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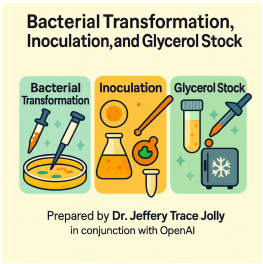
Bacterial Transformation, Inoculation, and Glycerol Stock (V1 08.10.25)



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We use this protocol and it's working

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Abstract

This protocol provides a complete workflow for *Escherichia coli* transformation, colony selection, inoculation, glycerol stock preparation, and revival for downstream molecular biology applications. It details best practices for selecting competent cell strains, performing heat-shock transformation, recovering in selective media, and initiating clonal overnight cultures. The method also includes procedures for generating long-term glycerol stocks and efficiently reviving them for reuse, along with preparation steps for plasmid extraction. Following this workflow ensures high transformation efficiency, consistent culture purity, and reliable plasmid yield, while preserving bacterial viability during long-term storage.

Image Attribution

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Guidelines

- Ensure all work with *E. coli* is conducted in accordance with your institution's biosafety regulations (BSL-1 or higher depending on strain and construct).
- Use sterile technique at all times to prevent contamination.
- Double-check plasmid maps and antibiotic resistance markers before starting to ensure appropriate selection.
- Prepare all required reagents, media, antibiotics, and sterile consumables in advance to minimize workflow interruptions.
- Keep competent cells on ice at all times prior to heat shock to maintain transformation efficiency.

Materials

- **One Shot Mach1 T1 chemically competent *E. coli*** – ThermoFisher C862003; Invitrogen C862003; Fisher Scientific C862003
- **One Shot TOP10 chemically competent *E. coli*** – ThermoFisher C404003; Invitrogen C404003; Fisher Scientific C404003
- **DH5α chemically competent *E. coli*** – ThermoFisher 18265017; NEB C2987H; Sigma-Aldrich 69450-3
- **BL21(DE3) chemically competent *E. coli*** – ThermoFisher C600003; NEB C2527H; Sigma-Aldrich 69450-4
- **XL1-Blue chemically competent *E. coli*** – Agilent 200249; NEB C2992H; Fisher Scientific NC0070931
- **Plasmid DNA** (10 pg–100 ng per transformation) – prepared in-lab or purchased
- **SOC medium** – ThermoFisher 15544034; NEB B9020S; Sigma-Aldrich S1797
- **Terrific Broth (TB)** – ThermoFisher R064211; Sigma-Aldrich T9179; VWR 90004-130
- **Agar plates (TB or LB) with appropriate antibiotic** – VWR 90004-302; ThermoFisher R064402; Sigma-Aldrich 11539
- **Ampicillin sodium salt** (example antibiotic) – Sigma-Aldrich A0166; ThermoFisher BP1760-25; VWR 97061-654
- **Sterile 14 mL culture tubes** – VWR 60818-734; Corning 430052; Falcon 352059
- **Sterile cryovials (1.5–2.0 mL)** – ThermoFisher 5000-1020; VWR 66021-604; Sigma-Aldrich CLS430488
- **Sterile 50% glycerol solution** (or glycerol to prepare in-lab) – Sigma-Aldrich G5516; ThermoFisher BP229-1; VWR BDH1172-1LP
- **Micropipettes and sterile filtered tips** – ThermoFisher 9402520; VWR 89079-462; USA Scientific 1122-7810
- **Microcentrifuge tubes (1.5 mL)** – Eppendorf 022363204; VWR 87003-294; ThermoFisher 05-408-129
- **Microcentrifuge** – Eppendorf 5424; ThermoFisher mySPIN 12; VWR Galaxy Mini
- **Heat block or water bath (42 °C)** – VWR 12620-938; ThermoFisher 88870001; Benchmark Scientific H3760
- **Shaking incubator (37 °C)** – New Brunswick Innova 44; Benchmark Incu-Shaker Mini; VWR Symphony 3900 Series

Troubleshooting

Safety warnings

- Do not exceed recommended DNA input volumes when transforming; excess salts or DNA can reduce transformation efficiency.
- Use appropriate PPE (lab coat, gloves, and safety glasses) when handling bacterial cultures, antibiotics, and glycerol.
- Ampicillin and other antibiotics are potential allergens; avoid inhalation and direct skin contact.
- Glycerol can be slippery if spilled; clean spills immediately to prevent accidents.

