Methodic Protocols & Materials

For everybody's guidance

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Updated: 13.10.2018

Content

A. In vitro

I. Cloning

a) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a precise method for exponentially amplifying a fragment of DNA (from a mixture of DNA molecules) in vitro.

Standard PCR is a method that is used to amplify DNA sequences of various lengths with a thermostable polymerase with proofreading function.

Touchdown PCR is a variation of Standard PCR. The annealing temperature for the primers is not constant during the PCR but decreases by 1 degree per cycle in the first cycles in order to avoid unspecific primer binding.

Mutagenesis PCR is used in order to introduce point mutations, insertions or deletions into DNA sequences of interest. For this purpose, specific primers are designed.

Taq® DNA Polymerase

Component	25 µl Reaction	50 µl Reaction	Final [Conc]:
10X Taq Reaction Buffer	2.5 µl	5.0 µl	1X
10 mM dNTPs	0.5 µl	1.0 µl	200 µM
10 µM Forward Primer	0.5 µl	1.0 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	1.0 µl	0.2 µM
Template DNA	variable	variable	< 1,000 ng
Nuclease-free water (MQ)	to 25 µl	to 50 µl	
OneTaq® DNA Polymerase	0.5 µl	1.0 µl	1.25 units/50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	0:30 min.	1 cycle
Denaturation	95 °C	0:15 – 0:30 min.	30 cycles
Annealing*	45 – 68 °C	0:15 – 1:00 min.	
Extension	68 °C	1:00 min. / kb	
Final Extension	68 °C	5:00 min.	1 cycle
Hold	4 – 10 °C	Indefinite	1 cycle

Phusion® High-Fidelity DNA Polymerase

Component	20 µl Reaction	50 µl Reaction	Final [Conc]:
5X Phusion HF or GC Buffer	4.0 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1.0 µl	200 µM
10 µM Forward Primer	1.0 µl	2.5 µl	0.5 µM

10 µM Reverse Primer	1.0 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3 %
Nuclease-free water (MQ)	to 20 µl	to 50 µl	
Phusion	0.2 µl	0.5 µl	1.0 units/50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0: 50 – 0:10 min.	
Annealing*	45 – 72 °C	0:10 – 0:30 min.	25 – 35 cycles
Extension	72 °C	0:15 – 0:30 min. / kb	
Final Extension	72 °C	5:00 – 10:00 min.	1 cycle
Hold	4 – 10 °C	Indefinite	1 cycle

Q5® High-Fidelity PCR

Component:	25 µl Reaction	50 µl Reaction	Final [Conc]:
Q5 Reaction Buffer 5x	5.0 µl	10 µl	1x
dNTPs (10mM)	0.5 µl	1 µl	200 µM
Forward Primer (10µM)	1.25 µl	2.5 µl	0.5 µM
Reverse Primer (10µM)	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	1 ng/µL
(Q5 High GC Enhancer)	(5.0 µl)	(10 µl)	(1x)
Nuclease-free water (MQ)	to 25 µl	to 50 µl	(to 50 µl)
Q5 DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0: 50 – 0:10 min.	
Annealing*	50 – 72 °C	0:10 – 0:30 min.	25 – 35 cycles
Extension	72 °C	0:20 – 0:30 min. /kb	
Final Extension	72 °C	2:00 min.	1 cycle
Hold	12 °C	Indefinite	1 cycle

* The annealing temperature for a specific amplification reaction will depend on the sequences of the two primers.

b) Restriction digest

In order to insert DNA fragments into plasmids via ligation, it is necessary to digest both components with restriction enzymes.

Single DNA Digestion

The following is an example of a typical analytical single restriction enzyme digestion.

1. Add up the following:
    - 500 ng DNA
    - 5 µl 10 x appropriate NEBuffer
    - 1 µl restriction enzyme (10 U)
    - Fill up to 50 µl with nuclease free H2O
2. Incubate for 30 minutes at 37°C
3. Heat inactivation: Incubate at 80 °C for 20 minutes

Larger or smaller scale DNA digestions can be accomplished by scaling this basic reaction proportionately.

Multiple Restriction Enzyme Digests

Use the optimal buffer supplied with one enzyme if the activity of the second enzyme is acceptable in that same buffer (Check table supplied by NEB, BioBrick Assembly: Buffer 2.1). Follow the single restriction enzyme digestion by using 1 µl of the additional enzyme and take off 1 µl from the nuclease-free water.

c) Dephosphorylation

Antarctic Phosphatase catalyzes the removal of 5'-phosphate groups of DNA/RNA and thus prevents re-ligation of cut vectors. It is used before ligation.

Procedure

1. Reaction Mix
    - Restriction product
    - 1/10 of reaction end volume 10 x Antarctic Phosphatase Reaction Buffer
    - 1 µl of Antarctic Phosphatase
2. Incubate at 37 °C for 30 minutes
3. Heat inactivation: Incubate at 70 °C for 5 minutes
4. Continue with ligation

d) DNA Ligation

DNA ligation is necessary to assemble digested DNA parts into a vector. The cut ends generated by restriction enzymes are put together by DNA ligase.

