Practical Molecular Biology and Genetic Engineering

From cDNA to the purified protein in 2 weeks

#	*Date*	*Topic*	*Seminar room*
1	**Mon 8.9.2014**	The basics: Enzymes, Plasmids, Cells, Transformation, Transfection	1-2
2	12.9.2014	Expression systems (E.coli, yeast, insect cells, mammalian cells, transient and stable expression)	1-2
3	19.9.2014	Protein detection & quantification (Western, Metabolic labeling, Coomassie, Silver staining, etc.)	7 (5th floor)
4	**Wed 24.9.2014**	Protein purification (tagged vs. untagged, what tag for what purpose)	3
	3.10.2014	**No seminar**	
	10.10.2014	**No seminar**	
5	17.10.2014	Mutagenesis and in-vitro evolution (considerations, types, methods, examples)	1-2
6	24.10.2014	The modern way to do it: Gibson Assembly/In-Fusion/SLICE, Recombineering, ZFN/Talens/CRISPR	3
7	31.10.2014	Bioinformatics for wetlab scientists: software, online tools, data sources (patent databases, ...)	1-2
8	7.11.2014	Sources of sequences (gene collections, cDNA libraries, de-novo synthesis, PCR/RT-PCR), designing expression vectors, Rapid/high thruput cloning systems: Gateway (Invitrogen), Creator/In-Fusion (Clontech), UniVector	3
9	14.11.2014	Transgenic animals, Design knock-out and knock-in constructs, conditional transgenes, BAC transgenes	1-2
10	28.11.2014	Viral vectors: Lentivirus, Retrovirus, Baculovirus, Adenovirus, AAV	1-2

Course requirements

- http://www.helisci.fi/hbgs-kurssit/practmolbiol2014/

Lectures

- 10 lectures: Fridays 3:00 - 3:45 pm (note exceptions), 80% participation
- one project: a cloning simulation (in groups of 1-4)
- Software: SnapGene, SerialCloner, whatever you wish (make sure everybody can access and verify your results!)

Lab work

- Max. 16 students: 2 weeks: Dec 1st – Dec 12th 2014 (3h/d: group 1 9-12 am, group 2 1-4 pm)
- Realization of the cloning simulation, using the results of the cloning to express (and, if time permits, to detect) the protein
- Deadline to register for the lab work: Oct 15th, project discussion until end of October, groups of ~3 students work on one project (if students don't form groups by themselves they will be put into groups at random by the end of October!)
- No own project? Ask the organizer!

Software

Recommended

- SnapGene: http://www.snapgene.com (Mac, Win, Linux/Wine)
- Serial Cloner: http://serialbasics.free.fr/Serial_Cloner.html (Mac, Win, Linux)

Any other software you prefer

- GENtle V2 (online tool! https://gentle.synbiota.com)
- ApE: http://biologylabs.utah.edu/jorgensen/wayned/ape (Mac, Win)
- pDRAW32: http://www.acaclone.com (Win only)
- GENtle: http://gentle.magnusmanske.de (Mac, Win, Linux)
- GeneConstructionKit http://www.textco.com/ (Mac, Win, Linux/Wine)
- CloneManager http://www.scied.com/index.htm (Win only)
- PlasmaDNA http://research.med.helsinki.fi/plasmadna/ (Mac, Win)

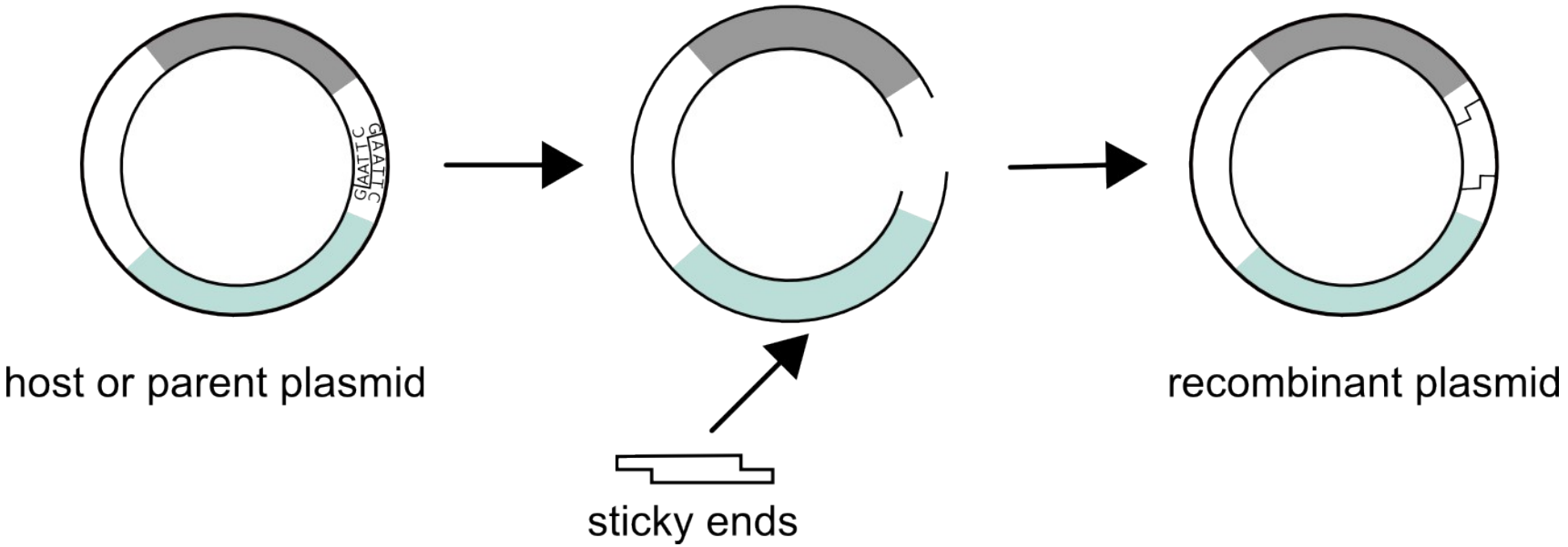
- free software (not only open source...)

