NOVOBIOTIC
PHARMACEUTICALS

March 14, 2007

Edward Hammond
The Sunshine Project
1920 Stuart St
Berkeley CA 94703

Dear MR. Hammond:

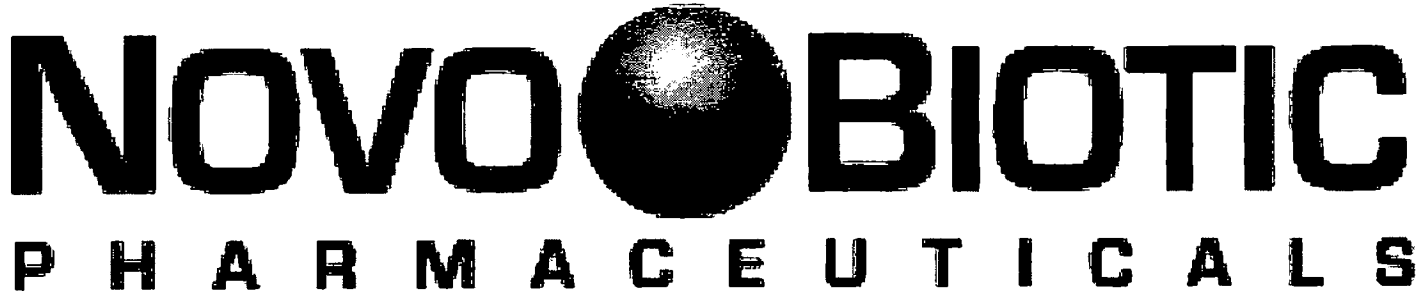
Enclosed please find the minutes to our IBC meetings, we have only met twice, in 2005 and 2006 as we were founded in late 2003.

If you have any additional questions please contact me in writing.

Sincerely yours,

Aram T. Salzman, CEO
NovoBiotic Pharmaceuticals

767C Concord Avenue • Cambridge, MA 02138
ph: (617) 864-2880 • fax: (530) 685-5083 • web: www.novobiotic.com


NOVOBIOTIC
PHARMACEUTICALS

NovoBiotic Pharmaceuticals, LLC
IBC Meeting and
Recombinant DNA Permit Application

Presentation to NovoBiotic
Institutional Biosafety Committee
and the Cambridge, MA Biosafety Committee

September 20, 2005

Aram Salzman, CEO
NovoBiotic Pharmaceuticals, LLC
767C Concord Avenue
Cambridge, MA 02138

NovoBiobiotic Pharmaceuticals, LLC

Founded 2003, currently 7 employees

- **Scientific management**
- Lucy Ling, Ph.D. Director of Biology
- Andrew Staley, Ph.D. Director of Chemistry
- Rob Nicol, Ph.D. Microbiologist

Mission

- Antibiotic Drug Discovery
- On-site rDNA research is necessary to support these efforts
- Research lab is operational but no current rDNA work at present

Research Facility

Existing Lab space at 767C Concord Avenue, Cambridge, 02138

- ~2,000 sq. ft. laboratory ~1,800 sq ft office and storage
- Previous occupants: Infimed
- Prior permit for BSL-1 and BSL-2 used by Infimed
- NovoBiotic will use existing space with minimal modification

Access Control

- I. Locked building entrance; II. Locked lab entrance
- Lab space and office space are segregated
- Alarm system

Location

- BSL-2 throughout main lab
- Authorized access only
- Autoclave in lab

NovoBiobiotic Pharmaceuticals, LLC

Mission: Develop New Antibiotics

NovoBiotic Research Plan

- Explore / Isolate previously uncultured microorganisms
- Use traditional microbiology techniques to screen for novel antibiotics
  - Screening techniques include overlay of isolates with test organisms including *B. subtilis* and *E. coli*
- Additional screening performed with extracts from organisms of interest.
- Utilize 16S rDNA analysis for typing of organisms
- All work to be done on an analytical scale, small volumes

Research at NovoBiotic

Natural sources (terrestrial soil)
into chambers

↓

Domestication / Isolation
(Ability to grow on petri dishes)

