AGILE
BIOMANUFACTURING

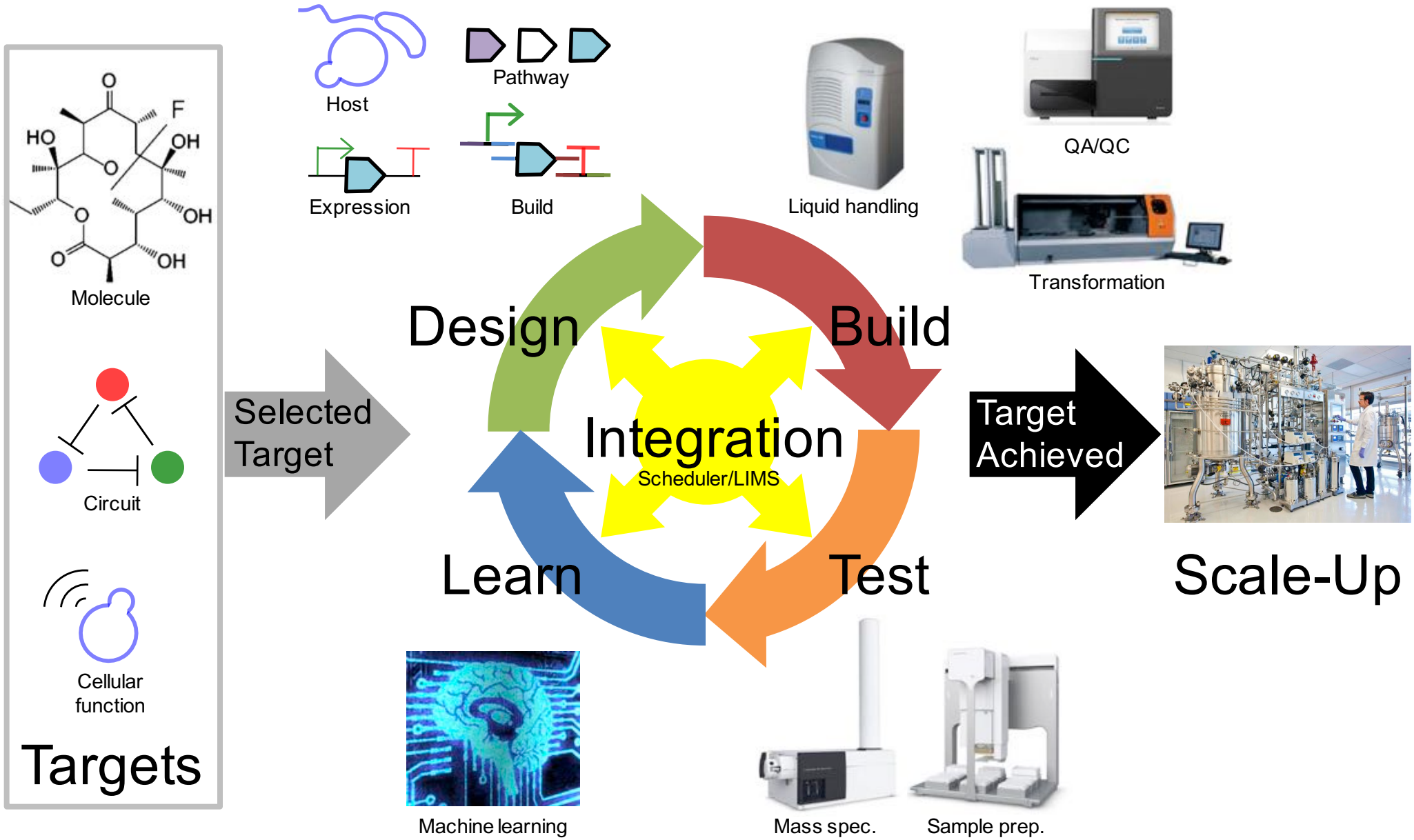
Foundry proof-of-concept

Nathan J. Hillson

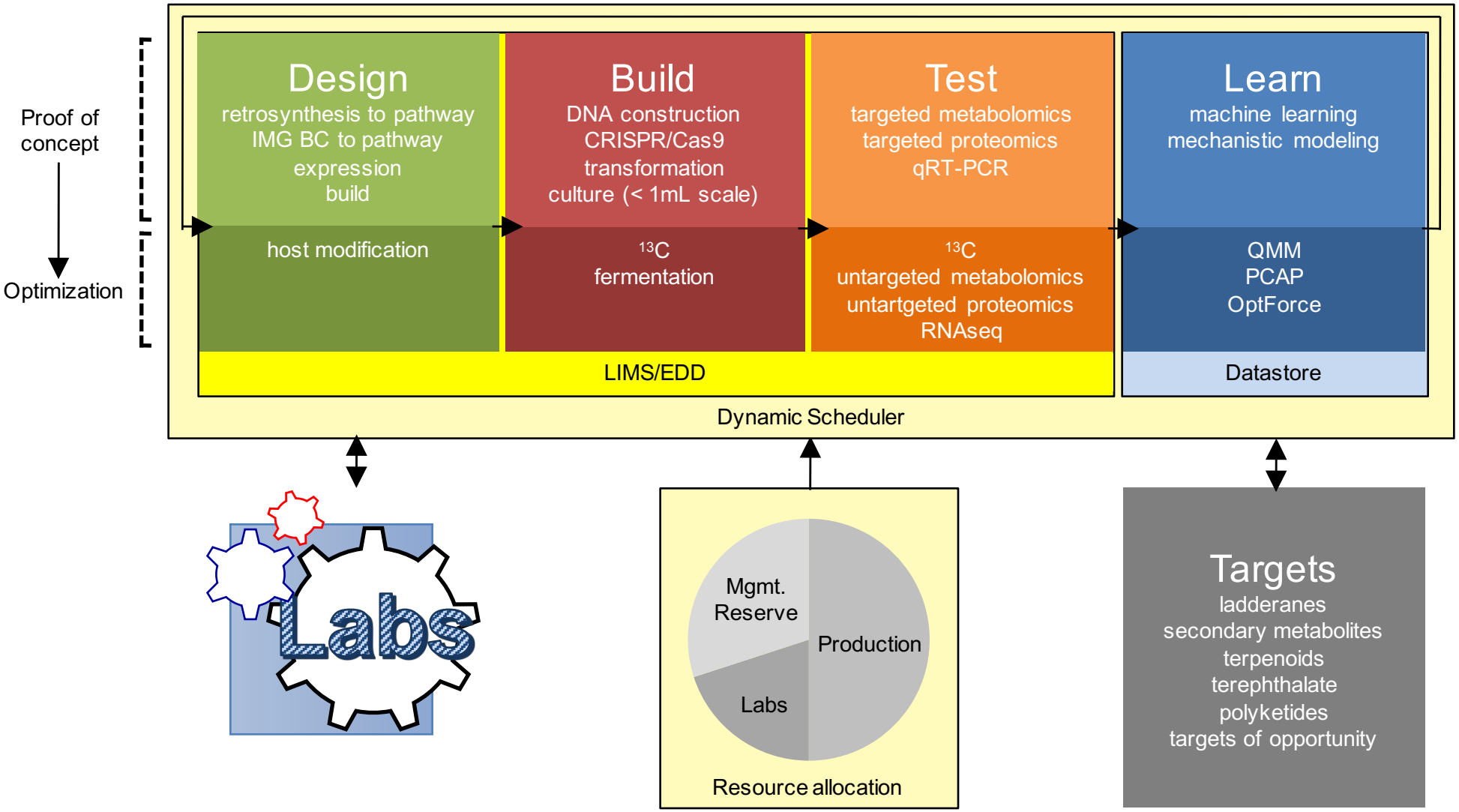
EERE Visit
Feb 19, 2016

The Ames Laboratory — Creating Materials & Energy Solutions — U.S. Department of Energy
Argonne National Laboratory
INL Idaho National Laboratory
Los Alamos National Laboratory EST. 1943
Berkeley Lab
NREL National Renewable Energy Laboratory
Oak Ridge National Laboratory
Pacific Northwest National Laboratory
Sandia National Laboratories

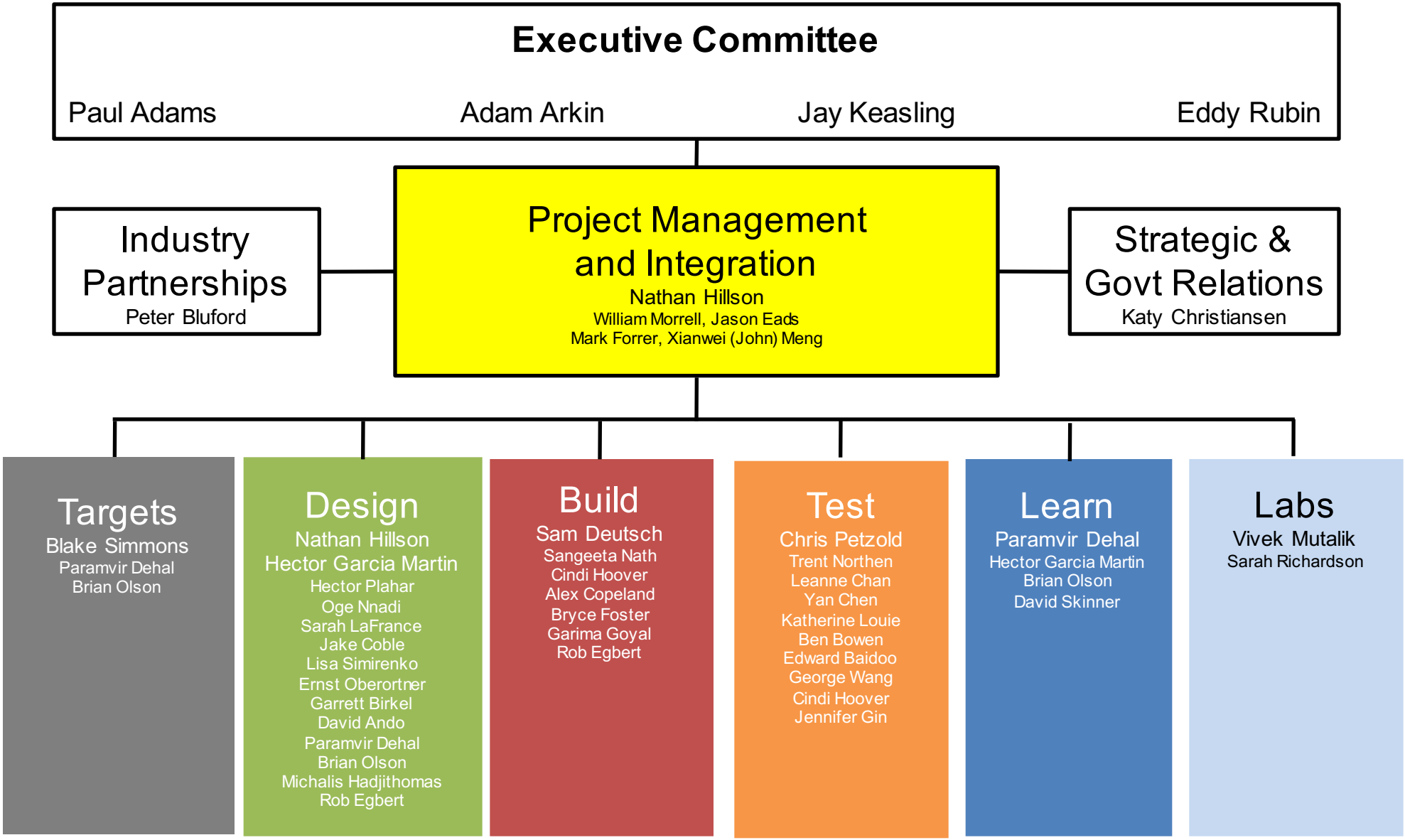
Integrated Design / Build / Test / Learn



Concept of operations



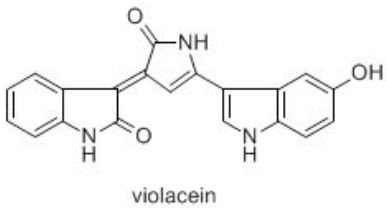
Organizational chart

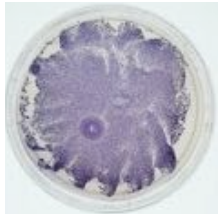


Representative target molecules

Violacein

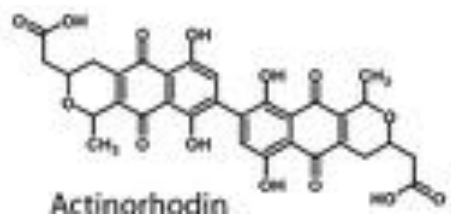
- Antibiotic, 5 gene pathway
- Violacein and side products detectable
- Great model for developing Learn





Actinorhodin

- Polyketide antibiotic, 22 gene pathway
- Complex, large, and high GC content
- Great model for refactoring



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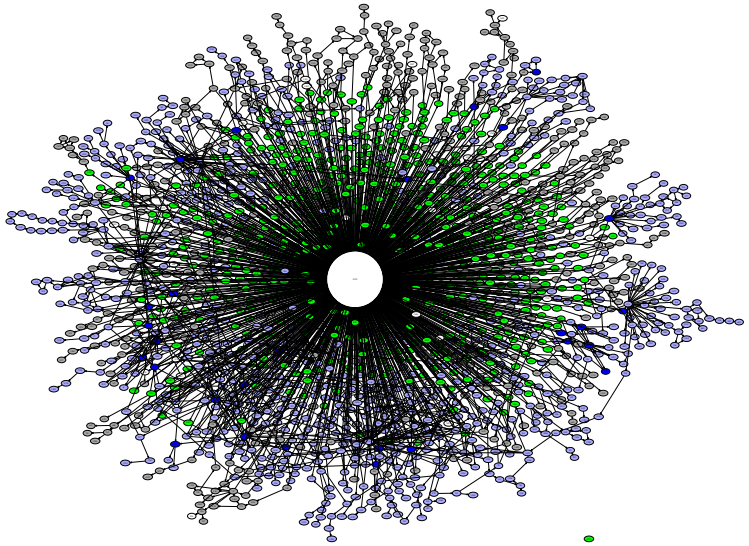
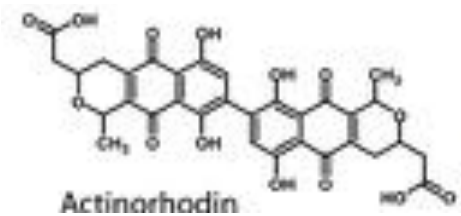


Actinorhodin

Actinorhodin: polyketide antibiotic produced by *Streptomyces coelicolor*, requiring 21 genes for biosynthesis. Cluster ~ 25 kb, %GC >70



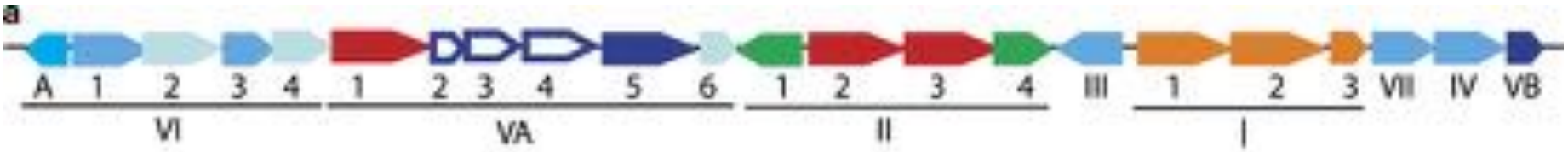
Why actinorhodin?

