

Einfluss von Zuckern auf lsr-GFP Expression

WEB 202

- 3 ml Kultiv LB-Medium (+ 150 µg/ml Amphotericin B und 1) + 0.4% Glucose Zugabe
 2) + 0.4% Lactose Zugabe
 3) + 0.4% Rhamnose Zugabe
 4) nur LB-Medium
- mit Einzelkolonie von A) E.coli JW1593 sydGH pUCB1 GFP-DAS+
 B) E.coli DH5α pUCB1 GFP-DAS+ angeimpft und über Nacht bei 37°C / 200 rpm inkubiert

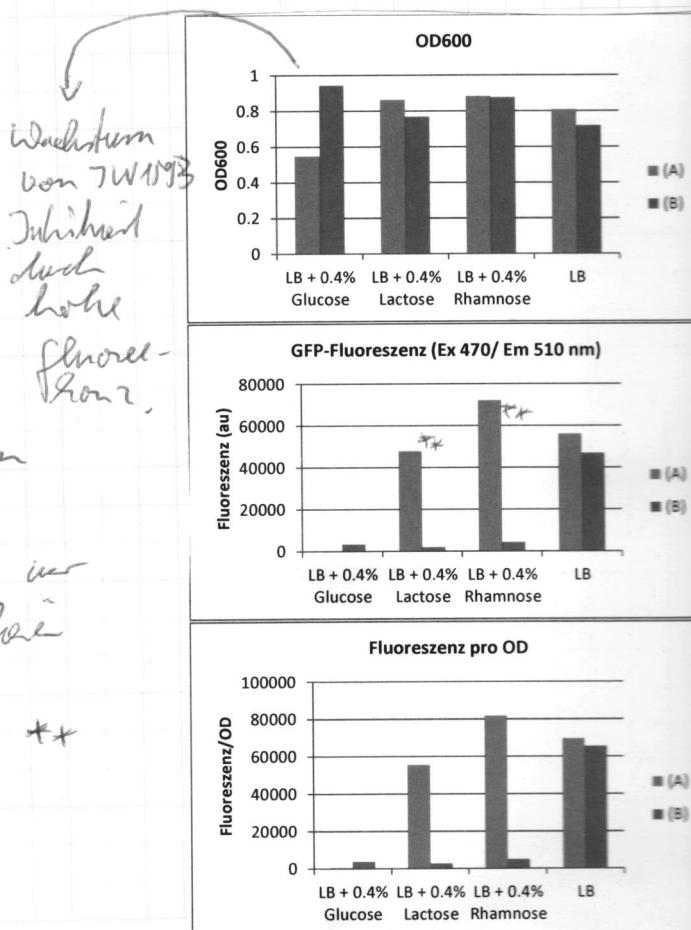
Auswertung:

- 200 µl Probe genommen und davon je 100 µl in NTP zur OD₆₀₀-Bestimmung und GFP-Fluoreszenz gegeben (Ex 470 nm, Em 510 nm)

→ Glucose inhibiert GFP-Expression (durch CRP)

→ Lactose & Rhamnose inhibieren die GFP-Expression bei DH5α → Inhibition durch A1-2? *

→ JW1593 nicht inhibiert, da kein A1-2 Snap-off notwendig



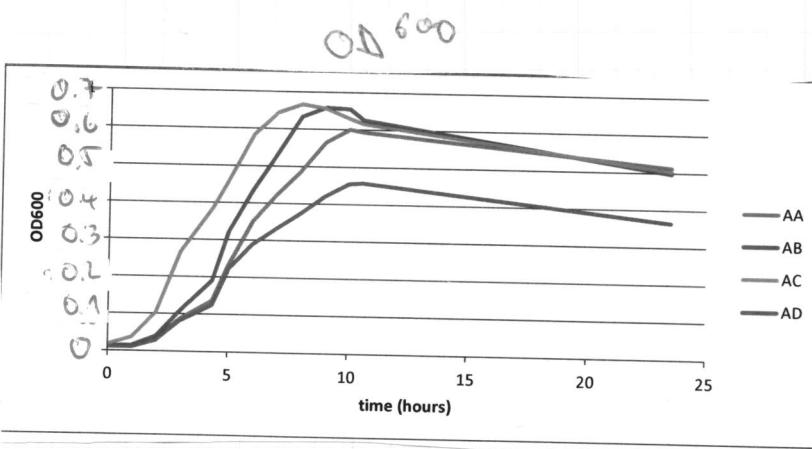
Coculturen (A) JM110 pheB1 sfGFP-DAS + 4 und

(B) JM110 pheB1 ♂ ; (C) JW1893 pheB1 ♂
(D) DH5 α pheB1 ♂ (λ -irresistible)

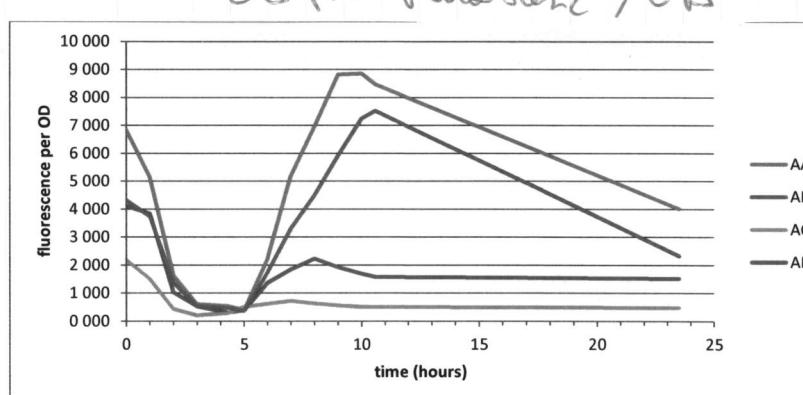
- 3ml Kultur über Nacht von (A) - (D) (37°C / 200 rpm)
- Zelle pelletiert (5min @ 5000 xg)
- 2x mit 2ml warmem PBS gewaschen
- in 1ml LB aufgezogen und OD⁶⁰⁰ bestimmt

	OD ⁶⁰⁰	VF für OD ⁶⁰⁰ = 0.05
A	3,717	74,3
B	3,805	76,1
C	5,703	114
D	3,885	77,7

→ @ 4°C gelagert, bis zum nächsten Tag



• 10 ml Kultur
(LB + 110 µg/ml Amp)
in 20 ml Kolbe
→ zu je 0,05 (OD⁶⁰⁰)
angemischt



Kultur	V(A)	V(X)
AA	134 µl	134 µl
AB	n	131 µl
AC	n	89 µl
AD	n	129 µl

→ bei 37°C / 200 rpm
in Subiel & Schmid
Kolbe pronate

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- ständliche hohe Fluoreszenz ab $00^{\circ\circ}$ & GFP-Fluoreszenz
im MFP gemessen
- Entwicklung wird ganz etabliert
Etabliert:
 - höchste Fluoreszenz \rightarrow AD der D A1-2 exponiert & A dient anfachend dann
 - geringste Fluoreszenz \sim AC, da A1-2 nicht exponiert werden kann wenn C \sim am wenigsten A1-2 vorhanden

AA
A1

Merke an A12:
im Kulturbürostaat

$$AD \Rightarrow AA = AB \Rightarrow AC$$

Fluoreszenz: $AD \Rightarrow AA > AB > AC$

~ aber AA größere Fluoreszenz als AD

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② phe81 sf GFP-DAS + 4 8-① pBETW1a pFront

② pBETW1a SspB pFront

E. coli

transformiert in JW1593 Δgdgb Zelle

→ ~ 80 µg ① & ~ 100 µg ② oder ③
für Transfektion

- in 500µl SOC → wachst plattiert

→ Colony PCR von Kolonien & Mask-plate:

Primer mix I für phe81

← sffyFP-fw
sffyFP-rv

Primer mix II für pBETW

- TT term
- pBETW-shield

Elongationszeit 2:10 min :)

→ N. fluorescens, aber zu lange lange lange

sfGFP
MT 2 3 4 5 1 6 7 8 9 10



sffyFP-Bande und dicht
in Tasche des nächsten
Kantens → ↗

→ aber: grüne Kolonien ↗

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Glucosebestimmung mit GOD / HRP

Glucoseoxidase horseradish perox.

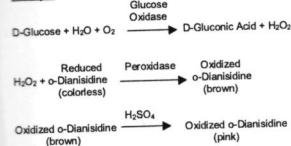
Assay abgewandelt nach Technical Bulletin von Sigma -
Aldrich

**GLUCOSE (GO) ASSAY KIT**

Product Number GAGO-20

TECHNICAL BULLETIN**Product Information****Product Description**

Enzymes, as analytical tools, have found widespread use in the food, biochemical, and pharmaceutical industry. Enzymatic methods are specific, reproducible, sensitive, rapid, and therefore, ideal for analytical purposes. Due to the high specificity and sensitivity of enzymes, quantitative assays may be done on crude materials with little or no sample preparation. This kit is for the quantitative, enzymatic determination of glucose in food and other materials.

Principle

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color measured at 540 nm is proportional to the original glucose concentration.

Reagents

- Glucose Oxidase/Peroxidase Reagent (Product No. G 3660)
Store the unopened kit reagent at 2-8 °C. Each capsule contains 500 units of Glucose Oxidase (*Aspergillus niger*), 100 Purpurallin units of peroxidase (horseradish) and buffer salts. In an amber bottle, dissolve the contents of the capsule in 39.2 ml of deionized water. The solution is stable up to one month at 2-8 °C and for at least 6 months frozen at -20 °C. Discard if turbidity develops.
- o-Dianisidine Reagent (Product No. D 2679)
Store the unopened kit reagent at 2-8 °C. Minimize exposure to light. Prewheighed vial contains 5 mg of o-dianisidine dihydrochloride. Reconstitute the vial

- of o-dianisidine with 1.0 ml of deionized water. Invert the vial several times to dissolve. Avoid exposing the reagent to light. Solution is stable for 3 months at 2-8 °C.
- Assay Reagent
Add 0.8 ml of the o-Dianisidine Reagent to the amber bottle containing the 39.2 ml of Glucose Oxidase/Peroxidase Reagent. Invert bottle several times to mix. Minimize exposure to light. Solution is stable up to 1 month at 2-8 °C. Discard if turbidity develops or color forms.
 - Glucose Standard Solution (Product No. G 3285)
D-Glucose, 1.0 mg/ml in 0.1% benzoic acid. Store reagent at 2-8 °C. Supplied ready to use. Solution is stable at 2-8 °C for at least six months. Discard if turbidity develops.

Reagents not included in kit:
Sulfuric Acid, ACS reagent (Product No. S 1526)
Reagent is 36 N sulfuric acid. Prepare a 12 N solution in deionized water.

- Apparatus**
1. Spectrophotometer or colorimeter suitable for measuring absorbance at 540 nm.
2. Cuvettes
3. Test tubes, 18 mm X 150 mm
4. Pipettes capable of accurately dispensing volumes from 20 µl to 2.0 ml.
5. Water bath capable of maintaining temperature at 37 ± 1 °C.

Precautions and Disclaimer
Refer to Material Safety Data Sheets for updated risk, hazard, or safety information.

Procedure
Sample Preparation

Liquids:
Dilute sample with deionized water to approximately 20 - 80 µg glucose/ml. Filter or deproteinize solution if necessary to clarify. Decolorize solutions that are strongly colored and that have a low glucose concentration. Degas carbonated or fermented products.

Solids:
Weigh out sample to nearest 0.1 mg. Extract sample with deionized water. The solution may be heated (>75 °C) to aid extraction. Dilute with deionized water to approximately 20 - 80 µg glucose/ml. Filter or deproteinize solution if necessary to clarify.

Determination
Method 1 - Glucose Concentration from Standard Curve

- Pipette the following solutions into the appropriately marked test tubes:

Tube	Water (ml)	Sample (ml)	Glucose Standard (ml)
Reagent Blank	1.00	—	—
Standard # 1	0.98	—	0.02
Standard # 2	0.96	—	0.04
Standard # 3	0.94	—	0.06
Standard # 4	0.92	—	0.08
Test	—	1.00	—

- At zero time, start the reaction by adding 2.0 ml of Assay Reagent to the first tube and mixing. Allow a 30 to 60 second interval between additions of Assay Reagent to each subsequent tube.

- Let each tube react exactly 30 minutes at 37 °C. Stop the reaction at 30-60 second intervals by adding 2.0 ml of 12 N H₂SO₄ into each tube. Carefully mix each tube thoroughly.

- Measure the absorbance of each tube against the reagent blank at 540 nm.

Method 2 - Glucose Concentration from a Single Standard

- Pipette the following solutions into the appropriately marked test tubes:

Tube	Water (ml)	Sample (ml)	Glucose Standard (ml)
Reagent Blank	1.00	—	—
Standard	0.95	—	0.05
Test	—	1.00	—

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CMH/MAM/KMR 04/02

→ buffer const.?
→ Agarose mit GOD
→ pH Optimum bei ~5.0 pH

→ Sigma: Enzymatic assay of Glucose
→ Rühr 50 mM NaOAc pH 8.1 @ 35°C
mit HCl eingestellt.

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honey

- ① • 500 U GOD (Sigma G-6125, G-7773 Typ II
 ↓
 (Cat 45H9506) von App. Nip
 $\approx 22 \text{ mg } (@ 23000 \text{ U/mg})$
 • 100 U HRP ($@ 273 \text{ U/mg}$) $\approx 0.4 \text{ mg}$

→ 500 U GOD & 100 U HRP in 33.7 ml $50 \text{ mM NaOAc pH 5.1}$
 lösen

② 5 mg O-Dianisidin HCl in 1 ml ddH₂O löse

③ 0.8 ml ② zu 33.7 ml ① gebe

↳ Glucose-Bestimmungsreakt. $\approx 0.15 \text{ mg/ml Dianisidin}$

Ablaufplanung: RN (6 h) H_2SO_4 in ddH₂O

→ für Mikrotiterplatte:

- 25 µl Probe + 50 µl Glucose-Bestimmungsreakt. ③ zusammengegeben
- 30 min @ 31°C / 200 rpm im Schüttler inkubiert
- mit 50 µl 6 M H₂SO₄ abstopfen

→ Absorption bei 540 nm (pink) messen

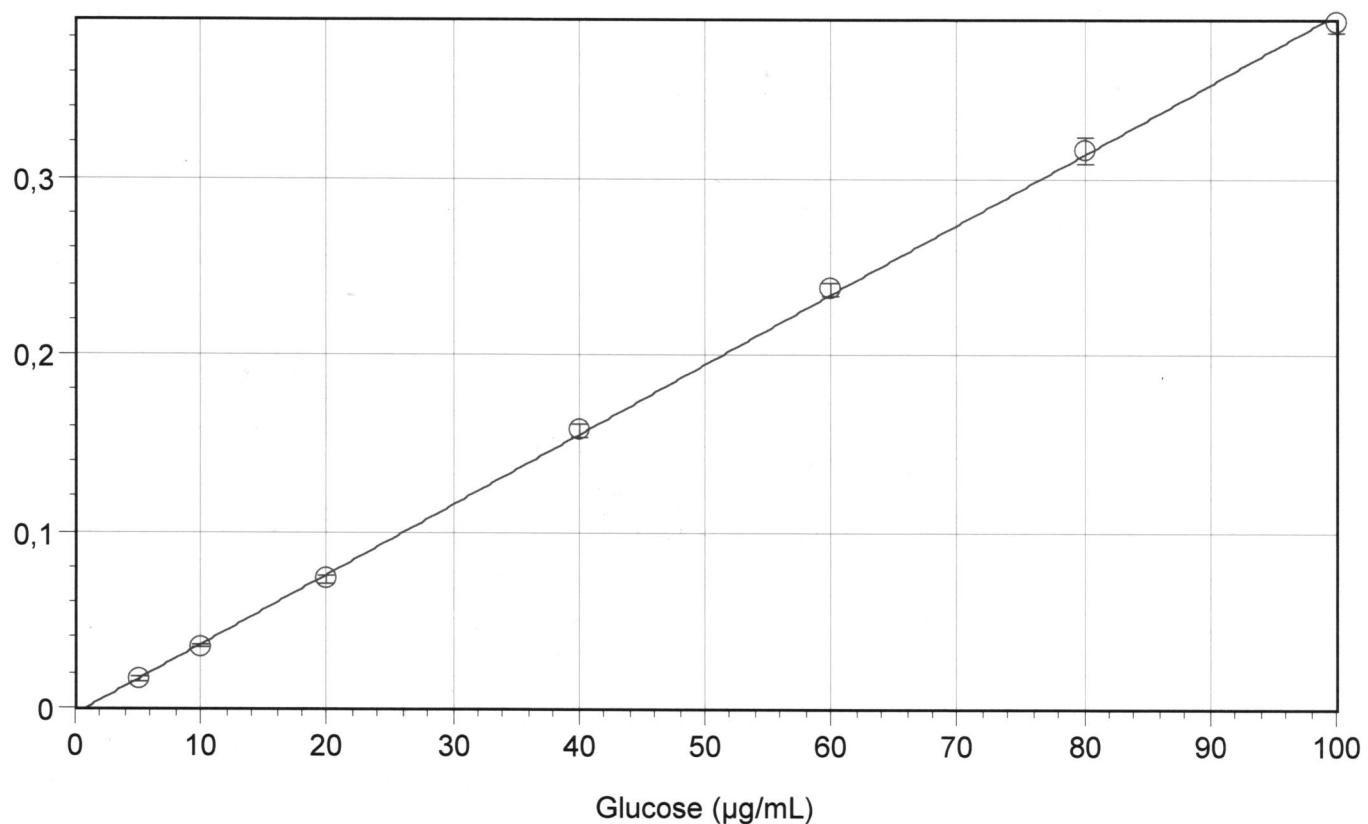
Für Glucose Eichkurve:

- 1 mg/ml D-Glucose in 0.1% Boraxsaure (H₂O) herstellen
- 100 µg/ml Glucose in Blank-Lösung (z.B. H₂O) herstellen
- Verdunstungsreihen 5-100 µg/ml Glucose herstellen

Glucose (µg/ml)	Glucose (µg/ml) zu jehe (µL)	Wasser (µL)
100	1000	0
80	800	200
60	600	400
40	400	600
20	200	800
10	100	900
5	50	950
0	—	1000

z.B.

Grafik bestimung

StandardLinear Fit: $y = A + Bx$:

$$\begin{array}{l} A \\ -0.00327 \end{array}$$

$$\begin{array}{l} B \\ 0.00396 \end{array}$$

$$\begin{array}{l} R^2 \\ 1 \end{array}$$

Eichgerade (A: Conc vs MeanValue)

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Wachstumskurve mit Fluoreszenzbestimmung

- 3 ml Starter-Kultur in LB + 100 µg/ml Amp von JW1593 sgdg67 pMEB1 sfGFP-DAS+4
- über Nacht @ 37°C / 200 rpm inkubiert
- Zelle pelletiert in 50 ml Tüte @ 5000 x g für 5 min
- 2 x mit 15 ml PBS (halt) gewaschen
- Pellet in 3 ml LB + 100 µg/ml Amp. aufgespült.
(durch Schütteln)
- ↳ OD⁶⁰⁰ gemessen → OD = 17.4

↳ 1:248 verdünnt für OD = 0.05

→ 203 µl / 50 ml Kultur

- Testmedien angeimpft

A	LB + 0.05% fluorescein	V(40%) fluorescenz @50 ml
B	LB + 0.1% fluorescein	
C	LB + 0.4% fluorescein	

↳ 50 ml Kultur in 500 ml Kolben je Dreistocher

- bei 37°C / 200 rpm inkubiert und stündlich Proben genommen
- Proben ~~bei~~ auf Eis gelagert bis zum Abend und OD⁶⁰⁰
↳ Fluoreszenz gemessen

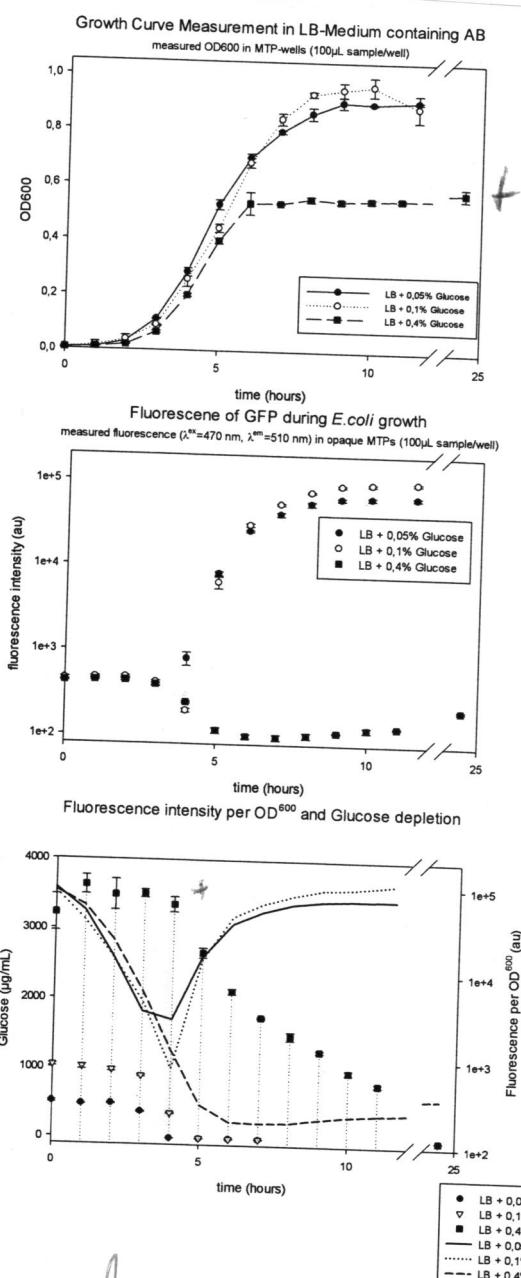
für Fluoreszenzbestimmung:

- 200 µl Aliquot von Probe in neuem Eppi unter Rührer und bei 14000 x g / 4°C für 3 min pelletiert

→ 100µl des gelösten Überstandes abgenommen und in neues Eppi überführt

↳ für GFP-GOD-Test in MTP:

- 2,5µl Probe in Well gegeben & mit 22,5µl ddH₂O auf 25µl aufgetallt
- dann wie (S.106-107)



+ → mit 0,4% Gluose wird das Wachstum gehemmt → Zellen wachsen nur noch halb so dicht

- 0,05 und 0,1% Gluose reicht nicht aus, um sTFFP-Expression zu unterstützen → bei 0,1% Gluose ist eine lag-Phase absehbar, trotzdem wird weiter sTFFP exprimiert
- bei 0,4% Gluose wird sTFFP nicht exprimiert → Gluose hemmt sTFFP Expression
- * Gluose retardiert Transkription der ~~tsr~~ Promoter tsrA-sTFFP (0,1% Gluose, → erst wenn Gluose aufgebricht, wird sTFFP exprimiert)

MTP - Layout:Glucose bestimmung:

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	Blank	AA 0 hours	AB 0 hours	AC 0 hours	AA 8 hours	AB 8 hours	AC 8 hours			
B	5	5	5	AA 1 hours	AB 1 hours	AC 1 hours	AA 9 hours	AB 9 hours	AC 9 hours			
C	10	10	10									
D	20	20	20									
E	40	40	40									
F	60	60	60									
G	80 µg/mL	80 µg/mL	80 µg/mL					Zeit = 0				
H	100 µg/mL	100 µg/mL	100 µg/mL							Zeit = max		

- 125 µl Gesamt-
 volumen:
 25 µl Probe
 50 µl Bestimmungs-
 reagenz
 50 µl Abstopp-
 lösung

- Blanks - Glucose - Eichreihe (3x)
 - glucose - Probenreihe A je 3x Bestimmung
 - ref. - Probenreihe B (3x)

OD600 & Fluoreszenz:

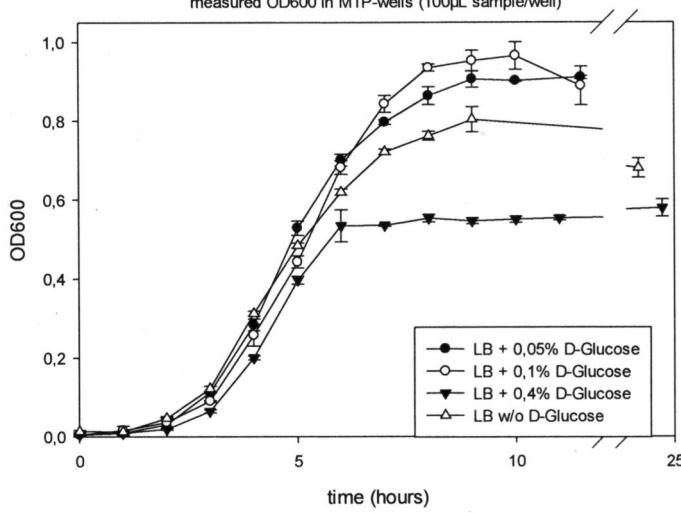
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank (LB Medium)	AA	AB Zeit = 0	AC								
B	blank											
C	blank											
D	blank				AA Zeit = max	AB	AC					
E	blank				BA	BB Zeit = 0	BC					
F	blank											
G	blank											
H	blank						AA Zeit = max	AB Zeit = max	AC			

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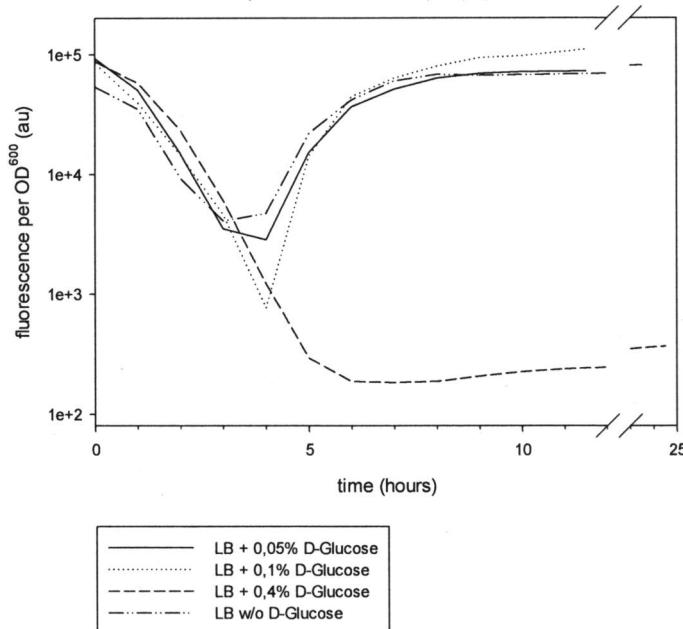
Vergleich der Wachstumskurve & Fluoreszenz von
E. coli JW1893 sydg_{gy} puerA sfgFP-D₁₄

- wird in LB medium und 150 µg/ml Ampicillin mit steigender Glucose Konzentration
- OD₆₀₀ nicht normal auf 1 an

Growth Curve Measurement in LB-Medium containing AB
measured OD₆₀₀ in MTP-wells (100µL sample/well)



Fluorescence of GFP during *E.coli* growth
measured fluorescence ($\lambda^{ex}=470$ nm, $\lambda^{em}=510$ nm) in opaque MTPs (100µL sample/well)

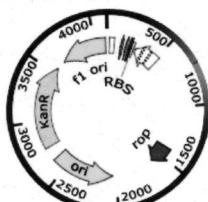
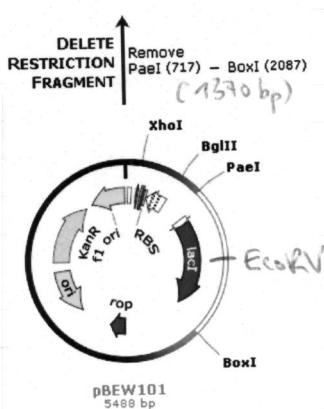


- steigende Konzentrationen an Glucose haben eine in unterschiedlichem Einfluss auf *IsrA'-sfgFP* Transkription und die Zellzahl

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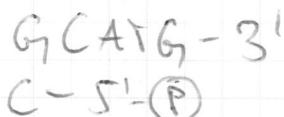
LacI - fer aus pBEW101 schneiden, dann es wird nicht benötigt

- Schneiden mit BoxI (PshAI) & PaeI (SphI)

pBEW102
4114 bppBEW101
5488 bp

SphI
↓

PshAI
↓



(P)-5'- blunt
3'- blunt

blunting with T4 DNA Polymerase

G - 3'
C - 5' - P

(P)-5'-
3' -

Injekt

Verdünnung von pBEW101

2 µl FD Buffer freien

1 µl FD SphI

1 µl FD PshAI

1 µl pBEW101 (17,5 µl @ 80 µg/ml)

ad to 20 µl dH₂O (8,5 µl)

→ 5 min @ 37 °C

20 min @ 80 °C (Enzymaktivierung) → abkühle auf 4 °C

+ 1 µl 2 mM dNTP-mix (von KOD Bl. Kit) zugehe *

+ 0,2 µl T4 DNA Polymerase zugegele

→ 20 min @ 22 °C inkubat (RT)

→ 10 min @ 75 °C inkubat

2 Reaktionen

(+) → mit dNTP *

(-) → ohne dNTPs

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N.D. M

→ auf 1% Agarose & ausgeschnitten

→ MN fel cleanup
mit 30 µl ddH₂O
durch

	ng/ml
(+)	8,76
(-)	13,8



01.08.2013 16:36:57

Blunt-End-Ligation

MM

20 µl T4 ligase buffer
2 µl T4 ligase ~~400 U~~
10 µl 50% PEG-4000
74 µl ddH₂O

48 µl Kulturmix
2 µl Lysisinits Flüssig
 $\left(\begin{array}{l} \text{(-) } \rightarrow 28 \mu\text{g} \\ \text{(+)} \rightarrow 18 \mu\text{g} \end{array} \right)$

→ 1h @ 22°C inkubiert

- 5 µl Ansatz → DNA & transformiert
- + 500 µl SOC → 200 µl plattiert
- LB @ 37°C

Kolonie

(+) →

> 50 Kolonie

(−) →

3 Kolonien

→ 3 Kolonien gezählt, ~~habt~~ 3 ml -Kultur und Miniprep → Testen mit EcoRV & SphI

<u>Festverdampf</u>		βEW101	βEW102	mit	erwartete Fragmente
β	XbaI	1 site	1 site	101	3954 bp & 1534 bp
	EcoRV	1 site	0 sites	102	4115 bp

