BioLamina
REVOLUTIONIZING CELL CULTURE

COATING CELL CULTUREWARE
WITH BIOLAMININ SUBSTRATES

INSTRUCTIONS FOR USE 001

BIOLAMINA DEVELOPS, MANUFACTURES AND DISTRIBUTES CELL CULTURE REAGENTS THAT MAKES IT POSSIBLE TO CULTURE PLURIPOTENT STEM CELLS, ADULT STEM CELLS AND TISSUE-SPECIFIC CELLS IN A CELL SPECIFIC AND PHYSIOLOGICALLY RELEVANT ENVIRONMENT. IN VIVO, LAMININS ARE KEY PROTEINS IN THE BASEMENT MEMBRANE THAT UNDERLIE ALL EPITHELIA AND ENDOTHELIA AND SURROUND INDIVIDUAL CELLS, WITH ESSENTIAL ROLES IN REGULATION OF MANY CELLULAR FUNCTIONS, SUCH AS ADHESION, DIFFERENTIATION, MIGRATION, PHENOTYPE STABILITY, AND RESISTANCE TO APOPTOSIS. BIOLAMINA'S HUMAN RECOMBINANT LAMININ CELL CULTURE MATRICES, BIOLAMININS, ARE CHEMICALLY DEFINED AND ANIMAL COMPONENT-FREE AND MAKES CELL CULTURE EASY AND STANDARDIZED.

IMPORTANT NOTES

- **All procedures should be done under sterile conditions using aseptic techniques**
- **Avoid prolonged exposure of the protein to ambient temperatures**
- **Repeated freeze/thaw should be avoided**
- **The Biolaminin stock solution is stable for 3 years when stored at -20°C to -80°C**
- **Thawed, undiluted Biolaminin stock is stable for at least 3 months when stored at +2°C to +8°C under aseptic conditions**
- **For your convenience, coated plates can be kept for up to 4 weeks when stored aseptically at +2°C to +8°C**

COATING PROTOCOL

1. Slowly thaw the Biolaminin stock solution at +2°C to +8°C before use.
   - *Thawed, undiluted Biolaminin stock is stable for at least 3 months when stored at +2°C to +8°C under aseptic conditions. Repeated freeze-thaw cycles should be avoided. For longer storage needs, we recommend dividing the thawed stock solution in smaller working aliquots and to store frozen. Frozen stock can be stored up to three years in -20°C to -80°C.*
2. Calculate the concentration and the amount of coating solution needed for the experiment. The MX and CTG matrix generally should be used in a slightly higher concentration (10-15 µg/mL) compared to the LN matrix (5-10 µg/mL). Once the cells are adapted, a lower coating concentration often can be used but should be optimized empirically for each cell line. Guidelines for surface coating calculations can be found in the table below.
   - *Lowering the coating concentration might affect the proliferation rate, extending the culture time with about 1 day. Make sure the coating concentration is high enough to support an even cell growth.*
   - *When culturing cells on the Biolaminin matrices for the first time, an initial coating concentration of 10-15 µg/mL is recommended for the first few cell passages. Some cell lines might need an adaptation period and a higher coating concentration is then recommended for the first few passages. Once the cells are adapted to the Biolaminin matrix, the coating concentration usually can be reduced. The coating should be optimized empirically for each cell line and cell type.*
3. Gently invert the vial to mix the Biolaminin stock solution. Do not vortex as this may cause fragmentation.

ORIGINAL

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4. Dilute the Biolaminin stock solution with 1xDPBS (Ca^{++}/Mg^{++}) and add the solution to the cultureware of choice. There is no need to pre-treat the cultureware. Make sure the entire surface is covered by the laminin coating solution. Uncoated surface will not support cell growth. Recommended coating volumes for different cultureware formats can be found in the table below.
   - *DPBS with Ca^{2+} and Mg^{2+} should be used since divalent cations are important for the protein structure and function.*
   - *The laminin matrices work well with most commercial cultureware brands (e.g. Falcon, Sarstedt, Corning).*
   - *The laminin matrix can easily be used for coating of glass. Overnight coating at +2°C to +8°C is recommended for a more reliable coating.*

