Methods in Structural Chemistry: A Lab Manual, 2015 Edition

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A Lab Manual

2015 edition

Andreas Hofmann

METHODS IN STRUCTURAL CHEMISTRY – A LAB MANUAL

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PREFACE

This Lab Manual intends to provide the experimental procedures as well as the fundamental background for methods used in a structural biochemistry laboratory. It has arisen as write-up of procedures used and developed during my postdoctoral years and later in my laboratory. The Lab Manual is a collection of methods and procedures routinely used in our laboratory and always a project under construction. The basic theoretical background of various techniques used in structural research of biological molecules is covered in our book "Methods of Molecular Analysis in the Life Sciences". For this current second edition, more work flow schemes, as well as some basics in protein crystallisation have been added.

Note for the Structural Chemistry Program:
The Lab Manual with its collection of routine procedures is an integral part of our laboratory and as such constitutes a write-up of standard operating procedures for reference and training purposes, as well as Health & Safety and Good Laboratory Practice.

Manuscript and figures for this book have been compiled entirely with open source and academic software under Linux, and I would like to acknowledge the efforts of software developers and programmers who make their products freely available.

I would also like to thank the various staff and students in my lab over the years who helped to update and expand this compendium: Ursula Broder, Natascha von Gnielinski, Lyn Mason, Adlina Mohd-Yusof, Calvin Shih, Conan Wang, Saroja Weeratunga, Anja Winter.

Andreas Hofmann

Brisbane, December 2014

0 GENERAL DATA

0.1 PERIODIC TABLE OF THE ELEMENTS

(Obtained from http://www.nist.gov/pml/data/periodic.cfm, 31.01.14)

0.2 UNITS AND CONSTANTS

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10^{-1}	deci	d	10	deka	da
10^{-2}	centi	c	10^{2}	hekto	h
10^{-3}	milli	m	10^{3}	kilo	k
10^{-6}	micro	m	10^{6}	mega	M
10^{-9}	nano	n	10^{9}	giga	G
10^{-12}	pico	p	10^{12}	tera	T
10^{-15}	femto	f	10^{15}	peta	P
10^{-18}	atto	a	10^{18}	exa	E

Table 0.1: Decimal factors.

A, α	alpha	I, ι	iota	Σ, σ	sigma
B, β	beta	K, κ	kappa	T, τ	tau
Γ, γ	gamma	Λ, λ	lambda	Y, υ	upsilon
Δ, δ	delta	M, μ	mu	Φ, ϕ	phi
E, ε	epsilon	N, ν	nu	X, χ	chi
Z, ζ	zeta	Ξ, ξ	xi	Ψ, ψ	psi
H, η	eta	Π, π	pi	Ω, ω	omega
Θ, θ	theta	P, ρ	rho		

Table 0.2: The Greek alphabet.

Symbol	Parameter	Dimension	Name
I	Electric current	1 A	Ampere
I	Light intensity	1 cd	Candela
l	Length	1 m	Meter
m	Mass	1 kg	kilogram
n	Molar amount	1 mol	Mol
t	Time	1 s	second
T	Temperature	1 K	Kelvin

Table 0.3: SI base parameters and units.

Symbol	Parameter	Dimension	Name
B	Magnetic induction	1 T = 1 kg s^{-2} A^{-1} = 1 V s m^{-2}	Tesla
c	Molar concentration	1 mol l^{-1}	
C	Electric capacity	1 F = 1 kg^{-1} m^{-2} s^4 A^2= 1 A s V^{-1}	Farad
E	Energy	1 J = 1 kg m^2 s^{-2}	Joule
ε	Molar extinction coefficient	1 l mol^{-1} cm^{-1}	
ε	Permittivity	1 F m^{-1}	
F	Force	1 N = 1 kg m s^{-2} = 1 J m^{-1}	Newton
Φ	Magnetic flux	1 Wb = 1 kg m^2 s^{-2} A^{-1} = 1 V s	Weber
G	Electric conductivity	1 S = 1 kg^{-1} m^{-2} s^3 A^2 = 1 $Ω^{-1}$	Siemens
H	Enthalpy	1 J = 1 kg m^2 s^{-2}	Joule
η	Viscosity	1 P = 0.1 kg m^{-1} s^{-1} = 0.1 Pa s	Poise
i	Current density	1 A m^{-2}	
j	Flux density	1 mol m^{-2} s^{-1}	
κ	Conductivity	1 S m^{-2}	
L	Magnetic inductivity	1 H = 1 kg m^2 s^{-2} A^{-2} = 1 V A^{-1} s	Henry
$Λ_m$	Molar conductivity	1 S m^2 mol^{-1}	
m_i	Molality	1 mol kg^{-1}	
M	Molar mass[a]	1 g mol^{-1} = 1 Da	(Dalton)
ν	Frequency	1 Hz = 1 s^{-1}	Hertz
p	Pressure	1 Pa = 1 kg m^{-1} s^{-2} = 1 N m^{-2}	Pascal
P	Power	1 W = 1 kg m^2 s^{-3} = 1 J s^{-1}	Watt
Q	Electric charge	1 C = 1 A s	Coulomb
ρ	Density	1 g cm^{-3}	
ρ*	Mass concentration	1 mg ml^{-1}	
θ	Temperature	1°C	Celsius
R	Electric resistance	1 Ω = 1 kg m^2 s^{-3} A^{-2} = 1 V A^{-1}	Ohm
S	Entropy	1 J K^{-1}	
u	Ion mobility	1 m^2 s^{-1} V^{-1}	
U (ϕ, E)	Electric potential (voltage)	1 V = 1 kg m^2 s^{-3} A^{-1} = 1 J A^{-1} s^{-1}	Volt
V	Volume	1 l	
V_m	Molar volume	1 l mol^{-1}	
v	Partial specific volume	1 ml g^{-1}	

w	Mass fraction (or volume fraction)	1 (typically given in %w/w, %w/v or %v/v)
x	Mole fraction	1
z	Charge number	1

Table 0.4: Important physico-chemical parameters and units. [a]Note that the molecular mass is the mass of one molecule given in atomic mass units (u, Da). The molar mass is the mass of 1 mol of molecules and thus has the units of g mol^{-1}.

Symbol	Constant	Value
c	Speed of light *in vacuo*	$2.99792458 \cdot 10^{8}$ m s^{-1}
e	Elementary charge	$1.6021892 \cdot 10^{-19}$ C
$\varepsilon_0 = (\mu_0 \cdot c^2)^{-1}$	Electric field constant	$8.85418782 \cdot 10^{-12}$ A^2 s^4 m^{-3} kg^{-1}
$F = e \cdot N_A$	Faraday's constant	$9.648456 \cdot 10^{4}$ C mol^{-1}
g	Earth's gravity near surface	9.81 m s^{-2}
$g_e = 2 \cdot \mu_e / \mu_B$	Landé factor of free electron	2.0023193134
γ_p	Gyromagnetic ratio of proton	$2.6751987 \cdot 10^{8}$ s^{-1} T^{-1}
h	Planck's constant	$6.626176 \cdot 10^{-34}$ J s
$k = k_B = R / N_A$	Boltzmann's constant	$1.380662 \cdot 10^{-23}$ J K^{-1}
m_e	Mass of electron	$9.109534 \cdot 10^{-31}$ kg
m_n	Mass of neutron	$1.6749543 \cdot 10^{-27}$ kg
m_p	Mass of proton	$1.6726485 \cdot 10^{-27}$ kg
μ_0	Magnetic field constant	$4\pi \cdot 10^{-7}$ m kg s^{-2} A^{-2}
$\mu_B = e \cdot h / (4\pi \cdot m_e)$	Bohr magneton	$9.274078 \cdot 10^{-24}$ J T^{-1}
μ_e	Magnetic moment of electron	$9.284832 \cdot 10^{-24}$ J T^{-1}
$\mu_N = e \cdot h / (4\pi \cdot m_p)$	Nuclear magneton	$5.050824 \cdot 10^{-27}$ J T^{-1}
N_A, L	Avogadro's (Loschmidt's) constant	$6.022045 \cdot 10^{23}$ mol^{-1}
p°	Standard pressure (IUPAC)	$1.00 \cdot 10^{5}$ Pa
R	Gas constant	8.31441 J K^{-1} mol^{-1}
R_∞	Rydberg's constant	$1.097373177 \cdot 10^{7}$ m^{-1}
ϑ°, T°	Standard temperature (IUPAC)	0°C, 273.15 K
$V_m^{\circ} = R \cdot T^\circ / p^\circ$	Molar volume of an ideal gas	22.41383 l mol^{-1}

Table 0.5: Important physico-chemical constants.

0.3 CONVERSION FACTORS

	J	cal	eV
1 J	1	0.2390	$6.24150974 \cdot 10^{18}$
1 cal	4.184	1	$2.612 \cdot 10^{19}$
1 eV	$1.60217646 \cdot 10^{-19}$	$3.829 \cdot 10^{-20}$	1

Table 0.6: Conversion factors for energy.

	Pa	bar	atm	mm Hg (Torr)	psi
1 Pa	1	10^{-5}	$9.869 \cdot 10^{-6}$	$7.501 \cdot 10^{-3}$	$1.450 \cdot 10^{-4}$
1 bar	10^{5}	1	0.9869	750.1	14.50
1 atm	$1.013 \cdot 10^{5}$	1.013	1	760.0	14.69
1 mm Hg (Torr)	133.3	$1.333 \cdot 10^{-3}$	$1.316 \cdot 10^{-3}$	1	$1.933 \cdot 10^{-2}$
1 psi	$6.895 \cdot 10^{4}$	$6.897 \cdot 10^{-2}$	$6.807 \cdot 10^{-2}$	51.72	1

Table 0.7: Conversion factors for pressure.

0.4 CONVERSION AND OTHER USEFUL FORMULAE

	Formula	Example
Double bond equivalents	$DBE = N(C) - \frac{1}{2}N(H) + \frac{1}{2}N(N) + 1$	pyrrole: C_4H_5N  $DBE = 4 - \frac{5}{2} + \frac{1}{2} + 1 = 3$
Energy to wavelength	$\lambda = \frac{1.24 \cdot 10^{-6}\ \text{m}}{E / 1\ \text{eV}}$	
Hydrogen rule	$N_{max}(H) = 2 \cdot N(C) + N(N) + 2$	
Nitrogen rule	If *m*/*z* is odd, N(N) is odd. If *m*/*z* is even, N(N) is even.	
Mass fraction to molar concentration	$c(\text{solute}) = \frac{w(\text{solute}) \cdot \rho(\text{solution})}{M(\text{solute})}$	w(HCl) = 32%, ρ(HCl)= 1.15 g cm^{-3} $c(\text{solute}) = \frac{0.32 \cdot 1.15 \cdot 1000}{36}\ \frac{\text{mol}}{\text{l}}$
Protein atom count (estimated) from residue count	$N(C) = 5 \cdot N_{res}$; $N(N) = 1.2 \cdot N_{res}$ $N(O) = 1.5 \cdot N_{res}$; $N(H) = 8 \cdot N_{res}$	

Table 0.8: Conversion and other useful formulae.

0.5 COMMONLY USED PH-BUFFERS

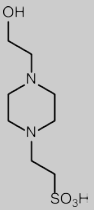
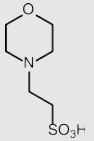
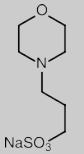
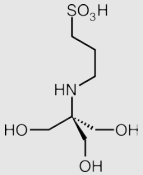
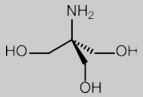
HEPES	4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid	
	$C_8H_{17}N_2NaO_4S$ (sodium salt)	
	260.3 g mol^{-1}	
	HEPES – NaOH buffer pH: 7.2 – 8.2	
	HEPES – NaOH – NaCl buffer pH: 6.6 – 8.5	
MES	4-Morpholine-ethanesulfonic acid, monohydrate	
	$C_6H_{13}NO_4S \cdot H_2O$	
	195.3 g mol^{-1} (monohydrate: 213.2 g mol^{-1})	
	MES – NaOH buffer pH: 5.6 – 6.8	
	MES – NaOH – NaCl buffer pH: 5.2 – 7.1	
MOPS	4-Morpholine-propanesulfonic acid, sodium salt monohydrate	
	$C_7H_{14}NNaO_4S \cdot H_2O$	
	231.3 g mol^{-1}(monohydrate: 249.3 g mol^{-1})	
	MOPS – NaOH – NaCl buffer pH: 6.3 – 8.2	
	MOPS – KOH buffer pH: 6.6 – 7.8	
TAPS	N-[Tris-(hydroxymethyl)]-3-aminopropanesulfonic acid	
	$HO_3S(CH_2)_3NHC(CH_2OH)_3$	
	243.28 g mol^{-1}	
	TAPS – NaOH – NaCl buffer pH: 7.5 – 9.4	
TRIS	Tris-(hydroxymethyl)-aminomethane	
	(2-Amino-2-hydroxymethyl-1,3-propanediol)	
	$NH_2C(CH_2OH)_3$	
	121.14 g mol^{-1}	
	TRIS – HCl buffer pH: 7.0 – 9.0	

Table 0.9: Commonly used pH buffers and their chemical properties.

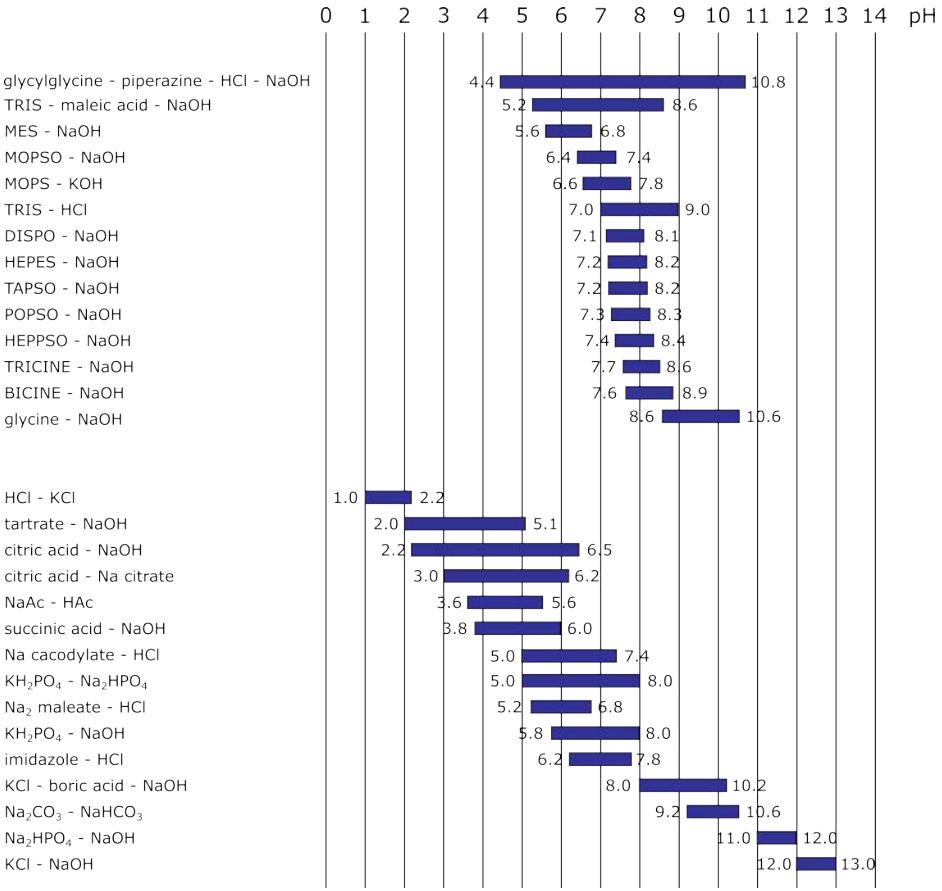


Figure 0.1: Commonly used pH buffers and their buffer ranges.

0.6 COMMON CONCENTRATIONS OF SELECTED ACIDS AND BASES

Acid / Base	Weight % (w/w)	Density ρ at 20°C (g l^{-1})	Molar concentration c (mol l^{-1})
Ammonia solution	25	0.91	13.5
Ammonia solution	30	0.88	15.5
Ammonia solution	35	0.88	18
Caustic potash	30	1.3	7
Caustic potash	47	1.5	12.5
Caustic soda	33	1.36	11
Formic acid	98 - 100	1.22	26
Glacial acetic acid	96	1.06	17
Glacial acetic acid (99 - 100%)	99 - 100	1.06	18
Hydrochloric acid	25	1.12	8
Hydrochloric acid conc. (1.16)	32	1.16	10
Hydrochloric acid conc. (1.18)	36	1.18	12
Hydrochloric acid smoking	37	1.19	12.5
Nitric acid conc.	65	1.40	14
Nitric acid smoking	100	1.52	21
Phosphoric acid conc. (1.71)	85	1.71	15
Phosphoric acid conc. (1.75)	89	1.75	16
Sulphuric acid dil.	25	1.18	6
Sulphuric acid conc.	95 - 97	1.84	18

Table 0.10: Common concentrations of selected acids and bases.

0.7 MOLECULAR MASS OF AMINO ACIDS

Amino acid			Molecular mass m (Da)	Residue mass m – m(H_2O) (Da)
A	Ala	alanine	89	71.079
C	Cys	cysteine	121	103.145
D	Asp	aspartic acid	133	115.089
E	Glu	glutamic acid	147	129.116
F	Phe	phenylalanine	165	**147.177**
G	Gly	glycine	75	57.052
H	His	histidine	155	137.141
I	Ile	isoleucine	131	**113.160**
K	Lys	lysine	146	**128.17**
L	Leu	leucine	131	**113.160**
M	Met	methionine	149	131.199
MSO	MetSO	metsulphoxide	165	**147.199**
N	Asn	asparagine	132	114.104
P	Pro	proline	115	97.117
Q	Gln	glutamine	146	**128.131**
R	Arg	arginine	174	156.188
S	Ser	serine	105	87.078
T	Thr	threonine	119	101.105
V	Val	valine	117	99.133
W	Trp	tryptophan	204	186.213
Y	Tyr	tyrosine	181	163.176

Table 0.11: Average molecular masses of amino acids, free and within peptides (residue mass). The numbers in bold indicate amino acids that may be ambiguous in sequencing by tandem-MS. Data taken from (Aitken, 2010).

0.8 NUCLEOTIDE PHYSICAL PROPERTIES

Nucleotide	M (g mol^{-1})	λ_{max} (pH 7.0) (nm)	Absorbance at λ_{max} 1 M solution (pH 7.0)
ATP	507	259	15400
CTP	483	271	9000
GTP	523	253	13700
UTP	484	262	10000
dATP	491	259	15200
dCTP	467	271	9300
dGTP	507	253	13700
dTTP	482	267	9600

Table 0.12: Physical properties of nucleotides.

0.9 GENETIC CODE

	U		C		A		G		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop	A
	UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

Table 0.13: The genetic code. Triplet codons and their corresponding amino acids.

0.10 ENDONUCLEASE CLEAVAGE CLOSE TO THE END OF DNA FRAGMENTS

Enzyme	Oligo Sequence	Chain Length	% Cleavage	
			2 hr	20 hr
*Acc*I	C**GTCGAC**C	8	0	0
	CG**GTCGAC**CG	10	0	0
	CCG**GTCGAC**CGG	12	0	0
*Afl*III	C**ACATGT**G	8	0	0
	CC**ACATGT**GG	10	>90	>90
	CCC**ACATGT**GGG	12	>90	>90
*Asc*I	**GGCGCGCC**	8	>90	>90
	A**GGCGCGCC**T	10	>90	>90
	TT**GGCGCGCC**AA	12	>90	>90
*Ava*I	C**CCCGGG**G	8	50	>90
	CC**CCCGGG**GG	10	>90	>90
	TCC**CCCGGG**GGA	12	>90	>90
*BamH*I	C**GGATCC**G	8	10	25
	CG**GGATCC**CG	10	>90	>90
	CGC**GGATCC**GCG	12	>90	>90
*Bgl*II	C**AGATCT**G	8	0	0
	GA**AGATCT**TC	10	75	>90
	GGA**AGATCT**TCC	12	25	>90
*Bss*HII	G**GCGCGC**C	8	0	0
	AG**GCGCGC**CT	10	0	0
	TTG**GCGCGC**CAA	12	50	>90
*Bst*EII	G**GGT(A/T)ACC**C	9	0	10
*Bst*XI	AACTGCAGAA**CCAATGCATTGG**	22	0	0
	AAAACTGCAG**CCAATGCATTGG**AA	24	25	50
	CTGCAGAA**CCAATGCATTGG**ATGCAT	26	25	>90
*Cla*I	C**ATCGAT**G	8	0	0
	CC**ATCGAT**GG	10	>90	>90
	CCC**ATCGAT**GGG	12	50	50

Enzyme	Oligo Sequence	Chain Length	% Cleavage	
*Eco*RI	G**GAATTC**C	8	>90	>90
	CG**GAATTC**CG	10	>90	>90
	CCG**GAATTC**CGG	12	>90	>90
*Hae*III	GG**GGCC**CC	8	>90	>90
	AGG**GGCC**CCT	10	>90	>90
	TTGG**GGCC**CCAA	12	>90	>90
*Hind*III	C**AAGCTT**G	8	0	0
	CC**AAGCTT**GG	10	0	0
	CCC**AAGCTT**GGG	12	10	75
*Kpn*I	G**GGTACC**C	8	0	0
	GG**GGTACC**CC	10	>90	>90
	CGG**GGTACC**CCG	12	>90	>90
*Mlu*I	G**ACGCGT**C	8	0	0
	CG**ACGCGT**CG	10	25	50
*Nco*I	C**CCATGG**G	8	0	0
	CATG**CCATGG**CATG	14	50	75
*Nde*I	GGGTTT**CATATG**AAACCC	18	0	0
	GGAATTC**CATATG**GAATTCC	20	75	>90
	GGGAATTC**CATATG**GAATTCCC	22	75	>90
*Nhe*I	G**GCTAGC**C	8	0	0
	CG**GCTAGC**CG	10	10	25
	CTA**GCTAGC**TAG	12	10	50
*Not*I	AAATAT**GCGGCCGC**TATAAA	20	10	10
	ATAAGAAT**GCGGCCGC**TAAACTAT	24	25	90
	AAGGAAAAAA**GCGGCCGC**AAAAGGAAAA	28	25	>90
*Nsi*I	TGC**ATGCAT**GCA	12	10	>90
	CCA**ATGCAT**TGGTTCTGCAGTT	22	>90	>90
*Pac*I	**TTAATTAA**	8	0	0
	G**TTAATTAA**C	10	0	25
	CC**TTAATTAA**GG	12	0	>90

Enzyme	Oligo Sequence	Chain Length	% Cleavage	
*Pme*I	G**GTTTAAAC**C	10	0	25
	GG**GTTTAAAC**CC	12	0	50
	AGCTTT**GTTTAAAC**GGCGCGCCGG	24	75	>90
*Pst*I	TGCA**CTGCAG**TGCA	14	10	10
	AA**CTGCAG**AACCAATGCATTGG	22	>90	>90
	AAAA**CTGCAG**CCAATGCATTGGAA	24	>90	>90
*Pvu*I	C**CGATCG**G	8	0	0
	AT**CGATCG**AT	10	10	25
	TCG**CGATCG**CGA	12	0	10
*Sac*I	C**GAGCTC**G	8	10	10
*Sac*II	G**CCGCGG**C	8	0	0
	TCC**CCGCGG**GGA	12	50	>90
*Sal*I	**GTCGAC**GTCAAAAGGCCATAGCGGCCGC	28	0	0
	GC**GTCGAC**GTCTTGGCCATAGCGGCCGCGG	30	10	50
	ACGC**GTCGAC**GTCGGCCATAGCGGCCGCGGAA	32	10	75
*Sca*I	G**AGTACT**C	8	10	25
	AAA**AGTACT**TTT	12	75	75
*Sma*I	C**CCCGGG**G	8	0	10
	CC**CCCGGG**GG	10	10	50
	TCC**CCCGGG**GGA	12	>90	>90
*Spe*I	G**ACTAGT**C	8	10	>90
	GG**ACTAGT**CC	10	10	>90
	CGG**ACTAGT**CCG	12	0	50
*Sph*I	G**GCATGC**C	8	0	0
	CAT**GCATGC**ATG	12	0	25
	ACAT**GCATGC**ATGT	14	10	50
*Stu*I	A**AGGCCT**T	8	.90	>90
	GA**AGGCCT**TC	10	.90	>90
	AAA**AGGCCTT**TT	12	.90	>90

Enzyme	Oligo Sequence	Chain Length	% Cleavage	
*Xba*I	C**TCTAGA**G	8	0	0
	GC**TCTAGA**GC	10	>90	>90
	TGC**TCTAGA**GCA	12	75	>90
*Xho*I	C**CTCGAG**G	8	0	0
	CC**CTCGAG**GG	10	10	25
	CCG**CTCGAG**CGG	12	10	75
*Xma*I	CC**CCCGGG**GG	10	25	75
	CCC**CCCGGG**GGG	12	50	>90

Table 0.14: Data for cleavage of restriction endonucleases close to the end of DNA fragments are taken from the Reference Appendix of the *New England Biolabs* catalogue.

0.11 MULTI-WELL MATRICES

	1	2	3	4	5	6
A	1	2	3	4	5	6
B	7	8	9	10	11	12
C	13	14	15	16	17	18
D	19	20	21	22	23	24

	1	2	3	4	5	6
A	25	26	27	28	29	30
B	31	32	33	34	35	36
C	37	38	39	40	41	42
D	43	44	45	46	47	48

	1	2	3	4	5	6
A	49	50	51	52	53	54
B	55	56	57	58	59	60
C	61	62	63	64	65	66
D	67	68	69	70	71	72

	1	2	3	4	5	6
A	73	74	75	76	77	78
B	79	80	81	82	83	84
C	85	86	87	88	89	90
D	91	92	93	94	95	96

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	1	2	3	4	5	6	7	8	9	10	11	12
F	13	14	15	16	17	18	19	20	21	22	23	24
G	25	26	27	28	29	30	31	32	33	34	35	36
H	37	38	39	40	41	42	43	44	45	46	47	48

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	98	99	100	101	102	103	104	105	106	107	108
B	109	110	111	112	113	114	115	116	117	118	119	120
C	121	122	123	124	125	126	127	128	129	130	131	132
D	133	134	135	136	137	138	139	140	141	142	143	144
E	145	146	147	148	149	150	151	152	153	154	155	156
F	157	158	159	160	161	162	163	164	165	166	167	168
G	169	170	171	172	173	174	175	176	177	178	179	180
H	181	182	183	184	185	186	187	188	189	190	191	192

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	194	195	196	197	198	199	200	201	202	203	204
B	205	206	207	208	209	210	211	212	213	214	215	216
C	217	218	219	220	221	222	223	224	225	226	227	228
D	229	230	231	232	233	234	235	236	237	238	239	240
E	241	242	243	244	245	246	247	248	249	250	251	252
F	253	254	255	256	257	258	259	260	261	262	263	264
G	265	266	267	268	269	270	271	272	273	274	275	276
H	277	278	279	280	281	282	283	284	285	286	287	288

1 TYPICAL WORKFLOWS

1.1 BACTERIAL EXPRESSION OF SOLUBLE RECOMBINANT PROTEINS

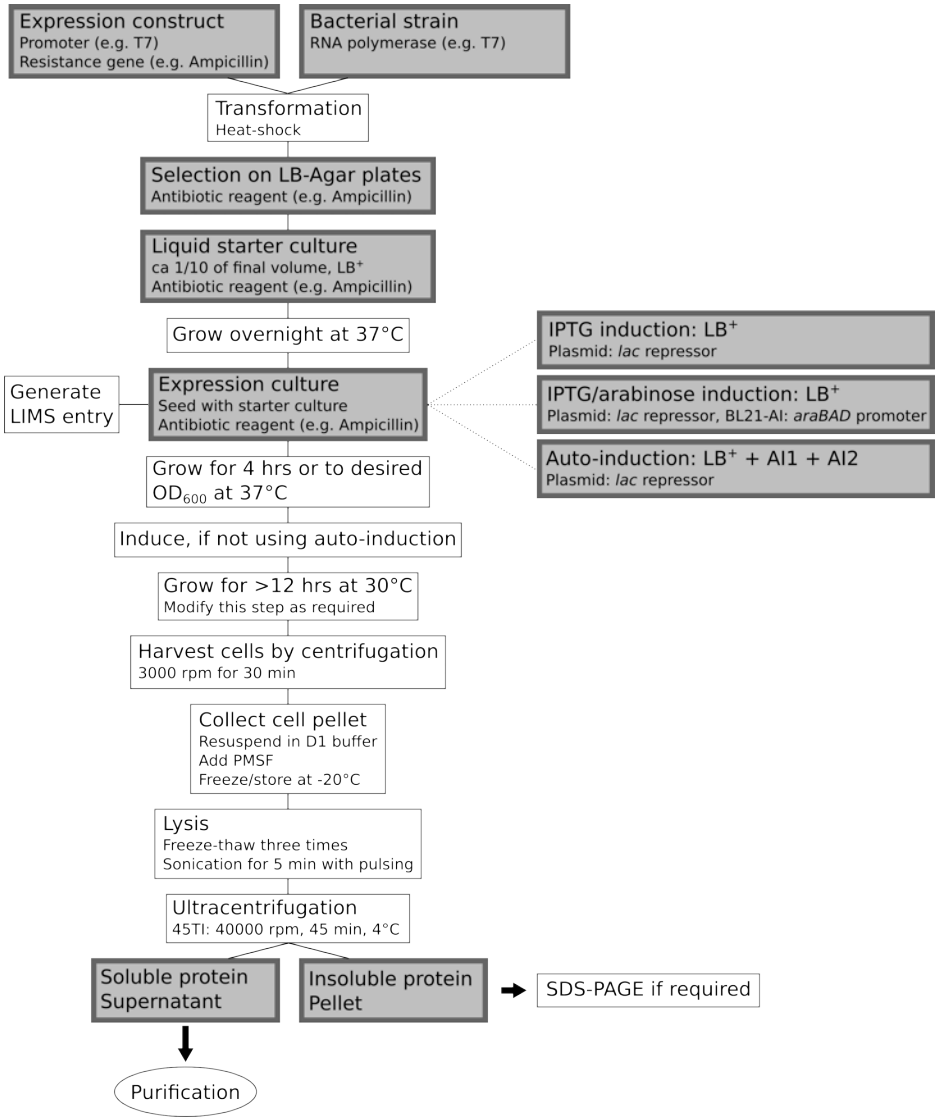


Figure 1.1: General workflow for bacterial expression of soluble recombinant proteins.

