PI: **MURPHY, CHERYL L**	FOA: PA06-006	Council: 05/2006
Received: 11/30/2005	FOA Title: SMALL BUSINESS INNOVATION RESEARCH PROGRAM PARENT ANNOUNCEMENT	Accession Number: 2913411
2 R44 AI060275-02A1	IPF: 10002385	Former Number:
Dual: EB		
IRG/SRG: **ZRG1 IDM-Q (10)B**	Delivery Location:	

APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION

❍ Pre-application ● Application
❍ Changed/Corrected Application

4. Federal Identifier
2 R44 AI060275-02

5. APPLICANT INFORMATION *** Organizational DUNS:** 1523533760000

* Legal Name: RiboNovix, Inc.
Department: Division:
* Street1: 8 Farrar Rd. Street2:
* City: Lincoln County: * State: MA * ZIP Code: 01773
* Country: USA

Person to be contacted on matters involving this application

Prefix:	* First Name:	Middle Name:	* Last Name:	Suffix:
	Alison		Taunton-Rigby	PhD

* Phone Number: 781-259-3183 Fax Number: 781-259-3183 Email: alison@ribonovix.com

6. * EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)*:
51-0495417

7. * TYPE OF APPLICANT
O: Small Business

8. * TYPE OF APPLICATION: ❍ New
● Resubmission ❍ Renewal ❍ Continuation ❍ Revision

Other (Specify):

Small Business Organization Type
❍ Women Owned ❍ Socially and Economically Disadvantaged

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)*:

9. * NAME OF FEDERAL AGENCY:
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
93.061
TITLE: Innovations in Applied Public Health Research

* Is this application being submitted to other agencies? ❍ Yes ● No
What other Agencies?

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Identification of E. coli anti-infective rRNA targets

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*
MA, MI, CA

13. PROPOSED PROJECT:

* Start Date	* Ending Date
07/01/2006	06/30/2008

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant	b. * Project
MA-007	MA-007

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix:	* First Name:	Middle Name:	* Last Name:	Suffix:
	Cheryl	Isaac	Murphy	PhD

Position/Title: Vice President, Technology Development * Organization Name: RiboNovix, Inc.
Department: Division:
* Street1: 8 Farrar Rd. Street2:
* City: Lincoln County: * State: MA * ZIP Code: 01773
* Country: USA
* Phone Number: 781-259-3183 Fax Number: 781-259-3183 * Email: cheryl@ribonovix.com

16. ESTIMATED PROJECT FUNDING	
a. * Total Estimated Project Funding	$2,055,875.00
b. * Total Federal & Non-Federal Funds	$2,055,875.00
c. * Estimated Program Income	$0.00

17. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

18. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● * I agree

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

19. Authorized Representative

Prefix:	* First Name:	Middle Name:	* Last Name:	Suffix:
	Cheryl	Isaac	Murphy	

* Position/Title: Vice President, Technology Development

* Organization Name: RiboNovix, Inc.

Department:

Division:

* Street1: 8 Farrar Rd.

Street2:

* City: Lincoln

County:

* State: MA

* ZIP Code: 01773

* Country: USA

* Phone Number: 781-259-3183

Fax Number: 781-259-3183

* Email: cheryl@ribonovix.com

*** Signature of Authorized Representative**

Cheryl Murphy

*** Date Signed**

11/30/2005

20. Pre-application File Name: Mime Type:

424 R&R and PHS-398 Specific
Table Of Contents

RESEARCH & RELATED Project/Performance Site Location(s)

Project/Performance Site Primary Location

Organization Name: RiboNovix, Inc.

* Street1: 8 Farrar Rd.		Street2:		
* City: Lincoln	County:	* State: MA	* Zip Code: 01773	* Country: USA

Project/Performance Site Location 1

Organization Name: RiboNovix, Inc.

* Street1:		Street2:		
* City: Lexington	County:	* State: MA	* Zip Code: 02421	* Country: USA

Project/Performance Site Location 2

Organization Name: Wayne State University

* Street1: 5047 Gullen Mall		Street2:		
* City: Detroit	County:	* State: MI	* Zip Code: 48202	* Country: USA

Project/Performance Site Location 3

Organization Name:

* Street1:		Street2:		
* City:	County:	* State:	* Zip Code:	* Country: USA

Project/Performance Site Location 4

Organization Name: Harvard Medical School/National Screening Laboratory

* Street1: 250 Longwood Ave.		Street2: SGM 610		
* City: Boston	County:	* State: MA	* Zip Code: 02115	* Country: USA

File Name	Mime Type

Additional Location(s)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 04/30/2008

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ❍ Yes ● No		
1.a. If YES to Human Subjects		
Is the IRB review Pending? ❍ Yes ❍ No		
IRB Approval Date:		
Exemption Number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6		
Human Subject Assurance Number		
2. * Are Vertebrate Animals Used? ❍ Yes ● No		
2.a. If YES to Vertebrate Animals		
Is the IACUC review Pending? ❍ Yes ❍ No		
IACUC Approval Date:		
Animal Welfare Assurance Number		
3. * Is proprietary/privileged information included in the application? ● Yes ❍ No		
4.a. * Does this project have an actual or potential impact on the environment? ❍ Yes ● No		
4.b. If yes, please explain:		
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No		
4.d. If yes, please explain:		
5.a. * Does this project involve activities outside the U.S. or partnership with International Collaborators? ❍ Yes ● No		
5.b. If yes, identify countries:		
5.c. Optional Explanation:		
6. * Project Summary/Abstract	2065-Abstract.pdf	Mime Type: application/pdf
7. * Project Narrative	2664-Narrative.pdf	Mime Type: application/pdf
8. Bibliography & References Cited	6355-biblio.pdf	Mime Type: application/pdf
9. Facilities & Other Resources	9598-Facilities.pdf	Mime Type: application/pdf
10. Equipment	6082-Equipment.pdf	Mime Type: application/pdf

Antibiotic resistance is a growing and increasingly serious public health problem. Infectious diseases caused by *Escherichia coli* and other bacteria are responsible for millions of deaths each year, and much of this mortality is due to the rise of antibiotic resistant organisms. Because antibiotic-resistant infections double the duration of hospital stay, mortality, and morbidity as compared with drug-susceptible infections, economic costs of antibiotic resistance are estimated to be in the billions of dollars. The overall goal of this project is to develop new anti-infectives that are highly effective and refractory to antibiotic resistance using a Combinatorial Genetic Technology (CGT) that allows the identification of new rRNA target sites and the specific nucleotides that are essential for functionality and viability, and RNA Homology Modeling software that allows accurate prediction of mutant RNA structures.

Phase I of this project was highly successful. A functional mutation library of *E. coli* 16S rRNA was constructed and ~5000 viable clones were sequenced. Using CGT, 67 regions of *E. coli* rRNA that contain nucleotides essential for viability were identified. The 67 functionally important regions include known binding sites for antibiotics, tRNAs, proteins, the large ribosomal subunit and initiation factors. Also included were a number of sites that are clearly essential for ribosome function, but for which no functional role has been identified to date. Some of the individual regions occur near each other in 30S subunit crystal structures and probably contribute to a single functional role.

The Phase II specific aims are 1) to select one RNA subdomain as a prioritized target from the four potential targets chosen from the RNA “regions of interest” identified in Phase I; 2) to use CGT to identify every mutation of the target that could lead to drug resistance, and use multidimensional NMR spectroscopy and homology modeling to determine the essential structural components of the target; 3) to screen compound libraries against the wild type target and its viable mutants; and 4) to carry out structural studies of target/hit complexes to allow optimization of hit compounds, and validate the target/compound using *in vitro* and *in vivo* assays of antibacterial activity. RiboNovix will complete the work necessary to develop drug candidates from the leads, and will move qualified candidates into pre-clinical development.

Anti-infectives developed against the target identified in this study will likely be highly effective against microbial pathogens and resistant to target site mutation, thus resulting in drugs refractory to antibiotic resistance.

Antibiotic resistance is a growing and increasingly serious public health problem. Infectious diseases caused by *Escherichia coli* and other bacteria are responsible for millions of deaths each year, and much of this mortality is due to the rise of antibiotic resistant organisms. The overall goal of this project is to develop new anti-infectives that are highly effective and refractory to antibiotic resistance.

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94. Ding, S., Wu, T. Y., Brinker, A., Peters, E. C., Hur, W., Gray, N. S. & Schultz, P. G. (2003). Synthetic small molecules that control stem cell fate. *Proc Natl Acad Sci U S A* **100**, 7632-7.
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98. Nelson, M. L., Ismail, M. Y., McIntyre, L., Bhatia, B., Viski, P., Hawkins, P., Rennie, G., Andorsky, D., Messersmith, D., Stapleton, K., Dumornay, J., Sheahan, P., Verma, A. K., Warchol, T. & Levy, S. B. (2003). Versatile and facile synthesis of diverse semisynthetic tetracycline derivatives via Pd-catalyzed reactions. *J Org Chem* **68**, 5838-51.
99. Bursavich, M. G. & Rich, D. H. (2002). Designing Non-Peptide Peptidomimetics in the 21st Century: Inhibitors Targeting Conformational Ensembles. *J. Med. Chem.* **45**, 541–558.
100. Winkler, F. K., Banner, D. W. & Bohm, H. J. (2001). Structure-Based Approaches in Modern Drug Discovery Research. *Ernst Schering Res. Found. Workshop* **34**, 123–142.
101. Lesley, S. A., Brow, M. A. & Burgess, R. R. (1991). Use of in vitro protein synthesis from polymerase chain reaction-generated templates to study interaction of Escherichia coli transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies. *J Biol Chem* **266**, 2632-8.
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103. Pelham, H. R. & Jackson, R. J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem* **67**, 247-56.

Facilities and Other Resources

Laboratory

RiboNovix currently leases approximately _ sq. feet of wet laboratory space from another biotechnology company in Lexington Massachusetts. This biotechnology company has excess laboratory space (and equipment) as it terminated most of its early stage R&D efforts to focus on its clinical stage products. RiboNovix' lab space is well equipped with general and some specialized lab equipment (see equipment). Two chemical hoods are available and one laminar flow hood for biological experiments. There is currently _ sq. ft of additional empty lab space available for RiboNovix to expand the area that it currently leases. The rental includes utilities and general support to meet safety and regulatory requirements.

At Wayne State University, Dr. Cunningham's labs occupy approximately 2200 sq. feet in the Biology building. The labs are fully equipped with chemical fume hoods, lab benches, distilled deionized water etc. to enable research in molecular biology, protein chemistry, enzymology, and organic chemistry. The lab is fully stocked with glassware, electrophoresis equipment, water baths, incubators etc. Dr. SantaLucia's 2000 sq. ft. laboratory in the Chemistry building is fully outfitted for organic chemistry and molecular biology with two 6 ft. fume hoods for synthesis reactions and radioactivity. Walk-in cold rooms and instrument rooms are available adjacent to the labs. Unrestricted access to multi-user facilities include: ultracentrifuges, scintillation counters, autoclaves, glass washing facilities, and digital gel documentation systems.

The NSRB screening laboratory at Harvard University is equipped with state-of-the-art robotic equipment and is approved for work with a variety of BSL-2 organisms. The NSRB supports any 96-or 384-well assay that is read using a plate reader (EnVision).

Office and Computers

RiboNovix has office facilities in Massachusetts equipped with both Macintosh and PC computers. At Wayne State University several Macintosh and PC computers are available with software for genetic analysis, homology modeling, and NMR processing. A Silicon Graphics workstation (Octane) with 40 GB hard drive is located at Wayne State.

Equipment

RiboNovix has use of or owns the following equipment: MJ thermacycler, heat blocks, autoclave, 2 variable temperature incubators (RT-50C), -80 freezer, cold box, 2 Eppendorf 5415C centrifuges, Perkin-Elmer Victor III plate reader, under counter refrigerator, UV Camera, New Brunswick shaker incubator, microwave, pH Meter, balance, heat/stir plate, -20 freezer, pipetmen, 2 drummond pipettors, 1 large and I small water bath, Beckman low and high speed centrifuges, UV Vis, 2 picofuges, BioRad electroporator, BioRad Micropulse, 2 power supplies and gel box equipment. Additional equipment necessary for execution of the project is requested in the budget including: a nanodrop spectrophotometer, speedvac, HPLC and columns, water purification equipment, a multidrop dispensor for 96/384 well plates, and an additional thermal cycler.

Specific major equipment available at Wayne State University includes: Sorvall RC-5 centrifuge with GSA rotor and SS34 rotors, Molecular Devices multi-well plate spectrophotometer and fluorometer, Licor Global IR automated DNA sequencer, French press, Biocomp gradient formation apparatus, 296-well MJ Research PTC200 thermal cyclers, 4 incubators, 3 New Brunswick shaking water baths, 1 New Brunswick air shaker, Perkin-Elmer Lambda Bio UV-Vis spectrophotometer, and Hitachi UV-Vis detector with flow cell.

A Bruker Avance-700 MHz NMR spectrometer equipped with cryoprobe 1H, 13C, 15N triple resonance probe with Z-axis PFG coil is located in the Chemistry building.

RESEARCH & RELATED Senior/Key Person Profile

PROFILE - Project Director/Principal Investigator

Prefix	* First Name	Middle Name	* Last Name	Suffix
	Cheryl	Isaac	Murphy	PhD

Position/Title: Vice President, Technology Development

Department:

Organization Name: RiboNovix, Inc.

Division:

* Street1: 8 Farrar Rd.

Street2:

* City: Lincoln County: * State: MA * Zip Code: 01773 * Country: USA

*Phone Number	Fax Number	* E-Mail
781-259-3183	781-259-3183	cheryl@ribonovix.com

Credential, e.g., agency login: CHERYLM1

*** Project Role:** PD/PI **Other Project Role Category:**

	File Name	Mime Type
***Attach Biographical Sketch**	7603-CM bio.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 1

Prefix	* First Name	Middle Name	* Last Name	Suffix
	Philip	R.	Cunningham	PhD

Position/Title: Associate Professor

Department: Biological Sciences

Organization Name: Wayne State University

Division:

* Street1: 5047 Gullen Mall

Street2:

* City: Detroit County: * State: MI * Zip Code: 48202 * Country: USA

*Phone Number	Fax Number	* E-Mail

Credential, e.g., agency login:

*** Project Role:** Other (Specify) **Other Project Role Category:** Consortium Head

	File Name	Mime Type
***Attach Biographical Sketch**	1859-pc bio.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 2

Prefix	* First Name	Middle Name	* Last Name	Suffix
	Ian	A.	MacNeil	PhD

Position/Title: Head, Assay Development
Department:

Organization Name: RiboNovix, Inc.
Division:

* Street1:
Street2:

* City: Lexington
County:
* State: MA
* Zip Code: 02421
* Country: USA

*Phone Number	Fax Number	* E-Mail
		ian@ribonovix.com

Credential, e.g., agency login: MacNeil

*** Project Role:** Other (Specify)
Other Project Role Category: Microbiologist

	File Name	Mime Type
***Attach Biographical Sketch**	8387-IM bio.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 3

Prefix	* First Name	Middle Name	* Last Name	Suffix
	John		SantaLucia	PhD

Position/Title: Associate Professor
Department: Chemistry

Organization Name: Wayne State University
Division:

* Street1: 5101 Cass Avenue
Street2:

* City: Detroit
County:
* State: MI
* Zip Code: 48202
* Country: USA

*Phone Number	Fax Number	* E-Mail

Credential, e.g., agency login:

*** Project Role:** Other (Specify)
Other Project Role Category: Structural Chemist

	File Name	Mime Type
***Attach Biographical Sketch**	6925-JSL bio.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 4

Prefix	* First Name	Middle Name	* Last Name	Suffix

Position/Title:
Department:
Organization Name:
Division:
* Street1:
Street2:
* City:
County:
* State:
* Zip Code:
* Country: USA

*Phone Number
Fax Number
* E-Mail

Credential, e.g., agency login:

*** Project Role:** Other Professional
Other Project Role Category: Consortium Head

	File Name	Mime Type
***Attach Biographical Sketch**		
Attach Current & Pending Support		

PROFILE - Senior/Key Person 5

Prefix	* First Name	Middle Name	* Last Name	Suffix
	Christine		Chow	

Position/Title: Professor
Department: Chemistry
Organization Name: Wayne State University
Division:
* Street1: 5101 Cass Avenue
Street2:
* City: Detroit
County:
* State: MI
* Zip Code: 48202
* Country: USA

*Phone Number
Fax Number
* E-Mail

Credential, e.g., agency login:

*** Project Role:** Other (Specify)
Other Project Role Category: Other Significant Contributor

	File Name	Mime Type
***Attach Biographical Sketch**	8580-CC bio.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 6

Prefix | * First Name | Middle Name | * Last Name | Suffix

Position/Title: | Department:

Organization Name: | Division:

* Street1: | Street2:

* City: | County: | * State: | * Zip Code: | * Country: USA

*Phone Number | Fax Number | * E-Mail

Credential, e.g., agency login:

*** Project Role:** Other (Specify) | **Other Project Role Category:** other significant contributor

File Name | Mime Type

***Attach Biographical Sketch**

Attach Current & Pending Support

PROFILE - Senior/Key Person 7

Prefix | * First Name | Middle Name | * Last Name | Suffix

Position/Title: | Department:

Organization Name: | Division:

* Street1: | Street2:

* City: | County: | * State: | * Zip Code: | * Country: USA

*Phone Number | Fax Number | * E-Mail

Credential, e.g., agency login:

*** Project Role:** Other (Specify) | **Other Project Role Category:** Other significant contributor

File Name | Mime Type

***Attach Biographical Sketch**

Attach Current & Pending Support

File Name | Mime Type

ADDITIONAL SENIOR/KEY PERSON PROFILE(S)

Additional Biographical Sketch(es) (Senior/Key Person)

Additional Current and Pending Support(s)

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Murphy, Cheryl Isaac	Vice President, Technology Development
eRA COMMONS USER NAME CHERYLM1	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Williams College, Williamstown, MA	B.A.	1978	Biology and Chemistry
Duke University, Durham, NC	Ph.D.	1982	Microbiology
Dana Farber Cancer Institute, Boston, MA	postdoctoral	1982-87	Molecular Virology

A. Positions and Honors.

Positions and Employment

1987-1992 Scientist, Molecular Biology, Cambridge Biotech Corp., Worcester, MA
1992-1994 Senior Scientist, Molecular Biology, Cambridge Biotech Corp., Worcester, MA
1994-1998 Head of Molecular Biology, Cambridge Biotech Corp. and Aquila Biopharmaceuticals, Worcester, MA
1998-2000 Associate Director, Technology Development, Aquila Biopharmaceuticals, Framingham, MA
2000-2003 Director of Business Development, Antigenics Inc., Woburn, MA
2003-present Vice President, Technology Development, RiboNovix Inc., Lincoln, MA

Other Experience and Professional Memberships

1995-1996 Visiting Scientist, Walter Reed Army Institute of Research, Washington, D.C.
1992-present Member, American Association for the Advancement of Science

Honors

1986 Aid for Cancer Research Fellowship
1996 Inventor U.S. Patent #5,516,657. Baculovirus vectors for expression of secretory and membrane-bound proteins.
2001 Inventor U.S. Patent #6,204,252 Characterization of granulocytic ehrlichia and methods of use.
2001 Inventor U.S. Patent #6,306,394 Nucleic acids, proteins, and methods of use of granulocytic ehrlichia.

B. Selected peer-reviewed publications (in chronological order).

1. Murphy, C.I., Bikel, I. and Livingston, D.M. 1986. Cellular proteins which can specifically associate with simian virus 40 small t antigen. J. Virol. 59:692-702.
2. Murphy, C.I., Weiner, B., Bikel, I., Piwnica-Worms, H., Bradley, M.K. and Livingston, D.M. 1988. Purification and functional properties of simian virus 40 large and small T antigens overproduced in insect cells. J. Virol. 82:2951-2959.
3. Murphy, C.I., Lennick, M., Lehar, S.M., Beltz, G.A. and Young, E. 1990. Temporal expression of HIV-1 envelope proteins in baculovirus-infected insect cells: Implications for glycosylation and CD4 binding. Gen. Anal. Tech. Appl. 7:160-171.

4. Wu, J.-Y., Gardner, B.H., Murphy, C.I., Seals, J.R., Kensil, C.R., Recchia, J., Beltz, G.A., Newman, G.W. and Newman, M.J. 1992. Saponin adjuvant enhancement of antigen specific immune responses to an experimental HIV-1 vaccine. J. Immunol. 148:1519-1525.
5. Murphy, C.I. 1992. Scale-up of protein production in an airlift fermenter. In: Baculovirus Expression Vectors/A laboratory manual. Ed. W.H. Freeman and Company, New York, pp. 249-256.
6. Newman, M.J., Wu, J.-Y., Coughlin, R.T., Murphy, C.I., Seals, J.R., Wyand, M.S. and Kensil, C.R. 1992. Immunogenicity and toxicology testing of an experimental HIV-1 vaccine in nonhuman primates. AIDS Red. Hum. Retro. 8:1413-1418.
7. Murphy, C.I., McIntire, J.R., Davis, D.vR., Hodgdon, H., Seals, J.R. and Young, E. 1993. Enhanced expression, secretion and large scale purification of recombinant HIV-1 gp120 in insect cells using the baculovirus egt and p67 signal peptides. Protein Expression and Purification 4:349-357.
8. Yeh, J., Seals, J.R., Murphy, C.I., van Halbeek, H. and Cummings, R.D. 1993. Site-specific N-glycosylation and oligosaccharide structures of recombinant HIV-1 gp120 derived from a baculovirus expression system. Biochemistry 32:11087-11099.
9. Newman, M.J., Munroe, K.J., Anderson, C.A., Murphy, C.I., Panicalli, D.L., Seals, J.R., Wu, J.-Y., Wyand, M.S. and Kensil, C.R. 1994. Induction of antigen-specific killer T-lymphocyte responses using subunit SIVmac251 gag and env vaccines containing QS-21 adjuvant. AIDS Res. Hum. Retro. 10:853-861.
10. Murphy, C.I. and Piwnica-Worms, H. 1994. Expression of proteins in insect cells using baculovirus vectors. In: Current Protocols in Molecular Biology. Ed. John Wiley and Sons, Inc., New York. pp. 16.9.1 - 16.11.19.
11. Daugherty, J.R., Murphy, C.I., Doros-Richert, L.A., Barbosa, A., Kashala, L.O., Ballou, W.R., Snellings, N.J., Ockenhouse, C.F.and Lanar, D.E. 1997. Baculovirus-mediated expression of *Plasmodium falciparum* erythrocyte binding antigen 175 polypeptides and their recognition by human antibodies. Infect. and Immun. 65:3631-3637.
12. Storey, J.R., Doros-Richert, L.A., Gingrich-Baker, C., Munroe, K., Mather, T.N., Coughlin, R.T., Beltz, G.A., and Murphy, C.I. 1998. Molecular cloning and sequencing of three granulocytic *Ehrlichia* genes encoding high-molecular weight immunoreactive proteins. Infect. and Immun. 66:1356-1363.
13. Murphy, C.I., Storey, J.R., Recchia, J., Doros-Richert, L.A., Gigrich-Baker, C., Munroe, K., Bakken, J.S., Coughlin, R.T., and Beltz, G.A. 1998. Major antigenic proteins of the agent of human granulocytic ehrlichiosis are encoded by members of a multigene family. Infect. and Immun. 66:3711-3718.
14. Massung, R.F., Owens, J.H., Ross, D., Reed, K.D., Petrovec, M., Bjoersdorff, A., Coughlin, R.T., Beltz, G.A., and Murphy, C.I. 2000. Sequence Analysis of the *ank* Gene of Granulocytic Ehrlichiae. J. Clin. Microbiol. 38**:**2917- 2922.
15. Ockenhouse, C.F., Barbosa, A., Blackall D.P., Murphy, C.I., Kashala, O., Dutta, S., Lanar, D.E., and Daugherty, J.R. 2001. Sialic acid-dependent binding of baculovirus-expressed recombinant antigens. From *Plasmodium falciparum* EBA-175 to Glycophorin A. Mol. Biochem. Parasitol. 113:9-21.

C. Research Support.

Ongoing Research Support

R43 AI066445-01 Murphy (PI) 7/01/05-12/31/05
NIH/NIAID
Three Fluorophore Assay for Antibiotic Inhibitors of Ribosome Assembly. The goal of this project is to optimize the assay and to adapt it for high throughput screening.
Role: PI

Completed Research Support

R41 AI49641-01 Chang (PI) 3/15/01-3/14/04
NIH/NIAID

A *P.falciparum* MSP1 p42/QS-21 malaria vaccine. The goal of this project was to produce cGMP p42 material and perform preclinical evaluation of the vaccine.
Role: Co-investigator

R43 AI060275-01 Murphy (PI) 4/01/04-3/31/05
NIH/NIAID
Identification of E. coli anti-infective rRNA targets. The goal of this project was to produce a mutation library of E. coli 16S rRNA, sequence 5000 clones and identify targets for anti-infective drugs.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Cunningham, Philip Roger	Associate Professor
eRA COMMONS USER NAME	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Murray State University	B.S.	1979	Biology
Southern Illinois University	Ph.D.	1987	Microbiology
Roche Institute of Molecular Biology		1987-1991	Molecular Biology

A. Positions and Honors.

Positions and Employment

1979-1981 Research Assistant - Murray State University, Murray, Kentucky
1981-1986 Research Assistant - Southern Illinois University, Carbondale, Illinois
1989 Visiting Scientist – Univ of Leiden, Gorlaeus Laboratory, Leiden University, Leiden, The Netherlands
1987-1991 Postdoctoral Fellow - Roche Institute of Molecular Biology, Nutley, New Jersey
1991-1997 Assistant Professor - Department of Biological Sciences, Wayne State University, Detroit, Michigan.
1997-present Associate Professor - Department of Biological Sciences, Wayne State University, Detroit, Michigan.
1998-present Adjunct Professor - Department of Chemistry, Wayne State University, Detroit, Michigan.
1999-present Associate Professor – Institute for Scientific Computing, Wayne State University, Detroit, Michigan.
2000-2002 Chair – Div of Mol Biology and Biotech, Dep. of Biological Sciences, Wayne State University, Detroit, MI
2000-present Associate Professor – Institute for Drug Design, Wayne State University, Detroit, Michigan.
2000-present Director, WSU Biotechnology Program, Wayne State University, Detroit, Michigan.
2003-present Founder and Chair of the Science Board, RiboNovix, Inc.

