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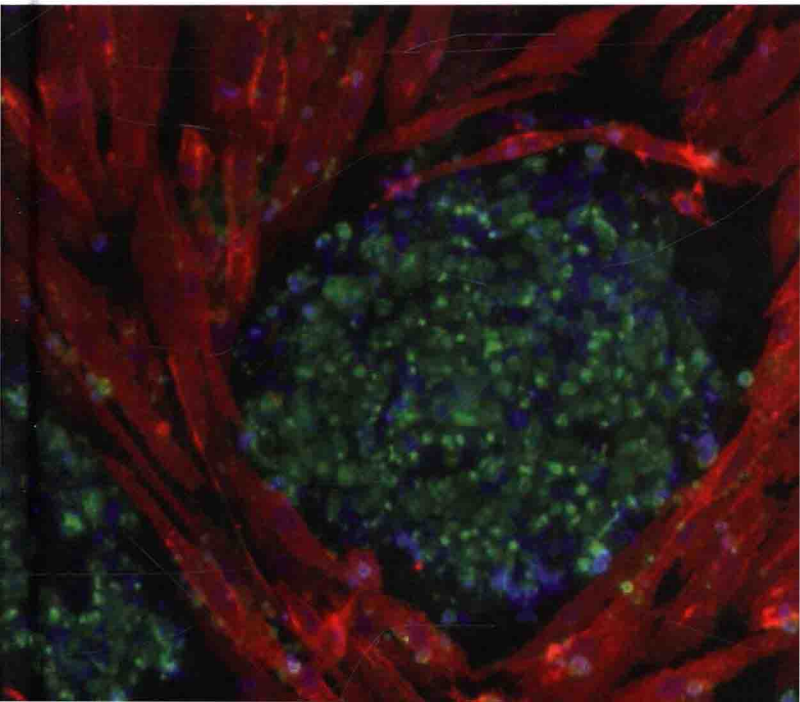
Human Pluripotent Stem Cells

A Practical Guide



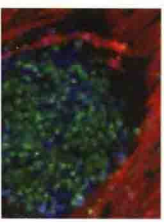
Comprehensive coverage of the entire induced pluripotent stem cell basic work flow

Pluripotent stem cells (PSC) can divide indefinitely, self-renew, and can differentiate to functionally reconstitute almost any cell in the normal developmental pathway, given the right conditions. This comprehensive book, which was developed from a training course, covers all of the PSCs (embryonic, embryonic germ, and embryonic carcinoma) and their functions. It demonstrates the feeder-dependent and feeder-free culture of hESC and hiPSC, which will be referred to in all protocols as PSCs. It also addresses the methods commonly used to determine pluripotency, as defined by self-renewal marker expression and differentiation potential.

Human Pluripotent Stem Cells: A Practical Guide offers in-depth chapter coverage of introduction to stem cell, PSC culture, reprogramming, differentiation, PSC characterization, and more.

- Includes helpful tips and tricks that are normally omitted from regular research papers
- Features useful images to support the technical aspects and results visually as well as diagrammatic illustrations
- Presents specific sections (i.e., reprogramming, differentiation) in a concise and easily digestible manner
- Written by experts with extensive experience in stem cell technologies

Human Pluripotent Stem Cells: A Practical Guide is an ideal text for stem cell researchers, including principal investigators, and others in university and industry settings, and for new graduate students in PSC labs.



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Introduction

Successful execution of any cell-based project relies on a setting up a robust cell culture laboratory. Guidelines under the Guidance on Good Cell Culture Practice provides an overview of the critical parameters in establishing facilities and training personnel [1]. This is even more important for a stem cell laboratory where primary cells from donor tissue or their derivatives are cultured for extended periods of time [2].

1.1 Biosafety

In addition to the safety risks common to most workplaces, such as electrical and fire hazards, a cell culture laboratory has a number of specific hazards associated with handling and manipulating human or animal cells and tissues, as well as toxic, corrosive or mutagenic solvents and reagents. The most common of these hazards are accidental inoculations with syringe needles or other contaminated sharps, spills and splashes onto skin and mucous membranes, ingestion through mouth pipetting, animal bites and scratches, and inhalation exposures to infectious aerosols.

The fundamental objective of any biosafety program is to reduce or eliminate exposure of laboratory workers and the outside environment to potentially harmful biological agents. The most important element of safety in a cell culture laboratory is strict adherence to standard microbiological practices and techniques.

Human Pluripotent Stem Cells: A Practical Guide, First Edition. Uma Lakshmipathy, Chad C. MacArthur, Mahalakshmi Sridharan and Rene H. Quintanilla.

1.2 Biosafety Levels

The regulations and recommendations for biosafety in the United States are contained in the document *Biosafety in Microbiological and Biomedical Laboratories*, prepared by the Centers for Disease Control (CDC) and the National Institutes of Health (NIH), and published by the US Department of Health and Human Services. The document defines four ascending levels of containment, referred to as biosafety levels 1 through 4, and describes the microbiological practices, safety equipment, and facility safeguards for the corresponding level of risk associated with handling a particular agent.