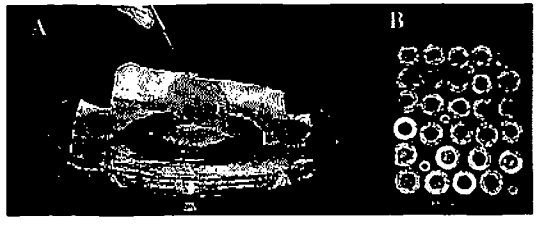
↓

Preliminary screen with test
organism

↓

Chemical Extraction → Secondary screening

Biological Identification → 16S rDNA typing



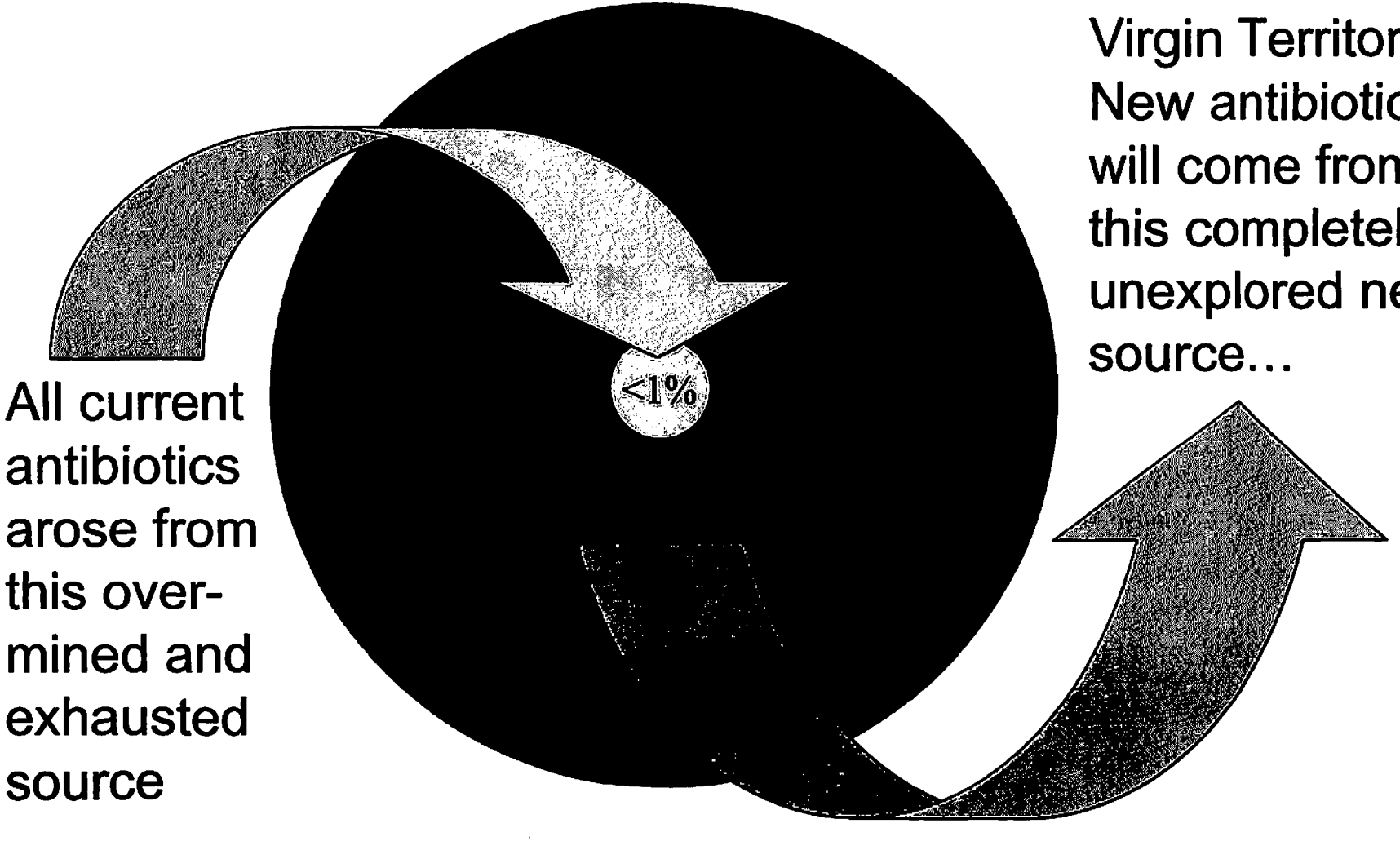
First Step

Explore / Isolate previously uncultured microorganisms

WHY/ HOW?

< 1 % of microorganisms are culturable on the petri dish

Antibiotic "Renaissance"



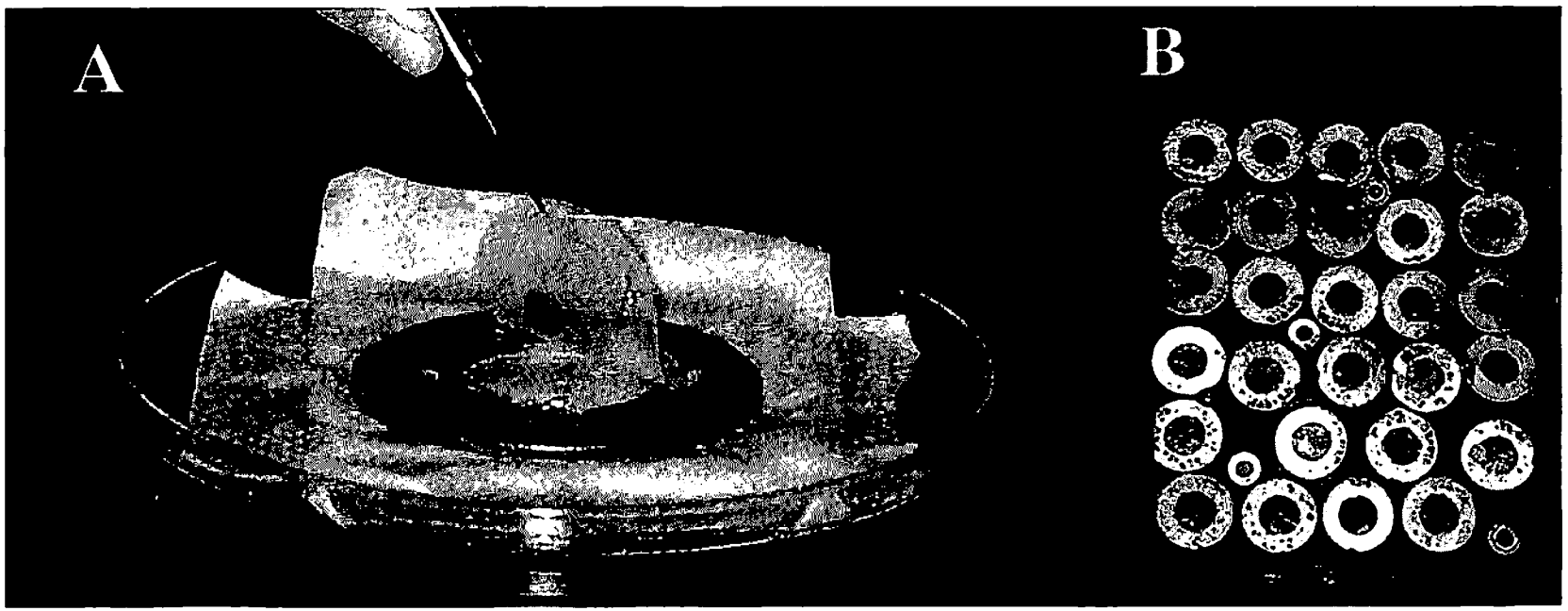
All current antibiotics arose from this over-mined and exhausted source

Virgin Territory: New antibiotics will come from this completely unexplored new source…

