Uppsala iGEM 2019

PROTOCOLS

Cloning Protocols

Buffers & Media

Protocol #1: 0.9% NaCl (10 mL)

Introduction:

Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

This solution is used for suspending cells to avoid early lysis by osmosis. Some laboratories use phosphate buffered saline (PBS) instead.

Materials:

- NaCl
- ddH2O

Procedure:

NaCl	0.09g
ddH_2O	10 mL

- Add the salt to a glass bottle and add water to dissolve.
- Make up to the final volume of 10 mL.

Protocol #2: 1 M CaCl2 (10 mL)

Introduction:

Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

$CaCl_2$ is used in a less concentrated form for making competent *E. coli* cells. Upon exposure to $CaCl_2$, the cell wall becomes fragile and at 42 °C *E. coli* takes up foreign plasmids efficiently.

Materials:

- **CaCl2**
- **ddH2O**

Liljeruhm, Josefine et al. *Synthetic Biology: A Lab Manual*. 1st ed., World Scientific, 2014.

Procedure:

$CaCl_2$	1.11 g
ddH2O	10 mL

- Weigh out the powder and add it to 8 mL water.
- Stir and shake until it dissolves.
- Make up to 10 mL final volume.
- Autoclave for 20 min.

Protocol #3: 10 x TBE solution

Introduction:

Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Na_2EDT or Na_4EDTA can be used since their different effects on pH can be neglected for this buffer. The concentration in the gel and running buffer should always be 1x TBE.

TBE stands for the three components tris, boric acid and EDTA. EDTA chelates Mg^{2+}, both inhibiting enzymatic degradation of nucleic acid and giving sharper bands on gels. There is no risk of contaminative growth in 10x stock solution, but over time the salt may precipitate, requiring preparation of fresh buffer.

Material:

- **Tris base**
- **Boric acid**
- **EDTA**
- **ddH_2O**

Procedure:

1. Dissolve the following reagents in 400 mL ddH_2O using a magnetic stirrer:

Components	**Volume/weight**
Tris base	53.9 g
Boric acid	27.5 g
EDTA	4.7 g
ddH_2O	500 mL

Tris to a final concentration of 0.89 M;

Liljeruhm, Josefine et al. *Synthetic Biology: A Lab Manual*. 1st ed., World Scientific, 2014.

Boric acid to a final concentration of 0.89 M.

2. Dissolve Na_2EDTA or Na_4EDTA to final concentration of 25 mM.

3. Adjust the volume to 0.5 L. The pH will be ~8.5.

4. Store at room temperature.

1x TBE working solution:

Dilute the stock solution by 10x with deionized water ($dH_2=$) in a new 1L bottle.

Protocol #4: (v/v) Glycerol, 50 mL

Introduction:
Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

This solution is to be used for making cell glycerol stocks of important bacterial strains.

Materials:

- **Glycerol stock**
- **ddH_2O**

Procedure:

1. Check which percentage glycerol is in the stock.

2. Calculate how much volume you need of glycerol and how much water you need to add to reach a final volume of 50 mL.

Components	Volume/weight
Glycerol stock (85% in stock)	29.4 mL
ddH_2O	20.6 mL

3. Measure the glycerol in a measuring cylinder.

4. Add to a glass bottle and add water to make 50% glycerol solution.

5. Autoclave for 20 min.

Liljeruhm, Josefine et al. *Synthetic Biology: A Lab Manual*. 1st ed., World Scientific, 2014.

Protocol #5: E.coli O/N in LB+Antibiotic (5/6 mL)

Introduction:
Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Antibiotics should not be added to LB before autoclaving the LB or after subsequent cooling to room temperature for storage because antibiotics are not sufficiently stable. Antibiotics solutions are stored pure at -15 ℃ and only added just before the addition of bacteria.

Material:

- **Ampicillin: 100 mg/mL;**
- **Chloramphenicol: 25 mg/mL;**
- **Kanamycin: 50 mg/mL.**

Procedure:

1. Quickly burn the neck of a bottle containing LB medium before pouring it out into a flask (or tube). Even the slightest contamination of LB will be visible the next day as an unwelcoming culture!
2. Add the antibiotic to give the appropriate concentration listed below.

