Chapter 6

IMMUNOASSAYS IN CLINICAL CHEMISTRY (PRINCIPLES OF IMMUNORADIOMETRIC ASSAYS)

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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

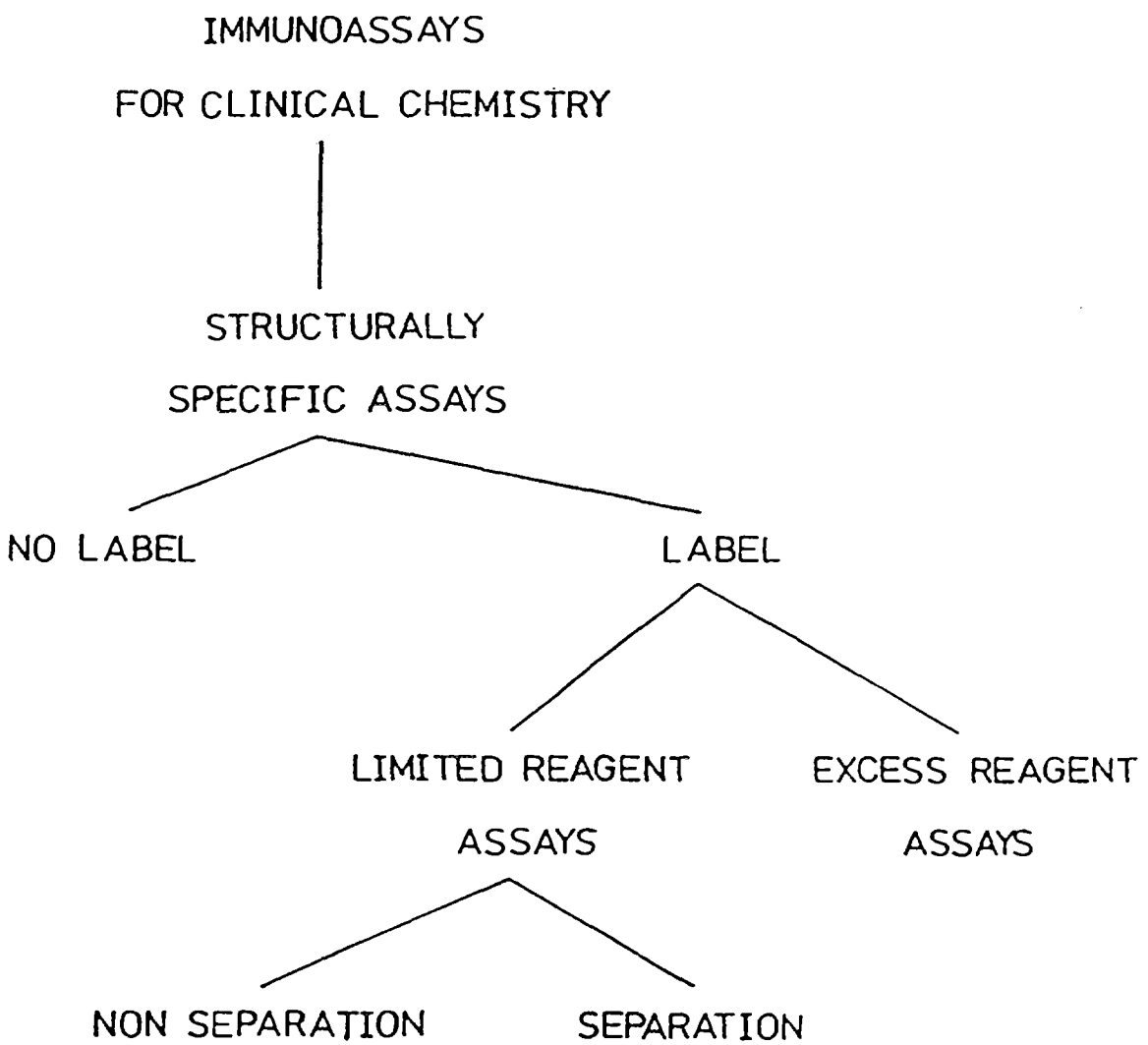
The minimum detection limit of the two site assay was restricted by the appearance of a 'bland' binding in the absence of analyte. The degree of 'blank' was a function of the quality of

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.

