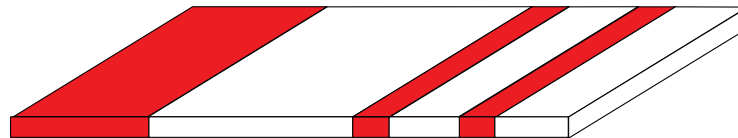


B E A D S • A B O V E T H E R E S T™



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I. ABOUT THE TECHNOLOGY

Immunochromatographic assays, also called lateral flow tests or simply strip tests, have been around for some time. They are a logical extension of the technology used in latex agglutination tests, the first of which was developed in 1956 by Singer and Plotz.¹ The benefits of immunochromatographic tests include:

1. User-friendly format.
2. Very short time to get test result.
3. Long-term stability over a wide range of climates.
4. Relatively inexpensive to make.

These features make strip tests ideal for applications, such as home testing, rapid point of care testing, and testing in the field for various environmental and agricultural analytes. In addition, they provide reliable testing that might not otherwise be available to developing countries.

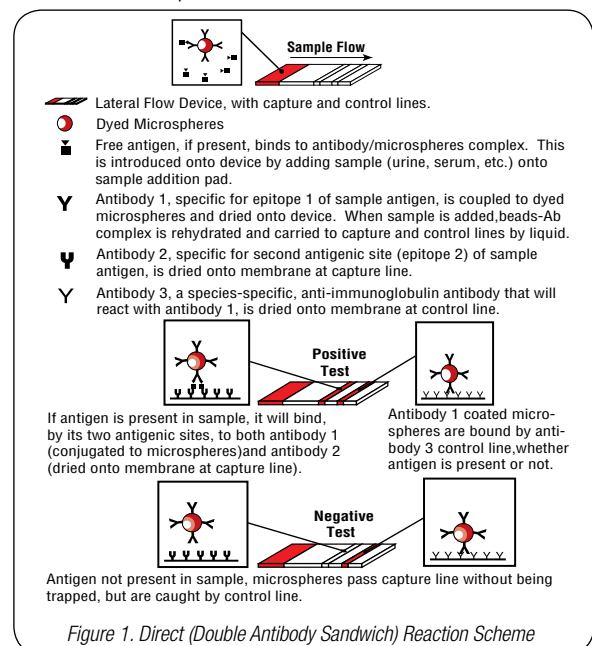
The principle behind the test is straightforward, and will be discussed in

greater depth in a subsequent section. Basically, any ligand that can be bound to a visually detectable solid support, such as dyed microspheres, can be tested for qualitatively, and in many cases, even semi-quantitatively. Some of the more common lateral flow tests currently on the market are tests for pregnancy, Strep throat, and Chlamydia. These are examples of conditions for which a quantitative assay is not necessary.

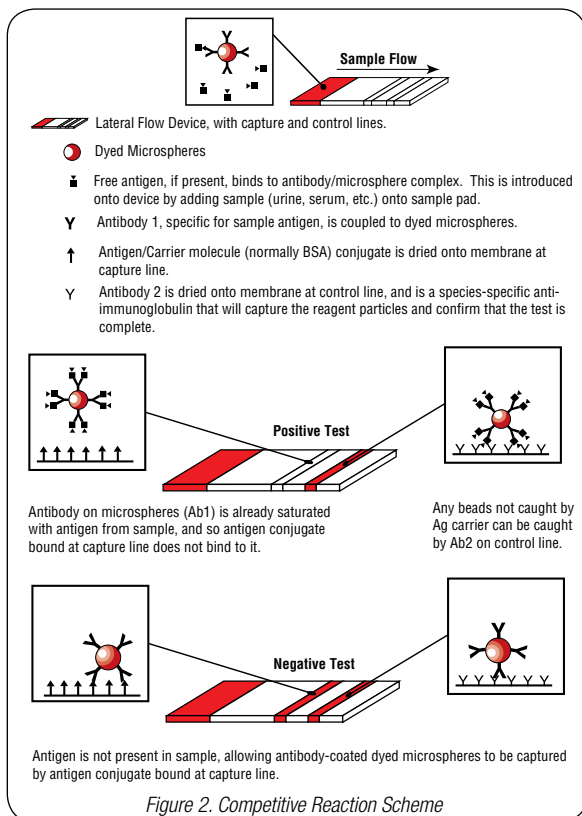
II. REACTION SCHEMES

The two predominant approaches to the tests are the Non-Competitive (or direct) and Competitive (or competitive inhibition) reaction schemes. These can best be explained graphically, as shown in Figures 1 and 2.

