VAN DE POEL 实验室克隆手册

Table of Contents (目录)

(注：为保持格式整洁，目录页码对应原PDF页码)

DNA extraction

DNA 提取

gDNA extraction from Arabidopsis for cloning

用于克隆的拟南芥 gDNA 提取

Extraction buffer:
提取缓冲液：

- 200 mM Tris-HCl pH 7.5
- 250 mM NaCl
- 25 mM EDTA
- 0.5 % SDS

> 💡 **注释 (Annotation):**
>
> - **Tris-HCl:** 维持 pH 值稳定，保护 DNA 不被酸碱破坏。

- **NaCl:** 提供离子环境，有助于维持 DNA 结构并帮助蛋白质沉淀。
- **EDTA:** 螯合镁离子 (Mg^{2+})。由于 DNase（DNA酶）需要 Mg^{2+} 才能降解 DNA，EDTA 能有效抑制 DNase 活性，同时破坏细胞膜的稳定性。
- **SDS:** 十二烷基硫酸钠，一种强效去污剂。用于裂解细胞膜和核膜，并使蛋白质变性，将其与 DNA 分离。

Protocol:
实验方案：

- Grow up desired genotype and take a small bit of plant material (e.g. ~ 50 mg of leaf) in a 1.5 mL ep.
  - 种植所需的基因型植株，取少量植物材料（例如约 50 mg 叶片）放入 1.5 mL EP 管（离心管）中。
- Flash-freeze in liquid nitrogen.
  - 在液氮中速冻。

  > 💡 **注释:** 液氮速冻可以瞬间停止所有酶的活性（防止内源性 DNase 降解 DNA），并使植物组织变脆，易于研磨破碎。
- First slightly crush with a sterile pestle (bleu), then add 400 µL of the extraction buffer and ground completely to fine powder with the pestle.
  - 首先用无菌研磨杵（蓝色）轻轻压碎，然后加入 400 µL 提取缓冲液，用研磨杵完全研磨成细粉。
- Centrifuge at maximum speed for 1 minute at 4°C.
  - 在 4°C 下以最高速度离心 1 分钟。

  > 💡 **注释:** 离心将细胞碎片（沉淀在底部）与含有溶解 DNA 的溶液（上清液）分离。
- Take ± 350 µL of the supernatant and transfer to a new tube.
  - 吸取约 350 µL 上清液并转移至新管中。
- Add 1:1 cold isopropanol (100 %) and mix thoroughly.
  - 加入 1:1 体积的冷异丙醇（100%）并充分混合。

  > 💡 **注释:** 异丙醇会改变溶液的介电常数，使 DNA 分子更容易相互聚集并从水中析出（沉淀）。低温有助于提高沉淀效率。
- Leave overnight at -20°C.
  - 在 -20°C 下放置过夜。
- Centrifuge at 4 °C at maximum speed for 2 minutes and discard the supernatant.
  - 在 4°C 下以最高速度离心 2 分钟，弃去上清液。
- Add ± 600 µl cold EtOH (70%).
  - 加入约 600 µl 冷乙醇（70%）。

> 💡 **注释:** 70% 乙醇用于洗涤 DNA 沉淀。其中的水成分可以溶解共沉淀的盐分（盐分会抑制后续酶反应），而乙醇成分则保持 DNA 处于不溶解的沉淀状态。

- Centrifuge again at 4°C at maximum speed for 2 minutes and discard the supernatant, be careful not to disturb the pellet.
    - 再次在 4°C 下以最高速度离心 2 分钟，弃去上清液，注意不要扰动沉淀。
- Keep the tubes open to dry the pellets and remove all the EtOH (around 10 minutes) at room temperature.
    - 保持管盖打开，在室温下干燥沉淀并去除所有乙醇（约 10 分钟）。

> 💡 **注释:** 残留的乙醇会严重抑制后续 PCR 或酶切反应，必须挥发干净。但也不能过度干燥（变透明），否则 DNA 难以重新溶解。

- Resuspend in ± 20 µL MilliQ
    - 用约 20 µL MilliQ 水（超纯水）重悬。

Rough gDNA extraction from Arabidopsis, Marchantia or Physcomitrium for quick genotyping

用于快速基因分型的拟南芥、地钱或小立碗藓粗糙 gDNA 提取

Solutions (both from sigmaaldrich.com):
溶液（均购自 sigmaaldrich.com）：

- (Extract-N-Amp Direct PCR Extraction Solution)
    - (Extract-N-Amp 直接 PCR 提取液)
- Dilution solution D5688-12mL (Dilution Solution, Plant Direct PCR Sigma-Aldrich)
    - 稀释液 D5688-12mL (植物直接 PCR 稀释液，Sigma-Aldrich)

Both solutions were originally intended to be used for the Extract-N-Amp Plant PCR kit (sigmaaldrich.com). However, a modified version of the manufacturer's protocol can be used to prep gDNA from different plant species for genotyping (up to 1000 bp fragments) and can be tested to be used for cloning (quality might not be good enough for difficult PCR amplifications or downstream cloning).
这两种溶液最初是用于 Extract-N-Amp 植物 PCR 试剂盒 (sigmaaldrich.com)。然而，可以使用制造商方案的修改版本来制备不同植物物种的 gDNA 用于基因分型（最高 1000 bp 片段），也可以测试用于克隆（但对于困难的 PCR 扩增或下游克隆，其质量可能不够好）。

Protocol:
实验方案：

- Aliquot 15-30 µL of extraction solution into a PCR tube.
    - 将 15-30 µL 提取液分装到 PCR 管中。
- Transfer a small piece of plant material into the tube (for Arabidopsis: small leaf from seedlings or a piece of a leaf from older plants; for Marchantia or Physcomitrium: small piece of plant material).

    - 将一小块植物材料转移到管中（对于拟南芥：幼苗的小叶或老植株的叶片碎片；对于地钱或小立碗藓：一小块植物材料）。
    - The plant material should not soak up all the extraction solution present in the tube; therefore, avoid taking too much plant material, but also not too little.
        - 植物材料不应吸干管中所有的提取液；因此，避免取用过多的植物材料，但也不要太少。

- Incubate the samples for 10 min at 90 °C in a thermocycler.
    - 在热循环仪（PCR 仪）中 90°C 孵育样品 10 分钟。

> 💡 **注释:** 高温用于裂解细胞释放 DNA。

- Add an equal amount of dilution solution (15-30 µL; 1:1 ratio of extraction to dilution solution).
    - 加入等量的稀释液（15-30 µL；提取液与稀释液比例为 1:1）。

> 💡 **注释:** 稀释液用于中和提取液中的抑制成分，使环境适合 PCR 酶。

- Mix by pipetting up and down or by vortexing.
    - 通过上下吹打或涡旋混合。
- Now, the gDNA is ready to be used for PCR.
    - 现在，gDNA 即可用于 PCR。

For genotyping (and subsequent sequencing): if a PCR volume of 50 µL is used, 4 µL of the gDNA should is recommended.
对于基因分型（以及随后的测序）：如果使用 50 µL 的 PCR 体积，建议使用 4 µL 的 gDNA。

DNA synthesis

DNA 合成

Synthetic genes:
合成基因：

Currently, GenScript offers the best price for synthetic genes with the FLASH Gene service (link provided in original).
目前，GenScript（金斯瑞）通过 FLASH Gene 服务提供最优惠的合成基因价格（原文附链接）。

Do not set-up an account directly; ask for an official recommendation from a colleague via the GenScript webpage, this will allow you to get some further discount for your quote.
不要直接自行注册账户；请通过 GenScript 网页向同事索取官方推荐，这将使您的报价获得更多折扣。

(If the prices change in the future, alternative companies could be: TwistBioscience or BioCat)
（如果未来价格变动，替代公司可以是：TwistBioscience 或 BioCat）

Codon optimisation
密码子优化

For ordering primers, oligos and gene fragments, IDT can be used (https://www.idtdna.com.).
订购引物、寡核苷酸和基因片段，可以使用 IDT (https://www.idtdna.com.)。

Primers are ordered as “Single-stranded DNA” in tubes, generally at a concentration of 25 nmole DNA.
引物通常以“单链 DNA”的形式订购于管中，浓度通常为 25 nmole DNA。

Plasmid miniprep

质粒小量制备（Miniprep）

Execute all steps at room temperature. We use the Thermo Scientific GeneJJET Plasmid Miniprep kit (K0502).
所有步骤均在室温下执行。我们使用 Thermo Scientific GeneJET 质粒小量制备试剂盒 (K0502)。

Before you start:
开始之前：

- Check if the Resuspension solution contains RNase A and is stored in at 4 °C and is not older than 6 months.
    - 检查重悬液（Resuspension solution）是否含有 RNase A，是否储存在 4°C 下，且时间不超过 6 个月。

> 💡 **注释:** RNase A 用于降解细菌 RNA。如果不去除 RNA，它会干扰后续的酶切和电泳分析，使条带模糊。

- Check if ethanol (96 %) is added to the Wash solution (in a ratio 1:1.75 or 35 mL EtOH per 20 mL Wash solution).
    - 检查洗涤液（Wash solution）中是否已加入乙醇（96%）（比例为 1:1.75，或每 20 mL 洗涤液加入 35 mL 乙醇）。
- Check if there are salt precipitates or not. If so, shake the bottle or heat it up to 37 °C, and let it cool down again to room temperature.
    - 检查是否有盐沉淀。如果有，摇动瓶子或将其加热至 37°C，然后让其再次冷却至室温。

> 💡 **注释:** 裂解液（Lysis buffer，通常含 SDS/NaOH）在低温下容易析出 SDS 白色沉淀。如果不溶解，裂解效率会大幅下降。

- In case you are making a freezer stock, take out an aliquot from your overnight culture before doing the mini-prep.
    - 如果需要制作冻存菌种，请在进行小量制备前从过夜培养物中取出一部分。
- Always make sure the bag of spin columns is airtight closed before and after use.
    - 始终确保离心柱的包装袋在使用前后都是密封的。

Protocol:
实验方案：

- Harvest overnight culture of 5 mL in a falcon, or take a 2 mL ep tube and centrifuge at 8000 rpm (6800 x g) for 2 min at room temperature.
    - 收集 5 mL 过夜培养物于 Falcon 管中，或取 2 mL EP 管，在室温下以 8000 rpm (6800 x g) 离心 2 分钟。
    - o To increase plasmid yield for low-copy numbers: take 2 or 3 x 2 mL culture (or spin down 10 mL culture in a falcon) and pool them after the resuspension step.
        - o 若要增加低拷贝数质粒的产量：取 2 或 3 管 2 mL 培养物（或在 Falcon 管中离心 10 mL 培养物），并在重悬步骤后将它们合并。
- Remover the supernatant by pipetting or decanting (make sure the LB medium is gone).
    - 通过移液或倒出法去除上清液（确保 LB 培养基已清除）。
- Resuspend the pellet in 250 uL Resuspension Solution by briefly vortexing the cells and the pellet is completely dissolved.
    - 用 250 uL 重悬液重悬沉淀，通过短暂涡旋细胞，直至沉淀完全溶解。
    - o If you have multiple pellets from the same culture, only use 250 uL Resuspension Solution once for all pellets.
        - o 如果你有来自同一培养物的多个沉淀，所有沉淀只使用一次 250 uL 重悬液。
- Add 250 uL Lysis buffer and mix immediately by thoroughly inverting the tubes 4-6 times (do not vortex) until the solution becomes viscous and clear.
    - 加入 250 uL 裂解缓冲液（Lysis buffer），并通过彻底颠倒试管 4-6 次立即混合（不要涡旋），直到溶液变得粘稠和澄清。

> 💡 **注释:** 裂解液呈碱性且含 SDS，能破裂细胞并使 DNA 变性。**切勿涡旋**，否则会物理剪切细菌基因组 DNA，导致其断裂成小片段污染质粒提取物。

- Add 350 uL Neutralization Solution and mix immediately by thoroughly inverting the tube 4-6 times (do not vortex).
    - 加入 350 uL 中和液（Neutralization Solution），并通过彻底颠倒试管 4-6 次立即混合（不要涡旋）。

> 💡 **注释:** 中和液使 pH 值恢复中性。质粒 DNA 较小，能迅速复性（重新形成双螺旋），而基因组 DNA 较大，无法复性并与变性蛋白质、SDS 形成白色絮状沉淀。

- Centrifuge for 5 min at >12.000 x g at room temperature.
    - 在室温下以 >12,000 x g 离心 5 分钟。
- Transfer the supernatant to the GeneJET spin column by decanting or pipetting.
    - 通过倾倒或移液将上清液转移到 GeneJET 离心柱中。
- Centrifuge the spin columns for 1 min at >12.000 x g at room temperature.
    - 在室温下以 >12,000 x g 离心柱子 1 分钟。

> 💡 **注释:** 质粒 DNA 在高盐环境下特异性结合到硅胶膜上。

- Add 500 uL Wash Solution to the GeneJET spin column.