The Basics: Enzymes, Plasmids, Cells, Transformation, Transfection

1. Recombinant DNA technology
2. Restriction enzyme cloning
3. Most important enzymes
4. Plasmids
5. Cells (E.coli)
6. Transformation & Transfection

Recombinant DNA technology

type II restriction endonucleases



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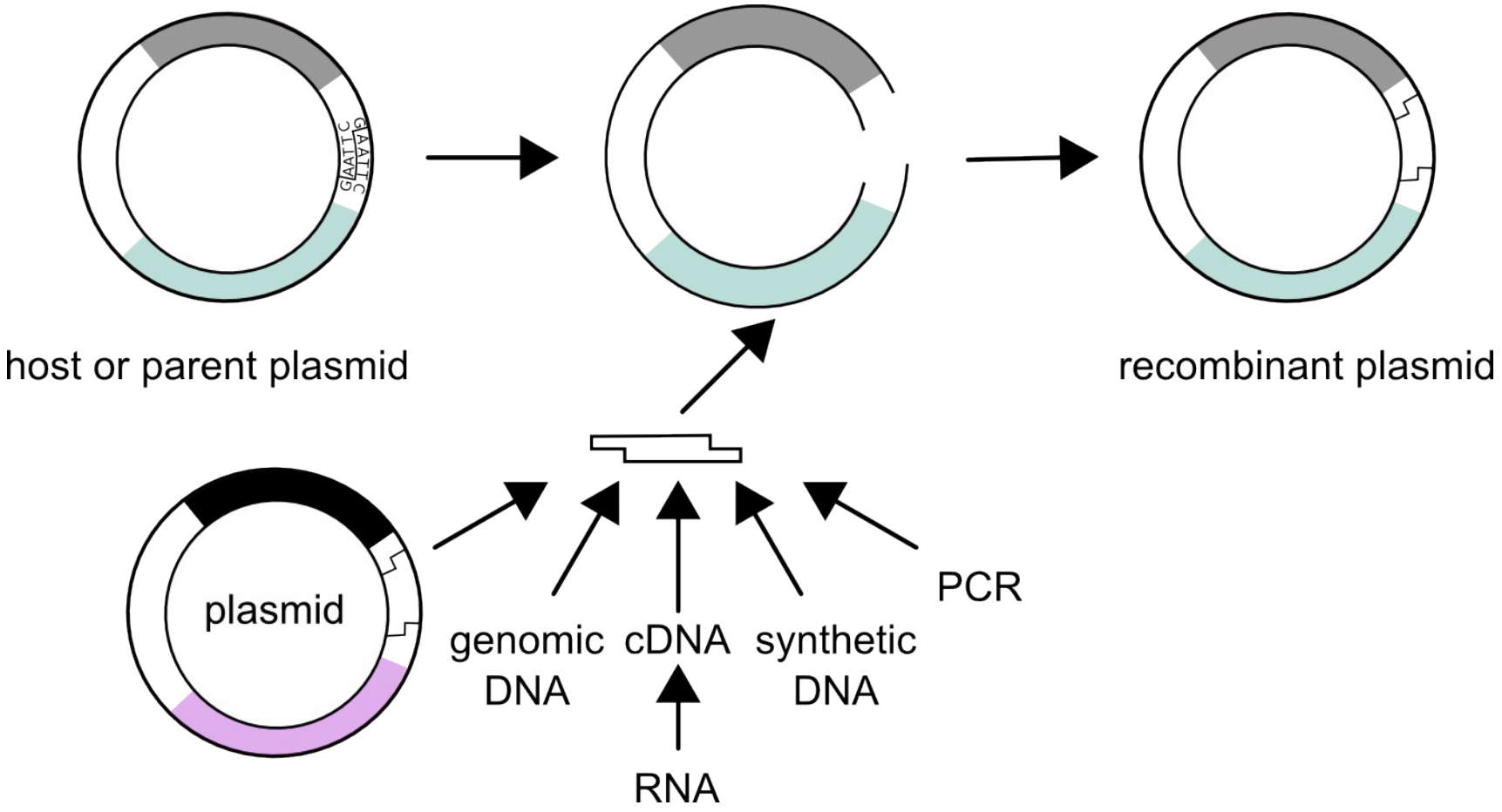
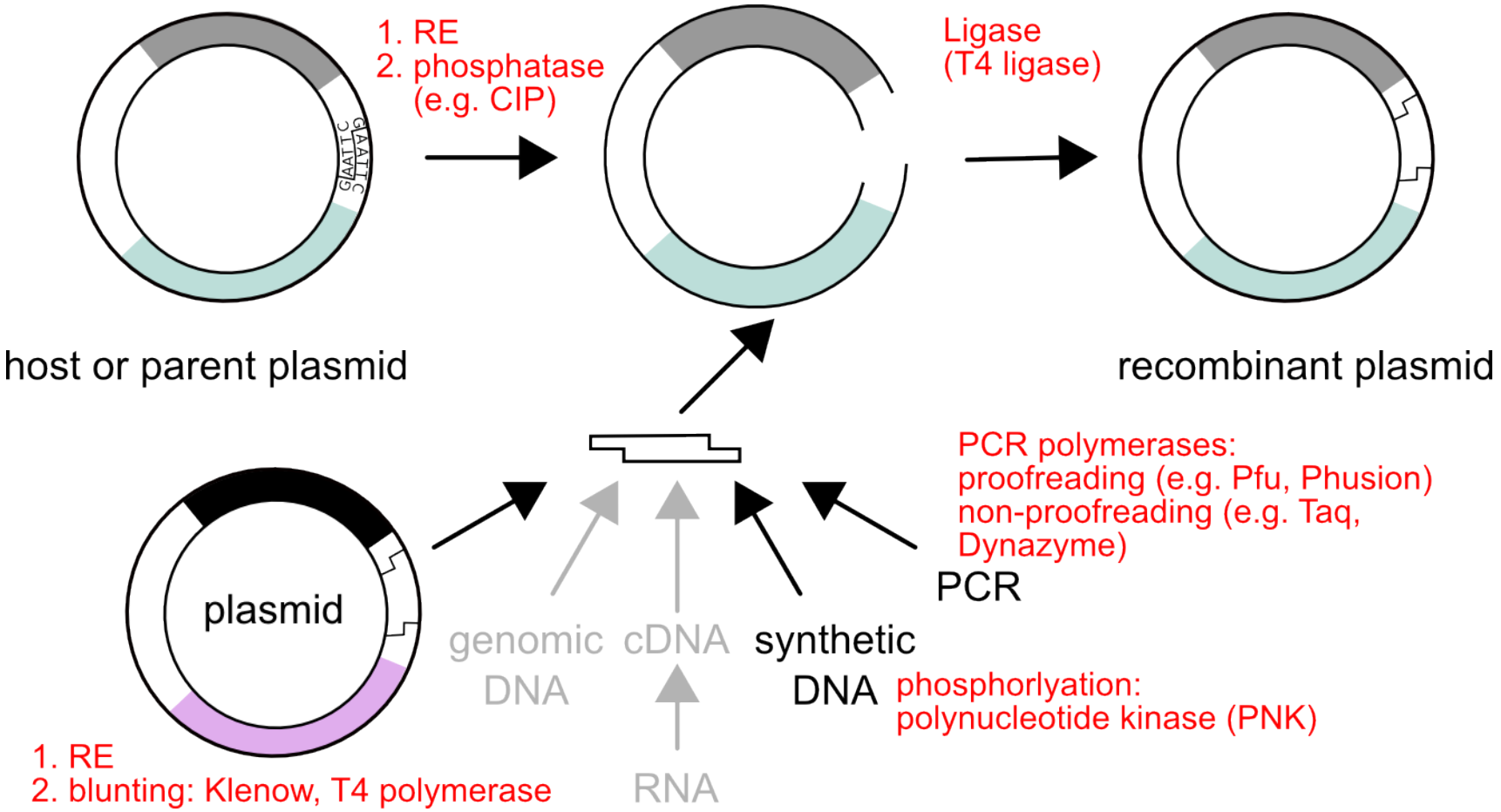


