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Breaking the DoE Bottleneck: How Definitive Screening Designs Enable Faster, Cheaper Biologics Process Development

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Abstract

Efficient bioprocess characterization is essential for both regulatory compliance and commercial viability of biologics. Traditional approaches using resolution III/IV screening designs followed by response surface methodology are time-consuming, costly, and not always effective in identifying the important experimental effects. Definitive screening designs (DSDs) represent a novel class of three-level screening designs that can simultaneously evaluate main effects and quadratic relationships. While DSDs are increasingly used in bioprocess development, practical implementation guidelines remain limited. This case study bridges this gap by introducing a model-based framework to identify critical process parameters (CPPs) and optimize operating ranges for robust biologics production using plasmid DNA (pDNA). Minimal 14-run DSDs evaluated six input parameters and successfully identified CPPs and optimal operating ranges. This approach reduces experimental requirement by >50% compared to traditional designs, providing an efficient and economical strategy for bioprocess characterization and optimization.

INTRODUCTION

Traditionally, a process is characterized by using a combination of resolution III/IV screening designs and response surface designs.[1] This two-step process is time-consuming and costly. Jones and Nachtscheim[1] developed definitive screening designs (DSDs) that can allow factor screening, quadratic effects estimation, and two-way interaction assessment in a single experiment. The advantages of these designs are as follows:

1. Each experimental factor is assigned three levels, which allows the estimation of quadratic effects to accommodate curvature in the relationships between the experimental factors and the responses.
2. The minimum number of runs is $2k+1$, where k is the number of factors and an additional centerpoint (CP) run is added to estimate the model intercept term.
3. For k even, the main effects are orthogonal, and for k odd, the main effects are nearly orthogonal and can be made orthogonal by the addition of only two runs.[2] An orthogonal design allows the effect of each factor to be assessed completely on its own, without any influence from the others.
4. The main effects are free of any aliasing, full or partial, with quadratic effects and two-way interaction effects. This means their estimates are not confounded with or indistinguishable from the effects of other factors.
5. No quadratic effect or two-way interaction effect is completely aliased with another quadratic effect or two-way interaction.

The DSDs are implemented in JMP statistical software. Statistical models for the responses are fitted using JMP and follow a two-stage model selection strategy to find a model containing only the active effects.[1] Two commonly used, modern measures of model performance are the Akaike Information Criterion *corrected* (AICc) and Bayesian Information Criterion (BIC).[3] For both measures, smaller values indicate a better prediction capability or performance. The two-stage modeling strategy involves *forward selection* followed by *all subsets regression* using both AICc and BIC statistics to determine a model that best predicts the performance of the process. The method assumes *heredity* and *sparsity*. Although it is unnecessary, DSDs are easily augmented in cases where some ambiguity exists in the identification of the significant effects. In these cases, the degree of augmentation still results in fewer total runs than would be necessary to complete a full response surface design. In general, augmentation should not be necessary unless an unusually large number of effects appear to be

significant indicating that the well-known *Pareto Principle* is not holding. For this design, the model is selected using the following steps:

1. Specify a full quadratic model and use the *stepwise platform*.
2. Use *forward selection* with the minimum BIC or AICc criterion to see how many effects might be important. These models are also candidates for final models.
3. Use *all possible models* with the maximum model size for a DSD set to a maximum value for which AICc can be estimated.
4. Sort the *all possible models* report in ascending order by AICc (or BIC) and turn the report into a data table.
5. Create an overlay plot of AICc and BIC by model size (number).
6. Interpret the graph to select a candidate model size or sizes.
7. Examine this set of models and select one or more of them for further investigation.

While DSDs are increasingly used in bioprocess development, practical implementation guidelines remain limited. This study bridges this gap by demonstrating a structured approach to model selection, critical process parameters (CPPs) determination, and process optimization. The approach is demonstrated through an *Escherichia coli* (*E. coli*) fermentation case study for plasmid DNA (pDNA) production, establishing a practical template for biologics process development.

