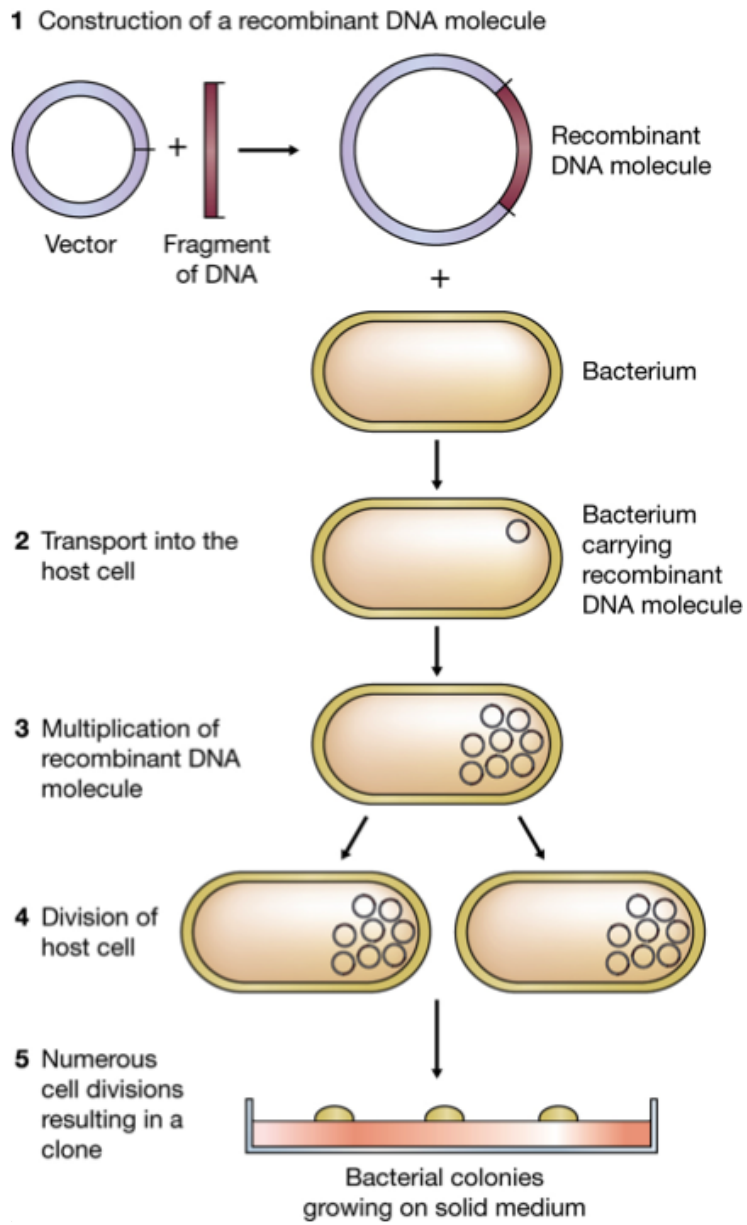
Engineering Genetic Circuits

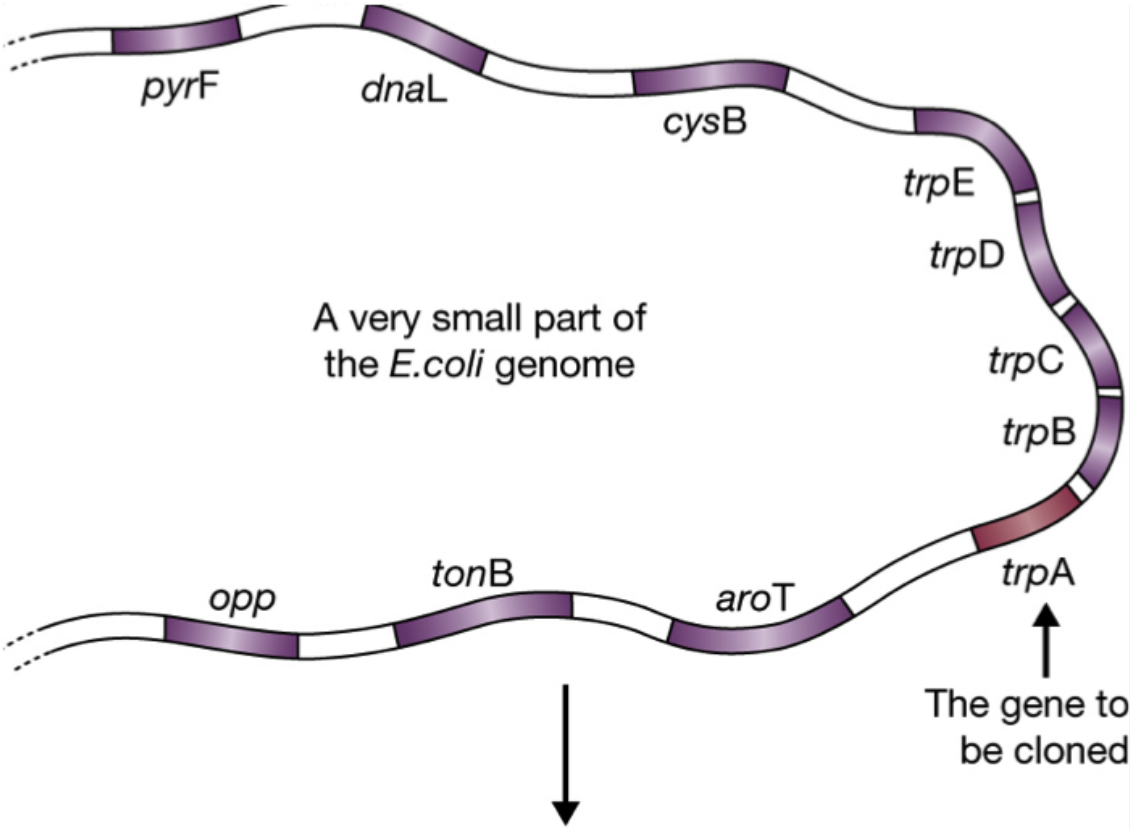
Chris J. Myers

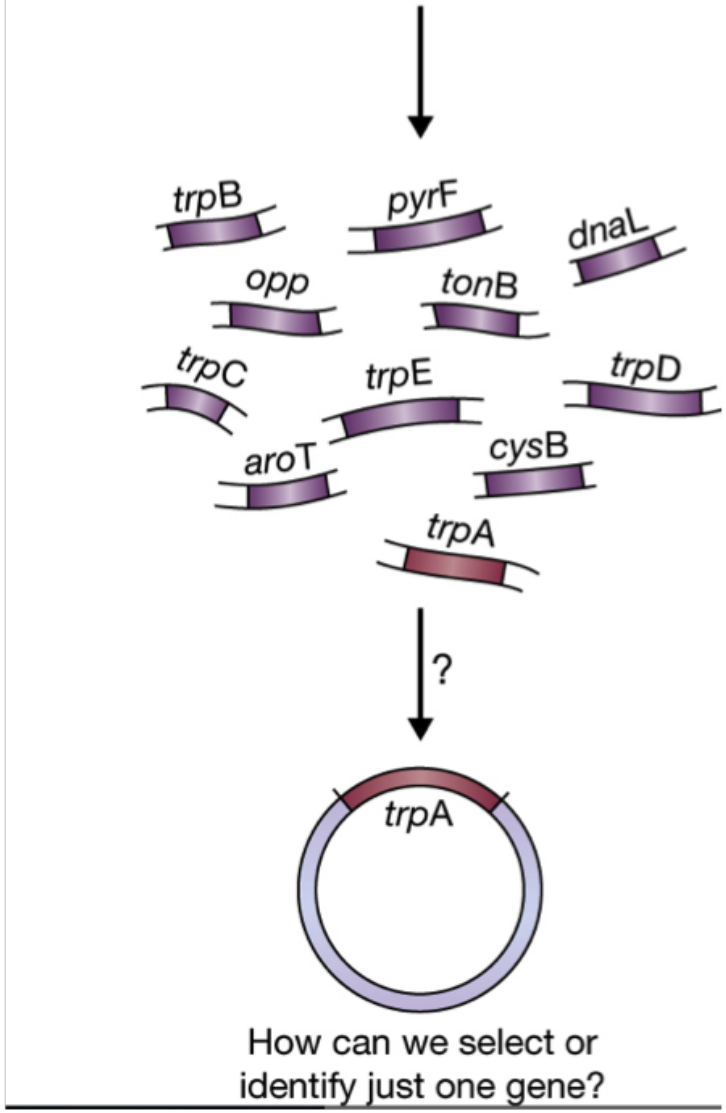
Lecture 4: Genetic Construction

Gene Cloning

