Adenovirus Experiment Record

SYSU-China 2019

19.6.20

Receive puncture bacteria containing our plasmids and store at 4 ° C

The maximum storage time is 2wks.

19.6.21

1. 8:30 Streak pAdEasy (AmpR) and pShuttle (KanR) puncture bacteria on LB plates and place them in 37 °C incubator.

2. Patch pAdEasy (AmpR) and pShuttle (KanR) bacteria to new plates.

19.6.22

1. Inoculate pAdEasy (AmpR) and pShuttle (KanR) bacteria in two tubes. Shake them at 37 °C shaker.

2. Extract the plasmids using the endotoxin-free plasmid extraction kit and measure the concentration by ultraviolet spectrophotometer.

pAdEasy 21.6ng/uL 41.4 ng/uL

pShuttle 32.1 ng/uL 29.6 ng/uL

19.6.23

Inoculate pAdEasy, pShuttle and pAdTrack bacteria in 20mL tubes, shake them at 37 °C shaker.

19.6.24

1. 10:00 Obtain BJ5183 strain and inoculate 3ml LB medium (Strep anti)

2. Extract plasmid pShuttle and pAdTtack (not endotoxin-free extraction), plasmid pAdEasy (endotoxin-free extraction), measure their concentration.

3. pAdEasy, pAdTrack, pShuttle preserve in 20% glycerin(glycerin :bacteria=1:1), keep in - 80°C.

19.7.12

Dispense $9x10^5$ 293T cells(3mL)/hole in 6-well plate.

19.7.13

1. Linearize pAdEasy.

The system is as follows:

pAdEasy	110ug
PacI	4uL
Cutsmart	25 uL
ddH2O	111 uL
Total	250 uL

Incubate at 37 ° C for 15 min.

Water bath at 65 ° C for 20 min to inactivate the enzyme.

2. Purify the product using the DNA purification column. Measure its concentration: 0.1μg / μl.

3. Transfect pAdEasy into 293T using Lipo2000 reagent

1)The system is as follows:

PacI-pAdEasy	40uL
DMEM	110 uL
Total	150 uL .....(1)

Lipo reagent	10uL
DMEM	140uL
Total	150uL .....(2)

Leave at room temperature for 5 minutes

2) Mix (1), (2) and leave at room temperature for 20 minutes.

3) Remove the cell culture medium, wash with 4 mL DMEM, add 2.5 mL DMEM, incubate at 37 ° C for more than 10 min.

4) Add mixed 300uL transfection solution and incubate at 37 °C

5) After 4 h, remove the culture solution, wash it with PBS, add 3 mL DC, and wrap the plate with the paper film.

19.7.15

19:00 Inoculate pAdEasy bacteria in 20mL tubes, shake them at 37 °C shaker.

19.7.16

17:00 Preserve pAdEasy bacteria at -80 ° C.

19.7.17

The medium in six-well plate was changed, and the supernatant was collected into a 15 mL centrifuge tube D4-293T-pAdEasy. Wrap the sealing film. The tips were soaked overnight with 84 disinfectant.

19.7.20

A large number of cells floated up and agglomerated cell debris was seen in the supernatant. The supernatant was collected in a 15 mL centrifuge tube D7-293T-pAdEasy and wrapped with paper film. Store at 4 ° C.

The tips and six-well plate were soaked overnight with 84 disinfectant.

19.7.25

1. Linearize pAdTrack.

The system is as follows:

pAdTrack	2μL
EcoRI HF enzyme	1μL
Cutsmart	2μL
ddH2O	15μL
total	20μL

Incubate at 37 ° C for 15 min.

Water bath at 55 ° C for 10 min to inactivate the enzyme.

2. Purify the linearized product with the DNA purification column.
3. Electroporate BJ5183 Ecoli.
1)Add 1μl AdEsay + 6μl linearized-pAdTrack into 20uL BJ5183 Ecoli. Mix thoroughly. Place on ice for 30min.
2)Transfer mixture into electroporation cuvette. Electroporate at 2kV.
3)Immediately add 500μl LB. Transfer it to a EP tube. Briefly and gently pipet up and down to mix thoroughly.
4)Incubate at 37° C for 1h.
5)Transfer 100μl to a Kan(working concentration: 100μg/mL) LB plate.

