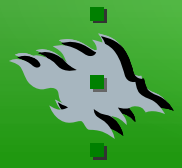
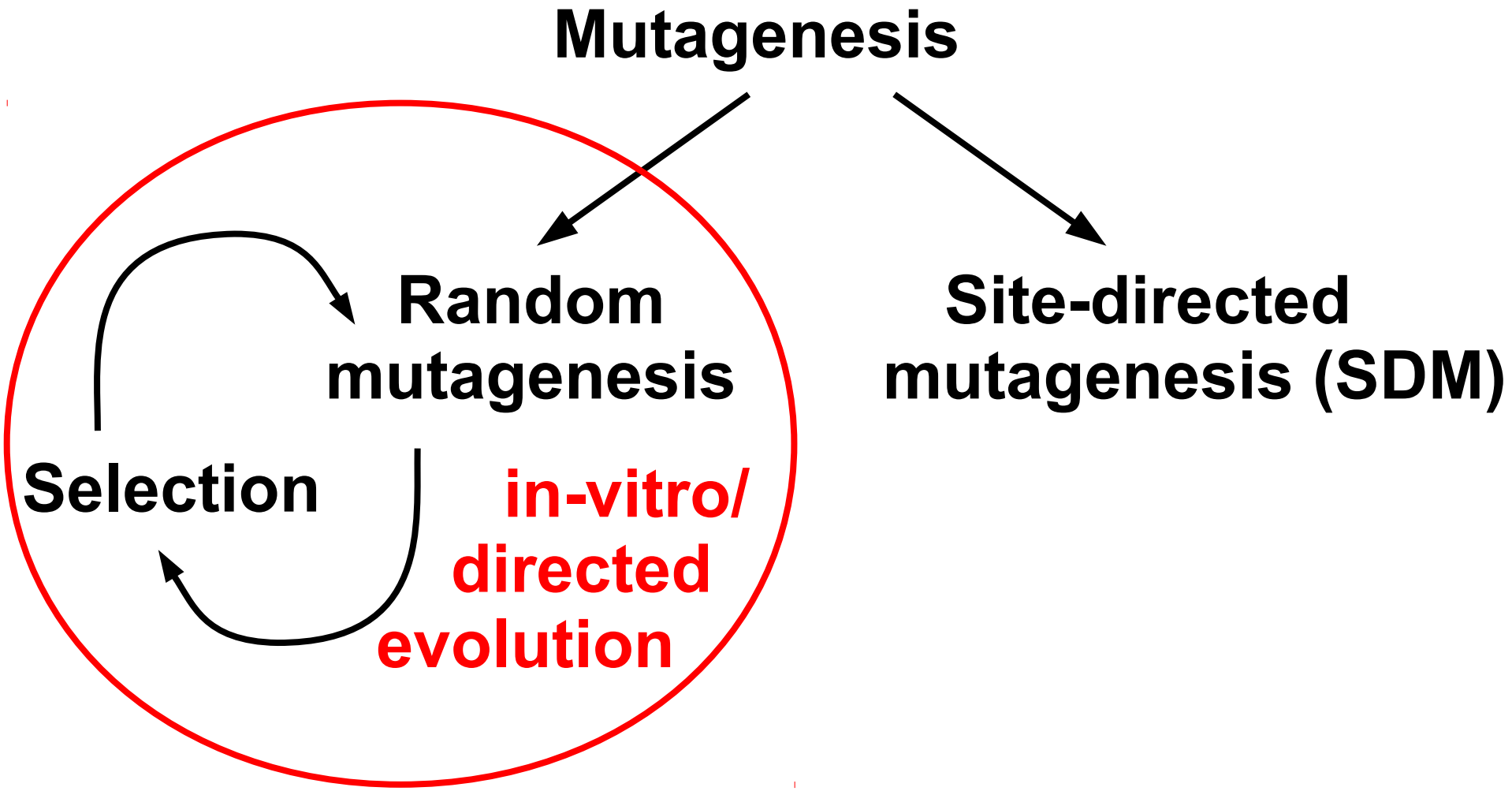


Mutagenesis & in-vitro evolution

The course schedule is subject to modifications!

Mutagenesis & in-vitro evolution

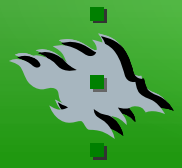


Site-directed mutagenesis & in-vitro evolution

Types of mutations you may want to introduce:

- Point mutations
- Deletions
- Insertions

Technically, cloning an insert into a vector is equivalent to insertional mutagenesis (“casette mutagenesis”)

