An Inexpensive Phagestat for Continuous Evolution Research

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# Abstract

Continuous evolution systems (PACE, PATHE) provide a powerful approach to protein engineering to increase Protein-DNA and Protein-Protein binding affinities and selectivity through directed evolution. Ad-hoc systems built from commercially available equipment have material costs on the order of $30,000 as well as high labor costs because of the requirement of continuous monitoring. Alternatively, by using inexpensive computational systems, commodity electronic and plumbing, and a few custom 3D-printed components, a bio-reactor can be built for approximately $1000 which reduces oversight requirements through non-contact sensing, image processing, and computer-controlled feedback. This inexpensive and easy-to-use apparatus will be a scalable platform for using directed evolution for protein engineering.

# Problem

Protein Engineering consists principally of finding DNA sequence modifications that result in a desired protein structure. The difficulty of predicting precise three-dimensional shape and charge distribution from a sequence change means that traditional engineering approaches are often impractical.

And even if a precise correspondence between sequence changes and conformational changes were possible, it would still require precise knowledge of the target's shape and charge distribution in-vivo, which might be significantly different from the structural information available through crystallography or NMR. However, if the protein engineering task is to create a protein with stronger and more selective binding to a particular target, we can use evolution to perform a hill-climbing search among randomly generated variations of a protein.

Directed evolution is a method whereby in-vivo interaction between a target protein and mutable protein can be used as a selection mechanism to discover novel sequences with the desired property. Given a target and a provisional protein which have some binding affinity, directed evolution provides an alternative approach to find those sequence changes which will increase binding and selectivity (Cf. Esvelt et al.).  
  
We give the name “phagestat” to an apparatus which maintains a population of bacterial virus (phage) which is undergoing directed evolution through interaction with a bacterial host that has been programmed to mutate, select, and propagate that virus.  
  
An inexpensive, highly automated, and reliable phagestat will allow more directed evolution experiments to be performed. Although the task of designing plasmids for the host cell generally is more difficult and may take longer than running the evolution experiment, the inexpensive phagestat opens the possibility of performing many parallel experiments with different starting sequences and sequentially evolving sequences for increased binding, selectivity, or negative selection pressure for unwanted properties.

# Current Status of Research

Prototypes of all individual components, including the turbidostat, host cell incubator (Cellstat), computer vision level monitoring, leakage alarm, and automatic sampling system have been built and tested. Reliability testing over several weeks has been performed on various subsystems including the heating/mixing/aeration cell culture system, phage lagoon mixing, heating, and lighting systems as well as the Bluetooth communications between these subsystems (Cellstat and Lagoons) and the main computer. Platform independent control software, written in Python and Prolog, and using OpenCV for image processing has been tested on Microsoft Windows (XP, 7) and Linux (Debian8, Ubuntu, and Raspian).  
  
We know of no similar work to reduce the cost of constructing and operating phage-based continuous evolution environments. We acknowledge that the capital and labor costs of running such experiments may be of minor importance to practitioners of academic biological research. Researchers currently involved in active research in this area probably do not regard a $30,000 capital cost and oversight requiring several lab technicians as a primary concern. However, the effort that goes into setting up and debugging these systems has not being fully quantified, and we believe that streamlining the mechanics of maintaining an evolving phage population will free these practitioners to concentrate on plasmid design and conduct many more experiments within their time and budget constraints.  
  
This work requires expertise in areas outside of molecular biology, and its goal is to lower the cost of entry into this area of research rather than to achieve any particular protein engineering task. That said, the current design is driven almost entirely by the requirements of ongoing work by Drs. Edgell, Charles, and Collier to enhance binding for protease inhibitors of bacterial extra-cellular virulence factors.

# Technical Goals

# Project Plan

# Evaluation

### Criteria for a Go/No-Go Decision, Including Metrics

Completed construction of three fully functional EvoStats with a parts cost of less than $2000.

All instances capable of maintaining environmental conditions continuously for four days.

Demonstrated ability to replace each of the major subsystems: Cellstat, Lagoon, and AutoSampler while maintaining a live culture.

Perform several complete evolution experiments on evolving phage.

## Discuss challenges

Image processing must be adapted to constantly changing hardware and laboratory conditions.

Active cooling must be added to low-cost USB cameras to avoid the expense of a cryogenic camera ($25,000 from Hammumatsu).

Equipment must be modular and easy to move due to space restrictions.

# Commercial Proposal Narrative

## Problem

Phage-based continuous evolution is a new technology with few practitioners. This is as much of an opportunity as it is a problem as we see this technology having ?????????????

## Commercial Goals

Provide consulting to entities

## Market

Pharmaceutical research organizations engaged in protein engineering.

