

Manufacturing-Related Issues

- Process Design - Choice of System Components
- Comparison of Protein Expression Systems
- Characterization Testing
- Scale of Manufacturing: Pilot vs. Commercial
- Process Development: Neural Nets and Edge of Failure
- Principle Component Analysis (PCA)
- Process Development: Overview of Mammalian Cells
- Bioprocessing Overview: Bioreactor Considerations
- Bioreactor Design
- Downstream Purification
- Comparability Studies
- Integration of Analytical Testing Profile to Manufacturing and Stability
- Summary of Key Points

- Inducible promoter is the best way to do the plasmid, since it will allow the cell growth to be maximized with minimum impact on plasmid shedding.
- E. coli sigma factors recognize promoters and important for efficient finding of RNA polymerase and efficient transcription and translation of product.
- Promoter selection has big impact on levels of protein
- Need to be careful of evaluating a fermentation by OD alone. OD can be plateaued, but temperature and induction time changes can have tremendous impact on plasmid stability (as assessed by antibiotic resistance).
- Watch out for licensing and patent issues when using certain promoters (e.g., T7)
- Fidelity of codon use and translation - note that E. coli and yeast may use different preferred codon sequences, so it's
- Fusion proteins: four good uses - secretion systems, good handle for affinity chromatography, stabilizing product intra- and extracellularly, enables activation of protein in a more controllable fashion (first, expression and then activation)
- Some people have used E. coli enterotoxins as fusion proteins for linking with specified product. Since E. coli is not known to secrete much, this mechanism is a way that you can secrete specified product from an E. coli system. But this has only been used for R&D to demonstrate 'proof of principle'. FDA hurdles for use in man may be too early/ much to tell if it can be used commercially.
- Runaway system is used to keep copy number low and then induce shooting the copy number up to an extreme amount and thus, product being produced. Known as **convergent transcription** uses a negative antisense. Very quick process (minutes to hours). Advantage is to boost growth phase from induction phase.
- Eukaryotic systems used for 4 major post-translational modifications: disulfide bonds, proteolytic cleavage into an active form, glycosylation (both amount and type), specific additions to amino acids within the protein (acetylation, phosphorylation, etc.).
- Three different classes of expression vectors used with yeast: plasmid vectors not that useful in yeast, so need to integrate the vectors into yeast system. Yeast artificial chromosome (YAC) sort of mimics chromosome - mostly used in R&D, not too much for commercial.
- Alpha factor is a key factor in yeast secretion of proteins. Key because only secreted proteins are glycosylated! To facilitate secretion, use a pre-pro-leader sequence with alpha factor built in. During the export process, the first step is disulfide bond formation, then proteolytic cleavage of pre-pro sequence, and then post-translational modifications (glycosylation).

Process Design - Choice of System Components

• Eukaryotic vs. Prokaryotic Organisms

- **Microbial (e.g., E. coli):** inexpensive and versatile in bioprocessing scenarios, but limited utility for products with post-translational changes such as glycosylation or certain types of protein folding
- **Mammalian cells (e.g., Chinese Hamster Ovary):** while tissue cultures are more expensive to maintain, grow, and harvest - it has advantages in efficiency for post-translational changes and secretion of desired product: limitations in that mammalian cells grow on surfaces or must be adapted for suspended cultures, making large scale production tricky
- **Insect cells (e.g., Drosophila species):** can be used in continuous circulation tanks since they can take more abuse in shear pressure
- **Yeast (e.g., Saccharomyces):** limited utility of plasmid vectors in yeast due to difficulty in integration; Yeast Artificial Chromosomes (YAC) used to mimic chromosome - mostly used in R&D but not too much for commercial
- **Plant cell cultures** can also be used (e.g., large scale fermentation of Taxol). Increased taxol levels from of taxol are < 0.1% (dry weight basis from Pacific Yew tree bark) and now by cell culture, can be up to 120 mg/ gram of dry weight basis.

