**Search for the optimal two-phase experimental designs using simulated annealing algorithm**

**Structure of this chapter**

1. Introduction
   1. With a given set of design parameters, the treatment and animal allocations from the phase 1 experiments to the second phase experiments can be many. Animal is the experimental units.
   2. The aim is to find an allocation that is said to be *optimal*, then describe the different optimality criteria from John and Williams.
   3. Briefly introduce the simulated annealing algorithm to the reader, and it will be further discussed in detail in Section 2).
   4. Theoretical ANOVA table is used to study the properties of designs
      1. Can a valid test for the treatment differences be conducted?
      2. Can the variances of treatment effects be estimated?
      3. Degrees of freedom remain in the between animals stratum for conducting the test.
      4. Amount of treatment information remaining, based on the average efficiency factor computed from the harmonic means of the canonical efficiency factors. Best and worst treatment contrasts???
   5. Aim is to develop a method for generating the optimal two-phase experimental designs focusing on the multiplex proteomics experiments. This chapter describes in generating the two-phase optimal designs for the following design parameters:
      1. Phase 1 experiment – completely randomised designs with 2-8 treatments, 2-10 biological replicates and 2 technical replicates,
      2. Phase 2 experiment – randomised block designs with 4/8 tags.
      3. Introduce an example to be used throughout this chapter.
2. Objective function
   1. Since the aim of the animals and treatments allocations to runs and tags is to minimise the level of confounding between animal and treatment effects with both runs and tag effects. Instead, we can find the design that has the most animal and treatment information within the runs and tags stratum. Hence, the second phase design is in fact the row-column design.
   2. The objective function is the weighted sum of the average efficiency factors of animals and treatments within runs and tags stratum. Explain why using the A-optimal criterion instead of MS-optimal, i.e. do not bother about the whether the animal component of the design is disconnected or completely confounded (i.e. losing degrees of freedom) with runs. Example?
   3. In addition, to avoid the treatment to be disconnected to the runs and tags, addition criterion on monitoring the degrees of freedom of the treatment effects is included in the objective function. Example?
3. Describe the Simulated annealing in detail and how it will be used for this experiments
   1. Generating the starting design - discusses the 2-by-2 grouping for designing experiment of 2 technical replicates, try to illustrate it with the tables or matrices.
   2. Temperature control – applied accelerated cooling method described by John and Whitaker (1993).
   3. Swapping method
      1. Discuss the swapping of treatment/animal pairs for the experiments with 2 technical replicates
      2. Two-stage swapping described by Williams and John (1996) was also applied by swapping the observations restricting to runs and tags alternatively. This will allow the reduction of the search space while applying the simulated annealing.
   4. How the simulated annealing algorithm described here is implemented by the optim function in R. Present the Pseudo Code and a **step-by-step** process of finding the optimal design.
4. Illustrate some examples and table of results
   1. Show few examples with different set of design parameters with their theoretical ANOVA tables,
      1. 6 treatment 3 biological replicates and 4-plex experiments to show how the simulated annealing algorithm described here improves the design.
      2. Another set of design parameters where simulated annealing cannot improve the starting designs.
      3. Another design parameter with 8-plex experiments
   2. Results – table
5. Conclusion

Print out the designs layout in the appendix.

**1. Introduction**

Studying different two-phase experiments can take a long time. This is because for a given experiment with a number of treatments, blocks and replicates, there can be many different combinations of treatments and blocks. This set of treatments, blocks and replicates is also known as the design parameters. With a given set of the design parameters, we should be able to generate at least one design that is optimal with respect to some statistical criteria. This design is known as the optimal design. There are many optimal criteria have been defined, as described by John and Williams (1998). The chapter focused only on the MS- and A-optimal criteria, which is further discussed in Section 2.

The method discussed in this chapter of generating the optimal designs is *simulated annealing algorithm*. The simulated annealing algorithm was introduced in the case of optimisation in the statistics field by Kirkpartick et al (1983) which is a good method to solve the global optimization problem. Here, the simulated annealing algorithm is used to search for the optimal design which maximised the objective function that is defined in Section 2. The starting designs and the swapping method are defined for the example of two-phase experiment is defined in Section 3. The simulated annealing is implemented by the optim function in R program.

The designs that generated from the simulated annealing algorithm are further investigated by studying their theoretical ANOVA tables, which is has the same structure of the regular ANOVA table expected is consists of the expected mean squares (EMS) instead of the mean square calculated from the experimental data. From studying the theoretical ANOVA table, we can determine whether the formal test for the treatment differences to be conducted. This can be confirmed by the theoretical ANOVA table, where the coefficients of the variance components of the treatment expected mean squares is identical to the residual expected mean squares. In addition, the theoretical ANOVA table allows us to check whether the variance of the treatment effects can be estimated from the residual EMS in the same stratum as the treatment EMS. We can also check for degrees of freedom (DF) associated with the treatment effects remains in the stratum for conducting the test, because some designs can cause confounding of the treatment with other random effects. The inforDecompuTE R package is used to produce the theoretical ANOVA table. The ANOVA table produced by inforDecompuTE R package also provides the average efficiency factor of the treatment effects which is computed by the harmonic means of the canonical efficiency factors.

The aim is to develop a method for generating the optimal two-phase experimental designs focusing on the multiplex proteomics experiments. This chapter describes in generating the two-phase optimal designs for the following design parameters:

* + 1. Phase 1 experiment – completely randomised designs with 2-8 treatments, 2-10 biological replicates and 2 technical replicates,
    2. Phase 2 experiment – randomised block designs with 4/8 tags and the number of runs, , depends on the total number of observations.

For the layout of this chapter, the construction of the objective function is explained in Section 2. Section 3 discusses two components of the simulated annealing algorithm, which are the new initial design and the modified swapping methods and show how these two components can improve the search for the optimal designs. Section 4 describes two examples using the objective function and simulated annealing algorithm. Many experiment examples are introduced throughout this chapter to aid in the explanation of how the objective function and simulated annealing algorithm are improved for searching the optimal two-phase experimental designs.

**2. The Objective Function**

The objective function plays an important role in finding the optimal designs using the simulated annealing algorithm. A well-chosen objective function will allow us to find a design that is able to conduct a valid test for the treatment differences.

The main differences between finding the optimal two-phase experiment design to find a general optimal design is that we need to allocate the random factors from the first phase experiment to the random factors from the second phase experiment.

This section considers three issues:

1. Compare MS and A-optimal designs based on the animal effects

2. Add the treatment effects into the A-optimal design using the weighted sum and how much weight

3. Add another component which monitors the degrees of freedom associated with the treatment effects.

The construction of the objective function that is applied here is shown, based on these three points, for finding the optimal two-phase designs.

**2.1 MS and A-optimality criteria**

Finding an MS-optimal design is a two-stage process. The first stage is to find the design with the highest trace of the information matrix. The second stage is to find the design with the lowest trace of the square of the information matrix out of the designs that were found in the first stage. This method is fast and easier to implement, because this method does not require computing of the eigenvalues. The objective function of finding the MS-optimal design is by adding the trace of the information matrix to the inverse of the trace of the square of information matrix.

The A-optimal design is the design with the highest average efficiency factor. The objective function of finding the A-optimal design is first compute the canonical efficiency factors from the eigenvalues divided by the replication number. The average efficiency factor is then calculated by the harmonic mean of the canonical efficiency factors.

**2.2 The information matrix**

It is important to define the information matrix, because it is where the optimality criteria are computed. This is also the initial step of the constructing the objective function.

For the case of iTRAQ experiments, the aim of the animals and treatments allocations to runs and tags is to minimise the level of confounding between animal and treatment effects with both runs and tag effects. Instead, we can find the design that has the most animal and treatment information within the runs and tags stratum. Hence, the second phase design can be consider as the row-column design. The orthogonal projector for the within runs and tags stratum can be written as

where and denotes the total number of runs and tags are used in the second phase experiment. The information matrix of animal within runs and tags stratum can be written as

where denotes the animal design matrix. For this animal design matrix, the rows correspond to the observations of the Phase 2 experiment and the columns correspond to the animals.

The information matrix of the treatments in the within runs and tags stratum can be written as

where denotes the treatment design matrix. The rows of this treatment design matrix correspond to the observations of the Phase 2 experiments and the columns corresponds the levels of the treatments.

Both the MS and A-optimal criteria can be tested from the information matrix defined here. With the orthogonal projector for the within runs and tags stratum stays the same, the aim is find the matrices and that generates the optimal design.

**2.3 Compare MS and A-optimal designs based on the animal effects**

The first comparison to constructing the objective is to decide whether the MS or A-optimal criterion is used for the assignments of animals of Phase 1 experiment to the runs and tags of Phase 2 experiment. For the purpose of comparing these two criteria, only the maximisation of the animal information is considered for this part.

The MS and A-optimal designs are compared using an experiment with following design parameters:

Phase 1 experiment - 2 treatments, 3 biological replicates,

Phase 2 experiment – 2 technical replicates, 3 runs and 4 tags.

Animals A, C and E are assigned to Treatment 1 and animals B, D and F are assigned to Treatment 2. Since there are 2 technical replicates, which mean there are total of 12 samples to be measured in the second phase experiment. Using the 4-plex experiment, three runs are required.

The theoretical ANOVA for this first phase experiment is as follows,

$ANOVA

DF Ani

Between Ani

Trt 1 1

Residual 4 1

$EF

Trt eff.Trt

Between Ani

Trt 3 1

All the treatment information is in the between animals stratum.

