Protocol

Rapid and Efficient Plasmid Construction by Homologous Recombination in Yeast

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The cloning of DNA fragments is a fundamental aspect of molecular biology. Traditional DNA cloning techniques rely on the ligation of an insert and a linearized plasmid that have been digested with restriction enzymes and the subsequent introduction of the ligated DNA into *Escherichia coli* for propagation. However, this method is limited by the availability of restriction sites, which often becomes problematic when cloning multiple or large DNA fragments. Furthermore, using traditional methods to clone multiple DNA fragments requires experience and multiple laborious steps. In this protocol, we describe a simple and efficient cloning method that relies on homologous recombination in the yeast *Saccharomyces cerevisiae* to assemble multiple DNA fragments, with 30-bp homology regions between the fragments, into one sophisticated construct. This method can easily be extended to clone plasmids for other organisms, such as bacteria, plants, and mammalian cells.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Agarose gel (1.2%) and electrophoresis reagents
Bacterial strains
Any standard Escherichia coli *strain such as Top10 will suffice.*
Deionized water (sterilized)
Dimethyl sulfoxide (DMSO)
dNTPs (10 mM)
Ethanol (70%)
Glass beads (0.4–0.6 mm)
Glycerol (10%)
Isopropanol
Lithium acetate (LiAc)

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TABLE 1. Sequence of the oligonucleotides used in this protocol

Name	Sequence (5′–3′)
ori-F	GATACTAACGCCGCCATCCAGTTTCCCGGGaaaggcggtaatacggtta
ori-R	CCCGGGttgataatctcatgaccaaaatcc
ampR-F	TGGTCATGAGATTATCAACCCGGGaaaggatcttcacctagatcct
ampR-R	GGGcacttttcggggaaatgtgcg
CEN-F	GTTCCGCGCACATTTCCCCGAAAAGTGCCCGGGtccttttcatcacgtgc
CEN-R	GGGcttaggacggatcgcttgc
LEU2-F	AGTTACAGGCAAGCGATCCGTCCTAAGCCCGGGaactgtgggaatactcaggt
LEU2-R	Cgtgtcgtttctattatgaatttc
LYS2-F	TTTATAAATGAAATTCATAATAGAAACGACCCGGGcttcaatagttttgccagcg
LYS2-R	GCTCCCGGGcatatcatacgtaatgctca
URA3-F	TTGAGCATTACGTATGATATGCCCGGGagcttttcaattcatcttttttttttttgttc
URA3-R	GGGtaataactgatataattaaattgaagc
HIS3-F	GCTTCAATTTAATTATATCAGTTATTACCCGGGcttcattcaacgtttcccatt
HIS3-R	GGGtgatgcattaccttgtcatc
kanR-F	TACTGAAGATGACAAGGTAATGCATCACCCGGGtagcccatacatccccatgt
kanR-R	CCCGGGTAAATCACGCTAACATTTGA

All oligonucleotides are desalted and <60 bp in length. The sequences shown in lower case are homologous to sequences upstream (F) or downstream (R) of the template gene or origin; the sequences shown in upper case are homologous to one of the other fragments; the SmaI sites are underlined.

To prepare a 1 M solution, dissolve 10.2 g of LiAc in 100 mL of deionized water, filter-sterilize, and store at room temperature.

Luria–Bertani (LB) medium plus ampicillin <R>
Lysis buffer for yeast <R>
Milli-Q water
Oligonucleotides (desalted)

For a list of all the oligonucleotides used in this protocol, see Table 1.

Phenol (saturated)
Phusion high-fidelity DNA polymerase and buffer (5×)

For example, consider using Phusion Hot Start II DNA Polymerase (Thermo Scientific); kit includes bespoke buffers.

Plasmid miniprep kit
Polyethylene glycol (PEG) 3350

Prepare 100 mL of a 50% solution by dissolving 50 g of PEG3350 in 50 mL deionized water, adjusting the volume to 100 mL with deionized water, followed by filter sterilization.

Single-stranded DNA (ssDNA; e.g., Sigma-Aldrich D8899)
SmaI restriction endonuclease
Synthetic amino-acid-dropout medium (SD-all) <R>
Synthetic lysine-dropout medium (SD-lys) <R>
Terrific broth (TB) medium <R>
Tris–EDTA (TE) buffer (10×)

Add 0.2 mL of EDTA (0.5 M, pH 8.0) and 1 mL of Tris–Cl (1 M, pH 8.0) to 99 mL of deionized water. Filter-sterilize. Store at room temperature.