Plasmid DNA is used in gene therapy and vaccine studies. As new gene therapies and DNA vaccines advance toward regulatory approval, the optimization and characterization of pDNA biomanufacturing is critical to the approval process and economic viability of new therapeutics based on pDNA. A risk-based assessment was used to identify parameters for evaluation in this study. A comprehensive list of fermentation input parameters was developed by a thorough examination of the process flow diagram and a review of historical process performance. The assessment evaluated several considerations including each parameter's potential impact on process yield and robustness, and product quality. Based on the assessment of the associated risk levels, culture temperature (T), pH, glycerol feed rate (FR), and the concentrations of calcium/magnesium (Ca/Mg), trace metals (TMs), and thiamine in the batch medium were identified to be potential critical parameters. Tejeda-Mansir and Montesinos[4] and Carnes and William[5] also reported that these parameters are the key factors that impact pDNA yield and quality.

Statistical modeling of a physical system typically only requires the inclusion of the first order (main effects), second order (quadratic), and two-way interaction terms. In the current experiment, the number of input factors was $k = 6$, and the full quadratic model contained six main effects, six quadratic terms, 15 two-way interactions, and an intercept for a total of 28 terms. Fortunately, the *Pareto Principle* dictates that only a fraction of the total terms are likely to be active. For any given experiment, the number of active effects is likely to be only a small subset, typically 20–30%, of the total number of potential effects. Without the *Pareto Principle*, screening designs, and to some degree, DoEs, in general have little chance of successfully characterizing a physical system.[6]

In this study, 14-run DSDs, including two CP runs, were performed to provide a clear guideline for characterizing and optimizing a biologics production process.

MATERIALS AND METHODS

Strain and pDNA

The *E. coli* host strain, DH5α (Thermo Fisher Scientific) was used to propagate the pDP8.ape plasmid (PlasmidFactory) in high cell density fermentation using the *E. coli* chemically defined protein medium 1-based formulation (ECPM1), as specified by Bernard and Payton.[7] The pDP8.ape contains the adenoviral helper genes AAV2 *rep* and AAV8 *cap* with a vector size of 22.0 Kbp.

Shake Flask

Fifty mL of ECPM1 medium[7] was aliquoted into 0.250 L baffled shake flasks and inoculated with 100 µL from seed vials (0.2% v/v inoculum ratio). The shake flask was agitated in a 1" throw incubator at 37 °C for at least 15 ± 1 hr, ensuring OD_{600} was 6 ± 4 absorbance units (AU). The culture was then transferred into a sterile syringe in a biosafety cabinet prior to inoculating fermenters.

Fermentation

DASGIP Bioblock fermenters (Eppendorf) were used to carry out 14-run DSDs and 4-run confirmation fermentations. Each fermenter contained 0.6 L ECPM1 batch medium supplemented with 1.0% (low), 1.2% (center), or 1.4% (high) Ca/Mg, TM, and thiamine. It was inoculated with the appropriate volume of seed culture for an initial OD_{600} of 0.02 AU. Batch-phase growth occurred for the first 7 ± 2 hrs. Aeration was initially set to 30 standard liters per hour (SLPH). The pH was maintained at 6.7 (low), 7.0 (center), or 7.3 (high) using NH_4OH and H_3PO_4. The T was kept constant at 35°C (low), 37°C (center), or 39°C (high). Dissolved oxygen (DO) was controlled at 30 ± 5% by an agitation/air flow/oxygen cascade. A feed solution containing 50% w/w glycerol with 1.0% yeast extract was initiated at a constant rate of 2.5 (low), 4.5 (center), or 6.5 (high) mL/h when a DO spike was observed. Samples were collected at the end of the fermentation (22 ± 0.5 hr post-feed initiation). The samples were stored at -80°C prior to analysis. The total fermentation time was 30 ± 1 hr.

Analytical Methods

Plasmid DNA was isolated by the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

Plasmid DNA concentration was determined by UV absorbance at 260 nm. Total pDNA concentration was determined by multiplying pDNA concentration with the final fermenter volume.

