Molecular Biology in Cellular Pathology

Edited by

John Crocker

Department of Cellular Pathology,
Heartlands Hospital, Birmingham, UK

Paul G. Murray

Department of Pathology,
The Medical School University of Birmingham, Birmingham, UK

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With love
to our families

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Preface

Since the publication of the original edition of this book, there have been rapid advances in our understanding of disease, mainly as a result of the impetus provided by some of the newer technologies. In particular, the rapidly developing fields of genomics and proteomics are enabling an understanding of gene expression both at the mRNA and protein level on a global scale (i.e. the whole transcriptome or proteome) not previously imaginable. Whereas gene expression studies in pathology have frequently relied purely on immunohistochemistry and *in situ* hybridisation, in their own right still immensely invaluable procedures, they could only essentially give information on a single gene in a single experiment. Now information on expression from the whole of the genome can be assessed in a single experiment. Proteomics, in particular, is providing the tools not only to examine global protein expression, but also to dissect protein function, through the development of approaches to study protein activity. Likewise, in genetics, there is an impending revolution. Comparative genomic hybridisation, for a long time a difficult alternative to conventional cytogenetics, will blossom with the advent of array approaches to, allowing high resolution mapping of chromosomal changes across the whole genome without the need for difficult interpretation of chromosome morphology.

The key question is to what extent these developments will impact on diagnostic pathology in the future. The polymerase chain reaction was heralded years ago with the view that its introduction into routine diagnostic pathology practice was only a matter of time. Events have not proved this assumption correct; although the polymerase chain reaction does have applications in routine pathology it has not impacted directly in a significant way on routine histopathology. It is the authors' view that the same will not be true of the newer technologies. At the very least these newer approaches may identify a whole host of disease-specific markers for use in conventional assays for disease. At the other end of the spectrum, they may change forever the morphological assessment of disease to be substituted by an entirely objective set of array data providing detailed information on chromosome changes and global gene expression. We hope that this new edition will give some insights into some of the developments

in molecular biology that provide us not only with immense opportunities for the future but also with considerable challenges.

We would particularly like to thank all those who have contributed to this text and also our families, to whom we are of course deeply indebted for allowing us to pursue this project, often at their expense. Mrs Ruth Fry supplied excellent secretarial support.

John Crocker
Paul Murray
21 October 2002

Preface to *Molecular Biology in Histopathology*

The past 20 years have witnessed numerous changes in the practice of histopathology, with many powerful techniques, such as immunohistochemistry and image analysis, aiding the accuracy and objectivity of diagnosis and research. However, perhaps the greater revolution, occurring in the past half decade, of the application of molecular methods, will be even more fruitful. Molecules related to, for example, hormones, immunoglobulins, infectious agents or chromosomes can be identified by means of gene probes. Furthermore, the molecular basis of cell replication has become more clearly understood, assisting in tumour prognosis. What, then, are these new methods? As the title of this book implies, the techniques included in it are those that can be performed on histological material, although not necessarily involving microscopic examination. The reader should not be led into the belief that 'molecular' must always imply 'DNA' and, as we can see in at least two chapters herein, 'molecular' should have a wider, more appropriate meaning.

The purpose of this series of volumes is to supply a guide to those just qualified and undertaking research or to those who have taken degrees some years in the past and who wish to glean new information rapidly. Thus, this is not a molecular 'recipe book'; such exist elsewhere.

In the first chapter, Mr Murray and Professor Ambinder have given an account of the methods available for the demonstration of infectious agents *in situ* in histological material. The applications of these techniques are also outlined. Chapters 2 and 4 by Drs Fleming, Morey and Yap, and by Drs Waters and Long, then describe these methodologies and others as applied to the examination of malignant tissues and chromosomes in histological material.

