IAEA-TECDOC-1001

In vitro radionuclide techniques in medical diagnosis

INTERNATIONAL ATOMIC ENERGY AGENCY IAEA

The IAEA does not normally maintain stocks of reports in this series. However, microfiche copies of these reports can be obtained from

INIS Clearinghouse
International Atomic Energy Agency
Wagramerstrasse 5
P.O. Box 100
A-1400 Vienna, Austria

Orders should be accompanied by prepayment of Austrian Schillings 100,– in the form of a cheque or in the form of IAEA microfiche service coupons which may be ordered separately from the INIS Clearinghouse.

The originating Section of this publication in the IAEA was:

Nuclear Medicine Section
International Atomic Energy Agency
Wagramer Strasse 5
P.O. Box 100
A-1400 Vienna, Austria

IN VITRO RADIONUCLIDE TECHNIQUES IN
MEDICAL DIAGNOSIS
IAEA, VIENNA, 1998
IAEA-TECDOC-1001
ISSN 1011–4289

Printed by the IAEA in Austria
February 1998

FOREWORD

The use of radionuclide based microanalytical techniques for medical diagnosis has been increasing in recent years. Several such methods are in routine use in advanced laboratories in the industrialized world, but are often beyond the capability of developing countries. The IAEA has organized several training activities and seminars to bring the benefits of such advances to laboratories in developing Member States. The most recent was an interregional training course in Tokyo, March 1996, in collaboration with the Japanese Atomic Energy Research Institute (JAERI). The course was conducted by international experts, local staff, and IAEA staff. With the intention of giving the main techniques used at the course to a wider distribution they have been compiled into the present technical document. It is hoped that laboratories in developing Member States that did not have participants at the training course will find the information provided useful and sufficiently detailed to enable their application in their home institutions.

EDITORIAL NOTE

In preparing this publication for press, staff of the IAEA have made up the pages from the original manuscript(s). The views expressed do not necessarily reflect those of the IAEA, the governments of the nominating Member States or the nominating organizations.

Throughout the text names of Member States are retained as they were when the text was compiled.

The use of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.

CONTENTS

INTRODUCTION 1

CHAPTER 1. TECHNIQUES FOR THE RADIOACTIVE DETERMINATION OF STEROID RECEPTOR CONTENT OF BREAST CANCER 3

1.1. Oestrogen and progesterone receptor assays 3
1.1.1. Tissue fractionation 3
1.1.2. Oestrogen receptor assay 4
1.1.2.1. Preparation of ^{3}H-oestradiol-17 solutions 4
1.1.2.2. Assay of receptors 4
1.1.2.3. Cytosol fraction 4
1.1.2.4. Nuclear fractions 5
1.1.3. Progesterone receptor assay 5
1.1.3.1. Preparation of ^{3}H-ORG 2058 solutions 5
1.1.3.2. Assay of receptors 5
1.1.3.3. Nuclear receptors 6

Appendix to Chapter 1 7

CHAPTER 2. OESTROGEN AND PROGESTERONE RECEPTOR ASSAYS 12

CHAPTER 3. STEROID RECEPTOR ASSAYS IN WHOLE 13

3.1. [^{3}H]-steroid solutions for ER and PR assays 13
3.1.1. Preparation of [^{3}H]-E_2 solutions 13
3.1.2. Preparation of [^{3}H]-ORG 2058 solutions 13
3.2. Whole cell [^{3}H]-steroid exchange assay for ER and PR 14
3.3. Cytosol assay for ER-Dextran-coated charcoal method 14
3.4. Cytosol assay for ER-Enzyme immunoassay method 14
3.5. Tissue collection and storage 15

CHAPTER 4. MODIFIED BURTON METHOD FOR ASSAY OF DNA CONTENT OF TISSUE 16

CHAPTER 5. PROTEIN ASSAY 17

CHAPTER 6. IMMUNOASSAYS IN CLINICAL CHEMISTRY (PRINCIPLES OF IMMUNORADIOMETRIC ASSAYS) 18

6.1. Limited reagent methods 18
6.2. Excess reagent methods 20
6.3. Monoclonal antibodies 20
6.4. Alternative labels 21

CHAPTER 7. LINKAGE OF BIOMOLECULES TO SOLID PHASES FOR IMMUNOASSAY 32

CHAPTER 8. RADIOLABELLING FOR IMMUNOASSAY 39

8.1. Introduction 39
8.2. Choice of radioisotope 39
8.3. Radioiodination 39

8.4. Mechanism of radioiodination 40
8.5. Radioiodination methods 40
8.5.1. Direct methods 40
8.5.1.1. Chemical oxidation 40
8.5.1.2. Enzymatic oxidation 41
8.5.2. Indirect methods 42
8.6. Post-iodination purification 43
8.7. Specific activity 43
8.8. Assessment of radiolabels 44
8.9. Storage of radiolabels 44

CHAPTER 9. INTERFERENCE IN IMMUNOASSAY 56

9.1. Introduction 56
9.2. Heterophilic antibodies 56
9.3. Human anti-mouse antibodies 61
9.4. Specificity in immunometric assay 61

CHAPTER 10. DETECTION OF HEPATITIS C VIRUS RNA USING REVERSE TRANSCRIPTION PCR 69

10.1. Introduction 69
10.2. HCV RNA assay 69
10.2.1.Specimen handling 69
10.2.2.Materials and reagents 70
10.2.3.Reverse transcriptase 70
10.3. HCV RT-PCR protocol 70
10.3.1.Sample preparation 70
10.3.2.Reverse transcription 71
10.3.3.Amplification 72
10.3.4.Detection 73

CHAPTER 11. TRAINING MANUAL ON THE ANALYSIS OF MICROSATELLITE REPEATS IN HUMAN DNA FOR DIAGNOSTIC APPLICATIONS 75
11.1. Introduction 75
11.2. Procedure 76
11.3. Analysis of PCR products on sequencing gels 82
11.4. Useful guidelines for designing PCR assays 84
11.4.1. Search for duplicate sequences 85
11.4.2 Search for vector sequences 85
11.4.3. Search for repetitive DNA sequences 85
11.4.4. Determine the length of the repetitive element 85
11.4.5. Selecting primer pairs or sets 86
11.4.6. Testing the primer pairs 86
11.4.7. Maximizing throughput 87

LIST OF CONTRIBUTORS 89

INTRODUCTION

In clinical pathological diagnosis it is essential to monitor the dynamic changes of ions, intermediate metabolites, proteins (product of phenotypic expression of genetic information) in the blood. Measurement of certain species of these analytes, present in nanogram concentration, requires the use of the sensitive radioimmunoassay (RIA) technique.

Since the introduction of in vitro competitive binder ligand assay by Yalow, Berson and Ekins 37 years ago, there have been tremendous advances in the field of RIA. New and specific binders can be produced in large quantities because of availability of technology to generate monoclonal antibody from hybridoma, heterohybridoma, recombinant phage, oligo-nucleotide array (for construction of antibody array), and lately synthetic antibody from polymer mould. Protein binder can also be engineered according to design by molecular modelling, point mutagenesis and humanized by the recombinant phage technique. The availability of non-isotopic labels such as silver/gold sol further stimulates the development of sensitive dipstick immunochromatographic assay which enables qualitative point-of-care testing and self-testing to be carried out in the clinics, wards and home. The recent serendipitous invention of conductive polymer and rapid advances in optics enable design of immunosensor, complemented by humanized antibody technology, will certainly contribute to the future development of in vivo immunosensor for real time measurement of chronobiological changes of whole blood analytes. In addition there have also been rapid advances in solid phase design, techniques of immobilization of chemicals such as self-assembly of monolayer, molecular visualization and micromachinery by atomic force microscopy. This will certainly be applied to immunoassay in future to increase assay sensitivity and specificity. All these new immunoassays methodologies (mostly protected by patents) have to be calibrated against the gold standard set by RIA which remains up till now the most robust and cost effective immunoassay methodology for routine diagnosis and method development of new analytes.

The performance efficiency of RIA can be further improved by the recent introduction of automation using the modular robotic system, complemented by global validation of results based on artificial intelligence assisted by a novel inference engine. In this TECDOC the basic principles of competitive binder ligand and labelled antibody assay are highlighted. This is particularly useful for training of laboratory personnel working with 'black-box' technology and wishing to understand in depth the fundamental principles of immunoassay within the black box.

To analyse the genetic information and to detect nucleic acid for diagnostic purposes, one has to resort to molecular biology techniques. Diagnosis by detection of nucleic acids sequences theoretically is applicable to diseases caused by infection or resulting from abnormalities in the genetic make-up of an individual. It is based on the recognition of short stretches of nucleic acids, which uniquely identifies a pathogenic organism or an abnormal piece of genetic information. From the point of view of technology transfer this unified methodological approach can contribute to efficient use of resources such as personnel training, equipment and the supply of reagent. This has obvious implications in terms of the sustainability of the transferred technology.

The initial method of nucleic acid detection is lengthy and requires sophisticated equipment and expensive reagents. The advent of the novel technique for the amplification of nucleic acid sequence based on the polymerase chain reaction (PCR) has shortened the time required for clinical laboratory diagnosis. The PCR technology uses relatively simple equipment, and the

whole process can be automated to increase sample throughput which further reduces the assay costs. As the nucleic acid sequence can be amplified to the order of million folds by PCR, the methodology is highly sensitive and can be further enhanced by use of the nested PCR technique if the need arises. The high sensitivity of nucleic acid analysis based on PCR allows early disease diagnosis and use of an extremely small sample volume e.g. blood collected from finger prick, which is distinctly advantageous for young children. With appropriate standardization the methodology also gives valuable quantitative diagnostic information in clinical practice.

Hybridization of the PCR products using radioisotopic nucleic acid probes can further improve the sensitivity of the assay, and also its specificity which is essential for quality assurance of this extremely sensitive method.

A number of infectious diseases such as hepatitis, tuberculosis, and AIDS currently can be diagnosed by such methods as well as inherited disorders such as fragile X syndrome, muscular dystrophy and the thalassaemias. Two such methods are illustrated in this publication: the diagnosis of hepatitis C using reverse transcriptase PCR followed by detection of the product by a ^{32}P labelled hybridization assay and the detection of tandem repeats for the diagnosis of thalassaemias again using PCR with one of the primers being labelled with 32P.

It is hoped that this publication will provide the basic practical reference protocols for RIA and molecular biology laboratories in the future.

Chapter 1

TECHNIQUES FOR THE RADIOACTIVE DETERMINATION OF STEROID RECEPTOR CONTENT OF BREAST CANCER

R.E. Leake
Biochemistry Department,
Glasgow University,
Glasgow, United Kingdom

Based on the methodology published in *Steroid Hormones: A Practical Approach* (B. Green & R.E. Leake, Eds), Oxford University Press, 1987.

1.1. OESTROGEN AND PROGESTERONE RECEPTOR ASSAYS

The standard assay procedure for breast cancer biopsies is as follows:

1.1.1. Tissue fractionation

A section of tumour is taken from the area adjacent to that taken for pathological examination. For full Scatchard analysis, 150–200 mg of tissue is required (rather less can be used if the assay is to be a simple 'two-point' assay or an Enzyme-immunoassay). All adhering fat, together with any obviously necrotic tissue, should be removed. The tissue is then homogenised at a concentration of 50 mg/mL in HEPES-EDTA BUFFER (2O mM HEPES, 1.5mM EDTA, pH7.4) made freshly (i:e. on the day of assay) 0.25 mM in dithiothreitol (DTT). This is HED buffer. Initial homogenisation is achieved by 2 × 10 second bursts at a setting of 150 on an Ultra-Turrax homogeniser (Model TP 18/2) — this is a mechanical homogeniser with rotating blades, other similar homogenisers will work equally well but it is essential to keep the homogenate cool. Finer homogenisation is achieved by transferring this initial homogenate into a glass/glass homogeniser (Kontes Duall). **Again it is essential to keep the homogenate cool.** Receptor is rapidly degraded in homogenised tissue at 8°C or above. Ensure that all the tumour is reduced to an even, fine suspension.

The homogenate is then centrifuged at 4°C at 5000g for 5 min to yield a crude cytosol supernatant and a 'nuclear' pellet. Both supernatant and pellet are retained. The pellet is washed once with 0.9% buffered NaCl (10 mM HEPES, 0. 15 M NaCl, pH7.4) and then resuspended in 0.9% buffered NaCl, using the same volume as that used for HED in the original homogenisation. The glass/glass homogeniser should be used to ensure even resuspension of the nuclear pellet. A purer nuclear pellet can be obtained by centrifuging at 100 000g but this is not normally required for simple oestrogen receptor determination.

Tissue may alternatively be homogenised in a micro-dismembrator. For this, the tissue should be cut into small pieces then all the pieces frozen in liquid nitrogen. The homogenisation vessel and the steel ball bearing should also be cooled in liquid nitrogen before the tissue and ball bearing are combined in the vessel. Micro-dismembrate in 10 sec. bursts until the tumour is reduced to a fine powder. This powder can then be used for the cytosol assay below.

1.1.2. Oestrogen receptor assay

1.1.2.1. Preparation of ^{3}H-oestradiol-17 solutions

Three stock solutions are prepared from the Amersham supply of ^{3}H-oestradiol-17. One is at 10^{-7} M, another at 5×10^{-7} M and a third at 5×10^{-7} M but also containing 5×10^{-5} M diethylstilbestrol 9DES) as competitor. These stock solutions are stored in absolute alcohol at –20°C. To prepare working solutions, take 10 small glass stock bottles and label them 1-10. To bottles 1-4, add 8, 12, 20 and 30 µL of the 10^{-7} Mstock. To bottles 5–7, add 12, 16 and 24 µL respectively of the 5×10^{-7} M stock, and to bottles 8-10 add 12, 16 and 24 µL of the 5×10^{-7} oestradiol + 5×10^{-5} M DES. Make the volume of ethanol in each stock bottle up to 30 µL and add 970 µL of HED buffer. These working solutions can be stored at 4°C for a **maximum** of one week. The accuracy of preparation of each batch of working solutions should be checked by measuring the radioactive content. The initial commercial supply should also be checked for purity and, if necessary, re-purified prior to use.

1.1.2.2. Assay of receptors

It is possible to assay receptor content only in the cytosol (soluble) fraction. However, to get most information it is a good idea to assay receptor content of both the soluble and nuclear fractions. The preliminary steps are the same for both the cytosol and pellet fractions of the tissue.

Fifty µL of each of the oestradiol solutions prepared as above is mixed with 150 µL aliquots of cytosol or nuclear suspension in a polystyrene tube (check steroids do not stick to the chosen tubes–coat with protein such as immunoglobulin if there is any problem). This gives final ^{3}H–oestradiol concentrations of 2, 3, 5, 7.5, 15, 20 and 30×10^{-10} M. Tubes 8–10 will each give a measure of non-specific binding and the final calculation of specific binding will be achieved by taking an average of this estimate of non-specific binding (see calculation of results). All tubes are incubated at 4°C for 18 h (preferred method) or 20°C for 2 h. A set of control tubes for the cytosol assay is also set up (to give totals and blanks) each time a new batch of oestradiol solutions is made up. This set contains, in addition to steroid as above, 150 µL of HED in place of cytosol.

After incubation, unbound steroid is removed in order to ascertain how much steroid has been bound by receptor. This is achieved by different methods for the cytosol and nuclear fractions.

1.1.2.3. Cytosol fraction

900 µL HEPES-EDTA buffer is added to each tube, and the tubes mixed. 200 µL aliquots are removed from the control tubes at this point into scintillation vials to act as a measure of the total available activity. 500 µL (or 400 µL in the control tubes) of dextran-coated charcoal solution (DCC), (0.15 % w/v Norit A charcoal, 0.0015% w/v dextran T70 in 0.25 M sucrose, 1.5 mM EDTA, 20 mM HEPES (pH7.4)) is added and tubes mixed. Charcoal treatment is continued at 0°C for 15min. with periodic mixing. At the end of this time, the charcoal is pelleted by centrifugation at 1000g and 4°C for 5 min. 1 mL aliquots of each supernatant are transferred to scintillation vials, 10 mL Triton-toluene scintillant (1400 mL toluene/PPO (5g/L)/POPOP (0.24g/L):600 mL Triton-X100:200 mL absolute alcohol) added and vials counted - if Ecoscint is available, then 4 mL of this will replace 10 mL of Triton-toluene.

1.1.2.4. Nuclear fractions

100 µL aliquots from each tube are added to 5 mL aliquots of 0.9% buffered NaCl **immediately prior** to pouring onto a pre-wetted Whatman GF/C filter held in a Millipore filter apparatus. The tube which had contained the saline plus nuclear suspension is washed out with 5 mL saline, and this poured also onto the filter. The chimney of the apparatus is then washed with 3 times 4 aliquots of saline, then removed and the very edge of the filter washed with 3 mL saline. The filters are placed in scintillation vials at 60°C overnight to dry. (A separate set of control counts may be obtained by putting dry filters into scintillation vials and adding 50 µL aliquots of each incubation mixture containing labelled steroid to give a separate measure of total available activity - these filters should be re-dried as above). 10 mL toluene/PPO(5g/L) scintillant (or 4 mL Ecoscint) is then added to each vial and the vials counted.

1.1.3. Progesterone receptor assay

When assaying for progesterone receptor, it is essential to include **15% (v/v) glycerol** in the initial homogenisation HED buffer used to generate the cytosol and nuclear fractions.

1.1.3.1. Preparation of ^{3}H-ORG 2058 solutions

There are several synthetic ligands which can be used for assay of progesterone receptor (e.g. R 5020). In our hands, the most consistent results have been obtained with ORG 2058.

Stock ^{3}H-ORG 2058 is prepared from the Amersham supply at 5×10^{-7} M and stored in absolute alcohol at –20°C. Aliquots of this are removed and added to separate glass stock bottles as follows: To bottles 1–7, add 4, 6, 8, 16, 24, 32 and 40 µL of 5×10^{-7} M stock. To bottles 8-10, first add 24, 32 and 40 µL of 5×10^{-5} M unlabelled ORG 2058 (in ethanol), then evaporate off the ethanol and add respectively 24, 32 and 40 µL of ^{3}H-ORG 2058 (5×10^{-7} M). Ensure that the unlabelled material is fully re-dissolved. Make the volume of ethanol in each stock bottle up to 40 µl. Add 960 µL HED buffer containing 10% glycerol to each bottle. These working solutions should be stored at 4°C for a maximum of 7 days.

1.1.3.2. Assay of receptors

As with the assay for the oestradiol receptor, 50 µL aliquots of the working solutions are added to 150 µL aliquots of the tissue fraction (cytosol or nuclear suspension) or buffer (for calculation of total counts available). Tubes are incubated for 18 h at 4°C or 2 h at 20°C. After incubation, unbound steroid is removed as described for the oestrogen receptor assay, except that the HEPES-EDTA is made 15% (v/v) in glycerol.

Calculation of Results

Calculation of results is normally done by computer (see, for example the chapter by Leake, Cowan and Eason). However, it is possible to calculate the results using a pocket calculator as follows:

Assume that the number of non-specifically bound counts is proportional to the number added. Then plot the numbers of non-specific counts measured in tubes 8–10 against concentration of added 'hot' steroid. You can now read off the value for non-specific counts at all other concentrations of added 'hot'.

For cytosolic oestrogen **and** progesterone receptors

(1) Multiply totals by 5.5.

(2) Multiply counts from all assay vials by 1.6.

(3) Subtract calculated 'non-specific' bound counts from total bound counts in each 'hot' vial. This gives the specific BOUND counts.

(4) Subtract BOUND counts from totals (counts derived from the 200 μL removed from the control (buffer in place of cytosol) tubes prior to the addition of charcoal times 5.5 to correct for volumes). These are the FREE COUNTS.

(5) Divide BOUND by FREE to the B/F ratio.

(6) Divide BOUND by total to give proportion bound.

(7) Multiply proportion bound by concentration of labelled oestradiol or progestin in each tube to give concentration bound in molar units.

(8) Convert concentration bound to pmolar quantities.

(9) Plot B/F against BOUND (pmolar).

(10) Fit a straight line using a minimum of five points for best fit.

(11) The slope of the line is $-1/K_d$ (the dissociation constant) for the binding (see the Appendix to Chapter 1 for details).

(12) Divide the intercept on the X axis by 0.75 (to correct for the dilution of cytosol when mixing 150 μL of cytosol with 50 μL of labelled steroid). This gives total receptor concentration in fmol/mL cytosol.

(13) Determine the protein concentration of the cytosol in mg/mL and divide the receptor concentration found in step 12 by this value to give receptor concentration in fmol/mg cytosol protein.

1.1.3.3. Nuclear receptors

(1) Using the values from tubes 8–10, draw a graph as before to calculate non-specific binding at each concentration. Calculate specifically BOUND counts.

(2) Subtract BOUND counts from totals (counts derived from 50 μL aliquots of the original incubation mixture placed on filter discs times 2 . This gives FREE COUNTS.

(3) Proceed as for cytosol receptors except that the DNA concentration of the nuclear suspension should be determined and the final result expressed as fmol/mg DNA.

Appendix to Chapter 1

COMPUTER PROGRAM FOR SCATCHARD ANALYSIS OF PROTEIN: LIGAND INTERACTION – USE FOR DETERMINATION OF SOLUBLE AND NUCLEAR STEROID RECEPTOR CONCENTRATIONS

R. Leake, S. Cowan, R. Eason

Steroid receptor concentration may be determined routinely in biopsy samples of breast and endometrial cancer by the competition method described in Chapter 2. This method yields data for both the soluble and nuclear fractions of the tissue. The data are usually subject to Scatchard [1] analysis. This Appendix describes a computer program written initially for a PDP-11. It has been modified for use with IBM, Apple Macintosh and BBC microcomputers. The nature of the correction for competition is described and examples of the printout are given. The program is flexible and its use for different receptors is explained. The program can be readily adapted to other assays in which Scatchard analysis is appropriate.

The basis for Scatchard analysis

Scatchard analysis can be applied to any saturation assay using a labelled ligand. For the interaction of hormone with receptor protein, let us assume that: H represents free steroid; R represents free receptor, and HR represents hormone–receptor complex then:

$$H + R = HR \qquad (1a)$$

At equilibrium the concentration of complex is B; the concentration of free hormone is F, and the concentration of free receptor is R_o–B, where R_o is total receptor concentration

$$\begin{matrix} H + R = HR \\ F[R_o–B]\ B \end{matrix} \qquad (1b)$$

The dissociation constant K_d can then be defined from Equation 1b as

$$K_d = \frac{F\,[Ro - B]}{B}$$

This can be re-arranged to give:

$$B/F = R_o/K_d - B/K_d$$

As seen in Fig. 1, plotting B/F against B should give a straight line of slope $-1/K_d$. R_o should be given by the intercept on the X axis. This derivation assumes:

(i) H:R = 1:1.
(ii) HR only breaks down to H and R.
(iii) H binds only to R.

Of these assumptions, (iii) is certainly incorrect. Hence the need to correct for lower affinity binding.

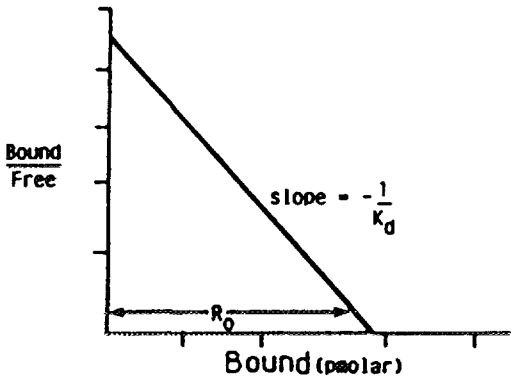


Fig 1 Standard Scatchard analysis of binding data. Concentration of bound steroid is shown on the X axis and the ratio of bound free steroid is shown on the y-axis

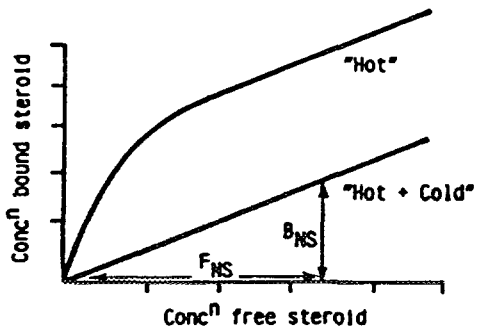


Fig 2 'Typical' binding data If binding is measured at increasing concentrations of free steroid, then the plot shown for the 'hot' data (assay in the presence of labelled steroid alone) would reflect one high affinity binding entity in the presence of many low affinity binding molecules It is implicit in this diagram that the low affinity binding sites are essential non-saturable over the concentration range of ligand required to saturate the high affinity site The specifically bound steroid (B_s) is indicated by the difference between the 'Hot' and 'Hot + Cold' lines

To achieve this, we must remember that a saturation assay of a single, high affinity binding component, present in conjunction with several other lower affinity components, would be expected to appear as the 'Hot' line in Fig. 2. The various parameters (concentrations) involved in the calculations are:

B_s specifically bound steroid
B_T total bound steroid
B_{NS} non-specifically bound steroid
F free steroid (as measured in tubes 1–7 of the assay)
F_{NS} free steroid (measured in competition tubes 8–10 of the assay).

Remember that the assay involves 20 incubation tubes of which 1–7 contain increasing concentrations of 'hot' steroid and tubes 8–10 contain the top three concentrations of labelled steroid together with excess unlabelled steroid as competitor.

```
**Cytosol data section**
 Current values of the constants
    Specific activity of ligand : 101.0 [in Ci/mmol (3.74 × 10³ GBq/mmol)]
    Efficiency of counting      : 0 32
    Dilution factor             : 5.0
    dpm–Curies*                 : 0.222E + 13
    Added multiplier            : 1.0
    Bound multiplier            : 2.0
    *For GBq it is 6E + 10
```

Fig 3 Memory input for standard assay conditions

Assuming then, that the lower affinity components are essentially non-saturable over the ligand concentration range studied, the plot of counts bound in the presence of competitor (hot + cold) against increasing concentration of labelled steroid should be a straight line of constant slope ($=B_{NS}/F_{NS}$) (Fig. 2) over the concentration range studied. Thus, this ratio should be a constant value at all concentrations of free steroid. Therefore, if B_{NS}/F_{NS} is determined at the top three ligand concentrations (tubes 8–10) and the average taken, then we get $[(B_{NS}/F_{NS})$av.].

The non-specific binding at any one point may then be computed as $B_{NS} = F \times (B_{NS}/F_{NS})$av. Now $B_S = B_T - B_{NS}$, for each of the seven points. Although this method of correction works well in practice, it still fails to incorporate a measure of the true concentration of 'available' steroid. Note that this procedure is only applicable where the lower affinity binding is effectively non-saturable. Where this is not the case, additional corrections must be introduced [2].

We can now calcualte B_S/F for each point. However, we need some standard information to convert 'bound' (c.p.m.) to 'bound' (mol/L). For any particular set of data, we need first to check these standard parameters (Fig. 3). Figs 3–6 refer to what is seen on the VDU of the computer. Where a value is followed by E+13 or E–3, this indicates the value is $\times 10^{13}$ or $\times 10^{-3}$. E0 indicates that the value is as stated (i.e. $\times 10^{0}$). Specific activity, of course, refers to the particular batch of [^{3}H]ligand. Counter efficiency should be checked regularly as should any 'built-in' conversion of c.p.m. to d.p.m. 'Dilution factor' converts the incubation volume to 1 mL. 'Added' and 'Bound' multipliers refer to relevant dilutions involved in aliquoting samples for counting. 'Added' refers to the aliquot taken to determine 'total' counts added to the incubation (the volume taken to determine totals is, in this method, exactly the same as that added to the incubation tubes and so the appropriate multiplier is × 1). 'Bound' refers to the aliquot taken to determine 'bound' counts (and is 200 µL out of 400 µl, i.e. the multiplier is 2).

The next step is to check the existing 'added c.p.m.' for each assay. If the same batch of working standard steroid solutions was used for the previous assay, then the added c.p.m. should be the same. If not, then new added c.p.m. are listed separately (values 1–7 and 8–10, respectively; see Fig. 4).

Once the particular assay has been identified (e.g. type in patient initials, hospital name, patient number and date of assay), the non-specifically bound values will be asked for. The programme is flexible and may be adapted for use with one, two or three 'hot + cold' tubes. However, where less than three tubes are used, the condition(s) selected should be that with the highest 'hot' steroid concentrations(s). If there are three non-specifically bound (NSB) tubes,

```
Current values of first 7 added c.p.m.
Value  1 = 2688.8
Value  2 = 4953.5
Value  3 = 6599.7
Value  4 = 10016.0
Value  5 = 21095.8
Value  6 = 28007.7
Value  7 = 41749.3

After first 7 added c.p.m. values? Y/N : N

Current values of last 3 added cpm
Value  8 = 20726.5
Value  9 = 27916.8
Value 10 = 40653.8

After last 3 added c p.m.? Y/N : N
```

Fig 4 Total 'counts per minute' (c p m) Added at each concentration

```
[Bound]   B/F
1.343E-10  2.602E0
2.051E-10  2 724E0
3.010E-10  1 945E0
3.414E-10  9.837E-1
3.885E-10  3.690E-1
4 046E-10  2.686E-1
4.099E-10  1.685E-1

Total specific receptors: 5.465E-10 moles/l
```

Fig 5 Data output for a single patient.

```
The points used to derive the data were 2 3 4 5 6 7
Total receptor concentration: 5.655E-10 moles/l
Dissociation constant: 7.657E-11M
Linear correlation coefficient: -9.861E-1
```

Fig 6 Scatachard analysis-derived results for a single patient

insert bound values from tubes 8–10. If competition was only done at the highest 'hot' concentration, enter the value as NSB value 10. The seven bound values are then entered and the average correction factor for non-specific binding (from tubes 8–10) will appear. Should the volume of soluble fraction (cytosol) added to the incubation tube not be 75% of the final incubation volume, then this appropriate correction should be made at this stage.

