

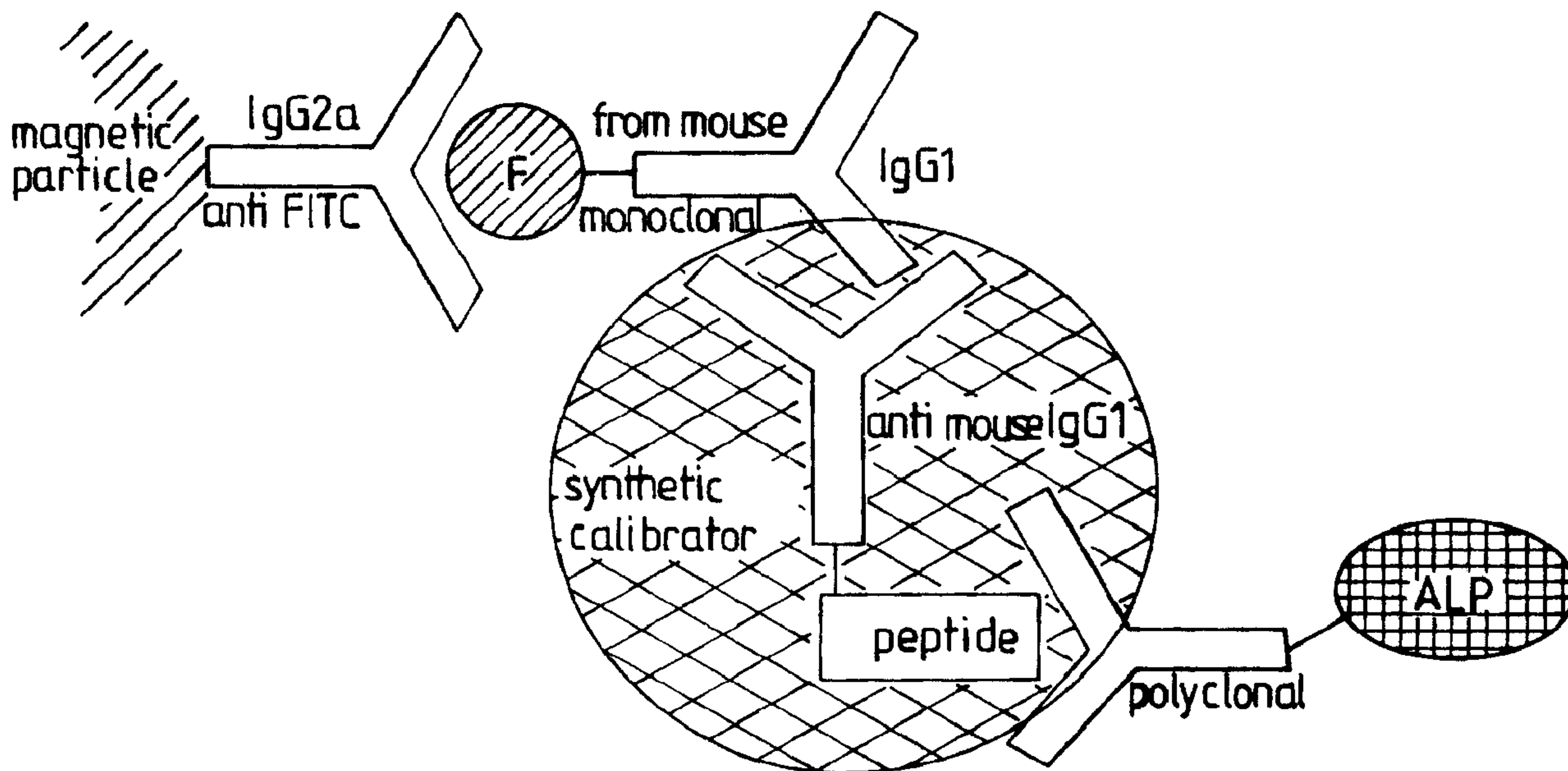


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(54) Titre : METHODE POUR PREPARER UN ETALONNEUR SYNTHETIQUE POUR IMMUNO-ESSAIS EN SANDWICH, L'ETALONNEUR ETANT CONSTITUE D'UN ANTICORPS DE L'UN DES ANTICORPS UTILISES DANS L'ESSAI ET D'UNE SEQUENCE DE L'ANALYSAT
 (54) Title: PROCESS FOR PREPARING A SYNTHETIC CALIBRATOR FOR USE IN SANDWICH IMMUNOASSAYS, WHICH CALIBRATOR CONSISTS OF AN ANTIBODY AGAINST ONE OF THE ANTIBODIES USED IN THE ASSAY AND OF A SEQUENCE OF THE ANALYTE