Using MS-optimality criterion in the objective function, the animal allocation of this design is shown as follows

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | E | F | C | D |
| 2 | B | C | A | E |
| 3 | A | D | F | B |

The animals are confounded with both runs and tags. The incidence matrix of animals and runs is as follow,

Run

Ani 1 2 3

A 0 1 1

B 0 1 1

C 1 1 0

D 1 0 1

E 1 1 0

F 1 0 1

which shows that this design is a binary design, where no treatments, in this case animals, occurs more than once in any block, in this case run. The concurrence matrix of animals and runs is

Ani

Ani A B C D E F

A 2 2 1 1 1 1

B 2 2 1 1 1 1

C 1 1 2 1 2 1

D 1 1 1 2 1 2

E 1 1 2 1 2 1

F 1 1 1 2 1 2

From this concurrence matrix, we cannot identify any grouping for the animals. Hence, the design of assigning the animals to the runs is connected. This design generated 5 canonical efficiency factors, of animals in the in the within runs stratum, which are 1, 1, 1, 0.75 and 0.75. The average efficiency factor is 0.8823529. This means all 5 DF for animals are all in the within runs stratum for this design, which means this design of assigning animals to runs is connected. However, 2 out of 5 DF for animal only have 0.75 of the information in the within runs stratum, this means 2 out 5 DF for animals have 0.25 of information in the between runs stratum.

The treatment allocation to runs and tags follows the assignment of treatments to the animals in the Phase 1 experiments which is shown as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 1 | 2 | 1 | 2 |
| 2 | 2 | 1 | 1 | 1 |
| 3 | 1 | 2 | 2 | 2 |

The theoretical ANOVA table that is generated from this design is as follows

DF e Ani Run

Between Run

Between Ani

Trt 1 1 1/2 4

Residual 1 1 1/2 4

Within

Between Ani

Tag 2 1 9/5 0

Trt 1 1 811/490 0

Residual 2 1 367/196 0

Residual

Tag 3 1 0 0

Residual 1 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Trt 1 1/6

Within

Between Ani

Tag 3/2 26/15 1/2 13/45

Trt 49/15 49/90

Residual

Tag 27/19 9/19

The theoretical ANOVA table shows that this design does not provide a valid F-test for the treatment differences, because the coefficients of between animals variance components, , are not identical. A valid F-test can be conducted by adjusted the coefficients of between animals variance components from the linear combination of the residual in the within animals within runs stratum. The DF for the newly estimated EMS is approximated using the mean squares from the experimental results. Hence, the variances of the treatment effects cannot be estimated directly for this design. This design is connected design where all five DF associated with the animal effects are in the within runs stratum. However, two of five DF have 0.75 of animal information in the within runs stratum. In additional, treatment is also confounded with tag, from the theoretical ANOVA table, there is 0.5444 of pure treatment information.

Using A-optimality criterion in the objective function, the animal allocation for this design is shown below

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | C | F | F | C |
| 2 | E | A | B | D |
| 3 | D | B | A | E |

Two groups of animals can be observed different set of runs can be observed. Run 1 comprised of Animals C and F. Run 2 and 3 comprised of Animals A, B, D and E.

The incidence matrix of animal and run is

Run

Ani 1 2 3

A 0 1 1

B 0 1 1

C 2 0 0

D 0 1 1

E 0 1 1

F 2 0 0

This matrix shows that this design is a non-binary design. The binary design is a design where no treatment, in this case animal, occurs more than once in any block, or in this case runs. The non-binary design can be observed for Animal A, B, D and E. The binary design can be observed for Animal C and F. Hence, animals can be split into two groups, which mean this design is disconnected.

The disconnectedness can also be observed in the concurrence matrix of animals and run which is

Ani

Ani A B C D E F

A 2 2 0 2 2 0

B 2 2 0 2 2 0

C 0 0 4 0 0 4

D 2 2 0 2 2 0

E 2 2 0 2 2 0

F 0 0 4 0 0 4

where the grouping of Animals A, B, D and E and Animals C and F can be seen.

This design generated 4 canonical efficiency factors all unity, for the animals in the within runs stratum. The average efficiency factor is also 1. This also means 1 out of 5 DF for animals are in the between runs stratum.

The treatment allocation to runs and tags follows the assignment of treatments to the animals in the Phase 1 experiments which is shown as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 1 | 2 | 2 | 1 |
| 2 | 1 | 1 | 2 | 2 |
| 3 | 2 | 2 | 1 | 2 |

The theoretical ANOVA table that is generated from this design is as follows

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 2 4

Residual 1 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 1 1 2 0

Residual 2 1 2 0

Residual

Tag 2 1 0 0

Residual 3 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Tag 3 2/3 1 1/9

Trt 16/3 8/9

Residual

Tag 3 1

The theoretical ANOVA table shows that this design provides a valid F-test for the treatment differences, because both the treatment and residual EMS, in the between animals within runs stratum, contains variance components of . The variances of the treatment effects can also be estimated directly from the residual mean squares divided by the treatment replication number. However, the treatment effects are still confounded with tag effects, but this shows that there is still 88.89% of pure treatment information remaining. In addition, this design is disconnected, because 1 DF of animals are in the between runs stratum which leaves 4 DF of animals in the with runs stratum.

Therefore, despite the MS-optimal design is connected, for the purpose of conducting an experiment and testing for the treatment differences, the A-optimal design is more preferable. In addition, the variance component estimates of the animals in the between runs stratum can be recovered using the restricted maximum likelihood method.

**2.4 Add the treatment effects into the A-optimal using the weighted sum and how much weight**

The objective function is to find A-optimal design instead of the MS-optimal design. This means that the current objective function for the simulated annealing method is to find the design with the highest average efficiency factor when allocating the animals of Phase 1 experiment to the runs and tags of the Phase 2 experiments. The next step is to also maximise the treatment information in the within runs and tags stratum similar to the animals.

The treatment component can be add into the objective function using the weighted sum of the average efficiency factor of animals and treatment in the within runs and tags stratum. Thus, the design criterion is to maximise the average efficiency factor, the aim is to find and that can compute the highest the average efficiency factor from both the information matrix. Note the order of matrix will affect the order of matrix. These two average efficiency factors can be combined as

,

where and are the average efficiency factors of treatments and animals, and are weights of treatments and animals for calculating the combined average efficiency factors.

To comparing different weights, an experiment with following design parameter is used:

Phase 1 experiment - 2 treatments, 2 biological replicates,

Phase 2 experiment – 3 technical replicates, 3 runs and 4 tags.

Animals A and C are assigned to Treatment 1 and animals B and D are assigned to Treatment 2. Since there are 3 technical replicates, which mean there are total of 12 samples to be measured in the second phase experiment. Using the 4-plex experiment, three runs are required. based

The theoretical ANOVA table for the first phase experiment is as follows,

$ANOVA

DF Ani

Between Ani

Trt 1 1

Residual 2 1

$EF

Trt eff.Trt

Between Ani

Trt 2 1

The objective function is set to have same weight for both average efficiency factor of animals and treatments in the within run and tags stratum. Using this objective function, the following animal allocation for this design is generated

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | B | A | C | D |
| 2 | C | D | A | B |
| 3 | A | B | D | C |

All three runs contain each of four animals; however, all four tags only have three of four animals.

The incidence matrix of animals and runs is

Run

Ani 1 2 3

A 1 1 1

B 1 1 1

C 1 1 1

D 1 1 1

which shows the assignment of animals to runs is binary, since all elements of matrix are one. This also indicates the assignment of animals to runs is complete block design where each run has each of four animals.

The incidence matrix of animals and tag is

Tag

Ani 1 2 3 4

AA 1 1 1 0

AB 1 1 0 1

AC 1 0 1 1

AD 0 1 1 1

which also shows the assignment of animals to tags is binary, since all elements of matrix are either zero or one. The concurrence matrix of animal and tag as follows

Ani

Ani A B C D

A 3 2 2 2

B 2 3 2 2

C 2 2 3 2

D 2 2 2 3

which shows that any pair of animal occur together in exactly 2 runs. This indicates the assignment of animals to tags is balance incomplete block design, which means all 3 DF associated with tag effects is confounded with the all 3 DF of animals.

The amount of un-confounded tag information can be calculated as follows,

The treatment allocation is based on the phase 1 experiment

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 2 | 1 | 1 | 2 |
| 2 | 1 | 2 | 1 | 2 |
| 3 | 1 | 2 | 2 | 1 |

The canonical efficiency factors of the animal in the within runs stratum are 1, 1 and 1; hence the average efficiency factor of animals in the within runs is also one.

The theoretical ANOVA table is as follows

$ANOVA

DF e Ani Run

Between Run 2 1 0 4

Within

Between Ani

Tag 3 1 3 0

Residual

Tag 3 1 0 0

Residual 3 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Within

Between Ani

Tag 1/3 6 1/9 1

Residual

Tag 8/3 8/9

From the fixed effects table, 1/9 of the tags information for all 3 DF is in the between animals within runs, which means 8/9 of the tags information for all 3 DF is in the within animals within runs stratum. From the random effects table, all three DF associated with tag effects is confounded with 3 DF of between animals within runs stratum. Therefore, the formal test for the treatment differences cannot be conducted. In addition, one of three DF associated with the tag effects is confounded with the treatment effects.

The objective function is set the weight for average efficiency factor of animals to be 0.75 and the weight for average efficiency factor of treatment to be 0.25.The animal allocation for this design is shown below

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | C | A | D | B |
| 2 | A | B | D | C |
| 3 | B | C | D | A |

All three runs again contain each of four animals. Tag 114, 115 and 117 contain animals A, B and C and Tag 116 contains Animal D. In fact, 3-by-3 section of Run 1, 2 and 3 and Tag 114, 115 and 117 is a Latin square design of Animal A, B and C.

The incidence matrix of animals and runs is

Run

Ani 1 2 3

AA 1 1 1

AB 1 1 1

AC 1 1 1

AD 1 1 1

which also shows the assignment of animals to runs is binary, since all elements of matrix are one. This also indicates the assignment of animals to runs is complete block design where each run has each of four animals which is same as the previous design. However, the incidence matrix of animals and tag is

Tag

Ani 1 2 3 4

AA 1 1 0 1

AB 1 1 0 1

AC 1 1 0 1

AD 0 0 3 0

which is not a binary design anymore, since Animal D only appears in one of four tags. The concurrence matrix of animals and tag is

Ani

Ani AA AB AC AD

AA 3 3 3 0

AB 3 3 3 0

AC 3 3 3 0

AD 0 0 0 9

which confirms the grouping of Animals A, B and C versus Animal D; hence, the allocation of animals to tags is disconnected.