1.2 PROTEIN CRYSTALLISATION

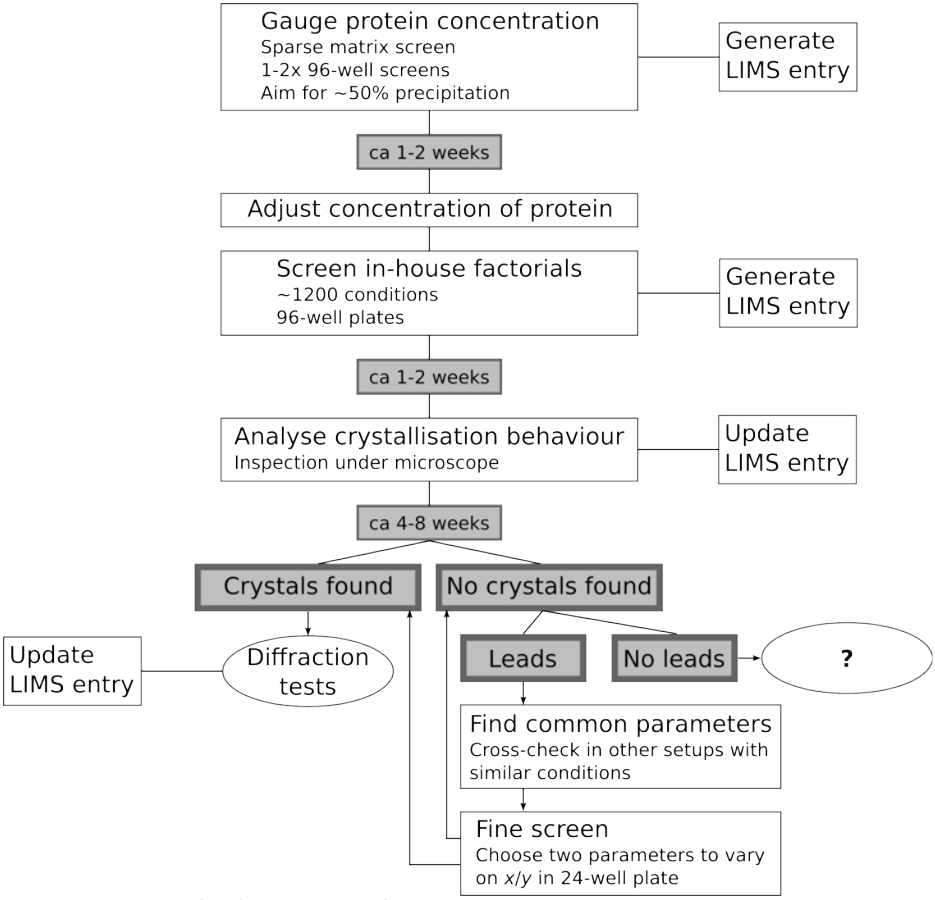


Figure 1.2: General workflow for crystallisation of an uncharacterised protein.

1.3 PROTEIN CRYSTAL SPACE GROUP DETERMINATION

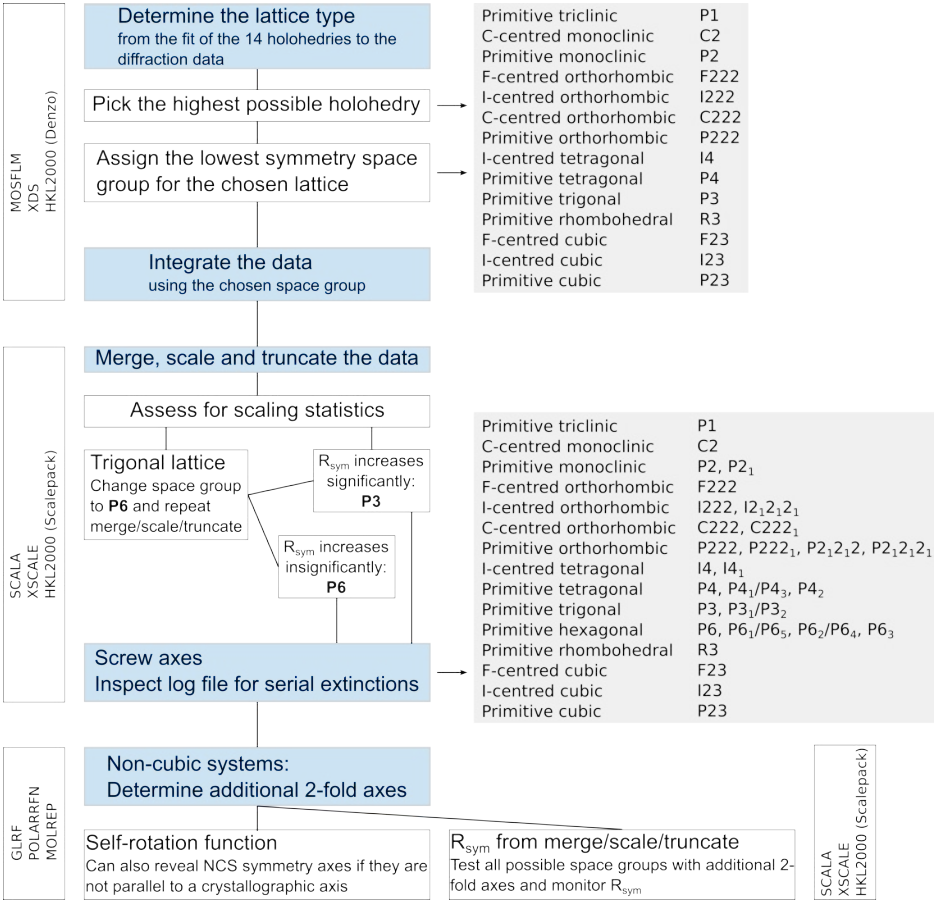


Figure 1.3: General (simplified) workflow for space group determination using protein single crystal diffraction data.

2 METHODS IN MOLECULAR BIOLOGY

2.1 STANDARD METHODS FOR WORK WITH BACTERIA

As a general rule, all solutions and tools involved in work with DNA and bacteria have to be sterile to avoid unwanted contamination (product protection). The work place has to be cleaned and disinfected (e.g. wiping with 70% EtOH) and gloves are to be worn (product protection, personal protection). Any equipment, which has been in contact or contaminated with bacterial cells has to be sterilised afterwards using appropriate methods (e.g. chemically, autoclaving, etc).

2.1.1 Cell strains

Escherichia coli bacteria are used for two different purposes in the lab, plasmid propagation and protein expression. Table 2.1 lists some commonly used *E. coli* strains. Importantly, for plasmid propagation, any strain can be used, although there are differences in DNA quality and yield when using the cells to isolate plasmids. Host strains such as DH5α and the slower growing XL1-Blue yield DNA of very high quality which works well for isolation and sequencing.

When choosing *E. coli* strains for protein expression, it is important to remember the concepts of transcription and translation. The information coded on the gene to be expressed is first transcribed by an RNA polymerase which needs to find the promoter site on the plasmid carrying the gene. The promoter type and RNA polymerase need to be matched; i.e. the commonly used T7 promoter plasmids require *E. coli* strains that express T7 polymerase.

Strain	Main usage	RNA polymerase / promoter required for expression	Comments	Resistance
ArcticExpress(DE3)	Expression	T7 / T7	Expression at low temperatures.	Tetracycline Gentamicin
B834(DE3)	Expression	T7 / T7	Met auxotroph; used for SeMet labelling.	
BL21-AI	Expression	T7 / T7	T7 RNA polymerase gene under the control of the *araBAD* promoter. Used for toxic proteins where tight regulation is required.	
BL21(DE3)	Expression	T7 / T7	The “standard' expression strain.	
DH5α	Propagation	host / tac		
DH10B	Propagation	host / tac		
ER2566 (T7 Express)	Expression	T7 / T7	T7 RNA polymerase gene inserted into *lacZ* gene.	
HMS174	Expression	host / tac		
JM109	Expression	host / tac		
K12 M5219	Expression	host / tac	Trp auxotroph; used for D_6-Trp labelling.	
TOP10	Propagation	host / tac		
Rosetta2(DE3)	Expression	T7 / T7	Strain to overcome *E. coli* codon bias.	Chloramphenicol
Rosetta-Gami B(DE3)	Expression	T7 / T7	Hybrid strains for addressing *E. coli* codon bias and disulphide bond formation.	Tetracycline Chloramphenicol Kanamycin
XL1-Blue	Propagation	host / tac		

Table 2.1: Commonly used *E. coli* cell strains.

2.1.2 Growth of bacterial cultures

Antibiotics

Stock concentrations of antibiotics are chosen such that they can be added to the final volume of media with a dilution factor of 1/2000 (e.g. 50 µl of antibiotic stock solution in 100 ml of media). Agar plates are marked with colours to indicate the presence of individual antibiotics.

Antibiotic	Abbreviation	Stock solution	Storage	Colour label
No antibiotic				(no label)
Ampicillin	Amp	100 mg ml^{-1} in 50% EtOH	-20°C	black
Kanamycin	Kan	50 mg ml^{-1} in H_2O	4°C	red
Chloramphenicol	Chl	68 mg ml^{-1} in 100% EtOH	-20°C	green
Gentamicin	Gent	20 mg ml^{-1} in H_2O	4°C	purple
Zeocine	Zeo			

Table 2.2: Antibiotic stock solutions and colour labels.

Plates

For plate cultures, transformed bacteria are transferred from a liquid culture to an LB-Agar plate, which usually contains an antibiotic reagent for selection. After incubation at 37ºC over night, single colonies should appear on the plate. If used for protein expression, these single colonies can be stored at 4°C for four weeks. After four weeks, cells should be freshly transformed.

LB medium (Luria-Bertani medium)	
10 g	tryptone
5 g	yeast extract
5 g	NaCl
ad 1l	H_2O

LB-Agar		
	500 ml	LB medium
15 g l^{-1}	7.5 g	bacto-agar
	microwave	

2× YT medium	
16 g	tryptone
10 g	yeast extract
5 g	NaCl
ad 1l	H_2O

Liquid cultures

LB medium with antibiotic reagent (e.g. 50 µg ml^{-1} ampicillin) is inoculated by transferring a colony of a plate culture (or resuspended cells from other liquid culture). Incubate over night in a shaker a 37ºC and 100-180 rpm min^{-1}.

For plasmid purification volumes of 1 ml to 100 ml are used, for protein expression volumes of 2 l – 10 l are typical. Latter ones are inoculated by a 1:10 ratio.

Glycerol cultures

Glycerol cultures are used for storing bacteria. A stationary liquid culture is mixed with (sterile) glycerol (final concentration: 35%) and shock frozen with liquid N_2 (T= 77K, θ= -196°C). These

cultures are stored at –80ºC.

2.1.3 Transformation of competent bacteria

The entry of plasmid DNA into bacteria (transformation) requires the cells to be transformation competent.

Preparation of competent cells

50 ml of a liquid culture in its exponential growth phase (OD_{600nm}= 0.6 – 0.8) is centrifuged (3000×*g*, 15 min, 4ºC), and the pellet resuspended in 5 ml sterile $CaCl_2$ solution (50 mM). Another centrifugation run yields the remaining pellet, which is resuspended in 1 ml $CaCl_2$ solution. Aliquots of 100 µl are taken and prepared as glycerol cultures.

Transformation by heat shock

One aliquot of competent cells is thawed on ice and 0.1 µg – 1 µg plasmid DNA is added. Incubate for 15 min on ice. Then heat cells for 3 min at 42 ºC and incubate another 5 min on ice. Add 800 µl LB medium and grow at 37ºC for approx. 45 min. Harvest cells (3000×*g*, 5 min, RT), resuspend in a small amount of LB and plate on agar plates (usually selection *via* resistance to antibiotic reagent). Incubate plates over night at 37ºC.

Transformation by electroporation

For electroporation, (chemically competent) cells have to be prepared in salt-free medium. Harvest the cells by centrifugation (3000×*g*, 5 min, 4°C), resuspend gently in 400 µl of cold 10% glycerol, and centrifuge again. Repeat this washing step two more times, making sure that the resuspension is carried out very carefully, as the cells are becoming more and more fragile.
Plasmid DNA (1 µl) is added to the cells which are then kept on ice for another 15 min. The cells are transferred into the pre-cooled cuvette which is placed in the electroporator (BioRad GenePulse II). The voltage is set to 2.5 kV, the resistance to 200 Ω, the capacitance to 25 µF, and the mode to “time constant”. Pulse the cells by pressing both red buttons at the same time. Ideally, there should be no sparks appearing during the pulsing.
Immediately after electroporation, the cells need to be transferred into 800 µl warm LB media followed by 45 min of incubation at 37°C. Harvest cells (3000×*g*, 5 min, RT), resuspend in a small amount of LB and plate on agar plates (usually selection via resistance to antibiotic reagent). Incubate plates over night at 37ºC.

2.2 STANDARD METHODS FOR WORK WITH YEAST

2.2.1 Preparation of competent cells

1. Grow a 5 ml culture of *Pichia pastoris* in YPD medium in a 50 ml conical tube at 30 °C overnight.
2. Inoculate 500 ml of fresh YPD medium in a 2 l flask with 0.2 ml of the overnight culture. Grow overnight to an OD_{600} of about 1.3 - 1.5.
3. Centrifuge the cells at 2000×*g* (3000 rpm, JA-14 rotor) for 5 minutes at 4°C. Resuspend the pellet in 100 ml buffer B1, and incubate at 30°C for 15 minutes without shaking.
4. Fill up to 300 ml with buffer B2.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 300 ml of ice-cold buffer B2.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold 1 M sorbitol. Transfer the content into a 50 ml conical tube.
7. Centrifuge the cells for 5 min at 1500×*g*, then resuspend the pellet in 0.5 - 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice to use that day.

Table 2.3: Preparation of competent yeast cells.

10× D (20% dextrose)		
	200 g	D-glucose
	ad 1 l	H_2O
		autoclave or filter sterilise
		shelf life ca. 1 year

YP		
	10 g	yeast extract
	20 g	peptone
	ad 900 ml	H_2O
		autoclave

1 M sorbitol		
	18.2 g	sorbitol
	80 ml	H_2O
		pH to 8.0 with NaOH
	ad 100 ml	H_2O

YPD		
	900 ml	YP
		autoclave, cool to ~60°C and add required amount of zeocin
	100 ml	10xD

B1		
200 mM	10 ml	2 M HEPES (pH= 8.0)
25 mM	2.5 ml	1 M DTT
	100 ml	YPD

B2		
10 mM	3.0 ml	2 M HEPES (pH= 8.0)
	600 ml	H_2O, autoclaved, cold

2.3 STANDARD METHODS FOR WORK WITH DNA

As a general rule, all solutions and tools involved in work with DNA have to be sterilised before usage (product protection).

2.3.1 Preparation of DNA from bacterial cells

Most methods for DNA purification follow the general principles of Birnboim & Doly (Birnboim & Doly, 1979). Depending on the volume of the over night liquid culture (mini prep.: 5 ml, midi prep.: 25 ml) the appropriate DNA kit (different suppliers: *QIAGEN*, *MN*, *JetSorb*; buffers below are cited from the *QIAGEN* kits) is used.

Cells are harvested by centrifugation (3000 rpm, 15 min, 4ºC), resuspended in buffer P1 and disrupted by alkaline lysis (buffer P2). Incubate for 15 min at RT. The resulting lysate is neutralised (buffer P3). Separate from cellular remains and proteins by centrifugation (14000 rpm, 45 min, 4 ºC). The DNA containing supernatant is applied to an anionic chromatography column (equilibrated with buffer QBT). Wash with buffer QC and elute DNA with buffer QF. Then precipitate DNA by adding *i*-PrOH (70%, v/v), centrifuge (14000 rpm, 45 min, 4ºC) and wash with EtOH (70%, v/v). Dry pellet completely (heat at 42ºC or use SpeedVac) and resuspend in 5 mM TRIS (pH= 8.0).

Buffer P1 (Resuspension)		
50 mM	5 ml (1 M)	TRIS (pH= 8.0)
10 mM	2 ml (0.5 M)	EDTA
100 mg/ml	10 g	RNase A
	ad 100 ml	H_2O

Buffer QBT (Equilibration)		
750 mM	15 ml (5 M)	NaCl
50 mM	5 ml (1 M)	MOPS (pH= 7.0)
15% (v/v)	15 ml	*i*-PrOH
0.15% (v/v)	0.15 ml	Triton X
	ad 100 ml	H_2O

Buffer P2 (Lysis)		
200 mM	4 ml (5 M)	NaOH
1% (w/v).....	1 g	SDS
	ad 100 ml	H_2O

Buffer QC (Wash)		
1 M	20 ml (5 M)	NaCl
50 mM	5 ml (1 M)	MOPS (pH= 7.0)
15% (v/v)	15 ml	*i*-PrOH
	ad 100 ml	H_2O

Buffer P3 (Neutralisation)		
3 M	29.4 g	KAc
2 M	11.5 ml (18 M)	HAc (glacial)
(pH= 4.8)	ad 100 ml	H_2O

Buffer QF (Elution)		
1.25 M	25 ml (5 M)	NaCl
50 mM.....	5 ml (1 M)	TRIS (pH= 8.5)
15% (v/v)	15 ml	*i*-PrOH
	ad 100 ml	H_2O

Buffer PB		
5 M		GuHCl
30%	30 ml	*i*-PrOH
	ad 100 ml	

QIAGEN buffer compositions as cited by http://openwetware.org/wiki/Qiagen_Buffers and US Patent 6,383,393.

2.3.2 Preparation of DNA from yeast cells

The following protocol for DNA preparation from yeast cells has been published by (Harju *et al.*, 2004).

1.	Transfer 1.5 ml of liquid culture of yeast grown for 20 - 24 hrs at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) into a microcentrifuge tube. Pellet cells by centrifugation at 20,000×*g* for 1-5 minutes.
2.	Add 200 µl of Harju- buffer.
3.	Immerse tubes in a dry ice-ethanol bath for 2 minutes.
4.	Transfer to in a 95°C water bath for 1 minute.
5.	Repeat the last two steps.
6.	Vortex 30 seconds.
7.	Add 200 µl of chloroform and vortex 2 minutes.
8.	Centrifuge 3 minutes at room temperature, 20,000×*g*.
9.	Transfer the upper aqueous phase to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol. Mix by inversion or gentle vortexing.
10.	Incubate at room temperature, 5 minutes. Alternatively, precipitate DNA at -20°C to increase yield.
11.	Centrifuge 5 minutes at room temperature, 20,000×*g*.
12.	Carefully remove the supernatant.
13.	Wash the pellet with 0.5 ml 70% ethanol.
14.	Centrifuge 5 minutes at room temperature, 20,000×*g*.
15.	Remove supernatant.
16.	Air-dry the pellets at room temperature or for 5 minutes at 60°C in a vacuum dryer.
17.	Resuspend in 25- 50 µl TE (pH 8.0) or water.

Table 2.4: Preparation of DNA from yeast cells.

Harju Buffer		
100 mM	2 ml (5 M)	NaCl
1 mM	0.2 ml (0.5 M)	EDTA
10 mM	1 ml (1 M)	TRIS (pH 8.0)
1%	10 ml (10%)	SDS
2%	2 g	Triton X-100
	ad 100 ml	H_2O

2.3.3 DNA Precipitation

A DNA precipitation step may be necessary in the following cases:

- to remove proteins e.g. after PCR or ligation reaction
- to purify DNA from a mishap purification
- to obtain a more concentrated DNA sample.

For this purpose, 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the sample (at least 100 µl), and the mixture is vigorously shaken. After centrifugation (13000 rpm, 10 min, 4°C) the upper (aqueous) phase containing DNA is carefully removed. NaAc is added from a 3 M stock solution to the sample to yield a final concentration of 0.3 M. Then 2.5 volumes of ice-cold EtOH (100%) are added to the mixture.

After incubation for about 30 min at -20°C, the sample is centrifuged (13000 rpm, 4°C, 15 min) and the supernatant is removed carefully. The DNA pellet is washed with 100 µl EtOH (70%) and the suspension is centrifuged again (13000 rpm, 4°C, 15 min). After carefully removing the supernatant, the pellet is air-dried over-night or at 60°C for 30 min.

Reagents	
25:24:1	phenol:chloroform:isoamyl alcohol
3 M	NaAc
100%	EtOH, cold

2.3.4 Measurement of DNA concentration

DNA concentration can be determined spectroscopically (Sambrook *et al.*, 1989). Assuming an averaged molecular mass of M= 500 g mol^{-1} for the nucleotides, the absorbance A of a DNA solution at 260 nm can be converted to mass concentration ρ* by:

$$\rho^* = 50 \frac{\mu g}{ml} \cdot A_{260\text{nm}} = 0.05 \frac{\mu g}{\mu l} \cdot A_{260\,nm}$$

The ratio A(260 nm)/A(280 nm) is an indicator for the purity of the DNA solution and should range within 1.8 – 2.0.

2.3.5 Isolation of DNA fragments by agarose gel electrophoresis

Mixtures of DNA fragments, originating e.g. from PCR, restriction digest or simply for characterisation, can be separated by using agarose gel electrophoresis. Since the charge of a DNA molecule is proportional to its size it can be separated from other DNA fragments by applying an electric field.

The gel is made of 1% agarose in TAE (or TBE) buffer which is melted in the microwave, mix with 8 µl of commercial SYBR-Safe solution and poured into a horizontal gel chamber. After the gel has solidified, the comb is removed and running buffer TAE (or TBE) is added. The samples are mixed with application buffer (1:6) and loaded into the slots. For a 10 cm × 15 cm gel a current of approx. 60 mA – 75 mA is used.

TAE buffer		
40 mM	40 ml (1 M)	TRIS
20 mM	1.2 ml	HAc (glacial)
1 mM	2 ml (0.5 M)	EDTA
(pH= 8.4)	ad 1 l	H_2O

TAE buffer (50×)		
2 M	121.1 g	TRIS
1 M	29 ml	HAc (glacial)
50 mM	9.7 g	EDTA
(pH= 8.4)	ad 500 ml	H_2O

Application buffer (6×)		
30% (v/v)	15 ml	glycerol
0.25% (w/v)	0.1 g	bromphenolblue
0.25% (w/v)	0.1 g	xylene cyanol
	ad 50 ml	H_2O

Extraction of DNA from an agarose gel is done by using the *QIAGEN* gel extraction kit. Excise the appropriate DNA band (UV trans-illuminator; operate carefully to avoid eye contact with UV light) and add 3 volumes of buffer QG (previously: QX1) to 1 volume of gel (100 mg ~ 100 µl). Incubate at 50ºC and carefully mix, until gel is completely dissolved. To increase yield of DNA fragments < 500 bp and > 4 kb add one gel volume iso-propanol. Apply the solution to a *QIAGEN* spin column and centrifuge (1 min, 15000 rpm) to adsorb the DNA. Discard flow through and wash column with 500 µl buffer QG, and then with 750 µl buffer PE. If DNA is used for salt-sensitive reactions let column stand for 2-5 min after applying buffer PE. To remove any residual ethanol from the last wash step discard flow through and spin again for 1 min. Put the spin column into a new centrifuge tube and elute DNA by applying 30 – 50 µl H_2O (alternatively: buffer EB or 5 mM TRIS, pH 8) directly onto the membrane. Incubate for 5 min, and spin for 1 min at 15000 rpm. Store DNA at –20ºC.

Buffer QX1	
7 M	Na_2HPO_4
10 mM	NaAc
(pH 5.3)	

Buffer PE	
70% (v/v)	EtOH
100 mM	NaCl
10 mM	TRIS (pH= 7.5)

Buffer EB	
10 mM	TRIS (pH 8.5)

QIAGEN buffer compositions as cited by http://openwetware.org/wiki/Qiagen_Buffers and US Patent 6,383,393.

2.3.6 Enzymatic reactions with DNA

Restriction digest

Digestion of plasmid DNA with restriction enzymes can be done analytically (for characterisation) and preparatively (preparation for ligation reaction). The appropriate enzymes are usually bought from New England Biolabs and come with their respective buffers (NEB1 – NEB4). Analytical reactions contain approx. 1 µg DNA in a volume of 20 µl with 1 – 5 units of enzyme. Preparative reactions are done with up to 30 µg DNA in a volume of 200 µl with 5 units of enzyme per 1 µg DNA. Note: Numbers may vary in individual applications; rule of thumb only.

Ligation of DNA fragments

The covalent linkage of DNA fragments (ligation) is carried out in a proper volume by use of T4 DNA ligase. The reaction is preferably done over night at 16ºC, but can also be carried out in 4 hrs at room temperature. 24 Weiss units correspond to 1600 u (Weiss *et al.*, 1968).

Ligation reaction		
10 µl	200 ng	DNA fragment (after restriction digest)
1 µl	200 ng	plasmid (after restriction digest)
2 µl		T4 DNA ligase buffer (10×)
3 µl		H_2O
4 µl	24 Weiss units	T4 DNA ligase

Ligation requires at least one phosphate group to be present per each connection to be formed. When inserting a PCR fragment into a vector via two different restriction sites there are two phosphate groups per connection (5' and 3' of vector, and 5' and 3' of insert). When inserting into a vector with the same restriction site on the 5' and the 3' side one might wish to dephosphorylate the vector before ligation in order to prevent re-ligation of the vector.

Dephosphorylation

Removal of the 5'-phosphate from DNA is carried out with calf intestinal alkaline phosphatase (CIP) or bacterial alkaline phosphatase (BAP). BAP is more active than CIP but also more resistant against inactivation by heat or detergents. Most commonly, CIP is used to dephosphorylate DNA. CIP can be inactivated by heating to 75°C for 10 min in the presence of 5 mM EDTA. The best way of CIP removal is DNA purification in agarose gel electrophoresis after the dephosphorylation reaction.

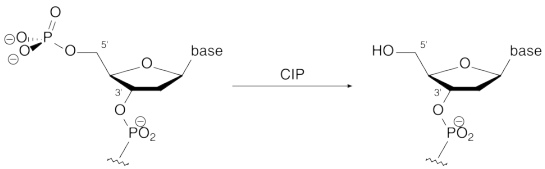


Figure 2.1: CIP-catalysed dephosphorylation of 5'-phosphate of DNA.

2.3.7 Amplification of DNA by polymerase chain reaction (PCR)

Polymerase chain reaction is based on thermo-stable polymerases which synthesise singular DNA strands starting from a primer and using DNA information from a template. A PCR comprises of the steps denaturation, annealing and elongation in a repeated fashion, which finally leads to amplification of the original DNA (template). A typical PCR protocol is as follows:

Standard PCR protocol			
Temperature	Reaction step	Repeat	Time interval
94°C	denaturation		5:00
94°C	denaturation		1:30
42°C – 66°C	annealing	25×	1:30
72°C	elongation		3:00
72°C	elongation		5:00
4°C	storage / end		

Annealing

Annealing temperature is used to tune the ratio between specific and non-specific binding of the primers to the template DNA. Higher temperatures reduce non-specific binding (preferred when working with DNA mixtures like e.g. libraries). Lower temperatures increase binding of the primers to the template DNA and therefore increase amplification efficiency (preferred in subcloning steps where the DNA template is very homogeneous). The ***melting temperature***s of primers depend on the A/T and G/C contents and generally should be between 55°C and 80°C for best results (Innis *et al.*, 1990). A formula to estimate the melting temperature T_m of a primer is (Thein & Wallace, 1986):

$$T_m = 2 \cdot (N_A + N_T) + 4 \cdot (N_G + N_C) \quad ,$$

where N is the number of primer adenine (A), thymidine (T), guanidine (G), or cytosine (C) bases. Other equations for estimation of melting temperatures have also been introduced (Rychlik *et al.*, 1990; Wu *et al.*, 1991). In ***primer design*** one should consider that primer pairs exhibit similar melting temperatures and are as complementary to the template as possible.

The annealing time has to be chosen in accordance to the lengths of the primers. For longer primers longer annealing intervals should be chosen, short primers require only short annealing intervals.

Elongation

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C (Chien *et al.*, 1976; Lawyer *et al.*, 1993), and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA

fragment.

Polymerases

Taq, Vent, Pfu, Pwo. Our in-house Taq polymerase has been working with the *Roche* HiFi-Taq buffer or, alternatively, the *Fermentas* buffer.

Roche HiFi-Taq buffer (10×)	
100 mM	TRIS (pH= 8.3)
500 mM	KCl
15 mM	$MgCl_2$

Fermentas Taq buffer (10×)		
750 mM	3.75 ml	2 M TRIS (pH= 8.8)
200 mM	0.5 ml	4 M $(NH_4)_2SO_4$
15 mM	150 µl	1 M $MgCl_2$
0.1%		Tween-20
	ad 10 ml	H_2O

Invitrogen buffer (10×)	
200 mM	TRIS (pH= 8.4)
500 mM	KCl
3.75 mM	$MgCl_2$

2.3.8 Mutagenesis

The two main methods for site-directed mutagenesis are the recombinant cyclic PCR (RCPCR; "Four-oligo-method") method and a "quick" method making use inverse PCR and *Dpn*I discrimination between parental and mutated DNA.

Recombinant cyclic PCR (RCPCR; "Four-oligo-method")

A method for site-directed mutagenesis using PCR and four oligo-nucleotides has been described by Jones and Howard as recombinant cyclic PCR (Jones & Howard, 1990; Jones & Winistorfer, 1991; Jones *et al.*, 1990). Two separate PCR reactions are performed with two primers, one of which contains the desired mutation. The amplified fragments from both reactions are purified via gel electrophoresis and then mixed, denatured and subjected to rehybridisation (see Figure 2.3). The last step yields double-stranded cyclic DNA with cohesive ends (still missing from Figure 2.3!). This product can be transformed into competent bacterial cells. The bacteria will perform *in vivo* recombination and replication.

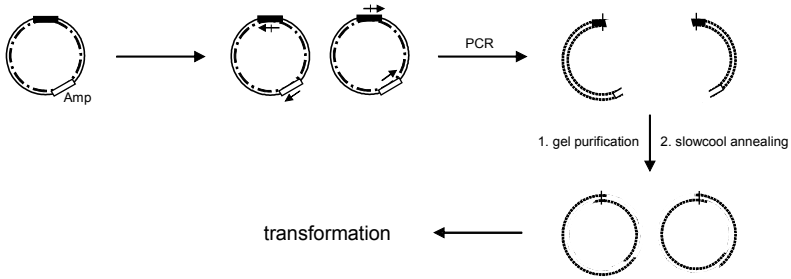


Figure 2.2: Schematics of site-directed mutagenesis using the four-oligo-method.

A typical protocol for hybridisation is as follows:

Hybridisation: Sample		
15 µl		purified PCR product 1
15 µl		purified PCR product 2
0.6 µl	100 mM	NaCl (5 M)

Hybridisation: Temperature protocol		
Temperature	Reaction step	Time interval
94°C	denaturation	3 min
50°C	hybridisation	2 hrs

QuickChange® method

This technique reported by Stratagene as QuickChange® method is based on inverse PCR (Stratagene, 2004). Two homologous primers carrying the mutation are designed, one for the coding, one for the non-coding strand. The number of mismatches between primer and parental DNA sequence should not exceed 3 and a sufficient overhang upstream and downstream of the mutation site should be provided. The usage of multiple primers annealing to either the coding or the non-coding strand is possible, which allows for introduction of several mutations in one run. In this case, only a single-stranded plasmid is produced and transformed; a special blend of polymerases seems to be required.

Plasmid DNA from almost all of the commonly used *E. coli* strains (*dam*$^+$) is methylated and suitable as a template for this mutagenesis method. Plasmid DNA isolated from *dam*$^-$ strains, such as JM110 and SCS110, is not suitable. The restriction enzyme *Dpn*I cleaves the sequence G^{Me}ATC where ^{Me}A means that the adenylate nucleotide is methylated. *Dpn*I will not cleave the unmethylated sequence GATC. The parental strand would be methylated at every GATC sequence (that is, approximately every 200 to 300 nt). The newly synthesised DNA, however, will be unmethylated. Therefore, there is a marked difference between the parental DNA (methylated) and the mutagenised DNA (unmethylated).

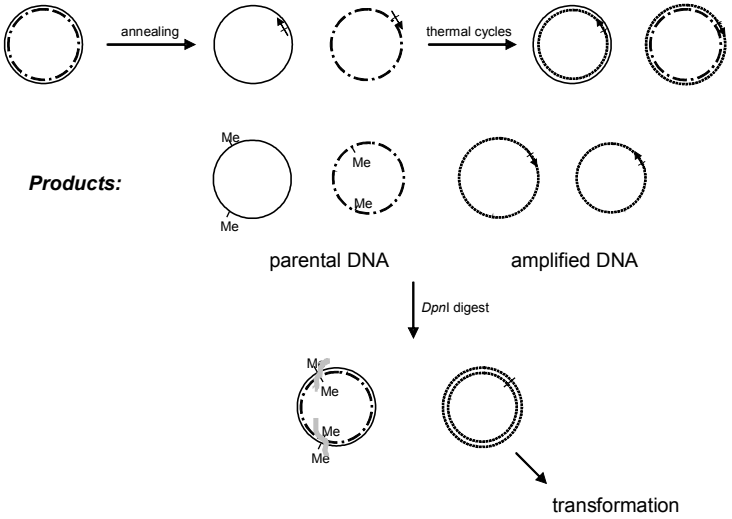


Figure 2.3: Schematics of site-directed mutagenesis using *Dpn*I discrimination between parental and synthetic DNA.

