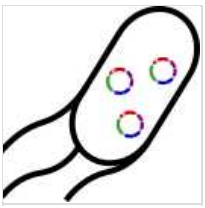

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Transforming E. coli (Instructor protocol)

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Yeast ORFans CURE

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ABSTRACT

This is the instructor protocol for

Transforming E. coli
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PREVIEW RUN

Setup for this lab can be pretty intensive if you're starting from scratch. It requires:

- Competent E. coli
- LB-agar + kanamycin plates
- SOC outgrowth media

Notably, commercial competent E. coli are *really* expensive. We use the Zymo kit, which brings the cost down to ~50 cents per transformation. This protocol makes about 100 transformations, and the cells are competent enough for subcloning, regular restriction-and-ligation cloning, and Golden Gates.

In an attempt to make this work more widely accessible, I have been *extremely* verbose in these protocols. If you are familiar with recombinant DNA work, you likely have your own protocols for making chemically competent E.coli, pouring plates, and making SOC -- use those!

PROTOCOL CITATION

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KEYWORDS

competent, E. coli, outgrowth, selection

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MATERIALS TEXT

Equipment

- Autoclave
- several 1L and 250 ml bottles
- A 250 ml baffled flask
- Shaking incubator
- Spectrophotometer & micro cuvettes
- Nanodrop (or similar instrument for measuring DNA concentration, such as a Qbit or Dynaquant)
- **Refrigerated** swinging-bucket or high-speed centrifuge
- Cold room (not strictly required but highly recommended)
- **-80 °C** freezer

Materials and Reagents

- Microcentrifuge tubes, sterile
- 15 ml and 50 ml conical centrifuge tubes

  Petri dish, 10cm, polystyrene **Fisher**
- **Scientific Catalog #FB0875712** In 2 steps
-

  Kanamycin **Research Products International**

  (rpi) Catalog #K22000-25.0 Step 2

  (or kanamycin from another vendor, or kanamycin solution at [M]**50 mg/mL**)

  10 mL syringes **BD**
- **Biosciences Catalog #BD 309695** Step 4

  0.2 µm syringe
- filter **Corning Catalog #CLS431212** Step 4

Tryptone **Fisher**

- **Scientific Catalog #BP1421-500** In 2 steps

  BD Bacto™ Yeast Extract **BD**

- **Biosciences Catalog #212750** In 2 steps

  Sodium Chloride **Fisher**

- **Scientific Catalog #S271** In 2 steps

  Potassium chloride **Sigma**

- **Aldrich Catalog #P9333** Step 8

- Magnesium chloride hexahydrate **Sigma Aldrich** Step 8

  Magnesium sulfate heptahydrate **Sigma**

- **Aldrich Catalog #M2773** In 2 steps

  α-D-Glucose **Sigma**

- **Aldrich Catalog #158968** Step 11 solution,

  [M]**40 Mass Percent** (autoclaved or filter-sterilized)

  Agar, bacteriological

- grade **Amresco Catalog # J637** Step 13

  Magnesium sulfate heptahydrate **Sigma**

- **Aldrich Catalog #M2773** In 2 steps

- LB Broth **Contributed by users** Step 24

- Mix & Go! E.coli Transformation Buffer Set **Zymo Research Catalog #T3002** Step 29

  Monarch Plasmid Miniprep

- Kit **NEB Catalog #T1010** (or equivalent miniprep kit)

- Monarch DNA Elution Buffer - 25 ml **New England Biolabs Catalog #T1016L** Step 44

  Glass beads 5 mm **VWR**

- **Scientific Catalog #26396-596** Step 45

SAFETY WARNINGS

Several of these chemicals are moderately hazardous, particularly the ones in the miniprep kit. Wear appropriate PPE, including gloves, safety glasses and a lab coat.

This protocol involves the creation or manipulation of genetically modified organisms. Make sure cultures and contaminated plastics are disposed of only after inactivating the GMOs, such as by autoclaving or treating with bleach.

Prepare kanamycin stock solution

1 Prepare 9 empty microcentrifuge tubes in a rack.

2 Weigh **0.5 g** of Kanamycin **Research Products International (rpi) Catalog #K22000-25.0** into a 15 ml conical centrifuge tube.

3 Add **10 mL** deionized water to make a stock solution with a concentration of **50 mg/mL** . Vortex to dissolve.

4 Mount a 0.2 µm syringe filter **Corning Catalog #CLS431212** on a 10 mL syringes **BD Biosciences Catalog #BD 309695** . Pull the plunger out of the back and pour the kanamycin solution in.

5 Holding the syringe filter over the first microcentrifuge tube, insert the plunger back into the syringe. Squeeze the syringe to filter the kanamycin into the waiting tubes. Put about **1.2 mL** into each tube. You don't have to be precise, but make sure there's at least **1 mL** in each.

