Master of Regulation
Tandem Promoters & dCas9-based Multi-level Promoters

2013 iGEM of Wuhan University
Proper Promoter is crucial to ideal performance of Genes

Just like Power Source to Equipments

25000V HighSpeed rail
375V Tesla S
4V iPhone
Brainstorming
Expression at any-amount, any-where
Expression Control in non-model species is hard

Algae

Flower

Fungus
Brainstorming

Sliding Scribing

Base of Rheostat

Resistance

Multilevel Resistance
The Base

Tandem Promoters

Sliding Scribing

Modeling

Human Practice
Tandem Promoters - The Base

Employ limited promoters to reach various expression levels

Promoter1 → Promoter2 → RFP

E X S P

P1:J23102  P2:J23106  P3:J23116
Employ **limited** promoters to reach **various** expression levels

**Tandem Promoters - The Base**

**BBa_K1081002** *

(* One of Our Seven Biobricks)
Employ limited promoters to reach various expression levels

Tandem Promoters - The Base

BBa_K1081002

BBa_K1081003

BBa_K1081004

BBa_K1081005

BBa_K1081006

BBa_K1081007

BBa_K1081008
Expression Assay

### Strength of Tandem Promoters

<table>
<thead>
<tr>
<th>Strength Value Combinations</th>
<th>Promoter Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: J23102</td>
<td>0.86</td>
</tr>
<tr>
<td>2: J23106</td>
<td>0.47</td>
</tr>
<tr>
<td>3: J23102-102</td>
<td>1.35</td>
</tr>
<tr>
<td>4: J23106-106</td>
<td>0.77</td>
</tr>
<tr>
<td>5: J23102-102</td>
<td>1.30</td>
</tr>
<tr>
<td>6: J23106-106</td>
<td>0.70</td>
</tr>
<tr>
<td>7: J23116-116</td>
<td>0.93</td>
</tr>
<tr>
<td>8: J23116-106</td>
<td>0.98</td>
</tr>
<tr>
<td>9: J23116-102</td>
<td>0.69</td>
</tr>
</tbody>
</table>

- **Diverse Combinations**
- **Broader Strength Range**
Primary Achievements

✓ Seven New Biobricks (From K1081002 to K1081008)

✓ Tandem Promoters with Higher Strength Threshold

✓ More Levels for Potential Regulations
The Base

Sliding Scribing

Cas9-based Regulation

Modeling

Human Practice
dCas9 - Sliding Scribing

Tandem Promoter

Sequence-specific Targeting

Multilevel Promoter
dCas9 - Sliding Scribing

CRISPR System

- Simple
- Convenient
- Stable System
dCas9 - Sliding Scribing

Repress RNAP binding & Initiation
dCas9 Construction & Expression

One of Our Biobricks: BBa_K1081000
Multi-level Regulation

Level 1

Promoter 1  Promoter 2  Reporter Gene

Level 2

dCas9
gRNA

Level 3

dCas9
gRNA
Multi-level Regulation Result 1

Double Promoter: J23106-116+dCas9
Partial Repression: J23106-116+dCas9 +gRNA (Repress J23106)
Complete Repression: J23106-116+dCas9 +gRNA (Repress J23116)

Fluorescence/OD600

- Double Promoters: J23106-116+dCas9
- Partial Repression: J23106-116+dCas9 +gRNA (Repress J23106)
- Complete Repression: J23106-116+dCas9 +gRNA (Repress J23116)
Multi-level Regulation Result 2

Double Promoters
J23106-102+dCas9

Partial Repression
J23106-102+dCas9
+gRNA (Repress J23106)

Fluorescence/OD600

Double Promoters
J23106-102 + dCas9

Partial Repression
J23106-102 + dCas9 + gRNA (Repress J23106)
Improve Regulation by aCas9
More levels