The [Bound] and Bound/Free values for each tube are then displayed (Fig. 5), as is the value for the receptor concentration (Total Specific Receptors) calculated simply from the competition data at 30×10^{-10} M [^{3}H]estradiol (i.e. tubes 7 and 10 – the highest concentration of

labelled ligand). This is expressed in mol/L cytosol and, therefore, is corrected for the difference between added cytosol and final incubation volume.

The data in Fig. 5 can be converted to a graphical plot. Appropriate minimum and maximum values for the X axis ([Bound] in pmol/L) and the y axis (B_S/F) are selected and entered. The best fit straight line is then calculated using all seven points, each incorporating the non-specific binding correction factor. Should there be one or two clear 'outliers', these can be discarded and the line redrawn. [Note, the discarded point(s) will still appear on the plot but will not have been taken into account in constructing the new line.] The receptor concentration (in mol/L cytosol) and K_d value, together with the correlation coefficient related to this line can then be printed out (Fig. 6).

The data from the nuclear incubation can now be analysed by putting in the appropriate constants, multiplication factors and experimental data.

This program can be equally well used if both soluble estrogen and soluble progesterone receptor assays are being carried out.

Some counter manufacturers (e.g. Packard) supply programs to generate Scatchard plots. Check that any particular program has an adequate correction mechanism for the non-specific binding.

Application of Scatchard analysis by routine biochemistry laboratories to assays of different steroid-binding proteins in many different tissues has greatly increased. A flexible computer program which can accommodate all the variables involved has, therefore, been developed. Laboratories with access to a PDP 11/34 with graphics terminal or appropriate IBM, Apple Macintosh or BBC microcomputers can now use this program for any steroid receptor assay which follows the general outlines described here. One of the past problems in reporting receptor data, particularly in a clinical context, has been the variable approach to data analysis [3]. Use of this program should make comparison of data from different laboratories more realistic. Alternatives to Scatchard methodology are also available for analysis of the binding data [4].

ACKNOWLEDGEMENTS

We are most grateful to several colleagues for valuable discussion. In particular we thank Ton Koenders for regular advice.

REFERENCES

[1] SCATCHARD, G., Ann. N.Y. Acad. Sci. **51** (1969) 660.
[2] BRAUNSBERG, H., HAMMOND, K.D. J. Steroid Biochem. **11** (1979) 1561.
[3] CHAMNESS, G.C., MCGUIRE, W.L., Steroids **26** (1975) 538.
[4] WILSON, D.W., RICHARDS, G., NICHOLSON, R.I., GRIFFITHS, K., Br.J. Cancer **50** (1984) 493.

Chapter 2

OESTROGEN AND PROGESTERONE RECEPTOR ASSAYS

R.E. Leake
Biochemistry Department,
Glasgow University,
Glasgow, United Kingdom

A standard procedure for steroid receptor assays:

Steroid receptors are inherently unstable above 8°C and **all solutions** must be kept on ice, unless otherwise stated.

Standard (quality control material) ER material is made up from recombinant material. Powder should be dissolved in 5 mL of HED buffer – remember to vent you vial before injecting buffer.

Preparation of stock solutions: Three stock solutions are prepared from the Amersham supply by diluting into absolute ethanol. One is 10^{-7} M, another 5×10^{-7} M and a third 5×10^{-7} M labelled oestradiol + 5×10^{-5} M unlabelled diethylstilbestrol (as competitor). These absolute ethanol solutions are stable at –20°C. To prepare your working solutions, take 10 small glass stock bottles and label them 1–10. To bottles 1–4, add 8, 12, 20 & 30 µL of the 10^{-7} M stock. To bottles 5–7, add 12, 16 & 24 µL of the 5×10^{-7} M stock, and to bottles 8–10 add 12, 16 & 24 µL of 5×10^{-7} M label + 5×10^{-5} M DES. Make the volume of ethanol in each stock bottle up to 30 µL and add 970 µL HED buffer. These working solutions can be stored at 4°C for a **maximum** of one week. The accuracy of preparation of each batch of working solutions should be checked by measuring the radioactive content of a small aliquote.

Assay of Receptor content

Fifty µL of each of the oestradiol stock solutions (prepared as above) is mixed with 150 µL aliquotes of the cytosol (quality control solution) in an RT30 (polystyrene) tube. This gives final concentrations of labelled oestradiol of 2, 3, 5, 7.5, 15, 20 & 30 $\times 10^{-10}$ M. Tubes 8–10 are included to measure the non–specific binding. All tubes are incubated at 4°C for 18 h. A set of control tubes, containing steroid + buffer in place of cytosol, should be set up to determine total counts (and also blank values).

Removal of unbound steroid

900 µL of HEPES–EDTA buffer is added to each tube and the contents mixed. 200 µL aliquots are removed from the **control** tubes only at this point into scintillation vials to give total counts available. 500 µL (or 400 for the control tubes) of dextran-coated charcoal solution is then added to each tube. Tubes are left to stand for 15 min. on ice with periodic mixing. At the end of this time, the charcoal is pelletted by centrifugation at 1000 × g and 4°C for 5 min. 1 mL aliquots of each supernatant are transferred to scintillation vials and counted in 4 mL Ecoscint.

Chapter 3

STEROID RECEPTOR ASSAYS IN WHOLE CELLS

R.E. Leake
Biochemistry Department,
Glasgow University,
Glasgow, United Kingdom

Note: If the attached methodology does not work, for any reason, the fall-back position is to incubate the cells (grown on cover slips in multi-well plates) in labelled steroid (concentration about 20% above K_d e.g. try 10^{-9} M for oestradiol) in triplicate. Run parallel triplicate incubations with 10^{-9} M label + 10^{-7} M 'cold' material (DES for oestrogen receptor assay).

Extract both lots with alcohol and add to scientillant to count directly. Calculate receptor content from specific counts. This method is not ideal since you are not saturating the receptor yet you will measure some type II receptors. However, it is OK if you are looking for changes. Note you should measure both DNA content and cell number of the cells in each well in order to express your results per unit DNA or per cell. If 100% recovery of cells is not possible, then grow parallel triplicate cultures purely to get average cell number/DNA content.

3.1. [^{3}H]-STEROID SOLUTIONS FOR ER AND PR ASSAYS

3.1.1. Preparation of [^{3}H]-E_2 solutions

Stock [^{3}H]-E_2 was prepared from the Amersham supply at 5×10^{-7} M and stored in ethanol at –20°C. A stock solution of unlabelled DES at 1.25 ti Mes 10^{-4} M was made up in ethanol and stored at –20°C. Two radiolabelled assay solutions were prepared as follows:

(a) 24 µL DES was aliquoted into a glass bottle and the ethanol evaporated under a gentle stream of N_2.

24 µL [^{3}H]-E_2 was then added and the volume of ethanol made up to 30 µl.

970 µL HED buffer was added, making the total volume 1 mL.

(b) 24 µL [^{3}H]-E_2 was aliquoted into a glass bottle and the volume of ethanol was made up to 30 µl.

970 µL HED buffer was added making the total volume 1 mL.

The final concentration of [^{3}H]-E_2 in the exchange assay was 30×10^{-10} M and DES was present in 250-fold excess.

3.1.2. Preparation of [^{3}H]-ORG 2058 solutions

Stock [^{3}H]-ORG 2058 was prepared from the Amersham supply at 5×10^{-7} M and stored in ethanol at –20°C. Unlabelled ORG 2058 was made up at a concentration of 5×10^{-5} M and stored at –20°C.

Two radiolabelled assay solutions were made up as follows:

(a) 40 µL unlabelled ORG 2058 was aliquoted into a small glass bottle and the ethanol evaporated under a gentle stream of N_2. 40 µL of labelled ORG 2058 was then added followed by 960 µL HED buffer containing 10% glycerol.

(b) 40 µL labelled ORG 2058 was aliquoted into a small glass bottle. 960 µL HED buffer containing 10% glycerol was added.

Final concentration of [^{3}H]-ORG 2058 in the exchange assay was 50×10^{-10} M and unlabelled ORG 2058 was in 100-fold excess.

3.2. WHOLE CELL [^{3}H]-STEROID EXCHANGE ASSAY FOR ER AND PR

Growth medium was discarded and the cell monolayers washed twice in ice cold PBS. Cells were scraped into PBS and pelleted by centrifugation at 1,000 rpm for 5 min at 4°C. The cells were resuspended in HED buffer containing 1.5 mM $MgCl_2$ and 10% glycerol, pH7.4.

150 µL cell suspension was incubated with 50 µL (radiolabelled ligand ± excess unlabelled ligand), (1.1. and 1.2.), for 2 hours at ambient temperature. 100 µL aliquots from each tube were then added to 5 mL aliquots of 0.9% (^{w}/v) NaCl immediately prior to pouring onto a pre-wetted Whatman GF/C filter disc (2.5 cm) held in a Millipore filter apparatus. The tube which had contained the saline was washed out with 5 mL saline, and this was poured onto the filter also. The chimney of the apparatus was washed with 3 × 4 mL aliquots of saline, then removed and the very edge of the filter washed with 3 mL saline. The filters were placed in scintillation vials and 4 mL Ecoscint added. Samples were counted in a LKB liquid scintillation counter with a counting efficiency of 35%.

3.3. CYTOSOL ASSAY FOR ER-DEXTRAN-COATED CHARCOAL METHOD

Growth medium was discarded and the cell monolayers washed twice in ice cold PBS. The cells were scraped into PBS from the plastic tissue culture flask and pelletted by centrifugation at 1 1000 rpm for 5 min at 4°C. The cells were resuspended in HED buffer, pH7.4 and sonicated for 3–5 min at 4°C in a Cole-Palmer ultrasonic cleaning bath (fixed, gentle setting). Cell debris was pelleted by centrifugation at 2 500 rpm for 5 min at 4°C. The supernatant was collected and tested for ER by [^{3}H]-E_2 exchange assay using dextrancoated charcoal to remove unbound steroid.

150 µL cell cytosol was incubated with 50 µL ([^{3}H]-E_2 ± 250-fold excess DES), (1.1.), for 18 h at 4°C. 200 µL dextran-coated charcoal solution (0.5% (^{w}/v) Norit A charcoal, 0.005% (^{w}/v) dextran T-70 in 10% (^{v}/v) glycerol, 1.5 mM EDTA, 20 mM Hepes, pH7.4) was added to each tube and the tubes mixed. Charcoal treatment was continued on ice for 15 min with periodic mixing. At the end of this time, the charcoal was pelleted by centrifugation at 2000 rpm for 5 min at 4°C. Aliquots of 200µL from each supernatant were transferred to scintillation vials, 4 mL of Ecoscint added and the vials counted in an LKB scintillation counter with a counting efficiency of 35%.

3.4. CYTOSOL ASSAY FOR ER-ENZYME IMMUNOASSAY METHOD

Cell cytosol was prepared as described in Section 3. Measurement of ER by the enzyme immunoassay (EIA) was exactly as described in the manual accompanying the Abbott EIA kit.

3.5. TISSUE COLLECTION AND STORAGE

Oestrogen receptors, like other receptors, are sensitive to protease degradation and tissue must be processed fresh or stored appropriately. If an estrogen receptor assay cannot be carried out on fresh tissue, then the receptor content is stable for several weeks if the tissue is stored in liquid nitrogen, in sucrose-glycerol buffer at -20^0C or in lyophilized form.

(i) Collect tissue from the experimental animal, or operating theatre in the case of clinical tissue, and section it into small pieces (1–2 cm^3).

(ii) Drop the sections directly into liquid nitrogen or immerse them in 0.25 M sucrose, 1.5 mM $MgCl_2$. 10 mM Hepes, pH7.4 made 50% (v/v) in glycerol.

(iii) Store tissue in sucrose–glycerol medium at –20°C, under which conditions it should not freeze. Thus there is little or no freeze–thaw damage when tissue is recovered for assay.

(iv) To prepare tissue prior to assay, remove it from the sucrose–glycerol and rehydrate it for 15 min in isotonic saline.

Chapter 4

MODIFIED BURTON METHOD FOR ASSAY OF DNA CONTENT OF TISSUE

R.E. Leake
Biocchemistry Department,
Glasgow University,
Glasgow, United Kingdom

(1) Set up a standard curve by, firstly, dissolving highly polymerised calf thymus DNA (Sigma Grade V), at 1 mg/mL, in 15 mM NaCl/1.5 mM Na_3 citrate (dissolve overnight on a stirrer in the cold room). Make a standard curve by aliquoting into concial centrifuge tubes sufficient DNA to give final concentrations to cover the range 0–300 μg/mL. Each tube should be made up to 1 mL with distilled water.

(2) Thaw out and pellet the unknown samples by centrifuging them (800 × g for 10 min. At 4°C). Pour off the supernatant and resuspend the pellet in 1 mL bovine serum albumin (BSA – a solution containing 0.1% w/v BSA in glass distilled water). 1 mL of this BSA solution is also added to each of the tubes containing DNA for the standard curve.

(3) To each tube, add the appropriate volume of 2.5 M perchloric acid (PCA) to give a final concentration of 0.25 M PCA. Leave to stand for 15 min. on ice, then centrifuge for 5 min. at 800 × g. Thoroughly resuspend the pellet in 1 mL of 0.3 M PCA and heat at 90°C for 30 min. Cool and centrifuge for 5 min. at 800 × g.

(4) Make up diphenylamine reagent by dissolving 1.5g of recrystallised diphenylamine in 100 mL glacial acetic acid, then add 1.5 mL concentrated sulphuric acid. This mixture may be stored **in the dark** at room temperature. Immediately prior to use, add acetaldehyde (a 16 mg/mL solution in water) at 0.1 mL/20 mL diphenylamine reagent.

(5) Transfer 250 μL aliquots, in duplicate, from each of the PCA supernatants (step 3) to clean test tubes, then add 500 μL diphenylamine reagent to each. Cover with aluminium foil (you may wish to seal this with parafilm but do not let the parafilm have direct contact with the diphenylamine vapour). Stand in the dark for 16 h. Read the absorbance at 600 nm.

Chapter 5

PROTEIN ASSAY

R.E. Leake
Biochemistry Department,
Glasgow University,
Glasgow, United Kingdom

Stock solutions: Pierce BSA standard – 2 mg/mL
Bio-Rad Protein Dye - concentrate.

Method:

The Bio-Rad dye concentrate is diluted 1 part to 5 parts i.e. 50 mLs to 250 mLs with dH_2O and then filtered through Whatman No.1 filter paper. Remember that some volume is lost in filtering, so make up sufficient.

Dilute the protein standard 1:1 with dH_2O to give a 1 mg/mL solution (final volume 2 mLs). Aliquot 100, 200, 300, 400 and 500 µL into a series of plastic tubes and make up to 1 mL with dH_2O i.e. add 900, 800, 700, 600 and 500 µL dH_2O. Mix each solution carefully. Set up the standard curve by aliquoting 100µL of each standard into a 10 mL tube. Remember to have a dH_2O blank. This gives a standard curve from 0 to 50 µg protein per 100 µL (0, 10, 20, 30, 40 and 50 µg). (You could set up a 5 µg point by taking 50 µL of the 100 µg/mL standard and adding it to 50 µL of dH_2O.)

Meanwhile thaw out the cytosols for protein determination. Mix the cytosols and then remove 50 µL from each cytosol and add to 450 µL of dH_2O. Mix each solution carefully. Instead of assaying the cytosols in duplicate, we assay at two dilutions. For each diluted cytosol, aliquots of 50 µL and 100 µL are taken and added to the glass tubes, 50 µL of dH_2O is added to the former to make the volume up to 100 µl. All the tubes now contain 100 µL of protein solution.

Add 5 mLs of the diluted dye to each tube gently so as to avoid frothing. The solution should not be vortexed. By adding the dye carefully down the inside of the tube, the solutions are mixed without shaking or vortexing. The tubes are left for 5–30 min before reading at 595 nm. Before removing an aliquot for reading, the solution is drawn up and down gently in a pasteur pipette twice.

Plot the standard curve on graph paper, and read off the unknown values. These values are in µg per 100 µL, but the unknowns were from 1/10 and 1/20 dilutions of the original cytosols, so the unknown values have to be multiplied by 100 or 200 to give values in mg/mL cytosol.

Chapter 6

IMMUNOASSAYS IN CLINICAL CHEMISTRY (PRINCIPLES OF IMMUNORADIOMETRIC ASSAYS)

R.S. Chapman
Division of Biochemical Endocrinology,
The Royal Postgraduate Medical School,
Hammersmith Hospital,
London, United Kingdom

The use of antibodies as reagents in clinical chemistry for the quantitation of a wide range of analytes has now become widely established. Initially antibodies were employed in precipitation techniques, usually for the analysis of serum proteins, in solution or in the form of antibody containing gels, e.g. immunoprecipitation, immunodiffusion, and immuno-electrophoresis. Further developments have led to the highly sensitive techniques of radioimmunoassay and recently immunometric assay for the measurement of drugs, tumour markers and hormones.

Indeed, the technique of immunoassay has been arbitrarily subdivided into numerous, often confusing, categories such that novices to the subject are presented with a bewildering list of apparently alternative methodologies. This situation results from the rapid commercialization into 'kit packaging' of the immunoassay technology. Each manufacturer wanting to provide a marketing label to identify its product leading to the appearance of the EMIT (enzyme inhibition technique), ELISA (enzyme linked immunosorbent assay) and FPIA (fluorescence polarisation immunoassay) and many others.

All immunoassays can be regarded as '**structurally specific**', the antibody reacting specifically with elements of the analyte structure to effect quantitative measurement. Consequently, immunoassays measure analytes in **units of mass**, i.e. number of molecules per unit volume. A simple classification system can be used to categorise all structurally specific immunoassays into initially **label** or **no label** techniques depending on the necessity to add a labelled probe, or tracer, to aid the measurement followed by subdivision of the labelled techniques into **Limited reagent methods** and alternatively **excess reagent methods** based upon the fundamental principles of measurement.

In general, those techniques without the addition of a label e.g. immunoprecipitation, immunodiffusion and immunoturbidimetry are the older techniques used for the measurement of serum proteins. These techniques are relatively insensitive, measuring at the g/L. level, and in the case of immunodiffusion are generally slow. Automation coupled with the development of chemistries to enhance precipitation has, however, reduced measurement times to minutes in modern laboratories. Nevertheless these methods have detection limits of the order of 1g/L.

6.1. LIMITED REAGENT METHODS

Limited reagent assays, the best example of which is radioimmunoassay, use antibodies at a limiting or saturable concentration, hence the alternative nomenclature of **saturation assays**. The analyte binding to the specific antibody according to the law of mass action is distributed into compartments of antibody bound and free and as a consequence, providing the specific antibody is at limiting concentration, the fraction of the analyte concentration bound will vary with the

total analyte concentration. The fraction bound may be conveniently monitored by the inclusion of a tracer quantity of labelled analyte, radioisotopically labelled in radioimmunoassay. The fraction of labelled analyte bound, assessed following physical separation of the bound and free fractions using an appropriate **separation system** will vary inversely with respect to the total analyte concentration. Quantitation can be made by comparison with a set of known analyte calibrators, or standards, set up under identical reaction conditions.

Limited reagent techniques have been devised which do not require a separation system e.g. EMIT (enzyme inhibition technique), FPIA (fluorescence polarisation immunoassay), FETIA (fluorescence excitation transfer immunoassay), SLFIA (substrate labelled fluoroimmunoassay), PGLIA (prosthetic group labelled immunoassay) and ARIS (apoenzyme reactivated immunoassay). These methods are all classified as limited reagent and due to the compromise of non-separation tend to be of limiting sensitivity in comparison with classical radioimmunoassay methodology. Nevertheless these methods are rapid and have found widespread usage in **therapeutic drug monitoring**, with a minimum detection limit of the order of about 2.5 nmol/L. However in the majority of applications drugs are measurable in the concentration range of umol/L.

Conventional radioimmunoassay uses a **second antibody separation technique** to separate the bound and free fractions. Here, the first antibody, specific antibody, reacts with the analyte forming the antibody bound and free fractions prior to the addition of a second antibody specific for the species of the first antibody and at carefully controlled concentration to effect precipitation of the first antibody. If the first antibody was raised in a rabbit then the second antibody would generally be a donkey anti rabbit Ig g serum. Following centrifugation and decantation of bound fraction remains separated and ready for radioactivity counting. This technique is slow of the order of 1–3 days with an additional incubation required for the second antibody stage.

Speed was not the only disadvantage of radioimmunoassay. The separation systems employed were never able to completely isolate the two fractions. In the second antibody systems there was always some of the free fraction trapped in the bound precipitation pellet leading to **misclassification errors** with the result that standard calibration curves never met the abscissa. This led to poor precision of measurement.

The development of **solid phase radioimmunoassay** led to improved sensitivity and precision by minimising the misclassification errors of the separation system. Various solid phase systems were developed. Antibodies were either covalently linked or physically adsorbed to microparticulate polymers such as cellulose, agarose, polyacrylamide, polystyrene and polymethacrylate. Alternatively beads, discs, fins, stars or the surface of polystyrene test tubes were used. In all cases because the antibody was already insolubilised the separation system was simplified, since no further reagents were required, and the assay incubation times were shortened. The misclassification errors were minimised because the contaminating free fraction could be effectively removed by successive washings of the solid phase.

Disadvantages of the solid phase radioimmunoassay resulted from the need to prepare the solid phase in advance often using complex chemistries which were expensive in antibody. The washing requirements, crucial to improved sensitivity and precision, were also tedious and disliked by many technicians particularly if multiple centrifugation was performed. Some particulate solid phase materials were designed to be paramagnetic **(magnetic solid phase)** so that separation was achieved by the application of a magnetic field removing the need for centrifugation.

6.2. EXCESS REAGENT METHODS

In excess reagent methods, the **immunometric methods**, the antibody is used in relative excess effectively forcing the reactions to equilibrium and therefore shortening the incubation times. Immunometric assays are therefore more rapid than their radioimmunoassay counterparts. The specific antibody, not the analyte, is labelled int he Immunometric assay. Calibration curves are therefore exponential since as the concentration of analyte increases more binding occurs and the more label signal is detected, within the constraint of the amount of reagents used. This is in contrast to radioimmunoassay where the calibration curves are inhibition curves, the fraction bound decreasing as the concentration of the analyte increases.

The earliest assays of this type of **immunoradiometric assays** used a radioisotopically labelled polyclonal antibody in excess, with a solid phase antigen **(immunoadsorbent)** preparation used to separate unreacted labelled antibody. The supernatant labelled antibody antigen complex was aspirated and counted following centrifugation of the immunoadsorbent. This assay was unfortunately also prone to the misclassification errors of the radioimmunoassay. A further disadvantage was the technical difficulty in preparation of labelled antibodies, a procedure which was also very time consuming.

A variant of this assay, the **two site immunoradiometric assay**, effectively addressed the misclassification problem by using twin analyte specific antibodies, one labelled with radioiodine and the other linked to a solid phase support. Two incubations were also used. In the primary incubation the biological sample of the analyte, usually serum or plasma, was incubated with the solid phase antibody alone, effectively extracting the analyte from the sample. Washing then removed non reactive serum components prior to the addition of the secondary specific antibody labelled with radioiodine. This antibody reacted with alternative antibody binding sites on the solid phase complex to form a solid phase antibody-analyte labelled antibody complex or sandwich. These assays are sometimes referred to as **sandwich assays**. A further washing step separated the complex from unreacted labelled antibody.

Although misclassification was minimised this was achieved at the expense of further tedious washing steps. Also, since more then one antibody determinant, **epitope**, was required for complex formation this assay was restricted to peptide, polypeptide and protein compounds. The simpler compounds thyroid and steroid hormones and drugs could only be assayed by the slower radioimmunoassay.

A further variant incubated the labelled antibody and analyte together as the primary incubation to take advantage of the more favourable reaction kinetics in solution prior to the delayed addition of the solid phase antibody, the **delayed addition two site immunoradiometric assay**. The extra washing step was removed in this variant and overall the assay was faster but the advantage of the removal of serum interferences was lost.

The major advantages of the two site immunoradiometric assays were **improved sensitivity and precision** and, as a consequence of the latter, a **wider working range** of precision than available in comparative radioimmunoassays.

6.3. MONOCLONAL ANTIBODIES

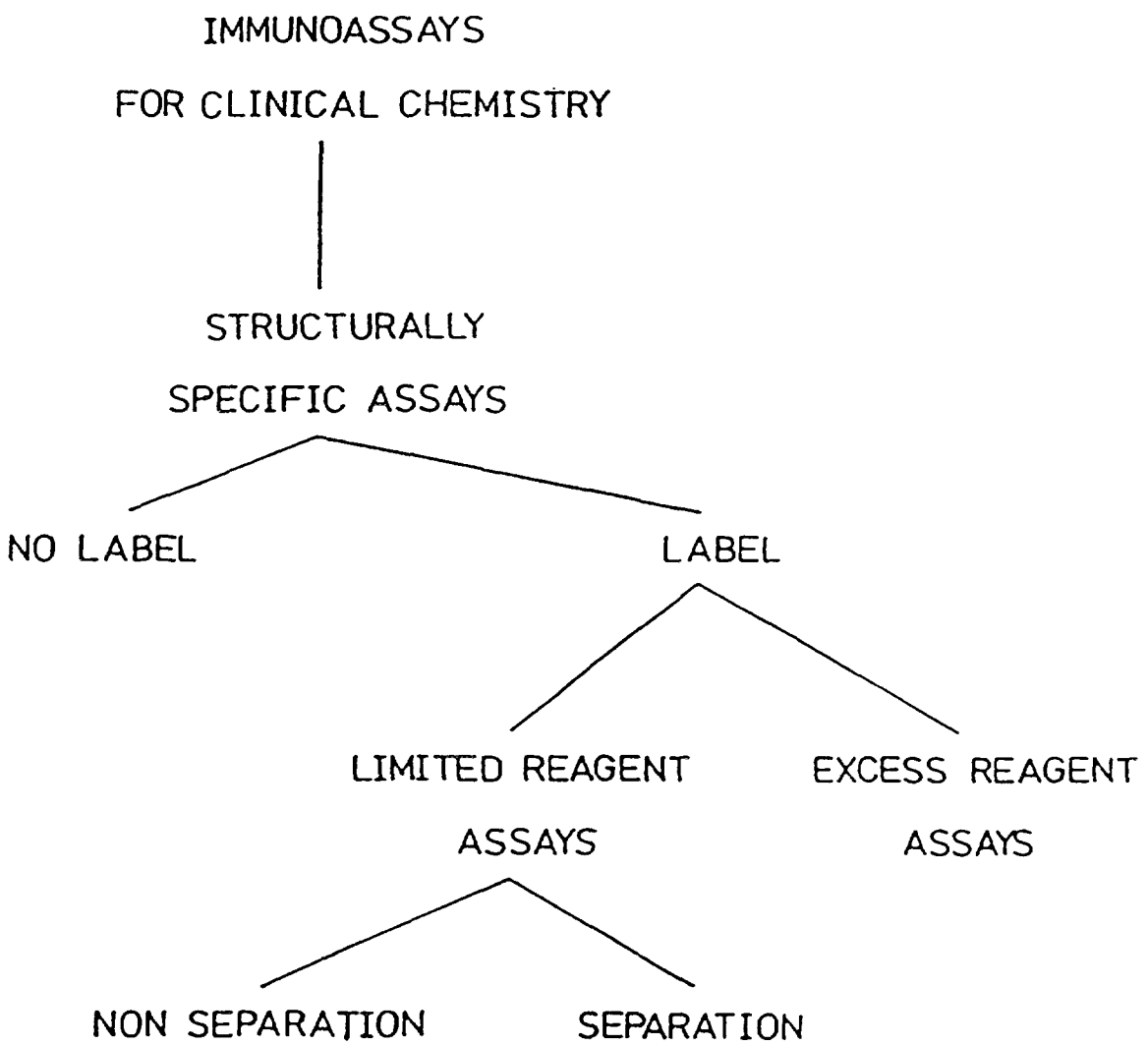
The minimum detection limit of the two site assay was restricted by the appearance of a 'bland' binding in the absence of analyte. The degree of 'blank' was a function of the quality of

labelled antibody, the non specific binding qualities of the solid phase polymer and the effectiveness of the separation washing procedures. Since the availability of monoclonal antibodies the 'blank binding levels' have been much reduced often to the order of 0.05% leading to a corresponding improvement in minimum detection limit. For thyroid stimulating hormone this has led to improvements in minimum detection limit from 0.5–0.05 mU/L allowing discrimination between the normal and suppressed levels of thyrotoxicosis.