Professional Activities

Memberships, American Association for the Advancement of Science, American Society for Microbiology, American Society for Biochemistry and Molecular Biology, RNA Society.
Ad hoc reviewer for the National Institutes of Health (PC Study Section-2002; SCOR-2002)

Honors

1998 College of Science Distinguished Teaching Award (Wayne State University)
2000 Howard Hughes Medical Institute Distinguished Lecture, Murray State University
2001 Career Development Chair Recipient, Wayne State University, Detroit, Michigan
2004 Presidents Award for Excellence in Teaching, Wayne State University

B. Selected peer-reviewed publications (in chronological order).

1. Cunningham, P.R., Nurse, K. Bakin, A., Weitzmann, C.J., Pflumm, M. and Ofengand, J. 1992. Interaction between the two conserved single-stranded regions at the decoding site of small subunit ribosomal RNA is essential for ribosome function. *Biochemistry,* **31**, 12012-12022.
2. Weitzmann, C.J. Cunningham, P.R., Nurse, K., and Ofengand, J. 1993. Chemical evidence for domain assembly of the *Escherichia coli* 30S ribosome. *Faseb J*., **7**, 177-180.
3. Leonardo, M.R., Cunningham, P.R., and Clark, D.P. 1993. Anaerobic Regulation of the *adhE* Gene, Encoding the Fermentative Alcohol Dehydrogenase of *Escherichia coli*. *J. Bacteriol.*, **175**(3), 870-878.
4. Santer, M., Santer, U., Nurse, K., Bakin, A., Cunningham, P.R., Zain, M., O'Connell, and Ofengand, J. 1993. Functional Effects of a G to U Base Change at Position 530 in a Highly Conserved Loop of *Escherichia coli* 16S RNA. *Biochemistry*, **32**, 5539-5547.
5. Cunningham, P.R., Nurse, K., Weitzmann, C.J., and Ofengand, J. 1993. Functional Effects of Base Changes at the Decoding Center of *Escherichia coli* 16S Ribosomal RNA: Mutation of C1400, G1405, G1497, and U1498. *Biochemistry*, **32**, 7172-7180.
6. Ringquist, S., Cunningham, P., Weitzmann, C., Formenoy, L., Pleij,C., Ofengand, J. and Gold, L., 1993. Translation initiation complex formation with 30S ribosomal particles mutated at conserved positions in the 3' minor domain of 16S RNA. *J. Mol. Biol*., **234**, 14-27.
7. Formenoy, L., Cunningham, P.R., Nurse, K. Pleij, CWA and Ofengand, J. 1994. Methylation of the conserved A1518-A1519 in *Escherichia coli* 16S ribosomal RNA by the ksgA methyltransferase is influenced by methylations around the similarly conserved U1512-G1523 base pair in the 3' terminal hairpin. *Biochimie* ,**76**, 1123-1128.
8. Lee, KS.,Holland-Staley, C. A., and Cunningham, P. R. 1996. Genetic Analysis of the Shine-Dalgarno Interaction: Selection of alternative functional mRNA-rRNA combinations. *RNA* , **2**, 1270-1285.
9. Lee, KS., Varma, S. SantaLucia, J. and Cunningham, P.R. 1997. *In vivo* determination of RNA structure-function relationships: Analysis of the 790 loop in ribosomal RNA. *J. Mol. Biol*. **269**, 647-946.
10. Morosyuk, SV, Lee, KS., SantaLucia, Jr.,J. and Cunningham, P. R. 2000. Structure and function of the conserved 690 hairpin in *Escherichia coli* 16S ribosomal RNA: Analysis of the stem nucleotides. *J. Mol. Biol.* **300** (1):113-26.
11. Holland-Staley, C. A. Lee, KS, Clark, DP and Cunningham, P. R. 2000. Aerobic Activity of *Escherichia coli* Alcohol Dehydrogenase is Determined by a Single Amino Acid. *J. Bacteriol.* **182**:6049-6054.
12. Morosyuk, S.M., Cunningham, P. R. and SantaLucia, J., Jr. 2001. Structure and Function of the Conserved 690 Hairpin in *Escherichia coli* 16 S Ribosomal RNA: II. NMR Solution Structure. *J. Mol. Biol.* **307** (1):197-210.
13. Morosyuk, S.M., SantaLucia, J., Jr. and Cunningham, P. R. 2001. Structure and function of the conserved 690 hairpin in *Escherichia coli* 16S ribosomal RNA: III. Instant evolution of the 690 loop *J. Mol. Biol.* **307** (1):211-28.
14. Lee, KS, Holland-Staley, C. and Cunningham, P. R. 2001. Genetic approaches to studying protein synthesis: Effects of mutations at ψ516 and A535 in *Escherichia coli* 16S rRNA. *J. Nutrition* **131**(11):2994-3004.
15. Belanger, F; Leger, M; Saraiya, A. A.; Cunningham, P. R.; Brakier-Gingras, L. 2002. "Functional Studies of the 900 Tetraloop Capping Helix 27 of 16S Ribosomal RNA", *J. Mol. Biol.*, 320, 979–989.
16. Chow, C. S.; Cunningham, P. R.; Lee, K.-S. 2002. Meroueh, M.; SantaLucia, J., Jr.; Varma, S. "Photoinduced Cleavage by a Rhodium Complex at G-U Mismatches and Exposed Guanines in Large and Small RNAs", *Biochimie*, 84, 859–868.
17. Belanger F, Gagnon MG, Steinberg SV, Cunningham PR, Brakier-Gingras L.. 2004. Study of the functional interaction of the 900 Tetraloop of 16S ribosomal RNA with helix 24 within the bacterial ribosome. *J Mol Biol* **338**(4):683-693.
18. Laios, E., Waddington M., Saraiya A.S., Baker K.A., O'Connor E, Paramathy ' D and Cunningham, P.R.. 2004. Combinatorial Genetic Technology for the Development of New Anti-infectives. *Arch Pathol.* **128**(12) 1351-1359.

C. Research Support

Ongoing Research Support

R21 (AI055822-01) Cunningham (PI) 4/1/03-3/31/05
NIH
Technology for New Tuberculosis Anti-Infectives
The goal of this project is to express functional *Mycobacterium tuberculosis* rRNA in *Escherichia coli*.
Role: PI

U01AI061192 Cunningham (PI) 7/15/04-7/14/08
NIH
Anti-infectives that target bacterial ribosome
The goal of this project is to identify new anti-infectives for the treatment of infections caused by *Yersinia pestis* and other potential bioterriorism agents.
Role: PI

Completed Research Support

R43 AI060275-01 Murphy (PI) 4/01/04-3/31/05
NIH/NIAID
Identification of E. coli anti-infective rRNA targets
Role: Co-investigator

RO1 (GM52896) Cunningham (PI) 5/1/96-4/30/2002
NIH
Genetic Analysis of Ribosomal RNA Function
Role: PI

RO1 (GM55745) Cunningham (PI) 12/1/97-11/30/02
NIH
Structure and Function of the 790 Loop in Ribosomal RNA
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
MacNeil, Ian A.	Associate Director
eRA COMMONS USER NAME MacNeil	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Pennsylvania State University	B.Sc.	1980	Microbiology and Immunol.
Temple University	M.Sc.	1982	Microbiology and Immunol.
University of Colorado Health Science Center	Ph.D.	1987	Microbiology and Immunol.

A. Positions and Honors.

Positions and Employment

1987-1989	Postdoctoral Fellow, HHMI, Stanford University Stanford, CA
1989-1993	Postdoctoral Fellow, DNAX Research Institute, Palo Alto, CA
1993-1995	Staff Scientist, Dept. of Biology, ARIAD Pharmaceuticals Cambridge, MA
1995-1999	Head, HTS Screening Facility, ARIAD Pharmaceuticals Cambridge, MA
1999-2000	Head, cDNA Cloning Group Aventis Pharmaceuticals Cambridge, MA
2000-2002	Head, Environ. Library Res., Aventis Pharmaceuticals, Cambridge, MA
2003-2005	Assoc. Dir. Assay Development, Activbiotics, Inc. Lexington, MA
2005-present	Head, Assay Development, RiboNovix, Inc., Lexington, MA
2005-present	Principal Investigator, Earthgenes, Inc., Lexington, MA

Other Experience and Professional Memberships

1989-present	Member, American Association for the Advancement of Science
1995-2002	Member, Society for Biomolecular Screening
1985-1995	Member, American Association of Immunologists
1987-1990	American Cancer Society Postdoctoral Fellowship
1980-1982	Temple University Fellowship

B. Selected peer-reviewed publications (in chronological order).

1) MacNeil, I.A., T. Suda , K.W. Moore, T.R. Mosmann, and A. Zlotnik. Interleukin 10: A novel growth cofactor for mature and immature T cells. *J.Immunol.* **145**:4167-4173, 1990.
2) Suda, T., I.A. MacNeil, M. Fischer, K.W. Moore, and A. Zlotnik. Identification of a novel thymocyte growth factor from B cell lymphomas. In *Mechanisms of Lymphocyte Activation and Immune Regulation III*, S. Gupta *et al.* (Eds.), Plenum Press, New York, 115-120, 1991.
3) MacNeil, I.A., T. Suda , K.W. Moore, T.R. Mosmann, M. Howard, L. Thompson-Snipes, and A. Zlotnik. Interleukin 10: A novel T cell growth cofactor. In *Cellular and Cytokine Networks in Tissue Immunity,* M. Meltzer and A. Mantovani (Eds.), Wiley-Liss, Inc., New York, 331-336, 1991.
4) Fischer, M., MacNeil, I.A., Suda, T., Cupp, J.E., Shortman, K. and A. Zlotnik. Cytokine production by mature and immature thymocytes. *J. Immunol.* **146**:3452-3456, 1991.

5) MacNeil, I.A., Kennedy, J., Godfrey, D.J., Jenkins, N.A., Masciantonio, M., Mineo, C., Gilbert, D.J., Copeland, N.G., Boyd, R.L., Zlotnik, A. Isolation of a cDNA encoding Thymic Shared Antigen-1, A new member of the Ly6 Family. *J.Immunol.* **151**:6913-6923, 1993.
6) Helaakoski, T., Annunen, P., Vuori, K., MacNeil, I.A., Pihlajaniemi, T., Kivirikko, K.I. Cloning, baculovirus expression and characterization of a second mouse prolyl 4-hydroxylase α-subunit isoform: Formation of an $\alpha_2\beta_2$ tetramer with the protein disulfide-isomerase/β subunit. *Proc. Natl.Acad. Sci.* USA **92**:4427-4431, 1995.
7) Zoller, K.E., MacNeil, I.A., Brugge, J.S. Protein Tyrosine Kinases Syk and ZAP-70 display distinct requirements for Src family kinases in immune response receptor signal transduction. *J.Immunol.* **158**:1650-9, 1997
8) Lynch, B.A., Loiacono, K., Tiong, C., Adams, S., MacNeil, I.A. A Fluorescence-Polarization Based Src-SH2 Binding Assay. *Anal Biochem* **247**: 77-82, 1997
9) MacNeil, I.A. and Zoller, M.J. Emerging Technologies for the Discovery of Small-Molecule Theraputics. In *Inflammation: Basic Principles and Clinical Correlates.* J.I. Galvin and R. Snyderman (Eds.), Lippincott Williams and Wilkins, Philadelphia. 1213-1226, 1999.
10) Buchanan, J.L., Chi B. Vu, Taylor J. Merry, Evelyn G. Corpuz,Selvaluxmi G. Pradeepan, Ukti N. Mani, Michael Yang, Hilary R. Plake,Vaibhav M. Varkhedkar, Berkley A. Lynch, Ian A. MacNeil, Kara A. Loiacono, Choi Lai Tiong and Dennis A. Holt. Structure-Activity Relationships of a Novel Class of Src SH2 Inhibitors. *Bioorganic and Medicinal Chemistry Letters* **9**: 2359-2364, 1999.
11) Vu, C. B., Corpuz, E. G., Merry, T. J., Pradeepan, S. G., Bartlett, C., Bohacek, R. S., Botfield, M. C., Eyermann, C. J., Lynch, B.A., MacNeil, I. A., Ram, M. K., van Schravendijk, M. R., Violette, S., Sawyer, T.K. Discovery of Potent and Selective SH2 Inhibitors of the Tyrosine Kinase ZAP-70. *J. Med. Chem.* **42**:4088-4098, 1999.
12) Lynch, B. A., Minor, C., Loiacono, K.A., Tiong, C. L., van Schravendijk, M. R., Ram, M. K.,Sundaramoorthi, R., Adams, S. E., Phillips, T., Lui, A., Holt, D., Rickles R. J., MacNeil I. A. Fluorescence Polarization Probes for Simultaneous Studies of Src SH2 and SH3 Domain Binding. *Anal. Biochem*. **275**:62-73, 1999.
13) Osburne M.S., Grossman T.H., MacNeil, I.A. Tapping Into Microbial Diversity for Natural Products Drug Discovery - A New Approach. *ASM News*. **66**:411-417, 2000.
14) Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K.A., Lynch, B.A., MacNeil, I.A., Minor, C., Tong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., Goodman, R.M. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *AEM*. **66**:2541-2547, 2000.
15) August P.R., Grossman T, Minor C., Draper M., MacNeil I.A., Pemberton, J.M., Holt D., Call K., Osburne, M.S. Sequence Analysis and Functional Characterization of the Violacein Biosynthetic Pathway from Chromobacterium violaceum . *JMMB*. **2**:1-7, 2000.
16) August, MacNeil, Grossman. *Novel vectors for improving cloning and expression in low copy number plasmids*. Patent No. WO0078977, 2000.
17) MacNeil, Zoller, Lynch. *In vitro fluorescence polarization assay.* Patent No. US6207397, 2001.
18) MacNeil I.A., Tiong, C. L., Minor C., August P.R., Grossman T., Loiacono, K.A., Lynch B.A., Aldrich T., Long H., Narula, S., Sundaramoorthi R.,Holt, D., Gilman, M., Osburne M. Expression and Isolation of Antimicrobial Small Molecules from Soil DNA Libraries. *JMMB*. **3**:301-308, 2001.
19) Martinez, A., Kolvek, S., Tiong, C. L., Hopke, J., Brown, K,. MacNeil, I. A., Osburne M. Genetically modified bacterial strains and novel shuttle BAC vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. AEM. **70**:2452-2463. 2004.
20) Martinez, A., Hopke, J., MacNeil, I. A., Osburne M. Accessing the Genomes of Uncultivated Microbes for Novel Natural Products. In *Natural Products: Drug Discovery, Therapeutics and Preventive Medicine.* Humana Press. In Press. 2004.

Ongoing Research Support

1 R43 AI66814-01 MacNeil (PI) 07/01/05 - 06/30/06
NIH/NIAID
Genetic Tool to Enhance Natural Product Gene Expression
The main goal of this project is to improve the host strains used in an environmental genomic antibiotic screening program so that a wider range of antibiotics will be discovered.
Role: PI

1 R43 AI066445-01 Murphy (PI) 7/01/05-12/31/05
NIH/NIAID
Three Fluorophore Assay for Antibiotic Inhibitors of Ribosome Assembly. The goal of this project is to optimize the assay and to adapt it for high throughput screening.
Role: Co-investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
SantaLucia, John, Jr.	Associate Professor

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Clarkson University, Potsdam, NY	B.S.	1987	Chemistry
University of Rochester, Rochester, NY	Ph.D.	1991	Physical Chemistry
University of California, Berkeley, CA	Postdoc	1991-94	Biophysical Chemistry

A. Positions and Honors.

Positions Held

Graduate Research Assistant: Department of Chemistry, University of Rochester, 1988–1991
N.I.H. Postdoctoral Fellow: Department of Chemistry, University of California, Berkeley, 1991–1994
Assistant Professor: Department of Chemistry, Wayne State University, 1994–2000
Associate Professor: Department of Chemistry, Wayne State University, 2000–present
Founder and Chief Scientist, DNA Software Inc., 2000 - present

Honors and Awards

W. D. Walters Teaching Award, 1988
C. F. Hutchison Fellowship, 1988–89
E. H. Hooker Fellowship, 1989–1990
The National Institutes of Health Postdoctoral Fellowship (UC Berkeley), 1991–1994
University Research Award (Wayne State University), 1995–1996
College of Science Teaching Award (Wayne State University) 1997–1998

Professional Activities

Ad hoc reviewer for the National Science Foundation, National Institutes of Health (SSSY-1999 to 2004; BBCA – 2002, P41 site visit - 2005)

Memberships: American Chemical Society (1987–present); American Society for the Advancement of Science (1988–present), RNA Society (1995–present), Karmanos Cancer Institute (1995–present)

B. Selected Publications (in Chronological Order).

SantaLucia, J., Jr.; Allawi, H. T.; Seneviratne, P. A. “Improved Nearest-neighbor Parameters for Predicting DNA Duplex Stability”, *Biochemistry* **1996**, *35*, 3555–3562.

Wu, M.; SantaLucia, J., Jr.; Turner, D. H. “Solution Structure of $(rGGCAGGCC)_2$ by 2-D NMR and the Iterative Relaxation Matrix Approach”, *Biochemistry* **1996**, *36*, 4449–4460.

Lee, K.-S.; Varma, S.; SantaLucia, J., Jr.; Cunningham, P. R. “*In vivo* Determination of RNA Structure-Function Relationships: Analysis of the 790 Loop in Ribosomal RNA”, *J. Mol. Biol.* **1997**, *269*, 732–743.

Allawi, H. T.; SantaLucia, J., Jr. “Thermodynamics and NMR of Internal G-T Mismatches in DNA”, *Biochemistry* **1997**, *36*, 10581–10594.

SantaLucia, J., Jr. “A Unified View of Polymer, Dumbbell, and Oligonucleotide DNA Nearest-Neighbor Thermodynamics”, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1460–1465.

Allawi, H. T. & SantaLucia, J., Jr. “NMR Solution Structure of a DNA Dodecamer Containing Single G•T Mismatches,” *Nucleic Acids Res.* 26, 4925-4934 (1998).

Peyret, N.; Seneviratne, P. A.; Allawi, H. T.; SantaLucia, J., Jr. “Nearest-Neighbor Thermodynamics and NMR of DNA Sequences with A-A, C-C, G-G, and T-T Mismatches”, *Biochemistry* **1999**, *38*, 3468–3477.

Blake, R.D., Bizzaro, J.W., Blake, J.D., Day, G.R., Delcourt, S.G., Knowles, J., Marx, K.A., & SantaLucia, J., Jr. “Statistical Mechanical Simulation of Polymeric DNA Melting with MELTSIM,” Bioinformatics 15, 370-375 (1999).

Bommarito, S., Peyret, N., and SantaLucia, J., Jr. “Thermodynamic Parameters for DNA Sequences with Dangling Ends,” Nucleic Acids Res. 28, 1929-1934 (2000).

Irani, R.; SantaLucia, J., Jr. “The Synthesis of 5-Iodocytidine Phosphoramidite for Heavy Atom Derivatization of RNA”, *Tet. Lett.* **1999**, *40*, 8961–8964.

Meroueh, M., Grohar, P.J., Qiu, J., SantaLucia, J., Jr., Scaringe, S.A., and Chow, C.S. “Unique Structural and Stabilizing Roles for the Individual Pseudouridine Residues in the 1920 Region of *Escherichia coli* 23S rRNA,” *Nucleic Acids Res.* **2000**, *28*, 2075-2083.

Morosyuk, S. M.; Lee, K.-S.; SantaLucia, J., Jr.; Cunningham, P. R. “Structure and Function of the Conserved 690 Hairpin in 16S Ribosomal RNA: Analysis of the Stem Nucleotides” *J. Mol. Biol.* **2000**, *300*, 113–126.

Morosyuk, S. M.; Cunningham, P. R.; SantaLucia, J., Jr. “Structure and Function of the Conserved 690 Hairpin in *Escherichia coli* 16S Ribosomal RNA: II. NMR Solution Structure”, *J. Mol. Biol.* **2001**, *307*, 197–211.

Morosyuk, S. M.; SantaLucia, J., Jr; Cunningham, P. R. “Structure and Function of the Conserved 690 Hairpin in *Escherichia coli* 16S Ribosomal RNA: III. Functional Analysis”, *J. Mol. Biol.* **2001**, *307*, 213–228.

Chow, C. S.; Cunningham, P. R.; Lee, K.-S.; Meroueh, M.; SantaLucia, J., Jr.; Varma, S. “Photoinduced Cleavage by a Rhodium Complex at G-U Mismatches and Exposed Guanines in Large and Small RNAs”, *Biochimie* **2002**, *84*, 859–868.

Irani, R.; SantaLucia, J., Jr. “The Synthesis of Anti-Fixed 3-Methyl-3-deaza-2'-deoxyadenosine and Other Imidazole [4,5-c]Pyridine Analogs”, *Nucleosides Nucleotides & Nucleic Acids* **2002**, *21*, 737-751.

Biswas, T.; Clos, L. J.; SantaLucia, J., Jr.; Mitra, S.; Roy, R. “Binding of Specific DNA Base-pair Mismatches by *N*-Methylpurine-DNA Glycosylase and its Implication in Initial Damage Recognition”, *J. Mol. Biol.* **2002**, *320*, 503–513.

SantaLucia, J., Jr. and Hicks, D., “The Thermodynamics of DNA Structural Motifs”, *Ann Rev. Biophys. Biomol. Struct.* **2004**, *33*, 415-440.

Dhimitruka, I. and SantaLucia, J., Jr. "Efficient preparation of 2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl chloride”, *SYNLETT* **2004**, *2*, 335-337.

Jang, S.B., Baeyens, K., Jeong, M. S., SantaLucia, J., Jr., Turner, D. and Holbrook, S. R. “Structures of two RNA octamers containing tandem G-A base pairs,” *Acta Cryst.* **2004**, 60D, 829-835.

Watkins, N. E. and SantaLucia, J., Jr. “Nearest-neighbor Thermodynamics of DNA deoxyinosine pairs,” *Nucleic Acids Res.* **2005**, 33, 6258-6267.

Dhimitruka, I. and SantaLucia, J., Jr. “Investigation of the Yamaguchi esterification mechanism. Synthesis of a Lux-S enzyme inhibitor using an improved esterification method” *Org. Lett.* **2005** (accepted).

C. Research Support.

Ongoing Research Support

Principal Investigator: SantaLucia, John, Jr.
Agency: NIH
Type: R01 (#GM073179)
Period: 2/01/05 – 1/30/09
Title: “Software for Structural Bioinformatics of Nucleic Acids”
The goal of this project is to develop new software homology modeling and *de novo* structure prediction of nucleic acids.

Principal Investigator: SantaLucia, John, Jr.
Agency: Michigan Life Science Corridor
Type: Infrastructure Award
Period: 11/1/00-10/30/06
Title: "Upgrade of 600 to 700 MHz. NMR plus cryoprobe"
This grant was used to purchase a 700 MHz NMR with cryoprobe accessory. This instrument is now available to complete the aims of this new proposal.

Principal Investigator: Cunningham, Philip (Co-investigator: Chow, Christine S., SantaLucia, John, and Spaller, Mark)
Agency: NIH
Type: U01 (AI061192)
Period: 07/15/2004 - 06/30/2008
Title: "Anti-Infectives that Target Bacterial Ribosomes"
The goal of this project is to identify new anti-infectives for the treatment of infections caused by *Yersinia pestis* and other potential bioterrorism agents.

Completed Research Support

Principal Investigator: SantaLucia, John, Jr.
Agency: Michigan Life Science Corridor
Type: LSC1653
Period: 2/1/01-1/30/04
Title: "Development of Software for Designing DNA Diagnostics"
The goal of this project was to develop software for predicting DNA folding and hybridization thermodynamics and apply this to software for DNA diagnostics.

Principal Investigator: Cunningham, Philip R. (Co-Investigator: SantaLucia, John, Jr.)
Agency: NIH
Type: R01 (#GM55745)
Period: 12/1/97-12/30/02
Title: "Structure and Function of the 790 Loop in Ribosomal RNA"

Principal Investigator: SantaLucia, John, Jr.
Agency: NIH
Type: R01 (#HG02020)
Period: 9/1/99-8/31/03
Title: "Addressing the Issue of Gap Regions in Genome Sequencing "

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY

A. Positions and Honors.

Positions and Employment

B. Selected recent peer-reviewed publications.

C. Research Support.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Chow, Christine S.	Professor
eRA COMMONS USER NAME	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Bowdoin College, Brunswick, ME	A.B.	1987	Chemistry
Columbia University, New York, NY	M.S.	1988	Organic Chemistry
California Institute of Technology, Pasadena, CA	Ph.D.	1992	Inorganic Chemistry
MIT, Cambridge, MA	Postdoc	1992-94	Inorganic Chemistry

A. Positions and Honors.

Positions Held

Graduate Research Assistant: Department of Chemistry, Caltech, 1989–1992
N.I.H. Postdoctoral Fellow: Department of Chemistry, M.I.T., 1992–1994
Assistant Professor: Department of Chemistry, Wayne State University, 1994–2000
Associate Professor: Department of Chemistry, Wayne State University, 2000–2004
Professor: Department of Chemistry, Wayne State University, 2004–present
Kyoto University, Institute for Advanced Energy, Visiting Professor, 2004

Honors and Awards

J. Malcom Miller Teaching Award (Columbia University), 1989
NRSA/N.I.H. Biotechnology Training Fellowship (Caltech), 1989–1990
Ralph M. Parsons Foundation Graduate Fellowship (Caltech), 1990–1992
The National Institutes of Health Postdoctoral Fellowship (M.I.T.), 1992–1994
University Research Award (Wayne State University), 1995–1997
Minority/Women Faculty Fellowship (Wayne State University), 1996–1997
College of Science Teaching Award (Wayne State University), 1998–1999
Career Development Chair Award (Wayne State University), 2001–2002
College of Science Teaching Award (Wayne State University), 2003–2004

Professional Activities

Ad hoc reviewer for the National Science Foundation, National Institutes of Health (Study Section B – 2000 and 2002; BNP – 2001 and 2003; Program on Bioterrorism-Related Research – 2002 and 2003), American Chemical Society, Research Corporation; NIH Study Section Member (SBCA (formerly BNP), 2004–present)

Memberships: American Chemical Society (1987–present); American Society for the Advancement of Science (1988–present), RNA Society (1995–present), Karmanos Cancer Institute (1995–present)

B. Selected Peer-Reviewed Publications (in Chronological Order).

(Publications selected from 38 peer-reviewed publications)

Chow, C. S.; Barton, J. K. “Shape-Selective Cleavage of $tRNA^{Phe}$ by Transition-Metal Complexes”, *J. Am. Chem. Soc.* **1990**, *112*, 2839–2840.

