

sequences representing all known high-risk HPV E6 were tested, and PDZ 88 interacted with all of them with greatest binding strength. Therefore, PDZ 88 was selected as the capture reagent. The recognition of PDZ 88 to only high-risk HPV E6 (e.g., HPV types 16, 18, 33, 35, 52, 58, and 66) but not low-risk HPV E6 (e.g., HPV types 77 and 57) provides a high degree of selectivity for the assay.

Antibodies to HPV E6 from high-risk HPV types were generated. The antibodies were screened for affinity and cross-reactivity with other HPV E6. The antibody with the strongest reactivity to HPV-16 E6 was selected as the detector.

The magnetic immunochromatographic strip was selected for evaluation as the assay platform on the basis of ease of use and acceptability in low-resource settings, inherent sensitivity of the technology, and the ability to quantify results. MagnaBioSciences has developed a magnetic immunochromatographic test platform including an assay cassette and reader that is suitable for developing quantitative assays for a variety of analytes. The assay described here was developed on this platform. Additional studies for the method have shown that strip-to-strip reproducibility yields a CV of <10%, and reader reproducibility yields a CV of 1.5% (data not shown).

The PDZ capture reagent was immobilized on a nitrocellulose membrane. Assays were assembled with a laminate backing, membrane with immobilized PDZ, hydrophilic sample pad, absorbent pad, and membrane cover tape. Test strips were cut to a width of 7.5 mm. The soluble-phase detection antibody was covalently conjugated to superparamagnetic particles.

Doubling dilutions of recombinant HPV-16 E6 from 332 to 5.2 $\mu\text{g/L}$ were prepared in the proprietary assay running buffer containing Tris, surfactants, and blocking agents. The detection reagent was mixed with the sample, and 250 μL was applied to the strip. On migration of the sample, the strips were inserted into the magnetic strip reader. The strip reader generated data curves. Peak height was selected, and data were reported as relative magnetic units (RMUs).

The assay cutoff was conservatively set at 3 times the mean RMU of the negative controls. The RMU value for each sample was divided by the cutoff. Samples >1 were considered positive.

Lower amounts of the target protein led to lower RMU values (Fig. 1B). Strips titrated with recombinant HPV-16 E6 and read on the magnetic strip reader exhibited a linear response.

An initial prototype of the magnetic immunochromatographic strip assay is capable of detecting recombinant HPV-16 E6 down to 2.6 ng/assay ($\sim 2\,600\,000$ cells). An early version of the assay in an ELISA format detected oncogenic E6 from cervical cancer cell lines down to 30 pg/assay ($\sim 30\,000$ cells). Future steps include further optimization to decrease detection limits and evaluation of cell cultures and cervical cell swabs from clients. Once utility with HPV16 is demonstrated, additional high-risk HPV types will be added to the assay.

Funding for this work was provided by the Bill and Melinda Gates Foundation and by National Institutes of Health SBIR award number 1 R43 CA103383-01.

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DOI: 10.1373/clinchem.2006.072884

Single-Molecule Detection for Femtomolar Quantification of Proteins in Heterogeneous Immunoassays, Eric A. Nalefski, Christina M. D'Antoni, Evan P. Ferrell, Janice A. Lloyd, Haoqun Qiu, John L. Harris, and Duncan H. Whitney* (U.S. Genomics, Woburn, MA; *address correspondence to this author at: U.S. Genomics, 12 Gill St., Suite 4700, Woburn, MA 01801; fax 781-938-0060, e-mail dwhitney@usgenomics.com)

There is growing interest in the development of ultrasensitive immunoassays to facilitate validation of novel biomarkers, especially serum biomarkers, and the application of those assays in clinical settings (1). Current assays focus predominantly on abundantly produced protein biomarkers, such as cardiovascular structural proteins that leak into the bloodstream, and less so on lower abundance cytokines and chemokines (2). Even rarer markers, such as those released from cancerous cells into the bloodstream, remain more elusive (3). Approaches that exploit recent technological advances in molecular imaging, especially at the single-molecule level (4), are likely to improve the detection limits of the immunoassay, and therefore enable improved detection of these markers.

Microfluidic sample delivery and confocal microscopic detection of labeled biomolecules are useful for detecting targets of interest (5, 6). Two-color correlation counting

derived from focused laser-induced excitation allows for high signal-to-noise detection even in the presence of large excesses of free probes, enabling measurement of single molecular events (6). Flow velocity and data acquisition rates can be matched to provide highly accurate quantification, even for rapid analyses on the order of seconds per sample, and analysis time can easily be extended to provide increased detection efficiency of rare targets.