Other, non-pharmaceutical, protein engineering.

Protein optimization services (for organizations that do not want to develop in-house phage/evolution capabilities). A protein optimization service would accept sequences for binding target and initial candidate proteins and design selection-plasmids and hybrid proteins, and perform the evolution process.

## Intellectual Property

Hardware designs and image processing software for flow-control, turbidity sensing, and detection of bioluminescence. A novel low-cost non-contact turbidometer design. Integrated communications architecture to reduce wiring and simplify set-up of equipment. Techniques for repair and replacement of subsystems during operation. Self-calibrating and continuous monitoring of all system parameters.

Data logging and alerts utilizing Internet capabilities.

## Project Plan

Completed design and construction of major sub-systems:

1. EvoStat Frame
   1. Three-level PVC support platform
   2. Insulated dark environmental chamber
   3. Camera
   4. Power supplies: 5-, 12-, and 36-volt
2. Cellstat
   1. Turbidostat
   2. Digital thermometer
   3. Heating element
   4. Meniscus light
   5. Magnetic mixer/Aeration
   6. Micro-controller + software
   7. Bluetooth communication module
3. Lagoon
   1. Thermometer
   2. Heating elements
   3. Meniscus light
   4. Magnetic mixer
   5. Pinch valve
   6. Microcontroller + software
   7. Bluetooth communication module
4. AutoSampler
   1. Rolling multi-valve
   2. Sample plate positioning platform
   3. Micro-controller, stepper, motor and sample-timing software
   4. Bluetooth Communication module
5. Luminometer
   1. USB microscopic camera
   2. Dark enclosure for camera
   3. Cooling unit for camera

Deliverables:

1. Manifest of parts costing less than $1000
2. Instructions for construction, maintenance, and modification of the device

(no more than one page): Provide an overview of the project, including a brief description of the technology, the name of the Commercialization Advisor, and a summary of the technical and commercialization goals.

Technical Proposal Narrative: The technical narrative should be no more than 4 pages long and include all sections below.   
  
   
  
Describe the work that has been done to date, both in the lab of the scientist and in the field.  
  
*-Technical Goals  
  
Explain the goals for the development of the technology to be achieved in this project.*

Develop software to assist calibration of the EvoStat using external references for luminosity and cell density, thus exploiting shared equipment while keeping the cost of each phagestat low.

Evaluate the use of several commodity USB and WiFi camera systems to accommodate hardware cost or performance improvements and availability.

Test operation over many hours of unattended operation. Goals include reliable operation for at least 100 hours (four days) and rapid detection and communication of problems (e.g., controlled suspension of operation and sending of text messages after leak detection).

Develop procedures for replacing any subsystem without interrupting the long-term experiment.

For example, the most difficult of these operations would involve cellstat replacement. First, a second cellstat would be filled with nutrient, brought to temperature, and innoculated from the current cellstat. Calibration data are supplied as the culture is brought to early log phase growth rate. Once achieved, the liquid flow through the system will be paused and the new cellstat swapped into the system. Restarting the system with the new cellstat name in the configuration file will complete the cellstat change. Each instance of cellstat, lagoon, or auto-sampler is given a unique name which will appear during Bluetooth discovery. The device names which appear in a configuration file define the components in that system. Auto-sampler replacement merely requires the controlled interruption and resumption of normal flow operations, while lagoon replacement will require a greatly reduced flow rate and possibly extra inducers to re-establish the viral population.

-Project Plan  
  
Three complete EvoStat systems will be built: One, nearing completion as of this writing, to support the work of Edgell et al. at the Genome Sciences building on the campus of UNC-Chapel Hill. A second will be constructed at the local makerspace in Durham, NC (Splatspace) where we have access to 3D printers, an electronic workbench, woodworking, and machine shop, and a third at the Museum of Life and Science, where principal construction of components for all three systems will occur.  
  
Patent application(s) will be completed covering various aspects of the project including the design of three custom components to perform turbidometer and auto-sampling, and software components for process control, communication, and image processing.  
  
Improve the design to exploit low-cost alternatives to specialized equipment, and allow for the construction and substantial modification of the device to be performed by the laboratory personnel who will be using it.  
  
Describe the studies that will be performed to meet the stated technological goals, including key technical milestones and a timeline narrative.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Aims |  | Months 1-3 | Months 4-6 | Months 7-12 |
| Level detection |  |  |  |  |
| Cell density |  |  |  |  |
| Luminosity |  |  |  |  |
| Auto-calibration |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |

Table 1 Project Timeline

We intend to use the devices to recapitulate the experiments done by Esvelt, Lui, Dickson, and others at Harvard and support the novel work being done by Dr. Edgell et al.  
  
