Form PTO-1595 (Rev. 09/04)
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U.S. DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

RECORDATION FORM COVER SHEET
PATENTS ONLY

Docket No.: 5864.050

To the Director of the U.S. Patent and Trademark Office: Please record the attached documents or the new address(es) below.

1. Name of conveying party(ies)/Execution Date(s):

Adam Zlotnick

Execution Date(s) 10/25/2006

Additional name(s) of conveying party(ies) attached? ☐ Yes ☐ No

2. Name and address of receiving party(ies)

Name: The Board of Regents of the Unversity of Oklahoma

Name (con't.):

Street Address: 660 Parrington Oval

Room 119

City: Norman

State: OK

Country: US Zip: 73019

Additional name(s) & address(es) attached? ☐ Yes ☐ No

3. Nature of conveyance:

☑ Assignment ☐ Merger

☐ Security Agreement ☐ Change of Name

☐ Government Interest Assignment

☐ Executive Order 9424, Confirmatory License

☐ Other

4. Application or patent number(s): ☐ This document is being filed together with a new application.

A. Patent Application No.(s)
11/546,796

B. Patent No.(s)

Additional numbers attached? ☐ Yes ☐ No

5. Name and address to whom correspondence concerning document should be mailed:

Name: DUNLAP, CODDING & ROGERS P.C.

Internal Address: P.O. Box 16370

Street Address: 1601 N.W. Expressway, Suite 1000

City: Oklahoma City

State: OK Zip: 73113

Phone Number: 405-607-8600

Fax Number: 405-607-8686

Email Address:

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 1.21(h) & 3.41) $ 40.00

☑ Authorized to be charged by credit card

☐ Authorized to be charged to deposit account

☐ Enclosed

☐ None required (government interest not affecting title)

8. Payment Information

a. Credit Card Last 4 Numbers 1013
Expiration Date 01/07

b. Deposit Account Number

Authorized User Name

9. Signature:

Signature — Date: 11/03/2006

Kathryn L. Hester, Ph.D.
Name of Person Signing

Total number of pages including cover sheet, attachments, and documents: 55

Documents to be recorded (including cover sheet) should be faxed to (703) 306-5995, or mailed to:
Mail Stop Assignment Recordation Services, Director of the USPTO, P.O.Box 1450, Alexandria, V.A. 22313-1450

OP $40.00 11546796

SENT BY FACSIMILE TO: **(571) 273-0140** **PATENT**
DATE SENT: **NOVEMBER 3, 2006**

ASSIGNMENT

WHEREAS:, Adam Zlotnick, an individual residing at 510 Stonewell Drive, Norman, OK 73072; has invented and owns a certain invention entitled: **METHODS FOR DETECTING INHIBITORS OF PROTEIN AGGREGATION UTILIZING AN *IN VITRO* FLUORESCENCE-BASED ASSAY**, application for Letters Patent of the United States was filed October 12, 2006, and assigned Docket No. 5864.050, U.S. Serial No. 11/546,796; and

WHEREAS: The Board of Regents of the University of Oklahoma, 660 Parrington Oval, Room 119, Norman, Oklahoma, 73019, a legal entity of the State of Oklahoma (hereinafter referred to as ASSIGNEE), is desirous of acquiring the entire interest in, to and under said invention and in, to and under Letters Patent or similar legal protection to be obtained therefor in the United States and in any and all foreign countries.

NOW, THEREFORE, TO ALL WHOM IT MAY CONCERN: in consideration of the receipt of good and valuable consideration, which is hereby acknowledged, and in reliance on the Patent Policy of The Board of Regents of the University of Oklahoma, adopted December 9, 1999, a copy of which is attached hereto as Exhibit A and made a part hereof, and in further reliance on the Proprietary Information/Invention Disclosure, a copy of which is attached hereto as Exhibit B and made a part hereof, ASSIGNOR has sold, assigned and transferred, and by these presents does sell, assign and transfer unto said ASSIGNEE, the full and exclusive right to the invention in the United States and its territorial possessions and in all foreign countries and the entire right, title and interest in and to any and all Patent Applications and Patents which may be granted therefor in the United States and its territorial possessions and in any and all foreign countries including the right to claim priority under any applicable provisions of the International Convention and the Patent Cooperation Treaty and to all Letters Patent or similar legal protection in the United States and its territorial possessions and in any and all foreign countries to be obtained for said invention by said application or any continuation, division, renewal, substitute or reissue thereof or any legal equivalent thereof in a foreign country for the full term or terms for which the same may be granted.

ASSIGNOR hereby authorizes and requests the Patent and Trademark Office Officials in the United States and any and all foreign countries to issue any and all of said Patents, when granted, to said ASSIGNEE, as the ASSIGNEE of ASSIGNOR'S entire right, title and interest in and to the same, for the sole use and enjoyment of said ASSIGNEE, its successors and assigns.

FURTHER, ASSIGNOR agrees that ASSIGNOR will communicate to said ASSIGNEE, or its representatives, any facts known to ASSIGNOR respecting said invention, and will testify in any legal proceedings, sign all lawful papers, execute all divisions, continuations, substitutions, renewal and reissue applications, execute all necessary assignment papers to cause any and all of said Patents to be issued to said ASSIGNEE, make all rightful oaths and generally do everything necessary or desirable to aid said ASSIGNEE, its successors and assigns, to obtain and enforce proper protection for said invention in the United States and in any and all foreign countries.

ASSIGNOR hereby covenants that no assignment, sale, agreement or encumbrance has been or will be made or entered into which would conflict with this assignment and sale.

Dated: 10/25/06

ADAM ZLOTNICK

Exhibit A

THE BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA

INTELLECTUAL PROPERTY POLICY

PREAMBLE

The people of the State of Oklahoma may reasonably expect that their investments in the University will create new industry and enhance existing industry within the State and Nation. Such new industry creates greater employment opportunities for citizens of the State and the Nation and an improvement in their standard of living.

The creation and development of intellectual property at the University encourages new business and is key to creating strong University and industry partnerships. It is the responsibility of University employees to disclose intellectual property and to foster an entrepreneurial attitude within the work force by involving students in the creation of intellectual property. Intellectual property development shall be pursued in concert with, but subject to, the University's principal responsibilities of education and knowledge creation.

Therefore, it is in the best interest of the University to adopt a policy that encourages disclosure of discoveries and inventions and rewards such creative activity. To do so, the University policy must insure that creators of copyrightable works or trademarks and inventors share in any financial success enjoyed by the University through the creation and commercialization of intellectual property. The basic objectives of the University's policy concerning creative works, trademarks, discoveries; and inventions (i.e., intellectual property) include the following:

1) To maintain the University's academic policy of encouraging research, publication, and scholarship independent of potential gain from royalties or other income.
2) To make patented materials created pursuant to University objectives available in the public interest under conditions that will promote their effective utilization and commercialization.
3) To provide adequate incentive and recognition to faculty and staff through proceeds derived from their creative works, trademarks, discoveries, and inventions.

PATENTS

(The text below appears in the Norman Campus and Health Science Center Faculty Handbooks)

1. OWNERSHIP

All discoveries and inventions, whether patentable or unpatentable, and including any and all patents (domestic and foreign) based thereon and applications for such patents, which are made or conceived by any member of the faculty, staff, or student body of the University, either in the course and/or scope of employment for the University or substantially through the use of facilities or funds provided by or through the University shall be owned by and be the property of the Board of Regents except as described below. Lab notebooks, records, drawings, sketches, photographs or other images, models, biological specimens or materials, chemical samples, or any other materials

needed to support the preparation, submission, prosecution, defense, or enforcement of a patent in the United States or other applicable jurisdiction, whether or not the University takes such action, shall also be owned by the Board of Regents.

Faculty, staff, and students having rights to discoveries/inventions prior to employment at the University of Oklahoma should notify the Office of Technology Development of such intellectual property so that ownership to any further development of that same intellectual property at the University of Oklahoma may be established, by written agreement, with the University Vice President for Technology Development. The Vice President for Technology Development shall consult with the Senior Vice President and Provost of the Health Sciences Center or his designee before entering a contract with faculty, staff, or students at the Heath Sciences Center.

In the event faculty or staff make discoveries or inventions outside the course of and/or scope of employment and using no University facilities, equipment, or supplies, or if using such reimburses the University for this use in accordance with a prior written agreement with the University; in accordance with University policy, title to such discoveries or inventions shall remain in the inventor, provided the University Vice President for Technology Development determines that the discovery or invention was made under these conditions. The inventor shall nonetheless submit a disclosure form to the Office of Technology Development. This disclosure shall contain sufficient information to enable the University Vice President for Technology Development to make a determination. If confidential information is required, the University will sign a nondisclosure agreement for purposes of this review. Should the University Vice President for Technology Development determine that the University does have a proprietary interest, a more complete disclosure may be required before making a decision in regard to title, the University Vice President for Technology Development shall consult with the Senior Vice President and Provost of the Health Sciences Center when the faculty member is based at the Health Sciences Center. If it is determined that the University has an interest, the provisions of this policy shall then be applicable. Appeals of such determinations may be made to the Faculty Appeals Board and then to the President.

All rights in and to discoveries and inventions described above shall be disclosed to and assigned to the Board of Regents as a specific condition of employment with the University and admission to and/or attendance at the University. Faculty, staff, and students shall execute any and all documents the University deems reasonably necessary to evidence such ownership, meet its legal obligations and effect patent protection, domestic and foreign, for the University or its nominee. All costs involved in obtaining and maintaining patent protection shall be borne by the University or its nominee.

The University agrees to act in good faith with respect to the determination of ownership.