Development and application of new assays are straightforward processes based on well-understood fluorescence methods. We describe application of the Trilogy[®] 2020 Single Molecule Analyzer for analysis of heterogeneous immunoassays. The human gonadotropin follicle-stimulating hormone (FSH), which plays a central role in fertility (7), was chosen as a representative analyte because of its clinical importance and current clinical requirements for high-sensitivity detection (8).

The Trilogy 2020 instrument uses laser beams shaped into stripes for fluorescence illumination and focuses the beams through a microscope objective into the center of a sample-delivering microcapillary (see the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol52/issue11>). Fluorescence collected through the objective lens is separated into channels and focused onto a charge-coupled device (CCD) camera. Photon counts are integrated over 110 μ s in each of 10 superpixels spanning the width of each laser stripe and stored in data bins. We developed a dynamic thresholding approach to identify and count fluorescence events arising from "in focus" probes (see the online Data Supplement).

We developed a direct immunometric (sandwich) assay for FSH based on a matched monoclonal antibody (mAb) pair specific for FSH (Medix Biochemica). An α -chain-specific capture mAb was biotinylated by reaction with EZ-Link[™] NHS-PEO₄-Biotin (Pierce Biotechnology), leading to 2 biotins/antibody based on biotin quantification by the avidin-2-4'-hydroxyazobenzene benzoic acid method. We Cy5-labeled a β -chain-specific reporter mAb by reaction with a FluoroLink[™] Cy5 reactive dye (GE Healthcare), leading to 5 dyes/antibody as measured with dye quantification by ultraviolet/visible spectrophotometry. The source of FSH was a purified preparation of FSH (Scripps Laboratories, cat. no. F0613) calibrated by the manufacturer. Capture antibody was coupled to Dynabeads[®] MyOne[™] Streptavidin C1 magnetic beads (DynaL Biotech) at 20 μ g per mg of beads in PBS/2% bovine serum albumin and washed extensively before use. Immunoreactions, performed in 96-well plates, consisted of a 100- μ L sample, 2.5 μ g prepared capture beads (50 ng capture antibody), and 2 nmol/L (30 ng) reporter antibody. After orbital shaking for 1 h at room temperature, we collected beads with a plate magnet and washed them 3 times with cold PBS/0.01% NP40. Reporter antibodies were eluted by addition of 30 μ L of 2 mol/L glycine, 0.15 mol/L NaCl, and 0.01% NP40 for 5 min; longer times did not lead to release of more molecules. After the magnetic beads were removed, we neutralized

the eluent with 3 μ L of 2 mol/L Tris, pH 8.6, and 0.01% NP40, and then analyzed the eluent on the Trilogy instrument.

To characterize the platform sensitivity of the Trilogy 2020 instrument, we collected data on control dilutions of the Cy5-labeled reporter antibody. Representative digitized data traces from a buffer control or a solution containing the Cy5-labeled antibody collected in the Red channel over a 10 ms period are shown in Fig 1A. To illustrate the increase in detected fluorescent events from reporter antibody, signals recorded on the CCD camera in each of the 10 pixels were gray-scaled on the basis of increasing intensity (from white to black). Histogram analysis of bin intensities collected in individual pixels (Fig. 1B) illustrates the increase in mean intensity with increasing antibody concentrations over a wide concentration range. A dynamic thresholding approach to analyze a dilution series of the Cy5-labeled antibody (Fig. 1C) reveals that the Trilogy 2020 device exhibits a lower limit of detection (LLOD) of 15 fmol/L, defined as the smallest antibody concentration that generates a mean (-2 SD) signal that is greater than the mean ($+2$ SD) signal of a buffer control. At this concentration of antibody, ~ 100 fluorescent events, which corresponds to less than a zeptomole, above background levels were counted in 12 s. The signal response in detecting dye-labeled antibodies was linear up to at least 125 pmol/L, above which there was a slight deviation downward from the prediction line, revealing a linear response of ~ 4 orders of magnitude. The signal response, however, continued to increase with even 1 nmol/L antibodies, indicating a dynamic response of $>6 \times 10^4$.