SxMh → 8_{pre} Mh
5 μl Buffer R 2_{pre} plasmid (~100 μg)

2 μl XbaI → 1h @ 37°C
2 μl EcoRV → 2 min @ 80°C

35 µl dH₂O → ant agarose gel

M 00112233
M-Marker (1KB ladder plus)

M - Marker (1KB Ladder Plus)
 Ø - pBEW101
 1-3 - pBEW102 Klon 1-3
 X → gelmitte



5000
4000
3000
1500

3. lant Testverdian ist
noch - sehr aus p. DEWMO
verbunnt

→ Laut Segmentation
(Order 305+968)
stimmt also

05.08.2013 13:23:55

```

          10   20   30   40   50   60   70   80   90   100
pBEW102 .....GTGGGAGGCCAACTGATCCTTCTGGGTTTGATCACGCCGATCCTAGCTGTTGTTGTTGTTGCTGAGGAGACCAAGTTCCTGCTCATAT
pBEW102_1 .....ACAGA-CCCCTGC
pBEW102_2 .....CCCC-CC-AACCTCTT
pBEW102_3 .....CAC-CC-TG-GAAT

          110   120   130   140   150   160   170   180   190   200
pBEW102 .....GGCTCCGCGGCGACACAGGCGCTCTGTTGATGAGTGAATGCTCCCTAACATAAGTACTCTTATTAATGTTGTTGCTTCTTAACTGGAGGAG
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          210   220   230   240   250   260   270   280   290   300
pBEW102 .....TTTTCAACAAATTGATTCCTCTTAAATTGTTGATCACAGCTAGGGCAATTACATATTCGCGCAATAGATCATTAACTTGTATTTGATT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          310   320   330   340   350   360   370   380   390   400
pBEW102 .....TCAGGTTGGTTTTCTAGCTGCTAGACTAACAGTGGTCCATCAGATATAAGACAGATTCAGATTCAAACTTGGATTTGATG
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          410   420   430   440   450   460   470   480   490   500
pBEW102 .....TTCAAAATGAGGAAGATATTAGAACATCACCCAGATCTGATCCTAACCGGAGCCTATGTTGGCTACATCAGGCGCCACAGTGGGTTGAT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          510   520   530   540   550   560   570   580   590   600
pBEW102 .....CGCTTATAGCGACATCACCGATGGGAGATGGGCTGACTCTGCTGAGCTGCGGCTACATCAGGCGCCACAGTGGGTTGAT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          610   620   630   640   650   660   670   680   690   700
pBEW102 .....GGGGAGCTTGGGGCGACCTCTCTGCTGCCCACCTTAACTGACTGTTCTTCATACATGAGAAGTGGCGCGAGCTCTGGGTCTAT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          710   720   730   740   750   760   770   780   790   800
pBEW102 .....TTTCGGCGAGGACCTTCTGCTGAGCGACGCCGATCTGGTATGGCTCTGGCTTGGTATGTTGAGATCTTCAGCGCTGCTAACTTCGAGTGT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

          810   820   830   840   850   860   870   880   890   900
pBEW102 .....CCCGACCAAAAGCTTGGCGAGAGACGCCGATCTGGCTGGCTCTGGCTTGGTATGTTGAGATCTTCAGCGCTGCTAACTTCGAGTGT
pBEW102_1 .....
pBEW102_2 .....
pBEW102_3 .....

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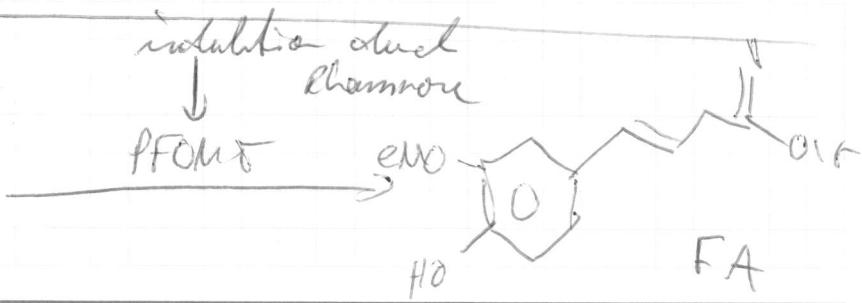
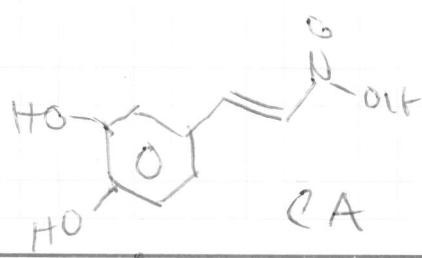
Wachstumskurve von E.coli JW1593WEB204mit phes1 sfGFP-DAS+6 & pBEW1a Test screening

2x 3ml Vorkultur in LB + 150 µg/ml Amp + 50 µg/ml Chloramph.
von JW1593 pMCB1 sfGFP-DAS+6, pBEW1a pROMT

- über Nacht @ 31°C / 200 rpm
- Zellen pelletten & 2x mit 1ml PBS gewaschen
(5 min @ 5000 rpm)
- in 3ml LB + Amp + Chlorampl. + 0.05% Fluore aufgenommen
↳ OD = 11,553

6x 50ml LB + 150 µg/ml Amp + 50 µg/ml Chloramph. + 0.05% Fluore
in 500ml Kolbe angeimpft zu OD¹⁰⁰ = 0.05 (216µl)

- standort 1,5 ml Probe aufgenommen
 - ↳ OD¹⁰⁰, Fluoreszenz, Fluore messen
 - ab Starke 5 und Expression (SDS-PAGE)
 - & HPLC messen → (1:100 aus 20% Rhamnose)
- bei OD=2 sollte mit 0,2% Rhamnose induziert werden
- A260R → erst bei OD = 3,702 mit Rhamnose
in oktaed & 250 µM Kalkessigsäure (250 mM stahl
in CH₃O) reagieren



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	41.07			

OD⁶⁰⁰ Kontrolle in Kuvette:

Zeit (h)	AA (OD ⁶⁰⁰)	Zeit (h)	AA (OD ⁶⁰⁰)
0	0.052	6	5.282
1	0.067	7	6.098
2	0.170	8	7.686
3	0.429	9	9.787
4	1.057	10	10.19
5	3.202	11	-
		74	-

• 0.2% (w/v) Phannose
in AA, AB, AC zugeben
(475 µl)

• 675 µl H₂O in BA,
BB, BC

⇒ 250 µM Kaffeesäure ((A))
(47,5 µl) in alle Proba geben

Durchgeführt von/
Performed by



Datum/
Date

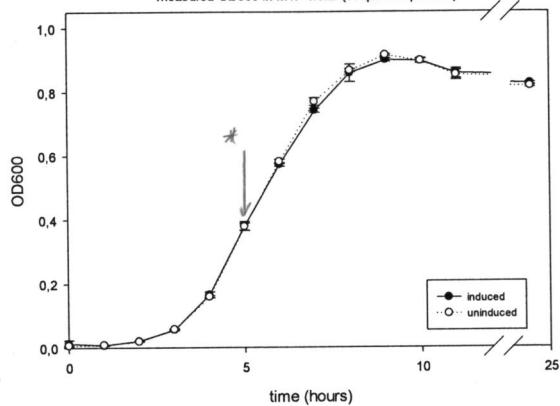
21.02.13

Bestätigt durch/
Approved by

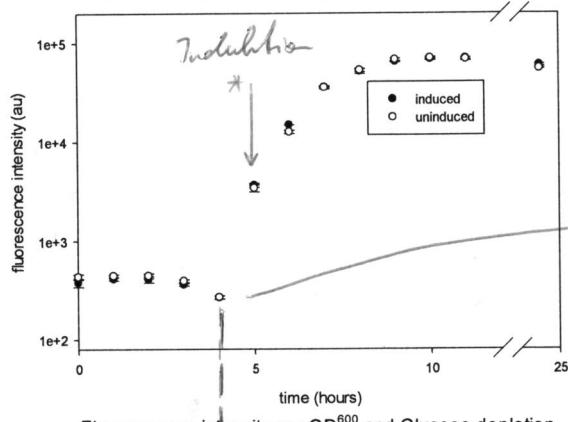
Datum/
Date

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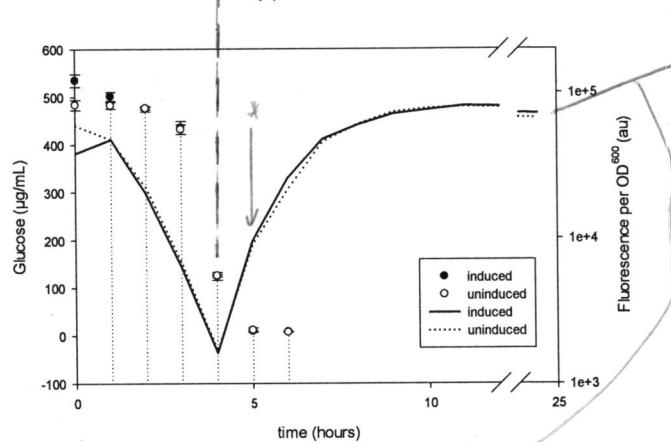
Growth Curve Measurement in LB-Medium containing 0,05% Glucose and 150 µg/mL Amp. + 50 µg/mL Chloramph.
measured OD⁶⁰⁰ in MTP-wells (100µL sample/well)



Fluorescence of GFP during *E.coli* growth
measured fluorescence ($\lambda^{ex}=470$ nm, $\lambda^{em}=510$ nm) in opaque MTPs (100µL sample/well)



Fluorescence intensity per OD⁶⁰⁰ and Glucose depletion



* Zugabe von 0,2% Rhamnose zu AA, AB, AC (had 5 study)
→ Zugabe von 250 µM CA zu Alle Probe
- Zellen wachsen etwas langsamer als Zellen ohne jektive Rhamnose

solange Fluoreszenz im Medium ist, ist es GFP expression reprimiert

→ Unterschied in Fluoreszenz/OD ist signifikant!

One Way Repeated Measures Analysis of Variance

Mittwoch, Juli 31, 2013, 19:13:34

Data source: Data 1 in 130730 WEB204.JNB

Normality Test (Shapiro-Wilk): Passed ($P = 0,293$)

Equal Variance Test: Passed ($P = 1,000$)

Treatment Name	N	Missing	Mean	Std Dev	SEM
A (+0,2% Rha + 250µM CA)	3	0	59251,656	1436,186	829,182
B (+ 250µM CA)	3	0	54551,715	431,789	249,293
Total	5	0			
Source of Variation	DF	SS	MS	F	P
Between Subjects	2	1843045,248	921522,624		
Between Treatments	1	33134168,105	33134168,105	24,959	0,038
Residual	2	2655096,825	1327548,413		
Total	5	37632310,179			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = 0,038$). To isolate the group or groups that differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0,050: 0,705

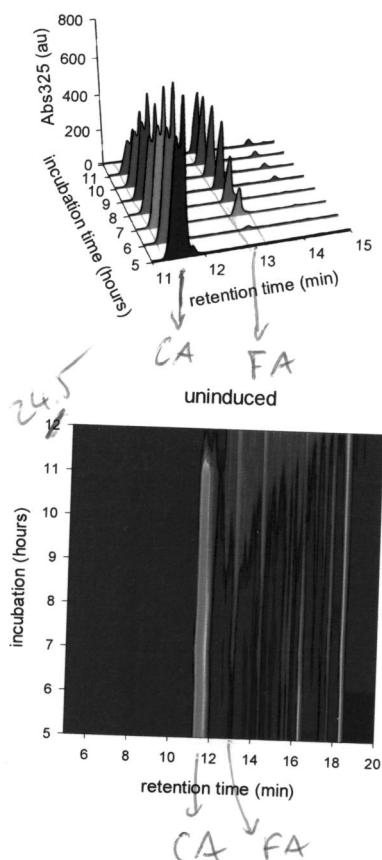
All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor:	Comparison	Diff of Means	t	P	P<0,050
A (+0,2% Rha vs. B + 250µM C		4699,941	4,996	0,038	Yes

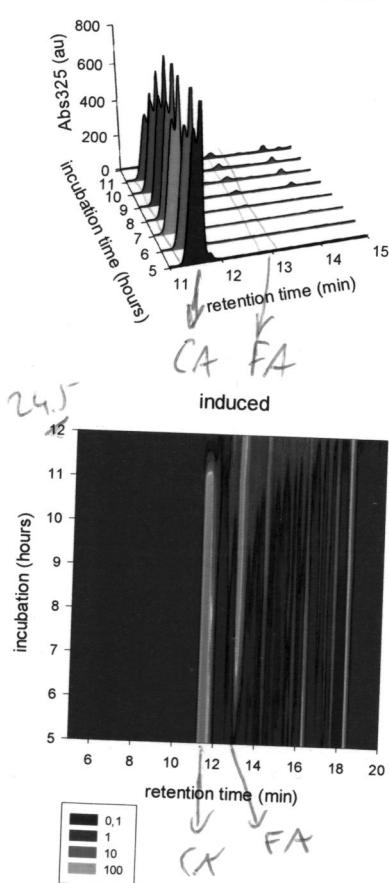
Durchgeführt von/ Performed by	Datum/ Date	Bestätigt durch/ Approved by	Datum/ Date	Fortsetzung auf Seite Nr./ Continued on page number
	31.07.13			

Umsetzung von Kaffeesäure zu Ferulasäure durch PFOMT *in vivo*
in E.coli JW1593 ΔYdgG pUCB1 sfGFP-DAS+4, pBEW1a PFOMT

Induktion der PFOMT durch 0,2% (w/v) Rhamnose nach 5 Stunden Wachstum,
gleichzeitige Zugabe von 250 µM Kaffeesäure



Keine Induktion der PFOMT.
Zugabe von 250 µM Kaffeesäure nach 5 Stunden Wachstum



CA - caffeic acid
FA - ferulic acid

→ Nach Induktion
von PFOMT wird
mit kurzer Verzögerung
auch Ferulasäure
gebildet

Samle:

Probenaufbereitung für HPLC:

- 1ml der Kultur → 2 mal mit 500 µl EtOHc (+1% für 30 sec extrahiere (vorsicht) Formic Acid)
 - die organische Phasen vereinigen und in der Speedvac das Lösungsmittel abdampfen
 - Rückstand in 200µl MeCN aufnehmen (115 vortelen)
 - 100µl von der Probe in Proben gläsche
- HPLC-Programm: A - H₂O 0%, FA 5% MeCN (0%), FA (4 min) → 100% MeCN (+0%) FA (5 min)
- 1 - H₂O 0%, FA 5% MeCN (0%), FA (4 min) → 100% MeCN (+0%) FA (5 min)
- 2 - MeCN +0%, FA 5% MeCN (0%), FA (4 min) → 100% MeCN (+0%) FA (5 min)

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Performed by

Datum/
Date

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Date

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Continued on page number

Expression profile von WEB204

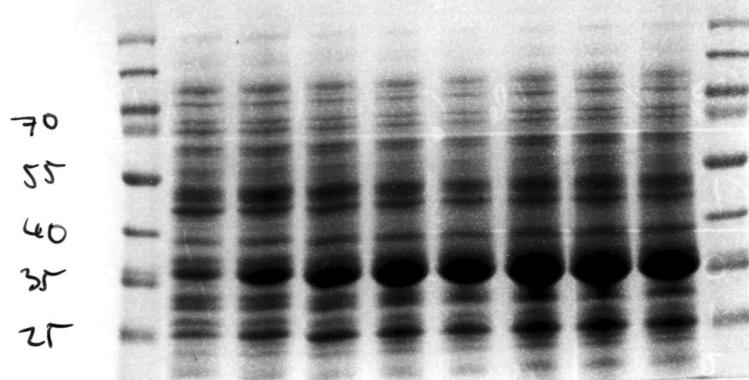
- 200µl Probe aus AA (mit Induktion) und BA (ohne Ind.)
pelletieren und in β x µl β -PER II aufnehmen
und lysieren

Bei einer OD^{600}
von 1 und
1µl Probe
 $\rightarrow 50\mu\text{l } \beta\text{-PER II}$

Vinyl
[β -PER]

$$\begin{aligned} & \frac{V(\text{Probe})}{1000\mu\text{l}} \\ & = OD^{600} \times 50\mu\text{l} \times \cancel{0.2 \text{ (Volumen)}} \\ & = OD^{600} \times 50\mu\text{l} \times 200\mu\text{l} \cdot 0.2 \end{aligned}$$

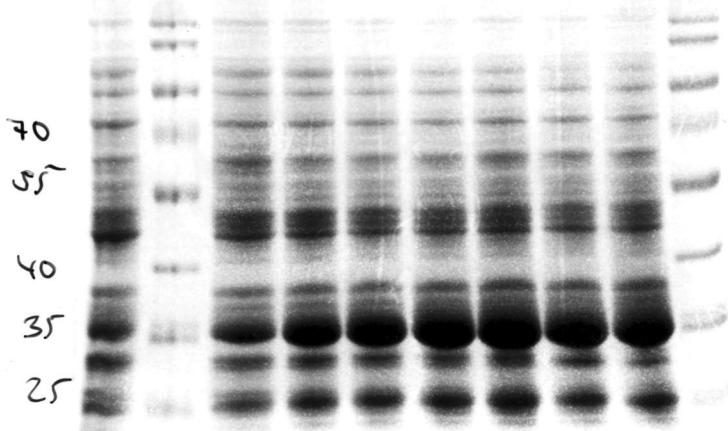
M 5 6 7 8 9 10 11 24 M



31.07.2013 18:58:50

O:\ABT\NWC\Weigel_WEB\BioDoc\130730_WEB204_
B(uninduziert).TIF

M 5 6 7 8 9 10 11 24 M



31.07.2013 18:58:19

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Date

31.07.13

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Datum/
Date

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Continued on page number

WEB207

Wachstumskurve JM 110
mit pNC81 stGFP-DNA & pBEVla RFOH

→ Durchführung wie WEB204 (S. 115 fl.)

- 3 ml VK JM110 pNC81 stGFP & pBEVla RFOH

→ in N. @ 31°C 200 rpm

- 2x mit PBS gewaschen

- in 3 ml ZB aufgenommen $OD^{600} = 8,013$

$\hookrightarrow 1:160$ für $OD^{600} = 0.05$

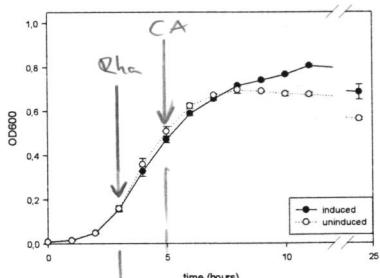
$\Rightarrow 31.7 \mu\text{l}$ pro 50 ml

OD^{600} Kontrolle (Kuvette) (AA):

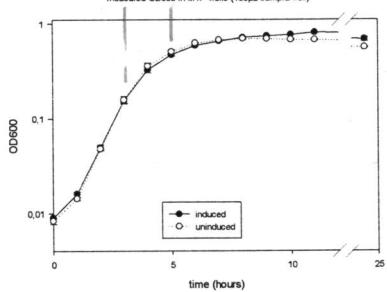
<u>t (h)</u>	<u>OD^{600}</u>
0	0,060
1	0,103
2	0,338
3	1,071
4	2,558
5	4,395
6	5,401
7	5,729
8	6,345
9	7,563
10	6,661
11	
24,5	

Glucose Konzentration bestimmt
 Zugabe von 0,2% Rha nach 3 Stunde
 Zugabe von 200 μM Caffeic Acid
 nach 5 Stunde
 HPLC Proben

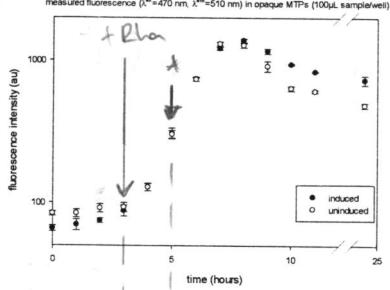
Growth Curve Measurement in LB-Medium containing 0,05% Glucose and 150 µg/mL Amp. + 50 µg/mL Chloramph.
measured OD₆₀₀ in MTP-wells (100µL sample/well)



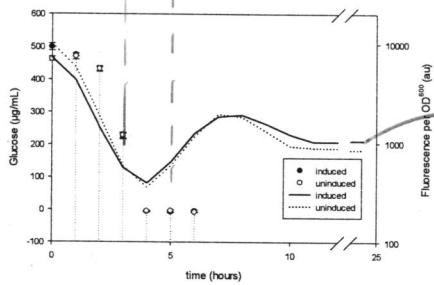
Growth Curve Measurement in LB-Medium containing 0,05% Glucose and 150 µg/mL Amp. + 50 µg/mL Chloramph.
measured OD₆₀₀ in MTP-wells (100µL sample/well)



Fluorescence of GFP during *E.coli* growth
measured fluorescence ($\lambda^* = 470 \text{ nm}$, $\lambda''' = 510 \text{ nm}$) in opaque MTPs (100µL sample/well)



Fluorescence intensity per OD₆₀₀ and Glucose depletion



t-test

Dienstag, August 13, 2013, 14:15:03

Data source: Data 1 in 130808 WEB207.JNB

Equal Variance Test: Passed ($P = 0,944$)

Group Name	N	Missing	Mean	Std Dev	SEM
A (+0,2% Rha + 250µM CA) - 24h	3	0	1033,705	9,903	5,717
B (+ 250µM CA) - 24h	3	0	899,071	10,895	6,290

Difference 134,634

$t = 15,839$ with 4 degrees of freedom.

95 percent two-tailed confidence interval for difference of means: 111,034 to 158,235

Two-tailed P-value = 0,0000929

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups ($P < 0,001$).

One-tailed P-value = 0,0000464

The sample mean of group A (+0,2% Rha + 250µM CA) - 24h exceeds the sample mean of group B (+ 250µM CA) - 24h by an amount that is greater than would be expected by chance, rejecting the hypothesis that the population mean of group B (+ 250µM CA) - 24h is greater than or equal to the population mean of group A (+0,2% Rha + 250µM CA) - 24h. ($P = <0,001$).

Power of performed two-tailed test with alpha = 0,050: 1,000

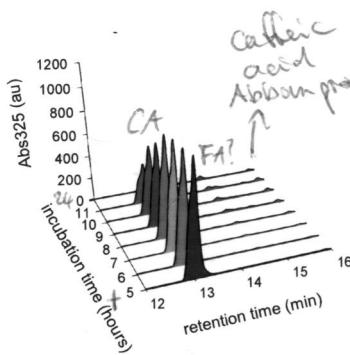
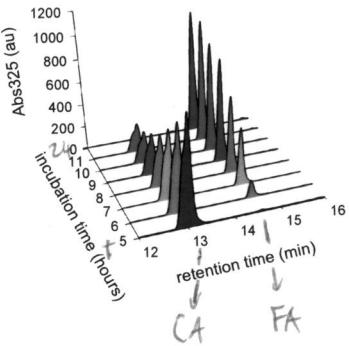
Power of performed one-tailed test with alpha = 0,050: 1,000

HPLC-Messung für WB107

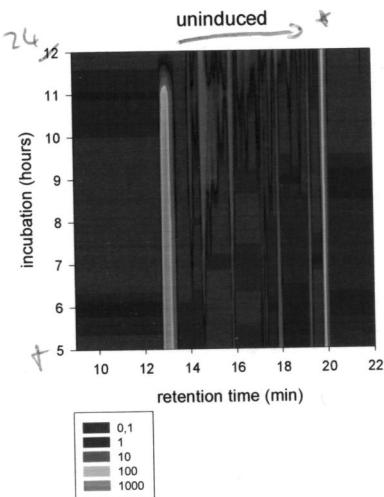
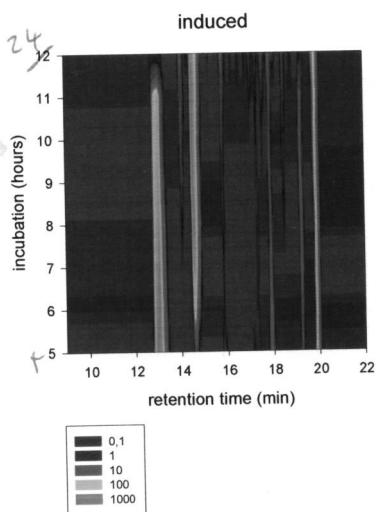
Umsetzung von Kaffeesäure zu Ferulasäure durch PFOMT *in vivo*
in E.coli JM110 pUCB1 sfGFP-DAS+4, pBEW1a PFOMT

Induktion der Expression nach 3h Wachstum mit 0.2% Rhamnose,
 Zugabe von 250 µM Caffeic acid nach 5h

keine Induktion der Expression,
 Zugabe von 250 µM Caffeic Acid nach 5h



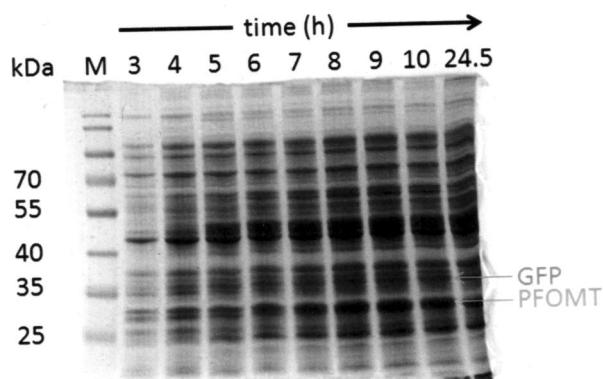
+ Zugabe von 250 µM
 Caffeic Acid



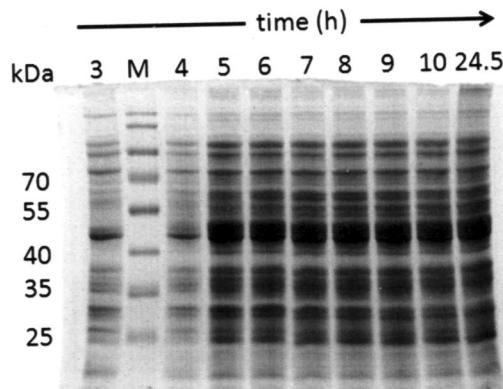
* Abbau von Kaffee säure
 erkennbar
 → sichtbar passiert ein
 wenig Methylierung zu
 Ferulic -
 Säure

SDS-PAGE WEB 207**Induced samples (AA)**

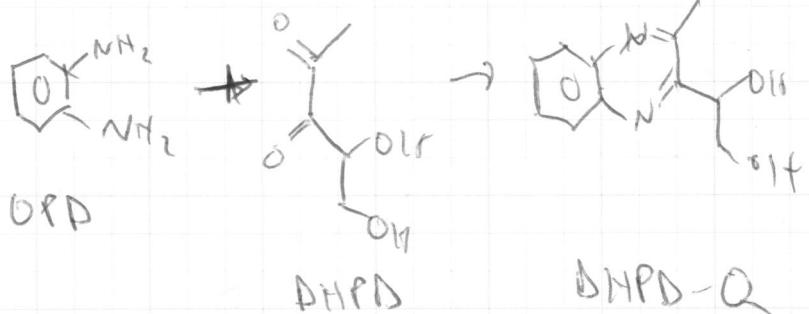
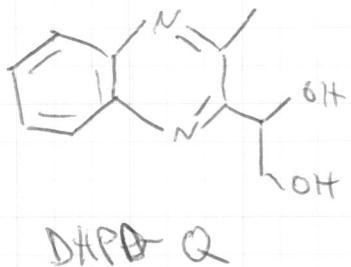
- Induction with 0.2 % Rhamnose after 3 hours growth
- Addition of 250 µM Caffeic acid after 5 hours growth

**Uninduced samples (BA)**

- No induction after 3 hours growth
- Addition of 250 µM Caffeic acid after 5 hours growth



→ PFOMT expression im induzierte Zustand relativ
gering
• auch GFP-Expression kaum vorhanden

WEB 208Synthese von Dihydroxyperoxidogenquinokali
(DHPD-Q)

- 3 ml VK von BL21(DE3) (LB)

Über Nacht @ 37°C

- 50 ml Kultur (LB) 7:250 mit VK eingesetzt
+ 0.4% glucose

→ bis OD = 4

→ dann Zellen pelletiert und Überstand abgezentrifugiert

→ 2 ml OPD (= 20 mg) in Überstand gegeben &

@ 35°C unter Ruhigstellung & Argonatmosphäre inkubiert

KPi-Buffer zugegeben: (pH 7.4)

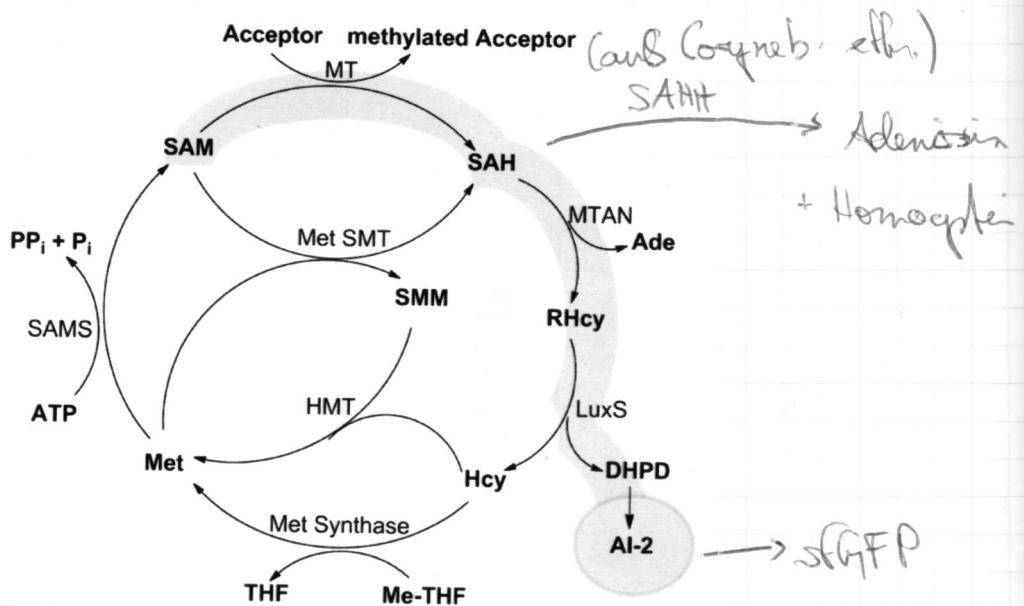
für ~16 h

- 80,2 ml K₂HPO₄ (1 h)

- 19,8 ml KH₂PO₄ (1 h)

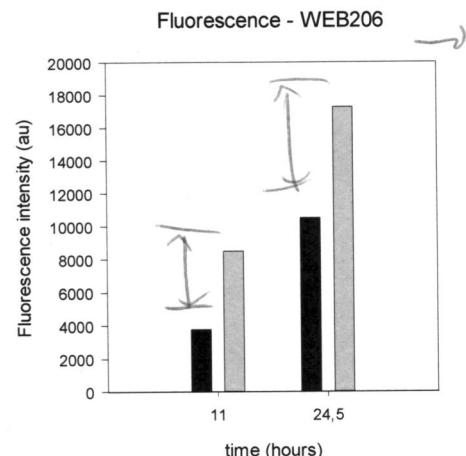
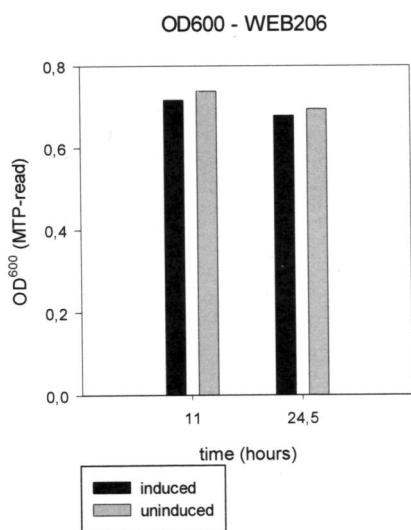
- ~~mit~~ Ansatz eingesetzt bis ca. 200 ml

- mit EtOAc extrahiert → ESI/MS von argon &
Wasserphase → gewünschte Masse (204) nicht da

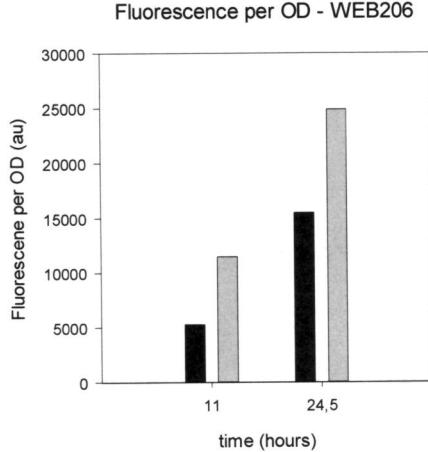
WEB 206Test pheB1 sfGFP + SANH~~pET28~~Theorie:

- Bei Expression von SANH dann nur die methylzyklische Kurzschlufe & es folgt eine komplexe AI-2-Konzentration und dadurch wenige sfGFP
- Transformation von pET28a(+) Cell SANH & pNCB1 sfGFP-DAS + 4 in E. coli BL21 (DE3) (Achtung! gleiche ORI & idemp.)

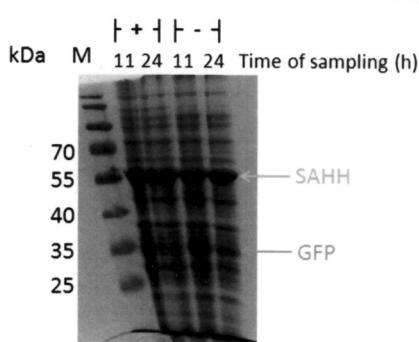
Durchführung: - in LB-Medium mit 100 µg/ml Kanamycin,
 (Autoreduktion) 150 µg/ml Ampicillin und 0,15% Glucose (C Repressor)
 → (+) in zuckerhaltiger Kultivierung außerdem 0,2% Lactose
 zur Induktion der Expression von SAHHt
 → @ 31°C / 200 rpm kultiviert → Probe @ 11 h &
 24 Stunden genommen → OD, Fluoreszenz, SDS-PAGE



→ Großer Unterschied
 um herauszufinden ob Synthese
 bestimmt ist



→ trotz uninduzierter SAHHt-Expression
 ist ein klarer Unterschied in der
 Fluoreszenzintensität erkennbar

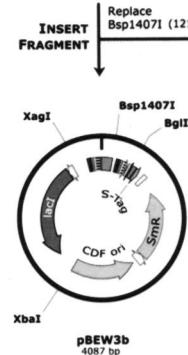
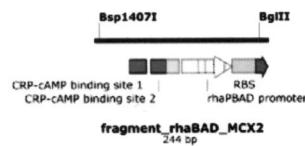
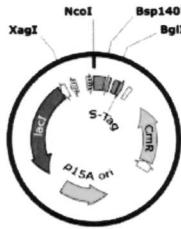
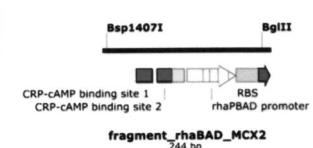
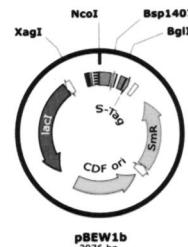


→ SAHHt Expression und uninduziertes Expression sehr leachy

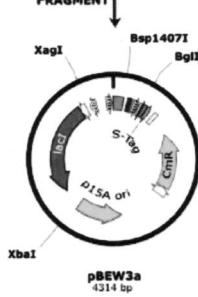
pUCB1 sfGFP-DAS+4 and pET28a(+) in E.coli BL21(DE3) – grown in LB-medium supplemented with 0,15% Glucose and (+) – induced sample (0.2% Lac) or (-) – uninduced sample (no lactose)
 Sampling at 11 hours and 24 hours of growth.

Klonierung von pBEW3a/3b & pBEW4a/4b

- aus pBEW1a/b durch einfache ~~des~~ des thermoph. RhaBAD
promotors mit künstl. RBS (transkript. initiat.-rate)

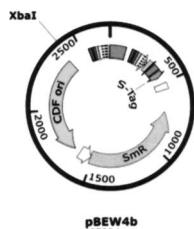


3



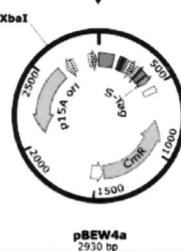
Insert Bsp1407I (7) - BglII (233)

DELETE RESTRICTION FRAGMENT
Remove XbaI (2408) - XbaI (3796)



4

DELETE RESTRICTION FRAGMENT
Remove XbaI (2635) - XbaI (4023)



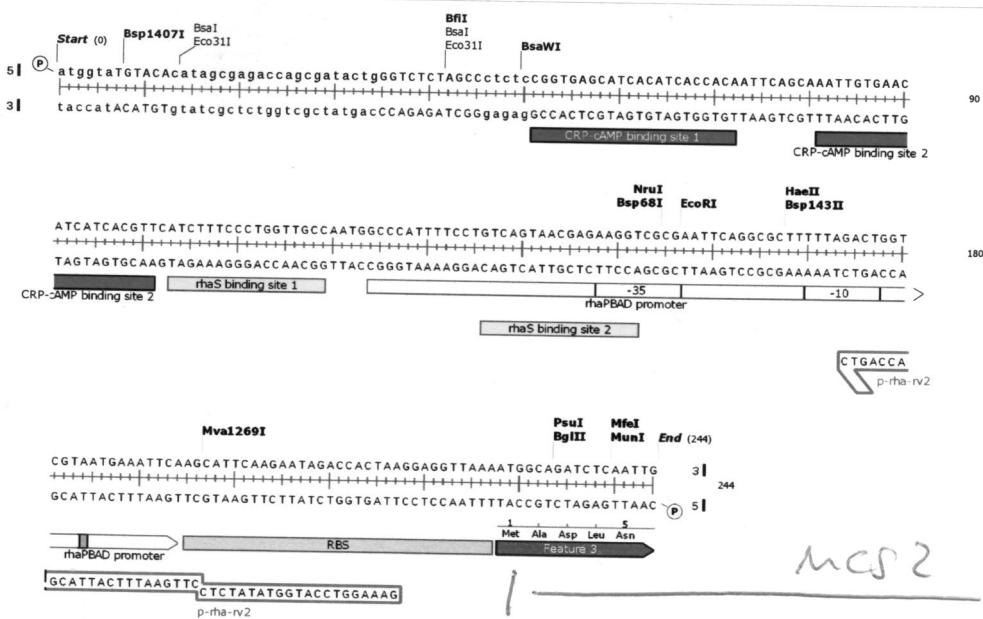
Fragment zum Klonieren:

Orde:

GeneArt Stomping DNA Fragment (Invitrogen) 13ABGYSR

Sequence: fragment_rhaBAD_MCX2.dna (Linear / 244 bp)
Enzymes: < Unused Enzyme Set > (15 of 646 total)
Features: 7 visible, 7 total
Primers: 1 visible, 1 total

Unique Cutters **Bold**



Vorhanden waren pBEW1a/1b & rhaP3AD-MCS2 und BgIII & Bsp1407I

Fragment

10 µl ~~pBEW1a~~ pBEW1a-MCS2 (@ 40µg/µl)

2 µl FD buffer

1 µl BgIII FD & Bsp1407I FD

6 µl ddH₂O

(20 µl final)

plasmids

3 x Mh

6 µl FD Buffer green

3 µl FastAP

3 µl BgIII FD & Bsp1407I FD
1 µl ddH₂O

→ 10 µl Mh + 10 µl pBEW1a
(~800 µg)

10 µl Mh + 10 µl pBEW1b
(~1100 µg)

- linearisiert Plasmide über MN PCR & gel cleanup gebraucht
& ausgeschwärkt
→ mit MN PCR & gel-cleanup gereinigt

Probe	C _N (μl)
1a	3,35
1b	13,98

- Fragment über MN PCR & gel-cleanup KI gereinigt
→ c = 8.27 μl

Ligation

Markermix

Ligation

3a

3b

0.5 μl T4 DNA ligase
4 μl T4 ligase Buffer
18.8 μl ddH₂O

11.65 μl MN
0.85 μl MCS2
7.5 μl 1a

11.65 μl MN
0.85 μl MCS1
1.8 μl 1b
8.7 μl ddH₂O

- 1h @ 22°C inkubiert, 4°C über Nacht
- 5 μl in 0.6% Agarose Lammel → ausplattiert
→ über Nacht @ 37°C inkubiert

Colony PCR

Primer:

Inet-NcoI-fw

Elongationszeit: 30 sec

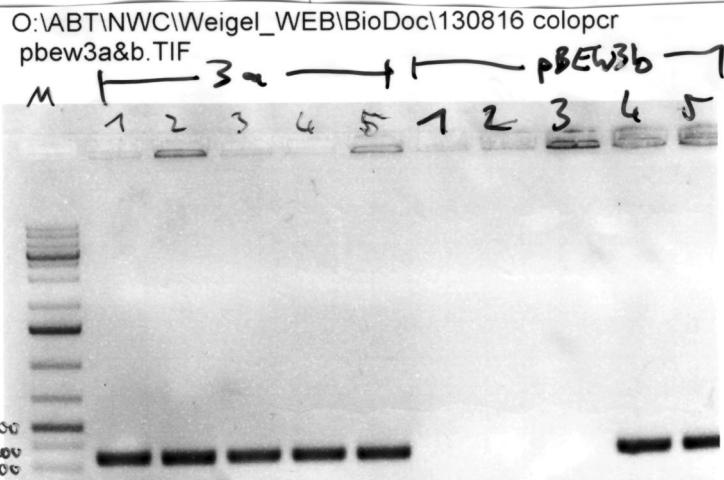
p-Ha-rev

→ 319 bp

Durchgeführt von/
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Date

15.08.13

Bestätigt durch/
Approved byDatum/
DateFortsetzung auf Seite Nr./
Continued on page number



lumiprep Klonen
3a 1 & 5
3b 2 & 5

sequenziert:

oder:

16.08.2013 15:15:39

MCS1 →

