Protocols

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Contents

Chapter 1

Making M9 Glucose 0.3%

1.1 What you need

Product	**Volume**
Water	34.07 ml
Salts (diluted 5x)	10 ml
$CaCl_2$ (1 mol)	5 µl
$MgSO_4$ (1 mol)	100 µl
B1 Vitamin (1%)	25 µl
Glucose (20%)	750 µl
Elements trace (diluted 1000x)	50 nl
Hepes (12%, 0.5 mol) (can be replaced with water)[1]	5 ml

1.2 Salts diluted 5 times

Product	**Mass**
$Na_2HPO_4(2H_2O)$	425 mg
KH_2PO_4	150 mg
NaCl	25 mg
NH_4Cl	50 mg

1.3 Elements trace

Product	**Volume**
H_2O	200 µl
$Na_2EDTA(2H_2O)$	100 µl
$ZnSO_4(7H_2O)$	100 µl
$CoCl_2(6H_2O)$	100 µl
$MnCl_2(4H_2O)$	100 µl
H_3BO_3	100 µl
$NaMoO_4(2H_2O)$	100 µl
$CuSO_4(5H_2O)$	100 µl
$FeSO_4(7H_2O)$[2]	5 µl

[1] Hepes: 5.96 g in 50 ml of water.

[2] **Warning: $FeSO_4(7H_2O)$ must be added to the solution just before use, from a stock solution of 0.03 g of $FeSO_4(7H_2O)$ in 1 ml of H_2O.**

Chapter 2

Gibson Assembly

2.1 Objective

Gibson Assembly was developed by Dr Daniel Gibson and his colleagues at the J. Craig Venter Institute and licensed to NEB by Synthetic Genomics, Inc. It allows for successful assembly of multiple DNA fragments, regardless of fragment length or end compatibility.

2.2 Materials and Reagents

- The Gibson assembly mix
- The DNA fragments

2.3 To be prepared in advance

- A 50 °C dry bath
- Calculation of DNA quantity

2.4 Compute DNA quantity

1. Nanodrop the DNA fragment to know the concentration : go on `http://www.promega.com/techserv/tools/biomath/calc05.htm` (or promega calc 05 on `http://www.google.com`). It will calculate the number of molecule of DNA in $\mathrm{pmol\,\mu l^{-1}}$ (if your nanodrop is over 1 µl).
2. See table 2.1 and table 2.2 for examples, the numbers are truncated.
3. Compute the ratio of the bigger strand over the smaller.
4. Multiply the concentration of the bigger strand by this ratio.
5. Divide this number by the concentration of the smaller strand. This gives you the ratio of volumes you will have to add. The question you will have to answer is *how can I add the same quantity in pmol of each DNA strand.*
6. Compute the volumes of DNA to add so that the sum is equal to 5 µl.

2.5 Protocol

In the tube containing the Gibson mix add the computed quantity of DNA and let sit for 1 h at 50 °C.

	Concentration in $\mathrm{pmol\,\mu l^{-1}}$	**Size of the strand in bp**	**Ratio #1 (Step 3)**
pZE	0.023	2155	
ParaBAD rpos	0.146	1559	1559/2155 = 1.4
	Step 4	**Step 5**	**Step 6**
pZE	0.023*1.4 = 0.0322		1 » 4 µl
ParaBAD rpos		0.0322/0.146 = 0.22	0.22 » 1 µl

Table 2.1: Example

	Concentration in $\mathrm{pmol\,\mu l^{-1}}$	**Size of the strand in bp**	**Ratio #1 (Step 3)**
pZE	0.023	2155	
ParaBAD rsd	0.012	1070	1070/2155 = 2
	Step 4	**Step 5**	**Step 6**
pZE	0.023*2 = 0.046		1 » 1 µl
ParaBAD rsd		0.046/0.012 = 4	4 » 4 µl

Table 2.2: Example

Chapter 3

PCR with *Phusion* Enzyme

3.1 Objective

The Polymerase Chain Reaction (PCR) is a biochemical technologyto amplify a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. *Phusion* is an high-fidelity DNA Polymerase that offers superior performance for PCR.

