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# Supporting Information for
Direct Single-Molecule Counting for Immunoassay Applications

Qiaoqiao Ruan†, Patrick J. Macdonald†, Kerry M. Swift, and Sergey Y. Tetin*

†These authors contributed equally to this work

*Corresponding author. Sergey Y. Tetin.
E-mail: **sergey.tetin@abbott.com**

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Table S1

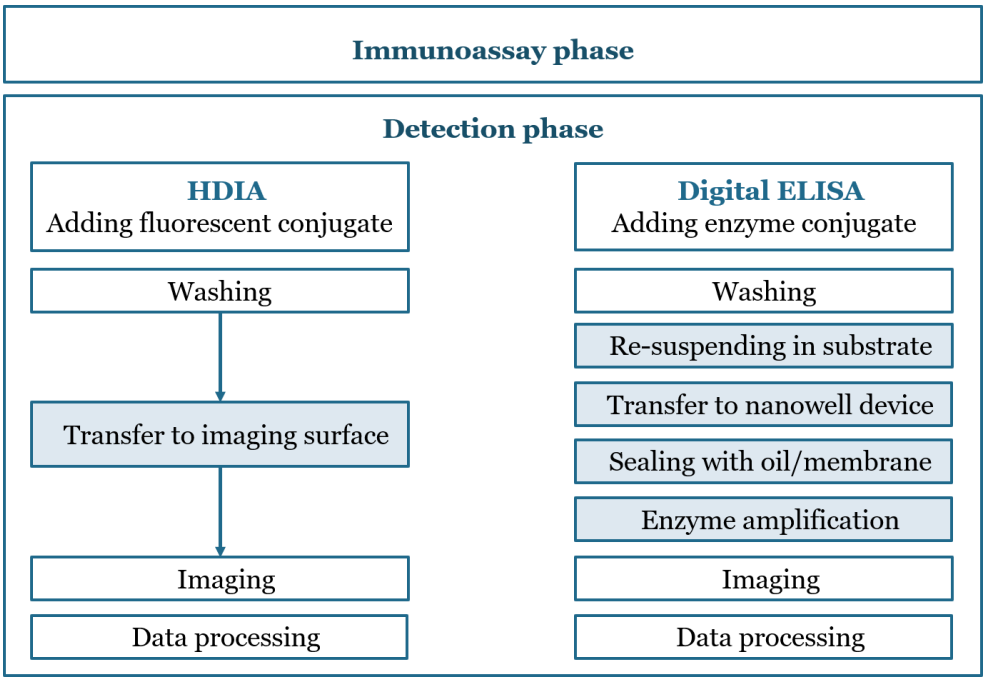


**Fig. S1.** HDIA and digital ELISA protocol comparison.

HDIA has a much simpler protocol with fewer steps and without the more complicated disposable required for digital ELISA. This simplicity makes HDIA both more practical and more robust for incorporation into high-throughput instrumentation.

