Application Note

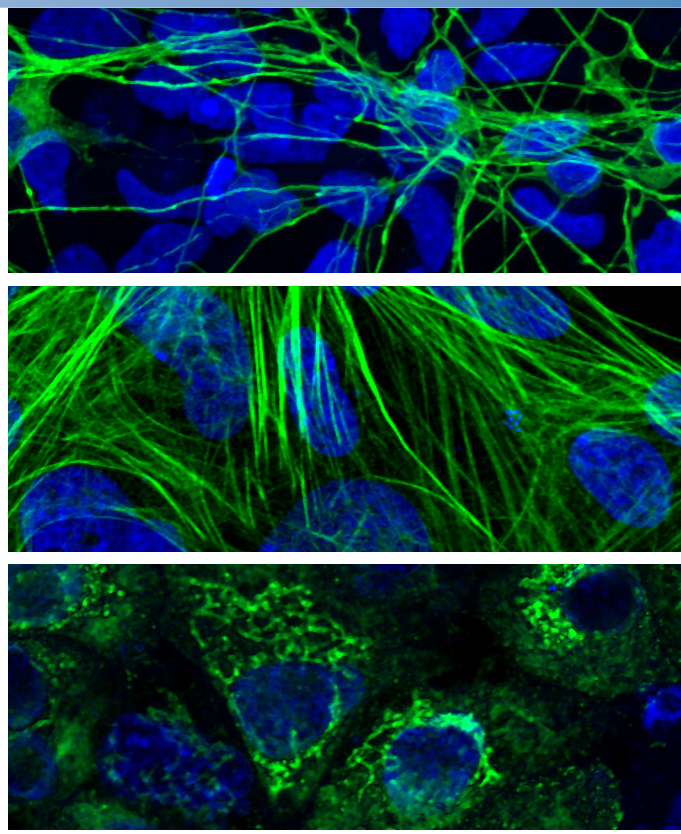
Embryoid body formation in Thermo Scientific Nunclon Sphera plate

Key Words

Nunclon Sphera, Essential 6 Medium, KnockOut Serum Replacement, Embryoid body, Spheroid, U-bottom 96-well plate, Stem Cell.

Introduction

The significance of stem cell research lies in the ability of these cells to become multiple different cell types. The formation of spheroids, such as embryoid bodies (EBs), is an important milestone in this differentiation process. Although several surfaces that offer properties of low adhesion are commercially available, spontaneous stem cell differentiation resulted from random cell attachment is still a challenge to many stem cell researchers. The variability seen in the spheroid culture has been linked to the inconsistent performance of the culture surface in different culture medium for different cell types. Here we introduce Thermo Scientific™ Nunclon™ Sphera™ that supports the EB formation of human embryonic stem cell (hESC). The Nunclon Sphera surface coating inhibits cell attachment to the culture dish by blocking the adsorption of extracellular matrix (ECM) proteins that usually mediate cell adhesion, thereby promoting cell-cell aggregation *in vitro*. It effectively and consistently allows for 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 EB in each well.



Differentiation of hESCs derived from EBs cultured on the Nunclon Sphera surface. Top image: Ectoderm marker ß-tubulin III. Middle image: mesoderm marker smooth muscle actin. Bottom image: endoderm marker alpha fetoprotein. Counter-stained with DAPI.

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Protocol

Key Materials	Source	Catalog Number
96U-well Nunclon Sphera plates	Thermo Scientific	174925 or 174929(Bulk)
Essential 8™ Medium	Life Technologies	A1517001
Essential 6™ Medium	Life Technologies	A1516401
DMEM/F12 with GlutaMAX™ -I	Life Technologies	10565-018
KnockOut™ Serum Replacement (KSR)	Life Technologies	10828010
MEM Non-Essential Amino Acids	Life Technologies	11140050
2-Mercaptoethanol	Life Technologies	21985-023
Recombinant human basic FGF (bFGF)	Life Technologies	PHG0264
TGF-ß	Life Technologies	PHG9204
D-PBS without calcium and magnesium	Life Technologies	14190-136
StemPro® Accutase	Life Technologies	A11105-01
Thiazovivin	Fisher Scientific	38-451-0
PrestoBlue® cell viability reagent	Life Technologies	A13261
LIVE/DEAD® Viability/Cytotoxicity Kit	Life Technologies	L3224

