

CRANFIELD UNIVERSITY

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AN ASSESSMENT OF THE SUITABILITY OF USING STREPTAVIDIN
COATED MICROSPHERES ON THE TEST LINE OF A LATERAL
FLOW IMMUNOASSAY WITH A HYDROPHILIC MEMBRANE.

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Abstract

Lateral flow immunoassays provide quick, qualitative detection of biomolecules. They are designed to be easy to use, cheap and suitable for applications away from the laboratory.

Most lateral flow assays detect a single analyte and use two analyte specific antibodies. One antibody is labelled, and physically separated from the other, which is bound to the test line of a hydrophobic membrane. When a liquid sample is applied, the sample and labelled antibodies flow towards the capture antibodies on the test line. If the analyte is present in the sample, a sandwich assay forms on the test line, giving a visual signal to indicate the presence of the analyte.

This research investigated using Streptavidin coated microspheres on the test line of a hydrophilic membrane and a mobile, analyte specific, biotinylated capture antibody upstream of the test line. A hydrophilic membrane material reduced the number of materials required, compared to a traditional design and the Streptavidin provided a method for designing a multi-analyte assay with an analyte independent test line.

The mobile biotinylated capture antibody and Streptavidin based test line gave variable results. Only one in four of the target assays developed could detect the analyte used (Rotavirus antigen) and only at higher concentrations, compared to traditional lateral flow designs. Better results were achieved by pre-incubating the biotinylated capture antibody with the Streptavidin microspheres, although it was unable to match the sensitivity of the traditional lateral flow assays. Furthermore, traditional lateral flow designs produced obvious results within ten minutes, while hydrophilic membrane based assays, with Streptavidin test lines, took approximately sixty minutes to produce less distinct signals. Experiments were performed to reduce the time taken to generate signals, but no significant improvement was achieved.

Further work should investigate the antibodies, buffers, choice of label, alternative test line capture methods and membrane materials.

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Abbreviations

BSA	Bovine Serum Albumin
ELISA	Enzyme linked immunosorbent assay
Fab	Antigen binding fragment of an antibody
Fc	Crystallisable fragment of an antibody
hCG	Human chorionic gonadotropin
ICA / ICG	Immunochromatographic assays
ICS	Immunochromatographic strip tests
IgG	Immunoglobulin G
LFIA	Lateral flow immunoassay
OTC	Over-the-counter
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
POC	Point-of-care
RO	Reverse osmosis (water)

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Chapter 1: *Introduction and Literature Review*

1.1 Immunoassay principles and classification

Immunoassays utilise the specific binding affinity found between antigens and their corresponding antibodies for the detection, and potential quantification, of biomolecules.

The three dimensional structure of the surface of an antigen gives rise to a number of specific epitopes (active binding sites). These epitopes specifically bind with the paratope (antigen binding site) of a complementary antibody in the immune response. The paratopes of an antibody are located at the tips of the antigen binding fragments (Fab) on the top of the Y-section shown in Figure 1. The lower portion of an antibody is known as the crystallisable fragment, or constant fragment, (Fc).

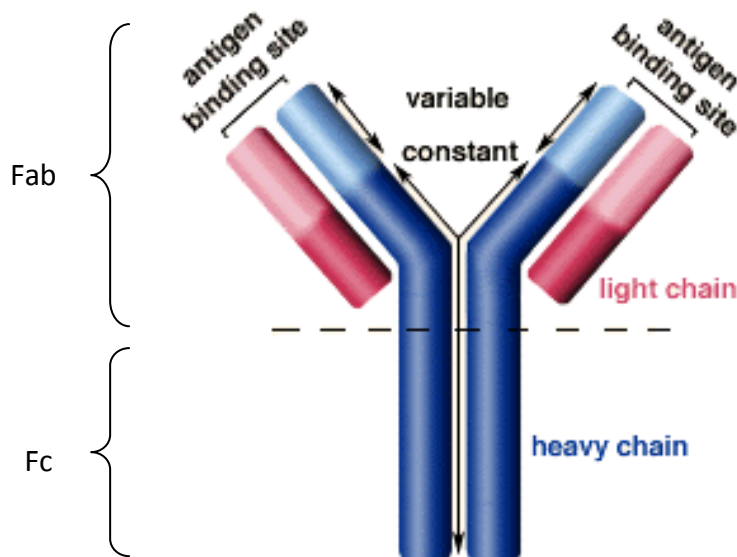


Figure 1 A diagrammatic representation of the structure of an antibody. The area above the dotted line represents the Fab section and the region below is the Fc portion (University of Arizona, 2000).

The Fc tail binds to T-cells *in vivo* but, in an immunoassay, it can be used to attach an antibody to a solid phase, or be conjugated with a label, or another antibody, in order to indirectly immobilise, isolate or identify an antigen via the exposed Fab region.

Immunoassays exploit the avidity of the antigen, antibody interaction and they can be classified according to the type of analyte, sample fluid or assay format. Price and Newman (1997) classify immunoassays according to three main criteria: the relative quantity of reagents, whether the assay uses a homogeneous or heterogeneous methodology and the presence and type of label used to produce a signal.

1.1.1 Relative quantity of reagents

Immunoassays make use of either restricted quantities of reagents (competitive assays) or an excess (non-competitive assays), depending on the analyte under investigation.

Competitive immunoassays use either a limited amount of labelled antigen or antibody. A known concentration of the labelled antigen (or antibody) is combined with a sample containing an unknown concentration of the antigen (or antibody) being tested. When the immunoassay is performed, the two reagents compete for binding sites and the competitive nature of this format results in an inversely proportional signal where the concentration of the unknown, unlabelled analyte can be determined indirectly by the signal generated due to the labelled reagent. Competitive, limited reagent, immunoassays are typically used when detecting weak concentrations of analyte, or those with a low molecular weight. Bangs Laboratories (2002) recommend the use of limited reagent, competitive type, assays for analytes with a molecular weight lower than 6000.

Immunoassays with an excess of antibody generate a signal that is directly proportional to the concentration of the analyte under test. Different non-competitive assays include direct, indirect and sandwich type methods:

The direct method involves the detection of an antigen with an excess quantity of a labelled antibody, specific for the antigen being analysed.

An indirect assay makes use of primary and secondary antibodies. Primary antibodies are chosen for their specific binding affinity for a particular antigen. Secondary antibodies are raised, in a different species, to be specific for the Fc fragment of the primary antibody. If the secondary antibody is labelled, it will indirectly signal the presence of the antigen, by binding the primary antibody, which has specific affinity for the antigen. Furthermore, since more than secondary antibody can bind to the Fc portion of the primary it gives an amplified, indirect way of detecting the antigen. Indirect assays are frequently used to detect the presence of a primary antibody when assessing autoimmune diseases or determining the immune response to treatment.

Sandwich immunoassays involve excess quantities of two separate antibodies. One of the antibodies is typically used to immobilise the antigen, while the second antibody is labelled and provides the signal. Each antibody binds with a different epitope on the antigen, so providing greater specificity and sensitivity.

1.1.2 Homogeneous and heterogeneous assays

The assay methodologies above require the generation of a signal that will indicate the presence, absence, or concentration of an analyte. In a homogenous assay, this signal is generated in a single reaction, without having to physically separate bound from unbound reagents. In contrast, heterogeneous assays require the separation of free reagents from specifically bound antigen-antibody interactions. This separation typically requires the use of a solid phase to allow the immobilisation of either a capture antigen or antibody, which indirectly produces a detectable signal in the presence of the target analyte, with any unbound reagents separated or removed *e.g.* with a washing step.

1.1.3 Signals and labels

Certain homogeneous assays may provide a signal without the need for a label, *e.g.* the assay may form a turbid solution which can be detected visually *e.g.* agglutination assays and nephelometry. However, the majority of immunoassays use a label to determine the presence, or absence, of the target analyte. Labels can be attached to

either an antigen or antibody and may be linked directly to the analyte or, in the case of competitive assays, to one of the other immuno-reagents.

Labels are used to give either a direct visual signal, or make use of an enzyme to mediate a visual response from a substrate. Alternatively a radioisotope such as ^{125}I can be combined with an antibody and used as a label and the presence or intensity of the signal measured using a scintillation counter. In his review of immunoassay methods, Gosling (1990) classifies labels into the following groups: enzyme based; fluorescent; ligand based; luminescent; microparticle; radioisotopic and vesicle based methods. Choice of label is dependent on the assay format chosen, ease of attachment, ease of measurement and needs to consider whether the use of radioactive materials is permitted or desirable.

More advanced methods may combine an immunoassay with a transducer so that the presence of the analyte indirectly produces an electrical signal on the surface of a transducer, creating an immuno based biosensor.

Combinations of the above criteria have led to a wide range of immunoassay methodologies used in medical and veterinary diagnostics, environmental applications, the detection of drugs of abuse and food safety analysis. The choice of assay depends on the analyte under test, whether a qualitative or quantitative result is required, the assay's sensitivity and specificity, the time required for the result and the conditions under which the test is being performed *i.e.* whether the assay requires incubation, refrigeration or instrumentation for detecting or measuring the label. Furthermore, some of these assays (and the results) will be performed and interpreted by medically trained professionals, while others can be obtained as an over-the-counter (OTC) product and used by patients themselves.

1.2 Lateral flow based immunoassays

Lateral flow immunoassays (LFIA) have a variety of alternative titles and abbreviations in academic journals and professional literature. They are also known as simply test

strips, immunochromatographic strip tests (ICS) and immunochromatographic assays (ICG or ICA).

LFIAs are designed to give a quick, qualitative response to the presence or absence of the target analyte. The signal generated is designed to be easily visualised and interpreted. They do not generally require any specialised equipment in their set up or in the analysis of results. They do not need incubation, electricity or refrigeration and can be used with little training or supervision. They can be produced comparatively cheaply in bulk and are designed to be disposable and used in, or away from, the laboratory, at point-of-care (POC), in the field, or as an OTC product *e.g.* pregnancy tests. A variety of samples may be tested in different assays, including urine, saliva, blood and serum.

Figure 2 depicts the functional zones of a typical LFIA. The main assay component is the membrane, which allows a sample containing the analyte (typically an antigen) to be applied at one end of the membrane, which then travels along the membrane by capillary flow. The membrane has two separate immuno-reagent zones along its length, which the sample passes as it wicks along the membrane. The immuno-reagents are dried down antibodies, which are specific for the analyte under test. The first zone comprises labelled antibodies, which get rehydrated by the sample and, if the analyte is present, their epitopes and paratopes bind and they wick along the strip together. The second zone consists of a second set of analyte specific antibodies that are immobilised onto the membrane. Their purpose is to capture and anchor the passing antigen by further specific immunological binding. Since the antigen has already bound the labelled antibody, a sandwich is formed and a visual signal is present on the test line.

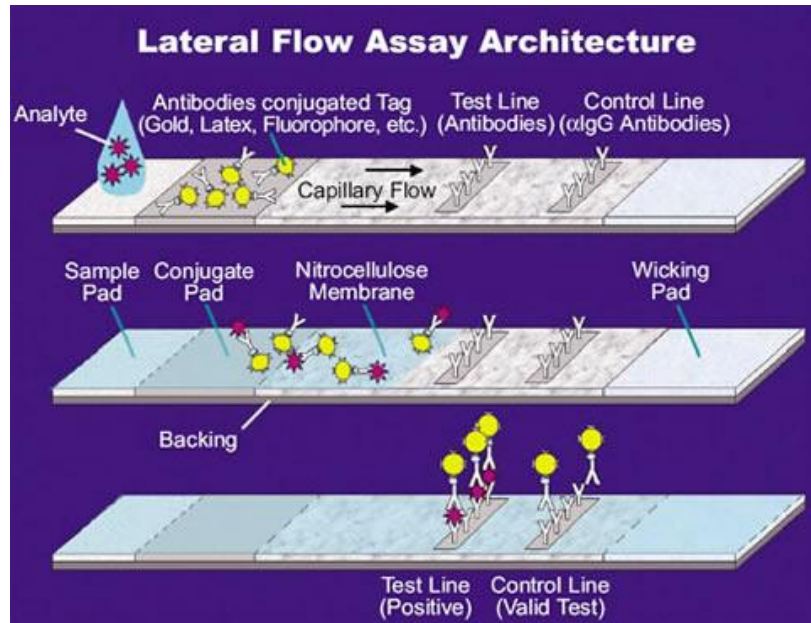


Figure 2 Schematic sequence of events in a typical lateral flow immunoassay (Guardian Biosciences, 2004).

Based on the classification described above, most LFIAs generally employ the excess reagent, sandwich assay format, although LFIAs designed to detect smaller antigens or drugs of abuse tend to use the competitive assay format (Millipore, 2008). Lateral flow assays are heterogeneous, since they use the membrane as a solid phase to separate the labelled antibody from the remote capture, immobilisation antibody, unlike homogenous assays where all of the reagents are mixed together. The antibody used for labelling the presence of the analyte is conjugated with microparticles, which are typically either coloured latex spheres or colloidal gold.

Components that demonstrate the correct functioning of an individual test strip, within the recommended assay time scale, known as a positive control, are often included in the LFIA format. These comprise additional reagents to those required by the analyte test itself. They indicate that the strip has functioned correctly and the assay has run for sufficient time.

Rather than using a simple coloured signal, some more recent LFIA devices have made use of a transducer based signalling method, enabling home pregnancy tests to have a

qualitative, digital display. While the majority of devices are designed for qualitative use, semi-quantitative and quantitative measurements are possible with design modifications. These adaptations may necessitate the inclusion of a “strip reader” system where imaging and quantification of the test line can occur. For example, Response Biomedical (2008) have developed a quantitative method (RAMP) that uses a fluorescent label. A strip reader measures the fluorescent signal at the test and control lines and compares the ratio of antigen bound label to unbound label to calculate the concentration of antigen present. Such systems increase the sensitivity of the LFIA approach but obviously require calibration and bring an increased cost per assay.

1.3 Lateral flow device components

A generic LFIA device has the components shown in Figure 3, which are often housed within a plastic case for protection. Each component has a specific function, as detailed below.

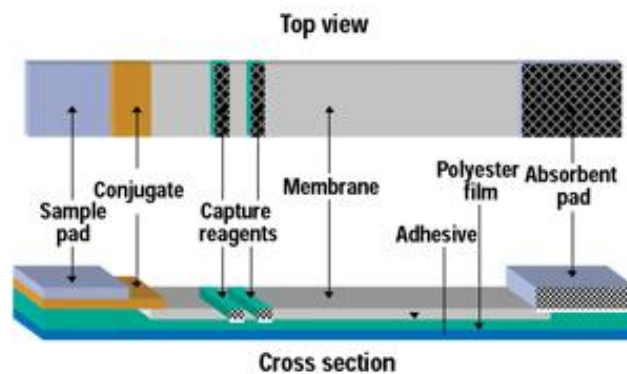


Figure 3 Components of a typical lateral flow device (IVD Technology, 2001).

1.3.1 Sample pad

The main function of the sample pad is to allow the sample to flow evenly over the conjugate area and potentially control the rate of flow of the sample, once applied. Cellulose based absorbent paper and woven mesh materials are frequently used as sample pads. Woven mesh materials tend to be stronger than cellulose papers, but have inferior liquid retention. The choice of sample pad also depends on the type of body fluid being used in the sample, *e.g.* cellulose paper is preferred for urine based assays (Millipore, 2008).

1.3.2 Conjugate pad

The conjugate pad is located between the sample pad and the membrane and overlaps the two zones. It has the dual role of channelling the sample analyte towards the membrane and holding the dried down immuno-labelled reagents in place, until the assay is performed. Once the sample enters the conjugate pad matrix, the dried down labelled antibodies are rehydrated and the solutions flow together along the membrane. The material chosen needs to be able to provide an even flow, unload the dried reagents readily and not cause any of them to bind to its fibres non-specifically. As with the sample pad, the material chosen depends on its relative strength, porosity and characteristics of the sample, but glass fibre and cellulose are commonly used.

1.3.3 Membrane

Different membrane materials are available depending on the sample, the analyte under test and the sensitivity required. For example, LFIA designers who want to detect analytes in serum may prefer an advanced material that can handle a whole blood sample, rather than having to separate blood from serum in advance. In addition, membranes that have the quickest capillary flow will be less sensitive than materials that take longer to run, since the reagents get less time to interact and produce a signal. For this reason, membranes with faster capillary flow frequently require greater volumes of immuno-reagents to be dried down, increasing production costs.

The choice of membrane is also dependent on its degree of protein binding, pore size and porosity, all of which determine the capillary flow or wicking rate of the applied sample. Millipore (2008) highlights the key difference between porosity and pore size. Porosity is the total volume of air in the three dimensional membrane structure, which may be independent of the pore size of the membrane material, since porosity takes account of pore density and distribution, not just diameter. A membrane's bed (or void) volume are used as alternative terms for porosity.

The test and control line reagents are positioned on the membrane and must remain in place during the assay and not wash down to the absorbent pad. Nitrocellulose based membranes are hydrophobic and bind proteins electrostatically, so that capture and control line antibodies are highly likely to remain in position. In contrast, cellulose acetate based membranes are more hydrophilic and consequently have reduced protein binding characteristics, but faster wicking rates than nitrocellulose based membranes. Capillary flow rates need to be optimised so that the sample can travel along the membrane easily and so that the immuno-reagents have sufficient time for the sandwich assay to form. Ultimately, the choice of membrane material and its inherent pore size and porosity, depends on the sample, analyte, type of assay and the capture methods being employed.

1.3.4 Adhesive and polyester film

Membranes are often fragile and are frequently coupled with a polyester backing to provide support and to help keep the liquid sample contained. The pads and membrane are held in position using an adhesive film, which may either run the full length of the strip or be positioned only at the ends of the membrane, to avoid the adhesive interfering with the wicking process, particularly with unsupported membranes.

1.3.5 Absorbent pad

The cellulose based absorbent pad is located at the opposite end to the sample pad and its function is to draw as much of the sample (and conjugate reagents) as possible through the membrane, passing the capture and control lines, into a final absorbent reservoir. Without the absorbent pad, the sample and conjugate may remain on these lines, inducing non-specific binding, which occurs when protein based materials in the assay stick to the membrane affecting the assay's sensitivity.

1.4 Lateral flow device reagents

The choice of reagents used in the conjugate area and on the capture line are crucial to overall sensitivity, specificity and the ultimate success of the whole assay.

1.4.1 Conjugate reagents

Both coloured latex spheres and gold are frequently used as labels in LFIAs. Analyte specific antibodies are conjugated with these coloured particles so that they will give a clear, visible signal on the test line following the formation of the sandwich assay.

Gold particles are cherry red in colour, giving an easily visible signal against a white coloured membrane. Gold particles can vary in size, although Chandler *et al.* (2000) suggest that 40nm particles are applicable to most assays, unless the antigen is particularly small, in which case, 20nm particles may be preferred, to avoid steric hindrance.

May *et al.* (1988) cite the first use of a coloured latex sphere in a lateral flow based OTC pregnancy test. Microspheres used in LFIAs are frequently made of polystyrene and come in a range of colours and sizes. Having a variable colour allows their use with different types of sample body fluid. Urine based pregnancy tests, for example, frequently use blue coloured beads to give a good visual signal. The size of the sphere is generally dependent on the porosity of the selected membrane. Bangs and Meza (1995) recommend that the ratio of sphere diameter to membrane porosity should be a minimum of 1:1 and optimally 1:10 to give optimum flow rates and suggest that spheres between 0.1 and 0.4µm in diameter are suitable for the majority of LFIAs, although the smaller sizes may give a less intense visual signal.

The conjugation involves the attachment of the microsphere or gold particles to the Fc portion of the antibody, freeing the Fab region for specific immunological binding reactions. The conjugation process should bind all of the available antibody in order to avoid any competition between labelled and unlabelled antibodies, otherwise, the sandwich assay may be completed, but with an unlabelled antibody, giving a falsely negative result. To achieve this, unlabelled antibody is removed by dialysis and centrifugation.

Chandler *et al.*, (2000) describe the forces involved in the conjugation of gold according to the amino acid sequence of the antibody's Fc portion, depicted below in

Figure 4. The negatively charged ion layer surrounding the gold particle attracts any positively charged amino acids in the Fc fragment of the antibody. Hydrophobic forces attract non-polar amino acids in the antibody, while amino acids with sulphur containing thiol side chains form covalent dative bonds with the gold.

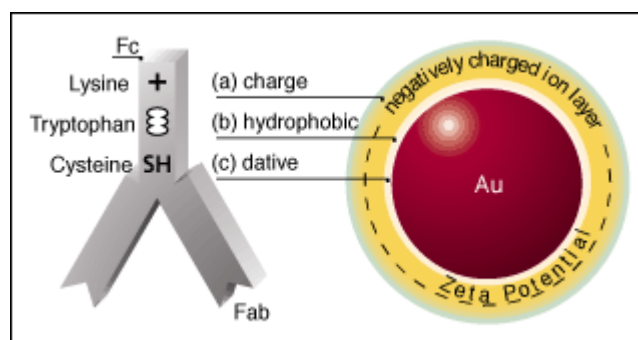


Figure 4 The different forces involved in the adsorption of colloidal gold to Fc region of an antibody (Chandler *et al.*, 2000).

While gold particles are attached via adsorption, latex microspheres can be attached to antibodies either by non-covalent adsorption or covalently. Attaching antibodies using adsorption requires careful adjustment of the pH of the buffer used with the spheres. Using a buffer solution with a slightly alkaline pH, compared to the isoelectric point of the chosen protein, favours adsorption of the antibody at the Fc region of the antibody, leaving the Fab end available for immunological recognition (Kawaguchi *et al.*, 1989). Bangs Laboratories (2002) cite eighteen scenarios where the covalent attachment of antibodies to latex beads may be preferable over adsorption methods, including increased sensitivity, better spatial alignment and improved coating levels for smaller antibody fragments or spheres.

1.4.2 Test and control line reagents

Antibodies specifically raised against the analyte are used to capture and immobilise their target on the test line. Similarly, control line antibodies are selected that are specific for the labelled antibodies in the conjugate zone. These reagents are pre-dried onto the surface of hydrophobic membranes and located downstream of the conjugate area. Their function is to identify and immobilise their specific targets in the

sample (or conjugate mix) and display a signal to indicate the presence, or absence, of the antigen (or labelled antibody). The reagents selected should be able to withstand the drying process and remain active after binding to the membrane and following rehydration.

Test line antibodies should be selected that have specificity for a different binding site (on the antigen/analyte) to that selected by the label conjugated antibody, so that on successful detection of the analyte, the sandwich assay format complex can be formed and bound to the substrate.

While the test line reagents' function is to immobilise the target analyte (or an immuno-reagent complex containing the analyte), the control line reagents detect an ingredient in the conjugate mix in order to give a "positive control" signal, regardless of the presence of the test analyte. This will indicate that the assay has run successfully and for long enough. Control line antibodies should therefore be from a different species, specifically raised against the host used in the label conjugation, see Figure 5.

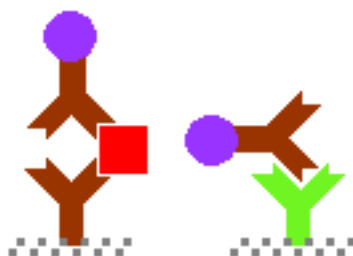


Figure 5 Capture antibodies on the membrane acting as a test line (left) and control line (right). The purple signal on the test line is only displayed when the capture antibody detects the (red) antigen, which has previously bound the labelled conjugate antibody upstream. The (green) control line antibody, raised against the labelled antibody in a different species, binds the conjugate, regardless of the presence of the antigen (adapted from WHO, 2005).

1.5 Optimisation of reagents

Numerous variations and combinations of reagents, materials and components are possible in order to optimise LFIAs for particular samples or analytes.