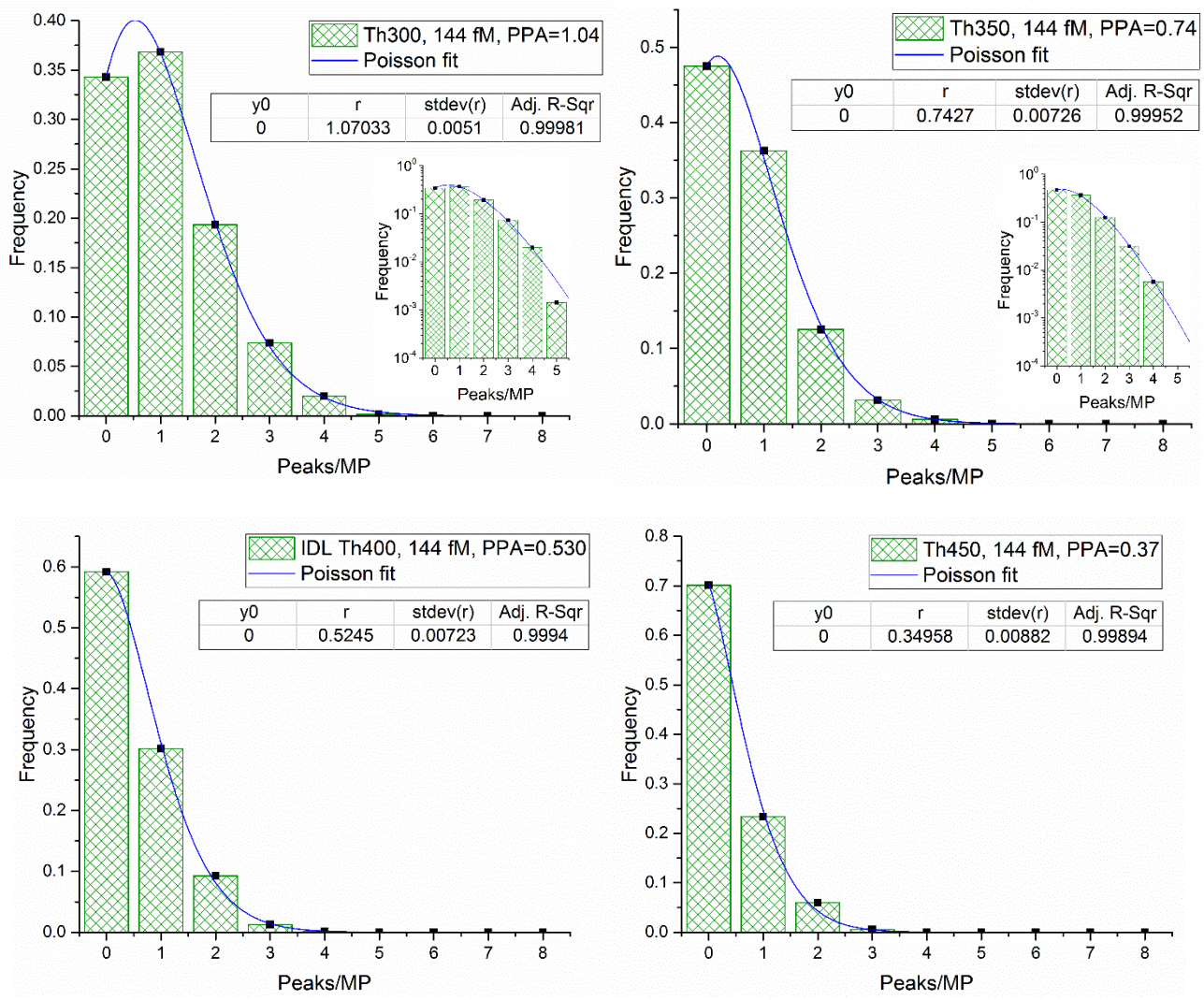


**Fig. S2.** Poisson distributions of same sample with varying analysis thresholds.

HDIA measurements of the same SAPE multimer sample (144 fM) captured on biotinylated microparticles were analyzed using different thresholds to determine positive intensity peaks. This provides an alternate way to probe the values of peaks/microparticle. Again, the Poisson model fits the data very well. Note, however, the semi-log inserts in the first two plots (lowest thresholds). While in both cases the data points at 4 peaks/MP align perfectly, at 5 peaks/MP the HDIA result either undershoots or is absent altogether. This indicates that, in the current setup, there is insufficient resolution to reliably detect more than 4 peaks/MP, and that for larger values—higher concentrations or lower thresholds—the analog analysis mode should be employed.

