Optimal Row-Column Designs in High-Throughput Screening Experiments

Saturated row-column designs are studied to eliminate row and column effects in primary high-throughput screening experiments. All paired comparisons of treatments in the designs recommended are estimable within each microplate in spite of the existence of row and column effects. The (M,S)-criterion is used to select optimal and eliminate inefficient designs. It turns out that all (M,S)-optimal designs are binary, i.e., no treatments appear twice in any row or column. Optimal designs are not unique with respect to design isomorphism. A series of (M,S)-optimal designs are constructed and all paired comparisons of treatments in the constructed designs are estimable regardless of the two-way heterogeneity. An (M,S)-optimal design for 8×12 microplates is provided and optimal designs of other dimensions can be constructed systematically.

KEY WORDS: Binary designs; (M,S)-optimality; Row-column designs; Saturated designs.

1. INTRODUCTION

High-throughput screening (HTS) is a large-scale process that screens hundreds of thousands to millions of compounds in order to identify pharmacologically active compounds. In an HTS process compounds are usually tested for binding activity or biological activity against target molecules. A key piece of HTS equipment is the microplate: a small container that features a grid of small, open divots called wells. Figure 1 presents 96 (8×12)-well microplates commonly used in HTS practices where solid and empty circles are wells. Other HTS microplates such as 384 (16×24)-well, 1536 (32×48)-well, and 3456 (48×72)-well ones are also used in HTS experiments. Even though the plating format and the number of compounds per plate vary, primary HTS operations typically measure a single observation from each compound incubated in a well of rectangular microplates.

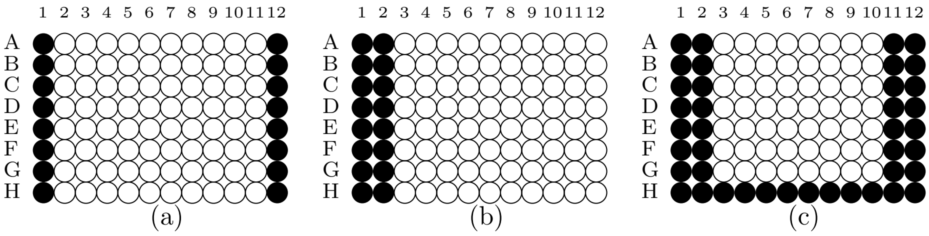

Figure 1. Current HTS Designs

There are many statistical challenges in data preprocessing and active-compound (also called hits) identification in primary HTS. Malo *et al.* (2006) reviewed statistical issues in the current HTS practice. Random and systematic errors due to aging, reagent evaporation, cell decay, or liquid handling, etc. contaminate HTS data and bias hit selections in almost every HTS experiment. Often, systematic errors result in spatial effects or row and column effects on microplates. That is, observations from the same compound may vary systematically as well as randomly from well to well on the microplate.

Figure 2 shows the heat map of data from an HTS experiment. In this experiment, one control compound is incubated to all 384 wells. Different levels of activity are observed from wells in different rows and columns. An analysis of variance shows row and column effects are statistically significant ($\alpha = 0.05$). Brideau *et al.* (2003) studied more than $1,000$ 384-well microplates and discovered that values situated in row one were, on average, 14% lower than those in row 16.

Due to the tremendous impact of row and column effects on false-positive and false-negative discovery rates in HTS, experimental remedies have been proposed to eliminate row and column effects in HTS. For instance, Lundholt, Scudder, and Pagliaro (2003) used a pre-incubation technique to reduce spatial effects in cell-based assays. However, experimental remedies usually increase the cost of inventory such as time and materials in HTS. On the other hand, analytic methods using experimental design are more cost-effective. Figure 1 displays three commonly used designs in HTS experimentation where solid circles contain controls and empty circles have compounds to be screened. As is shown later, not all paired comparisons of treatments in such designs are estimable and usually these designs are unable to decompose treatment effects from the contamination of row and column effects.

Arranging compounds or treatments on microplates to eliminate spatial effects is

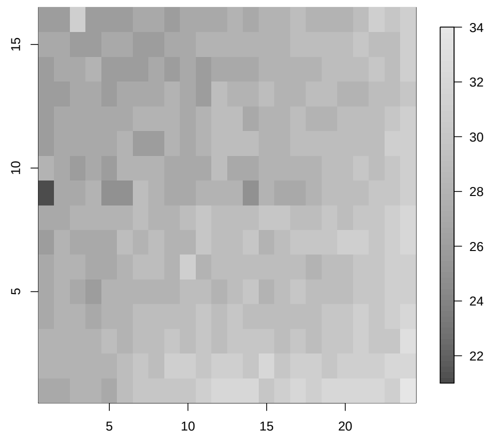

Figure 2: Row and Column Effects

the same as constructing row-column designs to eliminate two-way heterogeneity. Latin squares, Youden squares and their generalizations are classic row-column designs with various optimalities (Cheng, 1981, Kiefer, 1975). However, these classic designs usually have strictly combinatorial constraints on numbers of rows, columns, and treatments. For example, numbers of rows, columns as well as treatments have to be equal in Latin square designs; numbers of columns and treatments are the same in Youden squares, etc. Because of such constraints, classic designs with small number of treatments are not applicable to HTS experiments in drug discovery (Hüser *et al.*, 2006) and microarray experiments (Kerr, 2003), etc. HTS experiments are often characterized by a shortage of experimental materials to evaluate hundreds of thousands of compounds, or genes (called treatments, hereafter). Moreover, scientists in HTS are primarily interested in the selection and identification of superior treatments for further improvement as opposed to precise estimation or prediction of their effects. Therefore, multiple replications of all treatments are neither feasible nor cost-effective.

Two computer packages CycDesigN (Whitaker *et al.*, 2008) and Gendex DOE Toolkit (Nguyen, 1997) can generate row-column designs of any dimensions with all treatments equally replicated and occurring at least twice. Such designs are not popular in the early stage of selection processes (Lin and Poushinsky, 1983) because they can study, at most, $N/2$ treatments, where N is the number of wells. A class of row-column designs in which no constraints are imposed on numbers of rows and columns and the majority of treatments are not replicated is studied in this paper.

There are many criteria in selecting row-column designs. A design whose geometric mean of canonical efficiency factors is at least as large as that of any other design is D-optimal. Although D-optimality has proved to be useful in the context of linear regression analysis, it has a less meaningful interpretation in selecting row-column designs (John and Williams, 1995, p.32). E-optimality (Jacroux, 1990) is also a criterion. A design whose smallest canonical efficiency factor is at least as large as that of any other design is E-optimal. A-optimal designs have the largest harmonic mean of canonical efficiency factors. However, A-optimal row-column designs are intractable to find when the number of experimental units is not a multiple of the number of treatments. Sonnemann (1985) studied A-optimal row-column designs for two treatments. Morgan and Parvu (2007) solved the A-optimality problem for three treatments.

The (M,S)-optimality criterion is used in this paper. A design is said to be (M,S)-optimal among a class of designs if it has the maximum trace of the information matrix and has the minimum trace of the information matrix squared over all designs with the maximum trace. The rationale of using the (M,S)-criterion is as follows. First, it is desirable in HTS experimentation to estimate all paired comparisons with the same precision and it is known that all paired comparisons can be estimated with the same precision if and only if all nonzero eigenvalues of the information matrix are equal (Raghavarao, 1971, p.52). Let λ_i, for $i = 1, 2, \cdots, n$, be the nonzero eigenvalues of the information matrix. Since $\sum_{i=1}^{n}(\lambda_i - \bar{\lambda})^2 = \sum_{i=1}^{n}\lambda^2 - (\sum_{i=1}^{n}\lambda)^2/n$, $\sum_{i=1}^{n}\lambda^2$ is the trace of the information matrix squared, and $\sum_{i=1}^{n}\lambda$ is the trace of the information matrix, (M,S)-optimal designs have the least variable eigenvalues. Second, the (M,S)-criterion has huge computational advantages over other criteria. Such advantages have been popularly adopted in generating A- and E-optimal row-column designs by computers (Nguyen, 1997). Although (M,S)-optimal designs are not A- and E-optimal, they tend to have better A- and E-criterion performance (Cheng, 1978). Third, the (M,S)-criterion provides great insight in searching for A- and E-optimal designs because eigenvalues bounds of the information matrix can be obtained through its trace (Wolkowicz and Styan, 1980).

The paper is arranged as follows. Section 2 introduces the class of row-column designs and some preliminaries. Main results are presented in Section 3 and concluding remarks are given in Section 4.

