

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

FUJIREBIO DIAGNOSTICS, INC.,
Petitioner,

v.

QUANTERIX CORP.,
Patent Owner.

Case Unassigned
Patent 11,275,092
Issue Date: March 15, 2022

DECLARATION OF INGRID HSIEH-YEE, PH.D.

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I, Ingrid Hsieh-Yee, declare as follows:

I. INTRODUCTION AND QUALIFICATIONS

1. I am over the age of eighteen and otherwise competent to make this declaration.

2. I have been retained by counsel for Petitioner to provide my expert opinion in connection with the above-captioned proceeding (“IPR”) regarding the public availability of certain prior art references in relation to U.S. Patent No. 11,275,092 (“Challenged Patent”).

3. I am being compensated for my time in connection with this IPR at my current consulting rate of \$300/hr. I am also being reimbursed for any reasonable expenses associated with my work and testimony in this investigation. My compensation is not contingent on the results of my study, the substance of my opinions, or the outcome of this matter.

A. Qualifications and Professional Experience

4. My complete qualifications and professional experience are described in my academic curriculum vitae (**Appendix A**). The following is a brief summary of my relevant qualifications and professional experience.

5. I was a professor in the Department of Library and Information Science at the Catholic University of America for 32 years and was conferred the title of Professor Emerita in December 2022. I have experience working in an academic

library, a medical library, and a legislative library. I hold a Ph.D. and a Masters in Library and Information Studies from the University of Wisconsin-Madison.

6. I am an expert on library cataloging and classification and have published two editions on this subject, *Organizing Audiovisual and Electronic Resources for Access: A Cataloging Guide* (2000 and 2006). I taught a variety of courses, including Cataloging and Classification, Advanced Cataloging and Classification, Organization of Internet Resources, Organization of Information, Metadata, Digital Content Creation and Management, Internet Searches and Web Design, Information Literacy Instruction, Advanced Information Retrieval and Analysis Strategies, and The Information Professions in Society. I am familiar with metadata schema design and implementation. In my teaching, I covered the design and implementation of metadata in databases, search engines, digital repositories, digital libraries, and digital archives. I also covered how information organization affects the discovery and access to digital resources on the Internet. My research interests cover cataloging and classification, information organization, metadata, information retrieval, information architecture, digital collections, scholarly communication, social media, user interaction with information systems, and others

II. BACKGROUND OF LIBRARY CATALOGING

A. Machine-Readable Cataloging (“MARC”)

7. I am very familiar with a library cataloging encoding standard known

as the “Machine-Readable Cataloging” standard, also known as “MARC,” which became the national standard for sharing bibliographic data in the United States by 1971 and the international standard by 1973. MARC is the primary communications protocol for the transfer and storage of bibliographic metadata in libraries. Experts in my field reasonably rely upon MARC records when forming their opinions.

8. A MARC record consists of several fields, each of which contains specific data about the work. Each field is identified by a standardized, unique, three-digit code corresponding to the type of data that follows. **Appendix B** is a true and correct copy of Parts VII to X of “Understanding MARC Bibliographic: Machine-Readable Cataloging” (<https://www.loc.gov/marc/umb/um07to10.html>), a brief description and tutorial published by the Library of Congress in 2009 that explains commonly used MARC fields. For example, the personal author of a work is recorded in Field 100, the title is recorded in Field 245, publisher information is recorded in Field 260, the physical volume and characteristics of a publication are recorded in Field 300, and topical subjects are recorded in Fields 650. More details of MARC21 format for Bibliographic Data are available on the Library of Congress website at <https://www.loc.gov/marc/bibliographic/>.

9. I am very familiar with library cataloging and processing practices and the relationships between OCLC (Online Computer Library Center) and library catalogs, and how libraries create and share their records to help the public discover

and access information.

10. OCLC is the largest bibliographic network of the world, and its WorldCat database contains more than 559 million records from thousands of member institutions (many of which are libraries of some type) in more than 100 countries. OCLC was founded in 1967 to promote and support library cooperation. According to the third article of the “Amended Articles of Incorporation of OCLC [Online Computer Library Center], Inc.,” OCLC was created “to establish, maintain, and operate a computerized library network and to promote the evolution of library use, of libraries themselves, and of librarianship, and to provide processes and products for the benefit of library users and libraries, including such objectives as increasing availability of library resources to individual library patrons and reducing the rate of rise of library per-unit costs, all for the fundamental public purpose of furthering ease of access to and use of the ever-expanding body of worldwide scientific, literary, and educational knowledge and information” (source: <https://www.oclc.org/content/dam/oclc/membership/articles-of-incorporation.pdf>).

11. OCLC members can contribute original cataloging records in MARC to the system or derive cataloging records from existing records, an activity referred to as “copy cataloging.” When an OCLC participating institution acquires a work, it can create an original MARC record for the work in OCLC’s WorldShare system (a system for catalogers to create and share MARC records), and the system will

automatically generate a code for the date of record creation in the yymmdd format in the 008 field, and the creating library's OCLC symbol is recorded in subfield "a" of the 040 field. Once the MARC record is created in WorldShare, the record is stored in the OCLC WorldCat database and becomes available to other OCLC members for adoption to their local online catalogs (*i.e.*, copy cataloging). "WorldCat is the world's most comprehensive database of information about library collections" (source: WorldCat, <https://www.oclc.org/en/worldcat.html>).

12. After a MARC record is added to OCLC's WorldCat database, it also becomes searchable and viewable on WorldCat.org, a free web portal for the public to explore more than 10,000 libraries worldwide. Records in WorldCat.org, however, are not presented in MARC fields. Instead, the data elements are labeled to help users interpret the records. Thus, the information stored in MARC records in the WorldCat database is available to the interested public through the user-friendly WorldCat web portal. In addition, WorldCat database records can be searched on OCLC FirstSearch, a subscription-based search system that provides access to many proprietary databases, including the WorldCat database.

B. Library Online Catalogs

13. Library online catalogs gained acceptance in the early 1980s and many libraries migrated their systems to the World Wide Web in the mid-1990s. Library online catalogs are based on MARC records that represent their collections in order

to help the public understand what materials are publicly accessible in those libraries. Most libraries with online catalogs have made their catalogs freely available on the Web. These online catalogs offer user-friendly search interfaces. Strong user interest in keyword searches and the popularity of Google have led to the “googlization” of library search systems. As a result, many library catalogs nowadays provide a single search box for users to conduct keyword searches, with additional support for searches by author, title, subject terms, and other data elements such as ISBN (International Standard Book Number). Library catalogs these days also offer features for users to narrow their search results by language, year, format, and other elements. Many libraries display MARC records on their online catalogs with labels for the data elements to help the public interpret MARC records. Many libraries also offer the option to display MARC records in MARC fields.

14. Libraries create MARC records for works they acquire, including books, serials, motion pictures, and publications in other formats. Monograph cataloging is fairly common in libraries, and most libraries make a newly cataloged monograph available to the public soon after the cataloging work is completed, usually within a week.

15. Libraries can create original cataloging records or use an existing record in the OCLC WorldCat database to create a copy cataloging record. As soon as the cataloging record is completed, it is added to the library’s online catalog for

users. If the record is an original record, it is also entered into the OCLC WorldCat database. If it is a copy cataloging record, the library's holding symbol is attached to the existing original record in the OCLC WorldCat database to facilitate searching and interlibrary loan.

16. The cataloging of serials and the serial check-in process are discussed here to show how libraries usually provide access to newly received serial issues. According to the glossary of the *RDA: Resource Description and Access* cataloging standard, a serial is “a mode of issuance of a manifestation issued in successive parts, usually bearing numbering, that has no predetermined conclusion. A serial includes a periodical, monographic series, newspaper, etc.” (RDA Glossary at https://original.rdatoolkit.org/rdagloss_rdaregistry.info-termList-ModeIssue-1003_1.html (subscription required for access); cited in <https://www.ccslib.org/Catalogers/index.php/Category:Serials>). Because the publisher of a serial makes new issues of the serial available successively, a customary cataloging practice is to create one bibliographic record for the entire serial, and the serial record (encoded in MARC) typically provides information on the beginning date and frequency of the serial, not the dates of individual issues. In other words, libraries typically do not create MARC records for individual issues of a serial. Instead, they rely on a serial check-in system to track the receipt of new issues. A common check-in practice is to date stamp a new issue when it arrives. This

practice has become automated since the late 1990s, and libraries now vary in how they share the receipt date of a new serial issue with the public. Some libraries use a date stamp, some affix a label to indicate the receipt date, some pencil in the receipt date, and some do not provide the information to the public.

17. The serial check-in process usually takes less than an hour, and one of the steps involves placing a date stamp on the new issue to document the date the issue is checked in. After that, the holdings information of the serial is updated in the library's catalog so that users know which issues are available for request or access. After serial check-in is completed, the new issue is placed on the shelf with the previous issues of the serial. Libraries with a public periodical room typically place new issues in the periodical room for easy user access. Because information presented in serials often reflects latest discovery, a general practice of libraries is to make new issues of serials available for user access soon after they are checked in, usually within a week.

18. I am personally familiar with many online catalogs, databases, and search engines. In preparing for this declaration, I used the following authoritative information systems to search for records:

- Internet Archive (<https://archive.org/>)
- Libraries.org, a directory of libraries throughout the world (<https://librarytechnology.org/libraries/>)

- Online catalog of the Linda Hall Library
(https://catalog.lindahall.org/discovery/search?vid=01LINDAHALL_INST:LHL)
- Online catalog of the National Library of Medicine
(https://catalog.nlm.nih.gov/discovery/search?vid=01NLM_INST:01NLM_INST)
- PubMed (<https://pubmed.ncbi.nlm.nih.gov/>)
- WorldCat (<https://search.worldcat.org/>)

C. Scope of this Declaration

19. I have been asked to offer an opinion on the public availability date of each of the following documents:

- 1) Rissin, D. M., Kan, C. W., Campbell, T. G., Howes, S. C., Fournier, D. R., Song, L., ... & Duffy, D. C. (2010, May). Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations, *Nature Biotechnology*, 28(6), 595-599, **EX1005** (“*Rissin*”);
- 2) Todd, J., & Agee, S. J. (2008, Fall). Next generation immunoassay systems and the revival of protein biomarkers, *Drug Discovery World*, Fall 2008 issue, pp. 51 to 57, **EX1006** (“*Todd*”);

- 3) Webpage for INNOTESTTM hTAU Ag from Innogenetics, **EX1024** (“*INNOTEST*”);
- 4) Webpage for Alzheimer’s Disease (AD) from Quanterix, **EX1030** (“*AD*”);
- 5) Webpage for Assay Kits from Quanterix, **EX1034** (“*Assay*”);
- 6) Nalefski, E. A., D’Antoni, C. M., Ferrell, E. P., Lloyd, J. A., Qiu, H., Harris, J. L., & Whitney, D. H. (2006, November). Single-molecule detection for femtomolar quantification of proteins in heterogeneous immunoassays, *Clinical Chemistry*, 52(11), 2172-2175, **EX1035** (“*Nalefski*”);
- 7) Webpage for A β -42 (Amyloid beta protein 42) from Singulex, **EX1036** (“*A β -42*”);
- 8) Bourzac, K. (May 13, 2008). Next-Generation Diagnostics: A startup can detect tiny traces of cancer markers in blood samples, *MIT Technology Review*, **EX1011** (“*Bourzac*”).

20. I am informed by counsel and, understand that a given reference is “published” upon a satisfactory showing that such a document has been disseminated or otherwise made publicly available to the extent that persons interested and ordinarily skilled in the subject matter or art could locate it exercising reasonable diligence and obtain the document. I have also been informed by counsel and

understand that materials available in a library constitute “printed publications” if they are catalogued and indexed according to general library practices and protocols that make the references available and accessible to members of the interested public.

21. I have been informed by counsel that the Challenged Patent claims April 12, 2011 as its earliest priority date. I have assessed public accessibility of the documents referenced below as of this claimed priority date.

22. I have been informed by counsel that the subject matter of this proceeding generally relates to a method for producing a patient bodily fluid sample having an analytically quantified amount of endogenous tau protein using an assay that is highly sensitive.

23. I have been informed by counsel that a “person of ordinary skill in the art at the time of the invention” (POSA) is a hypothetical person who is presumed to be familiar with the relevant field and its literature at the time of the inventions. I have been informed by counsel that this hypothetical person is also a person of ordinary creativity, capable of understanding the scientific principles and literature applicable to the pertinent field.

24. I have been informed by counsel that a person of ordinary skill in the art as of April 12, 2011, would have knowledge of the scientific literature and have skills relating to art of molecular neuroscience that includes anti-tau antibodies, and assays that use these antibodies, especially for the detection or diagnosis of

neurological diseases (including AD). A person of ordinary skill would have possessed a Bachelor's degree in molecular, cellular, or developmental biology, biochemistry, biomedical sciences, immunology, or neuroscience such as molecular neuroscience, or an equivalent degree, coupled with six years of laboratory experience; or a Master's degree in such fields with four years of laboratory experience; or a PhD in such fields with at least two years of laboratory experience.

25. It is my opinion that such a person would have been actively engaged in learning about the field, possibly through formal instruction and through bibliographic resources. By at least **April 12, 2011**, such a person would have had access to a vast array of print and electronic resources, including at least the documents referenced below.

D. Evidence Considered in Forming My Opinion

26. In the preparation of this declaration, I have reviewed **EXs1005, 1006, 1024, 1030, 1034, 1035, and 1036** referenced in paragraph 19 above, and any other documents I reference herein. Each of these is a type of material that experts in my field would reasonably rely upon when forming their opinions. I provided a further description of materials I considered in **Appendix C**.

III. Authenticity and Public Availability of *Rissin* (EX1005)

A. Authentication

27. **EX1005** is a true and correct copy of "Single-molecule enzyme-linked

immunosorbent assay detects serum proteins at subfemtomolar concentrations,” (“*Rissin*”), by Rissin et al., *Nature Biotechnology*, 28(6), 595-599, that I received from counsel. This copy of *Rissin* is presented as **EX1005** in this declaration.

28. Page 1 (internal page 595) of **EX1005** shows the title, authors, and abstract of *Rissin*. The top of the page indicates this article is included in the Letters section of *Nature Biotechnology*. The bottom of the page shows the manuscript was received “1 February; accepted 29 April; published online 23 May 2010; doi:10.1038/nbt.1641” and the article appears in vol. 28, no. 6 of *Nature Biotechnology* that has an issue date of “June 2010.” **EX1005** has a total of six pages, including four figures and 30 references. Internal page 599 shows under “Methods” that “[m]ethods and any associated references are available in the online version of the paper at <http://www.nature.com/naturebiotechnology/> and supplementary information is available on the *Nature Biotechnology* website. Pages 7 to 10 are the supplementary information that includes three supplementary figures, three supplementary tables, and a supplementary discussion that covers “Sensitivity to Enzyme Labels as Sample Volume Increases” and “Optimization of Labeling of Captured Proteins in Digital ELISA.”

29. To authenticate **EX1005**, I searched Google Scholar for records for *Rissin* and a record led me to the publisher webpage for this article at <https://www.nature.com/articles/nbt.1641>. I then used the “Buy Now” link to obtain

a PDF of this article and followed the publisher's link for "Supplementary Text and Figures" at https://static-content.springer.com/esm/art%3A10.1038%2Fnbt.1641/MediaObjects/41587_2010_BFnbt1641_MOESM21_ESM.pdf to download the file. I combined the publisher copy of *Rissin* and the supplementary information to produce **EX1005A**. I have closely compared **EX1005** with **EX1005A**, and found them to be the same.

30. To further authenticate *Rissin*, I searched WorldCat.org, the free web portal for records, and a record for this article at <https://search.worldcat.org/title/672081431> showed the National Library of Medicine (NLM) was one of the holding libraries. After verifying the holdings information in the online catalog of NLM, I requested a copy of *Rissin* from this library through a document vendor. This NLM copy is presented as **EX1005B** in this declaration.

31. Page 1 of **EX1005B** is the cover that identifies this issue as vol. 28, no. 6 of *Nature Biotechnology* that has a "June 2010" issue date. The cover features "Single-molecule ELISA," "Human ES cells cultured on defined surfaces," and "Targeting influenza nucleoprotein A." It shows a date stamp label indicating this issue was received by NLM on "2010-07-13" (*i.e.*, July 13, 2010) and it is included in the General Collection with a call number of "W1 BL918LW." The cover includes a stamp indicating this issue is the "Property of the National Library of Medicine."

32. As discussed above, a customary library practice is to place a newly

received journal issue through the serial check-in process, make the new issue publicly accessible as soon as possible, usually within a week after serial check-in is completed. The date stamp on **EX1005** shows a receipt date of July 13, 2010. It is therefore my opinion that this issue containing *Rissin* was publicly accessible at NLM by July 20, 2010.

33. Page 2 of **EX1005B** is the copyright page that shows that “1087-0156” is the International Standard Serial Number (ISSN) for *Nature Biotechnology*, that the journal “is published monthly by Nature Publishing Group,” and that this issue has a “2010” copyright date with Nature America, Inc. as the copyright holder.

34. Pages 2 to 4 are the table of contents, and page 2 (internal page i) shows an image identified as “Artist’s impression of digital ELISA. *Rissin* et al. determine protein abundance by capturing beads bearing single analyte molecules in arrayed femtoliter-volume reaction chambers (p. 595). Credit: Ken Eward.” Page 4 (internal page v) shows *Rissin* is included in the “Letters” section of this issue and begins on internal page 595.

35. Pages 5 to 9 (internal pages 595 to 599) are the full text of *Rissin*. I have obtained the supplementary information from the publisher’s webpage for this article and attached the supplementary file to **EX1005B** as pages 11 to 14. I have closely compared **EX1005B** with **EX1005** and the publisher copy (**EX1005A**) and found these three copies to contain the same pages 595 to 599, and the same supplementary

information. One difference is **EX1005** and **EX1005A** include an unnumbered page after page 599 that shows “Online Methods” and reference no. 30. Since the NLM copy of *Rissin* (**EX1005B**) is a print publication, it is understandable why it does not include a page on “Online Methods” as **EX1005** and **EX1005A**.

36. **EX1005** is in a condition that creates no suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. In addition, **EX1005** is identical to the online version obtained from the publisher (**EX1005A**). Furthermore, **EX1005** and the NLM copy (**EX1005B**) have the same content for *Rissin* (pages 595 to 599), and **EX1005B** is scanned from an issue held by a national library. Based on my review and analysis, I see no reason to question the authenticity of **EX1005**.

B. Records of the National Library of Medicine

37. **EX1005C** is a true and correct copy of the bibliographic and MARC serial records for *Nature Biotechnology* whose vol. 28, no. 6 contains *Rissin*. I personally located, identified and obtained these records from the online catalog of NLM by searching for the journal title for records. They are the type of records experts in my field would reasonably rely upon when forming their opinions on the public availability of a document.

38. Pages 1 to 4 of **EX1005C** are the bibliographic record that shows

Nature Biotechnology is available online and in print at NLM. The journal title and the ISSN of the print version match the information presented in **EX1005B**, confirming this record represents *Nature Biotechnology* that contains *Rissin*.

39. Pages 5 and 6 are the MARC record. The first six digits of Field 008 show that this MARC serial record was created on “961010” (*i.e.*, October 10, 1996), and the code of “c19969999” following this creation date indicates that the journal is a continuing resource that began publication in 1996 and “9999” means the journal has no predetermined conclusion date of publication. Subfields “a” and “c” of Field 040 show that “DNLM” created the original MARC serial record. “DNLM” is the MARC organization code for the National Library of Medicine, according to the MARC Code List for Organizations at <https://www.loc.gov/marc/organizations/org-search.php>. Data from Fields 008 and 040 inform my opinion that NLM created the original MARC serial record for *Nature Biotechnology* in 1996.

40. As discussed earlier, a common library practice is to create a record for the entire serial, without creating records for individual issues of the serial. When new issues are received, they go through the serial check-in process, which usually takes less than an hour. After that step is completed, the newly received issue is promptly processed and placed on the shelf or in a periodical reading room for user access, usually on the same date of serial check-in or within a week after serial check-in. I know this is the customary library practice from personal experience as a

cataloger, cataloging instructor and researcher of library catalogs and information systems. The date stamp of “2010-07-13” on the NLM copy of *Rissin* (**EX1005B**) indicates NLM received the journal issue containing this article on July 13, 2010. Based on this date stamp and the NLM serial record for *Nature Biotechnology* (**EX1005C**), it is my opinion that this issue (and *Rissin* contained therein) became publicly accessible at NLM by July 20, 2010.

41. Field 022 of the MARC record (**EX1005C**) shows “1087-0156” as the ISSN for the print version of this journal. Field 245 shows the journal title, Fields 260 show publisher information, Field 362 shows the journal began with vol. 14, no. 3 in March 1996, and Field 310 shows the earlier publication frequency was thirteen numbers a year. Field 510 shows *Nature Biotechnology* has been indexed by MEDLINE, PubMed since 1996. These two indexes mean that interested users would be able to search for individual articles published by this journal. Field 710 shows the publisher Nature Publishing Company is included to help users discover this journal.

42. The subject of this journal is represented in Field 060 by a National Library of Medicine Classification number of “W1” that represents periodicals on “General Medicine. Health Professions” (source: National Library of Medicine, Classification 2025 Winter Edition at <https://classification.nlm.nih.gov/schedules/w>). Subjects are also represented in Field 650 by a Medical Subject Heading of “Biotechnology” and in Field 655 by a Medical

Subject Heading genre term of “Periodical” that indicates this publication is a journal. These subject representations enable users to conduct subject searches to discover this journal.

43. This MARC serial record (**EX1005C**) shows that *Nature Biotechnology* is a long-running journal, and the MARC serial record has made it discoverable in the online catalog of NLM since October 1996. Interested users would be able to discover this journal by conducting subject searches by the classification number and the subject headings. They would also be able to discover and access the journal by the journal title, ISSN, and publisher. This MARC record and the date stamp on **EX1005B** inform my opinion that the journal issue containing *Rissin* became publicly accessible at NLM by July 20, 2010.

44. As evidence that NLM had an online catalog in operation in 2010 when the library received the journal issue containing *Rissin*, I obtained a profile of NLM at <https://librarytechnology.org/library/2297> from libraries.org, a directory of libraries throughout the world. I personally located, identified, and obtained this profile. This is the type of record experts in my field would reasonably rely upon when forming their opinions. This profile is presented as **EX1005D** in this declaration.

45. The Technology Profile on page 1 of **EX1005D** shows NLM began using Voyager as their online catalog in 1998, and migrated to Alma in 2020. These

dates mean that in 2010 interested users would be able to use the Voyager system at NLM to discover the journal containing *Rissin*.

C. PubMed Record

46. To locate index records of *Rissin*, I searched PubMed by the article title for records. PubMed is an authoritative index system that contains “more than 38 million citations for biomedical literature from MEDLINE, life science journals, and online books” and PubMed records may “include links to full text content from PubMed Central and publisher web sites” (source: <https://pubmed.ncbi.nlm.nih.gov>).

47. **EX1005E** is a true and correct metadata record for *Rissin* (<https://pubmed.ncbi.nlm.nih.gov/20495550>) that I personally located, identified and obtained from PubMed. This is the type of record experts in my field would reasonably rely upon when forming their opinions. I saved the metadata record and the PubMed format of the record in PDF to show the record creation dates and other data by data fields.

48. Page 1 of **EX1005E** shows the title, authors, and abstract of *Rissin*, and indicates the article was published in “Nat Biotechnol. Jun;28(6):595-9. doi: 10.1038/nbt.1641. Epub 2010 May 23.” The top of the record shows a full text link to “nature portfolio” that leads to the publisher’s webpage for this article at <https://www.nature.com/articles/nbt.1641>, and a link for free full text access to this article at <https://pmc.ncbi.nlm.nih.gov/articles/PMC2919230/> on PMC (PubMed

Central). Data elements in this PubMed metadata record match the information contained in **EX1005**, confirming this metadata record represents *Rissin*.

49. Pages 3 to 5 of **EX1005E** are the PubMed metadata record saved in the PubMed format to show the data elements in specific fields. I use a copy of the “MEDLINE/PubMed Data Element (Field) Descriptions” (<https://www.nlm.nih.gov/bsd/mms/medlineelements.html>) archived by Internet Archive on July 9, 2010, to interpret this PubMed record with data fields. This archived copy is available at <https://web.archive.org/web/20100709164257/https://www.nlm.nih.gov/bsd/mms/medlineelements.html> and presented as **EX1005F** in this declaration.

50. The “PMID” field on page 3 of **EX1005E** shows “20495550” as the PubMed identifier for the metadata record for *Rissin*. The “STAT” field shows the status of the record is “MEDLINE” (meaning the record has been indexed with Medical subject headings and passed the quality assurance routines to be elevated to MEDLINE status, see page 25 of **EX1005F**). The DCOM (“Date Completed”) field is “the date processing of the record ends” meaning “MeSH Headings have been added, quality assurance validations are completed, and the completed record subsequently is distributed to PubMed and licensees” (see page 9 of **EX1005F**). The DCOM field shows the PubMed record for *Rissin* was completed on “20100913” (*i.e.*, September 13, 2010). The IS field shows the ISSN for the print version of the

journal containing *Rissin* is “1087-0156.” Fields VI (volume), IP (issue), DP (date of publication), Ti (title) and PG (pages) show *Rissin* was published in vol. 28, no. 6 (2010 Jun), pp. 595-9. The first FAU (full author) field shows “Rissin, David M” as the first author. The JT (journal title) field shows *Rissin* appears in *Nature Biotechnology*. The MH (MeSH terms) fields show seven medical subject headings were assigned to indicate the subjects of *Rissin*. Field “MHDA” (date of medical subject headings) shows subject index terms were added to this PubMed record on “2010/09/14” (*i.e.*, September 14, 2010). The “AID” (article identifier) field shows the doi of *Rissin* is “10.1038/nbt.1641.”

51. This PubMed record (**EX1005E**) made *Rissin* discoverable in PubMed on September 13, 2010, when the PubMed record was completed, and the record was enriched with medical subject headings on September 14, 2010. These dates show that by September 14, 2010, interested users were able to discover the PubMed metadata record for *Rissin* by authors, title, journal ISSN, journal title, abstract, and medical subject headings assigned to this article, and could follow the link to full text in the PubMed record to reach the publisher website to access this article.

52. As evidence that PubMed was in operation when the PubMed record for *Rissin* became discoverable in September 2010, I searched Internet Archive for records of PubMed. **EX1005G** is a true and correct copy of the PubMed home page archived by Internet Archive. I personally located, identified, and obtained these

archived webpages. They are the type of record experts in my field would reasonably rely upon when forming their opinions.

53. Page 1 of **EX1005G** is a screenshot of a Wayback Machine calendar that shows this home page of PubMed was archived 61,094 times between 1997 and 2025, and this particular copy was archived on “AUG 04 2010” and available at <https://web.archive.org/web/20100804041346/http://www.ncbi.nlm.nih.gov/PubMed/>. Page 2 is the PDF of this archived copy that shows PubMed is a service of the National Library of Medicine, and that as of August 4, 2010, users could conduct simple and advanced searches, limit search results, and use “Help” to find search guide. This page also shows that PubMed records “may include links to full-text content from PubMed Central and publisher web sites.”

54. The PubMed record for *Rissin* (**EX1005E**) and related PubMed documents (**EX1005F** and **EX1005G**) inform my opinion that PubMed was already in operation by August 2010, and *Rissen* became discoverable on PubMed in September 2010. As of September 14, 2010, interested users were able to use PubMed to locate the metadata record for *Rissin*, then follow the full text link to access the full article from the publisher’s website.

D. Internet Archive Record

55. Since *Nature Biotechnology* that contains *Rissin* is published online and in print, I searched Internet Archive for information on this journal. **EX1005H** is a

true and correct copy of the webpage for volume 28, number 6 of *Nature Biotechnology* archived by the Internet Archive. I personally located, identified, and obtained this record. This is the type of record experts in my field would reasonably rely upon when forming their opinions.

56. Internet Archive is a digital library that archives scholarly materials, cultural artifacts, and other web resources on the Internet and provides the public with free access to its collections. I have used the Internet Archive in the past and am familiar with their practice of assigning an extended URL to an archived resource in the format of `http://www.archive.org/web/[Year in yyyy][Month in mm][Day in dd][Time code in hh:mm:ss]/[Archived URL]` to show the point of time the archived file was captured. Since Internet Archive usually does not archive web resources the moment they appear on the Internet, and a web resource must be available first before it can be archived, the archive date of a file captured by the Internet Archive is typically later than the date when the file first became publicly accessible. In other words, the archive date means the archived file was available before, and definitely no later than, the archive date.

57. **EX1005H** is a true and correct copy of the online version of vol. 28, no. 6 (June 2010) of *Nature Biotechnology*. Page 1 is a screen capture of a Wayback Machine calendar that shows this webpage was archived 32 times between 2010 and 2017, and this particular copy was archived on “JUN 28 2010” and available at

<https://web.archive.org/web/20100628153945/http://www.nature.com/nbt/journal/v28/n6/index.html>. Pages 2 to 11 are the PDF of this archived copy that shows the same issue cover featuring *Rissin* as the cover included in the NLM copy (**EX1005B**). Pages 8 and 9 of **EX1005H** shows *Rissin* is included in the Letters section, and shows the title, authors, pages, doi, and links to “First Paragraph | Full Text | PDF (511 KB) | Supplementary information.” **EX1005H** informs my opinion that *Rissin* became publicly discoverable and accessible by June 28, 2010, on the webpage of *Nature Biotechnology*.

58. The “Full Text” link in **EX1005H** leads to a webpage on how to access *Rissin*. This access page is presented as **EX1005I** in this declaration. Page 1 is a screen capture of a Wayback Machine calendar that shows this webpage was captured 36 times between 2010 and 2018, and this particular copy was archived on “JUN 11 2010” and available at <https://web.archive.org/web/20100611073455/http://www.nature.com/nbt/journal/v28/n6/full/nbt.1641.html>. The top of page 1 states under “Access” that “[t]o access this article in full you may need to log in, make a payment or gain access through a site license” and the side panel explains that “[p]ersonal subscribers to *Nature Biotechnology* can view this article” and users without such access “can purchase the article ... or access it through a site license.” Pages 2 and 3 are the PDF of this archived copy.

59. Taken together, **EX1005H** and **EX1005I** inform my opinion that *Rissin* became discoverable and accessible on the Internet by June 2010. Personal subscribers to this journal would be able to access the full article from the journal webpage, and those without subscriptions could purchase the article from the publisher. Access through subscribing institutions was another option.

60. In addition to publisher webpages, Quanterix Corporation provided information on the public availability of *Rissin*. **EX1005J** is true and correct copy of the company's press release of May 23, 2010. I personally located, identified, and obtained this record. This is the type of record experts in my field would reasonably rely upon when forming their opinions.

