Form Approved Through 05/2004 — OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed character length restrictions indicated.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
Controlled transgenics via 3'UTR and site specificity

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME *(Last, first, middle)*: Cearley, Jamie A.

3b. DEGREE(S): PhD

3c. POSITION TITLE: Research Scientist

3d. MAILING ADDRESS *(Street, city, state, zip code)*
Open Biosystems
6705 Odyssey Drive
Huntsville, AL 35806

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Research and Development

3f. MAJOR SUBDIVISION:

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (256) 704-4848 FAX: (256) 704-4849

E-MAIL ADDRESS: jcearley@openbiosystems.com

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes

4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.

4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no.

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name: Open Biosystems
Address: 6705 Odyssey Drive
Huntsville, AL 35806

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☒ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER

DUNS NO.
Congressional District: Alabama District 5

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Mark Kershner
Title: Chief Finacial Officer
Address: 6705 Odyssey Drive
Huntsville, AL. 35806
Tel: (256) 319-1476 FAX: (256) 704-4849
E-Mail: mkershner@openbiosystems.com

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Mark Kershner
Title: Chief Financial Officer
Address: 6705 Odyssey Drive
Huntsville, AL. 35806
Tel: (256) 319-1476 FAX: (256) 704-4849
E-Mail: mkershner@openbiosystems.com

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a.
(In ink. "Per" signature not acceptable.) — DATE

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13.
(In ink. "Per" signature not acceptable.) — DATE

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The long-term goal of this proposal is to provide a reliable, repeatable, and comparable method for generating mouse transgenics that allows for exquisite control over expression level, genome location, and strain background. The tool set ultimately produced will be a collection of 6 bacterial artificial chromosome (BAC) vectors designed to work in complimentary *hprt* negative mouse embryonic stem cells. Transgenic mice can be made with much greater ease and efficiency when this modular system is combined with its stringent selectable properties. Defined placement of a transgene in the genome and control over mRNA expression will greatly impact the biological relevance of mouse transgenics bringing us closer to accurately assessing gene function and the consequences of mutation. The core technology relies on the modulation of mRNA stability using a collection of five defined 3' untranslated regions (UTRs) in conjunction with site-specific integration at the drug selectable *Hprt* gene locus. This system provides for a defined alteration in gene expression levels of up to 100-fold. Modification of this technology for use in BACs, opens the door for its use in making transgenics in a variety of mouse strains. A series of BACs will be constructed consisting of 5 vectors where a gene of interest can be placed under the influence of one of five different 3' UTRs, and an additional test vector. All vectors will undergo stringent testing to make sure they are performing appropriately. In phase II we will expand the technology to include additional levels of gene expression control based on the use of newly defined 3'UTR regions. A wide collection of mouse ES cell lines from different mouse strains will also be made available for use with this system.

PERFORMANCE SITE(S) *(organization, city, state)*
Open Biosystems, Inc. Huntsville, Alabama

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below.
Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Cearley, Jamie A., Ph.D.	Open Biosystems, Inc.	Principal Investigator
DuBreuil, Rusla M., Ph.D.	Open Biosystems, Inc.	Co-principal Investigator
TBD	Open Biosystems, Inc.	Technician II
Kershner, Mark F., M.B.A.	Open Biosystems, Inc.	Financial Management
Smithies, Oliver, Ph.D.	University of North Carolina	Consultant

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ **Yes**

Principal Investigator/Program Director (Last, First, Middle): Cearley, Jamie A.

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT
TABLE OF CONTENTS

	Page Numbers
Face Page	1
Description, Performance Sites, and Personnel	2
Table of Contents	3
Detailed Budget for Initial Budget Period (or Modular Budget)	4
Budget for Entire Proposed Period of Support **(not applicable with Modular Budget)**	6
Budgets Pertaining to Consortium/Contractual Arrangements **(not applicable with Modular Budget)**	
Biographical Sketch – Principal Investigator/Program Director (*Not to exceed four pages*)	8
Other Biographical Sketches (Not to exceed four pages for each – *See instructions*)	10
Resources	14
Research Plan	16
Introduction to Revised Application *(Not to exceed 3 pages)*	
Introduction to Supplemental Application *(Not to exceed one page)*	
A. Specific Aims	16
B. Background and Significance	17
C. Preliminary Studies/Progress Report/ relevant experience	20
Phase I Progress Report (SBIR/STTR Phase II ONLY)	
D. Research Design and Methods	22
E. Human Subjects	31
Protection of Human Subjects (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Women (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Minorities (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Children (Required if Item 4 on the Face Page is marked "Yes")	
Data and Safety Monitoring Plan (Required if Item 4 on the Face Page is marked "Yes" ***and*** a Phase I, II, or III clinical trial is proposed	
F. Vertebrate Animals	31
G. Literature Cited	31
H. Consortium/Contractual Arrangements	33
I. Letters of Support (e.g., Consultants)	33
J. Product Development Plan (SBIR/STTR Phase II and Fast-Track ONLY)	
Checklist	34

(Items A-D: not to exceed 25 pages*)

** SBIR/STTR Phase I: Items A-D limited to 15 pages.*

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited. ☐ Check if Appendix is Included

Number of publications and manuscripts accepted for publication *(not to exceed 10)*

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY	FROM 07/01/05	THROUGH 07/01/06

PERSONNEL *(Applicant organization only)*		TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT				SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Cearley, Jamie A., Ph.D.	Principal Investigator	12	50.0				
DuBreuil, Rusla M., Ph.D.	Co-principal Investigator	12	25				
Kershner, Mark F., M.B.A.	Finance	12	10.0				
TBD	Technician II	12	50				

Category	TOTAL
CONSULTANT COSTS Smithies, Olivier, Ph.D.	
EQUIPMENT *(Itemize)* Electro Cell Manipulator™ 630 $5,399 MJ Research Chromo4 $28,000	
SUPPLIES *(Itemize by category)* Consumables $ Enzymes $ Cell culture Media $ Sequencing $ Primers $	
TRAVEL	0
PATIENT CARE COSTS — INPATIENT	0
PATIENT CARE COSTS — OUTPATIENT	0
ALTERATIONS AND RENOVATIONS *(Itemize by category)*	0
OTHER EXPENSES *(Itemize by category)*	

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	0
	FACILITIES AND ADMINISTRATIVE COSTS	0
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* →		$
SBIR/STTR Only: FEE REQUESTED		

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (*from Form Page 4*)	ADDITIONAL YEARS OF SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*						0
CONSULTANT COSTS						0
EQUIPMENT						0
SUPPLIES						0
TRAVEL		0				0
PATIENT CARE COSTS	INPATIENT	0				0
	OUTPATIENT	0				0
ALTERATIONS AND RENOVATIONS		0				0
OTHER EXPENSES		0				0
SUBTOTAL DIRECT COSTS						0
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	0				0
	F&A	0				0
TOTAL DIRECT COSTS						0
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)*						
SBIR/STTR Only Fee Requested						
SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period (Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.)						

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel:

Jamie Cearley, Ph.D., Principal Investigator, (50% effort) will be responsible for the day-to-day organization and running of the project, especially with regards to the tissue culture needs, ES manipulation and vector modification.

Rusla DuBreuil Ph.D., Co-principal Investigator, (25% effort), will oversee the BAC modification and homologous recombination, as well as all chromosomal and molecular evaluations. She will also maintain quality assurance and ensure the potential customer usability of the final products.

Mark Kershner, M.B.A., (10% effort), will administer the funds on a weekly basis, ensuring that accurate documentation is routinely completed and submitted, bills are paid and a budget is maintained.

Research assistant, TBD (50% effort), will be responsible for maintaining the daily tissue culture requirements and carry out basic molecular manipulations to alter the vectors. This person will also assist in the more complex manipulations and techniques.

Consultant costs:

Oliver Smithies, Ph.D., will provide advise, collaboration and guidance to the overall project and some of the molecular specifics. As Dr. Smithies conceptualized the idea of using 3'UTRs to modulate transgene expression [1], he would be an obvious and useful ally to the proposed agenda.

Equipment:

Electro Cell Manipulator™ 630; although there is already an electroporator available at Open Biosystems, it will be used for bacterial manipulation during the BAC modification processes and thus would not be suitable to carry out the ES cell electroporation/manipulations as well, due to the potential/likely contamination of the ES cell culture facility with bacteria. Thus an additional electroporator dedicated to tissue culture would be a requirement.

MJ Research Chromo4; real-time quantitative PCR will be carried out to evaluate the efficacy of the 3'UTR modulation of gene expression. Open Biosystems already has in its possession numerous MJ Research thermal cyclers, which can be upgraded to carry out real-time PCR with just the addition of a chromo4 bonnet and software.

No additional equipment is required for the completion of phase II.

Supplies:

$14,850 is requested for the basic tissue culture and molecular modulation requirements, as well as vector verification by sequencing. Open Biosystems will provide any additional supplies required.

Fee:

A fee of 7% of total costs (direct and indirect) is requested ($8,474). This fee contributes to the growth of the small business concern by allowing expansion of resources and personnel development. The fee is consistent with a normal profit margin provided for research and development work.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Jamie A. Cearley	Research Scientist

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Lindenwood College, St. Charles, MO.	B.S.	1992	Biology
Southern Illinois University, Edwardsville, IL.	M.S.	1994	Biology
University of Alabama at Birmingham, AL.	Ph.D.	2001	Biochemistry and Molecular Genetics
University of Alabama at Birmingham, AL.			Postdoctoral Training

OFFICE ADDRESS: Open Biosystems, Inc.
6705 Odyssey Dr.
Huntsville, AL 35806
Telephone: (256) 704-4848
Fax: (256) 704-4849
Email: jcearley@openbiosystems.com

Research Experience/ Professional Appointments:

November 2003 – present: Research Scientist at Open Biosystems Inc., Research and Development Department, Huntsville Alabama.
Development and implementation of mouse transgenic technologies. Establishing methodologies, quality control procedures and production processes for the distribution of gene targeting vectors, transgenic mice and embryonic stem cells.

December 2001 – October 2003: Postdoctoral Fellow with Peter J. Detloff, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL.
Use of a mouse model to create an adult knockout of the wild type Huntington's Disease Gene.

