“BUILD A GENOME”
Designing and Synthesizing Sc2.0

Chris Von Dollen, Rose Xie, Yuan Guo, Pablo Lee, James DiCarlo, Ingrid Spielman
Johns Hopkins University
Outline

Chris  Why Build Synthetic Yeast?
Rose   The Build a Genome Course
Yuan   Improving Build a Genome Workflow / Side Project #1
James  Build a Genome Standard - RFC38, Genome Stabilization / Side Project #2, 3
Pablo  Custom Software / Side Project #4
Chris  Genome Shuffling
Ingrid  Deliverables
MEET Sc2.0

(Saccharomyces cerevisiae)
Not Your Average Yeast

• Custom blueprint
• Remove junk DNA
• Cut out introns
• Add loxPsym sites
• Reorganize tRNA genes
Replacing Native Yeast Sequence
With Synthetic DNA

Chromosome 9R
One 90 kb piece
Replacing Native Yeast Sequence With Synthetic DNA

Chromosome 9R
One 90 kb piece

- Non-essential ORF
- Uncharacterized ORF
- Removed pseudogene
- Removed Ty1 LTR
- TAG > TAA
- Essential ORF
- Dubious ORF
- Removed tRNA(I)
- PCRTag
- loxPsym
Uh... So Where is this Going?

• Fully synthetic eukaryotic genome (a first)
• Streamlined custom organism
• Minimal genome 'goal seek'
• BioBrick and Device chassis
• Map gene relationships
• Distill the rules of genome structure
BUILD A GENOME COURSE

Rose Xie
Goals
Goals

- Low cost undergraduate labor (i.e. FREE)
Goals

- **Low cost undergraduate labor (i.e. FREE)**
- Hands-on lab experience
- Exposure to cutting edge research
- Develop independence
- Gain presentation and speaking skills
Goals

• **Low cost undergraduate labor (i.e. FREE)**

• Hands-on lab experience

• Exposure to cutting edge research

• Develop independence

• Gain presentation and speaking skills

• **Build the starting materials for Sc2.0**
Lectures

• Fundamental genetics
• Bioinformatics
• Central concepts of synthetic biology: recombinant DNA technology, gene synthesis, etc.
• Bioethics
• Economics
Boot Camp

- 8 Sessions
- Master lab techniques
- Milestones
- First lab meeting
Independence!

• Each student assigned ~12 building blocks for a total of 10,000 bp

• Each student gets their own key to the laboratory

• Regular “lab meetings” held (mini-presentations, troubleshooting)
Working hard...
But really...
Evaluation/Results

- Ability to synthesize assigned building blocks: ~9 out of 12
- Number of perfect clones (“winners”): typically 3 out of 12
First pass of chromosome 3 completed!
Over 50 B-A-G Grads!
Beyond Build-a-Genome

• B-A-G Mentors!
• Side projects (up next!)
• Job opportunities!
• “The Mosh Pit” business competition: 2 BAG graduates won 2nd prize = $10,000!

Saturday, October 31, 2009
Beyond Build-a-Genome

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• **World domination**?!
Beyond Build-a-Genome

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- World domination?!

Make Build-a-Genome replicate
IMPROVEMENTS IN B-A-G PROTOCOLS

Zheyuan Guo
Chromosome 3 ~330 kbp

3L ~ 110 kbp

B1

B2

B3

B2.1 B2.2 B2.3 B2.4 B2.5 B2.6 B2.7

B2.16

4 X ~28 kbp

15 X ~750 bp BB

tPCR

AACTTCGTCAGTATCAGCTTTATCCTTTATCACCCACATCAGCCATAAATATTAGCTCCAAAAGTTTGAG

16 X ~70 nt (nucleotides)
Typical B-A-G Work Flow
Typical B-A-G Work Flow

1st week

3rd week

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Typical B-A-G Work Flow

PROBLEM 1

1 week

2-3 Iterations

1st week

3rd week

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Typical B-A-G Work Flow

PROBLEM 1
- tPCR
- fPCR
- GEL
- 2-3 Iterations
- 1 week
- 1st week
- 3rd week

PROBLEM 2
- TRANSFORMATION
- csPCR
- GEL
- SEQUENCING
- 5th week
- 6th week
Problem #1

t-PCR ("Templateless") Oligonucleotide Assembly

• Process of going from 70 bp oligos to 750 bp products

• Gapped Overlapping Regions:

• Good news!! Works ~70% of the time

• But when you don’t get desired product…

  • Go back and start over

  • More money for reagents

  • More HOURS are lost in the Lab!

Saturday, October 31, 2009
Alternative to t-PCR

LCR (Ligase Chain Reaction)

- **Good:** Complete overlap: higher frequency of successful building blocks increases
- **Bad:** Costs more due to more oligos

**Optimizations**
- Taq ligase
- T4 ligase
- 9 degrees north
- Pfu ligase
- Different PCR cycling times, annealing temperatures
- Different dilutions
- Enzyme concentrations
- DNA concentrations
Ligase Chain Reaction Method (LCR)

85% of failed standard PCR produced full length products by LCR

Helps save TIME and MONEY!
Problem #2

Point mutations from oligos

- Oligos have a 1% error rate per base
- Requires sequencing multiple (12-18) clones of BBs to identify “winners”
Taq MutS Protein to the Rescue!

- Taq MutS protein binds to mismatched pairs of heteroduplex DNA
- Enriches DNA population for homoduplexes (non-mutant DNAs)

Optimizations
- MutS
- Different tags
- DNA protein ratios
- Binding temperature
- Magnesium concentration in buffer
- Binding time
### Overall Fidelity

#### t-PCR/f-PCR vs LCR/f-PCR

<table>
<thead>
<tr>
<th></th>
<th># Mutations / 1,000 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PCR/f-PCR</td>
<td>2.6</td>
</tr>
<tr>
<td>LCR/f-PCR</td>
<td>2.1</td>
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</table>

#### Taq MutS

<table>
<thead>
<tr>
<th></th>
<th># Mutations / 1,000 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>MutS Untreated</td>
<td>4.4</td>
</tr>
<tr>
<td>MutS Treated</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Bottom line

• LCR leads to a higher success rate
• LCR increase fidelity slightly
• MutS may increase fidelity
Optimizations
Modifying Building Block Ends

\[ x = 3 - 11 \text{ bp} \]
Building Block Assembly

Promoter

AAAAT
TTTATTA

Terminator

AAATAAT
TTTATTA

Protein Coding Sequence

AAAAUG
TTTTAC

TAAGGT
AUTCCA

5' 3'

5' 3'

5' 3'

5' 3'

5' 3'

5' 3'

Saturday, October 31, 2009
After USER

**Protein Coding Sequence**

5' AAAAUG TTTTAC 3'

**Promoter**

5' AAATAAT TTTATTA 3'

**Terminator**

5' AAGGU TTCCA 3'

5' TAAGGT AUTCCA 3'
After USER

Protein Coding Sequence

5' AAAA G TTTTAC
3'

Promoter

5' AAATAAT TTTATTA
3'

AAAAT TTTA

5' TAAGGT A TCCA
3'

Terminator

5' AAGG TTCCA
3'

AAATAAT TTTATTA

Saturday, October 31, 2009
After USER

Protein Coding Sequence

5'
G
TTTAC
3'

Promoter

5'
AAATAAT
TTTATTA
3'

Terminator

5'
AAATAAT
TTTATTA
3'

AAAAT

TTCCA
Ligation and Repair

Protein Coding Sequence

Promoter

Terminator

5'
AAATAAT
TTTATTA

3'
AAAAT
TTCCA

TAAGGT

TGTTTAC

TAAGGT
Ligation and Repair

Promoter

Protein Coding Sequence

Terminator

5'
AAATAAT
TTTATTA

3'
AAAAT
TTTTAC

G
TAAGGT
TTCCA

TTATTA
AAATAAT
Ligation and Repair

5' AAATAAT TTTATTA 3'