2. SATURATED ROW-COLUMN DESIGNS

In an HTS experiment, wells in the microplate are experimental units and they are grouped by two blocking factors with one factor representing the rows of the microplate and the other representing the columns. The third factor of the experiment is treatments. An appropriate row-column design for the experiment increases the accuracy of estimating treatment comparisons by eliminating row and column effects.

In this section, a class of saturated row-column designs is introduced. Consider allocating $v = (b-1)(k-1)+1$ treatments, $T_1, T_2, \ldots, T_v$, to the bk experimental units in $b(\geq 3)$ rows and $k(\geq 3)$ columns. The class of designs, say, $\mathcal{D}$, studied in this paper is constructed as follows.

Table 1: Saturated row-column designs

Row\Column	1	2	3	$\cdots$	$k-1$	k
1	T_1	T_2	T_3	$\cdots$	T_{k-1}	η_1
2	T_k	T_{k+1}	T_{k+2}	$\cdots$	$T_{2(k-1)}$	η_2
3	T_{2k-1}	T_{2k}	T_{2k+1}	$\cdots$	$T_{3(k-1)}$	η_3
$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$
$b-1$	$T_{(b-2)k-(b-3)}$	$T_{(b-2)k-(b-4)}$	$T_{(b-2)k-(b-5)}$	$\cdots$	T_{v-1}	η_{b-1}
b	θ_1	θ_2	θ_3	$\cdots$	θ_{k-1}	T_v

a. Treatment $T_{(i-1)(k-1)+j}$ is arranged in the (i,j)th cell of the design for $i = 1, 2, \cdots, b-1$, $j = 1, 2, \cdots,$ and $k-1$.

b. Treatment T_v is placed at the (b,k)th cell of the design.

c. For $1 \leq i \leq b-1$, η_i is any treatment in $\{T_1, T_2, \cdots, T_v\}$ that has not appeared in the ith row.

d. For $1 \leq j \leq k-1$, θ_j is any treatment in $\{T_1, T_2, \cdots, T_v\}$ other than those that are already in the jth column.

The construction of designs in Table 1 has great feasibility in HTS experimentation where only treatments in the bth row and kth column are restricted, replicated, and all others are free of restrictions and non-repeated. There are no constraints on b and k.

Such designs were first proposed in Qu *et al.*. (2010). However, only squared ($b = k$) (M,S)-optimal designs are studied there. The results in this paper hold for all b and k. There is no loss of generality of taking $b \leq k$, since the design can always be rotated by 90^o to achieve this. It is also important to note that class $\mathcal{D}$ studied in this paper is a proper subclass of saturated row-column designs of b by k.

The following discussion is devoted to notations of row-column designs. More details can be found in John and Williams (1995, Chap. 5). Let Y_{ij} be the observation from the (i, j)th cell, $i = 1, 2, \ldots, b$, and $j = 1, 2, \ldots, k$. The following additive fixed-effect model is considered in this paper.

$$Y_{ij} = \mu + \alpha_i + \beta_j + \tau_\ell + \epsilon_{ij}, \tag{1}$$

where μ is the general mean, α_i is the effect of the ith row, β_j is the effect of the jth column, τ_ℓ is the effect of treatment T_ℓ if T_ℓ appears in the (i, j)th cell, $\ell = 1,\ 2,\ \cdots$, v, and ϵ_{ij}'s are independently and identically distributed random errors with mean zero and standard deviation σ.

Since treatment, row, and column effects in an HTS experiment form a three-way classification, equation (1) is the simplest linear model that separates treatment effects from random errors as well as row and column effects. However, this model has not been used for hit selection in the HTS literature. Currently, most selection procedures are ad hoc. Few procedures are able to de-alias treatment effects from the contamination of spatial effects and random errors. The popular B-score method (Brideau et al., 2003) eliminates spatial effects by estimating such effects through Tukey's median polish procedure and subtracting them from corresponding observations. Such elimination does reduce the influence of spatial effects but cannot separate treatment effects from random errors. In fact, the residuals used in the B-score procedure are sums of treatment effects and random errors. The design of HTS experiments is another reason that model (1) is not applied. In order to estimate treatment effects in model (1), all paired comparisons of treatments must be estimable in the row-column design, i.e., the design is treatment-connected. As is discussed below, most HTS designs in the literature are not treatment-connected. Zhang (2008) proposed a series of plate designs (e.g., C1 to C5) by arranging controls in certain patterns to offset row-column effects. It can be shown that designs C1 to C5 are not treatment-connected. Therefore, linear model (1) is not applicable to analyze data from those designs. Next, we introduce the concept of information matrix to describe the treatment-connectedness of a row-column design.

The information matrix C_d of a row-column design d has the following form under model (1)

$$C_d = R - \frac{1}{k}N_1N_1' - \frac{1}{b}N_2N_2' + \frac{1}{bk}\mathbf{r}\mathbf{r}', \tag{2}$$

or, equivalently,

$$bkC_d = bkR - bN_1N_1' - kN_2N_2' + \mathbf{r}\mathbf{r}', \tag{3}$$

where $\mathbf{r}' = (r_1, \ldots, r_v)$ is the treatment replicate vector, $R = \text{diag}(r_1, r_2, \ldots, r_v)$ is a diagonal matrix with entries $r_1, \ldots, r_v$, $N_1 = (n_{ij}^{(r)})$ of order $v \times b$ denotes the treatment-row incidence matrix, i.e., $n_{ij}^{(r)}$ is the number of times treatment T_i appears in the jth row, $N_2 = (n_{ij}^{(c)})$ of order $v \times k$ is the treatment-column incidence matrix, and $n_{ij}^{(c)}$ is the number of times treatment T_i occurs in the jth column. If all entries in N_1 and N_2 are either 0 or 1, design d is binary.

It is known that a row-column design is treatment-connected, i.e., all paired comparisons of treatments are estimable, if and only if the rank of C_d is $v - 1$. If all solid circles in design (a) are controls, there are 81 distinct treatments in each microplate and a straightforward QR decomposition shows that rank$(C_d) = 70$ which is smaller than 80 (i.e., $81 - 1$). Therefore, design (a) is not treatment-connected and not all paired comparisons of treatments are estimable. Similarly, design (b) is not treatment-connected either. If all solid circles in design (c) are controls rank$(C_d) = 56$ and the design is treatment-connected. As is pointed out next, the maximum number of treatments that can be arranged in an 8×12 treatment-connected design is 78.

Since there are bk observations in the design of Table 1 for each b and k, the total number of degrees of freedom is $bk - 1$. According to equation (1), $b - 1$ and $k - 1$ degrees of freedom are used to estimate row and column effects, respectively. There are $(b - 1)(k - 1) = v - 1$ degrees of freedom left for treatment effects. Thus, the design is saturated because $v = bk - b - k + 2$ is the maximum number of treatments that can be arranged in a row-column layout to eliminate non-negligible two-way heterogeneity. There are no degrees of freedom left to estimate the variance of the random error σ^2.

The analysis of data from a design in Table 1 using model (1) is challenging due to the saturation and nonorthogonality of the design. Though many analysis methods have been proposed for orthogonal, saturated designs (Hamada and Balakrishnan, 1998), only a few articles in the literature deal with nonorthogonal, saturated designs. Two methods in Kunert (1997) and Wang and Voss (2001) are good candidates for hit selection because effect sparsity, one of the key features of HTS, is used to estimate the variance of the random error. The LASSO (least absolute shrinkage and selection operator) method

(Tibshirani, 1996) can also be applied. We are currently assessing the performance of these procedures. Another challenge of linear regression using model (1) is that random errors in HTS experiments are usually nonnormal and heteroscedastic.

3. MAIN RESULTS

In this section, a general expression of $\mathrm{tr}(C_d)$ for design $d \in \mathcal{D}$ is given. The general formula is then used to explore the specific structure of (M,S)-optimal designs in $\mathcal{D}$. Proposition 1 provides an explicit formula of $\mathrm{tr}(C_d)$ in terms of the occurrences of treatments in the bth row and kth column. Proposition 2 gives an upper bound of $\mathrm{tr}(C_d)$. The proofs of Propositions 1 and 2 are given in Qu *et al.*. (2010).