Ethics statement

This protocol involves work with non-pathogenic laboratory strains of *Escherichia coli*. All work should be conducted in compliance with institutional biosafety and waste disposal regulations. No animal or human subjects are involved in this protocol; therefore, no IACUC or IRB approval is required. If adapting this method for pathogenic organisms, appropriate regulatory and ethical approvals must be obtained before proceeding.

Before start

- Competent cells are available and stored appropriately at −80 °C.
- All necessary antibiotics are prepared at correct stock concentrations and are fully thawed and mixed.
- Agar plates with the correct selection antibiotic are at the correct temperature (pre-warmed if required).
- Heat block or water bath is pre-heated to 42 °C for heat-shock transformation.
- All culture tubes, cryovials, pipette tips, and other consumables are sterile.

Part 1: Bacterial Transformation and Plating

2h

1 **Determine the appropriate chemically competent *E. coli* strain for your DNA type (e.g., cloning, lentiviral, recombinant protein expression, or Gateway cloning).**
Explanation: Different strains have genetic modifications that optimize transformation efficiency or plasmid stability for specific applications.

Note

Bacterial Strain Selection Guide:

One Shot Mach1 T1 (ThermoFisher, C862003)
- *Primary Purpose / Strengths:*
  • Extremely rapid growth (colonies visible ~8 h)
  • High transformation efficiency (> 1×10⁹ cfu/µg)
  • Phage-resistant (T1/T5
  • Blue-white screening
  • Reduced recombination
  • Enhanced plasmid yield
- *Recommended Use:* Fast turnaround cloning; ideal when time-sensitive, high yield, phage resistance, and screening are priorities.

One Shot TOP10 (ThermoFisher, C404003)
- *Primary Purpose / Strengths:*
  • Very high transformation efficiency (> 1×10⁹ cfu/µg)
  • Broad cloning utility
  • Compatible with methylated DNA
  • Blue-white screening
- *Recommended Use:* Standard cloning workflows, library construction, or work with genomic/methylated inserts.

DH5α *(available from multiple vendors, e.g., Invitrogen, NEB)*
- Primary Purpose / Strengths:
  • High transformation efficiency
  • recA1 and endA1 mutations improve plasmid maintenance and purity
  • Supports blue-white screening via lacZΔM15
- Recommended Use: Routine cloning requiring high plasmid yield and purity, such as sequencing or subcloning.

BL21(DE3) (ThermoFisher protein expression strains)
- *Primary Purpose / Strengths:*
  • Optimized for recombinant protein expression via T7 system
  • Lacks major proteases (Lon, OmpT) for higher yields
- *Recommended Use:* Protein expression from T7-promoter constructs, large-scale or high-yield production.

XL1-Blue / BW3KD (BW3KD info – PMC article)
- *Primary Purpose / Strengths:*
  • XL1-Blue: robust general cloning strain
  • BW3KD: extremely high transformation efficiency, ideal for large-fragment DNA assembly
- *Recommended Use:* XL1-Blue for standard cloning; BW3KD for complex assembly or large plasmid work.

2 **Thaw a tube of competent cells on ice for** 00:07:00 **.** 5m

Explanation: Keeping cells cold preserves membrane permeability and prevents premature uptake of DNA.

3 **Set the heat block to** 42 °C **.**

Explanation: This prepares the equipment for the heat shock step, which temporarily opens membrane pores.

4 **Add the desired amount of plasmid DNA to a** 50 µL **aliquot of competent cells.**

Flick the tube gently to mix. Do not pipette up and down.

Explanation: Gentle mixing maintains cell integrity; harsh pipetting can rupture fragile cell membranes.

Note

- The added amount of DNA must not exceed 3 µL to avoid disrupting the salt balance of the 50ul transformation.
- Most of the time 0.5 µL is optimal.
- You only need a very small amount of DNA (1 pg - 100 ng)
- If transforming something post LR reaction or if there are salts are other contaminants consider diluting the product 1:1-1:5 with DI water.

5 **Incubate the DNA–cell mixture on ice for** 00:07:00 **.** 5m

Explanation: This allows the DNA to bind to the bacterial cell surface before heat shock.

6 **Heat-shock the cells at** 42 °C **for** 00:00:40 **.** 40s

Explanation: The sudden temperature change creates a thermal imbalance that drives DNA uptake through transient membrane pores.

7 **Quickly place the cells back on ice for** 00:07:00 **.** 5m

Explanation: Rapid cooling closes the membrane pores and stabilizes the cells after heat shock.

