Protocols

DOMINGO, 01/01/2017

Culture mediums

- **Medium M17:**
  - 2.1 g of ready medium M17 in ~ 50 ml of distilled water.
  - Dissolve in the microwave and check pH 7.0.
  - Separate 10 mL into the regeneration medium.
  - Autoclave the rest.

- **Home-made M17 medium:**
  Our commercial M17 pre-mixed powder (Sigma) already contains lactose as carbon source. Therefore, we had to improvise a home-made M17 in order to test different carbon sources.
  - 0.5 gPancreatic Digest of Casein
  - 0.5 g Soy Peptone
  - 0.5 g Beef Extract
  - 0.25 g Yeast Extract
  - 0.05 g Ascorbic Acid
  - 0.025 g Magnesium Sulphate
  - 1.9 g Na_3PO_4*
  - Distilled water up to 90 mL.
  - **Procedure**:
    - Ajust pH to 7.0
    - Autoclave at 121°C for 15min. Cool down to room temperature.
    - Add 10mL sterile 10% carbon source solution (glucose, fructose, galactose, mannitol).
    - Add 1 µg/mL erythomycin for *L. lactis* transformed with pSEUDO plasmids.
    - *The original recipe indicates the use of 1.9g Disodium-β-glycerophosphate. But we didn't have. We did some search and concluded glycerophosphate most likely is a source for phosphate. So, we exchanged it for sodium phosphate. Apparently, maximum culture growth was not affected.

- **Recovery Medium (Groeningen team)**:
  - 10 µl 200mM MgCl2
  - 10 µl 200 mM CaCl2
  - 430 µl 1.1 M Sucrose
  - 50 µl 10% glucose
  - 500 µl 2X M17
  - Store on ice until use

- **Electroporation Solution (ES):**
  - To 400 mL:
    - 68.46 g sucrose + 40 mL glycerol + complete 400 mL with distilled water.
    - Separate 100 mL for SL.
    - Autoclave the remaining 300 mL. (Concentrations: 0.5M Sucrose, 10% glycerol)

- **Wash Solution (WS**):
  - To 100 mL:
    - 100 mL Electroporation Solution (SE) + 1.46 g EDTA.
    - Adjust pH 7.0 with solid NaOH.
    - Add each pellet and wait until the next one is dissolved.
      - EDTA may not dissolve until the pH reaches close to 7.0.
    - Autoclave. (Concentrations: 0.5M Sucrose, 10% glycerol, 50mM EDTA)

- **Growth medium (GM):**
  - LB
  - 0.5M sorbitol

- **Recovery medium:**
  - LB
  - 0.5M sorbitol
  - 0.38M mannitol

- **Electroporations medium:**
  - 0.5M sorbitol
  - 0.5M mannitol
  - 10% glycerol
  - mixed in ddH2O

- **LB-medium (medinalab):**
  - **To 1 L:**
    - 1. Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
    - 2. Adjust the pH of the medium to 7.0 using 1N NaOH and bring volume up to 1 liter.
    - 3. Autoclave on liquid cycle for 20 min at 15 psi. Allow solution to cool to 55°C, and add antibiotic if needed (50µg / mL of Amp or Kan).
    - 4. Store at room temperature or +4°C.

  - **LB agar-plates:**
    - 1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
    - 2. After autoclaving, cool to approx. 55°C, add antibiotic (if needed), and pour into petridishes.
    - 3. Let harden, then invert and store at +4°C in the dark.

Culture of the microbiota in a reactor

- Carbohydrate-based medium composition:
  - 3 g/L of starch
  - 2 g/L of pectin
  - 4 g/L of type III mucin from porcine stomach
  - 1 g/L of xylan
  - 1 g/L of peptone
  - 1 g/L of arabinogalactan
  - 0.4 g/L of glucose
  - 3 g/L of yeast extract
  - 0.5 g/L of L- cysteine

Miniprep (Promega)

1. Transfer 600µl of bacterial culture grown in LB medium to a 1.5ml microcentrifuge tube
2. Add 100µl of Cell Lysis Buff er, and mix by inverting the tube 6 times
3. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting the tube
   a. The sample will turn yellow when neutralization is complete, and a yellow precipitate will form. Invert the sample an additional 3 times to ensure complete neutralization.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn
   a. Do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette
6. Place the minicolumn into a PureYield™ Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the fl owthrough, and place the minicolumn into the same PureYield™ Collection Tube.
8. Add 200µl of Endotoxin Removal Wash to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds. It is not necessary to empty the PureYield™ Collection Tube.