5. Seal the plate (e.g. with Parafilm®) to prevent evaporation and contamination. Incubate at +2°C to +8°C overnight. If a more rapid coating is required, incubate at +37°C for 2 hours.
   - *Do not allow the coated surface to dehydrate as that will inactivate the Biolaminin coating.*
   - *For your convenience, the coated plates can be kept for up to 4 weeks when stored aseptically at +2°C to +8°C. Extra 1xDPBS (Ca^{++}/Mg^{++}) might have to be added after 1-2 weeks to prevent the plate from drying out.*

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Guidelines for surface coating calculations

CULTUREWARE	COATING CONCENTRATION (ug/mL)	COATING CONCENTRATION (ug/cm²)*	COATING SOLUTION		TOTAL COATING SOLUTION VOLUME
			LAMININ STOCK	1xDPBS (Ca^{++}/Mg^{++})	
6-well	5	0.45	50 uL/well	950 uL/well	1000 uL/well
12-well	5	0.51	25 uL/well	475 uL/well	500 uL/well
24-well	5	0.55	15 uL/well	285 uL/well	300 uL/well
48-well	5	0.49	7.5 uL/well	142.5 uL/well	150 uL/well
96-well	5	0.46	3.5 uL/well	66.5 uL/well	70 uL/well
T-25cm² flask	5	0.55	150 uL/flask	2850 uL/flask	3000 uL/flask
T-75cm² flask	5	0.51	400 uL/flask	7600 uL/flask	8000 uL/flask

* Calculations based on the entire surface area coated.

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BioLamina
REVOLUTIONIZING CELL CULTURE

INSTRUCTIONS FOR USE 003

CULTURING HUMAN ES AND IPS CELLS

ON HUMAN RECOMBINANT BIOLAMININ 521

BIOLAMINA DEVELOPS, MANUFACTURES AND DISTRIBUTES HUMAN RECOMBINANT LAMININ CELL CULTURE REAGENTS THAT MAKES IT POSSIBLE TO CULTURE PRIMARY CELLS AND CELL LINES IN A CELL SPECIFIC AND BIOLOGICALLY RELEVANT ENVIRONMENT. LAMININ 521 IS A KEY PROTEIN OF THE NATURAL STEM CELL NICHE AND ARE EXPRESSED IN THE DEVELOPING EMBRYO AND SECRETED BY HUMAN PLURIPOTENT STEM CELLS (hPSCs) IN CULTURE. THE DEFINED AND ANIMAL COMPONENT-FREE LAMININ 521 MATRICES, BIOLAMININ 521 LN, MX AND CTG, SUPPORTS FAST CELL EXPANSION AND IS THE RECOMMENDED MATRIX FOR CULTURE OF hPSCs. BIOLAMININ 521 CREATES A MORE AUTHENTIC CULTURE ENVIRONMENT AND MAKES HANDLING OF hPSCS RELIABLE AND STANDARDIZED. FURTHERMORE, EASY AND CONTROLLED SINGLE-CELL PASSAGING CAN BE PERFORMED, TOTALLY INDEPENDENT OF ROCK INHIBITOR (ROCKi). THE CELLS GROW IN A HOMOGENEOUS MONOLAYER WITHOUT NEED FOR MANUAL REMOVAL OF DIFFERENTIATED CELL AREAS.

TRANSITION PROTOCOL

This is a protocol for the transition of hPSCs to the Biolaminin 521 cell culture matrices (LN, MX and CTG) from another feeder-free matrix (e.g. Matrigel) or from feeders.

1. Coat new cultureware with Biolaminin 521 as described in **INSTRUCTIONS FOR USE 001.**

2. For the transition step, split you cells as you normally do and then seed the cells on the Biolaminin 521 coated plate. So, if you are culturing your cells as colonies, transition to the Biolaminin 521 substrates as colonies.
   - *It is important that the cells transferred to the Biolaminin 521 matrix are of high quality. Carefully select only undifferentiated cell areas for transfer.*
   - *It is to be expected that cell morphology will look different on Biolaminin 521 compared to cells cultured as colonies on feeder or on other feeder-free matrices. The cells flatten out on the Biolaminin 521 substrate and will look bigger compared to colony cultured cells. Cell morphology may also differ depending on the medium used.*
   - *It is not recommended to change both the medium and matrix brand at the same time. Preferably, transition to the Biolaminin 521 matrix before undertaking a gradual medium transition.*