Promoter

3' AAAAT TTTTAC

G

Protein Coding Sequence

5' TAAGGT A TTCCA

Terminator

AAATAAT TTTATTA

Bottom Line:
Building blocks can only go together in one way
Ligation and Repair

Bottom Line:
Building blocks can only go together in one way
BioBrick vs Building Blocks

• No restriction enzymes
• No scars
• Seamless
• Single step multi-fragment assembly
• Both are abbreviated BB
Building Blocks

• No restriction enzymes
• No scars
• Seamless
• Single step multi-fragment assembly
A New DNA Assembly Standard

RFC 38

RFC 38: Building Blocks - Standard Large DNA/Genome Construction

7 October 2009

1. Purpose

The physical assembly of standard parts is currently a non-standard process, which can either come from direct genome PCR with restriction enzyme sites incorporated into the PCR primers, or overlap assembly PCR. Furthermore, current Biobrick construction standards [RFC 10, 11, 12 etc.] rely heavily on restriction enzyme based methods, which can be sequence, cost and time restrictive. This is especially true of large DNA assembly, where 100% control of over DNA sequence may be mandatory, such as in whole genome assembly. We propose a novel standard, The Building Block Method, for both the construction of standard parts and their assembly.

This Building Block standard can be either A) an interchangeable or B) a non-interchangeable format, based on the desired use of the part.

2. Relations to other BBF RFCs

This RFC (Request for Comments) is a new proposal for standardized large DNA/genome construction. It describes a series of methods for construction and assembly that are unrelated to existing RFCs.
A New DNA Assembly Standard - RFC 38
Option of interchangeable or non-interchangeable building blocks

<table>
<thead>
<tr>
<th>Type of Building Block (Based on its contents)</th>
<th>5’ Standard End</th>
<th>3’ Standard End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter ONLY</td>
<td>AAATAAT</td>
<td>AAAAT</td>
</tr>
<tr>
<td>Protein Coding Region ONLY</td>
<td>AAAATg</td>
<td>tAAGGT</td>
</tr>
<tr>
<td>Terminator ONLY</td>
<td>tAAGGT</td>
<td>AAATAAT</td>
</tr>
<tr>
<td>Promoter + Protein Coding Region</td>
<td>AAATAAT</td>
<td>tAAGGT</td>
</tr>
<tr>
<td>Protein Coding Region + Terminator</td>
<td>AAAATg</td>
<td>AAATAAT</td>
</tr>
</tbody>
</table>
SIDE PROJECT #4
STREAMLINING THE GENOME

Genome Stabilization
Genome Stabilization

• Goals:
  • Stable genome structure
  • Controllable evolution
• Remove / Relocate:
  • Retrotransposon repeats
  • Sub-telomeric repeats
  • tRNA genes
# tRNA organization

<table>
<thead>
<tr>
<th></th>
<th>Sc1.0</th>
<th>Sc2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>274 tRNA genes stochastically placed throughout the yeast genome</td>
<td>Engineered array of ~300 tRNA genes</td>
</tr>
<tr>
<td></td>
<td>Many upstream repeats, some introns</td>
<td>No upstream repeats, no introns</td>
</tr>
</tbody>
</table>
tRNA Genes have upstream repetitive regions

Repetitive Regions (Ty Retrotransposons)

TFIIIB Complex

TFIIIC Complex

A Box

B Box

TTTTT

tDNA
Hybrid tRNA Genes

Repetitive Regions (Ty Retrotransposons)