Chow, C. S.; Barton, J. K. “Transition Metal Complexes as Probes of Nucleic Acids”, *Methods Enzymol.* **1992**, *212*, 219–242.

Chow, C. S.; Behlen, L. S.; Uhlenbeck, O. C.; Barton, J. K. “Recognition of Tertiary Structure in tRNAs by $Rh(phen)_2phi^{3+}$: A New Reagent for RNA Structure-Function Mapping”, *Biochemistry* **1992**, *31*, 972–982.

Chow, C. S.; Barton, J. K. “Recognition of Unusual RNA Structures by Tris(4,7-diphenyl-1,10-phenanthroline) Rhodium(III)”, *Biochemistry* **1992**, *31*, 5423–5429.

Chow, C. S.; Bogdan, F. M. “A Structural Basis for RNA-Ligand Interactions”, *Chem. Rev.* **1997**, *97*, 1489–1513.

Bogdan, F. M.; Chow, C. S. “The Synthesis of Allyl- and Allyloxycarbonyl-Protected RNA Phosphoramidites. Useful Reagents for Solid-Phase Synthesis of RNAs with Base-Labile Modifications”, *Tet. Lett.* **1998**, *39*, 1897–1900.

Llano-Sotelo, B.; Chow, C. S. “RNA-Aminoglycoside Antibiotic Interactions: Fluorescence Detection of Binding and Conformational Change”, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 213–216.

Wu, J.; Taila, R.; Lamsen, L.; Chow, C. S. “Investigation of Guanidinium Group Involvement in RNA Recognition by Parallel *in Vitro* Selection of Streptomycin- and Arginine-Binding RNAs”, *Nucleic Acids Sym. Ser.* **1999**, *41*, 192–195.

Meroueh, M.; Grohar, P. J.; Qiu, J.; SantaLucia, J., Jr.; Scaringe, S. A.; Chow, C. S. “Unique Structural and Stabilizing Roles for the Individual Pseudouridine Residues in the 1920 Region of *Escherichia coli* 23S rRNA”, *Nucleic Acids Res*. **2000**, *28*, 2075–2083.

Chui, H. M.-P.; Meroueh, M.; Scaringe, S. A.; Chow, C. S. “Synthesis of a 3-Methyluridine Phosphoramidite to Investigate the Role of Methylation in a Ribosomal RNA Hairpin”, *Bioorg. Med. Chem.* **2002**, *10*, 325–332.

Haddad, J.; Kotra, L. P.; Llano-Sotelo, B.; Kim, C.; Azucena, E. F., Jr.; Liu, M.; Vakulenko, S. B.; Lee, H.; Chow, C. S.; Mobashery, S. “Design of Novel Antibiotics that Bind to the Bacterial Ribosomal Acyltransfer Site”, *J. Am. Chem. Soc.* **2002**, *124*, 3229–3237.

Llano-Sotelo, B.;Azucena, E. F.; Kotra, L. P.; Mobashery, S.; Chow, C. S. “Aminoglycosides Modified by Resistance Enzymes Display Diminished Binding to the Bacterial Ribosomal Acyl-Transfer Site”, *Chem. Biol.* **2002**, *9*, 455–463.

Liu, M.; Amro, N. A.; Chow, C. S.; Liu, G.-Y. “Production of Nanostructures of DNA on Surfaces”, *Nanoletters* **2002**, *2*, 863–867.

Chow, C. S.; Cunningham, P. R.; Lee, K.-S-; Meroueh, M.; SantaLucia, J., Jr.; Varma, S. “Photoinduced Cleavage by a Rhodium Complex at G-U Mismatches and Exposed Guanines in Large and Small RNAs”, *Biochimie* **2002**, *84*, 859–868.

Chui, H. M.-P.; Desaulniers, J.-P.; Scaringe, S. A.; Chow, C. S. “Synthesis of Helix 69 of *E. coli* 23S rRNA Containing its Natural Modified Nucleosides, $m^3\Psi$ and Ψ”, *J. Org. Chem.* **2002**, *67*, 8847–8854.

Desaulniers, J.-P.; Ksebati, B.; Chow, C. S. "Synthesis of ^{15}N-Enriched Pseudouridine Derivatives", *Org. Lett.* **2003**, *5*, 4093–4096.

Esho, N.; Desaulniers, J.-P.; Davies, B.; Chui, H. M.-P.; Rao, M. S.; Chow, C. S.; Szafert, S.; Dembinski, R. "NMR Conformational Analysis of p-Tolyl Furanopyrimidine 2'-Deoxyribonucleoside and Crystal Structure of its 3',5'-Di-O-acetyl Derivative", *Bioorg. Med. Chem.* **2005***, 13*, 1231–1238.

C. Research Support.

Ongoing Research Support

Principal Investigator: Chow, Christine S.
Agency: NIH
Type: R01 (GM054632)
Period: 03/01/2003 - 11/28/2006
Title: "A Study of Site-Specifically Modified RNAs"
The goal of this project is to understand the role of the modified nucleoside pseudouridine.

Principal Investigator: Mobashery, Shahriar (Co-investigator: Chow, Christine S.)
Agency: NIH
Type: R01 (AI055496)
Period: 07/01/2003 - 12/31/2007
Title: "Ribosomal Function and Antibiotic Design"
The goal of this project is to understand the role of RNA dynamics in aminoglycoside binding, and to design new aminoglycoside antibiotics that avoid resistance.

Principal Investigator: Cunningham, Philip (Co-investigator: Chow, Christine S., SantaLucia, John, and Spaller, Mark)
Agency: NIH
Type: U01 (AI061192)
Period: 07/15/2004 - 06/30/2008
Title: "Anti-Infectives that Target Bacterial Ribosomes"
The goal of this project is to identify new anti-infectives for the treatment of infections caused by Yersinia pestis and other potential bioterrorism agents.

Completed Research Support

Principal Investigator: Chow, Christine S.
Agency: NIH
Type: S10 (RR16771)
Period: 05/01/02 – 04/30/03
Title: "Purchase of a MALDI-TOF Mass Spectrometer"

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education)*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY

Ongoing Research Support

BIOGRAPHICAL SKETCH

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NAME	POSITION TITLE
eRA COMMONS USER NAME	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY

A. Positions and Honors

C. Research Support

Ongoing Research Support

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY

A. Positions and Honors.

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ❍ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.		Cheryl	Isaac	Murphy	PhD	PD/PI		12					
2.		Ian		MacNeil	PhD	Microbiologist		12					

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** **179,685.00**

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Structural Chemist	8					
2	Research Associate	12					
3	**Total Number Other Personnel**					**Total Other Personnel**	**142,560.00**
						Total Salary, Wages and Fringe Benefits (A+B)	**322,245.00**

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 04/30/2008

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ○ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)
1. PCR cycler	7,500.00
2. Spectrophotometer (nanodrop)	7,000.00
3. SpeedVac	4,500.00
4. HPLC and columns	15,000.00
5. Milli-Q water system	4,000.00

Total funds requested for all equipment listed in the attached file

Total Equipment **38,000.00**

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	4,200.00
2. Foreign Travel Costs	
Total Travel Cost	**4,200.00**

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 1

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ○ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	40,480.00
2. Publication Costs	
3. Consultant Services	8,000.00
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	56,614.00
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Sample sequencing costs	15,000.00
9. Synthesis of RNA molecules	15,000.00
Total Other Direct Costs	**135,094.00**

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	**499,539.00**

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. Modified Total Direct Costs/will negotiate rate			
2. Wayne State F&A off-site			
3.			
		Total Indirect Costs	**439,325.00**

Cognizant Federal Agency None

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	**938,864.00**

J. Fee	**Funds Requested ($)**
	65,720.00

K. * Budget Justification File Name: 5383-justification.pdf Mime Type: application/pdf

(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

Personnel:
Cheryl Murphy (_ % effort in year 1, _ % in year 2) is the Principal Investigator. She has 16 years of experience in the biotechnology area, with expertise in drug discovery and development in the field of infectious disease (*S. pneumonia*, *S. aureus*, malaria, tuberculosis, HIV and ehrlichiosis). As PI, Dr. Murphy will be responsible for coordinating and supervising the work outlined in the specific aims of this project, and for writing the research summary reports. With other personnel, she will write and submit publications reporting the results of the work.

Ian MacNeil (_ % effort in years 1 and 2) is a cellular biologist with 18 years of experience in the pharmaceutical/biotechnology industry. He will be responsible for the saturation mutagenesis studies, assay development, high throughput compound screening, and *in vitro* and *in vivo* assessments of compound activity.

RiboNovix plans to hire a Ph.D. level structural chemist who will be responsible for the selection and preparation of chemical libraries, high throughput compound screening, structural analysis (in collaboration with Dr. Santa-Lucia at WSU), and compound optimization work. Two B.S. level scientists (one with a chemistry/screening and the other a biology/assay background) will be hired to perform assay development, high throughput screening and *in vitro* and *in vivo* assay work.

Equipment: We are requesting funds for the purchase of the following equipment:
PCR cycler $7,500; RiboNovix has partial use of a thermocycler in the facility it is currently leasing, but will need to have a dedicated instrument for this project.
Spectrophotometer (made by Nanodrop, especially for use with small volumes) $7,000
SpeedVac $4,500; This instrument is necessary for drying small samples. RiboNovix does not have access to one currently.
Used HPLC and columns $15,000; The equipment is necessary for sample purification.
Milli-Q water purification system $4,000; The equipment will be used to purify water used in experimental assays.
Total: $38,000

In year 2 we request funds for the following:
Microdrop dispenser $5,000; This will be used in sample preparation for pcr and fluorescence assays.
Total, $5,000

Travel: Travel costs for scientists between Boston and Detroit (airfare and per diem at $1,000 each, 3 people/ trips per year, one night stay), and Boston and _ (I airfare and per diem at $1,200 each year, 2 night stay). Total $4,200 per year. Travel will be for project meetings between RiboNovix scientists and scientists listed in each consortium.

Supplies:
General lab supplies includes all disposable items (pipettes, tips, 96 and 384 well plates, tubes, plastics, media, miscellaneous chemicals and reagents, enzymes, radioisotopes, oligonucleotides, etc.
Small lab equipment includes small items (<$1000) and glassware etc.
Total for supplies calculated at 20% of salary. Total year 1: $40,480, year 2: $54,912

Consultants:
Four consultants will provide services for this project as follows:
Christine Chow (Wayne State University) will provide advice for fluorescence assays and modified RNA synthesis. _ will provide RiboNovix with a small library of RNA-binding molecules and will provide advice on screening with RNA targets. _ will also provide us with molecules for screening and will provide advice on medicinal chemistry. _ will provide assistance in phage/peptide library screening. Each consultant will provide _ days of service at $_ per day.

Contractual Expenses: Much of the sequencing required to analyze each viable mutant of the target sites chosen will be done on a contract basis. We have worked with SeqWright (Texas) previously but will obtain additional quotes in order to ensure we get the best price. The cost for sequencing 3-500 mutant RNAs will be

about $15,000 and will take place in year 1 of the grant period. Synthesis of the target RNAs will be carried out by a third party contractor. We have worked with Dharmacon, but again quotes will be obtained from other RNA manufacturers in order to obtain the best price. Each RNA will cost $1000 -1500 to make, depending on the size and complexity. We estimate that total RNA synthesis costs will be $15,000 in year 1 as we will be evaluating a number of clusters, and $5,000 in year 2.

Consortium Costs: Two consortia will be formed, one with Wayne State with Dr. Cunningham as Head, and the other with _ .

In the WSU consortium funds are requested for _ , a research assistant in Dr Cunningham's laboratory, (_ % effort in year 1) to provide support for the saturation mutagenesis studies.
In addition, funds are requested for _ , a graduate student, (_ % effort in year 1 and _ % effort in year 2) who will help with the structural and NMR analysis of the RNA target and RNA target/hit complexes. In addition, WSU requires us to support tuition expenses for students. Drs. Cunningham and Santa-Lucia are funded at _ % each, for each of the 2 years to provide oversight to the work. The costs also include purchasing access to the 700 MHz NMR at WSU in both years, and supplies and tuition costs. We are requesting a total of $61,359 for the WSU consortium in year 1.
The WSU consortium costs for year 2 will total $38,205.

The total for both consortia is year 1, $91,014 and year 2, $38,205.

Indirect Costs and Fee:
RiboNovix does not yet have a negotiated F&A rate, but is using a provisional rate of _ % based on cost calculations.
In year 2, we have included a 4% cost of living increase. We request a 7% fee consistent with the profit margin of other projects of this scope.

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 2

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ❍ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.		Cheryl	Isaac	Murphy	PhD	PD/PI		12					
2.		Ian		MacNeil		Microbiologist		12					

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** **166,795.00**

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Structural chemist	12					
2	Research Associates	24					
3	**Total Number Other Personnel**					**Total Other Personnel**	**234,748.00**
						Total Salary, Wages and Fringe Benefits (A+B)	**401,543.00**

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 04/30/2008

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 2

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ○ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)
1. Microdrop dispenser	5,000.00

Total funds requested for all equipment listed in the attached file

Total Equipment **5,000.00**

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	4,200.00
2. Foreign Travel Costs	
Total Travel Cost	**4,200.00**

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	

Number of Participants/Trainees **Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 2

* ORGANIZATIONAL DUNS: 1523533760000

*** Budget Type:** ● Project ○ Subaward/Consortium

Enter name of Organization: RiboNovix, Inc.

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	54,912.00
2. Publication Costs	
3. Consultant Services	4,000.00
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	25,318.00
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Synthesis of RNA molecules	5,000.00
Total Other Direct Costs	**89,230.00**

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	**499,973.00**

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. Modified Total Direct Costs/will negotiate rate			
2. Wayne State University F&A off-site			
		Total Indirect Costs	**482,542.00**

Cognizant Federal Agency None

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	**982,515.00**

J. Fee	**Funds Requested ($)**
	68,776.00

K. * Budget Justification File Name: 5383-justification.pdf Mime Type: application/pdf

(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		**346,480.00**
Section B, Other Personnel		**377,308.00**
Total Number Other Personnel	6	
Total Salary, Wages and Fringe Benefits (A+B)		**723,788.00**
Section C, Equipment		**43,000.00**
Section D, Travel		**8,400.00**
1. Domestic	8,400.00	
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		**224,324.00**
1. Materials and Supplies	95,392.00	
2. Publication Costs		
3. Consultant Services	12,000.00	
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs	81,932.00	
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	20,000.00	
9. Other 2	15,000.00	
10. Other 3		
Section G, Direct Costs (A thru F)		**999,512.00**
Section H, Indirect Costs		**921,867.00**
Section I, Total Direct and Indirect Costs (G + H)		**1,921,379.00**
Section J, Fee		**134,496.00**

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.		Philip		Cunningham	PhD	Consortium Head		12					
2.		John		SantaLucia	PhD	Structural Chemist		12					

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** **9,263.00**

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
	Post Doctoral Associates						
1	Graduate Students	12					
	Undergraduate Students						
	Secretarial/Clerical						
1	Technician	12					
2	**Total Number Other Personnel**					**Total Other Personnel**	**21,962.00**
						Total Salary, Wages and Fringe Benefits (A+B)	**31,225.00**

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 03/31/2005

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)
Total funds requested for all equipment listed in the attached file	
Total Equipment	

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	
Number of Participants/Trainees **Total Participant/Trainee Support Costs**	**0.00**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	4,000.00
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. tuition	1,937.00
9. NMR time	3,500.00
Total Other Direct Costs	**9,437.00**

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	**40,662.00**

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. Total Direct Costs			
		Total Indirect Costs	**20,697.00**

Cognizant Federal Agency none

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	**61,359.00**

J. Fee	**Funds Requested ($)**

K. * Budget Justification	File Name: 9068-WSU justif.pdf	Mime Type: application/pdf
	(Only attach one file.)	

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

A. Senior/Key Person

	Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.		Philip		Cunningham	PhD	Consortium Head		12					
2.		John		SantaLucia	PhD	Structural chemist							

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** **9,633.00**

B. Other Personnel

* Number of Personnel	* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
	Post Doctoral Associates						
1	Graduate Students	12					
	Undergraduate Students						
	Secretarial/Clerical						
1	**Total Number Other Personnel**					**Total Other Personnel**	**8,216.00**
						Total Salary, Wages and Fringe Benefits (A+B)	**17,849.00**

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 03/31/2005

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	* Funds Requested ($)

Total funds requested for all equipment listed in the attached file

Total Equipment

Additional Equipment: File Name: Mime Type:

D. Travel	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	
Number of Participants/Trainees	**Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 2

*** ORGANIZATIONAL DUNS:** 0019622240000

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization: Wayne State University

*** Start Date:** 07-01-2007 *** End Date:** 06-30-2008 **Budget Period: 2**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	3,000.00
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. tuition	969.00
9. NMR time	3,500.00
Total Other Direct Costs	**7,469.00**

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	**25,318.00**

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. Total Direct Costs			
		Total Indirect Costs	**12,887.00**

Cognizant Federal Agency none

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	**38,205.00**

J. Fee	**Funds Requested ($)**

K. * Budget Justification	File Name: 9068-WSU justif.pdf	Mime Type: application/pdf
	(Only attach one file.)	

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		**18,896.00**
Section B, Other Personnel		**30,178.00**
Total Number Other Personnel	3	
Total Salary, Wages and Fringe Benefits (A+B)		**49,074.00**
Section C, Equipment		
Section D, Travel		
1. Domestic		
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		**16,906.00**
1. Materials and Supplies	7,000.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	2,906.00	
9. Other 2	7,000.00	
10. Other 3		
Section G, Direct Costs (A thru F)		**65,980.00**
Section H, Indirect Costs		**33,584.00**
Section I, Total Direct and Indirect Costs (G + H)		**99,564.00**
Section J, Fee		

Justification – Wayne State University

In the WSU consortium funds are requested for _ , a research assistant in Dr Cunningham's laboratory, (_ % effort in year 1) to provide support for the saturation mutagenesis studies.

In addition, funds are requested for _ , a graduate student, (_ % effort in year 1 and _ % effort in year 2) who will help with the structural and NMR analysis of the RNA target and RNA target/hit complexes. In addition, WSU requires us to support tuition expenses for students. Drs. Cunningham and Santa-Lucia are funded at _ % each, for each of the 2 years to provide oversight to the work. The costs also include purchasing access to the 700 MHz NMR at WSU in both years, and supplies and tuition costs. We are requesting a total of $61,359 for the WSU consortium in year 1.
. The WSU consortium costs for year 2 will total $38,205.

RESEARCH & RELATED BUDGET - SECTION A & B, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:**

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization:

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

A. Senior/Key Person

Prefix	* First Name	Middle Name	* Last Name	Suffix	* Project Role	Base Salary ($)	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits ($)	* Funds Requested ($)
1.												

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name: Mime Type: **Total Senior/Key Person** **1,958.00**

B. Other Personnel

* Number of Personnel		* Project Role	Cal. Months	Acad. Months	Sum. Months	* Requested Salary ($)	* Fringe Benefits	* Funds Requested ($)
	Post Doctoral Associates							
	Graduate Students							
	Undergraduate Students							
	Secretarial/Clerical							
1	Chemist		6					
1	**Total Number Other Personnel**					**Total Other Personnel**		**12,995.00**
						Total Salary, Wages and Fringe Benefits (A+B)		**14,953.00**

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Tracking Number:

OMB Number: 4040-0001
Expiration Date: 03/31/2005

RESEARCH & RELATED BUDGET - SECTION C, D, & E, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:**

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization:

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	*** Funds Requested ($)**
Total funds requested for all equipment listed in the attached file	
	Total Equipment

Additional Equipment: File Name: Mime Type:

D. Travel	**Funds Requested ($)**
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs	**Funds Requested ($)**
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other:	
Number of Participants/Trainees	**Total Participant/Trainee Support Costs**

RESEARCH & RELATED Budget {C-E} (Funds Requested)

RESEARCH & RELATED BUDGET - SECTIONS F-K, BUDGET PERIOD 1

*** ORGANIZATIONAL DUNS:**

*** Budget Type:** ❍ Project ● Subaward/Consortium

Enter name of Organization:

*** Start Date:** 07-01-2006 *** End Date:** 06-30-2007 **Budget Period: 1**

F. Other Direct Costs	**Funds Requested ($)**
1. Materials and Supplies	1,000.00
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
Total Other Direct Costs	**1,000.00**

G. Direct Costs	**Funds Requested ($)**
Total Direct Costs (A thru F)	**15,953.00**

H. Indirect Costs

Indirect Cost Type	**Indirect Cost Rate (%)**	**Indirect Cost Base ($)**	*** Funds Requested ($)**
1. Total Direct Costs			
		Total Indirect Costs	**13,703.00**

Cognizant Federal Agency none

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	**Funds Requested ($)**
Total Direct and Indirect Institutional Costs (G + H)	**29,656.00**

J. Fee	**Funds Requested ($)**

K. * Budget Justification File Name:

(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		**1,958.00**
Section B, Other Personnel		**12,995.00**
Total Number Other Personnel	1	
Total Salary, Wages and Fringe Benefits (A+B)		**14,953.00**
Section C, Equipment		
Section D, Travel		
1. Domestic		
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		**1,000.00**
1. Materials and Supplies	1,000.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1		
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		**15,953.00**
Section H, Indirect Costs		**13,703.00**
Section I, Total Direct and Indirect Costs (G + H)		**29,656.00**
Section J, Fee		

Justification –

SBIR/STTR Information

OMB Number: 0925-0001
Expiration Date: 09/30/2007

*** Program Type (select only one)**

● SBIR ○ STTR

○ Both *(See agency-specific instructions to determine whether a particular agency allows a single submission for both SBIR and STTR)*

*** SBIR/STTR Type (select only one)**

○ Phase I ● Phase II

○ Fast-Track *(See agency-specific instructions to determine whether a particular agency participates in Fast-Track)*

Questions 1-7 must be completed by all SBIR and STTR Applicants:

● Yes ○ No	* 1. Do you certify that at the time of award your organization will meet the eligibility criteria for a small business as defined in the funding opportunity announcement?
○ Yes ● No	* 2. Does this application include subcontracts with Federal laboratories or any other Federal Government agencies? * If yes, insert the names of the Federal laboratories/agencies:
○ Yes ● No	* 3. Are you located in a HUBZone? To find out if your business is in a HUBZone, use the mapping utility provided by the Small Business Administration at its web site: http://www.sba.gov
● Yes ○ No	* 4. Will all research and development on the project be performed in its entirety in the United States? If no, provide an explanation in an attached file. * Explanation:
○ Yes ● No	* 5. Has the applicant and/or Program Director/Principal Investigator submitted proposals for essentially equivalent work under other Federal program solicitations or received other Federal awards for essentially equivalent work? * If yes, insert the names of the other Federal agencies:
● Yes ○ No	* 6. Disclosure Permission Statement: If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?
	* 7. Commercialization Plan: If you are submitting a Phase II or Phase I/Phase II Fast-Track Application, include a Commercialization Plan in accordance with the agency announcement and/or agency-specific instructions. * Attach File: 4453-comm plan.pdf

SBIR/STTR Information

OMB Number: 0925-0001
Expiration Date: 09/30/2007

SBIR-Specific Questions:

Questions 8 and 9 apply only to SBIR applications. If you are submitting ONLY an STTR application, leave questions 8 and 9 blank and proceed to question 10.

○ Yes ● No	* 8. Have you received SBIR Phase II awards from the Federal Government? If yes, provide a company commercialization history in accordance with agency-specific instructions using this attachment. * Attach File:
● Yes ○ No	* 9. Will the Project Director/Principal Investigator have his/her primary employment with the small business at the time of award?

STTR-Specific Questions:

Questions 10 and 11 apply only to STTR applications. If you are submitting ONLY an SBIR application, leave questions 10 and 11 blank.

○ Yes ○ No	* 10. Please indicate whether the answer to BOTH of the following questions is TRUE: (1) Does the Project Director/Principal Investigator have a formal appointment or commitment either with the small business directly (as an employee or a contractor) OR as an employee of the Research Institution, which in turn has made a commitment to the small business through the STTR application process; AND (2) Will the Project Director/Principal Investigator devote at least 10% effort to the proposed project?
○ Yes ○ No	* 11. In the joint research and development proposed in this project, does the small business perform at least 40% of the work and the research institution named in the application perform at least 30% of the work?

Attachments

NonDomesticPerformanceExplanation

File Name	Mime Type

CommercializationPlan

File Name	Mime Type
4453-comm plan.pdf	application/pdf

SBIR

File Name	Mime Type

J. Commercialization Plan

1. Value of the SBIR Project, Expected Outcomes and Impact.

In 1996, 17 million people died from infectious diseases worldwide. The annual economic burden of infectious disease in the United States has been estimated to exceed $30 billion [NIAID fact sheet]. While antibiotics have proven to be remarkable drugs for the treatment of bacterial infections, the rapid rise in antibiotic resistance poses a threat to human health worldwide and has created a large market for the development of novel anti-infectives. Thus, there are significant humanitarian and economic incentives for developing new drug-discovery technologies. The goal of RiboNovix is to develop new anti-infectives that are safe and effective, and that specifically address the issue of antibiotic resistance.

