NUCLEIC ACID HYBRIDIZATION

Editor:

David Rickwood, Department of Biological and Chemical
Sciences, University of Essex, Colchester, Essex

CENTRIFUGATION

RADIOISOTOPES

LIGHT MICROSCOPY

ANIMAL CELL CULTURE

GEL ELECTROPHORESIS: PROTEINS

PCR, SECOND EDITION

MICROBIAL CULTURE

ANTIBODY TECHNOLOGY

GENE TECHNOLOGY

LIPID ANALYSIS

GEL ELECTROPHORESIS: NUCLEIC ACIDS

LIGHT SPECTROSCOPY

DNA SEQUENCING

MEMBRANE ANALYSIS

PLANT CELL CULTURE

NUCLEIC ACID HYBRIDIZATION

NUCLEIC ACID HYBRIDIZATION

M.L.M. Anderson

Institute of Biomedical and Life Sciences, Glasgow University,
Glasgow, UK

Taylor & Francis

Taylor & Francis Group

LONDON AND NEW YORK

© **Taylor & Francis Publishers Limited, 1999**

First published 1999

All rights reserved. No part of this book may be reproduced or transmitted, in any form or by any means, without permission.

A CIP catalogue record for this book is available from the British Library.

ISBN 1-85996-007-3

Published by Taylor & Francis

2 Park Square, Milton Park, Abingdon, Oxon, OX14 4RN

52 Vanderbilt Avenue, New York NY 10017

Transferred to Digital Printing 2009

ISBN10: 1-85996-007-3

Production Editor: Andrea Boshier

Typeset by Marksbury Multimedia Ltd, Midsomer Norton, Bath, UK

Publisher's Note

The publisher has gone to great lengths to ensure the quality of this reprint but points out that some imperfections in the original may be apparent.

Contents

Abbreviations	xi
Preface	xiii
1. Introduction to nucleic acid hybridization	1
Structure of nucleic acids	1
Forces stabilizing DNA structure	2
Mismatched base pairs	4
Stability of nucleic acids	5
Effects of pH	5
Effects of temperature	5
Renaturation and hybridization	9
2. Types of hybridization and uses of each method	11
Solution hybridization	11
Filter hybridization	12
<i>In situ</i> hybridization	13
Polymerase chain reaction	13
DNA chips	15
Use of more than one hybridization technique	16
References	16
3. Solution hybridization: reassociation of DNA	17
Reassociation kinetics	17
Experimental analysis of a reassociation reaction	18
Presentation of data	22
Relationship between the rate of reassociation and the sequence complexity of DNA	23
<i>C₀t</i> curves of eukaryotic DNA show the presence of repeated sequences	25
Determining the repetition frequency of an isolated sequence	29
Isolation of different components	29
Analysis of repetitive DNA	29

Examining the relatedness of sequences	30
Interspersion of unique and repetitive DNA	32
References	33
4. Solution hybridization: RNA:DNA hybridization	35
Introduction	35
Progress of reaction	36
DNA:RNA hybridizations with excess DNA	37
DNA:RNA hybridizations in RNA excess	38
Estimation of the number of genes being expressed by saturation hybridization	39
Estimation of complexity of an RNA population by kinetic analysis of RNA-excess hybridization	40
Detection of sequences differentially expressed in two cell types	44
References	47
5. Solution hybridization: reaction conditions	49
The rate of formation of duplexes	49
Temperature	49
Salt concentration	50
Fragment length	51
Other factors	51
Preventing thermal breakdown of nucleic acid	51
Speeding up the reaction	52
Lowering the incubation temperature	52
Factors affecting the stability of hybrids	53
Mismatches	54
References	55
6. Basic types of filter hybridization	57
Probing recombinant libraries	57
Southern blots	59
Northern blots	61
Dot blots and slot blots	61
References	64
7. Hybridization strategy: long probes	65
Introduction	65
Factors affecting the rate of hybridization and stability of hybrids	65
Concentration of nucleic acids	66
Length of the probe	67

Complexity of the probe	68
Base composition	68
Salt concentration	68
Temperature	69
Formamide	70
Imperfectly matched sequences	72
Hybridization accelerators	73
Enzyme-linked probes	74
Reaction volume	74
Time of hybridization	74
References	76
8. Hybridization strategy: oligonucleotide probes	77
Introduction	77
Factors affecting the hybridization of oligonucleotides and stability of hybrids	77
Melting temperature	77
Mismatches	79
Rate of hybridization	80
Salt concentration	80
Time period for hybridization	80
Hybridization accelerators	81
Washing	81
Control of stringency	82
Optimizing conditions	82
Probe design	83
Length of the probe	83
Single sequence probe	85
Mixtures of oligonucleotide probes	85
Reducing the complexity of an oligonucleotide pool	86
References	87
9. Choice of probe	89
Introduction	89
Characteristics of probes	89
DNA probes	89
RNA probes	90
Oligonucleotide probes	90
Characterizing the probe	91
Labeling the probe	91
Radioactive probes: general characteristics	91
Nonradioactive probes: general characteristics	92
Choosing a probe	94
Sensitivity required	94
DNA or RNA or oligonucleotide?	96

End-labeling or uniform labeling?	96
References	96
10. Basic techniques: binding of nucleic acid to filters	97
Types and properties of support	97
Transfer of nucleic acid to filters	98
Phage/colony transfer	98
Transfer of DNA from gels	98
Northern blots	101
Dot/slot blots	101
Immobilization of nucleic acid to filters	102
Baking	102
UV fixation	102
Alkali fixation	103
Hybridization in dried gels	104
Experimental procedures	104
Preventing degradation of RNA	104
Phage/colony transfer	105
Southern blots	106
Northern blots	109
DNA dot/slot blots	110
RNA dot/slot blots	112
References	114
11. Basic techniques: labeling of probes	115
Introduction	115
Radioactive probes	115
Uniform labeling	117
Labeling of DNA by random priming	117
Labeling of DNA by nick translation	120
Uniform labeling of RNA	120
Uniform labeling by chemical methods	126
End labeling of nucleic acids	126
Labeling at the 5' end	126
Labeling at the 3' end	128
Removal of nonincorporated nucleotides	129
Size exclusion	129
Sephadex spin columns	129
Ethanol precipitation	129
Adsorption to silica particles	130
Phenol extraction	130
Determining the efficiency of incorporation of label	132
Radioactive probes	132
Nonradioactive probes	134
References	136

12. Basic techniques: prehybridization, hybridization and washing	137
Equipment	137
Prehybridization	138
Hybridization	139
Washing	140
Protocols for prehybridization, hybridization and washing	141
References	146
13. Basic techniques: detection of hybrids	147
Radioactive hybrids	147
Autoradiography	147
Phosphor imaging	151
Detection of nonradioactive hybrids	151
Chromogenic substrates	152
Chemiluminescent substrates	155
Enhanced chemiluminescence	157
Reuse of filters and probes	161
Removal of products of nonradioactive detection systems	162
Stripping probe off filters	163
Reuse of the probe	164
References	165
14. Trouble shooting	167
15. Specific applications of techniques	171
Mapping DNA	171
Mapping repetitive sequences	171
Detection of related sequences	172
Discrimination between related sequences	172
Zoo/garden blots	172
Restriction fragment length polymorphisms	174
DNA fingerprinting by hybridization	176
Detection of mutations	178
Loss of enzyme restriction sites	178
Loss of genes	180
Expansion of repeated sequences	180
Use of allele specific oligonucleotide probes	181
Reverse dot blots	184
Ligase mediated gene detection	184

