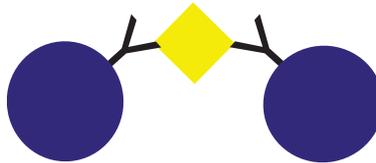


B E A D S • A B O V E T H E R E S T™



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Ag can be adsorbed onto them. These "sensitized" microspheres then act to magnify or amplify the Ag-Ab reaction which takes place when they are mixed with a sample containing the opposite reactant. In simple particle agglutination, a positive test results when uniformly dispersed milky-appearing Ab-coated particles in a drop of water or on a glass slide react with Ag in a drop of sample (whole blood, serum, urine, etc.) to cause particle agglutination (clumping of microspheres, to look like curdled milk) (Figure 1). Similarly, an agglutination test for Ab can be made with Ag-coated particles.

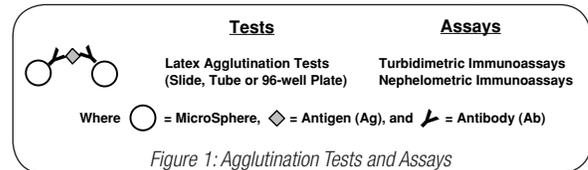


Figure 1: Agglutination Tests and Assays

Qualitative Agglutination (Tests): Microsphere or latex agglutination tests (LATs) have been around since 1956, when Dr. Jacques Singer developed a rheumatoid factor test.¹ Then, in 1957, he developed a pregnancy test. Since then, LATs have been applied to > 100 infectious diseases. Types and recent examples are as follows:

- Bacterial: leprosy, cholera, *Yersinia enterocolitica*, Lyme Disease, TB
- Viral: HIV, *Herpes simplex*, cytomegalovirus
- Fungal: aspergillosis, candidiasis, cryptococcosis
- Mycoplasmal: mycoplasmal pneumonia (*Mycoplasma pneumoniae*)
- Protozal: amoebiasis, toxoplasmosis
- Rickettsial: Rocky Mountain spotted fever

Commercial LATs exist for > 60 other chemical analytes, e.g. hCg, RF, CRP, ASO, FDP, and fecal occult blood. LATs are used for analyte detection in many other applications, including veterinary medicine (feline parvovirus and cryptococcosis), plant health (potato viruses), law enforcement (DAU, drugs of abuse in urine), food (antibiotics in milk), and the environment.

LATs are portable, rapid, efficient, and useful under even the most primitive conditions. Ideal for point-of-care use in the field, ambulance, or bedside, they can be run quickly and simply (2 minutes from sample preparation). Diagnosis and treatment can commence promptly, before the patient is transferred or discharged. Examples of such tests include those for FDP (fibrin degradation products), myoglobin (for heart attacks), rotavirus (to

isolate contagious pediatric patients), and for sexually transmitted disease clinics (test patients and treat them before they leave).²

Tests for new analytes are continually being added, such as TechLab's Leuko-Test, an LAT for lactoferrin released from fecal leukocytes in diarrheal stool specimens - ideal as a screening test for "traveler's diarrhea," or inflammatory diarrhea caused by *Shigella*, *Salmonella*, *Campylobacter*, and *Clostridium difficile*.³ By the way, this one was developed by an alumna of The Latex Course™.

Eiken Chemical, has a *dual* test (two LATs in one) - an occult blood test for hemoglobin and transferrin in feces. The microspheres have anti-hemoglobin and anti-transferrin bound to them, so that either hemoglobin or transferrin (or both) can cause agglutination for "a much higher detection rate compared to hemoglobin [alone]."⁴

Also new is an LAT for BFP in urine, a new tumor marker for bladder cancer.⁵

In 1996, new notable LATs were announced: one for mass screening for TB⁶ and another for systemic lupus erythematosus (SLE) - a three minute test for antinuclear Abs.⁷ In 1997, a new LAT for the herbicide 2, 4 D appeared.

Murex's Staphaurex Plus™ latex, using beads coated with human fibrinogen and IgG, can agglutinate *three* different ways - by encountering either clumping factor, surface antigen, or Protein A (found on most Staph A).

II. AGGLUTINATION TESTS

A. Active Agglutination Tests

1. Wet & White on Black

The earliest tests used *liquid* reagents made with plain, white microspheres and were run on washable, reusable glass slides, usually with a black background. Tests are now run on disposable plastic or coated paper cards. White slides are also available for colored microsphere tests. Most active LATs require the clinician to rock the slide or card for 2-5 minutes to mix sample and reagent and to speed up agglutination.

2. Slide Test "Automation"

Refinements of the "ordinary" slide test include novel devices designed to make the tests less technique dependent. In Wampole's (Carter-Wallace) Fast Trak™ (Figure 2) and Roche's OnTrak™ (Figure 3) devices, the sample and reagent with coated microspheres are mixed and guided into a "track" or capillary. As the reactants move down the track by capillary action, they mix themselves, and agglutination is read with transmitted light after they reach the end, 2-3 minutes later. No hand rocking or rotation is necessary, and the test is quite operator-independent.

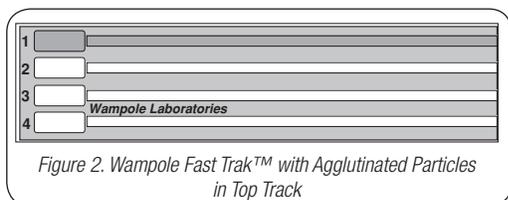


Figure 2. Wampole Fast Trak™ with Agglutinated Particles in Top Track

Note that the Roche DAU tests are run as inhibition tests, so a positive tests yields no agglutination (i.e., drug in urine inhibits the agglutination), while a negative test shows agglutinated microspheres.

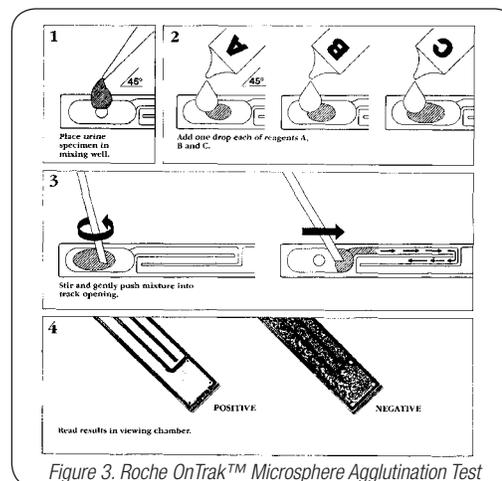


Figure 3. Roche OnTrak™ Microsphere Agglutination Test

3. Dried Microspheres

Several companies produce tests with reagent-coated microspheres dried on a card. In use, the microspheres are rehydrated with sample, stirred, and observed for agglutination. Excellent shelf-life should be possible with dried reagents.

4. Dyed Microspheres

Dyed microspheres provide different contrast (dyed microspheres observed against a white background). *Staphylococcus* tests by Carr-Scarborough (red microspheres) and REMEL (black) use dyed microspheres. REMEL also has a *black* LAT for *E. coli* O157:H7 (the strain implicated in the August 1997 Hudson Foods recall of 25 million pounds of ground beef). Two or three new mycobacterium LATs (for *M. tuberculosis* and *M. avium* or *M. kansasii*) also use dyed microspheres. The *E. coli* and mycobacterium tests were developed by Latex Course™ alumni, at different companies.

5. Mixed Colors

MUREX has a *Salmonella* test which uses antibodies to three different antigen groups bound to three different colored microspheres (red, blue, and green). By comparing the color of the combined agglutinated microspheres to a background color, one can determine which salmonella groups are present in the sample. They also have a *Shigella* test kit employing two colors of microspheres and two reagents, to differentiate between four different strains.

See *Microspheres as Markers and Stains* (Section XI) for more uses of dyed microspheres.