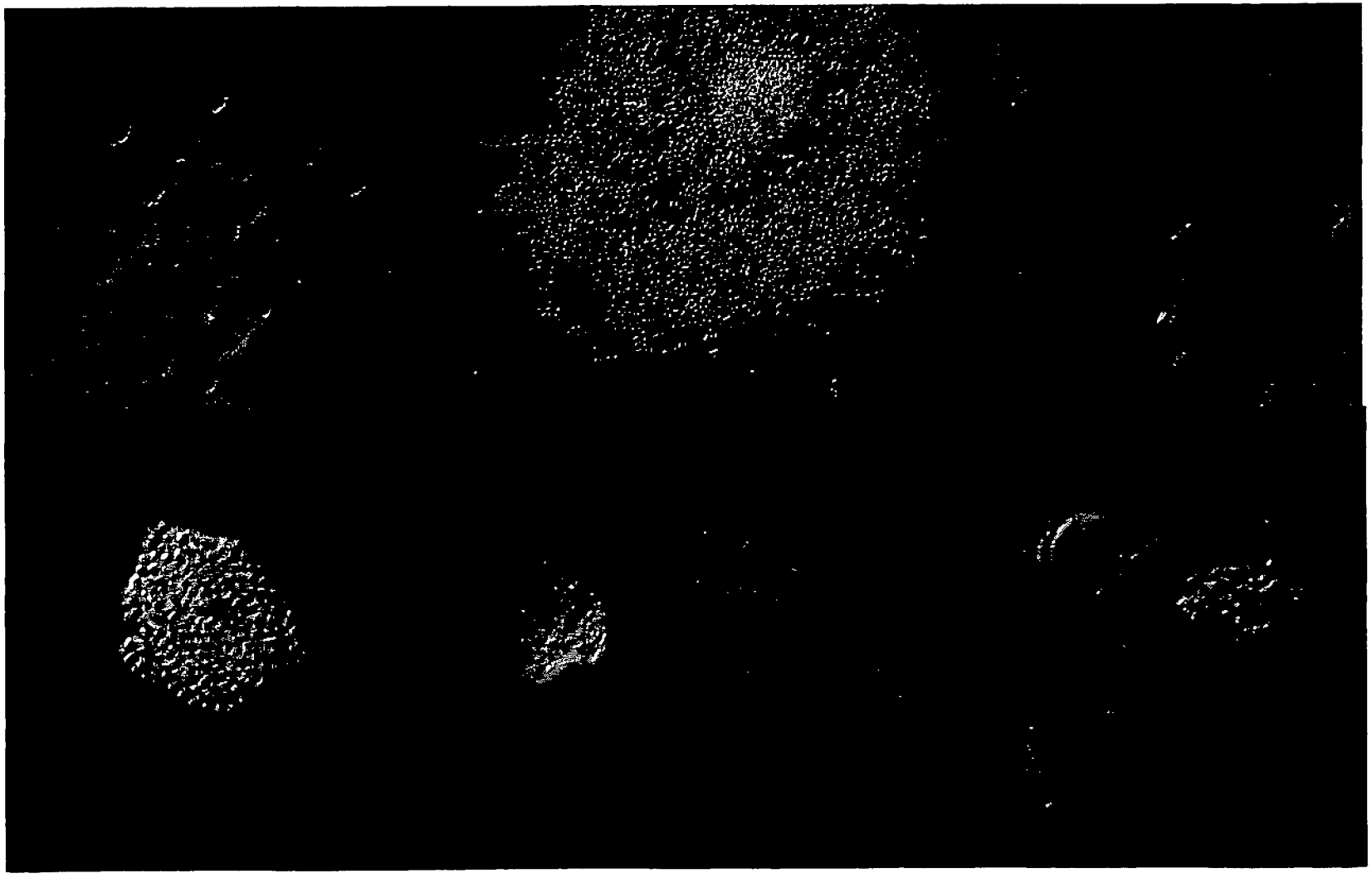
Collecting environmental samples

Making it Work

- Membranes – polycarbonate (0.03-µm pore-size)
- Glue 2 membranes between a metal washer
- Environmental sample in agar placed between membranes
- Put chamber back in environment



Diversity of Growth in the Chamber



Science Publication
Technology licensed from Northeastern University

Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment

T. Kaeberlein, K. Lewis,* S. S. Epstein*†

The majority (>99%) of microorganisms from the environment resist cultivation in the laboratory. Ribosomal RNA analysis suggests that uncultivated organisms are found in nearly every prokaryotic group, and several divisions have no known cultivable representatives. We designed a diffusion chamber that allowed the growth of previously uncultivated microorganisms in a simulated natural environment. Colonies of representative marine organisms were isolated in pure culture. These isolates did not grow on artificial media alone, but formed colonies in the presence of other microorganisms. This observation may help explain the nature of microbial uncultivability.

The number of existing microbial species is estimated at 10^6 to 10^8 (*1*, *2*), but only several thousand have been isolated in pure culture (*3*), because few microorganisms from environmental samples grow on nutrient media in Petri dishes (*4*–*16*). Attempts to improve the recovery of microorganisms from environmental samples by manipulating growth media have met with limited success (*6*, *13*, *17*–*19*), and the problem of uncultivability remains a major challenge (*4*).

We reasoned that uncultivable microorganisms might grow in pure culture if provided with the chemical components of their natural environment. To allow access to these components, we placed microorganisms in diffusion chambers and incubated the chambers in an aquarium that simulated these organisms' natural setting.

Intertidal marine sediment was used as a source of microorganisms (*20*). The upper layer of the sandy sediment harbors a rich community of microorganisms, primarily aerobic organoheterotrophs, which reach densities of $>10^9$ cells/g (*21*) and are mostly uncultivated (*22*, *23*). These microorganisms were separated from sediment particles, serially diluted, mixed with warm agar made with seawater, and placed in the diffusion chamber (*20*) (Fig. 1). The membranes allow exchange of chemicals between the chamber and the environment, but restrict movement of cells. After the first membrane was affixed to the base of the chamber, the agar with microorganisms was poured in, and the top was sealed with another membrane (Fig. 1A).

The sealed chambers were placed on the surface of the sediment collected from the tidal flat and kept in a marine aquarium (Fig. 1B). A thin layer of air was left between the agar and the top membrane. In the aquarium this space was filled with seawater. This design allowed us to observe the undisturbed agar surface after peeling off the top membrane.

A large number of colonies of various morphologies were observed after 1 week of incubation in the chambers (Fig. 2A). Most of these (>99%) were microcolonies invisible to the naked eye. Addition of 0.01% casein increased the number of colonies in the chamber, and this supplement appeared superior to starch or marine broth tested at a variety of concentrations (*20*).

In a series of microbial recovery experiments (*20*), we determined the fraction of cells that formed colonies inside the chambers compared with the standard Petri dish method (Fig. 2B). The greatest microbial colony recovery in the chambers represented 40 ± 13% of the cells inoculated and came from a sample obtained in June 2001. The number of microcolonies obtained in different months ranged from 2 to 60% of the cells inoculated, with an overall average of 22 ± 13%. This is likely an underestimate, because the total direct microbial count included dead cells, our colony-counting technique produced conservative estimates (*20*), and the fairly dormant March sample showed the recovery results. Representative microorganisms from the chambers were successfully isolated in pure culture by passage to new chambers. Of the 33 colonies passaged, 23 produced sustainable growth in the chambers at the first attempt.

Unexpectedly, a significant number of microcolonies appeared on the Petri dishes (6 ± 4%, of the number of cells inoculated). We investigated their ability to produce sustainable growth in three independent trials. Each time, 27 to 30 microcolonies were passaged to a new Petri dish. Most of the transfers (86 ± 7%) did not result in microbial growth. It seems that the majority of microorganisms from the sediment could only undergo a limited number of divisions on a Petri dish. The microcolonies that did grow after passage to Petri dishes (14%) appeared to represent mixed cultures, and only those that produced rapidly growing macrocolonies, visible to an unaided eye, seemed capable of sustained growth on Petri dishes. Counting visible colonies is the conventional method of performing microbial plate counts (*24*). Such Petri-dish macrocolonies made up 0.054 ± 0.051% of the inoculum, consistent with previous reports (*15*, *17*). Finally, ~300-fold as many microorganisms produced sustainable growth in the growth chambers as in standard Petri dishes.

We attempted to isolate into pure culture some of the microorganisms grown in the diffusion chambers (*20*). The isolates were considered pure if no contaminants could be detected microscopically or by polymerase chain reaction amplification of 16S ribosomal RNA (rRNA) gene (*20*). Several passages were required to achieve purity. Passages typically produced hundreds of macrocolonies per chamber, which was more than sufficient for the purposes of the present study.

To date, two isolates, MSC1 and MSC2 (Fig. 3), have been obtained; nine others are at different stages of isolation into pure culture. A 1400 base pair sequence of 16S rDNA from MSC1 indicates that it is a previously undescribed bacterium, with 93% sequence similarity (*20*) to its closest relative *Leeuwella persica* [*Herpetosiphon persicus* (*25*)]: Class Sphingobacteria, Phylum Bacte-

Biology Department, Northeastern University, Boston, MA 02115, USA, and Marine Science Center, Northeastern University, Nahant, MA 01908, USA.

*These authors contributed equally to the work. †To whom correspondence should be addressed. E-mail: s.epstein@neu.edu

Fig. 1. Diffusion growth chamber for in situ cultivation of environmental microorganisms. (**A**) The chamber is formed by a washer sandwiched between two 0.03-μm pore-size polycarbonate membranes (**B**). Growth chambers incubated on the surface of marine sediment.