```

810 820 830 840 850 860 870 880 890 900
pBEW3a ATACCAATCATACCAAGCCAGGATCCGAATTGAGCTCGGCCGCCTGAAAGTCGACAAGCTTCCGGCCGATAATGCTTAAGTCGAAAGAAAATGAA
pBEW3a_1_T7term -- u
pBEW3a_5_T7term -- u

910 920 930 940 950 960 970 980 990 1000
pBEW3a TCGTAGTGATACATAAGCCAGACCAAGCCATACTGGGTTCTAAGCCCTCTCCGGTGAAGCAATCACATACCAAAATTCAGCAAATTTGAGACATCATACG
pBEW3a_1_T7term -- u
pBEW3a_5_T7term -- u

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
pBEW3a TCACTTTCCCTGGTGGCCAATGGCCCATTTCTCTGTCAGTACGAGGTGGCGGAAATTCAGGCCGCTTTTAAAGCTGGTGTAAATGAAATTCAAGCATT
pBEW3a_1_T7term -- u
pBEW3a_5_T7term -- u

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
pBEW3a CAAAGAAAGACCACTTAAAGGGTTAAAAATGGCGAGATCTCAATTGGATATGGGATATGGGCGGCCACGGATTCGCTGAGTCGGTACCCCTGAGTCGGTAAAGGAA
pBEW3a_1_T7term -- u
pBEW3a_5_T7term -- u

1210 1220 1230 1240 1250 1260
pBEW3a CGCTGCTGGAAATTGAGCCAGCAGCATGGACTCGTACTAGCGCAGCTTAATTAACCTAGGC
pBEW3a_1_T7term -- u
A. TAGC. AA. G. CT
pBEW3a_5_T7term -- u
A. . . A. T. T

```

MCS2 →

→ Just in beide Klonen

i

MCS1 →

```

810 820 830 840 850 860 870 880 890 900
pBEW3b ATATACATGGGACAGCGCATACCAATCATACCAAGCCAGGATCCGAATTGAGCTCGGCCGCCTGAAAGTCGACAAGCTTCCGGCCGATAATGCTTAAGTCGAA
pBEW3b_4_T7term -- u
pBEW3b_5_T7term -- u

910 920 930 940 950 960 970 980 990 1000
pBEW3b TTAAAGTCGAAACAGAAAGTAATGCTGATACATACATAGGAGCTGGGTTCTAAGCCCTCTCCGGTGAAGCAATCACACCAAAATCAGCA
pBEW3b_4_T7term -- u
pBEW3b_5_T7term -- u

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
pBEW3b AATTGTGAAACATCATACGTTCTCTTCCCTGGTGGCCAATGGCCATTTCTCTGTCAGTACGAGGTGGCGGAAATTCAGGCCGCTTTTAAAGCTGGT
pBEW3b_4_T7term -- u
pBEW3b_5_T7term -- u

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
pBEW3b CCGTATGAAATTCAAGGATTCAGAAAGACCACTTAAAGGGTTAAAAATGGCGAGATCTCAATTGGATATCGGGCGGCCACGGATTCGCTGAGTCGGTAC
pBEW3b_4_T7term -- u
A. . . G. A. T.
pBEW3b_5_T7term -- u
A. . . G. A. T.

1210 1220 1230 1240 1250 1260 1270 1280
pBEW3b CCTCGAGCTCTGGTAAAGAAACCGCTGCTGGNAAATTGAGCCAGCAGCATGGACTCGTCTAGCGCAGCTTAATTAACCTAG
pBEW3b_4_T7term -- u
A. . . G. A. T.
pBEW3b_5_T7term -- u
A. . . G. A. T.

```

MCS2 →

→ Just in beide überlapt

i

Durchgeführt von/
Performed by

Datum/
Date

Bestätigt durch/
Approved by

Datum/
Date

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16.08.13

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Auslösung des lacZ-fors aus pBEW3a & pBEW3b

- Schneide mit XbaI & XbaI
 → danach blunting mit T4 DNA Polymerase

Ovalan von pBEW3a / 3b

2µl FD Buffer Green
 1µl FD XbaI (CONE)
~~~ 5µl~~ XbaI (normal, nicht FD)  
 1µg pBEW3a / pBEW3b  
 ad to 70µl ddH<sub>2</sub>O

→ 10 min @ 37°C  
 20 min @ 80°C (Inaktivierung)  
 4°C

- Normal Restrikptions-enzyme Funktionen sind in FD Buffer → aber unterschiedlich!

- 1µl 2mM dNTP-Mix (aus 400 µl Pol. Kit) recycle
- + 0,2 µl T4 DNA Polymerase recycle
- 10 min @ 22°C inkubiert
- 10 min @ 75°C inkubiert
- auf 1% Agarose gel getrennt & ausgespülte
- mit MN gel & PCR Cleanup Kit gereinigt

|        | cug/µl) |       |
|--------|---------|-------|
| pBEW3a | 1) 3,4  | 5) 15 |
| pBEW3b | 4) 9,5  | 5) 11 |

Blank End-ligation

4x Mh

20μl T4 ligase buffer

4μl T4 ligase

20μl 50% PEG-4000

148μl ddH<sub>2</sub>OReaktive

48μl MM

+ 2μl linearized, blanked  
plasmid

→ 1h @ 22°C inkubiert

→ 5μl des Ansatzes in DH10<sup>a</sup> transformiert + 500μl SOC

→ 200μl plakat → in N. @ 31°C inkubiert

| Klonen aus | (3) | Klonen |   |
|------------|-----|--------|---|
|            |     | 1      | 2 |
| pBEW4a     | 4   | ~30    |   |
| pBEW4a     | 5   | >70    |   |
| pBEW4b     | 4   | ~50    |   |
| pBEW4b     | 5   | ~30    |   |

→ je 4 Klonen von pEW4a ①  
& pEW4b ④ separiert & colony PCR

→ Miniprep von pEW4a 1,2 &amp; pEW4b 1,2 für Sequenzierung

→ his  
art  
Insertion  
in Ori  
passt  
die  
Sequenz

pBEW4a  
pBEW4a\_1\_pBEW4-seq -- unclippe  
pBEW4a\_2\_pBEW4-seq -- unclippe

10 20 30 40 50 60 70 80 90 100

TCTTATTTCAATTAGTGAAAGTGGACCTCTTAAGTGCGGATCACGGTCATTTCCCAAAGGTGGCCAGGCTTCCGGTATCACAGGACA  
... .AGC..CT..TCGAAGGGACT.G.A..T.....T.....T..G T.T.A.. CT...TC.  
....A..A.TAT...G.....A.....G T.....CT..C.....

110 120 130 140 150 160 170 180 190 200

CCAGGATTATTATTCGCGAAAGTGAATCTCGTCAAGGTATTATTCGGGC  
... .G.....A.....T.....T.....CA.....A.....G.....  
....A.....

210 220 230 240 250 260 270 280 290 300

ATGG TGTGTTGAGGT GCTCCAGTGCTCTGTTCTAGCTGAGGGTGTGGCTGACTGAGGGGTGCGTACGCGGAAACGGCTGACG  
... .G.....A..G..A.....  
....GA.....G.....

310 320 330 340 350 360 370 380 390 400

GGACATCGCGCTAGCGGAATGTAATGCTACTATGTTGGCATGATGAGGGTGTAGTGAAGTGTCTTCAATGTCAGGAGAAAAGGCGTGAACG  
... .  
....GA.....

410 420 430 440 450 460 470 480 490 500

GTCGTCAGCGATAATGTAATGCAAGGATAATTCGGCTTCGCTACTGACTGCTACGCTCGGTGTTGAGTCACTCCGGAAAGGCGTACG  
... .  
....GA.....

510 520 530 540 550 560 570 580 590 600

AACGGGGCGGAATTTCTCGAGAATGCCAGGAGATACTTAACAGGGAGTGAAGAATGGGCGCGGAAAGCGTTTCCATAGGCTCCCGCCCGCTGAC  
... .  
....GA.....

610 620 630 640 650 660 670 680 690 700

AAAGCATCACGAAATCTGCCTCAAACTGAGTGGCGAGAACCGAGGATTAAGAATCCAGGGTTTCCCC  
... .  
....C.....C.....

710 720 730 740 750 760 770 780 790 800

CTGTTCTGCCTTCGGTTACCGGTGTCATCCGGTGTATGGCGGCTGTCATTCAGCGACTGAGTCCGGTAGGCAGTGTGCTCA  
... .  
....GA.....

810 820 830 840 850 860 870 880 890 900

ACCTGGACTATCAGAACCCCCCGTTAGTCGACCGCTGGCGTATCCGGTACTATCGCTTGAATGCTAACCCCGAAAGACATTCAGAACAC  
... .  
....GA.....

910 920 930 940 950 960 970 980 990 1000

ACTGGCAGCGCAGCTGGTAAATGAGTGGAGCTAGAGAACCTTGGAAAACCGCGTGAAGGCTAACGAAAGGCAAGTTGGTGAATGGCTCC  
... .  
....A.....

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100

TCCAAAGCCATTCTCGGTCAAAGAGTGGAGCTAGAGAACCTTGGAAAACCGCGTGAAGGCTAACGAAAGGCAAGTTGGTGAATGGCTCC  
... .  
....A.....

1110 1120 1130 1140 1150

→ multiple Priming?  
→ last Chromatogram  
→ three Dots  
← on

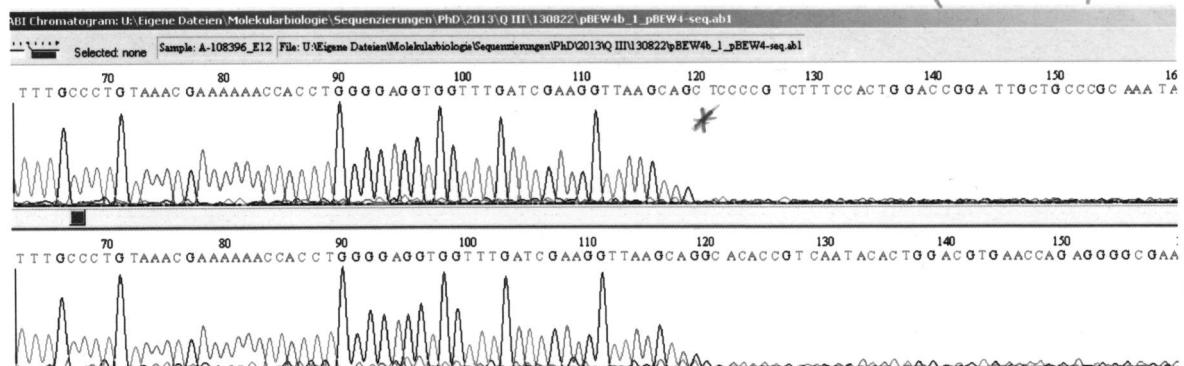
✓ on

10 20 30 40 50 60

pBEW4b AGCGGTTCAAGGAAAGATCAAAGGATCTTCTGAGATCCTTTCTGCGCGTAAATCT  
pBEW4b\_1 ...TC..T.....G.....C.....  
pBEW4b\_2 GC..TC.....G.T.....C.....

70 80 90 100 110 120

TTTGCCTGTAACGAAAAAACACCTGGGAGGTGGTTGATCGAAGGTTAAGTCAGTTGGGA  
... .  
....CAG.C....CCCCG  
....CAG.CACACCG



✓ Sequencing gut → bricht dann unverhofft ab  
→ vermutlich 2nd Run ab

| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
|                                   | MWB            |                                 |                |                                                        |

Klonierung der PFOMT-MutantenPCR Amplifikation der Mutanten3x Mutterkunst→ Rn\* pET28c (+) PFOMT  
Vorlage von SLM

15 µl KOD HS Buffer

1 µl 25 mM MgSO<sub>4</sub>

15 µl 2 mM dNTPs

+ 4.5 µl PFOMT-B11-fw  
PFOMT-B11-rv

3 µl KOD HS Polymerase

97.5 µl ddH<sub>2</sub>O495 µl H<sub>2</sub>O+ 0.5 µl Plasmid. Aktivator

1) wt

2) N181A \*

3) W184 M \*

wt

4x wt

~0

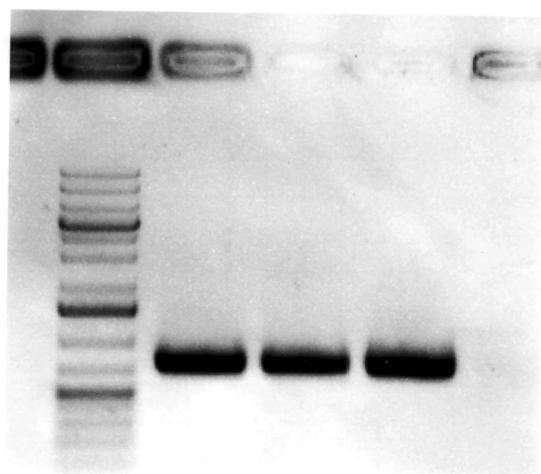
| Prote | eGy/µl) |
|-------|---------|
| wt    | 202     |
| N181A | 195     |
| W184M | 217     |

→ Elongationszeit beyon PCR: → siehe 1.65

→ Agarosegel zur Kontrolle

+ restlich Ansatz mit MN Gel & PCR  
Cleanup wird gereinigt (i 3 µl ddH<sub>2</sub>O  
eluiert)

O:\ABT\NWC\Weigel\_WEB\BioDoc\130820\_pfomt mutanten pcr 4 cloning.TIF



20.08.2013 13:26:07

Verdunnen der PCR-Produkte mit XbaI, PstIMM

3 µl XbaI FD

→ Rer

3 µl PstI FD

15 µl MM

6 µl FD Buffer

→ 5 µl PCR cleanup

33 µl H<sub>2</sub>O

→ 15 min @ 37°C

→ 20 min @ 75°C inkubiert

- PCR cleanup & i 30 µl ddH<sub>2</sub>O elutet

|       | (µg/µl) |
|-------|---------|
| wt    | 26      |
| N181A | 24      |
| W184M | 25      |

Ligation in pETM1aMM

0.5 µl T4 ligase

4 µl T4 ligase Buffer

10 µl pETM1a PstI, XbaI (=21 µg)

23,9 µl ddH<sub>2</sub>O

→ je 19,2 µl MM + 0.8 µl Fragment

→ 22°C für 1h, danach über Nacht @ 4°C

→ 5 µl in 100x Transformant

→ @ 37°C inkubiert

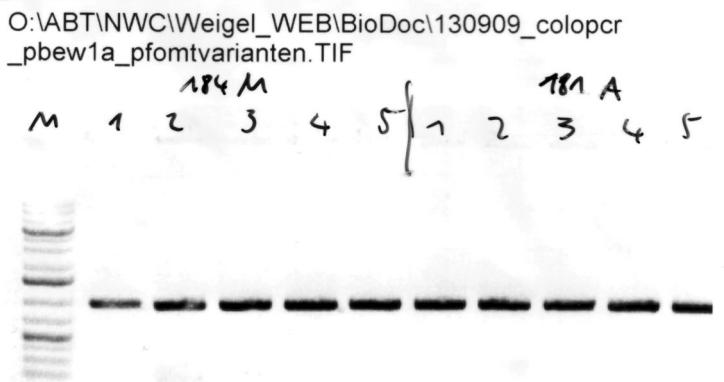
Colony - PCR:

Primer: T + kom

&amp; Duet NopI fw

→ Klonen 1 & 2 in  
LB angezogen & Miniprep  
gemacht

→ dann d. zur Sequenzierung



10.09.2013 11:21:30

• Klonen alle  
positiv auf Sonderhe  
Insert sequenziert

→ am für PFOMT\_181A-

→ verworfen

Sequence data for pBEW1a\_PFOMT, pBEW1a\_PFOMT\_181A\_1, pBEW1a\_PFOMT\_181A\_2, pBEW1a\_PFOMT\_184M\_1, and pBEW1a\_PFOMT\_184M\_2 across various positions (10 to 1100). The sequence shows a highly conserved region followed by a variable region with some mutations.

```

pBEW1a_PFOMT      GGAAACAAAT 10-100
pBEW1a_PFOMT_181A_1 C.G...TC.ACA.T 10-100
pBEW1a_PFOMT_181A_2 AC...G.CC.C 10-100
pBEW1a_PFOMT_184M_1 ..CG.T..GG.C..T 10-100
pBEW1a_PFOMT_184M_2 ...TT..TC.CC..T 10-100

pBEW1a_PFOMT      GGAATTGTCTACAGAGTGACGGATTAGCCAGTATATTCTCCGACTAGTGCTCATCTCCGQAACTCGAGGTTCTCAAGGAACTCGAGGAGCCAACTQAA 110-200
pBEW1a_PFOMT_181A_1 ..... 110-200
pBEW1a_PFOMT_181A_2 ..... 110-200
pBEW1a_PFOMT_184M_1 ..... 110-200
pBEW1a_PFOMT_184M_2 ..... 110-200

pBEW1a_PFOMT      GTCAACCCGAGCTCTTATATATGTCGACTTCACCACTTCTGTCGAAATTGATGTCATCTGCTTAAGAACTAGTGAATGAAAGAACTATTGAGCTGGAGT 210-300
pBEW1a_PFOMT_181A_1 ..... 210-300
pBEW1a_PFOMT_181A_2 ..... 210-300
pBEW1a_PFOMT_184M_1 ..... 210-300
pBEW1a_PFOMT_184M_2 ..... 210-300

pBEW1a_PFOMT      CTTAACGGATACTCTCTTACTCACTGCTCTTCAANCTCTGATGATGAACTGGAACTGATTTCGACAGAGGCCATAGAATGGGTGGAGT 310-400
pBEW1a_PFOMT_181A_1 ..... 310-400
pBEW1a_PFOMT_181A_2 ..... 310-400
pBEW1a_PFOMT_184M_1 ..... 310-400
pBEW1a_PFOMT_184M_2 ..... 310-400

pBEW1a_PFOMT      CCATTATTCGAAAAGCTGGTGTGAGGCAAACTAACTTCAATTGATGCGATGCTATGCTAGCTGCTTGCACATCTCTGCAAGGAAAGAACTGGGG 410-500
pBEW1a_PFOMT_181A_1 ..... 410-500
pBEW1a_PFOMT_181A_2 ..... 410-500
pBEW1a_PFOMT_184M_1 ..... 410-500
pBEW1a_PFOMT_184M_2 ..... 410-500

pBEW1a_PFOMT      GGAATTACQACTTTGGTTGTGATGGGGCAAACCTAACTACATCAAATGACATGAGGTTGATGAAACTAGTCAAGGTGGGTGGCTATGCTTCA 510-600
pBEW1a_PFOMT_181A_1 ..... 510-600
pBEW1a_PFOMT_181A_2 ..... 510-600
pBEW1a_PFOMT_184M_1 ..... 510-600
pBEW1a_PFOMT_184M_2 ..... 510-600

pBEW1a_PFOMT      TGACAAACATTAAGGGTGGAAACTGTAGGCCAACCTGAACTGAGATCCGAACTACCAATGATTTCAAGAAGAAAACAGAGGCTTATGAACTCAAACCTG 610-700
pBEW1a_PFOMT_181A_1 ..... 610-700
pBEW1a_PFOMT_181A_2 ..... 610-700
pBEW1a_PFOMT_184M_1 ..... 610-700
pBEW1a_PFOMT_184M_2 ..... 610-700

pBEW1a_PFOMT      CTTCCTGCTGATCTCTGATATGAGATTGACATCTCTCTTGGGTGATGGTATCACTTCTGAGGGCTCTTATGAAATTGAGGCTCCGTGCAAGGT 710-800
pBEW1a_PFOMT_181A_1 ..... 710-800
pBEW1a_PFOMT_181A_2 ..... 710-800
pBEW1a_PFOMT_184M_1 ..... 710-800
pBEW1a_PFOMT_184M_2 ..... 710-800

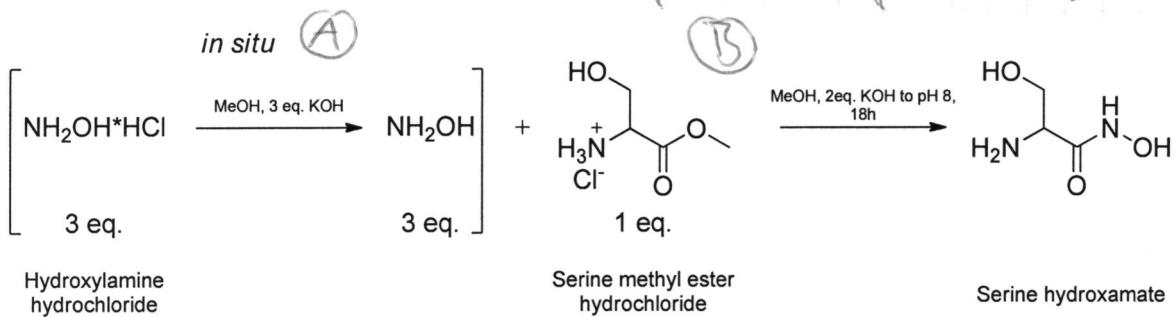
pBEW1a_PFOMT      TGGCGCCGCACTGAGCTGTGTTAAACGGAACTCTGCTGAAATTGAGACCCGAGCACTGGAACTCTGCTACTAGCCAGCTTAATTAACTTAGGCTC 810-900
pBEW1a_PFOMT_181A_1 ..... 810-900
pBEW1a_PFOMT_181A_2 ..... 810-900
pBEW1a_PFOMT_184M_1 ..... 810-900
pBEW1a_PFOMT_184M_2 ..... 810-900

pBEW1a_PFOMT      TGGCACCCTGAGCAAATAGCTAGCATAAACCCCTGGGGCTCTAAACGGGTCTTGAAGGGTTTTGCTGAACTCTGAGCTTAACTAGCCAGCTTAATTAACTTAGGCTC 910-1000
pBEW1a_PFOMT_181A_1 ..... 910-1000
pBEW1a_PFOMT_181A_2 ..... 910-1000
pBEW1a_PFOMT_184M_1 ..... 910-1000
pBEW1a_PFOMT_184M_2 ..... 910-1000

pBEW1a_PFOMT      CACACTGCTCCGGTAGCTAAACCGGTAAACCGCAATAAGACATAAGCCGATTTAACGACCCCTGGCTGAACTGAGCCAGACCGGGCTGAACTTGGCT 1010-1100
pBEW1a_PFOMT_181A_1 ..... 1010-1100
pBEW1a_PFOMT_181A_2 ..... 1010-1100
pBEW1a_PFOMT_184M_1 ..... 1010-1100
pBEW1a_PFOMT_184M_2 ..... 1010-1100

