Welcome

ISPE Boston Area Chapter Presents:

Process Scale-up & Technology Transfer

"Beyond *E. coli* and CHO:

Case Studies in Alternative Host Platforms"

HOST CELLS for Approved Recombinant Therapeutics in the US

- First recombinant therapeutic - 1982 Humulin (Lilly) - *E. coli*
- First mammalian – 1987 TPA (Genentech) – CHO
- First 10 years 1982-1991:
  - 12 microbial - *E. coli* or yeast and
  - 2 mammalian - CHO
- First 30 years 1982-2011:
  - 50+ microbial - *E. coli* or yeast and
  - 50+ mammalian - CHO, BHK (2), mMC/mHC (8+), HEK/human (3)
- First human – 2001 Xigris (Lilly) - HEK, and 2 since then:
  - 2006 Elaprase (Shire) - human cell
  - 2010 VPRIV (Shire) - human fibroblast cell

Escherichia coli, Chinese Hamster Ovary, Baby Hampster Kidney fibroblasts, Human Embryonic Kidney, murine Myeloma or Hybridoma

Statistics from Bioprocess International

A *Pseudomonas fluorescens* fermentation process for quality recombinant protein production

Lawrence Chew, PhD
Pfenex Inc, San Diego, CA
lchew@pfenex.com
www.pfenex.com

Boston Area Chapter
May 2011

Pfenex Inc.

Outline

- Pfenex background
- *Pseudomonas fluorescens* as an alternative expression system
- Background on properly folded protein expression
- Pfenex process development approach
- Safety and regulatory considerations
- *P. fluorescens* fermentation process
- Process transfer

Pfenex Inc.

Pfenex Overview

- Located in San Diego, California
- 30 employees
- 25,000 square feet of laboratory and product development space housing full capabilities for process development and non-cGMP protein production
- Core technology: *Pseudomonas fluorescens* expression platform

Businesses:

- Strain engineering and process development services
- Product Development
  - Subunit vaccines and biosimilars
- Reagent Proteins
  - Messenger proteins
  - Enzymes
  - Vaccine components

Pfenex Inc.

ISPE

Established Expression Systems for Pharmaceuticial Proteins

E. coli

- Non-glycosylated recombinant proteins
  - Growth hormones, insulin
  - Antibody fragments

Yeasts (*Saccharomyces cerevisiae, Pichia pastoris*)

- Limited complex (non)-glycosylated recombinant proteins
  - Growth hormones, insulin
  - Vaccines

Mammalian cell culture (e.g. CHO)

- Complex glycosylated recombinant proteins
  - Monoclonal antibodies

Pfenex Inc.

ISPE

Pseudomonas fluorescens as an Alternative Microbial Expression Platform

Similarities to *E. coli*

- Simple molecular cloning
- Short development timeline
- Good scalability
- Short cultivation time (24-48 hours)
- Simple defined media
- High titer (insoluble) inclusion body expression with difficult refolding

Differences from *E. coli*

- Ability to screen high number of production strains with similar amount of effort
- Secretion to the periplasm (supernatant)
- Soluble, active protein product, no refolding needed
- Less effort in downstream processing
- Correct N-terminus
- Titers @ g/L level
- Most advance product in Phase III clinical trial

Pfenex Inc.

ISPE

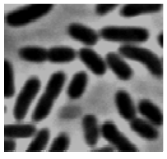
5

Pseudomonas fluorescens Background

History of industrial use

- Produced insecticidal proteins for application as biopesticides for >20 years (Mycogen)
  - High cell density fermentation at >100,000 liters scale
- Produced a GRAS food processing enzyme at >10,000 liters scale (Dow)
- Expression of biopharmaceutical proteins/biologics (Dow/Pfenex) transferred to various cGMP facilities and subsequently produced at >1,000 liters scale

Characteristics of *Pseudomonas fluorescens*



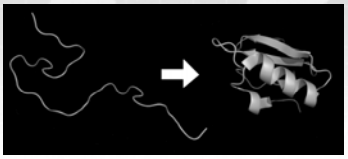
- Non-pathogenic Gram-negative aerobic bacterium
- Does not accumulate inhibitory acids
- Growth in a soluble defined mineral salts medium without the need for animal derived products or organic nitrogen
- Amenable to genetic manipulations
  - Genome sequenced and annotated
  - Genetic and recombinant gene expression tools developed
  - Periplasmic expression support proper N-terminus amino acid and disulfide bond formation → soluble expression of active proteins

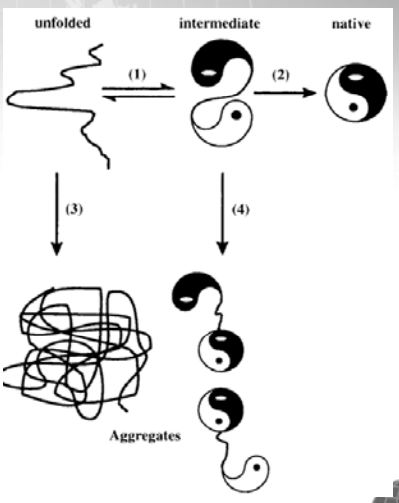
Pfenex Inc.

ISPE

6

Importance of Proper Protein Folding





Proper protein folding needed to
- achieve native structure or conformation to ensure full activity and stability
- avoid unwanted immune response
- prevent aggregation

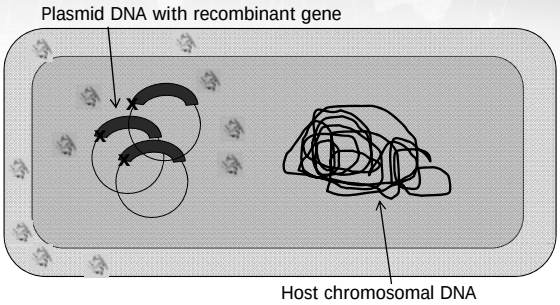
Pfenex Inc.

ISPE

7

Inducible Gene Expression → Properly Folded Protein

Induction **Protein expression** **Protein secretion**



Proper protein folding more likely to occur in periplasm due to the presence of machinery for disulfide bond formation

Pfenex Inc.

ISPE

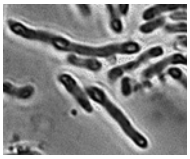
8

Purification Process Considerations

Insoluble inclusion body

- More unit operations
- Lower purification yields
- Longer process

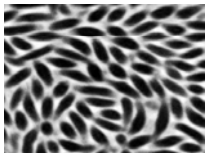
Fermentation
↓
Centrifugation
↓
Cell lysis
↓
Inclusion body recovery
↓
Solubilization & refold
↓
Chromatography 1
↓
Chromatography 2
↓
Chromatography 3



Soluble expressed protein ★

- Fewer unit operations
- High purification yields
- Simpler process

Fermentation
↓
Periplasmic release
↓
Centrifugation
↓
Chromatography 1
↓
Chromatography 2
↓
Chromatography 3



★ Pfenex preferred

Pfenex Inc.

