trations to the reference analyses for this set of test specimens are shown in Fig. 1. The IR-based analytical methods yielded creatinine concentrations with a $S_{y|x}$ [the root mean square difference between IR-predicted and reference analyte concentrations for the test set only] of 0.58 mmol/L (r = 0.98) for creatinine, 14.1 mmol/L (r = 0.98) for urea, and 0.48 g/L (r = 0.94) for protein.

The distribution of protein concentrations is skewed heavily, with the majority of specimens showing concentrations well below 1 g/L (Fig. 1, middle panel). As a result, the best approach to IR-based protein quantification is to use two models rather than one. A second PLS quantification model was optimized for those samples with concentrations <1 g/L, yielding $S_{y|x}$ = 0.13 g/L. although this still falls short of the performance required for accurate quantification at typical low protein concentrations, the method is sufficiently accurate to serve as a coarse screening test.

The ultimate accuracy of the IR-based methods is influenced in part by the accuracy of the reference methods used to calibrate them. This is not a factor for the protein analysis, where the reference method is clearly more accurate than the IR-based method, but it may play a role for both urea and creatinine. This possibility is suggested by the precision of the IR-based assays: SD_{dup} = 0.18 mmol/L for creatinine, 6.8 mmol/L for urea, 0.14 g/L for protein (including all samples), and 0.05 g/L for protein concentrations <1 g/L.[3] At least part of the gap between the precision and accuracy of the urea ($S_{y|x}$ = 14.5; SD_{dup} = 6.8 mmol/L) and creatinine ($S_{y|x}$ = 0.54; SD_{dup} = 0.18 mmol/L) assays may be attributable to scatter in the reference methods themselves.

The mid-IR quantification methods presented here match or exceed the performance of the near-IR methods presented previously *(12)*. Both approaches yield analyses that are accurate enough to serve as a routine method for urine urea and creatinine analyses. Although protein concentrations are too low for accurate quantification using IR spectroscopy, the method may serve as a screen to detect concentrations above ~0.5 g/L and to quantify at those concentrations.

The practical implementation of this and other clinical IR-based assays requires two key developments. One of these is the discovery of an inexpensive substrate to substitute for the costly BaF_2 windows that were used as part of this work. Although these windows can be cleaned and used repeatedly, this is probably impractical in high-volume laboratories. A surprising alternative has emerged recently, as we have shown recently that many analyses can be carried out using ordinary glass as the substrate, despite its limited transparency in the mid-IR region *(13, 14)*. The stumbling block that remains in place is a practical one, that being automation of the method. The practical benefits of IR-based methods are being realized in an extraordinary range of analytical applications *(15)*, and it would seem to be only a matter of time before these methods find their way into the clinical realm.

[3] $SD_{dup} = (\Sigma d^2/2n)^{1/2}$, where d is the difference between concentrations determined for duplicate aliquots and n is the number of samples *(11)*.

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Miniaturization of the Luminescent Oxygen Channeling Immunoassay (LOCI™) for Use in Multiplex Array Formats and Other Biochips, *Alan Dafforn,* Hrair Kirakossian, and Kaiqin Lao†* (Advanced Diagnostics Group, Dade Behring Inc., PO Box 49013, San Jose, CA 95161-9013; * author for correspondence: fax 408-239-2707, e-mail alan_dafforn@dadebehring.com; † present address: PE Biosystems, 850 Lincoln Centre Dr., Foster City, CA 94404)

Many of the emerging technologies in clinical chemistry and research require the ability to perform hundreds or thousands of measurements on a single sample such as amplified DNA, typically by contacting the sample with an array of different probes or other reagents. If these array approaches are to be practical, the underlying technology must be simple, robust, inexpensive, and amenable to automation. The Luminescent Oxygen Channeling Immunoassay (LOCI™) is a recently developed homogeneous assay method that should be suitable for arrays because of its simplicity. However, to perform large numbers of measurements on reasonable sample sizes (e.g., 500 different measurements on aliquots of a

50-μL volume), it must be possible to detect LOCI signals from very small volumes. Miniaturization has also become a central theme in other areas of clinical chemistry *(1)*. Accordingly, we have constructed a LOCI microscope and used it to demonstrate sensitive detection of analytes in small volumes for three types of assays of potential interest in arrays: detection of a single-stranded DNA fragment, detection of a double-stranded DNA amplicon, and an immunoassay for a protein.