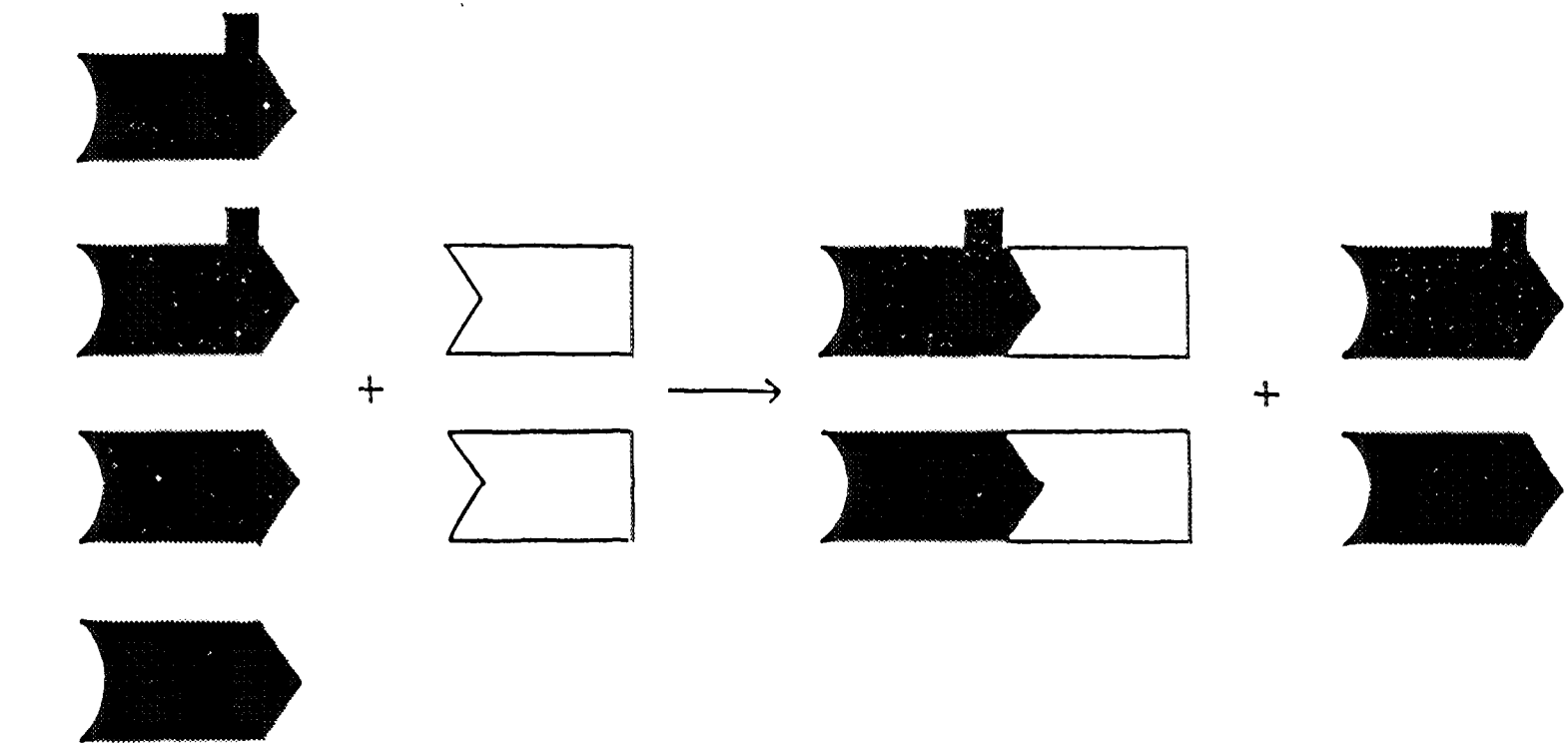
Since monoclonal antibodies are also epitope specific they can be used in simultaneous incubations significantly reducing incubation times.