Repeated sequences in probes	186
Suppressing hybridization of repetitive sequences	186
Using repetitive sequences as probes	187
Semi-quantitative analyses	188
Methylation	189
Analysis of transcripts	190
Preliminary characterization of transcripts	191
Refined mapping of transcripts	195
Quantitation of RNA transcripts	197
Sequences expressed in one cell type and not another	199
Hybrid selection of mRNA and hybrid arrest translation	202
References	203
16. Current trends	205
DNA arrays and chips	205
Preparation of DNA arrays	205
Preparation of oligonucleotide arrays	205
Gene expression analyses	206
DNA sequencing by hybridization	207
Probes	208
Developments in hybridization with oligonucleotide and peptide nucleic acid probes	208
Molecular beacons	209
References	210
Appendices	211
Appendix A: Glossary	211
Appendix B: Solution hybridization equations	215
Appendix C: Useful information	219
Appendix D: Composition of stock solutions	221
Appendix E: Suppliers	223
Index	229

Abbreviations

A	adenine
Ab	antibody
AMPPD	disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo-[3.3.1.1 ^{3,7}]decan]-4-yl) phenyl phosphate
AP	alkaline phosphatase
ASO	allele-specific oligonucleotide
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumen
C	cytosine
cDNA	complementary DNA
CDP-star	disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 ⁷]decan]-4-yl)-1 phenyl phosphate
CIAP	calf intestinal alkaline phosphatase
cps	counts per second
CSPD	disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 ^{3,7}]decan]-4-yl) phenyl phosphate
DABCYL	4-[4'-dimethylaminophenylazo]benzoic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleoside triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
G	guanine
HRP	horseradish peroxidase
kb	kilobases

MLP	multi locus probe
mRNA	messenger RNA
NET	1 x NET = 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.5% SDS
nm	nanometres
nt	nucleotide(s)
NTB	nitroblue tetrazolium
OD	optical density
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
RSP	restriction site polymorphism
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SLP	single locus probe
SSC	1 x SSC = 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0
SSPE	1 x SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA
SST	1 x SST = 0.15 M NaCl, 15 mM Tris HCl, pH 7.5, 5 mM EDTA
TCA	trichloroacetic acid
TE	Tris EDTA buffer = 10 mM Tris HCl, pH 8.0, 1 mM EDTA
T_m	melting temperature
tRNA	transfer ribonucleic acid
TTP	thymidine triphosphate
U	uracil
UV	ultraviolet
VNTR	variable number of tandem repeats

Preface

The first description of nucleic acid hybridization was by Marmur and Doty in 1961. They established that the two sequences involved in formation of a duplex must have a degree of complementarity and that the stability of the duplex depended on the degree of relatedness. These early experiments provided a means of studying relationships between nucleic acids that was quickly exploited. Application of nucleic acid hybridization to molecular biology has revolutionized our knowledge of gene structure, organization and expression. Many modifications have been made to the original technique that have extended its sensitivity and versatility.

There are many different type of hybridization currently in use. These include solution hybridization, *in situ* hybridization, filter hybridization, PCR and hybridization to DNA chips. Although the range of applications is wide, common features underlie them all.

This book is targetted at the beginner who has little or no knowledge of the principles or practice of hybridization. The field of hybridization is too large to be covered in a single book. Recent companion volumes give comprehensive accounts of PCR and *in situ* hybridization [1–2] and so these topics will not be covered at length here. The book will concentrate on solution and filter hybridization with a final chapter on current developments which includes DNA chips and advances in probe design.

To lay the framework for later discussions, the first chapter describes the structure and properties of DNA, particularly in relation to how they relate to hybridization. Chapter 2 describes the different types of hybridization that are in current use and the type of applications for which they are particularly suited. Some of the drawbacks are also discussed. Chapters 3–5 discuss the principles of reassociation kinetics and how they can be applied to answer biological questions. Many of the concepts that are used in hybridization (e.g. C_{ot} and R_{ot} , complexity and stringency) originated in studies of solution hybridization and are explained.

Chapter 6 introduces the basic types of filter hybridization on which all the applications are based. There is no single set of conditions that is

suitable for all hybridizations. The conditions need to be adjusted according to the purpose of the experiment. Chapters 7–8 discuss the factors which affect hybridization with long and oligonucleotide probes and the choices that have to be made at every step of a hybridization experiment. Chapter 9 discusses choices to be made in selecting and labeling the probe. Chapters 10–13 discuss all the steps in setting up a filter hybridization experiment and give practical details. The methods given are tried and tested so that they should be mastered by a beginner. Chapter 14 describes common problems encountered in filter hybridization, suggests reasons and offers advice on how to deal with them. Chapter 15 discusses a wide range of applications for filter and solution hybridization. The final chapter deals with developments such as hybridization to DNA chips in this fast evolving field.

It is hoped that through understanding the principles of hybridization the beginner can approach scientific literature without feeling intimidated and can begin to read critically. It is intended that the reader should gain sufficient basic knowledge to plan and carry out simple filter hybridization experiments, and should the experiments fail, it is hoped that there will be sufficient information herein with which to diagnose and remedy the problem.

Acknowledgements

I gratefully acknowledge the generosity of the following friends or colleagues who have contributed to this book.

Professor R.L.P. Adams, Dr A.M. Campbell, Professor J.R. Coggins, Miss A. Faichney, Dr G Lanyon, Professor D.M.J. Lilley, Dr A.-M. McNicoll, Dr V. Math and Dr M. Piper.

Figures 15.2 and 15.6 were provided by Dr G. Lanyon and *Figures 6.4 and 13.6* by Dr M. Piper, Boehringer Mannheim Customer Services. *Figures 6.6 and 15.9* are reproduced from *Nucleic Acid Research; A practical approach* (1985) with permission of IRL Press at Oxford University Press. *Figure 9.1* is reproduced from *Gene Probes 1: A Practical Approach* (1995) with permission of IRL Press at Oxford University Press.

References

1. Leitch, A.R., Schwarzacher, T., Jackson, D. and Leitch, I.J. (eds) (1994) *In Situ Hybridization*. BIOS Scientific Publishers, Oxford.
2. Newton, C.R. and Graham, A. (1994) *PCR*. BIOS Scientific Publishers, Oxford.

Safety

Attention to safety aspects is an integral part of all laboratory procedures and both the Health and Safety at Work Act and the Control of Substances Hazardous to Health (COSHH) regulations impose legal requirements on those persons planning or carrying out such procedures.

In this book every effort has been made to ensure that the recipes, formulae and practical procedures are accurate and safe. However, it remains the responsibility of the reader to ensure that all necessary COSHH requirements including completion of forms have been implemented. Any specific instructions relating to items of laboratory equipment must also be followed.

Neither the author, editors nor publishers accept any responsibility for loss or damage occasioned to any person or property through using the materials, instructions, methods or ideas contained herein, or acting or refraining from acting as a result of such use. While the author, editors and publishers believe that the data, recipes, practical procedures and other information as set out in this book are in accord with current recommendations and practice at the time of publication, they accept no legal responsibility for any errors or omissions, and make no warranty, express or implied, with respect to material contained herein.

Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

1 Introduction to nucleic acid hybridization

Nucleic acid hybridization describes a range of techniques in which single-stranded nucleic acids are incubated under conditions of temperature and ionic strength that favor pairing of complementary bases and duplex formation. Under these conditions a molecule of nucleic acid can reassociate with its complementary sequence even in the presence of a vast excess of unrelated sequences. The techniques are powerful and have had a major impact on our knowledge of gene structure and information flow in the cell. Since they all depend on the base-pairing properties of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), we will first consider the structure and properties of nucleic acids with special emphasis on properties that affect hybridization.

1.1 Structure of nucleic acids

All nucleic acids are composed of nitrogenous bases, pentose sugars and phosphate. In DNA, the bases are the purines adenine (A) and guanine (G) and the pyrimidines cytosine (C) and thymine (T). In RNA the major bases are adenine, guanine, cytosine and uracil (U). The sugars are deoxyribose in DNA and ribose in RNA (*Figure 1.1*). The basic building brick of a nucleic acid is the nucleotide in which the sugar is attached at carbon atom 1 to the base and at carbon 5 to the phosphate. In nucleic acids, nucleotides are joined into linear polymers via phosphodiester linkages (*Figure 1.2*).

Most DNAs are double-stranded with the individual strands twisting round each other and held together in a helical structure by hydrogen bonding between adenine and thymine and between guanine and cytosine (*Figure 1.3a*). An important consequence of the specificity of base-pairing in DNA is that the sequence of one strand determines the sequence of the other.

Figure 1.1. Base and sugar components of nucleic acids. Bases are attached via nitrogen 9 of purines and nitrogen 3 of pyrimidines to carbon 1 of the sugar.

Most RNAs are single-stranded, but contain double-stranded regions. the RNA molecule folds back on itself to form hairpin structures in which base-pairing occurs through complementary regions of the same strand. In RNA, hydrogen bonds are formed between A and U and between G and C.

1.1.1 Forces stabilizing DNA structure

Covalent bonds between adjacent nucleotides determine the primary structure of DNA and weak forces determine the three-dimensional shape. These forces are individually weak, but they act co-operatively and are collectively strong enough to maintain a stable structure while being weak enough to allow conformational flexibility. They stabilize the double-stranded helix in several ways.

- ***Hydrogen bonds between complementary bases.*** The base pairs A:T and G:C have similar dimensions and can fit similarly into the helical space.
- ***Hydrophobic effects.*** Purine and pyrimidine bases are flat rather hydrophobic molecules. Burying them inside the helix where they are protected from the aqueous environment, increases the stability of the helix.
- ***Stacking forces.*** When neighboring bases in the same strand are stacked on top of each other, the planar faces of the bases interact

Figure 1.2. Molecular structure of a strand of DNA. Nucleotides are linked by 5'–3' phosphodiester bonds. A free phosphate group is present at the 5' end and a free hydroxyl at the 3' end of the chain.

with each other in a complex way that involves weak van der Waals forces and dipole–dipole interactions.

- **Negative charges.** The negative charges on the phosphate groups of the sugar–phosphate backbone are a potential source of instability in

Figure 1.3. Base pairing in DNA. (a) Standard pairs: A pairs with T and C with G. (b) Nonstandard base pairs such as G–T and A–C can be accommodated within the DNA helix, but are less stable than standard base pairs. *Denotes a rare tautomer.

the helix. However, the charge repulsion is overcome by interaction with cations and positively charged side chains of amino acids in proteins.

1.1.2 Mismatched base pairs

The strictness of base-pairing rules (adenine with thymine and guanine with cytosine) produces a regular helix because each base pair is about the same size. Certain mismatches can be tolerated within a double-stranded structure, causing minimal local structural perturbations. Mismatches in base pairing are normally corrected *in vivo*. However, they can easily be created *in vitro* and have a profound effect on the stability of double-stranded nucleic acid. Among non-standard pairings is guanine with thymine and cytosine with adenine (Figure 1.3b).

1.2 Stability of nucleic acids

The noncovalent forces holding double strands together can easily be disrupted. Separation of the strands of double-stranded nucleic acid or the hydrogen-bonded regions of a single-stranded nucleic acid is known as denaturation. Total separation of strands does not occur *in vivo*, but local denaturation can occur in procedures such as DNA replication, transcription and DNA repair. *In vitro*, however, the environment of nucleic acids can be manipulated to alter the stability of nucleic acids and separate the strands.

1.2.1 Effects of pH

Changes in pH can alter the ionization state of bases, sugars and phosphates. At pH values above about pH 11–12 charge repulsion between base pairs causes the complementary strands of DNA to separate. Experimentally this is a very useful means of denaturing DNA. Alkali treatment also causes unfolding of RNA, but must be carried out under very mild conditions as the sugar–phosphate backbone of the RNA chain is hydrolyzed in alkali.

Reducing the pH to below about pH 3 also causes nucleic acid strands to dissociate. However, at low pH the linkage between purines and deoxyribose is broken leading to release of the base. This phenomenon is known as depurination. Pyrimidine/sugar linkages are more stable at low pH than those involving purines. Because of the loss of purines at low pH, acid treatment is not used experimentally for denaturation of nucleic acids.

1.2.2 Effects of temperature

One of the simplest ways of denaturing nucleic acids is to raise the temperature until all the hydrogen bonds and stacking forces have been broken. The strands then dissociate or ‘melt’. Single-stranded nucleic acid absorbs UV light more strongly than double-stranded DNA, a phenomenon called hyperchromicity, so as regions of the DNA melt, the absorption of light by those regions increases. The increase in absorption is proportional to the extent of denaturation so dissociation can be followed by monitoring absorbance as the temperature is being raised (*Figure 1.4a*).

When a solution of DNA is heated, there is initially little change in absorbance as the temperature increases, until over a very short temperature range there is an abrupt increase of about 40% followed by no further change. These three phases correspond to native double-

Figure 1.4. (a) Thermal melting curve for DNA. As the DNA is heated, the weak forces holding the double-stranded DNA together are broken. As regions of the DNA melt, the absorption of light of these regions increases so that the increase in absorption is proportional to the extent of denaturation. At T_m , the change in absorption is half the total change.

stranded DNA, breaking of the weak forces holding the two strands together and single-stranded DNA. If the DNA solution is heated very slowly, the structural changes are co-operative and reversible. The midpoint of the increase in absorption is defined as the melting temperature (T_m).

The T_m of a nucleic acid is a measure of its stability – the more stable a molecule is, the higher is the temperature required to dissociate the strands. T_m depends markedly on base composition. A GC-rich DNA melts at a much higher temperature than an AT-rich one because there are three hydrogen bonds to be broken for every G:C complementary base pair whereas there are only two for every A:T base pair. In addition, the stacking forces between GC neighbors is greater than those between AT neighbors.

So, more energy in the form of heat must be supplied to melt the strands of a GC-rich DNA. The dependency of T_m with GC content is linear and increases by about 0.4°C for each percent increase in GC content (*Figure 1.4b*). Under conditions where mammalian DNA (GC content = 40%) melts at about 87°C , a DNA with GC content of 50% will melt at 91°C . For an unknown sample, determination of the T_m can be used to determine the (G+C) content.