Procedure (20 µl batch)

1. Reaction mix
    - T4 Ligase Buffer 2 µl
    - T4 Ligase 1 µl
    - Digested Insert 6 µl
    - Digested Backbone 2 µl

Molar ratio: 3 : 1 (Insert : Vector)
For calculation: www.php.rsdnerf.com

- add ddH2O to 20 µl end volume

2. Incubate at 16 °C overnight or at room temperature for 30 minutes (results might be worse)
3. Inactivate at 65 °C for 10 minutes

e) Deletion (Leucin/KanMX cassette)

Leucin/KanMX deletion cassette amplification

- Plasmid (250 ng)..... 0.5 µl
  Leucin cassette in pUG73
  KanMX cassette in pUG6
- forward primer..... 2.5 µl
- reverse primer..... 2.5 µl
- Q5 MasterMix ..... 25 µl
- ddH_2O..... to 50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0:10 min.	
Annealing*	55 °C	0:30 min.	30 cycles
Extension	72 °C	0:30 min. /kb	
Final Extension	72 °C	2:00 min.	1 cycle
Hold	12 °C	Indefinite	1 cycle

II. Analysis and Purification

a) Agarose gel electrophoresis

Agarose gel electrophoresis is the most common used method to separate nucleic acids. Due to their negative charge DNA and RNA molecules can be moved through an agarose gel by an electric field (electrophoresis). Longer molecules move slower through the agarose matrix while short DNA fragments move faster and migrate further.

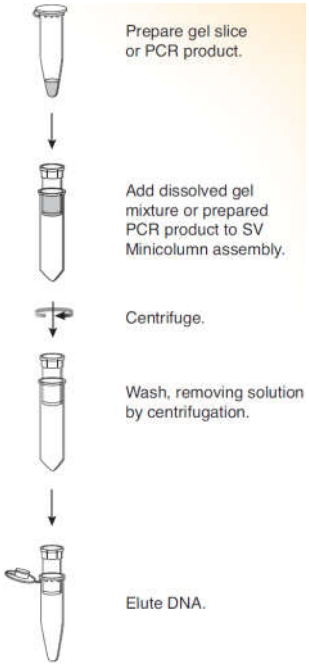
Procedure

In common we used 0.8 – 2.0 % agarose gels. Low concentrated gels lead to better results for large DNA fragments (2 – 6 kbp), while high concentrated gels lead to better results for small DNA fragments (0.3 – 0.7 kbp).

1. Mix desired amount of agarose with 1 x TAE-Buffer
2. Heat up liquid in microwave until whole agarose is dissolved
3. Let liquid cool down until you can touch the bottle with your hands
   Mix 2 µl Nancy-520 or HDGreen plus with 50 ml of agarose gel
4. Fill mixture into gel chamber and let it cool down (do not forget the well combs)
5. Fill-up chamber with 1 x TAE-Buffer
6. Take off well comb
7. Pipette 3 – 4 µl DNA ladder of choice into first pocket
8. Mix samples 5 : 1 with 6 x loading dye (5 µl sample with 1 µl loading dye) and pipette into pockets
9. Run electrophoresis with 120 V for 45 minutes.

b) Gel and PCR Clean-Up System (Wizard® SV)

Gel and PCR Clean-Up Systems are used to remove unincorporated primers, salts, and left-over dNTP´s from generated amplicons after PCR. This System also provides purification of amplicons from preparative agarose gel.



Procedure

Reference Wizard® SV Gel and PCR Clean-Up System Quick Protocol from Promega

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 ml microcentrifuge tube.
2. Add 10 µl Membrane Binding Solution per 10 mg of gel slice. Vortex and incubate at 50 – 65 °C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700 µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500 µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
6. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

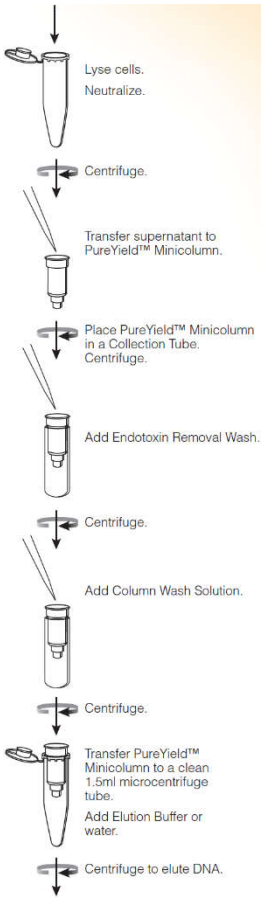
Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
9. Discard Minicolumn and store DNA at 4 °C or - 20 °C.

Source: https://www.promega.com/~/media/files/resources/protcards/wizard%20sv%20gel%20and%20pcr%20clean-up%20system%20quick%20protocol.pdf

c) Plasmid Miniprep (PureYieldTM System)

To get the plasmid out of the cells, we use the PureYield Plasmid Miniprep System Kit from Promega. It is a fast way to purificate plasmid DNA out of a bacteria culture by cetrifugation.



Procedure

The whole purification was done according to the "Quick Protocol" of the PureYield Plasmid Miniprep System from Promega.

Prepare Lysate

1. Add 600 µl of bacterial culture to a 1.5 ml microcentrifuge tube.
   Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3 ml of bacterial culture.
2. Add 100 µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350 µl of cold (4 – 8 °C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant (~ 900 µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet.

6. Place the Minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the flowthrough, and place the Minicolumn into the same Collection Tube.