6. Slide Test Sensitivity

Current LATs generally use 0.2-1.0µm diameter microspheres. My calculations show that larger microspheres should theoretically yield more sensitive tests; the assumptions are as follows:

- ~100 clumps must be seen to determine agglutination;
- Each clump must be ~50µm in size to be seen by the eye;
- ~10 bonds required per microsphere to agglutinate; and
- 10µL sample size.

If a single microsphere is 1µm in diameter, then ~50³ or ~10⁵ microspheres will be required to make one visible clump. To figure out how much Ag or Ab is required to detect agglutination, multiply 10 bonds per microsphere x 10⁵ microspheres per clump x 100 clumps, which equals ~10⁸ molecules or 10⁻¹⁶ mole of agglutinator.

If MW = 150,000 (\approx MW of IgG), then the necessary amount of Ag or Ab would be 15 picograms. If a 10 μ L sample is used, then sensitivity would be 10⁻¹⁶ mole / (10 x 10⁻⁶L) = 10⁻¹¹ mole/L = 10 picomoles/L. If microspheres are 0.1 μ m, then sensitivity will only be 10nM. If 10 μ m microspheres are used, sensitivity improves by 1000X to 10fM. These calculations are summarized below:

Table 1: Calculated Sensitivity

Microsphere Diameter	# of Microspheres in		Agglutinator Required		Calculated Sensitivity
	1 clump	100 clumps	Molecules	Moles wt., if IgG	
0.1 μ m	10 ⁸	10 ¹⁰	10 ¹¹	10 ⁻¹³ 15 ng	10 nM
1.0 μ m	10 ⁵	10 ⁷	10 ⁸	10 ⁻¹⁶ 15 pg	10 pM
10.0 μ m	10 ²	10 ⁴	10 ⁵	10 ⁻¹⁹ 15 fg	10 fM

If these calculations are close to being accurate, they lead to the prediction that you will get greater sensitivity by using larger particles. Please feel free to challenge this idea and report any results which would shed any light here.

Some limits to the theory are (1) clumps of larger microspheres may not be strong enough to withstand the hydrodynamic forces trying to break them apart and (2) it will take considerably longer for all the larger microspheres to form clumps. At an earlier course, Bob Veltri estimated that LATs could have sensitivity of 25ng with submicron particles, but what is possible with larger particles? Recent experimental data shows that an ordinary LAT can detect 600ng CRP/mL, while a LAT using careful technique and video microscope to detect agglutination can detect 300 ng/mL, but an ultrasound enhanced agglutination technique plus latex dilution and image analysis can detect 230 pg/mL.⁸

B. Passive Agglutination Tests

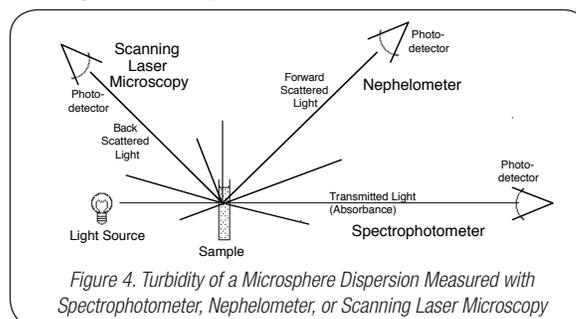
Classically, red blood cells (RBCs) (often "tanned" or specially processed to preserve them) have been used in agglutination tests. These *hemagglutination* tests are run by mixing samples with the coated RBCs in 96-well plates with "V"-shaped bottoms. They are called *passive* since there is no rocking involved. Agglutinated cells fall out of suspension and form a pink, lacy pattern covering the bottom of the wells; unagglutinated cells roll to the center of the "V" to form a dark red button.

Reverse passive latex agglutination (RPLA) tests use microspheres to replace RBCs. Dyed microspheres have been found to be more reproducible and shelf-stable than the RBCs. Tests using blue-dyed polystyrene microspheres have been made for 96-well plates,⁹ and generally use microspheres which are larger (> 1 μ m) and heavier (>> 1.05 g/mL) than those in regular LATs.¹⁰ Sawa International (Tokyo) has six RPLA tests for bacterial toxins which cause hemorrhagic colitis, hemolysis, toxic shock syndrome, food poisoning, and two other enterotoxins.¹¹

Sonologics (Hanover, NH) has an instrument, developed at the University of Wales, which uses a "focused ultrasound wave to accelerate agglutination." Normal two hour hemagglutination tests take only five minutes (two minutes for ultrasonic treatment in their "black box" and three minutes to develop the pattern). "Eighty different agglutination tests have been identified for which the technology is applicable," including LATs in capillary tubes.^{12, 13, 14} Ultrasonics is claimed to increase sensitivity to 40 pg/mL of *Candida albicans* mannan and 70 pg/mL of *Aspergillus fumigatus* galactomannan for a 250X and 500X increase in sensitivity over conventional LATs.¹⁵

III. AGGLUTINATION ASSAYS

Spectrophotometers and nephelometers have been used for years to measure protein precipitation directly. When these instruments, which measure transmitted, absorbed, or scattered light, are used in place of the human eye, it is possible to quantitate agglutination and to develop sensitive microsphere agglutination immunoassays (Figure 4). For a good discussion of light-scattering immunoassays, see Price and Newman.¹⁶



The intensity of light scattered particles dispersed in water varies with the number of particles, the diameter of particles, the wavelength of the incident light, the angle of the detector to the incident light, and a number of other variables. As agglutination starts, single particles first become doublets; thus, the number of light scatterers drops dramatically (decreases by half), and the apparent diameters increase rapidly up to 2X. After this point, the changes in numbers and diameters are less rapid. Microsphere immunoassays can be very sensitive, since the change of scattered light intensity is highest at the very beginning of agglutination or at the lowest concentrations of analyte.

Microspheres which scatter light best have diameters approximately equal to the wavelength of light being scattered. Therefore, for visible light ($\lambda = 390\text{-}760\text{nm}$), the best scattering microspheres have diameters of 390-760nm (0.39-0.76 μ m). Microspheres outside this range will not scatter visible light as well. In practice then, one can start with microspheres < 0.1 μ m which are poor scatterers. As they agglutinate, the clumps quickly grow to a size where they scatter light much better. Thus, change of scattered light versus analyte concentration can be the basis for very sensitive end-point or rate method immunoassays. UV light requires smaller microspheres (<< 100nm) and infrared light can use ~0.5 μ m microspheres. Conversely, one can also start with microspheres which scatter well (perhaps 0.5 μ m microspheres) and observe them clumping to sizes where they do not scatter as well, but most assay systems seem to use the principle of small, poorly-scattering microspheres clumping to form big, highly-scattering clumps.

One can use a nephelometer to follow scattered light directly^{17, 18} or a spectrophotometer to measure change of "absorbance" of light (measure scattered light indirectly).^{19, 20} DuPont calls these techniques particle enhanced turbidimetric immunoassay (PETIA) and particle enhanced turbidimetric inhibition immunoassay (PETINIA).

Behring's "latex-enhanced" nephelometric method measures forward scattered light of 840nm at ~20° angle from the light beam. Their assay for C-reactive protein (CRP) was judged good enough to have been proposed as the *reference method* for this assay.²¹

Modern 96-well plate readers can read a complete plate in about two seconds and can be used for end point or kinetic assays. One proposed lactogen assay measures microsphere agglutination turbidimetrically in such a plate reader.²²

These instrumental methods have now been applied to a wide variety of commercial assays,²³ and new assays and even new analytes are being added continually. This is truly one of the steady growth areas for the microsphere business. Orion Diagnostica (Finland) has a compact but sophisticated “doctor’s office” size turbidimeter for microsphere assays. They are steadily adding new assays.