Components	5 mL O/N
LB medium	5 µL
1000x Antibiotic	5 µL

3. Scoop one colony from the plate with a sterile inoculation loop (or micropipette tip).

4. Immediately stick the loop (or tip) into the flask (or tube) containing teh medium and antibiotic for a few seconds.

5. Cover the flask loosely with aluminum foil (or cap tube loosely) by taping it on to allow exchange of oxygen. Good oxygenation is required not only for maximal growth, but also for chemical maturation of the chromoprotein chromophores.

6. Incubate at 37 ℃ with shaking overnight.

Liljeruhm, Josefine et al. *Synthetic Biology: A Lab Manual*. 1st ed., World Scientific, 2014.

Protocol #6: LB Agar Plate (600 mL LA) and Addition of Antibiotics

Introduction:
Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Make sure that the chemicals below, including 5 M NaOH, and the autoclave are available before starting. These plates contain solidified lysogeny broth (LB), a rich growth medium for *E. coli*. In contrast to growth in liquid LB where the bacteria are mobile, growth on plats gives colonies originating from one single bacterial cell. Just before pouring the solution into petri dishes, an antibiotic can be added for resistance selection. Normal working concentrations are:

- Ampicillin: 100 µg/mL;
- Chloramphenicol: 25 µg/mL;
- Kanamycin: 50 µg/mL.

Normal stock concentrations are 1000-fold higher than above, respectively.
Note: Chloramphenicol stock is dissolved in ethanol.

Material:

- **LB media/powder**
- **Agar**
- **1000x antibiotic of choice**

Procedure:
1. Add the following to a 1 L bottle:

Components	Volume/weight
LB media (non-autoclaved)	600 mL
Agar	9 g
Antibiotics of choice	600 µL

2. Shake the bottle. It is unnecessary to dissolve all solids now because autoclaving will do this.

3. Autoclave for 20 min within 2 h.

4. Let it **cool to ~40-50 ℃** (touchable, so te antibiotics will not be destroyed by the high temperature).

5. **Add 600 µL of 1000x antibiotic of choice** (if any) and gently swirl the bottle to mix. Do not shake the bottle vigorously as this will create many bubbles that will be transferred to your plates!

6. Pour into empty petri dishes just enough to cover the surface (~20 mL per plate). If bubbles remain in the plates, heat the plates surface carefully with a burner to burst them. But make sure not to heat the solution in the plate too much since it might degrade the antibiotic.

7. Leave the plates at room temperature to solidify (~1 h).

8. Solidified plates should be turned upside down for a few hours at room temperature, then stored at 4 ℃.

Protocol #7: LB liquid media (600 mL)

Introduction:

Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Depending on the length of time spent in the lab, be prepared to make L several times, either due to high usage or contamination. Make sure that the chemicals below, including 5 M NaOH, and the autoclave are available before starting. Follow the autoclave instructions from the lab teachers. The finished media should be autoclaved within a couple of hours to prevent contaminative growth.

Lysogeny broth (LB) is one of the rich media for bacterial growth and is the standard choice for *E. coli*. LB was developed by G Berani and later optimized by Luria during 1950s. It subsequently acquired different names, including Luria broth, Luria-Bertani media and Lennox broth, some containing different salt concentrations.

Material:

- NaCl
- Bacto™ tryptone 1% (w/v)
- Yeast extract 0.5% (w/v)
- ddH_2O
- 5 M NaOH

Procedure:

1. Add the following to a 1 L bottle:

Components	Volume/weight
NaCl	5.9619 g

Liljeruhm, Josefine et al. *Synthetic Biology: A Lab Manual*. 1st ed., World Scientific, 2014.