Enzymes



Where to get cDNA from?

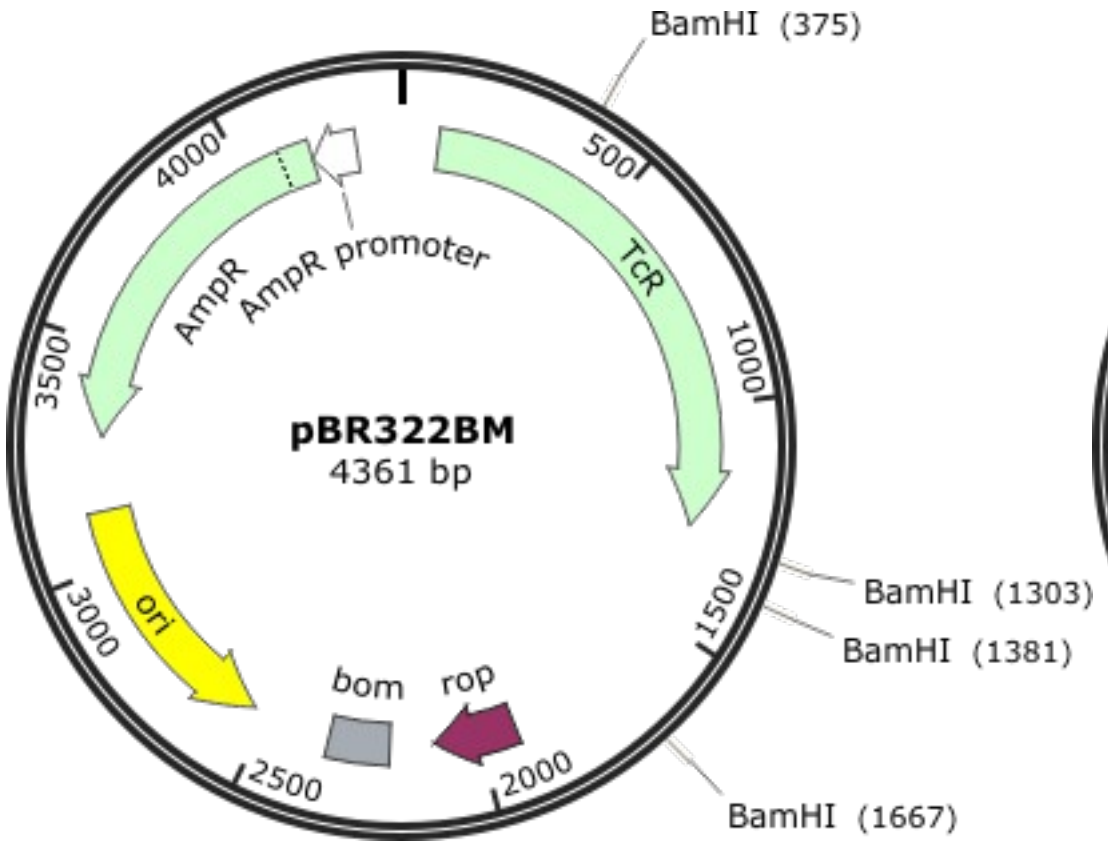
- http://mgc.nci.nih.gov/
- MGC/Orfeome (Genome Biology Unit, Biocentrum Helsinki)
  http://www.biocenter.helsinki.fi/bi/gbu/orf/

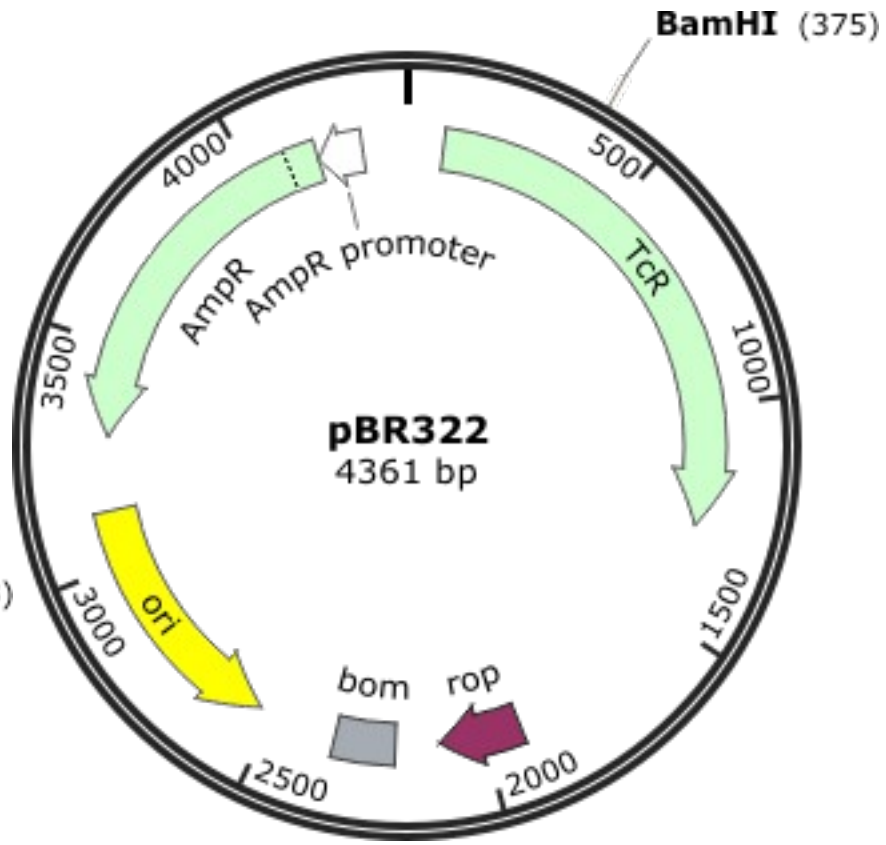
  Commercial suppliers
  GE Healthcare:
  http://dharmacon.gelifesciences.com/molecular-biology/gene-expression-cdna
  Invitrogen: https://clones.lifetechnologies.com/
  OriGene: http://www.origene.com/cDNA/
- Non-profit organizations for plasmid sharing