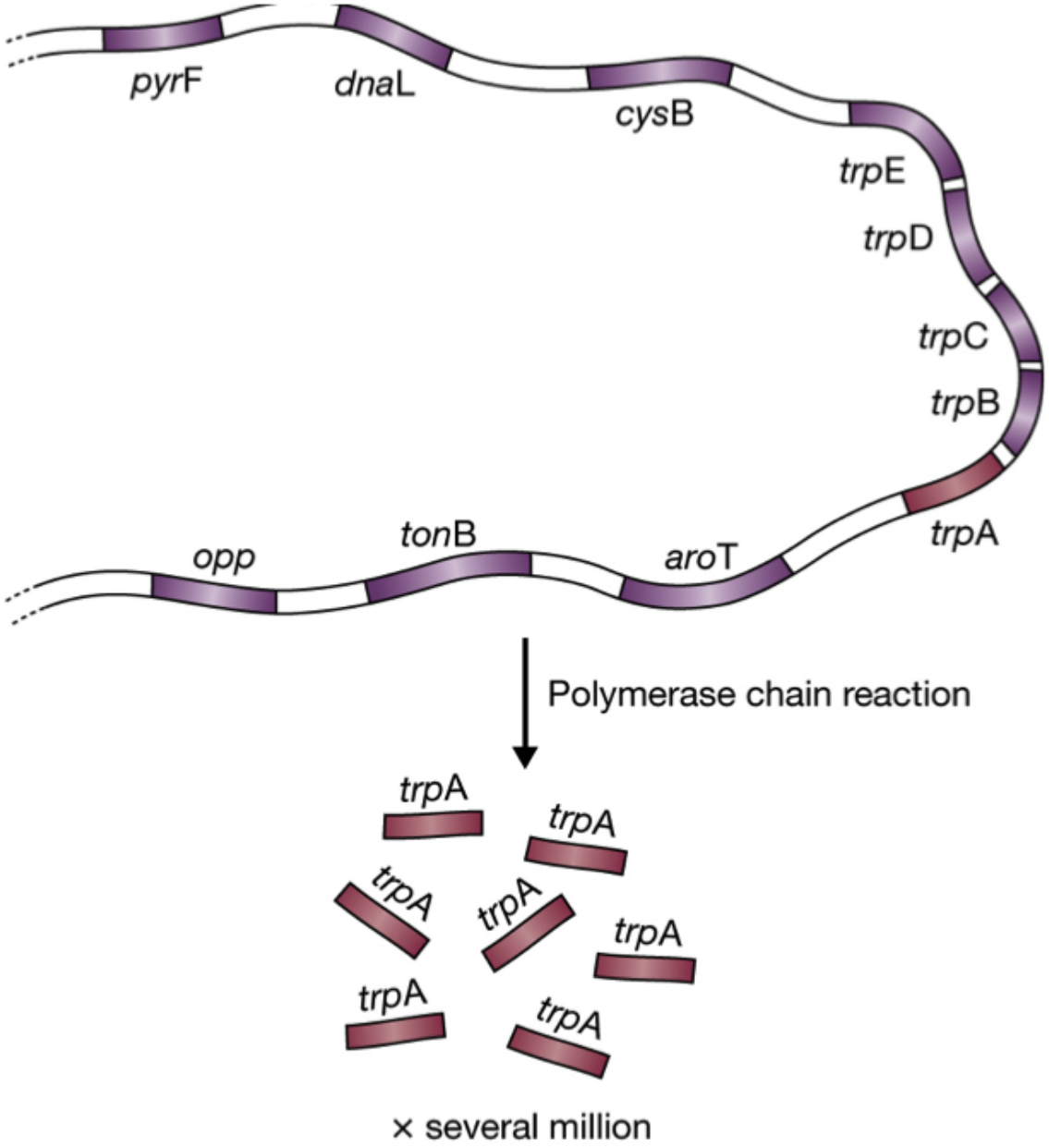


How Do We Clone the Correct Gene?





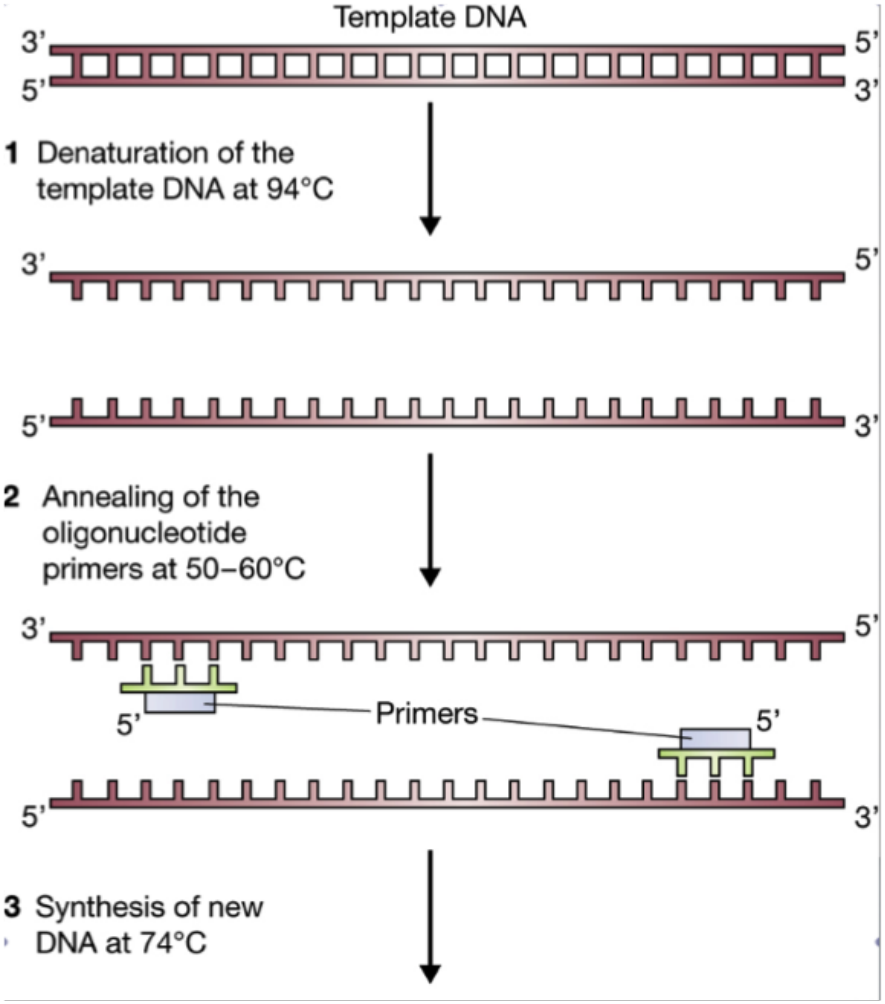
Polymerase Chain Reaction (PCR)

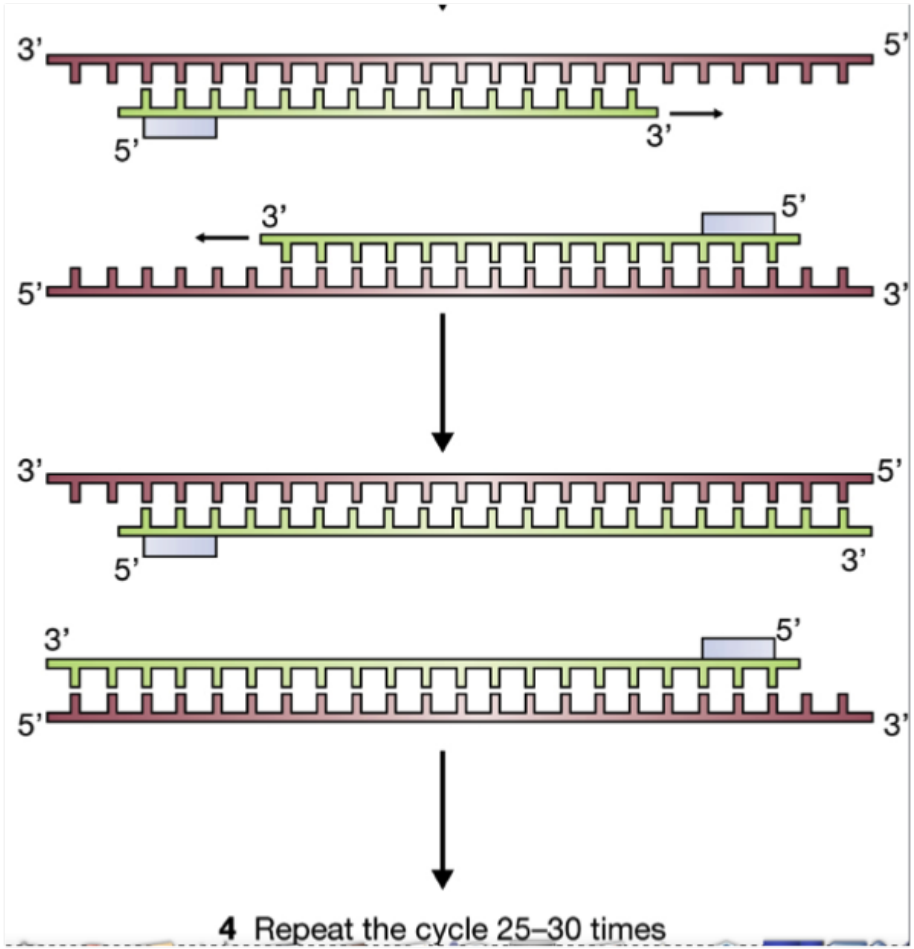


Polymerase Chain Reaction (PCR)