The Pfenex Integrated Approach

Optimal parallel process development for biopharmaceutical protein production - statistical design and data-driven

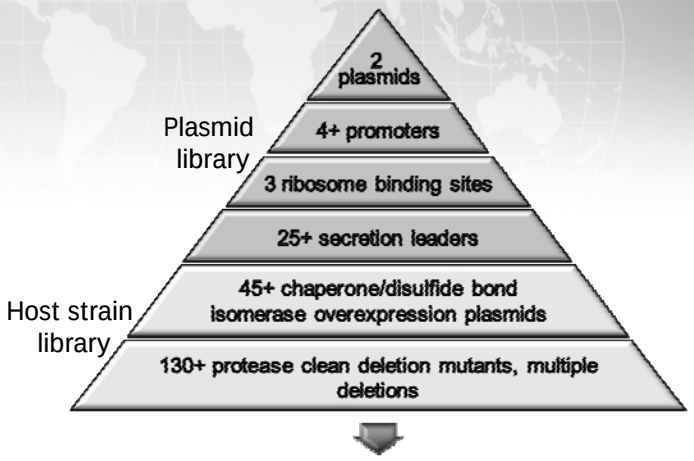
Rapid Strain Construction & Screening
↕
Fermentation Scouting and Optimization
↕
Primary recovery
↕
Purification
↕
Characterization and analysis

Automation enabled process analytical

Soluble properly folded protein expression

Pfenex Inc.

The Pfenex Strain Toolbox: The Next Generation for Bacterial Strain Engineering



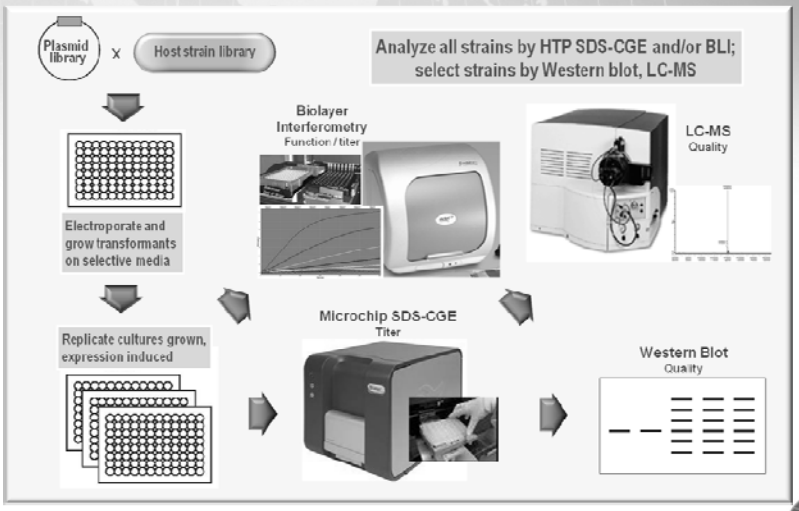
Thousands of unique combinations are seamlessly integrated to enable strain engineering for optimal protein production in *Pseudomonas fluorescens*

Pfenex Inc.

ISPE

11

High-Throughput Strain Development & Process Analytical



Pfenex Inc.

ISPE

12

Rapid Fermentation Development

- **Minibioreactors and parallel bioreactors are integral to Pfenex's rapid fermentation development process**

Minibioreactors (Micro-24)

Scalable high cell density process in 2 L glass or 20 L stainless steel bioreactors

- Multiple strains evaluated in multiple fermentation conditions in DoE
- Minibioreactor process can be predictably scaled

- Strain and process finalists scaled to higher cell densities in 2 L or 20 L bioreactors
- Production strain finalized and fermentation process further developed if needed

Pfenex Inc.

ISPE

13

Rapid Primary Recovery Evaluation

Standard Cell Lysis

- Homogenization

Solids Separation

- Continuous centrifugation
- Depth filtration units
- TFF

Periplasmic Release Toolbox

- Continuous osmotic shock process
- Heat treatment
  controlled temperature and time exposure
- Chemical treatment
- Solvent extraction
  use of glycol ethers
  phase-splitting to reduce volume

Screen at small scale

- Microtiter plates or small beakers
- Use of statistical DoEs
- JMP for design and analysis
- Assess step yield and purity

Optimize at bench scale

Evaluate at pilot scale

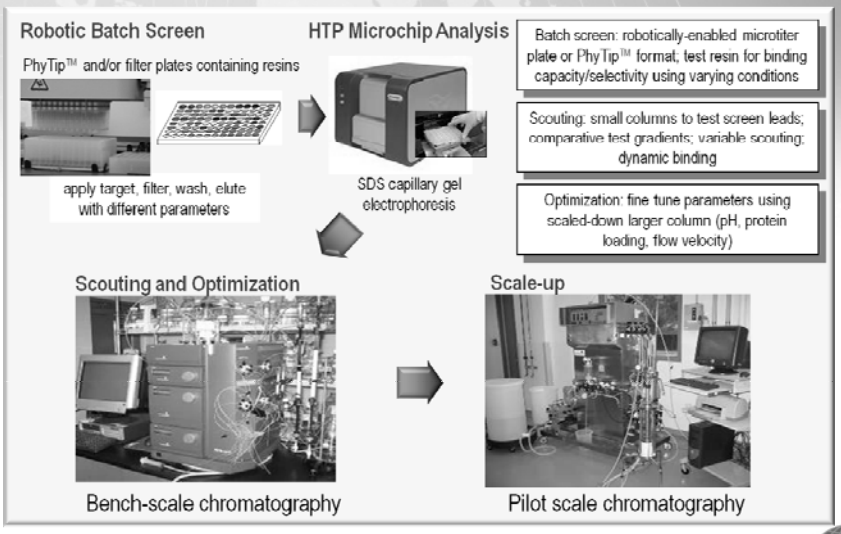
- Assess impurity profile

Pfenex Inc.

ISPE

14

Chromatography Development



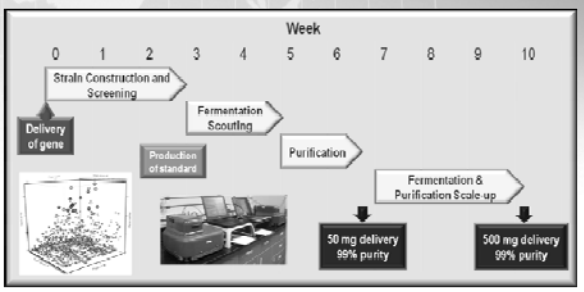
Pfenex Inc.

ISPE

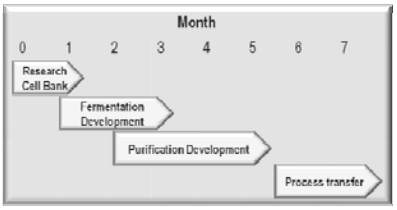
15

Project Examples and Timelines

- Lead protein strain engineering and delivery of purified protein for proof of concept studies



- Process development and transfer



Pfenex Inc.

