Monte Carlo comparison of rival experimental designs for two-agent combined action studies

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OBJECTIVE: The combined action of two or more chemotherapeutic agents and/or biological agents can be quantitatively described with empirical multidimensional concentration-effect response surface models. This intuitive statistical approach provides a framework for suggesting experimental designs for in vitro, in vivo and possibly clinical experiments of agent combinations. Five rival 32-point experimental designs for in vitro continuous response two-agent combined action studies were compared using Monte Carlo simulation.

DESIGN: The designs were: factorial; central composite; one-ray in duplicate; four-ray; and D-optimal.

SETTING: Datasets were simulated by generating ideal data with the authors' flagship two-agent combined action model, which includes six parameters: the control survival, \( \text{Econ}=100 \) (where Econ is the full range of response that can be affected by the drug); median effective concentrations, \( \text{IC}_{50,1}=10 \), \( \text{IC}_{50,2}=1 \) for drug 1 and drug 2, respectively; slope parameters, \( m_1=-1 \), \( m_2=-2 \) for drug 1 and drug 2, respectively; and the interaction parameter, \( \alpha=1 \) or \( \alpha=5 \). For each design, for each of four types of error (absolute, relative with 1% coefficient of variation [cv], relative with 10% cv, and relative with 10% cv plus a noise constant of 1% of Econ), for each of two values of the true \( \alpha \) (1, 5), 500 Monte Carlo datasets were generated, and then fit via weighted nonlinear regression with the flagship model.

MAIN RESULTS: For the \( \alpha \) parameter, for relative error-containing datasets, the D-optimal designs had the smallest variances.

CONCLUSION: The counterintuitive D-optimal designs may be useful for studies in which the experimental units are relatively precious, and frugal designs are essential. In addition, it may be fruitful to add the D-optimal design points to standard experimental designs.

Key Words: Combined action model, D-optimal design, Experimental design, Monte Carlo simulation, Synergy

Comparaison de modèles expérimentaux rivaux pour deux études sur l'action combinée de deux agents

OBJECTIF : L'action combinée de deux agents chimiothérapeutiques ou biologiques (ou de plusieurs) peut être décrite quantitativement à l'aide de modèles concentration-eflet empiriques pluridimensionnels. Cette approche statistique intuitive offre une base pour l'élaboration de modèles d'expérimentation in vitro, in vivo et possiblement, pour les essais cliniques sur les thérapeutiques d'association. Cinq études sur la réponse in vitro continue à des thérapeutiques d'association doubles, à l'aide de modèles expérimentaux en 32 points ont été comparées à l'aide d'une simulation.
and intensity of combined action of mixtures of agents may involve traditional pharmaceuticals, natural biologicals and/or modified biologicals. The quantitative assessment of the nature and intensity of combined action of mixtures of agents in vitro, in vivo, and especially in clinical studies, can be difficult. The most rigorous approaches to this problem involve the derivation of multiple agent concentration-effect combined action response surface models that include interaction parameters, and the fitting of these models to experimental data with modern statistical curve-fitting approaches. The authors have developed such a set of models and curve-fitting techniques (1-5), and have applied this paradigm to several in vitro studies of combinations of anticancer and other agents against cells in culture (6-9).

**Three-Dimensional Combined Action Models**

Our flagship combined action model is given as Equation 1. Equation 1 was derived with an adaptation of an approach suggested by Berenbaum (10), with the assumption of Equation 2, the Hill model (11), as the appropriate model for each agent alone.

In Equations 1 and 2, $E$ is the measured effect (response), $D$ is the concentration (or dosage) of drug, $E_{\text{Con}}$ is the full range of response that can be affected by the drug, $IC_{50}$ is the median effective dosage (or concentration) of agent, and $m$ is a slope parameter (additional subscripts, 1 and 2, refer to agents 1 and 2, respectively). When $m$ has a negative sign, the curve falls with increasing agent concentration; when $m$ is positive, the curve rises with increasing agent concentration. The interaction parameter is $\alpha$. When $\alpha$ is positive, Loewe synergism is indicated, when $\alpha$ is negative, Loewe antagonism is indicated, and when $\alpha$ is 0, Loewe additivity is indicated. This terminology is the central element of a recent consensus on combined action nomenclature and concepts [12]. The use of ‘Loewe’ as an adjective stresses the connection to the ‘no interaction’ reference model inherent in the classical isobologram approach to interaction assessment pioneered by Loewe [13]. The magnitude of $\alpha$ indicates the intensity of the interaction. Thus, although Equation 1 is not the model for Loewe synergism (or Loewe antagonism), it is a model for Loewe synergism (or Loewe antagonism).