Proposition 1. *For any design $d \in \mathcal{D}$,*

$$bktr(C_d) = bkv - b\sum_{i=1}^{v} {n_{ib}^{(r)}}^2 - k\sum_{j=1}^{v} {n_{jk}^{(c)}}^2 + \sum_{l=1}^{v} r_l^2, \tag{4}$$

where $n_{ib}^{(r)}$ is the number of times that treatment T_i appears in the bth row, $n_{jk}^{(c)}$ is the number of times that treatment T_j appears in the kth column and r_l is the replicate number of treatment T_l. If d is binary, then $bktr(C_d) = bk(v-2) + 1 + \sum_{l=1}^{v-1} r_l^2$.

Proposition 2 next shows that designs that attain the maximum trace of information matrices among all designs in $\mathcal{D}$ are binary. Therefore, (M,S)-optimal binary designs in $\mathcal{D}$ are also (M,S)-optimal in $\mathcal{D}$.

Proposition 2. *For any design $d \in \mathcal{D}$, let $n_{ib}^{(r)}$ be the number of times that treatment T_i appears in the bth row and $n_{jk}^{(c)}$ be the number of times that treatment T_j appears in the kth column. Then*

$$bktr(C_d) = bk(v+1)+b+k+2-(b-1)\sum_{i=1}^{v} {n_{ib}^{(r)}}^2-(k-1)\sum_{j=1}^{v} {n_{jk}^{(c)}}^2+2\sum_{i=1}^{v} n_{ib}^{(r)}n_{ik}^{(c)}-4n_{vb}^{(r)}-4n_{vk}^{(c)} \tag{5}$$

and

$$tr(C_d) \le bk - b - k + 1 + \frac{2|b-k| + 6min(b,k) - 6}{bk}. \tag{6}$$

The upper bound of $tr(C_d)$ in inequality (6) is attained if and only if $n_{ib}^{(r)} = 0\, or 1 (1 \le i \le v)$, $n_{jk}^{(c)} = 0\, or 1 (1 \le j \le v)$, $n_{vb}^{(r)} = n_{vk}^{(c)} = 1$, and $n_{ib}^{(r)} - n_{ik}^{(c)} = 0\, or 1$ when $k \ge b$ or $n_{ik}^{(c)} - n_{ib}^{(r)} = 0\, or 1$ when $b \ge k$.

Proposition 2 also highlights the structure of design $d \in \mathcal{D}$ whose information matrix attains the maximum trace. For $k \geq b$, it is observed that treatments appearing in the kth column must also appear in the bth row in order to maximize the trace of the information matrix. When $k \geq b$, proposition 2 implies that a design d with the maximum trace of information matrix has $b-1$ treatments appearing three times, $k-b$ ones appearing twice, and $bk-2k-b+3$ appearing once.

Without loss of generality, we assume $b \leq k$ throughout the following discussion. Theorem 1 provides the lower bound of $\mathrm{tr}(C_d^2)$ for any designs in $\mathcal{D}$ that attain the maximum $\mathrm{tr}(C_d)$ and its proof is given in the Appendix.

Theorem 1. *Let* $4 \leq b \leq k \leq 2b-1$. *For any design* $d \in \mathcal{D}$ *with the maximum trace of information matrix*

$$\begin{aligned} b^2k^2tr(C_d^2) \;\geq\;& b^3k^3 + b^2k^3 + 3b^3k^2 - 17b^2k^2 - 6b^3k - 4bk^3 \\ +\;& 40b^2k + 34bk^2 - 2b^3 - 2k^3 - 10k^2 - 50bk - 24b + 36. \end{aligned} \tag{7}$$

We now present an algorithm for constructing designs that attain the lower bound of inequality (7). For $b \geq 4$ and $k = b+s$ where $0 \leq s \leq b-1$, let $1, 2, \cdots, v = bk-b-k+2$ represent treatments in the design.

Construction Algorithm 1

1. Treatment $(i-1)(k-1)+j$ is in row i and column j for $i = 1, 2, \cdots, b-1$, $j = 1, 2, \cdots,$ and $k-1$, respectively;
2. Treatment v is in row b and column k;
3. Treatments in rows 1 to $b-1$ of column k are $k+1$, $2k+1$, $\cdots$, $(b-2)k+1$, and 1, respectively;
4. Treatments $k+1$, $2k+1$, $\cdots$, $(b-s-1)k+1$ are in columns 1 to $b-s-1$ of row b; those in columns $b-s$ to $b-1$ of row b are treatments $(b-s-1)(k-2)+2(b-1)$, $(b-s)(k-2)+2(b-1)$, $\cdots$, $(b-2)(k-2)+2(b-1)$; those in columns b to $k-1$ of row b are treatments 1, $(b-2)k+1$, $\cdots$, and $(b-s)k+1$, respectively.

Theorem 2 shows that designs from Construction Algorithm 1 are treatment-connected. Its proof is given in the Appendix.

Theorem 2. For $b \geq 4$ and $k = b + s$ where $0 \leq s \leq b - 1$, Construction Algorithm 1 produces designs that are (M,S)-optimal and treatment-connected.

Three important questions regarding (M,S)-optimal designs need to be addressed. First, are (M,S)-optimal designs in $\mathcal{D}$ unique with respect to design isomorphism? Two row-column designs are isomorphic if one can be obtained from the other by permuting rows or columns or relabeling treatments. Second, are all (M,S)-optimal designs treatment-connected? Third, are all treatment-connected, (M,S)-optimal designs isomorphic? Answers to these questions are negative.

Table 2 lists three (M,S)-optimal designs of 6×9 with 41 treatments labeled 1 to 41. Designs 1 and 2 are treatment-connected since their information matrices have ranks of 40. However, information matrices of designs 1 and 2 have different eigenvalues. Recall that information matrices of isomorphic designs have the same eigenvalues. It follows that designs 1 and 2 are not isomorphic. Design 3 is not treatment-connected because the rank of its information matrix is 39 instead of 40. Therefore, (M,S)-optimal designs are neither unique nor treatment-connected. There are nonisomorphic (M,S)-optimal designs that are treatment-connected.

Table 2: (M,S)-Optimal Designs of 6×9

Design 1								
1	2	3	4	5	6	7	8	10
9	10	11	12	13	14	15	16	19
17	18	19	20	21	22	23	24	28
25	26	27	28	29	30	31	32	37
33	34	35	36	37	38	39	40	1
10	19	24	31	38	1	37	28	41
Design 2								
1	2	3	4	5	6	7	8	10
9	10	11	12	13	14	15	16	19
17	18	19	20	21	22	23	24	28
25	26	27	28	29	30	31	32	37
33	34	35	36	37	38	39	40	1
10	19	24	31	38	28	1	37	41
Design 3								
1	2	3	4	5	6	7	8	10
9	10	11	12	13	14	15	16	19
17	18	19	20	21	22	23	24	28
25	26	27	28	29	30	31	32	37
33	34	35	36	37	38	39	40	1
19	31	38	10	24	1	37	28	41

When $b = k$, Theorem 3 shows that designs in Table 3 are (M,S)-optimal and treatment-connected. This result was proved in Qu and Ogunyemi (2009). We present a different but simpler proof in the Appendix.

Table 3: (M,S)-optimal square designs

1	2	3	$\cdots$	$b-2$	$b-1$	$b+1$
b	$b+1$	$b+2$	$\cdots$	$(b-1)+(b-2)$	$2(b-1)$	$2b+1$
$2b-1$	$2b$	$2b+1$	$\cdots$	$2(b-1)+(b-2)$	$3(b-1)$	$3b+1$
$\vdots$	$\vdots$	$\vdots$	$\ddots$	$\vdots$	$\vdots$	$\vdots$
$(b-3)(b-1)+1$	$(b-3)(b-1)+2$	$(b-3)(b-1)+3$	$\cdots$	$(b-3)(b-1)+(b-2)$	$(b-2)(b-1)$	$(b-1)^2$
$(b-2)(b-1)+1$	$(b-2)(b-1)+2$	$(b-2)(b-1)+3$	$\cdots$	$(b-2)(b-1)+(b-2)$	$(b-1)^2$	1
$b+1$	$2b+1$	$3b+1$	$\cdots$	$(b-1)^2$	1	$(b-1)^2+1$

Theorem 3. When $b = k \geq 4$, designs in Table 3 are (M,S)-optimal and treatment-connected.

Theorem 1 shows the lower bound of $\mathrm{tr}(C_d^2)$ when $4 \leq b \leq k \leq 2b-1$. Theorem 4 next provides the lower bounds in more general cases. Again, its proof is presented in the Appendix.