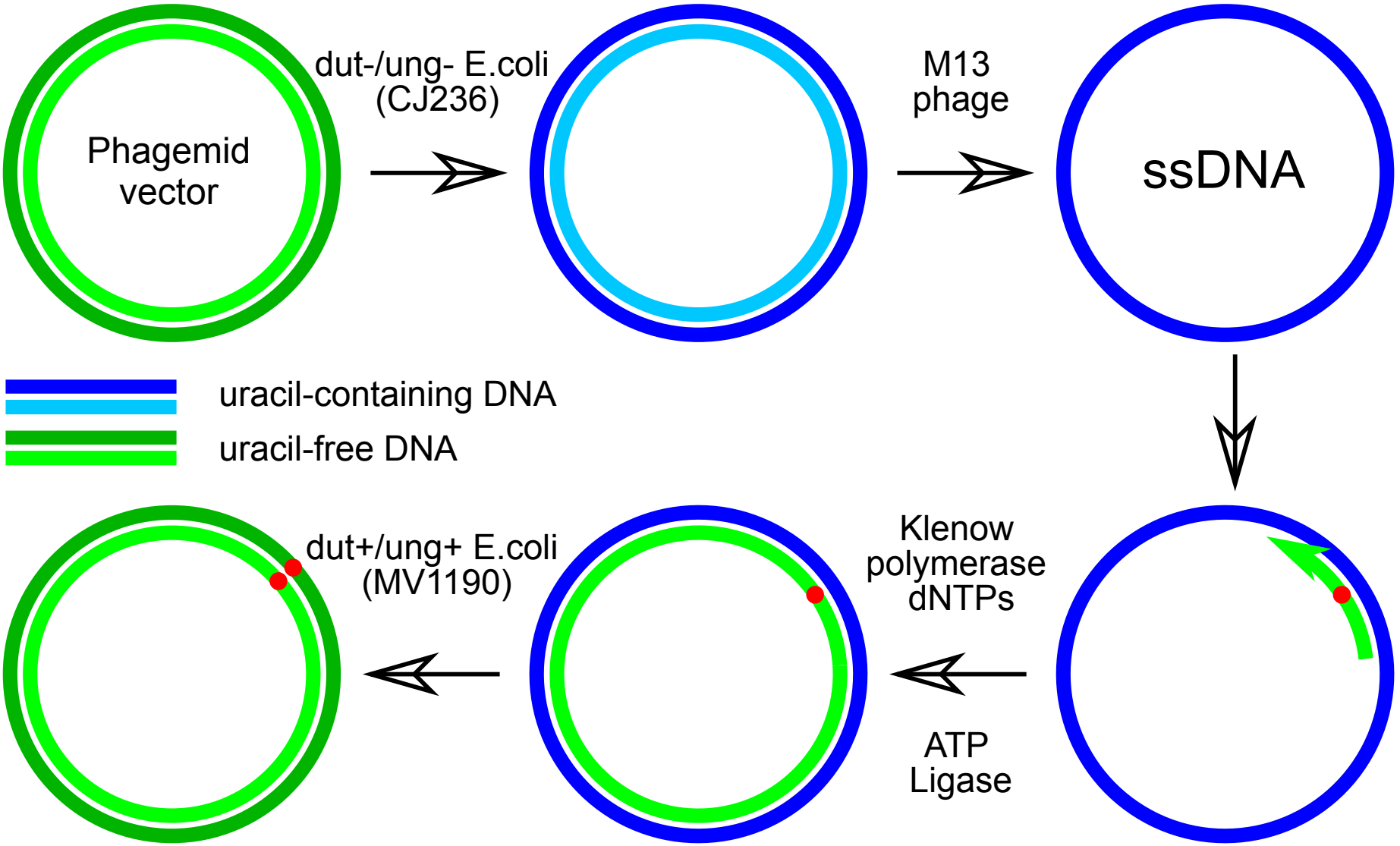


Site-directed mutagenesis & in-vitro evolution

Methods

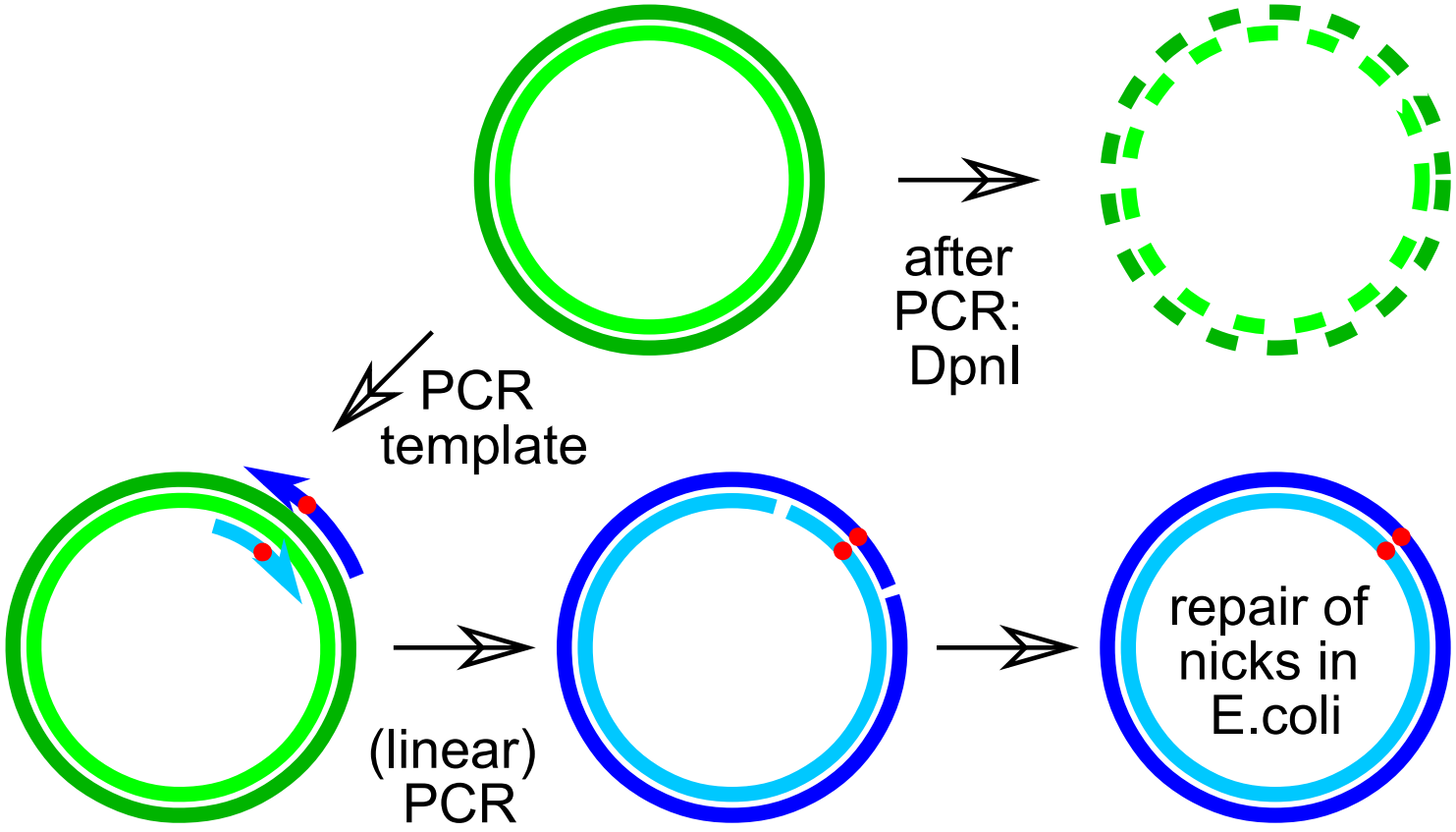
- Kunkel-Method, MutaGene® (Bio-Rad)
- Whole plasmid mutagenesis/Inverse PCR mutagenesis: QuikChange® (Agilent), Q5® SDM Kit (NEB), Phusion® SDM Kit (ThermoScientific)
- Insert PCR mutagenesis: Simple and overlap extension mutagenesis
- SDM by Gibson-Assembly or In-Fusion

Kunkel method