The treatment allocation is based on the phase 1 experiment is follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 1 | 1 | 2 | 2 |
| 2 | 1 | 2 | 2 | 1 |
| 3 | 2 | 1 | 2 | 1 |

Note that Tag 116 contains only Treatment 2.

The theoretical ANOVA table is as follows

$ANOVA

DF e Ani Run

Between Run 2 1 0 4

Within

Between Ani

Tag 1 1 3 0

Trt 1 1 3 0

Residual 1 1 3 0

Residual

Tag 2 1 0 0

Residual 4 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Within

Between Ani

Tag 3 2 1 1/3

Trt 4 2/3

Residual

Tag 3 1

From the random effects table, the valid test for the treatment differences can be conducted since the coefficients of the between animal variance components for the treatment and residual mean squares in between animals stratum is identical. In addition, only one DF associated with the tag effects is in the between animals within runs stratum. From the fixed effects table, the tag is confounded with 1/3 of the treatment information, this means there are still 2/3 of pure treatment information remains for conducting the test for treatment difference.

In summary, the weighted sum of the average efficiency factors of treatments and animals is follows

,

If the weights for both are identical, the results from objective function of the first design is

0.5 \* 8/9 + 0.5 \* 8/9 = 8/9.

However, for the second design, results from objective function becomes

0.5 \* 1 + 0.5 \* 2/3 = 5/6,

which is lower than the previous design, even though the second design is more preferable. The objective function is then set the weight for average efficiency factor of animals to be 0.75 and the weight for average efficiency factor of treatment to be 0.25. The results from objective function of the first design is still

0.75 \* 8/9 + 0.25 \* 8/9 = 8/9

However, for the second design, results from objective function becomes

0.75 \* 1 + 0.25 \* 2/3 = 11/12,

which is higher than the previous design. Thus, the second design can only be generated by having to be 0.75 and to be 0.25 for the objective function. What this objective function does is to put more emphasis on the average efficiency factor for assigning the animals to runs and tags to be close to 1 as much as possible while it maximises f both the animal and treatment information in the within runs and tags stratum.

**2.5 Add another component which monitors the DF associated with the treatment effects**

The current objective function gives a weighted sum of the average efficiency factors of animals and treatments. However, for an experiment consisting of more than 2 treatments, there will be more than 1 DF associated with the treatment effects in some stratum of the ANOVA table.

Using an example with 3 treatments; hence, there will be 2 DF associated with the treatment effects.

Phase 1 experiment - 3 treatments, 2 biological replicates, 2 technical replicates,

Phase 2 experiment – 3 runs and 4 tags.

Animals A and D are assigned to Treatment 1,animals B and E are assigned to Treatment 2 and animals C and F are assigned to treatment 3. Since there are 2 technical replicates, which mean there are total of 12 samples to be measured in the second phase experiment. Using the 4-plex experiment, three runs are required.

The Phase 1 theoretical ANOVA table is as follows

$ANOVA

DF Ani

Between Ani

Trt 2 1

Residual 3 1

$EF

Trt eff.Trt

Between Ani

Trt 2 1

Using the objective function mentioned with the weighted sum but does not monitor the DF of the treatment effects, the allocation of animals to runs and tags is found as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | B | B | D | D |
| 2 | C | C | F | F |
| 3 | A | A | E | E |

Three groups of animals to runs can be observed, where Run 1 contains Animals B and D, Run 2 contains Animals C and F and Run 3 contain Animal A and E. Two groups of animals to tags can also be observed, where Tag 114 and 115 contains only Animals A, B and C and Tag 116 and 117 contain Animals D, E and F.

The treatment allocation is based on the Phase 1 experiment.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 2 | 2 | 1 | 1 |
| 2 | 3 | 3 | 3 | 3 |
| 3 | 1 | 1 | 2 | 2 |

Two groups of treatments to runs Run 1 and 3 contain Treatment 1 and 2 and Run 2 has only treatment 3. All four tags have one of each 3 treatments.

The concurrence matrix of treatment to runs

Trt

Trt 1 2 3

1 8 8 0

2 8 8 0

3 0 0 16

which confirms the two groups of treatments.

The theoretical ANOVA table is as follows

$ANOVA

DF e Ani Run

Between Run

Between Ani

Trt 1 1 2 4

Residual 1 1 2 4

Within

Between Ani

Tag 1 1 2 0

Trt 1 1 2 0

Residual 1 1 2 0

Residual

Tag 2 1 0 0

Residual 4 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Trt 4 1

Within

Between Ani

Tag 3 1

Trt 4 1

Residual

Tag 3 1

From the random effects table, the valid test for the treatment differences can be conducted. However, the test is only based on one DF associated with the treatment effects in the between animals within runs stratum. Another one DF associated with the treatment effects is in the between animals between runs stratum. The treatment information for each of these two DF is 100%.

This means the given design found from the current objective function is disconnected in the assignment of treatments to runs. To avoid generating such the disconnected designs when searching for the optimal design using the simulated annealing algorithm, an additional component of the objective function is to add another term that monitors the DF of the treatment effects. Therefore, the modified objective function gives a weighted sum of the average efficiency factors of animals and treatments and the proportion of the DF of treatment of current design found during the simulated annealing algorithm to the total DF of treatment, i.e.

The weights for the average efficiency factors of animals and treatments, i.e. and , are set to 2/3 and 1/9, respectively, and the weight for the proportion of treatment DF, i.e. , is set to 2/9.

Using the newly modified objective function, the following design was found. The allocation of animals to runs and tag can be shown as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | C | C | D | D |
| 2 | B | A | E | F |
| 3 | A | B | F | E |

The allocation of animals to runs can be divided into two groups, where Run 1 contains Animals C and D. Run 2 and 3 contains Animal A, B, E and F.

Treatment allocation is again based on the Phase 1 experiment,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | 3 | 3 | 1 | 1 |
| 2 | 2 | 1 | 2 | 3 |
| 3 | 1 | 2 | 3 | 2 |

The concurrence matrix of treatment to runs

Trt

Trt 1 2 3

1 6 4 6

2 4 8 4

3 6 4 6

There is no obvious grouping of the treatment from observing this concurrence matrix, which suggests that the allocation of the treatments to runs may not be disconnected.

A different theoretical ANOVA table is shown as follows,

$ANOVA

DF e Ani Run

Between Run

Between Ani

Trt 1 1 2 4

Residual 1 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 2 1 2 0

Residual 1 1 2 0

Residual

Tag 2 1 0 0

Residual 3 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Trt 1 1/4

Residual

Within

Between Ani

Tag 3 1

Trt 24/7 6/7

Residual

Tag 3 1

From the random effects table, all 2 DF of treatment effects is in the between animals within runs stratum for conducting the test of the treatment group differences. However, one of DF of treatment effects has its 1/4 of information in the between animals between runs which remains 3/4 of the treatment information. The canonical efficiency factors for 2 DF are 1 and 3/4; hence the average efficiency factor which is the harmonic mean of the canonical efficiency factors gives 6/7.

The main idea of this objective function is to allow the animal to be disconnected to Runs and tags; hence the valid test for the treatment differences in the between animals within runs stratum can be conducted. In the meantime, this objective function will also preventing the treatment effects to be disconnected as much as possible, as most of the treatment information remains in the within runs stratum.

**3. Simulated annealing algorithm section**

Based on the objective function defined in the previous section, I need to address the each of following issues associated with the simulated annealing algorithm,

* The modified starting design versus any random starting design.
* The accelerated cooling method versus standard cooling method.
* Pair swapping method versus one-to-one swapping method.
* Two-stages swapping method versus standard swapping method.

These four issues are explored using a two-phase experiment comprised with one specific set of design parameters:

Phase 1 experiment - 6 treatments, 3 biological replicates, 2 technical replicates,

Phase 2 experiment – 9 runs and 4 tags.

For the Phase 1 experiment, the 6 treatments are denoted by “a”, “b”, “c”, “d”, “e” and “f”. Since 3 biological replicates are used, this means 3 animals are assigned to each treatment which gives a total of 15 animals. These 15 animals are denoted by upper case letters of “A” to “R”. The theoretical ANOVA of the Phase 1 experiment can be presented as follows,

$ANOVA

DF Ani

Between Ani

Trt 5 1

Residual 12 1

$EF

Trt eff.Trt

Between Ani

Trt 3 1

Since the animals is the observational and experimental units, all the information is in the between animals stratum for this first phase experiments. In the random effects table, there are 5 degrees of freedom (DF) associated with the treatment effects; hence, there are 12 DF remains associated with the residual mean squares in the between animals stratum. In addition, all treatment information is in the between animals stratum as shown in the fixed effects table.

**3.1 Comparing the modified starting design to a random starting design.**

The modified starting design for assigning the animals to the runs and tags is to group a pair of animals of the identical technical replicates and allocating them in a sector of 2 runs and 2 tags. For this experiment, since the total number of runs needed is 9; hence, the last pair of animals is assigned to the last run. The pair of animas can be Animals “A” and “B”, Animals “C” and “D” to Animals “Q” and “R”. The allocation of the animals to runs and tags can be shown as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | A | B | C | D |
| 2 | B | A | D | C |
| 3 | E | F | G | H |
| 4 | F | E | H | G |
| 5 | I | J | K | L |
| 6 | J | I | L | K |
| 7 | M | N | O | P |
| 8 | N | M | P | O |
| 9 | Q | Q | R | R |

The bold box in this animal allocation represents the pair of the animals. Note that the animal is confounded with both runs and tags. More specifically, the animal is confounded with a tag contrast of 114, 115 versus 116, 117. For the relationship between runs and animals, the runs can be separated into 5 groups according to the pairs of animals that are assigned. This means 4 DF associated with the animals are confounded with the runs, or we can also say that 4DF associated with the animals should be in the between runs stratum.

The treatment allocation to runs and tags is based on the assignments of treatments to animals of the Phase 1 experiments. The treatment design is shown as follows,

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Run | Tag | | | |
| 114 | 115 | 116 | 117 |
| 1 | a | b | c | d |
| 2 | b | a | d | c |
| 3 | e | f | a | b |
| 4 | f | e | b | a |
| 5 | c | d | e | f |
| 6 | d | c | f | e |
| 7 | a | b | c | d |
| 8 | b | a | d | c |
| 9 | e | e | f | f |

The bold box in this treatment allocation represents each pair of treatments from the pair of the animals. The treatment is also confounded with both runs and tags. The treatment is also confounded with the confounded with a tag contrast of 114, 115 versus 116, 117.

