EXPANSION OF PLURIPOTENT HPSC IN VARIOUS VITRONECTIN-BASED CULTURE SYSTEMS

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Abstract

Human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC), collectively referred as human pluripotent stem cells (hPSC), are able to differentiate into the three germ layers of the human embryo, and are presumed to have the capacity for self-renewal in vitro. Consequently, they possess great potential for cell-based therapy and differentiation studies.

Culture conditions, including culture media and matrix, have a substantial effect on pluripotency. The most common feeder-free matrices are Matrigel and recombinant proteins that support hPSC self-renewal such as laminin isoforms and vitronectin. NutriStem® V9 XF is a defined, xeno-free and serum-free medium specially designed to support the growth and expansion of hPSC using vitronectin matrix. The medium contains low concentration of human proteins and only the essential components required for long-term maintenance of hPSC.

The present study evaluates long term expansion of hPSC using NutriStem® V9 XF on Vitronectin ACF. Two Vitronectin-based culture systems were tested: pre-coated plates as well as precoating-free procedure by direct addition of the Vitronectin ACF to the culture medium.

Results show that NutriStem® V9 XF medium enables high proliferation rate in long-term culture, while maintaining stable karyotype, high pluripotency marker expression, and preservation of tri-lineage differentiation potential of hPSC.

Materials and Methods

Culture system

Medium: NutriStem® V9 XF (BI). A defined, xeno-free, serum-free medium specially formulated to support the growth and expansion of hPSC using vitronectin and enzyme-free passage with EDTA.
Cells: H1 hESC and ACS1019 hiPSC (ATCC).
Vitronectin pre-coating procedure: Tissue culture 6-well plate incubated with Vitronectin ACF diluted in DPBS w/o calcium and magnesium for an hour at room temperature.
Vitronectin precoating-free procedure: Vitronectin ACF (BI) added into equilibrated NutriStem® V9 XF medium prior to cell seeding.
Culture system: Cells expanded in NutriStem® V9 XF (BI) on pre-coated culture ware with Vitronectin ACF or using precoating-free procedure. Near confluent culture (70% confluency) harvested by non-enzymatic dissociation as small aggregates using 0.5mM EDTA solution (BI) (split ratio of 1:10-1:20) with a weekend-free feeding regime by complete medium change from the second day post-seeding followed by daily feed until culture reaches 70% confluency.

Nucleocount

Nucleocounts of hPSC performed on cell clumps suspension post-EDTA dissociation using a total aggregates count A100+B assay (Chemometec).

Embryoid bodies formation

Embryoid bodies (EBs) generated by harvesting H1 hESC using non enzymatic dissociation as small aggregates with 0.5mM EDTA solution (BI), suspended in NutriStem® V9 XF basal medium (BI) in ultra-low attachment T25 flasks on shaker platform where they spontaneously differentiate with medium change very 2-3 days.

Immunophenotyping

hPSC were expanded for several passages in NutriStem® V9 XF on Vitronectin ACF, harvested and labeled with antibodies against pluripotent markers. Internal staining; Oct-4 APC, NANOG PE and SOX-2 FITC or external staining; TRA-1-60 PerCP, SSEA-1 FITC, SSEA-4 APC. Flow cytometry data acquired using a Stratedigm flow cytometry system and FCS Express analysis software (De Novo).

Real-time PCR

Pluripotent hPSC expanded in NutriStem® V9 XF on Vitronectin ACF or EBs at 18 days of culture generated from matched passage were collected followed by RNA extraction, cDNA preparation. Quantitative real-time PCR performed using TaqMan® universal PCR master mix (Applied Biosystems), gene-specific TaqMan PCR probes and primers. Each sample was tested in duplicates, calibrated to Beta-actin (ACTB2) and GAPDH and as % expression from cells before expansion.

Immunofluorescence staining

hPSC were expanded for several passages in NutriStem® V9 XF on Vitronectin ACF, fixed and stained for the classical pluripotent markers Oct-4 Alexa Fluor 488 (Merck), NANOG Cy3 (Merck), TRA-1-60 (BioLegend), and DAPI counterstaining.

Histological assessment

EBs from 18 days of culture were fixed in 4% formaldehyde, trimmed, embedded in paraffin, sectioned at no more than 5 micron thickness, and stained with Hematoxylin & Eosin (H&E). Photos were taken using a microscope (Olympus BX60).

Karyotype

Genomic stability of hPSC was tested by G-banding karyotype analysis.