    - 向 GeneJET 离心柱中加入 500 uL 洗涤液（Wash Solution）。
- Centrifuge the spin column for 1 min at >12.000 x g at room temperature.
    - 在室温下以 >12,000 x g 离心柱子 1 分钟。
- Discart the supernatants and reuse the collection tube.
    - 弃去上清液并重复使用收集管。
- Centrifuge the spin column for an additional 1 min at >12.000 x g at room temperature.
    - 在室温下以 >12,000 x g 额外离心柱子 1 分钟。
- Make sure no ethanol residues remain or touch the spin column.
    - 确保没有乙醇残留或接触离心柱。

> 💡 **注释:** 此步骤非常关键，用于去除残留的乙醇。残留乙醇会抑制洗脱和后续的所有酶反应（如 PCR、酶切）。

- Transfer the spin column to a new collection tube (or 1.5 mL ep).
    - 将离心柱转移到新的收集管（或 1.5 mL EP 管）中。
- Add 50 uL Elution Buffer (or Mili-Q water) to the spin column membrane (in the center).
    - 向离心柱膜（中心）加入 50 uL 洗脱缓冲液（或 Mili-Q 水）。
- Do not touch the spin column membrane with the pipette tip.
    - 不要用移液枪头接触离心柱膜。
- You can also lower the elution volume to 25 uL to increase the plasmid concentration.
    - 你也可以将洗脱体积降低至 25 uL 以增加质粒浓度。
- Incubate the spin column for 2 min at room temperature.
    - 在室温下孵育离心柱 2 分钟。

> 💡 **注释:** 孵育可以让洗脱液充分渗透膜，溶解 DNA，提高产量。

- Centrifuge the spin column for 2 min at > 12.000 x g at room temperature.
    - 在室温下以 > 12,000 x g 离心柱子 2 分钟。
    - o You can resuspend the flow-through containing the plasmid on the same spin column membrane and repeat steps the last two steps to get a slightly higher plasmid yield.
        - o 你可以将含有质粒的流穿液重新加到同一个离心柱膜上，并重复最后两个步骤，以获得稍高的质粒产量。
- Store the plasmid at -20 °C.
    - 将质粒储存在 -20 °C。

Freezer stocks

菌种保藏（甘油菌）

- Take 0.75 mL of overnight bacterial culture and pipet in an autoclaved 1.5 mL screw-cap tube.
  - 取 0.75 mL 过夜细菌培养物，移液至高压灭菌的 1.5 mL 螺旋盖管中。
- Add 0.75 mL of 25 % Glycerol (v:v in water). The final glycerol concentration should be 12.5 %.
  - 加入 0.75 mL 25% 甘油（v:v 水溶液）。最终甘油浓度应为 12.5%。

> 💡 **注释:** 甘油作为抗冻剂，防止冰晶形成刺破细菌细胞膜，从而在低温下保护细菌。

- Write down the plasmid name, date, E coli strain and your name on the tube using a cryo-marker.
  - 使用耐低温标记笔在管上写下质粒名称、日期、大肠杆菌菌株和你的名字。
- Store in the -80 °C freezer and update the inventory list.
  - 存放在 -80 °C 冰箱中并更新库存清单。

PCR on DNA template

DNA 模板 PCR

For cloning, high fidelity DNA polymerases such as Q5 and Phusion are used.
对于克隆，使用高保真 DNA 聚合酶，如 Q5 和 Phusion。

Reaction Setup for Phusion Polymerase:
Phusion 聚合酶反应体系：

All components should be mixed and centrifuged prior to use. Assemble all reaction components on ice and quickly transfer the reactions to a thermocycler preheated to the denaturation temperature (98°C). Add the DNA Polymerase last to prevent any primer degradation caused by the 3′→ 5′ exonuclease activity.
所有组分在使用前应混合并离心。在冰上组装所有反应组分，并迅速将反应转移至预热至变性温度（98°C）的热循环仪中。最后添加 DNA 聚合酶，以防止由 3'→ 5' 外切酶活性引起的任何引物降解。

Component (组分)	20 µL Reaction	50 µL Reaction	Final Concentratio
Nuclease-free water (无核酸酶水)	to 20 µL	to 50 µL	
5 X Phusion HF or GC Buffer	4 µL	10 µL	1 X
10 mM dNTPs	0.4 µL	1 µL	200 µM
10 µM Forward Primer (正向引物)	1 µL	2.5 µL	0.5 µM
10 µM Reverse Primer (反向引物)	1 µL	2.5 µL	0.5 µM

Component (组分)	20 µL Reaction	50 µL Reaction	Final Concentratio
Template DNA (模板 DNA)	Variable (see below)	Variable (see below)	< 250 ng
DMSO (optional) (可选)	(0.6 µL)	(1.5 µL)	3 %
Phusion DNA Polymerase	0.2 µL	0.5 µL	1.0 units/50 µL PCR

To determine the amount of template DNA:
确定模板 DNA 的量：

DNA	Amount (量)
Genomic (基因组)	50 ng–250 ng
plasmid or viral (质粒或病毒)	1 pg–10 ng

Note: do not use more DNA/plasmid template as indicated: too much template can consume all primers.
注意：不要使用超过指示量的 DNA/质粒模板：过多的模板会消耗掉所有引物。

Thermocycling conditions for a routine PCR:
常规 PCR 的热循环条件：

Step (步骤)	T [°C]	Time (时间)
Initial Denaturation (初始变性)	98°C	30 seconds
25-35 Cycles (循环)		
Denaturation (变性)	98°C	5-10 seconds
Annealing (退火)	T annealing of the primers (45-72°C)	10-30 seconds
Elongation (延伸)	72°C	Depending on the length of t
Final extension (最终延伸)	72°C	5-10 minutes
Hold (保温)	4-10°C	

Trouble shooting (故障排除):

- **GC buffer:** replace the HF buffer with the GC buffer for DNA amplicons that have a high GC content (65-80 % GC content).
  - **GC 缓冲液:** 对于具有高 GC 含量（65-80% GC 含量）的 DNA 扩增子，用 GC 缓冲液替换 HF 缓冲液。
- **DMSO can be added:** it lowers the melting temperature and prevents annealing, thereby minimizing secondary DNA structures which can increase the specificity.
  - **可以添加 DMSO:** 它降低熔解温度并阻止退火，从而最大限度地减少 DNA 二级结构，这可以增加特异性。
- **Gradient PCR:** make a gradient in the PCR machine to test different annealing temperatures. Sometimes primers work better at a lower or higher temperature as the theoretical best temperature.

    - **梯度 PCR:** 在 PCR 仪中设置梯度以测试不同的退火温度。有时引物在比理论最佳温度更低或更高的温度下工作效果更好。
- **Quick-step PCR:** try omitting the annealing step in the PCR program if the annealing temperature of the primers is too high (>68 °C). Just run your sample by cycling between the denaturing (98°C) and extension (72°C) step.
    - **两步法 PCR (Quick-step):** 如果引物的退火温度太高 (>68 °C)，尝试省略 PCR 程序中的退火步骤。只需在变性 (98°C) 和延伸 (72°C) 步骤之间循环运行样品。
- **Touch-down PCR:** increasing specificity of PCR reactions (less nonspecific amplification) by starting with a higher annealing temperature and gradually reducing it through e.g. 10 cycles until it reaches the calculated annealing temperature of the primers or some degrees below. Follow the standard Reaction Setup for PCRs.
    - **降落 PCR (Touch-down):** 通过从较高的退火温度开始，并在例如 10 个循环中逐渐降低温度，直到达到引物的计算退火温度或更低几度，来提高 PCR 反应的特异性（减少非特异性扩增）。遵循 PCR 的标准反应设置。

Thermocycling conditions (Touch-down example):
热循环条件（降落 PCR 示例）：

Step	**T [°C]**	**Time**
Initial Denaturation	98°C	30 seconds
10 Cycles Auto delta: -0.5°C		
Denaturation	98°C	5-10 seconds
Annealing	T annealing + 2°C	10-30 seconds
Elongation	72°C	Depending on length / 15-30s/kb
25 Cycles		
Denaturation	98°C	5-10 seconds
Annealing	T annealing – 3°C	10-30 seconds
Elongation	72°C	Depending on length / 15-30s/kb
Final extension	72°C	5-10 minutes
Hold	4-10°C	

PCR clean-up

PCR 产物纯化

GeneJET PCR Purification Kit

GeneJET PCR 纯化试剂盒

All purification steps should be carried out at room temperature. All centrifugations should be carried out in a table-top microcentrifuge at >12000 x g.
所有纯化步骤应在室温下进行。所有离心应在台式微量离心机中以 >12000 x g 进行。

- Add a 1:1 volume of Binding Buffer to completed PCR mixture and mix thoroughly by pipetting up and down. (Color should be yellow. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix.)
    - 向完成的 PCR 混合物中加入 1:1 体积的结合缓冲液（Binding Buffer），并通过上下吹打充分混合。（颜色应为黄色。如果溶液颜色为橙色或紫色，加入 10 µL 3 M 乙酸钠 pH 5.2 溶液并混合。）

> 💡 **注释:** 结合缓冲液中含有 pH 指示剂。DNA 结合硅胶膜需要酸性高盐环境，黄色表示 pH 正确。

    - o Optional: if the DNA fragment is ≤ 500 bp, add a 1:2 volume of 100 % isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly.
        - o 可选：如果 DNA 片段 ≤ 500 bp，加入 1:2 体积的 100% 异丙醇（例如，向 100 µL PCR 混合物与 100 µL 结合缓冲液的混合液中加入 100 µL 异丙醇）。充分混合。
    - Note. If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
        - 注意：如果 PCR 混合物含有引物二聚体，建议不使用异丙醇进行纯化（有助于去除小分子二聚体）。但是，目标 DNA 片段的产量会降低。
- Transfer ≤ 800 µL of the solution from step 1 to the GeneJET purification column.
    - 将步骤 1 中的 ≤ 800 µL 溶液转移到 GeneJET 纯化柱中。
- Centrifuge for 30-60 s at >12000 x g at room temperature and discard the flow-through.
    - 在室温下以 >12000 x g 离心 30-60 秒，并弃去流穿液。
    - o Repeat this step, until the entire solution has been added to the column.
        - o 重复此步骤，直到所有溶液都已加入柱中。
- Add 700 µL of Wash Buffer to the purification column. Check if the Wash buffer was diluted with ethanol before use.
    - 向纯化柱中加入 700 µL 洗涤缓冲液。使用前检查洗涤缓冲液是否已用乙醇稀释。
- Centrifuge for 30-60 s at >12000 x g at room temperature and discard the flow-through and place the purification column back into the same collection tube.
    - 在室温下以 >12000 x g 离心 30-60 秒，弃去流穿液，并将纯化柱放回同一个收集管中。
- Centrifuge the empty GeneJET purification column for an additional 1 min at >12000 x g at room temperature to completely remove any residual wash buffer.
    - 在室温下以 >12000 x g 额外离心空的 GeneJET 纯化柱 1 分钟，以完全去除任何残留的洗涤缓冲液。
- Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube.
    - 将 GeneJET 纯化柱转移到干净的 1.5 mL 微量离心管中。
- Add 50 µL of Elution Buffer or Milli-Q to the center of the GeneJET purification column membrane and centrifuge for 1 min.

    - 向 GeneJET 纯化柱膜的中心加入 50 µL 洗脱缓冲液或 Milli-Q 水，并离心 1 分钟。
    - o Note. For low DNA amounts the elution volumes can be reduced to a volume between 20-50 µL.
        - o 注意：对于低 DNA 量，洗脱体积可以减少到 20-50 µL 之间。
    - o If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column.
        - o 如果 DNA 片段 >10 kb，在加到柱上之前将洗脱缓冲液预热至 65 °C。
    - o You can resuspend the flow-through containing the plasmid on the same spin column membrane and repeat steps the last two steps to get a slightly higher plasmid yield.
        - o 你可以将含有质粒的流穿液重新加到同一个离心柱膜上，并重复最后两个步骤，以获得稍高的质粒产量。

- Discard the GeneJET purification column and store the purified DNA at -20 °C.
    - 丢弃 GeneJET 纯化柱，并将纯化的 DNA 储存在 -20 °C。

Isopropanol method

异丙醇法

Extraction buffer:
提取缓冲液：

- 200 mM Tris-HCl pH 7.5
- 250 mM NaCl
- 25 mM EDTA
- 0.5 % SDS

Protocol:
实验方案：

- add 400 µL of cold extraction buffer to your DNA sample.
    - 向你的 DNA 样品中加入 400 µL 冷提取缓冲液。
- Centrifuge at maximum speed for 1 minute at 4°C.
    - 在 4°C 下以最高速度离心 1 分钟。
- Take ± 350 µL of the supernatant and transfer to a new tube.
    - 吸取约 350 µL 上清液并转移到新管中。
- Add 1:1 cold isopropanol (100 %) and mix thoroughly.
    - 加入 1:1 体积的冷异丙醇（100%）并充分混合。
- Leave overnight at 4°C.
    - 在 4°C 下放置过夜。
- Centrifuge at 4 °C at maximum speed for 2 minutes and discard the supernatant.
    - 在 4°C 下以最高速度离心 2 分钟，并弃去上清液。
- Add ± 600 µl cold EtOH (70%).
    - 加入约 600 µl 冷乙醇（70%）。
- Centrifuge again at 4°C at maximum speed for 2 minutes and discard the supernatant, be careful not to disturb the pellet.
    - 再次在 4°C 下以最高速度离心 2 分钟，弃去上清液，注意不要扰动沉淀。

- Keep the tubes open to dry the pellets and remove all the EtOH (around 10 minutes) at room temperature.
  - 保持管盖打开，在室温下干燥沉淀并去除所有乙醇（约 10 分钟）。
- Resuspend in ± 20 µL MilliQ
  - 用约 20 µL MilliQ 水重悬。

Gel extraction with a kit

试剂盒胶回收

E.Z.N.A Gel Extraction Kit (Omega Bio-tek)
E.Z.N.A 凝胶提取试剂盒 (Omega Bio-tek)

Before starting:
开始之前：

- Prepare SPW Buffer according to the manual (add 100 % ethanol).
  - 根据手册准备 SPW 缓冲液（加入 100% 乙醇）。
- Set heating block or water bath to 60 °C.
  - 将加热模块或水浴设置为 60 °C。
- Decontaminate the UV-illuminator with ethanol.
  - 用乙醇对紫外透射仪进行去污。
- Wear protective eyewear when using the UV-illuminator.
  - 使用紫外透射仪时佩戴护目镜。

Protocol:

- After adequate separation of bands in an agarose gel, carefully excise the DNA fragment of interest with a decontaminated scalpel or razor blade.
  - 在琼脂糖凝胶中充分分离条带后，用去污的手术刀或刀片小心切下感兴趣的 DNA 片段。
- Take the minimum size of agarose slice as possible (flip the excised band and remove the bottom part of the agarose that does not contain DNA).
  - 尽可能切取最小尺寸的琼脂糖切片（翻转切下的条带，去除底部不含 DNA 的琼脂糖部分）。
- Chop the agarose slice into smaller pieces.
  - 将琼脂糖切片切成更小的碎片。
- Weight the agarose slice in a clean 1.5 mL reaction tube.
  - 在干净的 1.5 mL 反应管中称量琼脂糖切片。
- Add 1 volume XP2 Binding Buffer (volume of XP2 buffer in mL = weight of agarose in mg).
  - 加入 1 倍体积的 XP2 结合缓冲液（XP2 缓冲液的体积 mL = 琼脂糖的重量 mg）。
- Incubate at 60 °C for 7 min or until completely melted; vortex/shake the tube every 2-3 minutes.
  - 在 60 °C 下孵育 7 分钟或直至完全融化；每 2-3 分钟涡旋/摇动试管。
  - o Monitor the pH of Gel/Binding Buffer mixture by color (if it turns orange/red, add 5 µM sodium acetate pH 5.2).
    - o 通过颜色监测凝胶/结合缓冲液混合物的 pH 值（如果变为橙色/红色，加入 5 µM 乙酸钠 pH 5.2）。