6 Use immediately or store at **-20 °C** .

Making SOB and SOC outgrowth media

7 In a 250 ml bottle, add approximately **200 mL** deionized H2O.

8 Add to the bottle:

- **5 g** Tryptone **Fisher Scientific Catalog #BP1421-500**
- **1.25 g** BD Bacto™ Yeast Extract **BD Biosciences Catalog #212750**
- **0.145 g** Sodium Chloride **Fisher Scientific Catalog #S271** (or **0.5 mL** of a **5 Molarity (M)** solution)
- **0.0475 g** Potassium chloride **Sigma Aldrich Catalog #P9333** (or **125 µL** of a **1 Molarity (M)** solution)
- **2.5 mL** **1 Molarity (M)** solution of Magnesium chloride hexahydrate **Sigma Aldrich**
- **2.5 mL** **1 Molarity (M)** solution of Magnesium sulfate heptahydrate **Sigma Aldrich Catalog #M2773**

9 Add deionized water to a final volume of 250 ml. (You can eyeball it -- no need to dirty a graduated cylinder).

10 Autoclave at **121 °C** on a liquid cycle for **00:30:00** (30m). (This media is SOB - "Super Optimal Broth")

11 Using good sterile technique, make **5 mL** aliquots into 15 ml conical centrifuge tubes, 1 per 4 people. To each aliquot, add **50 µL** **40 Mass / % volume** α-D-Glucose **Sigma Aldrich Catalog #158968**. (These aliquots are SOC - "Super

Optimal Broth, Catabolic")

Make several extra! These become contaminated really easily.

Pour LB-agar + kanamycin plates

12 Fill a 1 liter screw-cap bottle with approximately **900 mL** of deionized water.

13 Add:

- **5 g** BD Bacto™ Yeast Extract **Biosciences Catalog #212750**
- **10 g** Tryptone **Scientific Catalog #BP1421-500**
- **10 g** Sodium Chloride **Fisher Scientific Catalog #S271**
- **20 g** Agar, bacteriological grade **Amresco Catalog # J637**

14 Add water to a total volume of **1 L** (eyeballing is OK, no need to dirty a graduated cylinder). Cap and shake to mix.

Make sure you get all of the powder off of the bottom of the bottle. It doesn't have to be completely dissolved, just resuspended.

15 Loosen the cap and autoclave at **121 °C** for 30 minutes on a liquid cycle.

16 Swirl to mix well, then cool the bottle to at or below **60 °C** . You can do this in a water bath, or by swirling under a running cold water tap.

If the media is too hot when you add the antibiotic, it will break down.

My old grad student mentor used to say "if you can hold your hand against it for 60 seconds, it's cool enough." Or you could use an infrared thermometer gun. It's useful for the bottle to be cool enough to hold bare-handed, though!

17

Optional: pour several plates without any antibiotic. They'll be useful below!

18 Add **1 mL** of **50 mg/mL** kanamycin solution and swirl to mix well.

19 Option A: Pour ~15 ml of molten media into each Petri dish, 10cm, polystyrene **Fisher Scientific Catalog #FB0875712** , enough to cover the bottom . If you make 1 L of media, you'll use about 2 sleeves (25/sleeve).

20 Option B: Using a 25 ml pipette and a pipettor, pipette ~15 ml of molten media into each Petri dish, 10cm, polystyrene **Fisher Scientific Catalog #FB0875712** , enough to cover the bottom . If you make 1 L of media, you'll use about 2 sleeves (25/sleeve).

21 Leave the plates **Overnight** on the bench to cool.

22

Put the petri dishes back in their plastic bags and store **inverted** at **4 °C** . Plates are good for at least 3 months.

Making chemically competent E. coli

23 Two days before the prep, strike out the E. coli cloning strain (from a freezer stock) on an LB-agar plate (no antibiotics!). Incubate at **37 °C** **Overnight** . 30m

24 The afternoon before the prep, pick a colony off of the plate and start an overnight culture in **5 mL** LB Broth **Contributed by users** (in a round-bottomed test tube.) Shake **200 rpm, 37°C** **Overnight** 30m

25 Transfer 50 ml of SOB (above) to a 250 ml baffled flask.

26 Magnesium sulfate heptahydrate **Sigma**
Add **0.246 g** **Aldrich Catalog #M2773**

No, this isn't sterile -- but the culture won't be growing long enough for it to be a problem.

27 Add **0.5 mL** of the overnight culture.

28 Fold a piece of aluminium foil over the mouth of the flask. Shake **200 rpm at room temperature** until the OD600 of the culture reaches 0.4-0.6. This usually takes about 3 hours.

