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(54) Title: ASSAY METHOD

(57) Abstract: The present invention provides a method for assaying for an analyte in a sample, said method comprising: applying to a porous membrane to which optionally is bound a first specific binding partner for said analyte: (i) said sample; (ii) a water-binding polymer in aqueous solution; and (iii) one or both of a second, optionally labelled specific binding partner for said analyte in aqueous solution or dispersion and a labelled ligand for a said specific binding partner; flushing said membrane; and measuring a signal from or induced by a labelled material retained on the surface of said membrane whereby to generate an indication of the content of said analyte in said sample; with the provisos that the porosity of said membrane is such that, in the absence of any said first specific binding partner bound thereto, said second specific binding partner (where used) is able to be flushed therethrough but conjugates of said second specific binding partner and said labelled ligand (where used) are not able to be flushed therethrough, that at least one of said first and second specific binding partners is used, that at least one of a said labelled second specific binding partner and a said labelled ligand is used, and that where no first specific binding partner is used then both a said second specific binding partner and a labelled ligand are used.



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### Assay Method

5           This invention relates to an assay for an analyte in a sample, typically a sample of or derived from a body fluid or tissue.

          Axis-Shield ASA produces an assay kit for determination of D-dimer in plasma which is sold under  
10       the trade name NycoCard D-Dimer ST kit. The assay involves applying a sample onto a porous membrane to which is bound an anti-D-dimer antibody. A further anti-D-dimer antibody conjugated to colloidal gold particles is then applied to the membrane which is then  
15       washed to remove any unbound gold-antibody conjugate and the amount of membrane bound colloidal gold is then determined photometrically, detecting light reflected from the membrane. The measured signal is directly proportional to the concentration of D-dimer in the  
20       plasma sample. This assay is described in US-A-5958790.

          We have now found that the assay method may be improved by including a water-binding polymer (e.g. a polysaccharide or a polyalkylene oxide, for example PEG) in the liquid antibody conjugate reagent. If desired,  
25       the assay may be performed as a competitive assay by also including an antigen-particulate conjugate and using a non-antibody coated membrane with a pore size small enough to retain aggregates of the labelled antibody and the antigen-particulate conjugate.

30       Thus viewed from one aspect the invention provides a method for assaying for an analyte in a sample, said method comprising:

          applying to a porous membrane to which optionally is bound a first specific binding partner for said  
35       analyte: (i) said sample; (ii) a water-binding polymer in aqueous solution; and (iii) one or both of a second, optionally labelled, specific binding partner for said

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analyte in aqueous solution or dispersion and a labelled ligand for a said specific binding partner;

flushing said membrane;

5 and measuring a signal from or induced by a labelled material retained on the surface of said membrane whereby to generate an indication of the content of said analyte in said sample;

with the provisos

10 that the porosity of said membrane is such that, in the absence of any said first specific binding partner bound thereto, said second specific binding partner (where used) is able to be flushed therethrough but conjugates of said second specific binding partner and said labelled ligand (where used) are not able to be  
15 flushed therethrough,

that at least one of said first and second specific binding partners is used,

20 that at least one of a said labelled second specific binding partner and a said labelled ligand is used,

and that where no first specific binding partner is used then both a said second specific binding partner and a labelled ligand are used.

25 It will be appreciated that the assay may have a number of formats all employing the same basic principle of the use of the water-binding polymer to enhance assay performance.

Thus in one embodiment a membrane carrying the first specific binding partner (sbp) is used with a  
30 labelled second sbp but no labelled ligand. In this embodiment the labelled material retained on the membrane is a conjugate of first sbp, analyte and second sbp. In a second embodiment a first sbp carrying membrane is again used as is a labelled ligand but no  
35 second sbp. In this embodiment, the labelled ligand competes with the analyte and the labelled material retained on the membrane is a conjugate of first sbp and

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labelled ligand. In a third embodiment a membrane with no first sbp is used as is a labelled ligand and a second sbp (which may or may not be labelled but if the porosity of the membrane is such as to retain second  
5 sbp-analyte conjugates, the label on the ligand and the second sbp must be distinguishably different). In this embodiment the labelled species retained on the membrane is or includes the ligand-second sbp conjugate.

It is however especially preferred to use assay  
10 formats in which the membrane carries a first sbp.