Other factors such as the ionic strength of the solution in which the DNA is dissolved affects the T_m (*Figure 1.4b*). At low ionic strength, the negative charges on phosphate groups repel each other so the duplex is

Figure 1.4. (b) Dependence of melting temperature T_m on guanine + cytosine (G + C) content of DNA. There is a linear relationship between the G:C content of DNA and the T_m . Salt stabilizes the double-helix so as the ionic strength is lowered, the T_m decreases.

less stable than in high ionic strength solutions where the salt shields the electrostatic forces and stabilizes the helix. Less energy is, therefore, required for strand separation and the DNA melts at a lower temperature in low ionic strength solutions.

At the T_m of a long nucleic acid, 50% of the base pairs have been broken and the molecules contain both single-stranded and double-stranded regions (Figure 1.5a).

There are important differences between the T_m of a long duplex and that of a hybrid containing an oligonucleotide. In the latter the T_m is much lower than that for long DNA because there are far fewer weak forces to be disrupted. Oligonucleotide hybrids are too short to contain both single and double-stranded regions. Their T_m is the temperature at which 50% of the molecules have dissociated into single-strands (Figure 1.5b). At the T_m there is equilibrium between single-strands and duplexes.

RNA:RNA and RNA:DNA duplexes can be formed *in vitro*. By virtue of the angle at which the bases are tilted to the long axis of the RNA, these duplexes are more compact than DNA:DNA duplexes. As a consequence the RNA-containing molecules are more stable and have higher melting temperatures than DNA:DNA duplexes with the same base sequence. In general, the order of stability is (from greatest to least stable): RNA:RNA > RNA:DNA > DNA:DNA.

Figure 1.5. (a) Heat denaturation of 'long' DNA. As the temperature is raised, AT-rich regions of DNA melt first. At the T_m half the forces stabilizing the helix have been broken. (b) Heat denaturation of hybrids containing oligonucleotides. Oligonucleotide-containing hybrids melt over a very sharp temperature range. At T_m half the hybrids have dissociated.

Prolonged incubation of nucleic acids at high temperatures causes the sugar-phosphate backbone to break. RNA in particular undergoes rapid nonenzymic hydrolysis at high temperature, being degraded within 2 h at 80°C.

Substances such as urea, dimethyl sulfoxide (DMSO) and formamide break hydrogen bonds and thus reduce the T_m of nucleic acids. In their presence the T_m is lowered by an amount that depends on the concentration of denaturant. This property is very useful experimentally because when denaturants are present, the temperatures required for strand dissociation are lower and nucleic acids do not suffer the thermal degradation that occurs at higher temperatures.

1.3 Renaturation and hybridization

When heat-denatured DNA is cooled slowly, the complementary strands reassociate to form a double-stranded molecule. This is a two-step process (*Figure 1.6*). First, the DNA strands collide randomly with each other and during the process may form imperfectly base-paired stretches. However, these will not be very stable and will quickly dissociate. More collisions will occur until eventually one successfully brings the bases of complementary strands into correct register. This event is known as nucleation. The second step is a rapid zippering up that produces a correctly base-paired duplex. In most reassociations and hybridizations the rate-limiting event is nucleation and such processes are said to be nucleation limited. Slow cooling or incubation at a carefully determined temperature allows many initial base pairings to be sampled and broken until the most stable pairing is retained. On complete renaturation, the properties of the original DNA molecule are restored.

If denatured DNA is cooled quickly (e.g. by plunging the DNA-containing tube in ice), the single strands do not reassociate because there is not enough time for the correct nucleation event to occur.

Figure 1.6. Reassociation of DNA. Single strands of DNA undergo numerous random collisions until eventually a collision occurs that places complementary sequences in the correct register. Formation of a base-paired duplex quickly follows.

Instead the strands are trapped either in single-stranded or in imperfectly base-paired structures.

If denatured DNA or RNA is incubated so that the original base pairs are reformed, the nucleic acid is said to have renatured or reassociated. If the complementary strands come from different sources such as DNA from different species or a mixture of DNA and RNA, the process is known as hybridization.

Complementary strands do not have to be perfectly matched to form duplexes. Two strands of DNA with very similar, but not identical sequences will form hybrid double-stranded molecules, but these hybrids will be less stable (will have a lower T_m) than the perfectly matched parental DNAs. This is because in the mismatched hybrids there are fewer hydrogen bonds to be broken in order to dissociate the strands. At the sites of mismatching some combinations of bases are more stable than others, for example, G in one strand opposite T in the other is more stable than A opposite C.

The *in vitro* formation of double-stranded molecules from complementary strands underlies all techniques based on nucleic acid hybridization. The following chapter introduces the main types of hybridization that are currently in use.

2.6 Use of more than one hybridization technique

Experiments can involve more than one method of hybridization. For example:

- *In situ* hybridization may involve PCR on a microscope slide in order to amplify the hybridization signal.
- The products of PCR may be a mixture of sequences of the same size that have to be resolved by cloning and this may involve identifying the desired sequence by filter hybridization.
- Repetitive human DNA isolated by solution reassociation can be used as a molecular probe in filter hybridization, e.g. to identify clones containing human DNA from rodent–human interspecies hybrids.

References

1. **Leitch, A.R., Schwarzacher, T., Jackson, D. and Leitch, I.J.** (eds) (1994) *In Situ Hybridization*. BIOS Scientific Publishers, Oxford.
2. **McNicoll, A.M. and Farquharson, M.A.** (1997) *J. Pathol.* **182**: 250–261.
3. **Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Elrich, H.A. and Arnheim, N.** (1985) *Science* **230**: 1350–1354.
4. **Newton, C.R. and Graham, A.** (1994) *PCR*. BIOS Scientific Publishers, Oxford.
5. **McPherson, M.J., Hames, B.D. and Taylor, G.R.** (eds) (1995) *PCR 2: A Practical Approach*. IRL Press, Oxford.

will contain only unique fragments. The size of the repetitive fraction will be lower than either of the above situations. Conversely the size of the unique fraction will be larger than either of the above situations.

By carrying out reassociation experiments with different sizes of DNA fragments followed by analyses of the T_m of the duplex DNA, the pattern of interspersion of repeated and unique sequences can be investigated [8].

It follows from these considerations that the size of a component depends on the length of fragments into which the DNA has been broken.

For a single nucleic acid species reassociating with itself, the hybrid melts over a very narrow temperature range and the T_m is the same irrespective of the temperature of reassociation, T_i . However, if the same nucleic acid is incubated with a complex mixture of sequences which have varying degrees of homology, the T_m profile depends on the hybridization conditions. For hybrids formed at low criterion ($T_m - 25^\circ\text{C}$), the melting temperature is broad because both well-matched and poorly matched hybrids are formed. They melt at different temperatures, so the overall melting profile which is a composite of the contributions of all the hybrids, will reflect this. At high criterion ($T_m - 8^\circ\text{C}$), only hybrids with a high degree of homology form so they melt over a very narrow temperature range. The temperature profile is also broad when variable length fragments are used. This is most apparent at short average lengths of DNA in accordance with the empirical relationship:

$$T_n - T_m = 650/L$$

where L is the length of the probe in nucleotides, T_m is the melting temperature of the short hybrid and T_n is the melting temperature of long DNA molecules [1].