  ATCC: http://www.lgcstandards-atcc.org/
  Addgene: https://www.addgene.org/
- Other labs (via pubmed query „gene city“, try: „VEGF-C Helsinki“)

Restriction enzymes

1 unit RE cuts 1 µg DNA in 1 hour

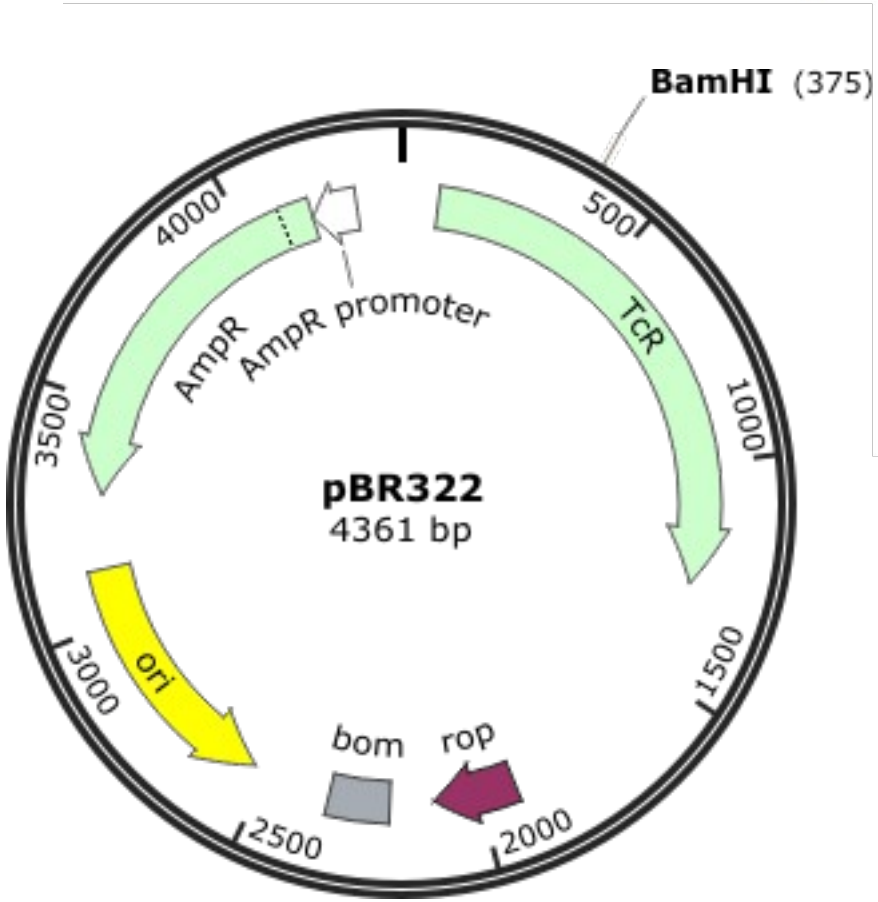




Restriction enzymes

1 unit RE cuts 1 µg assay DNA in 1 hour

$$units\,enzyme\,needed = \frac{\mu g\,DNA \cdot length\,of\,assay\,DNA \cdot cuts\,in\,target\,DNA}{length\,of\,target\,DNA \cdot cuts\,in\,assay\,DNA}$$



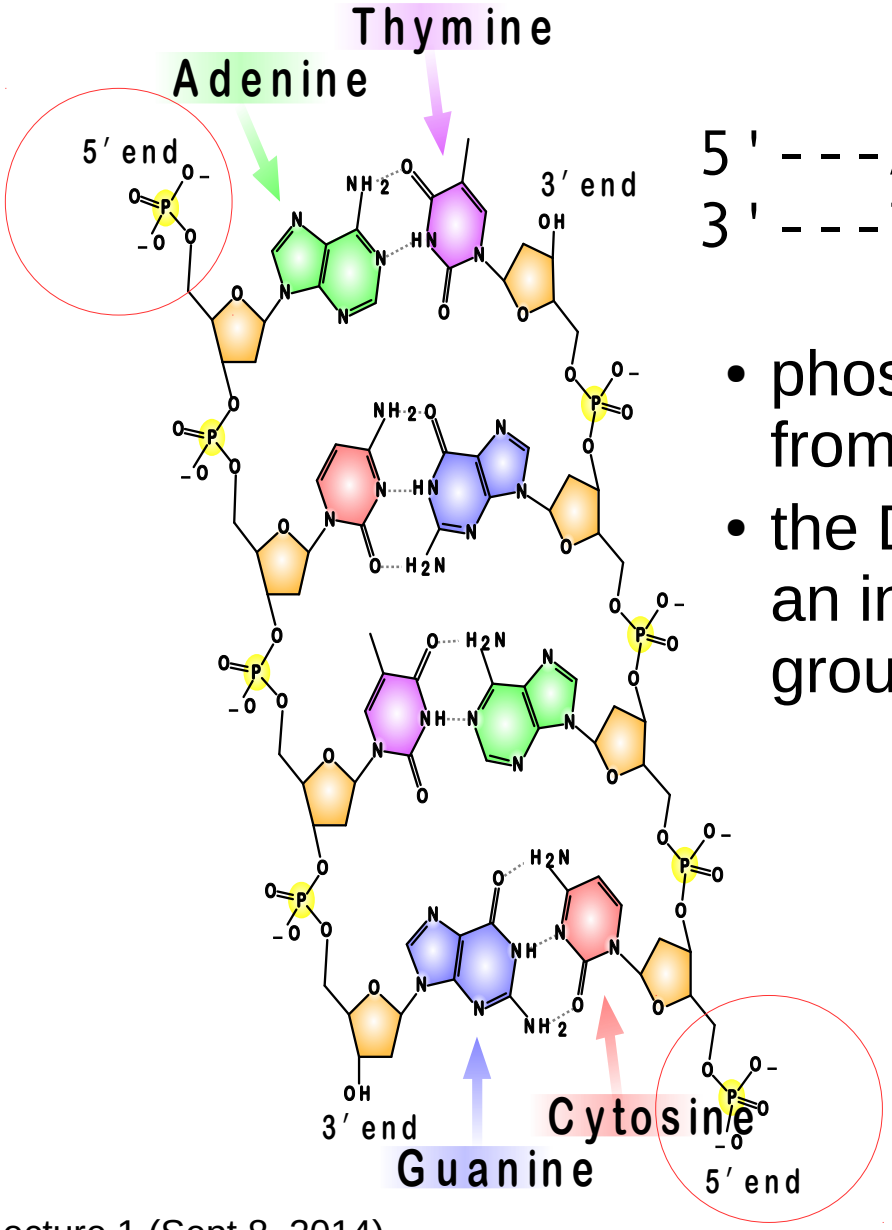
$$units\,enzyme\,needed = \frac{2 \cdot 48.5\,kb \cdot 1}{4.4\,kb \cdot 5} \sim 4.4$$

