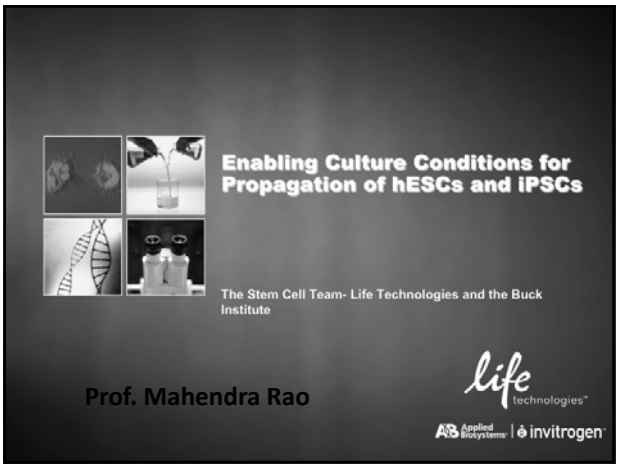
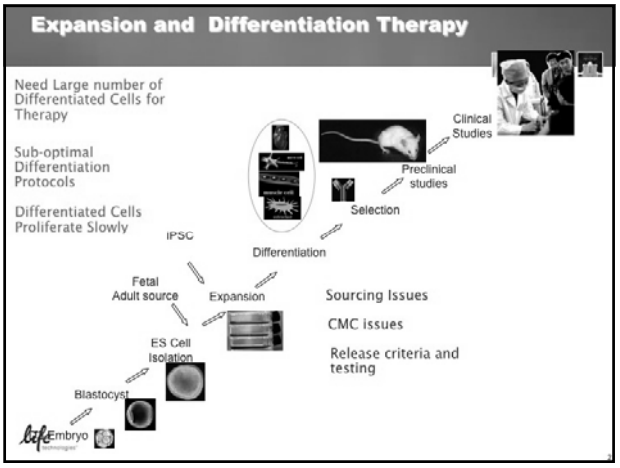
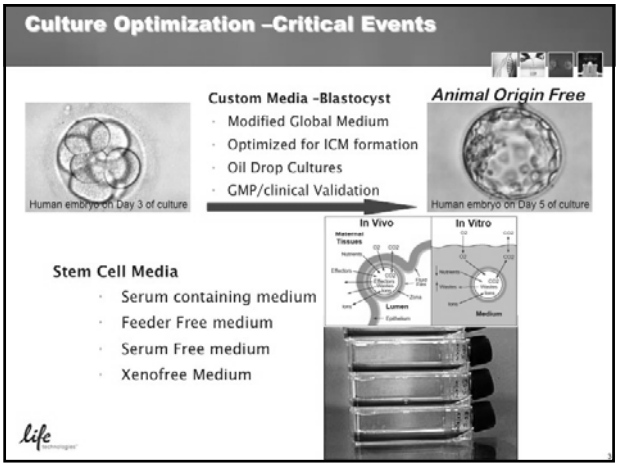
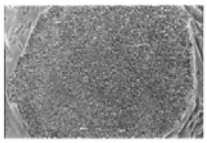

Enabling Culture Conditions for Propagation of hESCs and iPSCs
The Stem Cell Team- Life Technologies and the Buck Institute
Prof. Mahendra Rao
life technologies
Applied Biosystems | invitrogen


Expansion and Differentiation Therapy
Need Large number of Differentiated Cells for Therapy
Sub-optimal Differentiation Protocols
Differentiated Cells Proliferate Slowly
IPSC
Differentiation
Selection
Preclinical studies
Clinical Studies
Fetal
Adult source
Expansion
ES Cell Isolation
Blastocyst
Embryo
Sourcing Issues
CMC issues
Release criteria and testing

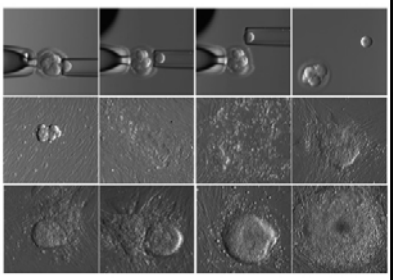

Culture Optimization –Critical Events
Human embryo on Day 3 of culture
Custom Media -Blastocyst
Modified Global Medium
Optimized for ICM formation
Oil Drop Cultures
GMP/clinical Validation
Animal Origin Free
Human embryo on Day 5 of culture
In Vivo
In Vitro
Stem Cell Media
Serum containing medium
Feeder Free medium
Serum Free medium
Xenofree Medium
life

Can we derive hESCs in defined medium?