TFIIIB Complex

A Box

B Box

TTTTT

tDNA
Hybrid tRNA Genes

500 bp *Ashbya gossypii* Flanking Region
Rox Recombination Site

- Similar to LoxP recombination
- Rox site is 32 bp, as opposed to 34 bp loxP site
- Utilizes Dre recombinase
tRNA Arrays

Glu- tRNA (UUC) -> Gly- tRNA (GCC) -> Lys- tRNA (CUU) -> Leu- tRNA (CAA) -> Met- tRNA (CAU)

Thr- tRNA (AGU) -> Ser- tRNA (CGA) -> Gin- tRNA (UUG) -> Pro- tRNA (AGG) -> Asn- tRNA (GUU)

Asp- tRNA (GUC) -> Glu- tRNA (CUC) -> Ile- tRNA (AAU) -> Ser- tRNA (UGA)
SOFTWARE TOOLS

Pablo Lee
Moodle
Modular Object-Oriented Dynamic Learning Environment
**The Dinosaur Machine**

**Shipping address - confirmed**
- Jef D. Boeke; Room 320
- Mudd Hall Loading Dock
- Dept of Biology, Johns Hopkins University
- Baltimore, MD 21218
- United States

<table>
<thead>
<tr>
<th>Description</th>
<th>Unit price</th>
<th>Qty</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Perkin Elmer GeneAmp PCR System 9600 Thermal cycler</td>
<td>$300.00 USD</td>
<td>3</td>
<td>$900.00 USD</td>
</tr>
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- Shipping and handling insurance - included
- **Total** $1,050.00 USD

-$300.00 USD
<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
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<tbody>
<tr>
<td>Sep 2</td>
<td>Lecture 1: Jef Boeke</td>
</tr>
<tr>
<td></td>
<td>Studying and Synthesizing Yeast</td>
</tr>
<tr>
<td></td>
<td>Mudd Hall 23</td>
</tr>
<tr>
<td>Sep 4</td>
<td>Lecture 2: Jef Boeke</td>
</tr>
<tr>
<td></td>
<td>Principles of gene synthesis</td>
</tr>
<tr>
<td></td>
<td>Introduction to the moodle: Pablo Lee</td>
</tr>
<tr>
<td></td>
<td>Mudd Hall 23</td>
</tr>
<tr>
<td>Sep 7</td>
<td>Labor day, no class!!</td>
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<tr>
<td>Sep 9</td>
<td>Boot Camp Day 1</td>
</tr>
<tr>
<td></td>
<td>Bacterial transformation, serial dilutions and pipetting skills</td>
</tr>
<tr>
<td></td>
<td>Biological Assays</td>
</tr>
<tr>
<td></td>
<td>Bacterial transformation</td>
</tr>
<tr>
<td></td>
<td>Pipetting skills &amp; serial dilutions</td>
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<td></td>
<td>Mudd 312</td>
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<tr>
<td>Sep 11</td>
<td>Boot Camp Day 2</td>
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<td>Templateless PCR</td>
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<td>PCR animation</td>
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<td></td>
<td>Mudd 312</td>
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<tr>
<td>Sep 14</td>
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<td></td>
<td>Finish PCR</td>
</tr>
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<td></td>
<td>Mudd 312</td>
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<tr>
<td>Sep 16</td>
<td>Boot Camp Day 4</td>
</tr>
<tr>
<td></td>
<td>Gel running</td>
</tr>
<tr>
<td></td>
<td>Mudd 312</td>
</tr>
<tr>
<td>Name: Zheyuan Guo Boot Camp CSPCR</td>
<td>Date: 8 October 2008</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>CS-PCR</td>
<td>Sample: CS-PCR</td>
</tr>
</tbody>
</table>

