living organism
In Vivo	Taking place within a living organism
Labware/Lab Supplies	Disposable cell culture items including conicals, serologicals, pipettes, syringes, filters, etc
Microcentrifuge Tubes/Eppitubes	Centrifuge tubes for volume ranging from 1 µl to 2 ml
Mr.Frosty	Container filled with isopropyl alcohol usedto control the freezing rate of cells (1°C/min)
Passage Number	Refers to the number of times that a cell population has been removed from a Growth vessel and undergone a subculture (passage) process

Phenol Red	A pH indicator (visual) where yellow indicates a pH<6.8 and magenta indicates a pH>8.2
Pipette Aid	Mechanical liquid transfer device used with pipettes/serologicals
Pipette	Instrument to transfer measured volumes
Reagent	Biological or non-biological substance/mixture
Serological	Graduated pipette to accurately transfer measured volumes
Serum	Plasma containing growth factors; example FBS
Subculture	Culture of daughter cells from passaging a growth vessel
Tissue Culture Flasks/Plates/Dishes	Containers with treated surfaces to promote cell attachment
Trypsin/EDTA	Enzyme/chelator agent used to detach cells from growth surface

Before You Get Started: General Cell Culture Equipment & Proper Use

Microorganisms are constantly present; they can be found on bench tops, objects, clothing, and skin as well as floating in the air. Cell cultures are highly susceptible to contamination by microorganisms such as bacteria, fungi, yeast, and mycoplasma. These microorganisms are easily transferred to cultures by incidental surface touches, simply falling off an object over an exposed culture, or moving along liquid pathways. Aseptic technique is the practice of sterile procedures to eliminate contamination sources. To safe guard cell cultures from unwanted contamination or cross contamination with other cell lines, aseptic techniques must be stringently followed.

PPE (Personal Protective Equipment)

* **ALWAYS** wear gloves when performing cell culture work.
* Wear a clean, barrier lab coat (optional: additional disposable sleeves) to eliminate exposure to contaminants residing on skin and clothing.
* **SPRAY YOUR GLOVES WITH 70% ETHANOL EVERY TIME YOUR HANDS ENTER THE HOOD**.

Why do we use 70% Ethanol?? Between 60-80% Ethanol is typically effective in destroying the cell membrane of most microbes/bacteria, thus killing it. Higher concentrations aren't as effective as it will evaporate fairly quickly, minimizing it's efficacy.

CENTRIFUGES

* Make sure caps are on tight before placing conicals in rotor buckets.
* Use safety caps on buckets to prevent accidental spills and contaminations.
* ALWAYS use the correct counterbalance(s) to balance across the rotor.

BIOSAFETY CABINETS/HOODS USE—BEFORE & AFTER CULTURING

BSCs are designed to protect individuals and the environment from potentially infectious aerosols and to maintain a sterile workspace inside the cabinet. Biosafety cabinets are certified once per year.

*Turn on the fluorescent light, and lift sash to marked location (the hood's alarm will stop beeping).
*Spray the hood down with 70% Ethanol PRIOR to working in the hood **AND AFTER** working in the hood (including the pipettes, vacuum knob and aspirator line)—**DO NOT SPRAY** the ceiling of the biosafety cabinet.
*Spray **ALL ITEMS** in the hood with 70% Ethanol PRIOR to placing in the hood **INCLUDING** your gloves—**DO NOT SPRAY** the caps of flask as to prevent wetting the filters.
*Turn the hood **OFF** when done working in it.
***DO NOT** cover the front or rear air grilles with paper, absorbent pads, or any other materials.
* When working in the biosafety cabinet/hood, leave gaps between items to allow proper air flow; do not build a "wall" which will block airflow.
*When finished, turn the fluorescent light off and close the sash to marked location (the hood's alarm will then stop beeping).

PIPETTE USE

Determine which pipette best suites your needs for the desired volume. **BE SURE YOU DO NOT EXCEED THE MAX VOLUME** as this will offset the calibration, as well as damage the pipette.

HANDLING LIQUIDS W/SEROLOGICAL PIPETTES…

***ALWAYS** label flasks, dishes, etc appropriately: Cell Type, Passage Number, Date and Initials.
***DO NOT** leave bottles, conical tubes, flasks, and plates open and exposed.
***DO NOT** cross over open conical tubes, bottles, flasks, or containers.
***DISCARD** serological/ tip if it touches any surface to minimize contamination.
* Caps can be placed faced up or on its side.

1. Banana peel serological wrapper and push pipette straight into pipette aid.
2. Orientate serological in pipette aid for ease of reading volumes.