The initial temperature is set at 100, this means the initial search for the optimal designs allows the values from the objective function to be worse than 100.

The simulated annealing algorithm was implemented using the optima function in R program for one million iterations. There is another parameter called tmax, which is the number of iteration at each temperature, this is set at 1000. This search takes just over nine minutes to complete.

After applying the simulated annealing using the modified starting design to start the search, this is shown that simulated annealing could not improve the designs since the result from the objective function was already at very high value of 97.62.

Using a random starting design, the simulated annealing algorithm does improve the design based on the objective function from 51.56 to 76.272. Note this value is still lower than using than objective function from the modified starting design.

**3.2 Comparing the accelerated cooling method described by John and Whitaker (1993) to standard cooling method.**

John and Whitaker (1993) mentioned that the convergence of the standard simulated annealing algorithm can be very slow and the solution may be far from optimal. This issue can be resolve from modifying the cooling schedule.

The cooling schedule of the current standard simulated annealing base on the optim function in R is

where temp is the initial temperature, t is the current iteration step and tmax is the number of iteration at each temperature. The operator “\” denotes the integer division, i.e. division removing the remainders. With initial temperature of 100 and tmax equals to 1000, after one million iterations the temperature has reduced to 7.239. This final temperature may still be too high to find the optimal design.

The modified revision of the cooling schedule separates the one million iterations into 10 levels of one hundred thousand iterations. At the first level, the initial temperature and tmax are still 100 and 1000, respectively; but the number of iterations is reduced to one hundred thousand iterations. Then, at the next level, the initial temperature is reduced by an half giving 50. The solution from the simulated annealing of the previous level is used as the starting design to start the search. The tmax and the number of iteration are remained the same of 100 and one hundred thousand. This process repeats again with initial temperature reduced by a half giving 25, 12.5 and till the tenth level of the simulated annealing algorithm is performed. The initial temperature of the tenth level is reduced to 0.1953125 and the final one hundred thousand iterations, the temperature is reduced to 0.01697941. Note that total number of the iteration is still one million, therefore the time required to complete this simulated annealing algorithm remain the same.

This approach to simulated annealing produce good solution by reducing the temperature quickly across the levels, but it also carries out the standard simulated annealing at each temperature level. The cooling schedule is known as *accelerated cooling* and the modified simulated annealing is also known as *nested simulated annealing*.

The nested simulated annealing starts a random walk across a surface with a high temperature to diversify the search. The accelerated cooling allows the search to intensify as it becomes a more local search. Therefore, gradually the random walks become more confined following the contours of the surface, with more restriction on accepting the worse designs.

Using the experiment, the accelerated cooling method again made no improvement on the modified starting design as the result form the objective function stays at 97.62. However, it does improve the result from the objective function with a random starting design with the result from the objective function of 78.647 after one million iterations. This is also shown to be better than the standard simulated annealing algorithm which obtains the design with objective function of 76.271.

**3.3 Comparing the pair swapping method to the one-to-one swapping method.**

With the modified starting design, both standard simulated annealing and nested simulated annealing could not improve the design.

The swapping method will need to modify to find the optimal design quickly and efficiently. The current swapping method is to swap any random pair of observations throughout the design. Note the modified starting design of the experiments with 2 technical replicates is to group a pair of animals and treatments and assigned them to a sector comprising 2 runs and 2 tags. Hence, the new swapping method is to swap any two random pairs of animals and treatments of the identical technical replicates.

With the pair swapping method, the optimal design, with the results of 98.189 from the objective function, has found fewer than ten thousand iterations. The nested simulated annealing was not required, because the optimal design was found within the first level of one hundred thousand iterations even with the initial temperature of 100.

**3.4 Comparing the two-stages swapping method to the standard swapping method.**

Williams and John (1996) described a two-stage swapping method for finding the optimal row-column design via simulated annealing. For this experiment, the runs and tags are considered as the rows and columns, respectively. In the first stage, the swapping only take place within runs, that means when the swapping of two observations, it has to be in the same run. The second stage is swapping within tags, which means when the two observations are swapped, these two observations have to be in the same tags. This method is attempted to reduce the search space of the simulated annealing algorithm; hence, it has ability to find a better designs more quickly.

The accelerated cooling and pair swapping methods were combined with the two-stage swapping method. Using same the accelerated cooling as described, it is achieved by separating the one million iterations into 10 levels of one hundred thousand iterations. To incorporate the two-stage swapping method, each level of one hundred thousand iterations are further separated for each of two stages; hence, each stage consists of fifty thousand iterations.

For this experiment, the pair swapping with two-stages swapping method on the modified starting design shown to be slower than the using pair swapping method alone. This is because this experiment with modified design is very easy to find the optimal design. The pair swapping method was able to find an optimal design required fewer than ten thousand iterations. As for the two-stage swapping, the optimal design could not be found within the first stage, but it only required another ten thousand iterations in the second stage to find the optimal design. Hence, the optimal designs can still be generated within sixty thousand iterations. Same using the pair swapping alone, the nested simulated annealing was not required, because the optimal design was found within the first level of one hundred thousand iterations even with the initial temperature of 100.

This may raise a question that the two-stage swapping method may not be useful, because it has no evidence on improving the design during the search. Thus, the accelerated cooling with two-stage swapping method was compared to the standard swapping method on the random starting design. After one million iterations, the design with result from the objective function of 84.97 was found which is the better design compare to using standard simulated annealing (76.27) and accelerated cooling method (78.64). Therefore, it shows that two-stage swapping method can generate a more optimal design than the standard swapping method based on the results from the objective function.

In conclusion, based on these results, I believe that using the modified starting design with accelerate cooling, pair swapping and two-stage swapping will allow user to find the optimal design more quickly and efficiently. The table of summary for comparing different method in simulated annealing is shown as follows.

**Summary of table for comparing different method in simulated annealing**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Methods | Modified Starting design | Accelerated cooling method | Pair swapping | Two-stage swapping | Objective function | | Iteration |
| Before | After |
| SA |  |  |  |  | 51.555453 | 76.271591 | 1e6 |
| SA on modified starting design | ˅ |  |  |  | 97.617991 | 97.617991 | 1e6 |
| Accelerated cooling method |  | ˅ |  |  | 51.555453 | 78.647182 | 1e6 |
| Accelerated cooling method on modified design | ˅ | ˅ |  |  | 97.617991 | 97.617991 | 1e6  ((10)1e5) |
| Pair swapping method with accelerated cooling method on modified starting design | ˅ | - | ˅ |  | 97.617991 | 98.189248 | 1e4 |
| Two-stages swapping method with Pair swapping method with accelerated cooling method on modified starting design | ˅ | - | ˅ | ˅ | 97.617991 | 98.189248 | 6e4 |
| Two-stages swapping method |  |  |  | ˅ | 51.555453 | 71.125898 | 1e6 (5e5) |
| Two-stages swapping method with accelerated cooling method |  | ˅ |  | ˅ | 51.555453 | 84.970056 | 1e6  ((10)1e5  (5e4)) |

**4. Example section**

This section illustrates two examples using the objective function and simulated annealing algorithm described in the previous two sections.

The first example is the same example of the simulated annealing algorithm section. The design parameter for both phases of the experiments is as follow,

Phase 1 experiment - 6 treatments, 3 biological replicates, 2 technical replicates,

Phase 2 experiment – 9 runs and 4 tags.

For the Phase 1 experiment, the 6 treatments are denoted by “a”, “b”, “c”, “d”, “e” and “f”. Since 3 biological replicates are used, this means 3 animals are assigned to each treatment which gives a total of 15 animals. These 15 animals are denoted by upper case letters of “AA” to “AR”. The theoretical ANOVA of the Phase 1 experiment can be presented as follows,

$ANOVA

DF Ani

Between Ani

Trt 5 1

Residual 12 1

$EF

Trt eff.Trt

Between Ani

Trt 3 1

The allocation of the animal for the starting design can be represented in the matrix notation as follows,

[,1] [,2] [,3] [,4]

[1,] "AA" "AB" "AC" "AD"

[2,] "AB" "AA" "AD" "AC"

[3,] "AE" "AF" "AG" "AH"

[4,] "AF" "AE" "AH" "AG"

[5,] "AI" "AJ" "AK" "AL"

[6,] "AJ" "AI" "AL" "AK"

[7,] "AM" "AN" "AO" "AP"

[8,] "AN" "AM" "AP" "AO"

[9,] "AQ" "AQ" "AR" "AR"

As described in the previous section, the assignment of animals to runs and tags is to group a pair of animals of the identical technical replicates and allocating them in a sector of 2 runs and 2 tags. This design generated 13 canonical efficiency factors all unity, for the animals in the within runs stratum. The average efficiency factor is also 1.

The allocation of the treatment for the starting design is also represented in the matrix notation as follows,

[,1] [,2] [,3] [,4]

[1,] "a" "b" "c" "d"

[2,] "b" "a" "d" "c"

[3,] "e" "f" "a" "b"

[4,] "f" "e" "b" "a"

[5,] "c" "d" "e" "f"

[6,] "d" "c" "f" "e"

[7,] "a" "b" "c" "d"

[8,] "b" "a" "d" "c"

[9,] "e" "e" "f" "f"

The pairs of treatments are always the same where are “a” and “b”, “c” and “d”, and “e” and “f”. This design generated 5 canonical efficiency factors, i.e. 1, 1, 0.976, 0.747 and 0.5, for the treatment elimination tags in the between animals within runs stratum. The average efficiency factor is 0.7856.

