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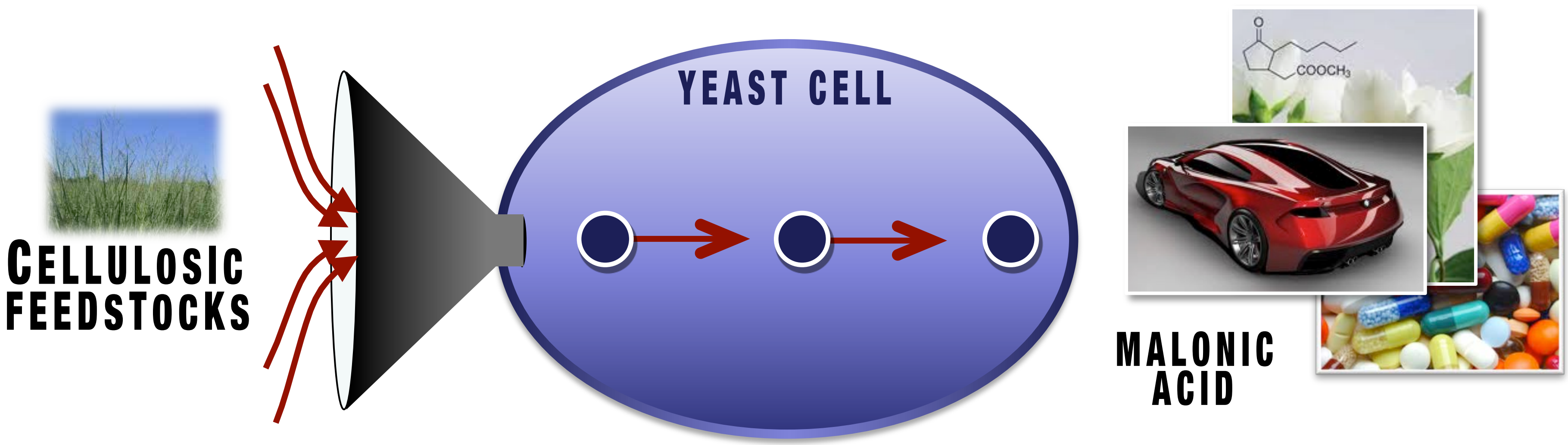
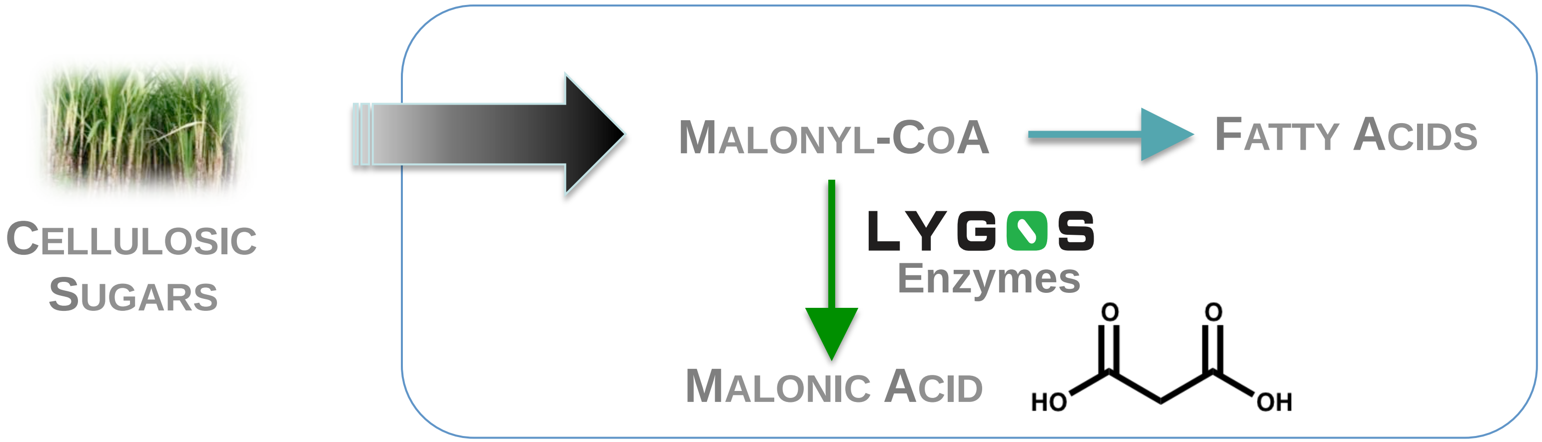
Design & Optimization of a Biochemical Production Platform with Biosensor-Guided Synthetic Evolution



March 9, 2017
DOE BETO Conversion Review

Eric J. Steen, Ph.D.
Lygos, Inc.