References

1. Britten, R.J., Graham, D.E. and Neufeld, B.R. (1974) *Methods Enzymol.* **29**: 363–418.
2. Wetmur, J.G. and Davidson, N. (1968) *J. Mol. Biol.* **31**: 349–370.
3. Britten, R.J. and Kohne, D.E. (1968) *Science* **161**: 529–540.
4. Young, B.D. and Anderson, M.L.M. (1985) In: *Nucleic Acid Hybridisation: A Practical Approach* (eds B.D. Hames and S.J. Higgins). IRL Press, Oxford, pp. 225–231.
5. Powell, J.R. and Caccone, A. (1990) *J. Mol. Evol.* **30**: 267–272.
6. Bonner, T.I., Brenner, D.J., Neufeld, B.R. and Britten, R.J. (1973) *J. Mol. Biol.* **81**: 123–135.
7. Caccone, A., DeSalle, R. and Powell, J.R. (1988) *J. Mol. Evol.* **27**: 212–216.
8. Graham, D.E., Neufeld, B.R., Davidson, E.H. and Britten, R.J. (1974) *Cell* **1**: 127–136.

To investigate the overlap in expression of mRNAs further, the cDNA of one tissue is fractionated into three kinetic components: fast, intermediate and slow hybridizing fractions. These are dissociated and the cDNA from each is recovered and hybridized to mRNA from a different cell type. These experiments show that the mRNAs that are abundant in one tissue are expressed at lower levels in other tissues. Similarly, results show that there is extensive overlap in the middle frequency cDNAs from the three tissues.

References

1. Birnsteil, M.L., Sells, B.H. and Purdom, I.F. (1972) *J. Mol. Biol.* **63**: 21–39.
2. Young, B.D. and Paul, J.P. (1973) *Biochem. J.* **135**: 573–576.
3. Hell, A., Young, B.D. and Birnie, G.D. (1976) *Biochim. Biophys. Acta* **442**: 37–49.
4. Bishop, J.O., Morton, J.G., Rosbach, M. and Richardson, M. (1974) *Nature* **250**: 199–204.
5. Young, B.D. and Anderson, M.L.M. (1985) In: *Nucleic Acid Hybridisation: A Practical Approach* (eds B.D. Hames and S.J. Higgins). IRL Press, Oxford, pp. 225–231.
6. Galau, G.A., Britten, R.J. and Davidson, E.H. (1974) *Cell* **2**: 9–20.
7. Axel, R., Felgelson, P. and Schutz, G. (1976) *Cell* **7**: 247–254.
8. Hastie, N.D. and Bishop, J.O. (1976) *Cell* **9**: 761–774.

References

1. Britten, R. J., Graham, D.E. and Neufeld, B.R. (1974) *Methods Enzymol.* **29**: 363–418.
2. Bonner, T.I., Brenner, D.J., Neufeld, B.R. and Britten, R.J. (1973) *J. Mol. Biol.* **81**: 123–135.
3. Nygaard, A.P. and Hall, B.D. (1964) *J. Mol. Biol.* **9**: 125–142.
4. Wetmur, J. and Davidson, N. (1968) *J. Mol. Biol.* **31**: 349–370.
5. Wetmur, J.R. (1971) *Biopolymers* **14**: 601–613.
6. Chang, C.-T., Hain, T.C., Hutton, J.R. and Wetmur, J.G. (1974) *Biopolymers* **13**: 1847–1858.
7. Wetmur, J.R. (1975) *Biopolymers* **14**: 2517–2524.
8. Kohne, D.E., Levinson, S.A. and Byers, M.J. (1977) *Biochemistry* **16**: 5329–5341.
9. Casey, J. and Davidson, N. (1977) *Nucleic Acids Res.* **4**: 1539–1552.
10. Hutton, J.R. (1977) *Nucleic Acids Res.* **4**: 3537–3555.
11. Young, B.D. and Anderson, M.L.M. (1985) In *Nucleic Acid Hybridisation: A Practical Approach*. (eds B.D. Hames and S.J. Higgins). IRL Press, Oxford, pp. 47–71.
12. Wetmur, J.G. (1991) *Crit. Rev. Biochem. Mol. Biol.* **4**: 227–259.
13. Vogelstein, B. and Gillespie, D. (1977) *Biochem. Biophys. Res. Commun.* **75**: 1127–1132.
14. Caccione, A., DeSalle, R. and Powell, J.R. (1988) *J. Mol. Evol.* **27**: 212–216.

Figure 6.7. Flow diagram showing the steps involved in filter hybridization.

There are many different ways of carrying out each step and the choices made can determine which hybrids form and which persist. Hybridization experiments need to be well planned in order to get satisfactory results. Chapters 7–9 discuss hybridization strategy and choice of the probe. Chapters 10–13 give experimental details for some of the most commonly used procedures.

References

1. **Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) In: *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Grunstein, M. and Hogness, D.** (1975) *Proc. Natl Acad. Sci. USA* **72**: 3961–3965.
3. **Benton, W.D. and Davis, R.W.** (1977) *Science* **196**: 180–182.
4. **Southern, E.** (1975) *J. Mol. Biol.* **98**: 503–517.
5. **Davis, P.S.** (1980) *Proc. Natl Acad. Sci. USA* **77**: 5201–5205.

overnight although this is probably much longer than is necessary. It should be noted, that in certain circumstances overlong hybridization may be detrimental as when discriminating between closely and distantly related members of a gene family. Discrimination is usually better at short hybridization times [2].

References

1. Flavell, R.A., Birfielder, E.J., Sanders, J.P. and Borst, P. (1974) *Eur. J. Biochem.* **47**: 535–543.
2. Beltz, G.A., Jacob, K.A., Eickbush, T.H., Cherbas, P.T. and Kafatos, F.C. (1983) *Methods Enzymol.* **100**: 266–285.
3. Flavell, R.A., Borst, P. and Birkfelder, E.J. (1974) *Eur. J. Biochem.* **47**: 545–548.
4. Wetmur, J.G. and Davidson, N. (1968) *J. Mol. Biol.* **31**: 349–370.
5. Bonner, T.I., Brenner, D.J. Neufeld, B.R. and Britten, R.J. (1973) *J. Mol. Biol.* **81**: 123–135.
6. Birnstein, M.L., Sells, B.H. and Purdom, I.F. (1972) *J. Mol. Biol.* **63**: 21–39.
7. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In: *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
8. Wetmur, J.G. (1991) *Crit. Rev. Biochem. Mol. Biol.* **26**: 227–259.
9. Howley, P.M., Israel, M.F., Law, M-F. and Martin, M.A. (1979) *J. Biol. Chem.* **254**: 4876–4883.
10. Casey, J. and Davidson, N. (1977) *Nucleic Acids Res.* **4**: 1539–1552.
11. Lathe, R. (1985) *J. Mol. Biol.* **183**: 1–12.
12. Caccone, A., DeSalle, R. and Powell, J.R. (1988) *J. Mol. Evol.* **27**: 212–216.
13. Wahl, G.M., Stern, M. and Stark, G.R. (1979) *Proc. Natl Acad. Sci. USA* **76**: 3683–3687.
14. Amasino, R. (1986) *Anal. Biochem.* **152**: 304–307.
15. Kohne, D.E., Levinson, S.A. and Byers, M.J. (1977) *Biochemistry* **16**: 5329–5341.
16. Kessler, C. (1995) In: *Gene Probes 1: A Practical Approach* (B.D. Hames and S.J. Higgins). IRL Press, Oxford, pp. 93–144.