2. REVENUE

The gross revenues (which shall include but not be limited to, cash and equity) received by the University from the licensing, sale, or commercialization of a University discovery or invention as described in Section 1, will be distributed among the discoverer(s)/inventor(s), his/her/their primary department(s), and the University, in accordance with the following formula:

35% of gross revenues to the discoverer(s)/inventor(s) (as submitted on the Invention Disclosure Form);

The remaining 65% to be used to reimburse the University for out-of-pocket expenses that it has or shall incur in connection with, but not limited to, patent filing, prosecution, maintenance, and defense;

After such expenses have been recouped, the balance will be distributed as follows:

31% to originating college(s), half of which to goes to the originating department

7% to President's discretionary fund

7% to the campus Vice President for Research

25% to OTD to apply to operational expenses with a pro rata share to go to the originating campus

30% to the Growth Fund maintained for each originating campus

Stock certificates issued to the University shall be held by the Controller's Office of the Norman Campus.

The right to receive such royalty revenue shall be extended to the inventor(s) in the event that the inventor is no longer an employee or student of the University. Such right shall also accrue to the estate of the inventor(s). Inventor(s) and/or their successors or assigns shall have the responsibility to provide the Office of Technology Development of the respective campuses with all the necessary information to make payments to the appropriate parties, including without limitation, current addresses; provided, failure to keep the University so informed shall permit the University to hold all such revenue for such parties for a reasonable time or until the lawful beneficiaries make claim thereto.

The University Vice President for Technology Development shall establish and maintain a Growth Fund for each originating campus to be used to stimulate general intellectual property disclosures as well as technology development and transfer. The fund will be used to further stimulate researchers on each campus to make proposals when there is a need for additional funding to provide prototypes, additional research results, and/or "gap" funding to keep a program in place during transition. Those accessing the Growth Fund will be expected to repay the Growth Fund at a target rate of two times the amount awarded from the Growth Fund. This fund will be administered under guidelines consistent with the research and scholarly missions of the University in consultation with the University Patent Committee. The Health Sciences Center Senior Vice President and Provost will confer with the University Vice President for Technology Development prior to the Senior Vice President and Provost's allocation of funds. If the University Vice President for Technology Development does not approve of the proposed allocation, the proposed allocation will be submitted to the President for final approval or disapproval.

When there are two or more discoverers/inventors, each shall share equally in the inventor's share unless all discoverers/inventors agree in writing to a different distribution of such share. Such originally-signed agreement shall be filed with the Office of Technology Development of the respective campuses of the discoverer(s)/inventor(s). No distribution of cash revenues will be made until this issue is resolved by the discoverer(s)/inventor(s) or their successors in interest.

The discoverer(s)/inventor(s) and his or her college shall be paid their share of the cash revenues upon receipt by the University and will be furnished with a statement of revenue derived from the commercialization of the invention at the time of payment. In the event of any litigation, actual or imminent, regarding patent rights, the University may withhold distribution until resolution of the particular matter.

The University does not act as a fiduciary for any person relating to consideration received under the terms of this policy.

3. ASSET MANAGEMENT COMMITTEE AND POLICY

The Asset Management Committee shall determine the disposition of equity assets obtained through the commercialization of University technology which is valued under $ 100,000.00; e.g., whether to sell, trade, or hold the assets, as it deems to be in the best interests of the University, considering, among other factors, the requirements of the University and risks associated with holding the particular equity/stock asset. For assets values $ 100,000.00 and above, the Committee shall make recommendations to the Board of Regents with regard to their disposition. Provided in such cases, should the Committee determine that University interests require a decision regarding the disposition of such assets prior to the next regularly scheduled meeting of the Board of Regents, it shall be authorized to do so, upon the approval of the President, and the results shall be reported at the next meeting of the Board of Regents.

At the call of the University Vice President for Technology Development, the Committee shall meet together in person, by teleconference or other acceptable means on an "as needed" basis in order to make decisions in a timely fashion regarding equity/stock as it is received by the University. However, such meetings shall take place not less than once each fiscal year. The Committee shall meet to review current assets, previous management actions taken and for any other purpose relate to management of the equity assets.

The Committee shall consist of two current members of the Board of Regents (to be appointed by the Board of Regents), the University Vice President for Technology Development, University General Counsel, a knowledgeable University employee who shall be appointed by the President, and two extra-mural members familiar with such matters by education, training, and/or experience. From recommendations by the President, the Board of Regents shall appoint the two non-employee members of the Committee, who shall serve at the convenience of the Board of Regents. Appointments shall be made on an annual basis, at the time of the Board of Regents' officer elections.

4. ADMINISTRATION

The President, after consultation with the University Vice President for Technology Development and appropriate campus officials, shall determine the disposition of University discoveries and inventions described in Section 1, as deemed prudent and consistent with the University's mission to ultimately convey the benefits of its research to the public for the general welfare of the State and Nation. In determining the proper disposition of University discoveries and inventions, the President shall consult as necessary with scientific and/or technical and/or business subject matter experts in fields appropriate to the discovery or invention under consideration. Among other choices, the President may:

License the discovery and/or invention to third parties to provide for the further development and/or commercialization of the property;

Transfer the discovery and/or invention for commercialization by entering into commission agreements with third parties to identify potential licensees to further develop and commercialize the property;

Transfer rights to the property to a patent service organization to further develop and commercialize the property;

Allow rights (U. S. and/or foreign) to the discovery or invention to revert back to the federal agency that funded the development of the discovery;

Transfer rights (U. S. and/or foreign) to the discoverer(s)/inventor(s) if requested by the discoverer(s)/inventor(s), and the President determines that the discovery/invention will not be pursued further by the Office of Technology Development.

If federal funds were used in the development of the discovery/invention, such transfer of rights is subject to a reversionary right in the Federal government as described in 37 CFR 401.

1) Such transfer also shall be subject to an irrevocable, non-exclusive, royalty-free, and world-wide right and license in the University to make, use, and/or practice the discovery or invention for University education, research, and/or service purposes. The University also reserves the right to publish and or present information and data obtained in the research project resulting in the discovery or invention being transferred, assuming such rights do not jeopardize the discoverer's/inventor's patent rights. Faculty, staff, and students shall execute any and all documents, as the University deems reasonably necessary to confirm or enforce such reserved right and license.

2) Such transfer shall be limited to the discovery or invention duly disclosed to the University, in writing, as of the time the transfer is requested by the discoverer/inventor;

3) Transfer rights to the discovery and/or invention to the person(s) or entity sponsoring the research in the course of which the discovery or invention was made if such action is required under the terms of the research agreement or is required by law; or

4) Transfer the discovery and/or invention into the public domain through publication of the invention by the discoverer/inventor.

5) All transfers of University discoveries or inventions shall be subject to and contingent upon any rights in third parties as may be governed and/or required by, among other things, sponsored research agreements, other third-party contracts, or law.

The University Vice President for Technology Development shall be responsible for administering the patent affairs of the University in a manner consistent with this policy. The University Vice President for Technology Development shall cooperate with the appropriate campus officers to establish written policies to be approved by the President and distributed to the faculty, staff, and students of the University, governing procedures to be followed in processing discoveries and inventions generated within the University. The Office of Technology Development shall provide information regarding disposition of specific discoveries/inventions to the inventor(s) no later than six months from the date the discovery/invention is marketed by the Office of Technology Development. If the Office of Technology Development is not going to pursue marketing and/or protecting the invention, rights in and to the discovery/invention shall revert to the inventor(s) upon his/her request.

When it is in the best interest of the University to get its technology into the marketplace, when the longer-term opportunity for returns to the University and the State exceed the short-term value of not taking equity, then the President will approve taking equity. As a matter of principle, some equity is desirable in all transactions to create the best opportunity for the University and the State of Oklahoma to get a fair return on the technologies transferred from the University to the marketplace.

5. DISCLOSURE

It is the responsibility of faculty, staff, or students of the University to report all inventions they may develop during their term of employment or registration as a student.

Any discovery/invention, whether or not patentable, must be reported to the University by filing an Invention Disclosure Form with the appropriate technology development office. Such Invention Disclosure Form shall provide sufficient information so that the Office of Technology Development, in conjunction with others, can determine its commercial potential and patentability. Although the maintenance of the laboratory notebooks that describe the discovery/invention is the responsibility of the discoverer/inventor, the Office of Technology Development may require access to such notebooks at any time throughout the prosecution and maintenance stage of patenting the discovery/invention.

6. APPLICATION

The provisions of this Patent Policy, as may be amended from time to time, shall apply to all faculty, staff, and students. Any licensing revenues received throughout the duration of such license shall be distributed in accordance with the distribution policy in effect at the time of the signing of

such license and can only be changed through mutual agreement of the parties receiving portions of such revenue.

7. UNIVERSITY PATENT COMMITTEES

The University shall have a Patent Committee for each campus that shall consider and investigate disputes among administrators, faculty, or staff, and students and shall recommend appropriate solutions to the President. It shall be convened as needed, but not less than three times per academic year. This Committee will consist of one student member appointed by the Graduate Student Senate for one year; two staff members, one appointed by the President and one appointed by the Staff Senate; and five faculty members, three appointed by Faculty Senate and one each by the President and the University Vice President for Technology Development. All staff and faculty appointments are for three-year terms. This Committee will assist the University Vice President for Technology Development in setting policy and procedures that will be implemented on a daily basis by the Office of Technology Development staff. The Committee will provide a forum for faculty, staff, and students to present any issues such as, but not limited to, revenue sharing, ownership, etc. The Committee will make recommendations to the University Vice President for Technology Development for the handling of these issues. The Director of the respective campus' Office of Technology Development, the respective campus' Vice Presidents for Research, and University Legal Counsel shall serve as ex-officio members of the Committee.

8. USE OF FACILITIES

As outlined in the Oklahoma Technology Transfer Act of 1998, use of the facilities of the University by a business enterprise or private business entity is allowed when that project involves the research or development of a University technology, whether or not the technology is protected pursuant to federal or state law governing intellectual property, the results of which have potential economic and academic value for the University. Such use of University facilities must be on a "space-available" arrangement in which normal University activities are not displaced. The use of such facilities by a business enterprise or private business will be done on a fee-for-service contract and in conjunction with projects where the University has a vested interest in the outcome of the transfer of University technology, through research and development of a discovery/invention for commercialization purposes or scholarly rewards, or where it furthers the University's teaching, research and creative/scholarly activity, and professional and University service and public outreach missions.

