Novel xeno-free, serum-free vitronectin-based culture system for hPSC

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Abstract

Human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC), collectively referred to 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.

Culture conditions, including culture media, 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, serum-free medium specially formulated to support the growth and expansion of hPSC using vitronectin and enzyme-free passage with EDTA. The medium contains a low concentration of growth factors and only the essential components required for long-term maintenance of hPSC.

NutriStem® V9 XF medium allows a high proliferation rate in long-term culture, while maintaining stable karyotype, high pluripotency marker expression, and tri-lineage differentiation potential of hPSC. In addition, NutriStem® V9 XF medium supports the culture of hPSC with direct addition of Vitronectin ACF (animal component-free) to the medium without the need for pre-coating of cultureware.

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-coated 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 cultureware with vitronectin or using precoating-free procedure. Near confluent culture (70% confluency) harvested using non-enzymatic dissociation as a small aggregate 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; OCT4-APC, NANOG-PE and SOX-2 FITC or external staining; Tra-1-60-PerCP, SSEA1-FITC, SSEA4-APC. Flow cytometry data acquired using a Stratedigm device 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 differentiation generated from matched passage were collected followed by RNA extraction, cDNA preparation and 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 (Merck), NANOG Cy3 (Merck), SSEA-4 (BioLegend), Tra-1-60 (BioLegend), and DAPI counterstaining.

Histological assessment

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

Karyotype

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

Abbreviations

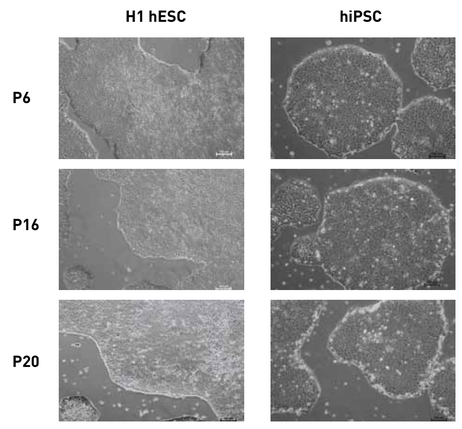
hESC	Human embryonic stem cells
hiPS	Human induced pluripotent stem cells
hPSC	Human pluripotent stem cells
ACF	Animal-component free
VTN	Vitronectin
RQ	Relative quantifications
EC	Ectoderm
ME	Mesoderm
END	Endoderm

Results

Validation of NutriStem® V9 XF and pre-coating procedure

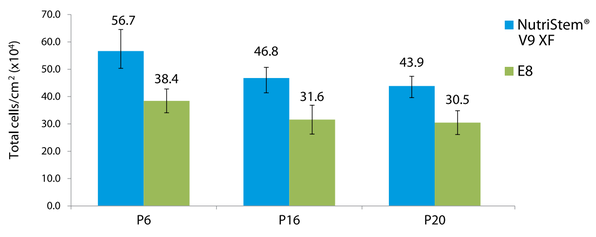
Expansion

Fig 1: **Typical hPSC colony morphology during long-term culture**



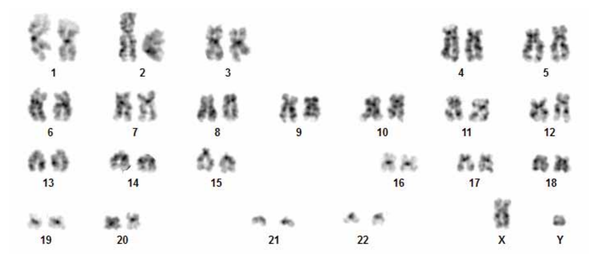
Phase contrast images of H1 hESC and hiPSC culture maintained in NutriStem® V9 XF using 0.5µg/cm² Vitronectin ACF-coated cultureware. Representative images from culture at passage 6 (P6), passage 16 (P16) and passage 20 (P20) (x100). **NutriStem® V9 XF maintains typical undifferentiated hPSC colony morphology during long-term culture expansion.**

Fig 2: **Cell counts during long-term culture expansion**



Nucleocounts during long-term expansion in NutriStem® V9 XF and E8 using 0.5µg/cm² Vitronectin ACF and passage as small aggregates every 3-5 days using 0.5mM EDTA solution. **NutriStem® V9 XF promotes high proliferation rates of hESC during long-term culture.**

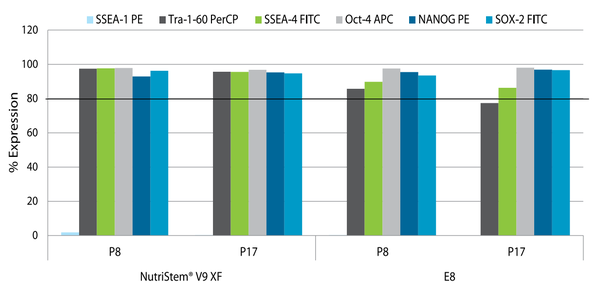
Fig 3: **Genomic Stability**



G-banding karyotype analysis of H1 hESC expanded for 17 passages in NutriStem® V9 XF on Vitronectin ACF.
H1 hESC grown in NutriStem® V9 XF medium maintained normal karyotype through multiple passages.

