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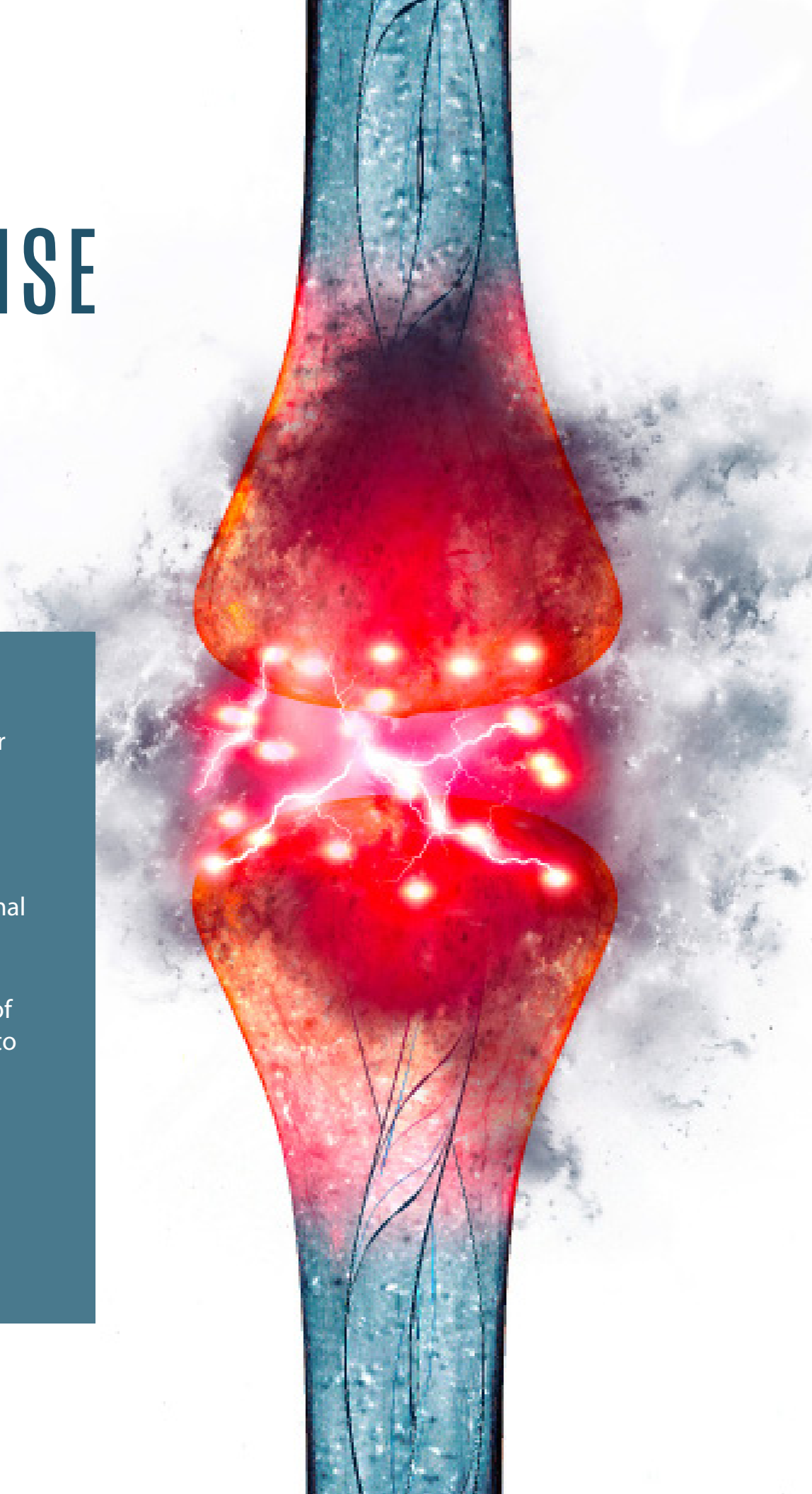
LIFE SCIENCE RESOURCES AND APPLICATIONS



Featuring

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New Uses for LAMP

(Loop-Mediated Isothermal Amplification)

Abstract

One of the newest methods of nucleic acid amplification, loop-mediated isothermal amplification (LAMP), is a quickly-evolving alternative to PCR that is simpler to perform and has great potential to provide quick point-of-care diagnostics and screening for multiple diseases. This adaptable technique often uses both LAMP-optimized reagents as well as the usual materials required for PCR procedures but without the need for a thermal cycler.

What is LAMP, and Why is it Important?

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method, similar to polymerase chain reaction (PCR). Both procedures work under isothermal conditions and have been used for multiple applications. LAMP methods are currently well-established, and are used across medicinal, agricultural, and food industries due to its simplicity, specificity, rapidity, and low-cost. These techniques are well-suited for point-of-care (POC) and field diagnostics, and assays have been designed for the detection of a wide range of RNA and DNA targets from all kinds of sample types (including feces, mucus, and saliva).

When compared, PCR has many limiting factors when it comes to field testing that LAMP can overcome. For example, a DNA extraction and purification step is required in PCR, making it difficult to perform at the bedside. LAMP also exhibits less sensitivity to inhibitory substances in biological samples when compared to PCR; this robustness makes LAMP, overall, a less expensive and more rapid technique. Not only is the cost of LAMP considerably lower than other molecular tests, results of a LAMP reaction can be analyzed by the naked eye, without the use of specialized equipment. LAMP experiments also do not require the use of a thermal cycler, raising its potential for use in low-resource settings.

LAMP vs. PCR

Basis of Differentiation	LAMP	PCR
Temperature requirements	Is an isothermal process, reaction occurs over a single consistent temperature of 60°C to 65°C throughout	Requires numerous cycles of heating and cooling - reaction occurs over varying temperatures
Number of primers used	Uses 4 to 6 primers to recognize 6 to 8 distinct regions	Uses two primers to recognize 2 regions
Reaction time	Rapid reaction, takes less than thirty minutes	Slow reactions, typically takes over one hour
Sensitivity to sample matrix inhibitors	More tolerant towards sample matrix inhibitors	Highly sensitive to sample matrix inhibitors
Visual detection	Amenable to visual detection based on certain factors such as turbidity among others	Not amenable to visual detection
Typical yield	Approx. 10 mg-20 mg	Approx. 0.2 mg
Equipment needed	Equipment is simpler	Equipment is more complex to accommodate the recurring heating and cooling cycles

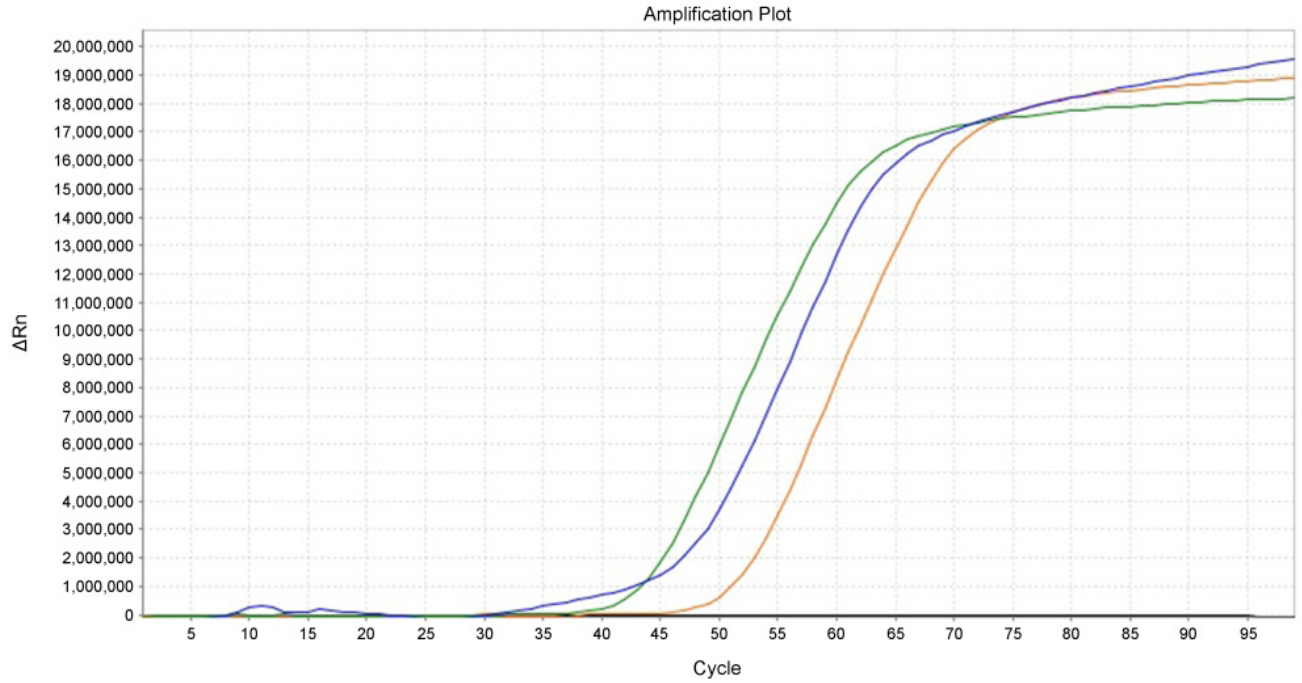

Figure 1. LAMP detection of BRCA1 in HeLa cells. 500 ng (Green), 50 ng (Blue), 5 ng (Orange), and NTC (Black) of gDNA in HeLa cells was used in LAMP reaction with LAMP Green™ fluorescent dye using ABI 7500 qPCR machine.

How Does LAMP Work, and What's a Typical Protocol?

LAMP uses 2-3 sets of specifically designed inner and outer primers (4-6 in total) to recognize 6-8 distinct regions of target DNA or RNA. These unique primers include two inner primers (FIP and BIP), two outer primers (F3 and B3), and/or two loop primers (LF and LB); each set contains one forward and one backward primer that bind to the antisense strand or sense strand of the target DNA, respectively. LAMP relies on the autocycling characteristics of DNA synthesis, performed by DNA polymerase, which maintains a high strand displacement activity. In the initial steps of LAMP all four primers are used, however, in later stages only DNA synthesis and the inner primers are needed to facilitate amplification through loop extension and primer annealing.

In a standard experiment, LAMP is carried out under isothermal conditions (around 65°C) and usually takes an hour to complete, from start to finish. First, the sample is thermally lysed to release DNA and/or RNA. The primer mix is then added to the reaction, containing FIP, BIP, F3, B3, LF and LB as well as DNA polymerase (typically, Bst DNA polymerase). In the case of reverse transcription-LAMP (RT-LAMP), a heat-stable reverse transcriptase enzyme also must be added to the LAMP mix to derive complementary DNA (cDNA) from RNA present. In conventional 1-step LAMP, all components can be added at once in the same reaction tube. The reaction is allowed to amplify at 65°C for one hour, and then the reaction mix can be denatured through heat and rapidly cooled on ice. The resulting DNA products are formed from repeats of the target sequence, and are extremely long (typically, over 20 kb), connected with single-stranded loop regions in long concatemers. Unfortunately, the amplified products are not usually appropriate for downstream manipulation, but the resulting amplification reaction is substantial enough that multiple modes of detection are possible.

