2017 Alliance Summer Workshop

Week 1
July 24- July 28

- Bioinformatics
- Scientific Grant Writing
- Recombineering: Making Gene Knockouts

Week 2
July 31- Aug 04

- Tissue Culture
- Protein Purification

Alliance for Global Health and Science
University of California, Berkeley

Berkeley
UNIVERSITY OF CALIFORNIA

WE BUILD FOR THE FUTURE

Seminar Speakers

- Jeffery Cox
- Nevan Krogan
- David Kateete

Mulago Guest House
9–11 AM

Schedule At-A-Glance

July 24—July 28

Monday, July 24	08:30 AM—09:00 AM	Workshop Check-In	Mulago Guest House
	09:00 AM—05:00 PM	Bioinformatic Workshop	Micro-Pathology Building
	09:00 AM—05:00 PM	Grant Writing Workshop	Clinical Research Building
	10:30 AM—11:00 AM	Break	
	11:00 AM—04:30 PM	Making Gene Knockouts Workshop	Micro-Pathology Building
	01:00 PM—02:00 PM	Lunch	
Tuesday, July 25— Friday, July 28	09:00 AM—05:00 PM	Bioinformatic Workshop	Micro-Pathology Building
	09:00 AM—05:00 PM	Grant Writing Workshop	Clinical Research Building
	10:30 AM—11:00 AM	Break	
	11:00 AM—04:30 PM	Making Gene Knockouts Workshop	Micro-Pathology Building
	01:00 PM—02:00 PM	Lunch	

July 31—Aug 04

Monday, July 31	08:45 AM—09:15 AM	Workshop Check-In	Mulago Guest House
	09:15 AM—09:30 AM	Jeffery Cox Welcome Speech	Mulago Guest House
	09:30 AM—10:30 AM	David Kateete's Seminar	Mulago Guest House
	10:30 AM—11:00 AM	Break	
	11:00 AM—12:00 PM	Protein Purification Lecture	Mulago Guest House
	11:00 AM—04:00 PM	Tissue Culture Workshop	Clinical Research Building
	12:00 PM—05:00 PM	Protein Purification Workshop	Micro-Pathology Building
	01:00 PM—02:00 PM	Lunch	
Tuesday, Aug 01— Wednesday, Aug 02	09:00 AM—10:00 AM	Jeffery Cox's Seminar	Mulago Guest House
	10:00 AM—11:00 AM	Nevan Krogan's Seminar	Mulago Guest House
	11:00 AM—11:30 AM	Break	
	11:30 AM—04:30 PM	Tissue Culture Workshop	Clinical Research Building
	11:00 AM—06:00 PM	Protein Purification Workshop	Micro-Pathology Building
	01:00 PM—02:00 PM	Lunch	
Thursday, Aug 03	08:00 AM—06:00 PM	Tissue Culture Workshop	Clinical Research Building
	09:00 AM—05:00 PM	Protein Purification Workshop	Micro-Pathology Building
	11:00 AM—11:30 AM	Break	
	01:00 PM—02:00 PM	Lunch	
Friday, Aug 04	09:00 AM—10:00 AM	Nevan Krogan's Seminar	Mulago Guest House
	10:00 AM—11:00 AM	David Kateete's Seminar	Mulago Guest House
	11:00 AM—11:30 AM	Break	
	11:30 AM—04:30 PM	Tissue Culture Workshop	Clinical Research Building
	11:30 AM—04:30 PM	Protein Purification Workshop	Micro-Pathology Building
	01:00 PM—02:00 PM	Lunch	
	05:30 PM	Banquet	Humura Resorts

Alliance for Global Health and Science

The Alliance for Global Health and Science (the Alliance) is a partnership between the UC Berkeley, Division of Biological Sciences and School of Public Health. The goal of the Alliance is to address threats posed by communicable and non-communicable diseases by strengthening the capacity for scientific and public health research of institutions in developing countries. The Alliance seeks to make a tangible, enduring impact by focusing on locally identified health research needs and strengthening collaborations between faculty at UC Berkeley and major research and teaching institutions in developing countries.

The vision of the Alliance is to sustain the indigenous generation of world-class research knowledge to help the understanding of the conditions and solutions to health problems and contribute to the advancement of people living in all regions of the world. The Alliance seeks to support a growing presence of researchers who are conducting robust research indigenously to ensure rootedness, sustainability, and ownership of the knowledge, as well as increase the likelihood for its relevance and applicability to local health challenges.

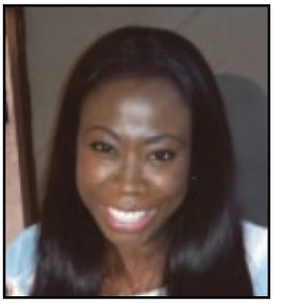
In the current Pilot Phase of the program, the Alliance seeks to build upon existing relationships between partner institutions in Africa and UC Berkeley. We will support training and research in fields such as molecular biology, molecular and field epidemiology, the design and development of vaccines, therapeutics and diagnostic tools, education, public health interventions, and agricultural development. The Alliance will draw upon the vast expertise and strengths of faculty and students at UC Berkeley to enhance scientific capacity to enable people and institutions in developing regions address health challenges important to them.

Contact Us

Alliance for Global Health Science is managed by the Henry Wheeler Center for Emerging and Neglected Diseases (CEND) at UC Berkeley. For more information, visit cend.berkeley.edu or contact CEND at cend@berkeley.edu or 510-666-3699.

The Team

Zilose Lyons | Program Manager | UC Berkeley



Prior to joining CEND in 2010, she was a sports journalist covering soccer, tennis, golf and darts for the Zambia Daily Mail in Lusaka. Zilose holds a Bachelors degree in International Development Studies from the University of California, Berkeley and a diploma in Journalism and Public relations from Evelyn Hone College, in Lusaka, Zambia.

Anny Lin | Program Coordinator | UC Berkeley

Anny completed her undergraduate degree in Sociology at University of California, Berkeley in 2014. She worked as a Faculty Assistant for four Professors at UCSF and now works as Executive Assistant for Dr. Jeffery Cox and is Program Coordinator for the Center for Emerging & Neglected Diseases (CEND). She enjoys baking in her free time.

Samuel Schildhauer, MPh | Project Coordinator |UC Berkeley

Samuel is a graduate of the Master of Public Health in Infectious Diseases and Vaccinology from UC Berkeley. Before beginning graduate school he worked as a medical assistant in a respiratory clinic. He has experience working in virology and immunology laboratories, focusing on influenza and dengue. He is interested in global health and expanding laboratory and diagnostic capacities in low-resource settings, as well as basic laboratory research.

Sarah Petnic, MPh | Project Coordinator | UC Berkeley

Sarah is a recent MPH graduate from the Infectious Diseases and Vaccinology program at UC Berkeley. Between her undergraduate and graduate degrees Sarah worked at UCSF in the Yamamoto Lab for a year and then the Madhani Lab for two years. In this next chapter, Sarah plans to pursue a career in development of sustainable global health initiatives and scientific infrastructure strengthening.

Ivan Mwebaza | Project Coordinator | Makerere University

Ivan is currently a pre-doctoral Researcher in Moses Joloba's lab studying Tuberculosis RDTs . He received a BS in Nursing in 2012, and his masters in Immunology and Clinical Microbiology in January 2016.

Welcome

Dear friends and colleagues,

On behalf of the Alliance for Global Health and Science, and the Center for Emerging and Neglected Diseases at the University of California at Berkeley, I am delighted and honored to co-host the first workshop that joins the research endeavors of Makerere University, the University of Harare, and the University of California. I believe that these two weeks will be an enriching experience for all of us, and hope that the workshops and faculty seminars will be a stimulating experience for everyone, from students to faculty. Moreover, I hope that these activities will not only accelerate our joint efforts to combat the most important diseases of mankind, but will also serve as the foundation for developing collaborations and sparking new research endeavors between our campuses.

Speaking for the American contingent, we are most excited about meeting you and learning about your research. Our hope is that we will not only provide new perspectives/methodologies that you can implement into your work, but that we will be inspired to catalyze great advances through fundamental research, and renew our commitment that together we can do great things.

These two weeks would not be possible without the dedicated work of a large number of people. These include the faculty leaders of the workshops who have given their time freely, the teaching assistants, and the administrators who have painstakingly organized our festivities. In particular, Anny Lin, Sarah Penic, Sam Schildhauer, Zilose Lyons, and Ivan Mwebaza have painstakingly spent hours coordinating all the logistics of this endeavor.

Most importantly, we are indebted to Steve Issacs, whose philanthropy and love of this great continent has made this all possible.

We are very much looking forward to meeting you all and creating new scientific partnerships.

Dr. Sarah Stanley
Assistant Professor
School of Public Health
UC Berkeley

Sarah received her BS in chemistry from Trinity University and her PhD in Biomedical Sciences from UCSF where she was a graduate student in Jeff Cox's lab. Sarah was a Helen Hay Whitney Postdoctoral Fellow at the Broad Institute of Harvard and MIT in Deborah Hung's laboratory. She started her group at UC Berkeley in 2012.

Dear friends and colleagues,

On behalf of Makerere University, I would like to welcome each of you to the 2017 alliance summer workshop series. A special welcome goes to our Colleagues from the University of California-Berkeley and the University of Harare. We would like to express our appreciation to the team from the University of California-Berkeley, Center for Emerging and Neglected Diseases (CEND) for having conceived this whole idea of collaboration among our Institutions. It is an exciting moment to see three academic and research institutions coming together to develop Biomedical Science Research skills. I would also like to extend my thanks to CEND for awarding seed grants to our students. I am very confident that these grants will incubate into bigger grants which will help a number of grantees to start off their research careers.

Whereas most of the collaborations target the already established researchers, this particular one has a very unique structure that it engages students and aims at developing capacity for upcoming researchers. I am also glad to inform you that this workshop series was suggested by students after identifying some gaps in their research skills.

We hope that these workshops will not only help in developing laboratory and research skills among participants but also build partnerships both at Institutional and individual levels. Such forums are always precursors of global research collaborations and I am very hopeful we will form a strong research partnership between Makerere University, the University of Harare, and the University of California-Berkeley.

Finally, we highly appreciate the generosity of Stephen Isaacs who made all this possible.

I encourage you to stay engaged, and feel at home.

Professor Moses Joloba
Dean
School of Biomedical Sciences
College of Health Sciences
Makerere University

Dr. Joloba's focuses on TB research, including bacterial quorum sensing, TB molecular epidemiology and drug resistance. In 2003, he established the Molecular Biology laboratory in Department of Medical Microbiology. He is also the director of the National TB Reference Laboratory which provides diagnostic services and conducts field trials of new diagnostic systems. Dr. Joloba completed his MBchB at Makerere University in 1994, did a Master of Science in Pathology and Clinical Microbiology, as well as a PhD in Molecular Microbiology in 2003 at Case Western Reserve University.

Seminar Series

Jeffery Cox, PhD | UC Berkeley

Jeff pursued his graduate studies at UC San Francisco in the laboratory of Dr. Peter Walter, where he made the initial discoveries of the unfolded protein response in yeast and received a PhD in Biochemistry and Biophysics. He moved to New York for a postdoc. fellowship with Dr. Bill Jacobs at Albert Einstein School of Medicine, where he developed new genetic strategies that allowed him to identify key virulence factors in *M. tuberculosis*. He subsequently returned to UCSF as an Assistant Professor in the Dept. of Microbiology & Immunology, and established his research program studying the mechanisms of *M. tuberculosis* pathogenesis during his 15-year career there. In January 2016, he returned to Berkeley as Professor of Molecular and Cell Biology and as Faculty Director of the Center for Emerging and Neglected Diseases (CEND). His vision for CEND is to harness the broad strengths of Berkeley and the greater Bay Area to promote innovative research that focuses on major infectious diseases of the developing world.

August 1 | 9 AM

Discrimination of pathogen vs. non-pathogen by macrophages

Innate immune cells discriminate pathogens from non-pathogens at the earliest stages of infection and tailor their responses to match the level of the threat. A fundamental way this is achieved is through sensing membrane perturbations mediated by bacterial virulence factors, either directly or via the recognition of specific bacterial molecules in the cytosol, leading to activation of cytosolic surveillance pathways characterized by elicitation of type I interferons (IFNs). Type I IFNs play a paradoxical role in immune defense as they are critical mediators of anti-viral defense, but their elicitation by bacterial pathogens can be detrimental to hosts. We have shown that the cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS), is required for activating IFN production via the STING/TBK1/IRF3 pathway during *M. tuberculosis* and *L. pneumophila* infection of macrophages, whereas *L. monocytogenes* short-circuits this pathway by producing the STING agonist, c-di- AMP. Upon sensing cytosolic DNA, cGAS also activates cell-intrinsic antibacterial defenses, promoting autophagic targeting of *M. tuberculosis*. Importantly, we show that cGAS binds *M. tuberculosis* DNA during infection, providing direct evidence that this unique host-pathogen interaction occurs in vivo. These data uncover a mechanism by which IFN is likely elicited during active human infections.

August 2 | 9 AM

Ubiquitin-mediated control of intracellular bacterial infection

Ubiquitin and ubiquitin-like proteins impart regulatory information during innate immune responses to infection, but the scope of their role in coordinating the responses of immune cells to infection is unknown. Recent advances in immmuno-affinity purification of ubiquitylated peptides, paired with high accuracy mass-spectrometry, have provided a powerful platform to measure global cellular ubiquitylation with extraordinary depth. We have applied this methodology to quantitatively measure changes in ubiquitylation of the macrophage proteome during infection with three intracellular bacterial pathogens: *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes*. Our results revealed thousands of ubiquitylation events that occur early after infection, most of which were previously unknown. Unlike transcriptional responses to bacterial infection, which are largely monotonic, our results reveal a surprising diversity in ubiquitin-mediated responses to each pathogen. Likewise, using well-defined attenuated bacterial mutants revealed a remarkable ability of macrophages to discriminate virulent from non-virulent bacteria, suggesting macrophages can integrate information about the nature of the engulfed bacteria and elicit unique responses. Many of these changes likely play functional roles in host resistance, as we have identified a novel ubiquitin-modified pathway, the antiviral OASL1-IRF7 pathway, which is critical for *Mycobacterium tuberculosis* growth *in vitro* and *in vivo*.

Nevan Krogan, PhD | UC San Francisco

As a graduate student at the University of Toronto, Nevan led a project that systematically identified protein complexes in the model organism, *Saccharomyces cerevisiae*, through an affinity tagging-purification/mass spectrometry strategy. This work led to the characterization of 547 complexes, comprising over 4000 proteins, and represents the most comprehensive protein-protein interaction map to date in any organism. To complement this physical interaction data, Nevan developed an approach, termed E-MAP (or epistatic miniarray profile), which allows for high-throughput generation and quantitative analysis of genetic interaction data. Nevan's lab at UCSF focuses on applying these global proteomic and genomic approaches to formulate hypotheses about various biological processes, including transcriptional regulation, DNA repair/ replication and RNA processing. His lab at UCSF is now developing and applying methodologies to create genetic and physical interactions between pathogenic organisms, including HIV, Mtb, and Dengue, and their hosts, which is providing insight into the human pathways and complexes that are being hijacked during the course of infection.

August 1 | 10 AM
From systems to mechanism: using unbiased approaches to study complex biology

Budding yeast is one of the simplest eukaryotes, and has been studied extensively as a model organism. It is easy to manipulate in a laboratory setting, yet shares many important pathways with mammalian cells. A wealth of information relevant to human biology has been derived from yeast, including understanding of basic biological processes, and diseases such as cancer and Parkinson's disease. However, the translation of findings from yeast to humans remains a challenge and requires an extensive mapping of conservation between these species. Over the last several years, we have worked towards extending our methods and knowledge from yeast to mammalian systems. As a first step in this direction, we genetically interrogated fission yeast, a distant relative of budding yeast. Networks that are conserved between these two organisms are typically of high importance and likely to be relevant in mammalian cells as well. We used our findings from these yeasts to hone in on the most critical pathways to target in human cells, and developed mammalian technological platforms inspired by our high-throughput methods in yeast. Our effort to create a stronger bridge between yeasts and mammalian systems is centered on three types of data: genetic interactions, protein-protein interactions and post-translational modifications. Protein structural information helps to prioritize and functionally understand these large-scale datasets; conversely global, unbiasedly collected datasets helps inform the more mechanistic studies.

August 2 | 10 AM
Probing the host-pathogen interface using quantitative biology

There is a wide gap between the generation of large-scale biological data sets and more-detailed, structural and mechanistic studies. However, recent work that explicitly combines data from systems and structural biological approaches is having a profound effect on our ability to predict how mutations and small molecules affect atomic-level mechanisms, disrupt systems-level networks and ultimately lead to changes in organismal fitness. Our group aims to create a stronger bridge between these areas primarily using three types of data: genetic interactions, protein-protein interactions and post-translational modifications. Protein structural information helps to prioritize and functionally understand these large-scale datasets; conversely global, unbiasedly collected datasets helps inform the more mechanistic studies. Our efforts in this respect have been focused on model organisms, but more recently in mammalian cells, with a particular focus on pathogenesis, as we use these tools, and a number of viruses and bacteria, to systematically and quantitatively study the host-pathogen interface.

August 3 | 9 AM
Using systems approaches for studying cancer

My lab uses network biology to derive mechanistic insights into cellular processes and disease conditions, with a particular emphasis on pathogenesis, cancer, and heart disease. Our pathogenesis studies make use of a variety of high-throughput methods to explore the changes made to host cell systems during infection. These include proteomics techniques, like affinity purification with mass spectrometry and post-translational modification profiling, as well as functional genomic techniques like CRISPR/Cas9 editing and genetic interaction mapping. By employing unbiased systems approaches to infectious disease, we can identify critical nodes for pathogenic persistence and infection in the host, which in turn can inform the design and development of new therapeutic strategies. Our Cancer Cell Map Initiative (CCMI) is aimed at comprehensively detailing complex interactions among cancer genes and proteins using a combination of physical interaction, genetic interaction, and computational approaches.

David Kateete, PhD | Makerere University

David obtained a Bachelor's degeree in Veterinary Medicine and a Master's degree in Molecular Biology at Makerere University, Kampala Uganda. In 1999, he was immediately recruited as Research Assistant in the then newly started Molecular Biology lab under Prof. George W. Lubega, at the Faculty of Veterinary Medicine, Makerere University, where he worked until 2005. David is one of the pioneer trainees and students of molecular biology locally trained in Uganda. In 2005, he joined another newly created Moleceular Biology lab in the department of Medical Microbiology, Makerere University College of Health Sciences, as a Research Associate, under Dr. Moses L. Joloba. He was later appointed as an Assistant Lecturer and obtained a 4-year Forgarty Scholarship for his doctoral studies. David has vast experience in molecular microbiology & medical parasitology, with exposure to various state of the art research laboratories across borders. His research interests are mainly in the area of pathogen biology, aiming at drug/vaccine target discovery and development and molecular diagnostics. For his PhD, he is studying the roles of quorum sensing genes in *Mycobacterium smegmatis*.