Check the culture every hour until the OD600 reaches 0.2, then ever 30 minutes until it is between 0.4 and 0.6. *Do not overgrow the cells; if they are overgrown, throw out the culture and begin again from step 27.*

29 While the culture is growing:

- Chill a swinging-bucket or high-speed centrifuge to **4 °C**
- Load 96 microcentrifuge tubes into a 96-position tube rack and place them in the cold room to chill (or on ice). Tent a paper towel over them to keep out contaminating microbes
- From the
  Mix & Go! E.coli Transformation Buffer Set **Zymo**
  Research Catalog #T3002 kit,
  mix 2.5 ml of 2X Wash Buffer and 2.5 ml Dilution Buffer to prepare 5 ml of Wash Buffer in a conical centrifuge tube. Keep on ice.
- From the
  Mix & Go! E.coli Transformation Buffer Set **Zymo**
  Research Catalog #T3002 kit,
  mix 2.5 ml of 2X Competent Buffer and 2.5 ml Dilution Buffer to prepare 5 ml of Competent Buffer in a conical centrifuge tube. Keep on ice.
- Pre-chill a 50 ml conical centrifuge tube on ice.

30 When the culture has reached an OD600 of between 0.4 and 0.6, transfer the baffled flask to an ice bucket and mound up the ice around the flask. Chill for 10 minutes.

From this point on, everything must be kept as cold as possible!

31 10m Transfer the culture from the 250 ml baffled flask to the 50 ml pre-chilled conical centrifuge tube. Centrifuge in a pre-chilled swinging-bucket centrifuge (or high-speed fixed-angle centrifuge) **2000 x g, 4°C, 00:10:00** .

Move the conical from the ice bucket, to the centrifuge, and back to the ice bucket.

32 In the cold room (if available), decant the media back into the baffled flask, then invert the 50 ml conical onto a paper towel for a minute to let the media drain away.

33 Resuspend in 5 ml 1X Mix&Go Wash Buffer by gentle votexing (ie, on a setting of 3-4). Be patient, it will take a few minutes for the cells to resuspend at this speed. Put the resuspended cells back on ice.

If you don't have a cold room, alternate between vortexing and incubating on ice. Remember, cold is key!

34 Centrifuge in a pre-chilled swinging bucket (or high-speed fixed-angle) centrifuge 5m
2000 x g, 4°C, 00:05:00

35 In the cold room, decant the wash buffer, then invert the 50 ml conical onto a paper towel for a minute to let the wash buffer drain away.

36 Resuspend the pellet in 5 ml 1X Mix&Go Competent Buffer by gentle votexing (ie, on a setting of 3-4). Be patient, it will take a few minutes for the cells to resuspend at this speed. Put the resuspended cells back on ice.

37 Pipette 50 ul aliquots into the prepared microcentrifuge tubes.

An electronic pipettor or a repeat pipettor can be a real time-saver here!

38 Optional but highly recommended - snap-freeze the cells in liquid nitrogen.

39 Transfer the tubes to a -80°C freezer, trying to minimize the time between cold room and freezer.

Competent cells prepared this way last for at least a year with no practical decrease in transformation efficiency.

Prepare the transformation control

40 Two days before: strike out the E. coli harboring the YTK96 plasmid on a LB agar + kanamycin plate

41 The day before: Pick one colony of the YTK96-harboring E. coli into 5 ml LB broth + 50 ug/ml

kanamycin.

42 Miniprep the plasmid, using ☒Monarch® Plasmid Miniprep Kit **New England Biolabs Catalog #T1010** or comparable.

43 Analyze the concentration and purity of the miniprep using a Nanodrop.

44 Prepare transformation controls by diluting the YTK96 miniprep to a concentration of 1 ng/ul in ☒Monarch DNA Elution Buffer - 25 ml **New England Biolabs Catalog #T1016L** or similar.

I generally make this easy - if the concentration is, say, 67 ng/ul, then I put **67 µL** of elution buffer in a tube and add **1 µL** of miniprep.

Prepare several tubes of control plasmid.

Miscellaneous preparation

45 ☒Glass beads 5 mm **VWR** Pour the **Scientific Catalog #26396-596** into several 15 ml conical centrifuge tubes.

Depending on the bottle, it may be easier to pour them into a 50 ml tube first, then into 15 ml tubes.

Instructor Tips & Common Student Errors

46 Instructor Tips

- *Do not decrease the incubation times, especially for the outgrowth.* I tried decreasing the outgrowth time to 30 minutes, once, and every single transformation failed. Kanamycin is not like ampicillin -- it doesn't just inhibit growth, it actually kills the cells. So they need to be expressing the resistance gene before they are challenged with the antibiotic.
- I recommend testing the competent cells to make sure they are actually competent before handing them to your students.
- Especially the first time, a two-hour lab may not be enough time for the 90 minutes of (total) incubation. I will often instruct students to label and prepare their plates with beads and leave them on the bench for me -- and then, after their incubations are done, I'll plate their cells for them. Timing isn't important here -- I've seen successful transformations after even 3 hours of outgrowth.
- Make sure the water bath and incubator are turned on well before lab starts -- they take a while to come to temperature!
- If you're using a dry bath instead of a big water bath, fill the dry bath's wells with water to increase the efficiency of heat transfer.
- If you don't have an incubating shaker, it's not the end of the world -- a **37 °C** incubator is probably fine.

47 Common Student Errors

- Didn't check the SOC and used a contaminated tube.
- Added the positive control AND their ligation to the competent E. coli.