Dr. Krtolica

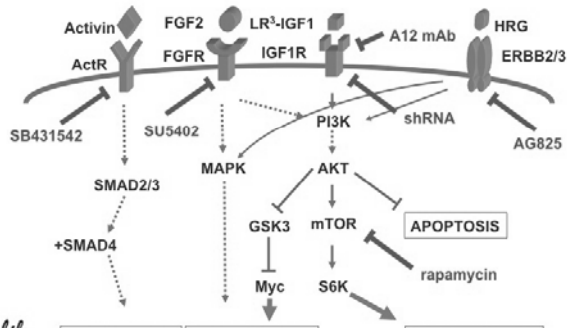


hESCs derived in serum-free conditions on human placental fibroblasts



Serum-free derivation from the biopsied blastomeres without embryo destruction on human feeders

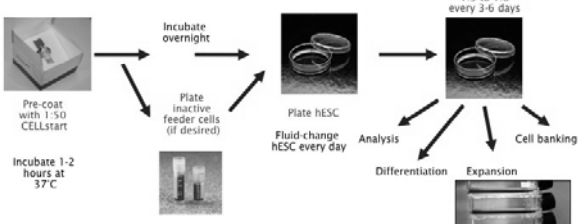
Inhibition of hESC Signaling Pathways



hESC Workflow for Xenofree Platforms

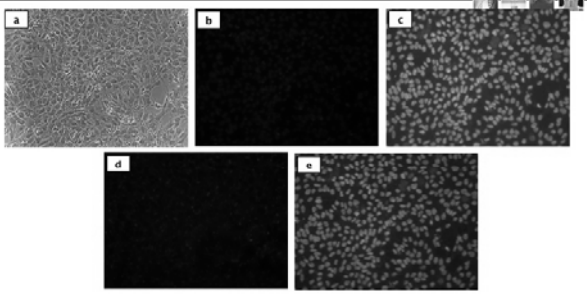
Isolation | Characterization | Expansion | Differentiation

Workflow of culturing hESC with KSR XenoFree



NOTE: Feeder protocols are available on the KSR XenoFree web pages.

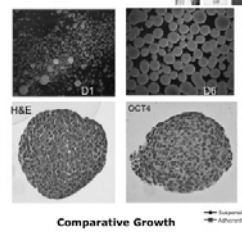
KSR XenoFree - Immunocytochemistry Data

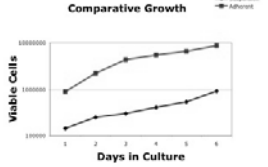


Feeder-free, Xeno-Free Growth of hESC. BG01v were cultured in KSR XenoFree Medium with KSR XenoFree Growth Factor Cocktail, directly on CELLstart Matrix. At passage 15, cells were fixed and stained for OCT-4 and TRA-1-60 expression. Data shows that the stem cell phenotype was maintained. **a.** phase contrast, **b.** Hoechst, **c.** OCT-4, **d.** TRA-1-60, **e.** Hoechst, OCT-4 & TRA-1-60.

hESC Suspension Cultures

- BG02 in suspension for >3 month
- Minimal spontaneous differentiation
- No obvious visceral endoderm layer
- Growth rate comparable to adherent culture
- Poor initial viability at low density
- > 30x expansion possible
- Applications
  - Massive expansion of hESCs
  - Suspension differentiation to endoderm





Demonstrating scalable production

- •Verify SOP's
- •Verify staff training
- •Verify receiving and storage process

→ •Receive Seed bank → •Store in validated storage facility

↓

•Prepare Master bank → •Test and store master bank

↓

•Prepare working bank → •Define passage number to be used

↓

•Prepare differentiated cells → •Define passage number to be used

↓

- •Use Growth Factors
- •Or
- •Expose to CM

→ •Initiate differentiation
•Optimize process → •Define time point for harvesting