Level 4

Level 5
aCas9 Construction
Applications of dCas9: Multi-level regulator

Constructed an aCas9 plasmid
Future Work

Changes of Number, Type and Order

More Regulatory Sites
The Base
Sliding Scribing
Modeling
Design your own Multilevel Promoter
Human Practice
Modeling

Design Your Own Multilevel Promoter (MP)

1. Determine the required expression levels
2. Design the tandem-repeat promoter (TRP)
3. Design the targeting sequence and gRNA
1. Expression Level of MP

- **Level 1**: No inhibition
- **Level 2**: Inhibit one sub-promoter
- **Level 3**: Inhibit both sub-promoters

**Example.**

<table>
<thead>
<tr>
<th>Target</th>
<th>TRP Before Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1:</td>
<td>0.06</td>
</tr>
<tr>
<td>Level 2:</td>
<td>0.33</td>
</tr>
<tr>
<td>Level 3:</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Promoter Strength**

- **Level 1**: P1-P2 + dCas9
- **Level 2**: P1-P2 + dCas9 + gRNA (Rep.P1)
- **Single Promoter**: P2 + dCas9
- **Level 3**: P1-P2 + dCas9 + gRNA (Rep.P2)
Modeling

Design Your Own Multilevel Promoter (MP)

1. Determine the required expression levels
2. Design the **tandem-repeat promoter** (TRP)
3. Design the **targeting sequence** and gRNA
2. Tandem-repeat Promoter Model

\[
\text{Strength}' = 1 - \prod_{i}^{n}(1 - p_i n^j)
\]

**Experiment Result versus Model Prediction**

- Error less than 10%
- Compared with published data
- Compared with our data
The Derivation of the model

**Kinetic part**

1. **Transcription-Translation analysis**

\[
\frac{d[\text{mRNA}]}{dt} = \alpha[\text{RP}] - \lambda[\text{mRNA}]
\]

\[
\frac{d[\text{protein}]}{dt} = \nu[\text{mRNA}] - k[\text{protein}]
\]

**Strength**

\[
\text{Strength} = \frac{\nu \alpha}{\lambda k} [\text{RP}] = \xi[\text{RP}]
\]

2. **Time scale separation** of Transcription initiation and RNAP binding

\[
\text{DNA} + \text{RNAP} \xrightarrow{K_1} \text{RP} \xleftarrow{k_2} \text{RP} \xrightarrow{k_3} \text{RP}_o
\]

\[
\text{DNA} \rightarrow \text{RNA} \rightarrow \text{protein}
\]

So \(p_i\) is proportional to \([\text{RP}]\)
2. Tandem-repeat Promoter Model

The Derivation of the model

Thermodynamic part

3. RNAP binding Boltzmann equilibrium probability analysis

\[
\frac{p_{ij}}{p_i p_j} = \frac{Z(P-2)Z_{tot}}{Z(P-1)^2} = \frac{N!}{(P-2)!(N-P+2)!} \times \frac{N!}{P!(N-P)!} = \frac{(N-P+1)(P-1)}{(N-P+2)P} = \frac{NP}{NP} = 1
\]

4. RNAP binding probability to tandem promoter

\[q_i = 1 - p_i; \quad p_{tot} = 1 - \prod_{i}^{n} q_i\]

5. Tandem promoter strength adjustment

\[p_{tot} = 1 - \prod_{i}^{n} (1 - p_i n^j) \quad \Rightarrow \quad \text{Strength} = \frac{\mu \xi}{V} [1 - \prod_{i}^{n} (1 - p_i n^j)]\]
Modeling

Design Your Own Multilevel Promoter (MP)

1. Determine the required expression levels
2. Design the tandem-repeat promoter (TRP)
3. Design the targeting sequence and gRNA
3. Cas9 Off-target Model