RESULTS AND DISCUSSION

Definitive Screening Designs Input and Output Data

The design matrix for the 14 runs and the resulting normalized total pDNA titer (volume [L] × titer [g/L]) for the DSDs are provided in **Table 1**. For confidentiality reasons, each pDNA titer was normalized relative to the lowest titer (run #13), yielding normalized titers ranging from 1.00 to 2.47 units. The design included two replicates of the central point (CP) (runs #4 and #11) to determine the experimental error. The normalized pDNA titer from the two CP runs (2.24 and 1.69 units) were within the expected experimental error range of ±0.5 normalized units. This variability is inherent to the measurement process, which uses a Qiagen kit for purification and titer analysis.

Model Selection for pDNA

The DSDs were analyzed using JMP version 18 statistical software (SAS Institute Inc.). The model-fitting strategy began by defining the full quadratic model, which in the present experiment contained 28 experimental effects. *Forward selection* using AICc and BIC fitting statistics was used to determine the total number of effects that were likely to be active. The maximum model size was set to ten and specified that only five models (for each model size) should be displayed in the output of the *all possible models* window (**Figure 1**). The *heredity restriction* box was checked to restrict models where interactions implied lower order effects. For this case, ten was the largest model that could be specified for AICc and BIC. Finally, an *all possible models* report was generated, which was then sorted in ascending order by AICc (**Table 2**) or BIC (**Table 3**) to determine which model had the best prediction capability in terms of process behavior.

From the *graph builder* overlay plot (**Figure 2**), one can conclude that the AICc model (with four to five terms) versus the BIC model (with five to seven terms) revealed better predictive models based on their smaller values. For comparison

TABLE 1. Input and output parameters for DSDs.

Run #	Input Parameters						Output Parameters
	pH	T (°C)	FR (mL/h)	Ca/Mg (%)	TM (%)	Thiamine (%)	Normalized Total pDNA (unit)
1	6.7	35	2.5	1.4	1.2	1.4	1.08
2	6.7	39	4.5	1.4	1.0	1.0	2.38
3	7.0	35	2.5	1.0	1.0	1.0	1.04
4*	7.0	37	4.5	1.2	1.2	1.2	2.24
5	7.0	39	6.5	1.4	1.4	1.4	2.31
6	7.3	35	4.5	1.0	1.4	1.4	2.11
7	7.3	39	6.5	1.0	1.2	1.0	2.47
8	6.7	35	6.5	1.2	1.4	1.0	1.51
9	6.7	37	6.5	1.0	1.0	1.4	1.72
10	6.7	39	2.5	1.0	1.4	1.2	1.12
11*	7.0	37	4.5	1.2	1.2	1.2	1.69
12	7.3	35	6.5	1.4	1.0	1.2	1.18
13	7.3	37	2.5	1.4	1.4	1.0	1.00
14	7.3	39	2.5	1.2	1.0	1.4	1.07

* = centerpoints

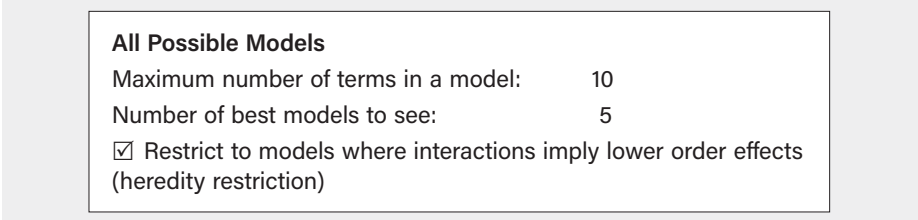


FIGURE 1. The JMP's *all possible models* window.

TABLE 2. Sorted models based on ascending AICc.

Model #	Model	Factor # in the Model	R^2	RSME	AICc	BIC
1	T, FR, T×FR, FR×FR	4	0.9162	0.1973	12.0988	3.9331
2	T, FR, T×T, T×FR, FR×FR	5	0.9357	0.1833	17.0642	2.8709
3	T, FR, FR×FR	3	0.7943	0.2933	18.1780	13.8733
4	T, FR, TM, T×FR, FR×FR	5	0.9266	0.1958	18.9065	4.7132
5	T, FR, Ca/Mg, T×FR, FR×FR	5	0.9225	0.2013	19.6854	5.4921
6	FR, FR×FR	2	0.6531	0.3632	20.4381	18.5499
7	T, FR, Thiamine, T×FR, FR×FR	5	0.9165	0.2089	20.7170	6.5237
8	pH, T, FR, T×FR, FR×FR	5	0.9162	0.2092	20.7638	6.5706
9	T, FR, T×T, FR×FR	4	0.8137	0.2942	23.2868	15.1211
10	T, FR, TM, FR×FR	4	0.8047	0.3013	23.9506	15.7849

T = temperature
FR = glycerol feed rate
Ca/Mg = calcium and magnesium concentration in batch medium
TM = trace metal concentration in batch medium

TABLE 3. Sorted models based on ascending BIC.