Bacto™ tryptone 1% (w/v)	6 g
Yeast extract 0.5% (w/v)	3 g
ddH_2O	600 mL
5 M NaOH	100 µL

2. Autoclave for 20 min within 2 h.

3. Store at room temperature,

Protocol #8: SOB Medium

Introduction:

SOB medium or Super optimal Broth, is used for preparing chemically competent cells. This protocol is adapted from Ausubel *et al. (1999).*

Materials:

- **Yeast Extract**
- **Bacto™ tryptone**
- **NaCl**
- **KCl**
- **ddH_2O**
- **5 M NaOH**

Procedure:

1. Add the following to a 1 L bottle:

Components	Weight/volume
Yeast extract	3 g
Bacto™ tryptone	12 g
NaCl	0.3507 g
KCl	0.1118 g
DDH_2O	~600 mL

5 M NaOH	120 µL

2. Autoclave for 20 min within 2 h.

3. Store at room temperature.

Assays

Protocol #9: Preparation of E. coli cells for transformation

Introduction:

E. coli is inefficient at taking up foreign plasmids. One cannot rely on its natural competence (ability to take up foreign DNA). To increase the competence, the cell wall is made permeable by treatment with $CaCl_2$. It is important that the whole process is kept chilled! Remember to be careful with the cells they become very fragile.

Materials:

- **SOB-medium**
- **Plate with single cell colonies**
- **0.1 M $CaCl_2$, ice-cold**
- **0.1 M $CaCl_2$ with 20% glycerol, ice-cold**
- **Liquid nitrogen**

Procedure:

1. Take one colony and start a 5 mL overnight culture at 37 °C, with shaking.

2. Dilute the overnight culture 1:100 into 50 mL SOB-medium.

3. Grow culture at 37 °C with shaking to an OD_{600} = 0.4.

4. Let the culture sit on ice for ~15 min, swirling occasionally. When the cells are properly chilled proceed to the next step.

5. Pour the culture into a 50 mL Flacon™ tube.

6. Centrifuge at 3500 rpm for 5 min at 4 °C.

7. Remove as much as possible of the supernatant without disturbing the pallet.

8. Resuspend the pellet in 100 µL 0.1 M $CaCl_2$ with the help of a sterilized loop.

9. Add **15 mL ice-cold 0.1 M $CaCl_2$**. Mix gently by pipetting up and down a few times. Do not vortex!

10. Incubate the cells on ice for 30 min.

11. Pellet the cells again at 3500 rpm for 5 min at 4 ℃.

12. Resuspend the cells in **2 mL of ice-cold $CaCl_2$/20% glycerol**.

13. Incubate for 45 min on ice.

14. Aliquote carefully in 50 µL amounts to chilled 1.5 mL. Note: Some gentle mixing is required as cells tend to fall to the bottom of the liquid. Also, competent cells give the highest transformation efficiencies when fresh compared with after freezing in step 15.

15. Snap freeze in **liquid nitrogen** any tubes that will not be used for transformation within a few hours.

16. Store at -80 ℃ Note: once the cells have been frozen and thawed they cannot be frozen again because this kills the cells.

Protocol #10: Transformation of Competent E. coli cells

Introduction:

Remember that competent cells are very fragile and should only be mixed gently. Transformation plating results can be very very difficult to interpret without positive and negative controls. A good way to plan controls is to think about how you want to interpret different possible results. Three plating controls are gently used to distinguish the results:

1) Negative control (ddH_2O).
2) Unligated, cut plasmid (negative control).
3) Positive control (intact plasmid), this will transform much more efficiently than ligated plasmid.

Materials:

- **$CaCl_2$ competent *E. coli* cells**
- **LB media**
- **DNA plasmids**
- **Agar plates**

Procedure:

1. Turn on a water bath or heating block to 42 ℃.

2. Thaw competent cells on ice for 15 min.

3 Add 15 µL of ligation reaction mixture or controls above to 50 µL of competent cells.

4. Incubate for 30 min on ice.

5. Heat shock for 45 s at 42 ℃.

6. Incubate for 5 min on ice.

7. Add 950 µL of LB media (pre-heated to 37 ℃)

8. Incubate for 1-1.5 h at 37 ℃, with occasional gentle mixing by inversion of the tubes.

9. For positive controls, mix gently and plate 100 µL only (=1/10th) on agar containing the appropriate antibiotic as in step 12.