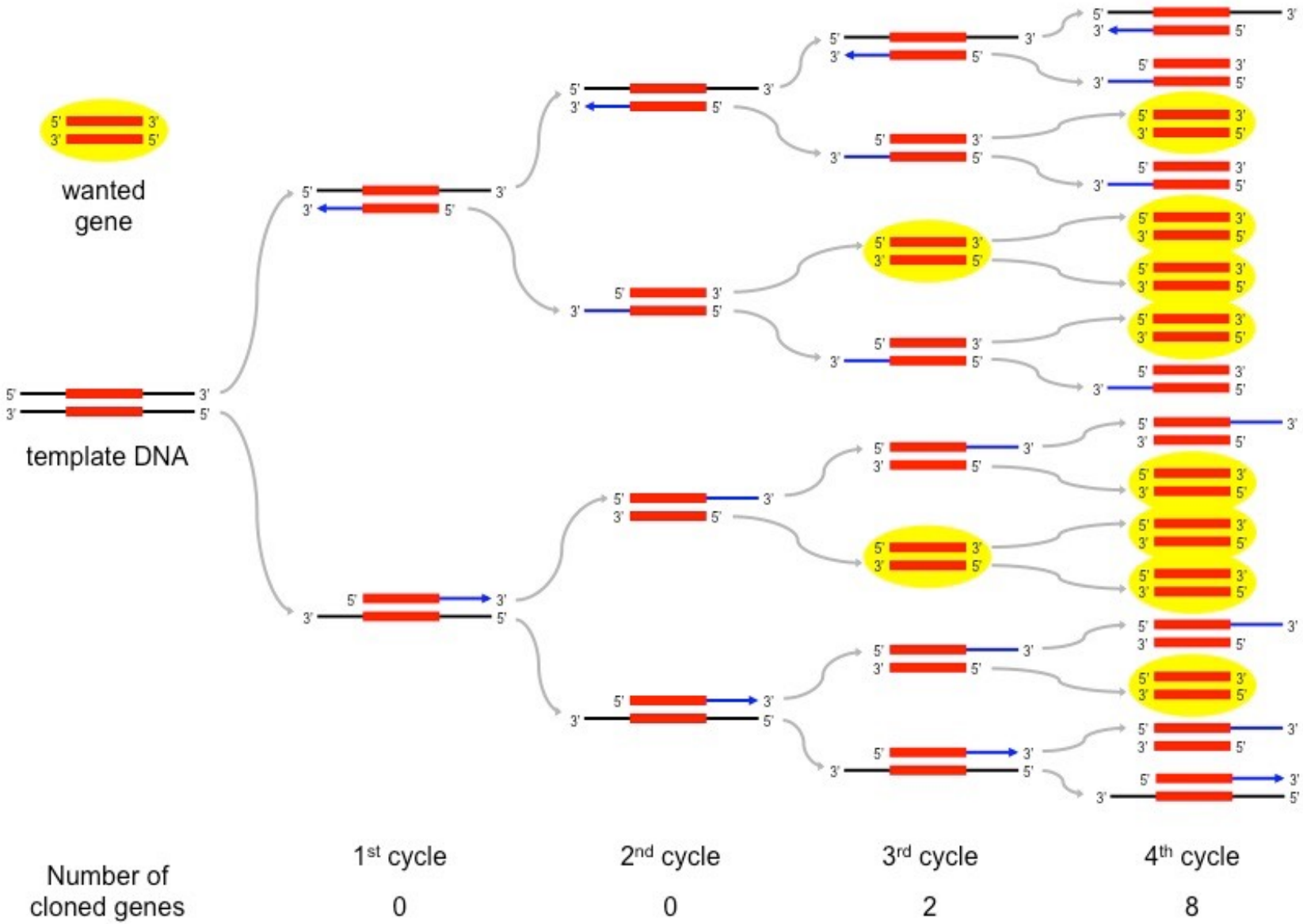
- Invented by Kary Mullis in 1983 (Nobel in 1993).
- Now a common and indispensible technique in molecular biology.
- Use to amplify selective fragments of DNA.
- Relies on thermal cycling and heat resistant enzyme DNA polymerase.
- `http://www.youtube.com/watch?v=_YgXcJ4n-kQ`

Polymerase Chain Reaction (PCR)

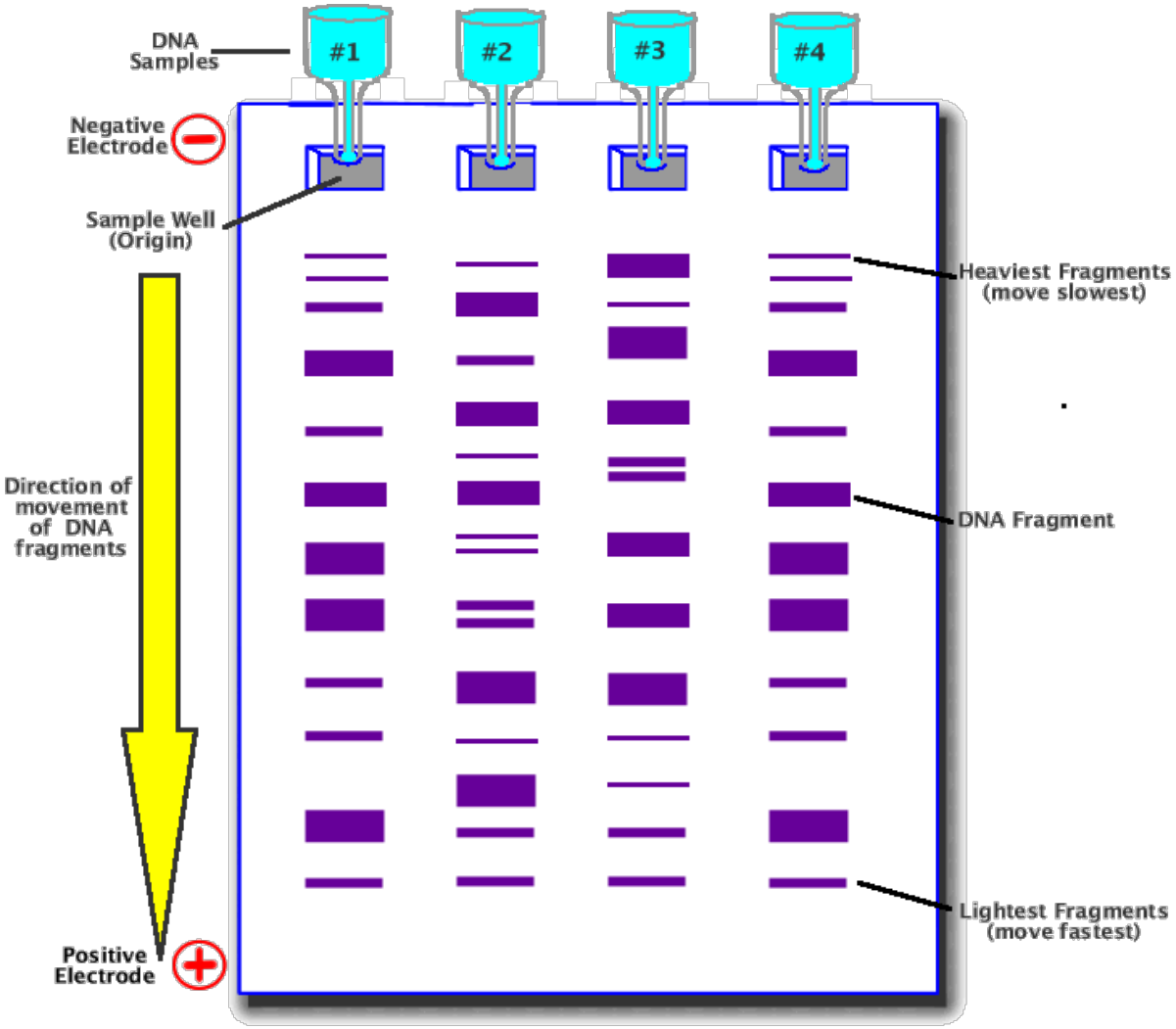




Polymerase Chain Reaction (PCR)



