

Design and analysis of a microplate assay in the presence of multiple restrictions on the randomization

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MeBiOS



Outline

1. What are microplates and why are we studying them ?
2. What are the randomization restrictions in the experiment?
3. How did we generate the design?
4. What did we learn from the experiment ?

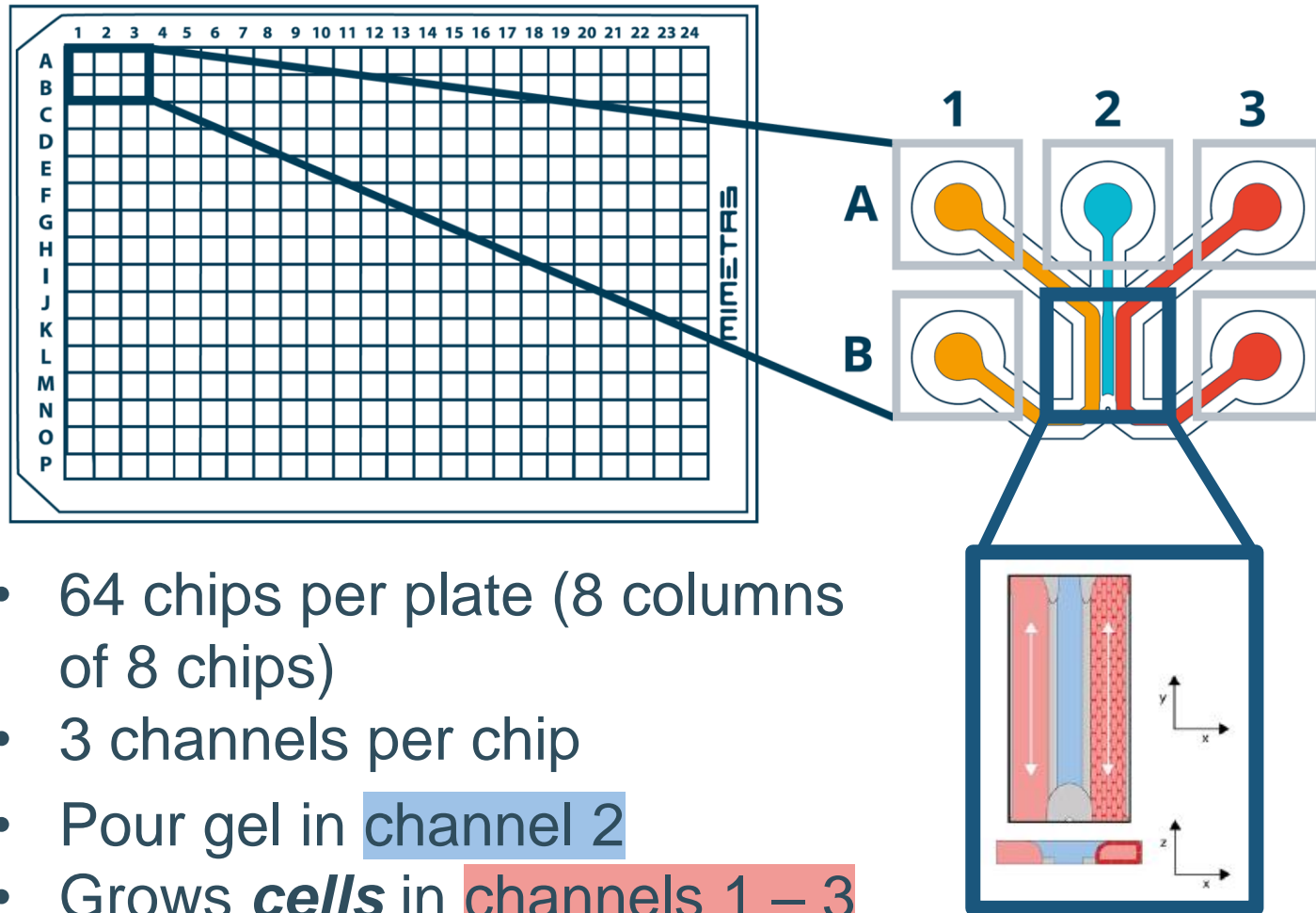
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Collaboration with Mimetis B.V.
(Leiden, NDL)

What are microplates and why are we studying them ?



OrganoPlate[®] by Mimetas



- 64 chips per plate (8 columns of 8 chips)
- 3 channels per chip
- Pour gel in **channel 2**
- Grows **cells** in **channels 1 – 3**

