TECHNIQUES IN MOLECULAR BIOLOGY

Edited by

John M. Walker

and

Wim Gaastra

TECHNIQUES IN MOLECULAR BIOLOGY

TECHNIQUES IN MOLECULAR BIOLOGY

Edited by JOHN M. WALKER and WIM GAASTRA

CROOM HELM
London & Canberra

Croom Helm Ltd, Provident House,
Burrell Row, Beckenham, Kent BR3 1AT

British Library Cataloguing in Publication Data

Techniques in molecular biology.
1. Molecular biology – Technique
I. Walker, John M. II. Gaastra, Wim
574.8'8'028 QH506

ISBN 0-7099-2747-9
ISBN 0-7099-2755-X (pbk)

Typeset by Leaper & Gard Ltd, Bristol
Printed and bound in Great Britain

CONTENTS

PREFACE

The last few years have seen the rapid development of new methodology in the field of molecular biology. New techniques have been regularly introduced and the sensitivity of older techniques greatly improved upon. Developments in the field of genetic engineering in particular have contributed a wide range of new techniques. The purpose of this book therefore is to introduce the reader to a selection of the more advanced analytical and preparative techniques which the editors consider to be frequently used by research workers in the field of molecular biology. In choosing techniques for this book we have obviously had to be selective, and for the sake of brevity a knowledge of certain basic biochemical techniques and terminology has been assumed. However, since many areas of molecular biology are developing at a formidable rate and constantly generating new terminology, a glossary of terms has been included.

The techniques chosen for this book are essentially based on those used in a series of workshops on 'techniques in molecular biology' that have been held at The Hatfield Polytechnic in recent years. In choosing these chapters we have taken into account many useful suggestions and observations made by participants at these workshops. Each chapter aims to describe both the theory and relevant practical details for a given technique, and to identify both the potential and limitations of the technique. Each chapter is written by authors who regularly use the technique in their own laboratories. This book should prove useful to final year undergraduate (especially project) students, postgraduate research students and research scientists and technicians who wish to understand and use new techniques, but who do not have the necessary background for setting-up such techniques. Although lack of space precludes the description of in-depth practical detail (e.g. buffer compositions, etc.), such information is available in the references cited.

J.M. Walker
W. Gaastra

ABBREVIATIONS

A_{260}	—	Absorbance at 260nm
ABM	—	Aminobenzyloxymethyl
amp-r	—	ampicillin resistant
amp-s	—	ampicillin sensitive
APT	—	Aminophenylthioether
bis	—	methylenebisacrylamide
bp	—	base-pairs
CBA	—	Cyanogen bromide activated
CCC	—	Covalently closed circular (DNA)
cDNA	—	Complementary DNA
Dansyl	—	dimethylaminonapthalene-5-sulphonyl
DBM paper	—	diazobenzyloxymethyl paper
DEAE	—	diethylaminoethyl
DNA	—	deoxyribonucleic acid
DNase	—	deoxyribonuclease
DPT	—	diazophenylthioether
ds-cDNA	—	double-stranded cDNA
EDTA	—	ethylenediaminotetraacetic acid
HART	—	hybrid-arrest translation
HFBA	—	heptafluorobutyric acid
HPLC	—	High performance liquid chromatography
IEF	—	isoelectric focussing
mRNA	—	messenger RNA
nt	—	nucleotides
ODS	—	octadecylsilane
PAGE	—	polyacrylamide gel electrophoresis
PITC	—	phenylisothiocyanate
poly (A)	—	poly(adenylic)acid
poly A^+ RNA	—	polyadenylated RNA
poly (U)	—	poly(uridylic)acid
PTH	—	phenylthiohydantoin
RNA	—	ribonucleic acid
RNase	—	ribonuclease
RNP	—	ribonucleoprotein
RPC	—	reversed phase chromatography
rRNA	—	ribosomal RNA
S	—	Svedberg unit (corresponding to a sedimentation coefficient of $1-10^{-13}$ seconds)
SDS	—	sodium dodecyl sulphate (sodium lauryl sulphate)
TCA	—	trichloroacetic acid

TEMED	—	N,N,N′,N′,-Tetramethylethylenediamine
tet-r	—	tetracycline resistant
TFA	—	trifluoracetic acid
Tris	—	Tris(hydroxymethyl)aminomethane
tRNA	—	transfer RNA
u.v.	—	ultra-violet