The derivation of Equation 1, the flagship equation for two-drug interactions, is provided in detail in Greco et al (6). Equation 1 allows the slopes of the concentration-effect curves for the two drugs to be unequal. It is this key feature that distinguishes Equation 1 from many other response surface models used by others to describe drug interactions (eg, 14). Since Equation 1 is in unclosed form, a one-dimensional bisection root finder (eg, 15) is used to calculate $E$ for simulations. Equation 1 was not derived from biological theory, rather it is an empirical equation which often matches the shape of real data (eg, 2.6-8).

**Statistical Experimental Design**

The main decisions that must be made regarding experimental design are: first, where to choose the concentrations; second, numbers of replicates; and third, numbers of experiments. These seemingly simple questions have spawned many full careers for statisticians, who have delved deeply into them to reveal their inherent complexity. The adoption of a response surface paradigm for the assessment of combined action of agents facilitates the understanding and use of formal statistical experimental design. First, the experimenter must decide whether he/she is in an exploratory or a confirmatory mode. Screening experiments (exploratory mode) should first include, for each agent individually, agent concentrations which span the anticipated response region. Logarithmic spacing of the concentrations over a thousand-fold to a million-fold range is probably necessary, depending upon the previous knowledge of the researcher about the concentration-effect behaviour of the compound. After the individual agent concentration-effect curves are well characterized, a combination experiment should be conducted...
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\[ 1 = \frac{D_1}{IC_{50.1} \left( \frac{E}{E_{\text{con}} - E} \right)^{\gamma_{D_1}}} + \frac{D_2}{IC_{50.2} \left( \frac{E}{E_{\text{con}} - E} \right)^{\gamma_{D_2}}} + \alpha D_1 D_2 \left( IC_{50.1} \right)^{\gamma_{D_1}} \left( IC_{50.2} \right)^{\gamma_{D_2}} \]

Equation 1

\[ E = \frac{D}{IC_{50}} \]

Equation 2

**TABLE 1**

Factorial design (5X5)

| Drug 2 concentration | 0  | 2  | 5  | 10 | 20 | 50 | 100 |
|----------------------|----|----|----|----|----|----|-----|
| 0                    | 4  | 1  | 1  | 1  | 1  | 1  | 1   |
| 0.2                  | 1  | 1  | 1  | 1  | 1  | 1  | 1   |
| 0.5                  | 1  | 1  | 1  | 1  | 1  | 1  | 1   |
| 1                    | 1  | 1  | 1  | 1  | 1  | 1  | 1   |
| 2                    | 1  | 1  | 1  | 1  | 1  | 1  | 1   |
| 5                    | 1  | 1  | 1  | 1  | 1  | 1  | 1   |
| 10                   | 1  |    |    |    |    |    |     |

Table entries are the number of replicates at each design point, i.e., each combination of drug 1 concentration and drug 2 concentration

that repeats single agent data points, and that includes a set of combination points. A full factorial (checkerboard) design (Table 1), a single ray (fixed ratio) design (Table 3) or a multiple ray design (Table 4), all with logarithmically spaced concentrations, may be appropriate. If a complex three-dimensional concentration-effect surface is anticipated, then the entire interesting region of agent 1 and agent 2 concentrations should be sampled, either with a checkerboard or multiple ray design. However, if a well-behaved three-dimensional concentration-effect surface is anticipated, and the specific combination being studied is only one of many candidates being screened, then a single ray may be sufficient. Composite designs consisting of a checkerboard and some rays might also be used. Of course, if the intended data analysis approach is firmly tied to a particular design, then that design will have to be used.