For example, among the available optimisation strategies, alternative membrane materials are used to reduce the incidence of non-specific binding giving false positive (or false negative) results. Furthermore, multi-analyte assays that simultaneously detect several antigens in the diagnosis of infectious diseases, or when detecting “drugs of abuse”, will require a variety of different reagents in the same LFIA.

1.5.1 Alternative immobilisation methods

Using a hydrophilic membrane, to reduce non-specific binding, requires an alternative immobilisation method on the test and control lines, since antibodies will show limited, if any, affinity for a hydrophilic surface. Instead of depositing antibodies directly on the capture lines, it is possible to attach antibodies to microspheres or use an indirect ligand-based recognition methodology. These techniques are of benefit when detecting smaller antigens since the use of microspheres provides a greater surface area for the analyte to bind.

a) Antibodies and microspheres

Bangs and Meza (1995) used the term “boulders in a stream” to describe a method of using large, colourless microspheres on the test and control lines to capture both the analyte and labelled antibodies respectively. The spheres are precoated with the capture antibody in order to trap and bind the target reagents as they flow passed the test and control lines. Tsuda *et al.* (1992) first adopted this technique in their, dipstick based, Rapid Immunofilter Paper Assay (RIPA) for detecting plant viruses. Bangs Laboratories Inc. (1999) report that Carter-Wallace Inc. were early pioneers of this approach in a LFIA, with their First Response 1-Step OTC pregnancy test, which got approval from the US Food and Drug Administration (FDA) in 1999.

As with the attachment of coloured spheres for labelling, antibodies can be attached to these larger spheres either by (non-covalent) adsorption, or covalently. In comparison to the coloured spheres used in labelling methods, these immobilisation spheres need to be larger, since they are designed to remain in position on the membrane during the assay. Bangs and Meza (1995) recommend that the ratio of the diameter of these spheres, compared to the membrane porosity, should be at least 1:1

and ideally 2:1 (or greater) and suggest a minimum diameter of 0.8µm depending on the membrane material.

b) Ligand based recognition

As an alternative to directly coating microspheres with antibodies, it is also possible to conjugate the spheres with other binding ligands, which will subsequently bond to pre-treated antibodies. This indirect method can help to avoid any steric hindrance issues and ensure that the Fc region of the antibody is anchored to the sphere, leaving the Fab section for immunological binding.

Avidin (a glycoprotein found in egg white) and biotin (a small water soluble vitamin) have a particularly strong affinity for each other. A molecule of avidin binds four molecules of biotin and their binding affinity is stronger than that found between epitope, paratope binding reactions. Avidin and biotin have a dissociation constant (K_D) of approximately 10^{-15} M compared to a K_D of 10^{-7} - 10^{-11} M for antibody, antigen interactions (Bayer and Wilchek, 1990).

Chalet and Wolf (1964) reported on a related binding protein, Streptavidin, from the bacteria *Streptomyces avidinii* which shows similar characteristics to avidin. They also calculated that, like avidin, a molecule of Streptavidin binds four molecules of biotin. Streptavidin has the same affinity and dissociation constant as avidin, but displays less non-specific binding due to a reduced isoelectric point and sugar content (Diamandis and Christopoulos, 1991). In addition to their strong mutual affinity, Streptavidin and biotin bind each other specifically and remain stable over a wide range of temperatures and pH.

Microspheres can be covalently coated with Streptavidin and used to attract biotinylated antibodies, which have biotin covalently attached to the Fc fragment of the antibody. The biotinylation process is carried out via the amino groups of lysine residues in the protein using a biotin ester such as N-hydroxysuccinimide (Dent, 2001).

Alternatively, this recognition method may be reversed by having microspheres coated with biotin which are used to attract Streptavidin labelled antibodies.

In addition to optimising the capture line reagents and immobilisation methods, the other key reagents, that can influence the sensitivity of the assay, are the antibodies themselves.

1.5.2 Antibody optimisation

a) Use of polyclonal and monoclonal antibodies

Polyclonal antibodies, produced by the B-cells of the host's immune system *in vivo*, are heterogeneous and so have a range of binding affinities and epitope specificities. However, this variety in their affinity and specificity can affect their performance in immunoassays, especially when batch to batch repeatability and consistency is essential in the commercial production of LFIA's.

The ability to be able to fuse B-cells with immortal myeloma cells enables the production of monoclonal antibodies. Producing monoclonal antibodies from hybridoma cells in tissue culture provides an unlimited supply of homogenous antibody which gives the uniform specificity required. Although monoclonal antibodies have higher specificity, their binding affinity are typically lower compared to polyclonal antibodies and they are more expensive.

Competitive (reagent limited) LFIA's tend to favour the use of polyclonal antibodies due to their increased affinity, while sandwich based, excess reagent tests (non-competitive) can make use of either a two monoclonal antibody based design or a combined monoclonal and polyclonal sandwich. Kane and Banks (2000) point out that many LFIA's use the combined monoclonal / polyclonal approach to avoid patent restrictions that may be in force on LFIA's based on two monoclonal antibodies.

b) Antibody fragments

Some immunoassays benefit from using antibodies that are divided into smaller functional fragments to reduce non-specific binding. Enzymes are used to cleave the structure of an antibody into separate chains or fragments. Papain, for example, separates the Fab section from the Fc region of an antibody. If antibodies are used on the capture line, cleaved Fab sections could be attached directly to the membrane.

However, LFIAAs that are dependent on the functionality of the Fc fragment for conjugation with a label, microsphere or ligand should use whole, uncleaved antibodies.

c) Recombinant antibodies

Recombinant DNA and gene expression techniques have made large supplies of immortal antibody libraries available. Light and heavy chain gene fragments are extracted and isolated from either spleen cells of immunised animals or lymphocytes from non-immune human donors. These can be cloned in phage display vectors to produce recombinant antibody fragments and the genes that code for them, allowing subsequent modification. Compared to the monoclonal, this method offers faster production and greater diversity in antigen specificity.

d) Synthetic antibodies

Polymerase chain reaction (PCR) techniques can also be used to replicate and manipulate the gene sequences of specific antibody fragments *in vitro*. This allows the production of synthetic antibodies, which have even greater purity and a wider range of epitope specificity.

1.6 Rotavirus

The target analyte used in this research is the Rotavirus antigen. Rotavirus infection is one of the primary causes of acute gastroenteritis in humans. It particularly affects babies, young children and the elderly and can lead to vomiting, diarrhoea, stomach cramps, fever, dehydration and loss of electrolytes, which can be fatal if not treated. The infection leads to the hospitalisation of approximately 55,000 children in the US each year (CDC, 2008). Dimmock *et al.* (2007) estimate that, worldwide, there are 140 million Rotavirus infections in children under five, per year. Of these, 527,000 are fatal, principally in the developing world (WHO, 2007).

There are seven groups of Rotavirus in the Reoviridae family (A to G), all of which have double stranded RNA. Groups A, B and C cause the infection in humans and the different groups can be identified according to differences in the antigenic properties

of the structural inner capsid protein, VP6 (Murray *et al.*, 2002). Other family groups lead to infection in other mammals and birds.

There are several potential diagnostic techniques that can provide a definitive diagnosis of Rotavirus infection and so distinguish it from other diseases that cause diarrhoea. Methods available include detection of the virus structure with electron microscopy, identification of a sequence of its RNA using reverse transcription-polymerase chain reaction (RT-PCR), or by studying the antigenic properties and interactions of the VP6 in immunological diagnosis methods. Immunological assay techniques include LFIA and enzyme linked immunosorbent assay (ELISA) based methodologies. Lateral flow methodologies are often preferred due to their portability, ease of use, speed and non-reliance on electricity or specialised equipment and reagents.

1.7 Aim, objectives and limitations of experiment

The aim of this research project was to assess the functionality of a lateral flow based immunoassay with a microsphere based capture line on a hydrophilic membrane material.

To achieve this aim, the experiments were designed with the following objectives. The first step was to recreate a working LFIA using a known methodology, with traditional lateral flow materials and immuno-reagents. Having optimised the assay for the reagents used, the materials and reagents are subsequently altered, one variable at a time, to achieve the target assay format. Finally, the new assay was compared with the original format.

1.7.1 The target assay format

The target assay format involved the use of Streptavidin conjugated microspheres on the test line, but positioned the biotinylated capture antibodies in the mobile phase with the labelled antibodies, (see Figure 6). This modification separates the capture phase of the assay from the immobilisation stage, *i.e.* the sandwich assay is completed before the reagents reach the test line. The Streptavidin conjugated microspheres on

the test line immobilise the sandwich assay as the reagents flow down the membrane (since one of the antibodies in the sandwich is biotinylated). However, the production of a signal relies on both the ability of the microspheres to remain *in situ* and the affinity of the Streptavidin and biotin to bind on the test line. Having the Streptavidin coated microspheres positioned in this way provides a potential means of producing a multi-analyte assay with a test line that is not antibody based and therefore not dependent on a single analyte.

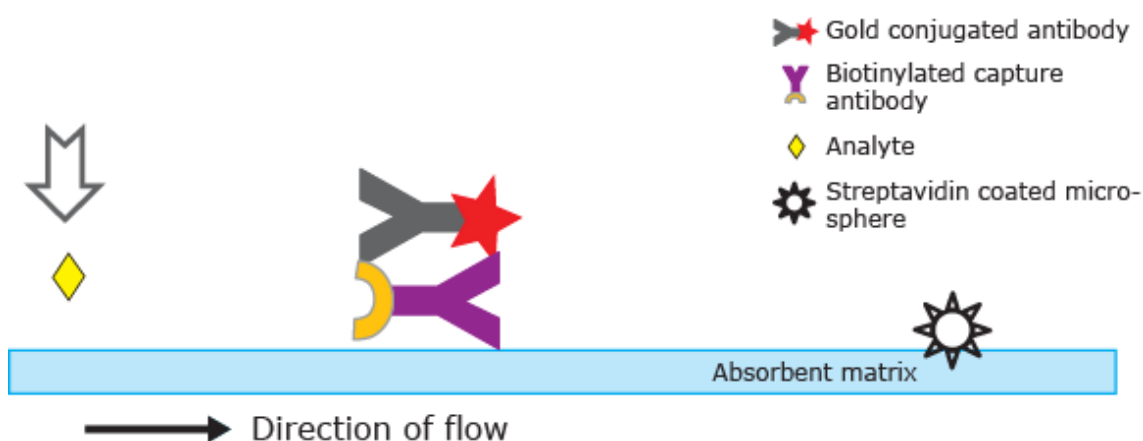


Figure 6 Diagram depicting the target assay with the separation of the biotinylated capture antibody from the test line. The capture antibody is located in the conjugate zone with the labelled antibody. The test line comprises Streptavidin coated microspheres to immobilise the biotinylated antibody. A signal is only produced at the test line if the analyte is present.

Furthermore, using a cellulose, or glassfibre based material for the membrane allows the possible replacement of the sample, conjugate and absorbent pads with a single material that will offer combined functionality and cheaper production costs. Fusion 5, a polymer treated glass fibre based membrane developed by Whatman is an example of such a multi purpose material, see Figure 7.

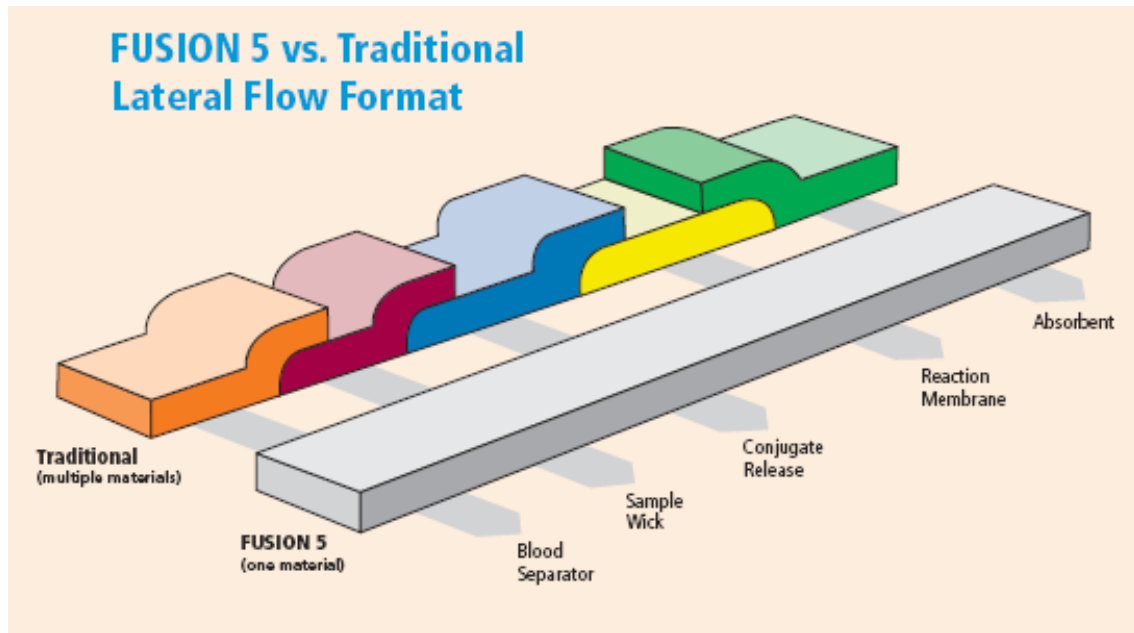


Figure 7 Schematic representation of the properties of Fusion 5 and the traditional lateral flow components it seeks to replace (Whatman, 2004).

The techniques employed in all the experiments described in this work were research based methods. The capture line reagents were applied by pipette, rather than by more sophisticated deposition methods, used in a production environment. Furthermore, to simplify the assay format and reduce the range of reagents on the strip, only test line reagents were applied to the immobilisation (test line) area and not control line reagents.

Chapter 2: Materials and Methods

2.1 Materials

A full description of all materials and reagents is listed in Appendix A.

2.1.1 Buffers

The buffers used to make up dilutions of the various reagents, or used in the preparation of the microspheres, are detailed below.

a) Sample dilution buffer

i) Proprietary constituents made up of saline solution, buffered to pH 7.5 with TRIS, plus EDTA, NaN_3 , detergent and charged proteins (Corus Bioconcept).

ii) Phosphate Buffered Saline with Tween (PBST), pH 7.4

One tablet (Sigma) in 200ml of reverse osmosis water comprises:

0.01M Phosphate buffer

0.0027M KCl

0.137M NaCl

plus 0.05% Tween 20 (Acros Organics)

b) Conjugate release buffer

0.1M Tris base, pH 8.0 (Fisher Bioreagents)

5% (w/v) Bovine Serum Albumin fraction V (Sigma)

5% (w/v) Sucrose (Sigma)

0.1% Tween 20 (Acros Organics)

c) Capture antibody dilution buffer

Phosphate Buffered Saline (PBS), pH 7.4

One tablet (Sigma) in 200ml of reverse osmosis water comprises:

0.01M Phosphate buffer

0.0027M KCl

0.137M NaCl

d) Microsphere binding buffer (pH 7.4) -

0.02M Phosphate Buffered Saline

1% (w/v) Bovine Serum Albumin fraction V (Sigma)

All reagent dilutions are shown from their original stock concentrations, for example:

1/4 dilution of antigen = rehydrated freeze dried form, 1/4 diluted in sample dilution buffer (Corus).

1/16 dilution of antigen = rehydrated freeze dried form 1/4 diluted in sample dilution buffer (Corus), followed by 1/4 dilution in PBST (2.1.1) or sample dilution buffer (Corus).

2.2 Using hydrophobic membranes in lateral flow immunoassays

The protocol for assembling the lateral flow strips assembly using the Predator membrane (Pall, see Appendix A) was adapted from Cranfield University, Medical Diagnostics laboratory practical (Taylor and Rixon, 2008).

Sections of absorbent and sample pad (Pall), each measuring 1cm x 0.5cm, were fixed to opposite ends of a 3.5cm x 0.5cm strip of laminated Predator membrane (Pall), using 1cm x 0.5cm pieces of adhesive film (Pall). The pads were arranged so that half of their length overlapped the membrane surface by 0.5cm, the other half remaining over the edge of the membrane.

The conjugate pad (Pall) was prepared by placing 10 μ l of gold conjugated anti-Rotavirus antibody (BB International), diluted 1/6 with the conjugate release buffer (2.1.1), on to a square section (0.4cm x 0.4cm) of conjugate release pad. The reagents were allowed to dry into the conjugate pad, which was then positioned between the sample pad and the membrane, as shown in Figure 8.

The capture (test) line reagents were pipetted directly onto the Predator membrane (Pall) at the half way point (1.75cm) or just beyond, towards the absorbent pad. The

test line reagents were applied 0.5µl at a time and allowed to dry down before the application of the next drop. The total volume applied was either 0.5µl, 1.0µl, 2.0µl or 4.0 µl.

The test sample (typically 80µl) containing the Rotavirus antigen (Corus) was added to the cap of a microcentrifuge tube and the sample pad end of the test strip was inverted and dipped into the sample such that it could wick along the membrane, via the conjugate area. Once the sample had wicked past the conjugate area, the strip was removed from the sample well and positioned horizontally to allow wicking to continue. The strip was checked for the presence of a pink coloured spot, in the test line area, within a fifteen minute period. In the majority of cases, each test assay format was performed in duplicate.

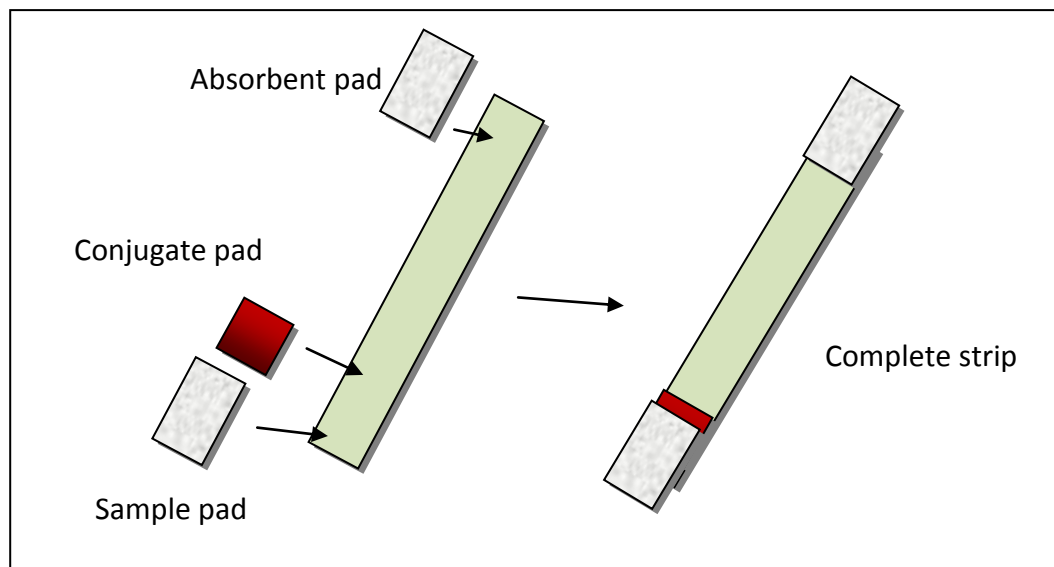


Figure 8 LFIA construction. Diagram showing position of the absorbent, sample and conjugate pads in relation to the membrane. Note the position of the conjugate pad, sandwiched between the sample pad and the membrane.

2.2.1 Positive control - Antigen viability assessment

Two different batches of Rotavirus antigen (Corus Bioconcept) were assessed and used as positive controls in all experiments described below. The antigen was activated, according to the manufacturer's specifications, by rehydration with 2ml of reverse osmosis (RO) water. One batch was further diluted by 1/4 using the supplied sample dilution buffer (Corus), while the second batch was not diluted and used (where indicated) as a stock concentration.

The activity of both batches of antigen was assessed by using a 0.5ml sample with the Rota-Strip Quick Test (Corus Bioconcept) and a 0.5ml negative control, using the proprietary dilution buffer (Corus), tested for comparison.

2.2.2 Initial optimisation - Gold label concentration

Optimal concentrations of the immuno-reagents were assessed for use on the Predator membrane (Pall) based LFIA. The gold conjugated anti-Rotavirus antibody (BB International), Rotavirus antigen (Corus), and the anti-Rotavirus capture antibody (Dako) were assessed by sequentially altering and comparing the concentrations of one reagent at a time.

Initially, two different gold conjugated anti-Rotavirus antibody concentrations (BB International) - diluted 1/3 and 1/6 with conjugate release buffer (2.1.1), were assessed, while the volume and concentration of Rotavirus antigen (Corus) and anti-Rotavirus capture antibody (Dako) remained constant, see Table 1.

The optimisation assay was repeated using sample dilution buffer (Corus), in place of the Rotavirus antigen (Corus), to act as a negative control.

Table 1 Gold label concentration optimisation experiment on Predator membrane. Description of immuno-reagents, their position on the strip and their relative dilutions.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/4 diluted in sample dilution buffer (Corus)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 and 1/3 diluted in conjugate release buffer (2.1.1)
Test line	Anti-Rotavirus antibody (Dako)	4µl	Stock concentration

2.2.3 Initial optimisation - Antigen concentration

The antigen concentration was serially diluted with the proprietary sample dilution buffer (Corus) by a factor of four each time (between 1/4 and 1/16384) and tested with a fixed 4µl volume of Anti-Rotavirus capture antibody (Dako) on the test line. The serial dilutions of antigen were assessed with two different gold antibody concentrations (BB International), diluted 1/3 and 1/6 with conjugate release buffer (2.1.1), see Table 2.

The optimisation assay was repeated using sample dilution buffer (Corus), in place of the Rotavirus antigen (Corus), to act as a negative control.

Table 2 Reagents used in experiments to assess the Rotavirus antigen sensitivity on the Predator based LFIA.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/4 – 1/16384 diluted in sample dilution buffer (Corus)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 and 1/3 diluted in conjugate release buffer (2.1.1)
Test line	Anti-Rotavirus antibody (Dako)	4µl	Stock concentration

2.2.4 Initial optimisation - Conjugate release buffer pH

The strips' performance was also assessed while varying the pH of the conjugate release buffer (2.1.1.). A range of conjugate buffers were made up with pHs ranging from 7.4 to 9.0 and used in a series of 1/6 dilutions of the gold conjugated anti-Rotavirus antibody. The volumes and concentrations of the Rotavirus antigen (Corus) and Anti-Rotavirus antibody (Dako) were the same as shown in Table 2.

2.2.5 Initial optimisation - Capture antibody volume

The number of 0.5µl applications of the Anti-Rotavirus capture antibody (Dako) sequentially added to the test line of the Predator membrane (Pall) was also adjusted, giving total volumes between 0.5µl-4.0µl, as shown in Table 3.

The optimisation assay was repeated using sample dilution buffer (Corus), in place of the Rotavirus antigen (Corus), to act as a negative control.

Table 3 Experiment to evaluate the effect of capture anti-Rotavirus antibody (Dako) volume on Predator membrane LFIA. Table of strip composition and reagents.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in sample dilution buffer (Corus)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
Test line	Anti-Rotavirus antibody (Dako)	1,2,4 or 8 x 0.5µl	Stock concentration

2.2.6 Optimal reagent volumes and concentrations for Predator based LFIA

After the initial optimisation, antigen samples were diluted in PBST buffer, rather than the proprietary sample dilution buffer (Corus) and the sensitivity compared.

All the optimisation strategies above were combined to obtain reproducible assay results using a LFIA with a Predator based membrane (Pall), the Rotavirus antigen (Corus), gold conjugated anti-Rotavirus antibody (BB International) and Anti-Rotavirus capture antibody (Dako).

2.2.7 Use of an alternative, biotinylated anti-Rotavirus capture antibody with Predator membrane

In preparation for the Fusion 5 membrane (Whatman) based assays, a goat based, biotinylated anti-Rotavirus capture antibody (Acris Antibodies) was used in place of the rabbit anti-Rotavirus capture antibody (Dako) and a serial dilution with PBS buffer (2.1.1) performed. The volumes and concentrations of all reagents are summarised in Table 4.