• Metabolic Engineering vs. Expressions Systems

- Metabolic engineering is continuous (cost-effective); expression systems are more on a batch basis (typically what FDA has seen)
- Focus is to minimize burden of plasmid on organism by using inducible enzyme system

•Form follows function. First, look to what others are doing - via patent searches and technology licensing agreements. Try to lock on to the best system for your needs and beware of using some tools that come with hefty licensing/ royalty streams or are particularly contentious. Also look out for companies that seem to be selling the technology to everyone for a stiff fee up front (e.g., marketing of MPL - an endotoxin-based adjuvant). Best bet is to advise with some person/ institution with lots of experience in scale-up and commercial applications of biotechnology (e.g., Colorado State University)

•Selection of system components is evaluation of (1) cost, (2) availability of technology (3) ease of scale-up or use in commercial applications, and (4) production efficiencies. Most companies explore several systems at once: microbial, mammalian cell culture, or other and make a decision point. Note that some products can be expressed in several systems (e.g., chymosin in bacteria, yeast, or mammalian cell lines).

•Microbial systems are cheap but limited utility for post-translational changes/ folding. Yet some companies live with inefficiencies (low yield on folding); Lilly uses an E. coli system for Humulin (recombinant human insulin) despite only a 30% yield on re-folding of protein to active moiety.

•Mammalian cells (tissue culture) offers higher front-end costs, but greater efficiencies on end-product of post-translational changes. Adherent requirements of cells limit them to surfaces or suspended cultures. Some firms use robot-controlled systems for the roller bottle operations for feeding. Large scale production with eukaryotic cells pose problems for large scale production - as compared to microbial. Some mammalian cell lines can be used in tanks when adhered to beads (usually adapted for suspended culture). Hollow fiber tanks can be used where the nutrients are pumped through the hollow fibers and diffuse across for feeding cells attached on the other side. Also known as cell retention tanks.

•Some novel applications include chimerics and transgenics (e.g., deriving protein in dairy cattle milk).

•Plant cell cultures can also be used (e.g., large scale fermentation of Taxol). Increased taxol levels from of taxol are < 0.1% (dry weight basis from Pacific Yew tree bark) and now by cell culture, can be up to 120 mg/ gram of dry weight basis. Donna Gibson (USDA) first isolated and crafted a cell culture for taxol cells. Hairy root cell culture is a technique that is used to transform cells into inducing the protein, causing cell immobilization.

•Metabolic engineering can (1) improve efficiency of existing metabolic pathway (e.g., eliminate rate-limiting steps for desired product), (2) introduce plasmids for known products (e.g., production of indigo in E. coli), or (3) produce novel combinations of biosynthetic pathways.

•Some companies will lock on to a certain expression system for pretty much all products (e.g., Genentech with E. coli or SKB with *Drosophila species*).

Comparison of Protein Expression Systems

Comparison of Heterologous Protein Expression Systems Available from Invitrogen						
SYSTEM	HOST	EXPRESSION LEVEL RANGE*	FUSION PARTNER	SECRETION	PURIFICATION	POSTTRANSLATIONAL MODIFICATIONS
ThioFusion™	<i>E. coli</i>	µg-mg	Thioredoxin (N-terminal)	No	Affinity purification (ThioBond™)	Phosphorylation, possible acetylation, no glycosylation
Xpress™	<i>E. coli</i>	µg-mg	Xpress™ Tag (N-terminal)	No	(His) ₆ affinity purification (ProBond™)	Phosphorylation, possible acetylation, no glycosylation
	Mammalian cells	ng-µg	Xpress™ Tag (N-terminal)	No	(His) ₆ affinity purification (ProBond™)	N-linked and O-linked glycosylation, phosphorylation, acetylation, γ-carboxylation
<i>Pichia pastoris</i>	Yeast	µg-g	None	Yes	Secretion to medium yields initial purification step	N-linked glycosylation†, phosphorylation, acetylation, and acylation possible
Baculovirus	Insect cells	µg-mg	Xpress™ Tag (N-terminal)	Yes	(His) ₆ affinity purification (ProBond™)	N-linked glycosylation (mannose), phosphorylation, acylation, possible acetylation
Sindbis	Eukaryotic cells	µg-mg	None	No	None	Determined by cell line used

* Expression levels will vary with every protein expressed and are dependent on many factors including protein size, structure, solubility, stability, and posttranslational modifications.