Yeast extract-peptone-dextrose (YEPD) <R>
Yeast strains

This protocol uses BY4709 MATα ura3Δ0 (ATCC200872), BY4712 MATα leu2Δ0 (ATCC200875), and BY4742 MATα his3Δ1 leu20 lys2Δ0 met15Δ0 ura3Δ0 (ATCC201389), but many other standard laboratory yeast strains can also be used.

Equipment

Agarose gel electrophoresis materials
Benchtop centrifuge/microcentrifuge

Electroporation system (e.g., MicroPulser by Bio-Rad)
Ice
Incubators (30°C and 37°C)
Microcentrifuge tubes (1.5-mL)
Orbital shaker
PCR tubes
Pipettes
Plates
Thermal cycler (PCR machine)
Vortex
Water bath (42°C)

METHOD

*To illustrate the cloning of multiple DNA fragments into one construct by homologous recombination, we describe the assembly of five different yeast selection markers (*HIS3, LEU2, LYS2, URA3, *and* kanMX6*) together with origins of replication for both yeast and* E. coli *(*CEN6/ARS4 *and* ori*) and an* E. coli *selection marker (*ampR*) into one plasmid (Fig. 1A).*

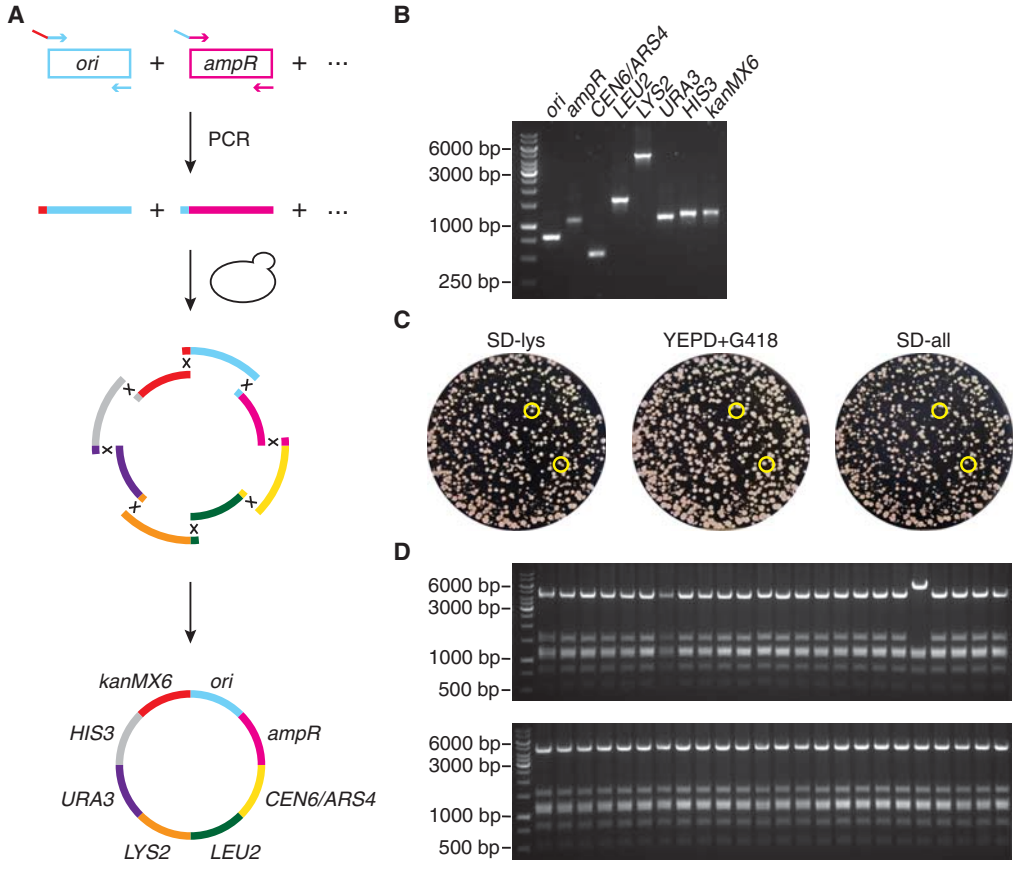

FIGURE 1. Rapid and efficient plasmid construction by homologous recombination in yeast. (*A*) A schematic representation of the described assembly of eight DNA fragments in yeast using 30-bp recombination sequences. (*B*) The PCR fragments used in the featured assembly. (*C*) The selection of yeast transformants on synthetic lysine-dropout medium (SD-lys) and subsequent confirmation of the presence of the six fragments containing yeast sequences by replica plating on YEPD + G418 and synthetic-dropout medium without amino acids (SD-all). Only two transformants fail to grow after replica plating (yellow circles). (*D*) The SmaI profiles of the final plasmids isolated from 24 yeast colonies (*upper* panel) or 24 *E. coli* colonies (*lower* panel).