Preparation in a few global steps

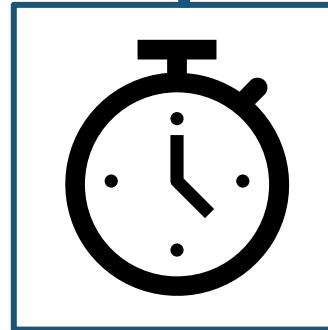
2. Pour into the chips



4. Measure fibrosity



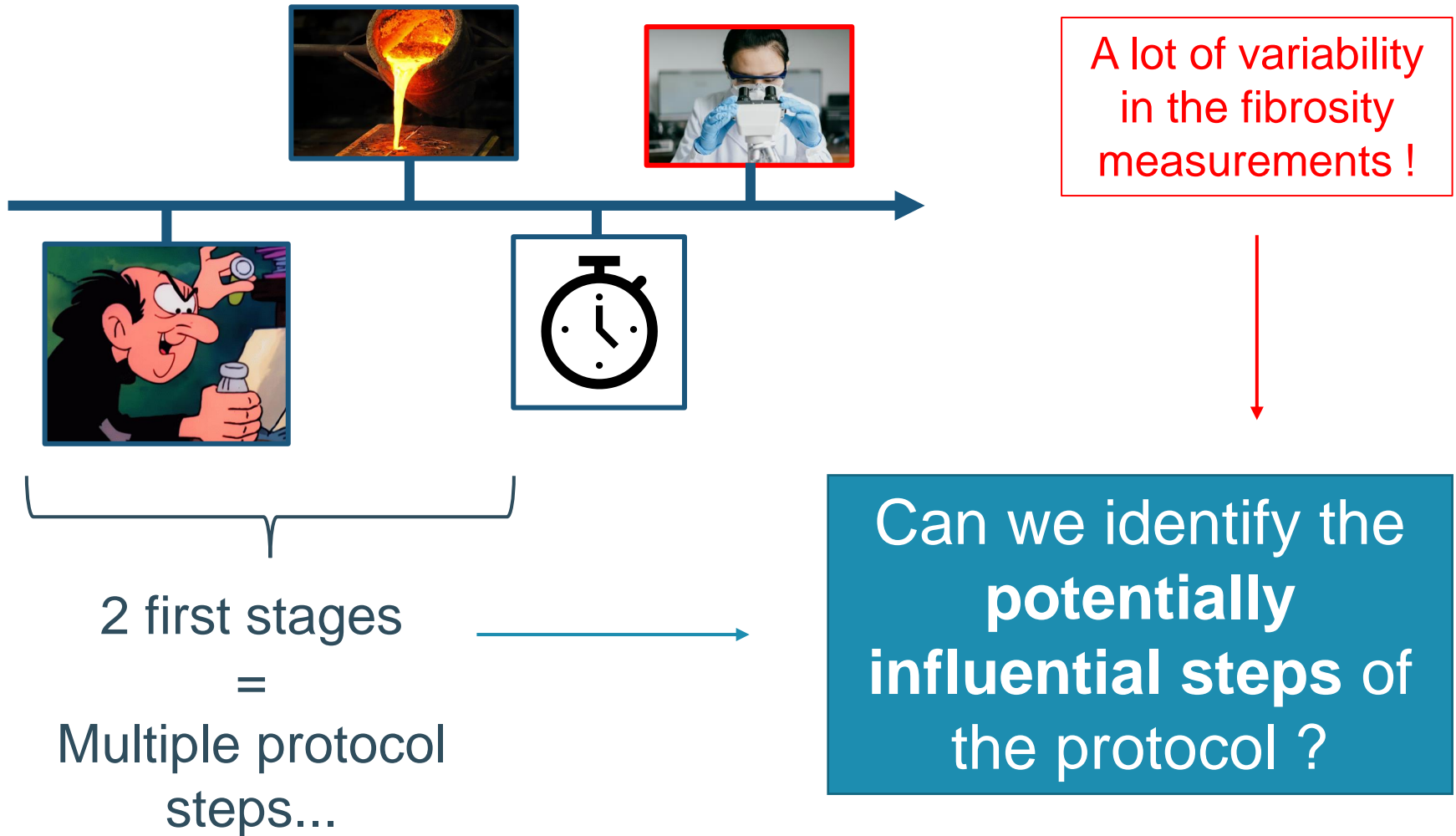
1. Prepare the gel



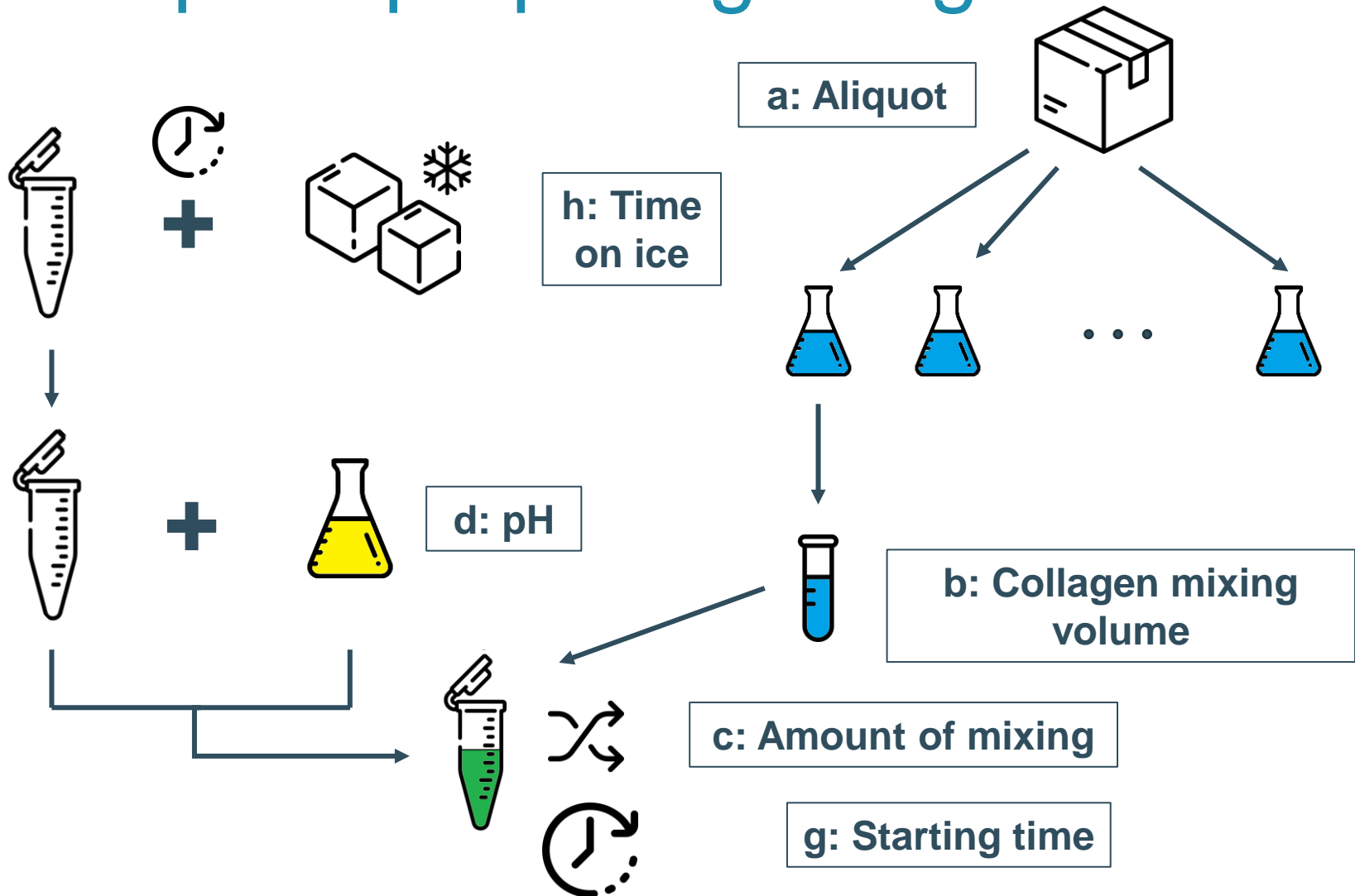
3. Wait for gel to set

...

What is the problem ?



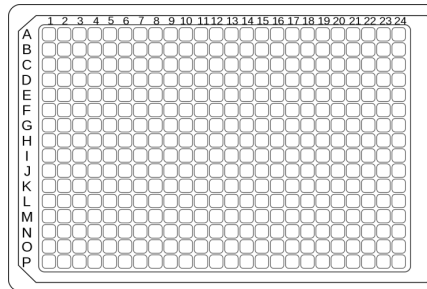
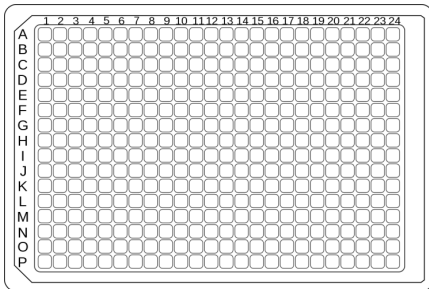
8 steps in preparing the gel...



... and finalizing the plate ...



e: HBSS Mg/Ca levels



f: HBSS removal

... means 8 factors studied...

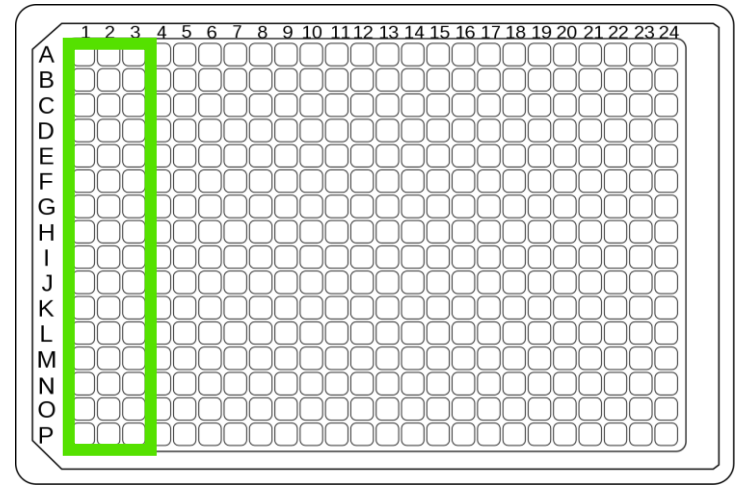
Stage	Letter	Factor	Settings	