Model #	Model	Factor # in the Model	R^2	RSME	AICc	BIC
1	T, FR, TM, T×T, T×FR, FR×FR, T×TM	7	0.9565	0.1741	41.9266	2.6781
2	T, FR, T×T, T×FR, FR×FR	5	0.9357	0.1833	17.0642	2.8709
3	T, FR, TM, T×T, T×FR, FR×FR	6	0.9461	0.1794	26.7236	3.0361
4	T, FR, Ca/Mg, TM, T×T, T×FR, FR×FR, T×TM	8	0.9627	0.1766	70.1002	3.1574
5	T, FR, Ca/Mg, TM, T×T, T×FR, FR×FR, FR×Ca/Mg	8	0.9611	0.1803	70.6771	3.7344
6	T, FR, T×FR, FR×FR	4	0.9162	0.1973	12.0988	3.9331
7	T, FR, Ca/Mg, TM, T×T, T×FR,FR×FR	7	0.9523	0.1823	43.2069	3.9584
8	T, FR, Ca/Mg, T×T, T×FR, FR×FR	6	0.9419	0.1863	27.7735	4.0859
9	T, FR, TM,T×FR, FR×FR, T×TM	6	0.9415	0.1870	27.8786	4.1910
10	T, FR, Ca/Mg, T×T, T×FR, FR×FR, FR×Ca/Mg	7	0.9507	0.1853	43.6688	4.4203

T = temperature
FR = glycerol feed rate
Ca/Mg = calcium and magnesium concentration in batch medium
TM = trace metal concentration in batch medium

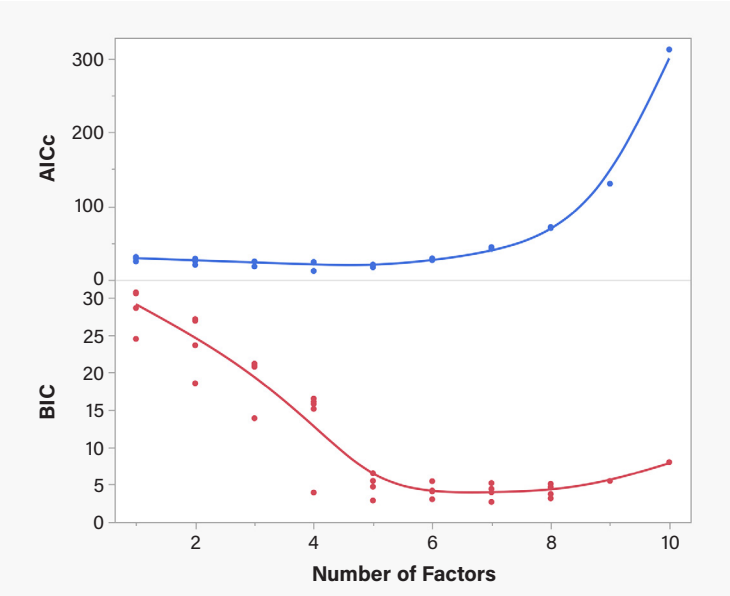


FIGURE 2. AICc and BIC vs. number of factors (*k*). AICc and BIC indicate models in the range of four to five terms, and five to seven factors are the best predictive models, respectively.

purposes, AICc models: #1 (with $k=4$ factors); #2 (with $k=5$ factors); and #3 (with $k=3$ factors) were selected based on their smaller AICc values, and BICc model #1 (with $k=7$ factors), shown in **Table 3** were identified as having the smallest BIC and root mean square error (RMSE).

