Translational Coupling:

A tool for evaluating the translation of heterologous proteins in *Escherichia coli*

Troubleshooting a limonene production pathway in *E. coli*
Let’s do Some Cloning!

pGFP
Let’s do Some Cloning!

pGFP
Let’s do Some Cloning!
Let’s do Some Cloning!

![Cell diagram with pGFP plasmid and GFP label]
Applications of Limonene
# Limonene as a Biofuel

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Flash point (°C)</td>
<td>Min: 38</td>
<td>51.1</td>
<td>14</td>
<td>83</td>
<td>43</td>
</tr>
<tr>
<td>Autoignition temp (°C)</td>
<td></td>
<td>210</td>
<td>365</td>
<td>203</td>
<td>237</td>
</tr>
<tr>
<td>Freezing Point (°C)</td>
<td>Max: −40 (−47)</td>
<td>−51</td>
<td>−114</td>
<td>−10</td>
<td>−74</td>
</tr>
<tr>
<td>Boiling Point (°C)</td>
<td>Max: 300 (340)</td>
<td>280</td>
<td>78</td>
<td>216</td>
<td>176</td>
</tr>
<tr>
<td>Density @ 15 °C (kg/L)</td>
<td>0.775-0.840</td>
<td>0.820</td>
<td>0.79</td>
<td>0.75</td>
<td>0.84**</td>
</tr>
<tr>
<td>Heat of Comb. (MJ/kg)</td>
<td>Min: 42.8</td>
<td>43.02</td>
<td>29.70</td>
<td>44.15</td>
<td>45.0</td>
</tr>
<tr>
<td>Energy density (MJ/L)</td>
<td></td>
<td>35.3</td>
<td>23.5</td>
<td>33.1</td>
<td>37.8</td>
</tr>
</tbody>
</table>

**Notes:** * British Petroleum; ** Density@20°C;
Enzymatic Production of Limonene

Acetyl-CoA $\xrightarrow{\text{AtoB}}$ HMGS $\xrightarrow{\text{tHMGR}}$ Mevalonate

IPP $\xrightarrow{\text{MVD1}}$ Mevalonate – 5 Phosphate

GPP $\xrightarrow{\text{ERG8}}$ Limmonene

DMAPP $\xrightarrow{\text{ispA}}$ FPP

Gene Source
- *E. coli*
- *S. cerevisiae*
- *Abies grandis*
- *Citrus Limon*

Production Vectors

Acetyl-CoA → HMG → Mevalonate → FPP → Limonene

AtoB → HMGS → pBbA5C → tHMGR

ISP → IPP → GPPS → Mevalonate – 5 Phosphate

OVERALL

Gene Source
E. coli
S. cerevisiae
Abies grandis
Citrus Limon
Production Vectors

pBbA5C with geranyl pyrophosphate synthase insertion

Acetyl-CoA → HMGS → tHMGR → ERG12 → ERG8 → MVD1 → idi → ispA

Acetyl-CoA

HMG

Mevalonate – 5 Phosphate

GPPS

OVERALL

FPP GPP

Gene Source
E. coli
S. cerevisiae
Abies grandis
Citrus Limon

Amorphadiene
Production Vectors

J23102 – Limonene synthase
J23102-BBa_I742111

Gene Source
- E. coli
- S. cerevisiae
- Abies grandis
- Citrus Limon
Production Vectors

Acetyl-CoA → HMGS → HMG CoA Reductase (tHMGR) → Mevalonate

Mevalonate → Mevalonate-5-Phosphate → IPP (Isopentenyl Pyrophosphate)

IPP + IPP → GPP (Geranyl Pyrophosphate)

GPP + IPP → FPP (Farnesyl Pyrophosphate)

FPP + IPP → DMAPP (Dimethylallyl Pyrophosphate)

Amorphadiene Synthase (ADS)

Amorphadiene Synthase (MVD1, ERG8) → Amorphadiene

Gene Source:
- E. coli
- S. cerevisiae
- Abies grandis
- Citrus Limon
Summary of Production Strains

AtoB → HMGS → tHMGR → ERG12 → ERG8 → MVD1 → idi → ispA

Amorphadiene synthase

J23102 – Limonene synthase

OR

+ pBbA5C

Amorphadiene

J23102

Limonene

ADS
Summary of Production Strains

pBbA5C with geranyl pyrophosphate synthase insertion

AtoB → HMGS → tHMGR → ERG12 → ERG8 → MVD1 → idi → GPPS

J23102 – Limonene synthase

J23102 + LimS1

Limonene
Production Assay

- 5 ml overnight cultures
- Diluted
- 40 ml cultures
  - IPTG induced
  - Dodecane overlay
  - Extracted Diluted
- Gas chromatography
  - Mass spectrometry
Production Assay Results

Target Compound (Limonene Standard)

Library Standard

Total Ion Count

Retention Time (min)

Total Ion Count

Limonene

Dodecane

pBbA5c + J23102-LIMS1

pBbA5c + J23102

(X10,000)

(X10,000,000)
Dissecting the Problem

Where do we start?