July 31 | 9:30 AM

A quest for the roles of rhomboid proteases in mycobacteria

Tuberculosis kills almost 2 million people every year. Despite being an ancient disease, the mechanisms underlying transmission in communities, emergence of drug resistance and latency are not fully understood. Rhomboids are newly discovered proteins that occur in all life kingdoms and they form a unique, evolutionary important superfamiliy of proteins that includes novel transmembrane serine proteases, some of which are responsible for vital roles like signaling and its regulation in drosophila, red blood cell invasion by apicomplexa parasites, and protein transport pathways in *Providencia stuartii*. Most sequenced mycobacterial genomes contain two rhomboid homologues (orthologous to Rv0110 and Rv1337 of *Mycobacterium tuberculosis* H37Rv), which appear evolutionary divergent and functionally distinct. Although rhomboids occur widely in prokarytotes, they have not been characterized in most bacterial species. In this seminar, I will discuss efforts at Makerere University to characterize bacterial rhomboids of an important genus, *Mycobacterium*, with focus on their roles in virulence and drug resistance in *M. tuberculosis.*

August 4 | 10 AM

Whole genome sequencing based characterization of genotypes and drug resistance conferring mutations in Mycobacterium tuberculosis isolates from Uganda

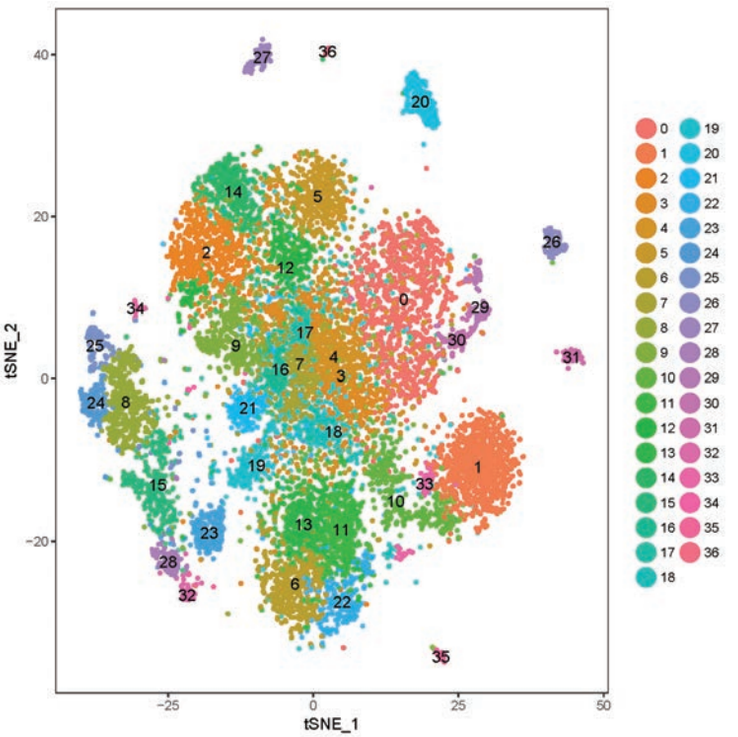
The transmission of tuberculosis in communities and drug resistance emergence are not clearly understood. We have routinely used common molecular approaches such as Restriction Fragment Length Polymorphisms (RFLP) analysis and PCR-based genotyping methods (such as Region of Difference [RD] analysis, Spoligotyping, and Mycobacterial Interspersed Repetitive Units-Variable-Number Tandem Repeats [MIRU-VNTR] analysis) for speciation of the *Mycobacterium tuberculosis* complex, strain identification, and inferring transmission patterns in Uganda. Whereas these methods remain useful in tuberculosis research and surveillance, they have low discriminatory power and they often fail to detect homoplasy (characteristics shared by a set of strains/species but not present in their common ancestor). It is now well established that whole genome sequencing (WGS) is superior to the common molecular methods when it comes to elucidating drug resistance and transmission dynamics of tuberculosis in communities. In this seminar, I will discuss progress on the application of modern genomic technologies particularly WGS to basic and applied tuberculosis research in Uganda.

Workshops

Bioinformatic: RNAseq

Description

This workshop will cover both theory and tools associated with command-line RNA-seq data analysis. Participants will explore experimental design, cost estimation, data generation, and analysis of RNA-seq data. Participants will explore software and protocols, create and modify workflows, and diagnose/treat prolematic data utilizing high performance computing services.



UC DAVIS
GENOME CENTER

Instructor

Matthew Settles, PhD | UC Davis

Over the past decade and a half, Matt has accumulated a significant amount of experience across a wide spectrum of research topics, both computational and biological, with the unifying theme of "big data". Today he considers himself a genomicist; meaning, he conducts research in the three primary facets of genome research: data generation, data analysis and new method development. His research path has progressed from evolutionary computation to applied bioinformatic analysis and genomics method development and finally to where he is today, as a genomics experimentalist and Director of a state of the art bioinformatics core facility at the UC Davis. His current research is on the generation, computational manipulation and interpretation of very large data sets across a wide range of biological questions, often applying techniques not originally designed for a particular data type or experiment in order to ask, and answer, new and interesting biological questions. He has had the pleasure of collaborating on projects in a wide range of subjects and problems including: functional genomics, evolutionary genomics, behavior genomics, comparative genomics, human and environmental microbiome, epi-genomics, whole genome association studies and many others.

Scientific Grant Writing

Instructors

Maria Elena Peñaranda, PhD | Sustainable Sciences Institute

Maria is the Scientific Director of Sustainable Sciences Institute (SSI), a non-profit organization dedicated to helping scientists in developing countries improve public health in their communities. Maria is responsible for all science related activities, including organizing capacity building workshops that provide education and training to scientists around the world. Maria started her professional training at the University of Costa Rica as a Clinical Microbiologist and in 1978 received a Master's degree, studying antibiotic resistant plasmids in enterobacteria. In 1983, she received her Ph.D. from the University of Texas, Houston for her work in the first characterization of plasmid mediated virulence factors. During her postdoctoral training at the Virology Department at Baylor College of Medicine (1983-1986), Dr. Peñaranda studied the molecular biology of rotavirus, and performed microinjection of dsRNA, in order to rescue viral reassortants by genetic manipulation.

Stephen Popper, PhD | Stanford University

Stephen is an Associate Fellow of Global Health at Stanford University, and also serves on the board of directors of the Sustainable Sciences Institute, a nonprofit dedicated to responding to the local needs of public health scientists and researchers in developing countries. His research uses genomic approaches to explore inter-individual variation in the human transcriptome, identify key features associated with protection and pathogenesis in patients with systemic infections, and develop host-based diagnostic and prognostic biomarkers. Stephen received his bachelor's degree from Swarthmore College, and then helped develop and field test the first nucleic-acid based test for species-specific diagnosis of leishmaniasis. He did his doctoral work at the Harvard School of Public Health, where he studied the epidemiology and pathogenesis of HIV-2 and developed a viral load assay for the disease. He completed a postdoctoral fellowship at Stanford in the Department of Microbiology & Immunology, using genome-wide gene-expression profiling to identify diagnostic and prognostic signatures for Kawasaki Disease, dengue, and other systemic infections. More recently he has used the host transcriptome to characterize the innate immune response to dengue vaccination, and is collaborating on a project to integrate next-generation sequencing and host-based biomarkers to diagnose infections in febrile patients.

Description

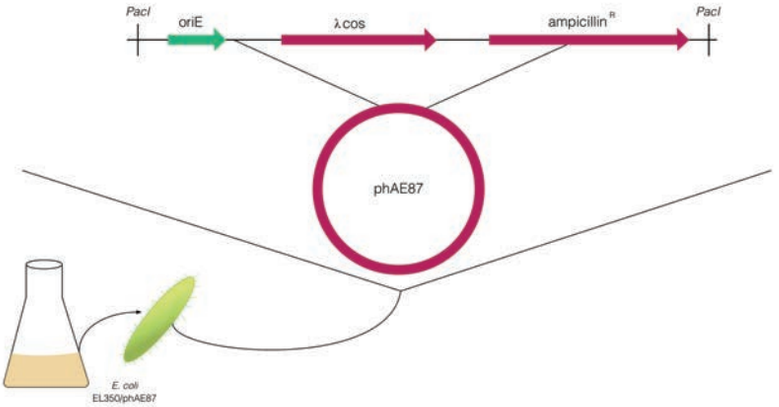
The grant proposal writing workshop will provide scientists with the skills and tools they need to compete successfully for scarce funding opportunities. By the end of the workshop, students will have a solid first draft of a grant proposal ready for submission. This workshop will focus on molecular epidemiology and laboratory-based science grant writing.

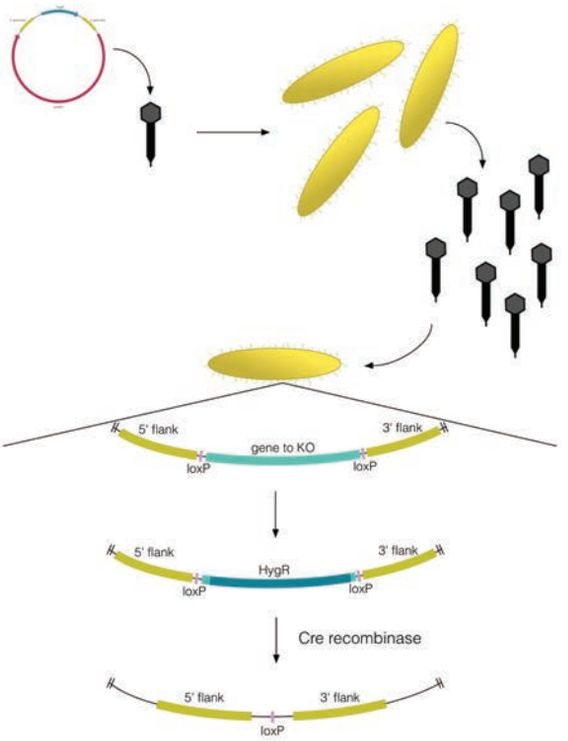
SUSTAINABLE SCIENCES INSTITUTE

Recombineering: Making Gene Knockouts in Mycobacterium smegmatis

Description

The Making Gene Knockouts in *Mycobacterium smegmatis* workshop will teach students the method of genetic recombineering in the context of *Mycobacterium* genetics. Recombineering is facilitated by phage-derived recombination proteins and allows for direct DNA manipulation without relying on *in vitro* techniques. The process will involve plasmid digestion, phage amplification, and *Mycobacterium* transduction.





Instructors

Zoe Netter | UC Berkeley

Zoe grew up in Chicago, IL and did her undergraduate work at the University of Rochester in upstate New York. As an undergraduate, she conducted research in a ribosome biochemistry lab studying the molecular motion of elongation factor G in translation, and a cytomegalovirus pathogenesis lab studying the genetic mechanism of tegument protein synthesis. Zoe currently works in Jeff Cox's lab at the University of California, Berkeley, studying genetic interactions between *Mycobacterium tuberculosis* and its host. She is interested in harnessing the power of microbial molecular communication to derive useful de novo systems.

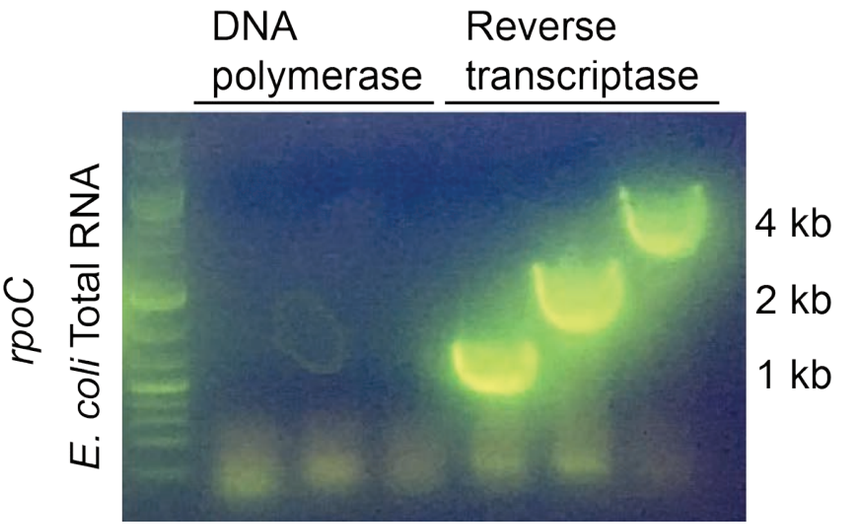
Katie Lien | UC Berkeley

Katie is a graduate student in Sarah Stanley's lab at UC Berkeley. She was born and raised in the rainy Washington state and attended Whitman College where she majored in Biochemistry, Biophysics, and Molecular Biology. At Berkeley she studies the pathogenesis of *Mycobacterium tuberculosis*. She is specifically interested in the role bacterial nanocompartments play during infection, but she loves talking about all things bacteria related!

Protein Purification for the Molecular Biology Laboratory

Description

The Protein Purification for the Molecular Biology Laboratory workshop will instruct students in expression, purification, and quantification of reverse transcriptase. Students will gain experience in plasmid transformation, RT expression and purification techniques, and learn to assay for enzyme activity.



Instructors

Oren Rosenberg, MD, PhD | UC San Francisco

Oren is an Assistant Professor of Medicine at University of California, San Francisco and an investigator at the Chan-Zuckerberg Biohub. He was an undergraduate at Vassar College and worked in Tanzania and Guatemala with the CDC as a Watson Fellow. This experience initiated his lifelong interest in public health and infectious diseases. He was an MSTP student at Yale and then went on to complete his internship and residency at Brigham and Women's Hospital. He was a clinical fellow in Infectious Diseases at UCSF and then a postdoc in the laboratory's of Jeffery Cox and Bob Stroud. His independent group, started in 2015, is using a multidisciplinary approach that combines structural biology and bacterial genetics to examine mechanisms used by intracellular bacteria to evade and exploit the host response to infection.

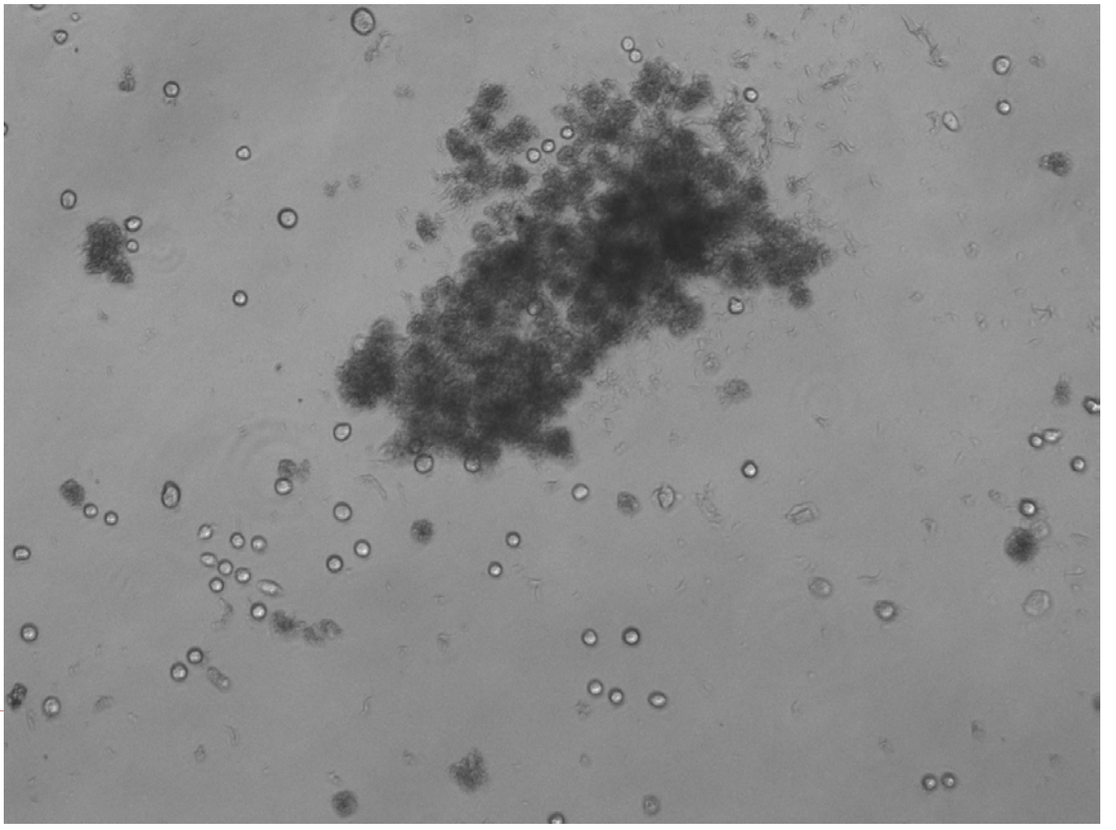
Nadine Czudnochowski, PhD | UC San Francisco

Nadine is a researcher in the laboratory of Professor Oren Rosenberg at the University of California, San Francisco. She did her graduate work at the Max Planck Institute of Molecular Physiology in Germany, where she studied the regulation of the transcription elongation machinery in eukaryotes and the mechanism by which the HIV-1 Tat protein hijacks this machinery to promote viral gene expression. She then moved to the United States to work as a postdoctoral fellow in the laboratory of Robert Stroud at the University of California, San Francisco, and elucidated the specificity of RNA-modifying enzymes by x-ray crystallography. In 2015, she joined the Rosenberg laboratory and is using her expertise in protein biochemistry and structural biology to help advance investigations into the molecular pathogenesis of *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis.

Tissue Culture and Intracellular Bacterial Growth Curves

Description

The Tissue Culture and Intracellular Bacterial Growth Curves workshop will train students in tissue culture technique and how to perform intracellular bacterial growth curves using J774 cells and *Listeria*. Students will learn to maintain a cell culture line, seed cells, and plot inracellular growth curves.