Preparation of DNA Fragments

This procedure should take 3–4 h on day 1.

1. Prepare genomic DNA from a mixture of the yeast strains BY4709 and BY4712 to serve as a template in the polymerase chain reaction (PCR) (see Steps 23–35).
2. Prepare a 20-µL PCR.

Sterile deionized water	13.4 µL
Buffer (5×)	4 µL
dNTP mixture (10 mM)	0.4 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Template DNA	1 µL
Phusion DNA polymerase	0.2 µL

3. Use the following PCR cycling conditions.

Initial denaturation	60 sec at 98°C
5 Cycles	10 sec at 98°C, 20 sec at 55°C, and 90 sec at 72°C
25 Cycles	10 sec at 98°C, 20 sec at 62°C, and 90 sec at 72°C

HIS3, LEU2, LYS2, and URA3 *can be amplified from ~100 ng of genomic DNA of a mixture of the yeast strains BY4709 and BY4712.* CEN6/ARS4, ampR, *and* ori *can be amplified from 1 pg of pRS416 (Sikorski and Hieter 1989), and* kanMX6 *can be amplified from 1 pg of pFA6-*kanMX6 *(Bahler et al. 1998).*

4. Run 2 µL of each PCR product on a 1.2% agarose gel (Fig. 1B).

If a PCR product contains multiple fragments, all fragments can potentially be assembled in the final construct. In this case, purification of the correct PCR product from the gel might be necessary. Alternatively, the unpurified PCR product with multiple fragments can be used, in which case the number of colonies that have to be screened for the expected construct has to be increased.

The PCR products can be stored at 4°C or –20°C.

Preparation of Competent Yeast Cells

The following procedure is a modified version of Gietz's method (Gietz and Woods 2002) and should take 5 min on day 1 and 10–15 min on day 2.

5. Patch the yeast strain BY4742 on a 2-cm^2 area on YEPD agar and incubate overnight at 30°C.

Alternatively, the yeast strain can be used to inoculate a 5-mL culture of liquid YEPD medium. Incubate overnight at 30°C while shaking on an orbital shaker at 200 rpm. Any standard laboratory yeast strain, such as BY4741 or W303, can be used instead of BY4742.

6. Scrape a 50-µL portion of yeast cells from the YEPD plate and resuspend the cells in 1 mL of sterile deionized water in a 1.5-mL microcentrifuge tube.

Alternatively, harvest 1–1.5 mL of a liquid culture.

7. Pellet the cells by centrifugation at 3000 rpm (800 rcf) for 1 min at room temperature in a microcentrifuge and discard the supernatant.
8. Wash the pellet once with 1 mL of 0.1 M LiAc.
9. Estimate the volume of the cell pellet and resuspend the cells in an equal volume of 0.1 M LiAc supplemented with 10% glycerol.

By using larger volumes of liquid culture in Steps 5 and 6, a large amount of competent cells can be made at once. These competent cells can be stored at –80°C in 0.1 M LiAc supplemented with 10% glycerol.

Yeast Transformation

This procedure is a modified version of Gietz's method (Gietz and Woods 2002) and should take 3 h (not including the time needed to grow the yeast cells) on day 2.

10. Prepare the transformation buffer by mixing 800 µL of 50% PEG3350, 100 µL of 1 M LiAc, 100 µL of 10× TE, and 50 µL of DMSO.
11. Boil the ssDNA (10 mg/mL) for 5 min and place it on ice.
12. Add ~100 ng of each DNA fragment, 2 µL of ssDNA, and 12 µL of competent yeast cells into a 1.5-mL microcentrifuge tube; mix gently by pipetting up and down.