19.7.27
Repeat the experiment on 19.7.25. But still fail to see colony on LB plate.

19.8.5
Prepare electroporation competent BJ5183.
1) Take -80° C preserved BJ5183 strain, and incubate it on a streptomycin (100 μg/mL) resistant LB plate overnight;
2) Pick a single colony and inoculate 2 mL of LB liquid medium, add 0.5 uL, place it at 37° C shaker, 200 rpm overnight; the next day, 1 ml of the bacterial solution is inoculated with 100 mL of LB liquid medium, and culture at 37 ° C, shaking at 200 rpm for 3-4 h. To OD600=0.4-0.6;
3) The culture was ice-bathed for 30 min, centrifuged at 4000 rpm for 10 min to harvest the cells; all supernatants were discarded, and the bacteria were resuspended in an equal volume of ice-cold WB solution at 4 ° C, 4000 rpm. Centrifuge for 10 min (WB = 10% glycerol, 90% double distilled water, filtered or autoclaved); repeat step2) two times;
4) Most of the WB was discarded, leaving the remaining volume to be 0.5% of the original culture, and resuspend to obtain BJ5183 electroporation competent cell. Each tube was dispensed with 100 μL and stored at -80 °C for later use.

19.8.6
Improve the conditions of electroporation.
1)Test linearization of pAdTrack

pAdTrack (212 ng / μl)	5 μL
EcoR I	1 μL
Cutsmart	1uL
ddH_2O	3uL
Total	10uL

Digest at 37 °C for 1 h.
Take 1μl of the product and dilute it to 5μl and test it by agarose electrophoresis. The linearization is thorough, which can eliminate the reason that the enzyme can not fully linearize pAdTrack.
The linearized plasmid was purified in DNA purification column, and finally 10 μl of ddH_2O was used to dissolve DNA.
2)Set control group of electroporation
Experimental group: 20 μl BJ5183 competent + 6 μl linearized pTrack fragment + 1 μl pAdeasy

(Kan+)
Positive control group: 20μl BJ5183 competent + 1μl pAdeasy (Amp+)
The electrical conditions are as follows: 1800V, 5ms

19.8.7
There was no result in the positive control group, and two small colonies were seen in the experimental group.
Inoculate 2ml LB with 25μg/μl Kan.

19.8.8
Pac I was digested and verified negative after plasmid extraction.
Re-inoculate BJ5183, ready to prepare chemical transformation competent bacteria.

19.8.9
Prepare chemical transformation competent BJ5183.
1) Pick a single colony and inoculate it into 2mL of LB medium, shake the bacteria at 37 °C overnight.
2) Take the overnight culture, inoculate it into LB medium at 1:50, and incubate at 37 °C (1-2 hr) until the bacterial concentration in the medium reaches OD600 of 0.2-0.4.
3) Take 1mL of bacterial solution into a 1.5mL centrifuge tube, place the culture on ice for 10min, 4000r/min, centrifuge for 2min, and collect the bacteria.
4) Suspend the cells in 500 uL of pre-cooled 0.1 mol/L CaCL2 and place on ice for 10 min (carefully mix it with a pipette)
5) Centrifuge at 4000r/min for 2min, discard the supernatant, gently suspend the bacteria in 100uL CaCl2, and place at 4 °C for more than 3hr to use.