The assay was repeated using PBST buffer (2.1.1), in place of the Rotavirus antigen (Corus), to act as a negative control.

Table 4 Experiments to evaluate the effect of a biotinylated anti-Rotavirus capture antibody concentration on LFIA performance and to assess the sensitivity on a Predator based LFIA.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
Test line	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1 – 1/256 diluted in PBS (2.1.1)

2.2.8 Effects of using Streptavidin coated microspheres on the test line with biotinylated anti-Rotavirus antibody pre-mixed with gold conjugated anti-Rotavirus antibody.

The LFIA format was modified so that the biotinylated anti-Rotavirus capture antibody (Acris) was premixed with the gold conjugated anti-Rotavirus antibody (BB International), see Table 5. The premixed solution was dried down in the conjugate zone of the Predator membrane, upstream of the test line. 4µl of the smaller (0.83µm) Streptavidin coated microspheres (Seradyn) were positioned on the test line. This assay would therefore resemble the target assay format (1.7.1) using a Predator (Pall) based LFIA.

The microspheres were prepared and washed according to the protocol in Appendix B, except that BSA was included in the binding buffer (2.1.1).

The optimisation assay was repeated in two negative control experiments. The first using PBST buffer (2.1.1), in place of the Rotavirus antigen (Corus) and the second omitting the biotinylated anti-Rotavirus capture antibody altogether.

Table 5 Reagents used in the evaluation of a Streptavidin coated microsphere test line method on a Predator membrane based LFIA.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)
Test line	Streptavidin coated microspheres (Seradyn)	4µl	

2.2.9 Preparation of microspheres

The test line in the above experiment and Fusion 5 (Whatman) based LFIA experiments utilised the Streptavidin coated microspheres.

Microspheres from both suppliers (Seradyn and Polysciences) were washed according to the Polysciences protocol (2005), see Appendix B, since a detailed washing protocol was not supplied with the microspheres supplied by Seradyn.

2.3 Assessment of the biotin binding affinity of the Streptavidin coated microspheres

The streptavidin coated microspheres were either used in isolation or pre-incubated with a biotinylated capture antibody, either biotinylated anti-Rotavirus antibody (Acris Antibodies) or, where shown, biotinylated anti-mouse IgG antibody (QED bioscience).

The incubation protocols for the two sources of microsphere differed. The smaller, 0.83µm diameter, microspheres required incubation with the selected biotinylated antibody at room temperature (which varied from 22-25°C) for one hour (Seradyn,

2004). The 2µm diameter microspheres were incubated with the chosen biotinylated capture antibody at 4°C for thirty minutes (Polysciences, 2005).

Following the incubation period, the microspheres (from both providers) were washed with three further cycles of centrifugation at 10,000g for five minutes.

2.3.1 Homogenous Rotavirus assay

In order to assess the binding affinity of the Streptavidin / biotin reaction, an 80µl sample of Rotavirus antigen (1/16 diluted with PBST buffer from the re-hydrated freeze dried form) was placed in an Eppendorf tube. Added to this were 10µl gold conjugated anti-Rotavirus antibody (Corus), diluted 1/6 with conjugate release buffer and 4µl of the larger 2µm Streptavidin beads (Polysciences), which had been pre-incubated with the biotinylated goat anti-Rotavirus antibody (Acris), 1/4 diluted with PBS buffer (2.1.1). Table 6 summarises the volumes and dilutions of the reagents used.

The negative control contained 80µl of PBS (2.1.1) in place of the diluted antigen.

Both positive and negative control Eppendorf tubes were centrifuged at 10,000g for two minutes and compared.

Table 6 Homogenous Rotavirus assay reagent quantities and concentrations used to verify the binding affinity of the Streptavidin-biotin reaction and the immuno-reagents.

Homogenous assay		Reagent	Volume	Dilution
Sample		Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Antibodies		Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
		Biotinylated anti-Rotavirus antibody (Acris) pre-incubated with Streptavidin coated spheres (Polysciences)	4µl	Antibody 1/4 diluted in PBS (2.1.1)

The experiment was also repeated using the stock concentration of the biotinylated anti-Rotavirus antibody (Acris) pre-incubated with the Streptavidin coated spheres (Polysciences).

2.4 Using a hydrophilic membrane in a lateral flow immunoassay

The hydrophilic Fusion 5 material (Whatman) was cut to the same dimensions as the Predator membrane (Pall), 3.5cm x 0.5cm (unless stated otherwise), but being a multi function material, it did not require the addition of the supplementary pads. Consequently, the gold conjugated anti-Rotavirus antibody was applied directly to the Fusion 5 membrane, 1.5cm from the sample end, and allowed to dry down.

The Fusion 5 based LFIA's used the Streptavidin coated microspheres on the test line. These were applied either as a single spot, or in two aliquot volumes spotted adjacently and positioned 3cm from the sample end of the Fusion 5 strip.

The sample application method for Fusion 5 based strips was the same as for the Predator membrane, except that the Fusion 5 strips were left inverted in the sample for longer, to allow the sample to wick past the test line area.

2.4.1 Microsphere immobilisation on Fusion 5

To assess the ability of the larger Streptavidin coated microspheres (Polysciences) to remain in position on the hydrophilic Fusion 5 membrane (Whatman), the spheres were pre-incubated with biotinylated capture antibodies and applied to the test line of a Fusion 5 based LFIA.

10µl of mouse based, gold conjugate anti-Rotavirus antibody (BB International), 1/6 diluted with conjugate release buffer (2.1.1), was applied to the conjugate zone, 1.5cm from the sample end of the Fusion 5 membrane and allowed to dry down.

Biotinylated goat anti-mouse IgG antibody (QED Biosciences), 1/4 diluted with PBS buffer (2.1.1), was pre-incubated with the microspheres (Polysciences) and separate strips prepared with both 2µl and 4µl of the pre-incubated solution pipetted onto the test line (3cm from the sample end of the Fusion 5 strip).

PBST buffer (2.1.1) was applied to the strip (80µl) as a sample and the strip inspected for the appearance of any pink spots after the buffer had wicked along the strip, past the dried down conjugate area. The reagents used are summarised in Table 7. Negative controls were run in parallel which omitted the biotinylated anti-mouse antibody and had only microspheres on the test line.

For comparison, strips were prepared in which the conjugate area was left bare and the gold-labelled mouse antibody (BB International) (10µl) was added to 80µl of PBST buffer and the combined sample applied to the strip. The test line comprised the microsphere and anti-mouse IgG pre-incubated mixture, as before.

Table 7 Reagent selection for the assay designed to assess whether the Streptavidin coated microspheres would remain in place during a Fusion 5 based LFIA.

Fusion 5 membrane	Reagent	Volume	Dilution
Sample	PBST (2.1.1)	80µl	-
Conjugate area	Gold conjugated, mouse based, anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
Test line	Biotinylated anti-mouse IgG pre-incubated with Streptavidin coated spheres (Polysciences)	2 or 4µl	Antibody 1/4 diluted in PBS (2.1.1)

2.4.2 Initial Fusion 5 methodology

The first experiment using the Rotavirus antigen (Corus) in a Fusion 5 (Whatman) based LFIA was a repeat of the experiment performed in section 2.2.8, but with Fusion 5 used in place of Predator (Pall) in the target assay (1.7.1).

It had the gold conjugate anti-Rotavirus antibody (BB International) premixed with the biotinylated anti-Rotavirus polyclonal antibody (Acris) the mixture was dried in the

conjugate area of the Fusion 5 membrane and the Streptavidin microspheres positioned on the test line.

The position, volumes and dilutions of the reagents are summarised in Table 8. The assay was carried out using the smaller Streptavidin coated microspheres and repeated with the larger size. The microspheres were washed in binding buffer containing BSA.

Table 8 Reagent volumes, position and dilutions used in an attempt to produce an LFIA with an analyte independent test line.

Fusion 5 membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)
Test line	Streptavidin coated microspheres (0.83µm or 2µm)	4µl	

Negative control experiments either used PBST (2.1.1) in place of the Rotavirus antigen or removed the biotinylated anti-Rotavirus antibody from the assay.

The experiment was also repeated using the proprietary sample dilution buffer (Corus) to dilute the Rotavirus antigen (Corus) and using the conjugate release buffer to dilute the biotinylated antibody rather than PBS.

2.4.3 Optimising the concentrations and combinations of reagents used with Fusion 5 membrane

The concentrations of the different immuno-reagents in Table 8 were adjusted sequentially, while using 4µl of the Streptavidin coated microspheres (Polysciences) on the test line of all the following Fusion 5 (Whatman) based assays.

The sample concentration was altered in the first series of Fusion 5 based assays, with dilutions of Rotavirus antigen (Corus) ranging from 1/4 to 1/60. All dilutions made with PBST (2.1.1). The concentrations of gold conjugated anti-Rotavirus antibody (BB International) and biotinylated anti-Rotavirus antibody (Acris) were as summarised in Table 8.

The concentration of the gold conjugated anti-Rotavirus antibody (BB International) was then adjusted, with dilutions ranging from 1/2 to 1/6. Dilutions were made using conjugate release buffer (2.1.1). The Rotavirus antigen (Corus) and biotinylated anti-Rotavirus antibody (Acris) were used as shown in Table 8.

The strength of the capture antibody was also optimised in a further series of Fusion 5 based assays by diluting the biotinylated anti-Rotavirus antibody (Acris) with PBS (2.1.1) in dilutions ranging from 1/4 to 1/10. The Rotavirus antigen (Corus) and gold conjugated anti-Rotavirus antibody (BB International) were kept constant, as listed in Table 8.

All of the above experiments were compared directly with negative controls which used PBST (2.1.1) in place of the Rotavirus antigen or omitted the biotinylated anti-Rotavirus antibody.

2.4.4 Pre-incubation of reagents (before immobilisation) using a Fusion 5 based membrane

A number of experiments were also performed involving pre-incubated combinations of the key immuno-reagents: Rotavirus antigen (Corus); Gold conjugated anti-Rotavirus antibody (BB International) and Biotinylated anti-Rotavirus antibody (Acris).

The position of the pre-incubated reagents in the Fusion 5 based assay were interchanged and assessed (see Table 9-13).

Two negative control experiments were carried out for each variation, PBST (2.1.1) was used in place of the Rotavirus antigen (Corus) and PBS (2.1.1) replaced the biotinylated anti-Rotavirus antibody (Acris).

a) Pre-incubated immuno-reagent sample

All of the three key immuno-reagents were pre-incubated and supplied together as the sample, with the Streptavidin coated microspheres (Polysciences) on the test line – see Table 9.

Table 9 All immuno-reagents were pre-incubated and supplied as a sample with Streptavidin coated spheres on the test line of a Fusion 5 based LFIA. Conjugate area left bare.

Fusion 5 membrane	Reagent	Volume	Dilution
Pre-incubated Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)
Conjugate pad	-	-	
Test line	Streptavidin coated spheres (Polysciences)	4µl	

b) Alternative pre-incubated sample strategy

The Rotavirus antigen (Corus) and biotinylated anti-Rotavirus capture antibody (Acris) were pre-incubated and applied as a sample to a Fusion 5 based LFIA (see Table 10).

Table 10 Pre-incubated Rotavirus antigen and biotinylated anti-Rotavirus capture antibody applied together as a sample, with gold conjugated anti-Rotavirus antibody dried in the conjugate area. Streptavidin coated spheres on the test line of a Fusion 5 based LFIA.

Fusion 5 membrane	Reagent	Volume	Dilution
Pre-incubated Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
Test line	Streptavidin coated spheres (Polysciences)	4µl	

c) Pre-incubated test line

The Streptavidin coated microspheres (Polysciences) were also pre-incubated with the biotinylated anti-Rotavirus capture antibody (Acris), using a range of antibody concentrations, see Table 11.

Table 11 Experiments using variable concentrations of biotinylated anti-Rotavirus capture antibody pre-incubated with Streptavidin coated microspheres.

Fusion 5 membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
Test line	Streptavidin coated spheres pre-incubated with biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 - 1/10 diluted in PBS (2.1.1)

d) Combined pre-incubated sample and test line strategy

A dual pre-incubation strategy paired up the Rotavirus antigen (Corus) with the gold conjugated anti-Rotavirus antibody (BB International), which were applied together as a sample. In addition, the test line combined biotinylated anti-Rotavirus capture antibody (Acris) pre-incubated with Streptavidin coated microspheres. The conjugate zone was left bare, see Table 12.

Table 12 Pre-incubated Rotavirus antigen and gold conjugated anti-Rotavirus antibody supplied as a sample with a pre-incubated test line comprising biotinylated anti-Rotavirus capture antibody pre-incubated with Streptavidin coated microspheres

Fusion 5 membrane	Reagent	Volume	Dilution
Pre-incubated Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
Conjugate pad	-	-	
Test line	Streptavidin coated spheres pre-incubated with Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)

e) Alternative test line pre-incubation strategy

An additional strategy had a test line that combined pre-incubated Rotavirus antigen (Corus) with the biotinylated anti-Rotavirus antibody (Acris) and this mixture was pre-incubated with the Streptavidin coated microspheres (Table 13). PBST (2.1.1) was applied as the sample to rehydrate the gold conjugated anti-Rotavirus antibody (BB International).

Table 13 Fusion 5 based assay using a pre-incubated Rotavirus antigen and biotinylated anti-Rotavirus capture antibody mixture combined with Streptavidin coated microspheres. PBST sample added to strip to rehydrate the dried down gold conjugated anti-Rotavirus antibody.

Fusion 5 membrane	Reagent	Volume	Dilution
Sample	PBST (2.1.1)	80µl	-
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
Pre-incubated Test line	Rotavirus antigen (Corus) pre-incubated with Biotinylated anti-Rotavirus antibody (Acris), then combined with Streptavidin coated spheres	4µl	Biotinylated anti-Rotavirus antibody 1/4 diluted in PBS (2.1.1)

2.4.5 Drying down all the reagents on Fusion 5

In additional experiments, where indicated, a more concentrated form of Rotavirus antigen (Corus) was used (as described in section 2.2.1), which enabled the Rotavirus antigen, to be dried down on the strip alongside the other immuno-reagents, in the conjugate zone, see Table 14. Each reagent was applied separately and allowed to dry down before the application of the next reagent. A sample of PBST (2.1.1) was then applied to the strip.

Conjugate position 1 in Table 14 corresponded with the location nearest the sample application end, while position 3 was mid-strip, nearest the test line. Position 2 was between positions 1 and 3.

Table 14 An assessment of pre-drying all immuno-reagents on to a hydrophilic membrane in a LFIA.

Fusion 5 membrane		Reagent	Volume	Dilution
Sample		PBST (2.1.1)	80µl	-
Conjugate position	1	Rotavirus antigen (Corus)	5µl	Stock conc
	2	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/4 diluted in PBS (2.1.1)
	3	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
Test line		Streptavidin coated spheres	2-4µl	

Negative control experiments run in parallel omitted either the Rotavirus antigen (Corus) or the Biotinylated anti-Rotavirus antibody (Acris) from the strip.

2.4.6 Use of a composite lateral flow strip with “dual test lines” using different membrane materials

Composite strips using sections of both Fusion 5 (Whatman) and Predator (Pall) membranes were also constructed. These were designed to have “dual test line” areas comprising dried down 2µm microspheres (Polysciences) on the Fusion 5 material and rabbit based anti-Rotavirus capture antibodies (Dako) on the Predator membrane. The remaining immuno-reagents (Rotavirus antigen, gold labelled antibody and biotinylated capture antibody) were individually applied to separate Fusion 5 pads, which after drying in isolation, were assembled on adhesive film (see Figure 9). This

was to ensure that the reagents dried without spreading into other zones of the strip and binding prematurely.

The volumes of reagents and their position on the strip matched the experiment above (Table 14). The Predator section had 4µl of the stock concentration of anti-Rotavirus capture antibody (Dako) applied.

Negative controls used blank membrane sections in place of either the Rotavirus antigen section, or one of the capture antibody test line sections *i.e.* the biotinylated anti-Rotavirus antibody (Acris) or the anti-Rotavirus antibody (Dako) sections.

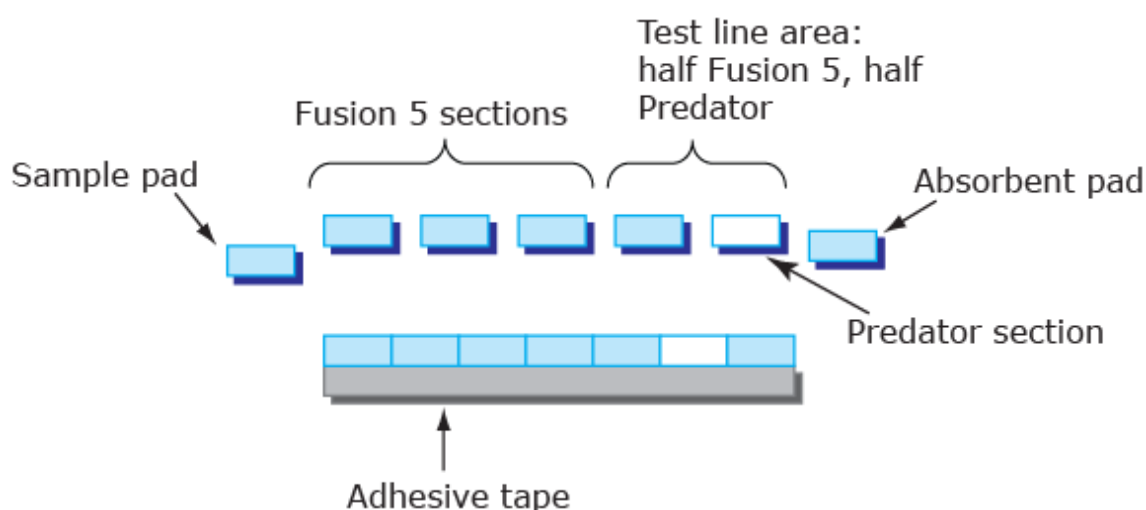


Figure 9 Composite strip, with separate sections, to allow isolated drying of reagents and dual Fusion 5 / Predator test line. Sections assembled using adhesive tape.

2.5 Optimisation of the Fusion 5 methodology to improve assay performance

In addition to having all the immuno-reagents dried on the strip, including the Rotavirus antigen (Corus), the assay methodology was further optimised, based on the reagent volumes and concentrations shown in Table 14 (unless otherwise indicated).

In the majority of cases the assays were carried out in duplicate and negative controls were tested in parallel, which involved removing the Rotavirus antigen (Corus) or the biotinylated capture antibody (Acris) from the assay.

The following modifications were made:

- The length of the Fusion 5 (Whatman) assay strip was varied
- The strips were dried in an oven (37°C) after the addition of the sample
- The assay was performed vertically, so that the reagents flowed down the strip towards the test line, after the sample had been applied.
- The order and position that the immuno-reagents were dried down on the Fusion 5 membrane (Whatman) was changed.
- The effects of using different dilution buffers (2.1.1) were also examined for their effects on assay performance.
- The concentration of the biotinylated anti-Rotavirus capture antibody was increased by using the stock concentration and a solution 1/2 diluted with PBS (2.1.1).
- The assay was repeated using alternative hydrophilic membranes (see Appendix A)

2.6 Optimised reagent selection for the target assay format on a Fusion 5 based LFIA

The final reagent selection used to try and achieve the target assay format (1.7.1), was selected following a number of the optimisation strategies listed above in section 2.5. The selection is summarised in Table 15.

The sample application method was changed by using two aliquots: first using a small volume of concentrated Rotavirus antigen (20µl), allowing the sample to reach the conjugate area, followed by 60µl of PBST (2.1.1).

Table 15 Final reagent conditions.

Fusion 5 membrane		Reagent	Volume	Dilution
Sample 1*		Rotavirus antigen (Corus)	20µl	1/4 diluted in PBST (2.1.1)
Sample 2*		PBST (2.1.1)	60µl	-
Conjugate position	1	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate buffer (2.1.1)
	2	Biotinylated anti-Rotavirus antibody (Acris)	4µl	1/2 diluted in PBS (2.1.1)
Test line		Streptavidin coated spheres	2µl	

*The sample was applied in two aliquots: 20µl 1/4 diluted Rotavirus antigen, followed by 60µl PBST (2.1.1).

Chapter 3: *Results*

3.1 Buffers

3.1.1 Use of alternative sample dilution buffers

Substituting PBST (2.1.1) in place of the supplied sample dilution buffer (Corus) to dilute Rotavirus antigen samples produced no noticeable change in the generation, or intensity, of any positive results produced on the Rota-Strip Quick Test (Corus), Predator (Pall) or Fusion 5 (Whatman).

3.2 Results from using hydrophobic membranes in lateral flow immunoassays

Both the Rota-Strip (Corus) and Predator (Pall) based lateral flow assays used hydrophobic membrane materials (Appendix A).

3.2.1 Positive Control - Antigen viability assessment

The Rotavirus antigen (Corus), 1/4 diluted in sample dilution buffer (Corus), was tested with a Rota-Strip Quick Test (Corus) and compared with a negative control, comprising sample dilution buffer (Corus) alone. The result is shown in Figure 10.

In the positive control strips (presence of Rotavirus), two pink lines were visible in each strip (A and B, left), while only a single line was visible in the negative control strips with no Rotavirus (C and D, right).

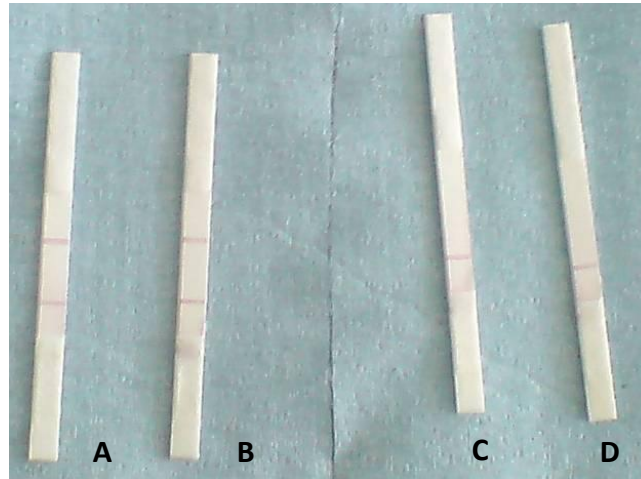


Figure 10 Rota-Strip test results using positive and negative control samples. Positive control sample of Rotavirus antigen 1/4 diluted in sample dilution buffer (A and B, left) and sample dilution buffer alone (C and D, right).

A second batch of Rotavirus antigen (Corus) was also tested and compared against the original batch using the Rota-Strip Quick Test. Equivalent dilutions of Rotavirus antigen produced slightly darker test lines with the second batch.

3.2.2 Initial optimisation results - Gold label concentration

The diagram in Figure 11 shows the positions of the key reagents in the initial Predator (Pall) based assay. The initial experiments compared the signal produced by the LFIA using two concentrations of the gold conjugated anti-Rotavirus antibody (BB International). The volumes and concentrations of all immuno-reagents are summarised in Table 1. Negative controls were performed using sample dilution buffer (Corus) in place of the Rotavirus sample (Corus).