ISPE

16

Safety and Regulatory Considerations

Clearance of *P. fluorescens* host contaminants

- Demonstrated clearance of *P. fluorescens* host contaminants to acceptable levels for injected drugs by standard purification processes
  - Host cell proteins (HCP)
  - Nucleic acids
  - Endotoxins – lipopolysccharide (LPS) components of outer cell membrane of Gram-negative bacteria

Assurance of clearance

- Development of ELISA for quantitation of *P. fluorescens* HCP
  - Generation and characterization of *P. fluorescens* HCP specific serum
  - Assay development and qualification
  - ELISA kit now commercially available
- Verification of applicability of standard assays for *P. fluorescens* nucleic acids
- Verification of applicability of standard assays for *P. fluorescens* endotoxin
  - *P. fluorescens* LPS was purified and determined to be assayable using standard LAL assays
  - rabbit pyrogenicity tests demonstrated equivalent response with reference LPS from *E. coli*

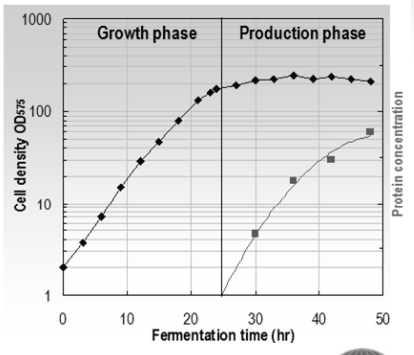
Pfenex Inc.

ISPE

17

P. fluorescens Fermentation Process

- Simple defined mineral salts medium with glycerol and ammonia as carbon and nitrogen sources
  - Recipe designed to ensure excess nutrients to support high cell density
  - No animal derived materials or antibiotics
- Fed-batch high cell density fermentation process
  - Cell densities >200 OD (>100g per liter dry cell weight or >2x 10^{11} cells/mL)
  - Minimal inhibitory acid production
  - High specific protein expression
  - 2 days or less
- Two-phase production process
  - Growth – biomass generation
  - Production – target protein expression
- Engineering considerations
  - Oxygen transfer rate of ~ 300 mmol/L/hr
  - Heat transfer of ~ 40 kcal/L/hr



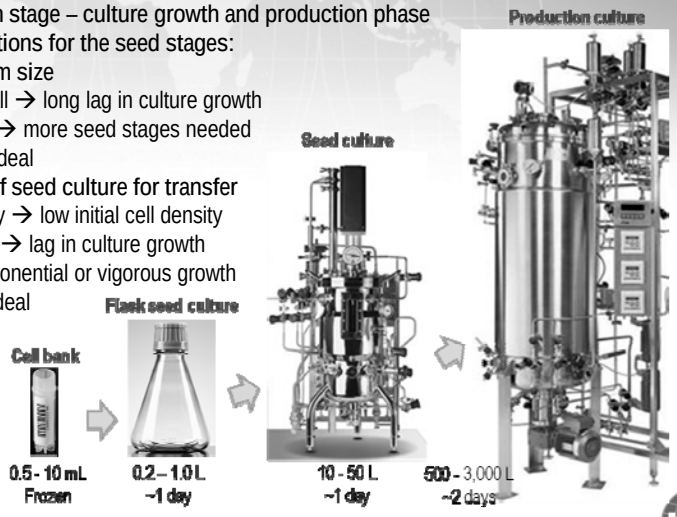
Pfenex Inc.

ISPE

18

Full Fermentation Process

- Seed stages – culture growth phase only
- Production stage – culture growth and production phase
- Considerations for the seed stages:
  1. Inoculum size
     - too small → long lag in culture growth
     - too big → more seed stages needed
     - 2-10% ideal
  2. Phase of seed culture for transfer
     - too early → low initial cell density
     - too late → lag in culture growth
     - mid-exponential or vigorous growth phase ideal



Pfenex Inc. **Frozen cell bank to flask to seed and production fermentors ~ 4 days**

ISPE

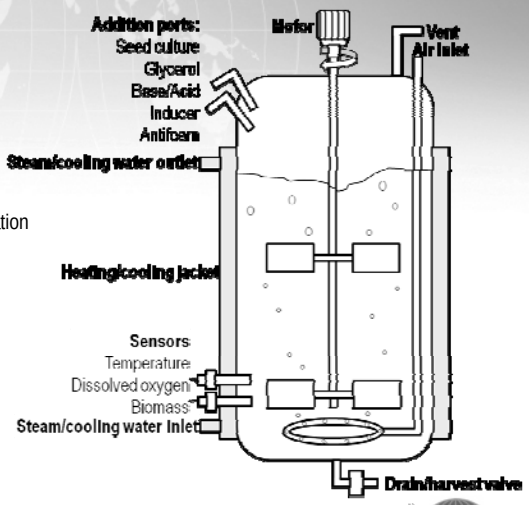
Elements of a Production Fermentor

Sterility

Sterile entries and exits
- Additions ports for inoculum, feeds
- Sterile air/gas inlet
- Ports for essential sensors

Engineering considerations
- Gas exchange and mixing
  - Aeration rates – air, oxygen supplementation
  - Sparger types
  - Motor power
  - Impeller types, size and numbers
  - Baffles
- Heat exchange through jacket or coils
  - Heating capability during sterilization
  - Cooling capability

Considerations for *P. fluorescens*
- Oxygen transfer rate of ~ 300 mmol/L/hr
- Heat transfer of ~ 40 kcal/L/hr



Pfenex Inc.

ISPE

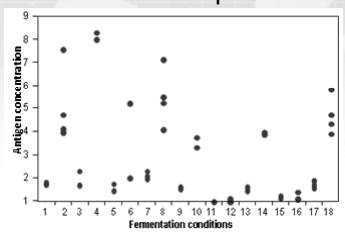
Typical Process Transfer

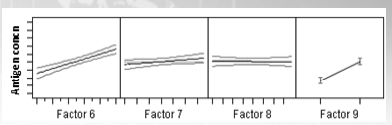
- Documentation of 20 L fermentation (and purification) process and associated analytical methods
  - Detailed process description
  - Process flow diagram
  - Sample plan
  - Bill of materials
  - Equipment list
  - Batch production records
  - Analytical protocols e.g. HCP ELISA
- Transfer of process documents
- Onsite training of plant personnel at Pfenex
  - Execute a 20 L fermentation process
  - Compile and transfer of batch production records along with appropriate analytical results
- Transfer of Research Cell Bank
- Shake-down run(s) in scale-down bioreactor at CMO pilot plant
  - Remote or on-site support from Pfenex personnel
- Preparations and shake-down run(s) in production bioreactor at CMO
  - Remote or on-site support from Pfenex personnel

Pfenex Inc.