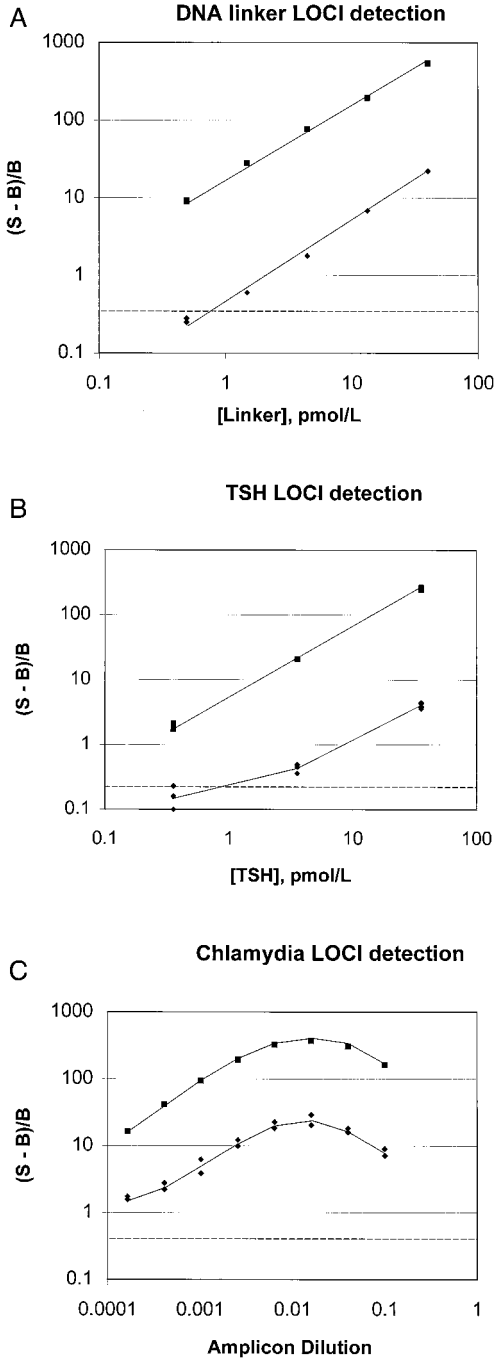

Fig. 1. Calibration curves for three assays determined in 10 nL (♦) or a large volume (■; 80 μL in *A* and *B*, 20 μL in *C*).

The ratio of specific signal (signal − background) to background in the absence of analyte [*(S − B)/B*] is plotted against the final concentration of analyte in the reaction (or dilution for *Chlamydia* amplicon). In each case, the *dashed line* represents the signal at 3 SD above background. (*A*), assay in which two beads are cross-linked by an oligonucleotide; (*B*), immunoassay for TSH; (*C*), assay for amplified *Chlamydia* DNA.

LOCI is a sensitive (femtomolar) detection method that uses chemiluminescence to quantify latex agglutination *(2)*. This technique utilizes one latex particle dyed with a photosensitizer and a second dyed with a chemiluminescent dye, both having binding ligands on their surfaces. Particle suspensions are mixed with the sample, and cross-linking by any analyte present leads to formation of bead pairs or higher aggregates. When the suspension is then illuminated, singlet oxygen is generated by the sensitizer particle, migrates to the chemiluminescent particle, and generates light. Nonspecific signals are low because singlet oxygen decays before it can reach unpaired particles.

A small-volume LOCI reader was constructed by modifying a fluorescence microscope to allow sample illumination with a 678 nm laser and monitoring of chemiluminescence with a photomultiplier tube. Illumination was accomplished either through the objective, using a beam splitter, or from below the stage, using a shutter to protect the photomultiplier; similar results were obtained in either mode. Samples were read by adding a 10-μL aliquot to a hemocytometer cell and imaging a single field. The volume imaged was defined by the depth of the cell (100 μm) and the diameter of the field (365 μm) as ~10 nL.

Three different assays were compared by performing incubations as described previously *(2)*, and then removing aliquots of the final mixture and reading either using the microscope or using our regular LOCI readers. These prototype readers illuminate and read 20- or 80-μL aliquots through standard optics. Reagents and procedures for two of the analytes, an oligonucleotide linker and thyroid-stimulating hormone (TSH), were essentially as described previously *(2)*. Briefly, in one assay an oligonucleotide linker included the sequences 5′-$(TACT)_5$ and T_{20} separated by a short spacer. This linker forms bead pairs between sensitizers with conjugated A_{24} and chemiluminescent particles with conjugated 5′-$(AGTA)_6$. In the other assay, TSH binds first to a chemiluminescent bead conjugated with one monoclonal anti-TSH antibody and to a second biotinylated monoclonal anti-TSH antibody. Streptavidin beads are then added to form bead pairs if TSH is present. Assays were read by three cycles (five for TSH) of illuminating for 1 s at 678 nm followed by 1 s of light collection.