↓

•Selection protocols
•Depletion protocols → •Validate process

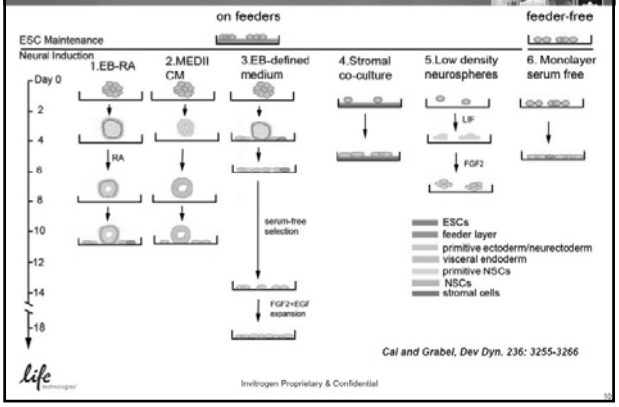
↓

- •Validate tests for
- •Lot release
- •Efficacy
- •Viability
- •Growth characteristics
- •Freeze thaw viability
- •Contaminants

←

•Transplantable product → •Shipping and delivery to site for use → •Endpoint testing

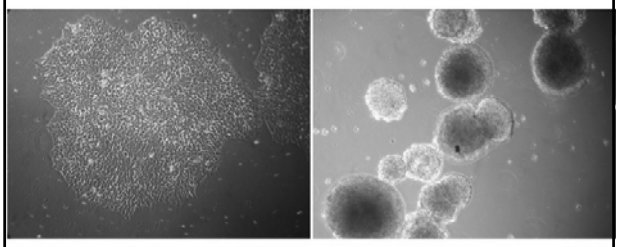
Neural differentiation of ESCs



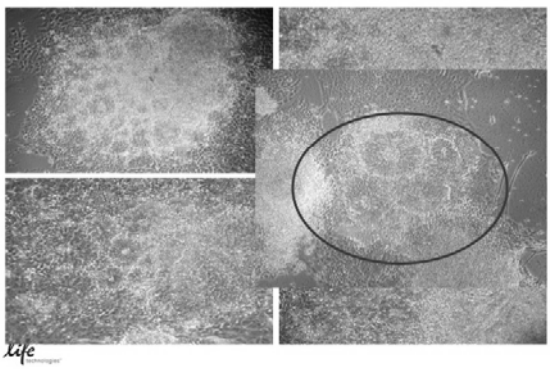
Cai and Grabel, Dev Dyn. 236: 3255-3266

I6 hESCs p42p8
cultured in defined media

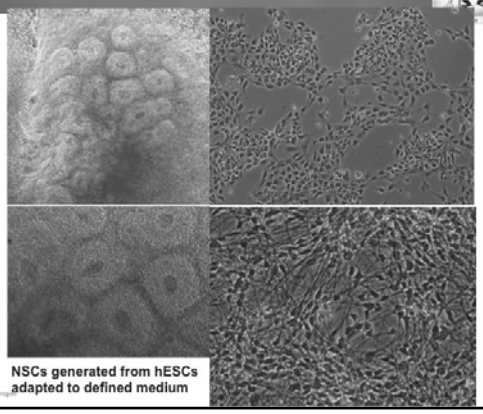
I6 Embryoid Bodies D2
cultured in ES media (–FGF2)



Derivation of I6 NSCs in defined media

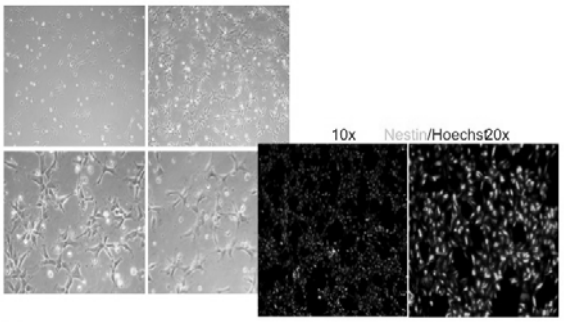


Can we generate NSC in defined medium?