19.8.10
1. Chemical transform a)pAdEasy; b)pAdEasy & pAdTrack.
1) Chemical transformation competent BJ5183 was taken out from -80 °C, placed in ice, and the bacteria were melted after 5 minutes.
(2) Add the linearized plasmid of target gene and pAdTrack, and gently mix by hand on the bottom of the EP tube (avoid to use a gun) and let stand for 25 minutes in ice.
(3) Heat shock at 42 ° C for 45 seconds, quickly put it back into the ice and let it stand for 2 minutes. Shaking will reduce the conversion efficiency.
(4) Add 700 μl of antibiotic-free sterile LB medium to the centrifuge tube, mix and resuscitation at 37 ° C for 60 minutes at 200 rpm.
(5) Centrifuge at 5000 rpm for one minute, and take 100 μl of supernatant. Gently blow and resuspend the pellet and apply it to LB medium containing the corresponding antibiotic.
(6) The plate was placed upside down in a 37 ° C incubator for overnight culture.

19.8.11
Colony PCR to verify both pAdEasy(Amp) and pAdEasy+pAdTrack(Kan) were positive. Pick 10 colonies of pAdEasy+pAdTrack into the same tube with 2ml LB and 0.5μL Kan, shake it at 37° C overnight.

19.8.12

1.Extract pAdEasy+pAdTrack (we name it pAdET) plasmid using Vazyme™ plasmid extraction kit.

2. Agarose gel electrophoresis to test the product

1)Digest pADET.

The system is as follows:

pAdET	10uL
PacI	1 uL
Cutsmart	25 uL
ddH2O	14 uL
Total	50 uL

2)Incubate at 37 ° C for 15 min.

3)Water bath at 65 ° C for 20 min to inactivate the enzyme.

4)0.8% agarose gel electrophoresis

No target strip.

We speculate that the plasmid was so large that could not be extracted successfully.

19.8.18

Repeat chemical transformation, colony PCR and overnight incubation of colonies to prepare for plasmid extraction.

19.8.19

Extract plasmid using another method, which is more suitable to extract large plasmids.

Bacmid extraction:

1)Pick a single colony containing Bacmid and inoculate 3-4 ml of LB liquid containing the corresponding antibiotic, culture at 37° C overnight;

2)Collect 2.5 ml of bacterial with 2 ml EP tube, 13,000 × g, 1 min, discard the supernatant;

3)Resuspend the cells by adding 200 solution Tl/RNaseA (vortex or blow);

4)Add 200 solution T2 to lyse the cells, mix gently, and lyse at room temperature for 5 min;

5)Add 200 solution T3, mix gently, ice bath for 5-10min;

6)Centrifuge at ⩾13,000 × g for 10 min at room temperature or 4 ° C, discard the precipitate;

7)Transfer the supernatant to a 1.5 mL EP tube, add 0.1 volume of ETR, and mix 7-10 times by inversion.

8)Ice bath for 10 min, reversing and mixing again during the ice bath;

9)Water bath at 42 ° C for 5 min, centrifuge at 10,000 × g for 3 min, and slow down at the end of centrifugation (soft).

10)Transfer the supernatant to a 2 ml EP tube containing an equal volume of pre-chilled isopropanol, mix by inversion, ice bath for10min.

11)Centrifuge at 13,000 × g for 15 min, discard the supernatant.

12)Add 500uL 70% ethanol to wash the precipitate, centrifuge at 13000 rpm for 5 min, discard the supernatant.

13)Repeat 12);

14)In the bechtop, discard the supernatant, air dry, add 40uL preheated sterile ddH20 to dissolve DNA, store at 4 ° C.

2. The measured concentration is 1924.6 ng/μl

3. 0.8% agarose gel electrophoresis

Result: a wide and fuzzy strip near 1 kb

19.8.21

Electroporate again.

	Experiment Group 1	Experiment Group 2	Control Group 1
pAdEasy	1μl	1μl	/
pAdTrack	/	6μl	6μl
electroporation competent BJ 5183	50μl	50μl	50μl
LB plate with antibiotic (working concentration: 100ug/mL)	Amp	Kan	Kan

19.8.22

Experiment Group 1 and Control Group 1: no colonies.

Experiment Group 2: positive colonies can be seen.

1. Colony PCR

pAdET: strips around 1.5kb can be seen(but not target strip)

2. Inoculate 8 colonies into 5mL LB in each 20 mL tube, incubate at 37° C shaker.
3. Use 7uL pAdEasy to electroporate again.