A typical protocol for a PCR reaction with Turbo *Pfu* polymerase is as follows:

Site-directed mutagenesis PCR reaction: Sample		
32 µl		H_2O
4 µl	7 ng	10× Pfu Turbo buffer
0.4 µl	3.2 pmol	dNTPs (25 mM)
1 µl		coding primer
1 µl		non-coding primer
0.6 µl		template DNA (dilution[1])
1 µl		Turbo *Pfu* DNA polymerase

Site-directed mutagenesis PCR reaction			
Temperature	Reaction step	Repeat	Time
94°C			0:30
94°C	denaturation		0:30
55°C	annealing	18×	1:00
68°C	elongation		5:00
68°C			5:00

[1]Template DNA dilution: When using cDNA from a MiniPrep, use a 1:20 and a 1:30 dilution of the original DNA. When working with template DNA from a MidiPrep, use a 1:30 and a 1:50 dilution.

The polymerase is always added last. Up to 10% DMSO might be included in the sample when working with Turbo *Pfu* polymerase (reduce water to keep the final volume at 40 µl). After the PCR, add 4 µl of *Dpn*I directly to each tube and incubate at 37°C for 5-6 hrs to digest the parental (methylated) DNA. After incubation, the DNA is precipitated by adding

1. NaAc to a final concentration of 0.3 M (5 µl of a 3 M NaAc stock solution)
2. 2.5 volumes (125 µl) of ice-cold EtOH (100%)

into each tube. Following 30 min of incubation at -20°C the tubes are centrifuged (13000 rpm, 4°C, 15 min) and the supernatant is carefully removed. To wash the DNA pellet, which might or might not be visible at this point), 100 µl of ice-cold 70% EtOH are used to dissolve the precipitate. Finally, the DNA is pelleted by centrifugation (13000 rpm, 4°C, 15 min). After carefully removing the supernatant, the pellet is air-dried over night.

For transformation, the pellet is dissolved in 7 µl sterile distilled H_2O. An aliquot of 100 µl of XL1-Blue competent cells is used per transformation. Each aliquot is incubated with 1.7 µl of β-mercaptoethanol for 10 min on ice. Then, the dissolved DNA is added and the cells are incubated a further 30 min on ice. The cells are exposed to a heat shock at 42°C for 45 sec and immediately put on ice for another 2 min. 900 µl of pre-heated NYZ medium are added and the cells are incubated at 37°C for 1 hr. The cells are harvested by centrifugation (3000 rpm, 5 min) and plated on agar plates containing suitable antibiotics for selection.

2.3.9 DNA sequencing

Sequencing protocol for dRhodamine kit

Sequencing PCR reaction: Sample		
4 µl		Terminator Reaction Mix
(1 µl)	7 ng	Template DNA
1 µl	3.2 pmol	Sequencing primer
14 µl		H_2O

Sequencing PCR reaction: PCR reaction			
Temperature	Reaction step	Repeat	Time interval
96°C	denaturation		0:20
50°C	annealing	25×	0:20
60°C	elongation		4:00

When sequencing DNA from a MiniPrep, use 5 µl template. For sequencing DNA from a MidiPrep, 1 µl is used.

3 METHODS IN PROTEIN BIOCHEMISTRY

3.1 PROTEIN EXPRESSION

Protein expression requires a suitable expression system, consisting of an expression vector and an expression organism. The most commonly used organisms are *E. coli* bacteria. Other expression systems (in order of increasing ‚difficulty') are yeast systems (*S. cerevisiae*, *P. pastoris*), baculovirus systems (host organism Sf9 insect cells) and *Drosophila* systems.

3.1.1 Bacterial expression using BL21(DE3) cells

BL21(DE3) cells are defective of several proteases, thus decreasing the proteolytic degradation of the protein of interest. They carry the T7 polymerase from λ phage DE3 in the cells. Therefore, one can use a variety of different vectors with the T7 promoter system. Usage of the *lac* repressor makes expression of recombinant protein inducible with isopropyl-1-thio-β-D-galactopyranoside (IPTG), or by auto-induction. Vectors used in this context include, for example, the pET series, the pRSET series (pBluescript derived vectors) etc.
A single colony of transformed bacteria is used to inoculate 1 l of LB medium containing 50 µg ml^{-1} ampicillin. This culture is incubated in a shaker overnight at 37°C. The next day, the overnight culture is used to inoculate further 7 l of LB medium (containing 50 µg ml^{-1} ampicillin). Incubation continues in a shaker at 37°C until OD(600 nm) exceeds 1. The culture is then induced by addition of 0.5 mM IPTG and incubation continues another 4-6 hrs.

3.1.2 Bacterial expression using ArcticExpress (DE3) RIL cells

Proteins that are expressed in a misfolded state in the usual bacterial expression protocols may be expressed correctly folded at lower temperatures. For this purpose, ArcticExpress cells (Agilent) have been derived from the high-performance BL21-Gold cells (Stratagene), in order to allow expression at low temperatures in the presence of the chaperonins Cpn60 and Cpn10 from psychrophilic bacterium *Oleispira antarctica*. The cells also provide correction for codon bias. A typical expression protocol is as follows:

1. Transform the plasmid of interest into ArcticExpress (DE3) competent cells using the established transformation protocol.
2. Plate the transformed cells onto LB agar plates containing the appropriate antibiotic for selection of the expression plasmid. It is not necessary to add gentamycin to the transformation plates.
3. Inoculate 100 ml of LB+ medium (containing 20 µg ml^{-1} gentamycin and the appropriate antibiotic for selection of the expression plasmid) with single colonies from the transformation plates. Incubate at 37°C with shaking at 200 rpm overnight.
   It may be necessary to test more than one colony as colony-to-colony variations in protein expression are possible.
4. Transfer the o/n culture into 1 l of LB+ medium (containing no antibiotics) and continue growth at 37°C with shaking at 200 rpm for 3 hours.
5. After 3 hours, reduce the temperature of the incubator to 10°C and let the culture equilibrate to 10°C.
6. After the culture has equilibrated to 10°C, add IPTG to each flask to a final concentration of 1 mM.

7.	Incubate the culture at 10°C, with shaking at 200 rpm for 24-48 hours.

Table 3.1: Bacterial expression with *E. coli* ArcticExpress cells.

3.1.3 Bacterial expression using auto-induction media

The principle of auto-induction depends on mechanisms used by bacteria to regulate the consumption of carbon and energy sources present in the growth medium.

In the T7 expression system, expression of the target protein is dependent on presence of chromosomally coded T7 RNA polymerase which is under control of the inducible *lacUV*5 promoter in host cells such as BL21(DE3). The coding sequence for the target protein is placed in a plasmid under the control of the T7 promoter, the binding site for the T7 polymerase which is able to transcribe the target gene. In order to prevent expression of the target protein when the cells are not induced, the T7 promoter is placed in close proximity to a negatively regulating *lac* operon, consisting of *lac*-repressor gene, operator and genes Z, Y and A. The *lac* repressor binds to the operator region next to the T7 promoter and thus prevents binding of T7 polymerase from binding to its promoter when the substrate lactose is missing. If glucose is present, catabolite repression and inducer exclusion prevent the uptake of lactose by lactose permease, the product of *lacY*. Once glucose is depleted, lactose can be taken up and converted to allolactose by β-galactosidase (product of *lacZ*). Gene *lacA* codes for thiogalactosid-transacetylase which is needed in this process although its physiological function is not fully understood at present. Once allolactose is produced, it binds to both the *lacUV*5 promoter and the *lac*-repressor displacing it from the lac operon, inducing production of the T7 polymerase and subsequently the target gene.

Auto-induction media have been designed to specifically enhance this naturally occurring mechanism (Studier, 2005). ZYM-5052 media consists of tryptone, yeast extract, salts and trace metals supplemented with glucose, glycerol and lactose. A good balance between the inducer lactose and other carbon sources such as glycerol or glucose is important to ensure fast cell growth before induction but without basal expression of target protein and high level production of target protein after induction. The commonly used inducer isopropyl-β-thiogalactosid (IPTG) is an allolactose-mimic being able to induce protein production that cannot be used as a carbon source by the cells.

The presence of 0.05% glucose blocks induction by lactose in the early stage of growth as it is the preferred carbon source. Therefore, it prevents basal expression of the target protein while the cells are growing up to a density suitable for induction. This is very important when expressing target proteins that are highly toxic to the host cell. In the absence of glucose, amino acids provided by a tryptic digest of casein (tryptone) appear to modulate or prevent induction of target proteins by lactose until cell growth slows down due to decreasing oxygen levels in the culture upon approach to saturation.

Having a carbon and energy source other than lactose to support continued growth is crucial to production of large amounts of target protein after induction. Glycerol does not induce production of target protein and was therefore chosen as primary energy source.

This technique can be used for any expression system in which elements driving expression of target proteins are induced by a change in metabolic state that is brought about by growth of cell culture. ZYM-5052 medium is suitable for usage with our BL21(DE3) and Rosetta2 cells whereas 0.1% L-arabinose needs to be added to the medium when using BL21-AI cells. Here, the T7 RNA polymerase is expressed from the chromosome by the arabinose-inducible pBAD

promoter. Benefits of this cell strain as compared to BL21(DE3) are a lower basal expression and greater tolerance of target proteins that are highly toxic to the cell.
For a more convenient work flow, we have recalculated the ZYM-5052 media composition and formulate and store the components in form of two mixes (AI1 and AI2). These are added to the final LB+ media of the production culture.

Recipes for 1000x metals

$FeCl_3$		
0.1 M	3.38 g	$FeCl_3 \cdot 6\ H_2O$
0.12 M	1.25 ml	12 M HCl
	ad 125 ml	H_2O

$CoCl_2$		
0.2 M	2.38 g	$CoCl_2 \cdot 6\ H_2O$
	ad 50 ml	H_2O

$CaCl_2$		
1 M	1.11 g	$CaCl_2$
	ad 10 ml	H_2O

$CuCl_2$		
0.2 M	1.71 g	$CuCl_2 \cdot 2\ H_2O$
	ad 50 ml	H_2O

$MnCl_2$		
1 M	1.98 g	$MnCl_2 * 4\ H_2O$
	ad 10 ml	H_2O

$NiCl_2$		
0.2 M	2.38 g	$NiCl_2 \cdot 6\ H_2O$
	ad 50 ml	H_2O

$ZnSO_4$		
1 M	14.4 g	$ZnSO_4 \cdot 7\ H_2O$
	ad 50 ml	H_2O

Na_2MoO_4		
0.2 M	2.42 g	$Na_2MoO_4 \cdot 2\ H_2O$
	ad 50 ml	H_2O

H_3BO_3		
0.2 M	0.620 g	H_3BO_3
	ad 50 ml	H_2O

Na_2SeO_3		
0.2 M	2.62 g	$Na_2SeO_3 \cdot 5\ H_2O$
	ad 50 ml	H_2O

1000× metals					
50 mM	125 ml	0.1 M $FeCl_3$	2 mM	2.5 ml	0.2 M $NiCl_2$
20 mM	5 ml	1 M $CaCl_2$	2 mM	2.5 ml	0.2 M Na_2MoO_4
10 mM	2.5 ml	1 M $MnCl_2$	2 mM	2.5 ml	0.2 M $CuCl_2$
10 mM	2.5 ml	1 M $ZnSO_4$	2 mM	2.5 ml	0.2 M Na_2SeO_3
2 mM	2.5 ml	0.2 M $CoCl_2$	2 mM	2.5 ml	0.2 M H_3BO_3
				ad 250 ml	H_2O

Recipes for auto-induction stocks and final media

2 M $MgSO_4$		
2 M	123.25 g	$MgSO_4 \cdot 7\ H_2O$
	ad 250 ml	H_2O

AI Mix 1 (25× M)		
0.625 M	178 g	Na_2HPO_4
0.625 M	170 g	KH_2PO_4
1.25 M	165 g	$(NH_4)_2SO_4$
0.125 M	29.2 g	NaCl
pH= 6.7	ad 2 l	H_2O
	autoclave	

AI Mix 2 (25× 5052)		
1.35 M (12.5%)	250 g	glycerol
70 mM (1.25%)	25 g	glucose
140 mM (5%)	100 g	α-lactose · 1 H_2O
	ad 2 l	H_2O
	autoclave	

LB+		
	40 g	tryptone
	20 g	yeast extract
	20 g	NaCl
2 mM	4 ml	2 M $MgSO_4$
1x	4 ml	1000x metals
	ad 4 l	H_2O
	autoclave	

Final media for expression		
	1 l	LB+
	40 ml	AI mix 1
	40 ml	AI mix 2

10% L-arabinose		
	1 g	L-arabinose
	ad 10 ml	H_2O

3.1.4 Expression of Se-Met-labelled protein using auto-induction

17aa mix (10 mg ml^{-1} each)		
59 mM	5 g	Glu
75 mM	5 g	Asp
55 mM	5 g	Lys-HCl
47 mM	5 g	Arg-HCl
48 mM	5 g	His-HCl · H_2O
112 mM	5 g	Ala
87 mM	5 g	Pro
133 mM	5 g	Gly
84 mM	5 g	Thr
95 mM	5 g	Ser
68 mM	5 g	Gln
67 mM	5 g	Asn · H_2O
85 mM	5 g	Val
76 mM	5 g	Leu
76 mM	5 g	Ile
61 mM	5 g	Phe
49 mM	5 g	Trp
5 µM	3.4 mg	Vitamin B_{12}
	ad 500 ml	H_2O
	sterile filter	

20× P		
50 mM	142 g	Na_2HPO_4
50 mM	136 g	KH_2PO_4
25 mM	66 g	$(NH_4)_2SO_4$
	ad 1 l	H_2O

25 mg ml^{-1} Met		
168 mM	250 mg	Met
	ad 10 ml	H_2O

50 mg ml^{-1} Se-Met		
254 mM	1 g	Se-Met
	ad 20 ml	H_2O

PASM-5052		
2 mM	1 ml	2 M $MgSO_4$
0.2x	200 µl	1000× metals
1x	40 ml	25× 5052
1x	50 ml	20× P
200 µg ml^{-1}	20 ml	17 aa mix
either		
10 µg ml^{-1}	400 µl	25 mg ml^{-1} Met
or		
125 µg ml^{-1}	2.5 ml	50 mg ml^{-1} Se-Met
	ad 1 l	H_2O

3.1.5 Expression of ^{15}N-labelled protein using IPTG-induction

M9		
50 mM		Na_2HPO_4
20 mM		KH_2PO_4
10 mM		NaCl
	200 ml	5× M9 salts
	6 ml	Vitamins
0.6%	12 ml	50% Glucose (w/v)
	1 ml	antibiotics
	1 ml	1000× metals
3 mM	3 ml	1 M $MgSO_4$
	1 g	NH_4Cl
	ad 1 l	H_2O

Vitamins		
	0.5 g	nicotinamide
	0.5 g	thiamine · HCl
	ad 100 ml	H_2O
	sterile filter and keep at 4°C	

5× M9 salts		
0.25 M	36 g	Na_2HPO_4
0.1 M	14 g	KH_2PO_4
0.05 M	2.9 g	NaCl
	ad 1 l	H_2O
	autoclave	

3.1.6 Expression of ^{15}N-labelled protein using auto-induction

20× NK		
1 M	71 g	Na_2HPO_4
0.8 M	54 g	KH_2PO_4
0.1 M	8.7 g	K_2SO_4
	ad 500 ml	H_2O
	autoclave	

Vitamins	
0.5 g	nicotinamide
0.5 g	thiamine · HCl
ad 100 ml	H_2O
sterile filter and keep at 4°C	

NG medium	
600 µl	vitamins
50 µl	2 M $MgSO_4$
100 µl	1000× metals
1 ml	glucose (50% w/v)
5 ml	20× NK
100 µl	1000× antibiotic
250 mg	$^{15}NH_4Cl$
ad 100 ml	H_2O

NK-5052 medium	
12 ml	vitamins
1 ml	2 M $MgSO_4$
2 ml	1000× metals
80 ml	25× 5052
100 ml	20× NK
2 ml	1000× antibiotic
5 g	$^{15}NH_4Cl$
ad 2 l	H_2O

This protocol describes the preparation of uniformly ^{15}N-labeled protein in auto-induction medium at the scale of 1 l.

1.	Inoculate 5 ml of LB medium containing the appropriate antibiotic with a single colony from a freshly transformed plate of *E. coli* BL21(DE3) cells containing plasmid for overexpression of the target protein.
2.	Grow at 37°C overnight with shaking at ~200 rpm.
3.	In a sterile 250 ml Erlenmeyer flask, prepare 50 ml of NG minimal medium from sterile concentrated stock solutions.
4.	Transfer 20 ml of the overnight culture in rich growth medium to 2 ml of NG minimal medium. Grow at 37°C for 6-8 hours until the culture is visibly turbid.
5.	Transfer the entire 2 ml minimal culture to the remaining 48 ml NG medium and continue growth at 37°C overnight. This provides a starter culture for large-scale expression the following day.
6.	Prepare 1 l of NK-5052 auto-induction medium from sterile concentrated stock solutions, and divide 500 ml aliquots into sterile 2 l Erlenmeyer flasks.
7.	Add 25 ml of the starter culture to each flask and grow in a refrigerated incubator at 30°C with shaking at 200 rpm for 24 hours.
8.	Harvest the cells by centrifugation at 3000 rpm, 4°C for 30 min.
9.	Purify target protein using the appropriate method.
10.	Prepare a sample for NMR analysis.

Table 3.2: Protocol for bacterial expression of ^{15}N-labelled protein using auto-induction.

3.1.7 Bacterial expression under osmotic shock

When in osmotic shock, bacteria employ an intrinsic system to protect proteins from denaturation. An idea has been put forward that uses this trick to increase the solubility of recombinant proteins in bacterial expression. The osmotic shock is induced by high external sugar concentrations (Barth *et al.*, 2000; Blackwell & Horgan, 1991).

Cells are grown as normal until they reach an OD(600 nm) of 0.6-1.0. After induction, the culture is left to grow for another hour. Then, 1 M sorbitol is added along with 5 mM betaine.

3.1.8 Bacterial cell harvest

The cells are harvested by centrifugation at 3000 rpm for 20 min, preferably in 1 l centrifuge tubes. The resulting pellet is resuspended in D1 buffer. The resuspended cells might be lysed directly or stored at –20°C.

Buffer D1		
100 mM	20 ml (5 M)	NaCl
1 mM	2 ml (0.5 M)	EDTA
20 mM	20 ml (1 M)	TRIS (pH= 8.0)
0.1%	1 g	Triton X-100
1 mM	add to sample!	PMSF
5 mM	0.8 g	Benzamidinium chloride
	ad 1 l	H_2O

PMSF (100×)		
100 mM		PMSF
	ad 100 ml	*i*-PrOH

BL21(DE3) cells are deficient of the Lon protease which helps protecting the over-expressed proteins from degradation. In the D1 buffer, benzamidinium chloride, EDTA, and PMSF are used as protease inhibitors. Benzamidine and its derivatives are inhibitors of serine proteases, including tryptase, thrombin, and trypsin-like enzymes. In humans, benzamidine inhibits the auto-activation of blood coagulation factor VII. EDTA chelates mainly two- and three-valent metal ions, and is therefore used as an inhibitor of metallo-proteases, such as calcium-dependent cysteine proteases. PMSF (phenylmethylsulphonylfluoride) is an inhibitor of serine proteases and acetylcholinesterase (Fahrney & Gold, 1963; Turini, 1969). It inhibits proteases such as chymotrypsin, trypsin, thrombin and thiol proteases such as papain. The mechanism of action is sulphonation of the catalytic serine residue in the active site (Gold, 1967; Gold & Fahrney, 1964). Since PMSF is also inhibiting acetylcholinesterase, it is a highly toxic compound. PMSF is kept as a 100 mM stock solution in *i*-PrOH at 4°C. In aqueous solution, PMSF is quickly hydrolysed. Therefore, the appropriate amount of 100× PMSF in *i*-PrOH is added to the resuspended cells immediately before the lysis procedure and not contained in the shelved D1 buffer.

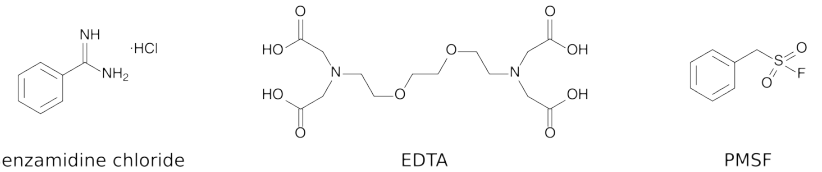


Figure 3.1: Chemical structures of frequently used protease inhibitors.

3.1.9 Bacterial cell lysis

The recommended procedure for cell lysis in the Structural Chemistry laboratory consists of a three-fold freeze-thaw cycle (lysis) and subsequent sonication (disruption of genomic DNA).

Cell lysis by repeated freeze-thaw cycles

Bacterial cells can be lysed by repeatedly subjecting them to freezing and thawing conditions (Johnson & Hecht, 1994). Resuspended cells are frozen at –80°C and then thawed in warm water three times each. Finally, the lysed cells are centrifuged at 20000×*g* for 45 min, or preferably at 40000×*g* for 30 min (ultracentrifuge) to separate the soluble from insoluble contents and cell debris.

Cell lysis by sonication

Cell membrane disruption by sonication is based on ultrasound-induced cavitation. Ultrasonics propagate in liquid media by pressure waves that alternatively expand and contract, thereby creating microbubbles or 'cavities.' Collapse of these cavities can produce extreme shear forces with the ability to disrupt membranes (Lee *et al.*, 2004; Miller *et al.*, 1996). Probe-type ultrasonication should be carried out rosette cells which possess a higher efficiency in circulation and cooling of the sample as well as enhancement of the cavitation process (Yasumitsua *et al.*, 2013). The efficiency of cell lysis by sonication is thought to be around 50-60%.

When using the method of repeated freeze-thaw for lysis, sonication of the resulting suspension may be required to break down the bacterial genomic DNA which can make the suspension stringy and viscous. In that case, the suspension after lysis is transferred into a rosette cell which is bedded in wet ice. Using the large probe, about 5 min of pulsing at 80% power is usually sufficient to obtain a reasonable fluid suspension for further processing.

Cell lysis using a French press

An alternative method to disintegrate biological samples is the use of a French laboratory press. The press can be used to disrupt cell walls of while leaving the cell nucleus undisturbed. A motor-driven piston inside a steel cylinder is used to generate pressures of up to 40000 psi. High pressure and rapid decompression can produce preparations with high yields of intact cell nuclei.

The pressure intra- and extra-cellular pressure of cells in the sample increases as the pressure generated by the press increases. When the sample is dispensed through the outlet tube, the external pressure on the cell walls drops rapidly to atmospheric pressure (decompression). However, the intra-cellular pressure does not drop at the same rate.The pressure differential causes the cell wall to burst, thus releasing the intra-cellular contents.

Typically, sample suspensions of up to 50 ml are bled through a needle valve at a rate of 1 ml min^{-1}. While the French press is thought to have a high efficiency of lysis (>90% with one pass) and produces a very uniform suspension, it is a very time-consuming process.

Sample	Pressure in psi
Bacteria	20000
Yeast	20000
Plant cell walls (cellulose)	40000
Nucleic materials	40000

Table 3.3: Typical pressures used for lysis of samples by a French press.

Cell lysis by treatment with lysozyme

Bacteria are classically divided into Gram-positive and Gram-negative organisms, according to their reaction to the Gram stain (crystal violet, iodine, safranine). Gram-positive cells have cell walls that contain very little lipid, whereas the cell walls of Gram-negative bacteria are rich in lipid.

In general, the cell wall of Gram-positive bacteria can be thought of as a rigid, brittle box, whereas the cell wall of Gram-negative organisms has a smooth soft, lipid-rich skin, with the peptidoglycan framework underneath. The biosynthesis of the cell wall occurs outside the cell membrane. The precursors are generated inside the cell, passed to the membrane and then assembled by enzymes.

Lysozyme causes lysis of many Gram-positive bacteria by dissolving the cell wall. It catalyses the hydrolysis of the $1 \rightarrow 4$-glycosidic linkages of the polysaccharide backbone peptidoglycan surrounding the bacterial cell. When Gram-positive bacteria are treated with lysozyme in the presence of high concentrations of an impermeant solute such as sucrose, the wall is removed to yield a protoplast. Protoplasts remain viable so long as the medium is hypertonic to prevent swelling and rupture of the membrane.

The walls of Gram-negative bacteria, such as *E. coli*, have somewhat more accessory material attached to the peptidoglycan framework. These accessory components consist of polypeptides, lipoproteins, and complex lipopolysaccharides. These materials contribute to the complex antigenic specificity of certain Gram-negative cells, as well as to their acceptor specificity for viruses and bacteriocins. The peptidoglycan skeleton of many Gram-negative bacteria can be broken down by treatment with lysozyme or other enzymes, but the wall material usually remains attached to the cell, which is then called a spheroblast.

3.1.10 Yeast expression of secreted proteins with Pichia pastoris

Yeast expression can provide a means for obtaining correctly folded protein in cases where bacterial expression fails. As a eukaryotic expression system, yeast provides the possibility of post-translational modification of the target protein, although this may not be desired in case of significant glycosylation. The *Pichia pastoris* expression system in conjunction with the pPICZα vector is being used successfully for the production of various recombinant heterologous proteins (Macauley-Patrick *et al.*, 2005). In this particular system, the target protein is secreted into the media due to an N-terminal fusion of a signal peptide. The signal peptide is typically (but not always) cleaved by *Pichia* upon secretion. Checking for successful protein production may involve concentration of the media due to low expression levels. Possible improvements in cases of low yields have been suggested (Huang *et al.*, 2008).

Preparation and test expression

1. Transform the expression construct in the pPICZα vector into XL1 Blue cells or another recA, endA strain. Select transformants on low salt (< 90 mM; pH 7.5) LB plates containing 25 μg ml^{-1} zeocin.
2. Pick transformants and grow cultures of 400 ml LB+ containing 25 μg ml^{-1} zeocin at 37°C for ~12 hrs.
3. Purify DNA by MidiPrep of 400 ml culture to obtain a significant amount of DNA (a large amount of DNA is required to transform into yeast cells; ~ 75 μg).
4. Linearise plasmid DNA using the restriction enzyme *Sac*I, *Pme*I or *Bst*XI (choose a restriction enzyme that does not cut inside the gene of interest):
   - 150 μl DNA solution (containing about 75 μg of DNA)
   - 20 μl NEB buffer 1 or 4
   - 2 μl BSA
   - 15 μl restriction enzyme
   - 13 μl H_2O (total volume is 200 μl)

   The volumes can be changed. However, the concentration of the reagents should be kept constant.
   Incubate the reaction mixture at 37°C overnight.
   Run a 1% agarose gel to confirm the linearisation of the plasmid DNA.
5. Purify the linearised DNA.
   - Add 200 μl (same volume as in the DNA digestion mixture) phenol:chloroform and shake vigorously.
   - Centrifuge for 30 min at 15000 rpm.
   - Remove the DNA containing upper layer and add 20 μl (1/10 of the initial volume) of 3 M NaAc and 500 μl (2-3 volumes of the initial volume) of 100% EtOH.
   - Mix and keep in the -80°C freezer for ~30 min.
   - Separate the precipitated DNA by centrifugation at 15000 rpm for 30 min.
   - Wash pellet with 70% EtOH.
   - Air-dry and resuspend the DNA in 25 μl of sterile water.
   - Measure the DNA concentration.
6. Mix 80 μl of X-33 and KM71H cells with ~25 μg of DNA in 5-10 μl sterile water in a pre-chilled Eppendorf tube. Then transfer the reaction mixture into a pre-chilled (5 min on ice) 0.2 cm electroporation cuvette.
7. Pulse the cells with 1.5 kV, 25 μF, 200 Ω and immediately add 1 ml of cold sorbitol in HEPES (10 ml of 1 M sorbitol + 200 μl 2 M HEPES, pH 8.0).
8. Transfer the cuvette contents into a sterile 15 ml tube.
9. Incubate the tubes at 30°C without shaking for 2 hrs.
10. Prepare 8 YPDS plates containing 100 μg ml^{-1} zeocin using the recipe below.

11. Carefully place nitrocellulose membranes on the YPDS plates without trapping any air bubbles between the agar slab and membrane (should be carried out under sterile conditions).
12. Apply ~200 µl of culture onto each YPDS plate (onto the nitrocellulose membrane). Spread the culture towards the center of the plate.
13. Incubate plates at 30°C for 24 hrs.
14. Prepare 8 YPD plates (4 plates for each strain) containing 100, 500, 1000, 2000 µg ml^{-1} zeocin using the recipe below.
15. After 24 hrs, transfer the nitrocellulose membranes from YPDS plates to YPD plates containing different zeocin concentrations.
16. Incubate plates at 30°C for 2-5 days until colonies are formed. Determine the highest concentration of zeocin to be used in the next step of colony isolation. For example, if there are any colonies found in the plate containing 2000 µg ml^{-1} zeocin, then use that concentration of zeocin for the next step. For multi-copy transformants (preferred) it is better to proceed with the colonies with the highest zeocin resistance.
17. Isolate single colonies in a fresh YPD plate containing the appropriate zeocin concentration for each strain.
    - Divide the plate into eight parts and streak one colony into each part.
    - Incubate the plates at 30°C until single colonies are formed.
18. Pick 6 colonies (3 from each strain) and grow them in 5 ml of BMGY medium containing 500 µg ml^{-1} of zeocin for 24 hrs (30°C, 200 rpm)
19. Next day, measure ODs (1 $OD_{600} \approx 5\times10^7$ cells ml^{-1}) and start 50 ml BMGY cultures with equal amounts of cells in 250 ml baffled flasks (approximately 2-3 ml pre-culture per 50 ml BMGY). It is not necessary to add zeocin at this step. After transferring the medium into flasks, cover them with two EtOH soaked KimWipes. Grow for 24 hrs (30°C, 200 rpm).
20. After 24 hrs, take samples from each flask to make glycerol stocks (final concentration of 15% glycerol).
21. Centrifuge 50 ml of BMGY culture of KM71H cells (2000×*g* for 8 min) and resuspend the pelleted cells in 10 ml of BMMY medium.
    Also, centrifuge 10 ml of BMGY culture of X-33 cells and resuspend the pelleted cells in 50 ml of BMMY medium. Grow these cultures for 24 hrs (30°C , 200 rpm).
22. After 24 hrs, induce the cultures by adding MeOH to a final concentration of 0.5% (KM71H: 50 µl MeOH; X33: 250 µl). Before adding MeOH, take 500 µl sample from each flask to run on a SDS-PAGE later. Continue the expression for 5-6 days, taking a sample for SDS-PAGE (optimum expression period analysis) and adding MeOH every 24 hrs.
    19.To store the samples, centrifuge for 2 min maximum speed. Transfer supernatant to a separate tube and store both fractions at -80ºC until assessing by SDS-PAGE.
23. After 5-6 days, collect cells (2000×*g* for 8 min). Check samples of cells and medium on

SDS-PAGE for expressed and secreted protein. If the protein secreted into the medium is not clearly visible in the gel, try concentrating the medium using any protein concentration procedure.

