Characterization of undifferentiated state of mouse ES cells

(Expression analysis of undifferentiated marker genes)

BY

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SCHOOL OF ENGINEERING

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

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ABSTRACT

Embryonic stem (ES) cells are very useful in regenerative medicine due to their unique properties including pluripotency, regenerative of tissues and organs, modeling diseases, drug testing and development, cell replacement therapy and potential of mass production.

In this project, our main goal is to understand the fundamentals of ES cell mechanisms. Additionally, we aim to understand how Leukemia Inhibitory Factor (LIF) affects these cells. We used two methods including electrophoresis and Western blot analysis to study genes and proteins in two different enviroments: one with the presence of LIF and the other without LIF. This allows us to compare and understand the impact of LIF on both genetic and protein components in our research.

From the results, STAT3, a transcription factor that plays a crucial role in maintaining pluripotency in ES cells, plays a key role in this process. Activating STAT3 leads to an increase in the percentage of GAPDH, DAX1, KLF, and ESRRB. STAT3, GAPDH, DAX1, KLF, and ESRRB help the ES cells to the maintenance of the pluripotent properties.

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LIST OF SYMBOLS/ ABBREVIATIONS

Symbols/Abbreviations	Terms
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
cDNA	Complementary Deoxyribonucleic acid
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetra Acetic acid
ESCs	Embryonic stem cells
ESRRB	Estrogen-related receptor beta
EtOH	Ethanol
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK293	Human embryonic kidney 293
iPSCs	Induced pluripotent stem cells
IL-6	Interleukimia
KLF 4	Krüppel-like factor 4
LIF	Leukemia inhibitory factor
mESCs	Mouse embryonic stem cells
Oligo dT	Deoxy-Thymidine nucleotide sequence
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl-sulfate polyacrylamide

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	gel electrophoresis
Stat3	Signal transducer and activator of transcription 3
SYBR	Sensitive Ethidium Bromide
TAE	Tris base, Acetic acid and EDTA

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CHAPTER 1
INTRODUCTION

This chapter introduces the overarching themes of this report and places the motivation for the work into context. Thereafter, the rationale and goals defined for the investigation of the project are discussed, followed by a summary of the overall project. Finally, an overview of the dissertation is given on a per-chapter basis.

1.1 Background and significance of the study

Nowadays, scientists are focusing on regenerative medicine for various compelling reasons. This field offers innovative solutions for conditions and diseases that currently lack effective treatments. Additionally, the global population is aging, contributing to an increase in chronic diseases. Successful regenerative medicine can offer sustainable therapeutic solutions, improving the quality of the aging population. This can lead to decreasing healthcare costs associated with long-term treatments.

Embryonic stem cells (ESCs) play an important role in regenerative medicine. They originate from the inner cell of embryos, and they have pluripotent qualities, meaning that they can form various cell types [1]. This unique feature makes ES cells highly valuable in regenerative applications, where they develop specific cell types for repairing and replacing damaged tissue. Nowadays, many researchers have ried to explore the potential of ESCs such as cell therapy, which ES cells involve in the cell transportation into patients or treating wide range diseases or the tissue engineering for developing artificial tissue for transplantation and regenerative purposes.

In this project, ES cells is used to test two different environments of Leukemia Inhibitory Factor (LIF) [2]— with LIF and without LIF because LIF plays a key role in maintaining the pluripotent state of embryonic stem cells, by activating STAT3. Analyzing the outcomes will provide valuable information on how LIF affects the pluripotency and self-renewal capacities of ES cells. Such knowledge is essential for optimizing the conditions required to keep ES cells in their undifferentiated state, a critical factor for their potential used in regenerative medicine applications.

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1.2 Objectives

- Understand how mouse ES cell can be cultured in an in-vitro environment to compare between the presence and absence of Leukemia Inhibitor Factors (LIF)
- Understand the role of LIF in maintaining or improving the undifferentiated state of cells
- Enhance the mechanism of controlling pluripotency and differentiation, especially in the leukemia environment and ES cell biology

1.3 Scope of the study

The research based on the knowledge of ES cell mechanism focuses on characterizing the undifferentiated state of mESCs with a specific focus on the role of STAT3 and the influence of LIF in two different conditions: with and without LIF through PCR and Western blot methods.

1.4 Report outline

This report contains all the information required to understand ES cell mechanisms and understand how LIF affects these cells. For easy reading, the rest of this report is organized as follows:

Chapter 2 reviews all the related literature that is required to comprehend this project. Covering the principles such as stem cells, signaling pathways, the importance of certain proteins, and explaining the importance of each step for this experiment.

Chapter 3 thoroughly describes the equipment and material required in this experiment. The initial step involves practicing with HEK293 cells in the practicing phase before proceeding to the actual testing with and without LIF and mESCs is our actual target experiment.

Chapter 4 demonstrates the results, as well as discussion and problems we encountered during the process with solutions.

Chapter 5 closes the report, reviewing the work undertaken and leads to conclusions about key parts of the work that was undertaken. Finally, future work is discussed with particular focus on how this experiment could be improved based on the problems that were faced throughout the project.

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CHAPTER 2
REVIEW OF THEORY RELATED

This chapter discusses the knowledge required for this project and provides the reader with a comprehensive review of the literature related to the problem under experiment. In section 2.1-2.4 focus on the regenerative medicine, HEK 293 and the characteristic of mouse embryonic stem cell, the self-renewal ability. In the second section, 2.5-2.10 are the part of methodology that is required in the experiment.

2.1 Regenerative medicine

Regenerative medicine constitutes an interdisciplinary realm in the medical sciences concentrating on devising strategies and treatments aimed at mending, substituting, or rejuvenating impaired, wounded, or afflicted tissues and organs within the human body. The principal objective of regenerative medicine is to reinstate the regular functionality of these tissues and organs, effectively revitalizing them to their prime condition. This domain encompasses a diverse array of approaches and methodologies, holding immense potential for treating a broad spectrum of medical ailments. A pivotal aspect in regenerative medicine involves the utilization of stem cells. Stem cells are unspecialized cells capable of evolving into various cell types within the body [3]. Stem cell therapy encompasses employing these cells to mend or substitute damaged or dysfunctional tissues and organs [4]. This method may involve transplanting stem cells, guiding their differentiation into specific cell types, or inciting the body's own stem cells to facilitate regeneration. Regenerative medicine stands poised to transform the treatment of an extensive range of conditions, including heart disease, diabetes, neurodegenerative disorders, spinal cord injuries, among others. Although numerous approaches in regenerative medicine are still in experimental or developmental phases, some therapies have already entered clinical usage. The field continually progresses through ongoing research and clinical trials, aiming to broaden the scope of treatable conditions and enhance patient outcomes. Nonetheless, it's critical to emphasize that considerations regarding safety, ethics, and regulatory standards hold utmost importance in the development and application of regenerative medicine techniques.

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2.2 HEK293 cells

HEK293, or Human Embryonic Kidney 293 cells, as shown in figure 1, is a commonly used cell line in biological and biomedical research. These cells were originally derived from the kidney tissue of a human female embryo in 1973. HEK293 cells are valuable in research for several reasons. First, HEK293 cells are immortalized, they can divide and replicate indefinitely under laboratory conditions. This feature makes them a consistent and reliable resource for experimental work, and this is the main reason why we choose HEK293 for our training experiments. HEK293 can be used to express foreign genes efficiently and can introduce specific genes into these cells to study gene's function and cell behavior [5]. The high transfection efficiency, ease of use, and reliability of HEK293 cells have made them a fundamental tool in molecular biology, genetics, and biotechnology research. However, it's important to note that the use of HEK293 cells is subject to ethical considerations and guidelines due to their human origin. Researchers must obtain proper approvals and adhere to ethical standards when working with HEK293 cells, especially in studies involving gene editing or the creation of genetically modified organisms [6].

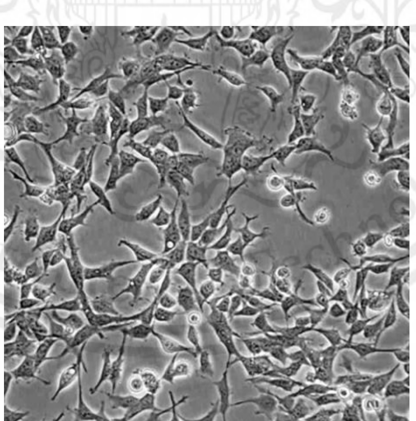

Figure 1 HEK293 cells observed under microscope

2.3 Mouse embryonic stem cells

Mouse embryonic stem cells (mESCs) are a type of pluripotent stem cells original from the inner cell mass during mouse embryo development. These cells possess the remarkable capability to transform into nearly any cell type within the adult mouse body. The distinctive pluripotent nature of mouse embryonic stem cells has been proven

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crucial in various scientific studies [7]. These studies encompass developmental biology, genetics, regenerative medicine, and the exploration of disease models. Their pluripotency, enabling them to develop into diverse cell types, positions them as an invaluable tool for examining cellular differentiation and tissue development. The inherent potential of mouse embryonic stem cells aligns with the objectives of our ongoing project, as we explore their capacity for tissue and organ regeneration [8]. This exploration offers promising prospects for potential medical treatments in the future. These investigations into the mechanics behind their self-renewal further support our research goals and aspirations.