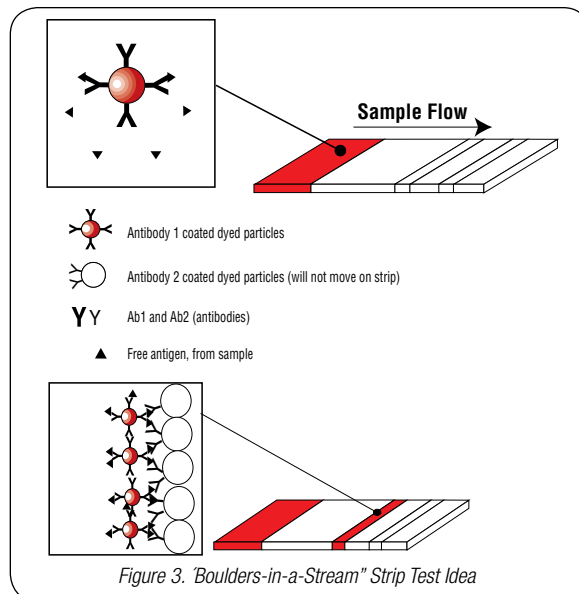
The double antibody sandwich format is used when testing for larger analytes with multiple antigenic sites, such as LH, hCG, and HIV. In this case, less than an excess of sample analyte is desired so that some of the microspheres will not be captured at the capture line, and will continue to flow toward the second line of immobilized antibodies, the control line. This control line uses species-specific anti-immunoglobulin antibodies, specific for the conjugate antibodies on the microspheres.



The competitive reaction scheme is used most often when testing for small molecules with single antigenic determinants, which cannot bind to two antibodies simultaneously. If this format is chosen, it is important to pay close attention to the amount of antibody bound to the microspheres, in relation to the amount of free antigen in the sample. If the sample does not contain an excess of free antigen, some of the microspheres will bind at the capture line, giving a weak signal and making the test result ambiguous.



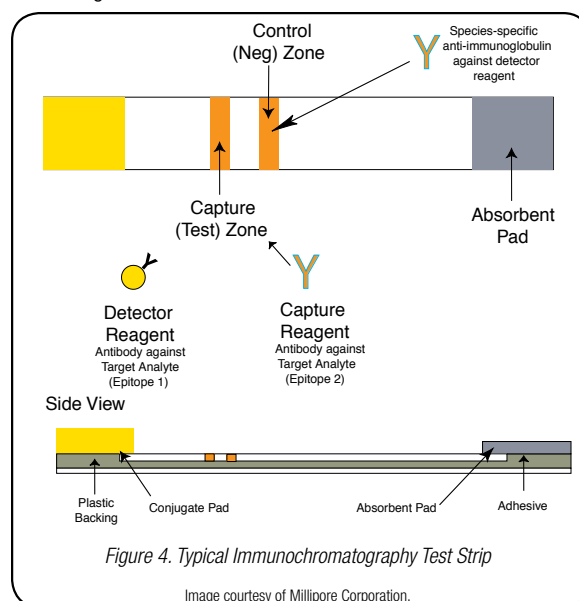
2. Add sample (with antigen). Sample flow moves dyed microspheres down membrane.
3. Capture antibodies at test line held in place by attachment to undyed microspheres, which are too large to migrate on the membrane.