ung- = uracil N-glycosylase-deficient => high intracellular levels of dUTP
dut- = dUTPase-deficient => prevents removal of uracil from DNA

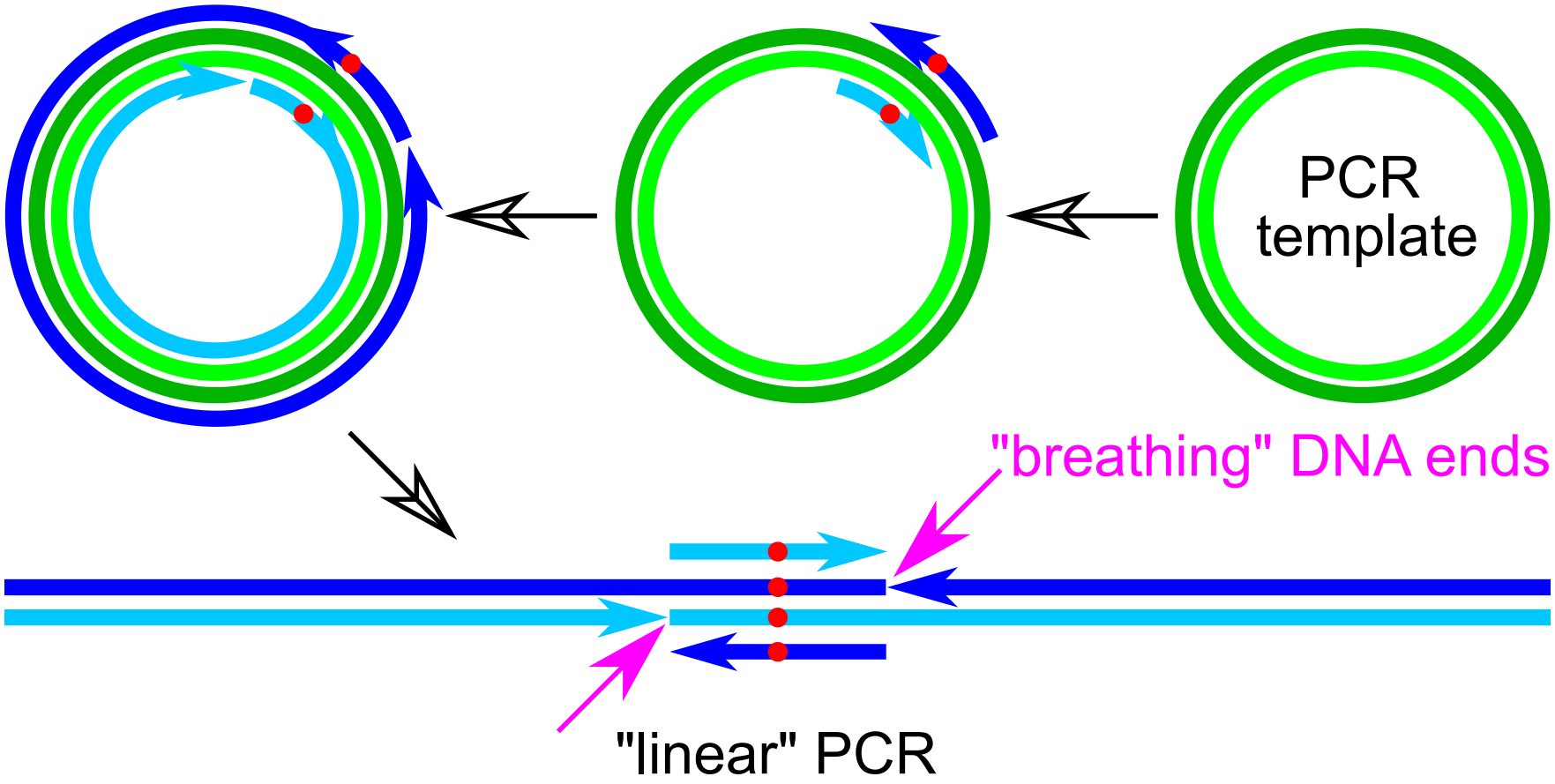
QuikChange



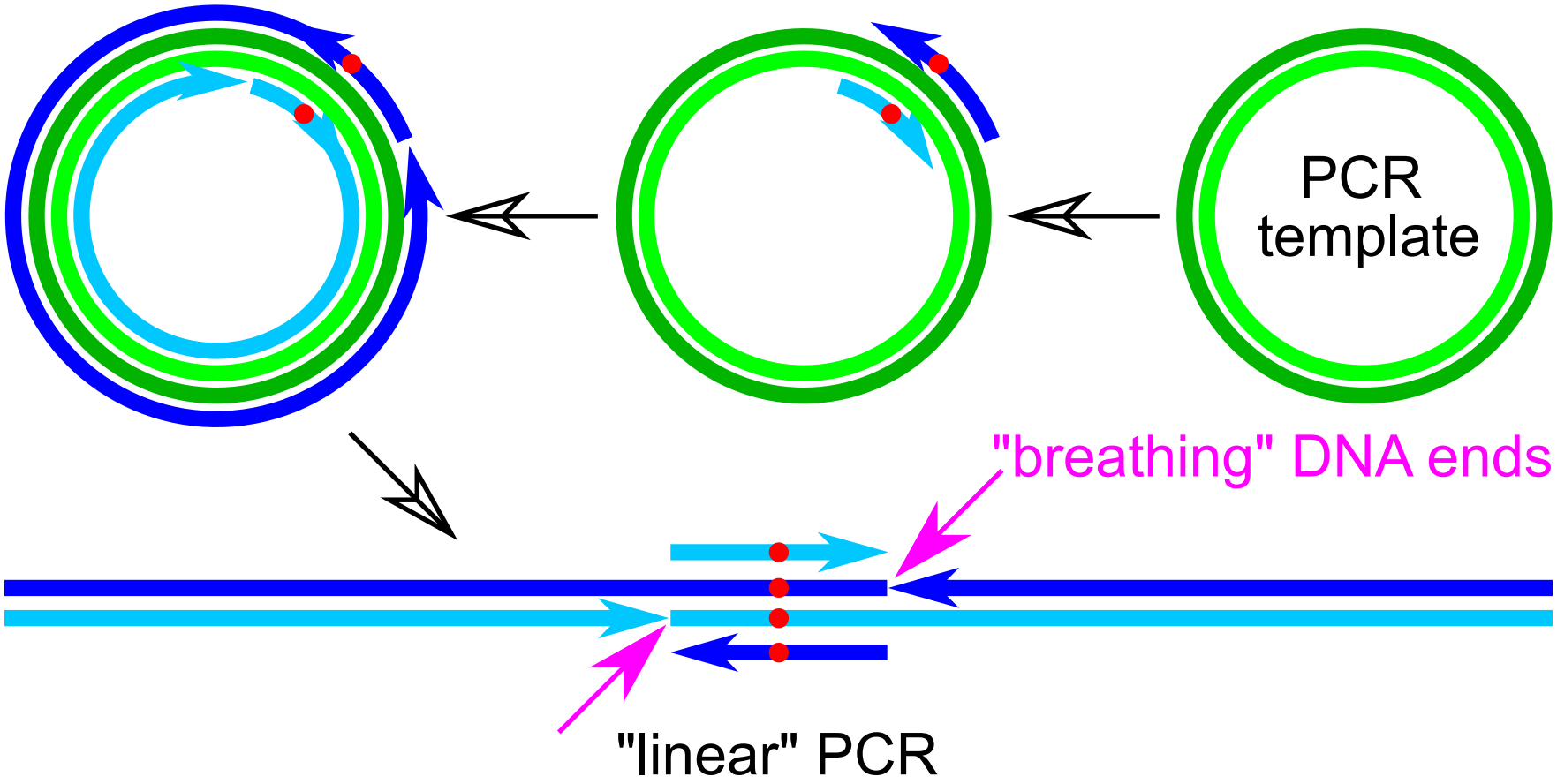
Limitations

- **PCR efficiency**
  Primer dimers
  Length of construct
  DNA secondary structure
  (very high/low GC content)
- **Type of mutation**
  Point mutations
  Small insertions & deletions