3. For the next passage, follow our protocol described in the passaging and culture instructions below. Some hPSC lines are more difficult to transition to the Biolaminin 521 matrix and might require an adaptation period (up to 5 passages) before they can be cultured as single-cells or small aggregates. To insure a successful transition, we recommend that you do the following:
   - add ROCK inhibitor (10 uM) for the first few passages
   - use a higher coating concentration (10 ug/ml)
   - seed at a higher cell density (50,000-100,000 cells/cm^2) for the first few passages

   Once the cells are adapted to the Biolaminin 521 matrix, the seeding density and coating concentration usually can be lowered and the hPSCs can routinely be cultured as single cells without need of ROCK inhibitor.

IMPORTANT NOTES

- **All procedures should be done under sterile conditions using aseptic techniques**
- **The protocols can easily be made totally defined and animal component-free with your choice of culture medium and dissociation reagent**
- **It is important that the cells transferred to the Biolaminin 521 matrix are of high quality**
- **Some hPSC lines transferred to the Biolaminin 521 matrix, might require an adaptation period before they can be cultured according to the single-cell passaging protocol**
- **Once adapted to the Biolaminin 521 matrix, hPSCs can routinely be cultured as single cells without ROCKi**
- **Biolaminin 521 facilitates long-term self-renewal of hPSC without weekend feeding. For reduced labor and cost, follow the reduced feed protocol described in APPLICATION NOTE 001**

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PASSAGING PROTOCOL

The following protocol is for expansion and easy single cell passage of hPSCs on the Biolaminin 521 matrices (LN, MX and CTG). This is a generic guideline that might require optimization for best results. Volumes given in this section are for 6-well plates and should be adjusted accordingly for different sized cultureware. Once successfully transitioned and adapted to the Biolaminin 521 matrix, hPSCs can be cultured as single cells without the addition of ROCKi. Cells cultured on the Biolaminin 521 matrix are ready to be passaged when cell culture is 60-90% confluent. Note that none of the other Biolaminin matrices (except for 511) support single-cell hPSC culture. For single-cell culture of hPSCs on another Biolaminin matrix than 521 or 511, ROCKi must be added. Alternatively, mix in one part of 521 with the other Biolaminin isoform (1:3 mix) and use for coating.

BEFORE START:

- Coat new cultureware with the Biolaminin 521 cell culture matrix as described in **INSTRUCTIONS FOR USE 001**.
- Aliquot sufficient amounts of all solutions needed for the protocol and pre-warm to +37°C.