Sandwich-Immunoassay with a synthetic calibrator



(57) Abrégé/Abstract:

A conjugate consisting of a sequence of the analyte and an antibody against one of the antibodies used in the test can be employed, in aqueous solution and in precisely known quantity, as a stable calibrator in a sandwich immunoassay for detecting the analyte.

Process for preparing a synthetic calibrator for use in sandwich immunoassays, which calibrator consists of an antibody against one of the antibodies used in the assay and of a sequence of the analyte.

Abstract

A conjugate consisting of a sequence of the analyte and an antibody against one of the antibodies used in the test can be employed, in aqueous solution and in precisely known quantity, as a stable calibrator in a sandwich immunoassay for detecting the analyte.

30725-95

-1-

Process for preparing a synthetic calibrator for use in sandwich immunoassays, which calibrator consists of an antibody against one of the antibodies used in the assay and of a sequence of the analyte.

5 FIELD OF THE INVENTION

The invention relates to synthetic calibrators for use in sandwich assays.

BACKGROUND OF THE INVENTION

On account of their particularly good specificity
10 and sensitivity, immunoassays are frequently employed for detecting proteins, for the purposes of medical diagnosis, in serum samples or urine samples. This requires, in addition to one or two specific antibodies, a calibrator, which is used as a comparison standard for quantifying the patient
15 samples. It is desirable to be able to store the calibrators at 4°C for periods of several weeks to months, particularly in the case of automated assays carried out in large analytical laboratories. Depending on the analyte, these demands placed on the stability of the calibrator formulation
20 can give rise to difficulties if, for example, there is no guarantee of solubility under physiological salt and pH conditions. As an example, mention may be made in this context of troponin I and troponin T, which are only adequately stable and soluble in denaturing solutions (6 M
25 urea, 0.01 M dithiothreitol). However, it is not possible to establish any immunoassay using this denaturing formulation, since the antibodies are damaged by this treatment.

It is known that proteins are relatively unstable in solution and that reagents containing them are frequently
30 sold in freeze-dried form, together with a solvent of suitable composition in which the experimenter has to

30725-95

-2-

dissolve them prior to use. If the solutions which are obtained in this way are stored at 4°C, they can be used for several days even if daily determination indicates that the concentration of the reagent is changing to some extent. In
5 general, therefore, it is recommended - in the case of troponin I and troponin T as well - that the comparison solutions which are obtained from the freeze-dried material be frozen in unit-dose form if they are to be stored for a relatively long period.

10 SUMMARY OF THE INVENTION

In one aspect, the invention provides a calibrator for use in a sandwich immunoassay for troponin I, wherein the troponin I is bound between a first antibody or fragment thereof and a labelled second antibody or fragment thereof,
15 said calibrator comprising: a third antibody or fragment thereof which specifically binds either to said first antibody or fragment thereof or to said labelled second antibody or fragment thereof; said third antibody or fragment thereof conjugated to at least one peptide which consists of
20 an antibody binding site of said troponin I and which specifically binds to whichever of either said first antibody or fragment thereof or said labelled second antibody or fragment thereof is not bound by said third antibody or fragment thereof.

25 The invention further provides the use of a calibrator as described above for preparing or calibrating a diagnostic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1 depicts a typical sandwich immunoassay for determining an analyte.