6.4. ALTERNATIVE LABELS

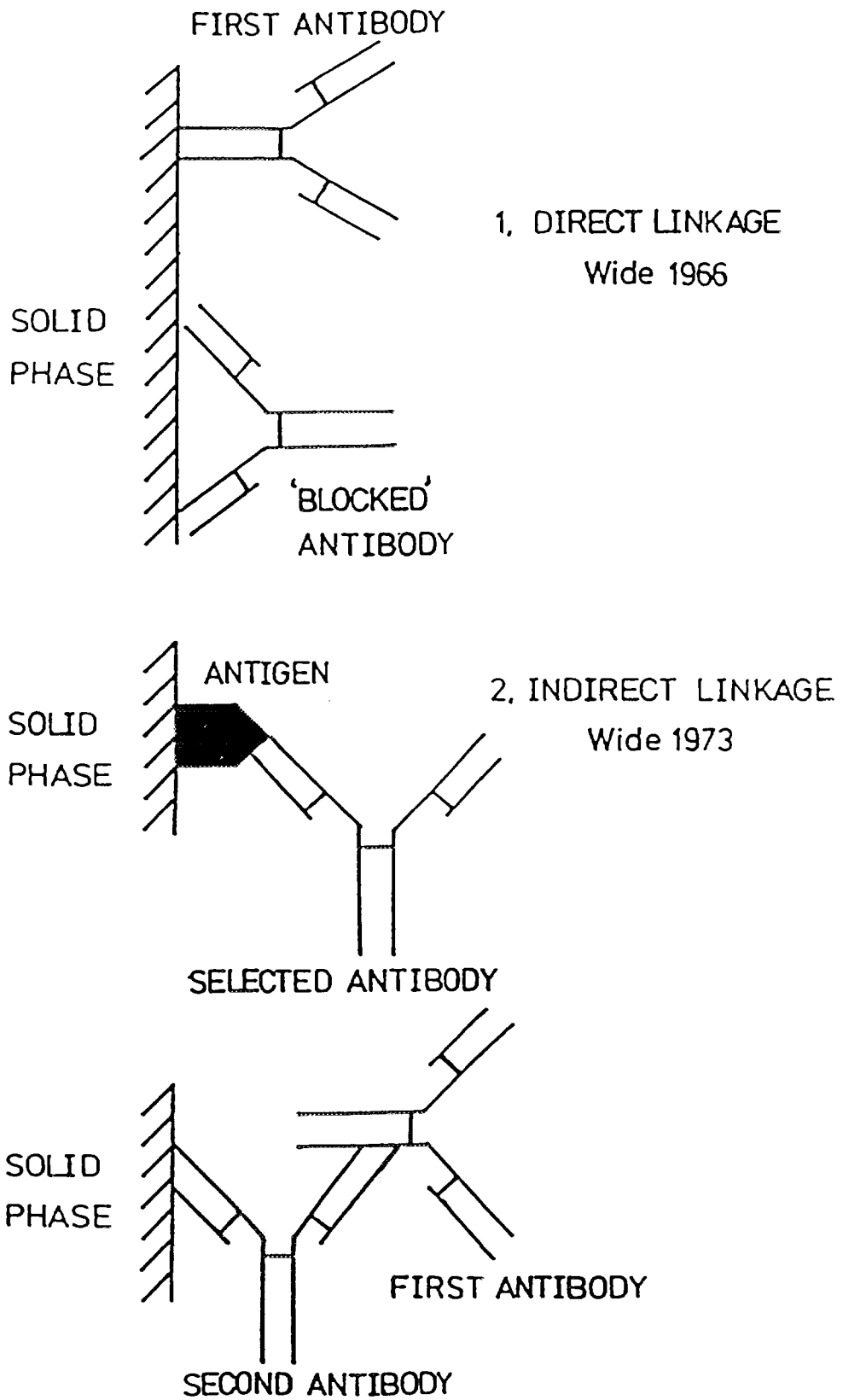
At this stage of development radioisotopically labelled antibodies are limiting improvements in minimum detection limits. If 100 000 cpm ^{125}I labelled antibody are present as total counts then a 'blank' level of 0.05% represents 50 cpm, a level difficult to discriminate from background radioactivity 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