1. Carefully remove the excess Biolaminin coating solution from a new plate without disturbing the coated surface. Immediately add 2 mL fresh culture medium of choice to each well. Let the plate equilibrate at +37°C, with 5% CO_2 and 95% humidity.
   - *Do not allow the coated surface to dehydrate as that will inactivate the Biolaminin coating.*
   - *Culture medium for different cell types and applications can be determined accordingly by the user. Biolaminin 521 work well in combination with most commercial media brands (e.g. NutriStem™, mTeSR™1, TeSR™2, Essential 8™ and iPS-Brew). It is to be expected that cell morphology will look different dependent on the medium used for culture.*
2. Aspirate the medium from the cells and rinse gently with 1xDPBS (Ca^{--}/Mg^{--}) (1 mL/well).
   - *DPBS without Ca^{2+} and Mg^{2+} should be used since divalent cations have negative effect on some dissociating enzymes.*
   - *Cells cultured on the Biolaminin 521 matrix can be grown to near 100% confluence but should not grow over-confluent. Too confluent cultures will be difficult to detach.*
3. Add 1 mL/well of dissociation reagent of choice (e.g. TrypLE™, EDTA, Accutase, Trypsin) and incubate at +37°C for 3-6 minutes.
   - *The incubation time is cell line and dissociation reagent dependent. Stem cells are sensitive and too much mechanical stress caused by extensive pipetting may result in low cell viability. A more confluent culture and the use of a high coating concentration could make the cells attach more tightly to the surface and a prolonged treatment time with the dissociation agent may be required. If it's still difficult to dissociate the cells, try lowering the coating concentration.*
4. Gently aspirate the cell dissociation solution and add 1-2 mL/well of pre-warmed fresh medium. Gently pipette up and down 2-4 times to achieve single-cell suspension or small aggregates. The mechanical force applied should be minimal not to cause significant physical damage to the cells.
   - *Use a microscope to verify that the cells are properly dissociated. When using Biolaminin 521, we recommend passaging pluripotent stem cells as single cells or as small aggregates. When using EDTA, try to achieve as small cell aggregates as possible without using too much mechanical force. Extensive pipetting may result in low cell viability. Rather, increase the incubation time to minimize pipetting.*
5. Collect the cell suspension in a 15 mL conical tube. Centrifuge at 100 x *g* for 4 minutes at room temperature (+15°C to +25°C). If you are using EDTA as a dissociation agent, you can skip step 5 and 6.
6. Aspirate and discard the supernatant and gently resuspend the cell pellet in fresh, pre-warmed culture medium of choice. For each well of hPSCs collected, add 1-2 mL of medium.
7. Count the cells and calculate the cell suspension volume needed for seeding. hPSC should be seeded with a density of 30,000-50,000 cells/cm^2 or with a split ratio of 1:10 to 1:30.
   - *Optimal seeding densities will vary from one cell line to another. The Biolaminin 521 culture system is flexible and the split ratio can be adjusted empirically for each protocol and need. Biolaminin 521 can support cell survival at a seeding density as low as 5,000 cells/cm^2.*
   - *When transitioning your cells to Biolaminin 521 from another feeder-free matrix (e.g. Matrigel) or from feeder cells, follow the instructions in the* **TRANSFER PROTOCOL** *above.*
8. Bring out the fresh Biolaminin 521 coated tissue culture plate containing fresh, pre-warmed culture medium and transfer the desired volume of cell suspension to each well. Immediately rock the plate to get an even cell distribution.
   - *If you are transitioning your cells to Biolaminin 521 from feeders or another feeder-free matrix, we recommend adding ROCK inhibitor to a final concentration of 10 uM for the first few passages. Once adapted to the Biolaminin matrix, pluripotent stem cells can routinely be cultured as single cells without the need for ROCK inhibitor.*
9. Place the plate into the incubator and culture the cells at +37°C, with 5% CO_2 and 95% humidity. Perform a complete medium change 24-48 hours after passaging and then feed cells on daily basis until the next passage. For reduced labor and cost, follow the weekend-free protocol described in APPLICATION NOTE 001.
   - *Within 60 minutes, the majority of the cells should have attached, evenly distributed as single cells across the well. The day after seeding the cells should have formed small colonies. hPSCs cultured on Biolaminin 521 should grow as a homogenous monolayer, without any differentiated areas. See representative pictures below.*

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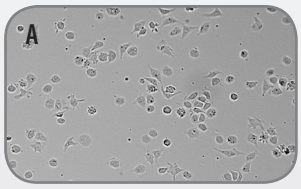
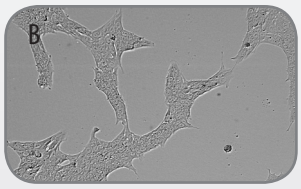
MORPHOLOGY OF hESC AT VARIOUS DAYS AFTER SEEDING

Representative pictures (10x magnification) of human embryonic stem cell (hESC) line HS181 seeded as single cells (30,000 cell/cm^2) in Nutristem™ medium onto 5 ug/mL of Biolaminin 521 (pictures A-C), or seeded as aggregates in mTeSR™ medium on Matrigel according to the manufacturer's instructions (picture D).

