Site-directed mutagenesis Taq'Ozyme

Introduction

DO NOT USE THIS PROTOCOL

Mix of two protocols :
- from the polymerase kit (https://www.ozyme.fr/gammes/ozy/pdf/ozy_taqozyme_hs_mix_1000_fiche_tech.pdf)
- from Wageningen iGEM team (http://2014.igem.org/wiki/images/b/b5/Wageningen_UR_protocols_Site_Directed_Mutagenesis.pdf)

Materials

- PCR
  - Taq'Ozyme HS Mix
  - Primers
  - Sterilized distilled water
  - Plasmid
  - Thermocycler
- Dpn1 digestion
  - Dpn1 enzyme 20 U/µl
  - PCR products

Procedure

Design of the primers

1. The primers should be designed in order to create a new restriction enzyme thanks to the mutation site to control it

PCR

2. Mix reagents in a PCR tube in the following order

   Sterilized distilled water qsp 50µl
   Forward primer 0.4µM (e.g. 1µl at 20µM)
   Reverse primer 0.4µM (e.g. 1µl at 20µM)
   DNA matrix (plasmid) 200ng
   Taq'Ozyme HS Mix 2X 25µl

3. Vortex then centrifuge briefly

4. Put the tube in the thermocycler and use the following program

PCR Program

	A	B	C	D
1	Step	T°	Duration	Nb of cycles
2	First denaturation	95°C	1 min	1
3	Denaturation	95°C	15 sec	25
4	Hybridisation	Tm-5°C	15 sec	
5	Elongation	72°C	1 min	
6	Final extension	72°C	5 min	1

5. Check with Qubit or Nanodrop

6. Store at 4°C if necessary

Dpn1 digestion

7. Add 1µl of Dpn1 enzyme (20U/µl) to the PCR product

8. Incubate 1h at 37°C

9. Check on agarose gel electrophoresis (cf. protocol Agarose gel electrophoresis) if the mutation was added after digesting some of the PCR product with the restriction enzyme

Transformation

10. Cf. protocol CaCl2 Competent cells/Transforming

11. Check with sequencing

Site-directed mutagenesis Pfu pol

Introduction

Protocol from https://france.promega.com/products/pcr/endpoint-pcr/pfu-dna-polymerase/?catNum=M7741#protocols
+ protocol from L. Bonnefond (Mutagenesis on mPRMT6 34-378 WT and C53S constructs to remove the stop codon before peptide insertion at the C-terminus, 2018-03-08)

Materials

- PCR
  - Pfu buffer (Promega)
  - DNA template
  - Primers 10µM
  - dNTP mix 10mM
  - Pfu pol 2.5U/µl (Promega)
  - Sterilized distilled water
  - Thermocycler
  - PCR tubes
- DpnI 20U/µl

Procedure

Design of the primers

1. The primers should be designed in order to create a new restriction enzyme thanks to the mutation site to control it

PCR

2. Mix reagents in a PCR tube in the following order

   Sterilized distilled water qsp 50µl
   Pfu buffer 10X 5µl
   Forward primer 10µM 1.5µl
   Reverse primer 10µM 1.5µl
   DNA template 50ng (<0.5µg/50µl)
   dNTP mix 10mM 1µl
   Pfu pol 2.5U/µl 1µl

3. Centrifuge briefly

4. Put the tube in the thermocycler and use the following program

PCR Program

	A	B	C	D
1	Step	T°	Duration	Nb of cycles
2	First denaturation	95°C	1 min	1
3	Denaturation	95°C	1 min	
4	Hybridisation	Tm-5°C	1 min	12
5	Elongation	72°C	2 min/kb	
6	Final extension	72°C	30 min	1

5. Store at 4°C if necessary

Dpn1 digestion

6. Add 2µl of Dpn1 enzyme (20U/µl) to the PCR product

7. Incubate 2h at 37°C

8. During this time, check 5µl of PCR product on agarose gel electrophoresis (cf. protocol Agarose gel electrophoresis)

Transformation

9. Cf. protocols CaCl2 Competent cells/Transforamtion

10. Check with sequencing and restriction enzyme

Agarose gel electrophoresis

Introduction

How to make an agarose gel to check the conformation and purity of plasmids.