Gel preparation	a	Aliquot	Late	Early
	b	Collagen mixing volume (μl)	100	300
	c	Amount of mixing	20	50
	d	pH of solution	7,1	8,3
	g	Starting time (min)	10	60
	h	Time on ice (min)	1	30
Loading	e	HBSS Mg/Ca levels	-	+
	f	HBSS removal	No	Yes



Time-related factors

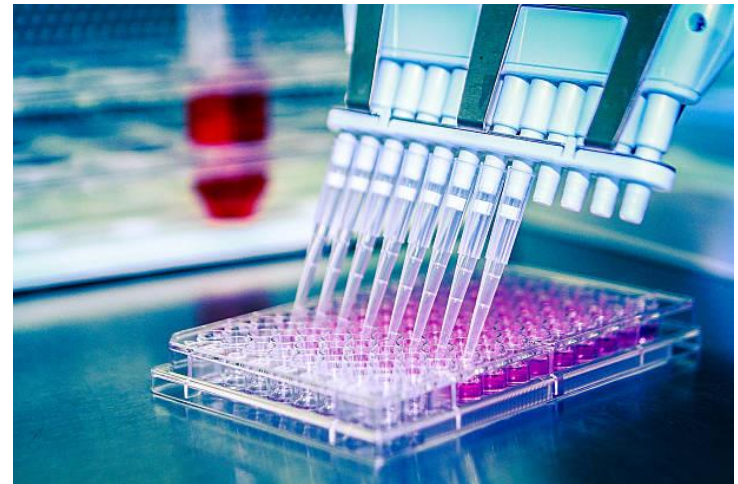
Four plates available

- 4 plates available:
→ $4 \times 64 = 256$ runs ?
- Factors can only be varied by **column**
→ only 8 runs per plate
- 4 plates \times 8 columns = **32 runs**



8 two-level factors
studied
over 32 runs

Should be easy, right ?