2.3.1 Mechanics behind pluripotency

In biology, the term "Pluripotent" means capable of developing into differentiated cells. So pluripotent cells are the embryonic stem cells that have the unlimited capacity to divide, self-renew and differentiate into cells of early primary germ cell layers, mesoderm, endoderm and ectoderm [9], as shown in figure 2. This is the reason why pluripotent stem cells are important, their ability to form all three of the basic body layers (ectoderm/endoderm/mesoderm) and even germ cells [10]. In other words: pluripotent stem cells can potentially produce any cell or tissue the body needs to repair itself. Indeed, many clinical trials are already underway in which cells and tissues made from pluripotent stem cells are being evaluated as treatments or cures for a variety of diseases, including diabetes, Parkinson's disease, spinal cord injury, blindness, thrombocytopenia, and many others [11].

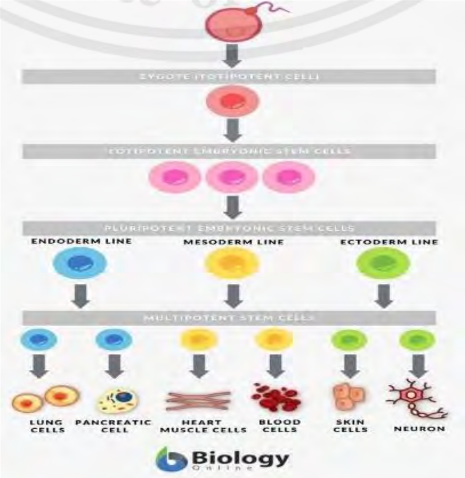

Figure 2 The development of embryonic stem cells [12]

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2.3.2 Mechanics of self-renewal

Self-renewal is the process by which stem cells divide to make more stem cells. Self-renewal is division with maintenance of the undifferentiated state. The self-renewal of ESCs is regulated by factors including signaling pathway proteins, transcription factors, epigenetic regulators, cytokines, and small molecular compounds [13]. These cell-intrinsic mechanisms are regulated by cell-extrinsic signals from the niche, the microenvironment that maintains stem cells and regulates their function in tissues. In response to changing tissue demands, stem cells undergo changes in cell cycle status and developmental potential over time, requiring different self-renewal programs at different stages of life [14]. Reduced stem cell function and tissue regenerative capacity during aging are caused by changes in self-renewal programs that augment tumor suppression.

2.4 Signaling pathways

The communication pathway involving Leukemia Inhibitory Factor (LIF) and Signal Transducer and Activator of Transcription 3 (Stat3) is a pivotal regulator in cellular signaling, especially concerning the preservation of stem cell pluripotency. This pathway commences with LIF binding to its specific receptor on the cell surface. LIF, a member of the interleukin-6 (IL-6) cytokine family, plays a vital role in overseeing the self-renewal and pluripotency of distinct stem cells like ESC and induced pluripotent stem cells (iPSCs) [14]. Upon binding, LIF induces a structural alteration in the receptor, activating internal signaling pathways. In ESCs and iPSCs, LIF usually binds to the LIF receptor (LIFR), as shown in figure 3, commencing the JAK/Stat3 (Janus kinase/Signal Transducer and Activator of Transcription 3) pathway [15]. This sequence involves the activation (phosphorylation) of Janus kinases (JAKs), followed by the phosphorylation and pairing of Stat3. Activated Stat3 translocate into the nucleus, a crucial event in the signaling chain. Within the nucleus, Stat3 attaches to specific DNA sequences in the promoter regions of target genes, thereby influencing their transcriptional activity—either activating or suppressing the transcription of these target genes. Stat3, in cooperation with other regulatory proteins and transcription factors, orchestrates the expression of genes linked to pluripotency and self-renewal. These regulated genes aid in sustaining the undifferentiated state of stem cells, enabling

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their continuous division and the generation of daughter cells that also maintain pluripotency.

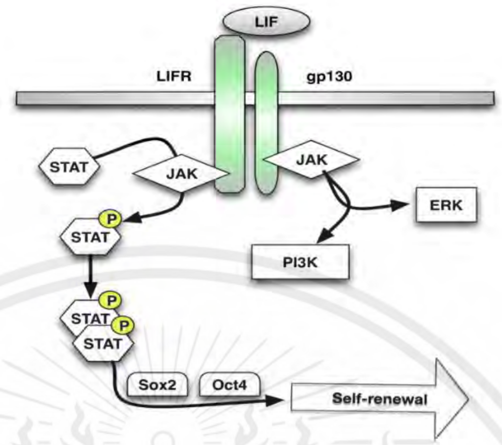

Figure 3 JAK/STAT3 signaling pathway [16]

2.5 Culture and passage

Passaging is the procedure of harvesting cells from a culture, transferring the cells to one or more culture vessels with fresh growth medium, and using those cells to start new cultures. It is also referred to as subculturing. As is relates to the phrase "passage number", it is the number of times the cells have been passaged in culture. Cell culture refers to the process of growing and maintaining cells outside their natural environment, typically within a controlled laboratory setting. This involves the cultivation of cells in an artificial environment that provides them with the necessary nutrients, temperature, humidity, and other conditions to support their growth and replication [17]. Cell culture is used in a wide range of biological and medical research, including studying cell behavior, developing vaccines, testing drugs, and producing biotechnological products [18].

There are several types of cell cultures, including primary cultures (cells directly isolated from a living organism), established cell lines (cultured cells that have been immortalized or transformed), and stem cell cultures (cultures of pluripotent or multipotent stem cells). Cell culture is an essential tool for understanding cellular processes, disease mechanisms, and developing medical treatments.

Figure 4 shows the removal of old medium by aspiration process which is the first process in cell culturing.

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Figure 4 The process during cell culture and passage

2.6 RNA and cDNA preparation

RNA preparation has various purposes in molecular biology, such as the gene expression analysis sequencing and functional for study the RNA molecules. The process for RNA preparation involves isolating and purifying RNA from the samples to have high-quality RNA for downstream application. The method of RNA preparation usually consists of 6 steps. First, collecting biological material or samples. The second step is RNA stabilization to prevent RNA degradation and stabilize the sample as long as possible [19]. The third step is RNA extraction. There are various methods of RNA extraction, but the purpose is to isolate RNA from the sample. The fourth step is purification step. This step removes contaminants and other impurities. To ensure a high-quality RNA. Next is quantification to assess the quantity and the quality of RNA. The last steps are to store the purified RNA to maintain its integrity for future use, which usually stores in extremely low temperatures (-80 °C).

For the purpose of cDNA preparation is to create a DNA from an RNA template for the study of gene expression and many downstream applications, such as PCR (polymerase chain reaction) [20]. It is very useful when studying gene expressions because it can represent the protein coding sequences without non-coding regions. The cDNA preparation consists of 5 steps.

2.7 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is a widely used molecular biology technique that allows for the amplification of a specific DNA segment, generating millions of copies of the target DNA. It was first developed in the 1980s by Kary Mullis [21] and

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has since become a fundamental tool in various fields of biology, including cell culture. The PCR process involves a series of temperature cycles, typically consisting of three main steps which are Denaturation: The double-stranded DNA sample is heated to a high temperature (usually around 94-98°C), as shown in figure 5. This causes the DNA strands to separate or denature, breaking the hydrogen bonds that hold them together, resulting in single-stranded DNA molecules. Annealing: The reaction mixture is cooled to a lower temperature (typically around 50-65°C) [22]. During this step, specific DNA primers, short single-stranded sequences of DNA that are complementary to the target DNA, anneal (bind) to their complementary sequences on the single-stranded DNA. Extension: The temperature is raised slightly (usually around 72°C), and a heat-resistant DNA polymerase enzyme begins to synthesize new DNA strands by extending from the primers. This results in the replication of the target DNA sequence, doubling the amount of DNA in each cycle. By repeating these temperature cycles multiple times (usually 20-40 cycles), the targeted DNA sequence is exponentially amplified, producing a substantial amount of DNA for analysis. So, PCR process is used to verify the identity of cell lines in culture. By amplifying and analyzing specific DNA markers and determining the genetic characteristics of cells in culture [23].