Materials

- Agarose gel
  - 1 g agarose
  - 100 ml water + TAE or TBE 1X
  - 5 µl BET
- Microwave
- Gel migration cuve
- Generator
- TE buffer
- Loading dye
- Transilluminator (Biorad, 4th floor)

Procedure

Gel preparation

1. Mix agarose with 100ml TBE or TAE 1X and heat with the microwave. When the solution starts to boil, take it out, mix it, let it cool down and heat again. Repeat until agarose is completely dissolved.
2. Add 5 µl of BET
3. Pour the gel in the cuve
4. When the gel is solidified, remove the comb, put the gel in the cuve filled with TE buffer.

Sample preparation

5. Mix 1 µl of plasmid solution with 1 µl of 6X loading dye and 4 µl of water

Running of the gel

6. Load the samples and the ladder on the gel
7. Run the gel for 40 min at 140 V.

Gel imaging

8. Take a picture of the gel using the transilluminator

CaCl2 Competent cells

Introduction

To obtain competent cells for transformation

Materials

- Cells
  - Any cell in a liquid culture
- Solutions
  - 0.1M CaCl2
  - 0.1M CaCl2 + 10% glycerol
  - LB medium
- Material
  - Incubator
  - Spectrophotometer
  - Ice
  - Centrifuge
  - Cryogenic tubes

Procedure

1. Dilute an overnight culture 1:200 in LB.
2. Aerate the culture at 30°C or 37°C until the OD600 is between 0.3 and 0.4. Record the OD600. Warning: Do not let the OD600 go above 0.5 or the culture will yield poor competent cells.
3. Chill the cells on ice for 1 hour.
4. Pellet the cells at 3200 g for 15 minutes at 4°C.
5. Resuspend the cells in 1/4 volume of ice cold 0.1 M CaCl2, and leave them on ice for at least 1 hour.
6. Pellet the cells at 3200 g for 15 minutes at 4°C.
7. Resuspend the cells in ice cold 0.1 M CaCl2; 10% w/v glycerol at a final concentration of 15 OD600 units/ml.

   Example: If the initial OD600 of a 50 ml culture was 0.3, then resuspend the cells in 1 ml. 8.
8. Store the new competent cells in 0.5 to 1 ml aliquots at -70°C. 100 µl of competent cells is needed for each transformation

Transformation of competent cells

Introduction

Protocol used with competent strains (CaCl2 treatment)

Materials

- Resuspended DNA to be transformed
- 10pg/µl Positive transformation control DNA (e.g. pSB1C3 w/ BBa_J04450, RFP on high-copy chloramphenicol resistant plasmid. Located in the Competent Cell Test Kit.)
- Competent Cells (50µl per sample)
- 1.5mL Microtubes
- LB Media (950µL per sample)
- Petri plates w/ LB agar and antibiotic (2 per sample)
- Ice
- 42°C water bath
- 37°C incubator
- Sterile spreader or glass beads

Procedure

Transformation

1. pre-chill 1.5ml tubes (one tube for each transformation, including your control).
2. **Thaw competent cells on ice:** This may take 10-15min for a 260µl stock. (Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.)
3. **Pipette 50µl of competent cells into 1.5ml tube:** 50µl in a 1.5ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.
4. **Pipette 1µl of resuspended DNA into 1.5ml tube:** Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
5. **Pipette 1µl of control DNA into 2ml tube:** Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
6. **Close 1.5ml tubes, incubate on ice for 30min:** Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
7. **Heat shock tubes at 42°C for 45 sec:** 1.5ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
8. **Incubate on ice for 5min:** Return transformation tubes to ice bucket.
9. **Pipette 950µl LB media to each transformation:** SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
10. **Incubate at 37°C for 1 hours, shaking at 200-300rpm**
11. **Pipette 100µL of each transformation onto petri plates** Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
12. **Spin down cells at 6800g for 3mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates** Spread with sterilized spreader or glass beads immediately. This increases the chance of getting colonies from lower concentration DNA samples.
13. **Incubate transformations overnight (14-18hr) at 37°C:** Incubate the plates upside down (agar side up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
14. **Pick single colonies:** Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.
15. **Count colonies for control transformation:** Count colonies on the 100µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of 1.5x10^8 to 6x10^8 cfu/µg DNA.