10. Spin cells from remaining 900 µL at 4000 rpm for 5 min.

11. Discard all but 100 µL of the supernatant and resuspend the pellet in the remaining 100 µL.

12. Spread the remaining suspension on an agar plate containing the appropriate antibiotic as following:

i) Dip the spreader into 95% ethanol.
ii) Put it into the flame for a second.
iii) Let the ethanol burn off outside the flame.
iv) Spread the bacterial suspension evenly out on an agar plate. Continue until all the inoculum has gone into the agar.
v) Put the plates at 37 ℃ overnight.

Following days

13. **Day 2:** Calculate transformation efficiency (colonies/µg) of the competent cells using the positive control plates.

14. In the evening, re-streak appropriate colonies.

15. **Day 3**: Set overnight cultures.

16. **Day 4**: Make glycerol cell stocks of strains worth saving and process the rest of the culture according to the instructions.

Protocol #11: 3A assembly-Digestion and ligation

Introduction:

Cut out the designated fragment and ligate them together through 3A assembly method or gel purification.

Materials:

- **DNA samples**
- **ddH_2O**
- **10 x reaction buffer for restriction enzymes provided by manufacturer**
- **Restriction endonucleases**
- **10x reaction buffer for T4 DNA ligase provided by manufacturer**

Digestion:

1. Make three mixes: Each containing 500 ng of one of the three plasmids and ddH_2O to 43 µL.

2. To each mix, add 5 µL of 10x reaction buffer for restriction enzymes.

3. Add 1µL each of the appropriate endonucleases (two per tube).

4. Tap on the tubes to mix. If necessary, centrifuge for a few seconds to spin down the liquid.

5. Incubate at 37 ℃ for 30 min.

6. Heat-inactivate the enzymes by incubating at 80 ℃ for 20 min.

7. At this point, samples may be stored at -20 ℃.

Gel analysis of digestion (recommended for first time)

8. Run 20 µL of each digestion mixture (200 ng) on a 1% agarose gel to measure the extent of digestion. Also run the three uncut plasmids (negative controls) directly beside their cut versions, and a DNA ladder marker should be loaded in a middle lane.

Ligation

9. Add 2 µL (20ng) of each of the three digestion mixtures to 11 µL of water.

10. Add 2 µL 10x reaction buffer for T4 DNA ligase.

11. Add 1 µL of T4 DNA ligase to give a final volume of 20 µL.

12. Incubate at room temperature (~22 ℃) for 30 min.

13. Heat-inactivate the enzyme by heating at 80 ℃ for 20 min.

14. At this point, samples may be stored at -20 ℃.

Protocol #12: Analytical Digestion and Agarose Gel Electrophoresis

Introduction:

Agarose gel electrophoresis is used for separation and analysis of large (>100 bases in length) nucleic acids under non-denaturing conditions. By adding the sample with loading buffer to the gel wells and applying a current over the anode and cathode. Analysis requires that the gel contains a DNA stain visible under UV light. Since the stain interacts with nucleic acids and is therefore potentially mutagenic, always wear nitrile gloves when working with agarose gels. If the stain is ethidium bromide, not Sybr®Safe, make sure that contaminated waste (pipette tips, gels, etc.) is disposed of in hazardous waste boxes. Use protective glasses when using the UV light box.

Materials:

- Agarose
- 1x TBE
- Sybr®Safe
- Loading dye mix
- DNA ladder size marker
- DNA samples
- Restriction enzymes
- 10x reaction buffer for restriction enzymes

Procedure:

1. Make a digestion reaction mixture in a 1.5 mL microtube according to the following graph:

Components	Volume
DNA sample (200 ng)	x

10x reaction mixture	2 µL
Restriction enzymes	1 µL each
ddH_2O	18 - n - x n=no. Of enzymes used
Total	20 µL

2. Incubate the mixture in 37℃ for 30 min, then heat-inactivate in 80 ℃ for 20 min.

Cast a gel

3. Insert the comb into the gel tray at one end.

4. For a 1% 50 mL agarose gel, weight 0.5 g of agarose on a 250 mL conical flask. Add 50 mL 1x TBE buffer.

5. To dissolve the agarose in the buffer, swirl to mix and microwave for a few minutes making sure not to boil the solution out of the flask. Remove the flask occasionally and check whether the agarose has dissolved completely. Be careful - the solution is very hot! Insulated gloves are too bulky to easily pull the flask out form the microwave, so a folded paper towel is suggested.