```

WEB 20g

Laut: Inorg. Chem., 2011, 50, 7707 - 7717  
(Phenylalanin hydroxamat.)

|                          | MW    | n        | m       |
|--------------------------|-------|----------|---------|
| NH <sub>2</sub> OH · HCl | 69.49 | 0.15 mol | 10.42 g |
| KOH                      | 56.11 | 0.15 mol | 8.4 g   |

- A
- Hydroxylamin HCl in MeOH gelöst (125 ml)
  - KOH in 50 ml MeOH gelöst
  - KOH-Lösung  $\Rightarrow$  unter Röhren b. N<sub>2</sub>-Fluss zugegeben bis pH 8-9
  - 5 min gerührt, danach KCl abfiltriert

(B)

|                          | MW     | n        | m      |
|--------------------------|--------|----------|--------|
| Serin methyl-ester · HCl | 155.53 | 0.05 mol | 7.78 g |
| KOH                      | 56.11  | 0.05 mol | 5.27 g |

- SerinOMe · HCl in wenig MeOH gelöst und zur Reaktion gegeben
- KOH in ~40 ml MeOH gelöst und zur Reaktion gegeben
- $\rightarrow$  KCl ausgetragen  $\rightarrow$  abfiltriert & Reaktion bei RT unter N<sub>2</sub> für 18 h fortgesetzt (Bei -5°C)

- am nächste Tag:

~~Test~~

- wenig ~~Nis~~ adhäsenter Niederschlag am Kolben

- Test für Fallung  $\rightarrow$  mit Hahn: Hophan (1:1) + Pentan (weil es da war)  
 $\hookrightarrow$  Weißer adhäsenter NS

- bei Zugabe von  $\text{Et}_2\text{O}$   $\rightarrow$  weißer NS

- Fallung des Produktes durch Zugabe von  $\sim 60 \text{ ml} \text{ Et}_2\text{O}$  unbr führen  $\rightarrow$  abfiltrieren von einer gelben Gummiatsche herau

$\hookrightarrow$  ESI (Ms) WEB 209 F1  
(Faktion 1)

$\rightarrow$  die base  $(\text{H}-\text{H}^+)$  von 104 pass zu sein (Ausgangsstoff nicht vollständig umgesetzt)

- Faktion 2: Fallung des restlichen Produktes durch Zugabe von  $\text{Et}_2\text{O}$  ( $\sim 1:1$ ) & Pentan bis keine Niederschlagswolke mehr vorhanden

- 1 h @  $-10^\circ\text{C}$  beläuft  $\rightarrow$  weißer, off-yellow NS

- NS abfiltriert (bleibt in Kochgeschälte) & mit wenig MeOH gewaschen

- NS in  $\text{H}_2\text{O}$  gelöst und lyophilisiert

|                            |                                                   |                                      |
|----------------------------|---------------------------------------------------|--------------------------------------|
| Laborbuch Nr./Notebook no. | Fortsetzung von Seite/<br>Continued from page no. | Seite Nr./ Page number<br><b>140</b> |
|----------------------------|---------------------------------------------------|--------------------------------------|

- getrocknete Substanz:

- Fraktion 1 (Dreieck) - 0,49 g

Fraktion 2 & - 4,27 g

Fraktion 3 - 0,7 g

|                                   |                |                                 |                |                                                        |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|                                   | 16.08.13       |                                 |                |                                                        |

Transformation von pFront Plasmid in  
Mb1655, JM110 & JW1593

- je 50 μg pNCB1 sfGFP-D4+4 und ..

(A) pBEW1a pFront N181A

(B) pBEW1a pFront W184 M

AB-Bisatz

in ① E.coli K12 Mb1655

② E.coli JW1593 s7alpha Kanam. ~~Streptomyces~~

③ E.coli JM110 ~~Streptomyces~~ Kanamycin

transformiert und auf Platte und LB + Amp + Chloram.  
ausplattet &

→ viele Kolonie Anzahl Kolonie :

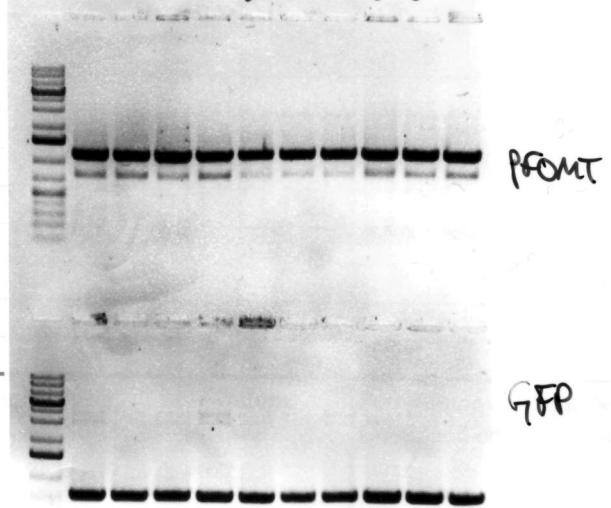
→ Colony PCR (siehe S. 85)

Mb1655 > JW1593 > JM110  
(~2000) (~500) (~7)

↳ nodivinal VD-Anteil auf LB + Strept. + Amp + Chl.  
(für ③) bzw. LB + Kan + Amp + Chl. (2)

WC\Weigel\\_WEB\BioDoc\130918\_colopcr  
riantien+gfp.TIF

M 1 2 3 4 5 6 7 8 9 10



213

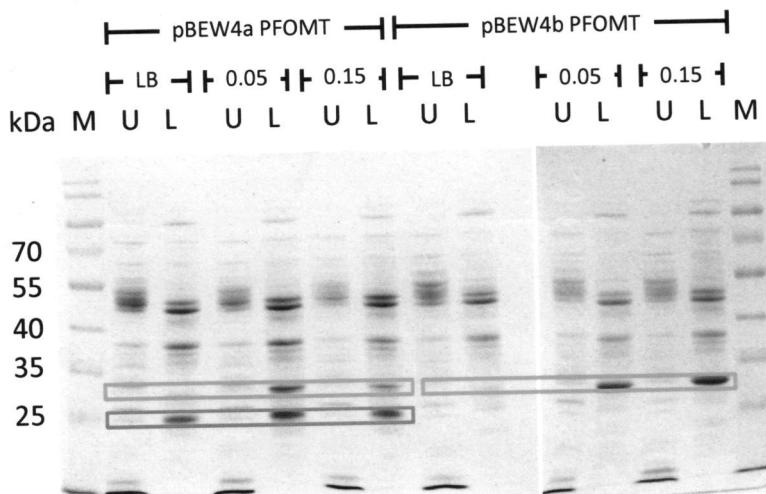
~~EB~~ Expression test pBEW4 PFOMT

### WEB210 - Expressiontest *E.coli* DH5 $\alpha$ mit pBEW4a/b PFOMT

- 2 ml Kultur (+ Antibiotikum) mit Einzelkolonie angeimpft und über Nacht bei 200 rpm und 37°C inkubiert
- Wachstum in LB, early Autoinduction (LB+0.05% Glu+0.2% Rha) and late Autoinduction (LB+0.15% Glu+0.2% Rha) media
- Bestimmung OD600 und Zellaufschluss mit B-PER II
- 10% SDS-PAGE von löslicher und unlöslicher Fraktion

#### Legende

- M** – Marker (PageRuler Prest. Protein Ladder)  
**U** – unlösliche Fraktion  
**L** – lösliche Fraktion  
**LB** – LB-Medium  
**0.05** – LB-Medium + 0.05% Glucose + 0.2% Rhamnose  
**0.15** – LB-Medium + 0.15% Glucose + 0.2% Rhamnose



PFOMT  
Chloramphenicol Acyltransferase

# ~~Agarose~~ Curiation von PFDMS & Mutationen in pBEW4a/b

Ossdare pBEW4a/b mit XbaI & SphI

2x Mm

2µl XbaI FD  
2µl SphI FD  
4µl FD Buffer Green  
2µl Fast AP  
10µl ddH<sub>2</sub>O

Run:

10µl Mm  
+ 10µl pBEW4a bzw pBEW4b  
- 15 min @ 37 °C  
- 20 min @ 75 °C

→ auf 1% Agarosegel &  
ausgeschnitten

→ mit MN PCR & gel cleanup kit  
abfascertet

pBEW4a | pBEW4b  
cby (µl)

## Curiation

7x Mm

14µl T4 Lysore Buffer  
1,4µl T4 Lysore  
86,1 µl ddH<sub>2</sub>O

Reactions:

4a

14,5µl Mm  
+ 4,35µl pBEW4a  
XbaI, SphI  
+ 1 µl fragment  
(Pfdm wt N181A,  
W184L)

4b

14,5µl Mm  
+ 4,8µl pBEW4b cat  
+ 1,1 µl fragment  
+ 2,6 µl ddH<sub>2</sub>O

Sequenzierung

→ Klon 2 von PForw-184M hat Frameshift  
durch A-inset → verwerfe

10 20 30 40 50 60 70 80 90 100  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

110 120 130 140 150 160 170 180 190 200  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

210 220 230 240 250 260 270 280 290 300  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

310 320 330 340 350 360 370 380 390 400  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

410 420 430 440 450 460 470 480 490 500  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

510 520 530 540 550 560 570 580 590 600  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

610 620 630 640 650 660 670 680 690 700  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

710 720 730 740 750 760 770 780 790 800  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

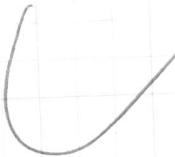
810 820 830 840 850 860 870 880 890 900  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

910 920 930 940  
 pBEW4a\_PFOMT  
 pBEW4a\_PFOMT\_wt\_3\_T7  
 pBEW4a\_PFOMT\_184M\_2  
 pBEW4a\_PFOMT\_184M\_1  
 pBEW4a\_PFOMT\_181A\_2

PFOMT

| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
|                                   | 12.05.19       |                                 |                |                                                        |

→ pBEW4b → alle Sequenzen sind korrekt und passen auf korrektes Inset



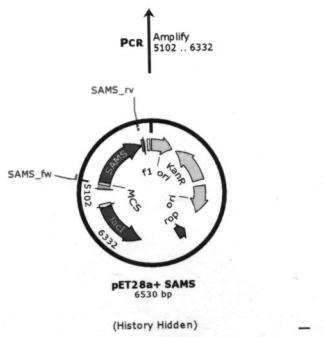
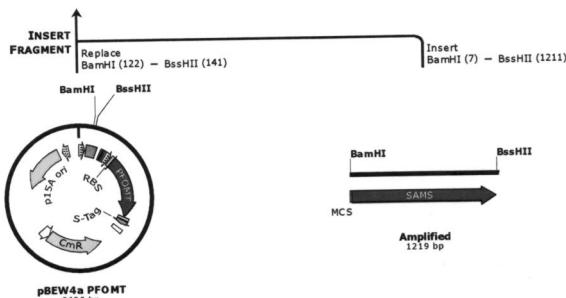
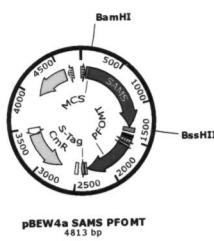
PFOMT

|                                                              |                                                                                                   |
|--------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| pBEW4b_PFOMT                                                 | CTAAAGGAGGTAAAAATGGCAGATCTCTTGGCTGTATGAAAGAGGTCAAATAACAGATTGCTACAGATGAGGAGTTATGCCAGTATTTCTCCGAAC  |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 10 20 30 40 50 60 70 80 90 100                               |                                                                                                   |
| pBEW4b_PFOMT                                                 | TAGTGCTCATCGGQGAAGCAGGGTCCCTAAGGAACTCAGGGAGCCAATGAAAGTCAACCAAGACTCTTATATGTCGACCTTCAACACTGCTGGACAA |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 110 120 130 140 150 160 170 180 190 200                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | TTGATGCTATTCTAAATAATTAGTGAATGAAAGAAAGACTATTGAGCTTGAAGTACTCCCTTACTACTGCTTTCAATTCTG                 |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 210 220 230 240 250 260 270 280 290 300                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | ATGATGAAAAGATTACGGCAATTGATTTCGACAGAGAGCCATAGAATTGGCTTCCATTATCAGAAAAGTGGTGTGGACACAAAATCACTTCAT     |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 310 320 330 340 350 360 370 380 390 400                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | ATGATGGATGCTATGCTAGCTCTGCAAACTTCTGCAAGGAAAGAAAGGGAGTTACAGACTTTGGCTTGTATGGGCAAAACCTTACTAC          |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 410 420 430 440 450 460 470 480 490 500                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | ATCAAATGACATGAGAAGGTGATGAAACTAGTCAGGTTGGCGCATAGTCGCTTATGAAACAACATTTGGGTGAGACTCTGAGCCAGCTGATTCG    |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 510 520 530 540 550 560 570 580 590 600                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | ATCAAATGACATGAGAAGGTGATGAAACTAGTCAGGTTGGCGCATAGTCGCTTATGAAACAACATTTGGGTGAGACTCTGAGCCAGCTGATTCG    |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 610 620 630 640 650 660 670 680 690 700                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | AAGTACCAATTCATGAGGAAAACAGAAGACCTGTTATGAACTCAACAAAGTTGCTCTGATCCCTGATTCGAATTGACATCTTCCCTTGGG        |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 710 720 730 740 750 760 770 780 790 800                      |                                                                                                   |
| pBEW4b_PFOMT                                                 | TGATGGTATCACTTCTGCAGGGCTCTTATTGAAATTGAGCTCCGTCGACAGCTTGGGGCCACTCGACTCTGTAAGAACCGCTGTCGMAAT        |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| 810 820 830 840 850                                          |                                                                                                   |
| pBEW4b_PFOMT                                                 | TTGAAACCCAGCACATGGACTCTGCTACTAGGGAGCTTAAATTAACTTAGGCT                                             |
| pBEW4b_PFOMT_wt_1_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_wt_2_T7                                         | .....                                                                                             |
| pBEW4b_PFOMT_181A_2                                          | .....                                                                                             |
| pBEW4b_PFOMT_181A_1                                          | .....                                                                                             |
| pBEW4b_PFOMT_184M_1                                          | .....                                                                                             |
| A. ACAT. GTC. AGC AT. GTC. AT. GC AT. GTT. A. AC. AA. GCC. C |                                                                                                   |

Klonierung von SAMS wt & 1317V in pBEW4a PFO MT

→ ~~for~~ für Biokonversion und STM & PFO MT in MG1655 & JM110

Sequence: pBEW4a SAMS PFO MT.dna (Circular / 4813 bp)  
Enzymes: restriction Cutters (75 of 646 total)  
Features: 22 visible, 22 total  
Primers: 15 visible, 15 total



### PCR - Programm

|      |       |
|------|-------|
| 95°C | 2 min |
| 95°C | 70 s  |
| 63°C | 10 s  |
| 70°C | 27 s  |
| 70°C | 2 min |
| 4°C  | ∞     |

30x

Primer zur SAMS Amplifizierung  
aus pET28a (+) SAMS:  
SAMS-fw & SAMS-rv

### PCR - Mastermix (2x)

|                         |                   |
|-------------------------|-------------------|
| 10x KOD HS Buffer       | 10 µl             |
| 25 mM MgSO <sub>4</sub> | 6 µl              |
| 2 mM dNTPs              | 10 µl             |
| 3 µl SAMS-fw            | 3 µl              |
| 3 µl SAMS-rv            | 3 µl              |
| KOD HS Polymerase       | 2 µl              |
| ad. H <sub>2</sub> O    | 99 µl             |
|                         | dH <sub>2</sub> O |

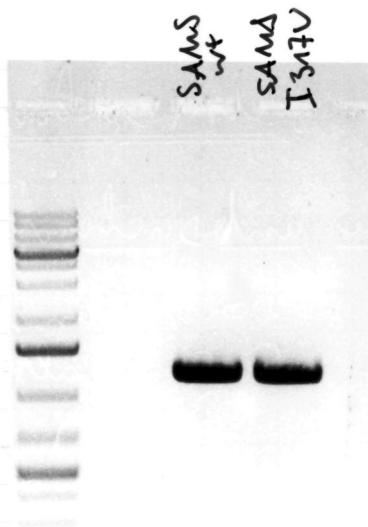
Rez

je 45.5 µl MM

+ 0.5 µl Plesund

(pET28a (+) BS SAMS,  
pET28a (+) STM 1317V)

→ 1.5% Agarosegel mit SceI PCR-Ran bp  
 → erweiterte Größe 1219 bp



### Oberan pBEW4a PFOMT & SAM I PCR

2 x MM

4 µl FD Buffer  
 2 µl BamH I FD  
 2 µl BssH I FD  
18 µl ddH<sub>2</sub>O  
 je 7 µl PCR-cleanup  
 + 13 µl MM

#### pBEW4a PFOMT

7 µl FD Buffer freien  
 1 µl BamH I FD  
 1 µl BssH I FD  
 15 µl pBEW4a PFOMT  
 (0.68 µg/µl)

1500  
1000  
500

;

15 min @ 37°C, 20 min @ 75°C inkubiert

(ng/µl) → Gel & ausprägen (Doctor) → Gel Cleanup  
 M 83.7 PFOMT  
 317V 41.7 PCR cleanup für Fragmente  
 216a 8.5

### Ligation

2 x MM

→ je 19.1 µl MM + 0.8 µl fragment

0.5 µl T4 DNA Ligase  
 4 µl T4 DNA L. Buffer  
 4,6 µl pBEW4a PFOMT  
 29,3 µl ddH<sub>2</sub>O

→ 1h @ 22°C, i. N. @ 6°C

inkubiert E. coli K12

→ 5 µl in MG1655 transformiert

→ Colony PCR & gel sieht S. 957  
Rinne: Diet-NcoI fw & SAM-I rv

WEB211Untersuchung PFOAT-Varianten

zu untersuchende Varianten: (A) WT  
von merken bzw. (B) F80A

\* „Screening“-Box

Konzent.

1,75 mg/ml  
3,1  
2,59

1,09  
2,57

1202W 0,51

(durch verdünnt)  
nl verdünnt

ark + 85,8 µl H<sub>2</sub>O

n + 100,64 µl H<sub>2</sub>O

n + 96,84 n

+ 64,96 µl n

+ 96,16 µl n

n STM (73,7 µl)

→ mischen, runterfügen

→ 12,5 h bei 30°C (Inkubator Autoklavenvolumen)

→ 2 x Extraktion mit 500 µl Ethylacetat  
(+ 2% HCOOH)

→ SpeedVac, Aufnahme in 100 µl ACN

Konz. PFOAT-Varianten

- A WT
- B F80A
- C W184R
- D Y51K
- E Y51R
- F Y51R N202W

1,75 mg/ml

3,10

2,59

1,09

2,57

0,51

ant 0,5 µg/ml  
→ pro Run 10 µl

Wahl.  
mit Y51R  
(100 µg)

m/

Fortsetzung auf Seite Nr./  
Continued on page number

WE211Untersuchung FOMT-VariantenVariantezu untersuchende Variantenvon Martin bzw.  
Susi erhalten

(A) WT

Konzent.

1,75 mg/ml

(B) F80A

3,1

(C) W184M

2,59

(D) Y51K

1,09

(E) Y51R

2,57

(F) Y51R, N202W 0,51

↓ (durch verd.)  
auf 0,5mg/ml verdünnt(A) 34,7 µl Variante + 85,8 µl H<sub>2</sub>O(B) 19,36 µl n + 100,64 µl H<sub>2</sub>O

(C) 23,16 µl n + 96,84 µl n

(D) 55,04 µl n + 64,96 µl n

(E) 23,34 µl n + 96,66 µl n

Ansatz: Doppelbestimmung  
(100 µl)

10 µl 10x SAM-Puffer

2,5 µl 0,1 M Glutathion

250 µM SAM → aufgefüllt 6,8 µl 5 mM SAM (73,5 µl)

5 µg Protein

2 µl 10 mM Substrat (in MeOH)

5 Substrate  $\times$  6 Proteine  $\rightarrow$  Doppelbestimmung  $\Rightarrow$  60 Reaktionen

Reaktionsmassemix ①

$\downarrow \times 5$  aliquoteen zu je...

②  $1056 \mu\text{l} +$  Zusatz von  $24 \mu\text{l}$  Substrat

$\downarrow \times 6$  aliquoteen zu

③  $90 \mu\text{l} \text{ MH}$  ②  
+  $10 \mu\text{l}$  Protein (@  $95 \mu\text{l}$ )

$\rightarrow 62 \times$  Mischung

$620 \mu\text{l}$  10x OMT-Buffer  
 $15 \mu\text{l}$  Glutathion (0.1M)  
 $421.6 \mu\text{l}$  SMH (5mM @ 73.5%)

4.26 ml ddH<sub>2</sub>O

5,4566 ml MH

④  $\rightarrow$  Reaktionsmix gemischt, rührer gefügt  
 $\rightarrow$  17.5 h @ 30°C inkubiert

$\rightarrow$  2x mit  $500 \mu\text{l}$  (EtOAc + 2% Formic Acid) extrahiert

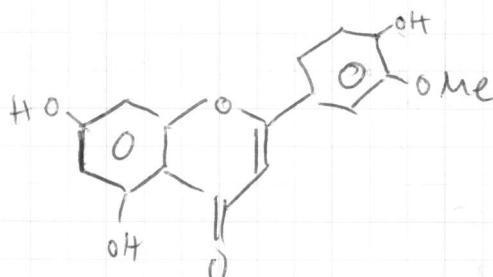
$\rightarrow$  org. Phase vereinigt & in Speedvac abgedampft

- in  $100 \mu\text{l}$  MeCN aufgenommen & per NPLC analysiert

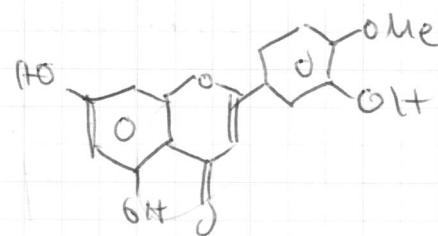
| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
|                                   | 19.03.13       |                                 |                |                                                        |

| Date       | Experiment | Sample | Substrate    | Enzyme             | Comments                                                                                                                               |
|------------|------------|--------|--------------|--------------------|----------------------------------------------------------------------------------------------------------------------------------------|
|            |            | 1A     |              | PFOMT b            |                                                                                                                                        |
|            |            | 1B     |              | PFOMT F80A         |                                                                                                                                        |
|            |            | 1C     |              | PFOMT W184M        |                                                                                                                                        |
|            |            | 1D     | Caffeic Acid | PFOMT Y51K         | Pipettierfehler! --> every reaction done with PFOMT Y51K was erroneously done with <u>Y51R</u> ... But with 11.8µg enzyme per reaction |
|            |            | 1E     |              | PFOMT Y51R         |                                                                                                                                        |
|            |            | 1F     |              | PFOMT Y51R / N202W |                                                                                                                                        |
|            |            | 2A     |              | PFOMT b            |                                                                                                                                        |
|            |            | 2B     |              | PFOMT F80A         |                                                                                                                                        |
|            |            | 2C     |              | PFOMT W184M        |                                                                                                                                        |
|            |            | 2D     | Eriodictiol  | PFOMT Y51K         | Pipettierfehler! --> every reaction done with PFOMT Y51K was erroneously done with <u>Y51R</u> ... But with 11.8µg enzyme per reaction |
|            |            | 2E     |              | PFOMT Y51R         |                                                                                                                                        |
|            |            | 2F     |              | PFOMT Y51R / N202W |                                                                                                                                        |
|            |            | 3A     |              | PFOMT b            |                                                                                                                                        |
|            |            | 3B     |              | PFOMT F80A         |                                                                                                                                        |
|            |            | 3C     |              | PFOMT W184M        |                                                                                                                                        |
| 19.09.2013 | WEB211     | 3D     | Luteolin     | PFOMT Y51K         | Pipettierfehler! --> every reaction done with PFOMT Y51K was erroneously done with <u>Y51R</u> ... But with 11.8µg enzyme per reaction |
|            |            | 3E     |              | PFOMT Y51R         |                                                                                                                                        |
|            |            | 3F     |              | PFOMT Y51R / N202W |                                                                                                                                        |
|            |            | 4A     |              | PFOMT b            |                                                                                                                                        |
|            |            | 4B     |              | PFOMT F80A         |                                                                                                                                        |
|            |            | 4C     |              | PFOMT W184M        |                                                                                                                                        |
|            |            | 4D     | Taxifolin    | PFOMT Y51K         | Pipettierfehler! --> every reaction done with PFOMT Y51K was erroneously done with <u>Y51R</u> ... But with 11.8µg enzyme per reaction |
|            |            | 4E     |              | PFOMT Y51R         |                                                                                                                                        |
|            |            | 4F     |              | PFOMT Y51R / N202W |                                                                                                                                        |
|            |            | 5A     |              | PFOMT b            |                                                                                                                                        |
|            |            | 5B     |              | PFOMT F80A         | sample 211_5B_2 was contaminated with 500 µl of the second extraction of 211_5C_2                                                      |
|            |            | 5C     | Naringenin   | PFOMT W184M        | sample 211_5C_2 was only extracted once with 500 µl EtOAc                                                                              |
|            |            | 5D     |              | PFOMT Y51K         | Pipettierfehler! --> every reaction done with PFOMT Y51K was erroneously done with <u>Y51R</u> ... But with 11.8µg enzyme per reaction |
|            |            | 5E     |              | PFOMT Y51R         |                                                                                                                                        |
|            |            | 5F     |              | PFOMT Y51R / N202W |                                                                                                                                        |

WEB211 → Variante Y51K (D) → wiederholt am



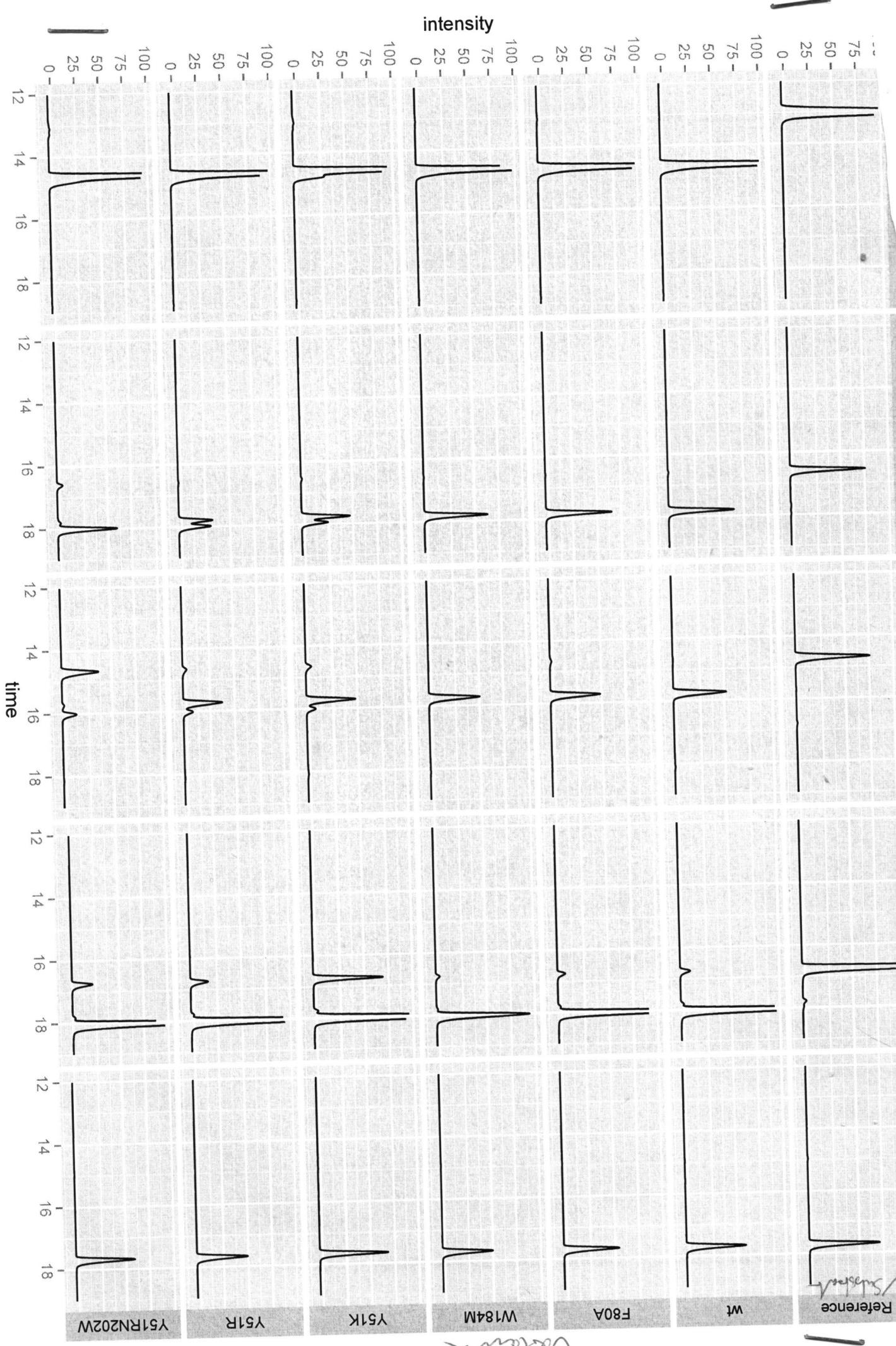
Chrysosoral



Diogenetin

Vat

Eriodictyol



# Ligation von ScOYEZ in pBEW102

Ran:

- 0,77 µl pBEW102 (= <sup>50</sup>~~100~~)  
 2 µl T4 ligase buffer  
 0,28 µl BsaI (@ 10 U/µl)  
 0,3 µl OYEZ Fragment (von DHH)  
 1 µl T4 ligase (@ 5U/µl)  
 ad to 70 µl ddH<sub>2</sub>O (15,6 µl)

Programm

vgl. S. 92

|                                   |                |                                 |                |                                                        |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|

Sequenzierung

pBEW4a - PFOMT - SAMS

→ SAMS

Primer

TTT TTT

&amp; pBEW\_rhahP1

10 20 30 40 50 60 70 80 90 100  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 A...T...ATTA.....A...GAT...CT...  
 ...GTC...T...C...

