Simple DNA Probe Assays Based on Particle Agglutination, William Bains (Merlin Ventures, 101 Beechwood Ave., Melbourn, Royston, Herts SG8 6BW, UK; e-mail william@wbains.u-net.com)

I describe the use of latex agglutination as a simple readout for DNA hybridization assays. Latex agglutination is a well-known readout for antibody-based diagnostics (1, 2) and uses “sandwich” assay chemistry for which analogous DNA assays are well known (3, 4). Latex agglutination is extremely simple and chemically robust, and latex particles have been used as a label in DNA hybridization (5). Here I show that simple agglutination of particles is an efficient measure of DNA hybridization, one that is insensitive to the presence of proteases, detergents, and solvents.

Cloned m13 DNAs from the cytochrome f gene of Phormidium laminosum were kindly provided by Chris Howe (Department of Biochemistry, Cambridge, UK) (6). m13 DNA was prepared essentially as described by Maniatis et al. (7). Polystyrene microparticles (5.6-μm polyvinylstyrene particles, density 1.060 kg/L; 4.5-μm polystyrene/divinylbenzene particles, density 1.100 kg/L) were obtained from Bangs Laboratories. Nitrocellulose filters were obtained from Schleicher & Schuell. All other reagents were obtained from Sigma Chemicals.

DNA was cross-linked onto polystyrene, using 1-ethyl-3,3-(3-dimethylaminopropyl)-carbodiimide (EDAC), according to the methods of Nikiforov et al. (8) and Vary (9). Two milligrams of DNA was incubated overnight with 2 mg of EDAC and 25 mg of beads in a total volume of 330 μL of water at 18 °C. Another 10 μL of 0.1 g/mL EDAC was added, and the incubation was continued for 1 h. Coupled beads were washed three times in 0.1× standard saline citrate (SSC; 1× SSC = 8.765 g of NaCl, 4.1 g of disodium citrate per liter of water), stored at 4 °C. Particles with a reduced surface density of DNA were prepared by reacting 2 mg of DNA with 2 mg of EDAC and 25 mg of beads in a total volume of 330 μL of water at 18 °C for 1 h; then the particles were washed. DNA was immobilized onto polystyrene plate surfaces using exactly the same protocol.

All hybridizations were carried out in 5× SSC, 0.1% sodium dodecyl sulfate (SDS) at 60 °C in an orbital shaker. A unified agglutination index (AI) was calculated from photomicrographs as follows:

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AI = \frac{(10. S + 2. T + 5. M + 100. L)}{(100. S + 5. T + M + L)}
\]

These constants were selected to reflect the growth kinetics of particle aggregates (10).

The density of particle aggregates was measured by centrifugation through a sucrose density gradient (1–12% sucrose in 5× SSC, 0.01% SDS) in 5-ml polypropylene tubes at 20 °C in a Sorvall (DuPont) OTD65B centrifuge equipped with a Sorvall AH627 swing-out rotor. Centrifugation times and speeds were not critical, providing that \( \omega^2 t > 6.4 \times 10^4 \text{revolutions}^2/\text{s} \) (e.g., 8000 rpm for 10 min).

The results of counting agglutinated particles with a microscope are given in Fig. 1. This measure is simple and allows sensitive, quantitative measurement of the degree of agglutination achieved. Many of these experiments also showed “clumping” of the particles, which was visible to the unaided eye. Particles that were shaken with DNA that hybridizes to both particles showed clear clumping into large aggregates. Particles shaken with DNA that does not hybridize to both particles or shaken without DNA showed only slight agglutination. Agglutination was dependent on the amount of DNA loaded onto the surface of the particles, as well as the amount in solution. Fig. 1 also illustrates that performing hybridization at lower temperatures results in a slightly lower AI. The rate of agglutination of particles is limited by particle collision kinetics (10), which are not markedly affected by temperature (for these large particles); therefore, the decrease in agglutination with temperature is not as extreme as would be expected from considerations of DNA hybridization kinetics (11). Decreasing temperature also increases nonspecific binding slightly. Most nonspecific binding is caused by static charging of the particles, as illustrated by the nonzero agglutination at zero DNA concentration in Fig. 1. Increasing reaction time increases the cumulative particle collision probability but also mechanically disrupts large aggregates; therefore, it only increases assay sensitivity when the target DNA concentration is low. At high concentrations, the agglutination is essentially complete in 25 min.

I also measured agglutination by detecting the binding of DNA-coated particles to the base of a DNA-coated microscope slide in the presence of complementary target DNA, an assay similar to that described by Vener et al. (5), and by measuring the density of aggregates that result when DNA-coated particles of two different densities hybridize to a target. The sucrose gradient separated the two original particles (high and low densities) from clusters of particles (intermediate density). The extent of hybridization is measured by the fraction of beads that have moved from the top and bottom bands into the central region of the tube.

The above experiments are summarized in Table 1. Both methods show agglutination caused by target DNA at \( 1 \times 10^{-8} \text{ g/L} \) in a 30-μL reaction volume, corresponding to \(~1.5 \times 10^4 \) genomes of target. This result is sensitive to the presence of DNase but not protease (Table 1); it is resistant to the presence of SDS (which was included in all hybridizations to reduce nonspecific particle agglutination), nonionic detergents, and 5% ethanol (data not shown).