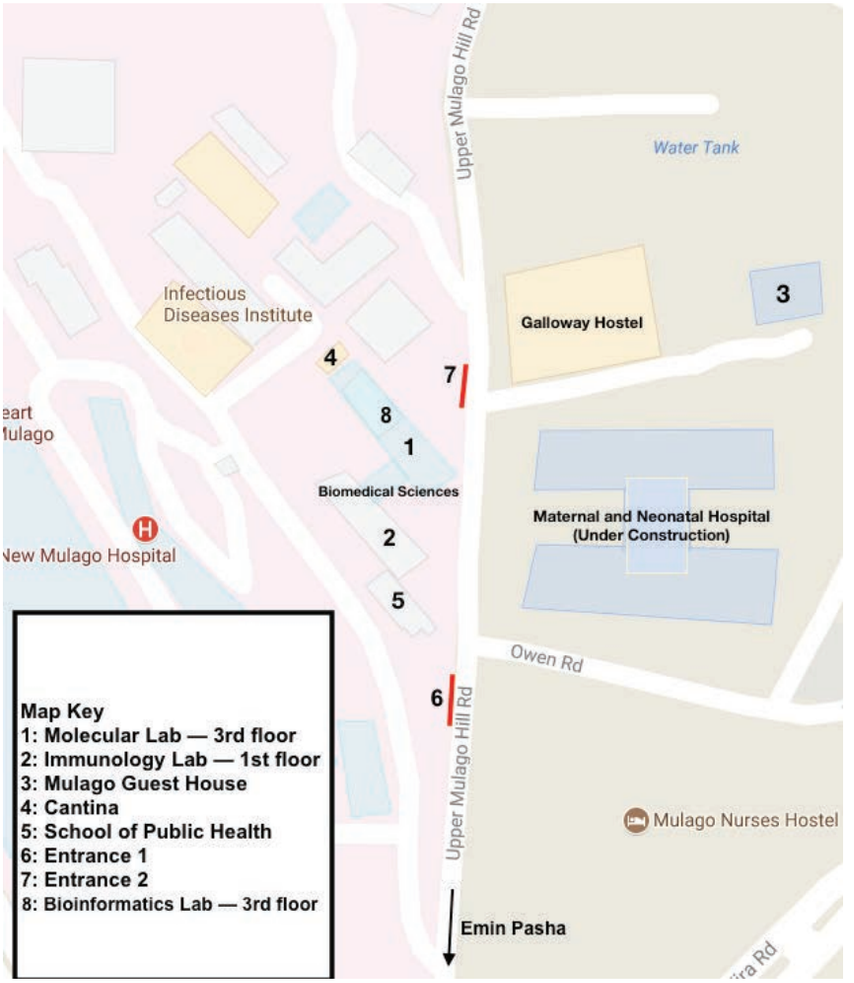


Instructor

Brittney Nguyen | UC Berkeley

Brittney is completing a PhD in Microbiology at UC Berkeley. She works in the Portnoy Lab, where she studies the interaction between *Listeria monocytogenes* and the immune system. Specifically, Brittney is interested in the effects of the Listeria pore-forming toxin LLO during infection, and in the immunosuppressive properties of Listeria that cannot produce LLO. Brittney grew up in San Diego, CA, and graduated from Stanford University in 2014 with a B.S. in Biology. When she's not doing science, Brittney enjoys baking, making pottery, and volunteering at NightLife at the California Academy of Sciences, where she gets the opportunity to teach the public about everyday applications of microbiology.

Map



Many Thanks To:

Ashaba Fred Katabazi
Edgar Kigozi
Hannington Tasimwa
Carol Musubika
Savannah Mwesiga
Gerald Mboowa
Willy Ssengooba

The Alliance for Global Health and Science

Protein Purification for the Molecular Biology Laboratory Workshop:

Reverse Transcriptase Purification, Quantification, and Activity Workshop

Oren Rosenberg
Assistant Professor, UCSF

Nadine Czudnochowski
Assistant Professional Researcher, UCSF

Welcome to the Alliance for Global Health and Science summer workshop in association with Makerere University and UC Berkeley! Thank you for participating in our Protein Purification for the Molecular Biology Laboratory Workshop led by Oren Rosenberg and Nadine Czudnochowski.

Introduction:

In this workshop students will learn how to express and purify the enzyme reverse transcriptase. Students will then test the activity of their purified enzyme for subsequent applications. Reverse transcriptase enzymes are common laboratory enzymes that use an RNA template to generate complementary DNA (cDNA) in a reaction called reverse transcription. The combination of reverse transcription and polymerase chain reaction (PCR), called RT-PCR, is commonly used in diagnostics and basic research. Important applications for RT-PCR include: the detection of RNA originating from pathogens in a biological sample and the quantification of gene expression levels. Although commercial RT kits are available, the cost per reaction is high and regular use of those kits can become a financial burden for research laboratories. Heterologous expression of enzymes in the laboratory provides a cost and time efficient way to obtain large quantities of material.

In this workshop, we will express and purify a hyper-thermostable, tagged version of a reverse transcriptase enzyme and subsequently test its activity. The RT enzyme will be overexpressed in *E. coli* BL21(DE)3 cells using the T7 expression system and purified using a heating step followed by affinity chromatography. Once purified, we will utilize various assays to test the activity of the recombinant RT enzyme. For example, using *E. coli* total RNA as substrate, we will reverse transcribe different-sized fragments of the rpoC gene. In another experiment, we aim to show the up-regulation of the IFN-β gene in a J774 murine macrophage cell line infected with *Listeria monocytogenes* compared to uninfected cells.

By the end of the workshop students will have a general understanding of protein expression and purification methods and will be equipped with the tools to purify proteins for their own research.

Reverse Transcriptase Purification, Quantification, and Activity Workshop

Overview:

Day 0: Solution preparations, start overnight culture
Day 1: Introduction lecture, induction of RT expression
Day 2: RT purification using Ni-NTA agarose
Day 3: Bradford Assay calibration curve for determining protein concentration
Day 4: Activity assay - RT-PCR
Day 5: Wrap-up

Reagents/stock solutions:

- Ampicillin stock solution: 10 mL, 100 mg/mL
- 2xYT media (For 1 L: 16 g Tryptone, 10 g yeast extract, 5 g NaCl, adjust to pH 7.0 with 5 M NaOH)
- IPTG stock solution: 1 M, 10 mL
- Lysozyme: 10 mg/mL in 10 mM Tris-HCl (pH 7.5), 5 mL
- NaCl stock: 5 M, 500 mL
- KCl stock: 1 M, 200 mL
- $MgSO_4$ stock: 2.5 M, 10 mL
- DTT: 1 M, 10 mL
- Imidazole: 5 M, 250 mL

Buffers (per purification):

- Equilibration Buffer (60 mL): 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM $MgSO_4$, 5 mM imidazole
- Wash Buffer (5 mL): 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM $MgSO_4$, 50 mM imidazole
- Elution Buffer (2.5 mL): 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 250 mM imidazole
- Storage Buffer (2 L): 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P40, 0.1% Tween20
- Storage Buffer + 50% glycerol (1L): 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P40, 0.1% Tween20, 50% glycerol
- 10x Assay Buffer (1 mL): 600 mM Tris-HCl (pH 8.5), 250 mM ammonium sulfate, 100 mM KCl

Procedure:

Day 0: Preparations (performed by techs):

1. Ampicillin stock solution
2. Equilibration, Wash, and Elution Buffers
3. Start 50 ml overnight culture in 2xYT supplemented with 100 µg/mL ampicillin using a glycerol stock or a single colony from a freshly transformed plate. Grow cells at 37°C.

Day 1: Induction of RT expression

1. Inoculate 250 ml 2xYT media supplemented with 100 µg/ml ampicillin with overnight culture in a 1 L flask to an initial OD_{600} of 0.05-0.1.
2. Grow cells at 37°C to OD_{600} 0.4 and lower temperature to 18°C.
3. Induce protein expression at OD_{600} 0.6-0.8 (mid-log phase) with 1 mM IPTG and grow cells at 18°C for 20 h.

Day 2: RT purification using Ni-NTA agarose

1. Harvest cells by centrifugation (20 min, 4,000 g)
2. Resuspend cells in 30 ml Equilibration Buffer
3. Add lysozyme to a concentration of 300 µg/mL (10 mg/mL stock solution)
4. Lyse cells by freeze/thaw
    a. Freeze cells in liquid nitrogen or dry ice and leave for 3 min
    b. Thaw at 42°C (water bath), mix well
    c. Repeat steps a) and b) 3 times
5. Pass supernatant through a syringe with attached needle 3-6x to reduce viscosity
6. Incubate supernatant at 85°C for 20 minutes (ideally shaking)
    a. Note: if you can't shake, swirl the tube every five minutes
7. Cool supernatant on ice for 20 minutes
8. Spin lysate in 50 mL Falcon tubes at at least 3,200 g for 30 minutes
9. Filter supernatant using 0.45 µm filter(s) (Note: may need to use more than one filter)
10. Pre-Equilibrate 0.5 mL Ni-NTA resin with 20 ml Equilibration Buffer (Note: be careful not to disturb resin - let buffer flow along the side of column)
11. Gravity flow supernatant over the Ni-NTA resin
12. Wash with 20 mL Equilibration Buffer (be careful not to disturb the resin bed)
13. Wash with 5 mL Wash Buffer
14. Elute column with 2.5 mL Elution Buffer (5 fractions of 0.5 mL each)
15. Transfer eluted protein to ~15 cm of dialysis tubing
    a. Wash 15 cm of tubing in ~25 mL of Storage Buffer
    b. Add eluted protein to tube leaving space for air (tubing needs to float)
16. Dialyze into 1 L Storage Buffer overnight
    a. Place clamped tube containing eluted protein into 1 L beaker of Storage Buffer
    b. Dialysis needs to take place in cold room while being stirred

Day 3: Bradford Assay calibration curve for determining protein concentration

1. Dialyze into 1 L Storage Buffer (to be done in the morning)
    a. Transfer tube into beaker with 1L Storage Buffer
    b. Dialysis needs to take place in cold room while being stirred
2. Dialyze into 1 L Storage Buffer + 50% glycerol
    a. Transfer tube into beaker with 1L Storage Buffer + 50% glycerol
    b. Dialysis needs to take place in cold room while being stirred
3. Bradford assay calibration curve (see Bradford assay manual, link below)
4. Preparation of RT-PCR Assay Buffer

Day 4: Activity assay - RT-PCR

1. Determine protein concentration of purified protein using Bradford standard curve
2. Perform activity assay of RT at different enzyme concentrations (see “RT-PCR Assay Protocol” below)
3. Perform activity assay using RNA from infected J774 macrophage cells as input material

Day 5

1. Wrap-up, questions?

Stock Solution Preparations:

1. Ampicillin stock: 100 mg/mL, 10 mL
    a. 1 g of ampicillin, 10 mL H_2O
    b. Filter with 0.22 µm filter
    c. 4°C for storage
2. IPTG stock: 1 M, 10 mL, 238.3 g/mol
    a. 2.38 g IPTG, 10 mL H_2O
    b. Filter with 0.22 µm filter
    c. Store at -20°C
3. NaCl stock: 5 M, 500 mL, 58.44 g/mol
    a. 146.1 g NaCl, 500 mL H_2O
    b. Filter using bottle top filter
    c. Store at RT
4. KCl stock: 1 M, 200 mL, 74.55 g/mol
    a. 14.91 g KCl, 200 mL H_2O
    b. Filter using bottle top filter
    c. Store at RT
5. $MgSO_4$ stock: 2.5 M, 10 mL, 120.366 g/mol
    a. 3.01 g $MgSO_4$ powder, 10 mL H_2O
    b. Filter with 0.22 µm filter
    c. Store at RT
6. DTT: 1 M, 10 mL, 154.253 g/mol
    a. 1.54 g, 10 mL H_2O
    b. Filter with 0.22 µm filter
    c. -20°C for storage
7. Imidazole: 5 M, 250 mL, 68.08 g/mol
    a. 85.1 g imidazole, 250 mL H_2O
    b. pH to 7.5
    c. Filter using bottle top filter
    d. 4°C for storage
8. Lysozyme: 10 mg/mL in 10 mM Tris-HCl (pH 7.5), 5 mL
    a. Make fresh
    b. Filter sterilize
9. Equilibration Buffer: 60 mL
    a. 20 mM Tris-HCl (pH 7.5): 1.2 mL of 1M stock
    b. 300 mM NaCl: 3.6 mL of 5 M stock
    c. 5 mM $MgSO_4$: 120 µL of 2.5 M stock
    d. 5 mM imidazole: 60 µL of 5 M stock
    e. Keep refrigerated
10. Wash Buffer: 5 mL
    a. 20 mM Tris-HCl (pH 7.5): 100 µL of 1 M stock
    b. 300 mM NaCl: 300 µL of 5 M stock
    c. 5 mM $MgSO_4$: 10 µL of 2.5 M stock
    d. 50 mM imidazole: 50 µL of 5 M stock
    e. Keep refrigerated
11. Elution Buffer: 2.5 mL
    a. 20 mM Tris-HCl (pH 7.5): 50 µL of 1 M stock

    b. 300 mM NaCl: 150 µL of 5 M stock
    c. 250 mM imidazole: 125 µL of 5 M stock
    d. Keep refrigerated
12. Storage Buffer: 2 L (RNase free)
    a. 50 mM Tris-HCl (pH 8.0): 100 mL of 1 M stock
    b. 50 mM KCl: 100 mL of 1 M stock
    c. 0.1 mM EDTA: 400 µL of 0.5 M stock
    d. 1 mM DTT: 2 mL of 1 M stock
    e. 0.1% Nonidet P40: 2 mL
    f. 0.1% Tween20: 2 mL
    g. Keep refrigerated
    h. Make sure RNase free**
13. Storage Buffer + 50% Glycerol: 1L (RNase free)
    a. 50 mM Tris-HCl (pH 8.0): 50 mL of 1 M stock
    b. 50 mM KCl: 50 mL of 1 M stock
    c. 0.1 mM EDTA: 200 µL of 0.5 M stock
    d. 1 mM DTT: 1 mL of 1 M stock
    e. 0.1% Nonidet P40: 1 mL
    f. 0.1% Tween20: 1 mL
    g. 50% glycerol: 500 mL
    h. Keep refrigerated
    i. Make sure RNase free**
14. 10x Assay Buffer (1 mL)
    a. 600 mM Tris (pH 8.5): 600 µL of 1 M stock
    b. 250 mM ammonium sulfate: 71.4 µL of 3.5 M stock
    c. 100 mM KCl: 100 µL of 1 M stock

Useful Equations:

Grams = Volume (L) * Molar Concentration * Molecular Weight

Ex: Grams = 0.01 L * 2.5 mol/L * 120.366 g/mol = 3.01g $MgSO_4$

$C_1V_1 = C_2V_2 \rightarrow Volume_{initial}\ (L) = [Volume_{Final}\ (L) * Molarity_{Final}] / Molarity_{initial}$

Ex: Volume = [0.015 L * 0.03 mol/L NaCl] / 5 mol/L = 0.00009 L = 90 µL of 5 M NaCl

Bradford Assay:
http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf

To set up a standard curve using 2 mg/ml BSA Standard kit, 1 mL cuvettes:

Pipet 20 µl of each of seven standard concentrations, unknown sample solution (20 µl of protein from elution before dialysis), and blank into separate clean cuvettes. Add 1 mL of 1x dye reagent to each tube (or cuvette) and vortex (or invert). Incubate at room temperature for at least 5 minutes (no more than 1 h). Take OD_{595} for each of the standards, blank, and sample. Create a standard curve by plotting the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). Determine the unknown sample concentration using the best-fit line for the standard curve. After completion of dialysis, take another OD_{595} with 20 µL of diluted dialyzed sample. Dialyzed sample will need to be diluted 1:10 into 50 mM Tris-HCl or dialysis buffer without glycerol.

RT-PCR assay protocol:

Ellefson, J. W., J. Gollihar, R. Shroff, H. Shivram, V. R. Iyer, and A. D. Ellington. "Synthetic evolutionary origin of a proofreading reverse transcriptase." Science 352.6293 (2016): 1590-593.

50 µL reverse transcription PCR (RT-PCR) reactions were set up on ice with the following reaction conditions: 1x Assay Buffer, 1 mM $MgSO_4$, 1 M Betaine (Sigma-Aldrich), 200 µM dNTPs, 400 nM reverse primer, 400 nM forward primer, 40 units RNasin Plus (Promega), 0.2 µg polymerase and 1 µg of Total RNA from Jurkat, Human Spleen or E. coli (Ambion). Reactions were thermal-cycled according to the following parameters: 68°C - 30 min, 25x (95°C- 30 sec, 68°C (63°C for rpoC) - 30 sec, 68°C - 30 s/kb).

Reagents	1x Reaction (50 µl Total)	
TOTAL		

Therefore, the potential exists for not only intraregional cortical transcriptomic differences, but also further intrasubtype heterogeneity. This might reflect a technical need for increased sampling depth for further subtype resolution, yet may also indicate the potential for even more diversity within subtypes associated with a broader range of individualized neuronal activities. Consistent with these observations, proportions of subgroup-variable genes were associated with neuronal subtype classification, postsynaptic function, and known regional expression variability (fig. S15C). These data support further local and regional functional heterogeneity existing among defined subtypes.

Our results demonstrate that postmortem SNS can identify expected and previously unidentified neuronal subtypes that provide insight into brain function through distinct profiles of activity-defining genes (fig. S16 and table S14). Furthermore, given that only a very small subset of layer-specific markers used in our analyses (*CARTPT*, *CHRNA7*, *PDYN*, and *RELN*) was found to have ISH differences between individual donors (*17*), our subtypes can be expected to be globally representative. Indeed, our subtypes remain highly conserved in mice (*3*), with differences highlighting evolutionary changes in potential orthologs (fig. S12). Our data sets reveal shared gene expression signatures that can distinguish subtypes and regional identity, supporting a transcriptional basis for well-known differences in cortical cytoarchitecture. Additional heterogeneity found within single neuronal transcriptomes may further reflect activities of complex neuronal networks that vary with function and time, as well as underlying genomic mosaicism that exists in human cortical neurons (*10*, *20*–*23*). Our study thus lays the groundwork for high-throughput global human brain transcriptome mapping using nuclei derived from readily available postmortem tissues for analyses of normal individuals, as assessed here, as well as myriad diseases of brain and mind.