† Differs from mammalian N-linked glycosylation

Characterization Testing

- **Host cell systems**
 - Source, relevant genotype and phenotype, as well as stability, purity, etc.
- **Gene Construct**
 - Gene construct and restriction enzyme digestion map, complete nucleotide sequencing with regulatory elements identified
- **Vector**
 - Source and function of vector components, origins of replication, antibiotic resistance, restriction enzyme digestion map, critical genetic markers identified
- **Expression system: Final Gene Construct**
 - Description of assembly, restriction enzyme digestion map, identity of critical sites, etc.
- **Cloning & Establishment of Cell Lines (MCB/ MWCB)**
 - Mechanism of transfer of final gene construct, copy number, physical state of final product (e.g., integrated vs. extra-chromosomal), selection criteria, etc.
- **Resulting Product & End-of-Production (EPC) Cells**
 - AAA, AA sequencing, peptide mapping, determination of disulfide linkage, SDS PAGE (reduced and non-reduced), isoelectric focusing, HPLC, SEC HPLC, RP HPLC, mass spectroscopy, assays to detect proteins including deamidated, oxidized, cleaved, and aggregated forms, AA substitutions, adducts/derivatives, assays to detect DNA, residual host cell proteins, and reagents, immunochemical analyses, bioburden, endotoxin, sterility; Post-translational modification testing such as glycosylation, sulfation, phosphorylation, or formylation; Additional testing for derivatization from/toxins, conjugates, radionuclides, etc.

A number of FDA guidance documents address characterization testing of recombinant system components, as well as the resulting product.

Plasmid components are as follows:

- **Ori** (origin of replication point for plasmid); **Par** locus (stabilizes plasmid distribution from mother to daughter cells. Loss of par locus means daughter cells can lose plasmids. **Reporter gene** (handle for the scientist to select, maintain, and identify plasmid in host cell). Usually done with antibiotic resistance (e.g., kanamycin, tetracycline). • **AUG** (start codon) with a spacer region between this and a ribosomal binding site (RBS) - key for initiation of transcription. Downstream from this is the product protein. There's a pre-protein and pro-protein segment that allow the cell to efficiently process the plasmid. Downstream is the stop codon (**UGA**). **BamI** and **SalI** are restriction enzymes used to create "cassettes" that can be used to analyse if the protein of interest is affected by manipulations. **Promoter** is evaluated by binding RNA polymerase activity (e.g., lac, trp, tac, cad, etc.). **Inducible promoter** is the best way to do the plasmid, since it will allow the cell growth to be maximized with minimum impact on plasmid shedding.
- E. coli sigma factors recognize promoters and important for efficient finding of RNA polymerase and efficient transcription and translation of product. Promoter selection has big impact on levels of protein
- Need to be careful of evaluating a fermentation by OD alone. OD can be plateaued, but temperature and induction time changes can have tremendous impact on plasmid stability (as assessed by antibiotic resistance).

Fusion proteins: four good uses - secretion systems, good handle for affinity chromatography, stabilizing product intra- and extracellularly, enables activation of protein in a more controllable fashion (first, expression and then activation). Some people have used E. coli enterotoxins as fusion proteins for linking with specified product. Since E. coli is not known to secrete much, this mechanism is a way that you can secrete specified product from an E. coli system. But this has only been used for R&D to demonstrate 'proof of principle'. FDA hurdles for use in man may be too early/ much to tell if it can be used commercially.

- Runaway system is used to keep copy number low and then induce shooting the copy number up to an extreme amount and thus, product being produced. Known as convergent transcription uses a negative antisense. Very quick process (minutes to hours). Advantage is to boost growth phase from induction phase.
- Eukaryotic systems used for 4 major post-translational modifications: disulfide bonds, proteolytic cleavage into an active form, glycosylation (both amount and type), specific additions to amino acids within the protein (acetylation, phosphorylation, etc.).
- Three different classes of expression vectors used with yeast: plasmid vectors not that useful in yeast, so need to integrate the vectors into yeast system. Yeast artificial chromosome (YAC) sort of mimics chromosome - mostly used in R&D, not too much for commercial.
- Alpha factor is a key factor in yeast secretion of proteins. Key because only secreted proteins are glycosylated! To facilitate secretion, use a pre-pro-leader sequence with alpha factor built in. During the export process, the first step is disulfide bond formation, then proteolytic cleavage of pre-pro sequence, and then post-translational modifications (glycosylation).

