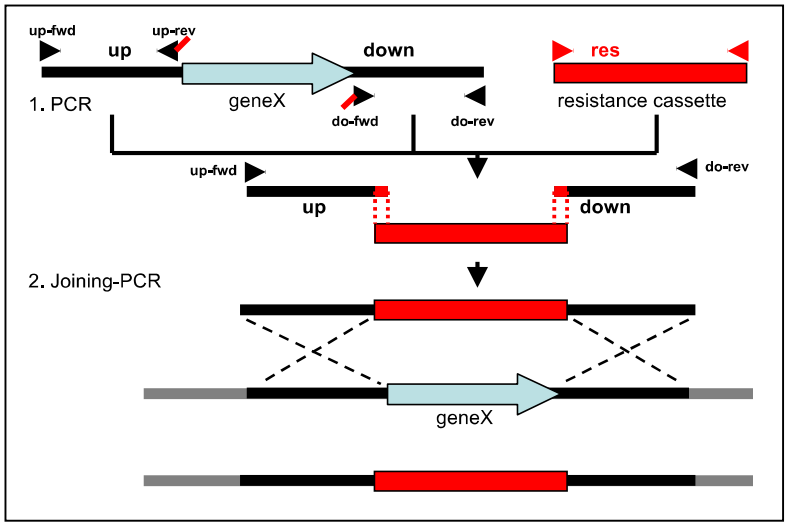
- LFH – Long-Flanking Homology PCR (by Anna, 2010)



- **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	No. in the vector collection	Primers
cat	pGEM-cat	#15	TM0135/TM0136
kan	pDG780 pDG783	#8 #9	TM0137/TM0138
mls	pDG647	#7	TM0139/TM0140
spec	pDG1726 pDG1727	#12 #13	TM0141/TM0142
tet	pDG1513 pDG1514	#10 #11	TM0143 or TM0144/TM0145

Comment [JR1]: Gives shorter fragment, still including promoter

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	-	30,5 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.

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

II Programme:

98°C 0:30
98°C 0:10
X°C 0:30
72°C Y:YY + 0:05/cycle
} 30x
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; 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	-*
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
72°C Y:YY
9x

X°C – annealing temperature

Y:YY – extension time (45 s/kb)

After the programme is finished, add primers.

II Programme:

94°C 2:00
94°C 0:20
X°C 0:20
72°C Y:YY + 0:05/cycle
30x
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	TM0146	Cat check rev	5'-end reverse
	TM0173	Cat check fwd	3'-end forward
Kan	TM0147	Kan check rev	5'-end reverse
	TM0056	Kan check fwd	3'-end forward
Mls	TM0148	Mls check rev	5'-end reverse
	TM0057	Mls check fwd	3'-end forward
Spec	TM0149	Spec check rev	5'-end reverse
	TM0058	Speck check fwd	3'-end forward
Tet	TM0150	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.