January 2003 – June 2003: Adjunct Professor at Samford University.
Instructor for Human Biology laboratory/class for non-biology majors.

September 1994 – November 2001: Graduate student in the laboratory of Peter J. Detloff, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL.
Devised a method to repeatedly target the Huntington's Disease (HD) locus. Characterization (molecular and phenotypic) of an HD knockout mouse and other models of trinucleotide repeat diseases.

July 1992 – August 1994: Masters Thesis, Department of Biology, University of Illinois, Edwardsville, IL.
Regeneration of *Solanum tuberosum* cv. Katahdin from leaf explants *in vitro.*

Publications:

Cearley, J.A. and P. J. Detloff (2001) Efficient repetitive alteration of the mouse Huntington's Disease gene by management of background in the tag and exchange gene targeting strategy. Transgenic Research 10, 479-488.

Lin C.H., S. Tallaksen-Greene, W. Chien, J. A. Cearley, W. S. Jackson, A. B. Crouse, S. Ren, X. Li, R. L. Albin and P. J. Detloff (2001) Neurological Abnormalities in a Knock-in Mouse Model of Huntington's Disease. Hum. Mol. Gen. 10, 137-144.

Ordway, J. M., J. A. Cearley, and P. J. Detloff (2001) Insights from mice carrying X-linked CAG-polyglutamine repeat mutations. In Glutamine Repeat s and Neurodegenerative Diseases: Molecular Aspects. P. S. Harper and M.F. Perutz eds. Oxford University Press.

Ordway, J. M., J. A. Cearley, and P. J. Detloff (1999) CAG-polyglutamine repeat mutations: independence from gene context. Phil. Trans. R. Soc. Lond. 354, 1083-1088.

Ordway, J. M., S. Tallaksen-Greene, C.-A. Gutenkunst, J. A. Cearley, E. M. Bernstein, H. W. Wiener, L. S. Dure IV, R. Lindsey, S. Hersch, R. S. Jope, R. L. Albin and P. J. Detloff (1997) Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. Cell 91, 753-763.

Cearley JA and Bolyard MG (1997) Regeneration of *solanum tuberosum* cv. Katahdin from leaf explants *in vitro* American Potato Journal 74:125-129.

Completed Research Support:

Hereditary Disease Foundation, Postdoctoral Fellowship - 2002 – Creation of an adult knockout of the wild type Huntington's Disease Gene.

Invited presentations:

University of Alabama, Birmingham Industry Roundtable – 2004 – Working For A Biotech Start-Up: The Ins and Outs.

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Du Breuil, Rusla M., PhD	Research Scientist

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Faculty of Science, University of the Witwatersrand, South Africa	BSc	1884	Science
Department of Genetics, University of the Witwatersrand, South Africa.	BSc (Hon)	1985	Genetics
Department of Molecular medicine and Haematology, School of Pathology, University of the Witwatersrand, South Africa Faculty Board approved conversion to PhD	MSc		Hematology
Department of Molecular Medicine and Haematology, School of Pathology, University of the Witwatersrand, South Africa	Ph.D.	2000	Molecular Medicine

OFFICE ADDRESS:

Open Biosystems, Inc.
6705 Odyssey Dr.
Huntsville, AL 35806
Telephone: (256) 704-4848
Fax: (256) 704-4849
Email: rusla@openbiosystems.com

Research Experience/ Professional Appointments:

July 2002- Present: Research Scientist, Open Biosystems, Inc. Development and implementation of transgenic technologies. Establishing methodologies, quality control procedures and production processes for the distribution of gene targeting vectors, transgenic mice and embryonic stem cells. Execution of high throughput systems and automation for molecular processes, such as cloning, PCR and tissue culture. Involvement in the setup and quality assessment of various expression-arrest systems e.g. Drosophila RNAi, morpholino's, and human and mouse shRNAs.

January 2003 – Present: Adjunct Associate Professorship, University of Alabama, Huntsville.

July 2001 –June 2002: Permanent Staff Member, Research Genetics, Invitrogen Corporation. Engaged in the research and development of several products related to molecular biology (commercial feasibility and patent applications filed and/or in progress – company confidential). Inventor of technologies disclosed in two families of patent applications.

January 2001 – July 2001: Postdoctoral fellowship and visiting scientist at Research Genetics, Invitrogen Corporation. Research and development on microarrays designed to detect molecular lesions indicative of diagnostic and prognostic factors in leukemia and lymphoma; as well as input in various other projects such as molecular bar-coding, chromosomal band specific probes and certain nested PCR methodologies.

1996 – 2000: Head of the Research and Development Program, Department of Molecular Medicine and Haematology, University of the Witwatersrand. Executed and supervised the implementation of molecular

techniques used in research projects and routine diagnosis and devised novel methods to achieve these goals especially in a developing country. Obtained and administered funding for Research and Development. Continued teaching duties stated below.

1988 – 1995: Medical Scientist, Leukaemia Research Unit, University of the Witwatersrand and the South African Institute for Medical Research (SAIMR). Taught undergraduate medical and science curricula as well as post-graduate lecturing, tutoring and supervision of Bsc, BSc(Hons), MSc, MMed and PhD projects. Developed and taught short molecular techniques courses for medical personnel. Designed novel primers for xeno-competitive PCR, sold commercially by Research Genetics, Inc. Alabama, USA:
β-Actin: XAHR 17, catalogue number: M502.10
XAHR 20, catalogue number: M502.11.

1986 – 1988: Assistant Researcher – Microbiologist, Council for Scientific and Industrial Research, South Africa, Division of Water Technology, Biotech Program. Research and Development of processes to biodegrade ore-bearing wood chips using vermiculture, anaerobic and aerobic techniques; biodegradation by micro-organisms of the polymer in drilling mud; luxury phosphate removal in waste processing plants.

Publications:

Richard L. Davis, Jr., Rusla M. DuBreuil, Shanker P. Reddy, Thomas P. Dooley. Epidermal Cells Methods and Protocols. Humana Press, Editor Turksen, Kursad. Methods for Gene Expression Profiling in Dermatology Research Using DermArray® Nylon Filter DNA Microarrays. October 2004, Chapter 38, pp. 399-412

C. Urbani, R. Dubreuil Lastrucci and B. Kramer. Sexing of Heat Treated Molars from Cadavers using DNA PCR. Journal of Forensic Odontostomatology 1999; 17(2), 35-39.

Rusla M Dubreuil Lastrucci, Debbie A Dawson, James H Bowden* and Marion Münster.
*Molecular Pathology, University of Virginia, Charlottesville, VA, USA.
Development of a Simple Multiplex PCR for the Simultaneous Detection of the Factor V Leiden and the Prothrombin 20210A Mutations.
Molecular Diagnosis, 1999, 4, (3), 247-250.

Rusla M Dubreuil Lastrucci, Debbie Dawson, Marion Münster. Development of an Internal Restriction Control in the PCR Detection of the Methylenetetrahydrofolate Reductase (MTHFR) C677T Mutation.
Molecular Diagnosis, 1999, 4 (2), 159-161

Rusla M Dubreuil Lastrucci, Debbie Dawson, Marion Münster. Development of an Internal Restriction Control in the PCR Detection of the Prothrombin 20210 A Mutation.
Clinical and Laboratory Haematology, 1999, 21(4) 281-283.

Rusla M Dubreuil Lastrucci, Wendy S Stevens, Barry V Mendelow. Extension of a Cold -Labelled Oligoprobe to Analyse Polymerase Chain Reaction Products.
Technical Tips Online. 5/8/98. http://tto.trends.com. T01419

R. Du Breuil, B. Mendelow. Quantitation of Specific mRNA Transcripts Using Xeno-Competitive PCR. PCR Methods and Applications, 1993, 3, 57-59.

Abstracts cited in International Journals:

R M Dubreuil Lastrucci, B V Mendelow. Molecular Medicine, the Next Millennium and the Developing World. The Association for Molecular Pathology Annual Meeting, St Louis, 1999.
Journal of Molecular Diagnosis, November 1999 p60, O3.

Lastrucci Rusla, Dawson Debbie, Münster Marion
Development of an internal restriction control in the PCR detection of the Prothrombin 20219A mutation.

American Journal of Pathology, 1998, 153 (5), 1648, G2.

Stevens W, Stevens G, Sherman G, Du Breuil R, Mendelow B.
The feasibility of a routine molecular diagnostic laboratory: A third world experience.
British Journal of Haematology, 1998, 102,1, 293

Rusla M D Lastrucci, N P Carter*, T L Coetzer and B V Mendelow
*Sanger Centre, Wellcome Trust Genome Campus, UK
Identification of Cancer Associated Chromosomal Translocations Using Differentially Labeled Chromosomes and Boolean Logic.
Blood, November, 1997, 90 (10) supplement 1, 217b

Invited presentations:

Hudson Biotechnology Seminar Series – Molecular Diagnostics: A Coming of Age. 20 February 2004.

Overview of research in the Department of Molecular Medicine and Haematology, SAIMR South Africa. Molecular Pathology Program, Department of Pathology, Georgetown University Medical Center, Washington DC, USA. 1999.

Molecular Diagnostics, Technology for a Developing Country. Department of Haematology, University of the Orange Free State, Bloemfontein, September 1998.

PCR Symposium. Boehringer Mannheim, South Africa, May 1995.
Poster topics:

Rusla MD Lastrucci and Marianne Pooler. Internal PCR controls for TB PCR.
Rusla MD Lastrucci and Marianne Pooler. Reverse Dot Blots using Non-Radioactive Methods to identify Mycobacterium.
Rusla MD Lastrucci and Marianne Pooler. ‘Spoligotyping’ to identify Mycobacterial strains.

CML PCR
Non-radioactive in situ Hybridisation Symposium, Boehringer Mannheim, South Africa, 18 August 1994.

Ongoing Research Support:

- NIH
  “Development of a Genome-wide Drosophila RNAi Resource”
  Principle Invstigator: Rusla M DuBreuil
  Agency: National Institutes of Heath
  Type: STTR grant (1 R41 HG003258-01), 2004 - 2006
  The objective of this project is to provide DNA and RNA constructs to facilitate specific gene inactivation in *Drosophila Melanogaster* via RNAi.