61. Page 1 of **EX1005J** is a screen capture of a Wayback Machine calendar that shows this press release was captured by the Internet Archive three times between 2010 and 2011, and this particular copy was archived on "JUN 01 2010" and available at <https://web.archive.org/web/20100601142509/http://quanterix.com/news/pdfs/natureBiotech.pdf>. This document is entitled "Groundbreaking Study Published in *Nature Biotechnology* Demonstrates Sensitivity of Single Molecule Test for Detection of Prostate Cancer." Page 2 is the PDF of this archived copy that shows that by this press release of May 23, 2010, "Quanterix Corporation, the single molecule diagnostics company focused on developing and commercializing high sensitivity

tests, announced the publication of a breakthrough study of the company's proprietary technology ... The research article, featured on the cover of the June issue (Volume 28, No. 6) of *Nature Biotechnology*, describes how researchers developed a single blood test using Quanterix's Single Molecule Array (SiMoA[™]) technology."

EX1005J informs my opinion that on the day when *Rissin* was published, Quanterix Corporation informed the public of this publication. Interested users could then locate *Nature Biotechnology* to access vol. 28, no. 6 that contains *Rissin*. This press release was first archived by the Internet Archive on June 1, 2010, meaning users would have known about the publication of *Rissin* by June 1, 2010, if not earlier.

E. Usage Record

62. Actual usage of a publication is reflected by the publications that make reference to it. The citation history on Google Scholar shows *Rissin* has been cited 2,246 times. I have reviewed the citations to identify five early citations. **EX1005K** shows citations from 2010, and the earliest citations were published in November 2010.

F. Summary of My Opinion on *Rissin*

63. **EX1005** is authentic because it contains no signs to raise questions about its authenticity, its content is identical to the publisher's copy (**EX1005A**) and the NLM copy (**EX1005B**), and the article is in the custody of a national library.

64. The date stamp on the NLM copy (**EX1005B**) shows the library

received the issue containing *Rissin* on July 13, 2010, and the NLM MARC record for *Nature Biotechnology* shows NLM created the original serial record for this journal in October 1996, making this journal discoverable from then on in this library. These NLM data inform my opinion that *Rissin* was publicly accessible at NLM from July 13, 2010 on, and the physical copy, after processing, would become publicly accessible by July 20, 2010.

65. The PubMed record for *Rissin* (**EX1005E**) and my knowledge of PubMed and MEDLINE inform my opinion that this PubMed record was completed on September 13, 2010, and indexed with medical subject headings on September 14, 2010, making this record discoverable on PubMed by subject terms and other data element from that date on.

66. **EX1005**, the publisher copy (**EX1005A**), and the NLM copy (**EX1005B**) show *Rissin* was published in vol. 28, no. 6 of *Nature Biotechnology* that has an issue date of June 2010, and the first page of this article carries a note indicating it was published online on “23 May 2010.” The webpage for vol. 28, no. 6 of this journal (**EX1005H**) archived by the Internet Archive on June 28, 2010, shows *Rissin* was discoverable and accessible by this date, and a webpage on how to access *Rissin* (**EX1005I**), archived by the Internet Archive on June 11, 2010, informs visitors that personal subscribers to *Nature Biotechnology* would be able to access the full paper of *Rissin*, and users without personal accounts could purchase the article

from the publisher or access the article through institutional subscribers.

67. On May 23, 2010, Quanterix announced a breakthrough study reported by *Rissin* (**EX1005J**) and provided enough information for interested users to access this article in vol. 28, no. 6 of *Nature Biotechnology*. Citation history on Google Scholar (**EX1005K**) shows *Rissin* has been cited 2,246 times and the earliest citations appeared in November 2010, supporting my opinions on the public accessibility of this article on the Internet, at the NLM library, and from the PubMed record.

68. Based on the documents and records I have reviewed, it is my opinion that the print copy of *Rissin* was publicly accessible at the National Library of Medicine by July 20, 2010. The online version was published on May 23, 2010, by *Nature Biotechnology*, and Quanterix informed the public of the significance of this article and its availability in this journal on May 23, 2010. Internet Archived records show that by June 11, 2010, interested users would be able to access *Rissin*. Citation records from 2010 show the earliest citations appeared in November 2010, supporting my opinion that the online version became publicly accessible by June 11, 2010, and the print version became publicly accessible by July 20, 2010.

IV. Authenticity and Public Availability of *Todd* (EX1006)

A. Authentication

69. **EX1006** is a true and correct copy of “Next generation immunoassay systems and the revival of protein biomarkers,” (“*Todd*”), by John Todd and Sara J.

Agee, *Drug Discovery World*, Fall 2008 issue, pp. 51 to 57, that I received from counsel. This copy of *Todd* is presented as **EX1006** in this declaration.

70. Page 1 (internal page 51) of **EX1006** shows the title, authors, and abstract of *Todd*. The top and bottom of the page indicate this article is included in the Immunoassays section of the Fall 2008 issue of the journal *Drug Discovery World*. **EX1006** shows this article has a total of seven pages, including four color figures and ten references.

71. To authenticate **EX1006**, I searched Google for copies for *Todd* and found the 2008 archive of *Drug Discovery World* (DDW) at <https://www.ddw-online.com/2008-archive/>. The “DDW Fall 2008” portion of the archive showed a “Download PDF” for *Todd*, so I obtained the PDF of this article located at <https://www.ddw-online.com/media/32/fall-08-immunoassay-systems.pdf>.

EX1006A is a true and correct copy of *Todd* from the DDW archive. I have included pages from the DDW 2008 archive to show my discovery path. I have closely compared **EX1006** with **EX1006A**, and found them to be the same.

72. To further authenticate *Todd*, I searched WorldCat.org, the free web portal for records, and a record for *Drug Discovery World* at <https://search.worldcat.org/title/894657237> showed the National Library of Medicine (NLM) was one of the holding libraries. After verifying the holdings information in the online catalog of NLM, I requested a copy of *Todd* from this library through a

document vendor. This NLM copy is presented as **EX1006B** in this declaration.

73. Page 1 of **EX1006B** is the cover that identifies this copy as the issue of “Fall 2008” of *Drug Discovery World* and shows www.ddw-online.com as the journal website. The cover carries a date stamp label identifying this issue as “v. 9, no. 4 (Autumn 2008)” and indicating it was received by NLM on “2008-11-20” (*i.e.*, November 20, 2008) and has been included in the General Collection with a call number of “W1 DR514BM.” The cover includes a stamp indicating this issue is the “Property of the National Library of Medicine.”

74. As discussed above, a customary library practice is to place a newly received journal issue through the serial check-in process, make the new issue publicly accessible as soon as possible, usually within a week after serial check-in is completed. The date stamp in **EX1006** shows a receipt date of November 20, 2008. It is therefore my opinion that this issue containing *Todd* was publicly accessible at NLM by November 27, 2008.

75. Page 2 (internal page 5) of **EX1006B** is the copyright page that shows RJ Communications & Media Ltd of London publishes *Drug Discovery World* four times a year, the journal’s ISSN (International Standard Serial Number) is “1469-4344,” and this issue has a “2008” copyright date with the publisher as the copyright holder.

76. Page 2 includes the table of contents that identifies this issue as

“Volume 9 Issue 4” with a “Fall 2008” issue date, and shows *Todd* is published in the Immunoassays section and begins on page 51. Pages 3 to 9 (internal pages 51 to 57) is the full article of *Todd*. I have closely compared **EX1006B** with **EX1006** and the publisher copy (**EX1006A**) and found these three copies to contain the same content for *Todd*.

77. **EX1006** is in a condition that creates no suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. In addition, **EX1006** is identical to the online version obtained from the publisher’s Fall 2008 archive (**EX1006A**). Furthermore, **EX1006** and the NLM copy (**EX1006B**) have the same content for *Todd*, and **EX1006B** is scanned from an issue held by a national library. Based on my review and analysis, I see no reason to question the authenticity of **EX1006**.

B. Records of the National Library of Medicine

78. **EX1006C** is a true and correct copy of the bibliographic and MARC serial records for *Drug Discovery World* whose vol. 9, no. 4 (fall 2008) contains *Todd*. I personally located, identified and obtained these records from the online catalog of NLM by searching for the journal title for records. They are the type of records experts in my field would reasonably rely upon when forming their opinions on the public availability of a document.

79. Pages 1 to 3 of **EX1006C** are the bibliographic record that shows *Drug Discovery World* is available online and in print at NLM. The journal title, publisher, publication frequency, and ISSN match the information presented in **EX1006B**, confirming this record represents the journal that contains *Todd*.

80. Pages 4 and 5 are the MARC record. The first six digits of Field 008 show that this MARC serial record was created on “060501” (*i.e.*, May 1, 2006), and the code of “c20009999” following this creation date indicates that the journal is a continuing resource that began publication in 2000 and “9999” means the journal has no predetermined conclusion date of publication. Subfields “a” and “c” of Field 040 show that “DNLM” created the original MARC serial record. “DNLM” is the MARC organization code for the National Library of Medicine, according to the MARC Code List for Organizations at <https://www.loc.gov/marc/organizations/org-search.php>. Field 500 shows the serial record was created based on vol. 7, issue 1 (winter 2005/6) and the title was taken from the cover. Data from Fields 008, 040 and 500 inform my opinion that NLM created the original MARC serial record for *Drug Discovery World* on May 1, 2006, based on vol. 7, issue 1 of this journal, making this journal discoverable in their online catalog from that date on.

81. As discussed earlier, a common library practice is to create a record for the entire serial, without creating records for individual issues of the serial. When new issues are received, they go through the serial check-in process, which usually

takes less than an hour. After that step is completed, the newly received issue is promptly processed and placed on the shelf or in a periodical reading room for user access, usually on the same date of serial check-in or within a week after serial check-in. I know this is the customary library practice from personal experience as a cataloger, cataloging instructor and researcher of library catalogs and information systems. The date stamp of “2008-11-20” on the NLM copy of *Todd* (**EX1006B**) indicates NLM received the journal issue containing this article on November 20, 2008. Based on this date stamp and the NLM serial record for *Drug Discovery World* (**EX1006C**), it is my opinion that this journal has been discoverable at NLM since May 2006, and the issue containing *Todd* became publicly accessible at NLM no later than November 27, 2008.

82. Field 022 of the MARC record (**EX1006C**) shows the ISSN. Field 245 shows the journal title and subtitle, Field 246 shows DDW as a variable title, Fields 260 show publisher information, Field 362 shows the journal began publication in 2000, and Field 310 shows the journal is published four issues a year. Field 500 indicates the print issues ceased with 2022 and Field 530 shows the journal is also issued online.

83. The subject of this journal is represented in Field 060 by a National Library of Medicine Classification number of “W1” that represents periodicals on “General Medicine. Health Professions” (source: National Library of Medicine,

Classification 2025 Winter Edition at

<https://classification.nlm.nih.gov/schedules/w>). Subjects are also represented in Fields 650 by two Medical Subject Headings of “Drug Industry” and “Technology, Pharmaceutical” and in Field 655 by a Medical Subject Heading genre term of “Periodical” that indicates this publication is a journal. These subject representations enable users to conduct subject searches to discover this journal.

84. This MARC serial record (**EX1006C**) shows that *Drug Discovery World* is a long-running journal, and the MARC serial record has made it discoverable in the online catalog of NLM since May 2006. Interested users would be able to discover this journal by conducting subject searches by the classification number and the subject headings. They would also be able to discover and access the journal by the journal title, variant title, and ISSN. This MARC record and the date stamp on **EX1006B** inform my opinion that the journal issue containing *Todd* became publicly accessible at NLM by November 27, 2008.

85. As evidence that NLM had an online catalog in operation in 2008 when the library received the journal issue containing *Todd*, I obtained a profile of NLM at <https://librarytechnology.org/library/2297> from libraries.org, a directory of libraries throughout the world. I personally located, identified, and obtained this profile. This is the type of record experts in my field would reasonably rely upon when forming their opinions. This profile is presented as **EX1006D** in this

declaration.

86. The Technology Profile on page 1 of **EX1006D** shows NLM began using Voyager as their online catalog in 1998, and migrated to Alma in 2020. These dates mean that in 2008 interested users would be able to use the Voyager system at NLM to discover the journal containing *Todd*.

C. Internet Archive Record

87. Since *Drug Discovery World* that contains *Todd* is also published online, I searched Internet Archive for information on this journal. **EX1006E** is a true and correct copy of the journal webpage archived by the Internet Archive. I personally located, identified, and obtained this record. This is the type of record experts in my field would reasonably rely upon when forming their opinions.

88. Internet Archive is a digital library that archives scholarly materials, cultural artifacts, and other web resources on the Internet and provides the public with free access to its collections. I have used the Internet Archive in the past and am familiar with their practice of assigning an extended URL to an archived resource in the format of `http://www.archive.org/web/[Year in yyyy][Month in mm][Day in dd][Time code in hh:mm:ss]/[Archived URL]` to show the point of time the archived file was captured. Since Internet Archive usually does not archive web resources the moment they appear on the Internet, and a web resource must be available first before it can be archived, the archive date of a file captured by the Internet Archive is

typically later than the date when the file first became publicly accessible. In other words, the archive date means the archived file was available before, and definitely no later than, the archive date.

89. **EX1006E** is a true and correct copy of the journal webpage at <https://www.ddw-online.com/>. Page 1 is a screen capture of this webpage with a Wayback Machine calendar that shows this webpage was archived 565 times between 2001 and 2025, and this particular copy was archived on “DEC 08 2009” and available at <https://web.archive.org/web/20091208050404/https://www.ddw-online.com/>. The navigation panel on the left side shows a link to “PDF Archive.” Page 2 is a screen capture that shows the submenus of “PDF Archive” include the “fall 2008” archive. Internet Archive has not archived this link to the fall 2008 archive. Nevertheless, **EX1005E** shows that this archive was available on the Internet by December 8, 2009, and interested users would have been able to use it to locate *Todd* by that date.

D. Summary of My Opinion on *Todd*

90. **EX1006** is authentic because it contains no signs to raise questions about its authenticity, its content is identical to the publisher’s copy (**EX1006A**) and the NLM copy (**EX1006B**), and the article is in the custody of a national library.

91. The date stamp on the NLM copy (**EX1006B**) shows the library received the issue containing *Todd* on November 20, 2008, and the NLM MARC

record for *Drug Discovery World* (**EX1006C**) shows NLM created the original serial record for this journal on May 1, 2006, making this journal discoverable from then on in this library. These NLM data inform my opinion that *Todd* was publicly accessible at NLM from November 20, 2008, and the physical copy, after processing, would become publicly accessible by November 27, 2008.

92. The PDF Archive on the *Durg Discovery World* website archived by the Internet Archive on December 8, 2009 (**EX1006E**), shows the Fall 2008 archive was publicly available on the Internet by that date. It is therefore my opinion that interested users would have been able to discover and access *Todd* on the Internet by December 8, 2009.

93. Based on the documents and records I have reviewed, it is my opinion that print copy of *Todd* was publicly accessible at the National Library of Medicine by November 27, 2008. The online version would be available from the PDF Archive on the *Durg Discovery World* website by December 8, 2009. In other words, interested users would be able to discover and access *Todd* on the Internet by December 8, 2009.

V. Authentication and Public Availability of *INNOTEST* (EX1024)

A. Authentication

94. **EX1024** is a true and correct copy of a webpage for “INNOTEST™ hTAU Ag,” (“*INNOTEST*”), from Innogenetics, that I received from counsel. The top

of page 1 shows “Innogenetics – Administration” and the first section shows “80005” as the article number, and “1 plate of 96 tests (8-well strips)” as the number of tests. This document has a total of four pages, including three color images and 23 references. It also includes links to 11 Innogenetics articles and information on customer support. This copy is presented as **EX1024** in this declaration.

95. To authenticate **EX1024**, I searched Internet Archive for records of this document. Internet Archive is a digital library that archives scholarly materials, cultural artifacts, and other web resources on the Internet and provides the public with free access to its collections. I have used the Internet Archive in the past and am familiar with their practice of assigning an extended URL to an archived resource in the format of [http://www.archive.org/web/\[Year in yyyy\]\[Month in mm\]\[Day in dd\]\[Time code in hh:mm:ss\]/\[Archived URL\]](http://www.archive.org/web/[Year in yyyy][Month in mm][Day in dd][Time code in hh:mm:ss]/[Archived URL]) to show the point of time the archived file was captured. Since Internet Archive usually does not archive web resources the moment they appear on the Internet, and a web resource must be available first before it can be archived, the archive date of a file captured by the Internet Archive is typically later than the date when the file first became publicly accessible. In other words, the archive date means that the archived file was typically available before, and definitely no later than, the archive date.

96. **EX1024A** is a true and correct copy that I personally located, identified, and obtained from Internet Archive. This is the type of material experts in my field

would reasonably rely upon when forming their opinions. I have closely compared **EX1024** and **EX1024A** and found them to be the same.

97. **EX1024** is in a condition that raises no suspicion about its authenticity. Specifically, *INNOTEST* does not miss any intermediate pages, the text flows logically from page to page, and there are no visible alterations to this paper. Furthermore, **EX1024** and an archived copy obtained from Internet Archive (**EX1024A**) have the same content. Based on my review and analysis, I have no reason to question the authenticity of **EX1024**.

B. Public Availability Date

98. Page 1 of **EX1024A** is a screen capture of a Wayback Machine calendar that shows the original webpage was archived seven times between 2003 and 2004, and this particular copy was archived on “SEP 14 2004” and available at https://web.archive.org/web/20040914040003fw_/http://www.innogenetics.be/site/prodview.asp?id=38&lang=E&print=true. The archive date of “20040914040003” means this webpage was archived on September 14, 2004, at 4 a.m., 0 minutes, and 3 seconds. It is therefore my opinion that **EX1024** became discoverable and accessible on the Internet by September 14, 2004.

VI. Authenticity and Public Availability of AD (EX1030)

A. Authentication

99. **EX1030** is a true and correct copy of a webpage for “Alzheimer’s

Disease (AD),” (“*AD*”), from Quanterix, that I received from counsel. The panel at the top of page 1 shows this webpage is about Applications of Quanterix products, and this particular webpage is about “Alzheimer’s Disease (AD).” This page presents the message of John Growdon, a physician of Massachusetts General Hospital, who states that “[f]or the first time, using Quanterix’s SiMoA technology, it is now possible to detect these same proteins with a simple blood test, even though they are present in the blood at exceedingly low concentrations, well below the limit of detection achieved by standard testing methods.” He further states that “[s]elected biomarkers from this test panel may also have clinical utility in diagnosing other neurological disorders including Parkinson’s disease, bipolar disorder, stroke and traumatic brain injury (TBI).” Page 2 shows this webpage has a “2010” copyright date and Quanterix Corporation is the copyright holder. This copy is presented as **EX1030** in this declaration.

100. To authenticate **EX1030**, I searched Internet Archive for records of this document. **EX1030A** is a true and correct copy that I personally located, identified, and obtained from Internet Archive. This is the type of material experts in my field would reasonably rely upon when forming their opinions. I have closely compared **EX1030** and **EX1030A** and found them to be the same.

101. **EX1030** is in a condition that raises no suspicion about its authenticity. Specifically, the text in *AD* flows logically, the author of the message is clearly

identified, and there are no visible alterations to this webpage. Furthermore, **EX1030** and an archived copy obtained from Internet Archive (**EX1030A**) have the same content. Based on my review and analysis, I have no reason to question the authenticity of **EX1030**.

B. Public Availability Date

102. Page 1 of **EX1030A** is a screen capture of a Wayback Machine calendar that shows the original webpage was archived three times between 2010 and 2011, and this particular copy was archived on “May 29 2010” and available at <https://web.archive.org/web/20100529185326/www.quanterix.com/applications/ad.html>. The archive date of “20100529185326” means this webpage was archived on May 29, 2010, at 6 p.m., 53 minutes and 26 seconds. Since the archive date on a web document archived by the Internet Archive indicates the original file was available before the archive date, it is my opinion that **EX1030** became discoverable and accessible on the Internet by May 29, 2010.

VII. Authenticity and Public Availability of *Assay Kits* (EX1034)

A. Authentication

103. **EX1034** is a true and correct copy of a webpage for “Assay Kits,” (“*Assay Kits*”), from Quanterix, that I received from counsel. The top image on this page shows this webpage is about Quanterix products, and the image captured in this copy is “Simoa.” Page 1 shows an image of the Simoa Reagent Kit, and states that

“Quanterix is developing a rapidly growing menu of validated assays in a number of research field,” including cardiovascular, inflammation, metabolic, multiplex panels, neurology, and oncology and vascular. It encourages interested users to visit “this page often as new assays will be added as they become available.” This page shows a “2013” copyright date with Quanterix as the copyright holder. This copy of *Assay Kits* is presented as **EX1034** in this declaration.

104. To authenticate **EX1034**, I searched Internet Archive for records of this document. **EX1034A** is a true and correct copy that I personally located, identified, and obtained from Internet Archive. This is the type of material experts in my field would reasonably rely upon when forming their opinions. I have closely compared **EX1034** and **EX1034A** and found them to be the same.

105. **EX1034** is in a condition that raises no suspicion about its authenticity. Specifically, the text in *Assay Kits* flows logically, and there are no visible alterations to this webpage. Furthermore, **EX1034** and an archived copy obtained from Internet Archive (**EX1034A**) have the same content. Based on my review and analysis, I have no reason to question the authenticity of **EX1034**.

B. Public Availability Date

106. Page 1 of **EX1034A** is a screen capture of a Wayback Machine calendar that shows the original webpage was archived 36 times between 2012 and 2019, and this particular copy was archived on “MAY 29 2013” and available at

<https://web.archive.org/web/20130529112628/http://www.quanterix.com:80/products/assay-kits>. The archive date of “20130529112628” means this webpage was archived on May 29, 2013, at 11 a.m., 26 minutes and 28 seconds. Since the archive date of a web resource captured by Internet Archive indicates the original web resource was available before the archive date, it is my opinion that **EX1034** became discoverable and accessible on the Internet by May 29, 2013.

VIII. Authenticity and Public Availability of *Nalefski* (EX1035)

A. Authentication

107. **EX1035** is a true and correct copy of “Single-molecule detection for femtomolar quantification of proteins in heterogeneous immunoassays,” (“*Nalefski*”), by Nalefski et al., *Clinical Chemistry*, 52(11), 2172-2175 that I received from counsel. This copy is presented as **EX1035** in this declaration.

108. Page 1 (internal page 2172) of **EX1035** shows the title and authors, and the top of the page indicates *Nalefski* is included in the “Abstracts of Oak Ridge Posters.” Page 2 (internal page 2173) shows this article appears in “*Clinical Chemistry* 52, no. 11, 2006.” **EX1035** shows *Nalefski* has a total of four pages including one figure, and nine references. Page 4 (internal page 2175) shows the DOI (digital object identifier) of this article is “10.1373/clinchem.2006.072850.”

109. To authenticate *Nalefski* (**EX1035**), I searched WorldCat.org, the free web portal, for records for this article and a WorldCat record at

<https://search.worldcat.org/title/264178851> showed that the Linda Hall Library (“LHL”) was one of the libraries holding this article. After verifying the holdings information in their online catalog, I obtained a copy of *Nalefski* from LHL via their Document Delivery Service. This LHL copy is presented as **EX1035A** in this declaration.

110. Pages 1 to 4 **EX1035A** show the full text of *Nalefski* (internal pages 2172 to 2175). I have closely compared this library copy with **EX1035** and found them to have the same content.

111. Page 5 is the front cover of vol. 52, no. 11. Page 6 is the title page that identifies this issue as “CLCHAU 52 (11) 1985-2175) (2006)” of *Clinical Chemistry*, which is an international journal of molecular diagnostics and laboratory medicine. This page carries a date stamp of “Linda Hall Library NOV – 7 2006” that indicates the library received this issue on November 7, 2006. Pages 7 to 10 are the table of contents that shows *Nalefski* is included in the “Abstracts of Oak Ridge Posters” section, is the last article in this issue, and begins on page 2172. Page 11 is the copyright page that shows “Clinical Chemistry (ISSN 0009-9147) is published monthly by the American Association for Clinical Chemistry” and this issue has a “2006” copyright date and the publisher holds the copyright.

112. As discussed above, a customary library practice is to place a newly received journal issue through the serial check-in process, make the new issue

publicly accessible as soon as possible, usually within a week after serial check-in is completed. The date stamp on **EX1005A** shows a receipt date of November 7, 2006. It is therefore my opinion that this issue containing *Nalefski* was publicly accessible at LHL by November 14, 2006.

113. **EX1035** is in a condition that creates no suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Furthermore, **EX1035** and the LHL copy (**EX1035A**) have the same content for *Nalefski* and **EX1035A** is scanned from a journal issue held by a well-known public research library. Based on my review and analysis, I see no reason to question the authenticity of **EX1035**.

B. Records of the Linda Hall Library

114. **EX1035B** is a true and correct copy of the bibliographic and MARC serial records for *Clinical Chemistry* whose vol. 52, no. 11 contains *Nalefski*. I personally located, identified and obtained these records from the online catalog of LHL by searching by the journal title for records. These records are the type of records experts in my field would reasonably rely upon when forming their opinions on the public availability of a document.

115. Pages 1 to 3 of **EX1035B** are the bibliographic record that shows *Clinical Chemistry* is available in print at LHL. The journal title, ISSN, and the

publisher match the information presented in **EX1035A**, confirming this record represents *Clinical Chemistry* that contains *Nalefski*.

116. Page 4 is the MARC record. The first six digits of Field 008 show that this MARC serial record was created on “750817” (*i.e.*, August 17, 1975), and the code of “c19559999” following this creation date indicates that the journal is a continuing resource that began publication in 1955 and “9999” means the journal has no predetermined conclusion date of publication. Subfields “a” and “c” of Field 040 show that “MUL” created the original MARC serial record. “MUL” is the MARC organization code for the University of Minnesota, Union List of Serial Cataloging Unit, according to the MARC Code List for Organizations at <https://www.loc.gov/marc/organizations/org-search.php>. Data from Fields 008 and 040 inform my opinion that LHL created a copy cataloging record for *Clinical Chemistry* based on the original MARC serial record created by MUL.

117. As discussed earlier, a common library practice is to create a record for the entire serial, without creating records for individual issues of the serial. When new issues are received, they go through the serial check-in process, which usually takes less than an hour. After that step is completed, the newly received issue is promptly processed and placed on the shelf or in a periodical reading room for user access, usually on the same date of serial check-in or within a week after serial check-in. I know this is the customary library practice from personal experience as a

cataloger, cataloging instructor and researcher of library catalogs and information systems. The date stamp of “NOV – 7 2006” on the LHL copy of *Nalefski* (**EX1005A**) indicates LHL received the journal issue containing this article on November 7, 2006. Based on this date stamp and the LHL serial record for *Clinical Chemistry* (**EX1035B**), it is my opinion that vol. 52, no. 11 (and *Nalefski* contained therein) became publicly accessible at LHL by November 14, 2006.

118. Field 022 of the MARC record (**EX1005B**) shows “0009-9147” as the ISSN for this journal. Field 245 shows the journal title, Field 260 shows the first publisher, Field 362 shows the journal began in February 1955 with vol. 1, no. 1, and Field 310 shows the publication frequency since 1983 has been “Monthly.” Fields 510 show this journal has been indexed by several indexing services. Field 550 shows the American Association of Clinical Chemists was the issuing body of this journal from 1955 and 1975, and the American Association for Clinical Chemistry has been the issuing body of this journal since 1976. These two issuing bodies are included in Field 710 to help users discover this journal.

119. Subjects are also represented in Field 650 by two Library of Congress subject headings, with “Clinical chemistry” and “Biochemistry” as the main topics, each followed by a form subheading “Periodical” encoded in subfield “v” to indicate the main topics are treated in a journal. These subject representations enable users to conduct subject searches to discover this journal.

120. This MARC serial record (**EX1035B**) shows that *Clinical Chemistry* is a long-running journal, and the MARC serial record has made it discoverable in the online catalog of LHL. Interested users would be able to discover this journal by conducting subject searches by the subject headings. They would also be able to discover and access this journal by the journal title, ISSN, and issuing bodies. This MARC record and the date stamp on **EX1035A** inform my opinion that the journal issue containing *Nalefski* became publicly accessible at LHL by November 14, 2006.

121. As evidence that LHL had an online catalog in operation in November 2006 when the library received the journal issue containing *Nalefski*, I obtained a profile of this library at <https://librarytechnology.org/library/2294> from libraries.org, a directory of libraries throughout the world. I personally located, identified, and obtained this profile. This is the type of record experts in my field would reasonably rely upon when forming their opinions. This profile is presented as **EX1035C** in this declaration.

122. The Technology Profile on page 2 of **EX1035C** shows LHL began using Horizon as their online catalog in 1994, and migrated to Voyager in 2010. These dates mean that in 2006 interested users would be able to use the Horizon system at LHL to discover the journal containing *Nalefski*.

C. PubMed Record

123. To locate index records of *Nalefski*, I searched PubMed by the article

title for records. PubMed is an authoritative index system that contains “more than 38 million citations for biomedical literature from MEDLINE, life science journals, and online books” and PubMed records may “include links to full text content from PubMed Central and publisher web sites” (source: <https://pubmed.ncbi.nlm.nih.gov>).

124. **EX1035D** is a true and correct metadata record for *Nalefski* (<https://pubmed.ncbi.nlm.nih.gov/18061992/>) that I personally located, identified and obtained from PubMed. This is the type of record experts in my field would reasonably rely upon when forming their opinions. I saved the metadata record and the PubMed format of the record in PDF to show the record creation dates and other data by data fields.

125. Page 1 of **EX1035D** shows the title, authors, and a full text link to Oxford Academic, and indicates the article was published in “Clin Chem. 2006 Nov; 52(11):2172-5” and its DOI is “10.1373/clinchem.2006.072850.” Data elements in this PubMed metadata record match the information contained in **EX1035**, confirming this metadata record represents *Nalefski*.

126. Pages 2 and 3 of **EX1035D** are the PubMed metadata record saved in the PubMed format to show the data elements in specific fields. I use a copy of the “MEDLINE/PubMed Data Element (Field) Descriptions” (<https://www.nlm.nih.gov/bsd/mms/medlineelements.html>) archived by Internet Archive on July 11, 2007, to interpret this PubMed record with data fields. This

archived copy is available at

<https://web.archive.org/web/20070711034105/https://www.nlm.nih.gov/bsd/mms/metadataelements.html> and presented as **EX1035E** in this declaration.

127. The “PMID” field on page 3 of the PubMed record (**EX1035D**) shows “18061992” as the PubMed identifier for the metadata record for *Nalefski*. The “STAT” field shows the status of the record is “MEDLINE” (meaning the record has been indexed with Medical subject headings and passed the quality assurance routines to be elevated to MEDLINE status, see page 23 of **EX1035E**). The DCOM (“Date Completed”) field is “the date processing of the record ends” meaning “MeSH Headings have been added, quality assurance validations are completed, and the completed record subsequently is distributed to PubMed and licensees” (see page 8 of **EX1035E**). The DCOM field shows the PubMed record for *Nalefski* was completed on “20071219” (*i.e.*, December 19, 2007). The IS field shows the ISSN for the print version of the journal containing *Nalefski* is “0009-9147.” Fields VI (volume), IP (issue), DP (date of publication), Ti (title) and PG (pages) show *Nalefski* was published in vol. 52, no. 11 (2006 Nov), pp. 2172-5. The first FAU (full author) field shows “Nalefski, Eric A” as the first author. The JT (journal title) field shows this article appears in *Clinical Chemistry*. The MH (MeSH terms) fields show five medical subject headings were assigned to indicate the subjects of this article. Field “MHDA” (date of medical subject headings) shows subject index terms were added

to this PubMed record on “2007/12/20” (*i.e.*, December 20, 2007). The “AID” (article identifier) field shows the doi of *Nalefski* is “10.1373/clinchem.2006.072850.”

