B. Subtilis Transformation

Introduction

This is the general procedure Dr. Ellermeier uses for Bacillus subtilis transformation.
Reference: Wilson, G.A. and Bott, K.F., 1968. Nutritional factors influencing the development of competence in the Bacillus subtilis transformation system. *Journal of Bacteriology*, *95*(4), pp.1439-1449.

Materials

- 10X MC media (for 100mL) - Mix everything in 40~50mL then adjust to 100mL. Filter Sterilize and freeze at -20˚C in 10mL aliquots.
  - K_2HPO_4 - $3H_2O$ (14.036 g)
  - K_2HPO_4 (5.239 g)
  - Glucose (20 g)
  - 300mM Trisodium Citrate (10mL) - see below
  - 22mg/mL Ferric Ammonium Citrate (1mL) - see below
  - Casein Hydrolysate (1g)
  - Potassium Glutamate (2g)
- 300 mM $MgSO_4$
- 300 mM Trisodium Citrate
  - 8.823 g of Trisodium Citrate dissolved in 100mL dH_2O
- 22mg/ml Ferric Ammonium Citrate
  - 2.2 g of ferric ammonium citrate dissolved in 100mL dH_2O
  - STORE IN DARK (Wrap in Foil)

Procedure

Transformation Procedure

1. Make 1X MC by adding 1mL of 10X MC to 9 mL of dH_2O **AND** 0.1 mL of 300 mM $MgSO_4$
2. Inoculate a single colony of the recipient into 2-5 ml of 1X MC in a 15mL test tube and incubate for 3-5 hrs at 37˚C
3. Transfer 250-500 µL culture to a small test tube and add 1-5 µL of chromosomal DNA and incubate for 1-2 hrs at 37˚C
4. Plate on selective media and incubate overnight at 37˚C

Colony PCR

Introduction

Colony PCR is commonly used to quickly screeen for plasmids containing a desired insert. This method eliminates the need to culture individual colonies and prepare plasmid DNA before analysis.

Materials

- Well isolated bacterial colonies (or streaks or pellets)
- yellow pipet tips
- forward and reverse PCR primers flanking the insert (prefix and suffix)
- 10X colony PCR buffer
  - 500 mM Kcl
  - 100 mM Tris-HCl (pH 8.5 or 9.0)
  - 1% Triton X-100
- 10mM dNTPS (10 mM each dATP, dTTP, dGTP, dCTP)
- 25 mM $MgCl_2$
- Taq polymerase (no need to use a high quality polymerase here, Taq is inexpensive)
- sterile ddH_20

Procedure

Prepare the reaction mixture master mix and add cells

1. Mix together the following on ice: always add the enzyme last. For multiple samples, make a large master mix and aliquot 50 µl per tube (also on ice).

   38 µl sterile ddH_20
   5 µl 10X colony PCR buffer (see materials)
   3 µl 25 mM $MgCl_2$
   1 µl 10 mM dNTPs (or 4 microliters of "dNTPs Master Mix")
   1 µl 20 µM forward primer
   1 µl 20 µM reverse primer
   0.2 µl Taq polymerase
   TOTAL VOLUME = 50 µl

2. To each cold PCR tube containing reaction mixture, add a small amount) of colony. **Important**: Spot or streak cells from the very colony to be analyzed to a labeled antibiotic plate to preserve it in case it has the plasmid you want.

   Barely dip a fine yellow pipette tip into a streak or colony to pick just enough cells to fill the tip opening. The tip should be attached to a pipetter set to 30 µl.

Pipet up and down to mix.
NOTE: too many cells will inhibit the reaction.
NOTE: Sufficient mixing will result in complete lysis of the bacteria and sufficient DNA to serve as template

Run the PCR reaction

3. Use the following PCR program:

1 cycle -	5′ at 95°C
30-40 cycles -	1′ at 95°C
	x′ at yy°C (adjusted as follows: 1′ per kb extension; 5 degrees lower than the lowest primer Tm)
	1′ at 72°C
1 cycle -	5′ at 72°C

Analyze the products

4. Mix 20 µl of each reaction with 4 µl of 6x gel loading dyes and analyze the products on an agarose gel of the appropriate percentage.

