Simplifying Progress

# A Versatile Animal-Component Free Medium For hPSC Monolayer And Mid-Scale Aggregate Suspension Culture

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## Introduction

In the last several years, the number of clinical trials with human pluripotent stem cells (hPSC)-based therapies is rapidly increasing. These advancements raise the need for safe a and efficient system towards large scale expansion. Production of hPSCs in large quantities for clinical applications using standard 2D adherent culture is inefficient and process scalability is difficult. A developing strategy to overcome these hurdles, is 3D suspension culture of pluripotent aggregates in environmentally controlled bioreactors. This approach enables reproducible production of high numbers of pluripotent stem cells that can be used for differentiation into mature cells of various tissue types.

Aiming for therapeutic applications, the quality of culture medium and its performance are particularly crucial, since hPSC properties can be affected significantly by medium composition and culture conditions. More than that, with the stringent regulatory requirements, the need for an Animal-Component Free (ACF) culture media is preferred to minimize risks associated with infectious agents' transmission and immune rejection of the transplanted cells.

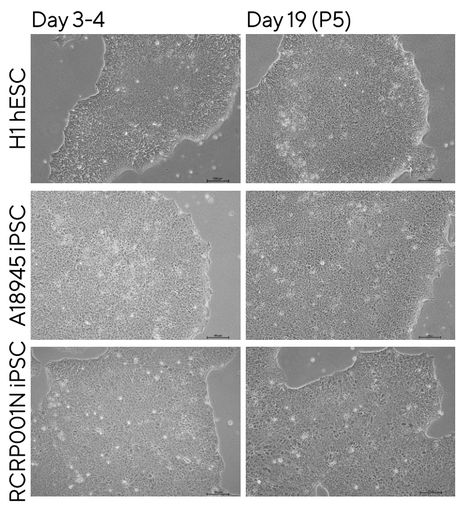
The present study describes the development of a versatile ACF medium suitable for hPSC expansion using recombinant matrices (e.g., Laminin, Vitronectin) in 2D culture for multiple passages and as aggregates in dynamic small-scale suspension culture (e.g., shaker flasks, spinner flasks). This ACF medium was used in design of experiment (DOE) studies implementing MODDE® software to identify optimal design space for aggregate formation and growth in the Ambr® 250 modular bioreactor. Results show that ACF medium enables high proliferation rate of hPSC, while maintaining high pluripotency marker expression and stable karyotype. This advanced culture system would greatly facilitate the development of a robust, clinically relevant culture process for generating quality-assured cells.

## 1. Monolayer hPSC expansion system using Nutri3D hPSC ACF

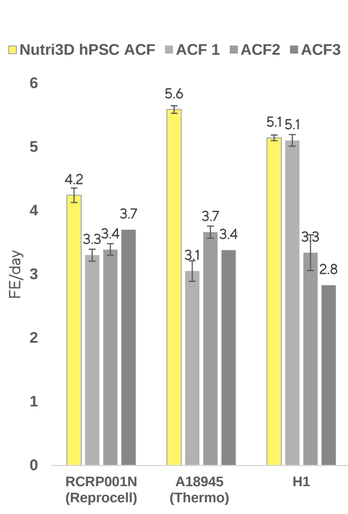
H1 hESC, A18945 (Thermo), and RCRP001N (Reprocell) hPSC lines were expanded for 5 passages (11-19 days) on Laminin521 or ACF Vitronectin coated-cultureware in Nutri3D hPSC ACF and a variety of ACF media. For Laminin based culture, cells were seeded at 4-15k cells/cm$^2$. Following expansion, cells were dissociated using recombinant Trypsin EDTA solution and evenly re-seeded at 4-15k cells/cm$^2$ every 3-4 days. For ACF Vitronectin based culture cells were dissociated to mini clumps using 0.5mM EDTA and spilt of 1:12-1:25 was done every 3-4 days. Cells from P5 were tested for pluripotency.