Theorem 4. Let $b \geq 4$. For $t(b-1)+1 < k \leq (t+1)(b-1)+1$, $t \geq 2$ and any design $d \in \mathcal{D}$ that maximizes the trace of the information matrix

$$\begin{aligned} b^2k^2 tr(C_d^2) &\geq b^3k^3 + 3b^3k^2 + b^2k^3 - 6b^3k - 17b^2k^2 - 4bk^3 + (36+4t)b^2k \\ &+ 26bk^2 + (2-2t-2t^2)b^3 + 2k^3 - (38+4t)bk \\ &- (4-4t^2)b^2 - 10k^2 - (24-2t+2t^2)b - 4k + 36. \end{aligned} \tag{8}$$

For $b \geq 4$ and $k = t(b-1)+s+1$ where $0 \leq s \leq b-1$ and $t \geq 2$, Construction Algorithm 2 next provides designs that attain the lower bound of inequality (8) where $1, 2, \cdots, v-1$ and $v = bk-b-k+2$ are treatments.

Construction Algorithm 2

1. Treatment $(i-1)(k-1)+j$ is in row i and column j for $i = 1, 2, \cdots, b-1$, $j = 1, 2, \cdots,$ and $k-1$, respectively;

2. Treatment v is in row b and column k;

3. Treatments in rows 1 to $b-1$ of column k are $k+1$, $2k+1$, $\cdots$, $(b-2)k+1$, and 1, respectively;

4. If t is even, treatments in columns 1 to $(t-1)(b-1)$ of row b are $2(b-1)$, $(k-1)+(b-1)+(b-2)$, $\cdots$, $(b-3)(k-1)+(b-1)+2$, $(b-2)(k-1)+(b-1)+1$; $(b-2)(k-1)+3(b-1)$, $(b-3)(k-1)+3(b-1)-1$, $\cdots$, $(k-1)+2(b-1)+2$,

$2(b-1)+1; \cdots; t(b-1), (k-1)+t(b-1)-1, \cdots, (b-3)(k-1)+(t-1)(b-1)+2,$ and $(b-2)(k-1)+(t-1)(b-1)+1$; those in columns $(t-1)(b-1)+1$ to $t(b-1)-s$ of row b are $1, (b-2)k+1, \cdots, (s+1)k+1$; those in columns $t(b-1)-s+1$ to $t(b-1)$ of row b are $(s-1)(k-1)+t(b-1)+s, (s-2)(k-1)+t(b-1)+s-1, \cdots, (k-1)+t(b-1)+2$, and $t(b-1)+1$; those in columns $t(b-1)+1$ to $k-1$ of row b are $k+1, 2k+1, \cdots, sk+1$ respectively;

5. If t is odd, treatments in columns 1 to $(t-1)(b-1)$ of row b are $2(b-1), (k-1)+2(b-1)-1, \cdots, (b-3)(k-1)+(b-1)+2, (b-2)(k-1)+(b-1)+1$; $(b-2)(k-1)+3(b-1), (b-3)(k-1)+3(b-1)-1, \cdots, (k-1)+2(b-1)+2, 2(b-1)+1$; $\cdots; (b-2)(k-1)+t(b-1), (b-3)(k-1)+t(b-1)-1, \cdots, (k-1)+(t-1)(b-1)+2$, and $(t-1)(b-1)+1$; those in columns $(t-1)(b-1)+1$ to $t(b-1)-s$ of row b are $k+1, 2k+1, \cdots, (b-1-s)k+1$; those in columns $t(b-1)-s+1$ to $t(b-1)$ of row b are $(b-s-1)(k-1)+t(b-1)+s, (b-s)(k-1)+t(b-1)+s-1, \cdots, (b-2)(k-1)+t(b-1)+1$; those in columns $t(b-1)+1$ to $k-1$ of row b are $1, (b-2)k+1, \cdots, (b-s)k+1$, respectively.

Theorem 5 shows that designs constructed by Construction Algorithm 2 are treatment-connected. Since its proof follows exactly the same steps as that of Theorem 2, it is omitted.

Theorem 5. *For* $b \geq 4$ *and* $k = t(b-1)+s+1$ *where* $1 \leq s \leq b-1$ *and* $t \geq 2$*, Construction Algorithm 2 produces designs that are (M,S)-optimal and treatment-connected.*

Note that, when $b = 3$, there is a $4b^2$ increase in $b^2\text{tr}(N_1N_1'N_1N_1')$. We have

Theorem 6. *For* $b = 3$, $2t+1 < k \leq 2t+3$, $t \geq 2$, *and any design* d *in* $\mathcal{D}$ *with the maximum trace of information matrix*

$$\begin{aligned} b^2k^2tr(C_d^2) &\geq b^3k^3 + 3b^3k^2 + b^2k^3 - 6b^3k - 17b^2k^2 - 4bk^3 + (36+4t)b^2k \\ &+ 26bk^2 + (2-2t-2t^2)b^3 + 2k^3 - (38+4t)bk \\ &+ 4t^2b^2 - 10k^2 - (24-2t+2t^2)b - 4k + 36. \end{aligned}$$

When $3 = b \leq k \leq 5$, Table 4 shows the unique (M,S)-optimal designs of 3×3, 3×4, and 3×5 with respect to design isomorphism, respectively. All designs in Table 4 are treatment-connected. The general construction of (M,S)-optimal designs of $3 \times k$ for $k \geq 6$ follows Theorem 5 and it can be shown that neither (M,S)-optimal designs are unique nor all (M,S)-optimal designs are treatment-connected for $b = 3$.

Table 4: (M,S)-optimal designs of 3×3, 3×4, and 3×5

3×3			3×4				3×5				
1	2	4	1	2	3	5	1	2	3	4	6
3	4	1	4	5	6	1	5	6	7	8	1
4	1	5	5	6	1	7	4	7	1	6	9

Agrawal (1969a, 1969b) constructed several row-column designs in which the number of treatments is larger than both the number of rows and the number of columns. However, compared to the saturated design in this paper, the number of treatments in an Agrawal design is much smaller and there are restrictions on the number of rows and columns as well.

Table 5 compares the proposed (M,S)-optimal designs with Agrawal designs and CycDesigN designs (generated by the demonstration version of CycDesigN 3.0 with the maximum number of treatments) in terms of the number of replicated treatments (NR), the number of unreplicated treatments (NU), the average variance of estimates of differences between any two treatments (AV$\times\sigma^2$), the average variance of estimates of differences between two unreplicated treatments (AVUU$\times\sigma^2$), the average variance of estimates of differences between one unreplicated treatment and one replicated treatment (AVUR$\times\sigma^2$), and the average variance of estimates of differences between two replicated treatments (AVRR$\times\sigma^2$). It can be seen that the (M,S)-optimal designs have much more treatments than others. For example, the (M,S)-optimal design of 6×25 has 121 treatments of which 97 are unreplicated and 24 are replicated while the maximum number of treatments in the CycDesigN design is 75 and the Agrawal design has only 30 treatments. The large ratio of the number of unreplicated treatments to that of replicated ones is an attractive feature in the early stage of HTS experiments.

It is observed that (M,S)-optimal designs have the highest AVs. For example, the AV of the (M,S)-optimal design of 6×25 is higher than those of CycDesigN and Agrawal designs by factors of 3 and 11, respectively. This is the trade-off between the number of treatments and the estimate precision. Saturated row-column designs are favored in situations when experimenters focus on screening rather than optimizing and therefore, the number of treatments in the design outweigh the precision of estimation. Moreover, precise estimates can also be obtained by one or two replicates of proposed (M,S)-optimal designs in HTS when the standard deviation of the random error σ is small.

Table 5: Variances of paired comparisons in row-column designs

Design	Agrawal		CycDesigN		(M,S)-optimal					
$b \times k$	NR	AV	NR	AV	NR	NU	AV	AVUU	AVUR	AVRR
4×9	12	0.85	18	1.56	8	17	4.01	4.99	3.43	2.07
5×6	10	0.82	15	1.45	5	16	3.96	4.81	3.02	1.30
5×16	20	0.61	40	1.49	15	46	4.96	5.64	4.25	3.01
6×10	15	0.58	30	1.39	9	37	4.41	5.05	3.42	1.87
6×25	30	0.47	75	1.42	24	97	5.82	6.34	5.03	3.84
7×8	14	0.59	28	1.36	7	36	4.44	5.01	3.24	1.48
9×10	18	0.45	45	1.31	9	64	4.75	5.20	3.40	1.60
11×12	22	0.37	66	1.27	11	100	4.97	5.33	3.51	1.69

4. CONCLUSIONS

We have studied a class of saturated row-column designs. These designs use the minimum number of experimental units to compare the maximum number of treatments in a row-column layout. All (M,S)-optimal designs in the class are binary, i.e., no treatments are repeated in any row or column. Therefore, (M,S)-optimal binary designs are also (M,S)-optimal in $\mathcal{D}$. For any b and k, treatment-connected (M,S)-optimal designs can be constructed systematically by Construction Algorithms 1 and 2. (M,S)-optimal designs are not unique with respect to design isomorphism. Some (M,S)-optimal designs are not even treatment-connected.

