How to: Golden Mutagenesis

This protocol shall provide a comprehensive overview on how to perform Golden Mutagenesis

General remark:

The following details regarding chemicals, enzymes and particularly the respective vendor are in accordance with all approaches that were performed to obtain the practical data presented within the publication. Of course enzymes, chemicals and kits of other suppliers can be used within the setup. However the cloning efficiency results can heavily depend on the chosen enzymes, buffers, kit combinations etc. used. Therefore to obtain optimal results the following vendors of the respective items are recommended.

What you need:

plasmids/enzymes

- pAGM9121 (Level 0; Spectinomycin Resistance; clone with BbsI):
  ***E.coli* universal cloning plasmid**
  pAGM22082_CRed (Level 2; Kanamycin resistance; clone with BsaI):
  ***E.coli* expression plasmid (T7 Promoter)**
  pICH86988 (Level2; Kanamycin resistance; clone with BsaI):
  Agrobacterium mediated expression in *A. thaliana* (35S Promoter)
- T4 DNA Ligase (1-3 units/µl; Promega)
- BsaI or BsaI-HFv2® (10 units/µl; New England Biolabs)
- BbsI (5 units/µl; ThermoFisherScientific)
- Phusion High-Fidelity DNA Polymerase (2 units/µl;ThermoFisherScientific)
- GoTaq® DNA Polymerase (Promega)

buffer/material

- 10x Ligase Buffer (Promega)
- dNTPS (10 mM Mix; ThermoFisher Scientific)
- 5x HF Phusion Buffer (ThermoFisherScientific)
- 5x GoTaq Buffer (Promega)
- Agarose (AppliChem)
- 1x fold TAE Buffer (40 mM Tris; 20 mM acetic acid; 1mM EDTA)
- Roti Stain (Carl Roth)
- NucleoSpin Plasmid Kit (Macherey-Nagel)
- NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel)
- 1 kb DNA ladder (Thermo Fisher Scientific)
- *E.coli* Competent cells (cloning strain: DH5α or DH10B; expression strain: BL21 DE3 pLysS)
- SOC Medium
- 75 % DMSO
- LB Agar
  + appropriate Antibiotic (50 µg × ml^{-1}Kanamycin or 100 µg × ml^{-1} Spectinomycin)
  + X-Gal (50 µg × ml^{-1})
  + IPTG (150 µM))
- sterile toothpicks

Step by step procedure:

a) Planning of Mutagenesis setup (tool-based)

- retrieve primer sequences out of Golden Mutagenesis planning tool
- Order Primer at vendor of choice (smallest amount; desalted purification grade sufficient)

Remark: Alternatively you can of course also design the Oligonucleotides by hand

b) The PCR reaction

- Primer arrival: Dilute Primer in stock tube to 100 µM (ddH_2O)
- Working solution (10 µM): 90 µl ddH_20 + 10 µl Pre-Diluted primer stock solution

Amplify gene fragments (keep in mind the respective sizes!)

PCR Mix (1 reaction; 50 µl):

Component	amount
5x HF Buffer	10 µl
DMSO (75 %)	2 µl
dNTPs	1 µl
Template (gene to be mutated)	100 ng per approach
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Phusion	0,2 µl
ddH_20	add to 50 µl final volume (depending on template concentration!)

PCR reaction (default):

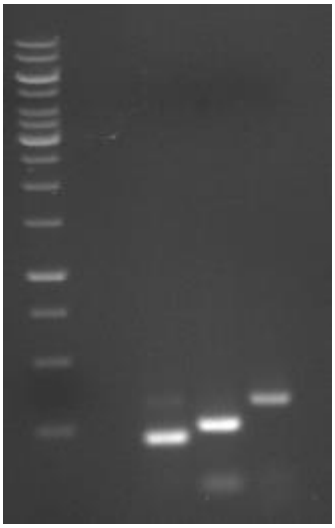
Step	Temperature, Time (passes)
initial denaturation	98 °C, 60s (1x)
denaturation	95 °C, 15 s (35x)
annealing	60 °C, 30 s (35x)
elongation	72 °C, 90 s per kb (35x)
final elongation	72 °C, 10 min (1x)
Cooling	12 °C, ∞

- approx. 30 min before the PCR is finished an Agarose Gel can be prepared (weigh in Agarose in TAE buffer); ranging from 1 (fragments >700 bp) to 2,5 (fragments down to 100 bp) % (w/v) Agarose shall be prepared
- boil Agarose in Buffer (Microwave; 2-3 min) till completely dissolved
- add DNA stain

  Example
  small gel: 90 ml 1x TAE buffer plus appropriate amount agarose + 1 µl RotiStain
  medium gel: 120 ml 1x TAE buffer plus appropriate amount agarose + 1,5 µl RotiStain

- after PCR is finished: Load 10 µl 1 kb ladder + 5 µl of each generated PCR fragment onto gel