-Evaluation  
  
The design should have a parts cost on the order of $1000 and could be constructed in a few weeks by an electronic technician with access to a 3D printer and machine tools. An important goal is to minimize the resources required for construction. Currently we drill press, hand drill, table saw, chop/miter saw, deburring tool, and thread tap. The 3D printer is not a requirement as high quality parts can be ordered through online services.  
  
1) Define the criteria for a go/no-go decision within the technical phase. Include defined metrics that will indicate or demonstrate a successful outcome.  
  
Achieving sufficient image sensitivity for bio-luminescence through the active cooling of commodity USB cameras (Logi-Tech, Dino-Lite USB microscope).  
  
Discuss the anticipated challenges within the technical aspects of the project.  
  
Commercial Proposal Narrative: The commercial narrative should be no more than 2 pages long and include all sections below. Use the headings provided.

The Commercialization Advisor should participate in the preparation of this section of the proposal.  
  
-Problem  
  
  
-Commercial Goals  
  
We want to encourage researchers with limited budgets to use these design ideas to perform research, possibly by requiring no licensing fee. This approach could result in the sharing of improvements to the design from an open community. We would then require licensing only for commercial exploitation of sequences resulting from the use of this device.  
  
As evolution-based protein modification matures into an industrial process, we expect to license this design to one or more manufacturers of laboratory equipment. Because the device itself does not produce therapeutic proteins or chemicals, but is a tool for sequence discovery, it will not be subject to any particular medical equipment or FDA requirements.  
  
One company (Innatrix) is already considering a business model to employ a number of these machines and a staff with expertise in selection-plasmid design to provide contract protein optimization. Such a service would allow a drug development company with a protein therapeutic to contract out the task of affinity or selectivity optimization without the need to build and maintain phage-stats or develop in-house expertise in directed evolution. This business model is of particular interest as it benefits directly from both the low build cost and the low operating cost of the device.  
  
Patent protection  
  
Ultimately, many practitioners may want to perform the continuous evolution process with requirements different from academic research. We see an opportunity to create a commercial version of the EvoStat with an emphasis on using evolution as an industrial process.  
  
  
Licensing  
  
See the FAQs online for examples of commercialization goals.  
  
-Market  
  
Phage-based directed evolution is a fairly recent development, but given the advantages of protein-based therapeutics, it seems likely that this method of protein modification may come into widespread use in pharmaceutical development.  
  
Manufacturers of liquid handling laboratory equipment such as Tecan, Aventics, Humphrey, Nordson EFD are all potential manufacturers of the EvoStat. Other companies include Becton Dickinson, Topac, Intellitech, and Pope Scientific.  
  
  
-Intellectual Property  
  
The PACE (Phage Assisted Continuous Evolution) technique is the subject of patents filed by Harvard University (ca. 2011). Modifications to this process, such as PATHE (Edgell et al. unpublished correspondence) represent improvements to this technique which themselves may or may not be constrained by the PACE patents. These patents relate exclusively to the molecular biology, phage selection, the use of evolving protein as a proxy for phage fusion protein, and other matters relating to the molecular biology of mutation and selection. This IP does not appear to impact phagestat design and our improvements are based upon well-known and largely open technologies. A description of the prior art can be found in Husimi (1989). The improvements represented in our IP are based primarily upon this non-patent prior art.  
  
As far as we know, none of the details of operation of this design are subject to any IP constraints.  
  
-Project Plan  
  
Support will provide funding for two days per week PI for six months: one day per week at the Museum of Life and Science for primary construction and software development of three complete EvoStats and one day per week on-site at the Genome Sciences building (biology department) at UNC-Chapel Hill integrating and maintaining the EvoStat for use by researchers.   
  
 and additional components (multiple extra instances of the lagoon units, one additional Cellstat, and one additional auto-sampler).   
  
Each EvoStat will consist of a main computer, one cellstat, four lagoons, and one auto-sampler. An additional cellstat and an auto-sampler along with a few additional lagoons may be constructed as backup units. Initially, we have no plans to run active cultures in the EvoStats located at the Museum of Life and Science or the makerspace. When the design stabilizes and the second and third EvoStat have demonstrated their reliability, they will be moved into space at UNC or another laboratory to be used for evolution experiments.

Peter Reintjes (PI) List of Publications  
  
  
U.S. Patent #5,728,963 for "Low-Power Music Synthesizer and Transmitter", technology for short-range radio-based toys and radio-location devices.  
  
 U.S. Patent #6,178,453 “Virtual circuit switching architecture for IP-telephony and collaborative computing applications”.  
  