- Representative of many biosynthetic clusters (size, complexity, GC content)
- Pathway is well characterized (but never before refactored)
- Actinorhodin is readily detectable (blue/red pigment depending on pH)

Actinorhodin pathway refactoring

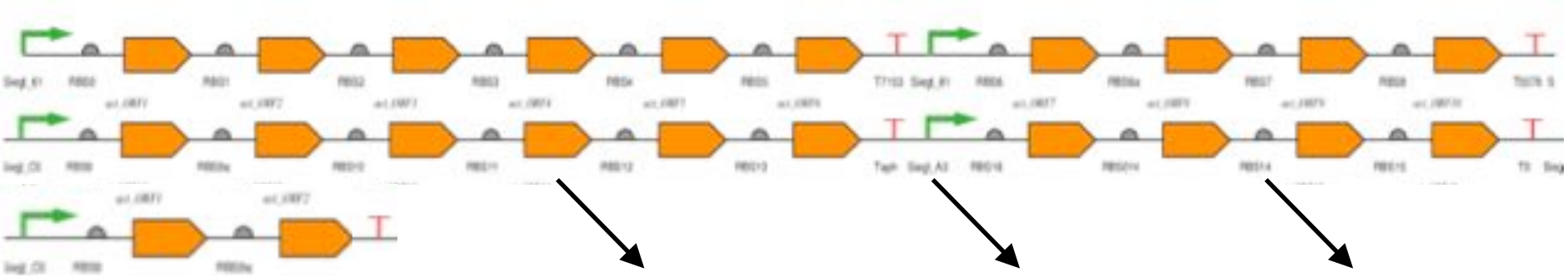
"Refactoring": Redesigning a complex set of operons that are highly regulated under native conditions to predictably achieve a phenotypic outcome under laboratory conditions

Native pathway:



Highly regulated expression; unknown control system

Refactored Design:



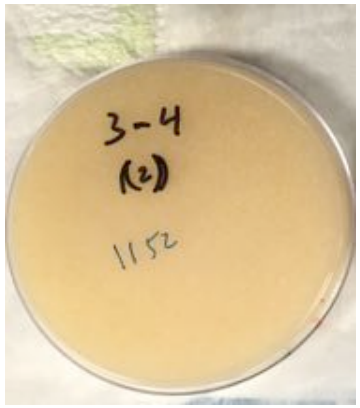
Codon optimized genes: lower GC, remove repeats & secondary structure, preserve high translational potential

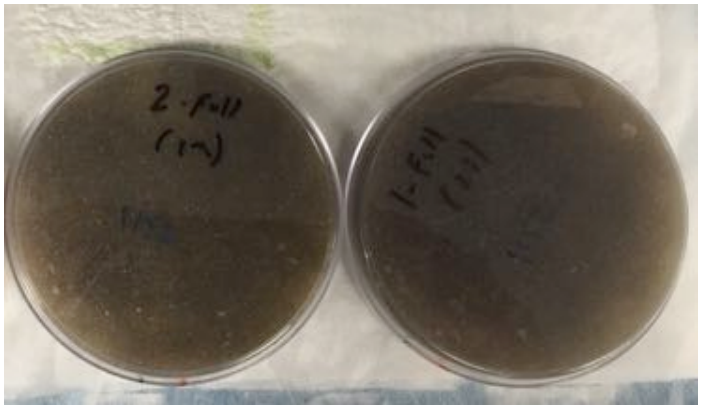
Previously validated constitutive promoters and terminators

Calculated optimal RBSs specific for each rCDS

Goal: predictable expression

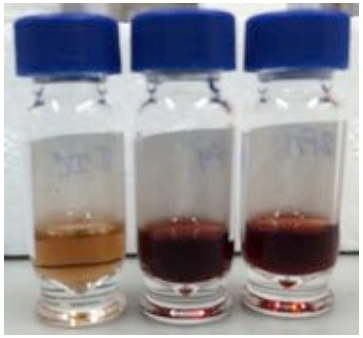
Actinorhodin strain results



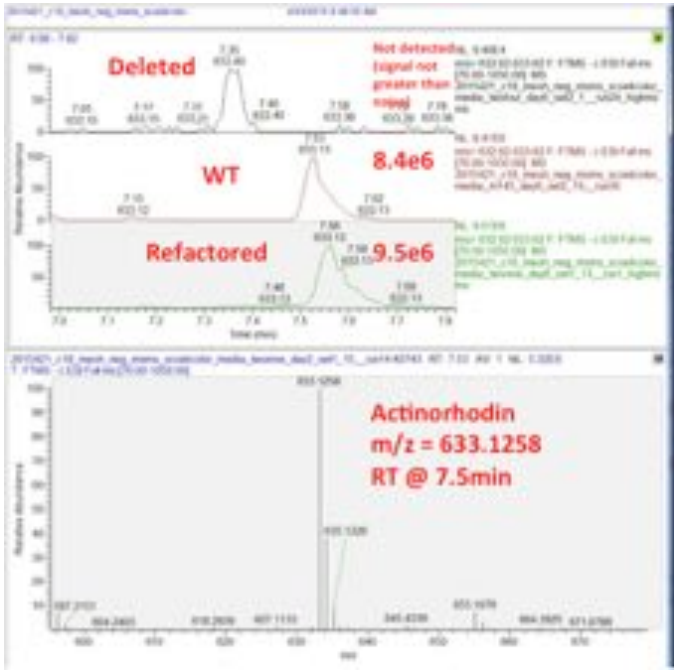


S. coelicolor Δ*act strain* (control)

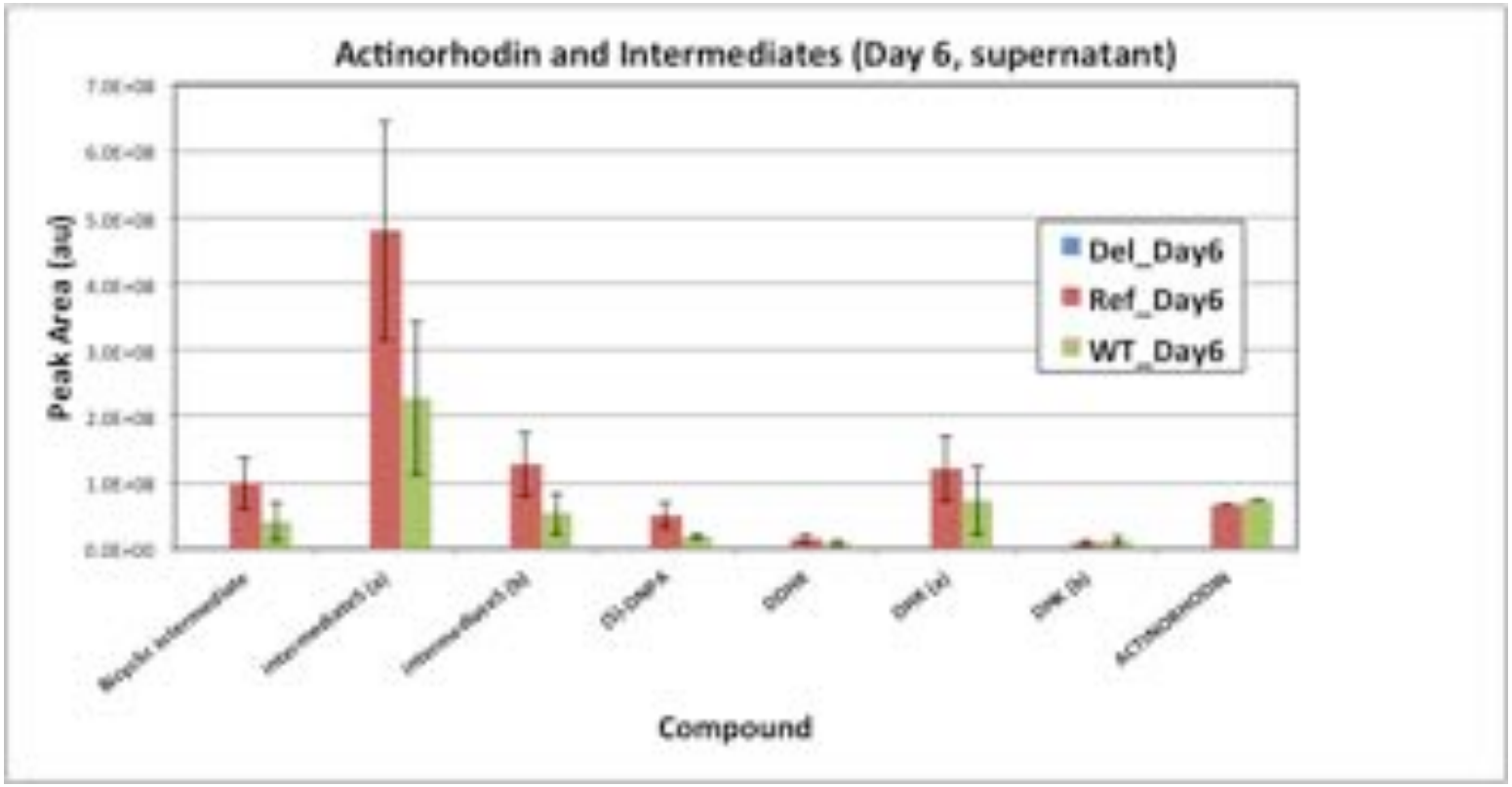
Refactored actinorhodin strains

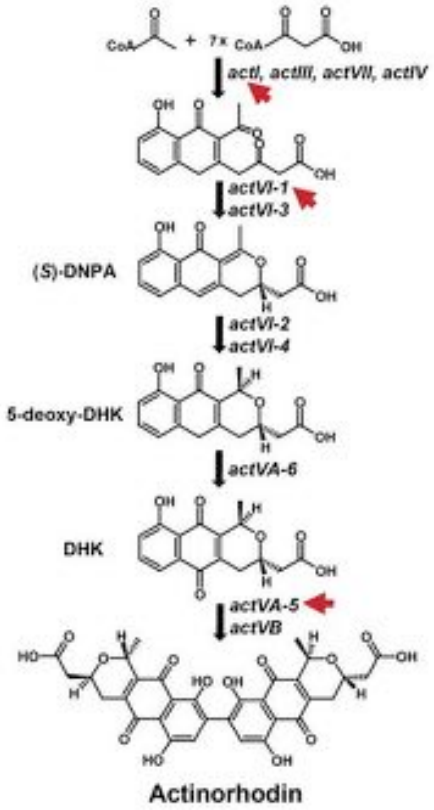


Extracts for LC-MS analysis



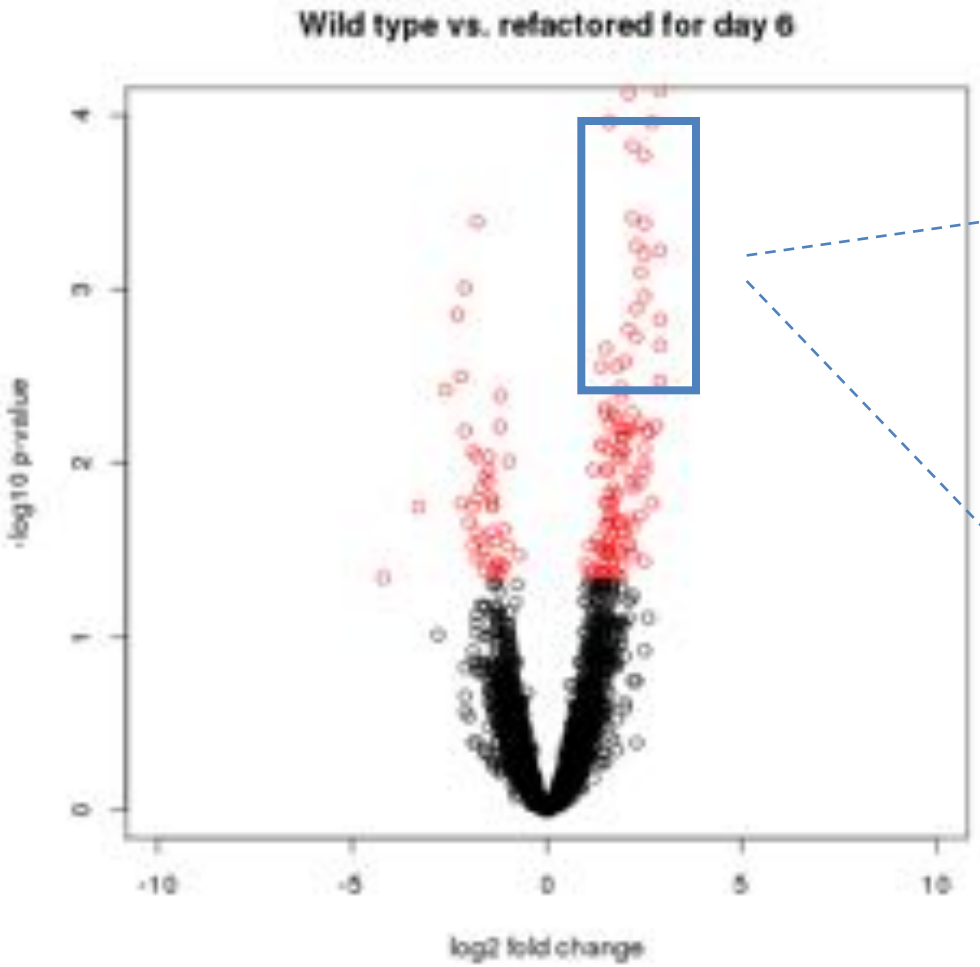
Pathway output is 100% of wildtype





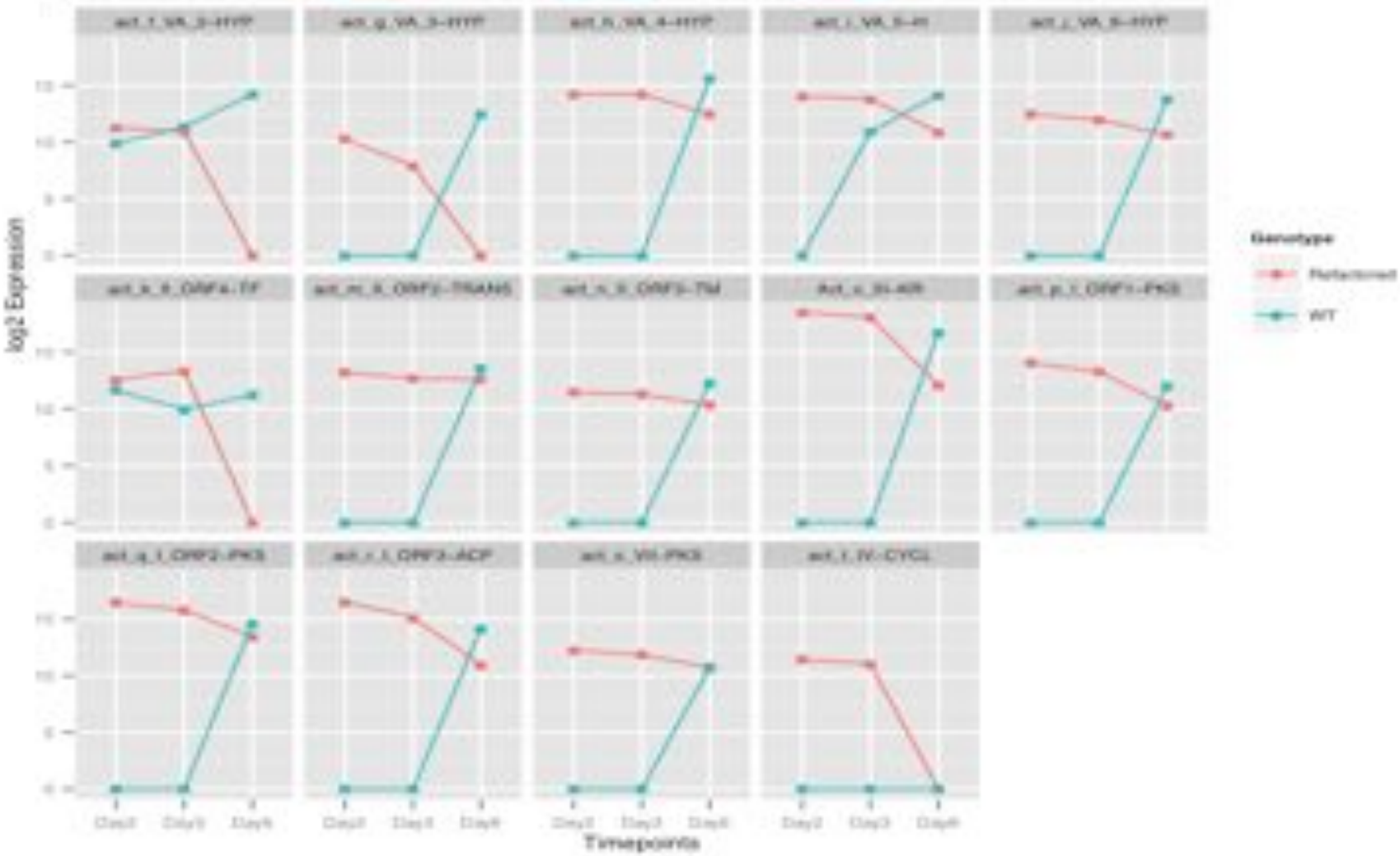
Constitutive actinorhodin production results in cell-stress response