128. This metadata record (**EX1035D**) made *Nalefski* discoverable in PubMed on December 19, 2007, when the record was completed, and the record was enriched with medical subject headings on December 20, 2007. These dates show that by December 20, 2007, interested users were able to discover the PubMed metadata record for *Nalefski* by the authors, article title, journal ISSN, journal title, abstract, and medical subject headings assigned to this article, and could follow the link to full text to Oxford Academic included in the PubMed record to reach the publisher website to access this article.

129. As evidence that PubMed was in operation when the PubMed record for *Nalefski* became discoverable in December 2007, I searched Internet Archive for records of PubMed. **EX1035F** is a true and correct copy of the PubMed home page archived by Internet Archive. I personally located, identified, and obtained these archived webpages. They are the type of record experts in my field would reasonably rely upon when forming their opinions.

130. Page 1 of **EX1035F** is a screenshot of a Wayback Machine calendar that shows this home page of PubMed was archived 61,109 times between 1997 and 2025, and this particular copy was archived on “MAR 29 2007” and available at

[https://web.archive.org/web/20070329092504/http://www.ncbi.nlm.nih.gov/PubMe](https://web.archive.org/web/20070329092504/http://www.ncbi.nlm.nih.gov/PubMed)

d. This screen capture shows PubMed is a service of the National Library of Medicine, and that as of March 29, 2007, users could search this system by entering one or more search terms, which may be topics, authors or journals. This page also informs visitors that PubMed records “include links to full text articles and other related resources.”

131. The PubMed record for *Nalefski* (**EX1035D**) and related PubMed documents (**EX1035E** and **EX1035F**) inform my opinion that PubMed was already in operation by March 2007, and *Nalefski* became discoverable on PubMed in December 2007. As of December 2007, interested users were able to use PubMed to locate the metadata record for *Nalefski*, then follow the full text link to access the full article from the publisher’s website.

D. Internet Archive Records

132. Since *Clinical Chemistry* that contains *Nalefski* is published online and in print, I searched Internet Archive for information on this journal. **EX1035G** is a true and correct copy of the webpage for vol. 52, no. 11 *Clinical Chemistry*, the webpage on how to access *Nalefski*, the full text of this article in HTML, and the PDF of this article. These web files were archived by the Internet Archive in January 2007, April 2007, and May 11, 2008, respectively. I personally located, identified, and obtained these files to produce **EX1035G**. They are the type of materials experts in

my field would reasonably rely upon when forming their opinions.

133. Internet Archive is a digital library that archives scholarly materials, cultural artifacts, and other web resources on the Internet and provides the public with free access to its collections. I have used the Internet Archive in the past and am familiar with their practice of assigning an extended URL to an archived resource in the format of `http://www.archive.org/web/[Year in yyyy][Month in mm][Day in dd][Time code in hh:mm:ss]/[Archived URL]` to show the point of time the archived file was captured. Since Internet Archive usually does not archive web resources the moment they appear on the Internet, and a web resource must be available first before it can be archived, the archive date of a file captured by the Internet Archive is typically later than the date when the file first became publicly accessible. In other words, the archive date means the archived file was available before, and definitely no later than, the archive date.

134. Page 1 of **EX1035G** is a screen capture of a Wayback Machine calendar that shows this webpage that presents the table of contents of “November 1 2006, Volume 52, Issue 11” of *Clinical Chemistry* was archived 27 times between 2007 and 2022, and this particular copy was archived on “JAN 28 2007” and available at <https://web.archive.org/web/20070128193542/http://www.clinchem.org/content/vol52/issue11/>. Pages 2 to 7 are the PDF of this archived copy that shows the articles included in this issue (each with links to Extract, Full Text, and PDF), and *Nalefski*

is the last article of this issue. It also shows this article is included in the “Abstracts of Oak Ridge Posters” section.

135. Page 8 is a screen capture of the PDF page for *Nalefski*. It includes a Wayback Machine calendar that indicates this webpage was archived five times between 2007 and 2009, and this particular copy was archived by the Internet Archive on “APR 26 2007” and available at <https://web.archive.org/web/20070426113525/http://www.clinchem.org/cgi/reprint/52/11/2172>. Page 9 is the PDF of this archived webpage that shows that this item “requires a subscription to Clinical Chemistry Online” and subscribers could sign in to access the PDF of *Nalefski*, pay to access this article “for 1 day for US\$15.00,” or subscribe “to the print and/or online journal.”

136. Page 10 of **EX1035G** is a screen capture that shows the full article in HTML was archived on May 11, 2008, and available at <https://web.archive.org/web/20080511153458/http://www.clinchem.org/cgi/content/full/52/11/2172>. Pages 11 to 14 are the PDF of this HTML file that identifies this article by title, authors, journal issue, pagination, and DOI, and indicates this article is included in the “Abstracts of Oak Ridge Posters.” The content of this HTML version is the same as the content of **EX1035**, except that the Internet Archive has not archived Figure 1, but the caption for this figure is included in the HTML version.

137. Page 15 of **EX1035G** is a screen capture that shows the PDF of *Nalefski*

was archived on May 11, 2008, and available at <https://web.archive.org/web/20080511152327/http://www.clinchem.org/cgi/reprint/52/11/2172> (follow the “Begin manual download” to reach the PDF presented in a frame). Pages 16 to 19 are the PDF of this article. I have closely compared this archived copy with **EX1035** and found them to have the same content for *Nalefski*.

138. Taken together, the four files in **EX1035G** inform my opinion that the webpage containing the table of contents of vol. 52, no. 11 of *Clinical Chemistry* became publicly discoverable by January 28, 2007, and interested users would be able to discover *Nalefski* from this webpage and follow the links to access the full article. The webpage for accessing the PDF of *Nalefski* was first archived on April 26, 2007, and it informed visitors to the webpage that subscribers to the journal could access the full articles right away and nonsubscribers could pay \$15 to access this article for one day. Access is further confirmed by the HTML version and the PDF version of the full article archived on May 11, 2008. Based on these findings, it is my opinion that *Nalefski* became publicly discoverable and accessible on the Internet by January 28, 2007. While the webpage for accessing the PDF of *Nalefski* and the full articles were archived in April 2007 and May 2008, respectively, it is helpful to keep in mind that there is usually a time lag between the time a web resource first became publicly accessible on the Internet and the time when it is archived by the Internet Archive. The archive dates of April 2007 and May 2008 serve to confirm that *Nalefski*

was accessible by April 2007, and definitely no later than May 2008 when the full text was archived. The January 2007 archived date of the table of contents of vol. 52, no. 11 of *Clinical Chemistry* shows that *Nalefski* was in fact discoverable and accessible by the January 2007 archive date.

E. Usage Record

139. Actual usage of a publication is reflected by the publications that make reference to it. The citation history on Google Scholar shows *Nalefski* has been cited 19 times. I have reviewed the citations to identify four early citations. **EX1035H** shows citations from 2008 and 2009, and the earliest citation was published in October 2008, supporting my opinion on the public availability of *Nalefski* in 2007.

F. Summary of My Opinion on *Nalefski*

140. **EX1035** is authentic because it contains no signs to raise questions about its authenticity, its content is identical to the LHL copy (**EX1035A**), and the article is in the custody of a public research library.

141. The date stamp on the LHL copy (**EX1035A**) shows the library received the issue containing *Nalefski* on November 7, 2006, and the LHL MARC record for *Clinical Chemistry* (**EX1035B**) shows the library created a copy cataloging record for this journal, making this journal discoverable in this library. These LHL data inform my opinion that *Nalefski* was publicly accessible at this library by at least November 7, 2006 if not earlier, and the physical copy, after processing, would

become publicly accessible by November 14, 2006.

142. The PubMed record for *Nalefski* (**EX1035D**) and my knowledge of PubMed and MEDLINE inform my opinion that this PubMed record was completed on December 19, 2007, and indexed with medical subject headings on December 20, 2007, making this record discoverable on PubMed by subject terms and other data element from that date on.

143. **EX1035G** presents four files to show that the webpage containing the table of contents of vol. 52, no. 11 of *Clinical Chemistry* became publicly discoverable on January 28, 2007, and users could use this webpage to access *Nalefski*. The webpage for accessing the PDF of *Nalefski*, archived on April 26, 2007, informed visitors that subscribers to the journal could access the full article after signing in, and nonsubscribers could pay \$15 to access the article. The HTML version and the PDF version of *Nalefski* were first archived on May 11, 2008, confirming the full article was publicly accessible no later than that date.

144. Based on the documents and records I have reviewed, it is my opinion that the print copy of *Nalefski* was publicly accessible at the Linda Hall Library by November 14, 2006. This article became discoverable on PubMed by December 2007, and interested users could use the PubMed record to access the full article from the publisher's website. This article actually became publicly accessible on the Internet by January 2007, because webpages of *Clinical Chemistry* archived by the

Internet Archive show that *Nalefski* became publicly discoverable and accessible by January 28, 2007, and by April 26, 2007, interested users were informed that journal subscribers could access the full article online, and nonsubscribers could pay a small fee to access the full article.

IX. Authenticity and Public Availability of $A\beta$ -42 (EX1036)

A. Authentication

145. **EX1036** is a true and correct copy of a webpage for “ $A\beta$ -42 (Amyloid beta protein 42),” (“ $A\beta$ -42”), from Singulex, that I received from counsel. The top of this page identifies $A\beta$ -42 as a “Singulex Technology” and this webpage includes a summary of the significance of “ $A\beta$ -40/ $A\beta$ -42 as a velocity biomarker in Alzheimer’s disease studies and to evaluate therapeutic interventions.” The webpage includes Biology and Diseases, Therapies, Unmet Need, Singulex Answer, and Application note with a link for download. This webpage shows a copyright date of “2006-2009” with Singulex as the copyright holder. This copy is presented as **EX1036** in this declaration.

146. To authenticate **EX1036**, I searched Internet Archive for records of this document. **EX1036A** is a true and correct copy that I personally located, identified, and obtained from Internet Archive. This is the type of material experts in my field would reasonably rely upon when forming their opinions. I have closely compared **EX1036** and **EX1036A** and found them to be the same.

147. **EX1036** is in a condition that raises no suspicion about its authenticity. Specifically, the text in *Aβ-42* flows logically, and there are no visible alterations to this webpage. Furthermore, **EX1036** and an archived copy obtained from Internet Archive (**EX1036A**) have the same content. Based on my review and analysis, I have no reason to question the authenticity of **EX1036**.

B. Public Availability Date

148. Page 1 of **EX1036A** is a screen capture of a Wayback Machine calendar that shows the original webpage was archived three times between 2007 and 2010, and this particular copy was archived on “SEP 12 2009” and available at https://web.archive.org/web/20090912044340/http://www.singulex.com:80/assay_details.html?id=2. The archive date of “20090912044340” means this webpage was archived on September 12, 2009, at 4 a.m., 43 minutes, and 40 seconds. Since the archive date on a web document archived by the Internet Archive indicates the original file was available before the archive date, it is my opinion that **EX1036** became discoverable and accessible on the Internet by September 12, 2009.

X. Authenticity and Public Availability of *Bourzac* (EX1011)

A. Authentication

149. **EX1011** is a true and correct copy of “Next-Generation Diagnostics: A startup can detect tiny traces of cancer markers in blood samples,” (“*Bourzac*”), by Katherine Bourzac, *MIT Technology Review*, May 13, 2008, that I received from

counsel. This copy is presented as **EX1011** in this declaration.

150. Pages 1 and 2 of **EX1011** are the full text of *Bourzac*. Page 1 shows the title, author, and publication date of this article, and indicates this article is available online at <https://www.technologyreview.com/2008/05/13/220548/next-generation-diagnostics/>. Page 1 includes a figure that has a caption title of “Lab on a tip” and a brief description of this figure. **EX1011** shows *Bourzac* includes 10 paragraphs.

151. Pages 3 to 5 are the online version of *Bourzac* that shows the article URL (<https://www.technologyreview.com/2008/05/13/220548/next-generation-diagnostics/>) at the bottom of each page. The content of this online version is the same as the content on pages 1 and 2 of **EX1011**, except that Paragraph 2 (“The premise is ...”) and the third paragraph from the end (“So little is known about ...”) were not fully saved in the capturing process.

152. To authenticate **EX1011**, I looked for *Bourzac* at <https://www.technologyreview.com/2008/05/13/220548/next-generation-diagnostics/> and downloaded the article from the MIT Technology Review website. This copy from the Internet is presented as **EX1011A** in this declaration. I have closely compared **EX1011A** with **EX1011**, and found that the content of **EX1011A** is the same as the content presented on pages 1 and 2 of **EX1011**.

153. **EX1011** is in a condition that creates no suspicion about its

authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. In addition, the content of pages 1 and 2 of **EX1011** is identical to the content of the online version obtained from the MIT Technology Review website (**EX1011A**). Based on my review and analysis, I see no reason to question the authenticity of **EX1011**.

B. Internet Archive Records

154. Since *Bourzac* was published online, I searched for records in Internet Archive to determine the earliest public availability date of this article. **EX1011B** is a true and correct copy of *Bourzac*. I personally located, identified, and obtained the files included in **EX1011B** from Internet Archive. These files are the type of material experts in my field would reasonably rely upon when forming their opinions. I used screen captures to produce this exhibit.

155. The top of Page 1 of **EX1011B** is a Wayback Machine calendar that shows *Bourzac* was archived 16 times by the Internet Archive between 2008 and 2022, and this particular copy was archived on “May 13 2008” and available at <https://web.archive.org/web/20080513175840/http://www.technologyreview.com/Biztech/20760/>. The archive date of “20080513175840” means this file was captured by the Internet Archive on May 13, 2008, at 5 p.m., 58 minutes and 40 seconds. Page 1 shows the title and author of *Bourzac* and indicates this article was published on

“Tuesday, May 13, 2008” on the website of Technology Review published by MIT. Pages 1 to 3 show the first six paragraphs of *Bourzac*, and page 2 shows a figure with a caption title of “Lab on a tip” and a brief description of the figure. Page 3 shows the rest of the article and includes a link for users to continue to page 2 of the article.

156. Pages 4 and 5 of **EX1011B** present the last four paragraphs of *Bourzac*. The top of page 4 is a Wayback Machine calendar that shows page 2 of *Bourzac* was archived three times, and this particular copy was archived on “May 17 2008” and available at <https://web.archive.org/web/20080517045755/http://www.technologyreview.com/Biztech/20760/page2/>. The “20080517045755” archive date means this file was captured by the Internet Archive on May 17, 2008, at 4 a.m., 57 minutes and 55 seconds. Under the article title on page 4, a note informs readers that this file is “[c]ontinued from page 1.”

157. I have closely compared the two files in **EX1011B** with **EX1011** and found the content of **EX1011B** is the same as the content presented by pages 1 and 2 of **EX1011**. As discussed earlier, the archive date of a web file captured by the Internet Archive is usually later than the first public availability date of the original web file because Internet Archive usually does not capture web files as soon as they appear on the Internet. **EX1011B** shows that page 1 of the online version of *Bourzac* was archived by the Internet Archive on May 13, 2008, and page 2 of the online

version of *Bourzac* was archived by the Internet Archive on May 17, 2008. While these two files were archived by the Internet Archive on two different days, the archive dates reflect the archiving activities of the Internet Archive, and do not mean that the online version of *Bourzac* was published on the MIT Technology Review website on two separate days. In fact, both files included in **EX1011B** show “Tuesday, May 13, 2008” as the publication date of this article. **EX1011B** informs my opinion that the online version of *Bourzac* became publicly accessible on the Internet by May 13, 2008.

C. Summary of My Opinion on *Bourzac*

158. **EX1011** is authentic because it contains no signs to raise questions about its authenticity, its content is identical to the copy obtained from the MIT Technology Review website (**EX1011A**) and the online version archived by the Internet Archive (**EX1011B**).

159. **EX1011B** shows the online version of *Bourzac* was archived by the Internet Archive on May 13 and May 17 of 2008. Since the two files in **EX1011B** show “Tuesday, May 13, 2008” as the publication date of this online version, it is my opinion that the online version of *Bourzac* became publicly accessible on the Internet by May 13, 2008.

CONCLUSION AND DECLARATION

My findings and opinions set forth in this Declaration are based on my work and examination to date. I may continue my examinations in view of additional documents or other factual evidence over the course of this proceeding that may necessitate supplementing and/or refining my opinions. I reserve the right to add to, alter, or delete my opinions and my Declaration upon discovery of additional information. I reserve the right to make such changes as necessary.

In signing this Declaration, I understand that the Declaration will be filed as evidence in a contested case before the Patent Trial and Appeal Board of the United States Patent and Trademark Office. I acknowledge that I may be subject to cross-examination in this case and that cross-examination will take place within the United States. If cross-examination is required of me, I will appear for cross-examination within the United States during the time allotted for cross-examination.

I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code.

[SIGNATURE ON FOLLOWING PAGE]

Date: May 28, 2015

By:

Ingrid Hsieh-Yee

Ingrid Hsieh-Yee, Ph.D.
Annapolis, Maryland

Appendix A Hsieh-Yee CV

Ingrid Hsieh-Yee
Professor Emerita
Dept. of Library and Information Science
Catholic University of America
Washington, D.C. 20064
E-mail: hsiehyee@cua.edu

Education

Ph.D. 1990. Library and Information Studies, University of Wisconsin-Madison
Minors: Sociology and Psychology

M.A. 1984. Library and Information Studies, University of Wisconsin-Madison.

M.A. 1983. Comparative Literature, University of Wisconsin-Madison.

B.A. 1980. Foreign Languages and Literature, National Taiwan University.

Work Experience

Professor, School/Dept. of Library and Information Science, Catholic University of America,
1990-2022 (Assistant Professor, 1990-1996; Associate Professor, 1997-2004; Professor
2004-2022; Professor Emerita 2022)

Co-Chair, Dept. of Library and Information Science, Catholic University of America, June 2015-
August 2016.

Acting Dean, School of Library and Information Science, Catholic University of America,
January 2010-June 2012.

Cataloger, Dept. of Legislative Reference Library, Annapolis, Maryland, 1989-1990.

Lecturer, School of Library and Information Studies, University of Wisconsin-Madison, 1988.

Teaching Assistant, School of Library and Information Studies, University of Wisconsin-
Madison, 1986-1988.

Cataloger, Health Sciences Library, University of Wisconsin-Madison, 1984-1986.

Areas of Teaching and Research Interests

Information Organization and Access; Metadata; Cataloging & Classification; Information
Architecture; Information Retrieval; Digital Collections; Social Media; Scholarly

Communication; Information Behavior; Health Informatics; Human Computer Interaction; Usability Studies

Grants & Honors

Cultural Heritage Information Management Project. IMLS grant. Amount: \$498,741. Period: Aug. 2012 to July 2015. Co-PI with Dr. Youngok Choi.

D.C. Health Information Technology (HIT4): Building Capacity & Providing Access in Our Nation's Capital. Dept. of Labor H2B Training Grant. Grant amount: \$4,175,500. Grant period: Nov. 2011 to Dec. 2015. Partner with the Metropolitan School of Professional Studies of the Catholic University of America, Children's National Medical Center, D.C. Department of Employment Services, Holy Cross Hospital, Howard University, Center for Urban Progress, Providence Hospital, and Sibley Memorial Hospital.

Capital Health Careers Project. Department of Labor Healthcare Sector and Other High Growth and Emerging Industries Grant. Grant amount: \$4,953,999. Grant period: March 2010 – February 2013. Awarded to a group of healthcare organizations and educational institutions in Washington, D.C. Providence Health Foundation of Providence Hospital (Lead institution). Part of the grant supported the development of a Master's degree program in Information Technology with a concentration in Health Information Technology offered by the School of Library and Information Science.

The Washington D.C. School Librarians Project. IMLS grant. Grant amount: \$412,660. Grant period: Aug. 2007 – June 2011. The School partnered with the District of Columbia Public Schools (DCPS) and the District of Columbia Library Association to educate and mentor school media specialists for the DCPS system. PI, Jan. 2010 to June 2011.

SIG Member of the Year, American Society for Information Science and Technology (2009).

Most Outstanding Paper of *OCLC Systems & Services* (2001).

ALISE Research Grant (2001).

Most Outstanding Paper of *OCLC Systems & Services* (2000).

Research Grant from ERIC (1999-2000).

Best Research Paper Award; Association for Library and Information Science Education (1998).

Research Grants, Catholic University of America. 1991, 1992, 1993, 1996, 1998, 1999, 2004, 2005, 2006, 2007, 2013-14.

Cooperative Faculty Research Grant, Consortium of Universities in the Washington Metropolitan Area (1993-1994).

Cooperative Research Grant, Council on Library Resources (1993-1994).

Journal of the American Society for Information Science Best Paper Award (1993).

ASIS/ISI Information Science Doctoral Dissertation Scholarship (1989).

HEA Title IIB Fellowship (Dept. of Education) (1989)

Chinese-American Librarians Association Scholarship (1987).

Beta Phi Mu (1985).

Vilas Fellowship, University of Wisconsin-Madison. 1984

Publications

Hsieh-Yee, I. (2021). Can We Trust Social Media? *Internet Reference Services Quarterly*, 1-15.
Published online: 28 July, 2021. 10.1080/10875301.2021.1947433.

Bailey, T., & Hsieh-Yee, I. (2020). Combating the Sharing of False Information: History, Framework & Literacy Strategies, *Internet Reference Services Quarterly*, 24 (1-2), 9-30.

Choi, Y., and Hsieh-Yee, I. (2010). Finding Images in an OPAC: Analysis of User Queries, Subject Headings, and Description Notes. *Canadian Journal of Information and Library Science*, 34(3): 271 – 295.

Hsieh-Yee, I. (2008). Educating Cataloging Professionals in a Changing Information Environment. *Journal of Education for Library and Information Science*, 46(2): 93-106.

Vellucci, S. L., Hsieh-Yee, I., and Moen, W.E. (2007). The Metadata Education and Research Information Commons (MERIC): A Collaborative Teaching and Research Initiative. *Education for Information*, 25(3&4): 169-178.

NISO Framework for Guidelines for Building Good Digital Collections. 3rd ed. Baltimore, MD: National Information Standards Organization, 2007. Also available online: <http://www.niso.org/framework/framework3.pdf> (NISO Working Group members: Priscilla Caplan (chair), Grace Agnew, Murtha Baca, Tony Gill, Carl Fleischhauer, Ingrid Hsieh-Yee, Jill Koelling, and Christie Stephenson.)

Choi, Y., Hsieh-Yee, I., and Kules, B. (2007). Retrieval Effectiveness of TOC and LCSH. *Proceedings of the Joint Conference on Digital Libraries*, pp. 233-234.

Vellucci, S., and Hsieh-Yee, I. (2007). They Didn't Teach Me That in Library School! Building

a Digital Teaching Commons to Enhance Metadata Teaching, Learning and Research. *Proceedings of the National Conference of the Association of College and Research Libraries, Baltimore, MD*, pp. 26-31.

Mitchell, Vanessa, and Ingrid Hsieh-Yee. (2007). Converting Ulrich's Subject Headings to FAST Headings: A Feasibility Study. *Cataloging & Classification Quarterly*, 45(1): 59-85.

Hsieh-Yee, I., Tang, R., and Zhang, S. (2007). User Perceptions of a Federated Search System. *IEEE Technical Committee on Digital Libraries Bulletin*, Summer 3(2) (URL = <http://www.ieee-tcdl.org>).

Tang, R., Hsieh-Yee, I., and Zhang, S. (2007). User Perceptions of MetaLib Combined Search: An Investigation of How Users Make Sense of Federated Searching." *Internet Reference Services Quarterly*, 12(12): 211-236.

Hsieh-Yee, I., Tang, R., and Zhang, S. (2006). User Perceptions of a Federated Search System. *Proceedings of the Joint Conference on Digital Libraries, June 11-15, 2006, Chapel Hill*, p. 338.

Hsieh-Yee, I. (2006). *Organizing Audiovisual and Electronic Resources for Access: A Cataloging Guide*. 2nd ed. Westport, Conn.: Libraries Unlimited.

NISO A Framework of Guidance for Building Good Digital Collections. 2nd ed. Bethesda, MD: National Information Standards Organization, 2004. Framework Advisory Group: Grace Agnew, Liz Bishoff, Priscilla Caplan (Chair), Rebecca Gunther and Ingrid Hsieh-Yee.

Hsieh-Yee, I. (2004). Cataloging and Metadata Education in North American LIS Programs. *Library Resources & Technical Services*, 48(1): 59-68.

Hsieh-Yee, I. (2004). Cataloging and Metadata Education. In Gary E. Gorman (Ed.), *International Yearbook of Library and Information Management 2003: Metadata Applications and Management*, (pp.204-234). London: Facet Publishing.

Yee, P. L., Hsieh-Yee, I., Pierce, G.R., Grome, R., and Schantz, L. (2004). Self-Evaluative Intrusive Thoughts Impede Successful Searching on the Internet. *Computers in Human Behavior*, 20(1): 85-101.

Hsieh-Yee, I. (2003). Cataloging and Metadata Education: A Proposal for Preparing Cataloging Professionals of the 21st Century. A report submitted to the ALCTS-Education Task Force in response to Action Item 5.1 of the *Bibliographic control of Web Resources: A Library of Congress Action Plan*. Approved by the Association for Library Collections and Technical Services. Web version available since April 2003 at <http://lcweb.loc.gov/catdir/bibcontrol/CatalogingandMetadataEducation.pdf>.

- Hsieh-Yee, I. (2002). Cataloging and Metadata Education: Asserting a Central Role in Information Organization. *Cataloging & Classification Quarterly* 34(½): 203-222.
- Hsieh-Yee, I., and Smith, M. (2001). The CORC Experience: Survey of Founding Libraries, Part I. *OCLC Systems & Services*, 17: 133-140. (Received "The Most Outstanding Paper of OCLC Systems & Services in 2001" award.)
- Hsieh-Yee, I., and Smith, M. (2001). The CORC Experience: Survey of Founding Libraries, Part II, Automated Tools and Usage. *OCLC Systems & Services*, 17: 166-177. (Received "The Most Outstanding Paper of OCLC Systems & Services in 2001" award.)
- Hsieh-Yee, I. (2001). ERIC User Services: Changes and Evaluation for the Future. *Government Information Quarterly*, 18: 31-42.
- Hsieh-Yee, Ingrid. (2001). Research on Web Search Behavior. *Library and Information Science Research*, 23: 167-185.
- Logan, E., and Hsieh-Yee, I. (2001). Library and Information Science Education in the Nineties. *Annual Review of Information Science and Technology*, 35: 425-477.
- Hsieh-Yee, I. (Ed.) (2001). *Library and Information Science Research*, 23 (2). A special issue in honor of the retirement of Douglas L. Zweizig.
- Hsieh-Yee, I. (2000). *ERIC User Services: Evaluation in a Decentralized Environment*. Washington, D.C.: Dept. of Education.
- Hsieh-Yee, Ingrid. (2000). *Organizing Audiovisual and Electronic Resources for Access: A Cataloging Guide*. Littleton, CO: Libraries Unlimited.
- Hsieh-Yee, I. (2000). Organizing Internet Resources: Teaching Cataloging Standards and Beyond. *OCLC Systems & Services*, 16: 130-143. (Received "The Most Outstanding Paper of OCLC Systems & Services in 2000" award.)
- Hsieh-Yee, I. (1998). The Retrieval Power of Selected Search Engines: How Well Do They Address General Reference Questions and Subject Questions? *Reference Librarian*, 60: 27-47.
- Hsieh-Yee, I. (1998). Search Tactics of Web Users in Searching for Texts, Graphics, Known Items and Subjects: A Search Simulation Study. *Reference Librarian*, 60: 61-85. (Received the 1997 Best ALISE Research Paper Award.)
- Hsieh-Yee, I. (1997). Access to OCLC and Internet Resources: LIS Educators' Views and Teaching Practices. *RQ*, 36: 569-86.

- Hsieh-Yee, I. (1997). Teaching Online and CD-ROM Resources: LIS Educators' Views and Practices. *Journal of Education for Library and Information Science*, 38: 14-34.
- Hsieh-Yee, I. (1996). The Cataloging Practices of Special Libraries and Their Relationship with OCLC. *Special Libraries*, 87: 10-20.
- Hsieh-Yee, I. (1996). Modifying Cataloging Practice and OCLC Infrastructure for Effective Organization of Internet Resources. In *Proceedings of the OCLC Internet Cataloging Colloquium*. [Online]. Available: <http://www.oclc.org/oclc/man/colloq/hsieh.htm>
- Hsieh-Yee, I. (1996). Student Use of Online Catalogs and Other Information Channels. *College & Research Libraries*, 57: 161-175.
- Hsieh-Yee, I. (1995). Ten entries in James S. C. Hu (Ed.), *Encyclopedia of Library & Information Science*, 913, 1028-29, 1036, 1037, 1145-46, 1514, 1575, 1763-64, 2216-27, 2378-79. Taipei, Taiwan: Sino-American Publishing. (Topics include "Advanced Technology/Libraries," "Information Ethics," "Instruction on Cataloging and Classification," "Instruction on Reference Services.")
- Hsieh-Yee, I. (1993). Effects of Search Experience and Subject Knowledge on Online Search Behavior: Measuring the Search Tactics of Novice and Experienced Searchers. *Journal of the American Society for Information Science*, 44: 161-174. (Received the 1993 Best JASIS Paper Award.)

Works in Progress

- Hsieh-Yee, I. Actionable lessons for debunking false information: A comparative analysis of literacy lessons in communication and library and information science
- Hsieh-Yee, I. Representing diverse voices and communities in cataloging and indexing: The evolution of controlled vocabularies
- Hsieh-Yee, I. Information Organization and Retrieval in Practice (a monograph scheduled for completion and publication in 2023)

Presentations

- Bailey, T. & Hsieh-Yee, I. (February 2020). The Phenomena of Sharing Misinformation and the Need for Information Literacy. Presented at the 2020 Bridging the Spectrum Symposium, Washington, D.C., February 14, 2020.