Results show that Nutri3D hPSC ACF supports high proliferation of hPSC on Laminin521 and ACF Vitronectin based 2D monolayer culture while maintaining classic morphology, high proliferation and high pluripotent marker expression. Significant advantage was observed when using Nutri3D hPSC ACF over other ACF media.

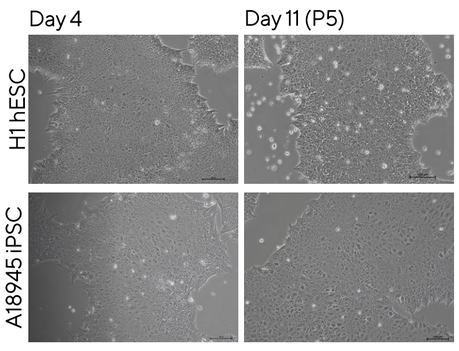
1A-1. ACF Vitronectin based 2D culture morphology



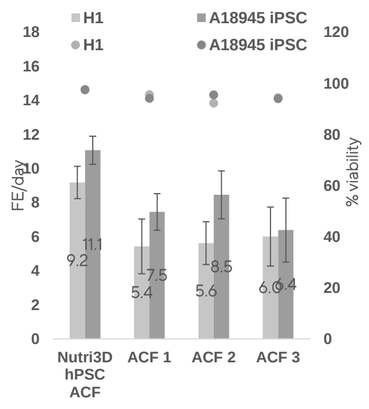

1A-2. Proliferation- Nucleocounts




1B-1. Laminin based 2D culture morphology




1B-2. Proliferation- viable cell counts




1C. Pluripotency assessment (FACS)

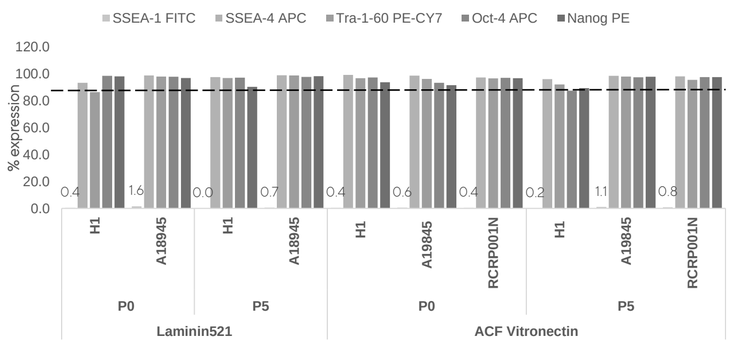



Figure 1: (A-1) Representative images (x100) of ACF Vitronectin based hPSC culture state at days 3-4 and 19 (P5). (A-2) Average FE/day of 5 passages for vitronectin culture (Nucleocounts ).
(B-1) Representative images (x100) of Laminin521 based hPSC culture state at days 4 and 11 (P5).
(B-2) Average FE/day and viability (%) during 5 passages expanded. (C) Immunophenotyping analysis for pluripotent markers of hPSC before expansion (P0) and at P5.

## 4. Conclusions

The data presented demonstrates that Nutri3D hPSC ACF is a versatile medium that allows a full ACF system for the expansion of hPSC cells in 2D monolayer culture using recombinant matrices; Laminin or Vitronectin and subsequent 3D suspension culture as aggregates in small- to mid-scale expansion systems.

Nutri3D hPSC ACF supports:

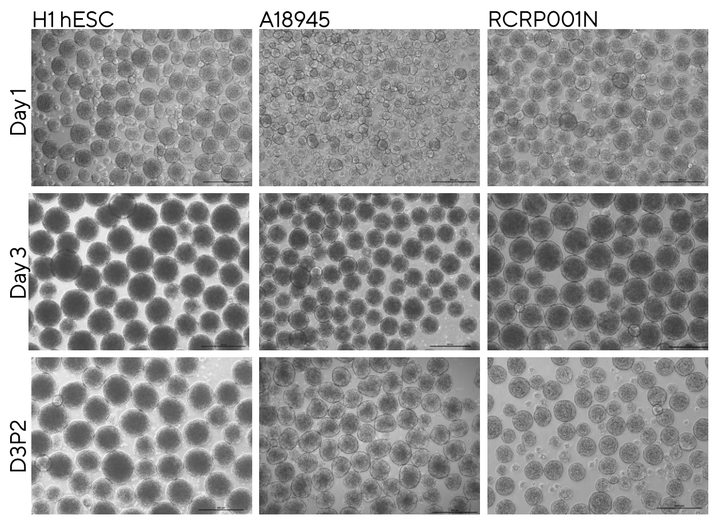
- Monolayer culture: High proliferation rates of hPSC on ACF Vitronectin and Laminin-coated cultureware, while maintaining typical colony morphology and high pluripotent marker expression.
- Small-scale aggregate suspension culture (EF, spinner): Efficient aggregation, high proliferation for multiple sequential passages while maintaining pluripotency state, trilineage differentiation potential and genomic stability.
- Mid-scale aggregate suspension culture: The Sartorius ambr 250 modular bioreactor platform in combination with Nutri3D hPSC ACF medium allows for the formation of highly viable iPSC aggregates, that reach high cell densities and importantly, maintain their stem cell relevant critical quality attributes including pluripotency.

## 2. Small- scale hPSC aggregates suspension culture using Nutri3D hPSC ACF

H1 hESC and A18945 iPSC from a Laminin521 based 2D culture were dissociated into single cells with recombinant Trypsin solution. RCRP001N iPSC from ACF Vitronectin based 2D culture was dissociated into single cells with 0.5mM EDTA solution. All cultures were counted and seeded at 120k cells per ml in total of 20ml in disposable Erlenmeyer shake flasks (EF) at 70rpm. 10µM ROCK inhibitor (Thiazovivin) was added to the single-cell suspension for the first 24 hours. Starting from day 1, at least 50% of the medium was removed and replaced with fresh medium. As aggregates diameter reached ~300µm, culture was dissociated using Accutase. Cells from P3 and P5 in the EF were characterized for pluripotency, differentiation potential (trilineage differentiation kit, Miltenyi) and genomic stabiity.

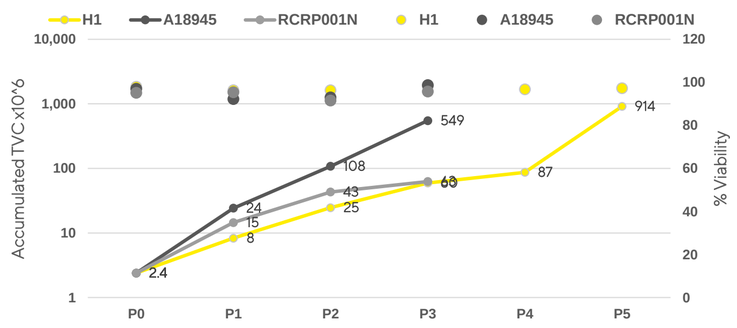
Results show that Nutri3D hPSC ACF supports high hPSC expansion rate as aggregates in EF suspension culture for multiple sequential passages, while maintaining high proliferation rate, pluripotency and differentiation potential.

2A. Aggregates culture morphology (x40)