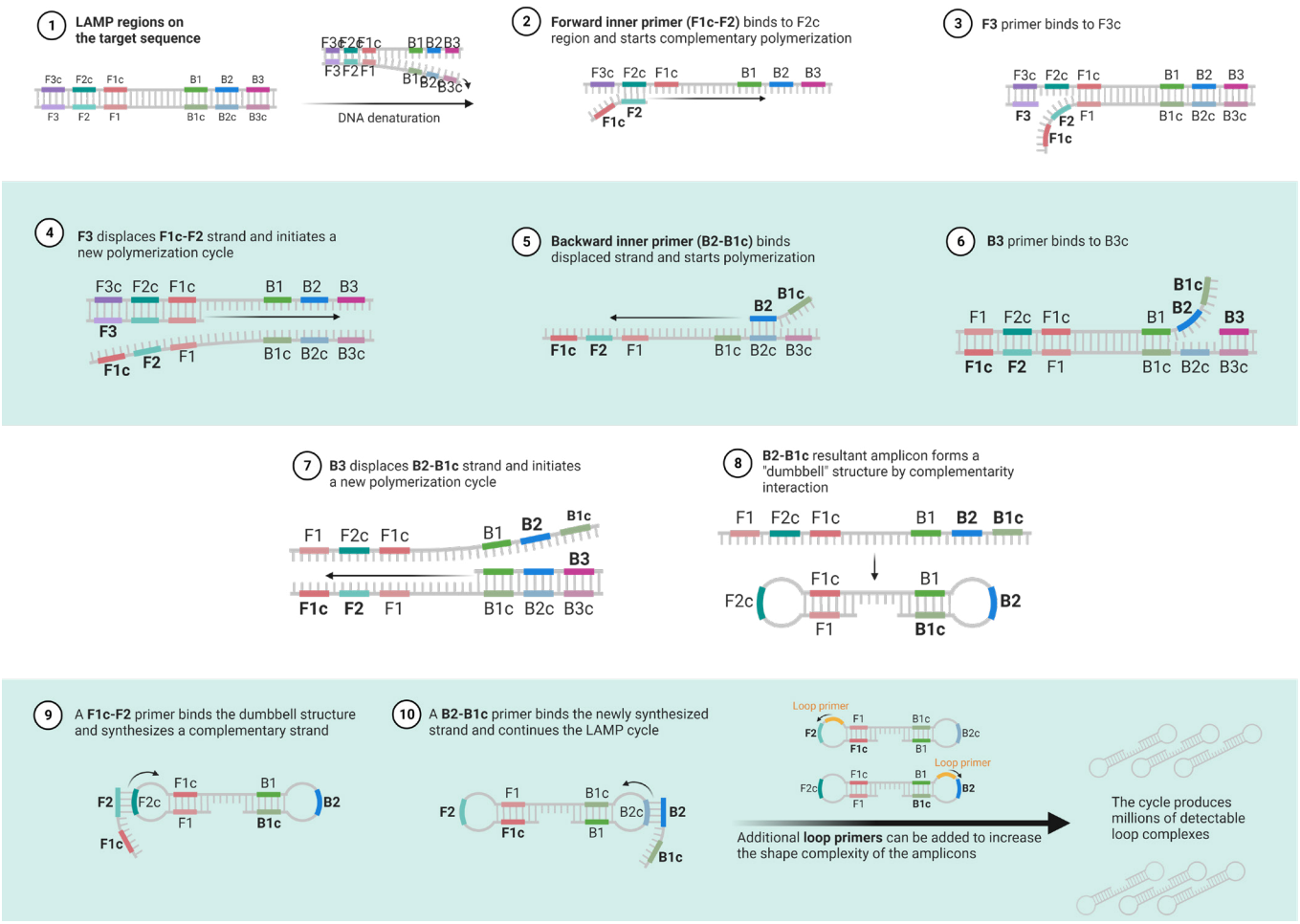

Figure 2. Illustrated principle of Loop-Mediated Isothermal Amplification (LAMP), from base target sequence to generation of quantifiable loop complexes produced by targeted primers and polymerization steps. Illustration made using Biorender.

How Can Amplified Products From LAMP be Visualized?

The detection of DNA from a LAMP reaction has been visualized using one of three methods, including agarose gel electrophoresis. Generally, 5 µL of LAMP products can be clearly visualized under UV light after electrophoresis in appropriate experimental conditions. Gel staining can also be carried out using different intercalating dyes, though regardless of dye used for the visualization, the amount of amplified product is represented by a ladder-like pattern on the gel. The laddered-pattern is due to nonuniformity in the size of the amplified products; sequentially inverted repeats of the target sequence tend to create many inconsistently sized, stemmed and looped DNA.

LAMP reaction products may also be viewed using fluorescence. In this technique, fluorescent dyes like ethidium bromide (EtBr), cyanine dyes (eg., SYBR Green I), or propidium iodide (PI) are also added to the reaction mixture. Amplification analysis can be performed through simple observation without opening the reaction tubes, decreasing the risk of contamination. Normally, a color change indicating the presence of the amplified product can easily be seen, negating the use for any fluorescent-detecting equipment. Moreover, fluorescent dyes normally do not affect the activity of other elements, like enzymes, in the reaction mixture. This method of LAMP analysis

includes using turbidity, through ion indicators, as a means of detecting amplified products. This can be accomplished as a major by-product of target amplification is magnesium pyrophosphate, which exhibits as a white precipitate, and can be measured using a turbidimeter or observed by the naked eye.

Historically, visualization methods have been end-based, meaning that determination of the presence of an amplicon is known at the end of an experiment. These techniques are all semi-quantitative, so deciphering the true abundance of an amplicon present in a sample is not always accurate. Further advances and developments in LAMP techniques have led to the creation of real-time technologies that now allow researchers to identify the load of an amplicon in a DNA sample, in real time. Such progress in fluorescent dye and ion indicator methods in particular have allowed for a more accurate quantification of amplified products thereby potentially providing vital information into the viral, parasitic, or even bacterial load on a sample, or more precisely an individual.

How Can LAMP be Used in Diagnostics?

LAMP diagnostic methods have been researched for detecting and diagnosing viral pathogens in humans including for human immunodeficiency virus (HIV), chikungunya virus, mumps, west Nile virus, Zika virus, human papillomavirus, dengue virus, MERS, H5N1 virus, SARS and SARS-CoV-2. LAMP is a significant assay in viral diagnosis because reverse transcription polymerase chain reaction (RT-qPCR) assays are not always equipped to satisfy the current demands of testing large numbers of persons during a viral outbreak or pandemic. Such viruses, if symptoms go untreated, could develop into more serious diseases for example in cervical cancer (from human papillomavirus), AIDS (from HIV), and lung function abnormalities (from MERS and SARS). This means there exists an urgent demand for a rapid, simple and sensitive POC assay that could be used at airports, public transit stations and hospitals, especially in rural areas, to expedite faster detection

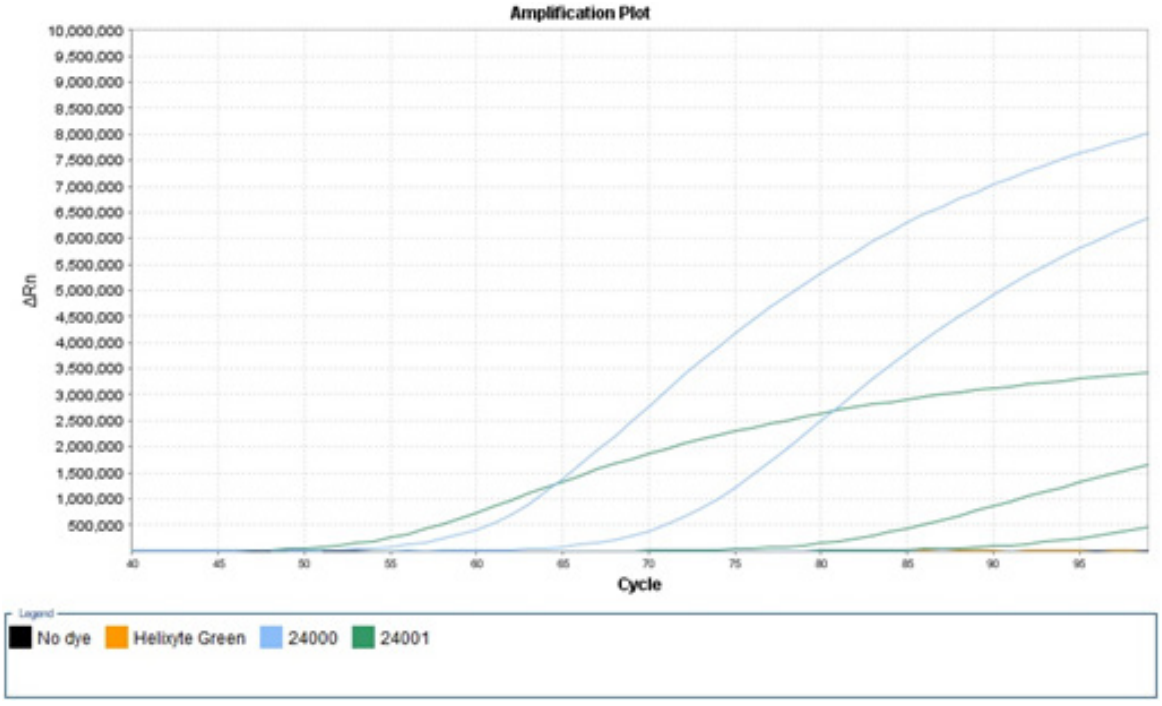

Figure 3. In a 96 well qPCR plate, HeLa cells gDNA (100, 10, 1, 0 ng) were mixed with IsoFAST buffer, BRCA primers mix, 5 mM MgCl2, IsoFAST enzymes, 1 μM DNA dyes (MycoLight™ Green JJ98 (#24000), MycoLight™ Green JJ99 (#24001), Helixyte™ Green (#17592)) and plates were incubated in Applied Biosystems® 7500 FAST Real-Time PCR System at 65 oC (100 cycles for 100 min) for real time detection of fluorescence. No dye negative control (NC): all the component except dyes.

of viral pathogens and help reduce or prevent spread.

Though LAMP has been used in research to diagnose malaria, LAMP may also be crucial for the diagnosis of other parasitic diseases. The World Health Organisation (WHO) has categorized twenty diseases into a group known as neglected tropical diseases (NTDs), twelve of which are parasitic by nature and are all contagious. These include American trypanosomiasis or Chagas disease, dracunculiasis or Guinea-worm disease, echinococcosis, foodborne trematodiases, human African trypanosomiasis or sleeping sickness, leishmaniasis, lymphatic filariasis or elephantiasis, onchocerciasis or river blindness, schistosomiasis or snail fever, soil-transmitted helminthiases, taeniasis and cysticercosis. Such parasitic diseases affect hundreds of millions of people in developing countries yearly where control is commonly diagnosis-based. As the most affected areas are usually far from laboratories with suitable infrastructure and/or sophisticated equipment, LAMP techniques could therefore significantly help parasite infection control and eradication programs.