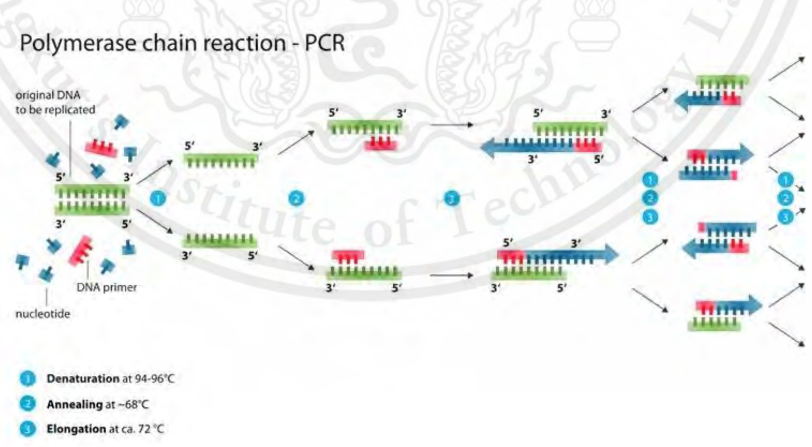

Figure 5 Polymerase chain reaction step [24]

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2.8 Protein primer

The protein primer that is used in the laboratory has two main reasons for being used. The first is for DNA synthesis and second is for DNA amplification and in our experiment is for DNA amplification. A protein primer for DNA amplification refers to a short amino acid sequence that initiates or promotes protein-related reactions or processes. A DNA primer in PCR for nucleic acid amplification, protein primer is a starting point for an amplification or generating specific protein products in laboratory technique. We used 4 protein primers in this experiment viz GAPDH, ESRRB, DAX1 and KLF4 [25].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is found in both differentiate and undifferentiated. It's a key enzyme in glycolysis, the main process that turns glucose into energy (ATP). In undifferentiated GAPDH is used to produce energy. As these cells transform into more differentiated types, such as muscle or nerve cells, GAPDH might change to match the new tasks these cells have. This ensures that each cell type gets the right amount of energy for its specific functions [26].

ESRRB (or Estrogen-Related Receptor Beta) is a transcription factor that is important for maintaining the pluripotent state of the embryonic stem cells [27]. It controls the expression of the genes involved in self-renewal and prevents differentiation [28]. DAX1 (or Dosage-Sensitive Sex Reversal, Adrenal Hypoplasia Critical Region, on Chromosome X, Gene 1) is another transcription factor. DAX1 also involves pluripotency maintenance and interacts with other factors to modify gene expression. It also plays a role of balancing self-renewal and differentiation [29]. KLF4 (Kruppel-Like Factor 4) is a transcription factor that helps ES cell to maintain the pluripotent. To sum up the role of those proteins, they are the key to the self-renewal and pluripotency of embryonic cells [30]. By using those protein primers in PCR, we can understand the expression pattern of each protein specifically, aimed to influence pluripotency in ES cells during culturing.

2.9 Western Blot Analysis

A Western blot test is a laboratory technique used for detecting and analyzing specific proteins. The purpose of them is to identify and quantify the presence of particular proteins based on their size. It is widely used to study protein expressions, to

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confirm the presence of specific protein samples and many. It is a fundamental tool to understanding various cellular processes and diseases. A western blot test consists of 5 steps process, shown in figure 6. First is protein separation from a sample based on their size through gel electrophoresis. The second step is to transfer from the gel to a solid membrane that is made from PVDF (polyvinylidene difluoride) [31]. The membrane will block to prevent nonspecific blinding of detecting reagent. Third step the membrane will incubate with the specific antibody and bind to the target protein. The fourth step is detection, a secondary antibody, mixed with a detectable marker applied to bind with primary antibody. It allows visualization of the target proteins. Last step the target protein is detected by the visualizing the marker that attached the secondary antibody though the chemiluminescence, fluorescence or colorimetric reactions. The resulting bands indicate the presence and quantity of the target protein [32].

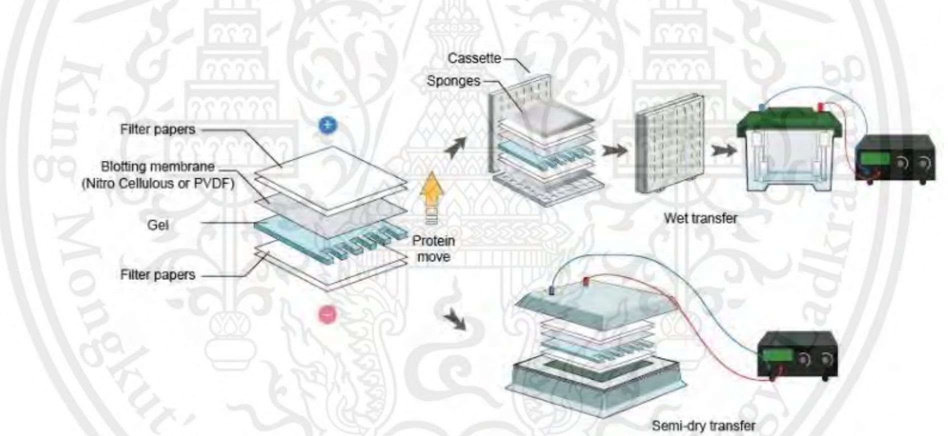

Figure 6 Western Blot Analysis [33]

2.10 Electrophoresis

Electrophoresis is a technique used in the laboratory, that is used to separate DNA, RNA or protein molecules based on their size and electric charge [34]. It works by applying the electric field. The interested molecules will move through the gel at different rates, as shown in figure 7, depending on the size and the charge, the result will show the bands or the pattern. For example, DNA fragments are loaded onto a gel, and an electric current is applied. Since DNA is negatively charged, it moves towards the positive electrode. Smaller fragments move through the gel more quickly than larger ones, leading to the separation of DNA fragments by size [35].

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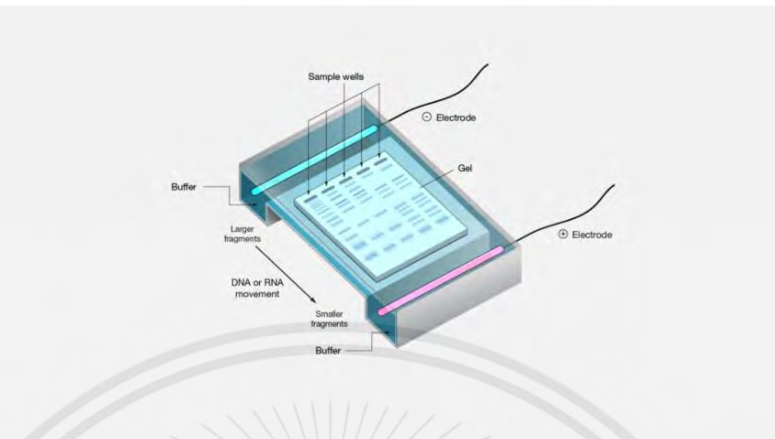

Figure 7 The component of Electrophoresis [36]

2.11 Bradford Protein Assay

The traditional method for calculating protein concentration of an unknown sample is to use a standard curve that is generated from known protein standards. The most reliable protein estimation is performed using reference or protein standard that has similar properties to the protein sample. However, it is difficult to find a perfect match for the unknown protein. Therefore, scientists use bovine serum albumin (BSA) or gamma globulin as standard. Using BSA or gamma globulin as references, there can be some differences between proteins, which means the result is an estimate, not an exact measurement.

When making a standard curve to measure protein samples, use at least 6 concentrations for generating the standard curve and adjust the dilutions of standards to cover the expected range of your unknown samples [37]. It is important to stay in the linear range of protein test for accurate results. The buffer of choice should be the same buffer with unknown protein standard.

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CHAPTER 3
METHODOLOGY

This chapter, methodology, explained about the method and equipment used for carrying out this experiment in depth. Starting with an overview of the overall project from practing by using HEK293 cells in the steps of culture and passage and RNA preparation and mESCs is our actual target experiment. Both of them are done with two conditions—one with LIF and the other without LIF (section 3.1), followed by the required equipment and materials in section 3.2. Section 3.3 describes the full procedure in this experiment. Closing this chapter with problems that occurred, their impact on the experiment, and solutions for problem that occur during the experiment.

3.1 Method overview

The experiment starts by growing mESCs through precess know as cell culture. Then, they are transferred into 4 dishes to proliferate in two difference environments: two dishes with LIF and two dishes without LIF. We have organized two sets of cells, each group contain two dished —one with LIF and the other without LIF—for the analysis of RNA with four specific primers (GAPDH, DAX1, KLF4, and ESRRB) using the process of electrophoresis. Then, protein analysis with two different anitbodies (tubulin and DAX1) using SDS-PAGE and Western blot anlysis. The overall steps are shown in figure 8 .

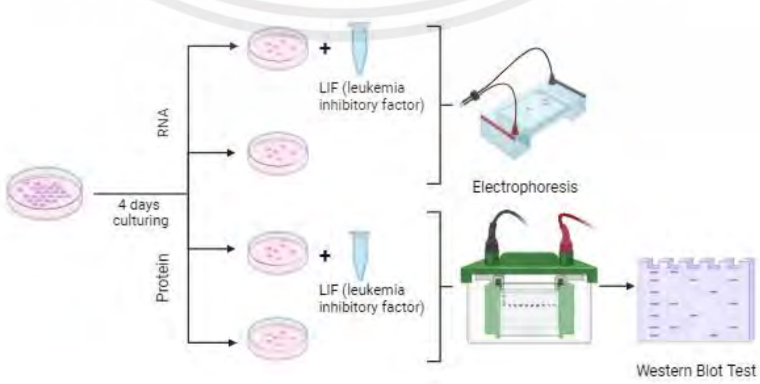

Figure 8 Overview methode

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3.2 Equipment and Materials

This section lists all the materials and equipment that were used to conduct this experiment.