An alternative means of reducing the complexity of an oligonucleotide pool is to design a single, longer oligonucleotide with an 'optimized' sequence. This takes into account features such as the known codon preferences of the species, the under-representation of the dinucleotide CpG in many genomes and the avoidance of coding sequences that involve leucine, arginine and serine, all of which have six codons. A detailed consideration of the theoretical and practical aspects of this approach and of methods to reduce the number of false positives are given in reference [12].

References

1. Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K. and Wallace, R.B. (1981) *ICN-ICLA Symp. Mol. Cell. Biol.* **23**: 682–693.
2. Bolton, E.T. and McCarthy, B.J. (1962) *Proc. Natl Acad. Sci. USA* **48**: 1390–1397.
3. Albertsen, C., Haukanes, B.-I., Aasland, R. and Kleppe, K. (1988) *Anal. Biochem.* **170**: 193–202.
4. Wood, W.I., Gitschier, J., Lasky, L.A. and Lawn, R.M. (1985) *Proc. Natl Acad. Sci. USA* **82**: 1585–1588.
5. Jacobs, K.A., Rudersdorf, R., Neill, S.D., Dougherty, J.P., Brown, E.L. and Fritsch, E.F. (1988) *Nucleic Acids Res.* **16**: 4637–4650.
6. Wallace, R.B., Shaer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura, K. (1979) *Nucleic Acids Res.* **6**: 3543–3557.
7. Cacccone, A., DeSalle, R. and Powell, J.R. (1988) *J. Mol. Evol.* **27**: 212–216.
8. Ikuta, S., Takagi, K., Wallace, R.B. and Itakura, K. (1987) *Nucleic Acids Res.* **15**: 797–811.
9. Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, H. and Itakura, K. (1981) *Nucleic Acids Res.*, **9**: 879–894 .
10. Wetmur, J.G. and Davidson, N. (1968) *J. Mol. Biol.* **31**: 349–370.
11. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12. Lathe, R. (1985) *J. Mol. Biol.* **183**: 1–12.
13. Nei, M. and Li, W.H. (1979) *Proc. Natl Acad. Sci. USA* **76**: 5269–5273.
14. Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y. and Matsubara, K. (1985) *J. Biol. Chem.* **260**: 2605–2608.

detect rare sequences. If the amount of nucleic acid available for binding is limited, then radioactive probes or probes that can be detected by enhanced chemiluminescence are probably the best choices.

9.4.2 DNA or RNA or oligonucleotide?

DNA probes are widely used as they are usually easy to prepare, easy to handle and give good levels of detection.

RNA probes can be used for the same applications as DNA probes. If screening Northern or RNA dot blots, care needs to be taken that the probe represents the antisense strand. DNA:RNA and RNA:RNA hybrids are more stable than DNA:DNA hybrids so hybridization and washing conditions can be more stringent. It can be difficult to remove RNA probes from filters because of the stability of RNA-containing hybrids.

Oligonucleotide probes are widely used for screening recombinant libraries, or hybridizing to dot and slot blots. Because they can discriminate between sequences that differ in a single nucleotide, they are particularly well-suited to detecting mutations in nucleic acid. Oligonucleotide probes are also very useful for probing nucleic acid in dried gels because their small size makes it easy for them to enter the gel.

9.4.3 End-labeling or uniform labeling?

Uniformly labeled probes are very widely used for screening libraries, probing Northern and Southern blots and for analyzing dot blots. More reporter molecules are available per probe molecule than when the probe is end-labeled, so less target material is needed to generate a detectable signal.

End-labeling is most useful with oligonucleotide probes or when mapping the ends of transcripts.

References

1. **Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. **Hames, B.D. and Higgins, S.J.** (1995) *Gene Probes 1: A Practical Approach*. IRL Press, Oxford.
3. **Ren, M. and Kurz, C.** (1984) *Nucleic Acids Res.* **12**: 3435–3444.
4. **Forster, A.C., McInnes J.L., Skingle, D.C. and Symons, R.H.** (1985) *Nucleic Acids Res.* **13**: 745–761.

PROTOCOL 10.6: RNA dot blots: glyoxal denaturation**Solutions**

Glyoxal denaturation solution For each 100 μ l denaturation solution, mix 34 μ l deionized glyoxal, 20 μ l 0.1 M sodium phosphate, pH 6.5 and 46 μ l RNase-free water.

Transfer by hand

1. To RNA ($\leq 8 \mu$ g) in 2 μ l water, add 2 μ l glyoxal denaturation solution.
2. Incubate for 1 h at 50°C.
Minimize evaporation by keeping the tube well submerged.
3. Spin briefly to bring contents to the foot of the tube.
4. Apply the samples to the filter in 2 μ l aliquots.
Dry in air between applications or apply cold air gently from a hair dryer.
5. For nitrocellulose filters, place the filter on a dry sheet of 3 MM paper and air dry. Bake at 80°C for 2 h in a vacuum oven
For nylon or charged nylon filters, either place the filter on a dry sheet of 3 MM paper air dry and bake at 80°C for 2 h;
or treat with UV light for a time determined empirically (see Section 10.3.2) and air dry.

Removing glyoxal group

6. Heat 200 μ l of 20 mM Tris-HCl, pH 8.0, to 100°C. Remove from the heat and carefully immerse the filter. Allow to cool to room temperature

The composition of the denaturation solution is 1.0 M glyoxal, 10 mM sodium phosphate, pH 6.5 and up to 2.0 mg RNA ml⁻¹.

Notes

1. Glyoxal is readily oxidized to glyoxylic acid which will degrade RNA. De-ionize it with a mixed-bed resin [1] and store in 100 μ l aliquots at -20°C. Do not re-use an aliquot once it has been opened.
2. DMSO can be included in the denaturation mixture at a final concentration of 50%. But should be omitted if nitrocellulose filters are used because DMSO dissolves nitrocellulose.
3. Serial dilutions can be prepared in 0.1% sodium dodecyl sulfate (SDS) so that each dilution is in a final volume of 4 μ l.

References

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Southern, E.M. (1975) *J. Mol. Biol.* **98**: 503–517.
3. Olszewska, E. and Jones, K. (1988) *Trends Genet.* **4**: 92–94.
4. Braman, J. and Dyaico, M. (1989) *Strategies* **2**: 59.
5. Bittner, M., Kupferer, P. and Morris, C.F. (1980) *Anal. Biochem.* **102**: 459–471.
6. Thomas, P.S. (1983) *Methods Enzymol.* **100**: 255–266.
7. Haas, M., Vogt, M. and Dulbecco, R. (1972) *Proc. Natl Acad. Sci. USA* **69**: 2160–2164.
8. Tsao, S.G.S., Brunk, C.F. and Pearlman, R.E. (1983) *Anal. Biochem.* **131**: 3665–3672.
9. Miyada, C.G. and Wallace, R.B. (1987) *Methods Enzymol.* **154**: 94–107.