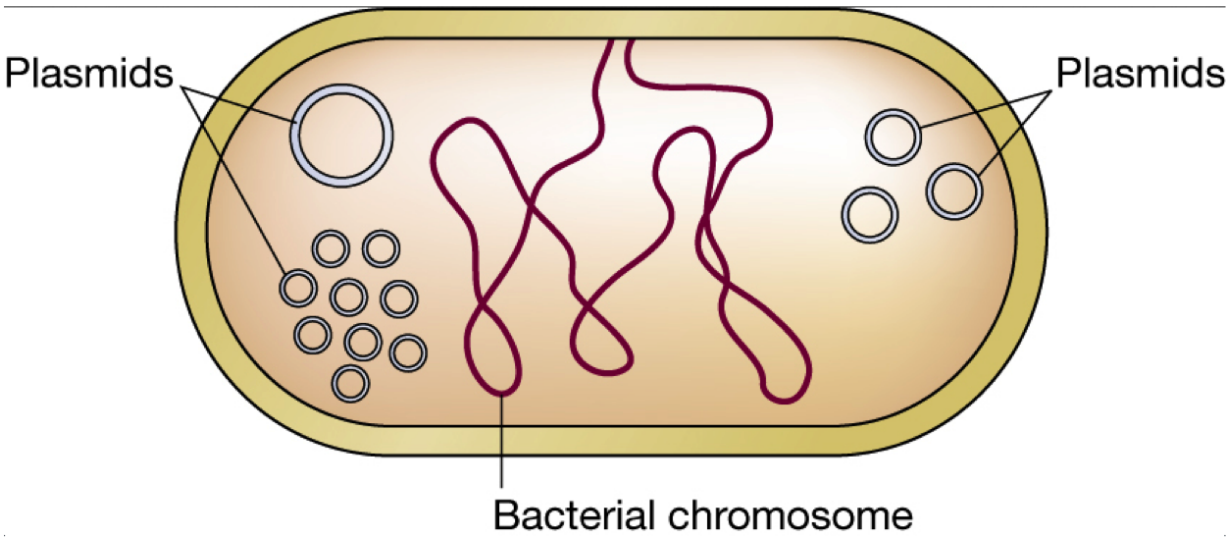
Gel Electrophoresis



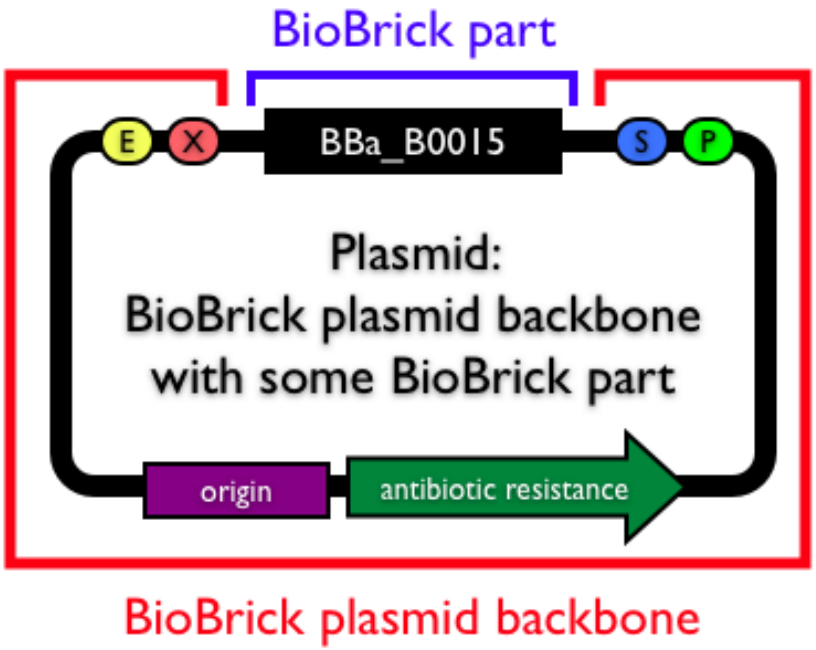
http://science.halleyhosting.com/sci/ibbio/biotech/electrophoresis.htm

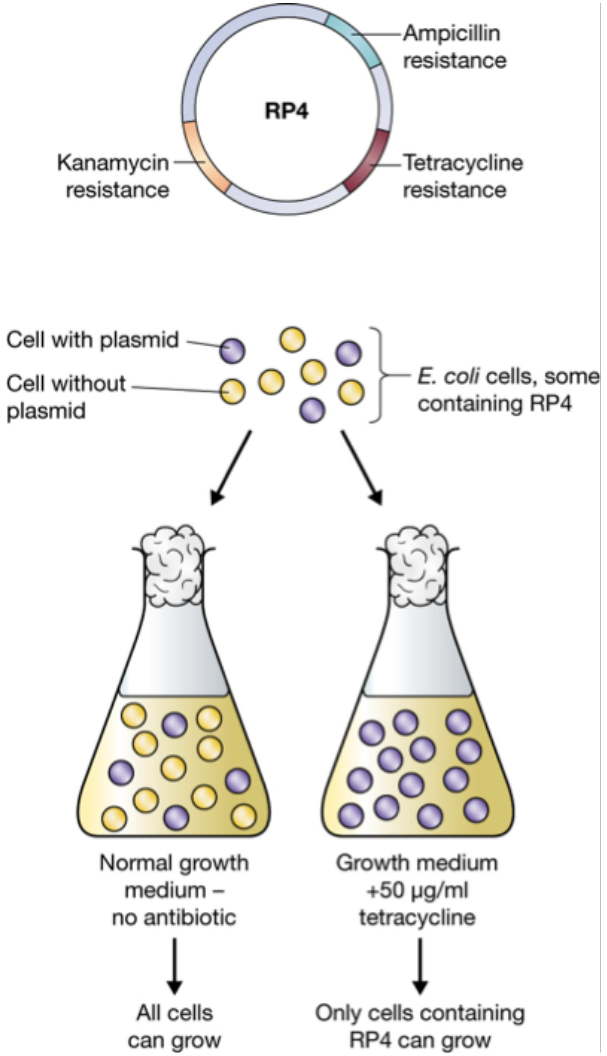
Vectors and Plasmids

- Sometimes termed backbone in synthetic biology.
- *Plasmids* most widely used.
  - Circular DNA molecules.
  - Independent genetic elements found in cells.
  - Have an *origin of replication* - can replicate.
  - Size and copy number (low, medium, high) are important.
- Bacteriophage vectors for E. coli most widely used.



Plasmid Backbones





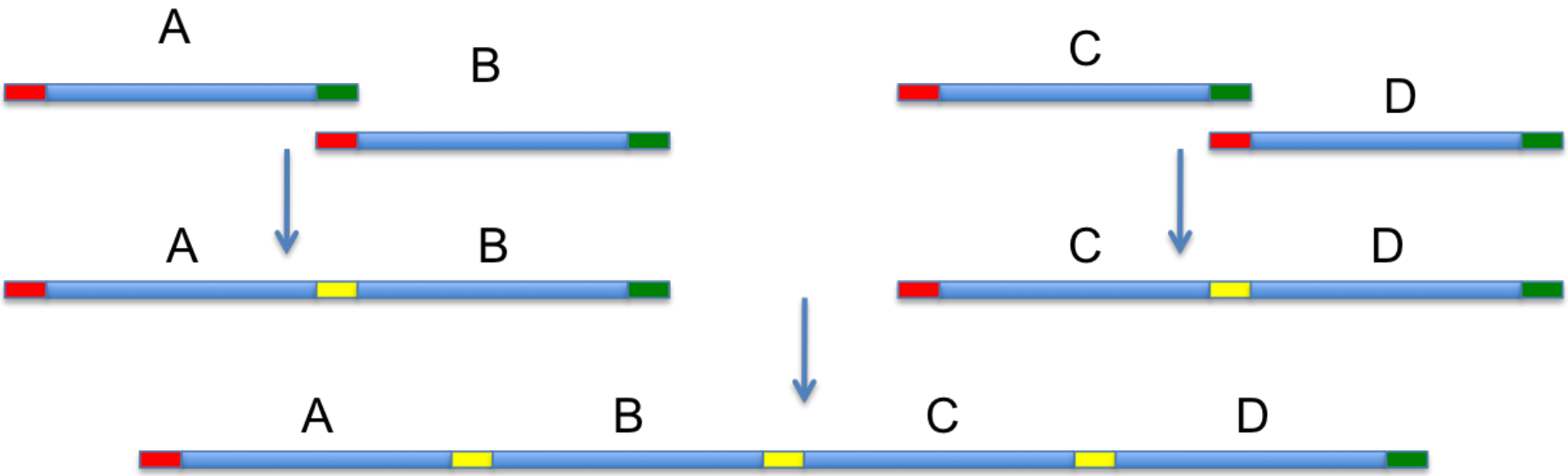
http://parts.igem.org/Plasmid_backbones

DNA Assembly Methods

- *Hierarchical assembly methods*, such as *BioBrick Standard Assembly*.
- *Type IIS restriction methods*, such as *Golden Gate* and *MoClo*.
- *End homology methods*, such as *Gibson Assembly*.
- Other assembly methods:
  - *Recombinase-based methods*.
  - *in vivo assembly*, used to create synthetic genomes.
  - *Hybrid linker-based methods*, such as *BASIC Assembly*.
  - *Bridging oligonucleotides*, such as *PaperClip Assembly*.