*What are the
randomization restrictions
in the experiment?*

Weeks and plates

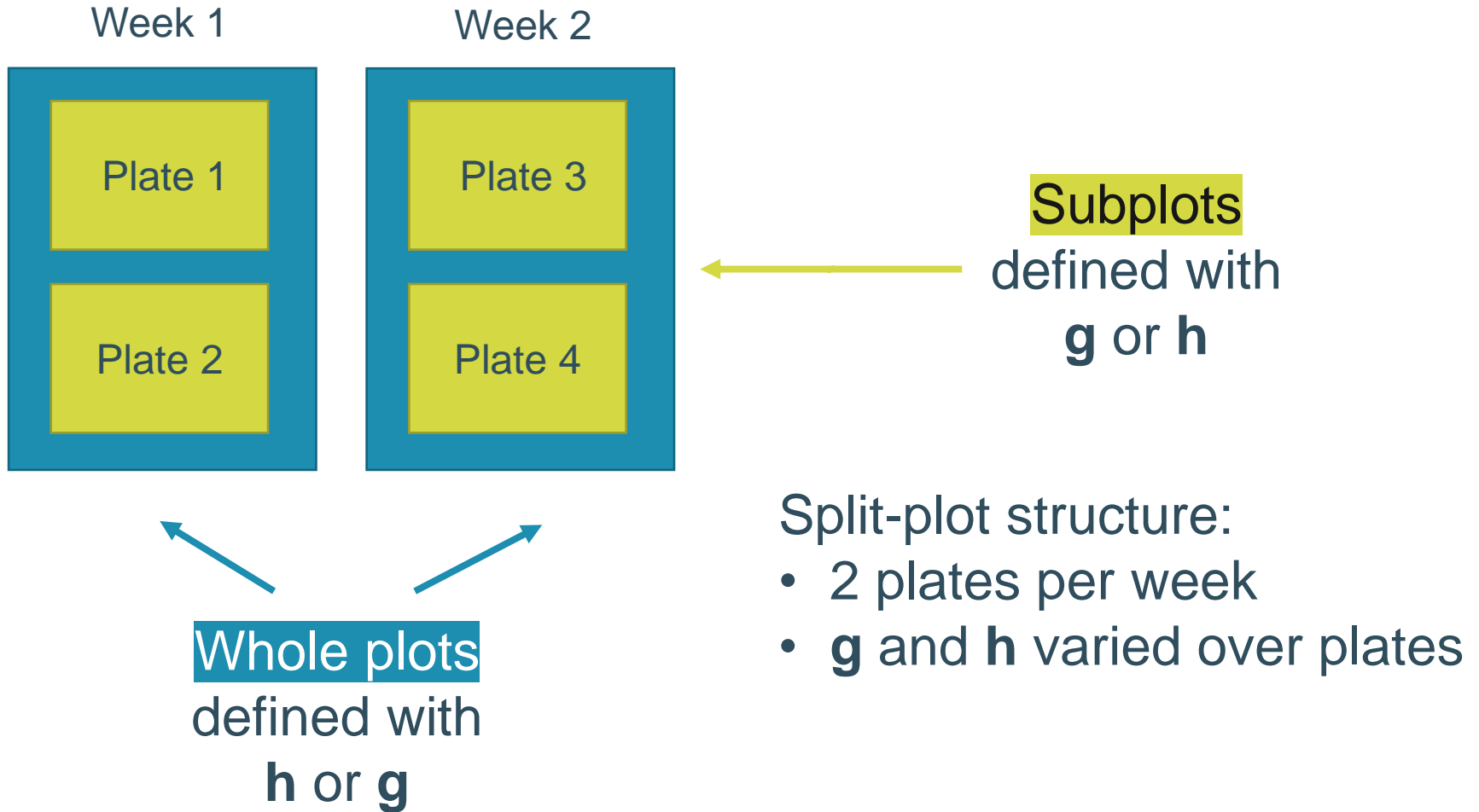
Time-related factors

- Factors **g** and **h** can only be varied between the plates
- Plate processing is *time-consuming*, so, we only have two plates processed per week
- Only **three options** to define the weeks and plates

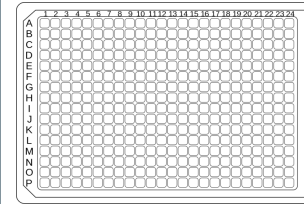
Plate	g	h
1	+	+
2	+	-
3	-	+
4	-	-

Week (h)	Week (g)	Week (gh)
1	1	2
2	1	1
1	2	1
2	2	2

Split-plot structure



Tube restrictions



Preparation						Loading	
a	b	c	d	g	h	e	f

- 4 factors can be varied between the tubes
- 32 runs so naturally → 32 tubes (one per run)
- Only 8 tubes feasible per week → 16 tubes in total

How to dispatch the 16 tubes over the 4 plates ?

