Clinical Chemistry 53:11
1990–1995 (2007)

Oak Ridge Conference

Ultrasensitive Flow-based Immunoassays Using Single-Molecule Counting

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Background: **Immunoassay (IA) technology has expanded the clinical utility of protein biomarkers, but demands for increased sensitivity, dynamic reporting ranges, and small sample volumes have limited the potential clinical usefulness of many biomarkers. We assessed the performance, including limits of detection (LODs) and the dynamic reporting range, of an IA-based technology, Erenna Immunoassay System, for a series of biomarkers, including cardiac troponin I (cTnI).**

Methods: **Erenna IAs were used with 10 different and clinically important biomarkers to ascertain the LOD with various sample sizes (10 μL to 200 μL).**

Results: **The Erenna Immunoassay System generated LODs of 10–100 pg/L using 100 μL of sample. For cTnI, the LOD was 0.2 ng/L and a 10% CV was seen between 0.78 and 1.6 ng/L.**

Conclusions: **The Erenna IA-based technology reproducibly measures protein biomarkers with detection limits of 10–100 pg/L, with a dynamic range of >4.5 logs in sample volumes of 50–150 μL.**

The increasing need for new therapeutics that target different mechanisms of action and methods for earlier diagnosis requires methods to increase understanding of disease processes and progression. Fundamental to gaining this understanding is the ability to distinguish between healthy and disease states. Biomarker measurement is important in the differentiation between these 2 states and the documentation of disease progression *(1)*. In some cases, protein biomarkers may provide clinically useful information that is dynamic and reflective of physiology. The clinical use of protein biomarkers, however, often necessitates the measurement of small changes at low concentrations in small volumes, which, in turn, demands technology that can provide sensitive, accurate, reproducible, and rapid measurements. Unfortunately, in the case of proteins these criteria can rarely be simultaneously achieved *(2, 3)*.

Since the introduction of immunoassay (IA)[1] technology and its application to the measurements of proteins in biological samples, significant improvements have been made in performance, including standardization, precision, and sensitivity *(2, 4–8)*. Today, the most robust and widely available IA platforms accurately measure proteins with a sensitivity of 1–10 ng/L, with CVs <20%. In certain instances, such sensitivity is adequate for research and diagnostic applications; in some cases, however, there is an evolving need for increasing sensitivity or the achievable sensitivity is insufficient *(2, 3)*. With available IA platforms, for example, cardiac troponins, proteins released after heart damage, are undetectable during early-stage and low-level cardiac injury when therapeutic intervention could be most effective *(9–11)*. Other examples include the inability to detect plasma cytokines in the absence of inflammation, although a certain normal level of cytokines is known to exist in the circulating plasma proteome of a healthy individual *(2)*, and p24, a viral protein present in HIV-infected individuals, which is present well before HIV antibodies are produced and can be detected. Unfortunately, current p24 antigen assays cannot detect p24 antigen in the early stages of HIV infection *(12)*. Detection at low and normal levels of protein biomarkers that cannot be measured with current technologies will be of the utmost importance in understanding the potential of such proteins for diagnosing disease, monitoring disease progression, and managing therapeutic intervention.

In addition to providing results with clinically relevant sensitivity, a clinically useful IA system must reliably and

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Received May 3, 2007; accepted August 29, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.091181

[1] Nonstandard abbreviations: IA, immunoassay; cTnI, cardiac troponin I; MP, microparticle; DE, detected event; PE, photon event; TP, total photon; IL, interleukin; LOD, limit of detection; NSB, nonspecific binding.

reproducibly detect the analyte in a potentially wide range from healthy to disease state. In this study, we report an IA-based technology that is designed to meet these requirements.