**CS Gel Electrophoresis**

- **Name:** Chris Von Dollen
- **Date:** 8 October 2008

**Rishi CSPCR D1**

- **Name:** Sunny Chen
- **Date:** 9 October 2008

**Rishi CSPCR D2**

- **Name:** Peter Pan
- **Date:** 8 October 2008
Progress Reports

9L.3_32.W3.02

<table>
<thead>
<tr>
<th>FPCR :</th>
<th>Number of white colonies:</th>
<th>Percent white colonies:</th>
<th>Number of positive CS-PCR clones:</th>
<th>Percent positive CS-PCR clones:</th>
<th>Number ready for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Successful</td>
<td>100</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Building block number 02 did not give me any problems. My first round of TPCR and FPCR worked perfectly and I got enough colonies from the first ligation/transformation to run CSPCR. I picked 24 colonies, 17 of which looked positive. After sending all 17 for sequencing, I received 6 PASS clones.

Mary Mallaney
15 December 2008

9L.3_32.X2.05

<table>
<thead>
<tr>
<th>FPCR :</th>
<th>Number of white colonies:</th>
<th>Percent white colonies:</th>
<th>Number of positive CS-PCR clones:</th>
<th>Percent positive CS-PCR clones:</th>
<th>Number ready for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Successful</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>33</td>
<td>15</td>
</tr>
</tbody>
</table>

Successful clone submitted. Though the first round of ligation/transformation produced a decent amount of white colonies (24/40) only ten of those colonies gave a successful CSPCR product. In a second round of ligation/transformation I diluted the FPCR product 1:3.3 and 1:10; I found the 1:10 worked the best yielding 170 total colonies with ~100 white.

Katrina Foelber
13 December 2008

9L.3_32.W2.10

<table>
<thead>
<tr>
<th>FPCR :</th>
<th>Number of white colonies:</th>
<th>Percent white colonies:</th>
<th>Number of positive CS-PCR clones:</th>
<th>Percent positive CS-PCR clones:</th>
<th>Number ready for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Successful</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

FAIL.

This clone failed sequencing. However, there appears to be no systematic errors. An attempt was made to grow up some more...
BioStudio
Graphical Representation
## Configure... BioStudio: Edit the Chromosome

**Enable Chromosome Editing**: yes

**Editor Name**

**Notes on Edit**: Delete tRNAφ and insert loxP

**This segment coordinates**: chrVI 158000..159000

### Sequence Manipulations
- **Nil replace all** AAA codons with AAA codons (annotated ORFs only)
- **Delete this segment** (and replace with loxP sites)
- **Propose this segment for deletion**

### Deletions
- **Delete** the tRNA features wholly contained within this segment (and replace with loxP sites)
- **Propose** the features wholly contained within this segment for deletion

### Insertion Position
- **Five prime of 158000**
- **Three prime of 159000**

### Insertions
- **Create and insert a new feature**
  - Orientation: NA
  - Unique Name
  - Upload and Go

### Site Specific Recombination
- **Flank** this segment with loxP sites

**This segment is contained within 1 feature**: chromosome chrVI (1..271188)

**2 features start upstream and terminate in this segment**: gene YFR006W (156397..158004)
  - CDS YFR006W_CDS_0 (156397..158004)
Oligo Generation
### Oligo Plate Layout

#### Plate 2

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>04.01</td>
<td>04.02</td>
<td>04.03</td>
<td>04.04</td>
<td>04.05</td>
<td>04.06</td>
<td>04.07</td>
<td>04.08</td>
</tr>
<tr>
<td>2</td>
<td>04.13</td>
<td>04.14</td>
<td>04.15</td>
<td>04.16</td>
<td>04.17</td>
<td>04.18</td>
<td>04.19</td>
<td>04.20</td>
</tr>
<tr>
<td>3</td>
<td>05.01</td>
<td>05.02</td>
<td>05.03</td>
<td>05.04</td>
<td>05.05</td>
<td>05.06</td>
<td>05.07</td>
<td>05.08</td>
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<td>05.13</td>
<td>05.14</td>
<td>05.15</td>
<td>05.16</td>
<td>05.18</td>
<td>05.19</td>
<td>05.20</td>
<td>05.21</td>
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<td>6</td>
<td>06.13</td>
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<td>06.16</td>
<td>06.17</td>
<td>06.18</td>
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<td>7</td>
<td>06.25</td>
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#### Plate 3