Environmental Chamber (Cross Section)

Research at NovoBiotic

Natural sources (terrestrial soil)
into chambers

↓

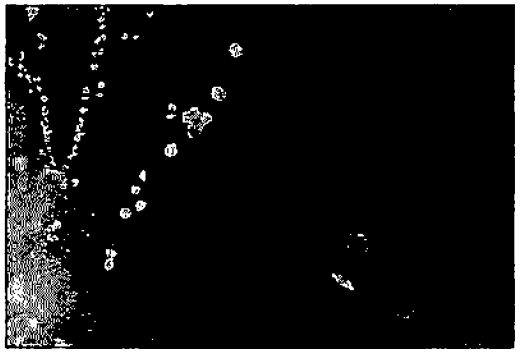
Domestication / Isolation
(Ability to grow on petri dishes)

↓

Preliminary screen with test
organism

↓

Chemical Extraction	Biological Identification
↓	↓
Secondary screening	16S rDNA typing



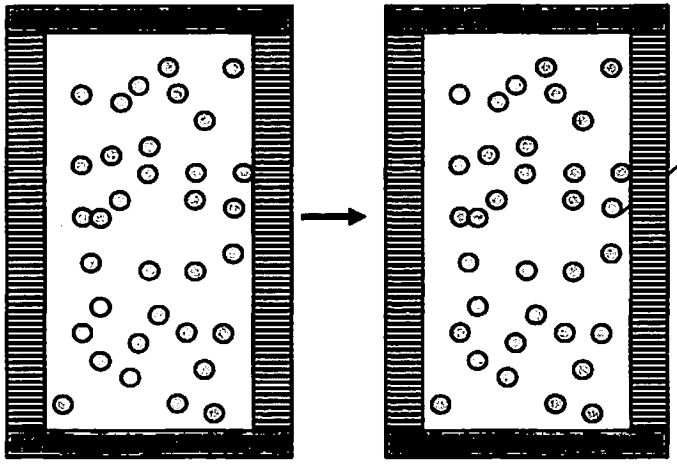
Second Step

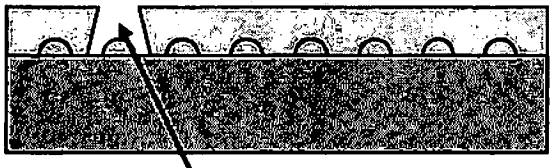
Use traditional microbiology techniques to screen for novel antibiotics

- Screening techniques include overlay of isolates with test organisms including *B. subtilis* and *E. coli*

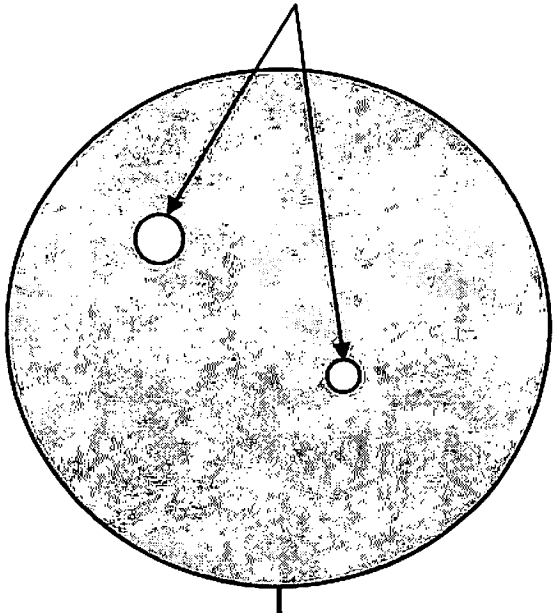
Agar overlay screening leading to specific isolation of producing strains