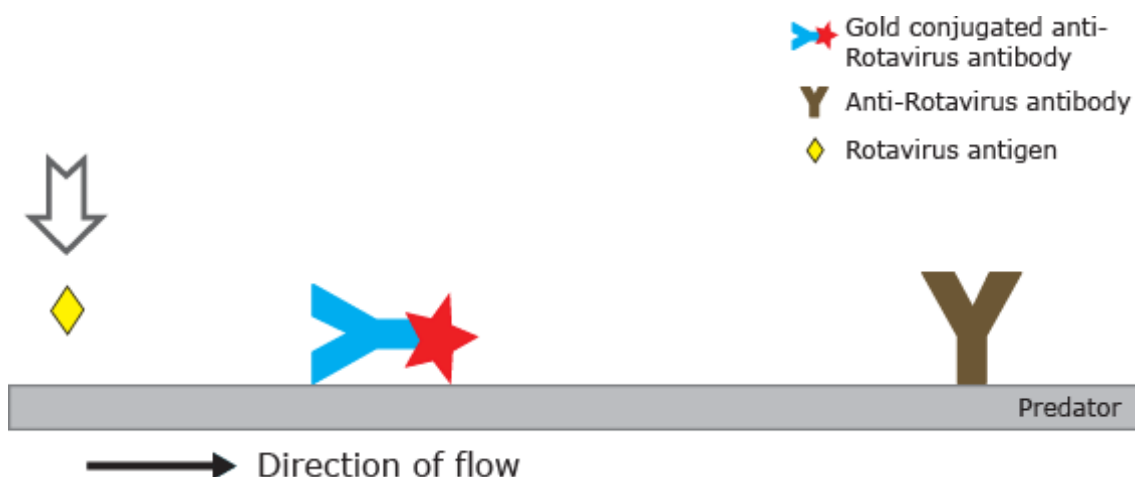


Figure 11 Diagram showing the relative position of assay reagents in the initial Predator (Pall) based lateral flow assay. The downward arrow on the left indicates the application of the sample.

Table 16 and Figure 12, show that a strong signal was produced in both cases where the antigen was present, but that there was no significant difference in the level of signal produced with the two concentrations of the gold conjugated anti-Rotavirus antibody tested. The signal results appeared quickly as the test strips dried, typically within ten minutes of applying the sample.

Table 16 Experiment to assess the difference in signal obtained using different concentrations of gold conjugated anti-Rotavirus antibody.

Predator membrane	Dilution factor*	Assay Result	Negative Control
Gold conjugated anti-Rotavirus antibody (BB International)	1/6	++	O
Gold conjugated anti-Rotavirus antibody (BB International)	1/3	++	O

* Gold conjugated anti-Rotavirus antibody diluted in conjugate release buffer (2.1.1).

Assay results key: ++ Strong Signal (+) Weak Signal
+ Signal O No signal

The strips in Figure 12 show the formation of a pink signal in the positive control examples (A and B), while the negative controls have no colour (C and D). The two positive control examples on the left show the test strips with gold conjugated anti-Rotavirus antibody (BB International) diluted 1/6 (A) and 1/3 (B) with conjugate release buffer (2.1.1).

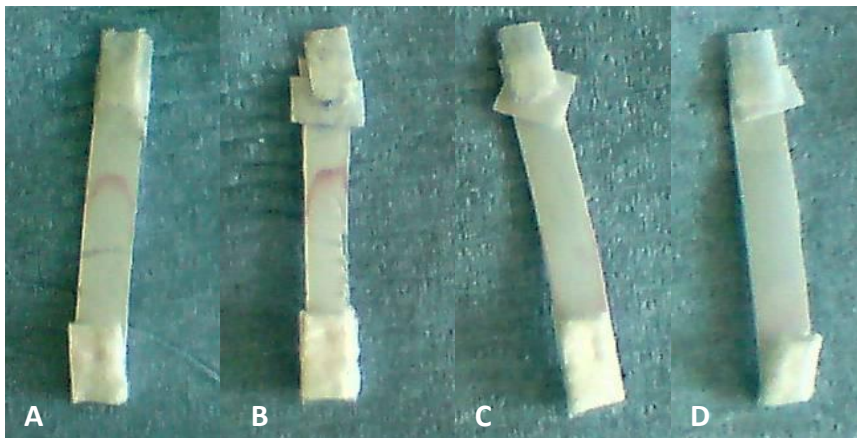


Figure 12 Initial LFIA results using the Predator based membrane. Two positive control examples using Rotavirus antigen (Corus) with gold conjugated anti-Rotavirus antibody (BB International) diluted 1/6 (A) and 1/3 (B) in conjugate release buffer (2.1.1). Two negative control samples used PBST (2.1.1) in place of Rotavirus antigen (C and D).

3.2.3 Initial optimisation results - Antigen concentration

The next optimisation assays varied the concentration of the Rotavirus antigen (Corus) using the proprietary dilution buffer (Corus) and compared the strength of signal produced using the gold labelled anti-Rotavirus antibody (BB International) at 1/6 and 1/3 dilutions of conjugate buffer (2.1.1). The experimental conditions are described in Table 2. The assay format remained the same as depicted in Figure 11.

The strength of the signal produced decreased as the concentration of the Rotavirus antigen became more dilute. Table 17 and Figure 13 show a weak signal when the sample was at a dilution of 1/64 in proprietary dilution buffer (Corus). The results from the 1/256 dilution were unclear.

Similar results were obtained using both test concentrations of gold conjugated anti-Rotavirus antibody (BB International) and the results for the 1/6 dilution (in conjugate

release buffer) are shown in Figure 13. The negative control was performed with the proprietary sample dilution buffer (Corus) in place of any diluted antigen.

Table 17 Signal intensity for LFIA on Predator membrane with serial dilutions of Rotavirus antigen (Corus) assessed with two different gold labelled anti-Rotavirus antibody (BB International) concentrations.

Rotavirus Antigen dilution [#]	1/4	1/16	1/64	1/256	1/1024	1/4096	1/16384	Negative control
Gold conjugated antibody (1/6 diluted*)	++	+	(+)	?	O	O	O	O
Gold conjugated antibody (1/3 diluted*)	++	+	(+)	?	O	O	O	O

[#]Rotavirus Antigen (Corus) diluted in sample dilution buffer (Corus)

*Gold conjugated anti-Rotavirus antibody (BB International) diluted in conjugate buffer (2.1.1)

Assay results key: ++ Strong Signal (+) Weak Signal ? Incomplete assay
+ Signal O No signal

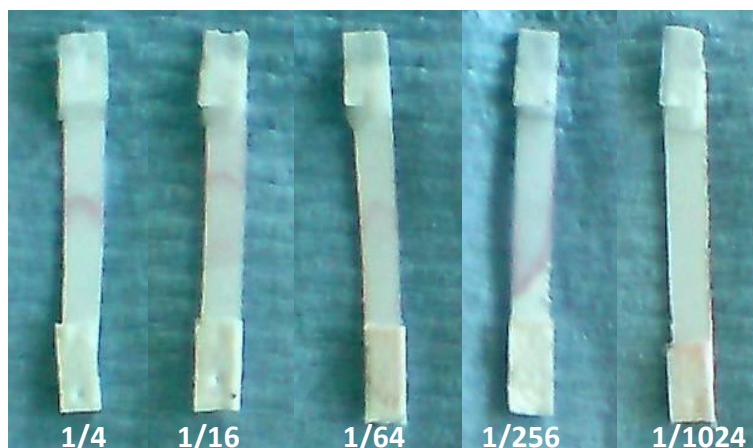


Figure 13 LFIA Predator membrane strips showing gold conjugated anti-Rotavirus antibody (BB International) diluted 1/6 with conjugate buffer (2.1.1) and Rotavirus antigen sample (Corus), serial dilutions (with Corus sample buffer) ranging from 1/4 dilution on far left, to 1/1024 dilution on the far right. Feint signal visible on middle strip (1/64 Rotavirus dilution).

3.2.4 Initial optimisation results - Conjugate release buffer pH

The effect of changing the pH of the conjugate release buffer (2.1.1) was assessed and the results shown in Table 18. The assay format remained the same as depicted in Figure 11. The signal remained visible at all the pH levels tested but gave a weaker signal at the most alkaline pH.

Table 18 Comparison of signal strength with pH of the conjugate release buffer (2.1.1) used to make a 1/6 dilution of the gold conjugated anti-Rotavirus antibody (BB International).

pH of conjugate release buffer (2.1.1)	7.4	7.8	8.2	8.6	9.0
Signal strength	+	+	+	+	(+)
<u>Assay results key:</u> ++ Strong Signal (+) Weak Signal + Signal O No signal					

3.2.5 Initial optimisation results - Capture antibody volume

The relationship between the volume of anti-Rotavirus capture antibody on the test line and LFIA signal intensity was demonstrated by changing the volume of the anti-Rotavirus capture antibody (Dako) applied.

The results produced are shown in Table 19 and illustrated in Figure 14. The colour intensity of the signal increases as the volume of the anti-Rotavirus capture antibody (Dako) rises.

Table 19 The change in signal intensity compared to the volume of anti-Rotavirus capture antibody applied to the test line of the Predator membrane.

Volume (µl) of anti-Rotavirus capture antibody (Dako)	0.5	1	2	4
Signal strength	(+)	+	+	++
<u>Assay results key:</u> ++ Strong Signal (+) Weak Signal + Signal O No signal				

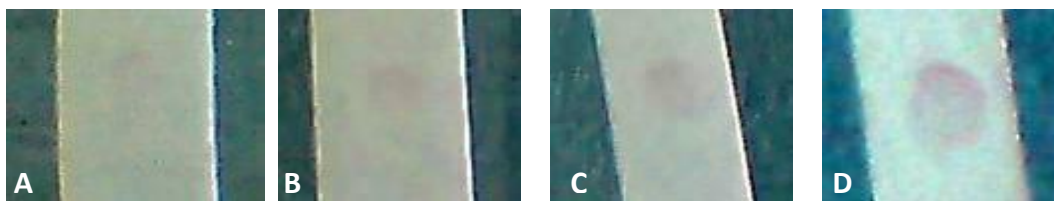


Figure 14 Photographs of LFIA strips constructed with increasing volumes of anti-Rotavirus capture antibody (Dako). A = 0.5µl, B = 1µl, C = 2µl and D = 4µl. All volumes added as 0.5µl aliquots.

3.2.6 Optimal reagent volumes and concentrations for Predator based LFIA

The results from optimising the concentrations and volumes of the key LFIA immuno-reagents are summarised in Table 20. Assays using these conditions produced clear, easily visible signals and reproducible results on a Predator based LFIA.

As indicated in 3.1.1, PBST (2.1.1) was shown to be a suitable replacement for the sample dilution buffer (Corus) when diluting the Rotavirus antigen (Corus).

Table 20 Optimum immuno-reagent selection for consistent assay performance on Predator membrane.

Predator membrane	Reagent	Volume	Dilution
Sample	Rotavirus antigen (Corus)	80µl	1/16 diluted in PBST (2.1.1)
Conjugate pad	Gold conjugated anti-Rotavirus antibody (BB International)	10µl	1/6 diluted in conjugate release buffer (2.1.1)
Test line	Anti-Rotavirus antibody (Dako)	4µl	stock

3.2.7 Results from using an alternative, biotinylated, anti-Rotavirus capture antibody with Predator membrane

The biotinylated goat anti-Rotavirus capture antibody (Acris Antibodies) was used in place of the anti-Rotavirus capture antibody (Dako), giving the assay configuration shown in Figure 15.

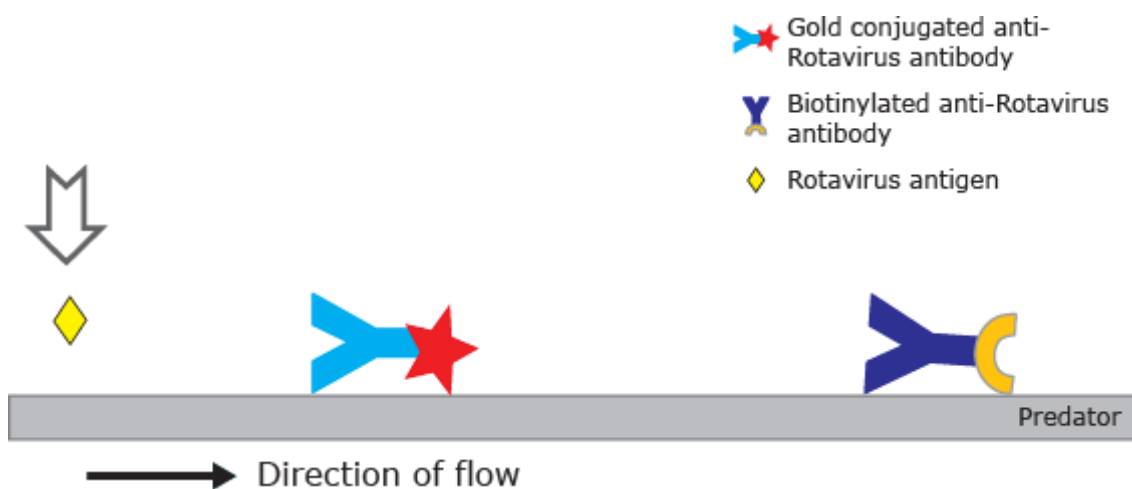


Figure 15 Original LFIA format, with biotinylated anti-Rotavirus capture antibody (Acris) substituted at the test line.

The biotinylated anti-Rotavirus antibody (Acris) was serially diluted using PBS (2.1.1) and combined with the optimal volumes and concentrations of the Rotavirus antigen (Corus) and gold conjugated anti-Rotavirus antibody (BB International) shown in Table 20.

The results of the experiments (carried out in duplicate) are shown in Table 21 and a selection of the results from the dilutions are pictured in Figure 16.

Table 21 LFI A performance compared with serially diluting the biotinylated anti-Rotavirus capture antibody (Acris) with PBS (2.1.1).

Concentration of biotinylated anti-Rotavirus antibody*	100%	1/4	1/10	1/16	1/64	1/256	Negative Control
Signal Strip 1	++	++	++	(+)	O	O	O
Signal Strip 2	++	+	+	O	O	O	O

*Biotinylated anti-Rotavirus capture antibody (Acris) diluted in PBS (2.1.1)

Assay results key: ++ Strong Signal (+) Weak Signal
+ Signal O No signal

Distinct signals were produced in the LFIAs which included the 1/4 and 1/10 dilutions of capture antibody (although the signals were weaker in one of the duplicates). A faint signal was visible in one of the 1/16 dilution strips, but no signal in the 1/64 dilutions or weaker concentrations. The negative controls, where PBST (2.1.1) was substituted for Rotavirus antigen, were also blank.

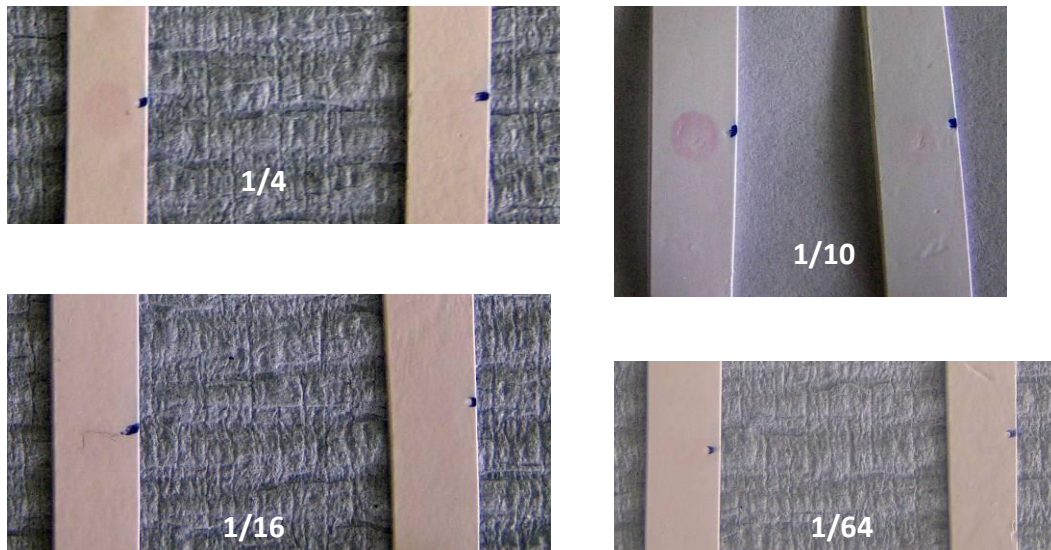


Figure 16 Photographs of Predator strips designed to investigate the effect of altering the concentration of biotinylated anti-Rotavirus capture antibody (Acris). Dilutions performed with PBS (2.1.1). Each dilution performed in duplicate, as shown.

3.2.8 Results from using Streptavidin coated microspheres on the test line with biotinylated anti-Rotavirus antibody pre-mixed with gold conjugated anti-Rotavirus antibody.

The biotinylated anti-rotavirus antibody (Acris Antibodies) was added to (and dried down with) the gold conjugate solution (BB International) and 0.83µm diameter Streptavidin coated microspheres (Seradyn) were positioned on the test line. The position of the immuno-reagents are depicted in Figure 17 and have the same format as the target assay (1.7.1)

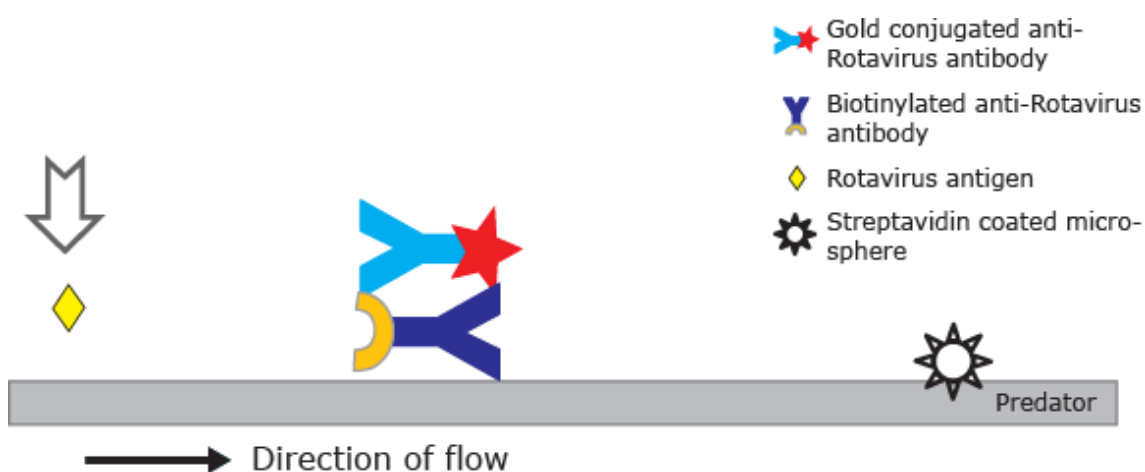


Figure 17 LFIA format on Predator membrane with Streptavidin coated microspheres (Seradyn) at the test line. Biotinylated anti-rotavirus antibody (Acris) premixed with the gold conjugate solution (BB International) and dried down together.

No signals were achieved in the positive control or either of the negative control assays. The negative controls were conducted with PBST (2.1.1) in place of the antigen and with no biotinylated capture antibody included.

3.3 Results from the assessment of the biotin binding affinity of the Streptavidin coated microspheres

The assessment of the biotin binding affinity of the Streptavidin coated microspheres, in both the homogenous assay and in the experiment to assess their mobility during the lateral flow assays, are summarised below.

3.3.1 Homogenous Rotavirus assay

The reagents used in the homogenous Rotavirus assay are shown in (Table 6). The contents of the positive and negative control Eppendorf tubes were identical, except the positive control contained the Rotavirus antigen sample (Corus), while PBS (2.1.1) was used in place of the antigen in the negative control tube.

The results of this experiment are shown in Figure 18, together with results using the stock concentration of the biotinylated anti-Rotavirus antibody (Acris). The use of the higher concentration of the biotinylated anti-Rotavirus antibody did not produce any noticeable change in colour intensity or agglutination (see B and D).

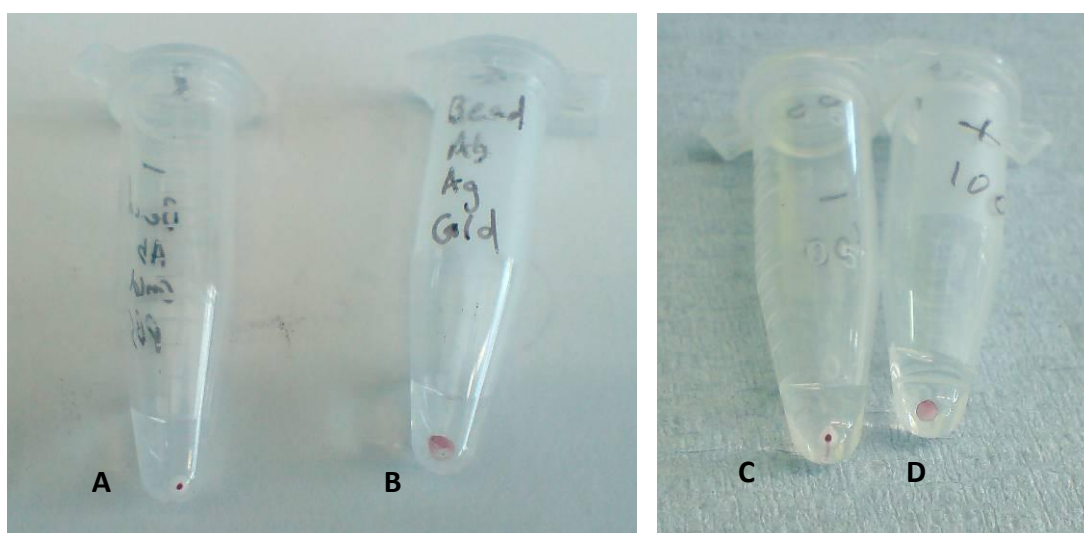


Figure 18 Photographs of Eppendorf tubes containing homogenous Rotavirus assay. Tubes A and B show the biotinylated antibody 1/4 diluted with PBS, while tubes C and D contained the stock concentration biotinylated antibody. The positive controls are tubes B and D, while tubes A and C represent the negative controls (which used PBS in place of the Rotavirus antigen).

3.4 Results of using a hydrophilic membrane in a lateral flow immunoassay

3.4.1 Microsphere immobilisation on Fusion 5

The assay format designed to assess whether the larger Streptavidin coated microspheres (Polysciences) would remain in position on the hydrophilic Fusion 5 membrane (Whatman) during a LFIA is summarised by Figure 19.

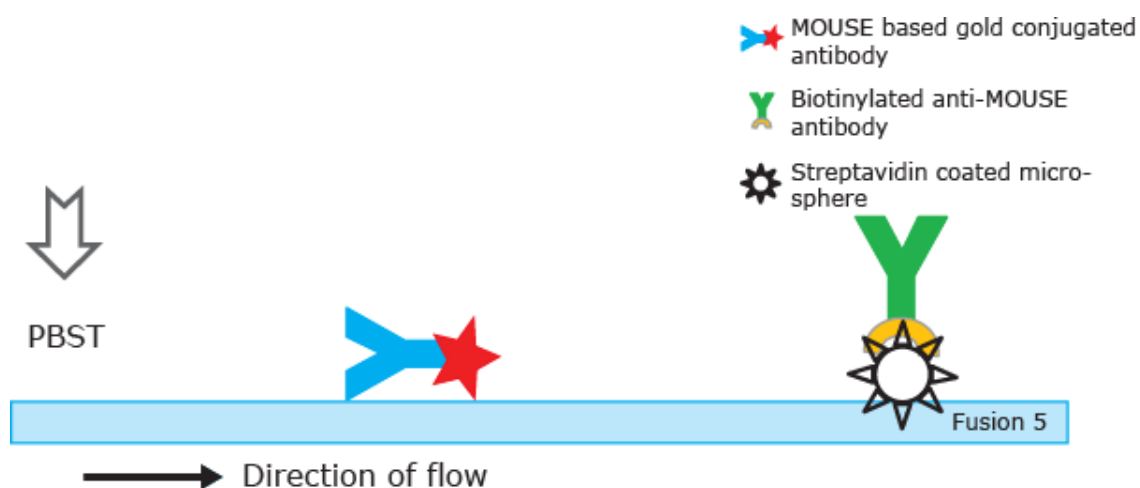


Figure 19 Assay configuration used to assess whether the microspheres remain in position during a Fusion 5 membrane based LFIA. The diagram shows the mouse based gold antibody (BB International) pre-dried in the conjugate area. PBST was used as the sample (2.1.1). The test line comprised 2µm diameter microspheres, pre-incubated with biotinylated anti-mouse antibody (QED Bioscience).