19.8.23

1. Extract pAdET using Bacmid extraction method. Gel electrophoresis shows no target strip.

2. 3 colonies on pAdEasy plate. Colony PCR and then gel electrophoresis. No target strip.

19.8.24-30

Repeat electroporation and optimize conditions.

19.8.31

PCR and gel electrophoresis to see results of previous electroporation.

	Negative1	Neg 2	Neg 3	Positive1	Pos2	Pos3
Template	BJ-pAdEasy （bacteria）	pAdEasy （7.18plasmid）	pAdEasy （7.18plasmid）	pAdET （8.28 plasmid）	pAdET （8.28 plasmid）	pAdET （8.28 plasmid）
Primer-F	AdEasy-F	AdEasy-F	AdET-F	AdET-F	AdET-F	AdEasy-F
Primer-R	AdEasy-R	AdEasy-R	AdET-R	AdET-R	AdEasy-R	AdEasy-R

Target product length	2020bp	2020bp	—	2508bp	Around 3000bp	—

Neg 1&2 show target strips. Others show no strips or wrong ones.

19.9.1

This month we continue to perform electroporation and optimize conditions. Design new primers to verify pAdEasy and pAdET.

19.9.5

pAdEasy colony PCR all positive.

pAdET colony PCR: one colony positive. Others appear clutter strips.

Electroporation condition: 2.5kV, 5.1ms, 10μL pAdEasy(100ng/μL), 50uL BJ5183

19.9.6

Extract positive plasmid and preserve corresponding bacteria in -80° C.

19.9.7

1. Prepare electroporation competent DH5α.
2. Prepare chemical competent BJ5183-pAdEasy.

19.9.8

1. Extract total RNA from HEK293

1)Take about 1×10^6 HEK293 cells (in DC), add them to a 1.5 mL centrifuge tube, centrifuge at 300 g for 5 min, discard the supernatant; add 100 μL of PBS, centrifuge at 300 g for 5 min, and discard the supernatant.

2)The DNase 2uL+10uL nucleic acid-free water was prepared on the clean bench.

3)Take the nuclease-free tips, pipets, and EP tubes. Wear the lab coat, wear a mask, spray the surrounding environment with a solid RNA degrading enzyme dilution solution, and turn on the 4 °C centrifuge.

4)RNA was then extracted according to the standard procedure of ES science's RNA Rapid Extraction Kit.

2. Determine the concentration of extracted RNA and absorbance

The concentrations of RNA in the two tubes were ~270 ng/uL, ~300 ng/uL, and 260/280 were slightly greater than 2.1, and the RNA fraction may have been degraded. Two tubes of RNA were labeled as HEK293 total RNA 1/2 and stored at -80 °C.

3. Reverse transcription of HEK293 total RNA

The system is as follows:

ddH_2O	0.6uL
5x Reaction buffer	4uL
$MgCl_2$	4uL
Oligo(dT)	0.5uL
Random primer	0.5uL
PCRmix	1uL

Inhibitor	0.4uL
RT	1uL
RNA template	2-3ug
Add H_2O	8uL-RNA
Total	20uL

Hold at 42° C for 30min, 70° C for 15min, and then store in 4° C for use.

4. Amplify E1A from HEK293 cDNA

cDNA	5uL
E1A-F	2uL
E1A-R	2uL
Phanta	25uL
dd H_2O	16uL
total	50uL

19.9.9

1. Gel electrophoresis E1A and use Omega gel extraction kit to extract E1A.

About 1000bp target strip is seen, but is a vague one.

2. E1A PCR

E1A was amplified by PCR for the second time using the E1A product today as template.

The system is as follows:

E1A	2uL
E1A-F	2uL
E1A-R	2uL
Phanta	25uL
ddH_2O	19uL
total	50uL

19.9.10

1. Gel electrophoresis E1A and extract E1A

900bp target strip is clearly seen. Extract it using Omega gel extraction kit.