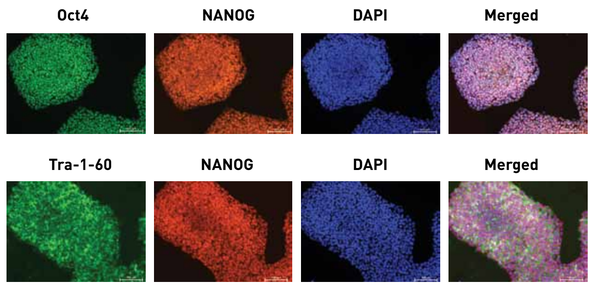
Pluripotency assessment

Fig 4: **Immunophenotyping**



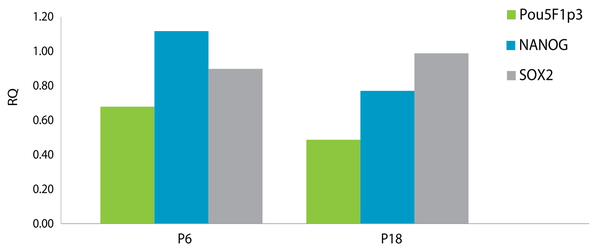
Immunophenotyping analysis for pluripotent markers of H1 hESC culture maintained in NutriStem® V9 XF using 0.5µg/cm² Vitronectin ACF at P8 and P17. Data presented as % expression from gated viable cells.
High expression of pluripotent stem cell markers preserved in hESC expanded in NutriStem® V9 XF medium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

Fig 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 (P8), fixed and stained for the classical pluripotent surface markers: Tra-1-60 (Alexa fluore) (green) and nuclear conjugated markers: OCT-4-Alexa fluore, Nanog-RRX, both counterstained with DAPI (blue). Scale bar 200µm.
Cells Cultured in NutriStem® V9 XF medium on vitronectin matrix express high levels of pluripotent markers.

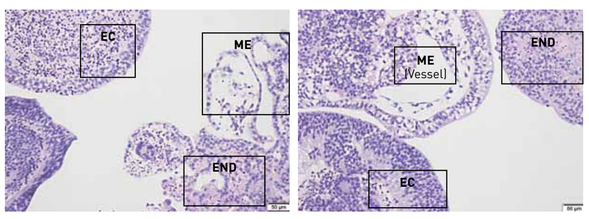
Fig 6: **Gene expression**



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

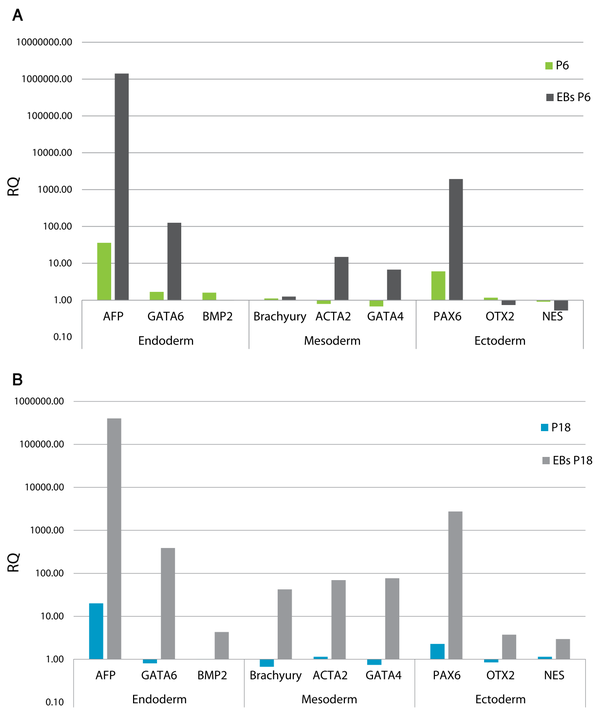
Tri-lineage differentiation potential confirmation

Fig 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. Cell types identified by examination of EBs histological sections stained with H&E. See letters EC=neural rosettes, ME=primitive vessels, ED=primitive parenchyma (X100)
NutriStem® V9 XF medium supports tri-lineage differentiation into the 3 germ layers.