Table 3.4: Protocol for test expression of secreted proteins with *P. pastoris*.

Large scale expression

1.	Using the test expression results, determine the strain which gives the highest amount of protein and use that strain for large scale expression.
2.	Inoculate transformed cells in BMGY medium (KM71H: 25 ml; X33: 200-250 ml) containing 500 µg ml^{-1} of zeocin with ~200 µl of the frozen stock of the strain to be used and grow for 24 hrs with vigorous shaking (30°C, 200 rpm).
3.	After 24 hrs, or when the mixture is very creamy, mix the 25 ml culture with 1 l of BMGY medium (no zeocin) and distribute the diluted culture into three 2 l baffled flasks. Culture volumes should be no more than 10-30% of the total flask volume.
4.	Cover the flasks with two EtOH-soaked KimWipes and incubate for 24 hrs (30°C, 200 rpm).
5.	If using KM71H cells: After 24 hrs, or when the mixture is very creamy, collect the cells by centrifugation (2000×*g*, 8 min, RT; Avanti J-E centrifuge, JLA-9.100 rotor: 3000 rpm) and resuspend the pellet in 1/5 - 1/10 of the original culture volume of BMMY medium.
6.	Cover the flasks with 2 layers of EtOH-soaked KimWipes and continue to grow (30 °C, 200 rpm).
7.	Induce the culture by adding MeOH to a final concentration of 0.5% (0.5 ml MeOH per 100 ml medium) every 24 hrs for the number of days determined in the small scale trial.
8.	Collect the cells by centrifugation (7500×*g*, 40 min, 4°C; Avanti J-E centrifuge, JLA-9.100 rotor: 4000 rpm). Separate the supernatant carefully and store at 4°C until use.

Table 3.5: Protocol for large scale expression of secreted proteins with *P. pastoris*.

Buffers

500× B (0.02% biotin)	
20 mg	Biotin
ad 100 ml	H_2O
	filter sterilise
	store at 4°C; shelf life ca. 1 year

10× YNB	
134 g	Yeast nitrogen base without amino acids
ad 1 l	H_2O
	heat to dissolve; filter sterilise
	store at 4°C; shelf life ca. 1 year

10×GY (10% glycerol)	
100 ml	glycerol
ad 1 l	H_2O
	autoclave
	store at RT; shelf life >1 year

10× M (5% methanol)	
5 ml	methanol
ad 100 ml	H_2O
	filter sterilise
	store at 4°C; shelf life ca. 2 months

10× D (20% dextrose)	
200 g	D-glucose
ad 1 l	H_2O
	autoclave or filter sterilise
	shelf life ca. 1 year

1 M sorbitol	
18.2 g	sorbitol
80 ml	H_2O
	pH to 8.0 with NaOH
ad 100 ml	H_2O

YP	
10 g	yeast extract
20 g	peptone
ad 900 ml	H_2O
	autoclave

YPD	
900 ml	YP
	autoclave, cool to ~60°C and add required amount of zeocin
100 ml	10xD

BMGY (Buffered glycerol-complex medium	
700 ml	YP
100 ml	1 M potassium phosphate buffer, pH 6.0
100 ml	10× YNB
2 ml	500× B
100 ml	10× GY

BMMY (Buffered methanol-complex medium)	
700 ml	YP
100 ml	1 M potassium phosphate buffer, pH 6.0
100 ml	10× YNB
2 ml	500× B
100 ml	10× M

YPDS Zeocin agar plates	
100 µg	1 l medium + 1 ml zeocin stock
100 µg	100 ml medium + 0.1 ml zeocin stock
500 µg	100 ml medium + 0.5 ml zeocin stock
1000 µg	100 ml medium + 1 ml zeocin stock
2000 µg	100 ml medium + 2 ml zeocin stock
2000 µg	40 ml medium + 800 µl zeocin stock

Zeocin stock
100 mg ml^{-1} zeocin

YPDS Zeocin agar	
10 g	yeast extract
20 g	peptone
182.2 g	sorbitol
20 g	agar
ad 900 ml	H_2O
	autoclave, cool to ~60°C and add required amount of zeocin
10 ml	zeocin stock
100 ml	10x D

3.1.11 Yeast expression of SeMet-labelled protein with Pichia pastoris

Yeast media	
90 mg	Adenine sulfate
90 mg	Uracil
300 mg	succinic acid
340 mg	thiamine
10 mg	inositol
90 mg	L-tyrosine
90 mg	L-leucine
90 mg	L-isoleucine
90 mg	L-lysine-HCl
90 mg	L-arginine-HCl
90 mg	L-tryptophan
90 mg	L-histidine-HCl
120 mg	L-cysteine
150 mg	L-phenylalanine
200 mg	L-proline
200 mg	L-alanine
300 mg	L-glutamic acid
300 mg	L-aspartic acid
300 mg	L-glutamine
450 mg	L-valine
600 mg	L-threonine
1200 mg	L-serine
13.4 g	Yeast nitrogen base without amino acids
100 mg	L-selenomethionine
ad 1 l	H_2O

Media for SeMet labelling in yeast	
90 mg	Adenine sulfate
90 mg	Uracil
10 mg	inositol
340 mg	thiamine
300 mg	succinic acid
100 mg	L-selenomethionine
	filter sterilise to 800 ml
100 ml	10× AA
100 ml	10× YNB

10× AA	
0.45 g	L-tryptophan
0.45 g	L-histidine-HCl
0.45 g	L-arginine-HCl
0.45 g	L-tyrosine
0.45 g	L-leucine
0.45 g	L-isoleucine
0.45 g	L-lysine-HCl
0.60 g	L-cysteine
0.75 g	L-phenylalanine
1.00 g	L-proline
1.00 g	L-alanine
1.50 g	L-glutamine
1.50 g	L-glutamic acid
1.50 g	L-aspartic acid
2.25 g	L-valine
3.00 g	L-threonine
6.00 g	L-serine
ad 500 ml	H_2O
	autoclave

3.2 CHARACTERISATION OF PROTEINS

3.2.1 Discontinuous gel electrophoresis by Lämmli's method

Polyacrylamid gel electrophoresis (PAGE) uses electrophoretic mobility as separating parameter (Lämmli, 1970). The mobility itself depends on molecular size and shape (extension), as well as charge. The discontinuous gel electrophoresis uses two gels, a separating gel and a stacking gel, to achieve sharp bands and high resolution of proteins in the sample. Each gel layer is made with a different pH to yield basic conditions in the separating gel and slightly acidic conditions in the stacking gel. The layers also differ in acrylamide and amine concentration.

SDS gels

are mostly used with denaturing (sample is heated) and reducing (loading buffer contains DTT) conditions. SDS acts as a charge carrier and the assumption is that every protein therefore has the same charge. This in turn eliminates charge as a separation parameter. Since the gel is also run under denaturing conditions one can assume that every protein is in the same unfolded state which eliminates contributions of special folding features to electrophoretic mobility. In summary, the gel should show a protein separation based on molecular size.

6 – 8 gels are prepared in a gel caster using the recipe below. The percentage of acrylamide should be chosen according to the size of the proteins to be separated. As a rough estimate one would use 12% gels for proteins in the range of 30 kDa – 40 kDa, 14% gels for proteins of 20 kDa – 30 kDa size, and 16% gels for 15 kDa – 20 kDa proteins. The focussing gel is usually a 5% matrix. The separation gel is prepared first; after pouring the gel matrix into the caster (check absence of bubbles!) some *n*-BuOH is applied on top to ensure a smooth surface during the polymerisation process. Polymerisation is usually completed after 1 - 2 hrs. Discard the butanol and wash gel surfaces with water excessively. Dry the slots well (one might use strips of blotting paper) and pour the focussing gel matrix. Insert the combs quickly. Gloves are mandatory, liquid acrylamide is neuro-toxic!

		Separation gel					Stacking gel	
Acrylamide		**16%**	**14%**	**12%**	**10%**	**8%**	**5%**	**3%**
H_2O	ml	24	30	36	42	48	41.6	45.6
2 M TRIS (8.8)	ml	17	17	17	17	17	-	-
1 M TRIS (6.8)	ml	-	-	-	-	-	7.5	7.5
SDS (10%)[1]	ml	0.9	0.9	0.9	0.9	0.9	0.6	0.6
AA / Bis-AA[2]	ml	48	42	36	30	24	10	6.0
APS[3]	µl	200	200	200	200	200	200	200
TEMED[4]	µl	100	100	100	100	100	100	100

[1]Sodium dodecylsulphate; [2]Acrylamide/Bis-acrylamide (30% / 8%); [3]10% $(NH_4)_2S_2O_8$ (w/v);
[4]N,N,N',N'-Tetramethylethylendiamine (alias TMEDA)

Mix samples with loading buffer (1:1) and heat them up to 90°C for 5 min. Electrophoresis is done at constant voltage of approx. 150 V – 200 V corresponding to a current amplitude of about 50 mA per gel.

Loading buffer (2×)		
100 mM	5 ml (1 M)	TRIS (pH= 6.8)
100 mM	0.77 g	DTT
4% (w/v)	20 ml (10%)	SDS
0.1% (w/v)	0.05 g	bromphenolblue
20% (w/v)	10 g	glycerine
	ad 50 ml	H_2O

Running buffer		
25 mM	3 g	TRIS
250 mM	18g	glycine
0.1% (w/v)	1g	SDS
(pH= 8.3)	ad 1l	H_2O

Native gels

are used in order to detect non-covalent protein aggregates (no SDS) or to subject proteins with functional disulphide bridges (no DTT) to gel electrophoresis. The recipe for 1 native gel, which is prepared by a "stand-alone" method (corners sealed with 1% agarose) is as follows:

		Separation gel					Stacking gel	
Acrylamide		**16%**	**14%**	**12%**	**10%**	**8%**	**5%**	**3%**
H_2O	ml	4.0	5.0	6.0	7.0	8.0	3.5	3.8
2 M TRIS (8.8)	ml	2.8	2.8	2.8	2.8	2.8	-	-
1 M TRIS (6.8)	ml	-	-	-	-	-	0.6	0.6
AA / Bis-AA[2]	ml	8.0	7.0	6.0	5.0	4.0	0.8	0.5
APS[3]	µl	100	100	100	100	100	100	100
TEMED[4]	µl	50	50	50	50	50	50	50

[1]Sodium dodecylsulphate; [2]Acrylamide/Bis-acrylamide (30% / 8%); [3]10% $(NH_4)_2S_2O_8$ (w/v);
[4]N,N,N',N'-Tetramethylethylendiamine (alias TMEDA)

Samples are prepared by mixing with native loading buffer (no heating). Apply to gel and run electrophoresis as described above.

Native loading buffer (2×)		
100 mM	5 ml (1 M)	TRIS (pH= 6.8)
0.2% (w/v)	0.1 g	bromphenolblue
20% (w/v)	10 g	glycerine
	ad 50 ml	H_2O

Native running buffer		
25 mM	3 g	TRIS
250 mM	18 g	glycine
	ad 1l	H_2O

Staining with Coomassie

Polyacrylamide gels are most commonly stained with Coomassie dye or with silver. Coomassie staining can be expedited by heating the gel in the staining solution for a short time (e.g. microwave: 10 sec, 1000 W) and incubate for 10 min. Destain the gel by repeatedly washing with water and destain solution (repeated heating in microwave possible: 30 sec, 1000 W). When heating stain and destain solutions in the microwave, make sure you observe the given time intervals and keep the door ajar afterwards to ventilate the inner compartment (MeOH vapour)!

Coomassie staining solution		
3 mM	2.5 g	Coomassie Brilliant Blue R250
10%	100 ml	HAc (glacial)
45% (v/v)	450 ml	MeOH
	450 ml	H_2O
	filtrate	

Destaining solution		
25% (v/v)	250 ml	MeOH
8%	80 ml	HAc (glacial)
	ad 1l	H_2O

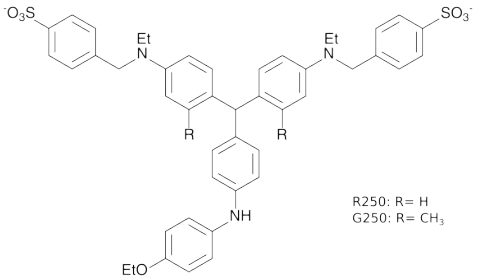


Figure 3.2: Structure of the Coomassie reagent. The intense blue colour is due to the extended conjugated π-electron system.

The di-sodium salt of the Coomassie reagent is available in form of two derivatives, R250 and G250. It forms non-covalent complexes with proteins, preferably *via* ionic interactions (sulphonic acid groups), but van der Waals interactions are possible as well. This type of staining enables the visualisation of about 0.1 µg protein in PAGE.
In the destaining step, the protein is fixated and hence not amenable to further analytical procedures (mass spectrometry, N-terminal sequencing). Therefore, standard Coomassie-stained gels cannot be used for in-gel digest/MS and other analytical techniques.
If protein bands on gels are subjected to further analytical experiments, they need to be blotted onto PVDF membranes (without prior staining of the gel), or special staining reagents need to be used. These staining reagents (e.g. GelCode Blue Stain, Thermo Scientific) are based on colloidal Coomassie dye G250. Protein bands can be viewed directly on the gel during the staining process, and , importantly, no further destaining other than a water wash is required.

Staining with silver

Silver stain requires fixation for 1 hr (fixation solution). Wash a few times with H_2O and incubate for 30 min in incubation solution. Wash again a few times with H_2O and transfer the gel to the staining solution (30 min). Wash with H_2O and develop the gel by incubating in developing solution (reduction of Ag^+ to Ag). When the desired degree of staining is reached, stop developing by washing with stop solution.

Fixation solution		
25% (v/v)	250 ml	MeOH
8% (v/v)	80 ml	HAc (glacial)
	ad 1l	H_2O

Staining solution		
6 mM	0.5 g	$AgNO_3$
0.01%	150 µl (37%)	HCHO
	ad 500 ml	H_2O

Incubation solution		
0.5 M	34.02 g.....	NaAc · 3 H_2O
8 mM	1 g	$Na_2S_2O_3$ · 5 H_2O
25% (v/v)	125 ml	EtOH
0.125%	2.5 ml (25%)	glutaraldehyde
	ad 500 ml	H_2O

Developing solution		
0.25 M	12.5 g	Na_2CO_3
0.01%	150 µl (37%)	HCHO
pH= 11.5		adjust
	ad 500 ml	H_2O

Stop solution		
60 mM	18.6 g	EDTA
	ad 1 l	H_2O

3.2.2 Single gel electrophoresis

The use of a stacking gel is a time-consuming task since it has to be prepared after the separating gel has polymerised. A new electrophoresis system named single gel electrophoresis has been introduced that eliminates the need for two different gel layers (Ahn *et al.*, 2001). It is thus simple and convenient to use. The elimination of the stacking gel provides an increased separation length with the same size cassette; this extra distance can be important for better separation with tightly resolved proteins.

Single gel PAGE has been demonstrated to yield protein and peptide resolution comparable to Lämmli's method, and the running buffer, gel staining/destaining procedures, as well as further usage in electroblotting is exactly the same as with the discontinuous gel systems (Ahn *et al.*, 2001; Yim *et al.*, 2002). Furthermore, the single gel does not contain any SDS, because it has been shown that the SDS contained in the running buffer populates the gel sufficiently to achieve the same functions as in conventional SDS-PAGE. This provides a second advantage of the single gel system, because the same gel can be used either as SDS, SDS/denaturing, or native gel, simply depending on the choice of sample preparation and running buffer. The electrophoresis system consists of acrylamide/bis-acrylamide, an amine with pH= 7.4, an acid, and three kinds of amino acids as electrolytes.

As before, when preparing the gels, gloves are mandatory, since liquid acrylamide is neuro-toxic!

2× SGN/TRIS		
0.2 M	6.8 g	glycine
0.2 M	9.9 g	serine
0.2 M	11.9 g	asparagine
Alternatively:	*13.5 g*	*asparagine · 1 H_2O*
167 mM	70 ml (1 M)	TRIS (pH= 7.5)
(pH= 7.4)	ad 420 ml	H_2O
		10 aliquots

		Single gel				
Acrylamide		**8%**	**10%**	**12%**	**14%**	**16%**
Total volume	ml	90	90	90	90	90
H_2O	ml	24	18	12	6	0
2x SGN/TRIS	ml	42	42	42	42	42
AA / Bis-AA[1]	ml	24	30	36	42	48
APS[2]	µl	400	400	400	400	400
TEMED[3]	µl	200	200	200	200	200

[1]Acrylamide/Bis-acrylamide (30% / 8%); [2]10% $(NH_4)_2S_2O_8$ (w/v);
[3]N,N,N',N'-Tetramethylethylendiamine (alias TMEDA)

3.2.3 Proteintransfer: Western Blot, Immunoblot

Further characterisation of proteins such as amino acid sequencing or immunochemical staining requires the proteins to be transferred from the polyacrylamide gel onto an accessible matrix. As matrix, a membrane made of polyvinyldifluoride (PVDF) is used.

Electroblot

The transfer (blot) is usually carried out in an electrical field and, like gel electrophoresis, uses the migration of protein:SDS complexes in the electric field. The most commonly used "semi-

dry" technique consists of two graphite electrodes, in between which several layers of blot paper (3M), the membrane and the polyacrylamide gel are arranged as a sandwich. The arrangement is such that the membrane is closer to the anode. All components of the "sandwich" are soaked in transfer buffer before the blot. The membrane itself has to be prepared with a few drops of MeOH before soaking in transfer buffer. Air bubbles between the different layers are eliminated by repeatedly rolling a glass bar over the stack of layers. Blotting is performed with a constant current amplitude of 0.65 mA per 1 cm^2 of membrane area. After the blot, the membrane might be stained with Coomassie Blue or Ponceau Red. Latter stain can subsequently be washed out, which is especially useful if the blot will be used for immunochemical staining.

Transfer buffer		
40 mM	3 g	glycine
50 mM	50 ml (1 M)	TRIS (pH= 8.3)
0.05%	0.5 g	SDS
20% (v/v)	200 ml	MeOH
	ad 1 l	H_2O

Ponceau Red		
0.1 % (w/v)	0.25 g	Ponceau Red
3 % (v/v)	7.5 ml	Cl_3C-COOH
	ad 250 ml	H_2O

Immunoblot

For immunochemical staining with antibodies, the blot membrane is incubated for 1 hr in blocking solution to saturate unspecific binding area. Then the first (primary) antibody is added and incubation is continued over night. After a first washing step with TBST and three more washing steps with TBS (15 min incubation each) the second antibody is added, usually at a dilution of 1/1000. If the primary antibody is polyclonal, an anti-rabbit IgG alkaline phosphatase conjugate is used as second antibody. For monoclonal primary antibodies an anti-mouse IgG is used. After incubation for 1 hr, four washing steps with substrate buffer (15 min each) are performed. The membrane is then transferred into staining solution. The stain reaction is stopped by washing with water.

Blocking solution		
0.5 mM	3 g	BSA
	ad 100 ml	TBS

NBT solution		
60 mM	50 mg	Nitro-blue tetrazolium chloride · H_2O
	1 ml	DMF/H_2O (70%, w/v)

TBST buffer		
0.02% (w/v)	0.1 g	Tween20
	500 ml	TBS

BCIP solution		
153 mM	50 mg	5-Bromo-4-chloro-3-indolyl phosphate
	1 ml	DMF/H_2O (70%, w/v)

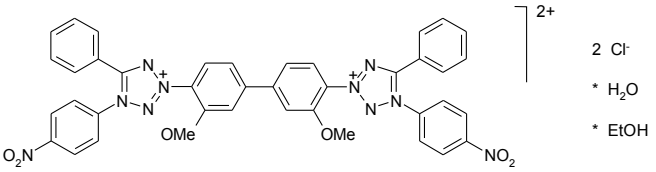
TBS buffer		
150 mM	15 ml (5 M)	NaCl
10 mM	5 ml (1 M)	TRIS (pH= 8.0)
	ad 500 ml	H_2O

Staining solution		
	10 ml	substrate buffer
36 mM	66 µl	NBT solution
46 mM	33 µl	BCIP solution

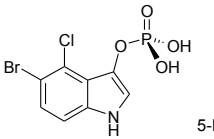
Substrate buffer		
100 mM	50 ml (1 M)	TRIS (pH= 9.5)
100 mM	10 ml (5 M)	NaCl
5 mM	2.5 ml (1 M)	$MgCl_2$
	ad 500 ml	H_2O

Alkaline phosphatase is a popular enzyme conjugate for of secondary antibodies. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) acts as a substrate for alkaline phosphatase, and is hydrolysed to form an intermediate that undergoes oxidative dimerisation. The RedOx partner in

this reaction is nitro-blue tetrazolium chloride (NBT), which is reduced to NBT-formazan. The end product of the oxidative dimerisation of BCIP is an insoluble black-purple indigo dye which visualises the substrate-antibody-complex.



Nitro Blue Tetrazolium chloride monohydrate



5-Bromo-4-chloro-3-indolylphosphate

Figure 3.3: Structures of NBT and BCIP, used in immunoblot staining.

3.2.4 Concentration of protein solutions

The preferred methods for concentration of protein solutions are based on ultrafiltration, as there is no inherent risk of contamination. When concentrating protein solutions, particular attention needs to be paid to the occurrence of precipitate (in which case stabilising measures such as higher salt concentrations, different pH, or addition of glycerol may be required). If multiple re-filling of the ultrafiltration device is required to process a sample, the contents from the previous ultrafiltration cycle should be recovered and pooled with the remaining sample to be processed, in order to minimise the risk of precipitation due to over-concentration.

Acetone precipitation of proteins is typically only used when the sample is subjected to denaturing analytical methods such as SDS-PAGE.

Ultrafiltration by centrifugation

Protein solutions can be concentrated by filtration through a membrane with the help of centrifugation. Depending on the size of the protein, ultrafiltration cartridges (Amicon Centricon and Centriprep; Pall Nanosep) with appropriate exclusion sizes (3 kDa, 10 kDa, 30 kDa, 50 kDa, etc) are used. The cartridges are centrifuged at 5000×*g*, corresponding to 4000 rpm in a 22 cm rotor (4°C). When using a swinging bucket rotor, the process needs to be closely monitored to ensure that the membrane does not run dry.

Ultrafiltration using a pressure cell

Ultrafiltration of large volumes (up to 400 ml) of protein solutions are carried out in a stirred pressure cell. Nitrogen gas is applied directly to the ultrafiltration cell which is operated at a pressure <55 psi (3792 hPa). Solutes above the membrane molecular weight cut-off are retained in the cell, while water and solutes below the cut-off pass into the filtrate and out of the cell. The

cell remains fixed in the retaining stand until the pressure-relief valve is opened and the cap depressed. The entire assembly is placed on a magnetic stirrer to maintain fluid movement during operation, to reduce the build-up of concentrated solutes on the membrane.

Osmosis-driven concentration

Large volumes of protein solutions can be concentrated by using osmosis. For this purpose, a dialysis tube is prepared by heating in H_2O and filled with the protein solution (note exclusion size!). The tubing is then placed in a trough filled with high molecular weight PEG (e.g. PEG20000) or sucrose. Osmosis results in a loss of H_2O within the tubing, and in the case of sucrose, yields a sucrose-saturated protein concentrate. While we have observed contamination of the protein solution when using PEG as the hygroscopic agent, the use of sucrose may require a second dialysis into an appropriate buffer to remove sucrose from the protein concentrate.
A similar approach is the use of AquaCide (Calbiochem) which can be used as hygroscopic substance surrounding the dialysis tube.

Chromatography

Proteins may be able to bind to particular chromatography resins under some conditions, and be eluted using other conditions. If the type of resin and the conditions required for adsorption and elution are known, chromatography can be used to concentrate proteins in solution. The chromatography types best suited for this task are affinity and ion exchange chromatography. A dilute protein solution can for example be loaded onto an anion exchange column and then be eluted with appropriate but small volumes of buffer that contains salt at a concentration sufficient for elution of this protein.
Concentration by chromatography may be a welcome effect during a purification protocol that has given rise to a sample with large volume. The method is less frequently used as a means of final concentration of a purified protein sample, but it presents an option for difficult protein samples.

Precipitation

Dilute protein samples that are to be subjected to analytic procedures, most commonly SDS-PAGE, may be concentrated by precipitation and isolation of the solid protein by centrifugation. The supernatant after centrifugation often contains contaminating substances that may have been present in the initial sample, therefore this procedure also provides a means of purification, albeit it is only useful for analytic purposes, since the protein is denatured in this process. The pellet obtained in the centrifugation step contains the precipitated, pure protein and can is re-dissolved in a buffer compatible with the downstream application. The most popular method of protein precipitation uses acetone.

1.	Place protein sample in acetone-compatible tube. Add four times the sample volume of cold (-20°C) acetone.
2.	Vortex tube and incubate for 1 hr at -20°C.
3.	Centrifuge 10 min at 13000-15000×*g* (4°C).
4.	Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
5.	Allow the acetone to evaporate from the uncapped tube at room temperature for 30 min.

6. Add the appropriate buffer for the next application and vortex thoroughly to dissolve the protein pellet.

Table 3.6: Acetone precipitation of proteins.

3.2.5 Measurement of protein concentrations

Determination of protein concentration can be carried out using either the intrinsic chromophores of proteins, like aromatic residues (280 nm) or peptide bonds (215 nm), or by using colorimetric assays, where the protein is treated with reagents to yield coloured or fluorescent products. The different methods have different ranges of sensitivity and depending on the purpose, either method can be used.

Method	Detection range
Biuret	1 mg – 5 mg
UV/Vis	1 µg – 2 mg
Lowry	5 µg – 40 µg
Dye binding	1 µg – 10 µg
Fluorescamine	0.1 µg – 2 µg

Table 3.7: Detection range for different protein assays based on a report by (Harris, 1987).

UV/Vis spectroscopically with extinction coefficient

Using the rule of Lambert-Beer the concentration of a purified protein sample can easily be calculated from its absorption:

$$A = \epsilon \cdot c \cdot l \quad \Rightarrow \quad c = \frac{A}{\epsilon \cdot l} \quad \Rightarrow \quad \rho^* = \frac{A \cdot M}{\epsilon \cdot l}$$

In the equations above A stands for the absorbance, c for the molar concentration, ε for the molar extinction coefficient, l for the cuvette thickness, M for the molar mass, and ρ* for the mass concentration. Note that A and ε are wavelength-dependent.

The molar extinction coefficient ε is either known or can be estimated from an increment system (software: EMBOSS *pepstats* (Rice *et al.*, 2000) or PCSB *Peptides* (Hofmann & Wlodawer, 2002)). Typically, the absorption value at 280 nm is determined from a UV/Vis spectrum recorded from 500 nm – 210 nm. One should always record a full spectrum to check for the presence (or absence) of prosthetic groups (or contaminants) absorbing in the Vis wavelength range. A slope in this range also indicates that the protein forms aggregates and the absorption values have to be corrected by extrapolation of the slope and extracting appropriate values at the desired wavelengths.

If the molar extinction coefficient is not known or cannot be estimated, the empiric formula of Warburg & Christian (Warburg & Christian, 1941) provides a simple way of determining protein concentrations:

$$\rho^* = (1.55 \cdot A_{280\text{nm}} - 0.76 \cdot A_{260\,nm}) \frac{mg}{ml}$$

Here, the absorbance A at λ= 280 nm and λ= 260 nm has to be determined in order to calculate the concentration.

Bradford reaction

The Bradford reaction (Bradford, 1976) (also called dye-binding method) is based on the Coomassie dye (see page 52), also used for staining gels from PAGE. Like all colorimetric assays, it requires the acquisition of a calibration curve, i.e. the measurement of protein samples with known concentrations. The assay can be done at micro scale (1 ml single cuvette) or multi-well plate format.

Upon binding to protein, the absorption maximum of Coomassie Brilliant Blue G-250 shows a bathochromic shift from λ_{max}= 465 nm to λ_{max}= 595 nm. The extinction coefficient of the dye:protein complex is nearly invariant over a concentration range spanning one magnitude. The Coomassie reagent is said to react primarily with arginine residues and less so with other basic and hydrophobic residues. The assay is said to exhibit different sensitivities with different proteins. Apparently, the sensitivity observed with bovine serum albumin is by about a factor of two higher than with other proteins.

Bradford solution		
0.12 mM	100 mg	Coomassie Brilliant Blue G-250
5% (v/v)	50 ml	EtOH
8.5%	100 ml	85% H_3PO_4
	ad 1l	H_2O
		filter through Whatman #1

For the calibration curve, prepare standard protein (albumin or immunoglobulin G) samples in the range of 5 µg to 100 µg in 100 µl volume. Add 900 µl of Bradford solution, incubate for 5 min at room temperature and measure the absorbance at λ= 595 nm. The calibration curve is constructed by plotting absorbance A versus molar concentration of protein.

Samples are measured in the same way, and their molar concentrations determined by using the calibration curve. The addition of an equal volume of 1 M NaOH solution to each sample was suggested by Stoscheck (Stoscheck, 1990) to allow the solubilisation of membrane proteins and reduce the protein-to-protein variation in colour yield.

Biuret reaction

This method can be used for concentration determination of colourless, soluble protein (Gornall *et al.*, 1949). Cu^{2+} ions in alkaline solution are coordinated by the nitrogen atoms of the peptide bonds in the protein, which produces a purple complex. To maintain Cu^{2+} in alkaline environment it is masked with tartrate as chelating reagent, which protects it from reaction with hydroxide ions. Decomposition of the colour reagent happens when Cu^{2+} is reduced to Cu^{+} (orange precipitate); however, the lifetime of the colour reagent solution can be prolonged by adding 0.1% (w/v) of KI.

Copper tartrate solution		
12 mM	0.75 g	$CuSO_4 \cdot 5\ H_2O$
43 mM	3 g	NaK tartrate $\cdot$ 4 H_2O
	ad 250 ml	H_2O

Colour reagent	
250 ml	Copper tartrate solution
150 ml (10%)	NaOH
ad 500 ml	H_2O

Typically, 25 µl of the protein sample (0.1 mg ml^{-1} – 5 mg ml^{-1}) is added to 275 µl of the colour reagent and incubated at room temperature for 2 h. Then, the absorbance at 540 nm is determined against a blank sample of 25 µl buffer and 275 µl colour reagent. As a rule of thumb, 1 mg of protein provokes an absorbance of $A_{540\ nm}$= 0.1. A calibration curve might be used as a more precise way of determining the protein concentration.
NH_4^+ ions at high concentrations might interfere with the assay by chelating Cu^{2+} ions. Also, reducing reagents must not be present to avoid reduction of Cu^{2+} to Cu^+; latter will form an orange precipitate.

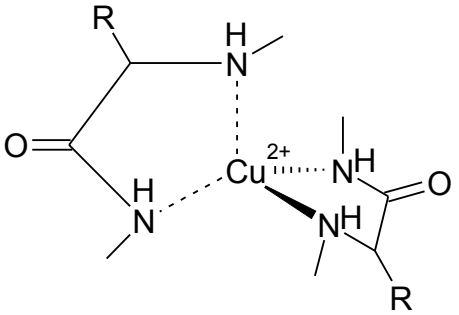


Figure 3.4: A possible tetrahedral coordination sphere for Cu^{2+} by peptide bond nitrogen atoms. The Cu^{2+}:peptide complex has a purple colour.