Detection of double-stranded DNA was demonstrated using an amplicon from *Chlamydia trachomatis.* A 518-bp sequence from cryptic plasmid pLGV440 was amplified by conventional PCR using the primers 5′-GGA CAA ATC GTA TCT CGG GTT ATT-3′ and 5′-GGA AAC CAA CTC TAC GCT GTT-3′. The final concentration of the amplified DNA was estimated as ~40 nmol/L by comparison to an earlier LOCI calibration curve. Various dilutions of the amplicon were mixed with 2 μg of each

LOCI bead (same as for single-stranded DNA detection above). Solutions also contained each of the following probes at a concentration of 50 nmol/L in a total volume of 40 μL of PCR buffer (70 mmol/L KCl, 10 mmol/L Tris-HCl, 4 mmol/L $MgCl_2$, 0.2 g/L acetylated bovine serum albumin, pH 8.2):

5′-CTC ACA GTC AGA AAT TGG AGT ACT TAC TTA CTT ACT TAC T-X
5′-TTT TTT TTT TTT TTT TTA GAC TTT TTC TAT TCG CAG CGC-X

Target-specific binding regions are underlined. X represents $OCH_2CH((CH_2)_4NH_2)CH_2OH$, a group introduced to block the 3′ end against possible enzymatic extension. Solutions were covered with 20 μL of mineral oil in 200-μL MicroAmp™ tubes (PE BioSystems), and then heated to 95 °C for 2 min to denature the amplicon, cooled to 50 °C for 15 min to allow probes to bind, then cooled to 37 °C for 70 min to allow formation of the complex with beads. Aliquots were then read as above.

The results for all three assays are summarized in Fig. 1. The ratio of specific signal (signal − background) to background is presented as a function of analyte concentration in each case to allow comparisons over many orders of magnitude. In general, the assays lost only 14- to 64-fold in signal/background over a 2000- to 8000-fold decrease in volume. As expected, ~1000-fold decreases in signal were partially compensated by decreased background. (Raw background counts for large volume and 10 nL are as follows: DNA Linker, 6506 and 60; *Chlamydia* DNA, 4777 and 68; and TSH, 14 934 and 183.) As they should be, the curve shapes in Fig. 1 are in general parallel and log-linear except when very little signal is present. The decreasing signal seen at high concentrations of *Chlamydia* amplicon is frequently observed when LOCI is used to detect DNA amplification in a closed tube. It is believed to result from saturation of bead surfaces, but it can be avoided by changes in protocol *(3)*.

Detection limits for each assay were defined as the concentrations at which observed signals exceeded background by 3 SD and were estimated from linear plots of low concentration points. The change in the lower limit of detection as a function of volume was larger than that in signal/background because the statistical CV of the background increased at small volumes. The limits for large volumes and for 10 nL were as follows:

Chlamydia amplicon: 1:1 000 000 and 1:12 000 dilutions (~3 pmol/L)
DNA Linker: 6 fmol/L and 0.8 pmol/L
TSH: 7 fmol/L and 2 pmol/L

Because most array applications are likely to involve detection and quantification of undiluted amplicons or of expressed proteins, LOCI has ample limits of detection for most applications even in very small volumes.

The imprecision of the specific signal (signal − background) was determined in the TSH assay as 82 ± 10 counts (n = 5; CV = 12%) at 3.57 pmol/L TSH and 731 ± 68 counts (n = 5; CV = 9%) at 35.7 pmol/L. The imprecision was also estimated using a single bead dyed with both sensitizer and chemiluminescent dye according to the basic procedures described previously *(2)*. Ten replicate aliquots of a bead suspension gave 2450 ± 328 counts (CV = 13%). The most likely source of the observed variability is manual positioning of each well of the slide under the microscope objective in this prototype instrument.

Realization of practical LOCI arrays will also require a sample cassette. This could be as simple as a matrix of closed wells, each containing LOCI reagents for a specific measurement and interconnected by channels to distribute the sample. Fortunately, the dimensions required are easily obtainable by inexpensive molding techniques *(4)*. For example, a well with a 50-nL volume could be 125 μm deep and 714 μm in diameter. If 0.5 cm of channel on the average was required to connect each well and the channel had a cross-sectional area of 10^4 μm^2, then another 50 nL per well would be required to fill the channel. Thus, a 50-μL sample volume would be sufficient to fill 500 wells and the necessary connecting channels.

In summary, LOCI offers several advantages for signal detection from arrays and other miniaturized devices: The assay retains ample sensitivity for analytes of likely interest in such devices. An oligonucleotide could be detected at ~1 pmol/L (6000 molecules), the protein TSH could be detected at 2 pmol/L, and a DNA amplicon could be detected even at a 1:10 000 dilution. In addition, arrays large enough for clinical diagnostic purposes should be feasible (500 or more measurements/sample). Homogeneous assay arrays should also be much simpler to manufacture than many types of arrays because no surface chemistry must be performed on a chip. The absence of surface chemistry or absorption should also give greater reproducibility compared with spotting technologies and simplify quality control. The use of generic reagents also simplifies preparation of large arrays. Finally, homogeneous assays offer relatively fast kinetics and simplicity of protocol.

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