$$units\,enzyme\,needed = \frac{2 \cdot 48.5\,kb \cdot 4}{4.4\,kb \cdot 5} \sim 17.6$$

$$\frac{length\,of\,assay\,DNA}{cuts\,in\,assay\,DNA} = hit\,rate$$

$$hit\,rate\,(BamHI) = \frac{48.5\,kb\,(\lambda)}{5} \sim 9.7$$

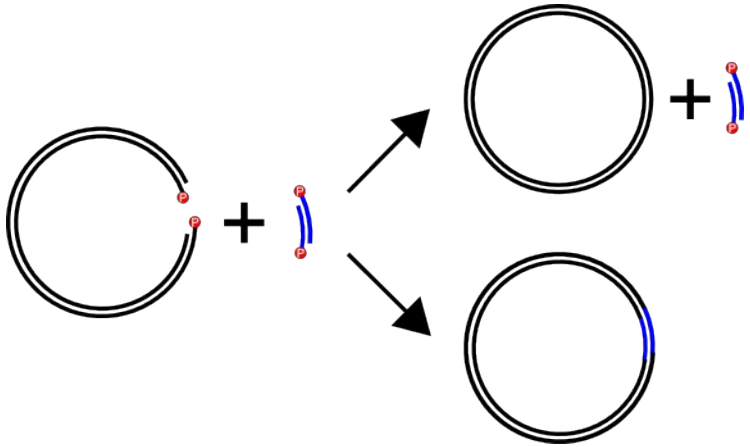
Phosphatases



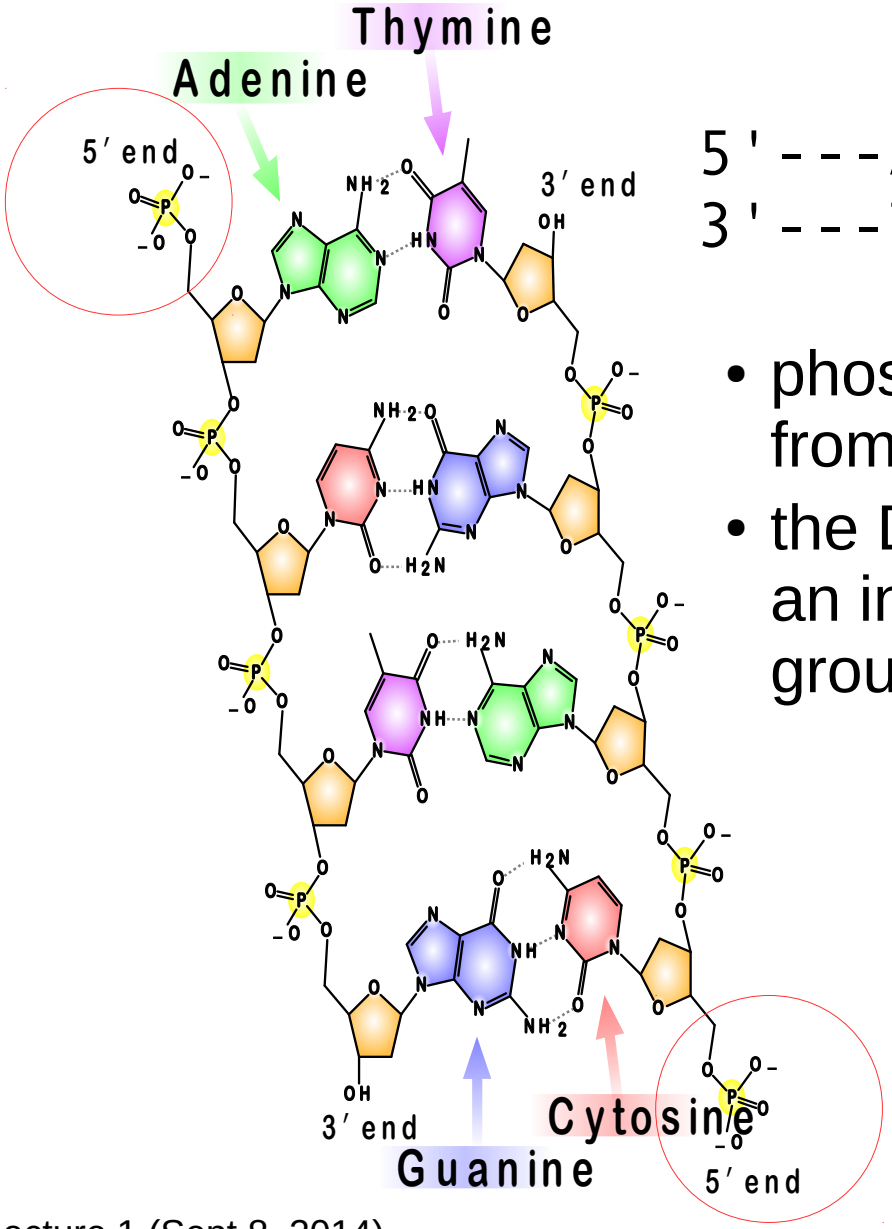
EcoRI

```
5'---AGCTGG-3'    5'-AATTCAGGAT---3'
3'---TCGACCTTAA-5'    3'-GTCCTA---5'
```

- phosphatases remove the phosphate group from the DNA 5' end
- the DNA cannot circularize again without an insert (which needs to carry a phosphate group at its 5'-ends!)