These principles are well-documented in the literature, and appear very straightforward. However, in order to maximize efficiency and minimize development and production costs, there are some guidelines which, if followed, could possibly reduce some of the hurdles normally associated with the development of a new technology.

III. MATERIALS

A good place to start is to examine the raw materials that will deliver the highest quality at the lowest cost. The constituents of a lateral flow test can be shown in Figure 4.



Specifically, the constituents are as follows:

Normally, the membranes used to hold the antibodies in place are made up of primarily hydrophobic materials, such as nitrocellulose. Both the microspheres used as the solid phase supports and the conjugate antibodies are hydrophobic, and their interaction with the membrane allows them to be effectively dried onto the membrane. These hydrophobic interactions are very reliable, so much so, that getting the hydrophobically bound antibody/microsphere complexes to enter into the mobile phase upon sample introduction can be difficult. One variation to the above reaction schemes which has been proposed is the "Boulders in a Stream" approach.² This gets around the problem of protein-coated microspheres sticking to the membrane nonspecifically by using a membrane that is inert, and does not bind antibodies. This makes migration of the mobile phase antibodies very efficient and reliable. The capture antibodies, rather than being physically bound by the membrane, are attached to large microspheres, which will be held in place physically, rather than chemically, while the sample passes by, much like boulders in a stream. This can be used for both of the above-mentioned reaction schemes, and is diagrammed in Figure 3.

Some considerations involved in this format are:

1. Choose a hydrophilic strip where Abs will NOT stick, with porosity so microspheres can move and boulders cannot.

A. Antibodies

There are three types of antibodies.

1. Stationary Phase
 - a. Capture Line Antibodies
 - b. Control Line Antibodies
2. Mobile Phase
 - a. Conjugate Antibodies (Antibodies on dyed microspheres, to which the sample analyte will bind initially). *Note:* If you are testing for small molecules using the competitive binding format, you will also need purified antigen, or an antigen / carrier molecule (BSA) conjugate, for attachment to test lines.)

B. Membranes

The membrane chosen is dependent upon the approach that you choose, as previously mentioned. Some options for this include:

1. Nitrocellulose (High Protein Binding)
2. Cellulose Acetate (Low Protein Binding)
3. Glass Fiber Membranes (Non-Protein Binding)

Membrane manufacturers generally offer a wide variety of material types and pore sizes, so it is a good idea to investigate several options before deciding which specifications most closely match your test objectives.

C. Microspheres

There are several sizes and polymers to choose from. Conjugate antibody or antigen is attached, and microspheres migrate down the membrane upon introduction of your sample. Some hints to choosing an appropriate particle follow.

1. Optimal flow rate is achieved by choosing microspheres 1/10 the pore size of the membrane through which they will migrate, or smaller.
2. Optimal colors for visualization in various types of samples:
 - a. Whole blood: Black or dark blue
 - b. Serum: Bright red or bright blue
 - c. Urine: Green, blue, red or black
 - d. Saliva: Any dark color
 - e. Cerebral spinal fluid: Any dark color
3. To minimize hindered flow caused by the inherent hydrophobic interactions between membrane and particle (in the case of a hydrophobic membrane), pretreatment of the membrane with a substance that will maintain a small distance between the microspheres and the membrane, yet which can be easily rehydrated, is often helpful (Figure 5). Examples of substances commonly used are sucrose, various water soluble inert polymers, and surfactants. The idea is to choose a substance that is stable in dry form, yet dissolves easily upon rewetting to allow the antibody bound microspheres to flow easily through the membrane upon addition of the sample. (A sample procedure for doing this is included later in this text).

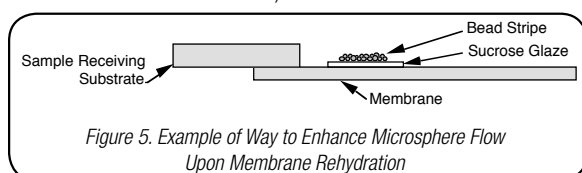


Figure 5. Example of Way to Enhance Microsphere Flow Upon Membrane Rehydration

In addition to treating the membranes, the microspheres themselves can also be pre-treated with surfactants, synthetic or protein-based blockers. If

done correctly, this can also help to reduce the problem of reagent mobility upon sample introduction. Much work has been done in developing optimum mixtures of these various polymers, detergents, and blockers. A list of suppliers of these materials and information on their use is listed at the end of this TechNote.

