#WTFrack is Up With Soluble Methane Mono-oxygenase: Controlling the Situation

Georgia Tech iGEM
November 1, 2014
• High pressure fluid injected into a gas well to generate small fractures
• Fractures release natural gas to be used as energy
• Extends the life of the wells to lower the need to create new wells
#FrackingProblems

*Flowback: The Consequences of Fracking*

- Waste water created from the process of fracking
- Collected in open air evaporation pools where water and volatile hydrocarbons including methane will evaporate

http://switchboard.nrdc.org/blogs/rhammer/frackings_after_math_wastewater.html
#MethaneGTFO

The Negative Impacts of Methane Contamination

- 95% of hydrocarbons in flowback is methane
- Methane is 21 times more harmful than CO₂
- Concentration of methane in the air has increased by 150% since 1750

[Source](http://therivardreport.com/eagle-ford-forum-ii-sustaining-the-boom-and-averting-the-bust/)
Current Treatments For Fighting Methane Contamination

- Flaring: combusts gaseous hydrocarbons (mandated in 2015)
  - releases carbon dioxide into the air
  - wastes potentially useful hydrocarbons
- REC (reduced emission completion technique)
  - extracts methane and other hydrocarbons from flowback for fuel
  - high capital cost of $500,000 per well prohibits widespread use

• Converting dissolved methane gas to liquid methanol would eliminate costly gas recovery arm of REC.
• Eliminates up to 25% of the capital cost
• Methanol recovered as condensate for use as fuel
# Shoutout2MyMethanotrophs

Utilizing SynBio to Solve the Methane Problem

- **Methylococcus capsulatus**
  - Most appreciable biological methane sink

- Contains multi-enzyme complex encoded in single operon
  - Soluble Methane Monooxygenase (sMMO)
Protein A (hydroxylase)
- Adds hydroxyl group to methane
- Composed of subunits: α, β, and γ
- Genes mmoX, mmoY, mmoZ

Protein B (effector)
- Regulates electron transfer
- Gene mmoB

Protein C (reductase)
- Acquire electrons from NADH
- Gene mmoC
Why not methanotrophs?

Scientists were able to express proteins B and C, however A was non-functional in E.coli.

- Scientists directly amplified the sMMO operon from the methanotrophs.
- Protein A could not fold properly due to incorrect subunit assembly (Simmons, 2002).
Producing full length antibodies in *E. coli* is a common issue.

- Complex proteins do not fold properly.

Research showed that titrating stoichiometric ratios of protein subunits increases functional protein yield.

- Produces full length antibodies.
#FollowtheBioBrickRoad

Designing/Developing Tools for Gene Titration

- Codon Optimization
- Identified Ideal RBS & Promoter Primers
- Generated Library of RBS & Promoter Combinations
- Validated Library in mCherry System
- Generated RBS & Promoter sMMO Combinations
- Will Test sMMO Activity

**Codon Optimization**

**Identified Ideal RBS & Promoter Primers**

**Generated Library of RBS & Promoter Combinations**

**Validated Library in mCherry System**

**Generated RBS & Promoter sMMO Combinations**

**Will Test sMMO Activity**
#CatchEmAll

Choosing RBS and Promoters of Varyingly Efficient Rates

- Chosen from Anderson Libraries for *E. coli*

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Relative Rate*</th>
<th>RBS</th>
<th>Relative Rates**</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH1</td>
<td>1</td>
<td>RH1</td>
<td>.914</td>
</tr>
<tr>
<td>PH2</td>
<td>.86</td>
<td>RH2</td>
<td>.914</td>
</tr>
<tr>
<td>PM1</td>
<td>.51</td>
<td>RM1</td>
<td>.471</td>
</tr>
<tr>
<td>PM2</td>
<td>.47</td>
<td>RM2</td>
<td>.471</td>
</tr>
<tr>
<td>PL1</td>
<td>.06</td>
<td>RL1</td>
<td>.007</td>
</tr>
<tr>
<td>PL2</td>
<td>.04</td>
<td>RL2</td>
<td>.007</td>
</tr>
</tbody>
</table>

* Rates are relative to the rate of the Anderson Promoter Consensus Sequence
** Rates were calculated from Salis Lab RBS Library Calculator and are relative to the theoretical T.I.R. of the Shine-Dalgarno Sequence
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

GAATTCCGCGGCCCCTTCTAGATG
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

EcoRI

GAAATTCCCGGGCCCGCTT

Xbal

Start of Part

CTAGATG
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

G A A T T C G C G G G C C G G C T T C T A G - R B S - A T G
#RBSThrowback
GT iGEM 2013’s RBS Primer Design

GAATTCCGGGCGCCGCTTCTAGAG-RBS-ATG
GT iGEM 2013’s RBS Primer Design

TGG AATT CGCGGGCGGCTT CTAG AG-RBS-TACTAG ATG
#WholeNewWorld

Expansion of RBS Primers to Promoter Primers

TGGAATTCCGCGGCGCGCTCTAGAG-RBS-TACTAGATG
ACCTTAAGCGCCGGCCGCAAGATCTC-RBS-ATGATCTAC
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