The designs considered in this paper are not equi-replicated. As is pointed out by Morgan and Parvu (2007), finding A- and E-optimal row-column designs with a large number of non-replicated treatments is mathematically intractable at the current stage. When it is not feasible to find A- and E-optimal designs, are (M,S)-optimal designs constructed in this paper good surrogates? In other words, do (M,S)-optimal designs also have high A- and E-efficiencies? Since it is insurmountable to answer the question for large b and k, Table 6 lists A- and E-optimal designs of 4×4 and 4×5 in $\mathcal{D}$, where A-efficiency (A-eff) is the harmonic mean of nonzero eigenvalues and E-efficiency (E-eff) is the minimum nonzero eigenvalue of the information matrix C_d with respect to R, respectively (John and Williams, 1995, pp. 39).

Table 6 shows that A-optimal designs are not necessarily E-optimal in general and vice versa. The complete search shows that A-optimal designs of 4×4 are also E-optimal but some E-optimal designs are not A-optimal. All (M,S)-optimal designs of 4×4 and 4×5 are not A- and E-optimal and vice versa. In fact, all A- and E-optimal designs are not treatment-connected while all (M,S)-optimal designs are treatment-connected. The

Table 6: Optimal designs of 4×4 and 4×5

			A-optimal					*E*-optimal					Connected, *A*- and *E*-optimal					(M,S)-optimal		
4×4	1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	5	
	4	5	6	1		4	5	6	1		4	5	6	8		4	5	6	9	
	7	8	9	10		7	8	9	2		7	8	9	3		7	8	9	1	
	10	9	8	10		6	9	1	10		5	9	1	10		5	9	1	10	
A-eff			0.7368					0.6667					0.6000					0.4884		
E-eff			0.5000					0.5000					0.5000					0.2500		
4×5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	6
	5	6	7	8	10	5	6	7	8	1	5	6	7	8	10	5	6	7	8	11
	9	10	11	12	13	9	10	11	12	13	9	10	11	12	3	9	10	11	12	1
	2	13	8	7	13	10	13	12	11	13	6	11	4	1	13	6	11	12	1	13
A-eff			0.6782					0.6780					0.5471					0.4721		
E-eff			0.3041					0.4000					0.2500					0.1836		

connected *A*- and *E*-optimal designs in Table 6 have the highest *A*- and *E*-efficiencies among all treatment-connected designs. The (M,S)-optimal designs in Table 6 are constructed by Construction Algorithm 1 and have the highest *A*- and *E*-efficiencies among all treatment-connected, (M,S)-optimal designs of 4×4 and 4×5, respectively. The high ratios of *A*-efficiencies of treatment-connected, (M,S)-optimal designs to those of treatment-connected, *A*-optimal designs, 0.814 for 4×4 and 0.8629 for 4×5, demonstrate that (M,S)-optimal designs constructed in this paper are good surrogates of *A*-optimal designs. It is also true that all (M,S)-optimal designs of 4×6 are treatment-connected and the design constructed by Construction Algorithm 1 is *A*- and *E*-optimal of all (M,S)-optimal designs. For $b \geq 5$ or $k \geq 7$, there are (M,S)-optimal designs that have higher *A*- and *E*-efficiencies than those from Construction Algorithms 1 and 2. We are studying *A*- and *E*-optimal designs among (M,S)-optimal designs of $b \times k$.

Unlike most row-column designs in the literature where many combinatorial restrictions have been put on numbers of rows and columns, designs considered in this paper can be constructed for any dimensions. This is extremely important to HTS practice because microplates of various dimensions have been manufactured and used in HTS experimentation. Table 7 presents a treatment-connected, (M,S)-optimal design of 8×12 for 96-well microplates. (M,S)-optimal row-column designs for 16×24, 32×48, and 48×72 microplates can be constructed accordingly.

Table 7: An (M,S)-optimal design of 8×12

1	2	3	4	5	6	7	8	9	10	11	13
12	13	14	15	16	17	18	19	20	21	22	25
23	24	25	26	27	28	29	30	31	32	33	37
34	35	36	37	38	39	40	41	42	43	44	49
45	46	47	48	49	50	51	52	53	54	55	61
56	57	58	59	60	61	62	63	64	65	66	73
67	68	69	70	71	72	73	74	75	76	77	1
13	25	37	44	54	64	74	1	73	61	49	78

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APPENDIX

In this appendix, proofs of Theorem 1, Theorem 2, Theorem 3, and Theorem 4 are presented. Lemma 1 (Gaffke, 1981) is used to facilitate the proof of Theorem 1

Lemma 1. *Let* $\mathbf{x} = (x_1, x_2, \cdots, x_n)'$ *and* $\mathbf{y} = (y_1, y_2, \cdots, y_n)'$ *be n-dimensional vectors with integer components. If* $\sum_{i=1}^n y_i = \sum_{i=1}^n x_i$ *and* $|y_i - y_j| \leq 1$ *for* $1 \leq i \neq j \leq n$ *then* $\sum_{i=1}^n y_i^2 \leq \sum_{i=1}^n x_i^2$.

Proof of Theorem 1

It can be shown that

$$\begin{aligned}
b^2k^2\mathrm{tr}(C_d^2) &= b^2k^2\mathrm{tr}(R^2) + \mathrm{tr}(\mathbf{r}\mathbf{r}'\mathbf{r}\mathbf{r}') + 2bk\mathrm{tr}(R\mathbf{r}\mathbf{r}') \\
&+ b^2\mathrm{tr}(N_1N_1'N_1N_1') + k^2\mathrm{tr}(N_2N_2'N_2N_2') + 2bk\mathrm{tr}(N_1N_1'N_2N_2') \\
&- 2b^2k\mathrm{tr}(N_1N_1'R) - 2bk^2\mathrm{tr}(N_2N_2'R) - 2b\mathrm{tr}(N_1N_1'\mathbf{r}\mathbf{r}') - 2k\mathrm{tr}(N_2N_2'\mathbf{r}\mathbf{r}') \\
&= b^2k^2\sum_{i=1}^v r_i^2 + (\sum_{i=1}^v r_i^2)^2 + 2bk\sum_{i=1}^v r_i^3 \\
&+ b^2\mathrm{tr}(N_1'N_1N_1'N_1) + k^2\mathrm{tr}(N_2'N_2N_2'N_2) + 2bk\mathrm{tr}(N_1'N_2N_2'N_1) \\
&- 2b^2k\sum_{i=1}^v r_i^2 - 2bk^2\sum_{i=1}^v r_i^2 - 2b\sum_{i=1}^b s_{\mathrm{row},i}^2 - 2k\sum_{j=1}^b s_{\mathrm{col},j}^2
\end{aligned}$$

where $s_{\mathrm{row},i}$ and $s_{\mathrm{col},j}$ are the sums of treatment replicates in the ith row and jth column, respectively.

