

Design of Experiment

A Powerful Tool to Optimise Sample Preparation in Bottom-up Targeted Protein LC-MS Workflows



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Conventional Optimisation



Changing a single factor at a time



- Does not always lead to real optimum
- Limited information
- Many experiments

Design of Experiment (DoE)



Conventional



DoE



- A strategically planed and executed series of experiments
- All factors (e.g. pH, solvent, temperature) are changed simultaneously

VS

- Allows to investigate multiple factors at the same time
- More information, model setup and predictive power
- Fewer experiments

Protein LC-MS Quantitation



Bottom-up approach Image: Section with the section of the sec

- Unique peptide selected
- Peptide analysis by LC-MS/MS





- 17 variables @ 2 levels at all combinations $\rightarrow 2^{17} = \sim 130,000$ experiments
- Full optimisation is not attempted
- Generic methods (empirical, historical) "Worked fine before"
- DoE for the help



1. Define objectives

2. Define factors

3. Selection of experimental design

4. Perform experiment

5. Process the data



1. Define objectives

- Model analyte: IgG1 antibody
- Spiked into rat plasma
- 4 abundant HC surrogate peptides selected:
 - DTLM FNWY TTPV

VVSV

2. Define factors

3. Selection of experimental design

Goal

4. Perform experiment

• Maximize the response for the 4 surrogate peptides selected

5. Process the data



What variable do we want to assess? At what levels? 1. Define objectives 2. Define factors Reaction buffer X • Chaotropic agent: guanidine, urea Reduction agent: DTT, TCEP Reduction agent concentration: 1 - 50 mM Reduction incubation time: 10 - 60 min ۲ 3. Selection of Reduction incubation temperature: 22 - 70°C experimental design Alkylation conditions X 4. Perform Protease enzyme type: methylated, non-methylated trypsin experiment Enzyme to protein ratio (amount of enzyme): 1:5 – 1:500 5. Process the data Digestion time: 1.5 hours - O/N ullet



1. Define objectives

Modde Go software package

2. Define factors

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Design Wizard

- Screening
 - Test a large number of factors
 - Normally 2 or 3 levels •
 - What factors have the most impact on the assay?
- Optimisation
 - Smaller number of factors
 - Min. 3 levels •
 - Model generation, prediction •
 - Find the best conditions
- **Output:** experiment table

Objec	tive 🔶	Responses	
hich type of des	ign do you want to do	?	
Q		Q	R
Screening	System	Optimization	Robustness
	Characterization	(RSM)	Verification

000										
	1	2	3	4	5	6	7	8	9	10
Ī	Exp No	Exp Name	Run Order	Incl/Excl	Enzyme	Chaotropic Agent	Reduction Agent	Reduction Agent Concentration	E/P ratio	reduction Temperature
1	1	N1	1	Incl v	Promega 🗸	Guanidine 🗸 🗸	DTT ~	5	50	22
2	2	N2	5	Incl v	Thermo 🗸	Guanidine 🗸 🗸	DTT v	5	100	22
3	13	N13	17	Incl v	Promega 🗸	Guanidine 🗸 🗸	TCEP 🗸	50	100	22
4	14	N14	14	Incl v	Thermo v	Guanidine 🗸 🗸	TCEP ~	50	50	22
5	11	N11	9	Incl v	Promega 🗸	Urea 🗸 🗸	DTT v	50	100	22
6	12	N12	13	inci v	Thermo v	Urea 🗸 🗸	DTT v	50	50	22
7	7	N7	4	inci v	Promega 🗸	Urea 🗸 🗸	TCEP ~	5	50	22
8	8	N8	2	inci v	Thermo v	Urea 🗸 🗸	TCEP ~	5	100	22
9	17	N17	3	inci v	Thermo v	Urea 🗸	DTT v	27.5	75	46
10	18	N18	19	inci v	Thermo v	Urea 🗸	DTT v	27.5	75	46
11	19	N19	22	inci v	Thermo v	Urea 🗸	DTT v	27.5	75	46
12	20	N20	20	Incl v	Promega 🗸	Urea 🗸 🗸	DTT v	27.5	75	46
13	21	N21	21	Incl v	Promega 🗸	Urea 🗸 🗸	DTT v	27.5	75	46
14	22	N22	18	Incl v	Promega 🗸	Urea 🗸 🗸	DTT v	27.5	75	46
15	9	N9	15	Incl v	Promega 🗸	Guanidine 🗸 🗸	DTT v	50	50	70
16	10	N10	10	Incl v	Thermo v	Guanidine 🗸 🗸	DTT v	50	100	70
17	5	N5	12	Incl v	Promega 🗸	Guanidine 🗸 🗸	TCEP ~	5	100	70
18	6	N6	7	Incl v	Thermo v	Guanidine 🗸 🗸	TCEP ~	5	50	70
19	3	N3	16	Incl v	Promega 🗸	Urea 🗸 🗸	DTT v	5	100	70
20	4	N4	6	inci v	Thermo v	Urea 🗸 🗸	DTT v	5	50	70
21	15	N15	8	inci v	Promega 🗸	Urea 🗸 🗸	TCEP ~	50	50	70
22	16	N16	11	inci v	Thermo v	Urea 🗸	TCEP ~	50	100	70



1. Define objectives

2. Define factors

3. Selection of experimental design

4. Perform experiment

Perform experiments

- Following experiment table
- Samples injected in a random order
- Waters Acquity Classic UPLC
 - Acquity UPLC peptide CSH C18 2.1x100 mm, 130 Å, 1.7 μm
- Waters Xevo TQS
 - Triple quadrupole



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5. Process the data

- Responses (peak area) imported from LC-MS data processing software
- Interpretation of results
- Visualisation
- Modelling
- Prediction of optimal conditions

Screening Design

DRUG DEVELOPMENT SOLUTIONS Part of Alliance Pharma, Inc.

- 6 factors at 2 or 3 levels
- Digestion time not included in the design performed twice: 1.5 hr and O/N digestion
- 3 replicates at center-point to assess variation



- Fractional factorial design
- 44 samples (injections)

Factor	Levels
Chaotropic agent	Guanidine Urea
Reduction reagent	DTT TCEP
Trypsin	Methylated Non-methylated
Reduction agent concentration (mM)	5 27.5 50
Reduction temperature (°C)	22 46 70
E/P ratio	1:50 1:75 1:100

Screening design – Results



Replicate plots for VVSV surrogate peptide



- Center-point replicates (blue squares) are very tight → high data quality
- Highest response is obtained by short digestion \rightarrow **O/N digestion not required**

Screening design - Results



What is significant? Coefficient plot for VVSV surrogate peptide

Coefficients (scaled and centered) (PLS) Screening_main factors-linear_LGC_1andHalf Hour digestion



Optimisation Design





- 4 factors at 3 levels
- Methylated trypsin not affordable at low E/P
- 3 replicates at center-point



- Reduced central composite face centered design
- 23 samples (injections)

Factor	Levels
DTT concentration (mM)	1 13 25
Reduction temperature (°C)	22 46 70
Reduction time (min)	10 35 60
E/P ratio	5 27.5 50

Optimisation – Results



Coefficient plot for VVSV surrogate peptide



- DTT concentration improves the response
- Reduction time has a negative effect
- Interaction effects detected: DDT conc. x temperature and temperature x time

Optimisation – Results



- Model generated
 Surface response plot for VVSV
- Prediction
 - Modde Optimizer for optimal sample preparation conditions

60

1e+06

800000

600000

1.2e+06

1.3e+06

1.2e+06

1.1e+06 1e+06

900000

800000

700000

600000

500,000

60

Outcome

- No single method fits for all
- 2 optimal methods for peptide pairs
 - 1. DTLM & FNWY:
 - Reduce with 1 mM DTT for 10 min @ 70°C

70

- Digest for 1.5 hours with E/P 1:50 (non-methylated enzymed)
- 2. TPEV & VVSV:
 - Reduce with 23mM DTT for 15 min @ 27°C 50
 - Digest for 1.5 hours with E/P 1:10 (non-methylated enzyme) Red. time (min) 6070

Next step: assess optimal methods vs a generic preparation

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Optimizer Dynamic Contour *			Pro Design s	Pro		Pro De		Design space				
profile Optimizer i	nterpretat	ion	Face -	explo	rer	explo	ration * S	etpo	oint analysis	Fa		
Replicate Plot Summar		y of F	it Plot	Work	csheet	F	Responses		Factors	Coefficient Pl	ot Opt	imizer ×
Setpoint	μ,	Obj	jective	Setpoint	(#3)	Alterna	tive setpoir	nts				
Selected cetroint: #2			Res	ponse Obje		ective	Value		Response range		log(D)	Prob. of failu
ociceted seepointing.	1		TPEV1		Maximize		279	993		•	-0.889239	
Alternative setpoints:		2	FNWY	2	Maxi	mize	13773.6			•	-1.23732	
# log(D) Prob.	of 🔨	3	DTLM	1	Maxi	mize	158	750		-	-1.03517	
1 -0.988		4	DTLM	ox1	Predi	cted	3759	.21		-		
2 -0.862		5	VVSV	1	Maxi	mize	1.07913e+	+06		-	-1.13045	
3 -1.05		6	VVSV	2	Predi	cted	190	918		•		
4 -1.04		7	% Oxi	dation	Predi	cted	2.67	479				
5 1.02		8	% Dez	midation	Predi	cted	6.56	669				

Optimisation – Prediction Validation

DTLM FNWY Sample113 : MRM of 8 Channels ES+ Sample113 2: MRM of 8 Channels ES 418.5 > 506.28 (DTLM 1) 560.3 > 765.87 (ENWY 2) 100-100 1.00e **2x 10x** 0.90 0.95 1.00 1.05 1.10 1.15 1.20 1.25 1 40 1.45 1.50 1.55 2.50 2.55 2.60 2.65 2.70 2.75 2.80 2.85 2.90 3.05 3 10 3.15 3.20 Sample111 2: MRM of 8 Channels ES+ Sample113 3: MRM of 4 Channels ES+ 714.1 > 472.28 (TPEV 1) 603.73 > 805.92 (VVSV 1) TPEV **VVSV** 100-100 1.20e7 **10x 50x** 3.20 3.30 3.40 3.50 3.60 3.70 3.80 3.90 4.00 5.50 5.60 5.70 5.80 5.90 6.00 6.10 6.20 6.30 6.40 6.50

DRUG DEVELOPMENT

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- Optimal preparation conditions for DTLM and FNWY by DoE (~3-hour prep.)
- Optimal preparation conditions for TPEV and VVSV by DoE (~3-hour prep.)
- Control generic preparation setup (2-day prep.)

Conclusions



- DoE excellent tool for protein sample preparation optimisation for LC-MS assays
- Achieved comprehensive optimisation within minimal experiments (~70 vs ~500)
- Reliable predictive power responses changed as predicted by the model
- Peptide yields from IgG1 increased by $\mathbf{10}\text{-}\mathbf{50x} \rightarrow \mathbf{increased}$ sensitivity
- Significant reduction of sample preparation time (~3 hours vs O/N)

\rightarrow higher throughput

• Challenge: difficult to execute in the lab \rightarrow looking at automation options



Special Thanks!



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Thank you for your attention

Any further questions?

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