8 **Add** 300 µL **SOC medium to the cells and gently resuspend by pipetting.**

Explanation: SOC medium is nutrient-rich, helping cells recover from transformation stress and begin expressing the antibiotic resistance gene.

Note

SOC media is preferred, but you can also use terrific broth as long as it is relatively clean. SOC might increase colony yield for finicky applications.

9 **Incubate to allow cells to recover on warm shaker** 300-500 rpm, 37°C, 01:00:00 1h

Explanation: Recovery time allows expression of the resistance marker before plating on selective media.

10 **Warm TB + antibiotic agar plates at** 37 °C **.**

Explanation: Bringing plates to temperature prevents condensation and helps colonies grow more evenly.

Note

Check your plasmid map to determine what type of antibiotic selection is required:

Antibiotic Concentrations

	Antibiotic	Recommended Stock Concentration	Recommended Working Concentration
	Ampicillin	100 mg/mL	100 µg/mL
	Bleocin	5 mg/mL	5 µg/mL
	Carbenicillin*	100 mg/mL	100 µg/mL
	Chloramphenicol	25 mg/mL(dissolve in EtOH)	25 µg/mL
	Coumermycin	25 mg/mL(dissolve in DMSO)	25 µg/mL
	Gentamycin	10 mg/mL	10 µg/mL
	Kanamycin	50 mg/mL	50 µg/mL
	Spectinomycin	50 mg/mL	50 µg/mL
	Tetracycline	10 mg/mL	10 µg/mL

Common concentrations of antibiotics used in the lab.

Notes:
Unless otherwise indicated, the antibiotic powder can be dissolved in dH_2O.
*Carbenicillin can be used in place of ampicillin. Carbenicillin is more stable, so it is potentially more effective at selecting only bacteria containing the plasmids of interest (for example, fewer satellite colonies will grow). However, it is also more expensive.

Over agar method (if needed):
Dilute antibiotic stock solution (above) 1:100 with LB/TB medium. Apply 150 µL of the antibiotic solution and spread evenly on the surface of the plate. Allow 00:30:00 for the antibiotic to be absorbed into the agar before plating.

11 **Spread** 300 µL **of transformed culture onto the plate (or split** 250 µL **on one plate and** 50 µL **on another). Incubate plates upside down at** 37 °C Overnight **.**

1d

Explanation: Inverting plates prevents condensation from dripping onto colonies, which can cause spreading. The 250 µL *and* 50 µL *protocol is always preferred in case of unpredictable growth patterns.*

12 **Remove plates the following morning to check for colony growth. Collect colonies 18-24 hours after adding to the plate.**

Explanation: Successful colonies indicate cells have taken up and maintained the plasmid. Exceeding 24:00:00 *can result in non-specific and satellite colony growth as the antibiotic is depleted.*

13 **Store plates upside down at** 4 °C **if using within a week.**

Explanation: Cool storage slows bacterial growth and preserves colony integrity.

Note

For storage at 4 °C, wrap the plate in parafilm to prevent drying out.

Part 2: Bacterial Culture Inoculation

1d

14 **Prepare/collect** 14 mL **culture tubes, Terrific Broth (TB), and the required antibiotic. Thaw antibiotic stocks if frozen. (See the note above regarding stock and working antibiotic solutions).**

Explanation: TB is a nutrient-rich medium that supports high-density bacterial growth; the antibiotic maintains plasmid selection.

15 **Add** 3-7 mL **TB and** [M] 3-7 µL/mL **antibiotic (1:1000 from the antibiotic stocks)to each culture tube.**

Explanation: The antibiotic concentration must be sufficient to prevent growth of non-transformed bacteria.

Note

Antibiotic stocks are prepared at 1000X for this purpose and kept in -20 °C. Be sure the stock is completely thawed and mixed well to prevent precipitation. Always double-check selection markers!