2. Amplify E1B55K from HEK293 cDNA

19.9.11

1. Gel electrophoresis E1B55K and extract E1B55K.

2. Dispense HEK293 cells in a 6-well plate, $9x10^5$ cells/well, 2wells.

3. Linearize pAdEasy

The system is as follows:

PacI enzyme	4uL
pAdEasy	5ug(110uL)
10x cutsmart	25uL
ddH_2O	111uL
total	250uL

Incubate at 37 ° C for 15 min.

Water bath at 65 ° C for 20 min to inactivate the enzyme.

4. Linearize pAdTrack

The system is as follows:

EcoRI HF	2uL
pAdTravk	15uL
10x cutsmart	4uL
ddH_2O	17uL
total	40uL

19.9.12

1. Gel electrophoresis to verify pAdEasy was thoroughly linearized.
2. Transfect linearize-pAdEasy into HEK293 using Lipo2000.

19.9.15

CPE can not be clearly seen. Change for new DC- culture medium.

19.9.16

1. Transfected cells are in poor condition. Adherent cells account for only about 10% of the well.
2. Linearized-pAdTrack transform into BJ5183-pAdEasy (using 9.12 linearized pAdTrack), coated with Kan plates. Another Amp plate was coated with BJ5183-pAdEasy as a control.

19.9.19

1. Linearize pAdEasy and transfect it into HEK293 again.

19.9.22

overlap PCR E1A and E1B 55K

19.9.23

1. Virus packaging: Cells in the transfection group begin to become round. A small amount of cells in control group became round too. There are not a lot of floating cells in both groups. Wash with PBS, chang the medium, and observe under the microscope, a large number of cells were washed away. The stock was collected in a 15 mL centrifuge tube 293-pAdEasy and placed at 4 °C.
2. Gel electrophoresis and extract OL-E1A using Omega gel extraction kit.

19.9.26

1. Collect adenovirus

Phenomenon: A large number of cells in the six-well plate are obviously round and floating. The control group also showed cell floating, but most of the floating cell appears to be aggregated cell debris.

1) The cells were washed with the medium in the well, and the collected cell suspension was dispensed into 2 preservation tubes.
2) Place at -80 ° C for 10 minutes, take out for a 37 ° C water bath, and after thawing, vortex to lyse the cells. Repeat for three times.
3) 13000×g centrifuge for 5 min, and the supernatant was dispensed into two tubes. Store at -80 °C.

19.9.28

Adenovirus titer detection (TCID50 method)

1. Collect 293 cells in the logarithmic growth phase and good state in the day before the experiment, and dilute with complete medium to 10^5 cells/mL. Inoculate 100 μL of cell suspension (10^4 cells/well) to per well in 96-well plate. Incubate overnight in a 5% CO2, 37 °C carbon dioxide incubator.

2. Prepare 12 sterile 2 mL EP tubes, add 100 μL of complete medium to the first tube (10^0), and 900 μL of complete medium to other 11 tubes.

3. Dilute the virus by limiting dilution. Take 100 μL of adenovirus stock solution to the second tube(10^{-1}). Tubes 10^{-2}~10^{-10} are likewise operated.

4. Remove the 96-well plate from the incubator and observe the growth of the cells. Aspirate the old medium in the well. Only use 10^{-5}~10^{-12} diluted virus, inoculate 8 wells per concentration, add 100 μL diluted virus per well. Add 100μL DC- in negative control group. Incubate in a 37 ° C, 5% CO2 carbon dioxide incubator.

5. Observe and record the results day by day, no need to change the liquid, generally need to observe 8~10 days.

6. Observe the CPE phenomenon of each hole under the inverted microscope for 10 days, and calculate the number of holes in which CPE appears in each row. As long as there is a small vacancy or some cells appear CPE is positive. If not sure, they can be compared with the negative control. It should be effective at the same time:

1) Significant CPE occurs in each of the 10 wells with a dilution of at least one sample.

2) At least 5 but no more than 9 of the 10 wells with at least one sample dilution show significant CPE.

