



BIOLAMINA DEVELOPS, MANUFACTURES AND DISTRIBUTES CELL CULTURE REAGENTS THAT MAKE 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 THE 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.

EB FORMATION PROTOCOL

The following protocol provides 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 the best results.

BEFORE START:

- Prepare the 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 an 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 (Ca²⁺/Mg²⁺) (1 mL/well).
 - DPBS without Ca²⁺ and Mg²⁺ should be used since divalent cations have a 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.

IMPORTANT NOTES

- All procedures should be done under sterile conditions using aseptic techniques
- The protocol can be made totally defined and animal origin-free with your choice of culture medium and enzyme
- For reproducible EB formation, it is essential that the cells used are in an 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.



- *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 prolonged treatment time with the dissociation agent may be required. If it's still difficult to dissociate the cells, try lowering the coating concentration.*
- 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.*
 - 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).
 - 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 μ M 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.*
 - 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 \geq 100 μ L.
 - *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.*
 - Centrifuge the plate at 600 x g for 5 minutes at room temperature (+15°C to +25°C).
 - Place the plate into the incubator and culture the cells at +37°C, with 5% CO₂ and 95% humidity.
 - After 24-48 h, one EB/well should be visible. The EBs may be surrounded by unaggregated single 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 an active proliferation phase when used for EB differentiation.*
 - Transfer the EBs to a conical tube using an open-end 1ml tip. Let the EBs sink by gravity (about 30 sec.).
 - Carefully aspirate and discard the supernatant containing single cells and replace it 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.
 - 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).*
 - 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.*

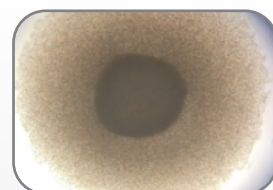


Figure 1. EB from hESC HS181 cultured on Biolaminin 521 LN (LN521), 24 h post-seeding. The EB is surrounded by unaggregated cells. 5x magnification.

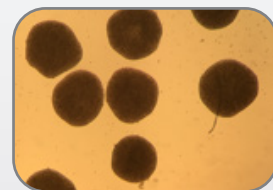


Figure 2. EBs from hESC HS181 cultured on LN521 just transferred to a flat-bottom, low attachment plate. 5x magnification.

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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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