In the method of the invention, where a labelled ligand and a labelled second sbp are both used and signals from or induced by the two labels are interdistinguishable, it is not necessary that the  
15 porosity of the membrane be such as to allow the ligand to pass through the membrane. However where a labelled ligand is used with an unlabelled second sbp, unless a further labelled species capable of binding to the second sbp or the second sbp:ligand conjugate is applied  
20 after retention of such conjugates on the membrane, then the porosity of the membrane should be such as to allow the ligand to pass through the membrane.

In the assay, the various materials which are applied to the membrane before the flushing step may be  
25 applied simultaneously or consecutively. It is preferred however that the water-binding polymer be present in the analyte-containing liquid and/or the second sbp-containing liquid, and/or the labelled ligand-containing liquid, especially the second sbp-  
30 containing liquid or a liquid containing the second sbp and the analyte. Premixing the water-based polymer with the sample and conjugate before application to the membrane is especially preferred. This has been shown to improve the sensitivity of the assay (see Examples 4  
35 and 5).

The application of liquids to the membrane preferably involves drawing the liquids through the

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membrane, e.g. under the action of gravity, or a pressure differential or by capillary action. Where a pressure differential is used, the liquid may be allowed to remain in contact with the membrane for an

5 "incubation period" if desired before the pressure differential is applied or in any case a liquid may be allowed to incubate before being contacted with the membrane. Such an incubation period may be desirable if the liquid contains species which it is desired should

10 form conjugates together, e.g. analyte and second sbp, etc. However we have found that such incubation periods are not always required.

The water-binding polymer used in accordance with the method of the invention is preferably one having an

15 exclusion or steric stabilization effect. Preferred polymers include those with relatively flexible backbones, e.g. incorporating etheric oxygens. Examples of such polymers include polysaccharides (such as for example pectin, carrageenan and dextran and polyalkylene

20 oxides (e.g. polyethylene glycol - PEG) (see Example 7)). Polyanionic polymers and hydrogen bond receiving but not donating polymers are preferred. The polymer clearly should not be used at concentrations which cause the liquid contacting the membrane to be unable to pass

25 through the membrane (e.g. due to gelling or excessive viscosity) and certain naturally occurring polysaccharides may require cleavage to reduce their gelling effect. Typically the polymer will be included in liquids contacting the membrane in a concentration in

30 the range 0.05 to 5% wt, especially 0.1 to 3% wt, particularly 0.5 to 2% wt. Subject to the polymer meeting the viscosity and gel-forming criteria mentioned above and to its retaining the necessary water-binding ability, the molecular weight of the polymer does not

35 appear to be particularly important. Polymers of weight average molecular weights of 1000 to 1000000 g/mole may typically be used, more especially 3000 to 600000,

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preferably 4000 to 100000. Where PEG is used, the molecular weight is preferably 6000 to 40000 g/mole and the concentration is preferably 0.5 to 2% wt, more especially 0.8 to 1.2% wt.

5           Although it has been shown that assay sensitivity is a function of membrane transit time (see Example 7), the improvement in assay performance achieved according to the invention is not simply the result of an increased liquid viscosity causing an increase in the  
10           membrane transit time. Comparative experiments using liquids of equal viscosity but where the viscosity derives from a non-polymer have shown no improvement in assay performance.

          The specific binding partners used in the method of  
15           the invention may be any substance capable of binding to the desired analyte in preference to any other materials in the sample. The sbp may for example be an antibody, an antibody fragment, an oligopeptide, an  
          oligonucleotide, or a small organic compound. Sbp's can  
20           be generated or identified using conventional techniques, e.g. antibody generation, library screening, combinatorial chemistry techniques, computer aided molecular design, etc.

          Surprisingly, the assay method of the invention is  
25           particularly suited for the use of relatively low affinity sbp's, e.g. sbp's having an affinity constant of  $10^5$  to  $10^9$  (e.g.  $10^5$  to  $10^8$ ), particularly  $10^6$  to  $5 \times 10^7$ . This is particularly the case when the same material is used as both first and second sbp. Indeed where the  
30           same material is used, the sbp affinity content is preferably low, e.g. as quantified above.

          Where a low affinity sbp is used, however, it is preferable to incubate the sbp and the analyte rather than to draw the sbp and/or analyte containing solutions  
35           through the membrane without a period of incubation.

          Where sbp's having different binding sites on the analyte are used as first and second sbp's, it may be

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desirable to use higher affinity sbp's.

