# Embryoid body formation in Nunclon Sphera plates

## Introduction

The significance of stem cells lies in the ability of these cells to become different cell types. The formation of spheroids such as embryoid bodies (EBs) is an important milestone in this differentiation process.

Although several surfaces offering properties of low adhesion are commercially available, spontaneous stem cell differentiation resulting from random cell attachment is still a challenge to many stem cell researchers. The variability seen in spheroid culture has been linked to inconsistent performance of the culture surface in different culture media for different cell types. Here we show that Thermo Scientific™ Nunclon™ Sphera™ plates support EB formation of human embryonic stem cells (hESCs). The surface coating on Nunclon Sphera plates inhibits cell attachment to the culture dish by blocking the adsorption of extracellular matrix (ECM) proteins that usually mediate cell adhesion; the inhibition of attachment to the plate surface promotes cell–cell aggregation *in vitro*. The surface effectively and consistently allows stem cells to grow in suspension with virtually no attachment. The Nunclon Sphera U-bottom 96-well plate provides a specialized format to drive cellular aggregation and generate single EBs in each well (Figure 1).

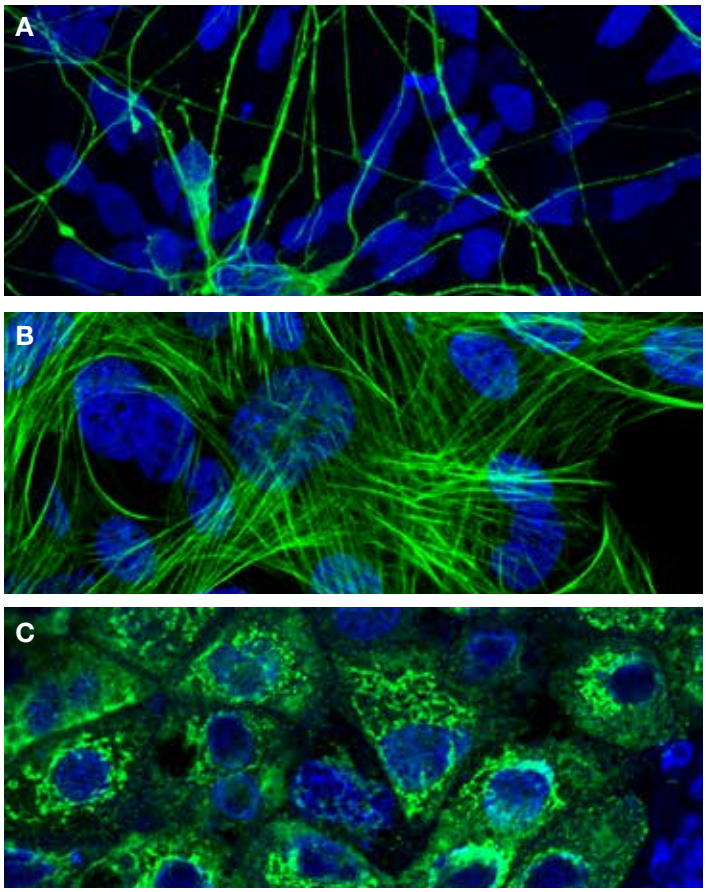


**Figure 1. Differentiation of hESCs derived from EBs cultured in Nunclon Sphera plates.** Cells were stained for the following markers (green) and counterstained with DAPI (blue): **(A)** ectoderm marker β-tubulin III; **(B)** mesoderm marker smooth muscle actin; **(C)** endoderm marker α-fetoprotein.

## Materials

| Product | Source | Cat. No. |
|---|---|---|
| Nunclon Sphera 96U-well plates | Thermo Fisher Scientific | 174925 |
| Essential 8 Medium | Thermo Fisher Scientific | A1517001 |
| Essential 6 Medium | Thermo Fisher Scientific | A1516401 |
| DMEM/F-12 with GlutaMAX Supplement | Thermo Fisher Scientific | 10565018 |
| KnockOut Serum Replacement (KSR) | Thermo Fisher Scientific | 10828010 |
| MEM Non-Essential Amino Acids (NEAA) Solution | Thermo Fisher Scientific | 11140050 |
| 2-Mercaptoethanol | Thermo Fisher Scientific | 21985023 |
| FGF-Basic (AA 1-155) Recombinant Human Protein | Thermo Fisher Scientific | PHG0264 |
| TGF-β1 Recombinant Human Protein | Thermo Fisher Scientific | PHG9204 |
| DPBS, no calcium, no magnesium | Thermo Fisher Scientific | 14190136 |
| StemPro Accutase Cell Dissociation Reagent | Thermo Fisher Scientific | A1110501 |
| Thiazovivin | Fisher Scientific | 38-451-0 |
| PrestoBlue Cell Viability Reagent | Thermo Fisher Scientific | A13261 |
| LIVE/DEAD Viability/Cytotoxicity Kit | Thermo Fisher Scientific | L3224 |