Malonic Acid Is An Ideal Biological Product



- **Malonyl-CoA lies on the carbon superhighway in biology**
  - Pathway is compatible with all available, low-cost feedstocks
  - Malonyl-CoA is basis of fatty acid production (90%+ yields reported)
- **Malonic acid is an ideal molecule to produce biologically**
  - Theoretical Yield (g/g-glucose): 1.73
  - $2/3\ C_6H_{12}O_6 + 2\ CO_2 \rightarrow 2\ C_3H_4O_4$

Goal Statement

Develop an integrated approach to biochemical pathway optimization for production of malonic acid & demonstrate path toward commercially-relevant fermentation metrics

Relevance to US & BETO mission

- **Reduce dependence on foreign oil used for malonic acid**
  - 130 million pounds produced in foreign countries
- **Advance the production of fuels & chemicals from lignocellulosic feedstocks; decreases price of biofuels**
- **Decrease greenhouse gas (GHG) emissions from malonic**
  - 100 million pounds of CO_2 sequestered
  - Eliminate 34 million pounds of sodium cyanide use
- **Major market utility in Advanced Manufacturing**
- **The Department of Energy identified malonic acid as one of the top 30 chemicals to produce from biomass[1]**

[1] "Top Value Added Chemicals From Biomass, Volume 1." US DOE (2004)

Quad Chart Overview

Timeline

- **Start**: Aug 31, 2013
- **Original End**: Apr. 1, 2016
- **End**: October 31, 2016
- Project is 100% complete

Barriers

- Ct-H: Cost effective production of bioproducts
- Fermentation productivity, yield
- Reduce cycle time for biocatalyst development
- Ct-A: feedstock variability

Budget, $2.06MM

	FY 13 Costs	FY 14 Costs	FY 15 Costs	FY 16 Costs
DOE Funded	$310K	$561K	$738K	$279K
Lygos Cost Share	$34K	$62K	$82K	$31K

Partners

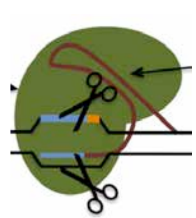
- Cellulosic sugars
- ABPDU: fermentation scaling

Project Overview & Technical Approach

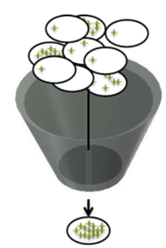
Overarching Goal: Develop an integrated approach to biochemical pathway optimization for production of malonic acid

- **Objective #1: Genome-scale metabolic node perturbation**
  - *In silico* biocatalyst design & management software
  - Multiplexed biocatalyst engineering, diversity creation, & targeting tools
- **Objective #2: Deploy biosensor screening method to enable faster identification of improved biocatalysts**
  - Biocatalyst evaluation via biosensor screen
- **Objective #3: Use statistical approach to guide biocatalyst design**
- **Objective #4: Translate benchtop fermentation to pilot scale**

***in silico* design**



biocatalyst construction tools



high throughput screening

scale up

Project Approach - Management

- Lygos was responsible for biocatalyst design, screening, and fermentation process development
- Leveraging expertise of partners
  - Commercial providers of lignocellulosic feedstocks (cellulosic sugars)
  - Fermentation scale-up at DOE-funded Advanced Biofuels Process Demonstration Unit (ABPDU)
- Materials & facility scheduling are planned in advance
- Go/No-Go decision points support objectives
  - Strain engineering capacity (genotype generation)
  - Screening capacity (phenotype confirmation)
  - Yield, titer, and productivity fermentation metrics
- Final validation performed at ABPDU with cellulosic sugars obtained from a commercial producer (i.e., real-world feedstock) at 50-liter scale