Materials and Methods

MATERIALS

Antibodies and analytes (recombinant) were obtained from R&D Systems. Manufacturer recommendations were followed for matched antibody pairs. Fluorescent dyes and biotin succimidyl ester, used to label antibodies, were obtained from Invitrogen. Rat, dog, and monkey human cardiac troponin I (cTnI) were purified from natural sources and obtained from Hytest. Human lithium citrate plasma specimens were purchased from Interstate blood bank. All blood and plasma samples were collected under institutional review board-approved collection protocols, and written informed consent was obtained from each participant. Streptavidin-coated paramagnetic microparticles (MPs) were obtained from Invitrogen (MyOne, no. 650-01). Antibodies were labeled with fluorescent dye (detection antibody, usually polyclonal) and biotin (capture antibody, usually monoclonal) according to the manufacturer's recommendations. MPs were coated with biotinylated antibody under saturation conditions (following the manufacturer's recommendations) and then washed and stored in assay buffer. Assay buffer consisted of 1% BSA, Tris-buffered saline, pH 7.4, with 0.5 mL Triton X-100/L, and heterophile/human antimouse antibody–blocking reagents (purchased from Scantibodies Laboratories) and was used per the manufacturer's recommendations.

ERENNA IAs

Unless stated otherwise, the typical Erenna IA was performed as follows. Samples or calibrators (in volumes of 50–100 μL) were diluted with assay buffer containing capture antibody-coated MPs (e.g., in 150 μL) and incubated in a 96-well plate for 1–2 h at 25 °C with shaking. All plasma or serum samples were tested undiluted without pretreatment. MPs were separated using a magnetic bed (Ambion). Supernatant was removed, MPs were washed once, and then 20 μL detection antibody (50–500 mg/L diluted in assay buffer) was added and incubated for 60 min at 25 °C with shaking. The MPs were again magnetically separated and washed 6 times using Tris-buffered saline with 0.5 mL Triton X-100/L. After removal of residual wash buffer, 20 μL elution buffer (4 mol/L urea) was added. This reagent disrupted antibody–analyte interactions and resulted in the release of detection antibody from the MPs. The solution in each 96-well plate was then transferred to a 384-well filter plate (0.2 μm, AcroPrep cat. no. 5070, Pall) and centrifuged at 1200*g* for 3 min to separate detection antibody in elution buffer from MPs. The eluted and filtered material in the 384-well plate was then placed into the Erenna Immunoassay System.

ERENNA IMMUNOASSAY SYSTEM

The Erenna Immunoassay System is based on single-molecule counting technology. Liquid is sipped from each well in the 384-well plate and pumped through a 100-μm diameter capillary flow cell. The liquid passes through an interrogation space within the capillary. As depicted in Fig. 1, light generated from a laser is directed via a dichroic mirror and a confocal microscope lens into the interrogation space. As dye-labeled antibodies pass through this space, they emit fluorescent light, which is measured via the confocal microscope lens and a photon detector. The output from the detector is a train of pulses, with each pulse representing 1 photon that was detected. These pulses are sent to counting electronics, where the pulses are counted in 1-ms bins. The >4.5-log dynamic reporting range is obtained by using a combination of output signals. First the background signal is determined, and based on this value a 5-SD threshold above background is created. Only flashes of light that are greater than this threshold are counted. These individual peaks (not signal intensity) are summed over either a 1-min interval or until 1000 peaks are obtained. The final signal is a sum of all such measured events and is termed detected events (DEs). The 2nd output is termed event photons and is the sum all the photons counted in all the DEs. This measure is used at higher concentrations, for which there is a significant probability that 2 molecules will pass through the detector in the same 1-ms counting bin. At the highest concentrations of analyte, photon events (PEs) begin to saturate and total photons (TPs), the sum of all PEs, is used. DE, PE, and TP signals are used to generate a weighted 4-parameter logistic curve fit for each signal type. To estimate the concentration of an unknown, the DE, PE, and TP signals are interpolated off each of the calibration curves to obtain 3 separate estimates of concentration. These 3 concentrations are combined by use of a weighted mean based on the slopes of the calibration curves.