9. Add 400µl of Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds
10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30µl of Elution Buff er directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge at maximum speed in a microcentrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.

Ligation Protocol with T4 DNA Ligase (M0202)

- Protocol:

1. Set up the following reaction in a microcentrifuge tube on ice. (T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator (http://nebiocalculator.neb.com/#!/) to calculate molar ratios.
2. *The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
3. Gently mix the reaction by pipetting up and down and microfuge briefly.
4. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
5. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours*(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
6. Heat inactivate at 65°C for 10 minutes.
7. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Table1

	A	B
1	COMPONENT	20 µl REACTION
2	10X T4 DNA Ligase Buffer*	2 µl
3	Vector DNA	20-100 ng
4	Insert DNA	3-5 x vector
5	Nuclease-free water	to 20 µl
6	T4 DNA Ligase	1 µl

Protocol for a Routine PCR with Phusion® High-Fidelity PCR Kit

1. Set up the appropriate reactions on ice (see Table 2)
2. Gently mix the reaction and spin down in microcentrifuge. *If the thermocycler does not have a heated lid, overlay the sample with mineral oil.*
3. Cycling Conditions for a Routine PCR (see Table 3)

Table2

	A	B	C	D
1	Component	20 µl Reaction	50 µl Reaction	Final Concentration
2	Nuclease-free water	to 20 µl	to 50 µ	
3	5X Phusion HF or GC Buffer	4 µl	10 µl	1X
4	10 mM dNTPs	0.4 µl	1 µl	200 µM
5	10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
6	10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
7	Template DNA	variable	variable	< 250 ng
8	DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
9	Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Table3

	A	B	C	D
1	Cycle step	Cycles	Temp	Time
2	Initial denaturation	1	98°C	30 seconds
3	Denaturation Annealing Extension	30	98°C 45–72°C 72°C	5 10 seconds 10 30 seconds 15 30 seconds per kb
4	Final extension	1	72°C	5–10 minutes
5	Hold	1	4°C	∞

Digestion (Addgene)

1. Samples: Plasmid + Insert
2. Materials:
    a. CIP
    b. Buffer
    c. Restriction enzymes
3. Select restriction enzymes to digest your plasmid.
4. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
5. In a 1.5mL tube combine the following: (Table4: A typical restriction digestion reaction)
    - DNA
    - Restriction Enzyme(s)
    - Buffer
    - BSA (if recommended by manufacturer)
    - dH_2O up to total volume

6. Mix gently by pipetting.
7. Incubate tube at appropriate temperature (usually 37 °C) for 1 hour. Always follow the manufacturer's instructions.
8. o visualize the results of your digest, conduct gel electrophoresis.

Table4

	A	B
1	DNA	1 µg
2	Restriction Enzyme	1 µL
3	10x Buffer	3 µL
4	10x BSA (if recommended)	3 µL
5	dH2O	x µL (to bring total volume to 30µL)

Gel Electrophoresis (Addgene)

- **Pouring a Standard 1% Agarose Gel:**

1. Measure 1 g of agarose
2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
4. Let agarose solution cool down to about 50°C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. *(Optional)* Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.
6. Pour the agarose into a gel tray with the well comb in place.
7. Place newly poured gel at 4°C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

- **Loading Samples and Running an Agarose Gel:**

1. Add loading buffer to each of your digest samples.
2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1xTAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.
5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel.
7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. *(Optional)* If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 5 mins.
9. Using any device that has UV light, visualize your DNA fragments.