30725-95

-3-

Figures 2a and 2b depict sandwich immunoassays using synthetic calibrators in accordance with the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a synthetic
5 calibrator which possesses very good stability and which can be used in sandwich immunoassays for determining medically relevant analytes in samples of blood, plasma, serum or urine. The distinctive feature of sandwich immunoassays consists in using two antibodies which are specific for the
10 analyte but which bind to different recognition sites (epitopes) so that the analyte comes to lie between these two antibodies (see Fig. 1). Accordingly, a calibrator for a sandwich immunoassay must also possess two binding sites: one for each of the antibodies employed. Often, the epitope
15 of at least one antibody is known in the form of the amino acid sequence, so that one binding site can consist of this peptide, i.e. of a constituent sequence of the analyte. If the epitope of the second antibody is not known, or if it has a three-dimensional structure, this recognition site cannot
20 be simulated by a peptide. In order to form a sandwich despite this, use can be made of the ability of the antibody to bind to a site other than the antigen recognition site. One possibility consists in employing antibodies, or antibody fragments, which are directed against the said antibody. If
25 this antibody is conjugated to the above mentioned peptide, a synthetic calibrator is obtained which carries a binding site for each of the antibodies used in the sandwich immunoassay (see Figs. 2a and b).

According to one aspect of the present invention
30 there is provided a calibrator for sandwich immunoassay, comprising a conjugate of an antibody with a peptide or peptide derivative which is specific for an analyte.

30725-95

-4-

The calibrator may be in the form of an aqueous solution which also comprises a buffer, stabilizer, preservative, detergent or cosolvent.

The chemical conjugation is carried out using known
5 methods which are described in the literature (S.S. Wong, Chemistry of protein conjugation and cross-linking, 1991, CRC Press Inc. ISBN 0-8493-5886-8).

The invention relates, in particular, to a synthetic calibrator material for cardiac troponin I, a
10 heart-specific protein which is of importance in diagnosing acute myocardial infarction. The calibrator consists in each case of a peptide of this analyte, which has been conjugated to antibodies. These antibodies react with the antibodies which are employed in the test for detecting the analyte.
15 The peptides are epitopes of the analyte-specific antibodies, that is, as a rule, protein sequences from the surface of the molecule. They can be prepared using commercially available synthesizers. Peptides are also to be understood as peptide derivatives in which one or more amino acids has been
20 derivatized by means of a chemical reaction. Examples of peptide derivatives according to the invention are, in particular, those molecules in which the backbone and/or reactive amino acid side groups, for example free amino groups, free carboxyl groups and/or free hydroxyl groups,
25 have been derivatized. Specific examples of derivatives of amino groups are sulphonamides or carboxamides, thiourethane derivatives and ammonium salts, for example hydrochlorides. Examples of carboxyl group derivatives are salts, esters and amides. Examples of hydroxyl group derivatives are O-acyl or
30 O-alkyl derivatives.

In addition, the term peptide derivative also encompasses those peptides in which one or more amino acids

30725-95

-5-

are replaced by naturally occurring or non-naturally occurring amino acid homologues of the 20 "standard" amino acids. Examples of such homologues are 4-hydroxyproline, 5-hydroxylysine, 3-methylhistidine, homoserine, ornithine, 5 β -alanine and 4-aminobutyric acid. The peptide derivatives must exhibit a specificity and/or affinity of binding to the antibodies which is essentially equivalent to that of the peptides from which they are derived. The length of the peptides is customarily at least 4 amino acids. Preferably, 10 the length is from 4 to 30, and particularly preferably from 4 to 15, amino acids.

A cysteine was attached to the C-terminal end of the peptides in order to facilitate conjugation. For the coupling, a method was selected which was known for 15 protein conjugation (S. Yoshitake et al., Eur. J. Biochem., 101:395, 1979). The antibodies were activated with succinimidyl 4-N-maleimidamethylcyclohexane-1-carboxylate (SMCC), by dissolving 20 mM SMCC in DMF and adding the solution, as a 25-fold excess, to the antibody. The mixture 20 was incubated at 25°C for 25 min. The reaction was terminated by adding 1 μ M glycine solution (25°C, 10 min). The peptides were bound to the activated antibody either by way of the sulphhydryl group in their sequence or by inserting such a group into them using 2-Iminothiolane 25 (2-IT). The number of peptides per antibody is customarily from 1 to 50, preferably from 1 to 10, and the number of the different peptide sequences is between 1 and 20, preferably 1-5, particularly preferably 1.