Inoculate cells from environmental sample, domesticate

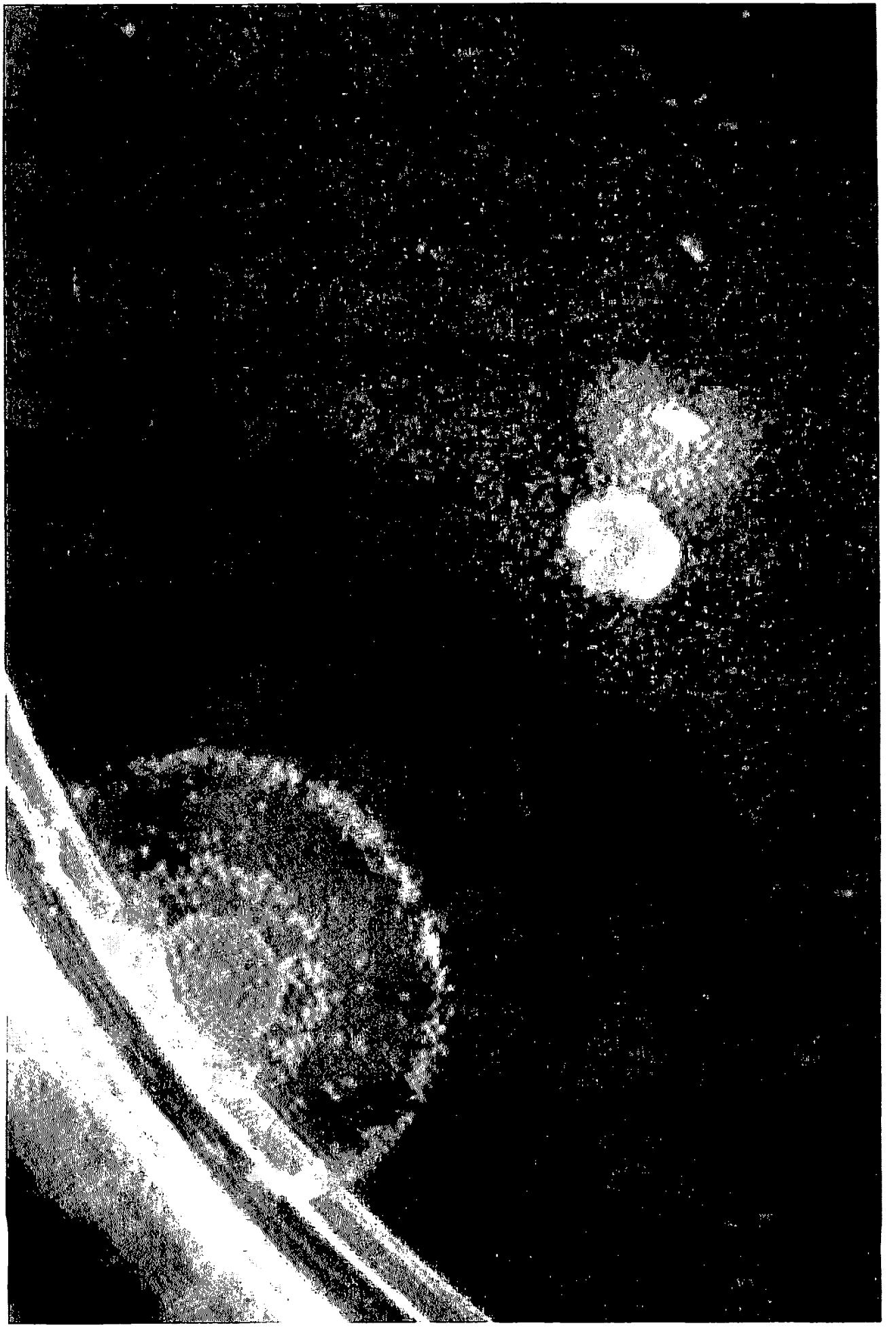




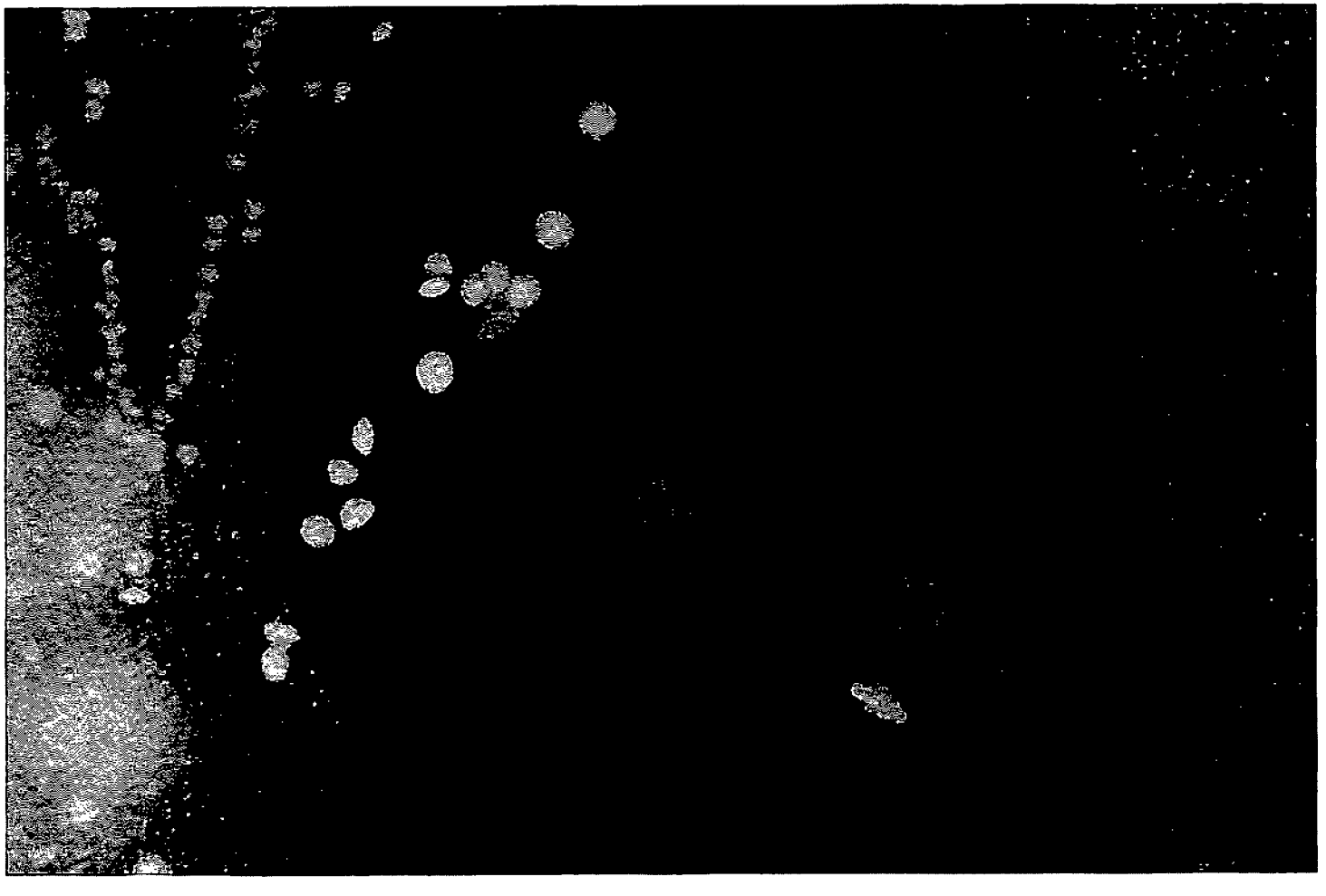
Growth inhibition zones of test cells indicate antibiotic production by underlying colonies which are then isolated.



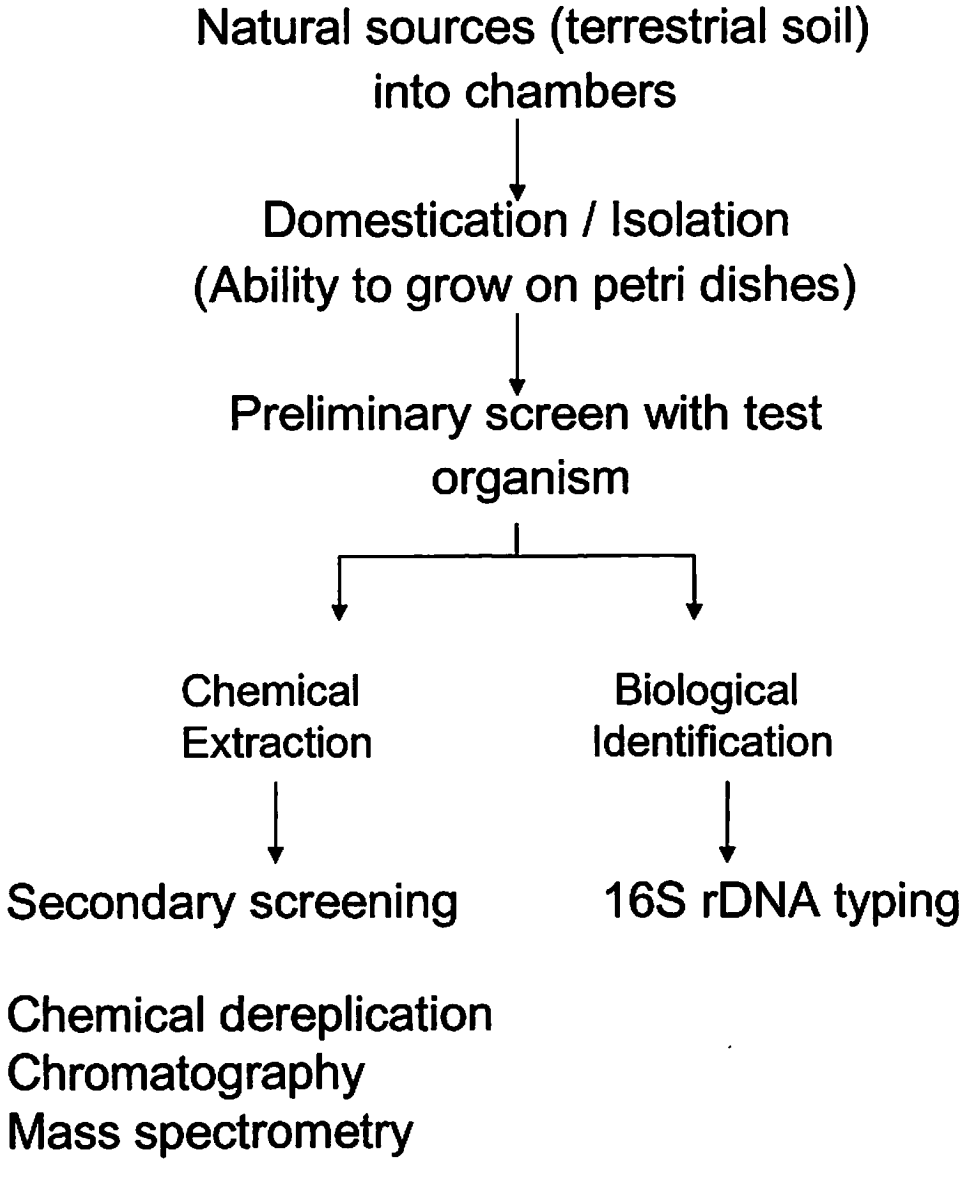
Overlay agar with test cells



Zone of inhibition



Research at NovoBiotic



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Natural sources (terrestrial soil)
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Domestication / Isolation
(Ability to grow on petri dishes)

