• Process Optimization Considerations include:
  - (1) Optimal protein yield,
  - (2) Optimal expression rate (cellular formation and specific productivity),
  - (3) Cellular processing of proteins to minimize proteolysis, maximize glycosylation, protein folding, and secretion,
  - (4) Stability, and
  - (5) Recovery.

Key areas of focus for regulators and development teams include: plasmid instability, selection of best promoter, mRNA stability and concentration. While efficient transcription and translation of product is vital, purity is often the determining factor.

• Potential systems for protein production include:
  - (1) Purification from natural sources (e.g., vaccines, plasma derivatives);
  - (2) Recombinant bacterial systems (e.g., E. coli);
  - (3) Recombinant mammalian cell lines (e.g., Chinese Hamster Ovary [CHO]);
  - (4) Recombinant yeast systems (e.g., Pichia pastoris);
  - (5) Plant transgenics (e.g., corn-based vaccines);
  - (6) Animal transgenics (e.g., proteins in milk);
  - (7) Gene therapy; and
  - (8) Synthetic methods (e.g., oligonucleotides, antisense, etc.).

Expression system and method of manufacture must allow for ease of scale-up (efficiency of scale) and robust/rugged steps that allow flexible production parameters without impacting product quality – as well as processes that step-wise and allow intensive testing for characterization and process controls.
• Eukaryotic vs. Prokaryotic Organisms
  - Microbial (prokaryotic) (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
  - Yeast (eukaryotic) (e.g., Pichia pastoris): great potential for large-scale manufacturing. Early use in Saccharomyces used plasmid vectors due to difficulty in integration. Yeast Artificial Chromosomes (YAC) used to mimic chromosome mostly used in R&D but not too much for commercial
  - Insect cells (eukaryotic) (e.g., Drosophila species): can be used in continuous circulation tanks since they can take more abuse in shear pressure
  - 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.
  - Mammalian cells (eukaryotic) (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
  - Focus is to minimize burden of plasmid on organism by using inducible enzyme system

• Form follows function. Look to what others are doing – via patent searches and technology licensing agreements. 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. 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 based on 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 are limited for post-translational changes/folding. Yet some companies live with inefficiencies (low yield on folding); Eli 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) have higher costs (e.g., testing, production), but greater efficiencies on post-translational changes and purity. Cell-adherent requirements limit them to roller bottles, suspended cultures on microbeads, or some proprietary disposable reactors. 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

<table>
<thead>
<tr>
<th>Source</th>
<th>Host</th>
<th>Expression Level Range</th>
<th>Promoter</th>
<th>Recombinants</th>
<th>Purification</th>
<th>Post-Translational Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThioExp™</td>
<td>E. coli</td>
<td>µg/kg</td>
<td>Thioextension (N-terminal)</td>
<td>No</td>
<td>Affinity purification (Thiodend™)</td>
<td>Phosphorylation, possible acetylation, no glycosylation</td>
</tr>
<tr>
<td>Xpress™</td>
<td>E. coli</td>
<td>µg/kg</td>
<td>Xpress™ Tag (N-terminal)</td>
<td>No</td>
<td>O-linked affinity purification (ProBond™)</td>
<td>Phosphorylation, possible acetylation, no glycosylation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&amp; Xpress™ Tag (N-terminal)</td>
<td>No</td>
<td>O-linked affinity purification (ProBond™)</td>
<td>W-linked and O-linked glycosylation, phosphorylation, acetylation, deamidation, p-carboxylation</td>
</tr>
</tbody>
</table>
| Piccolo pastoris | Yeast | µg/kg | None | Yes | Secretion to medium yields initial purification step | N-linked glycosylation, phosphorylation, acetylation, possible }
| Baculovirus | insect cells | µg/kg | Xpress™ Tag (N-terminal) | Yes | O-linked affinity purification (ProBond™) | N-linked glycosylation (common), phosphorylation, acetylation, possible acetylation |
| | Silkworm | µg/kg | 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.

