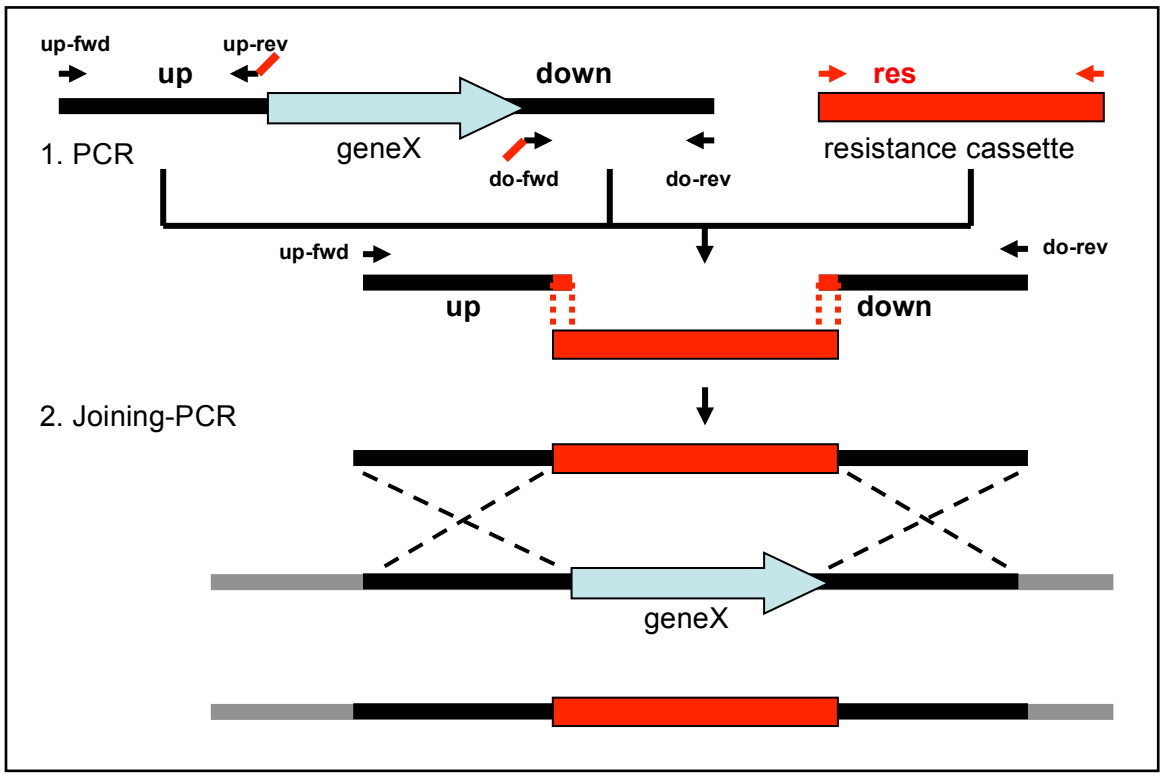
LFH – Long-Flanking Homology PCR



- **Step 1 : Choosing a cassette and designing primers:**

up fwd – about 1 kb upstream of the gene / region to be knocked out (about 20-25 nt)

up rev – within 10-50 bp downstream of the start of the gene to be knocked out.

At the 5' end of this oligo add the sequence corresponding to one end of the cassette being used from the following table. The cassette will be amplified with 5' and 3' overhangs that are the same for every cassette you use; therefore you can use more than one cassette with one set of up rev and do fwd primers.

do fwd - within 10-50 bp upstream of the end of the gene to be knocked out.

At the 5' end of this oligo add the sequence corresponding to the other end of the cassette being used from the primer table (see above).

do rev – about 1 kb downstream of the gene to be knocked out (about 20-25 nt).

* If an upstream or downstream gene overlaps the gene to be knocked out – the up-rev and do-fwd primers should start further within the gene to be knocked out.

Cassette	Source	Primers
cat	pGEM-cat	
kan	pDG780 pDG783	
mls	pDG647	
spec	pDG1726 pDG1727	
tet	pDG1513 pDG1514	

5' end of joining primer	
Up-rev	CCTATCACCTCAAATGGTTCGCTG
Do-fwd	CGAGCGCCTACGAGGAATTTGTATCG

- **Step 2: Amplification of fragments**

  We use Phusion Polymerase (see Standard PCR).

  primers (template):

  up region: up fwd, up rev (chromosomal DNA)

  down region: do fwd, do rev (chromosomal DNA)

  cassette: primers and template according to the table above

  Purify reaction using PCR purification kit – elute in 30-35 µl.

  Determine amount (Nanodrop or agarose gel).

- **Step 3: Joining PCR**

  Using Phusion Polymerase or PCR Extender (if Phusion doesn't work). Primers up-fwd, do-rev.