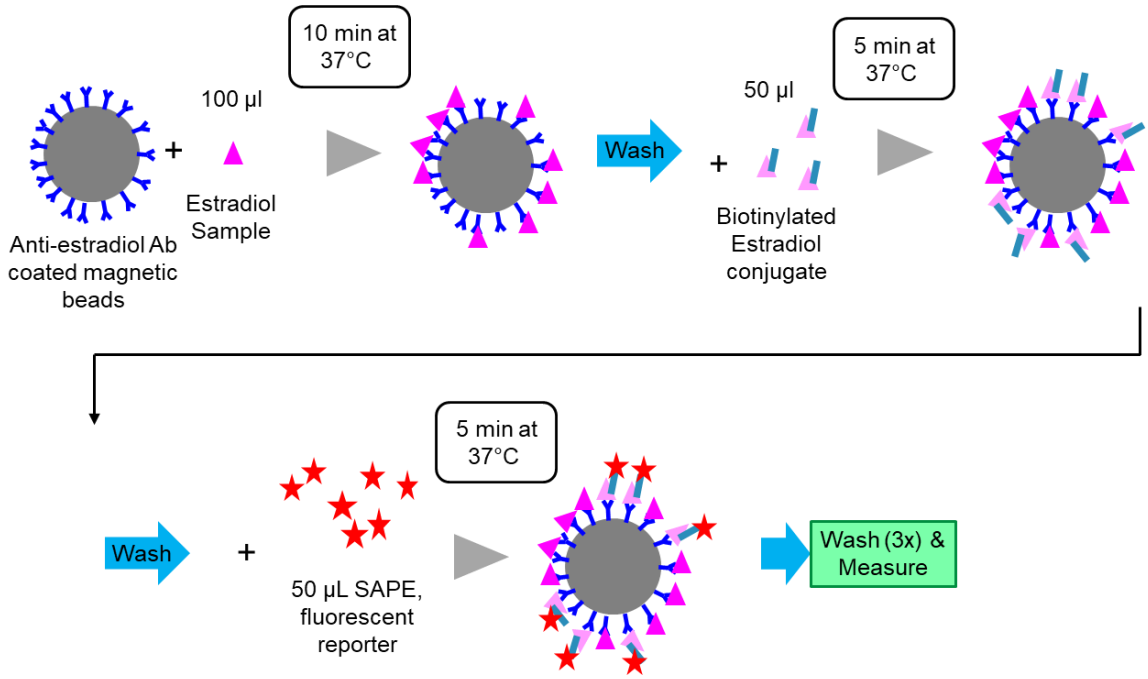


**Fig. S3.** Estradiol-HDIA assay, competitive format protocol.

The estradiol assay reaction was conducted on a KingFisher magnetic microparticle processor. First, 100 µL of the sample were mixed with 5 µL of anti-estradiol antibody coated magnetic microparticles, and then incubated at 37°C for 10 minutes. The microparticles were then washed in 100 µL of Architect wash buffer, before being transferred into 50 µL of estradiol-biotin (100 ng/mL) and incubated for an additional 5 minutes. After a second wash, the microparticles were incubated for a final 5 minutes in 50 µL of DBB, washed (3 x 100 µL), and measured.