110 120 130 140 150 160 170 180 190 200  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 GATTTTGATGAAATTAAAGAAACCCCTAACCGCGTGTGGTQAAAACATGTGACACAGGTGGTTCTTGTGAAGGGAGAATTCACAC

210 220 230 240 250 260 270 280 290 300  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 TTCTACGTTATGTTGACATTCGAAAGGCTGGCTGCAAAACATTAAAGAAATCGGATCACACCGTGAAGGAAATACGGATTGTGGGAACTTGTGCGGTT

310 320 330 340 350 360 370 380 390 400  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 TTAAACATCAATTGATGAGCATGTCGCTGATATGCGATGGCGTGAACCAAGCGCTGAGGCCGTAAGGCCAAATQAGCGACGAAAGAAATTGAAAGCGA

410 420 430 440 450 460 470 480 490 500  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 TTGGTGGGGTGGACCAAGGATTAATGTTGGTTATGGTGCGAACGAAAGAGCTTATGGCTCTTCACATTGACTGCCATAAAATTAGCCCGCC

510 520 530 540 550 560 570 580 590 600  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 CCTAACTGAAAGTCGCTAAAGAAATTTCTCCGTAACCCCTGACGGCAAAACACGGTAAACGGTAACTGATGAAATTAACAAACAGTCGC

610 620 630 640 650 660 670 680 690 700  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 ATTGACCGATGTTATTTCACTCGACATCCCTGAAATTACACTTGGAAATTTCAGGGCAAAATTAACAAAGCATGTAATCAATCCGGTTGTCTCG

710 720 730 740 750 760 770 780 790 800  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 AAAGAGCTGTTGATGAAAGAACCAAATTATTCATGAAACCTCAAGGGCTTTCTGTTAACTGGAGGCCCTAAAGGGAGCTTACAGGACGAA

810 820 830 840 850 860 870 880 890 900  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_I317V\_1  
 pBEW4a\_PFOMT\_SAMS\_1  
 CATCGTTGATCTGACGGCGTATCCACGCCACCGGGAGGGCGTTCTCAGGTTAAAGGAGGCAAGAACTGAGCCGTTCTGCAGCTTACGGCA

410 420 430 440 450 460 470 480 490 500  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 CGCCCTGAGGCCAAACACAGGTAACGGTTGAGTACGATGAAATTACAAACAGTCGGCATGAGCGATTGTTATTCACATCGCATACCCCTGAA

510 520 530 540 550 560 570 580 590 600  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 TTACACTTGACGAAATTGAGGCAACATTAAAGAACATGTTATCACTCCGTTGTTCCCTGAAAGCTGTTGATGAAAGAACAAATTATTCATCAACCC

610 620 630 640 650 660 670 680 690 700  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 TACAGGAACCTTCGTAATCGAGGCCCTAAAGGGATGGGGACTTACAGGACCGAAAATCATCGTTGATCTGAGGCCGTTATGACGCCACGGCGA

710 720 730 740 750 760 770 780 790 800  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 GGCGCGTTCTAGCTAAGGACGGACGAGCTGACGCCCTTCAGCTTGTGCTGAGCTTACGGCAAGATACGTTGCGAAATACTGTTGCGGCTGAGCTTGTGATT

810 820 830 840 850 860 870 880 890 900  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 CTTGGAGAATACAGCTTGTGACCGATGCTGTTGACACCGCTGTGTCATCTCAACATTCGTTTCAAGGAAAACCTCTGAGGAAAAACTGAT

910 920 930 940 950 960 970 980 990 1000  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 TGAAAGTTGTTGCAATACTTTGATTACGACCTCCGGCATATCAAAATCTTGTATTGCTGCGCTGAGCTTAAACAAACTGTCGTACGGCCAC

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 TTGGAGCTACGATGTTGACCTCCATGGAGGCCACAGACAAACCGGGAGCAGCTGCGTAAAGAGCTGTTAGGAAATAAGGCCGCTGAGGTCGA

1110 1120 1130 1140 1150  
 4a PFOMT SAMS  
 pBEW4a\_PFOMT\_SAMS\_1  
 pBEW4a\_PFOMT\_I317V\_1  
 AGCTTGGGCCGACATATGCTTAAGTCGAAACAGAAA...GTA...ATCGTATG...A...TC...AG...A...ATTC...A.G.CC

SAMS →

Klonierung Bl3 GVQ in pBew4a/b

# Scheman

Ursachen pBew4a/b & Bl3

3x MM

6 µl FD Buffer freien

3 µl FD Bam HI

10 µl Not I (non-FD)

11 µl ddH<sub>2</sub>O ..

30 µl total

→ p 10 µl MM + pBew4a/b

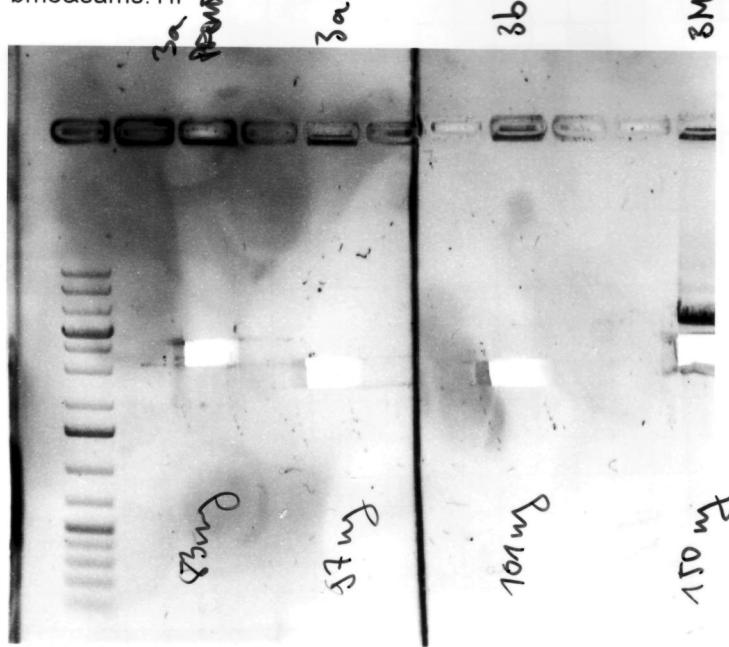
10 µl MM + 5 µl  
fETD<sub>C14</sub> Bl3  
GVQ  
+ 5 µl ddH<sub>2</sub>O

→ 15 min @ 37°C, 20 min @ 75°C  
in Linker

~~dig~~ → fel & Bande ausgewählt  
+ PCR Clean up.

after cleanup:

|        | <u>cly/µl</u> |
|--------|---------------|
| pBTh4a | 8.87          |
| pBTh4b | 15.15         |
| BM3    | 77.93         |

O:\ABT\NWC\Weigel\_WEB\BioDoc\131015\_verdau  
bm3&sams.TIFligation2x MM

0.5 µl T4 DNA ligase

4 µl T4 buffer

73 µl BM3 fragment (@ 17.9 µg/µl)

18 µl ddH<sub>2</sub>O

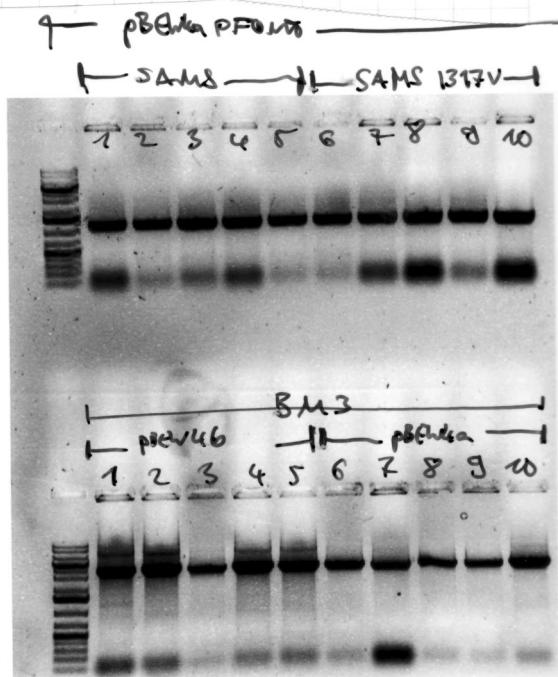
→ 17.75 µl MM

① + 2.25 µl pBTh4a

② + 1.3 µl pBTh4b  
+ 0.9 µl ddH<sub>2</sub>O→ 1h @ 22°C / in N<sub>2</sub> @ 4°C

→ 5 µl - M9 16S5 transformed

→ Colony PCR (Primer: 5'7 km &amp; Act-NaL-fw) ext. time: 3:30



| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
|                                   |                |                                 |                |                                                        |

# PfOMT Chrystallisation

alle Puffer durch  
0.45 µm Filter!

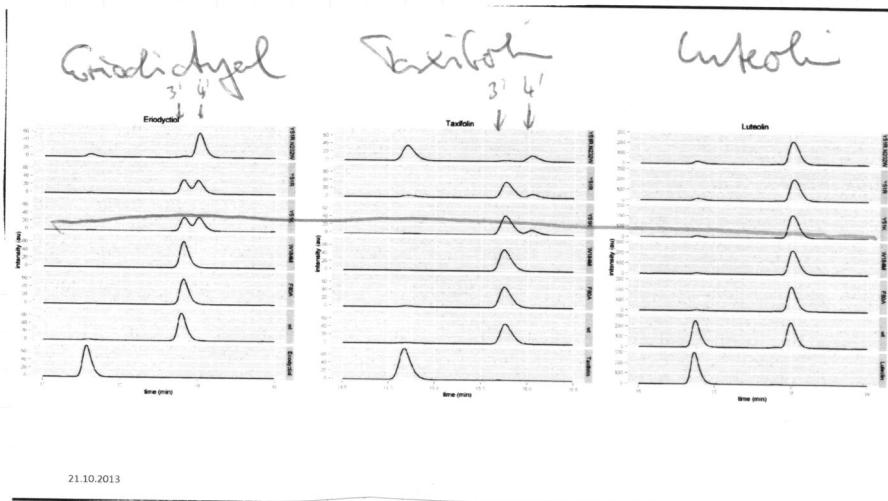
## PfOMT Kristallisation

- Goal: structure of variant with 4'-specificity with bound substrate
- Cocrystallization or soaking of wt PfOMT with SAM and a variety flavonoids was unsuccessful
  - Cocrystallize with SAM analog (e.g. SAE, SAP) and flavonoid
  - Or use inactive variant for crystallization
- To crystallize: wt, Y51R/N202W, perhaps inactive variant
- With substrates: eriodictiol, Taxifolin, Luteolin, SAE, SAP?
- Variant Y51R/N202W has reversed specificity (4'-methylation) for Eriodictiol and Taxifolin, but seemingly (as observed by HPLC)
  - On grounds of planarity?



- Dock different substrates?

21.10.2013



21.10.2013

- ~~10 mM Tris/HCl pH 7.5 für Dialysen → PfOMT Dr. Vavrova  
2x gegen 1x gegen 1L (1h @ RT), 1x gegen 0.5L (1h @ RT)  
und 1x gegen 0.5L (in N. @ 11°C) dialysiert~~
- 10 mM Tris / HCl pH 7.5 für ungelöster & konzentrierter Zentrifugalfilter

WEB 215**PFOMT-Kristallisation 28.10.2013**

Reservoir: 100 mM Hepes/NaOH, 0.2 M CaCl<sub>2</sub>,  
→ with pH 6.5-7.5 and 15-25% (w/v)PEG-4000

## Other solutions:

- 250 mM MgCl<sub>2</sub> in ddH<sub>2</sub>O
- 250 mM Quercetin in DMSO
- 250 mM Eriodictyol in DMSO

1. Concentrate Protein in centrifugal filter and rebuffer into 10 mM Tris/HCl pH 7.5  
2. Readjust concentration to 6.3 mg/mL with 10 mM Tris/HCl pH 7.5

For crystallization of:

- A) Original (Paper, only wt): add 1:1000 250mM MgCl<sub>2</sub> (final 250 µM), 1:10.000 250 mM Quercetin (final 25 µM), 1:20 5 mM SAM (final 250 µM)
- B) Without substrate (only wt): add 1:1000 250mM MgCl<sub>2</sub>
- C) SAE + ED (wt and variant): add 1:1000 250mM MgCl<sub>2</sub> (final 250 µM), 1:1000 250 mM Eriodictyol (final 250 µM), 1:20 5 mM SAE (final 250 µM)
- D) SAE + Q (wt and variant): add 1:1000 250mM MgCl<sub>2</sub> (final 250 µM), 1:1000 250 mM Quercetin (final 250 µM), 1:20 5 mM SAE (final 250 µM)
- E) SAE (wt): 1:1000 250mM MgCl<sub>2</sub> (final 250 µM), 1:20 5 mM SAE (final 250 µM)
- F) No effectors (only wt)

31.10.2013

nach Kopycki et al. J. Mol. Biol.  
(2008), 378, 154-164

Konzentration nach Konzektur

|                 | mg/ml | E                     |
|-----------------|-------|-----------------------|
| PFOMT wt        | 16.12 | 0.71                  |
| PFOMT SAE N202W | 14.81 | 0.85<br>bei<br>E=0.17 |

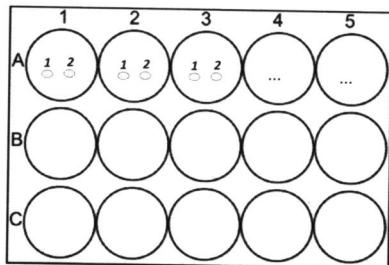
→ Enzym auf 12 mg/ml auf 10 mM Tris/HCl pH 7.5 verdünnen (Dilution)  
→ dann 1:1:1 bzw 1:1:2 (Enzym : Effektor : Reservoir)  
zum Kristallisieren wurde 1

## 4x Solution of effectors:

- A) 0.1 mM Quercetin, 1 mM MgCl<sub>2</sub>, 1 mM SAM
- B) 1 mM MgCl<sub>2</sub>
- C) 1 mM MgCl<sub>2</sub>, 1 mM Eriodictyol, 1 mM SAE
- D) 1 mM MgCl<sub>2</sub>, 1 mM Quercetin, 1 mM SAE
- E) 1 mM MgCl<sub>2</sub>, 1 mM SAE

## two different conditions/well:

- 1) 1:1:1 - Protein : Effectors : Reservoir → 1.33x final effector concentration
- 2) 1:1:2 - Protein : Effectors : Reservoir → 1x final effector concentration



31.10.2013

Effektordilution:

A) 1 µl 25 mM Quercetin

1 µl 250 mM MgCl<sub>2</sub>

50 µl 5 mM SAM (DIM)

add to 250 µl Tris/HCl pH 7.5

B) 1 µl 250 µM MgCl<sub>2</sub>

add to 250 µl Tris

C) 1 µl 250 mM MgCl<sub>2</sub>

1 µl 250 mM Eriodictyol

50 µl 5 mM SAE (DIM)

add to 250 µl Tris

D) 1 µl 250 mM MgCl<sub>2</sub>

1 µl 25 mM Quercetin

50 µl 5 mM SAE (DIM)

add to 250 µl Tris

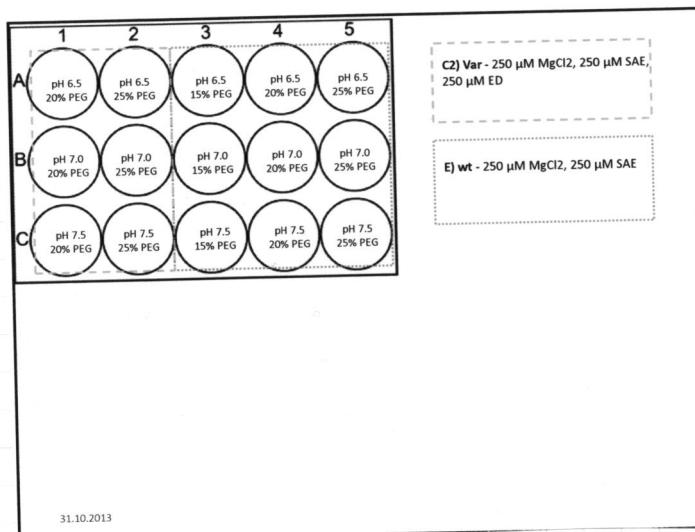
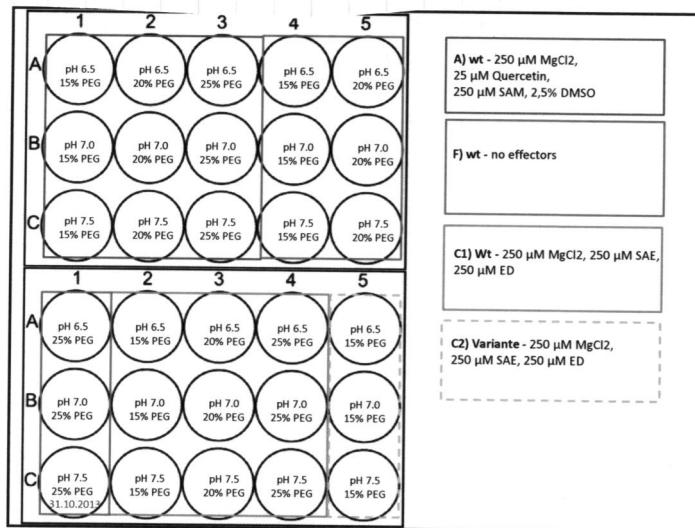
E) 1 µl 250 mM MgCl<sub>2</sub>

50 µl 5 mM SAE

add to 250 µl Tris

(C+D)

→ Eriodictyol & Quercetin sollte bei  
Zugabe aus 1 durch Spülung  
filtriert & zur Kristallisation genutzt!



PFORT - Reinigung:Exponent PFORT per Autoinduktiv

400 ml ZY

431 µl HgSO<sub>4</sub> (1M)

8.6 ml 50%2

86 µl 1000x Trace Metals

21.5 ml MRS

Binding - Buffer

50 mM Tris / HCl

500 mM NaCl

2.5 mM Imidazole

10% glycerin

pH 7

lysozyme → + 1% Tween 20Clatration buffer

- 250 mM Imidazole

→ mit ~~E. coli~~ 10 µl Transformationsansatz (BL21(DE3)  
ausgesetzt  
+ pET28c(+)/PROM)

→ in N. @ 37°C / 220 rpm gezüchtet

- Pelletet @ 10000 × g / 4°C / 15 min

- Pellet in circa 25 ml lysis - Buffer resuspended  
+ lysozyme (Geklopft) → 1h auf Eis

- 3x centrifugiert → + DNase → 15 min @ RT

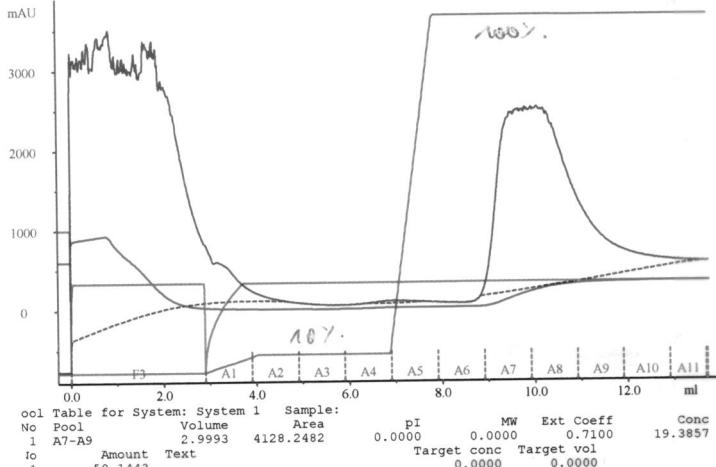
→ Zellkraft pelletiert (4°C / 10000 × g / 15 min)

→ mit ÄKTA über Talon (1ml grünig) → Run Queue  
Beachte: PFORT Talon Biore Queue 1 (5 × 5 ml injiziert)

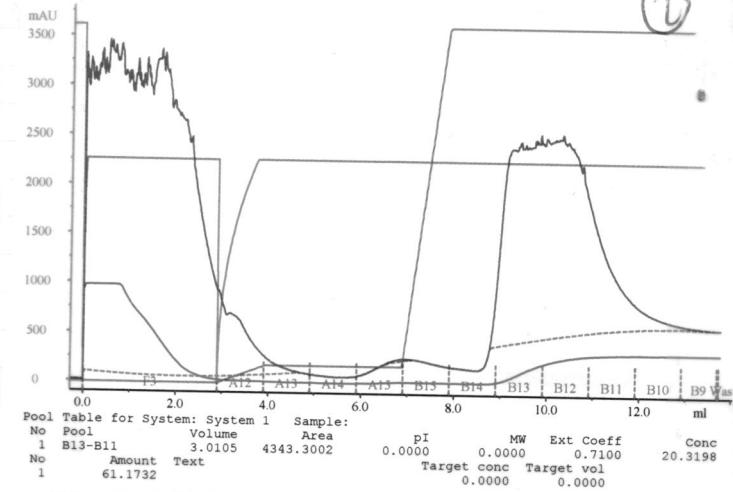
- Puffer A → Binding Buffer

- Puffer B → Elution Buffer

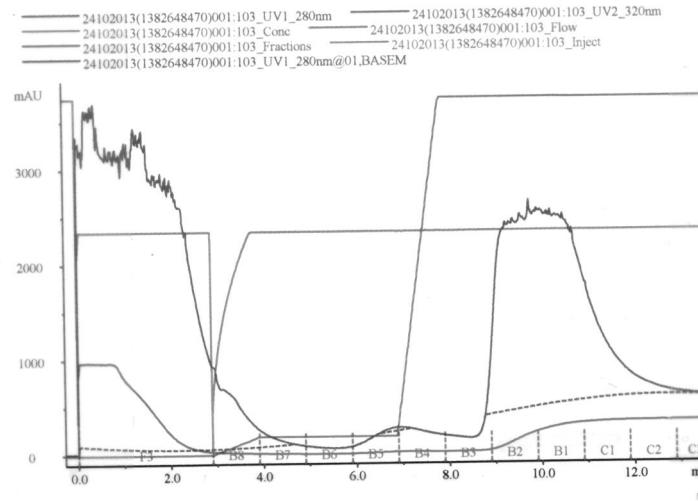
24102013(1382642832)001:10\_UV1\_280nm 24102013(1382642832)001:10\_UV2\_320nm  
 24102013(1382642832)001:10\_Conc 24102013(1382642832)001:10\_Flow  
 24102013(1382642832)001:10\_Fractions 24102013(1382642832)001:10\_Inject  
 24102013(1382642832)001:10\_UV1\_280nm@01,BASEM



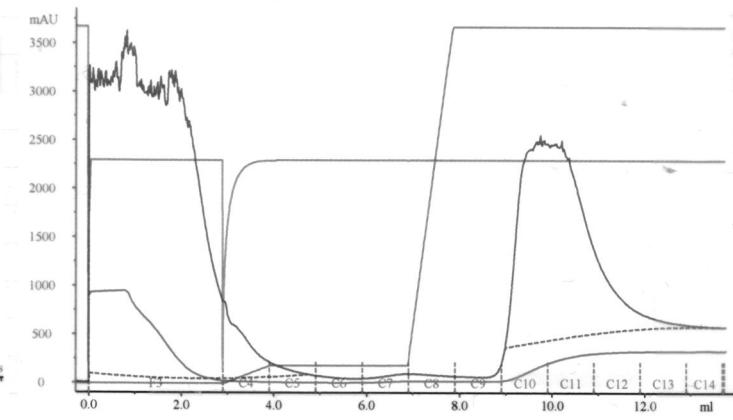
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 24102013(1382645872)001:104\_Conc 24102013(1382645872)001:104\_Flow  
 24102013(1382645872)001:104\_Fractions 24102013(1382645872)001:104\_Inject  
 24102013(1382645872)001:104\_UV1\_280nm@01,BASEM



UNICORN 5.31 (Build 743)  
 Result file: c:\...\WEB\24102013(1382642832)001



24102013(1382648470)001:103\_UV1\_280nm 24102013(1382648470)001:103\_UV2\_320nm  
 24102013(1382648470)001:103\_Conc 24102013(1382648470)001:103\_Flow  
 24102013(1382648470)001:103\_Fractions 24102013(1382648470)001:103\_Inject  
 24102013(1382648470)001:103\_UV1\_280nm@01,BASEM



24102013(1382655667)001:101\_UV1\_280nm 24102013(1382655667)001:101\_UV2\_320nm  
 24102013(1382655667)001:101\_Conc 24102013(1382655667)001:101\_Flow  
 24102013(1382655667)001:101\_Fractions 24102013(1382655667)001:101\_Inject  
 24102013(1382655667)001:101\_UV1\_280nm@01,BASEM

Fraktionen: (sehr nahezu)  
 gepoolt. (15ml)

| Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|----------------|--------------------------------------------------------|
|                |                                                        |

- 15 ml gepoolte Fraktionen ~~→ 1x gefroren~~  
dialysiert:

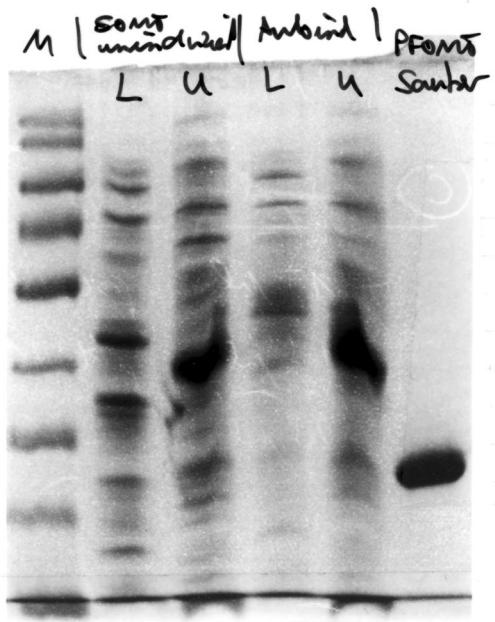
- 1x gegen 1L 25mM Hepes  
5% glycerin  
100mM NaCl  
1h @ RT dialysiert
- 1x gegen 500ml (1h @ RT)
- 1x gegen 500ml 11°C = Nachd.

