

# BIOCHEMISTRY

For 1st-year Informatics and Biology students



1 February – 25 April 2016

Instructors

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# PROGRAMMA

## Cursus Biochemie voor 1e jaarsstudenten I&B - 1 febr t/m 25 april 2016

In deze periode worden de colleges en practica gegeven op:

maandag 1<sup>e</sup> t/m 8<sup>e</sup> uur, dinsdag 1<sup>e</sup> en 2<sup>e</sup> uur, woensdag 1<sup>e</sup> t/m 4<sup>e</sup> uur (incidenteel 7<sup>e</sup> en 8<sup>e</sup> uur)  
donderdag 1<sup>e</sup> t/m 4<sup>e</sup> uur (incidenteel 7<sup>e</sup> en 8<sup>e</sup> uur). KIJK GOED OP JE ROOSTER!

De colleges worden gegeven in zaaltje 4T.01 (4e verdieping, lift uit: rechts)

Toetsen:

toets 1 donderdag 4 febr	11.15-13.00 uur
toets 2 donderdag 24 maart	09.00-12.00 uur
tentamen maandag 25 april	10.00-13.00 uur

### Week 1 (weeknummer 5)

#### Ma 1-2

- 09.00: *Inleiding cursus Biochemie*
- 10.00: *College buffers, pH, molariteiten*
- *Werkcollege: buffers, pH*

#### Di 2-2

- 09.00: Excel en Powerpoint

#### Wo 3-2

- 09.00: *College extractie methoden*
- 11.15: Installatie spectrofotometers
- 15.45: zelfstudie

#### Do 4-2

- 10.30-13.00: **Toets 1** sommen over pH en molariteiten

### Week 2 (weeknummer 6)

#### Ma 8-2

- 09.00-13.00: Absorptie spectrum en ijklijn NADH/BSA

#### Di 9-2

- 09.00: *Inleiding glycolyse en LDH*

#### Wo 10-2

- 09.00-13.00: Oplossingen maken (w.o. fosfaatbuffer)
- uitloop ijklijnen

#### Do 11-2

- Zelfstudie

### Week 3 (weeknummer 7)

#### Ma 15-2

- 09.00-17.00: Extractie LDH
- Ammonium sulfaat fractionering/opslag samples
- Dialyse

#### Di 16-2

- 09.00: *College Chromatografie*

#### Wo 17-2

- 09.00-13.00: Dialyse uithalen
- Eiwit concentratie bepaling

#### Do 18-2

- 09.00-13.00: Activiteitsbepaling fracties (afmaken 7<sup>e</sup> en 8<sup>e</sup> uur)

### Week 4 (weeknummer 8)

#### Ma 22-2

- 09.00-17.00: Affiniteitschromatografie LDH
- Samples doormeten, eiwitconcentratie/activiteit

#### Di 23-2

- Zelfstudie

#### Wo 24-2

- 09.00-13.00: Bepaling Km en Vmax van LDH

#### Do 25-2

- 09.00-13.00: Vervolg Bepaling Km en Vmax van LDH

### Week 5 (weeknummer 11)

#### Ma 14-3

- 9.00-17.00: Bepaling pH optimum LDH

#### Di 15-3

- 09.00: *College verslag schrijven*

#### Wo 16-3

- 09.00-13.00: Effect van inhibitors

#### Do 17-3

- 09.00-13.00: Terugreactie lactaat -> pyruvaat

### Week 6 (weeknummer 12)

#### Ma 21-3

- 09.00-17.00: Gelelectroforese van LDH isoenzymen

#### Di 22-3

- 09.00: *College Agrobacterium*

#### Wo 23-3

- 09.00-13.00: Labjournaal afmaken en deze onderling checken en beoordelen

#### Do 24-3

- 9.00-12.00: **Toets 2**

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## **Week 7** (weeknummer 13)

### **Di 29-3**

- 09.00-10.30: Aannten Agrobacterium stammen voor inoculatie op *N.glauc*a (prikproef)

### **Wo 30-3**

- 09.00-13.00: Prikproef
- Aannten Agrobacterium stammen voor inductie vir-promotors ( $\beta$ -galactosidase assay)

### **Do 31-3**

- 09.00-13.00:  $\beta$ -galactosidase assay

## **Week 8** (weeknummer 14)

### **Ma 4-4**

- 0.9.00-17.00: Overproductie Vir F (aannten stammen, induceren mbv temperatuurshift, samples opwerken dmv sonificatie)
- 10.00: *College Antilichamen en eiwitproductie in E. coli*

### **Di 5-4**

- 09.00-10.30: Evt niet opgewerkte samples sonificeren

### **Wo 6-4**

- 09.00-13.00: Eiwitgels runnen

### **Do 7-4**

- 09.00-13.00: Eiwitgels ontkleuren

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## **Week 9** (weeknummer 15)

### **Ma 11-4**

- 09.00-17.00: Eiwitgel runnen voor Western blotting
- Blocken van de blots

### **Wo 13-4**

- 09.00-13.00: Westernblots wassen en incuberen met AB
- Scoren prikproef

### **Do 14-4**

- 09.00-13.00: Blots wassen en kleuren

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## **Week 10** (weeknummer 16)

### **Ma 18-4**

- 09.00-17.00: Labjournaal afmaken
- Verslag schrijven over alles

### **Wo 20-4**

- 09.00-13.00: Presentatie voorbereiden over onderdelen/groep

### **Do 21-4**

- 09.00-13.00: **Presentaties**

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## **Week 11** (weeknummer 17)

### **Ma 25-4**

- 10.00-13.00: **Tentamen**
- 13.00: **Verslag inleveren**

# INTRODUCTION

The 1<sup>st</sup>-year course Biochemistry is organized by the Institute of Biology Leiden. Detailed knowledge of biochemistry is essential for performing molecular biological research and necessary for a good understanding of biological processes crucial for bioinformatics. For the course we have selected a topic that is closely related to ongoing research in the institute.

This course focuses on protein biochemistry. Proteins are macromolecules with complex properties and with complex interactions in cells. Protein biochemistry is a challenging matter. Although standard protocols exist, each individual protein or protein complex requires special techniques and specific variations in the protocols to enable their study. This optimization is done by trial and error. The aim of the course is to provide some insight in the intricacies of protein biochemistry.

In Chapters 2 and 3, step-by-step experimental protocols have been indicated by bulletts. Theoretical background pertaining to the experiments in these chapters, as well as the theory about protein biochemistry in Chapter 1, should be **learned by self-study**. In addition, several lectures will give theoretical background for the experiments.

The experiments are meant to demonstrate and practice a number of standard techniques in protein biochemistry, such as protein purification, protein quantification, protein separation by gel electrophoresis and protein detection by Western blotting.

These techniques will be used in different contexts and with different research goals:

1. Extraction and partial purification of an active enzyme from animal tissue, measurement of the enzymatic activity, study of iso-enzymes.
2. Isolation of recombinant proteins from *E. coli*, and in vitro protein-protein interaction studies.

## Rules for attendance

It is compulsory to attend all parts of the course. In case of absence due to sickness or due to other reasons the course instructors should be immediately notified. For each individual case the course instructors will determine how missed parts could be compensated.

## Presentation and reports

The main purpose of the presentation is to present the setup of the report. What was the research question, what was the experimental approach, what were the results and what is the conclusion? The reports should be written in the form of scientific articles according to the instructions in the manual, in Dutch or in English. Note that scientific articles are always written in English.

## Examination and marks

The marks will be based on (1) three written exams at the end of the first week, in the 6<sup>th</sup> week and at the end of the course, (2) on the presentation in week 10, (3) on the written report, (4) on the way in which the lab journal is maintained and (5) on the personal impression of the instructors, in the ratio 3:3:2:1:1 = average of exams : report : presentation : lab journal : impression. The exams will test the knowledge of theory and practice as described in the manual and as presented during the lectures, as well as the knowledge of selected parts of research articles.



# 1. METHODS IN BIOCHEMISTRY

## 1.1 Introduction

Biochemistry is the science that deals with the chemical properties of important biological molecules (proteins, carbohydrates, lipids, nucleic acids, and other biomolecules), in particular the chemistry of enzyme-catalyzed reactions happening in the living cells of organisms.

In order to study the chemical processes that the biomolecules are involved in, biochemistry aims at separating these molecules and obtaining them in purified form. The first step in purification of cellular components is the disruption of the cells. This is called cell lysis. The exact way to achieve cell lysis depends on the type of biomolecules under study, on the type of tissue and whether the cells possess a cell wall. Historically, physical lysis has been the method of choice for cell disruption; however, in recent years, detergent-based lysis has become popular due to ease of use, low cost and efficient protocols.

## 1.2 Cell Lysis, pH, Buffers

### 1.2.1 Cell lysis using physical methods

Animal cells are enveloped in an easily disruptable cell membrane, whereas bacterial, fungal and plant cells, in addition to the cell membrane, have rigid cell walls of interconnected polymers of peptidoglycan, cellulose or chitin that may require higher mechanical force to break. Several techniques for the physical disruption of cells are in use (Table 1.1).

**Table 1.1.** Techniques used for the physical disruption of cells.

Lysis Method	Apparatus	Description	
Mechanical	Waring Blender Polytron	Rotating blades grind and disperse cells and tissues	
Liquid Homogenization	Dounce homogenizer Potter-Elvehjem French press	Cell or tissue suspensions are sheared by forcing them through a narrow space	
Sonication	Sonicator	High frequency sound waves shear cells	

Freeze/Thaw	Freezer Dry ice/ethanol	Repeated cycles of freezing and thawing disrupt cells through ice crystal formation	
Manual grinding	Mortar and pestle	Grinding plant tissue, frozen in liquid nitrogen	

### **Mechanical Disruption**

Mechanical methods rely on the use of rotating blades to grind and disperse large amounts of complex tissue, such as liver or muscle. The Waring blender and the Polytron are commonly used for this purpose. Unlike the Waring blender, which is similar to a standard household blender, the Polytron draws tissue into a long shaft containing rotating blades. The shafts vary in size to accommodate a wide range of volumes, and can be used with samples as small as 1 ml.

### **Liquid Homogenization**

Liquid-based homogenization is the most widely used cell disruption technique for small volumes and cultured cells. Cells are lysed by forcing the cell or tissue suspension through a narrow space, thereby shearing the cell membranes. Three different types of homogenizers are in common use. A Dounce homogenizer consists of a round glass pestle that is manually driven into a glass tube. A Potter-Elvehjem homogenizer consists of a manually or mechanically driven Teflon pestle shaped to fit a rounded or conical vessel. The number of strokes and the speed at which the strokes are administered influences the effectiveness of Dounce and Potter-Elvehjem homogenization methods. Both homogenizers can be obtained in a variety of sizes to accommodate a range of volumes. A French press consists of a piston that is used to apply high pressure to a sample volume of 40 to 250 ml, forcing it through a tiny hole in the press. Only two passes are required for efficient lysis due to the high pressures used with this process. The equipment is expensive, but the French press is often the method of choice for breaking bacterial cells mechanically.

### **Sonication**

Sonication is the third class of physical disruption commonly used to break open cells. The method uses pulsed, high frequency sound waves to agitate and lyse cells, bacteria, spores and finely diced tissue. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles that form momentarily and implode, causing shock waves to radiate through a sample. To prevent excessive heating, ultrasonic treatment is applied in multiple short bursts to a sample immersed in an ice bath. Sonication is best suited for volumes <100 ml.

### **Freeze/Thaw**

The freeze/thaw method is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be quite lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols.

### **Grinding Using Mortar and Pestle**

Manual grinding is the most common method used to disrupt plant cells. Tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. Because of the tensile strength of the cellulose and other polysaccharides comprising the cell wall, this method is the fastest and most efficient way to access plant proteins and DNA.

## Additives/Facilitators

Cells can be treated with various agents to aid the disruption process. Lysis can be promoted by suspending cells in a hypotonic buffer, which cause them to swell and burst more readily under physical shearing.

Lysozyme (200 µg/ml) can be used to digest the polysaccharide component of yeast and bacterial cell walls. Alternatively, treating cells with glass beads can expedite processing in order to facilitate the crushing of cell walls. This treatment is commonly used with yeast cells. Viscosity of a sample typically increases during lysis due to the release of nucleic acid material. DNase can be added to samples (25-50 µg/ml) along with RNase (50 µg/ml) to reduce this problem. Nuclease treatment is not required for sonicated material since sonication shears chromosomes. Finally, proteolysis can be a problem whenever cells are manipulated; therefore, protease inhibitors should be added to all samples undergoing lysis.

### 1.2.2 Cell Lysis Using Detergents

Detergent cell lysis is a milder and easier alternative to physical disruption of cell membranes, although it is often used in conjunction with homogenization and mechanical grinding with a Polytron.

Detergents are polar lipids that are soluble in water. Detergents break the lipid barrier surrounding cells by solubilizing proteins and disrupting lipid:lipid, protein:protein and protein:lipid interactions.

Detergents, like other lipids, self associate and bind to hydrophobic surfaces. They are comprised of a polar hydrophilic head group and a nonpolar hydrophobic tail and are categorized by the nature of the head group as ionic (cationic or anionic), nonionic or zwitterionic (also called amphoteric) (Table 1.2). Their behavior depends on the properties of the head group and tail.

**Table 1.2.** Physical properties of commonly used detergents.

Detergent	Monomer, Da mw	Micelle, Da mw	CMC % (w/v)	CMC Molarity
<b>Anionic</b>				
SDS	288	18,000	0.23	8.0 x 10 <sup>-3</sup>
Cholate	430	4,300	0.60	1.4 x 10 <sup>-2</sup>
Deoxycholate	432	4,200	0.21	5.0 x 10 <sup>-3</sup>
<b>Cationic</b>				
C <sub>16</sub> TAB	365	62,000	0.04	1 x 10 <sup>-3</sup>
<b>Amphoteric</b>				
LysoPC	495	92,000	0.0004	7 x 10 <sup>-6</sup>
CHAPS	615	6,150	0.49	1.4 x 10 <sup>-3</sup>
Zwittergent 3-14	364	30,000	0.011	3.0 x 10 <sup>-4</sup>
<b>Nonionic</b>				
Octylglucoside	292	8,000	0.73	2.3 x 10 <sup>-2</sup>
Digitonin	1,229	70,000	----	----
C <sub>12</sub> E <sub>8</sub>	542	65,000	0.005	8.7 x 10 <sup>-5</sup>
Lubrol	582	64,000	0.006	1.0 x 10 <sup>-4</sup>
Triton X-100	650	90,000	0.021	3.0 x 10 <sup>-4</sup>
Nonidet P-40	650	90,000	0.017	3.0 x 10 <sup>-4</sup>
Tween 80	1,310	76,000	0.002	1.2 x 10 <sup>15</sup>

Unfortunately, there is no standard protocol available for selecting a detergent to use for membrane lysis. The ideal detergent will depend on the intended application. In general, nonionic and zwitterionic detergents are milder and less denaturing than ionic detergents and are used to solubilize membrane proteins where it is critical to maintain protein function and/or retain native protein:protein interactions for enzyme assays or immunoassays. CHAPS, a zwitterionic detergent, and the Triton-X series of nonionic detergents are commonly used for these purposes. In contrast, ionic detergents are strong solubilizing agents and tend to denature proteins, thereby destroying protein activity and function. Studies strictly assessing protein levels through gel electrophoresis and Western blotting typically use sodium dodecyl sulfate (SDS) to fully denature protein samples by boiling. It should be noted that antibodies usually retain their binding activity at 0.1% SDS or less. There are a few commonly used ionic detergents that are only mildly denaturing, including sodium cholate and sodium deoxycholate.

Two properties of detergents are important in their consideration for biological studies: the critical micelle concentration (CMC) and the micelle molecular weight. The CMC is the concentration at which monomers of detergent molecules combine to form micelles. Each detergent micelle has a characteristic molecular weight. Detergents with a high micelle molecular weight, such as nonionic detergents, are difficult to remove from samples by dialysis. The CMC and the micelle molecular weight vary depending on the buffer, salt concentration, pH and temperature. In general, adding salt will lower the CMC and raise the micelle size.

The choice of detergent for cell lysis also depends on sample type. Animal cells, bacteria and yeast all have differing requirements for optimal lysis due to the presence or absence of a cell wall. Because of the dense and complex nature of plant tissues, they require both detergent and mechanical lysis. In addition to the choice of detergent, other important considerations for optimal cell lysis include the buffer, pH, salt concentration and temperature. Consideration should be given to the compatibility of the chosen detergent with downstream applications. If the detergent used for lysis must be removed, then a dialyzable detergent should be selected.

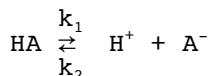
Several detergents have detrimental properties for some procedures:

1. Phenyl-group containing detergents (i.e., Triton X-100 and NP40) have a high absorbance at 280 nm and thus interfere with protein monitoring at that OD.
2. High micellar molecular weight interferes with gel filtration and removal by dialysis.
3. Sodium cholate and sodium deoxycholate are insoluble below pH 7.5. Above an ionic strength of 0.1%, SDS they will often crystallize.
4. Ionic detergents interfere with nondenaturing electrophoresis and isoelectric focusing.

## 1.3 pH AND BUFFERS

### 1.3.1 Equilibrium constant

A reaction continues until the rates of the forward and reverse reactions are the same. This is always the case for acid/base reactions. We can therefore write such equilibria as:



where  $k_1$  and  $k_2$  are the rate constants.

The reaction rates are:

$$v_1 = k_1 \cdot [\text{HA}] \quad \text{and} \quad v_2 = k_2 \cdot [\text{H}^+] \cdot [\text{A}^-]$$

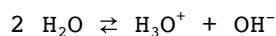
at equilibrium,  $v_1 = v_2$  or:

$$K_{\text{eq}} = \frac{k_1}{k_2} = \frac{[\text{H}^+] \cdot [\text{A}^-]}{[\text{HA}]}$$

### 1.3.2 Dissociation of acids and bases - the water equilibrium

According to the Brønsted-Lowry definition, an acid is a molecule that donates a proton, while a base accepts a proton. Strong acids and bases are molecules that are virtually completely dissociated in solution thereby producing either  $\text{H}_3\text{O}^+$  or  $\text{OH}^-$  ions.

When acids and bases are dissolved into water to form solutions they disturb an equilibrium of ions that already exists in the water. The water equilibrium forms because water dissociates to a small degree into  $\text{H}^+$  and  $\text{OH}^-$ . More correctly, it ionizes to form  $\text{H}_3\text{O}^+$  and  $\text{OH}^-$  ions according to the following equilibrium.



About 1 molecule in a billion ionizes in this way and therefore there are so few ions in water that it does not conduct a measurable current (unless you use very sensitive instruments).

Because the reactions are reversible and an equilibrium exists we can write an equilibrium constant expression for the above equation. It is:

$$K_i = \frac{[\text{H}_3\text{O}^+] \cdot [\text{OH}^-]}{[\text{H}_2\text{O}]^2}$$

Since the water cannot change concentration this term is not included in the above expression and the equation becomes:

$$K_w = [\text{H}_3\text{O}^+] \cdot [\text{OH}^-] \quad (K_w = K_i \cdot [\text{H}_2\text{O}]^2)$$

For pure water at 25°C this means:

$$[\text{H}_3\text{O}^+] = [\text{OH}^-] = 10^{-7} \text{ M} \quad (\text{with } 1 \text{ M H}_2\text{O} = 1000/18 = 55.6 \text{ moles/liter})$$

Substitution in the above formula makes:

$$K_w = 1.0 \times 10^{-14}$$

In (bio)chemistry the wide range in concentration of  $[\text{H}_3\text{O}^+]$  and  $[\text{OH}^-]$  in solution is expressed in the logarithmic form of pH. The following formula is commonly used in these calculations.

$$\text{pH} = -\log^{10} [\text{H}_3\text{O}^+] \quad \text{or} \quad [\text{H}_3\text{O}^+] = 10^{-\text{pH}}$$

For convenience,  $\text{H}^+$  is generally used to indicate  $\text{H}_3\text{O}^+$ . The notations for the water equilibrium and pH become:

$$K_w = [\text{H}^+] \cdot [\text{OH}^-] = 1.0 \times 10^{-14} \quad \text{and} \quad \text{pH} = -\log^{10} [\text{H}^+]$$

**Question:** What is the pH of a solution of 0.001 M HCl?

**Answer:** HCl is a strong acid, thus completely dissociated in water:

$$[\text{H}^+] = 10^{-3} \rightarrow \text{pH} = 3$$

Along the same reasoning a solution of 10<sup>-8</sup> M HCl would have pH = 8. However, this is impossible, an acid does not release  $\text{OH}^-$  and can never have a pH higher than 7. In fact we have to take into account the  $\text{H}^+$  from the dissociation of water and add those to the  $\text{H}^+$  from the HCl dissociation.

$$\text{Thus: } [\text{H}^+] = 10^{-8} + 10^{-7} = 1.1 \times 10^{-7} \rightarrow \text{pH} = 6.96$$

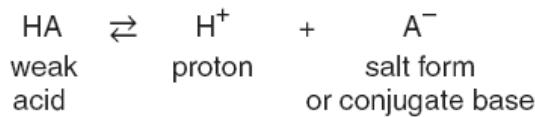
This is an approximation, since in the above calculation the shift in the dissociation of water due to the dissolved HCl is not considered. It is better to substitute the extra  $\text{H}^+$  from HCl into the water equilibrium (of which  $K_w$  always remains constant):

$$K_w = [\text{H}^+] \cdot [\text{OH}^-] = 10^{-14} \rightarrow (10^{-8} + a) \cdot (a) = 10^{-14} \rightarrow \text{pH} = 6.98$$

(a represents the number of moles  $\text{H}^+$  and  $\text{OH}^-$  from the dissociation of  $\text{H}_2\text{O}$ .)

### 1.3.3 Buffers

Consider the release of a proton by a weak acid represented by HA:



The “salt” or conjugate base,  $\text{A}^-$ , is the ionized form of a weak acid. According to the calculation above the dissociation constant of the acid,  $K_a$ , is

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

[Note: The larger the  $K_a$ , the stronger the acid, because most of the HA has been converted into  $H^+$  and  $A^-$ . Conversely, the smaller the  $K_a$ , the less acid has dissociated, and therefore the weaker the acid.]  
By solving for the  $[H^+]$  in the above equation, taking the logarithm of both sides of the equation, multiplying both sides of the equation by  $-1$ , and substituting  $pH = -\log[H^+]$  and  $pK_a = -\log K_a$ , we obtain the Henderson-Hasselbalch equation:

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

A buffer is a solution that resists change in pH following the addition of acid or base. A buffer is created by mixing equal concentrations of a weak acid (HA) and its conjugate base ( $A^-$ ).

[Note: If the amounts of HA and  $A^-$  are equal, the pH is equal to the  $pK_a$  ( $\log 1 = 0$ .)]

If acid is added to such a solution,  $A^-$  can neutralize it, in the process being converted to HA. If a base is added, HA can neutralize it, in the process being converted to  $A^-$ . A conjugate acid/base pair can serve as an effective buffer when the pH of a solution is within approximately  $\pm 0.5$  pH unit of the  $pK_a$  of the weak acid, whereas maximum buffering capacity occurs at a pH equal to the  $pK_a$ .

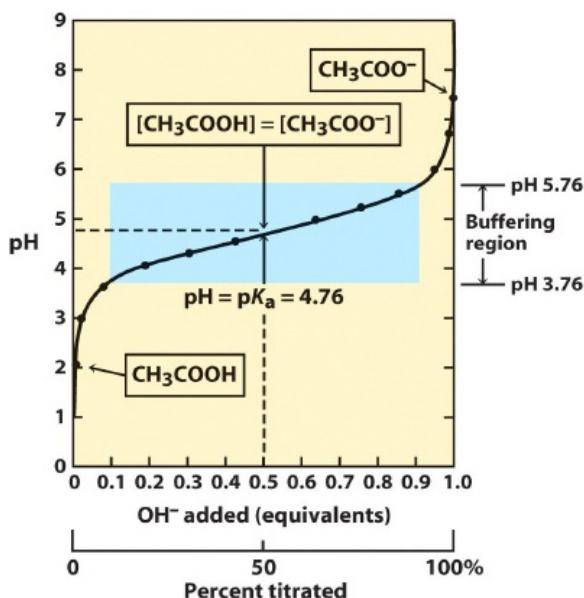


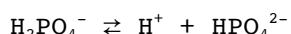
Fig. 1.1. Buffering capacity of acetic acid/acetate

As shown in Fig. 1.1, a solution containing acetic acid ( $HA = CH_3COOH$ ) and acetate ( $A^- = CH_3COO^-$ ) with a  $pK_a$  of 4.76 resists a change in pH from pH 3.76 to 5.76, with maximum buffering at pH = 4.76. [Note: At pH values less than the  $pK_a$ , the protonated acid form ( $CH_3COOH$ ) is the predominant species. At pH values greater than the  $pK_a$ , the deprotonated form ( $CH_3COO^-$ ) is the predominant species in solution.]

Most chemical reactions are affected by the acidity of the solution in which they occur. In order for a particular reaction to occur or to occur at an appropriate rate, the pH of the reaction medium must be controlled. Biochemical reactions are especially sensitive to pH. Most biological molecules contain groups of atoms that may be charged or neutral depending on pH, and whether these groups are charged or neutral has a significant effect on the biological activity of the molecule.

In all multicellular organisms, the fluid within the cell and the fluids surrounding the cells have a characteristic and nearly constant pH. The pH of mammalian blood is maintained close to 7.38 by the combined effects of a large number of buffer systems such as  $H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$ ,  $CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^-$ , many organic acids, organic bases, and proteins. In living plants, the normal range of pH in tissues is about 4.0-6.2.

The phosphate buffer system operates in the internal fluid of all cells. This buffer system consists of dihydrogen phosphate ions ( $H_2PO_4^-$ ) as hydrogen-ion donor (acid) and hydrogen phosphate ions ( $HPO_4^{2-}$ ) as hydrogen-ion acceptor (base). These two ions are in equilibrium with each other as indicated by the chemical equation below.



If additional hydrogen ions enter the cellular fluid, they are consumed in the reaction with  $HPO_4^{2-}$ , and the equilibrium shifts to the left. If additional hydroxide ions enter the cellular fluid, they react with  $H_2PO_4^-$ , producing  $HPO_4^{2-}$ , and shifting the equilibrium to the right. The equilibrium-constant expression for this equilibrium is

$$K_a = \frac{[H^+][HPO_4^{2-}]}{[H_2PO_4^-]}$$

The value of  $K_a$  for this equilibrium is  $6.23 \times 10^{-8}$  M at 25°C. From this equation, the relationship between the hydrogen-ion concentration and the concentrations of the acid and base can be derived.

$$[\text{H}^+] = K_a \frac{[\text{H}_2\text{PO}_4^-]}{[\text{HPO}_4^{2-}]}$$

Thus, when the concentrations of  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  are the same, the value of the molar concentration of hydrogen ions is equal to the value of the equilibrium constant, and the pH is equal to the  $\text{p}K_a$  ( $-\log K_a$ ), namely 7.21. Buffer solutions are most effective at maintaining a pH near the value of the  $\text{p}K_a$ . In mammals, cellular fluid has a pH in the range 6.9 to 7.4, and the phosphate buffer is effective in maintaining this pH range.

In order to study biochemical reactions in the reaction tube with enzymes isolated from their cellular background, it is evident that the reaction conditions must closely match the conditions in the cell. For the pH this means that it is buffered in the range of the enzyme's optimal pH. The use of buffers based on inorganic or organic salts is limited because of the interference of buffer cations and anions with the biological reaction under study. Therefore a number of zwitterionic (neutral molecules having positive and negative charge on different groups) buffers were developed that display low interference with biological processes and which have  $\text{p}K_a$ 's in the physiological buffer range between 6.15-9.55. Moreover, the zwitterionic nature of these buffers makes them very water soluble, while they do not absorb UV light (important for spectrophotometric analysis). Table 1.3 shows a number of zwitterionic buffers used in biological research.

**Table 1.3.** Zwitterionic buffers and their associated  $\text{p}K_a$  values and useful pH ranges.

Buffer	$\text{p}K_a$ at 25 °C	useful pH range
MES	6.15	5.50 – 6.50
Bis-Tris	6.50	5.80 – 7.30
PIPES	6.80	6.10 – 7.50
ACES	6.88	6.00 – 7.50
MOPS	7.20	6.50 – 7.90
TES	7.40	6.80 – 8.20
HEPES	7.55	6.80 – 8.20
HEPPS	8.00	7.30 – 8.70
Tricine	8.15	7.80 – 8.80
Bicine	8.35	7.60 – 9.00
CHES	9.50	8.60 – 10.00
CAPS	10.40	9.70 – 11.10

### 1.3.4 How to make a phosphate buffer

Because it contains three acidic protons, phosphoric acid ( $\text{H}_3\text{PO}_4$ ) has multiple dissociation constants and can be used to create buffers for either of the three corresponding pHs.

The three  $\text{p}K_a$  values for phosphoric acid are 2.15, 7.21 and 12.32. Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) and its conjugate base, dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ) are usually used to generate buffers of pH values around 7, for biological applications, as shown below.

#### Decide on the buffer properties

Before making a buffer you must know what molarity you want it to be, what volume to make and what the desired pH is. Most buffers work best at concentrations between 0.05 M and 10 M. The pH should be within approximately 0.5 pH unit of the acid/conjugate base  $\text{p}K_a$ . For simplicity, this sample calculation will be for 1L of 1 M K-phosphate buffer.

#### Determine the ratio of acid to base

Use the Henderson-Hasselbalch equation (below) to determine what ratio of acid ( $\text{KH}_2\text{PO}_4$ ) to base ( $\text{K}_2\text{HPO}_4$ ) is required to make a buffer of the desired pH. Use the  $\text{p}K_a$  value nearest your desired pH and the ratio will refer to the acid-base conjugate pair that corresponds to that  $\text{p}K_a$ .

$$\text{Henderson-Hasselbalch Equation: } \text{pH} = \text{p}K_a + \log([\text{Base}]/[\text{Acid}])$$

For a buffer of pH 7.4, choose the  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  acid/base buffer system ( $\text{pK}_a = 7.21$ )

Putting these values in the Henderson-Hasselbalch equation results in:

$$\log([\text{Base}]/[\text{Acid}]) = 0.19 \rightarrow [\text{Base}]/[\text{Acid}] = 10^{0.19} \rightarrow [\text{Base}]/[\text{Acid}] = 1.55$$

The desired molarity of the buffer is the sum of [Acid] + [Base].

For a 1 M buffer,  $[\text{Base}] + [\text{Acid}] = 1 \rightarrow [\text{Base}] = 1 - [\text{Acid}]$

By substituting this into the ratio equation, above, you get:

$$[\text{Acid}] = 0.392 \text{ moles/L and } [\text{Base}] = 0.608 \text{ moles/L}$$

Prepare just under 1 L of solution using the correct amounts of  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ .

Use a pH meter to confirm that the correct pH for the buffer is reached. Adjust slightly as necessary, using phosphoric acid or potassium hydroxide.

Once the desired pH is reached, bring the volume of buffer to 1 L.

This same buffer can be diluted to create buffers of 0.5 M, 0.1 M, 0.05 M or anything in-between.

## 1.4 Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is a method of protein purification by altering the solubility of protein. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed.

Proteins vary greatly in their solubility depending on their amino acid composition and the characteristics of the solvent such as ionic strength and temperature. A common approach for separating proteins is to add increasing concentrations of a salt in order to “salt out” the proteins. Salting out is often used as an early step in a purification strategy. Ammonium sulfate is commonly chosen as the salt since high concentrations can be achieved and since it does not usually denature proteins.

“Salting out” is thought to work by “dehydrating” the environment around the protein. When ammonium sulfate is added to the protein solution, a large number of water molecules bind to the sulfate ion, which reduces the amount of water available to bind to the protein. If a protein is not hydrated by binding to water molecules, it will precipitate. Different proteins precipitate at different concentrations of ammonium sulfate. We can take advantage of this difference to remove some contaminating proteins from our crude extract.

In a typical experiment, the ammonium sulfate concentration is increased stepwise, and the precipitated protein is recovered at each stage. Each protein precipitate is dissolved individually in fresh buffer and assayed for total protein content and amount of desired protein. The aim is to find the ammonium sulfate concentration, which will precipitate the maximum proportion of undesired protein, whilst leaving most of the desired protein still in solution.

The precipitated protein is then removed by centrifugation and then the ammonium sulfate concentration is increased to a value that will precipitate most of the protein of interest while leaving the maximum amount of protein contaminants still in solution. The precipitated protein of interest is recovered by centrifugation and after removal of excess ammonium sulfate by desalting procedures like dialysis, gel filtration or simply by dilution it can be analyzed or used for subsequent stages of purification.

This technique is useful to quickly remove large amounts of contaminant proteins, as a first step in many purification schemes. It is also often employed during the later stages of purification to concentrate protein from dilute solution following procedures such as gel filtration or affinity chromatography.

We will use ammonium sulfate precipitation to fractionate the protein mixture obtained upon homogenization of animal tissue to obtain a concentrated and enriched fraction specifically containing our protein of interest: lactate dehydrogenase.

Table 1.4 shows the weight (g) of ammonium sulfate to be added to one liter of ammonium sulfate solution to produce a desired change in the concentration (% saturation) of ammonium sulfate at 0°C.

**Table 1.4.** Ammonium sulfate solution calculator.

0°C	desired %-age																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	107	136	166	197	229	262	295	331	366	404	442	483	523	567	611	659	707
10	54	82	111	141	171	203	236	270	305	342	379	418	458	500	544	589	636
15	26	55	83	113	143	174	207	240	275	310	348	386	426	466	510	555	600
20		27	56	84	115	145	177	210	244	280	316	354	392	433	476	519	565
25			27	57	85	117	148	182	214	248	284	321	360	401	442	485	529
30				28	57	87	119	150	184	217	253	289	328	367	408	451	495
35					29	59	90	122	155	190	225	262	300	340	381	424	495
40						29	59	90	122	155	190	225	262	300	340	381	424
45							29	60	91	125	158	193	229	267	306	347	388
50								30	61	93	127	161	197	233	272	312	353
55									30	62	94	129	163	200	238	277	317
60										31	63	96	131	166	204	242	283
65											31	64	98	134	170	208	247
70												32	66	100	136	173	212
75													32	67	102	139	176
80														33	68	104	141
85															34	69	106
90															34	71	
95																35	

## 1.5 Desalting and Concentration

### 1.5.1 Dialysis

Dialysis is the most popular and conventional procedure for the separation of biomolecules from dissolved small molecules (such as salts) depending on their size. This technique involves placing an aqueous solution of a mixture of macromolecules and small molecules sealed in a porous membrane bag. This is then placed in a large container having a low ionic strength buffer, termed the dialysis buffer, which is slowly stirred. The pores of the dialysis membrane are of a specific size (called the “cut off” size expressed in Da or kDa), and they therefore allow only smaller molecules to pass through, while the bulkier molecules (such as proteins) remain within the sealed dialysis bag. The smaller molecules (e.g., ammonium sulfate) pass out freely through the pores and get diluted when they mix with the large amount of dialysis buffer outside. Frequent changes of the dialysis buffer help in reducing the salt concentration inside the bag to negligible levels. Dialysis typically requires several hours to days and is usually carried out at 4°C to minimize protein degradation.

### 1.5.2 Ultrafiltration

With ultrafiltration hydrostatic pressure forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained, while water and low molecular weight solutes pass through the membrane. This separation process is used for purifying and concentrating macromolecular ( $10^3$  -  $10^6$  Da) solutions, especially protein solutions. A common application of ultrafiltration employs filters that can be mounted in eppendorf tubes and centrifuged. Sample applied on top of the filter is pushed through the filter by the use of centrifugal force.

### 1.5.3 Gel filtration

Gel filtration is a type of size exclusion chromatography, a chromatographic method in which molecules in solution are separated based on their size. It is usually applied to large molecules or macromolecular complexes such as proteins or other polymers. Typically, molecules move through a bed of porous beads (a column), diffusing into the beads to greater or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not

at all and thus move through the bed more quickly. Both molecular weight and three-dimensional shape contribute to the degree of retention. Gel filtration may be used for analysis of molecular size, for separations of components in a mixture, or for salt removal or buffer exchange from a preparation of macromolecules. Frequently used gel filtration media for desalting purposes like the removal of ammonium sulfate from protein samples, are Sephadex G-10, G-15 and G-25, with size exclusion limits in the range of 700 to 5000 Da, meaning that macromolecules with higher molecular weights (like most proteins) can freely pass through the column and are collected immediately after the void volume, while salt ions with sizes smaller than 700 Da are retained in the column material (see also section 1.6.5).