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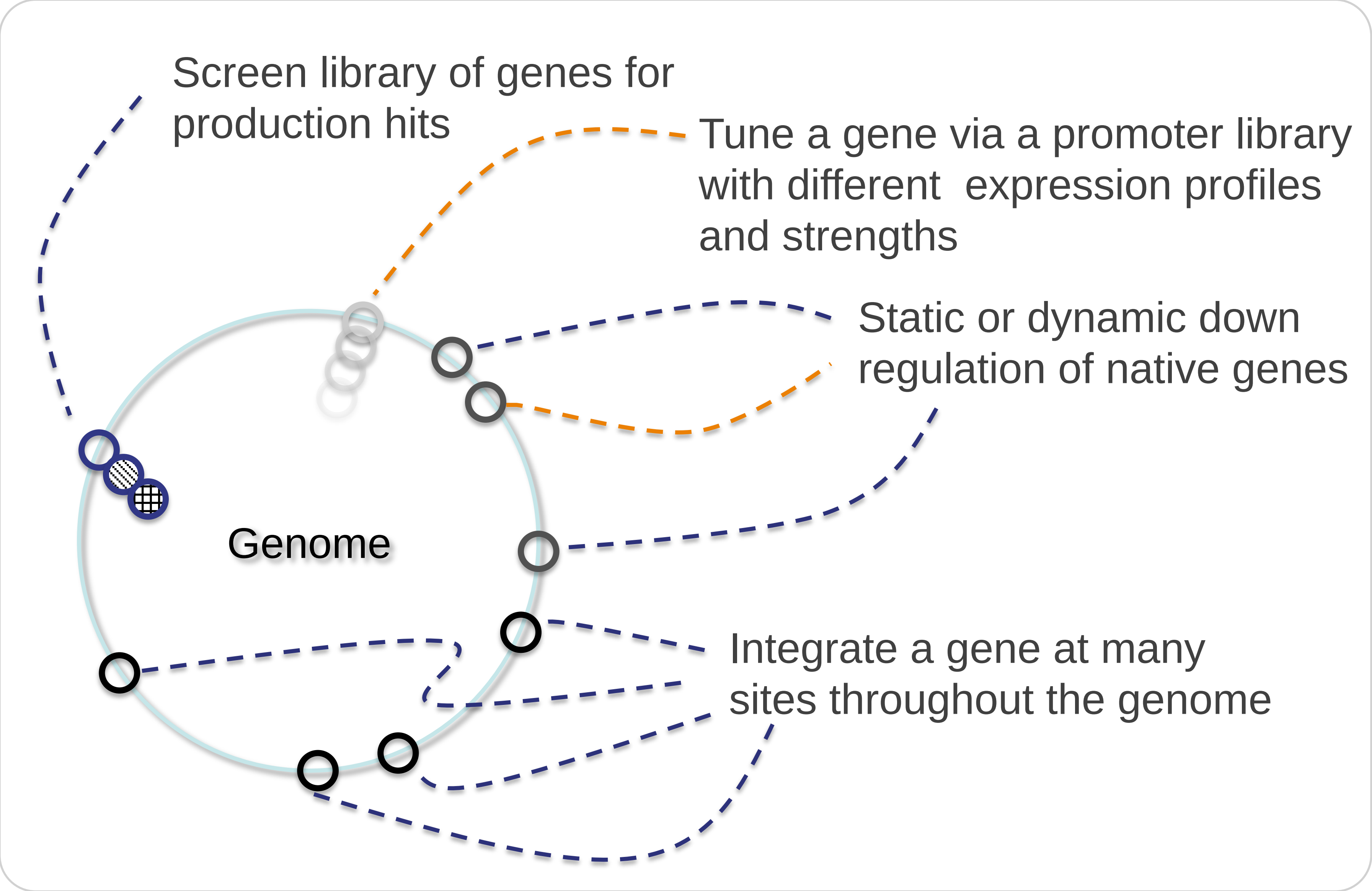
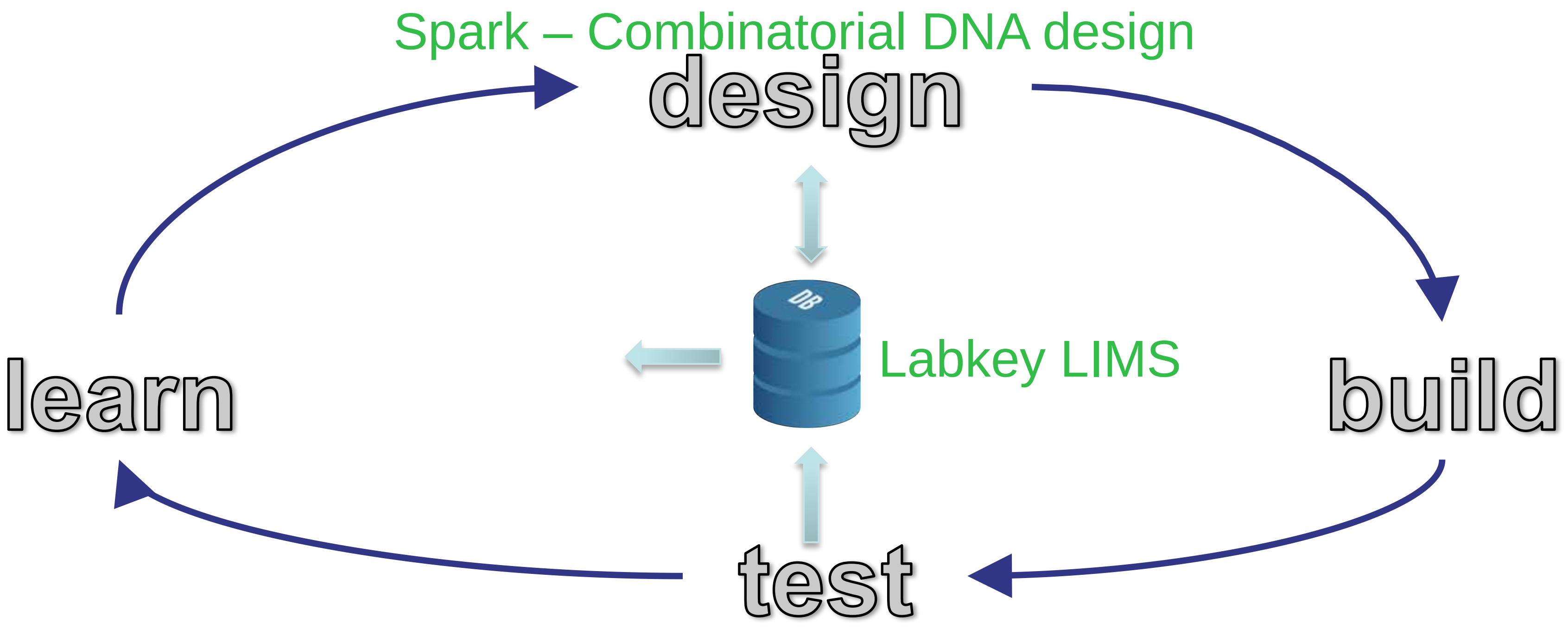
Task A: Software tools for designing & constructing biocatalysts