Requirement of regulation: Simplicity & Orthogonality

- If possible, choose a target that has at least 4bp difference with its most similar sequence.
- Otherwise, employ our model to find out a relatively better choice.
1. All target can be divided to two groups. The energy function $\Delta G' = F()$ may be a sigmoid function, result in insensitive to energy change at two extremes.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Single Mismatch tolerance</th>
<th>G/C</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCATGCTGTTTCATATGATC</td>
<td>low</td>
<td>7</td>
<td>[4]</td>
</tr>
<tr>
<td>AACTTTTCAGTTTAGCGGUCU</td>
<td>low</td>
<td>8</td>
<td>[3]</td>
</tr>
<tr>
<td>TGTGAAGAGCTTCACTGAGT</td>
<td>low</td>
<td>9</td>
<td>[1]</td>
</tr>
<tr>
<td>GATGCCGTTCTTCTGCTTGT</td>
<td>low</td>
<td>10</td>
<td>[8]</td>
</tr>
<tr>
<td>AGTCCTCATACTCCCTCAAGC</td>
<td>low</td>
<td>10</td>
<td>[1]</td>
</tr>
<tr>
<td>GAGATGATGGCCCCTTCTTTC</td>
<td>low</td>
<td>11</td>
<td>[2]</td>
</tr>
<tr>
<td>CTCCCTCAAGCCAGGGCCCCGC</td>
<td>low</td>
<td>15</td>
<td>[1]</td>
</tr>
<tr>
<td><strong>Ave. G/C 10.0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCAGATGTAGTGTTTCCACA</td>
<td>medium</td>
<td>9</td>
<td>[1]</td>
</tr>
<tr>
<td>GGTGGTGCGAGATGAACCTTCA</td>
<td>high</td>
<td>10</td>
<td>[8]</td>
</tr>
<tr>
<td>GGGGCCACTAGGGACAGGAT</td>
<td>high</td>
<td>13</td>
<td>[2]</td>
</tr>
<tr>
<td>GTCCCTCCACCCCCACAGTG</td>
<td>high</td>
<td>14</td>
<td>[2]</td>
</tr>
<tr>
<td>GGGCAGGGGCAGCTTGCCGG</td>
<td>high</td>
<td>16</td>
<td>[8]</td>
</tr>
<tr>
<td><strong>Ave. G/C 12.4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. The binding energy between gRNA and DNA determine the targeting efficiency. Different position on gRNA has different weight (importance).

Calculate $\triangle G(i)$ according to NN nearest neighbor model

\begin{align*}
\text{AT} \quad \text{terminal} &+ \quad \text{GG} + \quad \text{CG} + \ldots \quad \text{CC} + \quad \text{CC} + \quad \text{GG} \quad \text{terminal} \\
\text{TG} \quad \text{terminal} &+ \quad \text{CT} + \quad \text{GC} + \ldots \quad \text{GC} + \quad \text{GC} + \quad \text{CC} \quad \text{terminal} \\
\end{align*}

\triangle G(1), \quad \triangle G(2), \quad \triangle G(3), \quad \triangle G(17), \quad \triangle G(18), \quad \triangle G(19)

$$\Delta G' = F(\bar{\omega} \cdot \bar{a} + b) = F(\bar{\omega} \cdot [\Delta G(1), \Delta G(2), \Delta G(3), \ldots, \Delta G(19)]^T + b)$$
3. Cas9 Off-target Model

2. $\Delta G(i)$ determines the targetting efficiency in the mismatch sensitive case

Our result based on DNA thermodynamic model and data from [1]

The data of Single-nucleotide specificity of Cas9 from [7]
3. Derive the kinetic functions of Cas9 binding.