Gel chromatography (Superdex 200*) was carried out 30 in order to purify the calibrator substance, i.e. separate off the low molecular weight peptides. The concentration of

*Trade-mark

30725-95

-6-

the calibrator substance was then determined by UV spectrometry, and the substance was stabilized using 0.5% BSA/0.1% sodium azide. It is additionally possible to carry out an affinity chromatography purification, using the sequence-specific antibodies which are also employed in the immunoassay, for the purpose of separating off unlabelled antibody.

Examples

Example 1

10 A peptide having the sequence RAYATEPHAKKKS (SEQ ID NO: 1) was conjugated to an anti-mouse antibody. The immunoassay was carried out in accordance with the scheme depicted in Fig. 2a. The isotype of the monoclonal antibody was IgG1. Consequently, the anti-mouse calibrator antibody 15 must be directed against IgG1. It does not react with IgG2a (anti-fluorescein isothiocyanate (FITC) on the magnetic particles. The polyclonal antibody recognizes the peptide having the said sequence. This results in the formation of a sandwich in which the analyte troponin I (TnI) is replaced by 20 the synthetic calibrator.

Automated sandwich assay

The artificial calibrator was employed on the automated Immuno 1® Technicon Analyzer (Bayer Diagnostics). The assay format consisted of a sandwich which used the following antibodies: 1. monoclonal antibody against human 25 cardiac troponin I, 2. goat polyclonal antibody which has been affinity-purified against the sequence 1 peptide. The first antibody of the sandwich binds the anti-mouse IgG1 of the artificial calibrator. It is labelled with FITC and 30 is immobilized on magnetic particles by way of anti-FITC. The 2nd antibody of the sandwich reacts with the peptide on

30725-95

-7-

the synthetic calibrator. This latter antibody carries alkaline phosphatase and catalyses the colour reaction. The antibodies were incubated sequentially. In this test method, the colour intensity increased in proportion to the concentration of the calibrator substance.

Example 2

Another calibrator was formed from the sequence TGLGFAELQDLCRQIHARVD (SEQ ID NO: 2) and an anti-goat antibody (Fig. 2b). In this case, the monoclonal antibody recognizes the peptide. The anti-goat antibody of the calibrator binds to the goat polyclonal antibody, which latter carries the enzyme for the colour reaction.

Automated sandwich assay

The artificial calibrator as described in Example 2 was also employed on the automated Immuno 1® Technicon Analyzer (Bayer Diagnostics). The assay format was a sandwich which used the following antibodies: 1. monoclonal antibody against the sequence 2 of human cardiac troponin I, 2. goat polyclonal antibody against human cardiac troponin. The first antibody of the sandwich binds to the peptide having the sequence 2. This antibody is labelled with FITC and immobilized on magnetic particles by way of anti-FITC. The 2nd antibody of the sandwich reacts with the anti-goat antibody of the synthetic calibrator. It carries alkaline phosphatase and catalyses the colour reaction. The antibodies are incubated sequentially. In this test method, too, the colour intensity increased in proportion to the concentration of the calibrator substance.

30725-95

-10-

CLAIMS:

1. A calibrator for use in a sandwich immunoassay for troponin I, wherein the troponin I is bound between a first antibody or fragment thereof and a labelled second antibody or fragment thereof, said calibrator comprising: a third antibody or fragment thereof which specifically binds either to said first antibody or fragment thereof or to said labelled second antibody or fragment thereof; said third antibody or fragment thereof conjugated to at least one peptide which consists of an antibody binding site of said troponin I and which specifically binds to whichever of either said first antibody or fragment thereof or said labelled second antibody or fragment thereof is not bound by said third antibody or fragment thereof.
2. The calibrator according to claim 1 wherein said at least one peptide has a length of from 4 to 30 amino acids.
3. The calibrator according to claim 1 wherein said third antibody or fragment thereof is conjugated to 1 to 50 peptides, and wherein the peptides consist of 1 to 20 different amino acid sequences of said troponin I.
4. The calibrator according to claim 1 wherein said calibrator is in an aqueous solution.
5. The calibrator according to claim 1 wherein the at least one peptide has an amino acid sequence selected from the group consisting of SEQ ID NO.: 1 and SEQ ID NO.: 2.
6. The calibrator according to claim 1 further comprising one or more auxiliary substances selected from the group consisting of a buffer, a stabilizer, a

30725-95

-11-

preservative, a detergent and a cosolvent.