Completed Research Support:

- CANSA
  - 2001 for the analysis of cord blood using cell sorted populations, RNA and microarrays to elucidate why some babies born to HIV mothers don’t get AIDS.
  - 2001 for the analysis of CD19+ cells from various children with ALL to determine the cause of the difference in their disease progression.
- Freda and David Becker Trust Fund (for research in cancer in the aged)
  - 1998/9 for Boolean PCR
  - 2000 for telomeres and telomerase
  - 2000 for microarray technology
- CANSA – travel grant, 2000
- CANSA (Cancer Association of South Africa) - 1997 and 1998 for Boolean PCR

- SAIMR (South African Institute for Medical Research) 1996 - 2000
    - Haematology R and D
    - Boolean PCR
- Medical Faculty Research Endowment Fund
    - 1993 – Serum Modulation of Gene Expression
    - 1996 – Boolean PCR
    - 1999 – Determination of Sex using Skeletal Remains
- Stella and Paul Lowenstein Trust Fund Travel Awards.

Patents:

Inventor of two technologies disclosed in two families of patent applications filed with the United States Patent and Trademark Office (Invitrogen - company confidential).

Resources

Laboratory:

Open Biosystems will provide over 7,400 sq. ft. of molecular biology laboratory space for the development and processing of molecular constructs required to perform the described 3'UTR application. This space is currently equipped with ample electrical, air handling and lighting for standard laboratory operations. The laboratory has Milli-RO water purification equipment immediately accessible. Separated chemical and autoclave rooms comprise an additional 250 sq.ft. These rooms provide isolated areas for preparation of sterile medias, solutions, antibiotics and the treatment of biological waste. Currently in place are biological safety hoods and laminar flow cabinets as well as a Sorvall refrigerated high-speed centrifuge, microfuges, thermal-cyclers and a gel-documentation system.

Additionally, Open Biosystems maintains two cell culture laboratories with a shared preparation area comprising over 700 sq. ft. These rooms are maintained under limited access protocols and contain all necessary equipment for cell-based projects, including liquid nitrogen containers, five biological safety cabinets, three CO_2 incubators, inverted microscopes (Leitz Inverted epi-fluorescence phase contrast microscope), micro-manipulation capabilities, clinical centrifuge, electroporator and water baths.

Computer:

Open Biosystems has a broad range of computing infrastructure ranging from more traditional information technology (IT) systems to laboratory information systems. The IT and bioinformatics staff is composed of expertise in network administration, systems administration, security, software development and database administration.

Open Biosystems' data center has employed both physical and network security to protect its assets. Open Biosystems' network is connected to the Internet via a redundant fiber ring to maximize availability and uptime. Open Biosystems' business systems are Microsoft based, and are set on a foundation of Great Plains eEnterprise. In some instances, due to the unique nature of the products and services, Open Biosystems' programmers develop custom applications to meet these requirements. An internally developed e-commerce system that allows for ultimate flexibility when launching a new resource or product is utilized and is also used to develop our internal laboratory information management systems (LIMS). These open source platforms provide the greatest compatibility and broadest support for the research community that Open Biosystems serves. Both Drs. Cearley and DuBreuil and the technician on this project (TBH) have Windows-based Intel PCs.

Office:

Open Biosystems personnel have access to over 1000 sq. ft. of office space, adjacent to the laboratories, all under one roof. The office space is provided with appreciable amounts of lighting, air conditioning and desk space. Additionally, all scientists have access to the phone system and data connections.

All computer and laboratory equipment is located at Open Biosystems' facilities in Huntsville, AL.

Major equipment:

Tecan Genesis RSP100
Tecan Genesis 200
Two Tomtec Quadra96
One MJ Research PTC200s & three MJ Research Dyads thermal cyclers
UVP bio-imaging system GDS 8000
Leitz Inverted epi-fluorescence phase contrast microscope

Two Laminar flow hoods
Five Biological safety cabinets
Three CO_2 incubators
Electro Cell Manipulator VT600 (Bacterial use only, due to contamination issues)
Leica Microscope w/Eppendorf Transferman NK DMRIB
Isolation Table w/ Electrical Cover VH3036T-OPT
Inverted Microscope Zeiss
Cryogenic Storage Unit K Series
Two Leica Microscope MZ6
BioRad Fraction Collector 2110
Dionex AD20 Absorbance Detector (Plate reader)
Dionex ED40 Electrochemical Detector
Dionex GP40 Gradient Pump
Genevac Evaporator System Mega-980
5 orbital shaker incubators (New Brunswick Scientific)
Forty -80°C freezers
Leica DM IRB (for optimized flrorescence, visualization, software for quantitation)

A. Specific aims

An understanding of gene function and the effects of mutation are paramount to the discovery of disease treatments. A growing number of scientists are turning to transgenic mouse technology to gain this understanding. A qualified NCBI PubMed search from 1994-2003 around transgenic mice shows that the number of publications has increased each year with an overall increase in excess of 3-fold. The consequential increase in the number of transgenic animals has provided a wealth of biological information in many areas including diabetes and aging [2-4]. At the same time, many of these animals have highlighted the complexity of the genome and the need for a high standard in designing a mouse model in order to ensure biologically relevant results. Problems associated with unexpected effects of selectable markers, position effects, strain background, lack of ability to compare animal models to each other, and the inability to control gene expression levels are all common problems associated with today's transgenic animals [5-10]. Therefore, improved tools are needed that provide efficient targeting strategies offering predictable and comparable control over properties such as expression level, genome location, and strain background.

In order to meet these requirements, it is proposed to make a set of tools consisting of BAC-based targeting vectors and embryonic stem (ES) cell lines that will allow the researcher to alter the expression level of any transgene over a 100-fold range while maintaining control over marker effects, position effects and strain background. Using this one tool set, a user can create a single transgene, series of transgenes, or series of mutant alleles all expressing at predetermined levels and in a manner that allows the transgenes to be compared directly to one another. The availability of these types of standardized reagents provides a cost effective, time saving way for laboratories to enter this field of research with a high degree of likelihood that they will acquire meaningful results.

The core technology that will make this level of control possible is the modulation of mRNA stability using defined 3' untranslated region (UTR) moieties in conjunction with site-specific integration at the drug selectable hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene locus, both of which have been pioneered by the laboratory of Dr. Oliver Smithies [1, 11]. The following specific aims are proposed:

Specific aim 1: **Conversion of an existing plasmid-based vector system into one that is BAC-based.** This conversion will alleviate strain limitations as well as increase the ease and efficiency of modification as is already dictated by the current vector system.

- Five plasmid-based targeting vectors obtained from the laboratory of Dr. Oliver Smithies (Department of Pathology, University of North Carolina) will be converted to five new BAC-based vectors. During phase I the functional components of the five plasmid-based targeting vectors including a generic promoter (human β-actin), hrGFPn (humanized renilla Green Fluorescence Protein with a nuclear localization signal) reporter, 3' UTR, and the human promoter and exon 1 of the *Hprt* gene will be transported into a truncated *Hprt* containing BAC clone (modified RP23-173F3). Each vector contains a unique 3' UTR that will provide a defined level of expression. Collectively, the five vectors will deliver predictable levels of expression over a 100-fold range.
- The newly created hrGFPn containing BAC vectors mentioned above will be altered so that the hrGFPn reporter is removed, and replaced with a selection/counter selection marker (kanamycin/streptomycin) so that a sequence of interest can be easily substituted at the position the hrGFPn once occupied by a single step removal of the selection/counter selection marker, leaving the promoter, 3' UTR and *Hprt* components intact.

Specific aim 2: Creation of a BAC-based test vector that allows the end user to place the 3' UTR of their gene of interest (GOI) behind the hrGFPn reporter in a single step. This vector can be used to test the expression level of their GOI with respect to the range available with this system.

- During phase I, the 3' UTR in one of the BAC constructs will be exchanged for a selection/counter-selection marker (kanamycin/streptomycin). This will provide an efficient method for the end user to incorporate the 3' UTR of their GOI in a single step via homologous recombination in *Escherichia coli* (*E. coli*) (recombineering).
- The test vector will be validated by replacement of the selection/counter selection marker with the SV40+ 3' UTR followed by gene targeting. The SV40+ 3' UTR was chosen because in the plasmid-based system it produced expression levels toward the middle of the range of the overall system. The targeted ES cell line will then be assayed for hrGFPn expression on both the mRNA and the protein level as compared to the original complimentary ES cell line created via the plasmid-based targeting vector.

Specific aim 3: Functional testing of the newly created BAC vectors in ES cells.

- The five hrGFPn containing BAC vectors generated in phase I will be targeted to *hprt* negative ES cells from strain 129/Ola.
- Real-time PCR as well as a quantitative hrGFPn reporter assay using either a microtiter plate reader or computer software combined with fluorescent microscopy will be used to test the integrity of the vectors as compared to their predecessors.

The following products will be available at the end of phase I: five BAC targeting vectors each providing for expression of a transgene at a known level covering a 100-fold expression range as well as a test vector to determine the natural expression level of the end user's GOI with regard to the range of the system.

Plans for Phase II expansion:

During phase II, four ES cell lines from strains of mice other than 129/Ola will be converted to *hprt* negative by a gene targeted deletion of the promoter and exon 1.

- The four additional strains of ES cells will include: 129sv/Jae x 129sv/Jae, 129sv/Jae x BALB/c, C57Bl/6 x C57Bl/6, and 129sv/Jae x C57Bl/6 [12].
- The use of *hprt* negative ES cell lines provides a powerful selection strategy. The lacking *Hprt* promoter and exon 1 that are provided by the incoming BAC construct accomplish restoration of *Hprt* function. This approach provides for a high degree of targeting efficiency [11].

Also during phase II the repertoire of 3' UTRs carried in the BAC vectors will be expanded as dictated by results obtained during phase I. This could encompass as many as the full set of 28 originally described and tested as plasmid vectors by Dr. Oliver Smithies' laboratory as well as other potential modulators of gene expression.