- Hsieh-Yee, I. and Fragan-Fly, J. (May 2018) *Trends, Design & Strategies for Digital Scholarship Services*. Presented at the 2018 Maryland/Delaware Library Association Conference, Cambridge, MD.
- Hsieh-Yee, I. (February, 2018) *Research Data Management: What It Takes to Succeed*. Presented at the 10th Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee, I. (February, 2017) *Research Data Management: New Competencies and Opportunities for Information Professionals*. Presented at the 9th Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee, I. and Lawton, P. (February, 2017) *Enhancing Catholic Portal Searches with User Terms and LCSH*. Presented at the 9th Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee, I. (2016, October) *Visualizing Data for Information*. Presented at the 2016 Virginia Library Association Conference, Hot Springs, VA.
- Hsieh-Yee, I. (2016, August) *Religious Materials Toolbox for Archivists: Solutions to Problems Facing the Profession*. Presented at Archives * Records 2016, Atlanta, GA.
- Hsieh-Yee, I. and Lawton, P. (2016, March) *Enhancing Retrieval of Catholic Materials with LCSH Knowledge Structure*. Presented at the 2016 Catholic Library Association Conference, San Diego, CA.
- Fagan-Fry, J. and Hsieh-Yee, I. (2016, February) *Approaches to Digital Scholarship at Top Universities around the World: Scholarly Publishing in the Digital Age*. Presented at the 8th Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee and Fagan-Fry, J. (2016, January) *Innovative Services for Digital Scholarship at Top 100 Research Libraries of the World*. Poster presented at the 2016 Annual Conference of the Association for Library and Information Science Education, Boston, Mass.
- Hsieh-Yee, I. and Lawton, P. (2015, June). *Crowdsourcing terms for CRRA portal themes*. Poster presented at the third CRRA symposium and annual meeting, Bringing the created toward the Creator: Liturgical art and design since Vatican II. Catholic Theological Union, Chicago, Illinois.
- Hsieh-Yee, I. and Lawton, P. (2015, February). *Crowdsourcing terms for thematic exploration in the Catholic Portal*. Poster presented at the 7th Annual Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee, I., James, R., and Fagan-Fry, J. (2015, February). *Support for digital scholarship at top university libraries of the world*. Poster presented at the 7th Annual Bridging the Spectrum Symposium, Washington, D.C.

- Hsieh-Yee, I., Zhang, S., Lin, K., and Cherry, S. (2015, February). *Thus said the end users: Summon experience and support for research workflows*. Poster presented at the 7th Annual Bridging the Spectrum Symposium, Washington, D.C.
- Yontz, E., Hsieh-Yee, I., & Houston, S. (2015, February). *Healthy Heroes Summer Reading Club: Developing healthy youth at public libraries*. 11th Annual Jean Mills Health Symposium, Greenville, North Carolina.
- Yontz, E., Hsieh-Yee, I., and Houston, S. (2015, January). *Healthy youth and libraries: A pilot study*. Association for Library & Information Science Education (ALISE) Annual Conference, Chicago, Illinois.
- Hsieh-Yee, I. (2014, May). *Linking CRRA resources to portal themes via authority files*. Presented at the Catholic Research Resources Alliance 2014 Membership Meeting, Marquette, WI.
- Hsieh-Yee, I. (2014, April). *Enhancing subject access to CRRA resources*. Presented at the 2014 Catholic Library Association Conference, Pittsburgh, PA.
- Hsieh-Yee, I. (2014, January). *Health Information Technology Program: Educational entrepreneurship in action*. Presented at the 2014 annual Conference of the Association for Library and Information Science Education, Philadelphia, PA.
- Hsieh-Yee, I., Zhang, S., Lin, K., and Cherry, S. (2014, January). *Discovering information through Summon: An analysis of user search strategies and search success*. Paper presented at the 6th Bridging the Spectrum Symposium, Washington, D.C.
- Hsieh-Yee, I. (2012, December). *National Digital Stewardship Alliance and SLIS at CUA: An Educational Partnership*. Paper presented at Best Practices Exchange: Acquiring, Preserving, and Providing Access to Government Information in the Digital Era, Annapolis, MD.
- Choi, Y. and Hsieh-Yee, I. (2010, January). *Finding Images in an OPAC: Analysis of User Queries, Subject Headings, and Description Notes*. Paper presented at 2nd Annual Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I. and Coogan, J. (2010, January). *Google Scholar vs. Academic Search Premier: What Libraries and Searchers Need to Know*. Paper presented at 2nd Annual Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I. (2009, November). *Information Science Education: An LIS School's Perspective*. Paper presented at Annual Meeting of the American Society for Information Science and Technology, Vancouver, British Columbia, Canada.

- Hsieh-Yee, I., Menard, E., Ya-Ning Chen, A., Shu-Jiun Chen, S., Kalfatovic, M. R., Wisser, K. M. (2009, November). *Information Organization in Libraries, Archives and Museums: Converging Practices and Collaboration Opportunities*. Presented at Annual Meeting of the American Society for Information Science and Technology, Vancouver, British Columbia, Canada. (Organizer and moderator of this panel.)
- Hsieh-Yee, I. and Coogan, J. (2009, July). *Catching up to Google Scholar: The Retrieval Power of Academic Search Premier and Google Scholar*. Poster presented at American Library Association Conference, Chicago, Illinois.
- Hsieh-Yee, I., with the CUA Scholarly Communications Project Team. (2009, January). *Digital Scholarship@CUA: Developing an Institutional Repository for CUA*. Poster presented at 1st Annual Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.
- Wise, M., Cylke, K., and Hsieh-Yee, I. (2009, January). *Digital Talking Books: Meeting the Needs of the Blind and the Handicapped*. Paper presented at the Bridging the Spectrum Symposium, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I. (2009, January). *User Expectations of MERIC*. Presented at the Information Organization Competencies for the 21st Century Discussion Session of the 2009 Conference of the Association for Library and Information Science Education, Denver, Colorado.
- Choi, Y., and Hsieh-Yee, I. (2008, November). *Subject Access for Images in an OPAC*. Annual Meeting of the American Society for Information Science and Technology, Columbus, Ohio. (Also co-organized a panel on Retrieving and Using Visual Resources: Challenges and Opportunities for Research and Education.)
- Hsieh-Yee, I. (2008, June). *Educating Cataloging Professionals in a Changing Information Environment*. National Taiwan University, Taipei, Taiwan.
- Vellucci, S. L., Moen, W.E., Hsieh-Yee, I., Marson, B., and Wisser, K. (2008, January) *Building a Metadata Education and Research Community through MERIC (Metadata Education and Research Information Commons): Demo and Stakeholder Input*. A panel presented at the 2008 Conference of the Association for Library and Information Science Education, Philadelphia, Pennsylvania.
- Hsieh-Yee, I., Choi, Y. and Kules, B. (2007, October). *Searching for Books and Images in OPAC: Effects of LCSH, TOC and Subject Domains*. A poster presented at the American Society for Information Science and Technology Annual Meeting, Milwaukee, Wisconsin.
- Hsieh-Yee, I. and Coogan, J. (2007, August) *Google Scholar vs. Academic Search Premier: A Comparative Analysis*. Presented to the Faculty and Staff of the University of the District

of Columbia.

- Hsieh-Yee, I. and Coogan, J. (2007, June). *Google Scholar vs. Academic Search Premier: A Comparative Analysis*. Presented to the Washington Research Library Consortium Community, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I., Choi, Y., and Kules, B.. (2007, June). *What Users Need for Subject Access: Table of Contents or Subject Headings?* A poster presented at the 2007 American Library Association Annual Conference, Washington, D.C., June 2007.
- Choi, Y., Hsieh-Yee, I., and Kules, B. (2007, June). *Retrieval Effectiveness of TOC and LCSH*. A paper presented at the Joint Conference on Digital Libraries 2007, Vancouver, Canada.
- Vellucci, S. L., Hsieh-Yee, I., and Moen, W.E. (2007, May). *If We Build It, Will They Come? Building a Community of Practice for Metadata Stakeholders*. A poster presented at the Rutgers University Research Day, Bridgeton, New Jersey.
- Hsieh-Yee, I. (2007, May). *Federated Searching: User Experience & Perceptions*. International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.
- Hsieh-Yee, I. (2007, May). *Search Performance of Google Scholar and Academic Search Premier*. International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.
- Hsieh-Yee, I. (2007, May) *MERIC: Building a Digital Commons for Metadata Education & Research*. International Conference on Information Organization & Retrieval, National Taiwan University, Taipei, Taiwan.
- Hsieh-Yee, I., and Coogan, J. (2007, March/April). *A Comparative Analysis of Google Scholar and Academic Search Premier*. Poster presented at the Association of College & Research Libraries 13th National Conference, Baltimore, Maryland.
- Vellucci, S. L. and Hsieh-Yee, I. (2007, March/April) *They Didn't Teach Me That in Library School! Building a Digital Teaching Commons to Enhance Metadata Teaching, Learning and Research*. On-site presentation and Webcast by Elluminate. A contributed paper presented at the Association of College & Research Libraries 13th National Conference, Baltimore, Maryland. The acceptance rate for contributed paper was 20%. This paper was one of 10 conference papers chosen for live webcast during the conference.
- Moen, W., Hsieh-Yee, I. and Vellucci, S.L. (2007, January) *A DSpace Foundation for a Teaching & Research Commons: The Metadata Education and Research Information Commons*. A poster session presented at the Open Repositories Conference 2007, San Antonio, Texas.

- Tang, R., Hsieh-Yee, I., and Zhang, S. (2006, November) *User Perception of MetaLib Combined Search*. Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas, Nov. 2006.
- Hsieh-Yee, I. (2006, November). *Federated Searching: User Perceptions, System Design, and Library Instructions*. Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas. (Panel organizer, moderator, presenter).
- Hsieh-Yee, I. (2006, November). *Building a Digital Teaching Commons to Enhance Teaching and Learning: The MERIC Experience and Challenges*. Paper presented at the Annual Meeting of the American Society for Information Science and Technology, Austin, Texas. (Panel organizer, moderator, presenter)
- Hsieh-Yee, I. (2006, September). *Search Performance of Google Scholar and Academic Search Premier*. Paper presented at the ERIC Publishers Meeting, Washington, D.C.
- Hsieh-Yee, I., Zhang, S., and Rong Tang, R. (2006, June). *User Perceptions of a Federated Search System*. Poster presented at Joint Conference on Digital Libraries, Chapel Hill, North Carolina.
- Hsieh-Yee, I. and Zhang, S. (2006, June). *Preparing Users for Federated Search: Implications of a MetaLib User Perceptions Study*. Paper presented at the 2006 Ex Libris User Groups of North America Conference, Knoxville, Tennessee.
- Hsieh-Yee, I. (2006, January). *MERIC Organizations and Navigation*. Paper presented at the 2006 ALISE Annual Conference, San Antonio, Texas.
- Hsieh-Yee, I. (2006, January). *Metadata and Cataloging Education: Recommended Competencies*. Paper presented at the 2006 ALISE Annual Conference, San Antonio, Texas.
- Hsieh-Yee, I. (2005, November). *Digital Library Evaluation: Progress & Next Steps*. Presentation at the Annual Meeting of the American Society for Information Science & Technology, Charlotte, North Carolina.
- Hsieh-Yee, I. (2005, August). *Providing Access to Digital Content: Issues for DL Managers*. Presentation at MDK12 Digital Library Steering Committee Meeting, Columbia, Maryland.
- Hsieh-Yee, I. (2005, April). *Enhancing Teaching and Learning: The Role of School Library Media Specialists*. Presentation at Meeting of the Baltimore County Public School System School Media Specialists, Baltimore, Maryland.

- Hsieh-Yee, I. (2005, January). *Subject Access and Users: Insights & Inspirations from Marcia J. Bates*. Paper presented at the Historical Perspectives SIG, 2005 Conference of the Association for Library and Information Science Education, Boston, Massachusetts.
- Hsieh-Yee, I. (2005, January). *Electronic Resource Management: Practice, Employer Expectations, & CE Interests*. Paper presented at Technical Services Education SIG, 2005 Conference of the Association for Library and Information Science Education, Boston, Massachusetts.
- Hsieh-Yee, I. (2004, October). *Library Professionals for the Digital Age: Competencies & Preparation*. Paper presented at Bibliographic Access Management Team meeting, Library of Congress, Washington, D.C.
- Hsieh-Yee, I. (2004, January). *Cataloging and metadata expertise for the digital era*. Presented at Preparing 21st Century Cataloging and Metadata Professionals: A Workshop for Educators and Trainers, San Diego and sponsored by ALCTS, ALISE, LC, and OCLC.
- Hsieh-Yee, I. (2004, January). *Educating catalogers for the digital era*. Paper presented at the Technical Services SIG, 2004 Conference of the Association for Library and Information Science Education, San Diego.
- Hsieh-Yee, I. (2003, July). *Cataloging Education for the 21st Century*. A presentation at the Library of Congress, Washington, D.C.
- Hsieh-Yee, I. (2002, January) *Metadata Education and Research Priorities: A Delphi Study of Metadata Experts*. Presentation at the 2002 Conference of the Association for Library and Information Science Education, New Orleans.
- Hsieh-Yee, I. (2001, November). *A Delphi Study of Metadata: Preliminary Findings*. Poster session at the 2001 Annual Meeting of the American Society for Information Science & Technology, Washington, D.C.
- Hsieh-Yee, I. (2001, June). *Resources on Asian American Children: Analysis of Retrieval by Search Engines and WorldCat*. Presentation at the National Conference on Asian Pacific American Librarians, San Francisco.
- Hsieh-Yee, I. (2001, January). *Delphi Study on Metadata: Project Design*. Presentation at Research Awards Session, Association for Library & Information Science Education, Washington, D.C.
- Hsieh-Yee, I. (2000, May). *Web Search Behavior Research: Progress and Implications*. Presentation at the Symposium on Evaluating Library and Information Science Research, University of Wisconsin-Madison, Madison, Wisconsin.

- Hsieh-Yee, I. (2000, March). *ERIC User Services: Evaluation in a Decentralized Environment*. Presentation at the National ERIC Joint Directors/Technical Meeting, Arlington, Virginia.
- Hsieh-Yee, I. (2000, January). *Enhancing Learning with Web Technology*. Presentation at Faculty Conversations, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I. (2000, January). *From Surrogates to Objects: CUA's Approaches to Organizing Electronic Resources*. Paper presentation at the Annual Conference of the Association for Library and Information Science Education, San Antonio, Texas.
- Yee, P., and Hsieh-Yee, I. (1997, November). *Individual Differences in Search Behavior on the WWW*. A poster session presented at the 38th Annual Meeting of the Psychonomic Society, Philadelphia, Pennsylvania.
- Hsieh-Yee, I. (1997, April). *Research + Marketing + Preparation = Job!* Presented at the "Workshop on Resume and Interview Techniques," Special Libraries Association, Student Chapter, Catholic University of America, Washington, D.C.
- Hsieh-Yee, I. (1997, February). *Creating CyberCatalogers: Education and Training*. Presentation at ALA's Midwinter Meeting, Washington, D.C.
- Hsieh-Yee, I. (1997, February). *Search Tactics of Web Users in Searching for Texts, Graphics, Known Items and Subjects: A Search Simulation Study*. Presented at the Conference of the Association for Library and Information Science Education, Washington, D.C.
- Hsieh-Yee, Ingrid. "Beginning Your Special Library/Information Center Career." Presented at SLA's "Career Day," Jan. 11, 1997, Catholic University of America.
- Hsieh-Yee, I. (1996, September). *The Roles of Library and Information Scientists in Managing Electronic Information*. Presentation at Hamilton College, Clinton, New York.
- Hsieh-Yee, I. (1996, May). *The Future of Cataloging as a Profession*. Presented at "The Cataloging Forum, Library of Congress, Washington, D.C.
- Hsieh-Yee, I. (1994, October). *The Impact of the Internet on OPACs*. Presented at the Third Workshop on User Interfaces for OPACs, Library of Congress, Washington, D.C.

Reports

- Hsieh-Yee, I., with Knowledge Management Competencies and Performance Action Group of the Federal Knowledge Management Initiative. "From Knowledge Management Competencies to Improved Organizational Performance." April 9, 2009.

- Hsieh-Yee, I., with Knowledge Practices Action Group of the Federal Knowledge Management Initiative. "KM Practice in Government Agencies: Findings and Recommendations." April 9, 2009.
- Hsieh-Yee, I. "Delphi Study on Metadata." 2001. Three quarterly reports submitted to the Association for Library and Information Science Education.
- Hsieh-Yee, I. "College Students' Information Channels: Patterns of Use and Possible Factors in Channel Selection." 1995. Submitted to the Catholic University of America.
- Hsieh-Yee, I. "The Information-Seeking Patterns of Scholars and Their Use of an Online Information System." 1994. Submitted to the Council on Library Resources.

Book Reviews

- Review of *The Measurement and Evaluation of Library Services*, by Sharon L. Baker and F. Wilfrid Lancaster. *Information Processing and Management* 30 (1994): 450-52.
- Review of *Subject Access to Films and Videos*, by Sheila S. Intner and William E. Studwell; and *Cataloging Unpublished Nonprint Materials*, by Verna Urbanski with Bao Chu Chang and Bernard L. Karon. *Information Processing and Management* 30 (1994): 449-50.
- Review of *Automated Information Retrieval in Libraries: A Management Handbook*, by Vicki Anders. *Journal of Library and Information Science* 19 (1993): 98-100.
- Review of *Full Text Databases*, by Carol Tenopir and Jung Soon Ro. *Information Processing and Management* 28 (1992): 667-68.
- Review of *Descriptive Cataloging for the AACR2R And USMARC: A How-to-Do It Workbook*, by Larry Millsap and Terry Ellen Ferl. *Information Processing and Management* 28 (1992): 809-11.
- Review of *MARC Manual: Understanding and Using MARC Records*, by Deborah J. Byrne. *Information Processing and Management* 28 (1992): 537-38.

Service

Professional Associations and Societies

- Library of Congress. RDA Training Program for the Profession. Co-authored with Tim Carlton. 2013-2014.
- 2014 Digital Preservation Outreach & Education Survey. Contributed to the design of the survey, 2014.
- National Digital Stewardship Alliance. Outreach Committee. 2011-2014.
- National Digital Stewardship Residency Program. Advisory Group, 2012-2013.
- FEDLINK Health Information Technology Advisory Council, 2011-2015.

- 2012 Joint Conference on Digital Libraries. Program Planning Committee, Pre-Conference Proposals Review Committee, 2012
- Catholic Research Resources Alliance. Five-Year Strategic Plan Task Force, 2011-2012
- Institute of Museum and Library Services. Grant reviewer. 2004, 2005, 2010.
- Association for Library and Information Science Education.
 - * ALISE Bodan Wynar Research Paper Award Committee, 2015, 2016, 2017
 - * ALISE Eugene Garfield Dissertation Award Competition, Jury, 2013, 2014
 - * ALISE Research Grant Competition Committee. Chair, 2012
 - * Pratt-Severn Faculty Innovation Award. Chair, 2009, 2010
 - * ALISE Doctoral Poster Jury, 2012
 - * “Information Organization Competencies for the 21st Century” Discussion session leader. 2009 Conference of the Association for Library and Information Science Education.
 - * Assisted Technical Services SIG Convener in organizing a program, ““Building a Metadata Education and Research Community through MERIC (Metadata Education and Research Information Commons): Demo and Stakeholder Input” for the 2008 ALISE conference.
 - * Association for Library Collections and Technical Services/Association for Library and Information Science Education (ALCTS/ALISE) Metadata Education and Research Information Center (MERIC) Advisory Board, Co-Chair (with Sherry Vellucci), 2005-2007. Chair, 2008-2009 (leading the effort to build MERIC, a repository and collaborative space for metadata educators, practitioners, and researchers)
 - * Technical Services SIG, Convener, 2004-2005. Organized a program on “Electronic Resources Management: Current Practices, Employer Expectations, and Teaching Strategies” for the 2005 conference in Boston, Massachusetts.
 - * Technical Services SIG, Convener, 2003-2004. Organized a program on “Organizing Information with Metadata: Desired Competencies and Teaching Innovations” for the 2004 conference.
 - * Technical Services SIG, Convener, 1999-2000. Organized a program on "Teaching the Organization of Electronic Resources" for the 2000 conference.
 - * Curriculum SIG, Co-convener (with Sibyl Moses), 1996-97. Organized a program on “Government Information Policy” for the 1997 conference.
- American Society for Information Science & Technology.
 - * Reviewer, Conference program panel submissions and poster submissions, 2005, 2006, 2007, 2009, 2011, 2012, 2013, 2014, 2015, 2016, 2017
 - * Nomination Committee, 2009-2011
 - * Information Science Education Special Interest Group. American Society for Information Science and Technology. Chair-Elect, 2007-2008. Chair 2008-2009.
 - * Committee on Information Science Education. 1999-2006.
 - * Committee on Information Science Education. Organizing Committee for an orientation program for students at ASIS annual meetings, 1999-2001
 - * Committee on Information Science Education. Sub-committee on Student Welfare (focusing on issues related to master's education), 1998-2001

- * SIG ED. Organizing Committee for the "Seminar on Research and Career Development" for junior researchers. 1995-96 (chair), 1997-2001
- * ISI Doctoral Dissertation Proposal Scholarship Jury, 1997; 2001, 2002
- * Pratt-Severn Best Student Research Paper Award Jury. Chair. 1997
- * 1998 Midyear Meeting (referee of contributed papers), 1997
- * Organizer and moderator of the ASIS Doctoral Forum and the Doctoral Research Seminar 1994-1995
- * SIG Human Computer Interaction. Chair-Elect, Chair, 1993-1995
- * Doctoral Forum Award Jury, 1995
- * Best Student Paper Award Jury, 1995
- American Library Association.
 - * Committee on Accreditation, External Review Panelist, 2009- (site visiting team 2013-2014; site visiting team 2016-2017)
 - * Association for Library Collections and Technical Services Task Force on Competencies and Education for a Career in Cataloging, member, 2008-2009
 - * Facilitator for "What They Don't Teach in Library School: Competencies, Education and Employer Expectations for a Career in Cataloging," an Association for Library Collections and Technical Services Preconference, June 22, 2007 in Washington, D.C. Also a local liaison for bringing this program to the Catholic University of America.
 - * Facilitator for a discussion on "Effect of Electronic Resources on Technical Services" at ALA's Midwinter Meeting held in Feb. 1997 in Washington, D.C.
 - * International Relations Committee, Subcommittee Task Force for IFLA and China, 1994-1997
- Virginia Association of School Librarians. Scholarships and Awards Committee. 2010-2012
- Federal Knowledge Management Initiative, Knowledge Management Practices Action Group. Member. 2009 (leading the effort to build a knowledge management repository)
- Federal Knowledge Management Initiative, Knowledge Management Competencies & Learning Action Group. Member. 2009 (developing an action plan for helping government knowledge workers and government agencies to develop knowledge management competencies)
- National Center for Education Statistics. Technical Review Panel. 2008.
- External evaluator for a case of promotion to full professorship. University of Tennessee. 2008.
- National Information Standards Organization (NISO). Advisory Board, Revision of "IMLS Framework of Guidance for Building Good Digital Collections," 2004, 2007.
- Library of Congress, Bibliographic Control of Web Resources: A Library of Congress Action Plan. Principal Investigator of Action Item 5.1, focusing on cataloging and metadata education for students and new librarians, 2002-2003. (worked with the Association for Library Collections and Technical Services, Education Task Force)

- Chinese American Librarians Association
 - * Chinese American Librarians Association Outstanding Library Leadership Award in Memory of Dr. Margaret Chang Fung, Award Committee, 2016-2017
 - * Achievement Award Jury, 2000-2001
 - * CALA Goal 2000 Task Force, 1997
 - * Scholarship Committee, 1995, 1996-1997 (chair)
 - * Board of Directors, 1994-1997
 - * Publication Committee, 1993-1995
 - * International Relations Committee, 1993-1996
- SailorSM Assessment Advisory Group (An impact study of Sailor, Maryland's Public Information Network), 1995
- Editorial boards
 - Journal of Library & Information Science. Editorial Board, 2012-
 - Chinese American Librarians Association, *Occasional Papers Series*. Editorial Board, 2009-2016.
 - Library Quarterly*. Editorial Board, 2003-2008
 - Bulletin of the Medical Library Association*, 1994-97
 - Newsletter editor for the Chinese American Librarians Association, 1989-92
- Referee for the following journals
 - Information Processing and Management*
 - Journal of Digital Information*
 - Journal of Education for Library and Information Science*
 - Journal of Information Science*
 - Journal of Library & Information Science*
 - Journal of Library Metadata*
 - Journal of the American Society for Information Science & Technology*
 - Library and Information Science Research*
 - Library Quarterly*
- Expert reviewer, “Digital Library” course, Evaluation module, University of North Carolina, Chapel Hill, 2007-2008.
- Expert reviewer, “Information Organization” course, University of Michigan, Ann Arbor. 2007.

Catholic University of America

- Academic Senate Committee on Appointments and Promotions, 2005-2008
- Academic Senate Committee on Committees and Rules, 2009-2012, 2019-2022
- Academic Senate representative (for School of Arts & Sciences), 2017-2020

- Academic Senate representative (for School of Library & Information Science) 2003-2012
- Academic Senate Library Committee, Interim Chair (2007), Member, 2008-2012
- Academic Senate Committee on Computing, 1995-2003
- President's Administrative Council, 2010-2012
- Deans' Council, 2010-2012
- Academic Leadership Group, 2010-2012
- Graduate Board, 2010-2012
- School of Arts & Sciences, Committee on Appointments and Promotions, 2015-2019
- School of Arts & Sciences, Academic Council, 2015-2016.
- School of Arts & Sciences, Ordinary Professor Group, 2013-
- Doctoral Dissertation Defense Committee, Chair, Dept. of Psychology, 2016, 2017, 2018, 2019, 2020, 2021, 2022
- Doctoral Dissertation Defense Committee, Chair, Dept. of Education, 2014, 2015, 2017, 2018, 2019
- Doctoral Dissertation Defense Committee, Chair, National Catholic School of Social Services, 2019, 2020, 2021
- Doctoral Dissertation Defense Committee, Chair, School of Nursing, 2006, 2008
- CUA Scholarly Communication Project Team, Member (2007), Chair, 2008-2009
- Dean Search Committee, 1992-1994, 1998-1999, 2002-2003, 2006-2007
- Fulbright Review Panel, 2006
- CUA Service Learning Advisory Board, 2001-2002
- CUA Faculty Conversations on Enhancing Teaching and Learning through Technology, Planning Group, 1999-2001
- CUA Initiative on Technology and Teaching, 1998-2001

Dept. of Library and Information Science

- Admissions Committee, 2007-2009, Chair 2010-2012, Member 2013-2015, Member 2018-2022
- Appointments and Promotions Committee, 1991-2022
- Student advisement, 1990-2022
- 4+1 Bachelor to Master program, Program Advisor, 2014-2022
- LIS Minor, Advisor, 2017-2022
- Curriculum Committee, 2020-21 (Member), 2021-2022 (Chair)
- Scholarship & Awards Committee, 2016-2018, 2020-21
- Comprehensive examination editor, 2020-21
- Symposium, Colloquium, Lecture Series Committee, May 2018-May 2020 (Chair)
- Symposium and Colloquium Committee, fall 2016-May 2018
- Bridging the Spectrum Symposium session moderator, 2018, 2019, 2020, 2021

- Organization of Information Core Course Outcomes Assessment, 2019, 2020
- The Information Professions in Society Core Course Outcomes Assessment, 2019, 2020
- Community Services Librarianship, Course of Study Review and Revision, 2020
- Organization of Information, Course of Study Review and Revision, 2019
- Organization of Information, Competency and Course Offerings Review, 2018-2019
- Accreditation presentation, Chair, June 2015-August 2016
- Interim Co-Chair, June 2015-August 2016.
- Blended/OWL Learning Committee, spring 2016-2018
- Technology Committee, fall 2016-2017
- Comprehensive examination editor, 2016-2017, reader (every year since 1990)
- LIS Advisory Board, 2015-2016 (chair); fall 2016- May 2018 (member)
- Committee on Planning and Assessment, 2015-2016 (chair)
- Senior Faculty Committee, 2014-2016.
- Accreditation Steering Committee, 2014-2016 (Chair, 2015-2016)
- Accreditation Students Standard Committee, co-chair, 2014-2016
- Accreditation Mission, Goals, and Objectives Standard Committee, co-chair, 2014-2016
- Accreditation Curriculum Standard, member 2014-2-16
- Accreditation Administration and Finance Standard, member 2014-2016
- Cultural Heritage Information Management Project (IMLS-funded), Co-PI, 2012-2015
- Cultural Heritage Information Management Forum (scheduled for June 2015), Co-Organizer, 2013-2015
- Health Information Technology Interim Review Committee, 2015 (chair)
- Health Sciences Librarianship Advisory Group, 2015- (chair)
- Comprehensive examination editors, 2013-2014, 2016-2017
- National Digital Stewardship Alliance liaison, 2011-2014
- Advisory Board, Chair 2010-2012
- Academic Honesty Committee, Chair, 2008-2012
- Blended Learning Committee, 2010-2012
- Colloquium Committee, 2010-2012
- Comprehensive Examination Administration, 2010-2012
- Cultural Heritage Information Management Advisory Committee, 2010-2012 (chair), 2013-
- Curriculum Committee, 1991-2003, 2007-2009, Chair 2010-2012, member 2013-
- Curriculum Subcommittee on Comprehensive Examination, Chair 2009-2012
- Health Information Technology Advisory Board, Chair 2010-2012. Member 2013-
- Health Sciences Advisory Committee, 2009, Chair 2010-2012. Member 2013-
- HIT Expert Forum, Chair 2012. Member 2013-2015
- Health Information Technology Student Group Advisor, 2011-2012
- State Council for Higher Education of Virginia, SLIS Representative, 2010-2012
- Symposium Planning Committee, 2010-2012
- Website Management Team, Chair, 2010-2012
- Urban School Librarianship Project (IMLS-Funded), PI, 2007-2011 (chair, 2010-11)

- Failing Grades Committee, 1995-1997 (chair), 2000-2001 (chair), 2004-2005 (chair), 2007 (chair)-2011
- Faculty Search Committee, 1994-1998, 2002-2004, 2006 (chair), Fall 2007-2009, Chair fall 2009-2012
- Recruitment Committee, Chair 2010-2012
- Strategic Planning Committee, Chair 2010-2012
- Technology Committee, 2010-2012
- Accreditation Advisory Committee, 2007-2009
- Accreditation Coordinating Committee, 2007-2009
- Accreditation Steering Committee, 2007-2009
- SLIS Advisory Group, 2007-2009
- Accreditation Curriculum Standard Committee, Co-chair, 2007-2009
- Accreditation Faculty Standard Committee, Co-chair, 2007-2009
- LSC 551 Information Organization Review Team, Co-chair, 2008-2009, 2015-2016.
- Curriculum Subcommittee on Portfolios, 2009
- LSC 555 Information Systems in Libraries and Information Centers Review Team, contributor, 2008-2009
- Redesign of LSC 730 Use and Users of Libraries and Information. 2009-
- Development of a metadata institute that was taught as LSC 715 Organization of Internet Resources in 2008. The institute is being revised and will be offered in 2010 under a new course title.
- Development of lesson plans, assignments, and evaluation rubrics for LSC 606, Cataloging and Classification, for the School's NCATE accreditation. 2008
- Howard and Mathilde Rovelstad Scholarship Committee, Chair, 2004-2007
- Assistant Dean Search Committee, Chair, Fall 2007
- Liaison to the Association for Library Collections and Technical Services to bring its preconference program, Cataloging Education and Employer Expectations, to CUA during the 2007 American Library Association Annual Meeting in Washington, D.C.
- Organizer of the colloquium presentation and reception for Tamar Sadeh of Ex Libris on PRIMO June 2007
- Practicum review and design (work with potential supervisors, such as the American Indian Museum internship description revision) 2006-
- Comprehensive examinations (edits, proctoring, and grading), 1990-
- SLIS Web site redesign: Comments and suggestions. Fall 2007
- Conducted surveys of current students and alumni in preparation for the 2005 re-accreditation, 2004-2005
- Technology Committee, 1992-1999 (chair, 1996-1998), 2002-2003 (member)
- Colloquia Committee 1997-1999, 2002-2003.
- Advisor of the CUA Student Chapter of the American Society for Information Science and Technology, 2002-2003
- Visiting Professor Search Committee, 1999, 2000, 2001
- Leader, Participation in the CORC experiment, 1999-2000
- Advisor of the Special Libraries Association Student Chapter, 1993-1999; the group was recognized for outstanding leadership by SLA in 1999.

