Gene Collections

- MGC (Mammalian Gene Collection = human, mouse, rat, bovine): http://mgc.nci.nih.gov/ (most of these clones are available for a nominal fee from http://www.biocenter.helsinki.fi/bi/gbu/orf/)
  


- Xenopus gene collection: http://xgc.nci.nih.gov/

- Drosophila gene collection: https://dgrc.bio.indiana.edu/clones/Catalog

- MGC, honey bee, chicken, fugu, C. elegans: http://www.lifesciences.sourcebioscience.com/


- Long cDNAs (~4.5kb): Kazusa (http://www.kazusa.or.jp/huge/, KIAA designation) NEDO (http://www.kazusa.or.jp/NEDO, FLJ designation)
Cloning Club: Gateway and Golden Gate

- Host or parent plasmid
- Recombinant plasmid
- Plasmid
- Genomic DNA
- cDNA
- Synthetic DNA
- RNA
- PCR
How to make a cDNA library

1. mRNA isolation
2. reverse transcription
3. ligation into plasmids
4. transformation into E.coli
5. library amplification
6. clone amplification
7. plasmid DNA isolation
8. sequencing
Reverse transcription into cDNA

- **upstream regulatory region**
- **promoter**
- **transcriptional unit**
- **coding sequence (CDS)**
- **downstream regulatory region**

* = RNA polymerase binding site

- House keeping genes and small ORFs are overrepresented in cDNA libraries
- Priming: “radom hexamers”, ”oligo(dT)-primer”, gene-specific primers
- cDNA library vs. cDNA expression library
- ORF clones (no 5'- & 3'-UTRs), with sequence-specific primers
- cDNA libraries → cDNA collections
- Reverse transcriptase (error rate: 1:17,000-1:30,000, max. length ~15kb)
Gateway site-specific recombination cloning system

Based on λ phage integration system:

\[
\text{attB1xattP1} \rightarrow \text{attL1} \\
\text{attB2xattP2} \rightarrow \text{attL2} \\
\text{attL1xattR1} \rightarrow \text{attB1} \\
\text{attL2xattR2} \rightarrow \text{attB2}
\]

+/- stop codon

8 aa between GOI and tag (from att site)

ccdB containing plasmids must be propagated in a gyrA462+ strain!
In-Fusion/Creator Cloning System

In-Fusion + Cre recombinase

Figure from: Marsischky G & LaBaer J Genome Res. 2004; 14: 2020-2028
Univector site-specific recombination cloning system

Figure from: Marsischky G & LaBaer J. Genome Res. 2004; 14: 2020-2028
The RT step is not always needed!

RT-PCR: full-length cDNA as a goal; about 900 intronless genes in the human genome (notably histones and G-protein coupled receptors)

Non-CDS introns in 3'- and 5'-UTR

Genes with few exons (e.g. Prox-1: 5 exons (4 CDS, 575 of 737 aa encoded by exon 2)

Average: 8-9 exons per gene

Mega-Genes: Titin 35991 aa (363 exons)

Guide to the human genome:
http://www.cshlp.org/ghg5_all/section/gene.shtml
λ (Lambda) bacteriophage

E. coli genome

λ lysogen

Int + IHF

Int + IHF + Xis
Step 1: Generation of the Entry clone (BP reaction)

RT-PCR → PCR with attB-tagged gene-specific primers

attB1  \[\rightarrow\]  cDNA  \[\rightarrow\]  attB2

+  \[\rightarrow\]  attP1  \[\rightarrow\]  attP2

Donor vector  \[\rightarrow\]  BP reaction mix:
integrase (Int) + integration host factor (IHF) = BP clonase

attL1  \[\rightarrow\]  attL2  \[\rightarrow\]  Entry clone

attB: attachment site (Bacterial)
attP: attachment site (λ Phage)
attB1/attP1 and attB2/attP2 were engineered based on the E. coli /λphage wt attB/attP are incompatible with each other and thus guarantee directionality
Step 2: Generation of the Expression clone (LR reaction)

RT-PCR → PCR with attB-tagged gene-specific primers

attL1                            cDNA                             attL2

Donor vector

attR1 + attR2

In the LR reaction, the cDNA is excised from the donor vector and integrated into a destination vector.

Destination vector

LR reaction mix: excisionase Xis + integrase (Int) + integration host factor (IHF) = LR clonase

Expression clone

attL: left attachment site
attR: right attachment site
BP clonase: Int + IHF
LR clonase: Int + IHF + Xis

2 steps are not needed! LR clonase mix can operate on PCR product to directly generate expression clones.