3.2.1 Equipment

1. Safety cabinet
2. Standard laboratory equipment's (micropipettes, micropipette tips, pipette gun, test tubes, petri dish, incubator, hematocytometer, cover slip, and microscope, etc.)
3. PCR device (Figure 9)
4. Spectrophotometer (Figure 10)
5. Gel electrophoresis (Figure 11)
6. Western blot equipment's (gels, imaging systems, filter papers, tank blotting transfer systems, and semi-dry blotting systems)

Figure 9 PCR device

Figure 10 Spectrophotometer

Figure 11 SDS-PAGE

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3.2.2 Materials

1. HEK293 cells
2. HEK293 medium
3. PBS
4. Trypsin
5. LIF
6. mESCs
7. mESCs medium
8. Sepasol
9. Chloroform
10. Isopropanol
11. 75% EtOH
12. 5µM oilgo dT
13. 10mM dNTPs
14. Rever Tra Ace
15. H_2O
16. 5 x buffer
17. 2 x GoTaq
18. 10µM primer (GAPDH, DAX, KLF4, and ESRRB)
19. Agarose (Nakalai)
20. 1 x TAE buffer
21. SYBR Safe DNA gel stain
22. L.OB solution
23. CBB solution
24. BSA solution
25. Acrylamide gel
26. SDS-PAGE buffer
27. Protein marker
28. Western blot buffer
29. Antibody (tubulin and DAX1)

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3.3 Design Methodology

This research involves three key steps: cell culture and passage (3.2.1), gel electrophoresis, and Western blot analysis (3.3.7). Cell culture and passage includes growing and transferring cells into new dishes. Cells are kept in the presence of LIF every 3 days and moved to four-well dishes including two dishes with LIF and two dishes without LIF. We have set up two sets of cells, each group contains two dishes—one with LIF and the other without LIF.

For the first group, we extract RNA and convert it into cDNA, which will be used as template for the PCR. PCR is generated to amplify specific DNA sequences with four specific primers (GAPDH, DAX1, KLF4, and ESRRB). Then we employ gel electrophoresis to help us visualize and analyze our samples effectively [37].

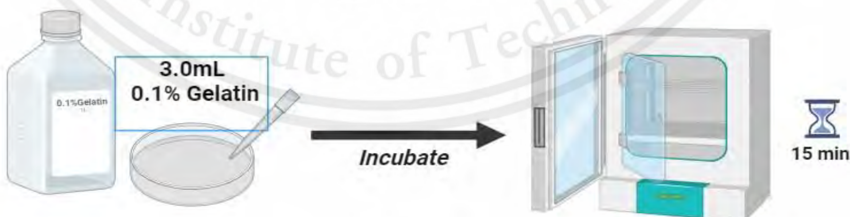
For the second group for protein analysis, we prepared a lysate from mESCs to measure protein concentration (3.3.6). Then, we performed SDS-PAGE and Western blot analysis.

Finally, we can observe the differences between the samples with LIF and those without LIF.

3.3.1 Culture and passage

Step for culture and passage:

1. Prepare gelatin-coated dish: For a 6-cm dish, add 3.0 ml 0.1% gelatin, and put in incubator at 37 °C.



2. Stand bottles/tubes of mESCs mediums and Phosphate-buffered saline medium at room temperature.
3. Wipe and sterilize the bottles/tubes with 70% EtOH, and then put it into a clean bench.
4. Remove culture medium from mECs cell culture dish by aspiration, add 4 ml PBS medium, and then remove the PBS by an aspiration.

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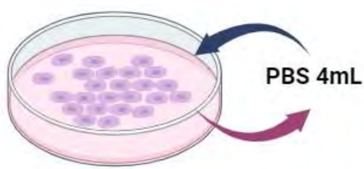
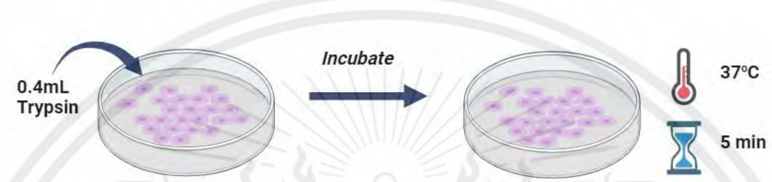
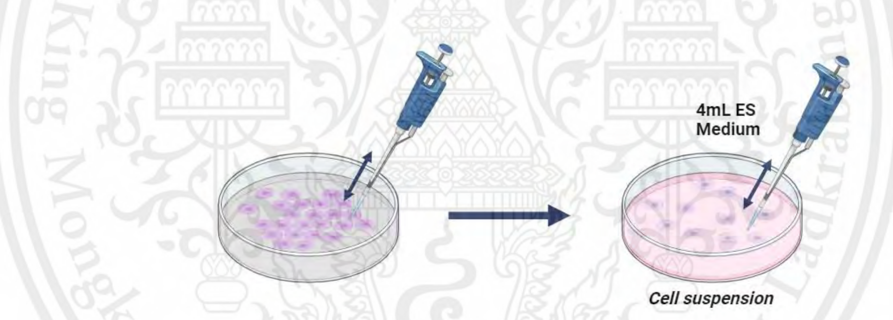


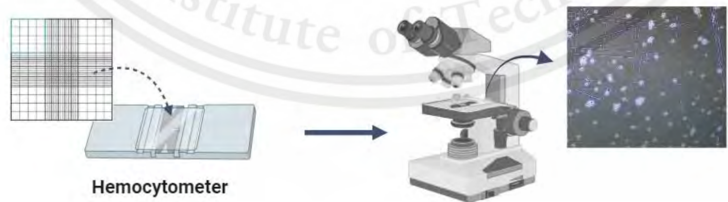
5. Add 0.4 ml of Trypsin/EDTA solution, and incubate at 37°C for 5 min.



6. Pipet up-down for 10 times to get cell suspension.
7. Add 4.0 ml of mESCs medium, pipet up-down



8. Count the number of ES cells.



9. Remove gelatin from a dish by an aspiration and transfer 0.5 x 10^5 mESCs cells into the 6-cm dish with 4 ml mESCs medium.
10. After that, add 1:1000 vol. of LIF (4μL).

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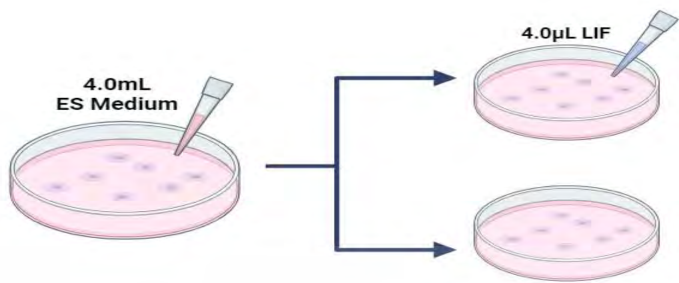


11. Culture the ES cells for 3 days.

Table 1 LIF concentration ratio for cell culture

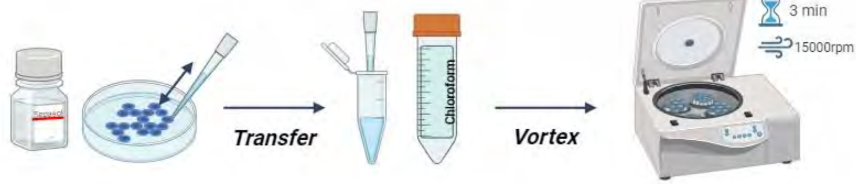
-LIF (2 days)	$1.5x10^5 ES\ cells\ in\ 6\ cm\ dish$
+LIF/-LIF(3days)	$0.5x10^5 ES\ cells\ in\ 6\ cm\ dish$
+LIF/-LIF (4 days)	$0.25x10^5 ES\ cells\ in\ 6\ cm\ dish$
-LIF (6 days)	$0.05x10^5 ES\ cells\ in\ 6\ cm\ dish$
-LIF (6 days)	$0.1x10^5 ES\ cells\ in\ 6\ cm\ dish$

This table shows the LIF ratio concentration for various days in cell culture.

3.3.2 RNA Preparation

Step for RNA preparation:

1. Remove culture medium from a cell culture dish by aspiration, after that add 4 ml PBS (-) and then remove the PBS (-) by an aspiration.
2. Add 500 µl of Sepasol into the dish.
3. Pipet up-down for several times to get all the cell suspensions completely.
4. Transfer the cell suspensions into the 1.5 ml tube.
5. Add 100 µL of chloroform and then vortex them well
6. Centrifuge the tube at room temperature, 15000 rpm for 3 minutes.
7. Transfer the aqueous phase into a new 1.5 ml tube.