After completing the analysis of the first mixture experiment in exploratory mode, the researcher may want to switch to confirmatory mode. The repeat of the combination experiment may use the same design as in the exploratory experiment, but probably the knowledge gained from the first run will help to refine the design for the second run. If a complex three-dimensional concentration-effect surface was found in the exploratory experiment, then agent concentrations in the interesting regions of the surface should be accentuated in the confirmatory experiment. Increasing the numbers of replicates probably also will be necessary. If a simple three-dimensional concentration-effect surface was found in the exploratory experiment, i.e., one with pure Loewe synergism or Loewe antagonism,
TABLE 5
D-optimal design for absolute error, \( \alpha=1 \) (OPTA)

| Drug 2 concentration | Drug 1 concentration | 0    | 3.52 | 6.32 | 28.4 |
|----------------------|----------------------|------|------|------|------|
|                      | 0                    | 7    | 5    | 5    |      |
|                      | 0.59                 | 5    |      |      |      |
|                      | 0.791                | 5    |      |      |      |
|                      | 1.69                 | 5    |      |      |      |

Table entries are the number of replicates at each design point, ie. each combination of drug 1 concentration and drug 2 concentration.

TABLE 6
D-optimal design for absolute error, \( \alpha=5 \) (OPTB)

| Drug 2 concentration | Drug 1 concentration | 0    | 3.13 | 3.52 | 28.4 |
|----------------------|----------------------|------|------|------|------|
|                      | 0                    | 7    | 5    | 5    |      |
|                      | 0.445                | 5    |      |      |      |
|                      | 0.59                 | 5    |      |      |      |
|                      | 1.69                 | 5    |      |      |      |

Table entries are the number of replicates at each design point, ie. each combination of drug 1 concentration and drug 2 concentration.

TABLE 7
D-optimal design for relative error, \( \alpha=1 \) (OPTC)

| Drug 2 concentration | Drug 1 concentration | 0    | 118  | 95   | 1000 |
|----------------------|----------------------|------|------|------|------|
|                      | 0                    | 7    | 5    | 5    |      |
|                      | 3.08                 | 5    |      |      |      |
|                      | 11.8                 | 5    |      |      |      |
|                      | 1000                 | 5    |      |      |      |

Table entries are the number of replicates at each design point, ie. each combination of drug 1 concentration and drug 2 concentration.

TABLE 8
D-optimal design for relative error, \( \alpha=5 \) (OPTD)

| Drug 2 concentration | Drug 1 concentration | 0    | 28.1 | 95   | 1000 |
|----------------------|----------------------|------|------|------|------|
|                      | 0                    | 7    | 5    | 5    |      |
|                      | 2.85                 | 5    |      |      |      |
|                      | 3.08                 | 5    |      |      |      |
|                      | 1000                 | 5    |      |      |      |

Table entries are the number of replicates at each design point, ie. each combination of drug 1 concentration and drug 2 concentration.

then a design that facilitates the estimation of parameters with the smallest variance may be appropriate. A D-optimal design (eg. 4) may be indicated with many replicates.

Interestingly, the number of design points in a D-optimal design (Tables 5-10) is equal to the number of estimable parameters. For example, if one assumes that Equation 1, which contains six parameters, will then a design that facilitates the estimation of parameters with the smallest variance may be appropriate. A D-optimal design (eg. 4) may be indicated with many replicates.

D-optimal designs may, at first, seem to be very strange and potentially noninformative. This type of frugal experimental design may have great potential for animal and human experiments, in which the experimental units are very dear. However, because D-optimal designs are very counterintuitive, a Monte Carlo computer simulation study was designed to compare several rival experimental designs, including D-optimal designs, for combined action studies.

METHODS

Monte Carlo simulations: For the Monte Carlo simulation, Equation 1 was assumed to represent the combined action of two agents, with realistic parameter values: \( Econ=100; IC_{50.1}=10; m_1=-1; IC_{50.2}=1; m_2=2; \) and \( \alpha=1 \) (slight Loewe synergism) or \( \alpha=5 \) (large Loewe synergism). Ideal continuous data were generated by inserting values of \( D_1 \) and \( D_2 \) for a specific design into Equation 1 and calculating \( E \) for each data point with a bisection root finder (15). The errors that were added to the ideal data to yield error-containing data were of four types: small absolute (SA); small relative with a 1% coefficient of variation (CV); large relative with a 10% CV; and large relative with a 10% CV plus a noise constant equal to 1% of \( Econ \) (LC). Relative (or proportional) error is commonly found in biological systems, and a noise threshold often fixes a lower limit for acceptable measurements. Of the four error types, the LC error type is the most realistic. To generate the errors, first standard normal random numbers \( \epsilon \) were generated via the Box-Müller Polar method (16) with the uniform \( 0,1 \) random deviates coming from the IMSL subroutine, GGBS (17). Small absolute errors were equal to \( \epsilon \), small relative errors were equal to \( r/1000 \), large relative errors were equal to \( r/10 \), large relative errors plus a noise constant were equal to \( r/10+\beta \), where \( \beta \) was the true error-free ideal dependent variable and \( \epsilon \) was the noise constant, set to be 1% of \( Econ \) (since \( Econ=100, \beta=1 \)).