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ACKNOWLEDGMENTS

Flow cytometry was performed both at the University of California, San Diego (UCSD) Human Embryonic Stem Cell Core and The Scripps Research Institute Flow Cytometry Core. Initial C1 runs were performed at the UCSD Stem Cell Genomics Core. The data tables accompanying this work are provided as Excel files in the supplementary materials. Clustering-and-Classification code used to identify neuronal subtypes and instructions (Readme.txt) for its operation in R are provided as supplementary files. We thank Fluidigm (M. Ray, R. C. Jones, and P. Steinberg) for instrument support and technical advice in adaptation of the C1 protocol for nuclei. Sequencing data has been deposited with dbGaP (accession phs000833.v3.p1), curated by the NIH Single Cell Analysis Program–Transcriptome (SCAP-T) Project (www.scap-t.org), and annotated in the supplementary materials (table S2). We thank G. Kennedy for help with RNAscope. Funding support was from the NIH Common Fund Single Cell Analysis Program (1U01MH098977). G.E.K. was additionally supported by a Neuroplasticity of Aging Training Grant (5T32AG000216-24).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6293/1586/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S16
Tables S1 to S16
Supplementary Files
References (*24*–*38*)

18 December 2015; accepted 19 May 2016
10.1126/science.aaf1204

BIOCHEMISTRY

Synthetic evolutionary origin of a proofreading reverse transcriptase

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Most reverse transcriptase (RT) enzymes belong to a single protein family of ancient evolutionary origin. These polymerases are inherently error prone, owing to their lack of a proofreading (3′- 5′ exonuclease) domain. To determine if the lack of proofreading is a historical coincidence or a functional limitation of reverse transcription, we attempted to evolve a high-fidelity, thermostable DNA polymerase to use RNA templates efficiently. The evolutionarily distinct reverse transcription xenopolymerase (RTX) actively proofreads on DNA and RNA templates, which greatly improves RT fidelity. In addition, RTX enables applications such as single-enzyme reverse transcription–polymerase chain reaction and direct RNA sequencing without complementary DNA isolation. The creation of RTX confirms that proofreading is compatible with reverse transcription.

The molecular basis for life rests on the information flow between DNA, RNA, and proteins (*1*). Early notions of a unidirectional central dogma were amended after the discovery of the reverse transcriptase (RT) enzyme (*2*, *3*). The RT family has a single ancient evolutionary origin based on amino acid homology and the presence of RT across multiple domains of life (*4*). RTs are involved in processes such as telomere addition, mitochondrial plasmid replication, transposition, and the proliferation of retroviral genomes (*5*). It is also hypothesized to be the catalyst in the transition of the RNA to DNA world by providing an avenue to copy RNA into more stable DNA genomes (*6*).

The progenitor of RT is postulated to be an RNA-dependent RNA polymerase. Because RNA polymerases generally lack an error-checking 3′-5′ exonuclease domain (*4*, *7*), proofreading activity is also not present across the RT family, resulting in low-fidelity reverse transcription and characteristic quasispecies behavior in organisms that rely upon it for replication (*8*). In contrast to RTs, other DNA polymerase families have evolved exquisite proofreading mechanisms to increase DNA synthesis fidelity during genome replication (*9*).

To determine whether the evolutionary divide between RTs and DNA polymerases is a matter of history or function, we have attempted to directly evolve a reverse transcription xenopolymerase (RTX; Fig. 1A) from an error-correcting DNA polymerase using a modified directed evolution strategy (*10*), reverse transcription–compartmentalized self-replication (RT-CSR) (Fig. 1B). RT-CSR enables the simultaneous screening of up to 10^9 polymerase variants for RT activity.

We chose the Archaeal family-B DNA polymerases (*polB*) for directed evolution of the RTX as they are monomeric, hyperthermostable, highly processive, and contain proofreading domains. Attempts to rationally design these enzymes to use RNA templates have met with limited success (*11*, *12*), and initial experiments confirmed that two common *polB* enzymes from *Pyrococcus furiosus* and *Thermococcus kodakarensis* (KOD) (*13*, *14*) failed to polymerize across five template RNA bases (fig. S1). Modeling to identify mutations enabling RT activity was deemed impractical, given the extensive contacts these polymerases make with the template (>50 direct interactions). We initiated evolution using low-stringency RT-CSR (10 RNA residues) with a random library

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(one or two amino acid mutations per gene) of KOD polymerase variants. As polymerases were enriched, we gradually increased RT-CSR stringency with the stepwise addition of RNA bases into primers (table S1). By cycle 18, primers were entirely composed of RNA—requiring reverse transcription of 176 residues to occur every thermal cycle to maintain exponential amplification in the emulsion polymerase chain reaction (PCR).

Profiling of polymerases revealed one variant, B11, which contained 37 mutations. RT-CSR enriched for RT activity, and B11 was capable of reverse transcription of at least 500 base pairs; however, sequencing and testing confirmed inactivation of the proofreading domain (fig. S2). Kinetic analyses established that B11 uses both DNA and RNA templates with similar efficiencies by greatly lowering the Michaelis constant (K_m) on RNA:DNA heteroduplexes. We attempted to restore proofreading by transplantation of the wild-type 3′-5′ exonuclease, which reactivated

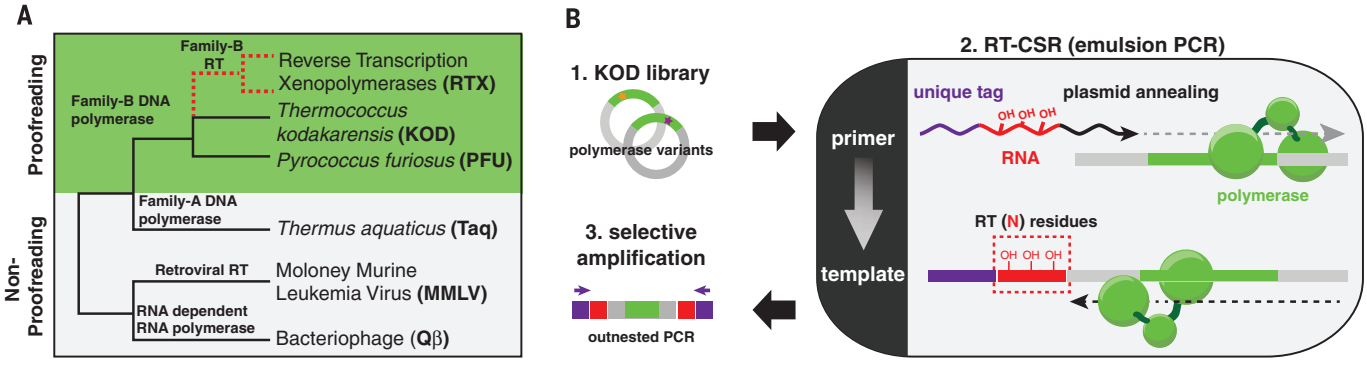

Fig. 1. Evolution of a synthetic family of reverse transcriptases by RT-CSR. (**A**) Polymerase phylogeny depicts reverse transcription xenopolymerases (RTX) as a second, evolutionarily distinct, origin of RT function. (**B**) Framework for the directed evolution of hyperthermostable RT using reverse transcription compartmentalized self-replication (RT-CSR). Libraries of polymerase variants are created, expressed in *Escherichia coli*, and in vitro compartmentalized. During emulsion PCR, primers flanking the polymerase enable self-replication but are designed with a variable number of RNA bases separating the plasmid annealing portion from the unique recovery tag.

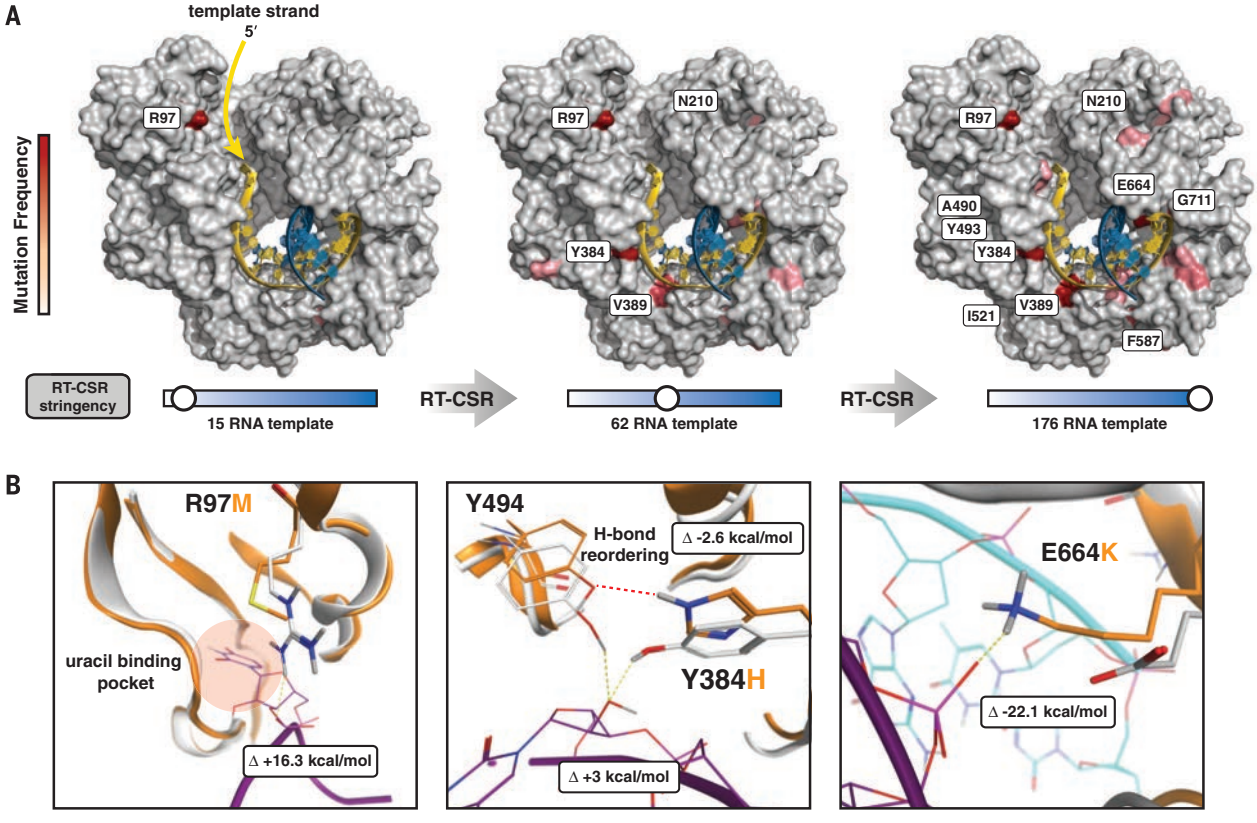

Fig. 2. Molecular checkpoints involved in template recognition. (**A**) Structural heat map of mutated residues over the RT-CSR process. Conserved mutations are colored incrementally darker shades of red to indicate frequency in the polymerase pool. Amino acid residues that were mutated in more than 50% of the population are labeled. [Figure was adapted from KOD structure PDB 4K8Z.] (**B**) Computer modeling of KOD (gray) and RTX mutations (orange) at checkpoints responsible for DNA and RNA template recognition at R97, Y384, and E664. Free-energy changes between wild-type KOD and RTX mutations are inlet displayed. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; M, Met; N, Asn; R, Arg; V, Val; and Y, Tyr.

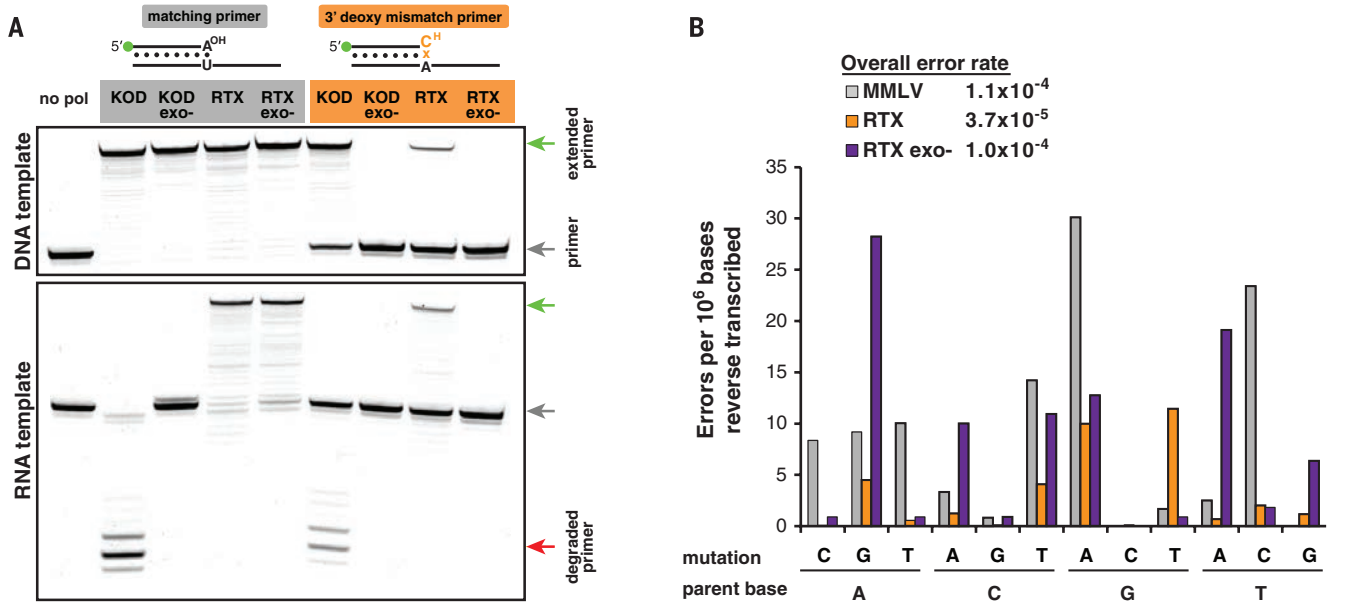

Fig. 3. RTX polymerase proofreads during reverse transcription. (**A**) Primer extension reactions of KOD and RTX polymerases and their proofreading-deficient counterparts (exo-), on both DNA and RNA templates. Extension reactions were performed with matched 3′ primer:templates (gray) or a 3′ deoxy mismatch (orange), which must be excised before extension can proceed. The primer is denoted by a gray arrow, extended product is in green, and exonuclease-degraded primer is in red. (**B**) Deep sequencing of reverse transcription reaction on *HSPCB* gene. The overall error rate was determined by dividing the sum of base substitutions and insertions or deletions by the total number of bases sequenced. The error profile of MMLV, RTX, and RTX exo- is shown as frequency of errors per million bases sequenced.

proofreading capabilities, albeit to barely detectable levels. Encouraged that minimizing extraneous mutations could restore proofreading activity, we sought to design polymerases with a minimal set of mutations.

To understand how our process reshaped KOD polymerase to use RNA templates, we deep-sequenced RT-CSR cycles to recapitulate the evolutionary path to RT activity (Fig. 2A and table S2). Mutations were identified throughout the polymerase and accumulated along the template-binding interface so as to progressively increase the length of RNA that could be accommodated. The mutated positions are hypothesized to be molecular checkpoints used to enforce strict DNA template utilization: as the template enters, near the active site, and at the nascent duplex. Given the likely importance of these regions, we used computer modeling to determine the molecular basis for RNA utilization (figs. S3 and S4).

The first selected mutation localized near the template entry site of the polymerase at position R97 (Fig. 2B). Proximal to this site, native *polB* scans for uracils (typically caused by cytosine deamination) by flipping template bases into a specialized pocket to halt polymerization until the mutation can be corrected by repair machinery (*15*, *16*). Evolved polymerases contained a variety of amino acid mutations at R97, all of which destabilize a salt bridge to the phosphate backbone that presumably regulates base flipping into the pocket.

As template residues near the active site, they encounter mutation Y384H, which prevents Y384 and Y494 from hydrogen bonding to the 2' hydroxyl of template RNA by reorganizing a hydrogen bonding network. After polymerization, in the thumb domain, the most prevalent mutations (E664K, G711V, and E735K) promote tighter homo- and heteroduplex binding in both A- and B-form conformations. The E664K mutation alone has been shown to increase binding to RNA:DNA heteroduplexes (*17*). To further validate that we had established an optimized set of mutations, we fully randomized several positions and repeated the RT-CSR. In support of our modeling, many amino acids solutions were viable at position R97, but other positions (Y384 and E664) had strong preferences for particular amino acids (fig. S5).

Modeled designs of several polymerases with favorable RT mutations were synthesized and tested empirically (fig. S6). The best-performing RTX contained fewer than half the mutations found in B11, without sacrificing catalytic efficiency or K_m on RNA (fig. S7). Mutations in RTX did not affect desirable properties of parental KOD polymerase. Thermostability was maintained, with optimal RT occurring at ~70°C, and consequently RTX was capable of single-enzyme RT-PCR (in which RTX performs both first-strand RT synthesis and PCR amplification). Across several RNA samples and gene loci, RTX demonstrated high processivity on RNA templates, performing RT-PCR on RNAs more than 5 kb in length (fig. S8).

Initial testing of RTX using dideoxy mismatch primers in PCR demonstrated robust proofreading activity on DNA template (fig. S9), but it was unclear whether the proofreading mechanism was compatible during reverse transcription because RNA:DNA heteroduplexes can adopt A-form helical structures (*18*). Primer extension reactions with a canonical matched base pair or a 3′ deoxy mismatched pair (preventing extension until terminator excision) were tested. Both wild-type KOD and RTX were capable of extending mismatched primers on DNA templates, unlike exonuclease-deficient mutants. When tested on an RNA template, KOD's exonuclease was stimulated—actively degrading the priming oligonucleotide. In contrast, RTX could extend the mismatched primer with activity indistinguishable from that of DNA templated proofreading (Fig. 3A).

Given that RTX is capable of proofreading during reverse transcription, we hypothesized that it may have increased RT fidelity compared to natural polymerases. Barcoded primers used during RT of several human mRNAs allowed multiple reads of a single cDNA during deep sequencing—reducing background sequencing errors by several orders of magnitude (fig. S10) (*19*). Sequencing analyses revealed that the control retroviral RT [Moloney murine leukemia virus (MMLV)] had an error rate of 1.1×10^{-4} to 4.8×10^{-4}, whereas RTX had an error rate of 3.5×10^{-5} to 3.7×10^{-5} (3- to 10-fold lower) (Fig. 3B). The mutational spectra of RTX favored G-to-A transitions and G-to-T transversions, which accounted for nearly half the observed mutations. Inactivating the RTX's proofreading capabilities increased error frequency nearly threefold, supporting evidence that active proofreading was occurring during RT. Inactivating the proofreading of RTX shifted the mutational bias (Fig. 3B and table S3). Given that the barcoding error detection limit is identical to the observed error of RTX (table S3) (*19*), we anticipate the true error rate for RTX to be even lower than reported.