We reuse the tubes

Each tube is used once on each plate within a week:

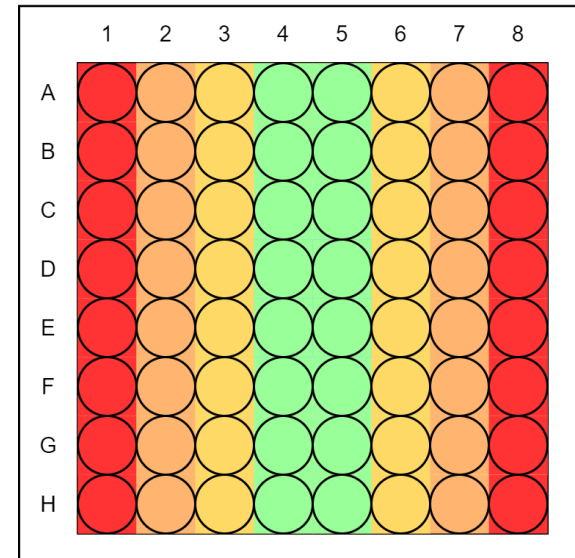
Split-strip-plot structure

→ Week / (Plate × Tube)

		Tube															
Week	Plate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1	●	●	●	●	●	●	●	●								
	2	●	●	●	●	●	●	●	●								
2	3									●	●	●	●	●	●	●	●
	4									●	●	●	●	●	●	●	●

Careful about the edge effects...

- Edge effect = uneven response depending on position on the plate
- Taking it into account:
→ Balancing the factor levels over the column positions on the plate
- We create **8 blocks for the 8 column positions** of a plate
- Equivalent to arrange design for factors **a** to **f** in 8 blocks



In summary: 8 factors and a complex structure

Preparation						Loading	
g	h	a	b	c	d	e	f

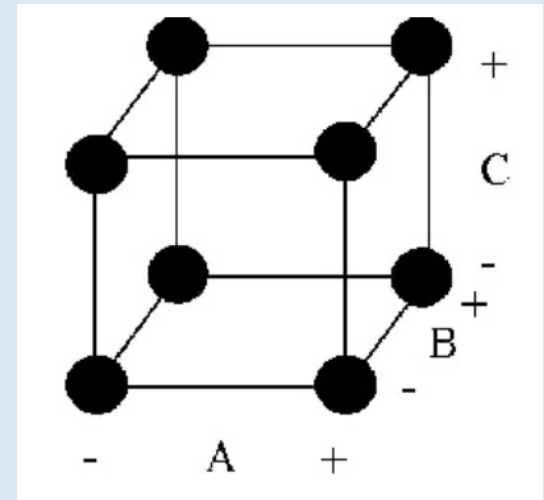
Week	Plate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1	•	•	•	•	•	•	•	•								
	2	•	•	•	•	•	•	•	•								
2	3									•	•	•	•	•	•	•	•
	4									•	•	•	•	•	•	•	•

Split-strip-plot

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

+ 8 blocks over the column positions

How did we generate the design?



Regular design created in 4 steps

1. 2^{6-1} design for factors **a** to **f**
2. Arrange the design in 8 blocks (= 8 column positions)
3. Define the two remaining factors **g** and **h**
4. Ensure that there are indeed 8 tubes per week, used once on each plate (split-strip-plot structure)

Steps 1 and 2

1. 2^{6-1} design for factors **a** to **f**:

Resolution VI design with **f = abcde**

2. Arrange the design in 8 blocks:

Blocking scheme from Mee (2009)
with:

$$p_1 = ab; \quad p_2 = ce; \quad p_3 = acf \quad \longrightarrow$$

→ minimizes confounding two-factor interactions with blocks

p_1	p_2	p_3	Col.
+	+	+	1
+	+	-	2
+	-	+	3
+	-	-	4
-	+	+	5
-	+	-	6
-	-	+	7
-	-	-	8

8 level combinations
=
8 blocks

Several options for Step 3

3. Define factors g and h

- Add orthogonal blocking over factors **a-f** using the OApackage :
→ only 3 regular options
- Determine **aliasing** between **a-f** and the four blocks
- Link these effects with factors **g** and **h**
- **Pick the best option**

Four blocks = four plates

Option	Factor	Aliasing
1	g	ace + bdf
	h	abc + def
	gh	be
2	g	cd
	h	ad
	gh	ac
3	g	ef
	h	ad
	gh	ce

Only two choices for Step 4

Among factors **g** and **h**, one is used for the weeks and the other for the two plates per week

Factor g for weeks

Week	Plate	Tube															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1	•	•	•	•	•	•	•	•								
	2									•	•	•	•	•	•	•	•
2	3	•	•	•	•	•	•	•	•								
	4									•	•	•	•	•	•	•	•

Each of the 16 tubes used once in each week



Factor h for weeks

Week	Plate	Tube															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1	•	•	•	•	•	•	•	•								
	2	•	•	•	•	•	•	•	•								
2	3									•	•	•	•	•	•	•	•
	4									•	•	•	•	•	•	•	•