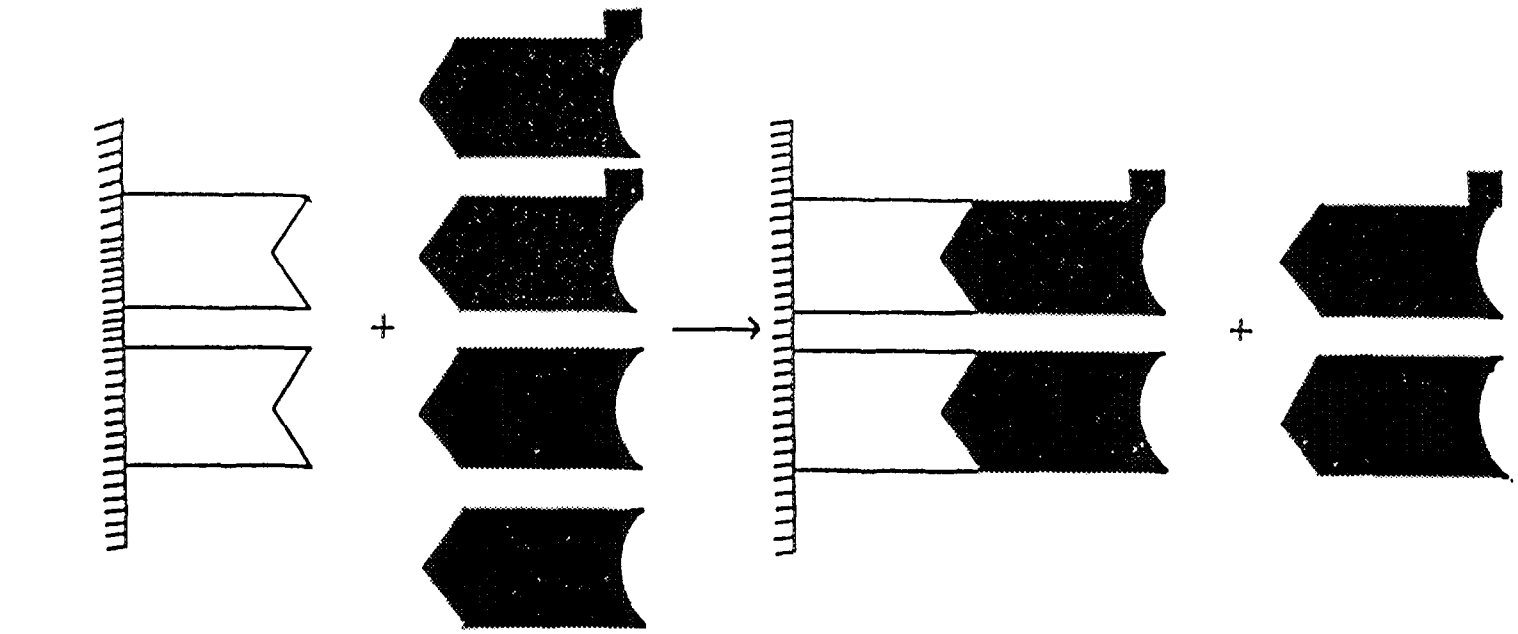
Limited Reagent Methods



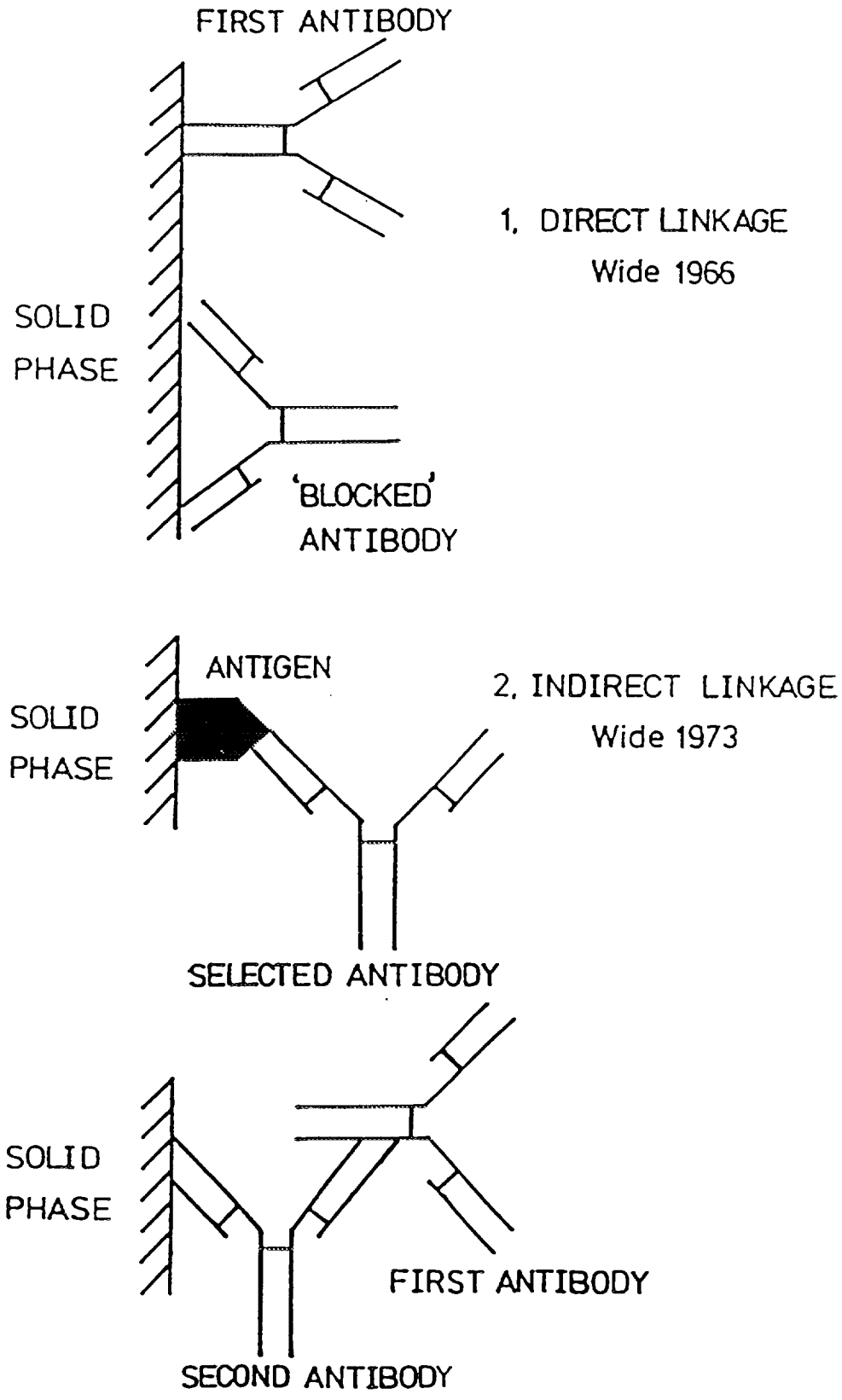
Saturation Assay ; RADIOIMMUNOASSAY ; Protein Binding Assay