Transcriptomics to detect and quantify over- and under-expressed genes



Constitutive actinorhodin pathway expression diminishes performance stability over time

Targeted proteomics to detect and quantify actinorhodin pathway enzymes



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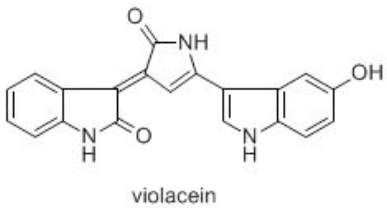

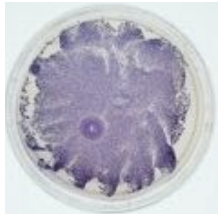

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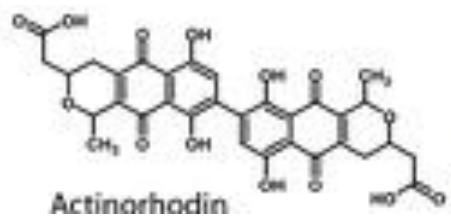

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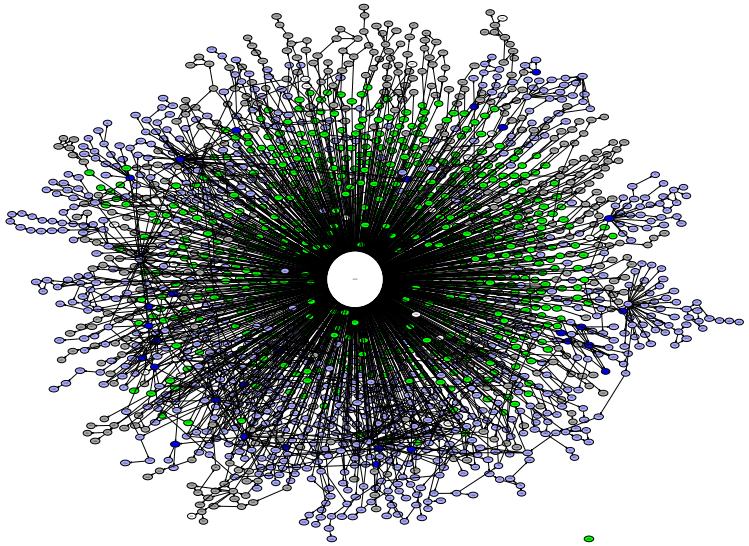
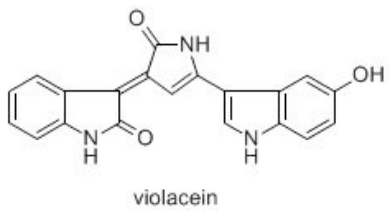
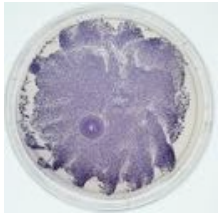


Violacein

Violacein: natural antibiotic produced by *Chromobacterium violaceum*, requiring 5 genes for biosynthesis. Cluster 8 kb, %GC ~50

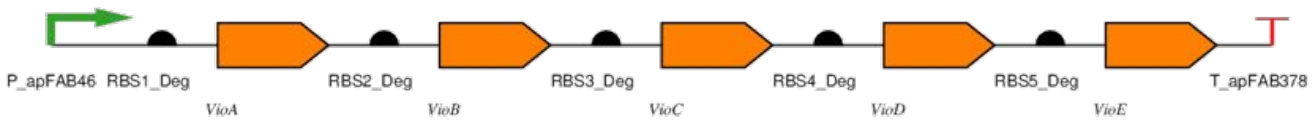


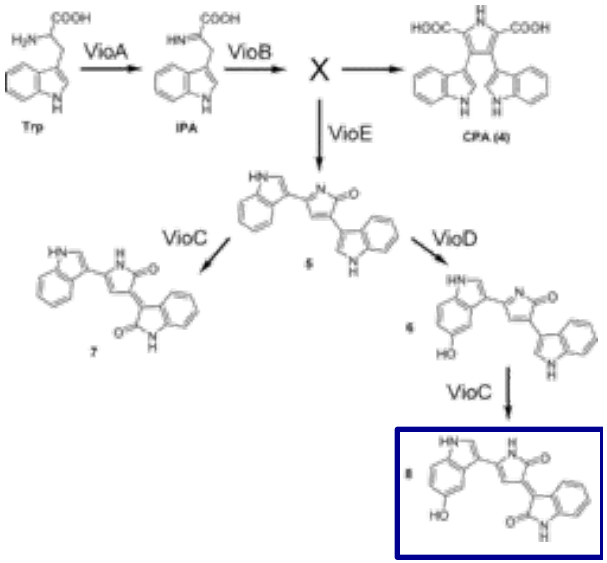


Goals:

- Generate large combinatorial combinatorial library
- Correlate molecule production to sequence and protein expression <u>to learn critical features</u> leading to optimized flux

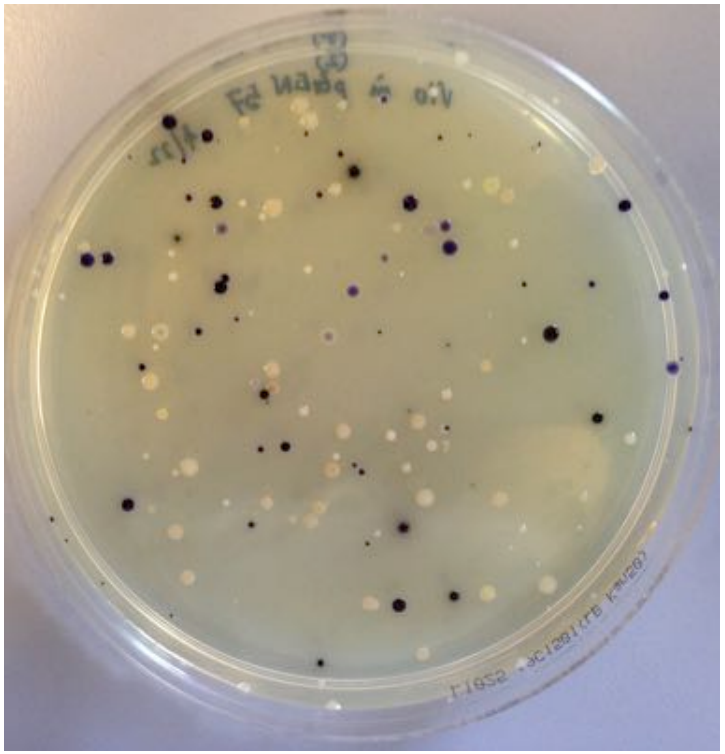
Violacein pathway design

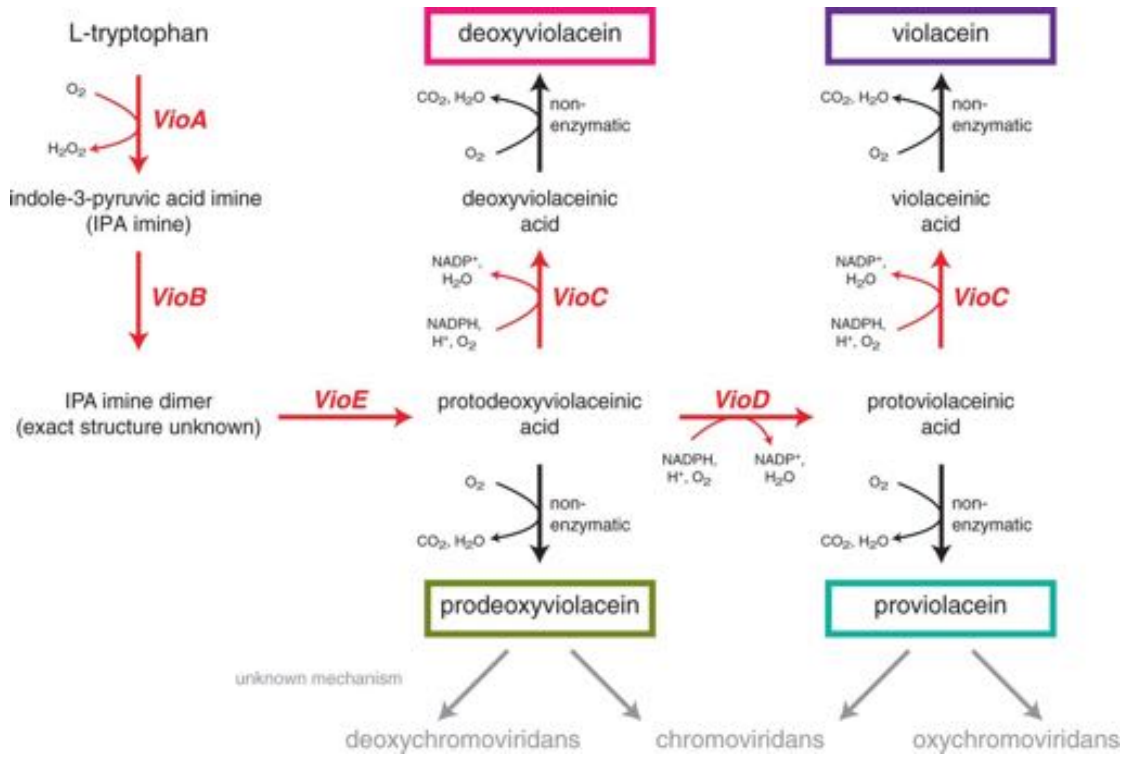




Design Features: Degenerate RBSs + multiple gene orthologs
50,000 strain variants

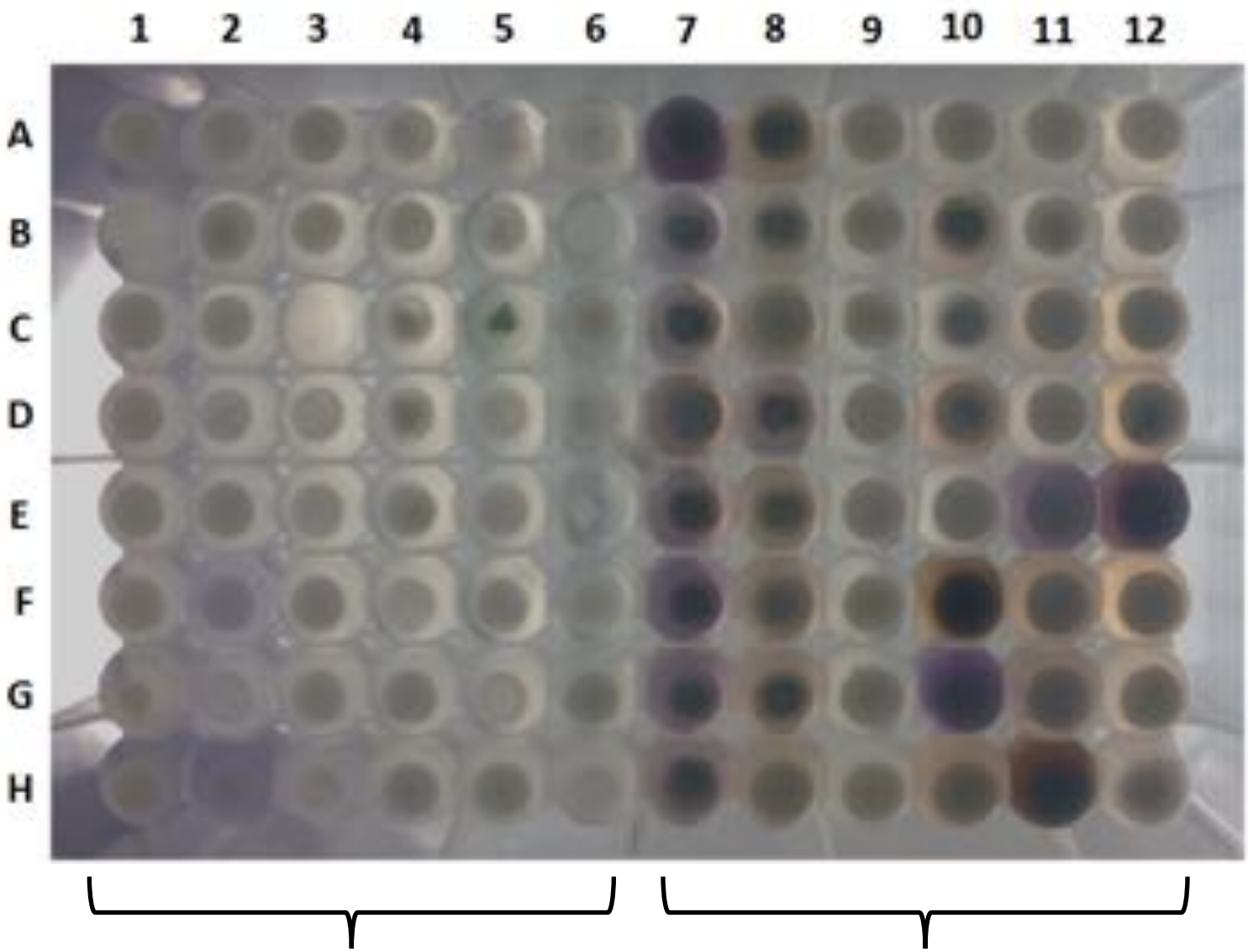
Violacein strain variants





Plan:
Select 500 strains based on phenotypes.
Sequence, metabolomics and proteomics to build pathway optimization model.