Chapters 3 and 5 give details of other methodologies, both of which are not histological (although one may become so). These techniques do, however, employ histological material, even of archival, paraffin wax-embedded type. Thus, Dr Young describes the value of the polymerase chain reaction in histopathology; indeed, this is already being adapted for use as an *in situ* method. Dr Camplejohn then describes the techniques of DNA flow cytometry and their

applications. One of the latter is that of the assessment of cell proliferative status. Leading on naturally from this, in Chapter 6 I have given an account of the molecular basis of the cell cycle and of some of the antibody probes which can be applied to visualize some of the components of the cell cycle. Also highly related to cell proliferation is the activity of the interphase nucleolar organizer region. The full significance of this structure is not yet fully understood but in the subsequent chapter, Professors Derenzini and Ploton give an account of the morphological and molecular corollaries of the nucleolar organizer regions.

Just as we are realizing and understanding the importance of cell proliferation in disease, so we are appreciating that cell death is also central to many physiological and pathological conditions. Accordingly, in Chapter 8, Drs Arends and Harrison tell us of the molecular basis of 'programmed cell death' or apoptosis in health and disease.

Thus, this volume gives an introduction to the currently available molecular techniques in histology and an account of the molecular basis of certain phenomena of importance in everyday histopathology.

John Crocker
Birmingham
1994

List of Contributors

S. Jane Astley	Division of Biomedical Sciences, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1SB, UK
Karl Baumforth	CRC Institute, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
Philip Bennett	Micropathology Ltd, University of Warwick Science Park, Barclays Venture Centre, Sir William Lyons Road, Coventry CV4 7EZ, UK
Werner Boecker	Gerhard Domagk Institute of Pathology, University Hospital Muenster, Germany
Horst Buerger	Gerhard Domagk Institute of Pathology, University Hospital Muenster, Germany
John Crocker	Department of Cellular Pathology, Birmingham Heartlands Hospital, Bordesley Green East, Birmingham B9 5SS, UK
Aoife Crowley	Department of Pathology, The Coombe Women's Hospital Dublin and The Department of Histopathology, Trinity College Dublin, Ireland
Michael J. Deery	Inpharmatica Ltd, 60 Charlotte Street, London W1T 2NU, UK
Massimo Derenzini	Università di Bologna, Dipartimento di Patologia Sperimentale, Via San Giacomo 14, 40126 Bologna. Italy
Paul I. van Diest	Department of Pathology, VU Medical Centre, Amsterdam, The Netherlands

Timothy Diss Histopathology Department, RF and UCL Medical School, University Street, London WC1E 6JJ, UK

Amanda Dutton Department of Pathology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Sara Dyer Regional Genetics Service, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK

David J. Harrison Department of Pathology, University of Edinburgh, Edinburgh, UK

Hermann Herbst Gerhard-Domagk-Institut für Pathologie, Westfälische Wilhems-Universität, Domagkstr. 17, 48149 Münster, Germany

Mario A.J.A. Hermsen Department of Pathology, VU Medical Centre, Amsterdam, The Netherlands

C. Simon Herrington Department of Pathology, Duncan Building, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

Lee B. Jordan Department of Pathology, University of Edinburgh, Edinburgh, UK

Ernst J. Kuipers Department of Gastroenterology and Hepatology, Erasmus University Medical Centre, Rotterdam, The Netherlands

Kathryn Lilley Cambridge Centre for Proteomics, University of Cambridge, Department of Biochemistry, Building O, Downing Site, Cambridge CB2 1QW, UK

Victor Lopez Department of Pathology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Fiona MacDonald West Midlands Regional Genetics Laboratory, Birmingham Women's Hospital NHS Trust, Edgbaston, Birmingham B15 2TG, UK

Cara Martin Department of Pathology, The Coombe Women's Hospital Dublin and The Department of Histopathology, Trinity College Dublin, Ireland

Gerrit A. Meijer Department of Pathology, VU Medical Centre, Amsterdam, The Netherlands

Paul G. Murray Department of Pathology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Paul N. Nelson Division of Biomedical Sciences, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1SB, UK