Wash

8. Add 200 µl of Endotoxin Removal Wash (ERB) to the Minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400 µl of Column Wash Solution (CWC) to the Minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

Elute

10. Transfer the Minicolumn to a clean 1.5 ml microcentrifuge tube, then add 30 µl of Elution Buffer or nuclease-free water directly to the Minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at – 20 °C.

Source: https://www.promega.com/~/media/files/resources/protcards/pureyield%20plasmid%20miniprep%20system%20quick%20protocol.pdf

d) DNA Quantification with NanoDrop

NanoDrop is a UV-Vis spectrophotometer which can be used to measure quantity and purity of DNA in a sample using only 1 – 2 µl.

Procedure

1. Start the program for the NanoDrop and choose "Nucleic Acids"
2. Pipette 1 µl of a water sample onto the lower measurement and click “OK” for initializing
3. Load your blank and click "Blank"
4. Load your DNA samples and click "Measure"

The system calculates automatically the concentration in ng/µl, the 260/280 value and the 230/260 value.

e) Sequencing of DNA (Eurofins Genomics)

Sequencing is a method to control if you have your construct of interest in your plasmid. With or without mutations.

Procedure

1. Pipette 15 µl of template into 1.5 ml reaction tube, DNA concentration needs to be between 50 & 100 ng/µl. Dilute if necessary.
2. Add 2 µl primer (10 µM) of choice (do only use one primer!).

Premixed Samples (a mixture of template and primer)

- Templates should consist of 15 µl purified DNA with the concentration given above.

- Add 2 µl of primer with a concentration of 10 pmol/µl (10 µM).
- Ensure that the total volume of your premixed sample is 17 µl.

Optimum Primer Conditions

- Primers must not contain phosphorylation or fluorescent dyes
- The optimum primer length is between 16 – 25 bases
- The primer melting temperature (Tm) should be 50 – 62 °C
- The GC content of the primer should be 35 – 60 %
- Ideally one G or C should be located at the 3' primer end
- The number of 3' Gs or Cs should not exceed 2 Gs or Cs
- If possible, avoid >3 identical bases in a row in the sequence

Primer Concentration and Volume

- Exactly 10 pmol/µl primer concentration is required per sequencing reaction
- Each primer must have a total volume of 15 µl (double distilled water or 5mM Tris-HCl); 5 µl of primer volume is required for every additional sequencing reaction
- Concentration of primers with wobble bases must be calculated according to the following formula: nX x ConcPrimer

n = number of bases within a wobble according to IUPC code, X = number of wobbles within the primer sequence. [e.g. 1 V (AGC) = 31 x 10 pmol/µl; 2 V (AGC) (AGC) = 32 x 10 pmol/µl]

Source:

http://www.eurofinsgenomics.eu/media/892645/samplesubmissionguide_valuereadtube.pdf

Address:

Eurofins Genomics | Anzinger Str. 7a | 85560 Ebersberg | Germany

f) SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is a widely used method to separate different proteins by mass.

Gels

	Separation gel (10 %)	Stacking gel (4 %)
MilliQ	1.975 µl	2.065 µl
Acrylamid/Bisacrylamid 37.5 : 1	1.670 µl	506 µl
Separation gel buffer	1.250 µl	
Stacking gel buffer		375 µl
SDS (10 %)	50 µl	30 µl
APS (10 %)	25 µl	15 µl
TEMED	2.5 µl	1.5 µl

Buffers

- Separation gel buffer (0.5 M Tris, 0.4% SDS, pH = 8.8)
- Stacking gel buffer (0.5 M Tris, 0.4% SDS, pH = 6.6)
- Running buffer (0.25 M Tris, 2 M glycine, 1% SDS , pH = 8.3)
- 2 x sample buffer “Laemmli-Buffer” (5 ml)

	Volume	Final concentration
Tris-HCL (1 M, pH 5.8)	650 µl	130 mM
SDS (10 %)	2000 µl	4 %
Glycerol (80 %)	1250 µl	20 %
β-Mercaptoethanol	500 µl	10 %
Bromphenolblue (pH = 6.75)	spatula tip	

- Staining buffer

	Volume/Amount
Coomassie brilliant blue G250	0.5 g
Coomassie brilliant blue R250	0.5 g
Methanol	100 ml
Acetate	20 ml
Aqua dest.	100 ml

Procedure

Load & run

1. Prepare separating gel and fill it into the chamber
2. Pour 1 ml isopropyl alcohol on the top of the gel to destroy air bubbles and prevent dehydration
3. Discard the isopropyl alcohol and pour the prepared stacking gel after separating gel is polymerized
4. Stick in the comb
5. If not used immediately, store the gel in wet cloth (to prevent dehydration) at 4°C
6. If used immediately, remove comb when the gel is fully polymerized and place it into SDS-PAGE chamber
7. Fill up chamber with running buffer
8. Heat up samples with 2 x sample buffer at 95 °C for 5 minutes and apply 20 µl to each pocket
9. Load one pocket with a commercial protein marker
10. Start the PAGE by applying 20 mA while stacking
11. Apply 40 mA while separation