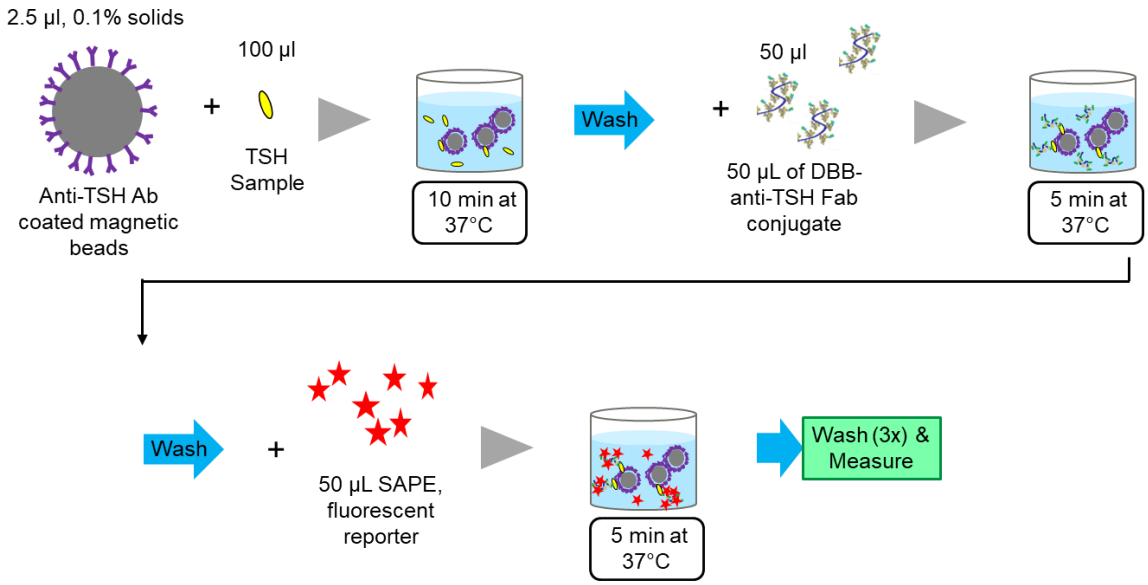


**Fig. S4.** TSH-HDIA assay protocol.

Magnetic microparticles were coated with anti-TSH antibody for antigen capture. DBB complexes, labeled with anti-TSH Fab were used as the primary conjugate, with 1:1 PE:SA labels as the secondary conjugate. 100 µL samples from two-fold serial dilutions of TSH calibrator (89 pM) diluted in Architect Calibrator 1 buffer matrix, and 2.5 µL of microparticles (0.1% solids) were pipetted into a 96-well plate, along with a buffer matrix only control. Following a 10-minute incubation, there was a 1-minute wash in Abbott blocking buffer, a second, 5-minute conjugate binding step with 50 µL of DBB in blocking buffer, a detergent wash step, a final 5-minute binding step with 50 µL of 12 nM SAPE conjugate, and 3 x 100 µL washes in detergent buffer. As before, the sample plate was processed on a KingFisher processor maintained at 37°C. The completed assay microparticles were transferred to an optical-bottom 96-well plate and imaged.

**Table S1.** Buffer matrix vs TSH deplete serum.

| **TSH diluents, nominal TSH concentration [fM]** | **HDIA [CPP]** (n≥18) |
|---|---|
| Cal A buffer matrix - 0 fM | 0.5 |
| In-house TSH microparticle, further depleted serum - 0 fM | 0.6 |
| Commercial TSH depleted serum - 0 fM | 2.9 |

The TSH titration experiments presented in the main text were not done in patient serum, because normal patient serum typically has TSH levels between 0.4 and 4.0 milliunits per liter. TSH levels that fall above or below this range are clinically relevant. Performing experiments on known TSH concentrations requires a diluent that is free of TSH. Normal patient serum can be stripped of TSH by repeated mixing with anti-TSH microparticles or surfaces, but it is very wasteful of reagents. In the table above, we show the signal levels detected by HDIA for a commercially available depleted serum, our own, in-house, re-depletion of the commercial depleted serum, and Abbott's Calibrator A buffer matrix. The results demonstrate that very carefully depleted normal patient serum is essentially equivalent to the buffer matrix as regards the HDIA background signal. Therefore, HDIA TSH experiments performed using the buffer matrix can reasonably approximate serum study results.