## 1.6 Chromatography

### 1.6.1 Overview

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm. Many different chromatographic methods exist.

#### Size exclusion chromatography

Chromatography can be used to separate protein in solution by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluant (solvent) before being collected at the other end of the column of gel.

In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly sized proteins.

#### Ion exchange chromatography

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon application of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations.

#### Affinity chromatography

Affinity chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces, which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

An example of a ligand often used in affinity chromatography is the blue dye Cibacron™ Blue F3G-A. The dye molecules are covalently attached to Sepharose CL-6B, resulting in the more commonly known Blue Sepharose. The structure of the blue dye makes it a very versatile tool for separating many proteins, e.g. albumin, lipoproteins and blood coagulation factors. It also binds several enzymes including kinases, dehydrogenases and most enzymes requiring adenyl-containing cofactors (including NAD<sup>+</sup> and NADP<sup>+</sup>). The cross-linked matrix provides a stable, rigid medium even in the presence of dissociating agents.

#### Metal binding

A common technique involves engineering a sequence of 6 to 8 histidines into the C-terminal region of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged

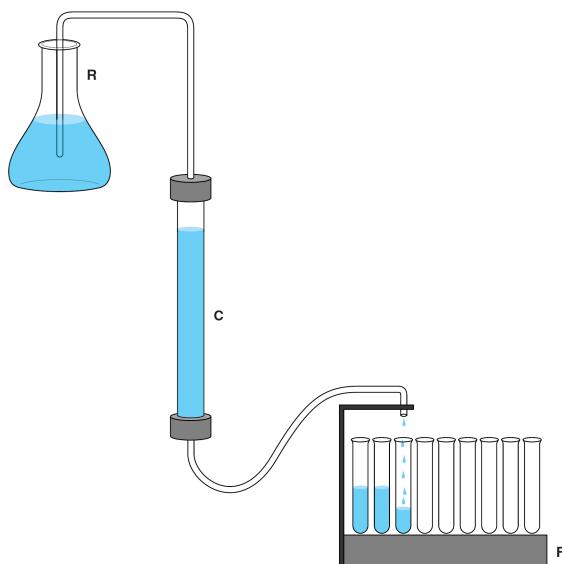
proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

### Immunoaffinity chromatography

Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources.

The following part is a detailed description of chromatography techniques taken from Amersham chromatography handbooks which are freely available on the GE Healthcare website.

#### 1.6.2 Introduction



**Fig. 1.2.** Components of a simple liquid chromatography apparatus. **R:** Reservoir of mobile phase liquid, delivered either by gravity or using a pump. **C:** Glass or plastic column containing stationary phase. **F:** Fraction collector for collecting portions, called fractions, of the eluate in separate test tubes. In some practical courses students are used as fraction collectors.

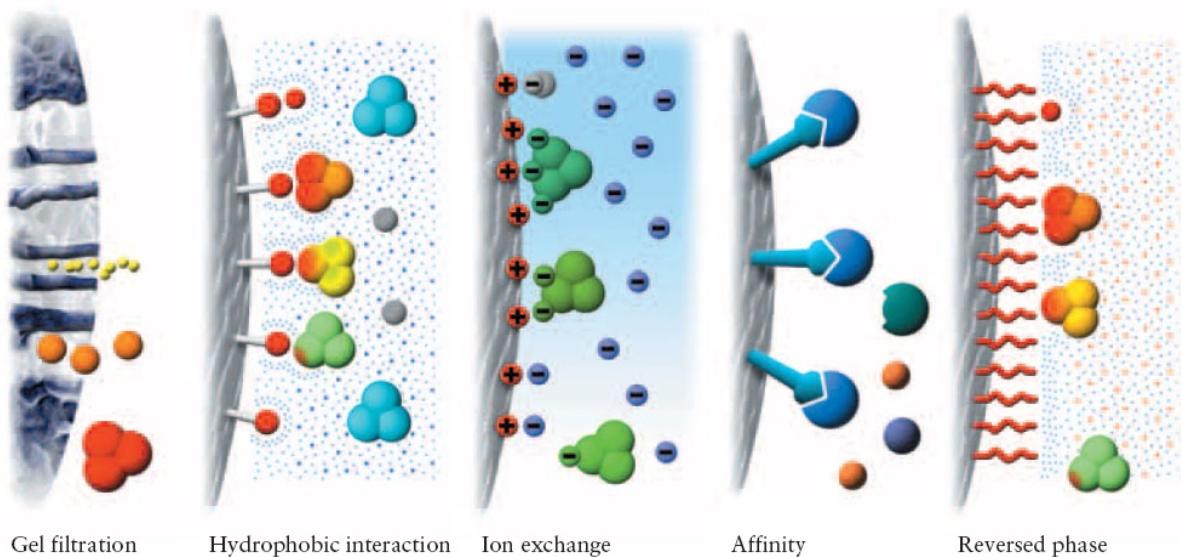
**Chromatography** (from Greek χρώμα: *chroma*, colour and γράφειν: "grafein" to write) is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a *stationary phase*, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated. An analogy which is sometimes useful is to suppose a mixture of bees and wasps passing over a flower bed. The bees would be more attracted to the flowers than the wasps, and would become separated from them. If one were to observe at a point past the flower bed, the wasps would pass first, followed by the bees. In this analogy, the bees and wasps represent the analytes to be separated, the flowers represent the stationary phase, and the mobile phase could be thought of as the air.

Chromatography may be preparative or analytical. Preparative chromatography seeks to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography normally operates with smaller amounts of material and seeks to measure the relative proportions of analytes in a mixture. The two are not mutually exclusive.

The most common form of chromatography is column chromatography (Fig. 1.2) in which the stationary bed is within a tube. But also paper and thin-layer chromatography are based on the same principles.

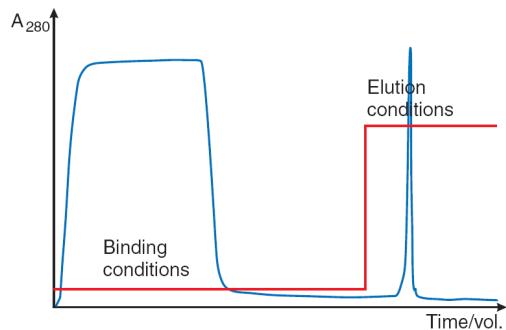
Biomolecules are purified using purification techniques that separate according to differences in specific properties, as shown in Fig. 1.3.

Property	Technique
Biorecognition (ligand specificity)	Affinity chromatography
Charge	Ion exchange chromatography
Size	Gel filtration (sometimes called size exclusion)
Hydrophobicity	Hydrophobic interaction chromatography Reversed phase chromatography

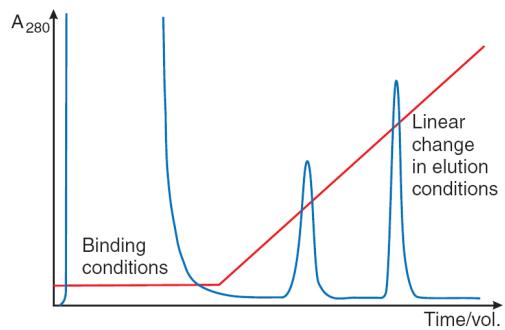


**Fig. 1.3.** Separation principles in chromatographic purification.

In all methods except gel filtration, molecules are bound to a solid matrix, and eluted with a solution that disrupts binding to the matrix. Elution can be performed stepwise by exchanging the binding or washing buffer for the elution buffer (Fig. 1.4A), or can be performed using a gradient (Fig. 1.4B). In contrast to stepwise elution, gradient elution allows separation of bound molecules based on their relative affinities for the solid phase and the mobile elution buffer.



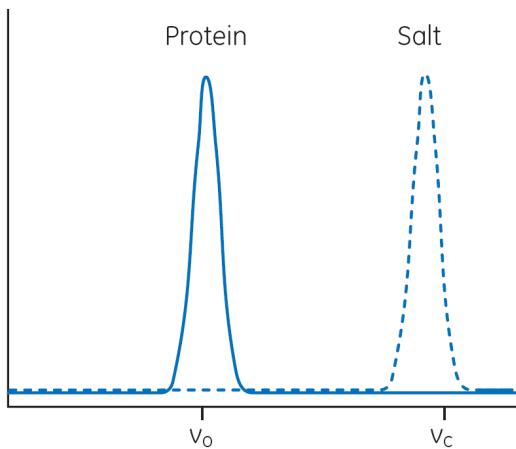
**Fig. 1.4A.** Step elution.



**Fig. 1.4B.** Gradient elution.

### 1.6.3 Buffer exchange and desalting after elution

After elution, it is often necessary for follow-up experiments to change the buffer of the sample, to remove salt and/or to remove small contaminants such as the ligands in the case of affinity chromatography (e.g. glutathione, imidazole). Dialysis is a common technique to remove salt or other small molecules and to exchange the buffer composition of a sample. Advantages are that dialysis is low-tech, inexpensive and does not require any hands-on time. However, dialysis is a very slow technique and requires large volumes of buffer. In addition, during handling or as a result of non-specific binding to the dialysis membranes, there is a risk of losing material. Another much faster technique is to use a desalting column, packed with for example Sephadex G-25, to perform a group separation between high and low molecular weight substances (Fig. 1.5). Proteins are separated from salts and other small molecules.



**Fig. 1.5.** Desalting by gel filtration.

#### 1.6.4 Affinity Chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix.

The technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. With high selectivity, hence high resolution, and high capacity for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable.

Target protein(s) is/are collected in a purified, concentrated form. Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity.

In a single step, affinity purification can offer immense time-saving over less selective multistep procedures. The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances.

Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography, are listed below:

- Enzyme  $\leftrightarrow$  substrate analogue, inhibitor, cofactor
- Antibody  $\leftrightarrow$  antigen, virus, cell
- Lectin  $\leftrightarrow$  polysaccharide, glycoprotein, cell surface receptor, cell
- Nucleic acid  $\leftrightarrow$  complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein
- Hormone, vitamin  $\leftrightarrow$  receptor, carrier protein
- Glutathione  $\leftrightarrow$  glutathione-S-transferase or GST fusion proteins
- Metal ions  $\leftrightarrow$  Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces

#### The matrix

The matrix is an inert support to which a ligand can be directly or indirectly coupled. The list below highlights many of the properties required for an efficient and effective chromatography matrix:

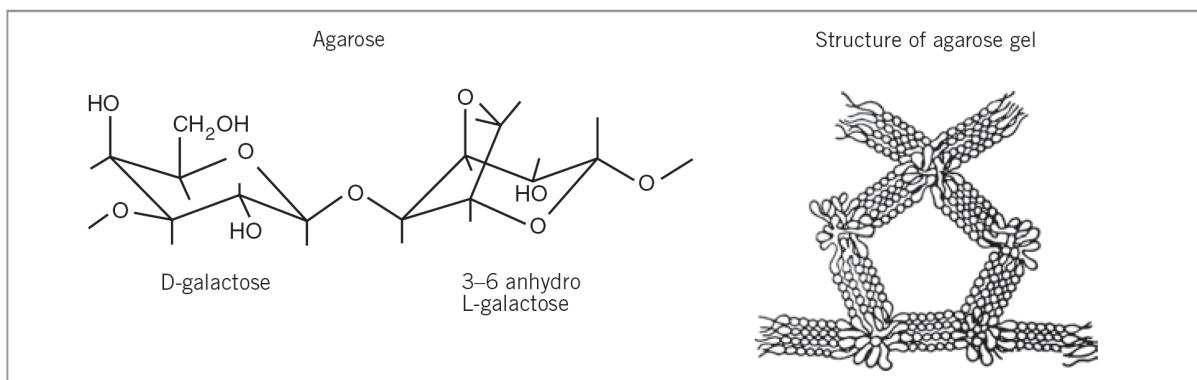
- Extremely low non-specific adsorption, essential since the success of affinity chromatography relies on specific interactions.
- Hydroxyl groups on the sugar residues are easily derivatized for covalent attachment of a ligand, providing an ideal platform for the development of affinity media.

- An open pore structure ensures high capacity binding even for large biomolecules, since the interior of the matrix is available for ligand attachment.
- Good flow properties for rapid separation.
- Stability under a range of experimental conditions such as high and low pH, detergents and dissociating agents.

Sepharose, a bead-form of agarose (Fig. 1.6), provides many of these properties. Sepharose media are based on chains of agarose, arranged in bundles and with different degrees of intra-chain cross-linking (Figure 5), to give a range of rigid, macroporous matrices with good capacity and low non-specific adsorption.

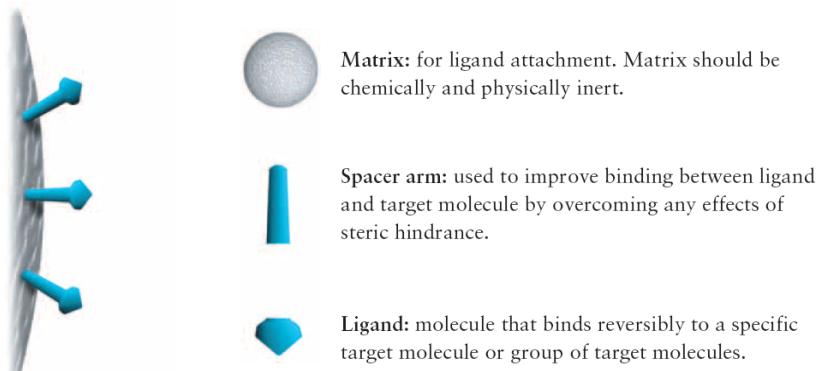
Affinity chromatography can also be used to remove specific contaminants.

The high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including, for example, common operations such as the purification of monoclonal antibodies or fusion proteins.



**Fig. 1.6.** Partial structure of agarose.

### Common terms in affinity chromatography



unbound substances from the column without eluting the target molecules or that re-equilibrate the column back to the starting conditions (in most cases the binding buffer is used as a wash buffer).

**Ligand coupling:** covalent attachment of a ligand to a suitable pre-activated matrix to create an affinity medium.

**Fig.1.7.** Common terms in affinity chromatography

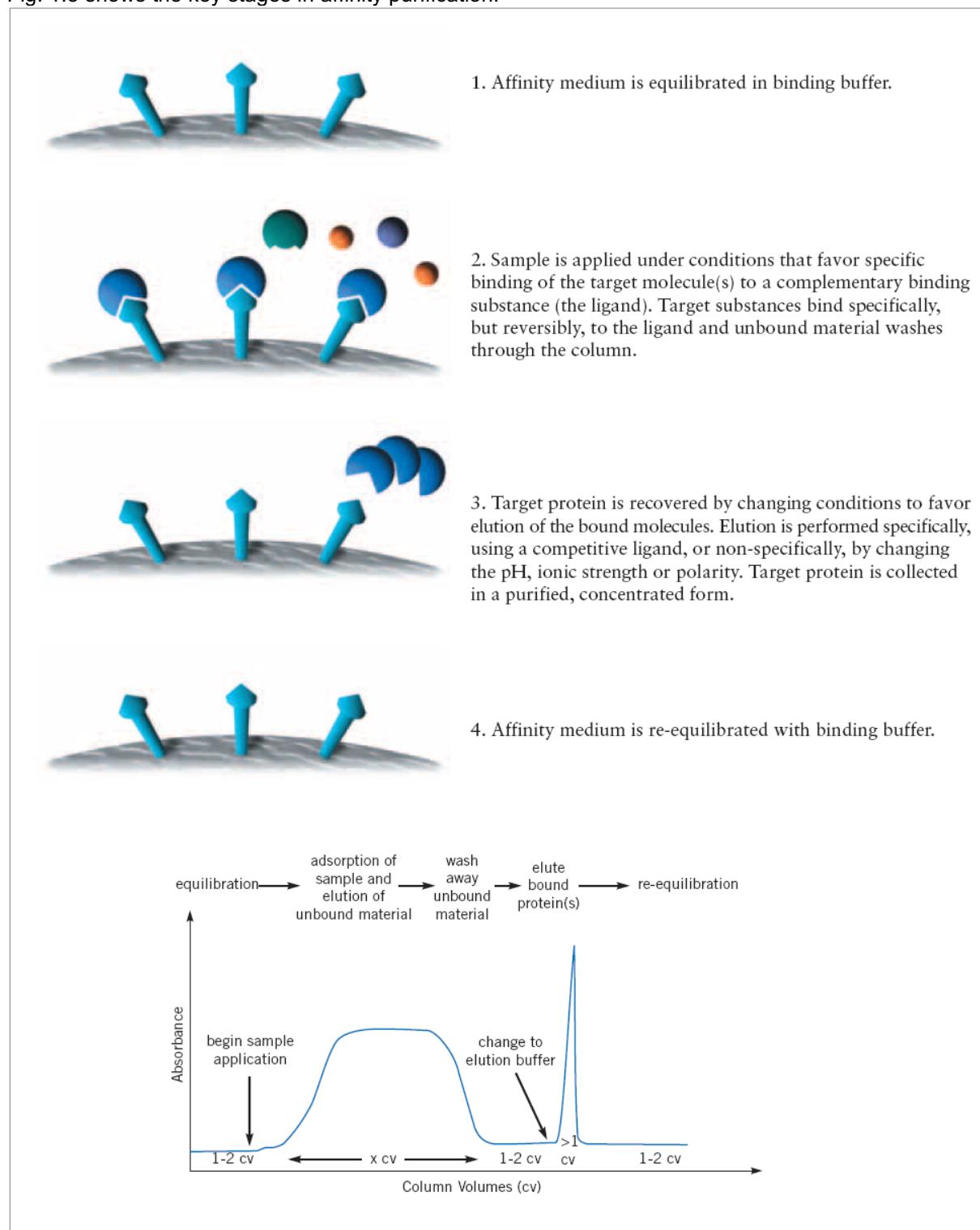
**Binding:** buffer conditions are optimized to ensure that the target molecules interact effectively with the ligand and are retained by the affinity medium as all other molecules wash through the column.

**Elution:** buffer conditions are changed to reverse (weaken) the interaction between the target molecules and the ligand so that the target molecules can be eluted from the column.

**Wash:** buffer conditions that wash

## Procedure for affinity purification

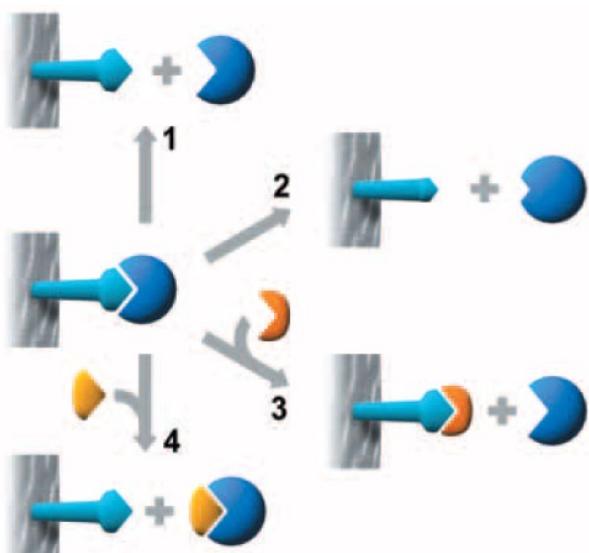
Fig. 1.8 shows the key stages in affinity purification.



**Fig. 1.8.** Typical affinity purification.

## Elution methods

An effective elution method elutes the target protein in a concentrated form. Elution methods may be either selective or non-selective, as shown in Fig. 1.9.



**Fig. 1.9.** Elution methods.

#### Method 1

The simplest case. A change of buffer composition elutes the bound substance without harming either it or the ligand.

#### Method 2

Extremes of pH or high concentrations of chaotropic agents are required for elution, but these may cause permanent or temporary damage.

#### Methods 3 and 4

Specific elution by addition of a substance that competes for binding. These methods can enhance the specificity of media that use group-specific ligands.

#### **pH elution**

A change in pH alters the degree of ionization of charged groups on the ligand and/or the bound protein. This change may affect the binding sites directly, reducing their affinity, or cause indirect changes in affinity by alterations in conformation.

A step decrease in pH is the most common way to elute bound substances.

#### **Ionic strength elution**

The exact mechanism for elution by changes in ionic strength will depend upon the specific interaction between the ligand and target protein. This is a mild elution using a buffer with increased ionic strength (usually NaCl), applied as a linear gradient or in steps. Enzymes usually elute at a concentration of 1 M NaCl or less.

#### **Competitive elution**

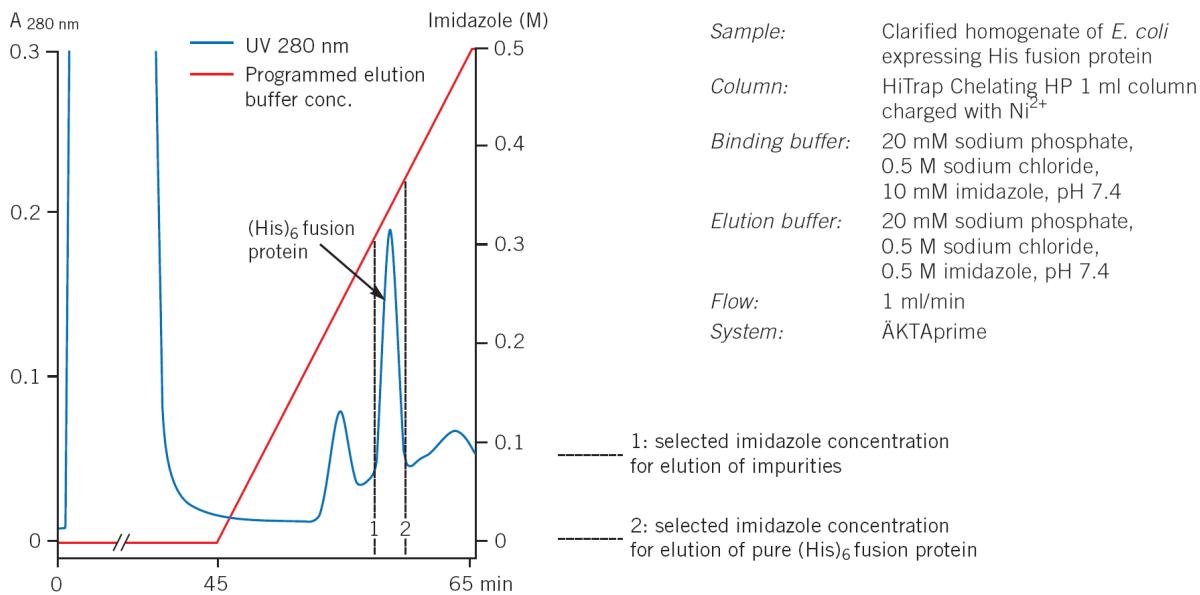
Selective eluents are often used to separate substances on a group specific medium or when the binding affinity of the ligand/target protein interaction is relatively high. The eluting agent competes either for binding to the target protein or for binding to the ligand.

#### **Reduced polarity of eluent**

Conditions are used to lower the polarity of the eluent promote elution without inactivating the eluted substances. Dioxane (up to 10%) or ethylene glycol (up to 50%) are typical of this type of eluent.

#### **Chaotropic eluents**

If other elution methods fail, deforming buffers, which alter the structure of proteins, can be used, e.g. chaotropic agents such as guanidine hydrochloride or urea. Chaotropes should be avoided whenever possible since they are likely to denature the eluted protein.

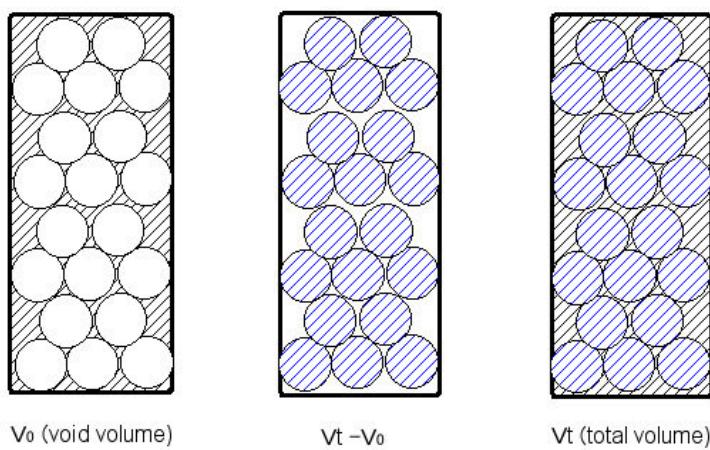


**Fig. 1.10.** Gradient elution of a  $(\text{His})_6$  fusion protein.

### 1.6.5 Gel filtration chromatography

Gel filtration chromatography is also known as size exclusion chromatography or gel permeation chromatography. Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks).

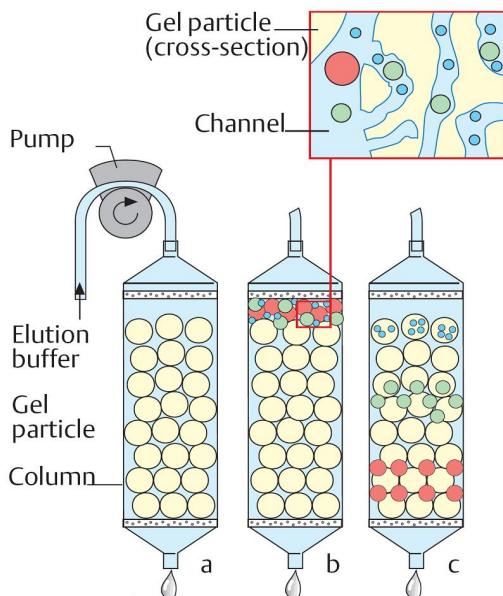
To perform a separation, gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles. The packed bed is equilibrated with buffer, which fills the pores of the matrix and the space between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase. It should be noted that samples are eluted isocratically, i.e. there is no need to use different buffers during the separation.



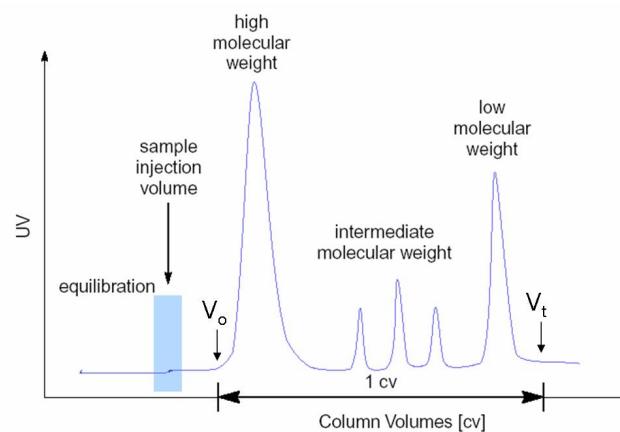
**Fig. 1.11.** Diagrammatic representation of  $V_t$  and  $V_0$ . Note that  $V_t - V_0$  includes the volume of the solid material which forms the matrix as well as the volume of buffer inside the matrix.

The sample is applied to the column. Buffer and sample move through the column. Molecules diffuse in and out of the pores of the matrix (also described as partitioning of the sample between the mobile phase and the stationary phase). Smaller molecules move further into the matrix and so stay longer on the column. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column. Smaller molecules diffuse into the pores and

are delayed in their passage down the column. Large molecules leave the column first followed by smaller molecules in order of their size. The entire separation process takes place as one total column volume ( $V_t$ ; equivalent to the volume of the packed bed) of buffer passes through the gel filtration medium.



**Fig. 1.12.** Size-exclusion chromatography. A mixture of larger molecules and smaller molecules are applied to the top of a gel filtration column. Upon entering the column, the smaller molecules enter pores in the stationary phase matrix from which the large molecules are excluded. As the mobile phase flows down the column, the large, excluded molecules have to travel only through the relatively small volume outside the beads ( $V_o$ ), while the smaller molecules move further in the matrix and so stay longer on the column.



**Fig. 1.13.** Theoretical chromatogram of a high-resolution fractionation (UV absorbance).

Results from gel filtration are usually expressed as an elution profile or chromatogram that shows the variation in concentration (typically in terms of UV absorbance at 280 nm) of sample components as they elute from the column in order of their molecular size.

Fig. 1.13 shows the theoretical elution profile (chromatogram) of a high resolution fractionation. Molecules that do not enter the matrix are eluted in the void volume,  $V_o$ , as they pass directly through the column at the same speed as the flow of the buffer. Molecules with partial access to the pores of the matrix elute from the column in order of decreasing size. Small molecules such as salts that have full access to the pores move down the column, but do not separate from each other. These molecules elute just before one total column volume,  $V_t$ , has passed through the column. The behaviour of each component can be expressed in terms of its elution volume,  $V_e$ , determined by direct measurement from the chromatogram.

### Gel filtration matrix

Gel filtration media are made from porous matrices. The size of the pores within a particle and the particle size distribution are carefully controlled to produce a variety of media with different selectivities. Today's gel filtration media cover a molecular weight range from 100 to 80,000,000, from a single amino acid to very large proteins and protein complexes.

The earliest gel filtration matrices were formed by cross-linking polymers to form a three-dimensional network, for example Sephadex is formed by cross-linking dextran. Controlling the degree of cross-linking and particle size made it possible to produce a broad range of media, each one having a selectivity over a narrow range of molecular weight values.

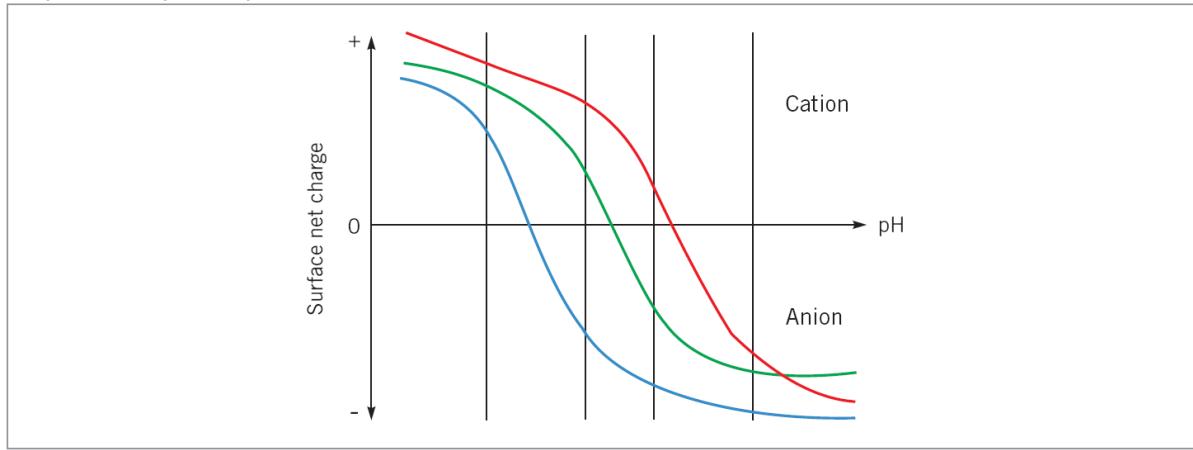
Alternative gel filtration media based on agarose which are able to withstand higher flow rates were later on developed, such as Sepharose and the more highly cross-linked Superose. A major advance in gel filtration technology occurred when composite gels could be prepared by grafting a second polymer onto a

pre-formed matrix, for example Sephadex (cross-linking allyl dextran with N, N'-methylene bisacrylamide), and the most recent, Superdex.

### 1.6.6 Ion exchange chromatography (IEX)

IEX separates molecules on the basis of differences in their *net surface charge*. Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different  $pK_a$  values depending on their structure and chemical microenvironment.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, their net surface charge will change gradually as the pH of the environment changes i.e. proteins are *amphoteric*. Each protein has its own unique *net charge versus pH relationship* (Fig. 1.14). IEX chromatography takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein.



**Fig. 1.14.** Theoretical protein titration curves, showing how net surface charge varies with pH.

In an IEX separation *reversible interactions* between *charged molecules* and *oppositely charged IEX media* are controlled in order to favour binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its *isoelectric point (pI)* will not interact with a charged medium. However, at a pH above its isoelectric point, a protein will bind to a positively charged medium or *anion exchanger* and, at a pH below its  $pI$ , a protein will bind to a negatively charged medium or *cation exchanger*. In addition to the ion exchange interaction, other types of binding may occur, but these effects are very small and mainly due to van der Waals forces and non-polar interactions.

#### Steps in an IEX separation

An IEX medium comprises a *matrix* of spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a *packed bed*. The bed is then equilibrated with buffer, which fills the pores of the matrix and the space in between the particles. Fig. 1.15 illustrates the separation process that follows. The pH and ionic strength of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind. The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample being loaded.

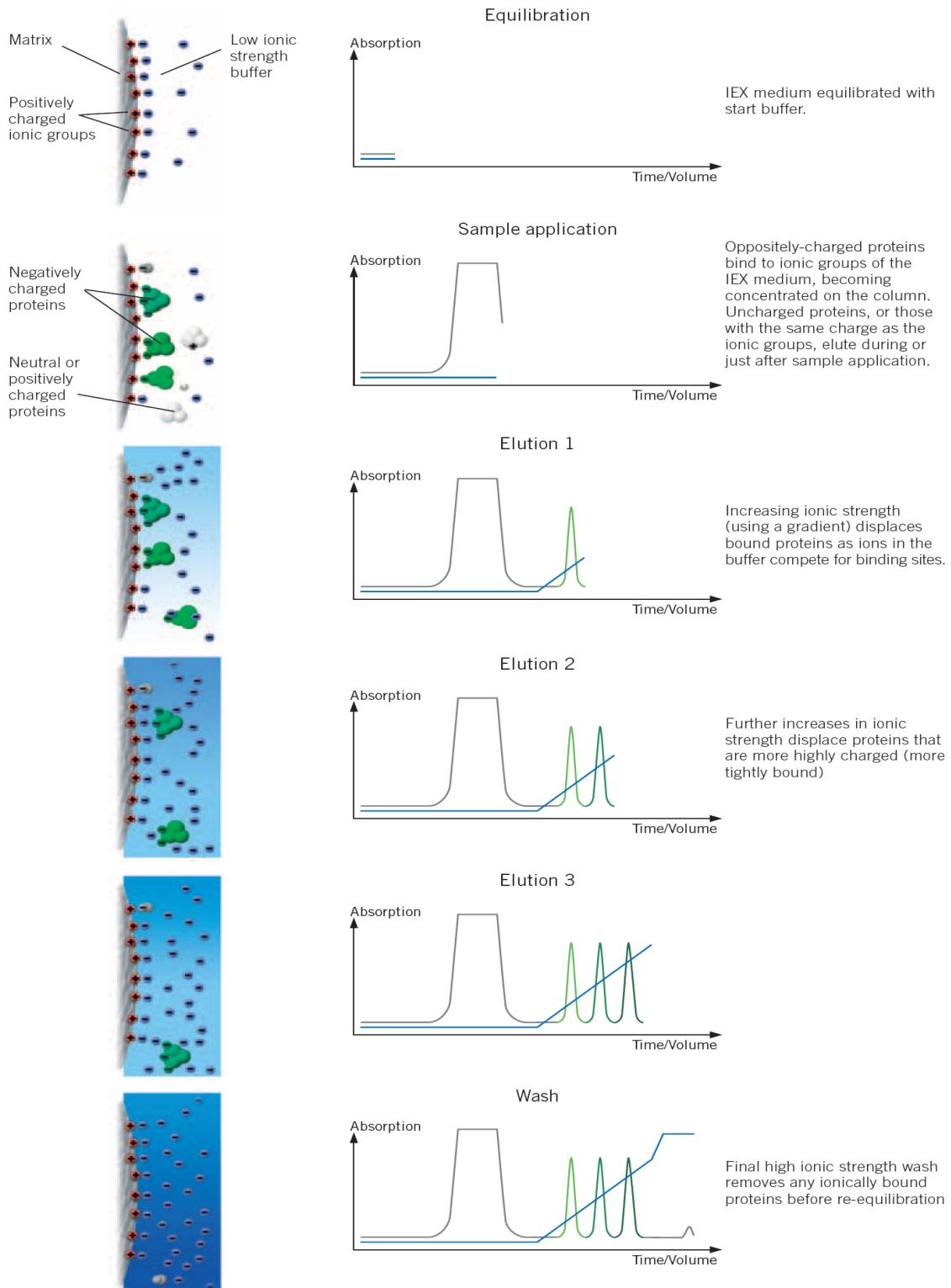
When the entire sample has been loaded and the column washed so that all non-binding proteins have passed through the column, conditions are altered in order to *elute the bound proteins*. Most frequently, proteins are eluted by *increasing the ionic strength (salt concentration)* of the buffer or, occasionally, by *changing the pH*. As ionic strength increases, the salt ions (typically  $\text{Na}^+$  or  $\text{Cl}^-$ ) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases.

Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the ionic strength that is needed for

elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form.

### Selectivity and pH

Good selectivity is achieved by performing IEX separations at pH values carefully selected to maximize the differences in net charge of the components of interest. Fig. 1.16 emphasizes the significance of pH. Optimum selectivity can be expected at a pH where there is maximum separation between the titration curves for the individual proteins (i.e. the difference in net charges between the species is greatest) and when using an ion exchanger with a charge opposite to the charge of the proteins at the particular pH.

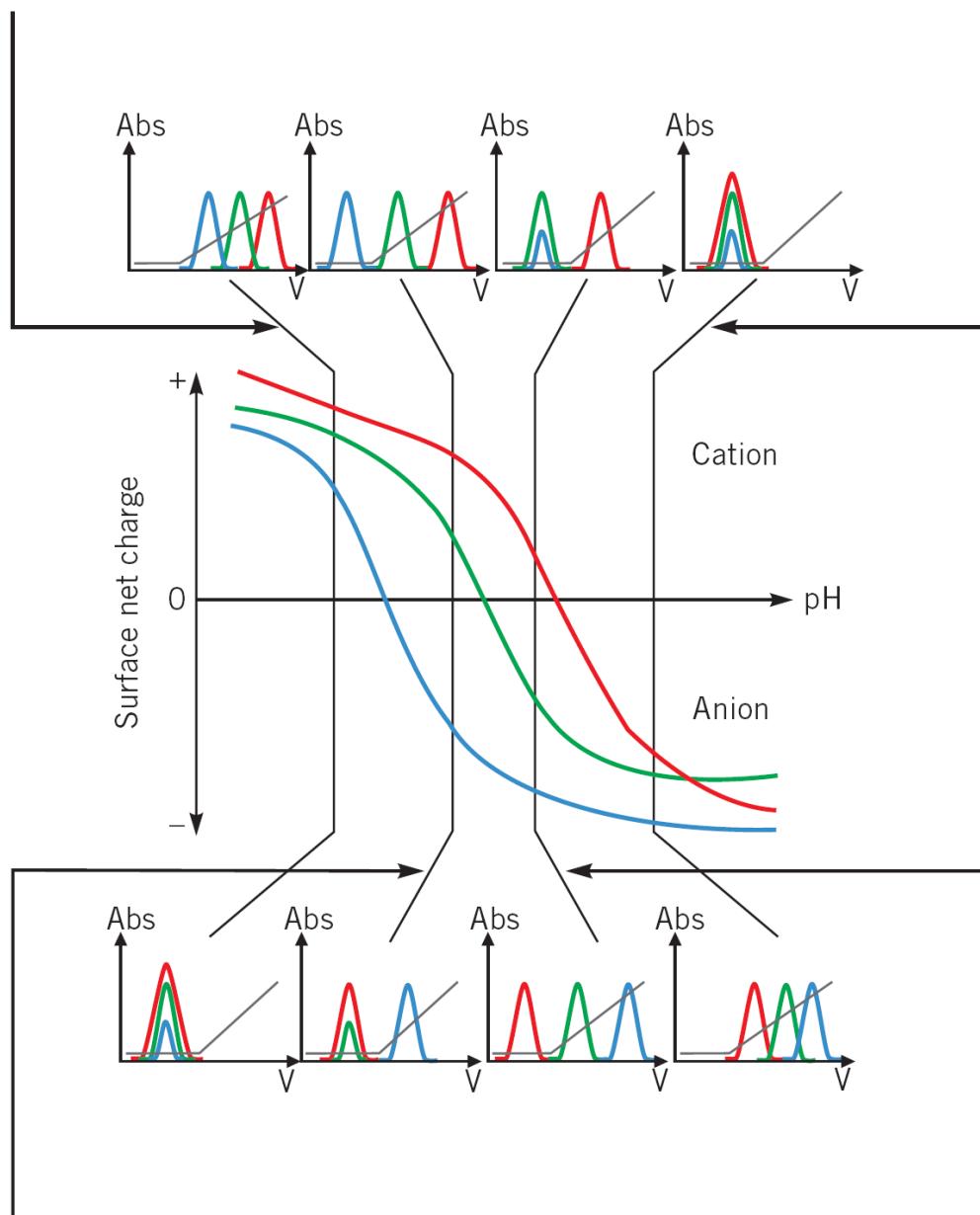


**Fig. 1.15.** Principles of an anion exchange separation.

## Selectivity and buffer pH

Most acidic pH: all three proteins are below their isoelectric point, positively charged, and bind only to a cation exchanger. Proteins are eluted in the order of their net charge.

Most alkaline pH: all three proteins are above their isoelectric point, negatively charged, and bind only to the anion exchanger. Proteins are eluted in the order of their net charge.



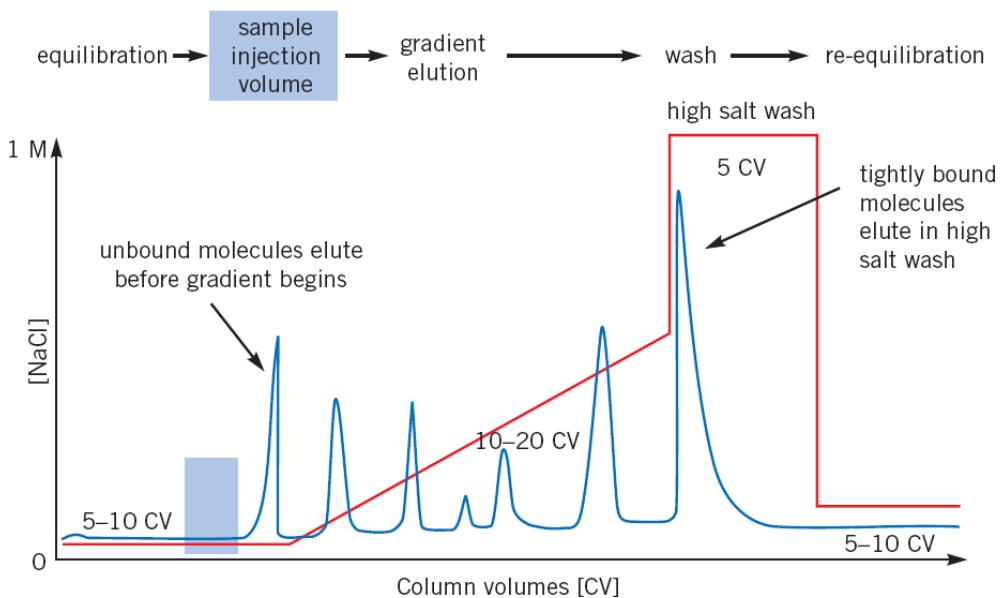
Less acidic pH: blue protein is above its isoelectric point, negatively charged, other proteins are still positively charged. Blue protein binds to an anion exchanger and can be separated from the other proteins which wash through. Alternatively, red and green proteins can be separated on a cation exchanger and the blue protein washes through.

Less alkali pH: red protein below its isoelectric point, positively charged. Red protein binds to cation exchanger and can be separated from the other proteins which wash through. Alternatively, blue and green proteins can be separated on an anion exchanger and the red protein washes through.

**Fig. 1.16.** Effect of pH on protein binding and elution patterns.

## Selectivity and elution

The figure below illustrates a common form of IEX separation in which proteins are eluted by increasing the ionic strength of a buffer (typically with NaCl) using a *linear gradient*. The UV absorbance and conductivity traces show the elution of protein peaks and the changes in salt concentration, respectively, during elution. Buffer volumes used during sample application, elution, washing and re-equilibration are expressed in *column volumes*, for example 5 CV = 5 ml for a column with a 1 ml bed volume. Linear ionic strength gradients, as shown in Fig. 1.17, are the most frequently used type of elution and should always be used when starting with an unknown sample (when as many components as possible are bound to the column and eluted differentially to see a total protein profile). At low ionic strengths, competition for charged groups on the IEX medium is at a minimum. Increasing the ionic strength increases competition and reduces the interaction between the medium and the bound substances which begin to elute. The elution buffer is usually the same buffer salt and pH as the start buffer, but contains additional salt, most often sodium chloride.



**Fig. 1.17.** Typical high resolution IEX separation using linear gradient elution (25–45 column volumes).

## Matrix

Modern IEX media use either polymer or agarose-based matrices to fulfil not only the requirements for high binding capacity, chemical and physical stability, but to generate media with suitable particle sizes for a range of applications.

## Functional groups

The functional groups substituted onto a chromatographic matrix (Table 1.4) determine the charge of an IEX medium i.e. a positively-charged anion exchanger or a negatively-charged cation exchanger.

**Table 1.4.** Functional groups used on ion exchangers.

Anion exchangers	Functional group
Quaternary ammonium (Q)	strong $-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak $-O-CH_2CH_2N^+H(CH_2CH_3)_2$
Diethylaminopropyl (ANX)*	weak $-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$
Cation exchangers	Functional group
Sulfopropyl (SP)	strong $-O-CH_2CHOHCH_2OCH_2CH_2SO_3^-$
Methyl sulfonate (S)	strong $-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM)	weak $-O-CH_2COO^-$

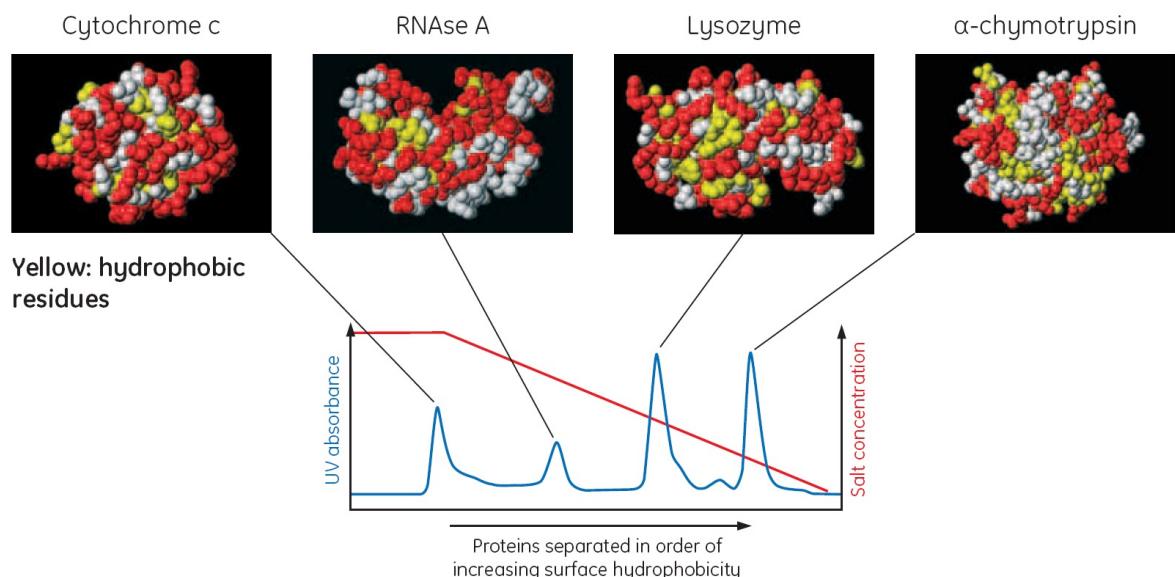
The terms strong and weak refer to the extent that the ionization state of the functional groups varies with pH. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins. Strong ion exchangers show no variation in ion exchange capacity with change in pH. These exchangers do not take up or lose protons with changing pH and so have no buffering capacity, remaining fully charged over

a broad pH range. The majority of proteins have isoelectric points within the range 5.5 to 7.5 and can be separated on either strong or weak ion exchangers. The counter-ions (salt ions) used in IEX are almost always  $\text{Na}^+$  for cation exchange and  $\text{Cl}^-$  for anion exchange.

### 1.6.7 Hydrophobic interaction chromatography (HIC)

HIC separates proteins according to differences in their surface hydrophobicity by utilizing a reversible interaction between these proteins and the hydrophobic surface of a HIC medium. There is no universally accepted theory on the mechanisms involved in HIC, even though a number of suggestions can be found in the scientific literature.

Fig. 1.18 shows how standard proteins with different degrees of surface hydrophobicity can be separated. The interaction between hydrophobic proteins and a HIC medium is influenced significantly by the presence of certain salts in the running buffer. A high salt concentration enhances the interaction while lowering the salt concentration weakens the interaction. In this example, all three proteins interact with the hydrophobic surface of the HIC medium, but, as the ionic strength of the buffer is reduced, the interaction is reversed and the protein with the lowest degree of hydrophobicity is eluted first. The most hydrophobic protein elutes last, requiring a greater reduction in salt concentration to reverse the interaction.



**Fig 1.18.** Proteins are separated according to differences in their surface hydrophobicity (yellow indicates hydrophobic and red hydrophilic amino acid residues).

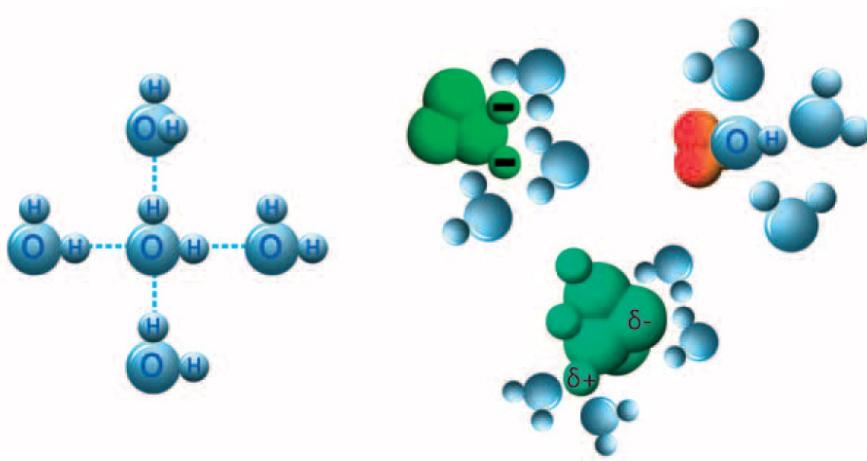
#### The role of water

Water is a good solvent for polar substances, but a poor solvent for non-polar substances. In liquid water a majority of the water molecules occur in clusters due to hydrogen bonding between themselves (Figure 18). Although the half-life of water clusters is very short, the net effect is a very strong cohesion between the water molecules, reflected, for example, by a high boiling point.

At an air-water interface, water molecules arrange themselves into a strong shell of highly ordered structure. Here, the possibility to form hydrogen bonds is no longer in balance, but is dominated by the liquid side of the interface. This gives rise to an ordered structure that manifests itself as a strong surface tension.

Anything that influences the stability of the water shell also affects the surface tension.

When a hydrophobic substance such as a protein or hydrophobic ligand is immersed in water something analogous to the surface tension phenomenon happens. The water molecules cannot “wet” the surface of the hydrophobic substance. Instead they form a highly ordered shell around the substance, due to their inability to form hydrogen bonds in all directions. Minimizing the extent of this shell leads to a decrease in the number of ordered water molecules, that is, a *thermodynamically more favourable situation in which entropy increases*. In order to gain entropy, hydrophobic substances are forced to merge to minimize the total area of such shells. Thus hydrophobic interaction depends on the behaviour of the water molecules rather than on direct attraction between the hydrophobic molecules (Fig. 1.19).



**Fig 1.19.** The solubilising properties of water reside in its ability to interact with dipoles and to form hydrogen bonds.

### Protein structure

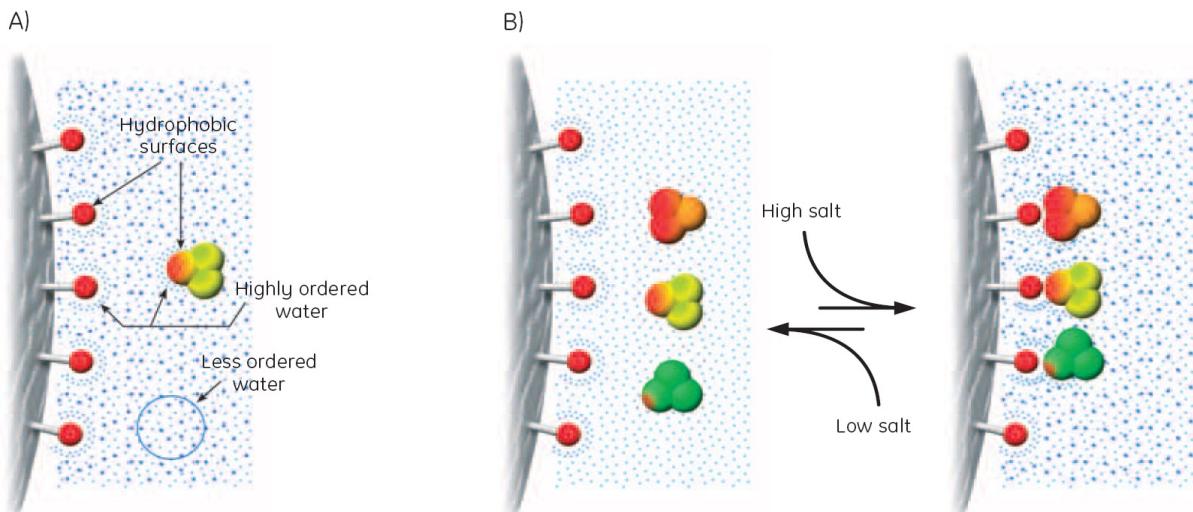
The three-dimensional structure of a protein is a result of intra-molecular interactions as well as interactions with the surrounding solvent. In the case of readily soluble proteins, this solvent is water, and hydrophobic side chains are therefore typically driven to the interior of the protein. The final structure is a result of a thermodynamic compromise that best suits the surrounding solution so that, although hydrophobic amino acid residues are most frequently buried in the interior of globular proteins, some are exposed, resulting in hydrophobic patches on the protein surface.

Since proteins carry both hydrophilic and hydrophobic areas on their surfaces they may precipitate at high concentrations of certain salts, mainly caused by *enforced hydrophobic interaction*. Changes in ionic strength, the presence of organic solvents, temperature and pH (especially at the isoelectric point, *pl*, when there is no net surface charge) can all affect protein structure and solubility and, consequently, the interaction with other hydrophobic surfaces, such as those in HIC media.

### Reversible interactions

The hydrophobic ligands on HIC media can interact with the hydrophobic surfaces of proteins. In pure water any hydrophobic effect is too weak to cause interaction between ligand and proteins or between the proteins themselves. However, certain salts enhance hydrophobic interactions, and adding such salts brings about binding (adsorption) to HIC media. For selective elution (desorption), the salt concentration is lowered gradually and the sample components elute in order of hydrophobicity (Fig. 1.20).

The final result of a HIC separation is based therefore on interplay between the prevalence and distribution of surface-exposed hydrophobic amino acid residues, the hydrophobicity of the medium, the nature and composition of the sample, and the type and concentration of salt used in the buffers.



**Fig 1.20.** A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

### Steps in a HIC separation

HIC media are composed of *ligands* containing alkyl or aryl groups coupled to an inert *matrix* of spherical particles. The matrix is porous, in order to provide a high internal surface area, while the ligand plays a significant role in the final hydrophobicity of the medium.

Interaction between the protein and the medium is promoted by moderately high salt concentrations, typically 1–2 M ammonium sulfate or 3 M NaCl. The type of salt and the concentration required in the start buffer are selected to ensure that the proteins of interest bind to the medium and that other less hydrophobic proteins and impurities pass directly through the column.

When sample loading is completed and the column has been washed so that all non-bound proteins have passed through, conditions are altered to begin elution.

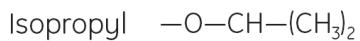
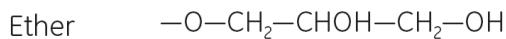
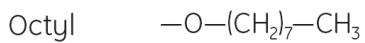
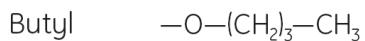
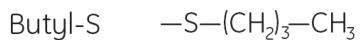
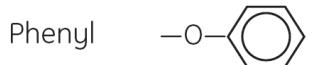
Proteins are eluted by *decreasing the salt concentration* in the elution buffer. As the level of salt decreases those proteins with the lowest hydrophobicity begin to elute from the column. By controlling changes in salt concentration using gradients, proteins are eluted differentially in a purified, concentrated form. Those proteins with the highest degree of hydrophobicity will be most strongly retained and will be eluted last (Fig. 1.21).

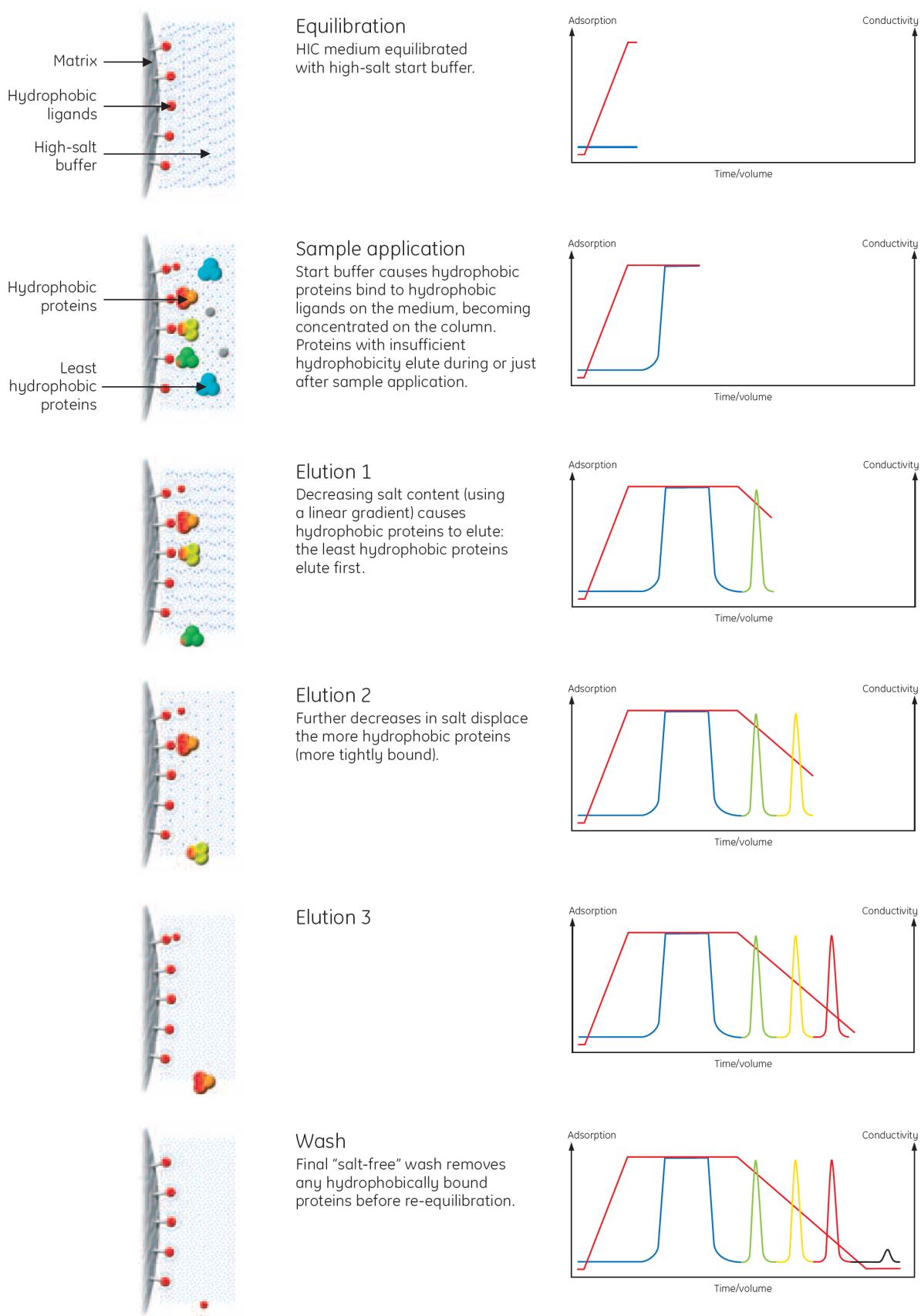
### Matrix

Modern HIC media use either polymeric or agarose-based matrices to fulfil the requirements for chemical and physical stability, high binding capacity and different particle sizes.

The most common hydrophobic ligands are shown in Table 1.4.

**Table 1.4.** Ligands substituted on HIC media.





**Fig. 1.21.** Steps in a HIC separation.

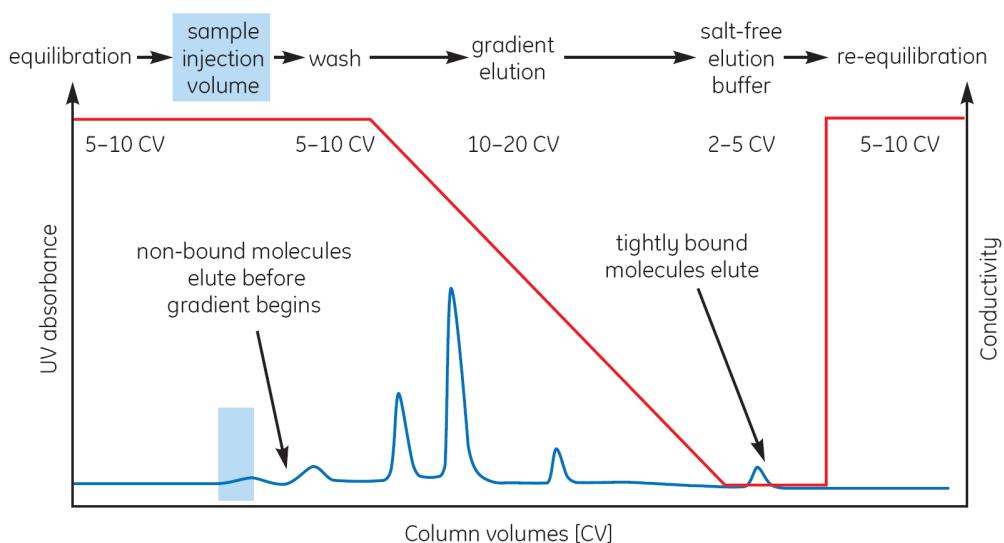
In general, HIC media fall into two groups, depending on their interactions with sample components. Straight alkyl chains (butyl, octyl, ether, isopropyl) show a "pure" hydrophobic character, while aryl ligands (phenyl) show a mixed-mode behaviour in which both aromatic and hydrophobic interactions, as well as lack of charge, play a role in the final chromatographic properties.

### Selectivity and elution

Fig. 1.22 illustrates a common form of HIC separation in which proteins are eluted by decreasing the salt content of a buffer using a *linear gradient*. The UV absorbance and conductivity traces show the elution of protein peaks and the changes in salt concentration, respectively, during elution.

Buffer volumes used during sample application, elution, washing and re-equilibration are expressed in *column volumes*, for example 5 CV = 5 ml for a column with a 1 ml bed volume.

Linear salt gradients, as shown in Fig. 1.22, are most frequently used for elution. Always use a linear gradient when starting with an unknown sample (when as many components as possible are bound to the column and eluted differentially to see a total protein profile). Decreasing the salt content of the running buffer weakens the hydrophobic interactions, and bound substances begin to elute. The elution buffer is usually the same buffer and pH as the start buffer, but without the high salt component.



**Fig 1.22.** Typical HIC separation using linear gradient elution.

### 1.6.8 Reversed phase chromatography (RPC)

RPC has become increasingly important for high-resolution separation and analysis of proteins, peptides and nucleic acids. The technique is ideal for applications such as peptide mapping or purity checking and is often used for final polishing of oligonucleotides and peptides.

RPC separates molecules according to differences in their hydrophobicity. In theory, HIC and RPC are closely related techniques since both are based upon interactions between hydrophobic patches on the surface of biomolecules and the hydrophobic surfaces of a chromatography medium. However, in practice, the techniques are very different. The surface of an RPC medium is usually more hydrophobic than that of a HIC medium. This leads to stronger interactions that, for successful elution, must be reversed using non-polar, organic solvents such as acetonitrile or methanol. HIC media offer an alternative way of exploiting the hydrophobic properties of biomolecules by working in a more polar and less denaturing environment.

### Terminology

Certain terminology occasionally associated with RPC reflects the developmental history of the technique. The term "reversed phase" derives from "normal phase" chromatography, a technique utilizing a hydrophilic *stationary phase* together with *mobile phases* consisting of organic solvents such as hexane or methylene chloride. In RPC the stationary phase is hydrophobic so that a water/organic solvent mobile phase is used, that is, the stationary phase is more hydrophobic than the mobile phase.

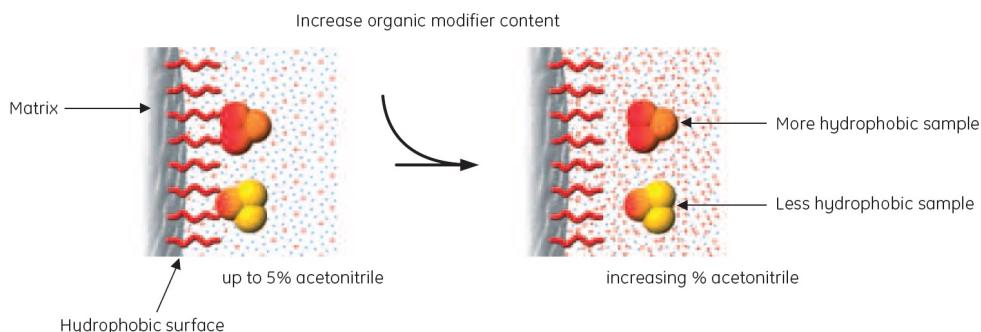
RPC media may be referred to as *adsorbents* while eluent solutions may be referred to as *mobile phases*.

### RPC in theory

The separation of biomolecules by RPC depends on a *reversible hydrophobic interaction* between sample molecules in the eluent and the medium. Initial conditions are primarily aqueous, favouring a high degree of organized water structure surrounding the sample molecule. Frequently, a small percentage of organic

modifier, typically from 3–5% acetonitrile, is present in order to achieve a “wetted” surface. As sample binds to the medium, the hydrophobic area exposed to the eluent is minimized.

Separation relies on sample molecules existing in an equilibrium between the eluent and the surface of the medium. The distribution of the sample depends on the properties of the medium, the hydrophobicity of the sample and the composition of the eluent (mobile phase), as illustrated in Fig. 1.23. Initially, conditions favor an extreme equilibrium state where essentially 100% of the sample is bound. Since proteins and peptides carry a mix of accessible hydrophilic and hydrophobic amino acids and are rather large, the interaction with the medium has the nature of a multi-point attachment.



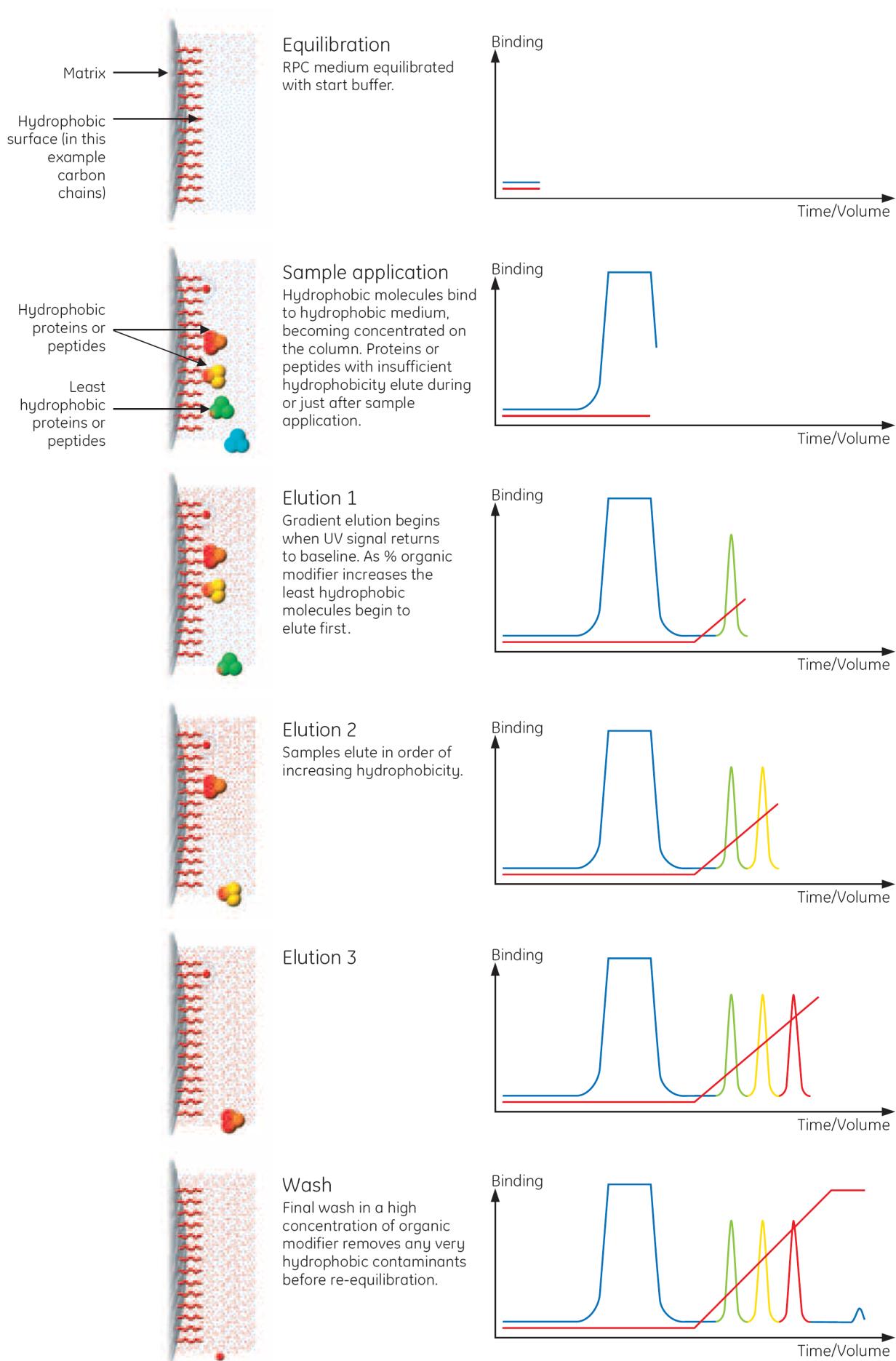
**Fig. 1.23.** Proteins and peptides bind to an RPC medium under aqueous conditions and elute as the eluent becomes more hydrophobic.

To bring about *elution* the amount of organic solvent is increased so that conditions become more hydrophobic. Binding and elution occur continuously as sample moves through the column. The process of moving through the column is slower for those samples that are more hydrophobic. Consequently, samples are eluted in order of *increasing hydrophobicity*.

#### Steps in an RPC separation

There are two main types of RPC media, one based on a hydrophilic matrix of *silica beads* covered with a bonded *hydrophobic* phase of carbon chains, typically n-alkyl or aromatic hydrocarbons, and one based on a naked, *hydrophobic polymer* matrix. Highly porous matrices provide a large internal surface area for high binding capacity. Matrices with uniform particle size can be used at higher flow rates. As with other chromatography techniques, an RPC medium is packed into a column to form a *packed bed*. The bed is then equilibrated with eluent to fill the matrix pores and the space in between the particles. A typical biological sample contains a complex mixture of molecules with a correspondingly diverse range of hydrophobicity. Most biomolecules are sufficiently hydrophobic to bind strongly to RPC media under aqueous conditions, in the presence of a low concentration of organic modifier, and to elute within a very narrow window of organic modifier concentration. *Gradient elution* is, therefore, the most practical method for RPC separation of complex biological samples.