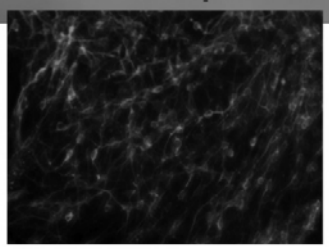


NSCs generated from hESCs adapted to defined medium

W10 NSCs



TH-positive neurons



- Defined media identified
- PA4 conditioned media
- Sorting protocol
- Promoters for lineage
- Growth in 96 well plates and in large scale

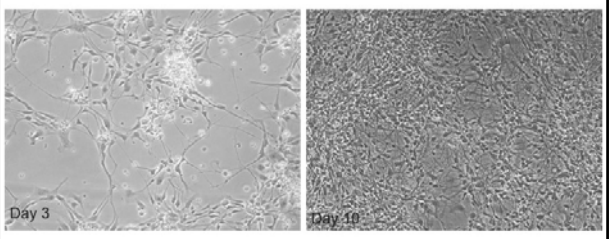
Four stage procedure

ESC → NSC

NSC → Neuronal

Neuronal ← Dopaminergic

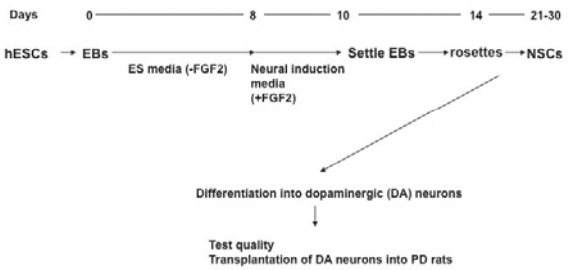
Can we store and thaw neurons?



Cells were frozen after 15 days of neuronal differentiation and thawed

life

A method to derive NSCs from hESCs in defined conditions

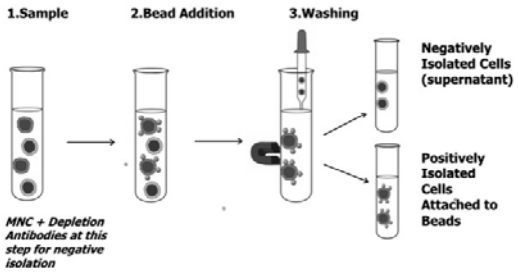


life

invitrogen

Bead Based Separation - Cell Isolation

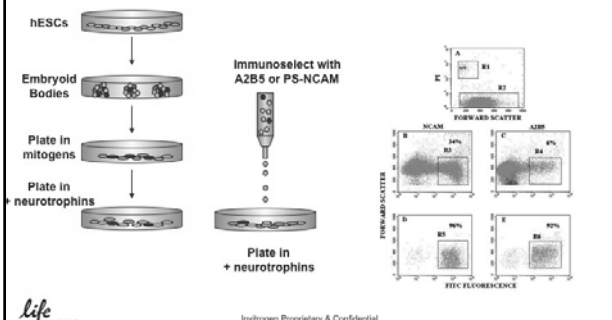
It's Easier and More Consistent: NO COLUMNS



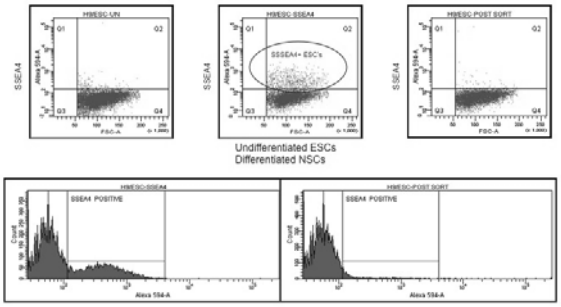
Fast, Easy, and Gentle

17

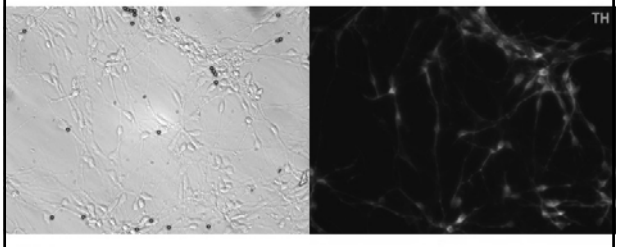
Developing sorting protocols and markers



Can we deplete pluripotent cells?



Can we enrich dopaminergic neurons?