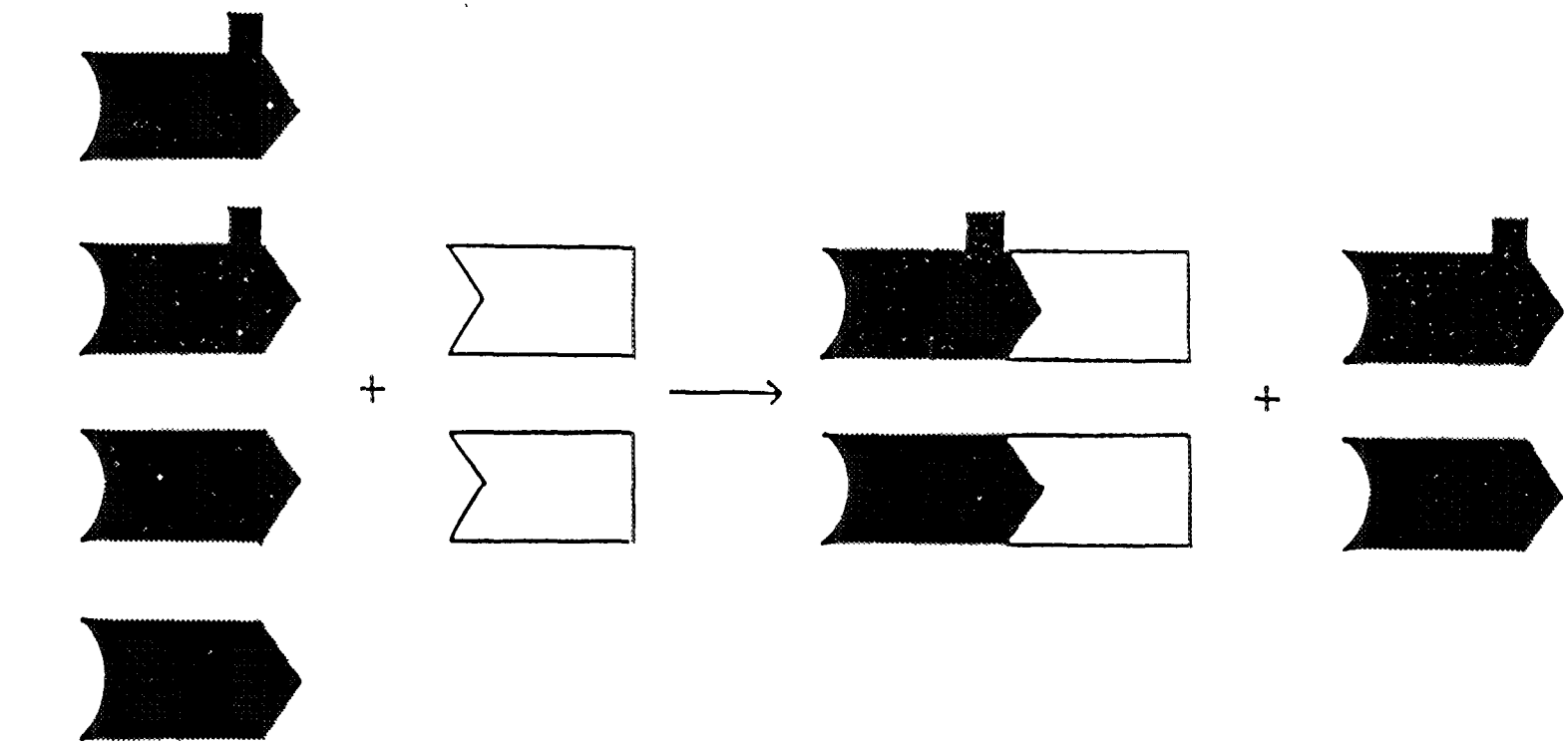
Since monoclonal antibodies are also epitope specific they can be used in simultaneous incubations significantly reducing incubation times.

6.4. ALTERNATIVE LABELS

At this stage of development radioisotopically labelled antibodies are limiting improvements in minimum detection limits. If 100 000 cpm ^{125}I labelled antibody are present as total counts then a 'blank' level of 0.05% represents 50 cpm, a level difficult to discriminate from background radioactivity levels with current radioisotope counting equipment. Alternative labels; enzyme amplification, time resolved fluorescence and chemiluminescence all offer higher specific activity labels and consequently are preferred for current developments. These labels also offer greater flexibility to commercial manufacturers in the design of automated systems an increasing number of which are becoming available.

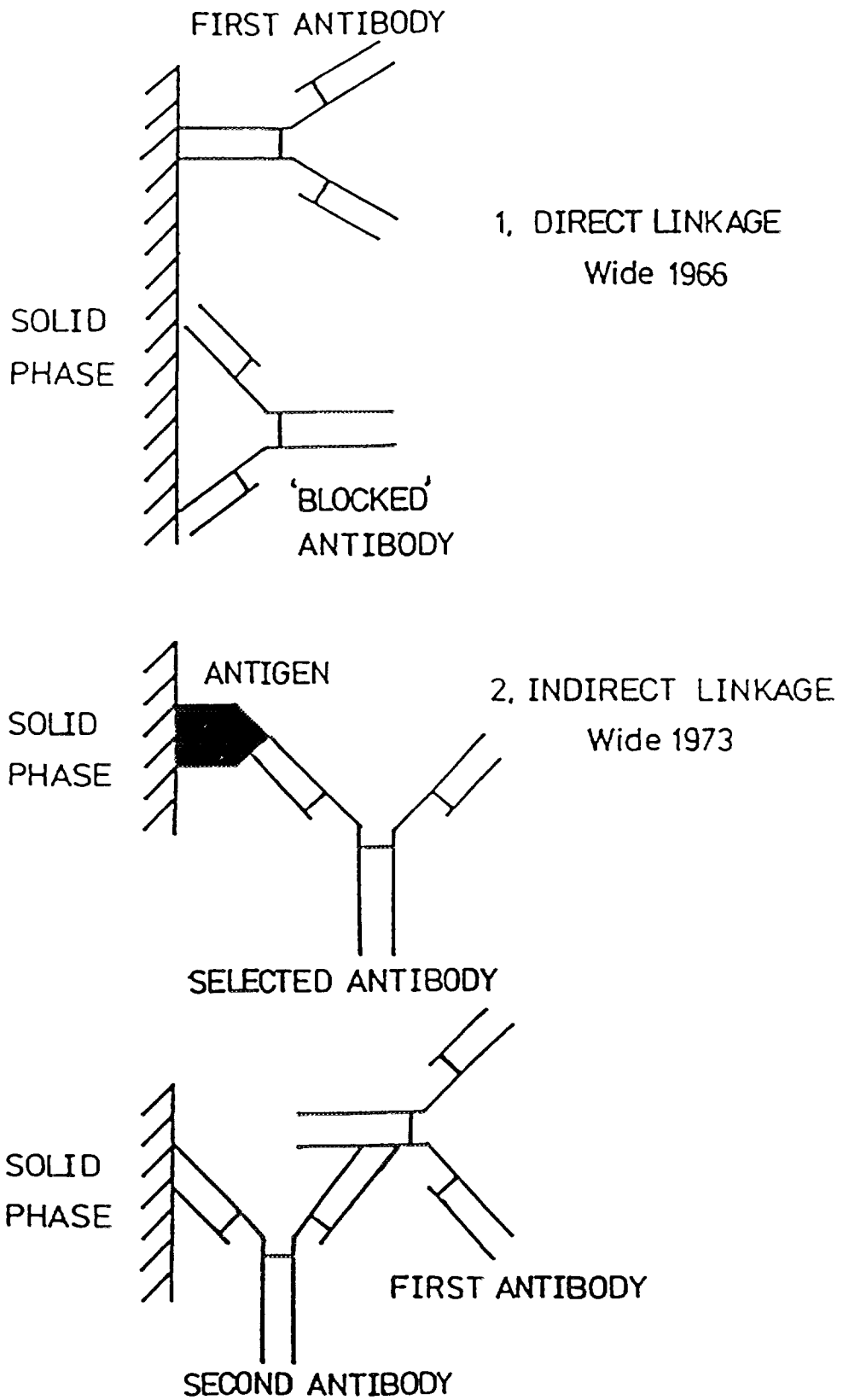

IMMUNOASSAYS
FOR CLINICAL CHEMISTRY
STRUCTURALLY
SPECIFIC ASSAYS
NO LABEL
LABEL
LIMITED REAGENT
ASSAYS
EXCESS REAGENT
ASSAYS
NON SEPARATION
SEPARATION

Limited Reagent Methods

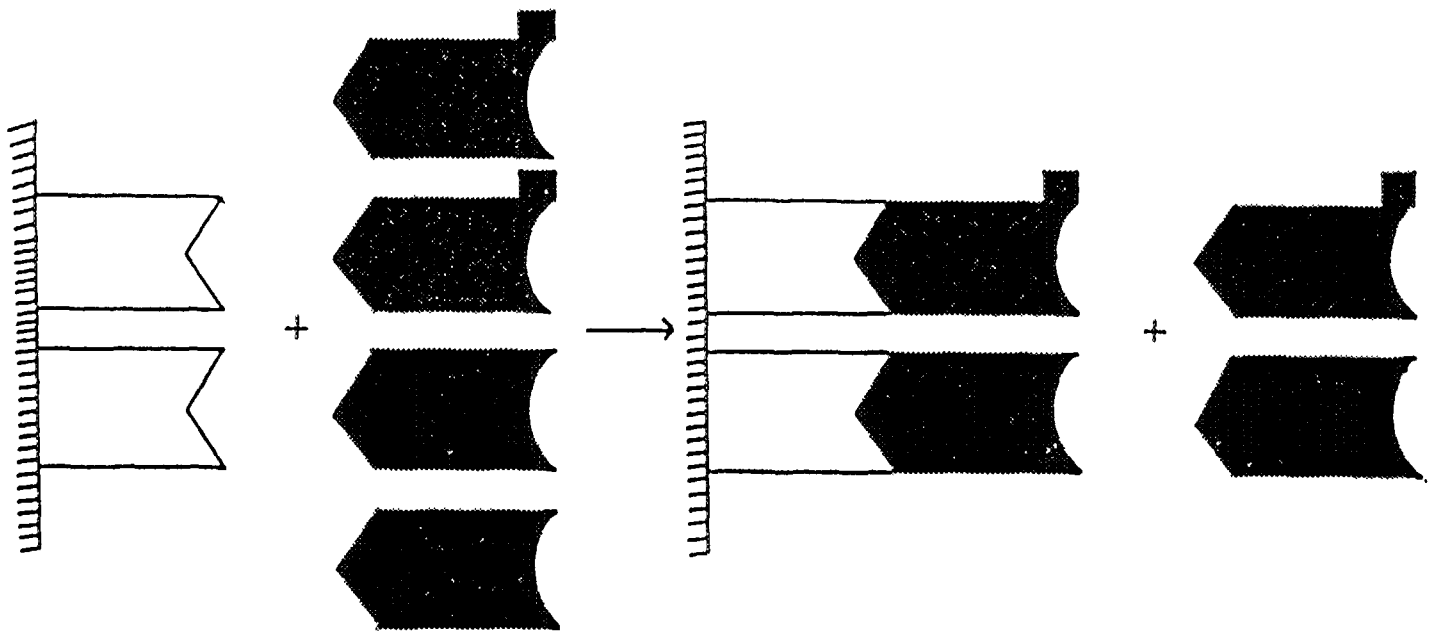


Labelled Antigen ('Limited' Antibody) → 'Bound' + 'Free'

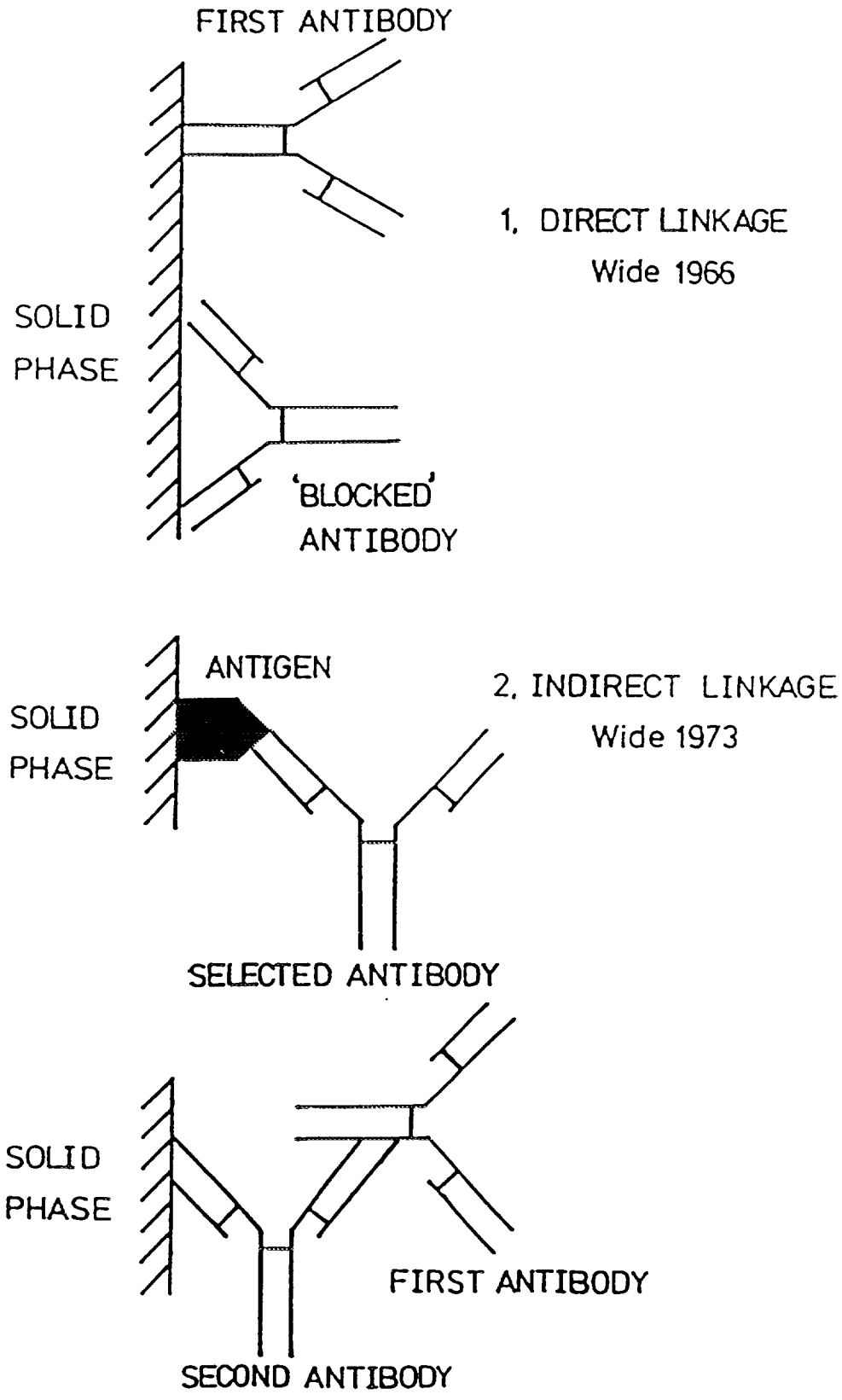
Saturation Assay ; RADIOIMMUNOASSAY ; Protein Binding Assay


FIRST ANTIBODY
SOLID
PHASE
'BLOCKED'
ANTIBODY
1, DIRECT LINKAGE
Wide 1966
ANTIGEN
SOLID
PHASE
SELECTED ANTIBODY
2, INDIRECT LINKAGE
Wide 1973
SOLID
PHASE
FIRST ANTIBODY
SECOND ANTIBODY

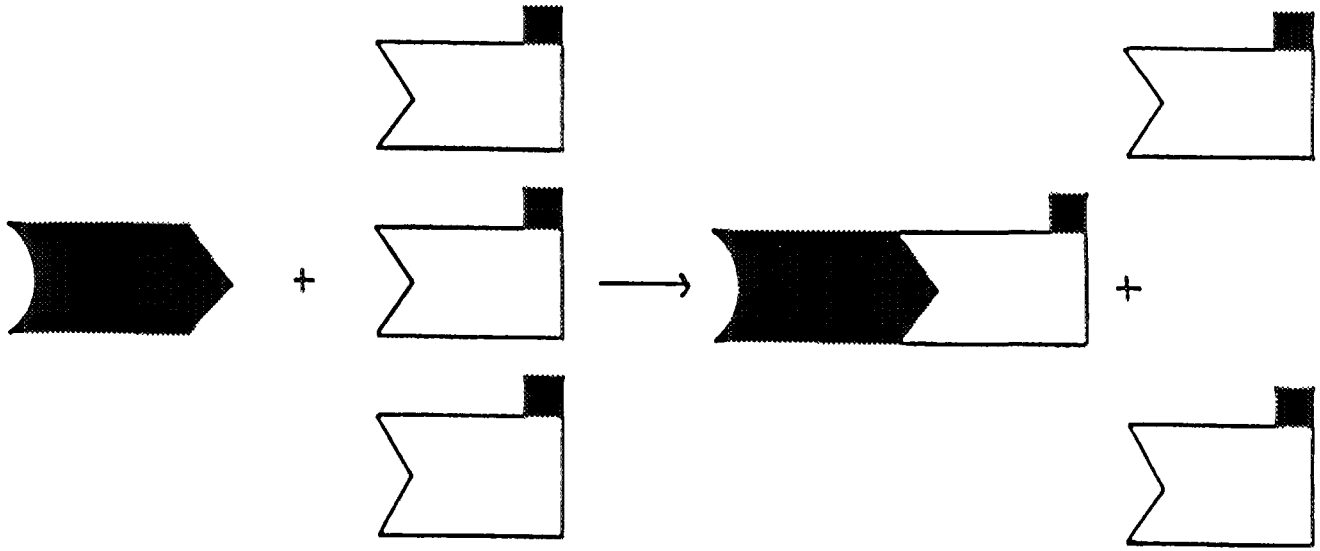
Limited Reagent Methods



SOLID PHASE RADIOIMMUNOASSAY

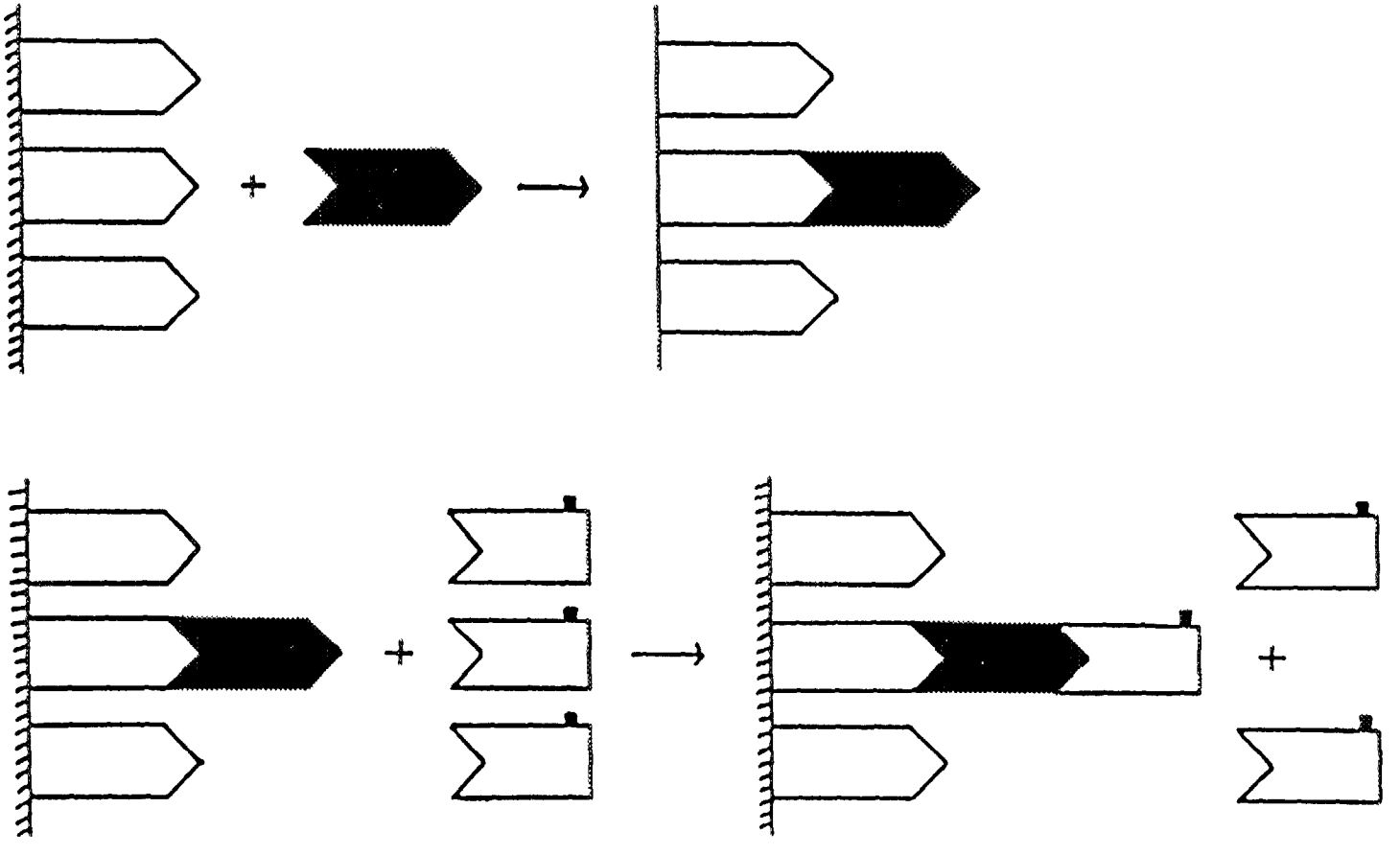

FIRST ANTIBODY
SOLID
PHASE
'BLOCKED'
ANTIBODY
1. DIRECT LINKAGE
Wide 1966
ANTIGEN
SOLID
PHASE
SELECTED ANTIBODY
2. INDIRECT LINKAGE
Wide 1973
SOLID
PHASE
FIRST ANTIBODY
SECOND ANTIBODY

Reagent Excess Methods



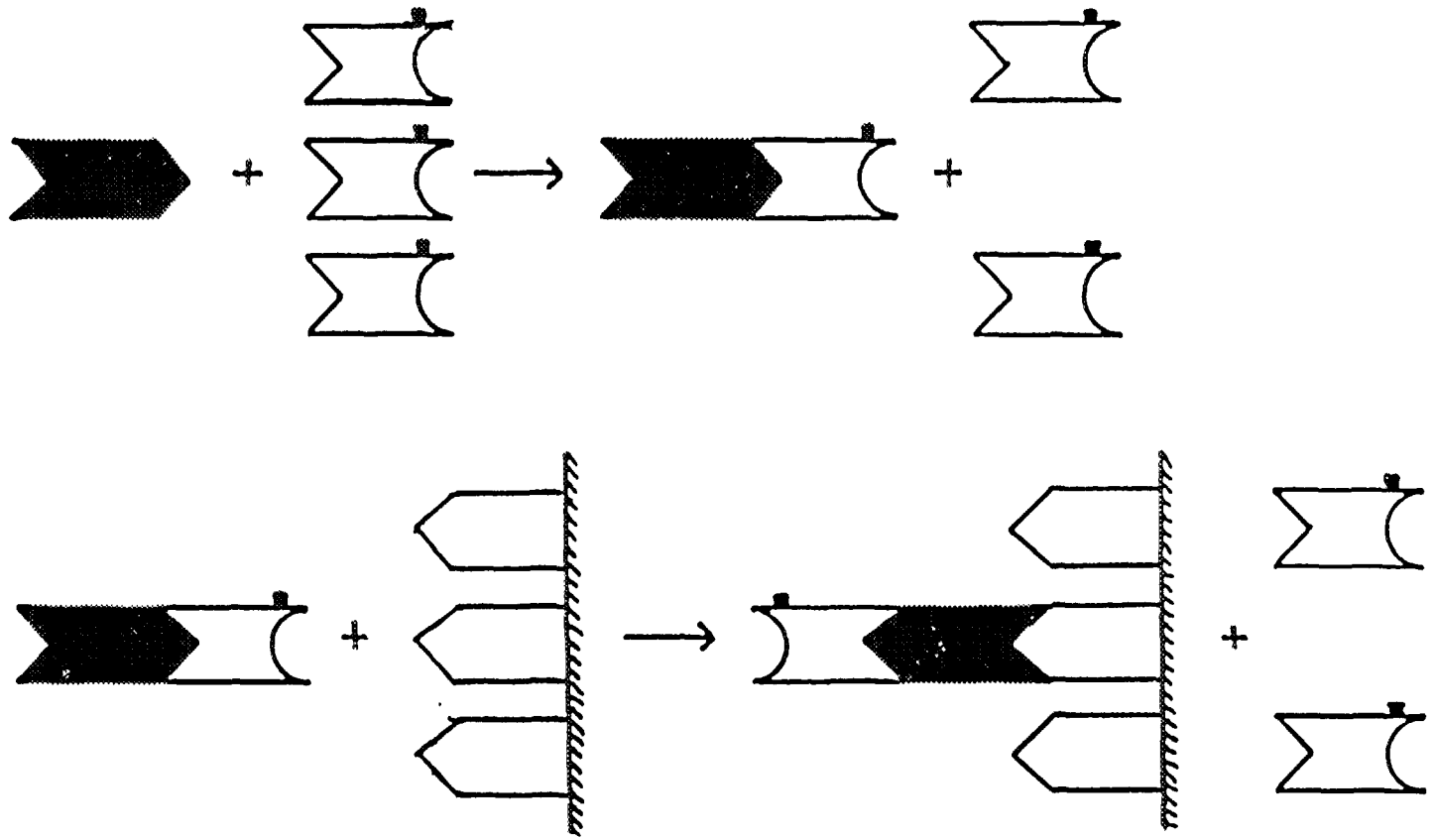
IMMUNORADIOMETRIC ASSAY

Reagent Excess Methods



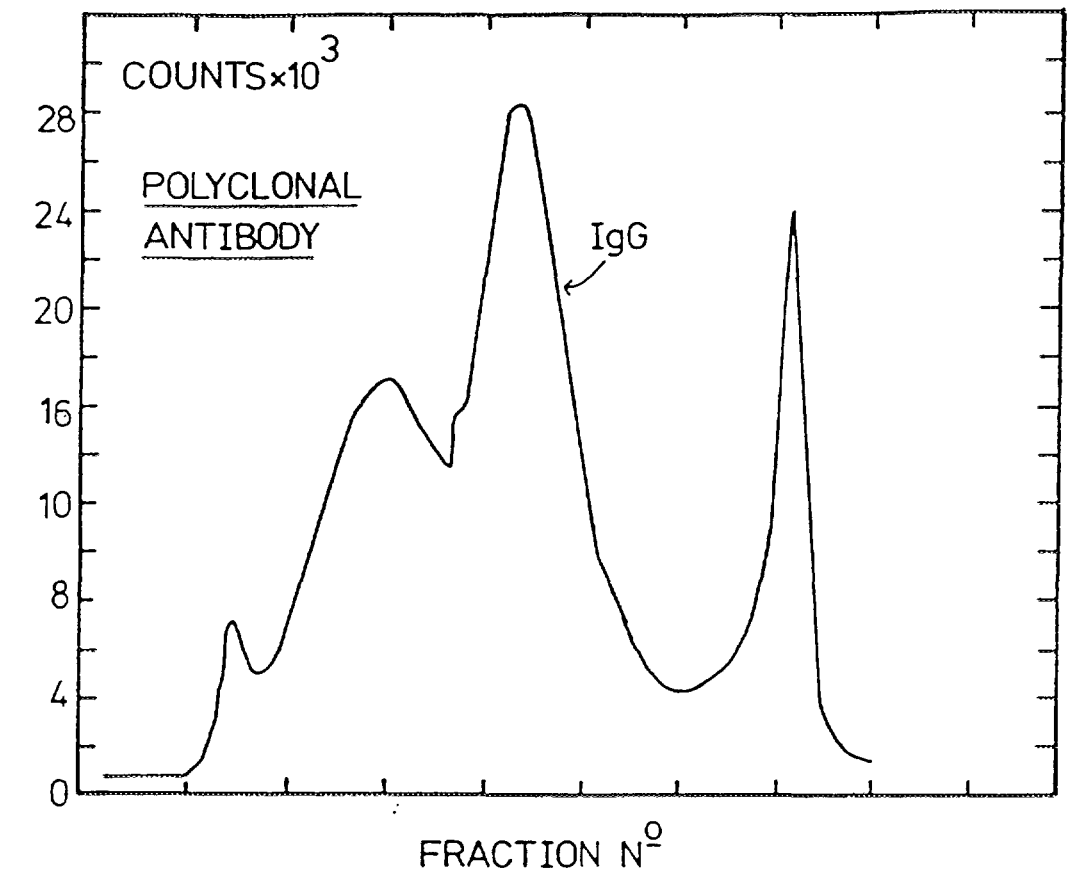
Two-site Immunoradiometric or sandwich assay