Task A: Lygos' software design and LIMS tools



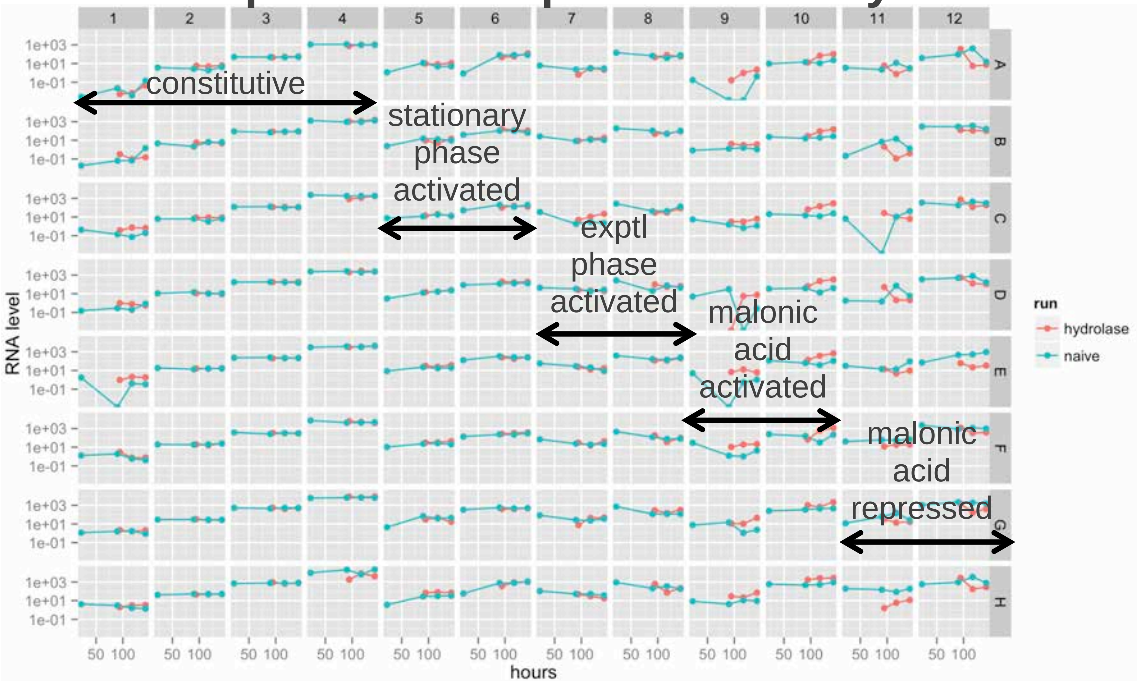
Spark proved critical to successful high-throughput strain engineering approach

- Automated design of heterologous DNA constructs enabling large-scale assays (design time reduced from weeks to hours)
- Demonstrated at 2100 constructs/person scale (Milestone completed)

Laboratory Information Management System (LIMS)

Task A: Lygos' software design and LIMS tools

Representative promoter study



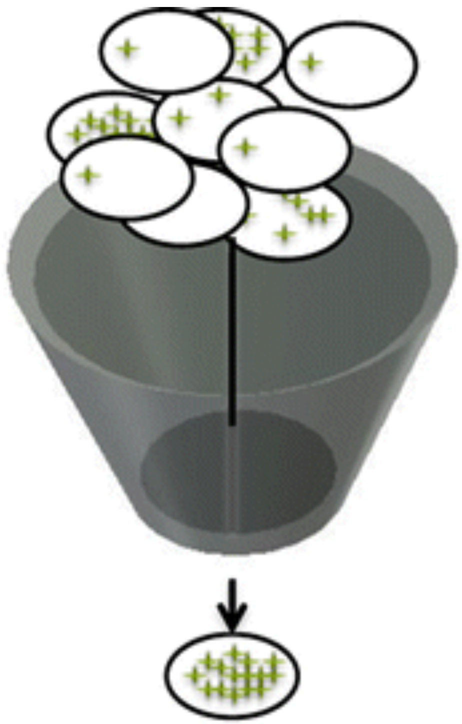
LIMS was critical to a successful data management plan

- Organized storage/analysis of data on thousands of strains, hundreds of assays
- Customized platform based on Labkey software (readily available)
- Expanded/revised project goals to include data analysis and visualization modules

Laboratory Information Management System (LIMS)

Task B: Biosensor-based screening

Genetic diversity is created which results in individual biocatalysts that produce different levels of malonic acid - but we don't know which ones.