↓

Preliminary screen with test
organism

↓

Chemical
Extraction

↓

Secondary screening

Chemical dereplication
Chromatography
Mass spectrometry

Biological
Identification

↓

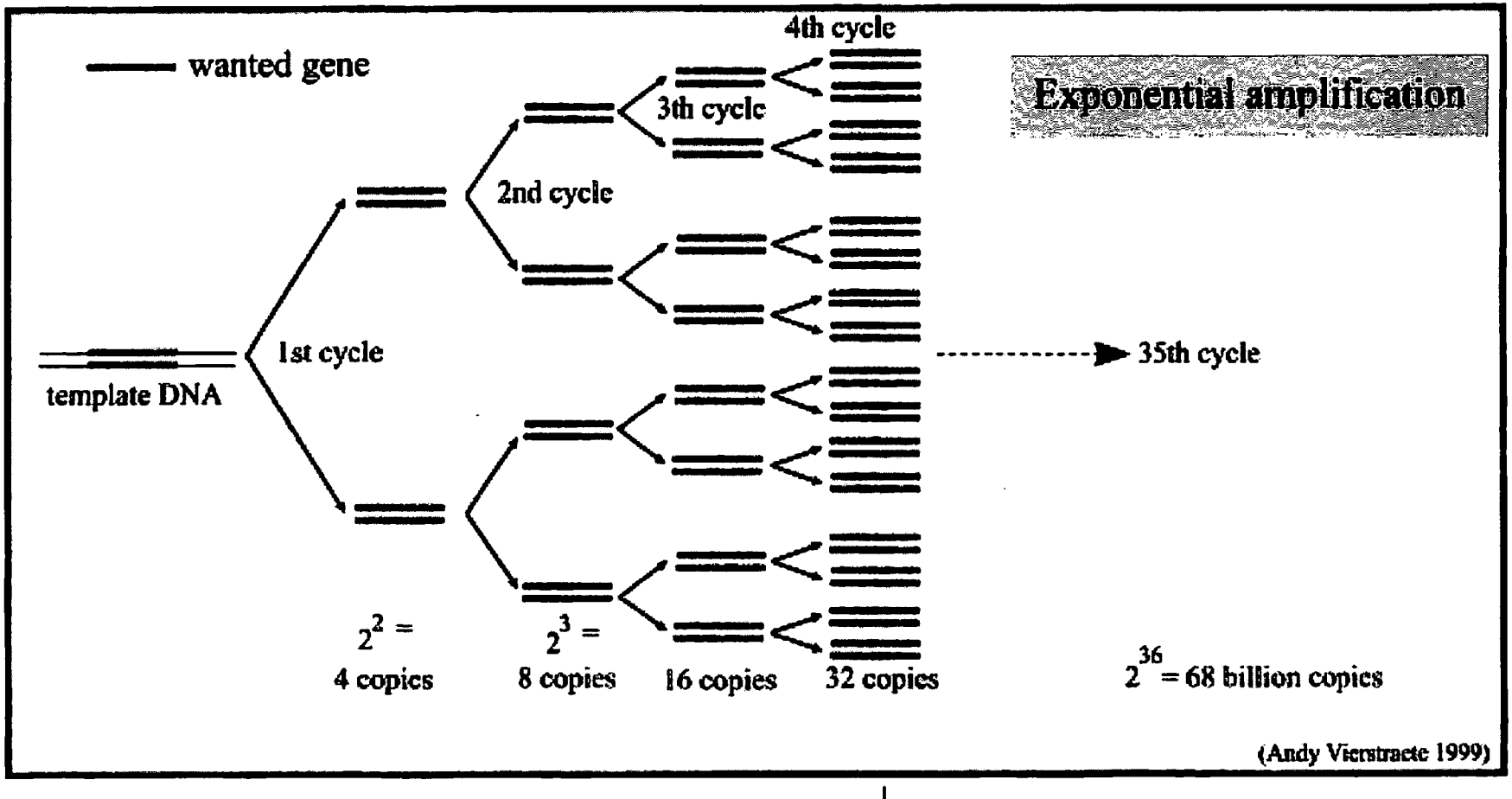
16S rDNA typing of
Environmental organisms

16S rDNA (Ribosomal DNA) analysis

- 16S rDNA is a region found in all bacteria
- A molecular method to distinguish / type bacteria
- Amplification of rDNA by PCR
- Sequencing outsourced
- Sequence Analysis
  - Comparison to microorganisms deposited in GenBank

16S rDNA analysis : Process

Step1: Polymerase Chain reaction (PCR): animated picture



Direct Sequencing of PCR products

16S rDNA analysis

- Sequencing outsourced
- Sequence Analysis
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Disposal of Biological Material

- Biological Culture in Liquid media
  - Add bleach directly, ratio of 1:10 (bleach: culture)
  - Sit for 1 hour
  - Dispose with plenty of running water
- Biological Culture in Solid media, all other solid waste such as pipette tips, tubes etc.
  - Seal tightly
  - Removed by Stericycle
- Surfaces wiped down with 70% ethanol

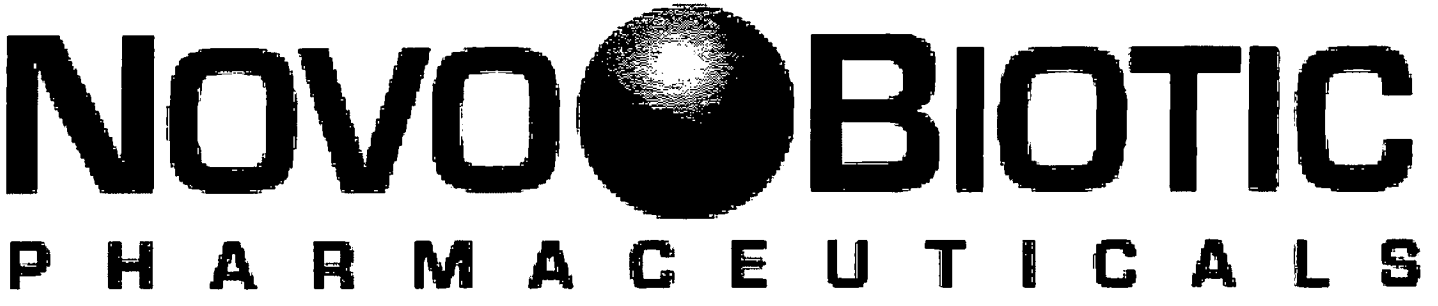
Permits and Timeline

Permit	Agency	Status
rDNA	Cambridge Public Health Dept.	In Progress
Wastewater	MWRA, & Camb. Water Department	October 2004
Water supply	Cambridge Water Department	Complete for Building
Fire Safety	Cambridge Fire Department	August 2004
Needle/Syringe	Mass. Dept. Public Health	June 2004
Haz. Waste	Mass Dept. Reg. Comp.	July 2004
	Biosafety Permit	In Progress
	health and safety manual	October 2004
	biosafety training program	Ongoing