The Binding Site has a new nephelometric immunoassay for β_2 -microglobulin using microspheres, and Instrumentation Laboratories has a new turbidimetric (spectrophotometric) assay for the same analyte for its Monarch centrifugal analyzer. (This analyte is an important prognostic indicator for AIDS.) Dako AS (Denmark) recently introduced a new “particle enhanced turbidimetric” (PET) assay for cystatin C - a new analyte described as a “better marker than serum creatinine for glomerular filtration rate.”²⁴

Spectrophotometric / nephelometric sensitivity should be better than LAT sensitivity and may be as good as 100 pg/mL (1 pg/10 μ L) for proteins.²⁵ Zolg estimated turbidimetric sensitivity at 5×10^7 molecules ($\sim 10^{-16}$ moles = 100 attomoles),²⁶ which would be ~ 1.5 pg/10 μ L [if MW = 150,000 (as for IgG) and 10 μ L sample volume were used]. He also estimated “quantitative agglutination” (like ACADE did, see next section) at 5×10^6 molecules, or one order of magnitude better than turbidimetry. Thus, the turbidimetric assay is estimated to be at least 10X more sensitive than a typical 1 μ m microsphere LAT (see above), and perhaps 10,000X more sensitive than a LAT test using 0.1 μ m microspheres. [Dako²¹ (previous paragraph) claims turbidimetric detection limit of 0.15 mg/L (=1.5 ng/10 μ L).]

The work by Price, Newman, et al.,^{16,17} has prompted interest in very small microspheres with higher refractive indices. For polyvinylphthalene, $n_D = 1.68$ vs. 1.59 for polystyrene ($n_D =$ refractive index using light at sodium D line or 589.26nm). These “brighter” microspheres scatter light better, especially when they agglutinate to the optimum scattering size. One can also get a higher refractive index for polystyrene by using shorter wavelength light: $n_{400nm} = 1.63$.²⁷

In another case, researchers chose “duller” microspheres of polybutylmethacrylate ($n_D = 1.43$), apparently because they do not scatter as well.²⁸ This property may be important in some instruments, for lower background scattering (a lower blank value for the calibration curve).

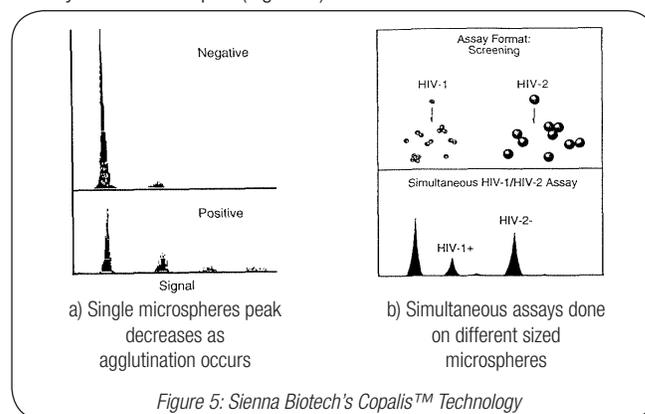
IV. OTHER INSTRUMENTAL AGGLUTINATION METHODS

Scanning laser microscopy instruments have been proposed to quantify agglutination.²⁹ This idea might offer advantages since, by using larger microspheres (> 1 μ m) and operating with concentrated microsphere suspensions, agglutination may occur more quickly.

Angular anisotropy (or “two-angle light scattering”)^{30,31} and quasi-elastic light scattering (also called “dynamic light scattering” or “photon correlation spectroscopy”)³² are more powerful techniques which have been investigated and patented for assay systems. These more sophisticated methods *might* deliver better sensitivity and should be considered after simpler methods have been exhaustively explored.

Sensitivity 10-15 times better than turbidimetry was claimed for work by Technicon and later by ACADE. They used particle counters to measure changes in numbers of single particles or clumps of particles during agglutination.³³

Sienna Biotech’s Copalis™ technology uses “optical sizing flow particle analysis,” a sensitive laser-based particle sizer / counter. They count single, Ab-coated microspheres ($\sim 1\mu$ m in diameter) before and after agglutination. As single particles become agglutinated by antigen in a sample, the signal in the single particles channel decreases, while the counts increase in the doublet, triplet, etc. channels. Thus, a *decrease* in single particles is proportional to analyte concentration. They can do several tests simultaneously using different sized microspheres coated with different Ab’s, and can use whole blood with no interference from cells. They claim a sensitivity for TSH = ~ 1 pM. (Figure 5)



Fujirebio Inc. introduced special microsphere-based reagents and an instrument to read agglutination by pattern recognition. It scans the bottom of 96-well plates and detects the difference between a central button of non-agglutinated microspheres and the typical diffuse, lacy pattern of agglutination. Researchers at the University of Wales are developing a similar instrument.^{10,11,12,13}

In fluorescence quenching analysis, fluorescent microspheres yield a lower fluorescent output on agglutination. When such microspheres clump together, they interfere with and absorb light from each other so that less light gets to the detector.

Magnets have been used to accelerate agglutination (pulling the particles together) in fluorescence quenching caused by agglutination of fluorescent magnetic particles.³⁴

Magnetic microsphere agglutination can be measured by magnetic moment analysis. As the microspheres pass through a magnetometer, agglutinated microspheres give a larger signal than single microspheres, so agglutination can be quantified (Hitachi patent³⁵). Magnetic particles can be coated with one Ab and mixed with plain particles coated with another Ab to the same Ag, then combined with a sample. If Ag is present in the sample, a magnet can be used to remove coagglutinated particles. The absorbance of the suspensions should be very different before and after sample addition.³⁶

A. Agglutination Made Colorimetric?

Indicia (France) offers “Spherotest,... a diagnostic system [for β_2 -microglobulin] based on quantitative microagglutination of calibrated and sterically stabilized synthetic microspheres.” “U.V.-visible absorption” results are read in a microplate reader.³⁷ Microspheres of diameter, d , dispersed in a liquid with closely matched refractive index will not scatter light, but will instead absorb a maximum amount of light at wavelength $\lambda_{max} \approx d/2$. [e.g., 740nm microspheres absorb at $\lambda_{max} \approx 340$ nm (blue) and appear yellow.] Upon agglutination, clumped particles appear as a different color and the loss of single microspheres results in a drop in $A(\lambda_{max})$ or absorbance at λ_{max} . Thus,

a plot of $A(\lambda_{max})$ vs. analyte concentration yields a linear curve of >5 orders of magnitude!³⁸

B. Beyond Simple Latex Agglutination

Filter Separation Agglutination Tests (and Assays): Kodak's earliest Surecell test kits used dyed agglutinated microspheres caught on a filter. Red microspheres coated with Ab were incubated with a sample and poured onto a filter. Single microspheres passed through the filter and no color appeared on the surface. If the sample contained the appropriate Ag, the microspheres agglutinated, and the agglutinated clumps were caught on the filter, resulting in a red (or pink) positive color test for the Ag (Figure 6).

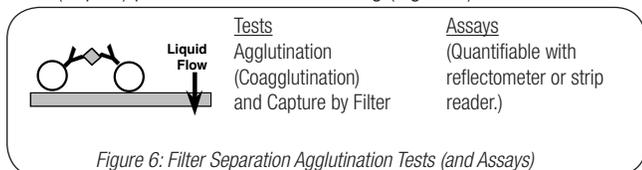


Figure 6: Filter Separation Agglutination Tests (and Assays)

Carter-Wallace's home pregnancy test, First Response®, uses plain microspheres (~1µm) and very small (< 50nm) red gold sol.

To Prepare the Test: The gold particles are coated with one antibody (Ab₁) to hCG (human chorionic gonadotropin); the plain microspheres are coated with an antibody to another hCG epitope (Ab₂); then, the particles are mixed and lyophilized.