- COA planning Committee, Task Force on Electronic Presentation of SLIS Reports (team leader) 1997-1998
- COA Planning Committee, Subcommittee on Technology 1996-1998
- NLM practicum coordinator, 1997-1998
- Computer Literacy Workshops: Assisted with the development and evaluation of the workshops, 1996-1998
- Leader, Participation in the InterCat project, 1995-1997

Appendix B Parts VII to X of *Understanding MARC Bibliographic*, available at <https://www.loc.gov/marc/umb/um07to10.html> from the Library of Congress.

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MARC 21 Reference Materials

[Part VII: A Summary of Commonly Used MARC 21 Fields](#)

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Part VII:

A Summary of Commonly Used MARC 21 Fields

This is a summary of the MARC 21 tags used most frequently by libraries in entering their own bibliographic records. For full listings of all MARC 21 tags, indicators, and subfield codes, see *MARC 21 Format for Bibliographic Data*.

In the explanations on these pages:

Tags -- The tags (3-digit numbers) are followed by the names of the fields they represent. In this summary, and in the *MARC 21 Format for Bibliographic Data*, if a tag can appear more than once in one bibliographic record, it is labeled repeatable (R). If it can only be used once, it is labeled non-repeatable (NR). For example, a catalog record can have several subjects, so the tags for subject added entries (6XX) are labeled repeatable (R).

Indicators -- The use of indicators is explained in fields where they are used. Indicators are one-digit numbers. Beginning with the 010 field, in every field -- following the tag -- are two character positions, one for Indicator 1 and one for Indicator 2. The indicators are not actually defined in all fields, however. And it is possible that a 2nd indicator will be used, while the 1st indicator remains undefined (or vice versa). When an indicator is undefined, the character position will be represented by the character # (for blank space).

Subfield codes -- All the data in each field (beginning with the 010 field) is divided into subfields, each of which is preceded by a delimiter-subfield code combination. The most common subfield codes used with each tag are shown. Each subfield code is preceded by the character \$, signifying a delimiter. The name of the subfield follows the code.

In general, every field MUST have a subfield 'a' (\$a). One exception that is often seen is in Field 020 (ISBN), when the ISBN information (subfield \$a) is unavailable but the price (subfield \$c) is known. Some subfields are repeatable. In this summary, repeatability is noted for only the more common repeatable subfields.

Examples: Examples follow the explanation for each field. For clarity, one space has been placed between the tag and the first indicator, one space has been placed between the second indicator and the first delimiter- subfield code, and one space has been inserted between the delimiter-subfield code and the subfield data.

010 Library of Congress Control Number -- (LCCN)

(NR, or Not Repeatable)

*Indicators undefined.**Subfield used most often:***\$a** -- Library of Congress control number

Example: 010 ## \$a ###86000988#

020 International Standard Book Number -- (ISBN)

(R, or Repeatable)

*Indicators undefined.**Subfields used most often:***\$a** -- International Standard Book Number**\$c** -- Terms of availability (often a price)**\$z** -- Cancelled/invalid ISBN (R)

Example: 020 ## \$a 0877547637

040 Cataloging source -- (NR)*Indicators undefined.**Subfields used most often:***\$a** -- Original cataloging agency**\$c** -- Transcribing agency**\$d** -- Modifying agency (R)

Example: 040 ## \$a DLC
 \$c DLC
 \$d gwhs

100 Main entry -- Personal name -- (primary author)

(NR; there can be only one main entry)

Indicator 1: Type of personal name entry element

0 -- Forename

1 -- Surname (this is the most common form)

3 -- Family name

Indicator 2 undefined.

Indicator 2 became obsolete in 1990. Older records may display 0 or 1

*Subfields used most often:***\$a** -- Personal name**\$b** -- Numeration

\$c -- Titles and other words associated with a name (R)
\$q -- Fuller form of name
\$d -- Dates associated with a name (generally, year of birth)

Example: 100 1# \$a Gregory, Ruth W.
 \$q (Ruth Wilhelme),
 \$d 1910–

130 Main entry -- Uniform title -- (NR)

Indicator 1: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Indicator 2 undefined.

Indicator 2 became obsolete in 1990. (See 100 above.)

Subfields used most often:

\$a -- Uniform title
\$p -- Name of part/section of a work (R)
\$l -- Language of a work
\$s -- Version
\$f -- Date of a work

Example: 130 0# \$a Bible.
 \$p O.T.
 \$p Psalms.

240 Uniform title (NR)

Indicator 1: Uniform title printed or displayed

0 -- Not printed or displayed

1 -- Printed or displayed (most common)

Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Subfields used most often:

\$a -- Uniform title
\$l -- Language of a work
\$f -- Date of a work

Example: 240 10 \$a Ile mystérieuse.
 \$l English.
 \$f 1978

245 Title Statement (NR)*Indicator 1:* Title added entry

(Should the title be indexed as a title added entry?)

0 -- No title added entry

(indicates a title main entry; i.e. no author is given)

1 -- Title added entry

(the proper indicator when an author given in 1XX; the most common situation)

Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present, including spaces; usually set at zero, except when the title begins with an article; e.g., for *The robe*, the second indicator would be set to 4. The letters *T*, *h*, *e*, and the space following them are then ignored in alphabetizing titles. The record will be automatically filed under "r" -- for *Robe*.

*Subfields used most often:***\$a** -- Title proper**\$h** -- Medium (often used for non-book media)**\$p** -- Name of part/section of a work (R)**\$b** -- Reminder of title (subtitles, etc.)**\$c** -- Remainder of title page transcription/Statement of responsibility

Example: 245 14 \$a The DNA story :
 \$b a documentary history of gene
 cloning /
 \$c James D. Watson, John Tooze.

246 Varying form of title (R)*Indicator 1:* Note/title added entry controller

1 -- Note, title added entry

3 -- No note, title added entry

Indicator 2: Type of title

-- No information provided

0 -- Portion of title

1 -- Parallel title

4 -- Cover title

8 -- Spine title

*Subfield used most often:***\$a** -- Title proper

Example: 246 3# \$a Four corners power review

250 Edition statement (NR)

Indicators undefined.

Subfield used most often:

\$a -- Edition statement

Example: 250 ## \$a 6th ed.

260 Publication, distribution, etc. (Imprint) (R)

Indicator 1: Sequence of publishing statements

-- No information provided

Indicator 2: Undefined

Subfields used most often:

\$a -- Place of publication, distribution, etc. (R)

\$b -- Name of publisher, distributor, etc. (R)

\$c -- Date of publication, distribution, etc. (R)

Example: 260 ## \$a New York :
 \$b Chelsea House,
 \$c 1986.

300 Physical description (R)

Indicators undefined.

Subfields used most often:

\$a -- Extent (number of pages) (R)

\$b -- Other physical details (usually illustration information)

\$c -- Dimensions (cm.) (R)

\$e -- Accompanying material (for example, "teacher's guide" or "manual")

Example: 300 ## \$a 139 p. :
 \$b ill. ;
 \$c 24 cm.

440 Series statement / Added entry--Title

This field was made obsolete in 2008 to simplify the series statement. See 490 and 830.

490 Series statement (No added entry is traced from field) (R)

Indicator 1: Specifies whether series is traced (whether an 8XX tag is also present)

0 -- Series not traced

1 -- Series traced (8XX is in record)

Indicator 2 undefined.

Subfield used most often:

\$a -- Series statement (R)

\$v -- Volume number (R)

Example: 490 1# \$a Colonial American craftsmen

500 General note (R)

Indicators undefined.

Subfield used most often:

\$a -- General note (Used when no specialized note field has been defined for the information. Examples: Notes regarding the index; the source of the title; variations in title; descriptions of the nature, form, or scope of the item.)

Example: 500 ## \$a Includes index.

504 Bibliography, etc. note (R)

Indicators undefined.

Subfield used most often:

\$a -- Bibliography, etc. note

Example: 504 ## \$a Includes bibliographical references.

505 Formatted contents note (R)

Indicator 1: Type of contents note

0 -- Complete contents

1 -- Incomplete contents (used with multivolume set when some volumes are not yet published)

2 -- Partial contents

Indicator 2: Level of content designation

-- Basic

Subfield used most often:

\$a -- Formatted contents note

Example: 505 0# \$a Pride and prejudice -- Emma
-- Northanger Abbey.

520 Summary, etc. note (R)

Indicator 1: Display constant controller

-- Summary

- 1 -- Review
- 2 -- Scope and content
- 3 -- Abstract

Indicator 2 undefined

Subfields used most often

- \$a** -- Summary, abstract, or annotation
- \$b** -- Expansion of summary note

Example: 520 ## \$a This basic guide to parliamentary procedure tells how to conduct and participate in a meeting properly.

600 Subject added entry -- Personal name (R)

Indicator 1: Type of personal name entry element

- 0 -- Forename
- 1 -- Surname (this is the most common form)
- 3 -- Family name

Indicator 2: Subject heading system/thesaurus (identifies the specific list or file which was used)

- 0 -- Library of Congress Subject Headings
- 1 -- LC subject headings for children's literature
- 2 -- Medical Subject Headings
- 3 -- National Agricultural Library subject authority file
- 4 -- Source not specified
- 5 -- Canadian Subject Headings
- 6 -- Répertoire de vedettes-matière
- 7 -- Source specified in subfield \$2

(Note regarding Sears subject headings: The MARC 21 format does not provide an assigned indicator for Sears subject headings. Therefore, an indicator of 7 is used, and the MARC defined code "sears" is placed in subfield \$2.)

Subfields used most often:

- \$a** -- Personal name (surname and forename)
- \$b** -- Numeration
- \$c** -- Titles and other words associated with a name (R)
- \$q** -- Fuller form of name
- \$d** -- Dates associated with a name (generally, year of birth)
- \$t** -- Title of a work
- \$v** -- Form subdivision (R)
- \$x** -- General subdivision (R)
- \$y** -- Chronological subdivision (R)
- \$z** -- Geographic subdivision (R)
- \$2** -- Source of heading or term (used with 2nd indicator of 7)

Example: 600 10 \$a Shakespeare, William,
 \$d 1564-1616
 \$x Comedies
 \$x Stage history.

Example: 600 10 \$a Shakespeare, William,
 \$d 1564-1616
 \$x Knowledge
 \$z Rome
 \$v Congresses.

Notice that subfields \$v, \$x, and \$z in the 600 field are repeatable. Subfields \$v, \$x, \$y, and \$z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

610 Subject added entry -- Corporate name (R)

Indicator 1: Type of corporate name entry element

- 0 -- Inverted name (not used with AACR2)
- 1 -- Jurisdiction name
- 2 -- Name in direct order

Indicator 2: Subject heading system/thesaurus.

See indicator 2 under 600

Subfields used most often:

- \$a -- Corporate name or jurisdiction name as entry element
- \$b -- Subordinate unit (R)
- \$v -- Form subdivision (R)
- \$x -- General subdivision (R)
- \$y -- Chronological subdivision (R)
- \$z -- Geographic subdivision (R)
- \$2 -- Source of heading or term (used with 2nd indicator of 7)

Example: 610 10 \$a United States.
 \$b Army Air Forces
 \$v Biography.

650 Subject added entry -- Topical term (Most subject headings fit here.) (R)

Indicator 1: Level of subject

- # -- No information provided

Indicator 2: Subject heading system/thesaurus

(identifies the specific list or file which was used)

- 0 -- Library of Congress Subject Headings
- 1 -- LC subject headings for children's literature

- 2 -- Medical Subject Headings
- 3 -- National Agricultural Library subject authority file
- 4 -- Source not specified
- 5 -- Canadian Subject Headings
- 6 -- Répertoire de vedettes-matière
- 7 -- Source specified in subfield \$2

Note regarding Sears subject headings: The MARC 21 format does not provide an assigned indicator for Sears subject headings. Therefore, an indicator of 7 is used, and the MARC defined code "sears" is placed in subfield \$2.)

Subfields used most often:

- \$a** -- Topical term
- \$v** -- Form subdivision (R)
- \$x** -- General subdivision (R)
- \$y** -- Chronological subdivision (R)
- \$z** -- Geographic subdivision (R)
- \$2** -- Source of heading or term used with 2nd indicator of 7)

Example: 650 #0 \$a Theater
 \$z United States
 \$v Biography
 \$v Dictionaries.

Notice that subfields \$v, \$x, and \$z in the 650 field are repeatable. Subfields \$v, \$x, \$y, and \$z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

651 Subject added entry -- Geographic name (R)

Indicator 1: undefined.

Indicator 2: Subject heading system/thesaurus.

See indicator 2 under 600

Subfields used most often:

- \$a** -- Geographic name
- \$v** -- Form subdivision (R)
- \$x** -- General subdivision (R)
- \$y** -- Chronological subdivision (R)
- \$z** -- Geographic subdivision (R)
- \$2** -- Source of heading or term (used with 2nd indicator of 7)

Example: 651 #0 \$a United States
 \$x History
 \$v Chronology.

Notice that subfields \$v, \$x, and \$z in the 651 field are repeatable. Subfields \$v, \$x, \$y, and \$z do not have to be in alphabetical order. They will be in the order prescribed by the instructions given by the subject heading system.

700 Added entry -- Personal name (R)

Indicator 1: Type of personal name entry element

- 0 -- Forename
- 1 -- Surname (this is the most common form)
- 3 -- Family name

Indicator 2: Type of added entry

- # -- No information provided (most common; co-authors, editors, etc.)
- 2 -- Analytical entry (The values for Indicator 2 changed in 1994 with Format Integration, and older records may display additional values. An analytical entry involves an author/title of an item contained in a work.)

Subfields used most often:

- \$a** -- Personal name
- \$b** -- Numeration
- \$c** -- Titles and other words associated with a name (R)
- \$q** -- Fuller form of name
- \$d** -- Dates associated with a name (generally, year of birth)
- \$e** -- Relator term (such as ill.) (R)
- \$4** -- Relator code (R)

Example: 700 1# \$a Baldrige, Letitia.

710 Added entry -- Corporate name (R)

Indicator 1: Type of corporate name entry element

- 0 -- Inverted name (not used with AACR2)
- 1 -- Jurisdiction name
- 2 -- Name in direct order

Indicator 2: Type of added entry.

- See Indicator 2 under 700
- # -- No information provided
- 2 -- Analytical entry

Subfields used most often:

- \$a** -- Corporate name or jurisdiction name as entry element
- \$b** -- Subordinate unit (R)

Example: 710 2# \$a Sunburst Communications (Firm)

740 Added entry -- Uncontrolled related/analytical title (R)

Indicator 1: Nonfiling characters

- 0-9 -- Number of nonfiling characters present (for initial articles, including

spaces)

Indicator 2: Type of added entry. See Indicator 2 under 700

-- No information provided

2 -- Analytical entry

(This field was redefined in 1994 with Format Integration. Prior to 1994, the field was also used for variant titles, such as a different wording on a spine title. In records created since Format Integration, those variant titles appear in a 246 field.)

Subfield used most often:

\$a -- Title

Example: 740 02 \$a Uncle Vanya.

800 Series added entry -- Personal name (R)

Indicator 1: Type of personal name entry element

0 -- Forename

1 -- Surname

3 -- Family name

Indicator 2 undefined.

Subfields used most often:

\$a -- Personal name

\$b -- Numeration

\$c -- Titles and other words associated with a name (R)

\$q -- Fuller form of name

\$d -- Dates associated with a name (generally, year of birth)

\$t -- Title of a work (the series)

\$v -- Volume number

Example: 800 1# \$a Fisher, Leonard Everett.
 \$t Colonial American craftsmen.

830 Series added entry -- Uniform title (R)

Indicator 1 undefined.

Indicator 2: Nonfiling characters

0-9 -- Number of nonfiling characters present (for initial articles, including spaces)

Subfield used most often:

\$a -- Uniform title

\$v -- Volume number

Example: 830 #0 \$a Railroads of America (Macmillan)

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Part VIII:

A List of Other Fields Often Seen in MARC Records

001	Control number
003	Control number identifier
005	Date and time of latest transaction
006	Fixed-length data elements -- additional material characteristics
007	Physical description fixed field
008	Fixed length data elements (See Part X)
022	International Standard Serial Number (ISSN)
037	Source of acquisition
041	Language code
043	Geographic area code
050	Library of Congress call number
060	National Library of Medicine call number
082	Dewey Decimal classification number (the one recommended by the Library of Congress; locally-assigned call numbers may appear elsewhere)
110	Main entry -- Corporate name (less frequent under AACR2 rules)
256	Computer file characteristics
263	Projected publication date (indicates a CIP -- Cataloging in Publication -- record)
306	Playing time
508	Creation/production credits note
510	Citation/references note (review sources)
511	Participant or performer note
521	Target audience note (first indicator: 0 = reading grade level, 1 = interest age level, 2 = interest grade level, 3 = special audience characteristics, 4 = motivation interest level)
530	Additional physical form available note
538	System details note
586	Awards note
656	Index term -- Occupation
730	Added entry -- Uniform title
852	Location
856	Electronic location and access
9XX	Reserved for local use. (They are used by vendors, systems, or individual libraries to exchange additional data)

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Part IX:

The Leader

There are 24 positions in the Leader, numbered from 00 to 23. For fuller explanation, see the *MARC 21 Format for Bibliographic Data*.

- 00-04 Record length (calculated by the computer for each record)
- 05 Record status
 - a = increase in encoding level
 - c = corrected or revised
 - d = deleted
 - n = new
 - p = increase in encoding from prepublication (previous CIP)
- 06 Type of record
 - a = language material
 - c = printed music
 - d = manuscript music
 - e = cartographic material
 - f = manuscript cartographic material
 - g = projected medium
 - i = nonmusical sound recording
 - j = musical sound recording
 - k = 2-dimensional nonprojectable graphic
 - m = computer file
 - o = kit
 - p = mixed materials
 - r = 3-dimensional artifact or naturally occurring object
 - t = manuscript language material
- 07 Bibliographic level
 - a = monographic component part
 - b = serial component part
 - c = collection
 - d = subunit
 - i = integrating resource
 - m = monograph/item
 - s = serial
- 08 Type of control
 - # = no specified type
 - a = archival
- 09 Character coding scheme
 - # = MARC-8
 - a = UCS/Unicode
- 10 Indicator count (always "2")
- 11 Subfield code count (always "2")
- 12-16 Base address of data (calculated by the computer for each record)
- 17 Encoding level
 - # = full level

- 1 = full level, material not examined
- 2 = less-than-full level, material not examined
- 3 = abbreviated level
- 4 = core level
- 5 = partial (preliminary) level
- 7 = minimal level
- 8 = prepublication level (CIP)
- u = unknown
- z = not applicable
- 18 **Descriptive cataloging form**
 - # = non-ISBD
 - a = AACR2
 - i = ISBD
 - u = unknown
- 19 **Multipart resource record level**
 - # = Not specified or not applicable
 - a = Set
 - b = Part with independent title
 - c = Part with dependent title
- 20 **Length of the length-of-field portion (always "4")**
- 21 **Length of the starting-character-position portion (always "5")**
- 22 **Length of the implementation-defined portion (always "0")**
- 23 **Undefined (always "0")**

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Part X:

Field 008 for Books

Field 008 is used for Fixed Length Data Elements ("Fixed Field Codes"). There are 40 character positions in field 008, numbered from 00-39. Undefined positions must contain either a blank (#) or a fill character (|). Positions 00-17 and 35-39 are defined the same way for all media.

The information shown here for positions 18-34 applies only to books. For explanation of all the positions below and for positions 18-34 for other media, see the *MARC 21 Format for Bibliographic Data*.

Note that field 008 has no indicators or subfield codes.

- 00-05 **Date entered on file (YYMMDD),**
where Y=year, M=month, and D=day
- 06 **Type of date/publication status:**
 - b = no dates given; B.C. date involved
 - e = detailed date
 - s = single known date/probable date
 - m = multiple dates
 - r = reprint/reissue date (Date 1) and original date (Date 2)

n = dates unknown
 q = questionable date
 t = publication date and copyright date
 | = no attempt to code

07-10 Date 1/beginning date of publication

11-14 Date 2/ending date of publication

Date fields contain the year(s) of publication. The type of date(s) in these elements are specified in fixed field element 06: Type of date/publication status. (For further details, see the field 008 description in the *MARC 21 Format for Bibliographic Data*.)

15-17 Place of publication, production, or execution

For example:

pk# = Pakistan

cau = California (US)

(For a full list of codes used in these positions, see the *MARC Code List for Countries*.)

18-21 Illustrations (up to 4 codes):

= no illustrations
 a = illustrations
 b = maps
 c = portraits
 d = charts
 e = plans
 f = plates
 g = music
 h = facsimiles
 i = coats of arms
 j = genealogical tables
 k = forms
 l = samples
 m = phonodisc, phonowire, etc.
 o = photographs
 p = illuminations
 | = no attempt to code

22 Target audience:

= unknown or not specified
 a = preschool
 b = primary
 c = pre-adolescent
 d = adolescent
 e = adult
 f = specialized
 g = general
 j = juvenile
 | = no attempt to code

23 Form of item:

= none of the following
 a = microfilm

b = microfiche
 c = microopaque
 d = large print
 f = braille
 r = regular print reproduction
 s = electronic
 | = no attempt to code

24-27 Nature of contents (up to 4):

= no specified nature of contents
 a = abstracts/summaries
 b = bibliographies (is one or contains one)
 c = catalogs
 d = dictionaries
 e = encyclopedias
 f = handbooks
 g = legal articles
 i = indexes
 j = patent document
 k = discographies
 l = legislation
 m = theses
 n = surveys of literature
 o = reviews
 p = programmed texts
 q = filmographies
 r = directories
 s = statistics
 t = technical reports
 u = standards/specifications
 v = legal cases and notes
 w = law reports and digests
 z = treaties
 | = no attempt to code

28 Government publication:

= not a government publication
 i = international intergovernmental
 f = federal/national
 a = autonomous or semi-autonomous component
 s = state, provincial, territorial, dependent, etc.
 m = multistate
 c = multilocal
 l = local
 z = other type of government publication
 o = government publication -- level undetermined
 u = unknown if item is government publication
 | = no attempt to code

29 Conference publication:

0 = not a conference publication
 1 = conference publication

- 30 **Festschrift:**
 0 = not a festschrift
 1 = festschrift
 | = no attempt to code
- 31 **Index:**
 0 = no index
 1 = index present
 | = no attempt to code
- 32 **Undefined (since 1990)** (Earlier records may contain the values 0 or 1)
 # = Undefined
 | = no attempt to code
- 33 **Literary form:**
 0 = not fiction (not further specified)
 1 = fiction (not further specified)
 c = comic strips
 d = dramas
 e = essays
 f = novels
 h = humor, satires, etc.
 i = letters
 j = short stories
 m = mixed forms
 p = poetry
 s = speeches
 u = unknown
 | = no attempt to code
- 34 **Biography:**
 # = no biographical material
 a = autobiography
 b = individual biography
 c = collective biography
 d = contains biographical information
 | = no attempt to code
- 35-37 **Language:**
 A three-letter code. For example: eng fre ger spa rus ita

(For a full list of codes used in these positions, see the [MARC Code List for Languages](#).)

- 38 **Modified record:**
 # = not modified
 x = missing characters (because of characters unavailable in MARC character set)
 s = shortened
 d = "dashed-on" information omitted
 r = completely romanized/printed cards in script
 o = completely romanized/printed cards romanized
 | = no attempt to code
- 39 **Cataloging source:**

= national bibliographic agency
c = cooperative cataloging program
d = other sources
u = unknown
| = no attempt to code

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Library of Congress

[Library of Congress Help Desk \(10/27/2009 \)](#)

Appendix C Materials Considered

Appendix C. Materials Considered

Exhibit	Document Description
1005	Rissin, D. M., Kan, C. W., Campbell, T. G., Howes, S. C., Fournier, D. R., Song, L., ... & Duffy, D. C. (2010, May). Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations, <i>Nature Biotechnology</i> , 28(6), 595-599, and supplementary information at https://static-content.springer.com/esm/art%3A10.1038%2Fnbt.1641/MediaObjects/41587_2010_BFnb1641_MOESM21_ESM.pdf (4 pages). (“ <i>Rissin</i> ”).
1005A	Copy of <i>Rissin</i> and supplementary information (4 pages) obtained from the publisher webpage for <i>Rissin</i> at https://www.nature.com/articles/nbt.1641 .
1005B	Copy of <i>Rissin</i> obtained from the National Library of Medicine.
1005C	Bibliographic and MARC records for <i>Nature Biotechnology</i> that contains <i>Rissin</i> at https://catalog.nlm.nih.gov/permalink/01NLM_INST/1o1phhn/alma998073023406676 from the online catalog of the National Library of Medicine.
1005D	Profile of the National Library of Medicine, available at https://librarytechnology.org/library/2297 .
1005E	PubMed metadata record for <i>Rissin</i> available at https://pubmed.ncbi.nlm.nih.gov/20495550/ .
1005F	“MEDLINE/PubMed Data Element (Field) Descriptions” archived by Internet Archive on July 9, 2010, available at https://web.archive.org/web/20100709164257/https://www.nlm.nih.gov/bsd/mms/medlineelements.html .
1005G	PubMed home page archived by Internet Archive on August 4, 2010, available at https://web.archive.org/web/20100804041346/http://www.ncbi.nlm.nih.gov/PubMed/ .

1005H	Publisher webpage for <i>Nature Biotechnology</i> , 28(6), archived by Internet Archive on June 28, 2010, available at https://web.archive.org/web/20100628153945/http://www.nature.com/nbt/journal/v28/n6/index.html .
1005I	Publisher webpage on how to access <i>Rissin</i> online, archived by Internet Archive on June 11, 2010, available at https://web.archive.org/web/20100611073455/http://www.nature.com/nbt/journal/v28/n6/full/nbt.1641.html .
1005J	Quanterix announcement of the publication of <i>Rissin</i> , issued on May 23, 2010, and archived by Internet Archive on June 1, 2010, available at https://web.archive.org/web/20100601142509/http://quanterix.com/news/pdfs/natureBiotech.pdf .
1005K	Citation history of <i>Rissin</i> obtained from Google Scholar.
1006	Todd, J., & Agee, S. J. (2008, Fall). Next generation immunoassay systems and the revival of protein biomarkers, <i>Drug Discovery World</i> , Fall 2008 issue, pp. 51 to 57. (“ <i>Todd</i> ”).
1006A	Copy of <i>Todd</i> obtained from the fall 2008 archive of <i>Drug Discovery World</i> at https://www.ddw-online.com/media/32/2304/fall-08-immunoassay-systems.pdf .
1006B	Copy of <i>Todd</i> obtained from the National Library of Medicine.
1006C	Bibliographic and MARC serial records for <i>Drug Discovery World</i> that contains <i>Todd</i> , available at https://catalog.nlm.nih.gov/permalink/01NLM_INST/1o1phhn/alma9912756903406676 from the online catalog of the National Library of Medicine.
1006D	Profile of the National Library of Medicine, available at https://librarytechnology.org/library/2297 .

1006E	Webpage of <i>Drug Discovery World</i> archived by Internet Archive on December 8, 2009, available at https://web.archive.org/web/20091208050404/https://www.ddw-online.com/ .
1024	Innogenetics webpage for INNOTESTTM hTAU Ag. (“ <i>INNOTEST</i> ”).
1024A	Webpage of <i>INNOTEST</i> archived by Internet Archive on September 14, 2004, available at https://web.archive.org/web/20040914040003fw_/http://www.innogenetics.be/site/prodview.asp?id=38&lang=E&print=true .
1030	Webpage for Alzheimer Disease (AD) from Quanterix. (“ <i>AD</i> ”).
1030A	Webpage for <i>AD</i> from Quanterix, archived by Internet Archive on May 29, 2010, available at https://web.archive.org/web/20100529185326/www.quanterix.com/applications/ad.html .
1034	Webpage for Assay Kits from Quanterix. (“ <i>Assay</i> ”).
1034A	Webpage for <i>Assay</i> from Quanterix, archived by Internet Archive on May 29, 2013, available at https://web.archive.org/web/20130529112628/http://www.quanterix.com:80/products/assay-kits .
1035	Nalefski, E. A., D’Antoni, C. M., Ferrell, E. P., Lloyd, J. A., Qiu, H., Harris, J. L., & Whitney, D. H. (2006, November). Single-molecule detection for femtomolar quantification of proteins in heterogeneous immunoassays, <i>Clinical Chemistry</i> , 52(11), 2172-2175. (“ <i>Nalefski</i> ”).
1035A	Copy of <i>Nalefski</i> obtained from the Linda Hall Library.
1035B	Bibliographic and MARC records for <i>Clinical Chemistry</i> that contains <i>Nalefski</i> , available at https://catalog.lindahall.org/permalink/01LINDAHALL_INST/191d

	a7s/alma992798393405961 from the online catalog of the Linda Hall Library.
1035C	Profile of the Linda Hall Library at https://librarytechnology.org/library/2294 .
1035D	PubMed metadata record for <i>Nalefski</i> , available at Pu at https://pubmed.ncbi.nlm.nih.gov/18061992/ and the record with field labels at https://pubmed.ncbi.nlm.nih.gov/18061992/?format=pubmed .
1035E	MEDLINE/PubMed Data Element (Field) Descriptions, archived by Internet Archive on July 11, 2007, available at https://web.archive.org/web/20070711034105/https://www.nlm.nih.gov/bsd/mms/medlineelements.html .
1035F	PubMed website archived by Internet Archive on March 29, 2007, available at https://web.archive.org/web/20070329092504/http://www.ncbi.nlm.nih.gov/PubMed .
1035G	<i>Clinical Chemistry</i> vol. 52, no. 11 Table of Contents, archived by Internet Archive on January 28, 2007, available at https://web.archive.org/web/20070128193542/http://www.clinchem.org/content/vol52/issue11/; PDF download page for <i>Nalefski</i>, archived by Internet Archive on April 26, 2007, available at https://web.archive.org/web/20070426113525/http://www.clinchem.org/cgi/reprint/52/11/2172; Full text of <i>Nalefski</i> in HTML, archived by Internet Archive on May 11, 2008, available at https://web.archive.org/web/20080511153458/http://www.clinchem.org/cgi/content/full/52/11/2172; and Full text of <i>Nalefski</i> in PDF, archived by Internet Archive on May 11, 2008, available at https://web.archive.org/web/20080511152327/http://www.clinchem.org/cgi/reprint/52/11/2172 and select “Begin manual download” to view the PDF version at https://web.archive.org/web/20080513140918/http://www.clinchem.org/cgi/reprint/52/11/2172.pdf.