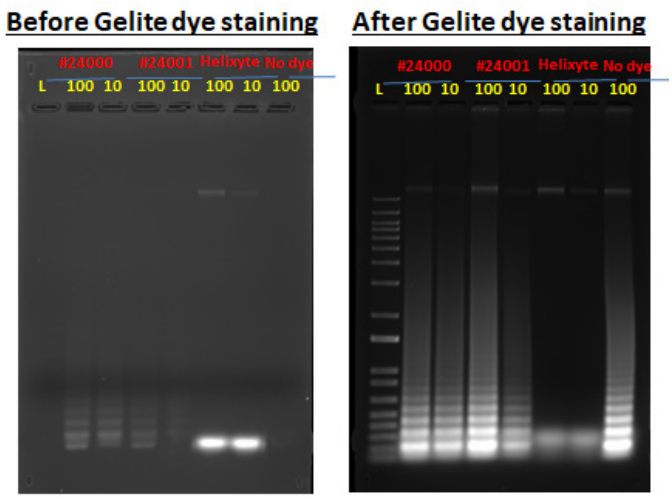

Figure 4. Same experiment, verified via gel electrophoresis run in 1% agarose gel, before and after Gelite™ Safe DNA Gel staining. Fluorescence intensity were increased over time in the presence of 1 μM of #24000 and #24001 dye, but not in Helixyte dye. Real time LAMP products were detected in the presence of 1 μM MycoLight dye (#24000,#24001). No amplified products were formed in the presence of 1 uM of Helixyte dye, indicating the Helixyte dye has an inhibitory effect on LAMP reaction. Being able to measure success, failure, and the relative rates of any LAMP reactions that do occur are essential for optimized experimental development.

Another significant concern related to these diseases is that parasites, once considered endemically stable, are now moving. For example, it has been reported recently that Schistosoma larvae, the cause of schistosomiasis and the second most devastating parasitic disease next to malaria, are infecting people in Europe. The movement of parasites across borders has been linked to, among other things, extreme population growth, urbanization, habitat loss, and seasonal migration. Additionally, climate change and global warming are increasing the dissemination of many vector-transmitted diseases, increasing the likelihood of parasitic outbreaks outside of only rural areas.

LAMP techniques also have the potential to be used in the food industry for testing of bacterial pathogens and fungal contaminants. Numerous outbreaks of bacterial pathogens have be linked to the consumption of food worldwide, and most common bacterial pathogens include *Escherichia coli, Staphylococcus aureus, Salmonella, Yersinia enterocolitica, Shigella, Brucella, Campylobacter jejuni, Clostridium, and Cronobacter sakazaki.* LAMP may play a significant role in bacterial detection to ensure the safety of food, and to prevent and control food-borne diseases.

Because LAMP is highly efficient, accurate, and sensitive even at low levels of burden, it has the potential to be used to prevent pathogenic transmission through the food chain at every step in the industry, from food production and processing, to marketing and surveillance. LAMP can also be used for bacterial disease diagnosis, as in the detection of meningitis that affects millions yearly, where clinical features alone may not always determine disease presence. In various studies, LAMP has successfully diagnosed meningitis from cerebrospinal fluid samples through detecting the key disease-causing bacteria; *Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis.* Still, the lack of available diagnostic tests in developing countries for bacterial diseases has been highly associated with widespread empiric antibiotic treatment and rising antibiotic resistance. There is an urgent need for effective, bacterial, diagnostic tests, like LAMP, that can help ensure global access to care in both developing and developed countries.

How is LAMP Evolving?

Over the years LAMP technologies continue to improve and integrate with other techniques to create simpler and more quantifiable diagnostic and detection systems. As LAMP continues to evolve, the potential ability and diversity of this technique increases. For example, in one study multiplex LAMP was created that was based on combining two sets of LAMP primers in tandem. A restriction enzyme cleavage site was inserted into the two pairs of each specific primers. This method allowed researchers to distinguish different pathogens simultaneously, using subsequent restriction enzyme analysis (Iseki et al., 2007).

Another variation includes digital LAMP, which was developed using a sample self-digitization chip. Digital LAMP demonstrated an on-chip, loop-mediated, DNA amplification technique that could detect relative changes in template concentration as well as absolute quantification of template numbers (Gansen et al., 2012). LAMP techniques have also been combined with lateral flow assays (LFA), based on DNA hybridization and antigen-antibody reactions. These assays are similar in sensitivity to the conventional agarose gel electrophoresis, but offer a higher level of simplicity, faster testing times, and do not require specialized equipment.

Another system, Lab-on-a-Disc (LoaD) LAMP, was developed that consisted of a fully integrated compact device for the automated detection and quantification of plant pathogens. The construct was a disposable cartridge disc which rotated to generate a centrifugal field. The pressure head pumped the sample from the center to the periphery of the disc, which was then processed, purified, and mixed with LAMP reagents. This instrument also incorporated modules for heating and fluorescent detection (Kinahan et al., 2018).

In another variation of the technique, lyophilized LAMP, all reagents can be combined into a single mixture in a closed amplification and detection system. Not only does this greatly speed up the assay, the user only needs to add water and the sample, or a DNA or RNA template, into the lyophilized mix before incubating. Many lyophilized LAMP kits are commercially available and are commonly associated with portable thermal cyclers that can be connected to a smartphone or tablet for viewing data in real time.

Research has also been done to integrate fluorescence resonance energy transfer (FRET)-based probes with RT-LAMP. These probes were labeled oligonucleotides that operated like a molecular zipper and allowed the quenching strand to displace from a partially complementary fluorescent strand during DNA synthesis. While the targeted double strand region was opened, its fluorophore emitted a fluorescent signal. In this method, pathogenic detection and quantification were performed in real time (Kubota et al., 2011). To date, there are several portable devices based on this technology commercially available.

Resources

Digital Catalog Page:

- Loop-Mediated Isothermal Amplification (LAMP)

Related AssayWise Letters:

- Low-cost, ultra-sensitive fluorescence detection of DNA by gel electrophoresis using environmentally benign Gelite™ Safe DNA Gel Stain
- Exploration of SYTO® 9 Variabilities of Labeling Live Bacterial Cells and Analysis of MycoLight™ Fluorophore Alternatives for Live Bacterial Labeling and Viability Assessment

Tools:

- qPCR Efficiency Calculator
- Gel Electrophoresis Annotator
- DNA Molecular Weight Calculator
- DNA Concentration Calculator

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Product	Unit Size	Cat No.
LAMP Green™ *50X DMSO Solution*	100 uL	17555
Gelite™ Green Nucleic Acid Gel Staining Kit	1 Kit	17589
Gelite™ Orange Nucleic Acid Gel Staining Kit	1 Kit	17594
Helixyte™ Green dsDNA Quantifying Reagent *200X DMSO Solution*	1 mL	17597
Helixyte™ Green dsDNA Quantifying Reagent *200X DMSO Solution*	10 mL	17598
Helixyte™ Green Fluorimetric dsDNA Quantitation Kit *Optimized for Broad Dynamic Range*	200 Tests	17645
Helixyte™ Green Fluorimetric dsDNA Quantitation Kit *Optimized for Broad Dynamic Range*	1000 Tests	17646
Helixyte™ Green Fluorimetric dsDNA Quantitation Kit *Optimized for Microplate Readers*	200 Tests	17650
Helixyte™ Green Fluorimetric dsDNA Quantitation Kit *High Sensitivity*	200 Tests	17651
Portelite™ Fluorimetric High Sensitivity DNA Quantitation Kit *Optimized for CytoCite™ and Qubit™ Fluorometers*	100 Tests	17660
Portelite™ Fluorimetric High Sensitivity DNA Quantitation Kit *Optimized for CytoCite™ and Qubit™ Fluorometers*	500 Tests	17661

Investigating Intercellular Channels

Focus on Gap Junctions

Abstract

One of the main three types of intercellular junctions, gap junctions are crucial for several cell communication pathways, and any dysregulation can have immediate and lethal effects. They have been implicated in tumor development and the inflammatory response in general, as well as standard cellular regulation. Given this importance, being able to investigate gap junction activity is useful for several fields of study. Using fluorescent labels is one of the simpler ways to experimentally determine gap junction activity within a cell population. Fluorescent labels can be adapted and optimized for various cell lines, experimental setups, and instrument platforms. Either a flow cytometer or fluorescence microscope can be used to analyze gap junction activity.

Overview of Intracellular Channels

There are many varieties of intercellular junctions, which are expressed to a greater or lesser extent depending on cell type. Three of the main categories of these, present on most epithelial and endothelial cells, are tight junctions, adherens junctions, and gap junctions. They are differentiated by both structure and function.

Tight junctions act as gates between cell surfaces, and are extremely dependent on cell polarity. Depending on subtype, they can be composed of varying proteins, such as claudin or occludin. Anchored within and through the plasma membrane, tight junctions do not directly transfer material between cells, but they are essential for protein signaling and intercellular crosstalk via protein-protein interactions. They also help regulate ion and other small molecular transfer between cells that provide a basis for cellular signaling. These signals control not only standard cell behavior such as migration, but also lifecycle actions like cell proliferation, differentiation, and death.

Adherens junctions are similar to tight junctions, as they are both anchored within the plasma membrane and transmit signals but not materials between cells. Adherens junctions are made primarily of E-cadherin transmembrane protein, and provide not only adhesive contacts for epithelial cells, but can bind to intracellular catenins. This facilitates the transfer of signals to organize the cytoskeletal structure as well as intracellular regulation of activities as important as gene transcription.

Gap junctions in particular are unique in that they are made of two interdocked cylinders made of connexin. Six of these molecules individually make up what is known as a hemichannel, which span the plasma membrane of each cell, facilitating easy transfer of small molecules when they meet and interlock to form a complete intercellular channel.

Unique Functionalities of Gap Junctions

Gap junctions are specialized intercellular membrane channels constituted with connexin that selectively facilitate the passage of small molecules of <1.2 kD across cells. They are tightly regulated by voltage, growth factors, cAMP, and retinoids, and they are modulated by phosphorylation. During cellular membrane depolarization (>+20mV) the channels will be

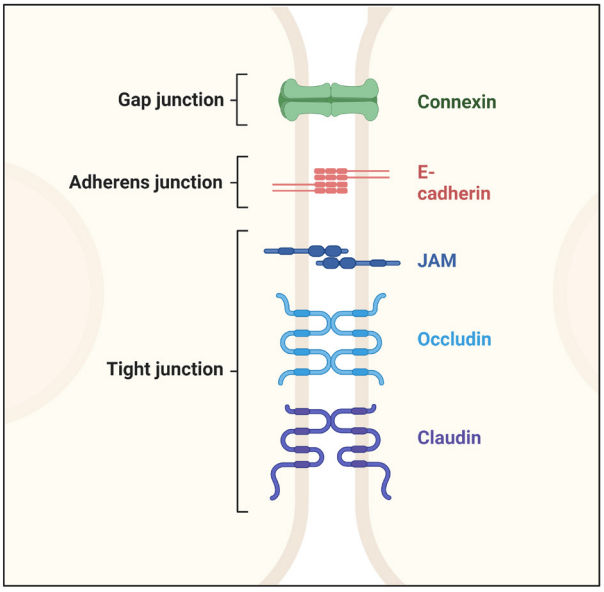


Figure 1. Common types of intercellular junctions, along with their primary composition material. Figure made in BioRender.

open, but closed otherwise. Gap junctions have been implicated in inflammatory diseases and tumor behavior, and severe dysfunction results in cell death and multiple problems with the organism as a whole.