Staining & washing of gel

1. Disconnect glass plates containing the already run gel
2. Cut off stacking gel
3. Put separation gel into staining buffer and shake at room temperature for at least one hour or heat it in the microwave
4. Put stained separation gel into distilled water and let shake for 10 minutes
5. Repeat previous step at least twice again with fresh distilled water

g) Western Blot

1. Place nitrocellulose membrane in a sandwich consisting of sponges, Whatman paper and the SDS PAGE.
2. Align the sandwich with the membrane facing the anode and the gel facing the cathode.
3. Blot the membrane for 1.5 hours with 200 mA in Western blot transfer buffer (25 mM Tris/HCL, 200 mM glycine, 3.5 mM SDS, 20 % (v/v) EtOH and 70 % H2O).
4. Use ponceau staining to determine whether the protein transfer was successful.
5. Block the membrane with 5% skimmed milk in TBS-T buffer (20 mM Tris, 150 mM NaCl and 0.05 % Tween 20) overnight.
6. Wash the membrane 3 times for 10 minutes.
7. Add primary antibodies (anti-6X His tag rabbit polyclonal antibody, Santa Cruz Biotechnology)
8. Wash the membrane again 3 times for 10 minutes
9. Incubate with secondary antibodies (rabbit- anti mouse IgG, HRP, Santa Cruz Biotechnology).
10. visualize the blot by using a detection reagent (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare).
11. generate image using ImageLab (Bio-Rad Laboratories, Inc.)

h) HPLC

B. In vivo

I. E. coli

a) Chemically competent cells

The transformation of E. coli with plasmid DNA via heat shock transformation requires chemically competent cells.

Procedure

Day 1:

1. Grow Top10 / BL21 (DE3) overnight in 5 ml LB at 37 °C.

Day 2:

1. Inoculate 100 ml LB with 1 ml of saturated overnight culture of E. coli cells
2. Incubate at 37 °C and 150 rpm until an OD600 0.4 – 0.6 (usually 2 – 3 h)
3. Incubate cells on ice for 5 minutes.

Note: After this point the cells should never touch anything that is warm – chill solutions, pipets, tubes, etc. beforehand.

4. Divide culture into 2 tubes with ~ 40 ml each
5. Centrifuge the culture at 4 °C and 3000 x g for 10 minutes
6. Gently resuspend each pellet with 15 ml of cold Mg2+/Ca2+ solution (Do not vortex!).
7. Incubate in an ice bath for 30 minutes
8. Centrifuge the culture at 4 °C and 3000 x g for 10 minutes
9. Resuspend each pellet with 1.6 ml of cold 100 mM CaCl2 solution
10. Incubate in an ice bath for 20 minutes
11. Combine cells to one tube
12. Add 0.5 ml cold 80 % glycerol and swirl to mix
13. Flash-freeze in liquid nitrogen as 100 µl aliquots
14. Store at - 80 °C.

Mixtures

- Mg2+/Ca2+
  - 3.25 g $MgCl_2 \cdot 6 H_2O$
  - 0.6 g $CaCl_2 \cdot 2 H_2O$
  - 200 ml ddH_2O
  - Autoclave
- $CaCl_2$ (100 mM)
  - 2.95 g $CaCl_2 \cdot 2H_2O$
  - 200 ml dH_2O
  - Autoclave
    Note: You can also make a 1:10 dilution of the 1 M stock

Source: http://openwetware.org/wiki/Griffitts:Chemocompetent_Cells

b) Bacterial cell culture

Bacterial cell culture is a method by which bacterial cells are cultivated under controlled conditions to multiply the number of cells.

Procedure

Starting culture: Under sterile conditions add about 5 ml of medium to a culture tube and insert the picked colony.

1. Cultivate the stock on agar plate e.g. until colonies grow (incubation usually at 37 °C)
2. Flame a glass pipette, open the bottle of medium and flame the mouth, measure out the amount you need to fill your tubes, flame the cap and recap the bottle as quickly as possible.
3. Remove the tube cap, flame the top of the culture tube, pipette in 5 ml, flame the top of the tube, and cap it. Pick a single colony (to assure the cells are from the same single clonal population) and transfer it to the medium by tapping a small (0.1 µl) pipette tip (held on a pipette) on the surface of the plate. Uncap a tube, flame the top, tip the tube so as to transfer cells from the pipette tip to the surface of the media without touching the inside of the tube with the non-sterile portion of the pipette, flame, cap.
4. Pipette the desired amount of antibiotic into each tube along the wall. Do not put the non-sterile part of the pipette inside the tube and use a new tip for each tube.
5. Vortex each tube for 1 – 2 seconds to mix well.
6. Take the tubes to incubate (usually at 37 °C) in an incubator or warm room.
7. Wait overnight or until your cells have reached the desired concentration.

Source: http://openwetware.org/wiki/Bacterial_cell_culture

c) Cell counting/plating

Cell counting/plating is a procedure to calculate the number of cells in a sample.

Procedure

1. Fill each tube in the dilution with 90 µl of LB.
2. Add 10 µl of the sample to the first tube and mix.
3. From the first tube, remove 10 µl and mix it in a second tube.
4. Repeat for the number of dilutions you wish to do (8 should be more than enough) [1].
5. Take 10 µl from each dilution and spread it out on an agar plate.
6. Allow droplet to dry and incubate.

The first dilutions will contain a thick lawn of cells and the last dilutions will contain no cells. There should be one drop which contains countable single colonies. From this, you can calculate the number of cells in the original sample. For example, if there 4 colonies on dilution 5, there are 44 cells/µl.