B. Background and Significance

Today, the employment of forward genetics to the discovery of new therapies for disease is proving to be a powerful approach. Transgenic mouse technology is being used to an ever-increasing degree to gain an understanding of gene function and the effects of mutation. Over expression as well as ectopic expression of a transgene has many times led to important discoveries [2-4]. However, transgenic mouse technology is not without its obstacles. Historically, pronuclear injection of DNA has been the method of choice in creating a transgenic mouse. Using this approach a DNA fragment containing the GOI is injected into the pronucleus of a developing embryo where it randomly integrates into the genome. Each animal born from an integration event is referred to as a founder. There are many problems inherent in

this technique that are well reviewed by Palmiter *et al* [7]. These problems include chromosomal deletions, inversions, and duplications which are common events occurring during integration [13]. These uncontrolled events can lead to unforeseen mutations in nearby genes making biologically relevant results unlikely. Random integration also leads to variable levels and patterns of expression in the founder animals. This is most often due to concatameric insertion or position effects [14]. Transgenics created by pronuclear injection may also exhibit mosaic expression of the transgene due to variegation, methylation, or intrachromosomal recombination within the transgene concatamer [15-18]. All of these possibilities require the analysis of multiple founders in order to increase the likelihood of meaningful results. There have been many attempts to correct for some of these problems such as the addition of locus control regions to the transgene, the use of insulator elements, and Cre-loxP to control for copy number [19-21]. In spite of these efforts there is at present no standard method of creating a transgenic mouse that will provide a high level of control over gene expression and gene locus that is efficient, reproducible, and will enable the comparison of one transgenic mouse directly to another. It would be of tremendous benefit to the mouse community to have a standardized system that is simple enough for the novice to utilize, yet powerful enough to answer the complex questions of specialists, where the GOI is controlled in every aspect by purposeful planning rather than chance. This proposal seeks to provide this standardized system by marrying what are perhaps three of the most powerful technologies available to date: targeted transgenics, 3' UTR modification and BAC recombineering.

Dr. Sarah Bronson first described targeted transgenics in 1996 [11]. This technique seeks to overcome many of the problems inherent in pronuclear injection by targeting a single copy transgene to a known locus with a high degree of efficiency. Dr. Bronson chose the *Hprt* locus to conduct her experiments. This locus has two fundamental characteristics that make it an ideal choice for targeted transgenesis. First, the *Hprt* gene lies on the X-chromosome and is drug selectable in both a positive and negative fashion. Therefore, in male ES cells that are hemizygous for *Hprt*, the gain or loss of *Hprt* function can be directly selected. Dr. Bronson developed a selection strategy that takes full advantage of this characteristic. She capitalized on a spontaneous deletion of the *Hprt* promoter and exon 1 by developing a targeting construct that would repair this deletion while simultaneously bringing in a transgene upstream. Because the transgene does not independently carry a functional selection marker there is virtually no background resulting from random integration. Additionally, since the original mutation is a large deletion, it is not spontaneously revertible. These two factors provide a powerful combination for the selection of homologous recombinants. The second characteristic making *Hprt* an ideal choice is that it is a housekeeping gene involved in the purine salvage pathway. This makes it unlikely that chromatin effects will impact the expression of a transgene inserted at that locus. Indeed, there are many examples of transgenes targeted to the *Hprt* locus that have shown endogenous expression patterns at endogenous levels [22-27].

Other loci are also amenable to targeted transgenesis, such as the one where the Rosa 26 gene trap lies. This locus has the properties of being on an autosome, a history of being gene targeted, and location in an open chromatin domain [28] . Additionally, there are loci that naturally exhibit high efficiencies of homologous recombination such as Rb and activin/inhibinβB [29, 30]. Furthermore, other selection strategies have proven to increase targeting efficiency such as using promoterless targeting vectors that are unlikely to express the drug selectable marker at sufficient levels to provide resistance when randomly integrated [31-33]. These systems and others each have their advantages and have provided a large degree of success in transgenesis. The *Hprt* locus has been chosen in this application because of its extensive history, selection advantages, and simplicity of use. There is no need to excise a marker in an additional step as with targeting at the Rosa26, Rb and activin/inhibinβB loci, the targeting efficiency equals or surpasses that of any other strategy, and no extraneous sequences are left behind in the final experimental

allele. Additionally, having a transgene on the X-chromosome provides for a natural mosaicism in heterozygous females that is not present in either hemizygous males or homozygous females. This may prove useful in studies where the overall expression of the transgene is lethal or where information on the effects of the transgene in specific tissues is desired. The culmination of all these attributes makes *Hprt* an ideal locus to pursue refined control of transgene expression using 3' UTR modification.

The 3' UTR of a gene has been shown to modulate mRNA stability [34]. Sufficient detail is known about the different elements of 3' UTRs such as, GU/U- rich sequence elements (GREs), AU-rich elements (AREs), stem loop destabilizing elements (SLDEs), and polyadenylation signals (polyAs) to envisage their effects on mRNA stability [35-38]. Oliver Smithies' laboratory used this body of knowledge to develop a novel plasmid-based vector set that would allow the researcher to predict and control the expression level of a transgene at a fixed locus using 3' UTR modifications [1]. His laboratory created targeting plasmids for the *Hprt* locus consisting of a human β-actin promoter driving an hrGFPn reporter followed by 28 natural or modified 3' UTRs. Since the vectors are standardized as a single copy transgene at a targeted locus, measurements of the hrGFPn fluorescence in ES cells can be used to stringently compare the effects of the various 3' UTRs. He further shows that this change in protein level correlates directly to the level of mRNA expression. By measuring the expression level of the reporter in ES cells it was determined that these 3' UTRs provided for a graded level of expression over a 100-fold range. Dr. Smithies went on to show that this 3' UTR modulation of expression level held true in ES cells that had undergone differentiation and in transgenic animals. His vector set can be used to increase or decrease the expression level of a transgene at a fixed locus with a level of precision and utility beyond what is currently available. A test vector was also developed that contains the human β-actin promoter driving the hrGFPn reporter with a cloning site for positioning a 3' UTR from a GOI. This test vector allows for the placement of a GOI under the range of expression control available with this system, thereby enabling the researcher to determine the utility of this technology for particular studies. While Dr. Smithies' set of constructs provides for a generally applicable and very powerful approach to the study of gene function, the use of the current vector set is somewhat limited. The number of restriction enzyme sites available for conventional cloning into the vectors is constrained. For example, cloning of a 3' UTR of interest into the test vector by restriction enzyme digestion and ligation is limited to a single *Mlu*I site. Additionally, the plasmids contain short arms of homology to the *Hprt* gene that are isogenic to the 129 strain of mouse. This makes it unlikely that these vectors will be able to target ES cells derived from strains other than 129 at a high degree of efficiency [29, 39]. These two limitations can be overcome by converting the plasmid-based vectors to those that are BAC-based.

There are two main advantages to a BAC-based system. Through the use of BAC recombineering, precise sequence alterations can be made to BACs with great ease through the enlistment of phage homologous recombination machinery in *E. coli* [40, 41]. Using this system, alterations can be made to sequences without any limitations such as restriction site availability and position, a common problem encountered when creating a targeting vector, as is the case with the single *Mlu*I site for 3' UTR positioning mentioned above. Second, unlike the smaller homology regions of plasmids, the much longer regions of homology that BACs provide permit homologous recombination to occur in strains of mice that are non-isogenic to the BAC [42]. This opens the door for use in many mouse strains. The demand and ability to use strains of mice other than 129 for transgenic production is growing. For example, many researchers are employing the use of tetraploid embryo complementation for the production of their transgenic mice, a technique that requires strains other than inbred 129 [12]. Furthermore, the US National Institute of Environmental Health Sciences is currently funding a project to sequence the genomes of 15 additional mouse strains [43]. Inbred mouse strains differ in their susceptibility to common diseases, behavior, and physiology. The ability to capitalize on these differences when designing a

transgenic mouse model would provide flexibility to study a disease or physiological system in a background with defined traits [44].

It is proposed to merge the use of targeted transgenics, modulation of expression level through the use of 3' UTR's, and BAC recombineering to generate and make available to the community a standardized series of vectors for generating transgenic mice. These vectors will make it possible to control the expression level of a transgene in virtually any strain of mouse to levels previously not possible. This technology also provides the potential for making reliable comparisons between animals with differing mutations due to the generic nature of the controlling elements involved in combination with targeted transgenics. A hypertension study conducted by Cvetkovic *et al* provided the proof-of principle for using targeted transgenics to assess a series of allelic variants. Cvetkovic states:

> ”We demonstrate that insertion of the single copy transgene upstream of *Hprt* does not affect the overall tissue- and cell-specific expression or hormonal regulation of human angiotensinogen (AGT). This study provides an important proof-of-principal that the functional significance of allelic variation in human AGT can be assessed by examining mice carrying transgenes targeted in a single copy to an identical insertion site [22].”

There are approaches other than 3' UTR modification available to the researcher who seeks to modify the expression of a transgene. Conditional alleles are one method of altering gene expression. While this system provides the researcher with a level of temporal control not available with the system proposed here, conditional alleles are either 'on' or 'off' [45]. No range of expression is provided as with the 3' UTR modification system. Another method for altering gene expression is gene duplication. In this case a gene is duplicated at its endogenous locus along with its regulatory elements [46, 47]. The ability to regulate gene expression at the endogenous locus, including all the natural controlling elements, is an appealing quality of this approach that is not present in the system proposed. One limitation of gene duplication is in the length of the region that can be duplicated, meaning it is not likely to be practical for larger genes. Another is that the genes expression level can be increased by a maximum of 4-fold (viz. two copies at each locus of a homozygous animal). While it is likely that the system proposed in this application will not be utilizable for every gene, the size of a gene is not a limiting factor as with the gene duplication method, since cDNAs can be used. Additionally, expression level increases of over 100-fold have been observed with the 3' UTR system.

Open Biosystems has many products on the market that would compliment this BAC vector set. These products include four strains of ES cells, three lines of embryonic mouse fibroblasts for use as feeder cells, mouse BAC libraries, cDNA libraries, and ORF libraries. Our clone collections contain greater than 11,000 mouse full-length cDNAs or ORF clones mapping to some 9,974 Unigene clusters, representing approximately 30% of the mouse genome. These clone sequences can be easily incorporated into the BAC-based targeting vectors, increasing the utility of this system as a whole.

In summary, it is envisaged that a set of tools will be provided to the researcher that will enable them not only to test the expression level of their GOI, but also to modulate its expression in many strains of mice.

