INTRODUCTION

The selection of methods included in the course reflects the techniques which members of the Antibiotics and Molecular Bacteriology Laboratory at the Department of Immunology, Microbiology and Parasitology considered essential for the basic practice of molecular biology. We have tried to include practical tips.

It should be emphasized that every molecular biologist evolves his or her own idiosyncratic variations on practical procedures. These often arise from the realisation that protocols should almost never be followed slavishly- a conceptual understanding of the biochemical features and properties of the systems being used far more important than the ability to replicate a procedure exactly. In other words, the practice of biochemistry is, like cooking, as much an art as a science. Perhaps the most important aspect of this is being able to evaluate the situations in which too much accuracy and precision are not appropriate (and when they are!).

BASIC PROCEDURES

This chapter covers a range of basic methods commonly used in molecular biology, including use and storage of bacterial growth media, chemicals and buffers, and frequently repeated nucleic acid manipulations such as phenol extraction, ethanol precipitation and electrophoresis.

Calculation of concentrations

Acids and bases: Glacial acetic acid with specific gravity 1.05 is 17.4 M
Acetic acid 36% with specific gravity 1.045 is 6.27 M
HCl with specific gravity 1.18 is 11.6 M
HCl with specific gravity 1.05 is 2.9 M
Ammonium hydroxide with specific gravity 0.898 is 14.8 M

Preparation of solutions and other basic things

It is remarkable how many biochemists and molecular biologist do not know the correct

methods of preparing solutions, using pipettes, etc. The following notes may be of help.

a) Solids dissolved in liquids occupy volume.

Thus, to make a 1M solution, weigh out 1 mole (the molecular weight in grams) of substance and dissolve it in less than 1 litre of solvent; make to 1 litre when it has dissolved. When making solutions to a specific pH, remember to leave space for the acid or alkali.

b) 1 mole is the molecular weight in grams ñ including the water of crystallization.

Thus, 82.1 g of anhydrous CH_3COONa contains the same amount of sodium acetate as 136.1 of $CH_3COONa.3H_2O$.

c) Dissolution of many compounds is endothermic - heat is required. This is important when making concentrated solutions of eg urea and guanidine hydrochloride. Heat can be provided by prolonged stirring at room temperature, or by gentle heating. Note that excess heating can spoil reagents: decomposition of guanidine hydrochloride can be dramatic!

d) When making mixtures of more than one component, it is generally easiest to make each component as a stock solution, and then mix these. Always add the water first ñ eg if you add NaCl and SDS before water, the SDS is likely to precipitate, depending on the concentrations of the NaCl and SDS and on the temperature. Generally, add salts and buffers before organic substances.

e) When making mixtures from stock solutions, dilute each component using the equation:

$V_1=V_2C_2/C_1$

where V_1 is the volume of stock solution of concentration C_1 to be added to a final volume (including all components) V_2 at a final concentration C_2. Note that this works with molar concentrations, with % concentrations and with weight/volume concentrations.

f) Some substances are supplied as pure liquids with no specification of concentration,

but only a specification of density - eg 2-mercaptoethanol. The concentration is calculated from the definition of a molar concentration as the number of moles of substance in 1 litre. The weight of 1 litre is obtained by multiplying the density in g/ml by 1000, and the number of moles in 1 litre by dividing this weight by the molecular weight in grams. For exmple, the weigh of 1 litre of water is 1000g, the molecular weight is 18, so the molar concentration is 1000/18= 55.555 M.

g) Use of pipettes. Air displacement pipettes can pose problems with both accuracy and with contamination. The correct way of using them is to depress the plunger to the second stop, suck up an excess of liquid, then displace to the first stop. This avoids losses, especially of viscous liquids, due to wetting on the inside of the tip. Tips should be placed into solutions as short a distance as possible to avoid carry-over on the inside of the tip, and to avoid generating aerosols which contaminate the barrel (an to avoid an inrush of liquid spouting to the barrel). It is remarkably easy to cross-contaminate experiments by bad technique - this can be monitored by carrying out your normal pipetting practice with labelled nucleotides and counting tips, barrels, tubes etc with a monitor.