7. Use of a calibrator according to any one of claims 1 to 6 for preparing or calibrating a diagnostic agent.

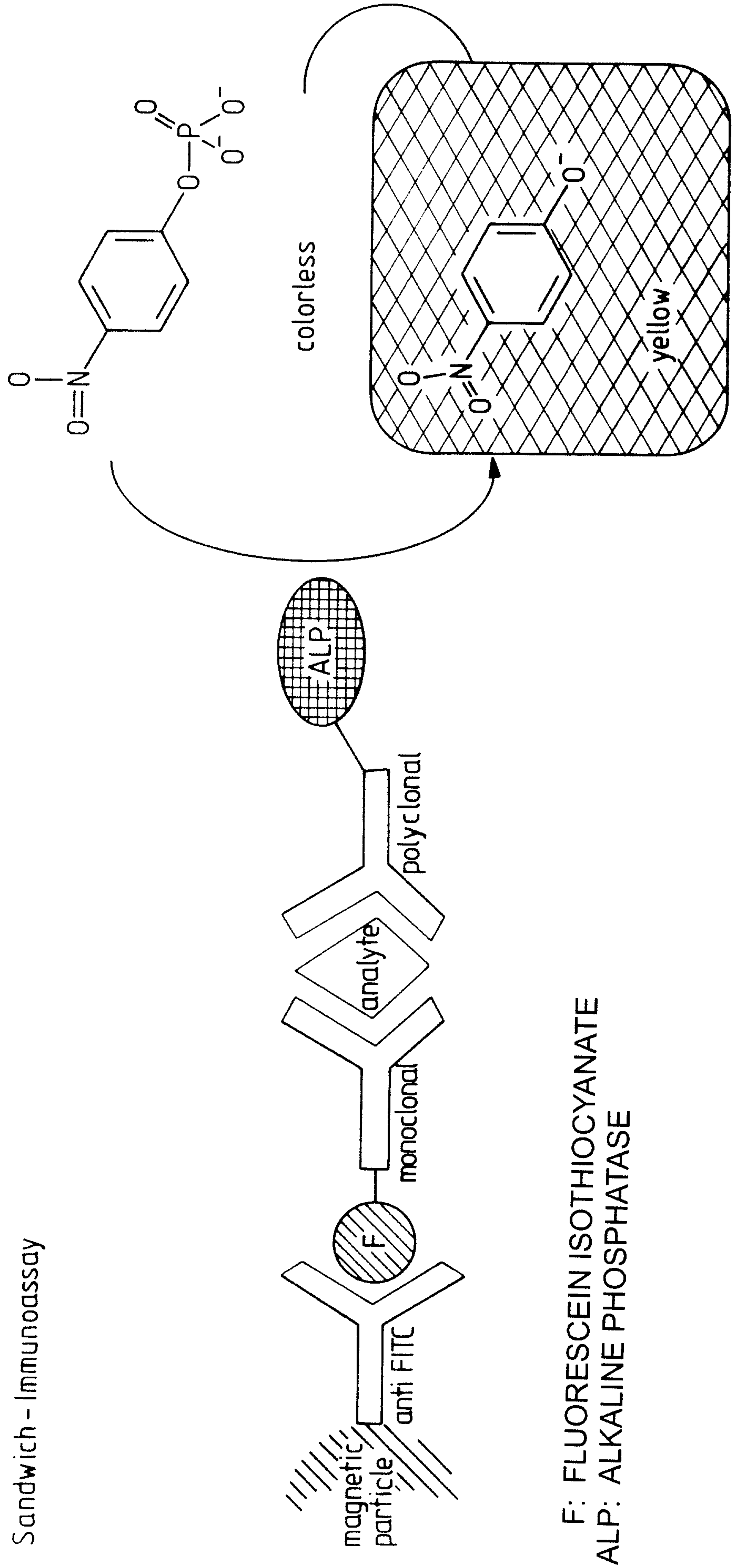
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OTTAWA, CANADA