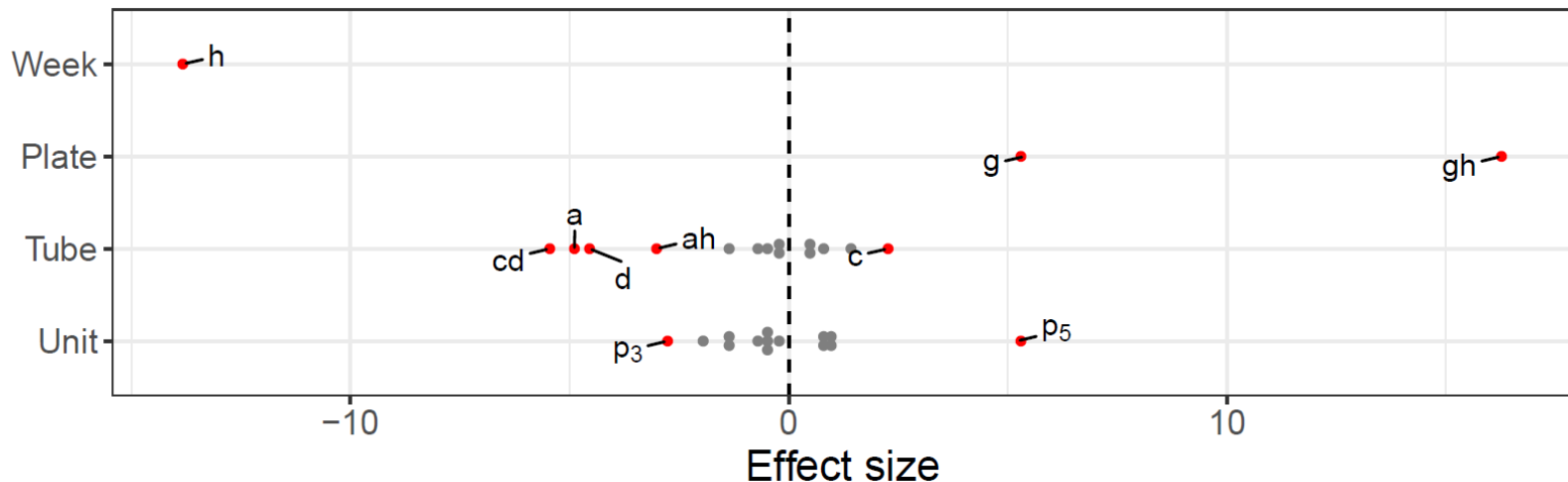
Each tube is used twice within a week



Effects divided in strata

- Effects assigned to the level at which they vary:
 - Level is called a **stratum**
 - *Ex: h varies between the weeks \rightarrow week stratum*
 - Four strata: *Week, Plate, Tube, Unit*

Stratum	df
Week	1
Plate	2
Tube	14
Unit	14



Error structure

<i>Week</i>	<i>Plate</i>	<i>Tube</i>	<i>Unit</i>
h	g	a	e
	gh + be	b	f
		c	$p_2 + ag + ce$
		d	p_3
		$p_1 + ab + ch$	$p_4 + bg + df + eh$
		p_6	p_5
		p_7	$ae + cg$
		$ac + bh + eg$	af
		ad	$bf + dg$
		$ah + bc$	cf
		$bd + fg$	$de + fh$
		cd	agh
		$dh + ef$	adg
		abd	cgh
1 df	2 df	14 df	14 df

- No main effects aliased with 2FI or 3FI
- Four 2FI not aliased with another 2FI
- Main effects of factors **g** and **h**, and their interaction **gh** are not testable (not enough df)

*What did we learn
from the experiment ?*

Conclusion

Conclusions

- Practical conditions impose restrictions on the randomization
- Even simple experiments can have a complex error structure
- Mimetas very satisfied with DOE → **better protocol implemented**

Thank you for your
attention

Extra slides on the data analysis and alternative scenarios

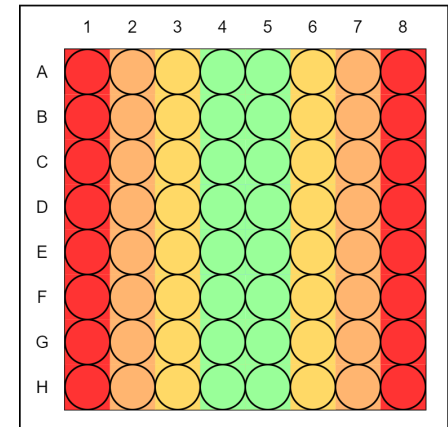
What if we could change things ?

Alternative scenarios

Four alternative scenarios

All scenarios make sense in practice:

1. Each tube is used twice on a single plate
2. 32 tubes are available instead of 16
3. All factors can be varied between the columns of the plates
4. Symmetric edge effect: only 4 blocks over the columns



Tubes used twice on a plate

- Back to the **split-split-plot** scenario discussed earlier
- Achieved by changing the allocation of tube to treatments
- Only change in error structure

Plate	Tube															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	•	•	•	•												
2					•	•	•	•								
3									•	•	•	•				
4													•	•	•	•

Stratum	df
Week	1
Plate	2
Tube	14
Unit	14




Stratum	df
Week	1
Plate	2
Tube	12
Unit	16

32 tubes available

- Each run gets its own tube
→ 16 tubes per week
- Similar to using factor **g** to define the weeks
- No variation at tube level
→ no more *Tube* stratum !
- 28 df in *Unit stratum*
→ Easier to detect active effects there