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

Limited Reagent Methods



SOLID PHASE RADIOIMMUNOASSAY


FIRST ANTIBODY
1. DIRECT LINKAGE
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2. INDIRECT LINKAGE
Wide 1973
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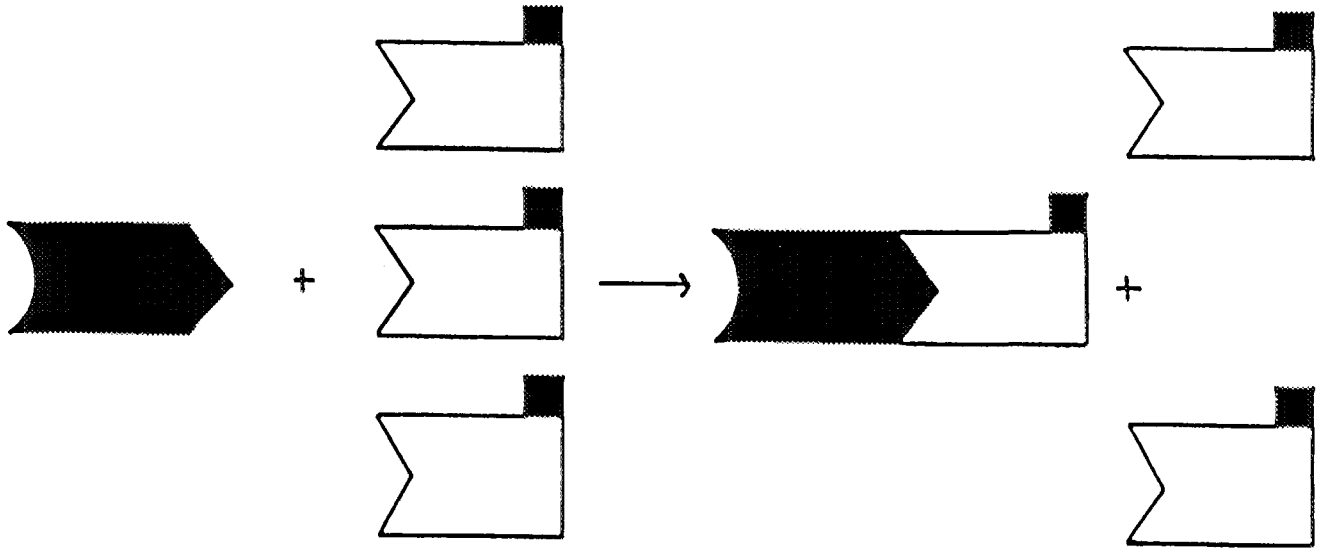
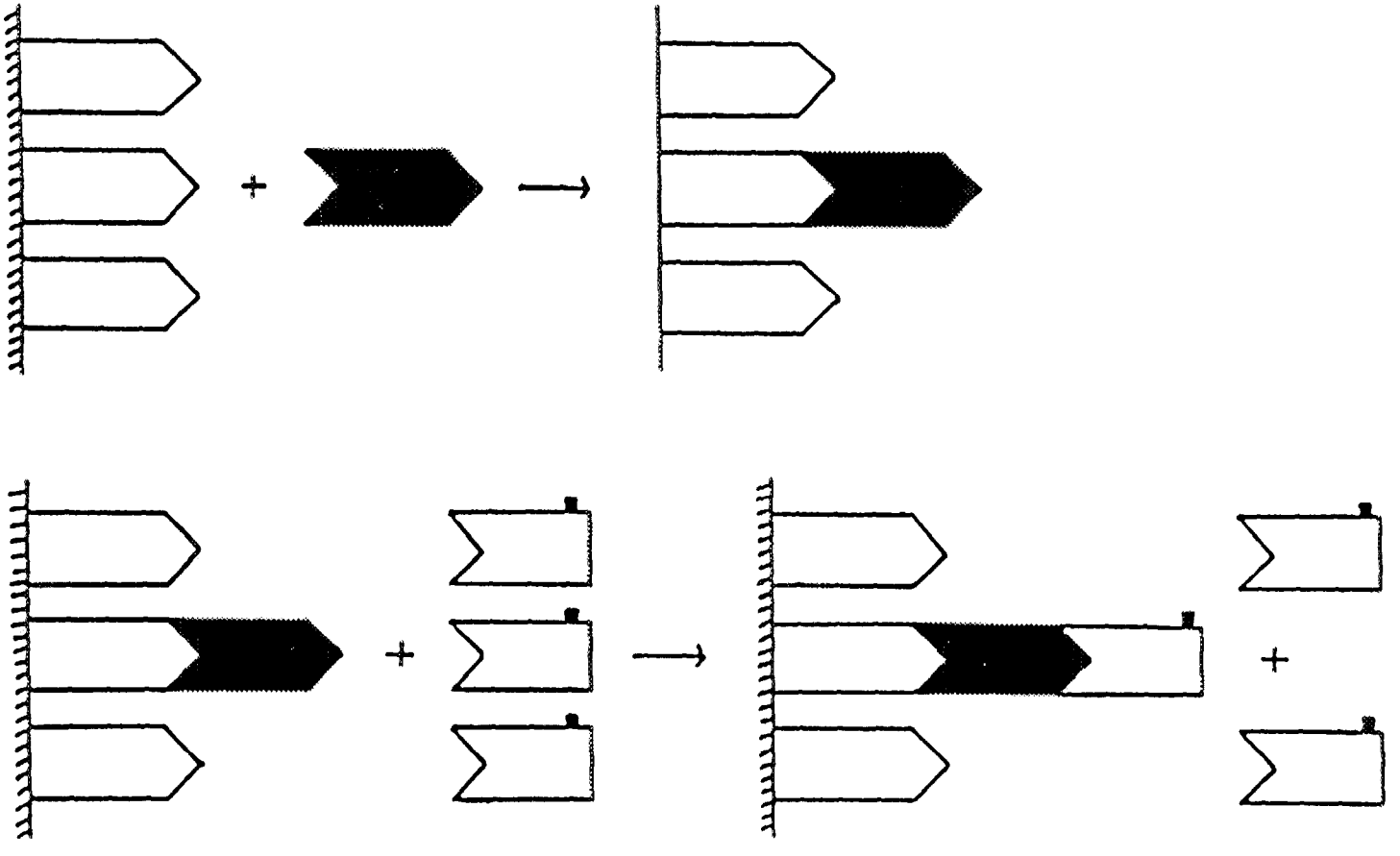
PHASE
SELECTED ANTIBODY
SOLID
PHASE
FIRST ANTIBODY
SECOND ANTIBODY