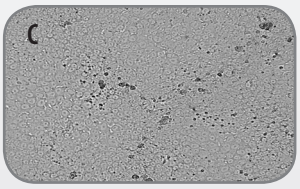
Within 1 hour after seeding on LN521, the majority of the cells should have attached, evenly distributed across the surface (A). The cells show high motility and will migrate to make contact with other cells, initiating proliferation. The day after seeding, the majority of the cells should have formed small colonies (B). Cells seeded on LN521 should grow as a homogenous monolayer. The cells should exhibit a cobblestone morphology with high nuclear-to-cytoplasm ratio and prominent nucleoli (C). Unlike colony passaging on other feeder-free matrices (D), cells cultured as single-cells on Biolaminin 521 can be cultured to near confluence without signs of spontaneous differentiation (C). It is to be expected that cell morphology will differ between single cell (C) and colony (D) cultured cells. Cell morphology may also differ depending on the medium used for culture.



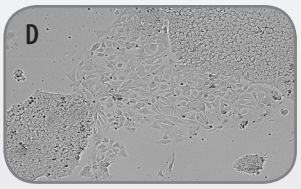
Day 0: hESC HS181 cultured on LN521, 1 hour after seeding. The cells have attached and are evenly distributed as single cells.



Day 1: hESC HS181 cultured on LN521, the day after seeding. The cells have formed small colonies.



Day 4: hESC HS181 cultured on LN521, just before passage. Confluent cell monolayer without differentiated cell areas.



hESC HS181 cultured as colonies on Matrigel, 3 days after seeding. Area of differentiation between 2 undifferentiated colonies.

THAWING CRYOPRESERVED hPSCs

Thawed hPSCs should be seeded into Biolaminin 521 coated wells. hPSCs cultured on another feeder-free matrix or feeders can be thawed directly onto Biolaminin 521. At the first passage, follow the single-cell **PASSAGING PROTOCOL**. When the cell transition to the Biolaminin 521 matrix is problematic, follow instruction in the **TRANSFER PROTOCOL** above.

BEFORE START:

- Coat new cultureware with Biolaminin 521 as described in **INSTRUCTIONS FOR USE 001**. If unsure of the number of cells / aggregates frozen down, a 6-well plate is recommended.
- Aliquot sufficient amounts of all solutions needed for the protocol and pre-warm to +37°C.

1. Carefully remove the Biolaminin coating solution from a new plate and immediately add 2 mL fresh culture medium of choice to each well. Let equilibrate at +37°C, with 5% CO_2 and 95% humidity.
   - *The coating does not require washing before use.*
   - *Do not allow the coated surface to dehydrate.*
   - *Culture medium can be determined accordingly by the user.*
2. Quickly thaw the hPSCs in a +37°C water bath by gently shaking the cryovial continuously until only a small frozen pellet remains.
3. Sterilize the cryovial with 70% ethanol and carefully transfer the cell suspension to a 15 mL conical tube.
4. Gently add 5-7 mL of pre-warmed medium of choice. Centrifuge at 100 x *g* for 4 minutes at room temperature (+15°C to +25°C).
5. Discard the supernatant and gently resuspend the cell pellet in 1-2 mL of fresh, pre-warmed culture medium.
6. Count the cell number (if applicable) and transfer the appropriate amount of cells suspension to a Biolaminin 521 coated well plate.
   - *About 1 million cells/well in a total volume of 2 mL is recommended if using a 6-well plate (adjusted accordingly for other well size).*
7. Place the plate into the incubator and gently rock the plate to distribute the cells evenly.
8. Culture the cells at +37°C, with 5% CO_2 and 95% humidity. Perform a medium change after 24-48 hours and then on daily basis.

CRYOPRESERVING hPSCs

hPSCs cultured on Biolaminin 521 should be cryopreserved as single cells or small aggregates when 60-70% confluent. The cryopreservation medium should be defined, serum-free and designed specifically for hPSCs. Volumes given in this section are for 6-well plates and should be adjusted accordingly for different sized cultureware.

BEFORE START:

- Aliquot sufficient amounts of all solutions needed for the protocol and pre-warm to +37°C. The cryopreservation medium should be handled according to the manufacturer's Instructions.
- Prepare and label cryovials.