- Add up to 700 µL of the Gel/XP2 Binding Buffer solution to a HiBind DNA Mini Column inserted into a 2 mL Collection Tube.
  - 将最多 700 µL 凝胶/XP2 结合缓冲液溶液加入插入 2 mL 收集管的 HiBind DNA 迷你柱中。
- Centrifuge at 10,000 x g for 1 minute at room temperature, discard the flow-through.
  - 在室温下以 10,000 x g 离心 1 分钟，弃去流穿液。
- Repeat these last two steps until all of the Gel/XP2 Binding solution has been transferred to the column.
  - 重复最后两个步骤，直到所有的凝胶/XP2 结合溶液都转移到柱中。
- Add 300 µL XP2 Binding Buffer.
  - 加入 300 µL XP2 结合缓冲液。
- Centrifuge at 13,000 x g for 1 minute at room temperature, discard the flow-through.
  - 在室温下以 13,000 x g 离心 1 分钟，弃去流穿液。
- Add 700 µL SPW Buffer. (Check if SPW Buffer is diluted with 100 % ethanol prior use).
  - 加入 700 µL SPW 缓冲液。（使用前检查 SPW 缓冲液是否已用 100% 乙醇稀释）。
  - o Check if SPW Buffer is diluted with 100 % ethanol prior use.
    - o 使用前检查 SPW 缓冲液是否已用 100% 乙醇稀释。
- Centrifuge at 13,000 x g for 1 minute at room temperature, discard flow-through.
  - 在室温下以 13,000 x g 离心 1 分钟，弃去流穿液。
- Repeat the washing step with SPW Buffer.
  - 重复 SPW 缓冲液洗涤步骤。
- Centrifuge the empty Column at 13,000 x g for 2 minutes at room temperature to dry the column.
  - 在室温下以 13,000 x g 离心空柱 2 分钟以干燥柱子。
- Transfer the Column to a clean 1.5 mL reaction tube.
  - 将柱子转移到干净的 1.5 mL 反应管中。
- Add 30-50 µL Elution Buffer or Mili-Q water to the center of the column membrane (do not touch the membrane with your pipette tip).
  - 向柱膜中心加入 30-50 µL 洗脱缓冲液或 Mili-Q 水（不要用移液枪头接触膜）。
  - o To increase the DNA yield, preheat the Elution buffer or Mili-Q water to 65 °C.
    - o 为增加 DNA 产量，将洗脱缓冲液或 Mili-Q 水预热至 65 °C。
- Incubate for 2 minutes at room temperature.
  - 在室温下孵育 2 分钟。
- Centrifuge at 13,000 x g for 1 minute at room temperature.
  - 在室温下以 13,000 x g 离心 1 分钟。
  - o To increase the DNA yield: repeat the last 3 steps by re-suspending the same DNA elution fraction onto the same column membrane.
    - o 为增加 DNA 产量：通过将相同的 DNA 洗脱液重新加到同一柱膜上来重复最后 3 个步骤。
- Store DNA at -20 °C.
  - 将 DNA 储存在 -20 °C。

Low melting agarose gel extraction

低熔点琼脂糖凝胶回收

(原文此标题下无内容，通常参考常规胶回收步骤或说明该步骤在特定实验中与常规方法类似)

Agarose gel electrophoresis

琼脂糖凝胶电泳

1 x TAE running buffer recipe can be diluted from a 50 X TAE stock solution with pH around 8.5:
1 x TAE 电泳缓冲液配方可以由 pH 约 8.5 的 50 X TAE 储备液稀释而成：

50 X TAE stock For 1 L:
50 X TAE 储备液 (1 L):

- Tris-buffer (121.14 g/mol): 242 g (Final: 2 M)
- 0.5 M EDTA stock solution (pH 8): 100 mL (Final: 50 mM)
- Acetic acid 100 %: 57.1 mL (Final: 1 M)

Note: 0.5 M EDTA is store-bought (do not attempt making this yourself). The 50 x TAE buffer is stored at room temperature. pH should not be adjusted normally.
注意：0.5 M EDTA 是购买的成品（不要尝试自己配制）。50 x TAE 缓冲液在室温下保存。通常不需要调节 pH 值。

DNA gel electrophoresis:
DNA 凝胶电泳：

- Weight out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution.
  - 称取适量的琼脂糖放入锥形瓶中。琼脂糖凝胶使用 w/v 百分比溶液制备。
  - The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5 %-2 %.
    - 凝胶中琼脂糖的浓度取决于要分离的 DNA 片段的大小，大多数凝胶的浓度范围在 0.5%-2% 之间。
  - ▪ For several general approaches in our lab, a 1 % gel is appropriate
    - ▪ 对于我们实验室的几种常规方法，1% 凝胶是合适的
  - ▪ For small DNA fragments (< 750 bp), try using a higher % (e.g. 2 %)
    - ▪ 对于小 DNA 片段 (< 750 bp)，尝试使用较高百分比（例如 2%）
  - ▪ For separating large DNA fragments (> 5.000 bp), try using a lower % (e.g. 0.8 %)
    - ▪ 对于分离大 DNA 片段 (> 5,000 bp)，尝试使用较低百分比（例如 0.8%）
- Add running buffer to the agarose (1 X TAE) inside the Erlenmeyer flask.
  - 向锥形瓶中的琼脂糖加入电泳缓冲液 (1 X TAE)。
  - ▪ Use around 30 mL for small gels
    - ▪ 小胶约用 30 mL
  - ▪ Use around 60 mL for medium gels
    - ▪ 中胶约用 60 mL
  - ▪ Use around 80 mL for large gels (two layers of combs)
    - ▪ 大胶（双层梳子）约用 80 mL
- Melt the agarose/buffer mixture in the microwave, make sure that the mixture is sufficiently dissolved (it can boil in the microwave).
  - 在微波炉中融化琼脂糖/缓冲液混合物，确保混合物充分溶解（它可以在微波炉中沸腾）。

- Pour the mixture into a prepared gel holder including the gel comb and add a droplet (1-2 µL) of Ethidium Bromide, mix thoroughly using the pipet tip.
  - 将混合物倒入准备好的插有梳子的制胶托盘中，并加入一滴 (1-2 µL) 溴化乙锭 (EtBr)，用移液枪头充分混合。
  - ▪ **BE CAREFUL** not to touch anything with your hands after working in the EtBr contaminated area! Always wear gloves when working with EtBr.
    - ▪ **小心**，在 EtBr 污染区工作后不要用手触摸任何东西！操作 EtBr 时始终佩戴手套。
  - ▪ Remove air bubbles attached to the comb wells.
    - ▪ 去除附着在梳孔上的气泡。
  - ▪ Make sure to have a tray underneath in case of spilling gel.
    - ▪ 确保下面有一个托盘，以防凝胶溢出。
- After solidification (30 min), place the gel into a gel chamber supplied with enough 1 x TAE running buffer.
  - 凝固后（30 分钟），将凝胶放入加有足够 1 x TAE 电泳缓冲液的电泳槽中。
  - ▪ Note: during warm summer days, gel solidification can take longer.
    - ▪ 注意：在炎热的夏季，凝胶凝固可能需要更长的时间。
- Gently remove the comb by lifting it upwards.
  - 通过向上提拉轻轻取下梳子。
- Load your samples.
  - 上样。
  - ▪ Samples contain loading dye to a 1 X end concentration.
    - ▪ 样品中含有终浓度为 1 X 的上样染料。
- Load a DNA ladder (e.g. 10 Kb-ladder: 4.5 µL, at one or both sides of your sample).
  - 上载 DNA Ladder（例如 10 Kb-ladder：4.5 µL，在样品的一侧或两侧）。
- Run the gel at manual settings (steady 120 V) for about 30-40 min or until your DNA is separated sufficiently.
  - 在手动设置下运行凝胶（恒定 120 V）约 30-40 分钟，或直到你的 DNA 充分分离。
- Visualize the gel at the Biorad GelDoc Gel Imaging System.
  - 在 Biorad GelDoc 凝胶成像系统中观察凝胶。

RNA gel electrophoresis:
RNA 凝胶电泳：

Note: RNA is easily degraded by RNases which are omnipresent in the gel tank or the buffers. Also use filter tips to prevent aerosols being blown in your samples. Make sure all products are clean by rinsing them with RNase-AWAY. Wear gloves at all times.
注意：RNA 很容易被凝胶槽或缓冲液中无处不在的 RNases（RNA 酶）降解。还要使用滤芯吸头以防止气溶胶吹入样品中。确保所有物品都通过 RNase-AWAY 清洗干净。全程佩戴手套。

- RNA gels are prepared similarly to DNA gels (1 % agarose), however, due to the instability of RNA, the devices (holders, combs, gel tank) should be cleaned with RNase AWAY prior use.
  - RNA 凝胶的制备类似于 DNA 凝胶（1% 琼脂糖），但是，由于 RNA 的不稳定性，设备（支架、梳子、凝胶槽）在使用前应使用 RNase AWAY 清洗。
- The running buffer (1 x TAE) is separate for RNA-gels (do not use same running buffer as for DNA gels).
  - RNA 凝胶使用单独的电泳缓冲液（1 x TAE）（不要使用与 DNA 凝胶相同的电泳缓冲液）。

- Prepare the gel as mentioned above with 1X TAE electrophoresis buffer diluted with DEPC H2O; do not use this buffer again.
    - 如上所述，使用经 DEPC 水稀释的 1X TAE 电泳缓冲液制备凝胶；不要再次使用此缓冲液。

> 💡 **注释:** DEPC 水处理可灭活水中的 RNase。

- Add loading dye to your samples prior loading them on the gel.
    - 在上样前向样品中加入上样染料。
- Add the normal DNA ladder.
    - 加入常规 DNA Ladder。
- Running conditions and visualization are identical as for DNA gels as indicated above.
    - 运行条件和可视化与上述 DNA 凝胶相同。

Restriction enzyme digestion

限制性内切酶消化（酶切）

A full list of restriction enzymes available in the PLT lab can be found in “Documents - BIOSYST - Van de Poel Lab/PPL-Lists/Restricton enzymes PPL.xlsx”
PLT 实验室中可用的限制性内切酶的完整列表可以在“Documents - BIOSYST - Van de Poel Lab/PPL-Lists/Restricton enzymes PPL.xlsx”中找到。

A typical restriction digest using ThermoFisherScientific restriction enzymes:
使用 ThermoFisherScientific 限制性内切酶的典型酶切反应：

Component	**Amount**
dH2O	Up to 20 µL
10X Buffer	2 µL (1X)
DNA	Up to 1 µg
Restriction Enzyme (10 units)	1 µL each

Mix gently and gently spin down for a few seconds. Note: the restriction enzyme should be the last added to the reaction and always kept on ice.
轻柔混合并短暂离心几秒钟。注意：限制性内切酶应该是最后添加到反应中的，并且始终保持在冰上。

- If you use several enzymes at once, the amount of enzymes should not exceed 1/10 of the total reaction volume.
    - 如果同时使用几种酶，酶的量不应超过总反应体积的 1/10。

> 💡 **注释:** 酶储存在甘油中，过多的甘油（>10%）会抑制酶活性或导致“星号活性”（Star activity，即非特异性切割）。

- If you use several enzymes at once, check for their efficiency in the diverse buffers (FD/B/G/O/R/Tango; Reaction Conditions for Restriction Enzymes | Thermo Fisher Scientific - BE).
    - 如果同时使用几种酶，请检查它们在不同缓冲液 (FD/B/G/O/R/Tango) 中的效率（参考 Thermo Fisher Scientific - BE 的限制性内切酶反应条件）。
- Incubation time can be 1-16 hours, typically 1-4 hours, but can be increased up to 16 hours with reduced units.
    - 孵育时间可以是 1-16 小时，通常为 1-4 小时，但如果减少酶单位，可以增加到 16 小时。
    - o Longer incubation times are needed when buffer compatibility is not optimal.
        - o 当缓冲液兼容性不是最佳时，需要更长的孵育时间。
- **Incubation temperature** is enzyme-dependent, typically at 37 °C.
    - **孵育温度**取决于酶，通常为 37 °C。
- **Stopping the digestion** reaction might be done by heat inactivation (65 °C or 80 °C for 20 minutes, depending on the enzyme).
    - **终止消化**反应可以通过热灭活（65 °C 或 80 °C 处理 20 分钟，具体取决于酶）来完成。

Ligation

连接

T4 ligation

T4 连接

- Set up the following reaction in a pcr-tube on ice
    - 在冰上的 PCR 管中设置以下反应

Component	**20 µL Reaction**
T4 DNA Ligase Buffer (10X)*	2 µL
Vector DNA (50 ng)	Use NEBioCalculator to calculate molar ratios
Insert DNA	Use NEBioCalculator to calculate molar ratios
Nuclease-free water	to 20 µL
T4 DNA Ligase	1 µL

Note: You can easily scale down the reaction to 10 uL.
注意：你可以轻松地将反应缩小到 10 uL。

T4 DNA Ligase should be added last.
T4 DNA 连接酶应最后加入。
T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.
T4 DNA 连接酶缓冲液应在室温下解冻并重悬。

> 💡 **注释:** 缓冲液中含有 ATP，为连接反应提供能量。ATP 遇冷易沉淀，需充分混匀，否则连接效率极低。

It is recommended to start with 3:1 insert:vector ratio and if unsuccessful to repeat with 1:1 and 5:1.
建议从 3:1 的 插入片段:载体 比例开始，如果不成功，则重复使用 1:1 和 5:1。

- Gently mix the reaction by pipetting up and down and briefly spin down.
  - 通过上下吹打轻柔混合反应液并短暂离心。
- Incubate in a PCR machine:
  - 在 PCR 仪中孵育：
  - o For cohesive (sticky) ends, incubate at 16 °C overnight or room temperature for 10 minutes.
    - o 对于粘性末端，在 16 °C 过夜孵育或在室温下孵育 10 分钟。

    > 💡 **注释:** 16°C 是连接酶活性和氢键稳定性之间的平衡点。室温下酶活性高但氢键不稳定，低温下氢键稳定但酶活性低。
  - o For blunt ends or single base overhangs, incubate at 16 °C overnight or room temperature for 2 hours. (high concentration T4 DNA Ligase can be used in a 10 minute ligation).
    - o 对于平末端或单碱基突出端，在 16 °C 过夜孵育或在室温下孵育 2 小时。（高浓度 T4 DNA 连接酶可用于 10 分钟连接）。
  - o Alternatively incubate at room temperature for 2 hours, followed by incubation at 16°C overnight.
    - o 或者在室温下孵育 2 小时，然后在 16°C 过夜孵育。
- Heat-inactivate at 65 °C for 10 minutes.
  - 在 65 °C 下热灭活 10 分钟。
- Chill on ice and transform 1-5 µl of the reaction into 50 µL competent cells.
  - 在冰上冷却并将 1-5 µl 反应液转化到 50 µL 感受态细胞中。

Restriction-based cloning

基于酶切的克隆

This is a classical method by combining the unique cut-sites of two different restriction enzymes to clone in a DNA fragment in the vector of choice. The RE cut-sites for a PCR product are typically added to the primers (extra base pairs after the template specific primer part).
这是一种经典方法，通过结合两种不同限制性内切酶的独特切割位点，将 DNA 片段克隆到选定的载体中。PCR 产物的酶切位点通常添加到引物中（位于模板特异性引物部分之后的额外碱基对）。

- Choose your backbone and insert so that they have compatible RE cut sites for restriction enzymes that allow your insert to be placed into the backbone in the proper orientation.
  - 选择你的骨架和插入片段，使它们具有兼容的限制性内切酶切割位点，从而允许你的插入片段以正确的方向放入骨架中。
- Note: take into account the orientation of the insert in your vector.
  - 注意：考虑载体中插入片段的方向。

