Split Plots Pros and Cons: Dealing with a Hard-to-Change Factor

There are many attendees today! To avoid disrupting the Voice over Internet Protocol (VoIP) system, I will mute all. Please use the Questions feature on GotoWebinar which we will answer during the presentation.

-- Pat

Presented by Pat Whitcomb, Founder Stat-Ease, Inc., Minneapolis, MN pat@statease.com

April 2021 Webinar

Agenda
Split Plot Pros and Cons

1. Restricting randomization
2. Factorial split plot
   (DNA Amplification)
3. Combined mixture process split plot
   (Reverse Phase HPLC)
4. Summary
Agenda
Split Plot Pros and Cons

1. Restricting randomization

2. Factorial split plot
   (DNA Amplification)

3. Combined mixture process split plot
   (Reverse Phase HPLC)

4. Summary

Split-Plot Designs

Problem:
Often in designed experiments some factors, e.g., temperature, are more difficult or expensive to vary than others. In some cases, conducting a completely randomized design isn’t practical.

Solution:
Restrict the randomization so it is practical to conduct the design. If you have to sort a factor to make a DOE easier to run, this restriction in randomization results in a “split-plot” design.

Do you have factors that are difficult to randomize?
Split-Plot Designs

The “split-plot” design originated in the field of agriculture. Experimenters applied one treatment (e.g. herbicide) to a large area of land, called a “whole plot” and other treatments (e.g. crop) to smaller areas of land within the whole plot called “subplot”.

Split-Plot Designs:

- Split plots have two types of factors: “Hard-to-change” (HTC) applied to the whole plots and “Easy-to-change” (ETC) applied to the subplots.
- The randomization of the HTC factor is restricted.
- Split plots naturally arise in many DOE studies.
- Building and analyzing a split-plot design is tricky, unless you have a good DOE package.

You are in luck, split plots are easy using Design-Expert software!
1. Restricting randomization

2. **Factorial split plot**  
   *(DNA Amplification)*

3. Combined mixture process split plot  
   *(Reverse Phase HPLC)*

4. Summary

---

**DNA Amplification**  
$2^{9-1}$ Factorial run as Split-Plot

Discussions with microbiologists identify 9 factors to study for the DNA amplification DOE:

- 3 factors are associated with the configuration of the thermocycler.
- 6 factors come with sample preparation on an automated liquid handling system.

The experiment is done with 96-well plates.
Key Points:

- Each combination of the 3 thermocycler factors \((2^3 = 8\) combinations\) must be run on a different plate; therefore 8 plates are required.
- Use a \(2^{9-1}\) fractional factorial (256 runs) for the 9 factors: 3 thermocycler + 6 sample preparation.

1. The 3 thermocycler factors, which are HTC, are varied between plates. They are “whole-plot” (or “whole-plate”).
2. The 6 sample preparation factors—easy to change (ETC) vary within the plates. They are the “subplot” (or “subplate”) factors.
DNA Amplification
$2^{9-1}$ Factorial run as Split-Plot

3 Thermocycler factors:

<table>
<thead>
<tr>
<th>Factor (HTC)</th>
<th>-1 level</th>
<th>+1 level</th>
<th>Factor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anneal Temperature</td>
<td>55°C</td>
<td>65°C</td>
<td>Inter-plate</td>
</tr>
<tr>
<td>Denature Temperature</td>
<td>90°C</td>
<td>97°C</td>
<td>Inter-plate</td>
</tr>
<tr>
<td>Denature Time</td>
<td>10 sec</td>
<td>20 sec</td>
<td>Inter-plate</td>
</tr>
</tbody>
</table>