8. Add equal volume of isopropanol, mix well and put them at room temperature for 10 minutes.
9. Centrifuges the tube at 4°C, 15000 rpm for 15 minutes.

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10. Discard the supernatant, add 150 µL of 75% EtOH.
11. Centrifuges the tube at 4°C, 15000 rpm for 5 minutes.
12. Discard the supernatant and then dry the pellet at 37C for 5 minutes.
13. Resuspend the pellet in 50 µL water

14. Mix them well and incubate the tube at 55°C for 10 minutes to resuspend completely.
15. Mix them well, spinddown and measure the RNA concentrations.
16. Store these RNA at -80 °C.

3.3.3 cDNA preparation

Step for cDNA preparation:

1. Mix all the solution together and spin-down
2. Incubate them in 42°C for 50 minutes and cover them with aluminum foil cap.
3. Continually incubate them with 100°C or 5 minutes.
4. Cool down on the ice for 3 minutes.
5. Add water 60 µL and mix well.

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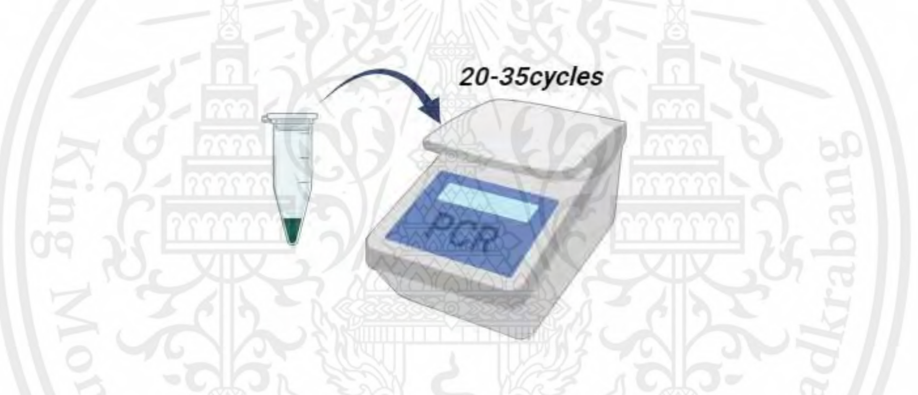
3.3.4 Go Taq

Step for making Go Taq:

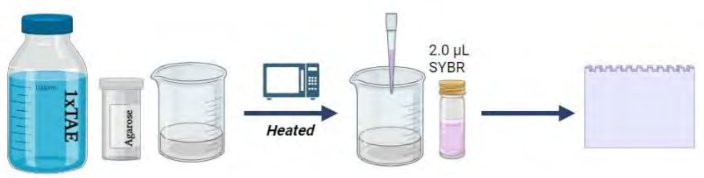
1. Mix the 2xGotaq, 10µM primer, water and template together
2. Then put the tube in the PCR machine and set the cycle from 25- 25 cycles.



3.3.5 Preparation of Agarose gel

Step for making Agarose gel:

1. Mix the agarose with 1xTAE buffer and then melt them in the microwave.
2. Cool down at room temperature.
3. Add 1.0 µL of SYBR to safe the DNA stain.
4. Pour the agarose solution into the cassette.



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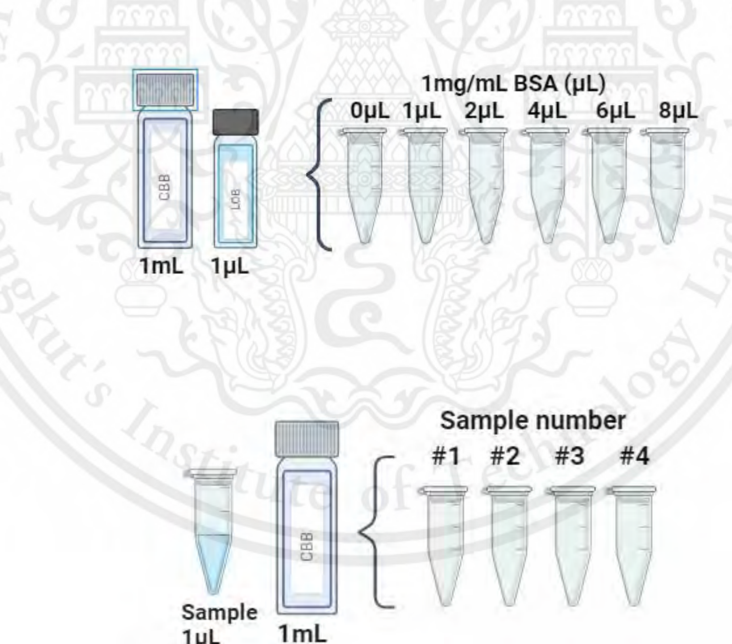
3.3.6 Determination of protein concentration

Step for detemation of protein concentration:

1. Prepare 1xCBB solution.
2. Transfer 1 ml of 1xCBB solution to several 1.5 ml tubes.
3. Add 0, 1, 2, 4, 6, or 8 µl of 1mg/mL BSA solution to each 1xCBB solution.
4. Add 1µL of L.O.B into each BSA tube.
5. Add 1 µl of sample to other 1 x CBB solution tubes.
6. Wait 10 minutes.
7. Measure the absorbance of each sample at 595 nm (100uL use for determination).
8. Then calculate the protein concentration of samples with BSA as a standard.
9. After calculate the protein centration, we will do the Bradford Protein assay, which we used to find a unknow concentration.



3.3.7 SDS-PAGE and Western Blot Analysis

Step for SDS-PAG and Western Blot Analysis:

1. Mix 40 µL of sample and 10 µL 5 x sample buffer.
2. Treat the samples at 100°C for 3 minutes.

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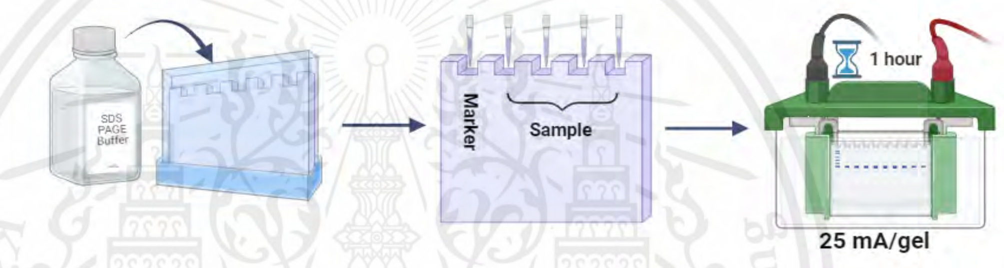
3. Cool down at room temperature, after that spin down.

4. Put acrylamide gel into an Electrophoresis Tank with SDS-PAGE buffer.
5. Apply 5.0 µl protein marker
6. Apply cell lysate samples (about 30-50 µg) into each well.
7. Start the electrophoresis process; 25 mA/gel for 1 hour condition.



8. Prepare 10-cm x 10-cm filter paper and 6.5-cm x 8.5-cm nitrocellulose filter.
   [For semi-dry method]
9. After electrophoresis, remove the one side gel-board, discard the stacking gel, and then transfer the separating gel into a container with Western Blot Buffer.
10. Put filter paper, the separating gel and a nitrocellulose filter on semi-dry electroblotting
11. Electroblotting in the condition of 160mA/gel for 1 hour.
12. Discard the filter paper, and then transfer the nitrocellulose filter into a container with 1% BSA/PBS-T solution, rotate the container for 10-30 minutes at room temperature.
13. Discard the 1% BSA/PBS-T solution
14. Incubate the filter with 1 ug/ml alfa-Myc antibody for 60 min at room temperature or 4°C for O/N with rotating [antibodies should be diluted in 1% BSA/PBS-T solution].
15. Wash the filter with PBS-T for 15 min at room temperature with rotating it 2 times.

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16. Incubate the filter with 5,000-fold diluted goat anti-mouse IgG HRP conjugate for 60 minutes at R.T. with rotating. [antibodies should be diluted in 1% BSA/PBS-T solution.]
17. Wash the filter with PBS-T for 15 min at R.T. with rotating 2 times.
18. Develop the signal with the ECL system (See manufacture protocol).

3.3.8 Cell lysate collecting

Step for Cell lysate collecting:

1. Harvested the starting-cell dish and then washed with PBS
2. Pellet the cells by centrifugation at a low speed
3. Discard the supernatant
4. Resuspend the cell pellet in an appropriate volume of ice-cold cell lysis buffer
5. Incubate on ice for about 15-30 minutes
6. Centrifuge the lysate at a high speed for 10-15 minutes at 4°C
7. Store the cell at -80°C

3.4 The problem and solution

3.4.1 HEK 293 cells

In this experiment, we used HEK293 as a practice sample for culture and passage. During the process, we encountered problems of cell clustering (figure 12) and presence of bubbles on the hemocytometer (figure 13).