- Depending on the restriction sites available in the backbone, add the corresponding sites to the start and end of the insert through PCR.
  - 根据骨架中可用的限制性位点，通过 PCR 将相应的位点添加到插入片段的起始和末端。
- Note: make sure these restriction sites are unique to the sites you need, avoid internal cut sites in both the vector and insert. In the case the RE can cut in your template, either use other RE or perform site directed mutagenesis.
  - 注意：确保这些限制性位点是你所需的唯一位点，避免载体和插入片段内部出现切割位点。如果酶在模板内部有切点，请使用其他酶或进行定点突变。
- Digest both backbone and insert with the correct RE(s) in separate reactions (see restriction digest).
  - 在单独的反应中用正确的限制性内切酶消化骨架和插入片段（见酶切消化）。
  - o The restricted insert can be isolated by using a PCR purification kit (see PCR clean-up).
    - o 酶切后的插入片段可以使用 PCR 纯化试剂盒分离（见 PCR 纯化）。
  - o The restricted vector backbone will have to be gel extracted, therefore it is recommended to use 1 µg of DNA template for its digestion.
    - o 酶切后的载体骨架必须进行胶回收，因此建议使用 1 µg DNA 模板进行消化。

Note: If you are going to use only one restriction enzyme for both ends, or enzymes that have compatible overhangs or no overhangs after digestion, you will need to use a phosphatase to prevent re-circularization of the backbone plasmid. Therefore, treat the vector with a phosphatase after RE digestion and prior to the ligation step (or prior to the gel purification step). CIP (calf alkaline phosphatase) or SAP (shrimp alkaline phosphatase) are commonly used phosphatases.
注意：如果你打算在两端只使用一种限制性内切酶，或者使用消化后具有兼容突出端或无突出端的酶，你需要使用磷酸酶来防止骨架质粒的自环化。因此，在酶切消化后和连接步骤之前（或胶纯化步骤之前），用磷酸酶处理载体。CIP（小牛碱性磷酸酶）或 SAP（虾碱性磷酸酶）是常用的磷酸酶。

> 💡 **注释:** 连接酶需要 5' 磷酸基团才能工作。磷酸酶去除载体末端的 5' 磷酸，使其无法自我连接（自连），只能与带有 5' 磷酸的插入片段（未处理过）连接。

- Ligate the two fragments with T4 DNA ligase (see ligation).
  - 用 T4 DNA 连接酶连接这两个片段（见连接）。
- After transforming and miniprepping the plasmid, use sequencing primers that cover the backbone and insert to confirm correctness of the orientation of the insert.
  - 转化并小量制备质粒后，使用覆盖骨架和插入片段的测序引物来确认插入片段方向的正确性。

Gateway cloning

Gateway 克隆

Official protocol for multiple Gateway cloning:
多重 Gateway 克隆的官方方案：
https://www.thermofisher.com/be/en/home/life-science/cloning/gateway-cloning.html

The protocol is described for cloning a promotor and gene of interest using two entry vectors: pDONRP4P1 for the promotor and pDONR221/pDONR207 for the coding sequence (CDS).
该方案描述了使用两个入门载体克隆启动子和感兴趣基因：**pDONRP4P1** 用于启动子，**pDONR221/pDONR207** 用于编码序列 (CDS)。

Primer design for cloning of promoter in pDONRP4P1:
在 pDONRP4P1 中克隆启动子的引物设计：

- Select a promoter region about around 3 Kb.
  - 选择约 3 Kb 的启动子区域。
- For the design of the primers, select DNA-specific sequences of 18-25 bp each and add Gateway-specific sequences.
  - 对于引物设计，选择各 18-25 bp 的 DNA 特异性序列并添加 Gateway 特异性序列。
- For promoter cloning, the attB4/B1r sites are compatible with pDONRP4-P1R.
  - 对于启动子克隆，attB4/B1r 位点与 pDONRP4-P1R 兼容。
- Gateway attachment sites plus 18-25 bp sequencing-specific (indicated with "X").
  - Gateway 附着位点加上 18-25 bp 测序特异性序列（用"X"表示）。

Primer	**Sequence**
attB4-Promoter-F	GGGGACAACTTTGTATAGAAAAGTTGxxxxxxxxxxxxxxxxxxxx
attB1r-Promoter-R	GGGGACTGCTTTTTTGTACAAACTTGxxxxxxxxxxxxxxxxxxxx

Note: the reverse primer should stop right before the ATG start site of the CDS.
注意：反向引物应在 CDS 的 ATG 起始位点之前停止。

Note: in some cases, there are regulatory elements in the first intron of the gene, and in this case, you include the first intron in the sequence of the promotor. Make sure the start site is non-functional, so that the actual start site is in the fusion protein.
注意：在某些情况下，基因的第一个内含子中存在调控元件，在这种情况下，你要将第一个内含子包含在启动子序列中。确保该起始位点无功能，以便实际起始位点位于融合蛋白中。

Primer design for cloning of coding sequences in pDONR221 or pDONR207:
在 pDONR221 或 pDONR207 中克隆编码序列的引物设计：

- Use cDNA (and not gDNA) for PCR-amplifying the gene of interest.
  - 使用 cDNA（而不是 gDNA）作为 PCR 扩增感兴趣基因的模板。
- For cloning a gene of interest via the Gateway cloning strategy, design primers consisting of 18-25 bp gene-specific sequences and the Gateway attachment sites.
  - 通过 Gateway 克隆策略克隆感兴趣的基因，设计由 18-25 bp 基因特异性序列和 Gateway 附着位点组成的引物。
- The attB1/2 sites are compatible with pDONR221 or pDONR207 as entry vector.
  - attB1/2 位点与作为入门载体的 pDONR221 或 pDONR207 兼容。
- For the gene-specific sequences:
  - 对于基因特异性序列：
- The forward primer starts with the start codon (ATG) of the CDS.
  - 正向引物从 CDS 的起始密码子 (ATG) 开始。
- For the reverse primer, it depends if you want to add a C-terminal tag (e.g. fluorescent marker like GFP).
  - 对于反向引物，这取决于你是否要添加 C 端标签（例如荧光标记，如 GFP）。
  - ▪ If you have no tag at the end of the gene: you include the stop codon in the sequence-specific part of your primer.
    - ▪ 如果你在基因末端**没有**标签：在引物的序列特异性部分中**包含**终止密码子。

- ▪ If you want to add a C-terminal tag, then do not include the stop codon in the sequence-specific part of your primer, and make sure the coding sequence is in frame with the C-terminal tag in the destination vector.
  - ▪ 如果你想**添加** C 端标签，则**不要**在引物的序列特异性部分中包含终止密码子，并确保编码序列与目的载体中的 C 端标签读码框一致。

Primer	Sequence
attB1-CDS-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgxxxxxxxxxxxx
attB2-CDS-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCxxxxxxxxxxxxxx

Before starting:
开始之前：

- For the BP and LR reactions, half the enzyme volume of the official protocol is sufficient (4 µL instead of 8 µL).
  - 对于 BP 和 LR 反应，官方方案的一半酶体积就足够了（4 µL 代替 8 µL）。
- Aliquot the BP and LR clonase enzyme mix and avoid freezing and thawing; prepare aliquots of about 2 µL, and store at -80 °C as this increases the lifespan of the enzymes.
  - 分装 BP 和 LR 克隆酶混合物，避免反复冻融；准备约 2 µL 的分装，并储存在 -80 °C，这可以增加酶的寿命。
- Treat the BP and LR clonase enzyme mix gently; do not vortex!
  - 轻柔对待 BP 和 LR 克隆酶混合物；**不要涡旋**！

BP reaction (Gateway™ BP Clonase™ II Enzyme mix):
BP 反应 (Gateway™ BP Clonase™ II Enzyme mix):
(功能：将 PCR 产物重组入入门载体 pDONR)

- Put 150 ng entry vector (e.g. pDONR221 or pDONR227 or pDONRP4P1) in a PCR tube.
  - 将 150 ng 入门载体（例如 pDONR221 或 pDONR227 或 pDONRP4P1）放入 PCR 管中。
- Add attB-PCR product (40-100 fmol).
  - 加入 attB-PCR 产物 (40-100 fmol)。
  - ▪ Optional use a pDEST with GOI already including attB-sites in the same amount as the pDONR.
    - ▪ 可选使用已包含 attB 位点的 GOI（感兴趣基因）的 pDEST，其量与 pDONR 相同。
- Add 2 µL TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
  - 加入 2 µL TE 缓冲液 pH 8.0。
  - ▪ Use at least 2 µL TE buffer; fill up to 4 µL with TE buffer if needed.
    - ▪ 至少使用 2 µL TE 缓冲液；如果需要，用 TE 缓冲液补足至 4 µL。
- Add 0.5-1 µL BP clonase enzyme.
  - 加入 0.5-1 µL BP 克隆酶。
  - ▪ for small inserts like oligo cloning, 0.5 µL BP clonase is sufficient; for longer sequences like promoter regions or coding sequences use 1 µL.
    - ▪ 对于像寡核苷酸克隆这样的小插入片段，0.5 µL BP 克隆酶就足够了；对于像启动子区域或编码序列这样的较长序列，使用 1 µL。
  - ▪ Add BP clonase gently (do not vortex), flick against the tube and then gently spin down the mixture quickly.
    - ▪ 轻轻加入 BP 克隆酶（不要涡旋），轻弹试管，然后迅速轻柔离心混合物。

- Incubate mixture at 25 °C for at least 1 h (preferably overnight) in a PCR machine.
  - 在 PCR 仪中于 25 °C 孵育混合物至少 1 小时（最好过夜）。
  - ▪ Reaction mixture can be also kept at room temperature (instead of 25 °C), unless the room temperature is unusually high or low (winter/summer).
    - ▪ 反应混合物也可以保持在室温（代替 25 °C），除非室温异常高或低（冬天/夏天）。
- Optional: Add 0.5 µL of Proteinase K solution to terminate the reaction; incubate for 10 min at 37°C. *works also without proteinase K treatment.
  - 可选：加入 0.5 µL 蛋白酶 K 溶液以终止反应；在 37°C 孵育 10 分钟。*没有蛋白酶 K 处理也可以。

For calculating the fmol PCR-product needed for the BP reaction, use the following formula:
计算 BP 反应所需的 fmol PCR 产物，使用以下公式：

$$ng = \frac{fmol \times N \times 660\ \text{fg}}{\text{fmol}} \times \frac{1\ \text{ng}}{10^6\ \text{fg}}$$

- N: size of DNA in bp (DNA 的大小，单位 bp)

For further details see: (Link in original)
更多详情请见：（原文中有链接）

LR reaction (Gateway™ LR Clonase™ II Enzyme mix):
LR 反应 (Gateway™ LR Clonase™ II Enzyme mix):
(功能：将入门克隆中的片段重组入目的载体 pDEST)

- Put 150 ng destination vector in a PCR tube.
  - 将 150 ng 目的载体放入 PCR 管中。
- Add 75 ng entry vector.
  - 加入 75 ng 入门载体。
- Add 2 µL TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
  - 加入 2 µL TE 缓冲液 pH 8.0。
  - ▪ Use at least 2 µL TE buffer; fill up to 4 µL with TE buffer if needed.
    - ▪ 至少使用 2 µL TE 缓冲液；如果需要，用 TE 缓冲液补足至 4 µL。
- Add 0.5-1 µL LR clonase.
  - 加入 0.5-1 µL LR 克隆酶。
  - ▪ for small inserts like oligo cloning, 0.5 µL LR clonase is sufficient; for longer sequences like promoter regions or coding sequences use 1 µL.
    - ▪ 对于小插入片段，0.5 µL LR 克隆酶足够；对于较长序列，使用 1 µL。
  - ▪ Add LP clonase gently (do not vortex), flick against the tube and then gently spin down the mixture quickly.
    - ▪ 轻轻加入 LR 克隆酶（不要涡旋），轻弹试管，然后迅速轻柔离心。
- Incubate mixture at 25°C for at least 1 h (preferably overnight) in a PCR machine.
  - 在 PCR 仪中于 25°C 孵育至少 1 小时（最好过夜）。
  - ▪ Reaction mixture can be also kept at room temperature (instead of 25 °C), unless the room temperature is unusually high or low (winter/summer).
    - ▪ 反应混合物也可以保持在室温（代替 25 °C），除非室温异常高或低（冬天/夏天）。
- Optional: Add 0.5 µL of Proteinase K solution to terminate the reaction; incubate for 10 min at 37°C. *works also without proteinase K treatment.
  - 可选：加入 0.5 µL 蛋白酶 K 溶液终止反应；37°C 孵育 10 分钟。*没有蛋白酶 K 处理也可以。

For both, BP and LR reactions: use 2 µL for transformation into chemically competent E. coli cells.
对于 BP 和 LR 反应：使用 2 µL 转化到大肠杆菌化学感受态细胞中。

Note: For multiple Gateway cloning:
注意：对于多重 Gateway 克隆：

- LR reaction that combines multiple entry vectors into one destination vector needs to be done overnight.
  - 将多个入门载体组合到一个目的载体中的 LR 反应需要过夜进行。
- In this case, commercially produced competent E. coli cells are preferential.
  - 在这种情况下，商业生产的感受态大肠杆菌细胞是首选。

GreenGate cloning

GreenGate 克隆

GreenGate cloning is based on *BsaI* mediated golden gate cloning, but adapted with a suite of plasmids for use in plants. The strategy consists of first making 6 entry plasmids (A – F modules, corresponding to promoter, N-terminal tag, CDS, C-terminal tag, terminator and a plant resistance gene) using *BsaI* cloning.
GreenGate 克隆基于 *BsaI* 介导的 Golden Gate 克隆，但通过一套质粒进行了调整以用于植物。该策略包括首先使用 *BsaI* 克隆制作 6 个入门质粒（A – F 模块，分别对应启动子、N 端标签、CDS、C 端标签、终止子和植物抗性基因）。

These entry modules are designed to give complementary overhangs to the neighboring plasmid in the scheme, so that they can only be ligated in the correct order A – F in the destination vector.
这些入门模块被设计为给方案中的相邻质粒提供互补的突出端，因此它们只能以正确的顺序 A – F 连接到目的载体中。

Then, these 6 plasmids and the destination plasmid are taken to a single tube Greengate reaction, where they are digested with *BsaI* and ligated with T4 DNA ligase. The standard GreenGate protocol results in a single expression casette, but it is also possible to have multiple expression casettes.
然后，将这 6 个质粒和目的质粒放入单管 Greengate 反应中，在那里它们被 *BsaI* 消化并用 T4 DNA 连接酶连接。标准的 GreenGate 方案产生单个表达盒，但也可以拥有多个表达盒。