The theoretical ANOVA table can be shown as follows,

$ANOVA

DF e Ani Run

Between Run

Between Ani

Trt 2 1 2 4

Residual 2 1 2 4

Residual 4 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 5 1 2 0

Residual 7 1 2 0

Residual

Tag 2 1 0 0

Residual 12 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Trt 3/2 1/4

Residual

Within

Between Ani

Tag 9 2/3 1 1/9

Trt 3540/751 590/751

Residual

Tag 9 1

From the random effects table, the formal test for the treatment differences can be conducted as the variance components of the between animals for the treatment and residual mean square in the between animal stratum are identical. From the fixed effects table, the treatment is confounded with runs and tags effects by 1/4 and 1/9 of treatment information, respectively. Hence, there is 590/751 = 0.7856 of pure treatment information remaining, note that this value is also the same as the average efficiency factor computed.

**Pseudo code of the simulated annealing**

Generate a starting design as discussed in Section 3.

Set the initial temperature of 100; then I use the optim function in R which implements the simulated annealing algorithm.

Repeat 50000 times{

Generate a new design using the stage 1 of swapping – swapping is restricted to within run.

Compare the new design to the previous design based on the objective functions.

If the result of the objective function is better for the new design than the previous design, then the new design is accepted for next iteration and store as the best design.

If the result of the objective function is worse for the new design than the previous design, then computes the acceptance probability using formula as follow

where and denote the result from the objective function of the current and previous design, respectively, and denote the temperature that is used for the ith iteration. The acceptance probability is compared to the probability of accepting this design, which is randomly generated between the numbers of zero and one.

If the acceptance probability is higher than the probability of accepting this design, then this design is accepted for next iteration.

If the acceptance probability is lower than the probability of accepting this design, then this design is rejected. Hence, the previous design is retain for the next iteration.

}

Extract the best design from the search and use this design as the starting design for the next search.

Repeat 50000 times{

Generate a new design using stage 2 of swapping – swapping is restricted to within tags.

Compare the new design to the previous design based on the objective functions as described above.

}

Extract the best design from the search and use this design as the starting design for the next search.

Apply the accelerated cooling method, which is to repeat process of these two stages of swapping with the initial temperature of 50, 25, 12.5 and till the tenth level of the simulated annealing algorithm is performed.

**Step-by-step illustration**

Note the starting allocation of the animals and treatments to runs and tags are represented in a matrix notation as,

[,1] [,2] [,3] [,4]

[1,] "AA" "AB" "AC" "AD"

[2,] "AB" "AA" "AD" "AC"

[3,] "AE" "AF" "AG" "AH"

[4,] "AF" "AE" "AH" "AG"

[5,] "AI" "AJ" "AK" "AL"

[6,] "AJ" "AI" "AL" "AK"

[7,] "AM" "AN" "AO" "AP"

[8,] "AN" "AM" "AP" "AO"

[9,] "AQ" "AQ" "AR" "AR"

[,1] [,2] [,3] [,4]

[1,] "a" "b" "c" "d"

[2,] "b" "a" "d" "c"

[3,] "e" "f" "a" "b"

[4,] "f" "e" "b" "a"

[5,] "c" "d" "e" "f"

[6,] "d" "c" "f" "e"

[7,] "a" "b" "c" "d"

[8,] "b" "a" "d" "c"

[9,] "e" "e" "f" "f"

The result of the objective function defined in Section 2 of this design is 97.61799.

The first swap of the first stage and first level is swapping any random pairs of animals and treatment within the same runs under the temperature of 100. For this illustration, the first swap is between Animals “AJ” and “AK”, the allocation of the animals and treatment becomes

[,1] [,2] [,3] [,4]

[1,] "AA" "AB" "AC" "AD"

[2,] "AB" "AA" "AD" "AC"

[3,] "AE" "AF" "AG" "AH"

[4,] "AF" "AE" "AH" "AG"

[5,] "AI" **"AK"** **"AJ"** "AL"

[6,] **"AK"** "AI" "AL" **"AJ"**

[7,] "AM" "AN" "AO" "AP"

[8,] "AN" "AM" "AP" "AO"

[9,] "AQ" "AQ" "AR" "AR"

[,1] [,2] [,3] [,4]

[1,] "a" "b" "c" "d"

[2,] "b" "a" "d" "c"

[3,] "e" "f" "a" "b"

[4,] "f" "e" "b" "a"

[5,] "c" **"e"** **"d"** "f"

[6,] **"e"** "c" "f" **"d"**

[7,] "a" "b" "c" "d"

[8,] "b" "a" "d" "c"

[9,] "e" "e" "f" "f"

The result of the objective function of this design is 96.31085, which is lower than the starting design of 97.61799. Therefore, this design is not as good as the previous design based on the objective function. The next step is check whether I should accept this design to continue the searching of the optimal design.

The probability of accepting this design is then randomly generated between the numbers of zero and one, for this case, the number 0.424 is picked.

The acceptance probability is calculated using the following formula,

where and denote the result from the objective function of the current and previous design, respectively, and denote the temperature that is used for the ith iteration. For this example, this formula gives 0.987 and is higher than the probability of accepting this design of 0.424. Hence, this design is accepted to continue the search, but the previous better design is stored to be compared throughout the search.

The second swap is between the animals “AM” and “AO”

[,1] [,2] [,3] [,4]

[1,] "AA" "AB" "AC" "AD"

[2,] "AB" "AA" "AD" "AC"

[3,] "AE" "AF" "AH" "AG"

[4,] "AF" "AE" "AG" "AH"

[5,] "AI" "AK" "AJ" "AL"

[6,] "AK" "AI" "AL" "AJ"

[7,] **"AO"** "AN" **"AM"** "AP"

[8,] "AN" **"AO"** "AP" **"AM"**

[9,] "AQ" "AQ" "AR" "AR"

[,1] [,2] [,3] [,4]

[1,] "a" "b" "c" "d"

[2,] "b" "a" "d" "c"

[3,] "e" "f" "b" "a"

[4,] "f" "e" "a" "b"

[5,] "c" "e" "d" "f"

[6,] "e" "c" "f" "d"

[7,] **"c"** "b" **"a"** "d"

[8,] "b" **"c"** "d" **"a"**

[9,] "e" "e" "f" "f"

The objective function of this design gives 96.50794, which is higher than the previous design of 96.31085, but it is still lower than the first design of 97.61799. The probability of accepting this design is then again randomly generated to be 0.752. The acceptance probability is computed to be 1.002; hence, this design is still accepted for the search. However, the best design is still the first design.

After 50000 swaps, the following design is generated

[,1] [,2] [,3] [,4]

[1,] "AB" "AD" "AA" "AC"

[2,] "AD" "AB" "AC" "AA"

[3,] "AE" "AG" "AF" "AH"

[4,] "AG" "AE" "AH" "AF"

[5,] "AK" "AJ" "AL" "AI"

[6,] "AJ" "AK" "AI" "AL"

[7,] "AO" "AN" "AP" "AM"

[8,] "AN" "AO" "AM" "AP"

[9,] "AR" "AR" "AQ" "AQ"

[,1] [,2] [,3] [,4]

[1,] "b" "d" "a" "c"

[2,] "d" "b" "c" "a"

[3,] "e" "a" "f" "b"

[4,] "a" "e" "b" "f"

[5,] "e" "d" "f" "c"

[6,] "d" "e" "c" "f"

[7,] "c" "b" "d" "a"

[8,] "b" "c" "a" "d"

[9,] "f" "f" "e" "e"

The objective function of this design gives 97.67238, which is higher than the first design of 97.61799. This means this search did find a design that is better than the first design.

The best design from the previous search is used as the starting design of the search for the second stage. The second stage of the swapping is restricted to within the same tags. For this example, this first swap is between the animals “AR” and “AN”, which gives the following allocation of animals and treatment to runs and tags,

[,1] [,2] [,3] [,4]

[1,] "AB" "AD" "AA" "AC"

[2,] "AD" "AB" "AC" "AA"

[3,] "AE" "AG" "AF" "AH"

[4,] "AG" "AE" "AH" "AF"

[5,] "AK" "AJ" "AL" "AI"

[6,] "AJ" "AK" "AI" "AL"

[7,] "AO" **"AR"** "AP" "AM"

[8,] **"AR"** "AO" "AM" "AP"

[9,] **"AN" "AN"** "AQ" "AQ"

[,1] [,2] [,3] [,4]

[1,] "b" "d" "a" "c"

[2,] "d" "b" "c" "a"

[3,] "e" "a" "f" "b"

[4,] "a" "e" "b" "f"

[5,] "e" "d" "f" "c"

[6,] "d" "e" "c" "f"

[7,] "c" **"f"** "d" "a"

[8,] **"f"** "c" "a" "d"

[9,] **"b" "b"** "e" "e"

The objective function of this design gives 97.87345, which is better than the previous design is 97.67238. Hence, this design is both accepted for the search and stored as the best design.

After another 50000 swaps, the following design is generated

[,1] [,2] [,3] [,4]

[1,] "AO" "AB" "AP" "AF"

[2,] "AB" "AO" "AF" "AP"

[3,] "AJ" "AE" "AM" "AI"

[4,] "AE" "AJ" "AI" "AM"

[5,] "AK" "AR" "AC" "AH"

[6,] "AR" "AK" "AH" "AC"

[7,] "AD" "AG" "AL" "AQ"

[8,] "AG" "AD" "AQ" "AL"

[9,] "AN" "AN" "AA" "AA"

[,1] [,2] [,3] [,4]

[1,] "c" "b" "d" "f"

[2,] "b" "c" "f" "d"

[3,] "d" "e" "a" "c"

[4,] "e" "d" "c" "a"

[5,] "e" "f" "c" "b"

[6,] "f" "e" "b" "c"

[7,] "d" "a" "f" "e"

[8,] "a" "d" "e" "f"

[9,] "b" "b" "a" "a"

The objective function of this design gives 98.1892, which is the best design that has been founded so far.

This process of is then repeated with the lower starting temperature of 50, 25, 12.5 and till the tenth level of the simulated annealing algorithm is performed. This is discussed in Section 3 as the method called “accelerated cooling”. However, for this example, the accelerated cooling could not improve the best design founded in the first level with starting temperature of 100. Therefore, the best design founded in the first level can be considered as the optimal design for the experiment of the design parameters of

Phase 1 experiment - 6 treatments, 3 biological replicates, 2 technical replicates,

Phase 2 experiment – 9 runs and 4 tags.