Since gap junctions transfer so many important molecular types (including second messengers as well as more typical yet essential ions) they are central in multiple regulatory cellular communication pathways that regulate cellular and tissue homeostasis. This importance is broadly termed gap junctional intercellular communication (GJIC) and has implications up to and including organ health as well as cell behavior and maintenance.

Principles of Gap Junction Analysis via Fluorescent Labeling

Simplicity is the key to experimental setup. The cell population is divided evenly, with one population incubated with a low-toxicity cytoplasmic dye. This dye will be contained within those living cells. The other population will be stained with a plasma membrane dye in an alternative color. A wide spectral separation between the colors is ideal, to minimize signal overlap. After washing and mixing, any of a variety of experimental stimuli can be applied (or simply a period of time will be permitted to pass) and the mixed population of cells can be either analyzed via flow cytometry or simply visualized with a fluorescence microscope.

The possible results can be grouped into four possibilities: double-positive cells showing both colors of dye are those that have exchanged cytoplasmic material via gap junction. Double-negative cells (no staining at all) are dead, and are a useful control group as well as a measure of the overall health of the population. Other populations will be stained only with the colors of the dyes they were incubated with, and these single-signal cells are then used to compare to the double-positive cells, with that proportion being a determination of gap junction activity.

Exemplifying this type of noninvasive gap junction investigation, the Cell Meter™ Fluorescence Gap Junction Tracing Kit provides a reliable and robust assay for the in vitro determination of gap junction function. The cell population under study is divided such that one fraction is loaded with a lipophilic cell plasma membrane permeable dye, Calcein UltraGreen™ AM, that is hydrolyzed upon cellular uptake by cytoplasmic esterases to yield Calcein UltraGreen, a highly fluorescent and well-retained and membrane-impermeable molecule.

Calceins are a fairly large family of dyes commonly used to determine cell viability and plasma membrane integrity. As a nearly neutral substrate, the am esters of these dyes can diffuse freely and noninvasively across the plasma membrane and permeate the cytosol of live cells. Once inside, cytosolic esterases in metabolically active cells rapidly hydrolyze the non-fluorescent calcein AM substrates into calcein products retained by cells with intact plasma membranes. Since dead cells lack esterase activity, only viable cells are labeled. Compared to other live-cell labeling reagents, such as BCECF AM or carboxyfluorescein diacetate, the brighter fluorescence, photostability, and low cytotoxicity of calcein AM is advantageous for a variety of studies, including cell tracking, cell adhesion, chemotaxis,multidrug resistance, cell viability, apoptosis, and cytotoxicity. Many colors are available, although the green spectra is the most commonly employed, for this kit as well as for other similar ones.

The other fraction is loaded DiD, which is a lipophilic membrane dye that diffuses laterally to stain the entire cell membrane in deep red fluorescence upon incorporation into membranes. DiI, DiO, DiD and DiR dyes are a family of lipophilic fluorescent stains for labeling membranes and other hydrophobic structures. The fluorescence of these environment-sensitive dyes is greatly enhanced when incorporated into membranes or bound to lipophilic biomolecules such as proteins although they are weakly fluorescent in water. They have high extinction coefficients, polarity-dependent fluorescence and short excited-state lifetimes. Once applied to cells, these dyes diffuse laterally within the cellular plasma membranes, resulting in even staining of the entire cell at their optimal concentrations. Among them DiD is well excited by the 633 nm He-Ne laser, and has much longer excitation and emission wavelengths than those of DiI, providing a valuable alternative for labeling cells and tissues that have significant intrinsic fluorescence.

Once the fractions have been loaded sufficiently according to the experimental cell type, the two fractions are mixed and incubated under coculture conditions. Calcein UltraGreen is transferred to the DiD-stained cells through gap junctions. The assessment of this uptake can be monitored by fluorescence imaging or flow cytometry.

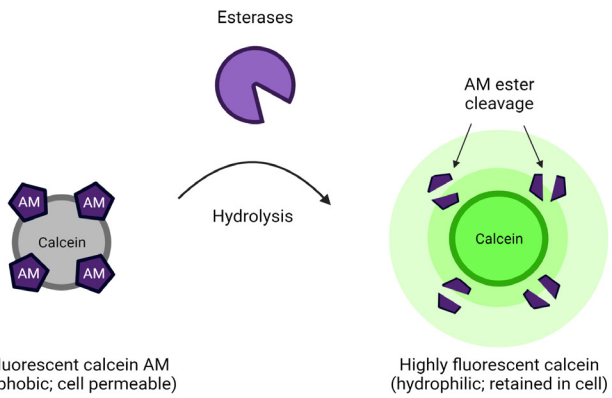


Figure 2. Calcein AM hydrolysis. Non-specific intracellular esterase cleavage of AM ester groups converts calcein AM into highly fluorescent calcein.

Sample Protocols

The following example protocols can be used as a guideline and should be optimized according to experimental needs.

Cell staining protocol for plasma membrane dye (Calcein Ultragreen AM)

1. Grow cells in cell culture medium in 6-well cell culture plates.
2. Remove the cell culture medium and add 0.5 mL of Calcein Ultragreen AM working solution.
3. Incubate cells at 37 °C for 10-20 minutes. Note Incubation time should be optimized for each cell line.
4. Remove the dye working solution and wash cells with GAP Junction Assay buffer.

Note: For the adherent cells, detach cells from the plate using rubber policeman.

5. Resuspend cells in cell culture medium

Note: The nonionic detergent Pluronic® F-127 can be used to increase the aqueous solubility of AM esters. Avoid long-term storage of AM esters in the presence of Pluronic® F-127.

Cell labeling protocol for lipophilic membrane dye (DiD)

1. Grow cells in cell culture medium in 6-well cell culture plates.
2. Remove the cell culture medium and add 0.5 mL of DiD working solution.
3. Incubate cells at 37 °C for 10-20 minutes. Note Incubation time should be optimized for each cell line.
4. Remove the dye working solution and wash cells with GAP Junction Assay Buffer.

Note: For the adherent cells, detach cells from the plate using rubber policeman.

5. Resuspend cells in cell culture medium.

GAP junction assay

1. Mix Calcein stained cells and DiD labelled cells with 1:1 ratio and plate them in wells.
   - For fluorescence microscopy, add 50 µL of each into the well of a 96-well plate and mix well.
   - For flow cytometry, add 500 µL of each into the well of a 6-well plate and mix well.
2. Incubate cells at 37 °C for 2-3 hours.
3. Assess and analyze the cells using either a flow cytometer or fluorescence microscope.

Details of Flow Cytometry Analysis

For flow cytometric analysis, ensure that the machine is fully cleaned and calibrated prior to beginning the procedure.

Note: For the adherent cells, detach cells from the plate using rubber policeman.

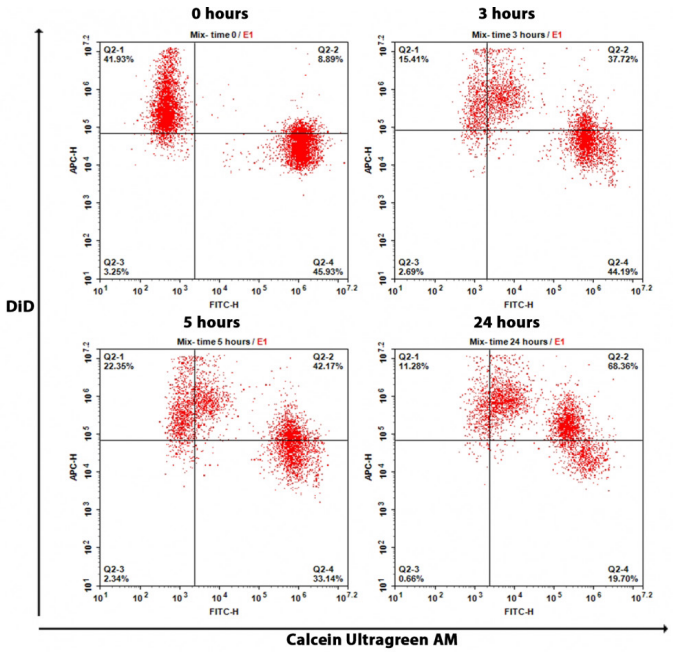


Figure 3. GAP junctions analyzed by flow cytometry. HeLa cells were stained with Calcein Ultragreen AM and DiD separately as per the protocol. Cells were mixed well with 1:1 ratio and replated with cell culture medium. Response was measured using NovoCyte flow cytometer (ACEA Biosciences) with FITC and APC channel. As time progress, Q2-2 population (double positive population) increases.

Once cells are in suspension or for cells in suspension, wash cells twice with DPBS or buffer of your choice. Resuspend cells in HHBS or DPBS or buffer of your choice and measure the response with 530/30 nm filter (FITC channel) and 660/20 nm filter (APC channel).

The success of the procedure will dictate how the data should be gated. Clear groupings indicate an ideal experiment, but a lack of clearly separated populations strongly implies one or more issues. As is typical for most cell activity experiments, use negative and positive controls to calibrate and discern meaningful data.

Details of Fluorescence Visualization

Monitor the cells with a fluorescence microscope using the FITC and Cy5 filter sets. In most cases, standard settings will be sufficient, but optimize as needed.

Most gap junction assays, including the one used here to demonstrate, have an incubation timeframe that has been tested and optimized to provide enough time for cells to begin displaying double positive signal (cells that will generate both green and red fluorescence) due to cytoplasmic interchange via gap junctions. Cell populations are likely to continue to increasingly show this double positive trend as time goes on, although cell type and experimental design will affect when this tendency will plateau and/or begin to decrease. The image above shows images at both 3 and 5 hours after initial cocultured incubation, as an example.