1. Differences from mammalian N-linked glycosylation.
Comparison of Protein Expression Systems: Prokaryotic Cell Lines: *E. coli*

**Advantages**
- Relatively high yield (0.1 - 10 g/L)
- Method of manufacture (fermentation) relatively low cost and facile scale-up
- Fast doubling times (allowing fed-fast batches to complete within 24-36 hours)
- Simple nutritional needs
- Process development pretty well established
- Relatively easy MCB & MWCB characterization
- Pervasive technology for genetic manipulation

**Limitations**
- Post-translational modifications limited to phosphorylation and possible acetylation, but no glycosylation (carbohydrate modification)
- Protein folding efficiencies lower than mammalian cell lines
- Protein usually isolated as part of a fusion peptide in an inclusion body
- Proteolytic enzymes can quickly reduce yield

- 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 (optical density) alone. OD can plateau, 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
- 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.
Comparison of Protein Expression Systems:  
Eukaryotic Cell Lines: Yeast (Pichia pastoris)

- Several strains used investigationally or commercially: Hansenula polymorpha, Kluyveromyces lactis, Pichia pastoris, Saccharomyces cerevisiae (also known as Brewer’s Yeast), Yarrowia lipolytica, and Schizosaccharomyces pombe
- P. pastoris - methyltrophic (uses methanol as sole carbon source) yeast that was used to degrade waste streams from the petrochemical industry
- Cloning via an ‘expression cassette’ (see details below)
- Post-translational modifications (e.g., glycosylation) occur during transfer across the Golgi complex; vesicles containing the protein merge with plasma membrane and are expelled into extracellular medium
- P. pastoris does not express the α-1,3-linked mannose terminals (which are residues associated with immunogenicity)
- Yeast Artificial Chromosomes (YAC): Used early on overcome size limitation for cloning large DNA segments. YAC have the telomere, replication origin, and centromere sequences of normal yeast chromosomes but with foreign DNA (up to a million base pairs) instead of yeast DNA

- Pichia pastoris enzyme alcohol oxidase catalyzes the first steps in biotransformation of methanol. Pichia pastoris produces two functional alcohol oxidase enzymes (I and II) expressed from separate genes AOX1 and AOX2 (which share 93% homology in their AA sequences). Production of both enzymes is regulated at the level of mRNA transcription. When Pichia pastoris is cultivated in other carbon sources like glucose, the AOX genes are repressed (no mRNA detectable).
- An expression cassette (going from 5′-3′) is comprised of: 5′ - AOX Promoter Region - Signal Sequence for secretion of expressed protein - Protein Coding Sequence - Transcription Terminator - 3′ Final elements of the expression cassette is a polyadenylation sequence that adds a poly-A tail to the mRNA, protecting it from degradation and from the transcription terminator (which halts mRNA transcription). The poly-A sequence may be from the AOX sequence itself, to assure easier recognition. Construction cassettes may be assembled head-to-tail to ensure high levels of protein expression.
- As with E. coli strains, mutant Pichia pastoris strains have been created that only grow correctly in the absence of a key metabolite – complementation of metabolic function.
- Pichia pastoris does not express the enzyme α-1,3 mannosyl transferase and consequently does not produce the α-1,3 mannose terminal residues – which are a common feature of glycosylation in S. cerevisiae and associated with highly antigenic responses. Notably, excessive glycosylation (e.g., hyperglycosylation) associated with expression in S. cerevisiae may result in decreased activity and serum retention (half-life). The N-linked high-mannose oligosaccharides are characteristic of yeast glycoproteins, but P. pastoris exhibits substantially shorter chain lengths (up to 20 units) vs. S. cerevisiae structures (50-150 units).
Profile of a Fed-Batch *Pichia pastoris* Fermentation

- Practical large-scale fermentation can be simple batch, fed-batch, or continuous modes. From an industrial perspective, continuous fermentation results in maximal productivity, but it’s operation is difficult at large scale over extended periods of time.

- Early stages of fermentation start with glycerol as carbon source, which is replaced by methanol. Production fermentor is inoculated with 5-10% of the initial volume in the receiving vessel.