Lowry method

The Lowry method (Lowry *et al.*, 1951) builds on the Biuret approach, but involves oxidation of many groups in the protein sample, including aromatic and cysteine side chain residues. The Bicinchoninic acid assay (Smith *et al.*, 1985; Wiechelman *et al.*, 1988) and the Hartree–Lowry assay (Hartree, 1972) are subsequent modifications of the original Lowry procedure. Cu^+ is produced by the oxidation of peptide bonds and reacts with the Folin-Ciocalteau reagent, a mixture of phosphotungstic and phosphomolybdic acid with the empirical formulae of $H_3PW_{12}O_{40}$ and $H_3PMo_{12}O_{40}$, respectively. It is generally believed that during the reduction of the Folin-Ciocalteau reagent, tryptophan, tyrosine and cysteine side chains are oxidised (Everette *et al.*, 2010). The absorbance of the reduced Folin-Ciocalteau reagent is monitored at a wavelength of 750 nm. The assay is best used with protein concentrations of 0.01 - 1.0 mg ml^{-1}. In order to achieve best sensitivity, it is recommended to vortex the reaction after addition of each component. It has also been reported that the addition of dithiothreitol 3 min after adding the Folin-Ciocalteau reagent improves the sensitivity (Larson *et al.*, 1986). The caveat with these types of assays is the fact that a range of substances, including buffers, drugs, nucleic acids, and sugars, can interfere thus leading to wrong readouts.

Fluorescamine method

The reaction of the non-fluorescent spiro compound fluorescamine with primary amines (lysine side chain, peptide N-termini) can be used to detect proteins and peptides due to formation of fluorescent products (Böhlen *et al.*, 1973; Stein *et al.*, 1973). The method is not very accurate,

apparently due to hydrolysis of the fluorescamine in solution. Alternative methods that also use conjugation of proteins with compounds to yield spectroscopically detectable derivatives make use of *o*-phthalaldehyde, the Ellman reagent (see section Error: Reference source not found) or epicocconone.

Densitometric by assessing SDS-PAGE results

While all previous methods for determination of protein concentration require a sample of purified protein, the densitometric method allows assessment of protein concentrations directly from SDS-PAGE. The intensity of a band on the stained gel is proportional to amount of this individual protein species in the sample loaded onto SDS- (or native) PAGE. With the software *ImageJ* (Rasband, 2005), density histograms of individual lanes on a scanned gel can be calculated, and the ares of interest (bands) can be integrated using graphical tools of the software.

In order to obtain absolute concentrations, standards of a protein with known concentration need to be run on the same gel, in order to construct a calibration curve. In many cases (e.g. liposome binding assays; see section Error: Reference source not found), the absolute concentration is not required, and it may be sufficient to determine the percentile concentration of protein bands with respect to a master sample (100%).

3.2.6 Test for free cysteine side chain residues (Ellman test)

Free thiol groups can be tested for with the Ellman reaction (Ellman, 1958; Ellman, 1959). The reaction is based on the RedOx reaction of free thiols with bis-(3-carboxy-4-nitrophenyl)-disulfid (DTNB) which renders a nitro-benzothiol anion. The prominent feature of this anion is its yellow colour, which can be analysed spectroscopically in a quantitative manner.

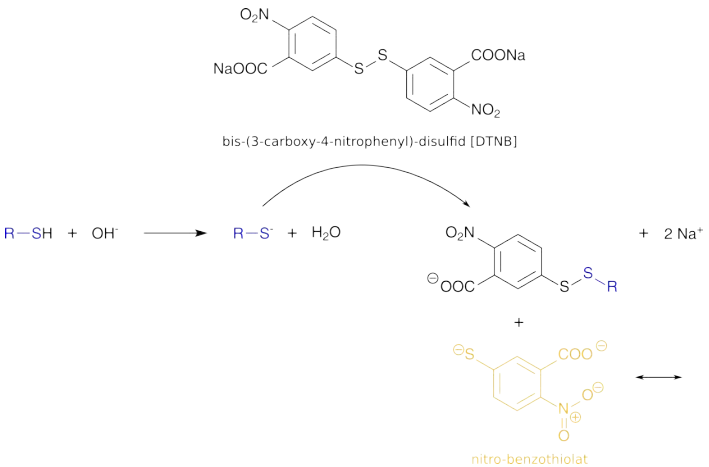


Figure 3.5: The Ellman reaction to test for free cysteine residues. Cysteine residues are deprotonated to yield thiolate anions in slightly basic environment. The Ellman reagent undergoes a RedOx reaction with the thiolate yielding a conjugate with one moiety and a liberated second moiety, which is coloured deeply yellow.

A sample with a total volume of 1 ml is composed of 960 µl buffer (Ellman A or Ellman B), 30 µl Ellman Reagent and 10 µl of protein solution. The absorbance at 412 nm is determined spectroscopically, and a sample without protein but 10 µl buffer is used as a control. For calibration, a dilution series (2 µM – 200 µM) with reduced glutathione (GSH; Glu-Cys-Gly with a γ-peptide linkage between Glu and Cys) is generated. In order to obtain reasonable absorbance measurements, the effective concentration of free thiols should be at least 1 – 5 µM.
If the protein under investigation is to be analysed for inter- or intra-molecular RedOx chemistry, the number of free thiol groups in the non-reduced state, the reduced state (achieved by reduction with DTT or TCEP), and the oxidised state (achieved by oxidation with H_2O_2) is assessed.

Solutions

Protein buffer		
100 mM	1 ml (5 M)	NaCl
20 mM	1 ml (1 M)	TRIS (pH= 8.0)
	ad 50 ml	H_2O

Phosphate buffer		
100 mM	3.45 g	NaH_2PO_4
pH= 8.0	ad 250 ml	H_2O

250 mM DTT solution		
250 mM	1.93 g	Dithiothreitol
	ad 50 ml	H_2O

Reduced glutathion stock solution		
5 mM	1.5 mg	GSH
	ad 1 ml	H_2O

10 mM DTT solution		
10 mM	10 µl (0.25 M)	DTT solution
	240 µl	Protein buffer

50 mM DTT solution		
50 mM	10 µl (0.25 M)	DTT solution
	40 µl	Protein buffer

10 mM H_2O_2 solution		
10 mM	1 µl (30%)	H_2O_2
	879 µl	Protein buffer

50 mM H_2O_2 solution		
50 mM	5 µl (30%)	H_2O_2
	875 µl	Protein buffer

Ellman A solution		
1 mM	200 µl (0.5 M)	EDTA
	ad 100 ml	Phosphate buffer

Ellman B solution		
1 mM	200 µl (0.5 M)	EDTA
7 M	42 g	urea
	ad 100 ml	Phosphate buffer

Ellman reagent		
2.5 mM	20 mg.....	Di-sodium-bis(3-carboxy-4-nitrophenyl)-disulfide (DTNB)
	ad 20 ml	Phosphate buffer

Calibration curve

A concentration series of glutathione (reduced) is subjected to the Ellman test (A and B) in triplicate. Calibration curves for Ellman A and B are determined using the final concentration range of 0.25 - 20 µM. The 10× glutathione concentration series can be established from the 5 mM stock solution as follows:

c (GSH) in µM	2.5	5.0	10	25	50	100	200
V_L (GSH @ 5 mM) in µl	0.5	1	2	5	10	20	40
V_L (protein buffer) in µl	99.5	99	98	95	90	80	60

Protein sample preparation

Aliquots of the protein sample are incubated with either 1 mM DTT or 1 mM H_2O_2 for 1 hr at RT, alternatively over night at 4°C. A sample without protein is run as control for each Ellman condition.

Reduced / oxidisied sample		
0.6 nmol	15 µl	36 kDa protein (2.4 mg ml^{-1})
0.9 or	1.5 µl	DTT or H_2O_2 (10 or 50 mM stock)

Untreated sample		
0.6 nmol	15 µl	36 kDa protein (2.4 mg ml^{-1})
	1.5 µl	Protein buffer

Purification by size exclusion

Using spin columns such as S-200 (GE Health Care) or Zeba Spin Desalting Columns, 7K MWCO (*Pierce*), excess reagents are removed from the protein samples. Times given below, especially for the prep steps, need to be re-checked.

- Remove storage buffer from spin-column by centrifugation (2400 rpm, 2 min)
- Add 150 µl phosphate buffer and centrifuge (2400 rpm, 2 min)
- Add 50 µl phosphate buffer and centrifuge (2400 rpm, 2 min), change collection tube
- Apply sample and centrifuge (2400 rpm, 2 min), should yield about 15 µl.

Purification by ultrafiltration

The sample is diluted with the appropriate Ellman buffer to a final volume of 2.5 ml and subjected to ultrafiltration with an appropriate ultrafiltration cartridge (2-5 ml size). Once the volume of 200 µl is reached, the cartridge is filled again with 2 ml of Ellman buffer and centrifuged. This step is repeated again, which finally yields a dilution by the factor of 50000 (1 mM → 20 nM).

Ellman reaction

The purified sample (15 µl or 200 µl) is mixed with the appropriate Ellman buffer (A or B) to yield a final volume of 485 µl. A UV/Vis spectrum is acquired and the protein concentration determined. The sample is then mixed with 15 µl of Ellman reagent and incubated for 2 min at RT (mix gently). The formation of cleaved Ellman reagent is quantified by measuring the absorbance at λ= 412 nm. For the UV/Vis spectrum, as well as the latter absorption measurement, a solution of 485 µl of Ellman buffer (A or B) and 15 µl Ellman reagent is used as blank.

Analysis

Using the calibration curves, the concentration of thiolates in the sample can be calculated from the A_{412} values. The concentration of thiolates is divided by the concentration of protein in the sample as determined by UV/Vis spectrometry to yield the number of reduced cysteine side chains per protein molecule.

3.2.7 Limited proteolysis for mass spectrometric finger-printing

For peptide mass fingerprinting, the protein is digested with trypsin, a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues with stringent specificity. The peptides gained from in-solution or in-gel digestion are purified and concentrated, then analysed by MALDI-TOF mass spectrometry to determine their molecular masses. The acquired mass spectra are analysed in a suitable programme to yield a list of peptide masses, which is then searched in the Mascot database (http://www.matrixscience.com/) or the peptide data base SwissProt (http://www.expasy.org/). Protein samples can be digested using two different protocols, in-solution or in-gel digestion. For in-solution digest, the incubation time is 2-18 hours at 37ºC, or 32ºC over night. For in-gel digest, longer incubation times are needed (typically over night).

Preparation of Trypsin solution

Dissolve 25 µg of trypsin (1 vial) in 250 µl of 1 mM HCl to yield a final trypsin concentration of 0.1 mg ml^{-1}. This solution is further diluted $^1/_{10}$ to $^1/_5$ in 25 mM NH_4HCO_3 immediately before use.

Protocol for in-solution digestion

For general in-solution digestion, the final protein concentration should be around 1 mg ml^{-1}. Since cysteine side chains may give rise to undesired inter- or intra-molecular disulphide bridges, they can be modified by a variety of reagents prior to protein analysis, due to the fact that they act as strong nucleophiles. For reductive alkylation, DTT is added to the protein solution at a final concentration of 5 mM, and the mixture is heated to 60ºC for 30 minutes (reduction). After cooling to room temperature, iodo-acetamide is added to a final concentration of 15 mM, and the mixture is incubated for 30 minutes in the dark at room temperature (alkylation). For the tryptic digest, 1/5 to 1/10 pre-made trypsin solution in 25 mM NH_4HCO_3 is added, and the mixture is incubated at 37ºC for 2 hrs, or at 32ºC over night. 1 µl of the digested sample and 1 µl of α-cyano-4-hydroxycinnamic acid are mixed in a tube and 1 µl is spotted onto the MALDI plate, left to dry, and then inserted into the mass spectrometer.

Protocol for in-gel digestion

For in-gel digestion, protein samples are separated by SDS-PAGE. When developing the gel, care must be taken not to fixate the protein to the gel matrix. Hence, the gel is stained with GelCode Blue Stain Reagent (*Pierce*) or Gel Stain (*Fermentas*), and then destained with water. The protein bands are cut out tight and placed in a microcentrifuge tube. 300 µl of buffer 1 are added to the samples, which are left to incubate at room temperature for 30 min. This is repeated twice to remove all SDS in the gel. The gel pieces are then incubated in 300 µl of buffer 2 for 1 hr to reduce oxidised cysteine residues in the protein. The gel pieces are then washed three times with 300 µl of buffer 1, before alkylating the thiolates by adding 100 µl of buffer 3, followed by incubation at room temperature in the dark for 20 min. The gel pieces are then washed three times with 500 µl of buffer 4, and cut into pieces of 2 mm × 1 mm. After centrifugation at 13000 rpm (4ºC), the supernatant is removed and the gel pieces covered with 100% acetonitrile. The gel pieces will shrink and turn white due to dehydration. Acetonitrile is then removed, and the remaining precipitate is allowed to dry. The dried pieces are swollen in pre-made $^1/_{10}$ to $^1/_5$ trypsin solution in 25 mM NH_4HCO_3 at 4ºC. Finally, the samples are incubated at 32ºC over night. On the next day, samples are sonicated shortly prior to mixing with the matrix and spotting onto the MALDI plate.

Buffer 1		
200 mM	0.79 g	$(NH_4)HCO_3$
50%	25 ml	acetonitrile
	ad 50 ml	H_2O

Buffer 2		
200 mM	1.54 g	DTT
200 mM	0.79 g	$(NH_4)HCO_3$
50%	25 ml	acetonitrile
	ad 50 ml	H_2O

Buffer 3		
50 mM	0.46 g	iodo-acetamide
200 mM	0.79 g	$(NH_4)HCO_3$
50%	25 ml	acetonitrile
	ad 50 ml	H_2O

Buffer 4		
20 mM	0.08 g	$(NH_4)HCO_3$
50%	25 ml	acetonitrile
	ad 50 ml	H_2O

Day 1	
1.	Separate protein mixture by SDS-PAGE.
2.	Wash gel in 200 ml water for 5 min. Repeat three times.
3.	Stain with 20 ml GelCode Blue Stain (*Pierce*) for l hr.
4.	Destain with water for 1-2 hrs.
5.	Cut out gel band tightly, with the minimum amount of acrylamide. Do not touch gel with fingers as keratin may contaminate.
6.	Gel pieces should be stored in Eppendorf tubes at -20°C.
7.	Incubate in 300 µl buffer 1 at RT for 30 min. Repeat twice.
8.	Incubate in 300 µl buffer 2 at 32°C for 1 hr.
9.	Wash three times with 300 µl of buffer 1.
10.	Alkylate cysteines by incubation with 100 µl of fresh buffer 3 (RT in dark for 20 min).
11.	Wash three times with 500 µl of buffer 4.
12.	Cut bands into 2 mm × 1 mm pieces.
13.	Spin at 13000 rpm for 2 min.
14.	Cover with acetonitrile - must turn white.
15.	Decant acetonitrile and allow gel pieces to dry.
16.	Swell gel pieces in 29 µl of 50 mM $(NH_4)HCO_3$ containing l µl trypsin at 4°C. For larger gel pieces, double the quantities. Initially, keep this solution at 4°C until the gel swells to avoid auto-digestion of trypsin. Seal the tops of the tubes with Parafilm®, then incubate at 32°C for 16-24 hrs.
Day 2	
17.	Sonicate the sample for 10 min prior to spotting onto the MALDI plate.
18.	Store digests long term at -80°C.

Table 3.8: Protocol for protein tryptic in-gel digestion.

3.2.8 Validation of full-length protein mass by ESI-MS

The full-length molecular mass of proteins can be determined by mass spectrometry that uses electrospray ionisation (ESI-MS). ESI produces multiply charged ions from macromolecules without generating significant amounts of fragments. It is thus the most commonly used technique to characterise intact full-length proteins. Additionally, because ESI produces multiply charged ions and the parameter measured in mass spectrometry is the mass-to-charge ratio m/z, it extends the use of MS analysers into the region of kDa/MDa.

This sort of characterisation can be done with samples containing more than one protein species and/or samples in standard protein buffers (containing salts), if combined with a chromatographic separation (LC-MS).

For direct injection of purified proteins in standard buffers, the samples need to be desalted and subjected to an acidic environment to help ionisation. This is typically done by reversed phase chromatography, either by spin column size exclusion chromatography, or by using disposable C_{18} (for proteins up to 100 kDa) or C_4 (for proteins over 100 kDa) resins built into pipette tips (*ZipTips*).

Sample preparation using spin columns

The following protocol is for *Zeba Spin* desalting columns with a 7 kDa cutoff; centrifugation speeds may vary for other spin columns. Note the molecular mass cutoff, as molecules less than 7 kDa cannot be processed with this type of resin.

1.	Mix contents of the spin column, remove the bottom closure and loosen the cap.
2.	Centrifuge at 1500 × *g* for 1 min to remove storage solution.
3.	Carefully apply ~100 µl protein solution of about 1 mg ml^{-1} concentration.
4.	Place spin column in the centrifuge such that the upward slanted resin faces outward.
5.	Centrifuge at 1500 × *g* for 2 min to collect the desalted sample.

Table 3.9: Protocol for preparation of purified protein samples for ESI-MS using *Zeba Spin* desalting columns.

Sample preparation using ZipTips

Activation solution		
70%	35 ml	acetonitrile
0.5%	0.3 ml (85%)	formic acid
	ad 50 ml	H_2O

Equilibration solution		
0.5%	0.3 ml (85%)	formic acid
	ad 50 ml	H_2O

Wash solution		
5%	2.5 ml	acetonitrile
0.5%	0.3 ml (85%)	formic acid
	ad 50 ml	H_2O

Elution solution		
80%	40 ml	acetonitrile
0.5%	0.3 ml (85%)	formic acid
	ad 50 ml	H_2O

1.	Activate ZipTip by aspirating 5× with 10 µl Activation solution.
2.	Equilibrate ZipTip by aspirating 5× with 10 µl Equilibration solution.
3.	Load 10 µl protein sample (ca. 1 mg ml^{-1}) by aspirating 5×.
4.	Wash ZipTip by aspirating 10 µl Equilibration solution for 5×.
5.	Wash ZipTip by aspirating 10 µl Wash solution for 5×.
6.	Elute sample with 5× 10 µl Elution solution.

Table 3.10: Protocol for preparation of purified protein samples for ESI-MS using *ZipTips*.

3.2.9 Cross-linking

Cross-linking reagents contain reactive ends that are able to form a covalent bond with specific functional groups (primary amines, sulfhydryls, etc.) on proteins. Subjecting covalently linked protein molecules to analytical methods such as trypsin digest and mass spectrometry allows determination of protein interaction interfaces.

Different oligomeric species can be separated by reducing denaturing SDS-PAGE, followed by in-gel digestion and mass spectrometric analysis (see page 64). If DSP was used for cross-linking, reducing electrophoresis conditions cannot be employed, since the cross-linker would be cleaved by DTT.

For cross-linking with glutaraldehyde, the stock solution of 25% (stored at -20ºC) is diluted with water, and cross-linking is performed at a final concentration of 0.1-1% glutaraldehyde for up to 30 min at room temperature. The reaction is stopped with TRIS.

Reagent		Reactant	Stock concentration	Final concentration	Buffer	Quenching with
BS	Bissulfosuccinimidyl	Lysine	8 mM		PBS pH 8.0	1 M TRIS pH 7.5
DMS	Dimethyl suberimidate	Primary amines	35.7 mM		PBS pH 8.0	1 M TRIS pH 7.5
DSP	Dithiobis[succinimidylproprionate]	Primary amines	10 mM		PBS pH 8.0	1 M TRIS pH 7.5
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride	Glutamic acid Aspartic acid	52.2 mM		MES pH 6.0	10% β-Mercaptoethanol
Sulfo-SMCC	4-(N-maleimidomethyl) cyclohexane-1-carboxylate, Sulfosuccinimidyl	Primary amines Sulfhydryl groups	10 mM		PBS pH 8.0	1 M TRIS pH 7.5
CMC	N-Cyclohexyl-N′-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate	Glutamic acid Aspartic acid	10 mM		MES pH 6.0	1 M TRIS pH 7.5
GA	Glutaraldehyde	Amines	25%	0.1% - 1%	PBS pH 8.0	1 M TRIS pH 7.5

Table 3.11: Specificity and experimental conditions of some common cross-linking agents.

3.3 CHROMATOGRAPHY

3.3.1 Ion exchange chromatography

Ion exchange chromatography is a technique for separating compounds based on their net charge. The resins contain negatively or positively charged functional groups covalently bound to a solid support, yielding either a cation or anion exchanger, respectively. Charged compounds are adsorbed and retained by an ion exchanger having the opposite charge, whereas compounds that are neutral or have the same charge as the media pass through the void volume and are eluted from the column. The binding of the charged compounds is reversible, and adsorbed compounds are commonly eluted with a salt or, less commonly, with a pH gradient.

Preparation of the sample for ion exchange chromatography requires to embed it in a buffer of low ionic strength, either by dialysis or by dilution. It is thus advisable to prepare dilute solutions of sample to be subjected to ion exchange chromatography, since concentrated protein solutions at low ionic strength have a tendency to precipitate.

A typical workflow consists of

- sample preparation — low ionic strength
- column equilibration — low ionic strength
- sample loading
- wash — low ionic strength
- elution — ionic strength gradient
- column cleaning — high ionic strength
- re-equilibration/storage — low ionic strength.

The charge of a protein molecule depends on its pI, typically estimated computationally rather than measured experimentally. The buffer pH at which the chromatography is run is chosen at least 2 pH units off the pI. For example, a protein with a pI of 5.5 can be subjected to anion exchange chromatography at a pH of 7.5 or 8.0. Likewise, a protein with a pI of 8.2, can be subjected to cation exchange chromatography at a pH of 6.

As a matter of experience, protein cation exchange tends to work less well than anion exchange. The latter method is thus preferred. In some cases, “reverse” chromatography can be applied, i.e. a positively charge protein may be purified by anion exchange chromatography under conditions where it “runs through” the column and is thus found in the flow-through and wash; negatively charged contaminants are removed since they bind to the chromatography resin.

Frequently used ion exchange media include *QA52* (an inexpensive, weak anion exchanger), *DEAE Sepharose* (a weak anion exchanger), *Q-Sepharose* (strong anion exchanger) and *SP-Sepharose* (strong cation exchanger).

3.3.2 Hydrophobic interaction chromatography

In general, hydrophobic amino acids of proteins are usually located away from molecular surfaces, but many proteins have some surface-exposed hydrophobic groups (hydrophobic patches) that allow interaction with hydrophobic ligands on media.

Different salts play a significant role in self-association or the association with hydrophobic surfaces. This has first been formulated by Franz Hofmeister (Hofmeister, 1888) who discovered that particular cat- and anions (lyotropic ions) have varying abilities to precipitate dispersed substances of lyophilic sols (“salting out”). Late members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules ("salting out"); in

effect, they strengthen the hydrophobic interaction. By contrast, early salts in the series increase the solubility of non-polar molecules ("salting in") and decrease the order in water; in effect, they weaken the hydrophobic effect. The well-known Hofmeister series describing decreasing abilities to salt out is as follows:

$$Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Li^{+}, Na^{+}, K^{+}, NH_4^{+}, Rb^{+}, Cs^{+}$$

$$\leftarrow \text{salt-out effect} \quad \text{salt-in effect} \rightarrow$$

$$\text{citrate}^{3-}, \text{tartrate}^{2-}, F^{-}, SO_4^{2-}, HPO_4^{2-}, \text{acetate}^{-}, Cl^{-}, Br^{-}, NO_3^{-}, ClO_3^{-}, ClO_4^{-}, I^{-}, SCN^{-}.$$

These effects are due to the different hydration properties of these ions, which also decreases in each of the above series. For example, in the monovalent cation series, Li^{+} possesses the smallest atomic radius and has the largest hydrated radius; the opposite is true for Cs^{+}.

It has been discovered later on, that these ions also have effects on the stability of protein secondary and tertiary structure, with the anions apparently displaying larger effects than the cations. Generally, it is believed that these effects are not due to changes in the water structure, but instead due to specific interactions between ions and proteins and ions and the water molecules directly in contact with protein (Zhang & Cremer, 2006).

Many of these salts also interact directly with charged groups and/or dipole moments on proteins or may even bind specifically at particular sites. Ions with a strong salt-in effect such as I^{-} and SCN^{-} are strong denaturants (chaotropic agents), because they salt in the peptide group, and thus interact much more strongly with the unfolded form of a protein than with its native form. Consequently, they shift the chemical equilibrium of the unfolding reaction towards unfolded protein (Baldwin, 1996).

These phenomena are the basis for hydrophobic interaction chromatography (HIC), where a chromatographic matrix containing hydrophobic groups, binds proteins delivered in aqueous solutions to different extents, depending on the protein structure and a range of environmental factors including salt concentration, pH, and temperature. Different HIC resins include *phenyl-Sepharose*, *butyl-Sepharose*, etc.

The interactions between protein and HIC resin is enhanced by buffers with high ionic strength, which makes this type of chromatography an excellent purification step after elution in high salt during ion exchange chromatography, or the rather rarely employed ammonium sulphate precipitation.

In the loading stage of this chromatography, sample components bind to the column in high ionic strength buffer, typically 1-2 M $(NH_4)_2SO_4$ or less commonly 3 M NaCl. As such high concentrations of salt may precipitate proteins, the suitability of these conditions for a particular target protein need to be evaluated prior to the chromatography. Elution is usually performed by decreasing the salt concentration, stepwise or by using a gradient. Where as all protein chromatography is performed at low temperatures (typically 4ºC), hydrophobic interaction chromatography with ammonium sulphate should be done at ambient conditions, since ammonium sulphate at high concentrations can easily precipitate at low temperatures in the chromatography tubing, and thus causing severe practical issues.

In some cases, the existence of significantly different conformations of proteins under different environmental conditions can be exploited for purification by HIC. For example, neuronal calcium sensor proteins possess two significantly different conformations, an open one in the presence of calcium, and a closed one in the apo state. The open conformation has more

surface-exposed hydrophobic patches and thus interacts more readily with HIC resins. Therefore, these proteins can be conveniently adsorbed to HIC resins in the presence of calcium, and eluted with EDTA-containing buffer (i.e. without the need for high salt concentrations).

3.3.3 Affinity chromatography

Immobilised metal affinity chromatography (IMAC)

Most natural proteins possess moderate affinities for transition metal ions, especially under conditions of high ionic strength where electrostatic interactions are suppressed. If a recombinant protein is engineered to create metal ion affinity, this can be used for purification purposes. IMAC was introduced as early as 1975 as a group-specific affinity technique for separating proteins (Porath *et al.*, 1975). The working principle is based on the reversible interactions between various amino acid side chains and immobilised metal ions. Depending on the immobilised metal ion, different side chains such as histidine, cysteine, and tryptophan have been implicated in protein binding (Hemdan *et al.*, 1989; Porath *et al.*, 1985; Zhao *et al.*, 1991; Zucali & Sulkowski, 1985). Histidine residues exhibit highly selective coordination of certain transition metal ions and are thus very useful as affinity tags. Under conditions of physiological pH, the imidazole nitrogen of histidine donates electrons to the electron-deficient orbitals of transition metals: metal coordination (see Figure 3.6).

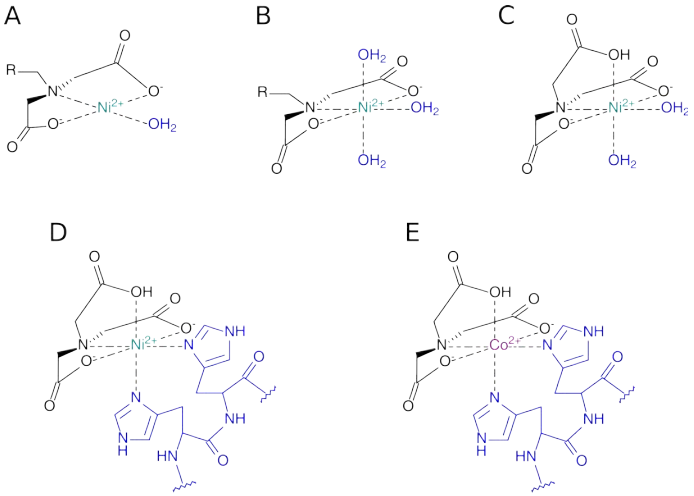


Figure 3.6: Metal ion complexes used in immobilised metal ion affinity chromatography (IMAC). ***A,B*** Iminodiacetic acid (IDA) as a chelating ligand for Ni^{2+}. A tetragonal-planar (***A***) and an octahedral (***B***) coordination geometry is possible with Ni^{2+}-IDA. IDA was the first used metal-chelator for IMAC, but has weak binding properties due to only three coordination sites. Subsequently, better chelators with more binding sites such as NTA have been employed for IMAC. ***C*** Ni^{2+}-NTA coordination. ***D*** The Ni^{2+}-NTA complex bound to a poly-His-fusion protein. ***E*** The Co^{2+}-complex of the *BD Talon*™ resin.

Although three consecutive histidine residues may be sufficient for coordination of metal ions under certain conditions, six contiguous histidine residues reliably bind to transition metal ions

even in the presence of chaotropic agents such as guanidinium chloride or urea (Hochuli *et al.*, 1987). Thus, a hexa-His-tag is commonly fused to either the N- or C-terminal region of a protein. The strength of protein adsorption for the immobilised transition metal ions increases with the order $Co^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+}$ on iminodiaceticacid containing resins (Winzerling *et al.*, 1996). The choice of a certain chelating chromatography resin determines the stability of the metal ion complex under different conditions and the affinity of the protein to be purified (Jiang *et al.*, 1998). The most commonly used resin to date is the tetradentate (η^4) chelator nitrilotetraacetate (NTA; Figure 3.6C,D). Tridentate chelators such as iminodiacetic acid (Figure 3.6A,B) exhibit less stability under drastic conditions (high salt, chaotropic agents). Furthermore, tridentate (η^3) chelators bind the metal ion less tightly which leads to low yields, impure products and metal ion contamination of isolated proteins due to the ions leaching. Therefore, tridentate chelating materials are less preferred. When performing immobilised metal affinity chromatography, the presence of metal chelating agents (like e.g. EDTA) or metal ion – reactive agents (like e.g. thiolates, DTT) should be avoided. Elution of the protein can be achieved by increasing amounts of ligands with higher affinity for the metal (like e.g. imidazole) or slightly acidic conditions where the imidazole nitrogen is protonated (pK_a= 6.0).

(i) Ni^{2+}-NTA. The coordination chemistry of nickel is exceptional, since it can be coordinated in planar, tetrahedral or octahedral geometry. Tetradentate chelators bind nickel ions in octahedral geometry with two water molecules occupying the remaining two sites (Figure 3.6). This is the most commonly used immobilised metal affinity chromatography and requires the presence of a histidine-tag (typically a His_6 sequence) at the very N- or C-terminus of the protein. The resin consists of Ni^{2+}-NTA agarose which can be reused multiple times and regenerated with 100 mM $NiSO_4$ solution to replace metal ions that have been washed off. In the active state, the resin presents Ni^{2+} ions with octahedral coordination sphere where two positions are occupied by water molecules. In the protein-bound state, the two water positions are occupied by the imidazole nitrogens of two consecutive histidine residues of the hexa-His-tag. The protein is typically eluted from the column by increasing amounts of free imidazole which possesses higher affinity for the immobilised Ni^{2+} ions the hexa-His-tag.
Ni^{2+}-NTA material is supplied as agarose (Ni^{2+}-NTA coupled to *Sepharose CL-6B*), superflow (bulk or pre-packed), spin columns (Ni^{2+}-NTA silica) and magnetic agarose beads. For high-throughput, a 96-well format of the Ni^{2+}-NTA magnetic agarose beads is available.

(ii) Co^{2+}-Talon™. Enhanced selectivity of poly-His-tagged protein to immobilised metal ions is observed with Co^{2+} bound to the *BD Talon*™ resin (*BD Biosciences*, *Clontech*). Cobalt-based resins allow only binding of adjacent or specially positioned histidine residues. This means a reduced unspecific binding of proteins to the resin and thus elution with less stringent conditions as compared to the Ni^{2+}-NTA chromatography. As cobalt requires a tetradentate chelator and the *BD Talon*™ resin is similar to nitrilotetraacetate, the common nickel-chelating resin, it is tempting to speculate about usage of the NTA-resin for cobalt-immobilisation , especially since nickel-based IMAC resins often bind unwanted proteins containing exposed histidine residues (Kasher *et al.*, 1993). Our in-house results show that the commercially available NTA-resin from *QIAGEN* (which is a η^4 ligand; see Figure 3.6C) can be used for cobalt-based IMAC as well (Winter & Hofmann, unpublished).