Results

With the intent of providing an overall perspective of the performance of the Erenna Immunoassay System, this

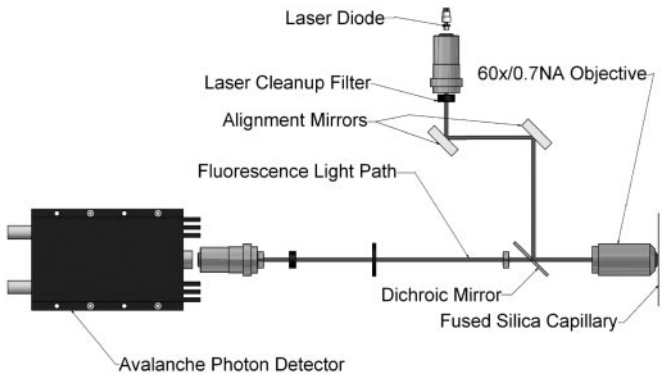

Fig. 1. Schematic representation of the Erenna Immunoassay System.

section presents representative data from a series of experiments using different analytes.

REPORTING RANGE, LINEARITY, ACCURACY, AND REPRODUCIBILITY

An example of typical signal data generated with a human interleukin (IL)-17 assay is presented in Table 1. The DE, PE, and TP signals that are used to construct the calibration curve are presented in a bold font. To determine goodness of the curve fit, the signal values in Table 1 were back-interpolated using the curve-fit algorithm, and the results are presented as measured vs expected concentrations (ng/L). The mean value of the (measured concentration)/(expected concentration) was 99% (range 84%–123%), and a linear response (R^2 = 0.99) was observed from 60 pg/L to 1 μg/L, representing a 4.3-log reporting range. The mean recoveries of analytes (IL-17, IL-6, and human cTnI) added into panels of human plasma at concentrations of 5 and 50 ng/L were between 90% and 110% at both concentrations (data not shown). The results of back-interpolating the calibration curves over 8 consecutive runs (during a 6-day period using 1 lot of reagents and freshly prepared calibrators each day) are presented in Fig. 2. In these experiments, the highest concentration calibrator used was 100 ng/L. A linear (R^2 = 0.99) response was observed at both the high and low ends of the calibration curve shown. The CV for the 8 assay runs for back-interpolated determinations of calibrators was <10% for all values >0.78 ng/L. The CVs were 16% and 23% for the 0.39 and 0.2 ng/L values, respectively (Table 2). Interassay and intraassay precision studies, using plasma or sera with known amounts of added analyte, were performed. As an example of results obtained with these experiments, the human IL-17 assay yielded intraassay (replicates of 4) CVs ranging from 3% to 8% (IL-17 >0.4 ng/L) and 10% (0.2 ng/L), as well as interassay (6 assay runs) CVs ranging from 3% to 9% (IL-17 >0.4 ng/L) and 11% (0.2 ng/L).