Source: http://openwetware.org/wiki/Bacterial_cell_culture

d) Heat Shock Transformation

Heat shock Transformation is a widely used technique to insert foreign plasmid DNA into chemically competent bacteria cells.

Procedure

1. Defrost stocks of competent cells (100 µl in 1.5 ml reaction tube) on ice.
2. Add DNA (2 – 6 µl) and incubate the suspension for 15 minutes on ice.
3. Heat shock is done by incubating the cells for 45 seconds at 42°C.
4. Put samples back on ice for 2 minutes.
5. Add 1 ml of LB medium and incubate for 1 hour at 37°C in order to obtain antibiotic resistance.
6. It might be useful to spin down cells at 5000 rpm for 5 minutes.
   Resuspend pellet in 100 µl LB.
7. Spread out cells on an agar plate

e) Colony PCR

E. coli

Colony PCR is used to analyze whether a sequence of interest is present on a plasmid in E. coli. Flanking primers are used to amplify DNA in between the primer binding sites that are located on the plasmid backbone.

Procedure

The colony PCR is a modified PCR program employed to verify transformation success by amplifying the insert or the vector construct used for transformation. This is necessary due to the fact that a transformation with the empty vector may lead to antibiotic resistance.

1. Reaction mixture 1x - (25 µl)
   - 12.5 µl 2x Taq Master Mix
   - 0.5 µl VF2 (10 µM)
   - 0.5 µl VR (10 µM)
   - ddH2O to 25 µl
2. Pick one colony with a sterile tip and suspend in reaction mixture
3. Start the PCR using the following program and 1 x mix.
4. Run a agarose gel to determine the product length

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	5:00 min.	1 cycle
Denaturation	95 °C	0:10 min.	
Annealing	55 °C	0:15 min	30 cycles
Extension	72 °C	1 min/kbp	
Final Extension	72 °C	1.5 min/kbp	1 cycle

Hold	4 – 8 °C	Indefinite	1 cycle

Yeast

f) Glycerol stock

In order to have a permanent culture of cells glycerol stocks can be made.

Procedure

1. Add 200 µl of sterilized glycerol or DMSO to 800 µl cell culture and mix well.
2. Shock freeze with liquid nitrogen
3. Store the stock at -80 °C.

g) Protein Expression (T7-promoter system)

(If working with different promotor system, use appropriate inducing chemical and take care of using appropriate strain)

Procedure (Choose volume appropriate to your desired amount of expressed protein)

1. Inoculation of 50 ml LB medium in a 100 ml flask with E. coli BL21 (DE3) strain transformed with desired plasmid
2. Incubation at 180 repulsion per minute (rpm) at 30 °C to an OD600 = 4
3. Transfer starter culture into 1 L LB medium in a 3 L flask resulting in an OD600 = 0.2
4. Incubation to an OD600 = 0.6 at 180 rpm and 30 °C.
5. Incubation for 15 minutes on ice.
6. Induction of protein expression with 20 ml of IPTG (stock concentration 1 M).
7. Incubation of the cell suspension over night at 180 rpm at 30 °C.

II. Yeast

a) Competent cells

Frozen-EZ Yeast Transformation II Kit, Zymo Research

- Preparation of an overnight culture with YPD medium
- Inoculation of 10 ml media with the overnight culture, to generate an OD_{600} of 0.4
- Let the cells grow until an OD_{600} of 0.8 – 1 (approximately 4 – 6 h)
- Centrifuge 1 – 5 ml culture at 500 x g for 4 min
- Discard the supernatant
- Wash the pellet in 1 ml EZ 1 solution
- Centrifuge at 500 x g for 5 min
- Discard the supernatant

- Resuspend the pellet in 100 µl EZ 2 Solution

f) Transformation

Frozen-EZ Yeast Transformation II Kit, Zymo Research

- Mix 50 µl competent yeast cells with 700 – 1000 ng plasmid DNA (or linear DNA for genome integration) and 500 µl EZ 3 solution
- Incubate at 30 °C for 60 – 90 min, vortex every 15 min
- Plate on SD or YPD medium with the respective selection marker

g) Overnight culture

- 10 ml medium (YPEG or YPD)
- 10 µl culture / small colony sample
- Shake over Night (~16 h at 200 rpm)

h) Cell disruption

- Centrifuge 10 ml overnight culture at 3000 x g for 3 min
- Discard supernatant
- Wash the pellet with 1 ml SE-Buffer
- Centrifuge at 3000 x g for 3 min
- Discard the supernatant
- Resuspend the pellet with 0.4 ml SE-Buffer and 5 µl Zymolyase
- Incubate for 1 h at 37 °C
- For the extraction of the genome DNA plasmid preparation is required

i)

C. Protein purification

I. His-tag purification

for ycdW (Glyoxylate reductase), aceA (Isocitrate lyase), ADH (alcohol dehydrogenases) and CHMO (cyclohexanone monooxygenase)

Cell resuspension and disruption

1. Inoculation of cell culture in 1 L LB media by an OD600 = 0.1,
2. Induction of cell culture with 0.5 mM IPTG by an OD600 = 0.5, growing over night
3. Centrifugation of cells (15 min, 6,140 x g, 4 °C) after 19 hours inducing
4. Thawing the cell pellet on ice, resuspend pellet in 50 ml ice-cold Resuspension buffer
5. Wash EmulsiFlex with water and ice-cold Resuspension buffer while adjusting the pressure to about 1500 bar
6. Add resuspended cells in EmulsiFlex
7. Collect disrupted cells in a 50 ml Flacon tube on ice
8. To ensure that all cells are crushed repeat the disruption step twice
9. Fill cell lysate into clean ultracentrifuge tubes and centrifuge cells (60 min, 60,000 x g, 4 °C)
10. Collect the supernatant into a new tube on ice. Perform this step carefully
11. If the supernatant is still cloudy, sonicate it for 30 s and then filter it through a 0.2 µm filter

Purification via ÄKTA

Supernatant of cell disruption is removed and purified with the ÄKTApure purifier with the software Unicorn and a HisTrap column with a volume of 1 ml by GE Healthcare. Elution is elaborated with a linear increasing concentration of imidazole by elution buffer.