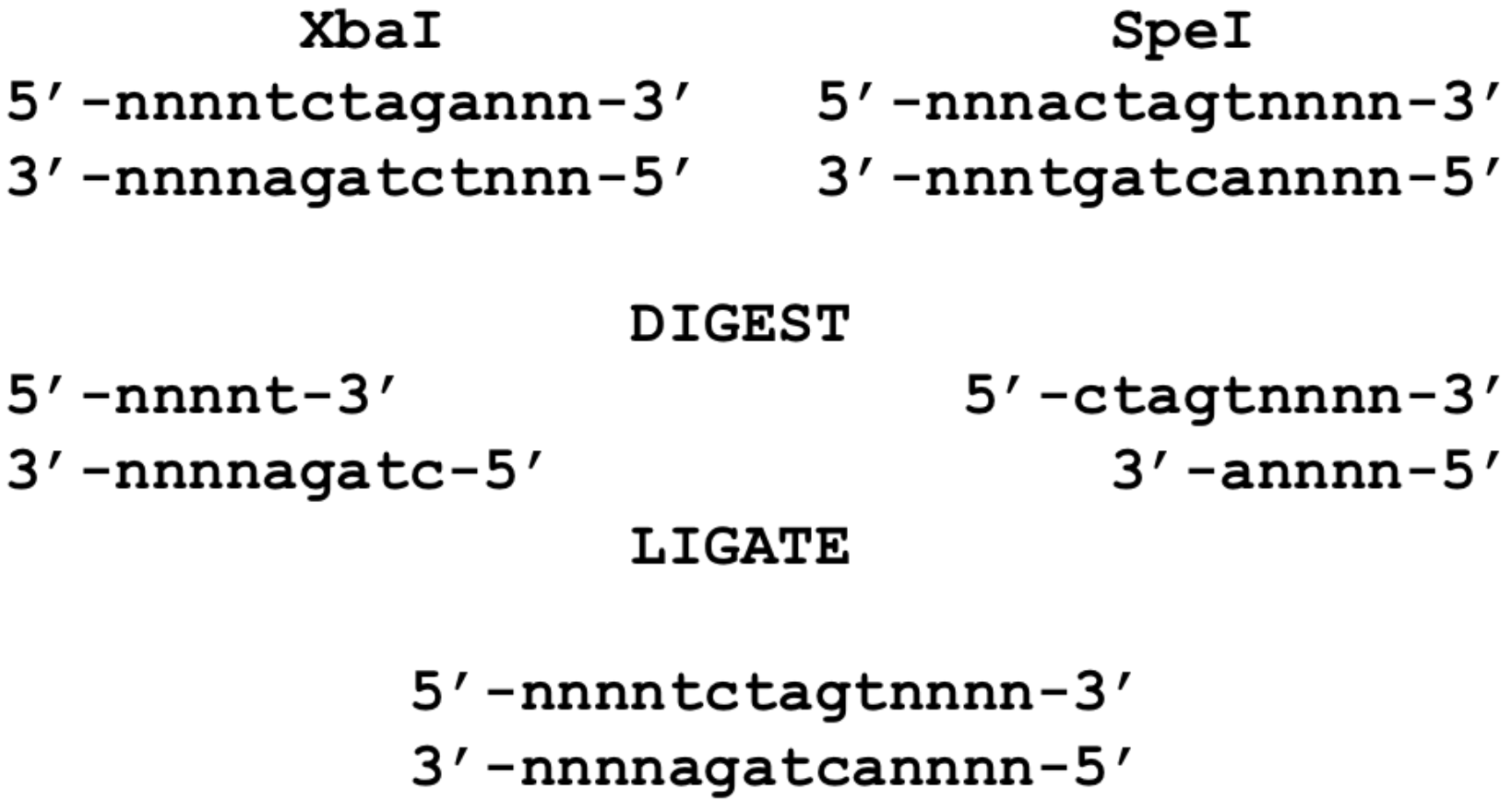
BioBrick Standard Assembly

- BioBrick RFC10 developed by Tom Knight (MIT) in 2003, used in iGEM.
- Two type II restriction enzymes which generate compatible sticky ends.
- On ligation, both sites are destroyed leaving a *scar*.
- Product can then be reacted with any other part.
- **Advantage**: modular composable parts, i.e., any combination of parts from a library can be assembled in any order.
- **Disadvantage**: since all parts have the same ends, parts need to be combined two at a time, with cloning in between, hence slow.



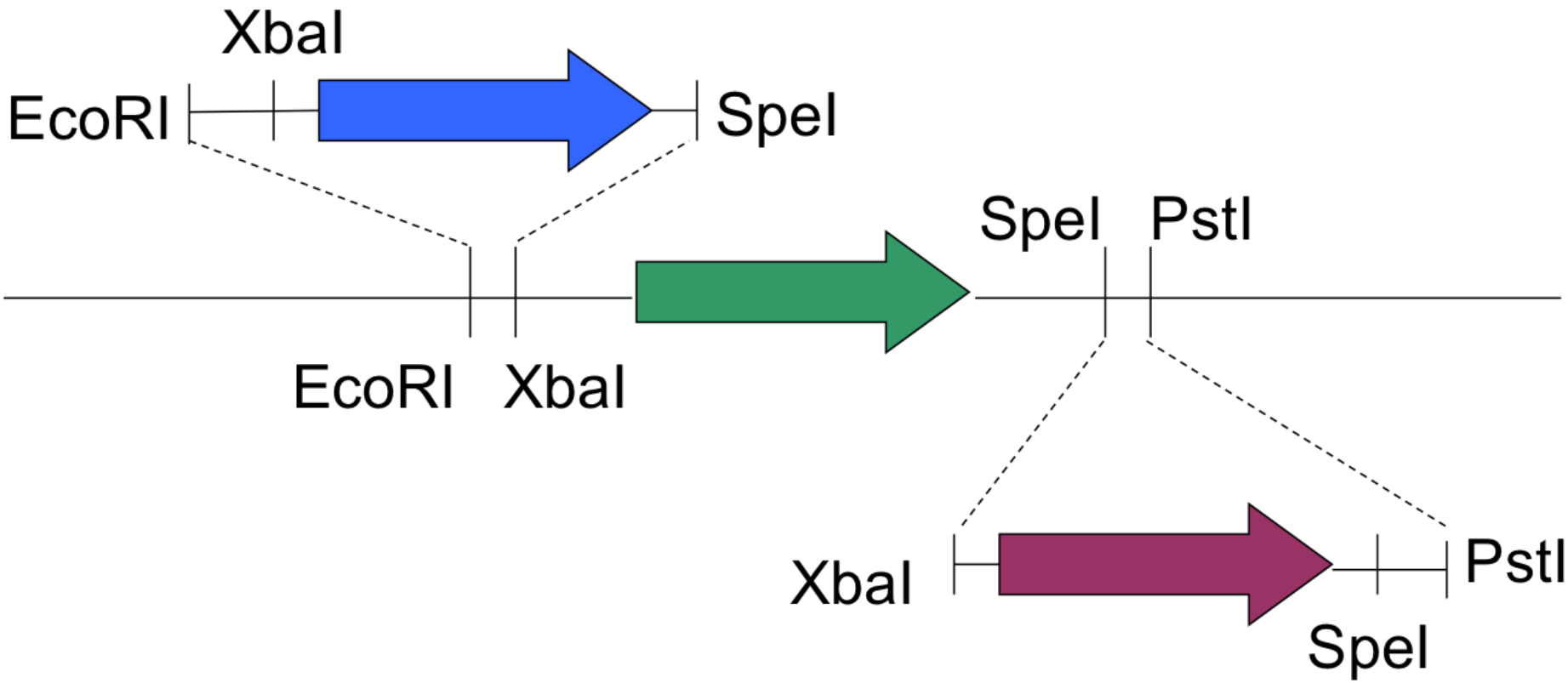
How BioBrick Assembly Works

- Each part has a Prefix (EcoRI, XbaI) and Suffix (SpeI, PstI).
- XbaI and SpeI generate compatible sticky ends, but on ligation neither site is regenerated.

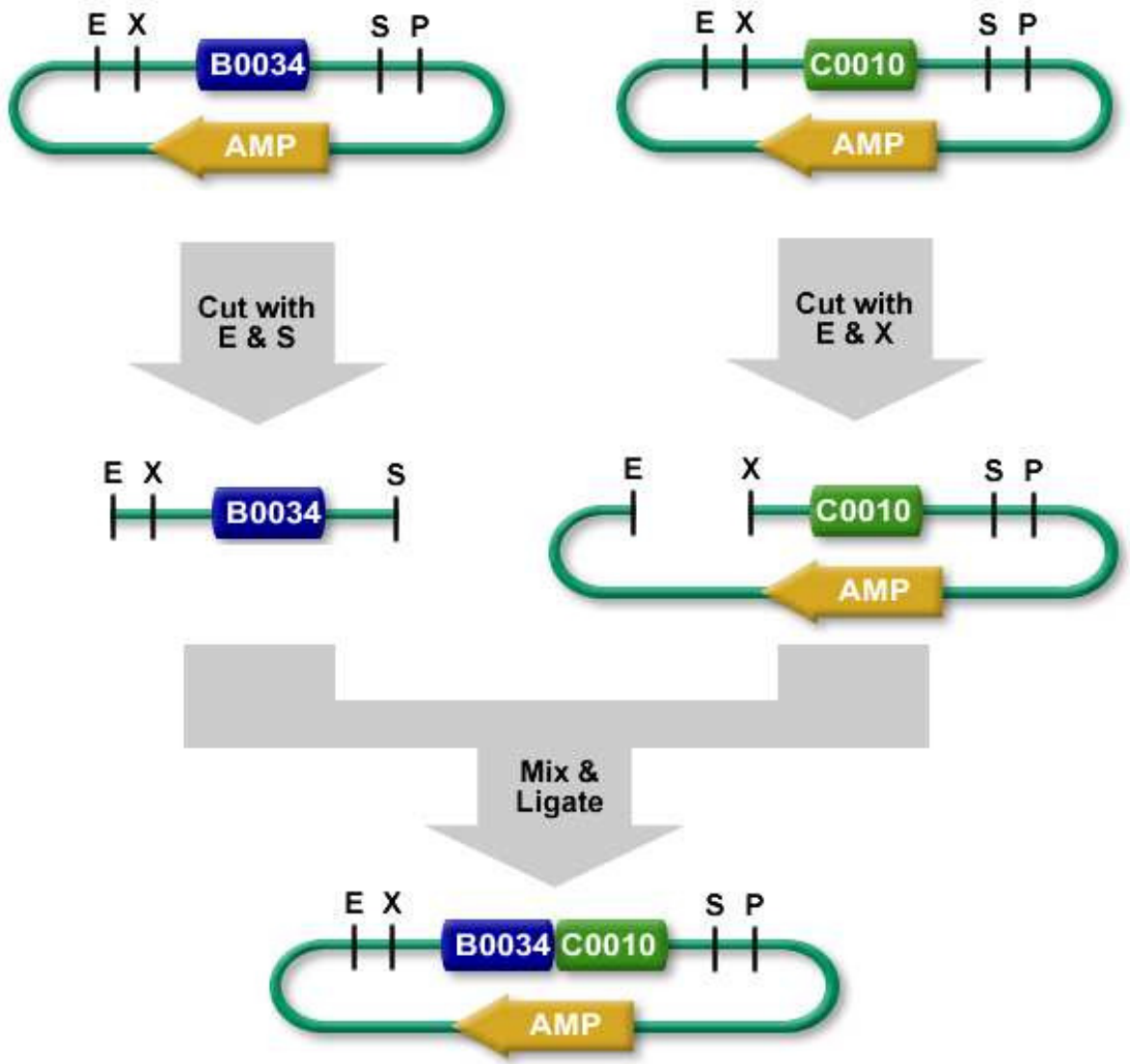


Combining BioBricks

- Any two BioBricks can be combined in either order, and the product is another BioBrick, which can then be combined with any other BioBrick (which might itself be made of smaller BioBricks).