E. coli Boiling DNA Miniprep

Introduction

This protocol yields plasmid DNA that is suitable for restriction digests and cloning purposes. This preparation method works well on *E. coli* strains containing the *endA* mutation, such as XL1-Blue, DH5-α, but not HB101.

Holmes, D.S. and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114, 193-197.

Materials

- Boiling water or temperature block set to 95-100°C
- STET (Store at room temperature)
  - 8% sucrose (19.5 mls of 1.2M sucrose (41%) per 100 mls buffer)
  - 5% Triton X-100,
  - 50 mM Tris pH 8.0,
  - 50 mM EDTA pH 8.0
- STETL:
  - add lysozyme (0.5-1.0 mg/ml final concentration) to STET. This can be made up in 10 ml batches stored at -20°C as ~1.25 ml aliquots (enough for ~10 minipreps).
  -

Procedure

Grow cultures carrying plasmids of interest

1. Inoculate a 2-3 ml culture in rich media + appropriate antibiotic using a purified colony or frozen stock. Incubate overnight on a shaker or rotator at 37°C.

Prepare DNA

2. Transfer 1.5 ml saturated culture to a microtube. Store the remaining culture at 4°C until needed.
3. Pellet the cells for 1-2 min. The short centrifugation time makes the pellet easier to resuspencd.
4. Pour off or aspirate the supernatant.
5. Vortex the cell pellet until a homogenous cell paste is obtained.
6. Add 110 µl STETL and vortex briefly.
7. Place tubes in boiling water bath or 95-100°C heat block for 45 sec. Move tubes to ice for 5 mins.

A boiling water bath can be fashioned from a large beaker on a hot plate. At least half the tube must be in the water. Use a floating tube holders for this purpose.

8. Spin for 10 min maximum speed in a microfuge immediately after boiling. A large stickly loose pellet should form. Centrifugation time can be increased if pellet is not well formed.

9. Remove pellet by fishing it out with a sterile toothpick and discard pellet. Because the pellet is quite slippery, it is useful to have a paper tissue at the top of the tue to catch the pellet and prevent it from slipping back into the tube.

10. Add 1-2 volumes of isopropanol (110-220 µl) and spin in a microfuge for 10 min at maximum speed. Pour off or aspirate supernatant

11. Wash the pellet briefly (vortexing and resuspension are NOT needed). Simply add 0.4 ml ice cold 70% ethanol then centrifuge 5mins at max speed.

12. Pour off or aspirate the ethanol wash solution and spin 30 sec in microfuge. This step is analogous to drying spin in the Qiagen miniprep protocol.

13. Remove residual liquid with a micropipet and dry pellet in open air for ~15 min.

    Pellet will be large and contaminated with protein. These proteins will not interfere with subsequent restriction digests, but may require removal for future cloning steps.

14. Dissolve pellet in 20 µl TE buffer.

    Use one to three microliters for restriction digests.
    Store plasmid prep at 4° for days-weeks or -20° for months-years.
    Note that RNA may also be isolated by this method. Therefore include 1 µl of 10mg/ml RNAse (DNAse free) during restriction digest reactions.
    This method does not produce sequenceable DNA.
    ***Pellet will be invisible, but assume it is hard to resuspend! Pipet up and down for several minutes, this is a gentler method than vortexing. Do not vortex. If bubbles occur, spin briefly to remove bubbles.

Extraction of DNA fragments from an Agarose Gel

Introduction

Enzyme contamination of DNA samples can interfere with subsequent downstream applications. Therefore DNA fragments from enzymatic reactions and agarose gels often must be "cleaned" or purified prior to further use. QIAquick Kits can be used for highly efficient removal of a broad spectrum of enzymes widely used in molecular biology. The Qiagen Gel Extraction kit allows recovery of fragments as small as 70 bp and as large as 10 kb. Buffer QG in the QIAquick Gel Extraction Kit solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane in the purification column. DNA adsorbs to the silica membrane in the presence of high concentrations of salt, while contaminants pass through the column. Impurities are efficiently washed away, and pure DNA is eluted with Tris buffer or water.