- *Biosafety Level 1 (BSL-1)*: the basic level of protection common to most research and clinical laboratories. Appropriate for agents that are not known to consistently cause disease in normal, healthy human adults (examples: *Bacillus subtilis*, *E. coli*).
- *Biosafety Level 2 (BSL-2)*: appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, and all stem cell labs have this as a requirement.
- *Biosafety Level 3 (BSL-3)*: BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections.
- *Biosafety Level 4 (BSL-4)*: BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high-containment laboratories.

For more information about the biosafety level guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition, which is available for downloading at www.cdc.gov/biosafety/.

1.3 Aseptic Technique

Successful cell culture depends heavily on keeping the cells free from contamination by microorganisms such as bacteria, fungi, and viruses. Non-sterile supplies, media, and reagents, airborne

particles laden with microorganisms, unclean incubators, and dirty work surfaces are all sources of biological contamination.

Aseptic technique, designed to provide a barrier between the microorganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

1.3.1 Maintaining a Sterile Work Area

The simplest and most economical way to reduce contamination from airborne particles and aerosols (e.g., dust, spores, shed skin, sneezing) is to use a cell culture hood.

- The cell culture hood should be properly set up, and located in an area that is restricted to cell culture, is free from drafts from doors, windows, and other equipment, and with no through traffic.
- The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area.
- Before and after use, the work surface should be disinfected thoroughly, and the surrounding areas and equipment should be cleaned routinely.
- For routine cleaning, wipe the work surface with 70% ethanol before and during work, especially after any spillage.
- Using a Bunsen burner for flaming is not necessary or recommended in a cell culture hood.
- Leave the cell culture hood running at all times, turning it off only when it will not be used for extended periods of time.
- Practice good personal hygiene. Wash your hands before and after working with cell cultures. In addition to protecting you from hazardous materials, wearing personal protective equipment also reduces the probability of contamination from shed skin as well as dirt and dust from your clothes.

1.3.2 Aseptic Work Area

The major requirement of a cell culture laboratory is to maintain an aseptic work area that is restricted to cell culture work. Although a separate tissue culture room is preferred, a

designated cell culture area within a larger laboratory can be used for sterile handling, incubation, and storage of cell cultures, reagents, and media. The simplest and most economical way to provide aseptic conditions is to use a cell culture hood (i.e., biosafety cabinet).

1.3.3 Cell Culture Hood

The cell culture hood provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. Three kinds of cell culture hoods, designated as Class II, III, and I, have been developed to meet varying research and clinical needs.

- *Class I* cell culture hoods offer significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques, but they do not provide cultures with protection from contamination. They are similar in design and airflow characteristics to chemical fume hoods.
- *Class II* cell culture hoods are designed for work involving BSL-1, -2, and -3 materials, and also provide an aseptic environment necessary for cell culture experiments. A Class II biosafety cabinet should be used for handling potentially hazardous materials (e.g., primate-derived cultures, virally infected cultures, radioisotopes, carcinogenic or toxic reagents).
- *Class III* biosafety cabinets are gas tight, and provide the highest attainable level of protection to personnel and the environment. A Class III biosafety cabinet is required for work involving known human pathogens and other BSL-4 materials.

A cell culture hood should be large enough to be used by one person at a time, be easily cleanable inside and outside, have adequate lighting, and be comfortable to use without requiring awkward positions. Keep the workspace in the cell culture hood clean and uncluttered, and keep everything in direct line of sight. Disinfect each item placed in the cell culture hood by spraying it with 70% ethanol and wiping clean.

The arrangement of items within the cell culture hood usually adheres to the following right-handed convention.

- A wide, clear workspace in the center with your cell culture vessels.
- Pipettor in the front right and glass pipettes in the left, where they can be reached easily.
- Reagents and media in the rear right to allow easy pipetting.
- Small container in the rear middle to hold liquid waste.

1.3.3.1 Airflow Characteristics of Cell Culture Hoods

Cell culture hoods protect the working environment from dust and other airborne contaminants by maintaining a constant, unidirectional flow of HEPA-filtered air over the work area. The flow can be horizontal, blowing parallel to the work surface, or vertical, blowing from the top of the cabinet onto the work surface.

Depending on its design, a horizontal flow hood provides protection to the culture (if the air is flowing towards the user) or to the user (if the air is drawn in through the front of the cabinet by negative air pressure inside). Vertical flow hoods, on the other hand, provide significant protection to both the user and the cell culture.

1.3.3.2 Clean Benches

Horizontal laminar flow or vertical laminar flow "clean benches" are not biosafety cabinets; these pieces of equipment discharge HEPA-filtered air from the back of the cabinet across the work surface toward the user, and may expose the user to potentially hazardous materials. These devices only provide product protection. Clean benches can be used for certain clean activities, such as the dust-free assembly of sterile equipment or electronic devices, and they should never be used when handling cell culture materials or drug formulations, or when manipulating potentially infectious materials.