Approximately half of all naturally occurring antibiotics affect protein synthesis, and most of these target ribosomes. Mutations in ribosomal RNA (rRNA) are often lethal because they affect the production of all cellular proteins. Importantly, human and bacterial rRNAs are sufficiently different to allow specific drug interactions. Thus, bacterial rRNA is an ideal drug target and the modular nature of rRNA facilitates the development of in vitro assays, structure determination, and molecular-modeling studies for drug design.

Naturally occurring compound classes that have been used as antibiotics include tetracyclines (targeting 16S rRNA), aminoglycosides (16S rRNA), lincomycins (23S rRNA), macrolides (23S rRNA) chloramphenicol (23S rRNA) and the newest class of antibiotics, the oxazolidinones (23S rRNA). Resistance to these naturally occurring antibiotics is typically acquired by one of two mechanisms, either by transferal of resistance from other bacteria or spontaneously through a mutation in the bacterial chromosome. For naturally occurring antibiotics that target rRNA, transferred resistance often involves a mobile genetic element such as a plasmid that encodes an enzyme that alters the antibiotic or, in some cases, pumps the antibiotic out of the cell before it can reach its target. Most spontaneous, chromosomal mutations that lead to resistance are mutations of the drug target that prevent its recognition by the antibiotic. Chemically synthesized antibiotics are also susceptible to development of drug resistance. However, resistance in this case is usually primarily due to chromosomal mutation of the drug binding site.

Although antibiotic resistance has been a problem for decades, only recently has the occurrence of resistance spread to virtually every common bacterial strain. More than 70 percent of the bacteria responsible for hospital-acquired infections are resistant to at least one of the drugs commonly used to fight them (IDSA, 2004). These resistant bacteria are not only a problem for hospitalized patients, but have spread to otherwise healthy individuals in communities in the United States and other countries. Infections caused by drug-resistant bacteria are also much more expensive to treat, resulting from increased hospitalization, use of higher cost antibiotics, lost work days, and death.

The development of drug resistance has led to significant investment in the discovery and development of new antibiotics. However, only two new antibiotic classes have been introduced in recent years - the oxazolidinones in 2000, and the lipopeptides in 2003. The majority of new drugs are next generation drugs that are structurally similar to existing antibiotics, but with an improved therapeutic index, such as increased potency or decreased toxicity. Since their therapeutic activity is the result of a common mode of action, they are very susceptible to the development of drug resistance by mechanisms common to the structural class. At the same time, many pharmaceutical companies have spun off, reduced, or halted their anti-infective discovery efforts. This has led to a dramatic decrease in the number of new antibiotics moving through the drug development pipeline. In 2004, two antibiotics were approved by the FDA – Xifaxan (rifaximin) and Ketek (telrithromycin).

Many groups have addressed the problem of antibiotic resistance by trying to discover new antimicrobials that work through novel mechanisms. There have been significant advances in the area of genomic information, molecular biology and screening methods, but these new technologies have yet to lead to a significant number of new drugs. More importantly, little new technology has been developed to address the critical issue of drug resistance. While drugs in development can be tested against known resistant clinical isolates, this does not give any information about what new mutants might arise when a new antibiotic is introduced to the market.

RiboNovix' proprietary technology provides, for the first time, a way to rapidly isolate, identify and characterize all mutations in rRNA drug targets that do not severely impair protein synthesis and might therefore lead to antibiotic resistance. If the viable mutations of a rRNA drug target are known, the components of the target required for function can be identified and new drugs developed to specifically recognize the essential components of the target. These drugs should be significantly less susceptible to resistance than those recognizing only the wild-type target.

While the lack of novel technology to address antibiotic resistance has led some pharmaceutical and biotechnology companies to decrease or abandon efforts in antibiotic research, there have been recent signs of increased interest with two significant acquisitions. In 2005, J & J acquired Peninsula Pharmaceuticals and Pfizer bought Vicuron Pharmaceuticals. A number of biotech companies have successfully launched new antibiotics for hospital-acquired infections – Cubist Pharmaceuticals and Oscient Pharmaceuticals. (See competitors below).

When venture capitalists evaluate investment in companies developing antibiotics, they want to know how the issue of drug resistance is being addressed. As a result, RiboNovix believes that SBIR funding will play an important role in allowing the company to advance its technology and achieve a proof of concept. The combination of the increased medical need for new antibiotics, and the need to combat antibiotic resistance, provides a compelling opportunity for RiboNovix to pursue its innovative drug discovery technology leading to the successful development of new antibiotics refractory to drug resistance.

2. Company.

RiboNovix, Inc., was founded in 2003 to discover and develop new anti-infectives that are less susceptible to antibiotic resistance, using a novel functional genomics platform technology developed in the laboratories of Dr. Philip R. Cunningham at Wayne State University. In 2004 the company began work developing Dr. Cunningham's technology under a Phase I SBIR grant. The Company broadened its access to technologies in the anti-infectives field through the establishment of a scientific board, bringing in leading researchers from other institutions including The Scripps Research Institute, Harvard Medical School and the University of Illinois. In 2005, a research collaboration was established with Scripps, under a second Phase I SBIR. RiboNovix established its own research laboratories in Massachusetts in 2005.

Current research efforts at RiboNovix involve the use of its combinatorial genetic technology to identify new ribosomal drug targets in *E. coli* and *Pseudomonas aeroginosa*. Research ongoing at Wayne State University in the laboratory of Dr Cunningham includes extension of the technology to other pathogens such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococci* and a group of biodefense pathogens, *Yersinia pestis*, *Bacillus anthracis* and *Francisella tularensi*. Programs are also planned for a series of third world diseases such as *Vibrio cholerae*, *Salmonella enterica*, and *Shigella dysenteriae*. Through its license with Wayne State, RiboNovix has rights to all inventions in the field developed as the result of the research in Dr. Cunningham's laboratory over a _ year period.

The Scripps program is comprised of research to develop a target specific assay. Again, RiboNovix has rights to use the inventions from this collaboration.

RiboNovix' board of scientific advisors includes leading researchers in the scientific disciplines of RNA structure and biology, functional genomics and medicinal chemistry, with specific experience in the fields of infectious diseases and antibiotic resistance. Dr. Cunningham is Chair of the Science Board. His laboratory specializes in microbial genetics in the field of protein synthesis, and in the areas of molecular biology, biochemistry and microbial physiology.

Other science advisors include Alexander Mankin, Ph.D., who is Professor, Center for Pharmaceutical Biotechnology, Department of Medicinal Chemistry at the University of Illinois, Chicago. He is an internationally recognized leader in the study of ribosome function, protein synthesis and the development of drug resistance. James Williamson, Ph.D., is a Professor in the Department of Molecular Biology and the

Skaggs Institute for Chemical Biology at the Scripps Research Institute. Dr. Williamson's laboratory uses a variety of biochemical and biophysical techniques, including NMR spectroscopy, to characterize structure and folding of RNA molecules and RNA/protein complexes. Gerald Beltz, Ph.D., is in the Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, where he is involved in Harvard's program on Biodefense. He has 20 years of experience in the development of products to treat and prevent infectious disease and cancers. John SantaLucia Jr., Ph.D., is Associate Professor, Department of Chemistry, Wayne State University. His laboratory specializes in solution structure determination by NMR and nucleic-acid folding thermodynamics. Christine Chow, Ph.D., Professor in the Department of Chemistry at Wayne State University, is developing new synthetic strategies for the site-specific incorporation of modified nucleosides into RNA and examining small molecule-nucleic acid interactions. Mark Spaller, Ph.D., Assistant Professor, Department of Chemistry, Wayne State University, specializes in the areas of combinatorial chemical synthesis, peptidomimetic chemistry and rational drug design. John Montgomery, Ph.D., Professor of Chemistry at the University of Michigan, is an expert in organic synthesis.

RiboNovix' business team is made up of experienced biotechnology executives. Alison Taunton-Rigby, Ph.D., O.B.E. is a Founder, Director, and serves as President and Chief Executive Officer. She has been a senior executive in the healthcare industry for over 25 years, and was previously CEO of Aquila Biopharmaceuticals, Cambridge Biotech Corporation, and Mitotix Inc., and has held senior roles at Genzyme Corporation and Biogen Inc. Cheryl Murphy, Ph.D., is Vice President, Technology Development. She has held senior technology and management positions at Antigenics, Inc., Aquila Biopharmaceuticals and Cambridge Biotech Corporation. Both Dr. Taunton-Rigby and Dr. Murphy have extensive experience with the drug development process. Aquila and CBC had multiple products approved and in clinical development, many in the field of infectious diseases. In addition, Dr. Taunton-Rigby led the team that developed and launched Ceredase® and Cerezyme® at Genzyme.

RiboNovix' core competency is drug development. The company's goal is to discover anti-infective drug candidates and develop them through proof of concept clinical trials (Phase II). RiboNovix is using a combination of grant funding and venture capital for initial funding, and will use licensing agreements, strategic alliances and partnerships with pharmaceutical firms and other biotechnology companies for late stage clinical trials (Phase III), FDA approval, and marketing and distribution of the drug products (see section 6).

3. Market, Customers and Competition.

3.1 Market Size. The market for antibiotics is large with current sales of $25 billion worldwide, and $8 billion in the US. There are over 150 products on the market, with a significant number of very successful drugs, each with sales in the hundreds of millions of dollars. Five antibiotics have revenues of over $1 billion annually. The market is growing at 9% per year and is expected to grow even faster in the next decade. RiboNovix believes that a new, novel, broad-spectrum antibiotic that is effective against resistant strains of bacteria, could achieve $1 billion in sales worldwide within a few years following launch by a pharmaceutical partner. A narrow-spectrum antibiotic that addressed a particular pathogen could achieve sales of $250 million on a worldwide basis, and is more likely to be marketed directly by a biotechnology company.

Of the antibiotics on the market today, cephalosporins make up the largest structural class with macrolides, penicillins and quinolones representing the bulk of the other classes. The drugs within each structural class act by a common mechanism. For example, penicillins and cephalosporins act by inhibiting cell wall synthesis. The quinolones act by blocking a key enzyme called DNA topoisomerase. Macrolides, tetracyclines and aminoglycosides inhibit protein synthesis. One of the new classes of antibiotics developed in the last few years are the oxazolidinones, which also inhibit protein synthesis, but through a different mechanism.

Top Antibiotics by FY2004 Sales (dollars in billions)

Antibiotic	FY2004 Sales	Indications
Ciprobay/Cipro (Bayer)	$1.09	Urinary tract infections (UTIs), cystitis, chronic bacterial prostatitis, gonorrhea, lower respiratory tract infections, infectious diarrhea
Augmentin (GlaxoSmithKline)	$2.57	Bacterial sinusitis, pneumonia, acute otitis media, lower respiratory tract infections, UTIs, skin and skin structure infections
Zithromax (Pfizer)	$1.85	Chronic obstructive pulmonary disease, pneumonia, pharyngitis/tonsillitis, skin and skin structure infections, urethritis, cervicitis, genital ulcer disease, acute otitis media
Rocephin (Roche)	$1.12	Lower respiratory tract infections, acute bacterial otitis media, skin and skin structure infections, UTIs, gonorrhea, pelvic inflammatory disease, bacterial septicemia, bone and joint infections, intra-abdominal infections, meningitis, surgical prophylaxis
Biaxin/Clarithromycin (Abbott)	$1.18	Pharyngitis/tonsillitis, sinusitis, bronchitis, pneumonia, skin and skin structure infections, acute otis media

Source: Company public SEC filings

In spite of the large number of very successful antibiotics, drug resistance is a significant issue that leads to a constant need to discover and develop new, effective antibiotics. Drug-resistant bacterial pathogens are a major public health concern. Many bacterial infections are becoming untreatable with existing antibiotics, and the majority of hospital-acquired infections involve drug resistant pathogens. The CDC has estimated that 28% of the bacteria that most often cause hospital-acquired infections are resistant to the preferred antibiotic. Bacteria have a fairly simple genetic composition, and because they replicate quickly, they evolve quite rapidly. Bacteria adapt quickly to each new environment, and a single gene mutation can affect a cell's ability to withstand an antibiotic.

The rapid development of resistance is primarily the result of overuse of antibiotics. The CDC has estimated that about 50% of antibiotic prescriptions are unnecessary; they are often ineffective prescriptions for viral infections given as a result of pressure from patients. Furthermore, many patients do not use their prescriptions properly and do not complete the course of treatment, allowing microbes to adapt rather than be killed. In addition, doctors rely on broad-spectrum antibiotics for treatment, rather than undertake the work to enable a definitive diagnosis and identification of the specific bacteria to treat. This provides a selective advantage to bacteria that harbor drug resistance. Other factors include the increased use of antibiotics in third world countries and the widespread use of antibiotics in animal feed, which has lead to the development of antibiotic resistant strains of *Escherichia* coli and *Salmonella*.

This market needs new technology, as there is a significant unmet medical need due to antibiotic resistance. Newer antibiotics typically cost more, and patients infected with drug resistant pathogens often require repeated treatment. Pharmaceutical companies traditionally prefer to market broad-spectrum antibiotics through their large sales forces. Interestingly, a number of biotechnology companies have developed and are now directly marketing narrow spectrum antibiotics for niche markets through their own sales and marketing organizations. Examples include Cubist Pharmaceuticals and Oscient Pharmaceuticals (see section 3.3).

The total annual cost of treating drug resistant infections is estimated by the Institute of Medicine to be $30 billion in the US. The CDC has estimated that the current costs related to the treatment of infections by antibiotic resistant pathogens, is over $4 billion annually. Newer antibiotics typically cost more and infected patients often require repeated treatments. Frequently, patients with resistant infections end up in the hospital with secondary infections. Side effects, such as allergic reactions, also add to the costs. By identifying and targeting the "signature" rRNA sequences that are unique to a particular bacterial species or group of bacteria, RiboNovix believes it can develop new anti-infectives that affect only disease causing bacteria and not helpful bacteria, thus reducing many of the side effects commonly observed when treating patients.

Market Applications for Target Pathogens

Pathogen	Infections	Patients per year, U.S.	Primary Antibiotics (% Resistance)
***Escherichia* coli**	Food poisoning, urinary tract infections	73,000	Ampicillin (30%), Bacterim
***Pseudomonas* aeruginosa**	Cystic Fibrosis, skin and lung infections	198,000	Cipro (23%), Ampicillin, Imipenem (21%)
Enterococci	Urinary tract and wound infections, bacteremia	175,000	Ampicillin or Amoxicillin, Vancomycin (25%)
***Helicobacter* pylori**	Stomach ulcers	3,600,000	Zithromax, Erythromycin
Salmonella	Food poisoning	39,574	Cipro, Bactrim, [Flagill]
***Staphylococcus* aureus**	Skin infections, endocarditis	2,000,000	Methicillin (34%), Vancomycin or Linezolid

Source: Centers for Disease Control, National Institutes of Health, National Foundation for Infectious Diseases

3.2 New Antibiotics. The first oxazolidinone, Zyvox®, was approved in 2000 and is marketed by Pfizer. The antibiotic is approved for the treatment of bacteremia caused by vancomycin resistant *Enterococcus faecium* (VREF), hospital acquired pneumonia, skin infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) and bacteremia associated with community-acquired pneumonia due to penicillin susceptible Streptococcus pneumoniae. While Zyvox has considerable activity against both Gram positive and Gram negative bacteria, it is only bacteriostatic, and does not show bacteriocidal activity.

Also notable was the approval in 1999 of Synercid®, which is a combination of quinupristin and dalfopristin. These two agents act synergistically to prevent protein synthesis by deactivating the bacterial ribosome. Synercid is approved for use against blood infections due to vancomycin resistant *Enterococcus faecium* (VREF) and skin infections caused by methicillin susceptible *Staphylococcus aureus* or *Streptococcus pyogenes*.

Aventis won approval in April 2004 for Ketek®, a member of the ketolide family of antibiotics. Ketolides are macrolide derivatives that act by attaching themselves to ribosomes and inhibiting protein synthesis While resistance to macrolides normally develops when the bacterium is able to alter the target site on the ribosome, Ketek® binds to two different sites on the ribosome and so the development of resistance may be more difficult. Abbott has a ketolide in Phase III (ABT-773).

In June 2005, Wyeth announced that the FDA approved Tygacil™ (tigecycline), a novel I.V. antibiotic with a broad spectrum of antimicrobial activity, including activity against the drug-resistant bacteria methicillin-resistant Staphylococcus aureus (MRSA). Tygacil is indicated for the treatment of complicated intra-abdominal infections (cIAI) and complicated skin and skin structure infections (cSSSI) in adults, and is described by Wyeth as a structurally distinct and re-engineered tetracycline. A modification of its core structure enables it to bind to the 30S ribosomal subunit with increased affinity compared with other tetracyclines. This allows it to overcome typical bacterial resistance to tetracyclines mediated by efflux pumps and ribosomal protection.

Other approaches in development target different mechanisms. Cubist is marketing Cubicin™, which acts by interfering with bacterial cell wall synthesis. Microbiotix is targeting enzymes involved in DNA replication such as Polymerase III. Several companies are developing efflux pump inhibitors to prevent the removal of antibiotics, lactoferrins that deprive bacteria of nutrients, and cationic peptides to compromise cell wall integrity. Oscient has recently begun selling Factive, an orally administered quilnolone that works by inhibiting two specific enzymes, DNA gyrase and DNA topoisomerase IV, which aid in bacterial DNA replication.

3.3. Competition

i. Pharmaceutical Companies. The market for anti-infectives has been dominated historically by the presence of many well-known pharmaceutical companies including Lilly, Pfizer, Bayer, Bristol-Myers Squibb, Glaxo Wellcome, Wyeth-Ayerst, and Ortho-McNeil. The products produced by these companies are equally well known and typically generate large returns because of their patent protection. Examples of these products include Fortaz (ceftazidime), Ceftin (cefuroxime axetil), Rochephin (cefuroxime axetil), Augmetin (amoxicillin), Biaxin (clarithromycin), Cipro (ciprofloxacin), and Zithromax (azithromycin). As the patents expire and drug resistance develops, limiting use of these drugs, pharmaceutical companies will look to the biotech companies to supply the next generation of novel anti-infective compounds. Significantly, while a number of pharmaceutical companies are investing strongly in new anti-infectives, a number of others, for example Lilly, are dropping out of the field.

In 2005, two significant pharmaceutical company acquisitions occurred.

Peninsula Pharmaceuticals was acquired by **Ortho MacNeil (J & J)** for $245 million**.** Peninsula is currently developing two product candidates: doripenem for injection, licensed from Shionogi and Co., Ltd., and PPI-0903 licensed from Takeda Chemical Industries, Ltd. Doripenem is a synthetic carbapenem antibiotic currently in six Phase III clinical trials for the treatment of serious bacterial infections in hospitalized patients. PPI-0903 is a member of the cephalosporin class of beta-lactam antibiotics and the company has recently completed a phase I clinical trial of the drug.

Vicuron Pharmaceuticals was acquired by **Pfizer** for $1.9 billion. Vicuron is developing antifungal and antibiotic agents. NDA's have been filed with the FDA for its lead products, Anidaulafungin and Dalbavancin. The company research is based on functional genomics, natural product discovery, mechanism based drug design and combinatorial and medicinal chemistry. Vicuron has facilities in Pennsylvania and California in the US and in Gerenzano and Pisticci in Italy.

ii. Biotechnology Companies. Start-up activity and investment in the biotech anti-infective space has been heavy over the past few years, with over 15 new companies started and over $400M of investment. Many of the biotech companies developing anti-infective drug candidates are concentrating on the ribosome. This fact is not a surprise given the large number of current antibiotics that target the ribosome and the scientific community's recent discoveries concerning its structure and function.

Rib-X, Inc. is a Yale University spinout founded in the fall of 2000. The company focuses on developing antibiotics through the use of structure-based research on the 50S subunit of the ribosome. The company is located in New Haven, Connecticut and has collaborations with Sun Microsystems, RX Johnson, and the Institute of Cancer Research. The company secured financing in January 2000 of $22M (first round) and in May 2003 of $63.5M (series B). Investors include Oxford Bioscience Partners, Axiom Venture Partners, Warburg Pincus, and Cardinal Partners.

Replidyne, Inc. is a private company based in Louisville CO, developing and commercializing innovative anti-infective products, particularly antibiotics. Replidyne's lead product, faropenem medoxomil, is an orally active antibiotic for the treatment of community-acquired infections such as respiratory tract infections (RTIs). The company expects to file an NDA late in 2005. Faropenem is a novel member of the penem class of β-lactam antibiotics, and is expected to be resistant to β-lactamase degradation and have lower gastrointestinal side effects. Replidyne is also developing a tRNA synthetase inhibitor for skin infections and staph eradication.

Ibis Therapeutics, a division of Isis Pharmaceuticals, has developed a novel program to discover low molecular weight orally-bioavailable drugs that work by binding to RNA. The company uses genomic mining software to identify structural motifs in the therapeutic targets of interest, and a massively parallel screening strategy to identify small molecules that bind RNA targets using high resolution Fourier Transform mass spectrometry.

Anadys was founded in May 2000 and is focusing on the ribosome and RNA for possible targets. The company's initial product development efforts are centered on the Hepatitis viruses, and it is collaborating with Amgen, Daiichi, Gilead and Roche. In 2004, the company went public raising over $45 million.

Paratek Pharmaceuticals, Inc. is another university spinout. The company was founded in 1996 and is headquartered in Boston, MA. Paratek's research is also concentrated in part on the ribosome with particular focus on tetracycline, and it recently executed an agreement with Bayer AG and Bayer HealthCare Corp. for the development of its lead tetracycline-derived antibacterial, PTK 0796. The company secured its fourth round of financing in May 2002 with funds supplied from Novartis Bioventures, Chinese Development Industrial Bank, BankInvest, and others.

Microbiotix, Inc. based in Worcester, Massachusetts, was founded in January 2000 as a University of Massachusetts Medical School spinout. The company concentrates on the development of antibiotics that target the bacterial DNA polymerase. The company licensed technology from Wayne State University and the University of Michigan as a part of its platform.

Cubist Pharmaceuticals, Inc. is based in Lexington, MA. Its flagship product, Cubicin™, a cyclic lipopeptide, was approved in September of 2003 for the treatment of complicated skin and skin structure infections (cSSSI) caused by specific Gram-positive bacteria. Cubicin has a distinct mechanism of action. The molecule binds to the bacterial cell membrane, causing rapid depolarization of membrane potential which then leads to inhibition of protein, DNA, and RNA synthesis, resulting in bacterial cell death. Cubist reported 2004 year-end net revenues of $58.6 million for Cubicin.

Oscient Pharmaceuticals Corporation, formerly Genome Therapeutics, is marketing its FDA-approved lead product, FACTIVE® (gemifloxacin mesylate) tablets. The company also has a novel antibiotic candidate, Ramoplanin (a glycolipodepsipeptide), in advanced clinical development for the treatment of *Clostridium difficile*-associated diarrhea (CDAD).

ActivBiotics, Inc. is a private biopharmaceutical company in Lexington, MA. The company's lead product candidate, rifalazil, is in Phase 2 clinical trials for the treatment of peptic ulcer disease caused by *Helicobacter pylori*. ActivBiotics is also investigating the use of rifalazil for *Clostridium difficile*-associated disease. The Company's lead pre-clinical compound in a pipeline of novel rifamycins, ABI-0043, shows significant potential for the treatment of infections caused by Gram-positive pathogens, including *Staphylococcus aureus* and MRSA (methicillin-resistant *Staphylococcus aureus*).

Arpida Ltd was founded in Muenchenstein, Switzerland in July 1997 and started research operations in January 1998. Arpida is a therapeutically focused "second generation" company using an integrated multidisciplinary approach: genomics-assisted selection of targets, innovative assays, high throughput screening, parallel synthesis for lead optimization, in vitro metabolism and cell lines for toxicity and absorption. The company's lead product, Iclaprim, was in-licensed from Roche and has successfully completed Phase II clinical trials.

Vertex Pharmaceuticals is using its structural biology expertise to develop dual-targeting antibiotics. As such, an antibiotic might show increased potency and slow the development of resistance, because more than one mutation would be needed to confer resistance. Vertex expects to name an antibiotic development candidate in 2005.

Other biotechnology companies with anti-infective programs include Basilea, Advancis, Athelas, PolyMedix, Kosan, Novexel, and Prolysis.

4. Intellectual Property Protection.

The RiboNovix technology is protected by patent applications filed by Wayne State University with the United States Patent and Trademark office in 2002 and 2005. RiboNovix has an exclusive, worldwide license to

these patent applications. Under the license, Wayne State received equity in RiboNovix, and the company has agreed to make specific milestone payments to the University, to reimburse them for costs incurred in connection with the prosecution of the patent rights, and to make specific royalty payments based upon net sales of products sold involving the patented technology.

To date the patent applications filed include:

1. "Methods and Compositions for the Identification of Antibiotics that are not Susceptible to Antibiotic Resistance." Filed 2002.
2. "Methods and Compositions for the Identification of Antibiotics that are not Susceptible to Antibiotic Resistance in Pseudomonas aeruginosa." Filed 2005.
3. "Novel Targets for the Identification of Antibiotics that are not susceptible to Antibiotic Resistance" Filed 2005.

The patent applications provide compositions and methods, which may be used to identify antibiotics that are less susceptible to the development of antibiotic resistance. The specific claims cover reagents, drug targets, and methods to discover drug candidates.

Additional intellectual property will be developed directly by RiboNovix scientists in the course of developing products based on the technology. Of particular importance will be composition of matter patents on drug candidates identified using RiboNovix technology.

5. Finance Plan.

RiboNovix is a privately held, development stage company. The company is committed to the development and commercialization of new anti-infectives that are refractory to antibiotic resistance, and has assembled a team of business and scientific leaders to reach its goals. RiboNovix is currently funded by a combination of private funds and SBIR grants.