Case Study: Vaccine Protein Fermentation

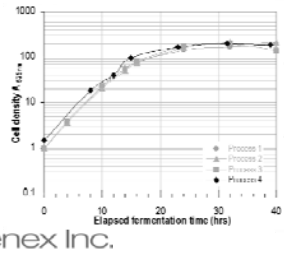
Nine fermentation parameters investigated in µ-24 minibioreactors

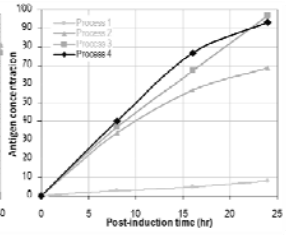


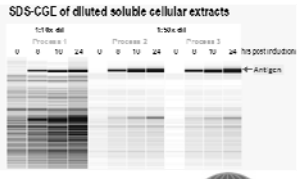


- Two factors have key effects on antigen production
- Optimal operating ranges defined

Improved antigen expression and quality confirmed in scalable 1 L

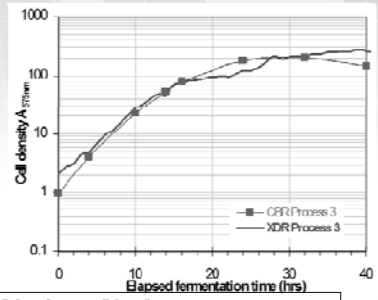


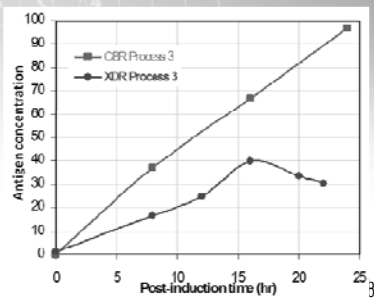




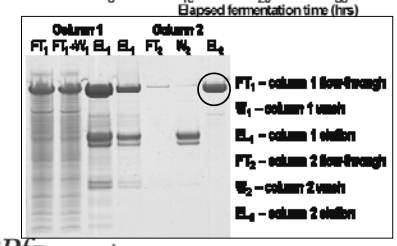
Pfenex Inc.

Scale-up and Transfer to Single-use Disposable Bioreactor





bioreactor
bioreactor



- Scale-up run in XDR-50 Prototype 2
- Growth rates identical, final cell density >200 OD
- Antigen expression can be improved in XDR process *but* 3MM doses already exceeded in a single batch
- Antigen relatively pure after two columns

Pfenex Inc.

Considerations for Successful Process Transfer

- Raw materials from plant qualified vendors
  - Test fermentations to ensure equivalent performance as Pfenex specified vendors
  - Glycerol example – chemically synthesized vs. plant derived
  - Antifoam
- Preparation of sterile mineral salts medium and feeds
  - Batch, continuous or filter sterilization
- Oxygen transfer capability to support high cell density *P. fluorescens* culture
  - Sparger/agitator design
  - Air with/without oxygen supplementation
- Heat exchange capability
  - Cooling fluid type, temperature and flow rates
  - Jacket, coils, etc.
- Control of glycerol feed delivery
  - Control loop based on on-line signal(s) specific to the plant bioreactor
- Extra care needed to ensure sterility of culture in the absence of antibiotics
- Detailed process understanding by plant staff achieved after a few shake-down runs

Pfenex Inc.

Acknowledgements

Colleagues at Pfenex

- Pat Lucy – Business Development
- Keith Haney - Fermentation
- Torben Bruck - Fermentation

Jeff Guertin et al. at Xcellerex

Pfenex Inc.

Abbreviations

BLI	biolayer interferometry (binding assay)
CMO	contract manufacturing organization
DNA	deoxyribonucleic acid
DoE	Design of Experiments
HCP	host cell protein
HTP	high throughput
JMP	statistical design of experiment software package
LAL	limulus amebocyte lysate assay for endotoxin or LPS
LC	Liquid chromatography
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption/ionization, a soft ionization technique used in mass spectrometry
MS	mass spectrometry
OD	optical density
QTOF	quadrupole time of flight mass spectrometer
RP-HPLC	reverse phase high-performance liquid chromatography
SDS-CGE	sodium dodecyl sulfate capillary gel electrophoresis
SEC	size exclusion chromatography
TFF	tangential flow filtration

Pfenex Inc.

Expression and Purification of Recombinant Proteins Using the PER.C6® Human Cells

Rachel Hoff

Associate Scientist II, Upstream Process Development

PERCIVIA, Cambridge MA

PERCIVIA

1

Why Mammalian?

- Mammalian cells
  - Mammalian cells dominate the field for manufacture of complex proteins (glycosylated) and monoclonal antibodies,
  - Since 1957, CHO was freely available and initially favored because of its robust growth and relatively high yields
- Microbial cells
  - Lack the machinery for proper folding and post translational modification of complex proteins, critical for pharmaceutical performance
- Yeast cells
  - Post-translational modifications differ from mammalian systems
- Others
  - Transgenics, plant systems, and microalgae are still in development

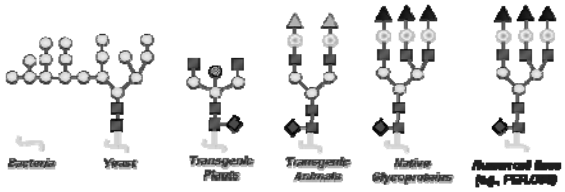
Morrow, K. John; Therapeutic Protein Production: A changing landscape. Cambridge Healthtech Institute, 2010.

PERCIVIA

2

Why Human?

- Accurate human post-translational modifications
  - Species-specific differences of protein glycan structures can have implications for function, clearance, and safety
- No possibility of non-human contaminants in biological products used for human therapy



PERCIVIA

High — Immune Rejection — Low

ISPE

3

Why Percivia?

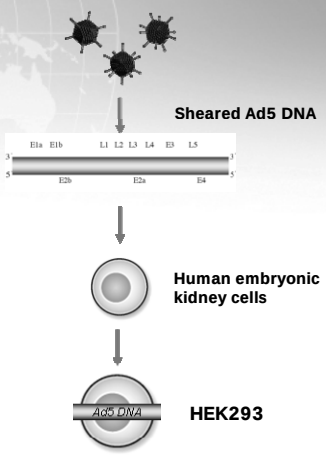
- The PER.C6® cell line was rationally designed for pharmaceutical manufacturing
  - Its creation, characterization, and safety testing are documented in a Biologics Master File submitted to the FDA
- Low cost of goods
  - Percivia has developed an efficient and high yielding protein production platform based on the PER.C6® cell line
- FDA-accepted expression system
  - Approximately 40 clinical trials have been initiated with no adverse effects reported