6 Sample preparation factors:

<table>
<thead>
<tr>
<th>Factor (ETC)</th>
<th>-1 level</th>
<th>+1 level</th>
<th>Factor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward [FOR] primer</td>
<td>200 nM</td>
<td>900 nM</td>
<td>Reagent</td>
</tr>
<tr>
<td>Reverse [REV] primer</td>
<td>200 nM</td>
<td>900 nM</td>
<td>Reagent</td>
</tr>
<tr>
<td>DNA probe</td>
<td>100 nM</td>
<td>400 nM</td>
<td>Reagent</td>
</tr>
<tr>
<td>MgCl2</td>
<td>3 nM</td>
<td>9 nM</td>
<td>Reagent</td>
</tr>
<tr>
<td>Tween (PCR) buffer</td>
<td>0.005%</td>
<td>0.020%</td>
<td>Reagent</td>
</tr>
<tr>
<td>Taq Gold polymerase</td>
<td>0.1 units/μL</td>
<td>0.25 units/μL</td>
<td>Reagent</td>
</tr>
</tbody>
</table>

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$2^{9-1}$ Factorial Split-Plot
8 whole plots each with 32 subplots

<table>
<thead>
<tr>
<th>Anneal temperature</th>
<th>Denature temperature</th>
<th>Denature time</th>
<th>Combinations of the 6 sample preparation factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 deg C</td>
<td>90 deg C</td>
<td>10 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>65 deg C</td>
<td>90 deg C</td>
<td>10 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>55 deg C</td>
<td>97 deg C</td>
<td>10 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>65 deg C</td>
<td>97 deg C</td>
<td>10 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>55 deg C</td>
<td>90 deg C</td>
<td>20 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>65 deg C</td>
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<td>20 sec</td>
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</tr>
<tr>
<td>55 deg C</td>
<td>97 deg C</td>
<td>20 sec</td>
<td>32 sample preps on a plate</td>
</tr>
<tr>
<td>65 deg C</td>
<td>97 deg C</td>
<td>20 sec</td>
<td>32 sample preps on a plate</td>
</tr>
</tbody>
</table>

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Summary:

- If you wanted to change the thermocycler factors every run (randomize every cell change), you would need 256 plates, instead of just 8 (8 plates * 32 cells per plate = 256 cells).
- This would amount to having only 1 DOE cell on the plate at a time, a waste of resources.
- Using the split-plot structure minimizes the number of plate changes.

Using the split-plot structure minimizes the number of plate changes.

DNA Amplification
29-1 Factorial run as Split-Plot

96-well plate factors:
- D: Forward Primer
- E: Reverse Primer
- F: DNA Probe
- G: MgCl₂
- H: Tween (PCR) buffer
- J: Taq polymerase

Thermocycler factors:
- A: Anneal Temperature
- B: Denature Temperature
- C: Denature Time

With only 8 whole-plot combinations (plates), we can get our 256 runs with 256/8 = 32 cells per plate.
That’s 32 times faster!
DNA Amplification
Building the $2^{9-1}$ Factorial Split-Plot

1. Open “PCR starter.dxpx” and re-build the design using previous info for a split-plot, regular, two-level, fractional factorial design for 9 total factors, 3 hard-to-change factors done with 32 runs (change from 16) per group.
2²⁻¹ Factorial Split-Plot
Building the Design (page 2 of 2)

2. Check the factor names and levels already entered:

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
<th>Change</th>
<th>Type</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>a [Numer]</td>
<td>Anneal_Temp</td>
<td>deg C</td>
<td>HarD</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>b [Numer]</td>
<td>Denature_Temp</td>
<td>deg C</td>
<td>HarD</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>c [Numer]</td>
<td>Denaturing_time</td>
<td>sec</td>
<td>HarD</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>D [Numer]</td>
<td>F1 primer</td>
<td>mM</td>
<td>Easy</td>
<td>200</td>
<td>900</td>
</tr>
<tr>
<td>E [Numer]</td>
<td>REV_primer</td>
<td>mM</td>
<td>Easy</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>F [Numer]</td>
<td>Probe</td>
<td>mM</td>
<td>Easy</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>G [Numer]</td>
<td>MG_C12</td>
<td>mM</td>
<td>Easy</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>H [Numer]</td>
<td>Thera</td>
<td>%</td>
<td>Easy</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>J [Numer]</td>
<td>Taq_Gold</td>
<td>Unit</td>
<td>Easy</td>
<td>0.04</td>
<td>0.03</td>
</tr>
</tbody>
</table>