After releasing the captured reporter antibodies into solution, we analyzed eluents from heterogeneous FSH immunoreactions on the Trilogy instrument. Short segments of representative trace files are provided in Fig. 1, D–E. Without added FSH, we detected only a few reporter antibodies over the dynamic threshold (Fig. 1D). In contrast, considerably more reporter antibodies were detected when FSH was included in the immunoreaction (Fig. 1E). A dose-response curve was generated in which FSH concentrations were increased over several logs, and we used the dynamic thresholding approach to determine the resulting antibody signal (Fig. 1F). Detailed analysis of the curve revealed that the LLOD for the immunoassay conducted under these conditions was 20 mIU/L FSH (~ 34 fmol/L), in this case defined as the smallest concentration of FSH that generates a mean signal that is 2 SDs above that of a buffer control. For this concentration of FSH, hundreds of fluorescent events, corresponding to zeptomoles of antibodies, were counted above background in 12 s. The signal response continued to increase to ≥ 200 IU/L FSH, yielding a total dynamic range >4000 . In contrast, commonly used enzymatic signal amplification assays are reported to be at least 1 order of magnitude less sensitive (8).

In conclusion, with the Trilogy platform, based on confocal microscopic detection in microcapillaries, photon bursts corresponding to fluorescently labeled antibodies

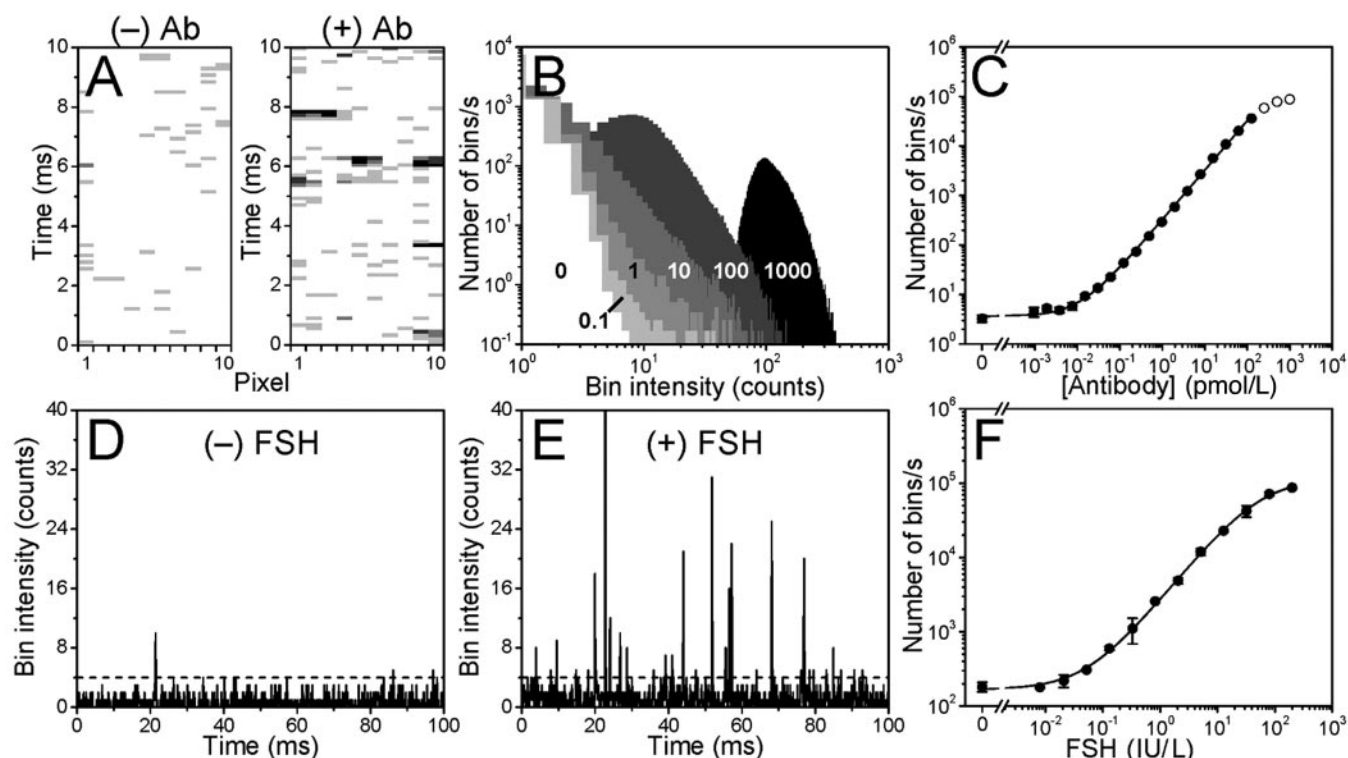


Fig. 1. Single molecule detection.