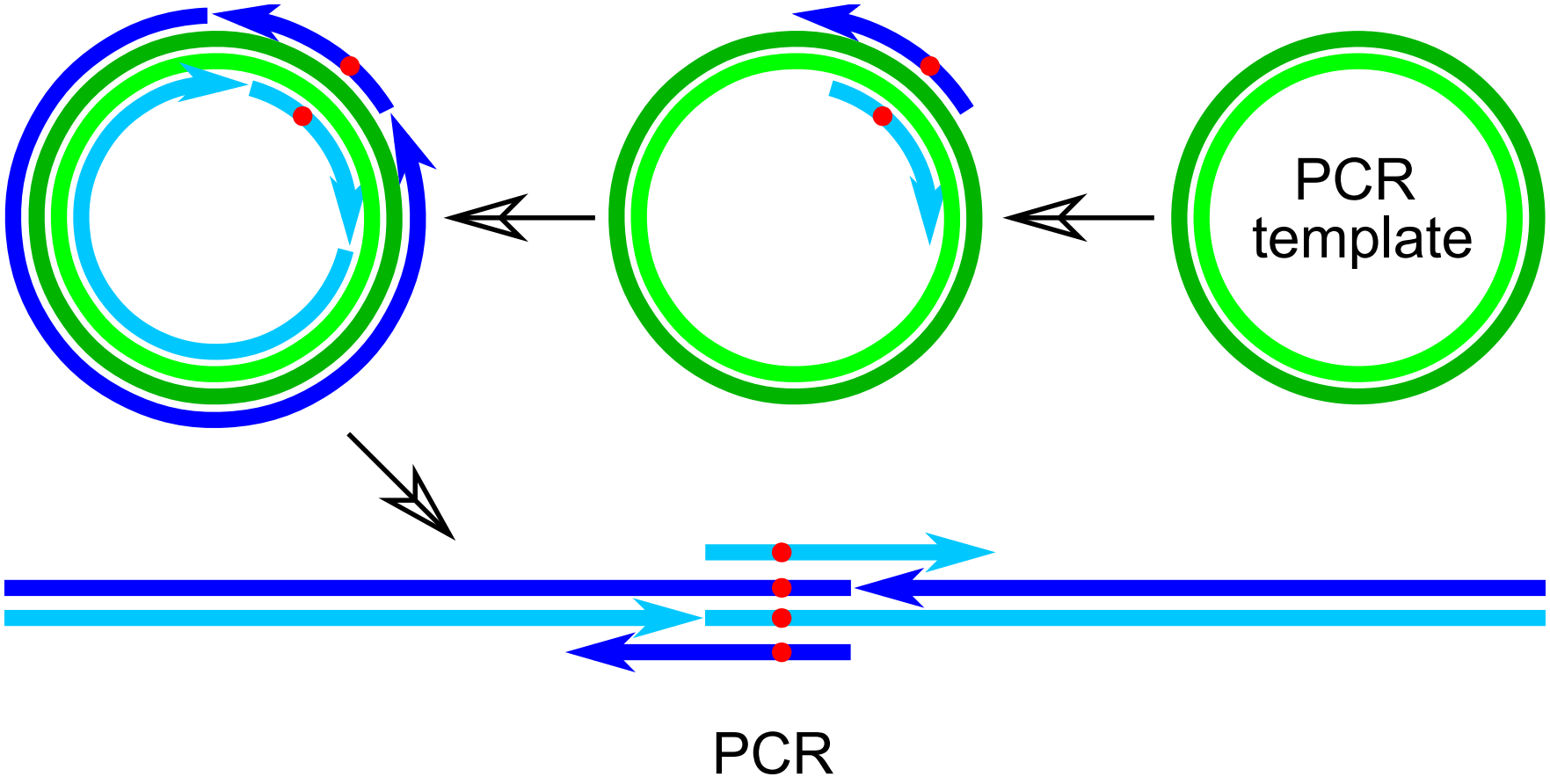
QuikChange



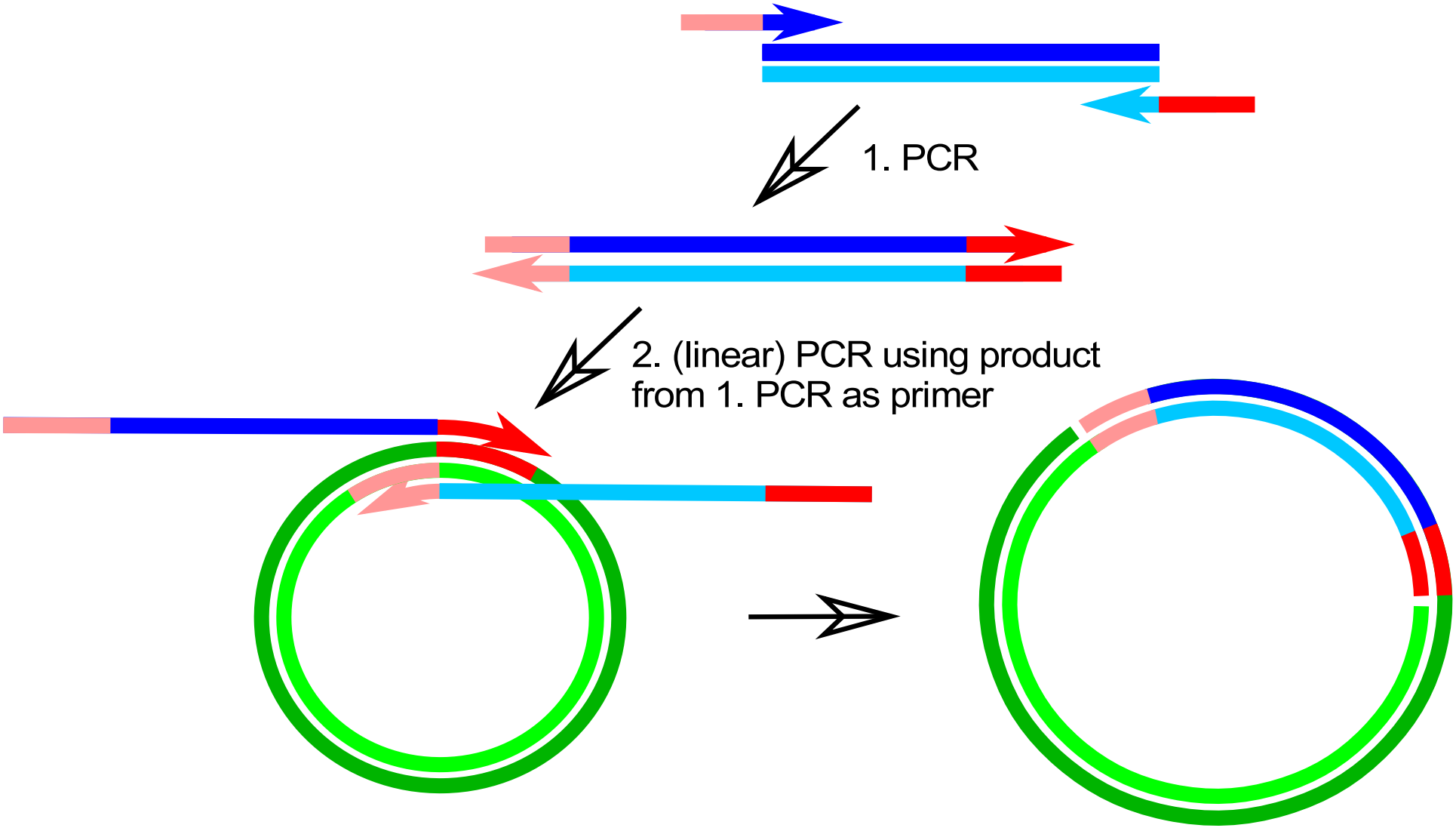
Improved QuikChange



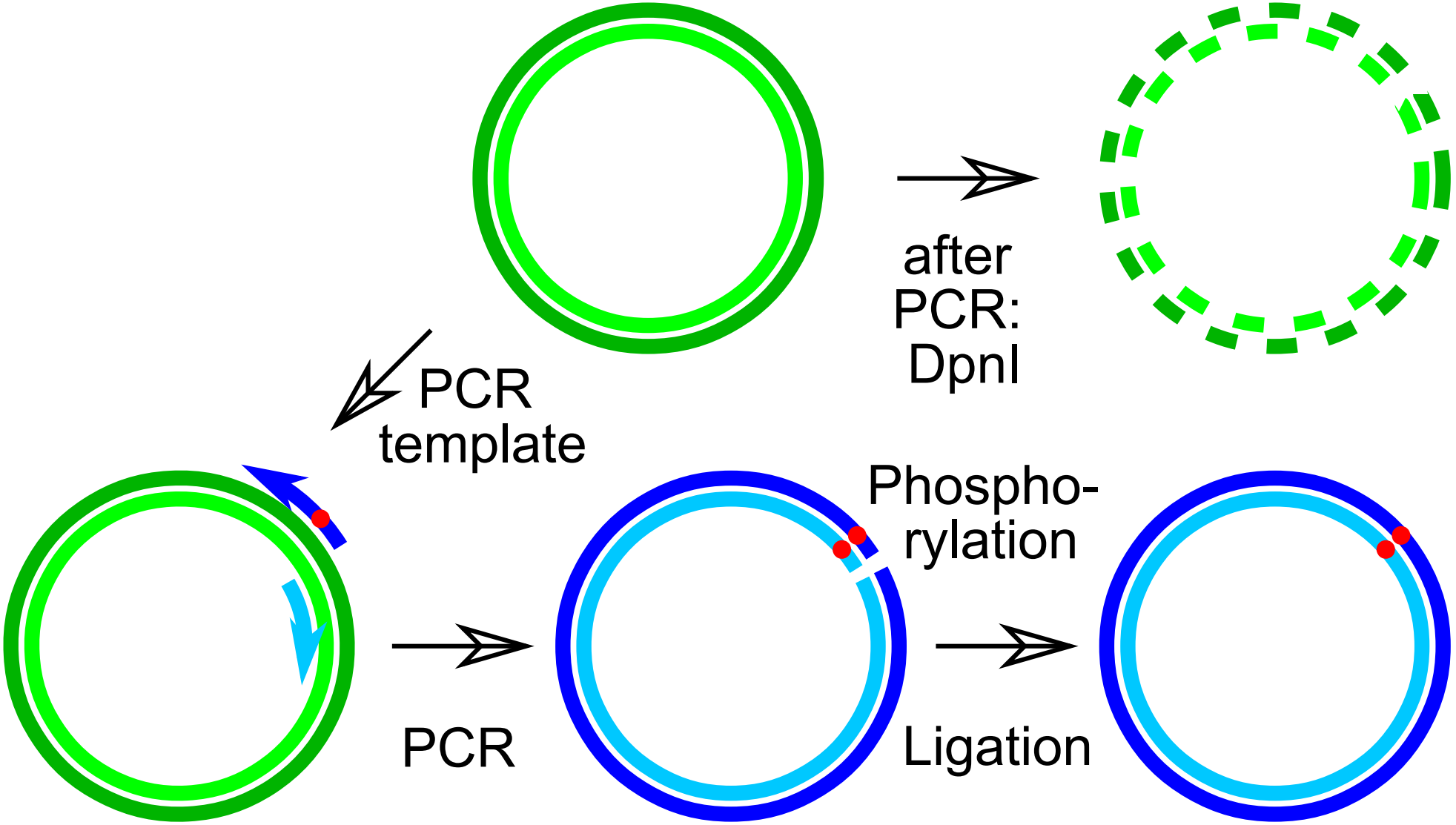
Improved QuikChange



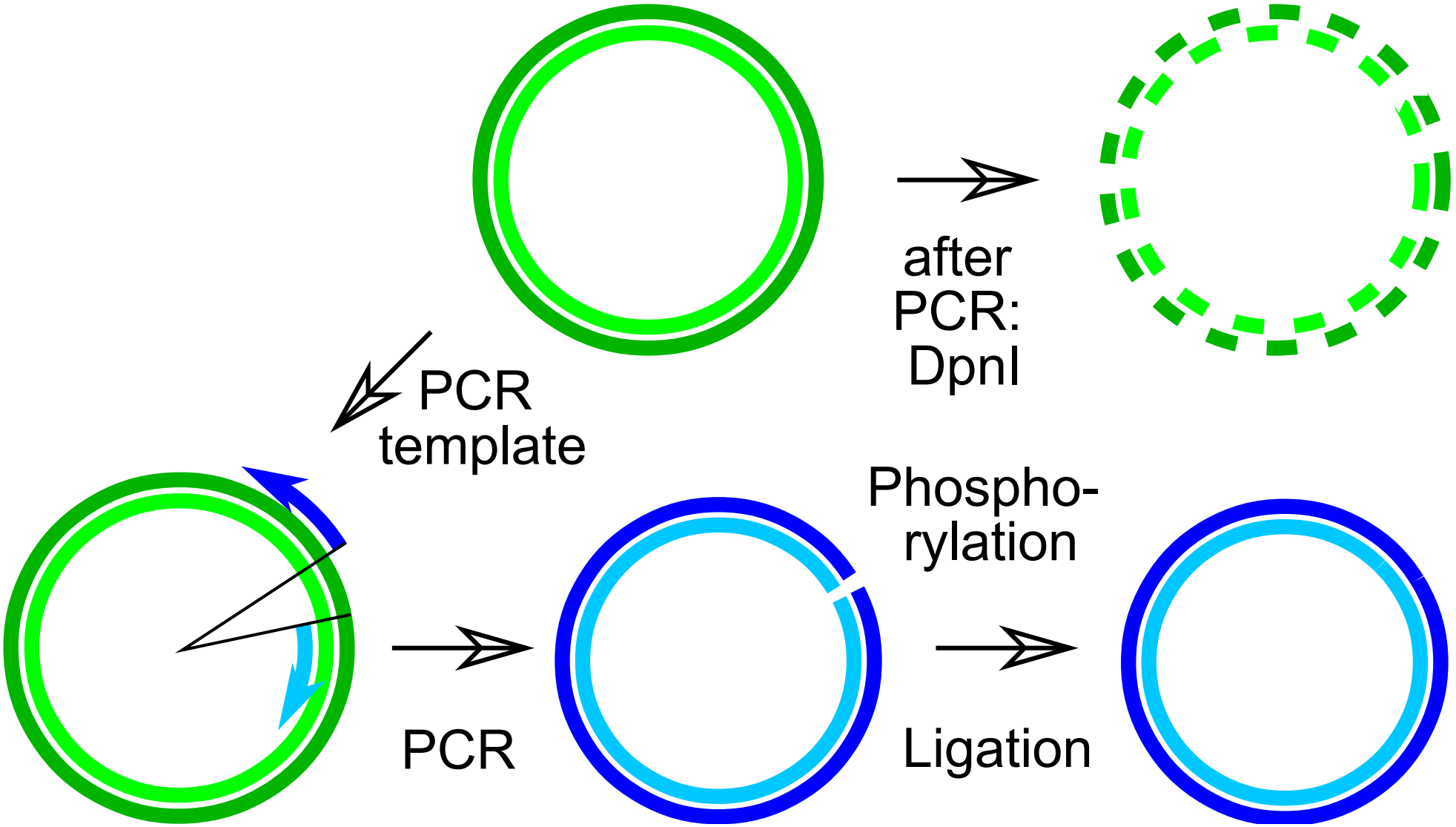
QuikChange Megaprimer (for large insertions)