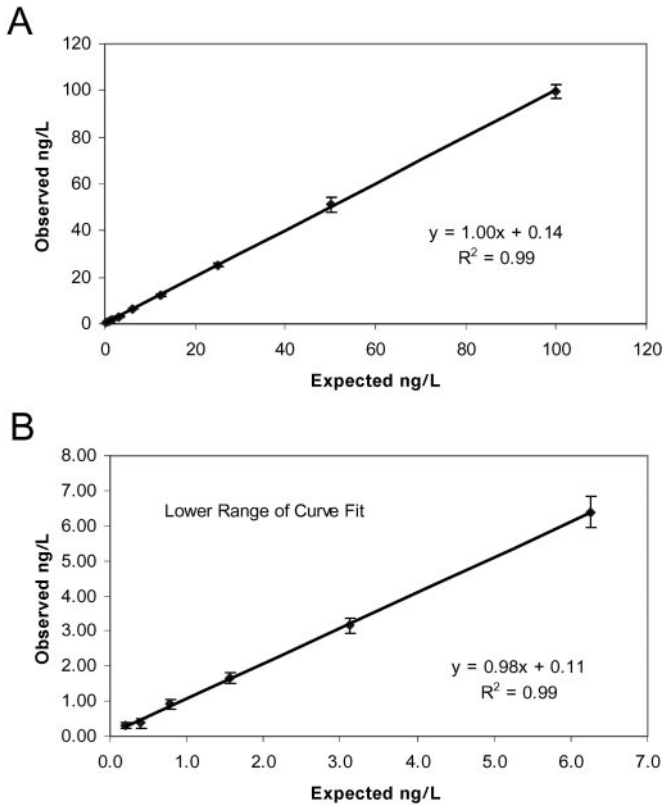

Fig. 2. Goodness of curve fit; back interpolation of cTnI calibration curves generated over 8 consecutive assay runs [full range (*A*) and low end range (*B*) of quantification].

Table 1. Signal, back-interpolated values, and recovery from a human IL-17 Erenna Assay Immunoassay System calibration curve.

	Detected events/min			Photon events/min			Total photons/min			Measured IL-17 ng/L			
IL-17, ng/L	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Found/ expected, %
1,000	8,920	2,268	25	13 879 788	157 439	1	**159 161 873**[a]	2 366 288	1	843	23.00	3	84
500	10 538	78	1	10 910 741	285 636	3	**88 854 604**	233 442	0	468	1.23	0	94
250	10 185	171	2	7 515 849	104 214	1	**46 946 733**	3 717 269	8	238	20.03	8	95
125	10 250	217	2	5 237 108	363 085	7	**25 753 075**	1 564 066	6	119	9.74	8	96
63	10 005	45	0	**3 485 980**	178 040	5	**14 781 797**	870 996	6	59	4.79	8	94
31	9,639	182	2	**2 406 459**	91 291	4	9 325 716	326 061	3	32	1.59	5	102
16	8,507	103	1	**1 564 471**	103 417	7	6 690 690	176 886	3	18	1.39	8	116
7.8	6,428	211	3	**915 104**	47 403	5	5 043 196	52 626	1	9.6	0.57	6	123
3.9	**3,999**	54	1	**459 594**	16 948	4	4 077 945	30 411	1	4.2	0.14	3	108
1.95	**2,111**	192	9	**212 996**	20 496	10	3 634 021	33 976	1	1.73	0.19	11	88
0.98	**1,189**	33	3	**113 214**	457	0	3 414 771	10 274	0	0.88	0.02	3	90
0.24	**342**	14	4	**31 199**	628	2	3 256 437	6,346	0	0.22	0.01	5	92
0.12	**211**	8	4	17 901	870	5	3 236 763	24 896	1	0.13	0.01	5	103
0.06	**125**	15	12	10 823	1,176	11	3 203 629	3,476	0	0.06	0.01	19	99
0	**64**	12	19	6,013	2,235	37	3 211 483	8,892	0				
Mean													**99**

[a] *Bold type* identifies signal data that were most heavily weighted in establishing the calibration curve.

Table 2. Reproducibility of the human cTnI assay calibration system over 8 assay runs (6 days).

cTnI, ng/L	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Mean, ng/L	SD	CV, %
100	101	101	104	96	97	99	99	98	100	3	3
50	46	55	50	53	51	51	51	50	51	3	6
25	26	24	24	26	26	27	24	25	25	1	4
12.5	11.4	12.3	12.5	12.7	12.7	12.2	12.6	12.6	12.4	0.5	4
6.3	6.6	6.1	6.2	6.2	6.5	6.5	6.6	6.3	6.4	0.2	3
3.1	3.0	3.1	3.2	2.9	3.4	3.2	3.2	3.1	3.2	0.2	5
1.6	1.7	1.7	1.6	1.6	1.8	1.8	1.4	1.5	1.7	0.1	8
0.78	0.93	0.91	1.16	0.94	0.66	0.87	0.97	0.74	0.92	0.15	16
0.39	0.38	0.49	0.30	0.40	0.25	0.44	0.32	0.49	0.37	0.08	23
0.2	0.12	0.50	0.19	0.29	0.34	0.44	0.34	0.52	0.32	0.13	42
0.1	0.00	0.03	1.02	0.26	0.11	0.05	0.05	0.23	0.22	0.36	168