Using trypsin incorrectly when passing cells can lead to cell clustering. Trypsin is importance tool that helps separate cells during cell culture. It works by breaking down proteins. In this case, we did not use the trpsin carefully, which lead to cell stress and damage, changes in cell behavior, cell aggregation, contamination and loss of pluripotency or differentiation. To avoid theses issues, it is importance to follow the right steps for using trypsin by using the right amoint of trypsin and suitable time of incubation time. Additionally, working quickly and efficiently during the detachment process can help decrease stress on the cells,

Bubbles on the hemocytometer is another issue. This problem occurs when we did not press the thin glass tightly with hemocytometer before placing solution, leading to inaccurate volume measurement, cell distribution disruption, difficulty in fousing,

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iconsistency in results. To avoid these problems, it is important to pipet carefully, place the tool properly and allow the bubbles to escape.

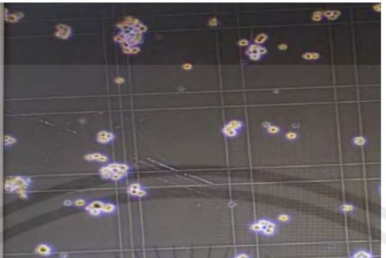

Figure 12 Dense cells observed on the hemocytometer under microscopy

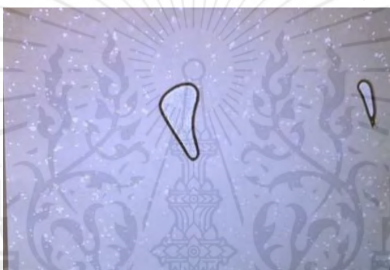

Figure 13 Bubbles observed on the hemocytometer under microscopy

3.4.2 Embryonic stem cells

Before we did the PCR, we must mix the reagents, which are RNA, Oligo dT, buffer, dNTPs, Rever Tra Ace and water together. The amount of RNA reagent must be calculated the ratio of RNA and water before by measured their concentration and then calculated the amount of them. The RNA preparation steps must do it carefully because the RNA was quite easy to derogate. From the result, they were RNA samples that had quite high concentration, when compared to others which are the +LIF sample of group 1 (1403.9 ng/µl). It had a ratio of absorbance higher than 2, which the ratio could tell us how pure the sample is, lower than 2 means the sample is pure [38]. But the samples had a higher absorbance ratio than acceptable criteria. The possible reason of this problem is we didn't do it gently with the RNA during the RNA preparation step then we made them derogated, and this is cause of the derogation of the RNA, so we must be more careful and gentler when do the RNA preparation [39].

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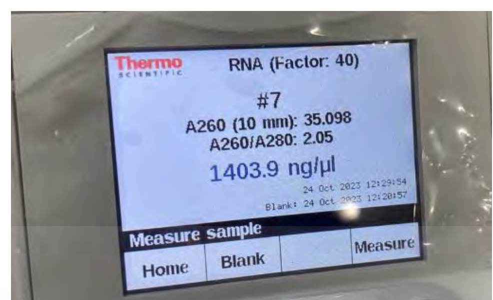

Figure 14 RNA measurement from Thermo Scientific

In the electrophoresis part, we had to prepare the agarose gel by pouring the gel mixture into the mold and letting them set for 15 minutes. After that we needed to pull out the mold barrier gently because the gel after set, it still easy to be torn. After that, we dropped the sample mixture into the hole on the gel, in this step we must carefully drop them and didn't let the solution leaked out from the gel mold.

Figure 15 The preparation of electrophoresis

The Western Blot Test is a method in which individual proteins are separated according to size by polyacrylamide gel electrophoresis. The electrophoresis is done by using the buffer to be the intermediary but during the process one of our tanks didn't have enough buffer and this affected with our result after we observed the result from the Amersham (observed under the UV light), the signal messed up and couldn't observed the result clearly, as shown in figure 16. We should add enough buffer and always observe the tank during electrophoresis [40].

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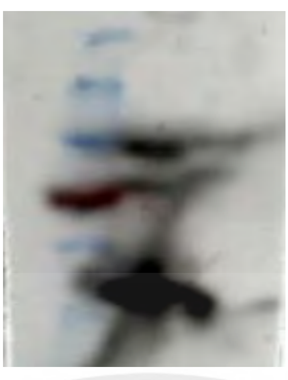

Figure 16 The messed signal result from Western Blot analysis of Tubulin

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CHAPTER 4
EXPERIMENTAL RESULT AND DISCUSSION

For this chapther, we show the calculation for cell counting, cDNA preparation, and determine protein concentration. The overall results are summarize in section 4.2. Then, we compared the result and discuss in 4.3 section.

4.1 Cell culture

After cell counting, can determine the average cell number using the following equation:

$$Average\ cell\ count = \frac{H_1+H_2+H_3+H_4}{No.counted\ chambers} \quad \textbf{(1)}$$

,where

$$H_x = number\ of\ counted\ cell\ in\ each\ square$$

Figure 17 cells under hemocytometer

$$Average\ cell\ count = \frac{40+47+42+41}{4} = 43$$

Next, we can use the answer from equation 1 to find Average concentration

$$Average\ concentration\left(\frac{cells}{mL}\right) = \left(\frac{Average\ cell\ count}{10} 10^5\right) \quad \textbf{(2)}$$

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Then, we used average concentration to calculate the required volume for transferring to new dishes for cell culture. The split ratio depends on the day needed to be cultured, as indicated in table 1. In this case 4 days of culture is required.

$$\frac{Average\ concentration}{1000} = \frac{0.25\ x\ 10^5 cells}{V} \quad \textbf{(3)}$$

when

$$V = Required\ concentration\ for\ cell\ culture\ (\mu L)$$

$$Average\ concentration\left(\frac{cells}{mL}\right) = \left(\frac{43\ cells}{10\ mL}\right)10^5 = (4.3\frac{cells}{mL})(10^5)$$

$$V = \frac{0.25\ x\ 10^5 cells}{4.3(10^5)} * \frac{cells}{mL} * \frac{1000\mu L}{mL}$$

$$V = 58\ \mu L$$

After we measured the absorbance of unknwon RNA concentration, we needed to convert the unit of measered RNA from ng to µg

$$1403.9\ \text{ng}/\mu\text{L}$$

$$\frac{1403.9\ \text{ng}/\mu\text{L}}{1000} = 1.4039\ \mu\text{g}/\mu\text{L}$$

Table 2 RNA concentration in ES cells

	$C_{RNA}(\frac{\text{ng}}{\mu\text{L}})$	$C_{RNA}\left(\frac{\mu\text{g}}{\mu\text{L}}\right)$
Sample 1 (+LIF)	1403.9	1.40
Sample 1 (-LIF)	1497.6	1.50
Sample 2 (+LIF)	1671	1.67
Sample 2 (-LIF)	1552	1.55
Sample 3 (+LIF)	1155.6	1.16
Sample 3 (-LIF)	738.3	0.7383

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This table shows the RNA concentration in ES cells, measured in the units of $(\frac{\text{ng}}{\mu\text{L}})$ and converted in to $\left(\frac{\mu\text{g}}{\mu\text{L}}\right)$.

when needed 3µg of RNA

$$1.40\ \mu g : 1\ \mu L$$
$$3\ \mu g : x\ \mu L$$

where

$$x = volume\ of\ 33\ \mu g\ of\ RNA$$
$$\text{x} = \frac{3\mu g * 1\mu L}{1.4039\ \mu g} = 2.14\ \mu L$$

Then, find total RNA volume for cDNA preparation

$$Total\ RNA\ volume = V_{RNA} + V_{H_2O} = 10 \quad \textbf{(4)}$$

where

$$V_{RNA} = V_{RNA} = 4.2\ \mu L$$
$$10\mu L - 4.2\mu L = 5.8\mu L$$

Table 3 Volume of RNA and water

	V_{RNA} (μL)	V_{H_2O}(μL)
Sample 1 (+LIF)	4.2	5.8
Sample 1 (-LIF)	2	8
Sample 2 (+LIF)	1.8	8.2
Sample 2 (-LIF)	1.9	8.1
Sample 3 (+LIF)	3.2	6.8
Sample 3 (-LIF)	4	6

This table shows the ratio of RNA and H_2O that we will use to mix the RNA in the cDNA preparation.

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4.2 Protein concentration

Table 4 Protein concentration of BSA

BSA (μL)	Absorbance of protein (A)
0	0.52
1	0.59
2	0.61
4	0.70
6	0.90
8	0.95

This table shows the absorbance of protein in BSA.