1035H	Citation records to <i>Nalefski</i> , obtained from Google Scholar.
1036	Webpage for “A β -42 (Amyloid beta protein 42)” from Singulex. (“A β -42”).
1036A	Webpage for A β -42 from Singulex, archived by Internet Archive on September 12, 2009, available at https://web.archive.org/web/20090912044340/http://www.singulex.com:80/assay_details.html?id=2 .
1011	Bourzac, K. (May 13, 2008). Next-Generation Diagnostics: A startup can detect tiny traces of cancer markers in blood samples, MIT Technology Review. (“ <i>Bourzac</i> ”).
1011A	Copy of <i>Bourzac</i> available at https://www.technologyreview.com/2008/05/13/220548/next-generation-diagnostics/ from MIT Technology Review.
1011B	Copy of <i>Bourzac</i> , archived by the Internet Archive on May 13, 2008 and May 17, 2008, available at https://web.archive.org/web/20080513175840/http://www.technologyreview.com/Biztech/20760/ and https://web.archive.org/web/20080517045755/http://www.technologyreview.com/Biztech/20760/page2/ .
App. A	Hsieh-Yee CV.
App. B	Parts VII to X of <i>Understanding MARC Bibliographic</i> , available at https://www.loc.gov/marc/umb/um07to10.html from the Library of Congress.

EX-1005A Copy of *Rissin* and supplementary information (4 pages) obtained from the publisher webpage for *Rissin* at <https://www.nature.com/articles/nbt.1641>.

Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations

David M Rissin^{1,3}, Cheuk W Kan^{1,3}, Todd G Campbell¹, Stuart C Howes¹, David R Fournier¹, Linan Song¹, Tomasz Piech¹, Purvish P Patel¹, Lei Chang¹, Andrew J Rivnak¹, Evan P Ferrell¹, Jeffrey D Randall¹, Gail K Provuncher¹, David R Walt² & David C Duffy¹

The ability to detect single protein molecules^{1,2} in blood could accelerate the discovery and use of more sensitive diagnostic biomarkers. To detect low-abundance proteins in blood, we captured them on microscopic beads decorated with specific antibodies and then labeled the immunocomplexes (one or zero labeled target protein molecules per bead) with an enzymatic reporter capable of generating a fluorescent product. After isolating the beads in 50-fl reaction chambers designed to hold only a single bead, we used fluorescence imaging to detect single protein molecules. Our single-molecule enzyme-linked immunosorbent assay (digital ELISA) approach detected as few as ~10–20 enzyme-labeled complexes in 100 μ l of sample (~10⁻¹⁹ M) and routinely allowed detection of clinically relevant proteins in serum at concentrations (<10⁻¹⁵ M) much lower than conventional ELISA^{3–5}. Digital ELISA detected prostate-specific antigen (PSA) in sera from patients who had undergone radical prostatectomy at concentrations as low as 14 fg/ml (0.4 fM).

The clinical use of protein biomarkers to differentiate between healthy and disease states, and to monitor disease progression, requires the measurement of low concentrations of proteins in complex samples. Current immunoassays typically measure proteins at concentrations above 10⁻¹² M⁶. The serum concentrations of the majority of proteins important in cancer⁷, neurological disorders^{8,9} and the early stages of infection¹⁰, however, are thought to range from 10⁻¹⁶ to 10⁻¹² M. For instance, a 1-mm³ tumor composed of a million cells that each secrete 5,000 proteins into 5 liters of circulating blood translates to a concentration of ~2 $\times$ 10⁻¹⁵ M (or 2 fM). Moreover, serum from individuals recently infected with HIV contains 10–3,000 virions per ml, resulting in estimated concentrations of the p24 capsid antigen ranging from 50 $\times$ 10⁻¹⁸ M (50 aM) to 15 $\times$ 10⁻¹⁵ M (15 fM)¹⁰. Attempts to develop methods capable of measuring these concentrations of proteins have focused on the replication of nucleic acid labels on proteins^{11,12}, or on measuring the bulk, ensemble properties of labeled protein molecules^{13–16}. The work of Mirkin *et al.*^{12,17} and others¹⁸ using labels based on gold nanoparticles and DNA barcodes has pushed the detection of proteins into the low femtomolar range; a recent report

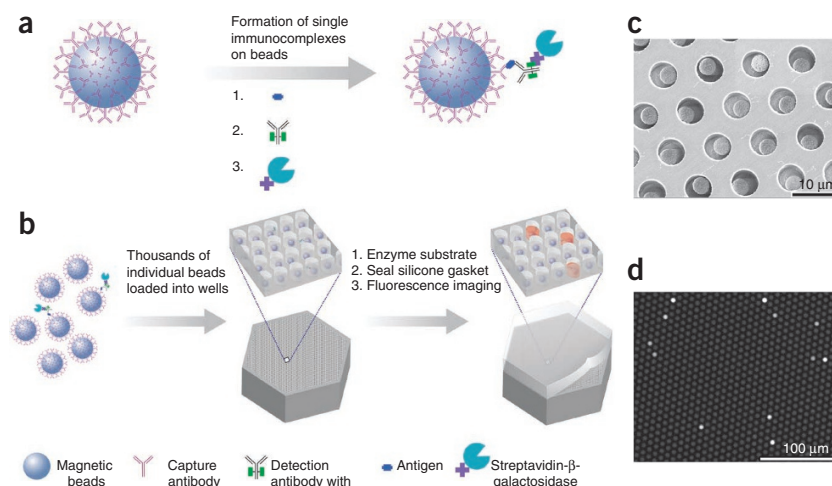
using this technology demonstrated the detection of 10 fM of PSA in serum¹⁷. Nonetheless, the sensitivities achieved by methods for detecting proteins still lag behind those for nucleic acids, such as PCR, limiting the number of gene products that have been detected in blood^{6,19}. The isolation and detection of single protein molecules provides a promising approach for measuring extremely low concentrations of proteins^{1,2}. For example, Todd *et al.*² have developed flow-based methods for serially detecting single fluorescently labeled detection antibodies that have been released from immunocomplexes formed on solid substrates. Here we report an approach for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for detecting proteins, namely, the ELISA. This method has been used to detect proteins in serum at subfemtomolar concentrations.

Our approach makes use of arrays of femtoliter-sized reaction chambers (Fig. 1)—which we term single-molecule arrays (SiMoAs)—that can isolate and detect single enzyme molecules^{20–24}. This approach builds from the work of Walt *et al.*^{20–23}, who used these arrays to study the kinetics²¹ and inhibition²⁰ of single enzymes. Our objective was to exploit the ability of SiMoAs to trap and detect single enzymes to detect single enzyme-labeled proteins. In the first step of this single-molecule immunoassay (Fig. 1a), a sandwich antibody complex is formed on microscopic beads (2.7 μ m diameter), and the bound complexes are labeled with an enzyme, as in a conventional bead-based ELISA. When assaying samples containing extremely low concentrations of protein, the ratio of protein molecules (and the resulting enzyme-labeled complex) to beads is small (typically <1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution. At low concentrations of protein, the Poisson distribution indicates that beads carry either a single immunocomplex or none. For example, if 50 aM of a protein in 0.1 ml (3,000 molecules) is captured and labeled on 200,000 beads, then 1.5% of the beads will carry one protein molecule and 98.5% will not carry any protein molecules (Fig. 1b)²². It is not possible to detect these low numbers of enzyme labels using standard detection technology (for example, a plate reader), because the fluorophores generated by each enzyme diffuse into a large assay volume (typically 0.1–1 ml), and it takes hundreds of thousands of enzyme labels to generate a

¹Quanterix Corporation, Cambridge, Massachusetts, USA. ²Department of Chemistry, Tufts University, Medford, Massachusetts, USA. ³These authors contributed equally to this work. Correspondence should be addressed to D.C.D. (dduffy@quantix.com).

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Figure 1 Digital ELISA based on arrays of femtoliter-sized wells. (a,b) Single protein molecules are captured and labeled on beads using standard ELISA reagents (a), and beads with or without a labeled immunocomplex are loaded into femtoliter-volume well arrays for isolation and detection of single molecules by fluorescence imaging (b). (c) Scanning electron micrograph of a small section of a femtoliter-volume well array after bead loading. Beads (2.7 μm diameter) were loaded into an array of wells with diameters of 4.5 μm and depths of 3.25 μm . (d) Fluorescence image of a small section of the femtoliter-volume well array after signals from single enzymes are generated. Whereas the majority of femtoliter-volume chambers contain a bead from the assay, only a fraction of those beads possess catalytic enzyme activity, indicating a single, bound protein molecule. The concentration of protein in bulk solution is correlated to the percentage of beads that carry a protein molecule.



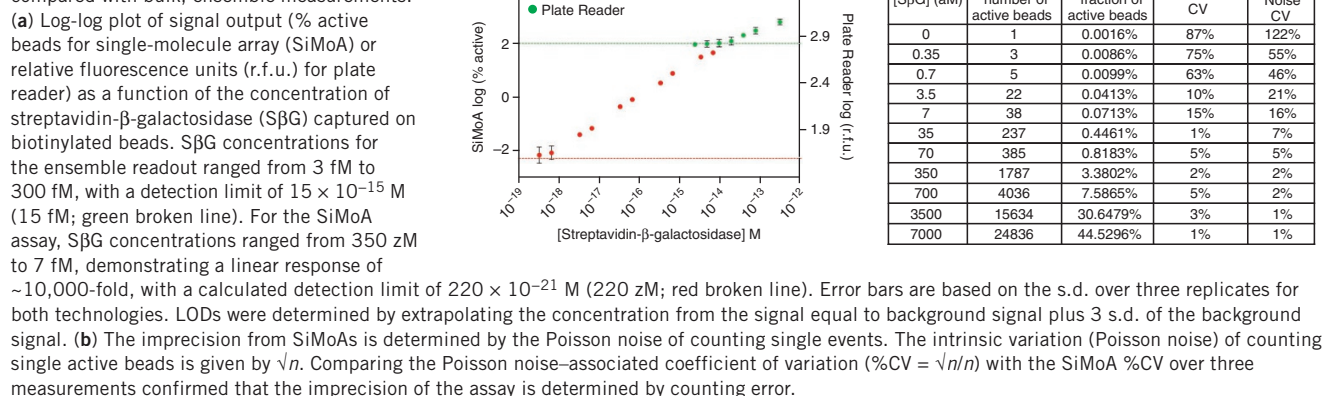
fluorescence signal above background. In contrast, SiMoAs permit the detection of very low concentrations of enzyme labels by confining the fluorophores generated by individual enzymes to extremely small volumes (~ 50 fl), ensuring a high local concentration of fluorescent product molecules. To achieve this confinement in our assay, beads are loaded into an array of femtoliter-sized wells; we used 2-mm-wide arrays with $\sim 50,000$ wells, each with a diameter of 4.5 μm and a depth of 3.25 μm (Fig. 1c). After sealing the loaded arrays against a rubber gasket in the presence of a droplet of fluorogenic enzyme substrate, each bead is isolated in a femtoliter-volume reaction chamber. Beads possessing a single enzyme-labeled immunocomplex generate a high concentration of fluorescent product that is restricted to the 50-fl reaction chamber (Fig. 1d). By acquiring time-lapsed fluorescence images of the array using standard microscope optics, it is possible to distinguish beads associated with a single enzyme molecule ("on" well) from those not associated with an enzyme ("off" well); **Supplementary Figure 1** shows histograms of fluorescence from "on" and "off" wells. Imaging the arrays allows simultaneous detection of tens to tens of thousands of single immunocomplexes. The protein concentration in the test sample is determined by counting the number of wells containing both a bead and fluorescent product relative to the total number of wells containing beads (Fig. 1d). As SiMoAs enable concentration to be determined digitally rather than by using the total analog signal, we call our approach to detecting single immunocomplexes digital ELISA.

We first assessed the intrinsic sensitivity of this strategy by creating populations of beads with well-characterized enzyme-to-bead ratios. We mixed 400,000 biotin-modified beads with a range of concentrations of the enzyme conjugate streptavidin- β -galactosidase (S β G). For convenience, biotinylated beads were provided by hybridizing biotinylated DNA with beads functionalized with complementary DNA. (We note that this experiment should not be construed as a sensitive DNA assay; the sensitivity of such an assay is limited by nonspecific interactions between the enzyme conjugate and surface-bound DNA as shown in **Supplementary Fig. 2**.) These beads were detected in two different ways. First, we assayed an ensemble of beads in 100 μl using a fluorescence plate reader after 1 h incubation with 100 μM resorufin- β -D-galactopyranoside (RGP), a fluorogenic substrate for β -galactosidase. The detection limit for enzyme on the plate reader was 15 fM of S β G (Fig. 2). Second, we loaded the beads into femtoliter-volume well arrays and, after sealing a solution of RGP

into the wells of the array, allowed the signal from single enzymes to accumulate in the reaction chambers for 2.5 min, acquiring fluorescent images every 30 s. At the end of the experiment we used an image of the array that was acquired in white light to identify wells that contained beads (bead-containing wells scatter light differently than empty wells). The fluorescent images were used to determine which of those beads had an associated bound enzyme (from increasing intensity in time-lapsed fluorescent images). **Figure 2** shows a log-log plot of the percentage of beads that contained an enzyme as a function of bulk S β G concentration. The lowest concentration of enzyme conjugate detected was 350 zeptomolar (zM) and the calculated limit of detection (LOD)—determined by extrapolating the enzyme concentration at a signal equal to background plus 3 s.d. of the background signal—was 220 zM. The sensitivity of SiMoAs to intrinsic label was, therefore, ~ 10 –20 enzymes in 100 μl , corresponding to an increase in sensitivity over ensemble measurements using a typical ELISA-plate reader of a factor of about 68,000. For comparison, chemiluminescent detection of alkaline phosphatase⁴, a highly sensitive enzyme reporter system widely used in clinical diagnostics, has an LOD of about 30 aM (see <http://www.turnerbiosystems.com/doc/appnotes/S_0096.php>). The high sensitivity that arises from the thermodynamic and kinetic efficiency of the capture and detection processes in our approach are discussed below and detailed in **Supplementary Table 1**.

The linear dynamic range of digital detection of enzyme labels by SiMoAs is determined by the ability to distinguish "on" and "off" wells. At ratios of enzyme to beads of less than $\sim 1:10$, Poisson statistics show that the only statistically significant populations of beads are those carrying either one enzyme or no enzyme. Single enzymes can be detected provided that sufficient beads are interrogated and the number of active beads rises above the Poisson noise of counting active beads. At ratios of enzyme to beads greater than $\sim 1:10$, the fraction of active beads becomes much higher, and Poisson statistics show that there are a significant number of beads with multiple enzymes. To quantify the number of detected enzymes and maintain linearity in the subpopulations of beads with multiple enzymes, we use Poisson statistics to convert the number of active beads to the number of detected enzymes (see **Supplementary Table 1**). As the percentage of active beads approaches 50% (ratios of enzyme to beads greater than $\sim 1:1.5$), however, distinguishing "on" and "off" wells using image analysis software becomes challenging, and we reach a practical upper limit of the digital dynamic range. For example, the

Figure 2 Digitization of enzyme-linked complexes greatly increases sensitivity compared with bulk, ensemble measurements.



signal at 7 fM ($\sim 45\%$ active) in **Figure 2** deviates from linearity. As a result, the digital, linear dynamic range demonstrated here using 50,000 wells spanned approximately four orders of magnitude (from 3.5 fM down to 350 zM). Provided that proteins are labeled using appropriate enzyme concentrations (see below), this dynamic range is sufficient for many clinical applications.

We have developed digital ELISAs for two clinically relevant proteins—PSA and tumor necrosis factor- α (TNF- α)—to determine the sensitivity of the approach for detecting proteins in blood. The critical parameters in developing these assays were the concentrations of the beads and the two labeling reagents (detection antibody and enzyme conjugate). The choice of bead concentration depends on several competing factors. First, a sufficient number of beads must be present to capture most of the target analyte from thermodynamic and kinetic perspectives. Thermodynamically, 200,000 beads in 100 μ l, each of which has $\sim 80,000$ antibodies²⁵ bound to it, equates to an antibody concentration of about 0.3 nM. The antibody-protein equilibrium at that concentration permits a high capture efficiency ($>70\%$) (D.M.R., E.P.F., D.C.D., unpublished work). Kinetically, for 200,000 beads dispersed in 100 μ l, the average distance between beads is about 80 μ m. Proteins the size of TNF- α and PSA (17.3 and 30 kDa, respectively) will diffuse 80 μ m in <1 min, suggesting that capture of the protein molecules will not be limited kinetically over a 2-h incubation. Second, a sufficient number of beads must be present to be loaded onto the arrays to limit Poisson noise. Loading 200,000 beads into 50,000-well arrays typically results in 20,000–30,000 beads being trapped in femtoliter-sized wells. For a typical background signal of

1% active beads (see below), this loading results in a background signal of 200–300 active beads detected, corresponding to an acceptable coefficient of variation (CV) from Poisson noise of 6–7%. Third, excessive bead concentrations can lead to both increases in non-specific binding that reduces signal-to-background and low ratios of analyte to beads that can result in high CVs from Poisson noise. The balance of these factors means that 200,000 to 1,000,000 beads per 100 μ l of test sample is optimal for digital ELISA. The concentrations of detection antibody and enzyme conjugate were also minimized to yield the acceptable background signal (1%) and Poisson noise (**Supplementary Discussion**).

Figure 3 shows data from digital ELISAs for PSA and TNF- α . The human forms of the proteins were spiked into 25% bovine serum to final concentrations representative of clinical test samples. A fourfold dilution factor is typically used to reduce matrix effects in immunoassays⁴. Using digital ELISA to detect PSA in 25% serum, we obtained an LOD of ~ 50 aM (1.5 fg/ml), which equates to an LOD in whole serum of ~ 200 aM (6 fg/ml). The lowest concentration tested and detected was 250 aM in 25% serum, corresponding to 1 fM in whole serum. As LOD is determined by extrapolating the concentration at background plus 3 s.d. of the background, LODs for different runs are dependent on the CV of the background. We obtained subfemtomolar LODs of PSA in whole serum over several experiments with typical background variances. For comparison, a leading commercial PSA assay (ADVIA Centaur, Siemens) reports an LOD of 3 pM (0.1 ng/ml) in human serum, and ultrasensitive assays have been reported with LODs in the range

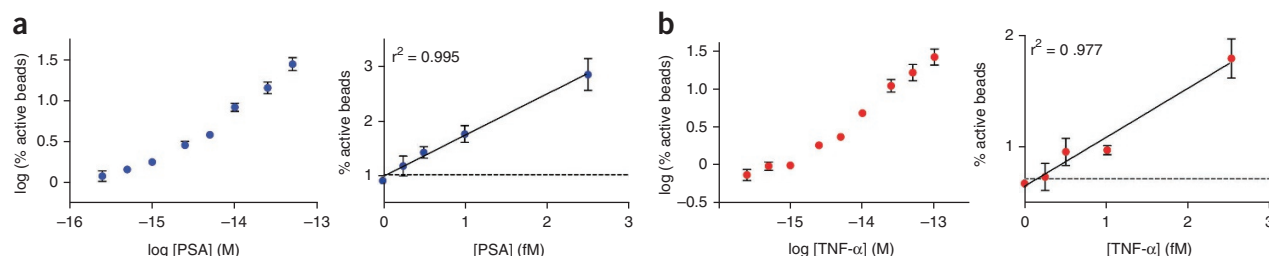
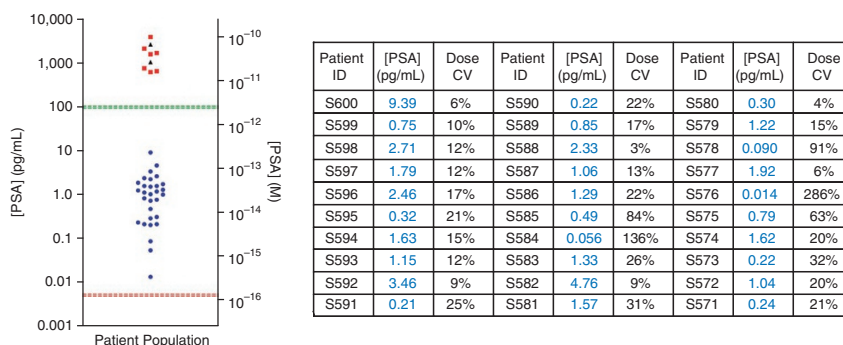


Figure 3 Subfemtomolar detection of proteins in serum using digital ELISA. (a,b) Changes in the percentage of active beads with changes in analyte concentration for human prostate-specific antigen (PSA) spiked into 25% serum (a) and human tumor necrosis factor- α (TNF- α) spiked into 25% serum (b). The concentrations plotted on the x axes refer to the final concentration of spiked protein in the diluted sample. The plots on the left-hand side show the assay response over the concentration range tested in log-log space. The plots on the right-hand side show the assay response in the femtomolar range in linear-linear space to illustrate the limit of detection (LODs) and linearity of response. LODs were determined by extrapolating the concentration from the signal equal to background signal plus 3 s.d. of the background signal. Broken lines, signal at the LOD. Error bars, s.d. over three replicates.

Figure 4 Digital detection of prostate-specific antigen (PSA) in serum samples of patients who had undergone radical prostatectomy. Concentrations of PSA in serum samples from radical prostatectomy patients (●), healthy control samples (■) and Bio-Rad PSA control samples (▲) determined using digital ELISA. Radical prostatectomy patient samples (SeraCare Life Sciences) all had undetectable PSA levels as measured by a leading clinical diagnostic assay (ADVIA Centaur); the green broken line represents the detection limit of the ADVIA Centaur PSA assay (100 pg/ml or 3 pM). All 30 patient samples were above the detection limit of the PSA digital ELISA, shown by the red broken line (0.006 pg/ml or ~200 aM), with the lowest patient PSA concentrations measured at 0.014 pg/ml (~400 aM) using digital ELISA. Patient samples with the lowest PSA levels approached the LOD of the assay, resulting in a large imprecision in the concentration determined (high dose %CV). The digital ELISA was validated for specificity to PSA using control standards (Bio-Rad) and serum from healthy individuals (ProMedDx) that had been assayed using the ADVIA Centaur PSA assay (**Supplementary Table 3**).



of 10–30 fM^{17,26}. The detection limit determined from the TNF- α digital ELISA was ~150 aM (2.5 fg/ml), corresponding to ~600 aM (10 fg/ml) in whole serum; the highest sensitivity commercially available ELISA for TNF- α has an LOD of 21 fM (0.34 pg/ml) in serum (**Supplementary Fig. 3**). The zero concentration spike of target protein for both assays provides a useful negative control for these experiments: 25% serum contains millimolar concentrations of a wide variety of proteins, and very low concentrations of target protein can be detected above these high-protein backgrounds. We also developed a proof-of-principle digital assay for DNA to show that low concentrations of nucleic acids can be detected without replication of the target (**Supplementary Fig. 2**).

The ability of digital ELISA to measure much lower concentrations of proteins than conventional ELISA derives from two effects: (i) the high sensitivity of SiMoAs to enzyme label and (ii) the low background signals that can be achieved by digitizing the detection of proteins. The sensitivity of any immunoassay is determined by the sensitivity of the detection technology to the label, the antibody affinity, the assay background and the variance (%CV) of the background measurement²⁷. The subattomolar sensitivity of SiMoAs to enzyme labels (**Fig. 2**) enables digital ELISA to detect subfemtomolar concentrations of labeled proteins. That said, for antibodies of given affinity, the sensitivity of the immunoassay will be determined by the assay background, and the high label sensitivity of SiMoAs helps reduce this background. Control experiments have shown that backgrounds in digital ELISA arise from nonspecific binding of detection antibody and enzyme conjugate to the capture bead surface (**Supplementary Table 2**). As SiMoAs provide superior label sensitivity over conventional assays, substantially less detection antibody (~1 nM) and enzyme conjugate (1–50 pM) are needed to detect binding events as compared with conventional assays (labeling reagent concentrations ~10 nM). The decreased label concentration reduces nonspecific binding to the capture surface, resulting in much lower background signals. For example, in the TNF- α and PSA digital ELISAs, the levels of nonspecific binding were equivalent to the signal produced by 1.8 fM and 1.2 fM of target protein, respectively (**Fig. 3**). The highest sensitivity commercial TNF- α assay has a level of nonspecific binding equivalent to 85 fM of TNF- α (**Supplementary Fig. 3**), which is 50 times more than we observe using digital ELISA. The ability to reduce backgrounds in digital ELISA by lowering the concentration of labeling reagents enables more sensitive immunoassays than are possible using conventional assays.

To demonstrate the possible diagnostic value of detecting very low concentrations of proteins in blood using digital ELISA, we measured PSA in serum samples from patients who had undergone radical prostatectomy. Levels of serum PSA are used both to screen for prostate cancer and to monitor recurrence of the disease in patients who have undergone radical prostatectomy²⁸. After radical prostatectomy the vast majority of PSA is eliminated, and concentrations fall below the detection limit of standard commercial assays (3 pM or 0.1 ng/ml). Although regular monitoring of these patients for increases in PSA concentrations can detect recurrence of prostate cancer, several years may pass after surgery for biochemical recurrence to be detected by current immunoanalyzers. The ability to accurately quantify PSA levels in patients who have undergone prostatectomy at very low concentrations (<300 fM or 10 pg/ml) should provide early indication of recurrence if PSA levels increase^{17,29}. **Figure 4** shows PSA concentrations measured using digital ELISA in the serum of 30 patients (age 60–89) who had undergone radical prostatectomy and whose blood was collected at least 6 weeks after surgery. The PSA concentrations in the sera of all 30 patients were below the detection limit of commercial assays. Whole-serum samples were diluted 1:4 in buffer and measured using the digital ELISA specific for PSA (**Fig. 3a**). PSA was successfully detected in all 30 patients using digital ELISA, with concentrations ranging from 14 fg/ml to 9.4 pg/ml, with an average of 1.5 pg/ml. Further clinical studies are required to establish the diagnostic benefit of measuring PSA at femtogram per milliliter amounts in patients following radical prostatectomy.

By isolating and detecting single immunocomplexes in arrays of femtoliter-volume wells, digital ELISA enables clinically important proteins in serum to be measured at subfemtomolar concentrations. We believe that the improvement in sensitivity shown by digital ELISA over previously reported approaches will translate into diagnostic benefits. For example, PSA concentrations in 9 of the 30 radical prostatectomy samples assayed in this work fell below the LOD of the previously highest sensitivity PSA assay based on nanoparticle labels¹⁷. An attractive feature of this approach is the ability to directly use reagents developed for standard ELISA for substantially more sensitive assays. We continue to improve the SiMoA technology in two key areas. First, based on the sensitivity to enzyme label (**Fig. 2**), it seems that the sensitivity of protein detection could be increased at least 100-fold if nonspecific interactions that cause background signals could be minimized. The ability to isolate and interrogate single molecules on individual beads provides avenues for distinguishing antibody-antigen

binding events from nonspecifically bound complexes. Second, we are simplifying the logistics of the assays. Even in its present form, however, we believe that digital ELISA has the potential to facilitate earlier diagnosis and treatment of disease.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturebiotechnology/>.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

D.M.R., C.W.K., D.R.F., D.R.W. and D.C.D. conceived the approach. D.R.F. built the imaging system. D.M.R., C.W.K., T.G.C., S.C.H., L.S., P.P.P., A.J.R., E.P.F., J.D.R. and G.K.P. conducted the experiments. T.P. wrote the image analysis software. L.C. prepared reagents. D.M.R. and D.C.D. wrote the manuscript. All authors were involved in designing experiments, reviewing and discussing data, and commenting on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>.

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ONLINE METHODS

Materials. Optical fiber bundles were purchased from Schott North America. Nonreinforced gloss silicone sheeting was obtained from Specialty Manufacturing. Hydrochloric acid, anhydrous ethanol and molecular biology-grade Tween-20 were all from Sigma-Aldrich. Carboxyl-terminated magnetic beads (2.7- μm diameter) were purchased from Varian. Monoclonal capture antibody to human TNF- α , polyclonal detection antibody to human TNF- α and recombinant human TNF- α were purchased from R&D Systems. Monoclonal capture antibody to PSA, monoclonal detection antibody to PSA and purified PSA were purchased from BiosPacific; the detection antibody was biotinylated using standard methods. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, N-hydroxysulfosuccinimide and SuperBlock T-20 Blocking Buffer were purchased from Thermo Scientific. Purified DNA was purchased from Integrated DNA Technologies. S β G was conjugated in the laboratory using standard protocols. RGP was purchased from Invitrogen. The fiber polisher and polishing consumables were purchased from Allied High Tech Products.

Preparation of magnetic beads presenting biotin-labeled DNA and capture of enzyme conjugate (Fig. 2). Beads functionalized with DNA capture probe (5'-NH₂/C12-GTT GTC AAG ATG CTA CCG TTC AGA G-3') were prepared according to the manufacturer's instructions. These beads were incubated with 1 μM of biotinylated complementary DNA (5'-biotin-C TCT GAA CGG TAG CAT CTT GAC AAC-3') overnight (16 h) in TE buffer containing 0.5 M NaCl and 0.01% Tween-20. After incubation the beads were washed three times in PBS buffer containing 0.1% Tween-20. The bead stock was distributed into a microtiter plate giving 400,000 beads per well in 100 μl . The buffer was aspirated from the microtiter plate wells; the beads were resuspended and incubated with various concentrations of S β G in Superblock containing 0.05% Tween-20 for 4 h. The beads were then separated and washed six times with 5 $\times$ PBS buffer containing 0.1% Tween-20. For detection of enzyme, the beads were either (i) resuspended in 20 μl of PBS containing 0.1% Tween-20, and 10- μl aliquots were loaded onto two femtoliter-volume well arrays for SiMoA detection; or (ii) resuspended in 100 μl of 100 μM RGP in PBS, incubated for 1 h at 23 $^{\circ}\text{C}$ and read on a fluorescence plate reader (Infinite M200, Tecan).

Capture of proteins on magnetic beads and formation of enzyme-labeled immunocomplex (Figs. 3 and 4). Beads functionalized with an antibody to the target protein were prepared according to the manufacturer's instructions. Test solutions (100 μl) containing the protein of interest were incubated with suspensions of 200,000 magnetic beads for 2 h at 23 $^{\circ}\text{C}$. The beads were then separated and washed three times in PBS and 0.1% Tween-20. The beads were resuspended and incubated with solutions containing detection antibody (typically ~ 1 nM) for 45 min at 23 $^{\circ}\text{C}$. The beads were then separated and washed three times in PBS and 0.1% Tween-20. The beads were incubated with solutions containing S β G (1–50 pM) for 30 min at 23 $^{\circ}\text{C}$, separated and washed six times in PBS and 0.1% Tween-20. The beads were then resuspended in 10 μl of PBS and loaded onto a femtoliter-volume well array. The total time of the assay was ~ 6 h.