Large, diverse population

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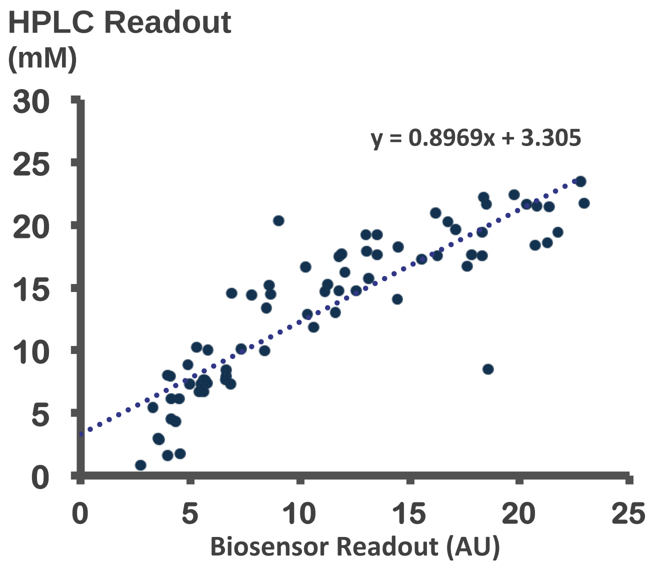
Identify improved production strain(s)

Biosensor screens can offer the most cost effective and direct measurement.

Dietrich JA et al. ACS Synthetic Biology 2013

Task B: Biosensor-based screening



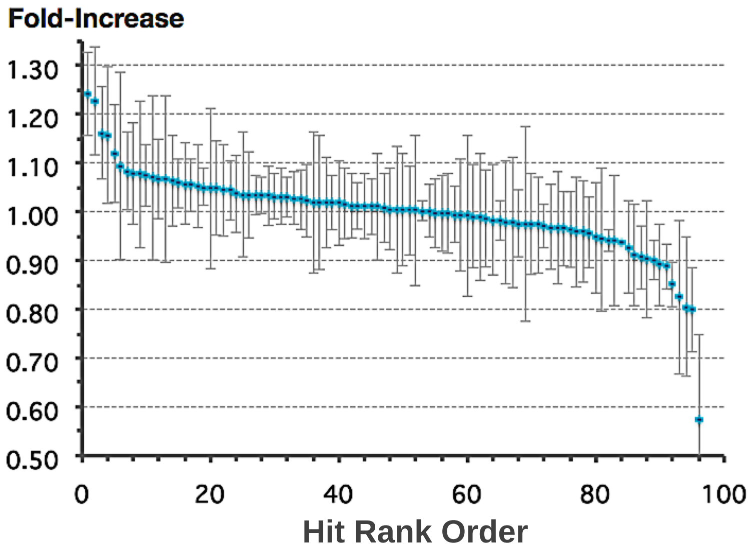
Biosensor output was well-correlated with chromatography output (gold-standard detection) within a range of concentrations well-suited for early optimization efforts

Lesson learned: 6 replicates were required to ensure no "hits" were missed

Biosensor was applied across multiple assays:

- **Transcription factor overexpression screening in 96-well plate**
- **2100 single gene overexpression assay**

Lesson learned: Labor requirements for running the screen were higher than estimated, decreasing value relative to HPLC. Data content lower.



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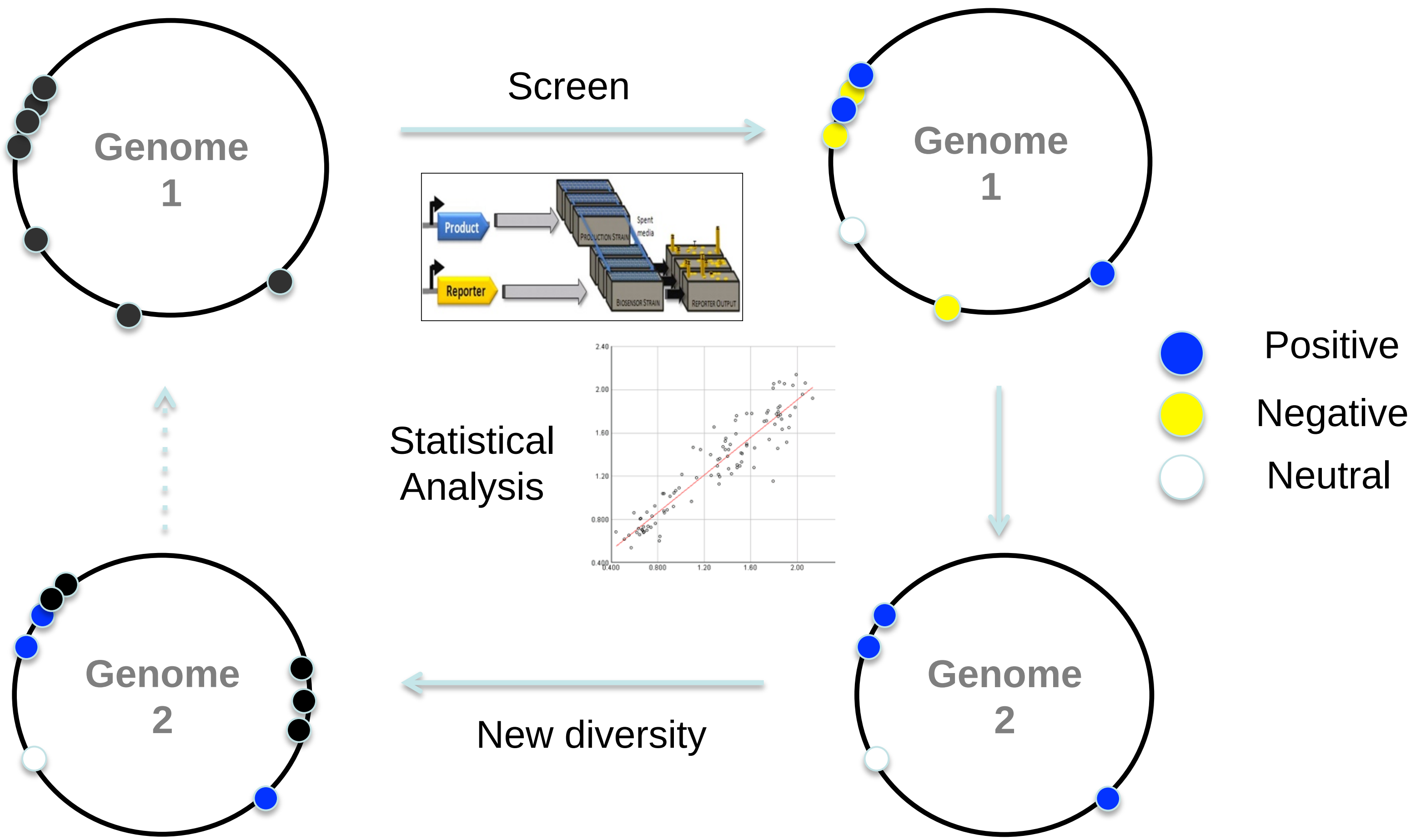
Task C: Metabolic hotspot ranking (original task objective)