D. Conjugate Pads

These are the parts of the lateral flow device to which the sample is added. Ideally, this is a pad made of a material that can absorb a large amount of sample, and will then release this sample into the membrane at a steady, controlled rate. A good source of information regarding these is Whatman, whose contact information is included in this TechNote.

E. Membrane Backing

This is added to give strength to the membrane, which is often very fragile. The membrane can be attached by adhesives or by a process called direct casting. This is advantageous, as it eliminates the possibility of adhesives interfering with the test, so when ordering the membranes, it is important to specify which type is preferred.

F. Desiccant

Desiccant can be added into the pouch separately or incorporated into the absorbent pad. It is used to keep ingredients dry during storage before use.

G. Plastic Housing

This is the case for the test, and the foil pouch in which the final product is presented. A good source of information regarding the various possibilities for each of these is Bio•Dot.

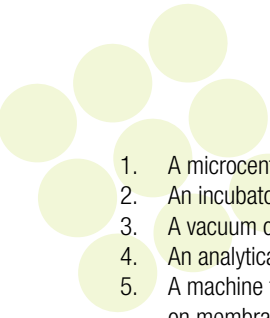
A look at the number of materials that are needed for these tests might be somewhat intimidating. However, the costs of mass producing these is surprisingly low.

IV. COSTS

Test Line Antibodies:	\$0.01 per test
Membranes:	\$0.02 per test
Control Antibodies:	\$0.01 per test
Absorbent Pad:	\$0.001 per test
Conjugate Antibodies:	\$0.02 per test
Sample Pad:	\$0.002 per test
Latex Microspheres:	\$0.01 per test
Membrane Backing:	\$0.01 per test
Desiccant:	\$0.02 per test
Pouch:	\$0.08 per test
Plastic Housing:	\$0.20 per test

Labor is generally estimated at approximately twice the raw material cost, bringing the total cost for materials and assembly to approximately \$0.38 per test. Some of the above-mentioned items are available in a range of prices, and in each case, the total is based on the high estimate. This does not take into consideration research costs, but considering that much of the information needed to develop these tests is readily available, there is really no need to allow cost to be a hindrance to a good strip test development idea.

The second consideration, of course, is the capital equipment needed to make these tests, which is relatively modest compared to other immunodiagnostic technologies. What is initially needed, and is often already found in most laboratories, is:

- 
1. A microcentrifuge capable of at least 15,000 rpm
 2. An incubator
 3. A vacuum oven
 4. An analytical balance
 5. A machine for spraying or printing antibodies/antigens on membranes

When in the research stage, it can be beneficial to try the process on some borrowed assembly equipment before investing in this for scaling up to manufacturing lots.

V. REACTION KINETICS

Now that we have looked at the principles behind these tests and some specifics regarding their manufacture, let's consider some of the factors involved in choosing the appropriate raw materials.

Test developers are often concerned with reaction kinetics. A faster test will not only be more attractive commercially, but often will be more accurate. On the other hand, the test must proceed slowly enough that antibody / antigen reactions are able to occur. Some principles that govern the kinetics of immunochromatographic assays follow:

1. The reaction rate decreases with the square of the increase in flow rate.
2. Assay time decreases with increasing flow rate.
3. Sensitivity decreases with the square of the increase in flow rate.
4. Reagent usage increases with increasing flow rate.
5. Background (streaking on the membrane prior to the capture antibody line) decreases with increasing flow rate.
6. Flow rate decreases as distance from the origin increases.
7. The amount of protein bound decreases (for nitrocellulose membranes) as the pore size increases.