Scale of Manufacturing: Pilot vs. Commercial

- **Pilot scale:**
 - use for process development (e.g., neural nets, edge of failure vs. process optimization)
 - may be used to demonstrate early efficacy for application and support sales until commercial facility is completed/ validated
- **Commercial scale:**
 - may be done at same site or with similar equipment but on larger scale;
 - should be material used in regulatory filing;
 - should support filing with at least 3 months stability from commercial run
 - compare against 6 months (or more) from pilot scale lots;
 - compare both against innovator stability profile

Process Development Strategies

- Expression and Growth factors
- Lots of ways; be creative about media, feed strategy, induction times, cell density, etc.
- pH becomes critical (avoid salting out protein of interest); there are lots of fermentations where expression changes with pH; pH can also be used for assessing plasmid stability.
- OxyR and SoxRS are two oxygen-sensitive proteins in E. coli that will help mediate O₂-mediated damage to proteins
- Arc A/B are two repressor/ regulatory genes that will shut down TCA (glycolysis) when activated, pushing organisms to anaerobic. PH goes up and product levels go down. FNR proteins may also be activated.
- Be careful when going above 20 generations - particularly for scale-up operations where you may hit 25-40 generations routinely.
- Need to achieve high cell density for commercial operations (fermentation) 50-80 g DCW/l.
- Secretion advantages (four): economical, avoid toxic impact on host cell, little/ no denaturation of proteins in inclusion bodies, and proper folding too.
- Yield of protein is a cumulative reflection of yield, aggregation, and folding.
- Some new approaches have used antibodies to aid in re-folding of proteins.

Process Development: Neural Nets & Edge-of-Failure

- Process focus is on biobatch parameters such as pH, time, temperature, fed-batch characteristics for translation of proteins, etc.
- Watch copy number, growth rates, and expressed product vs. plasmid stability
- Process Control: PID (Proportional Integral Detector) is integrated into commercial fermentation technology as programmable logic controller (PLC) language
- Principle Component Analysis (PCA): is part of the modeling used in neural net analysis
- PCA/ PLS (projection to latent structure) used to analyze several variables at once and assess covariance of each - relevance to "good vs. bad" batches

Plasmids place a burden on the host system, so a lot of engineering is focused on keeping the burden to a minimum, such as using an inducible enzyme system that may impact it at a precise moment in the growth phase. One of the most evident areas for this is ATP production levels for normal cells compared to rDNA-cells.

• Copy number of expressed plasmid can be manipulated, but impact on growth rate can be seen. As copy number goes up so does productivity, but growth rate comes down. Growth rate is also used as an indicator of strain stability. Any changes in growth rates will indicate lack of plasmid stability (e.g., growth rate going up may reflect shedding plasmid).

- Application of Neural Networks and PCA in Biotechnology
- PCA (principle component analysis)/ PLS (projection to latent structure) can be used to analyze several variables at the same time and assess the covariance of each. One can use PCA/ PLS to assess which data values are relevant to 'good batches' vs 'bad batches' Can then draw a confidence limit for certain variables. Based on a 95% CI, can then use Square Prediction Error (SPE) or Hotelling's T² statistic. Can use SPE to identify a given variable or fault protection to find out the errant variable.
- Program is Multi-DAT; PCA/ PLS software. Can buy the commercial software from Eigenvector, Inc. in Washington, (state or DC) Ask for Barry Wise. Sells PLS toolbox.
- Neural Network - based on data already obtained. A program that is designed (AI) to recognize certain features of a process. The input is sent through a processor (modelling predictions) and comes up on the output side with certain weighted features of the varying variables for the process.
- Inverse Network is where you can use PCA/ PLS to design the optimal media for fermentations.
- Visualization package available for another type of neural network: Self Organizing Maps (SOM) - also known as Kohonen's Network.