Metabolic hotspots are ID'd & ranked via statistical analysis in order to more efficiently identify subsequent engineering targets



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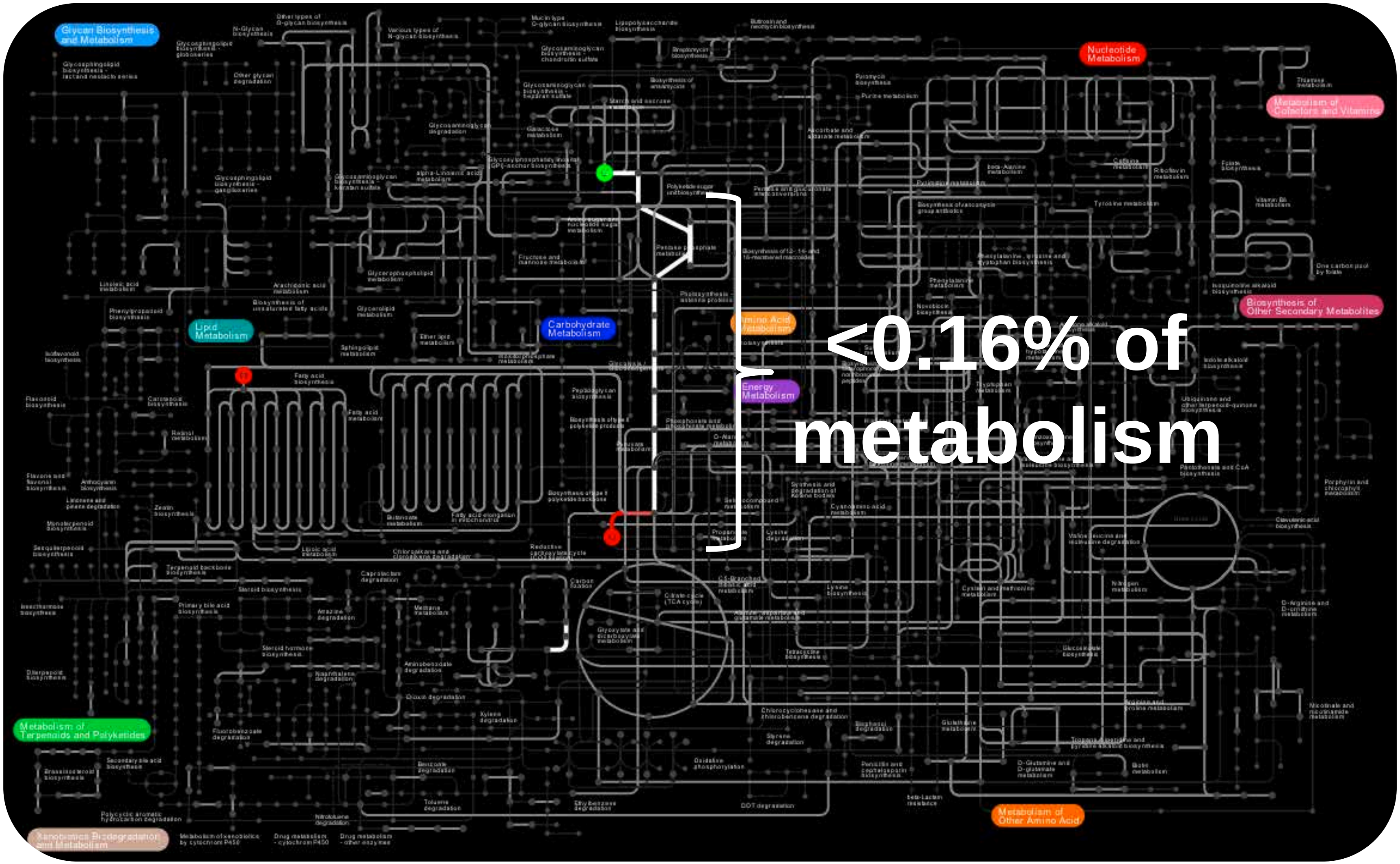