The label on the sbp or ligand may be any moiety capable of being detected directly or indirectly, e.g. a chromophore, fluorophore, radiolabel, enzyme, particle  
5 (e.g. colloidal gold), etc. Particulate labels and chromophores and fluorophores are especially preferred. The label can be conjugated to the sbp or ligand using conventional chemical techniques.

Where a labelled ligand is used, this is  
10 conveniently a labelled analog of the analyte or of a fragment or derivative of the analyte. The preparation and use of labelled analyte analogs is conventional in the field of competitive binding assays.

The sample may be any suitable body fluid or body  
15 tissue in which the analyte can exist. Typical body fluids include blood, serum, plasma, urine, saliva, mucous, etc. Particularly suitably the sample is plasma. Where the analyte exists in a precursor form in the sample, the sample may be pretreated to transform  
20 the precursor into the analyte. Thus for example where the assay is for homocysteine, the sample may be reduced and then treated with a homocysteine converting enzyme, e.g. S-adenosyl homocysteine hydrolase, to generate the detectable analyte, e.g. S-adenosyl-homocysteine.

25 The analyte in the method of the invention may be any biological compound of interest. Examples include D-dimer, S-adenosyl homocysteine, and prostate specific antigen (PSA).

Incubation in the method of the invention, if  
30 effected, is preferably for a minimum of 1 minute, e.g. up to 45 minutes, most preferably 2 to 14 minutes, particularly 3 to 13 minutes. The incubation is preferably effected at an elevated temperature, e.g. 30 to 45°C, especially 35 to 40°C.

35 The membrane used in the method of the invention may be any porous material, thus for example it may be a synthetic polymer or a cellulosic material, preferably

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nitrocellulose. Where the membrane does not have a first sbp for the analyte bound to it, the pore size or inter fibre spacing should be such as to trap conjugates of the second sbp (e.g. pore sizes of 150 to 1000 nm, especially 300 to 600 nm, particularly 400 to 500 nm). In this embodiment, it is especially preferred to perform the assay as a competitive binding assay including in an incubation mixture a particulate ligand with binding sites for the sbp bound to its surface. Such binding sites may for example be the analyte, or a fragment or analog thereof. In such a construct the particle may function as the label.

In one embodiment, this particulate ligand has a particle size greater than the pore size of the membrane, e.g. a mode particle diameter of 1  $\mu$ m or more, especially 1 to 10  $\mu$ m and is of a material which does not interfere with the reading of the label on a labelled second sbp. Typically the particle will be a polymer, e.g. an acrylate, polystyrene or latex. Such particulates are available commercially, e.g. from Dynal Biotech ASA, Oslo.

In one embodiment of the method of the invention the particulate ligand may be or contain superparamagnetic crystals. In this embodiment, before application to the membrane, a magnetic field may be applied to the sample, the liquid portion of the sample can then be separated off and the superparamagnetic material, optionally after being rinsed, may be resuspended in a liquid carrier before being applied to the membrane. In this embodiment, the rinsing of the membrane following application of the suspension may be omitted.

In the NycoCard system, the fluid is drawn through the membrane by placing an absorbent pad behind the membrane. The same system can be adopted for the method of the invention. Alternatively, the fluid may be drawn through the membrane by application of a pressure

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differential across the membrane. Thus the method of the invention can be performed in an assay device of the type described in WO 02/090995.

5 Viewed from a further aspect of the invention also provides an assay kit comprising a porous membrane, a water-binding polymer, and at least one of an sbp for an analyte and a labelled ligand for an sbp for said analyte (e.g. a particulate carrying antigens for said sbp), optionally a solution of said analyte of known  
10 concentration, and optionally a rinsing agent; wherein where said membrane does not have bound thereon an sbp for said analyte, said kit contains an optionally labelled sbp for said analyte.

Preferably the kit contains a set of standards,  
15 i.e. solutions of the analyte of known concentration. These may be used to calibrate the assay.

The invention will now be described further with reference to the following non-limiting Examples.

20 Example 1  
D-Dimer Assay

A gold-conjugated anti-D dimer antibody solution (50  $\mu\text{g/mL}$ ) from a NycoCard D-Dimer ST kit, with PEG 40000  
25 added to 2% wt is mixed with an equal volume of plasma. The mixture is incubated at 25°C for 2 minutes and 100  $\mu\text{l}$  is then applied to an anti-D-dimer antibody coated membrane (also from a NycoCard D-Dimer ST kit). (The antibody is the same as that in the antibody solution).  
30 50  $\mu\text{L}$  of washing solution from the NycoCard D-Dimer ST kit is applied to the membrane and the signal is measured using a NycoCard Reader II using the colour intensity option (K/S, green).