1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS AND NUCLEIC ACIDS

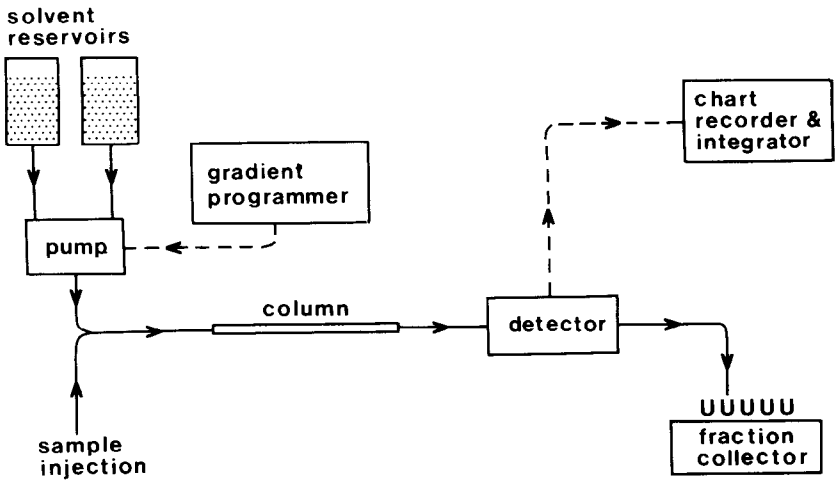
Keith Gooderham

CONTENTS

1. Introduction

High performance liquid chromatography (HPLC) provides an extremely effective method for separating complex mixtures of molecules. In addition to its high resolving power, HPLC also possesses at least three other major advantages over more traditional fractionation techniques: (i) separations are achieved very rapidly, usually in minutes rather than over a number of hours or days; (ii) only very small quantities of material are required for analysis, separations usually being obtained with nano- or picogram amounts rather than with micro- or milligram quantities; (iii) the fractionated molecules are usually detected by monitoring the u.v. absorbance of the column eluant and this passive detection method therefore allows fractions to be collected and the separated components recovered, on a preparative or at least semi-preparative scale.

Figure 1.1: A Schematic Representation of a High Performance Liquid Chromatography System



Note: Sample and solvent flow are shown by solid lines (————) and electronic signals by broken lines (– – – – –).

Although the basic layout of the HPLC system (Figure 1.1) is superficially similar to more traditional chromatography systems, there are a number of important differences. Extremely narrow bore tubing (1/64" internal diameter) and fine column packings (5–20 micron particles) are used in order to obtain the rapid, high-resolution separations characteristic of HPLC. As a consequence of these modifications there is a considerable resistance to the flow of solvent through the system. In order to overcome this back-pressure, the solvent has to be pumped through the system under high pressure (up to 8,000 psi, though more usually between 500 and 2,000 psi). The use of high pressures has frequently resulted in HPLC being referred to as high pressure liquid chromatography.

The most important component of any HPLC system is the column, or more precisely the packing material it contains. A variety of different forms of chromatography are used in HPLC but all of these methods depend upon the relative importance of the competing attractions of the packing material, or *stationary phase*, and the eluting solvent, or *mobile phase*, for the sample. These interactions can then be modified in a number of ways, for example by modifying the composition of the mobile phase, as in gradient chromatography, or by changing the flow rate (pressure) or temperature at which the chromatography is performed.

Currently three major types of HPLC are commonly employed. These are: size-exclusion chromatography; reversed-phase chromatography and ion-exchange chromatography. Ideally each of these methods will separate the sample on the basis of only one property, i.e. size, hydrophobicity and charge, respectively. However, ideal systems are rarely encountered in experimental work and any given separation is unlikely to be due to only one of these parameters. This situation is further complicated by the multiplicity of manufacturers producing HPLC columns. Differences in particle size and shape, as well as the choice of capping groups (see Section 3), can all have a profound effect upon the resulting chromatography.[1] Consequently, when attempting to follow a previously published method, it is important not only to reproduce the precise operating conditions but also to use an identical column.

Though commercially prepared columns are now relatively inexpensive, it is also possible to prepare satisfactory columns in the laboratory, using either commercially available packing machines or by using a home-made apparatus (for example as described by Manius and Tscherne[2]). In addition a number of companies now offer a highly competitive column packing service for a wide range of stationary phases. Once a suitable column has been obtained its working life can be considerably extended by filtering the mobile phase and sample prior to use, or by using a guard column positioned directly in front of the main column. Both of these precautions are designed to filter out any particulate matter, which otherwise would eventually block the column.