To Use the Test: The particles are redispersed with a sample of urine. If the sample contains hCG, the particles are coagglutinated, yielding red clumps. The mixture is poured through a filter which catches the red clumps to yield a pink-colored filter. With negative urine, unagglutinated red particles pass through the filter and no color develops.

The principles employed in these two tests could easily be applied to assays where the reflected color intensity (as measured, perhaps, in a dry strip reader?) would correlate with the sample's Ag content. Such assays would be comparable to the ELISA's below, but simpler to operate, and probably more stable (no enzymes).

Akers Research makes a series of tests where black-dyed antibody-coated microspheres are mixed with sample and poured onto a strip. If no antigen is present, the microspheres migrate up the strip where a black (grey) color is observed. Thus, if color is observed, it means a "negative" test result. But, if the microspheres are agglutinated by the sample, then the clumps are too large to travel up the strip, and no color develops at the observation point (no color = "positive").

V. PARTICLE CAPTURE ELISA / ELIST

To Prepare these Tests: Ab₁ is bound to microspheres, and the microspheres are caught on a filter and dried (Figure 7).



Figure 7: Particle Capture Enzyme Linked ImmunoSorbent Tests and Assays

To Use these Tests: First, a sample is passed through the filter, and any Ag is caught by Ab₁ on the microspheres. Second, Ab₂-enzyme reagent is put through the filter; Ab₂ is caught by the Ag-Ab on the microspheres to complete the sandwich. Third, enzyme substrate is passed through the filter

and reacts with enzyme to create an insoluble colored product (on the filter) which is proportional to the amount of Ag caught.

Various tests (like hCG, Strep A and others) using this principle have been made by Hybritech (ICON®), Abbott (Test Pack™), Novo Nordisk,³⁹ IDEXX, and others.

Murex's SUDS clinical tests use wet reagents (microspheres in suspension). Microspheres are captured on a filter after the sandwich reaction. Ab-coated microspheres + Ag (from sample) + second Ab-enzyme conjugate are mixed, then poured through the filter device to capture the microspheres, which are then reacted with an enzyme substrate to form color.

Assays have been made by Hybritech (ICON®, QSR), Abbott (IMx®, AxSym®), Neogen (Reveal®), and others. Using a reflectance meter, the colored spots caused by analyte are compared to a blank and a standard spot to yield a true quantitative analysis. Reveal is literally a "field" instrument (pocket-sized) for measuring plant pests, like fungal infestations of soybeans, and turf diseases on golf courses. A similar hand-held instrument would permit point-of-care quantitative assays in human diagnostics.

Strategic Diagnostics has a "Competitive Latex Enzyme Immunoassay" for industrial chemicals. Petroleum products (benzene derivatives) and explosives in soil and ground water are detected at parts per billion concentrations. Ab-coated 3µm microspheres are first caught on a 1µm filter (Figure 8). Then, a sample is passed through the filter, and Ag, if present, binds to the Ab. Next, an enzyme / Ag conjugate is passed through the filter. If there is no Ag in the sample, the Ag / enzyme will bind to the filter, and added enzyme substrate will result in color on the filter. If there is Ag in the sample, no enzyme will bind to the filter, and there will be no color on the filter from the added enzyme substrate.⁴⁰

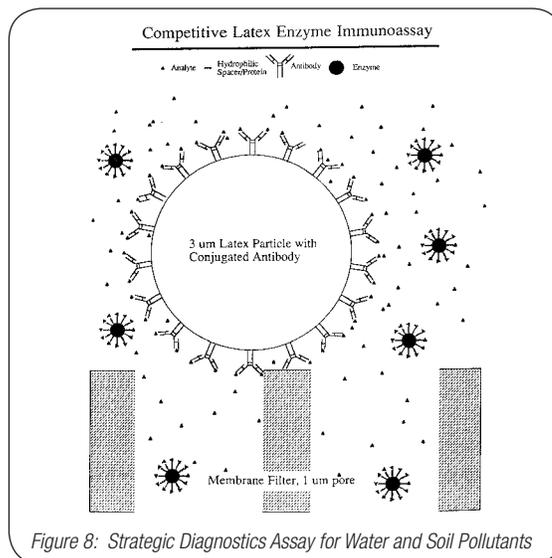


Figure 8: Strategic Diagnostics Assay for Water and Soil Pollutants

Costar Corporation has proposed a microsphere agglutination capture ELISA scheme.⁴¹ After reaction with chromogenic substrate, soluble color product is measured in a microplate reader.

V. LATERAL FLOW TESTS

A. Strip Tests

In 1988, a new over-the-counter pregnancy test (Clearblue Easy®, developed and patented by Unipath) revolutionized diagnostic immunological tests. The

test uses dyed microspheres in a sandwich format to give a one step test eliminating the need for unstable, color-generating enzymes.⁴² (Figure 9)

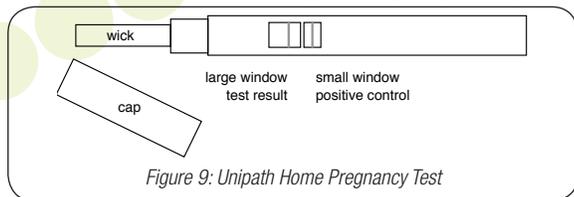


Figure 9: Unipath Home Pregnancy Test

To Prepare the Test: Small (dark blue) dyed microspheres (O) are first coated with primary antibody to hCG (Ab₁); the coated microspheres (O-Ab₁) are dried on one part of a nitrocellulose strip; another antibody to hCG (Ab₂) is immobilized on another section of the strip (Ab₂-I).

To Use the Test: The strip is wetted at one end with urine (Figure 10). As the urine moves by capillary action, it picks up the blue microspheres (O-Ab₁), and carries them downstream; any hCG in the urine reacts with the Ab₁ on the microspheres (O-Ab₁-hCG). When the flow reaches the immobilized (Ab₂-I), the dyed microspheres with hCG (O-Ab₁-hCG) are captured by Ab₂-I to form a blue line caused by the hCG sandwich (O-Ab₁-hCG-Ab₂-I). The blue line signals a positive pregnancy test.

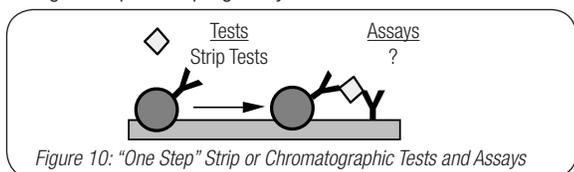
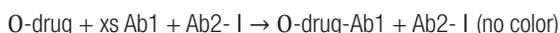


Figure 10: "One Step" Strip or Chromatographic Tests and Assays

Further downstream, there is another line of immobilized protein (Ab₃-I) which catches unconjugated O-Ab₁ (as O-Ab₁-Ab₃-I) (independent of hCG) to form another blue line, for a positive procedural control. If the second line does not form, the test results are invalid. Other home tests - for LH (luteinizing hormone) - and *clinical* tests for Strep A and Chlamydia are also available. Since the Unipath test, many other companies now offer laboratory single-analyte tests using this chromatographic principle. Examples are hCG, "popular" infectious diseases, and DAU⁴³ (Table 1). Eiken's hemoglobin test claims 50 ng/mL sensitivity.^{44,45} In March 1996, Quidel began shipment of its QuickVue *H. pylori* strip test. It is the first one-step, quick diagnostic for *H. pylori*-positive ulcer patients, and the first strip test, that we know of, which uses whole blood!