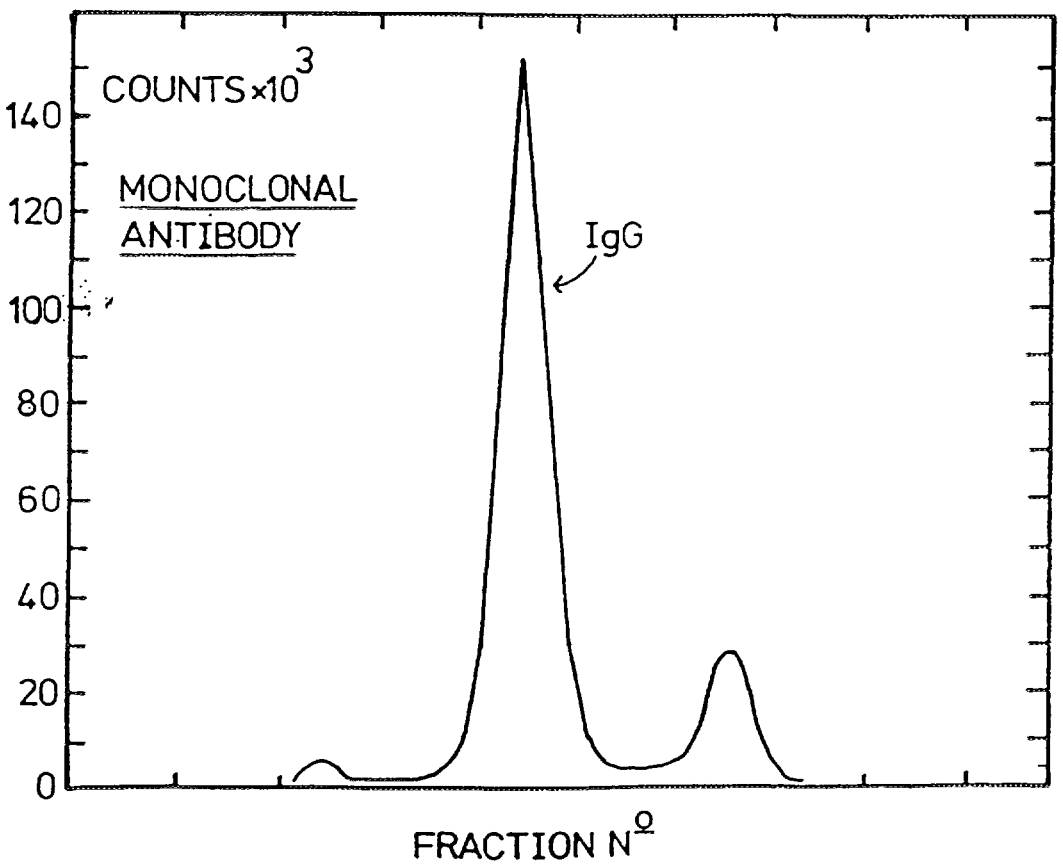
Reagent Excess Methods



Two-site Immunoradiometric or sandwich assay

(delayed solid phase addition)

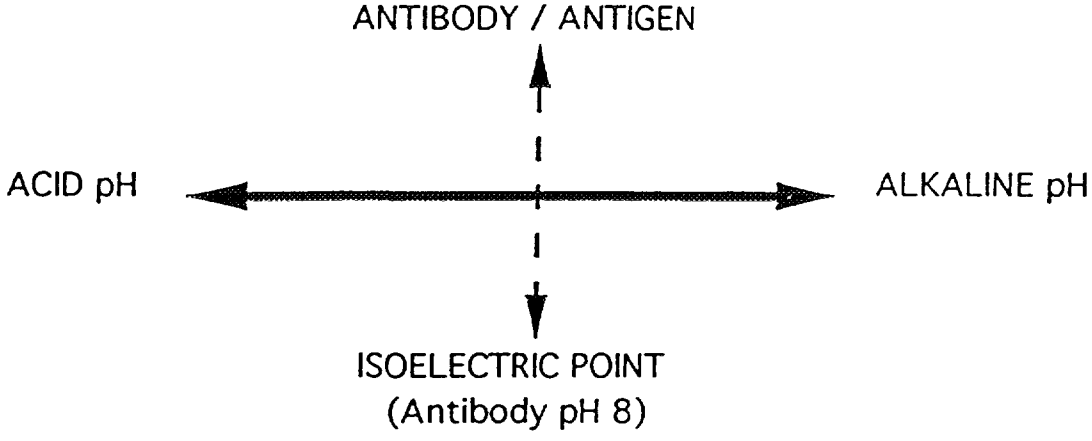

COUNTS×10³
28
24
20
16
10
8
4
0
POLYCLONAL
ANTIBODY
IgG
FRACTION Nº


COUNTS×10³
140
120
100
80
60
40
20
0
MONOCLONAL
ANTIBODY
IgG
FRACTION Nº

SOLID-PHASE MATERIALS

PHYSICAL ADSORPTION **COVALENT LINKAGE**

PHYSICAL ADSORPTION



SURFACE ATTRACTION

Hydrophobic Interaction
Hydrogen Bonding

For Antibodies usually coat at Alkaline pH 9-10 at Ambient Temperature for 18-24 hours .

Post coating unoccupied sites must be BLOCKED .

Chapter 7

LINKAGE OF BIOMOLECULES TO SOLID PHASES FOR IMMUNOASSAY

R.S. Chapman
Division of Biochemical Endocrinology,
The Royal Postgraduate Medical School,
Hammersmith Hospital,
London, United Kingdom

Topics covered by this lecture will include a brief review of the principal methods of linkage of biomolecules to solid phase matrices. Copies of the key self explanatory slides are presented as figures together with reprints of two publications by the author dealing with a preferred chemistry for the covalent linkage of antibodies to hydroxyl and amino functional groups and the effects of changes in solid phase matrix and antibody coupling chemistry on the performance of a typical excess reagent immunoassay for thyroid stimulating hormone.

Historically, prior to the development of monoclonal antibodies, covalent linkage of antibodies to particulate solid phases was widely used. Functional groups on its largely carbohydrate based polymers, cellulose and Sephadex were activated, originally by cyanogen bromide, and linked to IgG fractions of polyclonal antibodies for use in limited reagent, radioimmunoassay, methods. Solid phase antibodies could be used as primary reagents, or in the separation stge as solid phase second antibodies. The advantages in radioimmunoassay were originally perceived to be related to speed of assay. Primary antibodies attached to solid phase did not require time consuming and expensive second antibody precipitation. But, advantages were soon discovered of improved precision at lower antibody binding levels as a result of the introduction of multiple wash/separation systems. This had the effect of removal of misclassification error, washing away the proportion of free counts misclassified as bound, effectively removing the distortion caused at low binding levels from liquid phase radioimmunoassays and improving precision. As a result radioimmunoassays could be prepared using greater dilutions of primary antibody and often realised an improved sensitivity. However physical preparation of the solid phase tended to be wasteful of valuable antibody since linkage is random resulting in the steric hindrance of a proportion of the antibody coupled.

A further disadvantage was the physical form of the solid phase particle. Cellulose particles, size 20 μm, required shaking for incubation times greater than 1 hour. This stimulated research into the provision of more ideal particles of improved buoyant density. Smaller particles, silanised glass particles, later particles and uniform polystyrene particles with a shell of added methacrylate (Dynospheres) have since been developed which do not require agitation incubation.

A number of alternative chemistries, periodate, hydroxysuccinimide, carbonyldiimidazole, tresyl and tosyl, designed to extend the range of polymers and functional groupings available for antibody linkage have also achieved widespread application.

A novel development has been the production of magnetic solid phase particles, magnetic cellulose, silanised ferromagnetic particles, and spherical latex particles polymerized around a ferromagnetic core. These particles can be separated from immunoassay incubates by magnetism avoiding the requirement for centrifugation separation. This is however achieved at a loss of both

buoyancy, due to the greater density of the ferromagnetic component, and antibody coupling capacity.

The initial development of excess reagent assays was constrained by their use of polyclonal antibodies and as a consequence the poor capacity for antibody linkage of the cellulose solid phase particles. Since optimal assay performance required a high capacity for antibody linkage larger porous particles e.g. agarose, sepharose, sephacryl were often used. However, with the advent of monoclonal antibodies and their inherent mono-purity solid phases of lower capacity could readily be used. A change of emphasis could now be made to physical adsorption; binding of antibody by hydrophobic interaction to the surface of polymeric materials. Polystyrene tubes, beads and microtitre plates, the latter in strip or single well format became widely used. Consequently, the commercial quality of the polystyrene similarly improved. It is now possible to purchase tubes and plates with a guarantee of standard binding behaviour. This format was readily adapted to automated immunoassay instruments.

The evolution of solid phase technology was not however a series of random unconnected events but a logical progression related to improvements in immunoassay technical simplicity, precision and sensitivity. This technology particularly with excess reagent assay systems has led to dramatic advances in precision and sensitivity in comparison with limited reagent assays. Time permitting the lecture will illustrate these points with examples from immunoassays used in a busy clinical endocrinology department.

Solid phase TECHNOLOGY

Limited Reagent Immunoassay	**Excess Reagent Immunoassay**
RIA	**IRMA**
(Radioimmunoassay)	(Immunoradiometric Assay)
Solid phase Specific Antibody	Solid phase **Specific Antibody**
Solid phase **Separation Antibody**	

PHYSICAL ADSORPTION

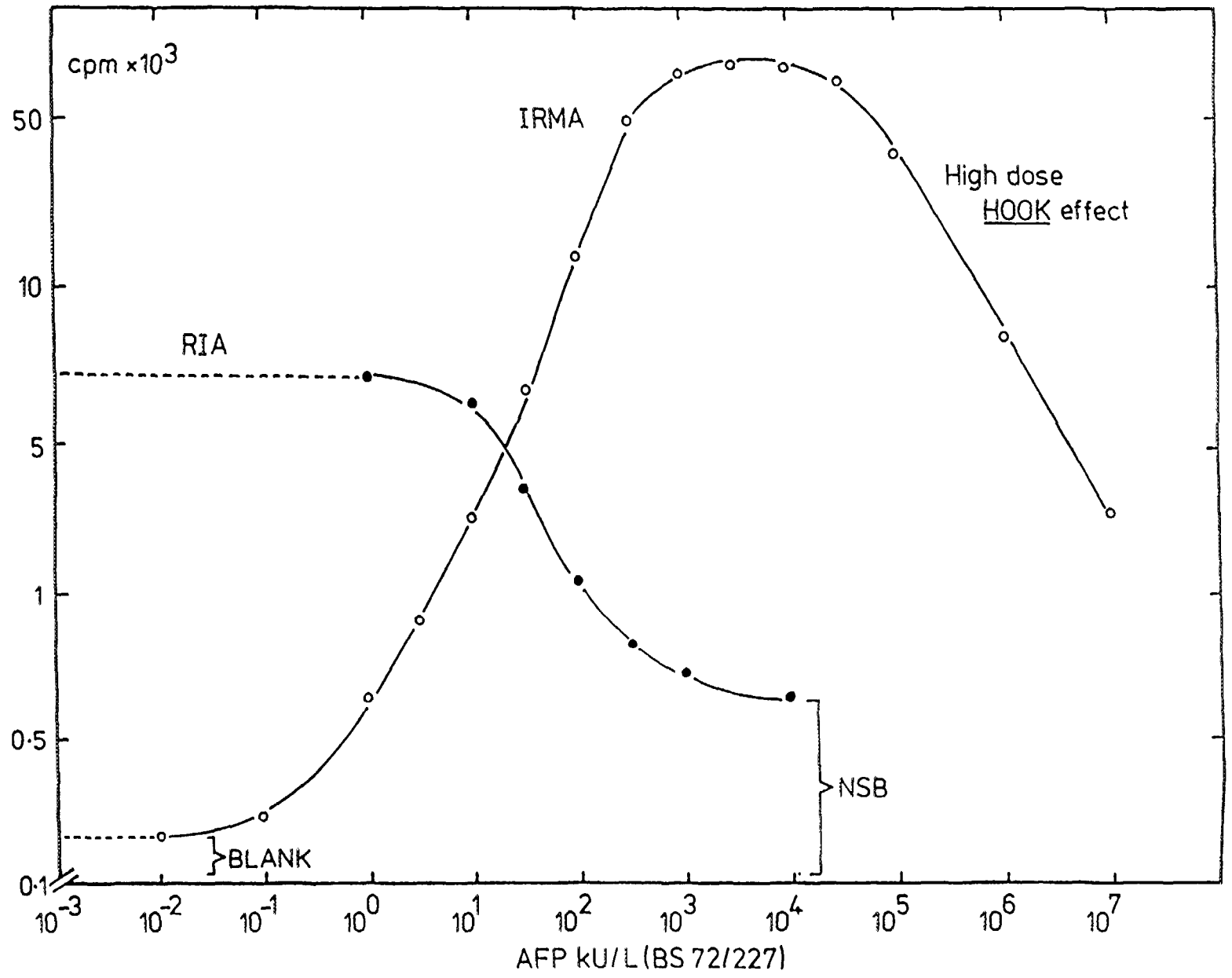
FORM	POLYMER
TUBE	POLYSTYRENE / POLYPROPYLENE
	STAR TUBES MAXISORB TUBES (NUNC) MINISORB TUBES
MICROTITRE PLATES STRIPS (8-well 12-well) WELLS (individual)	POLYSTYRENE/POLYVINYL CHLORIDE (Centrified surface quality)
BEADS/BALLS (Etched)	POLYSTYRENE
PARTICLES (Microspheres)	POLYSTYRENE POLYSTYRENE/METHACRYLATE (Dynospheres, Magnetisable)

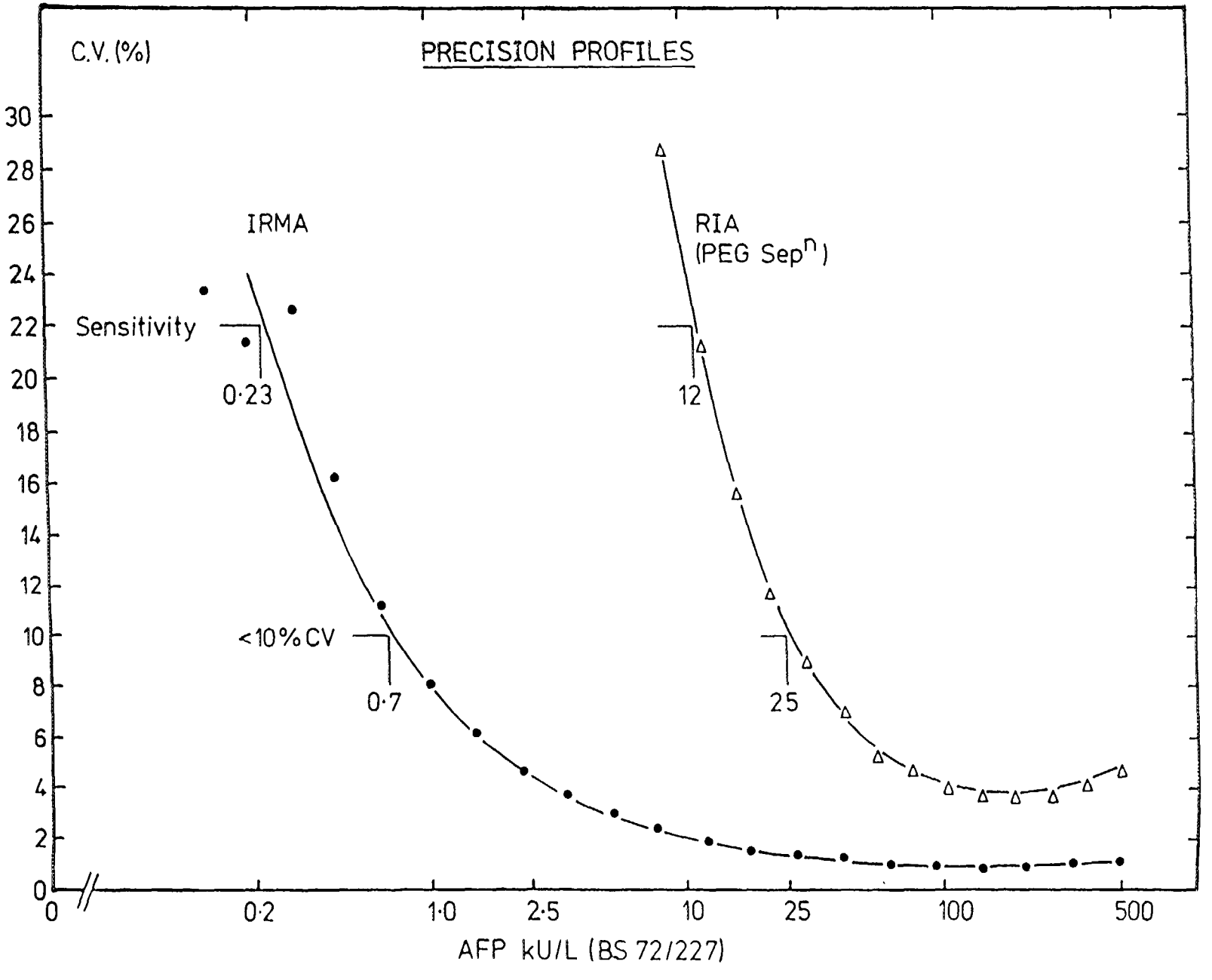
COVALENT LINKAGE

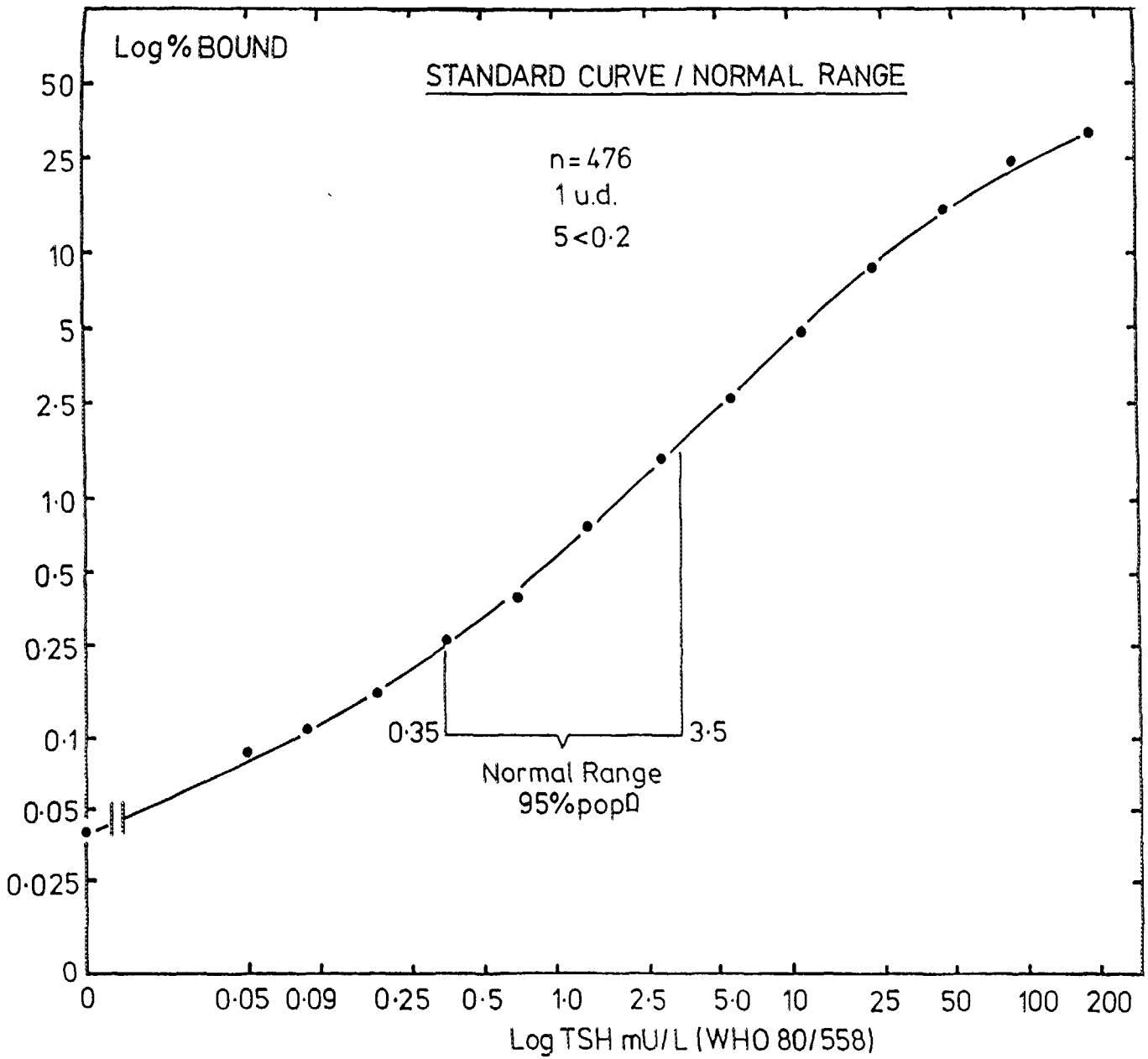
FUNCTIONAL GROUPS	COVALENT CHEMISTRY
AMINO	DIAZO GLUTARALDEHYDE CARBODIIMIDE
CARBOXYL	CARBODIIMIDE GLUTARALDEHYDE
HYDROXYL	CYANOGEN BROMIDE HYDROXYSUCCINIMIDE TRESYL TOSYL **CARBONYLDIIMIDAZOLE**
CIS-DIOL	PERIODATE

COVALENT LINKAGE

FORM	POLYMER
TUBE	POLYSTYRENE (Coated)
	POLYPROPYLENE (Glutaraldehyde)
MICROTITRE PLATES	POLYSTYRENE
STRIPS (8-well 12-well)	COVALINK (Nunc)
WELLS (individual)	COVALENT (Costar)
BEADS/BALLS	NYLON
	POLYSTYRENE
	(Surface Modified, Pierce)
PARTICLES	CELLULOSE
	BEADED DEXTRAN (Sephadex)
	BEADED AGAROSE (Sepharose)
	POLYACRYLAMIDE (Biogel, BioRad)
	CELLULOSE (Magnetisable)
(Microspheres)	POLYSTYRENE/METHACRYLATE (Magnetisable, Dynospheres)


cpm ×10³
50
10
5
1
0·5
0·1
IRMA
High dose
HOOK effect
RIA
BLANK
NSB
10⁻³
10⁻²
10⁻¹
10⁰
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
AFP kU/L (BS 72/227)


C.V. (%)
PRECISION PROFILES
IRMA
RIA
(PEG Sep^{n})
Sensitivity
0·23
12
<10% CV
0·7
25
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
0·2
1·0
2·5
10
25
100
500
AFP kU/L (BS 72/227)


Log % BOUND
STANDARD CURVE / NORMAL RANGE
n = 476
1 u.d.
5 < 0·2
50
25
10
5
2·5
1·0
0·5
0·25
0·1
0·05
0·025
0
0·35
3·5
Normal Range
95% popn
0
0·05
0·09
0·25
0·5
1·0
2·5
5·0
10
25
50
100
200
Log TSH mU/L (WHO 80/558)

Chapter 8

RADIOLABELLING FOR IMMUNOASSAY

R.S. Chapman
Division of Biochemical Endocrinology,
The Royal Postgraduate Medical School,
Hammersmith Hospital,
London, United Kingdom

8.1. INTRODUCTION

Since the early 1960s labelled compounds employed in immunoassay techniques, both radioimmunoassay and immunoradiometric assay, have involved radioisotopes typically ^{3}H (tritium) and ^{125}Iodine. With the advent of increasingly stringent governmental regulations regarding usage and disposal of radioisotopes and the impetus of research towards improved immunoassay sensitivity following the discovery of monoclonal antibodies and their application to excess reagent immunometric assay methodology, radioisotopic labels are gradually being replaced by non-isotopic labels: enzyme, fluorescence and chemiluminescence. Non-isotopic labels also have the attraction of longer shelf life but radioisotopic labels will continue to be widely applied particularly in limited reagent, radioimmunoassay methods for heptens (steroids, drugs, thyroid hormones) where non-isotopic labels do not confer analytical advantage. Similarly, laboratories may be unable to re-equip with the expensive capital equipment necessarily implied by the alteration in assay end point.

8.2. CHOICE OF RADIOISOTOPE (Figs 1, 2)

Radioisotopes have been central to biochemical research particularly the use of ^{14}C probes in metabolic studies and today the increasing usage of ^{32}P in genetic studies. However, immunoassay techniques have in the majority relied upon essentially three isotopes; ^{3}H (tritium), ^{131}I and ^{125}I (iodine). Organic compounds of biochemical interest could be labelled with tritium by isotopic exchange and remain useful today particularly in the immunoassay of steroid hormones. Unfortunately, ^{3}H, a emitting radionuclide, has a low specific activity and requires liquid scintillation counting which is expensive in terms of scintillation fluid and often requires tedious and time consuming preparation procedures. Radioiodine is available in two emitting isotopes ^{125}I and ^{131}I offering in addition to ease of counting, a much higher specific activity. Since ^{131}I is only available at 20% isotopic abundance with a counting efficiency of 30% and a half life of 8 days the alternative isotope, ^{125}I, is preferred in the preparation of high specific activity protein, polypeptide and peptide hormone labels in immunoassay. Radioiodine has also achieved importance in steroid immunoassay due to the advent of conjugation labelling. The preeminence of radioiodine isotopes is due to the relative ease by which they can be substituted into tyrosine and histidine amino acid constituents of proteins.

8.3. RADIOIODINATION

The process of radioiodination is dependent upon the nature of the compounds for labelling. If the compound contains stable atoms of iodine e.g. iodothyronines (thyroid hormones), then radioiodination is achieved either by isotopic exchange or the radioiodination of a suitable precursor. Labelled tri-iodothyronine and thyroxine can be prepared by labelling di-iodothyronine, T_2, or tri-iodothyronine, T_3, respectively. If the compound of interest is lacking

in stable iodine atoms then radioiodination can proceed by one or two routes. Either direct iodination by covalent linkage to carbon atoms, e.g. electrophilic substitution into tyrosine residues, or indirect iodination by covalent linkage of a prelabelled compound or a compound capable of post linkage labelling, e.g. Bolton and Hunter reagent for peptides and conjugation labelling for steroids.

8.4. MECHANISM OF RADIOIODINATION (Figs 3, 4)

The chemistry of radioiodination is complex. Radioiodine in the form Na ^{125}I is available in alkaline solution (0.1 mol.L NaOH) without carrier and reductant. Radioiodination proceeds via three phases; firstly, oxidation of iodide to molecular iodine, secondly, substitution of hydrogen at aromatic groups by electrophilic substitution at unsaturated carbon atoms (tyrosine and histidine residues of peptides and proteins) followed by reduction of excess oxidised iodine and oxidising reagent. Molecular iodine is highly reactive generating numerous complex chemical reactions which are poorly understood in dilute solution. However, it is thought that the H_2OI^+ cation is the principal reactive species in electrophilic substitution at unsaturated carbon atoms.

8.5. RADIOIODINATION METHODS

Methods of radioiodination can be categorised into direct methods dependent upon either chemical or enzymatic oxidation of iodide and indirect methods dependent upon conjugation labelling.

8.5.1. Direct methods

8.5.1.1. Chemical oxidation

(a) Chloramine T

Of the chemical methods chloramine T oxidant [1] was the first to gain widespread usage for the production of high specific activity radioiodinated compounds. (Fig. 5) Incorporation of radioiodine, hence high yield and high specific activity, is largely governed by the concentration of the reactants, consequently iodinations are performed at microlitre (μl) volumes. Small conical based reaction vessels are bet employed. Excess chloramine T is reduced by the addition of sodium metabisulphite ($Na_2S_2O_5$) and free iodine is reduced to iodide. Post iodination, excess unlabelled sodium iodide (NaI) or potassium iodide (KI) is added to act as a carrier for the high specific activity ^{125}I iodide together with carrier protein (usually 0.5–1.0% bovine serum albumin, protease free) in a suitable buffer (0.1 mol/L phosphate, pH7.4) prior to separation of iodinated and free iodine species by an appropriate technique. The chloramine T reaction has an optimum of pH7.5 with reduced yield above and below this Fig.. Consequently a strong buffer, usually 0.25–0.5 mol/L phosphate pH7.4, is required particularly since commercial radioiodine is supplied at alkaline pH. (NaOH at pH8–10). Iodination of histidine residues is favoured at higher pH (pH8.0–8.5).

The advantages of Chloramine T are a technically simple and rapid method widely applicable to the majority of peptides, giving high specific activity. One of the major disadvantages is that the compound to be labelled is in direct contact firstly with the oxidising agent and secondly with the reducing agent. This inevitably leads to a degree of iodination damage which may vary according to the nature of the species iodinated. This damage can take the form of polymerisation, (aggregation), and oxidative damage to sulphydryl groups

(methionine residues) and disulphide bonds (cystine residues). Reducing agents may also lead to reductive cleavage of disulphide bridges. Since the reaction is so rapid (15–30 s) it is difficult to exert any degree of control other than to reduce the concentrations of the various reactants. Variations of the chloramine T method have been to optimise the concentration of oxidant to the minimum required for the individual peptide and to substitute cysteine as reducing agent [2]. Both manoeuvres are claimed to reduce iodination damage.