1. Aspirate the medium from the cells and rinse gently with 1 mL/well of 1xDPBS (Ca^{--}/Mg^{--}).
   - *DPBS <u>without</u> Ca^{2+} and Mg^{2+} should be used since divalent cations have negative effect on some dissociating enzymes.*
2. Add 1 mL/well of dissociation reagent of choice (e.g. TrypLE™, EDTA, Accutase, Trypsin) and incubate at +37°C for 3-6 minutes.
   - *The incubation time is cell line and dissociation reagent dependant. It also depends on the coating laminin concentration used and the degree of cell confluence.*
3. Gently aspirate the dissociation solution and add 1-2 mL/well of pre-warmed fresh medium. Gently pipette up and down 2-4 times to achieve single-cell suspension or small aggregates.
   - *When using EDTA, try to achieve as small cell aggregates as possible without using too much mechanical force.*
4. Collect the cell suspension in a 15 mL conical tube. Centrifuge at 100 x *g* for 4 minutes at room temperature (+15°C to +25°C).
5. Aspirate and discard the supernatant. Gently resuspend the cell pellet in 2 mL cryopreservation medium of choice. Count the cells (if applicable) and transfer 0.5-1 mL of cell suspension (0.5 -1x10^6 cells/mL) into cryovials.
6. Freeze cells using a standard slow rate controlled protocol (approx. -1°C/min) and store at -135°C to -180°C.

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BIOLOGICALLY RELEVANT CELL CULTURE SUBSTRATES FOR DIFFERENT CELL APPLICATIONS

Most organized cells in the human body grow on a basement membrane that contains laminins, an interaction that is essential for their survival and tissue specific functions. With BioLamina's human, recombinant laminin cell culture matrices, Biolaminins, it is now possible to culture pluripotent stem cells, adult stem cells and tissue-specific cells in a cell specific and physiologically relevant environment. Our Biolaminin matrices are defined and consistent, making cell culture easy, standardized and more authentic. The Biolaminin cell culture matrices have shown to improves the expansion and maturation of many cell types, such as pancreatic beta cells, cardiomyocytes, different kind of neurons, neural stem cells, endothelial cells, cancer stem cells and many more. Below are a few examples.

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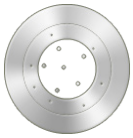

HUMAN PLURIPOTENT STEM CELL CULTURE

Robust derivation, reprogramming, expansion and differentiation of human ES and iPS cells under completely chemically defined, feeder-free and animal component-free conditions on Biolaminin 521. Biolaminin521 also increase survival and expansion of single cells at clonal densities.

RPE AND PHOTORECEPTOR CELL CULTURE

Culture retinal pigmented epithelial (RPE) cells, photoreceptors and other retinal cells on laminin isoforms 521, 511, 332 and 111, expressed in Bruch´s membrane and the neuroepithelium. Biolaminin 521 support efficient production of functional hESC-RPE cells.

HEPATOCYTE DIFFERENTIATON AND MATURATION

Biolaminin 521 and 111 support efficient specification and maturation of hESC-derived hepatocytes with significantly increased metabolic activity and functional organization.

PANCREATIC B-CELLS ISLETS

Efficient maintenance and expansion of primary pancreatic islets on pancreas-specific Biolaminin 521 and 421. Biolaminin 411 increase differentiation of mesenchymal stem cells into insulin producing beta cells.

CULTURE BEATING CARDIOMYOCYTES

Chemically defined isolation, expansion and differentiation of primary cardiac progenitors and hPSCs-derived cardiomyocytes is improved with cardiac laminins 521, 221 and 211.

SKIN AND HAIR CELL CULTURE

Laminin 332 and 511 is enriched in epithelial basement membranes and influences proliferation and migration of keratinocytes during wound healing. Laminin 332 and 511 is the major laminin of the hair follicle.

MAINTAINING ENDOTHELIAL CELLS IN CULTURE

Efficient differentiation of hESC to endothelial progenitor cells using Biolaminin 521. Human endothelial progenitor cells can also efficiently be maintained on endothelial-specific laminin.

CELL CULTURE OF NEURAL CELL LINEAGES

Cell culture of dopaminergic neurons, neural stem cells, motor neurons and other nerve cells on their respective laminins increase adhesion, neurite outgrowth, maturity and functionality.

