Schedule of basic technique for gene analysis

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Date
July 7, 2009, 13:00 – 19:00
Practical training I
July 14, 2009, 13:00 – 19:00
Practical training II

Place: Building A for clinical research, 2nd floor, department of medicine and biological science
Room number MC2-17

Contents: basic training to handle DNA, RNA, protein and culture cells.

Aim: Protein expression is translated from messenger RNA, which is transcribed from genomic DNA. Profiling of gene and protein expression and functional analysis of the products is basic as well as necessary for biological science. Therefore, we provide basic training program for techniques to handle these materials, or DNA, RNA and protein. The first curriculum includes (A) small scale preparation of plasmid DNA with spin columns (B) crude extraction of protein from cultured cells and SDS-PAGE and (C) basic technique to manipulate cultured cells under aseptic condition. The second program further includes (D) subcloning and (E) RNA extraction and electrophoresis.

Contents for the first day

A. DNA
(1) Plasmid preparation
Plasmid DNA will be extracted from transformed E. coli with spin columns (Qiagen).
(2) Digestion of plasmid DNA with restriction enzymes
Extracted DNA will be digested with restriction enzymes. Digested DNA will be run in an agarose gel and be visualized under UV.

B. Protein extraction and SDS-PAGE
(1) Crude extraction of protein
Protein will be extracted from cultured cells with lysis buffer.
(2) SDS-PAGE and protein staining
Extracted protein will be size-fractionated in an acrylamide gel and be stained with coomassie brilliant blue.

C. Cell culture
Preparation of cultured cell from frozen stock.
Study basic technique to keep aseptic condition. Thaw frozen stock, count cell number and transfer them to a dish.

Time table

13:00 14:00 15:00 16:00 17:00 18:00

lecture
mini-prep, Digest DNA, electrophoresis
Protein extraction, SDS-PAGE, gel staining
cell culuture
Summary

Contents for the second day

D. Subcloning

(1) Digest plasmid DNAs with restriction enzymes. The digested plasmid will be size-fractionated in an agarose gel by electrophoresis. The DNA fragments will be extracted from the gel with gel extraction kit (Qiagen).

(2) The extracted DNA (plasmid + insert) will be ligated with T4 ligase. Competent cells (E.coli) will be transformed with the ligated DNA by heat shock method.

E. RNA extraction and electrophoresis

Total RNA will be extracted from cultured cells with Trizol reagent (Invitrogen).

The extracted RNA will be size-fractionated in a denaturing agarose gel and be visualized under UV.

Time table

13:00	14:00	15:00	16:00	17:00	18:00
lecture					Summary
	Digest DNA, electrophoresis, gel extraction, ligation				
	Protein extraction, SDS-PAGE, gel staining				
			cell culuture		
		RNA extraction, electrophoresis			

Protocol

A. DNA

Small scale preparation of plasmid DNA (mini-prep)

1. Transfer 1 ml of bacterial cells to a microcentrifuge tube.
2. Centrifuge for 2 min at 3000 rpm, Decant the supernatatnt.
3. Resuspend the bacterial pellet with 250 µl of P1 buffer by vortex.
4. Add 250 µl of P2 buffer and gently invert the tube 5 times to mix.
5. Add 350 µl of P3 buffer and invert the tube immediatedly but gently 5 times.
6. Centrifuge for 10 min (5 min) at 13000 rpm.
7. Apply the supernatant to the QIAprep spin column by pipetting.
8. Centrifuge for 60 sec at 13000 rpm and Discard the flow-through.
9. Wash the column by adding 750 µl of PE buffer.
10. Centrifuge for 60 sec at 13000 rpm and Discard the flow-through.
11. Centrifuge for another 60 sec at 13000 rpm.
12. Place the QIAprep column in a clean microcentrifuge tube.
13. Add 50 µl of H_2O and let stand for 60 sec.
14. Centrifuge for 60 sec at 13000 rpm to harvest plasmid DNA.

Digest plasmid DNA

Mix reagents described below in a microcentrifuge tube. Incubate it at 37℃ for 60 min.

TA-vector	10 µl
10x buffer (H)	2 µl
EcoRI	1 µl
H_2O	7 µl
	20 µl

Electrophoresis in an agarose gel

1. Digested DNA 10 µl + 6x loading dye 2 µl
   Undigested DNA 5 µl + 6x loading dye 2 µl
2. Apply the samples to an agarose gel.
3. Run a gel at 100V for 20 min.
4. Visualize the DNA under UV.

B. Protein

Protein extraction and SDS-PAGE

1. Aspirate the culture medium. Add 2 ml of PBS and agitate it. Aspirate it again.
2. Add 150 µl of lysis buffer and scrape cells. Transfer the cell lysate to a microcentrifuge tube.
3. Vortex for 30 sec. Centrifuge for 10 min (5 min) at 13000 rpm at 4℃.
4. Take 5 µl of the supernatant and mix with 5 µl of loading dye.
   Positive control: BSA (1 µg/µl) 5 µl
5. Incubate the samples at 99 degree for 5 min and place them on ice for 5 min.
6. Apply the samples to an acrylamide gel.
7. Run a gel at 150V until the dye reaches the bottom.