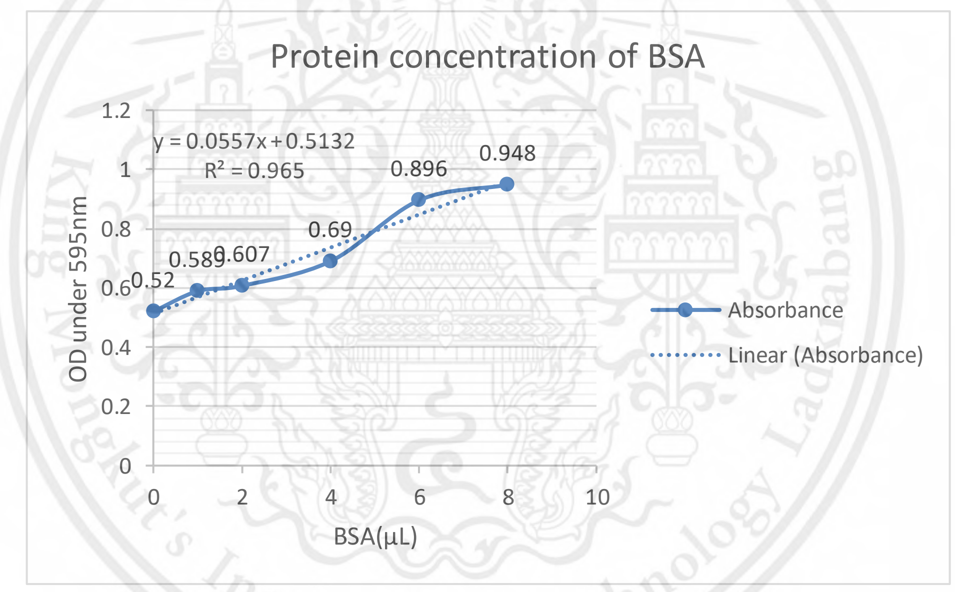

Figure 18 Protein concentration of BSA

After analyzing the standard curve, we can draw the conclusion that the relationship between y (protein absorbance) and x (BSA concentration) is represented by the equation $y = 0.0557x + 0.5132$. Then, we use these graph to compare with protein absorbance of ES cells in sample 4 and 5 in table 5 to find the concentration of ES cell.

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Table 5 Concentration and absorbance of ES cells

	Es cell concentration (µg/µL)	Absorbance of protein (A)
Sample 4 (+LIF)	6.04	0.85
Sample 4 (-LIF)	3.86	0.73
Sample 5 (+LIF)	4.83	0.78
Sample 5 (-LIF)	5.92	0.84

$$x = EScell\ or\ BSA\ cell\ concentration$$

$$y = protein\ absorbance\ of\ ES\ cell\ or\ BSA\ cell\ (A)$$

For this equation, we will use sample 4 with LIF condition as an example.

,when y = 0.85

$$0.85 = 0.0557x + 0.5132$$

$$x = \frac{0.85 - 0.5132}{0.0557}$$

$$x = 6.04\ \mu g/\mu L$$

Therefore, the ES cell concentration of sample 4 (+LIF) is equal to 6.04 µg/µL.

Find the 40 µg of protein of 80% ES cell concentration (µL) in SDS-PAGE and Western blot analysis.

Table 6 Summary concentration of ES cells

	ES cell concentration (µg/µL)	80% of ES cell concentration (µg/µL)	40 µg of protein of 80% ES cell concentration (µL)
Sample 4 (+LIF)	6.0	4.8	8.3
Sample 4 (-LIF)	3.9	3.1	12.9
Sample 5 (+LIF)	4.8	3.8	10.5
Sample 5 (-LIF)	5.9	4.7	8.5

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$$80\%\ of\ ES\ \text{cell concentration}\ \left(\frac{\mu g}{\mu L}\right) = Es\ \text{cell concentration} * \frac{80}{100}$$

$$40\ \mu\text{g of protein of } 80\%\ \text{ES cell concentration } (\mu\text{L}) = \frac{40\mu L}{80\%\ of\ ES\ cell\ concentration\ \left(\mu\frac{g}{\mu L}\right)}$$

,when $Es\ cell\ concentration = 6.0\ \mu g/\ \mu L$

$$80\%\ of\ ES\ cell\ concentration\ \left(\frac{\mu g}{\mu L}\right) = 6.0 * \frac{80}{100} = 4.8\ \frac{\mu g}{\mu L}$$

$$40\ \mu\text{g of protein of } 80\%\ \text{ES cell concentration } (\mu\text{L}) = \frac{40\mu L}{4.8\ \frac{\mu g}{\mu L}} = 8.3\ \mu L$$

4.3 Results

4.3.1 Cell culture

This is the result of ES cell culture in different culture day but same condition, +LIF condition. This is to observe the change of the ES cell. We observed that the number of cells increased directly with the culture day.

Table 7 Cell culture in ES cells

Culture day	+LIF
1 day	*Figure 19 ES cell 1 day culture*
3 days	*Figure 20 ES cell 3 days culture*

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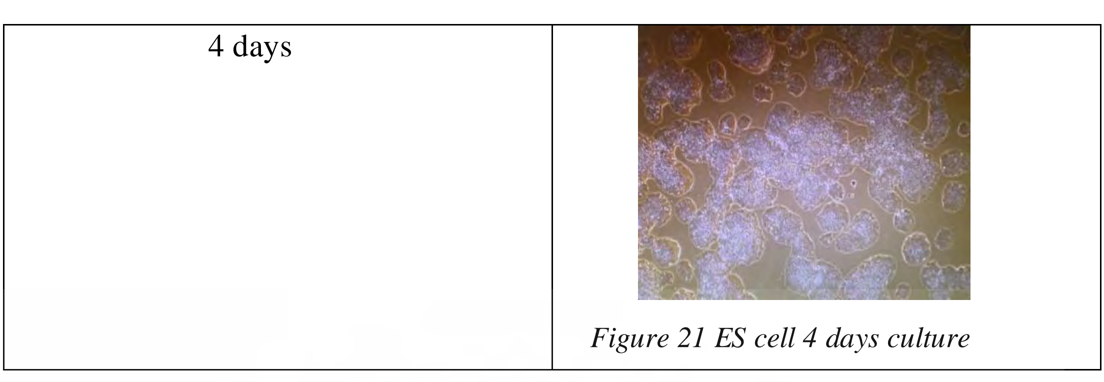

4 days	*Figure 21 ES cell 4 days culture*

The different between +LIF, –LIF and HEK29 cells

Figure 22 ES cells with +LIF condition

Figure 23 ES cells with –LIF condition

Figure 24 HEK 293 cells

This time we compared the results of ES cells in different conditions, with and without LIF, and HEK 293 cells to observe the different characteristics between them. We observed that the ES cells with LIF had more compact and uniform, while the –LIF had lower compact and peaked shape. For the highest intensity is the HEK 293 cells.

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4.3.2 RNA

We analyzed RNA using three groups of samples under two conditions: ES cells with LIF and without LIF. The concentration of RNA is shown in table 2. The analysis was done using electrophoresis with four different primers: GAPDH, DAX1, KLF, and ESRRB. Here are the results:

GAPDH

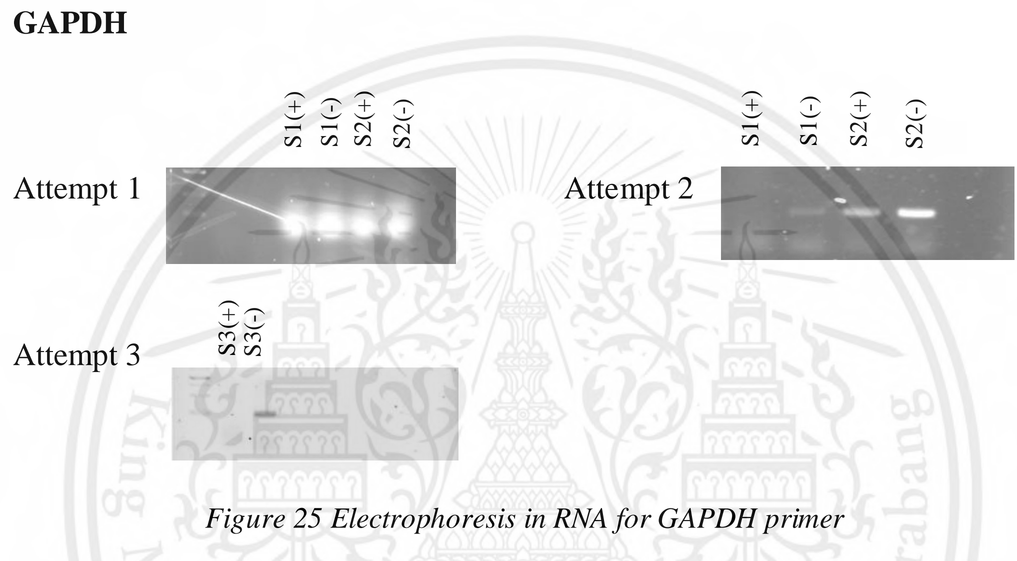

Figure 25 Electrophoresis in RNA for GAPDH primer

Regarding the GAPDH primer, we conducted 3 attempts. In the first try, we saw signal bands in samples 1 and 2 in both environments. In the second try, signal bands were in sample 1 in the environment without LIF and in sample 2 in both environments. Finally, in the third try, we only tested sample 3. The bands showed up only in the environment without LIF.

DAX1

Figure 26 Electrophoresis in RNA for DAX1 primer

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For DAX1, we conducted one attempt. The signal bands appeared in samples 2 and 3 in the environment with LIF.