h) Chemicals should be kept clean ñ ideally nothing should ever be put inside a chemical bottle, including spatulas, glass rods etc. A tiny bit of nuclease or protease getting into a restriction buffer can waste weeks of work.

i) Washing solids. Many protocols call for bacterial cells, ethanol precipitates of DNA, etc to be washed to remove substances which may interfere with subsequent manipulations. The most efficient way to wash an insoluble is to do three washes of equal volume; increasing the volume of a single wash makes very little difference. If a solid is pelleted from a solution containing, for example. 1 M salt, the concentration of salt in the residual liquid is 1 M: resuspending in wash solution dilutes this. In an Eppendorf tube, a typical small pellet may contain 10 µl of initial solvent, so each 1 ml wash represents a 100-fold dilution, giving a concentration after three washes of 1 µM. A single wash with 3 ml would reduce the concentration only to 3.3 mM; 2 and 4 washes with 1.5 ml and 0.25 ml respectively would reduce the concentration to 44.4 µM and 2.56 µM respectively.

j) Resuspending pellets: Many protocols call for pellets to be resuspended after centrifugation. This is either to remove the liquid phase, as in washing pellets, or to expose particles of the pellet to subsequent treatments (eg resuspension of bacteria prior to lysozyme digestion). Do not confuse resuspension with dissolution!! Complete resuspension separates the particles of the pellet completely, eg into individual bacterial cells; if you can see lumps, then you have not resuspended adequately. As long as the substance to be resuspended (or dissolved) is not going to be damaged by high shearing forces, resuspension can be assisted by vortexing the tube while holding a glass rod or plastic pipette against its inner surface, violently disrupting the liquid as it moves around the inside of the tube.

Spectrophotometric assay of nucleic acids

Nucleic acids absorb light in the ultraviolet region, with an absorbance peak at 260 nm. This is close to the protein absorbance peak at 280 nm, so that measurements of contaminated solutions at 260 nm only are not very useful. Measurement at both 260 and 280 nm allows the extent of contamination to be estimated: pure preparations of DNA and RNA have A260 / A280 ratios of 1.8 and 2.0 respectively. Contamination with UV-absorbing substances, including protein and phenol, result in lower values of this ratio. The molar extinction coefficients of different nucleic acids are different, so that:

- 1 A260 unit/ml of double-stranded DNA is about 50 µg/ml
- 1 A260 unit/ml of single-stranded DNA or RNA is about 40 µg/ml
- 1 A260 unit/ml of an oligonucleotide is about 33 µg/ml

Weight concentrations are related to molar concentrations as follows:

Oligonucleotides: x (g/(l = (x/n) x 3080 µM

Double-stranded DNA: x (g/(l = (x/n) x 1538 µM

Single-stranded DNA and RNA: x (g/(l = (x/n) x 3080 µM

where n = length in bases or base pairs

SAFETY IN THE LABORATORY

Basic laboratory safety rules are pretty obvious, and include the following:

- No eating, drinking, smoking, shaving, making up etc in ìwhite coatî areas.
- Always wear a white coat in the laboratory.
- No wearing white coats outside the laboratory.
- Wash hands frequently, especially before leaving the lab.
- No mouth pipetting.
- Treat all chemicals, solutions, isotopes, gases etc with respect.
- Familiarise yourself with the hazards of individual reagents and behave accordingly. Full details on all chemical hazards are now available from manufacturers under EU regulations.
- Always wear a radioactive badge in any lab doing radioactive work, even if you personally do not use isotopes.
- Store solvents in solvent cupboards or boxes.
- Never store ether in ordinary fridges; only use approved spark-free models.
- Always tell everyone in the lab and display a prominent sign when using ether.
- Keep the lab tidy, and clean up before going home. Everything has a place and should be returned immediately after use.
- Notify your local biological or radiological safety officer of all mishaps, however slight.

All labs have a local safety rules and an appropriate booklet should be available; read it.

Genetic Manipulation

The concept of genetic engineering induces panic in a large proportion of the populace; although the majority of genetic manipulations are probably harmless, it is essential to follow the correct procedures to avoid bad publicity and other problems.