The University may acquire an equity stake in such business enterprises or private businesses in consideration for the use of such facilities and/or the development of University discoveries/inventions which shall be managed in accordance with the University's policies on stock acquisition and management. Any cash revenues received from the sale or trade of such equity stocks shall be distributed as if received as royalty income. If the equity interest is acquired through an exchange of value other than money and the exchange of value is made in connection with the development of technology by the private business enterprise through the use of the facilities or resources or both of an institution within the Oklahoma State System of Higher Education, acquisition of an equity interest shall be permissible through the use of the facilities, premises, or assets of an institution within the Oklahoma State System of Higher Education through the use of faculty or staff expertise or student expertise, including the value of time expended by faculty, staff, or students upon developing a technology in connection with a private business enterprise or private

business entity. No state-appropriated monies shall be used or obligated to acquire an ownership interest in a private business enterprise except as authorized by the provisions of this section.

9. BACKGROUND

A patent is a grant issued by the U. S. Patent and Trademark Office (PTO) that provides the assignee of the patent the right to keep others from practicing or using its patented invention. Patents are issued for inventions that are novel (not published, sold, or utilized for more than one year), useful, and non-obvious to others equally skilled in the art. (In almost all foreign countries, to be patentable, an invention requires complete novelty. In others words, it cannot have been published, used, sold, or bartered publicly prior to the filing of a patent application either in the country itself or in the United States PTO.)

U. S. Patent Law has maintained the first-to-invent rule. What this means is that the first person to conceive and reduce to practice the invention shall be entitled to the patent. Foreign countries rely on the first-to-file concept in issuing patent rights. In the case of U. S. law, the laboratory notebooks of the discoverer(s)/inventor(s) may be crucial to the overall outcome of to whom the patent is issued. Laboratory notebooks should be bound notebooks where corrections are readily identifiable. The pages of the notebooks should be signed, dated, and witnessed on a daily basis and stored in a secure location. The term of patent applications filed in recent years is twenty years from the date of application. The laboratory notebooks should be maintained by the University researcher at least for that period of time. Such notebooks shall be owned by and be the property of the Board of Regents.

TRADEMARKS

A trademark identifies an item of intellectual property or an educational or training service. The University owns all right and title to any trademarks related to any item of intellectual property owned by the University. Any cash revenues received in exchange for the commercial use or sale of such trademark shall be distributed as those cash revenues received for discoveries/inventions.

COPYRIGHT

1. PREFACE

Copyrights are created by the Constitution and the laws of the United States to promote the progress of science and the useful arts by securing for limited times to authors the exclusive rights to their works and writings. The basic objectives of the University's policy concerning copyright include the following:

1) To maintain the University's academic policy of encouraging research and scholarship as such without regard to potential gain from royalties or other income.

2) To make copyrightable materials created pursuant to University objectives available in the public interest under conditions that will promote their effective utilization.

3) To provide adequate incentive and recognition to faculty, and staff through proceeds derived from their works.

4) To stimulate creativity across all media.

2. POLICY

It is the policy of the Board of Regents that all rights in copyright shall remain with the creator of the work unless the work is created with substantial use of University resources, is specifically assigned or commissioned by the University, is subject to non-University contractual or legal obligations, or is a "work made for hire" as that term is defined by U.S. Copyright Law.

3. OWNERSHIP

Scholarly/Aesthetic Works.

In keeping with traditional academic practice and policy, ownership of copyrights to works of artistry or scholarship in the creator's professional field such as textbooks, course materials, scholarly papers and articles, software and other computer materials when they are works of artistry or scholarship, novels, poems, paintings, musical compositions, or other such works of artistic imagination produced by University employees who have a general obligation to produce such works where the specific choice, content, course, and direction of the effort is determined by the employee without direct assignment or supervision by the University shall reside in the creators and the works shall not be deemed "works made for hire" under this policy unless they are also sponsored/contracted works or specifically assigned by the University. Copyrighted courseware and/or software that are not associated with traditional works as described above shall fall under and are subject to the Intellectual Property Policy. The general obligation of faculty to produce scholarly works does not constitute specific assignment. Upon request by the University, the creator(s) will grant the University a nonexclusive, free of cost, world-wide right and license to exercise all copyright rights in and to the work, except the right to commercially display, use, perform, or distribute copies of the work, unless to do so would impair the ability of the creator to have the work published or distributed. If a use of the work by University is reasonably determined by the creator to impair the exercise of such rights, the University shall discontinue the impeding use but otherwise shall remain free to use the work as provided in this paragraph. Subject to the approval of the University Vice President for Technology Development, the University will assist any University employee wishing to commercially exploit a scholarly/aesthetic work falling under this paragraph, through the Office of Technology Development and its respective campus officers. In such cases, the University will normally own the work and the provisions of the Intellectual Property Policy shall apply.

Personal Works.

Ownership of copyrights to works prepared outside the course and scope of University employment and without the substantial use of University resources (equipment, facilities, services, or funds--regardless of source--administered by and/or under the control of the University) shall reside with the creators; provided, the provision of office facilities, limited secretarial assistance,

library facilities for which special charges are not normally, made, or other resources which are made available to the public without charge, shall not be considered substantial use of University resources.

Sponsored Works.

Ownership of copyrights to works produced by or through the University in the performance of a written agreement between the University and a third-party/sponsor shall be governed in accordance with the agreement. If the agreement is silent in that regard, ownership shall be governed by the other provisions of this policy.

Commissioned Works.

Ownership of copyrights to works produced for University purposes by persons not employed by the University or by University employees outside their regular University employment (commissioned works) normally shall reside with the University. In all cases, copyright ownership shall be specified in a written agreement approved by University Legal Counsel signed by the parties. Any commissioned work agreement which provides for ownership by other than the University shall also provide, to the fullest extent possible, that the University will have an irrevocable, free-of-cost, non-exclusive, world-wide license to exercise all copyright rights in and to the work, except the right to commercially display, use, perform or distribute copies of the work unless to do so would impair the ability of the University employee creator to commercially or professionally exploit the work. If a use of the work by University is reasonably determined by the University employee creator to impair the exercise of such rights, the University shall discontinue the impeding use but otherwise shall remain free to use the work as provided in this paragraph.

University Works.

Except as otherwise provided in this policy, the University shall own all copyrights to works made by University employees in the course and scope of their employment and shall own all copyrights to works made with the substantial use of University resources. Provided, the University shall give due regard to the creator's interests in the quality and integrity of the work and, where appropriate, shall grant recognition for creation of the work. To the extent consistent with University rights under the U.S. copyright law, nothing herein shall be construed to prevent the creator from using his or her knowledge, expertise, research, and creative achievement in other employment.

Student Works.

Ownership of copyrights to works produced by enrolled students without the use of University funds (other than Student Financial Aid) or resources that are produced outside any University employment and are not sponsored or commissioned works, shall reside with the student creator(s). Provided, however, in all cases a student's graduate thesis or dissertation shall be deemed a student work under this policy. As a condition of enrollment and awarding a degree, the University reserves an irrevocable, non-exclusive, free-of-cost and world-wide right to reproduce in any media and distribute to the public, on a non-commercial basis, copies of said theses and dissertations, unless to

do so would impair the ability of the creator to commercially or professionally exploit the work. If a use of the work by University is reasonably determined by the creator to impair the exercise of such rights, the University shall discontinue the impeding use but otherwise shall remain free to use the work as provided in this paragraph.

Jointly Originated Works.

Ownership of copyrights to jointly originated works shall be determined by separately assessing the category of work of each creator under this Section 3. Rights between joint owners of a copyright shall be determined pursuant to copyright law or by agreement between the owners of the work.

4. REVENUE SHARING

The University may assign or license its copyrights to others. The University shall share royalty revenue derived from such assignment or license (excepting commissioned works and sponsored research funding) which it receives through copyrights with the creators, as provided for in the Patents section above.

Notwithstanding the above or anything else to the contrary herein, staff employees are not eligible to share revenues received from University-owned copyrights where such employees create copyrightable works as a part of their normal responsibilities of University employment. Provided, a staff employee may apply to the appropriate Senior Vice President and Provost to be treated as a faculty member for purposes of revenue sharing for a work resulting from a specific project upon a showing that his/her duties and responsibilities in that project are, in practical effect, substantially the same as those of a faculty member.

5. ADMINISTRATION

Release to the Creator.

An individual creator of a University-owned work may seek transfer of the University-owned copyright to him/herself by making written request to the appropriate Senior Vice President and Provost. If the University decides not to exploit such work, then it may transfer the copyright, by written agreement, to the individual creator to the extent consistent with any applicable third-party agreement or law. Provided, such transfer shall be subject to an irrevocable, non-exclusive, free-of-cost and world-wide license in the University to exercise all rights under the copyright in the work except the right to publicly distribute copies for commercial purposes or such other conditions as may be agreed upon in writing between the individual creator(s) and the Senior Vice President and Provost, unless to do so would impair the ability of the creator to have the work published or distributed. If a use of the work by the University is reasonably determined by the creator to impair the exercise of such rights as transferred in the agreement, the University shall discontinue the impeding use but otherwise shall remain free to use the work as provided in this paragraph.

Disclosure and Protection.

An individual creator of a University-owned copyrightable work shall protect the work by placing the following statutory copyright notice on all copies thereof: ("Copyright [insert year produced, e.g., 2000], the Board of Regents of the University of Oklahoma."). If the creator believes the work may have commercial value, he or she shall promptly provide written disclosure of the work to the appropriate Senior Vice President and Provost.

Legal Compliance

Any work created by a University employee or student shall not, to the best of the creator's knowledge and informed belief, infringe on any existing copyright.

Creators of copyrightable works subject to this policy and the University shall cooperate as reasonably necessary to effect the terms of this policy. For example, if copyright to a work of scholarship vests in the University by law, the University will, upon request and to the extent consistent with its legal obligations to third parties, promptly execute such documents as will transfer copyright to the faculty creator(s).