Therefore, while increased flow rate is generally desirable, and it is known that one of the major influences affecting this is the relationship between the microsphere and membrane pore size, there is a point of diminishing returns in trying to increase this variable. The above principles should be closely examined and weighed against each other in the research phase before deciding on the exact parameters for the final product.

VI. CALCULATIONS

Some of the variables to take into consideration when setting up a lateral flow test include:

A. Flow Rate of Membrane

This is determined empirically, and will vary according to the viscosity of the sample used. Data for the flow rates of specific membranes with specific sample types are supplied by the manufacturer.

B. Membrane Porosity

This describes the fraction of the membrane that is air (e.g., a membrane with a porosity of 0.7 is 70% air), and will have an impact on the flow rate of the membrane.

C. Membrane Capacity

By definition, this is the volume of sample that can pass through a given membrane per unit time, and is determined as a factor of the length (L), width

(W), thickness (T), and porosity (P) of the membrane:

$$L \times W \times T \times P = \text{Membrane Capacity}$$

A second important calculation is the determination of the amount of antibody that can be bound per unit area of membrane (pertaining to the capture and control lines). This calculation involves the following variables.

Dimensions of representative capture antibody line: $0.1 \text{ cm} \times 0.8 \text{ cm} = 0.08 \text{ cm}^2$. Binding capacity of membrane used for capture antibody (obtained from the membrane manufacturer). In this example, we will use $50 \mu\text{g}/\text{cm}^2$ - a low end estimate for nitrocellulose membranes. Therefore, the binding capacity of the membrane for the capture antibodies is simply a factor of these variables:

$$0.08 \text{ cm}^2/\text{line} \times 50 \mu\text{g}/\text{cm}^2 = 4.0 \mu\text{g}/\text{line}$$

This is a theoretical example, but from past experience we have learned that in practice, a tenth of this is normally sufficient. Therefore, as with all theoretical calculations, they can provide a baseline which is optimized for the specific conditions and reagents involved in each particular test.

There are other calculations involved in setting up this type of test, some of which are not within the scope of this text. However, the suppliers of the various raw materials are normally good sources for this information, and are generally happy to help ease the development process. For example, some important considerations involving the microspheres are the best type of binding (covalent attachment or simple adsorption), as well as the proportion of antibody to microspheres for best sensitivity in the final product.

Useful information regarding this can be found in our TechNote 204, *Adsorption to Microspheres*, and TechNote 205, *Covalent Coupling*, both of which can be downloaded from our website. Another good source for further information is our list of related references, which is supplied at the end of this TechNote.

VII. PROCEDURE (MEMBRANE PREPARATION)

The following is a procedure that has been used successfully in the past. This is specific for an hCG test in urine, using a nitrocellulose membrane, but can be easily modified to accommodate a wide range of analytes and test formats.

Preparation of Reagent Strip

1. A rectangular sheet of 3-10 μm pore size nitrocellulose membrane is cut with dimensions of 15cm x 8cm (representative dimensions for this type of test).
2. A reaction zone can be formed by applying a line of capture antibody (in this case anti- β hCG) across the long dimension of the strip, approximately 3cm from the top (arbitrary) of the strip.
3. The width of this antibody stripe should be approximately 2mm, and this can be controlled by using an airbrush or microprocessor controlled microsyringe.
4. Dry for 1 hour at room temperature.
5. Soak membrane with an aqueous solution of inert compound or polymer of your choice to block excess binding sites on the membrane (in this example, polyvinyl alcohol, 1% w/v in 20 mM Tris buffer, pH 7.4) for 30 minutes at room temperature.
6. Rinse membranes in distilled water, and dry for 30 minutes at 30°C.

A second membrane pre-treatment, allowing for better flow, can now be performed.

7. Prepare a solution of 30% sucrose in distilled water, and apply this to the membrane where the conjugate reagent is to be located (normally 1cm from the bottom, with a width of 3-5mm).
8. Bake membrane for 1 hour at 40°C.
9. Apply antibody-coated microspheres to membrane over sucrose glaze, keeping dimensions consistent with sucrose glaze.
10. Place bottom of membrane (approximately 0.5cm below reaction zone) between absorbent pads, saturate these with Synthetic Urine (containing purified antigen), and observe flow characteristics and color formation at capture zone.