Since any design with the maximum $\mathrm{tr}(C_d)$ have $b-1$ treatments appearing three times, $k-b$ ones appearing twice, and $v-(k-b)-(b-1)$ appearing once only, $\sum_{i=1}^{v} r_i^2 = bk+4b+2k-6$ and $\sum_{i=1}^{v} r_i^3 = bk+18b+6k-24$. It follows that

$$\begin{aligned}
b^2k^2\mathrm{tr}(C_d^2) &= b^3k^3+2b^3k^2-8b^3k-15b^2k^2-4bk^3+56b^2k+28bk^2 \\
&+ 16b^2-44kb+4k^2-48b-24k+36 \\
&+ b^2\mathrm{tr}(N_1'N_1N_1'N_1)+k^2\mathrm{tr}(N_2'N_2N_2'N_2)+2bk\mathrm{tr}(N_1'N_2N_2'N_1) \\
&- 2b\sum_{i=1}^{b} s_{\mathrm{row},i}^2 - 2k\sum_{j=1}^{b} s_{\mathrm{col},j}^2
\end{aligned}$$

Consider $\mathrm{tr}(N_1'N_1N_1'N_1)$. Let $\lambda_{[i],j}=1$ if the treatment in the (i,k)th cell comes from the jth row of the first $k-1$ columns and 0 otherwise. Then

$$N_1'N_1 = \begin{bmatrix}
k & \lambda_{[1],2}+\lambda_{[2],1} & \cdots & \lambda_{[1],b-1}+\lambda_{[b-1],1} & h_1+h_1'+1 \\
\lambda_{[2],1}+\lambda_{[1],2} & k & \cdots & \lambda_{[2],b-1}+\lambda_{[b-1],2} & h_2+h_2'+1 \\
\vdots & \vdots & \cdots & \vdots & \vdots \\
\lambda_{[b-1],1}+\lambda_{[1],b-1} & \lambda_{[b-1],2}+\lambda_{[2],b-1} & \cdots & k & h_{b-1}+h_{b-1}'+1 \\
h_1+h_1'+1 & h_2+h_2'+1 & \cdots & h_{b-1}+h_{b-1}'+1 & k
\end{bmatrix}$$

where h_i is the number of treatments in the kth column of the first $b-1$ rows that come from the ith row of the first $k-1$ columns and h_i' is the number of treatments in the bth row of the first $k-1$ columns except those appearing in the k column of the first $b-1$ rows that come from the ith row of the first $k-1$ columns. It is observed that $\sum_{i=1}^{b-1}(h_i+h_i')=k-1$, and for $i=1,2,\cdots,b-1$, $\sum_{j=1}^{b-1}\lambda_{[i],j}=1$.

Thus,

$$\mathrm{tr}(N_1'N_1N_1'N_1) = bk^2 + 2\sum_{i=1}^{b-1}(h_i+h_i'+1)^2 + 2\sum_{i=1}^{b-2}\sum_{j=i+1}^{b-1}(\lambda_{[i],j}+\lambda_{[j],i})^2.$$

When $b \geq 4$,

$$\sum_{i=1}^{b-2}\sum_{j=i+1}^{b-1}(\lambda_{[i],j}+\lambda_{[j],i})^2 \geq \sum_{i=1}^{b-2}\sum_{j=i+1}^{b-1}(\lambda_{[i],j}+\lambda_{[j],i}) \geq b-1.$$

By lemma 1, the lower bound is attained when $\lambda_{[i],j}+\lambda_{[j],i}=0$ or 1 for $i=1,2,\cdots,b-2$, and $j=i+1,\cdots,b-1$.

Similarly,

$$N_2'N_2 = \begin{bmatrix} b & \mu_{[1],2}+\mu_{[2],1} & \cdots & \mu_{[1],k-1}+\mu_{[k-1],1} & l_1+\psi_{[1],\Omega} \\ \mu_{[2],1}+\mu_{[1],2} & b & \cdots & \mu_{[2],k-1}+\mu_{[k-1],2} & l_2+\psi_{[2],\Omega} \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ \mu_{[k-1],1}+\mu_{[1],k-1} & \mu_{[k-1],2}+\mu_{[2],k-1} & \cdots & b & l_{k-1}+\psi_{[k-1],\Omega} \\ l_1+\psi_{[1],\Omega} & l_2+\psi_{[2],\Omega} & \cdots & l_{k-1}+\psi_{[k-1],\Omega} & b \end{bmatrix}$$

where $\mu_{[i],j} = 1$ if the treatment in the (b,i)th cell of the design comes from the jth column of the first $b-1$ rows and 0 otherwise; Ω is the set of all treatments in the kth column except T_v, and $\psi_{[i],\Omega} = 1$ if the treatment in the (b,i)th cell belongs to Ω and 0 otherwise; l_i is the number of treatments in Ω coming from the ith column of the first $b-1$ rows. Therefore,

$$\text{tr}(N_2'N_2N_2'N_2) = b^2k + 2\sum_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 + 2\sum_{i=1}^{k-2}\sum_{j=i+1}^{k-1}(\mu_{[i],j}+\mu_{[j],i})^2.$$

When $k \geq b \geq 4$,

$$\sum_{i=1}^{k-2}\sum_{j=i+1}^{k-1}(\mu_{[i],j}+\mu_{[j],i})^2 \geq \sum_{i=1}^{k-2}\sum_{j=i+1}^{k-1}(\mu_{[i],j}+\mu_{[j],i}) \geq k-1$$

and the lower bound is attained when $\mu_{[i],j}+\mu_{[j],i} = 0$ or 1 for $i = 1,2,\cdots,k-2$, and $j = i+1,\cdots,k-1$.

Let $\delta_{[i],j} = 1$ if the treatment in the (i,k)th cell is from the jth column and 0 otherwise; $\Delta_{[j],i} = 1$ if the treatment in the (b,j)th cell is from the ith row of the first $k-1$ columns and 0 otherwise. Then $N_1'N_2 =$

$$\begin{bmatrix} 1+\delta_{[1],1}+\Delta_{[1],1} & 1+\delta_{[1],2}+\Delta_{[2],1} & \cdots & 1+\delta_{[1],k-1}+\Delta_{[k-1],1} & 1+h_1 \\ 1+\delta_{[2],1}+\Delta_{[1],2} & 1+\delta_{[2],2}+\Delta_{[2],2} & \cdots & 1+\delta_{[2],k-1}+\Delta_{[k-1],2} & 1+h_2 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ 1+\delta_{[b-1],1}+\Delta_{[1],b-1} & 1+\delta_{[b-1],2}+\Delta_{[2],b-1} & \cdots & 1+\delta_{[b-1],k-1}+\Delta_{[k-1],b-1} & 1+h_{b-1} \\ 1+l_1+l_1' & 1+l_2+l_2' & \cdots & 1+l_{k-1}+l_{k-1}' & b \end{bmatrix}$$

where l_j' is the number of treatments in the bth row of the first $k-1$ columns that come from the jth column of the first $b-1$ rows and don't belong to Ω.

Note that, for $i = 1,2,\cdots,b-1$, $\sum_{j=1}^{k-1}\delta_{[i],j} = 2$ because the treatment in the (i,k)th cell of the design is in the first $b-1$ rows and $k-1$ columns and must also appear in the

bth row. Hence, $\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}\delta_{[i],j} = 2(b-1)$. Similarly, for any $j = 1, 2, \cdots, k-1$, $\sum_{i=1}^{b-1}\Delta_{[j],i} = 1$ since the treatment in the (b, j)th cell of the design must be from the first $b-1$ rows and the first $k-1$ columns. Thus $\sum_{j=1}^{k-1}\sum_{i=1}^{b-1}\Delta_{[j],i} = k-1 = (b-1)+(k-b)$. Therefore, $\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(\delta_{[i],j}+\Delta_{[j],i}) = 3(b-1)+(k-b)$.

It follows that

$$\mathrm{tr}(N_1'N_2N_2'N_1) = \sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(1+\delta_{[i],j}+\Delta_{[j],i})^2 + \sum_{i=1}^{b-1}(1+h_i)^2 + \sum_{j=1}^{k-1}(1+l_j+l_j')^2 + b^2.$$

By lemma 1, $\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(1+\delta_{[i],j}+\Delta_{[j],i})^2$ is minimized if the absolute difference between any two terms is 0 or 1. Since $(b-1)(k-1)-3(b-1)-(k-b) = (k-3)(b-2)-2 \geq 0$ when $k \geq 4$ and $b \geq 4$, $\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(1+\delta_{[i],j}+\Delta_{[j],i})^2$ is minimized when $3(b-1)+(k-b)$ and $(k-1)(b-1)-[3(b-1)+(k-b)]$ of $(\delta_{[i],j}+\Delta_{[j],i})$'s equal 1 and 0, respectively. Thus,

$$\begin{aligned}\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(1+\delta_{[i],j}+\Delta_{[j],i})^2 &\geq 3[3(b-1)+(k-b)]+(k-1)(b-1)\\ &= (b-1)(k+8)+3(k-b)\end{aligned}$$

It can be shown that

$$\sum_{i=1}^{b} s^2_{\mathrm{row},i} = \sum_{i=1}^{b-1}(2h_i+h_i'+k+2)^2+(2k+b-2)^2;$$

and

$$\sum_{j=1}^{k} s^2_{\mathrm{col},j} = \sum_{j=1}^{k-1}(2l_j+l_j'+\psi_{[j],\Omega}+b+1)^2+(3b-2)^2.$$