Human ESC Culture Systems- Testing

Lot release- pathogen, sterility, identity
functional quality, viability, consistency

Efficacy tests- Karyotype, epigenome, contaminating cells, HLA type, etc

Stage specific tests- In process testing

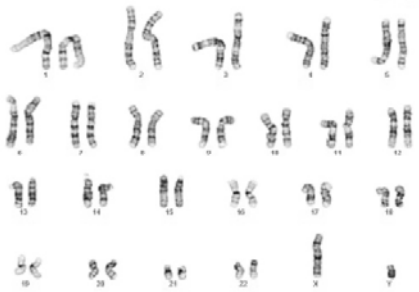
Can we assess function rapidly?

1. Markers: qPCR array

ESC	NSC	Dopaminergic progenitor (midbrain)	Dopaminergic neurons	Mature dopaminergic neurons	A9/A10
Oct4	Sox1, 2	En1, 2	Lmx1a, b	VMAT	Girk2
Nanog	Nestin	Pax2, 5	Nurr1	DAT	VMAT
Sox2	huD	Msx1, 2	Pitx3	Slit	DAT
Rex	Pax6	Lmx1a, b	En1, 2	Robo	
Tert	CD133	Ngn2	AADC	Netrin2	
	Otx2	FoxA2	TH	FGF20	
	Hox	Aldh1a1		Ret	
		Shh		NGFR	
		Wnt1		GDNF	
		Nkx2.2			
		Nkx6.1			

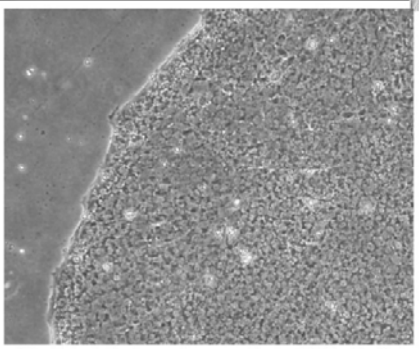
2. Antibodies

Karyotypic Stability Using KSR XenoFree



G-banding of Cyt49 hESC grown in KnockOut™ SR XenoFree, at p12. CyT49 grown in KnockOut™ SR XenoFree retain a normal karyotype. Data kindly provided by Tom Schulz at Novocell, Inc.

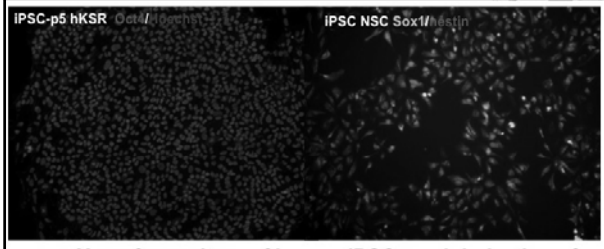
iPSC Derivation Using KSR XenoFree



Human iPS cells derived and grown to passage 5, using KSR XenoFree in feeder-free conditions on CELLstart. Data kindly provided by Dr. Akutsu at National Center for Child Health and Development.

iPSC and ESC protocols are similar

iPSC and ESC protocols are similar

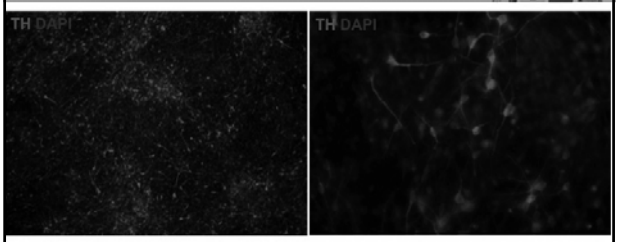


Xeno-free culture of human iPSCs and derivation of NSCs from iPSCs.

Expression Oct4 (left panel) in iPSCs that were cultured in xeno-free medium (hKSR with growth factors) for 5 passage. NSCs derived from xeno-free cultured iPSCs expressed NSC markers nestin and Sox1 (right panel).

Dr. Zeng- Buck Institute

iPSC and ESC protocols are similar



Differentiation of iPSC-derived NSCs into dopaminergic neurons.

NSCs derived from iPSCs can differentiate into dopaminergic neurons as efficiently as NSCs derived from hESCs. A high percentage of cells expressed TH after 23 days of differentiation.