PERCIVIA

ISPE

4

The Genesis of the HEK293 Human Cell Line

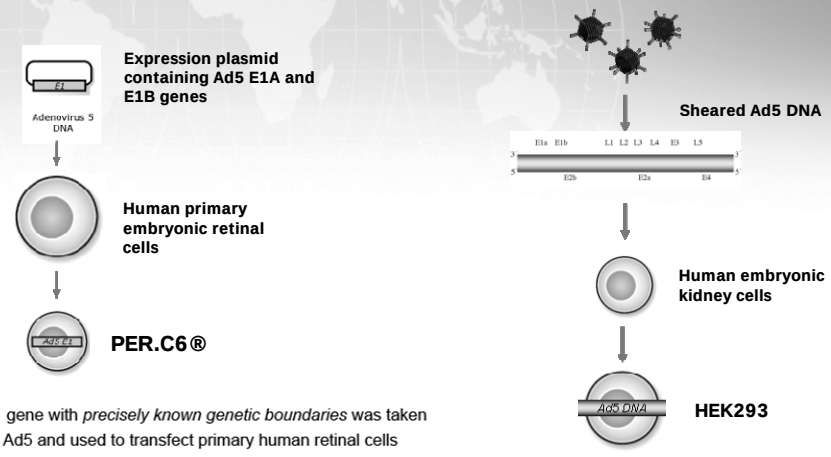


PERCIVIA

ISPE

5

The Genesis of the PER.C6® Human Cell Line



The E1 gene with *precisely known genetic boundaries* was taken from Ad5 and used to transfect primary human retinal cells

PERCIVIA

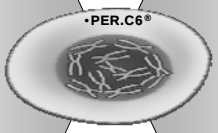
ISPE

6

Safety Testing

- **Viral/Bacterial Testing**
- Sterility
- Mycoplasma
- In vitro and in vivo adventitious viruses

- **Species Specific Viruses**
- HIV type 1 & 2
- Human T-lymphotropic virus 1 & 2
- Human cytomegalovirus
- Human herpes virus
- Human hepatitis B & C
- Simian virus 40
- Adeno associated virus
- Epstein-Barr virus



NO CONTAMINANTS FOUND

- **Bovine/porcine viruses**
- Bovine diarrhea virus
- Infectious bovine rhinotracheitis virus
- Para-influenza virus
- Porcine parvovirus

- **Retrovirus assays**
- Reverse transcriptase
- Transmission electron microscopy
- S+L- and XC plaque

- **Prions**
- PrPcSC negative
- No mutations in PrP
- 129 V/M (heterozygous)

PERCIVIA

ISPE

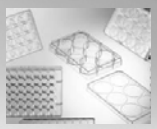
7

PER.C6® Cell Line Generation Process

1 **GOI Sequence Optimization & Vector Preparation**

2 **Transfection**

3 **Stable Pool & Material Generation**



LDC, Clone Screening & Expansion

4

FB Clone Screening & Selection

5

Create RCB

6

Stability Study

7

8

Create MCB

Process Development & Implementation

PERCIVIA

ISPE

8

Cell Culture Tool Box

Objective: Establish a tool box of platform processes from which the optimal process may be chosen for each program

Right tool for every job!

- Selection of process based on process requirements as dictated by product, clone characteristics, etc.
- Familiar and easy to implement

PERCIVIA

ISPE

9

PER.C6® Platform Production Processes

Available tools

- Batch - quick and easy
- Fed-batch - standard production work horse
- XD® - constant environment and extreme productivities

Safety and Consistency

- Free of animal-derived material or hazardous chemicals
- Chemically defined throughout

PERCIVIA

ISPE

10

PER.C6® Fed-Batch Historical View

Commercial batch medium (Xv_{max} ~ 5-6 million cells/mL, titer ~ 400 mg/L)

Combination of commercial feed and in-house designed cocktails

- Include hydrolysates, amino acids, trace element cocktails, etc.
- Multiple bolus shots applied throughout the process
- Xv_{max} ~ 15 million cells/mL
- Typical titer ~ 1.5 – 2 g/L in fed batch

PERCIVIA

ISPE

11

Platform Fed-Batch Attributes

"Generic", chemically-defined batch media intended for multiple cell lines with feed media a concentrated form of batch media components

Simple feeding strategy for multiple cell lines and scales

Small scale model which is predictive of fed-batch bioreactor performance

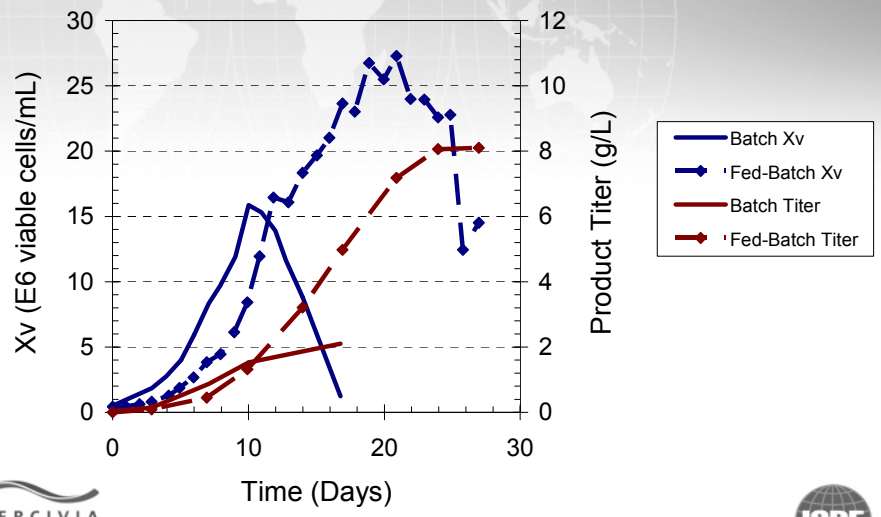
PERCIVIA

ISPE

12

Platform Batch and Fed-Batch Development

End Result



PERCIVIA

ISPE

13

Small-Scale Fed-Batch System

Easy to set up and manipulate - "high" throughput

- Small volume (50 mL in 250 mL Shake Flask)
- Minimal manual intervention/process analytical requirements (Feeding/sampling once daily or every other day)