Buffer QG contains an integrated pH Indicator allowing easy determination of the optimal pH for DNA binding. DNA adsorption requires a pH ≤ 7.5, and the pH Indicator in the buffers will appear yellow in this range. If the pH is >7.5, which can occur if during agarose gel electrophoresis, the electrophoresis buffer had been used repeatedly or incorrectly prepared, or if the buffer used in an enzymatic reaction is strongly basic and has a high buffering capacity, the binding mixture turns orange or violet. This means that the pH of the sample exceeds the buffering capacity of Buffer QG and DNA adsorption will be inefficient. In these cases, the pH of the binding mixture can easily be corrected by addition of a small volume of 3 M sodium acetate*, pH 5.0, before proceeding with the protocol.

Materials

- Buffer QG (yellow at pH less than or equal to 7.5)
- Buffer PE (Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%)
- a heating block or water bath at 50°C are required.

Procedure

Cut the fragment out of an agarose gel

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel on a UV box. Important: use eye and skin protection while doing this; the UV irradiation is harmful.

2. Weigh the gel slice in a colorless tube whose weight has been pre-determined.

Dissolve the agarose

3. Add 3 volumes Buffer QG to 1 volume gel

   Note 1: 100 mg gel ~ 100 µl.
   Note 2: The maximum amount of gel per spin column is 400 mg.
   Note 3: For >2% agarose gels, add 6 volumes Buffer QG.

4. Incubate at 50°C for 10 min or until the gel slice has completely dissolved. Vortex the tube every 2–3 min to help dissolve gel.

5. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix.

Bind the DNA to a Qiaquick 1column

6. Add 1 gel volume isopropanol to the sample and mix.

7. Place a QIAquick spin column in a provided 2 ml collection tube. Apply the sample to the QIAquick column and centrifuge for 1 min.

8. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin again.

9. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.

   Note 1: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt- ended ligation), let the column stand 2–5 min after addition of Buffer PE.

10. Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

    Note 2: This seems trivial and is easy to forget, but it is very important to remove all residual buffer.

Elute the DNA from the column

11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

12. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min.

    Note 1: For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.
    Note 2: After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

Evaluate Recovery

13. Option 1: If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. This, of course, uses up your precious sample, but is sometimes necessary to determine whether your DNA has been recovered and whether what was recovered is high quality.

14. Option 2: Alternatively, use the nanospec to get a DNA concentration.

Gibson Assembly® Protocol (E5510)

Introduction

This is the protocol for Gibson Assembly using the Gibson Assembly® Cloning Kit (E5510). More information from NEB can be found here.

Materials

- Gibson Assembly Cloning Kit
  - Gibson Assembly® Master Mix
  - NEBuilder® Positive Control
  - NEB® 5-alpha Competent *E. coli* (High Efficiency)
  - SOC Outgrowth Medium
  - pUC19 Transformation Control Plasmid
- DNA Polymerases (for generating PCR products)
  - Recommended: Q5® High-Fidelity DNA Polymerase, Q5 Hot Start High-Fidelity DNA Polymerase, or Q5 Hot Start High-Fidelity 2X Master Mix
- LB (Luria-Bertani) plates with appropriate antibiotic

Procedure

Set up the following reaction on ice:

1. Use Craig's program in the google drive in the protocol folder called Gibson Calculator. Set the vector volume to 5 microliters. Add the nanospecd concentrations for the vector and insert. If necessary, dilute the insert concentration to gain an appreciable insertion volume which is found in cell D11

2. Mix the 5 micro of vector and the quanitity in D11, then add 2.5 microliters of this solution to 2.5 microliters of the Gibson Assembly master mix. Proceed to step 3.