- run for 45 min (150 V)
- **How it could look like: Example for gene splitting (750 bp) into three fragments (222, 255 and 337 bp)**



- after confirmation of occurrence and correct size of PCR fragments: Subsequent purification of PCR fragments
- PCR product purification: Follow instructions of NucleoSpin® Gel and PCR Clean-up Kit (or other PCR purification kit)
- Measure absorbance at a wavelength of 260 nm (calculate ds DNA concentration in ng/µl)

c) The cloning

Golden Gate cloning reactions are always performed in 15 µl final volume consisting of:

Component	amount
10 x Ligase Buffer	1,5 µl
Acceptor plasmid	20 fmol
Insert(s) (1 to x)	20 fmol each
BsaI (pAGM22082; pICH86988) or BbsI (pAGM9121)	0,5 µl
T4 Ligase	1,0 µl
ddH_20	add to 15 µl

Crucial Tipps/Tricks:

a) calculate needed amount of insert(s):

$$\textbf{µl to pipet} = \frac{\textbf{20 (fmol)} \times \textbf{bp size of your PCR insert or donor plasmid}}{\textbf{1520 (factor)} \times \textbf{concentration of insert or plasmid (in } \frac{\textbf{ng}}{\textbf{µl}}\textbf{)}}$$

Calculation example: PCR insert of 750 bp size; stock concentration of 25 ng/µl

$$\textbf{0,4 µl} = \frac{\textbf{20 × 750 bp}}{\textbf{1520 × 25 ng/µl}}$$

Tipp: Often the volumes for pipetting are really small and therefore not handy at all; because of this it is recommended to prepare dilutions prior to GG reactions (in ddH_2O); so e.g dilute your insert to a working concentration that 1 µl of the dilution contains 20 fmol- so you can just add 1 µl of every insert to the GG mix (this is also very handy when it comes to acceptor plasmids!)

If you got an insert inside a GG plasmid, you have to calculate insert size + size of plasmid backbone (pAGM9121 backbone: 2057 bp) (same goes for acceptor plasmids → pAGM9121: 2659 bp; pAGM22082_cRed: 13411 bp; pICH86988: 9078 bp)

b) always add respective enzymes at the end!; transport them in a cryo rack to prevent warming of the stock enzyme

The Golden Gate reaction:

a) 1-2 fragments (constant temperature)

Step	Temperature, Time (passes)
Digestion/Ligation	37 °C, 3 or 4 h
Enzyme inactivation	80 °C, 20 min
Cooling	12 °C, ∞

b) 3 and more fragments (temperature cycling)

Step	Temperature, Time (passes)
Digestion	37 °C, 2 min (30 to 40 passes)
Ligation	16 °C, 5 min (30 to 40 passes)
Enzyme inactivation	80 °C, 20 min
Cooling	12 °C, ∞

- afterwards:

transform whole reaction setup into *E.coli* cloning strain (pAGM9121 constructs in general or pAGM22082_CRed/pICH86988 constructs for point mutagenesis) or *E.coli* expression strain (pAGM22082_CRed constructs for saturation mutagenesis)

Transformation:

- Thaw aliquot (50 µl; RbCl chemocompetent cells) of cells on ice (5 min)
- Add whole GG reaction setup to cells; flip the tube several times; cool on ice for 20 min
- heat shock (90 s; 42 °C): **Preheat thermoblock and SOC medium!**
- chill cells for 2 min on ice
- add 250 µl of SOC Medium
- Incubate for one hour (37 °C; 800 rpm in thermoblock)
- Plate on appropriate Agar plate (+appropriate antibiotic; + X-Gal & IPTG in case of pAGM9121/pICH86988 for blue/white selection screening)
- pAGM9121: plating 50 µl (1 fragment) up to 150 µl (up to four fragments) is recommended
- pAGM22082_CRed: plate the whole setup on a big Agar plate

General remarks:

Efficiency/colony numbers is strongly dependent on the grade of the utilized competent cells. Therefore serial dilutions for plating can be used, to test the colony amount that can be obtained with the respective plasmid and within the given setup.

For the creation of saturation libraries it is strongly recommended to use highly chemocompetent or even electrocompetent *E.coli* cells to obtain sufficient colony amounts that are covering the possible combinatorial space (2 NDT codons: 12 × 12 = 144 different combinations).