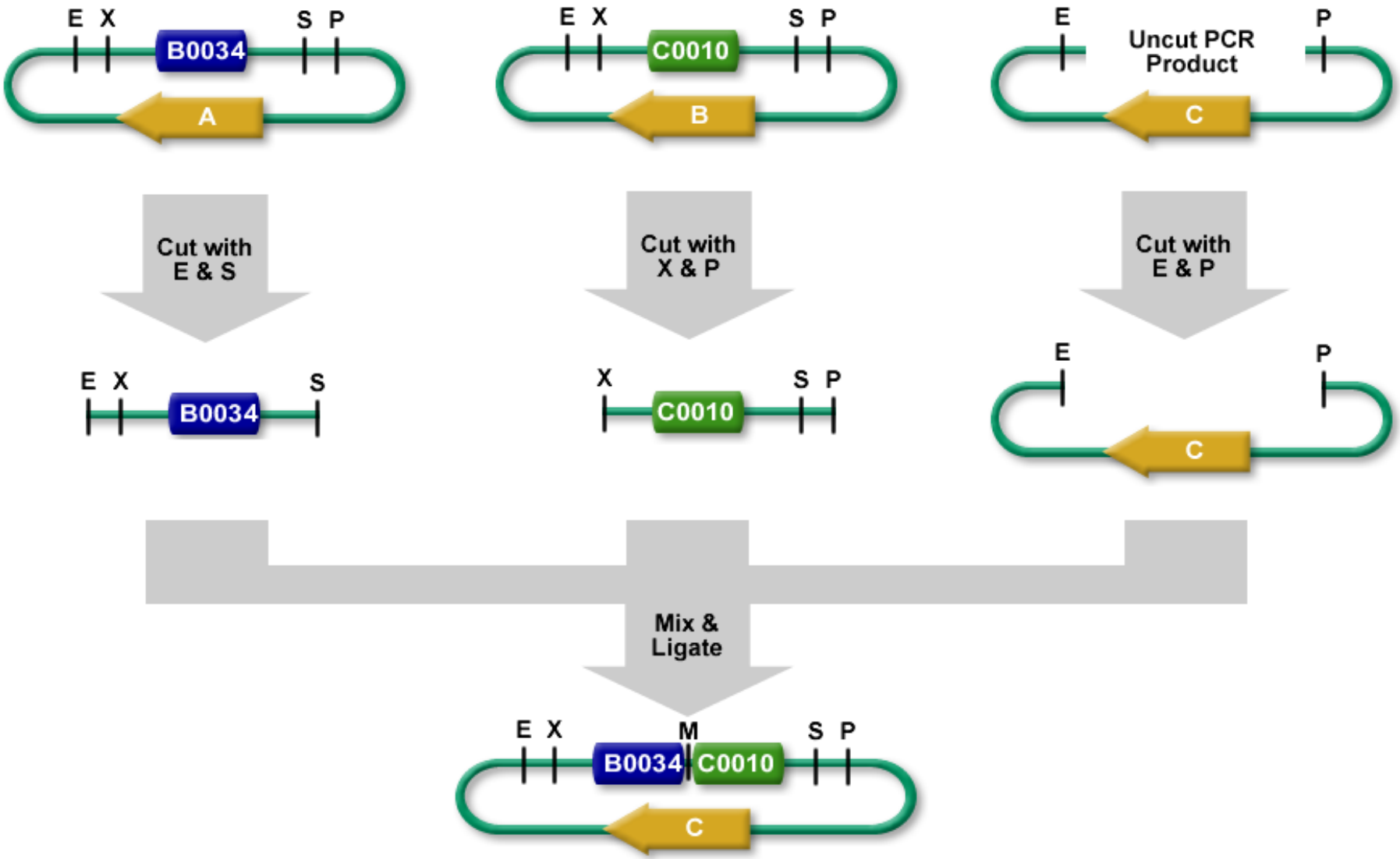


BioBrick Standard Assembly Summary



http://parts.igem.org/Assembly:Standard_assembly

BioBrick 3A Assembly Summary



http://parts.igem.org/Help:Assembly/3A_Assembly

BioBrick 3A Assembly Advantages

- No PCR
- No gel purification
- Higher success rate compared to Standard Assembly.

Golden Gate / MoClo Assembly

- Type IIS restriction enzymes cut outside their recognition site, leaving a user-definable overhang.
- Cycling restriction-ligation gives highly efficient assembly of multiple parts (up to ten or so) in a single reaction.

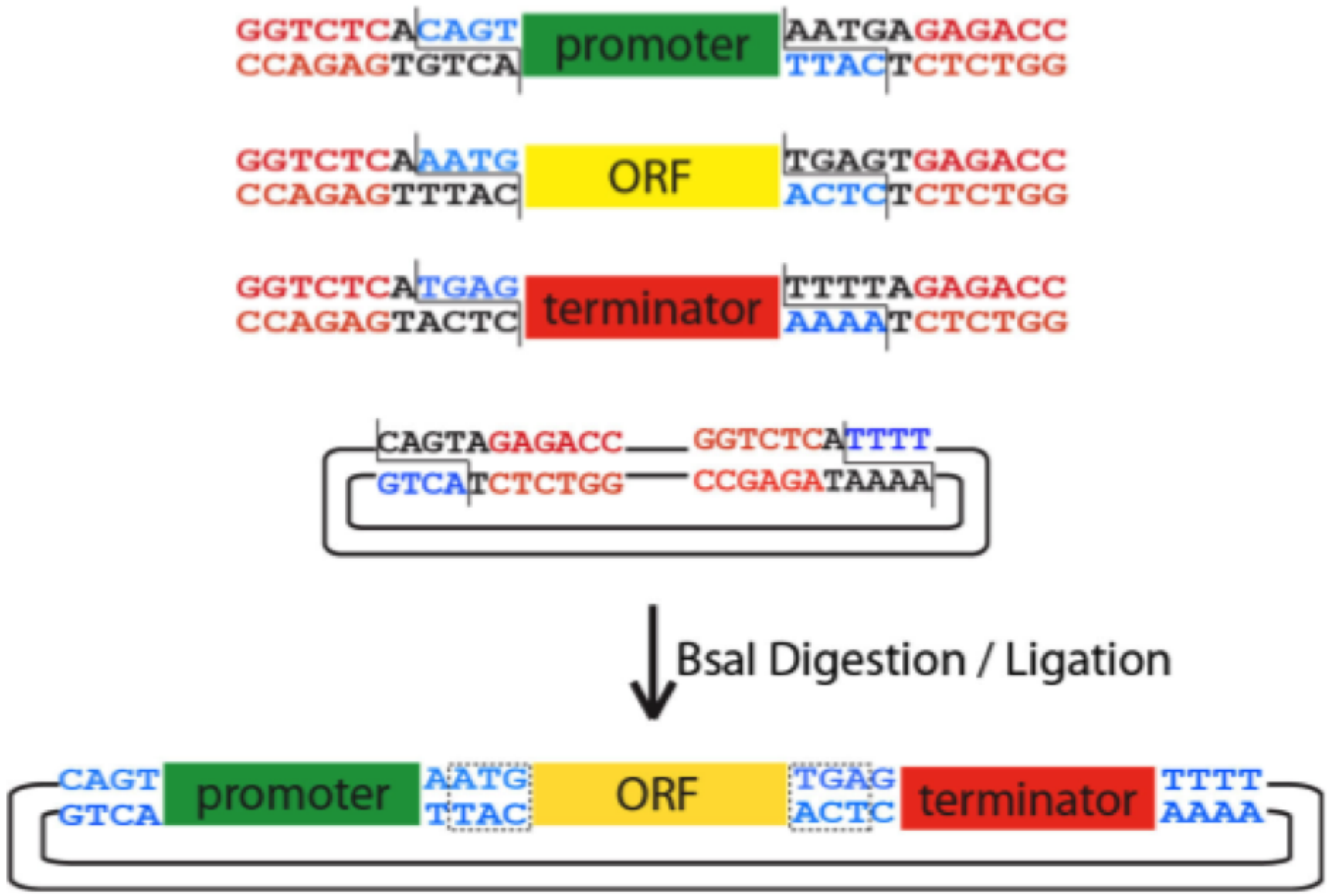
```
BsaI:  NGGTCTCN      NNNNNNN
       NCCAGAGNNNNN      NNN

EarI:  NCTCTTCN      NNNNNNN
       NGAGAAGNNNN      NNNN
```

Golden Gate Assembly

- Each part is cloned in a vector flanked by BsaI sites which generate a particular 4-base overhang at the upstream and downstream ends.
- Multiple parts can be assembled in a single reaction, with the order of part assembly dictated by the 4-base overhangs of each part.
- Correct ligation products have no BsaI site, whereas incorrect ones do, and can be recleaved - leads to high efficiency.
- **Advantage**: rapid, highly efficient multi-part assembly.
- **Disadvantage**: to change the order of parts, need to reclone in different vectors to give different 4-base overhangs.