3. Check the values for delta and sigma:

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
<th>Diff. to detect Delta/Signal</th>
<th>Est. Subplot Signal/Noise</th>
<th>Delta/ Sigma Signal/Noise Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification</td>
<td></td>
<td>3</td>
<td>4</td>
<td>0.75</td>
</tr>
</tbody>
</table>

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2²⁻¹ Factorial Split-Plot
Power Calculation

The power for the whole-plot factors (a, b & c) falls way off due to fewer resets (only 8); the whole-plot factors form a 2³ full factorial.

This is the cost associated with not randomizing all factors!

*Note: The whole-plot by subplot interactions have the error associated with subplot effects. If one of these interactions is selected, then the whole-plot term gets included for hierarchy, and its low power isn’t as much of an issue.*

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This split-plot design is really two designs:

1. The whole-plot factors (a, b, & c), for setting up the thermocycler to treat the whole plate.
2. The subplot factors (D, E, F, G, H & J), for creating the samples in each cell of the plate.

Since well-to-well, subplot variation occurs only within a plate, the plate-to-plate variation is not included in the subplot variance.

The error associated with resetting the thermocycler factors or, plate-to-plate variation, is included in the whole-plot variance.

Other considerations:

- Every run varies due to resetting factor levels.
- Whole-plot treatments only change by group.
- Subplot treatments may be altered on every run.
- To account for the differing errors, separate half-normal plots are created for the whole plot and subplot effects.
- The multiple error sources also influence the power calculations. The more the error and the fewer the changes the less the power.
DNA Amplification
Analyze and Optimize

Design-Expert 13 Software Demo

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DNA Amplification
$2^{3-1}$ Factorial run as Split-Plot

Recommended factor settings to maximize Amplification:

- a: Anneal_Temp = 55.0238
- b: Denature_Temp = 97
- c: Denature_time = 15
- d: FOR_primer = 200.468
- e: REV_primer = 550
- f: Probe = 100.074
- g: MG_C12 = 6
- h: Tween = 0.0125
- i: Taq_Gold = 0.01

Desirability = 0.785

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1. Restricting randomization
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Split-Plot Designs

In combined designs it’s common that either the process factors or the mixture components are hard to change (HTC).

Process Factors HTC

Mixture Components HTC

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High performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column’s stationary phase.

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase.
Reverse Phase HPLC
Four Mixture Components (one fixed)

Mobile phase:

A. water
   60 to 80 volume %
B. methanol
   0 to 35 volume %
C. acetonitrile
   0 to 20 volume %
D. tetrahydrofuran (THF)
   fixed at 5 volume %

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Reverse Phase HPLC
Two Process Factors

Column factors:

- particle size (3.5, 6 or 10 mm)
- pore size (100, 200 or 300 Å)
- bonded phase is fixed (C18)
- column dimensions are fixed (4.6 x 250 mm)

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Reverse Phase HPLC
Two Process Factors

Design considerations:

- The mobile phase is easy to mix and change.
- The column factors (particle and pore sizes) are difficult and time consuming to change.

The experiments will be easier, faster and less costly if the mixture components are specified as easy-to-change and the column factors are specified as hard-to-change.

*Use a split-plot combined design with Mix components as ETC and Numeric factors as HTC.*

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Reverse Phase HPLC
Build a Combined Design Split Plot

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Design-Expert 13 Software Demo
1. Choose “File”, “New Design”, “Custom Designs” and “Optimal (Combined)” with “4” Mixture 1 components and “2” Numeric factors:

```
Mix 1 components: 4  (0 to 20)
Mix 2 components: 0  (0 to 10)
Numeric factors: 2  (0 to 10)
```