Sequencing (abi prism 3100 genetic analyzer)

1. Materials:
    - Big Dye Mix
    - Bufffer 5x Terminator
    - Primers (3.2 uM)
    - DNA
    - Water
2. Reaction:

- PCR: **96°C (1min) → 96°C (10s) → Melting Temperature → 60°C (4 min) → 60°C (5 min) → 4°C (∞)**
- PCR purification:
  - Add 45 µL SAM
  - Add 10 µL Resin
  - Leave 30 minutes shaking
  - Add 20 µL of each sample in a 96-well plate
  - Read on the sequencer

Competent cells

- **Based on "Molecular Biological Methods for Bacillus"**

1. Streak out the strain to be made competent on an LB or TBABG [Trytose blood agar base (Difco) + 0.5% glucose] agar plate as a large patch and incubate overnight at30℃
2. The following morning scrape the cell growth off the plate and use to inoculate fresh, pre-warmed, SpC medium a (20 ml) to give an OD600 reading of about 0.5.
3. Incubate the culture at 37 with vigorous aeration and take periodic OD readings ℃(OD600) to assess cell growth.
4. When the rate of cell growth is seen to depart from exponential (i.e. no significant change in cell density over 20-30 min) inoculate 200 ml of pre-warmed, SpII mediumawith 2 ml of stationary-phase culture and continue incubation at 37 with slower ℃aeration
5. After 90 min incubation, pellet the cells by centrifugation (8,000 g, 5min) at room temperature.
6. Carefully decant the supernatant into a sterile container and save.
7. Gently resuspended the cell pellet in 18 ml of the saved supernatant and add 2 ml of sterile glycerol; mix gently
8. Aliquot the competent cell (0.5 ml) in sterile tubes, freezed rapidly in liquid nitrogen or a dry-iced/ethanol bath or ice/isopropanol bath and store -70 ℃.

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Table 1. Media for two-step transformation procedure

T base	per liter:	
	$(NH_4)_2SO_4$	2 g
	$K_2HPO_4 \cdot 3H_2O$	18.3 g
	KH_2PO_4	6 g
	trisodium citrate·$2H_2O$	1g
	→ Autoclave	
SpC	Made fresh on the day of use from the following sterile solution:	
	T base	20 ml
	50% (w/v) glucose	0.2 ml
	1.2% (w/v) $MgSO_4 \cdot 3H_2O$	0.3 ml
	10% (w/v) Bacto yeast extract	0.4 ml
	1% (w/v) casamino acids	0.5 ml
	Growth requirements (See table 3, if the strain is auxotroph)	
SpII	Made fresh on the day of use from the following sterile solutions:	
	T base	200 ml
	50% (w/v) glucose	2 ml
	1.2% (w/v) $MgSO_4 \cdot 3H_2O$	14 ml
	10% (w/v) Bacto yeast extract	2 ml
	1% (w/v) casamino acids	2 ml
	0.1 M $CaCl_2$	1 ml
	Growth requirements (See table 3, if the strain is auxotroph)	
SpII + EGTA	SpII (200 ml) with 4 ml EGTA (0.1 M, pH 8.0) but without $CaCl_2$. SpII + EGTA can be frozen at -20℃ in small aliquots, although repeated freeze-thawing should be avoided.	

- **Electrocompetent Cells (*L.Latis*)**

1. 1. Grow *L. lactis* overnight in 5 mL of M17 medium or MRS in tube (pre-culture)
2. Inoculate 2 mL of preculture in 200 mL of SGM17 medium in 250 mL erlen.
3. Grow overnight until OD600 = 0.3-0.5.
4. Place the erlen on ice.
5. Transfer cultures to 50 ml ice-cold Falcon tubes.
6. Centrifuge cells for 10 min at 5000 xg at 4 ° C.
7. Discard supernatant.
8. Resuspend cells in 200 mL of ice-cold SE (electroporation solution).Do not use vortex!
9. Centrifuge for 10 min at 7000 xg at 4 ° C.
10. Discard supernatant.