3.2 Materials and Reagents

- Ice
- 200 µl tubes
- Nuclease-free water
- *Phusion* buffer 5x
- sNTPs
- Primers
- Template DNA
- *Phusion* DNA polymerase

3.3 To be prepared in advance

- The ice you will have to work on.
- The Thermocycler, according to table 3.1. The Thermocycler must be 98 °C hot when the cycle begins (preheated).

No need to be under sterile condition, except when harvesting the template DNA.

Step	Temperature	Time
Initial denaturation	98 °C	30 s
25 to 35 cycles	98 °C	5 s to 10 s
	45 °C to 72 °C [1]	10 s to 30 s
	72 °C	15 s to 30 skb (calculation needed)
Final extension	72 °C	5 min to 10 min
Hold	4 °C to 10 °C	

Table 3.1: Thermocycler

[1] Depending on the primers, most of the time 55 °C.

3.4 Protocol

50 µl tubes: to obtain more copies of a region. 20 µl tubes: to check if the plasmid inserted is the good one by checking its size.

1. Prepare a single tube of preparation that you will divide afterward, in this tube you will add everything that is not specific to each PCR: water, buffer, dNTPs (DMSO optional) according to table 3.2 (in bold in the table).
2. Divide the mix in as many tube as needed and add the specific parts according to table 3.2.
3. ***Add the DNA polymerase at the very end !***
4. Put the tubes in the preheated thermocycler and begin the cycles.

Components	50 µl reaction	20 µl reaction
Nuclease-free water	up to 50 µl	up to 20 µl
Buffer 5x	10 µl	4 µl
10 mmol l^{-1} **dNTPs**	1 µl	0.4 µl
10 µmol l^{-1} Forward primer	2.5 µl	1 µl
10 µmol l^{-1} Reverse primer	2.5 µl	1 µl
Template DNA	variable	variable
DMSO	1.5 µl	0.6 µl
Phusion DNA polymerase	0.5 µl	0.2 µl

Table 3.2: Protocol

Chapter 4

TSS Treatment

4.1 Objective

This method aims to make cells competent using the TSS method, allowing subsequent transfection with an exogeneous plasmid.

4.2 Materials and Reagents

- Ice-cold water
- A preheated bath at 42 °C
- Bacterial culture
- LB medium
- TSS
- Sterile eppendorf tubes
- Sterile pipet tips

4.3 To be prepared in advance

- The day before prepare a culture of the given bacterium, it has to grow overnight.
- A little tank filled with ice and water.
- The 42 °C bath.
- **Sterile conditions !**

4.4 Protocol

1. Dilute 10 µl of the culture in 990 µl of LB.
2. Let it grow until the OD600 is about 0.3, or let it grow at 37 °C for 1.5 h.
3. Per wanted transformed strain add 50 µl of culture with 50 µl of TSS.
4. Let sit 45 min on ice.
5. In each tube add 10 ng of DNA.
6. **Heat-shock:** 10 min of ice-cold water, 1.5 min in 42 °C bath, 10 min on ice-cold water.
7. Add 900 µl of LB per tubes
8. Add the antibiotic (the concentration depends on the antibiotic of interest.).
9. Incubate 1 h at 37 °C.

Chapter 5

Plasmid Digestion

5.1 Objective

To extract a piece of DNA from a plasmid.

5.2 Materials and Reagents

- 100 ng of DNA
- Biolabs buffer #2 (5 µl)
- Water
- Restriction enzymes from Biolabs (1 µl per enzyme)

5.3 To be prepared in advance

- Dry bath at 37 °C
- Compute the volume of DNA according to its concentration.
- Compute the volume of water to go up to 50 µl

5.4 Protocol

- If you need more than one tube you can do a master mix.
- Put your tubes for 2 h at 37 °C.

Chapter 6

pBAD Characterization

6.1 Objective

pBAD promoter characterization.

6.2 Materials and Reagents

- L-Arabinose
- LB medium
- Antibiotics: Kanamycin (stock solutions (SS): $0.05\,\mathrm{g\,ml^{-1}}$)
- Black 96-microwell plate
- Multichannel pipet
- Spectrophotometer
- 15 ml Falcon tubes (3+8=11 tubes)
- 2 ml Eppendorf tubes (3x8=24)

6.3 To be prepared in advance

The day before (late evening)

- Prepare 3500 ml Erlenmeyer according to the table 6.1.
- Incubate overnight at 37 °C under agitation (350 rpm).