Federal funding:

Phase I SBIR grant # 1 43 AI060275-01
Title: Identification of *E. coli* anti-infective rRNA targets
Amount: $346,066. Period of support: 4/01/04 – 3/31/05

Phase I SBIR grant # 1 R43 AI066445-01
Title: Three Fluorophore Assay for Antibiotic Inhibitors of Ribosome Assembly
Amount: $272,869 Period of support: 7/01/05 – 6/01/06

RiboNovix will fund later stage R&D through venture capital investment and pharmaceutical and biopharmaceutical partnerships. In order to succeed in attracting venture investment, RiboNovix needs to develop its technology through several stages of proof of concept. The first is the identification of one or more potential drug candidates, that inhibit the bacterial activity of *E. coli*, and potentially other pathogens such as *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus,* in a dose-dependent manner. The next milestone will be the identification of a clinical candidate and its development for clinical testing. Subsequent milestones will involve Phase I and Phase II clinical trials.

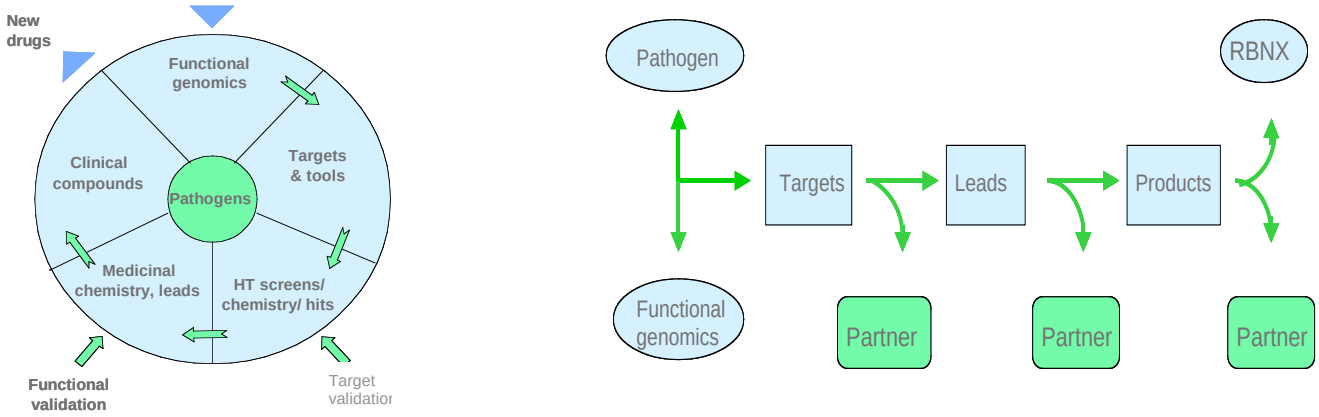
The company's technology can be used to develop both broad spectrum and narrow spectrum antibiotics, and over the long term RiboNovix intends to develop both. However, the initial target choice will be driven by the selection of the target that best fits our selection criteria. Our initial funding must be used to achieve proof of concept involving *in vivo* antibacterial activity, so that additional funding can obtained.

Since RiboNovix is developing new technology and entirely new chemical classes of antibiotics, the company will be able to establish significant intellectual property, which is important for attracting additional investment. An investment in the company is expected to generate significant returns, as technology and product advances

towards commercialization will increase the company's valuation. RiboNovix has initiated discussions with potential seed and Series A investors.

RiboNovix commercial strategy is to develop and commercialize products both independently and in partnership with other biotech and pharmaceutical companies. However, the timeline for drug development is very long and expensive. As a result, RiboNovix will focus most of its initial business model on collaborative efforts with pharmaceutical and biopharmaceutical companies. In order to be able to establish pharmaceutical and biotechnology partnerships, RiboNovix believes that it will be important to develop drug candidates that could lead to broad spectrum antibiotics which will be of interest to pharmaceutical partners, *and* narrow spectrum antibiotics for internal development or potential marketing by a biotechnology company partner.

RiboNovix' technology platform will be leveraged through a number of pharmaceutical partnerships, and by direct development for later partnering. License agreements typically will include up-front fees, milestone payments, success fees and royalty payments based on the sales of drug products.



RibNovix Development Strategy (left) and Partnering Approach (right).

6. Production and Marketing Plan.

Drug development timelines are long. While it is difficult to accurately predict the timelines for clinical development and commercialization, the typical timeline for drug development is 10-13 years based on data from Tufts and PHARMA. Using this data, we would anticipate that RiboNovix' drugs could be on the market in 2015 onwards.

Most antibiotics are small chemical molecules and as a result, manufacturing does not usually present large problems. Similarly, the regulatory issues with new chemical entities are well established and fairly easy to determine and resolve. Ribonovix plans to use contract manufacturing for its own products, and its pharmaceutical partners for production of licensed products.

The marketing rights of RiboNovix early products will be licensed to its pharmaceutical partners. However, RiboNovix intends to retain the rights to certain small or niche markets for direct or co-marketing. Biotechnology companies have shown that they can build sales and marketing capability for niche markets, especially in the field of anti-infectives. Cubist Pharmaceuticals and Oscient Pharmaceuticals are examples. Both companies have built sales and marketing organizations for products aimed at hospital-acquired infections. However, an antibiotic for community-acquired infections that require novel antibiotics because of resistance, would demand a larger pharmaceutical sales and marketing organization. In addition to the top tier pharmaceutical companies, the second tier companies outside the top ten, and several specialty pharma companies, are potential marketing partners. RiboNovix intends to market niche products directly and will partner with larger companies for other products.

7. Revenue Stream.

RiboNovix expects to achieve long-term financial revenues and earnings from royalties on the sale of new antibiotics developed and marketed by its commercial pharmaceutical partners, and receive revenues from the direct sales of its own specialized, niche anti-infectives. The company expects that it will require a significant number of years of research, development and clinical testing for each new anti-infective before FDA approval will be achieved. The company expects revenues in the near term from licensing and joint ventures with other companies.

The staffing plan for the company reflects the need to focus on research and development in the early years. While the company will build its own research organization, it will also use contract research organizations to supplement its resources in a cost effective manner.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Project Director / Principal Investigator (PD/PI)

Prefix:
* First Name: Cheryl
Middle Name: Isaac
* Last Name: Murphy
Suffix: PhD

* New Investigator? ● No ○ Yes

Degrees: Ph.D.

2. Human Subjects

Clinical Trial? ● No ○ Yes

* Agency-Defined Phase III Clinical Trial? ○ No ○ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:
* First Name: Alison
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* Last Name: Taunton-Rigby
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* Phone Number: 781-259-3183
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* Title: President and CEO

* Street1: 8 Farrar Rd.
Street2:
* City: Lincoln
County:
* State: MA
* Zip Code: 01773
* Country: USA

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 9/30/2007

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ●No ❍Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp . Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

OMB Number: 0925-0001
Expiration Date: 9/30/2007

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the research plan.

*Type of Application:

❍New ● Resubmission ❍ Renewal ❍ Continuation ❍ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	5835-Introduction.pdf
2. Specific Aims	4874-spec aims.pdf
3. Background and Significance	2910-Background.pdf
4. Preliminary Studies / Progress Report	3086-PhI report.pdf
5. Research Design and Methods	741-Research.pdf

Human Subjects Sections

Attachments 6-10 apply only when you have answered "yes" to the question "are human subjects involved" on the R&R Other Project Information Form. In this case, attachments 6-10 may be required, and you are encouraged to consult the Application guide instructions and/or the specific Funding Opportunity Announcement to determine which sections must be submitted with this application.

6. Protection of Human Subjects	
7. Inclusion of Women and Minorities	
8. Targeted/Planned Enrollment Table	
9. Inclusion of Children	
10. Data and Safety Monitoring Plan	

Other Research Plan Sections

11. Vertebrate Animals	
12. Consortium/Contractual Arrangements	9710-Consortium.pdf
13. Letters of Support	1489-letters.pdf
14. Resource Sharing Plan(s)	

15. Appendix

Attachments

IntroductionToApplication_attDataGroup0

File Name	Mime Type
5835-Introduction.pdf	application/pdf

SpecificAims_attDataGroup0

File Name	Mime Type
4874-spec aims.pdf	application/pdf

BackgroundSignificance_attDataGroup0

File Name	Mime Type
2910-Background.pdf	application/pdf

ProgressReport_attDataGroup0

File Name	Mime Type
3086-PhI report.pdf	application/pdf

ResearchDesignMethods_attDataGroup0

File Name	Mime Type
741-Research.pdf	application/pdf

ProtectionOfHumanSubjects_attDataGroup0

File Name	Mime Type

InclusionOfWomenAndMinorities_attDataGroup0

File Name	Mime Type

TargetedPlannedEnrollmentTable_attDataGroup0

File Name	Mime Type

InclusionOfChildren_attDataGroup0

File Name	Mime Type

DataAndSafetyMonitoringPlan_attDataGroup0

File Name	Mime Type

VertebrateAnimals_attDataGroup0

File Name	Mime Type

ConsortiumContractualArrangements_attDataGroup0

File Name	Mime Type
9710-Consortium.pdf	application/pdf

LettersOfSupport_attDataGroup0

File Name	Mime Type
1489-letters.pdf	application/pdf

ResourceSharingPlans_attDataGroup0

File Name	Mime Type

Appendix

File Name	Mime Type

Introduction to Revised Application.
The following is a response to the critiques provided by the reviewers of our first SBIR Phase II application and a summary of the changes that have been made in this revision.

General comments.
1. *Compound library description. All three reviewers noted that the compound libraries are not adequately described.* We have included a section (D, methods, specific aim 3) that describes each of the compound collections we will screen, and included relevant letters of support. Our goal in choosing libraries was to select compound collections enriched for RNA-binding molecules. Compared to compounds that bind proteins, few RNA-binding compounds have been characterized and relatively little is known about the general features that are important for RNA binding. We have therefore chosen libraries containing molecules that were either designed to bind to specific RNA structures or that have demonstrated specific RNA binding
ability. Although most of the compounds in the second category have been used to screen protein targets, many of the molecules are either known to interact with RNA, or have been identified as hits in other experimental screens using RNA targets.

As stated in the summary of our research plan (section D) the compound libraries we have selected for screening will give us both a large number of compounds (>100,000) and sufficient diversity to maximize the number of hits we identify. We also plan to screen phage and peptide libraries, as described in our original grant application. In addition to providing us with a source of hits, this will provide reagents for use in competition binding assays and for additional validation of our RNA targets.

2. *Information and clarification on screening and structural characterization methodology, and inclusion of in vivo studies.* In order to address these concerns and to more effectively characterize potential lead compounds in structural analysis and *in vitro* and *in vivo* validation studies, we have decided to focus on development of one rRNA target rather than two. We have examined the potential targets identified in our Phase I SBIR project for suitability as targets for small molecule screening based on a number of criteria including size and complexity, presence or absence of modified nucleotides, known interactions with proteins, RNA or other ligands, and differences from corresponding human sequences (see section D, research plan). After applying these criteria, we have narrowed our focus to 4 RNA targets. We will use 1-dimensional NMR analysis of the four candidates to eliminate targets that when isolated do not closely assume the correct structure as contained within the context of the ribosome and to identify targets that are most amenable to NMR structure determination in the absence and presence of a bound ligand (section D, methods, specific aim 1). One prioritized target will be chosen to take forward, and we will pursue additional targets as time and resources permit.

Specific concerns in this regard are addressed below:

a. *Compound screening description. The methods for compound screening are not adequately described*. We have added additional information under methods, specific aim 3 of the experimental plan to include a discussion of assay optimization, positive and negative controls, placement of fluorescent tags and ranking of hits. Some of these variables will depend on the final choice of target. Along with a detailed description of each target, we also include specific ways they could be used in high throughput screening assays.

Each library collection we have chosen contains compounds with known structures. We will confirm the integrity of any hits we obtain in the screening process by mass spectrometry or other suitable methods.

b. *Assessment of antibacterial activity, spectrum and selectivity*. We have included a new section under methods, specific aim 4 to address assessment of antibacterial activity. Specifically, *in vivo* antimicrobial activity will be evaluated for each "hit" by determining the minimum inhibitory concentration (MIC) of that compound required to inhibit bacterial cell growth of representative Gram positive and Gram negative bacterial genera.

Additional specific comments.

3. *Provide evaluation of sequence conservation of targets among pathogens*. For each rRNA target chosen for further evaluation in this grant proposal, we include a description of sequence conservation, comparing the target sequence to both the equivalent human sequence, and other bacterial sequences (Table 5). Figures 15-19 show the sequences of the RNA targets and equivalent human homologues if they exist.

4. *Methods for target mutagenesis should be described.* The section on target mutagenesis (D, methods, specific aim 2) has been updated with additional information.

5. *The goal of performing NMR structural analysis on two mutants per target is not adequately justified and seems arbitrary*. We have decided to focus on one target in Phase II, thus allowing more time and resources that can be committed to analysis of the target, mutants and potential hits. We intend to complete the structures of 4-6 of the key mutants of the chosen target that are most likely to affect binding of a small molecule to the target sequence. We will not know the exact details until we have chosen a target, completed the saturation mutagenesis, and identified the viable mutants.

6. *The responsibilities of the different consortium members are confusing*. In the justification section of the budget section and in figure 13 of the research plan we outline the responsibilities of RiboNovix and consortium personnel. In brief, RiboNovix will be responsible for the research work of the project and will select the target (in collaboration with Drs. Cunningham, SantaLucia and _), perform the saturation mutagenesis studies, assay development, preparation of libraries, screening experiments with most of the libraries, and *in vitro* and *in vivo* assessments of compound activity. The 1-dimensional NMR studies on the four target RNAs will be run at WSU, as will the homology modeling software studies and the NMR structural analysis by Dr. SantaLucia. Dr. Cunningham (WSU) will provide advice and support on the saturation mutagenesis methodology, and the GFP and protein synthesis assays.

7. *RiboNovix laboratory facilities are not established*. RiboNovix is currently leasing fully equipped laboratory space in Lexington, MA and this is described in detail in the facilities section of the revised application.

8. *Commercialization plan is vague*. We have revised our commercialization plan to include a better definition of the market for a putative product, a discussion of the future role of the research collaboration with Wayne State University, and a more thorough discussion of production and marketing plans.

9. *Description of tasks by various collaborators is uneven in detail*. We have delineated the responsibilities of the various groups involved in this program in #6 above, in figure 13 of the research plan, and in the justification section.

10. *Not enough detail in homology modeling and NMR methods.* We have added more detail on the homology modeling methods in section B, background and significance, and have added a figure (figure 3). We have also updated the description of the NMR methods in section D, methods, specific aim 2. One of the reasons we have changed our goal from 2 targets to 1 target is to be able to more fully characterize the structural properties of a target and its interaction with potential drug leads.

11. *Letters of collaboration are too similar*. New letters of collaboration are included with this application, detailing the role and expertise of the collaborator, and the specific tasks for which they will be responsible.

12. *Level of effort for Drs. Cunningham and SantaLucia appears low*. We have effectively increased the level of effort of Drs. Cunningham and SantaLucia by reducing the number of targets to one. We have outlined in more detail the responsibilities of various groups in the application (research plan, figure 13). While many of the technologies and research tools described in this application have been developed in the laboratories of our academic collaborators, our goal is to transfer these research tools to RiboNovix. The company is responsible for the development of new drugs using these techniques. Two areas of collaboration are the NMR and structural analysis work with Wayne State (Drs. Cunningham and SantaLucia), and the screening of one of the compound collections at _ .

Two consortia are planned to cover these aspects of the program. RiboNovix will be performing the majority of the experiments described in this application. Our scientific collaborators, Drs. Cunningham, SantaLucia, _ , Chow, and _ are all available for consulting and advice.

RESEARCH PLAN

A. Specific Aims

Antibiotic resistance is a growing and increasingly serious public health problem. Since 1998, the FDA has approved only 10 new antibiotics. Of these, only two are truly novel, with a new mechanism of action and no cross-resistance with other antibiotics. In addition, research in the field has stalled due to a lack of innovation and the inability to anticipate drug resistance. Currently, the only antibiotics in late stage development belong to two existing classes, the macrolides and quinolones. There is also a dearth of new narrow spectrum agents. Many of the antibiotics in development today are broad spectrum, which are more likely to contribute to the development of resistance and have unwanted side effects.

Our strategy for meeting this challenge is to use novel, complementary, proprietary technologies to develop new anti-infectives that are highly effective and refractory to antibiotic resistance. These include a Combinatorial Genetic Technology (CGT) that allows the identification of rRNA target sites and the specific nucleotides that are essential for functionality and viability, and a RNA Homology Modeling software that allows accurate prediction of mutant RNA structures.

Our Phase I goal, to find new drug targets in 16S rRNA, was extremely successful. Using CGT, we constructed a functional mutation library of *E. coli* 16S rRNA, sequenced ~5000 functional clones, and identified 67 regions in *E. coli* 16S rRNA that contain groups of nucleotides essential for viability. Some of them occur near each other in 30S subunit crystal structures, suggesting the involvement of several regions in a single functional role. The regions identified include known binding sites for antibiotics, tRNAs, proteins, the large ribosomal subunit, and initiation factors, as well as a number of sites that are clearly essential for ribosome function, but for which no functional role has yet been assigned. Based on a number of criteria including size and complexity, protein and ligand binding, and other properties (see section D) we have identified four rRNA sequences that are well suited as targets for compound library screening.

In Phase II, the prospective targets will be analyzed using NMR, and one will be selected as a top priority for drug development. We will use CGT to identify components of the target that are essential for protein synthesis, and to select viable mutants of the target that, together with the wild-type target, can be used to discover compounds that specifically recognize and bind the essential components of the target. Both chemical and phage libraries will be screened. RNA homology modeling software will be used to predict the structure of the wild type and viable mutant targets. In addition, we will complete NMR structures of the target/hit complexes, validate the targets using *in vitro* and *in vivo* assays, and lay the groundwork for the development of specific compounds into drug leads. To accomplish the Phase II SBIR, four specific aims are proposed:

1. **Target Selection.** One RNA subdomain will be selected as a prioritized target from the four potential targets chosen from the RNA “regions of interest” identified in Phase I.
   Milestone: One prioritized target, with backup lower priority targets, 2-3 months.

2. **Target refinement:** We will use CGT to identify every mutation of the target that could to lead to drug resistance, and use multidimensional NMR spectroscopy and homology modeling to determine the essential structural components of the target.
   Milestone: Structure information on prioritized target, 9-12 months.

3. **Target screening:** We will screen compound, phage and peptide libraries against the wild type target and its viable mutants.
   Milestone: Identification of hits that have specificity for the target and its viable mutants, 12-18 months.

4. **Validation of targets and compounds:** We will perform structural studies of target/hit complexes, optimization of hit compounds, and validation of the target using *in vitro* and *in vivo* assays.
   Milestone: One or more compounds that are potential leads, and target validated, 15-24 months.

RiboNovix will complete the work necessary to develop drug candidates from these leads, and will move qualified candidates into pre-clinical development.

B. Background and Significance

Antibiotic resistance is a growing and increasingly serious public health problem. Of particular concern are hospitalized patients, two million of whom contract bacterial infections during their hospital stay each year. According to CDC statistics, more than 70 percent of the bacteria that cause hospital-acquired infections are resistant to at least one of the antibiotics most commonly used to treat them. A need therefore exists for new antibiotics that are less susceptible to the development of antimicrobial resistance. While the primary sources of resistance for *natural* antibiotics are enzymatic inactivation and efflux pumps, resistance arising from the use of *synthetic* anti-infectives is often due to mutation of the drug target. If all viable mutations of a drug target were known, the components of the target required for function could be identified and new drugs developed to specifically recognize the essential components of the target. These drugs should be significantly less susceptible to the development of resistance than those recognizing only the wild-type target.

Antibiotic Resistance: Approximately half of all natural antibiotics affect protein synthesis and most are directed against ribosomes, a proven drug target. The primary sources of resistance for natural antibiotics are enzymatic inactivation and efflux pumps. However, a common mechanism for the occurrence of resistance to new chemical classes of antibiotics is through mutation of the drug-binding site. Mutations occur naturally in all bacterial cells and the wild-type form of any drug target is the product of natural selection of sequences that function best under normal circumstances. For target-site mutations to be clinically relevant, the mutated target must retain most of its original activity, since loss of function decreases the fitness and virulence of the pathogen.

Functional biological molecules usually interact with other cellular molecules (ligands), which is especially true of rRNA. Functionally, rRNA targets are composed of two types of nucleotides, each of which is important for viability. C*ritical nucleotides* are those involved in essential chemical interactions of the target with its natural ligand(s). *Structural nucleotides* form molecular motifs that result in proper positioning of the critical nucleotides so that they are able to interact with their ligand(s). Although both parts of the target are essential for function, they are quite different from a pharmaceutical perspective. Virtually any change to the critical nucleotides will prevent the target from performing its function because the interaction of the target with its ligand is usually disrupted. Unlike the critical nucleotides, however, there are usually several combinations of structural nucleotides that will maintain proper placement of the critical nucleotides [1; 2; 3; 4]. Antibiotic resistance develops when the structural nucleotides mutate to an alternative *viable* form that is no longer recognized by the drug. An example of this type of resistance recently occurred with the only new class of anti-infectives to be developed in 35 years, the oxazolidinones (linezolid) [5]. These compounds inhibit protein synthesis by binding to bacterial ribosomes. Approximately seven months after market introduction, linezolid-resistant strains of bacteria were isolated that contained mutations in ribosomal RNA [6; 7; 8; 9; 10; 11].

Target Identification: Traditionally, phylogenetic analysis has been used for rRNA target selection. Ribosomal RNA sequences from different species are compared to identify regions unique to bacteria [12]. The target sequences from several species of bacteria are then compared and those with the least sequence variability are selected for development of anti-infectives since these regions of the rRNA are the least likely to tolerate mutations that might lead to resistance.

Although phylogenetic analysis is widely used, there are several problems inherent to this approach. First, phylogenetic analysis depends on existing sequence information from bacteria and other species, including humans. Second, it requires that the target region be present in many different bacteria so that reliable comparisons can be made. As a result, drugs designed against these targets will inhibit all bacteria that contain the target sequence, including beneficial bacteria. And finally, analysis of drug resistant clinical isolates of bacteria has clearly shown that even highly conserved rRNA sequences can mutate to forms that are not found in any known organism, but which are nearly as viable as the original [13; 14].

Combinatorial Genetic Technology (CGT): We are using a genetic system in *E. coli* developed by Dr. Philip Cunningham at Wayne State University that allows the use of high-throughput, combinatorial genetics to identify all mutations in any given region of rRNA that affect ribosome function. Mutations in rRNA are often lethal because they affect the production of all cellular proteins. CGT allows mutational analysis of rRNA *in vivo* without affecting cell viability [4; 15]. In this system (Fig. 1), the cells produce two sets of ribosomes. One set

comes from genes present on the chromosome and carries out all of the normal protein synthesis functions in the cell. The second set of ribosomes is produced from rRNA genes on a plasmid (pRNA123); these ribosomes have been genetically engineered so that they only make two reporter proteins, green fluorescent protein (GFP) and chloramphenicol acetyltransferase (CAT).

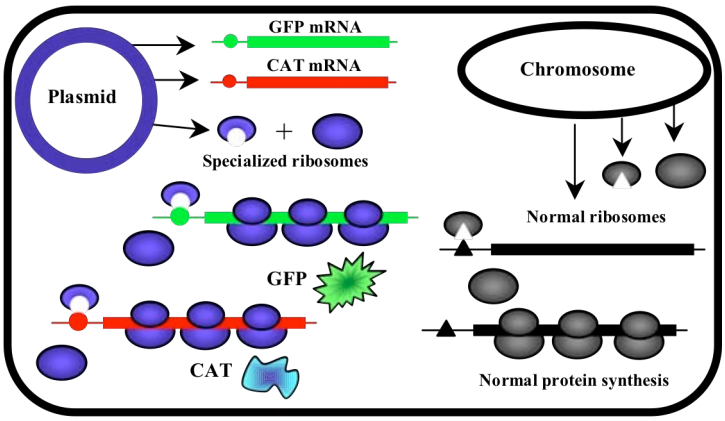


Figure 1: Genetic system for rRNA analysis

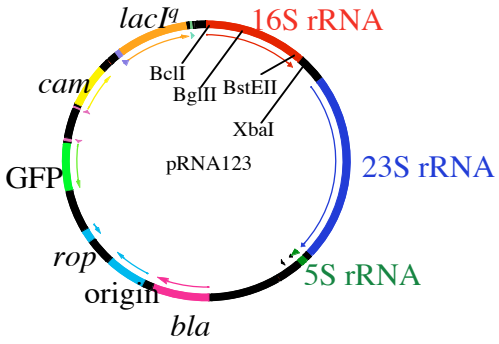


Figure 2. pRNA123.

The plasmid pRNA123 (Fig. 2) contains the *rrnB* operon, the *lac* repressor (*lacI*^{q}), the CAT gene (*cam*), the GFP gene, and the ampicillin resistance gene (*bla*). The key features of this system are: 1) Transcription of the plasmid-encoded *rrnB* operon is regulated by the inducible *lacUV5* promoter; 2) the presence of *lacI*^{q} insures an adequate supply of *lac* repressor protein to regulate rRNA transcription; 3) CAT and GFP transcription are constitutive, providing a constant supply of message; 4) the Shine-Dalgarno sequence of CAT and GFP have been changed from the wild-type, 5'-GGAGG, to 5'-AUCCC and the anti-Shine-Dalgarno sequence of the plasmid-encoded 16S RNA gene has been changed from 5'-CCUCC to 5'-GGGAU; 5) CAT and GFP mRNAs are not translated by wild-type ribosomes; 6) induction by IPTG renders cells resistant to chloramphenicol concentrations up to 550 µg/ml.