The benefits of Greengate are that it's modular, scar-free and easy to make single trascriptional units. The downside is that it's not ideal if the regions you are coding have internal *BsaI* sites, as ideally this would require mutagenesis to remove them before use.
GreenGate 的优点是模块化、无痕且易于制作单个转录单元。缺点是如果你编码的区域有内部 *BsaI* 位点，这就不太理想，因为理想情况下这需要在使用前通过诱变去除它们。

We have the original GreenGate kit (Addgene kit 1000000036; https://www.addgene.org/kits/lohmann-greengate/; Lampropoulos A, Sutikovic Z, Wenzl C, Maegele I, Lohmann JU, et al. (2013) GreenGate - A Novel, Versatile, and Efficient Cloning System for Plant Transgenesis. PLOS ONE 8(12): e83043. https://doi.org/10.1371/journal.pone.0083043). The full list of plasmids is available from Addgene or the shared –80 excel sheet. We also have added GFP in the B module.
我们拥有原始的 GreenGate 试剂盒 (Addgene kit 1000000036; 链接同上; 参考文献同上)。质粒的完整列表可从 Addgene 或共享的 –80 excel 表中获得。我们还在 B 模块中添加了 GFP。

GreenGate vector design and layout. The GreenGate system six modules represent the plant promoter (1), an N-terminal tag (2), the coding sequence of the gene of interest (3), a C-terminal tag (4), the plant terminator (5) and the plant resistance cassette (6).
GreenGate 载体设计和布局。 GreenGate 系统的六个模块代表植物启动子 (1)、N 端标签 (2)、感兴趣基因的编码序列 (3)、C 端标签 (4)、植物终止子 (5) 和植物抗性盒 (6)。

- **GreenGate entry vector (入门载体)**
  - The GreenGate cloning system uses six different types of pUC19 based entry vectors into which the individual elements are inserted.
    - GreenGate 克隆系统使用六种不同类型的基于 pUC19 的入门载体，单个元件被插入其中。
  - The multiple cloning site of pUC19 has been replaced by two *BsaI* recognition sites (magenta scissors), the respective overhangs for each module type, and a counter-selectable *ccdB* gene. DNA fragments can be cloned via the specific overhangs, via the BamHI and KpnI sites or via A-overhangs after XcmI digestion.
    - pUC19 的多克隆位点已被两个 *BsaI* 识别位点（品红色剪刀）、每种模块类型各自的突出端以及一个反向筛选的 *ccdB* 基因所取代。DNA 片段可以通过特定的突出端、BamHI 和 KpnI 位点或 XcmI 消化后的 A 突出端进行克隆。

> 💡 **注释:** ccdB 基因编码一种毒性蛋白。如果在克隆过程中未被插入片段替换，含有该基因的载体将杀死大肠杆菌。这保证了长出的菌落都是成功插入了片段的阳性克隆。

- **GreenGate destination vector (目的载体)**
  - The GreenGate cloning system uses a pGreen-IIS based destination vector.
    - GreenGate 克隆系统使用基于 pGreen-IIS 的目的载体。
  - The empty destination vector consists of a counter-selectable *ccdB*-cassette between the LB and RB sequences of pGreen-IIS, flanked by *BsaI* sites, with overhangs A and G.
    - 空的目的载体由 pGreen-IIS 的 LB 和 RB 序列之间的反向筛选 *ccdB* 盒组成，两侧是 *BsaI* 位点，具有 A 和 G 突出端。
  - Note: The pSa origin of replication (ori A. tum.) requires the presence of the helper plasmid pSOUP in agrobacteria.
    - 注意：pSa 复制起始位点 (ori A. tum.) 需要农杆菌中辅助质粒 pSOUP 的存在。

Making entry modules:
制作入门模块：

PCR is used to amplify the region of interest and add appropriate *BsaI* sites for cloning. The following sequences are added in front of the gene specific region of the F primer, and in front of the reverse complement of the gene specific region for the R primer.
PCR 用于扩增感兴趣的区域并添加适当的 *BsaI* 位点进行克隆。以下序列被添加到 F 引物的基因特异性区域之前，以及 R 引物的基因特异性区域的反向互补序列之前。

F: `5' AACAGGTCTCANNNN (nn) - Gene specific region -3'`
R: `5'-AACAGGTCTCANNNN – Gene specific region reverse complement 3'`

- `GGTCTC` is the *BsaI* recognition site.
  - `GGTCTC` 是 *BsaI* 识别位点。

- `AACA` was added because the enzyme does not cut if the restriction site is at the extreme ends of PCR products.
  - 添加 `AACA` 是因为如果限制性位点位于 PCR 产物的极末端，酶不会切割（保护碱基）。
- `NNNN` represents the module specific overhang with the coding triplet being underlined.
  - `NNNN` 代表模块特定的突出端，编码三联体带有下划线。
- and 2 nucleotides (nn) are needed in case of the coding sequence and C-tag modules to bring the modules into frame.
  - 对于编码序列和 C 标签模块，需要 2 个核苷酸 (nn) 使模块进入读码框。

PCR is conducted using standard protocols for high fidelity polymerase, and PCR product purified by gel extraction.
使用高保真聚合酶的标准方案进行 PCR，PCR 产物通过胶回收纯化。
Both PCR product and the corresponding entry module is digested with *BsaI*, and purified (PCR purification kit for PCR product, preferably gel extraction for entry module).
PCR 产物和相应的入门模块均用 *BsaI* 消化并纯化（PCR 产物用 PCR 纯化试剂盒，入门模块最好用胶回收）。
Then the digested entry PCR product and the corresponding entry vectors are ligated.
然后将消化的入门 PCR 产物和相应的入门载体连接。
The resulting entry vectors are then taken to the GreenGate reaction.
得到的入门载体随后用于 GreenGate 反应。

Greengate reaction:
Greengate 反应：

Set up the following in PCR tubes:
在 PCR 管中设置以下内容：

- 1.5 µl each entry vector 100 ng/µl (每个入门载体 1.5 µl)
- 1 µl destination plasmid 100 ng/µl (目的质粒 1 µl)
- 1.5 µl cutsmart buffer
- 1.5 µl ATP 10 mM
- 1 µl T4 DNA ligase
- 1 µl *BsaI*-HF
- To 15 µl with H2O

Cycle at 37 C for 2 mins and 16 C for 2 mins for 30 cycles, then 80 C for 5 mins. Transform 5 ul of the reaction into appropriate cells.
循环：37°C 2 分钟，16°C 2 分钟，循环 30 次，然后 80°C 5 分钟。将 5 ul 反应液转化到适当的细胞中。

> 💡 **注释:** 37°C 是 *BsaI* 酶切的最佳温度，16°C 是 T4 连接酶的最佳温度。循环进行可以不断切割-连接。Golden Gate 的特点是产物不再包含酶切位点，因此循环反应会使反应向产物方向进行。

Multiple transcriptional units

多转录单元

Two transcriptional units
两个转录单元

For two transcriptional units, two intermediate vectors, pGGM000 and pGGN000, and two adapter molecules are used.

对于两个转录单元，使用两个中间载体 pGGM000 和 pGGN000 以及两个接头分子。
The final construct is created in two steps: First, the two transcriptional units are assembled separately in two parallel reactions into the intermediate vectors.
最终构建体分两步创建：首先，两个转录单元在两个平行反应中分别组装到中间载体中。
The first of these two supermodules consists of a plant promoter, an N-terminal tag, a coding sequence, a C-terminal tag and a plant terminator.
这两个超级模块中的第一个由植物启动子、N 端标签、编码序列、C 端标签和植物终止子组成。
Furthermore, a pre-cloned adapter module with F and H overhangs is added to the reaction.
此外，一个预克隆的具有 F 和 H 突出端的接头模块被添加到反应中。
The intermediate vector has *BsaI* recognition sites remaining in the vector backbone with matching A and H overhangs and confers resistance to kanamycin.
中间载体在载体骨架中保留了 *BsaI* 识别位点，具有匹配的 A 和 H 突出端，并赋予卡那霉素抗性。
The elements are combined in a GreenGate reaction but because two *BsaI* sites remain in the final construct, an additional ligation reaction is performed before transformation.
元件在 GreenGate 反应中组合，但由于最终构建体中保留了两个 *BsaI* 位点，因此在转化前需要进行额外的连接反应。
The second supermodule is built similarly, but the adapter molecule has H and A overhangs and at the 39-end a plant resistance cassette is added.
第二个超级模块的构建类似，但接头分子具有 H 和 A 突出端，并在 39 端添加了植物抗性盒。
Thus, the resulting plasmid will have H and G overhangs.
因此，产生的质粒将具有 H 和 G 突出端。
The two supermodules are then combined with the destination vector in a second step in a standard GreenGate reaction yielding the final construct.
然后在第二步的标准 GreenGate 反应中，将这两个超级模块与目的载体结合，产生最终的构建体。

More than two transcriptional units
超过两个转录单元

An additional module that is a synthetic DNA duplex with unpaired ends, allows ligation to H and G overhangs during a GreenGate reaction to make it possible to have two transcriptional units.
一个额外的模块（具有未配对末端的合成 DNA 双链体）允许在 GreenGate 反应期间连接到 H 和 G 突出端，从而使拥有两个转录单元成为可能。
It contains two internal *BsaI* recognition sites, which cannot be cut by *BsaI* due to overlapping cytosine methylation.
它包含两个内部 *BsaI* 识别位点，由于重叠的胞嘧啶甲基化，这些位点不能被 *BsaI* 切割。
Thus, the element will behave as an ordinary H to G module in the first GreenGate reaction and replace the resistance cassette.
因此，该元件在第一次 GreenGate 反应中将表现为普通的 H 到 G 模块并替换抗性盒。
After transformation and miniprepping, this methylation will be lost as there are no dcm sites present in the oligo duplex.
在转化和小量制备后，这种甲基化将丢失，因为寡核苷酸双链体中不存在 dcm 位点。
In a second round of GG reaction, the two internal *BsaI* recognition sites are now targets for *BsaI*, giving rise to A and G overhangs, simulating an empty destination vector.
在第二轮 GG 反应中，这两个内部 *BsaI* 识别位点现在成为 *BsaI* 的靶点，产生 A 和 G 突出端，模拟一个空的目的载体。
Therefore, in each subsequent GreenGate round either the uncleavable oligo duplex can be used to prepare the plasmid for the insertion of another expression cassette or the plasmid can be finalized by adding a plant resistance cassette to the reaction.
因此，在随后的每一轮 GreenGate 中，可以使用不可切割的寡核苷酸双链体来准备质粒以插入另一个表达盒，或

者可以通过向反应中添加植物抗性盒来完成质粒。
In this approach, the transcriptional units are assembled serially, so it requires one additional step for each expression cassette to be added.
在这种方法中，转录单元是串行组装的，因此每添加一个表达盒都需要一个额外的步骤。
The FHadapter used along with the oligo duplex separates the individual expression units in the final construct and thus can be freely designed to act as a spacer of arbitrary length and sequence minimizing the risk of mutual interference of the expression behavior.
与寡核苷酸双链体一起使用的 FHadapter 分隔最终构建体中的各个表达单元，因此可以自由设计为任意长度和序列的间隔区，从而最大限度地降低表达行为相互干扰的风险。

Gibson cloning

Gibson 克隆

Gibson primer design

Gibson 引物设计

Protocol Samuel Miller Lab
Samuel Miller 实验室方案

Gibson assembly primers are broken down in two parts: primer sequence and overlap sequence. The primer sequence should be designed using traditional characteristics in mind (i.e. Tm° values, G/C ratio, and G/C anchors). The overlap sequence needs to have between 20 – 150 bp homology to insert or vector. A good recommended start is a primer of 60 bp in total length: 30 bp of vector and 30 bp of insert.
Gibson 组装引物分为两部分：引物序列和重叠序列。引物序列的设计应考虑传统特征（如 Tm 值、G/C 比和 G/C 锚点）。重叠序列需要与插入片段或载体具有 20 – 150 bp 的同源性。建议从总长度为 60 bp 的引物开始：30 bp 的载体序列和 30 bp 的插入序列。

- A: 5′ – (30 bp vector/ 30 bp insert) – 3′ and D: 5′ – (30 bp insert/ 30 bp vector) – 3′
- C: 3′ – (30 bp vector/ 30 bp insert) – 5′ and B: 3′ – (30 bp insert/ 30 bp vector) – 5′
- PCR-amplify (see section PCR from DNA) the vector and insert using the right primer combination (A + B for insert and C + D for vector).
    - 使用正确的引物组合（A + B 用于插入片段，C + D 用于载体）PCR 扩增（见 DNA PCR 章节）载体和插入片段。
- Note: If amplifying vector, digest with *DpnI* prior to Gibson assembly to remove parental vector.
    - 注意：如果扩增载体，请在 Gibson 组装前用 *DpnI* 消化以去除非突变的亲本载体。

    > 💡 **注释:** PCR 产物是非甲基化的，而细菌提取的质粒（模板）是甲基化的。*DpnI* 酶特异性降解甲基化 DNA，从而消除背景质粒。

- Note: PCR or gel-clean-up is not required.
    - 注意：不需要 PCR 纯化或胶回收（只要条带单一）。

Gibson reaction

Gibson 反应

Reaction Stock Preparation
反应储备液制备

Prepare 5x ISO buffer: 6 mL of this buffer can be prepared by combining the following:
制备 5x ISO 缓冲液：通过混合以下成分可制备 6 mL 此缓冲液：

- 3 mL of 1 M Tris-HCl pH 7.5
- 150 µL of 2 M MgCl2
- 60 µL of 100 mM dGTP
- 60 µL of 100 mM dATP
- 60 µL of 100 mM dTTP
- 60 µL of 100 mM dCTP
- 300 µL of 1 M DTT
- 1.5 g PEG-8000
  > 💡 **注释:** PEG-8000 是分子拥挤剂，模拟细胞内环境，增加分子碰撞概率，提高酶促反应效率。
- 300 µL of 100 mM NAD
  > 💡 **注释:** NAD 是 Taq 连接酶的辅因子。
- Add water to 6 ml
  - 加水至 6 ml

Aliquot in 100 µL aliquots and store at -20 °C.
分装成 100 µL 一份，储存在 -20 °C。

Prepare master mixture. Combine the following:
制备母液。混合以下成分：

- 320 µL 5x ISO buffer
- 0.64 µL of 10 U/µL T5 exonuclease
  > 💡 **注释:** T5 外切酶负责从 5' 端回切 DNA，产生 3' 单链突出端用于退火。
- 20 µL of 2 U/µL Phusion polymerase
  > 💡 **注释:** Phusion 聚合酶负责填补退火后的空隙。
- 160 µL of 40 U/µL Taq ligase
  > 💡 **注释:** Taq 连接酶负责连接缺口（Nick），形成完整的环状 DNA。
- Add water to 1.2 ml
  - 加水至 1.2 ml

Aliquot in 15 µL aliquots and store at -20 °C.
分装成 15 µL 一份，储存在 -20 °C。
This assembly mixture can be stored at -20 °C for at least one year.
此组装混合物可在 -20 °C 保存至少一年。

Reaction Procedure
反应步骤

- Thaw a 15 µL assembly mixture aliquot and keep on ice until ready to be used.