Principle Component Analysis (PCA)

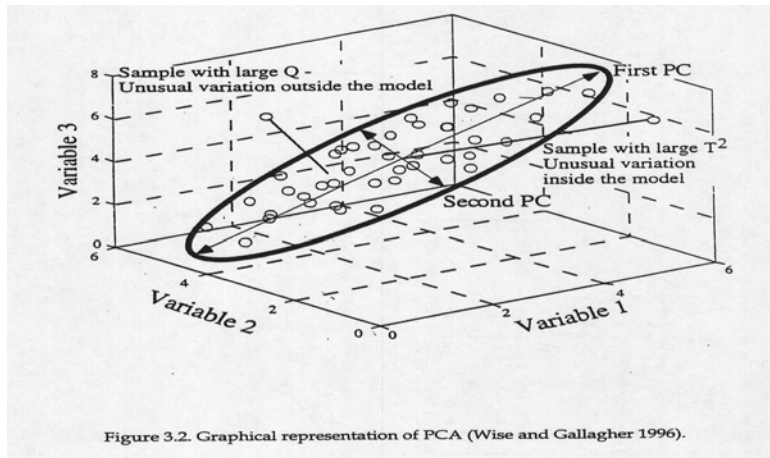


Figure 3.2. Graphical representation of PCA (Wise and Gallagher 1996).

Process Development: Overview of Mammalian Cells

- CHO is the most commonly used tissue culture line, but mouse myeloma cell lines are also becoming more popular
- Attachment-dependent cell lines are not the most optimal for production, since the attachment requirement is hard to implement on large scale
- The demand for non-essential amino acids becomes greater when you have TC under rapid growth, so most media come with a combined essential and non-essential amino acids.
- When shifting to serum-free media, you need to assess the minimum concentration of added components (e.g., insulin, transferrin) that will get you to the near maximal levels of cell growth.
- Osmolality: when adding nutrients in a fed batch scenario, it's important to use osmotically balanced solutions so that the continuous addition of solutes don't impact cell viability.
- Oxygen saturation is not toxic to the TC cells when done for a short time; useful to determine the duration of oxygen tension.
- Cell culture bioreactors: batch, fed batch, continuous, or perfusion it's important to simultaneously feed glucose and glutamine! Glutamine prolongs the stationary phase of growth - less cell die off.
- Productivity (e.g., Mab production) can be higher when cells are grown at specific growth rates less than the maximal. The theory is that Mab production is primarily in G1 growth phase of the cell and the slower growth rate allows a prolonged G1 cycle.

• Since most media were developed for particular cell lines and under static conditions - non production, the media don't have everything you need. You can buy non-essential AA to add during production. Also note that lots of media were developed with particular cell lines in mind.

• When using sodium bicarbonate as a buffering system, it's important to work quickly with TC outside of the incubator with the tops capped, since the CO₂ may quickly come out of solution, driving the pH up and injuring/ killing the TC.

• Can tell dead cells from live cells by size. Use cytometry to assess dead cells, which are smaller than live cells. Also the more dead cells, the slower the growth rate.

Bioprocessing Overview: Bioreactor Considerations

- **Metabolic Engineering**
 - Regulation of Carbon Flow
 - Optimization of Productivity: mutational & rDNA approaches
- **Process Development Strategies**
 - Factors Impacting Formation of Product: Productivity
 - Metabolic Demand on Expression System
 - Plasmid Instability
 - Promoters
 - Product Fate in cell & fermentation medium: Recovery

• Metabolic Engineering

- Improve efficiencies of existing metabolic pathways - usually rate-limiting steps or for undesired products
- Introduction of plasmid for known molecules (e.g., production of indigo in *E. coli* or B-carotene in *E. coli*)
- Novel combinations of biosynthetic pathways