However, the fact that alternative iodination methods were developed suggests that practitioners believed that better quality products could be obtained. Variations of the chemical oxidation methods were largely designed to overcome these limitations.

(b) Chlorine gas

Chlorine gas produced from the oxidation of sodium chloride by Chloramine T has been used to generate iodine from $Na^{125}I$ to effect radioiodination without direct contract with the oxidising agent [3]. Iodination by this method is achieved within 10 minutes but reducing agents are still required and any improvement in label quality would be achieved at the expense of increased radiation hazard due to the possible escape of volatile ^{125}Iodine.

Another chlorine containing compound 1, 3, 4, 6 tetrachloro–3a, 6a-diphenylglycoluril, trade name Iodogen, has also achieved wide usage [4] (Fig. 6). This material is insoluble in aqueous solution, but freely soluble in chloroform or dichloromethane. Consequently, the active compound can be deposited in solid phase form, following evaporation of the solvent, at the site of the iodination. Since subsequent reaction may only occur at the aqueous interface during radioiodination intimate contact between protein and oxidising agent is minimised.

Advantages of this technique are the use of a mild oxidising agent in sparingly soluble solid phase format and the absence of reducing agent since the reaction can be stopped by simple aspiration from the reaction tube. Higher yields and greater stability of iodinated compounds have been claimed for this method. Disadvantages are greater expense and the requirement for the preparation of solid phase reaction tubes together with a significantly longer incubation time.

Other workers have employed N-Bromosuccinimide a compound closer to iodine in the electrochemical series as a very mild chemical oxidative iodination reagent with good success rates in terms of high yields and retention of immunological activity at high specific activities [5].

8.5.1.2. Enzymatic oxidation

(a) Lactoperoxidase

An extremely gentle oxidation reaction is provided by the presence of a trace quantity of hydrogen peroxide together with the oxidoreductase enzyme lactoperoxidase, commercial preparations of which are prepared from bovine milk. [6] High specific activity radiolabelled proteins can be prepared and the method is widely used. Advantages again relate to negligible concentration of oxidising agent and the lack of a need for reducing agent since simple dilution can be used to halt the enzymic reaction. Both factors suggest this methods should minimise iodination damage. Also the rate of reaction, hence yield and specific activity, can be controlled effectively by manipulation of H_2O_2 addition. Further additions at intervals, c. 10 min, can improve specific activities. Good stability and consequently a longer shelf-life of product thought to be due to iodination only at surface tyrosine residues are claimed for this method.

Disadvantages are the pHdependency of the reaction and self iodination of the enzymes complicating post iodination purification. The method as a consequence of its gentle reaction is slow, 30 min.

(b) Solid phase lactoperoxidase

Prepration of the lactoperoxidase in a solid phase format can mitigate most disadvantages [7] (Fig. 7). Liquid phase lactoperoxidase iodinations were conventionally conducted at pH5–6 the optimum for activity of the enzyme but significantly suboptimal for the incorporation of radioiodine into tyrosine residues, pH7.5. Linkage of the enzyme to solid phase particles stabilises the preparation allowing radioiodination at the optimum, pH7.5, for radioiodine incorporation. A short post iodination centrifugation removes the solid phase enzyme and prevents contamination of product with iodinated free enzyme. The requirement for the preparation of solid phase lactoperoxidase may be regarded as a significant disadvantage but due to the low concentration of enzyme required to effect iodination a single preparation provides supply for a number of years in a busy clinical laboratory. This method has been used successfully for both the iodination of protein antigens in radioimmunoassay and monoclonal antibody preparations in immunoradiometric assay and is the preferred method of the author.

8.5.2. Indirect methods

Direct iodination methods cannot be used if the candidate protein or polypeptide either does not possess tyrosine or histidine or has these residues buried within the tertiary structure sterically hindered from reaction. Similarly iodination, due to the relatively large molecular size of the iodine atom, may reduce or abolish binding to the specific antibody as a consequence of incorporation at the epitope or alternation in tertiary structure of the native protein. Also the candidate protein may be irretrievably damaged by exposure to the oxidation and reduction environment of the reaction. Conjugation labelling procedures must then be contemplated, although in the latter case alternative iodination procedures should be investigated.

(a) Conjugation labelling of proteins - Bolton and Hunter reagent

Conjugation labelling of proteins and peptides can be conveniently undertaken using N-succinimidyl 3- (4hydroxy 5- (^{125}I) iodophenyl)propionate (Bolton and Hunter Reagent) [8] (Fig. 8). This material is available commercially overcoming the major disadvantage of complex synthesis, iodination and purification of the reactive ester. Separation of the labelled protein and unreacted labelled hydrolysis product (3- (4-hydroxyphenyl)propionic acid) can be accomplished gel filtration. However, since the hydrolysis product adsorbs to bovine serum albumin, 0.2% gelatin is preferred as a protein carrier during gel filtration.

(b) Conjugation labelling of steroids

Steroid hormones may also be iodinated by the conjugation of pre or post iodinated tyramine or histamine (Fig. 9). The linkage chemistry must be chosen to be compatible with that adopted for the preparation of the hepten conjugate during antibody production. Antibodies tend to have a high avidity for the linkage or 'bridge' groupings used in conjugation of carrier protein to the hapten to form the immunogen with the consequent result that the antibody has a greater avidity for the label that for the free steroid giving reduced assay sensitivity if the 'bridge' groupings. For example, antibody raised to progesterone 1 1α hemisuccinate and progesterone 1 1α glucuronide conjugated to iodinated tyramine as label. For further details and a review article the reader is referred to Corrie and Hunter, 1981 [9].

Conjugation labels are expensive to produce and time consuming giving lower yields and limited specific activity due to the complex chemistries involved. Similarly, complex post iodination purification is required and the extra manipulations inevitably lead to an increased radiation hazard. Conjugation labels have, however, provided a significant advance towards the efficient radioimmunoassay of steroid hormones, but with the advent of immunoradiometric assays using labelled antibodies for the analysis of proteins and peptides the method of Bolton and Hunter is rarely required.

8.6. Post-iodination purification

Post-iodination the iodinated protein, peptide, steroid must be isolated from the undesired original reactants and products. (Figs. 10, 11, 12) In its simplest form the iodinated species must be separated from the free unreacted ^{125}Iodine. Reality is rarely this simple due to the variable effects of original impurities in the compounds to be iodinated, particularly with proteins and peptides, and the degree of iodination damage. Also, it is at the purification stage that assessment of specific activity is calculated. A survey of the literature readily shows that the majority of physiocochemical separation procedures available to biochemistry have been used for post iodination purification; electrophoresis, gel-filtration, ion-exchange chromatography, thin layer adsorption chromatography and high performance liquid chromatography. In general, gel-filtration chromatography on an analytical grade gel appropriately chosen for the correct molecular weight range e.g. Ultrogel, Sephacryl, is used for proteins and polypeptides with thin layer chromatography or HPLC reserved for conjugated hapten labels e.g. steroids. Iodination of purified monoclonal antibody with solid phase lactoperoxidase results in very little higher molecular weight, aggregated, material. Here, antibody can be separated from free unreacted iodine in a form adequate for immunoradiometric assay by a small desalting column of Sephadex G25. These columns can be purchased prepacked and in a disposable format.

8.7. Specific activity

The specific activity of an iodinated material is a measure of the incorporation of radioiodine and quoted as unit radioactivity per unit mass, generally μCi/μg (SI units MBq/mg). As a general guide it is not recommended that incorporation greater than one iodine atom per protein molecule be exceeded. This degree of substitution represents the minimum alteration possible, providing a situation where theoretically the activity of the iodinated protein should be maintained post iodination. The immunoreactivity of proteins rapidly deteriorates as the incorporation of iodine is increased, the degree of which can be antibody dependent. Substitution levels of 1 atom ^{125}I per molecule will give specific activities the order of which is governed by their molecular weight e.g. growth hormone (20 000 daltons), 110 μCi/μg. (SI units, 4070 MBq/mg); IgG (150 000 daltons), 15μCi/μg (SI units 555 MBq/mg).

All methods of calculating specific activity of radioiodinated compounds make assumptions which have varying degrees of validity. It is essential that the mass of compound is known accurately together with the amount of ^{125}Iodine used in the iodination. The latter is best calculated by accurate pipetting of a known volume of radioiodine and using the commercial suppliers data for the activity taking into account any radioactive decay which may have occurred since the activity date. The specific activity of the iodinated compound can then be calculated by apportioning the mass of iodinated compound and amount of radioiodine incorporated from the separation profile of the iodination mixture. Obviously the more product identified on the separation profile the more questionable becomes the accuracy of the specific activity assigned to the purified iodination product, but a working figure has value since the comparison of data

between iodinations gives a good indication of the variability or alternatively the stability of the iodination procedure.

In an alternative procedure the specific activity of an iodinated compound can be calculated by self displacement in a radioimmunoassay. The principle is simple: increasing quantities of labelled antigen are incubated in a standard radioimmunoassay i.e. x, x, x the original quantity of labelled antigen added. The increment in mass of tracer and hence mass of tracer can then be interpolated from the standard curve. Providing the efficiency of the counting of radioiodine is known the activity can be equated with the mass of protein and the specific activity calculated [10].

8.8. ASSESSMENT OF RADIOLABELS

Choice of a particular iodination method in comparison with alternative methods for a particular compound, antigen or antibody, can only be made according to the quality of the iodinated product in which case an assessment of the immunological behaviour of the labelled compound is required. Essentially, the condition of identity in immunological behaviour must be established between labelled and unlabelled compounds. In radioimmunoassay systems for radioiodinated antigens the percentage binding from radioactivity counting after separation for increasing masses of labelled antigen, calculated form the specific activity, should be superimposable with the standard curve elaborated from increasing masses of unlabelled antigen in the normal manner. Any deviation from this ideal suggests a lack of identity in immunological behaviour.

In immunoradiometric assay the latitude for increasing masses of labelled antibody is reduced due to the high count rates often used, circa 100 00 copm, and a different approach must be used. Again the specific activity of the labelled antibody preparation must be known. A standard curve is prepared at approximately 10 times the usual quantity of radioactivity. The mass of antigen giving this count rate is known from the specific activity. A further standard curve prepared at the normal quantity of radioactivity (1 times the mass of antigen) plus 9 times the mass of unlabelled antigen should be superimposable if immunological identity is maintained.

8.9. STORAGE OF RADIOLABELS

As a general rule, loss of immunoreactivity and the accumulation of free iodide leaching from the iodinated compound are time dependent. Iodinated labels should be stored in the presence of protein (usually bovine serum albumin, protease free) and a bacteriostat (usually 0.05% sodium azide, or thiomersal), but the temperature of storage is a matter of controversy. Iodinated antibodies can be stored successfully at normal refrigerator temperatures, +4°C, but a wide variety of recommendations have been suggested for other iodinated compounds. It is not generally recommended to store at –20°C unless previously flash frozen in a solid CO_2/ethanol mixture and freezing and thawing during multiple usage should be avoided. Other recommendations are to store in 50% propylene glycol at –20°C, where freezing does not occur, or alternatively at –70°C. Laboratories involved in the distribution of labelled materials usually lyophilise to comply with transport and safety regulations. Since storage conditions are likely to vary with iodinated compounds it is best to determine optimal conditions by use of appropriate control experiments.

REFERENCES

[1] GREENWOOD, F.C., HUNTER, W.M., GLOVER J.S., The preparation of ^{131}I-labelled human growth hormone of high specific activity. Biochemical Journal **89** 114–123 (1963).

[2] BROWN, N.S., ABBOTT, S.R., CORRIE, J.E.T., "Some Observations on the Preparation, Purification and Storage of Radioiodinated Protein Hormones", Immunoassays for Clinical Chemistry, (Hunger, W.M., Corrie, J.E.T., Churchill Livingstone (1983) 267–276

[3] REDSHAW, M.R., LYNCH, S.S., An improved method for the preparation of iodinated antigens for radioimmunoassay. J. Endocrinol **60** (1974) 527–528.

[4] FRAKER, P.J., SPECK, J.C., Jr., Protein and Cell membrane iodination with a sparingly soluble Chloroamide 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril, Biochem., Biophys. Res. Comm. **80** (1978) 849–857.

[5] REAY, P., Use of N-bromosuccinimide for the iodination of proteins for radioimmunoassay, Annals of Clinical Biochemistry **19** (1982) 129–133.

[6] THORELL, T.I., JOHANSSON, B.G., Enzymatic iodination of polypeptides with ^{125}I to high specific activity, Biochem. Biophys. Acta. **251** (1971) 363–369.

[7] KARANEN, S.L., MORSKY, P., SIREN, M. SENDERLING, V., An enzymatic solid phase method for trace iodination of proteins and peptides with ^{125}Iodine, Analytical Biochemistry **67** (1975) 1–10.

[8] BOLTON, A.E , HUNGER, W.M., The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent: application to radioimmunoassay, Biochemical Journal **133** (1973) 529–539.

[9] CORRIE, J E.T , HUNTER, W.M., ^{125}Iodinated tracers for hapten specific radioimmunoassays, Methods Enzymol **73** (1981) 79

[10] ROULSTON, J.E , Validation of the self-displacement technique for estimation of specific radioactivity of RIA tracer, Annals of Clinical Biochemistry **16** (1979) 26–29.

RADIOISOTOPES IN BIOCHEMISTRY

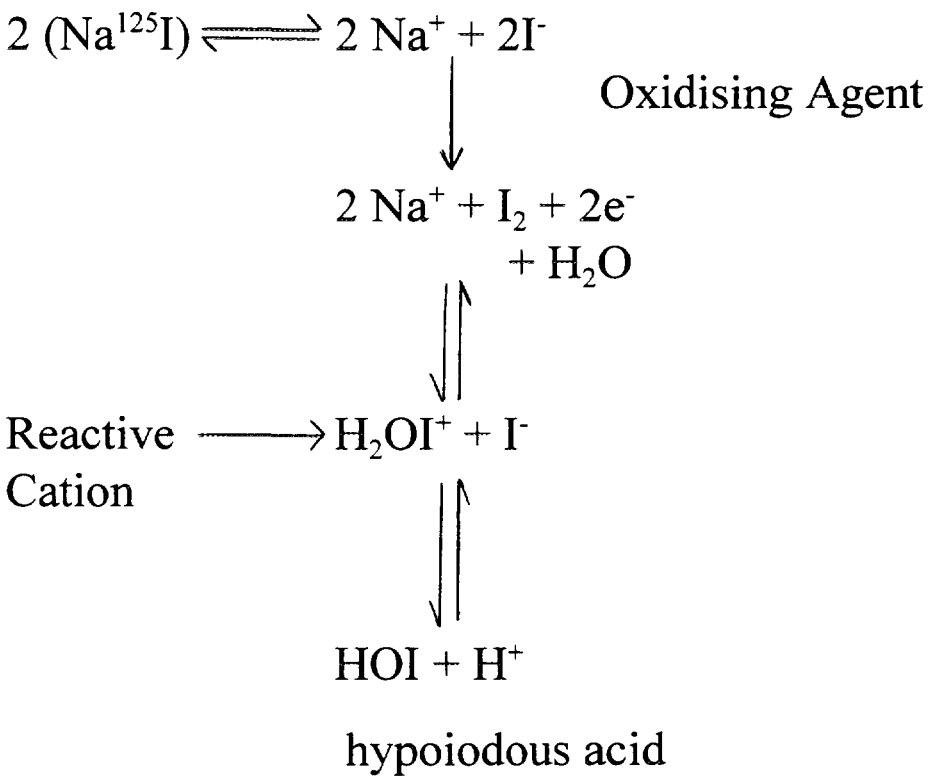
Radioisotope	Half Life	Specific Activity Ci/matom	Emission	Energy
^{14}C	5730y	0.062	β	0.156
^{3}H	12.2y	29	β	0.018
^{35}S	87d.	1500	β	0.167
^{32}P	14d.	9200	β	1.710
^{125}I	60d.	2200	γx	0.035 (γ)
^{131}I	8d.	16000	γβ	0.08-0.723 (γ)

RADIOIODINE

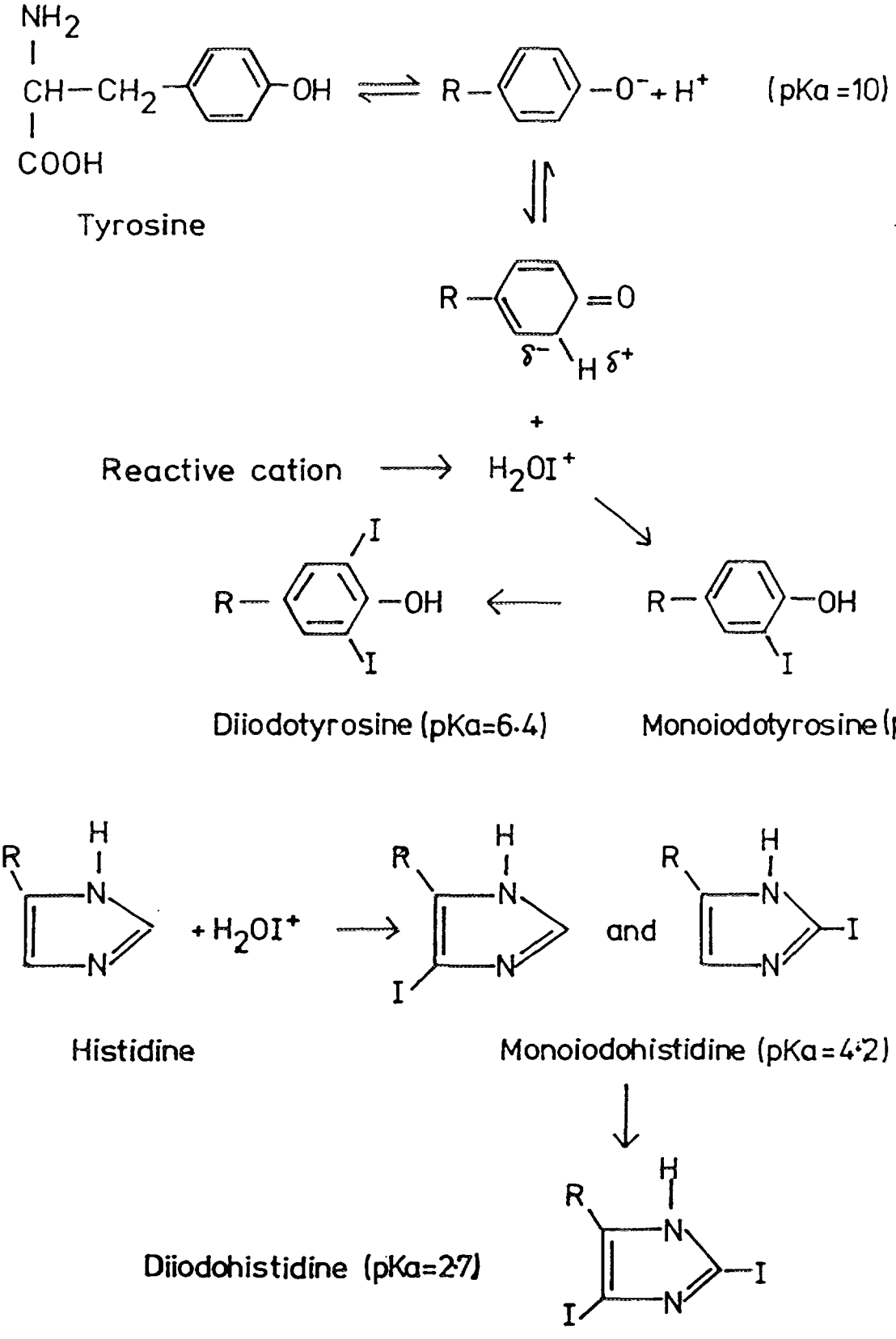
SOURCE	$Na^{125}I$ carrier free
DILUENT	0.1 molar NaOH
SPECIFIC ACTIVITY	17 Ci/mg (1mCi = 58.8ng)
CONCENTRATION	100mCi/mL (1mCi = 10 ul)
PURITY	99%
ISOTOPIC ABUNDANCE	95%
HALF LIFE	60 days
COUNTING EFFICIENCY	70–80%

MECHANISM OF RADIOIODINATION

1. OXIDATION OF RADIOIODINE:



2. ELECTROPHILIC SUBSTITUTION (AROMATIC GROUPS)



CHLORAMINE–T

	Vol
Protein (2–5 μg)	10 μl
Buffer (0.5 mol/L phosphate pH7.4)	10 μl
Na ^{125}I (0.5–2.0 mCi)	5 μl, 10 μl, 20 μl
Chloramine T (6–16 μg)	10 μl
Mix for 15–30 seconds	
Sodium metabisulphite (6–14μg)	10 μl–100 μl
Buffer (0.05 mol/L phosphate, 1% KI, 1% BSA, protease free)	100 μl
L–cysteine (56 μg) (Replacement for sodium metabisulphite $N_2S_2O_5$)	10 μl–100 μl

---

IODOGEN

A. Preparation of reaction tubes

1. Stock solution, **Iodogen**, 1 mg/ mL dichloromethane (AR)
2. Working solution 1:50 dilution in dichloromethane (20 μg/mL)
3. Add 150 μL (3 μg) to glass tubes, air dry.

B. Iodination	**Vol.**
Protein (10 μg, 0.1 mol/L phosphate buffer pH7.4)	100 μl
Na ^{125}I (1.0 mCi)	10 μl
Incubate, gentle agitation, 20 mins	
Aspirate incubate to: –	
Buffer (0.1mol/L phosphate, 1% KI, 0.5% BSA)	150 μl

SOLID-PHASE LACTOPEROXIDASE

A. Preparation of Solid-phase Lactoperoxidase

Lactoperoxidase is linked to a proprietory enzyme carrier, a copolymer of maleic anhydride and butanediol divinyl ether.

Karonen et al - Analytical Biochemistry 67, 1 - 10 (1975)

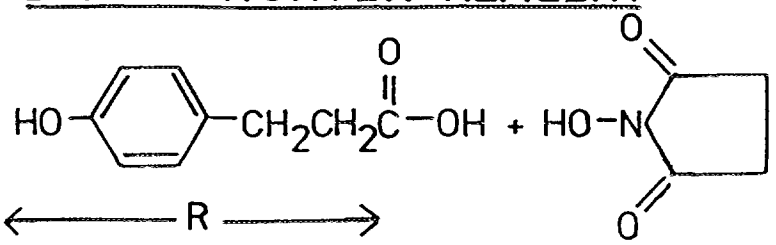
Preparation sufficient for 5-10 years iodinations in a busy laboratory.

B. Iodination	Vol
1. **Protein** (2 - 5µg), **Monoclonal Antibody** (20µg)	10 -20µl
2. **Buffer** (0.5mol/l Phosphate pH 7.4)	10µl
3. **Solid-phase lactoperoxidase** (1:50)	10µl
4. **Na ^{125}I** (0.5 - 1.0 mCi.)	5 -10µl
5. **H_2O_2** (100 vols; 1:100,000 in dist. H_2O	10µl

Vortex, incubate ambient temp. 30 min, revortex at 5-10 min intervals. (Further additions of H_2O_2 can be made at 5 - 10 min intervals.)

6. **Buffer** (0.2ml, 0.1mol/l phosphate, 0.5% BSA)

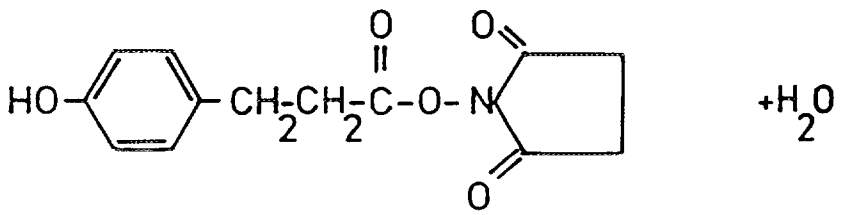
BOLTON-HUNTER REAGENT



3-(4-hydroxyphenyl) propionic acid

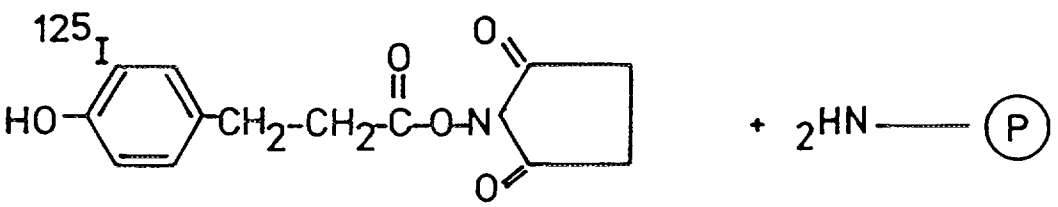
N-hydroxysuccinimide

carbodiimide
anhydrous solvent



(Ester)

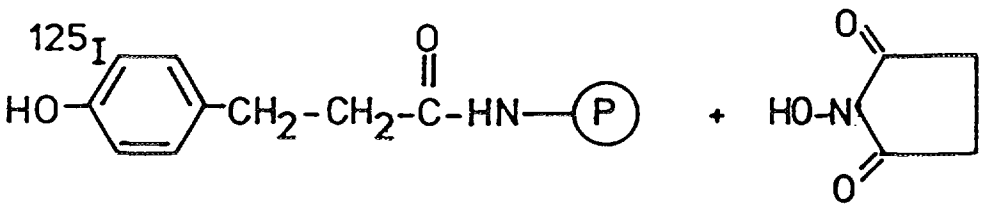
Chloramine-T iodination (^{125}I)



(Iodinated Ester)

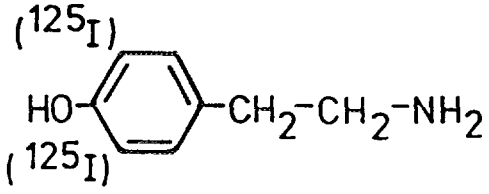
(Free Amine)

pH 7-9, 15-30 min.