Week = g	Plate = h	Tubes used
+	+	1 → 8
+	-	9 → 16
-	+	1 → 8
-	-	9 → 16

Would be
numbered 17 to
32



Stratum	df
Week	1
Plate	2
Unit	28

All factors varied over the columns

- Redo 4-step design generation process
 - $2^{6-1} \rightarrow 2^{8-3}$ (**a to h**)
 - *Week* stratum contains: **cf**
 - *Plate* stratum contains: **ade, ag**

→ Better allocation of effects to error strata

- All main effects can be tested
- Now **seven 2FI** are not aliased with other 2FI (four in the original scenario)

<i>Tube</i>	<i>Unit</i>
a	e
b	g
c	h
d	$p_3 + ae + bg + ch$
f	p_5
$p_1 + df$	$p_6 + de$
$p_2 + ad$	$p_7 + ef$
$p_4 + af$	$ah + ce$
$ab + eg$	$bh + cg$
$ac + eh$	dg
$bc + gh$	dh
bd	fg
bf	fh
cd	aef
14 df	14 df

Four-level blocking factor

- Start with the same 2^{6-1} design
- Add a four-level blocking factor instead of eight-level
- 4 levels \rightarrow 3 df
- Still only 6 main effects testable
- Now 5 2FI not aliased with another 2FI (compared to 4)
- More 3FI are unaliased with other effects (6 compared to 3)

<i>Tube</i>	<i>Unit</i>
a	e
b	f
c	ae + bg
d	af
p1 + ab + ch + eg	ag + be
p2	bf
p3	cf + dg
ac + bh	cg + df + eh
ad	de + fh
ah + bc	abf
bd	ace
cd	acf
dh + ef	acg
abd	ade
14 df	14 df

Conclusion on the scenarios

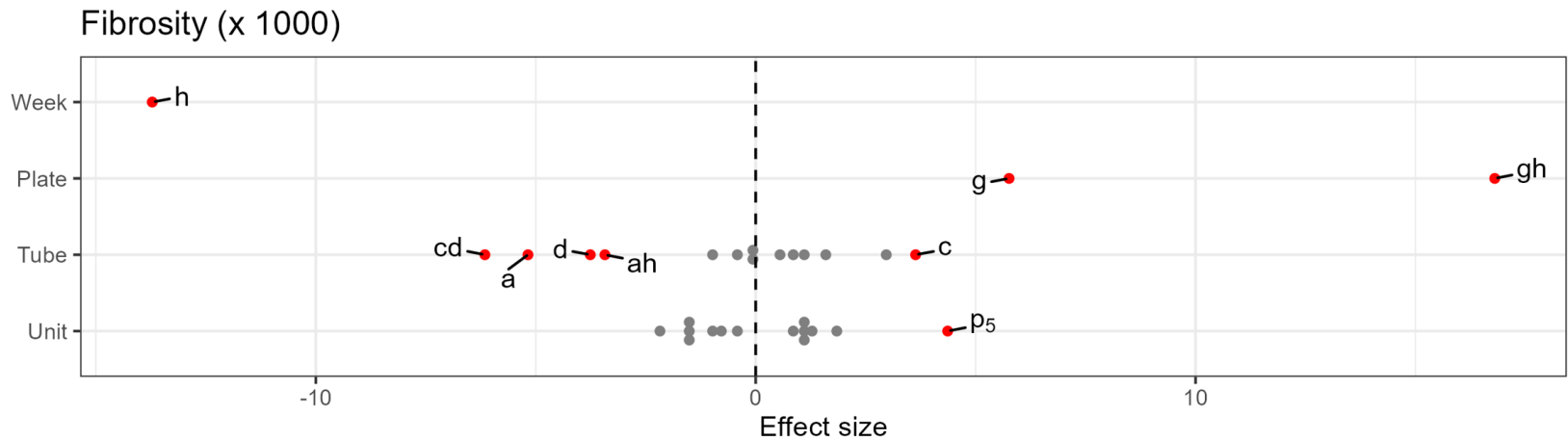
Scenario	+	-
Tube used twice on each plate	<ul style="list-style-type: none"> • More power in Unit 	<ul style="list-style-type: none"> • g, h, gh not testable • Less power in Tube
32 tubes	<ul style="list-style-type: none"> • Single Unit stratum with more power 	<ul style="list-style-type: none"> • g, h, gh not testable • 32 tubes needed
All factors varied	<ul style="list-style-type: none"> • All ME and more 2FI are testable 	<ul style="list-style-type: none"> • More complex logistically
Four-level blocking	<ul style="list-style-type: none"> • 4 df are free 	<ul style="list-style-type: none"> • g, h, gh not testable • Free df to 3FI • Assumption over edge-effect

Building the model

1. Estimate 31 coefficients
2. Separate them by error stratum
3. Compute a robust estimator of the standard error for the two strata with 14 *d.f.*
4. Determine active effects
5. Build a final model using the active effects

Modelling fibrosity

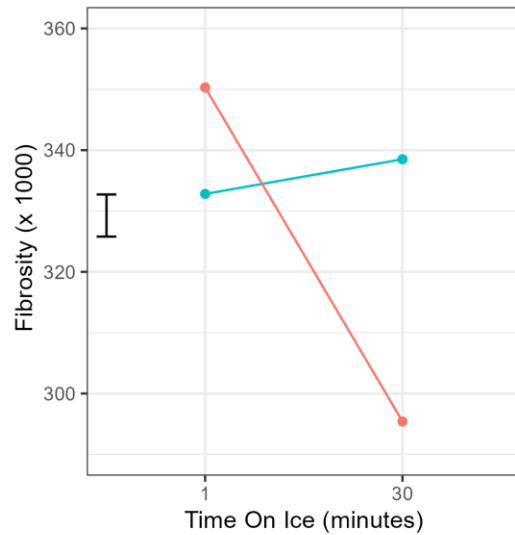
- Identify the factors that have an effect on fibrosity