Modifications can then be made as necessary to optimize the reaction kinetics discussed previously.

VIII. FUTURE TRENDS

The technology involved in these lateral flow tests is exciting in and of itself, in that it provides an accurate, easy-to-use, rapid diagnostic tool. Currently, the principles governing this test are being extended to allow for some exciting new possibilities for future tests. Some development possibilities that are currently being evaluated follow.

1. By using the same format for lateral flow tests and dyeing the solid support with a fluorescent dye, the possibility exists to create a truly quantitative test. If the spectral properties of the dyed microspheres to which the antibodies are conjugated are known, the amount of antibody bound at the capture line can be precisely quantified using a fluorometer. The benefits to this would include all of those lateral flow tests that currently exist. In addition, the tests could, theoretically, become truly quantitative assays.
2. By placing multiple lines of capture antibodies on the membrane, each for a different analyte, one can develop a single test for more than one analyte. An obvious application for this would be to create a drugs-of-abuse test panel. Biosite's Triage[®] is based on this format.³ Diagnostically, this principle could be used for panels for which multiple analytes can be tested, such as immune diseases, allergies, or even Multiple Chemical Sensitivity Disorder. Also, as the technology involved in preparing these tests continues to develop, it should be possible to combine both of these ideas (1. and 2.) to make a low-cost, rapid quantitative diagnostic assay for multiple analytes.
3. Another exciting application of this technology is in the environmental field. This format presents an opportunity to develop a rapid, reliable test that can be performed in the field for anything from water pollution to plant disease. Because these diagnostic tests must often be performed in harsh environments, the lateral flow format is ideal. With proper preparation and foil pouching, no refrigeration or special handling is required.
4. As knowledge in the field of molecular genetics continues to expand rapidly, the interest in using a simple format for detecting various genetic markers, and DNA- or RNA-related infectious disease pathogens will increase. The guiding principle behind this type of test, the ability to bind a ligand from solution to a solid support, can be performed on genetic material as well as proteins, making the application of this technology in this field almost limitless.
5. One development alternative that seems to have good potential for strip tests involves Printed Liquid Circuit Technology, which by definition is

a porous media element in which fluid flow is controlled by impervious barriers made using a printing process. This utilizes fluid flow in a set pattern that is analogous to simple electronic circuits, and offers the capability of threshold (qualitative), semi-quantitative, and true quantitative assays. Potential improvements include automatic reagent addition, lower costs, smaller sample size, and automatic sample metering. The intricacies of this technology are beyond the scope of this text, but companies currently working with printed liquid circuits are listed among the Manufacturers/Vendors at the end of this TechNote.

6. An idea, that we think can be advantageous in terms of reduced development time, would be to use protein-coated microspheres, such as our ProActive[®] Streptavidin-coated microspheres. By biotinylating the desired conjugate antibodies and taking advantage of the strong affinity that biotin has for streptavidin, the antibodies are easily attached to the microspheres. Alternatively, protein A-coated microspheres will bind many IgG's at the Fc region, allowing for optimized, directed antibody attachment. In this way, a series of tests could be developed rather quickly, using the same solid support, membrane, housing, etc. The only variable would be the conjugate and capture line antibodies used for each test. Additional information regarding these microspheres can be found in our TechNote 101, *ProActive[®] Protein-Coated Microspheres*.

IX. LIST OF MANUFACTURERS / VENDORS

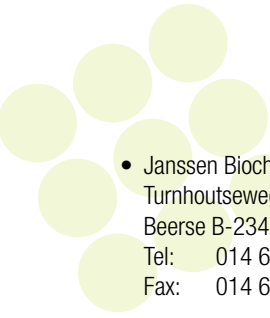
Note: This list of vendors is intended to help you find the appropriate reagents and procedures for developing your tests and does not constitute a product endorsement on our part. This list is not all-encompassing and we encourage you to explore several reagent vendors before committing your time and resources.