Abbreviations

hESC	Human Embryonic Stem Cells
hiPSC	Human Induced Pluripotent Stem Cells
hPSC	Human Pluripotent Stem Cells
ACF	Animal-Component Free
VTN	Vitronectin
RQ	Relative Quantifications
EC	Ectoderm
ME	Mesoderm
END	Endoderm

Results

Validation of NutriStem® V9 XF and pre-coating procedure using Vitronectin ACF

Expansion

Figure 1:
Typical hPSC colony morphology during long-term culture

Phase contrast images (x100) of H1 hESC and hiPSC maintained in NutriStem® V9 XF using 0.5µg/cm² Vitronectin ACF-coated culture ware. Representative images from culture at passage 6 (P6), passage 16 (P16), and passage 30 (P30).
NutriStem® V9 XF maintains typical undifferentiated hPSC colony morphology during long-term culture expansion.

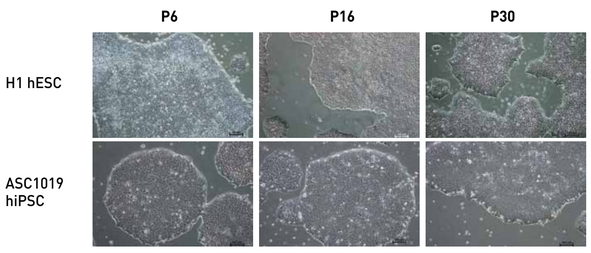


Figure 2:
Cell counts during long-term culture expansion

Proliferation during long-term expansion on 0.5µg/cm² Vitronectin ACF and passage as small aggregates every 3-5 days using 0.5mM EDTA solution. Nucleocounts performed on cell clumps suspension post-EDTA dissociation. Results presented as the average of accumulated counts from cell seeding up to the indicated passage (e.g.P6- from seeding up to P6).
(A) H1 hESC expanded in NutriStem® V9 XF or E8.
(B) H1 hESC and ACS 1019 hiPSC expanded in NutriStem® V9 XF.
NutriStem® V9 XF promotes high and consistent proliferation rates of hPSC during long-term culture.

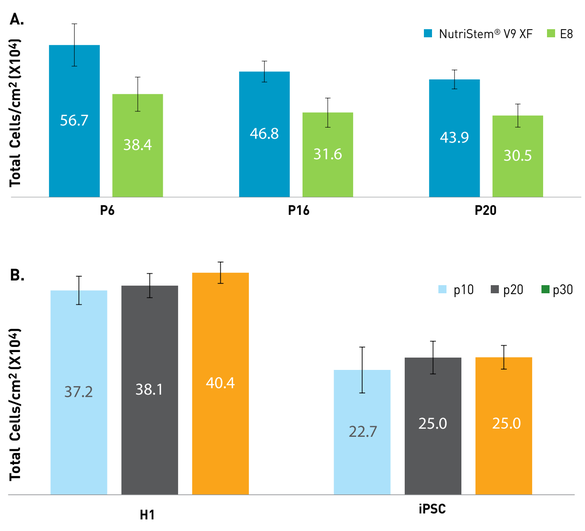


Figure 3:
Genomic stability

G-banding karyotype analysis of H1 hESC and ACS1019 hiPSC culture expanded for 30 passages in NutriStem® V9 XF on Vitronectin ACF.
H1 hESC cultured in NutriStem® V9 XF medium maintained normal karyotype through multiple passages.

Pluripotency assessment

Figure 4:
Immunophenotyping

Immunophenotyping analysis for pluripotent markers of hPSC culture maintained in NutriStem® V9 XF or E8 medium using 0.5µg/cm² Vitronectin ACF at P8 and P17. Data presented as % expression from gated viable cells. (A) H1 hESC expanded in NutriStem® V9 XF or E8. (B) H1 hESC and ACS 1019 hiPSC expanded in NutriStem® V9 XF.
High expression of pluripotent stem cell markers preserved in hPSC expanded in NutriStem® V9 XF medium on vitronectin matrix.