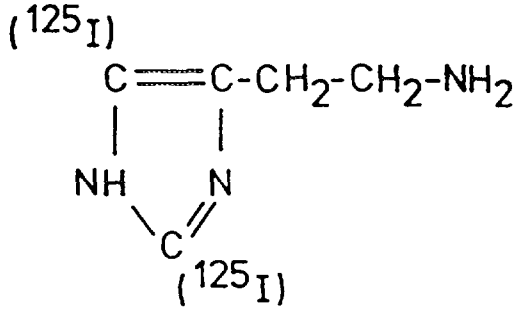


(Labelled Peptide)

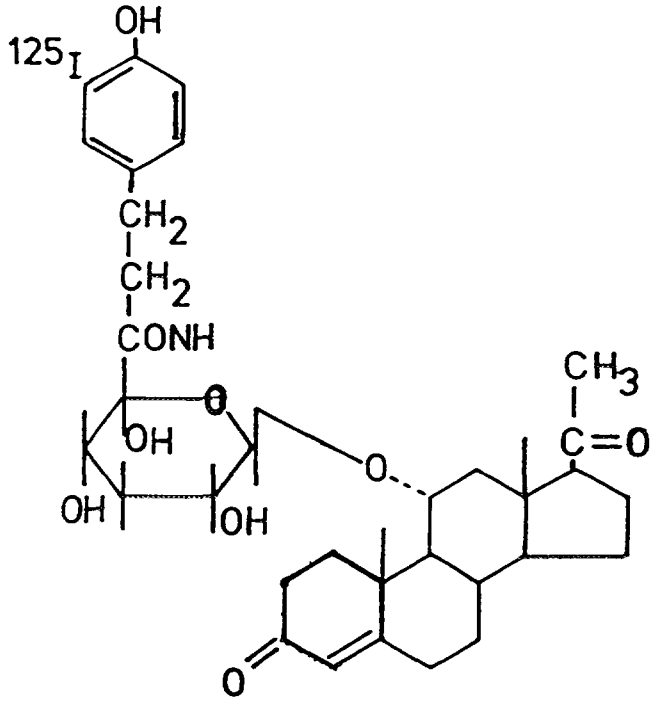
CONJUGATION LABELLING HAPTEN/STEROIDS



Tyramine

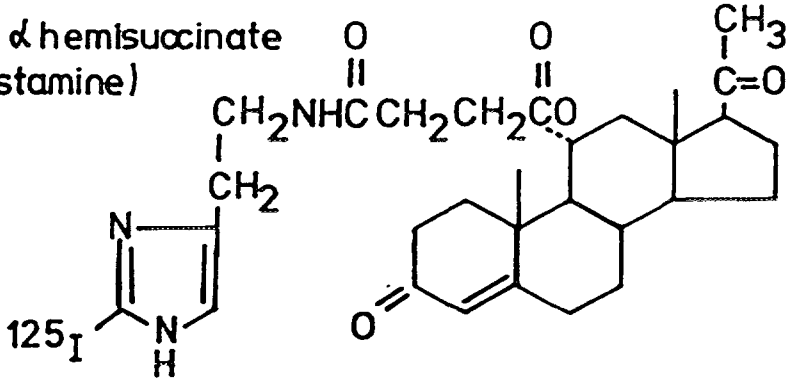


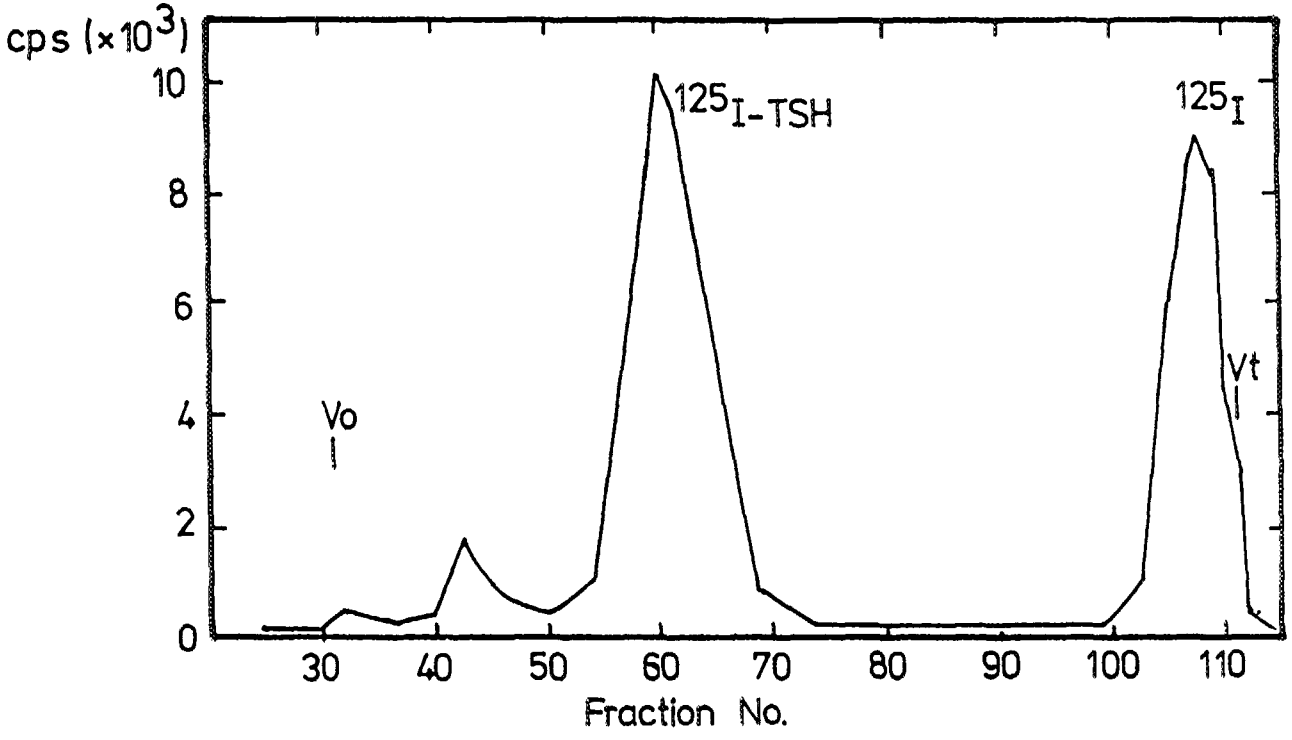
Histamine



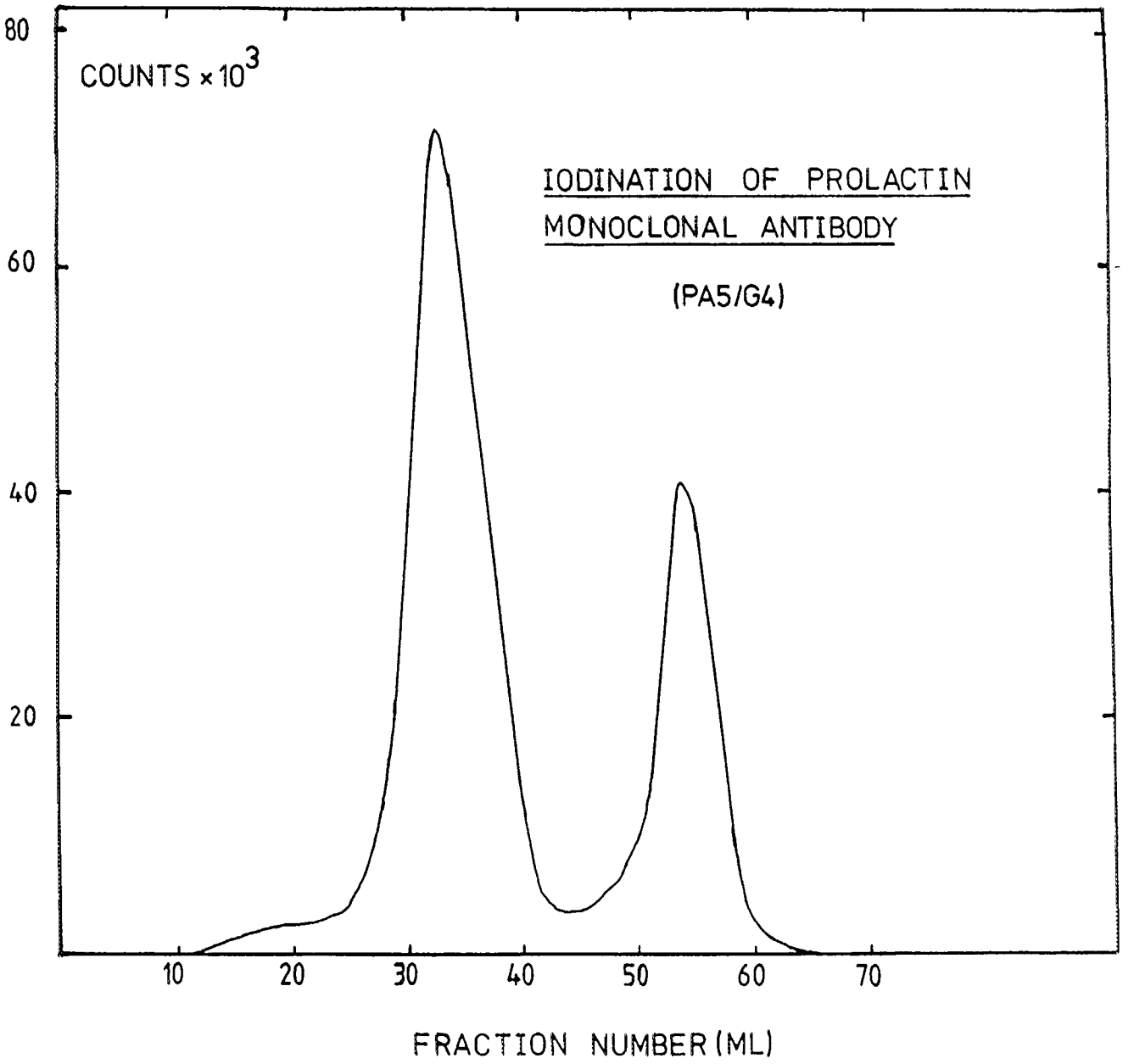
Progesterone 11α glucuronide (^{125}I) iodotyramine

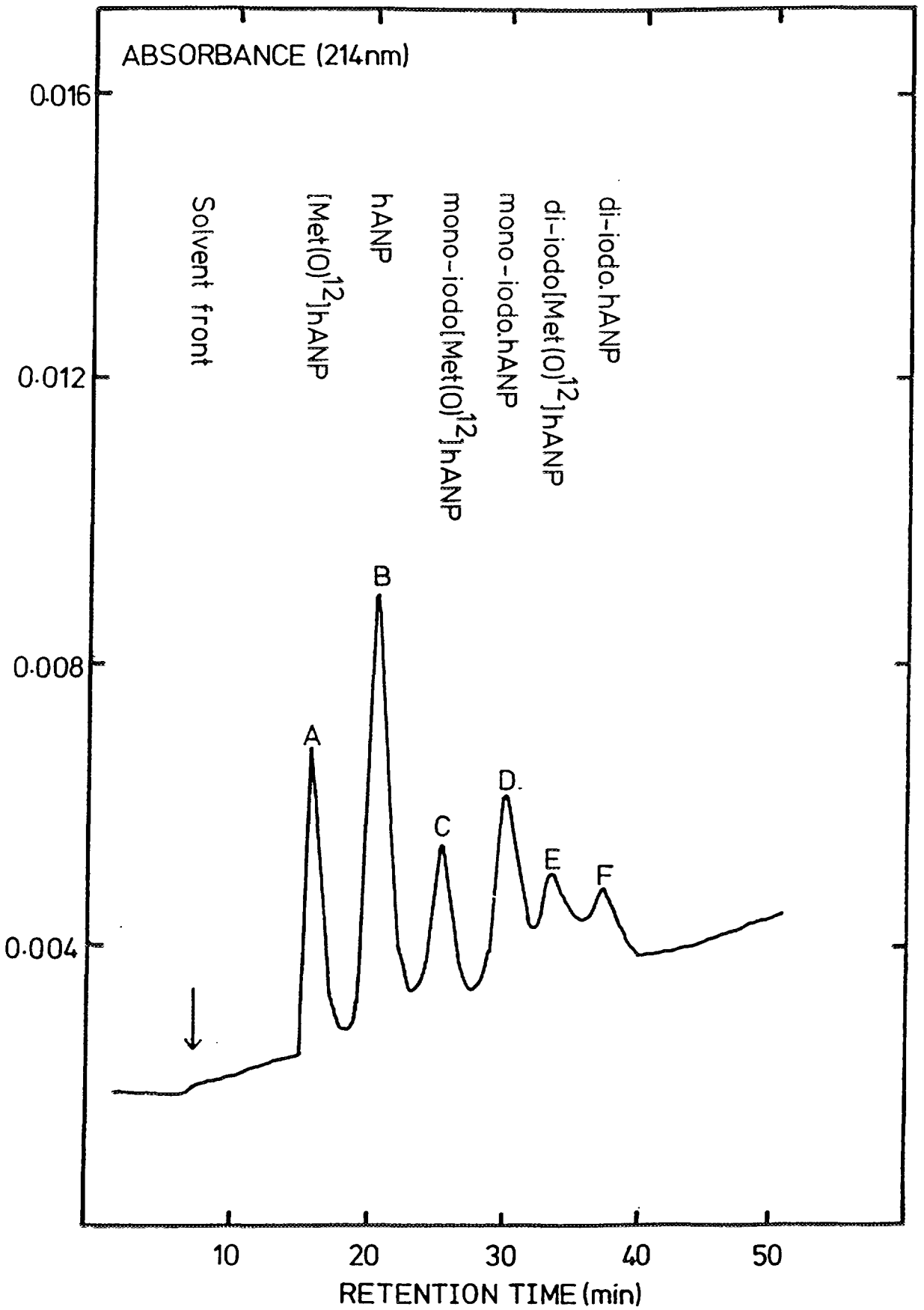
Progesterone 11α hemisuccinate (2-(^{125}I) iodohistamine)





TSH, Chloramine T, Ultrogel ACA-54


80
60
40
20
COUNTS × 10³
IODINATION OF PROLACTIN
MONOCLONAL ANTIBODY
(PA5/G4)
10
20
30
40
50
60
70
FRACTION NUMBER (ML)


ABSORBANCE (214nm)
0·016
0·012
0·008
0·004
Solvent front
[Met(O)12]hANP
hANP
mono-iodo[Met(O)12]hANP
mono-iodo.hANP
di-iodo[Met(O)12]hANP
di-iodo.hANP
A
B
C
D
E
F
10
20
30
40
50
RETENTION TIME (min)

Chapter 9

INTERFERENCE IN IMMUNOASSAY

R.S. Chapman
Division of Biochemical Endocrinology,
The Royal Postgraduate Medical School,
Hammersmith Hospital,
London, United Kingdom

9.1. INTRODUCTION

Interfering factors are evident in both limited reagent (radioimmunoassay) and excess reagent (immunometric assay) technologies and should be suspected whenever there is a discrepancy between analytical results and clinical findings in the investigation of particular diseases. The overall effect of interference in immunoassay is analytical bias in result, either positive or negative of variable magnitude. The interference maybe caused by a wide spectrum of factors from poor sample collection and handling to physiological factors e.g. lipaemia, heparin treatment, binding protein abnormalities, autoimmunity and drug treatments. The range of interfering factors is extensive and difficult to discuss effectively in a short review. Consequently, the presentation will be restricted to two major areas of direct interference in the immunological reaction. Firstly, the effect of heterophilic antibodies including human anti-mouse antibodies and secondly the complex issue of specificity assessment in immunoassay, particularly immunometric assay.

9.2. HETEROPHILIC ANTIBODIES

The presence of heterophilic antibodies in human serum is now widely recognised as a cause of interference, generally giving rise to positive bias, in assay systems. This subject was reviewed in 1988 by Boscato and is now included as a legitimate section in topical reviews of general immunoassay methodology; Gosling, 1990 [2].

One of the first instances of interference in immunoradiometric assay appeared in 1973 during clinical evaluation of an assay (Aus-RIA-Abbott laboratories) for hepatitis B antigen where a false positive rate of 80% was evident [3]. This was confirmed in a wider study, 5089 patients, with 91% false positive [4]. This effect, in the majority of cases, could be neutralised by the addition of normal guinea pig globulin antibodies. This effect was not specific to guineapig immunoglobulin since antisera to six different species of immunoglobulin similarly gave false positive effects.

However, this problem is not restricted to immunometric assay since analytical interference was reported in a solid phase radioimmunoassay using rabbit anti-TSH serum leading to falsely elevated thyrotrophin estimations [5] The interfering factor was again shown to be IgG and counteracted by the addition of rabbit IgG. A retrospective study indicated that 5% of hypothyroid patients were misclassified in the previous twelve months. A similar effect may occur in solid phase second antibody applications [6, 7] if non-immune carrier serum of identical species to the primary antibody is not included.

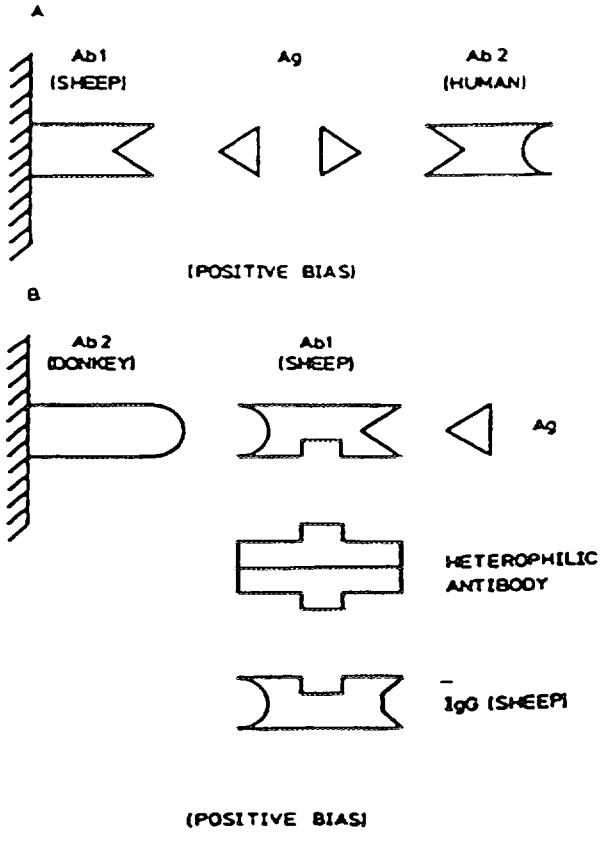


Fig. 1. Schematic diagram illustrating interference in radioimmunoassay (A) autoantibody; (B) beterophilic antibody.

The mechanism of radioimmunoassay interference can be visualised by referring to Fig. 1. Consideration of Fig. 1 (A) illustrates the particular case of a solid phase specific anti-analyte antibody in the presence of an interferant human anti-analyte antibody. If the human antibody were an anti-thyroglobulin or anti-thyroid hormone autoantibody, of relatively high frequency in patients with thyroid autoimmune disorders, and the analyte were thyroglobulin or either of the thyroid hormones, thyroxine or tri-iodothyronine, then the human antibody can be expected to sequester analyte lowering the binding of labelled analyte to specific antibody and resulting in spuriously elevated analyte levels [8]. This end result is however, dependent upon the design of the radioimmunoassay, being mirrored by a double antibody separation system provided that the second antibody is strictly specific for the first antibody and does not cross-react with the human antibody. Conversely, charcoal adsorption or -globulin precipitation by polyethylene glycolleads to an increased binding of tracer analyte and hance falsely low analyte estimates. Radioimmunoassays for thyroglobulin using double antibody separation procedures have however been designed to be free from interference from circulating autoantibodies [9] in contrast to those where marked interference occurs [10] Similarly, if the human antibody cross-reacted

with the specific analyte antibody preventing binding of the analyte the resultant inhibition of response would lead to a falsely elevated, or positively biased result. This is probably the mechanism responsible for the raised TSH estimations described earlier [5]. Addition of species specific carrier serum would be expected to neutralise this effect but would be ineffective for autoantibody interference.

Further consideration of Fig. 1 (B) illustrates the mechanism of interference in solid phase second antibody applications. Here the presence of a heterophilic antibody will lower the effective concentration of primary analyte antibody leading to a reduced immunoassay response, as a result of a similarly reduced binding to second antibody, and the consequence of positive bias. Again, addition of species specific carrier serum would be expected to mitigate this effect but at the expense of an increase in the optimal concentration of second antibody.

Imma interference

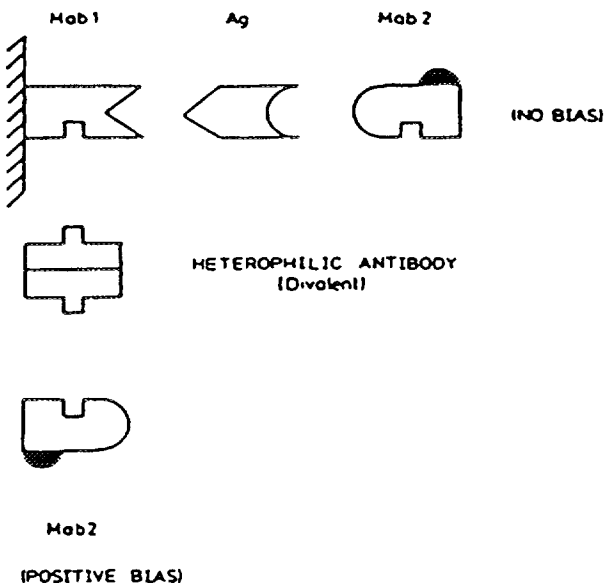


Fig. 2. Schematic diagram illustrating interference in immunometric assay-twin mouse monoclonal antibodies.

The more familiar situation, heterophilic antibody bridging between twin mouse monoclonal antibodies in immunometric assays causing spuriously elevated analyte estimations is illustrated in Fig. 2. A similar situation was apparent in earlier immunoradiometric assays employing identical species antibodies to form the requisite immunocomplex; guinea pig guinea pig for hepatitis B antigen [4]; rabbit : rabbit for thyrotrophin [11, 12] and mouse mouse for TSH, hCG, and creatine kinase -MB isoenzyme [13, 14, 15, 16, 17]. It has been advocated[6] that a change in species of one of the antibodies may abolish the interference although this has also been disputed [12]. This antibody arrangement, Fig. 3, does however introduce further complexity. If the heterophilic antibody recognises epitopes upon both solid phase specific analyte antibody (sheep) and the labelled antibody (mouse, monoclonal antibody) then the conventional bridging situation exists as in Fig. 2 and positive bias results. However, in a UK-EQAS experiment designed to simulate heterophilic antibody interference, a sheep anti-mouse IgG (SAM) was added to analyte samples at 0.005% and 0.05% for a range of analytes. The data for growth hormone is reproduced here as Table 1. The ALTM indicated that in general addition of SAM had little effect for a range of analytical immunoassays divided approximately, equally between radioimmunoassay and immunometric assay. Two of the immunometric assays demonstrated the

expected positive bias as a result of antibody interference. However, the immunoradiometric assay using solid phase sheep polyclonal anti-hGH operated by the Institute of Biochemistry at Glasgow Royal Infirmary exhibited a marked negative bias proportional to the concentration of added SAM. The mechanism for this finding, Fig. 3, must relate to the SAM action being specific to the monoclonal antibody, effectively sequestrating the monoclonal antibody and reducing the assay response with the consequence of marked negative bias. Therefore, in situations where the species of antibodies complexing the analyte are different the interference is far more problematic for the clinical chemist, causing either positive bias or negative bias dependent upon the spectrum of specificity of the heterophilic antibody.

Imma interference

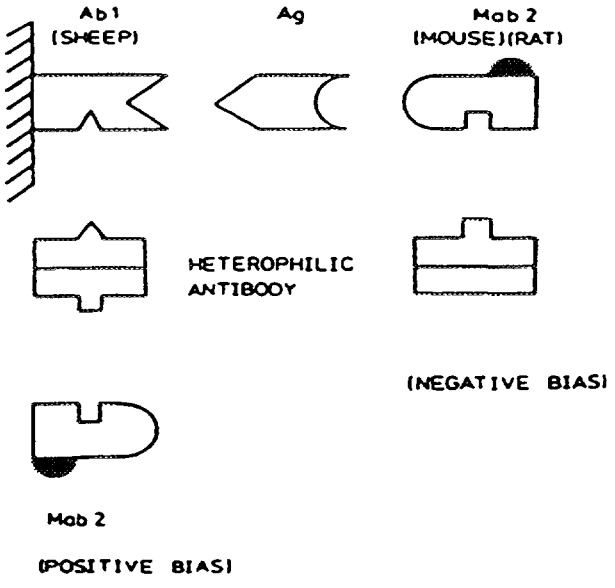


Fig. 3.Schematic diagram illustrating interference in immunometric assay — antibodies from different species.

The incidence of heterophilic antibody interference as reported in the literature is widely variable. In an assay devoid of any factors likely to prevent the discovery of heterophilic antibodies Boscato [16] demonstrated the likelihood that 50% of test sera could be implicated. A 15% false positive rate was detected in a normal population assayed by an 'in-house' two-site mouse monoclonal antibody assay for hCG. This is in contrast to lower incidence figures of 9.1% [17], 7% [6], 6.6% [3], and 1.2% [4] with several estimates lower than 1% [18,19,20]. The wide discrepancy in incidence undoubtedly relates to the success or otherwise of modifications to assay protocols designed to neutralise the interference.

Three patients sera with heterophilic antibody were studied in detail by Csako [21]. The conclusion of this study is detailed in Fig. 4. A two-site monoclonal antibody immunoradiometric assay for thyrotrophin was used with the addition of mouse whole IgG and fragments in an attempt to block the effect of heterophilic antibody. Addition of intact mouse IgG or Fc fragment successfully inhibited the interference, whereas Fab or F9ab)2 fragments failed int his respect since the expected positive bias was maintained. Rat and horse IgG and fragments were variable and ineffective respectively. These authors forwarded the view that if heterophilic antibodies were solely directed to epitopes coincident with the constant region of the analyte antibodies then

substitution of whole IgG by Fab or F(ab)2 fragments would be expected to prevent heterophilic antibody interference. However, this view must be regarded as simplistic since Boscato [1] demonstrated heterophilic antibodies directed against an epitope residing upon the F(ab)2 fragments of mouse IgG, which is common to a variety of species including bovine, ovine, equine, guinea pigt, rat and monkey immunoglobulin but not cat, dog or rabbit immunoglobulin. Heterophilic antibody directed to rabbit IgG epitopes was however clearly demonstrated by Howanitz [12].

TABLE 1. ADDITION OF SHEEP ANTI-MOUSE IGG TO AN HGH IMMUNOMETIC ASSAY

GROWTH HORMONE (mU/L)

Method	Sheep Anti-Mouse IgG			Comments
	0	0.005%	0.05%	RIA/IRMA
1. ALTM	6.1	6.7	5.9	21:27
2. RIA	5.5	5.5	5.3	
3. Netria (IRMA)	7.3	96–110.5	68.99	
4. IDS	8.3	12.8	8.2	
5. PHARMACIA	5.8	5.7	5.4	
6. CELLTECH	6.6	6.6	6.4	
7. GRI	6.1	4.0	1.3	

Imma interference

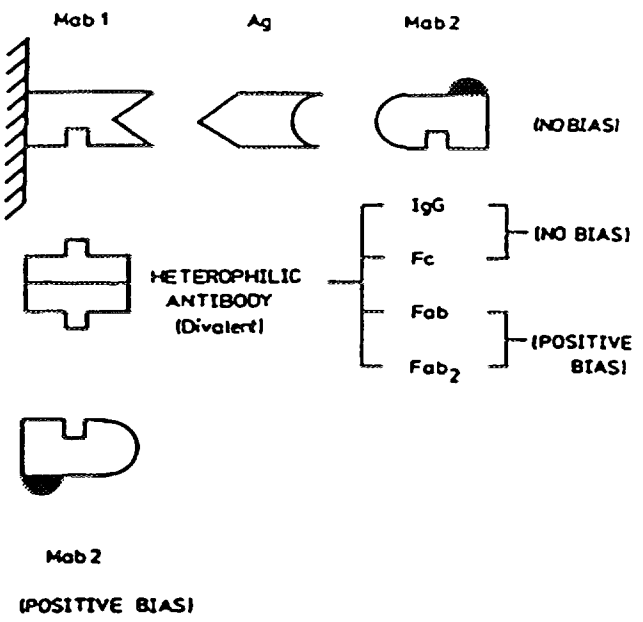


Fig. 4. Schematic diagram illustrating interference in immunometric assay — Diminution by IgG fragments.

As a result of these studies modifications to immunoassay protocols have been devised to neutralise the effect of heterophilic antibodies. Usually inclusion of normal animal serum, as a source of the appropriate IgG, of identical species to the specific antibodies involved in the analysis solves the problem [12, 15, 16]. Use of animal serum IgG unrelated to the species of specific assay antibody has proven of mixed success [6, 17]. It would seem prudent for clinical chemists to beware spuriously elevated or depressed results and question their clinical validity. Consequently a bank of sera with properties of assay interference could be established of obvious value to 'in-house' and commercial assay manufacturers in deciding appropriate strategies to fully counteract this interference.

9.3. HUMAN ANTI-MOUSE ANTIBODIES

The interference caused by heterophilic antibodies has now become enhanced by the increasing clinical application of mouse monoclonal antibodies for immunoscintigraphy and immunotherapy in oncology [22, 23]. Human antimouse antibody (HAMA) titres induced by the injection of murine monoclonal antibodies have been shown to be several fold greater than those observed for heterophilic antibodies [24]. False positive responses caused by HAMA were demonstrated in four immunometric assays for CEA, one 'in-house' and three commercial systems despite the inclusion of 1% mouse serum to prevent heterophilic antibody effects [24, 25]. Increasing the concentration of mouse serum in the 'in-house' assay, or heating the samples to 70°C, was ineffective in removing the HAMA interference. Extreme measures; polyethylene glycol precipitation, heat treatment to 90°C, adsorption with anti-human IgG or Protein A, were necessary to abolish the HAMA effect. Kricka et al [26] studied HAMA interference in a two site immunoenzymatic assay of hepatitis B surface antigen in two patients receiving mouse monoclonal immunoglobulins. Interference was blocked by inclusion of mouse immunoglobulin in one patient but in the other the specific therapeutic antibody required to be included to counteract the HAMA effect.

Other workers have clearly demonstrated HAMA interference to be due to human anti-idiotypic antibodies elicited in response to the injection of OC-125 monoclonal antibody raised against CA-125 in immunoscintigraphy for ovarian cancer [27, 28]. The interference caused here cannot be removed by the inclusion of mouse immunoglobulin in the CA-125 assays. These difficulties appear to be compounded in that the monoclonal antibodies used in clinical treatment and either side of the immunometric assay are identical. A remedial measure is maybe to ensure that the monoclonal antibodies used in clinical practice and immunoanalysis are different. In cases where this is not possible at current levels of scientific progress inclusion of non-specific mouse immunoglobulin allotypically and isotypically matched to the reagent antibody would seem to be good analytical practice. However, due to the continued existence of interference compounded by the clinical applications of monoclonal antibodies, the clinical chemist must remain vigilant in detecting analytically biased results to ensure that false clinical decisions do not ensue. At this stage it would seem prudent to study patients subjected to monoclonal antibody therapy separately, particularly if expensive and tedious pretreatment procedures become necessary to prevent HAMA interference. This policy if instituted would require the development of rapid HAMA screening assays to isolate those samples requiring pretreatment if clinical and diagnostic efficiency were to be maintained.

9.4. SPECIFIC IN IMMUNOMETRIC ASSAY

Interference in immunoassay techniques can be apparent if the antibody reagents employed are not entirely specific for the analyte. Assessment of adequate specificity therefore represents

a key factor in the optimisation of any immunoassay if interference is to be minimised. In limited reagent assays, radioimmunoassay, analysis of specificity is conventionally assessed by the substitution of standard analyte by potential interferant or cross-reactant. Any reactivity between antibody and cross-reactant causes a reduction in assay response (using % bound vs. Dose of analyte as coordinates) consequent to a potential overestimate in subsequent analyte estimation.

However, assessment of specificity in immunometric assays is more complex and unless particular care is taken in appropriate conclusions may be drawn. The majority of excess reagent assays, immunometric assays, use two separate antibodies recognising different epitopes on the analyte. Cross-reaction may therefore occur due to recognition by both antibodies together or either labelled or solid phase antibody alone assuming both antibodies are present in a single incubation. To a certain degree non-specificity can be improved by designing assays to include separate incubations interspersed with a wash step but this is tedious and abnormal in busy clinical chemistry laboratories particularly for 'in-house' immunoradiometric assays.