3. Carefully remove wrapper ensuring the tip does not contact anything including the wrapper—set aside for disposing of the tip once finished using it.
4. Remove cap from bottle/conical.
5. Place serological in the center of conical/bottle's neck. Avoid contact with the container's neck.
6. Place tip into solution; **DO NOT** submerge the shaft of the serological.
7. Press the top button on the pipette aid until the desired volume of liquid has been withdrawn. Follow the meniscus while pipetting to avoid drawing air.
8. Carefully remove serological from container without touching the interior.
9. Recap bottle/conical and uncap secondary container.
10. Insert serological in the center of secondary container. Slowly push bottom button on pipette aid to dispense liquid. Avoid blowing bubbles.
11. Remove serological from secondary container and recap.
12. To remove serological from pipette aid, pull straight out and re-sleeve pipette in wrapper and place in waste receptacle.

MICROSCOPES

* **ALWAYS** use gloves when using the microscopes and the computers connected to them.
* Periodically wipe down the microscopes with a germicidal wipe, alcohol prep pad, or a Kimwipe sprayed with 70% Ethanol.
* To clean the objective, only use lens paper and lens cleaning solution. Everything else will scratch the lens.
* Turn the microscope OFF after each use.

WATER BATH

* Maintain 37°C temperature at all times.
* Wipe down all bottles (coming from the water bath) **PRIOR** to placing in the hood **AND AFTER** coming out of the water bath.
* Spray **ALL ITEMS PRIOR** to being placed in the water bath with 70% Ethanol.
* Use ring weights to partially submerge bottles in water.
* Place cryovials and eppitubes in floating holders.

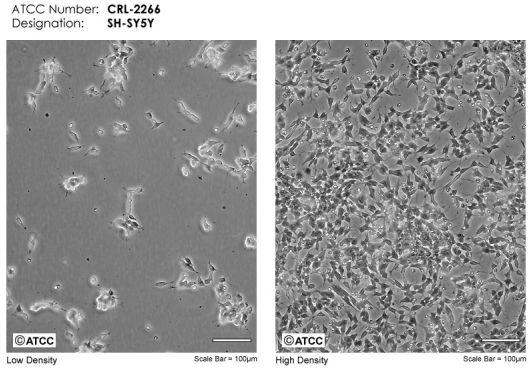
EXPERIMENTAL SYSTEM:

Human SH-SY5Y neuroblastoma cell line was purchased from ATCC® (American Type Culture Collection, Cat. No. CRL-2266™).

Characteristics

Growth properties	Mixed: adherent and suspension
Derivation	SH-SY5Y is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor.
Age	4 years
Gender	Female
Karyotype	modal number = 47; the cells possess a unique marker comprised of a chromosome 1 with a complex insertion of an additional copy of a 1q segment into the long arm, resulting in trisomy of 1q
Antigen expression	Blood Type A; Rh+
Comments	SH-SY5Y cells have a reported saturation density greater than 1 X 10^6 cells/cm^2. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity.

The SH-SY5Y neuroblastoma cells are cultured and used in undifferentiated, continuously proliferating, morphologically neuroblast-like form, non-polarized cell bodies with few, truncated processes, in monolayer 2D culture, although cells can be differentiated into more mature, neuron-like cells with retinoic-acid (RA) induction. Cell cultures include both adherent and floating cells, but the adherent population is used for the downstream applications, the floating cells are discarded during media changes.



NB: AS SUGGESTED FROM LITERATURE, CELLS WITHIN 5-6 PASSAGES WILL BE USED FOR EXPERIMENTS.
IT IS ADVISABLE TO KEEP THE CELLS BELOW 25 PASSAGES TO AVOID SENESCENCE.
EQUIPMENT

Fixed Equipment

- Humidified Cell Culture Incubator (37°C, 5% CO_2 in air)
- Laminar Flow Hood (BSL2)
- Inverted microscope with phase contrast (4x, 10x, 20x objectives)
- Cell counting chamber (Burker chamber)
- Centrifuge
- Water Bath
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively
- Pipette aid

Consumables

- Filter pipette tips 10, 200, 1000 µl
- 2, 5,10, 25 mL sterile serological pipettes
- 15 ml sterile tubes.
- 6-well Cell Culture Plate
- Gloves

MEDIA, REAGENTS, SERA, OTHERS

Basal medium:

- DMEM (Dulbecco's Modified Eagle's Medium) / Hams F-12 50:50 Mix

Other:

- Fetal Bovine Serum (FBS), heat-inactivated
- Penicillin-Streptomycin Solution, 100x
- Dulbecco's Phosphate-Buffered Saline (DPBS), without Ca^{2+} & Mg^{2+}, 1X
- Trypsin 0.25%, 0.53 mM
- Trypan blue solution 0.4%

PREPARATIONS

STOCK SOLUTIONS	FINAL CONCENTRATION	VF = 5 mL
Trypsin 10X 2.5%	0.25%	
EDTA 0.5 M	0.53 mM	
DPBS 1X		Up to 5

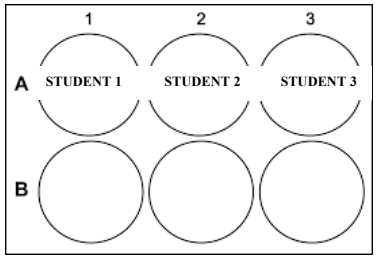
STOCK SOLUTIONS	FINAL CONCENTRATION	VF = 20 mL
FBS	10%	
Penicillin-Streptomycin Solution, 100X	1X	
DMEM/F12 Medium		Up to 20

NB Complete Medium and Solutions must be warmed to 37°C in the water bath before the use (almost for 15 minutes)

STEP 1: COUNTING CELLS

Each student has an aliquot of cells in suspension in a 15 ml sterile conical under the hood. Considering that:

- Each student of each group has to seed his/her cells in a well of a 6-well Cell Culture Plate



- For SH-SY5Y, a guide seeding density of $8x10^4$ cells/cm^2 is recommended.
- The surface area of each well is 10 cm^2.

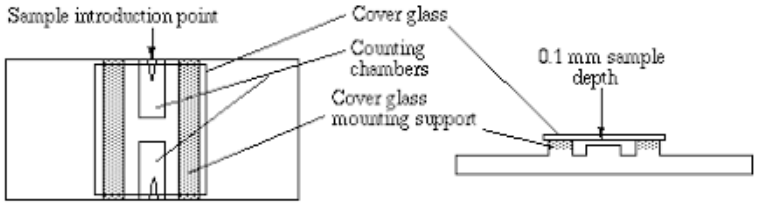
HOW MANY CELLS SHOULD BE SEEDED IN EACH WELL?

HOW MANY MICROLITERS OF YOUR SUSPENSION SHOULD BE PUT IN THE WELL TO HAVE THIS NUMBER OF CELLS?

The first step is to calculate the number of cells in each sample.

1.1 Preparing Burker Chamber

- Clean carefully Burker chamber with 70% Ethanol.
- Dry the chamber (preferably with a lens cleaning tissue).
- Wetten the cover slip support on either side with water ever so slightly.
- Slide on the coverglass using gentle pressure and you should see something called 'Newton's Rings' where the coverglass is in contact with the chamber (rings caused by an interference pattern).



1.2 Prepare the cell suspension

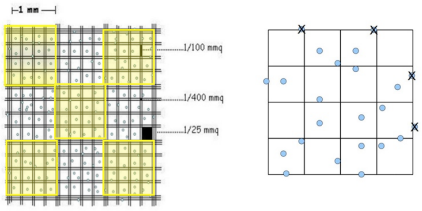
- Work under hood.
- The cells should be well mixed to provide a homogenous sample. You can do this by gently pipetting the cells up and down a pipette tip. Don't be tempted to use excessive pipetting or a vortex mixer as this may shear cells.
- Quickly remove a small volume (50 µl) of cells and transfer to a sterile Eppendorf tube.

1.3 Loading and counting cells

- Prepare a 1:1 dilution of the cell suspension in trypan blue. Approximately 10 microliters of cell suspension will be required to charge one chamber.

TRYPAN BLUE is an azo dye that is cell membrane impermeable and therefore only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins thereby rendering the cells a blue color. The trypan blue staining assay allows for a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population.

- With a pipette, carefully draw up 10 µl of the cell mixture. Place the pipette tip against the edge of the coverglass and slowly expel the liquid until the counting chamber is full. Capillary action will help to ensure that the counting chamber is full, but care should be taken not to overfill the chamber.
- Place the chamber on the microscope stage. Using the 10X objective, focus on the grid lines.



The Bürker chamber has 9 large squares, divided by double lines into 16 group squares. The double lines form small 0.0025 mm2s quares. The Chamber depth is 0.1 mm.

- It is advisable to use a hand tally to keep count of cells.
- Count the cells in 3 large squares (identified by the triple line). Count all of the clear cells within the squares and those touching the base and right hand grid lines.