In 1992, Biosite Diagnostics introduced Triage[®], an eight analyte DAU test panel.^{46,47} It is an *inhibition* test panel. In the *first* step, urine is used to reconstitute and react with a mixture of eight pairs of reagents. Each pair consists of very small colored (gold sol) particles conjugated to a drug (O-drug) + excess antibody (Ab₁) to that drug. In the *second* step, the urine and reagents mixture is allowed to migrate on a strip, whereon eight other antibodies to the eight drugs are bound in different locations, (Ab₂-I). If the urine is drug-free, then Ab₁ will bind all O-drug and none will be available for the second (solid-phase) reaction:



If a drug is present in the urine above a certain cut-off level (controlled by the amount of excess Ab₁ present), then O-drug will be free to migrate along the strip to become bound by Ab₂-I. If a colored line is found, then that drug was present in the urine at a level above the threshold level:

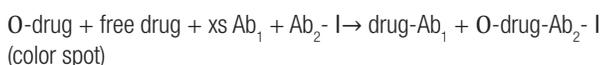


Table 1: Typical Dyed Particle Chromatographic Strip Tests

Analyte	Type	Company (Test Name)
hCG	OTC	Unipath (ClearBlue Easy™ or ClearBlue One Step™), Carter Wallace (One Step™ paddle, First Response® 1-Step), Johnson & Johnson (Fact Plus™, OEM by Abbott)
hCG	Lab	Unipath (ClearView™), Abbott (Test Pack Plus™), Pacific Biotech/Hybritech/Quidel (CARDS+-OST™ & Concise™), Sinovus
hLH	OTC	Unipath (ClearBlue™), Quidel (Conceive™)
Strep A	Lab	Unipath (ClearBlue™), Abbott (Test Pack Plus™), Pacific Biotech/Hybritech/Quidel (CARDS+-OST™ & Concise™)
Strep B	Lab	Pacific Biotech/Hybritech/Quidel (CARDS+-OST™)
Chlamydia	Lab	Unipath (ClearView™)
Mononucleosis	Lab	Pacific Biotech/Hybritech/Quidel (CARDS+-OST™)
Rotavirus	Lab	Sinovus
H. pylori	Lab	Quidel (QuickVue™, whole blood ulcer diagnostic)
DAU tests	Lab	Drug Screening Systems (several analytes), Biosite (Triage®, panel of 8 drugs)
Hemoglobin	Lab	Eiken (OC-Hemocatch Eiken, occult blood)
Brown Rot	Field	U.S. Department of Agriculture (wood decay)
Murine Ab type	Lab	Boehringer-Mannheim (ISO Strip™ mAb isotyping kit)
CPV*	Vet Lab	Sinovus
FelV**	Vet Lab	Sinovus

* Canine parvovirus

** Feline leukemia virus

B. Boulders in a Stream

An innovation in the Carter-Wallace First Response[®] 1-Step over-the-counter pregnancy test is the use of some Ab-coated larger microspheres on the membrane in the second and third Ab positions. (Figure 11 shows only the first and second Ab positions). These large microspheres are too large to move on the strip and therefore act as anchors to hold the second (and third) Ab stripe from moving with the liquid flow.

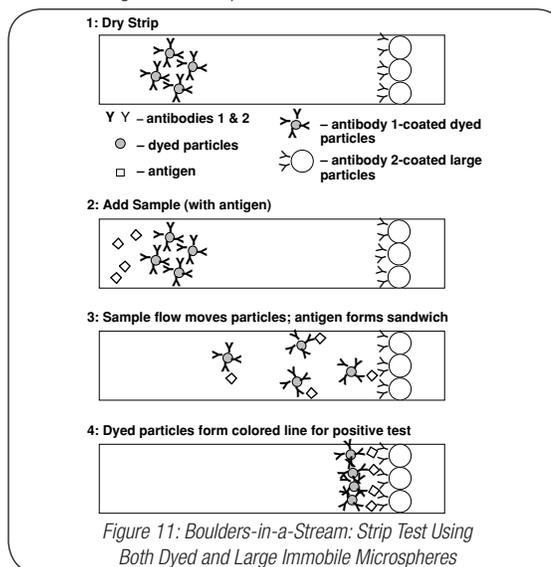


Figure 11: Boulders-in-a-Stream: Strip Test Using Both Dyed and Large Immobile Microspheres

The strip format is also being applied to non-human diseases, such as a USDA test for brown-rot decay in wood, which detects six different fungi which attack wood. Not fancy, this test was home-made by another Latex Course™ alumna using polyester cloth as the strip and Ab's specific to brown rot. It is the first immunological field test for detection of brown rot.⁴⁸

Boehringer-Mannheim has a test for murine antibody typing. This simple-looking, but sophisticated strip is useful for isotyping mouse mAb's and their light chains. On two sides of the strip, two bands will appear (out of eight

possible bands plus two positive control bands) for Ab class and sub-class (IgA, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM) and light chain (κ or λ).

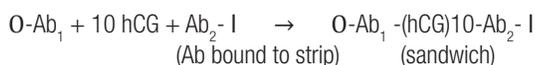
Another Latex Course™ alumnus at Sinovus (Sweden) has commercialized two veterinary strip tests: for CPV (canine parvovirus) and FeLV (feline leukemia virus), as well as tests for hCG and rotavirus. The Spring 1997 Clinical Ligand Assay Society Meeting⁴⁹ had a session on non-clinical immunoassays, including a strip test for a plant protein to identify cotton plants in the field which had been genetically altered.⁵⁰

Ian Wells says there are now > 250 different membrane-based tests.⁵¹

C. Sensitivity of Strip Tests

We calculated this, based upon the following assumptions:

1. The minimum line dimensions for visibility of the blue line are perhaps 0.5mm (500μm) wide, 5mm (5000μm) long, and 10 microspheres deep. With 0.25μm microspheres, the line would be 500μm / 0.25μm = 2000 microspheres wide; 20,000 microspheres long and 10 microspheres thick. Then, 2000 x 20,000 x 10 = 4 x 10⁸ microspheres (~7μg) per test.
2. It might take ~10 molecules of sandwich analyte (like hCG) reacting with each antibody-coated *dyed* microsphere and the second antibody immobilized on the strip in order to bind the dyed microspheres to the strip:



Some hCG will be wasted by binding to the wrong side of the dyed microsphere (the side away from the Ab₂ strip). In addition, it might require a ten-fold excess to bring about the reaction (90% of the hCG will be wasted and will not get to the microspheres or the strip-bound antibody).

3. It would probably take ~1 mL to thoroughly wet one of these strip tests and to move the particles to and past the immobilized Ab stripe. Therefore, sensitivity = 4x10⁸ x 10 x 10 = 4 x 10¹⁰ molecules (~6.7 x 10⁻¹⁴ moles) to cause a positive reaction. This is equivalent to (or, sensitivity *could* be) ~67 pg/mL for a positive test, if MW = 1000 or 0.67 ng/mL, if MW = 10,000.

Please feel free to challenge these assumptions and recalculate the possible sensitivity.

Independently, strip test sensitivity has been estimated at 0.1-0.2 fmol/mL for direct test and 1-2 fmol/mL for competitive tests.

Stability and ease of use are important features of these tests. Since no enzymes are used, the dried products should be stable for years - as long as freeze-dried IgG is stable. The tests are so easily run that one can conceive of many "dip and read" field tests. How about farmers diagnosing a wide variety of plant diseases, literally "in the field"?⁵² Water tests for pollutants? At the April 1996 course, I suggested an *E. coli* O157 test for FDA beef inspectors and Meridian announced one in August 1997.

All of these tests also have the promise of becoming true assays. If the intensity of color formed could be read by a dry strip reader, for example, then a quantitative result could be obtained. Different colors of dyed microspheres could be used and different analytes color coded. We know of folks who are working on quantitative strip tests. Maybe we can announce their existence at the next course?

VII. SOLID PHASE ASSAYS

Microspheres have unique properties - small enough to remain suspended for hours or longer at normal gravity, yet easily separated from suspension with a centrifuge, magnet, or filter. They have been used for years as solid supports for radioimmunoassays and other newer assays where solid / liquid (bound / unbound) separation is needed (Figure 12, Table 2).

