Protocol: Restriction cloning with BamHI and NdeI

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This protocol was taken from last year's Lund iGEM Team.

We have switched to Johan's protocol for restriction digestion, after we had some difficulties with this protocol.

Digestion of pET11 with NdeI and BamHI (Thermo Fisher FastDigest enzymes)

Materials

- Plasmid DNA (pET11)
- FastDigest NdeI (ThermoFisher)
- FastDigest BamHI (ThermoFisher)
- FastDigest Buffer (10X)
- FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher)
- Sterile Water
- DNA loading dye (typically 6X)
- Sterile 1.5 mL microcentrifuge tubes
- Heating blocks (37°C and 80°C)

Instructions

Note!

- The volumes may have to be adjusted depending on the concentration of DNA in your sample.
- Use two different heating blocks - one for 37°C and one for 80°C. They heat up fast but it takes a while for them to cool down.
- Always keep enzymes on ice while setting up.
- If you are digesting PCR products or genes as well (see below), you can do it at the same time as the vector, but please make sure to follow the separate protocol for PCR products.

Controls (prepare in parallel with your samples!)

Control Digest	***Enzyme***	***Purpose***
Single digest 1 (with NdeI)	NdeI only	Verifies NdeI activity and correct

		cutting
Single digest 2 (with BamHI)	BamHI only	Verifies BamHI activity and correct cutting
No digest (uncut)	None	Confirms plasmid integrity and migration

Digest all controls using the same buffer and plasmid amount (0.25 µg DNA). Adjust volumes to 21 µL total.

Step 1: Prepare the initial digestion mix

In a sterile 1.5 mL microcentrifuge tube, add the following:

Component	***Volume***
Sterile Water	Adjust based on volume of Plasmid DNA. The total volume should be 19 µL.
10X FastDigest Buffer *If you used the green 10X FastDigest Buffer: You do not need to add loading dye!*	2 µL
Plasmid DNA	x µL (calculate based on your DNA concentration to add 0.25 µg total)
FastDigest NdeI	1 µL
Total volume	**19 µL** *Final reaction volume after all additions (BamHI, and FastAP) will be ~21 µL.*

Step 2: Incubate

- Incubate at 37°C (heating block or thermocycler) for 45 minutes.
- Briefly spin down the tube after mixing to collect the liquid at the bottom.

Step 3: Add second enzyme

- Add 1 µL FastDigest BamHI directly to the reaction.
- Gently mix (pipette up and down or flick the tube).

Step 4: Second incubation

- Incubate at 37°C for an additional 10 minutes.

Step 5: Inactivate restriction enzymes

- Incubate at 80°C for 5 minutes.

- After heat inactivation, briefly centrifuge the tube to collect any condensation.

Step 6: Dephosphorylation (Dephosphorylation of the vector reduces self-ligation during ligation steps)

- Add 1 µL FastAP directly to the reaction.
- Mix gently.
- Incubate at 37°C for 30 minutes.

Step 7: Inactivate FastAP

- Incubate at 80°C for 20 minutes.

Step 8 (Optional): Prepare for gel electrophoresis

- If running on a gel, add the appropriate volume of DNA loading dye. It should often be diluted 6 times (please look if it says 6X on the tube), this means add approx 4µL if you have 20µL total volume.
- If you used **green 10X FastDigest Buffer**, you do **not** need to add additional DNA loading dye, it already contains tracking dyes and glycerol.

Digestion of PCR products

Use the same basic protocol as above. Digest 0.2 µg DNA, adjust H_2O accordingly. DO NOT dephosphorylate with FastAP (skip steps 6 and 7).

Purification of digestion products from gel

Step 1: Prepare and run agarose gel

- Run the agarose gel according to the separate protocol: *Gel Electrophoresis.* **If you plan to do gel extraction afterward**, use the **entire volume** of your digest (not just 5 µL). Don't split it, run the full sample in a single well to recover maximum DNA.
- Run all three controls on a gel next to your double digest.
- Agarose concentration should be based on the length of the DNA fragments.
    - For pET11a and our PET-hydrolyses, 1% works well.

Step 2: Excise DNA bands

- Visualize the DNA under UV light.
- Follow the gel imaging instructions as outlined in the *Gel Electrophoresis* protocol
- Important! Minimize exposure time to UV light to avoid DNA damage.
- Always wear UV-protective equipment (goggles, face shield, gloves).
- Use a clean scalpel to carefully cut out the DNA band of interest, following the instructions in *Gel Extraction from Agarose Gels Using Kit* protocol.

Step 3: Gel extraction (DNA purification)

- Follow the manufacturer's instructions for your gel extraction kit.

General tips for this step:

- When melting the gel slice:
    - Mix gently every 1–2 minutes to ensure even heating.
    - Make sure the agarose is *completely dissolved* before loading onto the column.
- Avoid bubbles when applying the sample to the column.

Step 4: Elution

- Elute purified DNA from the column using elution buffer (provided with the kit).

Important note on elution volume:

- The more elution buffer you use, the higher the total amount of DNA recovered, but the lower the DNA concentration.
- The less elution buffer you use, the higher the concentration, but you may recover slightly less total DNA.
- As a good compromise, 50 µL elution buffer usually works well for most applications.

Step 5: Measure DNA concentration

- Follow the protocol called *Plasmid Concentration Measurement*
- Use elution buffer as your blank.

ADD LIGATION STEPS HERE! - found in the E. coli transformation protocol