L11 DNA Assembly

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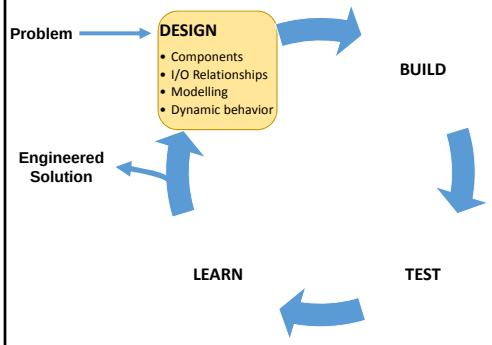
Recall…



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This lecture….

- Vector features
- Assembly basics
- BioBrick™ Standard

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Design fundamentally rooted in DNA

How do we deliver our constructs?

1. Plasmids – circular replicating DNA
2. Host genome
   - Integrated in natural chromosome
   - Synthetic chromosomes

DNA must be replicated for it to be useful

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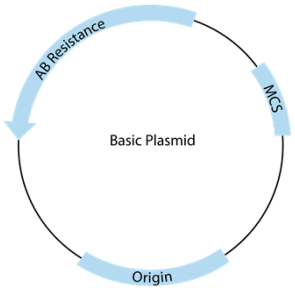
Plasmids contain at least 3 key components



AB Resistance or Selectable Marker – provides pressure for the cell to retain plasmid

Origin (ori) – site of replication initiation/essential for replication

MCS – region where you place your DNA design

Plasmid nomenclature
pXXX e.g. pUC19, pBAD24

idtdna.con

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Selection markers

Plasmids cost resources to maintain
- selection gives the cell reason to propagate your construct

Bacteria: Selection is typically with an antibiotic that inhibits or prevents growth
- ampicillin – bacteriostatic, inhibits cell wall synthesis (*bla*)
- chloramphenicol – inhibits protein synthesis/23s rRNA (*cat*)
- kanamycin – inhibits protein synthesis/30S subunit (*neo*)
- tetracycline – inhibits protein synthesis/tRNA::ribosome (*tetA*)

Eukaryotes (yeast): Selection is typically with a nutrient auxotrophy
- Uracil – *URA3* – needed for RNA synthesis
- Leucine – *LEU2* – needed for protein synthesis
- Histidine – *HIS3* – needed for protein synthesis

can also be used for counterselection – e.g. 5-FOA is assimilated by URA3 to make a toxic compound

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Origins of replication

Allows replication and ***sets plasmid copy number*** in the cells

How does plasmid copy number affect gene expression?

Yeast
- CEN ~ 1 copy per cell (centromeric)
- 2 micron ~ 50 copies per cell

*Yeast integrating plasmids have no ORI as they must be linearized and integrated into host for replication

Different yeast plasmids may be combined as long as each have a distinct selectable marker

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Origins of replication

Bacteria

Common Vectors	Copy Number+	ORI	Incompatibility Group
pUC	~500-700	pMB1 (derivative)	A
pBR322	~15-20	pMB1	A
pET	~15-20	pBR322	A
pGEX	~15-20	pBR322	A
pColE1	~15-20	ColE1	A
pR6K	~15-20	R6K*	C
pACYC	~10	p15A	B
pSC101	~5	pSC101	C
pBluescript	~300-500	ColE1 (derivative) and F1**	A
pGEM	~300-500	pUC and F1**	A

Only plasmids from different incompatibility groups with unique selection markers may be combined

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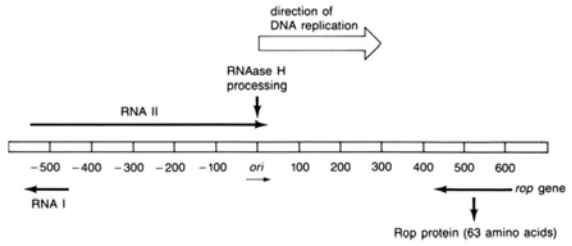
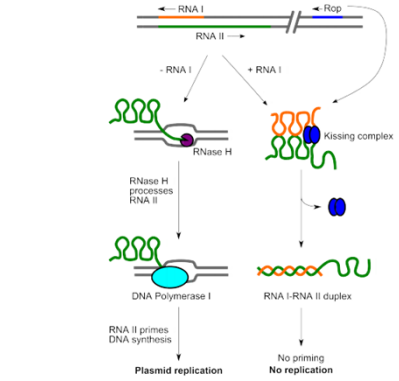
Incompatibility groups share replication mechanisms:
ColE1 type A plasmids in *E.coli*



Klumpp S (2011) Growth-Rate Dependence Reveals Design Principles of Plasmid Copy Number Control. PLoS ONE 6(5): e20403. doi:10.1371/journal.pone.0020403
http://journals.plos.org/plosone/article?id=info:doi/10.1371/journal.pone.0020403