1. The human ESCs were cultured under feeder free conditions in either hESC growth medium or Essential 8 medium.

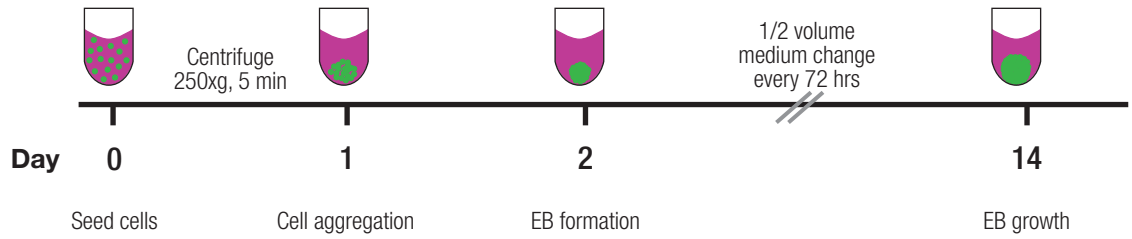
Prepare 100 ml hESC growth medium by mixing following reagents:

Reagent	Volume	Final concentration
DMEM/F12 with GlutaMAX-1	79 ml	1X
KSR	20 ml	20%
NEAA (10 mM)	1 ml	0.1 mM
2-Mercaptoethanol (55 mM)	100 µl	55 µM
bFGF (10 µg/ml)	40 µl	4 ng/ml

If stored at 4°C, hESC growth medium can be kept for up to 1 week.

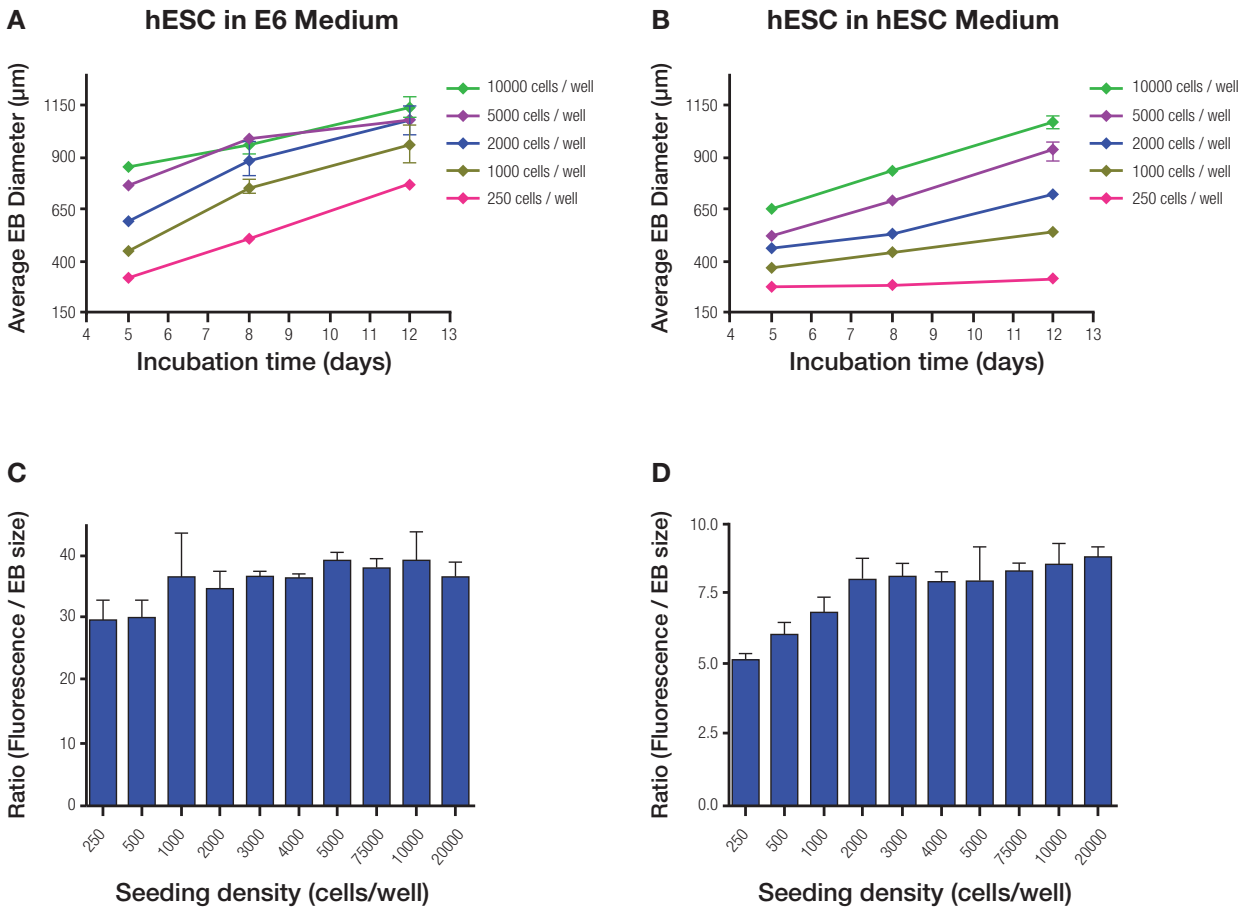
Warm the medium at room temperature before use.