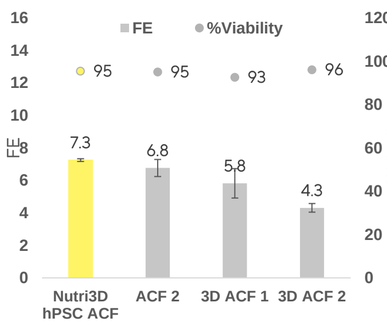



2B. Proliferation- cell counts of aggregates suspension culture

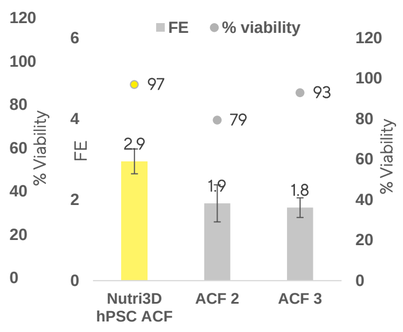
2B-1. Accumulated results



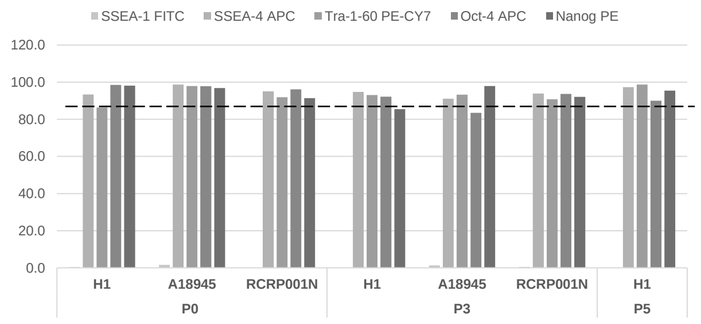

2B-2. RCRP001 iPSC- P3



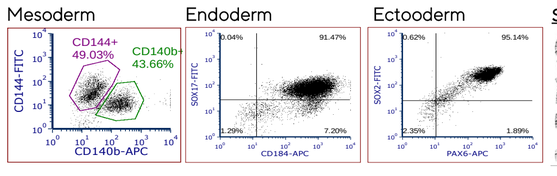

2B-3. H1 hESC- P3




2C. Pluripotency assessment (FACS)




2D. Tri-lineage differentiation potential (FACS)




2E. Genomic stability

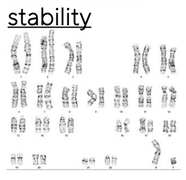


Figure 2: (A) Representative images (x40) of hPSC aggregates suspension culture at indicated day.
(B-1) Accumulated Total viable cells (x10^6), % viability over multiple passages. (B2-3) Average fold expansion and viability of hPSC in Nutri3D hPSC ACF and a variety of other ACF media at P3.
(C) Immunophenotyping analysis for pluripotent markers of hPSC at the indicated passage.
(D) Immunophenotyping analysis for germ layer specific markers. (E) G-banding karyotype analysis of H1 hESC aggregates suspension culture at P5 passages in Nutri3D hPSC 3D ACF.

## 3. Mid-scale hPSC aggregates suspension culture using Nutri3D hPSC ACF

A18945 hPSC were thawed and expanded in NutriStem hPSC XF medium supplemented with 40 ng/mL FGF for 3 passages (4 days per passage) on ACF Vitronectin coated-cultureware. Following expansion, the culture was dissociated into single cells using Accutase and seeded into the Ambr® 250 modular bioreactor system at 250mL per vessel. Seeding density, stir speed, vessel type and media type were varied as shown below. 10 µM ROCK inhibitor (ATCC) was added to the single cell suspensions for the first 24 hours. Starting from day 1, 80% of the medium was removed and replaced with fresh medium. Culture duration was 5 days, at which point aggregates were dissociated using Accutase.

Results show that Nutri3D hPSC ACF supports high hPSC expansion as aggregates with high viability and importantly, maintain pluripotency as measured by appropriate marker expression in the iQUE3. The Nutri3D hPSC ACF medium outperformed NutriStem hPSC XF medium in terms of both harvest density and fold expansion.