(A), digitized data recorded in 10 adjacent pixels, represented on the x-axis, of the Red channel over 10 ms, represented on the y-axis, from control buffer (left) or a 4 pmol/L solution of Cy5-labeled antibody (right). Bin intensities recorded on the CCD were scaled downward 20-fold and then gray-scaled equivalently in both panels from low intensity (white), to intermediate (grays), and to high intensity (black) for this representation. (B), histograms of bin intensities for data collected in single pixels of the Red channel over 12 s from increasing concentrations of Cy5-labeled antibody (0–1000 pmol/L), illustrating the increase in mean bin intensity. The number of bins was normalized to time. (C), dose dependence of the number of bins observed to exceed the dynamic fluorescence threshold, normalized to time, on the concentration of Cy5-labeled antibody. Symbols represent the mean of multiple replicates for no antibody ($n = 20$) or the indicated concentrations of antibody ($n = 3$). Standard deviations of replicates are indicated by bars, which are in most cases smaller than symbols. The solid line represents linear fitting of the data over 125 pmol/L; open symbols represent data deviating from linearity. LLOD is calculated here as 15 fmol/L. (D–E). Raw trace files collected in the Red channel over 100 ms in single pixels on eluents from immunoreactions containing no FSH (D) or 1 IU/L FSH (E). The dynamic threshold value of 4 counts used to detect antibody molecules is indicated by the dashed horizontal line. (F), FSH immunoassay dose–response curve, illustrating the dependence of observed numbers of bins that exceed dynamically applied thresholds, normalized to time, on the amount of FSH in the reaction. Symbols represent mean values for multiple replicates of no FSH ($n = 11$) or 12 different FSH dilutions ($n = 3$). Standard deviations of replicates are indicated by bars, which are smaller than symbols in most cases. The solid line represents fitting of data with a 4-parameter logistic equation. LLOD is calculated here at 20 mIU/L.

are readily distinguishable from background. Counting molecular events, in turn, provides a very sensitive and precise way to quantify biomolecules of interest. Analysis times of only a few seconds are required to detect as little as zeptomoles of protein analytes, leading to low femtomolar LODs.

In addition to several advantages offered by heterogeneous formats (9), analyte enrichment during the capture step provides a unique opportunity to capitalize on the exquisite sensitivity provided by single-molecule analysis. Because the interrogation zone is on the order of femtoliters, the inner diameter of the sample delivery microcapillary is on the order of tens of micrometers, suitable counting statistics are achieved in seconds, and only small volumes of captured and released reporter antibodies are required for analysis. For instance, at 10 $\mu\text{m}/\text{ms}$, the typical linear flow velocity, only a few microliters are consumed in 12 s, a small fraction of the tens of microliters of reporter antibodies eluted from immune reactions conducted on 100 μL of sample, as

reported here. Liquid handling procedures designed to deal with smaller volumes would further decrease the required sample size. This feature would be very useful in pediatric tests, for which only small sample volumes are dedicated per test. An added advantage of the single molecule approach is the requirement for only small quantities of probe reagents: for the FSH assay described here, only 30–50 ng of capture and reporter antibodies are consumed per sample per well, and even less would be needed with further miniaturization.

We used FSH as an example to demonstrate the principles and features of single-molecule, heterogeneous-immunoassays. The platform should be equally suitable for the analysis of other fluorescently labeled reporter antibodies and antigens. Although interference, antibody cross-reactivity, and matrix effects that potentially limit other immunoassays must be considered, they pose no particular challenge for the single-molecule approach. The present study used a capture approach involving biotinylated antibodies immobilized to streptavidin-con-

jugated beads, but the method is not limited to this approach; we have successfully quantified antigens with capture strategies involving antibodies covalently attached or nonspecifically adsorbed to beads and membranes (data not shown). Furthermore, with inclusion of additional sets of capture reagents and reporter antibodies matching the other fluorescent channels offered by the Trilogy instrument, the platform can simultaneously detect multiple analytes (data not shown). Finally, the platform can be used for precise and sensitive detection of fluorescently labeled probe molecules used in other assays, including those specific for particular nucleic acids (5).

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DOI: 10.1373/clinchem.2006.072850