2. Leave the Mix components as “Easy” to Change and enter the names, low and high values:

```
<table>
<thead>
<tr>
<th>Name</th>
<th>Change</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>A [Mixture]</td>
<td>water</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>B [Mixture]</td>
<td>methanol</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>C [Mixture]</td>
<td>acetonitrile</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>D [Mixture]</td>
<td>THF</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
```
3. Make the process factors “Hard” to change and enter their names, low and high values:

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>particle</td>
<td>mm</td>
</tr>
<tr>
<td>pore</td>
<td>Å</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard</td>
<td>Discrete</td>
<td>3</td>
<td>3.5</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Hard</td>
<td>Discrete</td>
<td>3</td>
<td>100</td>
<td>200</td>
<td>300</td>
</tr>
</tbody>
</table>

Discrete factors are numeric factors with specific fixed levels; i.e. discrete levels.

4. Design for a quadratic by quadratic model:

5. Enter the response Height of an Equivalent Theoretical Plate (HETP):

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>HETP</td>
<td>MM</td>
</tr>
</tbody>
</table>
Reverse Phase HPLC
Split-Plot Design

Nine groups defined by combinations of the two HTC process factors.

The three ETC active mixture components vary within the groups.

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Reverse Phase HPLC
Analyze and Optimize

Design-Expert 13
Software Demo

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Reverse Phase HPLC
Split-Plot Fixed Effects

After crossing the mixture model (ETC or subplot terms) with the process model (HTC or whole-plot terms), all terms have an ETC component, i.e., they are tested against the subplot error.

Reducing the model using Backward selection with the AICc criteria, and correcting for hierarchy yields:

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Reverse Phase HPLC
Split-Plot Variance Components

More experimental error is generated when changing the process factors (the Group Variance) than when the mixture components are changed (the Residual Variance):

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Minimize the Height of an Equivalent Theoretical Plate (HETP):

Desirability = 0.971
Solution 1 out of 37

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## Split-Plot Design Summary

### Advantages

**Pros include:**

- **Practical**: Randomizing hard-to-change (HTC) factors in groups, rather, than randomizing every run, is much less labor and time intensive. *(Carefully consider the cost savings of the split-plot design vs. the loss of power to detect HTC factor effects.)*

- **Flexible**: Factors that naturally have large experimental units can be easily combined with factors having smaller experimental units. *(Remember the DNA amplification: the plates are the “large units” and the 96 cells, on the plates, are the “small units”.*

- **More powerful**: Tests for the subplot effects from the easy-to-change (ETC) factors generally have higher power due to partitioning the variance sources.

---

### Disadvantages

**Cons include:**

- **Unfamiliar**: They result in differing errors for whole plot versus subplot terms. REML should be used for a proper statistical test. If you apply a standard ANOVA, it may select too many whole-plot factors and too few sub-plot factors.

- **Less powerful**: Tests for the hard-to-change factors are less powerful, having a larger variance to test against and fewer changes to help overcome the larger error.

- **Different**: HTC and ETC factor effects are tested against different estimated noise. This can result in large HTC effects not being statistically significant, whereas small ETC effects are significant even though they may not be practically important.
Split-Plot Design Summary
Facts to Remember

Things to remember:

- Not randomizing the groups increases the risk of confounding the group effects with a lurking variable.
- When a group change occurs, factors that are not changing should be reset.
- If factors are not reset, then it is another restriction on the randomization.

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Split Plots Pros and Cons:
Dealing with a Hard-to-Change Factor

Reminder, this presentation is posted at:
www.statease.com/webinar.html

If you have additional questions email them to:
stathelp@statease.com

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Stat-Ease Training:
Sharpen Up Your DOE Skills

- Modern DOE for Process Optimization
- Mixture Design for Optimal Formulations
- Designed Experiments for Pharma, Food, etc. (teams)

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Teams (6+ people)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improve your DOE skills</td>
<td>Choose your own date &amp; time</td>
</tr>
<tr>
<td>Ideal for novice to advanced levels</td>
<td>Customize via company case studies</td>
</tr>
</tbody>
</table>

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Thank You for Attending
Pat Whitcomb

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