The Senior Vice President and Provosts shall be responsible for administering the copyright affairs of the University in a manner consistent with this policy. They shall cooperate in consultation with the Copyright Committee on each campus to establish written directives to be approved by the President and distributed to the employees and students of the University, which shall govern the procedures to be followed in processing copyrighted works created within the University.

The University does not act as a fiduciary for any person concerning consideration received under the terms of this policy.

The University Vice President for Technology Development may negotiate ownership of copyrighted works with research sponsors when it is in the best interest of the University to do so. Otherwise, all rights are as described above.

Faculty having rights to copyrighted works prior to employment at the University should notify the Office of Technology Development of such intellectual property so that ownership to any further development of that same intellectual property at the University may be established, in a written agreement with the University.

6. APPLICATION

The provisions of this copyright policy, as may be amended from time to time, shall apply to all faculty, staff, and students.

7. RESOLUTION OF CONFLICT

Should disputes arise relative to the ownership of copyright between the creator and the University, the matter will be referred to the Copyright Committee, which will make recommendations to the President for proper resolution of the disputes. Either the University or creator may contact the Senior Vice President and Provost to arrange to have the Copyright Committee meet to consider such disputes.

8. UNIVERSITY COPYRIGHT COMMITTEES

The University shall have a Copyright Committee for each campus that shall consider and investigate disputes among administrators, faculty, staff, or students and shall recommend appropriate solutions to the President. The Committee's responsibilities shall include, but not be limited to, disputes concerning:

Ownership of copyright; and

Terms of commissions.

The Copyright Committee of each campus shall have as its members:

One member appointed by the President for a four-year term;

One student member appointed by the Graduate Student Senate for one year;

Two staff members, one appointed by the President, one appointed by the Staff Senate; all appointments are for three years; and

Three faculty members, two appointed by the Faculty Senate and one by the President. All appointments are for three-year terms. Tie votes will be settled by chair of Faculty Senate, who shall be an ex-officio member of the Committee.

Each member of the Copyright Committee shall have one vote. The Copyright Committee shall keep its own records, determine its own procedures, and elect its own chair, who shall report to the President. The Copyright Committee also may review this policy from time to time and may recommend changes to the President.

(RM, 11-13-80, pp. 16191-3; 10-14-82, p. 17246; 1-15-87, p. 19412; 1-16-89, p. 20834; 6-13-91, pp. 22461, 22458-59; 12-9-99, p. 26752; 12-7-2001, pp. 27808, 27809; 1-27-2004, p. 28924)

EXHIBIT B

NON-CONFIDENTIAL

OTD DISCLOSURE NO: 06H5C007 DATE RECEIVED OTD: AUG 17 2005

UNIVERSITY OF OKLAHOMA
PROPRIETARY INFORMATION/INVENTION DISCLOSURE

NOTE: This statement shall be treated as confidential information except for specific sections as noted. Except for individuals engaged in the evaluation and approval process, the information will not be divulged to others without proper confidentiality agreements in place, except as required by law. The objective of the form is to obtain the information necessary to determine whether to pursue patent protection for your invention.

SECTION I
NONCONFIDENTIAL INFORMATION

1. **Nonconfidential title of the work:**

   Generalizable Method for Searching for Assembly-directed Antiviral Compounds

2. **Nonconfidential lay abstract of invention:**

   Many viruses have a protein coat that is critical to virus function. Such viruses include hepatitis B virus (350 million chronic cases), hepatitis C virus (200 million chronic cases), and HIV (50 million chronic cases). Because the virus coat is critical to infection, interfering with normal assembly is potential therapeutic avenue. Because the assembly reaction must occur at the right time and/or place, either inhibiting or enhancing assembly are antiviral strategies. A great barrier to discovery of such assembly-directed molecules is the lack of an appropriate assay. This invention is a sensitive and reproducible method for observing assembly reactions in a format that is compatible with high throughput screening. Individual proteins are labeled with a fluorescent dye that self-quenches at high concentration. When assembly is induced, the fluorescence of the sample is decreased. Small molecules that inhibit assembly will prevent quenching; molecules that enhance assembly will increase quenching. Both elements of behavior have been demonstrated with hepatitis B virus capsid (coat) protein.

3. **Nature of work:** Machine ☐ Process ☑ Utility ☐ Software ☐

   If Software: Have proper copyright markings been utilized? Yes ○ No ○

OFFICE OF TECHNOLOGY DEVELOPMENT

660 Parrington Oval, Room 201
Norman, Oklahoma 73019
Telephone (405) 325-3800
Fax (405) 325-7162

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4. Please list all submitters below:

Submitters	Campus Address	Percent of Contribution*	Office Phone No.	Citizenship
a. Adam Zlotnick	BRC464	82.5%	1-9030	USA
b. Stephen Stray	BRC464	15%	1-3481	Australia
c. Jennifer M Johnson	BRC464	2.5%	1-9177	USA
d.				
e.				

*NOTE: This percent of contribution is an estimate providing for the division of proceeds and not an assessment of legal inventorship. If this column is blank, submitters will share equally any revenues generated based on information contained in this disclosure.

5. If you have any other affiliations (i.e. received salary from another party, housed in other facilities besides university facilities), please list below:

Submitters	Other Affiliations	% of Salary Other Affiliation Paid
a.		
b.		
c.		
d.		
e.		

6. Has a Conflict of Interest form been filed with the Provost's office? Yes ○ No ◉

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7. **List specific University research support as well as external funding. List all sources, including matching funds. You may attach additional pages as needed.**

   a. **Name of sponsoring agency, company, or internal funding**
   American Cancer Society

   b. **Principal Investigator**
   Adam Zlotnick

   c. **Co-Investigators; Consultants**

   d. **Grant or Project Number**
   RPG-MBC-98744; RSG-99-339-04-MBC

   e. **University Account Code**
   C5001301; C5016401

   f. **Please attach copy of grant or contract document.**

8. **Has a biological, chemical or physical material or substance obtained from others been used in the creation of this invention?** Yes (•) No ( )

   If Yes, was a Materials Transfer Agreement or similar document used to obtain the material or substance? Yes ( ) No (•)

   If Yes, attach a copy of the agreement.
   If No, identify source and explain.

GENERAL PATENT INFORMATION

In order to obtain patent protection, your invention must be

1. New (or novel): The invention must be new, that is, it has not been previously used, sold or described publicly.
2. Useful: The invention must have an actual use and not be just a subject for additional research.
3. Non-obvious: The invention must not be obvious at the time of conception to another person having ordinary skill in the art.

The patent laws set forth those classes of inventions eligible for patenting as follows:

1. Machines
2. Processes
3. Compositions of Matter
4. Manufacture

SECTION II
PROPRIETARY INFORMATION
DOCUMENTATION

OBJECTIVE: To define the nature, purpose and operation of the invention, a practical presentation is preferable to highly theoretical material.

EVENTS	DATE	SUPPORTING DOCUMENTATION
1. Initial idea	29 Sept, 1998	Grant proposal submitted to NIH for 1 Oct deadline.
2. First oral or written description of information. (Please provide copy of witnessed lab notebook showing date of discovery.)	29 Sept, 1998	Grant proposal submitted to NIH for 1 Oct deadline.
3. Level of testing completed (Lab scale, prototype, etc.)	27 July, 2001 2004 2005	Proof of principal that FRET quenching of a C-terminal fluorophore is an effective method for observing assembly. Demonstration of assay in 96-well format Demonstration of assay for assembly-directed molecules
4. Prototype completed	22 April, 2003	Use of Bo-DIPY dye demonstrated
5. First written or oral publication date (include date of printed abstracts, any oral presentations, or electronic publication dates of journals, poster presentations, presentations to industry etc.) Attach copies.	1 August, 2001	Using fluorescence to observe assembly (SURE presentation). This presentation only described single cuvet correlations between fluorescence and light scattering.
6. Other external oral or written disclosures.		

7. **Provide a full description of the proprietary information. This information should be in such detail as to "teach" the invention and to provide the basis for a patent application. Someone skilled in the art should be able to reproduce the invention within a reasonable period of time based on the information supplied here.**

a. **Complete detailed description of invention. Attach separate sheets if necessary.**

1. This invention is applicable to viruses where (i) there is an in vitro assembly system and (ii) either the C- or N-terminus of the capsid proteins are close to one another in the assembled capsid. The 2nd condition is typical for most viruses.
2. Genetically modify either the N- or C-terminus of the capsid protein (Cp) to include a cysteine. Other cysteines in the Cp should be mutated to alanine if they are not necessary for function.
3. Purify the modified Cp in a reducing environment.
4. After removing all reductant, specifically label the Cp with the maleimide derivative of a self-quenching fluorophore. BoDIPY-FL and fluorescein have been used successfully.
5. A series of assembly reactions are set up, with and without small molecules that affect assembly, in a 96-well plate with appropriate controls. We typically use 1 to 10μM Cp. For controls, we include no-drug reactions, standard curves of un-assembled labeled Cp, and purified labeled capsid.
6. Assembly is induced. For HBV NaCl to final concentrations of 150, 300, 500, and 1000mM.
7. Fluorescence is read at 20 minutes, 1 hour, and 24 hours.

b. **Provide a complete description of the State of the Art prior to your invention. Include a list of any literature references, patent applications, or issued patents you are aware of. Cite source of literature or patent search information. Attach separate sheets if necessary.**

See attached sheet.

c. **Describe the advantages, improvements and technical impact of your invention over existing practice (novel or unusual features).**

Capsid assembly is critical to virus propagation but has not been targeted by pharmaceuticals, in part because of the lack of good assays.

This in vitro fluorescence-based assay allows lead compounds to be identified independent of bio-availablity or toxicity, which may prevent use of some compounds in tissue culture or animal models.