Gerald Niedobitek Pathologisches Institut, Friedrich-Alexander-Universität, Krankenhausstr. 8–10, 91054 Erlangen, Germany

Marie-Françoise O'Donohue CNRS UMR 6142, Faculté de Médicine, Reims Cedex, France

John J. O'Leary Department of Pathology, The Coombe Women's Hospital Dublin and The Department of Histopathology, Trinity College Dublin, Ireland

Michael G. Ormerod 34 Wray Way, Reigate RH2 0DE, UK

Dominique Ploton CNRS UMR 6142, Faculté de Médicine, Reims Cedex, France

Azam Razzaq Cambridge Centre for Proteomics, University of Cambridge, Department of Biochemistry, Building O, Downing Site, Cambridge CB2 1QW, UK

Orla Sheils Department of Pathology, The Coombe Women's Hospital Dublin and The Department of Histopathology, Trinity College Dublin, Ireland

Antoine Snijders UCSF Cancer Centre, San Francisco, USA

Fiona J. Spence GlaxoSmithKline Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR

David Treré Università di Bologna, Dipartimento di Patologia Sperimentale, Via San Giacomo 14, 40126 Bologna. Italy

Philip Warren Division of Biomedical Sciences, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1SB, UK

Jonathan J. Waters NE London Regional Cytogenetics Department, Great Ormond Street Hospital, London WC1N 3BG, UK

Fiona Watson Department of Pathology, Duncan Building, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

Marjan M. Weiss Department of Gastroenterology, VU Medical Centre, Amsterdam, The Netherlands

Sophie E. Wildsmith GlaxoSmithKline Pharmaceuticals, The Frythe, Welwyn, Herts AL6 9AR

1

Blotting Techniques: Methodology and Applications

Fiona Watson and **C. Simon Herrington**

1.1 Introduction

The study of many different types of biomolecules has been advanced by the ability to attach the molecule to a membrane support. The technique used to transfer the biomolecules to the membrane is known as blotting and there are many variations of it. The basic steps in the procedure include the following: isolation of a cell-free mixture containing the biomolecule of interest; resolving the mixture into its component parts (if necessary); transfer (blotting) of the component parts onto a suitable membrane; and detection of the biomolecule of interest.

In general the blots are named according to the type of molecule that is blotted onto the membrane and include the Southern, Northern and Western blot which are used for the detection of DNA, RNA and protein, respectively. Variations of these, such as the Southwestern, Northwestern and Farwestern techniques, have been developed and there is also a lesser known technique called the Eastern. In this chapter we discuss both the methodology used to perform these techniques and the applications of their use.

1.2 Blotting techniques

The Southern Blot

This technique, which is used to detect specific sequences within mixtures of DNA, was first described by E.M. Southern in 1975 (Southern, 1975). In a