Fig 8: **EB formation- Gene expression analysis**



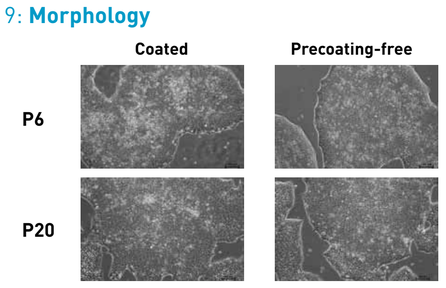
Embryoid bodies (EBs) were generated from H1 hES cells expanded for P6 (A) or P18 (B) in NutriStem® V9 XF medium on Vitronectin ACF as an evaluation of pluripotency. Cells were suspended in NutriStem® V9 XF basal, where they spontaneously formed EBs containing cells of embryonic germ layers. Real-time PCR analysis for differentiation genes from the 3 germ layers calibrated to ACTB2 and GAPDH. Results presented as % expression from H1 hES cells 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

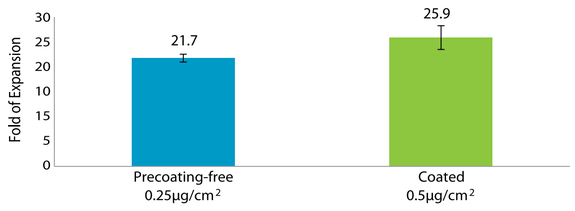
Novel procedure in which Vitronectin ACF added directly into NutriStem® V9 XF medium eliminating the need for pre-coating.

Fig 9: **Morphology**



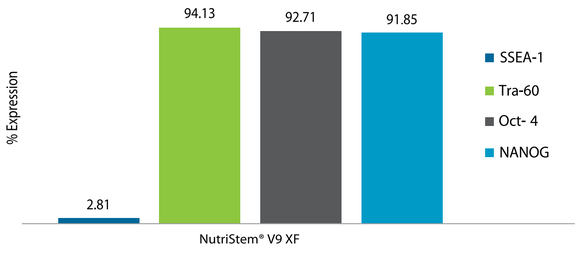
Phase contrast images (x100) of H1 hESC culture expanded in NutriStem® V9 XF for 20 sequential passages on Vitronectin ACF-coated and precoating-free protocol, passage as small aggregates using enzyme-free passage every 3-5 days. Representative images from culture at P6 and P20.
Typical undifferentiated H1 hPSC colony morphology maintained during long-term expansion in NutriStem® V9 XF using traditional coating and precoating-free culture procedure.

Fig 10: **Proliferation**



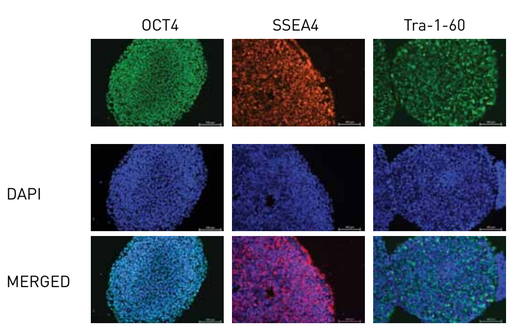
Nucleocounts of H1 hESC expanded in NutriStem® V9 XF for 6 passages on Vitronectin ACF-coated and precoating-free protocol and passaged as small aggregates using enzyme-free dissociation every 3-5 days. Results presented as fold of expansion (total cells/initial seeded).
NutriStem® V9 XF supports high proliferation rate of H1 hESC using both Vitronectin ACF-coated and precoating-free culture.

Fig 11: **Immunophenotyping**



Flow cytometry analysis of H1 hESC culture expanded in NutriStem® V9 XF for 6 sequential passages using Vitronectin ACF precoating-free protocol. Data presented as % expression from gated viable cells.
NutriStem® V9 XF supports high expression of pluripotent stem cell markers in hESC expanded using precoating-free procedure.

Fig 12: **Immunofluorescence staining**



Immunofluorescence analysis of human pluripotent markers of H1 hPSC expanded in NutriStem® V9 XF medium using the precoating-free procedure for 8 sequential passages. Cells from P8 were fixed and stained for the classical pluripotent surface markers: SSEA4 (RRX) (red), Tra-1-60 (Alexa fluore) (green) and nuclear conjugated markers: OCT-4-Alexa fluore, counterstained with DAPI (blue). Scale bar 200µm.
High levels of pluripotent markers maintained in H1 hESC expanded in NutriStem® V9 XF using the precoating-free procedure.

Summary

NutriStem® V9 XF supports high proliferation rates of hPSC on vitronectin-coated cultureware during long-term culture while maintaining:

- Typical pluripotent colony morphology
- High pluripotent gene expression
- High pluripotent marker expression
- Stable karyotype

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