6. Let the agarose solution cool down. Once the solution is touchable, add the DNA stain. Check the stock concentration and add the appropriate amount to give the desired final concentration. The working concentration for ethidium bromide is 0.5 µg/mL while for Sybr®Safe it is simply 1x.

7. Pour the gel solution into the gel tray. Remove any air bubbles with a pipette tip. Put in combs.

8. The gel will solidify while cooling down to room temperature, Depending on the initial temperature, this will take ~20 min.

9. Run the gel.

Running the gel

10. Release the gel tray from the tape or casting stand. Place the gel tray into the buffer chamber and remove the comb carefully.

11. Add 1x TBE buffer until the gel is completely covered.

12. Take part of your DNA sample (200 ng) and mix it with loading dye. This can be down either in 1.5 mL tubes or, if the volumes are very small, on a piece of parafilm.

13. Load the size marker.

14. Load your samples into the other wells while writing down which lanes have which samples.

15. Put the lid onto the buffer chamber and connect it to the power supply. Make sure to put it in the right direction so that your DNA runs towards the positive (red) electrode.

16. Run the gel at 100 V for 30-60 min. Neither of the two dyes should be run off the gel. If the electrophoresis runs correctly you will notice air bubbles coming from electrodes.

17. Stop the run and bring your gel to the UV table to visualize your gel bands. Use protective glasses. If sufficient separation was not achieved, put the gel back into buffer chamber and run it for longer.

18. Take a picture of your gel.

Protocol #13: Gibson assembly master kit

Introduction:

NEB recommends a total of 0.02-0.5 pmol of DNA fragments when 1 or 2 fragments are being assembled into a vector. To calculate the number of pmol of each fragment for optimal assembly, based on fragment length and weight, we recommend using NEB's online tool, NEBioCalculator, or using the following formula:

Pmols = (weight in ng) x 1000 / (base pairs x 650 daltons)

50 ng of 4000 bp dsDNA is about 0.015 pmol.

50 ng of 500 bp dsDNA is about 0.15 pmol.

The mass of each fragment can be measured using the nanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Materials:

- Fragments
- Gibson Assembly Master Mix (2x)
- Deionized H_2O

Procedure:

1. Set up the following reaction on ice:

	test group	Positive Control**
Total Amount of Fragments	0.02–0.5 pmol X µL	10 µL
Gibson Assembly Master Mix (2X)	10 µL	10 µL
Deionized H2O	10-X µL	0
Total Volume	20 µL	20 µL

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled.
3. Following incubation, store samples on ice or at -20°C for subsequent transformation.

Protocol #14: PCR (Dreamtaq)

Introduction:
Thermo Scientific™ DreamTaq™ DNA Polymerase is an enhanced Taq DNA polymerase optimized for all standard PCR applications. It ensures higher sensitivity, longer PCR products and higher yields compared to conventional Taq DNA polymerase.
Materials:

- **ddH2O;**
- **dNTPs, 2 mM;**
- **phosphorylated forward primer, 5 µM;**
- **phosphorylated reverse primer, 5 µM;**
- **DreamTaq DNA Polymerase**
- **10X DreamTaq Buffer**
- **DNA template.**

Procedure:

1. Prepare Reaction Mixture.

   Mix the components from the list below together in a PCR tube (0.2 mL).