The difficulties associated with specificity assessment in immunoradiometric assay were reemphasised on consideration of the results of a UK EGAS investigation into the effects of human placental lactogen (H.L.) addition to a growth hormone (HGH) [ambones]. Table 2 provides a summary of the data. Radioimmunoassay methods were shown to cross-react, identified as an expected overestimate of analyte concentration as described above. Examination of the data from excess reagent methods again demonstrated wide variation in cross-reactivity from a minimal 8% to a highest recorded level of 565%. However, the interference or cross reactivity always led to overestimates or positive bias. The immunoradiometric assay operated by the Institute of Biochemistry, Glasgow Royal Infirmary, was apparently free from cross-reaction and totally specific. This situation was received with some surprise since other investigations conducted during the optimisation phase of this assay, which used a sheep polyclonal anti-HGH IgG on solid phase and a radioiodinated mouse monoclonal antibody, has led us to the conclusion that a marked cross-reactivity, or interference, would have been expected from H.L. Indeed a project initiated by this situation to replace the mouse monoclonal antibody had been in place for some months prior to the UK-EGAS experiment. To investigate further three experiments were undertaken. Firstly, a classical specificity assessment where separate standard curves were constructed one with HGH standards and the other with the HGH standard substituted by H.L. Secondly, four standard HGH curves were examined three of which were subjected to constant H.L. additions of 50 250 and 10 000 μg/L respectively. Thirdly, the recovery of a constant concentration of HGH was estimated from a series of ten pregnancy sera of variable endogenous H.L. concentrations.

As a result of these studies modifications to immunoassay protocols have been devised to neutralise the effect of heterophilic antibodies. Usually inclusion of normal animal serum, as a source of the appropriate IgG, of identical species to the specific antibodies involved in the analysis solves the problem [12, 15, 16]. Use of animal serum IgG unrelated to the species of specific assay antibody has proven of mixed success [6, 17]. It would seem prudent for clinical chemists to beware spuriously elevated or depressed results and question their clinical validity. Consequently a bank of sera with properties of assay interference could be established of obvious value to 'in-house' and commercial assay manufacturers in deciding appropriate strategies to fully counteract this interference.

Fig. 5 illustrates the results of the cross reactant substitution experiment. The dose response curve for HGH behaved as expected with an increasing response directly proportional to the dose. However, a two phase calibration curve was obtained upon substitution with H.L. the signal

increasing up to a concentration of 250 μg/L which could be interpreted as cross-reaction equivalent to 5 μg/L (10 MU/L) HGH. AT higher concentrations the signal begins to fall eventually returning to baseline levels at an H.L. dose of 60 000 μg/L (60 mU/L). This should not however be taken as evidence that at elevated H.L. doses the cross reactivity improves!! Eventually achieving zero. Immunoassayists will recognise this phenomenon as the high dose 'hook effect' a potential hazard for the misclassification of analyte doses in single stage immunometric assays caused by the analyte dose exceeding the binding capacity of one or both of the constituent analyte antibodies. Nevertheless, standard substitution methods, widely used in the assessment of specificity in radioimmunoassay, are obviously inadequate for immunometric methods.

A clue to the resolution of this dilemma is given in the cross-reactant addition experiment, Fig. 6. At the lower concentrations of H.L. added a dramatic increase in responsivity occurs in those tubes not containing HGH standard, with the remainder of the standard curve exhibiting a flattened appearance. The flattened response curve is maintained at higher levels of H.L. addition but the overall responsivity is reduced as the capacity of the analyte antibodies is exceeded. The addition experiment demonstrates clearly that an assay with a serious H.L. cross-reaction would be unable to demonstrate analytical recovery of added HGH, depending upon the initial concentrations of both HGH and H.L. In the UK-EGAS experiment, Table 2, the apparent absence of H.L. cross-reaction in the Glasgow assay resulted from the addition of 6 mU/L H.L. to a sample of undetectable HGH concentration, where only increases in assay response quantified poor specificity. In this case the H.L. addition exceeded the binding capacity of either constituent antibody resylting in an inhibition of assay response with the resultant erroneous conclusion that specificity was appropriate when in fact the contrary was the case. Had the UK-EGAS experiment been performed on a serum containing 10 μg/L (20 mU/L) of endogenous HGH then the addition of 6000 μg/L (6 mU/L) H.L. would have caused substantial signal suppression consistent with a marked specificity problem.

TABLE 2. SUMMARY OF UK-EGAS CROSS REACTION EXPERIMENT

Summary of UK EGAS Cross Reaction Experiment
(Basal pool + 6 mU/L H.L. 73/545)

Method	GH Increment (mU/l)	% Cross Reactivity*
A. Limited reagent methods		
'In house' RIA	2.8	47
CIS RIA	6.6	110
B. Excess reagent methods		
'In house' non-isotopic	3.8	63
Celltech Sucrosep	33.9	565
Netria IRMA	1.5	25
Pharmacia	3.6	60
Hybritech Tandem-R	0.5	8
IDS Omnia	9.4	157
C. GRI 'in house' IRMA	0	0

*% Cross Reactivity = $\frac{\text{hGH increment (mU/l)}}{\text{100 hPL added (mU/l)}}$

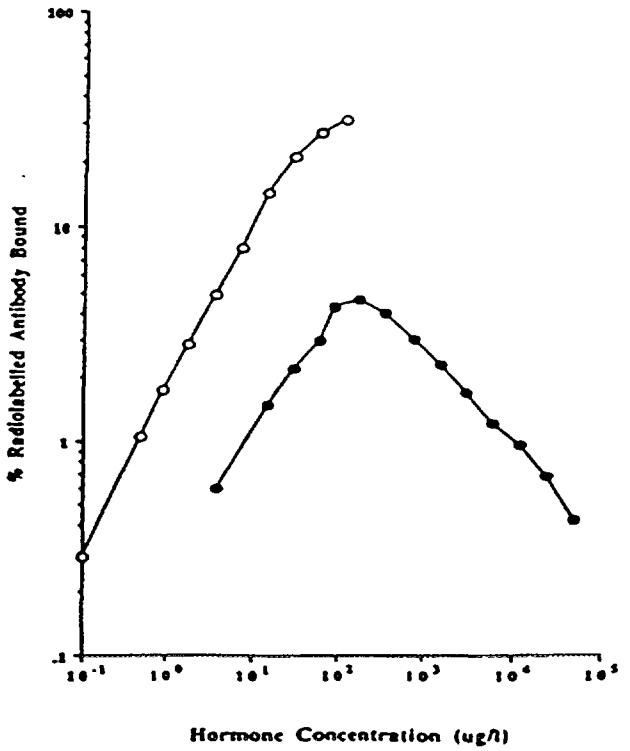


Fig. 5. Dose response curves for HGH (-) and H.L. (-) in a two site HGH immunoradiometric assay.

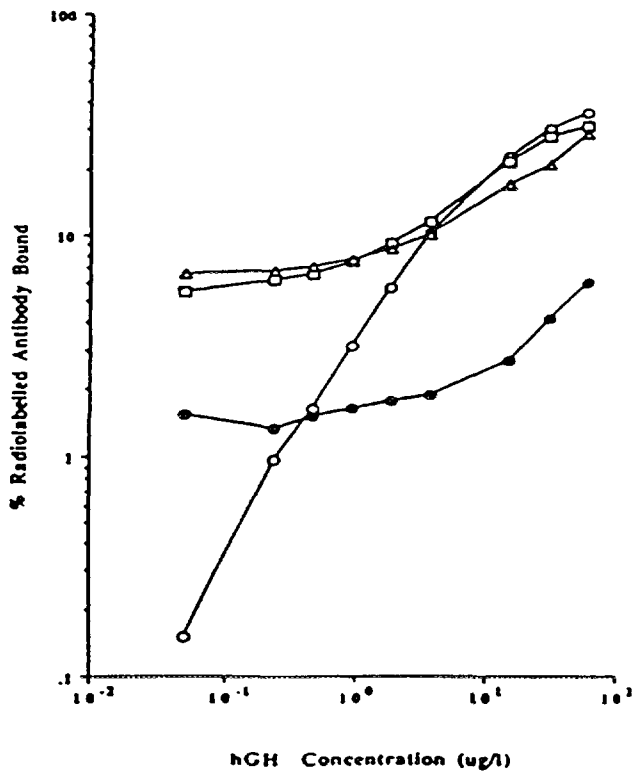


Fig. 6. The effect of H.L. addition ([] - []) 50 µg/L. - 250 µg/L and - 10.000 µg/L H.L.) to an HGH does response curve (-).

A recovery experiment with the addition of a constant HGH concemntration (11.7 mU/L) to a series of 10 pregnancy sera, with endogenous H.L. levels of 4–21 mU/L exhibited the expected lack of recovery secondary to poor specificity. (Table 3). Substitution of an alternative mouse monoclonal antibody of improved specificity for HGH and reassay of identical recovery samples gave a mean recovery of 104% (range 93–120%).

The importance of recovery estimations in the assessment of specificity was further confirmed by a study designed to determine TSH cross reactivity during the optimisation stage of an immunoradiometric assay for FSH [30]. Here the effect of addition of hTSH [IRP 80/558] at 0.25 and 100 mU/L to the FSH dose response curve was examined (Fig. 7).

The presence of increasing concentrations of hTSH standard caused a progressive reduction in assay response for each of the FSH standards studied suggesting an interference or lack of specificity. However, a recovery experiment was performed on 31 male sera, the majority of which were hypothyroid (TSH range 3.8–230 mU/L), with the addition of a constant 5 U/L FSH (IRP 78/549). The mean recovery of FSH was 101.3% demonstrating no interference or lack of specificity. The HTSH standard used in these studies must therefore have contained substances, not present in serum, which were capable of binding to one of the antibodies causing a reduced signal in the hFSH IRMA.

Specificity assessment in immunoradiometric assay is therefore more complex than radioimmunoassay. It is not appropriate to rely on conventional standard substitution or addition experiments since misleading conclusions may be drawn. Standard substitution data is widely used as a measure of specificity in the scientific literature, consequently it is prudent to question

Recovery of HGH added to the sera of pregnant subjects

TABLE 3. RECOVERY OF HGH ADDED TO PREGNANCY SERA

Basal hPL concentration (mU/L)	Basal hGH concentration (mU/L)	Basal +11.7 mU/L hGH (UK8) (mU/L)	% Recovery
4.0	5.87	6.38	4.3
7.0	1.67	2.35	5.8
8.1	2.30	2.79	4.2
9.0	2.87	3.29	3.6
10	2.37	2.88	4.4
11	2.44	2.80	3.1
12	2.26	2.72	3.9
15	2.08	2.27	1.6
15	1.75	1.94	1.6
21	1.02	1.15	1.1

Mean Recovery 3.36%

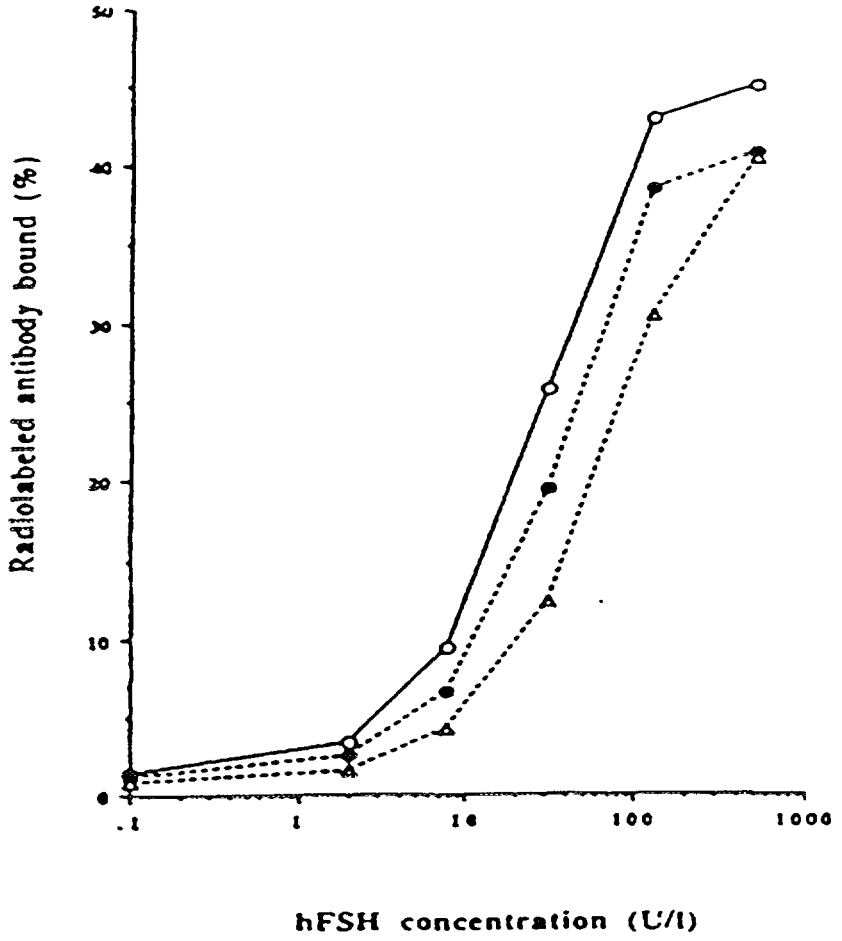


Fig. 7. The effect of hTSH [80/558] addition (..... 25 mU/L. -- .100 mU/L on an hFSH dose response curve (-).

the validity of specificity data in cases where interference causes analytical and clinical opinion to diverge. Problems may also be caused related to the quality of standard materials used as a basis for the specificity assessment. The assessment of recovery of hormone standard, preferably the international reference preparation, from patient samples containing Patho-physiological levels of potential cross-reacting species is recommended as currently the most reliable indicator of cross-reaction or interference problems.

ACKNOWLEDGEMENTS

I am indebted to my former colleagues M.G. McConway and D. Griffin of the Scottish Antibody Production Unit, together with B. Perry and G.H. Beastall of the Institute of Biochemistry, Glasgow Royal Infirmary for participation in the specificity studies. Also Dr. J. Seth for permission to reproduce data from the UK NEQAS for Peptide Hormones and Related Substances and Mrs. L. Currie for secretarial assistance.

REFERENCES

[1] BOSCATO, L.M., STUART, M.C., Heterophilic antibodies: a problem for all immunoassays. Clin. Chem 1988, 34: 27–33.

[2] GOSLING, J.P., A Decade of Development in Immunoassay Methodology. Clin.Chem. 1990, 36. 1408–1427.

[3] SGOURIS, J.T., Limitations of the Radioimmunoassay for Hepatitis B Antigen New Engl J Med. 1973; 288: 160–161.

[4] PRINCE, A M., BROTMAN, B , JASS D., IKRAM, H., Specificity of the Direct Solid–phase Radioimmunoassay for detection of Hepatitis B Antigen Lancet 1973; 16: 1346–1350.

[5] HEDENBORG, G., PETTERSSON, T., Carlstrom A., Heterophilic antibodies causing falsely raised thyroid stimulating hormone results. Lancet 1979; ii: 755.

[6] HUNTER, W.M., BUDD, P.S., Curculating antibodies to ovine and bovine immunoglobulin in healthy subjects: A hazard for immunoassays. Lancet 1980; ii. 1 1 36.

[7] SCHMIDT, N.A., DOCTER, R., Aberrant Results for Peptide Hormone Radioimmunoassays in the Presence of Antibodies to Rabbit IgG in Patients Sera. Clin. Chem. 1984; 30: 1427–1428 (Lett)

[8] SAKATA S., NAKAMURA, S., MIURA, K., Autoantibodies against thyroid hormones or iodothyronines. Implications in diagnosis, thyroid function, treatemtn and pathogenesis Ann Intern Med. 1985, 103: 579–589.

[9] BLACK, E.G., HOFFENBERG, R., Should one measure serum thyroglobulin in the presence of antithyroglobulin antibodies? Clin. Endocrinol. 1983; 19: 597–601.

[10] SCHNEIDER, A.B., PERVOS, R., Radioimmunoassay of human thyroglobulin effect of anti–thyroglobulin autoantibodies. J. Clin. Endocrinol. Metab. 1978; 47: 126–137.

[11] SCHAISON, G., THOMOPOULOS, P., MOULIAS, R., FEINSTEN, M.D., False hyperthyrotrophinaemia induced by heterophilic antibodies against rabbit serum. J. Clin. Endocrinol. Metab. 1981; 53: 200–202.

[12] HOWANITZ, P.J., HOWANITZ J.H., LABERSON, H.V., ENNIS, K.M., Incidence and Mechanism of Spurious Increases in Serum Thyrotrophin. Clin Chem 1982, 28. 427–431

[13] HAMBURGER, J.I., Factitious elevation of Thyrotrophin in euthyroid patients. New Engl. J. Med 1985; 314–521

[14] ZWEIG, M.N., CSAKO, G., BENSON, C.C., WEINTRAUB, B.D., KAHN, B.B., Interference by Anti-Immunoglobulin G. Antibodies in Immunoradiometric Assays for Thyrotrophin in euthyroid patients. New Engl. J. Med. 1985; 314–521.

[15] BOCK, J L., FURGINELE, J., WENZ, B., False positive immunometric assays caused by anti-immunoglobulin antibodies: a case report. Clin. Chimica. Acta. 1985, 147: 241–246.

[16] BOSCATO, L.M., STUART, M.C., Incidence and Specificity of Interference in Two site Immunoassays. Clin.Chem. 1986; 32: 1491–1495.

[17] THOMPSON, R.J., JACKSON, A.P., LANGLOIS, N., Circulating Antibodies to Mouse Monoclonal Immunoglobulins in Non-nal Subjects - Incidence, Species, Specificity and Effects

on a Two site Assay for Creatine-Kinase-MB Isoenzyme. Clin.Chem. 1986; 32: 476–481.

[18] JOHN, R., HENLEY, R., BARRON, N., Antibody interference in a two site immunometric assay for thyrotrophin. Ann. Clin. Biochem. 1989; 26: 346–352.

[19] GENDREL, D., FEINSTEIN, M.C., GREINER, J., Falsely elevated serum thyrotrophin (TSH) in newborn infants· Transfer from mothers to infants of a factor interfering in the TSH radioimmunoassay. J. Clin. Endocrinol. Metab. 1981; 52: 62–65.

[20] CZERNICHOW, P., VANDALEM, J.L., HENNEN, G., Transient neonatal hyperthyrotrophinaemia: A fictictous syndrom edue to the presence of heterophilic antibodies in the plasma of infants and their mothers. J. Clin. Endocrinol. Metab. 1981; 53: 387–393.

[21] CSAKO, G., WEINTRAUB, B.D., ZWEIG, M.H. The potency of immunoglobulin G fragments for inhibition of interference caused by anti-immunoglobulin antibodies in a monoclonal immunoradiometric assay for thyrotrophin. Clin. Chem. 1988; 34: 1481–1483.

[22] BALDWIN, R.W., BYERS, V.S., Monoclonal antibodies in cancer treatment. Lancet 1986; 1: 603–605.

[23] SCHROFF, R.W., FOON, K.A., BEATTY, S.M., Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. Cancer Res. 1985; 45: 879–885.

[24] HANSEN, H.J., LA FONTAINE, G., NEWMAN, E.S., et.al. Solving the problem of antibody interference in commercial 'sandwich'-type immunoassay of carcinoembryonic antigen. Clin Chem. 1989; 35: 146–151.

[25] PRIMUS, J., KELLEY, E.A., HANSEN, H.J., GOLDENBERG, D.M., Sandwich-type imunoassay of carcinoembryonic antigen in patients receiving murine monoclonal antibodies for diagnosis and therapy. Clin. Chem. 1988; 34: 261–264.

[26] KRICKA, L.J., SCHMERFELD-PRUSS, D., SENIOR M., GOODMAN, D.B.P., KALADAS P., Interference by human Anti-Mouse antibody in two site immunoassays. Clin. Chem. 1990; 36: 892–894.

[27] REINSBERG, J., HEYDWEILLER, A., WAGNER, U., PFEIL, K., OEHR, P., KREBS, D., Evidence for Interaction of human Anti-idiotypic antibodies with CA-125 determinaton in a patient after Radioimmunodetection. Clin. Chem. 1990; 36: 164–167.

[28] BOERMAN, O.C., SEGERS, M.F.G., POELS, L.G., KENEMANS, P., THOMAS, C.M.G Heterophilic Antibodies in Human Sera Causing Falsely Increased Results in the CA-125 Immunofluorimetric Assay. Clin. Chem. 1990; 36: 888–891.

[29] PERRY, B., CHAPMAN, R.S., MCCONWAY, M.G., GRIFFIN, D., BEASTALL, G.H., Specificity of two site immunometric assays. Ann. Clin. Biochem. 1991; 28: 83–86.

[30] MCCONWAY, M.G., CHAPMAN, R.S., PERRY, B., BEASTALL, G.H., Second IRP [80/558] hTSH standard composition influences specificity assessment of an hFSH immunometric assay Ann. Clin. Biochem. 1991; 28: 526–528 (Lett.).

Chapter 10

DETECTION OF HEPATITIS C VIRUS RNA USING REVERSE TRANSCRIPTION PCR

S.F. Yap
Department of Allied Health Sciences,
Faculty of Medicine, University of Malaya,
Kuala Lumpur, Malaysia

10.1. INTRODUCTION

Hepatitis C virus (HCV) is a major aetiologic agent of non-A, non-B (NANB) post-transfusion hepatitis. It is a positive stranded RNA virus of ~10 000 nucleotides. The viral genome comprises a 5' non-translated region (NTR) and a 3' NTR flanking a continuous single open reading frame (ORF) encoding a single polyprotein. The polyprotein is divided into a 5' structural region comprising putative core and envelope proteins, and a 3' region comprising non-structural (NS1 to NS5) proteins. Based on its genomic organization, the virus resemble most closely members of the flaviviridae family. HCV is classified into a series of 'genotypes' on the basis of differences in the nucleotide sequence of various regions of the genome particularly the core and NS5 regions. Some genotypes show worldwide distribution whereas others are more restricted geographically. The most common genotypes are types 1, 11, 111, IV and V (Mori/Okamoto) which corresponds to Simmonds types la, lb, 2a, 2b and 3a respectively.

Routine laboratory diagnosis is dependent on detection of anti-HCV antibodies that are produced in response to the infection. Commercially available diagnostic assays utilize a series of recombinant HCV antigens whose amino acid sequences are deduced from the nucleotide sequence of different regions of the viral genome. The objective of inclusion of several antigenic proteins in the assay, particularly those from regions that are less influenced by genotype variation, is improvement of the assay sensitivity in view of the significant genomic heterogeneity of the virus.

Detection of the viral genome (HCV RNA) is by a combination of cDNA synthesis and PCR followed by gel analysis and/or hybridization assay. In principle, cDNA is synthesized using the viral RNA as template and the enzyme, reverse transcriptase. The cDNA is then amplified by PCR and the product detected. Agarose gel electrophoresis provides a rapid and simple detection method; however, it is non-quantitative. Further, nested PCR is necessary to achieve the required sensitivity. To improve on the sensitivity as well as the specificity, various hybridization assays are employed for detection. Format that allow for quantitation of the viral load have also been described. HCV RNA detection has been applied to the diagnosis of acute HCV infection, to monitor response to anti-viral therapy and to supplement serological testing. The assay protocol described in this paper is adapted from that published by Chan et al. Comments on various aspects of the assay are based on experience with the method in our laboratory.

10.2. HCV RNA ASSAY

10.2.1. Specimen handling

Care must be taken when handling samples to ensure stability of the HCV RNA. Blood is collected into sterile plain tubes and centrifuged as soon as possible (within 4 hours) and the

serum stored in suitable aliquot. If analysis is delayed for more than 24 hours, it is recommended that the serum be stored frozen. Care must also be taken to ensure that there is no cross-contamination of specimens.

10.2.2. Materials and reagents

All materials and reagents employed must be sterile and RNase free. All reagents employed are molecular grade.

Primers are derived from the highly conserved 5' non-translated region of the viral genome. Primer sequences are as follows:

Primer	Region	5' base position	Polarity #	Sequence 5' to 3'
209	5'NTR	8	-	ATACTCGAGGTGCACGGTCTACGAGACCT
211	5'NTR	29	-	CACTCTCGAGCACCCTATCAGGCAGT
939	5'NTR	297	+	CTGTGAGGAACTACTGTCTT
940	5'NTR	279	+	TTCACGCAGAAAGCGTCTAG

Orientation of primer sequence (+ = sense; - = anti-sense)

10.2.3. Reverse transcriptase

Activity: 5' to 3' DNA polymerase

Substrate: RNA or DNA template with an RNA or DNA primer bearing a 3'hydroxyl group

Reaction:

$$DNA_{OH} \text{ or } RNA_{OH} \xrightarrow[Mg^{++}\ dATP,dTTP,dGTP,dCTP]{RT} DNA\text{-}({}_pdN)_n + nPP_i$$
$$RNA\text{-}({}_pdN)_n + nPP_i$$

Reference: Sambrook p 5.55

The enzyme employed in the assay is Superscript II RNase H Reverse Transcriptase from BRL. The enzyme is preferred as it is devoid of both RNase A and RNase H activity. If enzyme employed carry RNase H activity, it is necessary to include an RNase inhibitor in the reaction to prevent degradation of the template during reverse transcription.

10.3. HCV RT-PCR PROTOCOL

10.3.1. Sample preparation

Reagents required:
Trizol LS reagent from BRL
Chloroform

Isopropyl alcohol
75% ethanol
RNase free water

Equipment required:
Microcentrifuge
Microfuge tubes
Micropipettes and plugged tips

Procedure:

Unless otherwise stated, the following procedure is carried out at room temperatur:

(1) Add TRIZOL LS reagent to serum in a ratio of 3:1 and incubate for 5 minutes.

(2) Add 0.2 mL of chloroform for every 0.75 mL of TRIZOL LS reagent. Shake vigorously for 15 seconds and let stand for 2 to 15 minutes.

(3) Spin at 12000 × g for 15 min at 4°C.

(4) Transfer the upper clear aqueous phase to a fresh clean tube and precipitate the RNA with 0.5 mL isopropyl alcohol per 0.75 mL of TRIZOL LS reagent.

(5) Incubate for 10 min and centrifuge at 12000 × g for 10 min at 4°C.

(6) Discard supernatant and wash RNA pellet with 75% ethanol adding at least 1 mL of 75% ethanol for every 0.75 mL TRIZOL LS reagent. Mix and centrifuge for 5 min at 4°C.

(7) Discard ethanol and air-dry RNA pellet.

(8) Resuspend pellet in 20 µL RNase free water.

10.3.2. Reverse transcription

Reagents required:
Reverse transcriptase (Superscript II)
RNase free water
Primer for cDNA synthesis (primer 1, p2O9)
dNTPs

Equipment required:
Waterbath
Micropipette and plugged tips

Procedure:

(1) Transfer 2-5 µL of RNA into a fresh clean tube.

(2) Add primer I (0.3) µM) and water to 10 µl.

(3) Heat at 90°C for 2 min and then cool on ice.

(4) Add 4 µL 5X cDNA synthesis buffer; final conc.:

50 mM Tris-HCl pH8.3
75 mM Kcl
3 mM $MgCl_2$

2 µL 0.1 M DTT
3 µL dNTPs (10 mM stock) and
water to 19 µl

(5) Equilibrate at 37°C for 2 min and add 1 µL of reverse transcriptase, mix gently and incubate at 37°C for 45 min to 1 hour. Place on ice or freeze.

10.3.3. Amplification

Reagents required:
10 × PCR buffer (supplied with Taq)
dNTPs
Outer primers, 209 & 939 (for 1st round PCR)
Inner (nested) primers, 211 & 940 (for 2nd round PCR)
Taq DNA polymerase
RNase free water
Mineral oil

Equipment required:
Micropipette and filter tips
Thermal cycler

Procedure:

First round PCR:

1. Prepare PCR premix as follows:

Reagent	Final concentration
10 × PCR buffer	1x
dNTPs	0.2 mM each
Primers	0.3 µM each
Taq	1 unit
Water to 25 µl	

Final reaction volume = 30 µl

2. Overlay the premix with oil and add 5 µL of cDNA to the PCR mix and cycle using the following parameters:

Temperature (oC)	Time
94	10 min
94	1 min
50	1 min
72	2.5 min
72	10 min

Total number of cycles = 30 µl

Second round PCR:

Use 2-5 µL of the 1st round PCR product with the same cycling parameters and PCR mix except that the primers are the inner nested ones and the final volume is 50 µl.

10.3.4. Detection

Reagents required:
Agarose
Ethidium bromide (10 mg/mL)
1 × TAE or TBE buffer
Sample loading dye (bromophenol blue)
Molecular weight marker (eg. I 00 bp ladder)

Equipment required:
Gel tank and power pack
Magnetic stirrer with hot plate or microwave oven
UV transilluminator

Procedure:

(1) Prepare a 1.5–2% agarose gel using either 1 × TAE or TBE buffer.

(2) Add 4 parts of PCR product to 1 part of loading dye, mix and load into the gel.

(3) Load about 200 ng of molecular weight marker into the first lane of the gel.