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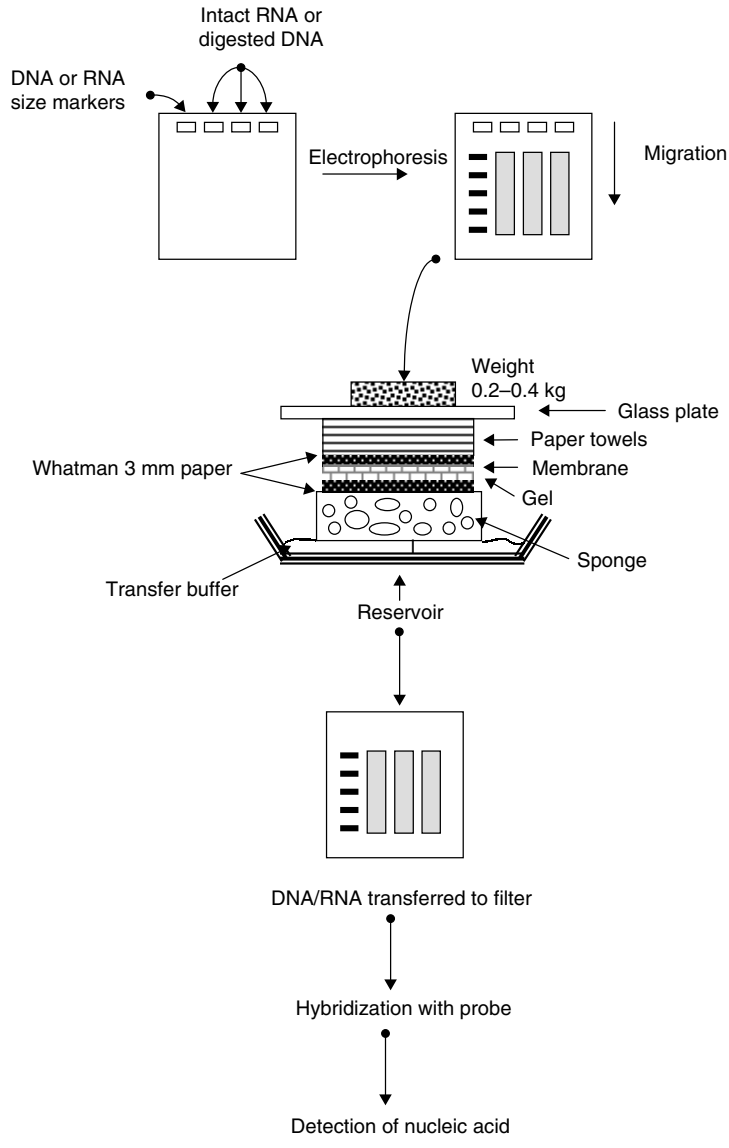


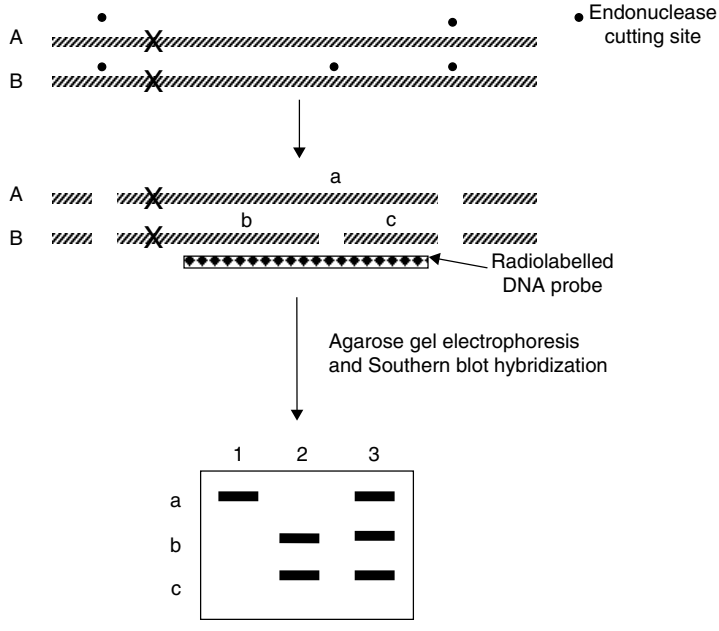
Figure 1.1 The steps involved in Southern and Northern blotting. Nucleic acids are resolved on a polyacrylamide gel prior to their upward capillary transfer onto a suitable membrane. The membrane is then hybridized with a probe which is suitable for the detection of the nucleic acid

Southern blot the DNA is size fractionated by gel-electrophoresis and then transferred by capillary action to a membrane (Figure 1.1). Membrane types and their uses are discussed in the section 'Membrane Types' below.

Non-specific binding sites on the membrane are then blocked and it is incubated with an appropriately labelled probe. Autoradiography or a phosphoimager

is used to detect nucleic acid/probe hybrids when a radiolabelled probe is used but non-isotopically labelled probes require detection with a non-radioactive reporter system (described in the section 'Detection Methods' below). The size of the DNA recognized by the probe is determined by the co-electrophoresis of DNA fragments of known molecular weight.