RTX has the potential to streamline workflows (combining RT and PCR steps) and increase the precision of transcriptomics, reducing biases and

errors introduced in the reverse transcription step of RNA-sequencing protocols (*20*). To demonstrate its utility, we introduced RTX into a commonly used platform for RNA sequencing. Analysis revealed nearly identical coverage and expression profiles (fig. S11), suggesting that RTX is compatible with established workflows. In addition, we developed a more streamlined protocol to directly sequence RNA. Using a traditional Sanger sequencing approach (*21*), we directly sequenced a $GATC_5$ RNA repeat (fig. S12). Direct RNA sequencing should be adaptable to single-molecule sequencing platforms, enabling high-throughput and high-fidelity sequencing of complex RNA samples by eliminating the biases created in cDNA synthesis and subsequent amplification.

The expanded template specificity of the RTX lineage may presage the ability to use entirely new chemistries in genetics. Primer extension reactions were performed on a ribose sugar analog [2' O-methyl (Me) DNA] that indicated that RTX reverse transcription could extend alternative templates but with much lower efficiency, indicating a preference for RNA substrates (fig. S13). However, RTX was still far more efficient at using 2'-OMe DNA than the parental wild-type, allowing the possibility of further optimization and, owing to 2'-OMe stability, potential therapeutic applications.

The RTX RNA reverse transcriptase function is fundamentally distinct from that of the retroelement lineage. Using RT-CSR, we have altered the substrate specificity of a high-fidelity DNA polymerase, highlighting the plasticity of highly conserved molecular machinery. Ostensibly, the mutations identified unlocked molecular checkpoints in the discrimination of DNA and RNA, but did not disrupt the proofreading capabilities of the polymerase. This was unexpected, especially given that RNA:DNA hybrid duplexes often form A-helical structures unlike DNA:DNA duplexes, and may provide insights into the transition from polymerization to editing modes of the polymerase.

Only a handful of mutations were required to impart RT activity, suggesting that the evolutionary hurdle for forming high-fidelity reverse transcription is relatively low. Nevertheless, all known retroelements use proofreading-deficient RTs, suggesting that high error rates are either a historical coincidence or an evolutionary strategy to promote diversity. Another possible explanation is that high fidelity was never required simply because RNA genomes are small as a result of their inherent instability (*22*). Given the plasticity of these polymerases for modified templates and the adaptability of the RT-CSR framework (as primers are simply programmed to contain modified bases), RTX evolution should be compatible with many base and sugar analogs (*23*–*26*). Combination with previously evolved XNA polymerases could enable synthesis of genomes entirely composed of artificial nucleic acids (*27*).

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ACKNOWLEDGMENTS

We thank the University of Texas Genomic Sequencing and Analysis Facility for DNA sequencing support. This work was supported by Defense Advanced Research Projects Agency (HR0011-12-2-0001), National Security Science and Engineering Faculty Fellowship (FA9550-10-1-0169), NASA (NNX15AF46G), and the Welch foundation (F-1654). J.W.E. conceived of the project. J.W.E., J.G., R.S. performed research with contributions from H.S and V.R.I. J.W.E, J.G., R.S., and A.D.E. wrote the manuscript. The authors declare competing financial interests. A.D.E. was a paid consultant for Enzymatics. A provisional patent has been filed on the sequence composition of the reverse transcriptase.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/352/6293/1590/suppl/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 to S3
Sequences

24 February 2016; accepted 31 May 2016
10.1126/science.aaf5409

Materials and Methods

Initial reverse transcription test for polymerases

30 pmol of 5' fluorescein labeled primer (25FAM) were annealed with 30 pmol of template (TEMP.A.DNA/1RNA/5RNA) and 0.4 µg of polymerase by heat denaturation at 90°C for 1 minute and allowing to cool to room temperature. Reactions were initiated by the addition of "start" mix which contained (50mM Tris-HCl (pH8.4), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 200 µM dNTPs. MMLV polymerase was treated according to manufacturers recommendations (New England Biolabs). Reactions were incubated for 2 minutes at 68°C until terminated by the addition of EDTA to a final concentration of 25 mM. The labeled primer was removed from the template strand by heating sample at 75°C for 5 minutes in 1x dye (47.5% formamide, 0.01% SDS) and 1 nmol of unlabeled BLOCKER oligonucleotide (to competitively bind the template strand). Samples were run on a 20% (7 M urea) acrylamide gel.

Reverse Transcription CSR (RT-CSR)

Briefly, *E. coli* cells containing a library of putative RTX variants are physically compartmentalized through a water-in-oil emulsion and subsequently thermal cycled. Primers are included in the emulsion to facilitate self-replication, but contain RNA residues which behave as a template in subsequent cycles of PCR to enforce reverse transcriptase activity. KOD polymerase libraries were created through error prone PCR (unless otherwise indicated) to have a mutation rate of ~1-2 amino acid mutations per gene. Libraries were cloned into tetracycline inducible vector and electroporated into DH10B *E. coli*. Library sizes were maintained with a transformation efficiency of at least 10^6, but more typically 10^7-10^8. Overnight library cultures were seeded at a 1:20 ratio into fresh 2xYT media supplemented with 100 µg / mL ampicillin and grown for 1 hour at 37°C. Cells were subsequently induced by the addition of anhydrotetracycline (typically at a final concentration of 200 ng / mL) and incubated at 37°C for 4 hours. Induced cells (200 µL total) were spun in a tabletop centrifuge at 3,000 x g for 8 minutes. The supernatant was discarded and the cell pellet was resuspended in RTCSR mix: 1x selection buffer (50 mM Tris-HCl (pH8.4), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$), 260 µM dNTPs, 530 nM forward and reverse RNA containing primers (detailed in Table S1). The resuspended cells were placed into a 2 mL tube with a 1mL rubber syringe plunger and 600 µL of oil mix (73% Tegosoft DEC, 7% AbilWE09 (Evonik), and 20% mineral oil (Sigma-Aldrich)). The emulsion was created by placing the cell and oil mix on a TissueLyser LT (Qiagen) with a program of 42Hz for 4 minutes. The emulsified cells were thermal-cycled with the program: 95°C - 3min, 20x (95°C- 30 sec, 62°C- 30 sec, 68°C- 2 min). Emulsions were broken by spinning the reaction (10,000 x g - 5 min), removing the top oil phase, adding 150 µL of H_2O and 750 µL chloroform, vortexing vigorously, and finally phase separating in a phase lock tube (5Prime). The aqueous phase was cleaned using a PCR purification column which results in purified DNA, including PCR products as well as plasmid DNA. Subamplification with corresponding outnested recovery primers ensures that only polymerases that reverse transcribed are PCR

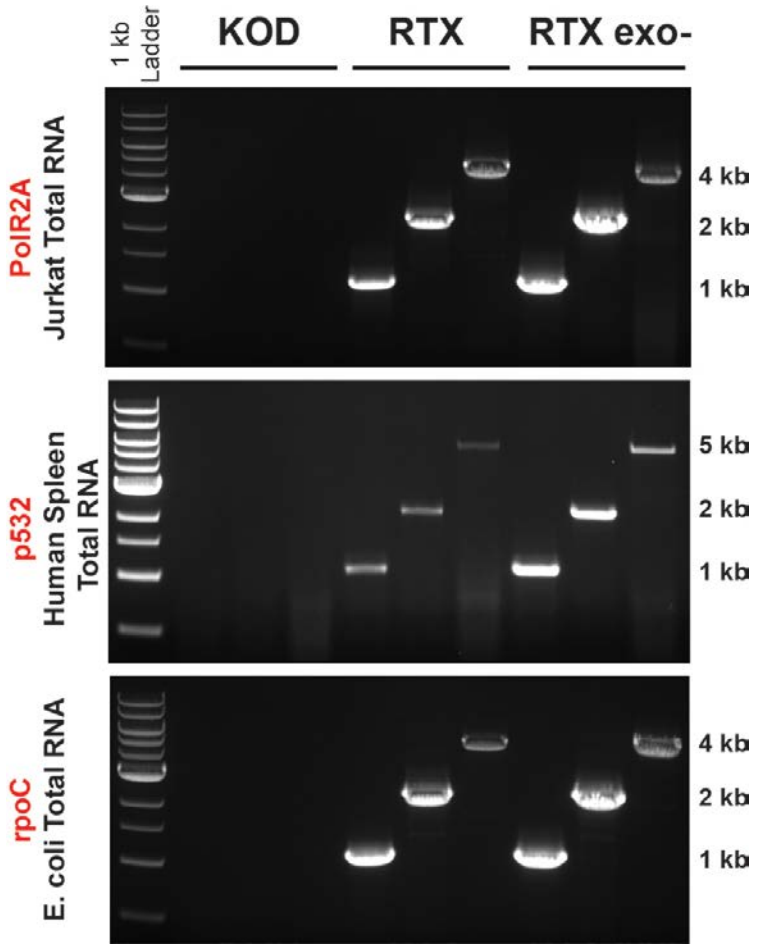

Fig. S8. Reverse transcription PCR (RTPCR) was performed using KOD polymerase, RTX, and the proofreading deficient version of RTX (N210D; exo-). Various genes were amplified (red), two human genes, PolR2A and p532, and rpoC from *E. coli* from various RNA sources.

The Alliance for Global Health and Science

Introduction to Molecular Cloning Workshop:

Cloning strategies and techniques for molecular biology

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Welcome to the Alliance for Global Health and Science summer workshop in association with Makerere University and UC Berkeley! Thank you for participating in our Introduction to Molecular Cloning Workshop led by David Savage and Robert Nichols.

Introduction: The goal of this workshop is to introduce students to the foundational principles of molecular cloning (the art/ science of designing and assembling recombinant DNA). Students will learn the history of molecular cloning including classical restriction enzyme cloning, as well as the latest technological advancements in the field such as Golden Gate cloning, Gibson cloning, and SLiCE cloning. Students will learn how to use software to design and synthesize DNA constructs containing their gene of interest into an expression vector. We will then perform the standard protocols for molecular cloning- PCR amplification of DNA of interest, gel electrophoresis, gel extraction/ DNA purification, DNA assembly, bacterial transformation, and sequence validation of the newly synthesized plasmid. The workshop will finish with the implementation of an advanced technique known as CRISPRi, to demonstrate how molecular cloning techniques learned in the course can be used to repress the expression of a gene of interest.

Alliance Cloning Workshop - Protocols Overview

Day 1 (Week 1: Monday)

Cloning for pMU1 (Beta-galactosidase Blue/White Screening)

1. Set up PCR reactions (See: "Polymerase Chain Reaction") to make DNA fragments for pMU1 construct.
2. Perform a gel electrophoresis (See: "Gel Electrophoresis") and DNA gel purification (See: "DNA Gel Purification") on PCR products.

Day 2 (Week 1: Tuesday)

Cloning for pMU1(Beta-galactosidase Blue/White Screening)

1. Use restriction enzymes to digest (See: "Classic Restriction Enzyme Cloning") purified DNA from Day 1.
2. Purify digested DNA (See: "DNA Purification" and note at end of protocol).
3. Set up a ligation of plasmid and insert (See: "Setting up a ligation reaction").
4. Transform ligated plasmid (See: "Setting up a transformation") into *E. coli*, and plate onto LB + Carb + IPTG + X-gal.

Day 3 (Week 1: Wednesday)

Cloning for pMU1 (Beta-galactosidase Blue/White Screening)

1. Start a 5 mL culture using a colony from Day 2 transformation plate.

Cloning for pMU2-4 (GFP, RFP, GFP/RFP fluorescence)

2. Set up PCR reactions (See: "Polymerase Chain Reaction") to make DNA fragments for pMU2-4 constructs.

Day 4 (Week 1: Thursday)

Cloning for pMU1 (Beta-galactosidase Blue/White Screening)

1. Miniprep pMU1 culture from Day 3 (See: "Miniprepping your sample").

Cloning for pMU2-4 (GFP, RFP, GFP/RFP fluorescence)

2. Perform Gel extraction clean-up for pMU2-4 DNA fragments (See: "DNA Clean-Up")
3. Perform SliCE assembly of pMU2-4 DNA fragments
4. Transform into DH5 alpha

Day 5 (Week 1: Friday)

Cloning for pMU1 (Beta-galactosidase Blue/White Screening)

1. Validate pMU1 plasmid via restriction enzyme digestion (See: "Restriction Enzyme digest validation)

Cloning for pMU2-4 (GFP, RFP, GFP/RFP fluorescence)

2. Flex time for pMU2-4 assembly and transformation

Day 6 (Week 2: Monday)
Cloning for pMU2-4 (GFP, RFP, GFP/RFP fluorescence)
1. Analysis of pMU2-4 transformants

Cloning for pMU5-6 (CRISPRi GFP/RFP repression)
2. Primer design for pMU5-7

Day 7 (Week 2: Tuesday)
Cloning for pMU5-6 (CRISPRi GFP/RFP repression)
1. PCR for pMU5-7 DNA fragments
2. Gel Excision of pMU5-7 DNA fragments

Day 8 (Week 2: Wednesday)
Cloning for pMU5-6 (CRISPRi GFP/RFP repression)
1. SliCE assembly of pMU5-7
2. Transform pMU5-7 into DH5 alpha

Day 9 (Week 2: Thursday)
Cloning for pMU5-6 (CRISPRi GFP/RFP repression)
1. Analysis of GFP/ RFP repression

Day 10 (Week 2: Friday)
Buffer day

Special notes for cloning round 1:
-We will plate our pMU1 transformants onto LB + Carbenicillin + IPTG + X-Gal.
-The next day we will look for blue or white colonies, to assay whether we have LacZ activity.

Special notes for cloning round 2 and 3:
-We will plate our pMU2, pMU3, pMU4, pMU5, transformants onto LB + Carb
-pMU6 will be plated onto LB + Chloramphenicol
-pMU5 + pMU6 double transformations will be plated on LB + Carb + Chloramphenicol
-We will determine whether we have GFP, RFP, GFP+RFP fluorescence using the blue light transilluminator.

Polymerase Chain Reaction (PCR)

1) Planning your PCR reactions ahead of time is extremely important and time should be spent determining the following:
   a) What do you want your final DNA plasmid to look like? What goal are you trying to achieve? i.e. Do you wish to insert a gene of interest that you are studying into an expression plasmid? What DNA fragment will be your vector (also called backbone), what DNA fragment(s) will be inserted into that vector and in what order?
   b) Design the assembly reaction using the free to use "Benchling" software at benchling.com. We will teach you how do this in class, but if you forget you can reference this Youtube video guide: https://www.youtube.com/watch?v=k0dnZ0in5IY
   c) Plan out which primers go with their respective template and what the proper annealing temperature of the primer pair is as can be calculated using this tool: http://tmcalculator.neb.com/
   d) Determine the expected length of the PCR product and use this to calculate how long of an extension cycle (the 72 ºC cycle) you will need for your reaction. The Q5 enzyme amplifies DNA at a rate of 1000 bases per 30 seconds.

2) Set up the PCR reactions for your DNA fragments according to the Q5 New England Biolabs kit protocol (*Note: Set up all reactions on ice.*):

COMPONENT (Final concentration)	**25 µl REACTION**	**50 µl REACTION**	**8.5x (25 µL rxn)**
5X Q5 Reaction Buffer (1X)	5 µL	10 µL	42.5 µL
10 mM dNTPs (200 µM)	0.5 µL	1 µL	4.25 µL
20 µM Forward Primer (0.5 µM)	0.5 µL	1 µL	4.25 µL
20 µM Reverse Primer (0.5 µM)	0.5 µL	1 µL	4.25 µL
1 ng/µL Template DNA (1 ng)	0.5 µL	1 µL	4.25 µL
Q5 High-Fidelity DNA Polymerase (0.02 U/µL)	0.25 µL	0.5 µL	2.13 µL
5X Q5 High GC Enhancer (optional) (1X)	(5 µL)	(10 µL)	(42.5 µL)
Nuclease-Free Water	to 25 µL	to 50 µL	to 212.5 µL

3) Set up thermocycler program as follows:

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
25–35 cycles	98 °C *50–72 °C 72 °C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72 °C	2 minutes
Hold	4–10 °C	

Gel Electrophoresis

1) While the PCR is running, make a 1% (weight/volume) agarose gel for gel electrophoresis.
    a. Make agarose TAE solution such that the final agarose concentration is 1% weight/volume (eg. 1 g agarose in 99 mL of TAE or TBE buffer).
        - We will make enough for the whole class at once and use 3 g of agarose in 300 mL of TAE.
    b. Microwave until buffer boils and agarose is fully dissolved.
    c. Let it cool for 1 minute, add 3 µL of SYBR Safe.
    d. Pour melted agarose solution into gel casting trays.
    e. Add well combs to the casting trays.
2) Once the PCR reaction is finished, add appropriate volume of 6X loading dye to the reaction.
    a. Since the loading dye is 6X (we want 1X final) and we made 50 µL PCR reactions, we will add 10 µL of loading dye to each 50 µL reaction.
3) Mix well by pipetting or inverting, then briefly spin down the liquid in a tabletop microcentrifuge.
4) Place your agarose gel into the gel box and fill with TAE buffer to the fill line (or at least to fully submerge the gel).
5) Add the DNA ladder to the first well and then add your samples in the following wells. Be sure to note in which order samples are loaded.
6) Run the gel box at 120 V for 15 minutes and monitor the position of the leading dye front. We will check our gels once the leading dye front is ~3/4 through the gel.
    a. For faster electrophoresis, gels can safely be run at 10 V/cm between electrodes (up to ~140 V for a small gel box and ~165 V for larger ones).
7) Turn off the gel rig system and remove the gel from the gel box.
8) Place the gel on the dark reader to visualize the DNA, checking that bands from each PCR reaction are of the expected size.
9) Using a clean razor blade, cut bands of interest out of the gel. (*Note:* Cut as close to the band as possible, so you do not have excess agarose going into the DNA purification reaction.)
10) Place gel slice into a clean 1.5 mL microcentrifuge tube. The gel slice may be stored at 4 ºC overnight or you may move on to the gel purification step.

DNA Clean-Up

We will follow the Zymo DNA recovery protocol.