Phosphatases



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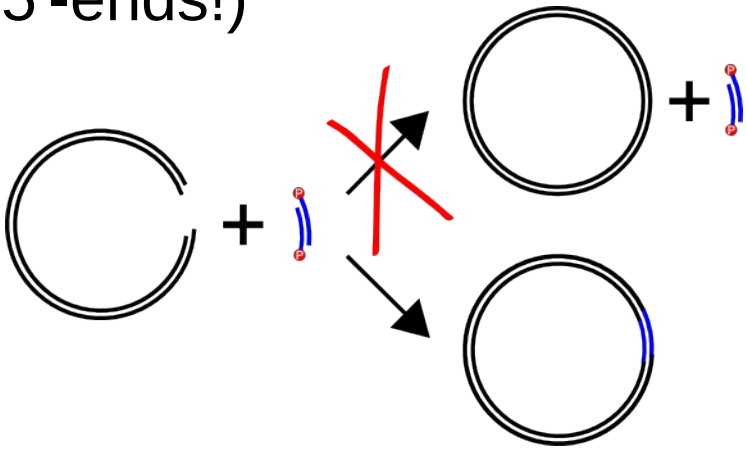
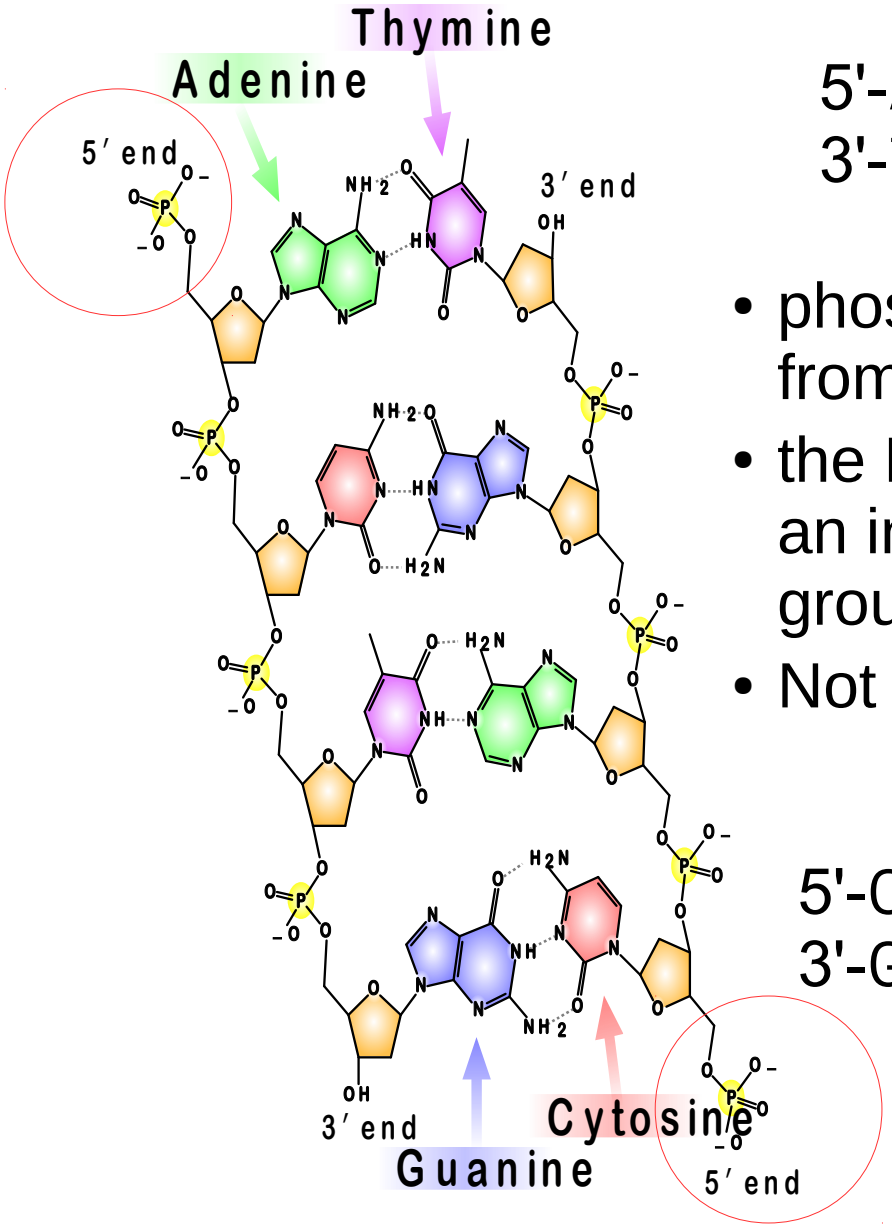


Phosphatases



EcoRI

5'-AGCTGG-3' 5'-AATTCAGGAT-3'
3'-TCGACCTTAA-5' 3'-GTCCTA-5'

- phosphatases remove the phosphate group from the DNA 5' end
- the DNA cannot circularize again without an insert (which needs to carry a phosphate group at its 5'-ends!)
- Not obligatory, sometimes not necessary

EcoRI **KpnI**

5'-CTGG AATTCAGGATGCGGGTAC CAT-3'
3'-GACCTTAA GTCCTACGCC CATGGTA-5'

„incompatible cohesive ends“

Blunting

parent plasmid insert

5'-GTTGG AATTCAGGA-3' 5'-CTGA AGCTTTGGATAGGTCGGGTAC CGC-3'
3'-CAACCTTAA GTCCT-5' 3'-GACTTCGA AACCTATCCAGCC CATGGCG-5'

EcoRI **HindIII** **KpnI**

DNA Polymerase I, Large (Klenow) Fragment („Klenow“)