    If the total volume of all the DNA fragments together is >20 µL, the overall transformation efficiency will decrease. The volume can be reduced by air-drying the DNA overnight at room temperature.
13. Add 100 µL of the transformation buffer from Step 10 and vortex for ~10 sec.
14. Incubate for 30 min at room temperature.
15. Incubate for 15 min in a 42°C water bath.
16. Incubate on ice for 5 min.
17. Pellet the cells by centrifugation at 3000 rpm (800 rcf) for 1 min and remove the supernatant.
18. Resuspend the cells in 1 mL of YEPD.
19. (Optional) Incubate the cells for 2 h at 30°C.

    This step allows the cells to produce the antibiotic resistance and/or auxotrophic marker proteins before applying selection and thereby increases the overall transformation efficiency.
20. Plate 250 µL of the cell suspension on SD-lys plates.

    Other media that select for one of the other cloned genes can also be used.
21. Incubate the plates for 2–3 d at 30°C.
22. Replica-plate the colonies on SD-all and YEPD + G418 plates (Fig. 1C).

    *The SD-lys plates select only for one of the yeast markers (*LYS2*) and for presence of the* CEN6/ARS4*. This replica-plating step tests whether the other yeast selection markers are present in the construct and functional. We have obtained 470 colonies on the SD-lys plate, only two of which failed to grow on the YEPD + G418 (which selects for* kanMX6*) and SD-all (which selects for* HIS3*,* LEU2*, and* URA3*) plates.*

Preparation of Yeast Genomic DNA

This procedure should take 1 h on day 4.

23. Wash all the colonies from the SD-lys plate from Step 21 using 5 mL of sterile water and transfer 1 mL of cells to a 1.5-mL microcentrifuge tube.
24. Pellet the cells by centrifugation at 3000 rpm (800 rcf) for 1 min and remove the supernatant.
25. Resuspend the pellet in 250 µL of lysis buffer for yeast.
26. Add 250 µL of saturated phenol and ~200 µL (~200 mg) of glass beads.
27. Close the cap tightly and vortex for >2 min at room temperature.
28. Centrifuge at 13,000 rpm (15,700 rcf) for 5 min.
29. Transfer 150 µL of the aqueous top layer to a new 1.5-mL microcentrifuge tube and centrifuge again at 13,000 rpm (15,700 rcf) for 5 min.
30. Transfer 100 µL of the top layer to a new 1.5 mL microcentrifuge tube, add 100 µL of 100% isopropanol and mix thoroughly by inversion.
31. Centrifuge at 13,000 rpm (15,700 rcf) for 10 min and remove the supernatant.
32. Wash the pellet once with 500 µL of 70% ethanol.
33. Briefly centrifuge at 13,000 rpm (15,700 rcf) for 10 sec and remove the remaining ethanol.
34. Dry the pellet at room temperature for ~10 min.
35. Dissolve the pellet in 40 µL of sterile water.

Preparation of Competent *E. coli* Cells

This procedure should take 10 min on day 4.

36. Inoculate 2 mL of TB using one bacterial colony and incubate overnight at 37°C while shaking at 250 rpm.

    In the example described here, we use Top10 cells.

37. Add 2 mL of TB and incubate for another 25 min at 37°C while shaking at 250 rpm in an orbital shaker.

38. Divide the culture into aliquots of 1 mL.

    This is approximately the amount of culture needed for one transformation.

39. Collect the cells by centrifuging at 13,000 rpm (15,700 rcf) for 1 min.

40. Wash the cells four times with Milli-Q water.

41. Estimate the volume of the cell pellet and resuspend the cells in an equal volume of Milli-Q water.

    More water (up to five times the volume of the cell pellet) can be used to obtain "more" competent cells. However, the transformation efficiency will slightly decrease because the cells are more diluted. By using greater volumes of liquid culture in Steps 36–41, a large number of competent cells can be made at once. These competent cells can be stored at –80°C in 10% glycerol.

    Other methods can be used to make competent E. coli *cells, but these will require longer preparation times. Also, with this method, all the steps after growing the culture can be performed at room temperature, which makes it more straightforward and robust.*

Plasmid Recovery from *Escherichia coli*

This procedure should take ~5 h on day 5 and day 6.