1. Calibrate ÄKTA system with 7 ml distilled water at a flow rate of 1 ml/min at a pressure of 0.5 MPa to remove remaining ethanol.
2. Apply 80 ml of the sample, put flow through in waste.
3. Wash column with 10 column volume Equilibration buffer to get rid of the remaining sample
4. Elute the protein with an increasing concentration of imidazole starting at 5 % and reach the final concentration of 100 % imidazole in 7 ml
5. Collect fractions of 0.5 ml
6. Wash apparatus with water and EtOH

The protein was collected in several fractions and an SDS-Page was performed to show if the purification has been successful.

II. Strep-tag purification

for GLYR1 (glyoxylate reductase)

Cell resuspension and disruption

1. Inoculation of cell culture in 0.5 L YPD medium by an OD600 0.7, growing overnight (30 °C, 170 rpm)

2. Centrifugation of cells (10 min, 4,000 x g, 4 °C)
3. Thawing the cell pellet on ice, resuspend pellet in 50 mL ice-cold resuspension buffer
4. Split the cell culture into two Falcons (25 ml each)
5. Centrifugation of cells (5 min, 4,000 x g, 4 °C)
6. Resuspend pellet in 20 ml ice-cold resuspension buffer and add 250 µl zymolyase
7. Incubate at 37 °C for 30 min
8. Cell disruption via sonication
    a. Amplitude 75 %
    b. Joules 3600
    c. Amplitude ON for 5 min
    d. Amplitude OFF for 5 min
9. Centrifugation of cells (60 min, 25000 x g)
10. Collect the supernatant into a new tube and then filter it through a 0.2 µm filter

Purification via ÄKTA

D. Assays

I. NADPH-dependent Assay

The glyoxylate reductase YcdW is a native *E. coli* gene, which is NADPH-dependent. It catalyzes the conversion of glyoxylate into glycolic acid. During the reaction NADPH is used as a cofactor and gets deprotonated into $NADP^+$. As NADPH levels can be determined by measuring absorption at 340 nm, the level of absorption at 340 nm is decreasing when YcdW is active. By measuring the absorption level over time, it is possible to quantify the enzyme activity and glycolic acid production

In *S. cerevisiae* we used AtGLYR1 from Arabidopsis thaliana. AtGLYR1 catalyze the conversion of glyoxylate into glycolic acid, comparable to the function of YcdW in *E. coli*.

Procedure:

- Use a 96-well plate for your measurement.
- Pipette 200 µl of reaction buffer into the wells.
- Add different concentrations of your enzyme, mix the reaction.
- Pipette every sample three times to ensure that there is no mistake and take the arithmetic mean.
- Add a positive sample consisting NADP+ in an additional well of reaction buffer and a negative sample consisting NADPH without any enzyme, as well.
- For E. coli
    - Measure the plate by 340 nm for 30 minutes in a Tecan Reader at 25 °C,
    - Repeat the measurement with a new approach/preparation at 21 °C as well as 30 °C.
- For S. cerevisiae
    - Measure the plate by 340 nm for 30 minutes in a Tecan Reader at 30 °C.

- Create a calibration curve by using different concentration of NADPH
    - The concentration of the stock solution amounts to 1 mM NADPH,
    - Prepare a dilution series with 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0 mM NADPH,
    - Measure it at one timepoint by 340 nm.

II. Phenylhydrazine-dependent Assay

The isocitrat lyase AceA is a native *E. coli* enzyme, which catalyzes the conversion of isocitrate into glyoxylate and succinate. As soon as AceA converts isocitrate into glyoxylate, the product reacts with phenylhydrazin to glyoxylate-phenylhydrazone, which possesses an absorption maximum at 324 nm. By measuring the absorption level over time, it is possible to quantify the enzyme activity by the production of glyoxylate-phenylhydrazone.

Procedure

- Use a 96-well plate for your measurement
- Pipette 200 µl of reaction buffer into the wells
- Add different concentrations of your enzyme, mix the reaction
- Pipette every sample three times to ensure that there is no mistake and take the arithmetic mean
- Add a negative sample without any enzyme in an additional well with reaction buffer, as well
- Measure the plate at 324 nm for 30 min in a plate reader at 21 °C
- Repeat the measurement with a new approach/preparation at 25 °C as well as 30 °C

- Create a calibration curve by using different concentration of glyoxylate and phenyl-hydrazine. Most important about this is that the molarity of phenylhydrazine needs to be twice as high as of glyoxylate.
    - The concentration of the stock solution amounts to 4 mM phenylhydrazine
    - Prepare a dilution series with 4 mM, 3 mM, 2 mM, 1.5 mM, 1 mM, 0.75 mM, 0.5 mM, 0.375 mM, 0.25 mM, 0.1875 mM, 0.125 mM and 0 mM glyoxylate
    - Measure samples at one time point at 340 nm

E. Chemistry

I. Anionic Polymerization

To perform an anionic polymerization, it is important to work in a completely water free environment. To achieve these conditions, the reaction vessel must be cleared of atmospheric air, which is humid, and filled with inert gas.