J23102 – Limonene synthase
Amorphadiene synthase

= Limonene Amorphadiene
Central Dogma

**Step:**
- DNA
- mRNA
- Protein
- Phenotype

**Confirmation:**
- Sequencing: J23102 well characterized
- Questionable
- Nothing
Codon Usage in *E. Coli*

Percentage of Codons

- Classic LimS1
- CO LimS1
- Native MalE

Codon Quality

0-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100
# Troubleshooting Translation

<table>
<thead>
<tr>
<th>Pros</th>
<th>Method</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Quantifiable</td>
<td>Mass Spectrometry</td>
<td>Expensive Multi-variable Functionality unknown</td>
</tr>
<tr>
<td>Sensitive Quantifiable</td>
<td>Western Blot</td>
<td>Expensive Functionality unknown</td>
</tr>
<tr>
<td>Protein functionality Enzymatic activity</td>
<td><em>In vitro assay</em></td>
<td>Purification purification Tags may alter activity</td>
</tr>
</tbody>
</table>
When your gene is being fully translated...

Ribosome helicase activity melts the hairpin...

The stop codon releases the ribosome...

Liberated RBS is available for binding...

Reporter gene is translated...
If the target gene is not translated...
Cloning Into the TCC

Is it translating?

Translation?

Yes

No

Gene of Interest

Reporter Gene (RFP)
Translational Coupling Cassette

5' - A C U A G U C - 3'

24 Mendez-Perez et al. Metab Eng 2012
Two genes were cloned into the cassette for characterization

- E0040: green fluorescent protein
  - Positive control

- Limonene synthase with an embedded stop codon
  - Negative control
96-Well Plate Reader Assay

5 ml overnight cultures → Diluted → 96 well plate → Tecan Fluorescence plate reader
96-well Characterization Data

- Strains: LimS1(NS), TCC-RFP, COLimS1(MS), COLimS1(NS), GFP-TCC-RFP
- Measurements: RFP / OD (au)
- Timepoints: 12 Hours, 18 Hours, 24 Hours

Graph showing the RFP/OD values for different strains at various timepoints.
Typhoon Imager Assay

1. Dropped culture
2. Incubated overnight
3. Scanned in typhoon imager

5 ml overnight cultures
Typhoon Plate Reader Data

- GFP-TCC-RFP
- LimS1-TCC
- COLimS1-TCC
- TCC-RFP
- LimS1-Stop-TCC
- COLimS1-Stop-TCC
Typhoon Plate Reader Data

- LIMS1 (NS)
- TCC-RFP
- CO-LIMS1 (MS)
- CO-LIMS1 (NS)
- GFP-TCC-RFP

Strains

Typhoon Pixel Density
Conclusions from Translational Coupling Cassette

- Limonene synthase (Bba_I742111) is not being translated
- Translation of codon-optimized limonene synthase
- The cassette works as designed
  - Available for all teams to use (BBa_K762000)
Further Production Assays

pBbA5C with geranyl pyrophosphate synthase insertion

AtoB → HMGS → tHMGR → ERG12 → ERG8 → MVD1 → idi → ispA

- pBbA5c + J23102-COLIMS1
- pBbA5c + J23102

Retention Time (min)

Total Ion Count

Limonene

Dodecane

(X10,000,000)
Conclusions and Future Steps

- **In vitro** assay after purification
- Western blot with His-Tag antibodies
- Different production techniques

Clone into TCC
- Run **in vitro** assay after purification
- Western blot with His-Tag antibodies

J23102 – Limonene synthase

**pBbA5C with geranyl pyrophosphate synthase insertion**

AtoB → HMGS → tHMGR → ERG12 → ERG8 → MVD1 → idi → GPPS

Limonene
Translational Coupling Cassette

A synthetic biology tool for troubleshooting the expression of heterologous proteins
iGEM Outreach
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James Madison Memorial High School advanced placement biology classes
Lab Visits and Brainstorming Sessions

Facilitating the formation of their high school iGEM team
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