FOR MORE INFORMATION ABOUT HOW OUR LAMININ CELL CULTURE MATRICES CAN BE USED FOR YOUR SPECIFIC CELL APPLICATION, PLEASE VISIT BIOLAMINA'S SCIENCE ROOM

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BIOLAMINA - REVOLUTIONIZING CELL CULTURE

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INSTRUCTIONS FOR USE 010

EMBRYOID BODY FORMATION

FROM hPSCs CULTURED ON BIOLAMININ 521

BIOLAMINA DEVELOPS, MANUFACTURES AND DISTRIBUTES CELL CULTURE REAGENTS THAT MAKES IT POSSIBLE TO CULTURE HUMAN PLURIPOTENT STEM CELLS (hPSCs), ADULT STEM CELLS AND TISSUE-SPECIFIC CELLS IN A CELL SPECIFIC AND PHYSIOLOGICALLY RELEVANT ENVIRONMENT. LAMININ 521 IS A KEY PROTEIN OF THE NATURAL STEM CELL NICHE AND WHEN USED AS COATING, IT RECREATES A MORE AUTHENTIC CULTURE ENVIRONMENT THAT SUPPORTS ROBUST AND STANDARDIZED SINGLE-CELL hPSC EXPANSION. THE hPSCs GROW IN A HOMOGENEOUS MONOLAYER ON BIOLAMININ 521 (LN521) WITHOUT NEED FOR MANUAL REMOVAL OF DIFFERENTIATED CELL AREAS. EMBRYOID BODY (EB) FORMATION IS A STANDARD APPROACH TO ASSESS THE DIFFERENTIATION POTENTIAL OF hPSCs AND FOR PRODUCING DIFFERENT CELL LINEAGES FOR FURTHER APPLICATIONS.

IMPORTANT NOTES

- **All procedures should be done under sterile conditions using aseptic techniques**
- **The protocol can be made totally defined and animal component-free with your choice of culture medium and enzyme**
- **For reproducible EB formation, it is essential that the cells used are in active proliferation mode**
- **EBs can be obtained in conical or round-bottom, non-treated or low attachment plates or as hanging drops**
- **Differentiation capacity is cell line and medium dependent. Lineage specific differentiation might require adjusted culture time or different medium.**

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EB FORMATION PROTOCOL

The following protocol provide instructions for a standardized, serum-free EB formation of hPSCs cultured as single-cells or as small aggregates on Biolaminin 521. hPSCs for EB formation should routinely be cultured on Biolaminin 521 according to **INSTRUCTIONS FOR USE 003**. Volumes given in this section are for 6-well plates and should be adjusted accordingly for different sized cultureware. This is a generic guideline that might require optimization for best results.

BEFORE START:

- Prepare appropriate differentiation medium of choice. For spontaneous EB differentiation, we recommend Knockout™-DMEM supplemented with 20% Knockout™ Serum Replacement, 1% L-glutamine, 1% MEM non-essential amino acids and 0.2% B-mercaptoethanol. 1% Penicillin-Streptomycin addition is optional. Store completed medium at +2°C to +8°C and use within 1 week.
- Aliquot sufficient amounts of all solutions needed for the protocol and pre-warm to +37°C.

1. hPSCs used for EB differentiation should be seeded on Biolaminin 521 coated plates in a cell density so that they reach 70-90% confluence after 3-4 days. The cells should be in active proliferation phase on the day used for EB formation.
   - *Proliferation rate will depend on the cell line, seeding density and the medium used, thus the seeding density should be adjusted accordingly.*
2. Aspirate the medium from the cells and rinse gently with 1xDPBS (Ca^{--}/Mg^{--}) (1 mL/well).
   - *DPBS without Ca^{2+} and Mg^{2+} should be used since divalent cations have negative effect on some dissociating enzymes.*
3. Add 1 mL/well of EDTA-based cell dissociation reagent and incubate at +37°C for 5-8 minutes.
   - *EDTA-based dissociation is recommended over enzyme-based dissociation for successful EB formation. When using enzyme-based dissociation, for successful EB formation it is essential that the cells used are in an active proliferation state and that ROCKi is added.*

