**Studying the patterns of the designs with 2 treatment groups with 4-plex experiments**

Using simulated annealing, some optimal designs have found for 2 treatment groups, 2, 3 and 4 technical replicates, 2-10 biological replicates and 4-plex experiments. This write-up describes the patterns in the allocation of animals and treatments to runs and tags of these designs. If patterns are identified, it can be used to develop and define a generic method for designing the two-phase MudPIT-iTRAQ experiments.

For designs with two treatment groups and two technical replicates with four-plex system, the number of biological replicates is identical to the number of MudPIT runs for the second phase experiments. For example, if a first phase experiment consists of 2 biological replicates, then the total number of samples to be assigned from the first phase experiment to the second phase experiment is 8. If the second phase experiment is using four-plex system, then only need 2 runs are needed to measure all 8 samples.

The patterns of animal and treatment allocations, for designs with two treatment groups and two technical replicates with four-plex system, can be divided into two main groups based on number of biological replicates: a) designs with even number of biological replicates and b) designs odd number of biological replicates.

For the rest of this write-up, the allocations of animals and treatments to runs and tags are shown as a matrix where the rows correspond to the runs and columns correspond to the tags. In addition, the upper case letter denotes the animal ID and the lower case letter denotes the treatments.

a) An example of a design with an even number of biological replicates is four biological replicates. Animals A, C, E and G are assigned to treatment “a” and Animals B, D, F and H are assigned to treatment “b”.

The animal allocation is as follows:

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

[1,] "A" "B" "C" "D"

[2,] "B" "A" "D" "C"

[3,] "E" "F" "G" "H"

[4,] "F" "E" "H" "G"

The animal effects is completely confounded with the run contrasts of 1, 2 versus 3, 4 and the tag contrasts of 114, 115 versus 116, 117.

The treatment allocation is as follows:

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

[1,] "a" "b" "a" "b"

[2,] "b" "a" "b" "a"

[3,] "a" "b" "a" "b"

[4,] "b" "a" "b" "a"

Since every run and tag has two of each treatment group, the treatment effects are orthogonal to both runs and tags.

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 2 4

Residual 2 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 1 1 2 0

Residual 4 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

Residual

Within

Between Ani

Tag 4 1

Trt 8 1

Residual

Tag 4 1

From the fixed effects table, the 100% of treatment information is in the between animals within runs stratum. However, from the random effects table, one DF associated with animal effects is in the between runs stratum and one DF associated with tag effects is in the within runs between animals stratum.

b) An example of design with odd number of biological replicates is five biological replicates. Animals A, C, E, G and I are assigned to treatment “a” and Animals B, D, F, H and J are assigned to treatment “b”.

The animal allocation is as follows:

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

[1,] "A" "B" "C" "D"

[2,] "B" "A" "D" "C"

[3,] "E" "F" "G" "H"

[4,] "F" "E" "H" "G"

[5,] "I" "I" "J" "J"

The first four rows of the animal allocation are identical to the previous design with four biological replicates. The animal allocation of the fifth row is assigned by “preserving” the confounding between the tags and the animals as mentioned in the previous design with four biological replicates, i.e. Animal I is assigned with the Animals A, B and E and Animal J is assigned with Animals C, D and H.

The treatment allocation is as follows:

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

[1,] "a" "b" "a" "b"

[2,] "b" "a" "b" "a"

[3,] "a" "b" "a" "b"

[4,] "b" "a" "b" "a"

[5,] "a" "a" "b" "b"

Due to the additional fifth run of the design, the treatment effect is confounded with the contrast of tag 114, 115 versus 116, 117.

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run

Between Ani 2 1 2 4

Residual 2 1 0 4

Within

Between Ani

Tag 1 1 2 0

Trt 1 1 2 0

Residual 5 1 2 0

Residual

Tag 2 1 0 0

Residual 6 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Tag 5 2/5 1 1/25

Trt 48/5 24/25

Residual

Tag 5 1

From the fixed effects table, there is 1/25 of treatment information confounded with tag effects; hence, there is 24/25 of pure treatment information remaining. In addition, two DF associated with animal effects are in the between runs stratum and one DF associated with tag effects is in the within runs between animals stratum.