1.3.4 Incubator

The purpose of the incubator is to provide the appropriate environment for cell growth. The incubator should be large enough, have forced air circulation, and should have temperature control to within ±0.2 °C. Stainless steel incubators allow easy cleaning and provide corrosion protection, especially if humid air is

required for incubation. Although the requirement for aseptic conditions in a cell culture incubator is not as stringent as that in a cell culture hood, frequent cleaning of the incubator is essential to avoid contamination of cell cultures.

1.3.4.1 Types of Incubators

There are two basic types of incubators, dry incubators and humid CO_2 incubators. Dry incubators are more economical, but require the cell cultures to be incubated in sealed flasks to prevent evaporation. Placing a water dish in a dry incubator can provide some humidity, but this does not allow precise control of atmospheric conditions in the incubator. Humid CO_2 incubators are more expensive, but allow superior control of culture conditions. They can be used to incubate cells cultured in Petri dishes or multiwell plates, which require a controlled atmosphere of high humidity and increased CO_2 tension.

1.4 Storage

A cell culture laboratory should have storage areas for liquids such as media and reagents, for chemicals such as drugs and antibiotics, for consumables such as disposable pipettes, culture vessels, and gloves, for glassware such as media bottles and glass pipettes, for specialized equipment, and for tissues and cells.

Glassware, plastics, and specialized equipment can be stored at ambient temperature on shelves and in drawers; however, it is important to store all media, reagents, and chemicals according to the instructions on the label.

Some media, reagents, and chemicals are sensitive to light; while their normal laboratory use under lighted conditions is tolerated, they should be stored in the dark or wrapped in aluminum foil when not in use.

1.4.1 Refrigerators

For small cell culture laboratories, a domestic refrigerator (preferably one without an autodefrost freezer) is an adequate and inexpensive piece of equipment for storing reagents and media at 2–8 °C. For larger laboratories, a cold room restricted to cell

culture is more appropriate. Make sure that the refrigerator or cold room is cleaned regularly to avoid contamination.

1.4.2 Freezers

Most cell culture reagents can be stored at −5 °C to −20 °C; therefore, an ultra-deep freezer (i.e., a −80 °C freezer) is optional for storing most reagents. A domestic freezer is a cheaper alternative to a laboratory freezer. While most reagents can withstand temperature oscillations in an autodefrost (i.e., self-thawing) freezer, some reagents such as antibiotics and enzymes should be stored in a freezer that does not autodefrost.

1.4.3 Cryogenic Storage

Cell lines in continuous culture are likely to suffer from genetic instability as their passage number increases; therefore, it is essential to prepare working stocks of the cells and preserve them in cryogenic storage. Do not store cells in −20 °C or −80 °C freezers, because their viability decreases when they are stored at these temperatures.

There are two main types of liquid nitrogen storage systems, vapor phase and liquid phase, which come as wide-necked or narrow-necked storage containers. Vapor-phase systems minimize the risk of explosion with cryostorage tubes, and are required for storing biohazardous materials, while liquid-phase systems usually have longer static holding times and are therefore more economical.

1.5 Contamination

1.5.1 Biological Contamination

Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories, sometimes with very serious consequences. Cell culture contaminants can be divided into two main categories: chemical contaminants, such as impurities in media, sera, and water, including endotoxins, plasticizers, and detergents, and biological contaminants, such

as bacteria, molds, yeasts, viruses, mycoplasma, as well as cross-contamination by other cell lines. While it is impossible to eliminate contamination entirely, it is possible to reduce its frequency and seriousness by gaining a thorough understanding of sources and following good aseptic technique.

1.5.2 Cross-Contamination

While not as common as microbial contamination, extensive cross-contamination of many cell lines with HeLa and other fast-growing cell lines is a clearly established problem with serious consequences. Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique will help you avoid cross-contamination. DNA fingerprinting, karyotype analysis, and isotype analysis can confirm the presence or absence of cross-contamination in your cell cultures.

1.5.3 Using Antibiotics

Antibiotics should never be used routinely in cell culture, because their continuous use encourages the development of antibiotic-resistant strains and allows low-level contamination to persist, which can develop into full-scale contamination once the antibiotic is removed from media, and may hide mycoplasma infections and other cryptic contaminants. Further, some antibiotics might cross-react with the cells and interfere with the cellular processes under investigation.

Antibiotics should only be used as a last resort and only for short-term applications, and they should be removed from the culture as soon as possible. If they are used in the long term, antibiotic-free cultures should be maintained in parallel as a control for cryptic infections.

1.6 Pluripotent Stem Cells

Pluripotent stem cells (PSC) can divide indefinitely, self-renew and can differentiate and functionally reconstitute into almost any cell in the normal developmental pathway, given the right conditions. There are several kinds of pluripotent stem cells.