Analytical limit of detection (LOD; defined as 2 SDs of the signal from the zero analyte background divided by the slope of the linear portion of DE signal) for the Erenna Immunoassay System varied from analyte to analyte and was dependent upon the volume of sample (or calibrator) used. Table 3 depicts the LOD for 10 different Erenna human IAs using 2 different sample volumes for each assay. Larger sample volumes consistently resulted in lower LODs. For example, sample volumes >50 μL resulted in LODs ranging from 0.01 ng/L (human IL-6) to 0.12 ng/L (human vascular endothelial growth factor). As sample volumes decreased, LODs increased in a proportional manner (mean proportional relationship 105%). The volumetric ratios of assay buffer to sample (plasma) must be varied, in an assay-specific manner, to achieve optimal sensitivity, recovery of added analyte, and dilutional linearity. The assay volumes presented in Table 3 represent such optimization. All of the assays were performed in a 2-step manner, with 1–2 h of capture and 1 h of detection.

To characterize the effect of assay incubation times on detection limit, incubation steps were combined into a single-step cTnI assay performed with 50 μL of sample (plus 150 μL assay buffer) and simultaneous capture and detection (10 μg/well MPs and 100 μg/L detection antibody) reactions. As incubation times increased, assay sensitivity improved, whereas assay background (DE signal of the zero analyte calibrator) remained constant over time. Of note, when the NIST reference material was used an LOD of 0.61 ng/L was achieved even with 15-min incubation (data not shown).

The sensitivity of the human cTnI assay was exploited to define the range of cTnI in human plasma obtained from 100 human blood donors (50-μL sample size). The results are presented in Fig. 3. The values ranged from <0.2 ng/L to 39 ng/L with a mean (SD) of 2.19 (4.1) ng/L. Three plasma had values <0.2 ng/L, which was the LOD. The value for the 99th percentile was 9 ng/L. With removal of the 1 apparent outlier of 39 ng/L from the data set, the mean (SD) for the population was 1.83 (1.9) ng/L with the 99th percentile at 8 ng/L.

Discussion

Previously, we reported use of the Erenna Immunoassay System (previously referred to as the Zeptyx System) to measure cTnI in serum from healthy individuals and IAs configured onto microplates *(9)*. In this report we show that the combination of ultrasensitive flow detection with MP-based IA technology provides protein IAs with analytical LODs in the picogram per liter range, which is approximately 10-fold lower than we previously demonstrated. We have applied this technology to create IAs for clinically useful protein biomarkers, such as cTnI and IL-6. This new development in IA technology provides

Table 3. The effect of sample volume on Erenna System immunoassay sensitivity (LOD).

Analyte	Volume, μL	LOD, ng/L	Volume, μL	LOD, ng/L	Proportionality, %
MCP-1[a]	200	0.03	20	0.25	120
RANTES	100	0.05	20	0.3	120
VEGF	100	0.12	20	0.55	109
IL-8	50	0.12	20	0.3	100
IL-1a	200	0.01	20	0.1	100
IL-7	200	0.02	10	0.3	133
Il-6	100	0.01	10	0.13	78
TNF-α	200	0.02	10	0.4	100
Il-1B	150	0.02	10	0.3	100
cTnI	100	0.11	10	1.2	92

[a] MCP-1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.

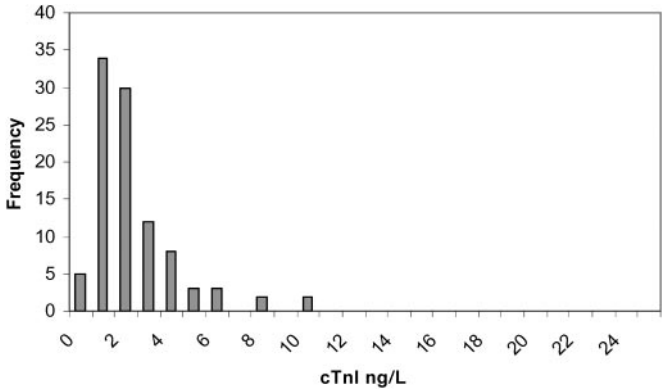

Fig. 3. Frequency distribution of cTnI in lithium heparin plasma specimens obtained from 100 blood donors.

sensitive and reproducible quantitative IAs with a broad dynamic range and analytical recoveries of 90%–110%.