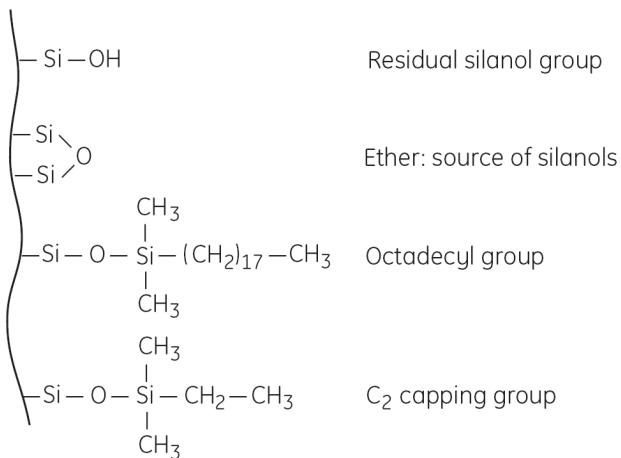
The key stages in a separation are shown in Fig. 1.24. Sample is applied under conditions that favour binding, typically using an aqueous solution containing an *ion-pairing agent*, such as trifluoroacetic acid (TFA), to enhance the hydrophobic interaction and a low concentration of organic modifier such as 5% acetonitrile. After application, and when all non-bound molecules have passed through, conditions are altered in order to elute the bound sample. Elution begins by increasing the concentration of *organic modifier*, such as acetonitrile. Molecules with the lowest hydrophobicity will elute first. By controlling the increase in organic modifier, molecules are eluted differentially. Those molecules with the highest degree of hydrophobicity will be most strongly retained and eluted last.



**Fig. 1.24.** Steps in an RPC separation using gradient elution.

### The silica matrix

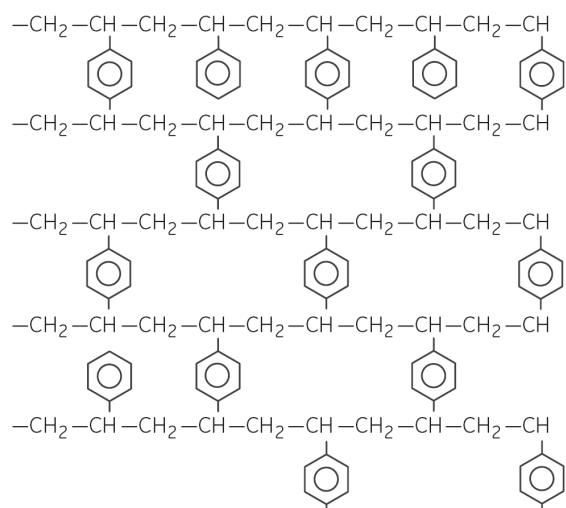
Any matrix used for a binding/elution technique must be chemically and physically stable and preferably porous to ensure adequate binding capacity. Historically, silica was one of the earliest materials to be used. Typically, silica particles are coupled via silanol groups to hydrophobic ligands of various chain lengths and surface density, as illustrated in Fig. 1.25. Most commonly, synthetic peptides, shorter peptides and oligonucleotides are separated on C18 ligands.



**Fig. 1.25.** Some typical structures on the surface of a silica-based RPC medium. The hydrophobic octadecyl group is one of the most common ligands.

### The polymer matrix

Synthetic organic polymers, such as beaded polystyrene, provide excellent chemical stability, particularly under strongly acidic or basic conditions (from pH 1 to pH 12). These stable matrices offer key advantages when separating complex mixtures of proteins or peptides (Fig. 1.26).



**Fig. 1.26.** Partial structure of a polystyrene-based RPC medium.

### Elution: organic modifiers

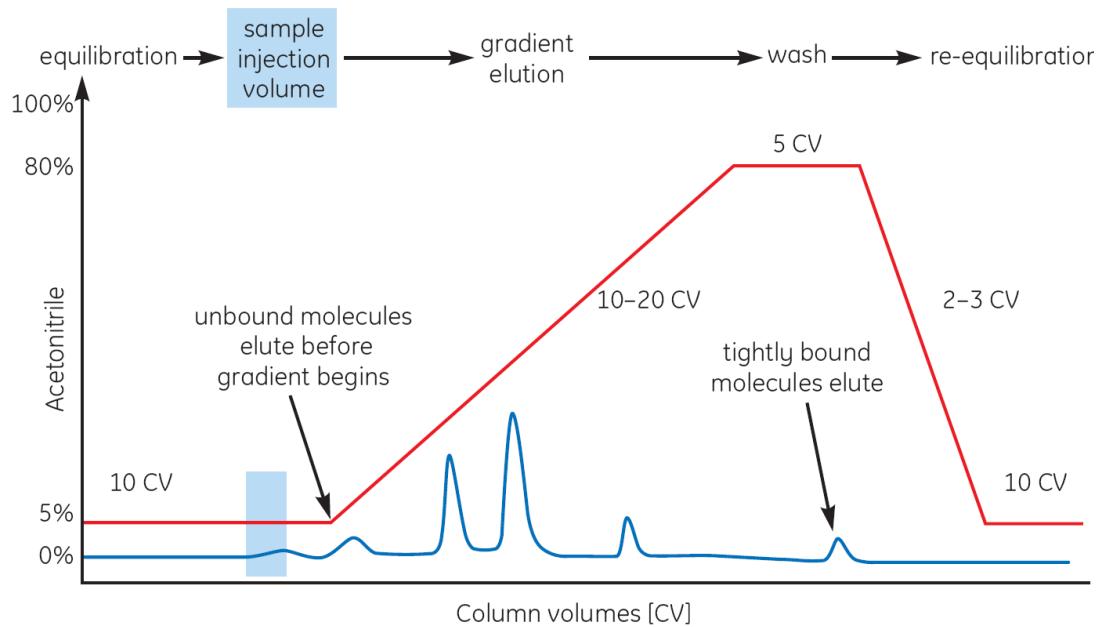
To bring about elution, an organic modifier is added to the eluent in order to increase the elution strength. The organic modifier must be miscible in water and UV transparent to enable detection of the eluting molecules. The boiling point must be sufficiently low to enable evaporation of the modifier after elution. Table 1.5 reviews the commonly used modifiers in terms of their suitability for protein and peptide separations.

**Table 1.5.** Acetonitrile is the preferred organic modifier for protein and peptide separations.

Organic modifier	Suitability	Boiling point (°C)	UV cut-off (nm)	Viscosity (cP at 20°C)	Comments
Methanol	Organic small molecules	65	210	Medium- low: 0.60	May destabilize protein structure.
Ethanol	Organic small molecules and peptides	78	205	Medium- low: 1.20	May destabilize protein structure.
2-propanol	Proteins Peptides	82	210	High: 2.30	Least effect on protein structure.
Acetonitrile	Organic small molecules Proteins Peptides	82	190	Low: 0.36	Most effect on protein structure. More powerful denaturant than alcohols. Toxic.

### Elution

*Gradient elution* is used most frequently for preparative and analytical, high-resolution separations of proteins and peptides, in order to minimize separation times. The UV absorbance and theoretical gradient traces shown in Fig. 1.27 represent the elution of sample components and the increase in concentration of organic modifier in the eluent (%B) during gradient elution.



**Fig 1.27.** Typical RPC separation using gradient elution.

## 1.7 Handling and Storage of Proteins

Proteins are only soluble when correctly folded. Protein folding is critically dependent on pH and salt strength. For example when a protein is resuspended in pure water, it is likely to denature and precipitate.

Proteins are subject to thermal denaturation and proteolysis by contaminating proteases. Therefore proteins are kept on ice where possible. In addition protease inhibitors can be added (see Table 1.6).

Additional stabilization of proteins to withstand conditions that lead to denaturation has been found necessary in many cases. A widely used method is the inclusion of glycerol in buffer solutions. Levels of glycerol from 10–50% v/v have been used with the higher concentrations reserved for storage. Protocols run in the presence of glycerol as a stabilizer are more time consuming due to the high viscosity of glycerol.

High salt concentrations stabilize enzymes and generally inhibit proteases. Many enzymes are typically supplied in 50% ammonium sulfate. If an ammonium sulfate fractionation is being carried out, leave

the protein in as high a concentration of ammonium sulfate as possible, that is to say, store the material as a "wet pellet".

Dilute enzyme solutions lose activity quickly. Therefore, try to store the protein at a concentration of at least 1 mg/ml. A small amount of purified protein can adsorb to the walls of the container. Extreme dilution of protein may lead to instability. The addition of bovine serum albumin (BSA) can act as an enzyme stabilizer. Used at concentrations up to 1 mg/ml, BSA will prevent the adsorption of the protein of interest onto container walls and increase the stability of proteins.

**Table 1.6.** Protease inhibitors

Inhibitor	specificity	effective conc
leupeptin	serine & cysteine proteases	10-100 $\mu$ M
antipain	serine & cysteine proteases	1-100 $\mu$ M
pepstatin A	acidic proteases	1-5 $\mu$ M
aprotinin	serine proteases	50 $\mu$ g/mL
PMSF	serine & cysteine proteases	0.1-2 mM
benzamidine HCl	serine proteases	100 $\mu$ g/mL
Na <sub>2</sub> -EDTA	metalloproteases	5-10 mM
Na <sub>2</sub> -EGTA	Ca(II)-Proteases	5-10 mM
Bestatin	aminopeptidases	1-150 $\mu$ M
MG132	26S proteasome	0.1-50 $\mu$ M

### **Frozen Storage**

During the freezing process many events occur. First, free water freezes and ice crystals grow which can be destructive to membranes and organelles. The least soluble solute will then precipitate. If the solute is one component of the buffer, the pH will markedly change before complete solidification takes place. The higher the protein concentration relative to buffer salts, the more it will be capable of acting as a buffer itself and counteracting drastic pH shifts that may occur during freezing.

If the temperature is low enough, all degradative processes stop, and the sample can theoretically be kept indefinitely. A temperature below -50°C reached as quickly as possible is recommended (snap freezing in liquid nitrogen). Normal deep freeze temperatures are usually suitable for overnight storage. Care should be taken when using a frost-free freezer as the defrost cycles could damage the protein. Freezing at -10° to -15°C is probably not better than not freezing at all. Long term storage of proteins should be done in a -80 °C freezer. When thawing, the rule is the faster the better. Immerse the container in warm 30-40°C water shaking frequently. Remove the tube when there is still a small piece of ice remaining.

Try to avoid repeated cycles of freezing and thawing. Store purified proteins in small portions and thaw individual samples once, as needed. Alternatively, the protein may be stored under conditions in which it does not freeze, such as high glycerol concentration. Many restriction endonucleases used in molecular biology are supplied as 50% v/v glycerol solutions.

### **Protecting Cysteine Residues**

Cysteine residues are susceptible to modification, especially oxidation, during purification and storage. Normally, within the living cell in a reducing atmosphere, the presence of other sulphhydryl-containing molecules like glutathione, protect these groups. However, when exposed to high oxygen tensions, several reactions are possible including disulfide bond formation, partial oxidation to a sulfinic acid, and irreversible oxidation to a sulfonic acid.

Formation of a disulfide bond requires another sulphhydryl group to be in the vicinity. Disulfide bond formation is accelerated in the presence of divalent cations which activate oxygen molecules and complex with sulphhydryls. For these reasons two protective actions are routinely taken. Metal ions are removed from the solution with the inclusion of the chelating agent EDTA. A sulphhydryl-containing reagent such as  $\beta$ -mercaptoethanol (2-ME) or dithiothreitol (DTT, Cleland's reagent) is added to the buffer. Routinely, 2-ME at 5–10 mM or DTT at 1–5 mM is added to the buffer along with 0.1–0.2 mM EDTA.

However, it should be noted that  $\beta$ -mercaptoethanol, DTT and EDTA should be avoided when performing Ni-NTA chromatography. The reducing agents react with Ni<sup>2+</sup> to form brown precipitates. EDTA will chelate the Ni<sup>2+</sup> and thereby strip the column of the active affinity group.

# 1.8 Spectrophotometry

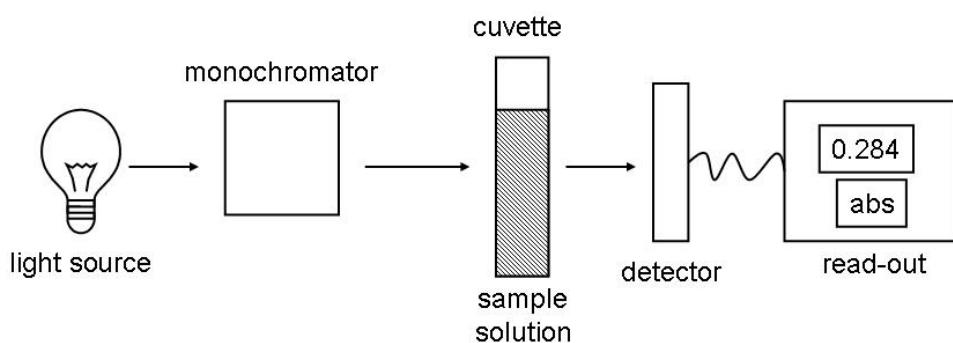
## 1.8.1 Introduction

Molecules in solution absorb light of a certain wavelength. This property allows to measure concentrations in solutions, and sometimes to identify (in)organic molecules by their absorbance spectrum. Absorbance of light of a certain wavelength selected from the UV, visible or infrared spectrum by molecules in solution can be accurately measured using a spectrophotometer. Pigments and colorants absorb light in the visible spectrum: 380-760 nm. Many other compounds absorb light from the ultra-violet or infrared spectrum.

Certain electron configurations in a molecule are responsible for absorbance of light of a particular wavelength. As a consequence each compound has a characteristic **absorbance spectrum**, which is the absorbance as a function of light wavelength. Based on the absorbance spectrum the type of compound and sometimes the exact identity can be determined. The wavelength at which a compound shows the highest absorbance is called the **absorbance maximum**. Light absorbance by a compound in solution increases with concentration. This allows the determination of concentrations using a spectrophotometer.

A spectrophotometer can also be used to measure light dispersion by particles in solution. These particles reduce the amount of light emitted at the other end of the cuvette and this reduction is proportional to the amount of particles. In this way the concentration of bacterial or yeast cells in a culture can be measured, which, e.g., makes it possible to measure the growth curve. Light dispersion does not only depend on particle concentration, but also on particle size. The bigger the particles, the higher the dispersion. Large protein molecules cause already a measurable light dispersion at 220 nm while the solution is still clear to the eye.

In this practical course we are using Thermo Scientific Genesys 20 Spectrophotometers. A schematic representation is shown in Fig. 1.28. The light source is a tungsten-halogen light bulb with an emission of 325-1100 nm. The light is focussed on a diffraction grating or monochromator. This device dissects the light in narrow wavelength portions with certain cut-offs (2 nm). Depending on the positioning of the monochromator a certain wavelength is selected, resulting in a beam of monochromatic light, which travels through the sample. The solution is placed in a cuvette, a container with certain dimensions made of plastic, regular glass or quartz glass. A standard cuvette has an optical length of 10 mm. Light that has not been absorbed by the solution in the cuvette is detected by a luminometer which converts radiation energy in electric energy. Measurements in the UV spectrum require a deuterium lamp, which has an emission between 200-400 nm. Measurements in the UV spectrum also require quartz cuvettes, since plastic and regular glass are impermeable to UV light. For measurements in the visible spectrum of aqueous or alcoholic solutions plastic cuvettes are suitable, whereas with other solvents glass cuvettes are necessary.



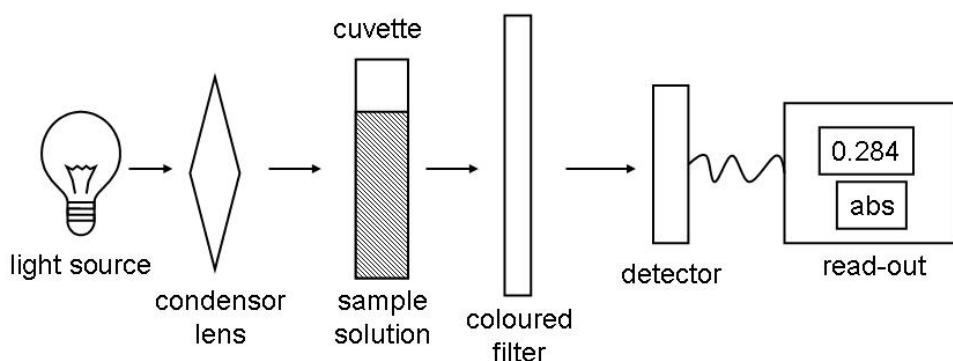
**Fig. 1.28.** Components of a spectrophotometer.

Often concentrations of compounds in solution can be measured with a photo- or colorimeter. This instrument is much simpler, since it does not possess a diffraction grating, but instead uses a set of exchangeable coloured filters that each selects a specific wavelength. Such an instrument is suitable for measurement of coloured solutions and suspensions, such as colour reactions in enzyme measurements and haemoglobin in blood. With a limited set of filters the concentrations of many standard lab chemicals can be measured.

The light source of a colorimeter is usually a tungsten light bulb (Fig. 1.29). The light is focussed by a condenser lens, which generates a parallel light beam. This light beam passes through the sample

solution, then through a coloured filter after which it is detected. The detector generates an electric potential, which is directly proportional to the amount of light. This electric potential is sent to a read-out unit, which displays the value in a logarithmic fashion.

The colorimeter is not suitable for the detection of a single compound in a complex mixture and also is less accurate than a spectrophotometer.



**Fig 1.29.** Components of a colorimeter.

### 1.8.2 Absorbance measurements

The relationship between the concentration of a dissolved compound and light absorbance is given by the law of **Beer-Lambert**:

$$A = \epsilon \cdot c \cdot l$$

Where

$A$  = absorbance

$\epsilon$  = molar absorbance coefficient of the compound ( $\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ )

$c$  = concentration of the compound ( $\text{mol} \cdot \text{L}^{-1}$ )

$l$  = length of the light path through the solution (cm)

(for standard cuvettes the length = 1)

To accurately determine the concentration of a compound, a **reference graph** is required. A reference graph is constructed by measuring the absorbance of a set of solutions with known concentrations of the compound of interest. To make the graph  $A$  is plotted against the concentration, resulting in a straight line with slope  $\epsilon$ . The concentration corresponding to the measured absorbance from an experimental sample can then be read from the graph. Besides absorbance, the Genesys 20 spectrophotometer can also read transmission. Therefore there are two scales, which can be read: the  $A$ - and the  $T$ -scale.

**Transmission** is the ratio between the intensity of the transmitted light beam and the intensity of the incoming light beam. The fraction of light that passes through the solution is called the transmission ( $T$ ):

$$T = I / I_0$$

where  $I$  is the intensity of the transmitted light beam and  $I_0$  the intensity of the incoming light beam.  $T$  is usually expressed as a percentage:

$$\%T = I / I_0 \times 100$$

The absorbance  $A$  (in the Netherlands also called extinction or  $E$ ), is a logarithmic function of  $T$ :

$$A = \log [1/T] = \log [I_0 / I] = \epsilon \cdot c \cdot l$$

At 100% transmission (transparent solution):  $A = \log 1 = 0$

When a solution transmits 10% light, then  $A = \log (1/0.1) = \log 10 = 1$

Absorbance measurements are only reliable if more than 10% and less than 90% light is transmitted. Absorbance measurements above 1.0 and below 0.1 are unreliable.

The law of Beer-Lambert only applies to monochromatic light, since the molar absorbance coefficient  $\epsilon$  depends on the wavelength. The same law can be used to approximate light dispersion by particles in solution. Because this is a fundamentally different process compared to absorbance, light dispersion is indicated as **optical density (OD)**.

Deviations from the law of Beer-Lambert occur at:

- high concentrations, above  $A = 0.8$
- low concentrations, below  $A=0.1$
- turbid solutions
- fluorescent compounds (absorbed energy is emitted again at another wavelength)
- reflection by the cuvette

Also light from outside or from a malfunctioning monochromator can be a source of errors. Light with other wavelengths than the selected wavelength is called stray light. Stray light results in a lower absorbance measurement.

## 1.9 Enzyme kinetics

### 1.9.1 Activity and units

Enzymes are proteins with catalytic properties. They lower the activation energy for chemical reactions, and thereby increase reaction speed. The compound that is converted is called the **substrate**. Each enzyme is very specific and only catalyzes a single reaction under specific conditions, like pH, temperature etc.

Enzymes often cooperate to perform a complex series of reactions, e.g. in the series of reactions that convert glucose in pyruvate (glycolysis).

Enzymes are classified in groups according to the reaction they catalyze. The name consists of the letters EC (Enzyme Commission), followed by 4 numbers. Each enzyme also has a trivial name, usually ending on -ase. For example, EC 1.1.1.1. is alcohol dehydrogenase, EC 1.1.1.27 is lactate dehydrogenase, EC 3.1.27.5 is Ribonuclease A, etc.

Enzyme activity is usually expressed in **katals**. One katal (abbreviation kat) is the amount of enzyme that converts under specific conditions per second 1 mole of substrate. This is a large unit, and usually people work with nanokatal ( $nkat = 10^{-9}$  kat) or picokatal ( $pkat = 10^{-12}$  kat).

The **reaction rate** of an enzyme, which is the amount of moles of substrate converted per second is indicated with the letter  $v$ , expressed in the units mol/s. It will be clear that  $v$  totally depends on substrate concentration. In addition other factors such as temperature, pH and salt strength play important roles. The **maximum reaction rate**, which is theoretically achievable under ideal conditions, is indicated with  **$V$**  (previously with  $V_{max}$ ). This value is still dependent on temperature and pH etc., but is independent of substrate concentration. Another important notion is **enzyme activity**, which is the amount of active enzyme present in a certain preparation (e.g. a commercial vial of pure enzyme, or in a self-prepared protein extract). Enzyme activity is also expressed as katals (with some exceptions, see below). Since enzyme activity in practice is dependent on substrate concentration, the nominal activity of an enzyme is expressed as the value  $V$ , which is independent of substrate concentration.

For enzymes with substrates with unknown or variable molecular weights, such as for example restriction enzymes that can cut DNA molecules of different sizes, the activity is expressed in Units (U). The definition of a unit depends on the enzyme. For a restriction enzyme, 1 unit is defined as the amount that cuts 1  $\mu$ g DNA of phage Lambda completely in 1 hour under the conditions specified by the manufacturer.

In addition to the total enzyme activity in a protein extract, the **specific activity** can be determined. Specific activity is defined as follows:

**Specific activity = amount of katals per mg protein.**

The specific activity is a measure for the purity of an enzyme. The higher the amount of other proteins in the extract besides the specific enzyme that you are interested in, the lower the specific activity. Specific activity is an important notion when different protein extracts are compared.

### 1.9.2 Enzyme kinetics

The kinetics of simple enzymatic reactions, which depend solely on substrate and enzyme concentrations and the specific properties of the enzyme, is described by equations (8) en (9) at the end, where (9) is the most useful and consequently the most used. Below an attempt is made to explain how these equations can be deduced in a relatively simple manner from the substrate and enzyme concentration [S] and [E] and by making a few simple assumptions.

To catalyze a reaction, the enzyme (E) and the substrate (S) must interact and form the enzyme-substrate complex ES:



Complex formation is reversible, and at the same time the ES complex dissociates into the initial enzyme E and the newly formed product P.

$k_{+1}$ ,  $k_{+2}$  en  $k_{-1}$  are the reaction constants of the respective reactions. The associated reaction rates are  $v_{+1}$ ,  $v_{+2}$  en  $v_{-1}$  respectively. Reaction  $R_{+1}$  leads to the formation of the ES complex, whereas reactions  $R_{+2}$  en  $R_{-1}$  lead to dissociation of the ES complex. Reaction  $R_{-2}$  does not occur at the beginning since there is no product P formed yet.

The total amount of enzyme,  $E_t$ , thus occurs in the form of the free enzyme E and as the complex ES:

$$[E_t] = [ES] + [E]$$

For the reaction rate  $v_{+1}$  the following is true:

$$v_{+1} = k_{+1} [E] \cdot [S] = k_{+1} ([E_t] - [ES]) \cdot [S] \quad (2a)$$

Similarly:

$$v_{-1} = k_{-1} \cdot [ES] \quad (2b)$$

$$v_{+2} = k_{+2} \cdot [ES] \quad (2c)$$

in which [ ] indicate that it concerns the concentration of the respective compounds.

Shortly after the start of the reaction, [ES] reaches a constant value dependent on the initial enzyme and substrate concentrations and on the intrinsic affinity of the enzyme for the substrate. In this so-called **steady state** the rate of formation of the ES complex equals the rates of dissociation.

$$v_{+1} = v_{-1} + v_{+2}$$

Substitution of (2a), (2b) and (2c) results in:

$$k_{+1} \cdot ([E_t] - [ES]) \cdot [S] = (k_{-1} + k_{+2}) \cdot [ES] \quad (3)$$

From this equation [ES] can simply be solved:

$$[ES] = \frac{[E_t]}{\frac{k_{+2} + k_{-1}}{k_{+1} \cdot [S]} + 1} \quad (4)$$

Substitution in (2c) results in the rate of product formation, or in other words the **reaction rate**  $v = v_{+2}$ :

$$v = \frac{k_{+2} \cdot [E_t]}{\frac{k_{+2} + k_{-1}}{k_{+1} \cdot [S]} + 1} \quad (5)$$

From (5) it follows that the **maximum reaction rate V** equals  $k_{+2} \cdot [E_t]$ , since the right part of the equation approximates  $k_{+2} \cdot [E_t]$  when [S] approximates infinity?

Therefore:

$$V = k_{+2} \cdot [E_t] \quad (6)$$

By definition the factor  $(k_{+2} + k_{-1}) / k_{+1}$  is called the **Michaelis-Menten constant  $K_M$** .

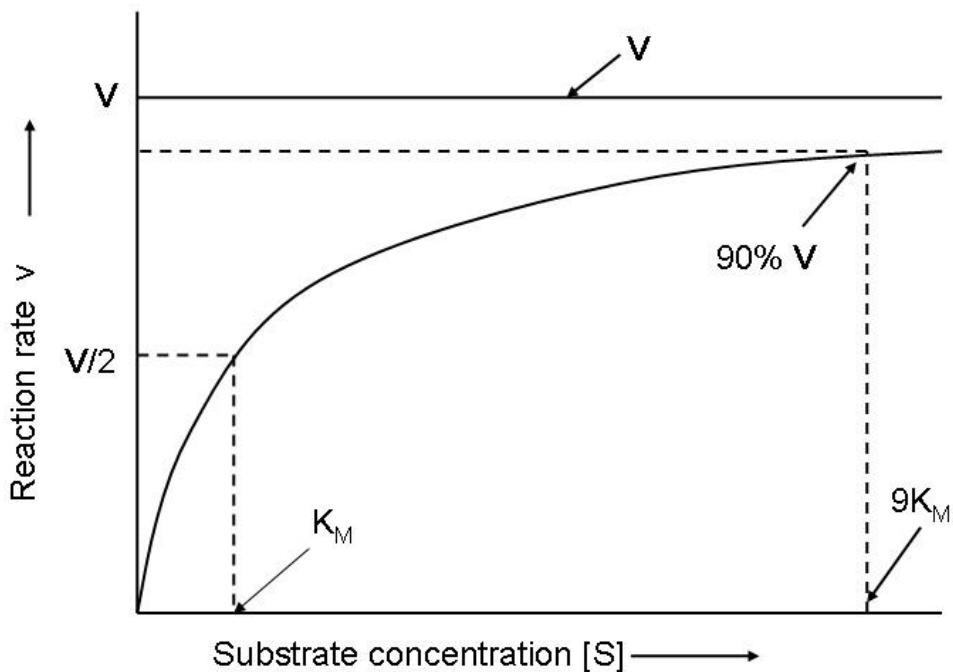
$$K_M = \frac{(k_{+2} + k_{-1})}{k_{+1}} \quad (7)$$

$K_M$  is often used as a characteristic value for an enzyme. It is a real constant, which is independent on  $[S]$  and  $[E]$ , and only dependent on pH, temperature etc.  $1/K_M$  is a measure of the affinity of an enzyme for its substrate.

We can write (5), by substituting (6) and (7), as:

$$v = \frac{V \cdot [S]}{K_M + [S]} \quad (8)$$

From (8) it follows that if  $v$  equals  $\frac{1}{2}V$ ,  $K_M$  equals  $[S]$ . Fig. 1.30 is a graph representing equation (8).



**Fig.1.30.** Relationship between the reaction rate  $v$  and the substrate concentration  $[S]$  in an enzymatic reaction with Michaelis-Menten kinetics.

The relationship between enzyme activity and substrate concentration is represented by a hyperbolic line. In practice one does not always find a hyperbolic relationship. In a mixture of isoenzymes the affinity of individual isoenzymes can differ strongly. Also at increasing substrate concentrations the phenomenon of substrate inhibition can occur. The more complicated kinetics of such cases will not be considered here.

From the hyperbolic line in Fig.1.30 one cannot precisely determine  $V$  and  $K_M$ . By converting equation (8) in the linear form  $y = ax + b$  this problem has been solved. **Lineweaver and Burk** have proposed in 1925 to plot  $1/v$  against  $1/[S]$ . Equation (8) is then transformed in:

$$\frac{1}{v} = \frac{K_M}{V} \cdot \frac{1}{[S]} + \frac{1}{V} \quad (9)$$

Since  $K_M$  and  $V$  are constants, this is the equation of a straight line, as shown in Fig. 1.31. The slope equals  $K_M/V$ , and  $1/V$  and  $1/K_M$  can be determined as the intercepts with the Y and X axes respectively.

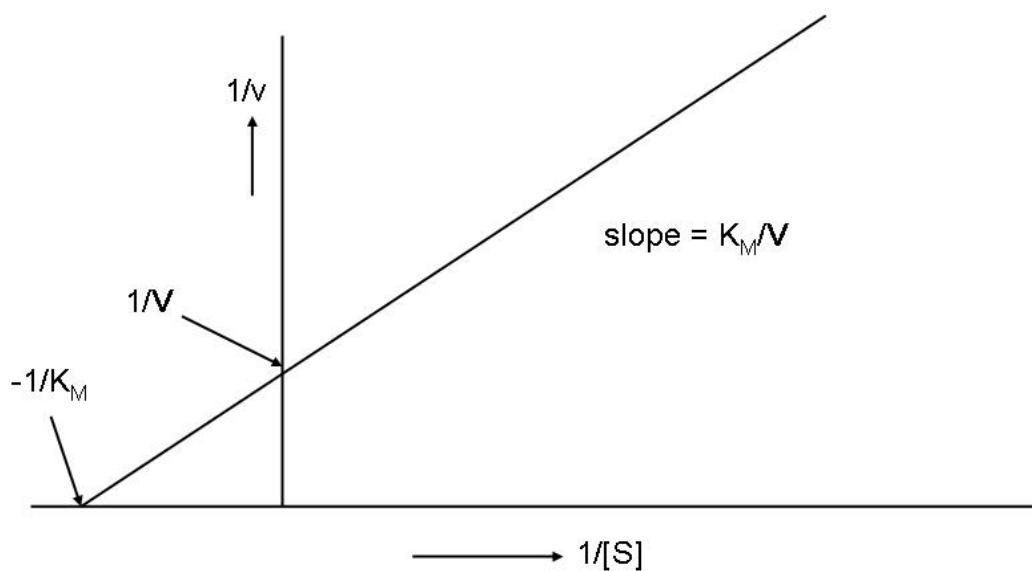


Fig. 1.31. Graph according to Lineweaver-Burk.

### 1.9.3 Enzyme inhibition

Specific compounds can inhibit enzyme activity. The catalytic functioning of the enzyme is reduced resulting in slower reaction rates. There are two main types of enzyme inhibition: **competitive** and **non-competitive** inhibition.

#### Competitive inhibition

Competitive inhibition occurs when substrate ( $S$ ) and inhibitor ( $I$ ) both bind to the same site on the enzyme. In effect, they compete for the active site and bind in a mutually exclusive fashion. Characteristic for competitive inhibition is that increasing the substrate ( $S$ ) concentration results in reduction of inhibition.

The Lineweaver-Burk plot offers a way to visualize the inhibition. In the presence of  $I$ ,  $V$  does not change, but  $K_m$  appears to increase. Therefore,  $1/K_m$ , the  $x$ -intercept on the plot will get smaller and  $-1/K_m$  gets closer to 0. Therefore, in comparison to the uninhibited Lineweaver-Burk plot, the plot will show a line, with the same  $y$  intercept ( $1/V$ ), but with the  $x$  intercept ( $-1/K_m$ ) closer to 0.

#### Non-competitive inhibition

A noncompetitive inhibitor is a substance that interacts with the enzyme, but usually not at the active site. The net effect of a non-competitive inhibitor is to change the shape of the enzyme and thus the active site, so that the substrate can no longer interact with the enzyme to result in a reaction. Non-competitive inhibitors may bind the enzyme in a reversible or irreversible fashion, but inhibition is not influenced by concentrations of the substrate, as is the case for a reversible competitive inhibitor.

Also non-competitive inhibition results in a characteristic Lineweaver-Burk plot. In the presence of  $I$ , just  $V$  will decrease. Therefore,  $1/V$  will get more positive while  $-1/K_m$  will stay the same.

Fig. 1.32 shows the influence of the different types of inhibitors on the Lineweaver-Burk plot.

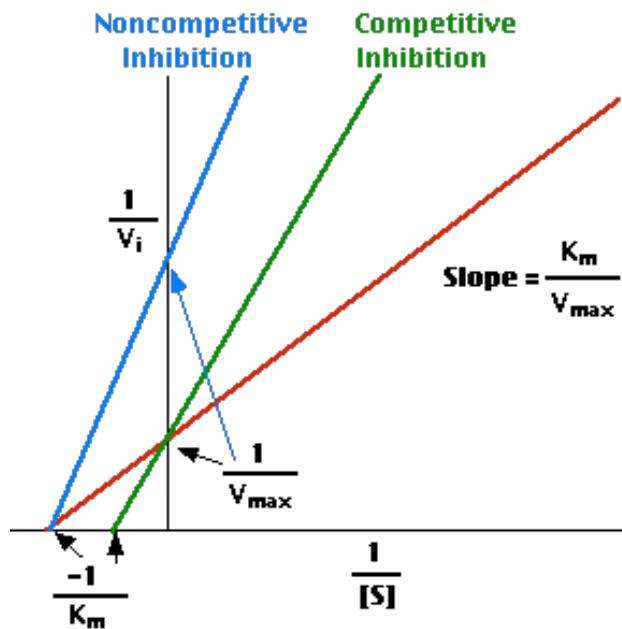


Fig. 1.32. Lineweaver-Burk plots of competitive and non-competitive inhibition.

## 1.10 Antibodies as Tools

Antibodies (also known as immunoglobulins, abbreviated Ig) are gamma globulin proteins that are found in blood or other bodily fluids of vertebrates, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses. They are typically made of basic structural units, consisting of two large heavy chains and two small light chains connected by disulfide bonds forming a Y-shaped molecule. Immunoglobulins can thus exist as monomers (one structural unit), dimers with two units or pentamers with five units.

Antibodies are produced by a type of white blood cell called a plasma cell, which is derived from a B-lymphocyte. B-cells do not produce antibodies until they become fully activated. Each B-cell has a unique receptor protein on its surface that will bind to one particular antigen. Once a B-cell encounters its cognate antigen and receives an additional signal from a T-helper cell, it can further differentiate into a plasma cell. B-cells exist as clones. All B-cells derive from a particular cell, and thus, the antibodies their differentiated progenies produce can recognize and/or bind the same components (epitope) of a given antigen. The great diversity in the immune response is the result of the fact that there are up to  $10^9$  clones, each specifically recognizing a different antigen.

Antibodies come in different varieties known as isotypes or classes. In placental mammals there are five antibody isotypes known as IgA, IgD, IgE, IgG and IgM, which differ in their biological properties, functional locations and ability to deal with different antigens. The antibodies in a typical antiserum used in molecular biology are of the IgG isotype.