Optimization and Troubleshooting Experimental Problems

Based on experimental need, either prepared kits such as demonstrated earlier in this article can be used, or the same principles can be applied on a case-by-case basis. When building a procedure in-house, each aspect must be tested and optimized. For either flow cytometry or fluorescence microscopy platforms, the **incubation time** and **dye concentrations** must be assessed for best results, and other platform-specific considerations may come into play as well as variation in

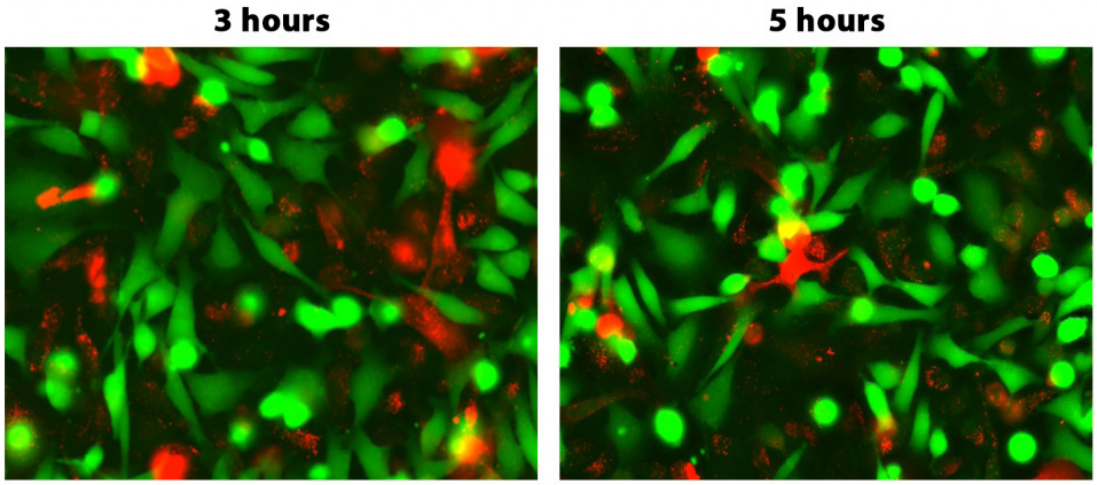
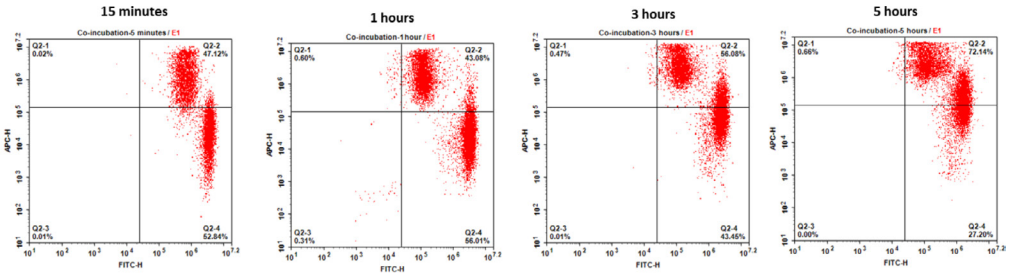
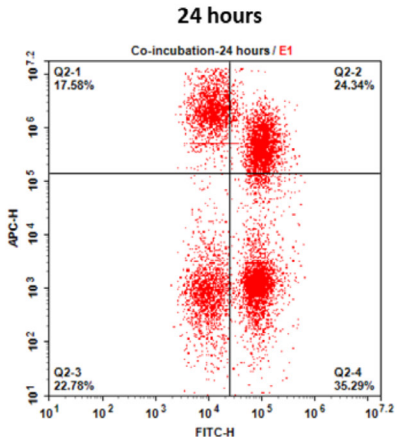


Figure 4. GAP junctions analyzed by fluorescence microscopy. HeLa cells were stained with Calcein Ultragreen AM and DiD separately as per the protocol. Cells were mixed well with 1:1 ratio and replated with cell culture medium. Images were acquired using a fluorescence microscope with the FITC and Cy5 filter sets. As time progresses, double positive population of Calcein Ultragreen AM and DiD increases.





Testing of HeLa cell behavior over time from 15 minutes onward, demonstrating increase of double-positive cell population up to the 5-hour mark, then stabilizing and decreasing until there is a smaller Q2-2 population at the 24-hour mark (shown on left)

Figure 5. Gap junction assay incubation time optimization testing in HeLa cells. Cells were washed with DPBS (No Ca+2 and Mg+2), then stained with Calcein AM (5 μM) and DiD (2.5 μM) for 10 minutes at 37C separately. Cells were washed with DPBS twice and mix it together and re plated with complete medium. Cells were collected at the indicated times, washed twice with DPBS, then resuspended in DPBS for flow cytometry procedure. Population of double-positive stained cells in quadrant 2 (Q2) increased as time increased up to the 5 hour mark, then steadied and began to decrease, until a net reduction of the Q2 population was shown at the 24 hour mark.

behavior based on cell lines.

Although upgraded reagents such as Calcein Ultragreen AM give better performance, cheaper classic options such as Calcein AM are still used frequently. In either case, cellular incubation time should be balanced between functionality and laboratory feasibility. For the image above, testing showed that any incubation longer than 5 hours provided little more data, and in fact less as time went on. This knowledge, despite the relatively low toxicity of the dyes, can inform not only procedure starting times (to permit adequate but not over-incubation) to achieve the best possible experimental results.

For determining ideal dye concentrations, the cells should be incubated with a range of molarities. Many kits and protocols will have recommended ranges, which are usually the best starting point. Even for dyes that have minimal cytotoxicity, high concentrations will frequently have deleterious effects to the health and proliferation rates of the cell population.

Note: For the adherent cells, detach cells from the plate using rubber policeman.

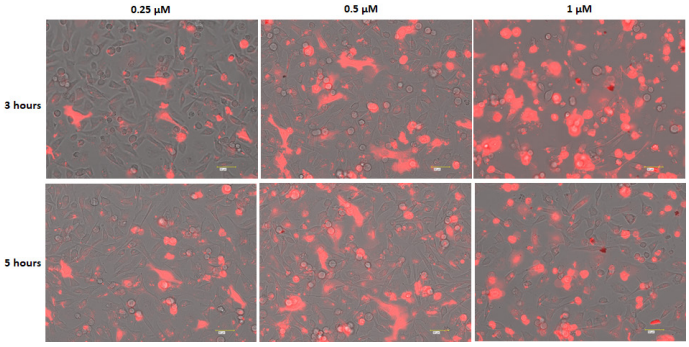


Figure 6. Gap junction assay DiD dye optimization. Cells were washed with DPBS (No Ca+2 and Mg+2). HeLa cells were stained with DiD at different concentrations (0.25, 0.5, and 1 μM) for 10 minutes at 37°C. Cells were washed with DPBS twice and re plated with complete medium on a 96- well plate. Images were acquired at indicated times and bright field (BF) and Cy5 images were overlapped. As is evident by the imaging, cells are healthy with both 0.25 and 0.5 μM concentrations, but significantly less so at 1μM.

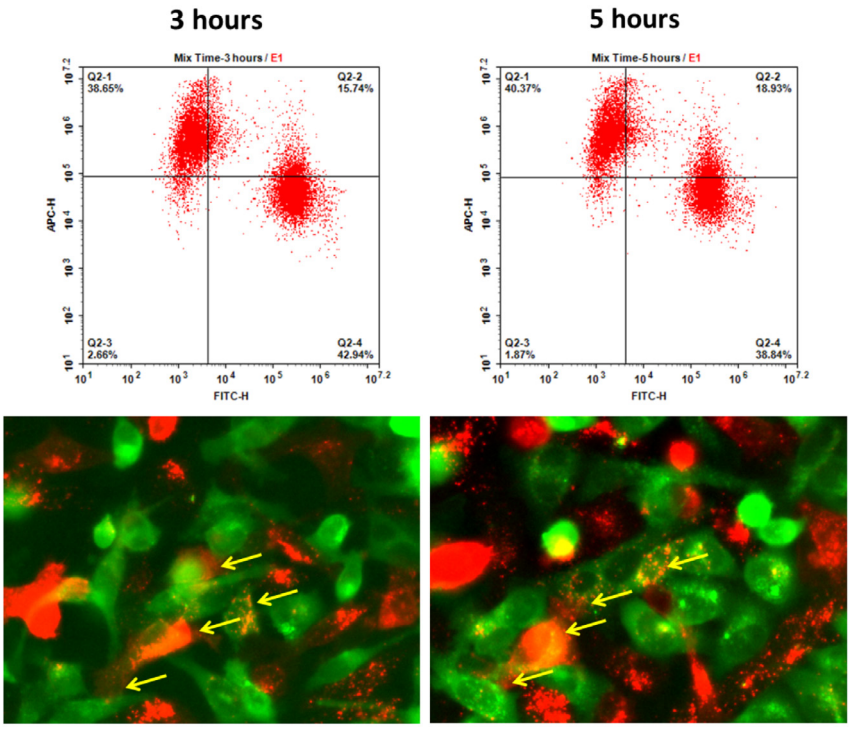


Figure 7. HeLa cells were washed with DPBS (No Ca+2 and Mg+2) and stained with Calcein Ultragreen AM (0.5 μM) and DiD (0.5 μM) for 10 minutes at 37°C separately. Cells were washed with DPBS twice and mix it together and re plated with complete medium. Cells were collected at indicated times, washed twice with DPBS, then resuspended in DPBS and flow cytometry was performed. Yellow arrows indicate double-positive tracked cells. Top row are flow cytometry analyses, and bottom row are fluorescence microscope images, taken at the listed incubation times.

Conclusion

Whether using a flow cytometer or a fluorescence microscope, visualizing and assessing gap junction activity has multiple possible applications. By using fluorescent labels, suitably optimized for a given procedure, cellular functions can be investigated on any platform. Gap junction analysis provides insight on many other aspects of cell health and behavior, and so tracking it is helpful in multiple fields of study, for a holistic understanding of intercellular communication.

Resources

Application Note:

- Calcein

Digital Catalog Pages:

- Calcein AM Viability Dyes and Assay Kits
- Cell Viability Assays

Selection Guides:

- Calcein

Webinar:

- Fundamentals of Flow Cytometry

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Product	Unit Size	Cat No.
Cell Meter™ Fluorescence Gap Junction Tracing Kit	100 Tests	23600

Unlocking the Potential of 3D Cell Culture

A Guide to Assay Optimization

Abstract

The exploration of three-dimensional (3D) cell cultures in contemporary biomedical research reveals their pivotal role as a bridge between conventional two-dimensional (2D) models and the intricacies of in vivo systems. It meticulously addresses the hurdles encountered during the transition to 3D cultures, emphasizing the imperative need for nuanced assay optimization strategies. Additionally, the integration of 3D cultures into high throughput screening is examined, alongside contemplations on the promising horizon of organ-on-a-chip and multi-tissue systems in the realm of future possibilities. Despite formidable challenges, 3D cultures offer an avenue to augment research depth and relevance within the domains of cellular research and drug discovery.

Introduction

Biomedical research has witnessed a remarkable transformation with the advent of cell culture techniques. The transition from traditional two-dimensional (2D) to three-dimensional (3D) cell culture systems marks a significant milestone in cellular studies. While 2D platforms have served as workhorses in research for decades, their limitations in replicating the complex in vivo environment have become increasingly apparent. The emergence of 3D cell cultures, closely mimicking physiological conditions, promises a more authentic representation of in vivo scenarios. However, this transition is not without its challenges, particularly when it comes to adapting and optimizing assays for 3D culture systems.