Photo credit: Arkin et al. http://glamm.lbl.gov/

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Task C: Metabolic hotspot ranking (original task objective)

Hypothesized improvements in malonic acid production would be obtained through combinatorial modification of metabolic nodes distant from central metabolism

Lesson learned: genes encoding central metabolism enzymes were more strongly correlated to pathway flux throughout the project

Further optimization as theoretical Y, T, P are approached require exploration of additional nodes.

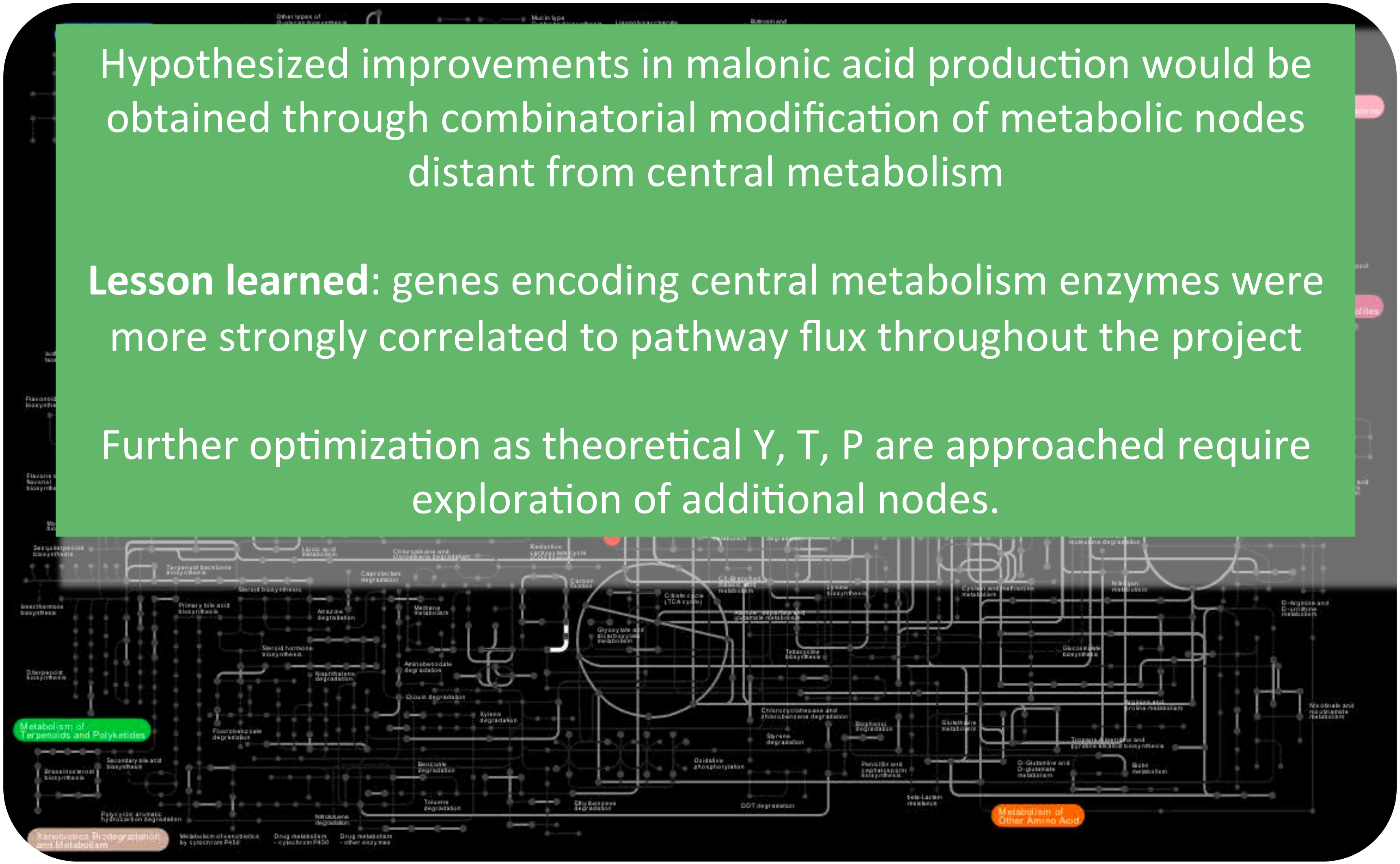
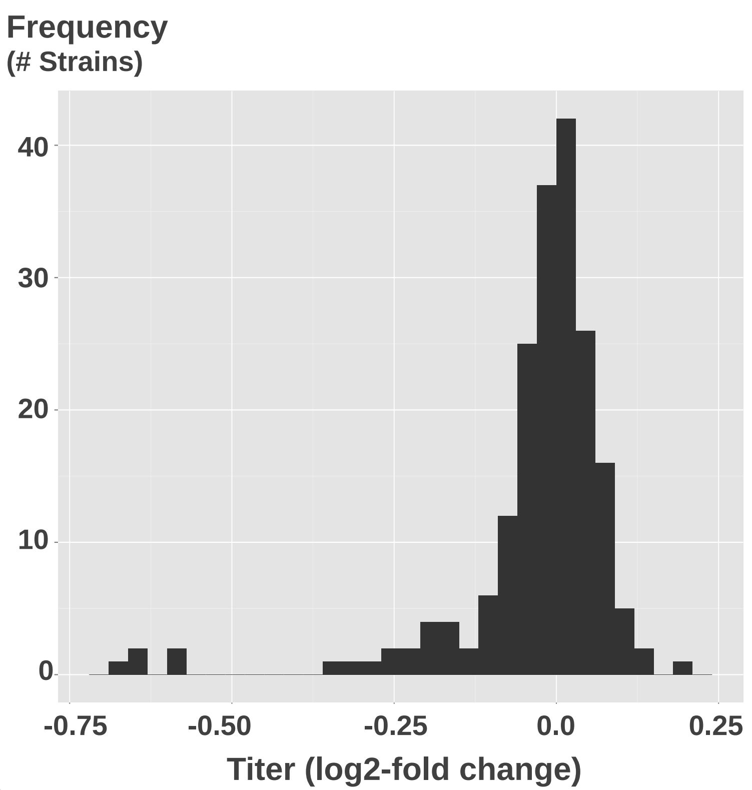


