TRADITIONAL CLONING PROTOCOL

During the Lab practice, protocol as below was used for traditional cloning purposes. “New England BioLabs - NEBcloner” webpage was used as a background for the purpose of these experiment.
Traditional cloning workflow consists of four parts as follow:
1. Restriction Digest -> 2. End Modification -> 3. Ligation -> 4. Transformation.

To improve cloning efficiency, simple rules were considered during the time of experiment. Before the actual beginning of each experiment the workflow was carefully planned. The attention was placed on the junction sequences and the effect on reading frames of translated sequences. The vector and insert were checked for internal restriction sites. the antibiotic selective marker in the vector compatibility with the chosen host strain was checked. The right concentrations of DNA and its cleanness was tested. Digestion reactions were set really carefully, with a concern of the DNA ends prepared for cloning. The DNA prior to vector:insert joining was cleaned up. Then the isolated material was quantitated. In all processes the manufacturer’s guidelines for the joining reaction were followed. The competent cells suited to needs were used.

Workflow steps protocols below:

MATERIAL:

- DNA
- 10X CutSmart Buffer (1X)
- EcoRI-HF
- SpeI
- Nuclease-free water
- 10X T4 DNA Ligase Buffer
- Vector DNA (3 kb)
- Insert DNA (1 kb)
- T4 DNA Ligase
- Agar plates with or w/o antibiotic
- SOC media

EQUIPMENT:

- Heat-block or PCR-machine
- Incubator (37C)
- Rota-mixer
- Eppendorf- or micro- tubes
- Microentrifuge (13000 rpm)

PROCEDURE:

1. **Restriction Enzyme Double Digestion:**
    - Reaction set up as follows for single 50 µL volume final:

Reagent	Quantities
DNA	1.0 µg
10X CutSmart Buffer	5.0 µL (1X)
EcoRI-HF	1.0 µL
SpeI	1.0 µL
Nuclease-free water	up to 50 µL

- Incubation performed at the PCR machine:
    - 20 min. in 37C
    - 20 min. in 80C
    - Hold in 4C

2. **End modification was optional** (no methylation).

3. **Ligation - T4 DNA Ligase:**

- Following reaction performed in a microcentrifuge tube on ice. T4 DNA Ligase added last. 20 µL of volume final:

Reagent	Quantities
10X T4 DNA Ligase Buffer	2 µl
Vector DNA (3 kb)	50 ng
Insert DNA (1 kb)	50 ng
T4 DNA Ligase	1.0 µL
Nuclease-free water	up to 20 µl

T4 DNA Ligase Buffer thawed and resuspended at room temperature.

- The reaction mixed by pipetting up and down, and briefly microcentrifuged.
- For cohesive (sticky) ends, sample incubated at 16°C overnight or room temperature for 10 minutes.
- For blunt ends or single base overhangs, sample incubated at 16°C overnight or room temperature for 2 hours (alternatively, high concentration T4 DNA Ligase used in a 10 minute ligation).
- Chilled on ice and transformed 1-5 µl of the reaction into 50 µl competent cells.

4. Transformation:

- Thaw a tube of Competent E. coli cells on ice for until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
- Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes. Do not mix.
- Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
- Place on ice for 5 minutes. Do not mix.
- Pipette 950 µl of room temperature SOC into the mixture.
- Place at 37°C for 60 minutes on rota-mixer.
- Warm selection plates to 37°C.
- Mix the cells thoroughly by flicking the tube and inverting.
- Spread 200 µl of each sample onto a selection plate and incubate overnight at 37°C, up to 48H.