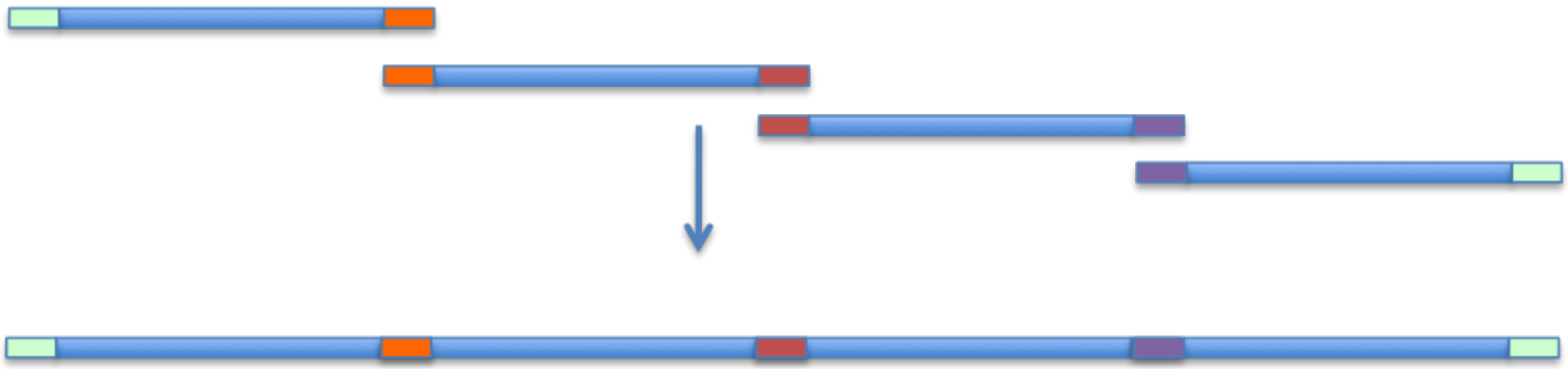
Yeast Golden Gate (RFC88)

- Jef Boeke lab, Johns Hopkins.
- Ideal for this application, since yeast expression cassettes always follow the same structure: promoter-ORF-terminator.



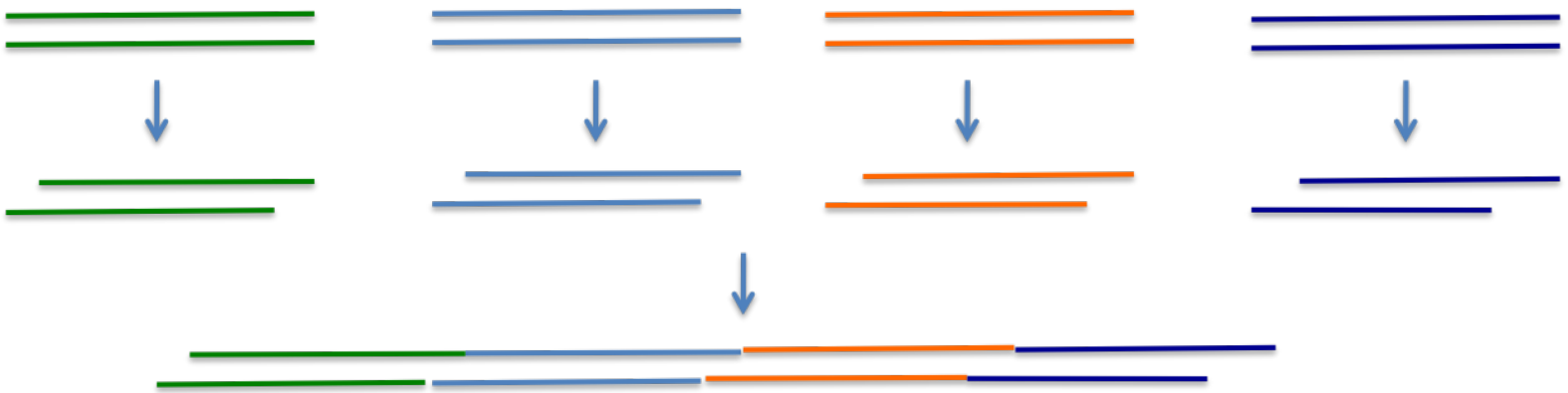
End Homology Methods

- Multiple parts are assembled simultaneously in a single reaction based on homology between the ends of adjacent parts.
- May be based on recombinases (eg InFusion, SLICE), PCR (CPEC), or single strand overlap regions (SLIC, USER, Gibson).
- **Advantage**: rapid, efficient assembly of multiple parts in a single reaction.
- **Disadvantage**: if you want to change the order of parts, need to use PCR to remake them with different homology ends.

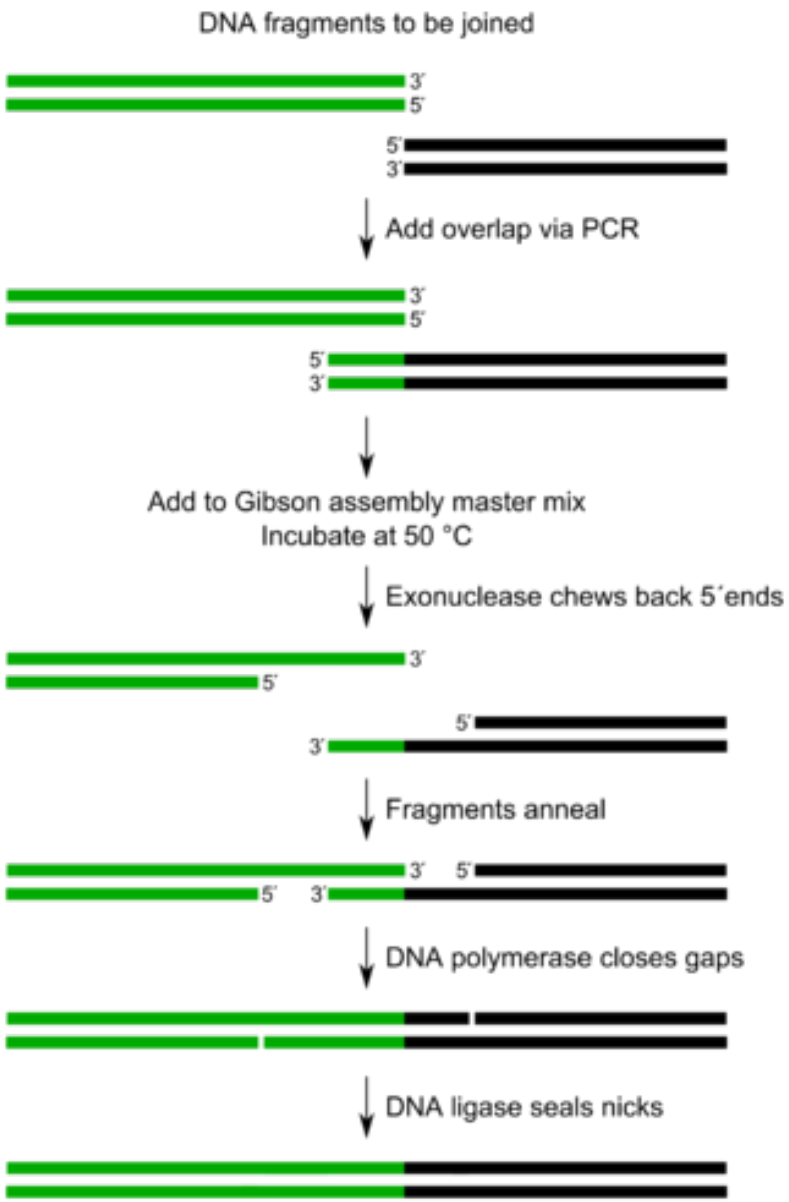


Gibson Assembly

- Parts are synthesised with 40 base overlap between adjacent parts.
- Incubate at 50°C with a mixture of T5 exonuclease, Phusion DNA polymerase, and Taq ligase for 1 hour.
- T5 exonuclease degrades one strand at each end leaving a single strand region, but is quickly inactivated by heat.
- Complementary single strand regions on adjacent parts anneal.
- Phusion polymerase fills in gaps, and Taq ligase ligates ends.