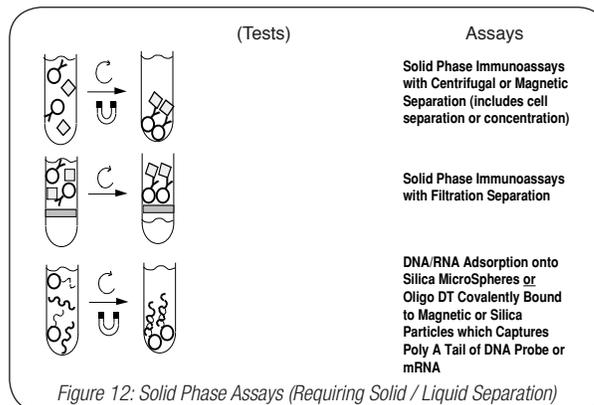


Table 2: Microsphere Use in Assays with Solid/Liquid Separation

Microsphere Type	Separation Method	Assay Examples
Large PS (>0.8μm)	Centrifugation	LDH-1 (Washington University)
Smaller PS (<0.5μm)	Centrifugal Filtration	LDL Separation (Genzyme)
Silica	Centrifugation	DNA / RNA Separations
<1μm PS / fluor. tag	Filtration	PCFIA (IDEXX Screen Machine)
Superparamagnetic	Magnet	Chemiluminescent immunoassays mRNA Purification (Novagen), PCR / QBC / LCR DNA assays (IGEN, Gene-Trak & Abbott), Template Prep / DNA Sequencing (MIT)

In a typical solid phase separation assay - for cardiac specific isoenzyme, lactate dehydrogenase, LD-1; antibody (D.8.1) is adsorbed on 0.8μm microspheres; the microspheres are mixed with serum; D.8.1 binds interfering isoenzymes LD-2, 3, 4, and 5; microspheres are centrifuged to remove the competing isoenzymes; and free LD-1 is left in solution to be reacted with substrate and measured in a spectrophotometer *without* interference.⁵³

Genzyme's "Direct Low Density Lipoprotein (LDL) Cholesterol Immunoseparation Reagent Kit" uses "...latex beads coated with affinity purified goat polyclonal antisera to specific human apolipoproteins, which facilitate the removal of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) in the specimen."⁵⁴ To use the kit, one mixes serum or plasma plus reagent (containing Ab-coated microspheres) and incubates in a separation device. After centrifuging the device (12,000 G's, 5 minutes) to filter the microspheres from the liquid, LDL cholesterol in the filtrate is measured using a conventional enzymatic cholesterol reagent.

Uniform *silica* microspheres will adsorb DNA or RNA to purify samples for PCR or assays. By adding chaotropic agents to nucleic acid solutions, the DNA / RNA can be made to adsorb onto silica. The density of the silica microspheres, 1.95 g/mL, makes them easy to centrifuge. One can also covalently bind to surface modified silica.⁵⁵

In particle concentration fluorescence immunoassay (PCFIA), particles

coated with one antibody trap a second antibody which traps an antigen or fluorescent-labeled antigen in a competitive binding assay. The particles are caught on a filter in the IDEXX "Screen Machine" and their fluorescence is measured. An internal assay from Eli Lilly & Company for tylosin (veterinary antibiotic) in animal feeds is an example.⁵⁶

Kodak researchers have covalently bound oligonucleotide probes onto 1µm microspheres and immobilized the microspheres in discrete locations on a membrane surface to capture biotinylated, PCR-amplified sample DNA. Each spot captures a different PCR sequence. These steps are followed by treatment with avidin-horseradish peroxidase, a wash step, and dye-precursor. The result is colored spots which are diagnostic for specific DNA markers for various infectious diseases.⁵⁷

The range of bead sizes for solid phase assays extends from < 1µm to > 100µm - the latter quite large by most standards. Sapidyne offers KinExA™, an immunoassay instrument based on the kinetic exclusion assay method. Typically, 100µm PMMA beads are coated with Ab or Ag. They are pumped into a flow cell built into the lens of a fluorescence analyzer and held in place by a screen for the duration of the reaction, then back-flushed out of the cell to complete the cycle.⁵⁸

Large, Ab *fragment*-coated polystyrene beads have been used to collect bacteria from milk, water, and food. When using single chain Ab absorbed onto polystyrene beads packed into a column, > 90% of *Pseudomonas* was removed from a sample.⁵⁹

VIII. SUPERPARAMAGNETIC MICROSPHERE BASED ASSAYS

"Magnetic" particles permit fast and easy separation of solid and liquid phases. Actually *superparamagnetic*, the particles respond to a magnet, but are not magnets themselves and retain no residual magnetism after removal of the magnet.

Magnetic particles are most commonly used in commercial solid phase RIA's, ELISA's and newer chemiluminescent assays by Amersham, Chiron, Merck/Biotrol⁶⁰ and Beckman (formerly Sanofi) (Figure 13). Over a dozen Sanofi papers have now appeared, both general⁶¹ and specific, e.g. their method for ferritin.⁶² One relatively new instruments is Nichols / Quest's Advantage™ instrument using magnetic microspheres with chemiluminescent assays.⁶³

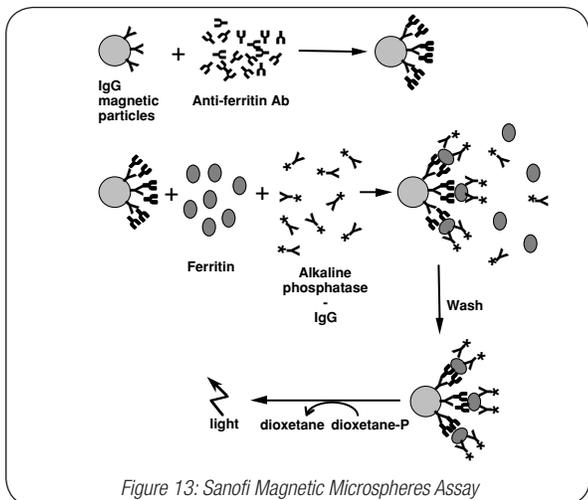


Figure 13: Sanofi Magnetic Microspheres Assay

"Space-resolved FIAs"⁶⁴ and immunoradiometric assays (IRMA's) can also be done with magnetic particles.⁶⁵ Reference Diagnostics (Bedford, MA)

adds magnetic particles to the conventional dextran sulfate - MgCl₂ reagent for HDL cholesterol separation from samples permitting more rapid HDL cholesterol assays.

Both animal and plant cells, as well as cellular components, are separated using magnetic microspheres. DYNAL sponsors much of the animal work, and there are many papers, for example.^{66, 67}

More recently, magnetics were used to sort and collect protoplasts of somatic potato hybrids.⁶⁸ They have also been used to collect and concentrate *Chlamydia trachomatis* from urine for subsequent analysis.⁶⁹ Cells can be positively or negatively selected using magnetic beads.