- One Biosafety Cabinet

Contract In Place or In Progress

- Sharps Disposal / Biowaste - Stericycle
- Pest Control - In-house management
- Plumbing/Backflow - West Cambridge Science Park
- Lab Coat Laundry Service - Northstar
- Water Testing for MWRA permit - Environmental Sampling Tech.
- Sprinkler System Testing - West Cambridge Science Park
- Hazardous Waste Disposal Group – Onyx Environmental
- Chip Tank Maintenance - West Cambridge Science Park
- Bio Safety Cabinet Certification (Yearly) - Airtest
- Air Balancing/Negative Pressure in BSL-2 TJ Heating
- Timeline for completing process/walkthrough date ASAP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NovoBiotic Pharmaceuticals, LLC
IBC Meeting and
Recombinant DNA Permit Application

Presentation to NovoBiotic
Institutional Biosafety Committee

October 15, 2006

Aram Salzman, CEO
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767C Concord Avenue
Cambridge, MA 02138

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- **Scientific management**
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- Charles Moore, PhD VP Lab Operations

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Mission: Develop New Antibiotics

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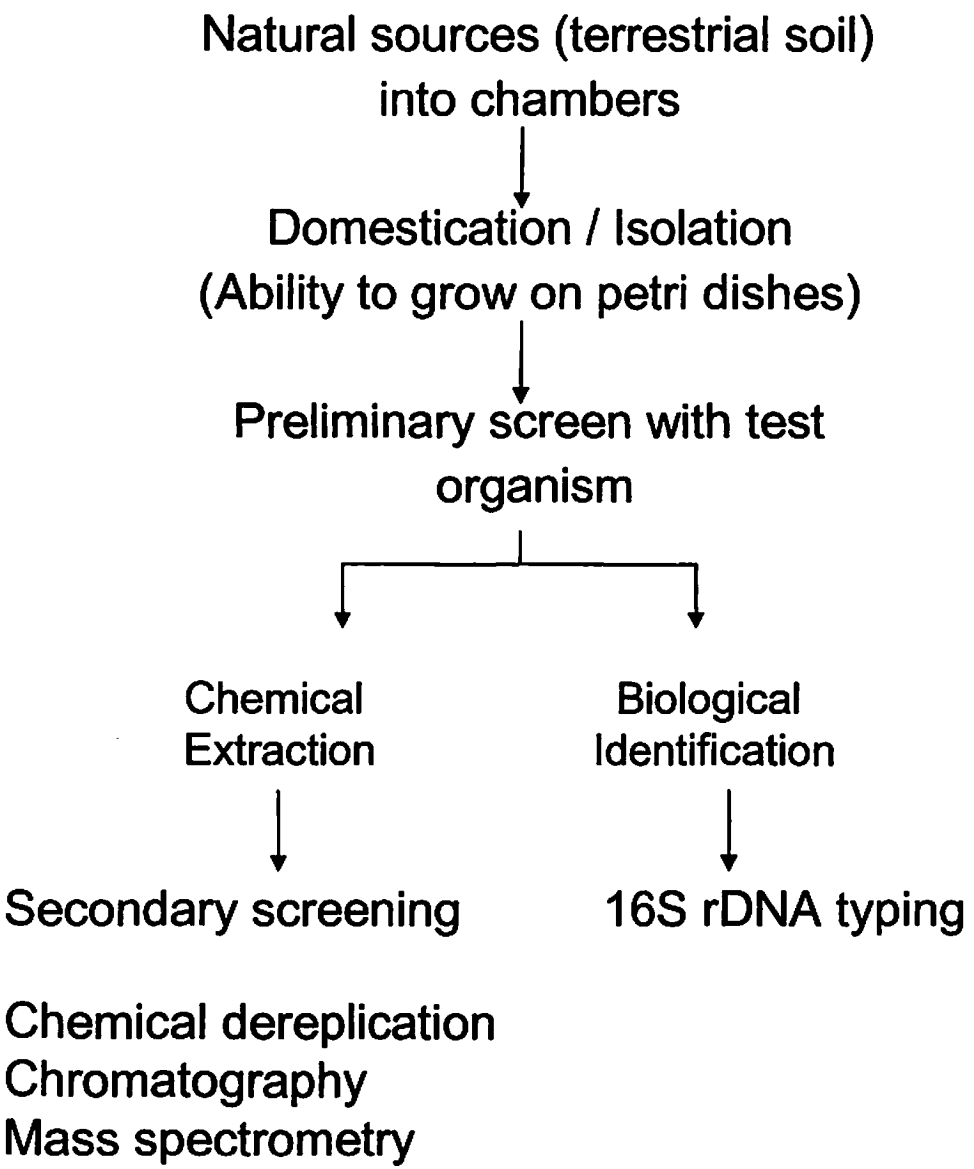

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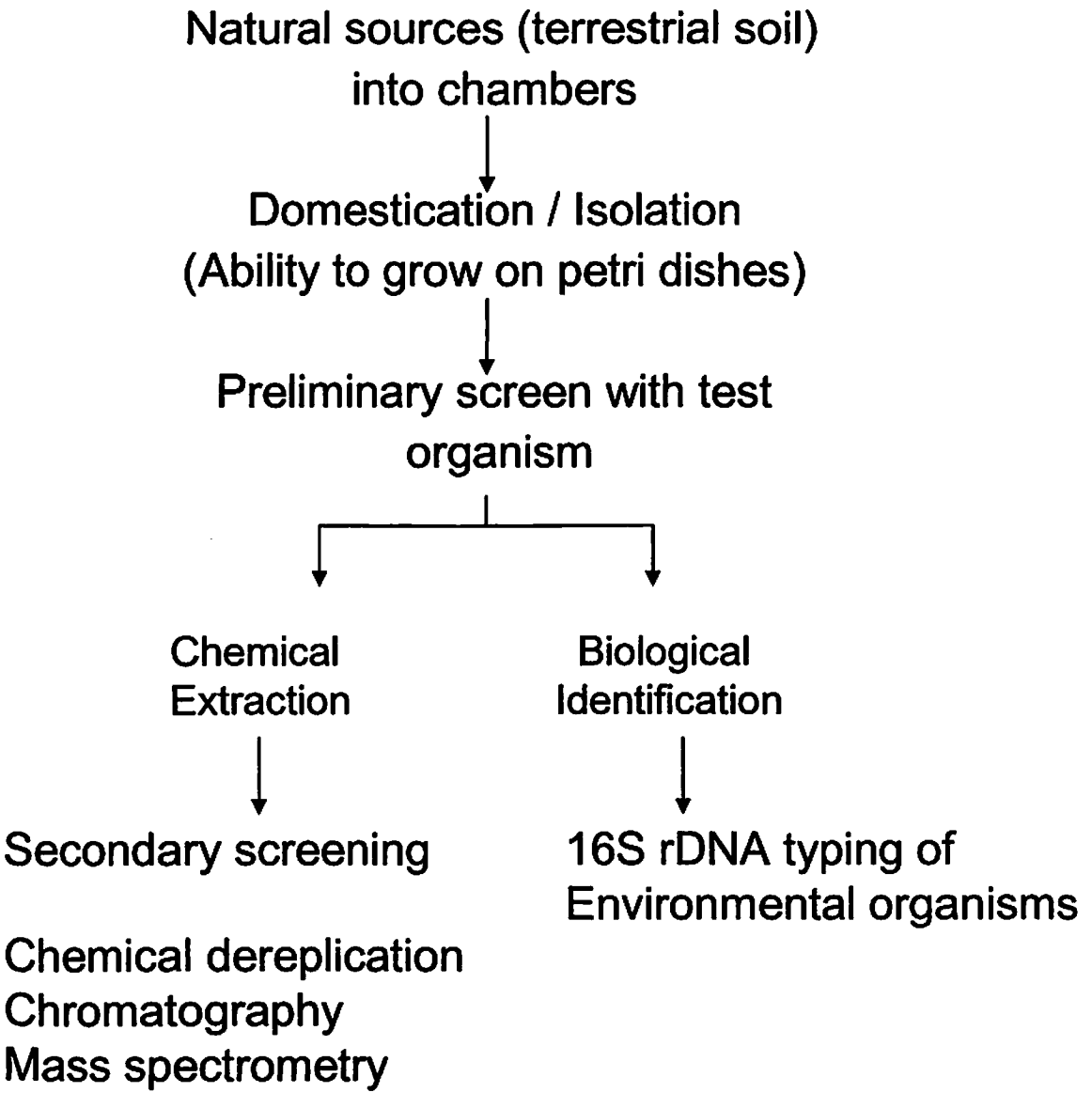