35 The gold conjugate used in this Example is produced as described in US-A-5691207, US-A-5650333 and EP-A-564449. The mode particle size is about 100 nm.

**Example 2****Homocysteine Assay**

10  $\mu$ L of plasma pretreated by addition of 70  $\mu$ L of a  
5 pretreatment agent (50  $\mu$ M adenosine, 5 mM Tris (2-  
carboxyethyl) phosphine:HCl (TCEP-HCl) and 3 U/mL S-  
adenosyl homocysteinase) is mixed with 50  $\mu$ L of a  
protein-SAH-gold conjugate, 50  $\mu$ L of an anti-SAH  
antibody-gold conjugate and PEG 40000 (1% wt final  
10 concentration). The mixture is incubated at 40°C for 2  
minutes. 100  $\mu$ L of the incubated mixture is then  
applied to a 0.45  $\mu$ m pore size nitrocellulose membrane  
(available as BA-S85 from Schleicher & Schuell) coated  
with an anti-SAH antibody (available from Axis-Shield  
15 ASA (the same antibody as in the gold conjugate). 50  $\mu$ L  
of phosphate buffered saline pH 7.4 is then applied to  
wash the membrane. The signal from the membrane is then  
read using a NycoCard Reader II using the colour  
intensity option (K/S, green).

20 In one version, the protein-SAH-gold conjugate is bovine  
serum albumin-SAH-gold, in a second it is  
polythyroglobulin-S-adenosyl-cysteine-gold. Conjugation  
is effected as described in US-A-5691207 and US-A-  
25 5650333.

**Example 3****Homocysteine Assay**

30 5  $\mu$ L plasma, pretreated with 40  $\mu$ L of a reducing agent  
(5mM TCEP and 50  $\mu$ M adenosine), 10  $\mu$ L of 150 U/mL S-  
adenosyl-homocysteinase, and 80  $\mu$ L Tris:HCl buffer  
(containing 2% wt PEG 40000) and incubated at 37°C for  
1.5 minutes. To the mixture is added 25  $\mu$ L of 2.75  $\mu$ m  
35 sized latex particles coated with S-adenosyl-cysteine  
(prepared as described in the manufacturer's (Estapor's)  
instructions). 50  $\mu$ L gold-conjugated anti-S-adenosyl

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homocysteine antibody (32-38  $\mu\text{g/mL}$ ) is added and the mixture is incubated for 5 minutes at 37°C. 100  $\mu\text{L}$  of the incubated mixture is placed on a flow-through device having a 0.8  $\mu\text{m}$  pore size Supor membrane (available from Pall), and washed with 50  $\mu\text{L}$  washing solution. The observed red signal is read using a NycoCard Reader II, using colour intensity option (K/S green).

#### Example 4

##### 10 PSA assay

###### a) Sequential assay:

To 50  $\mu\text{L}$  of a buffer solution (10mM Tris:HCl, pH 8.0, 1% BSA) was added PSA (0, 4.2, 8,4 and 42 ng/mL). This was placed in a flow-through device with a 0.45  $\mu\text{m}$  nitrocellulose membrane coated with anti-PSA antibodies (from Scripps). 50  $\mu\text{L}$  of a gold conjugated anti-PSA antibody (50  $\mu\text{g/mL}$ ) was applied to the same device when the sample had soaked through. 50  $\mu\text{L}$  of a washing solution was finally added to the membrane before reading the signal with a NycoCard Reader II (K/S/, green).

The same procedure was repeated adding PEG 40000 to a final concentration of 1% wt in the gold conjugate dispersion.

###### b) Mixing sample and gold-conjugate before applying to the membrane:

50 $\mu\text{L}$  of a buffer solution (10 mM Tris: HCl, pH 8.0, 1% BSA) was added PSA (0, 4.2, 8,4 and 42 ng/mL). This was mixed with 50  $\mu\text{L}$  of a gold conjugated anti-PSA antibody (50  $\mu\text{g/mL}$ ). The mixture was incubated at ambient temperature for 2 minutes. 100  $\mu\text{L}$  of the mixture was then applied to an anti-PSA coated membrane (as in section a) above). After the mixture had soaked through, 50  $\mu\text{L}$  of a washing solution was added. The

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coloured signal was read with a NycoCard Reader II (K/S, green).