- Bioreaction Kinetics:
 - In the Michealis-Menton (M-M) equation, the smaller the K_m , the quicker the V_0
 - Determination of M-M Model Constants: most common way is to invert the M-M equation which gives you a straight line, and the Y-intercept is the reciprocal of K_m , but a common problem is what starting concentration you go with (large error)
 - Note that in cell growth, product formation may occur during cell growth or it may happen after the cells are finished growing
 - Can use theoretical yield coefficients to determine if the actual yields are generating what you would expect. If not, you can use the theoretical yield to help adjust/ modify the process to come closer to the theoretical.
 - Product Formation Classification Schemes: Type I, II, and III: Type I is product formed as a result of primary energy metabolism (e.g. CO_2 or ethanol). Type II is product formed indirectly from reactions (e.g., citric acid or amino acids). Type III product has no direct relationship to energy metabolism (e.g., penicillin or other antibiotics) - done during the stationary phase. Sometimes with Type III metabolisms, the organism will deplete the primary substrate source and then switch into a secondary metabolic mode where the antibiotic components are made. If you then add more primary substrate again, the bacteria will stop making antibiotic and switch back to a straightforward Type III linear growth mode. With Type III, describing the first half (growth phase) is pretty straightforward; describing the second half (product formation) is the more difficult part.
 - Heat Generation: smaller numbers reveal more exothermic reactions. O_2 relationship also shows the constancy of the combined heat and O_2 on growth rates
 - Cultivation Measurements:
 - biomass is measured by dry cell weight (DCW), or by cells/ L or cell mass/ L. Be careful about OD measurements because spectrophotometers are not linear above certain levels of density (e.g., 0.3), so you have to dilute the sample down to make sure that you're on the linear part of your spectro measurement. Filtration sensors (mass concentration) used on line or during real time make more sense for use as an in-process control. DCW is too long to get feedback on fermentation. Filtration sensors are expensive (compared to DCW). Also laser sensors (about \$10 K) are available and validatable (pretty robust). The filtration sensors (direct product contact) aren't really being marketed, mostly R&D.
 - Direct estimate of cell number is by: microscope, Coulter counter, plate counting, or flow cytometry. It's also interesting to note that cell number relationship to cell mass and product formation needs to be assessed too during development. Can use McFarland standards for calibrating OD in spectrophotometry.
 - Indirect estimate of cell concentration:
 - Measurement of substrate and product concentrations:
 - Relationship of Measurements to Growth Reactions:
 - Kinetics of Microbial Growth:

Bioreactor Design

- **Design & Configuration**
 - Types of Bioreactors and Construction Materials
 - Typical Features and Operational Requirements
- **Bioreactor Design Equations**
 - Enzyme Bioreactors
 - Cell Bioreactors
 - Batch
 - Continuous Flow, well mixed
 - Continuous Flow, plug flow
 - Fed Batch
 - Comparison
- **Transport Phenomena in Bioreactors**
 - Oxygen Supply
 - Power Requirements
- **Immobilized Biocatalysts**
- **Operating Scale and Scale-up**

Factors impacting yield include:

PHYSICAL PARAMETERS: temperature, pressure, agitation speed, gas flow rate, and power input, liquid feed rate, liquid level, acid/base addition, antifoam agent addition, broth volume, color, density, gas humidity, osmotic pressure, and viscosity

CHEMICAL PARAMETERS (extracellular): pH, redox potential, dissolved oxygen, dissolved carbon dioxide, effluent oxygen, effluent carbon dioxide, respiratory quotient (RQ), nutrient composition, nitrogen, conductivity, cation level, ionic strength, amino acids, concentrations of substrates, product, precursors, phosphorus, intermediates, and inhibitors.

BIOCHEMICAL PARAMETERS (intracellular): amino acids, ATP/ ADP/ AMP; NAD/NADH, carbohydrates, cell mass composition, enzymes, intermediates, nucleic acids, total protein, vitamins, age distribution, aggregation, doubling time, genetic instability, generation number, morphology, mutation, size distribution, total cell count, viable cell count.

Bioreactor Design & Configuration

- Types include tank, column, or loop. Mixing includes stirring, multi-stage (cascade), or propellor loop. The choice depends on cultivation needs and size.
- Size of batch reactor does not impact the kinetics of the reaction, but does impact on the 'economy of scale' or 'scale of efficiency' = economics. There are mass transfer issues at larger scale - movement of substrate and oxygen in a larger reactor may impact efficiency and yields.
- Fed batch is used to overcome substrate inhibition. Making more product in the same amount of time.