Membranes:

- | | |
|---|---|
| <ul style="list-style-type: none"> • Bio•Dot
11781 Sky Park Circle
Irvine, CA 92614
Tel: 949-440-3685
Fax: 949-440-3694 | <ul style="list-style-type: none"> • Millipore
290 Concord Road
Billerica, MA 01821
Tel: 800-645-5476
Fax: 800-645-5439 |
| <ul style="list-style-type: none"> • Micron Separations, Inc.
135 Flanders Road
Westborough, MA 01581
Tel: 800-444-8212
Fax: 508-366-5840 | <ul style="list-style-type: none"> • Pall Biosciences
2200 Northern Blvd.
East Hills, NY 11548
Tel: 800-645-6532
Fax: 516-484-5228 |
| <ul style="list-style-type: none"> • Whatman, Schleicher, & Schuell
200 Park Avenue, Suite 210
Florham Park, NJ 07932
Tel: 800-WHATMAN
Fax: 973-245-8301 | |

Antibodies:

- | | |
|--|---|
| <ul style="list-style-type: none"> • Dako Corporation
6392 Via Real
Carpinteria, CA 93013
Tel: 805-566-6655
Fax: 805-566-6688 | <ul style="list-style-type: none"> • Sigma-Aldrich Chemical Co.
P.O. Box 14508
St. Louis, MO 63178
Tel: 800-325-3010
Fax: 800-240-4668 |
|--|---|



- Janssen Biochimica
Turnhoutseweg 30
Beerse B-2340 Belgium
Tel: 014 60 21 11
Fax: 014 60 28 41

- Jackson ImmunoResearch Laboratories, Inc.
872 West Baltimore Pike
West Grove, PA
Tel: 800-367-5296
Fax: 610-869-0171

There are many other good antibody sources. A reference is Linscott's Directory.

- Linscott's Directory
6 Grove Street
Mill Valley, CA
Tel: 415-389-9674
Fax: 415-389-6025

Hardware (Assembly, Finishing Equipment):

- Bio•Dot
11781 Sky Park Circle
Irvine, CA 92614
Tel: 949-440-3685
Fax: 949-440-3694
- Kinematic Automation, Inc
P.O. Box 69
Twain Harte, CA 95383
Tel: 209-532-3200
Fax: 209-532-0248

Polymers/Blockers to Enhance Membrane Flow:

- Pragmatics, Inc.
29477 County Road 16W
Elkhart, IN 46516
Tel: 800-213-1293
Fax: 219-262-3911
- RDI Div. of Fitzgerald Ind.
34 Junction Square Drive
Concord, MA 01742
Tel: 978-371-6446
Fax: 973-371-2266
email: antibodies@fitzgerald-fii.com

Printed Liquid Circuit Information Resources

- British Technology Group
101 Newington Causeway
London SE1 6BU
Tel: 0171 403 6666
Fax: 0171 403 7586
Contact: Dr. Ed Footitt
- Bio•Dot
11781 Sky Park Circle
Irvine, CA 92614
Tel: 949-440-3685
Fax: 949-440-3694

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10. **May, K., M.E. Prior, I. Richards.** November 1988. Immunoassays and devices therefore. International Patent: WO 88/08534.
11. **Rosenstein, R.W.** July 1988. Solid phase assay. European Patent: EP 0 284 232.
12. **Sommer, R.G.** October 1996. Quantitative detection of analytes on immunochromatographic strips. U.S. Patent: 5,569,608.
13. **Allen, et al.** November 1998. Electronic assay device and method. U.S. Patent: 5,837,546.

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3. **Bio•Site Company.** 11030 Roselle Street, San Diego, CA 92121.
4. **Jones, K.D.** 1999. Troubleshooting protein binding in nitrocellulose membranes. Part I: Principles, Part II: Common problems. *IVD Technology*, 5: 2-3, 32-41, 26-35.

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