All genetic manipulations must be assessed prior to the start of experimental work,

using a scheme designed to estimate the risk involved. On the basis of the results of this assessment , the conditions under which the work must be carried out are determined.

1. Access. This is a measure of the probability that a modified organism, or its DNA, will enter the human body and survive there. The access factor for bacteria with a known ability to colonise humans, eg wild type *Salmonella* and *E. Coli* is 1; non-colonising variants such as *E. Coli* K12, have an access factor of 10^{-3}; disables host-vector systems have a factor of $10^{-6} – 10^{-9}$; and genetically manipulated DNA in tissue culture cells has a factor of 10^{-12}. Note that if pathogens are being manipulated, the containment level specified for that pathogen is the minimum requirement; a lower overall risk factor is of lesser importance.
2. Expression. This is a measure of the anticipated or known level of expression. In unknown cases, the maximum level should be assumed. Deliberate in-frame insertions of expressible DNA downstream of a promoter with the intention of maximising expression gives an expression factor of 1; insertion of expressible DNA downstream of a promoter with no attempts to maximise expression gives a factor of 10^{-6}; and non-expressible sequences give a factor of 10^{-12}. Fusion proteins count as expressed; overall ratings are controlled by reduction in the damage factor rather than the expression factor.
3. Damage. This is an estimate of how much damage the expressed protein can cause. Expression of a toxic substance or pathogenic determinant under conditions where it is likely to have a significant deleterious biological effect gives a damage factor of 1; expression of a biologically active substance which might have a deleterious effect if delivered to a target tissue carries a damage factor of 10^{-3}; expression of a biological active molecule which is very unlike to have a deleterious effect, or where it could not approach more than 10% of the normal body level, gives a damage factor of 10^{-6}; use of a gene sequence where any biological effect is considered highly unlikely either because of the known properties of the protein or because of the high levels encountered in nature gives a damage factor of 10^{-9}; and cases of no foreseeable biological effect carry a damage factor of 10^{-12}.

The three risk factors are determined and are then multiplied together to give an overall

risk factor.

Other recommendations

In addition to the standard biosafety laboratory practises and good laboratory protocol you already perform in your laboratory, the following considerations apply specially to amplification techniques and should be monitored in order to ensure both your safety and the integrity of your assay.

- Always work in a one-way direction from Area 1 (reagent preparation) to Area 2 (specimen preparation) to Area 3 (amplification and detection) to avoid carryover contamination from amplified products.
- Specimens must be stored separately from reagents so as not to contaminate open reagents.
- When handling potentially contaminating material, always use a pipette with a plugged tip or a positive-displacement tip.
- All centrifuges should be kept at a distance from areas where you are preparing master mix and controls.
- Dry baths or blocks are preferable to water baths.
- Lab coats must be worn in all areas (the coat worn in area 3 must never be worn in areas 1 and 2)
- Gloves must be worn at all times and changed at each of the three work areas.

LABORATORY MANUALS

In order to obtain more detailed information about specific considerations, we recommend the following manuals:

- **Molecular Cloning, A Laboratory Manual**. Sambrook and Russell. Cold Spring Harbor Laboratory Press, New York.

- **Diagnostic Molecular Microbiology, Principles and Applications.** David H. Persing, Thomas F Smith, Fred C. Tenover, Thomas J. White. ASM Press,

- **PCR Protocols for Emerging Infectious Diseases**. David H. Persing. ASM Press,

- **PCR Strategies**. Michael A. Innis, David H. Gelfand, John J. Sninsky. Academic Press

- **PCR Primer, A Laboratory Manual**. Carl W. Dieffenbach, Gabriela S. Dveksler. Cold Spring Harbor Laboratory Press

- **PCR Protocols, A Guide to Methods and Applications**. Michael A. Innis, David H. Gelfand, John J. Sninsky, Thomas J. White. Academic Press Limited

- **Diagnostic Bacteriology Protocols. Methods in Molecular Biology, volume 46**. Jenny Howard and David M. Whitcombe. Humana Press Inc.

- **Short Protocols in Molecular Biology**. Fred Ausubel Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A. Smith, Kevin Struhl. John Wiley & Sons Ltd.