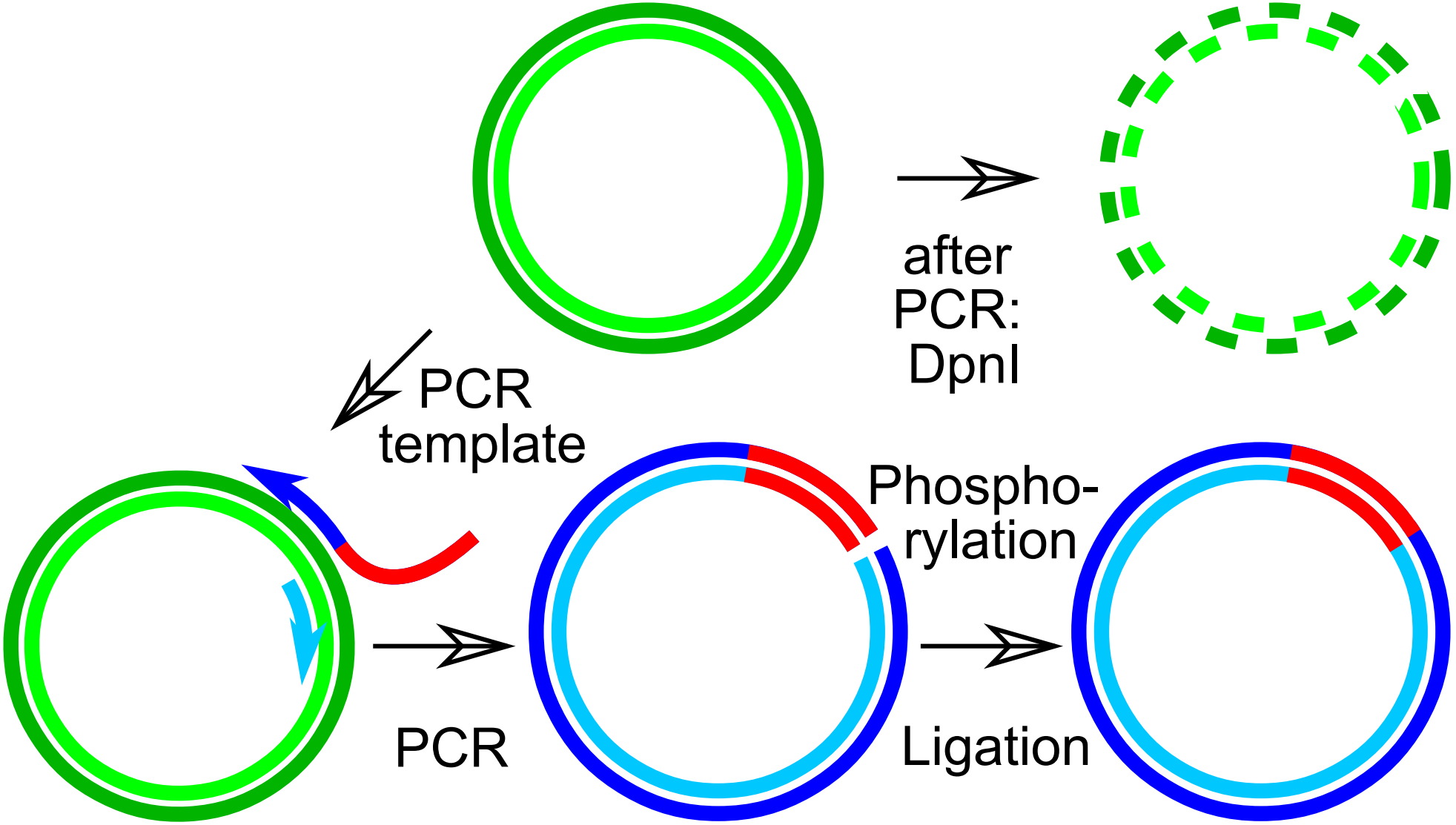
Q5®/Phusion® SDM: Point mutations



Q5®/Phusion® SDM: Deletions



Q5®/Phusion® SDM: Insertions

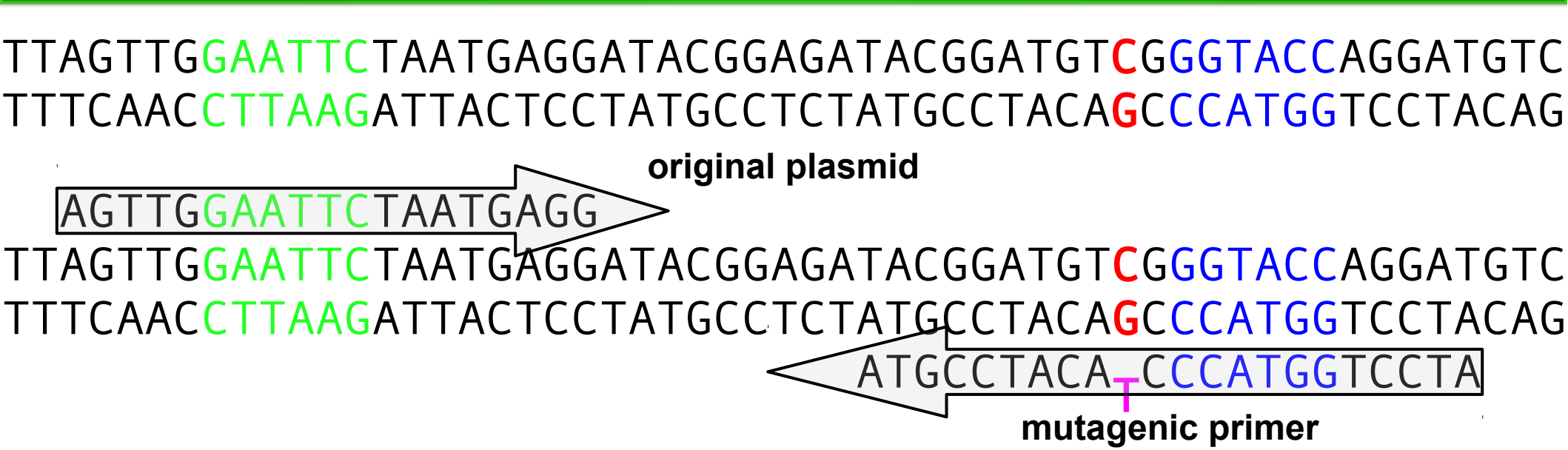


Whole plasmid mutagenesis: Limitations

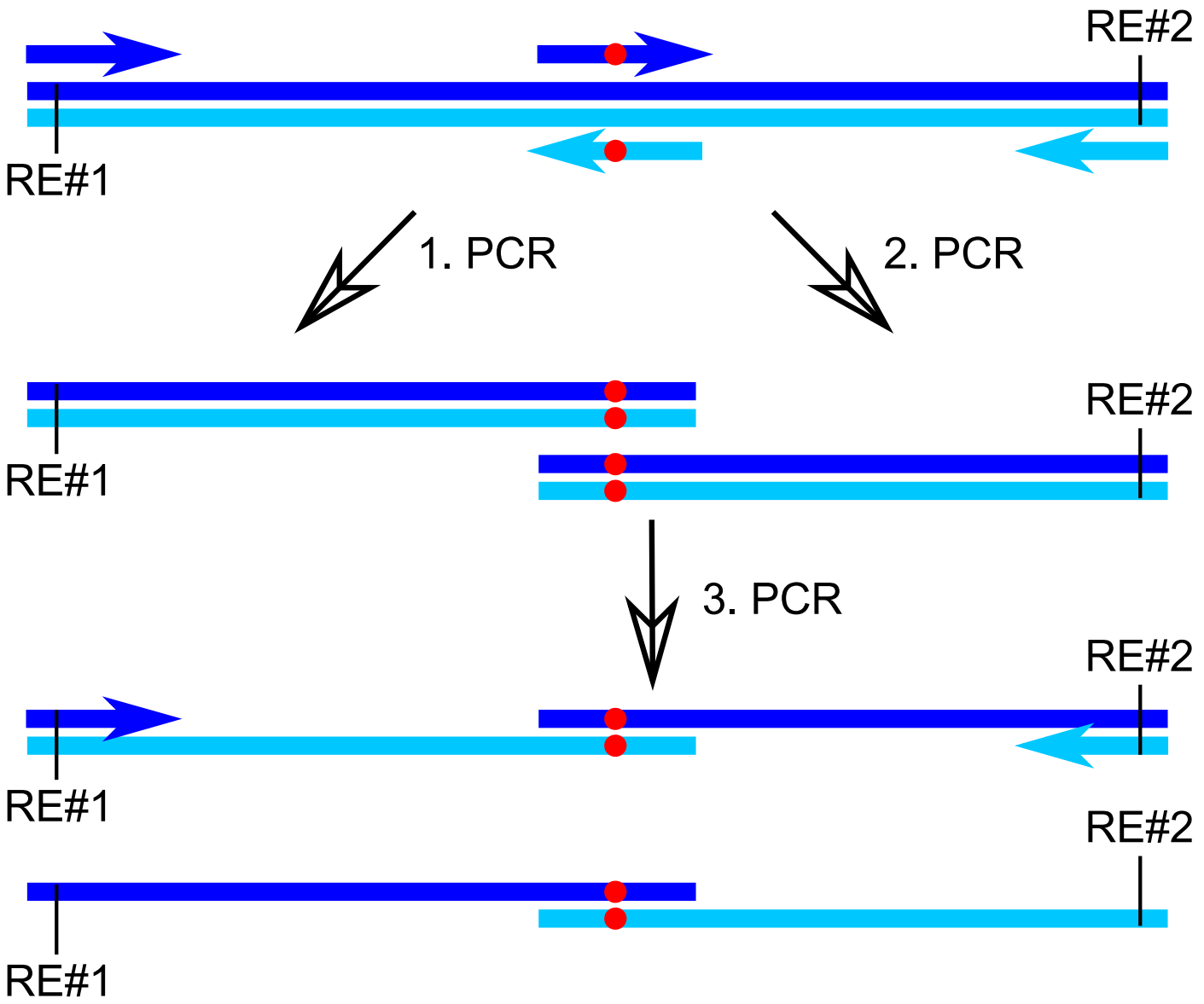
- **PCR efficiency**
  Primer dimers (solution: separate reactions)
  Length of construct (solution: subcloning into minimal vector)
  DNA secondary structure (very high/low GC content)
- **Type of mutation**
  Large insertions & deletions (solution: Megaprimer)
- **PCR error rate**
  All PCR-derived sequences contain potentially mutations and important sequences need to be either sequenced or functionally tested