1) Add 700 µL of ADB buffer (brown bottle found in kit) to each of your microcentrifuge tubes containing a gel slice.
2) Incubate microcentrifuge tube at 42 ºC for 10 minutes or until agarose gel is fully dissolved.
3) Pipette dissolved solution from microcentrifuge column into filter column with collection tube underneath.
4) Spin tube in a microcentrifuge at 18,000 x g for 30 seconds.
5) Discard flow-through in collection tube into your liquid waste.
6) Add 200 µL of wash buffer, spin at 18,000 x g for 30 seconds.
7) Add another 200 µL of wash buffer, spin at 18,000 x g for 30 seconds.
8) Discard flow-through in collection tube into your liquid waste.
9) Spin again at 18,000 x g for 30 seconds to remove any residual ethanol from the filter column.
10) Place filter column in new clean 1.5 mL microcentrifuge tube.
11) Add 13 µL of ddH2O, spin at 18,000 x g for 30 seconds.
12) Measure A260 of sample with Nanodrop to determine DNA concentration.

NOTE: If performing a DNA purification of ligation products use DNA binding buffer instead of ADB buffer, mix thoroughly, then proceed with protocol from Step 3. See instructions in kit for more information.

Classic Restriction Enzyme Cloning

Note: This protocol will only work if you designed your assembly with compatible restriction enzyme cut-sites/sticky ends. If your assembly is set up for SLiCE cloning, skip to the SLiCE cloning section below.

Setting up restriction enzyme digest:

1) In a PCR tube set up your digestion reaction for you insert and vector:
    a. *Calculating volume of DNA for digestion:* $\frac{1000\ ng\ total\ DNA}{[DNA\ extraction] ng/\mu L} = Total\ \mu L\ to\ digest$

Component	Volume
10X CutSmart buffer	5 µL
1 µg vector or insert	Calculate
SpeI	1 µL
XhoI	1 µL
Water	Fill to 50 µL

2) Place in thermocycler and run at 37 ºC for 20 minutes, then heat inactivate at 80 ºC for 20 minutes.
3) Purify the Insert and Vector using the Zymo DNA recovery kit protocol as listed in "DNA Gel Purification."

NOTE This time we will be using the DNA binding buffer instead of the ADB buffer since we are not dissolving our sample from a gel slice. See instructions in kit for more information

Setting up a ligation reaction:

1) Set up ligation reactions using PCR tubes:
    a. *See footnote if assembly calculator is not accessible.*

Component	Volume
10X T4 ligase	2 µL
20 fmol vector	Use assembly calculator
60 fmol insert	Use assembly calculator
T4 ligase	1 µL
Water	Fill to 20 uL

2) Let ligation reaction run at room temperature for 15 minutes, then heat inactivate in thermocycler at 65 ºC for 10 minutes.

Setting up a transformation:

1) Thaw your chemically competent *E. coli* cells while your reaction is running.
2) Add 3 µL of your assembly reaction to your chemically competent cells.
3) Let cells continue to incubate on ice for 10 minutes.
4) Heat shock cells in 42 ºC water bath for 30 seconds.

5) Place immediately back on ice for 2 minutes.
6) Add 500 µL of LB to heat shocked cells, then incubate at 37 ºC for 1 hour.
7) Plate onto LB + antibiotic (Concentration and antibiotic will vary depending on what antibiotic resistance genes are in your plasmid).
8) Incubate at 37 ºC for ~10 - 12 hours.

Footnote

DNA calculations can be performed manually using the following equations and constants:

$$Required\ mass\ insert\ (g) = \frac{desired\ insert}{vector\ molar\ ratio} * mass\ of\ vector\ (g) * ratio\ of\ insert\ to\ vector\ lengths$$

$$Moles\ dsDNA = \frac{mass\ of\ dsDNA\ (g)}{molecular\ weight\ of\ dsDNA\ \left(\frac{g}{mol}\right)}$$

$$Average\ molecular\ weight\ of\ a\ base\ pair = 617.96\frac{g}{mol}$$

For 20 fmol DNA:

$$Required\ volume\ of\ DNA\ (\mu L) = \frac{size\ of\ PCR\ product\ (bp) * 0.012359}{concentration\ of\ DNA\ (\frac{ng}{\mu L})}$$

For 60 fmol DNA:

$$Required\ volume\ of\ DNA\ (\mu L) = \frac{size\ of\ PCR\ product\ (bp) * 0.037078}{concentration\ of\ DNA\ (\frac{ng}{\mu L})}$$

SLiCE Cloning

Calculating how much of your DNA to add to the SLiCE assembly reaction:
- Use the DNA Assembly calculator Excel sheet to determine how much of each component you will need for your DNA assembly. If calculator is not available, see footnote in "Classic Restriction Enzyme Cloning" to calculate manually.

SLiCE DNA Assembly:

1) Set up components of your reaction in a PCR tube according to what you calculated with the spreadsheet.
2) Once reaction is set up, incubate samples at 37 ºC for 15 minutes (in a water bath, plate incubator, or thermocycler).

Setting up a transformation:

1) Thaw your chemically competent *E. coli* cells while your reaction is running.
2) Add 3 µL of your assembly reaction to your chemically competent cells.
3) Continue incubating cells on ice for 10 minutes.
4) Heat shock cells in 42 ºC water bath for 30 seconds.
5) Place immediately back on ice for 2 minutes
6) Add 500 µL of LB to heat shocked cells, then incubate at 37 ºC for 1 hour.
7) Plate onto LB + antibiotic (Concentration and antibiotic will vary depending on what antibiotic resistance genes are in your plasmid).
8) Incubate at 37 ºC for ~10 - 12 hours.

Miniprepping Your Sample

Preparing overnight cultures:

1) Pick a single colony from transformation plates and inoculate into 5 mL LB + antibiotic .
2) Incubate in 37 ºC shaker overnight.

Extracting plasmid using Qiagen columns:

1) Spin down overnight culture at 4000 x g for 10 minutes.
2) Pour off supernatant into liquid waste container.
3) Resuspend pellet in 250 µL P1 buffer, then transfer this solution into new, labeled 1.5 mL microcentrifuge tube.
4) Add 250 µL P2 buffer to your sample, then invert tube >5 times to fully mix the solution. **Do not vortex** as this will shear contaminating chromosomal DNA.
5) Add 350 µL N3 buffer to your sample, then invert tube >5 times to fully mix the solution. The lysate should turn from a slimy, viscous consistency to a low viscosity, homogenous suspension of an off-white flocculate.
6) Spin sample in a microcentrifuge 18,000 x g for 10 minutes.
7) Pour supernatant, or transfer using a 1000 µL pipette, into a new Qiagen column.
8) Spin at 18,000 x g for 30 seconds or until all the liquid passes through the column.
9) Discard flow-through in collection tube into your liquid waste.
10) Add 500 µL of PB buffer, then spin at 18,000 x g for 30 seconds.
11) Discard flow-through in collection tube into your liquid waste.
12) Add 750 µL of PE buffer, then spin at 18,000 x g for 30 seconds.
13) Discard flow-through in collection tube into your liquid waste.
14) Spin at 18,000 x g for 30 seconds to remove residual liquid from filter column.
15) Transfer filter column to a new 1.5 mL microcentrifuge tube.
16) Add 50 µL of water to column, then spin at 18,000 x g for 1 minute.
17) Discard filter column from microcentrifuge tube.
18) Seal cap on (plasmid-containing) microcentrifuge tube, then invert or vortex to mix.
19) Measure A260 of sample with Nanodrop to determine DNA concentration.

Supplementary questions:

1. What is a gene?

2. Draw a diagram of the typical "anatomy" of a bacterial gene. (Start codon, operon, etc.)

3. Describe the steps to a typical PCR thermocycler protocol including what temperatures each step is at, how long each step is, and what molecular events happen during each step.

4. List all of the components of a typical PCR reaction (ie. primers, DNA template), define them and describe how they are important to the overall reaction.

5. Draw a hypothetical "classical"/ type I restriction enzyme recognition and cut site.

6. Draw a hypothetical type II restriction enzyme (the type one would use for a Golden Gate reaction) recognition and cut site.

7. What are some advantages of using a Golden Gate cloning strategy versus classical restriction enzyme cloning.

8. Describe the Gibson Assembly cloning strategy (what enzymes are involved, how do you have to design your DNA pieces you wish to assemble). What are some advantages of using Gibson Assembly versus Golden Gate? What are some disadvantages of using Gibson Assembly versus Golden Gate?

9. What makes the SLiCE (Seamless Ligation Cloning Extract) a useful improvement to the Gibson Assembly strategy?

10. Why does our DNA run towards the positive end when performing gel electrophoresis?

11. How do we visualize our DNA bands after running our sample on an agarose gel? What stain do we use and how are we visualizing the stain?

12. We will do the "miniprep" protocol many times throughout this course in order to purify DNA. However, many modern laboratory kits these days have do not describe what the buffers and columns used in the kit are made of/ what they do for the purification process. It is important for us to understand what the different components of commercial kits are to fully understand the experiment we wish to do.
What do you suppose are the components of the P1, P2, and N3 buffers?

What do you think the DNA columns are made out of?

Why does the wash buffer contain ethanol?

13. What wavelength (in nanometers) is the absorbance maximum of DNA?

14. If you notice you have a have low 260/280 ratio (0.77), what is the likely contaminant you have in your sample?

15. What is CRISPR? What is Cas9? What is dCas9 What is a sgRNA?

16. What is CRISPRi? Give an example of a use of it.

17. We were able to use Benchling to help us design guide RNAs targeting our protein of interest. Not only does our guide RNA sequence need to be complementary to the sequence we wish to target, but there is an additional parameter to be considered when using Cas9 to target a specific sequence. What is this additional sequence parameter, what is this sequence known as and what are the nucleotides that comprise this sequence?

18. Your lab is studying a protein in baker's yeast that is believed to be a mitochondrial protein, however, recent data suggests it may reside in the endoplasmic reticulum at certain stages of the cell cycle. Describe a cloning strategy/ constructs you might design for a microscopy experiment that will help to uncover the cellular localization of this protein.

19. You are studying a newly discovered enzyme that has been shown to be capable of degrading a type of plastic known as poly(ethylene terephthalate) (PET). You would like to know which amino acids comprise the active site of the enzyme and are responsible for catalytic activity. Propose a cloning strategy that would allow you to generate mutations on your protein of interest (there may be more than one right answer).

20. In 1976, the city council of Cambridge, Massachusetts, USA (home of Harvard University and MIT) issued a moratorium on the use of recombinant DNA experiments. The technique of molecular cloning was brand new and the citizens of the city became concerned with the potential of recombinant DNA as a health and environmental risk to the community. Initial papers such as this one by Paul Berg's laboratory at Stanford University (http://www.pnas.org/content/69/10/2904.short) led to new questions of safety and ethics for the molecular biology community. At the time, it was unknown whether the virus the Berg lab was studying, SV40 was a causative agent for human tumors, yet the Berg lab sought to test whether they could clone the viral DNA into stable non-viral DNA sequences with the end-goal of transducing these non-viral DNA sequences such as the galactose operon from *E. coli* into mammalian cells.

Even to an educated individual at the time, this must have sounded like a project that had many risky unknowns that could cause potential danger to researchers or the public if proper safety measures were not considered. Many non-scientists were outraged including the Mayor of Cambridge, Alfred Vellucci, who interrogated scientists in a town hall meeting asking:
"Did anyone from this group at any time write to the mayor and the city council to inform us to that you intended to carry out these experiments in the city of Cambridge? You plan to use *E. coli* in your experiments, do I have *E. coli* inside my body right now? Does everyone else in this room have *E. coli* inside there bodies right now? Can you make an absolute 100% certain guarantee that there is no possible risk which might arise from this experimentation? Is there 0 risk of danger? Would recombinant DNA experiments be safer if they were done in a maximum security lab, a P4 lab, in an isolated non-populated area of the country? Would this be safer than using a P3 in one of the most densely populated cities in the nation? Is it true in the history of science that mistakes have been known to happen? Do scientists ever excersice poor judgement? Do they ever have accidents? Do you have enough foresight and wisdom to decide which direction the future of mankind should take? *Audience applauds*"
I highly encourage you to watch the riveting video of the town hall meeting:
https://youtu.be/uFjZQY8dAAI

Obviously, today scientists go about synthesizing recombinant DNA without too many people making a fuss. In the United States, there are now clear and agreed upon guidelines for the use of recombinant DNA in the laboratory: https://osp.od.nih.gov/biotechnology/nih-guidelines/

What are some ethical and safety concerns in the practice of research with recombinant DNA?

Today, there are many parallels to the ethical and safety concerns that surrounded initial research with recombinant DNA and the use of genome editing technologies such as CRISPR. Discuss some ethical and safety concerns you might imagine your friends or family who are not familiar with molecular biology and are hearing about CRISPR in the news. How would you respond to their concerns?

What are your own concerns about the use of CRISPR as a genome editing technology?

Reading/ videos to watch list:
-PCR videos:
https://www.youtube.com/watch?v=X2JuQHspT8w
https://youtu.be/Nl6eLez3CNI
https://youtu.be/mOKb0Pd_Rac
https://youtu.be/VD5qEVTsjTc
https://youtu.be/woYiV0KUUsk
https://youtu.be/DkT6XHWne6E

-Gibson assembly video:
https://youtu.be/KQoKT_4uoZA

-Enzymatic assembly of DNA molecules up to several hundred kilobases. (PMID: 19363495)
- A simple and ultra-low cost homemade seamless ligation cloning extract (SLiCE) as an alternative to a commercially available seamless DNA cloning kit. (PMID: 29124198)
- Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation (PMID: 26670017)

Week 1 Notes and calculations:

Week 1 Notes and calculations (continued):

Week 1 Notes and calculations (continued):

Week 2 Notes and calculations:

Week 2 Notes and calculations (continued):

Week 2 Notes and calculations (continued):

Primers:

	pnmer name	sequence	annealing temp (For Q5/ phusion)	extension time	final extension time	product (bp)	template (resistance)	Cloning Strategy
pMU1-LacZ-IPTG	RJN714-MU1-LacZ-insert-For	cacaccactcgagTTGACAATTAATCATCCGGCTCGTATAATGTG						
	RJN715-MU1-LacZ-insert-Rev	cacaccaactagtAAAAGGCCATCCGTCAGGATGG	69	1:00	3:00	1098	PSav31 (amp)	
	RJN716-MU1-Backbone-For	cacaccaactagtGCGTTTCTACAAACTCTTTTTGTTTATTTTTC						
	RJN717-MU1-Backbone-Rev	cacaccactcgagTCATTTCAGAATATTTGCCAGAACCGTTATGATG	65	2:00	5:00	3655	PSav31 (amp)	XhoI+SpeI
pMU2-GFP-IPTG								
	RJN718-MU2-Insert-For	AGGGGATAACAATTTCACACAAGGAGATATACCatgTCTAAAGGTGAAGAAC						
	RJN719-MU2-Insert-Rev	CTGTTTTATCAGACCGCTTCTtaTTTGTAGAGCTCATCCATGCCG	68	0:30	2:00	770	pRJN49 (amp)	
	RJN720-MU2-Backbone-For	TGGATGAGCTCTACAAAtaaGAAGGGGTCTGATAAAACAGAATTTGCC						
	RJN721-MU2-Backbone-Rev	TAGAcatGGTATATCTCCTTGTGTGAAATTGTTATCCGCTCACAATTCCACACAT	70	2:30	5:00	4086	pSav31 (amp)	
								SLiCE
pMU3-RFP-IPTG								
	RJN722-MU3-Insert-For	ACACAAGGAGATATACCatggtgagcaaggggaggaggataa						
	RJN723-MU3-Insert-Rev	CTGTTTTATCAGACCGCTTCTTAACCGCTctgtacagttcgt	68			754	pFP005 (amp)	
	RJN724-MU3-Backbone-For	aactgtacaagAGCGGTTAAGAAGCGGTCTGATAAAACAGAATTTGCC						
	RJN725-MU3-Backbone-Rev	tctctccgcttctgctcaccatGGTATATCTCCTTGTGTGAAATTGTTATCCG	69			4102	pMU2 (amp)	
pMU4-RFP-GFP-IPTG								
	RJN736-MU4-Insert-For	ACACAAGGAGATATACCatggtgagcaaggggaggaggataa						
	RJN737-MU-Insert-Rev	GTATATCTCCTTGTGTGAAATTAACCGCTctgtacagttcgtcc	69			754	pFP005 (amp)	
	RJN738-MU4-Insert2-For	aactgtacaagAGCGGTTAATTTCACACAAGGAGATATACCatgTCTAAAGGTGA						
	RJN739-MU-Insert2-Rev	CTGTTTTATCAGACCGCTTCtaTTTGTAGAG	67			778	pMU2 (amp)	
	RJN740-MU4-Backbone-For	TGGATGAGCTCTACAAAtaaGAAGGGGTCTGA						
	RJN741-MU4-Backbone-Rev	tctcctctgcccttgctcaccatGGTATATCTCCTTGTGTGAAATTGTTATCCG	69			4102	pMU2 (amp)	
pMU5-dCas9-IPTG								
	RJN742-MU5-Insert-For	TTCACACAAGGAGATATACCATGGATAAGAAATACTCAATAGGCTTAGCTATCG						
	RJN743-MU5-Insert-REV	CTGTTTTATCAGACCGCTTCTTAGTCACCTCCTAGCTCGACTCAAATCAAT	67			4147	pBlo1 (chloramphenicol)	
	RJN744-MU5-backbone-For	GTCAGCTAGGAGGTGACTAAGAAGGGGTCTGATAAAACAGAATTTGCCT						
	RJN745-MU5-backbone-Rev	ATTGAGTATTTCTTATCCATGGTATATCTCCTTGTGTGAAATTGTTATCCG	69			4099	pMU2 (amp)	
pMU6-gRNA-GFP								
	RJN746-MU6-insert-For	tttcgccagaatatcgacgtctggAATTCTAAAGATCTTTTGACACGCTAGCTCAGT						
	RJN747-MU6-insert-Rev	aatatatacccttaggtatataaaAGTTCACCGACAAACAACAGATAAAACG	68			563	pBlo2 (amp)	
	RJN748-MU6-backbone-For	CTGTTTGTTTTGTCGGTGAACTtttatacctaggatatatccgcttcctg						
	RJN749-MU6-backbone-Rev	CAAAGATCTTTAGAATTccagacgtcgatatctggcggaaaat	67			1767	pBlo1 (chloramphenicol)	

The Alliance for Global Health and Science

Recombineering: Making Gene Knockouts in *Mycobacterium smegmatis* Workshop

Katie Lien
PhD Candidate, UC Berkeley

Zoe Netter
UC Berkeley

Welcome to the Alliance for Global Health and Science summer workshop in association with Makerere University and UC Berkeley! Thank you for participating in our Making Genetic Knockouts in *Mycobacterium* workshop led by Katie Lien and Zoe Netter.