The Erenna Immunoassay System provides enhanced sensitivity via 2 mechanisms. First, unlike analog systems that count total light (e.g., a colorimetric 96-well plate reader), the Erenna Immunoassay System separates background fluorescent signal from antibody-tagged signal by thresholding out the background signal and measuring each fluorescently labeled detection antibody as a digital event *(9)*. Second, the MP solid phase has been designed to minimize nonspecific binding (NSB) of fluorescently labeled detection antibodies. Furthermore, incubations are performed in 96-well plates with polypropylene wells that have very low protein binding capacity and thus low NSB. With the Erenna Immunoassay System, when a fluorescently tagged detection antibody is released from the MPs, the NSB in reactions that contain no analyte is very low. These characteristics have allowed us to use relatively high concentrations of detection antibody (100–500 μg/L), which favor a steep assay response while maintaining a very low background.

The sensitivity of the Erenna Immunoassay System allows the measurement and quantification of biomarkers in healthy, nondisease states. The concentrations of cTnI that we measured in a panel of lithium-heparin plasma samples were similar to those reported previously for serum specimens *(9)* and also support the shape of the previously reported distribution curve. For cTnI, we demonstrated an analytical LOD of 0.2 ng/L and a 10% CV between 0.78 and 1.6 ng/L (based on back-interpolation of calibrators). Because healthy individuals have serum and plasma cTnI between 0.3 and 9 ng/L (mean 2–3 ng/L), increases from these concentrations can potentially be measured with high precision in most human serum and plasma samples.

The enhanced sensitivity of the Erenna Immunoassay System provides additional potential benefits in sample volume requirements and incubation times. For example, to achieve highest sensitivity, often sample volume is increased in IA applications. Because the Erenna Immunoassay System provides a more sensitive approach, less sample volume can potentially be used, thus enabling the preservation of precious specimens. This point is especially important when using clinical repository specimens or samples, such as sera, obtained from small animals such as mice and rats.

A limitation of rapid tests used today in near-patient settings is a lack of sensitivity compared with automated system counterparts. This limitation is attributable to technological features, sample volume, and incubation times. We demonstrated a proportional increase in sensitivity with incubation times. The Erenna Immunoassay System has LODs of 10–100 pg/L using 100 μL of sample and 1–2 h of incubation. Using a 1-step modification for the Erenna cTnI IA, we were able to reduce the incubation times to 15 min with an approximate LOD of 0.6 ng/L with 50 μL of sample. Taken together these data demonstrate the potential for the development of a rapid sensitive IA using single molecule counting as incorporated in the Erenna Immunoassay System. The clinical utility of such a platform warrants further investigation.

Approaches to enhance ELISA sensitivity have involved the use of a variety of technologies including signal amplification (e.g., double enzymatic amplification) *(8)*. Although high-sensitivity IAs have been demonstrated using this approach, often the dynamic range covers only 1.5–2.0 logs. If the analyte of interest exists in a wide biological range, such as for cytokines, then many samples will require dilution to achieve the correct concentration for measurement in the IA *(2)*.

In summary, the Erenna Immunoassay System builds single-molecule detection combined with MP IA technology to provide highly sensitive IAs. This system also provides a broad dynamic reporting range and flexible sample volume requirements. The flexibility of sample volume may allow determination of multiple quantifications from a small sample or to conserve samples. This feature could be especially important for analysis of rodent samples or human repository specimens.

Grant/funding support: None declared.
Financial disclosures: All authors are employees of Singulex and have a minority ownership position in Singulex.
Acknowledgments: We are grateful to Dr. Robert Puskas, founder of Singulex, for technical support and guidance.

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