Reagent Excess Methods



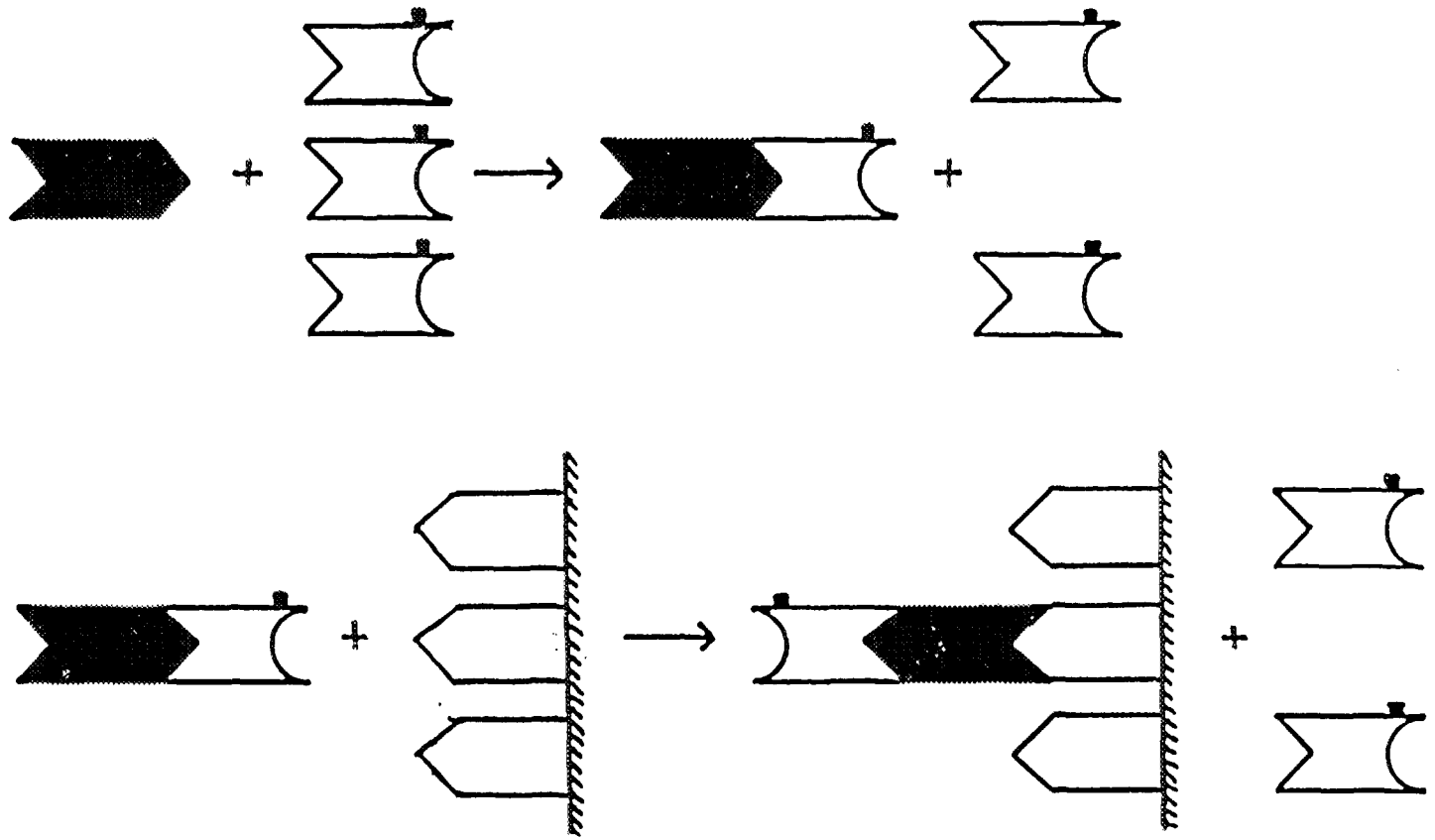
IMMUNORADIOMETRIC ASSAY