The Significance of 3D Cultures in Modern Biomedical Research

The shift from traditional 2D cell cultures to advanced 3D cell culture models represents a distinct leap forward in cell biology and tissue engineering. In 2D cultures, cells typically form monolayers on flat surfaces like culture dishes or flasks. While this approach is convenient and suitable for high-throughput screening, its limitations become evident when trying to replicate the intricate three-dimensional structures of tissues and organs. Consequently, 2D cultures fall short in faithfully mimicking in vivo conditions and studying complex, heterogeneous cell interactions.

Conversely, 3D cultures are designed to closely replicate the physiological environment. Cells within these cultures grow in three dimensions, often within a supportive matrix, allowing for realistic cell-cell and cell-matrix interactions. This unique feature makes 3D cultures invaluable for tissue-specific modeling, disease research, and drug testing, offering critical insights into how compounds behave within a tissue-like context. While their advantages are undeniable, it is crucial to acknowledge that 3D cultures can be more challenging to establish and require meticulous consideration of spatial factors. Nevertheless, they provide a more relevant platform for addressing research questions related to tissue development, drug efficacy, and disease mechanisms. Researchers must, therefore, choose between these two culture systems based on their specific research objectives and experimental requirements.

Navigating the Challenges of 3D Culture Assay Optimization

The transition from 2D to 3D cell cultures introduces a host of challenges. One particularly noteworthy challenge is the altered dynamics of diffusion within 3D environments. In 2D cultures, the diffusion of nutrients, gases, drugs, and assay reagents is relatively straightforward. However, in 3D cultures, the penetration of these substances becomes inherently more complex, leading to uneven gradients, especially concerning oxygen and essential nutrients. These uneven gradients can significantly impact cellular behavior and, consequently, assay outcomes.

Furthermore, the inherent depth of 3D structures can pose challenges when it comes to imaging clarity. Traditional microscopy techniques, optimized for observing shallow 2D layers, encounter difficulties when visualizing cells situated deeper within 3D structures. This can lead to potential misinterpretations or omissions in data acquisition, underscoring the importance of addressing these imaging challenges in the optimization of assays for 3D cell cultures.

Assay Optimization: Tackling the Challenges Head-On

The transition from a traditional 2D experimental setup to a more complex 3D environment represents a significant shift in the field of assay optimization. This shift is not merely a matter of adding an extra dimension but entails a complete reevaluation and redefinition of the entire experimental process. To successfully navigate this transition, tailored approaches to assay optimization become essential.

One critical aspect of this transition involves addressing challenges related to cell viability and proliferation assessment. In traditional 2D systems, commonly used colorimetric assays like the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay are relied upon to measure cell viability. However, in the context of 3D matrices, a new set of challenges arises. The formazan crystals produced during the MTT assay

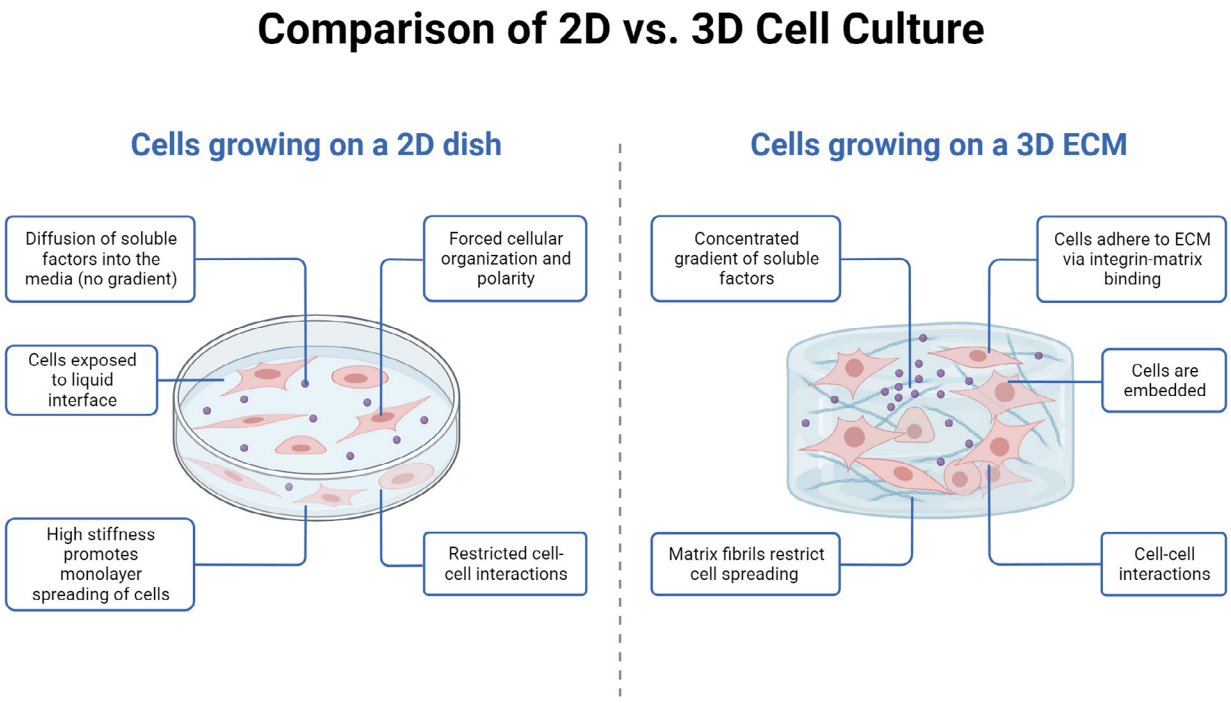

Figure 1. Comparison of 2D versus 3D cell culture models (figure made in BioRender).

may not solubilize effectively in the dense 3D environment. In response, ATP-based assays, such as the ReadiUse™ Rapid Luminometric ATP Assay Kit, the Cell Meter™ Live Cell ATP Assay kit, or the PhosphoWorks™ ATP Assays have emerged as a more suitable alternative, as they measure cellular ATP levels, offering increased sensitivity and the ability to penetrate deeper into the 3D cultures.

In drug studies and other research contexts, tracking cell apoptosis (programmed cell death) is of paramount importance. While conventional 2D assays for apoptosis often rely on colorimetric outputs, these may not provide optimal results within the intricate 3D matrix. Instead, assays utilizing fluorescence or luminescence have proven to be superior, delivering enhanced clarity and sensitivity, making them the preferred choice for 3D settings.

To further enhance our understanding of cellular behavior within 3D structures, researchers have turned to advanced imaging techniques. Among these techniques, confocal and multiphoton microscopy stand out due to their capacity to capture sequential z-stack images. When these images are reconstructed, they offer comprehensive 3D views, enabling researchers to gain deeper insights into cellular organization and behavior within the complex 3D environment.

Additionally, the choice of matrix material for 3D cultures plays a pivotal role in shaping cellular behavior. Whether using natural matrices like collagen or Matrigel or synthetic alternatives, the matrix composition significantly influences assay outcomes. Therefore, it is imperative to carefully fine-tune matrix components to strike the right balance, providing adequate support for cellular activities while avoiding unintended interference with assay results. This consideration underscores the multifaceted nature of assay optimization in the transition from 2D to 3D experimental setups, where researchers must address not only assay methods but also the intricacies of the matrix environment itself.

Scaling Up: 3D Cultures in High Throughput Screening

The realm of pharmaceuticals recognizes the immense promise held by 3D cultures, particularly in the context of drug screening. The inclusion of 3D models within high throughput screening (HTS) frameworks, however, necessitates a rigorous commitment to standardization. Even slight variations in critical parameters, such as spheroid size or seeding density, can

Table 1. Comparison of MTT and ATP-based assays commonly used in cell viability and proliferation studies.

Aspect	MTT Assay (2D)	MTT Assay (3D)	ATP-based Assay (2D)	ATP-based Assay (3D)
Principle	Measures mitochondrial activity (metabolic activity)	Measures mitochondrial activity (metabolic activity)	Measures ATP production (cell viability)	Measures ATP production (cell viability)
Advantages	• Widely used, well-established • Cost-effective • Simple protocol • Compatible with various cell types • Measures metabolic changes	• Applicable to some 3D cultures • Suitable for simple 3D models • Can capture metabolic changes • Provides insights into 3D-specific effects • Non-destructive to some 3D cultures	• Direct measurement of cell viability • Sensitive and rapid • Compatible with various cell types • Measures ATP production in real-time • Detects changes in real-time, indicating viability	• Direct measurement of cell viability • Suitable for some 3D models • Minimal interference from dead cells • Suitable for high-throughput screening • Provides insights into 3D-specific effects
Disadvantages	• Indirect measurement of viability • May yield false positives in some cases • Requires solubilization step for quantification • Requires solubilization step for quantification	• Limited applicability to complex 3D models • May require additional assays for accuracy • May underestimate viability in certain cases • Difficulties in uniform compound exposure	• Requires ATP extraction or lysis • Limited sensitivity in low ATP conditions • More expensive reagents • May not distinguish between viable and stressed cells	• Requires ATP extraction or lysis • Sensitivity to environmental factors • More expensive reagents • May not distinguish between viable and stressed cells

profoundly influence experimental outcomes. To navigate this terrain effectively, automation, particularly in the domains of imaging and data interpretation, must exhibit a high degree of proficiency in accommodating the unique intricacies inherent to 3D cultures.

To harness the potential of 3D cultures in pharmaceutical research, it is vital to establish standardized protocols. These protocols should encompass the formation of spheroids, the selection of suitable cell lines, and the optimization of culture conditions, including media composition, pH levels, and oxygen concentrations. Given that fluctuations in these parameters can significantly skew results, precision in control is paramount.

Automation plays a pivotal role in ensuring the reliability and consistency of 3D culture experiments. Liquid-handling robots, for instance, can meticulously control seeding density and spheroid size. Moreover, the imaging of 3D structures demands advanced technologies like confocal or multi-photon microscopy, and automating this process enhances efficiency in handling a substantial number of samples.

The challenge of interpreting data arising from 3D cultures is further accentuated by their three-dimensional nature. Specialized software and algorithms are imperative to accurately quantify parameters such as spheroid size, shape, and viability. Integrating this unique dataset into HTS workflows necessitates the development of databases and computational tools tailored to the specific demands of 3D assays, encompassing both imaging and molecular profiling data.

Incorporating high-content screening (HCS) systems is particularly beneficial when dealing with 3D cultures, as they furnish a comprehensive array of information extending beyond

Table 2. Critical parameters for high-throughput screening (HTS) in 3D cell culutres.

