Chapter 6

Worst-Case Approach To Validation of Operating Ranges

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A worst-case challenge is defined as executing a process under a set of conditions that leads toward process or product failure yet does not result in failure. The worst-case approach is attractive in that it allows examination of all of the critical process variables together, thus ensuring that additive effects and interactions are tested for. However running the process in this fashion poses a high risk of product or process failure. A more cautious approach is to first examine the effects of individual variables in separate experiments, e.g., in a factorial design, and then use that information to pose a worst-case which is tested experimentally. This approach was tested with a process for production and purification of a monoclonal antibody from cell culture. Process performance (antibody production and purification yield) was found to be reduced under worst-case conditions but was still within acceptable limits. Product quality characteristics were indistinguishable from product manufactured under standard conditions with the exception of the distribution of isoforms on isoelectric focusing gels. While the worst-case run demonstrated that the process can be successfully run with multiple variables at worst-case settings, it is doubtful that the same outcome would have been attained in the absence of prior knowledge of suitable ranges of critical process variables.

Operating range validation is an important component of process validation that identifies critical variables in the manufacturing process and characterizes their effect. Typically, the goal is to validate upper and lower limits on critical operating variables such that operation of the process within these limits has no adverse effect on product purity, potency, safety, or stability. The diagram in Figure 1 illustrates the concept of a process variable, for example pH in a fermentor, and its operating

In Validation of Biopharmaceutical Manufacturing Processes; Kelley, Brian D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1998.

ranges. Normally, a target setpoint is specified for the variable, denoted as "T". In practice, however, exact control of the variable at the setpoint is never achieved; rather, the variable is usually maintained within the range known as the "normal operating range". The limits of the normal operating range, known as "alert limits" (*1*), can be established by trending process performance over the production of clinical trial or qualification lots of a product. To cover occasional excursions outside of the normal operating range, it is desirable to establish a wider range known as the "maximum operating range", within which product quality attributes are shown to be acceptable. The limits of the maximum operating range, known as the "action limits" (*1*), are established through process validation studies or operating experience. Somewhere beyond the maximum operating range is the edge of failure at which process performance or product quality becomes unacceptable, however, it is not the objective of process validation to determine the edge of failure.

While the concept of operating range validation has been applied to traditional pharmaceutical manufacturing operations such as blending and tabletting, it has only recently been explored in the context of bulk biopharmaceutical manufacturing. As shown in Figure 2, the process for manufacture of a bulk biopharmaceutical can be extremely complex, consisting of numerous unit operations, each having multiple critical input variables. Thus, the task of identifying and characterizing the effects of the critical variables can be challenging and time-consuming. The situation is further complicated when interactions between variables are considered, both within a step and between steps. Additional constraints placed on the organization performing the validation studies are that limited time and resources are available to complete the validation effort. Finally, for the validation exercise to be meaningful, it is desirable to have reasonable degree of statistical confidence in the outcome, implying that some form of replication should be built into the design of the studies.

Worst-Case Approach. One approach to process validation is the so-called "worst-case" approach. The worst-case is defined in the FDA's *Guideline on General Principles of Process Validation* (*2*) as "a set of upper and lower processing limits and circumstances... which pose the greatest chance of process or product failure when compared to ideal conditions. Such conditions do not necessarily induce product or process failure." The worst-case approach is attractive for validating operating ranges in a complex process containing many critical variables because potentially only a single run is required to examine all critical variables at their worst-case setting. In addition, both intra- and inter-step interactions are accounted for in such a situation because the variables are changed simultaneously. However, the worst-case approach has a number of significant limitations such as:

- Only an upper or a lower limit can be established for a given variable in a worst-case run;
- Some knowledge (either theoretical or empirical) of each variable's effect is needed to properly design a worst-case;
- Worst-case runs represent a highly unlikely processing situation and have a high probability of failure.

Factorial Approach. An alternative approach to validating operating ranges is the "factorial approach" (*3*) in which one or more variables in a given process step are

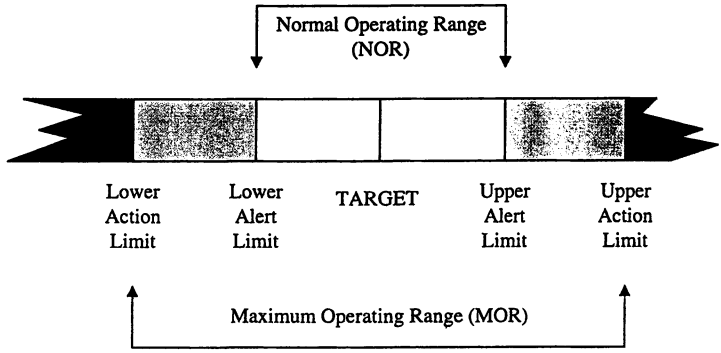

Figure 1. Operating Range for a Process Variable.

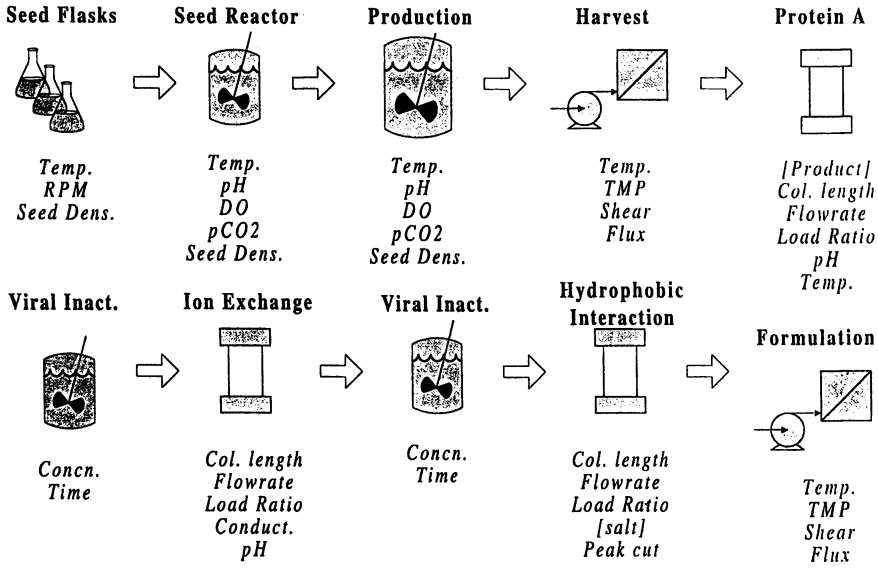

Figure 2. Typical Process Flow for Production of a Monoclonal Antibody.

manipulated in a full or partial factorial design to determine the effects on the step outcome. An advantage of the factorial approach is that, if properly designed, the effects of the individual variables and possibly some interactions between variables within a step can be ascertained.

It is possible to determine step-to-step interactions in factorial studies by including "forward linkage" variables in the design. Forward linkage variables are outputs of an upstream step that may impact the performance of the next downstream step in the process, e.g., purity of the product in the conditioned medium may impact performance or purity of the first purification step. The forward linkage variable can be handled in the same manner as any other factor in a factorial design by artificially creating feedstreams at high and low values of the linkage variable or by actually running the upstream step under different conditions to create the different feedstreams. The low and high limits of the linkage variables should represent, at a minimum, the likely ranges of the output to be encountered when the upstream step is run under its own validated conditions. If proper forward linkage variables are included for each major step in the process, then inter-step interactions will be carried throughout the entire process.

To determine whether the factorial approach with forward linkage can sufficiently predict intra- and inter-step interactions of variables, a worst-case challenge experiment was performed on a monoclonal antibody production process for which ranges of variables had been previously established via factorial experiments. In the event that comparable outcomes are achieved, it could be concluded that the factorial approach adequately considers these interactions, obviating the need for a worst-case experiment and its associated risks of failure in future validation exercises.

Materials and Methods

A recombinant Chinese Hamster Ovary (CHO) cell line was used to produce a humanized monoclonal antibody. The cell culture process consists of seed scale-up stages conducted in shake flasks and stirred-tank bioreactors, followed by a production stage conducted in a stirred-tank bioreactor in batch mode. The purification process consists of three chromatography steps, two viral inactivation steps, and a diafiltration/ concentration step, as illustrated in Figure 2. Factorial studies were performed in scaled-down models to determine ranges for critical process variables in the major steps of the process (cell culture seed and production stages, chromatography steps, and viral inactivations) (*4*,*5*). Acceptance criteria for the factorial studies were based on process performance (cell growth, antibody titer, column yield), and product quality (SDS-PAGE, isoelectric focusing) appropriate to the step being examined.

The worst-case study was performed at an intermediate scale between the benchtop scale that was used for the factorial experiments and full scale. Since the benchtop scale model had been qualified to adequately represent the full scale process for the purpose of establishing process ranges (*6*), it was assumed that the intermediate scale was also a qualified model. Table I lists the variables from the production bioreactor onward that were varied from setpoint. The direction (above or below setpoint) was chosen so as to lead to poorer process performance or product quality (the latter always taking precedence). The magnitude of the variation (not shown) was determined from the maximum acceptable range determined from the factorial studies.

Table I: Variables Examined in Worst-Case Run

Variable	Direction	Variable	Direction
Seed condition	–	Ion Exchange Flowrates	+
Seed density	–	Ion Exchange Equil. pH	+
Temperature	–	Ion Exchange Load pH	+
pH	+	Ion Exchange Load conductivity	+
Dissolved Oxygen	+	Ion Exchange Elution pH	+
Harvest Transmembrane Pressure	+	Ion Exchange Elution conductivity	+
Harvesting Temperature	+	Viral Inact. II Hold Time	+
Protein A Load Ratio	+	Hydrophobic Interaction Column Length	–
Protein A Flowrates	+	Hydrophobic Interaction Load Ratio	+
Protein A Wash Volumes.	–	Hydrophobic Interaction Flowrates	+
Elution pH	–	Hydrophobic Interaction gradient volume	–
Viral Inact. I Hold Time	+	Hydrophobic Interaction peak collection endpoint	+
Ion Exchange Col. Length	–	Hydrophobic Interaction [AmmSO4]	+
Ion Exchange Load Ratio	+		

+: factor run above setpoint; –: factor run below setpoint

Results

Figure 3 shows the performance of the cell culture production bioreactor as indicated by product titer, average specific productivity, the integral of viable cell density over time, and culture age (batch time) when the cellular viability reached 60 percent. The results of four different conditions are shown: results from full-scale runs under standard conditions; small-scale runs under standard conditions (the scale being that used to conduct the factorial range experiments); small-scale runs performed under conditions close to the worst-case run; and the worst-case run. It is seen that product titer and cell growth under worst case conditions were reduced from standard conditions, while specific productivity was elevated. When compared to the small-scale runs performed near worst-case conditions, titer and culture age at 60% viability were found to agree with the prediction, but specific productivity was unexpectedly

high and cell growth was somewhat low. The reason for this discrepancy is not known. Although the product titer was significantly reduced in the worst-case run, it was still within acceptable limits that allow successful recovery in the purification process based on the minimum validated loads of the full-scale columns.

Results of the purification process were compared in a similar manner for purification yield and protein purity. As seen in Figure 4, the yield over protein A was slightly lower than predicted from the scale-down run, while yields of the ion exchange and hydrophobic interaction steps were similar to the scale-down run and in fact were not significantly different from standard conditions at full scale. Figure 5 shows that purity of the process intermediates by quantitative Coomassie-stained SDS-PAGE was indistinguishable from standard conditions at full-scale in all cases. In Figure 6, the levels of protein A in process intermediates from the worst-case run were comparable to standard conditions within normal variation. These results indicate a very robust purification process.

Purified Bulk Biological Substance was characterized by a number of analytical methods normally used to release a batch. The Bulk was characterized shortly after preparation, and after one month storage at -70, 5, and 25 °C. Analysis by SDS-PAGE, Size Exclusion Chromatography, antigen binding, and cell-based potency assays all showed the Bulk to be indistinguishable from Reference Standard under all storage conditions. However, an abnormal pattern was seen on isoelectric focusing (IEF) gels in which the two most basic species were significantly higher in the worst-case samples (data not shown). To further investigate the source of this phenomenon, each process intermediate from the worst-case run was analyzed on IEF along with samples of Bulk Biological Substance from five standard runs at full scale and partially purified samples from the small-scale runs close to worst-case. The image of the IEF gel in Figure 7 shows that the shift towards the more basic species appeared in all process intermediates from the worst-case run and did not appear in any of the full-scale samples. Also, the trend was seen in small-scale samples as well. When scanned and quantitated, the shifts in isoform distribution were found to be significant in all worst-case process intermediates as shown in Figure 8. This result indicates that the phenomenon originated in the cell culture process, and that it is unlikely to be due to normal variation since it did not appear in any of the standard samples but it did appear in the small-scale samples run close to worst case. The shift towards basic pI at the small scale was also found to be statistically significant when compared to setpoint runs at that scale (*5*).

The identities of the more basic isoforms of the monoclonal antibody have not been determined. Modifications at the polypeptide level such as deamidation, cyclization of N-terminal glutamine to pyroglutamic acid, C-terminal processing, or translational read-through are known to affect charge properties that can be detected by high-performance ion exchange chromatography (HPIEC) or IEF (*7*, *8*). Changes in sialic acid content can also affect charge properties of glycoproteins. Since the amount of sialic acid on the antibody used in this study is extremely low under standard conditions (data not shown), changes in sialic acid content are unlikely to contribute to the appearance of isoforms on IEF. Therefore, the process conditions examined in the worst-case run most likely led to an increase or decrease in modifications of the polypeptide as compared to standard conditions. Before the worst-case conditions

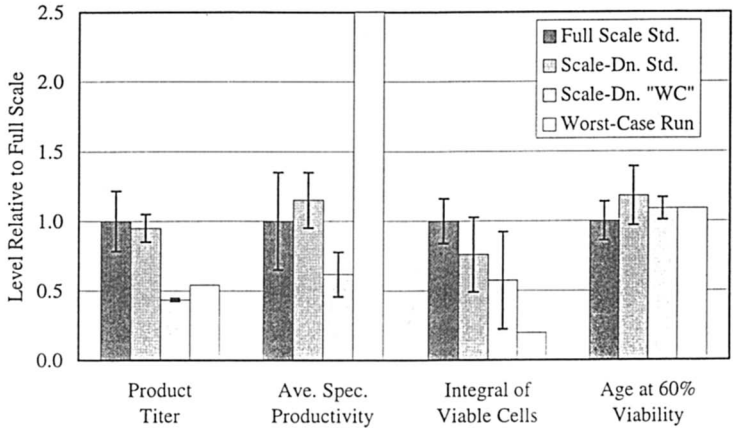

Figure 3. Performance of the Cell Culture Process. Error bars represent two standard deviations in cases where replicate experiments were run.

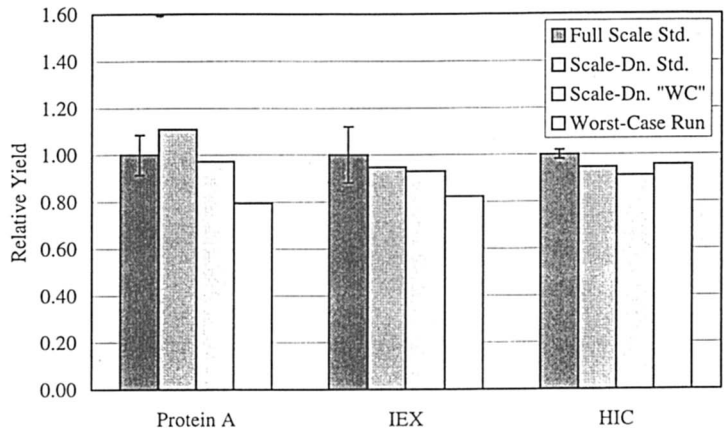

Figure 4. Yield of the Purification Process. Error bars represent two standard deviations in cases where replicate experiments were run.

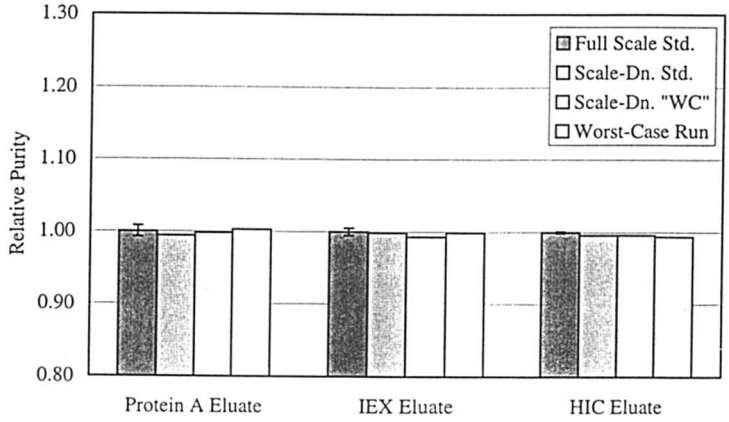

Figure 5. Purity of Purification Process Intermediates. Error bars represent two standard deviations in cases where replicate experiments were run.

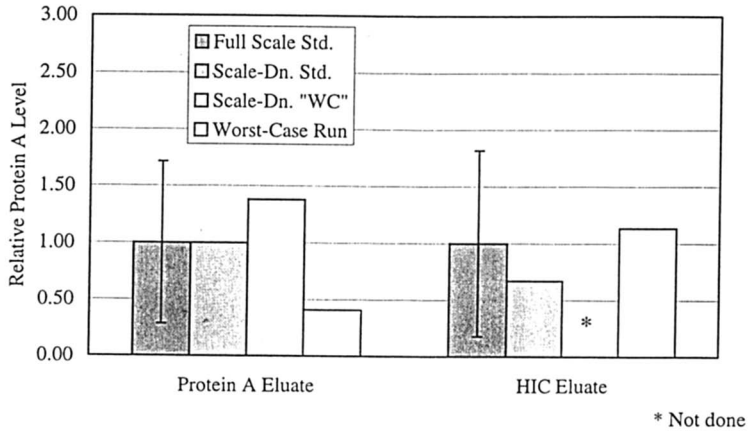

Figure 6. Protein A Levels in Purification Process Intermediates. Error bars represent two standard deviations in cases where replicate experiments were run.

Publication Date: July 23, 1998 | doi: 10.1021/bk-1998-0698.ch006

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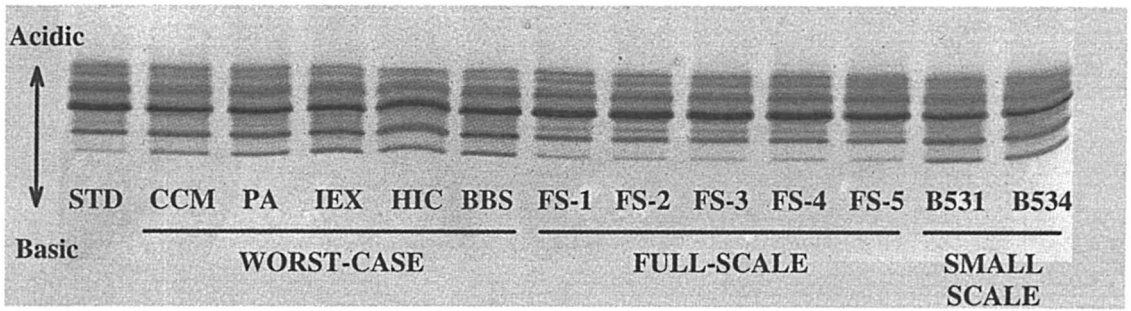

Figure 7. Isoelectric Focusing Gel from Worst-Case and Standard Conditions. STD: Reference standard; CCM: clarified conditioned medium*; PA: Protein A chromatography eluate; IEX: Ion exchange chromatography eluate; HIC: Hydrophobic interaction chromatography eluate; BBS: Bulk biological substance; FS-1 (etc): Bulk biological substance from full-scale runs at standard conditions; B531, B534: CCM from small-scale runs under worst-case conditions. *CCM samples were purified over a small-scale protein A column prior to IEF.

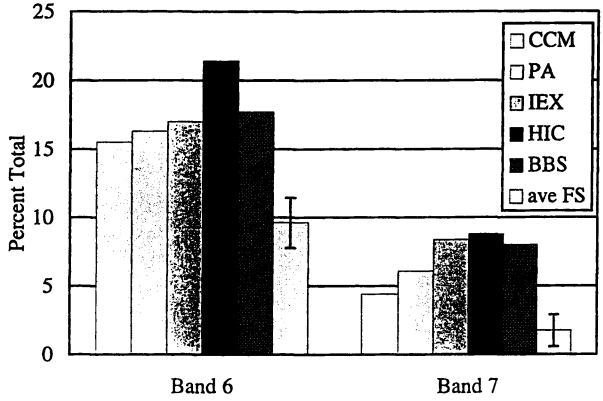

Figure 8. Quantitation of Isoelectric Focusing Gel. Error bars represent 2 standard deviations.

could be considered to be validated, these isoforms should be identified and any safety concerns should be addressed.

Conclusions

The monoclonal antibody production process, when run with its critical variables set towards worst-case conditions, yielded acceptable performance, although product titer in cell culture and the product yield over the protein A purification step were reduced from standard conditions. Product quality was indistinguishable from Reference Standard except for the distribution of isoforms on isoelectric focusing gels. Short-term product stability was also similar to Reference Standard. It is important to note that the IEF gel did not show the appearance of new isoforms, but rather a shift in the distribution of isoforms towards the more basic charge. To consider this result acceptable, the identity of the basic isoforms should be determined and any safety concerns should be addressed.

The outcome of the worst-case run was close to that predicted from the factorial experiments that were previously conducted, suggesting that a single worst-case run could be used to accomplish what was achieved in dozens of factorial experiments. However, the worst-case run was most likely successful because the critical variables had been previously identified and appropriate ranges had been established in the factorial experiments. Had the worst-case run been conducted without any prior knowledge of appropriate ranges, the probability of failure would have been much higher. Other drawbacks of the worst-case run were that only an upper or lower limit was established for each variable examined; that effects of individual variables could not be differentiated; and statistical confidence limits were not established. Furthermore, had the worst case run failed, then there would be little guidance from the data to suggest which variable(s) were responsible for the failure and by what

magnitude(s) their control range(s) would need to be adjusted to produce an acceptable result. A properly designed factorial approach can overcome all of these limitations, and inter-step interactions can be incorporated by including forward linkage variables.

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