Let

$$\begin{aligned}\Psi &= b^2\mathrm{tr}(N_1'N_1N_1'N_1)+k^2\mathrm{tr}(N_2'N_2N_2'N_2)+2bk\mathrm{tr}(N_1'N_2N_2'N_1)\\ &- 2b\sum_{i=1}^{b} s^2_{\mathrm{row},i} - 2k\sum_{j=1}^{k} s^2_{\mathrm{col},j}.\end{aligned}$$

We have

$$\Psi = b^2\left[bk^2+2\sum_{i=1}^{b-1}(h_i+h_i'+1)^2+2\sum_{i=1}^{b-2}\sum_{j=i+1}^{b-1}(\lambda_{[i],j}+\lambda_{[j],i})^2\right]$$

$$
\begin{aligned}
&+ \quad k^2\left[b^2k + 2\sum_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 + 2\sum_{i=1}^{k-2}\sum_{j=i+1}^{k-1}(\mu_{[i],j}+\mu_{[j],i})^2\right] \\
&+ \quad 2bk\left[\sum_{i=1}^{b-1}\sum_{j=1}^{k-1}(1+\delta_{[i],j}+\Delta_{[j],i})^2 + \sum_{i=1}^{b-1}(1+h_i)^2 + \sum_{j=1}^{k-1}(1+l_j+l'_j)^2 + b^2\right] \\
&- \quad 2b\left[\sum_{i=1}^{b-1}(2h_i+h'_i+k+2)^2 + (2k+b-2)^2\right] \\
&- \quad 2k\left[\sum_{j=1}^{k-1}(2l_j+l'_j+\psi_{[j],\Omega}+b+1)^2 + (3b-2)^2\right] \\
&\geq \quad 2b^2\sum_{i=1}^{b-1}(h_i+h'_i)^2 - 2b\sum_{i=1}^{b-1}(2h_i+h'_i)^2 + 2bk\sum_{i=1}^{b-1}h_i^2 \\
&+ \quad 2k^2\sum_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 - 2k\sum_{j=1}^{k-1}(2l_j+l'_j+\psi_{[j],\Omega})^2 + 2bk\sum_{j=1}^{k-1}(l_j+l'_j)^2 \\
&+ \quad b^3k^2 + b^2k^3 + 2b^3k - 2b^2k^2 - 24b^2k - 8bk^2 + 2b^3 + 2k^3 \\
&+ \quad 28bk - 8k^2 - 16b^2 + 16b + 6k \\
&= \quad 2b(b-2)\sum_{i=1}^{b-1}(h_i+h'_i)^2 + 2b(k-2)\sum_{i=1}^{b-1}h_i^2 + 2b\sum_{i=1}^{b-1}{h'_i}^2 \\
&+ \quad 2k(k-2)\sum_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 + 2k(b-2)\sum_{j=1}^{k-1}(l_j+l'_j)^2 + 2k\sum_{j=1}^{k-1}(\psi_{[j],\Omega}-l'_j)^2 \\
&+ \quad b^3k^2 + b^2k^3 + 2b^3k - 2b^2k^2 - 24b^2k - 8bk^2 + 2b^3 + 2k^3 \\
&+ \quad 28bk - 8k^2 - 16b^2 + 16b + 6k
\end{aligned}
$$

and the equality holds when $\lambda_{[i],j}+\lambda_{[j],i} = 0$ or 1 for $i = 1, 2, \cdots, b-2$, $j = i+1, \cdots, b-1$; $\mu_{[i],j}+\mu_{[j],i} = 0$ or 1 for $i = 1, 2, \cdots, k-2$, $j = i+1, \cdots, k-1$; and $\delta_{[i],j}+\Delta_{[j],i} = 0$ or 1 for $i = 1, 2, \cdots, b-1$, $j = 1, \cdots, k-1$.

When $4 \leq b \leq k \leq 2b-1$, $\sum_{i=1}^{b-1} h_i^2 \geq \sum_{i=1}^{b-1} h_i = b-1$ and the equality holds when all h_i's are 1, i.e., treatments in the (i,k)th cells of the design are from different rows, where $i = 1,\ 2,\ \cdots,\ b-1$. $\sum_{i=1}^{b-1} {h'_i}^2 \geq \sum_{i=1}^{b-1} h'_i = k-b$ and the equality holds if $k-b$'s h'_i's are 1 and others are 0. That is, treatments appear in the bth row but not in the kth column are from different rows. Thus,

$$\sum_{i=1}^{b-1}(h_i+h_i')^2 \geq 4(k-b)+2b-1-k = 3k-2b-1.$$

Similarly, $\sum\limits_{j=1}^{k-1}(l_j+l_j')^2 \geq \sum\limits_{j=1}^{k-1}(l_j+l_j') \geq k-1$, and equality holds when treatments in the bth row come from different columns. By lemma 1, $\sum\limits_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2$ is minimized when the absolute difference between any two terms of $(l_j+\psi_{[j],\Omega})$'s is at most 1. For $4 \leq b \leq k \leq 2b-1$ the minimum of $\sum\limits_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2$ is attained when $2b-k-1$ of $(l_j+\psi_{[j],\Omega})$'s take 2 and $2(k-b)$ have 1. This can be achieved by arranging $k-b$ treatments from Ω in those columns that don't have treatments in the kth column of the first $b-1$ rows. Hence, $\sum\limits_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 \geq 6b-2k-4$. Moreover, $\sum\limits_{j=1}^{k-1}(\psi_{[j],\Omega}-l_j')^2 \geq \sum\limits_{j=1}^{k-1}(\psi_{[j],\Omega}-l_j') = 2b-k-1$.

Therefore,

$$\begin{aligned}\Psi &\geq b^3k^2+b^2k^3+2b^3k-2b^2k^2-16b^2k+6bk^2-2b^3-2k^3 \\ &- 6bk-16b^2-14k^2+24b+24k,\end{aligned}$$

and it follows that

$$\begin{aligned}b^2k^2\mathrm{tr}(C_d^2) &\geq b^3k^3+b^2k^3+3b^3k^2-17b^2k^2-6b^3k-4bk^3 \\ &+ 40b^2k+34bk^2-2b^3-2k^3-10k^2-50bk-24b+36.\end{aligned}$$

Lemma 2 is used for the proof of Theorem 2 and it is from Shivakumar and Chew (1974).

Lemma 2. For a matrix $A=(a_{ij})_{n\times n}$ of real numbers with weakly diagonal dominance, i.e., $|a_{ii}| \geq \sum_{j=1,j\neq i}^{n}|a_{ij}|$ for all $i \in N=\{1,2,\cdots,n\}$. Let $J=\left\{i \in N \,\middle|\, |a_{ii}| > \sum_{j=1,j\neq i}^{n}|a_{ij}|\right\}$. If J is not empty and for each $i \notin J$, there is a sequence of nonzero elements of A such that a_{ii_1}, $a_{i_1i_2}$, $\cdots$, a_{i_rj}, and $j \in J$, then A is nonsingular.

Proof of Theorem 2

The (M,S)-optimality of the designs is obtained by observing that equality in inequality (6) and equalities in all inequalities in the proof of Theorem 1 are attained with the special arrangement of treatments in the bth row and kth column of the described design.

To prove the treatment connectedness of the design, it is sufficient to show that the difference between any treatment and treatment $v = (b-1)(k-1)+1$ is estimable. Note that any non-replicated treatment resides in a rectangle where three vertices are repeated treatments and any tetra-difference of treatment effects from such rectangle is estimable. For example, treatment 2 is in the rectangle with vertices 2, $k+1$, $2k+1$, and $k+1$. Tetra-difference $\tau_{2k+1}+\tau_2-\tau_{k+1}-\tau_{k+1} = (\tau_{2k+1}-\tau_v)+(\tau_2-\tau_v)-2(\tau_{k+1}-\tau_v)$ is estimable because $\tau_{2k+1}+\tau_2-2\tau_{k+1} = E(Y_{2,k}+Y_{1,2}-Y_{1,k}-Y_{2,2})$. Thus, $(\tau_2-\tau_v)$ is estimable if $(\tau_{2k+1}-\tau_v)$ and $(\tau_{k+1}-\tau_v)$ are estimable. Therefore, the design is treatment-connected if all paired differences between repeated treatments and treatment v are estimable.