    - 解冻一份 15 µL 组装混合物，并在使用前保持在冰上。
- Add 5 µL of PCR-amplified vector and insert DNA to be assembled to the master mixture.
    - 向母液中加入 5 µL PCR 扩增的载体和要组装的插入 DNA。
    - Note: The DNA should be in equimolar amounts.
        - 注意：DNA 应为等摩尔量。
    - Total DNA to be assembled should range between 20- 200 ng.
        - 组装的总 DNA 量应在 20-200 ng 之间。
    - For large DNA segments (> 150 kB): increased DNA amount should be used (e.g. 250 ng of each 150 kb DNA segment).
        - 对于大 DNA 片段 (> 150 kB)：应使用增加的 DNA 量（例如每个 150 kb DNA 片段使用 250 ng）。
- Thoroughly mix gently.
    - 轻柔地充分混合。
- Incubate at 50 °C for 15 to 60 min.
    - 在 50 °C 孵育 15 到 60 分钟。
- If cloning is desired, transform 1 – 5 µL to competent E. coli cell.
    - 如果需要克隆，将 1 – 5 µL 转化到大肠杆菌感受态细胞中。
    - Note: Dialyze prior to electroporation. Heat-shock transformation can be done right so.
        - 注意：电穿孔前需要透析（去除盐分）。热激转化可以直接进行。

Gibson limitations and obstacles
Gibson 的局限性和障碍

A major limitation (especially in high GC-rich templates such as in *P. aeruginosa*) to Gibson assembly is that the termini of the DNA sequence fragments to be assembled, should not have stable single stranded DNA secondary structure, such as a hairpin or a stem loop, or repeated sequences, as this would directly compete with the required single-stranded annealing/priming of neighboring assembly fragments.
Gibson 组装的一个主要限制（特别是在高 GC 含量的模板中，如铜绿假单胞菌）是，待组装的 DNA 序列片段的末端不应具有稳定的单链 DNA 二级结构，如发夹或茎环，或重复序列，因为这将直接与相邻组装片段所需的单链退火/引发竞争。
So make sure to run your sequences in Scitools to look for such nuisance areas (Delta G for hairpins should be more than -10 and with a lower annealing temperature than that of primer:primer duplex).
因此，请确保在 Scitools 中运行您的序列以查找此类麻烦区域（发夹的 Delta G 应大于 -10，且退火温度低于引物:引物双链体的退火温度）。

Site directed mutagenesis (SDM)

定点突变 (SDM)

Blunt end ligation-based SDM methods:
基于平末端连接的 SDM 方法：

SDM using blunt end ligation results in a blunt end PCR product, which is circularized by ligation. First, primers are designed to incorporate the desired mutation (substitution, deletion or insertion). The plasmid to mutagenize is used as a template for PCR, and the resulting blunt end PCR product is ligated.

使用平末端连接的 SDM 会产生平末端 PCR 产物，该产物通过连接环化。首先，设计引物以引入所需的突变（替换、缺失或插入）。将待诱变的质粒用作 PCR 模板，并将得到的平末端 PCR 产物进行连接。

Because the PCR product is missing the 5' phosphate, this needs to be added using a kinase. To avoid returning the original vector used as a template in the PCR, the sample should be digested using *DpnI*, which only cleaves methylated DNA and not the PCR product. Finally, the blunt end PCR product should be circularized using a ligase.
因为 PCR 产物缺少 5' 磷酸基团，所以需要使用激酶添加磷酸基团。为了避免 PCR 中用作模板的原始载体复原，样品应用 *DpnI* 消化，它只切割甲基化的 DNA 而不切割 PCR 产物。最后，平末端 PCR 产物应使用连接酶环化。

Independent enzymes SDM (独立酶法 SDM)

- o Perform PCR using a high-fidelity polymerase (Q5 or Phusion).
  - o 使用高保真聚合酶（Q5 或 Phusion）进行 PCR。
- o PCR clean PCR product.
  - o 纯化 PCR 产物。
- o Set up *DpnI* (Fast digest Thermo FD1703) digestion on PCR product:
  - o 对 PCR 产物设置 *DpnI* (Fast digest Thermo FD1703) 消化：

Component	**Amount**
DNA	Up to 1 µg
10X FastDigest buffer	2 µL
DpnI	1 µL
Nuclease free H2O	To 20 µL

- o Incubate 37 °C for 5 mins, inactivate at 80 C for 5 mins in a PCR machine.
  - o 在 PCR 仪中 37 °C 孵育 5 分钟，80 °C 灭活 5 分钟。
- o Clean digested product with a PCR clean up column.
  - o 用 PCR 纯化柱纯化消化后的产物。
- o Set up the T4 polynucleotide kinase (NEB M0201) reaction:
  - o 设置 T4 多核苷酸激酶 (NEB M0201) 反应：

Component	**Amount**
DNA	Up to 300 pmol of 5’ termini*
T4 PNK Reaction Buffer 10X	5 µL
ATP 10 mM	5 µL
T4 PNK	1 µL 10 units
Nuclease free H2O	To 50 µL

-
  - Use NEBioCalculator for calculating mass of DNA to add
    -
      - 使用 NEBioCalculator 计算需添加的 DNA 质量

- moles of dsDNA ends = mass of dsDNA (g)/((length of dsDNA (bp) x 615.96 g/mol/bp) + 36.04 g/mol) x 2

The T4 PNK buffer can be substituted for T4 DNA ligase buffer. The 1X T4 DNA ligase buffer contains 1 mM ATP, so ATP does not need to be added. In this case, the product does not need to be cleaned before ligation, but supplemental ATP should be added to the kinase reaction to a final concentration of 1 mM.
T4 PNK 缓冲液可以用 T4 DNA 连接酶缓冲液代替。1X T4 DNA 连接酶缓冲液含有 1 mM ATP，因此不需要额外添加 ATP。在这种情况下，产物在连接前不需要纯化，但应向激酶反应中补充 ATP 至终浓度为 1 mM。

- o Incubate at 37 °C for 30 minutes in PCR machine.
  - o 在 PCR 仪中 37 °C 孵育 30 分钟。
- o Inactivate the reaction at 65 °C for 20 minutes.
  - o 在 65 °C 下灭活反应 20 分钟。
- o Clean product with a PCR clean up column.
  - o 用 PCR 纯化柱纯化产物。
- o Set up T4 ligase (NEB M0202) ligation:
  - o 设置 T4 连接酶 (NEB M0202) 连接：

Component	Amount
DNA	1 µL of 50 ng
T4 Ligase Buffer 10X	2 µL
T4 Ligase	1 µL
Nuclease free H2O	To 20 µL

- o Incubate at 16 °C overnight or at room temperature for 10 minutes.
  - o 在 16 °C 过夜孵育或在室温下孵育 10 分钟。
  - ▪ The combination of 10 min at room temperature and 16 °C overnight is also possible.
    - ▪ 室温 10 分钟和 16 °C 过夜的组合也是可能的。
- o Heat inactivate at 65°C for 10 mins.
  - o 在 65°C 下热灭活 10 分钟。
- o Transform 1-5 µL into appropriate E. coli strain.
  - o 将 1-5 µL 转化到适当的大肠杆菌菌株中。

Q5 site-directed mutagenesis kit (E0554S)
Q5 定点突变试剂盒 (E0554S)

- o Primer should be designed with 5’ ends annealing back-to-back. It is recommended to use the NEB online design software (NEBaseChanger).
  - o 引物设计应使 5' 端背对背退火。建议使用 NEB 在线设计软件 (NEBaseChanger)。
- o Perform a PCR using a high-fidelity polymerase (Q5 or Phusion).
  - o 使用高保真聚合酶（Q5 或 Phusion）进行 PCR。

PCR MasterMix:

Component	25µL RXN	12.5µL RXN	Final conc.
Q5 Hot Start High-Fidelity 2X MasterMix	12.5µL	6.25µL	1X
10 µM Forward Primer	1.25µL	0.625µL	0.5 µM
10 µM Reverse Primer	1.25µL	0.625µL	0.5 µM
Template DNA (1-25 ng/µL)	1 µL	0.5µL	1-25 ng
Nulcease-free water	9µL	4.5µL	

Cycling conditions:

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec
25 Cycles		
(Denaturation)	98 °C	10 sec
(Annealing)	50-72 °C*	10-30 sec
(Extension)	72 °C	20-30 sec/kb
Final Extension	72 °C	2 min
Hold	4-10 °C	

- *For mutagenic primers, please use the Ta provided by the online NEB primer design software (NEBaseChanger).
    - *对于诱变引物，请使用 NEB 在线引物设计软件 (NEBaseChanger) 提供的 Ta（退火温度）。
- o Do a PCR clean-up using the kit.
    - o 使用试剂盒进行 PCR 纯化。
- o Set up the following KLD (Kinase, Ligase, DpnI) reaction on ice:
    - o 在冰上设置以下 KLD（激酶、连接酶、DpnI）反应：

Component	Amount
PCR product	1 µL
2X KLD reaction buffer	5 µL
10X KLD enzyme mix	1 µL
Nuclease free H2O	3 µL

- o Mix well by pipetting up and down and incubate at room temperature for 5 minutes.
    - o 通过上下吹打充分混合并在室温下孵育 5 分钟。
- o Transform 5 µL of KLD reaction to 50 µL appropriate competent cells.
    - o 将 5 µL KLD 反应液转化到 50 µL 适当的感受态细胞中。

Gibson-based SDM methods:
基于 Gibson 的 SDM 方法：

Gibson based SDM also requires designing primers incorporating the desired mutation, but the resulting PCR product should have complementary overlapping ends of 15-30 bp. Then an exonuclease chews back the 5' end, complementary regions anneal, DNA polymerase fills in the gaps within each annealed fragment, and ligase seals the nicks. The benefit of this approach is that it can also be used to assemble multiple fragments in one go.
基于 Gibson 的 SDM 也需要设计包含所需突变的引物，但得到的 PCR 产物应具有 15-30 bp 的互补重叠末端。然后外切酶回切 5' 端，互补区域退火，DNA 聚合酶填充每个退火片段内的空隙，连接酶封闭缺口。这种方法的优点是它也可以用于一次组装多个片段。

NEB HIFI (E2621)

- o Perform PCR using a high-fidelity polymerase (Q5 or Phusion)
  - o 使用高保真聚合酶（Q5 或 Phusion）进行 PCR
- o PCR clean the PCR product
  - o 纯化 PCR 产物
- o Set up the following reaction on ice:
  - o 在冰上设置以下反应：

Recommended Amount of Fragments Used for Assembly

Component	**2–3 Fragment Assembly***	**4–6 Fragment Assembly****
Recommended DNA Molar Ratio	vector:insert = 1:2	vector:insert = 1:1
Total Amount of Fragments	0.03–0.2 pmols* (X µL)	0.2–0.5 pmols** (X µL)
NEBuilder HiFi DNA Assembly Master Mix	10 µL	10 µL
Nuclease free H2O	10-X µL	10-X µL
Total Volume	**20 µL ††**	**20 µL ††**

-
  - Optimized cloning efficiency is 50–100 ng of vector with 2-fold excess of each insert. Use 5-fold molar excess of any insert(s) less than 200 bp. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20 %. To achieve optimal assembly efficiency, design 15-20 bp overlap regions between each fragment.
    -
      - 优化的克隆效率是用 50–100 ng 载体和 2 倍过量的每个插入片段。对于小于 200 bp 的任何插入片段，使用 5 倍摩尔过量。组装反应中未纯化 PCR 片段的总量不应超过 20%。为获得最佳组装效率，在每个片段之间设计 15-20 bp 的重叠区域。
- ** To achieve optimal assembly efficiency, design 20-30 bp overlap regions between each fragment with equimolarity of all fragments (suggested: 0.05 pmol each).
  - ** 为获得最佳组装效率，在每个片段之间设计 20-30 bp 的重叠区域，所有片段等摩尔（建议：每个 0.05 pmol）。
- † Control reagents are provided for 5 experiments.

    - † 提供的对照试剂可供 5 次实验使用。
- †† If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional NEBuilder HiFi DNA Assembly Master Mix.
    - †† 如果组装更多数量的片段，请增加反应体积，并使用额外的 NEBuilder HiFi DNA 组装母液。
- o Incubate samples in a thermocycler at 50 °C for 15 minutes (when 2 or 3 fragments are being assembled) or 60 minutes (when 4–6 fragments are being assembled).
    - o 在热循环仪中 50 °C 孵育样品 15 分钟（组装 2 或 3 个片段时）或 60 分钟（组装 4-6 个片段时）。
- o Store samples on ice or at –20 °C for subsequent transformation.
    - o 将样品储存在冰上或 –20 °C 以备后续转化。
- o Transform 2-5 uL of assembled product to appropriate cell strain.
    - o 将 2-5 uL 组装产物转化到适当的细胞菌株中。

Restriction enzyme-based SDM methods:
基于限制性内切酶的 SDM 方法：

Restriction enzyme based SDM makes use of type II restriction enzymes (we have *BsaI*, *AarI* and *BbsI*). These enzymes cut outside of their recognition site, meaning cloning can be scar-free.
基于限制性内切酶的 SDM 利用 II 型限制性内切酶（我们有 *BsaI*, *AarI* 和 *BbsI*）。这些酶在其识别位点之外切割，这意味着克隆可以是无痕的。
Primers can be designed to introduce the appropriate mutation with internal restriction sites. This can be designed so that the recognition sites are cut out, leaving complementary sticky ends which are ligated. The benefit of this method is that multiple products can be ligated together, but it is best if there are no internal sites for the restriction enzymes which are used.
可以设计引物以通过内部限制性位点引入适当的突变。设计时可确保识别位点被切除，留下互补的粘性末端进行连接。此方法的优点是可以将多个产物连接在一起，但最好是所用的限制性内切酶没有内部切点。

Type II restriction enzyme mutagenesis
II 型限制性内切酶诱变

- o Perform PCR using a high-fidelity polymerase (Q5 or Phusion). Set up the following reaction:
    - o 使用高保真聚合酶（Q5 或 Phusion）进行 PCR。设置以下反应：

Component	25µL RXN	12.5µL RXN	Final conc.
Q5 Hot Start High-Fidelity 2X MasterMix	12.5µL	6.25µL	1X
10 µM Forward Primer	1.25µL	0.625µL	0.5 µM
10 µM Reverse Primer	1.25µL	0.625µL	0.5 µM
Template DNA (1-25 ng/µL)	1 µL	0.5µL	1-25 ng
Nuclease-free water	9µL	4.5µL	

- o Cycling conditions:
    - o 循环条件：

Step	Temperature	Time
Initial Denaturation	98 °C	30 sec
25 Cycles		
(Denaturation)	98 °C	10 sec
(Annealing)	50-72 °C*	10-30 sec
(Extension)	72 °C	20-30 sec/kb
Final Extension	72 °C	2 min
Hold	4-10 °C	