KLF4

Attempt 1

Figure 27 Electrophoresis in RNA for KLF4 primer

For KLF4, we conducted one attempt. The signal bands appeared in samples 2 and 3 in the environment with LIF.

ESRRB

S1(+) S1(-) S2(+) S2(-) S3(+) S3(-)

Attempt 1

S1(+) S1(-) S2(+) S2(-) S3(+) S3(-)

Attempt 2

Figure 28 Electrophoresis in RNA for ESRRB primer

For ESRRB, we conducted two attempts. In the initial attempt, the signal bands did not appeared. In the second attempt, we detected signal bands in sample 2 and sample 3 in the environment with LIF.

4.3.2 Protein

We analyzed protein using two group of sample under two conditions: ES cells with LIF and without LIF. The concentration of RNA is shown in figure 17 and table 4.The analysis was done using electrophoresis and Western blot analysis with two different antibodies: α Tubulin and DAX1.

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α Tubulin

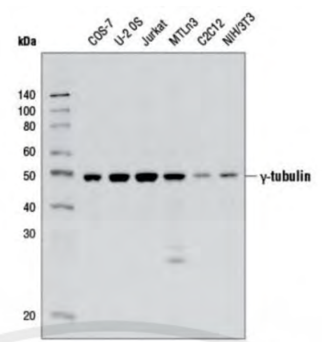

Figure 29 Tubulin molecular weight

Figure 30 Electrophoresis in protein for α Tubulin antibody

For α Tubulin, we conducted two attempts. The initial and second attempts, the signal bands appeared in both environments, with LIF and without LIF.

Dax1

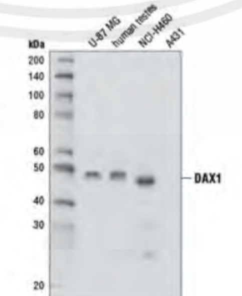

Figure 31 DAX1 molecular weight

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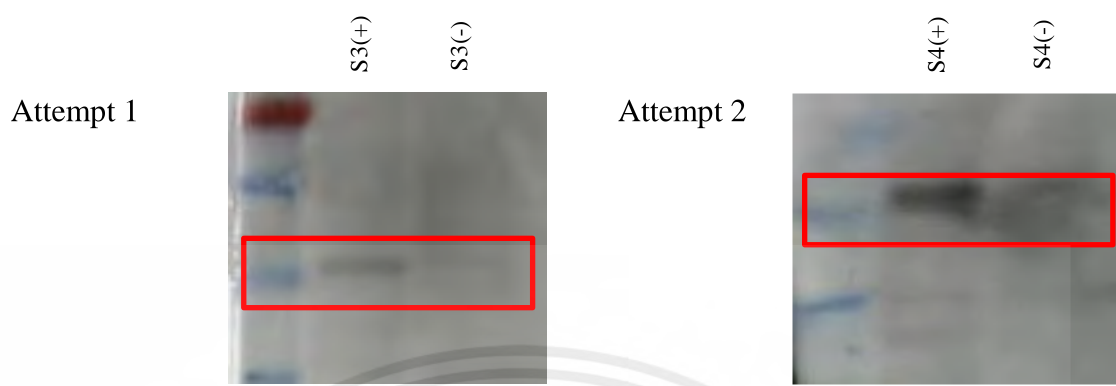

Figure 32 Electrophoresis in protein forl DAX1 antibody

For DAX1, we conducted two attempts. The initial and second attempts, the signal bands appeared in the environment of with LIF in both sample 3 and 4.

4.4 Explanation and discussion

From section 4.3.1 is a table to compare the ES cell culture in different time periods. It shows that the amount of the cell on the plate is direct variation with the culture day, the more culture day, the more amount of ES cell on the plate. On the 3-day culture plate has the highest cell intensity and the 1-day culture plate has lowest intensity. Next part is the comparison between the ES with and without Leukemia inhibitory factor (LIF). We can observe that the ES cell culture with LIF has a more compact and uniform appearance. The cell has a rounded shape with a high nuclear to cytoplasmic ratio. For the ES cell culture without LIF has peaked and slimmer shape than with LIF condition and it also has lower intensity than with LIF condition.

From RNA results (4.2.2 section), we used sample from all 3 three groups sample using the GAPDH primer, we made three attempts. In first attempt, the signal bands were not clear, spread out, and thick. This made it hard to compare with the marker for molecular weight, due to a high concentration that machine could not accurately detect [43]. Moreover, in the experiment, we discovered that we made mistakes using an inappropriate pipette for the specified volume, due to all these factors having an impact on the outcomes.

For the second attempt, we tried to dilute the concentration by adding 3.5 µL of water. The band was clearer compared to the first attempt except for sample 1 (+LIF)

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is still not visible, due to the A260/280 value was 2.05. The A260/280 values is direct DNA concentration measurement, and DNA purity assessment for the unknown samples. Typically, a ratio around 1.8 is considered pure for DNA, and approximately 2.0 is pure for RNA. This value of A260/280 in sample 1 suggests either RNA contamination or degradation. To fix this problem, adjustments can be made during the RNA preparation process such as implementing temperature control, swift tissue/RNA stabilization, proper sample handling, and other relevant measures to enhance the overall integrity of the RNA samples. For the third attempt, we tried to verify the result and find addtional resones for the absence of certain bands. This time we collected new cell samples and diluted the solution. From the result, we found out that sample 3 (+LIF) band did not appear. This could be associated with defective SYBR Safe DNA gel stain, which is very important to the gel electrophoresis process due to the property for visualizing the bands of DNA.

Based on the information provided, we can conclude that GAPDH is present in both environments (with LIF and without LIF). The fact that GAPDH bands were observed in both conditions suggests that this gene is detectable and present in the samples regardless of the presence of LIF. Therefore, we can tell that GAPDH can be detected and appears to be stable in both environments.

By observing the results obtained with the DAX1, KLF, and ESRRB primers, we noticed the presence of bands in sample 2 (+LIF) and sample 3 (+LIF), whereas the sample without LIF did not display any bands. Except for ESRBB primer, the signal did not appear at the end of 25 cycles. Therefore, we decided to increase the cycle number to 30 cycles, due to the possibility of lower abundance of the ESRRB target in comparison to the targets amplified by other primers. The reason behind increasing the number of cycles was to increase the chances of generating a detectable amount of the ESRBB target. To conclude, DAX1, KLF, and ESRRB primers are specifically present in ES cells with LIF. For sample 1 (+LIF), the absence of a visible signal band can be attributed to the same reasons we mentioned earlier, exceeding value of A260/280 [12].

In the protein analysis, we measured the absorbance of BSA at a wavelength of 595 nm. This standard serves as a reference for comparing the absorbance of proteins, helping us determine the concentration of unknown ES cells. We used two antibodies, α Tubulin and Dax1 for the analysis.

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For α Tubulin, we observed a strong signal in both attempt. However, in the second attempt, we noticed a messy signal, which was caused to an error in the SDS-PAGE process. The issue is due to not enough buffer in the tank, causing the gel to block the pathway for inserting the solution. Therefore, the solution leaked out of the system [37]. To conclude, the singal appeared in both environment.

In both attempts with DAX1, the band was visible in ES cells with LIF, while it was not visible in cells without LIF. This observation relates to the theory suggesting that DAX1 can maintain in an environment containing LIF. To conclude the signal appeared only in the environment of with LIF.

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CHAPTER 5
CONCLUSION

5.1 Conclusions

Referring back to the project's initial objectives, most of them have been achieved. Through extensive research and multiple trials, we have gained a clearer understanding of the mechanisms underlying ES cells and how LIF influences mouse embryonic stem cells (mESCs) [3]. It has become evident that regulating LIF is crucial for maintaining mESC pluripotency, primarily by sustaining important transcription factors like STAT3 by observing the results.

For RNA results of GAPDH signal bands appeared in both with LIF and without LIF. For DAX1, KLF, and ESRRB primers, the signal bands appeared only in ES cells with LIF. For protein results, α Tubulin appeared in both environment. However, in DAX1 the signal bands appeared in the environment of with LIF. Our understanding indicates that STAT3 plays a key role in this process. Activating STAT3 leads to an increase in the percentage of GAPDH, DAX1, KLF, and ESRRB [26]. This, in turn, contributes to the maintenance of the pluripotent properties of ES cells. This insight enhances our comprehension of the intricate regulatory networks involved in maintaining the pluripotency of ES cells, offering valuable information for future studies and applications in stem cell biology [40].

In conclusion, this experiment indicates that mESCs are well-suited to an environment enriched with LIF to preserve their pluripotency.

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5.2 Suggestion

1 Always make clear and detailed notes of all calculations during the experiment. This ensures that the information is easy to understand and prevents confusion when reviewing the notes later.

2 You need to handle the experiment with care and precision, especially in the RNA preparation process. Being too rough or careless could lead to issues, such as the degradation of RNA.

3 Always be cautious about not having enough buffer in the SDS-PAGE and electrophoresis tank, as this can affect the results.

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