Erlenmeyer 1	**Erlenmeyer 2**	**Erlenmeyer 3**
50 ml LB	50 ml LB	50 ml LB
50 µl Kanamycin SS		
1 clone of BW25113 pBAD-GFP	1 clone of BW25113 WT	

Table 6.1: Erlenmeyer preparation

6.4 Protocol

In the morning

1. Prepare your glucose solution by diluting 2 g of glucose powder in 10 ml LB.

2. Make a 100-fold dilution using the solutions of Erlenmeyer 1 and 2 and mesure optical density. Make sure they are similar and close to 0.3 uA.

3. If not, add some media in the more concentrated bacterial solution and re-perform the measurement until this condition is fulfilled.

4. Prepare the following set of standards:

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	**Tube 8**
[ara] (%)	20×10^{-3}	4×10^{-3}	8×10^{-4}	4×10^{-4}	1.6×10^{-4}	8×10^{-5}	1.6×10^{-5}	0
Vara	1980 µl							
V Erlen 1	20 µl							
V SS Kana	10 µl (final: 50 µg ml^{-1} Kanamycin)							
V Glucose	30 µl (final: 0.3% glucose)							

	Tube 1c	**Tube 2c**	**Tube 3c**	**Tube 4c**	**Tube 5c**	**Tube 6c**	**Tube 7c**	**Tube 8c**
[ara] (%)	20×10^{-3}							
Vara	1980 µl							
V Erlen 2	20 µl							
V SS Kana								
V Glucose	30 µl (final: 0.3% glucose)							

	Tube 1d	**Tube 2d**	**Tube 3d**	**Tube 4d**	**Tube 5d**	**Tube 6d**	**Tube 7d**	**Tube 8d**
[ara] (%)	20×10^{-3}							
Vara	1980 µl							
V Erlen 3	20 µl							
V SS Kana								
V Glucose	30 µl (final: 0.3% glucose)							

5. Add the content of tubes 1 to 8 in each channel of the pipet reservoir.

6. Using the multi-channel pipet, add 200 µl of those solutions in a row of the microplate.

7. Repeat this step 5 times (pentaplicate).

8. Wash the pipet reservoir with ethanol 70% and then rinse with deionized water.

9. Add the content of tubes 1c to 8c in each channel of the pipet reservoir.

10. Using the multi-channel pipet, add 200 µl of those solutions in a row of the microplate.

11. Wash the pipet reservoir with ethanol 70% and then rinse with deionized water.

12. Add the content of tubes 1d to 8d in each channel of the pipet reservoir.

13. Using the multi-channel pipet, add 200 µl of those solutions in a row of the microplate.

14. Add black pen spots in the bottom of two wells of the microplate.

15. Input your plate into the spectrophotometer and launch the required program.

Chapter 7

Transformation Efficiency for a Stock of Competent Cells

7.1 Objective

Stocks of competent cells are typically stored in 100 µl **aliquots. As certain geneticprocedures are difficult to do with less efficient cells, it is always of interest to know howefficient a stock of cells is so as to eliminate potential reasons for problems during experiments.**

Efficiency is typically measured in: number of viable colonies per µg **of DNA used during the transfection protocol. Research grade stocks average at** 10^8 colonies/µg **of DNA, whereas good self-made stocks average at about** 10^4 colonies/µg **to** 10^6 colonies/µg **of DNA.**

This protocol aims to evaluate the efficiency of an existing stock of cells for transfection.

7.2 Materials and Reagents

- Stocks of competent cells stored in 100 µl aliquots
- Plasmids with antibiotic resistance
  Concentration needs to be known if in solution, weight if purified for the purpose of this protocol.
- 2 sterile Petri Dishes
- A round-ended tube compatible with the waterbath (typically 14 ml)
- The corresponding antibiotic for the transfection plasmid

7.3 To be prepared in advance

- A 42 °C waterbath

7.4 Protocol

1. Place 10 ng of DNA in a 14 ml round-ended tube on ice.
2. Prepare a tube without DNA as a negative control.
3. Defrost the competent cells quickly by warming the aliquot with your hands.
4. Add the 100 µl of cells suspension into the tube.
5. Leave for 10 min on ice.
6. Place the tubes in the waterbath for 90 s.
7. Add 1 ml of LB medium.