PLGA

	Ratio 75:25	Ratio 66:33
• D,L-Dilactide	10.810 g	13.13 g
• Diglycolide	2.901 g	5.26 g
• Initiator (Stannous-Octoate)	14.8 mg	3.53 mg
• Coinitiator (1-Octadecanol)	16.2 mg	36.8 mg
• Total	13.7 g	18.43 g

PLGC

	Ratio 71:19:10	Ratio 46:44:10
• D,L-Dilactide	1.685 g	6.68 g
• Diglycolide	0.483 g	5.72 g
• ε-Caprolactone	0.240 g	1.17 g
• Initiator (Stannous-Octoate)	1 mg	2.6 mg
• Coinitiator (1-Octadecanol)	3 mg	27.3 mg
• Total	2.412 g	13.78 g

1. Preparation of the Schlenk flask. Heating out, evacuating, flood with inert gas.
2. Preparation of the initiator and coinitiator. Solving them in dry Tetrahydrofuran (THF).
3. Filling the Schlenk flask with monomer under Argon countercurrent.
4. Heating the mixture up to 140 °C
5. If everything is molten, add initiator and coinitiator.
6. Run reaction until magnetic stir bar stops regular movements.
7. Shut down and let it cool down.
8. Solve in THF.
9. Precipitate in Methanol.
10. Repeat solving (8.) and precipitating (9.).

nanospheres

II. Analysis

a) Gel Permeation Chromatography (GPC)

1. Weigh 3 mg of sample
2. Solve sample in 1 ml THF/toluene-buffer

3. Put solution in GPC and measure against polystyrene standard sample

Calculation for the expected result from the GPC:

- Amount of substance:
  $n = \frac{m}{M}$
- Amount of substance in the setting up:
  $n(setting\ up) = \sum n(monomer)$
- Ratio molecules per chain:
  $Ratio = \frac{n(setting\ up)}{n(initiator)}$
- Mole fraction:
  $x = \frac{n(monomer)}{n(setting\ up)}$
- Quantity of monomer molecules per chain:
  $N(monomer) = Ratio * x$
- Expected molecule weight of the polymer chain:
  $M(polymer) = \sum M(monomer) * N(monomer)$

b) Nuclear magnetic resonance (NMR) spectroscopy

1. Weigh 5-10 mg of sample
2. Solve in deuterized solvent
3. Give solution to NMR measure at 300 MHz

c) Dynamic light scattering (DLS)

1. Solve sample in MiliQ-water
2. Pipette 1.5 ml of the sample into a disposable plastic cuvette
3. Adjust DLS for PLA (standard closest to PLGA and PLGC)
4. Measure sample in DLS (Zetasizer Nano-ZS90) in three cycles of 15 times 10 s. The calibration time for every measurement was 180 s at 25 °C.

d) Transmission electron microscopy (TEM)

1. Suspend sample in methanol
2. A special carbon coated copper net is dipped into the suspension
3. Let methanol evaporate
4. Place copper net in TEM

e) Confocal microscopy

1. only nanosphere samples containing the fluorophore fluorescein were used for confocal microscopy
2. dissolve freeze-dried nanosphere sample in 1.5 mL ddH2O or use 1.5 mL sample directly from nanosphere synthesis
3. washing process to wash out any remaining fluorophore in the solvent: centrifugate shortly, discard supernatant, resuspend pellet in fresh ddH2O
4. repeat washing process until spheres contrast clearly from background solution

F. Materials

I. Buffers

a) Cloning

Tris (1 M, pH 7.5)

- Tris base.....60.5 g
- ddH_2O.....to 500 ml

adjust pH to 7.5 add the needed amount of 5 M HCl
store at room temperature

Tris Buffer (1000ml)

- Tris HCl.....100 mM
- NaCl .....150 mM
- EDTA.....1 mM
- ddH_2O.....to 1000 ml

Adjust to pH 8, add the needed amount of HCl

1 x PBS

- NaCl (140 mM) .....8.18 g
- KCl (2.7 mM) ..... 0.2 g
- Na_2HPO_4 (10 mM).....1.77 g
- KH_2PO_4 (1.8 mM).....0.24 g
- ddH_2O.....to 1000 ml

Adjust to pH 7.4 add the needed amount of HCl
store at room temperature

TE buffer

- Tris HCl.....10 mM
- EDTA.....1 mM
  (Ethylenediaminetetraacetic acid)

Adjust to pH 8.0 add the needed amount of HCl
store at room temperature

50 x TAE

- Tris base.....242 g
  mix Tris with stir bar to dissolve in about 600 ml ddH_2O
- glacial acetic acid.....57.1 ml
- EDTA solution (500 mM, pH 8.0) .....100 ml
  add EDTA and Acetic Acid
- ddH_2O.....to 1000 ml

store at room temperature

b) Protein purification

Resuspension/Equilibration buffer

His-tag

- NaCl .....500 mM
- NaPO4, pH = 7,4 .....20 mM
- Imidazol .....20 mM
- PMSF .....1 mM
  (only in Resuspension buffer, if you don't work with proteases later)