Dr. Zeng- Buck Institute

Current process

Receive control and working bank cells

Grow ESC/iPSC in defined media	ESC-SFM or hKSR-XF custom with Cell Start
Differentiate NSC from ESC	Differentiation Protocol with XF components
Propagate NSC	NSC SFM or NSC-XF custom with Cell Start with FGF
Initiate neurogenesis **Enhance dopaminergic differentiation**	Add growth factors in XF base media
Selection protocols **Depletion protocols**	Dynal positive selection Dynal depletion beads

Transplantable product

Multi-Parameter Human MSC Characterization

Flow cytometry combining

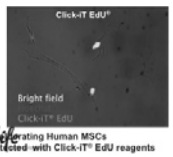
Click-iT® EdU proliferation analysis

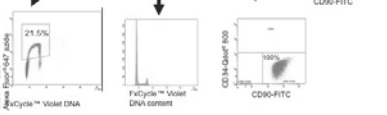
WITH

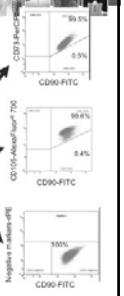
Antibody-based immunophenotyping

Simultaneous testing for percentage proliferation with phenotype characterization

Marker	Fluorophore
CD73	PerCP*
CD90	FITC
CD105	Alexa Fluor® 700
CD14	R-PE
CD19	
CD45	
HLA-DR	
CD34	Qdot® 800
Cell proliferation	Click-iT® EdU Alexa Fluor® 647 azide
DNA content	FxCycle™ Violet stain

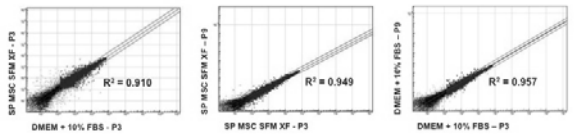






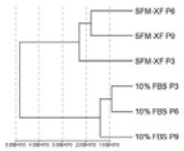
Proliferating Human MSCs detected with Click-iT® EdU reagents

StemPro® MSC SFM XenoFree: BM-MSC Characterization

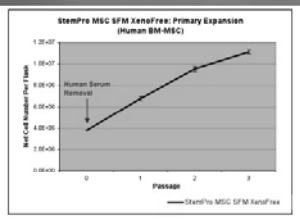


NOTES:

- Input cells = P5 MSC (4-donor pool)
- P3 = 13 days in culture
- P9 = 42 days in culture
- Beadchip = HumanWG-6 v3.0

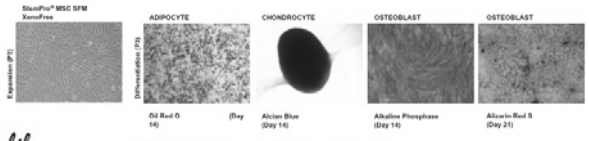


StemPro® MSC SFM XenoFree: BM-MSC Primary Isolation



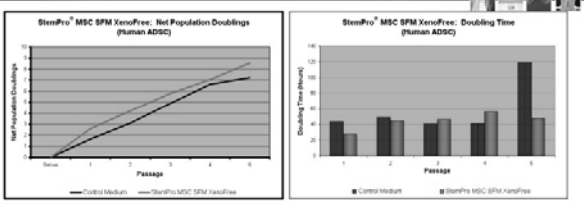
StemPro® MSC SFM XenoFree - Primary Isolation	
Marker	Passage 3 (% Positive)
CD73^{+}/NEG	99.8
CD90^{+}/NEG	99.9
CD105^{+}/NEG	99.7
CD34^{+}	0.4

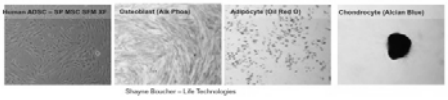
NOTE: NEG = multiplex analysis of CD14, CD19, CD45 and HLA-DR



Passage 3 Multi-lineage Mesoderm Differentiation - StemPro® Differentiation Reagents

StemPro® MSC SFM XenoFree: ADSC Expansion



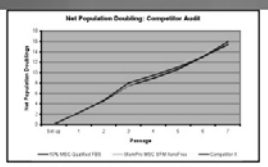


Shayne Boucher – Life Technologies

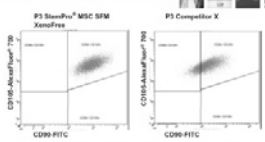
NOTES:

- Input cells = passage 4 StemPro® Human ADSCs
- Differentiation = Passage 3, Day 14 – StemPro® Differentiation Reagents

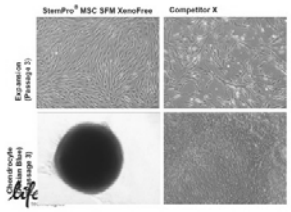
StemPro® MSC SFM XenoFree: Competitor Audit



NOTE: Input cells = passage 5 Human BM-MSCs (4-donor pool)



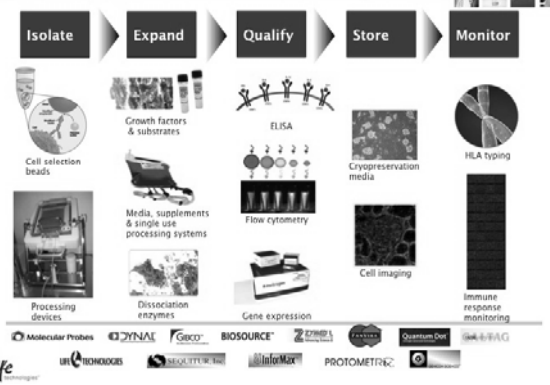
Flow cytometry data generated by Jolene Bradford – Life Technologies



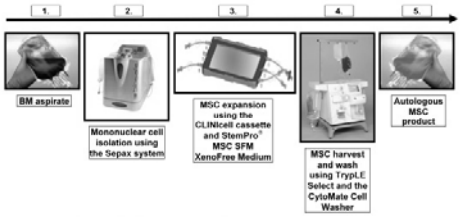
StemPro® MSC SFM XenoFree	
Marker	Passage 3 (% Positive)
CD73+/NEG-	98.8
CD90+/NEG-	100.0
CD105+/NEG-	100.0
CD34+	0.3
Competitor X	
Marker	Passage 3 (% Positive)
CD73+/NEG-	98.4
CD90+/NEG-	77.3
CD105+/NEG-	100.0
CD34+	0.4

NOTE: NEG = multiplex analysis of CD14, CD19, CD45 and HLA-DR

Cell therapy work flow



Closed System Xeno-Free MSC Isolation and Expansion



The detailed MSC isolation and expansion workflow was employed to provide an autologous MSC product from human bone marrow aspirate under completely closed conditions using xeno-free cell culture reagents.

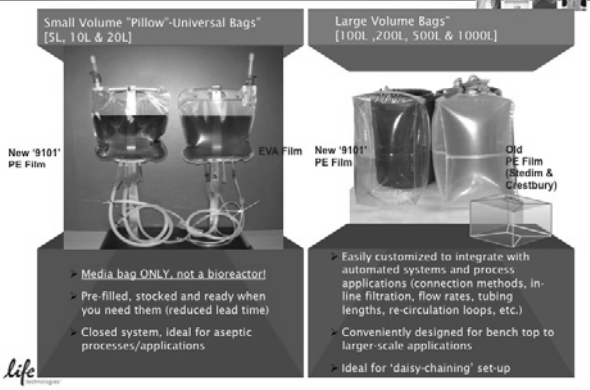
Data generated by Christian Prante, Wolfgang Prohaska and Knut Kleesiek - Ruhr-Universität Bochum

AGT Represents An Optimal Method To Disperse Add Backs and Trace Components Into Dry Powder Media

Reduce Process Steps And Enhance Operational Efficiency

Can achieve manufacturing runs equivalent to >200,000L of liquid media

Closed system considerations



- Media bag ONLY, not a bioreactor!
- Pre-filled, stocked and ready when you need them (reduced lead time)
- Closed system, ideal for aseptic processes/applications

- Easily customized to integrate with automated systems and process applications (connection methods, in-line filtration, flow rates, tubing lengths, re-circulation loops, etc.)
- Conveniently designed for bench top to larger-scale applications
- Ideal for 'daisy-chaining' set-up

CONFIDENTIAL: PATENT PENDING

Multi-Chambered AGT Bag: Work Flow-2

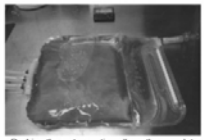
3: Apply pressure to break seal

Pressure is applied to the water section to break seal

4: AGT Rehydrates on contact

Seals break and water enters the powder section and instantly rehydrates the AGT

5: Massage bag to fully rehydrate content



Pushing the water section allows the complete powder to come back into main section. Stitched seal design enables better mixing

6: Filter content using supplied tubing set with filter

Mixing can be done by hand or by placing on a rocking plate. Finished media is ready within 10 mins.

invitrogen

Bioreactor – Single use bioreactors

Connection Methods

- Quick Connect (MPC)
- SCD Tubing
- Threaded Luers
- UNIVERSAL BAGS!!!!!