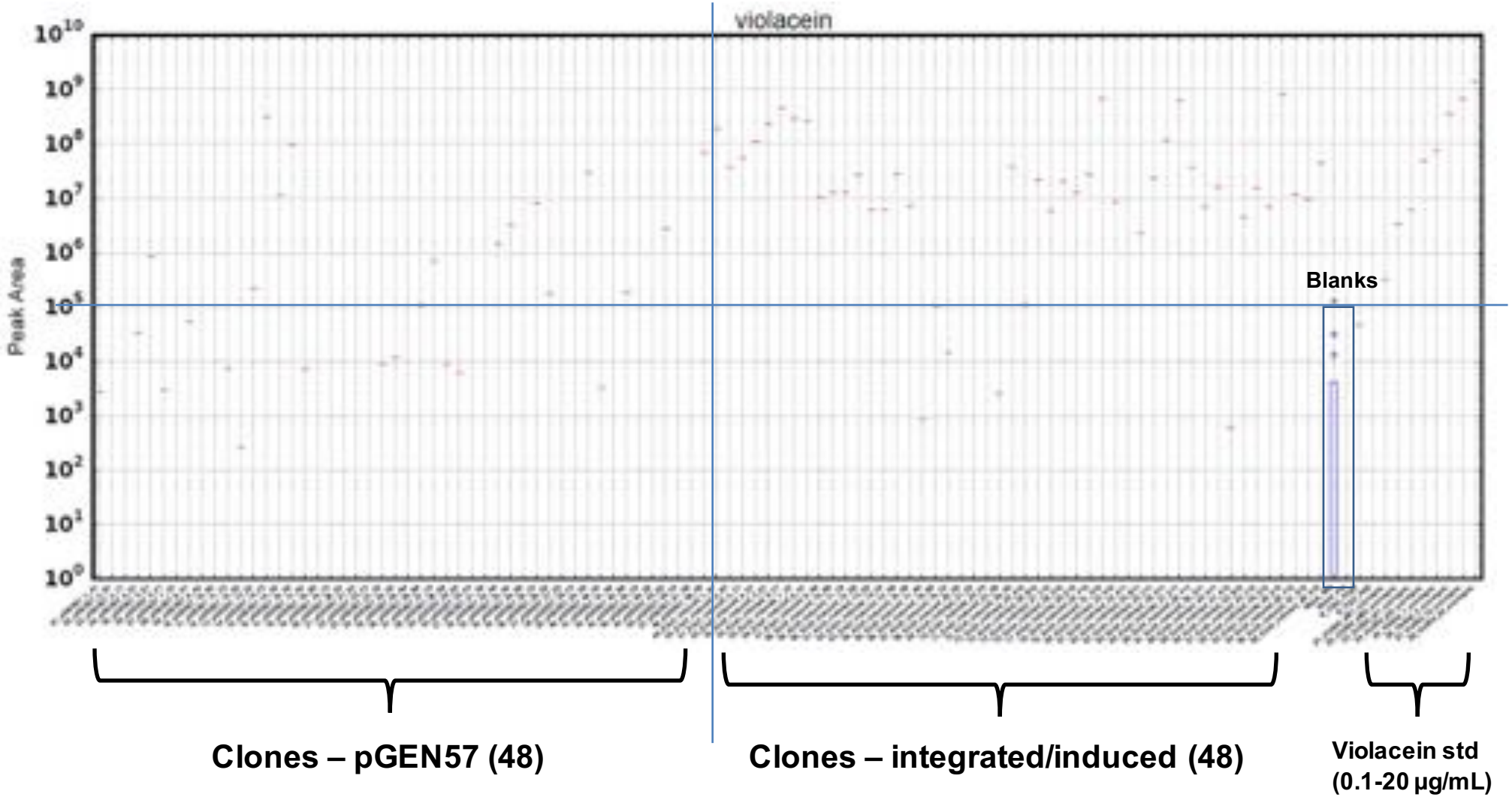
96-well plate (individual clones)



pGEN57 (1-48)

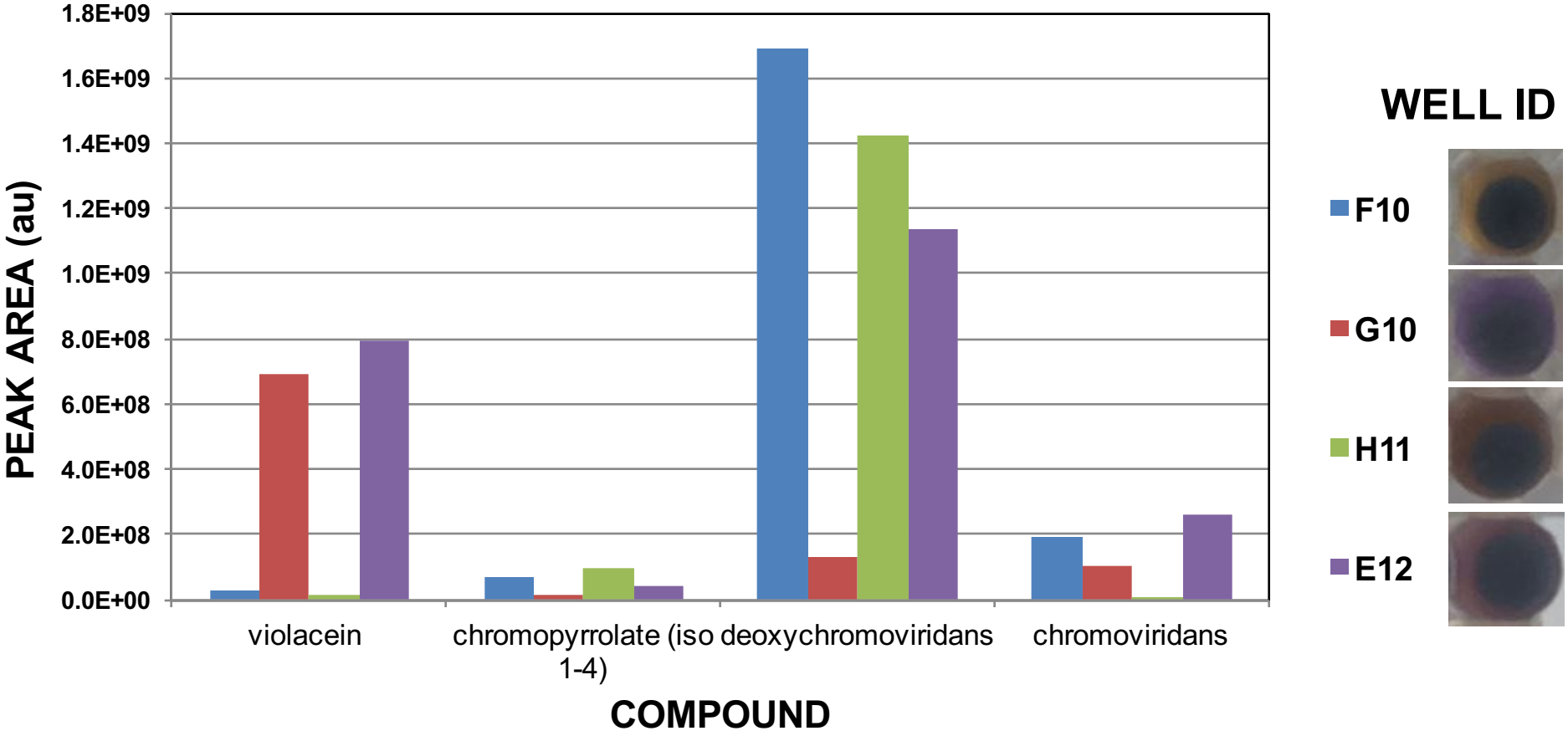
Integrated/induced (49-96)

Violacein detected in each clone

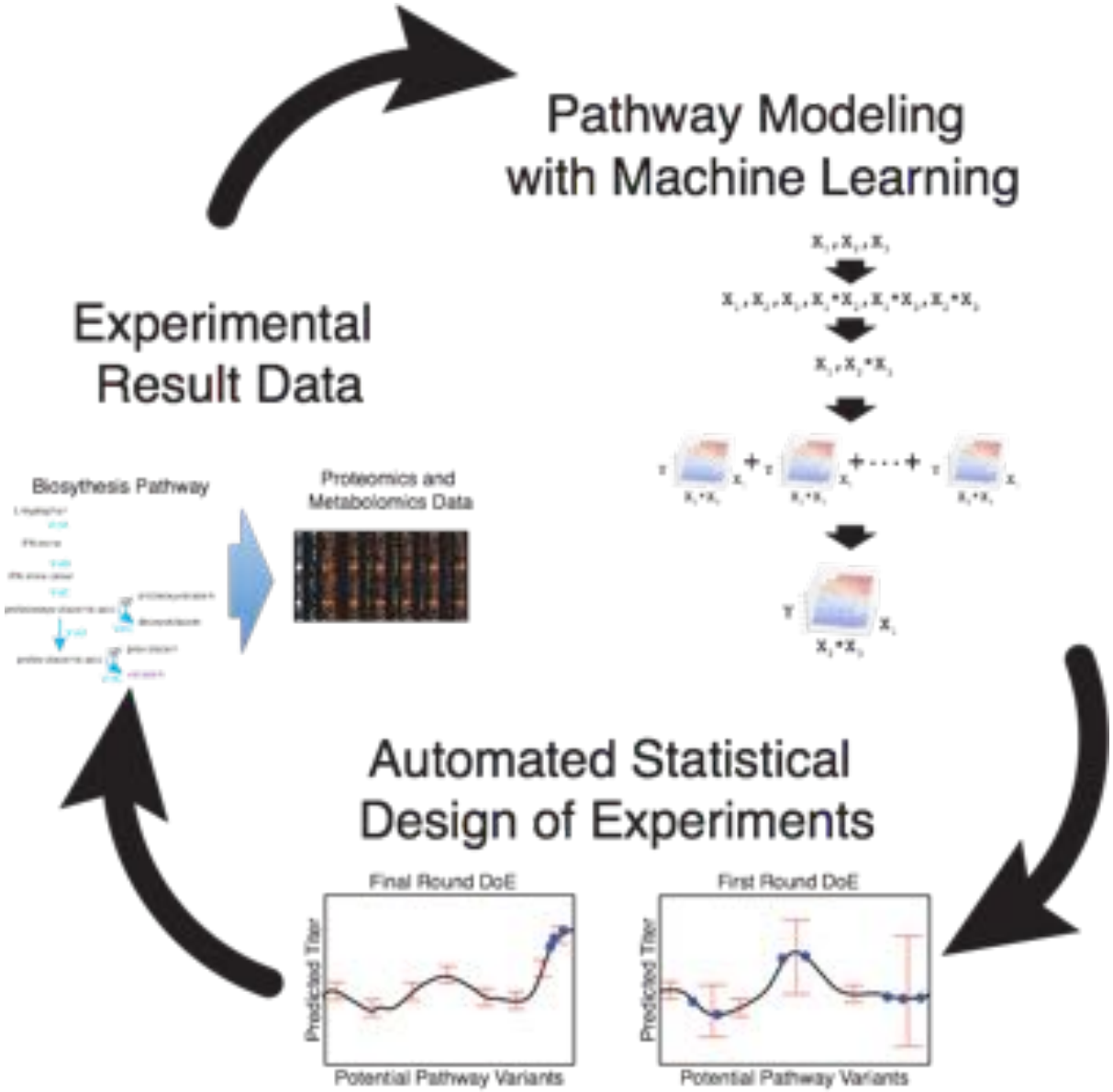


Select cut-off value for signal due to background / carryover from previous samples

Relative amount of violacein and shunt products



Design of experiments and machine learning

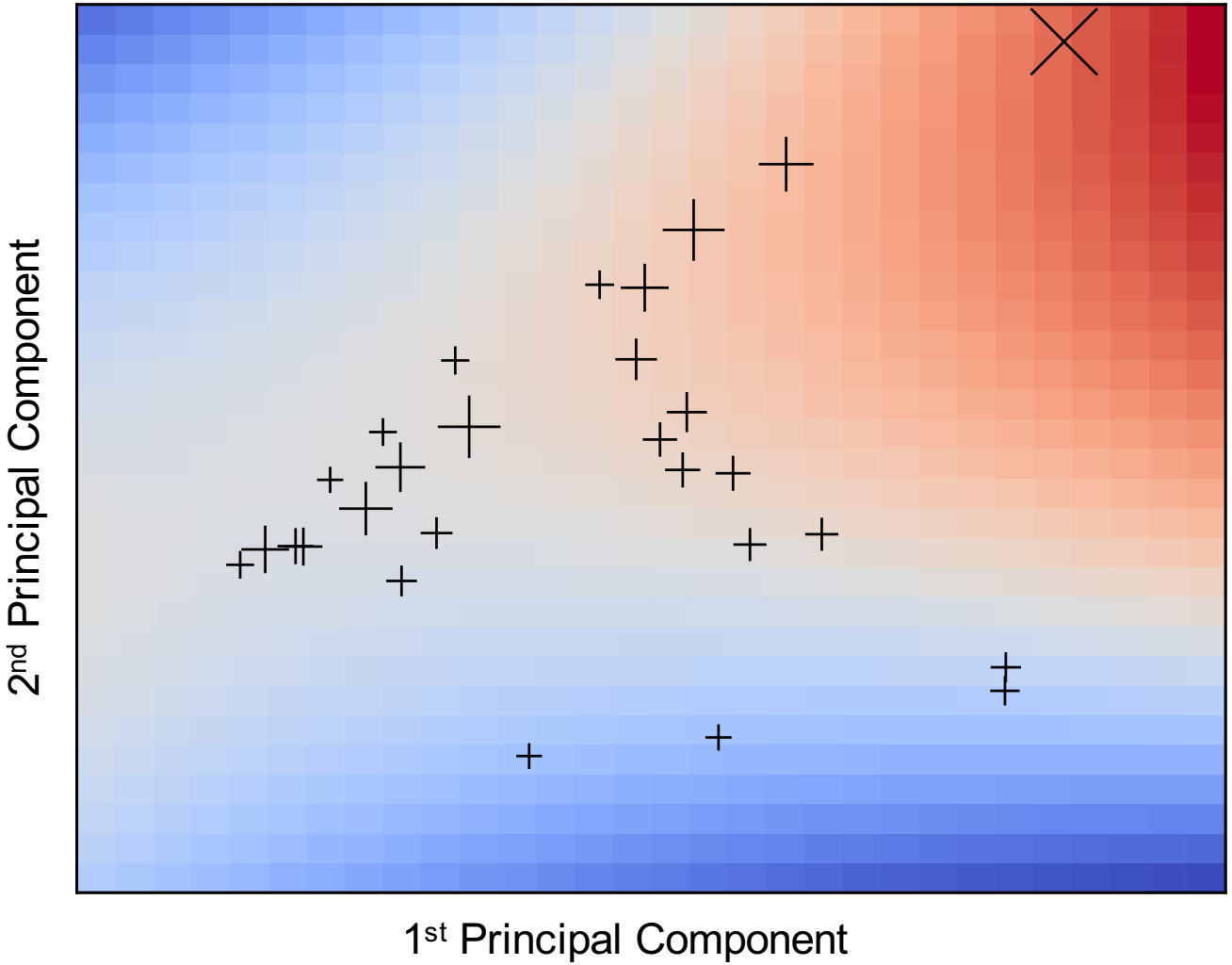


Case study: modeling limonene biosynthesis pathway

9 pathway genes divided into 3 clusters

3 promoter strengths per cluster
= 3^3 = 27 1st round strains

1 second round strain



Data Source

Alonso-Gutierrez et al., Metabolic Engineering, Volume 28, March 2015, Pages 123-133, ISSN 1096-717.