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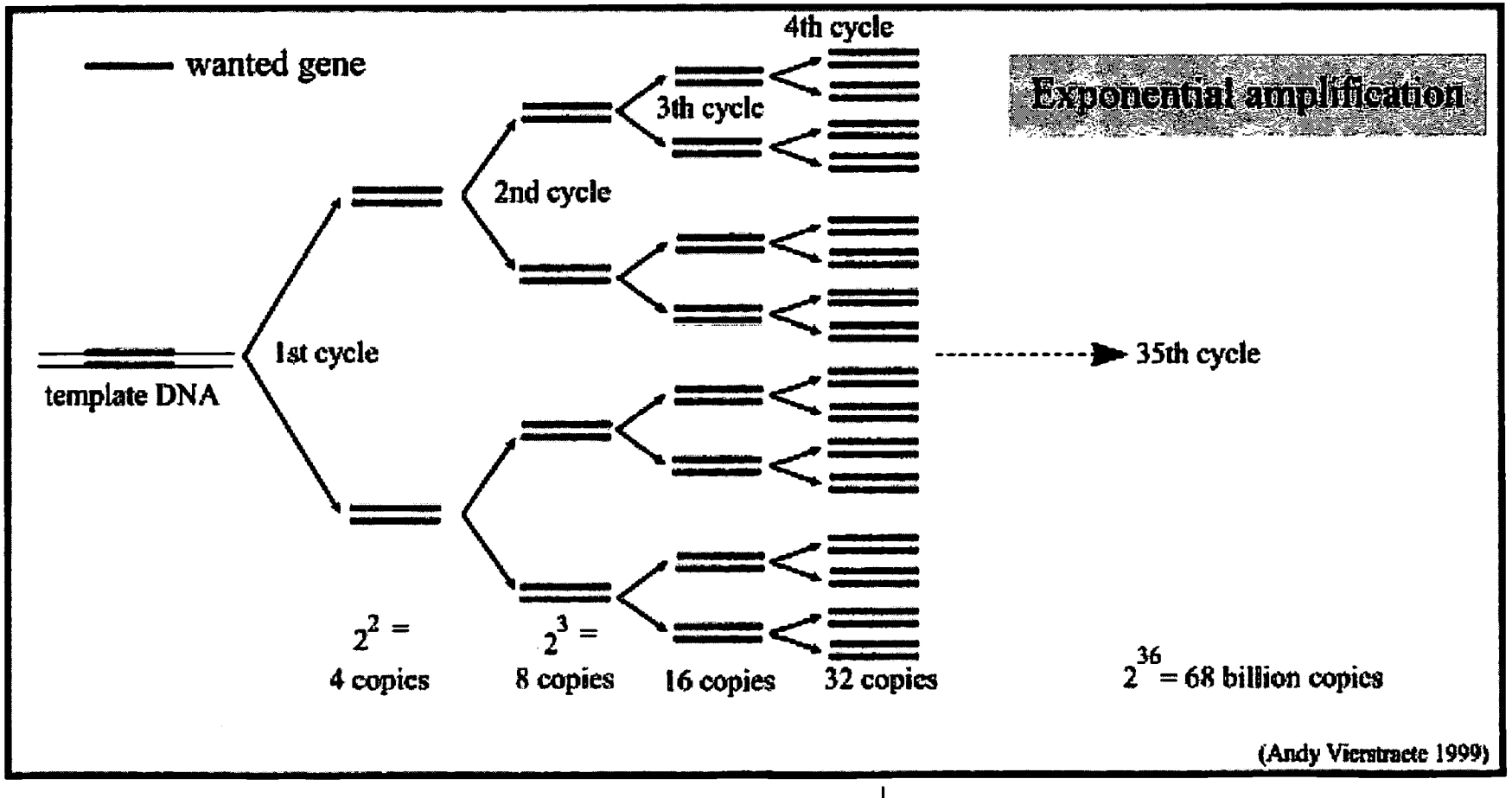
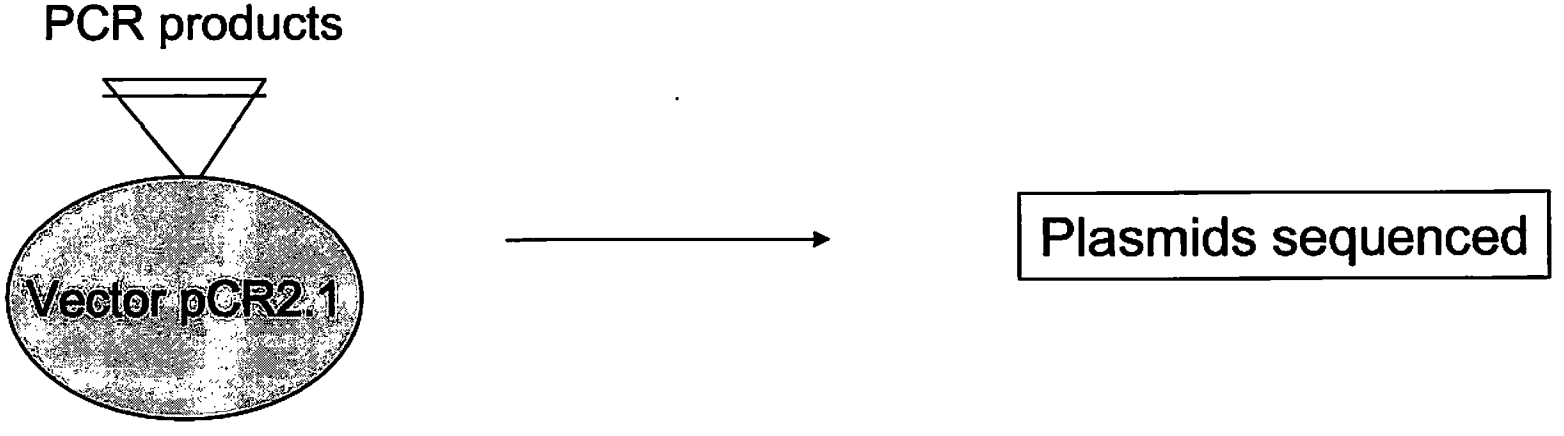


Direct Sequencing of PCR products

16S rDNA analysis : For problematic cases

Clone one copy of the 16S rDNA gene into vector pCR2.1
Increase copy number in E. coli TOP10



Recombinant DNA Technology

16S rDNA analysis

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