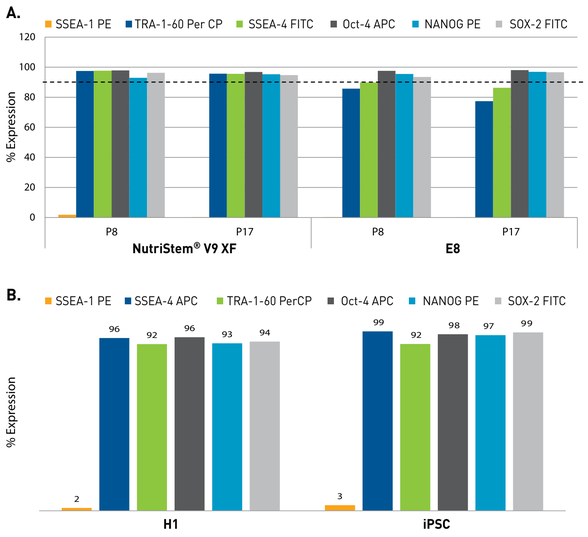


Figure 5:
Immunofluorescence staining

Immunofluorescence analysis of human pluripotent markers of H1 hESC expanded in NutriStem® V9 XF using Vitronectin ACF. Representative images of H1 cultured in NutriStem® V9 XF (P29), fixed and stained for the classical pluripotent surface markers: TRA-1-60 RRX (red) and nuclear conjugated markers: Oct-4 Alexa fluor 488 (green), NANOG cy-3 (red), both counterstained with DAPI (blue). Scale bar 200µm.
Cells cultured in NutriStem® V9 XF medium on vitronectin matrix express high levels of pluripotent markers.

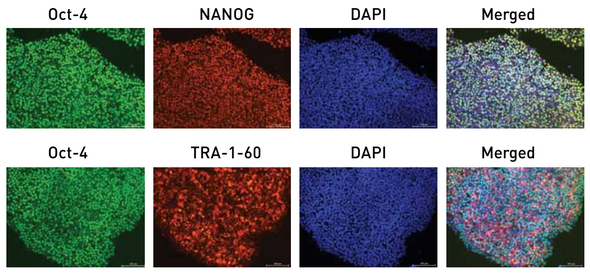
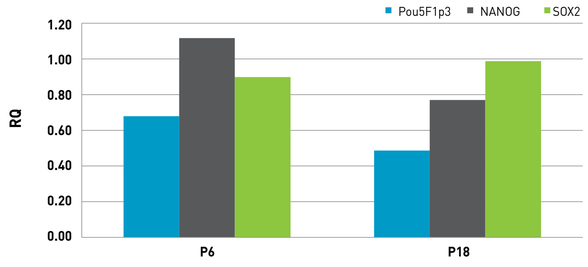


Figure 6:
Gene expression

Real-time PCR analysis for human pluripotent genes of H1 hESC maintained in NutriStem® V9 XF using 0.5µg/cm² Vitronectin ACF at P6 and P18. Results calibrated to ACTB2 and GAPDH and presented as relative expression from H1 hESC at P0 before long-term expansion.
NutriStem® V9 XF medium supports high pluripotent gene expression in cells during long-term culture.



Tri-lineage differentiation potential confirmation

Figure 7:
EB formation – Histological assessment

Embryoid bodies (EBs) were generated from H1 hESC expanded for 6 passages in NutriStem® V9 XF medium on Vitronectin ACF as an evaluation of pluripotency. Cells were suspended in NutriStem® V9 XF basal, where they spontaneously formed EBs for 18 days. Tissue types were identified by examination of EBs histological sections stained with H&E. (X10)
NutriStem® V9 XF medium supports tri-lineage differentiation into the 3 germ layers.

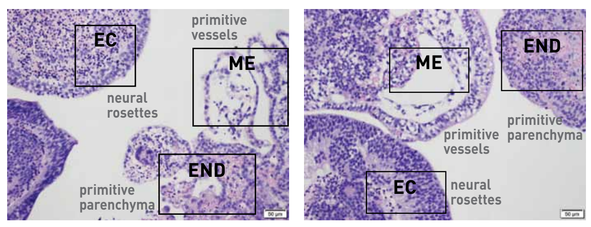
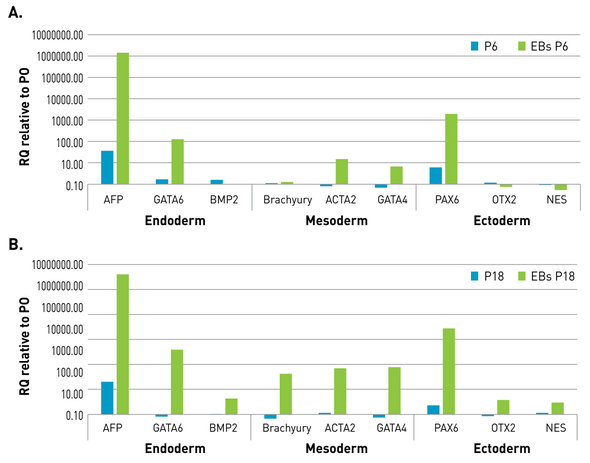


Figure 8:
EB formation – Gene expression analysis

Embryoid bodies (EBs) were generated from H1 hESC expanded for 6 passages (A) or 18 passages (B) in NutriStem® V9 XF medium on Vitronectin ACF as an evaluation of pluripotency. Cells were suspended in NutriStem® V9 XF basal, where they spontaneously formed EBs. Real-time PCR analysis for differentiation genes from the 3 germ layers calibrated to ACTB2 and GAPDH. Results presented as % expression from H1 hESC before long-term expansion.
NutriStem® V9 XF medium supports tri-lineage differentiation into the 3 germ layers.