For comparison, both 4µl and 2µl volumes of the pre-incubated microsphere, biotinylated anti-mouse IgG antibody mixture were applied to the test line. The results are summarised below in Table 22 and displayed in Figure 20. Negative controls included the microspheres (Polysciences) on the test line but omitted the biotinylated anti-mouse IgG.

Table 22 Assay results using the biotinylated anti-mouse IgG antibody pre-incubated on microspheres with mouse based gold label antibody (results show duplicate strips for each assay).

Fusion 5 membrane	Biotinylated anti-mouse with Streptavidin microspheres 4 μ l		Biotinylated anti-mouse with Streptavidin microspheres 2 μ l		Negative control 4 μ l microspheres	Negative control 2 μ l microspheres
Signal strength	++	++	+	++	O O	O O

Assay results key: ++ Strong Signal (+) Weak Signal
+ Signal O No signal

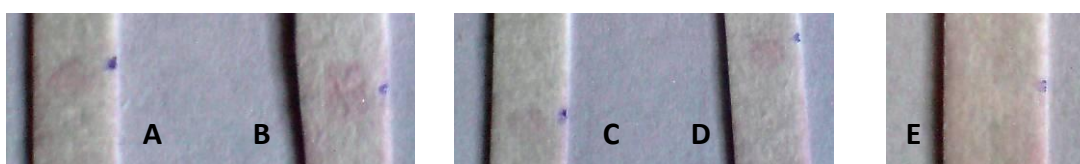


Figure 20 Photographs showing results from using the biotinylated anti-mouse IgG (pre-incubated with streptavidin covered microspheres) on the test line with the mouse based gold labelled antibody. Images A and B had 4 μ l of the Streptavidin coated microsphere and biotinylated IgG complex on the test line. Images C and D had 2 μ l of the same mixture applied. One of the negative controls (without the biotinylated IgG on the test line) is shown in photo E.

The signals displayed in strips A, B and D were slightly more intense than strip C. The signals appeared within fifteen minutes, but became more apparent as the strips dried. The pink colouration in the negative control (E) is due to the colloidal gold having run the full length of the assay, but no spot has formed at the blue ink mark on the right hand side.

The experiment was repeated by adding the mouse based gold conjugated antibody to the sample, rather than pre-drying it in the conjugate area of the Fusion 5 membrane, but all the results were negative.

3.4.2 Initial Fusion 5 results

Initial Fusion 5 based LFIA used the target assay format (1.7.1) shown in Figure 21. The volume of the reagents and their initial dilutions are shown in Table 8. The assay was performed using the smaller microspheres and repeated with the larger size.

The results, on both sized spheres proved to be negative. Similarly, the negative controls involving both the replacement of the Rotavirus antigen (Corus) with PBST (2.1.1) and the absence of the biotinylated anti-Rotavirus capture antibody (Acris), gave no signal.

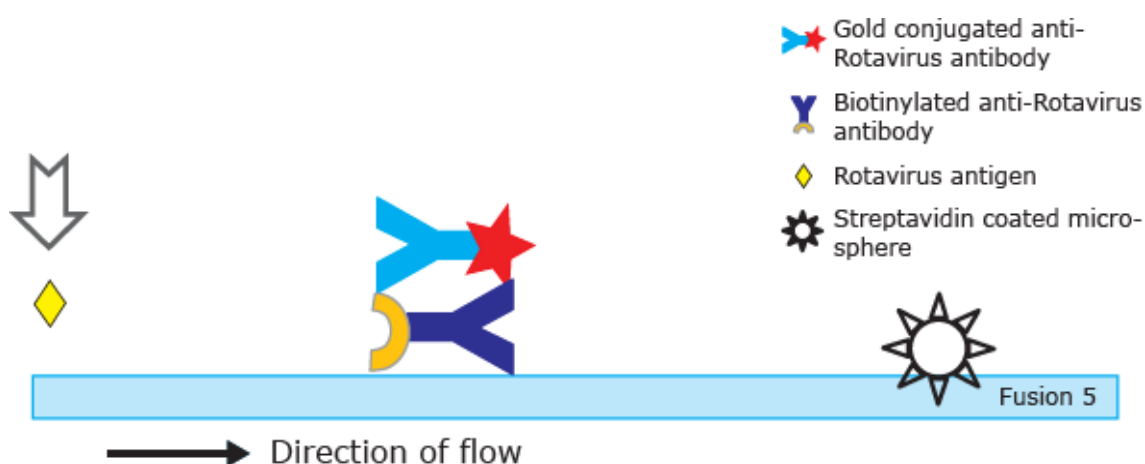


Figure 21 Target assay format on Fusion 5 membrane.

Replacing the PBST buffer (2.1.1) that was used to dilute the Rotavirus antigen (Corus) with the proprietary dilution buffer (Corus) made no difference to the results. Similarly, using the conjugate release buffer (2.1.1) to dilute the biotinylated anti-Rotavirus capture antibody (Acris), instead of PBS, also produced no signal.

3.4.3 Results from optimising the concentrations and combinations of reagents used with Fusion 5 membrane

The concentrations of the reagents used (Table 8) were altered one reagent at a time and used with the larger 2 μ m diameter Streptavidin coated microspheres (Polysciences) on the test line in a series of Fusion 5 strips.

The results from separately adjusting all three concentrations gave no signal. As expected, negative controls yielded no signal either.

3.4.4 Results from pre-incubating reagents (before immobilisation) using a Fusion 5 membrane

A variety of Fusion 5 based assays evaluated pre-incubation combinations of the Rotavirus antigen (Corus), gold conjugated anti-Rotavirus antibody (BB International), biotinylated anti-Rotavirus capture antibody (Acris) and the Streptavidin coated microspheres (Polysciences). Figure 22-26 depict the assay formats performed. The concentrations and volumes of reagents are summarised in section 2.4.4.

a) Pre-incubated immuno-reagent sample

All the immuno-reagents were pre-incubated together, so that that they could form the sandwich before running them all down the strip (see Figure 22). The test line consisted of the Streptavidin coated microspheres. No assays produced a signal, including negative controls.

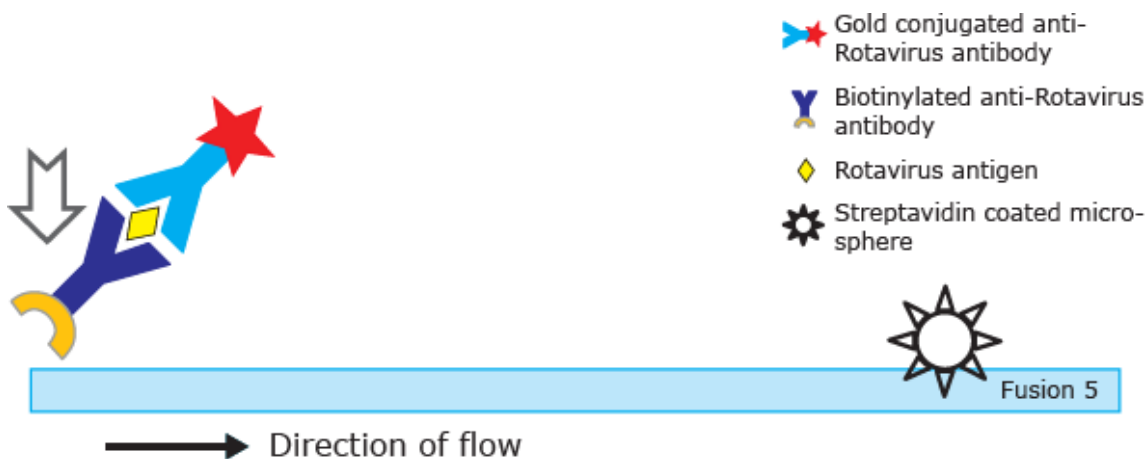


Figure 22 Image depicting the addition of a pre-incubated immuno-sandwich to a Fusion 5 based LFIA.

b) Alternative pre-incubated sample strategy

The configuration shown in Figure 23 was also tested, using the dilutions shown in Table 10. Although no signal was evident in any positive or negative controls.

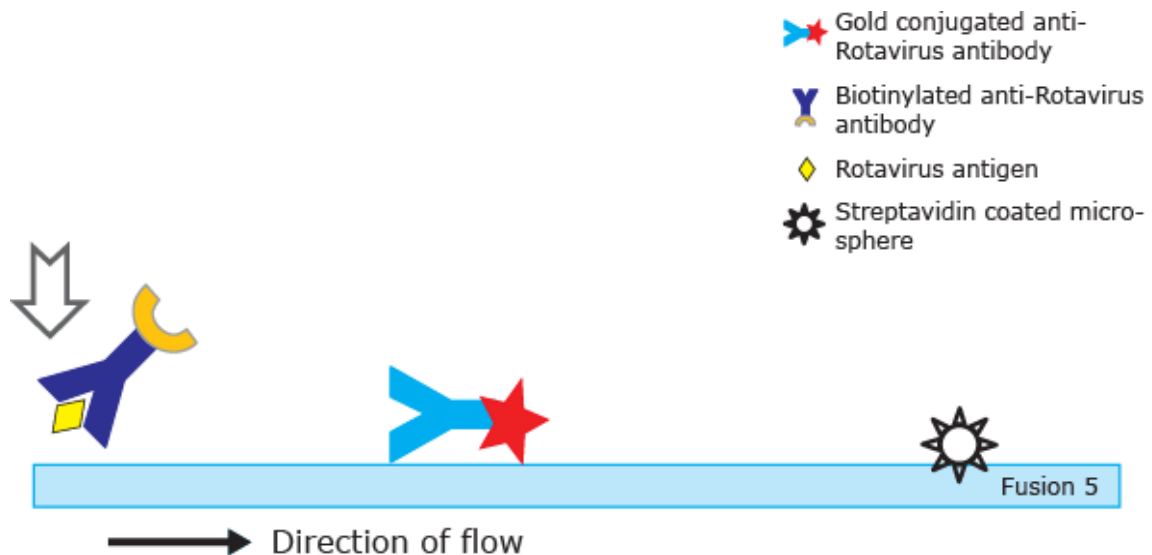


Figure 23 An assay format illustrating the Rotavirus antigen pre-incubated with the biotinylated anti-Rotavirus capture antibody and applied together as a sample.

c) Pre-incubated test line

Figure 24 illustrates an assay configuration where the biotinylated anti-Rotavirus capture antibody was pre-incubated with the microspheres, the gold conjugated anti-Rotavirus antibody was dried down in the conjugate area and the antigen supplied as a sample. This assay was performed using the reagents and dilutions shown in Table 11. The Fusion 5 based assay was repeated using various dilutions (1/4 to 1/10) of biotinylated anti-Rotavirus capture antibody. The capture antibody was diluted with PBS (2.1.1).

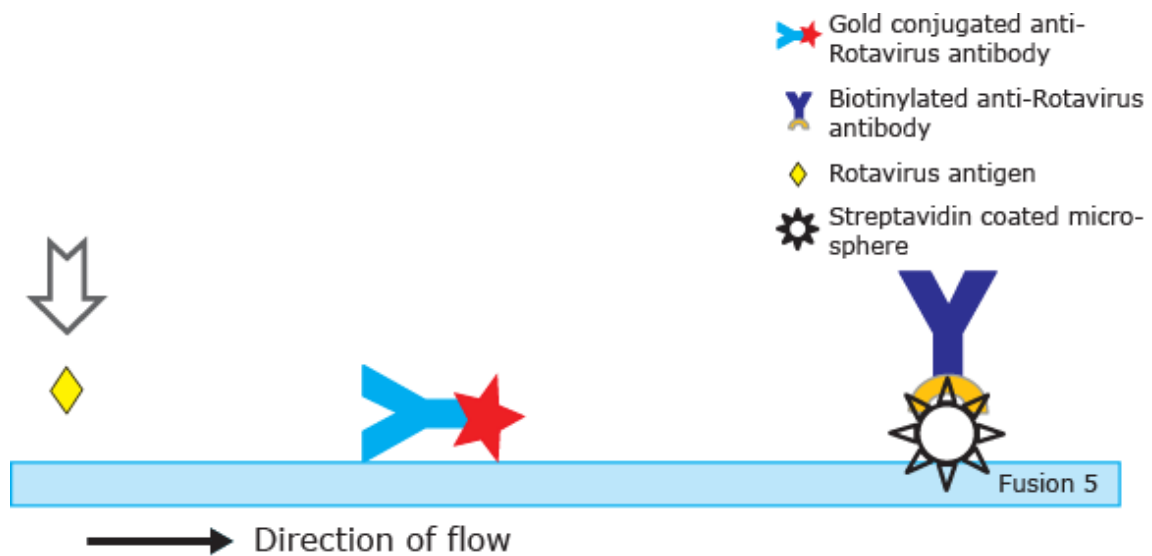


Figure 24 LFIA format using a Streptavidin coated microsphere test line, pre-incubated with biotinylated anti-Rotavirus antibody.

All results using these concentrations of biotinylated anti-Rotavirus antibody were negative, including the negative controls. However, after the strips had dried overnight, very feint pink spots were visible on the test line of the positive controls.

d) Combined pre-incubated sample and test line strategy

A further adaptation, shown in Figure 25 with the reagents summarised in Table 12, had a double pre-incubation methodology. However all LFIA results were negative.

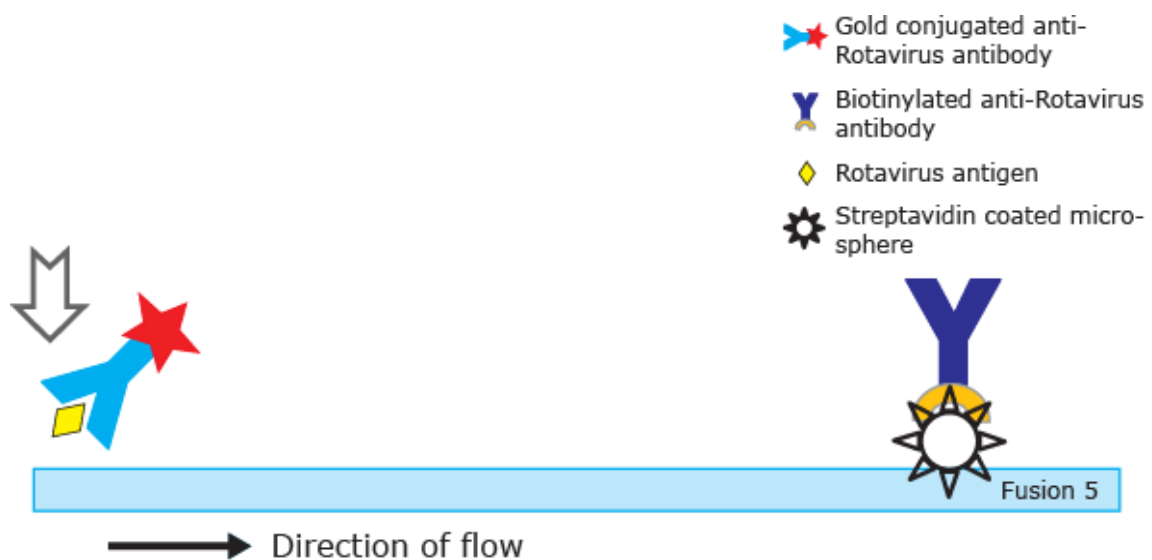


Figure 25 Dual pre-incubation methodology with a combined Rotavirus antigen and gold conjugated anti-Rotavirus antibody sample applied to a test line composed of biotinylated anti-Rotavirus antibody pre-incubated with Streptavidin coated microspheres.

e) Alternative test line pre-incubation strategy

The Rotavirus antigen was also pre-incubated with a mixture of biotinylated anti-Rotavirus antibody combined with the Streptavidin coated microspheres (see Figure 26). A sample of PBST was used to rehydrate the gold conjugated anti-Rotavirus antibody. Although all assays attempted in this format gave negative results.

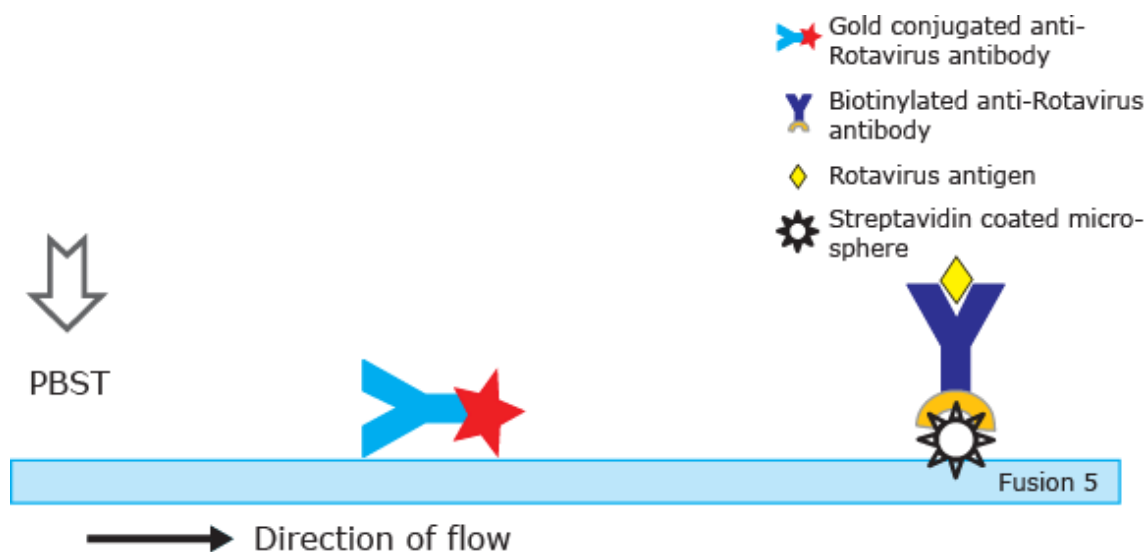


Figure 26 An alternative pre-incubated test line format that was designed to capture the one remaining immuno-reagent - the rehydrated gold conjugated anti-Rotavirus antibody.

3.4.5 The effect of drying down all the reagents on Fusion 5

Having all the immune-reagents dried down in the conjugate zone, using the reagents shown in Table 14, as depicted in Figure 27, produced no initial results. However, delayed signals did appear in the positive controls after sixty to seventy five minutes, as shown in Table 23.

Two negative controls, assays performed without either the Rotavirus antigen (Corus) or the biotinylated anti-Rotavirus antibody (Acris), remained blank.

The test line contained either 4µl or 2µl volumes of Streptavidin coated microspheres (Polysciences).

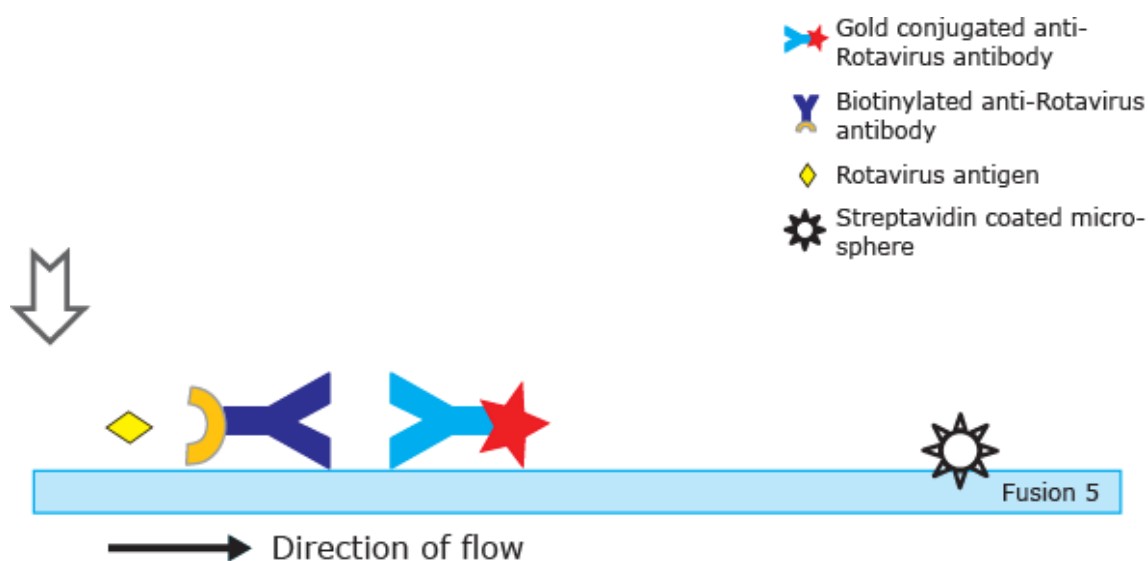


Figure 27 All reagents dried down separately, including the antigen, on the Fusion 5 membrane.

Table 23 Assays results from drying down all immuno-reagents on a Fusion 5 based LFIA. PBST applied to strip to rehydrate the reagents. Variable volumes of Streptavidin coated microspheres on the test line. Results show duplicate strips for each assay.

Fusion 5	4µl volume of Microspheres on test line	2µl volume of Microspheres on test line	Negative control 1	Negative control 2
Assay results	...+ ...(+)	...+ ...+	O	O

Negative control 1 = biotinylated anti-Rotavirus antibody omitted from assay

Negative control 2 = Rotavirus antigen omitted from assay

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal O No signal

Photographs showing the delayed positive results are shown in Figure 28. Note the double spot in images A and B, which represent the strips with a 4µl (two aliquot) volume of Streptavidin coated microspheres on the test line.

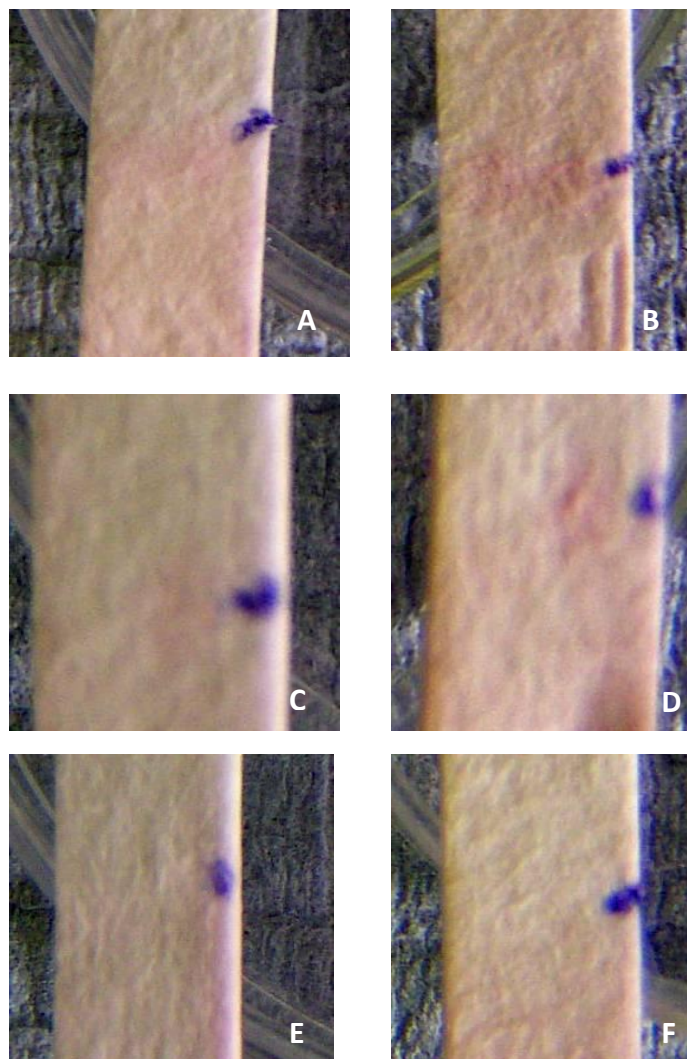


Figure 28 Delayed positive results displayed on a test line comprising 4 μ l Streptavidin coated microspheres (A and B). Additional delayed positive results (C and D) on a test line with 2 μ l Streptavidin coated microspheres. Negative controls are pictured at the bottom (E and F). Image E had no biotinylated anti-Rotavirus antibody in the assay, while image F had no Rotavirus antigen present.