This assay is fast. Assembly may be followed in real time, plates may be read in a fluorometer/plate reader minutes after initiating assembly. By comparison, tissue culture assays require at lest 3-5 days and complicated assays.

ELISAs require conformationally specific monoclonal antibodies and have a limited linear range.

The assay is robust over a wide range of assembly conditions including microtiter plates, unlike light scattering which is insensitive to small polymers and extremely sensitive to large aggregates and particulates.

d. List the areas of applications of invention indicating the problem solved in the area by your invention:

This invention is a fast, scalable method for observing capsid assembly. It can be directly applied to high throughput screening of molecules that alter assembly. Both inhibition of assembly and inappropriate acelleration of assembly are deleterious to virus propagation. This invention specifically examines assembly outside of a cellular mileu.

e. List the main advantages of the invention (list in the order as the advantages would relate to the list of applications in item d. above):

1. This invention specifically examines capsid assembly, which is critical to virus propagation but has not been targeted by pharmaceutical companies.
2. Capsid proteins and the assembly process are unique to viruses, without cellular analogs. Thus molecules that target assembly may be highly specific.
3. By examining assembly in vitro, we are able to identify lead compounds without the requirements regarding bioavailability and toxicity needed in tissue culture and animal systems.
4. The assay is rapid. Results may be examined minutes after mixing. Compare to several days for tissue culture.
5. The assay is scalable to microtiter plates and very small volumes for high throughput screening.
6. Inhibition of assembly and inappropriate enhancement of assembly are deleterious to the virus. Both inhibited and enhanced assembly are detectable in this assay.

f. List any known disadvantages using the same format as above:

This invention is strictly in vitro. Therefore, no information regarding toxicity or bio-availability will be evident.
An in vitro assembly system is required for this assay.
Modification of the capsid protein by mutation or the fluorescent label may affect assembly properties.

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8. **Describe the state of development of your invention. Is there a prototype or samples of materials available for viewing or testing? Include any test results available.**

The invention has been demonstrated in a 96-well format microtiter plate. We have shown that it is sensitive to assembly enhancers and inhibitors (see Fig 4 in the attached paper). It is ready to be applied to high throughput screens.

9. **Outline the further research and development, if any, that would greatly enhance the state of development of the invention.**

Alternative fluorescent labels for FRET quenching, or pairs of labels for FRET should be tested. Alternive chemistry for attaching labels may be of value.

MOST IMPORTANTLY, this assay should be applied to other proteins. HIV Gag is the most interesting target.

10. **List any funded or submitted proposals that have been submitted previously based on this invention. Were proprietary markings affixed to the proposal?**

NIH grant 1R01-AI067417-01, which will begin this December includes this assay. No proprietary markings were affixed as grant proposals are considered confidential. Preliminary reference to this assay were included in American Cancer Society proposals RPG-MBC-98744 and RSG-99-339-04-MBC.

11. **Attach any pertinent tables or drawings describing the invention.**

12. **Provide any other comments you may have regarding your proprietary information.**

13. **Provide the date and description of all planned publications, oral presentations, poster sessions, abstracts, preprints, or other public dissemination of the invention. (Check to see if the journal publishes electronically and provide OTD with the earliest of the dates.)**

DATE	DESCRIPTION
ms in preparation	Attached

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14. Submitter's signatures (this disclosure is submitted under the University of Oklahoma Intellectual Property Policy, Patents, Paragraph 1.1.)

In consideration of employment or a consulting relationship, and subject to any prior agreements with The University of Oklahoma Board of Regents, this invention/discovery is hereby assigned to The University of Oklahoma Board of Regents, together with all patents covering said invention/discovery.

Signature	Date	Home Address
	8/9/2005	510 Starr Well Dr.
		Norman, OK 73072
		City, State, Zip
	8/10/2005	2532 ½ NW 17th St
		Home Address
		Oklahoma City, OK 73107
		City, State, Zip
	8/11/05	2938 SW 53rd St.
		Home Address
		OKC OK 73119
		City, State, Zip
Signature	Date	Home Address
		City, State, Zip
Signature	Date	Home Address
		City, State, Zip

15. Invention Disclosed to and Understood By (Witness):

Signature	Date

16. Office of Technology Development

Signature	Date
	8/17/05

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SECTION III
COMMERCIAL POTENTIAL

NOTE: The information contained in this section will not be distributed to anyone outside of the University of Oklahoma, except as required by law.

1. **Where would your idea have commercial value?**

U.S.	☑	Central/South America	☑	Africa	☑
Canada	☑	Japan	☑	Australia	☑
Europe	☑	Asia	☑	Other	

2. **In your judgment, does the proprietary information require copyright or patent protection in order to be successfully marketed:**

a. Patent protection ☑
b. Copyright protection ☐
c. Know-How could be licensed ☑

3. **List any companies, and individuals within those companies, that you feel may have a commercial interest in licensing the invention. (Indicate by special note those companies that have contacted you regarding the invention.)**

Glaxo Smith Kline
Bristol Myers Squibb
Viropharma

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7b. Description of the state of the art

It is obvious that interfering with virus assembly is bad for the virus. The problem is identifying molecules that interfere with assembly and, by extension, developing methods for identifying such molecules. The invention described in this disclosure describes a method that is simple and efficient.

Early efforts to inhibit assembly were based on the hydrophobic molecule bis-ANS, which inhibits assembly of bacteriophage P22 assembly, probably by binding capsid or scaffold proteins (*1*). Bis-ANS was later shown to inhibit assembly of hepatitis B virus, a human pathogen; with HBV the mechanism is known (*2*); with HBV, bis-ANS can misdirect assembly as well as inhibiting formation of normal capsids. Using traditional screening methods, Bayer Pharma discovered the non-nucleoside HAP compounds, which interfere with capsid production (*3, 4*). HAPs were later shown to misdirect assembly (*5*); the mechanism of misdirection is based on deregulating assembly and stabilizing aberrant assembly products (*6*). An ongoing search for assembly inhibitors of HIV has also led to the discovery of a few small molecules (*7*).

Methods for searching for assembly inhibitors have been primitive. Bis-ANS effects were discovered by laborious assays (*1, 2*). The search for assembly inhibitors for retrovirus assembly have focused on light scattering assays (*7*). The HAP compounds were discovered in a tissue culture based screens that only searched for decreases in virus production (*3, 4*). The demonstration of assembly dependent changes in intrinsic protein fluorescence with HBV suggested a more efficient approach to observing assembly (*8*) which has been echoed with fluorescent fusions with HIV Gag (*7*). However, the latter fluorescence approach has not been applied to in vitro screens for assembly and assembly inhibitors.

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2. Zlotnick, A., Ceres, P., Singh, S., and Johnson, J. M. (2002) A Small Molecule Inhibits and Misdirects Assembly of Hepatitis B Virus Capsids. *J Virol* **76**, 4848--54.
3. Stoltefuss, J., Goldmann, S., Krämer, T., Schlemmer, K.-H., Niewöhner, U., Paessens, A., Lottmann, S., Deres, K., and Weber, O. (1998) pp 52 pp, Bayer Aktiengesellschaft, Germany, WO.
4. Deres, K., Schroder, C. H., Paessens, A., Goldmann, S., Hacker, H. J., Weber, O., Kramer, T., Niewohner, U., Pleiss, U., Stoltefuss, J., Graef, E., Koletzki, D., Masantschek, R. N., Reimann, A., Jaeger, R., Gross, R., Beckermann, B., Schlemmer, K. H., Haebich, D., and Rubsamen-Waigmann, H. (2003) Inhibition of Hepatitis B Virus Replication by Drug-Induced Depletion of Nucleocapsids. *Science* **299**, 893-6.
5. Hacker, H. J., Deres, K., Mildenberger, M., and Schroder, C. H. (2003) Antivirals Interacting with Hepatitis B Virus Core Protein and Core Mutations May Misdirect Capsid Assembly in a Similar Fashion. *Biochem Pharmacol* **66**, 2273-9.
6. Stray, S. J., Bourne, C., Punna, S., Lewis, W. G., Finn, M. G., and Zlotnick, A. (2005) A Heteroaryldihydropyrimidine Enhances and Can Misdirect Assembly of Hepatitis B Virus Capsid Protein. *Proc Natl Acad Sci U S A* **102**, 8138-43.
7. Tang, C., Loeliger, E., Kinde, I., Kyere, S., Mayo, K., Barklis, E., Sun, Y., Huang, M., and Summers, M. F. (2003) Antiviral Inhibition of the Hiv-1 Capsid Protein. *J Mol Biol* **327**, 1013-20.
8. Singh, S., and Zlotnick, A. (2003) Observed Hysteresis of Virus Capsid Disassembly Is Implicit in Kinetic Models of Assembly. *J Biol Chem* **278**, 18249-55.

8/11/05

Towards Developing Capsid Assembly as a Target for Antiviral Drug Development: a Simple, Sensitive, and Scalable Fluorescence-based Assay for Molecules Affecting Hepatitis B Virus Core Assembly

Stephen J Stray[1], Ben Kopeck[1], Jennifer M Johnson[1], and Adam Zlotnick[1,2].

Keywords: Hepatitis B Virus (HBV), virus assembly, virus inhibitors, high throughput assay, antiviral

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Abstract

Virus assembly is a critical step in the viral lifecycle, involving components unique to the infected cell. Virus assembly has not been specifically targeted for the development of antiviral drugs, largely because of the absence of tractable *in vitro* assembly systems. We have developed a fluorescence assay for Hepatitis B virus (HBV) capsid assembly *in vitro*, based on FRET quenching of labeled capsid protein, which can be used to test potential inhibitors of this essential step in the HBV lifecycle, and can be adapted for high throughput screening. Small molecule inhibitors of virus assembly may prevent, accelerate inappropriately, or misdirect capsid formation. An *in vitro* primary screen has the advantage of identifying promising lead compounds without requiring that they be absorbed by cells and be non-toxic. This system may be a paradigm for the development of similar screens for antivirals targeting viruses such as hepatitis C virus and HIV.