This report shows that particle agglutination can be used as a readout technology for DNA hybridization. This is a distinct application of DNA immobilization from other applications reported previously, such as the immobilization of DNA to beads for kinetic studies (12), for posthybridization labeling (5) or chromatographic separations.
rations (13, 14), or to 96-well microtiter plates (15) to aid PCR. In this study, as in latex-based immunoassays, the microparticles are both the solid support on which hybridization is carried out and the readout technology by which we determine that hybridization has occurred. The model reactions shown here provide clear indications of the presence of target DNA at concentrations of 100 pg (0.5 pmol) in times comparable with typical PCRs (16).

Test sensitivity is dependent on hybridization time, temperature, and probe density on the particles. Surprisingly, this simple readout technology can give greater sensitivity than a comparable sandwich hybridization using radiolabeled m13 probes (4).

Particle agglutination tests are simple to make and to operate. DNA probe-based tests using particle agglutination are thus attractive to smaller laboratories and less highly automated environments. Surveys of existing applications of DNA probe-based tests show that the sensitivity achieved in these model experiments is sufficient for

### Table 1. Summary of hybridization results.

<table>
<thead>
<tr>
<th>Format</th>
<th>Target DNA concentration, µg/L</th>
<th>Agglutination (CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90-min reaction</td>
<td>360-min reaction</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>93</td>
<td>8.4 (7.3–13.9)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4.2 (3.4–6.8)</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>1.3 (1.17–1.46)</td>
</tr>
<tr>
<td></td>
<td>0.0002</td>
<td>0.92 (0.73–1.11)</td>
</tr>
<tr>
<td>Density measurement c</td>
<td>9200</td>
<td>0.15 (0.14–0.16)</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.017 (0.0094–0.025)</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>0.019 (0.011–0.027)</td>
</tr>
<tr>
<td></td>
<td>0.009</td>
<td>0.045 (0.021–0.069)</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>0.019 (0.011–0.026)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.0096 (0–0.019)</td>
</tr>
<tr>
<td>With DNase f</td>
<td>9200</td>
<td>0.0085</td>
</tr>
<tr>
<td></td>
<td>9200</td>
<td>0.01527</td>
</tr>
<tr>
<td>With protease e</td>
<td>9200</td>
<td>0.1391</td>
</tr>
<tr>
<td></td>
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</table>

*Agglutination parameters with 95% confidence limits (CL) parentheses for hybridization reactions carried out for different times. Note that different experimental formats generated different agglutination parameters.

* DNA was chemically linked to the base of a 24-well microtiter plate as described, and the binding of DNA-coated beads to the plate was measured by direct counting. Figures are the ratio of beads bound to DNA-derivatized regions to beads in non-DNA-coated regions of the well base.

* Beads of two densities were hybridized in the presence of target DNA, and the fraction of beads forming clumps of intermediate density was counted.

* As above, but in the presence of 3000 U DNase I.

* As above, but in presence of 20 mg of proteinase K.

* Standard hybridization conditions.

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Fig. 1. Plot of AI vs DNA concentration.

(A) Several hybridization conditions are shown: Standard, as described in text; Control, with control (noncomplementary) DNA at 0.1 g/L; Low density, as for Standard but with lower density of probe DNA on beads; Low temperature, carried out at 45 °C. (B) and (C) Photomicrographs of agglutinated beads. (B) Beads agglutinated with 0.1 g/L of target DNA. (C) Beads shaken with 0.1 g/L nontarget DNA.
a substantial proportion of medical diagnostic applications of DNA probe-based tests (17).

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References

A new international reference material for 14 human serum proteins was certified by the Bureau Communautaire de Référence, Brussels, Belgium, in 1992 and released as CRM 470 (1). The same material was released in the United States by the College of American Pathologists (CAP) in 1993 as the Reference Preparation for Proteins in Human Serum, or RPPHS (2, 3). The large interlaboratory variance of values obtained on national quality control samples before 1990 was one of the major reasons for the preparation and introduction of this new material (3-5).

We undertook the present study to determine what effect, if any, the introduction of RPPHS has had on results of the CAP surveys for the major immunoglobulins IgG, IgA, and IgM. The overwhelming majority of clinical assays in the US are performed by immunonephelometry, utilizing the Beckman or Behring nephelometers. Therefore, we chose to compare only results from these two systems. Results were extracted from the Routine Immunology Survey Set Reports over the time period of 1992–96. The use of values referenced to RPPHS was first reported by Behring in February of 1994 and by Beckman in July of the same year. This transference corresponds closely with CAP survey reports that indicate a gradual conversion by Behring and Beckman users beginning with Survey Sets A and C of 1994, respectively. By 1995, the majority of laboratories were using the new values for all assays.

The CAP survey materials for the immunoglobulins include pathologic as well as nondiseased serum samples, with many samples containing myeloma proteins or M-components (MC), as detected on agarose gel electro-

Fig. 1. Average ratios (Beckman to Behring systems) for immunoglobulin results before and after introduction of the new RPPHS.
Results were extracted from the CAP Diagnostic Immunology Survey Reports for the period 1992–96. (MC, M-component).