- *For mutagenic primers, please use the Ta provided by the online NEB primer design software (NEBaseChanger)
    - *对于诱变引物，请使用 NEB 在线引物设计软件 (NEBaseChanger) 提供的 Ta
- o PCR clean the PCR product
    - o 纯化 PCR 产物
- o Digest PCR product with appropriate type II restriction enzyme and *DpnI* (to digest template DNA that is methylated). A typical restriction digest using ThermoFisherScientific restriction enzymes:
    - o 用适当的 II 型限制性内切酶和 *DpnI*（消化甲基化的模板 DNA）消化 PCR 产物。使用 ThermoFisherScientific 限制性内切酶的典型酶切反应：

Component	Amount
dH2O	Up to 20 µL
10X Buffer*	2 µL (1X)
DNA	Up to 1 µg
Restriction Enzyme (10 units)	1 µL each

- *If using Thermo *AarI*, be sure to add the 50X oligonucleotide mix as well as the buffer.
    - *如果使用 Thermo *AarI*，请务必添加 50X 寡核苷酸混合物以及缓冲液。
- o PCR clean PCR product
    - o 纯化 PCR 产物
- o Ligate using T4 DNA ligase
    - o 使用 T4 DNA 连接酶进行连接

Component	Amount
DNA	1 µL of 50 ng

Component	Amount
T4 Ligase Buffer 10X	2 µL
T4 Ligase	1 µL
Nuclease free H2O	To 20 µL

- o Transform 1 µL of ligation to appropriate cells.
  - o 将 1 µL 连接产物转化到适当的细胞中。

Escherichia coli transformation

大肠杆菌转化

Chemical transformation
化学转化

- Defrost chemical competent *E. coli* cells on ice.
  - 在冰上解冻化学感受态大肠杆菌细胞。
- Add 1-5 µL of DNA, mix by gently ticking the tube.
  - 加入 1-5 µL DNA，通过轻轻敲击试管混合。
  - o Do not vortex or pipet up and down!
    - o **不要涡旋或上下吹打！**

    > 💡 **注释:** 感受态细胞非常脆弱，剧烈震荡会破坏细胞。
  - o A lower volume of DNA will cause a lower dilution/contamination risk.
    - o 较小的 DNA 体积会降低稀释度/污染风险。
- Incubate 30 minutes on ice.
  - 在冰上孵育 30 分钟。
- Heat-shock cells for 45 seconds at 42 °C in a water bath, put back on ice.
  - 在 42 °C 水浴中热激细胞 45 秒，放回冰上。
  - o If you use bought competent cells, reduce incubation time to 30 seconds.
    - o 如果你使用购买的感受态细胞，将孵育时间减少到 30 秒。
- Add 950 µL LB medium.
  - 加入 950 µL LB 培养基。
  - o Alternatively, use SOC medium or low salt LB to increase efficiency.
    - o 或者，使用 SOC 培养基或低盐 LB 以提高效率。
- Incubate for 1 hour at 37 °C in a shaker.

- 在摇床中 37 °C 孵育 1 小时。

- Plate 100 µL of the cells on LB agar plates supplemented with the respective antibiotics.
  - 将 100 µL 细胞涂布在补充了相应抗生素的 LB 琼脂平板上。
- Centrifuge cells at 3,000 x g for 2 minutes, discard supernatant besides of about 100 µL.
  - 以 3,000 x g 离心细胞 2 分钟，除保留约 100 µL 外弃去上清液。

> 💡 **注释:** 此步骤用于浓缩细胞，以便在平板上接种更多菌量，适用于转化效率低的情况。

- Resuspend the pellet by gently vortexing or pipetting and plate left-over culture on LB agar plates supplemented with the respective antibiotics.
  - 通过轻轻涡旋或移液重悬沉淀，并将剩余的培养物涂布在补充了相应抗生素的 LB 琼脂平板上。
- Grow plates overnight at 37 °C.
  - 在 37 °C 下过夜培养平板。
- Check colonies the next day (they should not grow longer than max 24 h; these are false positives) and store plate at 4 °C.
  - 第二天检查菌落（生长不应超过 24 小时；长得太慢的可能是假阳性）并将平板储存在 4 °C。

> 💡 **注释:** 卫星菌落（Satellite colonies）通常在长时间培养后出现在主菌落周围，它们不含质粒，是依靠主菌落降解抗生素生存的。

Competent cells available in the lab:
实验室现有的感受态细胞：

- Depending on your plasmid of interest, choose between the following competent cells:
  - 根据你感兴趣的质粒，在以下感受态细胞中进行选择：
  - o **E. coli TOP10:** F- mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 ara 139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG
    - ▪ Similar to DH5a, streptomycin resistant, engineered for high transformation efficiency of high-copy number plasmids
      - ▪ 类似于 DH5a，对链霉素具有抗性，专为高拷贝数质粒的高转化效率而设计。
    - ▪ **NOT suitable for empty vectors containing the *ccdB* gene**
      - ▪ **不适用于含有 *ccdB* 基因的空载体。**

    > 💡 **注释:** Top10 对 CcdB 毒蛋白敏感。如果转化含有 ccdB 的空载体（如未重组的 Gateway 入门载体），细菌会死亡。
  - o **E. coli DB3.1:** gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20 glnV44 (=supE44) ara14 galK2 lacY1 proA2 rpsL20 xyl5 leuB6 mtl1
    - ▪ Streptomycin resistant
      - ▪ 链霉素抗性。
    - ▪ ***ccdB* resistant, so empty vectors can be transformed**

        - ▪ **对 *ccdB* 具有抗性，因此可以转化空载体。**

    > 💡 **注释:** DB3.1 含有突变的 gyrA 基因，使其对 CcdB 毒蛋白免疫。扩增带有 ccdB 基因的空载体时必须使用此菌株。

    - o **E. coli BL21:** fhuA2 [lon] ompT gal [dcm] ΔhsdS
        - ▪ Used for non-T7 expression
            - ▪ 用于非 T7 表达。
        - ▪ Suitable for transformation and protein expression
            - ▪ 适用于转化和蛋白质表达。

        > 💡 **注释:** BL21 缺乏蛋白酶（Lon 和 OmpT），适合表达外源蛋白，防止蛋白被降解。

Agrobacterium tumefaciens transformation

根癌农杆菌转化

Transformation of electrocompetent *A. tumefaciens* cells using electroporation:
使用电穿孔法转化农杆菌电击感受态细胞：

- Prepare electroporation cuvettes: they are re-usable; make sure they have been disifected with ethanol, washed with dH20, dried and cooled at -20 °C before usage.
    - 准备电击杯：它们是可重复使用的；确保在使用前已用乙醇消毒，用 dH20 清洗，干燥并在 -20 °C 下冷却。
- Defrost electrocompetent *A. tumefaciens* cells on ice.
    - 在冰上解冻农杆菌电击感受态细胞。
- Carefully add plasmid DNA (10-50 ng is sufficient).
    - 小心加入质粒 DNA（10-50 ng 即可）。
- **Do not add more than 100 ng of DNA; otherwise, salts might cause a short circuit.**
    - **不要加入超过 100 ng 的 DNA；否则，盐分可能导致短路。**

    > 💡 **注释:** 电击转化对盐分非常敏感。盐分过高会导致电弧（Arcing，即短路爆杯），不仅转化失败，还可能损坏仪器。

- Carefully pipet *A. tumefaciens* and plasmid DNA mixture up and down for 1-2 times.
    - 小心上下吹打 1-2 次混合农杆菌和质粒 DNA。
- Transfer cell-plasmid-mixture into pre-cooled (on ice) electroporation cuvettes.
    - 将细胞-质粒混合物转移到预冷（冰上）的电击杯中。
- Wipe ice/any residual water from the cuvettes before transferring it into the electroporator.

- 在将其转移到电穿孔仪之前，擦去电击杯上的冰/任何残留水。

> 💡 **注释:** 杯体外部的水会导致电流从外部通过，引发短路。

- Perform the electroporation using the electroporator from BioRad (located in the room on the 2nd floor with the laminar flows).
  - 使用 BioRad 电穿孔仪进行电穿孔（位于二楼层流室）。
  - o **Electroporation settings (电穿孔设置):** (原文此处留白，通常农杆菌设置为 2.2 kV, 25 µF, 200-400 Ω)
- After performing the electroporation, directly add 1 mL of LB or SOC medium for recovering the bacteria and pipette in a new ep.
  - 电穿孔后，直接加入 1 mL LB 或 SOC 培养基以恢复细菌，并移液到新的 EP 管中。
- Shake at 200 rpm at 28 °C for 2-4 hours.
  - 在 28 °C 下以 200 rpm 振荡 2-4 小时。

> 💡 **注释:** 农杆菌生长较慢，且电击造成细胞壁损伤，需要较长的复苏时间（2-4小时），比大肠杆菌（1小时）更长。

- Plate cells onto selection plates (LB agar plus appropriate antibiotics encoded by the plasmid and rifampicin).
  - 将细胞涂布在选择平板上（LB 琼脂加上质粒编码的相应抗生素和利福平）。
  - o Spin cell cultures down for 2 min at 3000 x g, remove supernatant and dissolve pellet in residual culture and plate the remaining culture on a separate selection plate.
    - o 将细胞培养物以 3000 x g 离心 2 分钟，去除上清液，用残留的培养物溶解沉淀，并将剩余的培养物涂布在单独的选择平板上。
- Incubate the plates for 2-3 days at 28 °C.
  - 在 28 °C 下孵育平板 2-3 天。

Making competent cells

制备感受态细胞

Heat-shock competent Top 10 *E. coli* cells:
热激 Top 10 大肠杆菌感受态细胞:

Media (培养基):

250 mL 2x YT broth in 500 mL flask:
500 mL 烧瓶中的 250 mL 2x YT 肉汤:

- 4 g Bacto tryptone (4 g 细菌胰蛋白胨)
- 2.5 g Bacto yeast extract (2.5 g 细菌酵母提取物)
- 1.25 g NaCl (1.25 g 氯化钠)
- Water up to 250 mL (加水至 250 mL)

- PH to 7.0 with NaOH (用 NaOH 调节 pH 至 7.0)
- Autoclave (高压灭菌)

50 mL 1x YT broth:
50 mL 1x YT 肉汤：

- 0.4 g Bacto tryptone
- 0.25 g Bacto yeast extract
- 0.125 g NaCl
- Water up to 50 mL
- pH to 7.0 with NaOH
- Autoclave

TS buffer (TSB; prepare fresh on the day, when the competent cells are being made):
TS 缓冲液 (TSB; 在制备感受态细胞的当天新鲜制备):

- 7.5 mL 2 x YT broth (final conc. 1X)
  - 7.5 mL 2 x YT 肉汤（终浓度 1X）
- 129 µL 5 M NaCl (final conc. 43 mM)
  - 129 µL 5 M NaCl（终浓度 43 mM）
- 150 µL 1 M MgCl2 (final conc. 10 mM)
  - 150 µL 1 M MgCl2（终浓度 10 mM）
- 1.5 g polyethylene glycol (PEG) 4000 (final conc. 10 % (v/v))
  - 1.5 g 聚乙二醇 (PEG) 4000（终浓度 10 % (v/v)）
- 750 µL DMSO (final conc. 5 % (v/v))
  - 750 µL DMSO（终浓度 5 % (v/v)）
- 3 mL glycerol (final conc. 20 % (v/v))
  - 3 mL 甘油（终浓度 20 % (v/v)）
- Water up to 15 mL
  - 加水至 15 mL
- Sterile filter the buffer and keep cool until needed
  - 除菌过滤缓冲液并保持冷却直到需要使用

For all steps, work in a laminar flow to keep the cells sterile
对于所有步骤，在层流罩中操作以保持细胞无菌

Day 1:

- Streak bacteria on an antibiotic free LB plate (streak bacteria from stock; in this case Top 10 cells, but also works for other *E. coli* strains such as DB3.1 or BL21).
  - 在无抗生素 LB 平板上划线接种细菌（从库存中划线接种细菌；此处为 Top 10 细胞，但也适用于其他大肠杆菌菌株，如 DB3.1 或 BL21）。
- Incubate the plate at 37 °C overnight.
  - 在 37 °C 下过夜孵育平板。

Day 2:

- Start an overnight culture in 4 mL YT broth and shake at 200 rpm and 37°C overnight.
  - 在 4 mL YT 肉汤中开始过夜培养，并在 37°C 以 200 rpm 振荡过夜。

Day 3:

From here on, keep the cultures/cells always on ice
从这里开始，始终将培养物/细胞保持在冰上

- Pre-cool big falcon centrifuge down to 4°C.
  - 预冷大型 Falcon 离心机至 4°C。
- Transfer 2 mL of the primary overnight culture into each 500 mL flask with 200 mL room temperature 2x YT broth.
  - 将 2 mL 初级过夜培养物转移到每个装有 200 mL 室温 2x YT 肉汤的 500 mL 烧瓶中。
- Shake at 200 rpm and 37 °C for about 2-3 hours until OD600=0.3-0.6 is reached.
  - 在 37 °C 下以 200 rpm 振荡约 2-3 小时，直到达到 OD600=0.3-0.6。

> 💡 **注释:** OD600 0.3-0.6 处于对数生长期（Log phase），此时细菌状态最活跃，细胞壁通透性最好，转化效率最高。

- Divide secondary culture into four 50 mL tubes, centrifuge for 10 min at 3000 rpm at 4 °C and discard supernatant.
  - 将次级培养物分成四个 50 mL 管，在 4 °C 下以 3000 rpm 离心 10 分钟，并弃去上清液。
- Resuspend the cells in 10 mL cold TSB (2.5 mL per falcon tube).
  - 用 10 mL 冷 TSB（每个 Falcon 管 2.5 mL）重悬细胞。
- Pool cells into a single falcon tube and incubate for 10 min on ice.
  - 将细胞汇集到一个 Falcon 管中，并在冰上孵育 10 分钟。
- Aliquot 50 µL of the cells into pre-cooled 1.5 mL tubes (autoclaved beforehand) and freeze immediately in liquid nitrogen.
  - 将 50 µL 细胞分装到预冷（预先高压灭菌）的 1.5 mL 管中，并立即在液氮中冷冻。
- Store cells at –80°C until use.
  - 将细胞储存在 –80°C 直到使用。
- Test an aliquot of the untransformed competent cells for antibiotic resistance by plating on LB plates containing ampicillin (100 µg/mL working conc.) and kanamycin (50 µg/mL working conc.), respectively.
  - 通过涂布含有氨苄青霉素（100 µg/mL 工作浓度）和卡那霉素（50 µg/mL 工作浓度）的 LB 平板，测试一份未转化的感受态细胞的抗生素抗性（作为阴性对照，确保细胞未受污染）。

Electrocompetent GV3101 Mp90 (pSoup-P19) Agrobacterium tumefaciens cells:
GV3101 Mp90 (pSoup-P19) 根癌农杆菌电击感受态细胞：