The proteins made by the "specialized" ribosomes are chloramphenicol acetyltransferase (CAT), which allows the cells to grow in the presence of the antibiotic chloramphenicol, and the green fluorescent protein (GFP) which allows high-throughput analysis of ribosome function. The CAT produced by the specialized ribosomes renders cells resistant to high concentrations of chloramphenicol. Mutations in the specialized ribosomes that decrease their ability to make protein result in lower CAT production and therefore increased sensitivity to chloramphenicol in a roughly linear manner [16]. Exposing cells to different concentrations of chloramphenicol allows direct selection of mutations in the specialized ribosomes that inhibit protein synthesis to different degrees [2; 4]. Since the same specialized ribosomes make both CAT and GFP, mutations in rRNA that affect CAT production have an equal effect on the production of GFP. The amount of GFP protein produced is easily quantified in whole cells using high-throughput fluorometry.

There are two important ways that CGT can be used to develop antibiotics refractory to resistance. One is for target identification, which involves random mutagenesis of rRNA genes from a specific pathogen followed by isolation and sequencing of the viable mutants. Under the Phase I SBIR, we used this approach to identify 67 "regions of interest" in the functional rRNAs that contain few or no mutations; these are potential drug targets. We can also use CGT to identify the critical components within each target, by doing saturation mutagenesis of the rRNA target region (target refinement). This process will identify the entire subset of mutations that are functional and which could lead to the development of drug resistance. We plan to use CGT in Phase II to refine the selected target and identify its viable mutants.

CGT Target Refinement. Early studies by Dr. Cunningham have identified the power of the genetic system described above to identify mutations that might lead to drug resistance. One example is the analysis of the 790 loop. Although not an appropriate target for antimicrobial therapy because of its similarity to human sequences, the 790 loop is an essential component of all ribosomes and shows very little sequence variability among bacteria (Table 1). Using CGT, the nine nucleotides of the 790 loop were randomly mutated *in vivo* and all of the 262,144 (4^9) possible sequences were cloned and screened by growth on chloramphenicol to isolate only the functional mutants[4]. A total of 78 mutants with ≥25% activity were characterized (Table 2.

Functional Mutants of the 790 Loop). Of these, 44 contained 4 to 6 mutations and retained 60-80% of the wild-type activity and no mutations were found at positions 789 or 791. Positions 789 and 791 are critical nucleotides, position 790 is structural, and positions 787, 788 and 792–795 can mutate quite freely without major loss of function.

Table 1: Phylogenetic analysis of the 790 loop from 3240 bacteria

Nucleotide	787	788	789	790	791	792	793	794	795
A	3231	3	3	3231	11	3233	10	3235	5
C	2	3	2	0	5	0	0	1	3233
G	5	0	1	6	3222	6	49	2	0
U	2	3234	3234	3	2	1	3181	2	2
Total	**3240**	**3240**	**3240**	**3240**	**3240**	**3240**	**3240**	**3240**	**3240**

Table 2: Analysis of the Functional Mutants of the 790 Loop

Nucleotide	787	788	789	790	791	792	793	794	795
A	54	24	0	69	0	15	18	35	16
C	2	16	0	8	0	24	26	5	34
G	22	21	0	1	78	16	4	9	7
U	0	17	78	0	0	23	30	29	21
Total	**78**	**78**	**78**	**78**	**78**	**78**	**78**	**78**	**78**

These viable mutants of the 790 loop could not have been predicted from the phylogenetic data (Table 1). If a new anti-infective were isolated by screening only the wild-type 790 loop, it is likely that one of the 78 functional mutants identified would not be recognized by the drug and would therefore lead to the development of drug resistance. By comparing the functional 790 loop mutants to several nonfunctional mutants, it was discovered that all of the viable mutants of the 790 loop share common features that place the key nucleotides 789 and 791 of the loop in the same three-dimensional conformation[4]. Similar experiments using CGT and NMR spectroscopy were recently performed to identify the key functional features of the 690 hairpin in *E. coli* 16S RNA [1; 2].

Homology Modeling of RNA Domains. We are planning to use software for RNA homology modeling developed by Dr. John SantaLucia at Wayne State University that allows prediction of mutant structures given the X-ray structure of a reference sequence. The accuracy of the predictions has been validated for several tRNAs with sequence identity as low as 50%, and predicted structures with all-atom RMSD near 2 Å are typically observed. For the ribosome, much higher levels of sequence identity are observed and thus the homology predictions should be highly accurate. We plan to use this modeling software in Phase II to predict the structures of the target and its viable mutants.

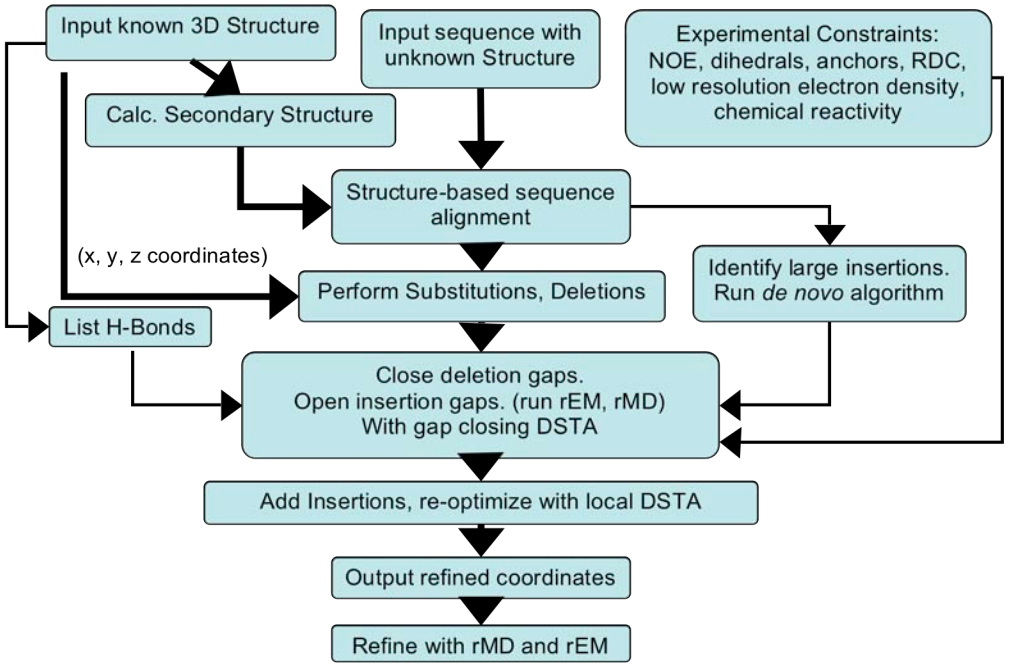


Figure 3. Flowchart for homology modeling of ribosomal RNA

Figure 3 shows the overall process for performing homology modeling of RNA. The basic steps are to input the coordinates of the known template structure (e.g. the *T. thermophilus* 30S structure 1J5E.pdb) and the sequence of the pathogen or other mutant (e.g. *E. coli*). The second step is to perform a sequence alignment that includes secondary structure constraints. The third step is to use the sequence alignment to guide the substitutions, deletions and insertions to be automatically made in the 3D structure. The final step is to optimize the

geometry of the structure using molecular dynamics and energy minimization. The ribosomal proteins can also be modeled by similar methods using public servers such as the expasy server; this approach is quite reliable since ribosomal proteins within the bacterial domain often have sequence identity >50%.

Figure 4 shows the results of homology modeling for the 690 loop from *Y. pestis* and from human derived by using the *T. thermophilus* X-ray structure of the 30S ribosomal subunit[17]. The models were produced by making the appropriate substitution, deletions, and insertions in the known *T. thermophilus* X-ray structure and performing energy minimization with a new force field developed by Dr. SantaLucia. Nucleotides that differ in *Y. pestis* and human are colored by atom type and identical residues are in red.

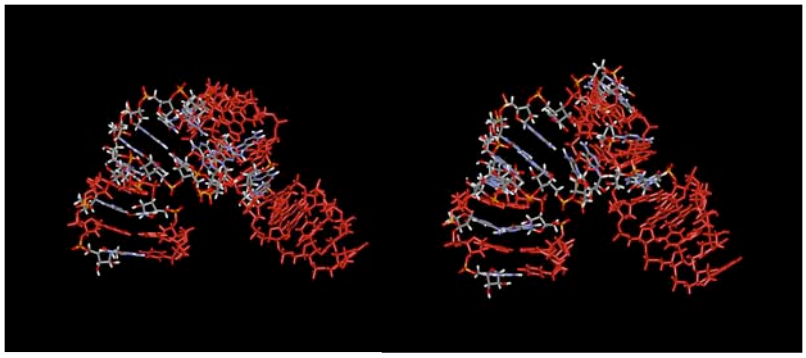


Figure 4. Homology models of *Y. pestis* (left) and human (right) H23 (nucleotides 677-713 in *E. coli* numbering).

Homology models were also produced for several mutants of the 690 region in *E. coli* as well as the A site (data not shown). The modeling software also allows substitution of modified nucleotides (m^3U, m^5C, etc.), which is particularly useful for the A site which contains several modifications.

The wild-type and mutant structures from NMR or homology modeling are superimposed to reveal the functional groups that are conserved in all functional mutants. Such an analysis was previously performed on the 690-697 GU mismatch and it was discovered that all of the functional mutants contain a H-bond acceptor (corresponding to U697-O2) at a conserved location in 3D space.[3] Subsequent analysis revealed that this functional group is required for function because it forms a crucial interaction with the 790 loop.

NMR Studies of RNA Domains. NMR spectroscopy can be used to determine key functional groups in RNA targets and provide several types of critical information. First, NMR can be used to verify whether the structure of *isolated* RNA targets taken out of the context of the ribosome resemble their structure in the ribosome and can therefore be valid targets for screening compound libraries. NMR spectroscopy will also be used to provide detailed stereochemical information on the mechanism of binding of small-molecule ligands with RNA targets. Lastly, NMR studies will be used to reveal crucial differences between the *E. coli* and the human small subunit rRNAs. Comparison of the wild-type and mutant structures will reveal the essential functional groups and structural folds required for ribosome function, thereby focusing design of drugs to these critical residues[4]. Structural characterization of RNA-ligand complexes will reveal how each compound recognizes the essential target motifs. Further, characterization of RNA dynamics by NMR in the presence and absence of bound drug leads will reveal the role of induced fit in RNA recognition[18].

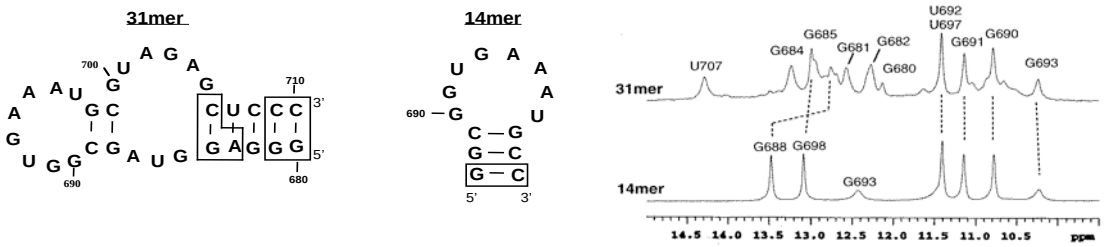


Figure 5. 14mer and 31mer NMR constructs of the 680-710 region of *E. coli* 16S rRNA. Boxed residues were changed to allow efficient transcription by T7 RNA polymerase for preparation of isotopically labeled samples. 500 MHz 1D imino proton NMR spectra of the 31mer and the 14mer. Note the similarity of the hairpin loop imino protons in the two constructs, indicating that they form the same structures.

As an example of the use of NMR, Figure 5 shows two 690 loop constructs and their NMR spectra. The imino-proton NMR spectra for these two sequences demonstrate that the structure of the hairpin loop region is the same in both constructs.

Figure 6 shows the 3D structure of the 690 loop14-nt hairpin. Both unlabeled and uniformly ^{13}C-^{15}N labeled 14mer samples were synthesized and a full set of 2D and 3D NMR experiments were performed. Importantly, the NMR structure agrees with the X-ray structure of the small-subunit[17] within 0.8 Å RMSD, indicating that the isolated 690 loop is valid for use in drug screening. The structure shows several novel features. Residues G693 through U697 form a novel recognition motif in which the Watson-Crick faces of all five residues are exposed to solvent in the minor groove and are pre-organized into a helical conformation, presumably for interaction with the 50S subunit. In the major groove, residues G690, G691, and U692 have their Watson-Crick faces partially exposed to solvent, which suggests they may also play a role in molecular recognition.

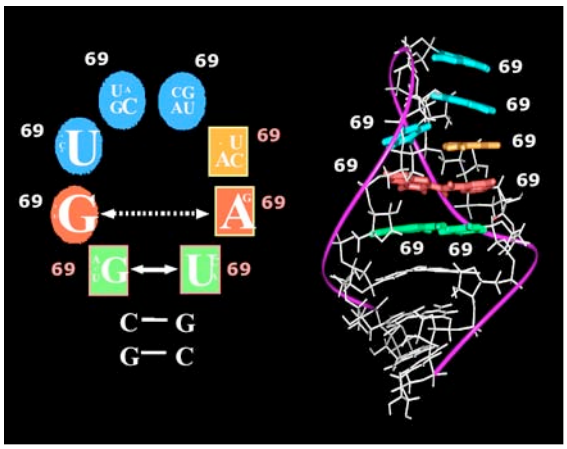


Figure 6. 3D structure of the 690 loop hairpin.

Comparison of data obtained using CGT with NMR structural studies and homology modeling allows correlation of specific sequence elements in rRNA targets with ribosome function. These methods are powerful tools in the ultimate discovery and design of new antibiotics.

Significance of these technologies. These data demonstrate the power of CGT to identify "regions of interest" with few or no mutations in the rRNA, and to identify all of the mutations of a drug target with the potential to lead to drug resistance. The data also show the usefulness of NMR and the RNA homology modeling software for structure determination. We plan to use these technologies in Phase II to choose and characterize one of the novel rRNA targets identified in Phase I, to guide the performance of compound screening experiments, and to determine the structures of target/hit complexes. These experiments should result in the discovery of compounds that are resistant to target site mutation and thus refractory to antibiotic resistance. The compounds will then be ready for optimization into clinical candidates.

***E.coli* Medical Significance.** Enteric diseases kill approximately 7000 children per day worldwide[19]. Antibiotic therapy is an effective treatment for most bacterial enteric diseases[20; 21; 22; 23; 24; 25]. However, many of these bacterial pathogens are already resistant to most of the antibiotics used to treat them, and a number of new multidrug-resistant pathogens have emerged[26; 27; 28; 29; 30; 31].

E. coli is a common cause of global bacterial diarrhea[32; 33; 34; 35]. All diarrheagenic strains of *E. coli* were initially termed enteropathogenic *E. coli* (EPEC) but based on different pathologies the additional groups of enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diarrhea-associated hemolytic *E. coli* (DHEC) and cytolethal distending toxin (CDT)-producing *E. coli* have been added[36]. The efficacy of antibiotic treatment for diarrheagenic *E. coli* varies with the specific etiology but in general antibiotic therapy is considered beneficial for the reduction of the course and severity of the disease[36] though some antibiotics may be contraindicated in the treatment of Shigatoxin-producing strains[37; 38; 39; 40]. As with the other etiologic agents of bacterial diarrhea, a number of antibiotic-resistant strains of diarrheagenic *E. coli* have emerged[37; 38; 39; 41].

Our mutation library will allow the identification of both narrow and broad-spectrum targets. Narrow spectrum targets are those present only in *E. coli* and other enterics such as *Salmonella* and *Shigella* species. Broad-spectrum targets are regions of the rRNA that are found in most bacterial species. We expect that broad-spectrum anti-infectives developed against the targets identified in this study will be effective against a large number of microbial pathogens including those for which the current arsenal of antibiotics is decreasing. These include Methacillin Resistant *Staphylococcus aureus* (MRSA)[42; 43] and vancomycin resistant *Enterococcus* (VRE)[44; 45; 46].

C. Preliminary Studies and Phase I Final Report

Beginning Date: 4/1/04
Ending Date: 3/31/05

Key Personnel	Title	Date of Service	Percentage Effort
Cheryl Murphy	Principal Investigator	4/1/04 – 3/31/05	_%
Philip Cunningham	Consortium Head	4/1/04 – 3/31/05	_%
_ _	Research Assistant	4/1/04 – 3/31/05	_%

The primary objective of the Phase I SBIR proposal was to use CGT to construct and analyze a mutation library of the *E. coli* 16S rRNA, and to identify subdomains essential for protein synthesis that contain few or no mutations, and therefore are potential drug targets

The **Phase I Specific Aims** were:

1. Construction of a functional mutation library of *E. coli* 16SrRNA using error-prone PCR.
2. Isolation and sequencing of 5000 functional mutants.
3. Functional analysis of each isolate and identification of important subdomains that contain few or no mutations and are "regions of interest".

Results
* Denotes proprietary information that RiboNovix requests not be released to persons outside the Government, except for purposes of review and evaluation.

AIM 1: Construction of a functional mutation library of *E. coli* 16S rRNA using error-prone PCR.

*Error-prone PCR[47] was used to create a pool of random mutations of the 16S rRNA gene from *E. coli*. The PCR reaction contained 200mM dATP, 200mM dCTP, 200mM dGTP, 200mM dTTP, 2.5mM $MgCl_2$, 0.45mM $MnCl_2$, 10nmol/mL pRNA123 (template), and 1mM of each primer. The PCR primers anneal to each side of the 16S rRNA gene. The PCR products were gel purified, digested with the *BclI* and *BstEII*, ligated into digested pRNA228 and transformed into *E. coli* DH5 cells using electroporation. PCR reactions, restriction enzyme digestions, DNA ligations and electroporations were performed according to standard procedures. A total of 3000 electroporations of the ligation mixtures were performed using 25 µL of competent cells at a time in an *E. coli* Porator (Gibco, Inc.). Approximately 600,000 transformants were plated on plates containing 25 µg/mL chloramphenicol, and 6000 chloramphenicol-resistant clones were isolated. Thus, only 1% of the initial mutant library produced functional ribosomes.

AIM 2: Isolation and sequencing of 5000 *functional* mutants.

*A total of 5640 chloramphenicol-resistant 16S rRNA mutants were isolated and individual colonies were sent to SeqWright Inc. (Houston, Texas) for DNA preparation and sequencing. Of these samples, 4234 completed and assembled sequences were returned. Either the DNA template preparation or the sequencing reaction failed for the other 1406 clones. Of the 4234 sequences received, 112 were wild-type, 686 contained one or more ambiguities or were partial sequences, and 827 were duplicates. Thus, 2609 usable sequences were obtained and analyzed.

*The resulting functional genomics mutation database contains a mean of 7.26 (St. Dev.= 6.08, Median = 6, Mode = 2, Min = 0, Max = 38) mutations per position of the 16S rRNA.

AIM 3. Identification of drug targets by assay of each isolate in the mutation library for function *in vivo,* and identification of functionally important subdomains that contain few or no mutations.

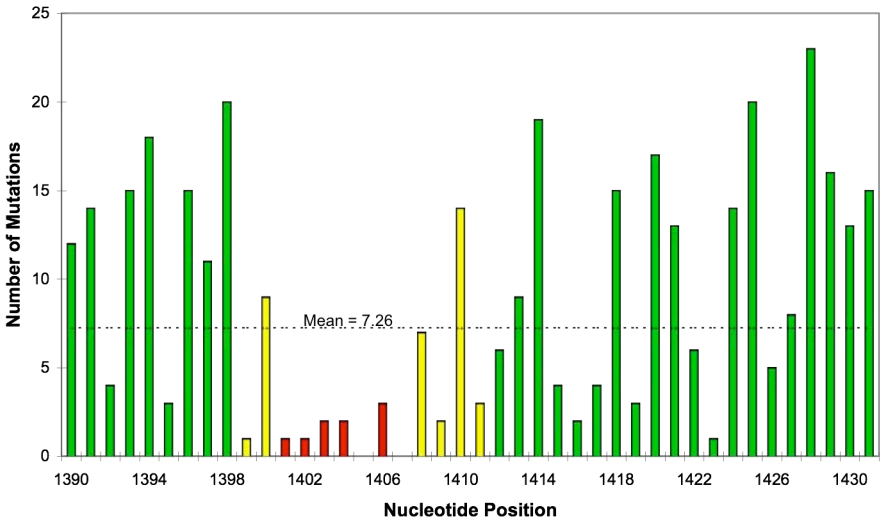
*The quality of our Phase I functional genomics mutation database far exceeded expectations. Based on the distribution of mutations throughout the entire 16S rRNA, adjacent nucleotides with three or fewer mutations were identified and analyzed to determine the probability that the observed conservation pattern was due to

random chance. Sites with a conservation pattern unlikely to have occurred by random chance (P<0.05) were identified as functionally important and as potential target sites for new anti-infectives. A total of 67 such regions in the 16S rRNA were identified (Table 3). The size of the regions varies from 2 to 28 nucleotides (mean = 6.9, median = 6, mode = 5). Some of the regions occur near each other in 30S subunit crystal structures[17; 48] and probably constitute a single target site.

*Among these 67 regions, _ are known antibiotic binding sites[49; 50; 51; 52], _ are tRNA binding sites[53], _ are ribosomal protein binding sites[49], _ form intersubunit bridges with the large ribosomal subunit[53], and _ are initiation factor binding sites[52; 54]. Remarkably, our data also implicate specific nucleotides within these functionally important regions that likely play key roles in each category. These residues will clearly be the focus of future target refinement experiments as described in our Phase II proposal. Several of the sites are

*__Table 3__. Functionally Important Regions

Figure 7. Mutations per Nucleotide in Decoding Region



involved in more than one functional activity (see decoding region below). In addition, there are 16 sites for which no functional role has been identified to date. Though no function has yet been assigned to these regions, it is clear that they are important functional components of the ribosome and are therefore also excellent potential drug targets.

*As an example of the data generated, one of the regions identified in our experiments and known to be important in ribosome

function is the ribosomal decoding region shown in Figure 7. This region of the ribosomal RNA is the place where the codons in mRNA pair with the anticodons in tRNA to translate genetic information into protein[55; 56]. Figure 7 shows the number of mutations per nucleotide in the region encompassing the decoding site (approximately nucleotides 1399-1411). Columns within the chart are color coded to indicate the degree of conservation (red denotes highly conserved nucleotides; yellow indicates residues that had fewer mutations as group; and a space indicates that 0 mutations occurred at that position).

*When the 16S rRNA regions in Table 3 are superimposed on an *E. coli* 30S subunit homology model (personal communication), many of them converge to form clusters of functional nucleotides (Table 4). This model was used to identify interactions among regions and interactions with other ribosomal components. Table 4 shows that most of the 67 regions form _ different functional clusters within the 30S subunit. When the data are analyzed in this way, some of the regions with no functions previously attributed to them are now shown to exist in close proximity with regions involved in ribosomal protein interactions. Whether the nucleotides in these regions directly bind to ribosomal protein remains to be determined.

*Table 4: Functional Clusters

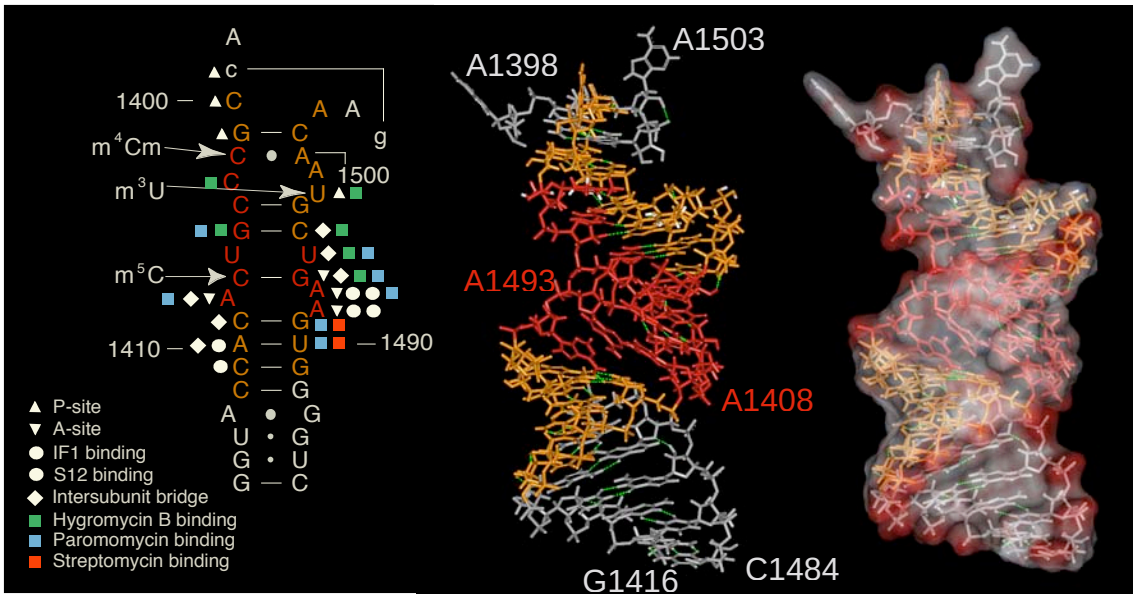
Figure 8. Three-dimensional model of the 16S rRNA showing residues identified as important targets (red) and residues that were statistically significant as a group (orange).

*More detail on some of these clusters is shown below as representative examples. Figure 9 shows the 2- and 3-dimensional structure of the ribosomal decoding region. Panel A shows the secondary structure of the ribosomal decoding region, Panel B shows the three-dimensional configuration of this region as superimposed on the 30S ribosomal crystal structure[17] and Panel C shows the solvent accessible surface of the decoding region. The residues identified in the mutation database as important and potential targets, as they are required for protein synthesis, are shown in red. Residues in orange were statistically significant as a group but not in individual clusters of three or less. Based on our current understanding of the roles played by the individual nucleotides of the decoding region, the nucleotides that play a direct role in protein synthesis in this region are the ones we identified as potential targets (in red) and the residues labeled in orange serve primarily a structural role.