A follow up experiment, where a combination of the Rotavirus antigen (Corus), the gold conjugated anti-Rotavirus antibody (BB International) and the biotinylated anti-Rotavirus antibody (Acris) were premixed and allowed to dry down in the conjugate area, gave negative results.

3.4.6 Results of using a composite lateral flow strip with “dual test lines” using different membrane materials

The composite strips that had separate membrane sections, each with individually dried immuno-reagents, and dual test lines (using both Fusion 5 and Predator) gave no signal in any of the positive controls. However, one of the negative controls, described in Figure 29, had an anti-Rotavirus capture antibody on the Predator based test line but excluded the biotinylated anti-Rotavirus capture antibody (Acris). This strip produced a pink spot on the Predator section of the strip – see Figure 30.

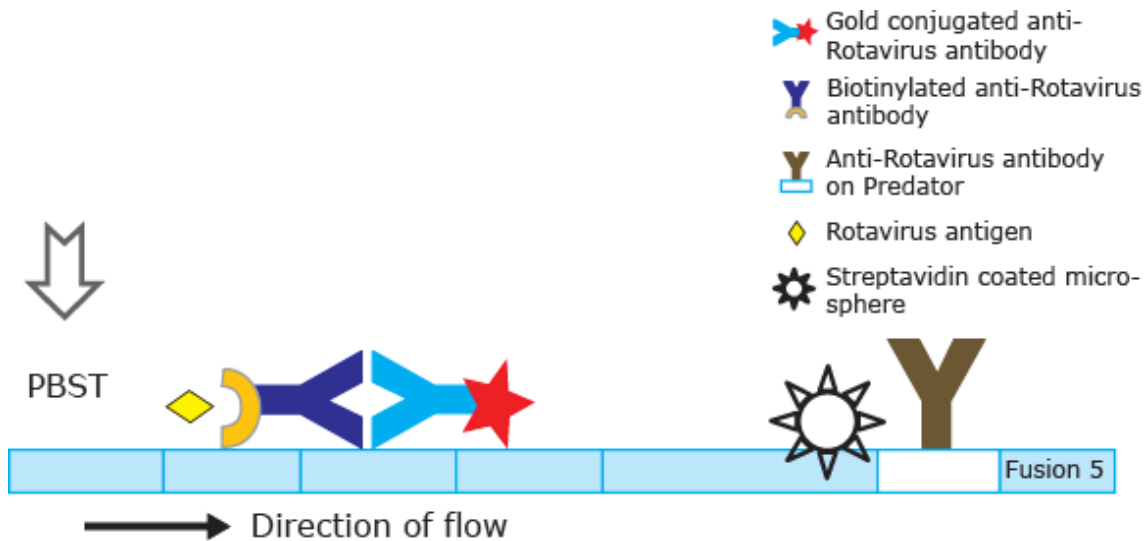


Figure 29 Schematic diagram depicting one of the negative control experiments using the composite strips. The strip had two test lines, one on the Predator section, the other on Fusion 5 material. All of the immuno-reagents were present, except the biotinylated anti-Rotavirus capture antibody (Acris).



Figure 30 A pink spot displayed in one of the negative control composite strips. The strip shown had a pink spot on the Predator section of the composite membrane (arrowed). This negative control had an anti-Rotavirus capture antibody on the Predator based test line, but no biotinylated anti-Rotavirus capture antibody present.

3.5 Results from optimisation of the Fusion 5 methodology to improve assay performance

Having achieved delayed positive signals on a Fusion 5 (Whatman) based LFIA the assay methodology was modified and optimised. The volumes and concentrations of the immuno-reagents were as shown in Table 14 unless otherwise indicated.

3.5.1 Altering strip dimensions and drying time

The assay format in Figure 27 was repeated with both longer and shorter strips (5cm and 2.5cm) which also gave delayed weak positive results, after at least sixty minutes, but the results were not consistent in duplicate strips. Drying the strips after the sample had wicked to the test line (at 37°C for an hour) also led to a mixture of delayed weak positive and negative signals, see Table 24.

Table 24 Results of altered strip dimensions and drying methodology, together with negative controls. Results show duplicate strips for each positive control assay.

Fusion 5 strip length	4µl volume of Microspheres on test line	2µl volume of Microspheres on test line	Negative control 1	Negative control 2
5cm	...(+) O	...(+) O	O	O
2.5cm	...(+) O	...(+) O	O	O
3.5cm*	...(+) ...(+)	O O	O	O

* = oven dried strip (37°C for 60 mins)

Negative control 1 = biotinylated anti-Rotavirus antibody omitted from assay

Negative control 2 = Rotavirus antigen omitted from assay

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal O No signal

3.5.2 Change of sample application methodology

The assay was performed vertically, so that the immuno-reagents were allowed to flow down the strip towards the test line. A faster result was produced in one strip (under ten minutes), although the duplicate strip took over an hour to develop and it had a weaker signal. The strips are shown in Figure 31.

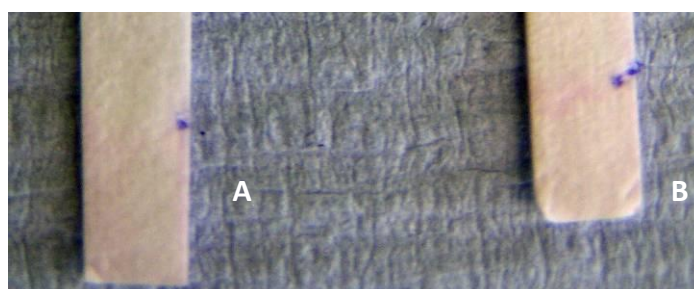


Figure 31 Results from an alternative method of sample application. The signal on the right was produced relatively quickly, although its duplicate on the left had a weaker signal and took much longer to appear.

3.5.3 Altering the order and position of the dried down reagents

The order in which the conjugate zone reagents (volumes and concentrations as Table 14) were dried down was varied and produced the results shown in Table 25. The assays repeated the vertical assay methodology and had a 2µl volume of Streptavidin coated microspheres (Polysciences) dried down on the test line.

Results appeared between forty and eighty minutes later. Some combinations gave no signal, *e.g.* when the gold conjugated anti-Rotavirus antibody (BB International) was in the first position (2.4.5). Other combinations generated a signal and having the Rotavirus antigen (Corus) first, the gold conjugated anti-Rotavirus antibody (BB International) in the middle and the biotinylated anti-Rotavirus capture antibody (Acris) in the third position gave consistent results in duplicate strips (see Table 25).

Table 25 Experiment results explored the importance of the relative positions of the separately pre-dried immuno-reagents on the Fusion 5 based strip. Position 1 was nearest the sample end of the strip. Position 3 was mid-strip, nearest the test line. Position 2 was between positions 1 and 3. All negative controls were blank.

Position 1	Position 2	Position 3	Fusion 5 Strip 1	Fusion 5 Strip 2
Gold conjugated antibody	Biotinylated capture antibody	Rotavirus antigen	O	O
Gold conjugated antibody	Rotavirus antigen (Corus)	Biotinylated capture antibody	O	O
Biotinylated capture antibody	Gold conjugated antibody	Rotavirus antigen (Corus)	...(+)	O
Biotinylated capture antibody	Rotavirus antigen (Corus)	Gold conjugated antibody	...+	O
Rotavirus antigen (Corus)	Biotinylated capture antibody	Gold conjugated antibody	O	...+
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody	...+	...+

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal O No signal

3.5.4 Buffer optimisation

The conjugate buffer was used to dilute the Biotinylated anti-Rotavirus capture antibody instead of the PBS buffer (2.1.1). The change of buffer did not lead to a noticeable difference in experimental results based on Figure 27, neither did a change of buffer reduce the time it took for any delayed positives to appear.

The proprietary sample dilution buffer (Corus) was reintroduced into the assay and used as the applied sample (in place of the PBST) in a repeat of the last two assay

formats in Table 25. The change of sample buffer produced the results shown in Table 26. The delay in signal varied from twenty to seventy minutes, but not consistently, and the use of the proprietary buffer (Corus) did not noticeably alter the signal intensity, or the assay time.

Furthermore, a reduced volume of microspheres was applied to the test line (2µl rather than 4µl) although this did not affect the sensitivity of the assay.

Table 26 The effects of changing the sample buffer on assay performance was examined together with the effects of altering the order of the reagents on the Fusion 5 membrane. Test lines consisted of 4µl or 2µl of Streptavidin coated microspheres. Results show duplicate strips for each positive control assay.

Sample buffer	Reagent position on Fusion 5 conjugate area			Test line volume		Negative Control 1
	1	2	3	4µl	2µl	
Corus	Rotavirus antigen	Biotinylated capture antibody	Gold conjugated antibody	...(+) ...+	...(+) ...+	...(+)
PBST	Rotavirus antigen	Biotinylated capture antibody	Gold conjugated antibody	...+ ...+	...+ ...+	O
Corus	Rotavirus antigen	Gold conjugated antibody	Biotinylated capture antibody	...+ ...+	...+ ...+	O
PBST	Rotavirus antigen	Gold conjugated antibody	Biotinylated capture antibody	...+ ...+	...+ O	O

Corus = Proprietary sample dilution buffer

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Negative control 1 = biotinylated anti-Rotavirus antibody omitted from assay

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal **O** No signal

Negative controls were completed in tandem, which either removed the biotinylated anti-Rotavirus antibody (Acris) or the Rotavirus antigen (Corus) from the LFIA format. As shown in Table 26, one of the negative controls, where the biotinylated anti-Rotavirus antibody (Acris) was omitted from the assay, gave a (false) delayed weak positive (see Figure 32). All other negative control experiments gave no signal.

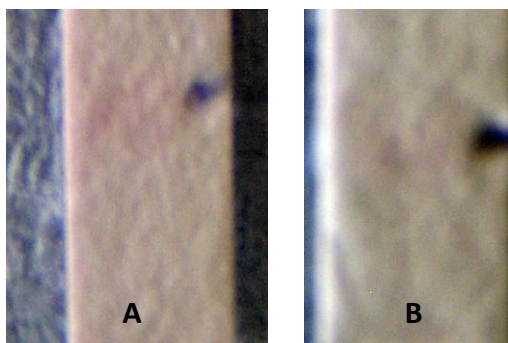


Figure 32 A comparison of the signal produced in a delayed true positive (A) in comparison to a delayed false positive (B).

3.5.5 Dried down reagents combined with a pre-incubated test line

The biotinylated anti-Rotavirus capture antibody (Acris) was pre-incubated with the Streptavidin coated microspheres (Polysciences) once again and both the Rotavirus antigen (Corus) and the gold conjugated anti-Rotavirus antibody (BB International) were separately applied and dried onto Fusion 5 (see Figure 33). Reagent volumes were as shown in Table 14 and produced the results shown below in Table 27.

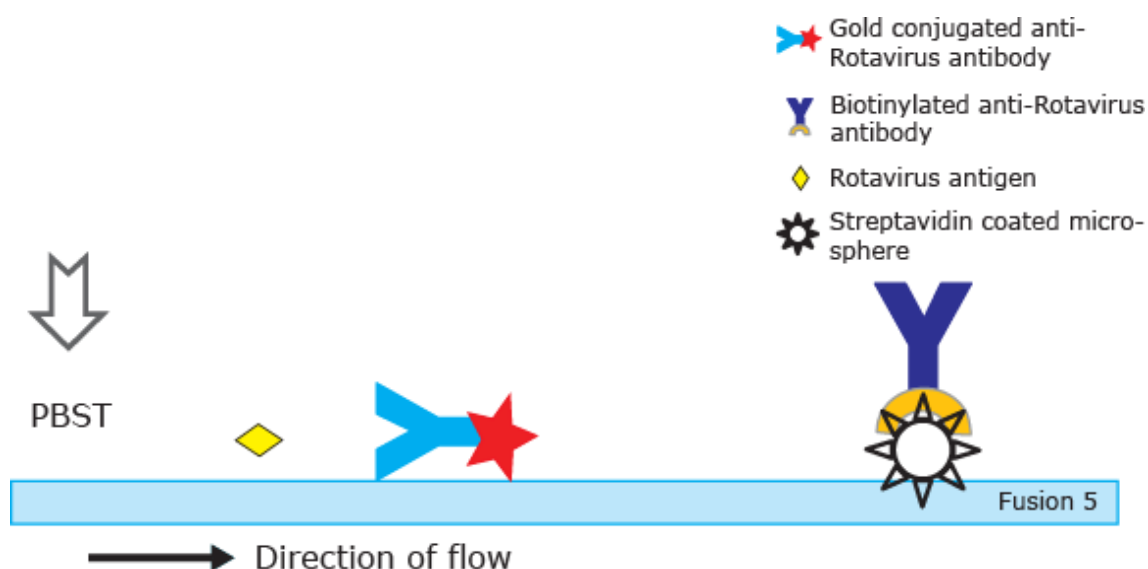


Figure 33 Fusion 5 assay format optimised to have a test line consisting of the biotinylated anti-Rotavirus capture antibody pre-incubated with the Streptavidin coated microspheres. Remaining reagents dried down in the conjugate zone and rehydrated with a PBST sample.

Table 27 Results from applying pre-dried Rotavirus antigen and gold conjugated antibody to the conjugate zone and a test line containing biotinylated capture antibody pre-incubated with Streptavidin coated microspheres.

Reagent position on conjugate area		Test line	Fusion 5	Fusion 5
1	2	reagents (2µl)	Strip 1	Strip 2
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody and Streptavidin coated microsphere	...++	...++

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Streptavidin coated microsphere = 2µm diameter (Polysciences)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal O No signal

Note the strong signal that was produced with this method. The delay in producing this signal was approximately seventy minutes. No signal was evident in either of the negative controls, which omitted either the Rotavirus antigen (Corus) or the biotinylated capture antibody (Acris).

In a repeat experiment, the Streptavidin coated microspheres (Polysciences) were washed in binding buffer with reduced salt content (1/4 strength PBS) and used during the incubation of the biotinylated anti-Rotavirus capture antibody (Acris). This also resulted in delayed positives, but the delay was still over an hour.

3.5.6 Readjustment of the biotinylated anti-Rotavirus antibody concentration

The concentration of the biotinylated anti-Rotavirus antibody (Acris) in the previous experiment (depicted in Figure 33) was altered. It was used at the stock concentration and a 1/2 dilution (with PBS buffer).

The assay produced the results shown in Table 28. There were four positive control strips and two negative control strips for each dilution. The delayed positive signals were apparent after sixty minutes. None of the negative controls produced a signal.

Table 28 Experimental results from using stronger concentrations of the biotinylated anti-Rotavirus capture antibody pre-incubated with the Streptavidin coated microspheres.

Reagent position			Fusion 5 Strips			
Position 1	Position 2	Test line (2µl)	1	2	3	4
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody (stock concentration) and Streptavidin coated microsphere	...++	...++	...++	...++
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody (1/2 diluted with PBS) and Streptavidin coated microsphere	...++	...++	...+	...+

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Streptavidin coated microsphere = 2µm diameter (Polysciences)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal ○ No signal

Photographs of the strips showing the delayed positive signals, for both concentrations of the biotinylated capture antibody, are pictured in Figure 34.

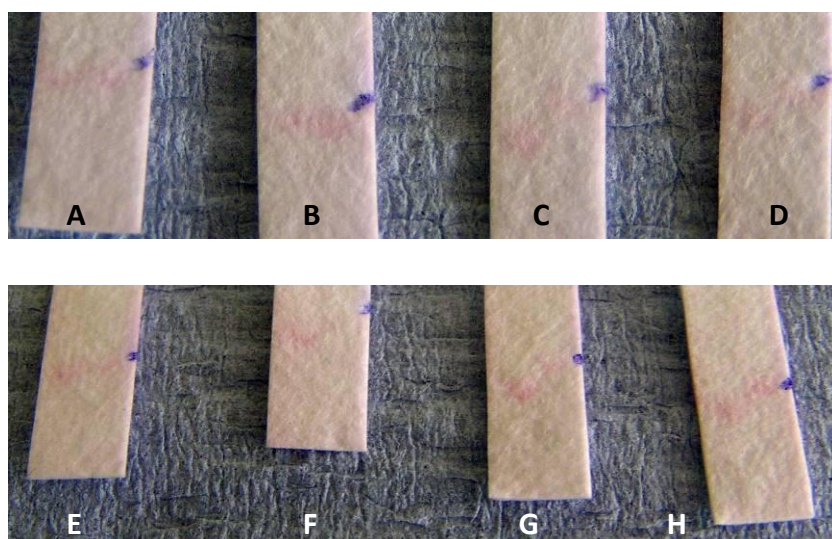


Figure 34 Delayed strong positive signals produced from using stronger concentrations of the biotinylated anti-Rotavirus capture antibody on the test line (pre-incubated with Streptavidin coated microspheres). The Fusion 5 strips pictured A-D show the assay performed with the stock concentration of the capture antibody. The lower four strips (E-H) represent the same assay performed with the biotinylated anti-Rotavirus capture antibody 1/2 diluted with PBS (2.1.1).

The higher concentrations of biotinylated anti-Rotavirus antibody (Acris) were also used dried down in the conjugate area, as described in the assay format shown in Figure 35. The immuno-reagent volumes were used as summarised in Table 14, except for the biotinylated anti-Rotavirus antibody (Acris) which was used at the stock concentration and 1/2 diluted with PBS (2.1.1).

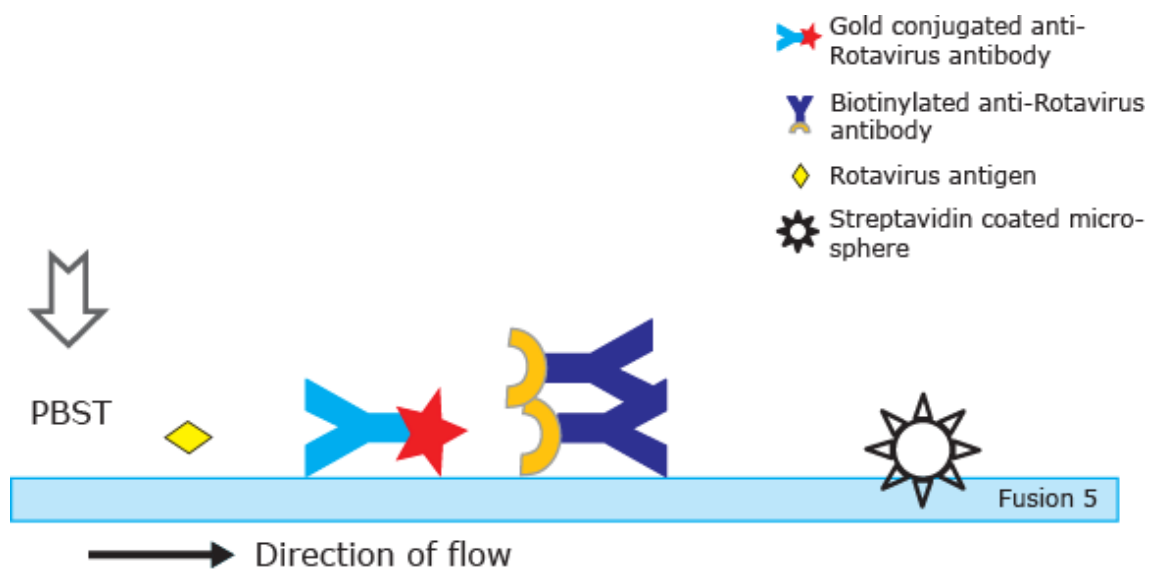


Figure 35 All immuno-reagents were separately dried down in the conjugate area, with stronger concentrations of the biotinylated capture antibody used than previously. The test line consist of Streptavidin coated microspheres (Polysciences).

There were four positive control strips and two negative control strips for each dilution. After a delay ranging from sixty to over one hundred minutes, the results shown in Table 29 appeared. All negative controls produced blank strips with no signal.

Table 29 Results from using an increased concentration of biotinylated anti-Rotavirus antibody dried down in the conjugate zone with the other immuno-reagents.

Reagent position in conjugate zone				Fusion 5 Strips			
Position 1	Position 2	Position 3	Test line (2µl)	1	2	3	4
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody (stock concentration)	Streptavidin coated microsphere	...(+)	...(+)	...+	...(+)
Rotavirus antigen (Corus)	Gold conjugated antibody	Biotinylated capture antibody (1/2 diluted with PBS)	Streptavidin coated microsphere	...+	...+	...+	...+

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Streptavidin coated microsphere = 2µm diameter (Polysciences)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal O No signal

Photographs showing the strips described by Table 29 are shown in

Figure 36. Note the comparison with Table 28 and Figure 34. Strips A-D represent the stock concentration of the biotinylated anti-Rotavirus antibody (Acris), while strips E-H are 1/2 diluted with PBS (2.1.1).

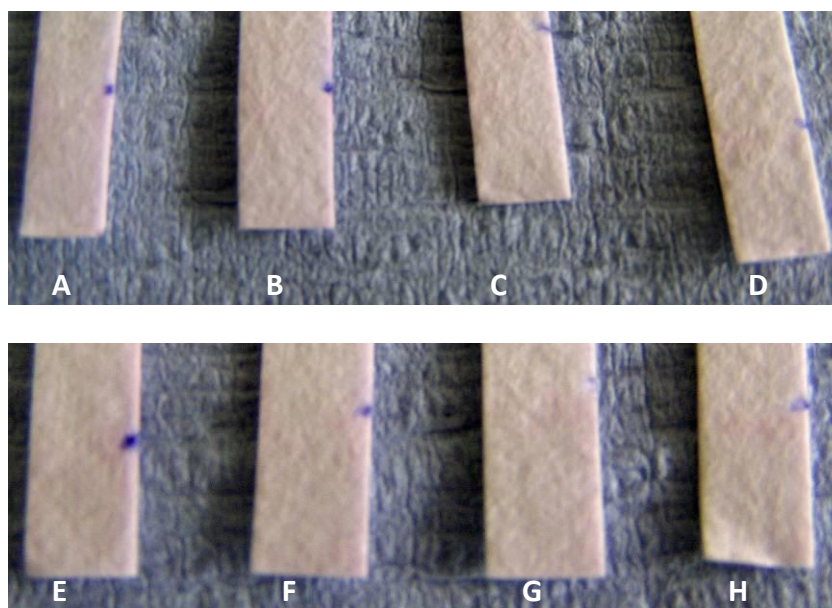


Figure 36 Photographs showing the results of using higher concentrations of the biotinylated capture antibody dried down in the conjugate area. Stock concentration used on strips A-D and 1/2 diluted capture antibody shown in strips E-H.

3.5.7 Alternative hydrophilic membranes

The assay format described by Figure 33 was tested using alternative hydrophilic membranes, but they did not produce faster results. Assays using VF1 and VF2 membranes (Appendix A) did not run successfully, while the MF1 (Appendix A) based LFIA took an hour for one strip and over ninety minutes in three other cases.

3.5.8 Applying Rotavirus antigen samples to optimised assay designs with a stronger concentration of biotinylated capture antibody

The assay was redesigned so that the Rotavirus antigen (Corus) was supplied as a sample, rather than it having pre-dried on the Fusion 5 based material.

The revised assay format is illustrated by Figure 37 and used the biotinylated anti-Rotavirus antibody (1/2 diluted in PBS) pre-incubated on the Streptavidin coated microspheres (Polysciences) on the test line once again.

