Fusion-PCR

- **Step 1 : Designing primers**

We used the same primers as described in the QuickChange Mutagenisis protocol.

- **Step 2: Amplification of fragments**

We use Q5 Polymerase (see Standard PCR).

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

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

- Purify reaction using PCR purification kit – elute in 30-35 µl.
- Determine amount (Nanodrop or agarose gel).

- **Step 3: Joining PCR**

Using Q5 Polymerase or PCR Extender (if Phusion doesn't work). →Primers up-fwd, do-rev.

- Master Mix Phusion (50 µl):

	Stock concentration	**Volume**	**Final concentration**
Q5 buffer (5 x)	5x	10 µl	1x
H_2O	-	33,5 µl – X µl – Y µl	-
Primer I (add later; see the programme below!)	10 µM (5 pmol/µl)	2 µl	0,4 µM
Primer II (add later; see the programme below!)	10 µM (5 pmol/µl)	2 µl	0,4 µM
dNTPs	10 mM	1 µl	200 µM each
DNA fragment up	-	X µl	-*
DNA fragment do	-	Y µl	-*
Q5 polymerase	2 U/µl	0,5 µl	0,02 U/µl

* 100-150 ng of each DNA fragment

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] 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	-*
Polymerase Mix	5 U/µl	0,5 µl	0,05 U/µl

* 100-150 ng of each DNA fragment.

** 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)

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

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

Protocol generously provided by the lab
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