2. In a sterile Biological Safety Cabinet wash the cells with D-PBS.
3. Add StemPro Accutase and incubate for 5-10 min.
4. Harvest and resuspend cells in either hESC growth medium or Essential 8 medium.
5. Centrifuge cells at 250 X g for 5 min.
6. Resuspend the cells in either the hESC growth medium without bFGF or the Essential 6 medium containing TGF-ß (1.8 ng/ml final concentration).
7. [Very Important] Add Thiazovivin to the cells (5 µM final concentration).



8. To monitor the growth of EBs on Nunclon Sphera surface, cells are plated in 200 µl/well at different densities, into a 96 U-well Nunclon Sphera plate. Seeding density may need to be optimized for each cell line.
9. Centrifuge the plate at 250 X g for 5 minutes.
10. Incubate the Nunclon sphera plate at 37°C and 5% CO2.
11. Monitor the EB formation up to 2 weeks.
12. Re-feed as needed every 72 hrs 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% CO2.
13. At time of harvest, EBs can be collected from the plate by simply pipetting out using wide bore pipette tips. Alternatively, many fluorescence- and colorimetric-based assays (e.g. PrestoBlue assay, LIVE/DEAD viability assay) can be easily performed on the Nunclon Sphera plates without needing to transfer the contents to another microplate.

EB Evaluation



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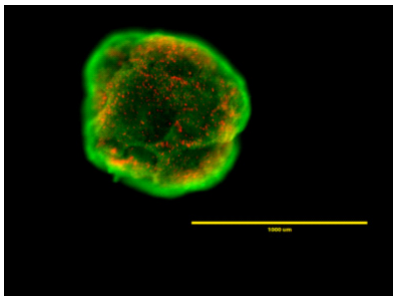

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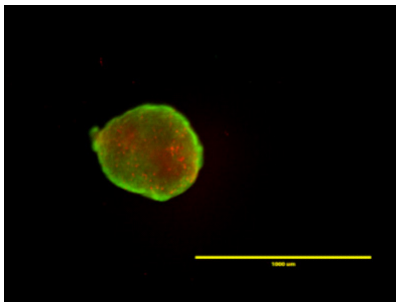

Assessment of human EB growth kinetics, EB health, and viability on Nunclon Sphera plate in either E6 medium (A, C, and E) or hESC growth medium (B, D, and F).

(A and B) Growth kinetics of hESC EB over period of 12 days is evaluated by size measurement. Data represents the mean ± SD of 3 replicates for each cell number.

(C and D) PrestoBlue assay are preformed to assess hESC EB health. The fluorescence reading was normalized against EB size for better quantitative comparison – higher ratio indicates healthier EBs. Briefly, 12-13 days after seeding, 20 µl of 10X PrestoBlue are added to each well. Plates are incubated at 37°C and 5% CO_2 for 2-5 hrs before being read on a fluorescence-based microplate reader (excitation and emission 560/590 nm or absorbance 570-600 nm).

(E and F) The viability of hESC EBs is evaluated by Live (green)/Dead (red) staining assay. Briefly, 12-13 days after seeding, the spheroids are incubated with Live/Dead staining solution (1 µM Calcein AM and 4 µM Ethidium bromide-1 in D-PBS) at room temperature for 30-45 min. The EBs are rinsed 2-3 times by half volume change of D-PBS before being imaged under fluorescence microscope (scale bar 1000 µm).

Conclusions

1. The Nunclon Sphera 96U-well plate format provides an excellent system to reproducibly generate single EB in each well.
2. 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 the Essential 6 medium as compared to that in the hESC growth medium.
3. The Nunclon Sphera plate provides an easy solution for evaluating the EB culture by colorimetric or fluorescence assays such as PrestoBlue assay and LIVE/DEAD Viability assay.

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