C. Relevant Experience

The principle investigator for the proposed research project will be Dr. Jamie Cearley. Dr. Rusla Du Breuil will assist the project as co-principle investigator. Dr. Oliver Smithies will participate as an advisor/consultant. Dr. Tim Townes (Chair Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham) has also provided his support for the project. A research assistant will help with experimental design and execution. The qualifications of the investigators are listed below.

Principal Investigator

Dr. Cearley (Open Biosystems) has 10 years of experience in mouse transgenics and draws on a vast theoretical and practical knowledge base to generate targeting vectors, transgenes, knock-outs and mouse models. Dr. Cearley graduated from Lindenwood College with a B.S. degree and from Southern Illinois University with a M.S. degree. She completed her Ph.D. at the University of Alabama, Birmingham, Department of Biochemistry and Molecular Genetics, where she also carried out her Postdoctoral studies. While at UAB Dr. Cearley mastered blastocyst injection and gene targeting to engineer multiple transgenic mouse lines. She optimized a gene targeting system using an *Hprt* selection strategy by which the Huntington's disease gene locus could be efficiently and repetitively targeted in mouse embryonic stem cells and characterized neurological abnormalities at a behavioral level in mice. After producing a classical knockout mouse of the Huntington's disease gene, Dr. Cearley engineered a recombinant protein used to generate an inducible knockout of the mouse Huntington's disease gene. During 2003 she was an Adjunct Professor for Samford University, teaching Human Biology to non-biology majors. Since coming to Open Biosystems in the fall of 2003 she has successfully brought products such as commercially available ES cells and feeder cells to market.

Unique areas of expertise that define Dr. Cearley's accomplishments include:

- Cloning (bacterial and mammalian), library screening, PCR, Real Time-PCR, gene targeting in mouse embryonic stem cells, blastocyst injection, Northern blotting, Southern blotting.
- Western blotting, recombinant protein expression in bacteria, immunoprecipitation.
- Primary neuronal, mammalian cell and plant tissue culture.
- Sectioning of brain tissue, antibody staining using HRP, DAB, and fluorescent visualization.
- Mouse handling, chimera production via blastocysts injection, live surgery, dissection, perfusion, stereotaxic injection, behavioral analysis. Maintenance of mouse colony containing greater than thirty lines.

Co-Principal Investigator

Dr. DuBreuil (Open Biosystems) has a broad professional history that extends between the research and clinical settings. Much of her work focused on the development of reproducible, robust methodologies that can be implemented in the field under stringent cost control. Her clinical training and the use of many animals as model organisms (such as drosophila, mouse and baboon), allows Dr. Du Breuil to evaluate technologies and develop tools for researchers to use these model organisms. Dr. Du Breuil has a strong background in developing products that require the optimization of protocols for the recurrent production of samples, and for their use in a wide variety of field conditions, often by non-expert operators. It is this type of thought process that will be applied to the development of the targeted transgenic tools proposed in this application. Dr. Du Breuil has experience in leading research and development efforts that have resulted in multiple commercial product releases at Open Biosystems, and previously Research Genetics (Invitrogen Corporation). These successes include the generation of the commercially available ES cells and feeders available from Open Biosystems, their quality assessment and preliminary production experiments. Additionally, her guidance over the Open Biosystems manufacturing team has scaled the generation of PCR products for use in custom microarray production from 14,400 to 86,400 per month. These capacity increases came through the implementation of good manufacturing practices, SOP driven processes, quality control vigilance, automated template setup, efficient scheduling and the continual improvement of methodologies.

Unique areas of expertise that define Dr. Du Breuil's accomplishments include:

- Experience in mouse IVT, and the cryostorage of mouse germline tissue and embryos.
- Experience in gene targeting, conditional knockout generation, and tissue specific promoter usage in mouse ES cells. As well as mouse husbandry, facility management and diagnostics.
- The use of RT-PCR (reverse transcriptase polymerase chain reaction) to quantify mRNA expression using novel competitor templates.
- Various PCR innovations for disease diagnosis and prognosis in leukemia and thromboembolic disorders, whilst insuring quality control and accuracy. These include the design of non-radioactive methods to validate PCR products, the development of internal restriction enzyme controls to assist in quick and accurate mutation analysis and the development of a simplified multiplex PCR allowing the simultaneous, cost-effective detection of more than one mutation and real-time PCR.
- The development of a method for the accurate sexing of heat damaged teeth (such as that which occurs in catastrophic events), using PCR and other molecular techniques.
- The use of complex molecular methodology to isolate, identify and characterize, chromosomal translocations involved in the generation of neoplasia. The use of Boolean logic and a matrix format allows the elucidation of these chromosomal translocations on a global basis, without any prior knowledge of the molecular lesion involved.
- The use of gene expression microarray technology and non-radioactive methodology to analyze the gene expression patterns in chronic lymphocytic and chronic myeloid leukemias (CLL and CML).
- The elucidation of telomere dynamics with regard to the progression of CML and CLL disease states.

Research Assistant (to be hired):
The research assistant that will be required to fulfill certain tasks within the project, should preferably have a Masters degree in Science, with strong molecular biological and tissue culture experience. ES cell culture experience would be an advantage. The research assistant would be required to do bacterial culturing, plasmid and BAC vector modifications and agarose gel analyses. Additionally the candidate would participate in the general ES cell culture requirements, and assist in the ES manipulation and targeting.

D. <u>Research Design and Methods</u>

Overview of Experimental Plan

The following aspects of construction are summarized in Figure 1.
An *Hprt* containing BAC clone will be modified by replacing the promoter and exon 1 region with a selection/counter selection marker. An existing set of five targeting vectors in plasmid form will be verified to contain the β-actin promoter, hrGFPn, a designated 3' UTR, the human *Hprt* promoter, and human *Hprt* exon 1. These functional components will then be exchanged for the selection/counterselection marker in the *Hprt* BAC. Next, the selection/counter selection marker will be re-inserted; however this time it will replace the hrGFPn reporter specifically. This will create a vector that will enable the end user to replace the marker with their GOI sequence in a single step. Additionally, one of the five newly created BAC vectors containing the functional components including the hrGFPn reporter will have the 3' UTR region replaced with the selection/counter selection marker in order to create a test vector where endogenous 3' UTRs of interest can be placed for testing their specific level of expression. The following is a proposed timeline for the completion of the specific aims outlined for phase I (Figure 2).

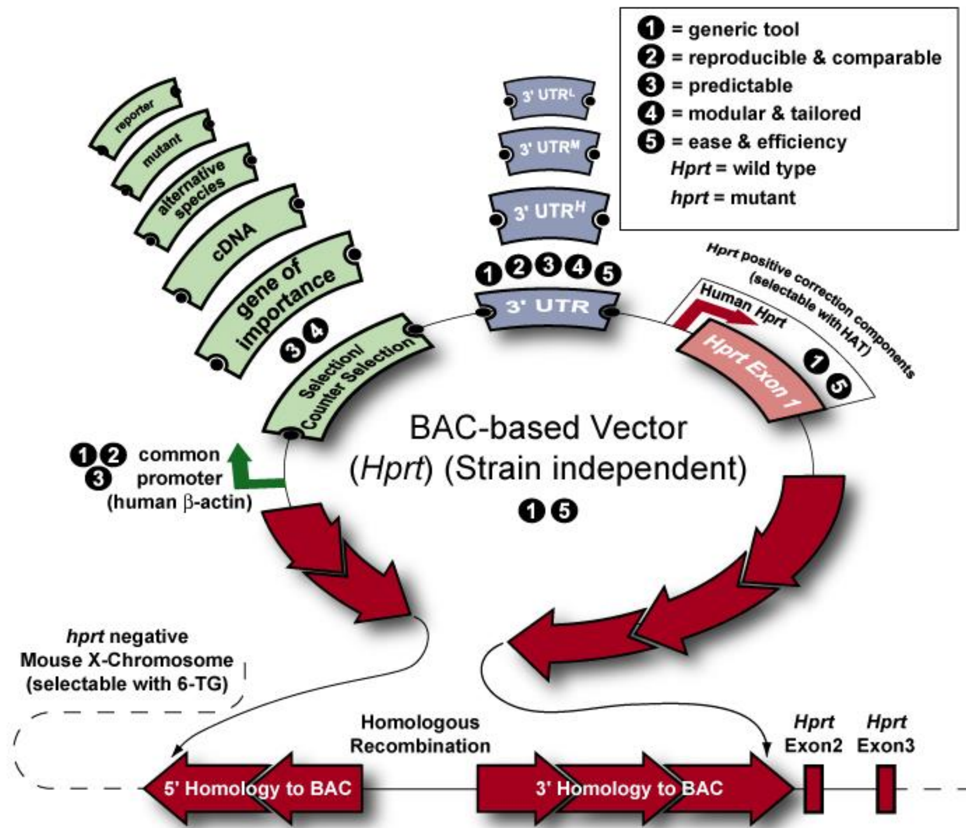

Figure 1. Summary schematic showing the various components of the BAC-based vector system and their respective advantages as well as potential uses.

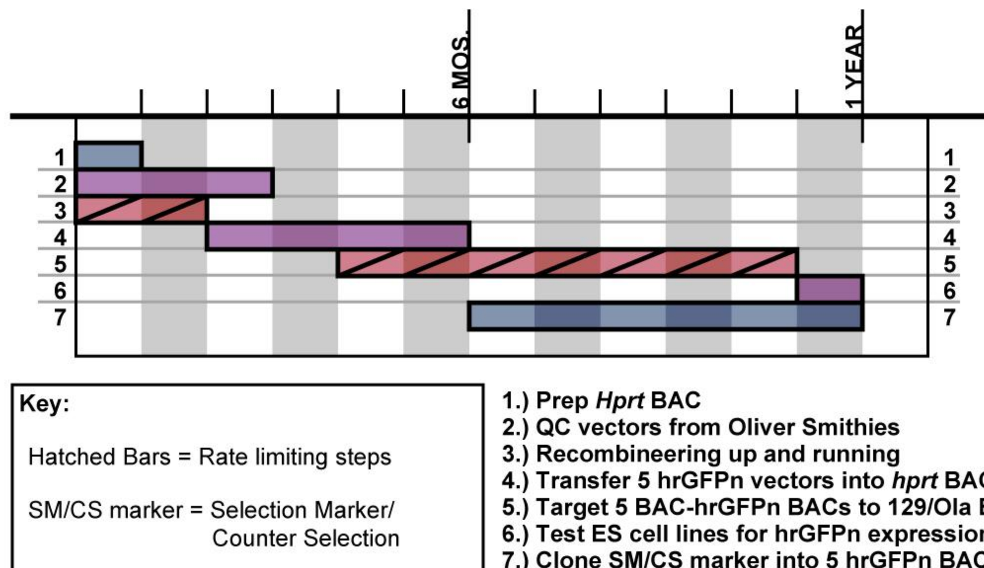

Figure 2. Timeline for completion of phase I specific aims.