GV3101 Mp90 (pSoup-P19) from ZellBio. (Link in original)
来自 ZellBio 的 GV3101 Mp90 (pSoup-P19)。（原文有链接）

For all the steps, work in a laminar flow to keep everything sterile.
对于所有步骤，在层流罩中操作以保持一切无菌。

Day 1:

- Inoculate *A. tumefaciens* colony (derived from a freshly streaked LB plate containing the appropriate antibiotics) in 3 mL LB culture with antibiotics at 28°C and 200 rpm (5 µg/mL rifampicin for *A. tumefaciens* in general, 30 µg/mL gentamycin and 5 µg/mL tetracyclin for pSoup-P19 helper plasmid).
  - 将农杆菌菌落（来自含有适当抗生素的新鲜 LB 平板）接种到 3 mL 含有抗生素的 LB 培养物中，在 28°C 和 200 rpm 下培养（一般农杆菌用 5 µg/mL 利福平，pSoup-P19 辅助质粒用 30 µg/mL 庆大霉素和 5 µg/mL 四环素）。
  - Rifampicin needs to be dissolved in methanol or DMSO (precipitation occurs more frequently with methanol; do not store for several month).
    - 利福平需要溶解在甲醇或 DMSO 中（甲醇更容易发生沉淀；不要储存几个月）。
  - Tetracyclin and Gentamycin can be dissolved in dH20; tetracyclin should be prepared freshly.
    - 四环素和庆大霉素可以溶解在 dH20 中；四环素应新鲜配制。

Day 2:

- Inoculate 150 mL LB with antibiotics with 2 mL of the overnight culture.
  - 用 2 mL 过夜培养物接种 150 mL 含有抗生素的 LB。
- Incubate at 28°C at 200 rpm until OD600 reaches 0.8-1.0.
  - 在 28°C 下以 200 rpm 孵育直到 OD600 达到 0.8-1.0。
- Start in the morning if you can clearly see that the 3 mL culture has grown; otherwise, wait till the evening.
  - 如果你能清楚地看到 3 mL 培养物已经生长，则在早上开始；否则，等到晚上。

Day 3:

- Pre-cool falcon centrifuge to 4°C.
  - 预冷 Falcon 离心机至 4°C。
- When the cultures reached the desired OD600, chill cultures for 15 min on ice and swirl regularly.
  - 当培养物达到所需的 OD600 时，将培养物在冰上冷却 15 分钟并定期摇晃。
  - **From here on, everything needs to happen on ice or at 4°C!**
    - **从这里开始，一切都需要在冰上或 4°C 下进行！**
- Distribute culture into 3x50 mL tubes and pellet by centrifugation at 4000 x g for 20 min at 4°C.
  - 将培养物分配到 3x50 mL 管中，并在 4°C 下以 4000 x g 离心 20 分钟使其沉淀。
- Discard supernatant and resuspend each pellet in 10 mL ice cold dH20 (autoclaved beforehand).
  - 弃去上清液，用 10 mL 冰冷 dH20（预先高压灭菌）重悬每个沉淀。
- Combine the 3 tubes into 2 tubes and further adjust volume to 50 mL each with ice cold dH20.
  - 将 3 个管合并为 2 个管，并用冰冷 dH20 将体积进一步调整为每个 50 mL。
- Vortex each falcon with 10 mL ice cold dH20, mix it and top up to 50 mL.

    - （原文可能有误，意为充分洗涤）用 10 mL 冰冷 dH20 涡旋每个 Falcon 管，混合并加满至 50 mL。
- Pellet cells by centrifugation at 4000 x g for 20 min at 4°C.
    - 在 4°C 下以 4000 x g 离心 20 分钟使细胞沉淀。
- Discard supernatant and resuspend each pellet in 10 mL ice cold dH20.
    - 弃去上清液，用 10 mL 冰冷 dH20 重悬每个沉淀。
- Combine the 2 tubes into 1 tube and further adjust to 50 mL with ice cold dH20 as described before.
    - 如前所述，将 2 个管合并为 1 个管，并用冰冷 dH20 进一步调整至 50 mL。
- Pellet cells by centrifugation at 4000 x g for 20 min at 4°C.
    - 在 4°C 下以 4000 x g 离心 20 分钟使细胞沉淀。

> 💡 **注释:** 这一系列繁琐的“离心-重悬”是水洗步骤，目的是彻底去除培养基中的盐分，防止电击时短路。

- Discard supernatant and resuspend pellet in 1.5 mL ice cold 10 % (v/v) glycerol (autoclaved beforehand).
    - 弃去上清液，用 1.5 mL 冰冷 10 % (v/v) 甘油（预先高压灭菌）重悬沉淀。
- Dispense 50 µL aliquots into pre-cooled 1.5 mL tubes (autoclaved beforehand) and flash freeze in liquid nitrogen.
    - 将 50 µL 分装液分装到预冷（预先高压灭菌）的 1.5 mL 管中，并在液氮中速冻。
- Store aliquots at -80°C until use.
    - 将分装液储存在 -80°C 直到使用。

Colony PCR

菌落 PCR

Colony PCRs can be used as an initial test to confirm success of the cloning approach before inoculating the transformants in liquid culture. For this, select a region for not more than 1 kb that you want to amplify. The primers that you use for amplifying the region of interest should be insert- and vector-specific (e.g. forward primer binds within the insert, reverse primer binds in the vector backbone).
菌落 PCR 可用作初步测试，在将转化子接种到液体培养物之前确认克隆方法是否成功。为此，选择一个不超过 1 kb 的区域进行扩增。你用于扩增感兴趣区域的引物应为插入片段和载体特异性的（例如，正向引物结合在插入片段内，反向引物结合在载体骨架中）。

> 💡 **注释:** 使用一条载体引物和一条插入引物可以验证插入片段的方向是否正确，并避免空载体的假阳性。

Depending on the number of colonies you want to test, prepare a master mix with the ThermoScientific DreamTaq Green PCR Master Mix (2X).
根据你要测试的菌落数量，使用 ThermoScientific DreamTaq Green PCR Master Mix (2X) 准备母液。

It is not necessary to prepare reaction volumes of up to 50 µL (according to the manufacturer's instructions), one quarter of the reaction volume (12.5 uL) is sufficient.
不需要按照制造商的说明准备高达 50 µL 的反应体积，四分之一的反应体积 (12.5 uL) 就足够了。

Always prepare a master mix for 1 reaction in excess.
始终多准备 1 个反应的母液。

MasterMix for colony PCR:
菌落 PCR 母液：

Component	Amount	Final conc.
DreamTaq Green PCR Master Mix (2X)*	6.25 µL	
Forward primer (10 µM stock conc.)	0.625 µL	0.5 µM final conc.
Reverse primer (10 µM stock conc.)	0.625 µL	0.5 µM final conc.
MilliQ water	Up to 12.5 µL	

- *no need to use a high fidelity polymerase (TAQ is sufficient)
  - *不需要使用高保真聚合酶（TAQ 就足够了）

Adding material of the bacterial colonies into the PCR vials containing the PCR mixture:
将细菌菌落材料加入含有 PCR 混合物的 PCR 管中：

- Aliquot the PCR mixture into PCR vials and transfer those into the laminar flow.
  - 将 PCR 混合物分装到 PCR 管中，并将其转移到层流罩中。
- Touch the bacterial colony with a sterile tip and transfer it into the PCR vials; gently pipette up and down to resuspend the bacteria in the PCR mixture.
  - 用无菌枪头接触细菌菌落并将其转移到 PCR 管中；轻轻上下吹打以将细菌重悬在 PCR 混合物中。
  - It is not necessary to visually see bacteria on the pipette tip, it is sufficient to gently touch the colony.
    - 不需要在移液枪头上肉眼看到细菌，只需轻轻接触菌落即可。

    > 💡 **注释:** 菌量过多会抑制 PCR 反应。

  - **Tip: Streak the colonies you want to test on a “Master” plate before adding it to the PCR mixture; then, you have more material for later inoculations and can organize them more carefully.**
    - **提示：在将其加入 PCR 混合物之前，将你要测试的菌落在“主”平板上划线（备份）；这样，你就有了更多材料供以后的接种使用，并且可以更仔细地组织它们。**
- Spin down the PCR reaction mixture and start the PCR in a thermo cycler.
  - 离心 PCR 反应混合物并在热循环仪中开始 PCR。

PCR settings:
PCR 设置：

- Perform the PCR according to the manufacturer's instructions, to the length of the fragment you want to amplify and the annealing temperature of the primers.

    - 根据制造商的说明、你要扩增的片段长度和引物的退火温度进行 PCR。
- 30 cycles are enough for colony PCRs; otherwise, you may proliferate artificial products.
    - 30 个循环对于菌落 PCR 来说足够了；否则，你可能会扩增出人造产物（非特异性产物）。
- **E. coli colony PCRs: for initial denaturation step at 98 °C for 3 min.**
    - **大肠杆菌菌落 PCR：初始变性步骤 98 °C 3 分钟。**
- **A. tumefaciens colony PCRs: for initial denaturation step at 98 °C for 10 min.**
    - **农杆菌菌落 PCR：初始变性步骤 98 °C 10 分钟。**

> 💡 **注释:** 高温初始变性除了激活热启动酶外，主要功能是裂解细菌释放质粒。农杆菌细胞壁更厚，需要更长时间。

After performing the PCR reaction in a thermo cycler, run the PCR in on a 1 % agarose gel together with a 1 kB DNA ladder. Afterwards, visualize the DNA bands with UV light. If the DNA bands are in accordance with the expected fragment size, you can inoculate the bacterial colony into LB medium with the respective antibiotic for further downstream processing (e.g. plasmid prep).
在热循环仪中进行 PCR 反应后，将 PCR 产物与 1 kB DNA Ladder 一起在 1% 琼脂糖凝胶上进行电泳。之后，用紫外光观察 DNA 条带。如果 DNA 条带与预期的片段大小一致，你可以将细菌菌落接种到含有相应抗生素的 LB 培养基中，进行进一步的下游处理（例如质粒制备）。

Sequencing (Sanger)

测序 (Sanger 法)

The results of our cloning approaches can be verified by Sanger Sequencing. We are sending our samples to LGC Genomics (Link in original). Results can be analyzed using Geneious.
我们的克隆结果可以通过 Sanger 测序进行验证。我们将样品发送给 LGC Genomics（原文有链接）。结果可以使用 Geneious 进行分析。

Step 1: Prepare your samples in 1.5 mL reaction tubes
第 1 步：在 1.5 mL 反应管中准备样品

Template requirements:
模板要求：

Template	**Concentration**
Plasmids	**100 ng/µL**
PCR products	
200 - 500 bp	10 ng/µL
500 - 1,000 bp	20 ng/µL
1,000 - 2,000 bp	40 ng/µL

- **FlexiRun:** 40 µL of template DNA

    - **FlexiRun:** 40 µL 模板 DNA
- **Ready2Run:** Pre-pipetted primer (10 µL DNA + 5 µL primer [5 µM])
    - **Ready2Run:** 预加引物（10 µL DNA + 5 µL 引物 [5 µM]）
- In room 00.34, you will find a cardboard box with LGC prepaid labels and envelopes.
    - 在 00.34 房间，你会发现一个装有 LGC 预付费标签和信封的纸板箱。
- Place one label per tube, collect all tubes in an envelope and put it together with the waybill number (secretary's office) in the LGC plastic box in the entrance of Biosystems II.
    - 每根管贴一个标签，将所有管收集在一个信封中，并将其与运单号（秘书室）一起放入 Biosystems II 入口处的 LGC 塑料盒中。

Step 2: Place your order at LGC
第 2 步：在 LGC 下单 (Link in original)

- At “sequencing ordering”, you will find below “single sample” the option for “Ready2 / Flexi Run”.
    - 在“测序订购”处，你会在“单一样品”下方找到“Ready2 / Flexi Run”选项。
- Enter your sample label ID, sample name and the service you want to use (Ready2 / Flexi Run).
    - 输入你的样品标签 ID、样品名称和你想要使用的服务 (Ready2 / Flexi Run)。
    - o In case you chose Flexi Run, indicate which primer should be used by LGC.
        - o 如果你选择 Flexi Run，请注明 LGC 应使用哪种引物。
- Continue with filling in the information for the pick-up box and your e-mail address on the next site, and confirm the order.
    - 继续在下一个站点填写取件箱信息和你的电子邮件地址，并确认订单。
- Schedule the pick-up with DHL until 12:00 via (Link provided).
    - 通过（提供的链接）在 12:00 之前安排 DHL 取件。
- Results will be available in about 2-3 working days (notification via e-mail) and can be downloaded at "Data Management – Order&Download Results”.
    - 结果将在大约 2-3 个工作日内提供（通过电子邮件通知），并可在“数据管理 – 订单和下载结果”处下载。

Step 3: Analyze your sequences with Geneious
第 3 步：用 Geneious 分析你的序列

- Book working station in advance via clustermarket.
    - 通过 clustermarket 提前预订工作站。
- Import .ab1 files to geneious, check sequence quality at the beginning and end of the sequence before aligning with your GOI.
    - 将 .ab1 文件导入 Geneious，在与你的 GOI（感兴趣基因）比对之前检查序列开始和结束时的序列质量。

Antibiotics

抗生素

- Prepare stock solutions of each antibiotic according to the desired concentration.
    - 根据所需浓度制备每种抗生素的储备液。
- Filter-sterilize in the laminar flow and aliquot into 1.5 mL tubes.

    - 在层流罩中进行除菌过滤，并分装到 1.5 mL 管中。
- Store at -20°C until use (keep lifetime of certain antibiotics in mind!).
    - 储存在 -20°C 直到使用（牢记某些抗生素的有效期！）。

For solid plates: Add antibiotics to the medium only, if it is hand-warm or cooler!
对于固体平板：仅在培养基手温或更凉时加入抗生素！

> 💡 **注释:** 培养基太热（>55°C）会使抗生素失效。

Antibiotic (抗生素)	**Stock concentration (储备浓度)**	**Working concentration (工**
Ampicillin (氨苄青霉素)	100 mg/mL	100 µg/mL
Carbenicillin (羧苄青霉素)	100 mg/mL	100 µg/mL
(alternative to ampicillin)		
Chloramphenicol (氯霉素)	25 mg/mL (dissolved in EtOH)	25 µg/mL
Kanamycin (卡那霉素)	50 mg/mL	50 µg/mL
Spectinomycin (大观霉素)	50 mg/mL	50 µg/mL
Gentamycin (庆大霉素)	50 mg/mL	30 µg/mL
Tetracycline (四环素)	10 mg/mL	10 µg/mL
Rifampicin (利福平)	50 mg/mL (dissolved in DMSO or MeOH)	5-20 µg/mL
Cefotaxime (头孢噻肟)	100 mg/mL	100 µg/mL

(完 / End of Manual)