The next set of designs to be described is consists of 2 treatment groups, 3 technical replicates and four tags. For this set of designs, the number biological replicate has to be even to able to fit into the four-plex experiments. For example, if a first phase experiment consists of 2 biological replicates, then the total number of sample to be assigned to the second phase experiment is 12; hence, 3 runs of experiments are needed to measure all 12 samples with four-plex system. However, if another first experiment consists of 3 biological replicates, then the total number of sample to be assigned to the second phase is 18. Using the four-plex system, 5 runs of experiments are require to measure all 18 samples, but 2 additional samples could have also be measured with 5 runs. In another word, 18 is not divisible by 4.

The first design contains two biological replicates where Animals A and C are assigned to treatment “a” and Animals B and D are assigned to treatment “b”.

The animal allocation is as follows:

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

[1,] "A" "B" "C" "D"

[2,] "C" "A" "B" "D"

[3,] "B" "C" "A" "D"

Note the first three columns of this animal allocation is a 3-by-3 latin square design of animals to runs and tags and the last column has only Animal D. This allocation allows the animal to be orthogonal with the runs, because every run contains all 4 animals. However, one tag contrast of 114, 115 versus 116, 117 is confounded with animal effects. The treatment allocation is as follows:

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

[1,] "a" "b" "a" "b"

[2,] "a" "a" "b" "b"

[3,] "b" "a" "a" "b"

The treatment effect is also confounded with the tag contrast of 114, 115 versus 116, 117.

The theoretical ANOVA table for the animal and treatment designs shown above 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 fixed effects table, there is 1/3 of treatment information confounded with the tag effects; hence, there is 2/3 of pure treatment information remaining. In addition, from the random effects table, one DF associated with tag effects is in the between animals stratum.

The second design contains four biological replicates where Animals A, C, E and G are assigned to treatment “a” and Animals B, D, F and H are assigned to treatment “b”.

The animal allocation is as follows:

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

[1,] "A" "B" "C" "D"

[2,] "C" "A" "B" "D"

[3,] "B" "C" "A" "D"

[4,] "F" "G" "H" "E"

[5,] "H" "F" "G" "E"

[6,] "G" "H" "F" "E"

The first three rows are identical to the animal allocation of previous design with two biological replicates. The next three rows are assigned in a same way expect the last column. In order for the treatment effects to be orthogonal to the tag effects, the animals that is perturbed by treatment “b” is assigned to the last column of last three rows, because Animal D is perturbed by treatment “a”. For this case, Animal E is assigned, because Animal E is perturbed by treatment “b”. The treatment allocation is as follows:

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

[1,] "a" "b" "a" "b"

[2,] "a" "a" "b" "b"

[3,] "b" "a" "a" "b"

[4,] "b" "a" "b" "a"

[5,] "b" "b" "a" "a"

[6,] "a" "b" "b" "a"

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 3 4

Residual 4 1 0 4

Within

Between Ani

Tag 1 1 3 0

Trt 1 1 3 0

Residual 4 1 3 0

Residual

Tag 2 1 0 0

Residual 10 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Tag 6 1

Trt 12 1

Residual

Tag 6 1

From the fixed effects table, the 100% of treatment information is in the between animals within runs stratum. However, from the random effects table, one DF associated with animal effects is in the between runs stratum and one DF associated with tag effects is in the between animals within runs stratum.

The next set of designs to be described is the designs with 2 treatment groups, 4 technical replicates and four tags. The patterns can also be divided into two main groups based on number of biological replicates from the first phase experiments: a) designs with even number of biological replicates and b) designs odd number of biological replicates.

a) An example of design with even number of biological replicates is with 2 biological replicates where Animal A and C are assigned to treatment “a” and Animal B and D are assigned to treatment “b”.