References

1. **Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. **Hames, B.D. and Higgins, S.J.** (eds) (1995) *Gene Probes 1: A Practical Approach*. IRL Press, Oxford.
3. **Feinberg, A.P. and Vogelstein, B.** (1983) *Anal. Biochem.* **132**: 6–13.
4. **Feinberg, A.P. and Vogelstein, B.** (1984) *Anal. Biochem.* **137**: 266–267.
5. **Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P.** (1977) *J. Mol. Biol.* **113**: 237–251.
6. **Langer, P.R., Waldrop, A.A. and Ward, D.C.** (1981) *Proc. Natl Acad. Sci. USA* **78**: 6633–6637.
7. **Sarker, G. and Sommer, S.S.** (1988) *Nucleic Acids Res.* **16**: 5197.
8. **Stoffet, E.S., Koeberl, D.D., Sarker, G. and Sommer, S.S.** (1988) *Science* **239**: 491–494.
9. **Kreig, P.A. and Melton, D.A.** (1987) *Methods in Enzymol.* **155**: 397–415.
10. **Kreig, P.A.** (1991) *Nucleic Acids Res.* **18**: 6463.
11. **Wahlberg, J., Hultman, T. and Uhlen, M.** (1995) In: *PCR2: A Practical Approach* (eds M.J. McPherson, B.D. Hames and G.R. Taylor). IRL Press, Oxford, pp. 71–86.
12. **Boyle, J.S. and Lew, A.M.** (1995) *Trends Genet.* **11**: 8.

Figure 12.1. Hybridization with an oligonucleotide probe. A blot containing restriction digests of a series of recombinant plasmids carrying human IgC $_{\lambda}$ genes was probed with a ^{32}P -labeled antisense J $_{\lambda}$ oligonucleotide probe. The sequence of the oligomer was 5'-TTGGTCCCTCCGCCGAACAC-3'. Hybridization was in 6 $\times$ NET, 5 $\times$ Denhardt's solution, 0.1% SDS, 50 μg calf thymus DNA/ml for 13 h, 42°C. Washing was twice in 6 $\times$ SSC at 4°C for 15 min each time, then three times in 6 $\times$ SSC at room temperature for 15 min each time.

References

1. Denhardt, D.T. (1966) *Biochem. Biophys. Res. Commun.* **23**: 641–645.
2. Singh, L. and Jones, K.W. (1984) *Nucleic Acids Res.* **12**: 5627–5638.
3. Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) *Genet. Anal. Tech.* **1**: 3–8.
4. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
5. Siegel, L.I. and Bresnick, E. (1986) *Anal. Biochem.* **159**: 82–87.
6. Stroop, W.G. and Schaefer, D.C (1989) *Anal. Biochem.* **182**: 222–225.
7. Albrechtsen, C., Haukanes, B.-I., Aasland, R and Kleppe, K. (1988) *Anal. Biochem.* **170**: 193–202.
8. Church, G.M. and Gilbert, W. (1984) *Proc. Natl Acad. Sci. USA* **81**: 1991–1995.
9. Amasino, R.M. (1986) *Anal. Biochem.* **152**: 304–307.
10. Budowle, B. and Baechtel, F.S. (1990) *Appl. Theor. Electro.* **1**: 181–187.

70°C for 30 min. The newly denatured probe can then be added to a second filter that has been prehybridized in the normal way.

References

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
2. Leary, J.J., Brigati, D.J. and Ward, D.C. (1983) *Proc. Natl Acad. Sci. USA* **80**: 4045–4049.
3. West, S., Schroder, J. and Kunz, W. (1990) *Anal. Biochem.* **190**: 254–258.
4. Conyers, S.M. and Kidwell, D.A. (1991) *Anal. Biochem.* **192**: 207–211.
5. Sheldon, E.L., Kellogg, D.E., Watson, R., Levensen, C.H. and Ehrlich, H.A. (1986) *Proc. Natl Acad. Sci. USA* **83**: 9085–9089.
6. Holtke, H.J., Ettl, L., Finken, M., West, S. and Kunz, W. (1992) *Anal. Biochem.* **207**: 24–31.
7. Nguyen, Q. and Heelfinger, D.M. (1995) *Anal. Biochem.* **226**: 59–67.
8. Thorpe, G.H.G., Kricka, L.J., Moseley, S.B. and Whitehead, T.P. (1985) *Clin. Chem.* **31**: 1335–1341.
9. Pollard-Knight, D., Read, C.A., Downes, M.J., Howard, L.A., Leadbetter, M.R., Phelby, S.A. *et al.* (1990) *Anal. Biochem.* **185**: 84–89.
10. Kessler, C. In: *Gene Probes 1. A Practical Approach* (eds Rickwood, D. and Hames B.D.). IRL Press, Oxford, pp. 93–144.
11. Lasky, L.A., Lev, Z., Xin, J-H., Britten R.J. and Davidson, E.H. (1980) *Proc. Natl Acad. Sci. USA* **77**: 5317–5321.
12. Xin, J-H., Brandhorst, B.P., Britten, R.J. and Davidson, E.H. (1982) *Dev. Biol.* **89**: 527–531.

These techniques are not easy and require a fair degree of technical competence. The hybridization of complementary mRNA to the filter-bound DNA must be maximized so that there is enough mRNA to be translated in the hybrid selection procedure. It must also be maximized for hybrid arrest translation or sufficient mRNA may remain in solution to allow translation of the polypeptide. Maximising hybridization requires that the amount of DNA on the filter is high, but the following must be remembered:

- Not all DNA bound to the filter is available for hybridization probably because of steric hindrance.
- If the source of DNA is a recombinant plasmid or phage, it is best to remove the vector sequences before binding the DNA to the filter. If linearized recombinant plasmid or phage is bound to the filter, the cloned complementary sequence will represent only a fraction of the bound DNA. For example, if a 4.7 kb recombinant plasmid having a 1 kb insert is used then a maximum of 21% of the bound DNA can be complementary to the mRNA. If the vector sequences are removed, all the bound sequences will be available to hybridize to the mRNA.
- Filter hybridizations tend not to go to completion so sufficient DNA must be present to ensure that in the time available for hybridization, enough mRNA is hybridized to give a signal on subsequent translation.

In practice, the amount of DNA to bind to the filter depends on the abundance of the mRNA of interest in the sample [26].

Other precautions should be taken when designing hybrid arrest or hybrid selection experiments. DNA should be bound to nylon filters in preference to nitrocellulose. This is in part because nylon has a better binding capacity and in part because DNA can be attached covalently. This is necessary to prevent losses of hybrids during hybridization and to prevent contamination of mRNA during elution which could inhibit translation.

It is most important to minimize RNA degradation. Hybridization is carried out in the presence of formamide to lower the T_m . This allows low incubation temperatures which is beneficial as RNA breakdown is less than at high temperatures. However, one of the main problems in these procedures is that contaminants in the formamide which accumulate on storage cause RNA degradation. It is, therefore, necessary to deionize the formamide.

References

1. Beltz, G.A., Jacobs, K.A., Eickbush, T.H., Cherbas, P.T. and Kafatos, F.C. (1983) *Methods Enzymol.* **100**: 266–285.
2. Jereys, A.J., Wilson, V. and Thein, S.L. (1985) *Nature* **314**: 67–73.