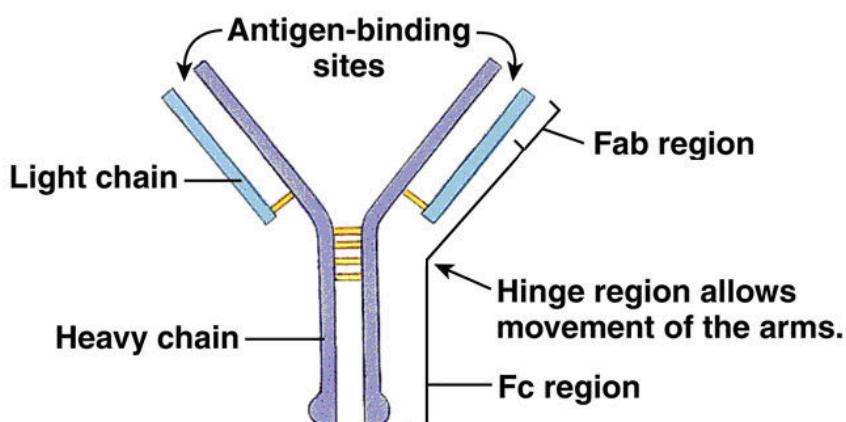


Fig. 1.33. Schematic representation of an antibody.

Although the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures, or antigen binding sites, to exist. This region is known as the hypervariable region. Each of these variants can bind to a different target, known as an antigen. This huge diversity of antibodies allows the immune system to recognize an equally wide diversity of antigens. The unique part of the antigen recognized by an antibody is called an epitope. These epitopes bind with their antibody in a highly specific interaction, called induced fit, which allows antibodies to identify and bind only their unique antigen in the midst of the millions of different molecules that make up an organism.

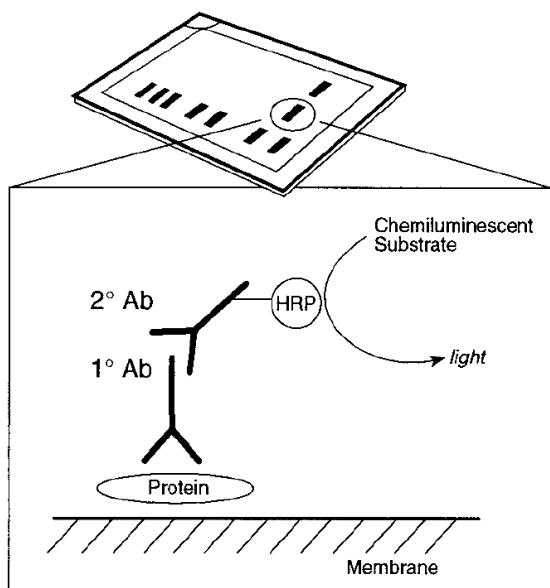
The large and diverse population of antibodies is generated by random combinations of a set of gene segments that encode different antigen binding sites (or paratopes), followed by random mutations in this area of the antibody gene, which create further diversity. The tips of the Y, containing the site that binds antigen is called the Fab (Fragment, antigen binding) region. It is composed of one constant and one variable domain from each heavy and light chain of the antibody (Fig. 1.33).

Antibodies are typically produced by immunization of a suitable mammal, such as a mouse, rabbit or goat. An antigen is injected into the mammal and is recognized by a single B-cell, which then divides to produce many B-cells. Most of these B-cells differentiate into plasma cells that secrete IgG immunoglobulins into the blood that bind the same epitope that elicited proliferation in the first place. The immune system of the animal usually produces a whole array of IgGs with different paratopes recognizing different surface regions of the antigen. Isolation of the IgGs from the mammal's serum therefore results in a so-called polyclonal antibody preparation.

Monoclonal antibodies are monospecific antibodies that are all identical because they are produced by one type of immune cell that is cloned and propagated from a single parent cell obtained from the immunized mammal's B-lymphocytes. It is possible to create monoclonal antibodies that specifically bind to almost any given substance. They can then serve to detect or purify that substance. This has become an important tool in biochemistry, physiology, molecular biology and medicine.

Antibodies are widely used as biological probes to identify specific molecules. The antibodies are linked to a label and introduced into the sample, where they bind to the targets of interest and provide a means of detection. Common labels include enzymes, fluorescent dyes, radioisotopes, or metal particles. This general technique has various applications. In an immunoassay, the goal is to detect and quantify specific targets. Tagged antibodies can also be used to separate target antigens selectively.

Common techniques to detect proteins with antibodies are ELISA and Western blotting. Enzyme-linked immunosorbent assay, also called ELISA, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In simple terms, in ELISA an unknown amount of antigen is affixed to the wells of a microtiter plate, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. Thus in the case of fluorescence ELISA, when light of the appropriate wavelength is shone upon the sample, an antigen/antibody complex will fluoresce so that the amount of antigen in the sample can be inferred through the magnitude of the fluorescence.



**Fig. 1.34.** Schematic representation of enzyme-linked antibody detection in Western blotting.

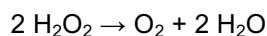
Similarly, enzyme-linked antibodies raised against a particular peptide (e.g. His6, GST or Strep) can be used to detect the presence of such a peptide (or a protein containing the peptide) on blots from protein gels (Western blots), as is schematically shown in Fig. 1.34.

Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are commonly used enzymes conjugated to antibodies. Linked to the IgG complex they are still able to convert particular substrates into easily detectable stains or fluorescent products.

The enzymes can either be conjugated directly to the specific antibody (the so-called "first" antibody), or to a non-specific antibody (called the "second" antibody), which recognizes the constant region of all first antibodies. For example, the first antibody is developed in rabbit against the antigen of interest, and the second antibody consists of commercially available anti-rabbit IgGs developed for example in goat or donkey which are conjugated to the enzyme. The use of first antibodies directly conjugated to the enzyme allows for a faster procedure since it involves only a single step and usually reduces undesired non-specific signals.

In this course we will use antibodies conjugated to alkaline phosphatase, which can enzymatically convert the colorless substrate BCIP into a blue-stained product in the presence of the NBT oxidant.

Alternatively, antibodies conjugated to horseradish peroxidase can be used. In this case, the substrate used for detection is luminol. Luminol ( $C_8H_7N_3O_2$ ) is a versatile chemical that exhibits chemiluminescence, with a striking blue glow, when mixed with an appropriate oxidizing agent. Luminol is used by forensic investigators to detect trace amounts of blood left at crime scenes as it reacts with iron found in hemoglobin. It is also used by biologists in cellular assays for the detection of copper, iron, and cyanides. To exhibit its luminescence, the luminol must first be activated with an oxidant. Usually, a solution of hydrogen peroxide ( $H_2O_2$ ) is used as the activator. In the presence of a catalyst such as an iron compound or a peroxidase enzyme, the hydrogen peroxide is decomposed to form oxygen and water:



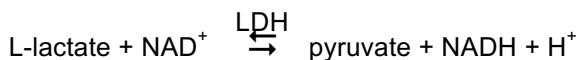
In the forensic detection of blood, the catalyst is the iron present in hemoglobin. When used in Western blotting the catalyst is the horseradish peroxidase conjugated to the antibodies. The peroxidase reaction yields luminol radicals, which then enter a complex chemical pathway to generate luminol hydroperoxide. This compound is very unstable and immediately decomposes to 3-aminophthalic acid (3-APA) with electrons in an excited state (indicated by  $3\text{-APA}^*$ ). As the excited state relaxes to the ground state, the excess energy is liberated as a photon, visible as blue light. p-Coumaric acid (4-hydroxycinnamic acid), a phenolic acid which has an important enhancing effect at low concentration on the luminol- $H_2O_2$ -horseradish peroxidase system, is also added to the reaction.



## 2 LDH ISOLATION AND ENZYME KINETICS

### 2.1 The Enzyme LDH

The enzyme **lactate dehydrogenase** (LDH) plays an important role in energy production in many animals, including man. It is essential for production of the cellular fuel ATP. In all animals the product of the reduction of pyruvate is L-lactate. LDH catalyzes the following reaction:



where  $K_{\text{eq}} = \frac{[\text{pyruvate}] \cdot [\text{NADH}] \cdot [\text{H}^+]}{[\text{L-lactate}] \cdot [\text{NAD}^+]} = 2.76 \times 10^{-12} \text{ M}$  (pH 7.0; 25 °C)

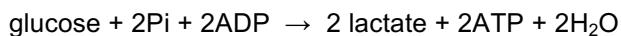
$K_{\text{eq}}$  (the equilibrium constant for this reaction) is very small and therefore the equilibrium lies strongly to the left side.

The name LDH refers to this reaction. The reaction is reversible, the enzyme works both ways. In LDH measurements the **substrate** pyruvate (pyruvic acid) or L-lactate (lactic acid) is used. In addition the **cofactor** NADH of  $\text{NAD}^+$  (nicotinamide-adenine-dinucleotide) is required. A cofactor is also called co-enzyme and can be viewed as a second substrate. The difference between a normal substrate and a co-enzyme is that the co-enzyme is all the time recycled. Co-enzymes are also less specific and function as substrates in a whole class of reaction (for example dehydrogenation reactions). Co-enzymes are often made from vitamins (organic compounds which are necessary in trace amounts and which are not made by your own body). The precursor (compound from which another compound is made) for NAD(H) is the vitamin nicotinic acid (B7), also called niacin.

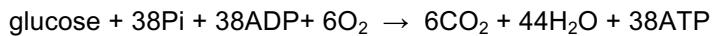
Glucose is converted in a multistep process called glycolysis to pyruvate (Campbell). If sufficient oxygen is present pyruvate is further converted to acetylcoenzyme-A, which is subsequently degraded via the citric acid cycle and oxidative phosphorylation to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This results in the production of a large amount of energy in the form of ATP (38 ATP/glucose). However, when there is oxygen shortage, oxidative phosphorylation and the citric acid cycle reactions are not possible, with the consequence that no ATP can be made via these pathways. Conversion of glucose into pyruvate still yields 2 ATP molecules, although at the cost of  $\text{NAD}^+$ , which is converted to NADH (see Fig. 2.1). The amount of  $\text{NAD}^+$  in the cell is limited and, if it would not be regenerated, the conversion of glucose to pyruvate would soon also block in the absence of oxygen, and no ATP would be made at all. The LDH reaction provides a solution to this problem. Reduction of pyruvate to lactate requires NADH, which regenerates  $\text{NAD}^+$  for another glucose molecule to be degraded to pyruvate, resulting in the production of again 2 molecules of ATP.

The LDH reaction is particularly important in muscle cells, which function often under limiting oxygen conditions, but still require energy. Under oxygen limitation lactate then accumulates, which leads to acidification of the muscles. In addition, an oxygen deficit is built up, because after an exercise additional oxygen is required to convert lactate back to pyruvate and to degrade the pyruvate via the citric acid cycle and oxidative phosphorylation. The LDH reaction is also important in certain bacteria, which grow under anaerobic conditions, such as lactic acid bacteria.

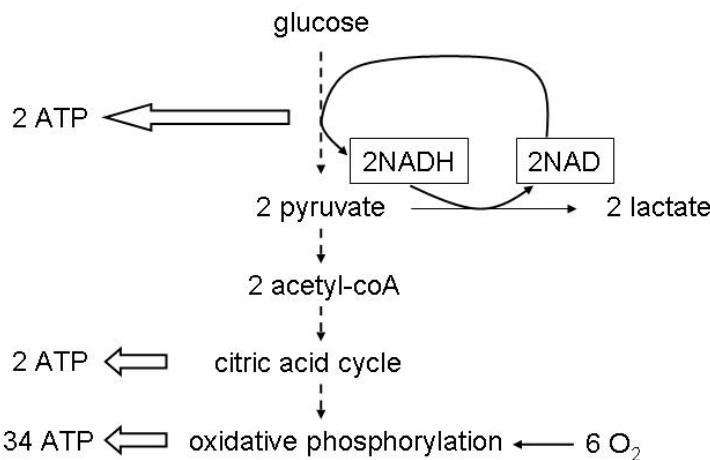
The overall equation of the glycolysis reaction to lactate is:



The overall equation of the complete degradation of glucose in carbon dioxide and water is:



The energy yield by glycolysis is therefore extremely poor compared to complete glucose degradation, but for the last process oxygen is an absolute requirement.



**Fig.2.1.** Degradation of glucose under (an)aerobic conditions.

## 2.2 LDH Isoenzymes

There are 5 different forms of the enzyme lactate dehydrogenase (LDH), which are called **iso-enzymes**. All 5 iso-enzymes have the same molecular weight of about 134,000, and they are all tetramers formed by 4 polypeptide chains with a molecular weight of about 33,500 each. There are two different types of these polypeptide chains, the H-(heart) and the M-(muscle)-type. These subunits are very similar but show some differences in their amino acid sequences that cause charge differences. These charge differences allow the electrophoretic separation of the enzymes. The 5 iso-enzymes are formed due to all possible combinations of the two enzymatically inactive subunits in enzymatically active tetramers. The iso-enzyme that is most abundant in muscle consists of 4 identical M chains and is indicated with M<sub>4</sub>. In heart the H<sub>4</sub> iso-enzyme is the most abundant.

The 5 iso-enzymes are:

LDH-1: H<sub>4</sub>  
 LDH-2: H<sub>3</sub>M<sub>1</sub>  
 LDH-3: H<sub>2</sub>M<sub>2</sub>  
 LDH-4: H<sub>1</sub>M<sub>3</sub>  
 LDH-5: M<sub>4</sub>

The 4 subunits are not covalently attached to each other, but assemble spontaneously due to protein-protein interactions. By hybridizing purified H and M chains it was shown that during catalysis each subunit functions independently of the others. The normal substrates of LDH are lactate and pyruvate.

The LDH iso-enzymes differ in their substrate affinities (especially for pyruvate) and V value. The M<sub>4</sub> iso-enzyme converts pyruvate relatively quickly to lactate, whereas H<sub>4</sub> performs this conversion relatively slowly and shows a stronger inhibition by pyruvate. The specific activities of the other iso-enzymes lie in between these two extremes. Muscle cells use glucose often anaerobically and convert it via glycolysis to lactate. This results in the energy in the form of ATP necessary for muscle labour under oxygen limitation (blood circulation cannot deliver oxygen fast enough at high muscle activity). Although the lactate produced is delivered as fast as possible to the blood stream, at high muscle activity lactate still accumulates in the muscle cells leading to acidification and impairment of muscle function.

The heart is an aerobically functioning muscle and does not convert pyruvate to lactate but degrades it via the citric acid cycle and oxidative phosphorylation. Obviously, it is of prime importance for the heart to function optimally and therefore it cannot afford acidification. The lactate transported from the muscle cells into the blood stream is taken care of by degradation in aerobically functioning tissues such the heart. In addition, it is re-converted to glucose in the liver in a process called gluconeogenesis.

The function of LDH iso-enzymes is therefore that they are adapted to let each specific tissue function optimally depending on the oxygen availability and energy requirement.

During the course we will isolate, characterize and determine the LDH isoenzymes in various tissues from different animals.

## 2.3 LDH Assay

LDH activity is measured by measuring NADH absorbance. The amount of NADH converted by LDH is equivalent to the amount of pyruvate converted or lactate. The absorbance spectrum of NADH shows a peak at 340 nm, whereas  $\text{NAD}^+$  has no absorbance at this wavelength (Fig. 2.2).

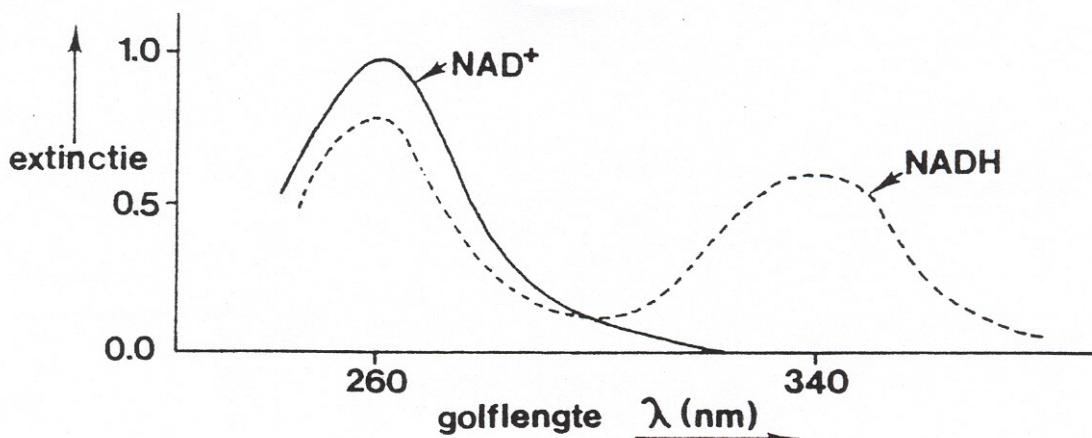


Fig. 2.2. Absorbance spectra of  $\text{NAD}^+$  and NADH.

By measuring the increase or decrease of [NADH] using pyruvate or lactate respectively as a substrate, the reaction rate and thereby the LDH activity can be measured. One should take into account that the reaction rate steadily decreases as more substrate is converted. The optimal situation for the measurements is therefore at the beginning of the reaction when almost no substrate has been converted. Therefore only the **initial reaction rate** is measured. If you are only interested in  $V$ , it is sufficient to perform a single measurement at an excess of substrate (assuming that there is no substrate inhibition). The excess of substrate required to obtain a reaction rate close to  $V$  can be deduced from the experimentally determined  $K_M$ . As shown in the Michaelis-Menten graph (Fig. 1.30), the reaction rate at a substrate concentration of  $10K_M$  exceeds 0.9 $V$ .

## 2.4 Protein Determination

(According to: Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254)

To determine the specific activity of an enzyme, that is the amount of katals per mg protein, you need to determine the protein amount. Most methods for protein measurement do not give an absolute protein amount, but require the use of reference protein solutions with known concentrations that need to be measured in parallel finally giving sample protein amounts relative to the standard.

To measure the protein amounts in our extracts we use a colorimetric (i.e. based on a color change) method according to Bradford. Bradford reagent contains the stain *Coomassie Brilliant Blue*. It binds to protein resulting in a colored product with an absorbance maximum of 595 nm. The absorbance values are compared with measurements carried out in parallel with a series of known protein standards. From the reference line generated by the standards the protein concentration in the extracts can be determined. A common reference protein is **bovine serum albumin** (BSA). This protein is isolated from cow's blood and is commercially available at high purity.

Usually, protein extracts have a complex composition. They contain multiple proteins with different molecular weights. Therefore, the concentration cannot be expressed in moles and the unit mg/ml is used, where the mg amount is related to the concentration of the standard BSA.

#### 2.4.1 Making a reference line for a Bradford determination

- Make a 5x dilution of the BSA stock solution (1 mg/ml) in demineralized water. Pipet 0, 2, 5, 10, 15, and 20 µg BSA from this solution in separate eppendorf tubes and add water to a total volume of 100 µl.
- Add 1 ml Bradford reagent to each of the BSA samples of the series and mix.
- The absorbance is measured in the Genesys20 spectrophotometer at 595 nm in a 1 ml cuvette.
- Open the Visionlite program. For protein measurements choose application *Quant*.
  - File menu, select: *New method*
  - Under Calibration-Curve type choose *Linear through zero*
  - *Measurement wavelength*: 595 nm
  - *Reference mode*: No reference line
  - *Unit*: µg/mL
  - In the table under *Concentration* enter the cells with the concentrations of the BSA dilution series (20, 50, 100, 150, 200).
- Fill a 1.0 ml cuvette with the “0” sample, place the cuvette in the spectrophotometer with the cuvette windows in North/South direction.
- Click button *Autozero*. The absorbance is set to “0”
- Remove the cuvette, pipette the “0” sample out and transfer it back into its eppendorf tube.
- Tap the cuvette upside down onto a piece of tissue to remove final traces of sample. Fill the cuvette with the first protein sample (20 µg/mL) and place it into the spectrophotometer.
- Click button *Measure standards*. The measured absorbance is displayed in the table.
- Repeat these steps for all BSA samples. You can use the same cuvette for each measurement as long as you go from lower to higher protein concentrations.
- After the last sample is measured Visionlite displays all measurements in a table and graph. Copy the data in the table to an Excel sheet for calculation of the conversion coefficient for protein quantification. This coefficient will be used for all future protein measurements.

#### 2.4.2 Protein determination

- Make 100-times dilutions of the samples from the LDH ammonium sulfate fractionation (crude, 40P, 40S, 70P and 70S). For each fraction pipette 10 µl into an eppendorf tube and add 990 µl K-phosphate buffer. Vortex briefly. Also make a blank of 1 ml K-phosphate buffer.
- Transfer 100 µl of the blank and of each dilution into new eppendorf tubes and add 1 ml Bradford reagent.
- Switch on the Genesys20 and wait till it finishes calibration.
- Open program Visionlite and choose application *Quant*.
- File menu: *Open method*, choose “BSA reference line”. Your reference graph is shown.
- Fill a cuvette with the blank sample, put it in the spectrophotometer and click button *Measure samples*.
- Next, measure all the LDH fractionation samples. Name each sample (crude, 40P, etc.) and enter 100 for dilution factor.
- After all samples have been measured, save the data to TIFF file (e.g. LDH fractionation) for later calculations.

## 2.5 Determination of NADH Absorbance Spectrum

#### 2.5.1 Make a dilution series

Make 2 ml of a 2.4 mM NADH solution in 50 mM K-phosphate buffer pH 7.2 (see 1.3.4). Check the calculated amount of NADH with a course instructor.

Make an NADH dilution series of 10 dilutions in K-phosphate buffer, including a blank sample, and with a highest concentration of 0.12 mM. Make 1 ml of each dilution. Make a table with the amounts to be pipetted

and have it checked by a course instructor. Before the reference line can be made, we need to know the optimal wavelength for the NADH absorbance. Therefore the NADH absorbance spectrum is determined.

### 2.5.2 Determination of the absorbance spectrum of NADH and NAD<sup>+</sup>

- Use the 0.06 mM solution of NADH to determine the absorbance spectrum.
- Start program Visionlite. Choose application Scan.
  - File Menu: *New Method*
  - Set *Start*: 325, *End*: 405 nM
  - *Measurement mode*: A (for absorbance)
  - *Y-axis maximum*: 0.5
  - *Y-axis minimum*: 0
  - *Threshold*: 0.01
  - *Maximum* checkmark on
- Insert cuvette with blank (Phosphate buffer)
  - click *Baseline*
- After the baseline scan is finished insert cuvette with NADH sample
  - click *Measure samples*
  - enter *Sample name*: NADH
  - enter *Description*: spectrum
- run scan

### 2.5.3 Making a reference line

- Determine the absorbance of all NADH solutions made above (2.5.1) at the previously determined absorbance maximum, using the Visionlite *Quant* application, similar to how the BSA reference line was made.
- Make a reference graph in Excel plotting the absorbance against the NADH concentration (see 1.8.2). Do you have a perfectly straight line? Determine  $\epsilon_{\text{NADH}}$ .

## 2.6 Isolation of LDH from Animal Tissue

### 2.6.1 Homogenization and ammonium sulfate precipitation

- Add 10 g of animal tissue cut into small pieces to 50 ml cold buffer (50 mM K-phosphate, pH 7.4) in a blender on ice.
- Homogenize the tissue.
- Transfer the homogenate to a 50 ml Falcon tube.
- Centrifuge 10 min at 4000 rpm to remove cell debris.
- Transfer the supernatant to an Erlenmeyer flask using a 25 ml pipet. Avoid the layer of fat floating on top of the supernatant by sticking the tip of the pipet underneath this layer. While pipetting, measure the volume of supernatant that ends up in the Erlenmeyer flask. Place the flask on ice.
- Dispose of the pellet in the Falcon tube and clean the tube.
- Transfer 1 ml of the supernatant to a fresh eppendorf tube, labeled “Crude”, and your group name, and place the tube on ice. This sample is for later analysis.
- Use the data of Table 1.4 to calculate the amount of  $(\text{NH}_4)_2\text{SO}_4$  to make the remaining volume of supernatant 40% saturated (start %-age = 0%, desired %-age = 40%  $\rightarrow$  229 g/L or 229 mg/ml). Weigh out this amount.
- Slowly add the ammonium sulfate to the supernatant in the Erlenmeyer flask under continuous swirling. Make sure that the ammonium sulfate is rapidly dissolved during addition.
- When all ammonium sulfate is dissolved, incubate the mixture on ice for 10 min.
- Transfer the protein suspension to the cleaned Falcon tube.
- Centrifuge 10 min at 4000 rpm. Meanwhile, clean the Erlenmeyer.
- Use the same 25 ml pipet as above to transfer as much as possible of the supernatant to the rinsed Erlenmeyer flask. Measure the volume while doing this.
- Again, transfer 1 ml to an eppendorf tube labelled “40S” and put this on ice.
- Resuspend the 40%  $(\text{NH}_4)_2\text{SO}_4$  pellet in 5 ml K-phosphate buffer and transfer 1 ml to a fresh eppendorf tube labeled “40P” and place the tube on ice. Measure the total volume of the resuspended pellet before discarding.

- Using Table 1.4, calculate the amount of ammonium sulfate needed to bring the supernatant in the Erlenmeyer flask to 70% saturation. Weigh out this amount of ammonium sulfate and slowly add it to the supernatant under continuous swirling. This will bring the mixture to 70% saturation.
- When all  $(\text{NH}_4)_2\text{SO}_4$  is dissolved incubate 10 min on ice.
- Transfer the mixture back to the rinsed Falcon tube.
- Centrifuge 10 min at 4000 rpm.
- Transfer 1 ml of the supernatant to an eppendorf tube labeled "70S" and place it on ice.
- Carefully decant the remaining supernatant. The pellet may be loose, so don't lose it!
- Estimate the volume of the pellet (should be around 2-4 ml) and add an equal volume of K-phosphate buffer.
- Dissolve the pellet until the mixture is clear.
- Add an equal volume (twice the previous volume of K-phosphate) of 100% saturated  $(\text{NH}_4)_2\text{SO}_4$  to the dissolved pellet and mix well.
- Incubate the mixture 10 min at room temperature.
- Centrifuge again for 10 min at 4000 rpm.
- Decant most of the supernatant but leave 2-3 ml in the tube.
- Resuspend the pellet in the remaining 2-3 ml supernatant. Write down the volume of the slurry, label the Falcon tube "70P" and place it on ice or in the refrigerator.
- This is the LDH stock preparation. It can be stored for several months in the refrigerator at 4°C.

Part of the LDH stock preparation will be dialysed to remove the excess ammonium sulfate that would otherwise interfere with the Blue sepharose affinity chromatography and isoenzyme gelectrophoresis experiments.

- Make a double knot at one end of a piece of pre-soaked dialysis membrane tubing. Pull the knot firmly without overstretching or tearing the tubing.
- Pipette 2 ml of the ammonium sulfate slurry into the tube. Push out as much of the air from the tube as possible to avoid snapping of the membrane when it takes up buffer during dialysis.
- Close the tube with a clamp.
- Dialyse overnight at +4°C against a large volume of 50 mM K-phosphate buffer, pH 7.2.
- After dialysis, collect the contents of the dialysis bags in eppendorf tubes labelled "70D" and store at 4°C until later use.

The above samples Crude, 40P, 40S, 70P, 70S and 70D will be used to measure the LDH fold-purification. Therefore, the protein concentration and the LDH activity of the samples will be determined. The protein concentration of the various fractions is determined as described under PROTEIN DETERMINATION above (see 2.4.2 on p.56).

#### THE LDH ENZYME ACTIVITY IS DETERMINED AS FOLLOWS:

- Open Visionlite application Rate
- *New method:* "LDH\_activity.mra"; Enter the following values: *Measurement time*=1 min; *Data interval*= 1 sec; *Wavelength*= 340 nM; *Measurement mode*= A; *Y-axis maximum*= 0.8; *Y-axis minimum*=0; *Rate calculation* from 0.08 to 0.45 min.
- Dilute the various fractions 50 times in K-phosphate buffer to a final volume of 1 ml. Keep the diluted samples on ice.
- Have available on ice a 2.4 mM NADH solution in K-phosphate buffer.
- Add in a 1 ml cuvette:
  - 0.85 ml K-phosphate buffer
  - 50  $\mu\text{L}$  12 mM pyruvate
  - 50  $\mu\text{L}$  50x diluted protein sample
- Close cuvette with parafilm and mix quickly by inversion without generating air bubbles.
- Place the cuvette in the spectrophotometer and choose *Autozero*.
- Next choose *Measure samples*, enter *Result filename*: LDH fractions and enter the *Sample name* of each sample: "crude", "40P", etc.
- Now add 50  $\mu\text{L}$  NADH, mix quickly, place cuvette back in spectrophotometer and choose *Measure*. The absorbance is measured at 1 sec intervals during a minute and the result is plotted in a graph with A from 0 to 0.8. Also the velocity is calculated as  $\Delta\text{A}/\text{min}$ .

- Make sure the graph is linear over the interval in which the velocity is calculated. If this is the case use the value under *Rate* [dA/min]. If not, select for *Rate calculation* an interval in which the graph is linear and choose *Recalculate*.
- For each next sample checkmark *Measure blank*, put in a cuvette with the next sample without NADH and push *Measure*. Next, add NADH, mix by inversion, *Measure*. Repeat the procedure for the other samples, entering the respective *Sample name* for each sample.
- Enter all data in Excel.

The decrease of NADH in time is reflected by the decline in absorbance. Fractions with highest LDH enzyme activity show the sharpest decline.

## 2.6.2 Blue Sepharose affinity column purification

- Pre-swollen Blue Sepharose has been prepared by the course staff. It was prepared as follows:
  - Weigh 0.2 g Blue Sepharose CL-6B and transfer it to a 10 ml plastic tube.
  - Add 10 ml P5G buffer (10 mM K-phosphate, pH 7.2, 5% glycerol), disperse the Sepharose and swell the Sepharose at least 5 min.
  - Decant the buffer. Don't lose any Sepharose!
  - Add another 10 ml P5G buffer, wait 5 min before decanting the buffer.
  - Repeat the washing step one more time.
  - Decant most of the buffer, but leave a slurry that can easily be poured or pipetted with a cut-off blue tip.
- Fit a plug of glass wool onto the bottom of a Pasteur pipette.
- Slowly pipette the Blue Sepharose slurry into the pipette, avoid trapping air bubbles, and let the column run dry. The volume of Blue Sepharose should be around 0.3-0.4 ml.
- Prepare a series of eppendorf tubes labeled 1 to 11
- Dilute 500  $\mu$ L "70D" LDH with 500  $\mu$ L P5G buffer. Mix by inversion.
- Add 500  $\mu$ L diluted LDH onto the column. Collect the flow-through in eppendorf tube 1. Place the tube on ice.
- Add the other 500  $\mu$ L onto the column and collect the flow-through in tube 2.
- Add 500  $\mu$ L of P5G buffer, collect flow-through in tube 3 and repeat this washing step 3 more times (until tube 6).
- Add 500  $\mu$ L 1 M KCl in P5G buffer and collect the flow-through in tube 7. Repeat the elution 4 times (until tube 11).
- Store all fractions on ice.

The protein concentration and the enzyme activity of the fractions will be determined as described above, but with 5-20x diluted samples instead of 50x (ask instructor).

## 2.7 Properties of LDH

### 2.7.1 Determination of LDH's $K_M$ and $V$

Although the optimum temperature of LDH is 37°C (body temperature), for convenience the reactions are carried out at room temperature.

- Out of a 12 mM pyruvate stock, make a dilution series in eppendorf tubes in 50 mM K-phosphate buffer (pH=7.2): 13 dilutions from 0 mM to 12 mM (1.2 ml each). Do not forget to write the concentrations on the eppendorf tubes.
- Dilute the dialysed 70P enzyme preparation 50 times in K-phosphate buffer to a final volume of 1 ml. Keep on ice.
- Have a 2.4 mM NADH solution in K-phosphate buffer ready on ice.
- Add in a 1 ml cuvette:
  - 0.85 ml K-phosphate buffer (of room temperature)
  - 50  $\mu$ L 12 mM pyruvate
  - 50  $\mu$ L 50x diluted enzyme preparation
- Close cuvette with parafilm and mix by inversion.
- Place the cuvette in the spectrophotometer set absorbance to 0.

- Add 50  $\mu$ L 2.4 mM NADH, mix, quickly place the cuvette in the spectrophotometer and measure directly. If the decline in absorbance is too fast, does not proceed linear and approaches zero within 30 seconds, the enzyme preparation should be further diluted.
- The LDH reaction rate at different pyruvate concentrations can now be measured.
- Repeat the measurements for each pyruvate dilution. Indicate the respective samples appropriately for later analysis.

### Data processing

All calculations are performed using Excel.

To convert  $\Delta A/\text{min}$  to  $S$ , the number of moles converted, use Lambert-Beer's law:

$$\begin{aligned} A &= \epsilon \cdot c \cdot l \\ \Delta A &= \epsilon \cdot \Delta c \cdot l \\ \Delta A &= \epsilon \cdot \Delta [S] \cdot l \end{aligned}$$

From the measured  $\Delta A/\text{min}$  you can therefore calculate  $\Delta [S]/\text{min}$ . To convert  $\Delta [S]$  (in mol/l) into  $\Delta S$  (amount of moles) you have to take into account the reaction volume in the cuvette (1 ml).

Put all the measured and calculated values in a clear graph.

- Enter the pyruvate concentrations and corresponding  $\Delta A/\text{min}$  values in an Excel spreadsheet (for each [pyruvate],  $\Delta A/\text{min}$  was obtained above).
- Using the  $\epsilon_{\text{NADH}}$  (in L/mol.cm) determined above, convert  $\Delta A/\text{min}$  into  $\Delta [S]/\text{min}$ , and then by taking into account the reaction volume into  $\Delta S/\text{min}$  (for a 1 ml cuvette divide by 1000). Divide  $\Delta S$  by 60 (sec/min), which results in the reaction rate  $v$  (this is the number of moles of substrate converted per second in one mL [pyruvate and NADH react in equimolar amounts]).
- Plot in a graph  $v$  against  $[S]$  (in the cuvette). Make sure you are using the correct units.
- Plot in a second graph (the Lineweaver-Burk plot)  $1/v$  against  $1/[S]$  and determine  $K_m$  and  $V$  by calculating the values where the linear trendline through the data points crosses the y- and x-axes (intercepts). You can also use the formula  $=\text{Intercept}(X\text{-values}, Y\text{-values})$  for the x-axis intercept and  $=\text{Intercept}(Y\text{-values}, X\text{-values})$  for the X-axis intercept.
- Calculate the specific LDH activity (kat/mg protein) in the protein extract.

### 2.7.2 Determination of LDH's pH optimum (this experiment may be skipped)

To determine the effect of pH on enzyme activity all measurements are done using the same pyruvate concentration. Therefore the optimal pyruvate concentration is chosen that was determined in the previous assay.

Use the same pipetting scheme as in the previous experiment, but use phosphate buffers of pH 4.3, 5.8, 7.2, 8.5, and 9.3

### Data processing

Make a graph of  $\Delta A/\text{min}$  against the pH. Draw a curve through the data points and indicate the pH optimum.

### 2.7.3 Effect of inhibitors

In this experiment we will study the effect of inhibitors oxalate and oxamate on the LDH enzyme.

- Prepare a dilution series of 0, 0.5, 1, 2, 4 and 8 mM pyruvate in 1 ml 50 mM K-phosphate buffer, pH 7.2 as described above and use the 2.4 mM NADH dilution.
- Prepare three series of reaction mixtures in eppendorf tubes (6 pyruvate concentrations per series a, b and c) according to the following scheme:

Use oxalate and oxamate solutions of 1 mM.

Series	a	b	c
Pyruvate ( $\mu$ L)	50	50	50
Phosphate buffer (ml)	0.85	0.8	0.8
Oxalate ( $\mu$ L)	-	50	-
Oxamate ( $\mu$ L)	-	-	50

- Open Genesys20 application Rate. Open method “LDH\_activity.mra”. Click *Measure samples*, enter a *Result filename* and a *Sample name*. For each tube separately, transfer the reaction mixture to a cuvette and add 50  $\mu$ L diluted enzyme. Mix by inversion, place cuvette in spectrophotometer and click *Measure*. For each sample, check mark *Measure blank* to set the absorbance to 0 without NADH.
- Next, click *Measure*, Add 50  $\mu$ L 2.4 mM NADH to the cuvette, mix by inversion and measure  $\Delta A/min$ .

#### **Data processing**

Calculate the end concentration pyruvate.