- Key process parameters are temperature, dissolved oxygen (DO), and pH. Fermentation is usually run 26-30°C. Later stages of fermentation (with high biomass) require rigorous control of heat. DO levels should be a minimum of 20% throughout fermentation. At the end of the run, the biomass is chilled (10-15°C) to decrease cellular mass and prevent autolysis of cells after discontinuation of oxygen supply and methanol feed. Where the protein may be secreted into the fermentation medium, cell removal by centrifugation or flocculation will generate a clarified filtrate.
Comparison of Protein Expression Systems: Eukaryotic Cell Lines: Insect (Baculovirus)

**Advantages**
- Cells grow at room temperature
- Do not require CO2
- Glycosylation patterns differences compared to mammalian cells show enhanced immunogenicity - which can be a benefit for vaccine production
- Can grow in suspensions and more tolerant of shear pressure than mammalian cell lines
- Fermentation parameters comparable to vertebrate cells, although lower pH ranges (6.0 - 6.9) and osmolality - which is high due to amino acids - as well as ion ratios (that vary from insect to insect);
- Some standard mammalian media formulations could be adapted for insect lines, but a number of firms have now developed specific serum-free and protein-free media for use with Sf9 and High Five™ cell lines.

**Limitations**
- Glycosylation pattern differences can be limiting when the desired profile is vital to structure-activity relationship (e.g., thrombopoietic factors)

- Glycosylation differences: insect cells produce mannosylated glycoprotein structures and lack their terminal sialic acid or penultimate galactose saccharides found on native mammalian proteins. This structure tends to decrease the effective half-life of proteins in vivo. However, this difference can be an advantage when the end result is to boost the immune system - such as with vaccine production. Hence, insect cell lines are finding increased use in commercial vaccine production.

- An historical - now superseded - cell line is S2 derived from a primary culture of late-stage *Drosophila melanogaster* embryos, which is used for heterologous protein expression - a cell line that is readily adaptable to suspension.

- Newest developed cell lines are Sf9 and Sf21 derived by USDA from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* - which are used extensively for the isolation and propagation of recombinant baculovirus stocks.

- The High Five™ cell line (BTI-TN-5B14) was developed at Boyce Thompson Institute for Plant Research (Ithaca, New York) from embryonic ovarian cells of the cabbage looper, *Trichoplusia ni* - a cell line that is easily grown in suspension and has increased protein production 5-10X higher than Sf9 cells.
Comparison of Protein Expression Systems:
Eukaryotic Cell Lines: Mammalian Cells

- Characteristics of an Ideal Mammalian Cell Line for Production
  - Can grow continuously in vitro without becoming senescent
  - Can grow in free suspension in culture medium
  - Is easily transfected with foreign genes
  - Produces low quantities of lactic acid and ammonia (which inhibit antibody production)
  - Grows and can be maintained in serum-free media (critical consideration with BSE and TSE testing/sourcing requirements)
  - Does not produce virus or viral enzymes
  - Has a stable and efficient secretory process
  - Is insensitive to shear forces in a mixing vessel
  - Has a high specific productivity (e.g., 3-5 pg/cell/min)
  - Production of the desired product is constitutive and does not destroy the cell
  - Exhibits an extended, viable stationary phase after the exponential growth phase

*It’s typical that microbial and cell culture/ or yeast systems are used in dual-track basis for R&D rDNA products, especially if it’s discovered that the microbial product has little or no biological activity.
Comparison of Protein Expression Systems:
Eukaryotic Cell Lines: Mammalian Cells

- **Advantages**
  - Able to perform post-translational modifications like phosphorylation, acetylation, glycosylation (carbohydrate modification), protein folding, and proteolytic processing
  - Easier to generate soluble, folded proteins
  - Protein secretion from cell eases isolation and purification
  - Improved success rate for creating complex proteins
  - Less troublesome contaminants (e.g., endotoxin, low RNA & DNA levels, no cell debris)
  - Protease activity is low

- **Limitations**
  - Lower cell growth rates means loner culture times & rich nutrient media susceptible to contamination
  - Bovine-derived raw materials must be sourced from BSE/TSE-free areas
  - Complex production methods means higher operating costs
  - More adventitious agent testing for mammalian cell lines
  - Tumor-derived host cells means oncogenes must be remove (and confirmed)
  - Relatively low production yields per volume of culture medium

While mammalian cell production is relatively higher cost than *E. coli* or *Pichia pastoris*, the downstream purification with mammalian cell lines tends to be more straightforward and there may be cost savings in scale-up due to reduced downstream purification.
Comparison of Protein Expression Systems: Cell and Gene Therapy Products