IX. PROXIMITY ASSAYS

A. Scintillation Proximity Assay

Amersham's scintillation proximity assay (SPA) system uses one microsphere coated with a β-emitter radio-labelled Ag and another microsphere dyed with scintillator and coated with Ab. When the microspheres are mixed together, an Ag-Ab reaction binds the microspheres together, and light is given off when β-rays emitted from the Ag-coated microspheres enter the Ab-coated, scintillator-dyed microspheres. When a sample is added to the mixture, any free Ag in the sample will interfere with the two microspheres coming together and decrease light output (Figure 14, top).⁷⁰

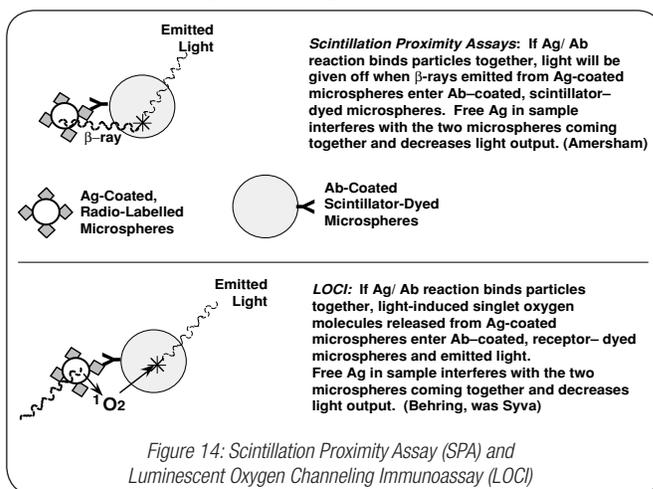


Figure 14: Scintillation Proximity Assay (SPA) and Luminescent Oxygen Channeling Immunoassay (LOCI)

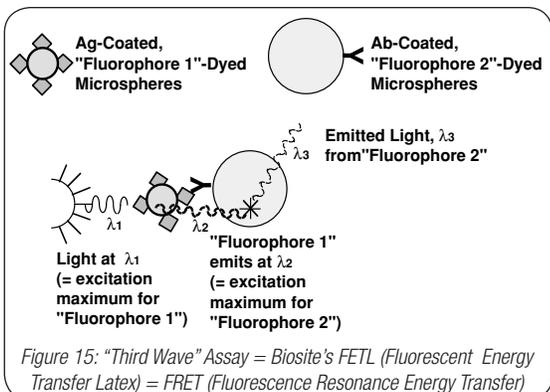
B. Luminescent Oxygen Channeling Immunoassay

Similar in concept to SPA, Behring (Syva)'s homogeneous immunoassay format, luminescent oxygen channeling immunoassay (LOCI), uses microspheres to measure TSH at 4 attomol! When Ag-coated and Ab-coated microsphere pairs bind together, molecular oxygen is released by a photosensitizer in one bead and diffuses to the other bead, which contains a high-quantum-yield chemiluminescent receptor. Again, Ag present in a sample will interfere with the two microspheres coming together and decrease light output (Figure 14, bottom).^{71, 72}

C. Third Wave Assay or Fluorescence Resonance Energy Transfer (FRET)

In 1994, borrowing on the ideas of fluorescent dye cascade (one day's emission exciting another) and the SPA idea, I suggested the idea of a "Third Wave" Assay (Figure 15). I imagined two microspheres dyed with different dyes - "Fluorophore 1" (F1), excited by a laser at λ₁ and emitting at λ₂ and "Fluorophore 2" (F2), excited by a laser at λ₂ and emitting at λ₃. If one microsphere is coated with Ab, and the other is coated with Ag, and if an

Ag/Ab reaction binds the microspheres together, then F2 in the Ab-coated (2nd) microsphere will emit light of λ_3 only if excited by λ_2 radiation, emitted by F1 in the adjacent Ag-coated (1st) microsphere. If there is competing Ag in a sample mixed with the two particles, then the particles will not get together, and no light will be emitted by the second particle.⁷³



Now Biosite Diagnostics (San Diego) has perfected this idea and calls it *Fluorescent Energy Transfer Latex (FETL)* using pairs of 0.2 μ m dyed carboxylate-modified beads, where one of the pair (the "donor" dye) is excited with 670nm light and in turn emits light of an intermediate wavelength which can excite the "acceptor" dye in another particle. The acceptor particle emits 760nm light. Now, imagine that the two different particles are each appropriately coated (via covalent coupling) with, for example, antibodies to two different epitopes on an antigen. If a sample with antigen is mixed with these particles, then a sandwich can occur and the 760nm emitted light (and antigen) can be measured quantitatively.^{74, 75}

Tosoh has a similar idea: Ab₁ and a fluorescer molecule are bound to one particle; Ab₂ and a quencher molecule are bound to another particle. The fluorescer will light unless the quencher is brought close by Ag in the sample, agglutinating or forming a sandwich between Ab₁ and Ab₂. Diminishing light signal is proportional to Ag content.⁷⁶

X. MICROSPHERES AND GENOMES

Enhanced chemiluminescence (ECL), robotics, and magnetic microspheres recently have been applied successfully to the human genome project. The microspheres are used in the first step of rapid DNA purification.⁷⁷ Novagen's Straight A's™ mRNA Isolation System uses their Magnetight™ Oligo (dT) Particles, which are superparamagnetic microspheres covalently coated with oligo (dT)₂₅. The protocol is designed to selectively extract and purify mRNA from a variety of sources. After magnetic separation, the purified mRNA is eluted off the magnetic beads for recovery or for a second round of purification.⁷⁸ Promega has a similar isolation procedure.⁷⁹

Other techniques using magnetic microspheres include oligonucleotide⁸⁰ and DNA template purification,⁸¹ "rapid genomic walking,"⁸² and sequencing.⁸³ Wilson used uncoated magnetic particles *twice* to purify ss-DNA - first to collect aggregated M 13 phage and later to collect its DNA from ethanol. Magnetic particles are cited as being relatively inexpensive raw materials in a method which reduces labor cost by half.

Streptavidin-coated magnetic particles are also used as a solid support in IGEN's human papilloma virus assay. This is yet another example of a DNA hybrid assay; it is based on polymerase chain reaction (PCR) and read by electrochemiluminescence.⁸⁴

PCR, QBR, LCR - (Q-Beta Replicase and Ligase Chain Reaction): These acronyms relate to molecular amplification techniques used for clinical lab identification of tiny amounts of various infectious agents. All these techniques use solid supports like microspheres and are explained in a good review article.⁸⁵

Gene-Trak Systems QBR technique uses "...d(T)-coated magnetic beads, which hybridize with the d(A) tail of the capture probe." The microspheres are used in the QB replication process. In the LCR method, Abbott uses an automated particle capture ELISA with small, protein-coated microspheres, as IMx® or AxSym® do, to detect the special hapten tags on the ends of ligated products after sample amplification.

The Human Genome Project is nearing the sequencing stage, and the Whitehead Institute/MIT has developed a method called Solid-Phase Reversible Immobilization (SPRI). DNA is captured onto carboxylate-modified encapsulated superparamagnetic microspheres. After the DNA is bound, the beads are washed with ethanol and then eluted from the beads in a low ionic strength solution. This method enables automatable, high quality DNA template purification, and can be used with all major templates and sequencing enzymes.⁸¹

XI. MICROSPHERES AS MARKERS AND STAINS

For years, microspheres, especially dyed ones, have been used as tags to identify cells or cell surface antigens on microscope slides. Most useful are those with the color or fluorescent dye *inside* the microspheres.

Because more dye can be loaded inside microspheres than on the surface, the color intensity is greater, and the dyes (especially the fluorophores) are well protected from photobleaching. The microsphere surface properties are not affected, so dyeing does not interfere with protein coating.

Ab-coated dyed microspheres will stick to cells and identify them. Dyed ~5 μ m microspheres, with appropriate Ab coatings, have been used as cell tags in rosette-type tests, where the microspheres cluster around certain cells to identify them in the microscope. The new technique called fluorescent *in situ* hybridization (FISH) involves labelling of intact cells using fluorescent microspheres.