Prepare Lineweaver-Burk plots as described above.

#### **2.7.4 Reverse reaction**

As we have seen above, LDH can also perform the reverse reaction from lactate to pyruvate, using  $NAD^+$  as electron acceptor.  $NAD^+$  is reduced to NADH, which can be measured by its absorbance at 340 nm.



In the following experiment we will determine the  $K_m$  and  $V$  for this reaction.

Because the reaction from pyruvate to lactate is much faster, the newly formed pyruvate and NADH could rapidly be converted back to lactate and  $NAD^+$  and this would interfere with the measurements. Therefore, the newly formed pyruvate is immediately removed by reaction with hydrazine.

The following stock solutions are available:

10 M lactate (as acid)

0.1 M Tris-HCl, pH 8.7

0.4 M hydrazine sulfate in 0.1 M Tris-HCl, pH 8.7

3.6 mM  $NAD^+$  (MW = 663.4) in 0.1 M Tris-HCl, pH 8.7

- Make a dilution series of 1 ml lactate ranging from 10 to 200 mM in 0.1 M Tris-HCl, pH 8.7
- For each concentration of lactate, transfer 0.1 ml lactate to a 1 ml cuvette
- Add 0.80 ml 0.4 M hydrazine sulfate in Tris buffer
- Start Visionlite, application *Rate*. Open “LDH\_activity.mra”.
- Add 50  $\mu$ L diluted\* 70D fraction, mix by inversion, place cuvette in Genesys20 and proceed as above to obtain the  $\Delta A/min$  for the different lactate samples (don't forget to set the absorbace to 0 for the samples before addition of  $NAD^+$ ).
- Add 50  $\mu$ L  $NAD^+$ , mix by inversion, place cuvette back in spectrophotometer, and measure the increase of absorbance during 1 min.

Reuse the cuvette for the next measurement after rinsing with water!

\*Dependent on the activity of the fraction, dilution will be between 5 and 100 times

#### **Data processing**

Calculate the concentrations of lactate in the reaction mixture.

Make a Lineweaver-Burk plot and determine  $K_m$  and  $V$ .

## **2.8 Separation of LDH Iso-Enzymes**

### **2.8.1 Basic principle of electrophoresis**

The word electrophoresis refers to the movement of ions in an electric field. Electrophoresis is a method that is used to separate charged molecules. In molecular biology the method is applied mainly to separate proteins or nucleic acids. All electrophoretic methods are based on the principle that charged molecules will migrate through a solution or a gel when electric tension is applied. The speed of migration depends on charge, size en three-dimensional shape of the molecule. Charged molecules that differ in one or more of these properties will migrate at different speeds and will eventually be separated in discrete bands.

During so-called zone electrophoresis de charged molecules move though a carrier with pores. This can be a humid solid material such as paper or cellulose acetate, or a gel. Common materials to make gels are agarose and polyacrylamide.

An electrophoresis unit consists of a power source that delivers direct current and an electrophoresis tank, which contains the carrier and the electrolyte solution. The carrier with the samples is placed between two buffer compartments that are connected to the cathode (negative electrode) and the anode (positive electrode), respectively. When voltage is applied, molecules will move towards the cathode or anode depending on their charge.

### 2.8.2 Electrophoresis of LDH iso-enzymes

LDH iso-enzymes in protein extracts can be electrophoretically separated in an agarose gel. Agarose is a polysaccharide, which forms a porous gel (it strongly resembles gelatine, but is plant- or algae-derived). Pore diameter is determined by the agarose concentration and it determines the separation range of the gel. The thin layer of agarose lies on top of a thin piece of polyester. The sample is applied using a cellophane mould with narrow slits. After the samples diffused in the agarose gel, the gel can be placed in the electrophoresis tank, with the samples flanking the cathode (negative black electrode). The LDH iso-enzymes are separated in the following order from anode to cathode: LDH-1, LDH-2, LDH-3, LDH-4 en LDH-5.

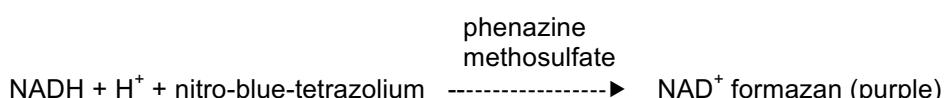
All mammals possess these 5 iso-enzymes, which can be separated in this way by electrophoresis. The H and M subunits of different mammals have highly similar amino acid sequences (with around 90-100 % amino acid identity), but small differences usually exist. If these differences affect the charge of the protein, the iso-enzymes from different mammals will show differences in migration speed. Therefore, usually when iso-enzyme patterns of two mammals are compared, in both cases 5 iso-enzymes will be detected, but several or all of the corresponding iso-enzymes will show differences in migration speed.

The protein extract does not only contain LDH iso-enzymes, but in addition thousands of other proteins and other molecules, which are all colourless, except the protein hemoglobin, which is red due to the bound heme group.

To visualize the LDH bands the gel is selectively stained based on LDH-mediated conversion of a colorless substrate in a colored product. The agarose gel is incubated in a mixture containing lactate,  $\text{NAD}^+$ , nitro-blue tetrazolium and phenazine methosulfate. Lactate is the LDH substrate and is converted to pyruvate, whereas  $\text{NAD}^+$  is reduced to NADH:



The hydrogen atoms from NADH are then via phenazine methosulfate transduced to nitro-blue tetrazolium, which is thereby reduced to an insoluble purple formazan compound.



In this way each LDH band stains purple.

#### Required

- A 1% agarose gel in 50 mM Tris-HCl pH 8.7 fixed on a sheet of polyester Gelbond (15 ml agarose/gel)
- A cellophane loading mold
- 0.5 L electrophoresis buffer (50 mM Tris-HCl pH 8.7)
- Loading buffer (12.5% Ficoll 400, 2 mM EDTA pH 8, 0.25% bromo phenol blue, 0.25% xylene cyanol)
- “Crude” and “70D” LDH protein extracts
- Gel tank and power supply
- Petri dish with 25 ml LDH reagent (prepare immediately prior to use)

#### Procedure

- Heat 20 ml 50 mM Tris-HCl pH8.7 with 0.2 g agarose (pronarose) in a 100 ml erlenmeyer in the microwave oven until the agarose is completely dissolved.

- Let erlenmeyer flask cool down until you can just hold it in your hand. Tape the corners of a sheet of Gelbond with tape such that is flat and has the hydrophilic side upwards. Using a pipette evenly spread 15 ml warm agarose over the Gelbond sheet. Let the gel solidify and dry out during 1.5 hours.
- Place the cellophane loading mold over the wide side of the gel such that the edges of the mold and the gel are level. There are at least 6 slits available for loading. Be careful, the agarose gel is soft.
- Each group will load several of its LDH fractions on the gel: e.g. "Crude", "40S", "70D", "fraction 8 of the BlueSepharose chromatography". Follow instructions of assistant for dilutions.
- Mix 20  $\mu$ L enzyme extract with 5  $\mu$ L loading buffer.
- Load 5-10  $\mu$ L of the extracts on top of the slits without touching the gel.
- Let the samples diffuse into the gel for 15 min.
- Remove remaining sample with a tissue, remove loading mold. Clean mold and return it to assistant.
- Carefully place the gel in gel tank with the samples facing the cathode (negative terminal). Two gels can be run simultaneously in one tank. Put about 250 ml electrophoresis buffer in each buffer compartment such that the edges of the gel are submerged
- Run at 75 V until the xylene cyanol (light blue) is about 2 cm from the edge (takes about 90 min). Check with assistant whether gel ran far enough.
- Put gel gel-side down in a glass petri dish with 25 ml LDH reagent and stain at 37°C until the LDH bands are visible.
- Write with black marker pen your initials, position of positive and negative terminals, and the loading order on the back (side without agarose) of the gel. Dry agarose-side up on a piece of paper in a 60°C oven.

## Appendix: solutions

LDH reagent 25 ml:

- solution 1: 5 mg nitroblue tetrazolium in 25 ml 50 mM phosphate buffer pH 7.2
- solution 2: 5 mg methylphenazoniummethosulfate in 5 ml 50 mM phosphate buffer
- solution 3: 25 mg NAD in 0.5 ml 50 mM phosphate buffer

Mix 25 ml solution 1 with 0.7 ml solution 2 and 0.5 ml solution 3 and 10 drops 100% Na-lactate solution. Adjust pH to 7.2 with 5 M KOH.

# 3 PROTEIN EXPRESSION, ISOLATION AND CHARACTERIZATION

## 3.1 Expression of Recombinant Proteins in *Escherichia coli*

Analysis of the properties of individual eukaryotic proteins is conveniently carried out using recombinant proteins expressed in the prokaryotic host *E.coli*. Purification of individual proteins from the eukaryotic organism under study is tedious and costly.

This has been solved by the development of efficient expression systems in the laboratory workhorse *E.coli*. The expressed proteins are provided with peptide affinity tags that allow easy purification often in a single step by affinity chromatography. In most cases the tag does not interfere with relevant protein properties. In those cases where it does, tags can be removed due to the presence of specific protease cleavage sites.

This all sounds ideal, but there are a few drawbacks. A common problem is that foreign proteins accumulate in non-crystalline aggregates called inclusion bodies due to misfolding. With some proteins it is possible to solve this problem by complete denaturation (remove all secondary and tertiary structure) of the proteins in inclusion bodies and subsequently, refold them. Another problem is that proteins expressed in *E.coli* in the soluble fraction are easily degraded leading to very small amounts of full-length protein. This can be circumvented by providing the expressed protein with different N-terminal and C-terminal peptide tags and use two sequential affinity chromatography steps. Although this approach does not solve the problem of the low yield of full-length protein, it does allow to separate the full-length protein from the degraded forms.

### 3.1.1 *E.coli* host for expression: BL21(DE3)pLysS

The *E. coli* B strain BL21 is deficient in the *lon* protease and lacks the *ompT* outer membrane protease that can degrade proteins during purification. Thus, some target proteins may be more stable in these strains than in host strains containing these proteases. BL21 is a widely used host for target gene expression. We use a derivative that also contains the pLysS plasmid carrying the T7 lysozyme gene. T7 lysozyme is a protein that cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall. T7 lysozyme is provided by a clone of the T7 lysozyme gene in the BamHI site of the plasmid vector pACYC184. The cloned fragment [bp 10,665–11,296 of T7 DNA] also contains the  $\phi$ 3.8 promoter for T7 RNA polymerase immediately following the lysozyme gene. A plasmid having this fragment oriented so that the lysozyme gene is expressed from the tet promoter of pACYC184 is referred to as pLysE; cells carrying this plasmid accumulate substantial levels of lysozyme. A plasmid having the fragment in the opposite orientation is referred to as pLysS; cells carrying this plasmid accumulate much lower levels of lysozyme. When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli* (i.e., no cell lysis occurs), apparently because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer. Neither lysozyme plasmid interferes with transformation of cells that contain it; pLysS has little effect on growth rate but pLysE causes a significant decrease in the growth rate of cells that carry it. The presence of pLysS or pLysE makes it easier to prepare cell extracts. After the target protein has accumulated, the cells are collected and suspended in a buffer such as 50 mM Tris-HCl, 2 mM EDTA, pH 8.0. Simply freezing and thawing, or adding 0.1% Triton X-100, will allow the resident T7 lysozyme to efficiently lyse the cells.

DE3 is a defective lambda phage integrated in the host genome. It carries the T7 RNA polymerase gene, which is necessary when using expression plasmids where recombinant protein expression is controlled by the T7 promoter (such as the pET series from Novagen; see below).

**Table 3.1.** Expression systems

Plasmid	Tag	Promoter	Repressor	Induction
pASK-IBA	Strep/His	tetA	tet	ah-Tc
pET	His	T7	lacI	IPTG

IPTG = isopropyl-D-thiogalactopyranoside

The lacI and tet repressors are carried on the plasmids, pET and pASK-IBA, respectively, to ensure efficient repression under non-induced conditions.

**Table 3.2.** Tags commonly used for recombinant protein production in *E.coli*

tag	length/ MW sequence	matrix/ elution	plasmids	supplier
GST	211 aa/ 25.5 kD	glutathione/ glutathione (GSH)	pGEX	GE Healthcare
Strep	WSHPQFEK	Strep-Tactin/ desthiobiotin, biotin	pASK-IBA	IBA biotagnology
His	6-10/ HHHHHH	nickel/ imidazole	pET pQE	Novagen Qiagen
MBP	396	amylose/ maltose	pMAL	NEB
BCCP biotin	100	streptavidin/	PinPoint	Promega

GST = glutathione S-transferase; MBP = maltose-binding protein; BCCP = biotin carboxyl carrier protein

### 3.1.2 Expression of foreign proteins in *E. coli*

The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells, which harbour the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer.

Commonly used inducible promoters are the lac promoter, a lac promoter derivative called lacUV5, and the tac promoter (a hybrid between the promoters controlling the tryptophan and lac operons). All these promoters are repressed by the lacI<sup>Q</sup> repressor. Induction is achieved by adding 0.1-1 mM of IPTG (isopropyl-D-thiogalactopyranoside), a metabolite of which binds to the lacI<sup>Q</sup> repressor and inactivates it. The series of pET vectors enable the quick production of a large quantity of any desired protein when activated. The pET16H plasmid used contains in addition to a ColE1 replication origin and an ampicillin resistance gene, several other important elements - a lacI gene which codes for the lac repressor protein, a T7 promoter which is specific to only T7 RNA polymerase (not bacterial RNA polymerase), a lac operator which blocks transcription from the T7 promoter under non-inducing conditions, and a polylinker into which the coding region for the protein of interest is cloned in such a way that it is translationally fused to a series of six histidine codons.

One of the most important parts of the pET expression system involves the fact that the gene of interest is not transcribed unless the T7 RNA polymerase is present. Usually, the host cell for this expression system is a bacterial strain, which has been genetically engineered to incorporate in its genome the gene for T7 RNA polymerase under the control of the lac promoter and the lac operator. When lactose or a molecule similar to lactose (i.e. IPTG) is added, transcription of the T7 RNA polymerase is activated. Typically, host cells used are *E. coli* BL(DE3) strains.

Another type of expression plasmids (one is used in this course) are thermo-inducible transcription vectors. Transcription is started by activation of the tandemly arranged  $P_R$  and  $P_L$  promoters of phage  $\lambda$  and terminated by the bacteriophage fd terminator. The transcriptional initiation is repressed at 28-30°C by the product of bacteriophage  $\lambda$  c lts857 gene, present on the vectors. Full induction is achieved by shifting the incubation temperature to 42°C. The combination of highly efficient transcriptional and translational signals on these vectors allows high-level expression of the desired protein in *E. coli*. In this course we use plasmid pJLA602.

### 3.1.3 Overview of affinity tags for protein purification

#### Polyhistidine

The polyhistidine affinity tag, also known as the His-tag or His6, usually consists of six consecutive histidine residues, but can vary in length from two to ten histidine residues. Polyhistidine is such a ubiquitous affinity tag that most companies providing expression vectors or protein expression and purification reagents offer products related to this tag (see Table 3.2 for examples). Histidine readily coordinates with immobilized transition metal ions. Immobilized  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Fe}^{3+}$  can all be used to purify polyhistidine fusion proteins, but  $\text{Ni}^{2+}$  is the most commonly used. Empirical determination of the most effective transition metal ion for purification of a specific polyhistidine fusion protein can be performed if purification by  $\text{Ni}^{2+}$  is unsatisfactory. There are several companies that offer IMAC (immobilized metal ion affinity chromatography) resin. Iminodiacetic acid agarose (chelating Sepharose, GE Healthcare), nitrilotriacetic acid agarose (Ni-NTA resin, Qiagen), and carboxymethylaspartate agarose (Talon resin, Clontech) are all used for the immobilization of transition metal ions. Commercially available IMAC resins are unaffected by protease or nuclease activity, and are appropriate for purification of fusion proteins from crude cell lysates. Most of these resins can be regenerated and reused indefinitely. Although polyhistidine does not usually cause a protein to be targeted to *E. coli* inclusion bodies, IMAC is amenable to denaturing agents (i.e., 8 M urea, 6 M guanidine.HCl, ionic and nonionic detergents, and low concentrations of reducing agents) for the purification of insoluble or membrane-bound proteins. High concentrations of reducing agents such as dithiothreitol (DTT) can reduce the immobilized metal ion and should be avoided. The relatively small size and charge of the polyhistidine tag rarely affects protein function, and elution by imidazole gradient is relatively mild, preserving the immunogenicity of polyhistidine fusion proteins. While purification of a highly-expressed polyhistidine fusion protein can lead to relatively (>80%) pure protein in one chromatographic step, purification from insect and mammalian cells, which contain a higher percentage of His residues in their proteins than *E. coli*, can lead to significant background binding to immobilized metal ions. This may be circumvented by using stringent wash conditions (e.g., 5 to 10 mM imidazole), although a stringent wash may cause premature elution of the protein of interest. The location of the tag (N terminal, C terminal, or internal) can also have an effect on IMAC. If a change in tag location does not increase the effectiveness of IMAC, a denaturing purification can be attempted. Primary antibodies have also been developed for the detection of polyhistidine fusion proteins in vitro. Again, because of the predominance of histidine residues in mammalian and insect systems, anti-polyhistidine antibodies are notoriously promiscuous.  $\text{Ni}^{2+}$  resin can also be used to precipitate a polyhistidine-tagged protein for the detection of protein-protein interactions.

#### Glutathione S-Transferase

The pGEX *E. coli* expression vectors encode for N-terminal glutathione S-transferase (GST) molecules followed by protease cleavage sites. pGEX vectors are available from GE Healthcare in all three reading frames and with three different protease cleavage sites (e.g., thrombin, factor Xa, and PreScission). GST fusion proteins can be purified by affinity chromatography on commercially available glutathione ( $\gamma$ -glutamylcysteinylglycine) Sepharose, which is affected by  $\gamma$ -glutamyl transpeptidase activity in crude cell lysates. Therefore, glutathione resin has a finite lifetime and can only be regenerated and reused between four and twenty times. Glutathione affinity chromatography is amenable to low concentrations of denaturing agents (2 to 3 M urea or guanidine hydrochloride), reducing agents (<10 mM  $\beta$ -mercaptoethanol or dithiothreitol), and nonionic detergents (2% v/v Tween 20), depending on the nature of the fusion protein. Elution of GST fusion proteins with 10 mM glutathione is relatively mild, often preserving protein function and antigenicity. A 70-kDa *E. coli* heat-shock-induced chaperonin often copurifies with eluted GST fusion proteins. This contaminant can be removed by treatment of cell lysates with 5 mM  $\text{MgCl}_2$  and 5 mM ATP prior to purification. Furthermore, GST can be cleaved from its fusion protein while still bound to glutathione agarose, providing a convenient method for separating the 26-kDa GST from the protein of interest. GST fusion proteins are often expressed at high levels in *E. coli* (typical yields ~10 mg/liter), which may result in accumulation of aggregated protein in inclusion bodies. Purification from inclusion bodies has both advantages and disadvantages. Some advantages are that protein targeting to *E. coli* inclusion bodies allows the high-level expression of toxic genes and the separation of inclusion bodies serves as a significant purification step from whole-cell lysate. Unfortunately, since glutathione affinity chromatography depends on the proper three-dimensional fold of GST, insoluble fusion proteins must be refolded and buffer exchanged before purification; however, some insoluble proteins may not refold correctly or into a soluble form. Another potential disadvantage of the GST tag is that the large size of the tag and its dimerization in solution may affect the properties of the fusion protein. GST fusion proteins can be detected by a colorimetric assay with the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) or with anti-GST antibodies (e.g., GE Healthcare, Sigma, BD Biosciences). Precipitations of GST fusion proteins with glutathione-coupled beads are commonly used for the detection of protein-protein or protein-DNA interactions.

#### Strep-tag/Strep-Tactin system

The *Strep*-tag II is a short peptide (eight amino acids, WSHPQFEK), which binds with high selectivity to *Strep*-Tactin, an engineered streptavidin. The binding affinity of *Strep*-tag II to *Strep*-Tactin ( $K_d = 1 \mu\text{M}$ ) is nearly 100 times higher than to streptavidin. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its bioactivity. The *Strep*-tag system can be used to purify functional *Strep*-tag II proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria. After application of the crude extract on a *Strep*-Tactin column and a short washing step, gentle elution of purified recombinant protein is performed by addition of low concentrations (2.5 mM) desthiobiotin. The *Strep*-tag/*Strep*-Tactin interaction is compatible with a variety of reagents making the system attractive for purifying metallo- and membrane proteins, large proteins and protein complexes. Binding capacity (25 - 100 nmol/ml) depends on the *Strep*-Tactin matrices and on the fused recombinant protein. Because of its small size, *Strep*-tag generally does not interfere with the bioactivity of the fusion partner. Thus, removal of the tag becomes superfluous. Comprehensive reviews and scientific publications giving an overview of various *Strep*-tag applications are listed at <http://www.ibago.com/>.

#### ***Strep*/6xHistidine system (double-tag)**

The *Strep*/6xHistidine system (double-tag) was developed to guarantee purification of full-length recombinant proteins at high purity. Recombinant proteins that carry 6xHistidine-tag at the N-terminus and *Strep*tag II at the C-terminus (or vice versa) are efficiently expressed in *E. coli*, yeast, insect, or mammalian cells. After cell lysis and clearing of the lysate, such recombinant proteins may be initially purified using IMAC (immobilized metal ion affinity chromatography) based on the 6xHistidine-tag/-Ni-NTA interaction. After elution from the Ni-NTA matrix with imidazole, the recombinant protein (which also carries the *Strep*-tag II epitope) is loaded directly onto a *Strep*-Tactin matrix. No buffer exchange is required. After a short washing step, the recombinant protein is eluted from the *Strep*-Tactin matrix using desthiobiotin.

### **3.1.4 Preparation and extraction of insoluble (inclusion body) proteins from *Escherichia coli***

High-level expression of many recombinant proteins in *Escherichia coli* frequently leads to the formation of highly aggregated protein commonly referred to as inclusion bodies. Inclusion bodies are normally formed in the cytoplasm; alternatively, if a secretion vector is used, they can form in the periplasmic space. Inclusion bodies recovered from cell lysates by low-speed centrifugation are heavily contaminated with *E. coli* cell wall and outer membrane components. The latter are largely removed by selective extraction with detergents and low concentrations of either urea or guanidine.HCl to produce so-called washed pellets. These basic steps result in a significant purification of the recombinant protein, which usually makes up to 60% of the washed pellet protein. The challenge, therefore, is not to purify the recombinant-derived protein, but to solubilize it and then fold it into native and biologically active protein.

Guanidine-HCl (8 M), urea and dithiothreitol (DTT) are commonly used to solubilize the washed pellet protein. Extraction with the denaturant simultaneously dissociates protein-protein interactions and unfolds the protein. As a result, the extracted protein consists (ideally) of unfolded monomers, with sulfhydryl groups (if present) in the reduced state.

The solubilized, denatured proteins are then folded with concomitant oxidation of reduced cysteine residues into the correct disulfide bonds to obtain the native protein.

### **3.1.5 Detection of protein-protein interactions by co-precipitation**

Coprecipitation of proteins is a valuable approach to test for physical interactions between proteins of interest. When a precipitating antibody is used, this method is referred to as co-immunoprecipitation. Coprecipitation can be used to study interactions between known proteins under a variety of conditions and as a means of identifying components of a complex. Coprecipitation may be the single method of choice, or may be used in combination with other methods that detect protein-protein interactions, such as two-hybrid analysis and tests of physical associations using purified proteins.

The bait protein is bound to a solid-phase affinity matrix and the precipitate is tested for the presence of a second specifically associated prey protein. The presence of an associated protein is detected by separating the precipitated proteins by SDS-PAGE and then immunoblotting with an antibody that recognizes the putative associated protein. Controls to test specificity of interaction are crucial. The first step is to generate reagents that detect the two proteins in the coprecipitate under nondenaturing conditions. If antibodies are available that can immunoprecipitate the proteins under nondenaturing conditions, then these can be used. Alternatively, the proteins can be differentially tagged in a variety of ways to allow their detection with commercially available antibodies or other affinity reagents. The tagged proteins are then expressed in the

*E. coli* host using expression vectors. A frequently used option is to add a short peptide or epitope that is recognized by a commercially available high-affinity antibody. The epitope is typically added at the amino or carboxyl terminus, although internal positions that do not disrupt function can also be used. Proteins can also be fused to small proteins or peptides that have high affinity to small molecules that can be attached to a solid support. Such alternative tagging methods include fusion to glutathione-S-transferase (to allow purification by a glutathione affinity matrix) or maltose-binding protein (to allow purification by a maltose affinity matrix).

### 3.1.6 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a pivotal procedure in protein characterization. A wealth of information can be obtained using PAGE. Molecular weight determination, purity of proteins, posttranslational modifications, subunit structure, enzyme activity, protein processing, and amino acid sequence are just a few areas that can be investigated using PAGE technology.

Acrylamide is the material of choice for the preparation of electrophoretic gels that are able to separate proteins by size. Acrylamide mixed with bisacrylamide forms a cross-linked network when the polymerizing agent ammonium persulfate (APS) is added. The addition of N,N,N',N'-tetramethylenediamine (TEMED) accelerates the polymerization and cross-linking of the gel. The pore sizes created in the gel are inversely proportional to the concentration of acrylamide. A 10% polyacrylamide gel will have a larger pore size than a 15% gel. Proteins migrate faster through a gel with large pore sizes. Ideally, the percentage gel selected will place the target protein at the center of the gel.

The most widely used electrophoretic system is called discontinuous or disc electrophoresis. In a discontinuous system a nonrestrictive large pore gel, referred to as a stacking gel, is layered on top of a separating gel. Each gel layer is made with a different buffer, and the running buffer is different from the gel buffers. The lower gel, also referred to as the separating gel or resolving gel, usually consists of from 5% to 20% acrylamide and is prepared with pH 8.8 buffer. It is in this gel that the proteins will separate. The separating gel is cast first and allowed to polymerize. Subsequently, a second layer of acrylamide solution, the stacking gel, which has a lower concentration of acrylamide (3–5%), is layered over the separating gel to a height of ~1 cm. The pH of the stacking gel is usually about 2 units lower than the pH of the separating gel. These differences allow the proteins in the sample to be concentrated into stacked bands before they enter the lower separating gel.

A sample well comb is inserted into the stacking gel and polymerization is allowed to proceed. When the comb is removed, wells for loading samples are formed.

Single percentage gels consist of one acrylamide concentration throughout the running length. Samples containing a narrow range of protein sizes are well resolved on single percentage gels. For general purposes, when the protein mixture is complex, single percentage gels will not be as useful as the gradient gel format. A gradient gel consists of a gradient of acrylamide concentrations from top (low % acrylamide) to bottom (high % acrylamide). The broad range of the gradient i.e. 4–20% makes it a good choice for the separation of proteins in a complex mixture containing a wide range of molecular weight polypeptides. Proteins to be electrophoresed are heated for 5 min in sample buffer containing sodium dodecyl sulfate (SDS), an anionic detergent, which denatures the proteins and binds to the uncoiled molecules. SDS binding also confers a negative charge on all of the proteins so that in an electric field they will migrate solely as a function of their molecular weights. With few exceptions, the charge conferred by the SDS will mask any charge that is normally present.

Therefore, when treated with SDS, polypeptides become rods of negative charges with equal charge densities or charge per unit length, and their separation is a function of size differences only. Most proteins contain a number of Cys-residues, often linked in pairs to form disulfide bridges. Therefore reducing agents are used such as  $\beta$ -mercaptoethanol (2-ME), dithiothreitol (DTT) or tributylphosphine (TBP). When a reducing agent is included in the sample buffer, the sample is run under reducing conditions. Conversely, if the sample does not contain a reducing agent, it is run under non-reducing conditions. Bromophenol blue, a low molecular weight pH-sensitive dye, is included in the sample buffer as a tracking dye. Bromophenol blue migrates at the front or leading edge of the electrophoretic run. The progress of the electrophoretic run can be visually monitored by following the movement of the blue dye through the gel. Typically the gel is electrophoresed until the bromophenol blue is within 1 cm of the bottom of the gel. Glycerol or sucrose is included in the sample buffer, increasing the density of the sample so that it will displace the running buffer when loaded into the sample wells.

### 3.1.7 Western blot analysis

Proteins are separated by gel electrophoresis and transferred to a membrane support by electroblotting. The membrane-bound proteins are challenged with primary antibodies, either polyclonal or monoclonal, followed by

secondary antibodies coupled to an enzyme whose activity causes the deposition of a colored or chemiluminescent product. Alternatively, primary antibodies can be used that are directly coupled to such an enzyme.

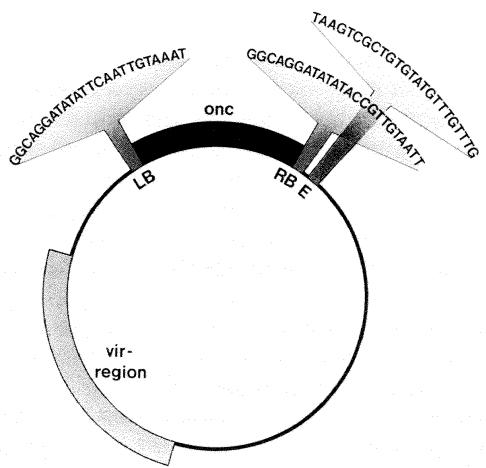
## 3.2 Agrobacterium-T-DNA Overdracht naar Planten

© Dit hoofdstuk is een onderdeel van de cursushandleiding "Genetica van Planten"

### 3.2.1 Introductie

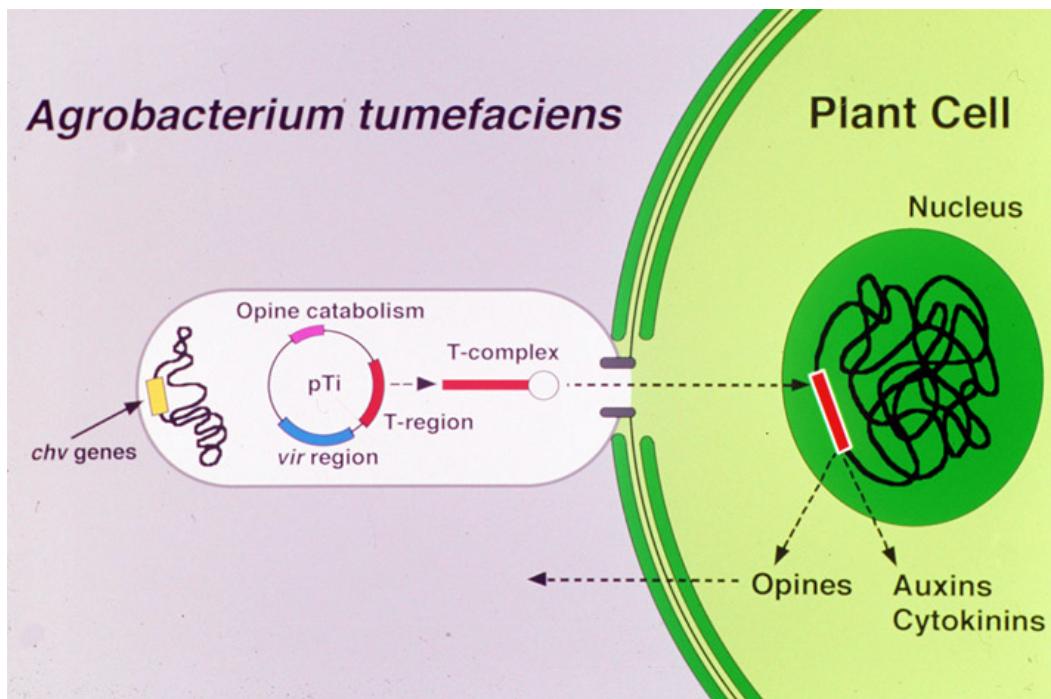
De gramnegatieve bacterie *Agrobacterium tumefaciens* is de veroorzaker van de zogenaamde "**crown gall**"

**tumoren** bij tweezaadlobbige planten. *Agrobacterium* is in staat op wondvlakken bij planten aan te hechten en vervolgens een gedeelte van het **tumorinducerend (Ti) plasmide** naar plantencellen over te brengen. De overdraagbare DNA regio van het plasmide, het "transferred" DNA ofwel **T-DNA**, wordt begrensd door imperfecte repeats van 24 baseparen, ook wel "**border repeats**" genoemd (Figuur 3.1). De border repeats bepalen welk stuk van het Ti plasmide gekopieerd wordt en als een **T-strand** of T-complex (T-strand + eiwit) naar de plantencel wordt overgedragen (Zie Figuur 3.2).



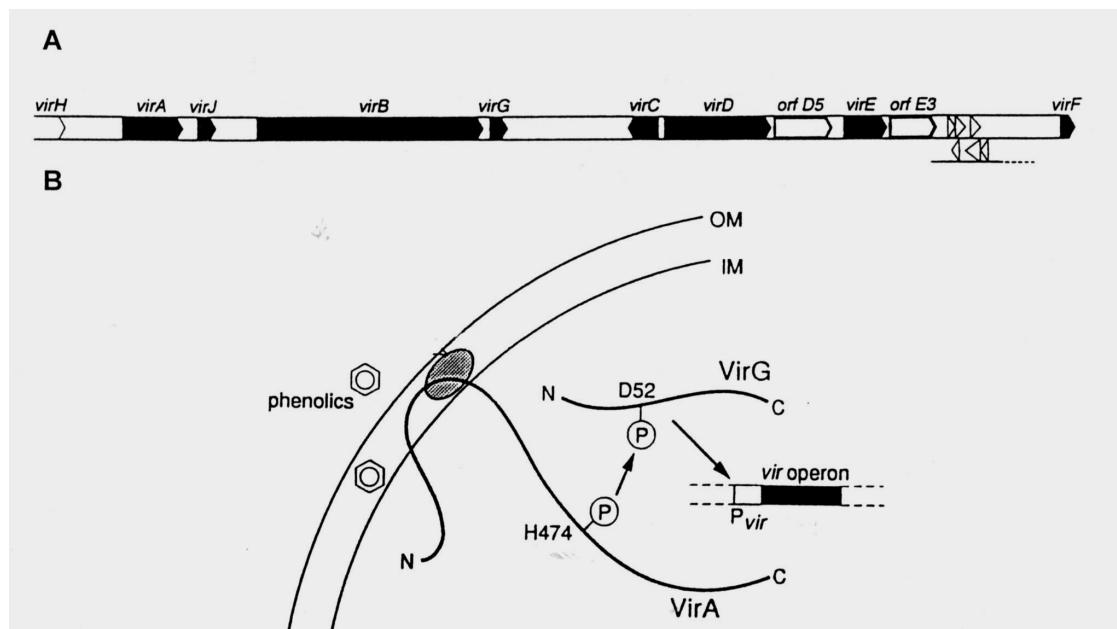
Figuur 3.1 Agrobacterium Ti plasmide.  
LB/RB: linker en rechter border repeat.

Op de T-regio liggen genen die in de plantencel tot expressie kunnen komen. De **onc** genen coderen voor enzymen die betrokken zijn bij de biosynthese van de plantenhormonen **auxine** en **cytokinine** (Figuur 3.1 & 3.2). Door de overproductie van plantenhormonen in de met het T-DNA **getransformeerde** plantencellen gaan deze cellen en hun buurcellen zich ongeremd delen en worden tumoren gevormd. Op de T-regio liggen ook genen die coderen voor enzymen die betrokken zijn bij de biosynthese van **opines**. Opines zijn van aminozuren afgeleide verbindingen die door de bacterie als koolstof- en stikstofbron gebruikt worden. Door tumoren te induceren creëert *Agrobacterium* op deze manier voor zichzelf een aangename leefomgeving waar de juiste voedingsstoffen in overvloed voor handen zijn. *Agrobacterium* stammen zijn in te delen naar het type opine dat in de geïnduceerde tumoren wordt geproduceerd.