10X DreamTaq Buffer	5 µL

dNTPs, 2 mM	5 µL
forward primer, 5 µM	5 µL
reverse primer, 5 µM	5 µL
DNA Templates	10 pg- 1 µg
DreamTaq DNA Polymerase	0.25µL 0f 5U/ µL
ddH_2O	up to 50 µL
Total	50 µL

2. Set up PCR programme

   Calculate the annealing temperature and the extension time. The extension time is about 1 min/kb. The annealing temperature can be calculated using the website of the manufacturer or locking at the container.

Initial denaturation	95	1-3 min	1x
Denaturation	95	30 s	25-40x
Annealing temp. - 5°C		30 s	
Extension	72		
Final extension	72	7 min	1x
Storage	4	∞	1x

Protocol #15: PCR for synthesizing DNA fragments

Introduction:
Taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Phusion®HF DNA polymerase has a little endonuclease activity, so the incubation time with the DNA should not be extended. The program is a version of touchdown PCR that increases specificity but still provides a good yield. Two different PCR

programs are recommended because long and short primers tend to have annealing temperatures above and below the extension temperature, respectively, in later cycles. The second program ensures that short primers can anneal for the extension reaction.

Materials:

- ddH2O;
- dNTPs, 2 mM;
- phosphorylated forward primer, 5 μM;
- phosphorylated reverse primer, 5 μM;
- 5x Phusion®HF buffer provided by manufacturer;
- Phusion®HF DNA polymerase;
- DNA template.

Procedure:

1. Prepare PCR reaction mix

   Mix the seven components below in a PCR reaction tube (0.2 mL tube, which is smaller than a typical

ddH2O	23.5 μL
2 mM dNTPs	5 μL
Forward primer (5 μM)	5 μL
Reverse primer (5 μM)	5 μL
5x Phusion® HF buffer	10 μL
Plasmid dilution (for genomic DNA we need 100~500ng, for plasmid DNA we need 1ng)	1 μL (plamid DNA_1ng/uL)10uL(Genomic DNA_10ng/uL)
Phusion® HF DNA polymerase	0.5 μL
Total	50 μL

2. Set up PCR programme

Description of Step	Temp (°C)	Time: (hh:mm:ss)	Number of cycles
Initial denaturation	98	00:00:30	1x
Denaturation	98	00:00:10	2x
Annealing temp. + 4°C		00:00:30	
Extension	72		
Denaturation	98	00:00:10	6x

Annealing temp. + 2°C		00:00:30	
Extension	72		
Denaturation	98	00:00:10	25x
Extension	72		
Final extension	72	00:07:00	1x
Storage	4	∞	1x

Calculate the extension time :

Length of amplicon: _____kb.

Phusion® pol extension time per kb: 15 s/kb for plasmid DNA, 30s/kb for Genomic DNA.

• Extension time (length × extension time per kb):_____ hh:mm:ss.

Use the manufacturer's Tm calculator and Phusion®information sheet to fill in the annealing temperatures in your chosen program grid above. Note: The recommended annealing temperature is a*bove or equal to Tm f*or Phusion® pol, depending on primer length, but b*elow Tm f*or Taq pol!

3. Run the PCR reaction
4. PCR products purification

   Analyse a 5µL aliquot by agarose gel electrophoresis.

   If a full-length band was visible, purify your PCR product using a PCR purification kit (or gel extraction kit) according to the manufacturer's protocol.

Protocol #16: Colorimetric Assay

Introduction:

This protocol is taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Chromoprotein expression level in bacteria can be qualitatively estimated by colors of bacteria.

Materials:

- 1 M Tris-HCl, pH 8.0;
- 0.5 M EDTA, pH 8.0;
- NaCl;
- Triton®X-100;
- lysozyme stock solution at 10 mg/mL in 10 mM Tris- HCl, pH 8.0;
- plate containing single colonies.

