



Nov 21, 2025

Preparation of chemically competent cells using modified Hanahan method

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvjepbngk5/v1>

Ainsley Lederer¹

¹University of Pittsburgh



Ainsley Lederer

University of Pittsburgh

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.n2bvjepbngk5/v1>

External link: <https://www.protocols.io/view/chemical-transformation-of-plasmid-into-e-coli-com-36wgqp675vk5/v1>

Protocol Citation: Ainsley Lederer 2025. Preparation of chemically competent cells using modified Hanahan method.
protocols.io <https://dx.doi.org/10.17504/protocols.io.n2bvjepbngk5/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working



Created: September 22, 2025

Last Modified: November 21, 2025

Protocol Integer ID: 227887

Keywords: original hanahan method, modified hanahan method, hanahan method, version of the original hanahan method, competent cells for routine cloning, use of rbcl2, protein purification, classic cac12 method, routine cloning, cells in the heat, shock transformation process, cell, competent cell, preparation, rbcl2, procedure, method

Abstract

This is a tried and true procedure for preparing highly competent cells for routine cloning and protein purification. This method improves the transformation efficiency compared to the classic CaCl₂ method. It is a modified version of the original Hanahan method, which requires the use of RbCl₂.

Please see the linked procedure for how to use these cells in the heat-shock transformation process.

Guidelines

I cannot emphasize enough the importance of not passing an OD of 0.4. Be very careful to remove the cells and place on ice to stop the division. Do not proceed with the procedure if OD goes beyond 0.4!



Materials

TYM broth recipe for 100 mL:

2 g bacto-tryptone
0.2 g yeast extract
0.58 g NaCl
0.5 mL 2.0M MgCl₂

TFB1 Buffer for 100 mL:

30 mM KAc - 0.294 g
50 mM MnCl₂•4H₂O - 0.989 g
100 mM KCl - 0.746 g
15% v/v glycerol

Fill to 95 mL with cell-culture grade water, adjust pH to 5.8 with 0.2M acetic acid. Fill to 100 mL and filter sterile.

*Be careful! If pH goes below 5.8, do not add base to increase pH, you will just form MnOH precipitate. If pH goes below 5.8, discard solution and make again.

TFB2 Buffer for 100 mL:

10 mM MOPS - 0.231 g
10 mM KCl - 0.0746 g or 1 mL of 1M solution
75 mM CaCl₂•2H₂O - 1.103 g
15% v/v glycerol

Fill to 95 mL with cell-culture grade water, adjust pH to 7.0 with HCl or NaOH. Fill to 100 mL and filter sterile.

*Be careful! Using too much HCl or NaOH will cause the pH to decrease to <2 or skyrocket to >12, respectively. Use tiny drops at a time.

Before start

Prepare your materials to work quickly, and in as cold as possible conditions (though above freezing). Use "wet ice" meaning ice-water mixture (but not so watery that your flasks and tubes sink below the water).

Place both buffers in the wet-ice before use. Pre-chill your pipette tips by putting in the fridge or in a cold room. Pre-chill your micro-centrifuge tubes by placing in the tube rack, then putting in the -20°C freezer for 1 hour, or prepare the day before and put in 4°C overnight.



Day 1

1

Take your stock of to-be competent cells, and streak on an LB agar plate with the correct antibiotics

Note

This is a general guideline for antibiotics in *my lab*. Please check if/which plasmid is transformed into the E. coli before you perform the procedure.

1. BL21 (DE3) - no starting plasmid, so **no antibiotic**
2. Rosetta and Rosetta2 (DE3) - contain the pRARE plasmid, with **chloramphenicol** resistance
3. BL21 pBirA (DE3) - contains pBirA plasmid for *in vivo* biotinylation, which has **chloramphenicol** resistance
4. BL21 pGroESL (DE3) - contains pGroESL, encoding for chaperone proteins, **kanamycin** resistance
5. BL21 pBirA pGroESL (DE3) - both **chloramphenicol** and **kanamycin** resistance
6. DH5alpha - no antibiotic
7. XL1-Blue - no antibiotic
8. BL21 Gold (DE3) - no antibiotic

Day 2

- 2 Select a well-isolated colony and inoculate into 3 mL LB broth with proper antibiotics, and shake overnight at 37°C at 200 rpm.

Day 3

- 3 Take 0.5 mL of your overnight culture and add to a 100 mL flask of TYM broth (recipe in materials). Put in shaker at 37°C.

Note

At this point, you can also make a glycerol stock (1:1 ratio of 50% glycerol solution to cell suspension) of this culture for long-term storage. You will still need to re-streak this stock in the future.

- 4 After 1 hour, check the OD of the culture. Take note of this OD, and understand that *E. coli* generally divide every 20-30 minutes, meaning the OD will double every 20-30 minutes.
- 5 After the initial 1 hour check, check the OD every 30 minutes after, and note the increase. It is absolutely essential to remove the cells from the shaker when the OD has reached 0.3-0.4. See notes below for suggestions.

Note

This step is absolutely critical - if the OD goes over 0.4, you should not proceed with the rest of this protocol, and instead start the day 3 protocol again. In order to not miss this OD window, calculate the approximate time your cells will reach the target OD based on the 1 hour OD.

For example - assume the cells divide every 20 minutes, and after 1 hour there is an OD of 0.05. Use this formula: $0.3 = 0.05 \times 2^x$. Solve for x. In this example, it would take ~2.5 doubling times (2.5 x 20 minutes) to reach the target OD.

While this calculation is helpful, do not rely on it entirely. I recommend after the initial 1 hour measurement to measure every half an hour.

If the cells are approaching the OD target, for example they are at OD = 0.27, remove the cells and proceed with the next step. They will continue dividing, albeit a bit slower. You will still hit the target OD.

- 6 After your cells have reached no higher than OD = 0.3-0.4, remove flask from incubator and place on wet ice. Swirl the flask on ice every few minutes until culture is cold. Let cells rest on ice for 20 minutes.
- 7 Divide 100 mL culture into two 50 mL pre-chilled conical tubes. For best results, if possible, perform the rest of this protocol in a 4°C room.
- 8 Spin the cells in a 4°C centrifuge at 1000 xG for 10 minutes using a fixed-angle rotor.
- 9 Carefully return cells to wet ice and pour off all media, aspirating if necessary but do not suck up the cells.
- 10 Gently add 20 mL ice-cold TFB1 buffer (recipe in materials) to each tube. ONLY swirl gently to resuspend the pellet. **Do not vortex, do not pipette up and down.**
- 11 Combine both tubes, so that you now have one tube with 40 mL cell suspension. Allow the tube to rest on ice for 30-60 minutes.



- 12 Spin cells in 4°C centrifuge at 1000 xG for 10 minutes.
- 13 Pour off supernatant, again being careful to not disturb the pellet. It is better to leave a small amount of TFB1 buffer than to aspirate the cells.
- 14 Add 4 mL ice-cold TFB2 buffer. Swirl gently to resuspend the cells.
- 15 Make 50 uL aliquots of cells into pre-chilled tubes in a pre-chilled tube rack, using pre-chilled pipette tips. If possible, use a multi-pipette to make this procedure faster. As soon as all tubes have been filled, close and transfer the tubes to ice.
- 16 Flash freeze the cells with liquid nitrogen and immediately put in -80°C freezer.
- 17 To test your competent cells, use a plasmid which you know transforms well, or use a pUC19 plasmid which is often used to quantitate efficiency of transformation.