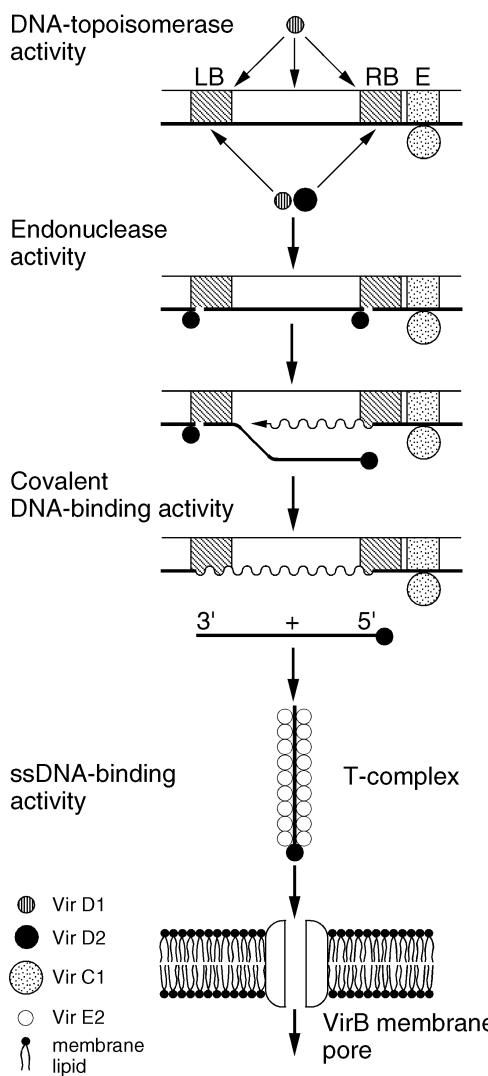


Figuur 3.2. T-DNA overdracht van *Agrobacterium* naar de plantencel.

### 3.2.3 Initiatie van T-DNA overdracht: inductie van virulentie genen.



Figuur 3.3. A. De organisatie van de vir operons van een octopine Ti-plasmide. B. Inductie van vir operon expressie door fenolische verbindingen verloopt via VirA en VirG.



Bij het proces van T-DNA overdracht zijn diverse bacteriële genen betrokken die de virulentie van *Agrobacterium* op een gastheerplant bepalen. Een aantal van de genen is gelegen op het chromosomale DNA. Deze zijn onder andere verantwoordelijk voor de aanhechting van de bacterie aan de plantencelwand (*chvA* en *chvB*). Daarnaast is op het Ti plasmide een regio te onderscheiden waar een achtal **virulentie-operons** (Figuur 3.3A), respectievelijk *virA* t/m *virH*, gelegen zijn. Met uitzondering van de genen *virA* en *virG* worden de *vir* operons niet overgeschreven gedurende normale vegetatieve groei van de bacterie. Pas wanneer de bacterie met verwonde plantencellen in aanraking komt, treedt er gecoördineerde inductie van expressie van de zes operons op. De inducerende stoffen zijn daarbij fenolachtige verbindingen, zoals **acetosyringon**, die zich op plaatsen van verwonding in de plant ophopen. De inductie van de *vir* operons is afhankelijk van de aanwezigheid van zowel ***virA*** als ***virG*** (Figuur 3.3B). Het *VirA* eiwit zit in het binnemembraan van de bacterie verankerd en dient als receptor voor de inducerende verbindingen. Na herkenning geeft *VirA* het signaal via fosforylatie door aan *VirG*, dat vervolgens als positieve regulator van de *vir* operons kan optreden.

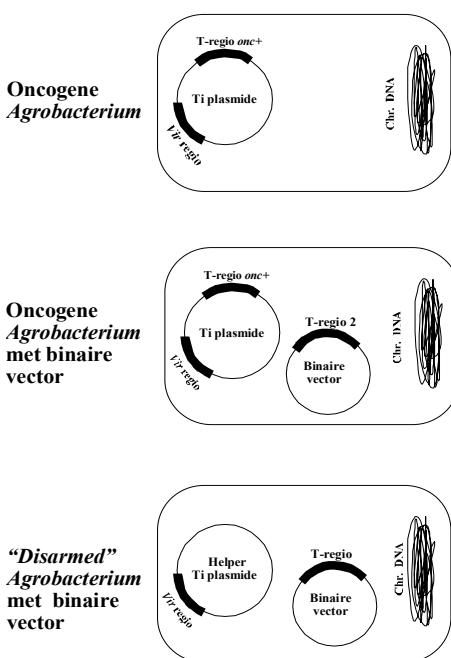
Figuur 3.4. Model voor T-DNA productie.  
NB: De binding van *VirE2* treedt waarschijnlijk pas op na overdracht. Zie ook tekst 3.2.6.

### 3.2.4 Het mechanisme van T-DNA overdracht

De productie van het T-DNA wordt geïnitieerd door twee eiwitten die gecodeerd worden door het **virD operon**, VirD1 en VirD2. Deze treden op als een **nickase**, dat op de plaats van de bordersequentie een enkelstrengs breuk introduceert (Figuur 3.1). Het VirD2 blijft daarbij covalent gebonden aan het 5' uiteinde van het DNA. Het vrije 3'OH einde dient als primer voor replacement DNA synthese waarbij de oude kopie als een enkelstrengs **T-strand** vrij komt. De T-strand vormt met één of meer Vir eiwitten, in ieder geval met VirD2, het **T-complex** wat via een **pilusstructuur** naar een eukaryote gastheer getransporteerd kan worden. Het *virB* operon codeert in combinatie met het *virD4* gen voor de eiwitten die de pilus vormen.

De functie van het covalent binden van VirD2 aan het T-DNA is tweeledig: het geeft bescherming tegen 5' ->3' exonuclease activiteit en ook bevat VirD2 signaalsequenties die verantwoordelijk zijn voor transport van het DNA-eiwit complex naar de kern van de plantencel. In de kern zal de T-strand weer een dubbelstrengs DNA molecuul moeten vormen, voor of na de integratie van het T-DNA in het genoom van de gastheer. In planten vindt integratie van vreemd DNA voornamelijk plaats via een proces van **illegitieme** (niet homologe) **recombinatie**. Het T-DNA integreert dan op een willekeurige positie in het chromosomale DNA, ook al is op het T-DNA homologie met het genomische DNA vorhanden. Daarbij kunnen meerdere T-DNAs op verschillende posities (loci) integreren, maar vaak worden meerdere inserties als tandem en/of "inverted" repeat op hetzelfde locus aangetroffen.

### 3.2.3 Genetische modificatie van planten



Figuur 3.5. Ontwikkeling van *Agrobacterium* als vector voor DNA transfer naar planten.

DNA isolaties. De meeste binaire vectoren repliceren niet stabiel in *Agrobacterium* of *E. coli* en bevatten een antibioticum resistantiegen (bijvoorbeeld tegen kanamycine) voor selectie van de plasmiden bevattende bacteriën.

### 3.2.4 Regeneratie en selectie van transgene planten

Om met behulp van *Agrobacterium* transgene planten te kunnen maken, moet er een methode voor regeneratie van planten uit de met T-DNA getransformeerde cellen worden ontwikkeld. De keuze van het juiste weefseltype blijkt daarbij zeer belangrijk. Zo kunnen voor *Nicotiana tabacum* (tabak) transgene planten geregenereerd worden uit bladschijfjes, zaailingen of bladprotoplasten. Bij aardappel worden juist weer knolsegmenten gebruikt. Voor de modelplant *Arabidopsis thaliana* (zandraket) is **regeneratie** van transgene

planten mogelijk uit zowel bladschijfjes als wortelsegmenten, maar het is bij deze plant veel gemakkelijker om bloeiwijzen in een *Agrobacterium* suspensie te dopen ("floral dip") waarna eicellen getransformeerd worden en zaden met transgene embryo's ontstaan.

Het bepalen van het juiste weefseltype en de samenstelling van het regeneratiemedium (hormonen als auxine en cytokinine, suiker, zouten etc.) is een empirisch proces. Zijn de regeneratiecondities eenmaal bepaald, dan zal bekijken moeten worden of deze condities ook optimaal zijn voor het samen kweken van weefsels met *Agrobacterium* en voor de T-DNA overdracht. Hiertoe wordt op de T-regio een zgn. **reporter gen** gebracht dat alleen na introductie in de plantencel tot expressie kan komen en waarvan het genproduct eenvoudig te detecteren is. In paragraaf 3.3.4 worden twee algemeen gebruikte reportergenen besproken.

Ongeveer 3 dagen na het samenbrengen van *Agrobacterium* met plantenweefsels wordt er een piek in de expressie van het reporter gen gevonden, de zgn. **transiënte expressie**. In eerste instantie werd verondersteld dat deze piek veroorzaakt wordt door genexpressie vanaf niet geïntegreerde T-DNAs die na verloop van tijd verloren gaan. Volgens een meer recente hypothese zijn de T-DNAs wel degelijk in het genoom geïntegreerd, maar wordt na verloop van tijd de genexpressie in de meeste cellen onderdrukt (**silencing**). Het aanwezig zijn van hoge aantallen T-DNA kopieën of inverted repeat structuren zou silencing bevorderen.

Een essentiële stap in het maken van transgene planten is de selectie van de transgene cellen gedurende het regeneratieproces. Op de T-regio is daarom meestal een **selectiemarker** aanwezig die de plantencellen resistent maakt voor een antibioticum of een herbicide. Door dit antibioticum/herbicide aan het regeneratiemedium toe te voegen, zullen alleen de transgene cellen tot scheuten kunnen regenereren. Veel gebruikt is het van Tn5 afkomstige *nptII* gen dat, codeert voor een aminoglycoside fosfotransferase. Dit enzym brengt een fosfaatgroep over naar antibiotica als neomycine en kanamycine en inactiviert hiermee de werking van het antibioticum (inhibitie van de translatie in prokaryoten of chloroplasten door binding aan de 30S ribosomale subeenheid). Vergelijkbaar in werking is het uit *E. coli* afkomstige *hpt* gen dat codeert voor hygromycine-B fosfotransferase. Naast deze twee genen wordt er veel gebruik gemaakt van het fosfinotricine acetyltransferase (*pat*) gen uit *Streptomyces hygroscopicus*. Dit gen maakt cellen resistent tegen het herbicide fosfinotricine (in het engels afgekort tot PPT). PPT is een aminozuuranaloog (ammoniumglufosinaat) en een irreversibele inhibitor van het glutaminesynthetase enzym van planten.

### 3.2.5 Genexpressie in planten

Allereerst een **definitie van genexpressie: het proces waarbij de informatie uit een gen tot uitdrukking komt als een functioneel genproduct**. Meestal is dat eiwit, maar het kan ook RNA zijn (denk aan rRNA, tRNA, etc.). De genen die op het wildtype oncogene T-regio liggen, dus zoals ze in *Agrobacterium* worden aangetroffen, komen in planten tot expressie. Dit betekent dat ze voorzien zijn van regulatorsequenties, zoals een promoter, een transcriptie terminatiesignaal en een translatiestart, die door de genexpressiemachinerie van planten herkend worden. De transcriptiesignalen van een aantal T-DNA genen zijn uitvoerig bestudeerd en worden gebruikt voor het tot expressie brengen van transgenen in planten. Aangezien de **promoter** voor een belangrijk deel de weefselspecificiteit en het tijdstip van expressie bepaalt, is met name aan deze regulatorsequentie veel aandacht besteed. Een aantal promoters van T-regio genen, zoals die van het nopalinesynthase gen en het octopinesynthase gen geven expressie in alle cellen van de plant en zijn daardoor veel gebruikt als zgn. **constitutieve promoters** voor de expressie van resistentiemarkers. Een alternatieve, zeer sterke constitutieve promoter is afkomstig van het Cauliflower Mosaic Virus (CaMV). Tijdens de replicatie van het DNA virus in de plant is deze promoter verantwoordelijk voor de transcriptie van het 35S RNA, een RNA kopie van het volledige virus genoom. De promoter wordt dan ook de **CaMV 35S promoter** genoemd.

De expressieniveaus van transgenen kunnen per transgene plantenlijn aanzienlijk verschillen. De expressie van het **transgen** blijkt afhankelijk te zijn van de positie van integratie van het T-DNA en ook van het aantal T-DNA inserties per plantenlijn. Een enkele T-DNA insertie blijkt de meest stabiele expressie te geven, terwijl bij meerdere inserties in repeat structuren er meestal na verloop van tijd **silencing** van het transgen optreedt.

Transgenen worden niet altijd constitutief tot expressie gebracht. Soms is het genproduct toxicisch of beïnvloedt het de ontwikkeling van de plant. In een dergelijk geval wordt gebruik gemaakt van een promoter die het gen op het juiste tijdstip in het doelweefsel tot expressie brengt. Een goed voorbeeld hiervoor is het bewerkstelligen van mannelijke steriliteit in planten door in de helmknop, tapetum-specifiek, een RNase gen tot expressie te brengen. Er is allerlei informatie over promoters en expressiesignalen beschikbaar.

### 3.2.6 Reporterogenen in planten

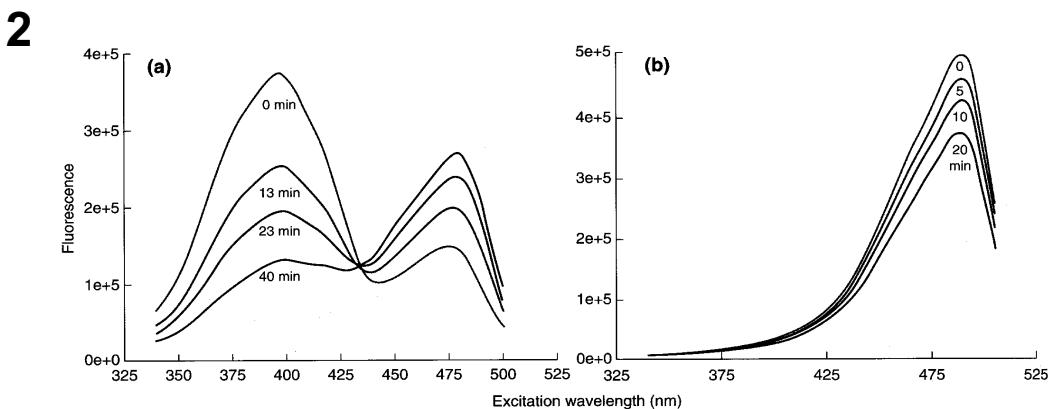
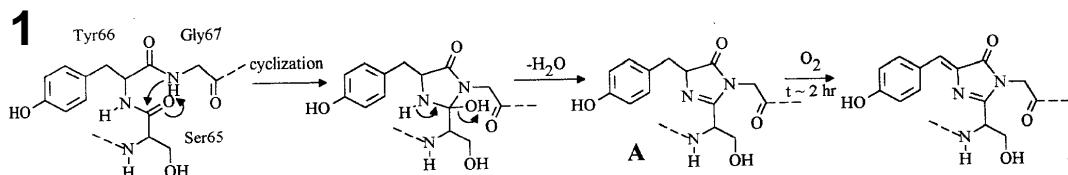


Figuur 3.6. Histochemische kleuring voor  $\beta$ -glucuronidase activiteit. X staat voor 5-bromo-4-chloro-3-indolyl.

Een veel gebruikte reporter in planten is een gen uit *E. coli* dat codeert voor het enzym  **$\beta$ -glucuronidase** (GUS). De enzymactiviteit is eenvoudig met een histochemische kleuring in plantenweefsels te detecteren (Figuur 3.6). Hier toe wordt het weefsel geïncubeerd in een buffer die 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide (X-gluc) bevat. De X groep wordt door het  $\beta$ -glucuronidase van het glucuronide afgesplitst en vormt na oxidatieve dimerisatie een blauw neerslag in de cel. Door een detergens aan de kleurbuffer toe te voegen dringt het X-gluc gemakkelijker de cellen binnen. Achtergrondkleuring door planteigen glucuronidase enzymen wordt onderdrukt, door het weefsel bij een pH te brengen die alleen voor het *E. coli* enzym optimaal is (pH=7). Een combinatie van ferri- en ferro-cyanide zorgt voor versnelde oxidatieve precipitatie van het reactieproduct. Hiermee wordt voorkomen dat het vrijgemaakte oplosbare X uit de cel kan diffunderen, waardoor een oneigenlijk patroon van kleuring kan ontstaan. Helemaal **celautonom** is de reactie echter niet.

Een alternatieve reporter die in planten ook veel gebruikt wordt is het *gfp* gen. Het gen is afkomstig uit de kwal *Aequorea victoria* en codeert voor het **groen fluorescerend eiwit** (Eng. afk.= GFP). Dit eiwit zendt groen licht uit ( $\lambda$  max = 509 nm) wanneer het aangestraald wordt met ultraviolet (UV) of blauw licht ( $\lambda$  max = 400 nm met een tweede piek bij  $\lambda$  max = 475 nm; Figuur 3.7.2). De **chromofoor**, het fluorescerend centrum van GFP, ontstaat door cyclisatie en oxidatie van het tot de aminozuurvolgorde van het GFP behorende tripeptide Ser65-Tyr66-Gly67 (Figuur 3.7).

Aangenomen wordt dat de chromofoor autokatalytisch gevormd wordt of dat bij dit proces algemeen aanwezige cellulaire factoren betrokken zijn. Het GFP-eiwit is reeds in verschillende organismen met succes tot expressie is gebracht. Om GFP als reporter in planten te kunnen gebruiken, bleek het nodig om de coderende regio aan te passen aan het codon-gebruik in planten. Tegelijkertijd is met deze aanpassingen



Figuur 3.7. Het *gfp* gen als reporter. 1. De vorming van het GFP-chromofoor door cyclisatie en oxidatie. 2. excitatiespectra van het orginele (a) en de S65T variant (b) van het GFP-eiwit. De emissie is gemeten bij 509 nm. Na lang aanstralen treedt er onomkeerbare fotobleking op (Cubitt et al., 1995, TIBS20, 448-445).

een cryptisch intron, dat in planten herkend wordt, verwijderd. Een andere belangrijke aanpassing is een mutatie in het *gfp* gen die een aminozuursubstitutie van Ser65 naar Thr65 tot gevolg heeft. De nieuwe variant heeft alleen nog een excitatiepiek rond 490 nm (blauw licht), maar geeft een 33-voudige emissie van groen licht vergeleken met het originele eiwit bij deze golflengte (Figuur 3.7.2).

Het *gfp* gen en het *gusA* gen hebben uiteraard hun eigen voor- en nadelen als reporters in planten. Het *gusA* blijkt in de praktijk een gevoeligere reporter. GUS-activiteit is zelfs bij lage expressieniveaus aan te tonen, met name doordat de enzymatische reactie als versterker van het signaal werkt. De assay is echter destructief en het materiaal kan na de histochemische kleuring niet meer verder gekweekt worden.

Tegenover de lagere gevoeligheid met het *gfp* gen staat dan ook dat GFP via een niet destructieve methode in de plantencel aangetoond kan worden. Dit geldt zeker voor de S65T-GFP-variant waarbij het weefsel met blauw licht in plaats van met het mutagene UV aangestraald wordt. Daarbij komt nog dat het GFP-eiwit een betere indicator is van celspecifieke genexpressie dan het GUS-eiwit, aangezien het GFP-eiwit minder snel van cel naar cel zal diffunderen dan het product X van de glucuronidase reactie. Om de voor- en nadelen van beide reporters te combineren zijn er genen beschikbaar die coderen voor een GUS-GFP of GFP-GUS fusie-eiwit. Recent zijn er op GFP gebaseerde technieken beschikbaar gekomen die gebruik maken van de vinding dat onderdelen van GFP pas na samenkomst een fluorescent eiwit opleveren.

### 3.3 *Agrobacterium*: Tumorvorming op *Nicotiana glauca*

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#### 3.3.1 Protocol

Tumorinductie door wildtype en mutante *Agrobacterium tumefaciens* stammen wordt getest op stengels van *Nicotiana glauca*. Door grootte en gewicht van tumoren te bepalen zal worden onderzocht of er aanwijzingen zijn voor overdracht van virulentie-eiwitten naar de plantencellen.

##### Opgroeien van planten

Ongeveer 2 maanden voor de geplande prikdatum worden zaden van *Nicotiana glauca* op aarde uitgezaaid en bij 24°C, 70% luchtvuchtigheid en 16 uur fotoperiode ontkiemd. Na 3 weken worden de zaailingen verpot en 2 à 3 weken voor het prikken worden de planten afzonderlijk opgepot.

##### Kweken van *Agrobacterium* stammen

Alle handelingen moeten onder Veilige Microbiologische Technieken (VMT) condities worden uitgevoerd, d.w.z. witte jas aan, werken bij een Bunsenbrander en in een VMT laboratorium (luchtververing + lichte onderdruk).

Voor lange termijn opslag worden *Agrobacterium* stammen als glycerolstock bij -80°C bewaard. Een glycerolstock wordt verkregen door 2 ml overnachtcultuur in een 2 ml Eppendorf buis af te draaien (6500 rpm) en de bacteriepellet in 1 ml 0.7% pepton / 26.1% glycerol te resuspenderen.

Let op: Gebruikte cuvetten / Falconbuizen / restanten van bacteriesuspensies moeten bij het afval voor de autoclaaf!

VRIJDAG, voorafgaand aan practicum (assistentie)

Strijk met een steriele entnaald (öse) de gewenste bacteriestammen vanuit de glycerolstock rein op LB-agar platen met de juiste antibiotica (zie Tabel 3.1).

Incubeer de platen 3 dagen bij 29°C.

##### De inoculatie op planten

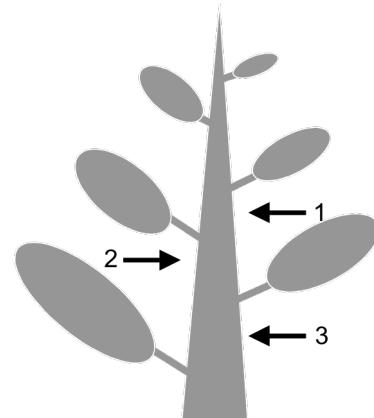
MAANDAG (week 7) worden voor de inoculatie gebruikte bacteriestammen in vloeibaar medium geënt.

Per paar worden 8 planten geïnfecteerd met de volgende stammen of combinaties

(1 plant per bacteriestam / combinatie):

1. LBA1100 (geen T-DNA)
2. LBA1010 (wt pTi)
3. LBA2572 (pTi deletie VirE2)
4. LBA2560 (pTi deletie VirF)
5. LBA1517 (pTi VirF::Tn831)
6. LBA2572 + LBA1100
7. LBA2560 + LBA1100
8. mock infectie

Inoculatieschema



Om een vergelijkbaar aantal cellen te gebruiken wordt de OD600 van een overnacht gegroeide bacteriesuspensie gemeten (ongeïnfecteerd LB medium is de referentie). Verdun in de cuvet 0.25 ml culture met 0.75 ml LB medium om in het traject tussen OD 0.4 en 0.8 te komen. Bereken hoeveel ml van de culture (al dan niet uit de cuvet) nodig is om 0.5 tot 1

ml bacteriesuspensie te maken met **OD 1.0**.

**Let op:** voor **gemengde inoculaties** (zoals nrs. 5 en 6) worden kort voor gebruik twee (denkbeeldige) suspensies van **OD 2.0** in 1:1 verhouding gemengd; per volume-eenheid zijn dan evenveel cellen van een bepaald type aanwezig als bij 1 t/m 4.

Pipetteer de benodigde hoeveelheid bacteriecultuur in een epje, centrifugeer 1 min bij 4000 rpm, verwijder het supernatans en neem het pellet op in de gewenste hoeveelheid fysiologisch zout oplossing (0.9% NaCl). Let goed op de aanwijzingen hoe de stengel van een *N. glauca* plant 3 keer moet worden verwond, te beginnen bij het 3e blad van de top (zie inoculatieschema). **Verwond niet meer dan 1 plant tegelijk.**

Pipetteer met een Gilson pipet 10 µl van een bacteriesuspensie (of mengsel) in een wondgat. De planten worden na inoculatie in de groeikamer bij 24°C, 70% RH teruggezet.

#### Score van tumorvorming

- Snijd het stuk stengel met alle wondgaten/tumoren uit de plant.
- Scoor de tumorvorming op het oog. Neem hierbij LBA1010 tumoren als 100% en controle als 0%.
- Maak ook een foto van het geheel.
- Snijd vervolgens **per plantenstengel** het tumorweefsel met een scheermes af en weeg het totaal aan tumorweefsel van één stengel. Gebruik een gevoelige weegschaal voor kleine tumoren.

De volgende punten komen aan de orde in het verslag:

- Vraagstelling, verwachting en opzet van de proef
- Het effect van de mutaties/stammen/mengsels op de T-DNA overdracht.
- Nut (en effect) van de stammen LBA1100 en LBA1010 bij de proef.
- Let op goede representatie (foto's/figuren/tabellen: ook voor presentatie te gebruiken))
- Statistiek gebaseerd op T-toets met n=...

#### Materiaal en Methode

##### Agrobacterium stammen:

	Stam	Ti plasmide	Antibiotica resistentie
1	LBA1100	pTiB6 (onc-)	rif, spc
2	LBA1010	pTiB6 (onc+)	rif
3	LBA2572	pTiB6 (onc+), <i>ΔvirE2</i>	rif
4	LBA2560	pTiB6 (onc+), <i>ΔvirF</i>	rif
5	LBA1517	pTiB6 (onc+), <i>virF::Tn831</i>	rif, spc

##### Media/oplossingen:

- LB-medium bevat per liter: 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, pH7.0 (NaOH)
- LB-agar: 16 g Bacto-agar per liter  
Autoclaveer voor 20 minuten bij 120°C
- Fysiologisch zout (9 gram NaCl per liter)
- Antibiotica voor groeien van Agrobacterium stammen:

Tabel 3.1

Antibioticum	stock	plaat	locatie bacterieel resistentiegen
rifampicine	10 mg/ml in methanol	20 µg/ml	chromosoom
specinomycine (spec)	125 mg/ml	250 µg/ml	Ti-helper plasmide/Tn1831/incP
kanamycine (kan)	50 mg/ml	100 µg/ml	binair plasmide

## 3.4 Agrobacterium: Inductie van Virulentie Genen

Bij de bestudering van inductie van virulentie genen maken we gebruik van een reporter gen. In deze cursus wordt het *lacZ* gen gebruikt. *LacZ* maakt deel uit van het lac-operon van *E.coli* en codeert voor enzym β-galactosidase. Dit eiwit is 120 kDa, en bevat 1024 aminozuren. De functie van het enzym in *E.coli* is om lactose te splitsen in glucose en galactose. Glucose kan door *E.coli* weer gebruikt worden als koolstofbron. De synthetische verbinding o-nitrophenyl-β-D-galactoside (ONPG) wordt ook door β-galactosidase herkend als substraat en splitst ONPG in galactose en o-nitrophenol (ONP). ONP heeft een gele kleur. Deze is te

meten mbv een spectrofotometer bij 420 nm. Is ONPG in overmaat aanwezig in de reactie dan is de productie van ONP een maat voor de concentratie van  $\beta$ -galactosidase. Bovenstaande test wordt de  $\beta$ -gal assay genoemd.

In de cursus worden een aantal *Agrobacterium* virulentie mutanten gebruikt die een translationele  $P_{virB}$ -virB-lacZ fusie bevatten. Door deze stammen te groeien in een inductiemedium met acetosyringon kan geanalyseerd worden welke *vir* genen essentieel zijn voor T-DNA overdracht.

Een lijst met *Agrobacterium* stammen zal tijdens het practicum gegeven worden.

### 3.4.1 Experimentele aanpak

- Ent *Agrobacterium* stammen aan in minimaal medium (MM) en groei deze overnacht (ON) bij 29°C. Meet de OD<sub>660</sub> van de overnacht cultures. Bereken hoeveel bacteriën je per stam nodig hebt om 2 cultures te starten van 5 ml OD<sub>660</sub> = 0,22. Centrifugeer de bacteriën in een eppendorfcentrifuge 5 minuten bij 8000 rpm. Resuspendeer de pellet in 0,5 ml inductie-medium (IM). Start 2 culturen per stam:
  - 1. 250  $\mu$ l bacteriën in 5 ml IM – acetosyringone (AS)
  - 2. 250  $\mu$ l bacteriën in 5 ml IM + AS (200  $\mu$ M)
- Groei o/n bij 28°C.
- Neem 1 ml samples uit de overnacht cultures. Meet de OD<sub>660</sub>.
- Let op: is de OD<sub>660</sub> > 0,8 verdun dan het sample (de waarden moet liggen tussen 0,2 en 0,8)
- Centrifugeer 1 ml van elke cultuur 5 minuten bij 8000 rpm. Resuspendeer de pellets in 1 ml Z-buffer.
- Voeg 40  $\mu$ l tolueen toe, vortex 30 seconden (voer dit uit in de zuurkast)
- Incubeer de samples 30 minuten bij 37°C (evt. kan dit ook o/n bij 4°C)
- Meng 100  $\mu$ l sample (vermijd de tolueen) + 900  $\mu$ l Z-buffer + 200  $\mu$ l ONPG in een 2 ml buis
- Incubeer 15 minuten bij 30°C. Stop de reactie door 500  $\mu$ l 1M Na<sub>2</sub>CO<sub>3</sub> toe te voegen
- Meet de OD<sub>420</sub> (gebruik hiervoor evt een 96-wells plaat)
- Let op: is de OD<sub>420</sub> > 0,8 verdun dan het sample (de waarden moet liggen tussen 0,2 en 0,8)
- Bereken de  $\beta$ -gal-units: OD<sub>420</sub> x1000 / v(ml) x15 (min) x OD<sub>660</sub>

#### Samenstelling medium 500 ml

minimaal medium (MM)	stocks	Inductie-medium (IM)	Z-buffer, 1 liter (zelf maken)
10 ml	M-N	10 ml	16,1 g Na <sub>2</sub> HPO <sub>4</sub> .7H <sub>2</sub> O
0,5 ml	1% CACl <sub>2</sub>	0,5 ml	5,5 g NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O
2,5 ml	Sporen (micro)	2,5 ml	0,75 g KCl
5 ml	FeSO <sub>4</sub>	5 ml	0,246 g MgSO <sub>4</sub> .7H <sub>2</sub> O
1,25 ml	NH <sub>4</sub> NO <sub>3</sub>	1,25	2,7 ml $\beta$ -mercaptoethanol
5 ml pH7 !!	1,25 M K-fosfaat	0,4 ml pH4,9 !!	
5 ml	20% glucose	5 ml	
470,75 ml	steriel H <sub>2</sub> O	452,85 ml	
-----	Glycerol	2,5 ml	
-----	1 M MES pH5,5	20 ml	
-----	0,2 M AS (in DMSO)	0,5 ml	
<b>ONPG (donker, 4°C)</b>			
4 mg ONPG in 1 ml Z-buffer			
<b>Stopoplossing</b>			
1M Na <sub>2</sub> CO <sub>3</sub>			

## 3.5 Overproduction of VirF Protein in *E.coli*

### 3.5.1 Growth of *E.coli* and induction of gene expression

- Overnight (o/n) cultures of *E.coli* pJLA602::VirF sense and antisense in different backgrounds are available.
- Dilute the strains 1:50 in LC<sub>cb</sub> 100, grow at 29°C until the OD<sub>660</sub>=0.2.
- Shift the temperature to 42 °C.
- Take samples at T=0, T=1 and T=3 hr
- To find out the presence of the protein in different cell fractions 2 samples are taken at each time point. Use 1.5 ml eppendorf tubes.  
For a total fraction: 0.5 OD unit.  
Preparation of total fraction:

- spin down in eppendorf at maximum speed
- add 150 µl sample mix
- boil for 5 minutes 100°C
- spin down for 5 minutes
- collect the supernatant in a new eppendorf tube
- use 8 µl for detection on a PAGE-SDS gel

For lysate/pellet fraction: 1 OD unit.

Preparation of lysate/pellet fraction:

- spin down in eppendorf maximum speed
- add 500 µl extraction buffer
- sonicate the samples on ice for 3 x 30 seconds (in between 15 seconds cooling)
- spin down for 10 minutes
- collect the supernatant (=lysate or soluble fraction)
- add 10 µl 5x sample mix to 40 µl lysate fraction
- boil for 5 minutes
- use 20 µl for detection on a PAGE-SDS
- wash the pellet with 500 µl extraction buffer
- spin down and add 75 µl sample mix
- use 15 µl of the pellet fraction after boiling for detection on a PAGE-SDS gel

## Solutions for protein isolation

### Extraction buffer

100 mM Tris-HCl pH 7.6

2 mM NaCl

70 mM  $\beta$ -mercaptoethanol ( $\beta$ ME) (add just before using the buffer)

### Sample mix

0,76 gram Tris pH 6.8 (adjust pH before adding  $\beta$ ME)

2 gram SDS

11.5 ml (v/v) glycerol (10% v/v final)

5 ml  $\beta$ ME

0.02 gram Bromophenol blue

## 3.5.2 Analysis of *VirF* protein on SDS-PAGE

- Clean glass plates with water and ethanol. Be extremely careful with glass plates, combs and all other parts of the assembly; parts are quite expensive.
- Assemble glass plates with 1 mm spacers according to the Mini-PROTEAN 3 assembly guide; seal bottom with 1% agarose.
- Pour running gel (see Table 3.2 below for solution and volumes)  
Running gel stocks: 40% acrylamide (37.5:1 = mono:bis)
  - 4x resolving gel buffer (1.5 M Tris-HCl pH8.8 / 0.4% SDS)
 Fill glass plates to about 1 cm under where the bottom of the comb will be.  
Gently overlay surface with water-saturated n-butanol.  
Let running gel polymerize for 45 min.
- Remove butanol, rinse with a bit of water, remove all fluid.
- Pour stacking gel (see Table 3.2)
  - stock: 4x stacking gel buffer
  - stacking gel buffer (0.5 M Tris-HCl pH6.8 / 0.4% SDS)
 Fill with stacking gel mix to the top, and gently insert 1 mm thick comb completely without air bubbles, and immediately put two black clamps on the glass plates over the teeth of the combs. Be careful not to break the glass plates. Work fast since the stacking gel mix polymerizes quickly.
- Let stacking gel polymerize for 45 min. Gently pull out comb, remove pieces of acrylamide with a tissue and clean wells with running buffer. Carefully inspect integrity of the wells.
- Carefully mount the gel in a gel tank and divide 0.25 L 1x running buffer over the upper and lower buffer compartments.
- The protein samples are already dissolved in sample mix. Boil samples for 5 min in a screw-lid rack.
- Load the indicated amount of protein on the gel see above.  
In one of the lanes load 2.5 µL Pageruler prestained protein marker (Fermentas).
- Run at 125 V until bromophenol blue reaches bottom of glass plates (takes around 1 hr).

### 3.5.3 Protein gel staining with Coomassie Brilliant Blue R-250 (CBB)

This staining method can detect down to 0.1 µg of protein in a single band.

#### Steps in the procedure

- Transfer the gel to a Boehringer box and cover with CBB stain solution (0.1% Coomassie Brilliant Blue R-250 / 40% methanol / 10% acetic acid) and agitate slowly for 2-4 hours. Stain solution can be reused, therefore collect it afterwards in a storage bottle. Wash with water.
- Destain the gel using destain solution (20% methanol / 10% acetic acid). Put a folded paper towel at one end of the box, it will bind the CBB stain. Agitate slowly. Refresh paper towel if necessary. Destain until blue protein bands are visible and the background is clear. Stained gels can be stored permanently in 10% acetic acid, or dried between sheets of porous cellophane in a gel dryer at low temperature (40°C).

#### Notes

- Always wear gloves when manipulating the protein gel.

### 3.5.4 Western blot detection of the virF protein using polyclonal antibodies

- Repeat the procedure described in section 3.5.3 for SDS-PAGE minigels. Use for this application stain-free acrylamide (Biorad) instead of the normal acrylamide. The stain-free acrylamide gives the opportunity to detect separate proteins using the Biorad imager before protein transfer to a membrane.
- Recalculate the protein concentration of the different samples to avoid overloaded lanes, based on the results of the CBB stained gels.
- After running the gels transfer proteins to ProTran (Schleicher&Schuell) membrane, at 25 V, 1.0 A, using the Trans-Blot Turbo System apparatus (Biorad). Cut filter paper and membrane to the exact size of the separation gel.
- See Appendix 4.7: “Transfer Using Traditional Semi-Dry Consumables” for treating the gel, the membrane and filter paper.
- After transfer, wash the membrane for 5 min in 1xTBST.
- Incubate the membrane for 1 hr at room temperature (rt) in 1xTBST + 5% non-fat dry milk on a rocking shaker.
- Add the first antibody (AB against VirF), diluted 1:5000 in 10 ml 1xTBST and incubate 3 hr at rt on a rocking shaker (or o/n).
- Wash 4 times for 5-10 min in 1xTBST at rt on a rocking shaker.
- Add the 2<sup>nd</sup> AB GAR-AP diluted 1:7500 in 7.5 ml TBST and incubate 1-2 hr at rt on a rocking shaker.
- Wash 4 times for 5-10 min in 1xTBST at rt on a rocking shaker.
- Wash 2 times with water.
- Pre-incubate the blot in AP buffer.
- Stain the blot in 5ml AP-buffer containing 33 µl NBT substrate (mix very good)+16.5 µl BCIP.
- Stop the staining by adding AP-stop buffer.