Capture of DNA on magnetic beads and formation of enzyme-labeled complex (Supplementary Fig. 2). A total of 200,000 beads functionalized with DNA capture probe were incubated with 100- μl solutions containing the target DNA (5'-TT GAC GGC GAA GAC CTG GAT GTA TTG CTC C TCT GAA CGG TAG CAT CTT GAC AAC-3') for 2 h. After incubation, the DNA target solution was removed and the beads were washed three times in 0.2 $\times$ SSC buffer containing 0.1% Tween-20. The beads were then resuspended and mixed with 10 nM biotinylated signal probe (5'-TAC ATC CAG GTC TTC GCC GTC AA/Biotin/-3') that is also specific to the target DNA for 1 h. The beads were then washed three times in 0.2 $\times$ SSC buffer containing 0.1% Tween-20 after removing the signal probe. A solution of 10 pM S β G was then added to the bead pellet, resuspended and mixed for 1 h. The beads were separated and washed six times in 5 $\times$ PBS buffer containing 0.1% Tween-20. The beads were then resuspended in 10 μl of PBS and loaded onto a femtoliter-volume well array.

Preparation of femtoliter-volume well arrays. Optical fiber bundles approximately 5 cm long were sequentially polished on a polishing machine using 30-, 9- and 1- μm -sized diamond lapping films. The polished fiber bundles were chemically etched in a 0.025 M HCl solution for 130 s and then immediately submerged into water to quench the reaction. The etched fibers were sonicated for 5 s in water, washed in water for 5 min and dried under vacuum. The differential etch rate of the core and cladding glass of the fiber bundle arrays caused 4.5- μm -diameter wells to be formed in the core fibers³⁰. Different etch times resulting in different well depths were initially investigated. If wells were too deep, then multiple beads were deposited in each well and sealing was disrupted; if wells were too shallow, then the beads were not retained in the wells and poor loading efficiencies were observed. Well depths of 3.25 ± 0.5 μm were optimal for retaining single beads in wells while maintaining good seals.

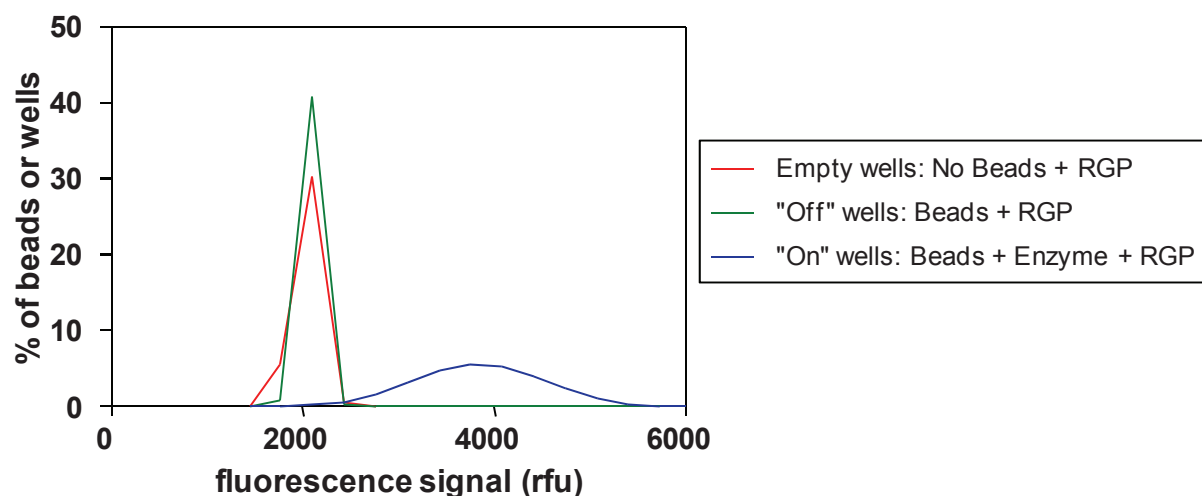
Loading of beads into femtoliter-volume well arrays. A short length of PVC tubing was placed on the etched end of a fiber bundle to create a reservoir to hold the bead solution. Ten microliters of the concentrated bead solution were pipetted into this reservoir. The fiber bundle was then centrifuged at 1,300g for 10 min to force the beads into the etched wells. The PVC tubing was removed after centrifugation. The fiber bundle was dipped in PBS solution to wash off excess bead solution, and the surface was swabbed with deionized water. In addition to well depth (see above), bead concentration was an important parameter for maximizing bead loading efficiencies. Above concentrations of 200,000 beads per 10 μl loaded, typically 40–60% of wells in a 50,000-well array were occupied by a single bead, resulting in percentage active beads with acceptable Poisson noise. At concentrations below 200,000 beads per 10 μl loaded, bead loading efficiency dropped, resulting in fewer active beads and higher Poisson noise. In these experiments, therefore, at least 200,000 beads per reaction were used and loaded onto the arrays.

Detection of beads and enzyme-labeled beads in femtoliter-volume well arrays. A custom-built imaging system containing a mercury light source, filter cubes, objectives and a CCD camera was used for acquiring fluorescence images^{21,23}. Fiber bundles were mounted on the microscope stage using a custom fixture. A droplet of β -galactosidase substrate (RGP) was placed on the silicone gasket material and placed in contact with the well arrays. A precision mechanical platform moved the silicone sheet into contact with the end of the fiber bundle, creating an array of isolated femtoliter-volume reaction vessels. Fluorescence images were acquired (558 nm excitation; 577 nm emission) with an exposure time of 1,011 ms. Five frames (at 30-s intervals) were taken for each femtoliter-volume well array. The product of the enzymatic reaction used in these studies—resorufin—has high photostability with a low photobleaching rate (rate of photobleaching, $k_{\text{ph}} = 0.0013 \text{ s}^{-1}$)²¹, making multiple exposures possible. We performed time-course fluorescence measurements (i) to allow stable fluorescent artifacts to be removed from images and (ii) to ensure that the signal from a beaded well was from an enzyme. For (i), the first fluorescent image was subtracted from fluorescent images acquired at each subsequent time point. This process removed light intensity that did not change with time; for example, fluorescence from dust and scattered light. For (ii), a positive or “on” well was identified only where fluorescence intensity in a beaded well increased in every frame and by at least 20% over four frames. This process removed false positives from random changes in fluorescence during image acquisition. **Supplementary Figure 1** shows histograms of fluorescence from wells with and without enzymes, showing the good distinction between “on” and “off” wells. Arrays were also imaged with white light to identify those wells that contain beads. After acquiring the fluorescence images, the arrays were illuminated with white light and imaged on the CCD camera. Due to scattering of light by the beads, those wells that contained a bead appeared brighter in the image than wells without beads. The fluorescence and white-light images were analyzed using customized software.

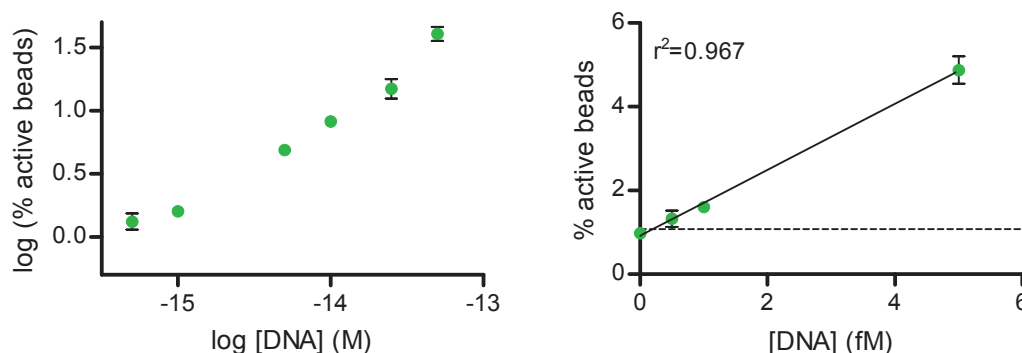
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Supplementary Information

1) Supplementary Figures and Legends

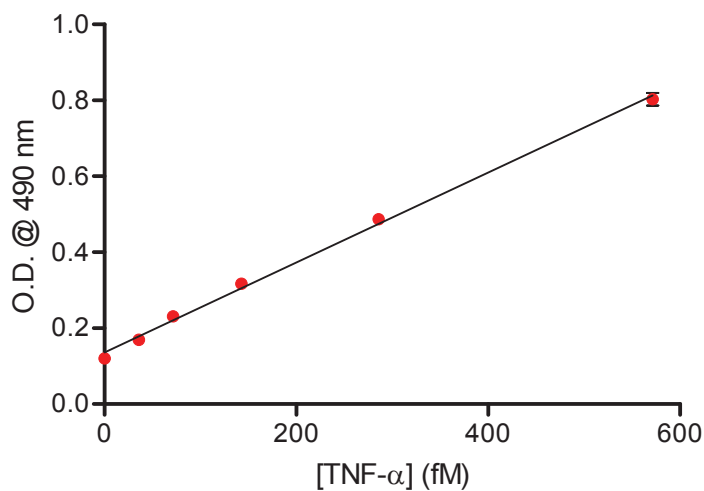


Supplementary Figure 1. Histograms of the average fluorescence intensity of wells in a SiMoA experiment. A representative set of images from a SiMoA experiment (Figure 1) were analyzed to determine populations of wells that: a) contained no beads; b) contained a bead (from white light scatter) but no enzyme (no increase in fluorescence intensity); and, c) contained a bead (from white light scatter) and an enzyme (increasing fluorescence intensity over four consecutive images, and an overall increase in fluorescence of at least 20%). Using this method of calling “on” wells, the occurrence of false positives in the absence of background proteins is very small (<1 in a 50,000-well array, see Figure 2). In the presence of background proteins, e.g., serum samples, there is a significant number of “on” wells from non-specific binding that sets the background signal and limits of detection (see discussion in main text). The fraction of beads within each population is plotted against the average intensity of wells with a bin size of 120 rfu. The red line represents data from empty wells; the green line represents wells with a bead but no enzyme (“off” well); and the blue line represents beads with both a bead and enzymatic activity (“on” well). While a range of enzyme activities are observed as anticipated²¹, there is good separation between on and off wells to allow digital quantification.



Supplementary Figure 2. Detection of DNA using digital counting of enzyme labels. Plots of % active beads against different concentrations of DNA spiked into buffer. Experimental details are provided in the Methods section. The plot on the left hand side show the assay response over the

concentration range tested in log-log space. The plot on the right hand side show the assay response in the femtomolar range in linear-linear space. Error bars are plotted as the standard deviation over three replicates. LODs were determined by extrapolating the concentration from the signal equal to background signal plus three standard deviations of the background signal; the dashed lines show the signal at the LOD. The LOD of the digital DNA sandwich assay was ~150 aM, corresponding to about 8000 copies. While these results are preliminary—the DNA was detected in buffer in the absence of non-target DNA—and the sensitivity does not approach that that can be achieved with PCR, they do illustrate that SiMoA can be used to detect nucleic acids without recourse to molecular replication.



Supplementary Figure 3. The R&D Systems HS-ELISA for TNF-α. A TNF-α calibration curve yielded an LOD of 21 fM in whole serum using a Tecan Infinite M200 plate reader. The non-specific binding (NSB) background in this high sensitivity assay was equivalent to about 85 fM of TNF-α.

2) Supplementary Tables

	Column A	Column B	Column C	Column D	Column E	Column F
[SβG] (aM)	Average % active beads observed (Figure 2)	Enzyme/bead ratio from Poisson distribution	Total # of enzymes on 400,000 beads	Background corrected # of enzymes on beads	Calculated # of enzymes in 100- μL sample	Capture Efficiency
0	0.0016%	0.000016	7	---	---	---
0.35	0.0086%	0.000086	34	28	21	132%
0.7	0.0099%	0.000099	40	33	42	79%
3.5	0.0413%	0.000413	165	159	211	75%
7	0.0713%	0.000713	285	279	421	66%
35	0.4461%	0.004471	1789	1782	2107	85%
70	0.8183%	0.008217	3287	3280	4214	78%
350	3.3802%	0.034387	13755	13748	21070	65%
700	7.5865%	0.078897	31559	31552	42140	75%
3500	30.6479%	0.365974	146390	146383	210700	69%
7000	44.5296%	0.589320	235728	235722	421400	56%

Supplementary Table 1. Calculations of the SiMoA capture efficiency of enzyme label from 0.35 aM to 7000 aM. From the fraction of beads that were active in Figure 2 (Column A), the enzyme/bead ratio (Column B) can be determined from the Poisson distribution²³. In this experiment, ~400,000 beads were incubated with each solution of enzyme (although only ~50,000 beads (~12.5%) on average were interrogated because of the current size of the femtoliter arrays (~50,000 wells)), so the number of enzymes on these beads is determined by multiplying Column B by 400,000 (Column C). After background subtraction, the total number of enzyme molecules captured on the beads can be determined (Column D). The ratio of this number to the total calculated number of enzymes in 100 μ L at the start of the experiment (Column E; Volume $\times$ Concentration $\times$ Avogadro's Number) yields a capture efficiency (Column F). The overall efficiency of capture and detection of enzyme using SiMoA is high (65-85%) in the range where Poisson noise is acceptable (<20%, from 3.5 aM to 3500 aM), and is probably only limited in this experiment by the slow diffusion of the large S β G conjugate (MW ~ 515 kDa). At 7000 aM, where the % active beads > 40%, the % active starts to deviate from linearity (giving rise to an apparent lower capture efficiency) because of difficulties in image analysis at these high fractions (see main text).

NSB Dropout Experiment (SiMoA PSA assay)					
	Average	SD	CV	% NSB	
No dAb; No PSA	0.110%	0.162%	147%	from SbG	20%
No SbG; No PSA	0.000%	0.000%	---	from dAb	80%
NSB	0.541%	0.194%	36%	Total NSB	100%

Supplementary Table 2. A dropout experiment isolating the sources of NSB in the PSA digital ELISA. By comparing 'no detection antibody NSB' (No dAb) and 'no S β G NSB' (No S β G) to total NSB (NSB), the contribution of detection antibody and S β G to the NSB of the assay was determined.

	Centaur ng/mL	SiMoA ng/mL
Bio-Rad Control 1	0.838	1.06 $\pm$ 0.21
Bio-Rad Control 2	2.47	2.66 $\pm$ 0.36
Normals		
ProMedDx S376	2.1	1.60
ProMedDx S378	2.3	1.70
ProMedDx S381	2.9	2.14
ProMedDx S388	4.1	3.95
ProMedDx S395	0.93	0.63
ProMedDx S396	0.9	0.77
ProMedDx S397	1.2	0.66

Supplemental Table 3. Specificity of the PSA digital ELISA. Specificity of the digital ELISA was confirmed using PSA samples from Bio-Rad (controls) and ProMedDx (serum from healthy individuals) that had previously been tested on a commercial immunoanalyzer (ADVIA Centaur, Siemens). While there was good correlation between the two methods ($r^2 = 0.96$), the PSA concentrations of the healthy

serum samples determined using SiMoA were (24±12)% lower than those originally determined on the ADVIA Centaur. There are two possible explanations for this systematic bias between the two technologies. First, the ADVIA Centaur values were obtained on fresh serum, whereas digital ELISA values were obtained after the sera had been frozen for extended periods of time and had experienced a freeze-thaw cycle. Second, there were likely differences between the PSA calibrators used to generate the calibration curves that would result in differences in PSA concentrations determined. We used Complex PSA from the World Health Organization (WHO) as calibrators; the ADVIA Centaur calibration PSA is unknown.

3) Supplementary Discussion

Sensitivity to Enzyme Label as Sample Volume Increases. For some applications, volumes larger than 100 μ L might need to be tested for very low concentrations of analyte. As the volume of sample increases, detecting small numbers of enzyme labels with high efficiency becomes more challenging technically. To achieve the same detection efficiencies in 1 mL and 10 mL of sample that we achieved in 100 μ L, would require 10 and 100 times more beads, respectively, both from thermodynamic and kinetic perspectives. These larger numbers of beads (2 million and 20 million) would require much bigger arrays and also larger imaging (CCD) chips or multiple images per array. While these experiments are feasible, they would require a much more complex instrument and longer read times, so might not be justified in a diagnostic setting.

Optimization of Labeling of Captured Proteins in Digital ELISA. The choice of the concentrations of detection antibody and enzyme conjugate (S β G) used to label the captured protein is important for optimizing digital ELISA. The relatively high background signals of ELISAs caused by the non-specific interactions between the capture beads and detection antibody and enzyme conjugate (see Supplementary Table 2), combined with the high sensitivity to label provided by SiMoA, means that often only a fraction of the capture proteins need to be labeled to avoid saturating SiMoA signals. For example, the background levels observed in digital ELISA are equivalent to ~1–2 fM of target protein (see main text), so the ratio of analyte to bead is about 0.3–0.6. At these ratios, the number of active beads would be 25–40% if every protein was labeled with an enzyme, i.e., at the upper end of the digital dynamic range. To ensure that these background signals are closer to the lower end of the digital dynamic range—1% active beads provides a reasonable noise floor for background in digital ELISA—appropriate labeling of the captured protein can be achieved by kinetic control of the labeling steps, either by minimizing the concentrations of both labeling reagents or by using shorter incubation times. We have chosen to minimize label concentrations and use standard ELISA incubation times; as a result, the total assay time is ~6 h. This length of time is acceptable for testing that tolerates a daily turnaround time for samples. For shorter turnaround times of, say, <1 h (e.g., for point-of-care applications), the assay could be performed with short incubations of higher concentrations of labeling reagents.

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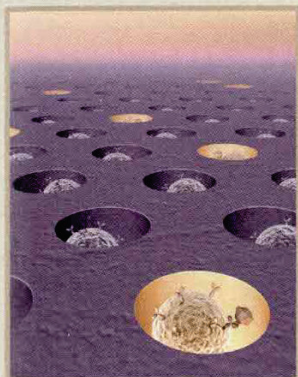
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Artist's impression of digital ELISA. Rissin *et al.* determine protein abundance by capturing beads bearing single analyte molecules in arrayed femtoliter-volume reaction chambers (p 595). Credit: Ken Eward © BioGrafx.



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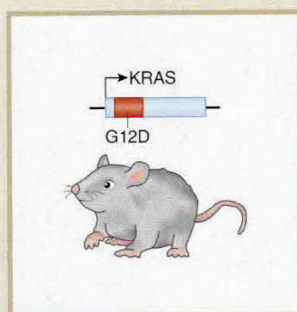
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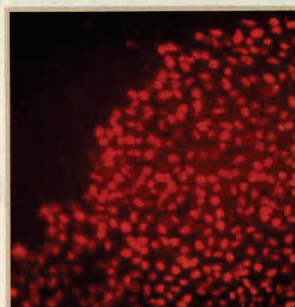


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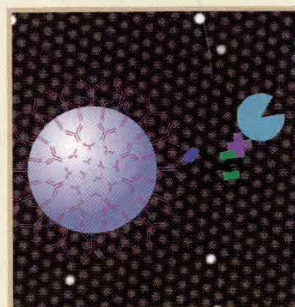
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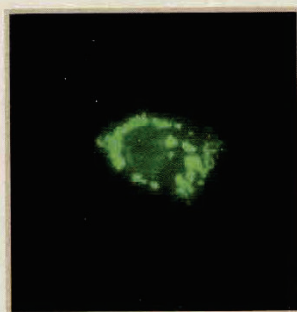
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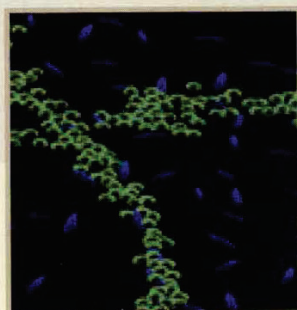
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Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations

David M Rissin^{1,3}, Cheuk W Kan^{1,3}, Todd G Campbell¹, Stuart C Howes¹, David R Fournier¹, Linan Song¹, Tomasz Piech¹, Purvish P Patel¹, Lei Chang¹, Andrew J Rivnak¹, Evan P Ferrell¹, Jeffrey D Randall¹, Gail K Provuncher¹, David R Walt² & David C Duffy¹

The ability to detect single protein molecules^{1,2} in blood could accelerate the discovery and use of more sensitive diagnostic biomarkers. To detect low-abundance proteins in blood, we captured them on microscopic beads decorated with specific antibodies and then labeled the immunocomplexes (one or zero labeled target protein molecules per bead) with an enzymatic reporter capable of generating a fluorescent product. After isolating the beads in 50-fl reaction chambers designed to hold only a single bead, we used fluorescence imaging to detect single protein molecules. Our single-molecule enzyme-linked immunosorbent assay (digital ELISA) approach detected as few as ~10–20 enzyme-labeled complexes in 100 μ l of sample (~10⁻¹⁹ M) and routinely allowed detection of clinically relevant proteins in serum at concentrations (<10⁻¹⁵ M) much lower than conventional ELISA^{3–5}. Digital ELISA detected prostate-specific antigen (PSA) in sera from patients who had undergone radical prostatectomy at concentrations as low as 14 fg/ml (0.4 fM).

The clinical use of protein biomarkers to differentiate between healthy and disease states, and to monitor disease progression, requires the measurement of low concentrations of proteins in complex samples. Current immunoassays typically measure proteins at concentrations above 10⁻¹² M⁶. The serum concentrations of the majority of proteins important in cancer⁷, neurological disorders^{8,9} and the early stages of infection¹⁰, however, are thought to range from 10⁻¹⁶ to 10⁻¹² M. For instance, a 1-mm³ tumor composed of a million cells that each secrete 5,000 proteins into 5 liters of circulating blood translates to a concentration of ~2 $\times$ 10⁻¹⁵ M (or 2 fM). Moreover, serum from individuals recently infected with HIV contains 10–3,000 virions per ml, resulting in estimated concentrations of the p24 capsid antigen ranging from 50 $\times$ 10⁻¹⁸ M (50 aM) to 15 $\times$ 10⁻¹⁵ M (15 fM)¹⁰. Attempts to develop methods capable of measuring these concentrations of proteins have focused on the replication of nucleic acid labels on proteins^{11,12}, or on measuring the bulk, ensemble properties of labeled protein molecules^{13–16}. The work of Mirkin *et al.*^{12,17} and others¹⁸ using labels based on gold nanoparticles and DNA biobarcodes has pushed the detection of proteins into the low femtomolar range; a recent report

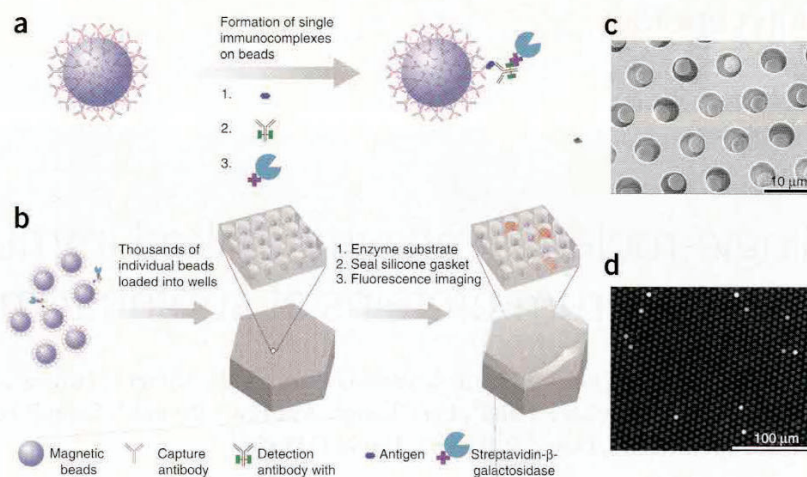
using this technology demonstrated the detection of 10 fM of PSA in serum¹⁷. Nonetheless, the sensitivities achieved by methods for detecting proteins still lag behind those for nucleic acids, such as PCR, limiting the number of gene products that have been detected in blood^{6,19}. The isolation and detection of single protein molecules provides a promising approach for measuring extremely low concentrations of proteins^{1,2}. For example, Todd *et al.*² have developed flow-based methods for serially detecting single fluorescently labeled detection antibodies that have been released from immunocomplexes formed on solid substrates. Here we report an approach for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for detecting proteins, namely, the ELISA. This method has been used to detect proteins in serum at subfemtomolar concentrations.

Our approach makes use of arrays of femtoliter-sized reaction chambers (Fig. 1)—which we term single-molecule arrays (SiMoAs)—that can isolate and detect single enzyme molecules^{20–24}. This approach builds from the work of Walt *et al.*^{20–23}, who used these arrays to study the kinetics²¹ and inhibition²⁰ of single enzymes. Our objective was to exploit the ability of SiMoAs to trap and detect single enzymes to detect single enzyme-labeled proteins. In the first step of this single-molecule immunoassay (Fig. 1a), a sandwich antibody complex is formed on microscopic beads (2.7 μ m diameter), and the bound complexes are labeled with an enzyme, as in a conventional bead-based ELISA. When assaying samples containing extremely low concentrations of protein, the ratio of protein molecules (and the resulting enzyme-labeled complex) to beads is small (typically <1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution. At low concentrations of protein, the Poisson distribution indicates that beads carry either a single immunocomplex or none. For example, if 50 aM of a protein in 0.1 ml (3,000 molecules) is captured and labeled on 200,000 beads, then 1.5% of the beads will carry one protein molecule and 98.5% will not carry any protein molecules (Fig. 1b)²². It is not possible to detect these low numbers of enzyme labels using standard detection technology (for example, a plate reader), because the fluorophores generated by each enzyme diffuse into a large assay volume (typically 0.1–1 ml), and it takes hundreds of thousands of enzyme labels to generate a

¹Quanterix Corporation, Cambridge, Massachusetts, USA. ²Department of Chemistry, Tufts University, Medford, Massachusetts, USA. ³These authors contributed equally to this work. Correspondence should be addressed to D.C.D. (dduffy@quanterix.com).

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Figure 1 Digital ELISA based on arrays of femtoliter-sized wells. (a,b) Single protein molecules are captured and labeled on beads using standard ELISA reagents (a), and beads with or without a labeled immunocomplex are loaded into femtoliter-volume well arrays for isolation and detection of single molecules by fluorescence imaging (b). (c) Scanning electron micrograph of a small section of a femtoliter-volume well array after bead loading. Beads (2.7 μm diameter) were loaded into an array of wells with diameters of 4.5 μm and depths of 3.25 μm . (d) Fluorescence image of a small section of the femtoliter-volume well array after signals from single enzymes are generated. Whereas the majority of femtoliter-volume chambers contain a bead from the assay, only a fraction of those beads possess catalytic enzyme activity, indicating a single, bound protein molecule. The concentration of protein in bulk solution is correlated to the percentage of beads that carry a protein molecule.



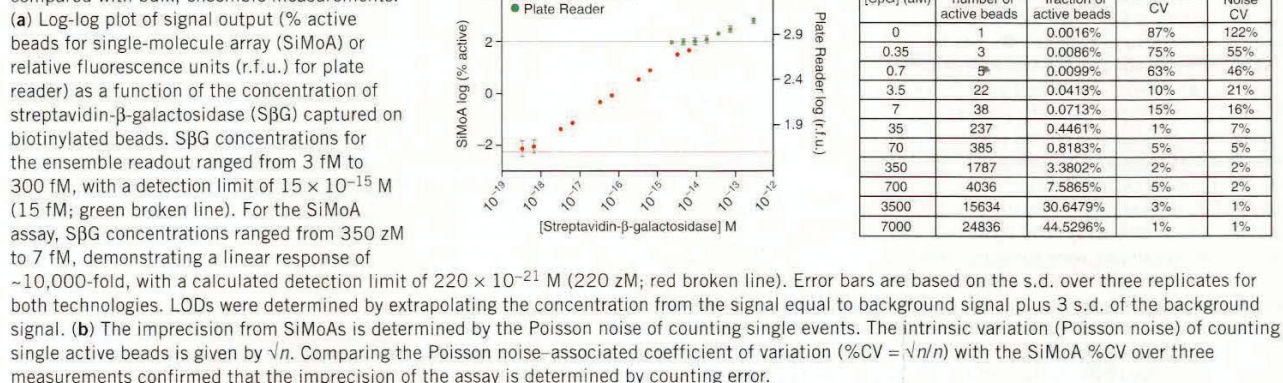
fluorescence signal above background. In contrast, SiMoAs permit the detection of very low concentrations of enzyme labels by confining the fluorophores generated by individual enzymes to extremely small volumes (~ 50 fl), ensuring a high local concentration of fluorescent product molecules. To achieve this confinement in our assay, beads are loaded into an array of femtoliter-sized wells; we used 2-mm-wide arrays with $\sim 50,000$ wells, each with a diameter of 4.5 μm and a depth of 3.25 μm (Fig. 1c). After sealing the loaded arrays against a rubber gasket in the presence of a droplet of fluorogenic enzyme substrate, each bead is isolated in a femtoliter-volume reaction chamber. Beads possessing a single enzyme-labeled immunocomplex generate a high concentration of fluorescent product that is restricted to the 50-fl reaction chamber (Fig. 1d). By acquiring time-lapsed fluorescence images of the array using standard microscope optics, it is possible to distinguish beads associated with a single enzyme molecule ("on" well) from those not associated with an enzyme ("off" well); **Supplementary Figure 1** shows histograms of fluorescence from "on" and "off" wells. Imaging the arrays allows simultaneous detection of tens to tens of thousands of single immunocomplexes. The protein concentration in the test sample is determined by counting the number of wells containing both a bead and fluorescent product relative to the total number of wells containing beads (Fig. 1d). As SiMoAs enable concentration to be determined digitally rather than by using the total analog signal, we call our approach to detecting single immunocomplexes digital ELISA.

We first assessed the intrinsic sensitivity of this strategy by creating populations of beads with well-characterized enzyme-to-bead ratios. We mixed 400,000 biotin-modified beads with a range of concentrations of the enzyme conjugate streptavidin- β -galactosidase (S β G). For convenience, biotinylated beads were provided by hybridizing biotinylated DNA with beads functionalized with complementary DNA. (We note that this experiment should not be construed as a sensitive DNA assay; the sensitivity of such an assay is limited by nonspecific interactions between the enzyme conjugate and surface-bound DNA as shown in **Supplementary Fig. 2**.) These beads were detected in two different ways. First, we assayed an ensemble of beads in 100 μl using a fluorescence plate reader after 1 h incubation with 100 μM resorufin- β -D-galactopyranoside (RGP), a fluorogenic substrate for β -galactosidase. The detection limit for enzyme on the plate reader was 15 fM of S β G (Fig. 2). Second, we loaded the beads into femtoliter-volume well arrays and, after sealing a solution of RGP

into the wells of the array, allowed the signal from single enzymes to accumulate in the reaction chambers for 2.5 min, acquiring fluorescent images every 30 s. At the end of the experiment we used an image of the array that was acquired in white light to identify wells that contained beads (bead-containing wells scatter light differently than empty wells). The fluorescent images were used to determine which of those beads had an associated bound enzyme (from increasing intensity in time-lapsed fluorescent images). **Figure 2** shows a log-log plot of the percentage of beads that contained an enzyme as a function of bulk S β G concentration. The lowest concentration of enzyme conjugate detected was 350 zeptomolar (zM) and the calculated limit of detection (LOD)—determined by extrapolating the enzyme concentration at a signal equal to background plus 3 s.d. of the background signal—was 220 zM. The sensitivity of SiMoAs to intrinsic label was, therefore, ~ 10 –20 enzymes in 100 μl , corresponding to an increase in sensitivity over ensemble measurements using a typical ELISA-plate reader of a factor of about 68,000. For comparison, chemiluminescent detection of alkaline phosphatase⁴, a highly sensitive enzyme reporter system widely used in clinical diagnostics, has an LOD of about 30 aM (see <http://www.turnerbiosystems.com/doc/appnotes/S_0096.php>). The high sensitivity that arises from the thermodynamic and kinetic efficiency of the capture and detection processes in our approach are discussed below and detailed in **Supplementary Table 1**.