Consider the $b-1$ linear equations produced by tetra-differences involving one treatment in the kth column twice, one treatment in the bth row or the kth column, and treatment v. For example, the linear equation involving treatment 1 is $2(\tau_1-\tau_v) - (\tau_{(b-2)(k-1)+b}-\tau_v) = E(Y_{b-1,k}+Y_{b,b}-Y_{b-1,b}-Y_{b,k})$ and the linear equation involving treatment $k+1$ is $2(\tau_{k+1}-\tau_v)-(\tau_1-\tau_v) = E(Y_{b,1}+Y_{1,k}-Y_{1,1}-Y_{b,k})$, etc. The coefficients in these $b-1$ equations are diagonally dominant because each of $\tau_1-\tau_v$, $\tau_{k+1}-\tau_v$, $\cdots$, and $\tau_{(b-2)(k-1)+b-1}-\tau_v$ has coefficient two in one of the $b-1$ linear equations and the coefficient of the other difference in that equation is -1.

There are s linear equations from tetra-differences involving twice a treatment from the bth row but not the kth column of the design, and two treatments from the kth column. For example, linear equation $2(\tau_{(b-2)(k-1)+b}-\tau_v)-(\tau_1-\tau_v)-(\tau_{(b-2)(k-1)+b-1}-\tau_v) = E(Y_{b-1,b}+Y_{b,b-1}-Y_{b-1,b-1}-Y_{b,b})$ is derived from the tetra-difference with treatments $(b-2)(k-1)+b-1$, $(b-2)(k-1)+b$, 1, and $(b-2)(k-1)+b$. The coefficients in these s equations are weakly diagonally dominant because each of $\tau_{(b-2)(k-1)+b}-\tau_v$ $\cdots$, and $\tau_{(b-s)(k-1)}-\tau_v$ has coefficient two in one of the s linear equations which is equal to the sum of the absolute values of the coefficients of other differences in that equation. Therefore, the coefficient matrix of the total $b-1+s$ linear equations is weakly diagonally dominant. Since every linear equation produced by a tetra-difference involving twice a treatment in the bth row but not in the kth column has a treatment effect in the kth column with coefficient -1, by Lemma 2, the coefficient matrix is nonsingular. Therefore, all paired differences between repeated-treatment effects and τ_v are estimable.

Proof of Theorem 3

The (M,S)-optimality is obtained by observing that equalities in inequality (6) and all inequalities in the proof of Theorem 1 are attained with the special arrangement of treatments in the bth row and kth column of the design in Table 3.

To prove the treatment connectedness of the design for a specific b, it is sufficient to show that the difference between any treatment effect and the effect of the vth treatment, where $v = (b-1)^2+1$, is estimable. Note that any off-diagonal or non-repeated treatment resides in a rectangle where three vertex treatments appear in the kth column and the b row and any tetra-difference of treatment effects from such rectangle is estimable. For example, treatment 3 is in the rectangle with vertices 3, $2b+1$, $3b+1$, and $b+1$. The tetra-difference $\tau_{b+1}+\tau_{2b+1}-\tau_{3b+1}-\tau_2 = (\tau_{b+1}-\tau_v)+(\tau_{2b+1}-\tau_v)-(\tau_{3b+1}-\tau_v)-(\tau_3-\tau_v)$ is estimable because it is the expectation of $Y_{1,k}+Y_{3,3}-Y_{3,k}-Y_{1,3}$. Thus, $(\tau_3-\tau_v)$ is estimable if $(\tau_{b+1}-\tau_v)$, $(\tau_{2b+1}-\tau_v)$, and $(\tau_{3b+1}-\tau_v)$ are estimable.

Consider the $b-1$ linear equations consisting of all tetra-differences involving treatment v, and two other treatments in the bth row and bth column where one such treatment appears twice.

$$
\begin{aligned}
&2(\tau_1-\tau_v)-(\tau_{(b-1)^2}-\tau_v) = E(Y_{b,b-1}+Y_{b-1,b}-Y_{b,b}-Y_{b-1,b-1})\\
&2(\tau_{b+1}-\tau_v)-(\tau_1-\tau_v) = E(Y_{b,1}+Y_{1,b}-Y_{b,b}-Y_{1,1})\\
&\vdots\\
&2(\tau_{(b-1)^2}-\tau_v)-(\tau_{b(b-3)+1}-\tau_v) = E(Y_{b,b-2}+Y_{b-2,b}-Y_{b,b}-Y_{b-2,b-2})
\end{aligned}
$$

The coefficient matrix of these linear equations is strictly diagonally dominant (the diagonal is 2, one off-diagonal element is -1, and all others are zeros in each row) when $\tau_1-\tau_v$, $\tau_{b+1}-\tau_v$, $\cdots$, and $\tau_{(b-1)^2}-\tau_v$ are diagonal. The Geršgorin circle theorem implies that the coefficient matrix is nonsingular (Berman and Plemnons, 1994, p.106) and the linear equations have a unique solution. Therefore, any treatment effect difference between a repeated treatment and v is estimable.

Proof of Theorem 4

Following notations in the proof of Theorem 1, we have

$$
\begin{aligned}
\Psi \;\geq\;& 2b(b-2)\sum_{i=1}^{b-1}(h_i+h_i')^2 + 2b(k-2)\sum_{i=1}^{b-1}h_i^2 + 2b\sum_{i=1}^{b-1}h_i'^2\\
+\;& 2k(k-2)\sum_{j=1}^{k-1}(l_j+\psi_{[j],\Omega})^2 + 2k(b-2)\sum_{j=1}^{k-1}(l_j+l_j')^2 + 2k\sum_{j=1}^{k-1}(\psi_{[j],\Omega}-l_j')^2\\
+\;& b^3k^2+b^2k^3+2b^3k-2b^2k^2-24b^2k-8bk^2+2b^3+2k^3\\
+\;& 28bk-8k^2-16b^2+16b+6k
\end{aligned}
$$

Note that $\sum_{i=1}^{b-1} h_i^2 \geq \sum_{i=1}^{b-1} h_i = b-1$. The equality holds when all h_i's are 1, that is, treatments in the k columns are from different rows. When $t(b-1)+1 < k \leq (t+1)(b-1)+1$ and $t \geq 2$, by lemma 1 $\sum_{i=1}^{b-1} {h'_i}^2$ is minimized when $[k-b-(t-1)(b-1)]$ h'_i's take the value of t and $[t(b-1)-(k-b)]$ h'_i's are $t-1$. Thus

$$\sum_{i=1}^{b-1} {h'_i}^2 \geq [k-b-(t-1)(b-1)]t^2 + [t(b-1)-(k-b)](t-1)^2.$$

Therefore,

$$\sum_{i=1}^{b-1}(h_i + h'_i)^2 \geq [k-b-(t-1)(b-1)](t+1)^2 + [t(b-1)-(k-b)]t^2.$$

Similarly, $\sum_{j=1}^{k-1}(l_j + l'_j)^2 \geq \sum_{j=1}^{k-1}(l_j + l'_j) \geq k-1$, and equality holds when treatments in the bth row come from different columns. $\sum_{j=1}^{k-1}(l_j + \psi_{[j],\Omega})^2 \geq \sum_{j=1}^{k-1}(l_j + \psi_{[j],\Omega}) \geq 2(b-1)$ and equality holds when no pairs of the treatments in the k column appear in any columns. $\sum_{j=1}^{k-1}(\psi_{[j],\Omega} - l'_j)^2 \geq \sum_{j=1}^{k-1}(l'_j - \psi_{[j],\Omega}) = k-2b+1$ and equality holds when treatments in the kth column are arranged in those columns that contribute treatments to the bth row but not kth column.

Therefore,

$$\begin{aligned}\Psi &\geq b^3k^2 + b^2k^3 + 2b^3k - 2b^2k^2 - (20-4t)b^2k - 2bk^2 + (2-2t-2t^2)b^3 \\ &+ 2k^3 + (6-4t)bk - (20-4t^2)b^2 - 14k^2 + (24+2t-2t^2)b + 20k.\end{aligned}$$

It follows that

$$\begin{aligned}b^2k^2\mathrm{tr}(C_d^2) &\geq b^3k^3 + 3b^3k^2 + b^2k^3 - 6b^3k - 17b^2k^2 - 4bk^3 + (36+4t)b^2k \\ &+ 26bk^2 + (2-2t-2t^2)b^3 + 2k^3 - (38+4t)bk \\ &- (4-4t^2)b^2 - 10k^2 - (24-2t+2t^2)b - 4k + 36.\end{aligned}$$

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