Introduction

This workshop is designed to teach students how to create genetic knockouts in *Mycobacterium* species. Genetic knockouts provide an effective way for researchers to study the function and essentiality of genes, and in regards to *Mycobacterium tuberculosis*, may identify new therapeutic targets. However, creating genetic knockouts in *Mycobacterium tuberculosis* is especially challenging due to its slow growth rate, difficulty to culture, poor transformation capacity, and low rate of recombination. To overcome these obstacles, a *Mycobacteria*-specific phage is used to deliver genetic fragments into the bacteria to induce recombination and replace a specific gene with an antibiotic resistance cassette.

To make the phage, students will construct a temperature-sensitive phagemid in a special strain of *E. coli* (the phagemid is a *Mycobacteria*-phage packaging plasmid). Using the Lambda recombination system in *E. coli*, homologous regions flanking the *Mycobacteria* gene of interest and the antibiotic resistance cassette are inserted into the phagemid. The phagemid is harvested from *E. coli* and used to transform *Mycobacterium smegmatis*. Inside of *M. smeg* cultured at 30°C, the phagemid is expressed to create lytic phage that replicates and forms plaques. The phage is then collected and amplified to create high titer phage for transduction of the desired *Mycobacterium* species. Following transduction, the phagemid is targeted to the gene of interest by the flanking sequences, and homologous recombination occurs. In this workshop, students will transduce *Mycobacterium smegmatis* with phage designed to knockout *Lsr2,* a gene important for cell wall maintenance. The resulting mutants have a defect in colony morphology.

Agenda

Day 1: Create linear recombineering substrate

Day 2: Induce expression of recombinase and electroporate *E. coli* to create phagemid

Day 3: Isolate phagemid from *E. coli*, transform *M. smeg* to produce phage

Day 4: Transduce *M. smeg* with high titer phage to create knockout

Day 5: Amplify and titer phage

Procedure:

Cloning:

1. Option 1: Clone the 5' and 3' flanking regions of your target gene of interest using the MCS on either side of the *loxP-hygR-loxP* region of pMSG360. Your homology regions should be 500-1000 base pairs in size.
   Option 2: PCR amplify the 5' flank, 3' flank, and *loxP-hygR-loxP* cassette separately with 20 base pairs of overlap between each fragment. Then combine all three by fusion PCR and clone into pMSG360 using appropriate restriction sites.

2. Following cloning, digest 1 ug of pMSG360 subclone with AflII and DraI:
   Per 50 uL reaction:
   x uL pMSG360
   5 uL 10X NEB CutSmart buffer
   1 uL AflII
   1 uL DraI
   x uL water

3. Digest for one hour at 37° C.

4. Run digest on 1% w/v agarose TAE gel.

5. Gel purify your linear recombineering substrate. Follow instructions included with gel purification kit and elute in 30 uL of ddH_2O. Adjust concentration to 25 ng/uL by dilution or using the speed-vac. Following steps 6-15 are from Qiagen Gel Extraction Kit:

6. Excise DNA fragment with a clean razor blade.

7. Weigh gel slice in microcentrifuge tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 uL). The maximum amount of gel per spin column is 400 mg.

8. Incubate sample at 50° C for 10 minutes (or until the gel slice has entirely dissolved). Vortex the tube every 2-3 minutes to help dissolve gel.

9. Add 1 gel volume isopropanol to the sample and mix.

10. To bind DNA, apply the sample to QIAquick column and centrifuge at 17,900 x g for 1 minute. Discard flow-through and place column in same tube.

11. To wash, add 750 uL buffer PE to column and centrifuge for 1 minute. Discard flow-through and place column in same tube.

12. Centrifuge the QIAquick column in tube for 1 minute to remove residual wash buffer.

13. Place QIAquick column into clean 1.5 mL microcentrifuge tube.
14. Add 30 uL of water to column and let stand for 4 min at room temperature.
15. Centrifuge the column for 1 min to collect sample.

Electroporate EL350/phAE87 to produce phagemid:

1. Streak EL350/phAE87 onto LB + Carbenicillin 100 ug/mL (LB + Carb100) plates and incubate at 30-32° C. Note: This strain is temperature sensitive. For each transformation (including controls), you will need 50 mL of logarithmically growing culture. Adjust the volume of overnight and log phase culture so that you have sufficient bacteria.
2. Pick a single colony of EL350/phAE87 into LB + Carb100 broth. Grow at 30-32° C shaking overnight.
3. The next day, dilute overnight culture 1:100 into 300 mL LB + Carb100 broth in a large flask and incubate at 30-32° C while shaking until the OD_{600nm} reaches 0.5-0.6. This will take anywhere from 3-8 hours depending upon temperature.
4. Pre-chill sterile ddH_2O, electroporation cuvettes, and 50 mL conicals before proceeding.
5. Transfer 150 mL of mid-log phase culture to a sterile flask and heat-shock at 42° C for 20 minutes in a shaking water bath to induce expression of the recombinase. Make sure to reserve 150 mL of the un-induced culture. Note: This is enough cells for 3 control and 3 recombination transformations. Scale up if you need more. While inducing recombinase, the un-induced cultures can remain at room temperature.
6. Cool the induced and un-induced culture by gently shaking the flasks in an ice water bath for 2 minutes.
7. Pour the culture into pre-chilled 50 mL conical tubes and place on ice for 10 minutes.
8. Pellet bacteria at 3,700 x g for 5 minutes at 4° C. Discard supernatant and gently resuspend pellet in 35 mL sterile ice-cold ddH_2O (you can combine 2-3 of the pellets as long as you keep the induced and un-induced cultures separate). Note: When resuspending pellet, only dispense ~2 mL of water initially and pipette water up and down to break up the cell pellet. You will be chasing your pellet around if you dispense the full 35 mL of water, or break up pellet with 10 mL strippette. Washing in water is essential to remove salt from the LB broth that may cause the electroporator to arc.

9. Pellet bacteria by centrifuging at 3,700 x g for 5 min. Discard supernatant and gently resuspend pellet in 35 mL sterile ice-cold water.

10. Pellet bacteria by centrifuging at 3,700 x g for 5 min. Discard supernatant and resuspend final cell pellet in 100 uL ice-cold water for each 50 mL of original culture volume (500X concentration).

11. Add 100 uL of bacteria to pre-chilled 0.2cm electroporation cuvette.

12. Add 500-100 ng of linear recombineering substrate directly to bacteria in cuvette. Note: do not add more than 5 uL of recombineering substrate or you may cause the electroporator to arc.

13. Tap the cuvette to mix and let cuvettes incubate on ice for 5 minutes.

14. Before electroporating, make sure there are no bubbles in the cuvette. Shock the cells using the electroporator at the following settings: 2.5 kV, 25 uF, 200 ohms.

15. Immediately recover the cells by adding 1 mL of LB broth to cuvette. Pipette to mix and then transfer all of the cells to a microcentrifuge tube.

16. Recover the cells by incubating at 30-32° C for one hour while shaking. Note: The un-induced cells look fluffy and clump after the recovery whereas the induced cells are more homogeneous.

17. After recovery, pellet the bacteria by spinning at full speed in microcentrifuge for 30 seconds. Discard supernatant and resuspend pellet in 500 uL of LB broth.

18. Plate 10 uL and 100 uL of induced bacteria on LB + Hygromycin 150 ug/mL (LB + Hyg150) plates. Divide the remainder of the induced bacteria between 3 LB + Hyg150 plates. It is only necessary to plate 100 uL of un-induced control. Note: Plating 10 and 100 uL allows you to gauge the transformation efficiency in case your other plates create a lawn of bacteria.

19. Incubate plates at 30° C for 2 days.

20. After 2 days of incubation, pool Hyg resistant colonies by scraping into 2 mL of LB. Expand bacteria onto 10 fresh LB + Hyg150 plates and incubate at 30° C overnight.

Isolate phagemid:

1. Scrape up lawn of Hyg resistant colonies into ~10 mL of LB broth and isolate phagemid DNA by following Qiagen mini-prep protocol through P1, P2, and N3 steps. Make sure

to adjust the volume according to the density of the harvested bacteria (usually increase volumes by 5X). Following steps 2-6 are from Qiagen Mini-prep Kit

2. Pellet bacteria by centrifugation at 3,500 RPM for 10 minutes at room temperature.
3. Resuspend pelleted bacterial cells in 250 uL (5X= 1.25 mL) Buffer P1 (make sure RNaseA has already been added to buffer P1) and transfer to a microcentrifuge tube.
4. Add 250 uL (5X= 1.25 mL) Buffer P2 and mix thoroughly by inverting the tube 4-6 times. Do not allow the lysis reaction to proceed for more than 5 minutes.
5. Add 350 uL (5X= 1.75 mL) Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
6. Divided sample into microcentrifuge tubes. Centrifuge for 10 minutes at 17,900 x g in microcentrifuge.
7. Collect supernatant and transfer to fresh tube.
8. Add equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) to supernatant to extract phagemid. Invert tube 20 times.
9. Separate aqueous phase (which contains phagemid) by spinning at 18,500 x g for 10 min.
10. Carefully transfer aqueous layer to fresh microcentrifuge tube. Do not disturb the organic bottom layer.
11. Re-extract by adding equal volume Chloroform:Isoamyl alcohol (24:1) to aqueous layer. Invert tubes 20 times.
12. Centrifuge samples at 18,500 x g for 10 min.
13. Carefully transfer aqueous layer to new microcentrifuge tube. Add 0.7x volume isoproponal. Invert tube to precipitate DNA and incubate on ice for 10 minutes.
14. Pellet phagemid DNA by spinning at 18,500 x g for 10 min. Discard supernatant and wash pellet with 70% EtOH.
15. Pellet phagemid DNA by spinning at 18,500 x g for 5 min. Discard supernatant and air-dry pellet.
16. Dissolve each phagemid DNA pellet in 30 uL 10 mM Tris pH 8.0

Transform *M. smegmatis* to produce phage:

1. Pre-chill 0.2 cm electroporation cuvettes.
2. Add 400 uL of electrocompetent *M. smegmatis* mc^2155 to each cuvette.
3. Add 1-5 uL of phagemid directly to bacteria in cuvettes. Tap cuvette to mix and incubate on ice for 5 minutes.
4. Transform *M. smeg* by electroporating at 2.5 kV, 25 uF, 1000 ohms. Immediately add 1 mL of 7H9 (no tween) to cuvette and transfer to microcentrifuge tube.
5. Pellet bacteria by centrifuging at maximum speed for 30 seconds in microcentrifuge.
6. Discard supernatant and resuspend pellet in 200 uL of 7H9 (no tween). Transfer bacteria to 15 mL conical.
7. To bacteria in conical, add 3.5 mL of 56° C top agar. Invert tube three times and immediately pour top agar + bacteria on to room temperature 7H10 plates. Rotate plate to ensure even spread of top agar.
8. Allow top agar to solidify and incubate at 30° C for two days.
9. Pick 2-3 single plaques into 200 uL of MP buffer by plugging the end of a sterile glass Pasteur pipette into the agar in the plaque. An agar plug including the plaque should end up in the pipette. Expel plug into MP buffer and incubate at RT for 2 hours.
10. Optional: If you want to verify that your flanks are present in phage prep, you can use 1-2 uL of it directly in a 25 uL PCR reaction (+DMSO) using primers that span your flanks and the hygR cassette.

Amplify and titer phage:

1. To amplify phage, mix 10 uL of phage/MP solution with 400 uL of M. smeg from overnight culture. Transfer to 15 mL conical.
2. Add 3.5 mL of 56° C top agar to each conical. Invert tube 3 times and immediately pour over room temperature 7H10 plates. Incubate at 30° C for 2 days. You want the plate to look “lacy”
3. Add 3 mL MP buffer to each phage plate and incubate for 2 hours at 4° C while rocking.
4. Collect phage stock from plates and pass through 0.2 um syringe filter. Store phage at 4° C.

5. To titer phage, grow a dense overnight culture of *M. smeg*. Take 200 uL of overnight culture and add to 15 mL conical.
6. Add 3.5 mL of 56° C top agar to bacteria. Invert tube 3 times and immediately pour onto room temperature 7H10 plate.
7. Make serial dilutions of phage in MP buffer and spot 5 uL of each dilution onto the solidified agar surface.
8. Incubate 10^{-4} – 10^{-8} dilutions at 30° C and 10^{0} – 10^{-4} dilutions at 37 C. Note: the latter is to verify temperature sensitivity of the phage. You'll see some lysis at the highest concentrations but there should be obviously less lysis at 37° C. Ideally want 10^{8} PFU/mL.

Transducing Mycobacteria with high titer phage:

1. Add 10 mL of overnight *M. smeg* culture to 15 mL conical.
2. Spin bacteria at 3,500 RPM for 5 min. Discard supernatant and wash with 10 mL MP buffer.
3. Spin bacteria at 3,500 RPM for 5 min. Discard supernatant and wash with 10 mL MP buffer.
4. Spin bacteria at 3,500 RPM for 5 min. Resuspend bacterial pellet with 950 uL MP buffer. Remove 50 uL of sample for use as a negative control.
5. Add 100 uL of high titer phage to remaining 900 uL of culture and mix.
6. Place tubes in shaking incubator at 37° C and allow transduction to incubate for 2 hours.
7. Centrifuge bacteria at 3,500 RPM for 5 min and wash bacteria with 1 mL PBS-T.
8. Centrifuge bacteria at 3,500 RPM for 5 min and resuspend bacteria in 1 mL PBS-T.
9. Plate 100 uL of transduced bacteria on 10 7H10 + Hyg50 plates. Incubate plates overnight at 37° C.

Recipes:

50X TAE Buffer (1 L)

500 mL water
242 g Tris Base
57.1 mL Glacial Acetic Acid
37.2 g EDTA
Water to 1L

Regular 7H9 (500 mL)

Add to bottle:
450 mL water
4 mL 50% glycerol
2.35 g 7H9
Stir bar

Loosely secure cap and cover cap with foil tightly so the cap doesn't fall off during autoclave cycle.

Put bottle of agar in autoclave bin. Add one inch of water to bin and place in autoclave. Autoclave on liquid cycle for 30-45 minutes.

Remove bottle from autoclave and cool by stirring on stir plate for 40 min.

Sterilely add 50 mL OADC
Sterilely add 1.25 mL sterile 20% Tween-80

Modified 7H9 (100 mL) only for phage preparation:

Add to bottle:
90 mL water
800 uL 50% glycerol
0.47g 7H9
Stir bar

Loosely secure cap and cover cap with foil tightly so the cap doesn't fall off during autoclave cycle.

Put bottle of agar in autoclave bin. Add one inch of water to bin and place in autoclave. Autoclave on liquid cycle for 30-45 minutes.

Remove bottle from autoclave and cool by stirring on stir plate for 40 min.

Sterilely add 10 mL ADC (similar to OADC but without oleic acid).

MP Buffer:

50 mM Tris HCl, pH 7.5
150 mM NaCl
10 mM $MgCl_2$
2 mM $CaCl_2$

Top Agar:

2 mM $CaCl_2$
0.6% w/v agarose in ddH_2O

Microwave to melt agar. Once melted, cool to 56° C before using

7H10 plates (1 L)

Add to bottle:
900 mL water
19 g 7H10 agar
10 mL 50% Glycerol
Stir bar

Loosely secure cap and cover cap with foil tightly so the cap doesn't fall off during autoclave cycle.

Put bottle of agar in autoclave bin. Add one inch of water to bin and place in autoclave. Autoclave on liquid cycle for 30-45 minutes.

Remove bottle from autoclave and cool by stirring on stir plate for 40 min.

Using sterile technique (by flame) add 100 mL of OADC. If using antibiotics, add now. Mix briefly and pour plates by flame or in biosafety cabinet.

Making Electrocompotent *Mycobacteria*

Protocol for electrocompotent cell prep of *M. smegmatis, M. marinum,* and *M.tuberculosis.*

M. smeg & *M. mar* preps should be conducted in the cold room if possible: pre-chill serological pipets, tubes, and other consumables, keep glycerol on ice, and do all spins at 4 degrees.

M. tb preps can be conducted at room temperature with room temp/warm glycerol.

Protocol details 50 mL culture scale, which yields ~10 x 200ul aliquots. Scale up or down based on needs

Reagents:
7H9 media
10% glycerol, sterile-filtered

Procedure:

1. Start a 3 mL bug culture in 7H9 and grow to saturation at correct temperature (37° C x 2 days for *M. smeg*, 32° C x ~3 days for *M. mar*. For *M. tb* a 3 mL starter culture is unnecessary and you can start a five day 50 mL culture)
2. Use the 3 mL starter culture to inoculate a 50 mL culture at OD600 = ~0.02 in a 250 mL culture flask and incubate at the correct temperature
3. When OD600 reaches 0.8-1.0 (next day for *M. smeg*, check every day for *M. mar*), bugs are ready to prep:
   Transfer culture to 50 mL conicals
   For *M. smeg* + *M. mar,* incubate cultures on ice 30 min-2hr and conduct all subsequent steps in the cold room with ice-cold glycerol.
4. Spin down culture(s) 3500 RPM for 5 min to pellet bugs and discard the supernatant.
5. Resuspend pellet in ½ volume (25 mL) 10% glycerol and spin 3500 RPM x 5 min, discard the supernatant.
6. Resuspend pellet in ¼ volume (12.5 mL) 10% glycerol and spin 3500 rpm x 5 min, discard the supernatant.
7. Resuspend pellet in ⅛ volume (6.25 mL) 10% glycerol and spin 3500 rpm x 5 min, discard the supernatant.
8. Resuspend pellet in 3 mL 10% glycerol
9. Use immediately or aliqout into microcentrifuge tubes
10. Snap-freeze aliquots in LN2 or 100% ethanol + dry ice, then store at -80.

The Alliance for Global Health and Science

Tissue Culture and Intracellular Bacteria Growth Curves Workshop

Brittney Nguyen
PhD Candidate in Plant & Microbiology, UC Berkeley

Welcome to the Alliance for Global Health and Science summer workshop in association with Makerere University and UC Berkeley! Thank you for participating in our Tissue Culture with Bacterial Growth Curve workshop led by Brittney Nguyen.

Introduction:

Tissue culture is a technique in which cells from an animal are harvested and grown in an artificial environment, allowing for scientists to conduct direct studies on many different cell types. Tissue culture techniques allow us to work with primary cells (cells harvested directly from an animal that have a limited life after harvest) and cell lines (cells that have been immortalized, so can be continuously grown *in vitro*). Tissue culture is very useful for studying the pathogenesis and immunology of infectious diseases, because it allows scientists to cultivate intracellular pathogens, observe the growth of those pathogens, and study the effects of those pathogens on the cell. Furthermore, by using different cell types, we can study the effects of cells on pathogens.