Using the objective function and simulated annealing described, a more improved design has been generated. The animals allocation is the resulting design can be represented in matrix notation as follows,

[,1] [,2] [,3] [,4]

[1,] "AK" "AD" "AG" "AN"

[2,] "AD" "AK" "AN" "AG"

[3,] "AA" "AP" "AQ" "AC"

[4,] "AP" "AA" "AC" "AQ"

[5,] "AI" "AH" "AJ" "AL"

[6,] "AH" "AI" "AL" "AJ"

[7,] "AM" "AR" "AO" "AB"

[8,] "AR" "AM" "AB" "AO"

[9,] "AF" "AF" "AE" "AE"

The arrangement of the animals still follows 2-by-2 setting but with the different animal pairs compare to the starting design. This design also generated 13 canonical efficiency factors all unity, for the animals in the within runs stratum. The average efficiency factor is also 1. This means the test for the treatment differences should be able to conduct.

The treatment allocation to the runs and tags based on the animal allocation and can be represented in matrix notation as follows,

[,1] [,2] [,3] [,4]

[1,] "e" "d" "a" "b"

[2,] "d" "e" "b" "a"

[3,] "a" "d" "e" "c"

[4,] "d" "a" "c" "e"

[5,] "c" "b" "d" "f"

[6,] "b" "c" "f" "d"

[7,] "a" "f" "c" "b"

[8,] "f" "a" "b" "c"

[9,] "f" "f" "e" "e"

For this new design, the treatment pairs are not always identical like before. The treatment pairs are “e” and “d”, “a” and “b”, “a” and “d”, “e” and “c”, “b” and “c”, “d” and “f”, “a” and “f”, “c” and “b” and “e” and “f”. This design also generated 5 canonical efficiency factors, but they are different than before, i.e. 0.9167, 0.9167, 0.8889, 0.75 and 0.75, for the treatment elimination tags in the between animals within runs stratum. The average efficiency factor is 0.837 which is higher than the previous design.

The theoretical ANOVA table can be shown as follows,

$ANOVA

DF e Ani Run

Between Run

Between Ani

Trt 4 1 2 4

Residual 4 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 5 1 2 0

Residual 7 1 2 0

Residual

Tag 2 1 0 0

Residual 12 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Trt 3/4 1/8

Residual

Within

Between Ani

Tag 9 2/3 1 1/9

Trt 7920/1577 1320/1577

Residual

Tag 9 1

From the random effects table, notable change compare the random effects table of the previous design is that there are 4 DF associated with treatment effects in the between runs stratum compare the 2 DF for the previous design. However, a valid test for the treatment differences can still be conducted. From the fixed effects table of the new design, the amount of treatment information in the between runs stratum become 1/8 which is lower than before There is still 1/9 of treatment information confounded with the tag. This means there is (1320/1577 =) 0.8370 of pure treatment information remaining in the between animals within runs stratum, that is 0.051 more than the previous design. Therefore, this suggests that the new design is better than the previous design. The treatment pairs have to be different to minimise the confounding of treatment with the runs, which will also maximise the treatment information in the between animals within runs stratum.

The second example uses the 8-plex system for the second phase experiment, the design parameters for both phase of experiment can be described as follows,

Phase 1 experiment - 8 treatments, 2 biological replicates, 2 technical replicates,

Phase 2 experiment – 4 runs and 8 tags.

The theoretical ANOVA table for the Phase 1 experiment can be shown as follows,

$ANOVA

DF Ani

Between Ani

Trt 7 1

Residual 8 1

$EF

Trt eff.Trt

Between Ani

Trt 2 1

The allocation of the animal for the starting design can be represented in the matrix notation as follows,

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] "AA" "AB" "AC" "AD" "AE" "AF" "AG" "AH"

[2,] "AB" "AA" "AD" "AC" "AF" "AE" "AH" "AG"

[3,] "AK" "AL" "AI" "AJ" "AO" "AP" "AM" "AN"

[4,] "AL" "AK" "AJ" "AI" "AP" "AO" "AN" "AM"

This design generated 14 canonical efficiency factors all unity, for the animals in the within runs stratum. The average efficiency factor is also 1.

The allocation of the treatment for the starting design is also represented in the matrix notation as follows,

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] "a" "b" "c" "d" "e" "f" "g" "h"

[2,] "b" "a" "d" "c" "f" "e" "h" "g"

[3,] "c" "d" "a" "b" "g" "h" "e" "f"

[4,] "d" "c" "b" "a" "h" "g" "f" "e"

This design generated 6 canonical efficiency factors all unity, for the treatment elimination tags in the between animals within runs stratum. The average efficiency factor is also 1.

The theoretical ANOVA table can be shown as follows,

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 2 8

Residual 2 1 0 8

Within

Between Ani

Tag 3 1 2 0

Trt 6 1 2 0

Residual 5 1 2 0

Residual

Tag 4 1 0 0

Residual 10 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Tag 4 4 1 1

Trt 4 1

Residual

Tag 4 1

From the random effect table, the valid test for the treatment differences can be conducted. Note that total number of DF associated with the treatment is seven. From the fixed effects table, both tag and treatment mean squares contain 100% of treatment information. In addition, there are 6 DF associated with the treatment mean square, this means One DF associated with the treatment effects is completely confounded with the tag effects. Therefore, despite there are 100% of the treatment information for conducting the test of the treatment differences, but, it is only based on the 6 DF associated with the treatment effects.

Apply the simulated annealing algorithm with the objective function, a following design was generated. The allocation of the animal for the starting design can be represented in the matrix notation as follows,

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] "AL" "AI" "AJ" "AO" "AM" "AN" "AP" "AK"

[2,] "AI" "AL" "AO" "AJ" "AN" "AM" "AK" "AP"

[3,] "AC" "AG" "AD" "AE" "AH" "AA" "AB" "AF"

[4,] "AG" "AC" "AE" "AD" "AA" "AH" "AF" "AB"

This design generated 14 canonical efficiency factors all unity, for the animals in the within runs stratum. The average efficiency factor is also 1.

The allocation of the treatment for the starting design is also represented in the matrix notation as follows,

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] "d" "a" "b" "g" "e" "f" "h" "c"

[2,] "a" "d" "g" "b" "f" "e" "c" "h"

[3,] "c" "g" "d" "e" "h" "a" "b" "f"

[4,] "g" "c" "e" "d" "a" "h" "f" "b"

Same as the previous example, for the improved design, the treatment pairs are not consistent like the previous design. This design also generated 7 canonical efficiency factors, but they are different than before, i.e. 1, 1, 1, 0.75, 0.75 and 0.5, for the treatment elimination tags in the between animals within runs stratum. The average efficiency factor is 0.8077 which is lower than the previous design, but it is based on seven canonical efficiency factors.

The theoretical ANOVA table can be shown as follows,

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 2 8

Residual 2 1 0 8

Within

Between Ani

Tag 3 1 2 0

Trt 7 1 2 0

Residual 4 1 2 0

Residual

Tag 4 1 0 0

Residual 10 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Tag 4 6/5 1 3/10

Trt 42/13 21/26

Residual

Tag 4 1

From the random effect table, the valid test for the treatment differences can be conducted. The test is conducted based on the seven DF associated with the treatment effects, however, the DF associated with the residual mean squares of the between animals within runs stratum is reduced to 4 from 5 of the previous design. Form the fixed effects table, there is 3/10 treatment information confounded with the tag effects; hence, there is still 21/26 = 0.8077 of the pure treatment information remaining for conducting the test for the treatment differences.

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] 0.0000 0.0000 0.0000 0.8660 0.0000 0.0000 0.0 -0.5

[2,] 0.0000 -0.2677 0.0000 -0.2887 0.0000 0.7714 0.0 -0.5

[3,] 0.0000 -0.5342 0.0000 -0.2887 0.0000 -0.6175 0.0 -0.5

[4,] 0.0000 0.8019 0.0000 -0.2887 0.0000 -0.1538 0.0 -0.5

[5,] 0.0000 0.0000 0.8660 0.0000 0.0000 0.0000 -0.5 0.0

[6,] -0.2677 0.0000 -0.2887 0.0000 0.7714 0.0000 -0.5 0.0

[7,] -0.5342 0.0000 -0.2887 0.0000 -0.6175 0.0000 -0.5 0.0

[8,] 0.8019 0.0000 -0.2887 0.0000 -0.1538 0.0000 -0.5 0.0

1; 8 vs 6 and 7

1; 4 vs 2 and 3

1; 5 vs 6, 7 and 8

1; 1 vs 2, 3 and 4

1; 6 vs 7 and 8

1; 2 vs 3 and 4

[,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8]

[1,] 0.0000 0.0000 0.0000 0.6124 0.0000 0.7071 0.0 -0.3536

[2,] -0.3800 0.4095 0.1460 -0.2041 0.7071 0.0000 0.0 -0.3536

[3,] 0.4712 0.2285 -0.5559 -0.2041 0.0000 0.0000 0.5 -0.3536

[4,] 0.6420 0.0420 0.4116 -0.2041 0.0000 0.0000 -0.5 -0.3536

[5,] -0.2621 -0.4514 -0.5575 -0.2041 0.0000 0.0000 -0.5 -0.3536

[6,] 0.0000 0.0000 0.0000 0.6124 0.0000 -0.7071 0.0 -0.3536

[7,] -0.3800 0.4095 0.1460 -0.2041 -0.7071 0.0000 0.0 -0.3536

[8,] -0.0913 -0.6379 0.4100 -0.2041 0.0000 0.0000 0.5 -0.3536

1; 3 and 4 vs 2, 5, 7 and 8

1; 2, 3, 4 and 7 vs 5 and 8

1; 2, 4, 7 and 8 vs 3 and 5

1; 1 and 6 vs 2, 3, 4, 5, 7 and 8

0.75; 2 vs 7

0.75; 1 vs 6

0.5; 3 and 8 vs 4 and 5

The second example utilised the last component of the objective function in monitoring the DF associated with the treatment effects.