Bioreactor Transport Phenomena

- Encompasses fluid mechanics in terms of heat transfer, substrate transfer.
- Oxygen supply: has low solubility in water which requires constant addition. Single largest way to improve is by increased mixing, increased pressure, and increased surface area of the O₂ bubbles.

Bioreactor Scale-up

- Typically the reaction is about 1% cell mass by volume (of the reactor)
- The larger the tanks, the less energy-efficient is the active agitators. Sparging and aeration are more commonly employed in larger systems. For instance, the 75,000 L tank used by ICI used loop kinetics for mixing.

Downstream Purification

- **Process Sequence and Engineering Principles**

- Product purity constraints
- Cost of production/ yield
- Scalability
- Reproducibility/ ease of implementation

- **Use processes with high purity factor, such as:**

- Affinity chromatography
- Inorganic adsorption
- Gel filtration
- Hydrophobic chromatography
- Ion exchange
- Detergent extraction
- Aqueous separation
- Precipitation

High purity factors are where the unit operation of before vs. after gives you a purity level. A 90% purity after a unit operation gives you a $90 \div 10 = 9$ purity factor.

- re-folding step usually included for rDNA products in inclusion bodies.
- For the insulin separation steps (12 steps for alpha chain alone), if each step was only 50% efficient, then the total PF of the 12 steps is 1 in 4000.
- Reverse osmosis (RO) membranes are often used for purifying water, but generally there is a high level of rejection of charged ions. However, if you just used acetate in a prior step, the RO membranes may not prevent the co-migration of acetate along with water.
- All filtration membranes 'foul' Mostly caused by smaller solute molecules going into the membrane layer, but not all the way through. Other solutes merely adsorb to the surface of the membrane. Lastly, 'gel concentration polarization' is when your solute of interest is concentrated in a localized fashion near the membrane, allowing a concentration of solute that clogs the membrane flow. Eventually, the membrane shuts down - as shown by using different concentrations of proteins (e.g., BSA). One can increase the filtration rate by increasing pressure, and when pressure and higher concentrations are involved, the filtration rate drops off considerably. 'Flux decline' is when the increased pressure causes a drop in filtration rate due to increased gel concentration polarization. Can try to overcome this with diafiltration by just refreshing the solvent. Also way to overcome it is by increasing membrane area. Increase pressure too - but only so much and then you'll burst the membrane.
- Stirring over the membrane will help keep the concentration polarization material from collecting too much. Also, can do a quick backflush (can have 10X increase in filtration) with that technique.
- Cross-flow is where the solvent travels perpendicular to the membrane channels will provide a shear force that will remove the adsorbed layer and reduce the gel concentration polarization layer.
- Hollow fiber filtration is the best choice for scale-up since there is a dramatic increase of surface area in a small area.
- Be aware that you need to achieve a 'steady state' with the permeate in a continuous loop around the retentate module. Once the steady state is achieved, then you can start with solvent replacement (e.g., dialysis)
- Since the smallest pores plug up first, the mean pore size of the membrane is changing.
- A trick for quick sedimentation is to add a flocculant that will help your particle of choice to make quicker centrifuge runs. May not want to use for ribosomes, but may want to get rid of bacterial debris. Same thing applies to ultrafiltration too. IF we know the particle size, one can do 'sigma analysis' to find the appropriate centrifugation speeds for purification.
- Fixed angle rotors have a smaller radius and thus, has less time to achieve the optimal time. Swinging buckets have a longer radius.
- Centrifugation may impact on biological activity and impact downstream processes or yields and efficiencies. For instance, mammalian cells will be shocked at high Gs, and some mixed cell populations (e.g., T cells and B cells) may be activated by centrifugation. Forcing greater concentrations and 'handshaking'. Some proteins and small particles can be impacted by very high Gs too (concentration of solutes along with proteins that inactivate them). Need to assess this on a case by case basis.