2. Size-exclusion Chromatography

Size-exclusion chromatography (also known as steric-exclusion, gel filtration and gel permeation chromatography), has long been an important method for the separation of proteins and peptides. However, it is only comparatively recently that this method has been successfully applied to HPLC. The principal reason for this delay has been the difficulty of obtaining a packing material which is both porous and yet of sufficient mechanical strength to withstand the pressures normally associated with HPLC. In addition, the majority of these column packings have a silica base, which is very reactive owing to the presence of negatively charged silanol groups. The stationary phase will therefore act as a weak cation-exchanger interfering with the size-dependent separation by binding positively charged molecules.

Advances in column technology have, however, largely overcome these problems. Modern columns are now capable of operating at relatively high pressures (up to 2000 psi or greater) and at a flow rate of 1 to 2 ml/minute. Ionic interactions between the stationary phase and the sample have also been reduced by the use of small organic 'capping' groups (see Section 3), though in some instances this has only replaced the ionic component of the chromatography by a reversed-phase element. The resulting chromatography can be further improved by a judicious choice of ionic strength and pH when selecting the mobile phase. In general, increasing the ionic strength of the buffer will minimise the degree of interaction between the sample and any remaining silanol groups. The pH of the mobile phase will similarly influence the binding of the sample to the charged stationary phase by controlling the degree of ionization and net charge of the sample.

Although ion-exchange and reversed-phase effects can both influence the separation, the resulting fractionation primarily depends on the molecular weight and shape of the sample molecules. Large molecules are excluded from entering the porous stationary phase and are carried straight through the column, while progressively smaller molecules are increasingly able to enter into the stationary phase and consequently have proportionately longer elution times. The porosity of the stationary phase therefore determines the molecular weight range which may be analysed and, as with the more traditional gel filtration media, a number of different fractionation ranges are required to meet the majority of possible applications. A list of the major size-exclusion columns suitable for protein and nucleic acid research, together with their theoretical fractionation ranges, is presented in Table 1.1.

While the majority of these columns are intended for use at high flow rates (0.5 to 2.0 ml/min) certain manufacturers (for example, LKB Instruments Ltd) are currently producing a series of columns which are intended to be operated at extremely low flow rates (~50 μl/min). The rationale behind this approach is that at low flow rates the individual

components of the sample have a greater opportunity to interact with the column and the resulting separation, though taking several hours, is therefore of considerably higher resolution.[3] Most workers at the moment, however, use the high flow rate columns and it remains to be seen whether the increased resolution obtained with these columns will lead to their wider use.

Table 1.1: Fractionation Ranges of some of the Major High Performance Size-exclusion Chromatography Columns

	Fractionation range (daltons)	
	Native globular proteins	Denatured random coil proteins
Group A[a]		
I–60	1,000–20,000	600–8,000
I–125	2,000–80,000	1,000–30,000
I–250	10,000–500,000	2,000–150,000
Group B[b]		
TSK G2000SW[c]	500–60,000	1,000–25,000
TSK G3000SW	1,000–300,000	2,000–70,000
TSK G4000SW	5,000–1,000,000	3,000–400,000

Notes:
a. Columns in group A are manufactured and supplied by Waters Associates Inc. (Milford, Mass. 01757, USA).
b. Group B columns are manufactured and supplied by Toyo Soda Manufacturing Co. Ltd (Tokyo, Japan).
c. LKB Instruments Ltd and Bio Rad Laboratories also use these TSK column packings for their Ultropac and Bio Sil columns respectively.

The principal application of size-exclusion HPLC, has been for the separation of proteins, using both non-denaturing and denaturing aqueous solvents. For non-denaturing chromatography, phosphate buffers (~ pH7), and usually containing 0.1–0.3M NaCl to minimise secondary ionic interactions, have been widely used (see Figure 1.2 and examples given in references[4,5,6,7]). Although satisfactory separations have been obtained under these conditions, the denaturing solvent systems generally produce superior separations and are preferred, providing it is not important to maintain the proteins in their native state. Denaturing solvent systems have the considerable advantage of permitting the analysis of a wider range of proteins by overcoming many potential problems caused by insolubility and aggregation of the proteins. Molecular weight determinations are also, in general, more accurate under these conditions because the denatured proteins assume an open random coil structure. In contrast, native proteins have a complex globular conformation and this frequently causes an anomalous rate of migration through the column. Two principal denaturants have been used: guanidine hydrochloride[8,9] and the ionic