Monarch® Plasmid DNA Miniprep Kit Protocol (NEB #T1010)

Introduction

Protocol used to purify plasmids

Materials

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› Monarch Plasmid DNA Miniprep Kit

Procedure

Miniprep protocol

All centrifugation steps should be carried out at 16,000 x g (~13,000 RPM).
If precipitate has formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.
Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

1. Pellet 5 ml of overnight bacterial culture (not to exceed 15 OD units) by centrifugation at **3260 g for 10 minutes**. (We don't have a centrifuge that goes higher for big tubes)

   Discard supernatant while keeping 1 ml to resuspend the pellet.
   Note: 1.5 ml of culture is sufficient for most applications. Ensure cultures are not overgrown (12-16 hours is ideal).

2. **Resuspend** the pellet in the residual supernatant and transfer in a microfuge tube. Centrifuge **30 seconds at 16,000g.**

3. Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1) (pink). **Vortex or pipet** to ensure cells are completely resuspended. There should be no visible clumps.

4. Add 200 µl Plasmid Lysis Buffer (B2) (blue/green). **Invert tube immediately** and gently 5–6 times until color changes to dark pink and the solution is clear and viscous. Do not vortex! Incubate for **one minute.**

5. Add 400 µl of Plasmid Neutralization Buffer (B3) (yellow). **Gently invert** tube until color is uniformly **yellow** and a precipitate form. Do not vortex! Incubate for **2 minutes**.

   Note: Sample is neutralized when color is uniformly yellow and precipitate forms.

6. Clarify the lysate by spinning for **2–5 minutes at 16,000 g.**

   Note: For culture volumes > 1 ml, we recommend a 5 minutes spin to ensure efficient RNA removal by RNase A. Pellet should be compact; spin longer if needed.

7. Carefully transfer supernatant to the **spin column** and centrifuge for **1 minute**. Discard flow-through.

8. Re-insert column in the collection tube and add **200 µl of Plasmid Wash Buffer 1.** Plasmid Centrifuge for **1 minute**. Discarding the flow-through is optional.

9. Add **400 µl of Plasmid Wash Buffer** 2 and centrifuge for **1 minute.** Discard flowthrough and repeat centrifugation.

10. Transfer column to a **clean 1.5 ml microfuge tube**. Use care to ensure that the tip of the column has not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute.

11. Add **40–50 µl DNA Elution Buffe**r to the center of the matrix. Wait for **10 minutes, then spin for 2 minutes to elute DNA.**

    Note: Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Additionally, yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated as a result of dilution. For larger plasmids (≥ 10 kb), heating the DNA Elution Buffer to 50°C prior to use can improve yield.

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. Reaction volumes: *Use this table to calculate reaction volumes and set up the reaction. Remember to* ***input your total DNA fragment volume in cells B3 and C3*** *for assemblies with 2-3 fragments and 4-6 fragments, respectively.*

   - NEB recommends a total of ***0.02–0.5 pmols of DNA fragments when 1 or 2 fragments*** *are being assembled into a vector and* ***0.2–1.0 pmoles of DNA fragments when 4–6 fragments*** *are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend using NEB's online tool, NEBioCalculator.*
   - 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.

Table1

	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)	8.5		10
4	Gibson Assembly Master Mix (2x) (µl)	10	10	10
5	Deionized Water (µl)	1.5	#VALUE!	0
6	Total Volume (µl) ***	20	20	20

   **Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.*
   *** Control reagants are provided for 5 experiments with the Gibson Asembly Kit.*
   **** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.*

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.

   Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section).
   **Select the pencil icon and change the timer to 60 minutes if working with 4-6 fragments*

   00:15:00

3. Store samples on ice or at –20°C for subsequent transformation.

4. Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the chemcial transformation protocol or electro competent cells transformation protocol