*Residues involved in binding and the proper positioning of tRNAs at both the P site and the A site, initiation factor binding, sites of interaction between the small and large ribosomal subunits (intersubunit bridges), and contact points for the binding of ribosomal protein S12 are all present in the decoding-region nucleotides we

identified (Figure 9A). Within the decoding region the binding sites for hygromycin, paromomycin and streptomycin were identified as potential antibiotic targets (Figure 9A).

*Figure 9. Mutation analysis of the *E.coli* 16S rRNA decoding region.



***Summary.** We have successfully created, sequenced and analyzed the 16S rRNA *E. coli* mutation library as described in our Phase I proposal. These data have already yielded a remarkable source of new potential anti-infective targets. In addition, the unexpected richness of the mutation library is providing insight as to which of the nucleotides within these targets are essential and which serve primarily structural roles.

*Analysis of the mutation database has revealed all of the regions in *E. coli* 16S rRNA that were already known to be essential for ribosome function and has also allowed us to identify several previously unidentified functionally important regions that are new potential targets for the development of anti-infectives. Within the previously known regions, we have preliminary data showing whether particular nucleotides are critical, structural or not essential for function. However, a few of the binding sites for antibiotics in the 30S subunit (as reported in the literature) were not identified in our search. These regions have no known function and are probably not the primary sites of action of the antibiotics. Some of these loci are also sites where mutations have been identified that lead to antibiotic resistance[57; 58]. The isolation of antibiotic resistance mutations at these positions *in vivo* clearly demonstrates that the mutations do not significantly disrupt ribosome function even though they do prevent binding of the antibiotic. These findings further support our prediction that the sites identified in our mutation library are composed of nucleotides that play critical roles in the translation process and therefore cannot be mutated without significant loss of ribosome function.

*The fact that previously identified functionally important regions and antibiotic binding sites were identified in our analysis of the mutation database provides validation that the regions of the rRNA identified through analysis of our mutation database are functionally important and will therefore be excellent candidates for new antibiotic targets.

*We have begun *in vivo* functional analyses (GFP assays) of each of the clones in the mutation library. Though the assay results are not complete, the clones that have mutations in the 67 regions of interest, identified as potential drug targets, appear to have the lowest function *in vivo*. This observation is consistent with our predictions since these regions were identified by their inability to tolerate mutations and retain function. When the GFP data are complete we will also perform covariation analysis to identify long-range sites of nucleotide interaction by identifying nucleotide pairs in which mutations tend to occur together in the functional mutants.

*In Phase II, we will use a number of criteria to evaluate the regions and prioritize sites for further development as drug targets. Criteria (described in detail in section D) will include: (1) size and complexity, (2) presence or absence of modified nucleotides, (3) known interactions with proteins or other parts of the rRNA, (4) accessibility to solvent, (5) differences from corresponding human sequences, (6) whether the target region

forms a structure that can be synthesized and used in the proposed assays, and (7) whether there is a known ligand. Further structural and mutational analysis of one prioritized target, selected using these criteria, will take place under the Phase II SBIR. In addition, we will screen small molecule inhibitors for binding to the selected target and its viable mutants in order to validate it as a drug target and develop potential drug candidates. The remaining targets will be back-up candidates to be developed in the event that technical difficulties arise with the first target, and will be developed as time and resources permit.

***Publications and Patents.** We plan to publish the data we have generated as the result of the Phase I SBIR, and have filed a patent application covering the 67 regions and _ clusters we have identified and the specific sequence data within each site.

Current employees.

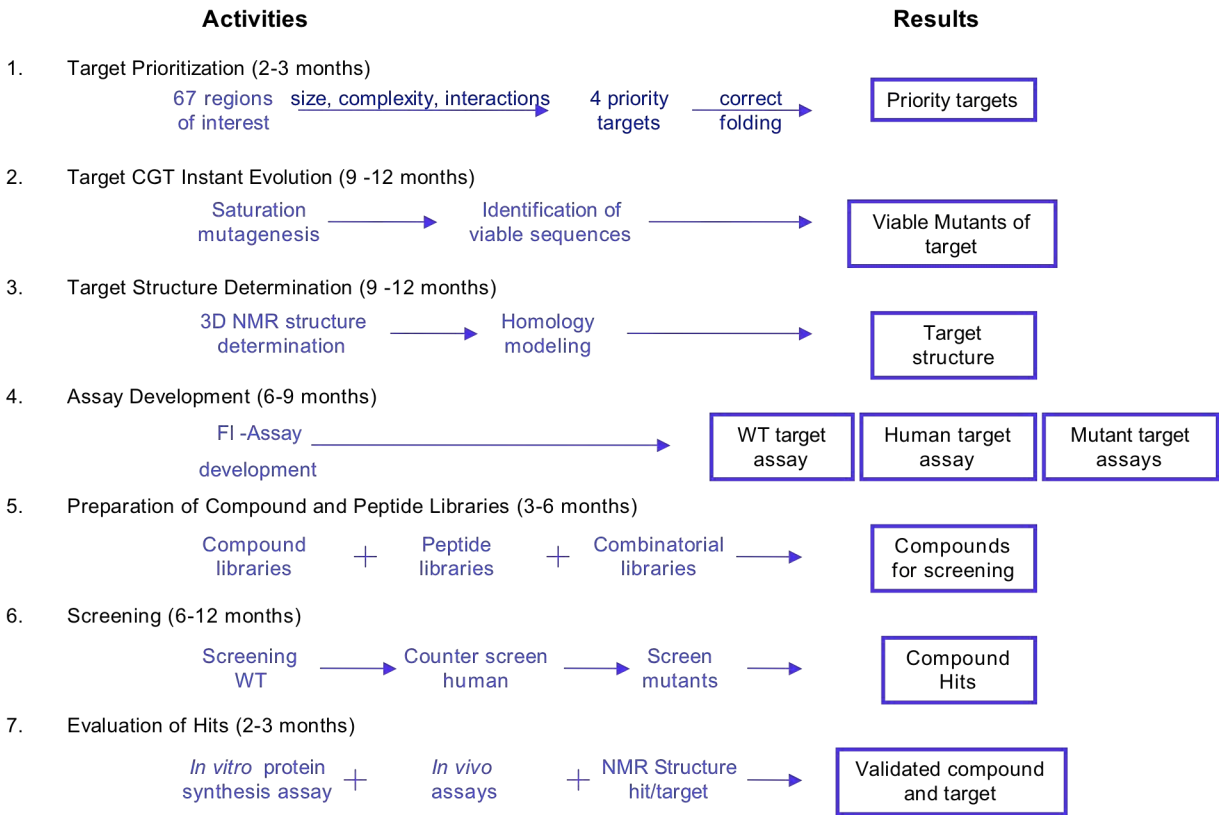
D. Phase II Experimental/Research Design and Methods.

Introduction. The long-term goal of this project is to develop new anti-infective drugs that are not susceptible to current resistance mechanisms and are less susceptible to the development of resistance *de novo*. The five steps towards this goal include: (1) selection of a potential drug target from the RNA "regions of interest" identified in Phase I, (2) refinement of the target using saturation mutagenesis to isolate all viable mutations, and structural analysis of the target using NMR spectroscopy and homology modeling, (3) development of high throughput assays for RNA target screening and acquisition of compound libraries, (4) screening to identify hits that recognize the target and its viable mutants, and (5) evaluation of the hits and validation of the target. Subsequent to this, RiboNovix will move these compounds through drug optimization and pre-clinical and clinical testing.

Phase II Proposed Project Plan

Strategy. We plan to integrate several technologies in Phase II to discover new classes of antibiotics to which bacteria cannot become resistant by target-site mutation, the most common mechanism for resistance to new synthetic anti-infectives. The activities are shown in Figure 12.

Figure 12: Project Scheduled Activities



Target Selection Criteria: Under Phase I we identified 67 RNA "regions of interest" with few or no mutations. Due to secondary and tertiary interactions of the 16S rRNA, many of these regions are located in close proximity with one another, forming loops and/or clusters. The actual number of potential drug targets, therefore, is more likely somewhere between _ (number of clusters) and _, depending on the number of nucleotides included. We have chosen four of these RNA subdomains (Table 5, Work Plan:Specific Aim 1) to investigate as potential anti-infective drug targets based on the following criteria:

1. *Size, complexity, and number of nucleotides with few mutations.* The target sites we choose must maintain the same native structure as they do within the context of the ribosome when synthesized as a separate RNA element. Those subdomains represented by hairpin loops or stem and loop type structures are thus more likely to maintain a native conformation apart from the ribosome. We have also chosen

target sites based on the number of nucleotides contained within these subdomains that have few or no mutations. Drugs targeted against such sites should be more effective at inhibiting protein synthesis and less susceptible to resistance through target site mutation. Thus, we have chosen RNA subdomains that are limited to less than 50 nucleotides per strand, form discrete structural elements, and contain at least two "regions of interest" that interact with one another.

2. *Presence or absence of modified nucleotides*. Several RNA subdomains contain modified nucleotides. Many of these are either available commercially or through our collaboration with Dr. Christine Chow at Wayne State University. One of the subdomains we have selected contains _ modified nucleotides (see Table 5), one of which is commercially available.

3. *Known interactions with proteins or other parts of the rRNA*. Many of the potential RNA targets we identified in our mutation analysis are known to interact with ribosomal proteins, other RNA molecules, or other parts of the rRNA. Because disruption of these known interactions by a small molecule are likely to affect the function of the bacterial ribosome, these particular RNA subdomains have the potential to be good targets. However, other RNA sites we identified have no known molecular interactions, but our data strongly indicates that these sites are also important for ribosome function. Therefore we have included one of these subdomains as part of our prioritzed list of targets (see Table 5).
4. *Accessibility to solvent*. Based on analysis of the target RNA regions in 3-dimensional space, we can make predictions as to whether the subdomains are likely to be accessible to solvent. However, final determination of accessibility will be based on experimentation.
5. *Differences from corresponding human sequences*. We have compared the sequences of the *E. coli* subdomains with the corresponding human sequence. One of the target subdomains has no mammalian homologue and three others have several differences from human (Table 5).

   Table 5 also shows the sequence conservation of each target compared to other bacteria.
   The more highly conserved targets (#2,3,4) can be used as broad spectrum drug targets, and those with more sequence variation (#1) can be used to develop narrow spectrum, organism-specific antibiotics. Although RiboNovix' long term plan is to develop both types of targets, the initial choice of target will be driven by the selection of the one that best fits our selection criteria.
6. *Whether the target region forms a structure that can be synthesized and used in the proposed assays*. We will determine by experimentation whether the RNA subdomains can be synthesized to form properly folded structures (see Specific Aim #1). Those RNAs that maintain native conformation when synthesized will be tested in pilot assays to determine suitability for high throughput screening.
7. *Whether there is a known ligand*. In addition to other *E. coli* molecules that interact with ribosomal RNA sites, there are antibiotics known to bind to specific regions of the 16S rRNA.

   Knowledge of such ligands may be useful in the development of competition screening assays.

More detailed descriptions of the targets can be found in Specific Aim 1 below. The RNA subdomains will be synthesized and evaluated using one-dimensional NMR analysis to assess whether they fold correctly when compared with the ribosome crystal structure. From this analysis the targets will be prioritized, tested in pilot screening assays, and one will be chosen for characterization and high throughput screening. The other sequences will be used as backups and will be developed later as resources permit.

Target Refinement: We will use CGT to make and analyze all of the viable mutants of the selected target. Concurrently, we will use NMR studies of the RNA domains, and the RNA homology modeling software to determine the 3D structures of the wild type target. In addition, we will determine the structures of the 4 - 6 viable mutants thought to be most likely to interfere with the binding of a compound to the target site.

Screening Strategy: Our technology allows identification of every target mutation with the potential to lead to drug resistance, and enables the determination of the critical, structural and non-critical nucleotides *before* the target is used to isolate new drug leads. It also allows us to generate a library of functional target mutations that can be used to screen compound libraries to discover new drug leads that recognize the wild-type *and* the viable mutants of the target. Our strategy is to screen first with the wild type target. Hits will be counter screened against the corresponding human target to eliminate compounds that would inhibit human

ribosomes. Compounds will then be screened against the viable mutants to select compounds that should be resistant to target site mutations. Hits identified by this strategy should mimic the target's natural ligand.

Most small molecule screening approaches are centered on the identification of molecules that bind to proteins. While much can be learned from the study of therapeutics that interact with proteins, our efforts require the use of RNA target-based drug design strategies. In order to identify molecules that will bind to the critical nucleotides in the RNA targets, we will use specific RNA-ligand based screening assays and a combination of diverse compound collections, including small libraries of RNA-binding compounds, large collections of known "drug-like" compounds, and peptide libraries.

We plan on using two parallel tracks for screening. For screening phage libraries we will use biotin labeled RNAs, streptavidin-coated beads and phage in a binding reaction, followed by bead recovery to isolate phage that bind to the RNA target. Secondly, we will use fluorescent assays to screen the RNA targets against various compound libraries. Initial pilot studies will use a small collection of RNA binding compounds to develop the assay system and controls, before moving on to high throughput screening.

RNA Screening Assays: One of our assays is based on the work of Dr. Christine Chow of Wayne State University, who has studied RNA-antibiotic interactions using fluorescence assays. The RNA molecules are tagged with reporter dye (fluorescein or other tag) and ligand-induced conformational changes in the RNA are monitored. The second approach involves the use of a three-fluorophore fluorescence resonance energy transfer (FRET) assay that can detect
both the conformational change of RNA fragments in the presence of a ligand and the disruption of a ribosomal protein/rRNA complex by a ligand. Depending on the specific target, one or the other type of assay may be more suitable.

The assay developed by Dr. Chow is fluorescence based, and has been used to study the conformational changes induced by aminoglycosides at the ribosomal A site[60; 61]. This assay was used to discover new antibiotics that bind to the A site, as well as to monitor the binding of enzyme-modified aminoglycosides[62; 63]. One of the aminoglycoside analogues exhibited a 45-fold enhancement in binding to 16S rRNA over human 18S rRNA (A1408 vs. G1408), demonstrating that a high specificity of ligand binding can be achieved, even for RNAs with almost identical sequences. In this assay, the 5' end of the target RNA fragment is tagged with fluorescein or another dye. An advantage of this assay is that only small volumes and concentrations of RNA and ligand are needed (µL, nM–µM).

The 3F-FRET assay has been used to screen for small molecule inhibitors of ribosome assembly. During the assembly of the 30S ribosomal subunit, the small subunit proteins bind to the 16S rRNA in a hierarchical manner. S15 is a primary binding ribosomal protein that interacts with a three-way junction in the central domain of 16S rRNA and induces a conformational change. In the assay, the RNA fragment containing the S15 binding site is labeled with two fluorophores and a third fluorophore is attached to the S15 protein. Compounds that bind to the junction and affect conformation can be identified (Fig. 13). The assay is highly sensitive and can be performed in 384-well microtiter plates for high throughput screening.

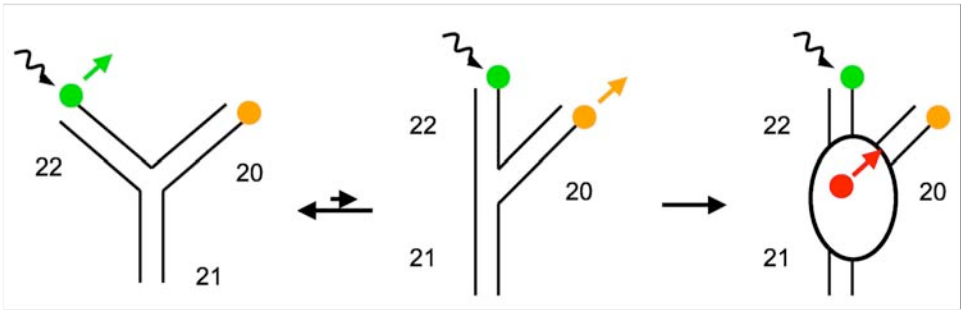


Figure 13**.** Schematic depiction of the central domain 3WJ of the 16S rRNA comprising helices 20, 21 and 22. In the absence of Mg^{2+} and S15, the junction is extended (left). Both Mg^{2+} and S15 induce the formation of a compact structure (middle), in which the helical arms 20 and 22 form a 60^{o} angle and helices 22 and 21 stack coaxially. S15 binds to the junction and stabilizes the folded form (right).

Compound Libraries: Much of our understanding of small molecule binding to macromolecules is largely derived by the study of small molecule-protein interactions. For these types of interactions, much of the binding energy is associated with the exclusion of water by hydrophobic contacts, typically involving an invagination of

the protein surface to form a hydrophobic binding pocket into which the small molecule ligand fits. In addition, the binding often entails some change in the orientation of the amino acids comprising the pocket ("induced fit").

These properties of small molecule-protein interactions indicate that RNA structures can be targets for small molecules as well because RNA shares many of the properties of proteins that make them attractive for binding small molecules. First, RNA frequently adopts a shape similar to proteins that bind small molecules. Second, the surface groups of RNA (sugars and bases) tend to present, thereby facilitating interactions of these groups with small molecules. Such interactions can include hydrogen bonding, van der Waals interactions, and electrostatic interactions, as well as hydrophobic interactions in certain orientations. Third, like proteins, RNA structures are plastic, which allows them to mold to the shape of a ligand (induced fit). Fourth, the sequence complexity of RNA, and particularly the complexity of higher order RNA structure, creates unique three-dimensional structures in specific RNA molecules that can be targeted with high specificity.

Most compound collections and combinatorial chemistries have been designed around the identification of molecules that bind to proteins. However, our interest is in small molecules that bind to RNA. The RNA molecules of the ribosome bind to many proteins and some of our rRNA targets may be involved in rRNA/protein interactions. Potential drug leads may alter the functional three-dimensional structure of the nucleic acid so that the interaction with the protein is inhibited, or they may prevent the formation of competent RNA-protein complexes. Since the natural ligands for our rRNA targets are likely to be the RNA binding domains of proteins, one of our strategies is to use phage and peptide libraries, to find weak binding hits. Besides providing information for developing small molecule lead compounds, peptide binders will also be useful for target validation studies and development of competition screening assays.

Phage-display methodologies and synthetic peptide libraries offer a convenient way to produce peptide ligands with high affinity for targets, such as rRNA regions. In addition: i) a number of peptides exhibit antibiotic activity[64]; ii) peptides have a proven ability to bind RNA molecules[65; 66]; iii) the level of molecular diversity using only the standard 20 amino acids is enormous; inclusion of 'unnatural' amino acids dramatically increases the diversity still further; iv) the generation of peptides (either through phage display or chemical synthesis) is routine and highly reliable; v) peptide leads can be used as the basis for generating non-peptide ligands and peptidomimetics[67], and vi) peptides and their derivatives have a history of use as therapeutic agents[68]. There is precedent that peptides and other organic ligands can be designed to bind RNA regions containing bulged residues and widened and accessible major grooves, structures that may be present in the proposed targets[18; 69; 70; 71; 72; 73].

While screening phage and peptide libraries will provide us with some useful information, our primary focus will be to screen several different types of compound libraries (for specific details see methods section). Two large collections of compounds are accessible through our collaborations with _ and Harvard University. Additional diversity will be obtained by screening small compound collections from _ and _. These smaller libraries contain compounds that have been selected for their ability to bind to RNA.

While we do not expect highly specific hits from these libraries, they will help in guiding us to the selection and development of compounds that bind to RNA. We intend to build up our compound collection with a view to collecting a specific library oriented around RNA-binding compounds.

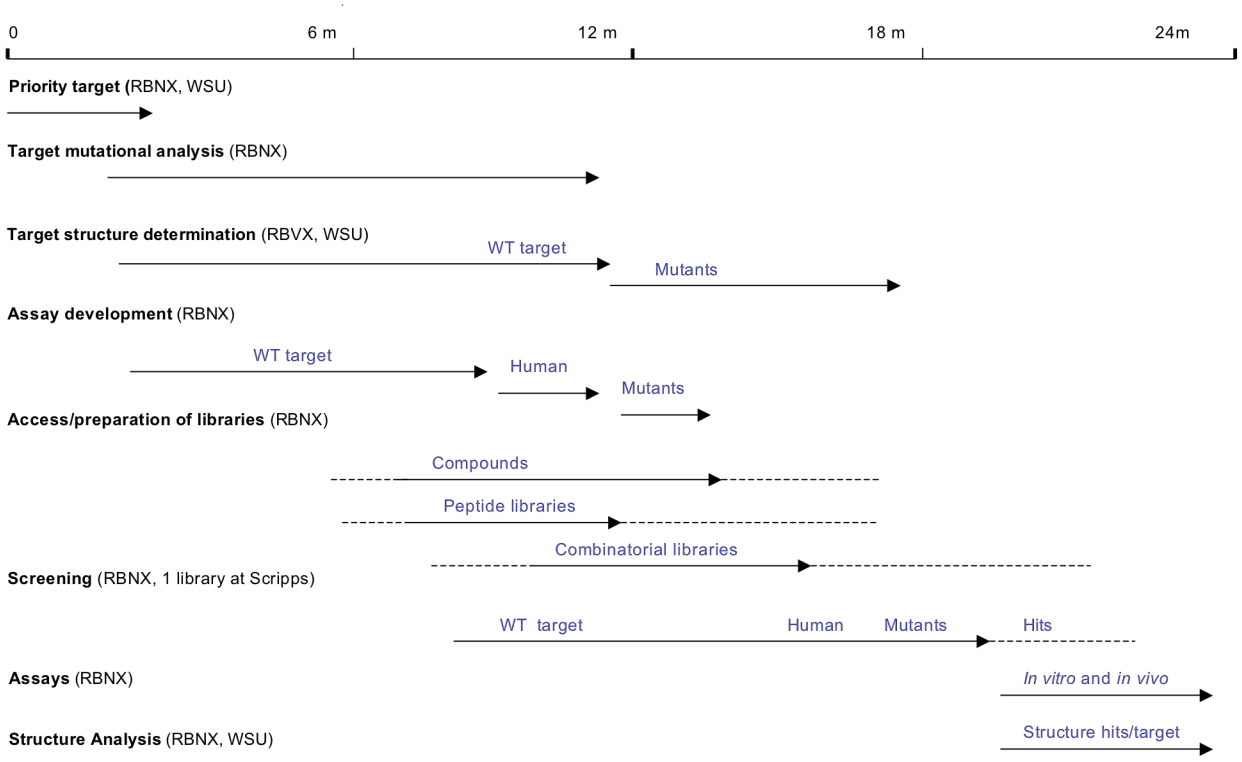
Once a collection of "hits" is generated, a focused library of compounds will be assembled using the structures of the peptides and compounds to guide the selection of new compounds for high throughput screening. This effort will be assisted by NMR studies of the RNA-ligand complexes, allowing rational decisions to be made, and fine-tuning of the binding interactions through site-specific chemical alterations to each ligand. We will use commercially available compounds and proprietary collections under licensing arrangements for this effort.

Structural Studies of Targets and Compounds: Interactions of the hits (peptides and compounds) and drug leads with their *E. coli* rRNA targets will be characterized using NMR spectroscopy to determine key functional groups important in the interaction. Later these data may be helpful in modifying the drug leads using rational drug design and medicinal chemistry to improve bioavailability, pharmacodynamics, and to reduce toxicity.

NMR studies will provide several types of critical information. First, NMR will be used to verify whether the structure of *isolated* RNA targets taken out of the context of the ribosome resemble their structure in the ribosome and will therefore be valid targets for screening compound libraries. NMR spectroscopy will also provide detailed stereochemical information on the mechanism of binding of small-molecule ligands with RNA targets. Lastly, NMR studies will reveal crucial differences between the *E. coli* and the human small subunit rRNAs. Comparison of the wild-type and mutant structures will reveal the essential functional groups and structural folds required for ribosome function, thereby focusing the design of drugs to these critical residues[4]. Structural characterization of RNA-ligand complexes will reveal how each compound recognizes the essential target motifs. Further, characterization of RNA dynamics by NMR in the presence and absence of bound drug leads will reveal the role of induced fit in RNA recognition[18].

Target Validation: Potential drug compounds will be tested in eukaryotic and bacterial *in vitro* protein synthesis assays. Compounds that inhibit wild-type and mutant bacterial ribosomes but not eukaryotic ribosomes will be tested for antibacterial activity *in vivo* by determining the minimum inhibitory concentration (MIC) of the compound required to inhibit cell growth of *E. coli* cells, as well as cultures of representative Gram positive and Gram negative organisms. Compounds active in the *in vitro* assay but with poor *in vivo* activity will be modified using established medicinal chemistry techniques to increase cell wall permeability.

Figure 14. Schedule of Activities and Responsibilities



WORK PLAN

SPECIFIC AIM #1. Target Selection. One RNA subdomain will be selected as a primary target from the four potential targets chosen based on the RNA "regions of interest" identified in Phase I.
Milestone: One target, with backup targets, 2-3 months.

Summary. Four target RNAs will be evaluated for their ability to fold correctly and have the same natural conformation as they do in the complete ribosome. To evaluate this we will synthesize small samples of each RNA subdomain and use 1D NMR to determine which regions fold correctly (as compared with the region in the context of the full ribosome), and which give the best NMR spectra and are therefore amenable to NMR structure determination. We will also evaluate the targets for changes on exposure to solvents, which will tell us if the targets are accessible.

Description of the Targets. Based on the criteria outlined earlier, four target subdomains were chosen for further analysis. All of the potential target RNAs form some type of discrete structural element, such as a stem and loop, and contain between 28 and 43 nucleotides (Table 5).

Table 5. Target Descriptions

a. Target #1.

b. Target #2.

c. Target #3.

d. Target #4.

Methods: RNA Synthesis. *E. coli* targets will be synthesized using standard phosphoramidite chemistries and can be obtained commercially from Dharmacon or generated using in vitro transcription.

Method: Verification of homology models. We will perform a series of relatively simple experiments to confirm the homology structures of the RNAs. For each RNA subdomain, chemical probing experiments will be performed on the isolated domains as well as complete 30S ribosome to determine if they have similar reactivity patterns and thus similar structures. Regions that show similar patterns will be studied by 1D imino proton NMR and 2D NOESY. The availability of the assignments for the wild-type *E. coli* domains will allow us to match the pattern of NOEs and chemical shifts to determine qualitatively if the structures of the wild-type and isolated regions are similar.