\[
\begin{align*}
\frac{[\text{measurement}_1]}{[\text{measurement}_2]} &= \frac{[TF_1]}{[TF_2]} = \frac{[E_1 S]}{[E_2 S]} = \frac{K_{d_2}}{K_{d_1}} = \frac{\Delta G'_i}{e^{RT}} = \frac{\Delta G'_i - \Delta G'_f}{RT} \\
p_{bw} &= \frac{[E_0]}{[E_0] + K_{dw}} \\
p_{br} &= \frac{[E_0] + K_{dr}}{[E_0] + K_{dr}} = \frac{[E_0] + K_{dr}}{[E_0] + K_{dr}} = \frac{\Delta G'_i}{e^{RT}} \quad \omega = [0.21, 0.25, 0.30, 0.39, 0.36, 0.32, 0.35, 0.39, 1.04, 1.19, 1.20, 1.05, 1.22, 2.80, 1.83, 1.92, 2.30, 2.36, 2.09]
\end{align*}
\]

Model prediction vs. data from [3] and [4]
4. Kinetic analysis show expression time of Cas9 is also crucial for off-target control in editing.

\[
\text{Cas9+DNA} \xleftrightarrow{\text{Reversible binding}} \text{Cas9-DNA} \xrightarrow{\text{Irreversible enzymatic reaction}} \text{Double strand break DNA} + \text{Cas9}
\]

\[
\therefore [C] = [A_0][1 + \left(\frac{1}{k_a - k_b}\right)(k_b e^{-k_a t} - k_a e^{-k_b t})]
\]

\[
k_a = \frac{k_{\text{cat1}}[E]^*}{K_M}, \quad k_b = \frac{k_{\text{cat2}}[E]^*}{K_M}, \quad K_M = \frac{k_{-1} + k_{\text{cat}}}{k_1}, \quad K_a = \frac{k_1}{k_{-1}} = e^{-\frac{\Delta G'}{RT}}
\]

Boundary conditions were set as \([A_0]=1.0, [B0]=[C0]=0, ka=0.2 \text{ min}^{-1}, kb=0.1 \text{ min}^{-1}\) for blue line; and \([A0]=1.0, [B0]=[C0]=0, ka=0.1 \text{ min}^{-1}, kb=0.05 \text{ min}^{-1}\) for red line.
Modeling Conclusion

- Produce designed expression level
- Switch between several expression levels
- Explore the best output in a systematic way
iGEM Popularization

Communication with 2013-HUST & HZAU

Forum in Wuhan

Display during the Science Festival

Communication with CAU team

Communication with USTC team

Lectures for Bio students

Lectures for Chem students
To iGEM-2013
Shenzhen_BGIC_ATCG

Helping Others

Constructed a functional dCas9 plasmid for their project
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• Prof. Zhixiong Xie, Prof. Xiangdong Gao, Prof. Yu Chen
• College of Life Sciences, WHU
Thank You!
References

Expression level of MP

90% inhibition
1. Inherit inaccuracy by employing "free DNA thermodynamic model" to mimic "protein influenced DNA-RNA binding"

2. Unable to calculate "b" as the unavailable of enzymatic data.

3. Unable to predict the variation in the "platform" area of binding energy.

However,

They said "it is difficult to define simple rules for gRNA design based on the results of the four studies" --Dana Carroll, *Nature Biotechnology*, 2013

And we provided our insights
changing the operon order of GGPP synthase and taxadiene synthase affect taxadiene synthase expression by 20% (GGPP synthase plus its RBS is ~1kb)

Why not IPTG, etc.

- Just two platform stage, Noise

- Non-model organism that IPTG may not work - traditional Chinese medicine, fungi, algae...

![Fluorescence Intensity vs. Concentration of IPTG](chart1.png)

![Comparison of Strength with gRNA](chart2.png)
Novel aCas9 Platform

- Gal11 interacts with Gal4-1/-2 & VP16
- CI repressor interacts with CI repressor
Normalized gRNA Assembly

Three cycles of Overlap PCR
TALE-Based Multi-Level Regulator?

TALE-Based Multi-Level Regulator?

TALE

- Lower Cost
- Different gRNAs+dCas9
- Normalized gRNA Assembly

dCas9

Targeting Efficiency
Explore the Best Output in a Systematic Way