``Logic and Language: Stretching Homologies to the Breaking Point'', Banquet Speech, ICPAP/PACT, Paris, April 1995.  
  
``Logic Programming for Manufacturing and Engineering'', Advanced Software Technology Seminars, London, December 1994.  
  
``Confessions of a Logical Programmer'', Keynote Address, International Conference on the Practical Applications of Prolog, London, April 1994.  
  
``MULTI/PLEX: Tools for Formal Languages'', Peter Reintjes and Suresh Rajgopal, Programming Environments Workshop, International Conference and Symposium on Logic Programming, Vancouver, November 1993.  
  
``Elegant Technologies'', Invited Talk, International Conference on the Practical Applications of Prolog, ALP, London, April 1992.  
  
``A Set of Tools for VHDL Design'', International Conference on Logic Programming, Paris, June 1991. MIT Press  
Also in Logic Programming in Action: Proceedings of the Second International Logic Programming Summer School, Zurich, September 1992, Springer-Verlag.  
  
``BIOSCAN: A VLSI-Based System for Biosequence Analysis'', White et. al.. 1991 IEEE International Conference on Computer Design, October 1991, IEEE Computer Society Press  
  
``PREDITOR: A Prolog-based VLSI Editor'', The Practice of Prolog, Leon Sterling, Editor, pp.21-72, November 1990, MIT Press  
  
``A VHDL Parser in Prolog'', MCNC Technical Report 90-41, March 1990, Microelectronics Center of North Carolina  
  
``AUNT: A Universal Netlist Translator'', 1987 Symposium on Logic Programming, September 1987, IEEE Computer Society Press, also in Journal of Logic Programming, 1990:8:5-19 North Holland.  
  
``A Proposal for Symbolic Supercomputing'', MCNC Technical Report 89-13, March 1990, Microelectronics Center of North Carolina  
  
``AI Methodology as a Key for Software Reusability'', Tools for Artificial Intelligence - TAI-89, October 1989, IEEE Computer Society Press  
  
``AI Languages and Software Engineering'', AAAI Spring Symposium, March 1989, Stanford University.  
  
``A VLSI Design Environment in Prolog'', Logic Programming: The Proceedings of the Fifth International Conference and Symposium, August 1988, MIT Press  
  
``A History of Machine Translation in Word, Phrase, and Fable'', Videotape for IBM Corporation, Information Development Education, 1988.  
  
``AI Applications in VLSI CAD'', Artificial Intelligence Applications Symposium, February 1987, North Carolina State University.  
  
``Network Tools: Ideas for Intelligent Network Software'', Byte Magazine, October 1981.  
  
``UNIX/C Seminars'', 1980, Eatoin Corporation.  
  
``Phase-Locked Waveform Generator'', Electronics, February 1978, McGraw Hill.  
  
``Self-gating Sample-and-Hold controls Oscillator Frequency'', Electronics, June 1977.

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| --- | --- | --- |
| Quantity | Price | Description |
| 2 | $7.51 | Charlotte Pipe 2-in x 10-ft 280-PSI Sch 40 PVC |
| 3 | $7.51 | Charlotte Pipe 1-1/2-in x 10-ft 280-PSI Sch 40 PVC |
| 3 | $2.96 | LASCO 2-in Dia 90-Degree PVC Sch 40 Tee |
| 3 | $15.58 | Charlotte Pipe 4-in x 10-ft Sch 40 PVC |
| 4 | $12.48 | Charlotte Pipe 4-in x 4-in x 2-in dia PVC Sanitary Tee |
| 16 | $12.48 | Charlotte Pipe 4-in x 4-in x 1-1/2-in dia PVC Sanitary Tee |
| 10 | $6.56 | Nipple 1/2In Thrd Both Ends 3-1/2In 304 |
| 10 | $9.09 | Nipple 1/2In Thrd Both Ends 3-1/2In 316 |
| 2 | $8.31 | Cross 1/2 In 316 Stainless Steel 150 PSI |
| 8 | $3.56 | Elbow 90Deg 1/2 In 304 Stainless Steel |
| 0 | $19.69 | Elbow 90 Deg 1/2 In 304 Stainless Steel |
| 1 | $5.00 | Drip Pan |
| 1 | $5.00 | Grill |
| 2 | $70.00 | Foam Box |
| 4 | $5.00 | Floor flange |
| 4 | $25.00 | Caster |
| 4 | $5.00 | Floor flange |
| 100 | $10.00 | 1/4-20 Socket cap Stainless steel 18-8 |
| TOTAL | $230.73 |  |

Table 2: Parts for EvoStat Frame