11. Resuspend cells in 100 mL (total volume) of ice-cold SL (wash solution). Do not use vortex!
12. Incubate on ice for 15 min.
13. Centrifuge for 10 min at 7000 xg at 4 ° C.
14. Discard supernatant.
15. Resuspend cells in 50 mL (total volume) of ice-cold SE (electroporation solution). Do not use vortex!
16. Centrifuge for 10 min at 7000 xg at 4 ° C.
17. Discard supernatant.
18. Resuspend cells in 4 mL (total volume) of ice-cold SE (electroporation solution). Do not use vortex!
19. Clot 200 µL in 1.5 mL eppendorfs.
20. Freeze at -80 ° C

Transformation

- **Standard heat-shock transformation of chemically competent bacteria (addgene)**

1. Take competent cells out of -80°C and thaw on ice (approximately 20-30 mins).
2. Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell/DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
5. Put the tubes back on ice for 2 min.
6. Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.
7. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.
8. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
9. Incubate plates at 37°C overnight.

- **Electroporation Protocol (*L.Latis*)**

1. Place a 2 mm cuvette on the ice, plasmid to be eluted in water + competent L. lactis cell (expect to thaw on ice).
2. Add to a sterile eppendorf 100 µl of competent cells + 1-5 µl of plasmid (200 to 1000 ng). Shake gently.
3. Transfer the cells to the cuvette
4. Perform the electroporation at 2000 V, 25 µF, 200 Ω. A normal pulse should last 4.5 to 5 msec.
5. Add 1000 µL of Regeneration Medium (M17 + MgCl2 + CaCl2).
6. Leave the bucket for 10 min on ice
7. Transfer the contents to a 1.5 ml eppendorf.
8. Incubate for 2h at 30 ° C without shaking
9. Centrifuge at 3000 rpm for 3 min
10. Discard ~ 900 µL of supernatant and resuspend the remaining cells
11. Add a petri dish the entire contents on antibiotic M17-agar medium (1-5 µg / ml erythromycin)
12. Incubate the plates at 30 ° C for 1-3 days

- **Electroporation competent Bacillus subtilis 168 (DTU-iGEM 2015)**

1. Prepare an O/N culture of bacillus subtilis 168 LB media with appropriate antibiotic incubate at 37°C.
2. Make a 16 fold dilution of the O/N culture Growth medium (GM), incubate in 37°C shaken at 200rpm.
3. Grow till the OD reaches 0.85-0.95, then add threonine, glycine and Tween 80 to an final concentration of 1.0, 2.0, 0.03% respectively.
4. Incubate for 1 hour
5. Cool the culture on ice on ice for 5 min.
6. Centrifuge the culture at 5000g for 10 min and resuspend in 1/20 of original volume in electroporations medium.
7. The cells can now be stored at -80°C (for up to one month) or directly used for electroporation.

- **Bacillus subtilis Transformation Procedure Based on “Molecular Biological Methods for Bacillus”**

1. Thaw competent cells rapidly by immersing frozen tubes in a 37 water bath ℃
2. Immediately, add one volume of SpII + EGTAa to the Thawed cells; mix gently

3. In a sterile test tube add competent cell (0.2~0.5 ml) to the DNA solution (<0.1 ml) and incubate in a roller drum at 37 . ℃
4. Dilute the transformed cells as appropriate in T base containing 0.5% glucose and plate immediately onto selective media.

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Table 2. Antibiotic concentration for direct selection

Antibiotics	Abbreviation	Selective concentration	Solute
Chloramphenicol	Cm	5 μg/ml	95% ethanol
Erythromycin[a]	Em	1 μg/ml	95% ethanol
Lincomycin[a]	Lm	25 μg/ml	95% ethanol
Neomycin	Nm	5 μg/ml	dH_2O
Kanamycin	Km	10 μg/ml	dH_2O
Phleomycin	Pm	0.1-0.5 μg/ml	dH_2O
Tertracycline	Tc	20 μg/ml	50% ethanol
Spectinomycin	Spc	100 μg/ml	dH_2O

a. When selecting for MLS^r, both Em and Lm are used.

Lugol test

Used to confirm if occurred *genome integration*

1. Make a plate with LB medium and starch without antibiotic
2. Divide the plate into areas
3. Add in one of the areas a negative control
4. Add the samples in the other areas
5. leave overnight
6. add lugol (1 - 2 mL)
    - in samples that have made HR there will be no halo.