Introduction to *Hprt* gene correction as a method for high efficiency gene targeting. The method used in this proposal to accomplish high efficiency gene targeting was originally described by Dr. Sarah Bronson [11]. *Hprt* is an endogenous gene that is directly selectable in both a positive and negative fashion. Proper *Hprt* function can be selected positively using HAT media and negatively with the addition of 6-thioguanine (6-tg). Dr. Bronson used this selectable property to develop a system which first made use of a spontaneous 55kb deletion in the *Hprt* gene that includes the promoter and exon 1, thereby rendering it dysfunctional and selectable with 6-tg (Figure 3a and b). Next, a targeting vector was designed that contained the necessary components to repair the *hprt* gene while simultaneously transporting in sequences of interest upstream (Figure 3c). Those cells correctly targeted were selected with HAT media. The original mutation, a large deletion, is irreversible. The correction of the gene by homologous recombination is made possible only by double crossover. These two properties ensure that background from non-homologous recombination or random integration, the largest obstacle to finding homologous recombinants, is virtually absent. An unprecedented 96% of the colonies resulting from HAT selection are correctly targeted using this strategy [11]. An additional benefit of this system is that the targeting event does not leave behind a selectable marker or require later excision with Cre leaving a loxP site in the sequence. We know from studies on other genes that the presence of selectable markers in a locus can have profound effects on the phenotype of the resulting line [5, 6, 9, 10]. Because of this, many researchers now delete the marker after the targets are selected via the Cre-loxP system. However, evidence suggests the Cre-loxP technique may also cause undesired alterations at other endogenous sites in the mouse genome that share homology to loxP sites [8]. The presence of such sites could lead to unwanted and undetectable deletions of various portions of the genome when Cre is expressed. The *Hprt* correction mechanism of selection leaves no marker sequences behind and does not require Cre expression, thereby providing a pure insertion of the transgene. The spontaneous deletion utilized in Dr. Bronson's studies has been recreated via gene targeting [48]. It is proposed to recreate this deletion in strains of ES cells other than 129/Ola during phase II, thereby opening the door for the expanded use of this technology.

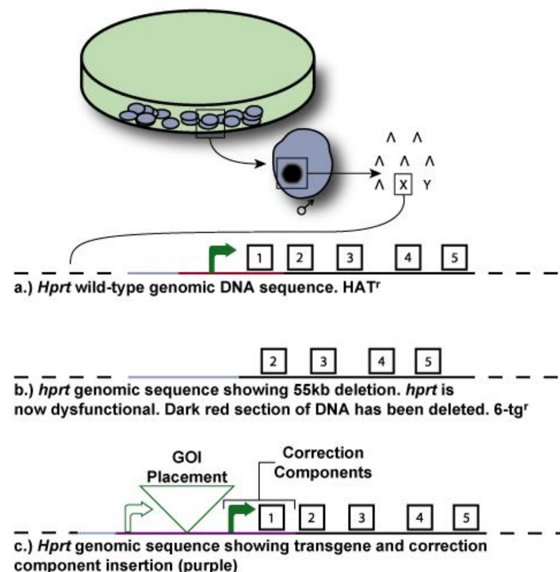

Figure 3. a.) Wild type *Hprt* locus of a male ES cell. b.) *hprt* locus showing the spontaneous 55kb deletion rendering the cell 6-tg^r. c.) *Hprt* locus showing the correction of the *Hprt* gene along with the insertion of a GOI upstream. The insertion of the correction components renders the cell HAT^r. 6-tg^r - 6 thioguanine resistant, HAT^r - HAT resistant.

Specific aim 1: **Conversion of an existing plasmid-based vector system into one that is BAC-based.** As a first step to this conversion process, all plasmid-based vectors obtained from the laboratory of Dr. Oliver Smithies will be verified using restriction enzyme digestion and, when necessary, DNA sequencing. Each vector should contain the β-actin promoter, hrGFPn, a designated 3' UTR, the human *Hprt* promoter, and human *Hprt* exon 1. The specific 3' UTRs that will be used and their relative expression levels are shown in figure 4 (Figure 4). A sufficiently annotated *Hprt* containing BAC (RP23-42J4) of C57Bl/6 origin obtained through Roswell Park will be used as a backbone to create the BAC targeting vectors. A Selection/Counter Selection BAC engineering kit available from Gene Bridges GmbH., will be used to modify the BAC. The kit utilizes the technology of recombineering via Red®/ET® driven homologous recombination in *E.coli*. The direct placement of a DNA fragment of interest into a BAC via recombineering is accomplished by designing two primers that flank the fragment to be inserted. These primers will have tails extending beyond the primer binding region that contain sequences homologous to the region of insertion into the BAC (Figure 5). The primers are then used to create a PCR product representing the insert. The *E.coli* containing the BAC to be modified are transformed with a Red®/ET® plasmid containing an exonuclease, DNA binding protein, and an inhibitor of the BCD system, all of phage origin. This plasmid, when induced by arabinose and a temperature shift will provide the components needed to drive homologous recombination in the *E.coli* while inhibiting the bacterial

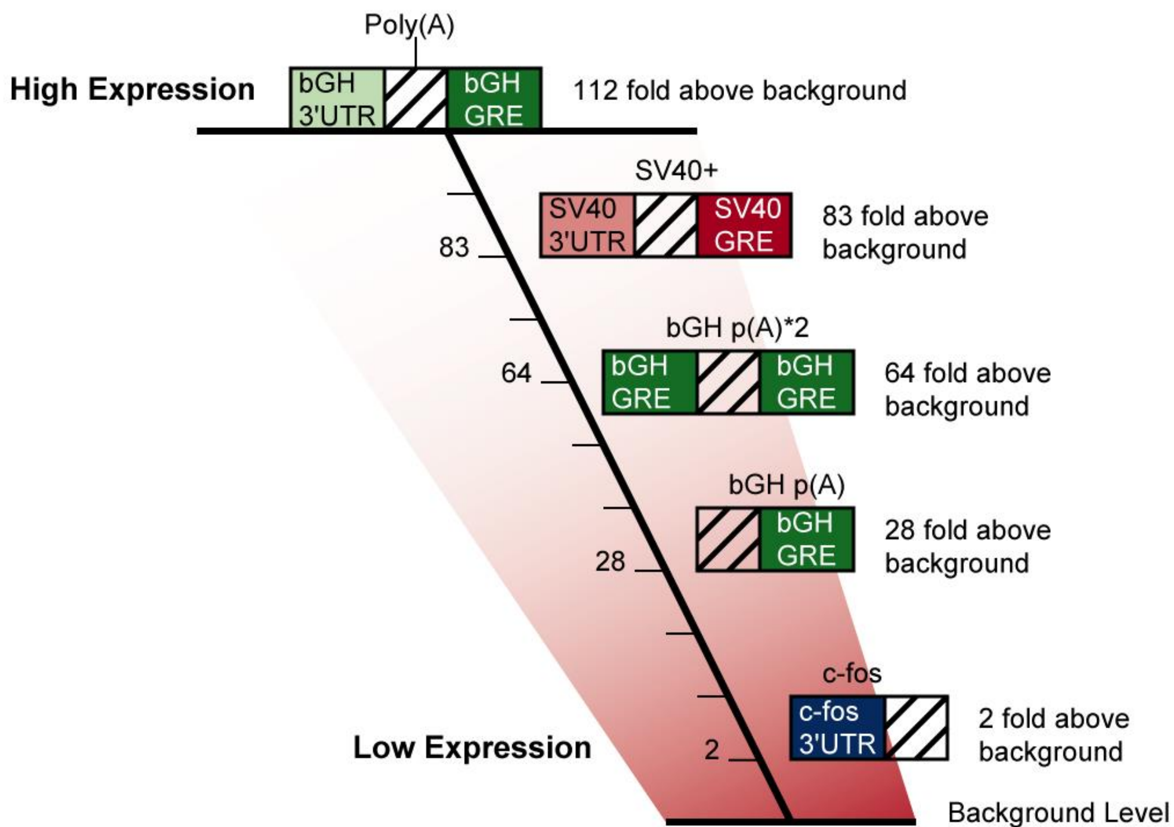

Figure 4. The five 3' UTRs that will be used and their relative expression levels. bGH- bovine growth hormone, GRE-GU/U-rich sequence elements.