→ **Minimize the PCR-derived sequences**

PCR mutagenesis



PCR mutagenesis: Overlap extension



SDM by Gibson Assembly

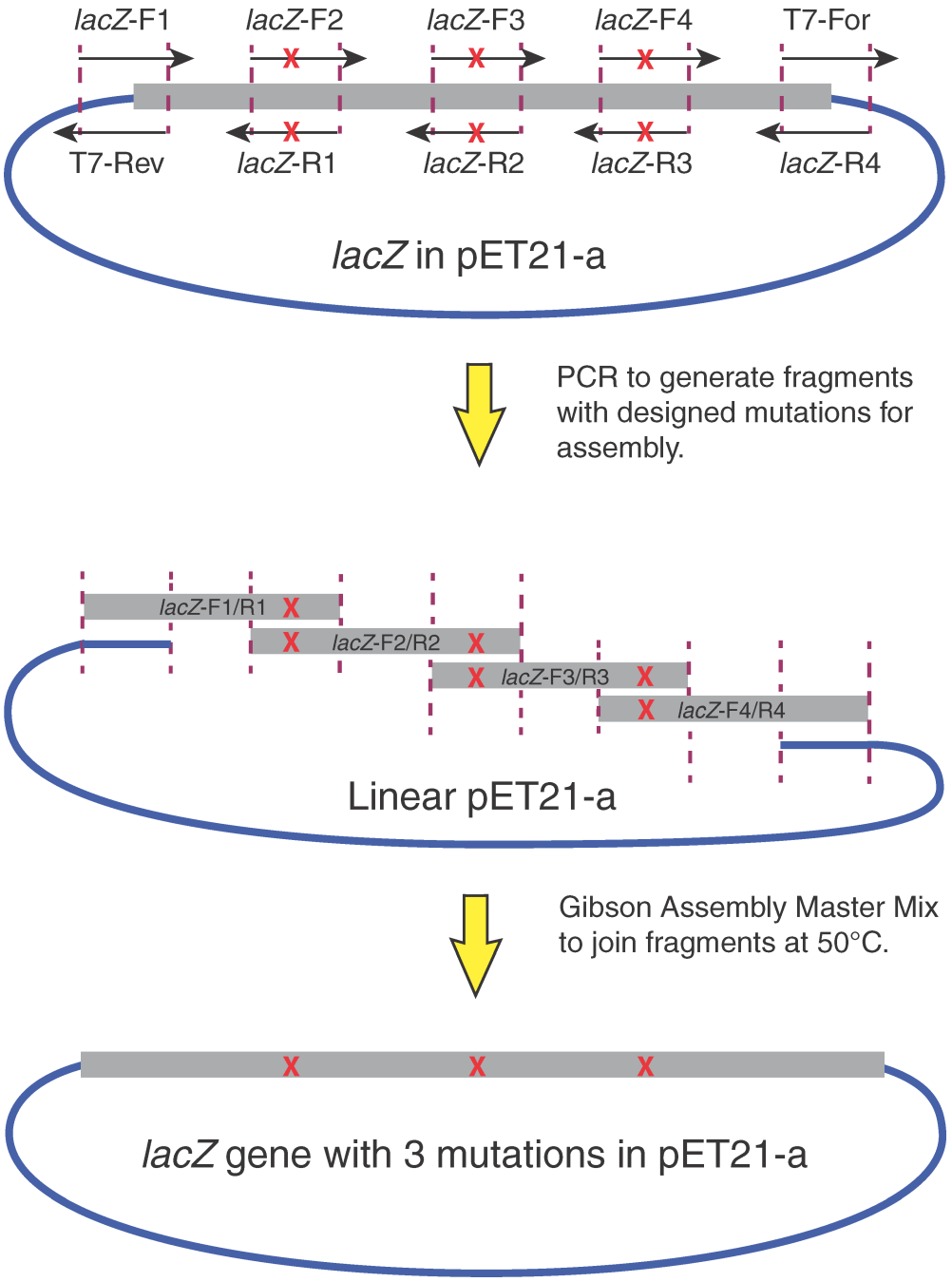
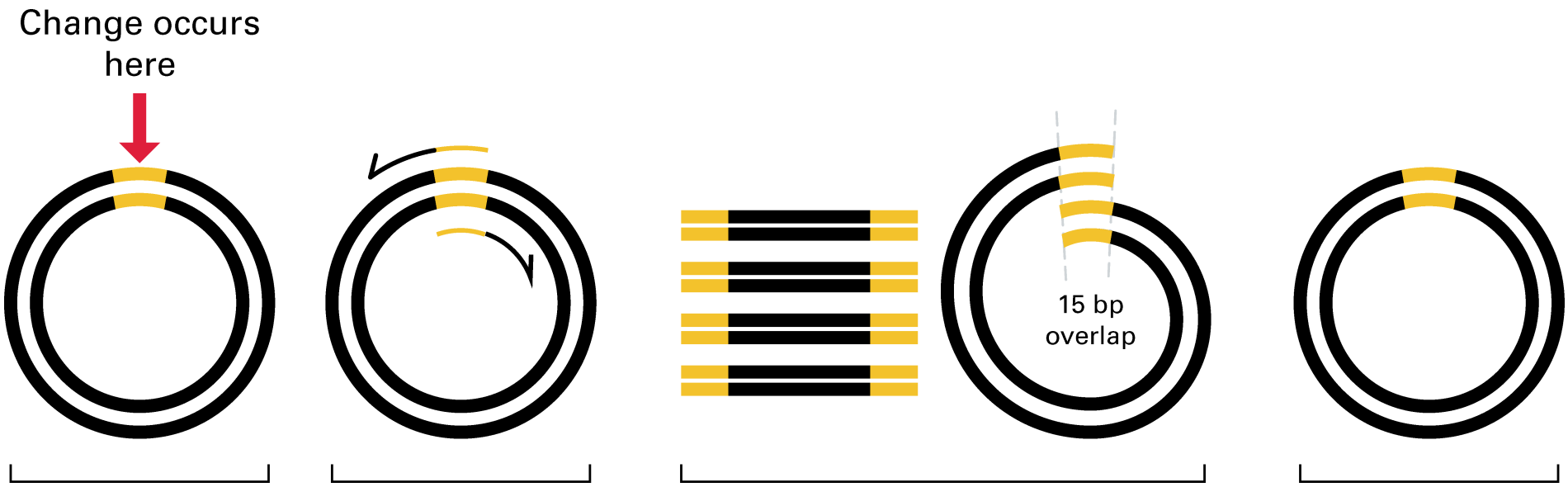


Figure from NEB

SDM by In-Fusion



1. **Envision final construct**
2. **Design primers**
3. **Perform In-Fusion protocol**
   - Amplify linear construct
   - Perform In-Fusion reaction to re-circulize vector at 15 bp overlap
   - Transform into Stellar Competent Cells
4. **Recover final construct**

Figure from Clontech

In-vitro evolution

Methods

Random mutations

- Error-prone PCR (Mn^{2+} or special polymerase)
- Chemical mutagenesis
- Combinatorial cassette mutagenesis
- Mutator strains
- Random Insertion/ Deletion (RID)
- Sequence saturation mutagenesis (SeSaM)
- Saturation mutagenesis
- UV irradiation

Homology-based recombination

- DNA shuffling
- DOGS
- Family shuffling
- Family shuffling with restriction enzymes
- RACHITT
- RPR
- StEP
- Genome shuffling

Homology-independent recombination

- Exon shuffling
- DHR
- ITCHY
- THIO-ITCHY
- RM-PCR
- SCRATCHY
- SHIPREC
- SISDC
- YLBS

In-vitro evolution

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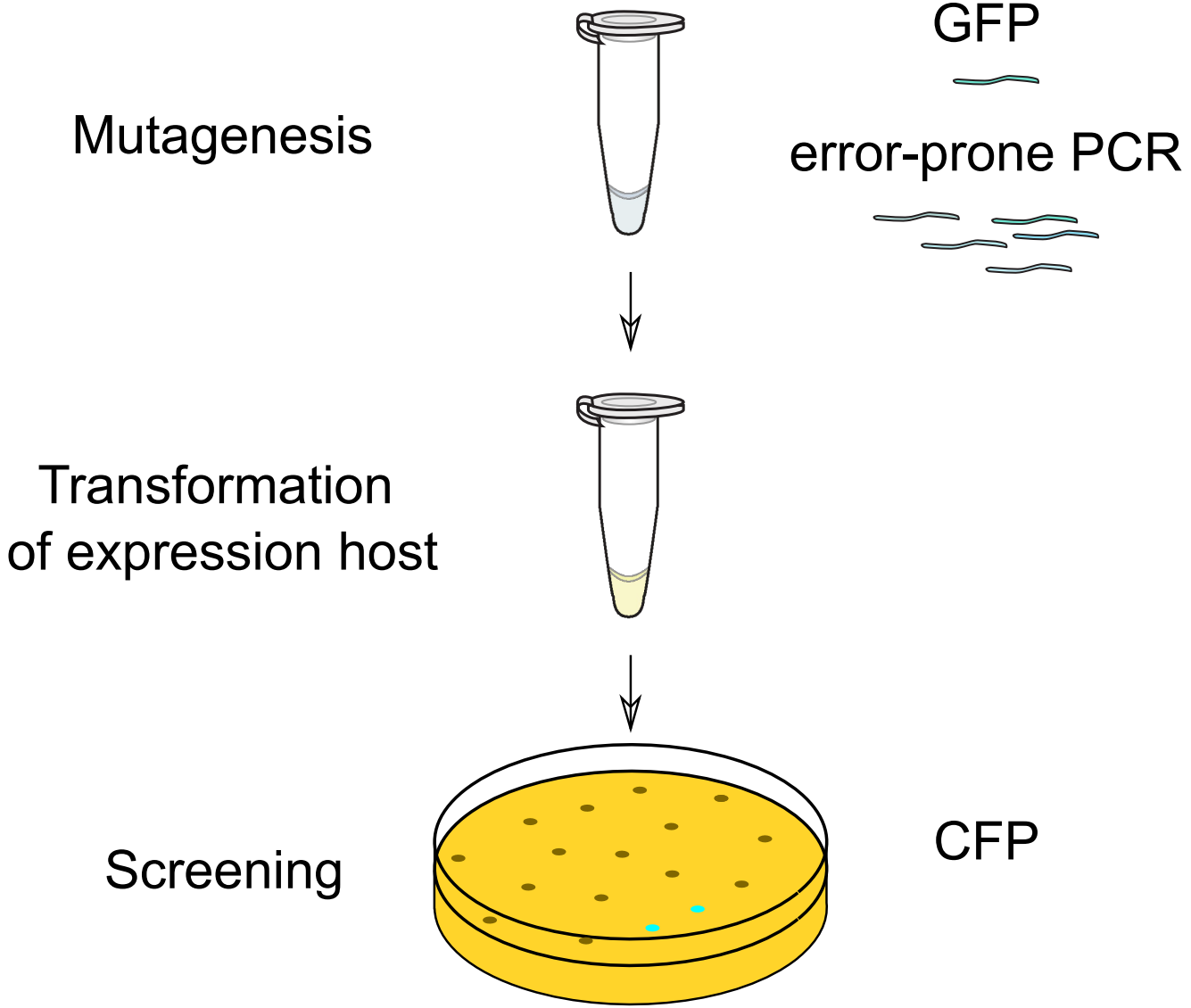
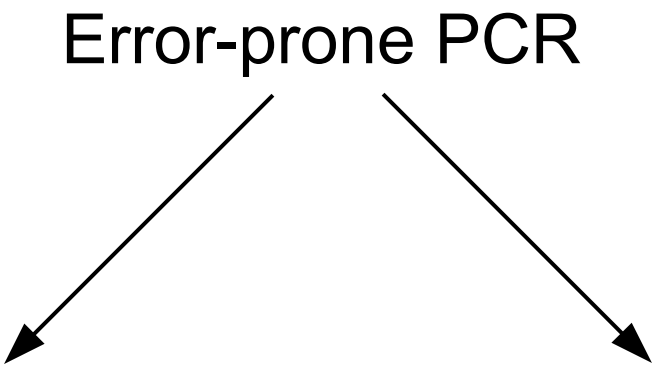
In-vitro evolution