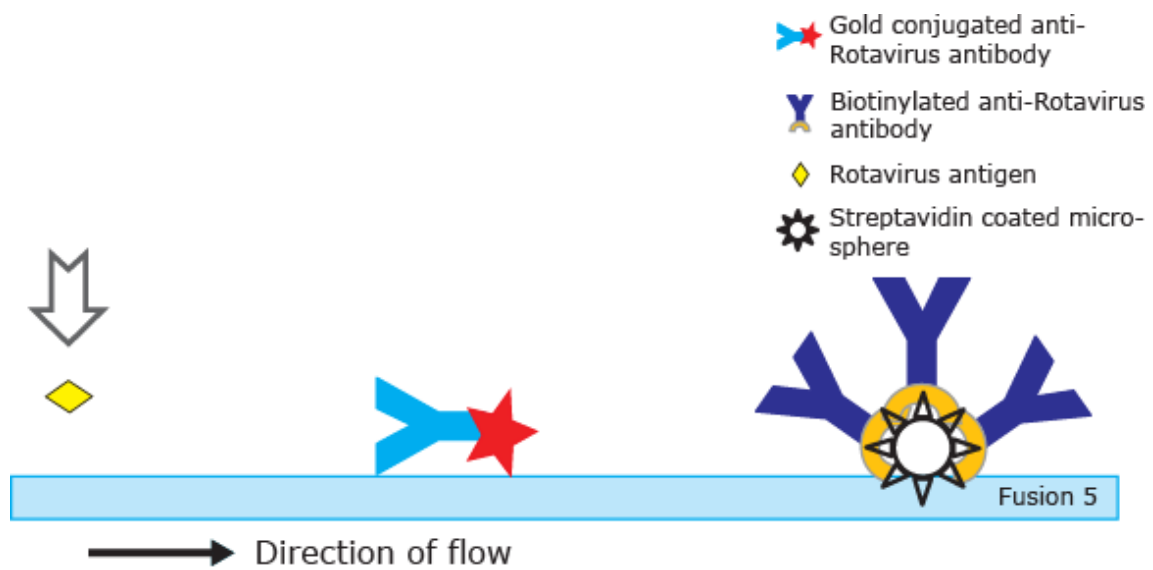


Figure 37 Revised assay format using biotinylated anti-Rotavirus antibody at 1/2 stock dilution, pre-incubated with Streptavidin coated microspheres on the test line.

The antigen sample was applied both as a single volume (80µl, 1/16 dilution in PBST) and also in two aliquots: a more concentrated, smaller volume (20µl, 1/4 diluted in PBST) followed by 60µl PBST. The results are shown in Table 30.

Table 30 Results from applying the Rotavirus antigen in a single 1/16 diluted sample and two aliquots (a 1/4 diluted sample, followed by PBST). Test line contains 1/2 diluted stock concentration of biotinylated anti-Rotavirus pre-incubated with Streptavidin coated microspheres.

Reagent position			Fusion 5 Strips				Negative
Sample	Conjugate area	Test line (2µl)	1	2	3	4	control 1
Rotavirus antigen (80µl)	Gold conjugated antibody	Biotinylated capture antibody (1/2 diluted with PBS) and Streptavidin coated microsphere	...++	...++	...+	...+	...(+)
Rotavirus antigen (20µl) PBST (60µl)	Gold conjugated antibody	Biotinylated capture antibody (1/2 diluted with PBS) and Streptavidin coated microsphere	...+	...++	...+	...++	0

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)
 Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)
 Streptavidin coated microsphere = 2µm diameter (Polysciences)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal 0 No signal

The delayed signals for the single application of the Rotavirus antigen (Corus) appeared after ninety minutes, while applying the Rotavirus antigen in two aliquots, produced the results shown in thirty to ninety minutes. Photographs showing the intensity of the signals are displayed in Figure 38.

Two different negative control experiments were run in parallel. The first set of negative controls omitted the biotinylated anti-Rotavirus antibody (Acris) from the assay (which produced one false positive result - not pictured). The second negative controls used PBST as a replacement for Rotavirus antigen (Corus) and all results were negative.

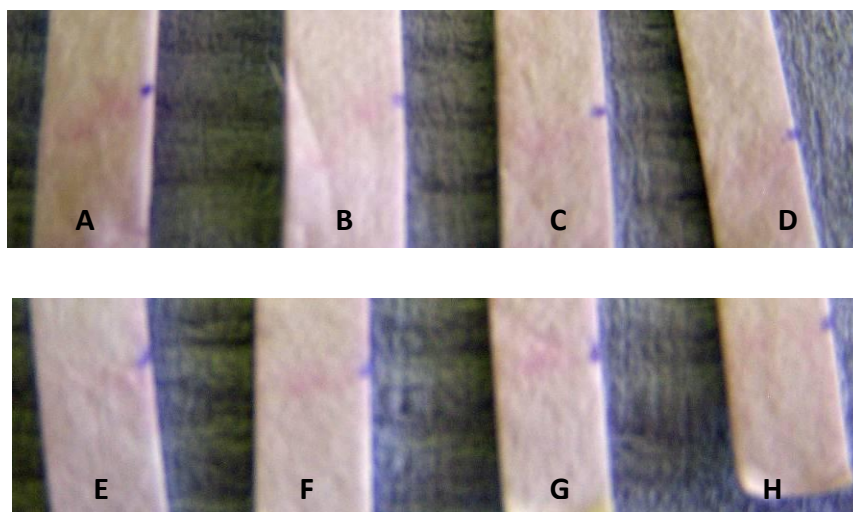


Figure 38 Results from applying a Rotavirus antigen sample to 1/2 diluted stock concentration of biotinylated anti-Rotavirus. The sample was applied as a single aliquot (A-D) and repeated using two aliquots (E-H).

3.6 Repeating the target assay using the stronger capture antibody concentration

The results from a variety of the optimisation experiments in section 3.5 were combined and are summarised in Table 15.

The target assay (1.7.1) was repeated using the 1/2 diluted (PBS) stock concentration of biotinylated anti-Rotavirus antibody (Acris) dried down in the conjugate area (Figure 39), next to the gold conjugated anti-Rotavirus antibody (BB International). The Rotavirus antigen sample (Corus) was applied using both the single and two aliquot application method. The test line consisted of Streptavidin coated microspheres (Polysciences).

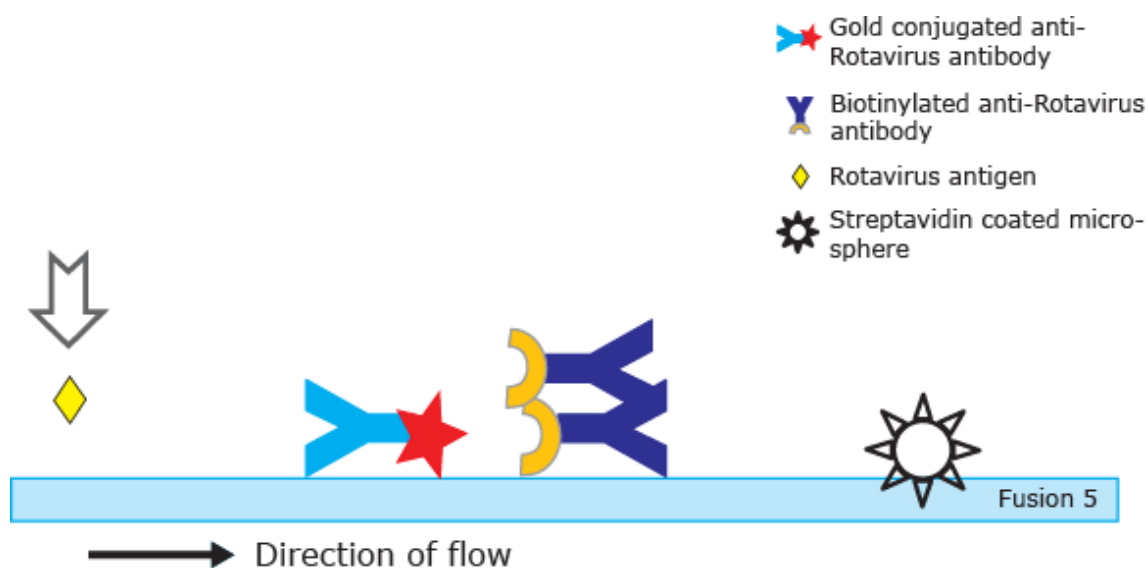


Figure 39 Target assay format using the stronger biotinylated capture antibody dried down in the conjugate area of a Fusion 5 based LFIA.

The results are tabulated below in Table 31. Negative controls, which either replaced the Rotavirus antigen with PBST (2.1.1), or omitted the biotinylated anti-Rotavirus capture antibody, gave no signal.

Table 31 LFIA results from optimised target assay on Fusion 5 based membrane with Streptavidin coated microsphere test line.

Reagent position on Fusion 5				Strips			
Sample	Conjugate area		Test line (2µl)	1	2	3	4
Rotavirus antigen (80µl)	Gold conjugated antibody	Biotinylated capture antibody	Streptavidin coated microsphere	○	○	○	○
Rotavirus antigen (20µl) PBST (60µl)	Gold conjugated antibody	Biotinylated capture antibody	Streptavidin coated microsphere	...(+) ○	○	○	○

Gold conjugated antibody = Gold conjugated anti-Rotavirus antibody (BB International)

Biotinylated capture antibody = Biotinylated anti-Rotavirus capture antibody (Acris)

Streptavidin coated microsphere = 2µm diameter (Polysciences)

Assay result key: ...++ Delayed Strong Signal ...+ Delayed Signal
 ...(+) Delayed weak signal ○ No signal

The delayed weak positive result achieved in one of the strips appeared after 120 minutes. The assays were repeated using an antigen sample that was further diluted by 1/4 in PBST (equating to 1/64 dilution), but all the results were negative.

Chapter 4: *Discussion*

4.1 Buffers

4.1.1 Use of alternative sample dilution buffers

The use of PBST (2.1.1), in place of the proprietary buffer (Corus) for diluting the Rotavirus antigen, made no apparent difference to the assays performed on Predator. The use of Tween in the PBST was to ensure that both sample dilution buffers contained a detergent to help the sample flow along the membranes.

4.2 Results from using hydrophobic membranes in lateral flow immunoassays

Both the commercially available Rota-Strip and the laboratory constructed Predator based assays used strips with hydrophobic membranes.

The Predator (Pall) based Rotavirus assays performed reasonably well and within a convenient timeframe, that fell within the recommended fifteen minute time limit suggested by the Rota-Strip manufacturers.

The use of a test line spot rather than a stripe has limitations. It was not always obvious that a spot had formed, especially if there was non-specific binding or excess gold at the side of the strips. Employing the double spot method, with a spot on each side of the strip (later employed on the Fusion 5 strips) would have been advantageous. Furthermore, the application of the capture antibody on the test line was time consuming, since each 0.5µl aliquot needed to dry before subsequent applications.

4.2.1 Positive control - Antigen viability assessment

The viability of the Rotavirus antigen (Corus) material was ascertained with commercial Rota-Strip (Corus) tests.

The two pink lines present in each of the commercial Rota-Strip tests on the left of Figure 10 (A and B) indicate positive results and confirm that the antigen is active and demonstrates the visual effectiveness of a colloidal gold based label on a white

background. The presence of the control lines in all four of strips show that the LFIAs have run for long enough and the assays have functioned correctly. The two strips on the right hand side (C and D) show only control lines, confirming that no antigen was present in the negative control sample.

The stronger Rota-strip signals with the second batch of antigen (2.2.1) implied that the concentration of this source was stronger than the original (although no quantitative test was carried out to compare the two concentrations). This demonstrates the potential quantitative nature of LFIAs. A calibration measurement methodology would be required to substantiate this further.

4.2.2 Initial optimisation results – Gold label concentration

There was no significant difference observed when the concentration of the gold conjugated anti-Rotavirus antibody was changed from 1/6 to 1/3 (dilutions performed in conjugate release buffer). Since the weaker concentration gave a clearly visible pink signal on the white Predator membrane, the 1/6 dilution was selected for subsequent assays.

4.2.3 Initial optimisation results – Antigen concentration

The Predator strips were able to detect the Rotavirus antigen at a dilution of 1/64 in the proprietary buffer (Corus). Unfortunately, both strips designed to detect the Rotavirus antigen dilution at 1/256 failed to perform and would need to be repeated before the true sensitivity could be ascertained.

4.2.4 Initial optimisation results – Conjugate release buffer pH

Adjusting the pH of the conjugate release buffer, did not noticeably alter the sensitivity of the Predator strips, although acidic and more extreme alkaline pH values were not tested. The pH of the other buffers used were not altered and therefore not evaluated.

4.2.5 Initial optimisation results – Capture antibody volume

Changing the volume of anti-Rotavirus capture antibody (Dako) applied to the strips, had an effect on the intensity and size of the signal produced. The results suggest that

a minimum of 2µl were required. This method of application saved time in strip preparation and would have commercial implications in any manufacturing process.

4.2.6 Optimisation summary

The initial experiments were designed to optimise the volumes and concentrations of reagents required for subsequent assays. An important consideration was the generation of detectable and reproducible signals on Predator membranes with minimal reagents, saving resources and costs.

4.2.7 Results from using an alternative, biotinylated, anti-Rotavirus capture antibody with Predator membrane

Using the optimal concentrations and volumes of two out of three reagents (as determined in the initial experiments) helped to rapidly introduce the biotinylated anti-Rotavirus capture antibody (Acris) into a working assay and to assess its sensitivity. The results obtained from the serial dilution of the biotinylated capture antibody, on Predator, were not consistent on each strip tested (see Figure 16) and the only signal achieved at 1/16 dilution (in PBS) was weak. However, clear signals were evident, in both strips, when the biotinylated antibody was at 1/10 and 1/4 dilutions (in PBS) and both of these dilutions were used in subsequent assays on Fusion 5 (Whatman) membranes.

Using the biotinylated anti-Rotavirus capture antibody on the Predator membrane may not have been an accurate test of its ultimate sensitivity. The chemical fusion of biotin to the antibody is via the Fc portion of the antibody molecule. It is therefore conceivable that this modification impairs its binding to the Predator membrane and its overall antigen binding capability. However, if the biotinylation of the molecule was responsible for reducing its sensitivity for target antigen on the Predator membrane, this should not have affected its performance when bound to Streptavidin in the subsequent Fusion 5 based assays.

4.2.8 Results from using Streptavidin coated microspheres on the test line with biotinylated anti-Rotavirus antibody pre-mixed with gold conjugated anti-Rotavirus antibody

Attempts to try the target assay format (1.7.1) on a Predator membrane, using the biotinylated capture antibody with the smaller Streptavidin coated microspheres (Seradyn), on the Predator membrane, were not successful. This could have been because of the presence of the microspheres, rather than directly due to the assay format itself. The microspheres may not have bound to the Predator membrane effectively and may have been washed away with the sample (although on visual inspection they appeared to remain in place). Alternatively, they may have been too large for the membrane and either not presented enough Streptavidin on their surface for the biotinylated capture antibody to bind, or they may have impeded the flow of the sample and the performance of the assay.

4.3 Results from the assessment of the biotin binding affinity of the Streptavidin coated microspheres

The aim of the homogenous assay was to validate that the Streptavidin immobilised on the microspheres would bind the biotinylated anti-Rotavirus capture antibody, and test the formation of the sandwich assay.

4.3.1 Homogenous Rotavirus assay

The pink colouration in tubes B and D of Figure 18 was due to the agglutination of the microspheres and reagents following a successful immuno-reaction. This not only confirmed that the sandwich assay had formed, due to the presence of all three immuno-reagents, but also that the biotin had demonstrated its affinity for the Streptavidin coated microspheres. It also showed that the relative concentrations of the reagents (antigen, antibodies and the biotin / Streptavidin complex) were suitable for a successful homogenous assay format which, although not directly transferable, could then be used as a baseline for subsequent LFIAs.

Tube A and C in Figure 18, contained no antigen, so were not able to complete the sandwich structure and so no agglutination occurred. The small pink speck in tube A and C indicated the presence of the colloidal gold following the centrifugation. The gold being heavier than the microspheres fell to the bottom of the tube. Experiments with increased concentrations of the biotinylated capture antibody gave similar results, but there was no obvious increase in agglutination or colour intensity, suggesting either that the biotinylated capture antibody was already in excess and unable to bind additional antigen, or that it was unable to access additional Streptavidin binding sites.

4.4 Results of using a hydrophilic membrane in a lateral flow immunoassay

The larger 2µm Streptavidin coated microspheres (Polysciences) were preferred over the 0.83µm microspheres, for use in assays on the Fusion 5 material, following recommended advice by Whatman (2004).

4.4.1 Microsphere immobilisation on Fusion 5

In order to evaluate the suitability of using the Streptavidin coated microspheres on the test line of the Fusion 5 membrane, an assay format was devised that exploited the affinity of an anti-mouse IgG antibody (QED Biosciences) for the mouse based, gold conjugated antibody (BB International). When the mouse based, gold conjugated antibody was dried down onto the conjugate area of the Fusion 5 membrane, with the biotinylated anti-mouse IgG antibody (pre-incubated with the Streptavidin coated microspheres) positioned on the test line, a signal was produced when PBST buffer was run on the strip to rehydrate the gold conjugate reagent. This not only demonstrated that the microspheres remained in position following the application of a sample and did not get washed away, but also that the concentration of gold was sufficient to produce a visible signal and that the relative concentrations of Streptavidin and biotin was appropriate for this antibody. As expected, in the negative control, where no biotinylated capture antibody was attached to the microspheres, no signal was present.

If the investigation into this assay later requires a control line, the biotinylated anti-mouse IgG antibody / Streptavidin complex is a potential candidate.

In contrast, when the biotinylated gold conjugated antibody was not dried down, but applied as a liquid sample, no signal was produced, so this assay format on Fusion 5 required the gold conjugated antibody to be fully dried down and rehydrated by the sample solution.

4.4.2 Initial Fusion 5 results and observations

This LFIA was an attempt at producing the target assay (1.7.1) on a Fusion 5 membrane. However, the assay yielded no signal, despite using the larger sized microspheres as recommended by Whatman.

It was apparent that the mobility of the gold conjugated antibody was not the reason for failure, as it was able to complete the journey along the membrane, as a pink residue was visible at the end of the strip - an observation reinforced by the results of using the anti-mouse based IgG antibody on the test line (3.4.1). Visual inspection also indicated that the microspheres were remaining *in situ* and not being washed away by the sample.

4.4.3 Results from optimising the concentrations and combinations of reagents used with Fusion 5 membrane

The concentrations of all the immuno-reagents were adjusted, one reagent at a time, in order to try and generate a signal using the target assay format (1.7.1), although, at this stage, no signal was produced.

The strongest concentration of Rotavirus antigen used at this stage (while the antigen was being applied as a liquid sample) was 1/4 diluted with PBST buffer. The strongest, stock based concentration was not used until later in the optimisation process. Furthermore, weaker concentrations (1/16 diluted in PBST) had been sufficient to yield a signal on Predator membrane and in the homogenous assay.

Changing the concentration of the gold-conjugated anti-Rotavirus antibody, from 1/2 to 1/6 dilutions (with conjugate dilution buffer), had no effect. The stronger concentration was attempted in order to match a methodology used by Jones *et al.* (2004), who successfully used a 40nm gold conjugate, diluted to OD5 (equivalent to 1/2 dilution) on Fusion 5 membrane. The conjugate was not diluted to more than 1/6, since this concentration had been sufficient to give a signal with the same reagents in the Predator membrane assay and in the homogenous assay test.

Similarly, the range in concentration of the biotinylated capture antibody (1/4 to 1/10 dilution with PBS) was selected to match the other successful assays used with the same reagents (on Predator and in the homogenous assay).

4.4.4 Pre-incubation of reagents (before immobilisation) using a Fusion 5 membrane

Pre-incubation of two, or more, of the immuno-reagents, before application to the Fusion 5 strip, allowed at least one of the binding reactions to take place in advance. This was designed to reduce the number of binding steps taking place during the test, and so try to further optimise the format.

In an attempt to demonstrate the capture affinity of the immobilised Streptavidin for a mobile biotinylated reagent, all three of the immuno-reagents (Rotavirus, biotinylated anti-Rotavirus antibody and gold conjugated anti-Rotavirus antibody) were pre-incubated together. When this mixture was applied and allowed to wick along the strip to the Streptavidin based test line, no visible result was produced. This indicated that either the reagents did not flow down the Fusion 5 matrix or that the Streptavidin based test line was not suitable for capturing the biotinylated sandwich as it flowed down the strip.

When the biotinylated capture antibody was pre-incubated with the microspheres (see Figure 24), faint signals were apparent on the Fusion 5 strips after fifteen hours. It was assumed that this was due to non-specific binding, since, as described in the Rota-Strip commercial test, results generated after 15 minutes are considered false positives.

However, the assay format being investigated, was similar in design to the successful biotinylated anti-Rotavirus capture antibody serial dilution experiment (3.2.7) on Predator, (*i.e.* the Rotavirus antigen was supplied as a liquid sample, the gold conjugated anti-Rotavirus antibody was dried down in the conjugate area and the biotinylated anti-Rotavirus capture antibody was positioned on the test line (compare Figure 15 and Figure 24). The dilutions of the reagents were also the same. The only differences were the choice of membrane (Fusion 5, rather than Predator) and the method used to attach the capture antibody to the membrane. On reflection, further investigation and optimisation at this stage, may have led to more successful assays sooner.

All of the other pre-incubation strategies failed to produce a signal.

4.4.5 *Drying down all the reagents on Fusion 5 membrane*

Separately drying down all the immuno-reagents in the conjugate area (including the antigen), with the microspheres on the test line, gave positive results that appeared after a delay of approximately one hour. Faster results were achieved, but not consistently. In contrast, pre-incubating the three immuno-reagents and drying the mixture down led to negative results. This implies that the separation of the reagents (and drying them down) is key to a delayed signal being generated.

The use of the second batch of antigen may have contributed to the generation of the delayed positives, however, when it was used in a repeat of the target assay (1.7.1) and also pre-incubated with the biotinylated capture antibody (and applied together as a sample, Figure 23) no signal was generated.

4.4.6 *The effect of using a composite lateral flow strip with “dual test lines” using different membrane materials*

The strips that used dual test lines (a combination of both Fusion 5 and Predator membranes, with the reassembled Fusion 5 based reagent pads) failed, largely due to their design. The sample did not transfer from one reassembled section to another very easily. However, the presence of a signal (on the Predator section) in the negative

control that contained no biotinylated capture antibody, confirmed that the Rotavirus antigen and gold conjugated antibody were at least able to wick along the Fusion 5 matrix and reach the end of the strip.

4.5 Results from optimisation of the Fusion 5 methodology to improve assay performance

The concentration of the immuno-reagents, their positions on the strip and the overall methodology were optimised to try to speed up the assay and improve the sensitivity.

Assessing the presence of a signal was occasionally a rather subjective process, particularly for the delayed weak positives. Holding the strips up to the light and comparing them with negative controls helped to determine whether spots were present. In addition, applying the test line reagents in two spots, rather than one, did help to identify the formation of a positive signal and distinguish it from unbound gold that had run to the end of the strip.

4.5.1 Altering strip dimensions and drying time

Optimising the drying down methodology, by using longer strips, (designed to give the immuno-reagents more time to bind before reaching the test line) did not reduce the delayed responses.

It was noted that the strips, being hydrophilic, took longer to dry than the Predator membrane strips and the signal was difficult to identify when the strips were wet. Using shorter strips, that would require a smaller sample and so dry quicker, and drying the strips in an oven at 37°C made no difference to the outcome. Furthermore, the shorter strips made the separation of the dried down reagents more difficult as the volumes of reagents used, caused them to spread out when dry, making the strip surface crowded.

4.5.2 Change of sample application methodology

Applying the sample by standing the strip in the sample well and then later inverting it, so that the sample could flow down towards test line helped to ensure that as much of

the sample as possible would reach the test line area and potentially contributed to the change from negative results to delayed positives. One strip gave a very quick signal (ten minutes), but this result could not be repeated.