Introduction

It is estimated that two billion people have been infected with Hepatitis B virus (HBV) at some time in their lives [1]; approximately 400 million people worldwide are chronically infected. Acute HBV infections are potentially fatal, and chronic HBV infection is the major risk factor for hepatocellular carcinoma (HCC).

HBV is a small enveloped DNA virus with an icosahedral core. The core is composed of the viral nucleic acid and reverse transcriptase enclosed in the protein capsid [2]. Core assembly is essential for HBV replication, as reverse transcription of the RNA pregenome occurs exclusively within the core particle, yielding a partially double-stranded circular genomic DNA [3]. The DNA-filled core then either buds into the endoplasmic reticulum, where it acquires its lipid envelope and surface antigen, or is imported into the nucleus to maintain infection. Recently, inhibition of HBV virion formation in model culture systems has been demonstrated using heteroaryldihydropyrimidines (HAPs) [4,5] discovered in the course of tissue culture-based screens. HAPs have also been shown to inhibit HBV capsid assembly *in vitro* [6,7]. Better assays for capsid assembly will be critical to exploiting this new target area for antivirals.

We have studied HBV capsid assembly extensively *in vitro* using a truncated form of HBV core protein (Cp), comprising the N-terminal assembly domain (amino acids 1-149) of strain *adyw* Cp expressed in *E. coli* (Cp149) [8]. This protein lacks the C-terminal nucleic acid binding domain (amino acids 150-183), which is not required for assembly of empty capsids [9]. HBV Cp is a dimer in solution [8,10]. The efficiency of *in vitro* assembly is a function of protein concentration, NaCl concentration, pH, and temperature [8,11,12]. Assembly is nucleated by a trimer of Cp149

dimers, followed by rapid addition of subsequent dimers [12]. A network of surprisingly weak intersubunit contacts holds HBV capsids together [11], a property that may be a feature of many virus systems [13]. Capsids persist even under unfavorable conditions because disassembly and reassembly reactions compete, leading to a kinetic barrier to dissociation, hysteresis [14]. A growing body of evidence leads to the hypothesis that HBV capsid assembly is allosterically regulated. We have shown that the HAP compound, HAP-1, deregulated HBV capsid assembly *in vitro* [7]: HAPs may act as synthetic allosteric activators, disrupting the assembly process by allowing assembly to occur inappropriately. HAP-1 also induced conversion of preassembled capsids into non-capsid polymers, presumably by interaction of HAP with dimers freed during capsid "breathing" [7].

We describe a simple fluorescence-based *in vitro* system allowing us to assess the ability of small molecules to alter HBV capsid assembly. Previous studies of potential HBV therapeutics have used either a transgenic mouse model or tissue culture systems expressing the HBV genome, limiting the features of potential drugs that can be tested. By screening compounds *in vitro* prior to the use of a cell-based system, lead compounds will be identified which would otherwise be discarded due to toxicity or inability to enter the cell. Using our method, we were able to demonstrate the effect of two distinctly different small molecules, which prevent normal HBV capsid assembly either by inhibiting capsid formation (urea [14]) or by accelerating and misdirecting assembly (HAP-1 [7]).

Results

Design of C150BO. Cryo-EM studies, using a gold label attached to an engineered C-terminal cysteine (residue 150), showed that the C-termini of the assembly domain were located on the

interior of the assembled capsid and close together in space [15]. This was supported in a crystal structure where density halted short of the actual C-termini (PDB entry 1QGT) [16]. Measuring from the last modeled amino acids of the X-ray structure (residue 142 or 143), distances between C-termini of adjacent subunits range from 12.3 Å to 14.8 Å. The distances across the fivefold and sixfold vertices in the HBV capsid crystal structure range from 17.3 Å to 21.6 Å. By comparison, the last ordered C-terminal residues in a dimer from1QGT are about 50 Å apart.

We chose the mutant described above, C150 (Cp149: C48A, C61A, C107A C150) for fluorescent labeling. We reasoned that assembly of capsids from HBV Cp dimers would cause Cp C-termini separated in space to be brought close together, allowing fluorescence resonance energy transfer (FRET) to occur between dye molecules attached to the C termini. The efficiency of energy transfer has a 6^{th} power dependence on the distance between donor and acceptor molecules; FRET efficiency is 50% at the Förster distance. The Förster distance for self-quenching by BODIPY-FL is approximately 57 Å [17]. Thus, we expected to see substantially enhanced quenching whenever dye-labelled dimers were present at the same vertex in a capsid.

By using a Cp149 mutant lacking the three native cysteine residues but adding a cysteine at the C-terminus (C150), we were able to uniformly and nearly quantitatively label C150 with a single BODIPY-FL maleimide, as determined by mass spectrometry (data not shown); this material was designated C150BO. Dimeric C150BO was highly fluorescent, but fluorescence was markedly reduced when C150BO was induced to undergo assembly (Fig. 1). We compared BODIPY-FL and fluorescein conjugates, BODIPY-FL gave a much stronger assembly-dependent change in fluorescence (data not shown). We also compared the effectiveness of

specific labeling via the unique C-terminal cysteine residue in C150 with maleimidyl conjugates to random labeling of α and ε amino groups with succinimidyl conjugates. Labeling with succinimides was inefficient (less than one dye molecule per monomer), and the resulting labelled proteins showed little change in fluorescence on assembly (data not shown).

Monitoring assembly by FRET. We have previously characterized assembly of HBV capsid protein (Cp) using techniques including real-time 90° light scattering (LS) and size exclusion chromatography (SEC) [7,11,18,19]. Assembly of wild-type Cp149 was found to be dependent on protein concentration, NaCl concentration, and temperature. C150BO was tested for assembly by LS using a range of NaCl concentrations (**Fig. 1a**). BODIPY-FL labeling did not measurably alter the ability of C150 to assemble. As seen with the wild-type, the rate and extent of assembly increased with increasing NaCl. We tested the same protein for FRET quenching, performing an identical panel of assembly reactions, but this time monitored BODIPY-FL fluorescence (Fig 1b). We observed that fluorescence decreases in response to the addition of NaCl, and the degree to which fluorescence is quenched depends on assembly (below).

Comparison of the time course of assembly by LS and F (**Fig. 1a** and **b**) shows that the LS and F curves had very similar shapes, even during the earliest phases of the reaction (**Fig. 1c**). Data from panels (a) and (b) were compared from 100 s to 600 s by plotting LS vs. F (**Fig. 1d**). For each case shown, most data points lie on a diagonal line, with the few outlying points correlating with "bumps" in the LS curves. The slopes of the F vs. LS curves were essentially the same for [NaCl] ≤ 0.75 M, indicating a strong correlation between the signals. The correlation between F and LS was weaker for assembly reactions above 1 M NaCl (data not shown), where there was

significant kinetic trapping and the accumulation of aggregates [12], both of which strongly influence LS but not F.

Assembly of C150BO at equilibrium. We next wished to compare the sensitivity of fluorescence quenching to the extent of assembly for reactions at 24 hours, as they approach equilibrium (**Fig. 2**). Fluorescence and LS were measured for reactions assembled overnight under the same conditions as described for Figure 1. Once F and LS had been determined, each sample was subjected to SEC over a superose 6 column. Previous studies showed that all of the cysteine residues of the HBV core protein were dispensable for core assembly, DNA replication, and particle production [20]. Surprisingly, a greater proportion of C150BO dimers were assembled than was seen previously for Cp149 under the same assembly conditions [11]; the apparent K_D ($K_D$app) can be calculated from the association energy or estimated from assembly isotherms as the point where dimer and capsid concentrations are equal [21] (**Table 1**). This indicated that assembly is more complete for the mutant than for wild-type, thus association energy is slightly higher for C150BO. Fluorescence, LS, and SEC measurements of assembly at equilibrium were in good agreement. Assembly increased over the range 0-0.75 M NaCl, while fluorescence decreased over this same range due to FRET quenching of the dye-labelled molecules.

At salt concentrations above 1 M, fluorescence was almost completely quenched. Unlike F, LS increased with increasing [NaCl] at 1 M NaCl and above, probably due to aggregate formation. SEC of C150BO reactions showed that the assembly products included a significant proportion of intermediates not usually seen in wild-type assembly reactions (**Supplementary Fig. 1**), intermediates were less prevalent at lower [NaCl] but were still more evident than in wild-type.

Similar profiles of intermediates and aggregates were also seen with unlabelled C150 These results suggest that the mutations in C150 affect the nucleation of assembly, leading to kinetic traps. Kinetic trapping is predicted by assembly models when association energy is increased and/or nucleation is no longer effectively limits the rate of assembly [12,19].

Quenching occurs mainly *in trans*, and can occur across vertices. To better understand the interactions that contribute to quenching in C150BO assembly, we set up a series of assembly reactions containing the same amount of total protein but different proportions of dye-labelled and unlabelled protein (**Fig. 3a**). Assembly results in little quenching at a 1:9 mixture of labelled and unlabelled dimer; at this ratio there are very few vertices with more than one fluorophore. This result also indicates that intra-dimer quenching (*cis* quenching) has not changed during assembly, i.e. quenching is not sensitive to the hypothesized conformational change from assembly-inactive to -active forms of dimer [12,19]. The fact that quenching is dependent on the proportion of dye-labelled molecules shows that quenching is dominated by interactions between dye molecules conjugated to neighboring dimers (*trans* quenching).

We suggest that quenching *in trans* (**Fig. 3b**) is due to two or more fluorophores at a vertex. In the context of a capsid, a dimer extends from a fivefold to a sixfold vertex or between two sixfolds, so that a protein at a vertex will have 4 or 5 neighbors. This hypothesis predicts that some quenching will occur as long as at least one other dye-labelled subunit (gray) is among the subunits meeting the black subunit at a vertex. Thus, a ratio of greater than 1:4 labelled to unlabelled dimer is needed for substantial quenching. We observe quenching with the 1:4 mixture of labelled and unlabelled protein. If quenching could only occur between adjacent

molecules, nearest neighbors, at least two labelled subunits in five would have to be labeled to induce significant FRET quenching.