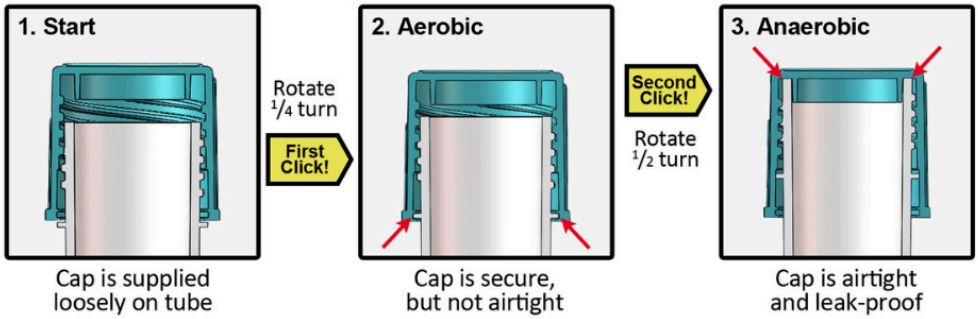
16 **Pick a single colony with a sterile pipette tip and drop the tip into the culture tube. Repeat for each colony. Vortex to disperse the bacteria.**
Explanation: Starting from a single colony ensures a genetically uniform culture.

17 **Incubate the cultures** 150-200 rpm, 37°C Overnight **(18-24 hours, depending on volume of broth).**
Explanation: Shaking aerates the culture, ensuring sufficient oxygen for rapid bacterial growth.

1d

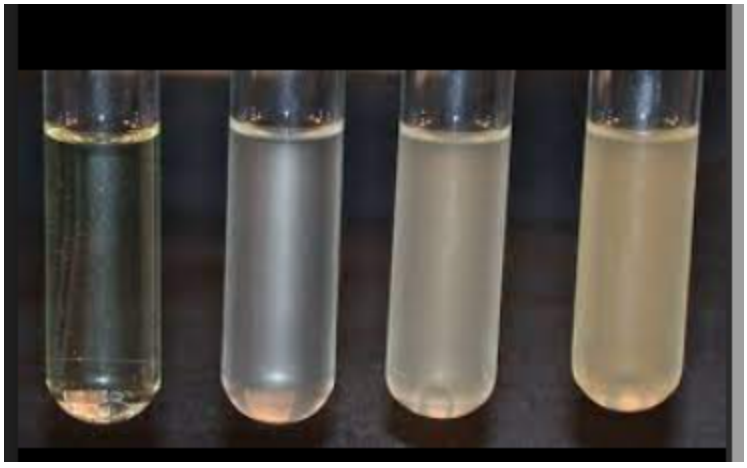
Note

Optimal OD at time of collection in Culture tube notes:
It's best to loosen the caps on the 14ml tubes to allow gas exchange.



Notes regarding caps of the culture tubes.
https://alkalisci.com/cellpro-culture-tubes-polypropylene-pp/?srsltid=AfmBOoqJzPafE29mbkHxzSkWsQkKksPmycQO81tiEtzv6mcDQXw5vbHC

Growth notes:
Collect bacteria when the solution becomes adequately cloudy. Do not overgrow to prevent cell death.



Stages of turbidity increase from left to right.
Bacteria must be collected as soon as the turbidity on the right is reached.
Optimal OD for the culture at time of collection is between 2.0 and 2.4 at 600 nm (OD600)
Most people do not quantify OD and just collect the culture if it is cloudy after O/N growth
https://www.fbioyf.unr.edu.ar/evirtual/pluginfile.php/108402/mod_resource/content/1/Ca

Part 3: Preparation of Glycerol Stocks

18 **Grow the bacterial culture in the appropriate medium (with antibiotics) at the desired conditions until it reaches mid- to late-log phase (typically OD_{600} of 0.6–1.0).**
Explanation: Cells in the log phase are metabolically active and more likely to survive freezing without loss of viability.

Note

Most people do not quantify OD and just take the sample for the stock the morning after inoculation. It's not that critical, as only a few viable cells are required to revive the culture later. If you are concerned that the culture has been in brief storage in 4 °C while plasmid sequences are being verified, you can revive the culture 1:1 with fresh TB and antibiotics, and allow it to re-enter the log phase by culturing 150-200 rpm, 37°C for ~ 01:00:00.

It's best practice to store the inoculations at 4 °C until the sequence of the plasmid has been confirmed. You can store the "empty" tubes if you have collected all the TB for plasmid isolation, to revive them at a later time by adding TB and antibiotics.

The temporary inoculation can be stored for up to 2 weeks at 4 °C. Be sure caps are closed to prevent contamination and evaporation.

19 **Label sterile cryovials with strain name, date, and any relevant plasmid or antibiotic information.** *Explanation: Proper labeling ensures accurate strain tracking and prevents mix-ups during retrieval. A printer label is highly preferred for proper organization.*

20 **Pipette 500 µL of well-mixed culture into a labeled cryovial.**
Explanation: Using mixed culture ensures an even distribution of cells in the glycerol stock.