Special case: subcloning via Level 0 before reassembly into pAGM22082_cRed/pICH86988

- case: subclone individual gene fragments into pAGM9121, then reassemble gene fragments into the expression plasmid → **in general the efficiency of reassembly from Donor plasmids is higher than in direct comparison with the similar approach from PCR fragments**

Day 1:

- clone individual fragments into pAGM9121; e.g. three fragments: three parallel GG approaches (see above); use BbsI as restriction enzyme
- Perform transformation in the same way, **but plate 50 µl of the transformation mixture for Quality Control (check amount and blue/white ratio) and inoculate the remaining transformation mixture directly into a 4 ml overnight liquid culture (TB-Medium (+Spec)) to maintain genetic diversity within randomization sites!**

Day 2:

- prepare Plasmid mixtures (NucleoSpin Plasmid Kit)
- measure plasmid concentrations and calculate amounts for GG reaction
- set up second Golden Gate reaction (into pAGM22082_cRed or piCH86988; +BsaI)

pAGM22082_cRed:

- transform into expression strain (e.g. BL21 DE3 pLysS cells) → when creating a randomization library
- plate on LB Agar (+Kanamycin)

pICH86988:

- transform into *E.coli* cloning strain
- plate on LB Agar (+Kanamycin, +X-Gal, +IPTG)

d) The "quick" analysis

- based on the presence of a colour selection marker (pAGM9121; pICH86988: lacZ = blue and pAGM 22082_cRed: Canthaxanthin = orange) as a placeholder within the multiple cloning sites, a distinction between recombined colonies (white) and unmodified acceptor plasmid (blue or orange) can be performed
- check for anticipated, correct insert size via Colony PCR (standard approach: 24 colonies; one negative control = unmodified plasmid)

Colony PCR Mastermix (27x reactions):

PCR Mix (27 reactions; 657 µl total- 25 µl per approach):

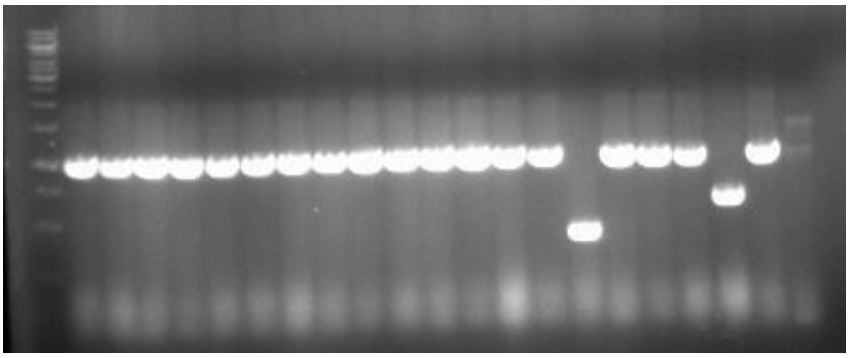
Component	amount
5x GoTaq Buffer	135 µl
dNTPs	10 µl
Forward Primer (10 µM)	20 µl
Reverse Primer (10 µM)	20 µl
Taq Polymerase	6 µl
ddH_20	484 µl

PCR reaction (default):

Step	Temperature, Time (passes)
initial denaturation	98 °C, 120 s (1x)
denaturation	95 °C, 15 s (35x)
annealing	55 °C, 30 s (35x)
elongation	72 °C, 60 s per kb (35x)
final elongation	72 °C, 6 min (1x)
Cooling	12 °C, ∞

- Prepare and label PCR tubes, add 25 µl of Mastermix to every respective tube
- **under sterile hood:** with sterile toothpicks scrape off single bacterial colonies and inoculate the colonies into the individual tubes; start PCR
- analyse results via Agarose Gel electrophoresis (instructions as above)

How it could look like: Example for colony testing, expected size ~1 kb (22 positive colonies; 2 negative colonies)

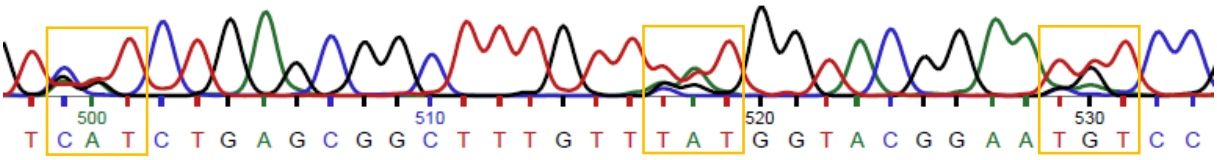


- efficiency of correct gene reassembly can be calculated afterward

e) The real analysis

- in case of saturation libraries:
- pAGM22082/pICH86988 + Gene for expression: scrape of >100 colonies of plate (can be easily done with an inoculation loop) and inoculate the cells into 4 ml TB medium (+Kan)
- next day: prepare plasmid mixture and send two samples for sequencing (forward and reverse primer of plasmid)

How the result could look like (three close NDT randomization sites):



- the .ab1 file can be uploaded into the tool and analyzed accordingly (pie chart distributions)

Appendix:

Primer sequences

Description	Sequence (5´→ 3´)
pAGM9121_for	CCTGTCGGGTTTCGCCACCT
pAGM9121_rev	GCCGTTACCACCGCTGCGTT
pAGM22082_for	GAAGGAGATATACCATGGGCAGCAG
pAGM22082_rev	CGTTTAGAGGCCCCAAGGGGTTATG
pICH86988_for	CTATCCTTCGCAAGACCCTTC
pICH86988_rev	GTAAGGATCTGAGCTACACATGC