42. Add 20 µL of competent *E. coli* cells to a 200-µL PCR tube and add 0.5 µL of yeast genomic DNA.

43. Mix the DNA with the competent cells by pipetting up and down.

44. Transfer the mixture to an electroporation cuvette (1 mm gap).

45. Electroporate the cells according to the manufacturer's manual.

46. Add 250 µL of LB and plate all the cells on an LB plate containing ampicillin.

    Note that kanMX6 *is functional in both* S. cerevisiae *and* E. coli. *Therefore, transformants can also be selected on LB media containing kanamycin.*

47. Incubate the plates overnight at 37°C.

48. Purify the plasmid DNA with a plasmid miniprep kit.

49. Confirm the correct assembly of the fragments by a SmaI digestion (Fig. 1D).

    In the example described here, SmaI restriction sites were added to each fragment by means of the primers to show the accuracy of homologous recombination in yeast. However, the addition of restriction sites is not necessary for this method.

DISCUSSION

Homologous recombination in yeast has been successfully used in the cloning of both natural and synthetic DNA fragments (Ma et al. 1987; Wang 2000; Chen et al. 2005; Gibson et al. 2008a,b; Gibson 2009; Shao and Zhao 2009; Tan and Tan 2010; Tsvetanova et al. 2011; Liang et al. 2012; Shao and Zhao 2013). However, many of these methods are based on yeast–bacterial shuttle plasmids, which rather restricted the technology to a small niche within molecular biology. For an introduction to these technologies, see Introduction: **Construction of Multifragment Plasmids by Homologous Recombi-**

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nation in Yeast (van Leeuwen et al. 2015). Recently, however, Kuijpers et al. (2013) have presented an efficient strategy for assembling a plasmid from mutiple DNA fragments by using overlapping homology regions of 60 bp. As this method does not make use of a vector backbone, it can easily be extended to construct plasmids for other organisms.

In this protocol, we have described the use of yeast as a host to assemble eight DNA fragments, with short homology regions (30 bp) between the fragments, into one plasmid (Fig. 1A). Equal amounts of PCR products were cotransformed into yeast, and transformants were selected on synthetic media lacking lysine (SD-lys), which confirms the presence and functionality of *LYS2* and the *CEN6/ARS4* origin of replication. The presence and activity of *HIS3*, *LEU2*, *URA3*, and *kanMX6* were confirmed by replica plating (Step 22). Only two out of 470 transformants selected on SD-lys failed to grow after replica plating. The genomic DNA was individually purified from 24 randomly picked yeast colonies from the SD-lys plate and from 24 randomly picked *E. coli* colonies from Steps 46–48, followed by recovery of the plasmids. SmaI sites were introduced between all the fragments when the primers were designed, which enabled confirmation of the correct assembly of the construct by a SmaI digestion. In one out of the 48 isolated plasmids, a SmaI site was missing, which could be caused by impurities in the primers used, whereas the other 47 plasmids all showed the expected DNA bands (Fig. 1D). Based on the growth phenotypes on the different media and the SmaI digestion profiles, we can conclude that the overall assembly efficiency is >95%.

This protocol uses short (<60 bp), desalted oligonucleotides and unpurified PCR products. This makes the method highly cost- and time-efficient, especially when cloning multiple fragments. We modified the yeast and bacterial transformation methods, which facilitates high-throughput cloning. However, this method has the common disadvantage that inserts that are toxic to either yeast or bacteria cannot be cloned. Also, because homologous recombination is so efficient in yeast, the cloning of inverted or repetitive sequences can be problematic.

Interestingly, we have found that the *kanMX6* selector module is functional both in yeast and bacteria, which reduces the number of required selection markers from two (one for yeast and one for *E. coli*) to one. Although, in the example described here, separate fragments are used for the *E. coli* selection marker and origin of replication (*ampR* and *ori*), we have routinely used the shortest backbone of pBlueScript II (Alting-Mees and Short 1989), which contains both *ori* and *ampR*, as one fragment in our assemblies. Finally, this method can be extended to clone constructs for other organisms by combining one or more fragment(s) with the genes or sequences of interest with a fragment containing a linearized nonyeast plasmid, and another fragment containing a *CEN/ARS* origin and yeast selection marker.

RECIPES

Luria–Bertani (LB) Medium Plus Ampicillin

Reagent	Quantity
Agar	20 g
NaCl	10 g
Tryptone	10 g
Yeast extract	5 g

Prepare the above-listed ingredients in 1 L of deionized water. Adjust the pH to 7.0 with 5 N NaOH. Autoclave for 20 min at 15 psi (1.05 kg/cm^2). Cool to ~60°C and add ampicillin (final concentration 120 µg/mL). Pour the medium into Petri dishes (~25 mL per 100-mm plate). Store the LB plates at 4°C; they will keep for at least 4 mo.