Procedures:

1. Start overnight cultures of appropriate test and con- trol strains of *E. coli*.
2. Prepare the lysis buffer from the first four components in the list above to give these final concentrations:
   10 mM Tris-HCl, pH 8.0; 1 mM EDTA;
   100 mM NaCl;
   0.5 % (v/v) Triton®X-100.
3. Pellet 2 ml of an overnight culture in a 1.5 ml tube by centrifugation twice at 5000x g for 5 min. Remove the supernatants.
4. Resuspend the cell pellet in 300 µL of the lysis buffer.
5. Add 25 µL of lysozyme stock solution.
6. Mix by vortexing for a couple of seconds.
7. Incubate the sample at 37°C for 30 min.
   Note: After this incubation, the sample may also be freeze-thawed a couple of times to test if this improves lysis.
8. Centrifuge the sample at max speed for 3 min to pellet the cell debris. Check the color of the pellet as it may tell the effectiveness of the cell lysis.
9. Take the supernatant and quantify its color using a spectrophotometer.

Protocol #17: Colony PCR

This protocol is taken from Synthetic Biology: A Lab Manual. (Liljeruhm et al.)

Introduction

The primary goal of colony PCR is to prepare DNA sam- ples from several colonies to screen for the desired mutant by sequencing (Fig. 34). DNA samples for sequencing are more easily prepared by colony PCR reac- tions than by overnight cultures followed by plasmid preps. There is no need for a cell lysis step because the DNA template is released from the bacterial colony dur- ing a PCR reaction. Before

sending for sequencing, PCR products are sized and quantified on an agarose gel to verify that the PCRs worked. Also, if a large deletion or insertion was desired, this size difference may be visible on the gel as a preliminary screening step before sequenc- ing (provided that you also run a **control PCR reaction** for comparison!).

Taq DNA polymerase is preferred for colony PCR.

Materials:

- A plate with re-streaked, single colonies;
- ddH_2O;
- dNTPs, 2 mM;
- Forward primer;
- Reverse primer;
- 10x Taq PCR buffer provided by manufacturer;
- Taq DNA polymerase.

Procedure

Screening requires testing several different colonies at once, *i.e.* several PCRs. In such cases, instead of preparing several reaction mixes individually, the number of pipetting steps can be reduced and the reproducibility of the reactions increased as follows: prepare just one "**master mix**" that includes all components except the varia- bles (in this case, the cells). For example, if you decide to perform six PCRs (including your original control colony and two negative controls), then you will need to make up a volume of master mix equivalent to seven reaction mix volumes, not six volumes, because there never seems to be enough volume in the last aliquot! So instead of using the volumes given for the reaction mix in Step 6 below, you would use 7x as much of each volume to make up your master mix, then aliquot 49 µL six times into different 0.2 mL tubes. The remaining volume- deficient "seventh aliquot" is then discarded.

1. Calculate the extension time in your lab note book:
    - Length of amplicon: _____ kb.
    - Taq extension time per kb: 1 min/kb.
      • Extension time (length × extension time per kb): _____ hh:mm:ss.
2. Fill in the extension time in the program grid:

Initial denaturation	95	1-3 min	1x
Denaturation	95	30 s	25-40x
Annealing temp. - 5°C		30 s	

Extension	72		
Final extension	72	7 min	1x
Storage	4	∞	1x

3. Program the PCR machine (unless an identical pro- gram already exists in the machine).
4. Number the colonies you wish to test by marking on the backs of test and control plates.
5. Using a sterile loop, pick a small portion of each of these colonies and suspend individually in 30 µL of water. Mix thoroughly.
6. Calculate the volumes for making up your pre-mix for several reactions (as described above) based on the volumes provided for just one reaction here:

10X DreamTaq Buffer	5 µL
dNTPs, 2 mM	5 µL
forward primer, 5 µM	5 µL
reverse primer, 5 µM	5 µL
DNA Templates	10 pg- 1 µg
DreamTaq DNA Polymerase	0.25µL 0f 5U/ µL
ddH_2O	up to 50 µL
Total	50 µL

7. Prepare your pre-mix and divide into 49 µL aliquots on ice.
8. Add 1 µL of each cell suspension to each aliquot and mix.
9. Run the PCR reactions.
10. Analyze a 5 µL aliquot of each PCR by agarose gel electrophoresis.
11. For reactions where a full-length band was visible, purify the PCR product using a PCR purification kit according to the manufacturer's protocol.

12. Measure the concentrations of your PCR products.