Error-prone PCR



Non-proofreading polymerase,
Mn^{2+},
dNTP concentration

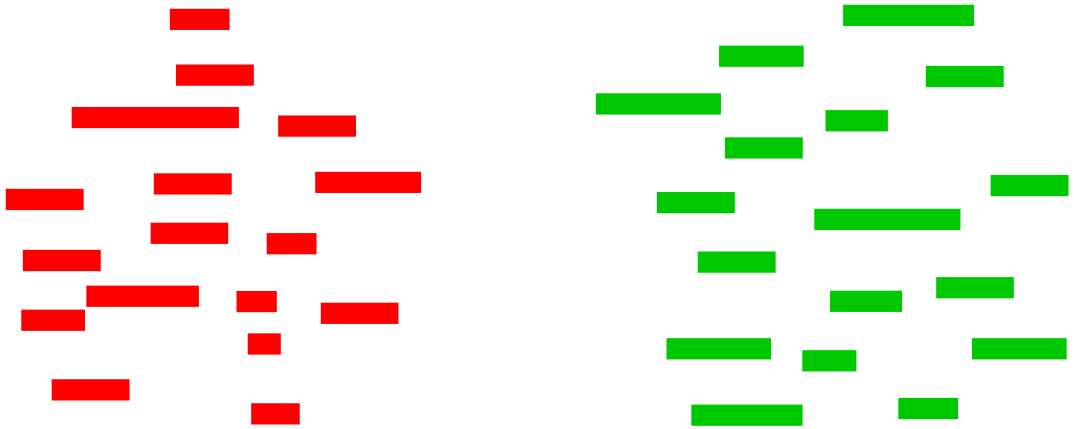
Mutating PCR enzymes ("Mutazyme")

Polymerase bias, limited diversity (e.g. changing Tryptophan (TGG) → Isoleucin (AT[TCA]) by error-prone PCR is almost impossible)

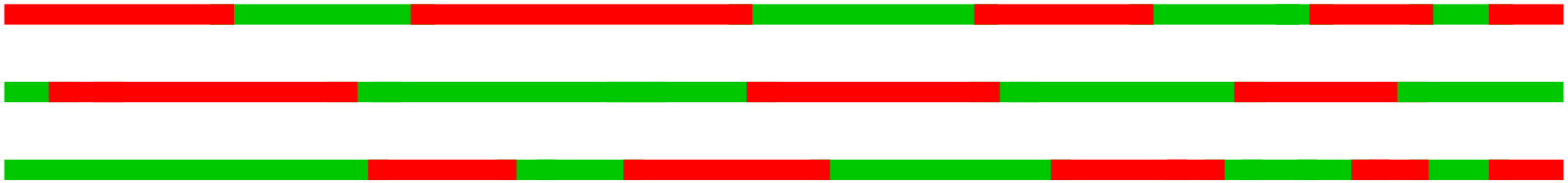
DNA shuffling

Two (or more) homologous sequences

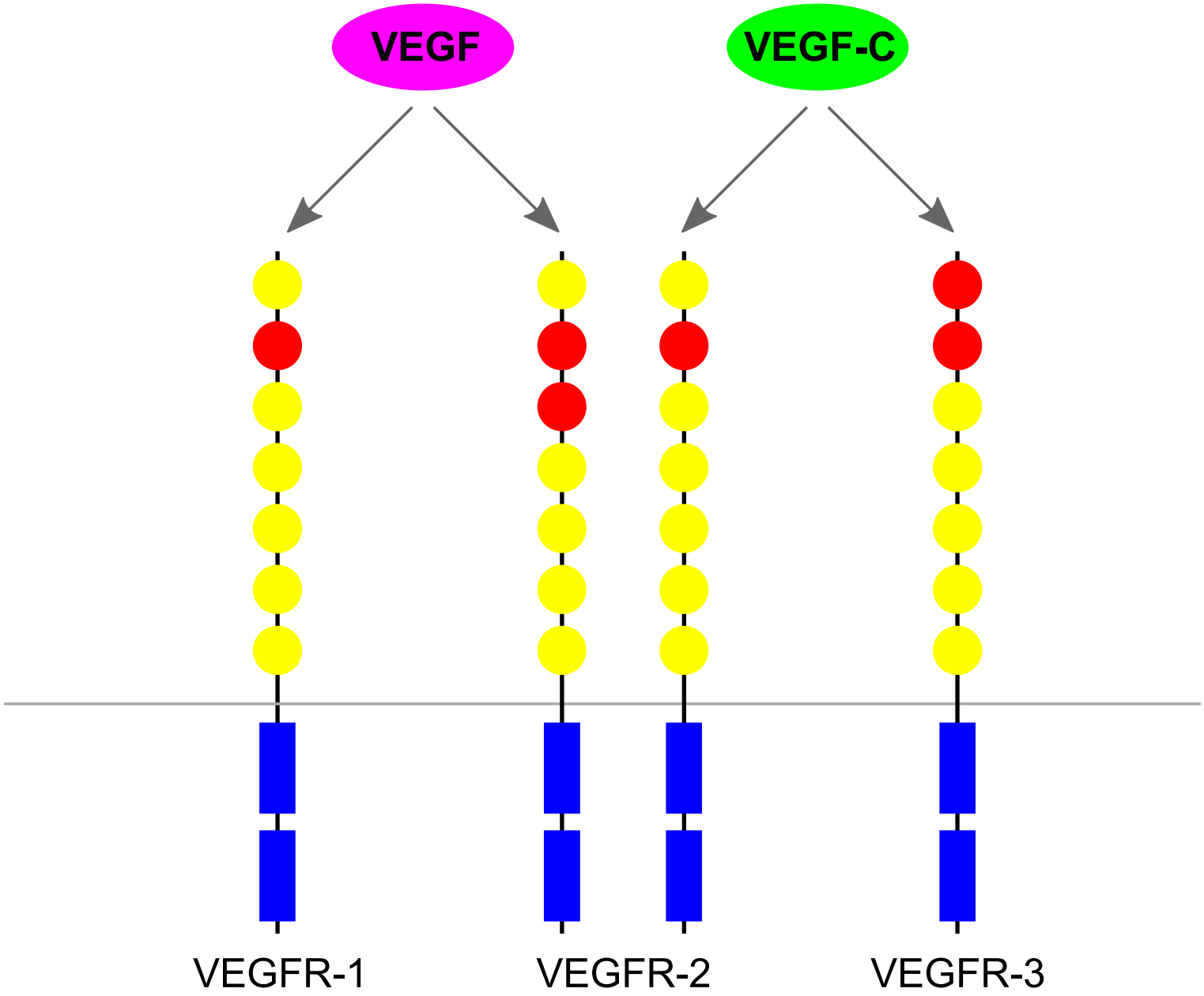
Random fragmentation (DNaseI)