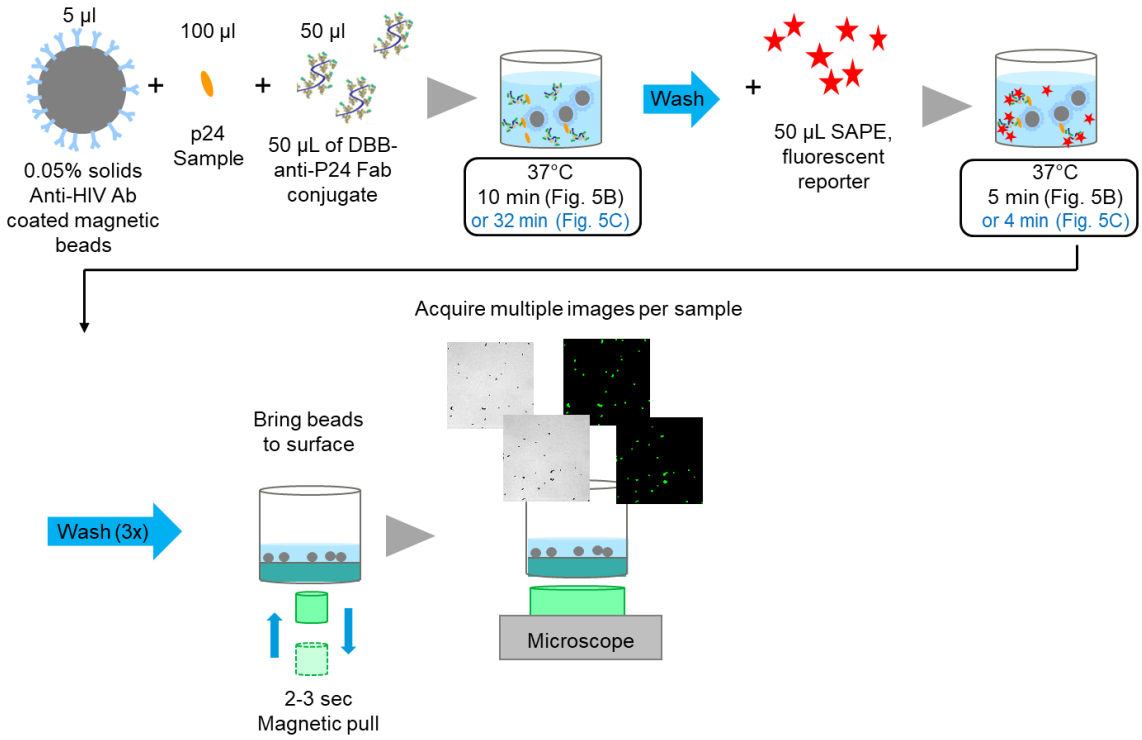


**Fig. S5.** HIV-HDIA assay protocols (10-5 min & 32-4 min).

Magnetic microparticles were coated with anti-p24 antibody for antigen capture. DBB complexes, labeled with anti-p24 Fab were used as the primary conjugate, with 1:1 SAPE binding to the DBB as the secondary conjugate. A patient sample with high HIV p24 levels was serially diluted into pooled viral-negative human plasma, generating a panel of known p24 concentrations ranging from 83 pM to 200 fM, along with a plasma-only background control. Using a protocol similar to the TSH assay above, 100 µL of p24 sample, 5 µL of microparticles (0.05% solids), and 50 µL of DBB diluted in a blocking buffer were incubated at 37°C for 10 (or 32) minutes, washed, incubated 5 (or 4) minutes with SAPE, washed 3x more, and imaged.