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<th>C</th>
<th>D</th>
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<td>08.01</td>
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<td>09.18</td>
<td>09.19</td>
<td>09.20</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
18 Clones
Building Block
\[
\frac{18 \text{ Clones}}{\text{Building Block}} \times \frac{12 \text{ Building Blocks}}{\text{Student}}
\]
216 Sequences
Yuan’s Clones
<table>
<thead>
<tr>
<th></th>
<th>BAG2008F</th>
<th>4_22_A06</th>
<th>9L3_32.X1.13</th>
<th>750</th>
<th>PASS</th>
<th>PASS</th>
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<td>4_22_A07</td>
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Saturday, October 31, 2009
Master Database
Sign up for our Mailing List!

http://www.syntheticyeast.org
GENOME SHUFFLING

Christopher Von Dollen
shuffling w/out thumbs

no good
So where are we now?

• Entire right arm of synthetic chromosome 9 completed (9R)

• Substitutes for the native chromosome arm

• LoxPsym + Cre system seems to work
So where are we now?

• Entire right arm of synthetic chromosome 9 completed (9R)

• Substitutes for the native chromosome arm

• LoxPsym + Cre system seems to work
Cre - loxPsym System

- Recombineering
- Ruled by sex... hormone (estradiol)
- Symmetrical
- ~Random

Otin, Frontiers in Bioscience, 2006
Lox-Cre survivors show phenotypic diversity

Native chromosome

Syn 9R strain

1x

100x
Quick, hide! It’s the FUZZ!

Walton et al, Mol Bio Cell, 2006
Pseudohyphae (Fuzz) Experiment

• Diploid yeast with Synthetic “9R”

• Add some fuzziness \((\textit{FLO8})\) and Cre

• Add some \textbf{SEX}... hormone

• Should be visible changes in phenotypes
Why are we doing this again?

• Test our knowledge of genome structure

• Streamlined, eukaryotic chassis

• Discover gene relationships/pathways

• **Create novel tools for synthetic biology**
Building Blocks sent to Parts Registry

Chromosome 3

Chunk # BB’s

A1 (12)
A2 (14)
B1 (14)
B2 (12)
B3 (9)
C1 (9)
C2 (9)
C3 (14)
D1 (11)
D2 (17)
D3 (12)

TOTAL BUILDING BLOCKS SUBMITTED = 86

300 More Ready to Go!

Please Join Us!

10/20/09
Acknowledgements

• Sarah Richardson
• Eric Cooper
• Katrina Foelber
• Jessica Dymond
• Lisa Scheifele
• Heloise Müller
• Jennifer Tullman
• Joel Bader
• Marc Ostermeier
• Srinivasan Chandrasegaran
• Jef Boeke
• Deborah Mathews

• National Science Foundation
• Krieger School of Arts and Sciences, JHU
• Whiting School of Engineering, JHU
• HiT Center, JHU School of Medicine
• Microsoft
• Department of Energy
• Department of Biology, JHU
• Department of Biomedical Engineering, JHU
• Department of Chemical and Biomolecular Engineering, JHU
1. **Would any of your project ideas raise safety issues in terms of: researcher safety, public safety, or environmental safety?**

   *Saccharomyces cerevisiae* = GRAS (generally regarded as safe; FDA). No serious safety concerns

2. **Is there a local biosafety group, committee, or review board at your institution?**

   Yes, JHU Institutional Biosafety Committee. We also have a personal bioethic consultant: Deborah Mathews

3. **What does your local biosafety group think about your project?**

   Since yeast is “exempt” from recombinant DNA regulations, no serious safety concerns. BL2 lab

4. **Do any of the new BioBrick parts that you made this year raise any safety issues?**

   No