alt calculator

	A	B	C	D
1		**2-3 Fragments Assembly**	**4-6 Fragments Assembly**	**Positive Control ****
2	**Concentration Range of DNA fragments**	02 - .5 pmols*	.2 - 1.0 pmols*	0 pmols
3	**Total Volume of Fragments (µl)**			10
4	**Gibson Assembly Master Mix (2x) (µl)**	10	10	10
5	**Deionized Water (µl)**	10	10	0
6	**Total Volume (µl) *****	20	20	20

3. Incubate samples in a thermocycler at 50°C for 30 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled.

4. Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with all of the assembly reaction, following the chemcial transformation protocol or electro competent cells transformation protocol

Linearized Backbone Protocols

Introduction

Digest, ligation, PCR - from iGEM

Materials

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›

Procedure

Digest

1. Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)

   5 ul NEB Buffer 2
   0.5 ul BSA
   0.5 ul EcoRI-HF
   0.5 ul PstI
   0.5 ul DpnI
   18 ul dH20

2. Digest Plasmid Backbone

   Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
   Add 4 ul of Enzyme Master Mix
   Digest 37C/30 min, heat kill 80C/20 min

Ligation

3. Add 2ul of digested plasmid backbone (25 ng)
4. Add equimolar amount of EcoRI-HF SpeI digested fragment (< 3 ul)
5. Add equimolar amount of XbaI PstI digested fragment (< 3 ul)
6. Add 1 ul T4 DNA ligase buffer (Do not use quick ligase)
7. Add 0.5 ul T4 DNA ligase
8. Add water to 10 ul
9. Ligate 16C/30 min, heat kill 80C/20 min
10. Transform with 1-2 ul of product

Note: For linearized plasmid backbones provided by iGEM HQ, a plasmid backbone with an insert of BBa_J04450 was used as template. As a result any red colonies that appear during your ligation may be due to the template as a background. Digesting with Dpn1 before use should reduce this occurrence.

Single Reaction PCR

PCR mix

11. 100 ul PCR Supermix High Fidelity
12. 0.7 uL each of primers

    gccgctgcagtccggcaaaaaa, SB-prep-3P-1
    atgaattccagaaatcatccttagcg, SB-prep-2Ea
    --> Diluted to 30 pmol/ul
13. 0.5 ul template DNA at 10 ng/ul

    Notes:
    Do not use a sample of linearized plasmid backbones (PCRed) as a template,
    The Registry uses BBa_J04450 as a template

PCR program

14. "backbonepcr" on thermocycler

    94C/2min
    94C/30s, 55C/30s, 68C/3min x 36
    68C/10min
15. Digest with DpnI enzyme: 2ul in 100ul reaction, incubate 37C/hour; heat kill 80C/20min

PCR cleanup - QIAquick PCR Purification

16. Add 500 ul Qiagen buffer PB
17. Spin through a column twice, discard flowthrough
18. Wash 1x with 700 ul buffer PB
19. Wash 2x with 760 ul buffer PE
20. Discard liquid, spin dry at 17000g for 3 min
21. Elute into a new tube twice with 50 ul of TE (100 ul total)

PCR Clean-up

Introduction

The QIAquick system is suitable for fast cleanup of up to 10 µg of DNA fragments from enzymatic reactions. Enzyme contamination of DNA samples can interfere with subsequent downstream applications. QIAquick Kits can be used for highly efficient removal of a broad spectrum of enzymes widely used in molecular biology. The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of contaminants in each specific application. DNA adsorbs to the silica membrane in the presence of high concentrations of salt, while contaminants pass through the column. Impurities are efficiently washed away, and pure DNA is eluted with Tris buffer or water.