Assembly misdirectors and inhibitors are readily assessed by FRET. We tested the utility of our FRET assay for compounds altering HBV Cp assembly *in vitro* by comparing fluorescence data to SEC data for assembly reactions containing either HAP-1 [7] or urea [14]. We recently characterized the effect of HAP-1 on the assembly of wild-type HBV Cp *in vitro*; we found that this molecule enhanced the rate and extent of HBV Cp assembly over a broad range of concentrations. At higher concentrations ($\geq 10\mu M$, > 1 drug molecule bound per protein dimer), HAP-1 lead to the formation of aberrant particles due to its preference for Cp hexamers rather than pentamers [7]. Fluorescence and SEC assays of C150BO assembled with HAP-1 showed that HAP-1 increased the degree of assembly and decreased the fluorescence (increased FRET, **Fig. 4a**). HAP-1 had a dramatic effect on both the rate and extent of C150BO assembly even at substoichiometric levels, as seen for wild-type Cp.

Urea inhibits HBV capsid assembly at concentrations above 0.75 M and causes reversible dissociation of wild-type HBV capsids at concentrations between 2.5 and 3.5 M without denaturing Cp dimers [14]. We observed a decrease in assembly by SEC and an increase in fluorescence (loss of FRET quenching) in the presence of urea. C150BO still eluted as an apparent dimer by SEC at urea concentrations ≥0.75 M, demonstrating that the inhibition of assembly observed was not due to denaturation of the protein.

To control for the possibility that either HAP-1 or urea was affecting BODIPY-FL fluorescence, we assayed the fluorescence of mock-assembled C150B0 dimer without NaCl. For HAP-1, these controls showed no effect of drug on fluorescence at early times (up to 2 h), even though the same amount of drug had a very strong effect on fluorescence of NaCl-induced assembly at the same times (data not shown). Therefore, the effect on fluorescence was due solely to increased assembly rather than the drug quenching the fluorescence directly. After longer incubations (24 h), BODIPY-FL quenching was observed in the HAP-1 mock-assembled controls. Analysis by SEC at 24 h revealed that the protein had, indeed, assembled at the higher HAP-1 concentrations even at low salt, consistent with our previous observation of slow assembly of wild-type Cp under normally non-permissive conditions [7]. There was neither assembly nor change in fluorescence due to urea in the mock-assembled controls.

Discussion

Virus assembly is an integral part of the viral lifecycle but there are no analogous proteins in the uninfected cell [15]; it should provide a specific target for antiviral therapy. Assembly has not been intentionally exploited because the molecular details are only now being elucidated and the detection methods generally used have not been amenable to large-scale screening of potentially active compounds. Viral enzymes such as polymerases and maturational proteases have been targeted by antiviral drugs for several decades, but targeting other aspects of the viral life cycle has only recently begun (see [22] for review). Part of the problem of specifically targeting such processes as virus entry, virion assembly and maturation, particle release, and capsid uncoating is that they are less readily assayed than enzymatic processes such as nucleic acid polymerization or proteolytic cleavage. Here we demonstrate a simple, rapid, and sensitive fluorescence assay

for HBV capsid assembly that is readily adaptable to a microtiter plate format (see Fig. 2 and 4) and scalable to high-throughput screening. This assay can be used to detect both inhibitors, which prevent or prematurely terminate capsid assembly, and misdirectors, which can hyperactivate capsid assembly leading to loss of regulation and/or the formation of aberrant products.

The availability of an *in vitro* system has the advantage of rapidity (2-24 h as opposed to 5-7 days) and cost compared to the currently available tissue culture model. A completely *ex vivo* system gives the tremendous advantage of identifying lead compounds which otherwise would be rejected because of problems with uptake or toxicity, but which may be useful after further chemical modification.

Assembly can be addressed *in vitro* by other techniques such as light scattering/turbidity measurements and size exclusion chromatography. Many of these techniques cannot be conveniently performed on large numbers of replicate samples. Light scattering and turbidity measurements are extremely sensitive to particulates and large aggregates. ELISA assays may useful in some cases, but are only possible for systems where assembly-specific antibodies (such as anti-HBcAg) have been characterized.

The FRET assay described here requires an efficient in vitro assembly system. Even in the absence of detailed structural information, it is a safe generalization that N- and/or C-termini of the capsid protein(s) will be close to one another [23] and are therefore the most likely sites for fluorescent labeling. The FRET assay is rapid, works in a microtiter plate format, and is not

sensitive to particulates, intermediates, or aggregates. As the molecular details of assembly are understood for more viruses, our system may provide a template for the development of screens for assembly inhibitors of other viruses including influenza [24], alphaviruses [25], hepatitis C virus [26], and HIV [27].

Materials and Methods

Mutagenesis. For simplicity of chemical labeling, we mutated the cysteine residues (C48, C61, and C107) in the assembly domain of HBV strain *adyw* core protein (amino acids 1-149[28]) to alanine residues, inserting a unique cysteine residue at the C-terminus (C150). This allowed us to label the protein with cysteine-active dye conjugates such as fluorescein or BODIPY-FL maleimide. Mutagenesis was performed using QuikChange Multi (Stratagene, La Jolla, CA). Mutagenic primers are described in Table 2 (IDT DNA technologies, Davenport, IA). Mutations were confirmed by sequencing (DNA Sequencing Core, Oklahoma Medical Research Foundation). We refer to the resulting mutant protein as C150.

Protein expression, purification, and dye labeling. Wild-type and mutant truncated HBV capsid protein dimers were expressed and purified from *E. coli* as described [28,29]. Protein was quantitated by absorbance at 280 nm (ε= 60,900 M^{-1} cm^{-1}). DTT levels were maintained at 5 mM throughout purification and storage. Immediately prior to derivatization, C150 protein was removed from storage buffer by chromatography over a G25 PD10 desalting column (Amersham Biosciences, Piscataway, NJ) equilibrated with ice-cold 50 mM HEPES pH 7.5 without DTT. Peak fractions were collected and reacted with BODIPY-FL maleimide (Invitrogen/Molecular Probes, Eugene, OR) on ice at a final concentration of 4 mM from a 20 mM BODIPY-FL stock

in DMSO. Most complete labeling was achieved by overnight reaction, although significant labeling could be achieved by reaction for as little as 10 min. Unreacted dye was removed by separation over a G25 PD10 desalting column equilibrated in ice-cold HEPES 50 mM pH 7.5. The degree of dye labeling was determined using absorbance at 504 nm (ε504=73,000 M^{-1} cm^{-1}, ε280=1300 $M^{-1}cm^{-1}$). The extinction coefficient was calculated by determining the degree of labeling of BODIPY-FL-labeled C150 preps by MALDI-TOF mass spectrometry. Yield of BODIPY-FL-labelled C150 was typically on the order 1.9 mole dye per mole of dimer.

Fluorescence and Light scattering. Assembly was monitored by fluorescence using a SPEX Fluoromax-2 fluorometer (Horiba Jobin Yvon, Edison, NJ), using a 0.3 cm path length cuvette (Hellma, Forest Hills, NY) or in black 96-well COSTAR fluorescence plates (Corning, Corning, NY) using a MicroMax adaptor. Assembly reactions were performed at 21 °C. Assembly was initiated manually by mixing Cp149 in 50 mM HEPES pH 7.5 with buffered NaCl (Sigma, St Louis, MO) as appropriate. Fluorescence was excited at 504 nm and emission was measured at 509 nm (1 nm band pass for each).

Where assembly was monitored by 90° light scattering, excitation and emission were set at 400 nm with a 3 nm band pass a 0.3 cm path length cuvette as previously described [7,12]. Light scattering was measured at 400 nm, rather than 320 nm as previously, to minimize the effect of absorbance of both HAP and BODIPY at the shorter wavelength. The intense scattered light was attenuated by a neutral density filter. Light scattering was determined either after 24 h incubation under assembly conditions, or monitored in real time during an assembly reaction.

Size exclusion chromatography (SEC). Assembly reactions were examined by SEC on a Superose 6 10/30 column (Amersham Biosciences, Piscataway, NJ) mounted on a Shimadzu HPLC system equipped with an auto injection module (Shimadzu, Columbia, MD). The column was equilibrated with 50 mM HEPES pH 7.5, 50 mM NaCl. Recovered protein was assigned either to the void (6.5 – 7.0 ml), capsid (7.0 – 8.3 ml), dimer (15 – 16.5 ml), or intermediate elution (8.3 – 15 ml).

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Abbreviations

Cp – core protein; Cp149 – assembly domain of core protein (amino acids 1-149); C150 – Cp149, C48A, C61A, C107A, C150; F – fluorescence; FRET – fluorescence resonant energy transfer; HAP – heteroaryldihydropyrimidine; HBV – Hepatitis B virus; LS – light scattering; SEC – size-exclusion chromatography.

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Figure Legends

Figure 1. Strong correlation of real time light scattering and fluorescence signals for assembly of BODIPY-labelled C150. (a) Assembly of 3 μM C150BO was measured by 90° light scattering at room temperature. All measurements were carried out in a cuvette. Assembly was initiated manually at 90 s. Final [NaCl], in molar, are indicated to the right of the panel. (b) Assembly monitored by fluorescence (excitation at 504 nm, emission at 509 nm). (c) Alignment of LS and F signals from assembly reactions at 0.25 M NaCl. To allow direct comparison, F data were scaled by applying the linear best fit of LS vs. F curve (panel d) to the primary F data. Note that scale for F has been inverted. (d) Comparison of fluorescence and light scattering from 100 s–500 s of assembly reactions using different [NaCl]. Molar [NaCl] is indicated next to each data set. Note that the F scale has been inverted.