## Protocol

1. Have ready a monolayer culture of hESCs. hESCs can be cultured under feeder-free conditions in either Gibco™ Essential 8™ Medium or hESC growth medium.
2. Prepare 100 mL hESC growth medium by mixing the following reagents:

| Reagent | Volume | Final concentration |
|---|---|---|
| DMEM/F-12 with GlutaMAX Supplement | 79 mL | 1X |
| KSR | 20 mL | 20% |
| NEAA (10 mM) | 1 mL | 0.1 mM |
| 2-Mercaptoethanol (55 mM) | 100 µL | 55 µM |
| FGF-Basic (10 µg/mL) | 40 µL | 4 ng/mL |

   - If stored at 4°C, hESC growth medium can be kept for up to 1 week. Warm the medium at room temperature before use.

3. In a sterile biological safety cabinet, wash the monolayer culture of hESCs with DPBS.
4. Add Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent and incubate for 5–10 min.
5. Harvest and resuspend the cells in either Essential 8 Medium or hESC growth medium.
6. Centrifuge the cells at 250 x *g* for 5 min.
7. Resuspend the cells in either Gibco™ Essential 6™ Medium containing TGF-β1 (1.8 ng/mL final concentration) or hESC growth medium without FGF-basic.
8. Very important: Add thiazovivin to the cells (5 µM final concentration).
9. Seed cells into the wells of a Nunclon Sphera plate (Figure 2). To monitor the growth of EBs on the Nunclon Sphera surface in this study, cells were plated in 200 µL/well at different densities into a Nunclon Sphera 96U-well plate. Seeding density may need to be optimized for each cell line.
10. Centrifuge the plate at 250 x *g* for 5 min.
11. Incubate the plate at 37°C and 5% $CO_2$.
12. Monitor EB formation for up to 2 weeks.
13. Refeed as needed every 72 hr by carefully removing 100 µL of medium from each well and replenishing with 100 µL of fresh medium. Continue to incubate the plate at 37°C and 5% $CO_2$.
14. At the time of harvest, EBs can be collected from the plate by simply pipetting them out using wide-bore pipette tips. Alternatively, many fluorescence and colorimetric assays (e.g., Invitrogen™ PrestoBlue™ assay, LIVE/DEAD™ viability assay) can be easily performed on the Nunclon Sphera plates without the need to transfer the contents to another microplate.

## Results

Growth kinetics of hESC EBs over a period of 12 days were evaluated by size measurement (Figure 3A, B). Data represent the mean of 3 replicates for each starting number of cells.

The PrestoBlue assay was also performed to assess hESC EB health (Figure 3C, D). The fluorescence reading was normalized against EB size for a better quantitative comparison; a higher ratio indicates healthier EBs. Briefly, 12–13 days after seeding, 20 µL of 10X PrestoBlue Cell Viability Reagent was added to each well. Plates were incubated at 37°C and 5% $CO_2$ for 2–5 hr before being read on a fluorescence-based microplate reader (excitation/emission at 560/590 nm).

The viability of hESC EBs was evaluated using the LIVE/DEAD Viability/Cytotoxicity Kit, which stains live cells green and dead cells red (Figure 3E, F). Briefly, 12–13 days after seeding, the spheroids were incubated with LIVE/DEAD staining solution (1 µM calcein-AM and 4 µM ethidium homodimer-1 in DPBS) at room temperature for 30–45 min. The EBs were rinsed 2–3 times by half-volume changes of DPBS before being imaged under a fluorescence microscope (scale bar of 1,000 µm).

## Conclusions

- The Nunclon Sphera 96U-well plate format provides an excellent system to reproducibly generate single EBs in each well.
- The Nunclon Sphera surface, in combination with Essential 6 Medium, provides a completely defined solution for EB generation; the EBs grow faster and are healthier in Essential 6 Medium than in hESC growth medium.
- The Nunclon Sphera plate provides an easy solution for evaluating EB cultures using colorimetric or fluorescence assays such as PrestoBlue and LIVE/DEAD viability assays.