3) At least 1 but no more than 5 of the 10 wells with at least one sample dilution show significant CPE.

4) There are no significant CPE phenomena in the 10 wells with at least one sample dilution. The results were calculated according to the Reed-Muench method.

19.9.29

1. PCR amplify E1A, E1B 55K(cDNA template), E1B 55K(previous E1B 55K template)
2. Gel electrophoresis and extract them.
3. RCR again using the extracted gene prouduct.

19.9.30

1. CPE observation

CPE appeared in the 10^0~10^{-7} group. No CPE in the control group.

19.9.11

1. PCR to obtain insert-E1A, BamHI-EcoRI-E1A and insert-E1B, BamHI-EcoRI-E1B55K

	1	2	3	4	5	6	7	8	9	10
template	E1A 9.29	E1A 10.8	E1B 55K9.11 Purified	E1B 55K9.29 Purified twice	E1B 55K9.29	E1A9. 29	E1A 10.8	E1B 55K9.11 Purified	E1B 55K9.29 Purified twice	E1B 55K9.29
Fw Primer	E1_I nsert_Pri mer F	E1_Insert _Primer F	E1B_Inse rt_Primer F	E1B_Inse rt_Primer F	E1B_Inse rt_Primer F	BamHI -E1A-F	BamHI -E1A-F	BamHI-E 1B55K-F	BamHI-E 1B55K-F	BamHI-E 1B55K-F
Re Primer	E1A _Ins ert_ Prim er R	E1A_Inse rt_Primer R	E1_Insert _Primer R	E1_Insert _Primer R	E1_Insert _Primer R	EcoRI- E1A-R	EcoRI- E1A-R	EcoRI-E1 B55K-R	EcoRI-E1 B55K-R	EcoRI-E1 B55K-R

3,5 Target strip can be seen. Run gel electrophoresis and extract them.

19.10.7

1. Reduces viral gradient to test adenovirus tilter

Take a new tube of virus and melt it on the ice. Dilute to 10^{-7}, use 10^{-7} to 10^{-12} diluted virus solution to make a gradient, virus stock solution as a positive control, DC- as a negative control, 100uL per well.

19.10.12

1. PCR to obtain insert-E1A and insert-E1B

	1	2	3	4	5	6
template	E1A9.9	E1A9.9	E1A 10.8	E1B 55K 9.11	E1B 55K 9.29 (purified twice)	E1B 55K9.29
Fw Primer	E1_Insert_Pri mer F	BamHI-E1A -F	BamHI-E1A -F	BamHI-E1B5 5K-F	BamHI-E1B55K-F	BamHI-E1B55K -F
Re Primer	E1A_Insert_P rimer R	EcoRI-E1A- R	EcoRI-E1A- R	EcoRI-E1B55 K-R	EcoRI-E1B55K-R	EcoRI-E1B55K- R
Target product length (bp)	930	930	930	1530	1530	1530

1,2,3,5 Target strip can be seen. Run gel electrophoresis and extract them.

2. pTRE-E1B 55K Gibson assembly

E1B 55K(60ng), pTRE(127ng)

19.10.13

1. pTRE-E1A Gibson assembly

E1A(36ng), pTRE(127ng)

2. pTRE-E1B 55K colony PCR.

7 positive colonies. Inoculate them to 5mL LB medium with 5uL Amp.

3. Adenovirus titer data!

	CPE Positive	CPE Negative	Accumulative Positive	Accumulative Negative	CPE Portion （%）
10^-7	8	0	25	0	100.0
10^-8	8	0	17	0	100.0
10^-9	5	3	9	3	75.0
10^-10	3	5	4	8	33.3
10^-11	1	7	1	15	6.3
10^-12	0	8	0	23	0.0

19.10.14

1. pTRE-E1A colony PCR

All eight colonies appear positive. There are faint bands around 1k.

2. Inoculate pTRE-E1A, pTRE-E1B 55K to 5mL LB.

19.10.15

Extract plasmids of pTRE-E1A and pTRE-E1B 55K, send for sequencing.