Five hundred Monte Carlo datasets were generated for each type of error for each design. Equation 1 was fit to each dataset with iteratively reweighted nonlinear regression via the Nash variant (18) of the Marquardt algorithm (19). Initial parameter estimates for \( Econ \) were generated by taking an average of effects at the \( 0,0 \) design point. Initial parameter estimates for \( IC_{50,1}, m_1 \), and for \( IC_{50,2}, m_2 \), were generated by fitting single agent data for drug 1 and drug 2, respectively, with a linearized transformation of Equation 2 via weighted linear regression. Initial parameter estimates for \( \alpha \) were generated by rearranging Equation 1 to isolate \( \alpha \) on the left side of the equals sign, then for each combination point, plugging in raw data and the other five initial parameter values to solve for \( \alpha \), and finally, calculating an average \( \alpha \) for the set of combination points. The
**TABLE 9**  
D-optimal design for relative error with intercept, $\alpha=1$  
(OUTE)  

| Drug 2 concentration | Drug 1 concentration 0 | Drug 1 concentration 11.4 | Drug 1 concentration 12.6 | Drug 1 concentration 107 |
|----------------------|------------------------|---------------------------|--------------------------|-------------------------|
| 0                    | 7                      | 5                         | 5                        | 5                       |
| 1.07                 | 5                      | 328                       | 5                        | 3.28                    |
| 1.40                 | 5                      | 328                       | 5                        | 3.28                    |
| 3.28                 | 5                      | 328                       | 5                        | 3.28                    |

Table entries are the number of replicates at each design point, i.e., each combination of drug 1 concentration and drug 2 concentration.

**TABLE 10**  
D-optimal design for relative error with intercept, $\alpha=5$  
(OPTF)  

| Drug 2 concentration | Drug 1 concentration 0 | Drug 1 concentration 11.4 | Drug 1 concentration 12.6 | Drug 1 concentration 107 |
|----------------------|------------------------|---------------------------|--------------------------|-------------------------|
| 0                    | 7                      | 5                         | 5                        | 5                       |
| 0.743               | 7                      | 5                         | 5                        | 5                       |
| 1.07                | 5                      | 328                       | 5                        | 3.28                    |
| 3.28                | 5                      | 328                       | 5                        | 3.28                    |

Table entries are the number of replicates at each design point, i.e., each combination of drug 1 concentration and drug 2 concentration.

**Figure 1** Box plots for the sets of 500 $\alpha$ estimates for Monte Carlo data sets containing small absolute error (SA), for each of five rival designs, for each of two values for the true interaction parameter ($\alpha = 1$, **left**; $\alpha = 5$, **right**). The box plot for each design includes a central bar for the median, upper and lower edges of a box for the 25th and 75th percentiles of the data, upper and lower lines extending to short capped bars for the 10th and 90th percentiles, and upper and lower circles which indicate the 5th and 95th percentiles.

weights used for each data point were appropriate for each type of error, and were equal to 1 for SA, $1/E^2$ for SR and LR, and $1/[100E^2+\hat{E}^2]$ for LC, where $\hat{E}$ is the predicted value of $E$ for each data point at each iteration. The 100 in the denominator for the weighting factor for the LC error type is necessary to properly scale the noise constant relative to $\hat{E}$. The final parameter estimates, six for each fit of 500 datasets, were placed in files for further analyses. The generation of Monte Carlo datasets and their fitting with Equation 1 via nonlinear regression was performed with computer software written by this group in the C programming language, and run on MSDOS-compatible 80486- and 80386-based microcomputers.