Predictive of fed-batch bioreactor performance

Applications

- Clone screening
- Media/process development
- Process troubleshooting

PERCIVIA

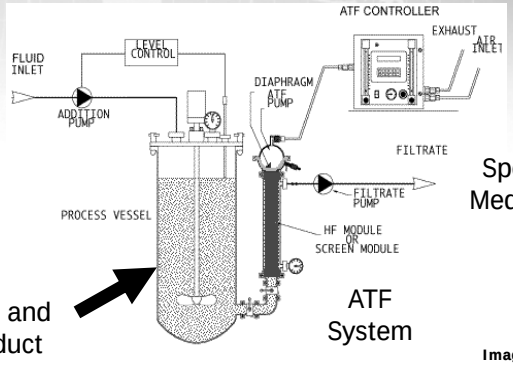
ISPE

14

The XD® Process

Extreme Density Cell Culture

Fresh Medium



Spent Medium

Cells and Product

ATF System

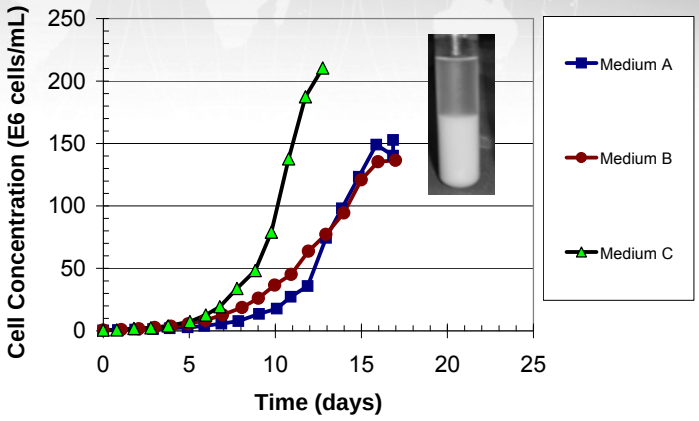
Image from www.magellaninstruments.com

PERCIVIA

ISPE

15

XD® Process Development

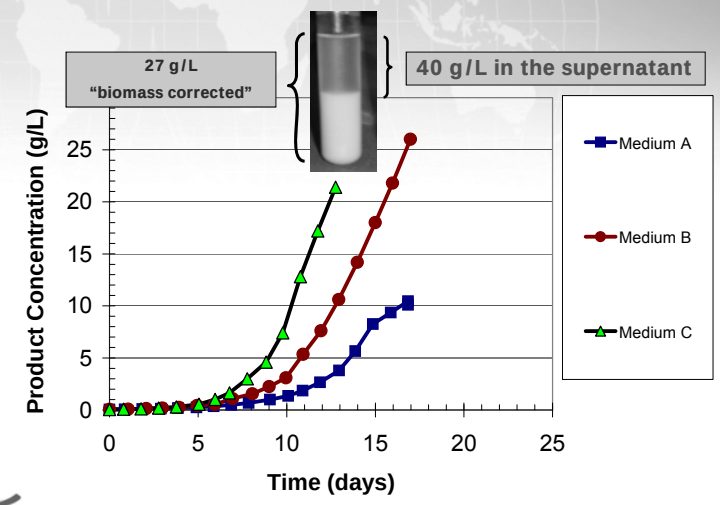


PERCIVIA

ISPE

16

XD® Process Development



PERCIVIA

ISPE

17

From 250 mL Shake Flask to 250 L Bioreactor

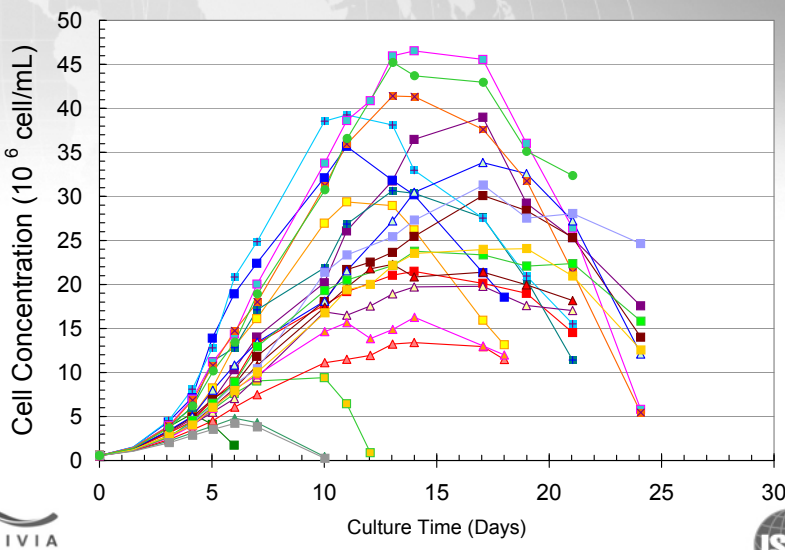
EXPERIENCES WITH THE PER.C6® PLATFORM FED-BATCH PROCESS

PERCIVIA

ISPE

18

PER.C6® Fed-Batch – 250 mL Shake Flask – Growth

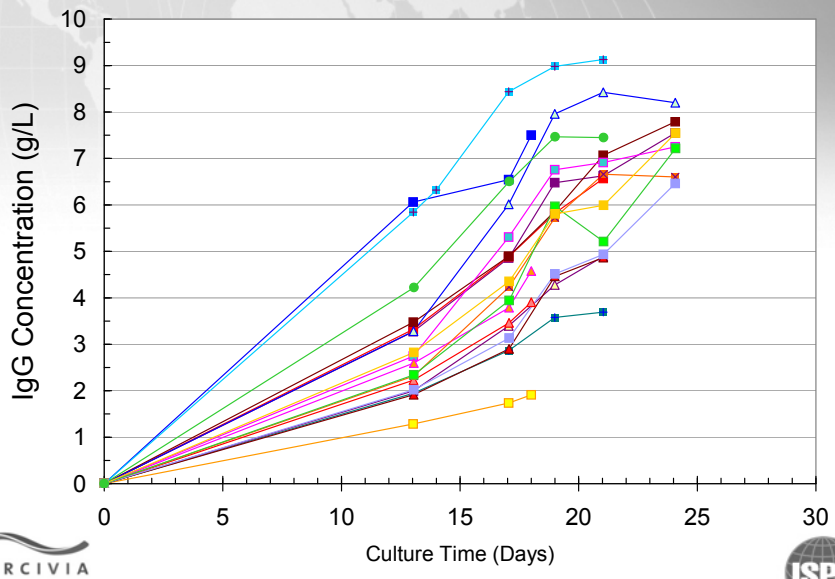


PERCIVIA

ISPE

19