Gel staining

1. Remove the gel from the plates and stain it with CBB.
2. De-stain it with de-staining solution until protein bands become visible.

C. Cell culture

1. Thaw a frozen stock tube containing 293T cells at 37℃.
2. Transfer the cells to a tube containing 5 ml of pre-warmed medium.
3. Centrifuge for 5 min at 1000 rpm.
4. Aspirate the medium and leave the cell pellet.
5. Re-suspend the pellet with 5 ml of medium.

6. Count the cell number with hemocytemeter.
7. Transfer the cells to 60 mm dish. Place the dish in an incubater at 37℃.

D. Subcloning

Digest plasmid DNA and extract fragmented DNA from an agarose gel

1. Mix reagents described below in a microcentrifuge tube. Incubate it at 37℃ for 60 min..
   Two kinds of plasmids (Vector + insert)

DNA (1.0 µg/µl)	5 µl
10x buffer (M)	2 µl
EcoRI	1 µl
BamHI	1 µl
H2O	11 µl
	20 µl

2. Size-fractionate the digested DNA in an agarose gel at 100V. Excise the DNA fragment with a razor blade.
3. Transfer the agarose block to a microcentrifuge tube. Add 3 volumes of buffer QG and dissolve the agarose at 50℃ for 10 min.
4. Place a QIAquick spin column in a collection tube.
5. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min at 5,000 rpm.
6. Discard flow-through, place QIAquick column back in the same collection tube.
7. To wash, apply 0.75 ml of buffer PE to QIA quick column and centrifuge for 1 min at 5,000 rpm.
8. Discard flow-through, place a QIAquick spin column back in the same collection tube and centrifuge for another 1 min at 14,000 rpm.
9. Place a QIAquick spin column in a new eppendorf tube.
10. To elute DNA, add 50 µl of H2O to the center of the QIAquick membrane. Let the column stand for 1 min and then centrifuge for 1 min at 14,000 rpm.

Ligation and transformation

1. Mix reagents described below in a microcentrifuge tube. Incubate it at room temperature for 10 min..

Insert DNA	9 µl
Plasmid Vector	1 µl
2x buffer	10 µl
T4 ligase	1 µl
	21 µl

2. Thaw competent cells on ice for 10 min.
3. Mix 60 µl of the competent cells with 6 µl of the ligation mix. Incubate on ice for 20 min.
4. Heat the tube at 42℃ in a water bath for 45 sec, rapidly transfer it to a crashed ice and leave it for 2 min.
5. Add 300 µl of LB medium and incubate for 60 min at 37℃.
6. Transfer the appropriate volume of transformed competent cells onto LB agar gel and incubate at 37℃ over night.

E. RNA extraction and electrophoresis

1. Aspirate medium from cultured cells. Add 500 µl of Trizol, scrape cells and transfer it to a microcentrifuge tube.
2. Add 100 µl of chloroform, vortex 10 sec, and centrifuge at 14000 rpm for 10 min at 4℃.
3. Tranfer 200 µl of the supernatant to a microcentrifuge tube and add 200 µl of isopropanol. Vortex briefly.
4. Centrifuge at 14000 rpm for 10 min at 4℃.
5. Decant the supernatant. Add 500 µl of 70% ethanol and centrifuge at 14000 for 3 min at 4℃.
6. Decant the supernatant and dry the pellet briefly.
7. Dissolve the pellet with 30 µl of DEPC treated water.
8. Mix 4 µl of RNA and 16 µl of loading dye.
9. Apply the RNA samples to a denaturing agarose gel. Run the gel at 100V for several hours. Check ribosomal RNA under UV.

前日・当日の準備

7月7日
会議室
プロジェクター
アガロースゲル確認　　枚
アクリルアミドゲル作成　　枚

スクレーパー　　本
サイズマーカー（DNA, protein）
Loading Dye（DNA, protein）
Distilled water
PBS 40ml x 8 本
CBB
Destaining solution
Heat block 99度
ビーカー
お湯
ミニプレプカラム 5本 x 8組
エッペンドルフチューブ
プラスミド（TA-PPARgamma 4.39）0.1 γ/μl, 60 μl x 8 本
バクテリア 100 ml
DMEM 2 bottle (培養室、実験室)
Buffer M
EcoRI　20 μl x 3 本

スクレーパー
変性ゲル
ローディングバッファー
DNA vector + insert
Buffer M
Restriction enzyme
RNA
Trizol
クロロフォルム
MOPS
T4 buffer
T4 ligase
LB medium
LB plate
Competent cell
Isopropanol

購入するもの
アガロース
カラム
Trizol