3. Jereys, A.J., Wilson, V. and Thein, S.L. (1985) *Nature* **316**: 76–79.
4. Edwards, A., Civitello, A., Hammond, H.A. and Caskey, C.T. (1991) *Am. J. Hum. Genet.* **49**: 746–756.
5. Gill, P., Ivanov, P.L. Kimpton, C., Piercy, R., Benson, N., Tully, G., Evett, I., Hagelberg, E. and Sullivan, K. (1994) *Nature Genet.* **6**: 130–135.
6. Kidd, V.J., Wallace, R.B., Itakura, K. and Woo, S.L.C. (1983) *Nature* **304**: 230–234.
7. Conner, B.J., Reyes, A.A., Morin, C., Itakura, K., Teplitz, R.L. and Wallace, R.B. (1983) *Proc. Natl Acad. Sci. USA* **80**: 278–282.
8. Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1986) *Nature* **324**: 163–166.
9. Dattagupta, N., Rae, P.M.M., Huguenel, E.D., Carlson, E., Lyga, A., Shapiro, J.A. and Albarella, J.P. (1989) *Anal. Biochem.* **177**: 85–89.
10. Serre, J.L., Taillandier, A., Mornet, E., Simon-Bouy, B., Boue, J. and Boue, A. (1991) *Genomics* **11**: 1149–1151.
11. Landegren, U., Kaiser, R., Sanders, J. and Hood, L. (1988) *Science* **241**: 1077–1080.
12. Wu, D.Y. and Wallace, R.B. (1989) *Genomics* **4**: 560–569.
13. Barany, F. (1991) *Proc. Natl Acad. Sci. USA* **88**: 189–193.
14. Sealey, P.G., Whittaker, P.A. and Southern, E.M. (1985) *Nucleic Acids Res.* **13**: 1905–1922.
15. Witt, M. and White, R.L. (1985) *Proc. Natl Acad. Sci. USA* **82**: 6206–6210.
16. Nisson, P.E., Watkins, P.C., Menninger, J.C. and Ward, D.C. (1991) *BRL Focus* **13**: 42.
17. Flavell, R.A., Birfielder, E.J., Sanders, J.P. and Borat, P. (1974) *Eur. J. Biochem.* **47**: 535–543.
18. Lasky, L.A., Lev, Z., Xin, J.-H., Britten, R.J. and Davidson, E.H. (1980) *Proc. Natl Acad. Sci. USA* **77**: 5317–5321.
19. Anderson, M.L.M. and Young, B.D. (1985) In: *Nucleic Acid Hybridization: A Practical Approach* (eds B.D. Hames and S.J. Higgins). IRL Press, Oxford, pp. 73–111.
20. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In: *Molecular Cloning: A Laboratory Manual, 2nd Edn.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
21. Hames B.D. and Higgins, S.J. (eds). (1995) *Gene Probes 2 A Practical Approach*. IRL Press, Oxford.
22. Williams, J.G. and Mason, P.J. (1985) In: *Nucleic Acid Hybridization: A Practical Approach* (eds Hames, B.D. and Higgins, S.J.). IRL Press, Oxford, pp. 139–160.
23. Miller, K.G. and Solner-Webb, B. (1981) *Cell* **27**: 165–174.
24. Sagerstrom, C.G., Sun, B.I. and Sive, H.L. (1997) *Annu. Rev. Biochem.* **66**: 751–783.
25. Paterson, B.M., Roberts, B.E. and Ku, E.L. (1977) *Proc. Natl Acad. Sci. USA* **74**: 4370–4374.
26. Mason, P.J. and Williams, J.G. (1985) In: *Nucleic Acid Hybridization: A Practical Approach* (eds Hames, B.D. and Higgins, S.J.). IRL Press, Oxford, pp. 113–137.

not fluoresce because the base-pairing in the stem keeps these groups close to each other so that the fluorescence of the fluorophore is quenched by the quenching group. When the beacon hybridizes, a conformational change occurs which disrupts the base-pairing in the stem and as a consequence the quenching group is too far away to quench the fluorophore. Hence, the hybrid fluoresces.

These molecules can discriminate between targets that differ by a single base [16]. Molecular beacons have some useful properties. It is not necessary to remove nonhybridizing molecules to detect hybrids so this saves time. These molecules are potentially useful for introducing into cells and following the synthesis of RNA in real time. By attaching different fluorophores to different probes, many different sequences can be detected simultaneously.

References

1. Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) *Science* **270**: 467–470.
2. Shena, M. (1996) *BioEssays* **18**: 427–431.
3. Adams, M.D. (1996) *BioEssays* **18**: 261–262.
4. Southern, E.M., Maskos, U. and Elder, J.K. (1992) *Genomics* **13**: 1008–1017.
5. Southern, E.M. (1996) *Trends Genet.* **12**: 110–115.
6. Ferguson, J.A., Boles, T.C., Adams, C.P. and Wall, D.R. (1996) *Nature Biotechnol.* **14**: 1681–1684.
7. Lockhardt, D.J., Dong, H., Byrne, M.C., Follettie, T., Gallo, M.V. *et al.* *Nature Biotechnol.* **14**: 1675–1680.
8. Stimpson, D.I., Hoijer, J.V., Hsieh, W.T., Jou, C., Gordon, J., Theriault, T. *et al.* (1995) *Proc. Natl Acad. Sci. USA* **92**: 6379–6383.
9. de Saizieu, A., Certa, U., Warrington, J., Gray, C., Keck, W. and Mous, J. (1998) *Nature Biotechnol.* **16**: 45–48.
10. Wallace, R.W. (1997) *Molec. Med. Today* **3**: 384–389.
11. Hacia, J.G., Brody, L.C., Chee, M.S., Fodor, S.P.A. and Collins, F.S. (1996) *Nature Genet.* **14**: 441–447.
12. Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C. Stern, D. *et al.* (1996) *Science* **274**: 610–613.
13. Jensen, K.K., Ørum, H., Nielsen, P.E. and Norden, B. (1997) *Biochemistry* **36**: 5072–5077.
14. Ørum, H., Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O. and Stanley, C. (1993) *Nucleic Acids Res.* **21**: 5332–5336.
15. Tyagi, S. and Kramer, F.R. (1996) *Nature Biotechnol.* **14**: 303–308.
16. Tyagi, S., Bratu, D.P. and Kramer, F.R. (1998) *Nature Biotechnol.* **16**: 49–53.

Appendix C

Useful information

Sources to check for oligonucleotide design and melting temperatures

Many software packages are available for designing an oligonucleotide probe, checking the proposed probe against sequence databases and for determining the T_m of hybrids. It is beyond the scope of this book to list them all. Excellent discussions of the hardware and software available and the databases containing probe information can be found in [1,2]. A programme, OLIGO, for selecting an optimal oligonucleotide probe and determining the T_m is described in [3]. Some software is available on the Internet at low or nominal cost.

Useful programmes include:

T_m determination:

<http://alces.med.umn.edu/rawtm.html>

Pedro's tools:

<http://www/nwfcs.noaa.gov/protocols/pedro/rt-1.html>

Oligonucleotide determination:

<http://www/nwfcs.noaa.gov/protocols/oligoTMcalc.html>

The PRIME programme of the Genetics Computer Group Inc's Wisconsin sequence analysis package.

References

1. **Fuchs, R. and Cameron, G.N.** (1995) In: *DNA Cloning 3 – Complex Genomes: A Practical Approach*. (D.M. Glover and B.D. Hames eds), IRL Press, Oxford.
2. **Woollard, P.M. and Williams G.** (1997) In: *Genome Mapping: A Practical Approach*. (P.H. Dear ed.), IRL Press, Oxford.
3. **Rychlik, W. and Rhodes, R.E.** (1989) *Nucl. Acids Res.* **17**: 8543–8551.