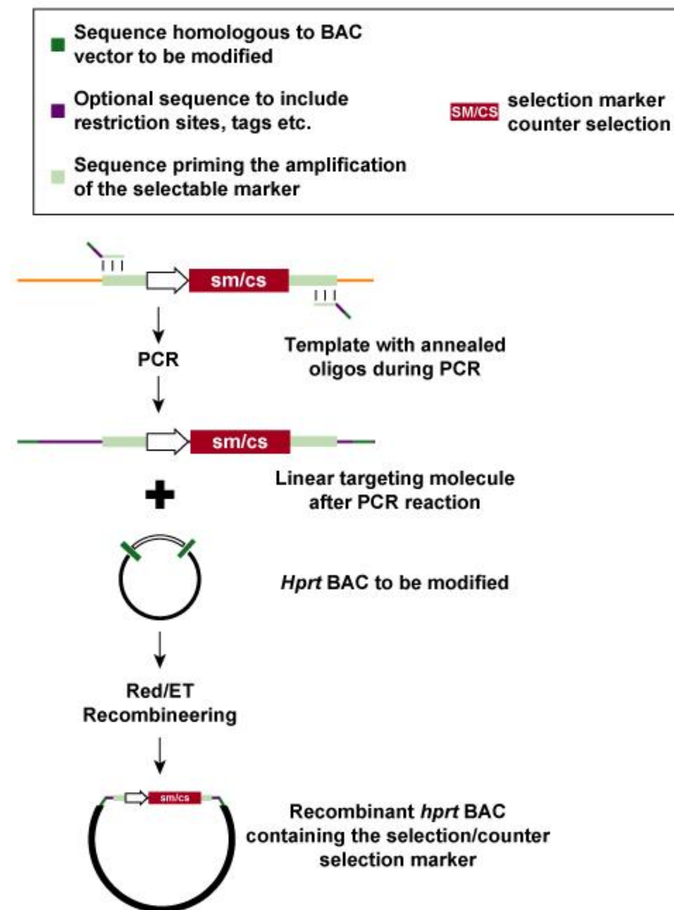

Figure 5. Insertion of the selection/counter selection marker into the *Hprt* BAC via recombineering.

exonuclease. The Selection/Counter Selection BAC kit makes use of a marker that contains both a kanamycin resistance gene for selection and a streptomycin sensitivity gene for counter selection. This allows the researcher to place sequences of DNA into a BAC that are not directly selectable. This is done by first inserting the selection/counter selection marker into the region where modification is desired. The resulting cells will be selected for with kanamycin resistance. In a second step, the selection/counter selection marker is exchanged for the sequence of interest leaving behind only those changes desired. The resulting cells are thus rendered streptomycin resistant due to the absence of the counter selection marker.

Stage 1. Insertion of the selection/counter selection marker into the *Hprt* BAC. The *E.coli* containing the *Hprt* BAC will first be tested for streptomycin sensitivity. Since streptomycin will serve as the counter selection marker it is important that the host bacteria are streptomycin sensitive otherwise the correct clones will not be recoverable from the background. This next step involves primer design, PCR, and incorporation of the selection/counter selection (kanamycin and streptomycin) marker into the *Hprt* BAC (Figure 5). Once the targeting PCR product is obtained it will be co-electroporated, along with the Red®/ET® plasmid, into *E.coli* containing the *Hprt* BAC. The Red®/ET® system will then be induced with arabinose and the bacteria incubated at 37°C. The Red®/ET® plasmid carries a tetracycline resistance marker while the selection/counter selection marker contains a kanamycin resistance marker. Consequently, the cells will be plated onto kanamycin/tetracycline containing plates with no arabinose and grown at 30°C. The switch in temperature from 37°C to 30°C as well as the removal of arabinose will shut down the recombination machinery preventing any further recombination events. DNA

minipreparation followed by restriction enzyme analysis and/or PCR will be used to confirm the successful integration of the selection/counter selection marker. In addition, positive clones will be streaked onto streptomycin plates to confirm their sensitivity and get an idea of possible background levels for the next step. This step is important since the marker has been generated via PCR making the chance of a mutation rendering the gene dysfunctional a distinct possibility that must be guarded against. Only those clones that are sensitive to streptomycin will be used further. The most common problem encountered with this procedure is the failure to select homology arms for the primers that match 100% to the BAC sequence to be targeted. If necessary the pertinent sections of the BAC clone will be re-sequenced to ensure that exact matches can be made. At this point an *Hprt* BAC vector with the selection/counter selection marker positioned at the promoter and exon1 will be ready for exchange with non-selectable incoming sequence.

Stage 2. Transfer of the functional components of the plasmid-based vectors to the *Hprt* BAC thereby removing the selection/counter selection marker and conferring streptomycin resistance. With a similar approach to Stage 1, PCR primers will be designed that flank the generic promoter, hrGFPn, and 3' UTR sequences in the plasmid-based vector. The sequence of these primers will be extended to have tails that represent regions of homology to the *Hprt* BAC that flank the outside of the selection/counter selection marker (Figure 6). The *E.coli* will still have the Red®/ET® plasmid, which has been maintained at 30°C with no arabinose, re-induced by addition of arabinose to the media and a temperature shift to 37°C. After induction, the cells will be prepared for electroporation with the promoter, hrGFPn, and 3' UTR containing PCR fragment. Only those colonies that have had the selection/counter selection marker replaced with the β-actin promoter, hrGFPn, and 3' UTR will grow on streptomycin. DNA minipreparation followed by restriction enzyme analysis and/or PCR will be used to confirm the successful integration. At this point five vectors that have had the selection/counter selection marker exchanged for the β-actin promoter, hrGFPn, designated 3' UTR, human *Hprt* promoter, and human *Hprt* exon 1 will have been completed.

Stage 3. Replacement of the hrGFPn reporter with the selection/counter selection marker. In this stage the hrGFPn containing BAC vectors will be altered so that the hrGFPn reporter is removed, and replaced once again with the selection/counter selection marker so that a sequence of interest can be easily substituted at the position the hrGFPn once occupied. The marker will be replacing the hrGFPn reporter sequence specifically, leaving the generic promoter and 3' UTR in place so that when a GOI is inserted it will be under the control of the promoter and 3' UTR of the vector. This stage will be conducted in the same fashion as stage 1 with the exception of primer choices. It is worth noting the ease with which a series of alleles could be created using these vectors. The series could be designed to express each allele at equal levels, or at levels of predictable variation. Each allele would be directly comparable to the next enabling powerful and accurate assessments to be made regarding the effects of a mutation or the quantity of protein. All relevant components of these vectors will be sequence verified. Upon completion of specific aim 1 five vectors containing the human β-actin promoter, selection/counter selection marker, unique 3' UTR, human *Hprt* promoter and exon 1 will be available. Using these vectors the researcher can easily exchange their GOI for the selection/counter selection marker.

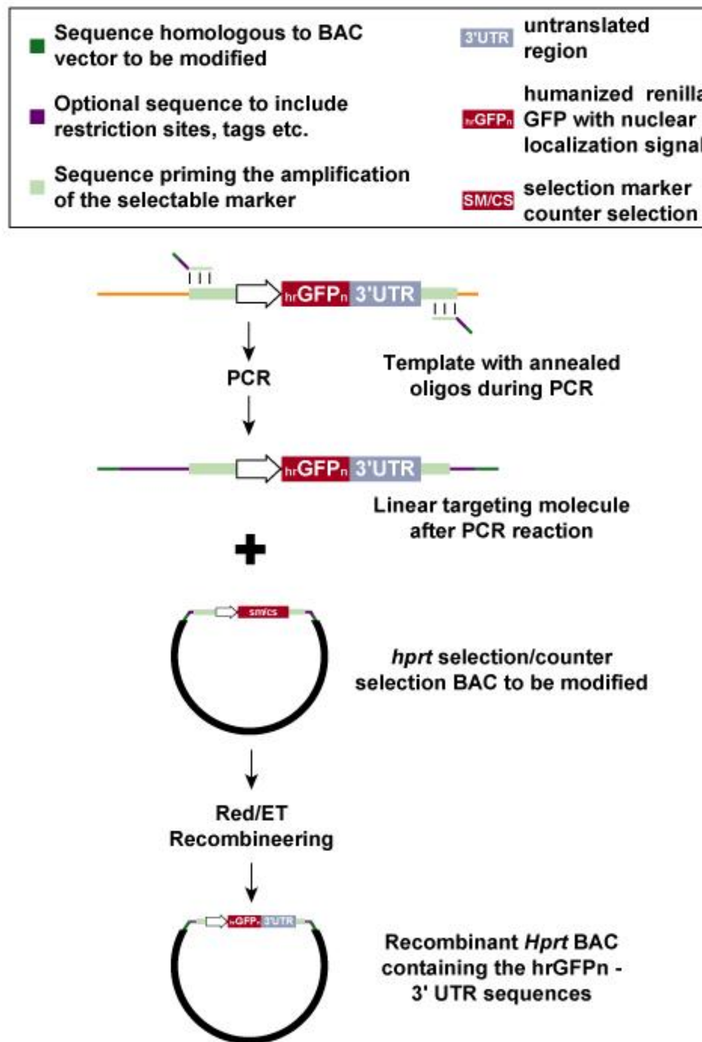

Figure 6. Replacement of the selection/counter selection marker with the hrGFP + 3' UTR reporter sequences via recombineering.

Specific aim 2: Creation of a test vector that allows the end user to place the 3' UTR of their gene of interest (GOI) into the system in a single step. The highest level of expression obtained using the original plasmid-based vector system was found using the bovine growth hormone (bGH) 3' UTR/bGH GRE and the lowest level with the c-fos 3' UTR [1]. There was a 100-fold difference in expression of hrGFPn when these two 3' UTRs were compared. While this range of influence is impressive there is a point of caution: If an end users' GOI were endogenously expressed at a level above that seen with hrGFPn+bGH 3' UTR/bGH GRE it would be futile to use this system in an attempt to increase the expression level of that GOI. To help aid the end user in making these judgments a test vector will be constructed that will allow the placement of the 3' UTR of their GOI behind the hrGFPn reporter in a single step. This will enable the researcher to directly compare the endogenous expression of their GOI to that of the various 3' UTRs. Also provided, will be ES cells expressing the hrGFPn reporter with the various 3' UTRs to aid this direct comparison. Construction of the test vector will proceed by replacing the 3' UTR region of one of the hrGFPn control BACs with the selection/counter selection marker via recombineering as described above. This test vector will be evaluated by incorporating the 3' UTR SV40+ (Figure 4) followed by restriction digest with *Sal*I and targeting of *hprt* negative 129/Ola ES cells. The

hrGFPn expression levels of targeted ES cells will then be compared to ES cells targeted with the plasmid-based hrGFPn vector from Dr. Oliver Smithies containing the 3' UTR SV40+. The results from this test will confirm that the selection/counter selection marker can be replaced with a 3' UTR of interest and expression of hrGFPn obtained. Levels of hrGFPn expression are expected to be closely comparable between the two ES cell lines, one from the BAC targeting and the other from the plasmid-based targeting. If the results differ significantly between the two cell lines it will remain acceptable so long as a consistent graded range of expression is established using the hrGFPn expressing BAC vectors with the varying 3' UTRs in specific aim 3. It is not necessary to obtain the exact range of expression seen by Dr. Smithies so long as a sufficient range of expression is obtained. Upon completion of specific aim 2, five experimental BAC vectors allowing insertion of a GOI will be constructed, and 1 test vector allowing insertion of a 3' UTR sequence to be tested, will be constructed and tested.