21 **Add 500 µL of sterile* [M] 50 % (v/v) glycerol to the cryovial (final concentration [M] 25 % (v/v) glycerol).**
Explanation: Glycerol acts as a cryoprotectant by preventing ice crystal formation, which can puncture cell membranes during freezing.

Note

***50% Glycerol Solution:**
- 1:1 glycerol + sterile water
- Can be stored for years
- Protocol says sterile, and this is good practice, but not required
- Make a stock (250 mL) and try to keep it clean by opening/closing quickly and storing it at 4 °C for the long term. Discard if the solution becomes cloudy or precipitates.

22 **Mix thoroughly by gentle pipetting to evenly distribute glycerol. Try to avoid bubbles and inverting, as this would push the bacteria into the lid.**
Explanation: Even mixing ensures that all cells are exposed to glycerol protection during freezing.

23 **Immediately freeze the cryovials at** -80 °C **.**
Explanation: Rapid freezing in glycerol preserves cell structure and viability for long-term storage (years).

Part 4: Revival of Glycerol Stock

1h

24 **Retrieve the bacterial glycerol stock from the** -80 °C **freezer and keep it on ice during handling.** *Explanation: Keeping the stock cold minimizes thawing, which can reduce viability and risk of contamination.*

25 **Using a sterile pipette tip, remove a small speck of frozen stock from the surface.**
Explanation: Taking only a small amount preserves the rest of the stock for future use and avoids repeated freeze–thaw cycles.

26 **Inoculate the speck directly into** 1 mL **of Terrific Broth (TB) without antibiotics in a sterile culture tube or microcentrifuge tube.**
Explanation: Providing a short antibiotic-free recovery phase allows cells to repair membranes and resume active growth before selective pressure is applied.

27 **Incubate** 150-200 rpm, 37°C **for** 01:00:00 **.**
Explanation: This "wake-up" period allows cells to enter exponential growth and produce the antibiotic resistance protein before exposure to antibiotics. Note that some people skip this step and inoculate the whole culture directly with antibiotics.

1h

28 **After the recovery period continue inoculation as described above in Part 2 (** go to step #17 **) by adding additional TB to reach** 3-7 mL **with appropriate antibiotics for overnight culture.**

1d

Explanation: Increasing culture volume and adding antibiotic at this stage promotes selective growth of the desired strain while providing sufficient nutrients for robust expansion.

Part 5: Preparation for Plasmid Extraction

29 **Pellet** 1.5 mL **of the bacterial culture at** 6000 rcf, Room temperature, 00:05:00 **in a microcentrifuge tube. Discard supernatant and repeat until all bacteria are collected.**

Explanation: This RCF and time efficiently collect E. coli without shearing or premature lysis, while avoiding over-compaction that makes pellets difficult to resuspend. If the pellet is loose, increase to 6,000×g for 10 minutes. If the pellet is too tight or resuspends poorly, reduce to 4,000×g for 5–10 minutes.

30 **Proceed directly with plasmid extraction (preferred) or store bacterial pellets in the freezer for later use.**

Explanation: Immediate plasmid extraction minimizes cell lysis and nuclease activity, preserving DNA yield and integrity. If extraction is delayed, pellets can be stored at -20 °C *for short periods or* -80 °C *for longer-term preservation, but repeated freeze–thaw cycles should be avoided to prevent plasmid degradation.*

Note

Best practice for freezing pellets:

- If you are not doing the plasmid prep immediately, **freeze the pellet at** -20 °C **or** -80 °C .
- -80 °C is preferred for longer-term storage because it halts nuclease activity more completely.
- For short-term (hours to 1–2 days), -20 °C is fine.

How long before plasmid yield or quality is affected?

- **At** -80 °C **:** Pellets are generally stable for weeks to a few months without noticeable plasmid degradation, especially if frozen promptly after pelleting.
- **At** -20 °C **:** Best to use within a few days; longer storage can lead to partial cell lysis during freeze–thaw, releasing nucleases that may damage plasmid DNA.
- Repeated freeze–thaw cycles are more damaging than extended frozen storage.

Extra protection tip:

- Remove as much supernatant as possible before freezing — residual medium can promote ice crystal damage and reduce plasmid quality.
- You can also snap-freeze the pellet in liquid nitrogen before storing at −80 °C to minimize ice crystal growth.