Parameter	Description
3D Culture Model Selection	Choose an appropriate 3D culture model (spheroids, organoids, hydrogels, etc.) based on your research objectives.
Cell Type and Source	Select relevant cell types and sources that mimic the target tissue or disease.
Culture Conditions	Optimize culture conditions including media composition, pH, oxygen levels, and temperature for 3D growth.
Assay Development	Develop assays compatible with 3D cultures, ensuring they provide meaningful readouts for your screening goals.
Automation	Implement automated liquid handling and robotics to streamline handling of 3D cultures and assays.
Plate Format	Choose appropriate microplate formats (e.g., 96-well, 384-well) for your HTS to match the scale of your screening.
Assay Readouts	Select relevant endpoints, such as viability, proliferation, apoptosis, or specific biomarkers, based on your research objectives.
Replicates and Controls	Ensure sufficient replicates and positive/negative controls to assess assay robustness and reproducibility.
Compound Libraries	Prepare or acquire compound libraries for screening, considering the size and diversity of the library.
Data Management	Establish data storage, management, and analysis workflows to handle the large volume of data generated in HTS.
Hit Confirmation and Validation	Confirm hits from primary screens using secondary assays and validate potential drug candidates.
Data Standardization	Implement standardized data formats and quality control measures to enhance data comparability.
Hit Prioritization	Develop criteria for prioritizing hits based on potency, specificity, and other relevant factors.
Hit Characterization	Characterize identified compounds for pharmacological properties and mechanism of action.
Data Visualization and Reporting	Create user-friendly data visualization tools and reports to facilitate decision-making.
Screening Assay Miniaturization	Optimize assays for miniaturization to reduce reagent and sample consumption, as well as screening costs.
Throughput and Screening Capacity	Determine the desired throughput (e.g., screens per day) and screening capacity of the HTS platform.
Data Integration with External Sources	Integrate data from external sources, such as literature, to augment screening results and prioritize compounds.
Quality Control and QC Metrics	Establish quality control metrics and monitoring procedures to ensure assay performance and data reliability.
Ethical Considerations and Compliance	Adhere to ethical guidelines and regulatory compliance, especially when using patient-derived samples or clinical data.
Hit Validation in Relevant Models	Validate hits in more complex, physiologically relevant models, including in vivo studies if applicable.

conventional viability assays. By marrying automated imaging with advanced data analysis, HCS extracts intricate details about cellular morphology and protein localization, among others.

Sustaining the integrity of 3D cultures over time mandates rigorous quality control measures. Regular assessments, including cell viability checks and validation of spheroid morphology, are indispensable to uphold consistency. Likewise, validation studies are essential to ascertain the reproducibility and reliability of 3D HTS assays, often entailing comparisons with established 2D assays and in vivo data.

Additionally, it is vital to consider regulatory requirements when employing 3D cultures in drug development, as these may diverge from those applicable to 2D assays. By embracing these challenges and rigorously addressing them, the integration of 3D cultures into high throughput screening platforms promises to enhance the precision and relevance of pharmaceutical drug development processes.

Peering into the Future of 3D Cell Cultures

Advancements in cellular biology suggest that the future of 3D cultures lies in even more sophisticated models. Concepts like organ-on-a-chip and multi-tissue systems are on the horizon. These intricate models, designed to simulate multiple organ systems simultaneously, promise to deliver unparalleled physiological relevance. Yet, their increased complexity will bring about new challenges in assay optimization, necessitating an interdisciplinary melding of cell biology, materials science, engineering, and computational analytics.

Conclusion

3D cell cultures represent a monumental evolution in cellular research, bridging the gap between the simplicity of 2D cultures and the elaborate dynamics of in vivo systems. They promise a closer approximation to physiological reality, making them indispensable tools in modern research. While the journey to full optimization might be laden with challenges, the potential benefits in terms of research depth, clarity, and relevance make it a journey worth undertaking. As we march into a future brimming with technological advancements, 3D cultures, with their blend of complexity and physiological relevance, are poised to be at the forefront of cellular research and drug discovery.

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Product	Unit Size	Cat No.
Cell Meter™ Colorimetric MTT Cell Proliferation Kit	1000 Tests	22768
Cell Meter™ Colorimetric MTT Cell Proliferation Kit	5000 Tests	22769
Cell Meter™ Live Cell ATP Assay Kit	100 Tests	23015
PhosphoWorks™ Colorimetric ATP Assay Kit	100 Tests	21617
PhosphoWorks™ Fluorimetric ATP Assay Kit	100 Tests	21620
PhosphoWorks™ Luminometric ATP Assay Kit *Extended Luminescence*	1 Plate	21609
PhosphoWorks™ Luminometric ATP Assay Kit *Extended Luminescence*	10 Plates	21608
PhosphoWorks™ Luminometric ATP Assay Kit *Maximized Luminescence*	1 Plate	21610
PhosphoWorks™ Luminometric ATP Assay Kit *Maximized Luminescence*	10 Plates	21621
PhosphoWorks™ Luminometric ATP Assay Kit *DTT-Free*	1 Plate	21612
PhosphoWorks™ Luminometric ATP Assay Kit *DTT-Free*	10 Plates	21613
ReadiUse™ Rapid Luminometric ATP Assay Kit	100 Tests	21601
ReadiUse™ Rapid Luminometric ATP Assay Kit	1000 Tests	21602
ReadiUse™ Rapid Luminometric ATP Assay Kit	10000 Tests	21603

Comprehensive Guide to Cell Health Assays

in Flow Cytometry: A Multidimensional Exploration

Abstract

Flow cytometry stands as an invaluable tool in biomedical research, facilitating nuanced cell health assessments across a spectrum of assays. This precise evaluation of cellular vitality, viability, apoptosis, and stress responses has profound implications in diverse scientific domains, encompassing drug development, immunology, and cancer research. In this comprehensive guide, we not only emphasize the pivotal role of cell health assays in flow cytometry but also embark on an in-depth exploration of best practices, supported by illustrative examples. Our central focus is on enhancing robustness, reproducibility, and accuracy to empower researchers in optimizing their experimental protocols and deriving meaningful insights from cell health assessments.

Introduction

The contemporary landscape of biomedical research is characterized by a fundamental pursuit: understanding the health of individual cells. Flow cytometry has revolutionized our capacity to delve into this pursuit by enabling comprehensive cell health assessments. This guide delves deep into the crucial role of cell health assays within the realm of flow cytometry. We navigate the intricate nuances and best practices, anchored by illustrative examples, to assist researchers in refining their experimental methodologies.

Unraveling the Essence of Cell Health Assessment

Cell health assessments serve as the bedrock of biomedical research, allowing us to explore the intricate workings of cells and unearth insights that transcend cellular boundaries. Among the various methodologies, flow cytometry emerges as the linchpin unlocking the secrets of cell health. This scientific analysis provides a holistic perspective on cell vitality and well-being, rendering it indispensable in numerous fields, including drug discovery, immunology, cancer research, neurodegenerative disease studies, and microbiology.

Viability Assessment

The foundation of cell health assessments lies in the discrimination between living and deceased cells. This critical distinction is achieved through the application of specialized dyes, such as propidium iodide (PI), calcein AM, and 7-AAD. These dyes act as indicators of cell survival, highlighting the integrity of the cell membrane and intracellular structures. In drug discovery, for instance, viability assessment plays a pivotal role in evaluating the cytotoxic effects of novel compounds. Researchers employ these dyes to gauge how various pharmaceutical agents impact cell health. A decrease in cell viability, as indicated by increased staining with dyes like PI or 7-AAD, hints at potential toxicity of the tested compounds. This insight guides the selection of promising drug candidates while safeguarding the well-being of healthy cells.

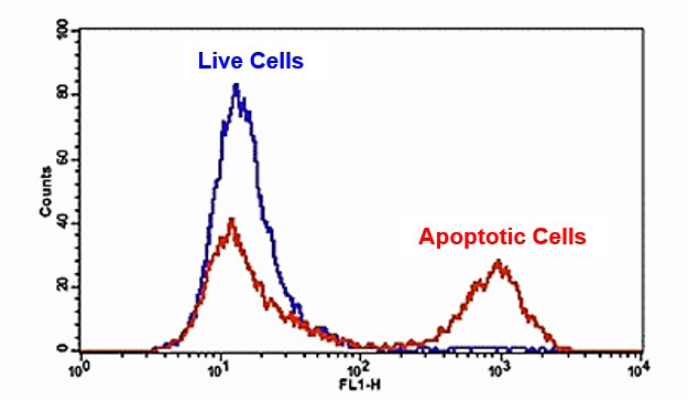

Figure 1. The detection of binding activity of FITC-Annexin V to phosphatidylserine in Jurkat cells using Cell Meter™ FITC-Annexin V Binding Apoptosis Assay Kit. Jurkat cells were treated without (Blue) or with 1 μM staurosporine (Red) in a 37ºC, 5% CO2 incubator for 4-5 hours, and then dye loaded for 30 minutes. The fluorescence intensity of FITC-Annexin V was measured with a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer using the FL1 channel.

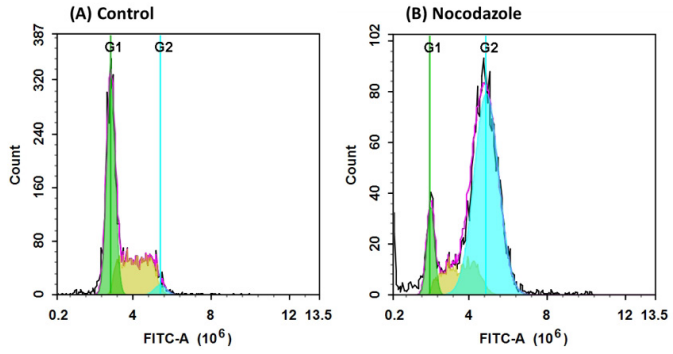

Figure 2. DNA profile in growing and nocodazole treated Jurkat cells. Jurkat cells were treated without (A) or with 100 ng/mL Nocodazole (B) in a 37 °C, 5% CO2 incubator for 24 hours, then incubated with Nuclear Green™ LCS1 for 30 minutes. The fluorescence intensity of Nuclear Green™ LCS1 was measured with an ACEA NovoCyte flow cytometer in the FITC channel. In growing Jurkat cells (A), nuclear staining with Nuclear Green™ LCS1 shows G1, S, and G2 phases. In Nocodazole treated G2 arrested cells (B), the frequency of G2 cells increased dramatically, while G1 and S phase-frequency decreased significantly.

Apoptosis Profiling

Apoptosis, the programmed cell death mechanism, stands as a vital component of cellular homeostasis. To dissect and comprehend this process, researchers rely on Annexin V-FITC/PI staining, a classic assay in the field of cell health assessment. This staining technique enables the precise differentiation of apoptotic cells from those undergoing necrosis or maintaining their health. In immunology, apoptosis profiling assists in characterizing immune cell responses to infections. For instance, during a viral infection, the immune system may trigger apoptosis in infected cells as a defense mechanism. Annexin V-FITC/PI staining empowers scientists to precisely quantify the extent of apoptosis among immune cells, shedding light on the immune response's intricacies.