8. Place 1/10 of the volume on the first Petri Dish and spread evenly to facilitate counting afterwards.
9. Place the remaining 9/10 of the volume on the second Petri Dish in the case that the cells aren't competent enough to produce colonies on the first dish.
10. Incubate the Petri Dishes for 12 h to 16 h.
11. Count the number of colonies for the Petri Dish where this is easier.

Transformation Efficiency is: $\frac{numberofcolonies*10}{100}$ for the first dish. Divide by 9 if counting on the second.

Chapter 8

Preparation and Transformation of Freezeable E.Coli Cells

8.1 Objective

Transform previously frozen competent E. Coli cells with a plasmid.

8.2 Materials and Reagents

All solutions are considered sterile. Make sure to work in sterile conditions.

Product	**Amount**
$MgCl_2$ 100 mmol l^{-1}	500 ml
$CaCl_2$ 100 mmol l^{-1}	250 ml
$CaCl_2$ 85 mmol l^{-1} - 15% glycerol	50 ml
LB medium	50 ml
500 ml Erlenmeyer [1]	50 ml
50 ml Falcon tubes	$2 - N$ [2] (make sure number is even)
500 µl Eppendorf tubes	$10 * (1 - N)$ [2]
DNA (plasmid to be transfected)	$N * 10$ ng (pure) or $N * (1 - 10)$ µl (ligation product)
14 ml round-ended tubes	N [2]

8.3 To be prepared in advance

1. Prepare a 50 ml E. Coli preculture in LB medium until saturated.
2. Resuspend 1 ml of the preculture in N x 50 ml LB and incubate until OD600 reaches 0.3 to 0.4 (corresponding to about 2 h incubation).
3. *During incubation:* prepare the centrifuge by setting its temperature to 4 °C.
4. Place the Falcon tubes used for centrifugation on ice.
5. Put the $MgCl_2$ and $CaCl_2$ solution-containing tubes on ice.
6. Resuspend the bacterial culture in 1/4 the initial culture volume of $MgCl_2$ 100 mmol l^{-1} **over** 5 min **and on ice.**

[1] Chose the erlenmeyer size according to your culture volume with the following rule: the erlenmeyer rated volume must be 1/10 of the culture volume. Use bigger erlenmeyers to prepare more competent cells.

[2] Depending on the amount of competent cells that you wish to prepare, calculate the amount of tubes and erlenmeyers needed with the following rule: 50 ml of initial culture yields 10 Eppendorf tubes with 100 µl in each. One Eppendorf tube is needed every time you wish to transform cells with a new plasmid. Maximum N is 40 according to prepared $MgCl_2$ and $CaCl_2$ solutions.

7. Centrifuge once again at 4000 rpm (3220 g) at 4 °C for 10 min.
8. Resuspend the new bacterial culture in 1/20 the initial culture volume of $CaCl_2$ 100 mmol l^{-1} **over** 5 min **and on ice.**
9. Add 9/20 the original culture volume of $CaCl_2$ 100 mmol l^{-1}.
10. Leave on ice for at least 20 min.
11. Centrifuge once again at 4000 rpm (3220 g) at 4 °C for 10 min.
12. Resuspend the new bacterial culture in 1/50 its original volume of sterile $CaCl_2$ 85 mmol l^{-1} – 15% w/v glycerol **over** 5 min **and on ice.**
13. Place the resulting volume in 100 µl aliquots to be frozen at −80 °C.
14. Tag appropriately.

8.4 Protocol

1. Prepare the waterbath by placing its temperature at 42 °C exactly.
2. Place the DNA in a 14 ml round-ended tube on ice.
3. Prepare a tube without DNA as a negative control.
4. Defrost the competent cells quickly by warming the aliquot with your hands.
5. Add the 100 µl of cell suspension into the tube.
6. Leave for 10 min on ice.
7. Place the tubes in the waterbath at 42 °C for 90 s.
8. Add 1 ml of LB medium.
9. Incubate the cells for 1 h at 37 °C in an incubator.
10. Spread 20 µl (when using pure purified plasmid) to 20 µl (when using ligase products) of the culture on a Petri Dish containing the appropriate antibiotic(s).
11. When dry, turn over and incubate 12 h to 16 h at 37 °C.
12. Harvest colonies using sterile tools.