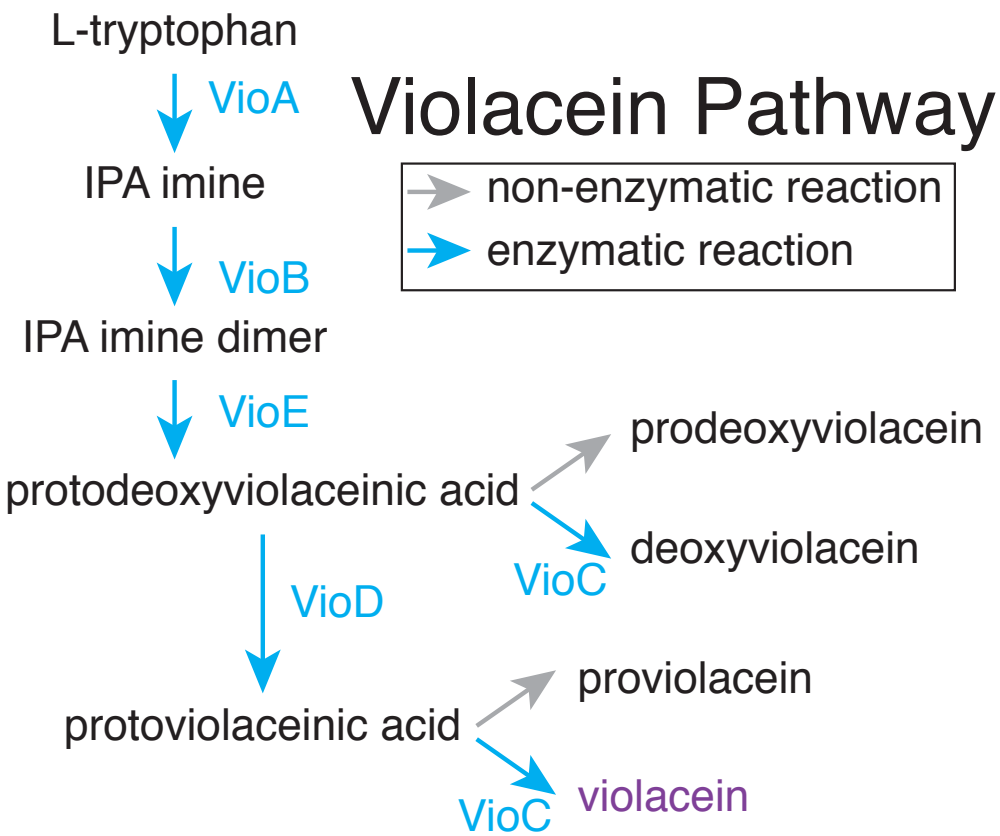
+ 1st Round Strains

× 2nd Round Strain

low high

Predicted Limonene Titer

Iterative improvement for violacein model



5 promoter levels for 5 Genes
= 5^5
= 3125 possible strains

187 randomly selected strains build and tested

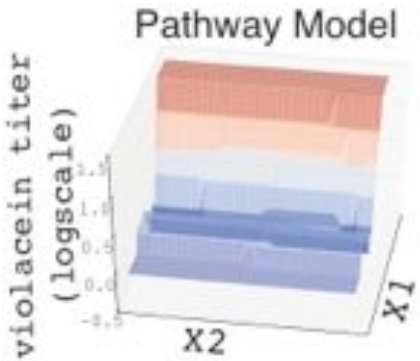
randomly divide strains into 5 sets

Data Source

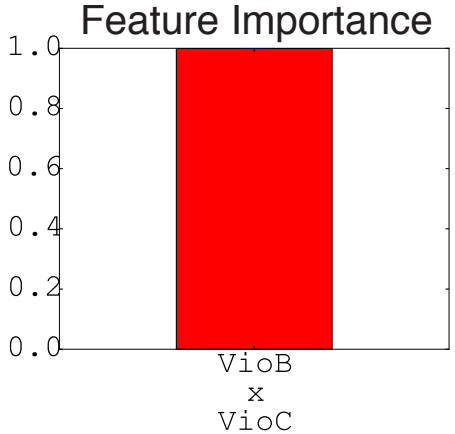
Lee ME, Aswani A, Han AS, Tomlin CJ, Dueber JE. Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay. Nucleic Acids Research. 2013;41(22):10668-10678.

Iterative improvement for violacein model

Round 1



R^2: .31



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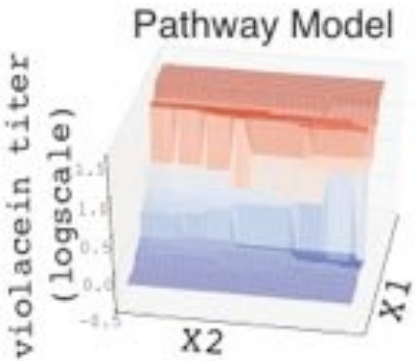
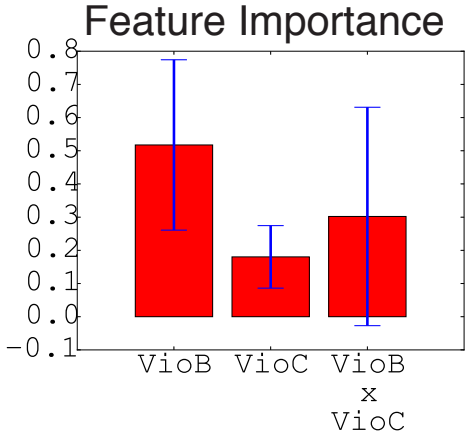
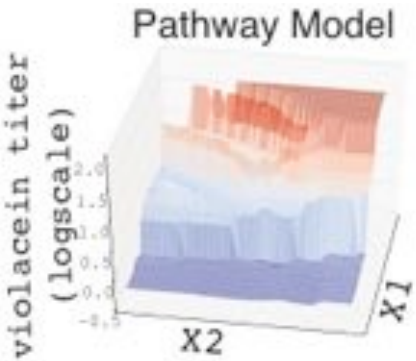


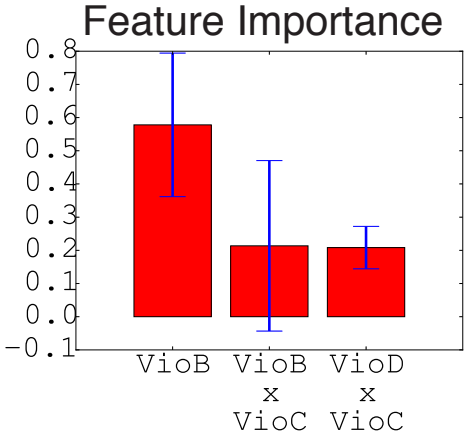
R^2: .74



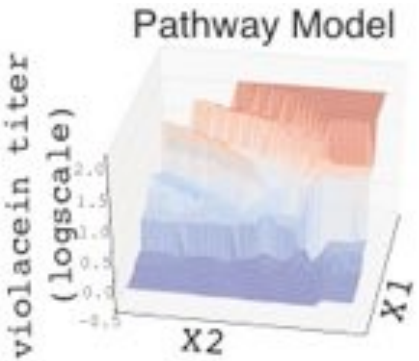
Round 3



R^2: .75



Round 4



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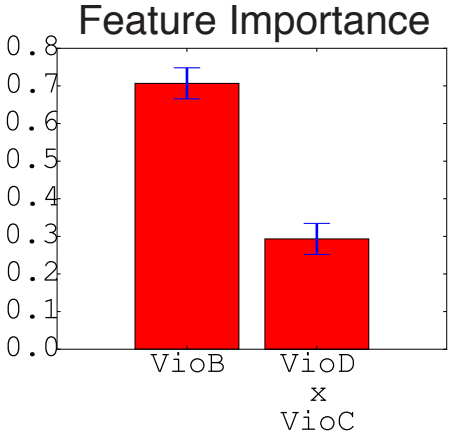

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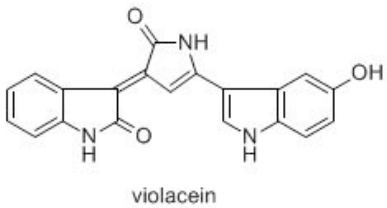

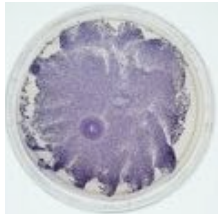

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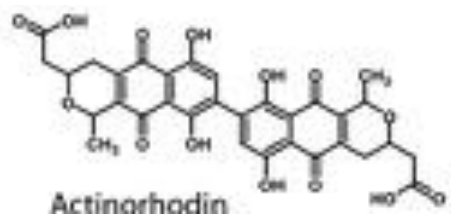

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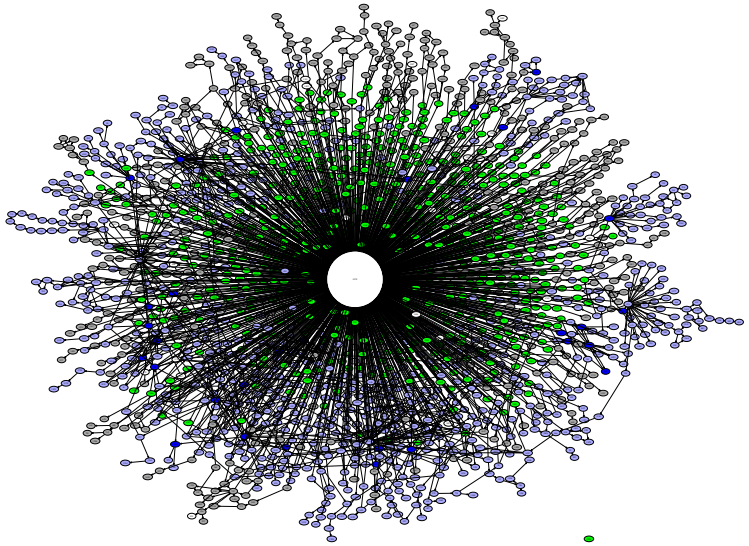
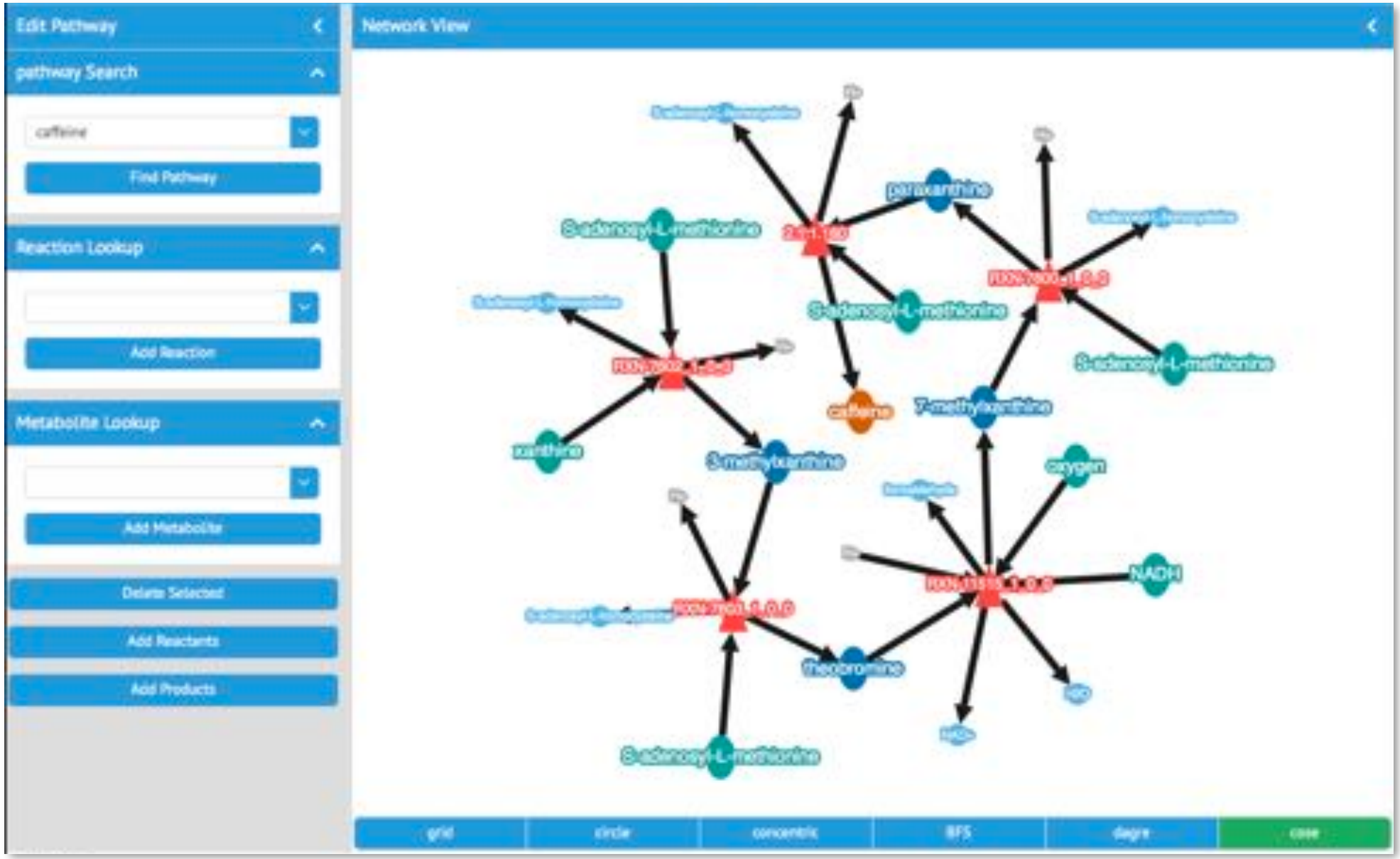


Retrosynthesis design



Retrosynthetic design space

Biochemical Reactions

METACYC

+

Chassis Organism

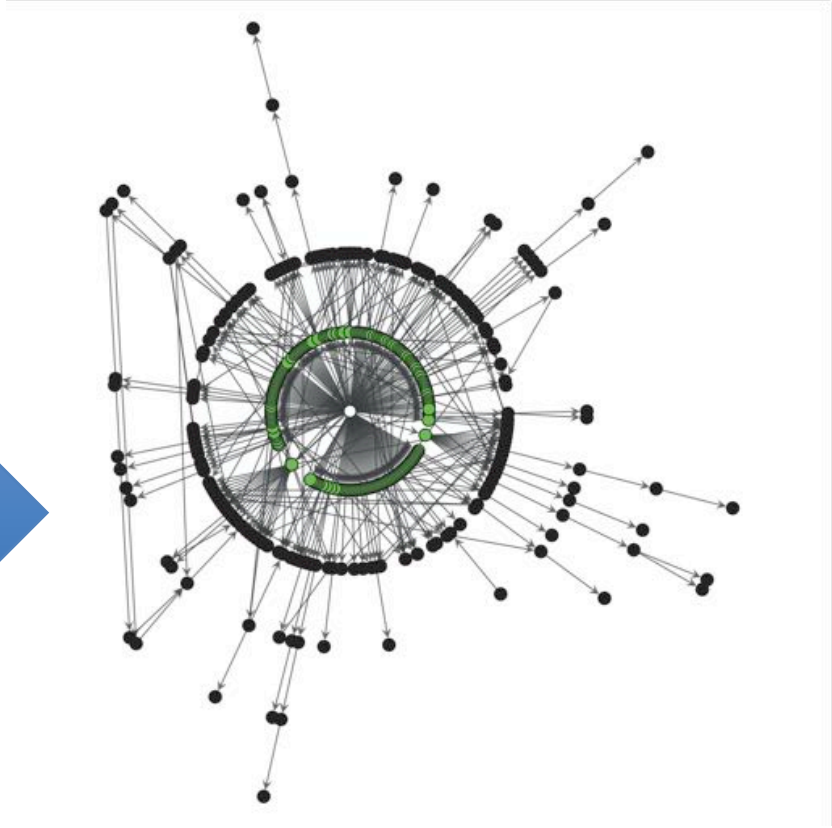
E. coli

Pathway Discovery

Heterologous Pathways

Pathway Constraints

Retrosynthetic Design Space (RDS)