Applications of Cell Therapy Products

- **Bone Marrow Transplantation**: devices and reagents to propagate or select stem and progenitor cells; to remove/replace diseased cells
- **Cancer**: T cells, dendritic cells, or macrophages exposed to cancer-specific peptides to elicit an immune response; autologous or allogenic cancer cells injected with cytokine and irradiated to elicit an immune response
- **Pain**: Endorphin-secreting cells encapsulated in a hollow fiber
- **Diabetes**: Encapsulated β-islet cells secreting insulin in response to glucose levels
- **Wound Healing**: sheet of autologous keratinocytes or allogenic dermal fibroblasts on a biocompatible matrix (e.g., collagen)
- **Tissue Repair**:
  - Focal defects in knee cartilage with autologous chondrocytes; cartilage-derived structures repaired with autologous or allogenic chondrocytes in biocompatible matrix;
  - bone repair via mesenchymal stem cells in a biocompatible matrix;
- **Neurodegenerative diseases**: via allogenic or xenogeneic neuronal cell implants
- **Liver disease**: allogenic or xenogeneic hepatocytes in an extra-corporeal hollow fiber system (used for bridging until liver transplant)
- **Infectious Disease**: activated T cells

- Cell therapy – also known as somatic cell therapy – is via non-germ cell lines, which may be combined with biocompatible materials for cell protection or enhanced functionality of the matrix support (e.g., collagen support for burns).
- Three sources of donor cells: autologous (from the patient’s own cells), allogenic (from another human donor), and xenogeneic (derived from animals such as pigs, primates, or cows).
  - Autologous cells are not rejected by the donor but they may not be available for many treatments due to: (1) missing, (2) dysfunctional, or (3) diseased.
  - Allogenic cells are more widely available but more likely to have a rejection reaction associated with them. However, they are less likely to trigger a rejection reaction than xenogeneic cells
  - Xenogeneic cells used when human cells (with desired characteristics) are not available or human donor supply too limited.
- Cell therapy products modified by treatment with DNA are known as **ex vivo gene therapy product**.
Gene Therapy Vectors

- Two types of vectors: viral or non-viral
  - Viral: adenovirus or retrovirus
  - Non-viral: plasmid-liposomes

- Attributes of an Ideal Vector (viral or non-viral)
  - capable of efficiently delivering an expression cassette with one or more genes, specific for its target,
  - not recognized by the immune system,
  - stable and easy to reproduce,
  - purified in large quantities at high concentrations,
  - would not induce inflammation, safe for the recipient and the environment
  - would express the gene as long as required in an appropriately regulated fashion.

- Clinical experience suggests retrovirus, adenovirus, and plasmid-liposome vectors all need refinement, but each is relatively well suited for clinical targets. Design hurdles include increase the efficiency of gene transfer, increase target specificity, and to enable targeted genes to be regulated.
- Some of the vector-specific hurdles include reducing risk of insertional mutagenesis in retrovirus vectors, minimization of immunity and inflammation evoked by adenovirus vectors, and enhancement of the translocation of the gene to the nucleus for the plasmid-liposome complexes.
Comparison of Protein Expression Systems: Cell and Gene Therapy Products

- Limitations of Cell Therapy Products
  - **Sterilization**: cells cannot be terminally sterilized or filtered, so removal/inactivation of microbes or viruses is problematic
  - **Raw materials**: purity is key since any raw material could contaminate the finished product
  - **Storage & Stability**: While some products can withstand freezing, some cell-based therapies cannot be frozen without losing key efficacy characteristics – especially those for differentiated functions. Such products must be administered within hours or days of production.
  - **Urgent clinical need**: Often, the product is administered quickly after production.
  - **Batch size = dose**: Some batch sizes equal the amount of the dose (small quantities), which limits the traditional analytical methods (e.g., sterility, mycoplasma, potency, etc.). Often, methods are not rapid enough to be done prior to use of the batch.
Comparison of Protein Expression Systems: Cell and Gene Therapy Products