PER.C6® Fed-Batch – 250 mL Shake Flask – IgG Titer

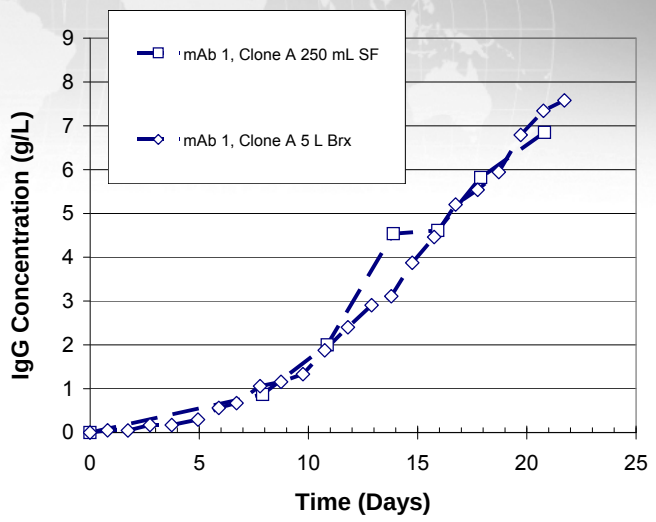


PERCIVIA

ISPE

20

PER.C6® Fed-Batch Titer – Benchmark at 5 L scale

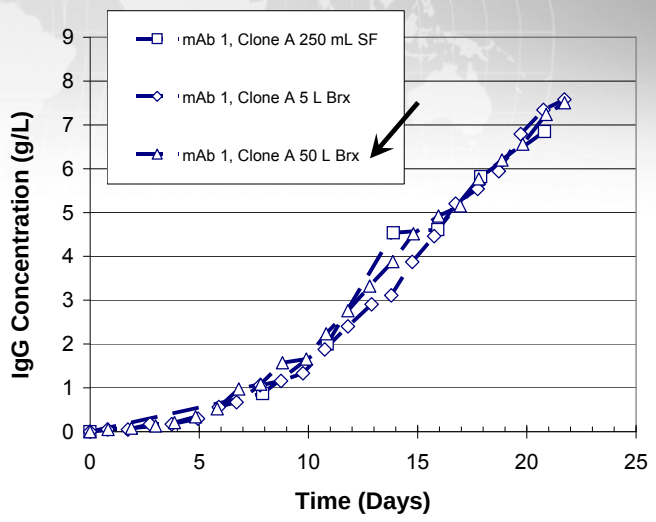


PERCIVIA

ISPE

21

PER.C6® Fed-Batch Titer – Tech Transfer, 50 L Scale

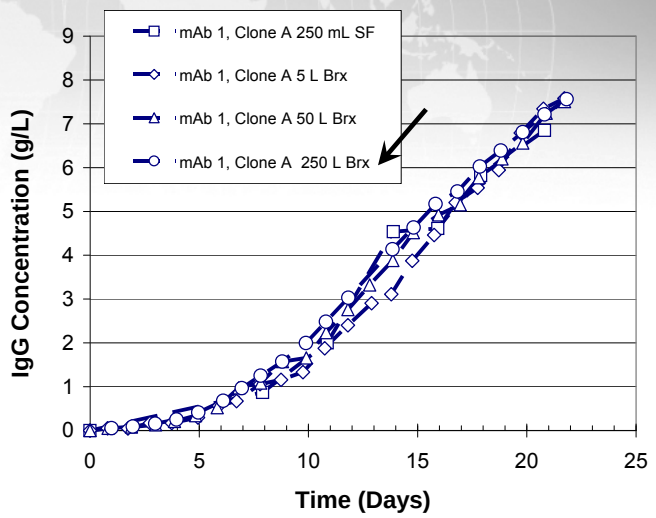


PERCIVIA

ISPE

22

PER.C6® Fed-Batch Titer – Scale-up to 250 L Bioreactor

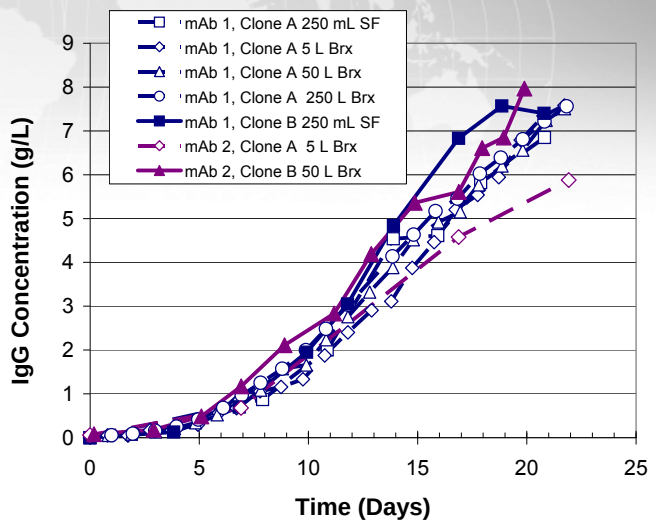


PERCIVIA

ISPE

23

PER.C6® Fed-Batch Titer – Other Clones, Proteins, Scales



PERCIVIA

ISPE

24

Three Processes:
Protein A Capture Process
CEX Capture Process
Disposable Process

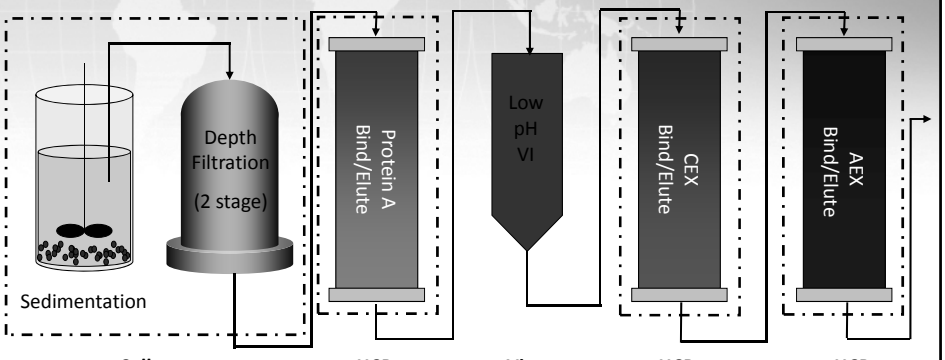
CASE STUDY: COMPARISON OF TRADITIONAL AND NON-TRADITIONAL DOWNSTREAM PROCESSES

PERCIVIA

ISPE

25

Process Overview – Base Case (Protein A Capture)