Specific aim 3: **Functional testing of the newly created BAC vectors in ES cells.** All newly created BAC vectors will have the functional components sequence-verified. The five hrGFPn containing vectors will be restriction digested with *Sal*I, releasing the BAC insert. Each vector will then be independently electroporated into 129/Ola *hprt* negative ES cells (E14Tg2a) in order to verify the gene targeting strategy and its efficiency. The E14Tg2a ES cells have been used numerous times in conjunction with the *Hprt* correction mechanism for successful gene targeting [22-25]. Colonies will be picked and expanded after 12 days under HAT selection. A PCR assay will be used to screen for homologous recombinants. The primers will flank the insertion site. Since *Hprt* is on the X-chromosome and the ES cells are male the absence of the smaller native PCR product along with the appearance of the larger insertion product will strongly indicate that gene targeting has occurred. ES cells targeted using the original plasmid-based targeting vectors and *hprt* negative 129/Ola ES cells will be used as controls.

Those clones that are confirmed to be gene targeted will be analyzed for hrGFPn mRNA expression using Real-Time PCR. In order to correlate mRNA levels with protein levels the targeted clones will be further analyzed for hrGFPn protein levels using a cell lysate reader or software combined with fluorescent microscopy. *Hprt* negative 129/Ola ES cells will be used as a control for background fluorescence as well as background mRNA expression. To make sure the system has retained the level of performance originally shown using the plasmid-based system by Dr. Oliver Smithies, those ES cells targeted with the BAC-based hrGFPn vectors will be directly compared to ES cells targeted with the plasmid-based hrGFPn vectors. With the functional components of the newly created BAC vectors having been sequence verified, in combination with the rigorous comparison of GFP expression levels on both the mRNA and protein levels to Dr. Smithies targeted ES cells, the continued integrity of the BAC-based system will be ensured. This testing is necessary in order to definitively show the expression level that the researcher can expect to achieve using our vectors. While we expect the expression levels to be very consistent to that produced by the plasmid-based vectors, a degree of unexpected difference in no way negates the utility of the system, so long as the levels are defined.

Further testing of the systems functionality in transgenic animals is not within the timeframe of phase I. With this said, Dr. Smithies has shown that the level of gene expression control provided by 3' UTR modification that is seen in ES cells is faithfully sustained in the mouse [1]. Furthermore, the differences between the BAC-based vectors and the original plasmid-based vectors are present only in the pre-target stage. After gene targeting has occurred, there should be no functional difference between an ES cell targeted with a BAC-based vector vs. a plasmid-based vector. There is the exception of potential for polymorphic differences between an ES cell line generated from a plasmid-based vector and one generated from the BAC-based vector. This is due to the plasmid backbones origination from the 129 strain of mouse while the BAC vector originates from C57Bl/6J. While these differences are not likely to

affect function, all ES cell lines will be germline tested for continued functionality in the mouse during phase II. At this point it bears restating that the advantages of the BAC-based vectors, include ease of manipulation, speed, and strain versatility far outweigh the issues mentioned above. Upon completion of phase I the five experimental BAC vectors and the test vector will have been completed and tested in ES cells.

Plans for phase II expansion: To expand the use of the BAC targeting vectors created during Phase I, ES cell lines from the following strains will be converted to *hprt* negative via gene targeting: 129sv/Jae x 129sv/Jae, 129sv/Jae x BALB/c, C57Bl6/J x C57Bl6/J, and C57Bl6/J x 129sv/Jae. Both inbred and hybrid lines are included. The hybrid lines once converted to *hprt* negative, will be the first of their kind available to the research community, making tetraploid embryo complementation compatible with the use of *Hprt* as a selectable marker. Although *hprt* negative cell lines from strain 129 are available to the academic community there is no commercial source for these ES cells nor are any of them from the 129sv/Jae strain to our knowledge. Open Biosystems currently has possession of these four ES cell lines originally acquired from the laboratory of Dr. Rudolf Jaenisch at the Whitehead Institute [12]. All four ES cell lines currently carry a functional *Hprt* gene conferring resistance to HAT medium.

Also during phase II the repertoire of 3' UTRs carried in the BAC vectors will be expanded according to the needs indicated by phase I results; up to the full set of 28 tested by Dr. Oliver Smithies' laboratory. Investigation of new 3' UTRs or further modification of these 28 by the addition of control elements or modification of miRNA binding elements all hold the potential for fine tuning expression levels.

The following is a description of the final products anticipated by harnessing the innovative technologies of targeted transgenesis at the *Hprt* locus, BAC modification via recombineering, and gene expression modulation via 3' UTR moeities:

Tool Set Components:

- 5-28 *E.coli* cultures containing a BAC targeting vector with a unique 3’ UTR sequence and a selection/counter selection marker positioned for single step replacement by a GOI. These vectors span over a 100-fold range of hrGFPn expression.
- 5-28 ES cell lines expressing hrGFPn under the control of a 3’UTR complimentary to the BAC targeting vectors supplied.
- 1 test BAC vector with the selection/counter selection marker positioned in the area of the 3’ UTR for single step placement of a test 3’ UTR from the end user's GOI.
- 1 *hprt* negative germline competent ES cell line of choice from the following strains: 129sv/Jae x 129sv/Jae, 129sv/Jae x BALB/c, C57Bl/6 x C57Bl/6, and 129sv/Jae x C57Bl/6

Available Separately:

- Five *hprt* negative ES cell lines from the following strains: 129sv/Jae x 129sv/Jae, 129sv/Jae x BALB/c, C57Bl/6 x C57Bl/6, and 129sv/Jae x C57Bl/6. Each sold independently for general use in gene targeting.

E. Human Subjects

No human subjects are involved in this project.

F. Vertebrate Animals

There are two areas of this proposal that require the housing and use of mice. Both of these requirements will be outsourced. No animals will be housed at Open Biosystems. The first area is the requirement for embryonic mouse fibroblasts for use as feeder cells in the culture of ES cells. Open Biosystems is currently housing mice for this purpose at Charles River Laboratories and will continue this practice. The second area of animal requirement lies in the germline testing of embryonic stem cell lines during phase II. Open Biosystems currently plans to outsource germline testing to a reputable company such as Ozgene.

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H. Contractual Arrangements

None.

I. Consultants

Dr. Oliver Smithies has agreed to provide consultation to Open Biosystems (see letter of support from Dr. Smithies). Dr. Smithies pioneered gene targeting, site-specific-integration, and modification of transgene expression by 3' UTR modification.

Principal Investigator/Program Director (last, First, Middle): Cearley, Jamie A.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☒ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant __________ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant __________ ☐ STTR Fast Track

☐ REVISION of application number: __________

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: __________

(This application is to extend a funded grant beyond its current project period.)

☐ SUPPLEMENT to grant __________

(This application is for additional funds to supplement a currently funded grant.)

INVENTIONS AND PATENTS
(Competing continuation appl. and Phase II only)

☐ No ☐ Previously reported

☐ Yes. If "Yes," ☐ Not previously reported

☐ CHANGE of principal investigator/program director.
Name of former principal investigator/program director: __________

☐ FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)
NONE		

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/ certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and

•Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☐ DHHS Agreement dated: NONE ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with __________ Regional Office.

☐ No DHHS Agreement, but rate established with __________ Date __________

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential*

a. Initial budget period:	Amount of base $ ______	x Rate applied ______	% = F&A costs $ ______
b. 02 year	Amount of base $ ______	x Rate applied ______	% = F&A costs $ ______
c. 03 year	Amount of base $ ______	x Rate applied ______	% = F&A costs $ ______
d. 04 year	Amount of base $ ______	x Rate applied ______	% = F&A costs $ ______
e. 05 year	Amount of base $ ______	x Rate applied ______	% = F&A costs $ ______
			TOTAL F&A Costs $

*Check appropriate box(es):

☐ Salary and wages base ☐ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*

4. SMOKE-FREE WORKPLACE ☒ Yes ☐ No *(The response to this question has no impact on the review or funding of this*

Place this form at the end of the signed original copy of the application.
Do not duplicate.

PERSONAL DATA ON PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitor the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director.

To provide the PHS with the information it needs for this important task, complete the form below and attach it to the signed original of the application after the Checklist. **Do not attach copies of this form to the duplicated copies of the application.**

Upon receipt of the application by the PHS, this form will be separated from the application. This form will **not** be duplicated, and it will **not** be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant/Contract Information)." The PHS requests Social Security Numbers for accurate identification, referral, and review of applications and for management of PHS grant programs. Provision of the Social Security Number is voluntary. No individual will be denied any right, benefit, or privilege provided by law because of refusal to disclose his or her Social Security Number. The PHS requests the Social Security Number under Sections 301(a) and 487 of the PHS Acts as amended (42 U.S.C 241a and U.S.C. 288). All analyses conducted on the date of birth and race and/or ethnic origin data will report aggregate statistical findings only and will not identify individuals. If you decline to provide this information, it will in no way affect consideration of your

DATE OF BIRTH (*MM/DD/YY*)	06/12/70	SEX/GENDER
SOCIAL SECURITY NUMBER	416-21-5109	☒ Female ☐ Male

ETHNICITY

1. Do you consider yourself to be Hispanic or Latino? (See definition below.) Select one.

Hispanic or Latino. A person of Mexican, Puerto Rican, Cuban, South or Central American, or other Spanish culture or origin, regardless of race. The term, "Spanish origin," can be used in addition to "Hispanic or Latino."

☐ **Hispanic or Latino**

☒ **Not Hispanic or Latino**

RACE

2. What race do you consider yourself to be? Select one or more of the following.

☐ ***American Indian or Alaska Native.*** A person having origins in any of the original peoples of North, Central, **or** South America, and who maintains tribal affiliation or community attachment.

☐ ***Asian.*** A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian **subcontinent**, including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific

☐ ***Black or African American.*** A person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black" or African American."

☐ ***Native Hawaiian or Other Pacific Islander.*** A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or **other** Pacific Islands.

☒ ***White.*** A **person** having origins in any of the original peoples of Europe, the Middle East, or North Africa.

☐ Check here if you do not wish to provide some or all of the above information.