The Southern blot technique has many applications. It has provided information about the physical organization of single and multicopy sequences in complex genomes, has expedited cloning experiments with eukaryotic genes, and was directly responsible for the discovery of introns (Doel *et al*., 1977). Southern analysis is used to study the structure and location of genes by identifying restriction length polymorphisms (RFLPs) (Figure 1.2) and with the use of a pair of isoschizomers recognizing methylated and non-methylated nucleotides, gene methylation patterns can also be determined (Botstein *et al*., 1980; Shaw *et al*., 1993). Southern blotting has led to an increased understanding of the genomic rearrangements that are important in the formation of antibodies and T cell receptors, has identified numerous rearranged genes that are associated with disease, and has been used in the prenatal diagnosis of genetic disease



Lane 1: Pattern observed if individual homozygous for A
Lane 2: Pattern observed if individual homozygous for B
Lane 3: Pattern observed if individual heterozygous for A and B

Figure 1.2 The principle of RFLP analysis. DNA restriction enzymes are used to create DNA fragments which are resolved by agarose gel electrophoresis. Differences in the size of the restriction fragments are detected by Southern blotting

(Chen *et al*., 1999; Davies, 1986; Kramer *et al*., 1998; Moreau *et al*., 1999; Yu *et al*., 2000). Activation of oncogenes by gene rearrangement, amplification or point mutation and inactivation of tumour suppressor genes by DNA rearrangements, point mutations, or allelic deletions can all be detected using Southern analyses (Munger, 2002). In addition, analysis of the allelic pattern of several polymorphic variable number of tandem repeats (VNTR) loci in an individual using the RFLP method can yield probabilities for investigating biological relationships or for matching forensic material found at a crime scene (Jeffreys *et al*., 1985). The Southern blotting technique is also being utilized within the Human Genome Mapping Project to determine the order of the genes along the chromosome.

The Northern Blot

In a Northern blot, RNA is the target molecule blotted onto the membrane. The methodology is similar to that used in a Southern blot (Figure 1.1) but precautions should be taken to prevent RNA degradation that can be caused by the presence of ribonucleases, which are stable and active enzymes (Alwine *et al*., 1977). To avoid RNase contamination, glassware should be baked and plasticware rinsed with chloroform prior to use. When possible, disposable plasticware should be used since it is essentially RNase free. Solutions that are made with ultrapure reagents that are reserved for RNA work in general are treated with diethylpyrocarbonate (DEPC) to 'inactivate' RNases and autoclaved prior to use. Tris-containing solutions are an exception. These solutions are made in DEPC-treated water and then autoclaved. It is important to remember that skin can be an important source of RNase contamination. For isolation of high-quality RNA, the starting material should be as fresh as possible and, if tissue or cells cannot be used immediately after harvesting, they should be flash frozen in liquid nitrogen and stored at -70°C until use. Commercial RNA isolation kits are available: these involve lysis of the cells with resultant inactivation of the ribonucleases as the first step.

A different type of gel from that used for Southern blotting is used for Northern blotting. In contrast to DNA, which usually is found as a double stranded molecule that migrates as a function of hybrid length, RNA has a significant secondary structure and must be electrophoresed under denaturing conditions if it is to migrate as a function of nucleotide length. The secondary structure associated with RNA would also reduce the efficiency of transfer to the membrane support. Thus RNA is denatured with either glyoxal and dimethylsulphoxide or formaldehyde and formamide (Lehrach *et al*., 1977; Thomas, 1980). The formaldehyde gel is more common but both techniques are equally efficient. Estimation of the size of RNA can be achieved by comparing its migration with that of the 18S and 28S ribosomal components or with commercially available RNA markers of known size. Northern blots are used both to detect changes in gene expression

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