Lysis Buffer for Yeast

Reagent	Quantity	Final concentration
Triton X-100	10 mL	2% (v/v)
SDS (10%)	50 mL	1% (w/v)
NaCl (5 M)	10 mL	100 mM
Tris-Cl (1 M, pH 8.0)	5 mL	10 mM
EDTA (0.5 M, pH 8.0)	1 mL	1 mM

Prepare in 500 mL of deionized water. Autoclave for 20 min at 15 psi (1.05 kg/cm^2). Store at room temperature; it will keep for at least 1 yr.

Supplements for SD-Lys

Reagent	Quantity
L-Leucine	0.84 g
L-Histidine HCl	0.42 g
L-Methionine	0.42 g
Uracil	0.25 g

In separate tubes, prepare 100× stocks of each of the four reagents by dissolving the quantities indicated in 100 mL of deionized water and then filter-sterilizing. Store all stocks at room temperature; they will keep for at least 6 mo.

Synthetic Amino-Acid-Dropout Medium (SD-All)

Reagent	Quantity	Final concentration
Difco yeast nitrogen base without amino acids	6.7 g	6.7 g/L
Agar	20 g	20 g/L
Dextrose (40%)	50 mL	20 g/L

Add 950 mL of deionized water to 6.7 g Difco yeast nitrogen base without amino acids and 20 g of agar. Autoclave for 20 min at 15 psi (1.05 kg/cm^2). After autoclaving, add 50 mL of a 40% dextrose solution. Cool the medium to ~60°C and pour into Petri dishes (~25 mL per 100-mm plate). Store the SD-all plates at 4°C; they will keep for at least 6 mo.

Synthetic Lysine-Dropout Medium (SD-Lys)

Reagent	Quantity	Final concentration
Difco yeast nitrogen base without amino acids	6.7 g	6.7 g/L
Agar	20 g	20 g/L
Supplements for SD-lys:		
L-Leucine (100×)	10 mL	8.4 mg/L
L-Histidine HCl (100×)	10 mL	4.2 mg/L
L-Methionine (100×)	10 mL	4.2 mg/L
Uracil (100×)	10 mL	2.5 mg/L
Dextrose (40%)	50 mL	20 g/L

Combine 10 mL of each of the SD-lys supplements (100× L-leucine, 100× L-histidine HCl, 100× L-methionine, 100× uracil) with 6.7 g of Difco yeast nitrogen base without amino acids and 20 g agar, and add 950 mL of deionized water. Autoclave for 20 min at 15 psi (1.05 kg/cm^2). After autoclaving, add 50 mL of a 40% dextrose solution. Cool the medium to ~60°C and pour into Petri dishes (~25 mL per 100-mm plate). Store the SD-lys plates at 4°C; they will keep for at least 6 mo. (Note that this recipe is optimized for BY4742 strains. It should be adjusted if another yeast strain is used as a recombination host.)

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Terrific Broth (TB) Medium

Reagent	Quantity	Final concentration
Yeast extract	24 g	24 g/L
Tryptone	20 g	20 g/L
Glycerol	4 mL	4 mL/L
Phosphate buffer (0.17 M KH_2PO_4, 0.72 M K_2HPO_4)	100 mL	0.017 M KH_2PO_4, 0.072 M K_2HPO_4

Add 900 mL of deionized water to 24 g of yeast extract, 20 g of tryptone, and 4 mL of glycerol. Shake or stir until the solutes have dissolved and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2). Allow the solution to cool to ~60°C and add 100 mL of sterile phosphate buffer. Store TB at room temperature; it will keep for at least 1 yr.

Yeast Extract-Peptone-Dextrose (YEPD)

Reagent	Quantity	Final concentration
Bacto peptone	20 g	2% (w/v)
Yeast extract	10 g	1% (w/v)
Dextrose	20 g	2% (w/v)
Agar (optional)	20 g	2% (w/v)
G418 (200 mg/mL; optional)	1 mL	200 mg/L

Add 1 L of deionized water to 20 g bacto peptone, 10 g yeast extract, and 20 g dextrose (and 20 g of agar for YEPD plates). Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2). To prepare YEPD plus G418 plates, allow the solution to cool to ~60°C, add 1 mL of G418 stock solution, and pour into Petri dishes (~25 mL per 100-mm plate). Store YEPD medium without G418 at room temperature, and store YEPD containing medium G418 at 4°C.

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