Photo credit: Arkin et al. http://glamm.lbl.gov/

Task C: Engineering central metabolism (revised task)

Communicated with DOE and mutually agreed to modify the Task C work plan and corresponding quantitative milestone

Construction and assaying a combinatorial library of 28 enzymes associated with central metabolism (784 strains)



- **Majority of engineered strains resulted in a titer decrease**
- **13 hits identified, under non-stringent hit-selection conditions designed to capture "fairly weak" hits[1]**

Hits were subsequently incorporated into optimized malonic acid producing strain and demonstrated in fermentations

1. Zhang et al. J Biomol Screen, 16, 2275-785 (2011)

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Task D: Translate plate metrics to pilot scale metrics

3rd party validation of final project metrics was performed at the ABPDU (50-liter scale)

- **Problems were encountered obtaining cellulosic sugars within allowed budget, limiting fermentation scale**
- **Demonstrated successful scaleup of fermentation process with real-world cellulosic sugars***

Parameter	As % of Control Fermentations
Yield	120%
Titer	99%
Productivity	99%

* Confidential commercial provider

Relevance

The work in this project promotes:

- Tools for cost effectively building and validating biocatalysts
- Replacing petro products with lignocellulosic-derived products
- High value bioproducts help drive down biofuel costs in integrated biorefineries
- Technoeconomic analysis suggests that upon successful commercialization could result in production cost of $1.60/lb; down from project initiation of >$600K/lb
- The potential to reduce carbon dioxide emissions related to petroleum produced malonic acid
- Identification of pathway targets may aid in development of other bio-products & fuels
- Synthetic evolution process could be applied to other products

Summary

- **Overview:** designing & constructing biocatalysts to consume cellulosic sugars to produce bioproducts (malonic acid)
- **Approach:** deploying synthetic biology techniques to accelerate the path toward commercialization (eg., design tools, screening tools, and validation).
- **Technical Accomplishments:**
  - Design software & tools; Biosensor screen
  - Continuously improved strains are being developed (Y,T,P)
  - Successful initiation of pilot scale fermentations
- **Relevance**
  - Ct-H Cost-effective production of bioproducts
- **Future Work**
  - Attain YTP milestones; expand workflow capacity

Additional Slides

Responses to Previous Reviewers' Comments

- Project was reviewed in 2015
- Overall Impressions:
  - "good example of product development & tools for other products. It fits well with DOE's desire for alternative products."
  - Successful and cost-effective production of biocatalysts that produce chemicals such as malonic acid as a platform molecule are promising
  - Interesting project focused on novelty in multiple aspects (e.g., pursuing target molecule that no other known microbe produced, running a HT screening tool). Integration w TEA to drive research is appreciated & has provided good results to date.
  - Good use of resources, although it would be good to see screening tool commercialized, the failure to do so because of resource allocation indicates a high level of dedication ot the project.
  - Innovative approach to strain engineering and signficant progress to developing robust organism
  - Excellent program that will help BETO achieve short and long range goals

Publications, Patents, Presentations, Awards, and Commercialization

Patents:

- None published relating to this grant, but we are developing inventions previously described in PCT Pub. No. 2013/134424 in work supported by the grant.

Presentations:

- Steen EJ. *Synthetic biology for brewing.* Synbiobeta Lecture: Synthetic Biology for Computer Programmers. October 8, 2013, San Francisco, CA.
- Steen EJ. *An industrial perspective on synthetic biology.* Synbiobeta Lecture: Synthetic Biology for Computer Programmers. November 31, 2013 San Francisco, CA.

Commercialization:

- **Financing**
- **Offtake**
- **Internal product development effort**