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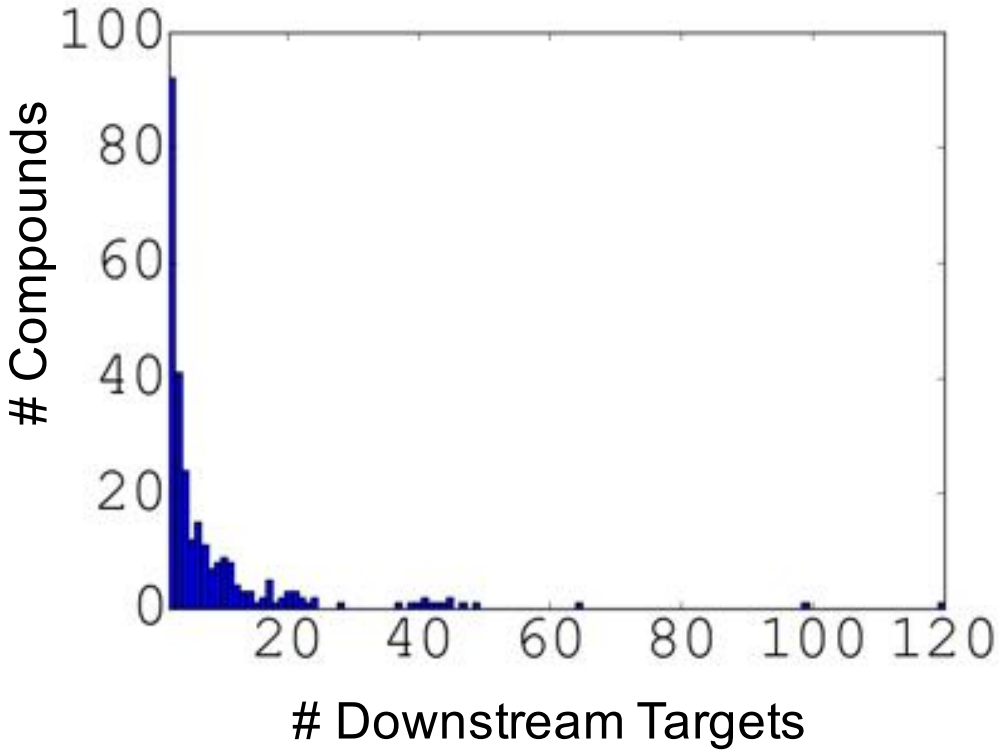

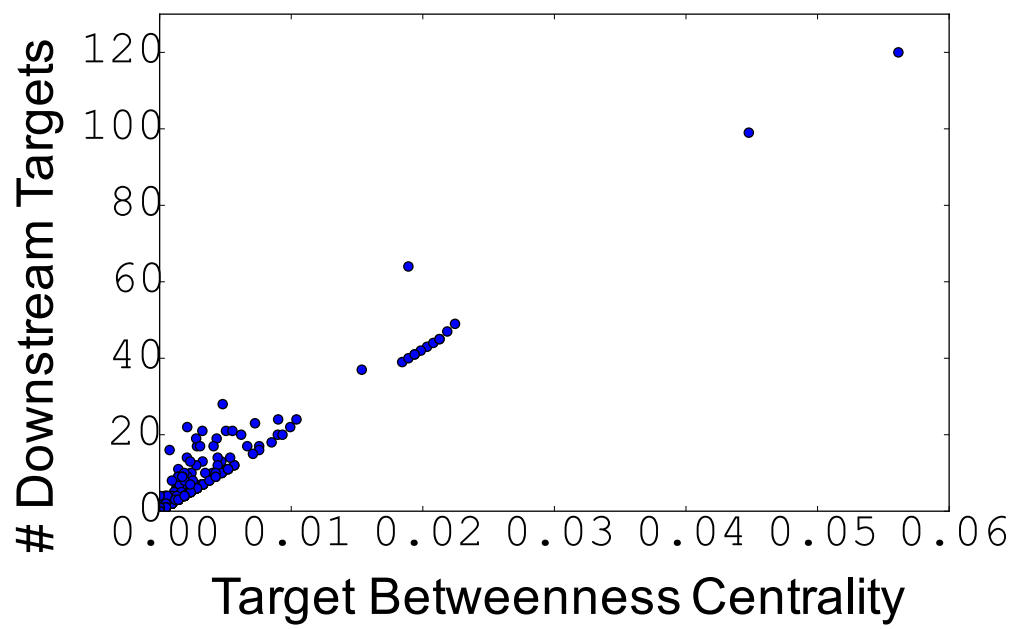

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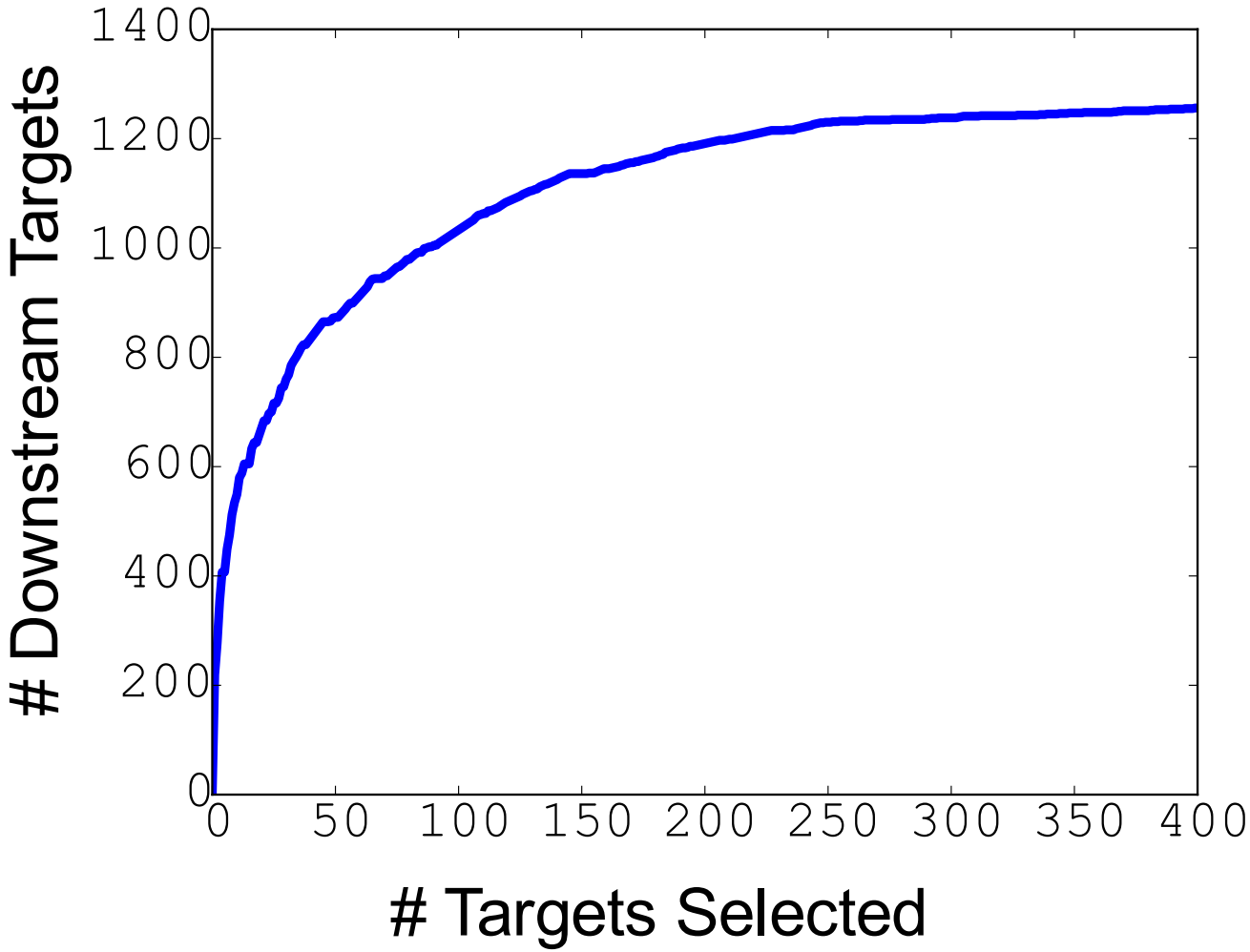
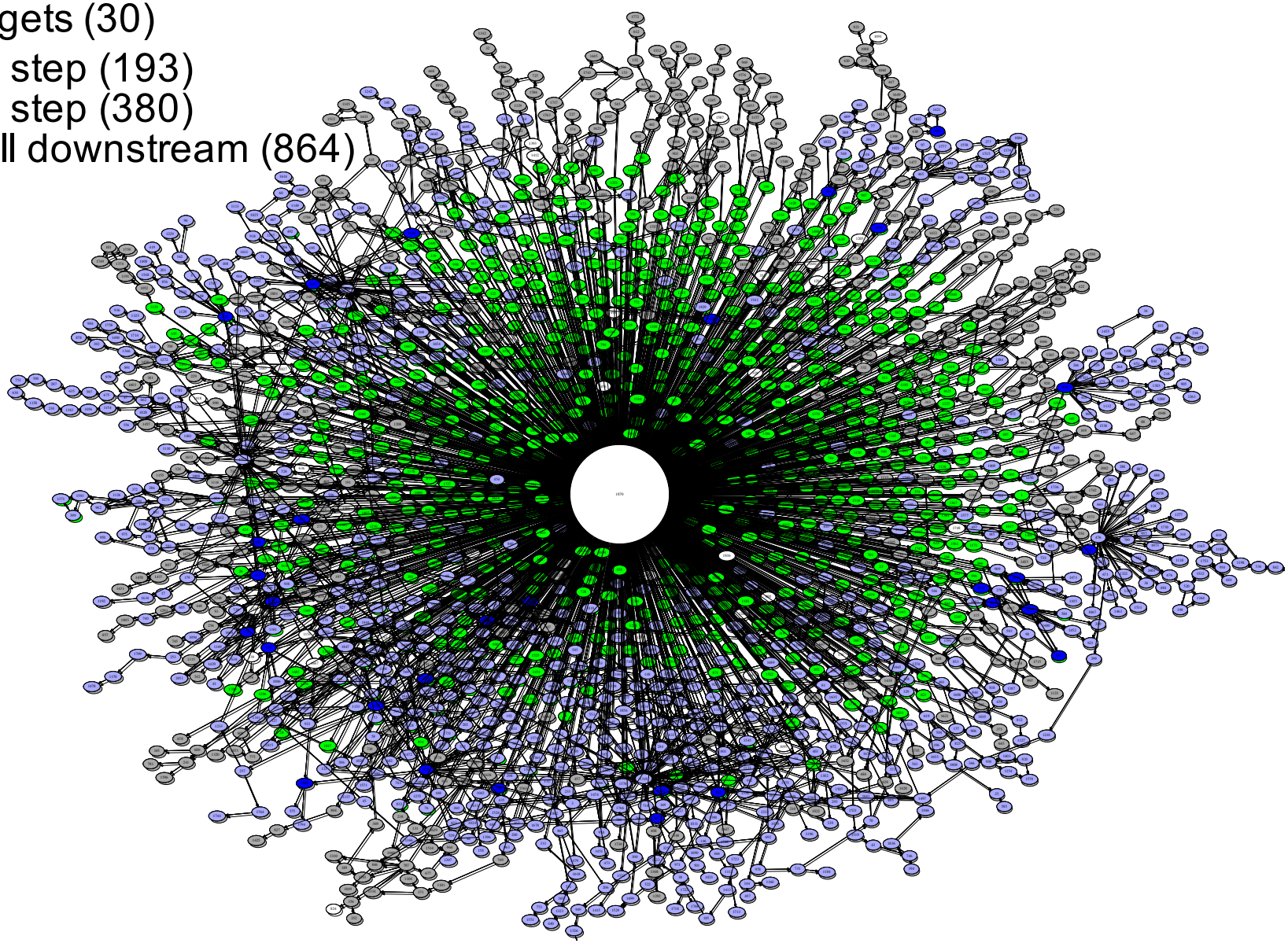


Targets of Opportunity

- 1 step from *E. coli*
- Selected Targets (30)
- Selected + 1 step (193)
  Selected + 2 step (380)
  Selected + all downstream (864)

E. coli RDS

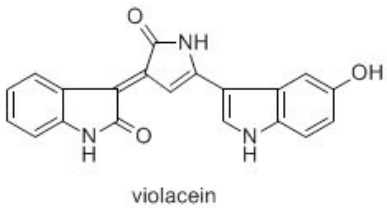


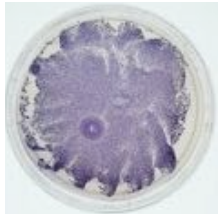
Public infrastructure enables industry

Summary of Progress to Date

Violacein

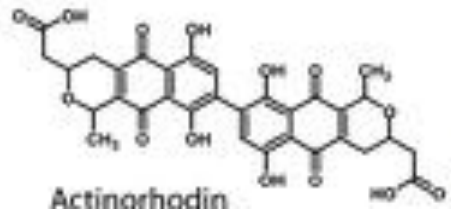
- Antibiotic, 5 gene pathway
- Violacein and side products detectable
- Great model for developing Learn





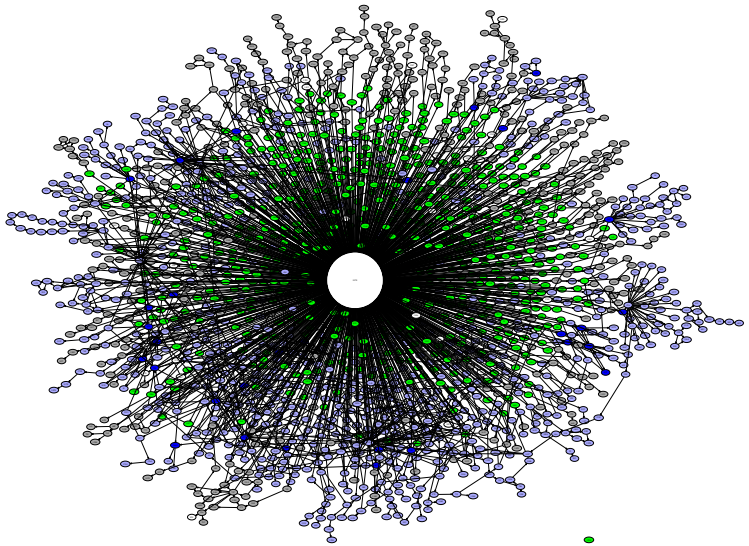
Actinorhodin

- Polyketide antibiotic, 22 gene pathway
- Complex, large, and high GC content
- Great model for refactoring