Note 1: During the DNA adsorption step, unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, and detergents (e.g., DMSO, Tween® 20) do not bind to the silica membrane but flow through the column. Salts are quantitatively washed away by the ethanol-containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Note 2: Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer. Contrary to adsorption, elution is most efficient under basic conditions and low salt concentrations. DNA is eluted with 50 µl or 30 µl of the provided Buffer EB (10 mM Tris·Cl, pH 8.5), or water. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water to elute, make sure that the pH is within this range. In addition, DNA must be stored at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent.

NOte 3: DNA yield depends on the following three factors: the volume of elution buffer, how the buffer is applied to the column, and the incubation time of the buffer on the column. To completely cover the QIAquick membrane, use 100–200 µl elution buffer. This ensures maximum yield, even when not applied directly to the center of the membrane. Elution with ≤50 µl requires the buffer to be added directly to the center of the membrane, and if elution is done with the minimum recommended volume of 30 µl, an additional 1 minute incubation is required for optimal yield. DNA will be up to 1.7 times more concentrated if the QIAquick column is incubated for 1 minute with 30 µl of elution buffer, than if it is eluted in 50 µl without incubation (Figure 4, page 13).

Materials

- Spin columns
- Buffer PE - check that ethanol has already been added (10mM Tris-HCl pH7.5, 80% ethanol)
- Buffer PB - (5M Gu-HCl, 30% isopropanol)
- Microfuge
- Sterile dd H_2O

Procedure

Adsoption

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.

   For example, add 500 µl of Buffer PB to 100 µl PCR sample.

2. If pH Indicator I has been added to Buffer PB, check that the color of the mixture is yellow.

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge (high or 13,000 rpm) for 30–60 s.

5. Discard flow-through. Place the QIAquick column back into the same tube.

   Collection tubes are re-used to reduce plastic waste.

Wash

6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge (high or 13,000 rpm) for 30–60 s.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

Recover DNA

9. To elute DNA, add 50 µl sterile dd water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column (high or 13,000 rpm) for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Recycle Qiagen and other DNA purification columns

Introduction

Save research $$ by reusing DNA mini, midi and maxi-prep columns. Use this simple and cheap method to regenerate and reuse the columns up to (guessing...) 20 times.
Note also that the blue and purple Qiagen columns are identical in formulation.
Unused columns can be cheaply purchased in bulk from Epoch Biolabs.

Materials

- Used Columns
- 1M HCl
- QBT buffer
  - 750 mM NaCl
  - 50 mM MOPS (pH 7.0)
  - 15% (v/v) isopropanol
  - 0.15% (v/v) Triton X-100
  - **PROCEDURE**: Dissolve 43.83g NaCl, 10.46g MOPS (free acid) in 800mL dH_2O. Adjust the pH to 7.0. Add 150mL pure isopropanol and 15mL 10% Triton X-100 solution. Adjust the volume to 1 liter with dH_2O.

Procedure

Save the columns

1. Wash the column with water
2. Spin it dry
3. Store in a cold dry place until it's ready for HCl treatment (*or*...proceed directly to HCl treatment)

Destroy Residual DNA

4. Fill the column with 700 µl of 1 M HCl and incubate overnight. If the columns are uncapped, cover them with a glass plate.

   HCl works by physically destroying the residual nucleic acid from your last prep.

5. Wash the columns and collection tubes in a large beaker of water (ddH_2O).
6. Assemble the column and collection tube and wash the column with 700 µl of ddH_2O.

7. Repeat the water wash 2 more times (step 6).

Regenerate the Column

8. Fill the column with 700 µl of buffer QBT and spin down, discarding the buffer.

9. Place the columns in an airtight plastic bag for storage

10. Wash the collection tubes, air dry, and store them for reuse

Restriction Digest, iGEM style

Introduction

The iGEM protocol for restriction digests is highly standardized. It uses a single buffer for all digests and a PCR machine to handle all temperature changes.