Strep-tag

- Tris-Cl, pH = 8 .....500 mM
- NaCl .....150 mM
- EDTA .....1 mM
  (only if it doesn't interfere the assay reaction)

Elution buffer

His-tag

NaCl .....500 mM
NaPO4, pH = 7,4 .....20 mM
Imidazol .....350 mM

Strep-tag

Tris-HCl, pH = 8 .....500 mM
NaCl .....150 mM
Desthiobiotin .....2.5 mM

c) Assays

NADPH-depended Assay

Reaction buffer for E. coli

- potassium phosphate .....100 mM
  (pH = 7.0 standard reaction mixtures)
- Glyoxylate .....1.5 mM
- NADPH .....0.25 mM
  positive sample $NADP^+$ instead of NADPH .....0.25 mM

Reaction buffer for S. cerevisiae

- trisodium phosphate (pH = 7.0) .....50 mM
- Glyoxylate .....2.0 mM
- NADPH .....0.20 mM
  positive sample $NADP^+$ instead of NADPH .....0.25 mM

Phenylhydrazine-dependent Assay

Reaction buffer

- 10 mM Tris-HCL buffer (pH = 7.5)
- 4 mM Phenylhydrazine
- 5 mM Magnesium chloride
- 2 mM DL-Isocitrate

II. Media

a) 1.7-DYT Media (500 ml)

- NaCl..... 2.5 g
- Tryptone ..... 8.0 g
- yeast extract ..... 5.0 g

add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

b) LB Media (1000 ml)

- Tryptone ..... 10 g
- Yeast extract..... 5 g
- NaCl ..... 10 g
- Agar (for plates) ..... 15 g

add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

c) SOB Media (1000 ml)

- KCl..... 0.186 g
- MgSO4 ..... 2.4 g
- NaCl ..... 0.584 g
- Tryptone ..... 20 g
- Yeast extract..... 5 g

add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

d) SOC Media (1000 ml)

- SOB ..... 980 ml
- 1 M glucose ..... 20 ml

e) YPD Media (1000 ml)

- yeast extract ..... 10 g
- peptone..... 20 g
- glucose ..... 20 g
- agar (for plates) ..... 20 g
- ddH_2O ..... to 1000 ml

If required for selection:
G418 (Geniticin) ..... 200 mg
after autoclavation and cooling down to < 50 °C

f) YPEG Media (1000 ml)

- yeast extract ..... 10 g
- peptone..... 20 g
- glycerol ..... 30 g
- ddH_2O ..... to 970 ml

Ethanol ..... 30 ml
after autoclavation and cooling down to < 50 °C

Autoclave all media after mixing!

III. Stock solutions

a) Antibiotics

- Ampicillin
  1. Mix
     - 4 g ampicillin (100 mg/ml)
     - add 40 ml ddH2O
  2. Sterile filtration
  3. Aliquot in 1 ml stocks and store at -20°C
  4. Use 1 µl per 1 ml medium
- Chloramphenicol
  1. Mix
     - 1 g chloramphenicol
     - add 40 ml ethanol
  2. Aliquot in 1 ml stocks and store at -20°C
  3. Use 1 µl per 1 ml medium
- Kanamycin
  1. 1.Mix
     - 3 g kanamycin (75 mg/ml)
     - add 40ml ddH2O
  2. Sterile filtration
  3. Aliquot in 1 ml stocks and store at -20°C
  4. Use 1 µl per 1 ml medium
- Tetracycline

b) Induction chemicals

- IPTG (Isopropyl-beta-D-thiogalactopyranoside)
  1. Dissolve 238 mg IPTG in 10 ml water
  2. Store in 1 ml aliquots at -20°C

IV. Recipes

a) 9:25 Pfu MM (450 µl)

- 10x Pfu buffer (BSA) ..... 250 µl
- Pfu polymerase ..... 50 µl
- dNTPs (10 mM) ..... 50 µl
- MgCl2 (50 mM) ..... 50 µl
- DMSO ..... 50 µl

b) 2 x Taq MM (1250 µl)

- 10 x Taq buffer ..... 250 µl
- Taq polymerase ..... 25 µl
- 50 mM MgCl2 ..... 50 µl
- 10 mM dNTPs ..... 50 µl
- DMSO ..... 75 µl
- ddH2O ..... 800 µl

c) 10 x Pfu buffer (100 ml)

- 200 mM Tris-HCl (pH 8.8 at 25 °C) ..... 2.42 g
  100 mM (NH4)2SO4 ..... 1.32 g
  100 mM KCl ..... 0.75 g
  20 mM MgSO4 x 7 H2O ..... 0.49 g
  1 % Triton X-100 ..... 1 ml
  ddH2O .....up to 100 ml
- Autoclaving/sterile filtration after mixing.
- add 1 ml 100 mg/ml BSA (-> end conc. 1 mg/ml)

d) 10 x Taq buffer (100 ml)

- 500 mM KCl ..... 3.7 g
  0.8 % Triton X-100 ..... 800 µl
  100 mM Tris-HCl (pH 8.8 at 25 °C) .....up to 100 ml
- Autoclaving/sterile filtration after mixing.