Targets of Opportunity

- Biosynthetic route hubs (high betweenness centrality)
    - maximize access to downstream targets
- Constraints:
    - energetically unfavorable steps <= 1
    - toxic intermediates <= 1
    - molecular weight of metabolites > 100 daltons
    - pathway steps <= 5



2.5.3.104 Agile Biomanufacturing – Adipic Acid Production in *P. putida* and *S. venezuelae*

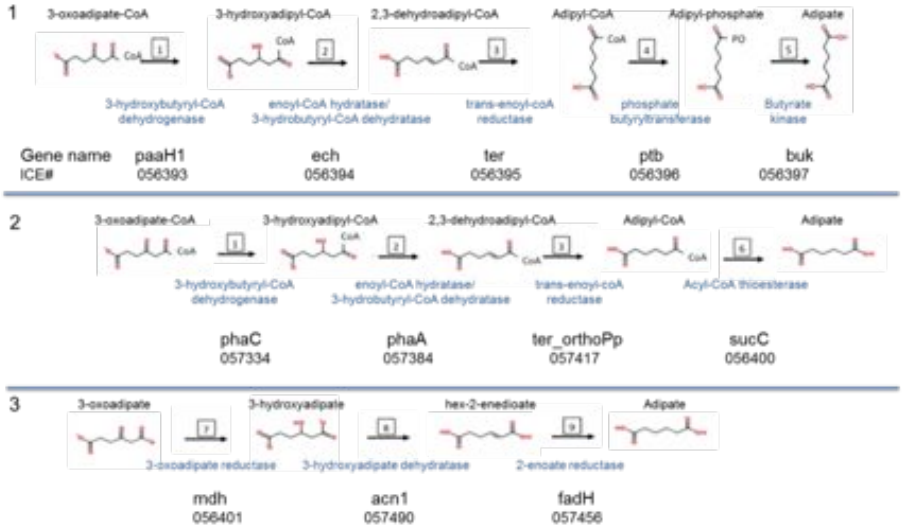
Project Objective

- Rapidly on-board new hosts and improve molecule production titers.
- > 100-250 mg/L adipic acid by FY16 Q4
- > 1-10 g/L adipic acid or precursor by FY17 Q2
- Precursor to a larger Foundry effort to address biomanufacturing challenges through advanced design, build, test, learn cycles leveraging synthetic biology and predictable scale-up.

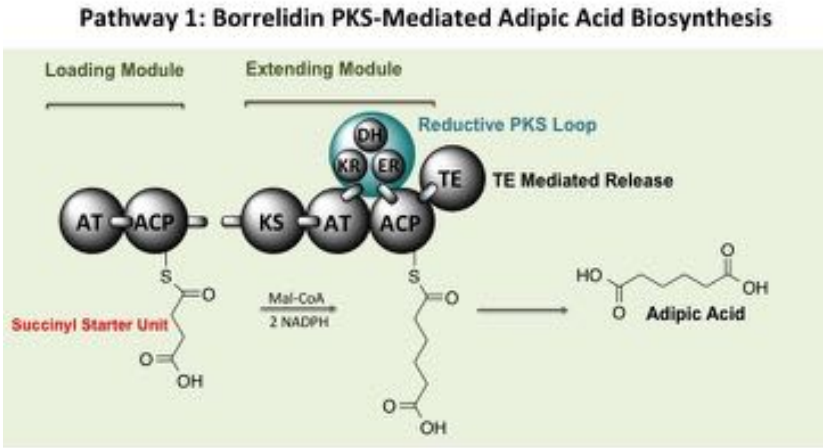
FY16 Q1 and Q2 Milestones

- Q1 - Design and build initial *P. putida* and *S. venezuelae*. Configure multi-omic test pipelines for *P. putida* and *S. venezuelae*.
- Q2 - Conduct bioreactor tests on at least one *P. putida* strain containing Pathway 1 and/or 2 and shake flask tests of *S. venezuelae* strains containing the PKS system to determine baseline titer, rate, and yield. Initiate ^{13}C metabolic flux and multi-omic analysis of *P. putida* and *S. venezuelae* strains to identify rate-limiting steps in baseline engineered strains.

Adipic acid production in *P. putida* from 3-oxoadipyl-CoA (Pathways 1/2) or 3-oxoadipate (Pathway 3)



Engineered PKS system to produce adipic acid in *S. venezuelae*



Organizational chart

Executive Committee

Nathan Hillson (Berkeley Lab)

John Gladden (Sandia National Laboratories)

Gregg Beckham (NREL)

Mark Butcher (Pacific Northwest National Laboratory)

Design
Gregg Beckham
Satoshi Yuzawa
Jamie Meadows
Jesus Barajas
Chris Johnson

Build
Sam Deutsch
Nathan Hillson
Jennifer Chiniquy

Test
John Gladden
Chris Petzold
Brenna Black
TBD (PD)
Davinia Salvachua
Holly Smith
Erica Gjersing
Scott Baker
Robby Robinson
Tom Metz
Young-Mo Kim
Wei-Jun Qian
Yuqlan Gao
Tom Fillmore
Megan Burnett

Learn
Hector Garcia Martin
Paramvir Dehal
Marcin Joachimiak
Corey Hudson
TBD (PD)
Jon Magnuson
Nancy Washton
TBD (PD)

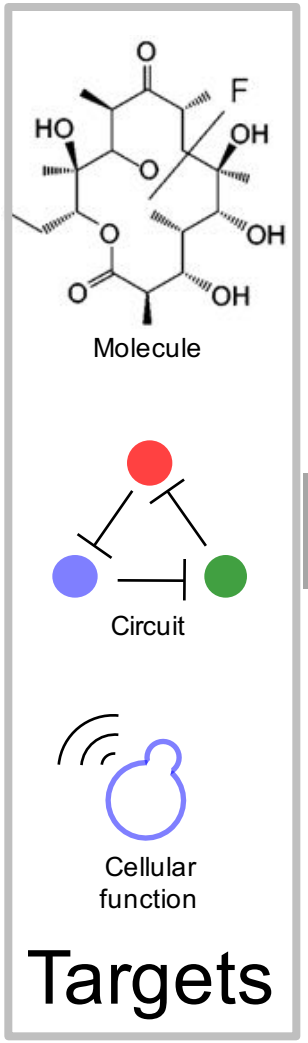
Host Onboarding
Vivek Mutalik
Thomas Ruegg
Payal Khanna

AGILE
BIOMANUFACTURING

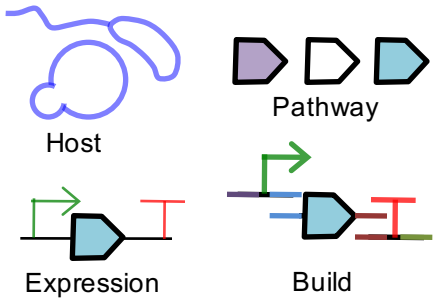
Integration and Predictive Scale-Up

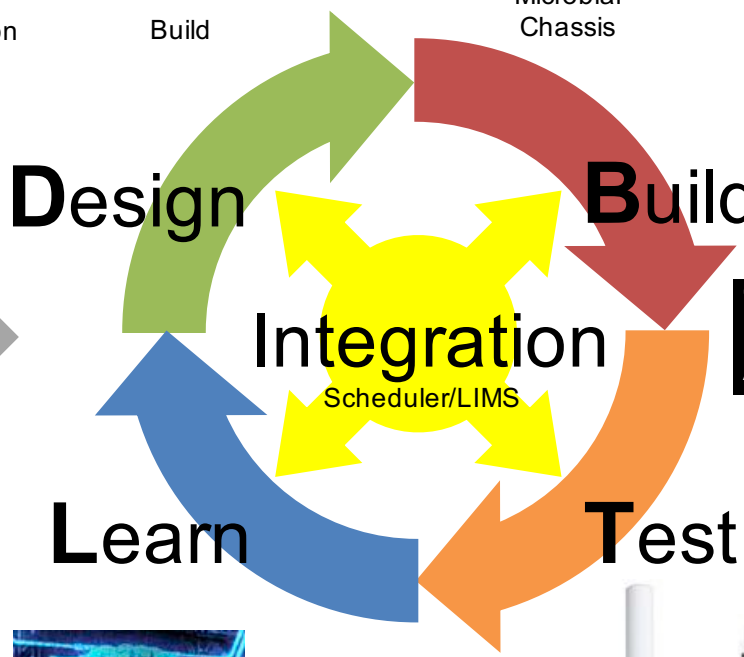
The Ames Laboratory — Creating Materials & Energy Solutions — U.S. Department of Energy
Argonne National Laboratory
INL Idaho National Laboratory
Los Alamos National Laboratory EST. 1943
Berkeley Lab
NREL National Renewable Energy Laboratory
Oak Ridge National Laboratory
Pacific Northwest National Laboratory
Sandia National Laboratories

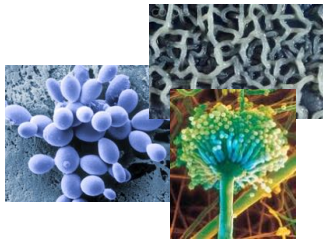
Scale-up & Validation in the BioFoundry



Selected Target







Microbial Chassis

QA/QC

Transformation

Target Achieved

Scale-Up & Validation

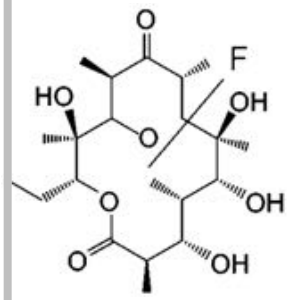
1-1000 L
(LBNL, NREL, et al.)
Predictive toolkits

Machine learning

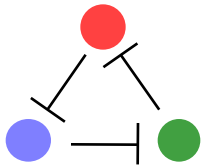
Mass spec.

Sample prep.

Goal for predictive scale-up



Molecule



Circuit

Cellular function

Targets

Integration of models across length scales to inform development

Optimize biomanufacturing processes from molecule to metabolic pathway to cell to fermenter to process

Infrastructure

THE Ames Laboratory
Creating Materials & Energy Solutions
U.S. DEPARTMENT OF ENERGY

Argonne NATIONAL LABORATORY

BERKELEY LAB
Lawrence Berkeley National Laboratory

INL
Idaho National Laboratory

Los Alamos NATIONAL LABORATORY
EST. 1943

NREL
NATIONAL RENEWABLE ENERGY LABORATORY

OAK RIDGE National Laboratory

Pacific Northwest NATIONAL LABORATORY

Sandia National Laboratories

National laboratories have the capabilities to conduct bench- and pilot-scale R&D to validate predictive scale-up toolkits

Biomass Feedstock National User Facility

Idaho National Laboratory

Production
- Feedstock characterization (chemical, solid fuel properties)
- Pairing feedstock to pathways
- Storage
- Pellet properties

Logistics
- Techno-economic assessments

Preprocessing
- Independent testing, development, & scale-up of preprocessing technologies

Conversion
- Feedstock Supply
- Scale-up & integration of preprocessing systems
- Feedstock Feasibility Studies

Feedstock Supply/Logistics Demonstration

Biomass Feedstock National User Facility

- Full-scale, integrated biomass processing system
  - Hammer mill grinding
  - Rotary drying and torrefaction
  - Pelleting and cubing
  - Multiple packaging options
- In operation since October 2013
  - Toll processing & characterization
  - Process Development
  - Preprocessing RD&D
- More than 1,000 tons of feedstock processed
  - Ag residues (corn stover, sugarcane bagasse)
  - Energy Crops (switchgrass, miscanthus)
  - Woody biomass (clean and whole tree chips)
  - Municipal Solid Waste
  - Cellulosic co-product

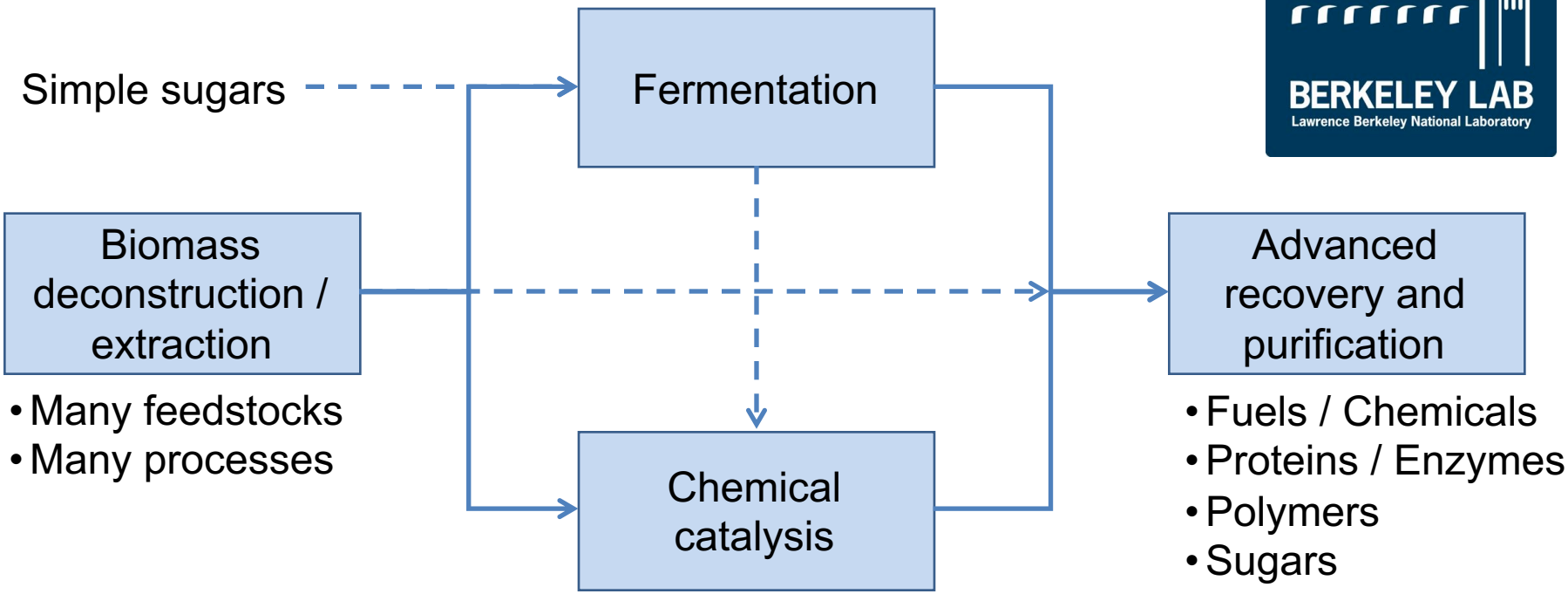
Reconfigurable PDU is located in 27,000-ft^3 high bay at INL's Energy Systems Laboratory

Idaho National Laboratory

Pilot facility -- ABPDU

Pilot facility -- ABPDU

- Process demonstration, integration and techno-economic / sustainability analysis across varied bio-process configurations, feedstocks and products