T4 DNA Polymerase

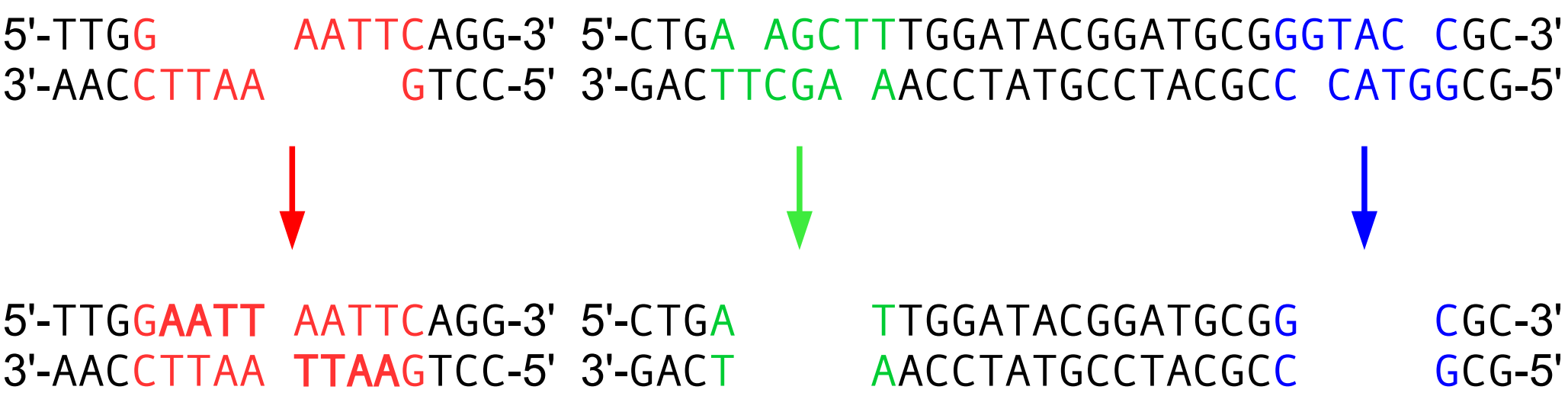
Mung Bean nuclease

Blunting

DNA Polymerase I, Large (Klenow) Fragment („Klenow“)

Mung Bean nuclease

T4 DNA Polymerase



Both Klenow and T4 polymerase have both 5'→3'-polymerase and 3'→5' exonuclease activities (but T4 polymerase's exo-Nuclease activity is much stronger than Klenow's)

Ligation

```
AGTTGGAATT     TTAATGAGGATACGGAGATACGGATGCGGGTAC       CAGG
TCAACCTTAA     AATTACTCCTATGCCTCTATGCCTACGCC      CATGGTCC

GGGTTAGTTGGAATTTTAATGAGGATACGGAGATACGGATGCGGGTACCAGGATGT
CGGTTTCAACCTTAAAATTACTCCTATGCCTCTATGCCTACGCCCCATGGTCCTACA
```

- T4 Ligase
- “Quick Ligation Kits“ = highly concentrated T4 Ligase
- ATP needed!

Polynucleotide kinase (PNK)

- Does undo the action of phosphatases, adds a 5'-phosphare group
- Needed when the 5'-end of an insert is derived from a synthetic oligonucleotide (e.g. when two oligo-nucleotides are annealed to create an insert or when a PCR product is used for cloning (without trimming the ends by a restriction enzyme)
- Can be added during oligo synthesis

PCR enzymes

Proofreading („High Fidelity“) vs. non-proofreading enzymes

	Error rate	**Reference**	
Taq, Dynazyme	1 in ~3500	NEB https://www.neb.com/tools-and-resources/feature-articles/polymerase-fidelity-what-is-it-and-what-does-it-mean-for-your-pcr	~50% errorfree molecules (400bp, 25 cycles)
	1.1 x 10^{-4} base substitutions/bp	Tindall, et. al. (1988) Biochemistry 27, 6008	
	2.4 x 10^{-5} frameshift mutations/bp	Tindall, et. al. (1988) Biochemistry 27, 6008	
	2.1 x 10^{-4} errors/bp	Keohavong, et. al. (1989) PNAS 86, 9253	
	7.2 x 10^{-5} errors/bp	Ling, et. al. (1991) PCR Methods Appl 1(1), 63	
	8.9 x 10^{-5} errors/bp	Cariello, et. al. (1991) Nucleic Acids Research 19(15), 4193	
	2.0 x 10^{-5} errors/bp	Lundberg, et. al. (1991) Gene 108, 1	
	1.1 x 10^{-4} errors/bp	Barnes, et. al. (1992) Gene 112, 29	
Pfu	1 in ~50,000-400,000		
Phusion	1 in 0.1-0.5 Mio.		
Q5	1 in 1 Mio.		99.5% errorfree molecules (1kb, 25 cycles)

PCR

- PCR conditions: dNTP concentration, Mg^{2+} concentration
- Number of cycles: as low as possible (14-25), relatively large amount of template (1ng - 0.5µg)
- PCR-derived sequences should be checked by sequencing
- Primer quality issues (DNA synthesis error rate: 1 in ~160)
- T/A overhangs or not? Non-proof-reading enzymes attach a single A-overhang to the 3'-end of the PCR product (exploited by T/A cloning)!

```
 AATGAGGATACGGAGATACGGATGCGGGTACCAGGATTCGGGAGATACGA
ATTACTCCTATGCCTCTATGCCTACGCCCATGGTCCTAAGCCCTCTATGC
```

Choosing a vector

- Insert size
- Copy number
- Incompatibilities
- Selectable marker
- Cloning sites
- Specialized vector functions

Insert size

- Max. ~15 kb
- Larger inserts require special vectors:
  Cosmids (28-45 kb)
  λ vectors (8-24 kb)
  Bacterial Artificial Chromosomes (up to 350kb)
  Yeast Artificial Chromosomes (up to 1Mb)

Copy number

- More is usually good
- Exception: toxic genes
- Dependent on origin of replication:

  ColE1 ori: 15-20 copies/cell (e.g. pBR322, „low copy plasmid“)

  pUC ori (= mutated ColE1): 500-700 copies/cell („high copy plasmid“)

  f ori (BACs): 1 copy/cell
- pUC ori is temperature sensitive!
- Amplification of low-copy plasmids with chloramphenicol
- Stringent (a few copies per cell) vs. relaxed plasmids (many copies per cell)

Incompatibilities

- Only of concern, if you need to maintain more than one vector in the same host cell
- E.g. ColE1 ori is incompatible with another ColE1 ori

Selection markers

- Mostly antibiotics: Ampicillin, Kanamycin, Chloramphenicol; Tetracyclin, Zeocin, Streptomycin, etc.
- Possible other selection markers:

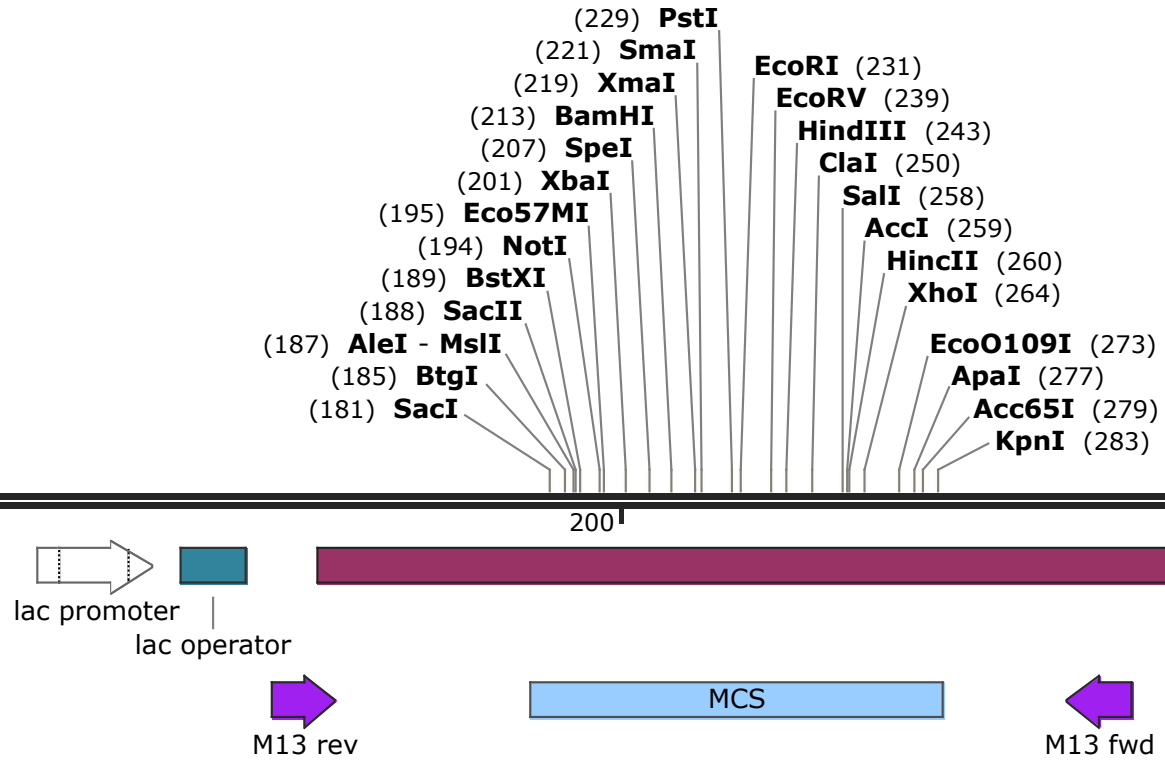
  GalK/galactokinase: in gal-operon-mutated strains like SW102; possibility for counterselection with 2-deoxy-galactose/DOG)

  SupF (mutated tRNA gene) enables expression of AmpR/amber TetR/amber by „UAG readthrough“, Tyr replaces termination), inefficient due to amber revertants!
- Selection markers do not have to be identical (different genes can confer resistance to the same antibiotic, e.g. for kanamycin)
- Mechanisms of antibiotic action and resistance are different

  (with consequences: amp inhibits cell wall synthesis, but not protein synthesis; amp is inactivated by enzymatic cleavage)
- E.coli strain: e.g. XL1 is tet^r, DB3.1 is $strep^r$, etc.
- Price!

Multiple Cloning Site (MCS)

- „Good“ selection of RE sites in the „right“ order
- LacZ screening for inserts
- Primer annealing sites for sequence verification



pBluescriptSK+

Special features

- Downstream applications
- Sp6/T7: Transcription by RNA polymerase
- bacteriophage ori (f1, M13): production of single stranded DNA (+/-)
- Promoters & other regulatory sequences (next seminar)

Different E.coli strains

- All K-12 derived: DH5α, DH10β („TOP10“)
- Dam-/dcm- (restriction enzymes are methylation sensitive, no routine maintanance of plasmids!)
- DB3.1 – for growth of ccdB(=“kill gene“)-containing plasmids
- Specialized E.coli:

  Protein expression – BL21(DE3)

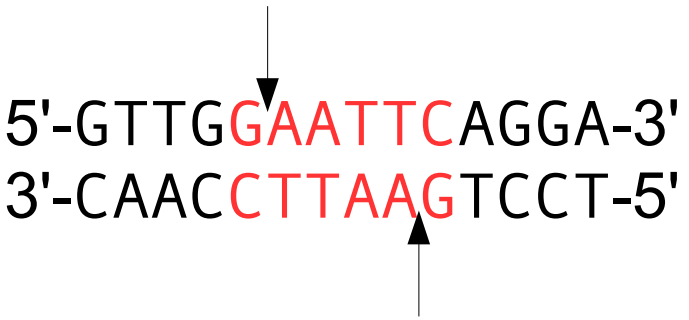
  Adenoviral DNA production – AdEasy

  Baculoviral DNA production – DH10BAC

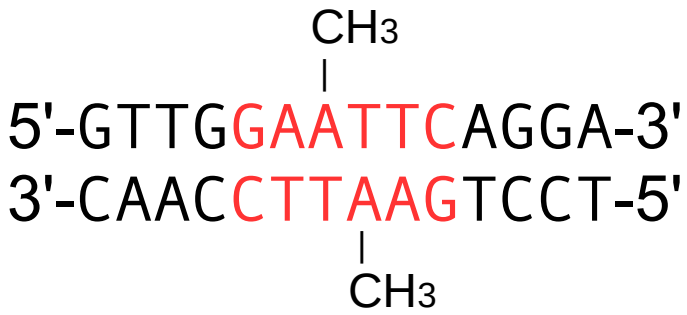
  Recombineering – DY380, EL350, SW102

Restriction Modification Systems

- Defense mechanism against foreign DNA
- EcoRI (first RE identified in E.Coli RY13 strain)



EcoRI
restriction endonuclease



EcoRI
methyltransferase

- Dam: GATC, Dcm: CC(A/T)GG

  XbaI: TCTAGANN (blocked if NN=TC)
  BclI: TGATCA (always blocked)

Transformation

- One way to introduce exogenous DNA into E.coli (other possibilities: conjugation („bacterial sex“, transduction by viruses)
- natural vs. artificial competence
- linear vs. circular DNA (exonucleases eat free DNA ends!)
- chemically competent vs. electrocompetent (chemical stress & low temperature)
- self-made vs. commercial

Transfection

- One way to introduce exogenous DNA into eukaryotic cells
- Chemical, electroporation, injection, gene gun, magnetofection, sonoporation

Science or Fiction

- Craig Venter's 1st synthetic cell had neither synthetic cytoplasm nor synthetic DNA, only the DNA fragments that were assembled by a yeast cell in a multistep process were synthetic.
- The NIH originally wanted to confine all recombinant DNA technology to class 3 facilities.
- A company has produced a DNA laser printer that uses lasers to select error free oligonucleotides for assembly into contigous DNA streches.
- The first type II restriction enzyme was HindIII. It was discovered in 1959 by Arber and Meselson.

Origin of plasmids

- Extrachromosomal circular ds DNA (after integration „episome“)
- Types of plasmids: Bacterial conjugation, antibiotic resistance, toxin/virulence, metabolism
- Horizontal gene transfer by (natural) transformation and transduction