Materials

- Ice and bucket/container
- 8-tube strip, or individual 0.6ml thin-walled tubes
- BioBrick Part in BioBrick plasmid (Purified DNA, > 16ng/ul) or other DNA
- sterile dH2O
- NEB Buffer 2 or CutSmart Buffer for HF enzymes
- BSA
- Restriction Enzymes: EcoRI, SpeI, XbaI, PstI (Choose a High-Fidelity (HF®) restriction enzyme, which has been engineered for reduced star activity, rapid digestion (5-15 minutes) and 100% activity in CutSmart Buffer).
- Thermal cycler with heated lid: set it to carry out the incubation step at 37°C (4 Hours) and the enzyme inactivation step at 80°C (20 min). This could be followed by an extended incubation at 4°C to hold the reaction until you can get back to it.

Procedure

Set up the Reaction (note: if setting up several digests with a single DNA sample, make a single reaction mix on ice and aliqot it to individual tubes, also on ice - add the enzymes last)

1. Add 250ng of DNA to be digested, and adjust with dH20 for a total volume of 16.5 ul.
2. Add 2.0 ul of Buffer (CutSmart buffer for HF enzymes) NEBuffer 2. (or universal buffer for HF enzymes)
3. Add 0.5ul of BSA.
4. Add 0.5ul of EcoRI (or Enz 1).
5. Add 0.5ul of PstI (or Enz 2).
6. There should be a total volume of 20ul. Mix well and spin down briefly.

Digest for Linearized Backbone Only(25ul total, for 5 rxns)

7. Make Backbone Enzyme Master Mix

    5 ul NEB Buffer 2
    0.5 ul BSA
    0.5 ul EcoRI-HF
    0.5 ul PstI
    0.5 ul DpnI (Used to digest any template DNA from production)
    18 ul dH20

8. Digest Plasmid Backbone

    Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
    Add 4 ul of Enzyme Master Mix
    Digest 37C/30 min, heat kill 80C/20 min

Carry out the Digestion and Inactivation of Enzymes

9. Incubate the restriction digest at 37°C for 4 -hrs in PCR Machine and inactive at 80°C for 20 min.

    Use the Restriction Digest iGEM style setting which carries out both steps.
    !!!!!!!! If digesting to run dna through a gel, digest for 45 mins plus 20 mins of inactivation.

Check the products of the reaction by Agarose Gel Electrophoresis

10. Run a portion of the digest on a gel (8ul, 100ng), to check that both plasmid backbone and part lengths are accurate.

Small scale DNA miniprep (Qiagen Kit)

Introduction

The Qiagen kit

Materials

- Qiagen Kit Buffers (can be homemade)
  - Buffer P1: 50 mM Tris HCl (pH 8), 10 mM EDTA, 100, 100 micrograms/ml (DNase free - RNaseA); store in the refrigerator
  - Buffer P2: 200 mM NaOH, 1% SDS (store at RT)
  - Buffer N3: 4.2 M Gu-HCl, 0.9M potassium acetate (N3 pH 4.8) (store at RT)
  - Buffer PB: 5M Gu-HCl (store at RT)
- Qiaprep spin columns
- Microfuge
- Ethanol

Procedure

Prepare Buffer P1

1. Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000
2. Add the provided RNase A 100 µg/mL to Buffer P1, mix, and store at 2–8°C.
3. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

Prepare the Cell Lysate

4. **RESUSPEND THE CELLS.** Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x *g*) for 3 min at room temperature (15–25°C).
5. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
6. **LYSE THE CELLS USING ALKALINE CONDITIONS.** Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. **Note:** If using LyseBlue reagent, the solution will turn blue. This is the
7. **NEUTRALIZE THE LYSATE.** Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.

8. **CLEAR THE LYSATE.** Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.

Purify the DNA

9. Apply the supernatant from step 8 to the QIAprep spin column by decanting or pipetting. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

10. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.

    Note: This step is only required when using *endA+* strains or other bacteria strains with high nuclease activity or carbohydrate content.

11. Wash the QIAprep spin column by adding 0.75 ml Buffer PE.

    Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source. Transfer the QIAprep spin column to the collection tube.

12. Centrifuge for 1 min to remove residual wash buffer. **Note:** Important - Do not skip this step.

13. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.