5 The same procedure was repeated adding PEG 40000 to a final concentration of 2% wt in the gold conjugate.

The results of the assay carried out by method a and method b, with or without a water-binding polymer (PEG 40000) are set out in Table 1 below.

10

Table 1

| Assay procedure: | a     | a with PEG | b     | b with PEG |
|------------------|-------|------------|-------|------------|
| PSA conc.        |       |            |       |            |
| 15 0 ng/mL       | 0.062 | 0.070      | 0.097 | 0.088      |
| 4.2 ng/mL        | 0.073 | 0.108      | 0.101 | 0.120      |
| 8.4 ng/mL        | 0.098 | 0.132      | 0.116 | 0.163      |
| 42 ng/mL         | 0.235 | 0.384      | 0.206 | 0.523      |

20

Table 1 shows the improved sensitivity of the assay when performed with the water-binding polymer (PEG 40000) and by premixing together with addition of the water-binding polymer.

25

Example 5

PSA assay

30 The procedure of Example 4 was repeated using an anti PSA antibody available from Medix Oy in place of the Scripps' antibody.

35 The results of the assay carried out by method a and method b, with or without PEG 40000 (as in Example 4) are set out in Table 2 below.

Table 2

| Assay procedure: | a     | a with PEG | b     | b with PEG |
|------------------|-------|------------|-------|------------|
| PSA conc.        |       |            |       |            |
| 0 ng/mL          | 0.055 | 0.067      | 0.085 | 0.070      |
| 4.2 ng/mL        | 0.153 | 0.255      | 0.136 | 0.223      |
| 8.4 ng/mL        | 0.255 | 0.433      | 0.212 | 0.413      |
| 42 ng/mL         | 0.922 | 1.58       | 0.545 | 1.40       |

The affinity constants of the antibodies used in Examples 4 and 5 are  $1 \times 10^{11}$  L/mol PSA and  $1 \times 10^{10}$  L/mol PSA.

Table 1 and Table 2 can be compared. Table 2 shows the results of the assay when antibodies with lower affinity constants are used than the antibodies used in the assays of Example 4. Comparing the results of Table 1 and Table 2 it can be seen that there is a much higher sensitivity gain in the assay when antibodies with lower affinity constants are used.

### Example 6

#### D-dimer Assay

The D-dimer assay of the NycoCard D-Dimer ST kit was performed using standards containing 0, 0.06, 0.3, 1, 2 and 8 mg/L D-dimer and according to the instructions in the package insert but with and without premixing of sample and antibody conjugate dispersion and with and without inclusion of PEG 40000 as 1% wt in the mixture (or in the antibody dispersion if no premixing occurred). The affinity constant of the antibodies used in this Example is in the range of from  $8.7 \times 10^7$  to

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8.6x10<sup>8</sup>. The same antibody was used as capture antibody as was used as signal-giving antibody. The results of the assay carried out using the sequential assay or by premixing, with or without inclusion of PEG 40000, are set forth in Table 3.

Table 3

| Test sequence | Sequential | Sequential with 1% PEG in antibody | Premixing sample-antibody | Premixing with 1% PEG in mixture |
|---------------|------------|------------------------------------|---------------------------|----------------------------------|
| D-Dimer       |            |                                    |                           |                                  |
| 0 mg/L        | 0.140      | 0.184                              | 0.166                     | 0.233                            |
| 0.06 mg/L     | 0.184      | 0.233                              | 0.218                     | 0.382                            |
| 0.3 mg/L      | 0.248      | 0.384                              | 0.492                     | 1.02                             |
| 1 mg/L        | 0.498      | 0.881                              | 1.22                      | 2.92                             |
| 2 mg/L        | 0.847      | 1.55                               | 2.07                      | 4.41                             |
| 8 mg/L        | 2.42       | 4.06                               | 3.95                      | 8.09                             |
| Ratio 0.3/0   | 1.77       | 2.09                               | 2.96                      | 4.38                             |

Table 3 shows that an increased sensitivity of the assay was achieved using both the sequential assay with a water-binding polymer and the premixing assay with a water-binding polymer. The greatest increase in sensitivity was achieved by using the premixing assay with a water-binding polymer.

When comparing Tables 1 to 3 it can be seen that the sensitivity gain of the assays was greatest using the premixing assay with the water-binding polymer in Example 6. The antibody used in this example had a lower affinity constant than those used in Examples 4 and 5.

