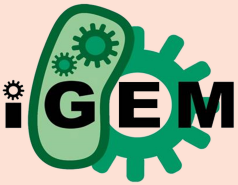
2019 IGEM RDFZ-China

Laboratory Manual



2019 iGEM RDFZ-China

Laboratory Manual

Team：RDFZ-China

刘若宸 潘旻佳 李明阳 李靖文 /编稿

贾泽然 赵倚晴 /主审

潘旻佳 王奕桥 /美工

李明阳 许航 辛浩然/翻译

鸣谢 陶庆华教授与朱薛辰学长的无私帮助

Introduction

This handbook serves as the experiment guidance for the iGEM team RDFZ-China 2019 and other RDFZ teams in the future and offers instructions on the use of some simple instruments in molecular biology labs. This handbook is designed for participants from high schools and readers who are learning simple molecular cloning experiments. The lab described in this handbook is Professor QingHua Tao's lab in Tsinghua University, and most of the content is specifically designed for the project "Fragrance Library" in 2019. Thus, there are some limitations of the content. We hope that all the readers of this handbook can adjust their methods and parameters according to their own experiments.

Table of Content

Room 307: managers' office. For consultation.

Room 306: the lab we use.

Address: Renhuan Building, Tsinghua University, Haidian District, Beijing
Lab manager info
Name: Xuechen Zhu
Phone Number: 1*********2

Laboratory Safety Management Institution

1. The representative of this lab is the person in charge of technical safety, security, and fire safety of this lab and is responsible for the implementation of relevant security and fire safety regulations. The representative receives supervision, examination and instruction from the security, fire safety and lab management departments.

2. The representative of this lab must regularly conduct technical safety, security and fire safety education for the student and teachers of the lab, especially those who have just entered the lab in order to improve everyone' s awareness of safety and self-protection. The representative of the lab must, as required, equip the lab with containers that recycle toxic substances, glass and metal sharps and waste liquid and instruments fire safety and security. The representative must be able to use these instruments adeptly, check them regularly and apply for maintenance in time.

3. There must be a fire safety representative for every room of the lab. They are fully responsible for the fire safety work in the room. Every day, before leaving work, the representatives must check the instruments and make sure that the water and electricity supply and doors and windows are in safe condition. They cannot leave before confirming that everything is safe. If there are personnel changes, fire safety representatives must be changed and the report must be handed to the academy office.

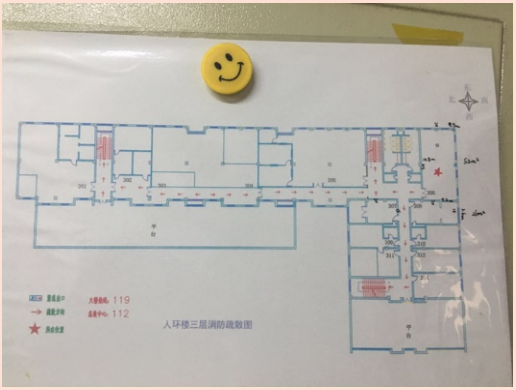
4. Every lab must conduct safety check frequently in order to detect problems and eliminate security and fire safety dangers. Temporary safety measures must be taken in order to address problems that are hard to solve, and the academy and the school must be informed in order to solve the problems.

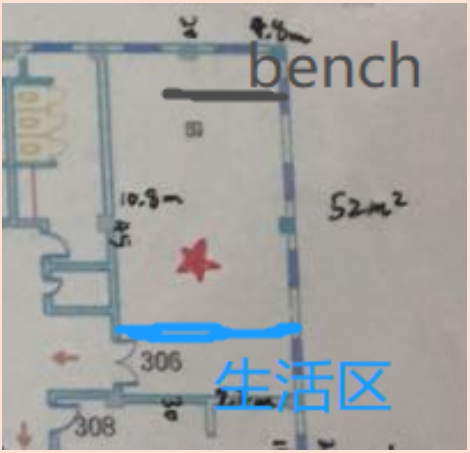
5. When conducting research, "*Radioactive Substance Safety Management Institution*" "*Experimental Animals Safety Management Institution*" and other relevant technical institutions must be followed. Toxic, combustible, explosive and radioactive substances must be kept and used properly. Large instrument must be taken care of by assigned people. Toxic wastes, waste liquids, solutions and containers must be classified before recycle, and should all be processed by the school lab. Animal carcasses and organs must not be thrown away.

6.Electricity safety awareness must be improved. It is not allowed to connect private wires. Open fire electric furnace must not be used in the lab.