The animal allocation is as follows,

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

[1,] "A" "B" "C" "D"

[2,] "B" "A" "D" "C"

[3,] "C" "D" "A" "B"

[4,] "D" "C" "B" "A"

This design is perfect design for allocating 4 animals into a four runs and four tags, where every run and tag has all four unique animals; hence, the animals is orthogonal to both runs and tags. The treatment allocation is as follows,

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

[1,] "a" "b" "a" "b"

[2,] "b" "a" "b" "a"

[3,] "a" "b" "a" "b"

[4,] "b" "a" "b" "a"

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run 3 1 0 4

Within

Between Ani

Trt 1 1 4 0

Residual 2 1 4 0

Residual

Tag 3 1 0 0

Residual 6 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Within

Between Ani

Trt 8 1

Residual

Tag 4 1

From the fixed effects, the 100% of treatment information is in the between animals within runs stratum. In addition, from the random effects table, all one DF associated with animal effects is in the within runs stratum.

Another example of design with even number of biological replicates is with 4 biological replicates where Animal A, C, E and G are assigned to treatment “a” and Animal B, D, F and H are assigned to treatment “b”. The animal allocation is as follows

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

[1,] "A" "B" "C" "D"

[2,] "B" "A" "D" "C"

[3,] "C" "D" "A" "B"

[4,] "D" "C" "B" "A"

[5,] "E" "F" "G" "H"

[6,] "F" "E" "H" "G"

[7,] "G" "H" "E" "F"

[8,] "H" "G" "F" "E"

The first four rows of this animal allocation are identical to the previous design with two biological replicates. The next four rows of design are assigned in a same way as the first four rows with Animal E, F, G and H.

The treatment allocation is as follows,

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

[1,] "a" "b" "a" "b"

[2,] "b" "a" "b" "a"

[3,] "a" "b" "a" "b"

[4,] "b" "a" "b" "a"

[5,] "a" "b" "a" "b"

[6,] "b" "a" "b" "a"

[7,] "a" "b" "a" "b"

[8,] "b" "a" "b" "a"

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 4 4

Residual 6 1 0 4

Within

Between Ani

Trt 1 1 4 0

Residual 5 1 4 0

Residual

Tag 3 1 0 0

Residual 15 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Trt 16 1

Residual

Tag 8 1

From the fixed effects, the 100% of treatment information is in the between animals within runs stratum. However, from the random effects table, one DF associated with animal effects is in the between runs stratum.

b) An example of design with odd number of biological replicates is with 3 biological replicates where Animal A, C and E are assigned to treatment “a” and Animal B, D and F are assigned to treatment “b”. The animal allocation is as follows,

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

[1,] "A" "B" "C" "D"

[2,] "B" "A" "D" "C"

[3,] "C" "D" "A" "B"

[4,] "D" "C" "B" "A"

[5,] "E" "E" "F" "F"

[6,] "F" "F" "E" "E"

The first four rows are identical to the previous design with two biological replicates. The remaining Animal E and F are assigned to the last two rows while making sure the treatment effects is still orthogonal to runs and tags. The treatment allocation is as follows,

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

[1,] "a" "b" "a" "b"

[2,] "b" "a" "b" "a"

[3,] "a" "b" "a" "b"

[4,] "b" "a" "b" "a"

[5,] "a" "a" "b" "b"

[6,] "b" "b" "a" "a"

The theoretical ANOVA table for the animal and treatment designs shown above is as follows:

$ANOVA

DF e Ani Run

Between Run

Between Ani 1 1 4 4

Residual 4 1 0 4

Within

Between Ani

Trt 1 1 4 0

Residual 3 1 4 0

Residual

Tag 3 1 0 0

Residual 11 1 0 0

$EF

Tag Trt eff.Tag eff.Trt

Between Run

Between Ani

Residual

Within

Between Ani

Trt 12 1

Residual

Tag 6 1

From the fixed effects, the 100% of treatment information is in the between animals within runs stratum. However, from the random effects table, one DF associated with animal effects is in the between runs stratum.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 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 (4) | 1 | 0.8889 |
| 4 | 16 | 4 | 1 | No (1 DF) | 4 | Yes | 1 (6) | 1 | 1 |
| 5 | 20 | 5 | 2 | No (1 DF) | 5 | No | 1 (7) | 1 | 0.96 |
| 6 | 24 | 6 | 2 | No (1 DF) | 7 | Yes | 1 (9) | 1 | 1 |
| 7 | 28 | 7 | 3 | No (1 DF) | 8 | No | 1 (10) | 1 | 0.9796 |
| 8 | 32 | 8 | 3 | No (1 DF) | 10 | Yes | 1 (12) | 1 | 1 |
| 9 | 38 | 9 | 4 | No (1 DF) | 11 | No | 1 (13) | 1 | 0.9877 |
| 10 | 40 | 10 | 4 | No (1 DF) | 13 | Yes | 1 (15) | 1 | 1 |
| 2 | 3 | 12 | 3 | 0 | No (1 DF) | 1 | No | 1 (3) | 1 | 0.6667 |
| 4 | 24 | 6 | 1 | No (1 DF) | 4 | Yes | 1 (6) | 1 | 1 |
| 6 | 36 | 9 | 2 | No (1 DF) | 7 | No | 1 (9) | 1 | 0.9630 |
| 8 | 48 | 12 | 3 | No (1 DF) | 10 | Yes | 1 (12) | 1 | 1 |
| 10 | 50 | 15 | 4 | No (1 DF) | 13 | No | 1 (15) | 1 | 0.9867 |
| 2 | 4 | 16 | 4 | 0 | Yes | 2 | Yes | 1 (3) | 1 | 1 |
| 3 | 24 | 6 | 1 | Yes | 3 | Yes | 1 (4) | 1 | 1 |
| 4 | 32 | 8 | 1 | Yes | 5 | Yes | 1 (6) | 1 | 1 |
| 5 | 40 | 10 | 2 | Yes | 6 | Yes | 1 (7) | 1 | 1 |
| 6 | 48 | 12 | 2 | Yes | 8 | Yes | 1 (9) | 1 | 1 |
| 7 | 56 | 14 | 3 | Yes | 9 | Yes | 1 (10) | 1 | 1 |
| 8 | 64 | 16 | 3 | Yes | 11 | Yes | 1 (12) | 1 | 1 |
| 9 | 72 | 18 | 4 | Yes | 12 | Yes | 1 (13) | 1 | 1 |
| 10 | 80 | 20 | 4 | Yes | 13 | Yes | 1 (15) | 1 | 1 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 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 (10) | 1 | 0.8889 |
| 8 | 32 | 4 | 1 | No (3 DF) | 10 | Yes | 1 (14) | 1 | 1 |
| 10 | 40 | 5 | 2 | No (3 DF) | 13 | Yes | 1 (17) | 1 | 0.96 |
| 4 | 3 | 24 | 3 | 0 | No (3 DF) | 3 | No | 1(7) | 1 | 0.6667 |
| 8 | 48 | 6 | 1 | No (3 DF) | 10 | Yes | 1(14) | 1 | 1 |
| 12 | 72 | 9 | 2 | No (3 DF) | 17 | No | 1(21) | 1 | 0.9630 |
| 2 | 4 | 16 | 2 | 0 | No (1 DF) | 1 | Yes | 1 (3) | 1 | 1 |
| 3 | 24 | 3 | 1 | No (1 DF) | 2 | No | 1 (4) | 1 | 0.8889 |
| 4 | 32 | 4 | 0 | No (1 DF) | 5 | Yes | 1(7) | 1 | 1 |
| 5 | 40 | 5 | 1 | No (1 DF) | 6 | No | 1(8) | 1 | 0.96 |
| 6 | 48 | 6 | 1 | No (1 DF) | 8 | Yes | 1(10) | 1 | 1 |
| 7 | 56 | 7 | 2 | No (1 DF) | 9 | No | 1 (11) | 1 | 0.9796 |
| 8 | 64 | 8 | 1 | No (1 DF) | 12 | Yes | 1 (14) | 1 | 1 |
| 9 | 72 | 9 | 2 | No (1 DF) | 13 | No | 1 (15) | 1 | 0.9877 |
| 10 | 80 | 10 | 2 | No (1 DF) | 15 | Yes | 1 (17) | 1 | 1 |