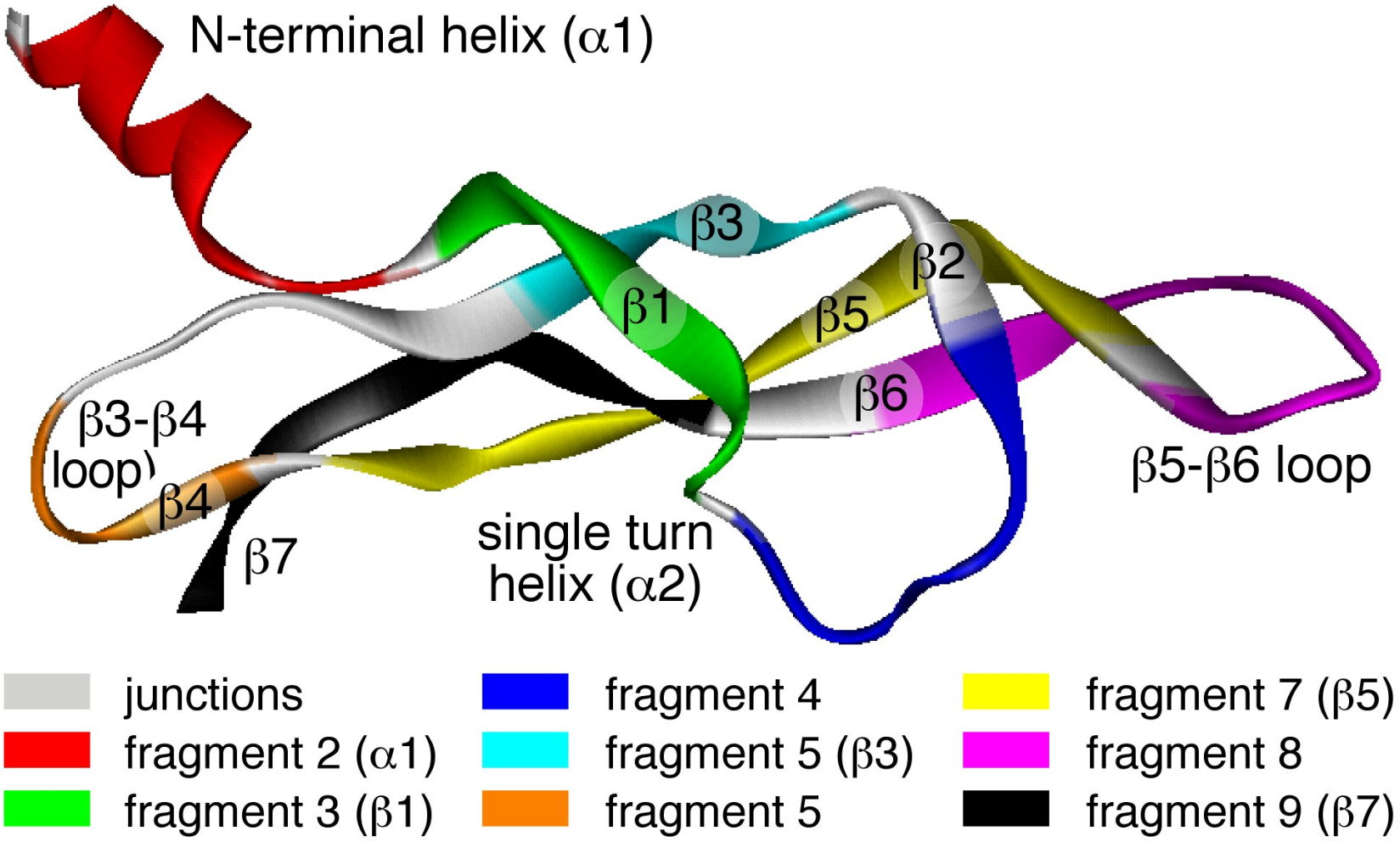
Annealing and repair by PCR



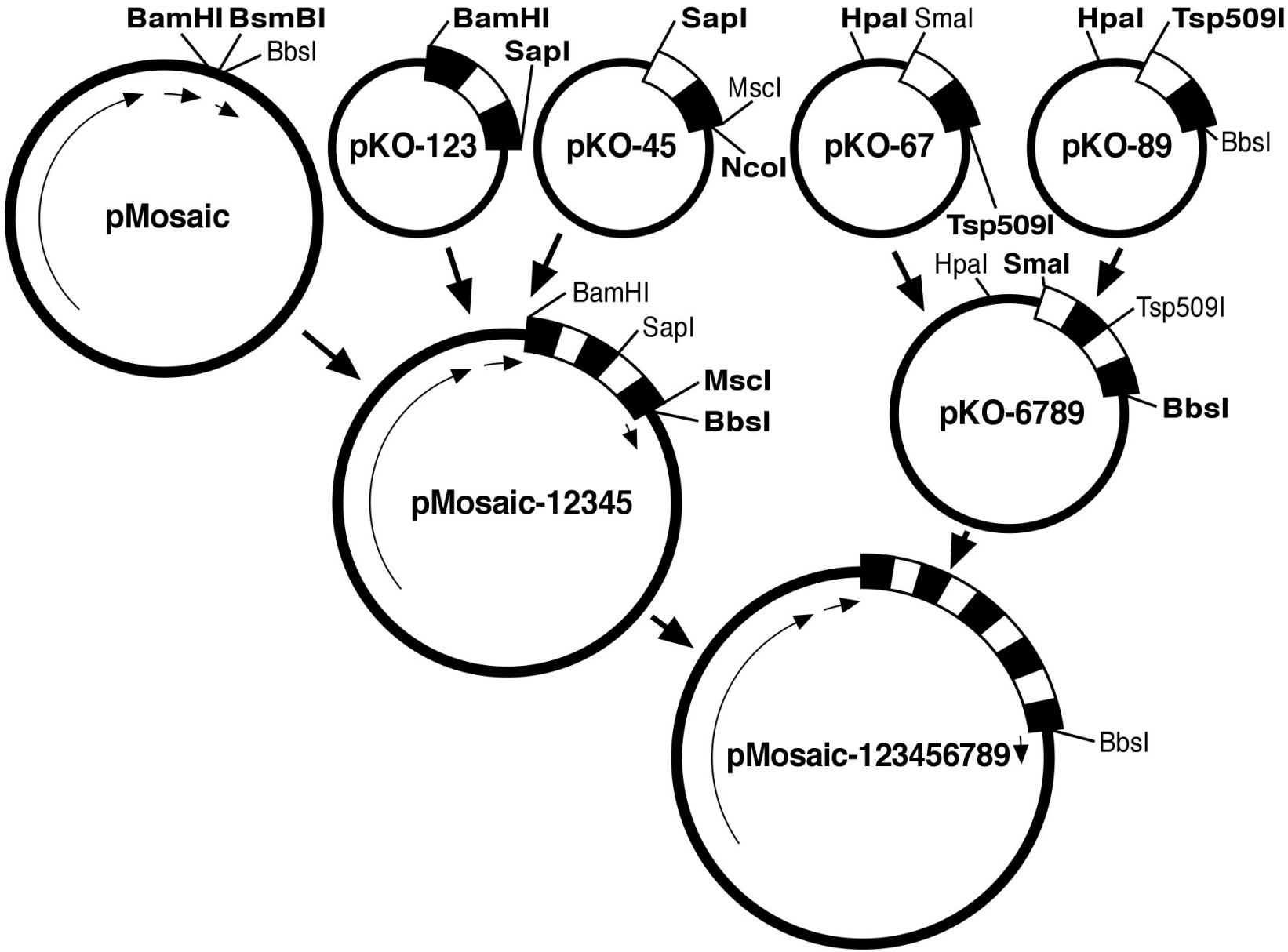
Family shuffling between VEGF-A and VEGF-C



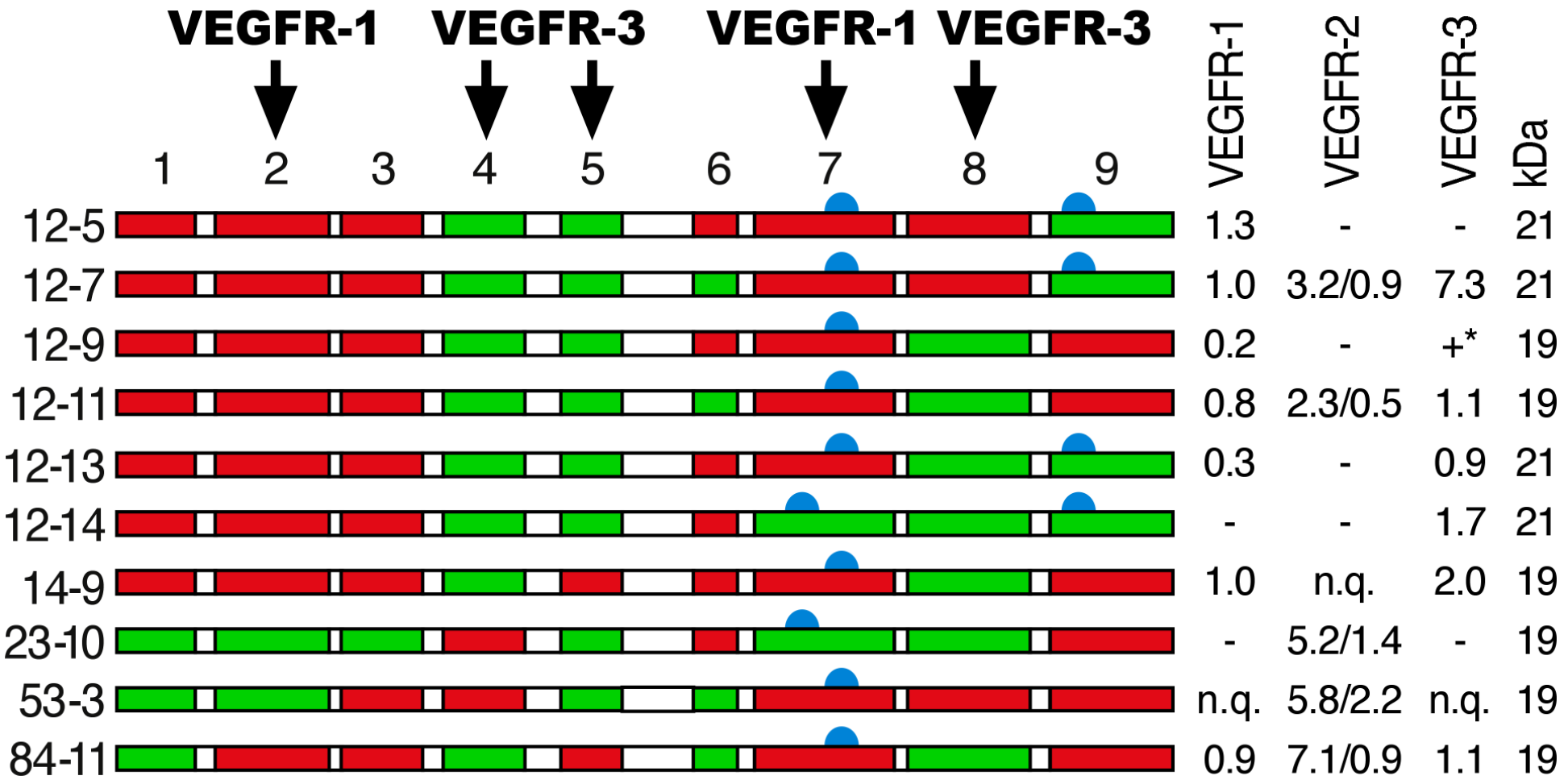
VEGF-A structure



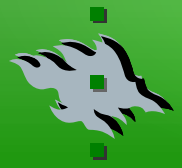
Non-random DNA shuffling



Novel receptor binding profiles in the 2^9 (= 512)-size assorted library



	VEGFR-1	VEGFR-2	VEGFR-3	kDa
12-5	1.3	-	-	21
12-7	1.0	3.2/0.9	7.3	21
12-9	0.2	-	+*	19
12-11	0.8	2.3/0.5	1.1	19
12-13	0.3	-	0.9	21
12-14	-	-	1.7	21
14-9	1.0	n.q.	2.0	19
23-10	-	5.2/1.4	-	19
53-3	n.q.	5.8/2.2	n.q.	19
84-11	0.9	7.1/0.9	1.1	19



Practical Course

- Participants will be contacted via e-mail
- Participants should form groups of 2 or 4 (if you don't have a group yet, I will assign you into one)
- Meetings with all groups within the next two weeks to discuss, plan and prepare the projects