Prefilled Wave O-Series Cellbag®

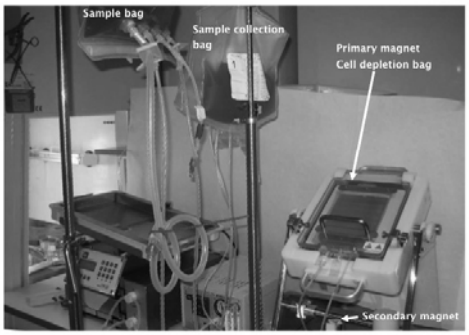
- Ready to ship - media formulations include Sf-900II and Freestyle™ 293
- Convenient - Cellbag® chambers are filled and ready to use
- Customizable - you choose the GIBCO® medium formulation and volume

Size	Working Volume
Cellbag10L/O	0.5L – 5L
Cellbag20L/O	1L – 10L

Wave BIOTECH

life technologies

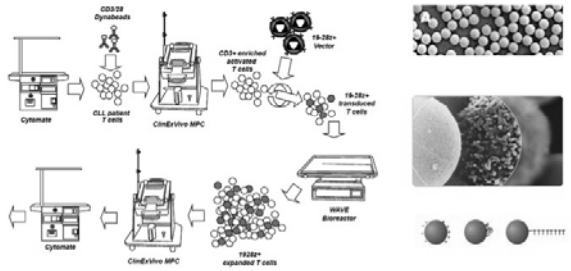
Processing using Dynal® ClinExVivo™ MPC



life technologies

DYNAL® Magnetic Beads

Combining magnetic particles and single use cell expansion technologies



Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy.

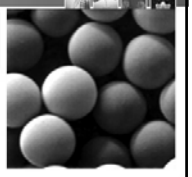
Hollyman D. et al. **J Immunother**. 2009 Feb-Mar;32(2):169-80.

life technologies

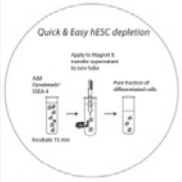
Phase I Clinical Trial underway at Memorial Sloan Kettering

Dynabeads® SSEA-4

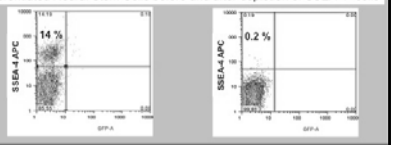
- Undifferentiated hESCs are efficiently depleted from the sample
- Higher purity of differentiated cells
- Differentiated cells remain unaffected after purification



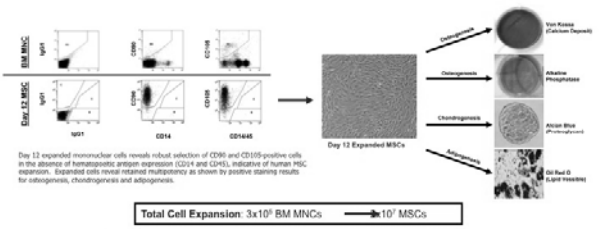
Dynabeads® SSEA-4 are designed to remove undifferentiated human embryonic stem cells from a culture, providing you with highly pure and differentiated stem cells for your translational applications



Differentiated Neural Stem Cells before and after depletion of SSEA-4+ cells



Closed System Xeno-Free MSC Isolation and Expansion



Day 12 expanded mononuclear cells reveals robust selection of CD90 and CD105-positive cells in the absence of hematopoietic antigen expression (CD14 and CD45), indicative of human MSC expansion. Expanded cells reveal retained multipotency as shown by positive staining results for osteogenesis, chondrogenesis and adipogenesis.

Total Cell Expansion: $3x10^5$ BM MNCs → $x10^7$ MSCs

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Others