Comparability Studies

- **Links: Impact of Manufacturing Controls to** -
 - Analysis of Product Characteristics: Comparability testing
 - Surrogate Equivalence: Impact on activity
 - Stability: Emergence of undetected impurities
 - Analytical Methods: Consistency of methodology/ data
 - Setting Rational Specifications

Integration of Analytical Testing Profile to Manufacturing and Stability

- FDA Guidance on Drug & Biologics Stability (6/98)
 - Length of stability studies with pilot scale material
 - Length of stability studies with commercial scale material (or representative of commercial scale)
 - Analytical links to manufacturing
 - Analytical links to innovator stability performance
 - Adequate sampling

- This is perhaps the most critical aspect of the program. Look carefully at both the FDA 1997 stability guidance and biotech guidances appropriate to your product.
- Set several lots of both innovator and your product on stability from various manufacturing scales. 6-months minimum for pilot scale material to assess everything's OK to go on for commercial scale. Although it sounds longer than most drug formulation prototype developments, it will go fast. You will need the 6 months to prepare for commercial manufacturing scale.
- During packaging and labelling runs, be sure to sample heavily noting when and where the specific samples came from during the process. For instance, if pre-filled syringes from a 5-line apparatus, know which line and what time certain samples were collected (e.g., 15 minutes after line start, 60 minutes?). When OOS data appear, you may find mechanical reasons - attributable reasons to exclude those data.
- When OOS data occur, be prepared to quickly make more lots and put them on stability - as soon as investigation recommendations are known. May not want to wait for entire investigation to conclude to find an attributable reason. May want to employ a shotgun method in terms of improved aseptic process handling, quicker holding times, more IPC checks, and additional training/ supervision of process.
- Need to do extensive linking to manufacturing processes and comparability protocol. Have organized stability protocols with numbering systems that will allow quick identification of samples and test data.

Summary of Key Points

- Regulatory strategy can only be defined after analytical and therapeutic equivalence is established
- Therapeutic equivalence criteria will probably be a mixture of innovator criteria + industry/ clinical experience with those classes of products
- Analytical equivalence must be established using the most current and wholistic approaches
- Analytical data must be extensive to support bioassay variability, manufacturing flexibility, microheterogeneity, stability changes, etc.
- Surrogate endpoints may be a repeat of innovator or a completely different approach, depending upon FDA acceptance
- Manufacturing process controls must reflect product parameters well within 'edge of failure'
- Stability data must be supported by extensive characterization testing to show subtle changes do not impact purity, potency, or safety
- Stability testing should include innovator/ reference product as well as comparator lots
- Manufacturing scale and process development changes must be supported by extensive comparability testing

List of References

- **FDA Guidelines, Guidances, & Points to Consider (PTC)**
 - CMC Information for a Therapeutic rDNA-derived Product or a Monoclonal Antibody Product for *in vivo* Use (August 1996)
 - PTC in the Characterization of Cell Lines Used to Produce Biologicals (1993)
 - PTC in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1994)
 - PTC for the Evaluation of Combination Vaccines: Production, Testing, and Clinical Study (1995)
 - PTC on Plasmid DNA Vaccines for Preventive Infectious Disease Indications (1996)
 - CMC and Establishment Description Information for a Vaccine or Related Product (1998)
 - CMC and Establishment Description Information for Human Blood and Blood Components Intended for Transfusion or for Further Manufacture (July 1998)
 - CMC and Establishment Description Information for Human Plasma-derived Biological Products or Animal Plasma-derived Products (Dec 1997)
 - Regulation of Placental/ Umbilical Cord Blood Stem Cell Products Intended for Transplantation or for Further Manufacture into Injectable Products (Dec 1995)
 - Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products
 - Stability Testing of Drug Substance and Drug Products (June 1998)
- **Code of Federal Regulations (CFR)**
 - 21 CFR 600 - 680
- **Federal Statutes**
 - Food and Drug Administration Modernization Act of 1997 (FDAMA)
 - Drug Price Competition and Patent Term Restoration Act of 1984 (Waxman-Hatch)
 - Food Drug & Cosmetic Act