20     Example 7  
          D-Dimer Assay

The D-dimer assay of Example 1 was performed using, in place of PEG, 0.1% wt pectin (Fluka, ref. 76280), 0.2% wt pectin (Fluka), 0.1% wt carrageenan (Sigma, C-1263), 1% wt dextran ( $M_w$  513000, Sigma, P-8136), and 2% dextran. The samples tested were standards containing 0, 0.3 and 3.0 mg D-dimer/L.

The results of the assay using polymer / additives other than PEG are set out in Table 4 below.

10     Table 4

| Polymer/<br>additive | water | Pectin | Pectin |
|----------------------|-------|--------|--------|
| Final conc.          | -     | 0.1%   | 0.2%   |
| 0 mg/L               | 0.153 | 0.211  | 0.292  |
| 0.3 mg/L             | 0.364 | 0.770  | 1.38   |
| 2 mg/L               | 1.47  | 3.29   | 6.11   |
| Ratio 0.3/0          | 2.38  | 3.65   | 4.74   |
| Flow time(s)         | 38    | 63     | 82     |

20

| Polymer/<br>additive | Carrageenan | Dextran | Dextran |
|----------------------|-------------|---------|---------|
| Final conc.          | 0.1%        | 1%      | 2%      |
| 0 mg/L               | 0.205       | 0.217   | 0.248   |
| 0.3 mg/L             | 0.848       | 0.861   | 1.34    |
| 2 mg/L               | 4.07        | 3.79    | 4.62    |
| Ratio 0.3/0          | 4.14        | 3.97    | 5.40    |
| Flow time(s)         | 73          | 60      | 72      |

30

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Table 4 shows that the increased sensitivity of the assay is not only a function of the flowtime through the membrane but also of the polymer used.

Claims

1. A method for assaying for an analyte in a sample, said method comprising: applying to a porous membrane to  
5 which optionally is bound a first specific binding partner for said analyte: (i) said sample; (ii) a water-binding polymer in aqueous solution; and (iii) one or both of a second, optionally labelled specific binding partner for said analyte in aqueous solution or  
10 dispersion and a labelled ligand for a said specific binding partner;  
flushing said membrane;  
and measuring a signal from or induced by a labelled material retained on the surface of said  
15 membrane whereby to generate an indication of the content of said analyte in said sample;  
with the provisos  
that the porosity of said membrane is such that, in the absence of any said first specific binding partner  
20 bound thereto, said second specific binding partner (where used) is able to be flushed therethrough but conjugates of said second specific binding partner and said labelled ligand (where used) are not able to be flushed therethrough,  
25 that at least one of said first and second specific binding partners is used,  
that at least one of a said labelled second specific binding partner and a said labelled ligand is used,  
30 and that where no first specific binding partner is used then both a said second specific binding partner and a labelled ligand are used.
2. A method as claimed in claim 1 wherein said sample,  
35 water-binding polymer and one or both of a second, optionally labelled specific binding partner for said

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analyte in aqueous solution or dispersion and a labelled ligand for a said specific binding partner are applied to said membrane consecutively.

3. A method as claimed in claim 1 wherein said sample,  
5 water-binding polymer and one or both of a second,  
optionally labelled specific binding partner for said  
analyte in aqueous solution or dispersion and a labelled  
ligand for a said specific binding partner are applied  
to said membrane sequentially.

10

4. A method as claimed in any of claims 1 to 3,  
wherein said first specific binding partner (sbp) is  
used with a labelled second sbp but no labelled ligand.

15

5. A method as claimed in any of claims 1 to 3,  
wherein said first sbp is used with a labelled ligand  
but no second sbp.

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6. A method as claimed in any of claims 1 to 3,  
wherein said labelled ligand and second sbp is used, but  
said first sbp is not.

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7. A method as claimed in any of the preceding claims  
wherein said water-binding polymer is chosen from  
pectin, carrageenan, dextran and polyethylene glycol.

8. A method as claimed in any of the preceding claims  
wherein said sbp is a low affinity sbp.

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9. An assay kit comprising a porous membrane, a water-  
binding polymer, and at least one of an sbp for an  
analyte and a labelled ligand for an sbp for said  
analyte (e.g. a particulate carrying antigens for said  
sbp), optionally a solution of said analyte of known  
35 concentration, and optionally a rinsing agent; wherein  
where said membrane does not have bound thereon an sbp

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for said analyte, said kit contains an optionally  
labelled sbp for said analyte.