**Overall summary of finding the optimal designs for situation of 2 technical replicates**

The canonical efficiency factors of animals in the within runs and tags stratum is always 1, hence the average efficiency factor is also 1.

Both the DF associated with the animals effects in the between runs stratum and residual mean squares in the between animals within runs stratum increases as the number of biological replicates increases.

The tag effects confound with the treatment effects if the number of biological replicate is odd.

4-plex

1 DF associated with the tag effects always in the between animals within runs stratum.

8-plex

3 DF associated with the tag effects always in the between animals within runs stratum.

**2 treatments**

4-plex

The even biological replicates the average efficiency factor of treatment effect is always 100%.

The odd biological replicates the average efficiency factor of treatment effect is always , hence, the higher the biological replicates the higher the average efficiency factor of treatment effects.

8-plex

Only can be used when the numbers of biological replicates are even, because it is when the total number of the observation is divisible by eight for 8-plex experiments.

The even biological replicates the average efficiency factor of treatment effect is always 100%.

The odd biological replicates the average efficiency factor of treatment effect is always .

In summary, if the number of biological replicate is odd, then 4-plex experiment has to be used. If the number of biological replicates is even, then 4-plex is preferable as the test is applied on the 100% of pure treatment information. However, if the number of biological is high, then the 8-plex can be a good option, because the runs required is halved compared to 4-plex, and the average efficiency factor of treatment effects will also increases to close to 100% with high number of biological replicates.

**3 treatments**

Treatment is confounded with the between runs.

4-plex

If the biological replicates number is divisible by 4, the amount of pure treatment information is always 15/16 = 0.9375. Note that the canonical efficiency factors are also 15/16.

The other biological replicates, the amount of pure treatment information does not follow an obvious pattern, but it does increase when the number of biological increase.

8-plex

Only the number of biological replicates that is divisible by 4 can be used.

The pure treatment information for 4 and 8 biological replicates are 30/31 = 0.9677 and 63/64 = 0.9843, respectively. Both cases are higher than using the 4-plex experiments of 0.9375. Note that 4 biological replicates only have 5 DF for the residual MS which is one DF lower than before, but 8 biological replicates have 16 DF for the residual MS which is one DF higher than before.

**4 treatments**

The treatment is orthogonal to runs

4-plex

The amount of pure treatment information is always 100% if the biological replicate is even.

If the biological replicates is odd, then the canonical efficiency factors of treatment effects is always 1, 1 and .

8-plex

If the biological replicates is odd, then the amount of pure treatment information is always .

The amount of pure treatment information is always 100% if the biological replicate is divisible by 4.

For the other biological replicates, the canonical efficiency factor is always, 1, and .

For this case, using the 4-plex system is always better in terms of amount of pure treatment information remaining for conducting the test compared to the 8-plex system.

**5 treatments**

Same as the case with 3 treatments, except for 8 biological replicates in 8-plex system where the pure treatment information is reduced to 0.9763, but is still higher than 15/16.

The DF of the residual MS is the same for 4 biological replicates, but higher for the 8 biological replicate to 4-plex for both cases.

**6 treatments**

The treatment is confounded with runs for both cases.

4-plex

If the biological replicates is even, then there is some treatment effects in the between runs stratum. The amount of treatment information gradually increases as the number of biological replicates increases.

If the biological replicates is odd, then not only some treatment effects is in the between runs stratum, the treatment effects are also confounded with the tag effects. The amount treatment information is confounded with the tag mean square can be calculated to be .

8-plex

Apart from the experiment with 2 biological replicates, the amount of pure treatment information and DF associated residual mean squares are generally higher than the 4-plex experiments.

**7 treatments**

Similar as the cases with 3 or 5 treatments, the treatment is confounded with runs for both cases.

4-plex

If the biological replicates number is divisible by 4, the amount of pure treatment information is always 7/8= 0.875. Note that the canonical efficiency factors are also 7/8.

The other biological replicates, the amount of pure treatment information does not follow an obvious pattern, but it does increase when the number of biological increase.

8-plex

Only the number of biological replicates that is divisible by 4 can be used.

The pure treatment information for 4 and 8 biological replicates are 0.9666 and 0.9844, respectively. Both cases are higher than using the 4-plex experiments of 0.875. The DF of the residual MS are higher to 4-plex for both cases.

**8 treatments**

For the 4-plex experiments, some treatment effects is always in the between runs stratum. If the number of biological replicates is odd, then tag effect is also confounded with the treatment effects, which will further reduce the amount of pure treatment information. The amount treatment information is confounded with the tag mean square can be calculated to be .

For the 8-plex experiments, there is no treatment information in the between runs stratum. If the number of biological replicate is not divisible by 4, then tag effect is confounded with the treatment effects.

However, the 8-plex experiment performs better in both the amount of pure treatment information and the DF in the residual mean squares in the between animals stratum.

Apart from the experiments with 2 or 4 treatment groups, using 8-plex system is preferable, because it will not only halved the number of runs require, it also allow the test to be conducted on the higher amount of pure treatment information. However, number DF of residual mean squares for 8-plex experiments can sometimes be smaller than the 4-plex experiments.

**Conclusion**

(To be completed)

**Appendix: Summary table of optimal designs**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment |
| Treatment | Bio Rep | Runs | Tags | Canonical Eff Factor | Average Eff Factor | Average Eff Factor |
| 2 | 2 | 2 | 8 | 2 | 4 | 0 | No (1 DF) | 1 | Yes | 1 (3) | 1 | 1 |
| 3 | 12 | 3 | 1 | No (1 DF) | 2 | No (1/9) | 1 (4) | 1 | 8/9 |
| 4 | 16 | 4 | 1 | No (1 DF) | 4 | Yes | 1 (6) | 1 | 1 |
| 5 | 20 | 5 | 2 | No (1 DF) | 5 | No (1/25) | 1 (7) | 1 | 24/25 |
| 6 | 24 | 6 | 2 | No (1 DF) | 7 | Yes | 1 (9) | 1 | 1 |
| 7 | 28 | 7 | 3 | No (1 DF) | 8 | No (1/49) | 1 (10) | 1 | 48/49 |
| 8 | 32 | 8 | 3 | No (1 DF) | 10 | Yes | 1 (12) | 1 | 1 |
| 9 | 38 | 9 | 4 | No (1 DF) | 11 | No (1/81) | 1 (13) | 1 | 80/81 |
| 10 | 40 | 10 | 4 | No (1 DF) | 13 | Yes | 1 (15) | 1 | 1 |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment |
| Treatment | Bio Rep | Runs | Tags | Canonical Eff Factor | Average Eff Factor | Average Eff Factor |
| 2 | 4 | 2 | 16 | 2 | 8 | 0 | No (3 DF) | 3 | Yes | 1 (7) | 1 | 1 |
| 6 | 24 | 3 | 1 | No (3 DF) | 6 | No (1/9) | 1 (10) | 1 | 8/9 |
| 8 | 32 | 4 | 1 | No (3 DF) | 10 | Yes | 1 (14) | 1 | 1 |
| 10 | 40 | 5 | 2 | No (3 DF) | 13 | No (1/25) | 1 (17) | 1 | 24/25 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 3 | 2 | 2 | 12 | 3 | 4 | 1 (1 Trt) | No (1 DF) | 1 | Yes | 1 (4) | 1 | 1, 3/4 | 6/7 |
| 4 | 24 | 6 | 2 (2 Trt) | No (1 DF) | 6 | Yes | 1 (9) | 1 | 15/16(2) | 15/16 |
| 6 | 36 | 9 | 4 (2 Trt) | No (1 DF) | 10 | Yes | 1 (13) | 1 | 23/24, 7/8 | 0.9148 |
| 8 | 48 | 12 | 5 (2 Trt) | No (1 DF) | 15 | Yes | 1 (18) | 1 | 15/16 (2) | 15/16 |
| 10 | 60 | 15 | 7 (2 Trt) | No (1 DF) | 19 | Yes | 1 (22) | 1 | 19/20, 9/10 | 0.9243 |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 3 | 4 | 2 | 24 | 3 | 4 | 1 (1 Trt) | No (3 DF) | 5 | Yes | 1 (10) | 1 | 1, 15/16 | 30/31 |
| 8 | 48 | 6 | 2 (2 Trt) | No (3 DF) | 16 | Yes | 1 (21) | 1 | 63/64 (2) | 63/64 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 4 | 2 | 2 | 16 | 4 | 4 | 1 | No (1DF) | 2 | Yes | 1 (6) | 1 | 1 | 1 |
| 3 | 24 | 6 | 2 | No (1DF) | 5 | No (1/9) | 1 (9) | 1 | 1(2), 8/9 | 24/25 |
| 4 | 32 | 8 | 3 | No (1DF) | 8 | Yes | 1 (12) | 1 | 1 | 1 |
| 5 | 40 | 10 | 4 | No (1DF) | 11 | No (1/25) | 1 (15) | 1 | 1(2), 24/25 | 72/73 |
| 6 | 48 | 12 | 5 | No (1DF) | 14 | Yes | 1 (18) | 1 | 1 | 1 |
| 7 | 56 | 14 | 6 | No (1DF) | 17 | No (1/49) | 1 (21) | 1 | 1(2), 48/49 | 0.9931 |
| 8 | 64 | 16 | 7 | No (1DF) | 20 | Yes | 1 (24) | 1 | 1 | 1 |
| 9 | 72 | 18 | 8 | No (1DF) | 23 | No (1/81) | 1 (27) | 1 | 1(2), 80/81 | 0.9959 |
| 10 | 80 | 20 | 9 | No (1DF) | 26 | Yes | 1 (30) | 1 | 1 | 1 |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 4 | 2 | 2 | 16 | 2 | 8 | 0 | No (3DF) | 2 | No (1/2) | 1 (7) | 1 | 1,1/2(2) | 3/5 |
| 3 | 24 | 3 | 1 | No (3DF) | 4 | No (1/9) | 1 (10) | 1 | 8/9 (3) | 8/9 |
| 4 | 32 | 4 | 1 | No (3DF) | 8 | Yes | 1 (14) | 1 | 1 | 1 |
| 5 | 40 | 5 | 2 | No (3DF) | 11 | No (1/25) | 1 (17) | 1 | 24/25(3) | 24/25 |
| 6 | 48 | 6 | 2 | No (3DF) | 15 | No (1/18) | 1 (21) | 1 | 1, 17/18(2) | 51/53 |
| 7 | 56 | 7 | 3 | No (3DF) | 18 | No (1/49) | 1 (24) | 1 | 48/49(3) | 48/49 |
| 8 | 64 | 8 | 3 | No (3DF) | 22 | Yes | 1 (28) | 1 | 1 | 1 |
| 9 | 72 | 9 | 4 | No (3DF) | 25 | No (1/81) | 1 (31) | 1 | 80/81(3) | 80/81 |
| 10 | 80 | 10 | 4 | No (3DF) | 29 | No (1/50) | 1 (35) | 1 | 1, 49/50(2) | 0.9866 |