- Master Mix Phusion (50 µl):

	Stock concentration	**Volume**	**Final concentration**
Phusion buffer**	5x	10 µl	1x
H_2O	-	29,5 µl – X µl – Y µl – Z µl	-
Primer I (add later; see the programme below!)	5µM (5 pmol/µl)	4 µl	0,4 µM
Primer II (add later; see the programme below!)	5µM (5 pmol/µl)	4 µl	0,4 µM
dNTPs	10 mM	1 µl	200 µM each
DNA fragment up	-	X µl	-*
DNA fragment do	-	Y µl	-*
Cassette	-	Z µl	-*
Phusion polymerase	2 U/µl	0,5 µl	0,02 U/µl

* 100-150 ng of each DNA fragment, 200-300 ng of cassette (It seems to work best if you keep about a 1:2 ratio between flanking region:cassette). It also works well (or even better) to use 10 ng of each DNA fragment and a 3x molar excess of cassette.

** Two buffers are provided with the enzyme: 5x Phusion HF Buffer and 5x Phusion GC buffer. The error rate of Phusion DNA Polymerase in HF Buffer ($4.4x10^{-7}$) is lower than that in GC Buffer ($9.5x10^{-7}$). Therefore the HF Buffer should be used as the default buffer for high-fidelity amplification. However, the GC Buffer can improve the performance of Phusion DNA Polymerase on some difficult or long templates, i.e. GC-rich templates or those with complex secondary structures. Use of GC Buffer is recommended for those cases where amplification with HF Buffer has failed.

I Programme:

98°C 0:30
98°C 0:10
X°C 0:30 } 9x
72°C Y:YY

X°C – annealing temperature

Y:YY – extension time. Extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid DNA) use extension time 15 s per 1 kb. For high complexity genomic DNA 30 s per 1 kb is recommended.

After the programme is finished, add primers.

II Programme:

98°C 0:30
98°C 0:10
X°C 0:30 } 30x
72°C Y:YY + 0:05/cycle
72°C 10:00
15°C ∞

Run ~ 5µl of reactions out on gel, purify remaining 45µl using PCR purification kit (very important as buffer contains detergents!).

- Master Mix PCR Extender (50 µl):

	Stock concentration	**Volume**	**Final concentration**
Phusion buffer**	10x	5 µl	1x
H_2O	-	35,5 µl – X µl – Y µl – Z µl	-
Primer I (add later; see the programme below!)	5µM (5 pmol/µl)	4 µl	0,4 µM
Primer II (add later;	5µM (5 pmol/µl)	4 µl	0,4 µM

see the programme below!)			
dNTPs	10 mM	1 µl	200 µM each
DNA fragment up	-	X µl	-*
DNA fragment do	-	Y µl	-*
Cassette	-	Z µl	-*
Polymerase Mix	5 U/µl	0,5 µl	0,05 U/µl

* 100-150 ng of each DNA fragment, 200-300 ng of cassette (It seems to work best if you keep about a 1:2 ratio between flanking region:cassette)

** For targets smaller than 2 kb use HighFidelity Buffer. For targets ranging between 2 – 10 kb it is recommended to try both buffers and then choose the one with the best ratio of yield to specificity.

I Programme:

94°C 2:00
94°C 0:20
X°C 0:20] 9x
72°C Y:YY

X°C – annealing temperature
Y:YY – extension time (45 s/kb)

<u>After the programme is finished, add primers.</u>

II Programme:

94°C 2:00
94°C 0:20
X°C 0:20] 30x
72°C Y:YY + 0:05/cycle
72°C 10:00
15°C ∞

Run ~ 5 µl of reactions out on gel (PCR Purification not necessary).

- **Step 4: Transformation**

  For *Bacillus* transformation use 10-15 µl of (purified) product. Follow the standard *Bacillus* transformation procedure.

- **Step 5: Screen colonies for verification (using colony-PCR)**

  See Standard PCR (Taq polymerase for fragments ≤1,2 kb, HotStar for >1,2 kb)

  Primers: up fwd primer + proper check rev primer
  do rev primer + proper check fwd primer

Cassette	Primer #	Primer name	Localization
Cat		Cat check rev	5'-end reverse
		Cat check fwd	3'-end forward
Kan		Kan check rev	5'-end reverse
		Kan check fwd	3'-end forward
Mls		Mls check rev	5'-end reverse
		Mls check fwd	3'-end forward
Spec		Spec check rev	5'-end reverse
		Speck check fwd	3'-end forward
Tet		Tet check rev	5'-end reverse

  Run on gel - should give about 1 kb (depending on the size of your up and down fragments) fragment in positive clones and no product in negative control.

- **TIPS:**
  - When using the Cat or Tet cassette the colonies seem to take about 2 days to come up
  - You don't have to use 2 rounds of joining PCR (one without primers), as described in the original protocol. However, if you are unable to get product with one round 2 rounds might work.
  - Increasing the amount of cassette template might help if you don't get joining product.

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