(4) Run at 80–100 V.

(5) View the bands on a UV transilluminator.

Quality control

(1) Include in each run a known positive and negative control serum.

(2) Include a 'reagent control' that contains water instead of sample.

(3) Observe all recommended procedures and precautions to avoid amplicon contamination.

BIBLIOGRAPHY

CHAN, S.W., MCOMISH, F., HOLMES, E.C., et al., Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants, J. Gen. Virol. (1992) 73, 1131–1141.

OKAMOTO, H., KURAI, K, OKADA, S., et al., Full length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes, Virology (1992) 188, 331–341

SAMBROOK, J., FRITSCH, E.F., AND MANIATIS, T., Molecular cloning: a laboratory manual, second edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

SIMMONDS, P., Variability of hepatitis C virus, Hepatology (1995) 21:570-583.

SIMMONDS, P., HOLMES, E.C., CHA, T.A., et al., Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS5 region, J. Gen. Virol (1993) 74 2391-2399.

Chapter 11

TRAINING MANUAL ON THE ANALYSIS OF MICROSATELLITE REPEATS IN HUMAN DNA FOR DIAGNOSTIC APPLICATIONS

P. Ioannou
Cyprus Institute of Neurology and Genetics,
Nicosia, Cyprus

11.1. INTRODUCTION

Until recently, the development of genetic maps of eukaryotic genomes has been limited by the availability of markers. The earliest maps of human chromosomes were developed using a variety of markers, including blood group antigens, serum protein polymorphisms, and erythrocyte enzymes. These markers were generally quite uninformative and required a diverse range of biochemical and immunological techniques for analysis. This changed rapidly after the discovery of widespread restriction fragment length polymorphisms (RFLPs), which can be analyzed using Southern blot hybridization. Markers based on RFLPs proliferated rapidly during the early 1980s and were soon supplemented by the more informative minisatellite or variable number of tandem repeat (VNTR) polymorphisms. Low-resolution maps of the human and mouse genomes have been completed using RFLPs and VNTRs but are limited in their usefulness because of the relatively low informativeness of most RFLPs and the non-random distribution of VNTRs in the human genome.

The recent discovery that simple sequence length polymorphisms (SSLPs), or microsatellites, are highly polymorphic has provided a rich source of genetic markers for the development of high-resolution maps. SSLPs are ideal markers because they are widely distributed throughout eukaryotic genomes and can be efficiently analyzed using the polymerase chain reaction (PCR). Each SSLP is based on the variable numbers of di-, tri-, or tetranucleotide repeats at a particular location and can easily be characterized (genotyped) using PCR primers that anneal to single-copy DNA flanking the repetitive element. Thousands of SSLPs have now been characterized for the human genome. In contrast to Southern blotting, PCR technology is easily automated and permits very-high-throughput typing of the many samples necessary for development of high-resolution maps of eukaryotic genomes. The recent development of moderate-resolution maps of both human and mouse genomes built entirely with SSLPs reflects the rapid conversion from manual Southern blot-based markers to semi-automated PCR-amplified markers during the last few years. Furthermore, these markers can also be used as 'sequence-tagged sites' (STS) in physical maps and provide a direct connection between the genetic and physical maps of eukaryotic chromosomes.

From the diagnostic point of view, the large numbers of highly polymorphic SSLP markers provide an extremely useful tool for the diagnosis of genetic disorders in the absence of any detailed knowledge of the exact molecular defect in each family. However, the use of such markers in diagnosis requires careful consideration of a number of factors, including:

(1) The markers should be closely linked to the gene of interest to minimize the risks of recombination. Intragenic SSLP markers are the most useful, and the risks of recombination can be reduced to a minimum when such markers are used in combination with markers at the 5' and 3' ends of the gene. However, quite often, only a limited number of markers may

be available at some distance from the gene, so a careful evaluation of the chances of recombination may be necessary.

(2) Highly polymorphic SSLPs tend to show some instability, with the emergence of new alleles not found in either parent. Sufficient family members should be analysed to detect such errors and avoid taking them into consideration for diagnosis.

(3) Most importantly, the use of SSLP markers in diagnosis depends on the accuracy of the clinical diagnosis and on the analysis of sufficient family members to establish unambiquously linkage between the markers being used and the disease under study.

Despite these difficulties, the identification of an ever- expanding number of genes and the disorders associated with them, forces diagnostic laboratories to look for gene-specific rather than mutation-specific approaches, since the latter approach requires a detailed knowledge of the exact molecular defect in each family. The ability to automate to a large extent the analysis of microsatellites means on the other hand that different families may be analysed simultaneously with the same SSLP markers. It is likely that in the future a minimum number of microsatellite repeats will be available for disease causing genes, while mutation specific approaches will be used only in those cases where particular mutations are found at high frequency, or where the family structure doe not allow the use of SSLP markers.

This unit describes basic methods for analysis of SSLP markers. Additional methods more commonly used for the analysis of mutations at disease-related loci may also be used for genotyping families. These include analysis of specific mutations by allele-specific hybridization, analysis of single-stranded conformation polymorphisms (SSCP), denaturing gradient gel electrophoresis (DGGE) and restriction analysis of PCR products (RFLPs).

11.2. PROCEDURE

Typical time schedule

Day 1

Labelling of one PCR primer from each pair to be used with [-^{32}P]-ATP using polynucleotide kinase. Setting up of PCR reactions, to be run overnight on thermal cycler. Preparation of sequencing gel, preferably left to polymerize overnight.

Day 2

Preparation and loading of PCR samples for electrophoresis on sequencing gel. Electrophoresis of samples. Recovery of sequencing gel and autoradiography.

Day 3

Visual analysis of autoradiograms and evaluation of the results.

Labelling of PCR primers by [-^{32}P-ATP] and setting up of PCR reactions

Specific simple sequence length polymorphisms (SSLPs) are amplified from genomic DNA using a -^{32}P-end-labelled primer in the PCR mix. PCR products are denatured and resolved on a

denaturing polyacrylamide gel, which is then used to expose autoradiographic film. SSLP genotypes are determined by visual examination of band patterns on the autoradiogram.

Radiation precautions: [-^{32}P]ATP is used in the procedures described below. Observe precautions for radiation handling diligently, so as to avoid contamination of yourselves, equipment and lab facilities. Use safe pipetting procedures to minimize creation and spreading of microdroplets and geiger counters for monitoring radioactivity in the working area. Use double gloves and dispose outer pair as soon as any contamination on hands is detected.

Reagents/Consumables for labelling of PCR primers with [-^{32}P]-ATP

- 20 µM solution for each primer to be labeled
- 10x T4 polynucleotide kinase buffer (no ATP)
- 500 µCi [-^{32}P]ATP (10 mCi/mL, 3000 Ci/mmol)
- T4 polynucleotide kinase (10 U/µl)
- Sterile H_2O
- One set of automatic pipettes: P2, P20, P200, P1000
- Radiation badge
- Sterile eppendorf tubes and tube opener
- Radiation protection block for holding 1.5–2.0 mL tubes
- Radiation shield
- Benchkote or similar bench cover
- Solid radiation waste container
- Gloves
- Ice bucket and supply of fresh ice
- Heating block (water bath) at 37°C
- Heating block (water bath) at 65°C

Thaw [-^{32}P]ATP in hood behind radiation screen and put on ice as soon as it is ready. Thaw other reagents on ice. Label tubes for PNK reaction. Place in perspex blocks for radiation protection. PNK reaction mix, order of addition of reagents (labelling oligo sufficient for 10 PCR reactions, allow 10% excess for wastage during pipetting):

- 2.0 µL 5x T4 polynucleotide kinase buffer
- 4.0 µL H_2O (adjusted as necessary to give final volume of 10 µl)
- 2.0 µL forward or reverse primer (20 µM) (adjust volume as necessary according to concentration)
- 1.0 µL 10 mCi/mL [-32P]ATP (3000 Ci/mmol) (1 µCi/reaction, fresh)
- 1.0 µL 10 U/µL T4 polynucleotide kinase (PNK) (Total volume 10 µL)
- Water bath, incubate 30 min. at 37°C.
- Heat block, incubate 10 min. at 65°C to heat inactivate polunucleotide kinase.
- Transfer to ice.

It does not usually matter which primer is end labelled. If one primer has sequence homology to a repetitive DNA element (e.g., Alu), the PCR products detected may include numerous inter-repeat PCR products that interfere with the analysis of the desired SSLP. In such cases end labelling the other primer will often produce a cleaner banding pattern.

The labelled primer may be stored at –20°C for a few days, but it is better to use it as soon as possible. No further purification of the labelling reaction mixture is necessary prior to PCR.

Reagents/consumables for setting up of PCR

- Genomic DNA: 5 to 20 ng/µL DNA in TE buffer or H_2O (not denatured)
- 20 µM solutions of two pairs of forward and reverse primer, one from each pair labelled by [-^{32}P]ATP as above
- 10x PCR amplification buffer
- 1.25 mM 4dNTP mix
- 5 U/µL Taq polymerase
- light mineral oil
- 2x formamide loading dye
- Sterile 0.2 mL or 0.5 mL PCR tubes (preferably 0.2 mL)
- Radiation protection block for holding 0.2–0.5 mL tubes
- DNA Thermal Cycler (preferably with heated lid, e.g. Perkin Elmer 9600 model or MJR, capable of holding 96 × 0.2 mL samples or 48 × 0.5 mL samples)

Prepare PCR mix (sufficient for 10 × 25 µL PCR reactions, allow 10% excess for wastage during pipetting) in PNK reaction tube:

- 10 µL PNK reaction mix with labelled forward or reverse primer (1 µL per sample)
- 25 µL 10x PCR amplification buffer (2.5 µL per sample) (no Mg)
- 7.5 µL $MgCl_2$ solution (50 mM)
- 15 µL 1.25 mM 4dNTP mix (1.5 µL per sample, final 75 µM)
- 2 µL 20 µM unlabelled reverse or forward primer, (adjust volume as necessary according to concentration)
- 2 µL 5 U/µL Taq polymerase (1 U per sample)
- 137.5 µL H_2O (adjust volume as necessary to give 20 µL per sample)
- Mix gently

Label clearly required number of 0.5 mL or 0.2 mL PCR tubes. Pipet 5 µL template DNA into each tube. It is good practice to keep DNA samples in numerical order.

Transfer 20 µL of each PCR mix to appropriate tube already containing the DNA. **Follow good pipetting practice, avoid creation of microdroplets.**

Overlay each tube with 60 µL light mineral oil (not necessary for 0.2 mL tubes on thermal cyclers with heated lid). Centrifuge the tubes briefly at 1000 to 2000 rpm, 4°C to ensure that all reagents are below the oil layer.

Program thermal cycler

Program thermal cycling parameters in accordance with the primers selected and the thermal cycling device used. Typical PCR conditions for a reaction containing primers with a Tm of 60°C are shown below. Usually, an annealing temperature 5°C below the minimum Tm of the PCR primers should be tried. This can be gradually adjusted upwards if nonspecific bands are identified.

Step	**Conditions**
1. Initial denaturation	94°C/5 min
2. Cycling	94°C /30 sec 55°C/1 min 72°C/1 min 24 cycles
3. Last cycle	94°C /2 min 55°C/2 min 72°C/5 min
4. Soak at 4°C	

Start thermal cycler without tubes. When block temperature reaches 94°C, add tubes to thermal cyclers quickly, press down well **(caution, block is hot!)** and complete PCR reactions overnight. Adding the tubes to the block after it is hot reduces the chance of primer misannealing, which can lead to artifacts.

Once PCR is completed, transfer PCR reactions from thermal cycler into Plexiglass block. Open very gently. Add 25 µl of 2x formamide loading buffer to each well. Because of its higher density, the buffer will descend below the oil layer and mix with the PCR reaction. There is no need to remove the oil or to add the buffer below overlay. Store tubes containing PCR reactions at –20°C in a Plexiglas box before or after addition of formamide loading buffer. Maximum signal is obtained when samples are run on gel immediately, but sample remains usable when stored <=2 weeks at –20°C.

Formamide loading buffer, 2x

Deionized formamide
0.05% (w/v) bromphenol blue
0.05% (w/v) xylene cyanol FF
20 mM EDTA
Do not sterilize
Store at –20°C

CAUTION: Formamide is hazardous!

Preparation of denaturing polyacrylamide gel

Reagents/consumables for making and running sequencing gels and for autoradiography:

- PCR samples for electrophoresis in formamide loading buffer
- 70% ethanol or isopropanol in squirt bottle
- 5% (v/v) dimethyldichlorosilane (Sigma) in $CHCl_3$ or equivalent lint-free paper towel
- 40% denaturing acrylamide gel solution (19:1) (see recipe)
- TEMED
- 10% (w/v) ammonium persulfate (make fresh weekly and store at 4°C)
- 1x TBE buffer, pH8.3 to 8.9 (see recipe)
- 30× 40-cm front and back gel plates
- 0.4 mm uniform-thickness spacers
- Large book-binder clamps
- 60 mL syringe
- 0.4 mm shark's-tooth or preformed-well combs
- Sequencing gel electrophoresis apparatus
- Power supply with leads
- 95°C heating block or water bath

- 46 × 57-cm gel blotting paper (e.g., Whatman 3 mM)
- Kodak XAR-5 X ray film or other comparable film
- Automatic or manual X ray film processor
- **10xTBE electrophoresis buffer, 1 liter**
- 108g Tris base (890 mM)
- 55g boric acid (890 mM)
- 40 mL 0.5 M EDTA, pH8.0 (20 mM)

CAUTION: Dimethydichlorosilane, acrylamide gel solution, TEMED, and formamide are hazardous!

Assemble the gel sandwiches

Meticulously wash front and back 30 × 40-cm gel plates with soap and water. Rinse well with deionized water and dry. Wet plates with 70% ethanol or isopropanol in a squirt bottle and wipe dry with Kimwipe or other lint-free paper towel.

Apply a film of 5% dimethyldichlorosilane in $CHCl_3$ to one side of each plate by wetting a Kimwipe with the solution and wiping carefully. After the film dries, wipe plate with 70% ethanol or isopropanol and dry with a Kimwipe. Check plates for dust and other particulates. (Siliconizing of bottom plate is usually sufficient in most cases and ensures that gel sticks to the top plate during plate separation (see below)).

Assemble gel plates according to manufacturer's instructions, with the silanized surfaces facing inward. Use 0.2 to 0.4 mm uniform-thickness spacers and large book-binder clamps, making certain side and bottom spacers fit tightly together.

Prepare denaturing acrylamide gel solution as indicated in the following table:

Preparation of denaturing acrylamide gel solution

Reagent	**Acrylamide concentration**		
	4%	**6%**	**8%**
Urea (ultrapure, gr)	25.2	25.2	25.2
38% acrylamide/2% bisacrylamide (mL)	6.0	9.0	12.0
10x TBE (mL)	6.0	6.0	6.0
H_2O (mL)	27	24	21
Total volume (mL)	60	60	60

To speed dissolution of urea, the gel mix can be heated; however, to prevent degradation of acrylamide and urea, do not heat over 55°C. Filter solution through Whatman no. 1 filter paper. Store 2 to 4 weeks at 4°C. Quantities are for a single sequencing gel. If gels are poured daily, make solution in large quantities (e.g., make 1 liter by multiplying above quantities by 16.7). Solutions of acrylamide deteriorate quickly, especially when exposed to light or left at room temperature.

CAUTION: Acrylamide and bisacrylamide are hazardous; observe proper guidelines on handling, storage, and disposal.

Thoroughly mix 60 µL TEMED, then 0.6 mL of 10% ammonium persulfate, into 60 mL acrylamide solution of desired concentration immediately before pouring gel. To achieve slower polymerization, reduce amounts of TEMED and ammonium persulfate to 40 µL and 0.4 mL, respectively, or use solution at 4°C.

Pour gel immediately. Gently pull acrylamide solution into a 60 mL syringe, avoiding bubbles. With short plate on top, raise upper edge of gel sandwich to 45 angle from the benchtop and slowly expel acrylamide between plates along one side. Adjust angle of plates so gel solution flows slowly down one side.

When solution reaches top of short plate, lower gel sandwich so that the top edge is ~5 cm above benchtop. Place an empty disposable pipet-tip rack or stopper underneath the sandwich to maintain the low angle. Insert flat side of a 0.2 to 0.4 mm shark's-tooth comb into the solution 2 to 3 mm below top of short plate, being very careful to avoid bubbles. Use book-binder clamps to pinch combs between plates so that no solidified gel forms between combs and plates. Layer extra acrylamide gel solution onto comb to ensure full coverage. Cover gel top with saran wrap. Put weight over the comb and allow to polymerize overnight.

Alternatively, insert teeth of preformed-well comb into gel solution and clamp as above. Rinse syringe with water to remove acrylamide.

11.3. ANALYSIS OF PCR PRODUCTS ON SEQUENCING GELS

Set up the electrophoresis apparatus

When gel polymerizes, remove bottom spacer or tape at bottom of gel sandwich. Remove extraneous polyacrylamide from around combs with razor blade. Clean spilled urea and acrylamide solution from outer plate surfaces with water. Remove shark's-tooth comb gently from gel sandwich without stretching or tearing top of gel. Clean comb with water so it will be ready to be reinserted as below.

If preformed-well comb was used, take care to prevent tearing of polyacrylamide wells. This comb will not be reinserted.

Fill bottom reservoir of gel apparatus with 1x TBE buffer so that gel plates will be submerged 2 to 3 cm in buffer. Place gel sandwich in electrophoresis apparatus and clamp plates to support. Sweep out any air bubbles at bottom of gel by squirting buffer between plates using syringe with a bent 20-G needle.

Pour 1x TBE buffer into top reservoir to ~3 cm above top of gel. Rinse top of gel with 1x TBE buffer using a Pasteur pipet.

Reinsert teeth of cleaned shark's-tooth comb into gel sandwich with points just barely sticking into gel. Using a Pasteur pipet, rinse wells thoroughly with 1x TBE buffer to remove stray fragments of polyacrylamide. If a preformed-well comb is used, this step is omitted.

Preheat gel ~30 min by setting power supply to 45 V/cm, 1700 V, 70 W constant power.

Load and run the gel

Rinse wells with 1x TBE buffer just prior to loading gels, to remove urea that has leached into them.

Samples containing 50% formamide loading dye are heated prior to electrophoresis to denature the DNA. The simplest way to heat samples is to program the thermal cycler to hold at 94°C for the specified period of time, then place the tubes on ice. Load 2 to 3 μL sample per well. Keep sequence of samples as for original DNA samples. Rinse sequencing pipet tip twice in lower reservoir after dispensing from each reaction tube.

Run gels at 45 to 70 W constant power. Maintain a gel temperature of ~55–60°C. Observe migration of marker dyes to determine length of electrophoresis as indicated in the following table.

DNA fragments (in bp) that migrate with dyes in denaturing polyacrylamide gels

%gel	BPB	XC
5	35	130
6	26	106
8	19	75
10	12	55
20	8	28

Temperatures >65°C can result in cracked plates or smeared bands; too low a temperature can lead to incomplete denaturation. To ensure even conduction of the heat generated during electrophoresis, an aluminum plate (0.4 cm thick, 34 × 22 cm) can be clamped onto the front glass plate with the same book-binder clamps used to hold the gel sandwich to the apparatus. The aluminum plate must be positioned so that it does not touch any buffer during electrophoresis. Newer versions of sequencing equipment from most companies include a back plate in direct contact with a water or buffer chamber for better heat uniformity.

Process and dry the gel

Fill dry ice traps attached to gel dryer (if required) and preheat dryer to 80°C.

After electrophoresis is complete, drain buffer from upper and lower reservoirs of apparatus and discard liquid as radioactive waste.

Remove gel sandwich from apparatus and place under cold running tap water until surfaces of both glass plates are cool. Lay sandwich flat on paper towels with short plate up. Remove excess liquid and remaining clamps or tape. Remove one side spacer and insert long metal spatula between glass plates where spacer had been. Pry plates apart by gently rocking spatula.

If only the back plate was siliconized, the gel should stick to the top plate. Slowly lift top plate from the side with inserted spatula, gradually increasing the angle until the top plate is completely separated from the back plate.

Once plates are separated, remove second side spacer and any extraneous bits of polyacrylamide around gel.

Hold two pieces of dry 46 × 57 cm blotting paper together as one piece. Beginning at one end of gel and working slowly towards the other, lay paper on top of gel. Take care to prevent air bubbles from forming between paper and gel.

Peel blotting paper up; gel should come off plate with it. Gradually curl paper and gel away from plate as it is being pulled away.

Wrap gel and support with Saran wrap, so that gel is completely covered in plastic. Monitor the gel with a Geiger counter. Carry out autoradiography for 2 to 24 h at –70°C using one intensifying screen. It is not necessary to dry the gel when ^{32}P is used. This is necessary only for ^{35}S and ^{33}P. Autoradiography at –70°C increases signal and also prevents labeled DNA molecules from diffusing in the gel during exposure.

If it is necessary to dry the gel, place paper and gel on preheated gel dryer. Cover with plastic wrap. Remove any bubbles between plastic wrap and gel by gently rubbing covered surface of gel from middle toward edges with a Kimwipe. Dry gel thoroughly 20 min to 1 h at 80°C. When gel is completely dry, the plastic will easily peel off without sticking.

Remove plastic wrap and place dried gel in X ray cassette with Kodak XAR-5 film in direct contact with gel. Autoradiograph at room temperature. After sufficient exposure time (usually overnight), remove X ray film and process.

A labelled DNA size marker should be loaded on each gel to orient the film and to permit comparison of specific alleles on different gels. A simple ladder that is suitable for most purposes is a -^{32}P-labelled MspI digest of pBR322; this contains 12 bands in the 100 to 250 bp range, which is appropriate for most SSLPs.

To compare PCR products for a large set of DNAs run on different gels, either the PCR products of one or two reference DNAs or a 1-bp-resolution -^{32}P-labelled M13 sequencing ladder should be loaded on each gel.

After autoradiography, visually examine autoradiogram, determine alleles present in each sample with each marker examined and place on the pedigree to construct the genotype.

11.4. USEFUL GUIDELINES FOR DESIGNING PCR ASSAYS

One of the most important elements in designing PCR assays is the selection of primer-pair sequences. A systematic approach in selecting primer pairs-to improve the percentage of functioning assays and to standardize conditions for amplification-is particularly crucial for generating a large number of PCR assays for physical and genetic mapping. A variety of computer programs (that can be operated on personal computers), both commercial and freely distributed, are available to assist in primer selection. Some programs are designed solely to assist in the design of PCR primers, and some include this capacity as part of a larger set of programs for DNA analysis. Current computer-assisted strategies for primer selection routinely produce assays that are >90% successful. Important features for computer-assisted analysis of DNA sequences prior to primer selection are described below.

Analyzing DNA sequence

An ideal PCR assay for genetic and physical mapping amplifies a DNA product that corresponds to a unique DNA site. DNA sequences obtained from genomic or chromosome-specific clones usually represent random tracts of DNA derived from either unique or repetitive DNA sequences. PCR assays derived from repetitive DNA sequences (e.g., Alu and LINE in the human genome) will usually give a multitude of products that do not allow detection of a specific locus. Therefore, the DNA sequence obtained from a specific clone should be analyzed as follows before proceeding with primer-sequence design.

11.4.1. Search for duplicate sequences

When clones obtained from a particular genomic, short-insert, or chromosome-specific library are sequenced, duplicate sequences may often be identified. Several factors can introduce bias in the selection of clones that are used to generate sequences for sequence-tagged sites (STSs). Repeated growth of the library selects for faster-growing clones, thus enriching the library for this subset of clones. PCR amplification of the DNA insert using Alu primers or linker primers selects for all Alu- or linker-containing sequences, which may include similar sequences in different clones; and picking the same clone more than once produces duplications.

11.4.2. Search for vector sequences

Sequences that contain no insert or short inserts must be identified systematically to avoid designing primers that include vector sequences.

11.4.3. Search for repetitive DNA sequences

Analysis of DNA sequence for repetitive sequences (e.g., Alu or LINE) requires computer assistance. Current data obtained from sequencing random clones from short-insert human genomic DNA libraries shows that ~15% of sequences contain known repetitive DNA sequences (e.g., Alu, L1, or alphoid repeats). Specific regions of the human and other genomes can contain a higher proportion of repetitive sequences. Screening of known repeats using homology-identification programs (e.g., FASTA or BLASTN) will significantly reduce the proportion of unusable assays.

11.4.4. Determine the length of the repetitive element

The length of the repetitive element is predictive of the informativeness of the SSR. Informativeness-as measured by heterozygosity or polymorphic information content (PIC)-is correlated with the minimum length of uninterrupted repeats. Therefore, it is desirable to screen out sequences with short or interrupted repeats before developing PCR primers. Analysis of large numbers of human (CA)n repeats (the best-characterized SSR in human genomic DNA) has demonstrated that repeats with <12 dinucleotides are usually monomorphic and that most (CA)n repeats with n>20 have heterozygosities >70%. The repeat length of tri- and tetranucleotide repeats also appears to be related to informativeness but there is still insufficient data to establish the predictiveness of repeat length for each class of repeat. The average length of (CA)n repeats appears to be greater in the mouse genome than in the human. However, ~90% of murine (CA)n repeats with n>10 are informative in M. musculus x M. spretus backcrosses and 50% are informative in crosses between inbred strains of one species.

11.4.5. Selecting primer pairs or sets

The ideal set of PCR primers should all amplify using identical PCR conditions. Designing primers for use in assays that employ uniform conditions permits routine testing of numerous samples and multiplex amplification of PCR products in the same reaction. Primer design is much facilitated by computer-assisted analysis of DNA sequences. The analysis should take the following elements into account:

1. Annealing temperature (Tm)

Simply determining the GC content of specific sequences does not provide an accurate estimate of Tm; the program should compute the Tm of the proposed primer sequences and identify those of suitable length that have a Tm in the desired range. If many forward and reverse oligonucleotides are possible from a given sequence, primer pairs with the most similar Tms are recommended.

2. Complementarity

Complementarity within or between primer sequences can lead to unwanted secondary structures or primer-dimer formation, both of which can interfere with the assay. The program should recognize unwanted complementarity of the oligonucleotides to themselves or each other.

3. Product length

The program should select primers that generate PCR products of the desired length.

4. Position of primer relative to repeat sequence

Polymorphic markers derived from microsatellite repeat sequences (SSLPs) require that the primers be derived from the sequence that flanks either side of the repeat. It is useful for the program to be able to recognize simple sequence [e.g. (CA)n] repeats and target them to be included within the PCR product. Primers selected >=50 bp from the repeat have a lower tendency to produce 'stutter' bands.

Extensive testing of parameters for SSLP and STS primers has indicated that 18 to 24 mer primers with Tms of 58–62°C routinely work well. Such primers are capable of generating 100– to 250 bp PCR products for SSLPs analyzed on polyacrylamide gels and 150 to 300 bp products for STSs analyzed on agarose gels.

11.4.6. Testing the primer pairs

Testing of new primer pairs involves an initial screening of genomic DNA by PCR amplification at several annealing temperatures, Mg2+ concentrations, and cycling times to determine the optimal reaction conditions for each primer pair. Thermal cyclers used for PCR have different operating characteristics (ramping times, tendency to overshoot or undershoot temperatures, and accuracy of temperature settings) and these influence optimal conditions for a given assay. Once optimal conditions are determined by experimental trials, they are usually reproducible for assays designed using similar primer parameters. Assays that do not work under standard conditions are unsuitable for laboratories interested in high-throughput PCR testing. It may be possible to improve weak or negative PCR assays by increasing or decreasing annealing temperature by 5°C or modifying the Mg2+ concentration of the reaction buffer. It is advisable to abandon assays that are not improved by these modifications because it is unlikely that the

assay will be robust, even with optimized conditions, when experimental DNA preparations are tested.

11.4.7. Maximizing throughput

Large-scale use of SSLPs is facilitated by the use of two simple and inexpensive items that can increase throughput dramatically - a 12-channel pipettor and 96-well microtiter plates. A more expensive but extremely useful tool is an electronic, programmable multichannel pipettor. Routinely used genomic DNA templates can be stored at the concentration used in the PCR reactions (e.g. 5 to 20 ng/μL) in 96-deep-well microtiter plates with appropriate covers (Beckman). These DNA templates can then be transferred to 96 well PCR plates using a 12 channel pipettor. PCR mix can be loaded using a repeating pipettor. Mineral oil and 2x formamide loading buffer can be added from a reservoir using a 12-channel pipettor. Gel loading is more rapid and accurate using a 12 channel Hamilton syringe and shark's-tooth combs (Owl Scientific) that are correctly spaced for the 96 well format.

LIST OF CONTRIBUTORS

CHAPMAN, R.S.	Division of Biochemical Endocrinology, Department of Metabolic Medicine, The royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, United Kingdom
COWAN, S.	Biochemistry Department, Glasgow University, Glasgow, United Kingdom
EASON, R.	Biochemistry Department, Glasgow University, Glasgow, United Kingdom
IOANNOU, P.	Cyprus Institute of Neurology and Genetics, P.O. Box 3462, Nicosia 1683, Cyprus
LEAKE, R.E.	Biochemistry Department, Glasgow University, Glasgow, United Kingdom
YAP, S.F.	Department of Allied Health Sciences, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia