GGNB Method Course

PCR:
self-made enzymes,
helpful additives
and insights into the reactions

PRACTICAL PART

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I. Expression

	PfuS-Polymerase	Pab-PPase	Pab-dUTPase
o/n culture	pSF421 in BL21 DE3 Star pRosetta, 0,5 l 2YT/ 300µg/ml Amp/ 34µg/ml CAM, 37°C	pSF318 in BL21 DE3 Star pRosetta, 0,5 l 2YT/ 300µg/ml Amp/ 34µg/ml CAM, 37°C	pSF323 in BL21 DE3 Star pRosetta, 0,5 l 2YT/ 2% glycerol/ 300µg/ml Amp/ 34µg/ml CAM, 37°C
Dilution	To 3,5l with prewarmed (37°C) TB/Amp/Cam final OD_{600}: 0.5-0.8 distribute in 4x 850ml in 5l flasks	To 3,5l with prewarmed (37°C) TB/Amp/Cam final OD_{600}: 0.5-0.8 distribute in 4x 850ml in 5l flasks	To 3,2l with prewarmed (37°C) 2YT/Amp/Cam + 400mM NaCl final OD_{600}: 0.3 distribute in 4x 800ml in 5l flasks
Shaking	37°C, 100rpm	37°C, 100rpm	37°C, 100rpm
Induction	at OD_{600}=3.0 add 0.2mM IPTG final	at OD_{600}=3.0 add 0.2mM IPTG final	at OD_{600}=0.8 add 1mM IPTG final
Harvest	3-4h after induction add 1mM PMSF (stock 100mM in EtOH) centrifuge 7min, 7000rpm	3-4h after induction add 1mM PMSF (stock 100mM in EtOH) centrifuge 7min, 7000rpm	5h after induction add 1mM PMSF (stock 100mM in EtOH) centrifuge 7min, 7000rpm
Resuspension	200 OD_{600}/ml in HS-buffer + 1mg/ml Lysozyme + 5mM DTT + 5mM Imidazole	200 OD_{600}/ml in LS-buffer + 1mg/ml Lysozyme + 5mM DTT + 5mM Imidazole	200 OD_{600}/ml in HS-buffer + 1mg/ml Lysozyme + 5mM DTT + 5mM Imidazole + 0.1% TX-100
Lysis	freece in liquid N2 thaw in warm water sonification 2-3x 1min on ice	freece in liquid N2 thaw in warm water sonification 2-3x 1min on ice	freece in liquid N2 thaw in warm water sonification 2-3x 1min on ice
Centrifugation	40000rpm, 60min	40000rpm, 60min	40000rpm, 60min
Storage	add 1/9 volume 2.4M sucrose freece in aliquots of ≈10ml	add 1/9 volume 2.4M sucrose freece in aliquots of ≈10ml	add 1/9 volume 2.4M sucrose freece in aliquots of ≈10ml

II. Purification

	PfuS-Polymerase	Pab-PPase	Pab-dUTPase
thawing	RT water	see polymerases	see polymerases
sample "native"	5µl + 95µl SDS-buffer	see polymerases	see polymerases
heat denaturation	in 5x 2ml tubes 15min, 90°C in Thermomixer with 2ml-rack	see polymerases	see polymerases
centrifugation	5 min, 13000rpm, RT pool supernatant	see polymerases	see polymerases
sample "heated"	5µl + 95µl SDS-buffer	see polymerases	see polymerases
nickel-binding	add 1g Nickel-Silica 24% 500Å pre-equilibrated with HS-buffer, rotate on wheel 30min at RT	add 1g Nickel-Silica 24% 500Å pre-equilibrated with LS-buffer rotate on wheel 30min at RT	add 1g Nickel-Silica 24% 500Å pre-equilibrated with HS-buffer rotate on wheel 30min at RT
sample "flow through"	sediment beads take sample 5µl + 95µl SDS-buffer	see polymerases	see polymerases
wash 1	remove supernatant wash 2x with 10ml HS-buffer containing 5mM DTT and 5mM Imidazole	remove supernatant wash 2x with 10ml LS-buffer containing 5mM DTT and 5mM Imidazole	remove supernatant wash 2x with 10ml HS-buffer containing 5mM DTT and 5mM Imidazole
wash 2	wash 2x with 10ml LS-buffer containing 5mM DTT	wash 2x with 10ml LS-buffer containing 5mM DTT	wash 2x with 10ml HS-buffer containing 5mM DTT
transfer to column	transfer to plastic column	see polymerases	see polymerases
wash 3	wash 1x with 2ml LS-buffer containing 5mM DTT	see polymerases	wash 2 HS-buffer containing 5mM DTT
elute	elute with 10x 300µl LS-buffer + 5mM DTT + 400mM imidazole collect fractions between each fraction wait ≈ 2 min check protein content by amido black staining (0.5µl each)	see polymerases	elute with 10x 300µl HS-buffer + 5mM DTT + 600mM imidazole collect fractions between each fraction wait ≈ 2 min check protein content by amido black staining (0.5µl each)

	PfuS-Polymerase	Pab-PPase	Pab-dUTPase
pool	pool 5-7 fractions with highest protein content	see polymerases	see polymerases
sample "eluate"	5µl + 20µl SDS-buffer	see polymerases	see polymerases
protein determination	measure OD_{280} with nano-drop against elution buffer PfuS: 1 OD_{280}= 0.73mg/ml	measure OD_{280} with nano-drop against elution buffer 1 OD_{280}= 0.70mg/ml	measure OD_{280} with nano-drop against elution buffer 1 OD_{280}= 0.75mg/ml
dilution	dilute to 1 mg/ml with 20mM Tris-HCl pH 7.5 100mM KCl 1 mM DTT 0.1 mM EDTA 0.5 % NP40 0.5 % Tween20 100µg/ml BSA 50% Glycerol	see polymerases	see polymerases

in groups of 3-4 students: prepare mixtures in dilution buffer

	PfuS	PfuS Tripple
Volume	**1ml**	**a lot!**
Polymerase	100ng/µl PfuS	100ng/µl PfuS
Pab PPase	-	15ng/µl
Pab dUTPase	-	5ng/µl

III. Activity Assay

Questions:
Effects of helper enzymes?
Speed of Polymerase mixtures?

Test-PCRs:

Template: mixture various plasmids with different insert sizes
pSF1350/787/1387/1568/961
Primers: pQE-F /pQE-R
PCR product lengths: 0.5/1kb/1.7kb/2.4kb/3kb

PCR master-mix:

50µl	5x Phusion HF buffer
25µl	dNTPs 2.5mM each
2.5µl	pQE-F primer
2.5µl	pQE-R primer
2.5µl	template-mixture (20ng/µl of each plasmid)
168µl	water

PCR reactions:
aliquot 4x 50µl (on ice)
add 0.5µl polymerase mix per reaction

1: no polymerase
2: PfuS tripple from stock
3: newly-made PfuS
4: newly-made PfuS tripple

PCR program:

1	pre-heating of block	pause (∞)	98.5°C
2	initial denaturation	1 min	98.5°C
3	denaturation	20 sec	98.5°C
4	annealing	20 sec	62°C
5	elongation	30 sec	72°C
	repeat steps 3 to 5	25 times	
6	final elongation	30 sec	72°C
7	cooling	∞	10°C

IV. Quality control:

SDS gel
boil samples 5 min
load 5µl of each sample

1	empty
2	native
3	heated
4	flow through
5	marker
6	eluate
7	empty

run 65min at 50mA, 400V
stain with Coomassie

Agarose gel
pour 1% agarose gel containing Ethidium Bromide
to each PCR reaction add 50µl DNA loading buffer
load 10µl per slot
run for 20-30min at 180V

V. Buffer compositions:

HS-buffer:
2 M NaCl
50 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$

LS-buffer:
44 mM Tris-HCl (pH 7.5)
290 mM NaCl
4.4 mM $MgCl_2$

polymerase dilution buffer:
20mM Tris-HCl pH 7.5
100mM KCl
0.1 mM EDTA
0.5 % NP40
0.5 % Tween20
100µg/ml BSA
50% Glycerol
1 mM DTT

prepare all stocks without DTT, always add DTT freshly from 1M stock.

SDS Sample buffer (1 to 2-fold)
for 250ml
62,5 ml 0.5M Tris-HCl pH 6.8
37.5 ml 20% SDS
1.93 g DTT (solid)
85.5 g sucrose (solid)
a bit of Bromphenol-Blue

Amido black solution:
0.2% Naphtol Blue Black
2% acetic acid

Anleitung für Expression, Zusammensetzung etc ist im Anhang zu finden. Wir verwenden den PCR-Puffer für Phusion (HF) von NEB.

Wichtig ist, bei der PCR die Denaturierung bei 98,5°C durchzuführen. Die Polymerase macht dann bis zu 6kb pro Minute. Ich rechne routinemäßig mit 2,5-3kb/min.