Plate Reader

The readings were performed on the ***Infinite® M200 (Tecan)***

- **Optical density (OD)**
    - Absorbance: Wavelength 600 nm
- **Luminescence**
    - Attenuation: NONE
    - Integration Time: 10000 ms
    - Settle Time : 0 ms
- **Flourescence**
    - Excitation Wavelength: 485 nm
    - Emission Wavelength: 535 nm

SDS-PAGE

- **Running (lower) gel: 10 ml (2 MINIgels) - 15% acrylamid**
- Water 2.2 ml
- 30% acrylamide 5.0 ml
- 1.5M Tris base, pH 8.8 2.6 ml
- 10% sodium dodecylsulfate (SDS) 100 ul
- 10% ammonium persulfate 100 ul
- TEMED 10 ul
- **Stacking (upper) gel: 4 ml (2 MINIgels) - 4% acrylamid**
- Water 2.65 ml
- 30% acrylamid 0.67 ml
- 1M Tris base, pH 6.8 0.65 ml
- 10% SDS 40 ul
- 10% ammonium persulfate 60 ul
- TEMED 6 ul

- **2x loading buffer**
- 100 mM Tris-Cl pH 6.8
- 4% SDS
- 0.2% bromophenol blue
- 20% glycerol
- **Tris-glycine running buffer**
- 5 mM Tris
- 50 mM glycine pH 8.3
- 0.02% SDS
- **Coomassie stain (1L)**
- 2.5 g Coomassie dye
- 500 ml methanol
- 400 ml water
- 100 ml glacial acetic acid
- **Destain solution (1L)**
- 500 ml ethanol
- 400 ml water
- 100 ml glacial acetic acid.
- **Procedure:**
    - Running gel:
        - Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
        - Prepare the gel solution (as described above) in a separate small beaker. Swirl the solution thoroughly.
        - Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates. Pipet ethanol on the top of it.
        - Wait for 30min to let it solidify.
    - Stacking gel:
        - Remove the ethanol.
        - Pipet in stacking gel.
        - Insert the well-forming comb without trapping air under the teeth. Wait for 30min.
        - Pace the gel into the running chamber.
        - Pour the running buffer into the inner chamber and keep pouring after overflow untill the buffer surface reaches the required level in the outer chamber.
    - Prepare the samples:
        - Mix your samples with sample buffer (loading buffer).
        - Heat them in boiling water for 5-10 min.
        - Load prepared samples and protein ladder into wells.
        - Run the gel at 90V for 20 min and 120V until the end.
    - Stainning:
        - Cover the gel with stanning solution and incubate for 1h.
    - Destanning:
        - Remove the stanning solution.
        - Cover the gel with destanning solution and incubate for 1h.
        - Remove the destainning solution and cover the gel with destilled water.

Western-blot

After running the samples in a 15% acrylamide SDS-PAGE gel, separated proteins bands were transferred to a PVDF membrane.

1. Transfer sandwich was set up bottom to top as follows: 3 pieces blotting paper, gel, membrane, 3 pieces blotting paper, all of them soaked in Towbin buffer*.
2. *Towbin buffer: 3g Tris, 14.4 g glycine, add Mili-Q water up to 800 mL, add 200 mL methanol.
3. A semi-dry transfer system was used. Settings: constant 0.04 A for 60 min.
4. After transference, the membrane was:
5. Washed 3 times with TBST buffer** for 10 min each. ** TBST buffer: 2.42 g Tris-base, 8.76 g NaCl, adjust pH to 7.2 - 7.4 with HCl, 1 mL Tween 20, add Mili-Q water up to 1L.
6. Blocked with fresh 10% milk in TTBS for 1h at room temperature

7. Washed twice with TBST for 10 min each time
8. Incubated with anti-Histag antibody from mouse diluted 1:20.00 in 10% milk TBST solution for 1h
9. Washed 4 times with TBST 10 min each
10. Incubated with anti-mouse antibody diluted 1:20.00 in 10% milk TBST solution for 1h
11. Washed 4 times with TBST 10 min each
12. Perform ECL using a suitable detection reagent.
    - Add (1: 1) luminol and oxygen peroxide to the gel for 1,5 minutes
13. A C-digit equipment was used to visualize labelled bands in the membrane.