→ Konzentration bestimmt am NanoLoop

bei  $E^{280\text{ nm}}$  ~~ca.~~  $\epsilon = 0.71 \text{ mg/ml}$  bei  $E=1$

$c = 3.49 \text{ mg/ml}$  → zu 1ml aliquotiert und @ -80°C gelagert

O:\ABT\NWC\Weigel\_WEB\BioDoc\131107\_express  
ionstest\_SOMT2&PFOMT\_Xtallization.TIF



11.11.2013 13:13:19

PCR SOMT-2 für Umbroming in pET22b(m), SOMT2,  
rhaP<sub>BAD</sub> & Autogenous p<sub>EW103</sub>

3 x MM

|                         |         |
|-------------------------|---------|
| 10 x KOD HS Buffer      | 15 µl   |
| 25 mM MgSO <sub>4</sub> | 9 µl    |
| 2 mM dNTPs              | 15 µl   |
| <del>2 x</del> KOD Pol. | 3 µl    |
| ddH <sub>2</sub> O      | 97.5 µl |

→ je 46.5 µl Mastermix +

A) ~~pEW103~~ - Autogenous rhaP<sub>BAD</sub>

- + 1.5 µl p<sub>EW103</sub>-tha-fw
- + 1.5 µl p<sub>EW103</sub>-tha-rv
- + 0.5 µl p<sub>EW4</sub>b

B) SOMT-2

- + 1.5 µl SOMT2-Moclo-fw
- + 1.5 µl SOMT2-Moclo-rv
- + 0.5 µl pQE30 SOMT2

C) ~~1.5 µl~~ p<sub>EW103</sub> Autogenous

- + 1.5 µl p<sub>EW103</sub>-fw
- + 1.5 µl p<sub>EW103</sub>-rv
- 0.5 µl p<sub>EW102</sub>

(zu einem Future eine Bam HI -  
SphI Stelle)

→ für p<sub>EW104</sub> Umbroming  
(Rhto für Moclo und rhamnose  
Promoter)

PCR - Programm

95°C 2 min

(A) 224 bp      (C) 4,2 kbp  
(B) 1,1 kbp

95°C 20 s

• (A) 50°C 10 s / (B) 55°C 10 s

70°C (A) - 3 s / (B) - 18 s / (C) - 1 min 42 s

70°C 2 min

4°C ∞

(A)+(B) → PCR cleanup

④ 25x  
③ 30x  
⑤ 20x

(C) + 5 µl FD Buffer  
+ 1 µl QnT FD  
+ 4 µl ddH<sub>2</sub>O

→ 15 min @ 37°C

→ 20 min @ 80°C

→ 1 µl in XL10 gold  
Inv. K12 MG1655

transformiert

~ am nächsten Tag

4 Klone gepickt

(3 von XL10 gold, 1 von MG1655)

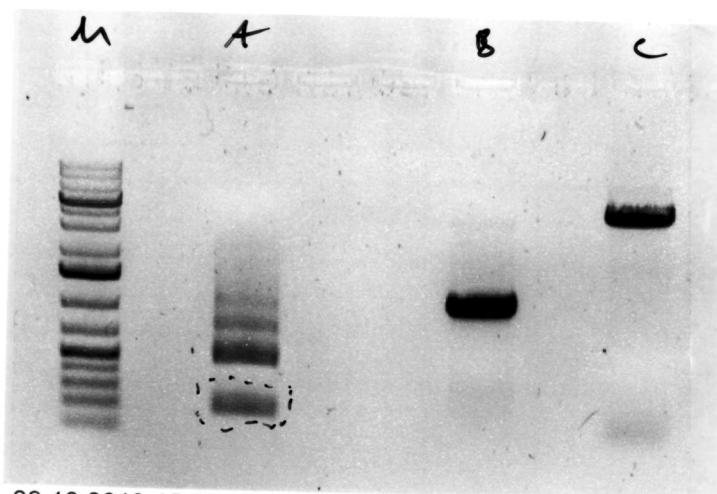
→ 3 µl Plasmid verdaut mit XbaI &amp; BamHI

4x M

4 µl FD Buffer freien  
2 µl BamHI FD  
2 µl XbaI FD  
20 µl H<sub>2</sub>O

→ 7 µl M  
+ 3 µl plasmid (~10 µg)

Agarosegel 1,5% mit 10 µl PCR

O:\ABT\NWC\Weigel\_WEB\BioDoc\131029\_pcr\_som  
t-pbew104.TIF

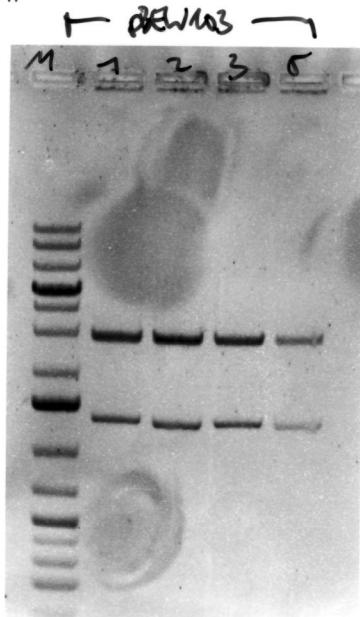
29.10.2013 15:55:51

Testreihen pBEW103

pBEW103 (1x XbaI, 1x BamHI)  
 → 1266 bp & 2848 bp

pBEW102 → keine Bande mit

O:\ABT\NWC\WeigelWEB\BioDoc\131101\_testverdau pbew103.TIF

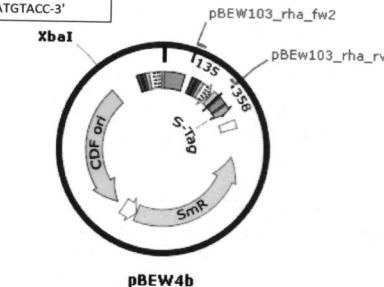
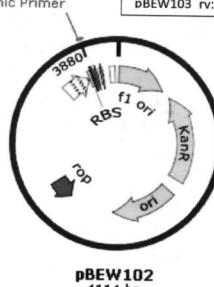
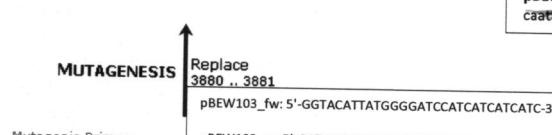
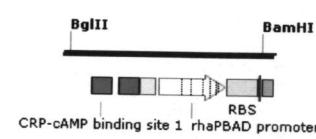
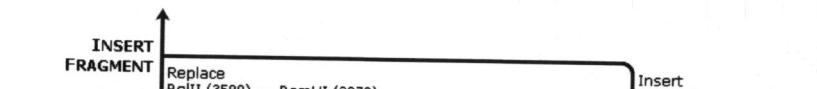
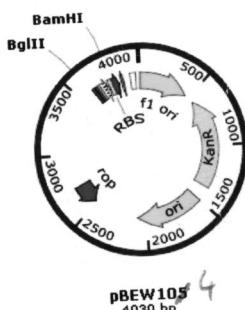


→ alle Klone zum Sequenzieren mit T7 kompatibel

→ Klone 2, 3, 5 positiv  
 & 100% korrekt

→ Klon 1 konnte nicht sequenziert werden

01.11.2013 09:06:14



Durchgeführt von/  
Performed by

Datum/  
Date

Bestätigt durch/  
Approved by

Datum/  
Date

Fortsetzung auf Seite Nr./  
Continued on page number

1.11.13

Ligation SOMT2 in pBEW102 & pET28a McClO

2 x MM  
 4 µl T4 Ligate Buffer  
 0.5 µl Bsa I  
 2 µl T4 ligase  
 0.2 µl SOMT2 ( $\approx$  50 ng)  
 + 31.3 µl ddH<sub>2</sub>O

$\rightarrow$  je 19 µl MM  
 + (A) 3 µl pBEW102 ( $\approx$  129 µg)  
 + (B) 1.2 µl pET28a ( $\approx$  120 µg)

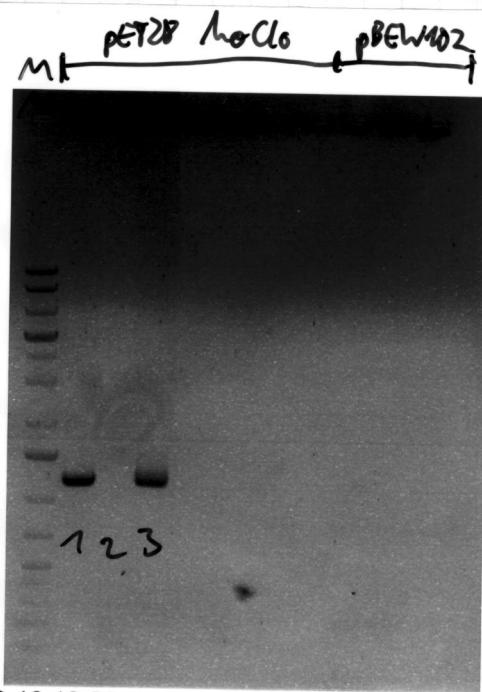
McClO - Pyramide: Siehe S. 92

- 10 µl      ①      ②  
 → Transformation in XL10-gold und MG1655  
 → @ 37°C in N.  
 → Kolone  $\rightarrow$  nur ~~mit~~ mit MG1655 Zellen gewachsen!

Colony - PCR

11 x MM  
 4,4 µl SOMT2\_McClO-fw  
 4,4 µl T7 kom  
 4,4 µl dNTPs  
 22 µl Dream Tag Buffer  
 1,1 µl Dream Tag  
 183,7 µl ddH<sub>2</sub>O

$\rightarrow$  PCR  $\rightarrow$  Extension time = 7:30



$\rightarrow$  Laut PCR nur in Klon 1 & 3 von pET28a McClO positive Test

$\rightarrow$  Klone 1 & 3 mit T7 & T7 kom sequenziert

↑ *SOMT2*

```

pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... T
..... GAGCGATAACAAATGCCCTAGAAAATTGGTTAACCTTAGAAGGAGGATACAGGAGCAGGATTTCAAGCTTGCAGGTGTTGGG
..... CGCTTACCTTCCTTAACTTGAGTTTAACTTAAACGAACTGGCGTAAGCAGTGGATTTCAGGTCAGGCTCTCTACAACTGGGTT
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... A
..... CCGTGCCGGGAGCATATGGTTTTCAATTAAAATAGGCGTAAGCAGTGGATTTCAGGTCAGGCTCTCTACAACTGGGTT
..... GGGGAAATTTCTAACTTAACTTAACTTGAGTTGAGTTAACATAACGACATAACAGCAGTGGCGAACCTTACTTCAGAGT
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... G
..... GGGGGAAATTTCTAACTTAACTTAACTTGAGTTGAGTTAACATAACGACATAACAGCAGTGGCGAACCTTACTTCAGAGT
..... TGAGTCATACAGTGGCGAACCTTACTTCAGAGTAACTAGACACAGTGGAGTTTGAGTAGAGATGGATC
..... TGAGTCATACAGTGGCGAACCTTACTTCAGAGTAACTAGACACAGTGGAGTTTGAGTAGAGATGGATC
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... C
..... 410 420 430 440 450 460 470 480 490 500
..... TGACACATGAAGCATATGCTTCAGCTGAGCTGAGTACTTCAGAGGATGCTTCAGAGCTGAGGAACTTCAGAGGAACTTCAGAGG
..... AATTTGAAAGTCAGGTCAGGCAAGTGAAGAGGTTGGTCAAGMGGAGATCTCAAGTAACTGGGTTGGGTTGGGTTGGGTTGGG
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... T
..... 510 520 530 540 550 560 570 580 590 600
..... AATTTGAAAGTCAGGTCAGGCAAGTGAAGAGGTTGGTCAAGMGGAGATCTCAAGTAACTGGGTTGGGTTGGGTTGGGTTGGG
..... ATTAAGAGAACTCATACAAAGTCATTCAAGGCAAGCTGAGGAACTGGCTTGGTGGATTCAGAGTGTGAGATTTAGAGATTCACATTGGGTTGGG
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... A
..... 710 720 730 740 750 760 770 780 790 800
..... ACTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGG
..... AAATTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGG
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... G
..... 810 820 830 840 850 860 870 880 890 900
..... AAATTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGG
..... CTTCAATTAAGGCAATGGCTTGATTCAGATTTGAAGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGG
..... TTGAGAGATGGGTGAGGAGATGTCAGAGTATTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGGTTGGG
pET28a SOMT
pET28_mocio_SOMT2_3
pET28_mocio_SOMT2_1_... C
..... 910 920 930 940 950 960 970 980 990 1000
..... ATTCGATGAAAGATATTGAAAAATTGAAAAAGCTATTTCAGTAAAGGAAAAGGATGTTGGATGAAAGGAAAGTGGATGAAAGGAAAGTGGATGAAAGG

```

↑ *SOMT2*

```

pET28_mocio_SOMT2_1_
pET28_mocio_SOMT2_3_... T
..... 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
..... TAAGGCCAAAGTACTGAACTAACACTCTTATGGAGTACACATGCAAGTATTAATGAAATAAGGAAAGAGAGATTTGGAGAAAATCTTC
pBEW102 SOMT
pET28_mocio_SOMT2_1_
pET28_mocio_SOMT2_3_... A
..... 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
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pBEW102 SOMT
pET28_mocio_SOMT2_1_
pET28_mocio_SOMT2_3_... G
..... 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
..... CAACATGAGATCTGGTACAGTGGGGAAATGGGAAAGGAAATGGGAAAGGAACTTGCTGCTGCTGCTGCTGAGAACACACAC
pBEW102 SOMT
pET28_mocio_SOMT2_1_
pET28_mocio_SOMT2_3_... C
..... 1310 1320 1330 1340
..... GGCTCTGAGGGTTTTGCTGAAAGGAGGACTTATATCGAT

```

— region d. Überlappung

→ Sequenzen 100% berecht  
mit His-Tag & ohne  
überdeckende Tag

→ bereekte verschleierung in  
pQE30 SOMT2

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PCR rha P<sub>BAD</sub>

011101

S. 165 → aus PCR-Gel → bands bei ~200 bp ausgeschnitten & PCR clean up

→ c = 4,5 µl

5 µl 10x KOD HS Buffer

3 µl 25 mM MgSO<sub>4</sub>

5 µl 2 mM dNTPs

1 µl KOD HS-Pol.

jeweils 1,5 µl prim. pBEW108-kanFw & rv

~~34~~ 34 µl ddH<sub>2</sub>O

→ je 25 µl MM

+ ① 1 µl rha P<sub>BAD</sub> (~4,5 µg)

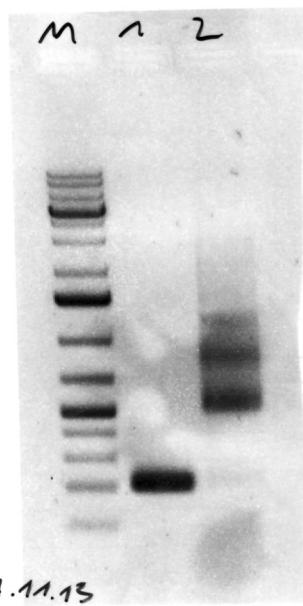
② 1 µl PCR Run (S. 165)

→ PCR Programm wie S. 115, aber Anwärme temp 52°C

→ Agarosegel → sparsame Bands bei ①, aber nicht bei ②

→ PCR-Clean up mit ~~25~~ 20 µl ddH<sub>2</sub>O elutet

→ c = 32 µl



7.11.13  
6:57:02

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|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
|                                   | 07.11.13       |                                 |                |                                                        |

Vorstan pbew113 & rba PBAO

mit gII & SamHC

pbew113

2 μl FD Buffer freen  
 $\geq$  1 μl SamHC FD & gII FD  
 15 μl pbew113 ( $\approx 940 \mu\text{g}$ )  
 1 μl FastAP

rba PBAO

2 μl FD Buffer  
 $\geq$  1 μl SamHC FD & gII FD  
 10 μl RR ( $\approx 130 \mu\text{g}$ )  
 6 μl ddH<sub>2</sub>O

→ 15 min @ 37 °C, 20 min @ 80 °C

J  
 gel & PCR  
 cleanup  
 $c(\mu\text{g}/\mu\text{l}) = 8.6$

J  
 PCR cleanup  
 $c = 14 \mu\text{g}/\mu\text{l}$

Ligation

2 μl T4 DNA ligase Buffer  
 0.25 μl T4 Ligase  
 2.91 μl pbew113 ( $\approx 25 \mu\text{g}$ )  
 0.45 μl rba PBAO  
 14.4 μl ddH<sub>2</sub>O

→ 20 °C für 1h  
 danach 4 °C über Nacht  
 $\rightarrow 5 \mu\text{l} \approx 1 \mu\text{g} 1115 \mu$   
 XL10 gold transformiert

→ ü. N. @ 37 °C

→ 5 Kolonien mit M9 1655; > 50 Kol.  
 mit XL10 Gold

→ Colony PCR

Primer pBTW-vhauP & T7korn

Taq Buffer 27 µl

dNTPs 4.4 µl

Primer 8 6.4 µl

DreamTaq 1.1 µl

183.7 µl ddH<sub>2</sub>O

→ 30 s elongationszeit

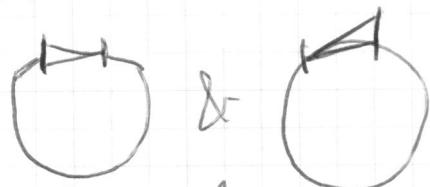
→ 1.5% Agarose gel

- B<sub>gl</sub>I & BamH I

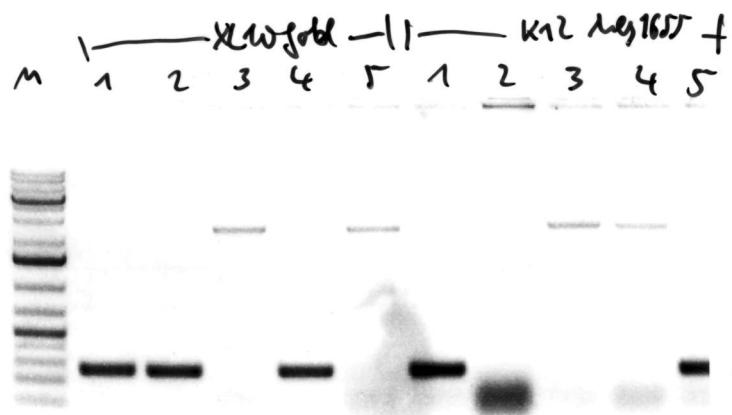


↳ erneute kompatible  
Ende → daher

50:50



eingebaut



11.11.2013 12:50:13

→ Klone 1 & 2 (XbaI gel)

gepreppt (~50 µg/ml)

→ und mit T7korn zum Soprene

⇒ Soprene zu 100% korrekt → 1+2 vereinf

→ lyophilisiert & in 25 µl ddH<sub>2</sub>O aufgenommen

$$c = 75 \frac{\mu\text{g}}{\mu\text{l}}$$

# Ligation SOM2 in pBEW102/104

100 ng vector

1x T4 DNA ligase Buffer

2.5 U BsaI

(1:1) same Molarity of fragment

TU T4 DNA ligase

@ 20 nl

2x MM

3 μl T4 DNA-ligase Buffer

0.5 μl BsaI ( $\approx 10 \mu$  μl)

2.7 μl SOM2 fragment ( $\approx 55.2 \mu$  g)

2 μl T4 DNA ligase ( $\approx 10 \mu$  l)

18.3 μl ddH<sub>2</sub>O

↓

→ 13 μl MM + ① 2 μl pBEW104 ( $\approx 100 \mu$  g)

② 1.8 μl pBEW102 ( $\approx 113 \mu$  g)

→ je 7.5 μl in XL10 gold & MG1655 transformiert

→ nur bei pBEW102 SOM2 (XL10 gold) ~~zelle~~ Kolonie gewachsen

→ ~~#~~ Colony PCR

# WEB214 Expressionstest SOMT2

## WEB214 - Expressionstest *E.coli* BL21(DE3) pET28a(+) MoClo SOMT-2

- 2 ml Kultur (+ Antibiotikum) mit Einzelkolonie angeimpft und über Nacht bei 200 rpm und (18-37°C) inkubiert
- Wachstum in Autoinductionsmedium (ZYP-5052, 0,05% Glucose, 0,2% Lactose, 0,5% Glycerin)
- Bestimmung OD600 und Zellaufschluss mit B-PER II
- 10% SDS-PAGE von löslicher und unlöslicher Fraktion

$$(V = 50 \text{ ml} \times \text{OD} \times V_{\text{Probe}}) \text{ in ml}$$

M - Marker (PageRuler Prest. Protein Ladder)

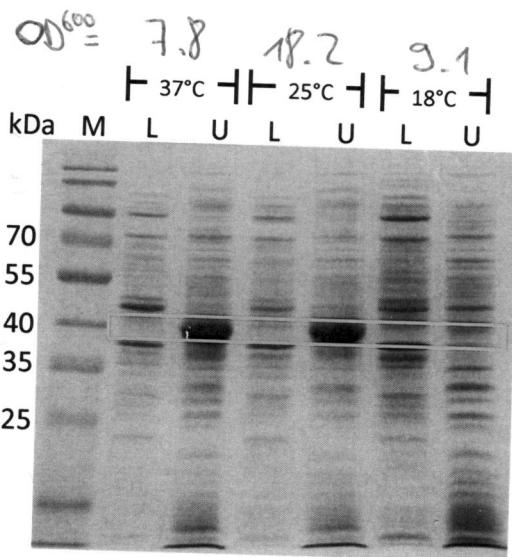
U - unlösliche Fraktion

L - lösliche Fraktion

37°C - Wachstum bei 37°C (über Nacht)

25°C - Wachstum bei 37°C (übers Wochenende)

18°C - Wachstum bei 18°C (3 Tage)



Number of amino acids: 358

Molecular weight: 40425.6 Da

Theoretical pl: 5.37

Extinction coefficients are in units of M<sup>-1</sup> cm<sup>-1</sup>,  
at 280 nm measured in water.

Ext. coefficient 51045 Abs 0.1% (=1 g/l) 1.263 (Cys oxidized)

Ext. coefficient 50420 Abs 0.1% (=1 g/l) 1.247 (Cys reduced)

**Instability index:** The instability index (II) is computed to be 40.02  
This classifies the protein as unstable.

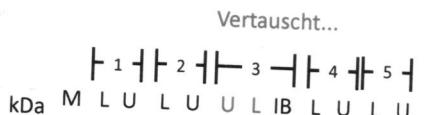
**Aliphatic index:** 94.75

**Grand average of hydropathicity (GRAVY):** -0.066

## Expressionstest *E.coli* SOMT-2

(28.11.2013)

- 3 ml Kultur (+ 200 µg/mL) mit Einzelkolonie angeimpft und ~20h bei 220 rpm und (25°C) inkubiert
- Wachstum in Autoinductions-Medium
- Bestimmung OD600 und Zellaufschluss mit B-PER II
- 10% SDS-PAGE von löslicher und unlöslicher Fraktion



Legende

M - Marker (PageRuler Prest. Protein Ladder)  
U - unlösliche Fraktion  
L - lösliche Fraktion  
IB - inclusion body

1 - C41 (DE3) pET28 SOMT2 (lactose autoinduction)  
2 - C41 (DE3) pBEW102 SOMT2 (true autoinduction)  
3 - Rosetta (DE3) pET28 SOMT2 (lactose autoinduction)  
4 - Rosetta (DE3) pBEW102 SOMT2 (true autoinduction)  
5 - DH5α pBEW104 SOMT2 (rhamnose autoinduction)

| OD (abs ~260) |              |
|---------------|--------------|
| 1             | 11.514 (19h) |
| 2             | 10.184 (19h) |
| 3             | 9.57 (20h)   |
| 4             | 7.46 (19h)   |
| 5             | 2.825 (21h)  |

→ alles Protein  
mit oder ohne  
unlöslich

→ ungepunktet, wieviel  
Protein unlöslich ist  
über native &  
denaturierende SDS-PAGE

↓ 19h

[3.901 (22h)]

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Date

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|                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                               |                                                                                                                                                                                                             |                                                           |                                                                                  |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|----------------------------------------------------------------------------------|
| ZY-Medium:<br>10 g Tryptone/Peptone<br>5 g Hefeextrakt<br>925 ml H <sub>2</sub> O                                                                                                                                                                                                                                                                                                                        | 20xNPS:<br>0.5 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub><br>1M KH <sub>2</sub> PO <sub>4</sub><br>1M Na <sub>2</sub> HPO <sub>4</sub> | 50x5052 [5052=0.5% Glycerin, 0.05% Glu, 0.2% Lac]:<br>25% (w/v) Glycerin<br>2.5% (w/v) Glucose<br>10% (w/v) α-Lactose                                                                                       | 50x5052 -Lac:<br>25% (w/v) Glycerin<br>2.5% (w/v) Glucose | 50x5052 Rha:<br>25% (w/v) Glycerin<br>2.5% (w/v) Glucose<br>10% (w/v) L-Rhamnose |
| Trace Metal Mix (100 ml):<br>36 ml H <sub>2</sub> O<br>50 ml 0.1 M FeCl <sub>3</sub> in 0.1 M HCl<br>2 ml 1 M CaCl <sub>2</sub><br>1 ml 1 M ZnSO <sub>4</sub><br>1 ml 0.2 M CoCl <sub>2</sub><br>2 ml 0.1 M CuCl <sub>2</sub><br>1 ml 0.2 M NiCl <sub>2</sub><br>2 ml 0.1 M Na <sub>2</sub> MoO <sub>4</sub><br>2 ml 0.1 M Na <sub>2</sub> SeO <sub>3</sub><br>2 ml 0.1 M H <sub>3</sub> BO <sub>3</sub> |                                                                                                                                               | Autoinductionsmedium für Expressionstest (1l):<br>~ 928 ml ZY<br>1000 µl 1M MgSO <sub>4</sub><br>20 ml 50x5052 entsprechender Sorte (z.B ohne Lac, mit Lac, mit Rha)<br>200 µl Trace Metal Mix 50 ml 20xNPS |                                                           |                                                                                  |

- 1B von ③ (siehe S. 173) von 500µl Kultur gepumpt
- 500 µl Kultur pelletiert & in 150 µl B-PER II suspendiert
- Zellabtrennung (feiner -/Tan) → pelletiert
- ÜS verwirkt
- Pellet (unlösliche Proteine) in 150 µl ZPER II aufgesponnen
- & mit 6 µl ~~10~~ 10 µl Hypromix versetzt
- 1 ml 1:20 B-PER (in ddH<sub>2</sub>O) zugehen, 1000 µl
- & zentrifugieren
- Pellet wird 1:20 Z-PER gewaschen (7x)
- Pellet in 150 µl H<sub>2</sub>O suspendiert & 10 µl auf gel gegeben  
(Protokoll aus Z-PER II manual)

|                                   |                |                                 |                |                                                        |
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|                                   |                |                                 |                | S177                                                   |

Colony PCR pbew102 somt2

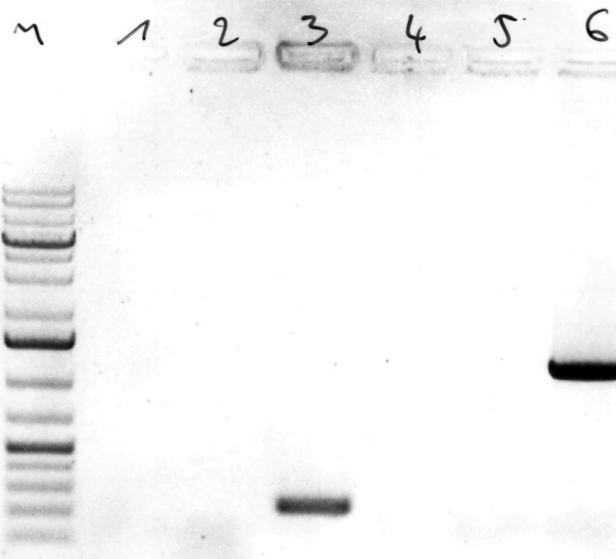
Probholl aus S. 167 fine somt2-MacLc-fw  
77 form

→ nur eine positive Kolonie

O:\ABT\NWC\Weigel\_WEB\BioDoc\131113\_colopcr  
pbew102 somt2.TIF

→ Separat mit pBEW-lsr-up k  
77 form

→ Klon zu 100%  
korrekt



13.11.2013 16:46:11

---

Klonierung somt2 in pBEW104  
 hochmaut ableitbar

Rxn

1.5 μl T4 ligase 50U

1.33 μl pBEW104 (@ 75 ng/μl)

1.15 μl somt2 fragment (@ 24 ng/μl)

0.25 μl Bsa-I

1 μl T4 ligase

9.77 μl ddH<sub>2</sub>O

→ ligation (1h @ 22°C, 4°C i.b.) → transformiert in DH5<sub>2</sub>

→ Colony PCR (wie S. 171)

→ Klon 2 + 3

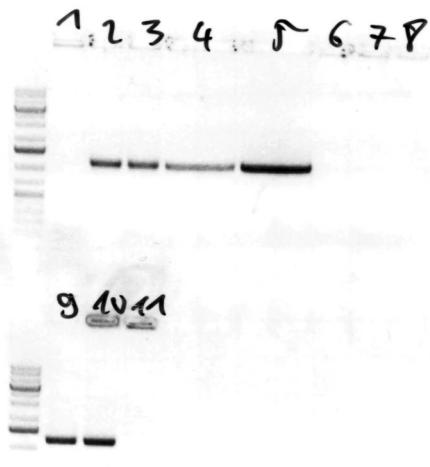
Miniprep  
& Sequenznet

→ Klon 2 100%  
korrekt

→ Dosen austausch  
in Klon 3

→ ist aber schwer  
zu sys aufgrund  
der Sequenzierung

O:\ABT\NWC\Weigel\_WEB\BioDoc\131126\_pbew104  
somt-colopcr.TIF



26.11.2013 18:55:45

# Analyse von bilden & unklarem Protein aus WT8/14

## Purification of proteins on mini-scale using His SpinTrap columns

29.11.13

### His SpinTrap

His SpinTrap™ is a pre-packed, single-use spin column for purifying histidine-tagged proteins by immobilized metal ion affinity chromatography (IMAC). The column allows fast and simple protein purification and is a valuable tool for screening purposes and high-throughput applications. His SpinTrap is used with a standard microcentrifuge and one purification run takes approx. 10 min.

#### His SpinTrap allows:

- High protein binding capacity—up to 750 µg pure histidine-tagged protein per column
- Direct purification of unclarified, as well as clarified cell lysates
- Short purification times—approx. 10 min per run

His SpinTrap columns contain Ni Sepharose® High Performance, which has negligible nickel leakage and is compatible with denaturing and reducing agents, as well as a wide range of additives.

Table 1 lists the main characteristics of the column.



Fig 1. Purifying histidine-tagged protein with His SpinTrap in a simple, four-step procedure. The process can be performed in 20 min using a microcentrifuge. After placing the column in a tube, add sample (1 ml) followed by adding binding buffer and centrifuge. Add sample (1 ml) with binding buffer (1 ml elute the target protein with elution buffer).

| Table 1. His SpinTrap characteristics |                                                                      |
|---------------------------------------|----------------------------------------------------------------------|
| Column material                       | Polypropylene barrel, polyethylene tips                              |
| Medium                                | Ni Sepharose High Performance                                        |
| Medium volume                         | 100 µl                                                               |
| Average bead size                     | 34 µm                                                                |
| Protein binding capacity <sup>a</sup> | Approx. 750 µg histidine-tagged protein/column                       |
| Compatibility during use              | Stable in all commonly used buffers and reducing agents, see Table 2 |
| Avoid in buffers                      | Chelating agents, e.g. EDTA, EGTA, citrate. (See table 2)            |
| Storage                               | 0.1% formalin <sup>b</sup> CG                                        |
| Storage temperature                   | -4 to +30°C                                                          |

<sup>a</sup> Measured at a protein concentration of 1 mg/ml.