- **Applications of Gene Therapy Products**
  - **Gene Replacement – Short Term:**
    - Cardiovascular Disease: growth factor vector on a biocompatible scaffold
  - **Gene Replacement – Long Term:**
    - Cystic Fibrosis: transmembrane conductance regulatory vector
    - Hemophilia: Factor VIII or IX vector
    - Sickle Cell Anemia: defective hemoglobin expression
  - **Immunotherapy:**
    - Cancer or arthritis using autologous tumor cells or lymphocytes transduced with cytokine genes; SCID (Severe Combined Immunodeficiency Disease) - also known as "Bubble-Boy Disease"
  - **Conditionally Lethal Genes:**
    - Cancer (solid tumor) via introduction of thymidine kinase (TK) or cytosine deaminase (CD) vectors into tumor cells;
    - Graft vs. Host Disease (GVHD): TK or CD vector transduced into donor T cells
  - **Antisense:**
    - Cancer therapy via anti-oncogene vector; Cytomegalovirus (CMV) retinitis therapy via antiviral vector
  - **Ribozyme:**
    - HIV therapy via antiviral ribozyme vector into autologous lymphocytes
  - **Intrabodies:**
    - Cancer or HIV therapy via single-chain antibody to tumor protein or viral protein

*For details of production and quality control, see USP General Chapter <1046> Cell and Gene Therapy Products.*
Comparison of Protein Expression Systems: Plant Transgenics

- **Corn:**
  - Monoclonal antibodies
  - Vaccines
  - Contraceptive agents

- **Tobacco:**
  - Interferon
  - Antibodies

- **Mother's Milk Sprouts:**
  - Antibiotics

**Advantages:**
- Plant expression systems can eliminate safety concerns regarding exposure to animal viruses, prions, or animal proteins in the finished product.
- May be more amenable oral delivery system for oral vaccines
- Storage stability is enhanced with seeds, allowing scheduling flexibility not seen with unstable intermediates: seeds stored under controlled conditions are viable up to 7 years. Clients have demonstrated stability of MAb in corn seed up to 2 years.
- Genetic stability of multiple genes over several generations has been well established.

**Limitations:**
- Potential for cross-contamination of food supply. For instance, StarLink's genetically engineered corn (grown on less than 1% of US corn acreage) cross-pollinated widely to 430 million bushels - triggering nationwide recalls of taco shells, corn chips, and other foods.

MAbs grown in corn:

Glycosylated and non-glycosylated versions of huNR-LU-10 MAb were developed by NeoRx Corporation. Non-glycosylated BR96 was developed by Bristol-Meyers Squibb and Seattle Genetics. Clinical comparability studies have shown therapeutic equivalency for mammalian cell-derived vs. transgenic products. Physico-chemical comparability was also shown, as were PK parameters (serum and urine clearance and tissue binding).

FDA Guidance for Industry: Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals (draft guidance) was issued September 2002. See guidance for details on testing and production considerations. Key areas cover host and source plant characterization, environmental considerations, manufacturing and process-related considerations, preclinical considerations, and clinical testing.
MCB & MWCB: 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/to toxins, conjugates, radionuclides, etc.

A number of FDA and ICH guidance documents address characterization testing of recombinant system components, as well as the resulting product. See Attachment 1 enclosed in this section for articles detailing MCB characterization testing, validation, and adventitious agent testing. See Attachment 2 enclosed in this section for an article regarding vector design for optimal protein expression. Regulatory aspects of MCB creation and characterization testing will be covered in (1) Biotechnology Development Milestones and (2) Regulatory Considerations in Biotech presentations.

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 analyze 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 are important for efficient finding of RNA polymerase and efficient transcription and translation of product. Promoter selection has big impact on levels of protein.

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.).
Flowchart of Expression with the ThioFusion™ Expression System

1. **pTrxFus**
   - Cloning of cDNA into pTrxFus vector to generate pTrxFus::cDNA.
   - Introduction of cDNA into mammalian cell line, e.g., Chinese hamster ovary (CHO) cells.

2. **Transduction with pTrxFus**
   - Transfection of CHO cells with pTrxFus using DEAE-dextran or lipofectamine.
   - Selection of transfected cells using Geneticin (G418, 600 μg/ml).

3. **Transcription and Translation**
   - Transcription and translation of cDNA in mammalian cells.

4. **pTrxFus**
   - Expression of cDNA in transfected cells.

5. **pTrxFus**
   - Purification of expressed protein from cell lysate.

6. **Biological Activity**
   - Characterization of expressed protein for biological activity.