4.5.3 Altering the order and position of the dried down reagents

The order in which the reagents were dried down also had an effect. Placing the gold conjugated antibody nearest the sample application end of the strips did not generate any positive signals. The reagent order that gave the strongest signals involved placing the antigen first, then the gold labelled antibody, followed by the biotinylated capture antibody.

This sensitivity to the order of deposition of the reagents implies that the antigen may have needed more time to bind one of the antibodies than the other. Since the gold conjugated antibody was monoclonal, it is likely to be this antibody that required more time, due to its increased specificity in looking for a particular epitope.

4.5.4 Buffer optimisation

Changing the buffers used to dilute the immuno-reagents did not alter the strength of the signal or reduce the time to generate them.

4.5.5 Dried down reagents combined with a pre-incubated test line

The strongest (delayed) signals were obtained when the biotinylated antibody was pre-incubated with the microspheres (before deposition on the test line). This suggests that the biotinylated antibody can capture more Rotavirus antigen (and indirectly, gold) when it is already on the test line, separate from the gold-conjugated antibody and not having to migrate through the membrane. Positioning the capture antibody on the microsphere may position the antibody more favourably, since the Fc portion of the antibody is anchored to the Streptavidin microsphere (via the biotin), potentially aligning the Fab region facing outwards, with its paratopes directed towards an oncoming antigen. Alternatively, the production of stronger signals could be because the monoclonal gold conjugated antibody has longer to interact with the Rotavirus antigen as they wick along the membrane together. Another consideration that may

account for the improved signal in this format is that the monoclonal gold conjugated antibody does not have to compete for binding sites with the polyclonal capture antibody.

4.5.6 Readjustment of the biotinylated anti-Rotavirus antibody concentration

Increasing the concentration of the biotinylated capture antibody on the test line (with the microspheres) also produced strong signals and yet the delay in the response remained. Drying down these higher concentrations of capture antibody in the conjugate area, conversely, produced weaker signals than when the capture antibody was on the test line (see Figure 34 and 36). Furthermore, the results of using the stronger stock concentration of capture antibody dried down in the conjugate area were weaker compared with the strips where 1/2 diluted capture antibody was used. This implied that the capture antibody concentration was too strong and that it was out competing the gold conjugated antibody (for Rotavirus antigen binding sites) and therefore reducing the signal.

4.5.7 Alternative hydrophilic membranes

The results of changing the strip membrane to an alternative hydrophilic material were inconclusive. Two of the membranes (VF1 and VF2) were comparatively thick and the assays failed, since the gold conjugate did not wick up and reach the test line area. A greater sample volume may have solved the issue. The MF1 alternative did produce a signal, but it took even longer to form compared to Fusion 5 membrane results.

4.5.8 Applying Rotavirus antigen samples to the optimised assay designs with stronger concentration of biotinylated capture antibody

Avoiding the need to dry down the Rotavirus antigen was paramount, since an LFIA that requires the analyte to be pre-dried is neither a rapid test nor a suitable approach for use at POC or OTC.

Applying the antigen to the strip, with a higher concentration of biotinylated capture antibody (pre-incubated with the microspheres) on the test line, did produce delayed positive results, reinforcing the notion that either the capture antibody or the gold

conjugated antibody have a greater binding affinity for the antigen when they are positioned separately on the strip.

Applying the liquid antigen in either a single dose or two aliquots (comprising a small volume of concentrated antigen, followed by sufficient PBST buffer to allow the reagents to travel the full length of the strip) produced no change in the intensity of the spots formed, but the double hit method did produce some quicker results (thirty minutes in some strips compared to ninety). This two aliquot method allowed the gold conjugated antibody early access to the antigen, at a higher concentration, before being washed towards the test line by the PBST.

4.6 Repeating the target assay using the stronger capture antibody concentration

Attempting to apply the antigen as a liquid sample to the test strip using the target assay format (1.7.1) using the 1/2 diluted stock concentration of capture antibody (with both the gold conjugated antibody and capture antibody dried down in the conjugate area) did produce one delayed weak positive, when using the two aliquot method. However, the delay was much longer than achieved in other assay formats (two hours) and the signal only appeared in one out of four strips in the target assay configuration.

4.6.1 Comparison of assay format and sensitivity

No direct comparison of the sensitivity of the two main assay formats was performed. However the Predator membrane based LFIA could detect the Rotavirus antigen at a 1/64 dilution within a few minutes. The target assay (1.7.1) using the Fusion 5 membrane could not detect the antigen at the same dilution (1/64) and only one strip out of four could detect the antigen at 1/16 dilution after a two hour period.

Quicker Fusion 5 based LFIAs, using the biotinylated capture antibody bound to the Streptavidin covered microspheres were achievable, although reproducible results and consistently strong signals took sixty to seventy minutes to form.

Overall, the Fusion 5 based strips are easier to prepare, since they do not require a different material for the membrane or separate sample, conjugate and absorbent pads. Furthermore, being hydrophilic the Fusion 5 assays do not suffer from non-specific binding as much as hydrophobic protein binding materials and no additional blocking reagent is required. However, the signals on Fusion 5 are more difficult to interpret, take longer to appear and are not as clearly defined as the signals produced on Predator.

4.7 False positives

There were two instances of false positives in the negative control experiments, see Table 26 and 30. Both instances occurred in negative controls where the capture antibody was the reagent that was omitted, but the assay configurations were different (one had the antigen dried down, while the other had antigen supplied as a liquid sample).

The false positives are likely to have been caused by either the gold particles themselves, or the combined antigen and gold conjugate immuno-complex, binding non-specifically with the microspheres on the test line.

Since the polystyrene based microspheres are hydrophobic, they may have formed a direct bond with the hydrophobic surface of the gold particles, see Figure 4 (Chandler *et al.*, 2001) to produce false positive signals.

However, the incidence of false positives was low and in other negative controls on both Fusion 5 and Predator membranes, (which lacked the same biotinylated antibody), false positive signals were not observed.

4.8 False negatives

The tendency for false negative results to occur was significant with the initial experiments using Fusion 5 membrane. The cause of the absence of signal, (when all the required reagents were present) was not immediately obvious, but approaching the problem systematically helped to reduce the incidence of false negatives. The

potential reasons for the false negatives could have been due to one, or more, of the reagents, the assay methodology, membrane material or buffer conditions.

4.8.1 Gold conjugated antibody

Pink residue from the gold conjugated antibody could be observed at the end of the Fusion 5 membrane in almost all cases, which indicates that the gold was successfully reaching and passing the test line. It is possible that the gold conjugated antibody was not completing the immuno-sandwich due to insufficient antigen (although a greater concentration of antigen did not generate a signal either).

The successful experiment using the mouse based gold conjugated antibody and the biotinylated anti-mouse antibody (pre-incubated with the Streptavidin microspheres) also suggests that the concentration of gold conjugated antibody was sufficient to give a visible signal within the Fusion 5 membrane matrix.

These factors suggested that the cause of the false negatives was unlikely to be down to the performance, or concentration, of the gold conjugated antibody. However, as the gold labelled antibody was monoclonal and recognised a specific epitope on the antigen, the monoclonal specificity may have been competing with the polyclonal biotinylated capture antibody. This situation could have reduced the ability of the gold conjugated antibody to bind the antigen and thus produce a signal.

Chandler *et al.* (2001) suggest that a lack of sugar in the conjugate release buffer or insufficient drying of the conjugate can lead to false negatives, however the same conjugate release buffer was used in all three assay methods tested (Predator, Fusion 5 and the homogenous test). A longer drying period (over a weekend) did not significantly alter the success of the assay, although Chandler *et al.* (2001) go on to suggest that the process of drying and rehydrating the gold conjugate could harm subsequent activity. It may be possible that the Fab portion of the gold conjugated antibody was being damaged or was otherwise inactive when used on Fusion 5 membrane, even though it survived being dried down in the glass fibre based conjugate release pad in the Predator assays.

4.8.2 Capture antibody

The assay format was modified so that the position of the capture antibody was changed. The effects of placing it in the conjugate zone and (in other experiments) on the test line, still resulted in false negatives, suggesting that mobility within the membrane was not the cause of the false negatives.

With the Predator membrane and in the homogenous assay, the capture antibody was diluted to 1/4 strength and positive assay results were achieved. However when the same concentrations, of all reagents, were repeated on Fusion 5 membrane, no signal was visible unless all the reagents were dried down. It is possible that the Fusion 5 membrane matrix performs better when the capture antibody is at a higher concentration and placed at a different position on the test strip. Placing the capture antibody on the test line gave more time for the monoclonal gold conjugated antibody to bind the antigen and a higher concentration of capture antibody may have then helped to bind the antigen-gold complex once it had flowed down the strip.

4.8.3 Immobilisation method

The biotin concentration in both the biotinylated anti-mouse antibody and the biotinylated anti-Rotavirus capture antibody were unknown and therefore no correlation from the successful binding between the anti-mouse antibody and the Streptavidin coated microspheres can be transferred to the Rotavirus assay. However, the biotin on the anti-Rotavirus capture antibody was shown to bind the Streptavidin coated microspheres in the homogenous assay and in the delayed positives (in the assay format where all the immuno-reagents were dried down on Fusion 5 membrane). Since the Streptavidin-biotin bond had the strongest affinity constant of all the reagents used, the relative concentrations of the biotin and streptavidin should have been the least crucial variable and not the cause of the false negatives.

4.8.4 Buffer conditions

As well as a lack of sugar, Chandler *et al.* (2001) also suggest that insufficient surfactant (Tween) in the conjugate buffer may be responsible for false negatives. However, since

the gold conjugated antibody gave a signal with the anti-mouse IgG antibody, the conjugate buffer was judged to be working appropriately. Chandler *et al.* also propose that excess, or a lack of, salt in the assay buffers may be the cause, together with incorrect pH.

The same buffers (sample dilution buffer, conjugate release buffer and capture antibody dilution buffer) were used on both the hydrophobic and hydrophilic membranes. Adjustment of the ionic strength and pH may have altered the solubility of the protein, in relation to the physical properties of the membranes, and reduced the incidence of the false negatives on Fusion 5 membrane.

In addition to adjusting the ionic strength and pH of the buffers, Jones (1999) also recommends the addition of alcohol in order to improve protein binding to hydrophobic membranes, which may have improved the sensitivity of the Predator assays. Millipore (2008) also propose adding alcohol to the buffers to reduce viscosity, resulting in improved fluid flow and to promote the drying down of reagents.

Using the microsphere binding buffer without the recommended addition of BSA was designed to reduce any blocking action the BSA may have been having on the microspheres (reducing affinity for the biotinylated antibody).

4.8.5 Membrane

The key difference between the two LFIA formats tested in this research was due to the physical properties of the membranes. The hydrophobic Predator membrane permitted the use of an antibody based attachment method at the test (and control) line, while the hydrophilic nature of Fusion 5 membrane required an alternative immobilisation method. However, the immuno-reagents were able to wick along both membrane types and pass the test line, so their mobility was eliminated as a possible reason for assay failure.

The reagents dried down on Fusion 5 membrane may not have been truly dry in some of the early experiments, and this may have contributed to some assay failures, but

the properties of the Fusion 5 membrane proved to be adequate when used with the gold conjugated antibody and anti-mouse antibody assay.

4.8.6 Antigen sample

The concentration of the Rotavirus antigen was both increased and decreased in the initial Fusion 5 assays, with no apparent influence on signal generation. The second batch of Rotavirus antigen also failed to generate any positives on two assay formats tested.

4.9 Delayed positives

When all the immuno-reagents were dried down, including the Rotavirus antigen, the assay appeared to give positive results but only after a substantial delay, typically after one hour and far in excess of the fifteen minutes recommended by commercial suppliers (who caution against interpreting results obtained after this period, since the signal may be due to non-specific binding or other artefacts).

However, the delayed positives became consistent and reproducible, suggesting that they may have been genuine positive signals after all.

4.9.1 Gold conjugated antibody

The biotinylated anti-mouse antibody successfully detected the mouse based gold conjugated antibody (BB International), and gave a comparatively rapid signal.

The successful binding in this immuno-reaction was due to the biotinylated anti-mouse antibody specifically targeting Fc fragments of the mouse based antibodies (QED Bioscience, 2003) and did not depend on the Fab section of the gold conjugated antibody.

In contrast, when binding the Rotavirus antigen, the same reagent, the gold conjugated monoclonal antibody (BB International) was dependent on the Fab portion binding a specific epitope of the Rotavirus antigen. This reduced affinity may have required a longer reaction time, which contributed to the delay in the production of a

signal, but it is unlikely to be the sole cause of the delay, since the same reagents were present in the Predator membrane based assays.

4.9.2 Capture antibody

Pre-incubating the biotinylated capture antibodies and Streptavidin microspheres, before deposition on the test line, reduced the number of binding reactions required during the assay run time. However, this step did not significantly reduce the time taken to generate a signal, although the signal generated was stronger. This was probably because the gold conjugated antibody had longer to bind the Rotavirus antigen, uninhibited by the capture antibody.

Increasing the concentration of the capture antibody did not speed up the assay either, although it did allow the antigen to be correctly applied as a liquid sample rather than being pre-dried.

4.9.3 Immobilisation method

Both the Polysciences and Seradyn protocols for attaching biotinylated antibodies to Streptavidin microspheres recommended an incubation period (thirty minutes at 4°C for the Polysciences 2µm microsphere and an hour at room temperature for the smaller Seradyn microsphere). This implies that although the binding affinity is strong, getting optimal (or maximal binding) takes time, which may be another reason that the LFIA signals took time to develop. In addition, the polystyrene microspheres are inherently hydrophobic which may be initially inhibiting the immuno-reagents from displaying a signal on the test line.

4.9.4 Buffer conditions

Using the conjugate release buffer to make up dilutions of the capture antibody, in place of PBS, gave the strip a small additional volume of Tween and BSA (which helps to avoid non-specific binding). These additions may also have helped to transform false negative responses into delayed positive results.

However, using the proprietary sample dilution, in place of PBST, for diluting the sample had no obvious benefit. Neither did reducing the salt content of the Streptavidin microsphere binding buffer speed up assay results.

4.9.5 Membrane

While the pore size of the two membranes tested was different by an order of magnitude (Appendix A), it is the difference in porosity that plays a more important role in determining the wicking rate, which, in turn, determines the time the reagents have to interact on the membrane before they reaching the test line. The Fusion 5 membrane had greater porosity than the Predator membrane, resulting in a faster wicking rate. This may have contributed to the delayed signal since there may not have been sufficient time for the immuno-reagents to react and form the sandwich, particularly the gold conjugated antibody and the antigen, as indicated earlier.

The use of alternative hydrophilic membranes did not improve the assay. Two of the membranes were too thick to perform with the volumes of reagents used and the MF1 membrane took as long, or longer, for a signal to appear.

4.9.6 Antigen sample

Applying the second batch of Rotavirus antigen, dried down on the Fusion 5 membrane, helped to achieve (delayed) positive results. This second batch of antigen was judged to be more concentrated than the original batch (as determined by Rota-Strip Quick tests) and either the drying down process or the increased concentration contributed to the success of getting (delayed) positive signals.

Experiments to determine whether the delay in obtaining signals could have been reduced by altering the concentration of the second batch, when dried down, were not carried out.

Using the more concentrated, second batch may, in fact, have contributed to a reduced, possibly delayed, signal. In his review of factors that can lead to interference in immunoassays, Selby (1999) describes an effect described as the “hook effect”. This occurs when the antigen is in excess and separately binds the paratopes of both the

labelled antibody and the capture antibody, thus reducing the likelihood of them sharing a single antigen and forming the immuno-sandwich.

4.9.7 Time

Attempts to speed up the assay by reducing the drying time of the strip had no effect. It was thought that the length of time the strips took to dry may have been masking the formation of any pink spot. Using longer strips (to give the reagents more time to interact) and shorter strips, to speed up the assay also had no immediate benefit. Drying the strips after the sample had been applied did not reduce the delay either.

Inverting the strip after the sample application, allowed the reagents to run down the strip towards the test line and ensure that all of the assay components reached the test line. This potentially reduced the assay interaction period but appeared to help in the generation of signals.

Chapter 5: Conclusions and Suggestions for further work

5.1 Conclusions

The Fusion 5 material potentially simplifies the design and fabrication of lateral flow strips and reduces the range of materials required, when compared to more traditional hydrophobic based lateral flow assays. The hydrophilic properties of Fusion 5 require an alternative test line strategy and large Streptavidin coated microspheres were used to attract biotinylated capture antibodies to this zone. This test line modification on Fusion 5 did lead to positive results, but only after a substantial delay compared to a similar lateral flow assay on Predator, a hydrophobic membrane. The target assay format (1.7.1) was designed to include an analyte independent test line so that one assay could be modified to detect multiple analytes. However, the results with the strongest signals and most consistent results required the capture antibody to be pre-incubated with the microspheres. These results also required the assay to run for between thirty and ninety minutes.

5.2 Improvements and suggestions for further work

5.2.1 Alternative label

Improved sensitivity in LFIAs, when detecting low concentrations of antigen, has been reported by Chandler *et al.* (2000) who propose adding a silver based reagent to the conjugate zone. The silver forms a black deposit around each gold particle, improving visualisation. Alternatively, Carney *et al.* (2006) indicate that using gold particles with a diameter of 60nm can improve visibility and sensitivity. Using a coloured latex sphere as a signalling method may also give improved sensitivity compared to gold.

5.2.2 Use alternative antibodies

Using monoclonal antibodies for both the labelled antibody and the capture antibody would improve the specificity of the assay, although the problem with delayed positives would likely remain and may get worse if both antibodies have reduced affinity for the antigen.

Using a polyclonal antibody for the gold conjugate and a monoclonal antibody for capture may speed up the signal produced in a positive control, but the concentration and position of the reagents will need to be optimised in order to reduce competition for antigen binding sites.

5.2.3 Alternative immobilisation methods for hydrophilic membranes

Alternative immobilisation methods, on hydrophilic membranes, that allow an analyte independent test line, include:

a) Biotin coated microspheres

Rather than using Streptavidin coated microspheres and a biotinylated capture antibody, the reagents could be reversed, *i.e.* using biotin coated microspheres with Streptavidin bound capture antibody. The relative concentrations of biotin on the spheres and the Streptavidin bound capture antibody would need to be carefully optimised to avoid any steric hindrance with the tetravalent Streptavidin preventing sufficient capture antibody reaching the biotin on the sphere based test line.

Alternative microsphere coatings include the use of Protein A and anti-mouse IgG antibodies. Use of these coatings will require careful selection of the antibodies used in the LFIA.

b) Protein A coated microspheres

Protein A, found in the cell wall of *Staphylococcus aureus*, binds the Fc region of IgG antibodies of certain species, *e.g.* rabbit, human and mouse; but not others, *e.g.* goat and chicken (Bangs Laboratories, 1999).

Protein A coated microspheres could therefore be used, as a replacement for the biotinylated microspheres, in the target assay (1.7.1) provided that, for example, the capture antibodies used were rabbit or mouse based (which would bind to the Protein A coated spheres) and the labelled antibody were goat based (which would not bind to the spheres).

c) Anti-mouse IgG coated microspheres

Microspheres can also be supplied coated with anti-mouse IgG (Bangs Laboratories, 1999). In which case, the target assay could be adapted so that the analyte specific capture antibody used is mouse based (and so attracted to the spheres) and the labelled antibody is non-mouse based, so as not to bind non-specifically with the test line.

5.2.4 Adjust buffers

A more thorough investigation into the pH, salt concentration and the use of buffer supplements such as alcohol, BSA, Tween and sugar content could be performed both in additional lateral flow experiments and in other diagnostic procedures such as ELISA.

5.2.5 Alternative membrane

Using an alternative hydrophilic membrane to those already tested, may provide a solution that generates signals in a suitable timeframe. Furthermore, an analyte independent test line could be developed on a hydrophobic membrane by using anti-mouse IgG on the test line. A mouse based, analyte specific capture antibody in the conjugate pad could then be combined with an analyte specific, non-mouse based, labelled antibody.

5.2.6 Alternative test line deposition methodology

The Rota-Strip, with its professionally striped test and control lines, demonstrates the advantages of using industrial based techniques to give a clearly defined, unambiguous signal.

5.2.7 Alternative antigens

The antibodies used in these assay formats were not tested with other antigens that can cause gastroenteritis and so the specificity for Rotavirus antigen cannot be assumed.

5.2.8 Alternative reagents

The molecular weight, physical structure or surface chemistry of the Rotavirus antigen may not be appropriate for use on a hydrophilic membrane such as Fusion 5. Using a different analyte, such as hCG (human chorionic gonadotropin) and corresponding antibodies may lead to improved assay performance.

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Appendices

A) Materials and Reagents

Lateral flow strip components

- a) Sample pad – Cellulose Absorbent Paper, Type 111 (Pall). Part number S70006
- b) Absorbent pad – Cellulose Absorbent Paper, Type 133 (Pall)
- c) Conjugate release pad – Glassfibre, Type 8980 (Pall)
- d) Adhesive – Adhesive Polyester Film (Pall). Part number S86875
- e) Membranes –
 - i) Laminated Predator (Pall) – polyethersulphone membrane modified to have nitrocellulose properties, part number 580590 (now discontinued). Pore size 0.45µm (Soufla, 2007).
 - ii) Fusion 5 (Whatman) – polymer treated glass fibre, maximum pore size 11µm. Average pore size 4.6-5.6 µm (Jones and Cox, 2008).
 - iii) VF1 (Whatman) – unbound, glass fibre based membrane
 - iv) VF2 (Whatman) – bound, glass fibre based membrane
 - v) MF1 (Whatman) – bound, glass fibre based membrane

Reference Lateral Flow Immunoassay

Rota-Strip Quick Test – Immunochromatographic test for the detection of rotavirus in human faeces. Nitrocellulose based positive control strip (Corus BioConcept).

Immuno-Reagents

- a) Antigen – Freeze dried form of bovine Rotavirus, UV inactivated. Code: C-1081 (Corus BioConcept). Concentration estimated to be between 3×10^8 and 3×10^9 viral particles / ml (Taylor and Rixon, 2008).
- b) Colloidal gold labelled antibody – Immunogold Conjugate, Monoclonal Anti-Rotavirus raised in Mouse, 40nm, OD=10.1 at 520nm (BB International). Concentration 8.4µg/ml.

c) Capture antibodies (all polyclonal)

i) Rabbit Anti-Rotavirus (Human) – Code B0218 (Dako). Activity directed towards VP6 antigen of group A rotaviruses. Reacts with group A rotaviruses from humans, calves and pigs. Concentration 0.2 mg/ml.

ii) Biotinylated Goat Anti-Rotavirus (Bovine) – Catalog number BP1055B (Acris Antibodies). Reacts with inner capsid proteins of bovine and human rotavirus. Concentration 4-5mg/ml.

iii) Biotinylated Goat anti-Mouse IgG – catalog number 83400 (QED Bioscience Inc.) Fc fragment specific antibody. Concentration 1.3 mg/ml.

Immobilisation Reagents

Microspheres (donated by Diamatrix Ltd.)

a) Power-Bind microspheres covalently coated with Streptavidin. 0.83 μ diameter. Catalog number 2900-1701 (Seradyn)

b) Microspheres covalently coated with streptavidin, 2 μ m diameter. Biotin binding capacity 7 μ g/mg. Catalog number 24160 (PolySciences Inc.)

B) Microsphere preparation and washing

Microspheres from both Seradyn and Polysciences were washed according to the Polysciences protocol (Polysciences, 2005).

This involved placing 500µl of the microsphere solution into a centrifuge tube and spinning at 10,000g for five minutes. The supernatant was then removed and the pellet resuspended in 500µl of microsphere binding buffer (2.1.1) - although the Bovine Serum Albumin (BSA) was omitted from the buffer solution, unless otherwise indicated. The spheres were then spun, the supernatant discarded and resuspended in binding buffer (2.1.1) a further three times in order to clean the spheres thoroughly.