Model Analysis

The *fit model report* (**Table 4**) provides details for the $k=4$, $k=5$, and $k=3$ AICc, and $k=7$ BIC effects models. The R^2 statistic indicated that all models fit the data well. Analysis of variance (anova) results demonstrated that the models were significant (p-value <0.05). The *lack of fit* test indicated that all AICc and BIC models adequately fit the data as p-value >0.05.

The parameter estimates of four models were compared in **Table 5**. All four models agreed on the important effects. FR and T were statistically more significant than the other parameters.

Confirmation Run

Four confirmation runs, including one CP condition (run #4), were performed to determine the best model among the four models selected. The input and output parameters are shown in **Table 6**. The mean and standard deviation of the residuals from the four model predictions are summarized in **Table 7**. Overall, the four-effects AICc model (model #1 with $k=4$) exhibited the smallest estimated mean bias and was selected as the best predictive model. Note that with the addition of run #4, a total of three CP runs were available to examine any experimental error.

Prediction Profiler and Assessing Process Capability

The four-effects AICc model indicates that T, FR and its squared term, and FR and T interaction are statistically

TABLE 4. Statistical analysis.

	AICc			BIC
Model #	1	2	3	1
Factor # in the model	$k=4$	$k=5$	$k=3$	$k=7$
Summary of Fit				
R^2	0.9162	0.9357	0.7943	0.9565
R^2 adjusted	0.8790	0.8955	0.7326	0.9057
RMSE	0.1973	0.1833	0.2933	0.1741
Mean of response	1.6371	1.6371	1.6371	1.6371
Observations	14	14	14	14
Anova for pDNA				
Prob >F	<0.0001	0.0001	0.0009	0.0011
Lack of fit test for pDNA				
Prob >F	0.6057	0.7815	0.1337	0.9958

TABLE 5. Sorted parameter estimate.

Parameters	Prob >|t|			
	AICc, Model #1, $k=4$	AICc, Model #2, $k=5$	AICc, Model #3, $k=3$	BIC, Model #1, $k=7$
FR	0.0002	0.0002	0.0019	0.0004
FR×FR	0.0003	0.0003	0.0036	0.0018
T	0.0036	0.0030	0.0256	0.0045
T×FR	0.0056	0.0046	N/A	0.0052
T×T	N/A	0.1583	N/A	0.2001
TM	N/A	N/A	N/A	0.2759
T×TM	N/A	N/A	N/A	0.2765

T = temperature; TM = trace metal concentration in batch medium
FR = glycerol feed rate; N/A = model does not incorporate this parameter

TABLE 6. Confirmation run input and output parameters.

Run #	Input Parameters						Output Parameters
	pH	T (°C)	FR (mL/h)	Ca/Mg (%)	TM (%)	Thiamine (%)	Normalized Total pDNA (unit)
1	6.7	37	4.5	1.0	1.0	1.0	1.77
2	7.3	39	6.5	1.4	1.4	1.4	2.81
3	7.0	38	4.5	1.2	1.2	1.2	2.32
4	7.0	37	4.5	1.2	1.2	1.2	1.88

TABLE 7. Summary statistics for confirmation run residuals.

	AICc			BIC
Model #	1	2	3	1
Factor # in the model	$k=4$	$k=5$	$k=3$	$k=7$
Mean	0.0025	0.0489	0.0656	0.0875
Standard deviation	0.3645	0.3074	0.4781	0.2672
Standard error mean	0.1823	0.1537	0.2391	0.1336
Upper 95% mean	0.5825	0.5380	0.8264	0.5127
Lower 95% mean	-0.5775	-0.4401	-0.6952	-0.3377
N (# of runs)	4	4	4	4
N missing	0	0	0	0