Validation of Vitronectin ACF precoating-free procedure

Precoating-free Procedure

Novel procedure in which Vitronectin ACF matrix is added directly into NutriStem® V9 XF medium, eliminating the need for pre-coating.

Figure 9:
Morphology

Phase contrast images (x100) of H1 hESC and hiPSC ACS1019 culture expanded in NutriStem® V9 XF for 30 sequential passages using precoating-free procedure by adding Vitronectin ACF directly to NutriStem® V9 XF medium before cells seeding. Representative images from culture at P6, P16, and P30.
Typical undifferentiated hPSC colony morphology maintained during long-term expansion in NutriStem® V9 XF using a pre-coating-free culture procedure.

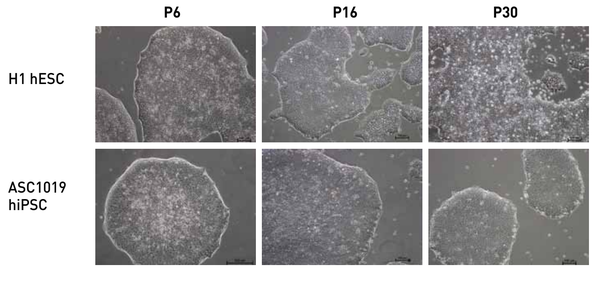


Figure 10:
Proliferation

Cell counts of hPSC during long-term culture (30 passages) in NutriStem® V9 XF using Vitronectin ACF precoating-free protocol by adding Vitronectin ACF directly to NutriStem® V9 XF medium before cells seeding. Nucleocounts performed on cell clumps suspension post-EDTA dissociation. Results presented as the average of accumulated counts from cell seeding up to the indicated passage (e.g.P10- from seeding up to P10).

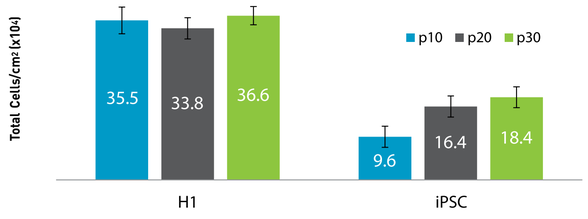


Figure 11:
Immunophenotyping

Flow cytometry analysis of H1 and hiPSC ACS1019 cultured in NutriStem® V9 XF for 30 sequential passages using pre-coating and precoating-free procedure by adding Vitronectin ACF directly to NutriStem® V9 XF medium before cells seeding. Data presented as % expression from gated viable cells.
NutriStem® V9 XF supports high expression of pluripotent stem cell markers in hPSC expanded using a precoating-free procedure.

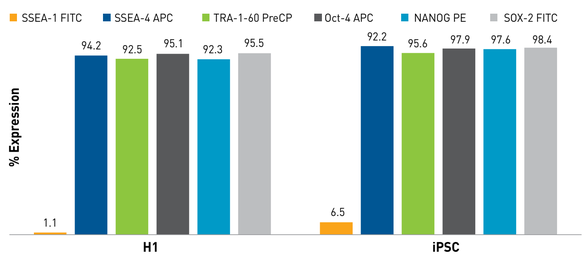
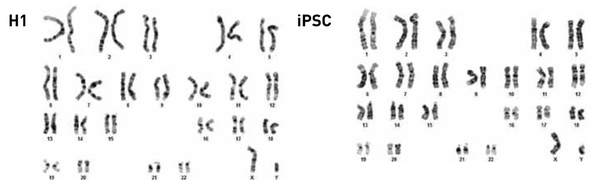


Figure 12:
Genomic stability

G-banding karyotype analysis of H1 hESC and ACS1019 hiPSC expanded for 30 passages in NutriStem® V9 XF using Vitronectin ACF precoating-free procedure.
hPSC cultured in NutriStem® V9 XF medium maintained normal karyotype through multiple passages using precoating-free procedure.



Summary

NutriStem® V9 XF is a versatile medium that allows the expansion of hPSC in both standard Vitronectin ACF pre-coating procedure as well as a precoating-free procedure by adding the vitronectin directly to the medium just before cell seeding.

NutriStem® V9 XF supports high proliferation rates of hPSC during long-term culture while maintaining typical pluripotent colony morphology, high pluripotent gene expression, high pluripotent marker expression, and stable karyotype.