**Rival experimental designs:** The 10 experimental designs that were compared are shown in Tables 1 to 10. All rival designs contain 32 data points. Table 1 shows a factorial or checkerboard design, with logarithmically spaced concentrations. Table 2 shows a central composite design. Table 3 shows a single fixed ratio or single ray design, in which the single fixed ratio is 10:1 for $D_1:D_2$ (the ratio of their IC₅₀ values). Table 4 shows a
RESULTS

Figures 1 to 4 show box plots of the distributions of the sets of estimated \( \alpha \) parameters. Each figure is for a different error type. Figure 1 is for SA, Figure 2 is for SR, Figure 3 is for LR and Figure 4 is for LC. These figures were made with the Sigma Plot graphics package (22). Table 11 lists the variances for the set of 500 \( \alpha \) estimates for each design for each type of error (SA, SR, LR or LC) with each true \( \alpha \) (1 or 5). For each type of error and each value of the true \( \alpha \), the designs are listed in the order of increasing variance for the sets of 500 \( \alpha \) estimates. Each set of 500 estimates was also divided into 20 consecutive subsets of 25 values, and the variance for each subset was calculated. Then these sets of 20 variance values were used to make pairwise comparisons of variance among all rival models in a group using the Wilcoxon rank sum test via the SAS statistical package (23).

Since each group of designs involved 10 comparisons, the type I error rate of 0.01 was chosen for making decisions of statistical significance for pairwise design differences, for an overall Bonferroni conservative type I error rate of 0.1 per group. For each group, the upper designs with the smallest variance form a subgroup, for

fixed ratio design with four different ratios (20:1, 10:1, 5:1 and 2:1). Tables 5 to 10 show D-optimal designs and are directly dependent upon the predicted values of the six parameters of Equation 1 and on the four different error structures. For each D-optimal design, the first five optimal concentration pairs (\( D_1 \), \( D_5 \)) (those not associated with the estimation of the interaction parameter, \( \alpha \)) were generated with formulas and concepts published by Bezeau and Endrenyi (20) for the precise estimation of the three single agent parameters for Equation 2. A discussion of the generation of D-optimal designs for the sixth optimal concentration pair (the one associated with the estimation of \( \alpha \)) for combined action studies with numerical function minimization methods has been published by the present group (4). However, the D-optimal designs in Tables 5 to 10 were generated with faster algorithms, which relied heavily on partial analytical solutions of the determinants of the variance-covariance matrix of parameters, for which the details will be published elsewhere. The D-optimal formulas were coded in the Mathematica mathematical programming language (21). All concentrations calculated to be greater than 1000 were capped at 1000 to keep the D-optimal designs realistic.
which the top design was not found to be significantly different from the other designs in the subgroup. For each subgroup, the lower designs with the largest variances form a subgroup, for which the bottom design was not found to be significantly different from the other designs in the subgroup. Significant biases were determined by comparing the mean of each set of 500 $\alpha$ estimates with the true $\alpha$ via the Student's $t$ test with a type I error rate of 0.01 with SAS.

A few general conclusions can be derived from Figures 1 to 4 and Table 11. First, for every group, the D-optimal designs were always among the design subgroup with the smallest variance. The superiority of the D-optimal designs over all of the other designs is clearly seen for the SR, LR and LC error types, but is less clear for SA, especially for the case in which the true $\alpha=1$. Second, the FR2X design (Table 4) was always in the subgroup with the largest variance. Third, there was a tendency for the FR2X design (Table 3) to be a relatively precise design and for 5X5 (Table 1) to be a relatively imprecise design. Fourth, the CEN design (Table 2) tended to be very precise for the SA error type, but had medium precision for the SR, LR and LC error types. Even though OPTC (Table 7), OPTD (Table 8), OPTE (Table 9) and OPTR (Table 10) had the smallest variances for the SR, LR, and LC error types, respectively, these designs resulted in a significant bias.

**DISCUSSION**

Among the five rival experimental designs, the seemingly counterintuitive D-optimal designs appear to result in the smallest variance for the interaction parameter, $\alpha$. From this result, one can infer that the D-optimal designs may be superior for assessing quantitatively the nature and intensity of combined action of two agents. The convenient single fixed ratio in duplicate design (FR2X, Table 3) was also very good, and ranked second.