SPECIFIC AIM #2. Target refinement: We will use CGT to identify every mutation that could to lead to drug resistance in the selected target, and use multidimensional NMR spectroscopy and homology modeling to determine the essential structural components.
Milestone: Structure information on target, 9-12 months.

Summary: Saturation mutagenesis of the target RNA will be performed using CGT followed by chloramphenicol selection to identify viable mutants. The viable mutants will be sequenced, assayed, and analyzed to identify the essential components of each target. GFP production in each mutant will be determined by measuring GFP fluorescence in whole cells. The essential sequence and structural motifs identified in the saturation mutagenesis experiments will be validated by constructing site-directed mutations that either disrupt or maintain the proposed motif in the target RNA and measuring the effects of the mutations on protein synthesis *in vivo*.

RNA homology modeling software developed by Dr. John SantaLucia at Wayne State University will be used to predict the structures of the wild-type target, viable mutants, and the corresponding human sequences. The software requires the input of a known 3D structure, followed by a sequence alignment to determine where in the 3D structure substitutions, deletions or insertions will be made. The sequence alignment for each domain will be done with a new alignment program called SBSA (structure based sequence alignment), which automatically performs the sequence alignment subject to the constraint that the paired residues from both secondary structures are in the proper register. The phylogenetically determined secondary structures of the 16S rRNA from *E. coli* and the human 18S rRNA are available.

Methods: Random mutagenesis and selection of viable mutants. Saturation mutagenesis will be carried out by PCR mutagenesis essentially as described by Higuchi[77]. Partially randomized mutations will be introduced using recombinant PCR, cloned in pRNA123 (Fig. 2) and used to transform *E. coli* DH5 cells by electroporation. The number of viable mutants of a given target depends on the stringency of the selection and on the functional constraints of the target. To isolate viable mutants, transformants will be plated on LB agar containing 100 µg/ml ampicillin, 1 mM IPTG and 50, 100, or 200 µg/ml chloramphenicol. This is significantly below the minimum inhibitory concentration (MIC) of chloramphenicol for cells expressing wild-type ribosomes in our system (700 µg/ml) to avoid eliminating mutations with reduced activity that may lead to resistance through the sequential accumulation of second-site complementation mutations[78; 79]. PCR reactions, restriction enzyme digestions, DNA ligations and electroporations will be performed according to standard procedures. Electroporations will be performed using a high capacity electroporation chamber to provide sufficient transformants for the isolation of viable mutants.

The number of survivors required to identify the essential sequence motifs in the target is dependent on the size of the target. We will initially isolate and characterize 300 viable mutants for the target. The final number of mutants to be analyzed will depend upon our initial evaluation of the data from these mutants. Based on our preliminary experiments, 300 mutants is probably in excess of the total number of viable mutants at each site and should be sufficient to allow identification of the components of the target that are essential for function. We will use resampling statistics[4; 80] to determine the probable total number of viable mutants based on the data from our initial 300 isolates. If the predicted number of viable mutants is greater than the 300 initial isolates, we will isolate and characterize enough additional mutants to make sure that we have isolated most of the possible target-site mutants. Mutant constructs will be sequenced through both external ligation junctions to check for the presence of unprogrammed mutations inserted during the amplification reaction.

Methods: GFP assays. Overnight cultures will be diluted in LB-Amp100 medium and grown at 37^{o}C with shaking until OD_{600}=0.1. The cultures will be induced with IPTG (1 mM) and incubated with shaking for an

additional three hours. Following incubation, 1 ml of each culture will be removed, pelleted, washed twice, and resuspended in buffer. Cell density (OD_{600}) will be determined and fluorescence (excitation=395 nm, emission=509 nm) measured using a microplate fluorometer. For each culture, fluorescence will be divided by OD_{600} and presented as a percentage of the wild-type *E. coli* construct. Assays for each construct will be performed at least three times and averaged.

Method: Homology modeling. RNA homology modeling software described earlier will be used to predict the structures of the wild-type target, viable mutants, and human domains. The sequence alignment for the target will be done by comparing the known phylogenetically determined structures of the 16S rRNA from *E. coli*, and human 18S. Structures will also be predicted for a number of the functional mutants. The wild-type and mutant structures will be superimposed to reveal the functional groups that are conserved in all functional mutants.

Method: Determination of high-resolution structures. We will focus our initial efforts on the wild-type target and its human equivalent. An average structure takes about 6 months to complete. This will be followed by analysis of the structures of the 4-6 highly functional mutant sequences that are most likely to affect the binding of a small molecule to the target sequence. We will not know the exact details of the sequences of these mutants until the saturation mutagenesis experiments are complete. NMR is well adapted to solving mutant structures once assignments for a wild-type sequence are known.

Method: NMR sample preparation. NMR samples will be synthesized using standard phosphoramidite chemistries or generated using run-off transcription from a synthetic template or from a linearized plasmid. Transcription reactions will be carried out as described previously[82]. Transcripts will be purified on denaturing polyacrylamide gels and recovered by electroelution. To obtain efficient transcription it is often necessary to mutate the first several nucleotides that form part of the T7 promoter to guanines and cytosines. If this is necessary for our sequence, then we will confirm their biological activity by making the same mutants in the whole ribosome encoded on pRNA123, as was done for the studies of the 790 loop[4].

Method: Preparation of labeled NMR samples. Uniform ^{13}C and ^{15}N labeling of RNA will be carried out using published methods[83; 84]. Uniform labeling facilitates resonance assignments by resolving resonances in multiple dimensions and by relying on through-bond transfers of magnetization. Such labeling also provides significant additional information through heteronuclear J-couplings measured by E. COSY type methods and residual dipolar couplings.

SPECIFIC AIM #3. Target screening: We will screen compound, phage/peptide libraries against the wild type target and its viable mutants.
Milestone: Identification of hits that have specificity for the target and its viable mutants, 12-18 months.

Summary: Compound and phage libraries will be screened using selection and fluorescence assays to isolate lead compounds with high affinity for the wild-type rRNA target and the viable mutants of the rRNA target, but low affinity for human ribosomes. The nature of the interaction of the hits with their targets will be determined by NMR analysis of lead-target complexes and other biophysical studies. After a collection of initial hits is generated and the sequence information obtained, compound libraries containing RNA-binding molecules will be synthesized and accessed from commercial and non-commercial sources. By using a limited set of input building blocks with structural and functional characteristics that resemble the monomers of the initial leads, the binding interactions with the RNA targets will be further enhanced and optimized.

Methods: RNA synthesis. RNA will be synthesized chemically or cloned into a plasmid that facilitates run-off transcription as described earlier. The method of synthesis will depend on the specific target chosen for screening, and whether it is being used for peptide screening or high throughput library screening.

Phage Library Screening

Methods: Phage display libraries. Two commercial phage-display systems will be screened, M13 and T7. One is constructed in filamentous phage, M13 (Ph.D.-7, New England Biolabs), which generally contain fewer contaminating bacterial proteins than lytic phage preparations. This is important when screening for RNA-

binding peptides due to RNase degradation[85]. The library is cloned into M13KE (a M13mp19 derivative[86]) as an N-terminus fusion to the M13 coat protein, pIII, displaying 5 copies of the peptide. The standard heptapeptide library will be used in our initial screens. The complexity of the pool is 2.8×10^9 transformants. This is greater than the number of sequences obtained if 7 amino acids are randomized (1.28×10^9). We will also try other M13 libraries. The Ph.D.-C7C library (New England Biolabs) contains cyclic peptides and will produce tighter binding peptides. Both the linear and the cyclic M13 libraries will be screened initially.

The second type of phage-display library is constructed in the lytic phage, T7, (T7Select 415-1, Novagen). Here, the diversity of peptides on the surface of lytic phage is not restricted by their inability to pass through the bacterial membrane[87]. The library will produce C-terminal fusions to the T7 capsid protein 10B and is present at 415 copies per phage. The 10B protein is normally produced as a frameshift protein of the 10A gene but in this construct the frameshift signals have been removed so that only one type of capsid protein is produced. A strain with the RNase I gene deleted will facilitate its use in screening RNA targets.

Methods. Preparation and labeling of RNA targets. The target sequences will be designed so that the transcript will form a stable clamp sequence in the stem with an RNA tail at the 3' end. RNA targets generated by transcription will be annealed with a 5' biotin-linked DNA or targets will be synthesized with a 5' biotin tag.

Methods: Screening RNA targets with phage libraries. We will first screen the wild-type *E. coli* target sequence. Phage that bind will be isolated and amplified. The phage pools derived from screening the wt target will be counter-screened against the corresponding human rRNA and competitor RNA with duplex sequences lacking the internal loops or bulges. Phage lacking affinity for the human or competitor RNAs will be retained as potential hits and amplified. The amplified pool will then be used to isolate phage that also bind to the viable mutant RNAs. After each binding reaction, the phage will be amplified and used to screen the next target. Phage that bind all of the bacterial sequences will be retained for further study. The resulting hits should therefore recognize the components of the targets that are essential for viability in bacteria.

Binding reactions with streptavidin-coated beads will contain RNA (10–120 nM), phage ($1–5 \times 10^9$ pfu), *E. coli* tRNA, and RNase inhibitor. After the binding reaction is complete, the beads will be recovered by centrifugation and the bound phage (without release from the beads) will be used to infect *E. coli*. Plaque assays will be performed to titer the phage. After each round of panning, a portion of the phage pool will be sequenced and an aliquot of the lysate will be used for the next round of selection. Phage will also be screened using human and control RNAs to eliminate any peptide sequences that bind nonspecifically to the RNA targets.

Compound Library Screening

Methods: Fluorescence-based assay. Fluorescein-tagged RNAs will be synthesized by chemical methods and deprotected by standard procedures as developed by Dr. Christine Chow at Wayne State. Fluorescein can be added to the 5' end of the RNAs during the last step of the chemical synthesis by means of a six-carbon linker. Remote placement of the dye molecule at the end of the substrate RNA allows detection of drug or ligand-induced, conformational changes in the RNA. Binding of a ligand can be monitored by a decrease in the fluorescence emission intensity of the attached fluoroscein dye[62; 88].

An alternative to end labeling the RNA molecule is to insert a fluorescent nucleoside analogue within the RNA target. This would most likely increase the sensitivity in detecting a binding event. A recent study using 2-aminopurine fluorescence was successfully used to monitor binding of HIV-1 Tat protein to the TAR region of the RNA genome[89]. The fluorescent analog was selectively substituted for nucleotides within the stem of the TAR hairpin. Binding of the Tat protein was monitored by changes in fluorescent intensity. In a similar fashion, this approach could be used to detect binding of a compound to one of the designated 16S target RNAs. The addition of an internal label within the target would also allow the design of a FRET based assay using a labeled ribosomal protein that binds to the target (see below). The ability of a ribosomal protein to bind the target RNA would also be an indicator of a correctly folded RNA molecule.

Methods: The 3F-FRET Assay: The assay uses
the binding of the ribosomal protein S15 to the three way junction (3WJ) formed by helices 20, 21 and 22 in

16S rRNA[90; 91]. The binding of S15 to the junction is a key step in the formation of functional bacterial 70S ribosomes in the early stages of 30S subunit assembly. Binding of S15 induces a conformational change in the 16S rRNA at this junction (Fig. 11) and serves as a prerequisite for subsequent binding events during ribosome assembly[92]. Similar conformational changes occur when other ribosomal proteins bind to RNA.

Several of the targets described above have the potential to bind to a ribosomal protein. The binding requires that the target assumes the correct secondary structure and, in some cases, additional nucleic acids need to be added.
Labeling of both the target and the ribosomal protein will
allow the use of a FRET assay analogous to the one described for S15.

Methods: Compound libraries. We have identified and have access to several sources of compound collections in order to achieve both sufficient number and diversity for rRNA target screening. These are described below.

b. NSRB at Harvard University. The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at Harvard University provides access to small molecule collections and screening facilities for investigators pursuing drug discovery efforts related to pathogens relevant to biodefense and emerging infectious disease. The NSRB currently has access to over 160,000 compounds, including commercially available compound libraries plus synthetic and natural product libraries that are not available elsewhere. Compound categories include known bioactive compounds, selected natural product extracts, and commercial compounds. The known bioactives library contains approximately 2500 molecules. Many of the compounds in the collection are FDA approved and others - which include some controlled substances such as cannabinoids - are known to influence brain activity. There are a number of known antibiotics in the collection, as well as anti-inflammatory, anti-neoplastic, and analgesic compounds, pure natural products and their derivatives, simple and complex oxygen heterocycles, alkaloids, sequiterpenes, diterpenes, pentercyclic triterpenes, and sterols. The selected natural product extracts library contains partially purified extracts from endophytic fungi (fungi that live in higher plants) that grow in Costa Rica. Crude extracts from the fungi were obtained and further fractionated to eliminate nuisance compounds, especially those of high molecular weight or high polarity. Other natural products include extracts of plants from the Philippines.

The commercial compounds available for screening were chosen for favorable physico-chemical properties such as solubility, decreased toxicity, and increased stability, as well as to assure “diversity and drug-likeness” of the compounds.

e. Additional sources. Other potential sources of compound libraries include collections owned by biotechnology companies . We are currently in discussions with _ and the other companies in order to broaden the number and scope of molecules to be screened using our rRNA targets. In addition, a number of companies have generated libraries of _ , which we may be able to access for screening.

Methods: Pilot studies. The design of the fluorescence assay that will be used for high throughput screening is dependent on the final choice of target RNA determined in Specific Aim 1. One critical issue we will investigate is the type and placement of fluorescent molecules in the reagents used in the assay. The placement of the analogue must satisfy several criteria including 1) non-disruption of the native structure, 2) proximity to the proposed binding site of the compound or, 3) in the case of FRET, proximity to the ribosomal protein which binds to the target, allowing for efficient energy transfer. In the case of the assay described by Chow et al., the placement of a fluorescent group at the end of the RNA target should have the least effect on the structural integrity of the target. However, its placement may be too far to detect all potential binding events (including proximity to a labeled ribosomal protein for FRET to occur). As has already been described, 2-aminopurine fluorescein can be inserted into various RNA targets and used as a readout, whether directly (using changes in intensity as a readout) or in a FRET-based system with a labeled ribosomal protein. The placement of the fluorescent analogue will be assessed by NMR and binding of the target RNA to specific ribosomal partners.

Methods: Fluorescence-based assays for high throughput screening.
Fluorescence-based assays, and FRET in particular, provide a robust system for high throughput screening and have distinct advantages over other detection methods. In the case of our nucleic acid targets, the assays we propose can be run as direct inhibition, or with the appropriate molecule, in an indirect competition assay. In the former case, it is possible to derive affinity measurements. However, for screening a compound library

where affinities and concentrations may vary, it is more practical to initially derive a ranking measurement using a competition-based assay. In this case, peptides (discovered in the phage display system), competing RNA molecules or known ligands , could serve as useful
competitors in a FRET-based system. Another advantage is that the FRET-based assays are homogeneous (no phase separation required). This can be critical in the testing of diverse compound libraries where affinity and sensitivity are key factors in the identification of binding molecules. In addition, the homogeneity of the assay, and the stability of many fluorescent molecules, allows for a relatively straightforward progression from a medium throughput assay to HTS.

Solution-based fluorescence assays will be adapted for use in high-throughput assays with the compound libraries described above. A fluorescence plate reader will be employed, such that many compounds can be assessed in a short period of time. Interactions of prospective new compounds will be analyzed, and further high-throughput binding assays with selected compound and mutant target sequences will be carried out, followed by lead optimization. Negative controls (human and non-specific RNA molecules, Fig. 15-18) will be assayed in parallel. Integrity of hits will be evaluated by mass spectrometry or other suitable methods.

All of the viable target mutants isolated *in vivo* will have chemical and structural properties that are essential for proper interaction of the targets with their ligand(s). Even without knowing what these properties are, the viable mutants can be used to identify drug leads that bind specifically to them. Screening will be continued with new targets and lead compounds through an iterative process until tight-binding molecules (nM or better) are identified.

SPECIFIC AIM #4. Validation of targets and compounds: Structural studies of target/hit complexes, optimization of hit compounds, and validation of the target using *in vitro* and *in vivo* assays.
Milestone: One or more compounds that are potential leads, and target validated, 15-24 months.

Summary*:* Interactions of the drug leads with their *E. coli* rRNA targets will be characterized using NMR spectroscopy. This information will enable optimization of hits by determining which functional groups of the compounds are essential for binding and which functional groups may be modified using rational drug design and medicinal chemistry to improve bioavailability, pharmacodynamics, and to reduce toxicity. In addition, the compounds isolated above will be characterized for their ability to inhibit protein synthesis *in vitro*. The compounds identified by screening should inhibit protein synthesis of bacterial ribosomes including those with target mutations. To test this hypothesis, peptides and compounds will be added to eukaryotic and bacterial *in vitro* protein synthesis assays. Bacterial protein synthesis assays will be performed using wild-type ribosomes. We will also test several of the functional target mutants that are not used in the screen to evaluate the ability of the compounds to recognize all viable target mutations.

Information from the NMR structural studies will complement the assays, and will be used to improve the design of compounds for pre-clinical development. These functionally and structurally characterized compounds represent starting points for structure-based design approaches to optimize drug leads[99; 100].

Methods: Bacterial *in vitro* protein synthesis assays*.* To determine whether the compounds have the ability to inhibit protein synthesis, they will be tested in a bacterial *in vitro* protein synthesis assay. A commercially available bacterial *in vitro* protein synthesis assay kit (*E. coli* S30 Extract System for Linear Templates, Promega) will be used. This kit contains an S30 extract of an *E. coli* B strain (F-, hsdS, gal, OmpT, lon, recBCD)[101] that is capable of coupled transcription and translation. It also contains S30 premix that provides all components (NTP's, tRNA's, ATP regenerating system, IPTG, and salts) except for amino acids; several amino acid mixtures that lack cysteine, methionine, and leucine are needed in the reaction. The provided control DNA of pBESTLuc (Promega) will be used as the transcription template. This plasmid contains the firefly luciferase gene under transcriptional control of Ptac and an ampicillin^r marker. To measure inhibition, a series of reactions containing varying amounts of the peptide will be incubated and the ^{35}S-methionine-labeled product will be quantified[102]. Inhibition will be indicated by the loss of product formation in a dose-dependent manner compared to the addition of buffer only.

Methods: Bacterial *in vitro* protein synthesis assays with mutant S30 extracts. The Shine-Dalgarno sequence of the luciferase gene in pBESTLuc will be modified to the same sequence used by CAT and GFP in

the specialized ribosome system (Fig. 1) using site-directed mutagenesis. The new construct will be substituted for pBESTLuc in the *in vitro* protein synthesis assays but will only be able to be translated by the mutant specialized ribosomes. Otherwise, the assay will be identical to the bacterial protein synthesis assay described above.

Methods: Eukaryotic *in vitro* protein synthesis assays. The compounds identified should not inhibit eukaryotic ribosomes. Compounds that inhibit bacterial protein synthesis will be tested in eukaryotic *in vitro* protein synthesis assays. A commercially available rabbit reticulocyte *in vitro* protein synthesis assay kit (Proteinscript II Linked Transcription:Translation Kit, Ambion) will be used. The transcription and translation reactions are separate. The kit contains all the reagents needed for both transcription and translation including the T7 enzyme and rabbit reticulocyte lysate[103].

Methods: Antibacterial activity assays (MIC). A major obstacle in the development of antibacterial agents is the discovery of compounds that are active *in vivo.* The bacterial cell wall is impermeable to certain types of compounds. It is, however, relatively simple to evaluate the antimicrobial activity of compounds against *E. coli* and other common laboratory strains. Molecules that inhibit protein synthesis *in vitro* will be evaluated for antibacterial activity *in vivo* by determining the minimum inhibitory concentration (MIC) of the compound required to inhibit cell growth of *E. coli*, *Pseudomonas*, *Staphylococcus*, and *Enterococcus* cells. MICs will be determined according to standard procedures. Inhibition of growth in a dose-dependent manner will indicate antimicrobial activity. Compounds active in the *in vitro* assay but with poor *in vivo* activity will be modified using established medicinal chemistry techniques to increase cell wall permeability.

Summary: Phase I of this project was highly successful. We identified 67 regions of *E. coli* 16S rRNA containing nucleotides that are essential for viability. Among these 67 regions, _ are known antibiotic binding sites, _ are tRNA binding sites, _ are ribosomal protein binding sites, _ form intersubunit bridges with the larger ribosomal subunit, and _ are initiation factor binding sites. There are _ sites for which no known functional role has been identified to date.

Under Phase II funding we plan to further investigate a number of these sites and develop one as a potential drug target. We will use saturation mutagenesis with CGT to identify the specific elements required for ribosome function, and NMR and the RNA homology modeling to determine the structure of the site. High throughput assays will be used to screen the targets against compound collections composed of peptides, RNA binding molecules, and other drug-like compounds. Compounds that bind to the target RNA will be used for target validation using *in vitro* protein synthesis and *in vivo* assays.

RiboNovix will complete the work necessary to develop drug candidates from lead molecules using *in vitro* and *in vivo* testing for anti-microbial activity, and will move qualified candidates into pre-clinical development. We expect that antibiotics developed using CGT will be highly effective and refractory to drug resistance.

Contractual Arrangements

RiboNovix has already licensed the Combinatorial Genetic Technology (CGT) from Wayne State University, and has initiated discussions concerning the RNA homology modeling software.
RiboNovix, Inc. intends to establish
additional consortium agreements with both Wayne State University and _
for the work under this Phase II program.

WAYNE STATE UNIVERSITY

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Cheryl Murphy, Ph.D.
RiboNovix, Inc.
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November 20, 2005

Dear Cheryl,

I am very pleased to offer my consulting services to you and RiboNovix as you collaborate with Dr. Philip Cunningham and Wayne State University on developing new antibiotic drug leads for ribosomal RNA targets in *E. coli*. The studies that you propose in your Phase II SBIR grant application entitled "Identification of *E. coli* anti-infective rRNA targets" will be important for not only finding new antibiotic drug leads effective against *E. coli*, but will also lead to the extension of the RiboNovix genetic technology for use with other pathogens.

As you know, I have over ten years of experience in the field of small molecule-RNA interactions, and I worked previously on DNA-protein interactions for my postdoctoral project. We have recently been using phage display methods in my lab to identify peptides with affinity for 23S rRNAs, and also developed fluorescence assays for monitoring RNA-ligand interactions. In addition, my lab is now using ESI mass spectrometry and ITC to examine peptide-RNA interactions. We also have experience in synthesizing modified RNAs, so will be happy to collaborate in order to generate modified rRNA targets.

I am looking forward to working with you and RiboNovix, and will be happy to assist you in your efforts.

Sincerely,

Christine S. Chow, Ph.D.

NSRB The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases

Harvard Medical School
250 Longwood Ave.
Boston, MA 02115

November 21, 2005

Dr. Cheryl Murphy, Ph.D.
Vice President of Technology Development
RiboNovix, Inc.
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Lincoln, MA 01773

Dear Dr. Murphy:

We are pleased to write this letter of support for your Phase II SBIR proposal on screens for small molecules that may serve as leads for the development of novel antibiotics directed against ribosomal RNA targets. I understand that your laboratory has begun developing high-throughput screening assays that are compatible with our facility, and we will be happy to work with you on this project, subject to approval of your screening application by our Advisory Committee.

The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB) was established as part of the Regional Centers of Excellence program to provide access to a chemical screening laboratory, equipment and chemical libraries for U.S. investigators conducting research on pathogens of biodefense and emerging infectious disease interest. The laboratory is housed on the Harvard Medical School campus and is managed by the New England RCE.

The NSRB facility is fully capable of performing the screens you have in mind. We can provide you with access to over 160,000 small molecules, advanced high-throughput technology, technical expertise, informatics, and medicinal chemistry. We have a full-time screening staff who will assist you in running the screens, and an informatics group who will assist you in comparing the results of your screen with results from the ~150 other screens performed at the NSRB/ICCB-Longwood by in-house screeners or collaborators. This is often useful in determining the specificity of hits. We will be happy to help you evaluate your hits and share our experience on follow-up approaches. We also have medicinal chemistry capabilities for subsequent studies, although access to a full medicinal chemistry effort requires approval by the NSRB Advisory Committee after your primary screen is completed.

There will be no charges for using the NSRB's resources and staff time, and the NSRB will supply you with all generic consumables and reagents that are required to screen up to 50,000 compound wells in duplicate. However, you will be responsible for the costs of screening beyond this level, and for any travel expenses for personnel from your laboratory who will be conducting screens in the NSRB facility.

We look forward to working with you on this exciting project,

Sincerely,

Su L. Chiang, Ph.D.
Assistant Director of Screening
The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB)

PHS 398 Checklist

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

❍ New ● Resubmission ❍ Renewal ❍ Continuation ❍ Revision

Federal Identifier: 2 R44 AI060275-02

2. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

❑ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ❍ No ❍

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ❍ No ❍

OMB Number. 0925-0001
Expiration Date: 9/30/2007

4. Program Income

Is program income anticipated during the periods for which the grant support is requested?

❍Yes ●No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. Assurances/Certifications (see instructions)

In agreeing to the assurances/certification section 18 on the SF424 (R&R) form, the authorized organizational representative agrees to comply with the following policies, assurances and/or certifications when applicable. Descriptions of individual assurances/certifications are provided at: http://grants.nih.gov/grants/funding/phs398/PolAssurDef.doc

*Human Subjects; *Research Using Human Embryonic Stem Cells; *Research on Transplantation of Human Fetal Tissue; *Women and Minority Inclusion Policy; *Inclusion of Children Policy; *Vertebrate Animals; *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); *Prohibited Research; *Select Agents; *Smoke-Free Workplace; *STTR ONLY: Certification of Research Institution Participation.

If unable to certify compliance, where applicable, provide an explanation and attach below.

Explanation:

Tracking Number:

Attachments

CertificationExplanation_attDataGroup0

File Name	Mime Type