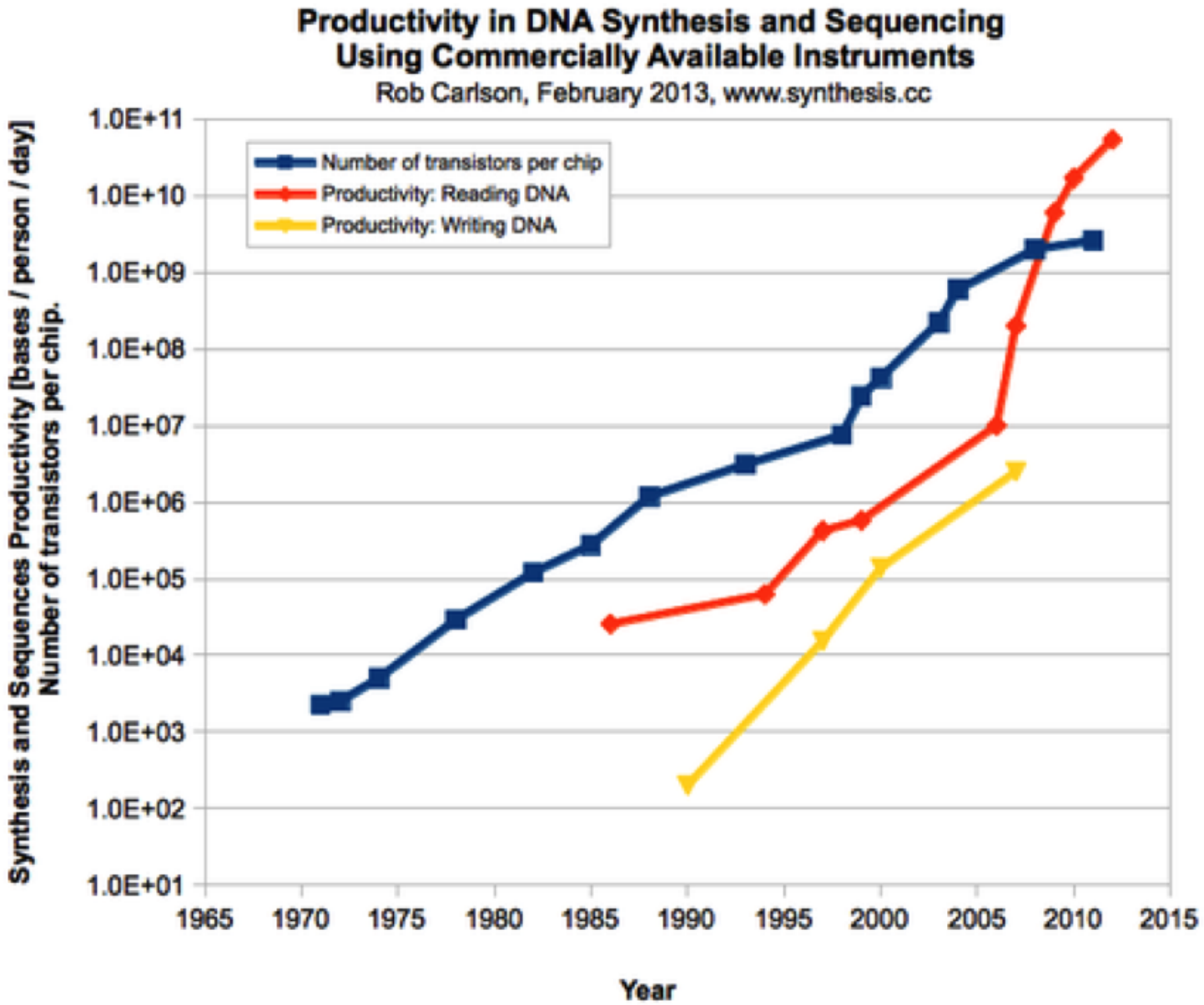
Gibson Assembly Overview



DNA Synthesis

- Several companies now offer synthesis of double stranded DNA up to about 5000 base pairs.
- Carefully designed pools of oligonucleotides will anneal and overlap.
- These are synthesized, then eluted in a mixture.
- Enzymes are used to fill in gaps and ligate ends.
- Mismatch repair proteins (eg MutS) may be used to prevent incorrectly annealed oligos from being incorporated.
- Difficult to make repetitive DNA, DNA with strong secondary structure.
- Recent improvements: pools of oligos are synthesised on chips and eluted together, reducing costs (Gen9, Twist Bioscience).

Carlson Curve



Biosecurity

- Biosecurity issues: bioterrorists may seek to order DNA encoding viruses, toxins, etc.
- Recently consortia have been established to ensure good practice.
- **International Association of Synthetic Biology (IASB)**: ATG:Biosynthetics GmbH, Entelechon GmbH, Eurofins MWG, Sloning Biotechnology GmbH.
- **International Gene Synthesis Consortium (IGSC)**: DNA2.0, GenScript, Integrated DNA Technologies, Blue Heron Bio, Life Technologies (owns GeneArt).

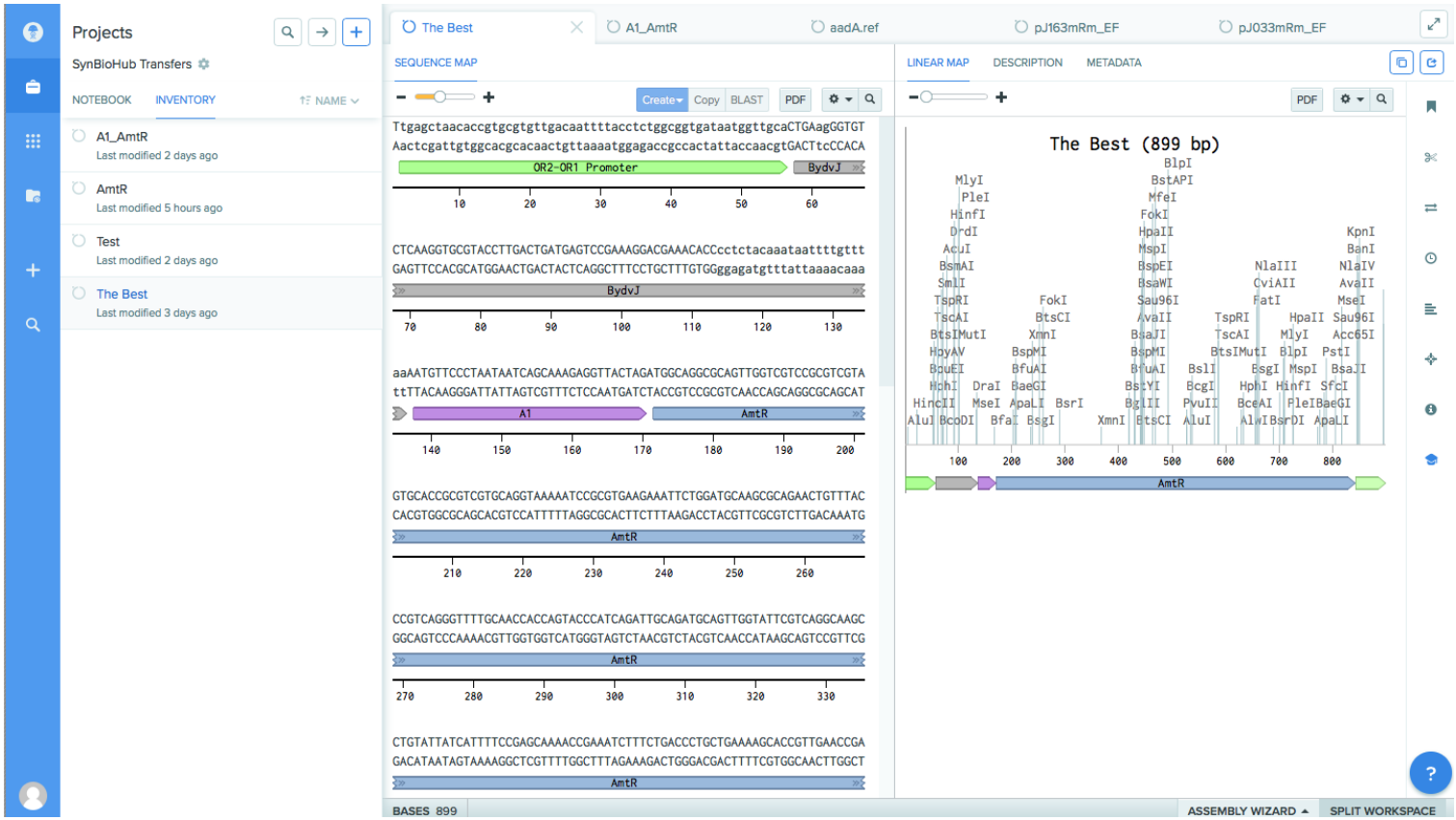
Conclusions

- Current methods involve chemical synthesis of single stranded oligonucleotides of around 100 bases, which are annealed to make blocks of around 1-5 kb, which are assembled using enzymatic methods to make larger constructs.
- Improvements may soon lead to the ability to 'print' long pieces of double-stranded DNA (or single-stranded, then generate second strand using DNA polymerase).
- Alternatively, a biological method might be developed to allow an enzyme to synthesise DNA for a user-defined sequence without a template.

Sources

- Gene Cloning & DNA Analysis - T. A. Brown
- Assembly slides from Prof. Chris French (U. of Edinburgh)
- Ellis T, Adie T and Baldwin G. 2011. DNA Assembly for synthetic biology: from parts to pathways and beyond. Integrative Biology 3, 109-118. doi: 10.1039/c0ib00070a
- `http://j5.jbei.org/j5manual/pages/1.html` (first section: introduction to DNA assembly).

Benchling (http://benchling.com)



Other sequence editors include:

ApE, Geneious, SnapGene, TeselaGen, Thumper

Sequence editors that support SBOL:

DeviceEditor, J5, VectorEditor (JBEI), DNAPlotLib (MIT/UW/Bristol), Eugene (Boston), GenoCAD (VBI), BOOST (JGI), etc.

Assignment #3

- Using BioBrick Standard Assembly, create an assembly plan for the genetic toggle switch.
  - Locate the plasmid backbone for each part required for your genetic toggle switch in the iGEM 2018 distribution. Note you may only use parts from this distribution in this assignment.
  - Attach the plasmid backbone to your parts using SBOLDesigner, and upload plasmids for each part to a private collection on `https://synbiohub.utah.edu`.
  - Obtain an account on `http://benchling.com`, request to join the Utah6760 organization.
  - Using `https://synbiohub.utah.edu`, copy your plasmids to Benchling.
  - Use Benchling to plan an assembly, and save each plasmid that will be constructed to a project that is shared with Utah6760.
  - Copy your plasmids to your private collection.
  - Document your private collection describing your assembly plan, and include a share link to your private collection with your plasmids.
- Briefly sketch an assembly plan for your genetic circuit. You may use any assembly method you like, and you do not need to design the plasmids.