Glutathione-Sepharose

Glutathione (GSH) is a tripetide Glu-Cys-Gly with a γ-peptide bond between the amine group of cysteine and the carboxyl group of the glutamate side chain. GSH is a commonly used RedOx buffer and, in many cells, it acts as a detoxification agent. The RedOx equilibrium

$$2\,GSH \rightleftarrows GS-SG\ +\ 2\,H^{+}\ +\ 2\,e^{-}$$

can supply or consume electrons and thus keep cellular components or proteins in their native oxidation state. GSH is linked to many toxins and chemical insults as an initial step in their detoxification and export. The thiole side chain of reduced GSH is easily deprotonated and then acts as a nucleophile that can react with peroxides and radicals to prevent oxidation and chain reactions.

Glutathione S-transferase (GST) is a 27 kDa enzyme that recognizes glutathione (GSH), and will thus bind readily to matrices with immobilised GSH. If fused to other proteins, GST can be used as an affinity tag for purification. The fact that it constitutes a globular soluble protein can also help to keep "difficult" payload protein in solution. GST fusion proteins are typically expressed using bacterial expression plasmids that encode for N-terminally fused GST from *Schistosoma japonicum* (such as e.g. pGEX vectors).

Amylose resin

The 43 kDa maltose-binding protein (MBP) from *E. coli* is a part of the maltose/maltodextrin system of these bacteria, and responsible for the uptake and efficient catabolism of maltodextrins. Originally developed by NewEngland Biolabs in the 1980s (pMAL expression vector), MBP has been used as a carrier protein fused N-terminally to the target protein in order to achieve high level recombinant protein production from bacterial cell culture. MBP fusion proteins are typically highly soluble, and MPB itself is thought to help folding of the target protein, albeit this effect is passive (Nallamsetty & Waugh, 2006). The affinity matrix for purifying MBP-fusion proteins utilises amylose covalently attached to agarose, thus exploiting the natural affinity of MBP for alpha-(1→4)-maltodextrins in the stationary phase. Due to its significant size, MBP needs to be proteolytically cleaved from the fusion construct, whereby solubility issues with the target protein may re-surface.

Multiple affinity tags

Especially when affinity tags are to be removed by proteolytic digest, it may be beneficial to use multiple tags (also called tandem tags). For example, a protein construct where the target is fused to an N-terminal His_6-GST tandem tag, linked by a TEV cleavage site, can be conveniently processed by His_6-TEV protease. The proteolytic digest will yield the free target protein, His_6-GST and His_6-TEV. This mixture can be separate by Ni-NTA chromatography where the target protein is to appear in the flow-through and all other components remain on the affinity column. The Structural Chemistry Program has obtained several tandem affinity tag vectors from EMBL (http://www.embl.de/pepcore/pepcore_services/cloning/choice_vector/ecoli/embl/index.html) and also has in-house produced His_6-TEV protease.

Dye resins

Dye affinity chromatography is a protein purification procedure based on the high affinity of immobilised dyes for the binding sites on many proteins (Stellwagen, 1990). The affinity for reactive dyes to proteins may be due to substrate/cofactor similarities as well as hydrophobic

and ion exchange properties (Haeckel *et al.*, 1968; Kopperschläger *et al.*, 1968), but the affinity of a specific dye for a particular nucleotide binding site on a protein cannot be predicted. Therefore, in many cases a screening procedure with several types of immobilised reactive dyes is required. The addition of divalent cations may be required for some proteins to bind to the dye resin (Scopes, 1986). Commonly used dye resins include Cibacron Blue 3GA, Reactive Brown 10, Reactive Red 120, Reactive Yellow 2, 3 and 86, Reactive Blue 4, 72, Reactive Green 5, 19.

3.3.4 Size exclusion chromatography

Gel filtration or size exclusion chromatography (SEC) is a separation technique where the retention time of proteins on the separation matrix depends on their size and extension (shape). The smaller the protein, the longer its retention time on a gel filtration matrix. Gel filtration columns have to be packed with care and thus are rather sensitive and costly devices. It is therefore necessary to treat them carefully and avoid the entry of air bubbles when connecting and running them in a chromatography system. Also, buffers used need to be filtered and degassed.

Analytical size exclusion chromatography

The main application of analytical size exclusion chromatography is the estimation of the molecular mass of proteins, and/or testing for existence of protein oligomers under certain buffer conditions. Commonly, the molecular mass of proteins are determined by comparison with protein standards that have been used to calibrate the column. The calibration is valid only for a defined set of chromatography conditions (flow rate, temperature) and the chosen running buffer.

A commonly used buffer consists of 100 mM NaCl, 20 mM HEPES (pH= 7.5). The proteins used to calibrate a column are globular, very stable and of known size. The following standards are used to calibrate the column: cytochrome C (12.4 kDa), myoglobin (17 kDa), carbonic anhydrase (29 kDa), bovine serum albumine (BSA; 68 kDa), and aldolase (160 kDa). Blue dextran (approx. 2 MDa) is used to determine the void volume.

For convenience, some proteins can be run simultaneously, e.g. aldolase can be combined with carbonic anhydrase, or BSA with cytochrome C (see Figure 3.7).

All proteins except aldolase are commercially available as powders and can thus be weighed in. Aldolase (SIGMA), however, is stored as a crystalline suspension and has to be dialysed to dissolve. Therefore, 700 µl of aldolase suspension is centrifuged for 5 minutes at high speed in a bench top centrifuge to remove the supernatant. The aldolase pellet is then dissolved in 700 µl IKMD buffer and dialysed against 300 ml IKMD buffer at room temperature for several hours. Afterwards, the dialysis bag is transferred into a new solution of IKMD buffer (300 ml) and dialysed over night at 4ºC. Finally, the dialysed enzyme is transferred into a micro-centrifuge tube, remaining aggregates are spun down for 5 minutes at high speed in a bench top centrifuge and the soluble enzyme is transferred into a new tube.

IKMD buffer		
40 mM	4 ml (2 M)	KCl
10 mM	2 ml (1 M)	Imidazole
1 mM	200 µl (1 M)	$MgCl_2$
0.5 mM	100 µl (1 M)	DTT
	ad 200 ml	H_2O

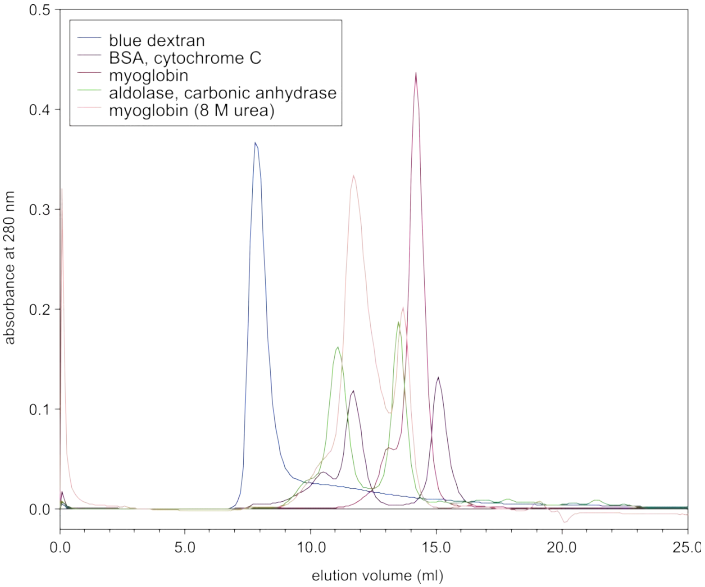


Figure 3.7: Calibration of a Superose-12 size exclusion column with six protein standards in fivedifferent runs. The different runs are coloured blue, magenta, red, green and orange. Note the combination of two proteins with clearly different sizes in the magenta and green runs. The denatured species of a protein elutes earlier than the folded species due to their vastly enlarged extension/shape (see folded and unfolded myoglobin in the red and orange curves, respectively).

The elution profiles are analysed to determine the centres of each peak, e.g. using the programme *PeakFit* or our in-house developed programme *SDAR* (Weeratunga *et al.*, 2012). These values are then translated into K_{av} values using the formula

$$K_{av} = \frac{V_e - V_0}{V_t - V_0},$$

where V_e is the volume at which the protein elutes, V_0 is the void volume, and V_t the total column volume (bed volume). K_{av} values are plotted against the respective molecular masses on a logarithmic scale (K_{av} vs. ln M), and can then be fitted with a linear equation (see Figure 3.8); alternatively, the non-transformed data (K_{av} vs. M) are fitted using an exponential equation.

From the fit, the molecular mass of the protein in question can be estimated. Notably, the protein might not be globular, in which case its mass might appear larger in the size exclusion experiment which separates by the extent of the molecules. Accordingly, size exclusion under denaturing conditions (8 M urea or 6 M guanidine chloride) results in an earlier elution of the protein when compared to its folded state (see myoglobin in Figure 3.7). The elution volume of the denatured species is close to the void volume of the column.

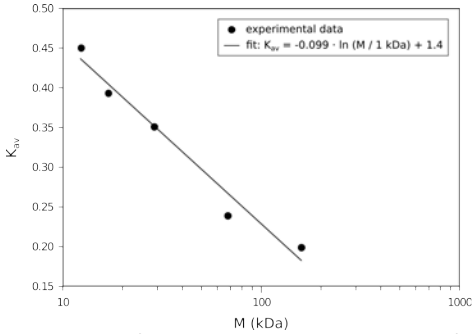


Figure 3.8: Fit of K_{av}-M data obtained during calibration of a size exclusion column (Superose-12; 0.5 ml min^{-1}; 100 mM NaCl, 20 mM HEPES, pH 7.5). The resulting equation enables the calculation of the molecular mass of a protein based on its retention value K_{av}.

3.3.5 Analytical chromatography

Batch experiments

In preliminary experiments, chromatographic conditions and elution behaviour of samples can be checked out in batch experiments. A small amount of the chromatography material (1 ml of the suspension) is transferred into an Eppendorf tube and centrifuged at low speed (3000 rpm, 4°C) for 5 min. Several resuspension steps with the appropriate buffer are used to equilibrate the resin. The sample is applied, and eluted again under appropriate conditions by centrifugation. The different supernatants are usually subjected to SDS-PAGE and analysed.

Batch experiments of this kind can conveniently be used when analysing expression of target proteins from cell lysate, e.g. by immobilised metal ion affinity chromatography with Ni-NTA resin (see Table 3.12).

1.	Prepare 10 ml of equilibration buffer: 100 mM NaCl, 20 mM TRIS (pH 8.0).
2.	Pipette 1 ml of well agitated Ni-NTA slurry into en Eppendorf tube.
Equilibration	
3.	Centrifuge the Ni-NTA resin for 1 min at 3000 rpm and carefully discard the supernatant.
4.	Add 1 ml equilibration buffer to the Ni-NTA resin and mix by inverting the tube a few times.
5.	Repeat steps (3) and (4) three times.
6.	Carefully discard the supernatant.
Loading	
7.	In a separate tube, mix 0.5 ml of bacterial lysate and 0.5 ml of equilibration buffer. Add the diluted sample to the Ni-NTA resin. Mix by inverting the tube a few times.
8.	Centrifuge the Ni-NTA resin for 1 min at 3000 rpm and carefully remove the supernatant.

	Save the supernatant and label “Flow-Through”.
Washing	
9.	Add 1 ml equilibration buffer to the Ni-NTA resin and mix by inverting the tube a few times.
10.	Centrifuge the Ni-NTA resin for 1 min at 3000 rpm and carefully remove the supernatant. Save the supernatant and label “Wash”.
Elution	
11.	Prepare four elution buffers with 50 mM, 100 mM, 200 mM and 500 mM imidazole.
12.	Pipette 25 µl, 50 µl, 100 µl, 250 µl of 2 M imid into an Eppendorf tube each and add 975 µl, 950 µl, 900 µl and 750 µl equilibration buffer, respectively. Mix by inverting the tubes a few times.
13.	Add the 50 mM imid elution buffer to the Ni-NTA resin and mix by inverting the tube a few times.
14.	Centrifuge the Ni-NTA resin for 1 min at 3000 rpm and carefully remove the supernatant. Save the supernatant and label appropriately.
15.	Repeat steps (13) and (14) for the remaining concentrations of imidazole.
Analysis	
16.	Analyse the following fractions on SDS-PAGE: bacterial lysate, “Flow-Through”, “Wash”, 50 mM, 100 mM, 200 mM, 500 mM elution fractions, Ni-NTA resin after last elution step.
17.	The Ni-NTA resin may be washed with equilibration buffer a few times and kept for further use.

Table 3.12: Analytical batch purification protocol to check bacterial cell lysate for presence of His-tagged proteins.

3.3.6 Preparative chromatography

Batch experiments

Usually, the batch approach is not used for preparative purposes, since it requires several centrifugation steps. There are some protocols, however, using a combination of batch and low-pressure modes; for instance, GST fusion proteins can be purified by adding the *Glutathion Sepharose* to the cell lysate. The suspension is then poured into a column and washing and elution steps performed as with normal low-pressure chromatography.

Gravity flow

Column chromatography without pumps (pressure) is usually not used for preparative purposes.

Peristaltic pump

A very common setup in the past was the usage of peristaltic pumps for applying pressure on a chromatography system. The pump consists of a plastic tubing which is clamped between a static unit and a rotating wheel system. The speed of the rotating unit controls the elution rate

and can usually be varied from 0.1 ml min^{-1} to 5 ml min^{-1}. The advantage of these systems is its high versatility and robust performance while being relatively inexpensive.

Gradient maker

If a non-automated low pressure chromatography system such as one with a peristaltic pump is used, elution gradients are typically generated by a gradient maker. A common used gradient maker type consists of two concentric cylindrical volumes that are connected at the bottom by a valve. In the "open" position, the valve will connect the outer to the inner chamber; in the "off" position, this connection is closed. The outlet is resourced from the inner chamber and needs to be manually opened and shut by (un-)clamping the tubing connected to the outlet. The buffer with starting conditions is filled into the inner chamber, the buffer with the endpoint conditions is filled into the outer chamber. The steepness of the gradient is determined by the volumes of buffer provided in the inner and outer chambers. A magnetic stirring bar is placed in the inner chamber to allow for mixing of the solutions during operation. For a linear gradient, the starting and end point buffers should be supplied as equal volumes; the filling height in the outer chamber should be a few millimeters higher than in the inner chamber to prevent back-flow. To start operation, place the prepared gradient maker on a magnetic stirrer, connected the tubing. Then open the outlet and start the chromatography pump and the stirrer. Open the connection valve and watch for successful flow from the outer to the inner chamber by observing the mixing region (refractive index differences will result in visible 'clouds'/Schlieren). If no flow can be observed, an air bubble may be trapped in the connection pipe and need to be removed.
Logarithmic gradients can be formed by sealing the inner chamber with an air-tight lid. A small volume in the inner chamber will produce a steeper gradient than a larger volume, at a given volume in the outer chamber. Because the buffer volume in the inner chamber will remain constant during operation with an air-tight lid, the total volume delivered is equal to the volume provided in the outer chamber (V_o). The volume of buffer in the inner chamber (V_i) required to generated a logarithmic gradient with starting concentration c_i and end point concentration c_e, is calculated as per:

$$V_i = \frac{V_e}{\ln\left(\frac{c_i}{c_e}\right)}$$

FPLC/ÄKTA

A commonly used chromatography system in the 1990s from Pharmacia was the so-called Fast Protein Liquid Chromatography System (FPLC). It works with higher pressure than systems with peristaltic pumps. A valve system and two pumps are regulated via keyboard. The columns used for this system are usually pre-packed and have to have special connectors. In the recent past, the ÄKTA system by the same company has begun to replace FPLC systems. With ÄKTA, moderate (FPLC) and high (HPLC) pressure tasks can be performed. Data acquisition and systems control is entirely computer-based.

3.3.7 Spin-column chromatography

Spin-column chromatography offers many advantages over "fixed-column" chromatography (Amersham Biosciences, 2002):

- Time: Preparation, application of sample and chromatography of the sample is done in about 5 min
- Convenience: No column packing, lengthy equilibration or fractionation of eluate
- Sample: Almost no dilution of sample occurs. In the preparation step, most of the storage buffer is removed. The sample elutes in a volume equivalent to the applied sample volume
- High throughput: In principle, numerous samples can be processed simultaneously
- Capacity: a range of sample volumes and quantities can be processed
- Versatility: Spin-column chromatography allows for a large number of applications

The most common chromatography resins in spin-columns are gel filtration resins, like for example Sephacryl resins. The resins do not exhibit fixed exclusion limits in spin-columns, since these rely on a continuous flow process where the molecules have sufficient time to reach an equilibrium between the time spent in the pores of the resin and the time spent in the eluent. In spin-column chromatography, exclusion properties of a given resin depend on experimental factors, like depth of the resin bed, sample volume, product size, and centrifugal force.

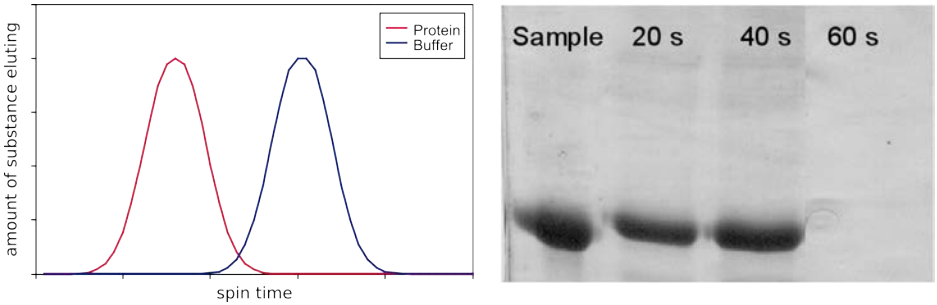


Figure 3.9: ***Left***: Elution profile of sample (red) and eluent (blue) in spin-column. ***Right***: Elution of a 36 kDa protein from an S-200 spin column centrifuged at 3000 rpm. The applied sample elutes between 20 s and 40 s.

Spin-columns with gel filtration resin were originally developed for nucleic acid purification applications. However, a variety of other applications is possible, such as simple desalting of protein samples or purification of liposome preparations (Hofmann & Huber, 2003). Other resins in spin-columns include Ni^{2+}-charged chelating agarose for rapid purification of His-tag fusion proteins and *Glutathione Sepharose* for purification of GST fusion proteins.

Usage

Best results with gel filtration spin-columns will be obtained when the smallest product to be purified is at least 20 times larger than the largest "contamination". Generally, purity has an inverse proportionality to yield. Larger samples will provide higher yield but lower purity, and vice versa. Resins with larger pore size will yield samples with higher purity, but lower yield. The non-specific binding properties are usually insignificant, which allows purification of samples in the nano-gram range. Naturally, there will be a uniform proportional loss of sample. For a given sample volume, the retention of a molecule is proportional to its size. A general

protocol involves the following five steps, again illustrated using the example of Zeba Spin Desalting columns with a 7 kDa cutoff (same as in Table 3.9):

1.	Mix contents of the spin column, remove the bottom closure and loosen the cap.
2.	Centrifuge at 1500 × *g* for 1 min to remove storage solution.
3.	Carefully apply ~100 µl protein solution of about 1 mg ml^{-1} concentration.
4.	Place spin column in the centrifuge such that the upward slanted resin faces outward.
5.	Centrifuge at 1500 × *g* for 2 min to collect the desalted sample.

Table 3.13: Protocol for preparation of purified protein samples for ESI-MS using Zeba Spin Desalting columns.

The centrifugation speeds may vary for other spin columns. Note the molecular mass cutoff, as molecules less than 7 kDa cannot be processed with this type of resin.

3.4 CENTRIFUGATION

The rotation velocity (rotational speed) ν_{rpm} on most centrifuges is displayed in "revolutions per minute" (rpm). A related measure is the so-called relative centrifugal force (RCF), an acceleration experienced by the sample in the centrifuge which is measured in multiples of the earth's gravitational constant *g*. Given the radius of the rotor, the rotational speed can be converted into RCF according to:

$$RCF = 11.18 \cdot \frac{r}{1\,cm} \cdot \left(\frac{\nu_{rpm}}{1000\,min^{-1}} \right)^2 \cdot g$$

where r is the radius of the rotor in cm and ν_{rpm} is the centrifugation speed in rpm. A graphical chart (nomogram) to convert rpm values into RCF is given in Figure 3.10.

Note: Centrifugation tubes have to be balanced extremely carefully due to the huge forces involved in the process!
Also, all tubes must be filled to at least ²/₃ – ¾ to avoid implosion of tubes and subsequent imbalance of the rotor.

As an example, if two tubes with 30 ml water each are centrifuged at 50000 rpm in a rotor with a radius of 8 cm, the force on the sample is 66000 N compared to 0.3 N due to earth gravity (1×*g*) at the bench top. Thus, the force felt by the sample in the centrifuge is about 220000×*g*. This is equivalent to a truck weighing 6000 kg at earth gravity.
The spindle (rotor shaft) cannot suspend the force equivalent to 6000 kg; however, it is only the total force which is experienced by the rotor shaft. Therefore, two samples of exactly the same mass have to sit in the rotor at opposite positions to reduce the resulting force on the spindle to zero.

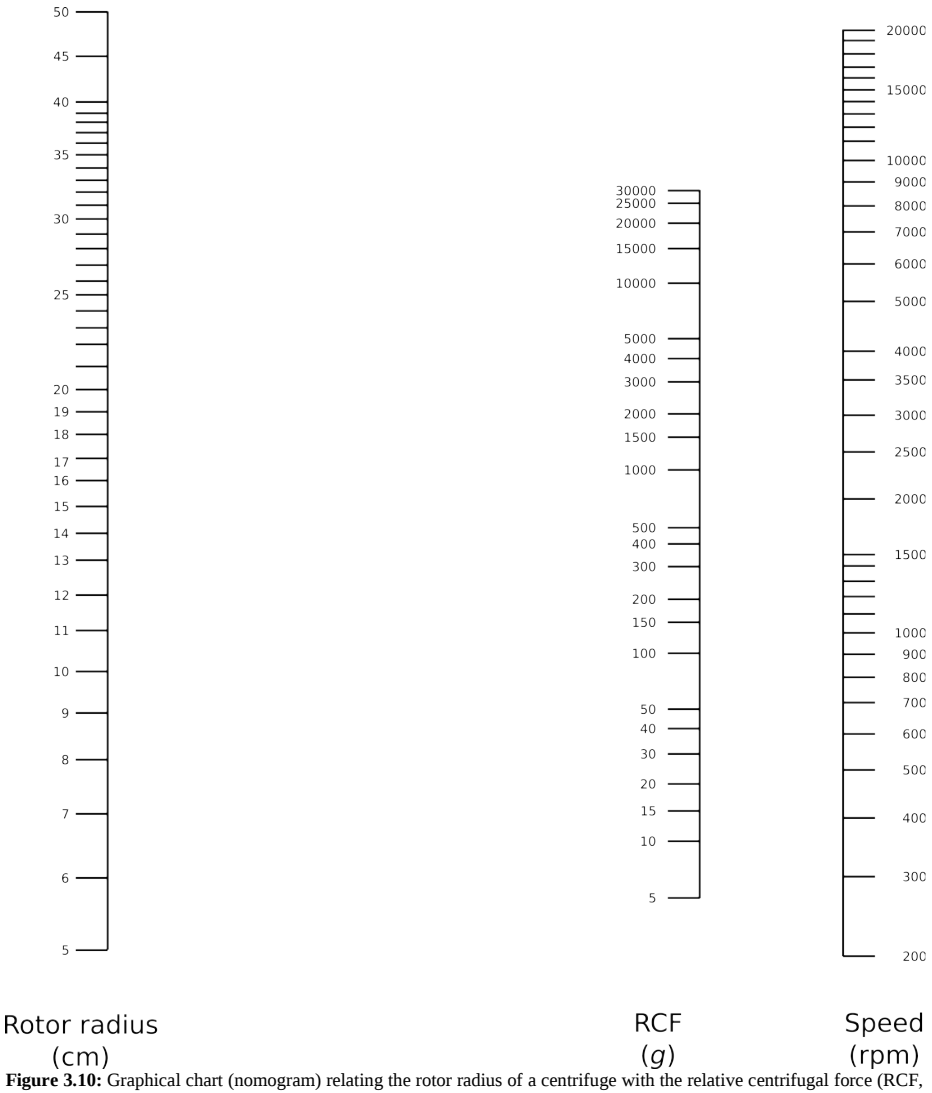


Figure 3.10: Graphical chart (nomogram) relating the rotor radius of a centrifuge with the relative centrifugal force (RCF, measured in multiples of *g*) and the rotational speed (in rpm). *Usage*: Measure the distance from the centre of the rotor to the outermost part in the bucket or tube rack, or read the radius off the rotor/prospectus. Position the left end of a ruler at that radius and the right end at the desired RCF. Then interpolate to obtain the rotational speed in rpm.

4 ASSAYS

4.1 LIPOSOME ASSAYS

4.1.1 Liposomes

Large unilamellar vesicles (LUVs) by the Reeves-Dowben method

Reeves & Dowben provided a protocol for the preparation of large unilamellar vesicles, which is also known as dehydratisation/rehydratisation method (Reeves & Dowben, 1969). For a 1 µmol batch of PS/PE (3:1) liposomes, 60 µl PS and 20 µl PE (10 mg ml^{-1} in $CHCl_3$; Avanti Polar Lipids, Alabaster) are mixed in a 250 ml Erlenmeyer flask, covered with a few milli-liters of $CHCl_3$/MeOH (2:1) and dried under a smooth stream of N_2. The precipitate is resuspended with a few amounts of Et_2O and again dried under N_2 (dehydratisation). For rehydratisation, the precipitate is then exposed for 30 min to an H_2O-saturated N_2 stream. The remaining lipid film is covered with the appropriate buffer and incubated for 2 – 3 hrs at 37°C or over night at room temperature. The liposomes are collected by centrifugehydratisation). For rehydratisation, the precipitate is then exposed for 30 min to an H_2O-saturated N_2 stream. The remaining lipid film is covered with the appropriate buffer and incubated for 2 – 3 hrs at 37°C or over night at room temperature. The liposomes are collected by centrifugation of the final suspension at 12000×*g*, 10 min, 10°C (use 1.5 ml Eppendorf tubes).

For a 10 µmol batch, use more incubation buffer (10 ml – 20 ml) and centrifuge at least two times for 30 min.

4.1.2 Liposome binding assay

The following co-pelleting assays are always accompanied by a suitable protein aggregation assay (see page 90) to ensure that the readout is due to to liposome binding rather than mere protein aggregation.

Centrifugation assay monitored by UV/Vis

Liposomes are prepared as described in section 3.1 using incubation buffer F4. A sample with a total volume of 500 µl contains a 20 µl aliquot of the liposome suspension, the protein of interest, additives (optional) and liposome buffer. After addition of the protein, the sample is incubated for 15 min at room temperature. Then centrifuge 200 µl of the sample for 30 min at 130000×*g* in a Beckman AirFuge (also works at 15000 rpm in standard bench-top centrifuge). Take 100 µl of the supernatant and measure the absorbance at 280 nm in a UV/Vis spectrometer. The baseline correction of the spectrometer should be carried out with a protein-free sample, which was subjected to the same incubation/centrifugation procedure.

Sample	
20 µl	liposome suspension
x µl	additive
100 µg	protein
ad 500 µl	Liposome buffer

Liposome buffer		
180 mM	3.1 g	sucrose
50 mM	0.5 ml (5 M)	NaCl
10 mM	0.5 ml (1 M)	HEPES (pH= 7.5)
	ad 50 ml	H_2O

Centrifugation assay monitored densitometrically

A 3 µmol batch of PC/PS (3:1) liposomes is prepared as described above and divided into three aliquots. Liposome buffer is added to each aliquot to yield a final volume of 1 ml in each tube. From each of these three tubes, five aliquots with 200 µl (corresponding to 0.2 µmol lipids) are prepared, yielding 15 individual samples. Different calcium concentrations can be established by following a pipetting scheme, e.g.:

Sample		0	1	2	3	4
V_L (100 mM $CaCl_2$)	µl	0	0	2.5	5	25
V_L (Liposome buffer)	µl	300	290	287.5	285	265
V_L (protein)	µl	0	10	10	10	10
c($CaCl_2$)	mM (in 500 µl)	**0**	**0**	**0.5**	**1**	**5**

Table 4.1: Pipetting scheme for calcium-dependent liposome co-pelleting assay.

0.4 nmol of protein solution is added to samples 1-4 yielding a final protein concentration of 0.8 µM. A control sample containing the same amount of protein in 50 µl buffer is prepared as well. Samples are incubated at room temperature for 30 min and then centrifuged for 45 min at 13000 rpm (4°C). The supernatant is separated from the pellets. Pellets are dissolved in 50 µl SDS solution (10%) and subjected to SDS-PAGE where one lane is used for the control sample that marks 100% protein (the maximum amount of protein that can be bound to the liposomes). Gels are stained with Coomassie, developed and scanned. Densitometric analysis is carried out with *ImageJ* (Rasband, 2005) and samples are normalised with respect to the 100% control present on each gel (see also Table 4.7).

FRET using dansyl-labelled liposomes

Since the dansyl group can be excited at 340 nm and emits at around 480 nm, it is a suitable tool for fluorescence resonance energy transfer using intrinsic protein fluorescence as donor (Hofmann & Huber, 2003).

For usage of PC/PS (3:1) liposomes with about 1% dansyl label, one prepares PC/PS/dansyl-PS liposomes in a ratio of 3:1:0.05 according to the procedure described above (dansyl-PS: 1 mg ml^{-1} in $CHCl_3$; PC, PS: 10 mg ml^{-1} in $CHCl_3$).

A sample for fluorescence titration is constituted by 150 µl of liposome suspension (corresponding to 0.5 µmol of lipids) and 150 µl of liposome buffer. Firstly, the liposomes are tested for performance by acquiring an emission spectrum with λ_{exc}= 340 nm, λ_{em}= 360 nm – 600 nm. If an emission peak can be detected, the liposomes are labelled.

The assay is carried out by performing a Ca^{2+} titration series for a sample with protein and a titration series for a sample without protein (correction). In all cases, acquire a spectrum without protein and calcium first in order to be able to normalise against the individual series. Emission spectra are recorded with λ_{exc}= 280 nm, λ_{em}= 400 nm – 550 nm and analysis is carried out by integration of spectra from 450 nm to 540 nm.

4.1.3 Liposome stability assay (carboxyfluorescein efflux assay)

The destabilising effect of proteins, peptides, ions or organic compounds with respect to liposome integrity can be assessed using the carboxyfluorescein (CF) efflux assay reported by Wiltschut and colleagues (Wiltschut *et al.*, 1980). The water-soluble fluorescence dye carboxyfluoresceine diacetate (CF) is enclosed in liposomes at self-quenching concentrations. Rupture of the membranes leads to efflux of CF into the bulk buffer where it is then present at diluted concentrations. The efflux process can thus be monitored by an increase in fluorescence intensity. CF liposomes are prepared from standard lipid films (1 µmol batch or more) in 300-500 ml wide-neck flasks, and incubated over night with CF buffer. Liposome buffer is added as needed to ensure coverage of the entire floor of the flask (but keep the overall volume to a minimum). Liposomes are harvested the next morning (12000×*g*, 45 min, 4°C), and purified by washing with liposome buffer. Excess dye not enclosed in the liposomes is removed by spin chromatography using S-200 spin columns. The pellet is resuspended in 60 µl of liposome

buffer and used the same day.