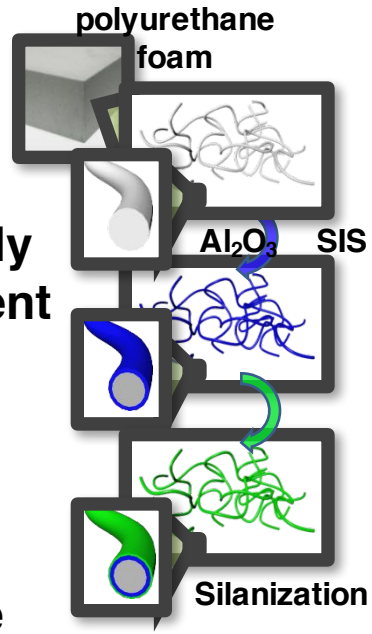


- Can focus on individual unit operations or several processes in succession

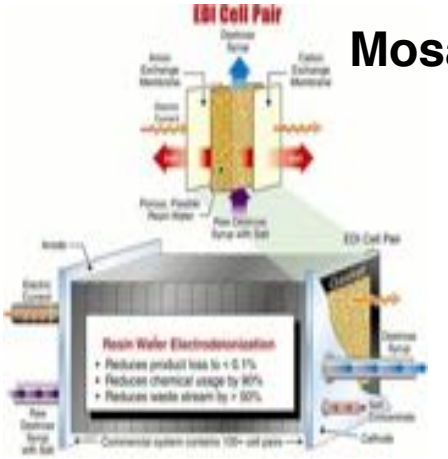
Separations capabilities

Suite of technologies reducing cost of feedstock preparation and bioproduct separations

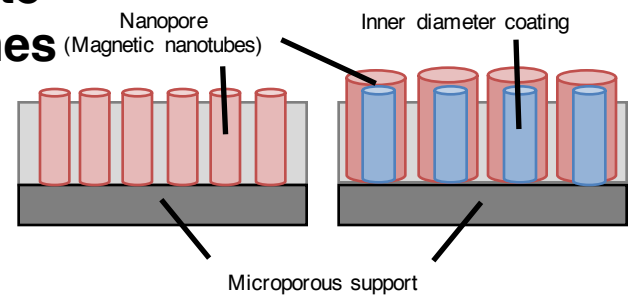
Reusable, environmentally benign adsorbent foams

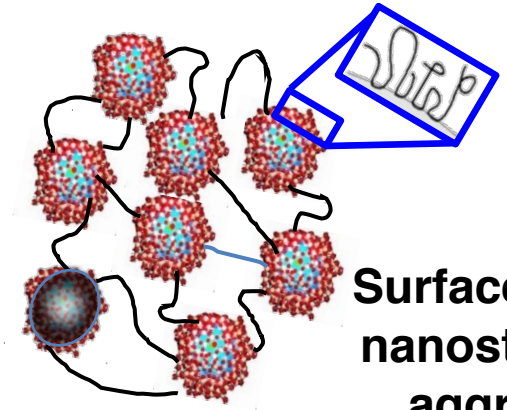


Mosaic ion-exchange resin wafer



Magnetic nanotube composite membranes

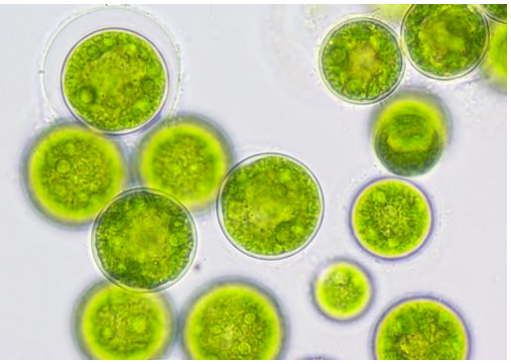




Surface-treated, nanostructured aggregates

Expertise in assessment and development of novel materials and processes improves the economics of biochemical production by streamlining separations steps

Algae capabilities



Pacific Northwest NATIONAL LABORATORY

Los Alamos NATIONAL LABORATORY EST. 1943

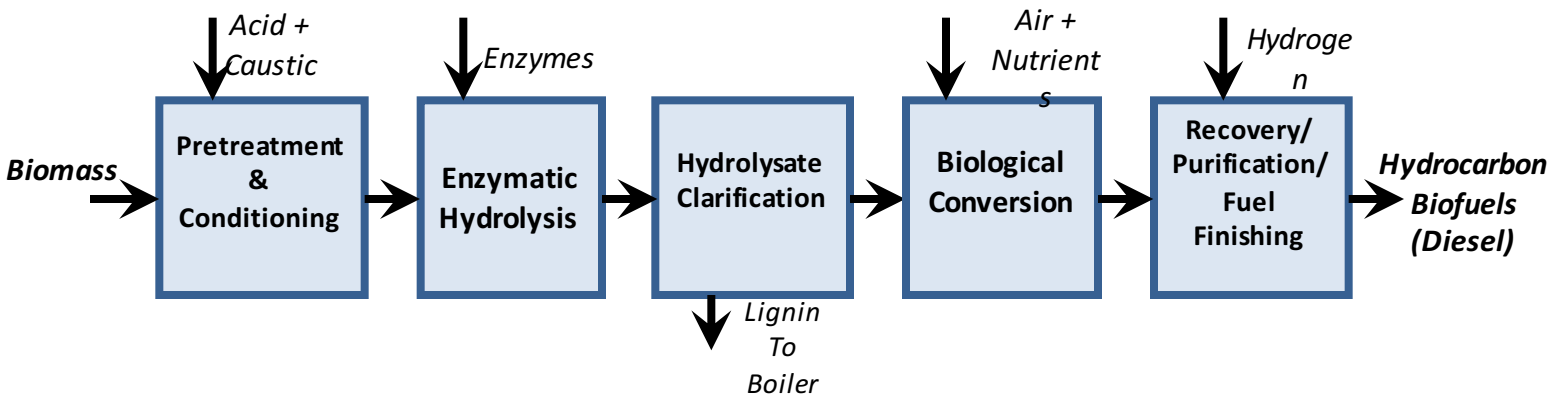
Argonne NATIONAL LABORATORY

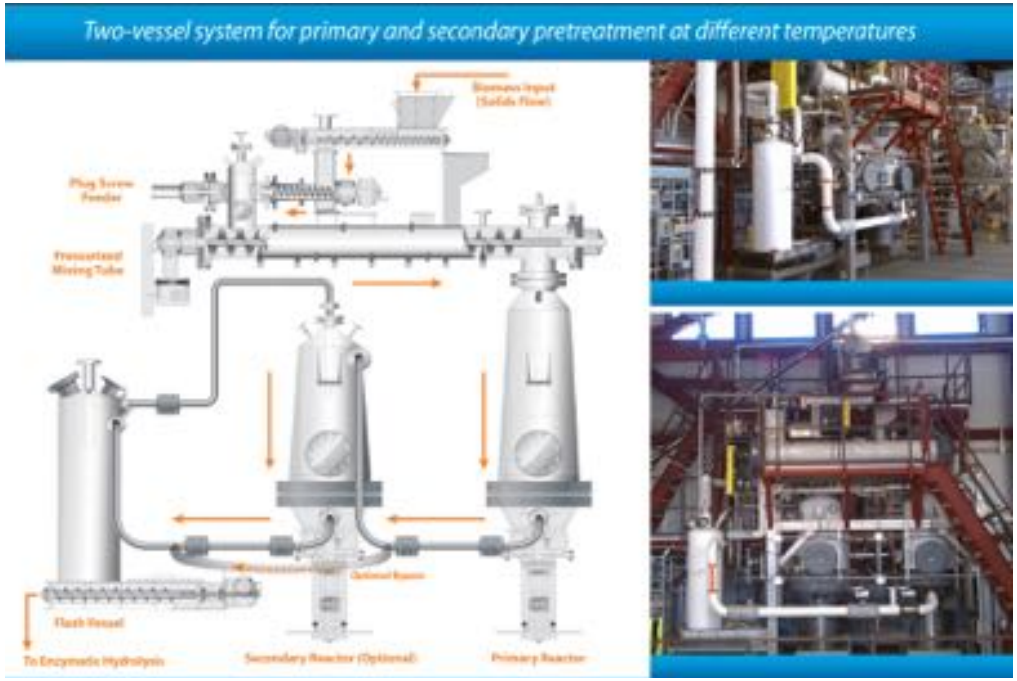
Sandia National Laboratories

Multiple core laboratory capabilities in algal cultivation, processes, and genetic engineering

- Environmental photobioreactors
- 100 L MicroBio mini-ponds
- Multiple scales of algae ponds across multiple laboratories

Full pilot-scale integration

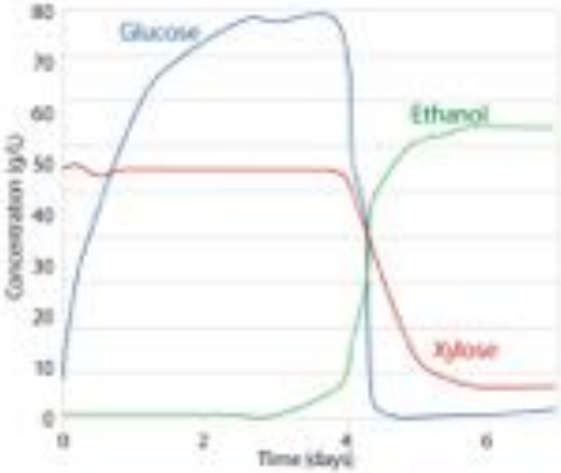




Large-scale fermentation

Fermentation

Bench-scale to pilot-scale capabilities for the conversion of biomass to sugars, fuels, and chemicals



Enzymatic hydrolysis and fermentation of pretreated corn stover

Fermentation applications

- Enzymatic hydrolysis and fermentation testing
  - Different enzyme mixtures
  - Pretreated lignocellulosic feedstocks at low and high solids concentrations
  - Aerobic and micro-aerophilic cultivation processes
- Microorganism evaluation and development
  - Evaluating microorganisms for production of biofuels and bioproducts
  - Producing inhibitor-resistant and high-product-tolerant microorganisms for biofuels production
- Aerobic and heterotrophic algal process development
  - Studying the effect of the environment on algae growth and lipid production
  - Using algae residue as a feedstock for biofuels production

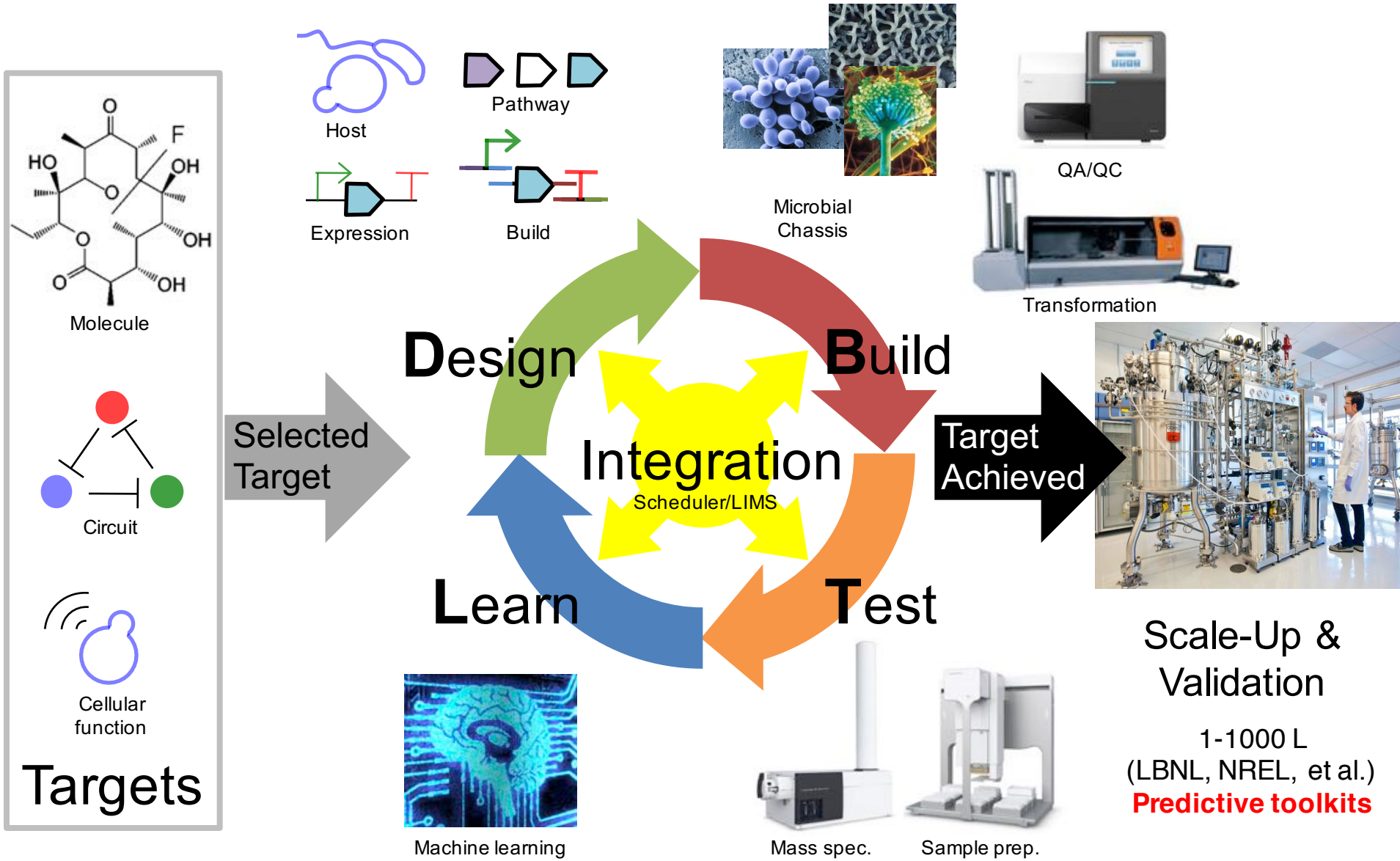
Bench-scale fermentation capabilities

- Fermentation systems with pH, temperature, and dissolved oxygen control
- Data acquisition and recipe control for the Biostat Q and Q-plus systems
- CO_2 and redox measurement capabilities
- At-line analytical equipment capable of monitoring glucose, xylose, ethanol, ammonium, and potassium ion with flexibility to measure other compounds
- Custom built bioreactor for high-solid lignocellulosic biomass slurries

Pilot-scale fermentation capabilities

- Multiple stirred bioreactor systems from 10 L to 100 L
- Two 160-L stirred vessels; two 1,500-L stirred vessels; four 9,000-L stirred vessels
- Feed and support vessels ranging in size from 100 L to 9,000 L

Questions?


Molecule
Circuit
Cellular function
Targets
Selected Target
Host
Pathway
Expression
Build
Microbial Chassis
QA/QC
Transformation
Design
Build
Integration
Scheduler/LIMS
Learn
Test
Target Achieved
Machine learning
Mass spec.
Sample prep.
Scale-Up & Validation
1-1000 L
(LBNL, NREL, et al.)
Predictive toolkits

Biomass Feedstock National User Facility

Expertise and capabilities to meet a customer's lab- and pilot-scale testing needs

- Sourcing for common and unique feedstocks
- Process development, testing, and design
- Feedstocks processed to partner specifications
- Feedstock characterization datasheets
- Packaging and shipping for partner testing

Working with industrial feedstocks during process design, scale-up, and integration can accelerate commercial-ization and prevent costly delays during commissioning and start-up

Idaho National Laboratory