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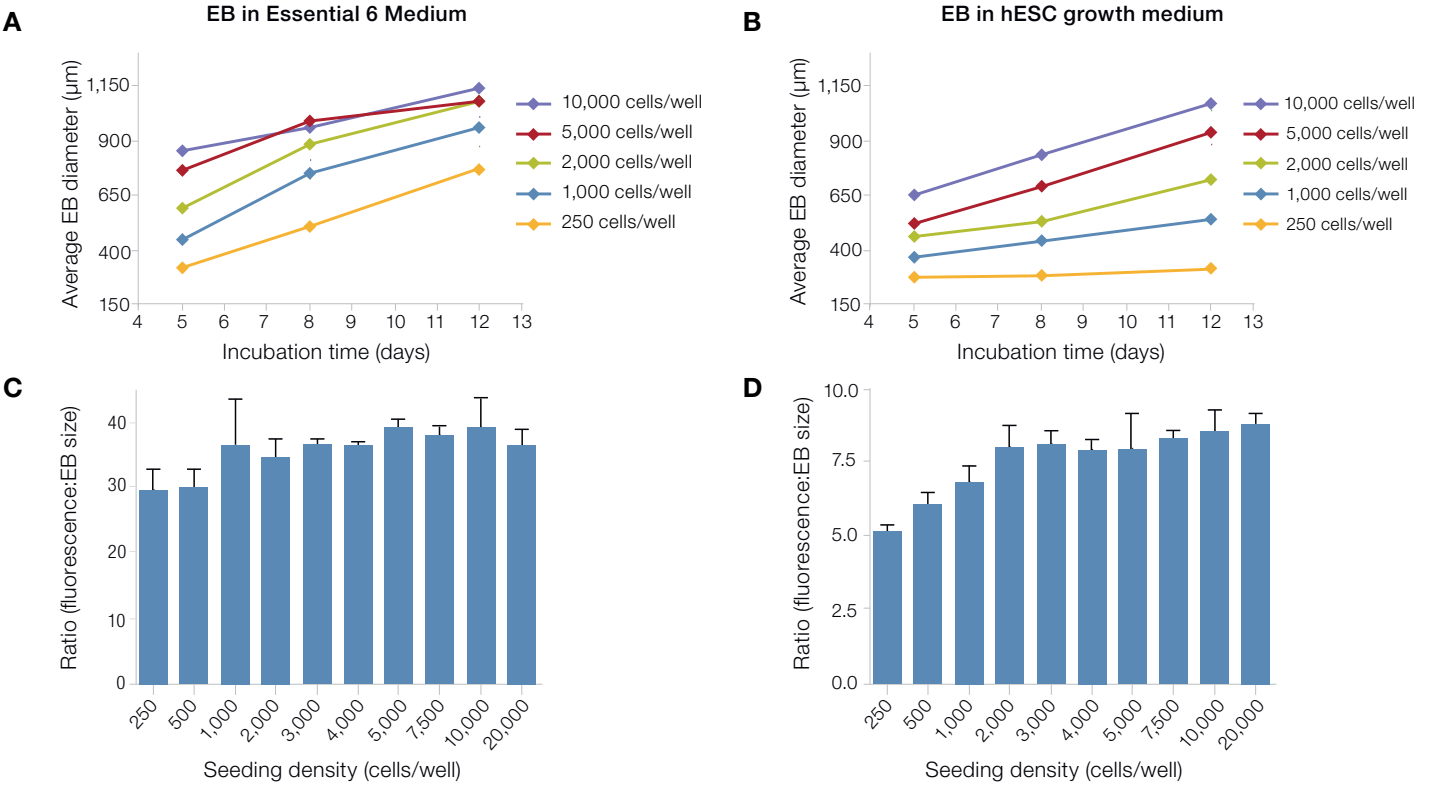
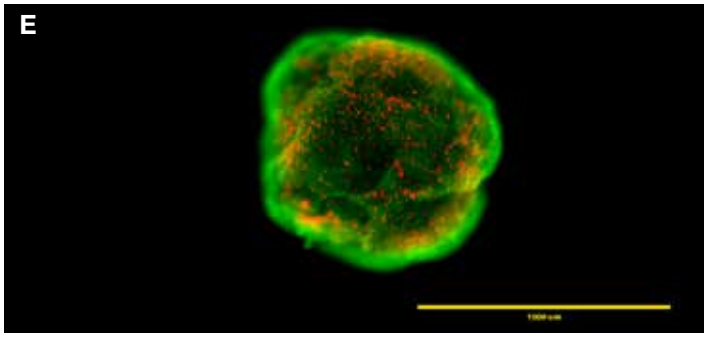
**Figure 2. Seeding of hESCs for EB formation.**



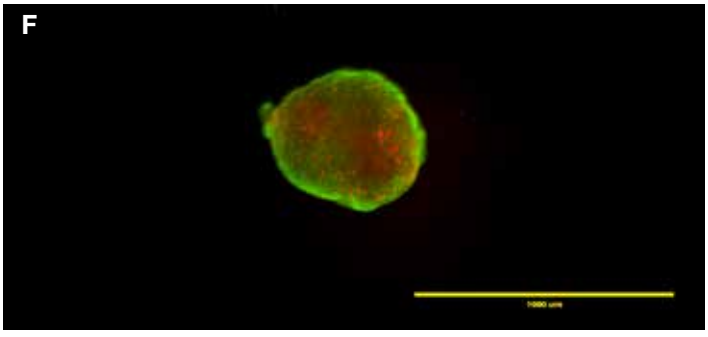




**Figure 3. Assessment of human EB growth kinetics, health, and viability.** EBs were grown on Nunclon Sphera plates in either Essential 6 Medium **(A, C, E)** or hESC growth medium **(B, D, F)**.

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