Bartels (formerly Zynaxis) has an assay method for enumeration of CD4+ and CD8+ T lymphocytes using mAb-coated fluorescent dyed microspheres and mAb-coated magnetic microspheres. The mixed microspheres form rosettes around the appropriate T cells. A magnet separates rosettes from unrosetted cells and permits quantitation of fluorescence.⁸⁶

A ciguatoxin test for fish offers a novel use for dyed microspheres as immunomarkers. A roughened wooden paddle is inserted into a cut in the fish. When it is removed, some fish flesh adheres to it. Next, the paddle is dipped in a suspension of antibody-coated, dyed microspheres and washed. A colored paddle shows a positive test for ciguatoxin. This simple test enables fishermen to keep and eat only safe fish.⁸⁷

Other applications for dyed microspheres include regional blood flow studies in animals. Multi-colored 10 and 15 μ m microspheres, injected into an animal's circulatory system, become lodged in the tissues during circulation. After tissue biopsies, the colored or fluorescent spheres are recovered and counted or analyzed for size and fluorescence intensity. The E-ZTrac® products from Interactive Medical Technologies Ltd. and other similar systems by Triton Corporation and Molecular Probes are examples of these products,

and they are replacing radio-labelled microspheres in this field.⁸⁸ Consult the Fluorescent Microsphere Resource Center at the University of Washington. They have developed a technical manual describing fluorescent microsphere technology for regional blood flow applications.⁸⁹

Roche Molecular Systems' new "Ultra Direct" technique for processing plasma with exceptionally low HIV-1 titers, involves high-speed centrifugation, followed by lysis of the virions and direct PCR amplification. Red 0.2 μ m microspheres are added to the sample and spin down with the virions, greatly improving visibility of the pellet.⁹⁰

Plain and dyed microspheres are used as standards for flow cytometry. There is growing interest in "designer" microspheres- dyed with "fluorochromes," "fluorophores" (fluorescent dyes with particular spectral properties), and scintillators. Often only a small amount of these dyes is required to give an intense signal. Stains and flow cytometry are obvious applications of these microspheres, and there will be others.

Molecular Probes has microspheres with several dyes in each, yielding spheres which are excited at one wavelength and which emit at a wavelength far removed from the excitation wavelength. In fact, there is a cascading of the excitation and emission wavelengths of a series of dyes, so fluorescent light emission from one dye excites the second dye, etc.⁹¹ Thus, it is much easier to separate the two wavelengths for detection; sensitivity can be higher and interferences are minimized.

XII. SUMMARY AND FUTURE

There is a wide selection of existing ways to use particles in diagnostics from LAT's to DNA probe assays. New developments continue to promise an exciting future.

A. Microsphere Manipulation

Researchers have used magnets, optical traps, and "laser tweezers" to manipulate microspheres (plain, silica, and magnetic) and stretch molecules linked between them. Some have even measured and sequenced a DNA molecule with an atomic force microscope.^{92, 93, 94}

Since 1981 (or before), microsphere-based assays run in flow cytometers have been actively studied,^{95, 96, 97} and researchers have predicted that many immunoassays would be done on cells and single microspheres in flow cytometry instruments, as use of these instruments became more widespread.⁹⁸ Antibody-coated microspheres are mixed with sample and if antigen is present, the laser light is scattered (or the microsphere fluoresces) differently. The difference in light scattered (emitted) by microspheres with and without Ag can be used to quantify the antigen (Figure 16). Now Luminex is making the dream come true.

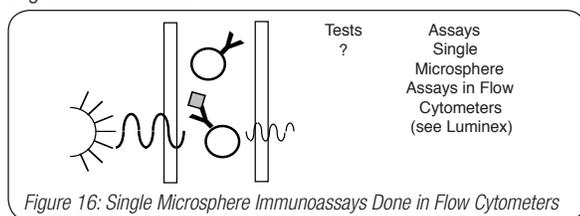


Figure 16: Single Microsphere Immunoassays Done in Flow Cytometers

B. Luminex FlowMetrix™ System

This new assay system can perform ≤ 48 discrete assays in a single tube with the same sample at the same time. Up to 48 different colors of microspheres carry the assay reactants. A flow cytometer (capable of

discriminating microspheres by size and fluorescent color) simultaneously performs real-time digital analysis of all the different assays on the surfaces of the colored microspheres.^{99, 100, 101}

C. Latex Test for Latex?

An example of these single microsphere assays is one for natural latex proteins (NLP), found in impure natural latex products. NLP's can cause severe allergic reactions in many spina bifida cases, certain other patients, and health workers who are often exposed to, and have become sensitized to, rubber products like shunts and gloves. An NLP Ab assay was constructed using microspheres with NLP coupled to the surface. In use, the microspheres were mixed with suspected samples containing NLP Ab's, then biotinylated IgE was added, followed by avidin-labelled fluorescein isothiocyanate (FITC). Microspheres were put through the flow cytometer and fluorescence measured after activation with 488nm light. Fluorescence is directly correlated with NLP ab levels.¹⁰²

XIII. NEW IDEAS

- Try agglutination tests using silica microspheres. More hydrophilic and with a higher density (~ 2 g/mL) than PS, they will yield different kinds of tests and assays.
- "Clear-to-cloudy" Test: If very small (< 50 nm) microspheres are diluted to $\sim 1\%$ solids, the suspension is transparent; after agglutination to clumps > 300 nm, the clumps are large enough to scatter light, and the suspension becomes turbid. This phenomenon could lead to a simple (OTC?) test: a change of appearance from clear to cloudy signalling a positive result.
- The field of biosensors has been "promising" for several years. An excellent review of immunosensors appeared recently in Clinical Chemistry.¹⁰³ These promises may soon be delivered and microspheres may be able to help in amplifying the signal from optical and electronic based sensors.

For example, try microspheres as amplifiers of the signal in the new evanescent-wave-based sensor technology (Figure 17). Ideally, on an antibody-coated biosensor, if antigen in a sample is bound by antibody, there will be a detectable signal change. However, if the signal is not strong enough, one can add a second antibody, perhaps with a microsphere attached. Then, surely, there will be a large change in the signal, with amplification brought about by the attached microspheres. For example, a press release from Fisons (UK) for their IAsys biosensor system with evanescent field technology built into the cuvettes claims it can analyze "a wide range of sample types and even cell or particle suspensions."

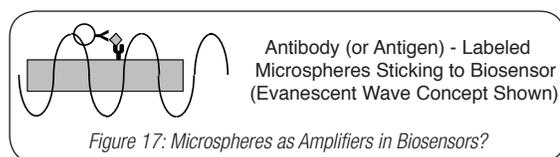


Figure 17: Microspheres as Amplifiers in Biosensors?

Piezoelectric / microsphere immunosensors are now possible. The signal from an Ab-coated piezoelectric crystal will change dramatically when Ag-coated particles are captured on its surface. Free Ag in a sample would probably not create as large a signal change on binding to the same surface, but free Ag would inhibit particle binding and thus moderate the signal change. A sandwich format should also work (Ab/crystal + Ag + Ab/particles gives large signal change; no Ag, no signal change). [Inspired by a recent paper.¹⁰⁴] Dr. Ben Felman of UCSF reports, "The idea has been tried before with limited success."^{105, 108} It is also possible to detect agglutination with no

immobilization of Ab or Ag to crystal.¹⁰⁷

Optical tweezer-based immunosensors using microspheres have been reported recently with femtomolar sensitivity.¹⁰⁸

- A Wall Street Journal article about Affymax work on DNA detection described chopping a patient's single stranded DNA into ~50 fragments and tagging it with fluorescent dye.¹⁰⁹ Why not use *fluorescent-dyed* microspheres to amplify the signal?
- Rapid Autonomous Self-Contained Miniature Assay (RAMSA, Organon Teknika): MicroELISA with unique fluidic circuits and special hydrophilic, reactive core-shell microspheres.^{110, 111}
- "Nanotechnology" (Oak Ridge Conference, 1994) dealt with micromachines, and similar very small applications of clinical chemistry and immunoassays,¹¹² such as the use of ~6µm microspheres to demonstrate and test effectiveness of a 5µm micromachined filter.¹¹³ Similarly,ACHEMA (Frankfurt) 1994 had a session on microtechnology (analysis in small volumes and instruments) and "nanotools" are being discussed in the trade press.¹¹⁴ Microspheres are the ball-bearings to keep those micromachines running smoothly!

This is just the beginning!

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