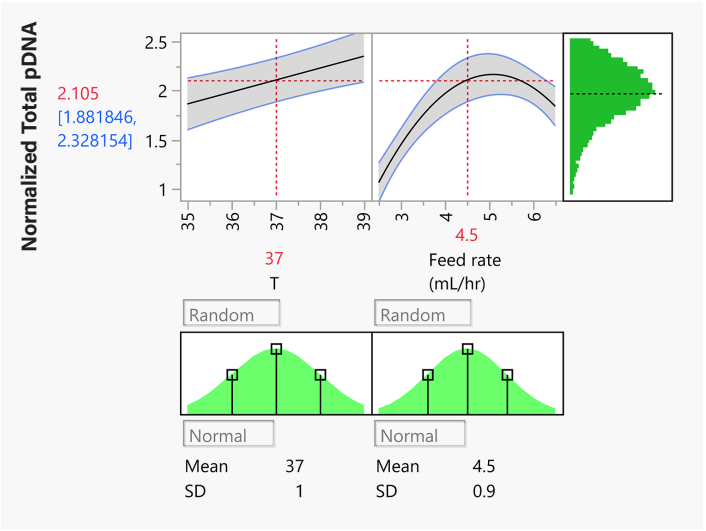
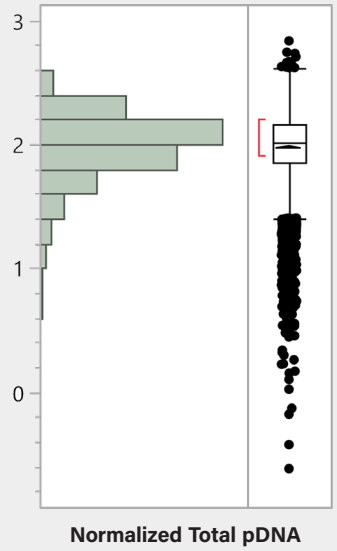


FIGURE 3. Prediction profiler and simulation at centerpoint setting for a four-effects AICc model.

significant. *Prediction profiler* (**Figure 3**) shows that T has a strong positive correlation with pDNA titer. FR appears as an important factor through its quadratic effect. The optimal settings of the profiler in **Figure 3** suggest that fermentation with the T of 39°C and FR of 5.5 mL/h provides the highest normalized total pDNA titer of 2.5 units.

Using the pDNA predictive model from the four-effects AICc model and the *simulator* in the JMP prediction profiler, the sensitivity of the pDNA response to variation in the T and FR was examined. T and FR, with the standard deviation of 1°C and 0.9 mL/h, respectively, were assessed. Using the results of the simulation, a 95%–95% tolerance interval (TI) was estimated using the *distribution platform*. The TI indicates, with 95% confidence, that at least 95% of the batches will have a normalized total pDNA titer in the range of 1.4–2.6 units at the CP settings for the selected process factors (**Figure 4**).

Histogram



Quantiles

100.0%	Maximum	2.8110033
99.5%		2.5225862
97.5%		2.3997444
90.0%		2.2668561
75.0%	Quartile	2.1511796
50.0%	Median	2.0130523
25.0%	Quartile	1.8505382
10.0%		1.6038229
2.5%		1.1886235
0.5%		0.6993580
0.0%	Minimum	-0.6818680

Summary Statistics

Mean	1.9651505
Standard deviation	0.2955764
Standard error mean	0.0029558
Upper 95% mean	1.9709444
Lower 95% mean	1.9593566
N (# of runs)	10,000
N missing	0

Tolerance Intervals (TIs)

Proportion	Lower TI	Upper TI	1-Alpha
0.950	1.378978	2.551323	0.950

FIGURE 4. TIs for a four-effects AICc model.

CONCLUSIONS

This study provides a step-by-step framework for implementing DSDs to select accurate predictive models, identify CPPs, and define robust operating ranges. Using pDNA production in *E. coli* fermentation as a case study, we demonstrated how to:

- evaluate model fit (selecting a four-effects model as optimal based on AICc),
- statistically confirm that T and FR are CPPs (p-value <0.05), and
- establish a design space defined by a 95% TI of 1.4–2.6 units at 37 ± 1°C and a FR of 4.5 ± 0.9 mL/h.

Compared to traditional two-stage DoE (screening + response surface method), which requires 30–50 experiments to characterize a single unit operation with six factors, our approach achieves the same objectives with just 14-run DSDs—reducing the experimental workload by >50%.

By providing clear guidelines for model selection, CPP determination, and design space optimization, this work demonstrates that DSDs represent an efficient and cost-effective approach for bioprocess characterization, particularly for multi-parameter optimization.

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