1.4 Calculating the number of cells

- Add the three counts together and divide by 3 to give an **average** over the whole counting chamber.
- Each large square of the chamber, with cover slip in place, represents a total volume of 0.1 mm^3 (1.0mm X1.0mmX 0.1mm) or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the total number of cells per ml will be determined using the following calculations:

  Viable Cells/ml = Average viable cell count x 2 x 10^4

  Total Viable Cells = Viable Cells/ml x mL of cells suspension

  %Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100

  Considering the total viable cells number, you can now calculate the microliters of cells you have to take from your suspension to respect the seed density.

STEP 2: SEEDING CELLS

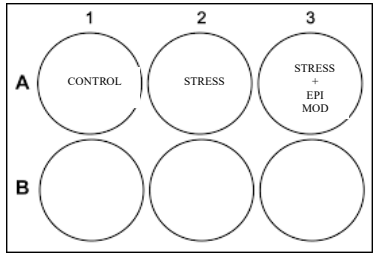
In this step you must work under the hood, in a sterile work area.

- With a permanent marker, write the cell type (SH-SY5Y), the passaging number (P7), the date and initials of your group on the plate's cover.
- Lift the plate cover being careful not to touch it internally
- With a pipette aid or a pipette put the calculated volume of cells in your well.
- With a new serological, add (2 - volume of cells) mL of complete medium to the well.
- Close the plate and distribute the cells on the surface of the well by gently moving the plate.
- Check the plate under the microscope.
- Place the plate in the incubator.

DAY 2: SH-SY5Y STRESS INDUCTION AND TREATMENT WITH EPIGENETIC MODULATORS

	STRESS INDUCED BY:	**TREATMENT WITH:**
GROUPS 1-3	ETHANOL	HDAC HINIBITOR: SODIUM BUTYRATE
GROUPS 4-6	H_2O_2 (OXIDATIVE STRESS)	METHYL DONOR: SAM (s-adenosylmethionine)
GROUPS 7-10	STARVATION	METHYL DONOR: SAM (s-adenosylmethionine)

IN EACH GROUP **3 CONDITIONS**:



REAGENTS

- Complete Medium
- Medium without FBS
- Ethanol 1 M in complete medium (from Absolute Ethanol)
- H_2O_2 0.1 M in complete medium (from H_2O_2 30%)
- Sodium Butyrate 0.01 M in complete medium
- SAM 32 mM
- Medium without FBS + SAM 100 µM
- Ethanol 40 µM + Sodium Butyrate 1 µM in complete medium
- H_2O_2 400 µM + SAM 100 µM in complete medium
- DPBS 1X

PREPARATIONS

STOCKS SOLUTIONS

- **Ethanol 1 M, VF 1 mL**

  Absolute ethanol: MW= 46,1 g/mol Density 0,79 g/ml

- **H_2O_2 0.1 M, VF 1 mL**

  H_2O_2 30% w/w : MW=34,01 g/mol Density 1,11 g/ml

STRESS CONDITION 1: ETHANOL

STOCK SOLUTION	FINAL CONCENTRATION	V = 2.5 mL
Ethanol absolute, 1 M	40 mM	
Complete Medium		Up to 2.5

STOCK SOLUTION	FINAL CONCENTRATION	V = 2.5 mL
Ethanol absolute, 1 M	40 mM	
Sodium Butyrate, 0.001M	1 µM	
Complete Medium		Up to 2.5

STRESS CONDITION 2: OXIDATIVE STRESS

STOCK SOLUTION	FINAL CONCENTRATION	V = 2.5 mL
H_2O_2 0.1 M	400 µM	
Complete Medium		Up to 2.5

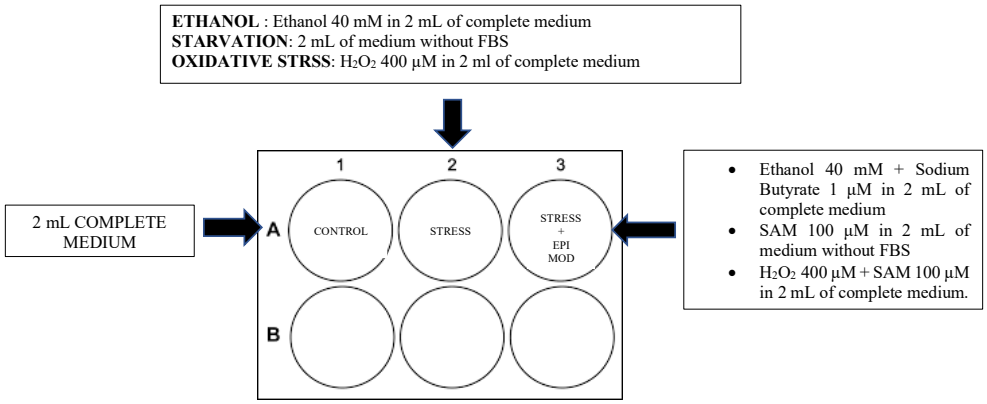
STOCK SOLUTION	FINAL CONCENTRATION	V = 2.5 mL
H_2O_2 0.1 M	400 µM	
SAM 32 mM	100 µM	
Complete Medium		Up to 2.5