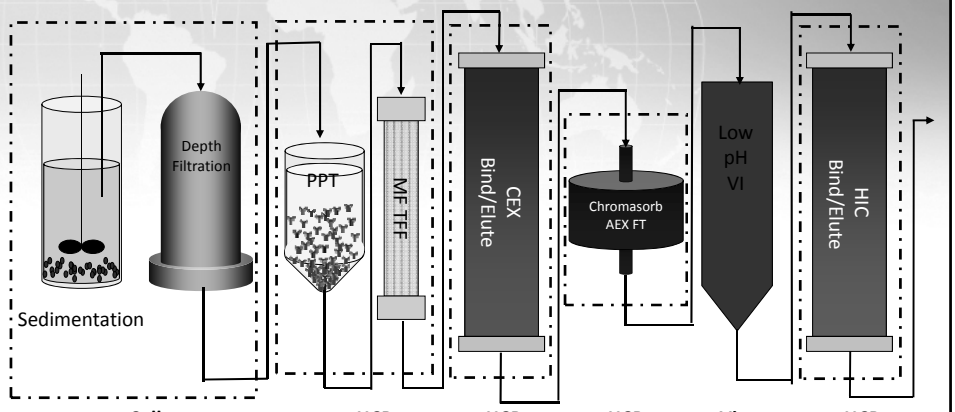


PERCIVIA

ISPE

26

Process Overview – CEX Capture



Cells
Cell debris

HCP
Concentration
Buffer exchange

HCP
DNA
Virus

HCP
DNA
Virus

Virus

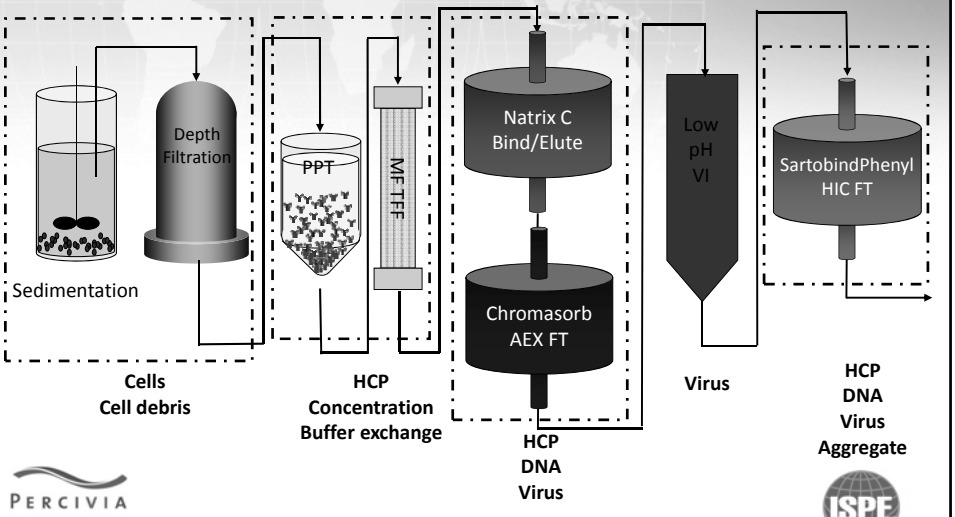
HCP
DNA
Virus
Aggregate

PERCIVIA

ISPE

27

Process Overview – Disposable Process



Cells
Cell debris

HCP
Concentration
Buffer exchange

HCP
DNA
Virus

Virus

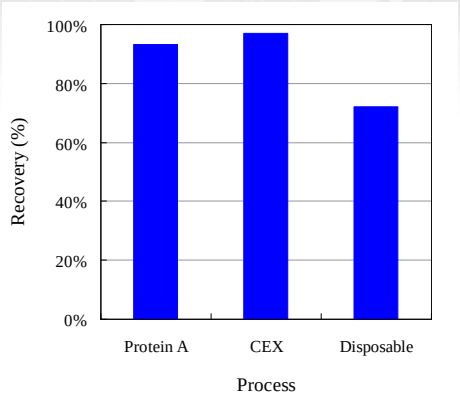
HCP
DNA
Virus
Aggregate

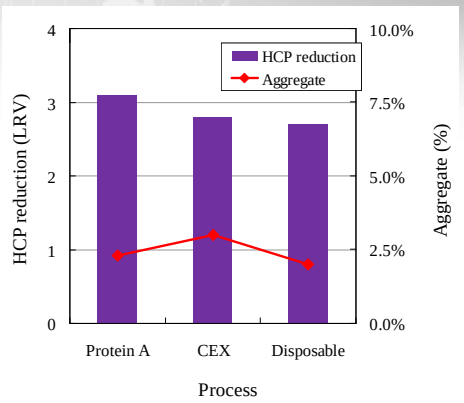
PERCIVIA

ISPE

28

Process Performance – Recovery and Aggregate Removal



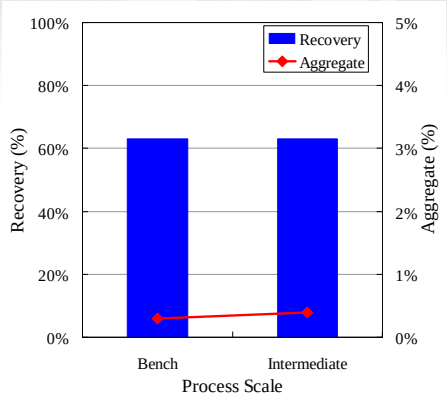


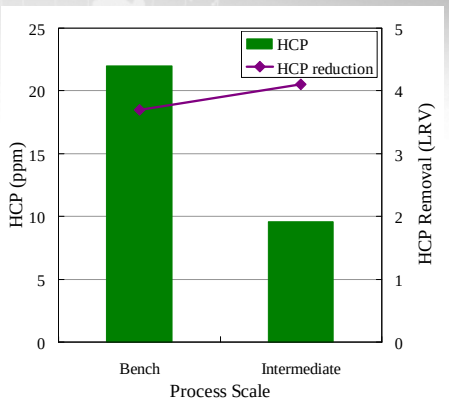
PERCIVIA

ISPE

29

Scalability of Disposable Process





Note: Starting material was from a different reactor than previous case study

PERCIVIA

ISPE

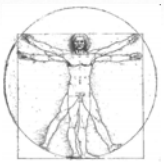
30

Summary

PER.C6® Platform Technology

Industrially-Relevant Cell Line Generation Timeline and Yields

Small-Scale Fed-Batch System Predictive of Bioreactor Performance



Scalable and Disposable Downstream Processes

High-yielding, Reproducible Production Processes

PERCIVIA

ISPE

31

PER.C6® Based Products in Clinical Trials

Sponsors	Products	# Studies	# Subjects (incl. placebo)	Countries
AME/Eli Lilly, AERAS, Ark Therapeutics, Berlex/Schering, FLUPAN, Genvec, IAVI, Merck&Co, ML Laboratories, Neotropix, NIH-VRC, NIH-NIAID, NIH-IPCAVD, Sanofi pasteur, Selective Genetics, Transgene	Gene Therapy Vaccines mAbs Recombinant Proteins	~40*	>5,500	US, Canada, Belgium, Finland, France, Germany, Hungary, Kenya, Norway, Netherlands, Sweden, Switzerland, UK, Malawi, South Africa, India, Thailand, Australia, Brazil, Peru, Philippines, Puerto Rico, Dom. Rep, Haiti, Jamaica

*** No PER.C6®-related AEs reported in any of the studies**

PERCIVIA

ISPE

32

Acknowledgements

Cell Line Generation
- Yevgenya Akselband
- Jessica Bosc
- David Chirgwin
- Bernie Eng
- Alyssa Handler
- Robert Lu
- Alec Murillo
- Anna Tchoudakova
- JR Warfield

Upstream Process Development
- John Chon, Sr. Director
- Carissa Bragg
- Marketa Foley
- Kathryn Golden
- Kelly Kral
- Nate Ostberg

Downstream Process Development & Protein Analytical Sciences
- Gregory Zarbis Papastoitsis, Sr. Director
- Emily Schirmer
- Mike Kuczewski
- Blanca Lain
- Ryan Beck

Contact:
Percivia
Rhoff@percivia.com
www.percivia.com

PERCIVIA

ISPE

33

List of Abbreviations

Ad5	Adenovirus serotype 5
HEK	Human Embryonic Kidney
CHO	Chinese Hamster Ovary
GOI	Gene of Interest
LDC	Limited Dilution Cloning
FB	Fed-Batch
RCB	Research Cell Bank
MCB	Master Cell Bank
Xv	Viable Cell Concentration
ATF	Alternating Tangential Flow
HF	Hollow Fiber
IgG	Immunoglobulin G
SF	Shake Flask
BRX	Bioreactor
HCP	Host Cell Protein
PrA	Protein A
PPT	PEG Precipitation
MF TFF	Microfiltration Tangential Flow Filtration
CEX	Cation Exchange
AEX	Anion Exchange
FT	Flow Through
VI	Viral Inactivation
HIC	Hydrophobic Interaction Chromatography
AE	Adverse Effects

PERCIVIA

ISPE

34