The linear dynamic range of digital detection of enzyme labels by SiMoAs is determined by the ability to distinguish "on" and "off" wells. At ratios of enzyme to beads of less than $\sim 1:10$, Poisson statistics show that the only statistically significant populations of beads are those carrying either one enzyme or no enzyme. Single enzymes can be detected provided that sufficient beads are interrogated and the number of active beads rises above the Poisson noise of counting active beads. At ratios of enzyme to beads greater than $\sim 1:10$, the fraction of active beads becomes much higher, and Poisson statistics show that there are a significant number of beads with multiple enzymes. To quantify the number of detected enzymes and maintain linearity in the subpopulations of beads with multiple enzymes, we use Poisson statistics to convert the number of active beads to the number of detected enzymes (see **Supplementary Table 1**). As the percentage of active beads approaches 50% (ratios of enzyme to beads greater than $\sim 1:1.5$), however, distinguishing "on" and "off" wells using image analysis software becomes challenging, and we reach a practical upper limit of the digital dynamic range. For example, the

Figure 2 Digitization of enzyme-linked complexes greatly increases sensitivity compared with bulk, ensemble measurements.



signal at 7 fM ($\sim 45\%$ active) in **Figure 2** deviates from linearity. As a result, the digital, linear dynamic range demonstrated here using 50,000 wells spanned approximately four orders of magnitude (from 3.5 fM down to 350 zM). Provided that proteins are labeled using appropriate enzyme concentrations (see below), this dynamic range is sufficient for many clinical applications.

We have developed digital ELISAs for two clinically relevant proteins—PSA and tumor necrosis factor- α (TNF- α)—to determine the sensitivity of the approach for detecting proteins in blood. The critical parameters in developing these assays were the concentrations of the beads and the two labeling reagents (detection antibody and enzyme conjugate). The choice of bead concentration depends on several competing factors. First, a sufficient number of beads must be present to capture most of the target analyte from thermodynamic and kinetic perspectives. Thermodynamically, 200,000 beads in 100 μ l, each of which has $\sim 80,000$ antibodies²⁵ bound to it, equates to an antibody concentration of about 0.3 nM. The antibody-protein equilibrium at that concentration permits a high capture efficiency ($>70\%$) (D.M.R., E.P.F., D.C.D., unpublished work). Kinetically, for 200,000 beads dispersed in 100 μ l, the average distance between beads is about 80 μ m. Proteins the size of TNF- α and PSA (17.3 and 30 kDa, respectively) will diffuse 80 μ m in <1 min, suggesting that capture of the protein molecules will not be limited kinetically over a 2-h incubation. Second, a sufficient number of beads must be present to be loaded onto the arrays to limit Poisson noise. Loading 200,000 beads into 50,000-well arrays typically results in 20,000–30,000 beads being trapped in femtoliter-sized wells. For a typical background signal of

1% active beads (see below), this loading results in a background signal of 200–300 active beads detected, corresponding to an acceptable coefficient of variation (CV) from Poisson noise of 6–7%. Third, excessive bead concentrations can lead to both increases in non-specific binding that reduces signal-to-background and low ratios of analyte to beads that can result in high CVs from Poisson noise. The balance of these factors means that 200,000 to 1,000,000 beads per 100 μ l of test sample is optimal for digital ELISA. The concentrations of detection antibody and enzyme conjugate were also minimized to yield the acceptable background signal (1%) and Poisson noise (Supplementary Discussion).

Figure 3 shows data from digital ELISAs for PSA and TNF- α . The human forms of the proteins were spiked into 25% bovine serum to final concentrations representative of clinical test samples. A fourfold dilution factor is typically used to reduce matrix effects in immunoassays⁴. Using digital ELISA to detect PSA in 25% serum, we obtained an LOD of ~ 50 aM (1.5 fg/ml), which equates to an LOD in whole serum of ~ 200 aM (6 fg/ml). The lowest concentration tested and detected was 250 aM in 25% serum, corresponding to 1 fM in whole serum. As LOD is determined by extrapolating the concentration at background plus 3 s.d. of the background, LODs for different runs are dependent on the CV of the background. We obtained subfemtomolar LODs of PSA in whole serum over several experiments with typical background variances. For comparison, a leading commercial PSA assay (ADVIA Centaur, Siemens) reports an LOD of 3 pM (0.1 ng/ml) in human serum, and ultrasensitive assays have been reported with LODs in the range

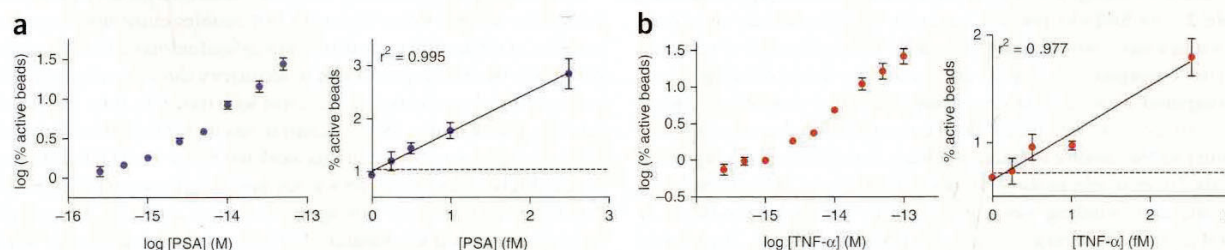
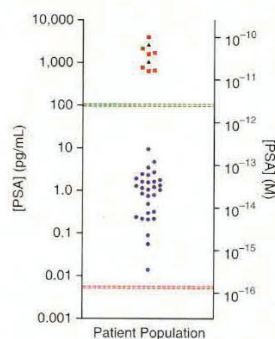


Figure 3 Subfemtomolar detection of proteins in serum using digital ELISA. (a,b) Changes in the percentage of active beads with changes in analyte concentration for human prostate-specific antigen (PSA) spiked into 25% serum (a) and human tumor necrosis factor- α (TNF- α) spiked into 25% serum (b). The concentrations plotted on the x axes refer to the final concentration of spiked protein in the diluted sample. The plots on the left-hand side show the assay response over the concentration range tested in log-log space. The plots on the right-hand side show the assay response in the femtomolar range in linear-linear space to illustrate the limit of detection (LODs) and linearity of response. LODs were determined by extrapolating the concentration from the signal equal to background signal plus 3 s.d. of the background signal. Broken lines, signal at the LOD. Error bars, s.d. over three replicates.

Figure 4 Digital detection of prostate-specific antigen (PSA) in serum samples of patients who had undergone radical prostatectomy.

Concentrations of PSA in serum samples from radical prostatectomy patients (●), healthy control samples (■) and Bio-Rad PSA control samples (▲) determined using digital ELISA. Radical prostatectomy patient samples (SeraCare Life Sciences) all had undetectable PSA levels as measured by a leading clinical diagnostic assay (ADVIA Centaur); the green broken line represents the detection limit of the ADVIA Centaur PSA assay (100 pg/ml or 3 pM). All 30 patient samples were above the detection limit of the PSA digital ELISA, shown by the red broken line (0.006 pg/ml or ~200 aM), with the lowest patient PSA concentrations measured at 0.014 pg/ml (~400 aM) using digital ELISA. Patient samples with the lowest PSA levels approached the LOD of the assay, resulting in a large imprecision in the concentration determined (high dose %CV). The digital ELISA was validated for specificity to PSA using control standards (Bio-Rad) and serum from healthy individuals (ProMedDx) that had been assayed using the ADVIA Centaur PSA assay (Supplementary Table 3).



Patient ID	[PSA] (pg/mL)	Dose CV	Patient ID	[PSA] (pg/mL)	Dose CV	Patient ID	[PSA] (pg/mL)	Dose CV
S600	9.39	6%	S590	0.22	22%	S580	0.30	4%
S599	0.75	10%	S589	0.85	17%	S579	1.22	15%
S598	2.71	12%	S588	2.33	3%	S578	0.090	91%
S597	1.79	12%	S587	1.06	13%	S577	1.92	6%
S596	2.46	17%	S586	1.29	22%	S576	0.014	286%
S595	0.32	21%	S585	0.49	84%	S575	0.79	63%
S594	1.63	15%	S584	0.056	136%	S574	1.62	20%
S593	1.15	12%	S583	1.33	26%	S573	0.22	32%
S592	3.46	9%	S582	4.76	9%	S572	1.04	20%
S591	0.21	25%	S581	1.57	31%	S571	0.24	21%

of 10–30 fM^{17,26}. The detection limit determined from the TNF- α digital ELISA was ~150 aM (2.5 fg/ml), corresponding to ~600 aM (10 fg/ml) in whole serum; the highest sensitivity commercially available ELISA for TNF- α has an LOD of 21 fM (0.34 pg/ml) in serum (Supplementary Fig. 3). The zero concentration spike of target protein for both assays provides a useful negative control for these experiments: 25% serum contains millimolar concentrations of a wide variety of proteins, and very low concentrations of target protein can be detected above these high-protein backgrounds. We also developed a proof-of-principle digital assay for DNA to show that low concentrations of nucleic acids can be detected without replication of the target (Supplementary Fig. 2).

The ability of digital ELISA to measure much lower concentrations of proteins than conventional ELISA derives from two effects: (i) the high sensitivity of SiMoAs to enzyme label and (ii) the low background signals that can be achieved by digitizing the detection of proteins. The sensitivity of any immunoassay is determined by the sensitivity of the detection technology to the label, the antibody affinity, the assay background and the variance (%CV) of the background measurement²⁷. The subattomolar sensitivity of SiMoAs to enzyme labels (Fig. 2) enables digital ELISA to detect subfemtomolar concentrations of labeled proteins. That said, for antibodies of given affinity, the sensitivity of the immunoassay will be determined by the assay background, and the high label sensitivity of SiMoAs helps reduce this background. Control experiments have shown that backgrounds in digital ELISA arise from nonspecific binding of detection antibody and enzyme conjugate to the capture bead surface (Supplementary Table 2). As SiMoAs provide superior label sensitivity over conventional assays, substantially less detection antibody (~1 nM) and enzyme conjugate (1–50 pM) are needed to detect binding events as compared with conventional assays (labeling reagent concentrations ~10 nM). The decreased label concentration reduces nonspecific binding to the capture surface, resulting in much lower background signals. For example, in the TNF- α and PSA digital ELISAs, the levels of nonspecific binding were equivalent to the signal produced by 1.8 fM and 1.2 fM of target protein, respectively (Fig. 3). The highest sensitivity commercial TNF- α assay has a level of nonspecific binding equivalent to 85 fM of TNF- α (Supplementary Fig. 3), which is 50 times more than we observe using digital ELISA. The ability to reduce backgrounds in digital ELISA by lowering the concentration of labeling reagents enables more sensitive immunoassays than are possible using conventional assays.

To demonstrate the possible diagnostic value of detecting very low concentrations of proteins in blood using digital ELISA, we measured PSA in serum samples from patients who had undergone radical prostatectomy. Levels of serum PSA are used both to screen for prostate cancer and to monitor recurrence of the disease in patients who have undergone radical prostatectomy²⁸. After radical prostatectomy the vast majority of PSA is eliminated, and concentrations fall below the detection limit of standard commercial assays (3 pM or 0.1 ng/ml). Although regular monitoring of these patients for increases in PSA concentrations can detect recurrence of prostate cancer, several years may pass after surgery for biochemical recurrence to be detected by current immunoanalyzers. The ability to accurately quantify PSA levels in patients who have undergone prostatectomy at very low concentrations (<300 fM or 10 pg/ml) should provide early indication of recurrence if PSA levels increase^{17,29}. Figure 4 shows PSA concentrations measured using digital ELISA in the serum of 30 patients (age 60–89) who had undergone radical prostatectomy and whose blood was collected at least 6 weeks after surgery. The PSA concentrations in the sera of all 30 patients were below the detection limit of commercial assays. Whole-serum samples were diluted 1:4 in buffer and measured using the digital ELISA specific for PSA (Fig. 3a). PSA was successfully detected in all 30 patients using digital ELISA, with concentrations ranging from 14 fg/ml to 9.4 pg/ml, with an average of 1.5 pg/ml. Further clinical studies are required to establish the diagnostic benefit of measuring PSA at femtogram per milliliter amounts in patients following radical prostatectomy.

By isolating and detecting single immunocomplexes in arrays of femtoliter-volume wells, digital ELISA enables clinically important proteins in serum to be measured at subfemtomolar concentrations. We believe that the improvement in sensitivity shown by digital ELISA over previously reported approaches will translate into diagnostic benefits. For example, PSA concentrations in 9 of the 30 radical prostatectomy samples assayed in this work fell below the LOD of the previously highest sensitivity PSA assay based on nanoparticle labels¹⁷. An attractive feature of this approach is the ability to directly use reagents developed for standard ELISA for substantially more sensitive assays. We continue to improve the SiMoA technology in two key areas. First, based on the sensitivity to enzyme label (Fig. 2), it seems that the sensitivity of protein detection could be increased at least 100-fold if nonspecific interactions that cause background signals could be minimized. The ability to isolate and interrogate single molecules on individual beads provides avenues for distinguishing antibody-antigen

binding events from nonspecifically bound complexes. Second, we are simplifying the logistics of the assays. Even in its present form, however, we believe that digital ELISA has the potential to facilitate earlier diagnosis and treatment of disease.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturebiotechnology/>.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

D.M.R., C.W.K., D.R.F., D.R.W. and D.C.D. conceived the approach. D.R.F. built the imaging system. D.M.R., C.W.K., T.G.C., S.C.H., L.S., P.P.P., A.J.R., E.P.F., J.D.R. and G.K.P. conducted the experiments. T.P. wrote the image analysis software. L.C. prepared reagents. D.M.R. and D.C.D. wrote the manuscript. All authors were involved in designing experiments, reviewing and discussing data, and commenting on the manuscript.

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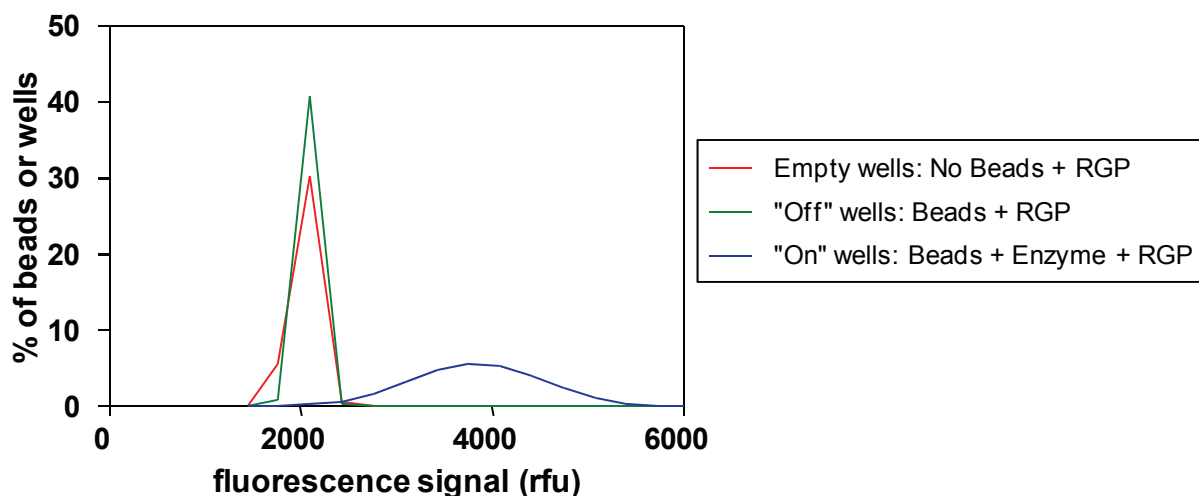
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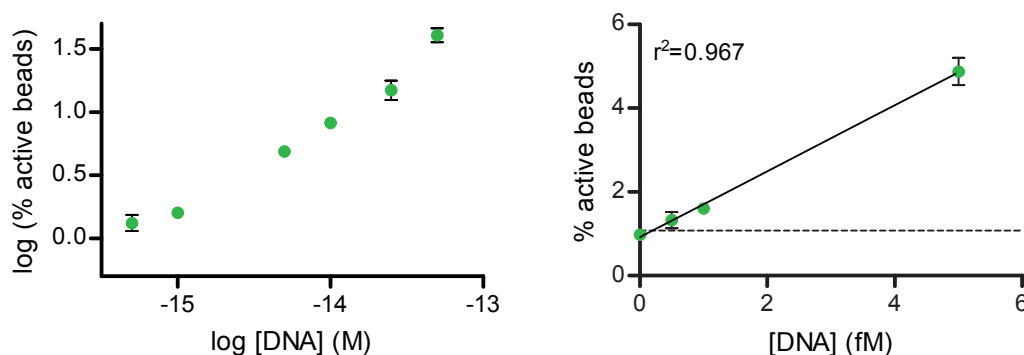
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Supplementary Information

1) Supplementary Figures and Legends

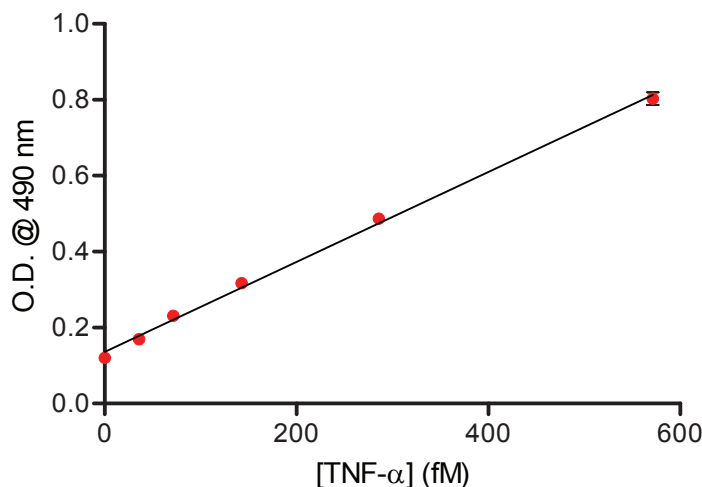


Supplementary Figure 1. Histograms of the average fluorescence intensity of wells in a SiMoA experiment. A representative set of images from a SiMoA experiment (Figure 1) were analyzed to determine populations of wells that: a) contained no beads; b) contained a bead (from white light scatter) but no enzyme (no increase in fluorescence intensity); and, c) contained a bead (from white light scatter) and an enzyme (increasing fluorescence intensity over four consecutive images, and an overall increase in fluorescence of at least 20%). Using this method of calling “on” wells, the occurrence of false positives in the absence of background proteins is very small (<1 in a 50,000-well array, see Figure 2). In the presence of background proteins, e.g., serum samples, there is a significant number of “on” wells from non-specific binding that sets the background signal and limits of detection (see discussion in main text). The fraction of beads within each population is plotted against the average intensity of wells with a bin size of 120 rfu. The red line represents data from empty wells; the green line represents wells with a bead but no enzyme (“off” well); and the blue line represents beads with both a bead and enzymatic activity (“on” well). While a range of enzyme activities are observed as anticipated²¹, there is good separation between on and off wells to allow digital quantification.



Supplementary Figure 2. Detection of DNA using digital counting of enzyme labels. Plots of % active beads against different concentrations of DNA spiked into buffer. Experimental details are provided in the Methods section. The plot on the left hand side show the assay response over the

concentration range tested in log-log space. The plot on the right hand side show the assay response in the femtomolar range in linear-linear space. Error bars are plotted as the standard deviation over three replicates. LODs were determined by extrapolating the concentration from the signal equal to background signal plus three standard deviations of the background signal; the dashed lines show the signal at the LOD. The LOD of the digital DNA sandwich assay was ~150 aM, corresponding to about 8000 copies. While these results are preliminary—the DNA was detected in buffer in the absence of non-target DNA—and the sensitivity does not approach that that can be achieved with PCR, they do illustrate that SiMoA can be used to detect nucleic acids without recourse to molecular replication.



Supplementary Figure 3. The R&D Systems HS-ELISA for TNF- α . A TNF- α calibration curve yielded an LOD of 21 fM in whole serum using a Tecan Infinite M200 plate reader. The non-specific binding (NSB) background in this high sensitivity assay was equivalent to about 85 fM of TNF- α .

2) Supplementary Tables

	Column A	Column B	Column C	Column D	Column E	Column F
[SβG] (aM)	Average % active beads observed (Figure 2)	Enzyme/bead ratio from Poisson distribution	Total # of enzymes on 400,000 beads	Background corrected # of enzymes on beads	Calculated # of enzymes in 100- μL sample	Capture Efficiency
0	0.0016%	0.000016	7	---	---	---
0.35	0.0086%	0.000086	34	28	21	132%
0.7	0.0099%	0.000099	40	33	42	79%
3.5	0.0413%	0.000413	165	159	211	75%
7	0.0713%	0.000713	285	279	421	66%
35	0.4461%	0.004471	1789	1782	2107	85%
70	0.8183%	0.008217	3287	3280	4214	78%
350	3.3802%	0.034387	13755	13748	21070	65%
700	7.5865%	0.078897	31559	31552	42140	75%
3500	30.6479%	0.365974	146390	146383	210700	69%
7000	44.5296%	0.589320	235728	235722	421400	56%

Supplementary Table 1. Calculations of the SiMoA capture efficiency of enzyme label from 0.35 aM to 7000 aM. From the fraction of beads that were active in Figure 2 (Column A), the enzyme/bead ratio (Column B) can be determined from the Poisson distribution²³. In this experiment, ~400,000 beads were incubated with each solution of enzyme (although only ~50,000 beads (~12.5%) on average were interrogated because of the current size of the femtoliter arrays (~50,000 wells)), so the number of enzymes on these beads is determined by multiplying Column B by 400,000 (Column C). After background subtraction, the total number of enzyme molecules captured on the beads can be determined (Column D). The ratio of this number to the total calculated number of enzymes in 100 μ L at the start of the experiment (Column E; Volume $\times$ Concentration $\times$ Avogadro's Number) yields a capture efficiency (Column F). The overall efficiency of capture and detection of enzyme using SiMoA is high (65-85%) in the range where Poisson noise is acceptable (<20%, from 3.5 aM to 3500 aM), and is probably only limited in this experiment by the slow diffusion of the large S β G conjugate (MW ~ 515 kDa). At 7000 aM, where the % active beads > 40%, the % active starts to deviate from linearity (giving rise to an apparent lower capture efficiency) because of difficulties in image analysis at these high fractions (see main text).

NSB Dropout Experiment (SiMoA PSA assay)					
	Average	SD	CV	% NSB	
No dAb; No PSA	0.110%	0.162%	147%	from SbG	20%
No SbG; No PSA	0.000%	0.000%	---	from dAb	80%
NSB	0.541%	0.194%	36%	Total NSB	100%

Supplementary Table 2. A dropout experiment isolating the sources of NSB in the PSA digital ELISA. By comparing 'no detection antibody NSB' (No dAb) and 'no S β G NSB' (No S β G) to total NSB (NSB), the contribution of detection antibody and S β G to the NSB of the assay was determined.

	Centaur ng/mL	SiMoA ng/mL
Bio-Rad Control 1	0.838	1.06 $\pm$ 0.21
Bio-Rad Control 2	2.47	2.66 $\pm$ 0.36
Normals		
ProMedDx S376	2.1	1.60
ProMedDx S378	2.3	1.70
ProMedDx S381	2.9	2.14
ProMedDx S388	4.1	3.95
ProMedDx S395	0.93	0.63
ProMedDx S396	0.9	0.77
ProMedDx S397	1.2	0.66

Supplemental Table 3. Specificity of the PSA digital ELISA. Specificity of the digital ELISA was confirmed using PSA samples from Bio-Rad (controls) and ProMedDx (serum from healthy individuals) that had previously been tested on a commercial immunoanalyzer (ADVIA Centaur, Siemens). While there was good correlation between the two methods ($r^2 = 0.96$), the PSA concentrations of the healthy

serum samples determined using SiMoA were (24±12)% lower than those originally determined on the ADVIA Centaur. There are two possible explanations for this systematic bias between the two technologies. First, the ADVIA Centaur values were obtained on fresh serum, whereas digital ELISA values were obtained after the sera had been frozen for extended periods of time and had experienced a freeze-thaw cycle. Second, there were likely differences between the PSA calibrators used to generate the calibration curves that would result in differences in PSA concentrations determined. We used Complex PSA from the World Health Organization (WHO) as calibrators; the ADVIA Centaur calibration PSA is unknown.

3) Supplementary Discussion

Sensitivity to Enzyme Label as Sample Volume Increases. For some applications, volumes larger than 100 μ L might need to be tested for very low concentrations of analyte. As the volume of sample increases, detecting small numbers of enzyme labels with high efficiency becomes more challenging technically. To achieve the same detection efficiencies in 1 mL and 10 mL of sample that we achieved in 100 μ L, would require 10 and 100 times more beads, respectively, both from thermodynamic and kinetic perspectives. These larger numbers of beads (2 million and 20 million) would require much bigger arrays and also larger imaging (CCD) chips or multiple images per array. While these experiments are feasible, they would require a much more complex instrument and longer read times, so might not be justified in a diagnostic setting.

Optimization of Labeling of Captured Proteins in Digital ELISA. The choice of the concentrations of detection antibody and enzyme conjugate (S β G) used to label the captured protein is important for optimizing digital ELISA. The relatively high background signals of ELISAs caused by the non-specific interactions between the capture beads and detection antibody and enzyme conjugate (see Supplementary Table 2), combined with the high sensitivity to label provided by SiMoA, means that often only a fraction of the capture proteins need to be labeled to avoid saturating SiMoA signals. For example, the background levels observed in digital ELISA are equivalent to ~1–2 fM of target protein (see main text), so the ratio of analyte to bead is about 0.3–0.6. At these ratios, the number of active beads would be 25–40% if every protein was labeled with an enzyme, i.e., at the upper end of the digital dynamic range. To ensure that these background signals are closer to the lower end of the digital dynamic range—1% active beads provides a reasonable noise floor for background in digital ELISA—appropriate labeling of the captured protein can be achieved by kinetic control of the labeling steps, either by minimizing the concentrations of both labeling reagents or by using shorter incubation times. We have chosen to minimize label concentrations and use standard ELISA incubation times; as a result, the total assay time is ~6 h. This length of time is acceptable for testing that tolerates a daily turnaround time for samples. For shorter turnaround times of, say, <1 h (e.g., for point-of-care applications), the assay could be performed with short incubations of higher concentrations of labeling reagents.

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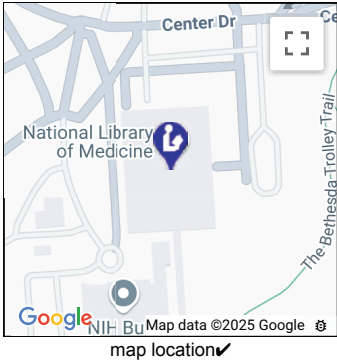


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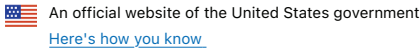
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Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations

David M Rissin ¹, Cheuk W Kan, Todd G Campbell, Stuart C Howes, David R Fournier, Linan Song, Tomasz Piech, Purvish P Patel, Lei Chang, Andrew J Rivnak, Evan P Ferrell, Jeffrey D Randall, Gail K Provuncher, David R Walt, David C Duffy

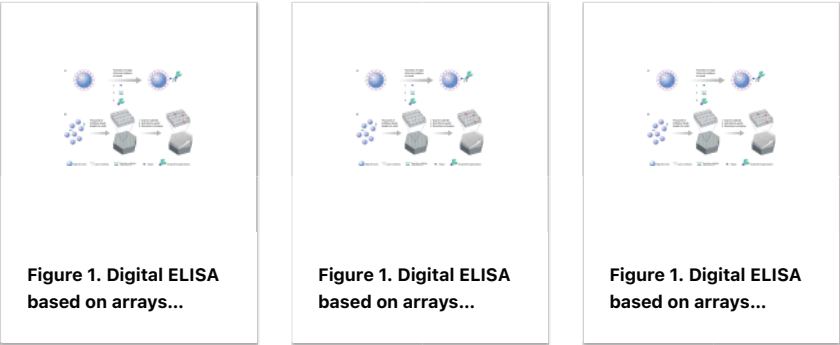
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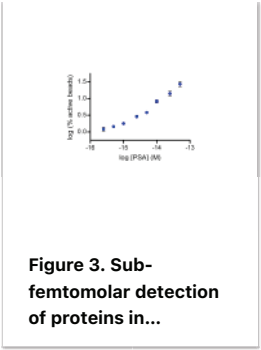
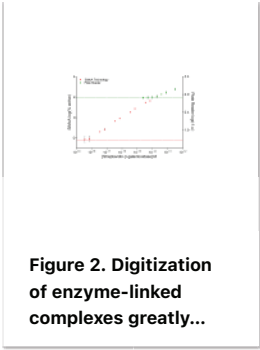
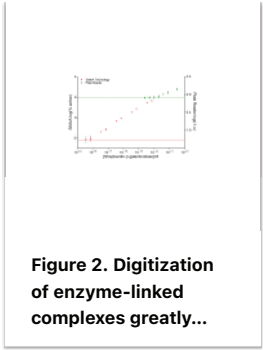
Abstract

The ability to detect single protein molecules in blood could accelerate the discovery and use of more sensitive diagnostic biomarkers. To detect low-abundance proteins in blood, we captured them on microscopic beads decorated with specific antibodies and then labeled the immunocomplexes (one or zero labeled target protein molecules per bead) with an enzymatic reporter capable of generating a fluorescent product. After isolating the beads in 50-fl reaction chambers designed to hold only a single bead, we used fluorescence imaging to detect single protein molecules. Our single-molecule enzyme-linked immunosorbent assay (digital ELISA) approach detected as few as approximately 10-20 enzyme-labeled complexes in 100 microl of sample (approximately 10⁻¹⁹ M) and routinely allowed detection of clinically relevant proteins in serum at concentrations (<10⁻¹⁵ M) much lower than conventional ELISA. Digital ELISA detected prostate-specific antigen (PSA) in sera from patients who had undergone radical prostatectomy at concentrations as low as 14 fg/ml (0.4 fM).

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