<sup>b</sup> Melting point is protein dependent.

### Protocol:

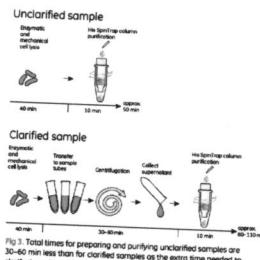


Fig 3. Total times for preparing and purifying unclarified samples are 30–60 min less than for clarified samples as the extra time needed to clarify the cell lysate by centrifugation is eliminated.

#### 6. Sample pretreatment protocol

This is the recommended sample pretreatment protocol. However, other established sample pretreatment procedures may also work. Use standard 2 ml microcentrifuge tubes.

#### 6.1. Dilute the cell paste

- Add 1 ml binding buffer to resuspend cell paste obtained from 20 to 50 ml cell culture volume depending on expression level.
- Note: To prevent binding of host cell proteins it is essential that the sample and binding buffers contain the same concentration of imidazole.

#### 6.2. Enzymatic lysis

- Add the following substances to specified final concentrations in the cell suspension:
  - Lysostaphine: 0.2 mg/ml
  - DNAse: 20 µg/ml
  - MgCl<sub>2</sub>: 1 mM
  - Tris-HCl, pH 7.5 or MOPS: 1 mM
  - Vortex the tubes gently and incubate at room temperature for 50 min.

Note: Chemical lysis kits can also be used, but make sure that they do not contain any chelating agent.

#### 6.3. Mechanical lysis

##### • Repeated freeze/thaw or sonication

#### 6.4. Clarify the lysate

- Spin at full speed in a microcentrifuge for 10 min to remove insoluble material.
- Collect supernatants and purify on His SpinTrap.

Note: Cell culture lysates may be directly applied to the column without prior clarification (i.e. omit step 4).

#### 7. Purification protocol

Run purifications on His SpinTrap using a standard microcentrifuge. Place the column in a 2 ml microcentrifuge tube to collect the liquid during centrifugation.

Use one 2 ml tube for every step.

##### 1. Remove storage solution

- Invert and shake the column repeatedly to resuspend the medium.
- Loosen the top cap one-quarter of a turn and open off the bottom closure.
- Place the column in a 2 ml microcentrifuge tube and centrifuge for 30 s at 70 to 100 × g.



##### 2. Column equilibration

- Add 600 µl binding buffer.
- Centrifuge for 30 s at 70 to 100 × g.



##### 3. Sample application

- Add up to 600 µl sample in one application.
- Centrifuge for 30 s at 70 to 100 × g.

Note: Several sample applications can be performed as long as the capacity of the column is not exceeded.

##### 4. Wash

- Add 600 µl elution buffer.
- Centrifuge for 30 s at 70 to 100 × g.



##### 5. Elution

- Add 200 µl elution buffer.
- Centrifuge for 30 s at 70 to 100 × g and collect the purified sample.
- Add 200 µl elution buffer.
- Centrifuge for 30 s at 70 to 100 × g and collect the purified sample.

Note: The first eluted 200 µl will contain the majority of the target protein.

## Bünde puffer (Chatur)

50 mM Tris/HCl  
500 mM NaCl  
2.5 mM Imidazol  
10% glycerol  
pH 7

## Elution Chatur

wie Bünde puffer,  
aber 250 mM Imidazol

## Sinkpuffer (Chatur) [50 ml]

50 mM ~~NaF~~ KP, (2ml 1M K<sub>2</sub>HPO<sub>4</sub>)  
6 M Guan~~SC~~ Cl (78.6) 0,5ml 1M K<sub>2</sub>HPO<sub>4</sub>  
300 mM NaCl  
pH 7.2

## Centrifuge (Chatur) [50 ml]

45 mM ~~NaF~~ NaPi (45 mM 98% HPO<sub>4</sub><sup>2-</sup>)  
5.4 M Guan~~SC~~ Cl (75.8) 2153 µl  
270 mM NaCl (0.7)  
150 mM Imidazol (0.5) und NaOH ant pH 7.1

Präparation der Proben

- 1 ml Zellsuspensionen halber pelletiert
- Pellet in 1ml Liposolubel (Sulfolysin + 1% Tween 20) + antipronomine
- mit > 10 µl 100 mM PhAT
  - 0,2 mg/ml Lysozyme
  - 0,02 mg/ml DNase I
 vorsichtig & 30 min @ 4°C inkubiert
- 3, 4 sind klar geworden (lyse)
- 1, 2, 5 nicht klar → immuno Fraktion  
→ lyse nicht vollständig?
- für Reinigung der körnchen Fraktion → pelletiert & überstand über His Spin Drop wie im Protokoll (S. 177) gereinigt
- Pellet in 1ml denat. Liposolubel ~~gewaschen (xx)~~ + antipronomine, zentrifugiert & unlösliches Material verworfen
- Überstand gereinigt mit Protokoll (eluiert in denat. Antipronomine)
- Eluate von native & abrahmante Fraktion getrennt für Gelanalyse
- Fällung: native Fraktion: + 1/10 Volume 1% NaDOC  
+ 1/50 V 50% TCA

→ 15 min gekühlt  
 - 5 min @ 8000 x g zentrifugiert (RT)  
 - Überstand abwärmen  
 - 1x mit 200 µl H<sub>2</sub>O aufgenommen geworfen  
 → bei -20°C für 1L inkubiert  
 → wieder pelletiert  
 → 10 min. @ max rpm (16°C) zentrifugiert  
 → Überstand abgehoben  
 → Pellet in 10 µl ddH<sub>2</sub>O aufgenommen & auf SDS-PAGE übertragen

### unlösliche Fraktion

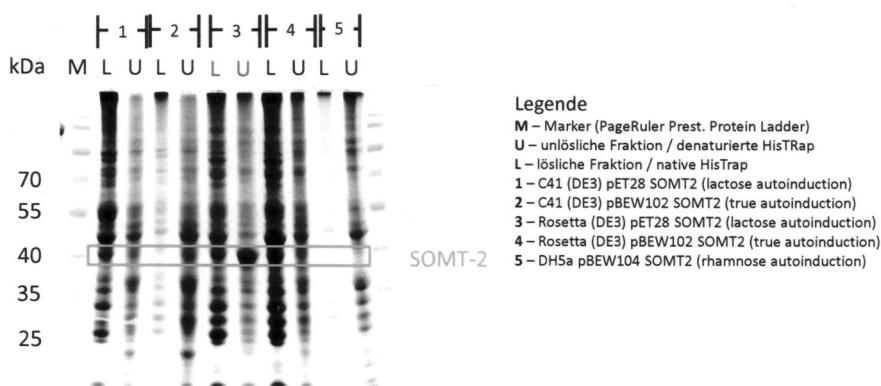
- 1/5 Volume 50% TCA zugeben
- 15 min inkubiert
- pelletiert
- 1x in Auto, 1x in 100% EtOH (je 200 µl) geworfen
- in 25 µl ddH<sub>2</sub>O aufgenommen & 10 µl in SDS-PAGE

His SpinTrap (Ni-NTA) SOMT-2 bei 25°C / autoinduction

(04.12.2013)

- 1mL Zellen pelletiert und in 1 mL Lysispuffer aufgenommen
- Zugabe von je 1 mM PMSF, 0.2 mg/ml Lysozym, 0.02 mg/ml DNase I
- 30 min bei RT inkubiert → 10 min @ 10000xg zentrifugiert → Überstand (lösliche Fraktion),
- Pellet in 1ml denat. Bindepuffer aufgenommen und gevortext → 30 min @ RT
- Zentrifugiert → Überstand (solubilisierter IB) → auf Säule

→ auch in der  
löslichen Fraktion  
ist SDS-PAGE 2,  
welche der  
Ni-NTA angeheftet  
werde kann



## ITC Binding Experiments PFOMT

07.01.2014

Determining binding constants (of substrates and inhibitors) via ITC.  
 Measure substrates by themselves and also with other co-substrates

Substrates/analogs to measure:

SAM and -analogues: SAM, SAH, SAE, SAP, SAB, dcSAM, dcSAH  
 caffeic acid etc.: caffeic acid, ferulic acid, ethyl ferulic acid

## Sample preparation

- 1) Dialyzed PFOMT against **25 mM HEPES, 100 mM NaCl, 5% (v/v) Glycerine pH 7.5** after IMAC and stored dialysate
- 2) Substrates to be measured were lyophilized (SAM, SAE etc.) from aqueous solutions and dissolved in dialysate
- 3) Substrate and protein concentration was measured by UV absorption  
 $(\text{SAM/SAH etc. (Adenine)} \rightarrow \epsilon^{260}=15400 \text{ M}^{-1}\text{cm}^{-1}, \text{Caffeic Acid} \rightarrow \epsilon^{312}=11200 \text{ M}^{-1}\text{cm}^{-1})$

## MicroCal ITC200

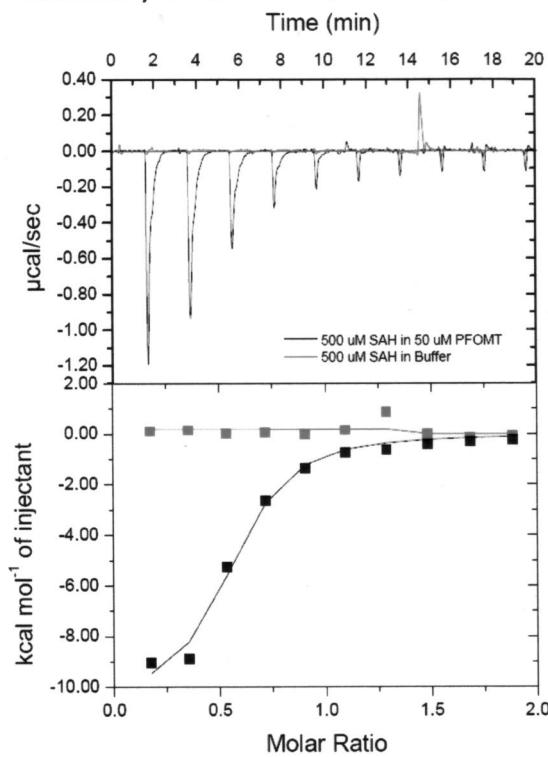
- 1) 50 uM PFOMT protein in the cell, 500 uM substrate in the syringe

~~0.77 ml~~  $\approx 1.437 \text{ mg/ml}$

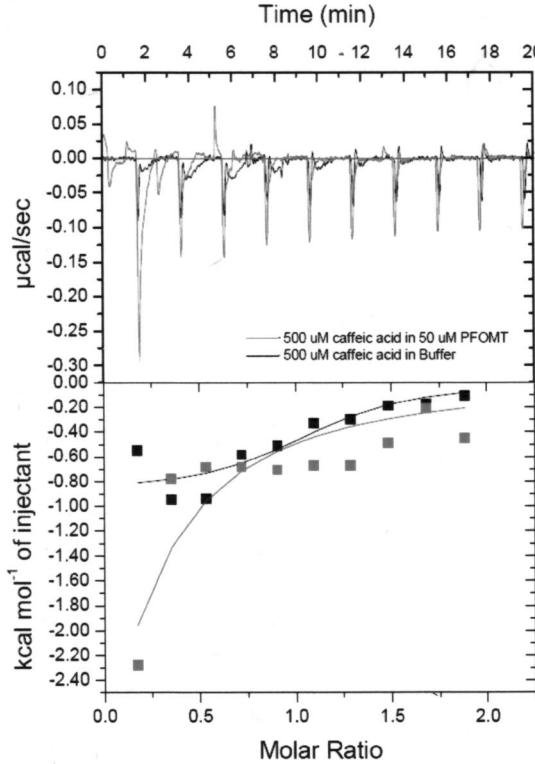
$MW = 28717.8$

## Preliminary data

10 Injections á 4 µl, 2 minute spacing



13.-14.11.2013



Binding curve measurable with SAH but not with caffeic acid → this data is according to literature (sequential bi-bi-mechanism, where **SAM binds first and SAH leaves active site pocket last**)  
 Caffeic acid should not bind without cosubstrate (SAM)

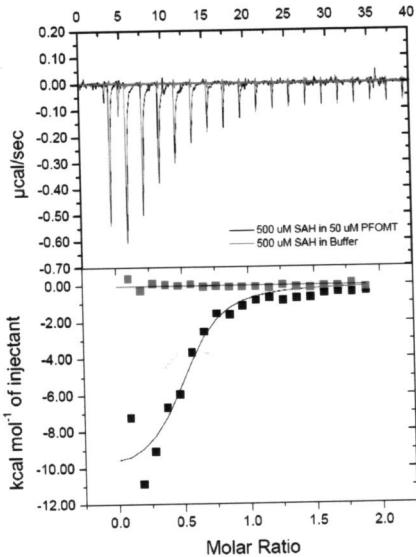
## Preliminary data 2

18.-19.11.2013

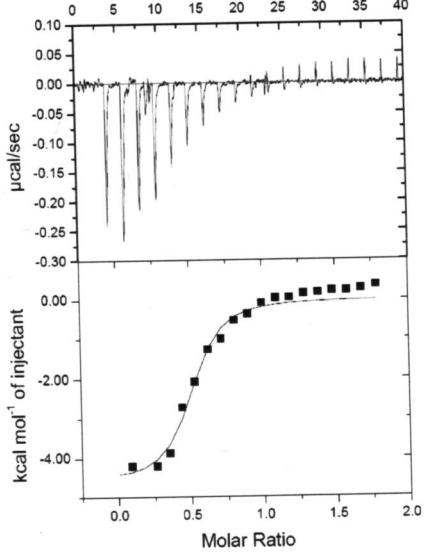
**Injectant:**  
**(500  $\mu$ M)**

**SAH**

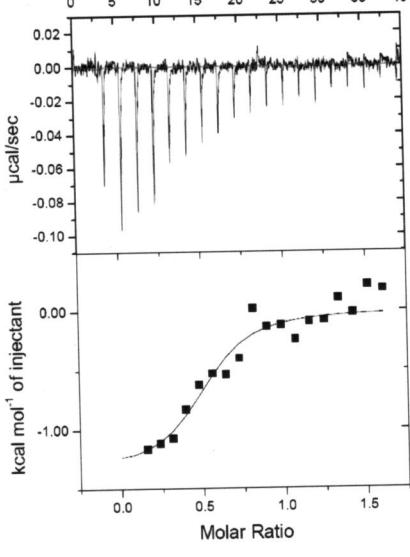
Time (min)

**SAM**

Time (min)

**SAE**

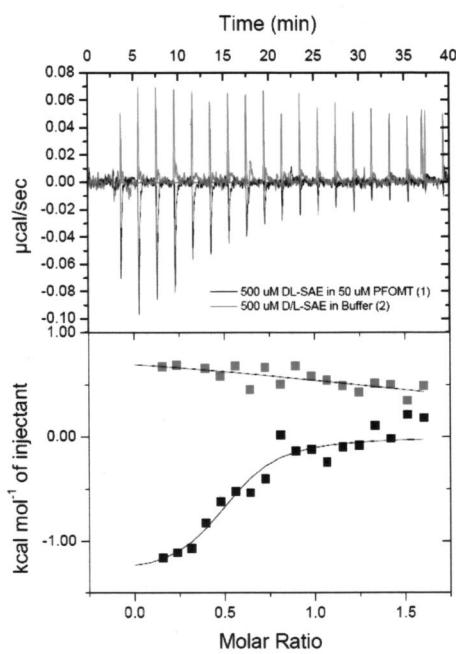
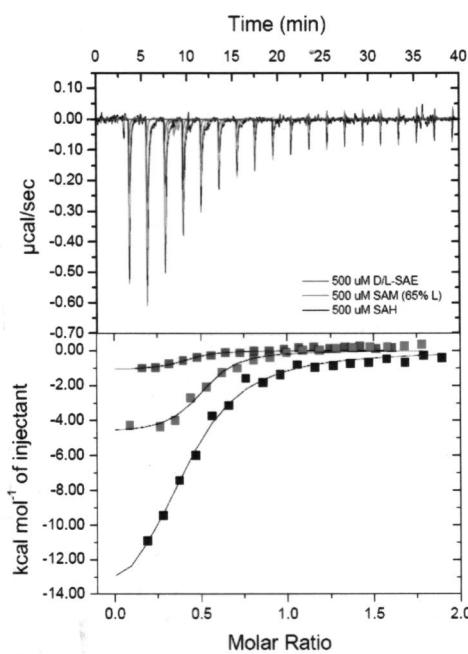
Time (min)



Charge on sulfur  
/net charge:  
**0**

**0****+1****+1****Length of sidechain**

Binding of SAM analogues produces less heat with increasing sidechains.  
→ Measure binding via competition (SAH and PFOMT in cell, titrated with competitor)



SAE injected in buffer shows positive heat → dirty SAE ??

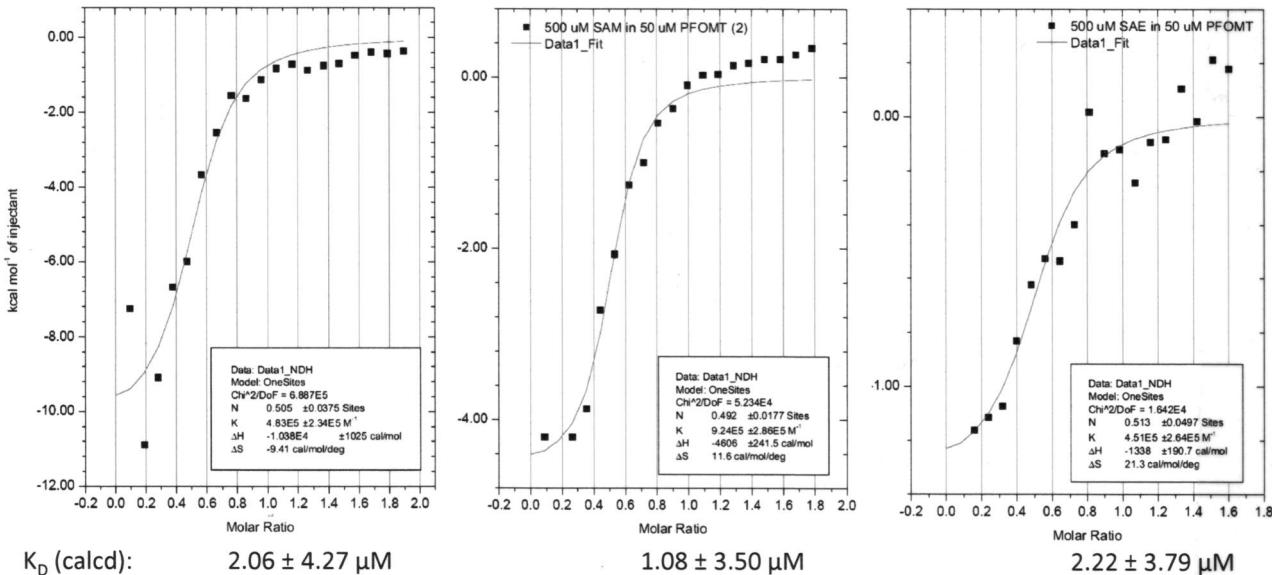
## Preliminary data – Fit for One Site Binding Model

Injectant:  
(500  $\mu$ M)

SAH

SAM

SAE

**Annahme: D- und L-SAM/SAE binden im aktiven Zentrum**Bindungsstöchiometrie aller Verbindungen an PFOMT beträgt ~0.5 (Bindung eines Substratmoleküls pro Dimer),  
Binding constant  $K_D$  (~2  $\mu\text{M}$ ) is also about the same for each compound.

The entropic contribution to binding increases from SAH &lt; SAM &lt; SAE

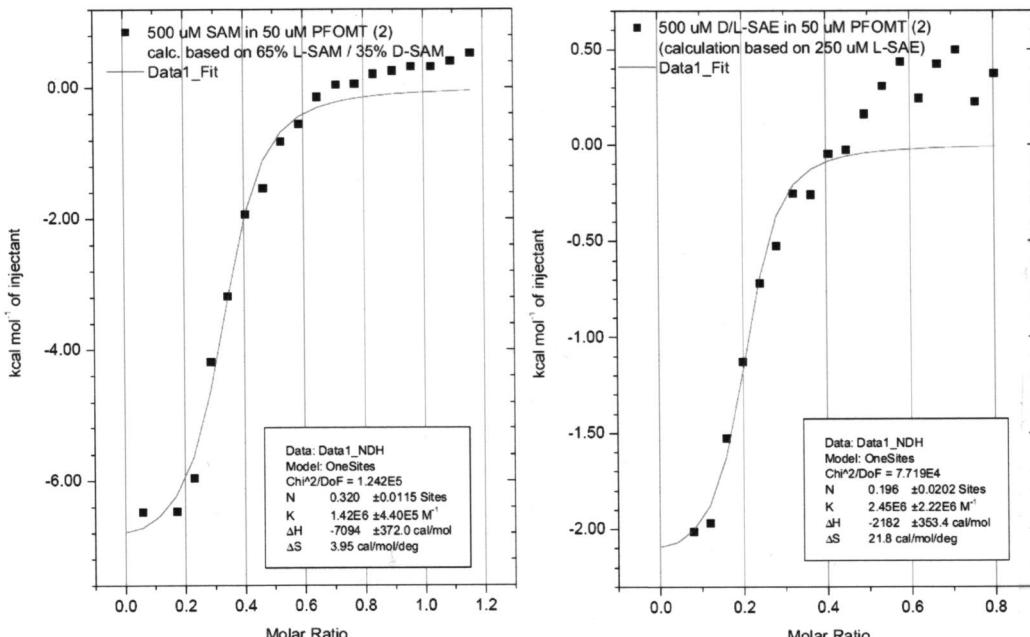
## Preliminary data – Fit for One Site Binding Model

18.-19.11.2013

**Assuming only binding of the L-form**Injectant:  
(500  $\mu$ M)

SAM

SAE

Du  
Per  
Binding stoichiometry is really screwed up when assuming that only the L-form of either SAM or SAE binds  
→ Therefore it is unlikely that this happens → rather it is likely, that both forms bind to PFOMTte Nr./  
number

~~Bestells~~ Bestells für → *Ricinus communis* Castene Synthese  
Re CAS

→ in ~~FFF~~ ddH<sub>2</sub>O aufgezogene & in M12 & BL21 transformiert

→ Colony PCR

3 x Mu

6 µl Taq Buffer

je 1.2 µl TT & RT Koen (~2500bp)

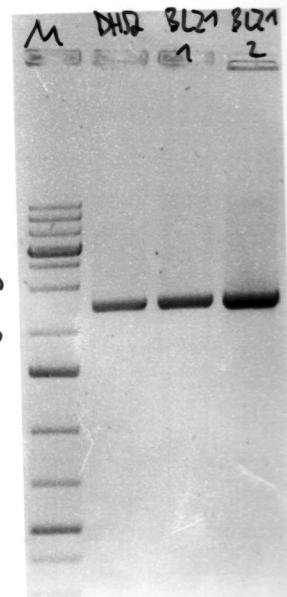
12 µl dNTPs

0.3 µl DreamTaq

50.1 µl ddH<sub>2</sub>O

→ 2.30 elongation time

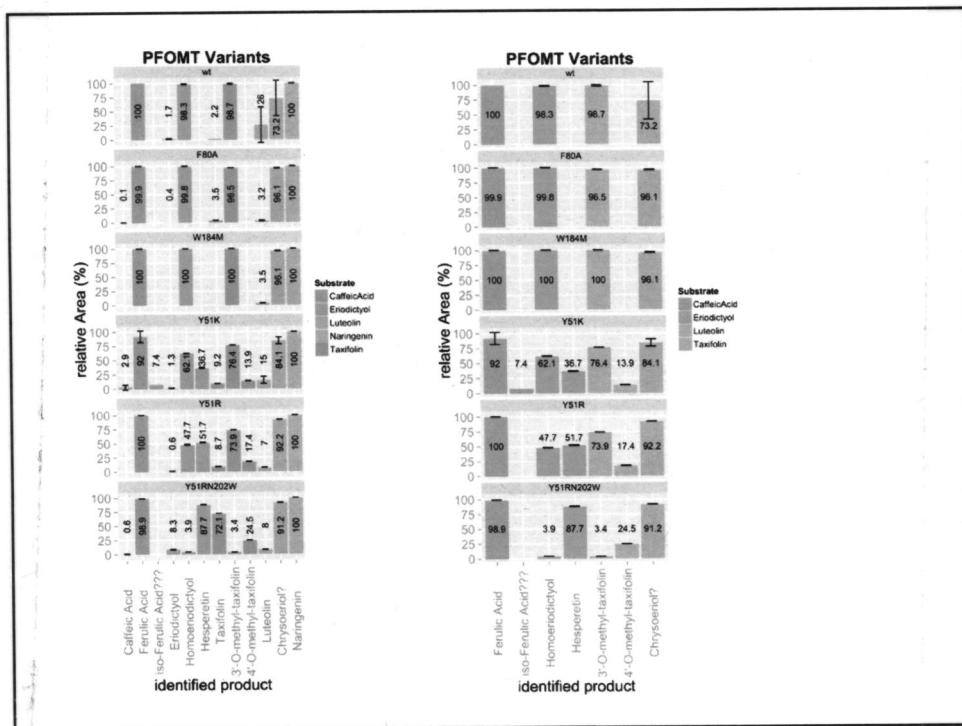
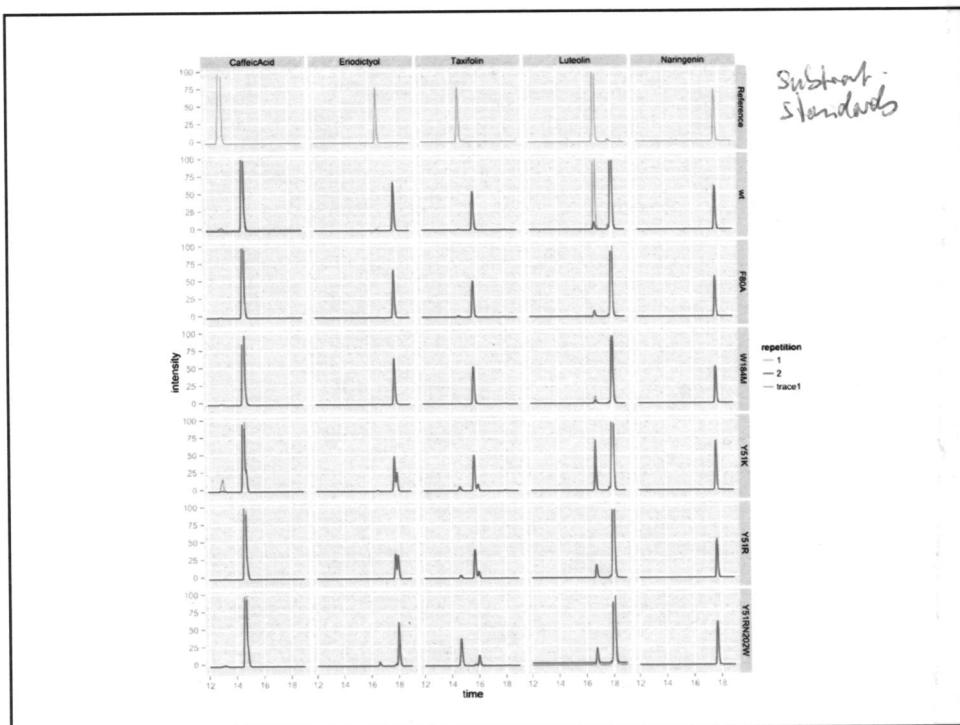
→ Glycerolstock !



08.01.2014 16:18:51

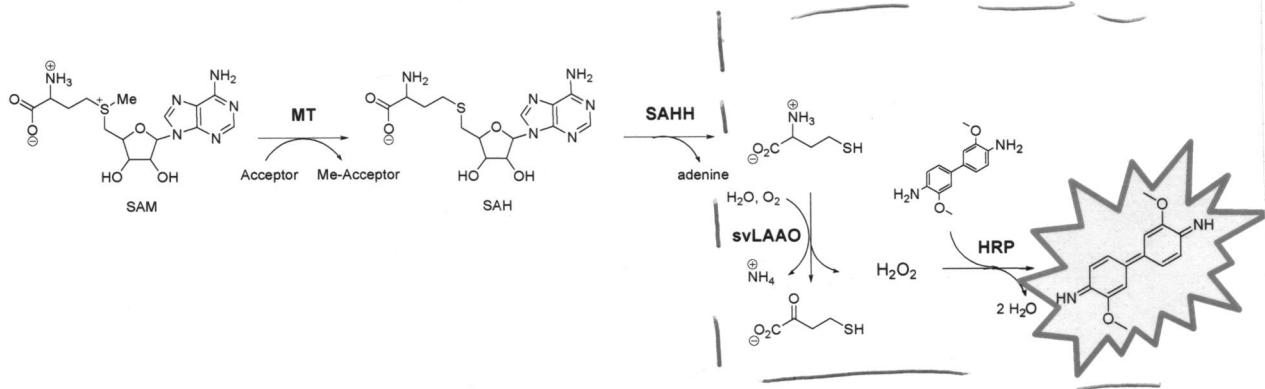
WZL11

PFOMT Variante - SIM

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Continued on page number

Aktivitätsfest für LAAOfür HRP-Aktivitätsfest von HPs

(letzter)

Puffer:LAAO-Speise

100 mM Tris/HCl

100 mM KCl

pH 7.5

Reaktion Puffer:

100 mM Tris/HCl

pH 7.5

- 5 mg/ml Tetramethylbenzidine Hydrolase in H<sub>2</sub>O gelöst

- HRP abgewogen

$\frac{200 \text{ U} (0.5 \text{ mg})}{\text{zu } 3-5 \text{ U/ml}} \text{ in } 10 \text{ ml}$  Reaktion Puffer gelöst

- 1 mg/ml LAAO in LAAO-Speise Puffer
- Lagerung @ 4°C!

Reaktion-Solution:

- 5-10 U/ml HRP

- 0.5 mg/ml TMH HCl

- 0.01-0.5 mg/ml LAAO (Crot. adamantis)

Solution

4.9 ml HRP-Puffer  
+ 0.1 ml 5 mg/ml TMH

- Substrate (Lysine, Methionin, SAM, SAH, Homocysteine) in 5 mM in ddH<sub>2</sub>O gelöst (5 mM stock solutions)

= 75 µl Re

Reaktion: (Volume = 100 µl)

- 80 µl Reaction solution (5-10 U/ml HRP, 0.5 mg/ml TMB)
  - 10 µl Substrate (5 mM)
  - 10 µl 1 mg/ml LAAO
- Inkubation @ 30°C im MTPs
- Absorptionsmessung @ 652nm (Blau)

#### Aktivitätstest L-amino acid oxidase aus *Crotalus adamanteus*

In MTPs:

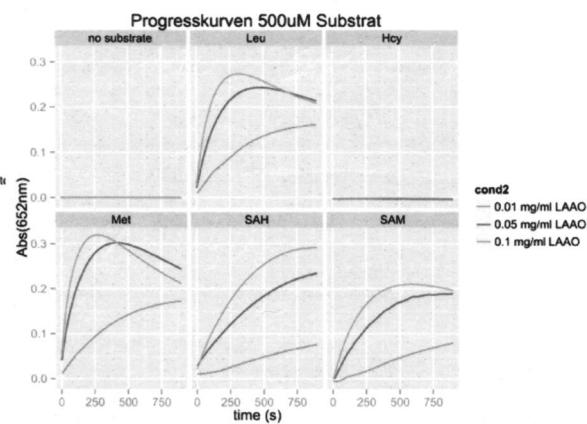
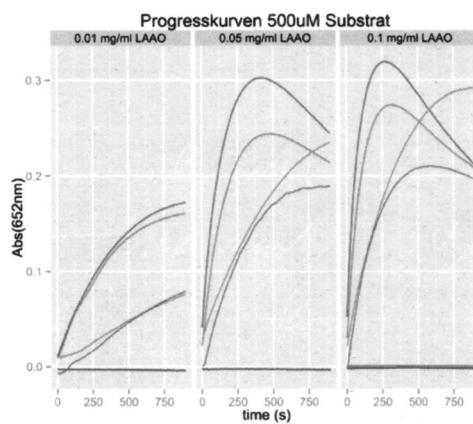
80 µl Reaction solution (3-5 U/ml HRP, 0.1 mg/ml TMB-HCl in 100 mM Tris/HCl pH7.5)

10 µl 5 mM substrate solution

1, 5, 10 µl 1mg/ml LAAO

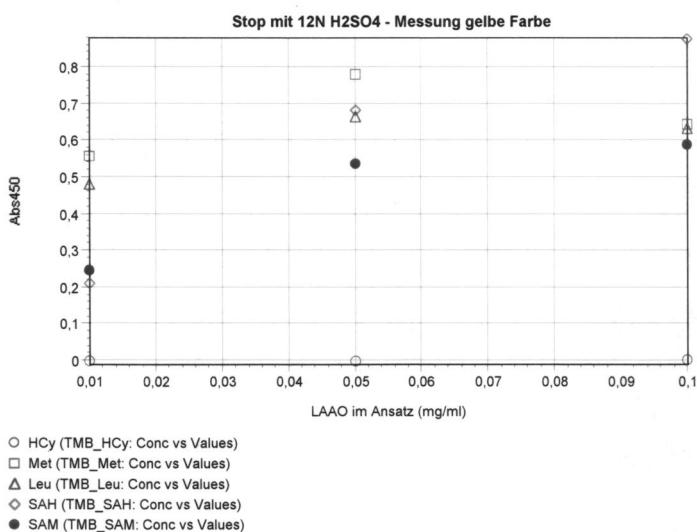
ad to 100 µl ddH<sub>2</sub>O

Incubate at 30°C and read continuous Absorption at 652nm (blue)



→ Homocystei nicht oxidiert, aber SAH & SAM  
 in → kein Assay

→ nach Reaktion (15 min) mit 50 µl 12N H<sub>2</sub>SO<sub>4</sub>  
 gestoppt → Farbe @ 450nm (gelb)



WEB223

LAAO-Assay zur Quantifizierung von SAH?-Erfgrade:

| Endkonz.<br>(SAH)<br>nM | V<br>SAH<br>(500µM) | V<br>H2O |
|-------------------------|---------------------|----------|
| 500                     | 100 µl              | -        |
| 250                     | 50 µl               | 50       |
| 125                     | 25                  | 75       |
| 50                      | 10                  | 90       |
| 25                      | 5                   | 95       |
| 10                      | 2.5                 | 97.5     |
| 5                       | 1                   | 99       |

500µM SAH - Stock - Substrat

→ bei 260nm Konz. bestimmt

$$E^{260} = 6.71 \Rightarrow \underline{44 \text{ nm}}$$

= 75 Reaktion:

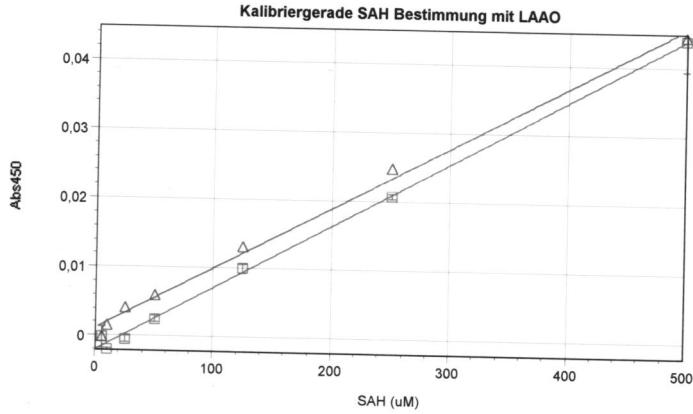
75 µl HRP + TMB ( $\frac{5 \text{ mg}}{0.5 \text{ ml}} \text{ HRP}$ )  
 25 µl Substrat (S.O.)  
 1 µl LAAO ( $\frac{1 \text{ mg}}{1 \text{ ml}}$ )

→ Inkubation bei 30°C für 10 min

→ Stop mit 50 µl 1M H2SO4

→ Ext. bei 450 nm

| SAH_TMB (uM) |       |         |        |           |         |       |
|--------------|-------|---------|--------|-----------|---------|-------|
| Sample       | Wells | Sample# | Values | MeanValue | Conc    | SD    |
| SA01         | B1    | 1       | -0.000 | -0.000    | 5,000   | 0,000 |
|              | B2    |         | 0.000  |           |         |       |
|              | B3    |         | -0.001 |           |         |       |
| SA02         | C1    | 2       | 0.001  | 0.001     | 10,000  | 0,001 |
|              | C2    |         | 0.003  |           |         |       |
|              | C3    |         | -0.000 |           |         |       |
| SA03         | D1    | 3       | 0.003  | 0.004     | 25,000  | 0,001 |
|              | D2    |         | 0.005  |           |         |       |
|              | D3    |         | 0.005  |           |         |       |
| SA04         | E1    | 4       | 0.005  | 0.006     | 50,000  | 0,001 |
|              | E2    |         | 0.008  |           |         |       |
|              | E3    |         | 0.004  |           |         |       |
| SA05         | F1    | 5       | 0.015  | 0.013     | 125,000 | 0,001 |
|              | F2    |         | 0.013  |           |         |       |
|              | F3    |         | 0.011  |           |         |       |
| SA06         | G1    | 6       | 0.026  | 0.025     | 250,000 | 0,001 |
|              | G2    |         | 0.025  |           |         |       |
|              | G3    |         | 0.023  |           |         |       |
| SA07         | H1    | 7       | 0.046  | 0.044     | 500,000 | 0,001 |
|              | H2    |         | 0.043  |           |         |       |
|              | H3    |         | 0.044  |           |         |       |



| SAH_Dianisidin (uM) |       |         |        |           |         |       |
|---------------------|-------|---------|--------|-----------|---------|-------|
| Sample              | Wells | Sample# | Values | MeanValue | Conc    | SD    |
| SA01                | B4    | 1       | 0.002  | -0.000    | 5,000   | 0,002 |
|                     | B5    |         | 0.001  |           |         |       |
|                     | B6    |         | -0.003 |           |         |       |
| SA02                | C4    | 2       | -0.002 | -0.002    | 10,000  | 0,000 |
|                     | C5    |         | -0.002 |           |         |       |
|                     | C6    |         | -0.002 |           |         |       |
| SA03                | D4    | 3       | -0.001 | -0.000    | 25,000  | 0,000 |
|                     | D5    |         | 0.000  |           |         |       |
|                     | D6    |         | -0.001 |           |         |       |
| SA04                | E4    | 4       | 0.002  | 0.003     | 50,000  | 0,000 |
|                     | E5    |         | 0.003  |           |         |       |
|                     | E6    |         | 0.002  |           |         |       |
| SA05                | F4    | 5       | 0.011  | 0.010     | 125,000 | 0,001 |
|                     | F5    |         | 0.011  |           |         |       |
|                     | F6    |         | 0.009  |           |         |       |
| SA06                | G4    | 6       | 0.021  | 0.021     | 250,000 | 0,000 |
|                     | G5    |         | 0.021  |           |         |       |
|                     | G6    |         | 0.020  |           |         |       |
| SA07                | H4    | 7       | 0.035  | 0.044     | 500,000 | 0,004 |
|                     | H5    |         | 0.046  |           |         |       |
|                     |       |         | 0.050  |           |         |       |

→ Kurve ist linear  
→ Prinzipiell möglich eine zu bestimmen

|                                   |                |                                 |                |                                                        |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
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|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|

~~Foto~~ WEB 223 |

Aktivitätsst/ SAHH

### Reaktion

20 µl 5,6 mM DNB (Ellman Reagenz)

10 µl 1 mM SAH

50 µl SAHH (3,2 mg/ml)

30 µl Tris (100 mM Tris-HCl, pH 7,5)

→ Inkubation bei 37°C in NMRs

Opferstet durch Enzymreakt

Wegen neu

R-Bright: Enzymkinetik -  
Padre g

(WEB224)

LAAO-Kineth für Dataset zum Teste

Reaktion-Solutio: 5.10 U/ml HRP

+ 0.1 0.5 mg/ml TMB-HCl

\* 0.06 mg/ml LAAO

+ 1 mg/ml LAA O-Stab  
 $\rightarrow A^{280} = 3,85$

$\rightarrow$  10 U/ml HRP (14.567 ml) / Dunkel!  
+ 333  $\mu$ l 1 mg/ml LAAO \*  
+ 0.1 ml 5 mg/ml TMB

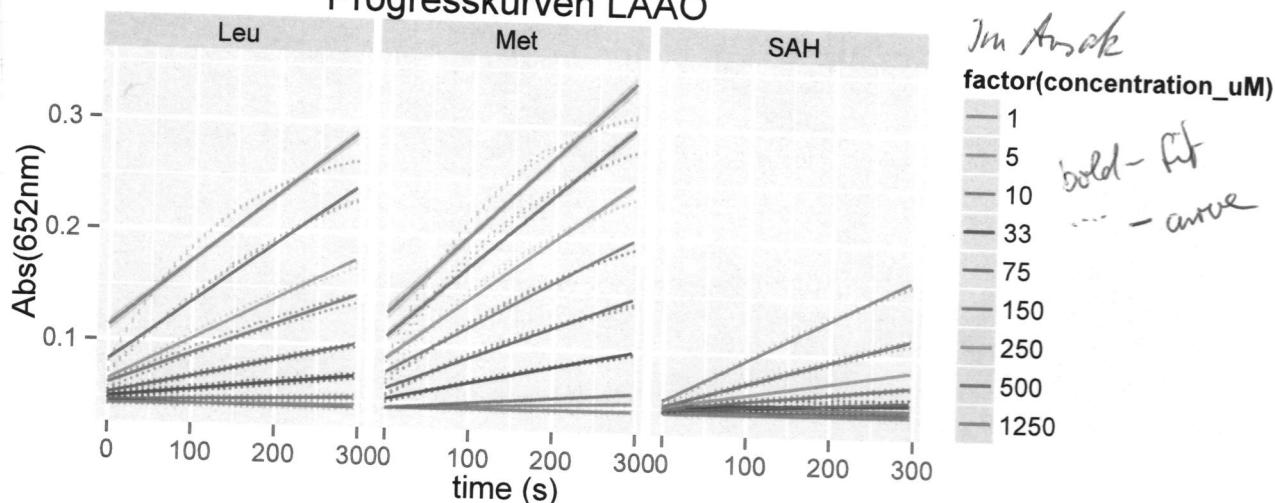
TMB  
lichtstabil

$\rightarrow$  in MTP  $\rightarrow$  Reaktion

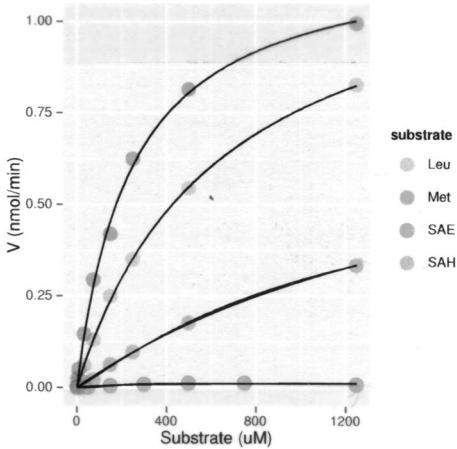
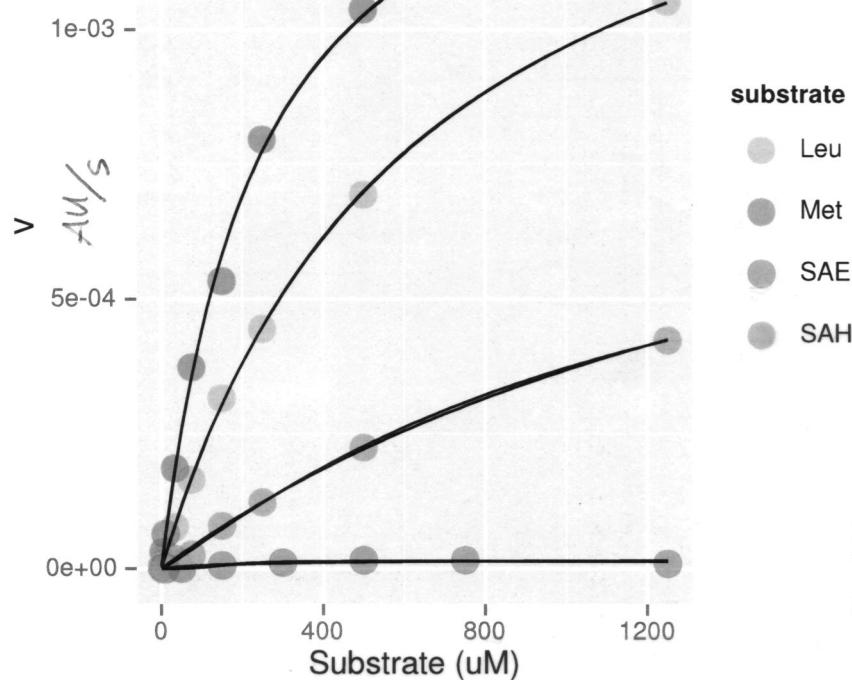
75  $\mu$ l Reaktion-solutio  
25  $\mu$ l Substratlösung  
+ 75  $\mu$ l Reaktion Solutio (stabil)

$\rightarrow$  @ 30°C  $\rightarrow$  measured @ 652nm

Progresskurven LAAO



Crookshanks adam, LAAO Oxidation of Different Substrates  
 (Amine-Oxidation)



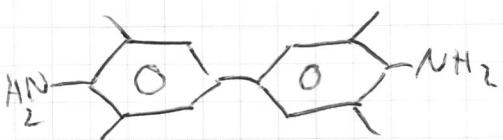
Fit Table

| Substrat | Km (uM) | Vmax (AU/s)          |
|----------|---------|----------------------|
| L-Leu    | 631.2   | $1.58 \cdot 10^{-3}$ |
| L-Met    | 244.4   | $1.52 \cdot 10^{-3}$ |
| SAE      | 211.8   | $1.71 \cdot 10^{-5}$ |
| SAH      | 2006.5  | $1.1 \cdot 10^{-3}$  |

|     | nmol/min |
|-----|----------|
| Leu | 1.24     |
| met | 1.20     |
| SAE | 0.013    |
| SAH | 0.87     |

|       | Vmax (AU/s)          | k <sub>cat</sub> (min <sup>-1</sup> ) | h    |
|-------|----------------------|---------------------------------------|------|
| L-Leu | $1.61 \cdot 10^{-3}$ | $656.6$                               | 0.98 |
| L-Met | $1.51 \cdot 10^{-3}$ | $240.1$                               | 1.01 |
| SAE   | $1.36 \cdot 10^{-5}$ | $763.2$                               | 3.45 |
| SAH   | $8.17 \cdot 10^{-4}$ | $7166.7$                              | 1.13 |

|     | nmol/min |
|-----|----------|
| Leu | 1.26     |
| met | 1.19     |
| SAE | 0.011    |
| SAH | 0.64     |

~~TMB~~

3,3',5,5'-Tetramethylbenzidine

$$\epsilon_{\text{charge transfer complex}} = 39,000 \text{ M}^{-1}\text{cm}^{-1} (@ 652 \text{ nm})$$

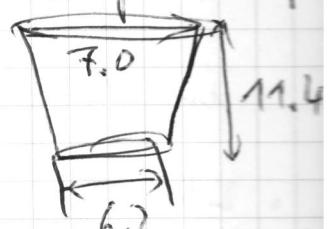
$$\epsilon_{\text{Diamine}} = 59,000 \text{ M}^{-1}\text{cm}^{-1} (@ 450 \text{ nm})$$

- 1 mol Diamine bzw. 0.1 mol charge Transfer-Komplex pro ~~per~~ 1 mol ~~H2O2~~ ~~reduktive~~ von HRP reduziertem  $\text{H}_2\text{O}_2$



$$V = \pi r^2 \cdot h, \text{ aber MTP eiförmig stromper Kp}$$

$$\rightarrow \text{Nahraug: } r = 6.6 \text{ mm} \quad (\text{mittlerer})$$

NUNC:  
36-MTP

$$\rightarrow \text{bei } 100 \mu\text{l} = 100 \text{ mm}^3$$

$$h = \frac{100 \text{ mm}^3}{\pi \cdot (3.3 \text{ mm})^2} = 2.92 \text{ mm} \quad \text{höhe der Lösung}$$

$$E = \epsilon \cdot c \cdot d$$

$$c = E / \epsilon \cdot d = E /$$

$$39000 \text{ M}^{-1}\text{cm}^{-1} \times 0.392$$

$$\frac{c}{\epsilon} = \frac{(E)}{(\epsilon \cdot d)} / \epsilon$$

$$V(\text{AU/s})$$

~~$V = V(\text{AU/s})$~~   
 ~~$E(\text{TMB-CK})$~~

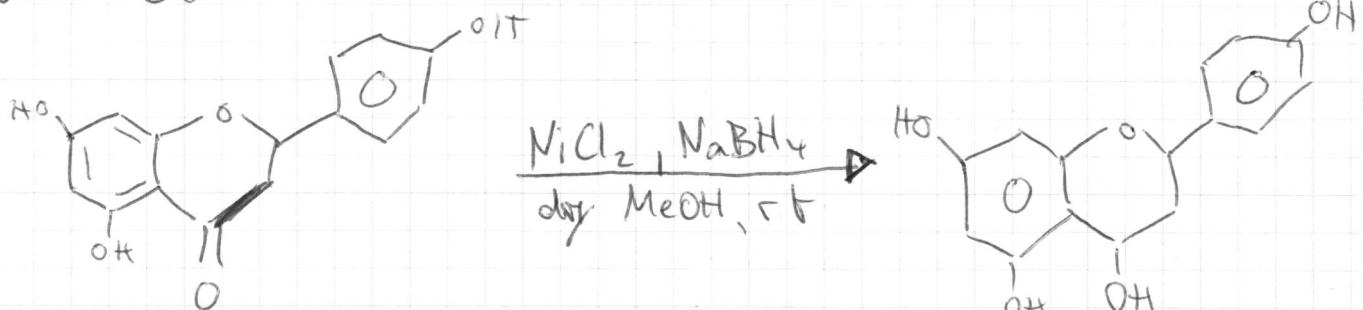
$$V = \frac{V(\text{TMB-charge.complex})}{E(\text{TMB-charge.complex}) \times d (0.392 \text{ mm})} = M/\text{s}$$

$$V = M/\text{s} \times 1000 \times 1000 \times 1000 \times 60 \times 0.000$$

(mM)      ( $\mu\text{M}$ )      (nM)      ( $\text{min}^{-1}$ )      (100)

$$= 44 \text{ nmol / min}$$

WEB 276



|            | MW (g/mol) | n            | PM         |
|------------|------------|--------------|------------|
| Naringenin | 272,26     | 0,5 mmol (1) | 272 mg     |
| NiCl₂      | 129,6      | 0,5 mmol (1) | 65425,6 mg |
| NaBH₄      | 37,83      | 1,5 mmol (3) | 57.214 mg  |

in 10ml MeOH

- 2h @ RT
- nochmal Zugabe von 0,5mmol NiCl₂ & 1,5mmol NaBH₄
- 15 min @ RT
- Zugabe von 7M HCl um NaBH₄ zu zerstören
- Extraktion mit Et₂O (2x 10ml)
- Et₂O-Phase mit mehr Et₂O versetzt  
→ Niederschlag → 1x mit Et₂O gewaschen
- NS abrentrifugiert
- Etherphase eingeeignet → Roter Rückstand  
→ mit Et₂O gewaschen \* (nicht löslich in Et₂O)

|                                   |                |                                 |                |                                                        |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
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|                                   |                |                                 |                |                                                        |

- Rindstiel an Cys gebacken

→ orange Rose Verzweigt

→ NMR → WEB 226 - a

\* Ether Waschfaktionen einzeln Galten für alle

Sequenzierung Smart Seq - Kit

Smart seq Nr.

Name

Punkt

|   |              |                         |   |         |
|---|--------------|-------------------------|---|---------|
| 1 | FR 0232 8966 | pET28a PFOMT Y51K K157D | ① | PFOMT_X |
| 2 | FR 0232 8955 | _____                   | ② | _____   |
| 3 | FR 0232 8975 | pET28a PFOMT Y51R K157D | ① | _____   |
| 4 | FR 0232 8973 | _____                   | ② | _____   |

→ Sequenzierung abnimmt bei 1, 3, 4  
 → keine Mutter in ②

→ Trafo in BL21 (DE3) & Expressie

(WT2P)

200 ml ZY

215 µl MgSO<sub>4</sub> (1M)

4.5 ml T052

43 µl 1000x Trace Dabbs

10.75 ml NPS

+ 100 µl/ml Kanamycin

→ p ~ 200 ml und EK von BL21 (DE3) pET28c (+) PA  
 angepflanzt u.N. @ 37°C/220 rpm  
 in flasche

① Y51K K157D  
 ② Y51R K157D

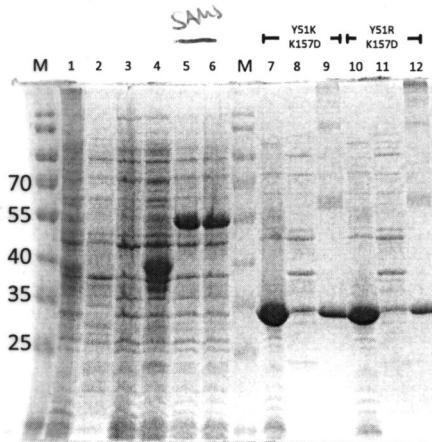
→ OD600 messen (am nächsten morgen)

① YKKD 7.587

② YRKD 7.327

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|-----------------------------------|----------------|---------------------------------|----------------|-------------------------------------|
| S. OZ. M                          |                |                                 |                |                                     |

→ lysiert mit & geäußert wie S.161-163  $A^{280}$   
 YK 1.57 c(ug/ml)  
 fel:  
 • Konzentrationsanalyse YK 4.08



| Nr. | Sample               |                                      |
|-----|----------------------|--------------------------------------|
| 1   | LiCCR insoluble      | pBEW102 LiCCR Expression test (RKS)  |
| 2   | LiCCR soluble        |                                      |
| 3   | SOMT2 pre-induction  | WEB230                               |
| 4   | SOMT2 post-induction |                                      |
| 5   | SAMS1                | WEB229                               |
| 6   | SAMS2                |                                      |
| 7   | Insoluble            | WEB228                               |
| 8   | Soluble              | BL21(DE3) pET28a(+) PFOMT YS1K K157D |
| 9   | Purified             |                                      |
| 10  | Insoluble            | WEB228                               |
| 11  | Soluble              | BL21(DE3) pET28a(+) PFOMT YS1R K157D |
| 12  | Purified             |                                      |

### Aktivitätsfest: voluntary

#### Mischung

24 µl 10x OAT-Buffer  
 24 µl Cystein Acid (10 mM in 20% DMSO)  
 6 µl 0.1M Glutathion  
 60 µl SAM (76% 5 mM)  
 81 µl ddH<sub>2</sub>O

| Extinction                                    |            |
|-----------------------------------------------|------------|
| Abs. 0.1% (-1 <sup>st</sup> 1 <sup>st</sup> ) | [hotparam] |
| PFOMT<br>YS1K<br>K157D                        | 0.663      |
| YS1R<br>K157D                                 | 0.662      |

#### Reaktionen:

① 65 µl Mischung +

② 11 µl PFOMT YS1R K157D (1.78 µg/ml)  
 4 µl H<sub>2</sub>O

③ 3.5 µl PFOMT YS1K K157D (5.76 µg/ml)  
 11.5 µl H<sub>2</sub>O

Blank - ③ + 15 µl H<sub>2</sub>O

→ 16h Inkubation @ RT → kein Unterschied zwischen blank und Probe →

|                                   |                |                                 |                |                                                        |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
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SAMS - Expression & Reinigung

WEB229

aus: Biochimica et Biophysica Acta (BBA) -  
Proteins and Proteomics, Vol. 1784 (12), 2008, 1949-1958

- 2x 200 ml LB-Medium (+50 µg/ml Kan.) und BL21(DE3) pET28c (+) SAMS B17V angeimpft
- bei  $OD^{600} = 0.6 - 0.8$  Z. Induktion mit 50 µM IPTG
- auf 20 °C gehalten und 2L bei 20°C / 200 rpm inkubiert

|                       |         |       |
|-----------------------|---------|-------|
| → $OD^{600}$ gemessen | Flask 1 | 1.984 |
|                       | Flask 2 | 2.091 |

→ Zellente

Reinigung:

- pelletet in insgesamt 25ml Lysis-Buffer ausgetragen
  - + 0.2 mg/ml DNaseI  
+ 0.2 mg/ml Lysozyme
  - 15 min @ RT inkubiert (umgewärmt)
  - 3x mit 70% Amphykohle sonisiert (je 30s / 15s on / 15s off)
  - Zentrifugiert & filtriert
  - gereinigt mit Talon (AKTA)
- 50 mM Tris/HCl  
 500 mM NaCl  
 20% Glycin  
 20 mM Imidazol  
 1% Tween  
 pH 7.5

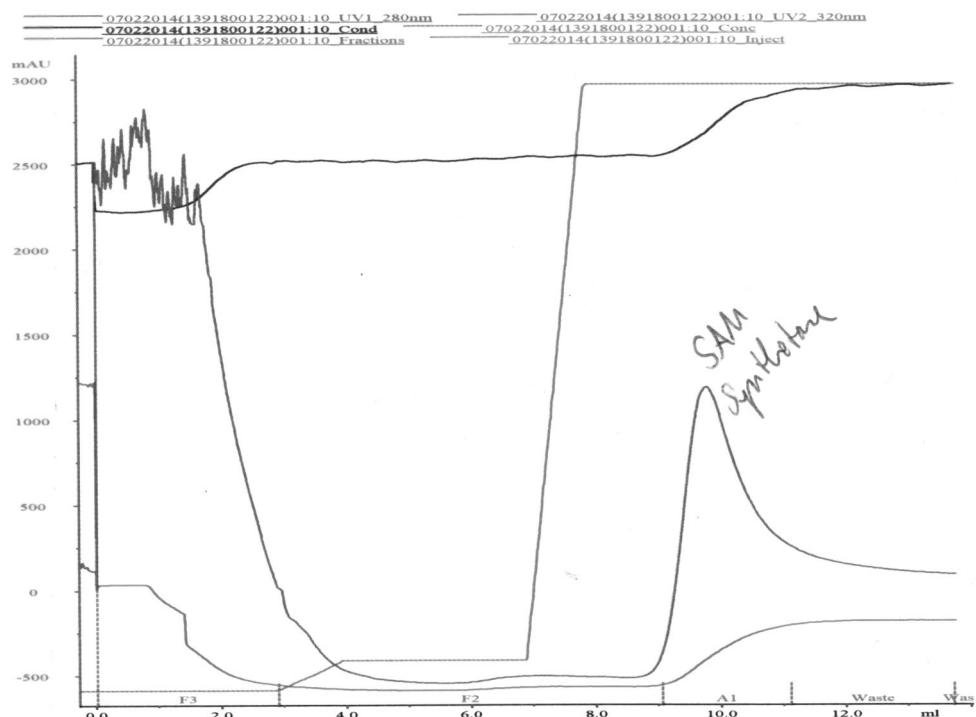
| Durchgeführt von/<br>Performed by | Datum/<br>Date | Bestätigt durch/<br>Approved by | Datum/<br>Date | Fortsetzung auf Seite Nr./<br>Continued on page number |
|-----------------------------------|----------------|---------------------------------|----------------|--------------------------------------------------------|
| b.214                             |                |                                 |                |                                                        |

Bindenpuffer

50 mM Tris-HCl, 0,5M NaCl, 10% (v/v) Glykول,  
20 mM Linderazol pH 7,4

Elutionspuffer

wie Bindenpuffer, aber 300 mM Linderazol



UNICORN 5.31 (Build 743)  
Result file: c:\...\WEB\07022014(1391800122)001

- 2 mal je 10 ml auf Teller Säule geelangt
- Fraktionen vereint ( $\geq 3.5 \text{ ml}$ ) & ~~je 200 ml~~ genommen & 2x je 500 ml ~~zur~~ glykolierte 200 ml für  $\lambda^{280}$
- 1 mg/ml  $\rightarrow \lambda^{280} = 0.57 \quad \lambda^{320} = 4$
- Konzentration @ 280 nm  $c = 4/0.57 \approx 7 \frac{\text{mg}}{\text{ml}}$
- bestimmt