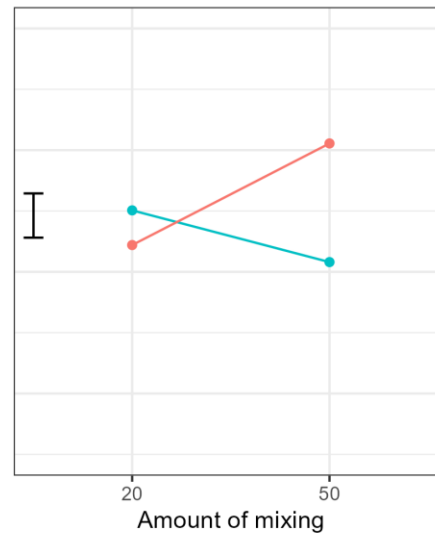
Letter	<i>a</i>	<i>c</i>	<i>d</i>	<i>g</i>	<i>h</i>	<i>p_x</i>
Factor	Aliquot	Mixing	pH	Start Time	Time on Ice	Column differences

Effect plots

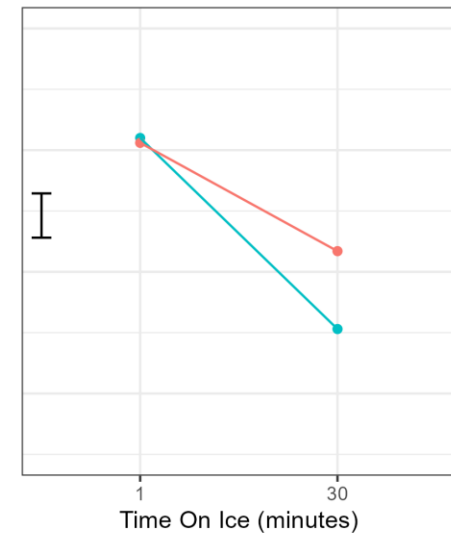
3 active interactions detected



Starting Time (minutes) — 10 — 60



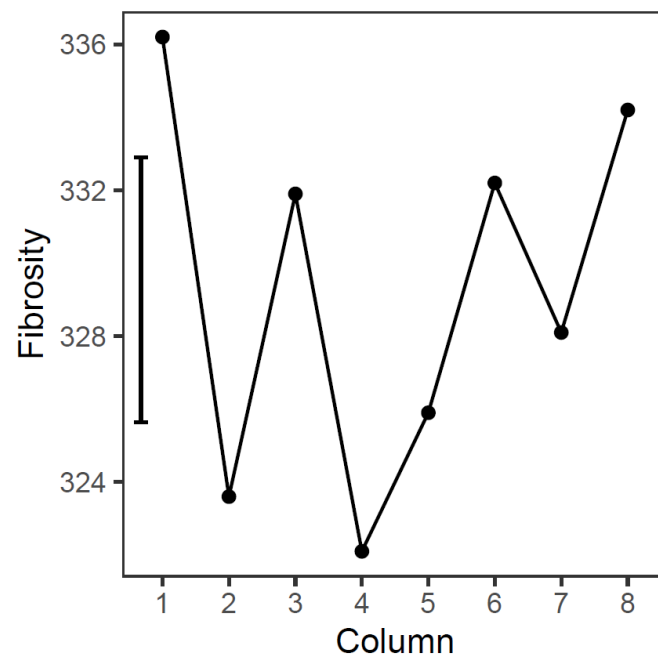
pH — 7.1 — 8.3



Aliquot — Early — Late

Column effect

High variation between columns and potential edge effects



All factors varied over the columns

Redo 4-step design generation process:

- Choose the MA 2^{8-3} design for factors **a** to **h**:
 $f = abcd$; $g = abe$; $h = ace$
- Arrange it in 8 blocks using Mee (2009):
 $p_1 = abc$; $p_2 = ad$; $p_3 = ae$
- Add 4-level factor using `OApkg` to define the weeks and the plates
- Arrange the design in 8 tubes per week:

Option	Factor	Aliasing
1	b_1	$ade + bdg + cdh$
	b_2	$cf + abd + deg$
	b_3	$ag + be + dfh$
2	b_1	$bc + gh + adf$
	b_2	$cf + abd + deg$
	b_3	$bf + acd + deh$
3	b_1	$bc + gh + adf$
	b_2	$ac + eh + bdf$
	b_3	$ab + eg + cdf$

Weeks = b_2 (abd)
 Plates = b_1 (ade)

Four-level blocking factor

- Start with the same 2^{6-1} design
- Add a four-level factor using Mee (2009):

$$p_1 = ab; \quad p_2 = acd$$
- 3 regular options to add a four-level factor to define factors **g** and **h**:
 1. Same as scenario 1
 2. Resolution III design
 3. OK with weeks = **h** = **abc**

Option	Factor	Aliasing
1	g	abd + cef
	h	abc + def
	gh	cd
2	g	adf + bce
	h	ac
	gh	abe + cdf
3	g	abe + cdf
	h	abc + def
	gh	ce

Stratum	df
Week	1
Plate	2
Tube	14
Unit	14

Summary of treatments allocation