If you want to pick simultaneously entry clones and expression clones, you need to select with different antibiotics.

If you want to increase the chance of generating entry clones, a ratio of BP clonase:LR clonase of 1:3 is advised!
Five mutually incompatible attachment sites:
attB1, attB2, attB3, attB4, attB5
Seamless/Scarless cloning

Problem

Gateway cloning always leaves sequences behind (typically 25 bp from the attB sites).

“Normal” restriction enzyme cloning leaves always the restriction site behind (typically 6 bp).

Solutions

• Gibson Assembly (or similar)
• Golden Gate Assembly
Example: pSecTagA

Start (0) NheI
CTGCTTACTGCTTATCGAAATTAAATACGACTCACATATAGGAGACCCAAAGCTGGCTAGCCACCATGAGACAAGACACACTCCTGCTATG
GACGAAATGACCGAATAGCCTTATATGCTAGTGAAGCTATCCCTCTTGGTTGTCGAGCATCGGTTGACTCCTCTGCTGTGTGAAGACGATAC

P(CMV) T7

SfiI HindIII KpnI SacI BamHI
GGTACTGCTCTCTGCTGGTTCCAGTGACGGCCACAGCGGCGGCGGCGGATCGAACAGTTGTTACGAGACTCGAGTCGAGATCC
CCATGACGAGCGAGCACCCAGAGTCCAGCTGCGGCGGTGCGGCGCTCCGCGCGCGATGCTTCAACATGCTCAGAGCTAG

10 V L L L W V P G S T G D A A Q P A R A R A V R S L V P S S D P
Ig K SP (in frame with Ia K SP)

SoeI EcoRI PstI EcoRV NotI XhoI XbaI AflII
ACTAGTCCAGTTGTTGGAATTCTGCAAGATATCCAGCAGACTTCGGCGGCGCTGAGTCAGAGGCGGCAACAAACTCATCTCAGAAG
TGATCGAGTCACACACCATTAGACAGTCTAATGAGCTGTCACGCCGCGGAGCTACGATCTCCCGGGGCTTGGTTTGAGTAGACTCTC

15 L V Q C G G I L Q I S S T V A A A R V
(in frame with Ia K SP)

End

AGGATCTGAATAGCCGCGTCGACCATCATCATTACTCATCATCATTAGTGTATAACCAGTCACTAGGCTCAGCTGGCTGCTGACTCTGCTGCCTTCTGATGTTGACGC
TCCTAGACTTTATCGCGCGAGCTGAGTAGTAAGTAGTAAGTAGAATCAATTTGGGCGAGCTGAGCTGACACGAGAAGATCAACCAGGTCG

10 E D L N S A V D
myc

H6
BGH pA

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pSecTagA: MCS
Type IIP restriction enzymes

- EcoRI (451): GAATTTC
- EcoRV:

```
GTGGAATTCTGCTGAGATATCCAGCACAGTG
CACCTTAAGACGTCTATAGGTCGTGTCAT
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Gly Gly Ile Leu Gln Ile Ser Ser Thr Val
Type IIS restriction enzymes

Sticky ends from different SapI sites may not be compatible. SapI gradually settles in solution, so a tube of SapI should be mixed before removing an aliquot.
Modified pSecTagA-SapI
Golden Gate Assembly

Possible restriction enzymes (selection):
BbsI/Bpil, BbvI, BfuAI, BspMI, Bsal, BsmAl, BsmBI, BsmFI, BtgZI, FokI, SapI, SfaNI

Additional advantages:
• Multiple fragments can be assembled with one restriction enzyme
• Cleavage and ligation can be performed simultaneously (the ligation cannot be cleaved anymore by the restriction enzyme; this can be perceived also a disadvantage:
• screening of minipreps by restriction digest is not possible)

Disadvantages:
• Especially larger vectors/cDNAs contain often type IIS sites
Modular Cloning (MoClo)
Literature (Gateway Cloning)

- Original description of the Gateway cloning system by Invitrogen
  http://genome.cshlp.org/content/10/11/1788.full
- Many Paths to Many Clones: A Comparative Look at High-Throughput Cloning Methods
  http://genome.cshlp.org/content/14/10b/2020.long
- Multigateway protocol
  https://tools.thermofisher.com/content/sfs/manuals/multisitegatewaypro_man.pdf
- Lambda attachment sites
  http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/phage/lambda-att-sites.html
- Single step Gateway cloning
- Golden Gate Assembly
  http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003647
- Modular Cloning
  http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0016765
Next meeting

- Group 3 presents its cloning plan
- Room KOK7 (7th floor)