Figure 2. Fluorescence quenching correlates with size exclusion chromatography at equilibrium. Assembly reactions containing 3 μM C150BO and NaCl were analyzed by fluorescence, 90° light scattering, or size exclusion chromatography at 24 h. Assembly reactions appeared to have equilibrated by this time. Note that fluorescence and light scattering data were normalized to their maximum values (at 0 NaCl for fluorescence, at 1 M NaCl for LS) for ease of comparison. Assembly data are expressed as total protein present in assembly products (capsid plus intermediate). Data are average ± standard deviation of three replicates, including samples from Figure 1. All fluorescence measurements were made in black 96-well fluorescence plates.

Figure 3. Quenching is dominated by interactions between dimers *in trans*, and occurs across vertices. (a) Assembly reactions (in triplicate) containing a mixture of dye-labelled and

unlabelled C150 were assayed by fluorescence at 24 h. Total protein concentration (labelled plus unlabelled) for each was 3 μM; final [NaCl] was 1 M, where assembly was expected to be almost complete (see Table 1). To account for the differing amount of dye-labelled protein in each condition, fluorescence of assembled material was normalized to fluorescence of the mock-assembled control for each condition (dimeric C150 and C150BO without NaCl,). The dotted line indicates the fluorescence of the mock-assembled control. (b) Cartoon of FRET quenching, using on a T=1 30-dimer assembly model [30]. Dye-labelled subunits are depicted in black or gray, with stars indicating the approximate position of the dye label. Any two dye labelled subunits meeting at a vertex will quench, i.e. the fluorescence of the black dimer can be quenched by any dye-labelled dimer (gray) meeting it at a vertex (solid-edged rectangles). A T=4 or T=3 HBV capsid would have 12 fivefold and 30 or 20 sixfold vertices, respectively.

Figure 4 FRET quenching is a useful tool for detecting assembly misdirection and inhibition. (a) Assembly reactions containing 3 μM C150BO and 150 mM NaCl at 21 °C plus HAP-1 were assayed by fluorescence or size exclusion chromatography at 24 h. HAP-1 enhanced assembly, leading to enhanced FRET quenching. (b). Assembly reactions containing 3 μM C150BO and 0.5 M NaCl at 21 °C plus urea, which inhibited assembly, were assayed by fluorescence or size exclusion chromatography at 24 h. No reduction in fluorescence due to either inhibitor was observed in mock assembly reactions at early times (2h, data not shown), demonstrating that neither HAP nor urea had intrinsic quenching activity. Analysis of mock assembly reactions by SEC showed that HAP alone at low salt was able to induce slow C150BO assembly (data not shown), as seen for Cp149wt [7]. Urea treatment at the levels used here was not

sufficient to denature dimer into monomer (data not shown). All fluorescence measurements were made in black 96-well fluorescence plates.

Table 1. Comparison of capsid stability ($K_D$app) of Cp149 wild-type and C150BO

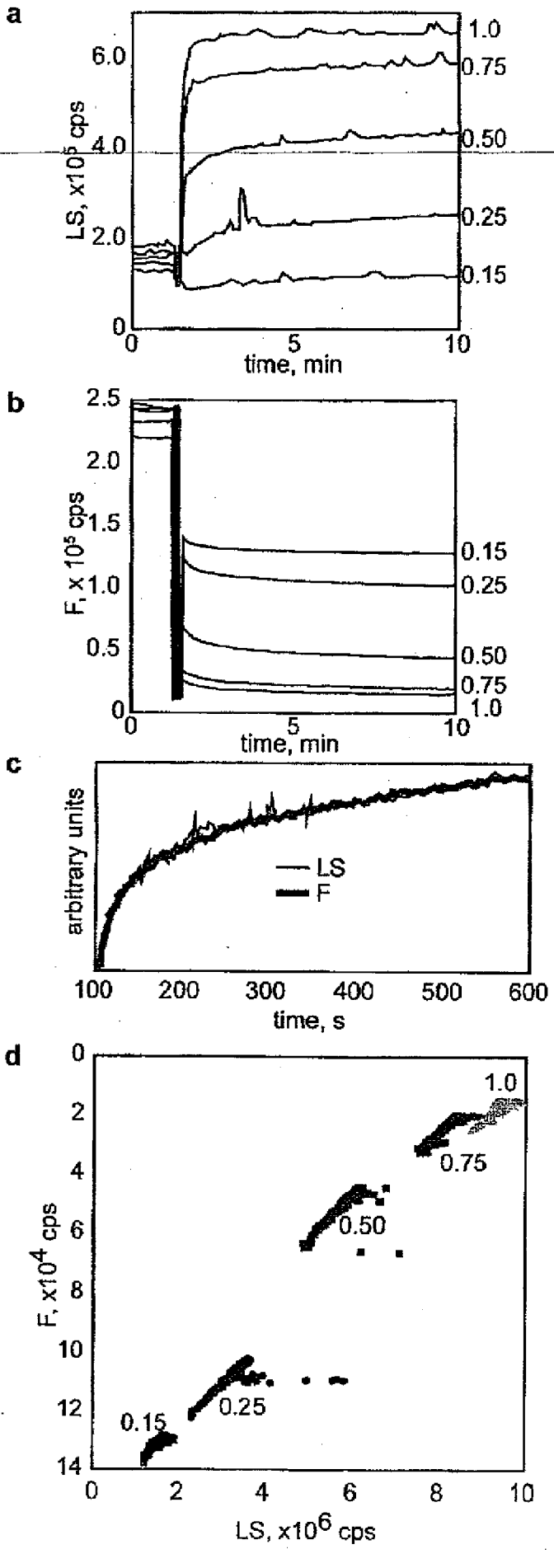
[NaCl], mM	150	300	500	700	750
Cp149†	14	1.9	1.8	0.77	
C150BO	2.4 ± 0.2	1.3 ± 0.1	0.5 ± 0.4		0.1 ± 0.05

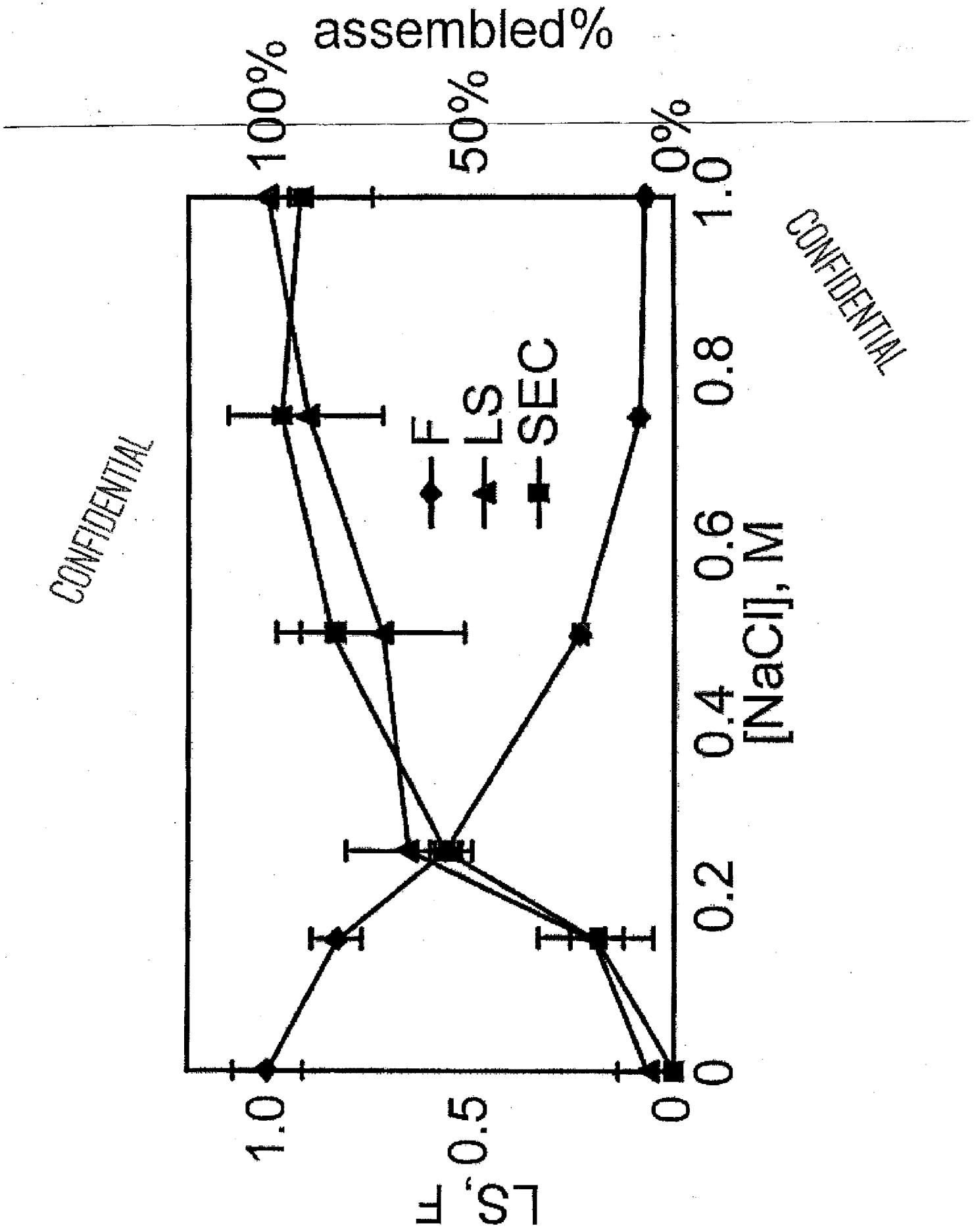
† Data from reference 11

Table 2. Mutagenic primers

Mutation	primer
C48A	CTCCTGAGCACGCCAGCCCTCACCATAC
C61A	GCAATTCTTGCCTGGGGAGACTTAATGACTC
C107A	GTGGTTTCACATTTCTGCTCTCACTTTTGGAAG
*ins*C150	GGAGACTACGGTTGTT ***TGC*** AAGGATCCGGCTGC

Mutated Codons are underlined. Inserted codons are shown in ***bold italic***.

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