*Note: The flowchart depicts the process of expressing a protein using the ThioFusion™ Expression System, which involves cloning, transfection, expression, and purification of the protein.*
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

- Timing of Scale-up:
  - Key consideration is progress of clinical studies vs. ease of scale-up or transfer to new site. For instance, significant manufacturing changes post-Phase III may require significant comparability studies prior to marketing. Scale-up/ transfers prior to Phase III offers the best balance of CMC vs. clinical milestones.

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 ferments 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 organism 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 & Optimization:  
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
- Principal 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 (modeling 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).
CHO is the most commonly used tissue culture (TC) 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 Michaelis-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.

- Heat Generation: smaller numbers reveal more exothermic reactions. O2 relationship also shows the constancy of the combined heat and O2 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:

batch, continuous flow, or fed batch types of cultivations.

Phases of batch cultivation include: lag, exponential, stationary, and death. Interest in each phase depends upon when the product of interest is formed.

Specific growth rate ($\mu$) is a way of normalizing or providing a constant for the doubling time of a single organism, whereas doubling time is more of a general description. Given a larger $\mu_{\text{MAX}}$, the cells will grow faster with more substrate available.

Complication in determining theoretical $\mu$ is when maintenance rates impact

Cultures may produce certain inhibitory proteins/substances that impact cell growth (e.g., enzyme activity changed or lost, cell permeability changed). See Haldane models. Monod model allows for single substrate, but when several growth-limiting nutrients are present, select one with lowest Ks for use in model equation. Note that Ks can change as cell growth situations change. The most common "multiple Monod" scenario is when glucose or oxygen are rate-limiting.

Unstructured model is one that uses the cell as a 'bag of stuff' - don't care about DNA, protein, etc. Structured is when the cell components are important. Unsegregated model is when all the cells in my cultivation - they are all the same. Segregated model is when you care about if cells have one copy of DNA or two copies. Most Monod models are written for unstructured and unsegregated models. What type of model you write depends on how complex you need to know the amount of recombinant protein. Need to write models that are appropriate for your application!!

Product Formation Classification Schemes

- **Type I**
  - product formed as a result of primary energy metabolism (e.g., CO2 or ethanol).

- **Type II**
  - 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.
  - **NOTE:** Sometimes with Type III metabolisms, the organism depletes the primary substrate source and then switches 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.
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, respiration 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.

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**Bioreactor Design & Configuration**

- Types include tank, column, or loop. Mixing includes stirring, multi-stage (cascade), or propeller 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.

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**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.

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**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.
Process Development: Batch Type
Process Development: Bioreactor Types

Figure 8.19: Column fermentors: (a) bubble column, (b) tapered bubble column, (c) annular bubble column, (d) multiple-stirred column, (e) piping column with external pumping, and (f) packed-bed columns with internal pumping.

Figure 8.20: Loop fermentors: (a) air-lift, (b) O2 pressure cycle, (c) stirred loop, and (d) jet loop.
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 $90 = 10 = 9$ 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 may 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 back flush (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. Some 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 sheared 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.
- By reducing the pH, one can reduce the charge – neutralizing it (increasing K) and improves solubility in a particular phase (increases extractability to the upper solvent)
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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

There are bound to be lots of process changes and comparisons for optimization. Comparability studies can only work when some fundamental SAR activity is known about the compound and its relationship to binding and activation. Thus, forced degradation studies that link physico-chemical aspects with bioactivity will complement the development project splendidly when major changes come up. It’s vitally important to understand the relative sensitivity of analytical methods (e.g., is 20% aggregated picked up in the bioassay - or 5% oxidized seen with purity method). When you can correlate the physico-chemical with the bioassay … which may already be correlated with surrogate endpoints, then comparability studies become very focused and scientifically defensible.
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

- Process design requires integrated approach of protein SAR, protein expression systems, and characterization testing – all linked to bioassay and manufacturing parameters.
- Scale of manufacture will change depending on process development data and clinical/market needs. Try to arrange significant changes prior to Phase III to avoid costly delays in comparability studies.
- Process development can be mapped and optimized via PCA (principle component analysis) and links back to the protein SAR.
- Downstream purification may vary widely depending on whether you use microbial or mammalian cell lines.
- Comparability studies will be vital to supporting consistency across manufacturing and process development changes.
- 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.