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 5 | 2 | 2 | 20 | 5 | 4 | 2 (2 Trt) | No (1 DF) | 2 | Yes | 1 (7) | 1 | 1(2), 7/8, 5/8 | 0.8434 |
| 4 | 40 | 10 | 4 (4 Trt) | No (1 DF) | 10 | Yes | 1 (15) | 1 | 15/16(4) | 15/16 |
| 6 | 60 | 15 | 7 (4 Trt) | No (1 DF) | 17 | Yes | 1 (22) | 1 | 23/24(2), 11/12 5/6 | 0.9137 |
| 8 | 80 | 20 | 9 (4 Trt) | No (1 DF) | 25 | Yes | 1 (30) | 1 | 15/16(4) | 15/16 |
| 10 | 100 | 25 | 12 (4 Trt) | No (1 DF) | 32 | Yes | 1 (37) | 1 | 19/20(2), 37/40, 7/8 | 0.9240 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 5 | 4 | 2 | 40 | 5 | 8 | 2 (2 Trt) | No (3 DF) | 10 | Yes | 1 (17) | 1 | 1(2), 15/16(2) | 30/31 |
| 8 | 80 | 10 | 4 (4 Trt) | No (3 DF) | 28 | Yes | 1 (35) | 1 | 0.994 (2), 0.959(2) | 0.9763 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 6 | 2 | 2 | 24 | 6 | 4 | 2 (2 Trt) | No (1 DF) | 3 | Yes | 1 (9) | 1 | 1(3), 3/4(2) | 0.8824 |
| 3 | 36 | 9 | 4 (4 Trt) | No (1 DF) | 2 | No (1/9) | 1 (4) | 1 | 11/12(2), 8/9, 3/4(2) | 0.8370 |
| 4 | 48 | 12 | 5 (4 Trt) | No (1 DF) | 12 | Yes | 1 (18) | 1 | 1, 15/16(2), 13/16(2) | 0.8937 |
| 5 | 60 | 15 | 7 (5 Trt) | No (1 DF) | 16 | No (1/25) | 1 (22) | 1 | 0.953, 0.9, 0.8836, 0.8235, 0.8 | 0.8686 |
| 6 | 72 | 18 | 8 (4 Trt) | No (1 DF) | 21 | Yes | 1 (27) | 1 | 1,  0.875 (4) | 0.8974 |
| 7 | 84 | 21 | 10 (5 Trt) | No (1 DF) | 25 | No (1/49) | 1 (31) | 1 | 0.9286, 0.9164, 0.8571(2), 0.8489 | 0.8803 |
| 8 | 96 | 24 | 11 (5 Trt) | No (1 DF) | 30 | Yes | 1 (36) | 1 | 0.9375 (2), 0.875 (3) | 0.8990 |
| 9 | 108 | 27 | 13 (5 Trt) | No (1 DF) | 34 | No (1/81) | 1 (40) | 1 | 0.9272, 0.9167, 0.8872, 0.8611, 0.8399 | 0.8852 |
| 10 | 120 | 30 | 14 (5 Trt) | No (1 DF) | 39 | Yes | 1 (45) | 1 | 0.9 (5) | 0.9 |

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| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 6 | 2 | 2 | 24 | 3 | 8 | 1 (1 Trt) | No (3 DF) | 2 | No (1/3) | 1 (10) | 1 | 1, 3/4,  2/3(3) | 0.7317 |
| 4 | 48 | 6 | 2 (2 Trt) | No (3 DF) | 13 | Yes | 1 (21) | 1 | 1(3), 15/16(2) | 0.9740 |
| 6 | 72 | 9 | 4 (4 Trt) | No (3 DF) | 23 | No (4/81) | 1 (31) | 1 | 0.9792,  0.9601, 0.9421 0.9375 0.9033 | 0.9438 |
| 8 | 96 | 12 | 5 (4 Trt) | No (3 DF) | 34 | Yes | 1 (42) | 1 | 1, 63/64(2), 61/64(2) | 0.9746 |
| 10 | 120 | 15 | 7 (5 Trt) | No (3 DF) | 44 | No (4/225) | 1 (52) | 1 | 0.975, 0.974, 0.962, 0.95, 0.949 | 0.9618 |

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| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 7 | 2 | 2 | 28 | 7 | 4 | 3 (3 Trt) | No (1 DF) | 3 | Yes | 1 (10) | 1 | 1(3), 7/8, 5/8, 1/2 | 0.7749 |
| 4 | 56 | 14 | 7 (6 Trt) | No (1 DF) | 14 | Yes | 1 (21) | 1 | 7/8 (6) | 7/8 |
| 6 | 84 | 21 | 10 (6 Trt) | No (1 DF) | 24 | Yes | 1 (31) | 1 | 7/8(5),  19/24 | 0.8599 |
| 8 | 112 | 28 | 13 (6 Trt) | No (1 DF) | 35 | Yes | 1 (42) | 1 | 7/8 (6) | 7/8 |
| 10 | 140 | 35 | 17 (6 Trt) | No (1 DF) | 45 | Yes | 1 (22) | 1 | 7/8(5), 33/40 | 0.8663 |

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| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 7 | 4 | 2 | 56 | 7 | 8 | 3 (3 Trt) | No (3 DF) | 15 | Yes | 1 (24) | 1 | 1(3),  31/32(2), 7/8 | 0.9666 |
| 8 | 112 | 14 | 13 (6 Trt) | No (3 DF) | 40 | Yes | 1 (49) | 1 | 63/64 (6) | 0.9844 |

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| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 8 | 2 | 2 | 32 | 8 | 4 | 3 (3 Trt) | No (1 DF) | 4 | Yes | 1 (12) | 1 | 1(4), 3/4(2), 1/2 | 0.8077 |
| 3 | 48 | 12 | 5 (6 Trt) | No (1 DF) | 10 | No (1/9) | 1 (18) | 1 | 1, 11/12(2), 8/9, 3/4(2), 2/3 | 0.8261 |
| 4 | 64 | 16 | 7 (7 Trt) | No (1 DF) | 16 | Yes | 1 (24) | 1 | 0.963 (2), 0.875 (2), 0.7866 (2), 0.75 | 0.8498 |
| 5 | 80 | 20 | 9 (7 Trt) | No (1 DF) | 22 | No (1/25) | 1 (30) | 1 | 9/10(3), 43/50, 4/5(3) | 0.8489 |
| 6 | 96 | 24 | 11 (6 Trt) | No (1 DF) | 28 | Yes | 1 (36) | 1 | 1, 5/6 (6) | 0.8537 |
| 7 | 112 | 28 | 13 (7 Trt) | No (1 DF) | 34 | No (1/49) | 1 (42) | 1 | 6/7(6), 41/49 | 0.8542 |
| 8 | 128 | 32 | 15 (7 Trt) | No (1 DF) | 40 | Yes | 1 (48) | 1 | 0.9192(2), 0.875, 0.8308(2), 0.8125(2) | 0.8545 |
| 9 | 144 | 36 | 17 (7 Trt) | No (1 DF) | 46 | No (1/81) | 1 (54) | 1 | 8/9(2), 71/81 , 5/6 (3) | 0.8546 |
| 10 | 160 | 40 | 19 (7 Trt) | No (1 DF) | 52 | Yes | 1 (60) | 1 | 0.9, 0.8854(2), 0.85(2), 0.8146(2) | 0.8559 |

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| Phase 1 Experiment | | Technical Rep | Number of observation | Phase 2 Experiment | | DF of Animal in the between Runs stratum | Tag orthogonal to Animal in the within runs stratum | DF of residual in between animals stratum | Tag orthogonal to Treatment | Animal | | Treatment | |
| Treatment | Bio Rep | Runs | Tags | Can Eff Factor | Ave Eff Factor | Can Eff Factor | Ave Eff Factor |
| 8 | 2 | 2 | 32 | 4 | 8 | 1 | No (3 DF) | 4 | No (3/10) | 1 (14) | 1 | 1(4), 3/4(2), 1/2 | 0.8077 |
| 3 | 48 | 6 | 2 | No (3 DF) | 11 | No (1/9) | 1 (21) | 1 | 1(4), 8/9(3) | 0.9492 |
| 4 | 64 | 8 | 3 | No (3 DF) | 18 | Yes | 1 (28) | 1 | 1(7) | 1 |
| 5 | 80 | 10 | 4 | No (3 DF) | 25 | No (1/25) | 1 (35) | 1 | 1(4), 24/25(3) | 0.9825 |
| 6 | 96 | 12 | 5 | No (3 DF) | 32 | No (1/30) | 1 (42) | 1 | 1(4), 35/36(2), 17/18 | 0.9837 |
| 7 | 112 | 14 | 6 | No (3 DF) | 39 | No (1/49) | 1 (49) | 1 | 1(4), 48/49(3) | 0.9912 |
| 8 | 128 | 16 | 7 | No (3 DF) | 46 | Yes | 1 (56) | 1 | 1(7) | 1 |
| 9 | 144 | 18 | 8 | No (3 DF) | 53 | No (1/81) | 1 (63) | 1 | 1(4), 80/81(3) | 0.9947 |
| 10 | 160 | 20 | 9 | No (3 DF) | 60 | No (3/250) | 1 (70) | 1 | 1(4), 99/100(2),  49/50 | 0.9942 |