Resources for Apoptosis

- Annexin V Staining
- Cell Meter™ Annexin V Binding Apoptosis Assay Selection Guide
- Annexin-V FAQs

Cell Cycle Analysis

Understanding how cells progress through the different phases of the cell cycle—G1, S, G2, and M—is pivotal for comprehending cell proliferation. DNA-binding dyes, including propidium iodide (PI), are deployed to illuminate the nuances of cell cycle dynamics. In cancer research, cell cycle analysis holds paramount importance. Chemotherapeutic agents designed to target rapidly dividing cancer cells often impact cell cycle progression. By employing PI staining, researchers can discern alterations in the distribution of cells across different cell cycle phases, providing critical insights into the efficacy and mechanisms of these treatments. For example, an increase in cells arrested in the G2 phase may indicate that a drug disrupts cell division, potentially inhibiting cancer growth.

Mitochondrial Health Assessment

Mitochondria, the cellular powerhouses, play a pivotal role in overall cell health. Preserving the integrity and function of mitochondria is essential for the cell's energy production

and overall well-being. Mitochondrial membrane potential (ΔΨm) assays, utilizing dyes such as JC-10™, JC-1, TMRE and TMRM, offer a window into mitochondrial function. In the field of neurodegenerative diseases, monitoring ΔΨm changes provides valuable insights into mitochondrial dysfunction, a common feature of conditions like Alzheimer's disease. For instance, in Alzheimer's research, alterations in mitochondrial membrane potential can signify early cellular stress, which may precede neuronal damage. By using JC-10™ staining, researchers can identify potential targets for therapeutic intervention aimed at preserving mitochondrial health.

Oxidative Stress Measurement

Reactive oxygen species (ROS) are molecules produced within cells in response to various stressors, including environmental toxins and pathogens. ROS can serve as critical indicators of oxidative stress levels within cells, a phenomenon linked to cellular damage and disease. To measure oxidative stress, researchers employ ROS assays with probes like OxiVision™ Green, dihydroethidium (DHE) and DCFH-DA. In microbiology, these assays are particularly valuable for assessing the impact of antimicrobial agents on bacterial cells. When exposed to antimicrobial compounds, bacteria may generate ROS as part of their defense mechanisms. Monitoring ROS production using DHE or DCFDA allows scientists to evaluate the efficacy of antimicrobial agents and gain insights into their mode of action. This information is essential for the development of new antimicrobial strategies to combat drug-resistant bacteria.

Resources for Oxidative Stress

- Selecting the right ROS probe
- Detection of Reactive Oxygen Species (ROS) Using Multicolor ROS Brite™ Reagents
- Detection of Reactive Oxygen Species (ROS) in Live Cell Mitochondria

Best Practices in Cell Health Assays for Flow Cytometry

Ensuring the reliability and reproducibility of cell health assessments through flow cytometry necessitates a

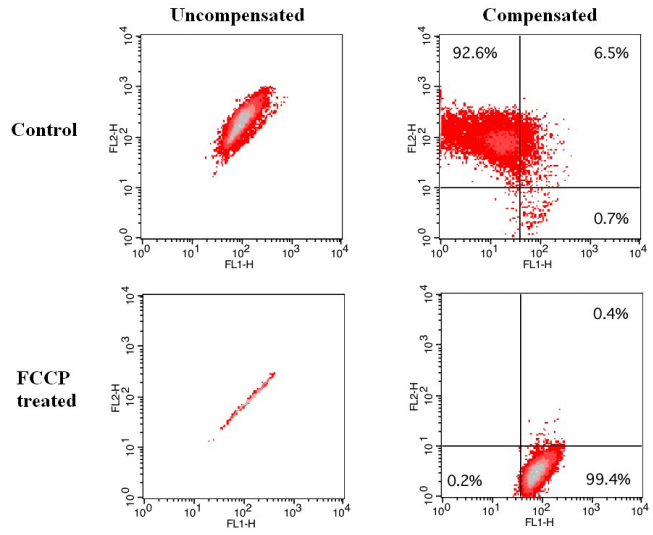

Figure 3. Effect of FCCP induced mitochondria membrane potential change in Jurkat cells. Jurkat cells were dye loaded with JC-10 dye working solution along with DMSO (Top) or 5 µM FCCP (Low) for 10 minutes. The fluorescence intensities for both J-aggregates and monomeric forms of JC-10 were measured with a FACSCalibur (Becton Dickinson) flow cytometer using FL1 and FL2 channels. Uncompensated data (left column) were compared with compensated data (right column).

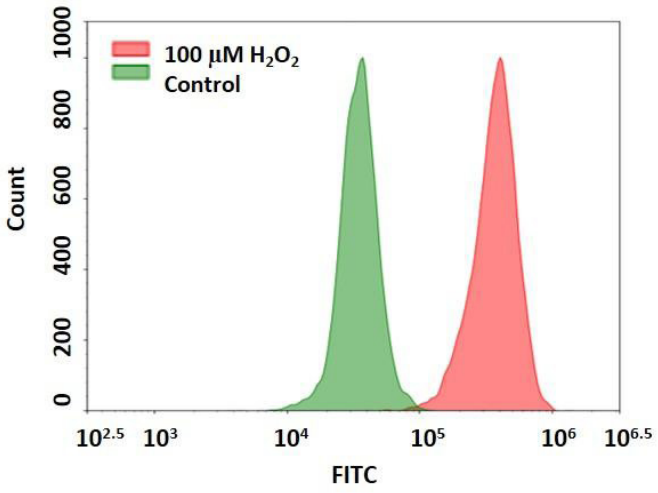

Figure 4. Detection of hydrogen peroxide in Jurkat cells using Cell Meter™ Intracellular Fluorimetric Hydrogen Peroxide Assay Kit (Cat#: 11506). Jurkat cells were stained with OxiVision™ Green peroxide sensor for 30 minutes and treated with 100 µM hydrogen peroxide at 37 °C for 90 minutes. Cells stained with OxiVision™ Green peroxide sensor but without hydrogen peroxide treatment were used as control.

multifaceted approach:

- **Sample Preparation:** Gentle sample handling emerges as the foundational layer to prevent cellular stress. Optimizing cell concentration is essential, and the meticulous exclusion of dead cells is pivotal. For instance, in stem cell research, maintaining the pluripotent state and integrity of stem cells during sample preparation is critical to the success of downstream experiments.
- **Appropriate Assay Selection:** The selection of an assay should harmonize with the research question at hand and the biological context. In drug development, the judicious choice of a viability assay can spell the difference between the advancement or abandonment of therapeutic compounds.
- **Control Panels:** A comprehensive control panel emerges as the keystone for precise data interpretation. This panel includes unstained cells, single-stained cells, and untreated cells—serving as North Stars for accurate data interpretation. In immunology, these controls ensure that observed changes in cell health align with vaccine-induced immune responses.
- **Staining Optimization:** The careful titration of staining reagents is a meticulous process that determines the optimal concentration, optimizing signal-to-noise ratios. In cancer research, the accurate measurement of cell cycle phases hinges on precise staining optimization, allowing the detection of subtle changes in cell cycle progression.
- **Incubation Conditions:** Adherence to recommended incubation conditions—time, temperature, and humidity—is paramount for staining procedures. Consistency ensures reproducibility, especially in studies that explore the effects of temperature on cell health, such as in virology research.
- **Data Acquisition:** The acquisition of a sufficient number of events is the bedrock for achieving statistical significance. Proper flow cytometer settings, including laser and detector configurations, are vital for the accurate acquisition of data. In neuroscience, meticulous data acquisition ensures that subtle changes in mitochondrial membrane potential are accurately detected in neuronal cells.
- **Gating Strategy:** The construction of a robust gating strategy, guided by controls and based on scatter properties and fluorescence intensity, is critical to focus on the cell population of interest. In hematological research, gating is essential to differentiate between normal and abnormal blood cell populations.
- **Instrument Calibration:** Regular calibration and maintenance of the flow cytometer are prerequisites for data accuracy. This entails meticulous checks on lasers, detectors, and fluidics. In microbial ecology studies, maintaining precise instrument calibration ensures the detection of subtle shifts in ROS levels in microbial populations exposed to environmental stressors.
- **Data Analysis:** Flow cytometry analysis software becomes the fulcrum for extracting pertinent parameters, such as viability percentages, cell cycle phases, or mitochondrial membrane potential. In the context of autoimmune diseases, precise data analysis aids in understanding the impact of immunosuppressive therapies on immune cell health.
- **Validation:** Results should be validated using complementary assays or biological controls, ensuring alignment with expected outcomes based on experimental design. In pharmacological research, validating cell health assay results with additional viability assays confirms the reliability of drug screening data.
- **Documentation and Record Keeping:** Comprehensive records encompassing protocols, instrument settings, and data files are the bulwark of data reproducibility. In regenerative medicine, meticulous record-keeping ensures the reproducibility of stem cell viability assessments.

Conclusion

Cell health assays, when strategically harnessed within the framework of flow cytometry, ascend as indispensable

tools for unraveling the intricacies of cellular well-being. The adherence to best practices spanning from sample preparation to data analysis fosters the generation of accurate and reliable results. Researchers, armed with this knowledge, navigate the labyrinthine terrain of cell health assessment, unlocking deeper insights into the dynamics of cellular processes in health and disease. Cell health assays in flow cytometry emerge not merely as laboratory techniques but as the quintessential instruments that empower scientists to illuminate the mysteries of cellular dynamics in the expansive domain of biomedical research. The journey of biomedical discovery is, indeed, a voyage into the very essence of life—cell by cell, insight by insight.

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Calcein Red™ AM	1 mg	21900
Calcein Red™ sodium salt	1 mg	21901
Calcein Deep Red™	1 mg	21902
CytoCalcein™ Violet 660, sodium salt *Excited at 405 nm*	1 mg	21903
CytoCalcein™ Violet 660, AM *Excited at 405 nm*	10x50 ug	21904
Calcein UltraGreen™ AM	10x50 ug	21905
Calcein UltraBlue™ AM	10x50 ug	21908
Calcein UltraBlue™ sodium salt	1 mg	21909
Calcein *UltraPure Grade* *CAS 207124-64-9*	10 mg	22001
Calcein, AM *CAS 148504-34-1*	1 mg	22002
Calcein, AM *UltraPure grade* *CAS 148504-34-1*	1 mg	22003
Calcein, AM *UltraPure grade* *CAS 148504-34-1*	20x50 ug	22004
Calcein Blue *CAS 54375-47-2*	25 mg	22006
Calcein Blue, AM *CAS 168482-84-6*	1 mg	22007
Calcein Orange™ sodium salt	1 mg	22008
Calcein Orange™ diacetate	1 mg	22009

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CytoCalcein™ Violet 500 *Excited at 405 nm*	1 mg	22013
JC-1 [5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide] *CAS#: 3520-43-2*	5 mg	22200
JC-1 [5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide] *CAS#: 3520-43-2*	50 mg	22201
JC-10 *Superior alternative to JC-1*	5x100 uL	22204
TMRE [Tetramethylrhodamine ethyl ester] *CAS#: 115532-52-0*	25 mg	22220
TMRM [Tetramethylrhodamine methyl ester] *CAS#: 115532-50-8*	25 mg	22221
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