Formal statistical experimental design often includes an interesting paradox: to design an experiment well, one has to know the final answer well. However, if the final answer is well known, i.e., both the correct model and the true model parameters, then one would not have to conduct the experiment. This paradox is solved with sequential experimentation; each experiment in a sequence provides better information for the
**Figure 4** Box plots for the sets of 500 estimates for Monte Carlo data sets containing large relative error with a noise constant (LC), for each of five rival designs for each of two values for the true interaction parameter (\(a = 1\), left; \(a = 5\), right). The box plot for each design includes a central bar for the median, upper and lower edges of a box for the 25th and 75th percentiles of the data, upper and lower lines extending to short capped bars for the 10th and 90th percentiles, and upper and lower circles which indicate the 5th and 95th percentiles.

**TABLE 11**
Variances for distributions of \(\alpha\) parameter estimates

| True \(\alpha\) | Small absolute (SA) | Small relative (SR) | Large relative (LR) | Large relative + noise constant (LC) |
|---------------|---------------------|---------------------|---------------------|-------------------------------------|
|               | Design | Variance | Design | Variance | Design | Variance | Design | Variance | Design | Variance |
| 1             | CEN\(^T\) | 0.00619  | OPTC\(^T\) | 0.000409 | OPTC\(^T\) | 0.00380  | OPTE\(^T\) | 0.0629  |
|               | OPTA\(^T\) | 0.00659  | FR2X  | 0.000363 | FR2X  | 0.0246  | FR2X  | 0.0913  |
|               | FR2X\(^T\) | 0.00763  | CEN   | 0.000301 | CEN   | 0.0307  | CEN   | 0.0962  |
|               | FR4X\(^T\) | 0.00900  | 5X5\(^T\) | 0.000356 | 5X5\(^T\) | 0.0384  | 5X5\(^T\) | 0.121   |
|               | 5X5\(^T\) | 0.0110   | FR4X\(^T\) | 0.000427 | FR4X\(^T\) | 0.0411  | 5X5\(^T\) | 0.129   |
| 5             | OPTE\(^T\) | 0.0463   | OPTD\(^T\) | 0.00134  | OPTD\(^T\) | 0.129   | OPTE\(^T\) | 0.727    |
|               | CEN\(^T\) | 0.00605  | FR2X\(^T\) | 0.00395  | FR2X\(^T\) | 0.345   | FR2X\(^T\) | 0.937    |
|               | FR2X\(^T\) | 0.00611  | 5X5   | 0.00409  | 5X5   | 0.406   | 5X5   | 1.15     |
|               | FR4X\(^T\) | 0.00764  | CEN   | 0.00430  | CEN   | 0.449   | CEN   | 1.26     |
|               | 5X5\(^T\) | 0.00790  | FR4X\(^T\) | 0.00723  | FR4X\(^T\) | 0.752   | FR4X\(^T\) | 1.44     |

\(^{*}\text{Significant biases.} \quad \text{1Upper designs with the smallest variances.} \quad \text{2Lower designs with the largest variances.} \quad \text{5X5 Factorial design; CEN Central composite design; FR2X fixed ratio design with single ratio in duplicate; FR4X fixed ratio design with four different ratios; OPTE D-optimal design for absolute error, } \alpha = 1; \quad \text{OPTB D-optimal design for absolute error, } \alpha = 5; \quad \text{OPTC D-optimal design for relative error, } \alpha = 1; \quad \text{OPTD D-optimal design for relative error, } \alpha = 5; \quad \text{OPTE D-optimal design for relative error with Intercept, } \alpha = 1; \quad \text{OPTE D-optimal design for relative error with Intercept, } \alpha = 5\)
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planning of the subsequent experiment. Based upon the results of the present study, for confirmatory two-agent combined action Loewe synergism studies for pairs of agents with simple three-dimensional concentration-effect surfaces (appropriately modelled with Equation 1), one should: first, understand the concentration-effect curves of each individual agent well, i.e., obtain precise estimates of $IC_{50,1}$, $m_1$, $IC_{50,2}$, and $m_2$; second, use a single ray fixed ratio design for combined action to get a fair estimate of $a$; and, finally, use a D-optimal design for a precise estimate of $a$. Alternatively, one might want to use a standard fixed ratio or factorial design routinely, but then also add the D-optimal design points to the final experimental design.

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