K.WANG
8 April 2021

Molecular cloning methods in our hands (Version 1.0)

1. Materials and reagents

High-Fidelity PCR polymerase: 2× Hieff Canace® PCR Master Mix (Yeasen, Cat# **10136ES01**)
High-Fidelity PCR polymerase: PrimeSTAR max 2× Master Mix (Takara, Cat# **R045Q**)
Colony PCR polymerase: 2xEs Taq MasterMix (CWBIO, Cat# **CW0690**)
DNA ligase: T4 DNA Ligase (NEB, Cat# **M0202M**)
DNA purification (Small-scale): BBI DNA purification Kit
DNA purification (Large-scale): 96-Well Plate PCR Purification Kit (BBI, Cat# **B518145**)
DNA gel recovery: QIAquick Gel Extraction Kit (QIAGEN, Cat#**28706**)
Fragments recombination: Hieff Clone® Plus One Step Cloning Kit (Yeasen, Cat# **10911ES20**)

 # You may need to read through the commercial protocol before cloning.

2. Gene isolation via PCR method

- PCR reaction Mix

Template DNA (1 ng/μL)[#1]	2 μL
FP (10 μM)	2 μL
RP (10 μM)	2 μL
DMSO[#2]	4 μL
2x Canace Mix[#3]	50 μL
PCR grade water	to 100 μL[#4]

#1 Plasmid DNA should be diluted to <1 ng/μL, high concentration of template may inhibit PCR reaction.

#2 DMSO is a frequently-used PCR additive. 1-3% of DMSO may increase the specificity or may help you to deal with high GC-contained templates.

#3 We have 2×ready-to-use master mix which contains polymerase, dNTPs, Buffers. Or you need to add 1 U/50 μL of Canace polymerase additionally.

#4 Commonly-used thermo cyclers are applicable for 50μL per hole, EXCESS IS FORBIDDEN.

- PCR cycling condition[#4]

98°C	3min	
98°C	10sec[#2]	31-35 cycles
60°C[#1]	20sec	
72°C	30sec/kb[#3]	
72°C	5min	

#1 The optimization of annealing temperature should be started from 60°C.

#2 Longer denaturing time may help you to deal with high GC-contained templates.

#3 For simple, low GC, plasmid DNA, the extension time can be decrease as 10 sec/kb.

#4 For PrimeSTAR max, we have another well-established protocol to use.

3. Plasmid linearization via restriction digestion

- Digestion reaction Mix

- DNA 1 μg
- 10×Buffer 50 μL
- Enzyme 1 μL (or 10 U)
- Nuclease-free water To 50 μL

➢ Incubate the reaction mix at suitable temperature.

 # 5–10 units of enzyme per μg DNA in a 1 hour digest. Overnight incubation may need to guarantee totally digesting

4. DNA electrophoresis

➢ Mix 1μL of loading dye with 4 μL of DNA sample for electrophoresis

➢ Gel preparation.

- Prepare gel mould and combs
- Boiling gel, add **10000×** of gel dye, mix well
- Pooling gel, cooling for 30 min

 # 1% of agarose is suitable for detecting DNA fragments >100bp

5. DNA purification

➢ In a 0.2 mL PCR tube, add 5 volumes of **Binding Buffer** (with 25% of isopropanol) to each volume of DNA sample.

➢ Mix well by pipet, transfer sample mixtures to the wells of 96-well **DNA purificator**.

➢ Centrifuge at ≥ 3,000 × *g* (4000 rpm) for 5 minutes until sample mixtures have been completely filtered. Discard the flow-through.

➢ Add 750 μL **Wash Buffer** to each well. Centrifuge at ≥ 3,000 × *g* for 5 minutes.

➢ Using air-dryer to dry the columns.

➢ Add 30-40 μL pre-heated water or **Elution Buffer** directly to the column matrix in each well. Incubate the plate in the 55℃ incubator.

➢ Transfer the DNA purificator onto an Elution Plate and centrifuge at ≥ 3,000 × *g* for 3 min to elute the DNA.

 # This protocol is modified for large-scale assay (>10 samples)

 # For small-scale assays, please refer to kit manual

6. DNA ligation

➢ Carefully DNA cleaning-up should be performed to avoid high false positive. Gel purifying the backbone and insert DNA if it is possible.

➢ Mixture condition (backbone : insert = 1:3, for 2 fragments rec.)

- Backbone: [0.02 × **basepairs**] ng
- Insert: [0.06 × **basepairs]** ng
- Add up to 4 μL, mix with 4 μL of 2 × Enzyme mix.

 # Normally, we use 40 ng of backbone for recombination.

7. Transformation

➢ Take 1 μL of ligation mixture from above, add 10 μL of competent cells into each well.

➢ Mix well and incubate on ice for 20 min. Thermal shock under 42℃ for 1 min, incubate on ice for 2 min. Add 160 μL of LB (without antibiotics), incubate for 1 h under 37℃.

➢ Take 20μL[#1] of cell suspensions for inoculation.

#1 For huge constructs like Huc, GFAP, inoculating more.