Copy number set by Negative Feedback:
ColE1 type plasmids in *E.coli* (Feedback via antisense RNA)



https://en.wikipedia.org/wiki/ColE1

Copy number set by relative expression of RNAII and RNAI and their interactions

e.g. pUC vs pMB1
Rop⁻ vs Rop⁺
~300 vs ~20

Other plasmid incompatibility groups have other mechanisms

Plasmid types

Expression plasmids will typically include:

- promoters: P_{T7}, P_{Tet}, P_{lac}, P_{BAD}, GALP
- Regulatory proteins: *lacI*, *tetR*, *araC*
- Terminators: $T7_{term}$, rrnB

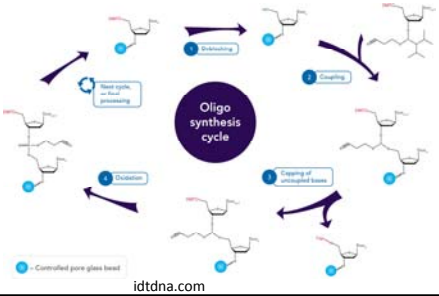
Shuttle vectors will have features for multiple species to "shuttle" DNA between them (e.g. ori and selecton)

- E.g pRS314 – bacterial features for cloning, yeast features for expression

Methods for DNA assembly

How do we put together our circuits?

1) Chemical addition (oligonucleotide/primer synthesis)



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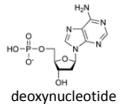
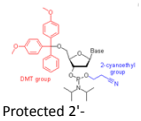


deoxynucleotide



Protected 2'-deoxynucleoside phosphoramidite

Methods for DNA assembly

How do we put together our circuits?

2) Homologous Recombination

e.g. CRISPR, Recombineering (Genome integration)

"Nucleotide sequences are exchanged between similar sequences of DNA"

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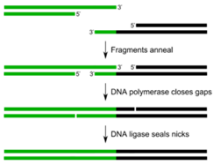
Methods for DNA assembly

How do we put together our circuits?

3) Anneal + Polymerization (Gibson CBA, SLIC, commercial DNA synthesis)

Exploits the ability of single stranded DNA to anneal (stick) to complementary strands



Wikipedia.com

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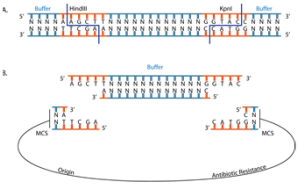
Methods for DNA assembly

How do we put together our circuits?

4) Digestion and Ligation

Exploits restriction enzymes to create 'sticky' overhangs that can be annealed to join pieces of DNA



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Restriction Enzymes

Most restriction enzymes recognize and cut <u>unique</u> 6nt **<u>palindromic</u>** sequences

→ 5' – 3' on either strand is exactly the same

<u>EcoRI</u>

```
5′ GAA TTC 3′
3′ CTT AAG 5′
```

Res often make staggered cuts yielding overhangs

```
5′ G     3′
3′ CTT AA 5′
```

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Digestion + Ligation

DNA cut with the same RE have the same overhang and anneal through complementary H-bonds (palindromic!)

H-bond immobilizes fragments long enough for permanent ligation by ***ligase***

How do we standardize this?

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BioBrick™ Standard

Standardizing on a *single* RE doesn't allow you to add assemble parts into devices and other higher order structures

Why?

Biobricks use 4 REs:

EcoRI	5′...G▼AATTC...3′ 3′...CTTAA▲G...5′	5′...A▼CTAGT...3′ 3′...TGATC▲A...5′	**SpeI**
PstI	5′...CTGCA▼G...3′ 3′...G▲ACGTC...5′	5′...T▼CTAGA...3′ 3′...AGATC▲T...5′	**XbaI**

SpeI and XbaI have complementary overhangs that destroy the restriction site after ligation

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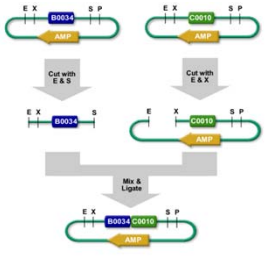
BioBrick™ Assembly

All parts have a prefix (EcoRI and XbaI, EX) and a suffix (SpeI and PstI, SP)



How would you do this if you wanted to put C0010 in front of B0034?

Ligation site has a ***scar***: **A**CTAGA **SpeI** XbaI
TGATCT

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BioBrick™ Assembly

Advantages	Disadvantages
- idempotent/scalable - Easily automatable - Robust - Only 4 REs used	- Need to remove internal Biobrick RE sites from all parts - Have a fixed scar (not good for protein fusions) - Can't add parts between parts

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Next time….

- More advanced high-throughput assembly techniques

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