Simply Cloning

A step-by-step video guide for making DNA constructs

Molecular Cloning Protocols

Cloning Strategies
www.cloningstrategies.com

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1 | Planning

- Create experiment outline in PowerPoint. Include:
  - Name of starting vector
  - Insert template
  - Restriction enzymes
  - Primers
- Build vector maps
- Design primers:
  - Select first/last 20 nucleotides of the insert sequence
  - Add a restriction site at the 5' end of the forward primer and at the 3' end of the reverse primer
  - Add six random nucleotides to enable nuclease cleavage close to the end of DNA fragment
  - For the forward primer: insert one or two nucleotides between the restriction site and the insert sequence to maintain the reading frame of your fusion protein
  - For the reverse primer: add a stop codon if necessary
  - For the reverse primer: transform to complimentary sequence

Forward primer: Random 5' CGATGA *Bam*HI GGATCC Frame GT Insert sequence GGTAGCAAGGGCGAGGAGC 3'

Reverse primer: Insert sequence 3' GTACCTGCTCGACATGTTC Stop TGA *Spe*I ACTAGT Random ACATAG 5'

Reverse complim.: 5' CTATGTACTAGTTCAGAACATGTCGAGCAGGTAC 3'

2 | PCR

- PCR mix:
  - 37.5 µl ddH_2O
  - 5 µl 10X PCR buffer
  - 4 µl dNTPs (10mM each)
  - 1 µl Primer (forward), 10 µM
  - 1 µl Primer (reverse), 10 µM
  - 1 µl DNA template
  - 0.5 µl Polymerase (1.25 units)

  Note: If you are using a plasmid miniprep as your DNA template, dilute it 50 times and take 1 µl of diluted solution

- PCR program:
  1. 94 °C – 2 min
  2. 94 °C – 30 sec
  3. 55 °C – 30 sec
  4. 72 °C – 1 min (or 1 min/kb)
  5. Go back to 2. Repeat 29 times
  6. 72 °C – 5 min
  7. 4 °C – forever (insert 0 on most thermal cyclers)

3 | Vector Restriction Digest

- Restriction mix:
  - 39 µl ddH_2O
  - 5 µl 10X enzyme buffer
  - 5 µl DNA (approximately 1 µg)
  - 0.5 µl restriction enzyme A (5 units)
  - 0.5 µl restriction enzyme B (5 units)
- Mix up by pipetting up and down
- Incubate at 37 °C for 45 min

4 | Gel Purification

- Prepare 1% agarose gel:
  - Add 1 g of agarose to 100 ml of TEA buffer
  - Bring to boil in a microwave, make sure the agarose completely dissolved
  - Cool at room temperature for 10 min
  - Add 2 µl ethidium bromide, pour into a tray
  - Leave for 30 min to solidify
- Add 5 µl of loading buffer (60% glycerol in TAE, trace amount of bromophenol blue) to 50 µl of your restriction digest/PCR
- Load gel:
  - Molecular weight marker
  - 1 µl of undigested vector (mixed with loading dye)
  - Vector restriction digest
  - Insert restriction digest
- Run gel until bromophenol blue dye reaches the end
- Take gel picture
- Cut out desired DNA fragments
- Purify with a gel purification kit. Elute with 50 µl of ddH_2O

5 | Insert Restriction Digest

- Restriction mix:
  - 44 µl of gel purified insert from step I-2
  - 5 µl 10X Buffer
  - 0.5 µl restriction enzyme A (5 units)
  - 0.5 µl restriction enzyme B (5 units)
- Mix up by pipetting up and down
- Incubate at 37 °C for 45 min
- Deactivate the enzymes:

  A. Clean on a GFX column (enzyme removal kit)
  B. Check the enzymes you are using if they can be heat deactivated. For most enzymes, incubate 30 min at 65 °C

6 | Ligation

- Ligation mix:
  - 4 µl H2O
  - 1 µl 10X ligation buffer
  - 1 µl of plasmid digested with restriction enzymes A and B
  - 3 µl of insert digested with restriction enzymes A and B
  - For the ligation control, use 3 µl of water instead of insert
  - 1 µl DNA ligase
- Incubate at 16 °C for 15 min - 24 hours
  Note: longer incubation times increase efficiency

7 | Transformation and Plating

- Thaw chemically competent cells on ice for 15 min
- Add ligation mix to the competent cells, mix by tapping gently
- Leave the cells on ice for 30 minutes
- Heat-shock cells at 42 °C for 60 seconds
- Put cells back on ice for 5 minutes
- Add 4 volumes of LB medium without antibiotics (for example, to 200 µl of competent cells add 800 µl of LB)
- Shake for 45 min at 37 °C for transformed cells to develop antibiotic resistance
- Spin down at 13000 rmp for 1 min, remove 800 µl of supernatant, resuspend pelleted cells in the remaining LB
- Plate on LB agar plate with antibiotics
- Incubate the plate at 37 °C overnight

8 | Plasmid Miniprep

- Prepare numbered cell culture tubes with 4 ml of liquid LB medium and appropriate antibiotics
- Pick colonies from the transformation plate with autoclaved toothpicks or pipette tips and inoculate the corresponding media in the numbered tubes
- Incubate the tubes in a shaker at 37 °C overnight
- On the next day, save 1 ml of culture in a separate eppendorf tube (for clone library)
- Spin down the remaining 3 ml of culture and proceed with your plasmid miniprep kit

9 | Clone Verification by Restriction Digest

- Restriction mix:
  - 16.6 µl H2O
  - 1 µl miniprep DNA
  - 2 µl 10X restriction buffer
  - 0.2 µl restriction enzyme A (2 units)
  - 0.2 µl restriction enzyme B (2 units)
- Incubate at 37 °C for 45 min
- Run on an agarose gel, take a picture

10 | Depositing Clones in a Library

- Create an Excel or Word file with library information Include:
  - Clone number and name
  - Clone description
  - Primers used
  - Date created
  - Anything else you find relevant
- For each clone, prepare two tubes with clone name and number
- Add 0.9 ml of overnight E. coli culture and 0.1 ml of DMSO to one of the tubes, store at -80 °C
- Put plasmid DNA into another tube, store at -20 °C