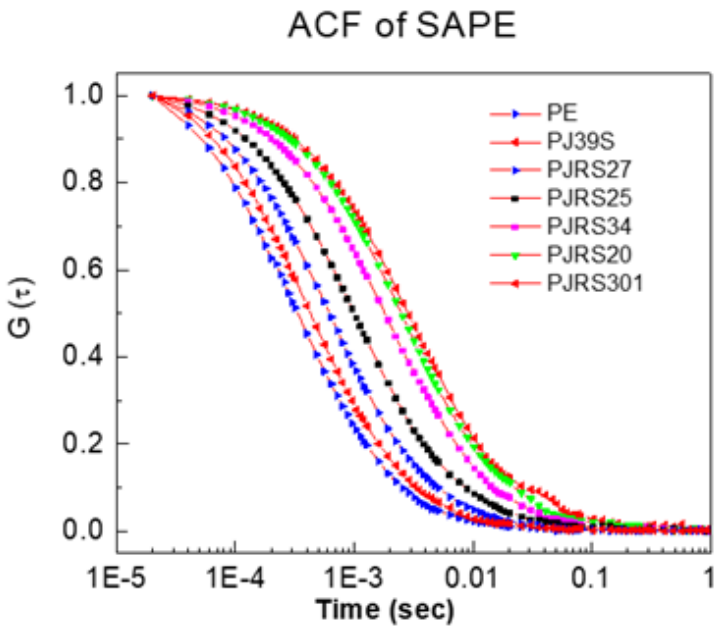


**Fig. S6.** FCS autocorrelation curves – streptavidin-phycoerythrin multimers.

The characterization of a series of streptavidin (SA)-linked phycoerythrin (PE) dye multimers, manufactured to have different numbers of PE per SA. FCS measurements were performed to determine the average number of PE dyes/SA for each product. Shown above are the autocorrelation curve data for each of the tested multimer variants. Curves which are shifted further to the right, indicate longer diffusion times and thus larger multimers.

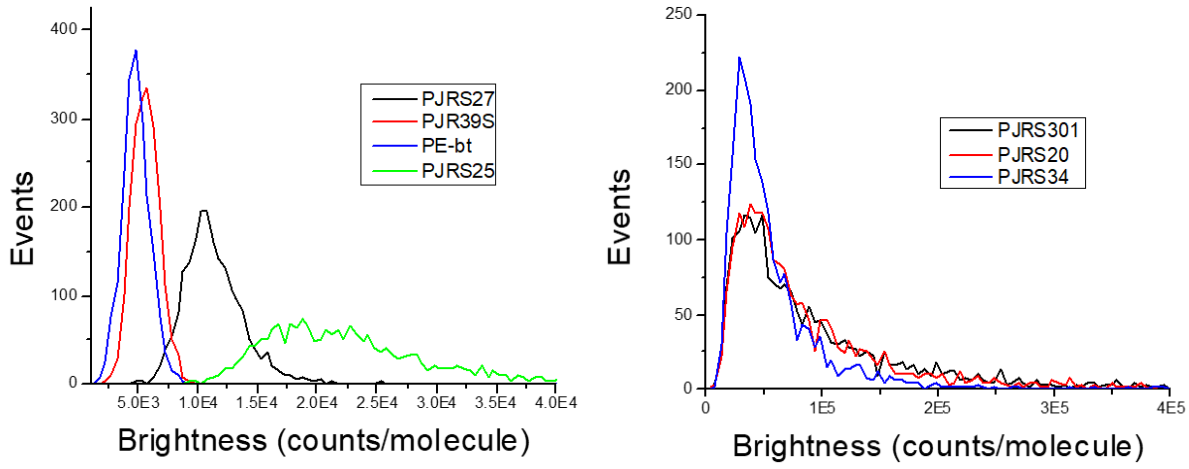


**Fig. S7.** Histograms of SAPE multimers by G1 analysis.

A series of differently sized SAPE multimer variants were analyzed using FCS G1 analysis. Autocorrelation analysis uses the variations in FCS signal amplitude over time to calculate the brightness (or number or diffusion rate) of the objects diffusing in and out of the excitation volume. When there is a distribution of object sizes, the result returns an apparent brightness which is a weighted average of the subpopulations. However, by breaking the data set into short segments and performing the analysis in a piece-wise fashion, more information about the subpopulations can be identified. $\boldsymbol{G(1)}$ is defined as the first actual correlation point, where $\boldsymbol{G(\tau)}$ is the autocorrelation function and $\boldsymbol{\tau}$ is the time shift index. $\boldsymbol{G(1)}$ is used instead of $\boldsymbol{G(0)}$, which is extrapolated from the fit equation, because $\boldsymbol{G(1)}$ is less sensitive to error when using the limited number of data points in the shorter segments.

Plotting the brightness values as a histogram, obtained from each segment by G1 analysis provides more information about the distribution of brightness species in the sample solution. The left plot shows the brightness distributions of the smaller SAPE multimer samples compared to a control (biotinylated-phycoerythrin). The right plot does the same for the larger SAPE multimer samples.