STRESS CONDITION 3: STARVATION

STOCK SOLUTION	FINAL CONCENTRATION	V = 2.5 mL
Medium without FBS		Up to 2.5
SAM 32 mM	100 µM	

- Remove the plate from the incubator.
- Check the plate under the microscope.
- **Under the hood**, lift the plate cover being careful not to touch it internally.
- Gently tilt the plate at a 45° angle and with the pipette aid and a serological remove the medium from each well.
- With a new serological add 1 mL of DPBS 1x in each well and then remove it.
- Repeat the wash a second time.

FOR THE TREATMENTS:



- Check the plate under the microscope.
- Place the plate in the incubator.

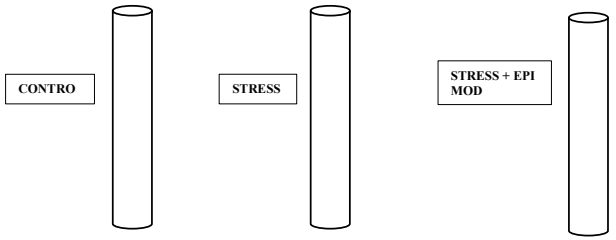
DAY 3: CELL PELLET PREPARATION & MOLECULAR ANALYS-I PART

REAGENTS

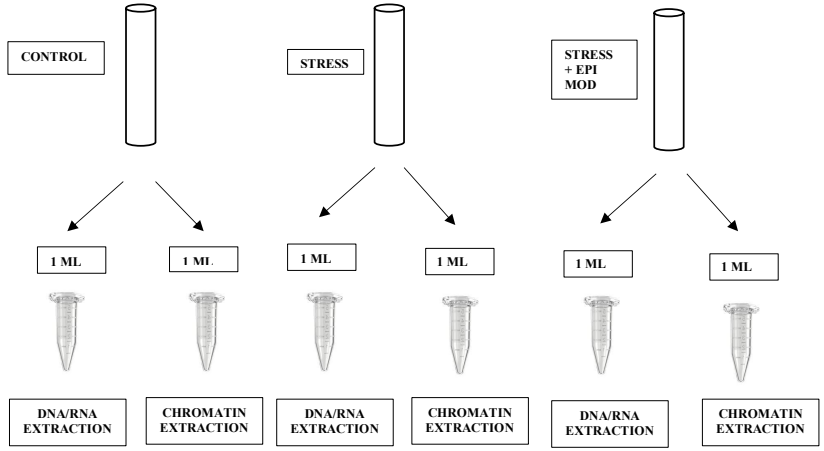
- DPBS 1X
- 0.25% trypsin, 0.53 mM EDTA solution
- Complete Medium

- Remove the plate from the incubator.
- Check the plate under the microscope.
- **Under the hood**, lift the plate cover being careful not to touch it internally.
- Gently tilt the plate at a 45° angle and with the pipette aid and a serological remove the medium from each well.
- With a new serological add 1 mL of DPBS 1X in each well and then remove it.
- Repeat the wash a second time.
- With a new serological add 1 mL of 0.25% trypsin, 0.53 mM EDTA solution in each well and put the plate in the incubator for 3 minutes.

- Check the plate under the microscope to see distached cells
- With a new serological add 2 mL of complete medium in each well to inactivate trypsin.
- Collect the total volume of each well in a 15 ml sterile tube → 3 conditions 3 tubes, each of which must be marked with the corresponding condition and the initials of the group.



- Centrifuge the tubes 125xg, 7 minutes.
- Discard the supernatant and resuspend the cell pellet in 2 mL of DPBS 1X.
- Split the content of each 15 mL tube in 2 eppendorf tubes (1 mL for Eppendorf) each of which must be marked with the corresponding condition and the initials of the group.



- Put the Eppendorf tubes in centrifuge, 8500xg 7 minutes.
- Discard the supernatants, removing all the DPBS 1X.
- Freeze one pellet of each condition and start the next step of the experiment with the other one.