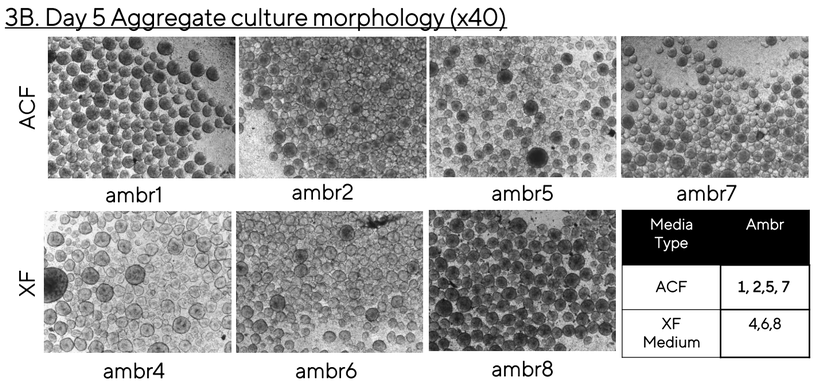
3A. DOE Matrix of Conditions

| Parameter | ambr 1 | ambr 2 | ambr 4 | ambr 5 | ambr 6 | ambr 7 | ambr 8 |
|---|---|---|---|---|---|---|---|
| Seeding Density, cells/mL | 2.5e5 | 2.5e5 | 2.5e5 | 4e5 | 4e5 | 4e5 | 4e5 |
| Stir Speed, RPM | 250 | 400 | 400 | 400 | 400 | 250 | 250 |
| Vessel Type | B | U | B | B | U | U | B |
| Media Type | ACF | ACF | XF | ACF | XF | ACF | XF |

ACF = Nutri3D hPSC ACF
XF = NutriStem hPSC XF
B = Baffled, dual impeller
U = Unbaffled, single impeller

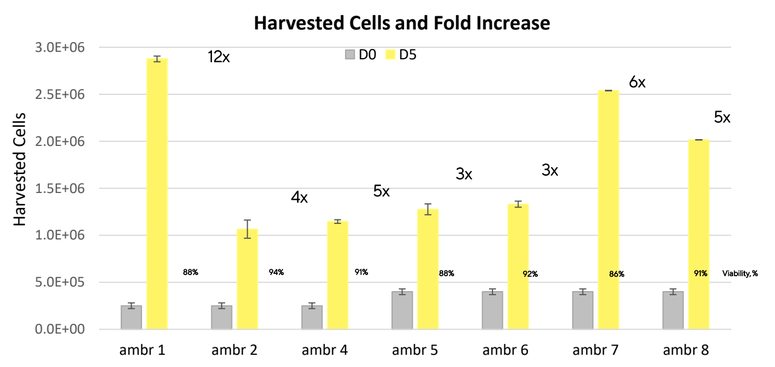
Ambr3 not included in study.

3B. Day 5 Aggregate culture morphology (x40)




| Media Type | Ambr |
|---|---|
| ACF | 1, 2,5, 7 |
| XF Medium | 4,6,8 |

3C. Proliferation- Viable Cell Counts & Fold Increase




3D. Pluripotency assessment (FACS)

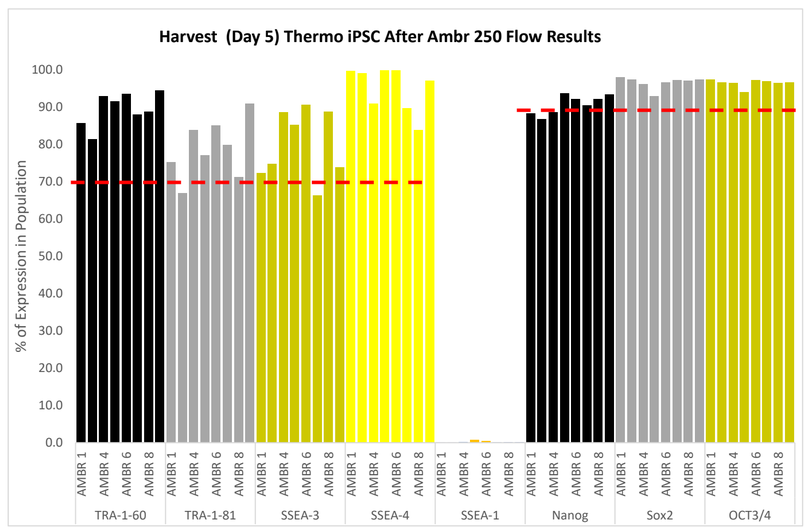



Figure 3: (A) Parameters utilized in the study. (B) Representative images (x40) of hPSC aggregate suspension cultures at day 5. (C) Cell Yield and fold induction achieved. (D) Flow cytometry results of aggregate cultures.

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