PATENT AGENTS

Fig. 1 (PRIOR ART)

Sandwich - Immunoassay



F: FLUORESCCEIN ISOTHIOCYANATE
ALP: ALKALINE PHOSPHATASE

Fig. 2a

Sandwich-Immunoassay with a synthetic calibrator

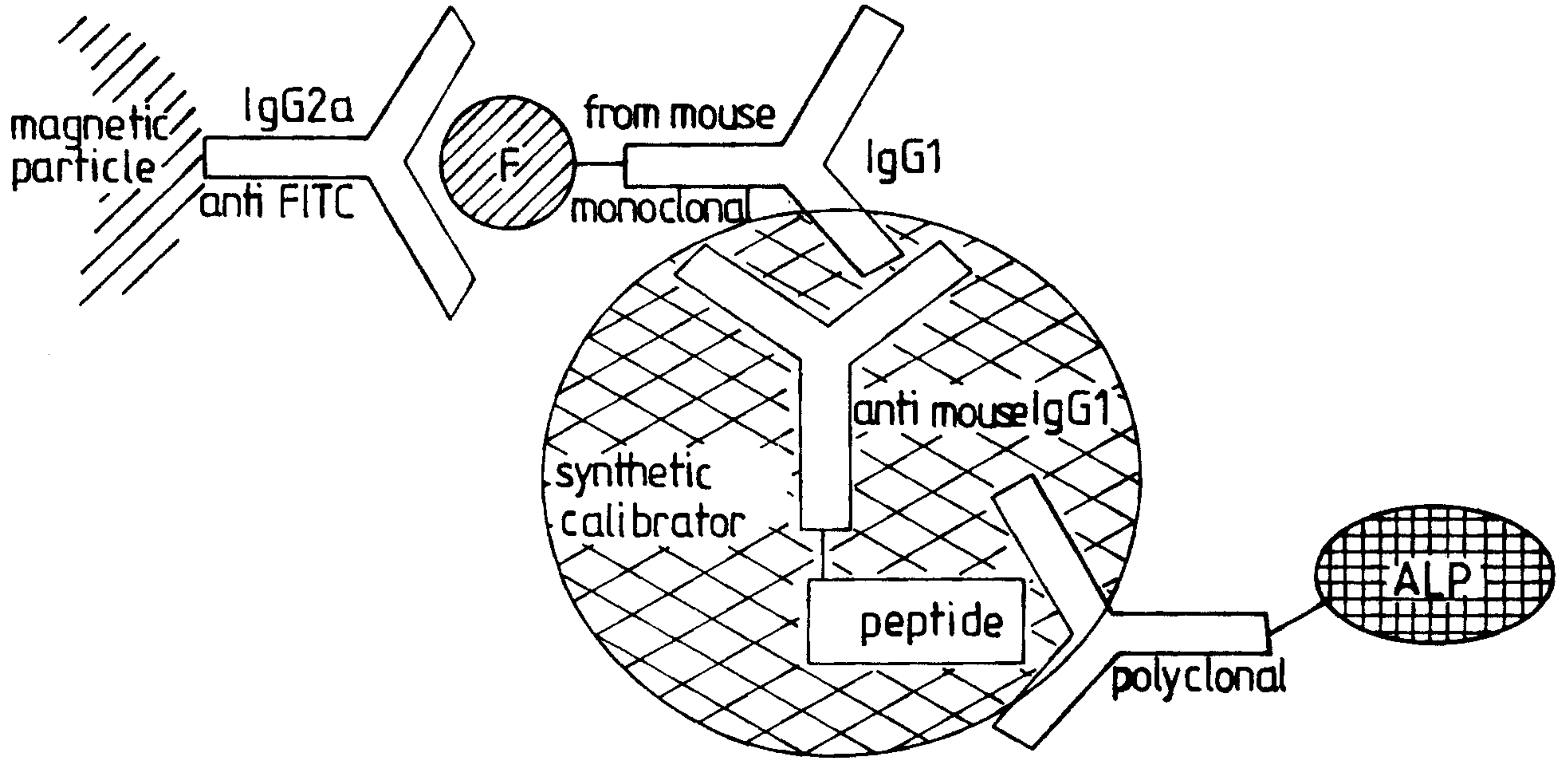
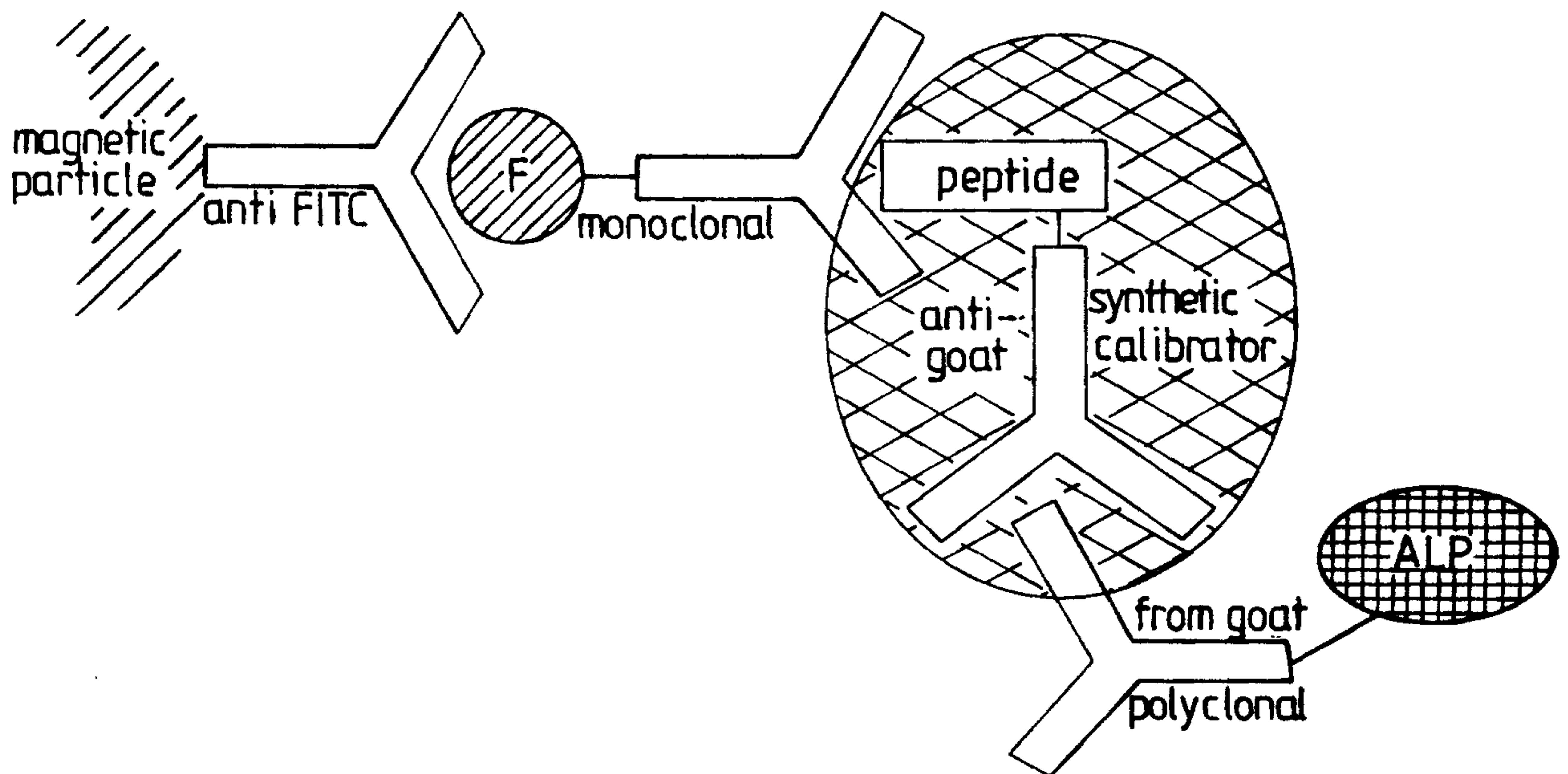


Fig. 2b

Sandwich-Immunoassay with a synthetic calibrator



Sandwich-Immunoassay with a synthetic calibrator