Liposome buffer		
180 mM	3.1 g	sucrose
0.1 mM	10 µl (0.5 M)	EDTA
50 mM	0.5 ml (5 M)	NaCl
10 mM	0.5 ml (1 M)	HEPES (pH= 7.5)
	ad 50 ml	H_2O

CF buffer (for 1 µmol batch lipsomes)		
	1 mg	5(6)-CF
0.3 mM	1 µl (0.5 M)	EDTA
160 mM	83 mg	sucrose
10 mM	15 µl	HEPES (pH= 7.5)
	ad 1.5 ml	H_2O

Fluorescence measurements are done using an excitation wavelength of 480 nm and monitoring the emission intensity at 520 nm. After recording the baseline for four minutes, the compound to test is added at t= 5 min. At t= 20 min, the maximum possible fluorescence intensity is determined by addition of 0.1% (final concentration) Triton X-100 (measurement until t= 25 min).

CF assay: sample		
	20 µl	CF liposome suspension
1 µM		substance to be tested
	ad 500 µl	Liposome buffer

4.2 STABILITY MEASUREMENTS

4.2.1 Equilibrium denaturation in urea gradient gels

Urea gradient gel electrophoresis is a simple method for monitoring the denaturant-induced unfolding of proteins and was first introduced by Creighton (Creighton, 1979). Urea gradient gels are slab gels with a horizontal gradient of urea concentration, usually 0 – 8 M. A single sample of protein is applied across the top of the gel and subjected to electrophoresis in a direction perpendicular to the urea gradient. As the protein migrates in the electric field, molecules at different positions across the gel are exposed to different urea concentrations. At positions where the concentration is high enough to promote unfolding, the mobility of the protein decreases because of the increased hydrodynamic volume of the unfolded form. When the gel is stained, the electrophoretic pattern can be interpreted directly as an unfolding curve.
Because urea tends to retard the electrophoretic mobility of proteins, even in the absence of unfolding processes, urea gradient gels are usually prepared with a compensating acrylamide gradient.

Gel preparation

8 gels are prepared in a gel caster using a pump with a constant flow of 1 ml min^{-1}. First, the running front is marked by pumping in 15 ml of marker solution. For the 0 M side pump in 25 ml of 0 M solution with 18 µl $(NH_4)_2S_2O_8$ and 6 µl tetramethyl-ethylen diamine (TEMED). Then pump in the gradient with a linear gradient former; 27.5 ml of 0 M and 8 M solution, respectively, are put in the two chambers of the gradient former and 18 µl $(NH_4)_2S_2O_8$ and 6 µl TEMED are added to each component. The 8 M side is created by pumping in 25 ml of 8 M solution with 18 µl $(NH_4)_2S_2O_8$ and 6 µl TEMED. Make sure, that the solutions in the gradient former are well stirred and add the radical start mix immediately when starting to pump in the respective component. Let polymerise over night at room temperature. Do not store the gels for longer than 3 – 4 days (fridge).
Prior to running the gel, unfix it by carefully pulling apart the two glass plates. Rotate gel by 90° and cut off the loading ramp. Re-fix the gel and run at a current amplitude of 50 mA.

Marker solution		
30%	4.5 ml	EtOH
	2 drops	Bromphenolblue
	ad 15 ml	H_2O

0 M solution	
7 ml (0.5 M)	TRIS (pH= 8.0)[1]
10.5 ml	AA/Bis-AA[2]
ad 70 ml	H_2O

8 M solution	
7 ml (0.5 M)	TRIS (pH= 8.0)[1]
33.63 g	urea
7. 64 ml	AA/Bis-AA[2]
ad 70 ml	H_2O

[1]TRIS-HAc; [2]Acrylamide/Bis-Acrylamide (30% / 0.8%)

4.3 PROTEIN LIGAND BINDING BY DIFFERENTIAL SCANNING FLUORIMETRY

Differential scanning fluorimentry (DSF) can be used to screen for interactions between a protein of choice and ligands such as small molecule compounds or peptides. Thermal denaturation ("melting") of the protein is monitored by using extrinsic fluorescence by a dye that measures surface hydrophobicity, i.e. shows no emission in hydrophilic environment, but strong fluorescence emission in hydrophobic environment (Hawe *et al.*, 2008). As a protein unfolds, hydrophobic patches are being exposed to which the dye will bind and concomitantly show increased fluorescence emission. The sigmoid thermal denaturation curves are analysed by determining the point of inflection, which is designated the melting temperature (T_m). In general, proteins are more resistant to denaturation in their ligand-bound states, and the melting temperatures between the ligand-bound protein and the apo protein will thus show a positive difference:

$$\Delta T_m = T_m(\text{ligand-bound}) - T_m(\text{apo})$$

Evaluation of the ΔT_m values therefore allows for clues when evaluating binding of ligands to proteins.

The dye used in DSF experiments is SYPRO Orange (λ_{exc}= 472 nm; λ_{em}= 569 nm) which is kept as a 5000× stock in DMSO. A working stock at a concentration of 100× is used for experiment setup.
There is a caveat, though, if experiments are carried out in the presence of metal ions, as some metals quench fluorescence emission of the dye or interfere otherwise with the fluorescence experiment. Metals we have previously found to interfere with this experiment include Co^{2+} and Hg^{2+}.

4.3.1 Experiment optimisation

Protein and dye concentrations need to be optimised for each protein to be tested. Typical protein concentrations range from 0.5 µM to 10 µM. Typical SYPRO Orange concentrations range from 5× to 20× for the final reaction. The following matrix is used to determine the optimal setup:

	1	2	3	4
A	8.0 µM protein, 5× dye	8.0 µM protein, 6.7× dye	8.0 µM protein, 10× dye	8.0 µM protein, 20× dye
B	4.0 µM protein, 5× dye	4.0 µM protein, 6.7× dye	4.0 µM protein, 10× dye	4.0 µM protein, 20× dye
C	2.0 µM protein, 5× dye	2.0 µM protein, 6.7× dye	2.0 µM protein, 10× dye	2.0 µM protein, 20× dye
D	1.0 µM protein, 5× dye	1.0 µM protein, 6.7× dye	1.0 µM protein, 10× dye	1.0 µM protein, 20× dye
E	0.5 µM protein, 5× dye	0.5 µM protein, 6.7× dye	0.5 µM protein, 10× dye	0.5 µM protein, 20× dye

Table 4.2: Matrix for DSF optimisation experiment.

The standard reaction volume in a 96-well plate is 20 µl, and the standard buffer consists of 100 mM NaCl, 20 mM HEPES (pH= 7.5). The following pipetting scheme can be used to obtain the conditions of the optimisation matrix in Table 4.2:

Well	c(protein) µM	Dye concentration	V_L (100 µM protein) µl	V_L (100× SYPRO Orange) µl	V_l (buffer) µl
A1	40	5×	8.00	1.00	11.0
A2	40	6.5×	8.00	1.30	10.7
A3	40	10×	8.00	2.00	10.0
A4	40	20×	8.00	4.00	8.00
B1	20	5×	4.00	1.00	15.0
B2	20	6.5×	4.00	1.30	14.7
B3	20	10×	4.00	2.00	14.0
B4	20	20×	4.00	4.00	12.0
C1	10	5×	2.00	1.00	17.0
C2	10	6.5×	2.00	1.30	16.7
C3	10	10×	2.00	2.00	16.0
C4	10	20×	2.00	4.00	14.0
D1	5.0	5×	1.00	1.00	18.0
D2	5.0	6.5×	1.00	1.30	17.7
D3	5.0	10×	1.00	2.00	17.0
D4	5.0	20×	1.00	4.00	15.0
E1	2.5	5×	0.50	1.00	18.5
E2	2.5	6.5×	0.50	1.30	18.2
E3	2.5	10×	0.50	2.00	17.5
E4	2.5	20×	0.50	4.00	15.5

Table 4.3: Pipetting scheme for DSF optimisation matrix.

Choose the optimal condition such that sigmoid shaped curves are obtained with a single peak/valley when inspected in the `LC480 Tm Calling Analysis`.

4.3.2 Program setup and data analysis

First, start the computer, then the *Roche LC480* real time PCR instrument.

Start up *LC480 Software* → Open `New experiment from template` → Choose `SYPRO Orange protein melt` → insert plate and press `Start run`.

Upon completion of a run, perform a `Tm calling analysis for all samples` in the

`Analysis` tab, right-click into the graphical display window, and select `Copy` from the right-click menu. Save the data in ASCII format. The raw data can be analysed using our in-house developed *DMAN* software (Wang *et al.*, 2012).

Alternatively, the data can be exported to a `*.ixo` file. This file can be opened *LC480 Protein Melting* software for analysis. (Note: Data from the *LC480 Protein Melting* software can also be used for analysis in *DMAN*. Right click on icon in task bar and open `*.ixo` file to generate a `*.csv` file. The `*.csv` file can be imported into *DMAN*).

4.4 HEPARIN BINDING ASSAY

Lectin properties of a protein can often be tested by evaluating binding to conveniently available carbohydrates. In this context, heparin immobilised on an agarose resin serves as a target carbohydrate that can be used in batch chromatography mode.

Heparin binding assay

The following protocol lists the steps to assess calcium-dependent binding of proteins to heparin:

1.	Clean heparin resin (each cleaning for 10 min):
1a.	2 M NaCl , 10 min
1b.	0.1 M NaOH , 10 min
1c.	0.5% Nonidet, 10 min
2.	Wash/equilibrate resin 3 times with buffer (100 mM NaCl, 20 mM Tris, pH 8).
3.	Resuspend resin in 4 ml buffer and distribute into 7 Eppendorf tubes (500 µl).
4.	Centrifuge at 3000 rpm, 3 min; discard supernatant.
5.	Add 390 µl protein buffer with $CaCl_2$, add 10 µl protein (1-5 mg/ml) and incubate for 10 min.
6.	Centrifuge at 3000 rpm, 3 min; keep flow through.
7.	Wash with 400 µl buffer with $CaCl_2$, centrifuge and keep supernatant.
8.	Elute with 400µl buffer with 30 mM EDTA, centrifuge and keep eluate.
9.	Run eluate on SDS gel together with positive control/master (390 µl buffer + 10 µl protein).
10.	Run flow through on SDS gel together with positive control/master (390 µl buffer + 10 µl protein).
11.	Optional: run irreversibly bound protein (resin). Pipet out 20 µl resin or resuspend the resin in 400 µl protein buffer. Run on SDS-PAGE together with positive control/master.

Table 4.4: Protocol for heparin binding assay.

Sample	**0**	**1**	**2**	**3**	**4**	**5**	**6**
c($CaCl_2$) in mM	**0**	**0.25**	**0.5**	**1**	**2**	**5**	**10**
V_L (0.1 M $CaCl_2$) in µl	0	2.25	4.5	9	18	45	90
V_L (0.5 M EDTA) in µl	1.8	-	-	-	-	-	-
V_L (buffer) in µl	898	898	896	891	882	855	810

Table 4.5: Pipetting scheme for calcium-dependent heparin binding assay.

Protein aggregation

To ensure that the protein does not aggregate under the different calcium concentrations used, an aggregation assay needs to be performed as control. This follows the same protocol as the one assay used as control for calcium-dependent liposome binding (see section 4.1.2).

1.	Incubate 10 µl (1-5 mg/ml) protein into 390 µl protein buffer with different amount of calcium concentration as in the above table for 10 min.
2.	Centrifuge at 15,000 rpm for 20 min at 4ºC, discard supernatant and dry the pellet.
3.	Dissolve pellet into 20 µl of running dye together with master sample (390 µl buffer + 10 µl protein, load 10 µl protein and 10 µl running dye).
4.	Check on 12% SDS-PAGE.

Table 4.6: Protocol for calcium-dependent protein aggregation assay.

Densitometric analysis

Protein amounts found in various samples are assessed by integrating the staining marks (Coomassie staining) obtained on gels after electrophoresis. The program of choice is *ImageJ* (Rasband, 2005).

1.	Analyse gels using *ImageJ*, compare each band at different additive concentration to the master sample (= 100%).
2.	The total amount of protein (= 100%) added to the sample should be recovered when considering the following fractions: • eluate (reversibly bound) • flow through (unbound) • aggregated protein (to be determined in separate aggregation assay) • non-eluted protein (irreversibly bound)

Table 4.7: Generic protocol for densitometric analysis of stained gels.

4.5 ENZYME KINETICS BY STOPPED FLOW EXPERIMENTS

4.5.1 General SFM procedure

All solutions to be prepared freshly before the experiment. The general procedure for the SFM MOS-LED is as follows:

Prior to experiment	
1.	Turn on SFM, MOS-LED control and computer.
2.	For both entry ports, wash with 30 ml H_2O using the wash syringe with multi-valve in `waste` position.
3.	Fill the two SFM syringes with H_2O and wash with multi-valve in `run` position.
4.	In the Spec-Lab software, set the number of shots to be acquired (e.g. 10), total recording time (10 s) and the recording period (e.g. 10 ms; data pitch). Set acquisition mode to `Wait for trigger`. For 300 µl shots, adjust stopper block at 5 cm distance from the stop syringe holder.
5.	Fill the two SFM syringes with appropriate buffers and connect to entry ports. Syringe 1, filled with dissolved substrate, is connected to the top port. Syringe 2 is connected to the bottom port and holds the sample buffer without protein.
6.	Set multi-walve to `waste` position. Inject mixture into the cell (ca 1/2 turn).
7.	To set the sensitivity of the detector, click the `Do ref.` button.
8.	Take reference curves (buffers without protein): Click `Run`, select folder and file name, click `OK`. Set multi-walve to `run` position. Start the reaction by turning the wheel counter-clockwise. Note that the absorbance of the final solution in the mixing cell should be less than 0.7 (may require checking in a UV/Vis spectrometer).
Experiment	
9.	Disconnect the SFM syringes. For the bottom port, wash with 30 ml H_2O using the wash syringe with multi-valve in `waste` position.
10.	Fill the two SFM syringes with appropriate buffers and connect to entry ports. Syringe 1, filled with dissolved substrate, is connected to the top port. Syringe 2 is connected to the bottom port and holds the protein.
11.	Inject mixture into the cell (ca 1/2 turn).
12.	Take reaction curves: Click `Run`, select folder and file name, click `OK`. Set multi-walve to `run` position. Start the reaction by turning the wheel counter-clockwise. Set multi-walve to `purge` position and empty stop syringe. Repeat as set under (4), i.e. 10 times.
13.	Watch out for leakage at entry ports, the multi-valve and the stop syringe. If leakage occurs, traces may not be interpretable. Aggregated sample can lead to blockage and leakage of the system.
14.	Continue experiments from (9).

Record keeping
15. Save files with sensible names (short, preferably numbers only, lower case, underscores instead of spaces) in a directory coding today's date (e.g. `20130118`) belonging to your directory tree.
16. Establish and update a `README` file with all experimental parameters and annotations of each produced trace.
After experiment
17. For both entry ports, wash with 30 ml H_2O using the wash syringe with multi-valve in *waste* position.
18. For both entry ports, wash with 30 ml MeOH using the wash syringe with multi-valve in *waste* position.
19. Clean both SFM syringes with ample H_2O and MeOH.
20. Fill the two SFM syringes with H_2O and wash with multi-valve in *run* position.
21. Fill the two SFM syringes with MeOH and wash with multi-valve in *waste* position.
22. Fill the two SFM syringes with MeOH and wash with multi-valve in *run* position.
Analysis
23. Traces can be fit with the *Bio-Logic Spec-Lab* software (online or offline).
24. All fit parameters are to be recorded in spreadsheets.
Archiving
25. All relevant traces belonging to one set of experiments, together with the spreadsheet analysis are to be archived on digital media.
26. Relevant dossiers are to be updated and the archives need to be cross-referenced.

Table 4.8: General procedure for conducting experiments with the SFM MOS-LED.

5 MONOLAYER ADSORPTION EXPERIMENTS

5.1 LANGMUIR SURFACE FILM BALANCE NIMA MODEL 301A

5.1.1 Cleaning the instrument

The lower metal hook is taken off the pressure sensor and put down at the border of the trough; do not touch or contaminate the hook during any procedure. Use only fluff-free *KimWipes*; wear surfactant-free nitrile or poly-ethylene gloves.

All tools (scissors, tweezers, aspirator tip, etc) are to be wiped off with a chloroform-soaked tissue. The trough, the inlay, the magnetic stirrer and the barrier are cleaned in three sequential steps: firstly, with a chloroform-soaked tissue; secondly, with a tissue soaked with ethanol; and thirdly, with a special fluff-free tissue (*Kimberley Clarke Professional Precision Wipe*) to soak up all remaining liquids. The cleaning steps need to be done before and after each experiment.

5.1.2 Getting started

An isotherm of buffer (e.g. 100 mM NaCl, 20 mM TRIS, pH 7.5) is recorded as baseline to ensure that no contaminations remained on the surface. If the pressure rises above 1mN/m, fluff from the surface is sucked away when the barrier is still closed using the provided nozzle and the isotherm is run again. The pressure is measured using a Wilhelmy plate electrobalance and a filter paper protruding into the monolayer which is renewed for each experiment. The barrier which is placed on top of the trough is used to limit the area of the monolayer, and can be controlled using the provided software. All experiments are conducted at ambient temperature. Hamilton micro-syringes used for applying lipids and protein are also cleaned with chloroform or buffer before and after usage.

The original Teflon inlay provided with the instrument provides a working volume of 100 ml. We also have a custom-made inlay for a buffer volume of 60 ml. However, the monolayer formation in this smaller trough has not been thoroughly tested yet.

Wilhelmy plates are made of Whatman filter paper, cut to ca. 2.5 cm × 1 cm. To prepare for usage, fold the filter paper strip length-wise and cut a small triangle near one side. This will become the whole for hanging the plate onto the metal hook. Using tweezers, attach the hook with the Wilhelmy plate on the pressure sensor. The filter paper only needs to dip into the aqueous phase and must not touch the bottom of the trough.

5.1.3 Preparing and conducting an experiment

A solution of lipids DOPC:DOPS (1:1) is prepared dissolving 2.23 mg DOPC and 2.30 mg DOPS in 10 ml chloroform. The average molecular weight (798 g mol^{-1}) and concentration (0.45 mg ml^{-1}) of the lipids are used as input into the software (`Monolayer` menu) which then calculates area-per-molecule values for data analysis.

Cleaning remaining contaminats

Choose the item `Isotherm` under `Barrier`, and `Close` the barrier. Rising surface pressure indicates that there are remaining particles on the buffer surface. Gently remove the very top of the compressed side using the aspirator and then open the barrier again. Repeat this step until the increase in surface pressure upon closing the barrier is less than 0.5 mN m^{-1}.

Spread lipids

The lipid solution is applied to the buffer surface and incubated for 10 min to allow the

chloroform to evaporate and the monolayer to form. For the trough size of 100 ml (original inlay), ca. 18-22 µl of lipid solution are required. For the trough size of 60 ml, 9-12 µl of lipid solution are required. Acquire an isotherm for the lipids, and determine the transition from the expanded to the compressed phase (e.g. 15 mN m^{-1}). This surface pressure will become the target pressure in subsequent experiments. Within a close series of experiments and a set lipid mixture, this step does not have to be repeated every time. If an isotherm has been acquired, a new monolayer will have to be prepared for the following experiments, since the current one may have collapsed upon compression.

Area control measurements

To examine binding events at the monolayer, either the area or the pressure of the monolayer has to be maintained constant, whilst a change in the other parameter is monitored. Typically, we carry out measurements under area control and interpret the rising surface pressure as binding event.

After preparing a new lipid monolayer as described above, `clean` the screen and `auto-zero` the pressure reading (`p&A vs t` under `Graphs`). Choose target pressure and enter the value determined from the isotherm. Then apply `Pressure control` under `Barrier`, and wait until the system has equilibrated. Read off the area value and stop the instrument. Then select `Target area` under `Barrier` and enter the area value; then start the measurement by choosing `Area control` under `Barrier`.

After starting the area control measurement, let the system equilibrate for ca. 30 min and record the surface pressure change. Ideally, there should be no significant change in surface pressure; however, further evaporation of solvent may result in slow decrease of surface pressure. Individual judgement will be required to assess whether a given monolayer preparation is suitable for further experimentation. Stop the recording and save the data for the record.

If the monolayer is deemed suitable, re-start the recording, wait another 5 minutes and then inject the protein (or other sample) underneath the subphase, avoiding introduction of air bubbles as well as any sort of mechanical disturbance of the monolayer. Between 10 and 50 nmol of protein is required for 100 ml trough volume. If binding occurs, an increase in surface pressure is expected. Recordings should initially be done for 60 min. In the past, we have had stable monolayers and recordings for 12 hours.

Prepare a `README` text file that will be saved together with the surface pressure recordings. For all experiments, the names and contents of files with the recordings should be noted, as well as buffer composition, protein concentration and volume, etc. The molar amount of sample injected (n_{sample}), the molar concentration (c_{sample}) and the monolayer area A should be recorded. The ratio A/n_{sample} (in nmol cm^{-2}) should be calculated and noted. In the past, we have worked at ratios between 0.3 and 3 nmol cm^{-2}.

Data processing

The *SDAR* program (Weeratunga *et al.*, 2012) from the *PCSB* suite (Hofmann & Wlodawer, 2002) is ideally suited for data processing. The time of injection can easily be identified visually by a spike in the surface pressure recording and conveniently be set to $t_0 = 0$. Minor drifts can also be corrected at this stage.

5.1.4 Charge-neutral PIP-containing membranes

When incorporating lipid-conjugated head groups into mono- or bilayers, the net charge on the membrane surface may be an important parameter to consider, especially when unspecific binding due to electrostatic interactions needs to be distinguished from specific binding to particular charged head groups.

DMPS/DMPC (3:1), 1% PI(4,5)P_2

	ρ^* (mg ml^{-1})	M (g mol^{-1})	charge
DMPS	0.5	702	-1
DMPC	0.5	677	0
PI(4,5)P_2	0.5	1071	-3

Table 5.1: Properties of components for DMPS/DMPC/PI(4,5)P_2 membranes.

Amounts for DMPS:DMPC (3:1)

$$V_L(DMPS) = 75\,\mu l$$

$$V_L(DMPC) = 25\,\mu l$$

$$n_0(DMPS) = \frac{\rho^* \cdot V_L}{M} = \frac{0.5 \cdot 0.075 \cdot mg \cdot ml \cdot mol}{702 \cdot g \cdot ml} = 53.4 \cdot 10^{-6}\, mmol$$

$$n_0(DMPC) = \frac{\rho^* \cdot V_L}{M} = \frac{0.5 \cdot 0.025 \cdot mg \cdot ml \cdot mol}{677 \cdot g \cdot ml} = 18.4 \cdot 10^{-6}\, mmol$$

$$n_0(lipids) = n_0(DMPS) + n_0(DMPC) = (53.4 + 18.4) \cdot 10^{-6}\, mmol = 71.8 \cdot 10^{-6}\, mmol$$

Amounts for DMPS:DMPC (3:1), 1% PI(4,5)P_2

$$n(PIP_2) = n_0(lipids) \cdot 0.01 = 71.8 \cdot 10^{-6} \cdot 0.01 = 0.718 \cdot 10^{-6}\, mmol$$

$$n(DMPS) = n_0(DMPS) - 2 \cdot n(PIP_2)$$
$$n(DMPS) = 53.4 \cdot 10^{-6}\, mmol - 2 \cdot 0.718 \cdot 10^{-6}\, mmol = 51.96 \cdot 10^{-6}\, mmol$$

$$n(DMPC) = n_0(DMPC) + n(PIP_2)$$
$$n(DMPC) = 18.4 \cdot 10^{-6}\, mmol + 0.718 \cdot 10^{-6}\, mmol = 19.12 \cdot 10^{-6}\, mmol$$

Volumes for DMPS:DMPC (3:1), 1% PI(4,5)P_2

$$V_L(DMPS) = \frac{M \cdot n}{\rho^*} = \frac{702 \cdot 51.96 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 73.0\,\mu l\,(72\%)$$

$$V_L(DMPC) = \frac{M \cdot n}{\rho^*} = \frac{677 \cdot 19.12 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 25.9 \mu l\,(27\%)$$

$$V_L(PIP_2) = \frac{M \cdot n}{\rho^*} = \frac{1071 \cdot 0.718 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 1.54 \mu l\,(1\%)$$

DMPS/DMPC (3:1), 1% PI(3,4,5)P_3

	ρ^* (mg ml^{-1})	M (g mol^{-1})	charge
DMPS	0.5	702	-1
DMPC	0.5	677	0
PI(3,4,5)P_3	0.5	1171	-4

Table 5.2: Properties of components for DMPS/DMPC/PI(3,4,5)P_3 membranes.

Amounts for DMPS:DMPC (3:1)

$$V_L(DMPS) = 75 \mu l$$

$$V_L(DMPC) = 25 \mu l$$

$$n_0(DMPS) = \frac{\rho^* \cdot V_L}{M} = \frac{0.5 \cdot 0.075 \cdot mg \cdot ml \cdot mol}{702 \cdot g \cdot ml} = 53.4 \cdot 10^{-6}\, mmol$$

$$n_0(DMPC) = \frac{\rho^* \cdot V_L}{M} = \frac{0.5 \cdot 0.025 \cdot mg \cdot ml \cdot mol}{677 \cdot g \cdot ml} = 18.4 \cdot 10^{-6}\, mmol$$

$$n_0(lipids) = n_0(DMPS) + n_0(DMPC) = (53.4 + 18.4) \cdot 10^{-6}\, mmol = 71.8 \cdot 10^{-6}\, mmol$$

Amounts for DMPS:DMPC (3:1), 1% PI(3,4,5)P_3

$$n(PIP_3) = n_0(lipids) \cdot 0.01 = 71.8 \cdot 10^{-6} \cdot 0.01 = 0.718 \cdot 10^{-6} mmol$$

$$n(DMPS) = n_0(DMPS) - 3 \cdot n(PIP_3)$$
$$n(DMPS) = 53.4 \cdot 10^{-6}\, mmol - 3 \cdot 0.718 \cdot 10^{-6}\, mmol = 51.25 \cdot 10^{-6}\, mmol$$

$$n(DMPC) = n_0(DMPC) + 2 \cdot n(PIP_3)$$
$$n(DMPC) = 18.4 \cdot 10^{-6}\, mmol + 2 \cdot 0.718 \cdot 10^{-6}\, mmol = 19.84 \cdot 10^{-6} mmol$$

Volumes for DMPS:DMPC (3:1), 1% PI(3,4,5)P_3

$$V_L(DMPS) = \frac{M \cdot n}{\rho^*} = \frac{702 \cdot 51.25 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 72.0 \mu l\,(71\%)$$

$$V_L(DMPC) = \frac{M \cdot n}{\rho^*} = \frac{677 \cdot 19.84 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 26.9 \mu l\,(28\%)$$

$$V_L(PIP_3) = \frac{M \cdot n}{\rho^*} = \frac{1171 \cdot 0.718 \cdot 10^{-6} \cdot g \cdot mmol \cdot ml}{0.5 \cdot mol \cdot mg} = 1.68 \mu l\,(1\%)$$

6 PROTEIN CRYSTALLOGRAPHY

6.1 CRYSTALLISATION

The fact the atoms and molecules form an ordered three-dimensional array in the crystalline state is visibly at the macroscopic or microscopic level where crystals typically appear as regular shapes. An object with regular shapes is distinguished from an amorphous object by the existence of regular faces. In general, molecules pack in crystals with a distinct orientation (determined by the crystal symmetry), but there is also an occasional disorder. Crystals to be used for X-ray diffraction purposes are typically grown in a process that slowly navigates through the phase diagram. In most cases, this starts with nucleation whereby a submicroscopic aggregate of molecules in appropriate orientation has built up. Further substance then can adsorb to the surface and give rise to an ordered growth which ultimately results in formation of a crystal. In some cases crystallisation may need to be promoted by adding nuclei. This may involve addition of seed micro-crystals of the same material or “mechanical” ways of inducing nucleation (see below).

Crystallisation describes the transition of molecules from their state in solution to the solid state, and represents a reversible equilibrium process that is governed by the minimisation of the free energy of the system (Weber, 1991). Using various different methods (see section 6.1.2), the concentration of molecules in a given system can be subjected to an ongoing change, which may result in a situation where there are insufficient solvent molecules available to provide full solvation of the dissolved molecules. This state is called supersaturated and no longer at equilibrium.

The process of crystal growth can be described in a two-dimensional phase diagram as shown in Figure 6.1 (Ducruix & Giege, 1992).

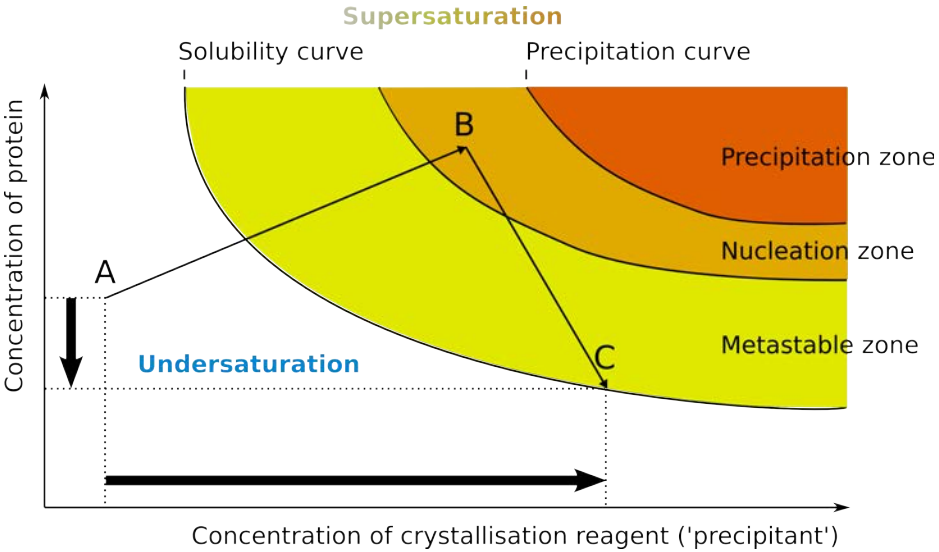


Figure 6.1: Crystallisation process by vapour diffusion in the phase diagram. Starting at point **A**, characterised by the initial concentrations of protein and precipitant, the protein concentration will increase from an unsaturated to a supersaturated state (**B**). As crystals are being formed, the concentration of protein in solution will decrease as more and more molecules will join the solid state. Crystals will grow until the protein concentration reaches the solubility curve (**C**).

In the phase diagram in Figure 6.1, the solubility curve separates the space into two areas, the undersaturated and supersaturated zones. Each point on the solubility curve corresponds to a concentration at which the solution is in equilibrium with the precipitating agent. Crystallisation cannot occur in the undersaturated zone (white). In the supersaturated zone, the protein concentration is higher than that at equilibrium for a given concentration of precipitant. This region can be subdivided into three zones:

- The metastable zone, where crystal growth occurs but no nuclei are formed.
- The nucleation zone, where protein molecules aggregate in a crystalline form. Near the precipitation zone, crystallisation may occur to yield large numbers of micro-crystals, which should not be confused with precipitate.
- The precipitation zone, where the excess of protein molecules immediately separates from the solution to form amorphous aggregates (precipitate).

The growth of well-ordered crystals should begin with the formation of a preferably single nucleus in the nucleation zone just beyond the metastable zone (point B). As the crystals grow, the solution will return to the metastable zone and no more nuclei can be formed. The remaining nuclei will grow until equilibrium is reached (point C).

Thermodynamically, the system will be driven to a new equilibrium state to minimise the free energy. This involves molecules losing rotational and translational degrees of freedom as they engage in non-covalent interactions with neighbouring molecules ($\Delta G_{cryst} = -(12\text{-}25)$ kJ mol^{-1}) (Drenth & Haas, 1992). This aggregation results in the appearance of a solid state of the molecules which can appear either as amorphous precipitate or crystal nuclei. Since amorphous precipitates are kinetically favoured, they tend to dominate the solid phase and thus inhibit the formation of ordered crystals.

6.1.1 Methods of nucleation

In an effort to promote crystallisation, nuclei can be added to a crystallisation experiment. If microcrystals of the same substance have already been obtained in preliminary experiments, these can be harvested and added as seeds. This process is called seeding and is used in small molecule as well as protein crystallisation experiments (Ducruix & Giege, 1992). Physically, growth of crystals on a seeding nucleus follows the process of epitaxy (see section 6.1.2). It is thus also possible to use microcrystals of unrelated compounds if they possess a similar unit cell shape (McPherson & Shlichta, 1988).

Other common methods in small molecule crystallisation include

- scratching the interior of the glass test tube used for the crystallisation experiment with a glass rod; or
- adding boiling chips which cause cavitation, i.e. the existence of liquid-free zones (voids), at the sites of their surface pores.

In protein crystallisation experiments, a commonly used nucleation technique is streak seeding. After composing the crystallisation droplet, a cat whisker is streaked through the drop a few times (1-3×).

6.1.2 General crystallisation methods

Controlled temperature change

Accurately controlled slow temperature change can be used to crystallise substances:

- for small molecules, slow cooling of a saturated solution may lead to crystal growth, if

the solubility of the substance decreases with temperature

- most proteins possess higher solubility at lower temperatures, therefore slow warming may be effective for crystallisation of proteins.

The formation of microcrystals typically indicates that the applied temperature change was too rapid.

Slow solvent evaporation

During evaporation of a solution, its volume decreases, and the solubility of a substance is reduced since its concentration increases during the process. At a particular point, the supersaturated solution required for crystal growth will be achieved.
This method is widely used for crystallisation of small molecules. It may be based on single solvent systems where the slow removal of the solvent drives the process, or mixtures of solvents where the different solubility of the substance in the solvents used brings about the desired effect. Technically, the setup consists of a glass beaker with the substance-solvent mixture which is left to stand undisturbed. To control the rate of evaporation (typically adjusted to yield crystals in a matter of days, not minutes or months), the beaker can be covered with a watch glass, or Parafilm® or aluminium foil with some holes punched into.

Vapour diffusion

The technique of vapour diffusion is the most commonly used crystallisation method for proteins. Conceptually, it is based on two spatially separated solutions in a sealed compartment. One solution (the crystallisation droplet) contains a mixture of the sample to be crystallised and the solution with precipitant. The other solution (well solution) has a ~50× higher volume and comprises only the solution with precipitant. Within the sealed compartment, a solvent equilibrium through the vapour phase is established between the two spatially separated components. Solvent will diffuse from the component with lower concentration of precipitant (the crystallisation drop) to the compartment with the higher concentration (the well solution). During this process, the volume of the crystallisation drop will decrease and the concentrations of all components increase. Eventually, supersaturation will be achieved, and crystals may grow. Since the amounts of purified macromolecules are typically very small (5-20 mg ml^{-1}), microcrystallisation techniques are employed to minimise the consumption of purified material. The hanging and sitting drop methods (Figure 6.2) are adaptations of the vapour diffusion technique suitable for micro-crystallisation, and the most commonly employed methods in protein crystallisation. In the hanging drop method, a mixture of protein and precipitant (2-6 µl) is suspended from a siliconised glass cover slip (or plastic cover slip) over a well reservoir containing the precipitant solution (300 µl). As suggest by the name, the sitting drop method has the mixture of protein and precipitant (1-3 µl) sitting in the a compartment that also holds the precipitant solution in the well (100 µl). In both cases, multi-well plates are used to set up 24 (hanging drop) or 96 experiments (sitting drop) in parallel. Most modern 96-well crystallisation plates offer the possibility of 2 or 3 sitting drops per well.
The smaller drop sizes in sitting drop experiments, makes this technique the method of choice for screening large numbers of different crystallisation conditions. However, the crystallisation results obtained with either hanging or sitting drop experiments can differ (which makes the choice of method yet another parameter in the screening space). In many cases, larger crystals are obtained in hanging drop setups, and in some cases protein crystals are only obtained in either one of the two methods.

Setting up hanging and sitting drop experiments can be automated by employing pipetting robots available from different manufacturers. In cases of very limited availability of purified protein samples, the use of robotics allows nano-crystallisation with drop sizes of less than 1 µl, and the use of 384-well plates.

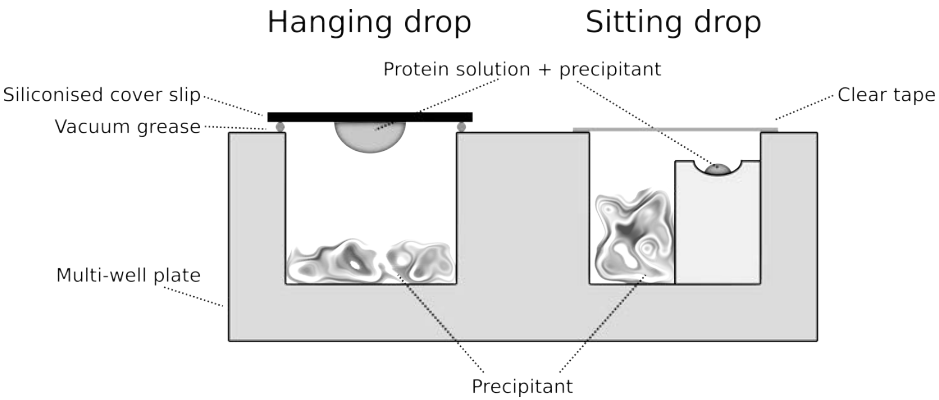


Figure 6.2: Schematics of hanging and sitting drop experiments.

Dialysis techniques

Supersaturation of a protein solution to be crystallised can gradually be achieved by dialysing against a solution containing high concentration of precipitant. The dialysis devices used for this procedure may be small dialysis bags or cells, dialysis buttons (see Figure 6.3).

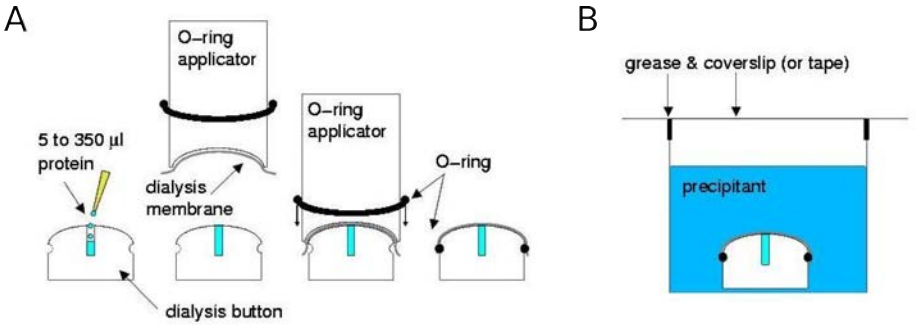


Figure 6.3: ***A*** Preperation of a dialysis button for protein crystallisation. ***B*** 24-well plates used for hanging drop experiments can be used for the dialysis experiment. Pictures taken from http://www-structmed.cimr.cam.ac.uk/Course/Crystals/Theory/methods.html

Batch crystallisation

Here, the precipitant and protein are mixed directly under oil.

Epitaxy

Epitaxy (oriented overgrowth) describes the process of oriented crystal growth of a substance on an entirely different substance acting as a support. The repeat distances in the two crystalline substances should be similar. These process are of eminent importance for the manufacture of microelectronic components.

Strictly speaking, crystallisation nuclei are aggregates of several molecules of the substance to be crystallised, oriented in a particular fashion. However, nucleation can also be occur at contaminating particles of unspecified composition or dust. This type of crystal growth happens by epitaxial growth.

It has also been suggested to that minerals insoluble in the supersaturated solutions of macromolecules can be used as heterogeneous nuclei for crystallisation of these macromolecules (McPherson & Shlichta, 1988; McPherson & Shlichta, 1987). Experience with proteins shows that a close match of unit cell dimensions between the support and the protein crystal is not necessary. Therefore, crystals of inorganic substances can be used to initiate epitaxial protein crystal growth (Berman *et al.*, 1988; Mann, 1988; Weiner, 1986).

Notably, the use of micro-crystals of the protein to be crystallised as seeds presents a special case of epitaxial growth: the substance of the support crystal and the substance to be grown on it are identical.

6.1.3 Strategies for protein crystallisation

In crystallisation experiments of macromolecules, the concentrations of the precipitant (mostly consisting of one, but sometimes more components), the macromolecule under study, the pH as well as the temperature all constitute an individual parameter ('dimension') that needs to be adjusted. Obviously, considering different conditions for each of those parameters will quickly lead to an extensive (hundreds to thousands) of individual crystallisation experiments to be prepared. Inevitably, a selection will need to be made as it is not possible to rigorously screen all possible combinations.

Secondly, the change from one batch of purified protein to another presents a variation in the sample as different batches frequently are not identical. Lastly, the time it takes for nucleation and crystal growth to occur can vary significantly (overnight, several days to several months) for different samples and crystallisation conditions, thus making the time required for crystallisation experiments of uncharacterised samples unpredictable.

Conceptually, different screening strategies can be applied:

Screening at the isoelectric point

Since a macromolecule has a net charge of zero at its isoelectric point, the pI constitutes a point of low solubility in polar solvent. It is thus assumed that crystallisation may occur at this point. Screening approaches at the pI can be done either by

- dialysis of the sample against low concentrations (≤ 20 mM) of pH buffers adjusted to or around the calculated pI, or
- conventional screening with precipitants but using pH conditions at or around the calculated pI.

Grid screening

Grid screens are systematic screens where each multi-well plate constitutes a two-dimensional

matrix, with two different parameters varied along the axes. This method is ideally suited for automated setups using dispensing robots (Cox & Weber, 1988). In many cases, this can be an iterative process, whereby one starts out with a coarse grid covering a broad range of the parameter space and subsequently refines conditions to end up with a narrow sampling around selected parameters.
Often, the first screen in this method tests a concentration range of a selected precipitant against pH. Analysis as to the presence or absence of precipitation, amorphous or single crystalline objects then determines the next screen at finer grid points. The procedure is repeated until the results converge to a best condition. Further improvements by testing additives or changing the temperature may be required.

Incomplete factorials
A statistical approach has been suggested by way of screening with incomplete factorials (Carter & Carter, 1979). From a set of about 20 crystallisation parameters, randomly chosen combinations are assigned to individual experiments. The observations made in each experiments are then graded, and the effects of each of the 20 parameters are statistically evaluated (Carter *et al.*, 1988). This should yield an objective assessment of the effects of the different parameters (precipitant, pH, cations, temperature).
The original reports used crystallisation experiments in dialysis buttons so that the precipitant could be removed and protein samples be recycled. The two series reported by Carter and coworkers found high quality crystals with just 35 trials.

Sparse matrix screening
Initial screening with crystallisation conditions that were previously known to work with protein crystallisation has first been reported by Kim & Jancarik who then suggested a sparse matrix screen (Jancarik & Kim, 1991; McPherson, 1992) that was then commercially sold as *CrystalScreen* by *Hampton Research*. Conceptually, sparse matrix screens sample through the space of chemical parameters in random fashion. If the sampling space is broad enough, initial crystallisation leads or crystals may be obtained that can be improved upon.

6.1.4 Crystal improvement
Following on from initial small crystals or crystallisation leads, conditions need to be further tuned to improve crystal growth, i.e. enhance their size and quality. An illustrative crystal improvement is shown in Figure 6.4.

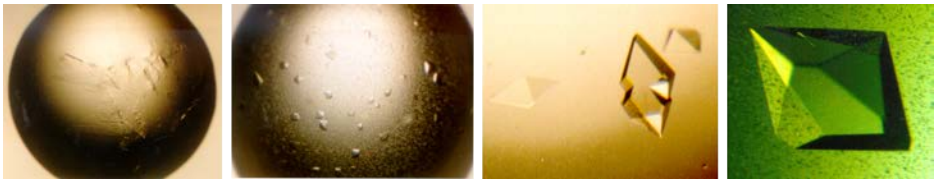


Figure 6.4: Crystal improvement. From left to right: ***1***: 30% MPEG, pH 6.0, 4°C; ***2***: 30% MPEG, pH 6.0, 17°C; 3: 30% MPEG, pH 5.6, 17°C; ***4***: 31% MPEG, pH 5.6, 17°C. MPEG: monomethoxypolyethylene glycol.

6.1.5 Generic crystallisation approach in the Structural Chemistry Program

In most cases, we apply a generic approach to crystallise an unknown protein in our laboratory. We maintain a collection of pre-made crystallisation buffers (factorials) which are organised into different screens (see Table 6.1). In order to evaluate a useful concentration of the protein sample to be used for crystallisation, a sparse matrix screen (e.g. CSI/CSII or N-Screen) is carried out in 96-well sitting drop plates. Initially, the concentration of the protein stock solution is chosen between 8 and 15 mg ml^{-1}. After a few days, the crystallisation drops are assessed and an estimate of the relative number of drops with significant precipitation is made. If the fraction of drops with precipitation is around 50% (± 10-15%), the chosen concentration appears about right; otherwise subsequent crystallisation experiments should be carried out with lower or higher protein concentration.

A typical workflow for crystallisation of an un-characterised protein is shown in section 1.2 .

Collection	Type	Based on	Components
CS I-Screen	Sparse matrix	*Hampton Research* Crystal Screen I (Jancarik & Kim, 1991)	
CS II-Screen	Sparse matrix	*Hampton Research* Crystal Screen II (Jancarik & Kim, 1991)	
E-Screen	Systematic	Proprietary	$(NH_4)_2SO_4$
F-Screen	Systematic	Proprietary	PEG
G-Screen	Systematic	Proprietary	MPD/Ca^{2+}
H-Screen	Systematic	Proprietary	PEG/Ca^{2+}
I-Screen	Additive	Proprietary	
MF-Screen	Sparse matrix	*Hampton Research* MembFac Screen	
MP-Screen	Sparse matrix	MacPherson Screen (Cudney *et al.*, 1994)	
N-Screen	Sparse matrix	Huber Lab, Martinsried	
NX-Screen	Sparse matrix	Natrix Screen (Scott *et al.*, 1995)	
OA-Screen	Orthogonal array	(Kingston *et al.*, 1994)	
P-Screen	Systematic	*Jena Biosceince* PACT Screen (Newman *et al.*, 2005)	PEG/anions/cations
SM-Screen	Orthogonal array		
SilverBullets	Additive	(McPherson & Cudney, 2006)	
SilverBullets Bio	Additive	(McPherson & Cudney, 2006)	

Table 6.1: Summary of the in-house crystallisation screens with ready-made factorials.

6.1.6 Observations in protein crystallisation experiments

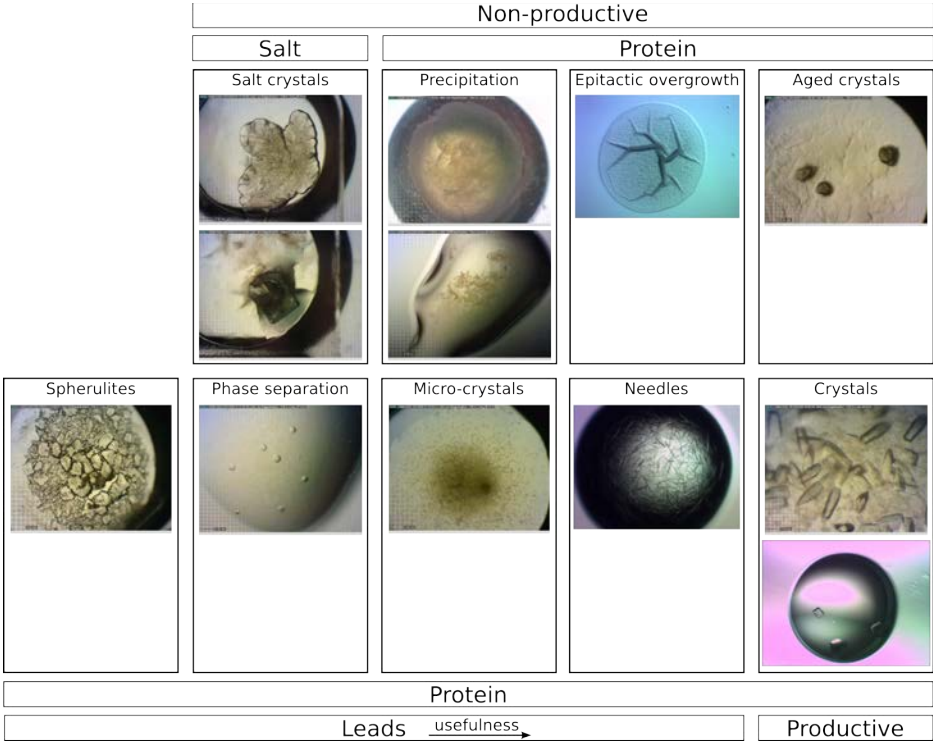


Figure 6.5: Typical observations in protein crystallisation experiments.

7 REFERENCES

Ahn T., Yim S., Choi H. & Yun C. (2001) Polyacrylamide gel electrophoresis without a stacking gel: use of amino acids as electrolytes. *Anal. Biochem* **291**, 300-303.

Aitken A. (2010) Mass spectrometric techniques. In *Principles and techniques of biochemistry and molecular biology*, Wilson K. & Walker J. (Eds.), Cambridge University Press, 352-398.

Baldwin R.L. (1996) How Hofmeister ion interactions affect protein stability. *Biophys. J.* **71**, 2056-2063.

Barth S., Huhn M., Matthey B., Klimka A., Galinski E. & Engert A. (2000) Compatible solute-supported periplasmic expression of functional recombinant proteins under stress conditions. *Appl. Env. Microbiol.* **66**, 1572-1579.

Berman A., Addadi L. & Weiner S. (1988) Interaction of sea-urchin skeleton macromolecules with growing calcite crystals - a study of intracrystalline proteins. *Nature* **331**, 546-548.

Birnboim H. & Doly J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acid Res.* **7**, 1513-1523.

Blackwell J.R. & Horgan R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Lett.* **295**, 10-12.

Böhlen P., Stein S., Dairman W. & Udenfriend S. (1973) Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* **155**, 213-220.

Bradford M.M. (1976) A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Carter C.W., Baldwin E.T. & Frick L. (1988) Statistical design of experiemnts for protein crystal growth and the use of precrystallissation assay. *J. Cryst. Growth* **90**, 60-73.

Carter C.W.J. & Carter C.W. (1979) Protein crystallisation using incomplete factorial experiments. *J. Biol. Chem.* **254**, 12219-12223.

Chien A., Edgar D.B. & Trela J.M. (1976) Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol.* **127**, 1550–1557.

Cox M. & Weber P.C. (1988) An investigation of protein crystallisation parameters using successive automated grid searches (SAGS). *J. Cryst. Growth* **28**, 318-324.

Creighton T. (1979) Electrophoretic analysis of the unfolding of proteins by urea. *J. Mol. Biol.* **129**, 235-264.

Cudney R., Patel S., Weisgraber K., Newhouse Y. & McPherson A. (1994) Screening and optimization strategies for macromolecular crystal growth. *Acta Crystallogr. D* **50**, 414-423.

Drenth J. & Haas C. (1992) Protein crystals and their stability. *J Cryst Growth* **122**, 107-109.

Ducruix A. & Giege R. (1992) *Crystallisation of nucleic acids and proteins. A practical approach*, Oxford University Press, New York.

Ellman G. (1958) A colorimetric method for determining low concentrations of mercaptans. *Arch. Biochem. Biophys.* **74**, 443-450.

Ellman G. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77.

Everette J.D., Bryant Q.M., Green A.M., Abbey Y.A., Wangila G.W. & Walker R.B. (2010) Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *J. Agric. Food Chem.* **58**, 8139-8144.

Fahrney D. & Gold A. (1963) Specific trypsin and chymotrypsin inhibitors. *J. Am. Chem. Soc.* **85**, 997.

Gold A.M. (1967) Sulfonylation with sulfonyl halides. *Meth. Enzymol.* **11**, 706-711.

Gold A.M. & Fahrney D. (1964) Sulfonyl Fluorides as Inhibitors of Esterases. II. Formation and Reactions of Phenylmethanesulfonyl alpha-Chymotrypsin. *Biochemistry* **3**, 783-791.

Gornall A., Bardawill C. & David M. (1949) Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751-766.

Haeckel R., Hess B., Lauterborn W. & Wüster K.H. (1968) Purification and allosteric properties of yeast pyruvate kinase. *Hoppe Seylers Z. Physiol. Chem.* **349**, 699-714.

Harju S., Fedosyuk H. & Peterson K. (2004) Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnol.* **4**, 8.

Harris D. (1987) Spectrophotometric assays. In *Spectrophotometry & spectrofluorimetry: a practical approach*, Harris D. & Bashford C. (Eds.), IRL Press, Oxford.

Hartree E.E. (1972) Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**, 422-427.

Hawe A., Sutter M. & Jiskoot W. (2008) Extrinsic Fluorescent Dyes as Tools for Protein Characterization. *Pharm. Res.* **25**, 1487–1499.

Hemdan E.S., Zhao Y.J., Sulkowski E. & Porath J. (1989) Surface topography of histidine residues: a facile probe by immobilized metal ion affinity chromatography. *Proc. Natl. Acad. Sci.* **86**, 1811-1815.

Hochuli E., Dobeli H. & Schacher A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **411**, 177-184.

Hofmann A. & Huber R. (2003) Liposomes in assessment of annexin-membrane interactions. *Methods Enzymol.* **372**, 186-216.

Hofmann A. & Wlodawer A. (2002) PCSB - a program collection for structural biology and biophysical chemistry. *Bioinformatics* **18**, 209-210.

Hofmeister F. (1888) Zur Lehre von der Wirkung der Salze. *Arch. Exp. Pathol. Pharmacol.* **24**, 247-260.

Huang D., Gore P.R. & Shusta E.V. (2008) Increasing yeast secretion of heterologous proteins by regulating expression rates and post-secretory loss. *Biotechnol. Bioengineer.* **101**, 1264-1275.

Innis M.A., Gelfand D.H., Sninsky J.J. & White T.J. (1990) *PCR protocols: A guide to methods and applications*, Academic Press, San Diego, California.

Jancarik J. & Kim S.H. (1991) Sparse-matrix dampling - a screening method for crystallization of proteins. *J. Appl. Cryst.* **24**, 409-411.

Jiang W., Graham B., Spiccia L. & Hearn M. (1998) Protein selectivity with immobilized metal ion-tacn sorbents: chromatographic studies with human serum proteins and several other globular proteins. *Anal. Biochem.* **255**, 47-58.

Johnson B. & Hecht M.H. (1994) Recombinant proteins can be isolated from E. coli cells by repeated cycles of freezing and thawing. *Biotechnology* **12**, 1357-1360.

Jones D. & Howard B. (1990) A rapid method for site-specific mutagenesis and directional subcloning by using polymerase chain reaction to generate recombinant circles. *BioTechniques* **8**, 178-183.

Jones D. & Winistorfer S. (1991) Site-specific mutagenesis and DNA recombination by using PCR to generate recombinant circles in vitro or by recombination of linear PCR products in vivo. *Methods* **2**, 2-10.

Jones D., Sakamoto K., Vorce R. & Howard B. (1990) DNA mutagenesis and recombination. *Nature* **344**, 793-794.

Kasher M., Wakulchik M., Cook J. & Smith M. (1993) One-step purification of recombinant human papillomavirus type 16 E7 oncoprotein and its binding to the retinoblastoma gene product. *Biotechniques* **14**, 630-641.

Kingston R.L., Baker H.M. & Baker E.N. (1994) Search designs for protein crystallization based on orthogonal arrays. *Acta Crystallogr. D* **50**, 429-440.

Kopperschläger G., Freyer R., Diezel W. & Hofmann E. (1968) Some kinetic and molecular properties of yeast phosphofructokinase. *FEBS Lett.* **1**, 137-141.

Lämmli U. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Larson E., Howlett B. & Jagendorf A. (1986) Artificial reductant enhancement of the Lowry method for protein determination. *Anal. Biochem.* **155**, 243-248.

Lawyer F., Stoffel S., Saiki R., Chang S., Landre P., Abramson R. & Gelfand D. (1993) High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Meth. Appl.* **2**, 275–287.

Lee E., Gallagher R., Campbell A. & Prausnitz M. (2004) Prediction of Ultrasound-Mediated Disruption of Cell Membranes Using Machine Learning Techniques and Statistical Analysis of Acoustic Spectra. *IEEE Transactions on Biomedical Engineering* **51**.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Macauley-Patrick S., Fazenda M.L., McNeil B. & Harvey L.M. (2005) Heterologous protein production using the Pichia pastoris expression system. *Yeast* **22**, 249-270.

Mann S. (1988) Molecular recognition in biomineralisation. *Nature* **332**, 119-124.

McPherson A. (1992) Two approaches to the rapid screening of crystallisation conditions. *J. Cryst. Growth* **122**, 161-167.

McPherson A. & Cudney B. (2006) Searching for silver bullets: An alternative strategy for crystallizing macromolecules. *J. Struct. Biol.* **156**, 387-406.

McPherson A. & Shlichta P. (1988) Heterogeneous and epitaxial nucleation of prtoein crystals on mineral surfaces. *Science* **239**, 385-387.

McPherson A. & Shlichta P.J. (1987) Facilitation of the growth of protein crystals by heterogeneous/epitaxial nucleation. *J. Cryst. Growth* **85**, 206-214.

Miller M., Miller D. & Brayman A. (1996) A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. *Ultrasound Med. Biol.* **22**, 1131-1154.

Nallamsetty S. & Waugh D.S. (2006) Solubility-enhancing proteins MBP and NusA play a passive role in the folding of their fusion partners. *Protein Expr. Purif.* **45**, 175-182.

Newman J., Egan D., Walter T.S., Meged R., Berry I., Ben Jelloul M., Sussman J.L., Stuart D.I. & Perrakis A. (2005) Towards rationalization of crystallization screening for small- to medium-sized academic laboratories: the PACT/JCSG+ strategy. *Acta Crystallogr. D* **61**, 1426-1431.

Porath J., Carlsson J., Olsson I. & Belfrage G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **258**, 598-599.

Porath J., Maisano F. & Belew M. (1985) Thiophilic adsorption--a new method for protein fractionation. *FEBS Lett.* **185**, 306-310.

Rasband W. (2005) ImageJ, version 1.30. *National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/.*

Reeves J. & Dowben R. (1969) Formation and properties of thin-walled phospholipid vesicles. *J. Cell. Physiol.* **73**, 49-60.

Rice P., Longden I. & Bleasby A. (2000) EMBOSS: The European Molecular Biology Open Software Suite. *Trends Gen.* **16**, 276-277.

Rychlik W., Spencer W.J. & Rhoads R.E. (1990) Optimization of the annealing temperature for DNA amplification in vitro. *Nucl. Acids Res.* **18**, 6409-6412.

Sambrook J., Fritsch E. & Maniatis T. (1989) *Molecular cloning: A laboratory manual*, 2nd edition,CSH Laboratory Press, Cold Spring Harbor.

Scopes R.K. (1986) Strategies for enzyme isolation using dye-ligand and related adsorbents. *J. Chromatogr.* **376**, 131-140.

Scott W.G., Finch J.T., Grenfell R., Fogg J., Smith T., Gait M.J. & Klug A. (1995) Rapid Crystallization of Chemically Synthesized Hammerhead RNAs using a Double Screening Procedure. *J. Mol. Biol.* **250**, 327-332.

Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J. & Klenk D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Stein S., Böhlen P., Stone J., Dairman W. & Udenfriend S. (1973) Amino acid analysis with fluorescamine at the picomole level. *Arch. Biochem. Biophys.* **155**, 202-212.

Stellwagen E. (1990) Chromatography on immobilized reactive dyes. *Methods Enzymol.* **182**, 343-357.

Stoscheck C.M. (1990) Quantitation of protein. *Methods Enzymol.* **182**, 50-69.

Stratagene (2004) *QuikChange® Multi Site-Directed Mutagenesis Kit*, Instruction Manual, Catalog #200514, Revision

#113002e.

Studier F. (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **41**, 207-234.

Thein S.L. & Wallace R.B. (1986) *Human genetic diseases: A practical approach*, IRL Press, Herndon, Virginia.

Turini P., Kurooka S., Steer M., Corbascio A.N. & Singer T.P. (1969) The action of phenylmethylsulfonyl fluoride on human acetylcholinesterase, chymotrypsin and trypsin. *J. Pharmacol. Exp. Ther.* **167**, 98-104.

Wang C.K., Weeratunga S.K., Pacheco C.M. & Hofmann A. (2012) DMAN: a Java tool for analysis of multi-well differential scanning fluorimetry experiments. *Bioinformatics* **28**, 439-440.

Warburg O. & Christian W. (1941) Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* **310**, 384-421.

Weber P.C. (1991) Physical principles of protein crystallisation. *Adv. Prot. Chem.* **41**, 1-36.

Weeratunga S.K., Hu N.J., Simon A. & Hofmann A. (2012) SDAR: a practical tool for graphical analysis of two-dimensional data. *BMC Bioinformatics* **13**, 201.

Weiner S. (1986) Organisation of extracellularly mineralised tissues: a comparative study of biological crystal growth. *CRC Crit. Rev. Biochem.* **20**, 365-408.

Weiss B., Jacquemin-Sablon A., Live T., Fareed G. & Richardson C. (1968) Enzymatic breakage and joining of deoxyribonucleic acid. IV. Further purification and properties of polynucleotide ligase from Escherichia coli infected with bacteriophage T4. *J. Biol. Chem.* **243**, 4543-4555.

Wiechelman K.J., Braun R.D. & Fitzpatrick J.D. (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal. Biochem.* **175**, 231-237.

Wiltschut J., Düzgünes N., Fraley R. & Paphadjopoulos D. (1980) Studies on the mechanism of membrane fusion: kinetics of calcium ion induced fusion of phopsphatidylserine vesicles followed by a new assay for mixing of aqueous vesicle contents. *Biochemistry* **19**, 6011-6021.

Winzerling J., Pham D., Kunz S., Samaraweera P., Law J. & Porath J. (1996) Purification of recombinant insect transferrin from large volumes of cell culture medium using high capacity Ni(2+)-dipicolylamine gel. *Protein Expr. Purif.* **7**, 137-142.

Wu D.Y., Ugozzoli L., Pal B.K., Qian J. & Wallace R.B. (1991) The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. *DNA Cell Biol.* **10**, 233-238.

Yasumitsua T., Liua G., Levequec J., Aonumab S., Duclaux L., Kimura T. & Komatsu N. (2013) A rosette cooling cell: More effective container for solubilization of single-walled carbon nanotubes under probe-type ultrasonic irradiation. *Ultrasonics Sonochemistry* **20**, 37-39.

Yim S., Ahn T., Kim J. & Yun C. (2002) Polyacrylamide gel electrophoresis without a stacking gel: application for separation of peptides. *Anal. Biochem.* **305**, 277-279.

Zhang Y. & Cremer P.S. (2006) Interactions between macromolecules and ions: The Hofmeister series. *Curr. Opin. Chem. Biol.* **10**, 658-663.

Zhao Y., Sulkowski E. & Porath J. (1991) Surface topography of histidine residues in lysozymes. *Eur. J. Biochem.* **202**, 1115-1119.

Zucali J. & Sulkowski E. (1985) Purification of human urinary erythropoietin on controlled-pore glass and silicic acid. *Exp. Hematol.* **13**, 833-837.

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