- *The incubation time is cell line and dissociation reagent dependent. A more confluent culture and the use of a high coating concentration could make the cells attach more tightly to the surface and a prolonged treatment time with the dissociation agent may be required. If it's still difficult to dissociate the cells, try lowering the coating concentration.*

4. Gently aspirate the dissociation solution and add 2 mL/well of pre-warmed fresh medium. Gently pipette up and down 2-4 times to achieve small cell aggregates. Try to achieve as small cell aggregates as possible without using too much mechanical force.
   - *The mechanical force applied should be minimal not to cause significant physical damage to the cells. Rather, increase the incubation time to minimize pipetting.*
5. Collect the cell suspension in a 15 mL conical tube. Centrifuge at 100 x *g* for 4 minutes at room temperature (+15°C to +25°C).
6. Aspirate and discard the supernatant and gently resuspend the cell pellet in 1-2 mL fresh, pre-warmed differentiation medium of choice supplemented with 10 uM ROCKi.
   - *If using a ROCKi-free EB formation protocol, a higher seeding density in step 7 is recommended (50,000-200,000 cells/well). Optionally, culturing the cells on a mix of Biolaminin 521 and 111 (1:1 ratio) a few days prior to the EB formation might increase the chance of ROCKi-free EB formation.*
7. Count the cell number and plate the cells into non-treated or low attachment surface conical or round-bottom 96-well plates. hPSC should be seeded with a density of 5,000-100,000 cells/well in a volume of ≥100 uL.
   - *The size of individual EBs is highly dependent on the cell Line and on the number of starting cells and might influence the general differentiation result.*
   - *Conical-bottom plates (e.g. Nunc™ 96 Well Polystyrene Conical Bottom MicroWell™ Plate) or U-bottom plates (e.g. Corning® 96 Well Clear Round Bottom Polystyrene Not Treated Microplate) can be used.*
   - *EBs can also be obtained as hanging drops.*
8. Centrifuge the plate at 600 x *g* for 5 minutes at room temperature (+15°C to +25°C).
9. Place the plate into the incubator and culture the cells at +37°C, with 5% CO_2 and 95% humidity.
10. After 24-48 h, one EB/well should be visible. The EBs may be surrounded by unaggregated sigle cells (Figure 1.)
    - *EB formation efficiency will vary between different cell lines. If EBs does not form, try increasing the EB seeding density, or start EB formation one day earlier when cells are less confluent. It is important that the cells are in active proliferation phase when used for EB differentiation.*
11. Transfer the EBs to conical tube using a open-end 1ml tip. Let the EBs sink by gravity (about 30 sec.).
12. Carefully aspirate and discard the supernatant containing single cells and replace with fresh, pre-warmed differentiation medium. Transfer the EB suspension to low attachment flat-bottom plate (e.g. Costar® 6 Well Clear Flat Bottom Ultra Low Attachment Multiple Well Plates) or a petri dish.
13. Place the plate into the incubator and gently rock the plate to distribute the EBs evenly.
    - *The individual EBs should have a condensed, rounded three-dimensional structure of equal size (Figure 2).*

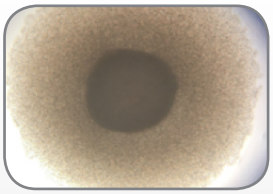

Figure 1. EB from hESC HS181 cultured on Biolaminin 521 LN (LN521), 24 h post-seeding. The EB is surrounded by unaggregated cells. 5x magnification.

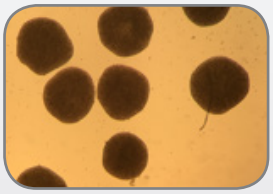

Figure 2. EBs from hESC HS181 cultured on LN521, just transferred to a flat-bottom, low attachment plate. 5x magnification.

14. Change medium every third day for 2-3 weeks. The EBs will grow in size over time and undergo spontaneous differentiation.
    - *Differentiation capacity is cell line and medium dependent. Lineage specific differentiation might require adjusted culture time or different medium.*

REFERENCE

A High Proliferation Rate is Critical for Reproducible and Standardized Embryoid Body Formation from Laminin-521-Based Human Pluripotent Stem Cell Cultures.
Dziedzicka D., et al., *Stem Cell Rev and Rep.*, 2016

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