Reagent Excess Methods



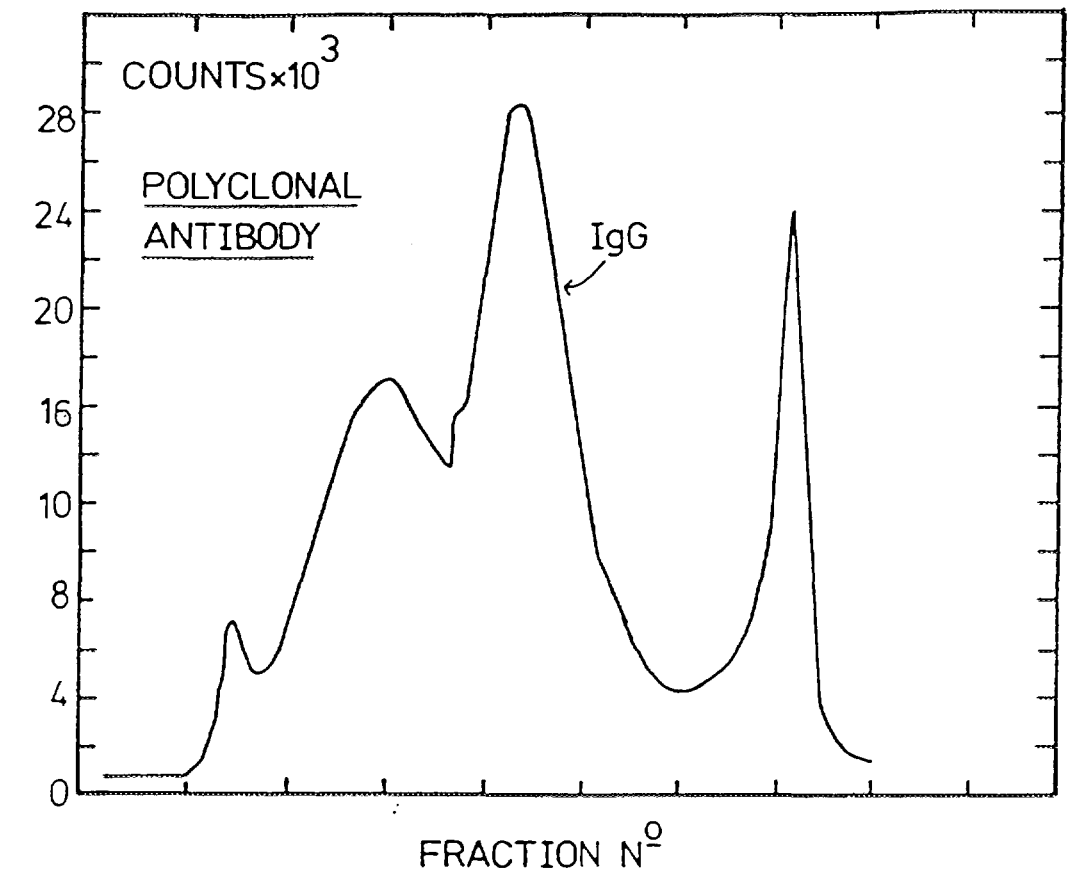
Two-site Immunoradiometric or sandwich assay