#### Buffers and Reagents

##### 10x SDS electrophoresis buffer (TGS)

0.25 M Tris-base (no pH adjustment)  
1.92 M glycine  
1%SDS

##### Blotting buffer

25 mM Tris  
192 mM glycine pH 8.3  
20% methanol

##### 10x TBS (1L)

24.2 g Tris  
80 g NaCl  
pH 7.6 with HCl

##### 1x TBST (1L)

add 0.5 mL Tween-20

**AP-buffer**

100 mM Tris-HCl pH 9.5  
 100 mM NaCl  
 5 mM MgCl<sub>2</sub>

**AP-stop**

20 mM Tris-HCl pH 8.0  
 5 mM EDTA

**NBT** = Nitro blue tetrazolium (Sigma)

50 mg/ml in 70% DMF (dimethylformamid)

**BCIP** = 5-bromo-4-chloro-3-indolylphosphate (Promega)

50 mg/ml in DMF

Volumes for preparing 1-4 gels of 10% acrylamide.

<b># of 10% resolving gels</b>	<b>1 (5 mL)</b>	<b>2 (10 mL)</b>	<b>3 (15 mL)</b>	<b>4 (20mL)</b>
40% acrylamide 37.5:1	1.25 mL	2.5 mL	3.75 mL	5 mL
4x resolving buffer	1.25 mL	2.5 mL	3.75 mL	5 mL
water	2.5 mL	5 mL	7.5 mL	10 mL
10% APS	50 µL	100 µL	150 µL	200 µL
TEMED	5 µL	10 µL	15 µL	20 µL

<b># of stacking gels</b>	<b>1 (2.5 mL)</b>	<b>2 (5 mL)</b>	<b>3 (7.5 mL)</b>	<b>4 (10 mL)</b>
40% acrylamide 37.5:1	0.25 mL	0.5 mL	0.75 mL	1 mL
4x stacking buffer	0.625 mL	1.25 mL	1.875 mL	2.5 mL
water	1.6 mL	3.2 mL	4.7 mL	6.3 mL
10% APS	50 µL	100 µL	150 µL	200 µL
TEMED	5 µL	10 µL	15 µL	20 µL

## 4 APPENDICES

### 4.1 Safety

In the course we use chemicals that can be toxic. Please read this section and stick to the rules. Dispose of all waste in the appropriate manner.

Notify the course instructors immediately when an accident occurs.

If in doubt: ask one of the instructors.

#### Compulsory is:

- wearing a white lab coat and safety goggles
- washing hands before leaving the course
- performing manipulations with volatile compounds (organic solvents, strong acids) in the fume hood
- cleaning up spilled chemicals and broken glassware

#### Furthermore it is not allowed to:

- smoke, eat, drink or apply cosmetics in the course room
- pipette using your mouth

### 4.2 Chemicals

Toxicity of a chemical is usually indicated on the label. Details can be looked up.



#### Irritant or Harmful (e.g. nitro blue tetrazolium)

This symbol covers a wide range of (sometimes relatively minor) hazards - with precautions such as avoid contact with the skin, do not breathe, etc.



#### Corrosive (e.g. hydrochloric acid)

Avoid contact with skin or eyes.



#### Poisonous (e.g. methanol)

The poison symbol is self-explanatory. Whereas most chemicals are fairly dangerous if ingested or inhaled, many of these are dangerous even on contact.



#### Oxidizing chemical

Oxidizing chemicals are materials that spontaneously evolve oxygen at room temperature or with slight heating, or that promote combustion. To be kept away from flammable chemicals at all costs!



#### Flammable or extremely flammable (e.g. methanol or ethanol)

Chemicals to be stored in flame-resistant cupboards.



#### Biohazard

Use your common sense when working with chemicals. Do not add water to concentrated acids (explosive heat generation), and do not warm up ethanol using an open flame. Do not cool down autoclaved bottles under a running tap or even worse in ice (bottle will likely explode).

## 4.3 Waste

### Yellow waste bin

Non-chemical lab waste (paper, anything that is not sharp, autoclaved bacterial waste).

### Sharp waste

Sharp waste (pipette tips, pasteur pipettes, needles, broken glass) is disposed of in the white container labeled "Sharp waste"

### Liquid chemical waste

Liquid waste is disposed of in plastic containers. Aqueous solutions of non-toxic salts, sugar etc. are disposed of in the sink. Chemical waste is disposed of in separate colour-coded containers depending on the type of compounds.

**black:** anorganic compounds in solution (heavy metals, diluted acids/bases, photographic developer)

**red:** organic halogen-free liquids (ether, methanol, other organic solvents)

**blue:** organic halogen-containing liquids (chloroform, or other F-, Cl-, Br-, or I-containing liquids)

**green:** oils (e.g. oil from a vacuum pump)

### Instructions for disposal of liquid chemical waste

- First neutralize reactive chemicals
- Never put solid waste in liquid waste containers
- do not fill container completely, leave a head space of about 5 cm
- After use close container with appropriate cap
- remove liquid spilled on the outside with a tissue

### Solid chemical waste

Dispose of appropriately. Ask course instructors for instructions.

## 4.4 How to keep lab notes

Planning, setup, results and conclusions from experiments are recorded in a lab journal. The importance of keeping good lab notes should not be underestimated. Notes are not supposed to be secret and only decipherable by you. First, good notes will help you while you are doing the experiments. Second, at the end of a research phase, for example after a 1-year master research training or after a 4-year PhD research training, good notes will help you enormously with reconstructing details of experiments when writing a report, thesis or research paper. Third, your material is often used by the research group after you have left, and then it is useful if your notes (which are left behind in the group) can be easily read by someone else in case something needs to be looked up about materials made by you. But there is more. In case of suspected fraud, a commission will go through your notes to find out whether the data were collected in a regular way. If your notes are unreadable for an outsider, your position will be very weak in disproving fraud. And finally, if you are working for a company, your notes are the most important link between a product made by you and your way of manufacturing it. In fact, in most companies you have to sign your notes at the end of the day and a copy is locked up in an archive.

Here are some general guidelines for keeping notes. The general idea is that any (professionally educated) outsider must be able to understand all details of your notes without further explanation.

- Notes are kept in English language in legible handwriting.
- Start your notes with a short descriptive title of the particular experiment.
- Put the date in the left margin in the format dd/mm/yy.
- Shortly note the goal of the experiment and the setup or approach.
- Write down details of your experiment, such as volumes, particular sources of chemicals etc.
- If you paste a result in your notes, such as the picture of a DNA gel, number the lanes and identify for each numbered lane what was loaded.
- Write down if there is something unexpected about the result and write down a possible cause if known.
- Write down what action you undertook based on the experiment, for example with a gel containing restriction enzyme digests from a cloning experiment, which clone you selected for further experiments.
- Shortly state the conclusion from the experiment, and any ideas about follow-up experiments.

## 4.5 How to write a report or paper

The generally preferred way to write a report (such as a bachelor or master report or a PhD thesis) is to write it in the form of a scientific article such as you can find in journals like Cell, Genes&Development, EMBO Journal, Plant Cell and Plant Physiology. Articles in Nature and Science are written in a different way with many details left out and do not form a good format for a report. The guidelines below are therefore applicable for scientific papers in most Molecular Biology journals. Other journals, such as ecological journals for example, have other ways of describing the experiments, and are usually difficult to read for a molecular biologist. The order of sections of a manuscript to be submitted to a journal is: Title page, Abstract, Introduction, Results, Discussion, Materials and Methods, Acknowledgements, References, Tables, Figure Legends, Figures. These sections are submitted in this order for publication. For a report or thesis, the text is integrated with the figures, figure legends and tables in an article-like format (page-wide without text columns). Switch on your English spell checker, and check all green and red underlined words with the right mouse button for suggestions.

- Identify the journal that you would like to send it to. Carefully read the instructions for authors regarding text and figures, and study the layout of recently published articles.
- Don't make the writing process too complicated, for example do not read too many articles, it will just confuse you. In fact, the most efficient is to write the chapter without reading any articles, and check later on whether essential information is missing. As a guideline for time investment, if you have a rough idea about the outline of a chapter/manuscript, a first draft can be written in one evening.
- In general, the best way if you start from scratch is to first organize your **Figures** in a logical manner so that it forms a story. Pay already serious attention to the layout (see below for more detailed instructions).
- Then write the **Figure Legends**. A Figure legend has a short title. These titles will also be used as subheadings in the Results section. Try to make a conclusive title if possible. For example "The expression of the Rubisco gene is induced by light" instead of "Analysis of the expression of the Rubisco

gene after light treatment". Explain details and abbreviations shown in the Figure. The Figure should be understandable with the legend only, without the need for reading the main text.

- **Tables:** Tables are numbered independently from the Figures. They have a short title and a short explanatory legend above the Table. At the bottom of the Table footnotes can be provided that explain certain aspects of columns in the Table, such as abbreviations used, the specific statistical test employed etc. In contrast to the Figures and their legends, the Table is presented as a whole including the legend and footnotes.
- Now write the **Results section**. Divide the Results in subsections. In principle each subsection describes the results from one Figure. The heading of the subsection is the same as the title of the Figure. For each subsection, start with shortly stating the goal of the experiment. Then describe the setup if necessary, and then describe the result with referral to the Figure. At the end of the subsection draw a short one-sentence conclusion.
- Write the **Introduction**. Now that you have the results, you should write an introduction that makes it possible to understand the results and the discussion with a minimum of prior knowledge. You should make clear why it was so important to perform the experiments in the results section. Do not give any information in the introduction that is not absolutely required to meet the requirements mentioned before. An introduction is not a review, and does not treat matters exhaustively; it only describes the need-to-know things. Generally the introduction starts by determining the wide area of research that your experiments fit in, and then stepwise focuses in on the precise research area of our experiments. You give an update of the relevant state-of-the-art of the particular research area of your manuscript/chapter/report. The reader should be able to understand what was known before your new results, to be able to understand why your experiments were done and which new information they provide. At the end of the introduction you state the aim of your research and you finish with a very short summary of the main results (similar to the Abstract). Typically, an introduction consists of 5-6 paragraphs, each consisting of 4-5 sentences. Every paragraph should have a clear topic, typically announced by the first sentence, followed by a couple of sentences that add information and provide links to the literature, before the last sentence closes the topic and potentially brings up a lead to the next paragraph. A single sentence paragraph is not a paragraph.
- Write the **Discussion**. As with the other parts of the manuscript (the Introduction and the Results), the reader should be able to understand the essence of the Discussion without having read the other parts. Start therefore with a very short summary of the main new findings described in your manuscript. Then sequentially discuss relevant discussion points. Before starting to write the discussion make a list of the main points that you want to discuss. Arrange them in a logical order. Discussion points can for example be speculations on farther reaching hypotheses about molecular mechanisms. You can/should underpin such speculations if possible by supporting evidence from other publications. You can also put forward experimental approaches to test such hypotheses. Disagreements with data published by others should also be pointed out, with possible explanations for the discrepancies. If your manuscript lends itself for it, you can summarize your new data and your conclusions in a cartoon-like model (your final Figure), which you discuss at this point. Finish with one or a few sentences putting your manuscript in a broader perspective with some outlook on future experiments or developments or giving your opinion about the impact on other research areas.
- Write the **Materials and Methods** section. Here you should describe all relevant details about your experiments in a manner that allows other scientists to exactly reproduce your experiments. This section is divided in subsections usually according to the different techniques used. If methods were performed as described in another paper, general methods book or in the instructions of a kit or product, you can refer to that source and include possible modifications. Include the manufacturer of critical material such as special chemicals or instruments, in the form (Sigma-Aldrich, St. Louis, MO).
- The **Acknowledgements:** Here you thank people that have significantly contributed to the research in a manner that exceeds the normal contributions from your lab members, but not significant enough to earn a co-authorship. For example critically reading the manuscript can qualify as such. People that contributed unpublished material or information should be thanked here. One does not acknowledge people that contributed published material in this section. Such material is described in the Materials and Methods section with the appropriate literature reference and without thanking anybody. Sources of financial funding are also acknowledged here.
- **Author Contributions:** as a relatively new development, some journals have now included such a section where it is explained shortly what each author contributed to earn co-authorship. This is done to ensure that each author deserves co-authorship and avoid unnecessary inclusion of persons as some groups tend to do. The format is something like this: H.J.M.L. designed the experiments, performed and interpreted the experiments, and wrote the paper, J.M. picked his nose and downloaded music from internet.
- **References:** Here all references cited in the text are listed usually in alphabetical order, although in some journals references are ordered according to appearance in the text. See the selected journal for

the exact lay-out of the references. The format is in general something like this: Linthorst, H.J.M., and Memelink, J. (1976) Plants, like animals, use DNA for storage of genetic information. *J. of Adv. Plant Research*: 204-256. Books have a different way of being cited, with a format like: Memelink, J., and Linthorst, H.J.M. (1977). Efficient extraction of DNA from plants. In: *Plant Molecular Biology Manual*, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Kluwer Academic Publishers, Dordrecht), pp. 1-23. You can only refer to published or accepted papers, the latter are cited as: in press. If you refer to unpublished data, these should be cited in the text as (Memelink J., unpublished results), and are not listed in the reference list. Personal communications are similarly only cited in the text as (Linthorst H.J.M., personal communication). In the text, the references are cited as (Memelink, 1978), (Memelink and Linthorst, 1979) or (Memelink et al., 1980) for more than two authors. Try to keep the total number of references at about 25-35 by careful selection for importance, and by citing relevant review papers. On the other hand, make sure that all key papers about your research topic (usually less than 5) are cited, and preferably include 1 or more references from all your main competitors. The manuscript will end up on the desk of several of them for peer review, therefore it is a good idea to not directly piss them off.

- **The Abstract:** there is a limitation on the number of allowed words (about 250); see the journal instructions for details. An abstract is a shortened version of the paper/chapter/report and should contain all information necessary for the reader to determine what the objectives of the study were, how the study was done, what results were obtained, and the significance of the results. Start by shortly introducing the subject with shortly stating the state of the art, then describe the aim of the studies, the main results and then summarize shortly the new contribution to the research field.
- **Title page:** Contains the title, names of authors including full first name and all other initials, and the affiliations. Often other information is also listed depending on the journal, such as present addresses of authors and who is the corresponding author with phone number and e-mail address. The order of authors is very important, at least in the field of Molecular Biology. The first author is the person (PhD student or postdoc) that was mainly in charge of the research and performed the majority of the experiments, the next authors did part of the experiments and are listed in order of importance of their contribution, and the last author is the supervisor of the research group. Sometimes the one before last author is a staff member in the group that personally supervised the first author and other co-authors. Sometimes the order of these last two authors is switched around. Usually you can tell who is really in charge of the research by being listed as corresponding author. In order to deserve co-authorship, a person should have performed a significant part of the experiments or contributed significantly intellectually. The rule is that a co-author should be able to understand and explain his/her contribution. For example a technician that performed experiments but really has no idea what the research is about (not uncommon) does not qualify as a co-author. Providing the money via writing a successful grant application is not a contribution that earns co-authorship unless the ideas in the grant proposal were crucial for the paper. Just being the main group leader is insufficient for co-authorship, that person should have contributed significantly intellectually or in writing the paper. Affiliation is important in that if an institute is listed as an affiliation it can put the publication on its yearly publication list. Therefore if you have contributed to a paper for example by working temporarily in another lab while you were employed somewhere else, make you sure that your affiliation is listed.
- **Making high-resolution figures.** Journals demand a certain resolution and format for figures. For example TIF files with a resolution of 1000 dpi for black&white line drawings (graphs etc) and 600 dpi for half-tone and color photographs. For making Figures act as follows. Make your drawing in powerpoint. Graphs can be copied from excel. After pasting, ungroup the graph and remove all extra lines, ticks etc that you don't want. What remains can be treated as powerpoint items. Paste in photographs or scans as 600 dpi or higher items. When you are finished, save your figure as a pdf file (requires Adobe Acrobat Professional). Open the pdf file, enlarge file size to 400%, and copy the figure with the snapshot tool and paste it in a graphic program such as Adobe Photoshop or Corel Paint Shop Pro. In this program you can adjust the desired resolution and save it as a TIF file. For the layout of the Figure use the following rules of thumb. Carefully study the details of figures and publications that are similar to the one you want to make. Make letters as large as you can without messing up the Figure. Use Helvetica or Arial letter type. Experimental results have a precise way of ordering according to European (and not Arabic) logic, which is that Figures are read from left to right and from top to bottom. When you do experiments with a certain treatment, or with mutants, the control is shown at the left and the treatment at the right. Similarly, the wild-type is shown at the left and the mutant at the right. A progressive time series of treatment or development is shown from left to right and from top to bottom. When printed, Figures are placed either in a single column of 84 mm wide, or over the whole page width (two columns) of 168 mm. Determine whether your Figure can be (preferably) placed in a single column or whether it will need the whole page width. Print the Figure at the exact size that it is intended to appear in print, and carefully check whether letter sizes and other features are large and clear enough.
- **Length of the manuscript:** many journals have limitations for the total number of words or pages. This means that you should write in a concise manner, omit irrelevant information and avoid unnecessary repetition. Common lengths for concise papers are 500-1000 words for the Introduction, 1500-2500

words for the Results, and 500-1000 words for the Discussion. The Introduction, Results and Discussion should be divided in paragraphs in a logical manner.

- **One more tip:** be careful throughout the whole text not to exaggerate or over-interpret your results. After submission your manuscript will be peer-reviewed by at least two scientists active in your field, and they are looking exactly for this and this raises the chance that your manuscript will be rejected. Stick to what your results actually show. Make sure that you have all the controls to demonstrate that your results really show what they show. If you go into the adventure to interpret your results in a wider context, be extremely careful in your wording and certainly do not make any statements that hint that your paper contains some form of proof for such far-reaching conclusions.
- **And here is the best tip of all:** copy as much text as you can from published articles written by American or North-West European (not French) groups. These publications have already been extensively edited (hopefully) and should therefore have impeccable English sentences. The best way is to identify a couple of publications that are exactly like the one you intend to write, and copy as much as possible. Don't forget to change the names of genes and proteins etc. This is NOT plagiarism.

## 4.6 General instructions for high-speed centrifuges

### Selection of tubes

Always ask yourself two questions in this order of importance: (1) can the tube withstand the speed that I want to use (every tube has an upper speed limit) and (2) can the tube withstand the solvent that I am using (never a problem with aqueous solutions). Even when the answer to question 1 is "yes" according to the manufacturer's specifications, you should carefully inspect the tube whether it does not show micro-fissures. Immediately discard a cracked tube.

### Starting a centrifuge run

- the rotor should be in perfect balance. Therefore carefully distribute tubes such that tubes with equal weights face each other. Equal in this context means less than 0.1 gram difference. Tubes should close well, loss of liquid will cause imbalance.
- Check that the speed that you would like to use is within the limits of the rotor that you have selected. All rotors have an upper speed limit.
- Stay with centrifuge until it reaches 4000 rpm. Immediately switch off centrifuge if you hear strange noises or feel strange movement.

## 4.7 Transfer Using Traditional Semi-Dry Consumables

### 3.7 Transfer Using Traditional Semi-Dry Consumables

Typical procedure utilizing the Trans-Blot Turbo system and conventional semi-dry western blotting consumables is detailed below:

1. Equilibrate the gel in Towbin transfer buffer (25 mM Tris, 192 mM glycine pH 8.3, 20% MeOH) for 10 min.
2. Soak two pieces of extra-thick (2.4 mm) filter paper in transfer buffer. Six pieces of thick (0.8 mm) filter paper can be used if extra-thick paper is not available.
3. While the gel is equilibrating, prepare a transfer membrane. Wet a nitrocellulose membrane briefly in transfer buffer or PVDF membrane in methanol or ethanol for 30 sec, then wash in water for 1–2 min, and equilibrate in transfer buffer for at least 10 min with agitation.
4. Assemble the transfer sandwich on the cassette base (anode) by placing one piece of wet extra-thick or 3 pieces of thick filter paper on the bottom, then the membrane, the gel, and finally, the remainder of the wet filter paper on top. Use the blot roller to remove air from between the assembled layers (Figure 19).
5. Once the stacks are positioned in the cassette base, place the cassette lid on the base. The lid is reversible, but ensure that the electrical contacts fit closely into the slots in the base. Press the lid down firmly and turn the dial clockwise to engage the lid pins into the locking slots.
6. Load a second cassette if desired. Refer to Table 1 for the appropriate combinations of gels that can be combined in a single run.
7. Slide the cassette (with the dial facing up) into the bay until it makes contact with the magnetic interlock and you hear a click. Cassettes can be inserted into the bays in any order, with or without power to the system.
8. Select the LIST button from the Home menu and the **STANDARD SD** transfer protocol from the Bio-Rad preprogrammed protocols or the user-defined protocol of choice.
9. To initiate the run, press the navigation button that corresponds to A:RUN for the cassette in the upper bay or B:RUN for the cassette in the lower bay.

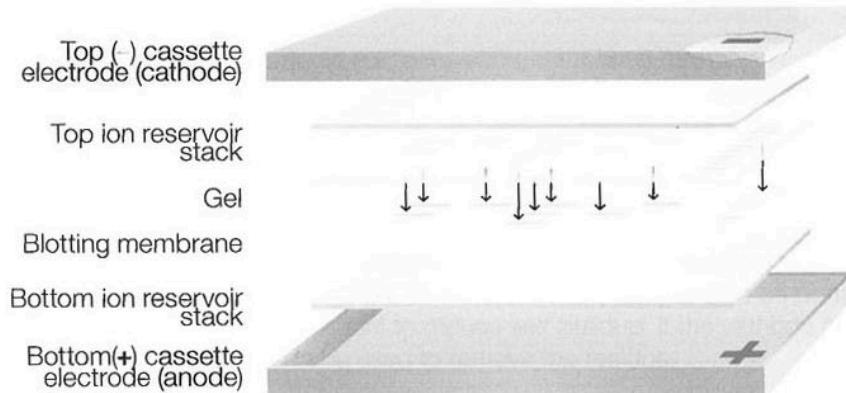


Fig. 19. Proper assembly of blotting sandwich using traditional consumables.

For further information, refer to the Bio-Rad Protein Blotting Guide: A Guide to Protein Detection and Blotting, bulletin 2895, for detailed information. Bulletin 2895 can be downloaded from our website, [www.bio-rad.com](http://www.bio-rad.com), as a PDF file, or contact Technical Support at 1-800-424-6723.

### 3.8 Optimizing Transfer Conditions

The following techniques, alone or in combination, will increase transfer efficiency:

- Use a low-percentage gel or a gradient gel. High-percentage gels retard protein transfer, especially with large proteins
- High molecular weight proteins may require increased transfer times, particularly when using thick gels. Increase the transfer time or power conditions
- With long transfer times or high power conditions, some very low molecular weight proteins may transfer through the membrane to the lower ion reservoir stack. Use a shorter transfer time or reduce power conditions for most efficient transfers
- Use the blot roller to remove any air bubbles when assembling the transfer sandwich. Air bubbles between layers of the assembled sandwich will prevent protein transfer, producing blank spots on the membrane.

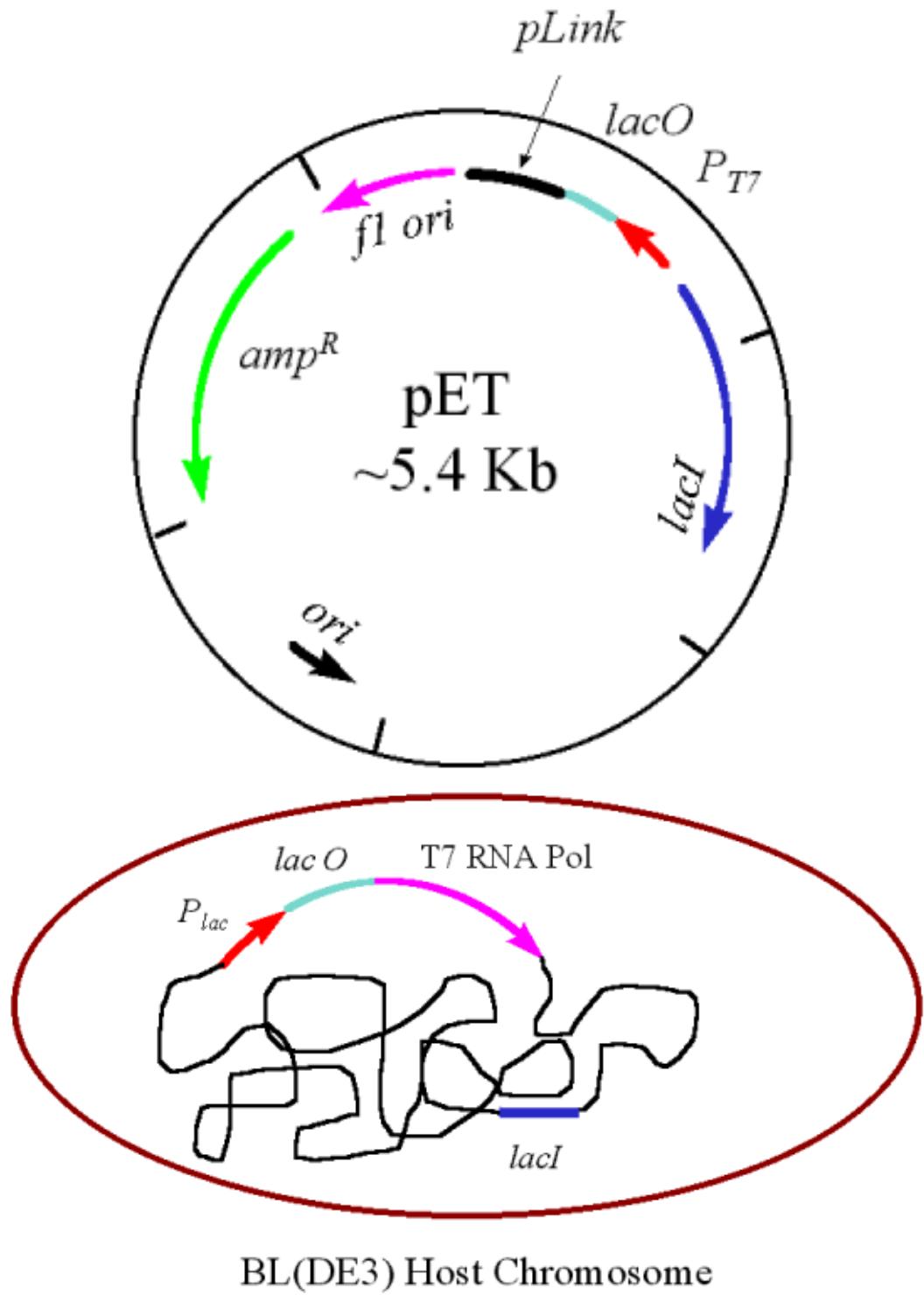
**Table 4. Recommended power conditions for transfer using the Trans-Blot Turbo system.**

	Single Mini Gel	Two Mini Gels or One Midi Gel
With Turbo Transfer Packs	25 V, 1.3 A, 7 min	25 V, 2.5 A, 7 min
With Standard Semi Dry Components		25 V, 1.0 A, 30 min

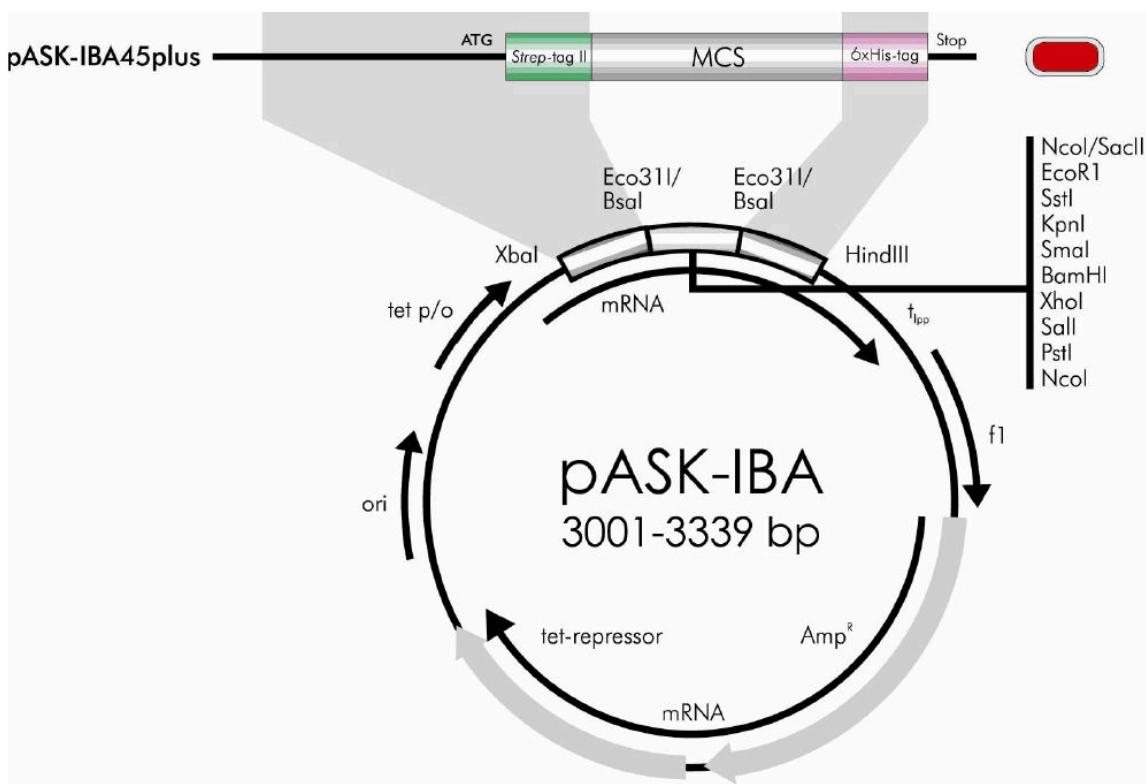
Refer to the Bio-Rad Protein Blotting Guide, bulletin 2895, for more information on optimizing electrophoretic transfer. The Protein Blotting Guide can be downloaded from our website, [www.bio-rad.com](http://www.bio-rad.com), as a PDF file, or call Technical Support at 1-800-424-6723.

## 4.8 Plasmid maps

### 4.8.1 General structure of *pET* plasmids and *BL(DE3)* *E. coli*

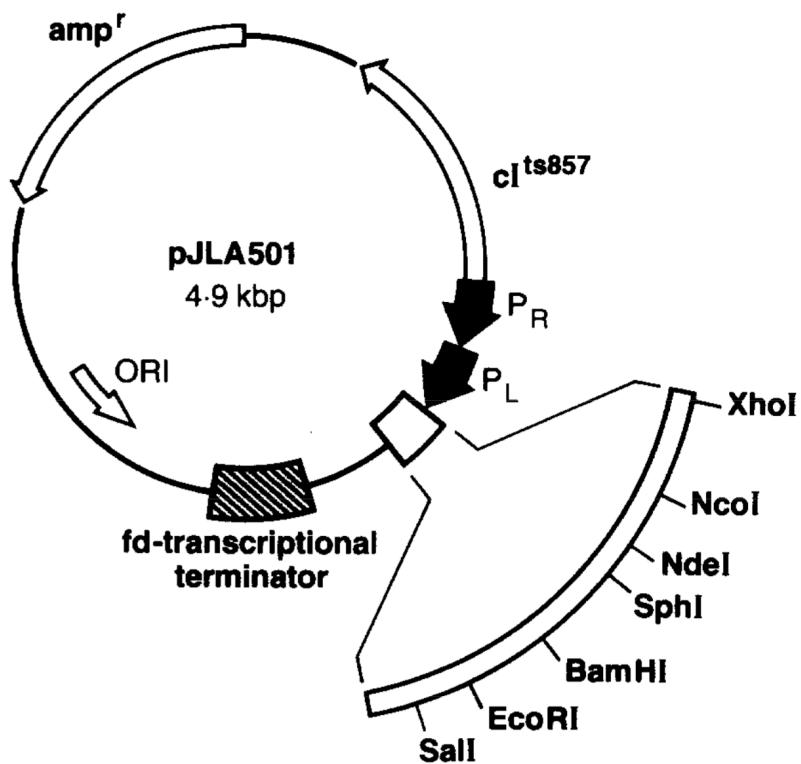


#### 4.8.2 Map of pASK-IBA45plus



#### 4.8.3 Map of pJLA501

The polylinker region of pJLA602 differs from the one shown here.



## 4.9 Hanna HI 2211 - pH meter calibration

### Two-point calibration

- The last set temperature is displayed blinking. Set the temperature to the appropriate temperature of the calibration buffers (ambient temperature, approximately 20-22°C) using the [ $\nabla^{\circ}\text{C}$ ]/[ $\Delta^{\circ}\text{C}$ ] buttons.
- Submerge water-rinsed electrode into pH 7 calibration buffer.
- Press [CAL]. "CAL" and "Buf 1" messages will appear and "7.01" (small print) will be displayed on the secondary LCD.
- The  symbol will blink until the reading is stable.
- When blinking stops "READY" and blinking "CFM" messages appear.
- Press [CFM] to confirm calibration.
- The calibrated value is now displayed on primary LCD (big print) and secondary LCD displays expected buffer pH "4.01" and "Buf 2". This will calibrate the meter for measurements in pH range 4-7.
- For measurements in pH range 7-10, press [ $\nabla^{\circ}\text{C}$ ]/[ $\Delta^{\circ}\text{C}$ ] buttons until secondary LCD displays pH "10.01"
- Submerge water-rinsed electrode into the pH 4 or pH 10 calibration buffer.
- Wait till  stops blinking and the "READY" and blinking "CFM" messages appear.
- Press [CFM], the pH meter will return to measuring

### How to activate electrode

A "slow" electrode can be reactivated by immersion in 0.1 M HCl for 15 sec. After rinsing immerse the electrode in 0.01 M NaOH. Repeat this three times.

## 4.10 Installation of Genesys20

The Genesys20 spectrophotometer will be used with several of the experiments during the course. This spectrophotometer is controlled by the Visionlite program that runs under Windows (tested with XP, 7 and 10. Version 8 was not tested, but will probably work too). Therefore, the Visionlite program plus the driver for the serial-USB cable with which the Genesys20 is connected to the computer must be installed on your laptop. There is no Visionlite for the Mac. When you have a Mac you can install VirtualBox and run the Visionlite program from a virtual Windows installed on VirtualBox. VirtualBox is freeware and can be downloaded from: <https://www.virtualbox.org/wiki/Downloads>. In addition, you need a legitimate Windows installation DVD. Ask the course leader for details.

### Installation of Visionlite:

- Download file *Visionlite.zip* from Blackboard and unzip the file.
- Double-click file *Setup.exe*. Select *Genesys20* when asked what device to install, and select port *COM3*.

### Driver installation:

- Download file *PL2303\_Prolific\_DriverInstaller\_v\_11\_0.zip* from Blackboard and unzip the file.
- Double-click file *PL2303\_Prolific\_Driverinstaller\_v1.11.0.exe*
- This will install a driver for the serial-to-USB device that connects the spectrophotometer to your computer.

### Connection of Genesys20 with the laptop (only on first use):

- Connect the Genesys20 to your laptop through the serial-to-USB conversion cable. From now on use the same USB port on your laptop.
- Switch on Genesys20 and wait until it is finished calibrating. This may take a minute or two!
- Check on which port the Genesys20 is connected in *control panel – system – device manager – ports. Prolific USB-to-Serial COMM PORT* should be indicated under COM3.
- Open the Visionlite program by clicking on its desktop icon and choose the required application. On error, right-click on the Visionlite icon and choose *File locator*, then select the program of choice

(`scan.exe`, `rate.exe`, etc.).