Tissue culture is an extremely powerful tool because we can learn through direct observation (visual signs of cell health, changes in cell appearance and behavior over time--live-cell microscopy) and indirect observation (changes in gene transcription, translation, etc.).

Bacterial growth curves can provide scientists with much information about a bacterial strain or species. They can help define rates of nutrient consumption, the different environments that favor the bacterium, and differences in virulence. By determining the growth rates of different strains of bacteria using tissue culture, one can infer the virulence of that specific strain or species.

This workshop is designed to teach the basics of tissue culture along with a bacterial growth curve. We will be using the established cell line of mouse derived J774 macrophages, which we will be infecting with the bacterium *Listeria monocytogenes*. You will be working with different strains of *Listeria*, which will have varying degrees of pathogenicity.

Vocabulary

Confluent/Confluency - The percentage of the flask that is covered in cells

Split or Passage – To reduce the number of cells in a flask and give them fresh media, this often needs to be done every few days to keep cells from becoming overgrown

Seed - To take cells from one source and transfer them to another (ex. For this experiment, we will take cells from a T75 and seed them into a 60mm dish)

T25, T75, T225, etc. - The usable surface area of a flask, in cm2. This is essentially the size of the flask

Recover – Thaw frozen cells and seed them into a flask to grow

Workshop Overview	
Day 1	Intro to Tissue Culture, Passaging cells
Day 2	Growth Curve Overview, Flaming coverslips
Day 3	Seeding cells, Starting bacterial cultures
Day 4	Growth Curve Experiment
Day 5	Counting plates and Wrap-up

Recipe for J774 Cell Culture Media	For 500 mL	For 1 Liter
10% Fetal Bovine Serum (**FBS**) 1% L-glutamine 1% Sodium-pyruvate 88% Dulbecco's Modified Eagle Medium (**DMEM**)	50 mL FBS 5 mL L-glutamine 5 mL Sodium Pyruvate 440 mL DMEM	100 mL FBS 10 mL L-glutamine 10 mL Sodium Pyruvate 880 mL DMEM

Day -2 – Recovering frozen cells

*We will begin this workshop with a confluent flask of J774 cells. However, cells are often received frozen in a cryovial. The following (Day -2) protocol is for seeding a flask with cells from a cryovial.

Materials	Equipment
(1) vial of frozen cells	Refrigerated Centrifuge
(1) T75 flask	37 degree C water bath
(29) mL of cell culture media	Biosafety cabinet
(1) 5mL stripette	500mL – 1L glass or plastic container for liquid cell waste
(3) 10mL stripettes	37 degree C, 5% CO2 incubator
(1) 25mL stripette	
(1) 15mL conical tube	
70% Ethanol in spray bottle	

*Prior to starting, warm cell culture media in 37 degree C water bath

Steps 2-3, and 5-8 should be performed in a biosafety cabinet. All materials should be sprayed with 70% Ethanol before entering biosafety cabinet.

1) Thaw cell cryovial quickly in 37 degree water bath (1-2 min).

2) Using a 1mL stripette, transfer 1mL of cells from cryovial to 15mL conical tube.

3) Using a 10mL stripette, add 9mL of cell culture media to conical tube with cells.

4) Centrifuge cells for 8 min at 300 rpm at 4 degrees C. Cells should have formed a pellet at the bottom of the tube.

5) Use a 10mL pipette to remove the liquid, leaving the cells undisturbed. Discard liquid waste in waste container. It is important to remove the media that the cells were frozen in because cells are frozen in 10% DMSO. The DMSO helps prevent the formation of ice crystals during the freezing process, which prevents the cells from lysing. However, DMSO is not healthy for cells that are not frozen.

6) Using a 25mL stripette, add 16mL of fresh cell culture media to a new T75 flask

7) Using a 10mL stripette, add 4mL of fresh cell culture media to the tube containing the cell pellet. Resuspend the cells by pipetting up and down quickly. Break up clumps of cells by expelling the cells against the side of the tube. Do this until there are no visible clumps of cells.

8) Transfer the 4mLs of cells into the T75 flask with the 16mL of cell culture media.

9) Incubate the flask at 37 degrees C in a 5% CO2 incubator.

Day 1 – Passaging Cells

Today, we will "split" or "passage" the cells from one T75 flask into a new T75 flask. For J774 cells, which double about once every 24 hours, if we "seed" the new flask with 25% of the cells the flask should be 100% "confluent" in 2 days. The ratio of cells that you seed into a new flask and the frequency with which you split will vary depending on the cell type. Because these are adherent cells, we will use 0.05% Trypsin-EDTA to remove the cells from the flask surface.

Materials	Equipment
(1) T75 that is 80-100% confluent	
(1) T75 flask	37 degree C water bath
(25.5) mL of cell culture media	Biosafety cabinet
(1) 5mL stripette	500mL – 1L glass or plastic container for liquid cell waste
(1) 10mL stripette	37 degree C, 5% CO2 incubator
(4) 25mL stripette	
20mL Phosphate-Buffered Saline (**PBS**)	
70% Ethanol in spray bottle	
2mL 0.05% Trypsin-EDTA	

The following steps should be performed in a biosafety cabinet. Cell culture media and PBS should be warmed to 37 degrees C before starting.

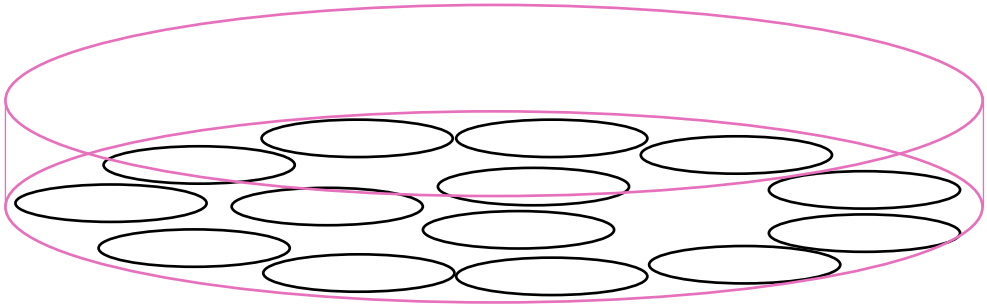
1) Using a 25mL stripette, remove the media from the flask. Discard the media in waste container.
2) Using a 25mL stripette, add 20mL of pre-warmed PBS. Rotate the flask so that the PBS covers the cells, and rock the flask back and forth to wash the cells.
3) Using a 25mL stripette, remove the PBS. Discard the PBS in the waste container.
4) Using a 5mL stripette, add 2mL trypsin. Again, rock the flask to cover the cells in the trypsin.
5) Place the flask in a 37 degree C incubator.* This step is optional, but Trypsin is more active at 37 degrees than at room temperature. **After approximately 5 minutes, and no longer than 10 minutes after addition of the trypsin, check the cells to see if they have detached. Extended exposure to Trypsin can cause the cells to die.** At this point, most adherent cells will have completely detached from the flask. J774s are extremely adherent, so you will need to bang the flask against a surface. I recommend using a padded chair.
6) When the cells have completely detached, use a 10mL stripette to add 8 mL of cell culture media to the flask and rock the flask to make sure all the cells come in contact with media. The FBS in serum inactivates trypsin.
7) Use the 10mL stripette to break up clumps of cells by expelling them quickly against the side of the flask repeatedly
8) Use the 10mL stripette to transfer ¼ of the cells (2.5mL) into a new T75 flask.
9) Use a 25mL stripette to add 17.5mL pre-warmed cell culture media to the flask with the cells.

Day 2 – Growth Curve Overview, Preparing Coverslips

The goal of the intracellular growth curve is to determine the number of bacteria within a cell population over a period of time. To control for possible variation in the number of cells measured and other factors, we will infect a large population of cells and take samples at different times.

For each strain of bacteria, we will need the following: one 60mm dish with (14) 12mm glass coverslips. To perform the growth curve, we will seed cells into the 60mm dish and infect the dish with one strain of bacteria. This method helps to ensure that each coverslip will have a similar number of cells and receives the same infection dose. At each time point, we will take and measure three coverslips from the dish. Thus, we are able to sample the infection with technical triplicates.

Materials	Equipment
(2) 60mm dishes (28) glass coverslips (5) mL of cell culture media (2) 5mL stripette 70% Ethanol in spray bottle	Biosafety cabinet 37 degree C, 5% CO2 incubator



Today, we will prepare the coverslips. It is important that we sterilize them before putting them into the dish. There are two methods to do this: (1) dip them in 90% ethanol and run them through a flame. (2) Autoclave. We have found that flaming increases the ability of cells to stick to the coverslips. However, you should determine which method is most convenient for your lab and will work best for your cells.

Each person should prepare one dish. Do this in the biosafety cabinet.

1) Place 14 sterilized glass coverslips into a 60mm dish.
2) Add 5mL of cell culture dish, and swirl so that all of the coverslips are covered in media.
3) Using a sterile object (forceps, stripette), push the coverslips down so that they are submerged in media and arrange them so that no coverslips are overlapping. Once they are arranged, tap firmly on each coverslip two or three times to get out as much of the air that is trapped underneath as is possible.
4) Place the dishes in the 37 degree C, 5% CO2 incubator to “degas” overnight. This step is necessary because the coverslips tend to float after some time, and we do not want them to float once we add the cells. If we let them “degas” overnight, then the next morning we can push down any floating coverslips before we seed the cells. Once pushed down they do not tend to float again.

Day 3 – Seeding cells, Preparing for Growth Curve

The cells need to be seeded the night before performing the experiment. This allows them to adhere to the coverslips and equilibrate to their new environment. Before seeding the cells, we will need to count them to make sure that we seed the correct number of cells.

Materials	Equipment
(1) T75 that is 80-100% confluent (2) 60mm dishes with coverslips (1) 15mL conical tube (~30) mL of cell culture media (5) 5mL stripette (1) 10mL stripette (3) 25mL stripette p20 Pipette and tips 70% Ethanol in spray bottle 15ul Trypan Blue 1.5mL Eppendorf tube or 96 well plate 20mL Phosphate-Buffered Saline (**PBS**) 2mL 0.05% Trypsin-EDTA Hemacytometer	37 degree C water bath Biosafety cabinet 500mL – 1L glass or plastic container for liquid cell waste 37 degree C, 5% CO_2 incubator

The following steps should be performed in a biosafety cabinet. Cell culture media should be warmed to 37 degrees C before starting.

1) Using a 25mL stripette, remove the media from the flask. Discard the media in waste container.
2) Using a 25mL stripette, add 20mL of pre-warmed PBS. Rotate the flask so that the PBS covers the cells, and rock the flask back and forth to wash the cells.
3) Using a 25mL stripette, remove the PBS. Discard the PBS in the waste container.
4) Using a 5mL stripette, add 2mL trypsin. Again, rock the flask to cover the cells in the trypsin.
5) Place the flask in a 37 degree C incubator for 5 minutes. Then, bang the flask to detach cells.
6) When the cells have completely detached, use a 10mL stripette to add 8 mL of cell culture media to the flask and rock the flask to make sure all the cells come in contact with media. The FBS in serum inactivates trypsin.
7) Use the 10mL stripette to break up clumps of cells by expelling them quickly against the side of the flask repeatedly.
8) Transfer the cells to a 15mL conical tube

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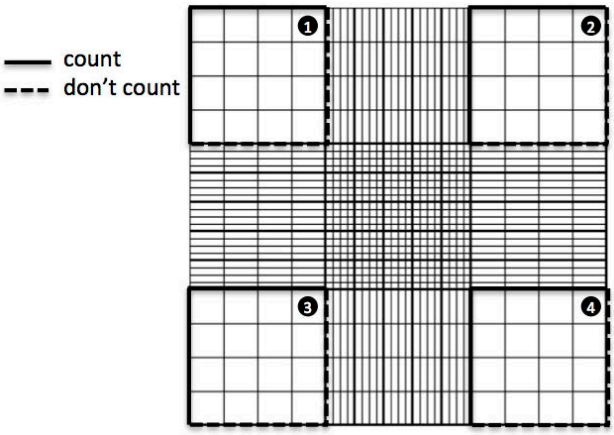
Day 3 continued - Counting Cells

We will take a tiny sample of the cells and stain it with trypan blue. When we look at the cells under the microscope, live cells will look white/clear and dead cells will be dark blue. We will count the cells using a hemacytometer.

1) Use a p20 with a sterile tip to remove 5uL of cells. Transfer the cells to a tube or 96 well plate.
2) Add 15uL of trypan blue and mix well. Then load 10ul into the hemacytometer.
3) Using a microscope, count the cells in the boxes labeled 1-4. The volume of cells in each of these boxes is $1/10^4$ mL.



To calculate the number of cells you have, use the following formula:

	x	1/4	x	4	x	10^4	=			
Cells counted	x	Dilution Factor	x	# of Boxes Counted	x	10^4	=	Cells/mL.		

Because you have 10mLs of cells, you can multiply your cells/mL by 10 to determine total cells. For this experiment we want to have 3x 10^6 cells/dish at the time of infection. The J774 cells will double overnight, so today we want to add 1.5 x 10^6 cells to each dish in a total volume of 5mL. Sometimes your cells will be to dilute, such that you need to add more than 5mL to a dish to get 1.5 x 10^6 cells. In this case, you can centrifuge your cells and resuspend them in a smaller volume.

	+		=	5
Number of mLs the contain 1.5 x 106 cells	+	Cell culture media	=	5mL / 60mm dish

4) Use a 5mL stripette to add 1.5 x 10^6 cells to a 60mm dish
5) Use a 5mL stripette to add cell culture media to the dish, to a total volume of 5mL
6) Incubate the cells overnight at 37 degrees C, 5% CO2.

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Day 3 continued – Preparing for growth curve

Materials	Equipment
(24) BHI + Strep Agar Plates (24) 15 mL conical tubes (120) mL autoclaved water (10) mL BHI + Strep (2) 25 mL stripettes (2) 10mL stripettes Autoclaved glass beads (2) 14mL culture tubes (2) Listeria strains Marker Sterile loop, stick, or pipette	30 degree C incubator

Each group will receive two strains so that each person in a group has their own strain. The strains are wildtype Listeria, ΔactA Listeria, and Δhly Listeria. Each person should do the following for the strain they are assigned.

You assigned strain is: _____

1) Label your 12 agar plates. For each time point (0.5 hpi, 2 hpi, 5 hpi, and 8 hpi) you will need 3 plates. Also write down the strain on each plate.
2) Pour ~25 glass beads onto each plate. Now set these aside because they are ready for tomorrow.
3) Fill 12 15mL conical tubes with 5mLs of autoclaved water.
4) Label each tube as you labeled the agar plates, with the time point and strain. These can now be set aside.
5) Add 5mL of BHI + Streptomycin to a 14 mL culture tube. Label your tube with your strain.
6) Using a sterile loop, stick, or pipette, take one colony of you strain from the agar plate and inoculate the 5mLs of BHI.
7) Incubate your tube of bacteria overnight at 30 degrees C.

Day 4 – Growth Curve Experiment

Today we will perform the growth curve experiment! Prior to doing anything, it's always a good idea to check your cells for contamination. The media should be clear and the same pink color that it was when you made it. If it is orange, yellow, or cloudy, that is a clear sign of contamination.

Materials	Equipment
(2) 5mL overnight cultures of Listeria	37 degree C incubator
(2) Dishes of seeded cells	37 degree C water bath
(10) mL PBS	Biosafety cabinet
(10) mL cell culture media	500mL – 1L glass or plastic container for liquid cell waste
(50) ul gentamicin (10 mg/mL)	37 degree C, 5% CO2 incubator
(6) 5mL stripettes	Vortex
p200 pipette and tips	
(24) 15mL conicals with 5mL water	
(24) BHI + Strep Agar plates with beads	
Forceps	
Diffquik stains	
Permount	

1) Dilute your overnight of bacteria 1:9 with PBS, in a total volume of 1mL in a 1.5mL Eppendorf tube.
2) Using a p20, add 5uL of the diluted bacteria to your 60mm dish with cells. Move the dish in a cross pattern to distribute the bacteria. Swirling the dish will concentrate the bacteria in the middle of the dish, so it should be avoided.

At 0.5 hours post infection:

3) Using a 5mL stripette, remove the 5mL of media containing bacteria and discard.
4) Using a 5mL stripette, add 5mL of PBS to wash the cells. Then remove and discard the PBS.
5) Using a 5mL stripette, add 5mL pre-warmed cell culture media to the cells.
6) Using a p200, Add 25ul gentamicin to each dish.

At each time point (0.5 hpi, 2 hpi, 5 hpi, 8 hpi):

7) Remove 3 coverslips and put each coverslip into a separate 15mL conical with 5mL water. Return the cells to the incubator.
8) Vortex the tubes for 15 seconds.
9) Use a p200 to plate the bacteria in water from the tubes on BHI + Strep plates. Refer to the following table for the amount of water to plate. Incubate plates at 37 degrees C overnight.

Strain	0.5 hpi	2 hpi	5 hpi	8 hpi
WT	50 ul	50 ul	5 ul*	5 ul*
ΔactA	50 ul	50 ul	5 ul*	5 ul*
Δhly	50 ul	50 ul	50 ul	50 ul

*To plate 5 ul, first add 50ul of PBS to the agar plate before adding 5ul of the water containing the bacteria. This helps the beads fully spread the bacteria around the plate.

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It can be helpful to visualize the infection. To do this, we can take coverslips and fix and stain the cells. We use a stain called diff-quik.

At 5 hpi and 8 hpi, take one coverslip for staining.

1) Dip the coverslip 15 times in each of the three solutions (1) methanol to fix, (2) Orange stain to stain bacteria, (3) purple stain to stain cells.
2) Rinse the coverslip by dipping in three cups of water.
3) Let the coverslip dry on a paper towel.
4) Mount the coverslip on a slide using a drop of permount.

Day 5 – Counting Colonies

It takes about 24 hours for Listeria to form colonies that are an easy size to count. To count them, use a pen to mark counted colonies on the lid of the agar plate as you go. I find it is easiest to count in tens. Fill out the following table and then graph your results. Congratulations on finishing the Tissue Culture and Intracellular Growth Curves workshop!

	Plate 1	Plate 2	Plate 3	Average	Dilution Plated	Total colonies/coverslip
0.5 hpi						
2 hpi						
5 hpi						
8 hpi