-Promoter-

ACCTTAAGCGCCGGGCAGAAGATCTC-RBS-ATGATCTAC
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

-Promoter- AAAGAG

ACCTTAAGCGCCGGGCGGAAGATCTC-RBS-ATGATCTAC
#RBSThrowback

GT iGEM 2013’s RBS Primer Design

CGCGGCCGCTTCTAGAG-Promoter-AAAGAG

ACCTTAAGCGCCCGGCGAAGATCTC-RBS-ATGATCTAC
#winning

Benefits of Our Universal Primers

<table>
<thead>
<tr>
<th>Qualities</th>
<th>Traditional 3A Assembly</th>
<th>Universal Primer Assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universality</td>
<td>Yes!</td>
<td>Yes!</td>
</tr>
<tr>
<td>Cost</td>
<td>Plasmid: $10s-100s</td>
<td>Primer &lt; $10</td>
</tr>
<tr>
<td>Time to Clone</td>
<td>5-7 days</td>
<td>3-4 days</td>
</tr>
<tr>
<td>Gel Screening</td>
<td>Differences Are Small</td>
<td>PCR Product is Obvious</td>
</tr>
</tbody>
</table>

gel confirmation of RBS primer Insertion
Validating the Primer Library and Design with mCherry

- Proof of concept with mCherry:
  - Test the effectiveness of promoter primer
  - To experimentally validate the promoter and RBS libraries

mCherry fluorescing cells (Image from University of Leicester)
• Different combinations of promoters and RBS were made successfully using primers.

• Flow cytometry shows that PH2/RH2 has a higher steady state than PL1/RL1.
# ItsPrimerTime

Applying Our Primers to the sMMO Genes

<table>
<thead>
<tr>
<th>sMMO part</th>
<th>Subunit α</th>
<th>Subunit β</th>
<th>Subunit γ</th>
<th>Protein B</th>
<th>Protein C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inserted in pSB1C3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>HE*RBS (promoterless)</td>
<td>In Progress</td>
<td>In Progress</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>LE*RBS (Promoterless)</td>
<td>In Progress</td>
<td>In Progress</td>
<td>✓</td>
<td>✓</td>
<td>In Progress</td>
</tr>
<tr>
<td>HE RBS/ He Promoter</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>HE RBS/ LE Promoter</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td>✓</td>
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</tr>
<tr>
<td>LE RBS/ LE Promoter</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

Gel confirmation of subunit γ (of protein A) and protein B with the PH2/RH2, PL1/RH2, PH2/RL1, PL1/RL1 promoter and RBS combination inserted
#ToInfinityAndBeyond

*Future Work with sMMO*

- Complete combinations of promoters and RBSs with sMMO genes
- Assemble the 5 genes of various combinations into an operon
- Test for sMMO activity to determine the optimal ratio of rates between genes.

sMMO Colometric Activity Assay (Image from Graham 1992)
Use *E. coli* in temporary flowback storage tanks to convert methane to methanol prior to REC.
#ReachingOut

Outreach and Collaboration

• Lambert High School
• Helped with iGEM Wiki and Jamboree Presentation

• Georgia State University
• Used our primers to express Mambalgin using pSB1C3
• Expanded our primers to CTG start codon

• University of Virginia
• Attended Human Practice Meet-Up
• Online Synthetic Biology Course
#GoingforGold

Checklist Requirements for Gold

✓ Document at least one new standard BioBrick Part or Device:
  ✓ BBa_K1539001- HE_Prom->HE_RBS->mCherry
  ✓ BBa_K1539002- LE_Prom->LE_RBS->mCherry
  ✓ BBa_K1539003- Beta subunit
  ✓ BBa_K1539005- Gamma subunit
  ✓ BBa_K1539008- sMMO B
  ✓ BBa_K1539013- sMMO C
  ✓ BBa_K1539021- High Efficiency RBS Primer
  ✓ BBa_K1539034- Low Efficiency RBS Primer
  ✓ BBa_K1539055- High Efficiency Promoter Primer
  ✓ BBa_K1539089- Low Efficiency Promoter Primer
#GoingforGold

**Checklist Requirements for Gold**

- Experimentally validate that at least one new BioBrick Part or Device of your own design and construction works as expected and submit to iGEM Parts Registry
  - LE and HE RBS Primers
  - LE and HE Promoter Primers
- Broader implications beyond the bench
  - Universal library of promoter and RBS primers for modulatory expression
  - Efficient processing of fracking waste with reduced cost
#GoingforGold

**Checklist Requirements for Gold**

- Improve the function of an existing BioBrick Part or Device
  - Improved on GT iGEM 2013 RBS Primer technique
  - Added promoter primers and created libraries
- Help any registered iGEM team from another school or institution
  - Lambert HS, Georgia State, University of Virginia
#ThanksForTheMemories

Special Thanks

IDT
INTEGRATED DNA TECHNOLOGIES

NEW ENGLAND BioLabs Inc.

GenScript
genious

MACROxGEN

Georgia Tech
Wallace H. Coulter Department of Biomedical Engineering
at Georgia Tech and Emory University

Georgia Tech Chemistry and Biochemistry
College of Sciences


References

**Traditional 3A**

**Day 1**
- Destination
- Day 2: Colony Expansion
- Day 4
- Day 5: Colony Expansion
- Day 6-7: Sequencing

**PCR Amplification**
- PCR Round 1: ~hours
- Dpn1 Digest + Cleanup: 1.5 hours
- PCR Round 2: ~hours
- E/P Digest + Cleanup: 1.5 hours
- Day 2: Colony Expansion
- Day 3-4: Sequencing

**Universal Primers**

**Day 1**
- Gene
- Day 2: Colony Expansion
- Day 3-4: Sequencing
- Day 6-7: Sequencing

**RBS**

**Pro**

**Destination**

**Pro**

**RBS**

**Gene**