

Timothy N. Breece, Ellen Gilkerson, and Charles Schmelzer

FDA requires that the performance of chromatography procedures be validated, and if possible and appropriate, FDA prefers prospective process validation. Fine-tuning the validation process means maximum flexibility and reduced costs while meeting GMP requirements. Part 2 of this case study shares the results from the tests used to develop a validation process that meets the goals of both FDA and the company accounting department.

Corresponding author **Timothy N. Breece** is a senior research associate in recovery sciences, **Ellen Gilkerson** is a senior biostatistician in the biostatistics department, and **Charles Schmelzer** is a senior scientist in recovery sciences at Genentech, Inc., One DNA Way, South San Francisco, CA 94080, 650.225.3292, fax 650.225.5275, tnb@gene.com, www.gene.com.

Validation of Large-Scale Chromatographic Processes, Part 2

Results from the Case Study of Neuleze Capture on Macrorep High-S

New drugs that use chromatography for purification must have the performance of those processes validated. This requires demonstrating that the chromatography media can be reused, cleaned, and sanitized; that the process, as defined, is robust; and that clearance can be shown for both impurities and adventitious and endogenous viruses. A well-defined validation process for production-scale manufacturing enables a company to set acceptable parameters, reduce costs, and meet GMP requirements.

Part 1 of this case study followed the development of a validation process that combined reuse and characterization studies for a cation-exchange column, Macrorep High-S, used as the production-scale capture step in the purification of Neuleze, a nerve growth factor (1). In this conclusion, we discuss the results from the tests used to develop performance criteria and discuss the approach this study took to GMP compliance.

Results

Reuse validation. Table 1 presents the mean and standard deviation of the Neuleze % yield and the log clearance of impurities — Chinese hamster ovary protein (CHOP), DNA, and a proprietary media component (PMC) for six large-scale production runs (LSP) and the 13 small-scale control runs from the reuse study (reuse control runs, RCR). The results clearly show no significant difference between LSPs and RCRs in impurity log clearance. Differences in mean % yields were within three standard deviations of each other and were deemed insignificant. To further guarantee that the resin was unaffected by 42 uses, the % yield and the CHOP log clearances for RCRs were plotted against cycle numbers (Figures 1 and 2). Linear regression performed on the % yield (slope=0.002, $P=0.9863$) and CHOP (slope=0.002,

$P=0.2369$) indicated no evidence of change with 42 uses (the P values indicate that variation is probably random). Linear regression was not performed for the DNA and PMC plots because they had fewer data points. Based on the results, 42 uses had no effect on % yield or impurity clearance, and the interspersed characterization runs had no effect on column performance.

Column cleaning. Table 2 contains the results from the large-scale and small-scale carry-over studies. For both the large and small-scale runs, no carry over was measurable, and the sum of the limits of detection was below 1,000 ng/mg, which is acceptable for total carry over. These results validate the cleanability of the large-scale apparatus and of the resin after 42 uses.

Column characterization. Before analyzing the characterization runs, it was necessary to determine the effect of fermentation lot on the results.

Effect of fermentation. Of the 13 center-point runs, six were from fermentation lot one, and seven from lot two. Yields were 81.1% (SDEV=6.1) and 78.1% (SDEV=4.1) respectively, showing no significant difference. Likewise, CHOP log clearance means (1.98 and 1.99 respectively) showed no significant difference. The levels of DNA and PMC were tested for only four center-point runs (two from each fermentation lot), and all were within the large-scale ranges. For all measured factors, each fermentation lot produced equivalent results.

To minimize the required number of assays, only the eight extreme conditions and the two specially added runs were originally assayed in addition to selected center-point runs. Table 3 shows those characterization results in bold.