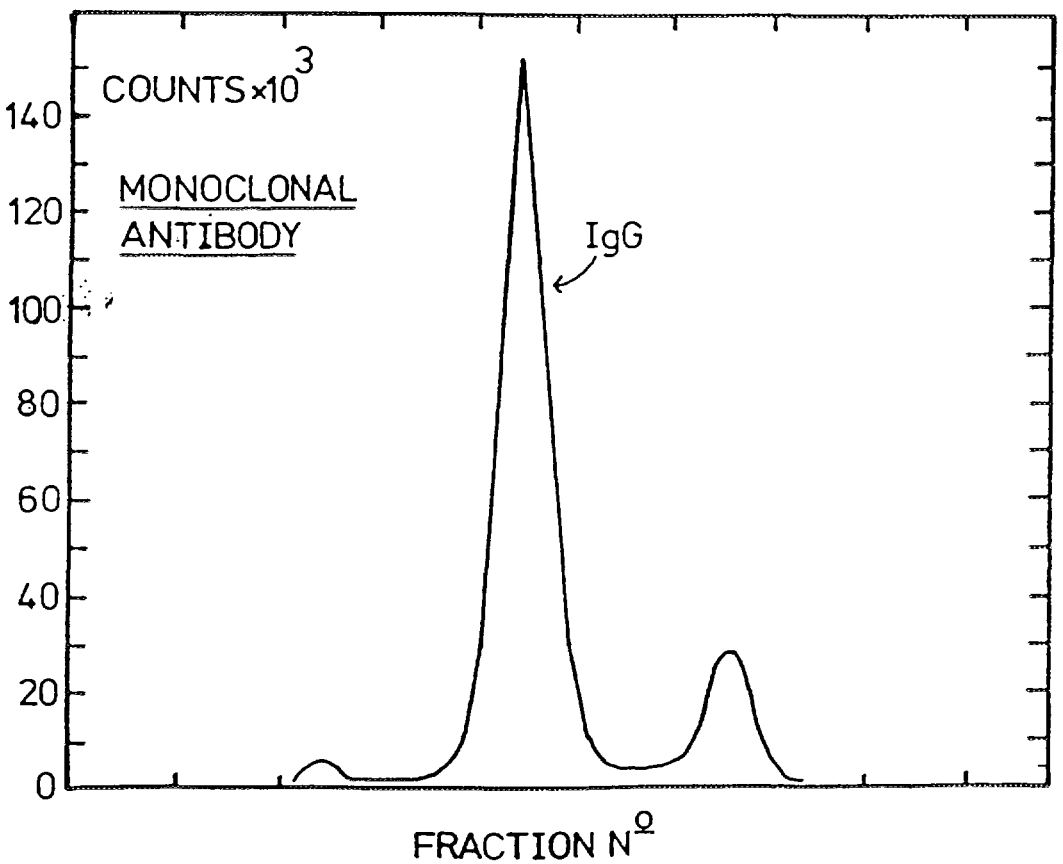
Reagent Excess Methods



Two-site Immunoradiometric or sandwich assay

(delayed solid phase addition)

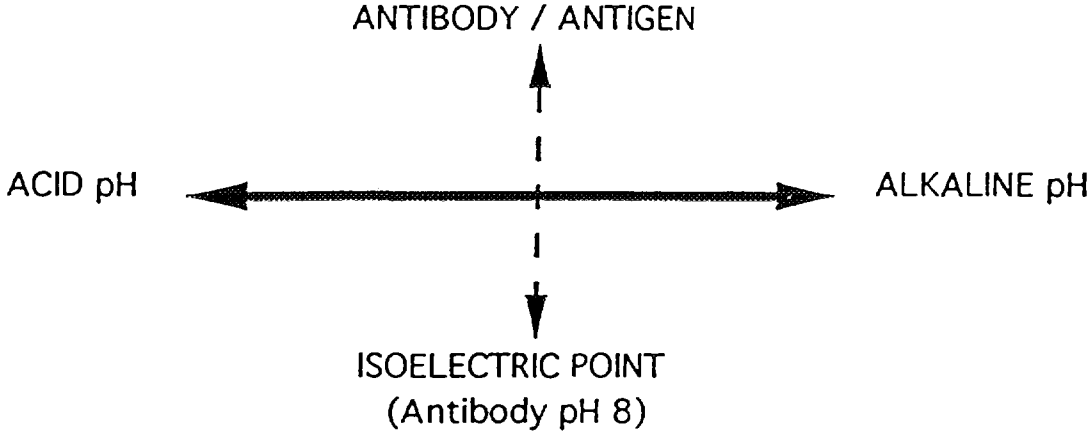

COUNTS×10^3
28
24
20
16
10
8
4
0
POLYCLONAL
ANTIBODY
IgG
FRACTION N°


COUNTS×10^3
140
120
100
80
60
40
20
0
MONOCLONAL
ANTIBODY
IgG
FRACTION N°

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 .