Percent yield was calculated for every run and graphed in Figure 3. Only cycle 27 produced a yield (51.3%) that was

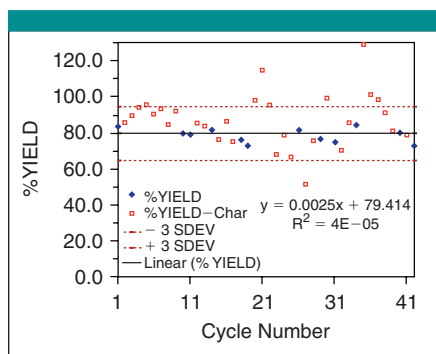


Figure 1. Effect of the number of uses on % yield

significantly lower than the mean. Cycle 27 was run with all factors set at the intermediate-high condition. Cycle 23 was run at high conditions but with low elution, so it might be expected to produce a yield worse than that of cycle 27. Instead, cycle 23 produced a yield of 67.8%. Cycles 22 and 24 had corresponding conditions but with low optical density (OD) cuts and produced yields of 78.5% and 94.9%. Yield loss appears to be caused by high OD cut. Because the higher flow rate and elution conditions would tend to make the peak elute earlier, the switching speed of the collection valve may have caused this effect.

Three cycles (20, 32, and 33) produced yields over 100% that are unexplained by the assay's coefficient of variance of 1.0%. There is no readily apparent correlation among these results, so repeating the assay was warranted to rule out potential assay error. Further analysis using the modeling function of the JMP program (Figure 3) resulted in a p value of 0.0001 (probability>F), which indicates that the model does have some explanatory value for % yield. Stepwise regression reduced the factors in the model, eliminating all factors involving elution condition.

The parameter estimates show equilibration level and its quadratic terms to be the most significant. From the Pareto chart, it is clear that the dominating factors are the same terms. The parameter prediction profile also shows a steady decline with elevating flow rate, but the only significant response was the effect of equilibration level. Prediction profiles are most useful as an interactive tool (rather than a static display) for determining the effect of variables on each other. It shows here mainly as evidence of the curvature in yield response.

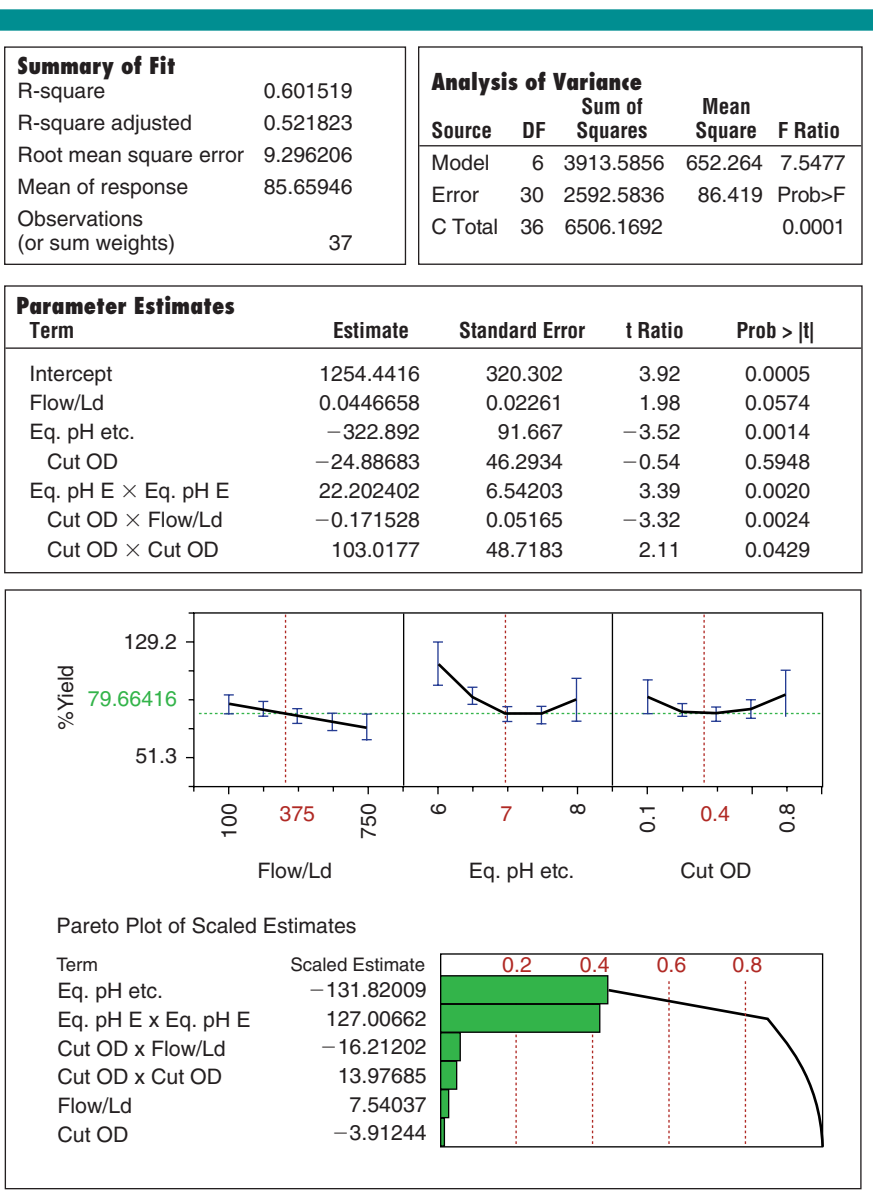


Figure 2. Screening model fit for percent yield

Table 1. Percent yield and impurity log clearance from large-scale runs (LSP) and small-scale reuse control runs (RCR)

Run Scale	Percent Yield	CHOP	DNA ^a	PMC ^a
LSP	93.3 ± 5.9	1.95 ± 0.08	2.99 ± 0.15	2.83 ± 0.16
RCR	79.5 ± 5.1	2.00 ± 0.10	3.08 ± 0.11	2.90 ± 0.16

^aOnly five cycles were analyzed for DNA and four cycles for PMC levels

Table 2. Results of column carry over studies

Scale	Resin Use (cycles)	Neuleze (ng/mg)	CHOP (ng/mg)	DNA (ng/mg)	PMC (ng/mg)
Production	10	<200	<80	<1.0	n/a ^a
Small	42	<170	<67	<0.03	<30

^aNot applicable

Table 3. Percent yield and impurity clearance

Cycle #	Pattern	Ferm. #	Flow (cm/h)	Equil. pH	Elute pH	Cut OD	% Yield	CHOP Log Clr	DNA Log Clr	PMC Log Clr
1	0000	2	375	7.0	7.0	0.4	83.6	1.98	3.18	3.10
3 ^a	++++	2	600	6.5	6.5	0.2	89.3	1.94		2.62
4	----	2	150	6.5	7.5	0.2	93.7	1.77		2.41
5	+-+-	2	600	6.5	7.5	0.2	95.5	2.09		2.41
6	----	2	150	6.5	6.5	0.2	89.8	1.78		2.44
7	----+	1	150	6.5	6.5	0.6	92.9	1.94		
8	+++++	1	600	6.5	6.5	0.6	84.0	2.08		
9	---++	1	150	6.5	7.5	0.6	91.4	1.98		2.32
10	0000	1	375	7.0	7.0	0.4	79.5	2.08	2.95	
11	0000	1	375	7.0	7.0	0.4	78.9	2.09		
12	+++++	1	600	6.5	7.5	0.6	84.9	2.25		2.69
13	-+---	2	150	7.5	6.5	0.2	83.6	1.67		
14	0000	2	375	7.0	7.0	0.4	81.4	1.94		
15	----+	2	150	7.5	7.5	0.2	76.1	1.76		
16^b	-000	2	100	7.0	7.0	0.4	86.3	1.74	3.18	3.33
17	+000	2	750	7.0	7.0	0.4	75.1	2.01	3.11	3.09
18	0000	2	375	7.0	7.0	0.4	75.8	1.84		
19	0000	2	375	7.0	7.0	0.4	72.8	2.02	3.18	2.94
20	---++	1	150	7.5	6.5	0.6	97.7	1.71		
21	+++++	1	150	7.5	7.5	0.6	114.5	1.79		
22	++--	1	600	7.5	6.5	0.2	94.9	1.77		
23	+++++	1	600	7.5	6.5	0.6	67.8	1.72		
24	+++++	1	600	7.5	7.5	0.2	78.5	1.78		
26	0000	2	375	7.0	7.0	0.4	81.6	2.07		
27	+++++	2	600	7.5	7.5	0.6	51.3	2.08		
28	00-0	2	375	7.0	6.0	0.4	75.4	1.89	2.78	2.99
29	0000	2	375	7.0	7.0	0.4	76.6	2.03		
30	00+0	2	375	7.0	8.0	0.4	99.2	1.97	3.11	3.22
31	0000	2	375	7.0	7.0	0.4	74.8	2.03	3.11	
33	0+00	1	375	8.0	7.0	0.4	85.4	1.59	3.28	3.12
34	0000	1	375	7.0	7.0	0.4	84.4	1.94		
35	0-00	1	375	6.0	7.0	0.4	129.2	1.82	3.04	1.22
36	000-	1	375	7.0	7.0	0.1	101.1	1.86		2.62
37	000+	1	375	7.0	7.0	0.8	98.6	1.99		2.91
38	0000	2	375	7.0	7.0	0.4	91.1	1.95		2.78
39^c	000	2	375	7.0	7.0	0.4	80.9	1.98	3.23	2.93
40	0000	1	375	7.0	7.0	0.4	79.9	1.90		
41^d	000	1	150	7.0	7.0	0.4	78.2	1.88	2.61	2.87
42	0000	1	375	7.0	7.0	0.4	72.8	1.92	2.99	2.76

^aThe pool from cycle 2 was spilled and unavailable for assay.
^bAxial runs are in bold.
^cCycle 39 was loaded with 124 CVs (two times the normal load) and run at the normal flow rate.
^dCycle 41 was loaded to the normal level and flowed at 150 cm/h.

Levels of CHOP were also measured for every cycle (Figure 2). Only two cycles had clearance levels below that expected at large-scale. Those were cycles 13 and 33, high-intermediate equilibration with all other factors low and the axial high equilibration run, respectively. In Figure 2, the circled runs were performed at either the intermediate or high equilibration condition. The equilibration level appears to cause a

significant effect (confirmed by the JMP modeling). First- and second-degree terms involving equilibration dominate the model. Analysis of eight runs at intermediate-high equilibration show a mean clearance of 1.79 (SDEV=0.13). Once again, the results are lower than the control values, but they are acceptably within three standard deviations. The JMP model shows that explanatory values are significant for all the parameter estimates (P<0.05).

Table 4. Log viral clearance for new and used Macrorep High-S

Resin Condition	SV40	MVM	XMuLV
New	1.26	1.9	1.58
Used	1.39	n/a ^a	n/a

^aNot applicable

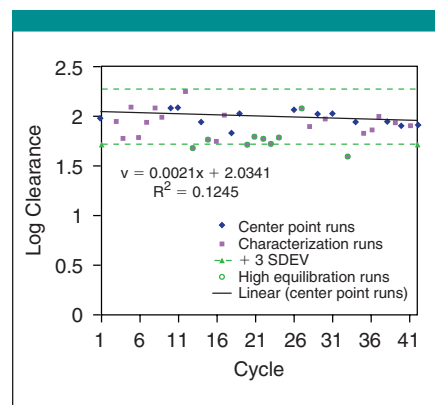


Figure 3. Chop log for every cycle analyzed using the modeling function of JMP software

Reuse Runs

A total of 42 reuse runs were performed under the following conditions:

- 13 runs used center-point conditions (operational conditions used in the manufacturing process scaled to the smaller column size),
- 16 runs used 2⁴ factorials with all factors varied at an intermediate-low and intermediate-high condition,
- Eight runs were at axial points (each axial point run had three center points and a very low or high point for the fourth),
- Two runs were repeated because the pools were not collected, and
- Two runs were added in which the flow rate and loading variables were separated (high loading at a normal flow rate and normal loading at a low flow rate).

The prediction profile in Figure 4 shows the curvature in response to equilibration level, and no significant effect from the linear responses to flow, OD, and elution. Induced hydrophobic interaction at higher salt concentrations probably accounts for this effect, which could be verified by

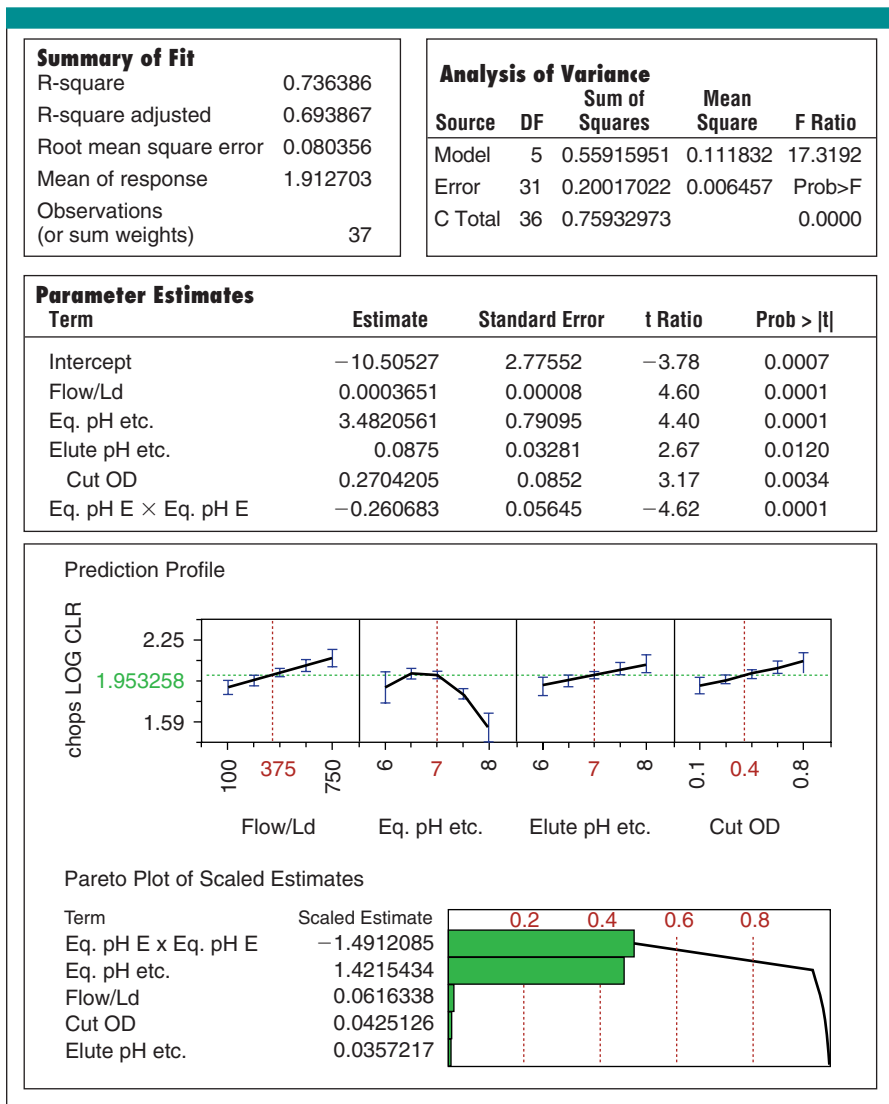


Figure 4. Screening model fit for CHOP clearance

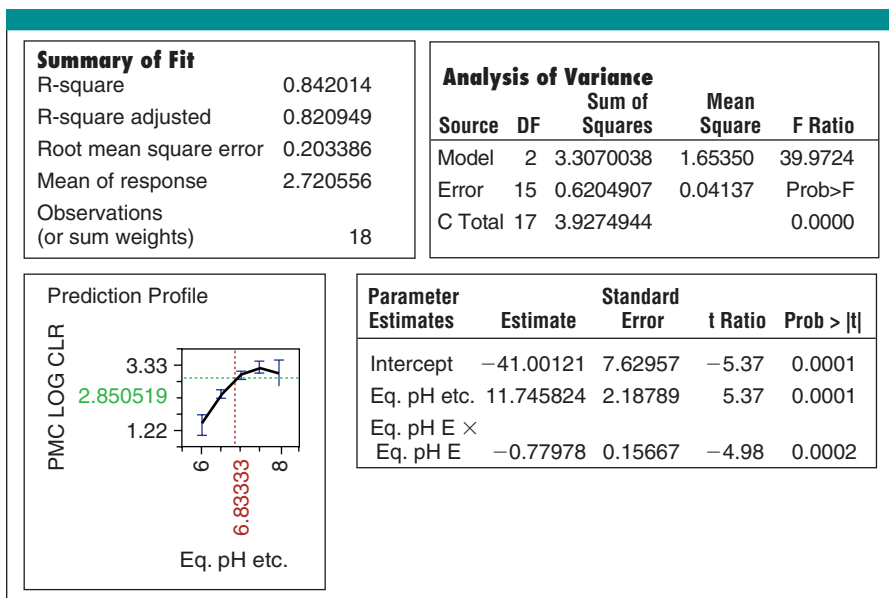


Figure 5. Screening fit for PMC clearance

deconfounding the pH and conductivity in further experiments. The Pareto chart further confirms the importance of equilibration level on CHOP clearance.

DNA clearance. As Table 3 shows, DNA clearance did not vary significantly with any of the conditions tested. Stepwise regression also found no significant levels, confirming the randomness of the data.

PMC results. After the initial measurements for PMC, it was clear that the low equilibration level had a significant effect (Table 3). So, we analyzed six of the runs at the intermediate equilibration condition (samples were exhausted for two of the runs). The mean for those runs was 2.48 (SDEV=0.14), which is just within three standard deviations of the control runs, and is therefore acceptable. When those results were modeled, stepwise regression eliminated all but the equilibration single and squared terms. Analysis of variance shows P=0.0131, and parameter estimates confirm, as expected, that the only significant factor is equilibration level. This can be explained by the molecular structure of PMC, which includes a pyridine ring that should become protonated at the low pH condition making it more likely to bind to the resin.

Viral clearance. Table 4 shows viral validation results from new and used Macroprep High-S resin. FDA advises that clearances less than one log should be considered negligible. Reuse had no apparent effect on clearance of SV40 (only SV40 was tested completely). Because other steps in the process provided greater clearance of the other viruses, the MuLV end-of-use was not tested for MVM and XMuLV to reduce costs.

Resin sanitization and storage. Test data for Macroprep High-S media exposed to a 0.5 N NaOH sanitizing solution indicates that 30-minutes of exposure result in greater than four log reductions in titers for all organisms tested. Those log reductions demonstrate the effectiveness of 0.5 N NaOH as a sanitizer for Macroprep High-S.

Recovery of the challenge organisms was reduced by greater than 3.0 logs within one day, and subsequent time points had the same results throughout the remaining test period. That adequately demonstrates the

SUCCESS in the study

highlights the value of combining characterization and reuse studies. And the unexpected multifactor effects suggest that factorial design should be used throughout the development process.

effectiveness of 0.1 N NaOH as a storage solution for Macroprep High-S media.

Meeting GMP Requirements

This validation study fulfills the basic requirements for use of the procedure in GMP manufacturing. The reuse study demonstrates that Macroprep High-S continues to perform after 42 uses. The continued performance also showed that the medium was not permanently affected by any of the conditions used for the characterization study. Carry-over results confirm that the regeneration procedure adequately cleans large-scale equipment, and that there was no build up of product or impurities at small scale after multiple uses. The sanitization and storage study demonstrates adequate bactericidal and fungicidal and bacteriostatic and fungistatic conditions during regeneration and storage. The process also demonstrates significant viral clearance, although we didn't use that in the final analysis.

Based on the characterization study, the limits of operation can be set at axial extremes for all single variable excursions except for equilibration conditions, which are limited on high conditions by CHOP clearance and on low conditions by PMC clearance. For

multiple variable excursions at intermediate levels, all impurity clearances were acceptable, but yields were unacceptable in cases with three or four factors all at high levels. Because it is suspected that low yields were caused by a combination of high flow rate, high pooling OD, and valve switching speed, this effect would need to be verified by further experimentation before strict limitations are enjoined. Also, before narrower parameters are set, the clearance achieved in other process steps might support retention of wider limits.

Sequential, stepwise regression followed by screening fit successfully differentiates random from significant effects and in this study, demonstrates that equilibration level was the most important variable. Interactive use of the prediction profile in JMP is especially useful in evaluating the effects of multiple changes in variables. The two additional runs, which deconfounded flow rate and load amount, extended the allowable parameters, doubling the allowable load to 124 CVs (cycle 39) at normal conditions and decreasing the flow to 150 cm/hr (cycle 41) with normal loading. Those extra runs demonstrate the versatility of the technique; additional runs can be performed to elucidate particular facets of your purification scheme without affecting the rest of the study's validity.

Improvements to the study. From our experience with this study, we can suggest some improvements that might be incorporated when designing reuse-characterization protocols. An initial, limited screening study can be used to determine important system variables, ranges, and appropriate variables to confound. Those variables can then be studied more extensively in the larger characterization study. Both studies can be incorporated into the reuse study, or the screening study can be done at an earlier point in development. If many cycles are required to validate a column (Protein A, for example), repetitions of each point tested can be included, and easily controlled variables (such as flow rate and load level) can be explored more completely. In this study, initially measuring only the axial extremes for the more difficult and expensive assays reduced the total number of assays required without missing any

effects. Including two worst-case runs that simultaneously vary all factors at the high and low levels of extreme conditions could enhance the reliability of this strategy.

We set abnormally wide ranges for buffers on an ion-exchange column because Neuleze appears to bind to the resin by hydrophobic interaction at equilibration, whereas the majority of impurities are eluted. In this case, confounding of equilibration pH and conductivity may have been inappropriate, but using the variable did not invalidate either the reuse or the characterization studies. Because a few additional runs could have deconfounded pH and conductivity, the protocol should be flexible enough to allow such runs if needed. In a factorial study such as this, it does not matter that variables are confounded as long as confounding relationships are known and the resulting process limits are set accordingly.

Although single variable development studies suggest the ranges we used are acceptable and the goal of the study was achieved, the near failure of some conditions supports setting ranges from the results of a factorial screening study. The success of this study (despite the wide ranges) highlights the value of combining reuse and characterization studies. Fewer characterization runs would have been performed, if not done as part of the reuse study. If we had not varied multiple factors in single runs, the high axial conditions would have appeared acceptable, and the apparent multiple factor effect of the high-intermediate conditions would never have been discovered. The unexpected multifactor effects suggest that factorial design should be used throughout the development process.

Reference

- (1) Center for Biologics Evaluation and Research, *Guideline on General Principles of Process Validation* (FDA, Rockville, MD, May 1987).
BP