7. Laws must be followed. National secrets, school's intellectual properties and network security must be protected. Harmful information from all avenues must be rejected. The good image of the country and the school must be maintained. The representative of the lab must educate all stuff members of the lab to follow all regulations of the school and academy and protect public safety and order.

8.All members in the lab must remember and be proficient in using frequently used telephones.


119
112
人环楼三层消防疏散图


bench
10.8m
52m²
306
生活区
308

Instructor: Yiqing Zhao,
1*********4(phone number)
Advisor: Zeran Jia, 1*********4
Team Leader: Ruochen Liu,
1*********8
These phone numbers are private.
For non-urgent communication,
please use WeChat.

*Contaminated Zone: Electrophoresis dye is carcinogenic, please be careful. When using it, you must wear a pair of PE gloves outside the

Centrifuge

Micropipette tips

Refrigerator that works at -20 °C. All DNA segments are stored here.

Micropipettes

Waste Liquid Container

Vortex mixer

Our drawer. Don't touch other drawers.

*Living Zone: Do not touch anything with gloves. You can put your backpack here.

Instructions on Simple Instruments Operation

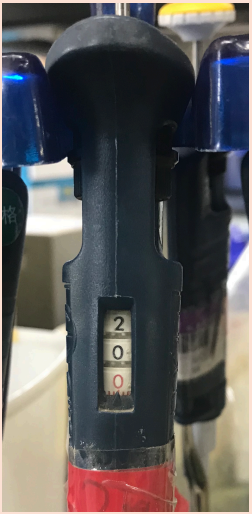
Micropipettes

Correct posture

As shown in the picture, this side should face you. You should use specific tips according to the ranges of micropipettes.

Ranges of Micropipettes

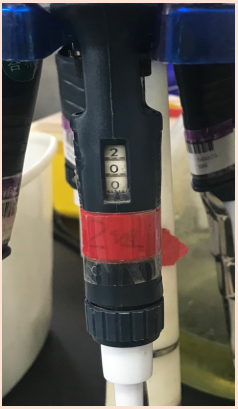
Noted at the top of the pipette. Always remember to put the scale back to its largest range of measurement after use. The last digit changed is marked red on the scale.



Type 1
Red readings at the bottom (eg: 0):
As shown in the picture, the reading is 20.0 µl

Type 2
Red readings at the top (eg: 1) As shown in the picture, the reading is 1000µl.

Ranges of Micropipettes

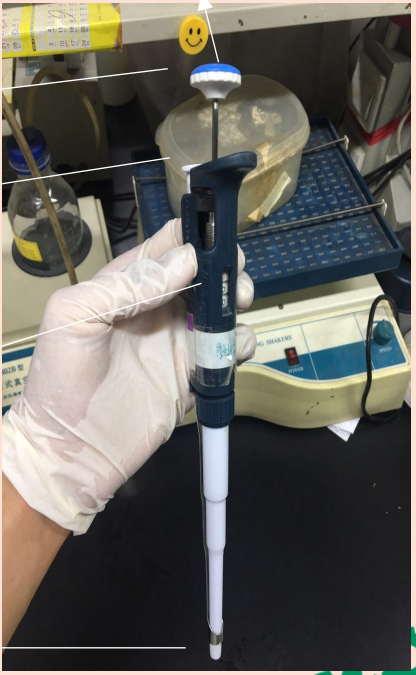


Type 3

No red readings. As shown in the picture, the reading is 200μl.

Absorb / Release liquids
The range of volume is shown here.
When pushing the button, please remember that there will be two stops.
You will reach and strongly feel the first stop before you draw up / after you release the liquid.

Use the push-button to set the volume.
After using the instrument, you must set it to the biggest volume.
Make sure you have chosen the appropriate volume before using the instrument.
You should never use the micropipette to measure volumes outside of its range.

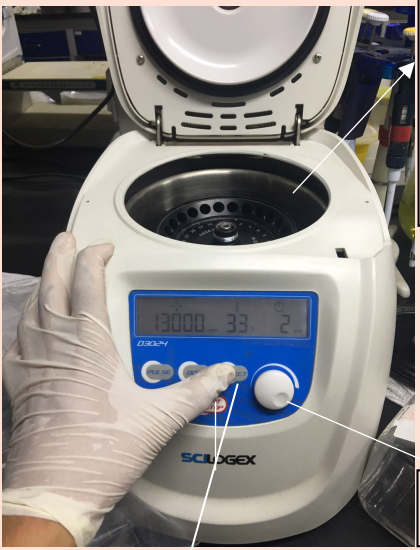


Tip ejector

The volume you set is shown here. The red readings of the pipettes in the lab represent the nest level of counting unit. The smaller counting unit has the readings at the bottom.

This part must not touch other things in the lab.
You must not hold this instrument upside-down.

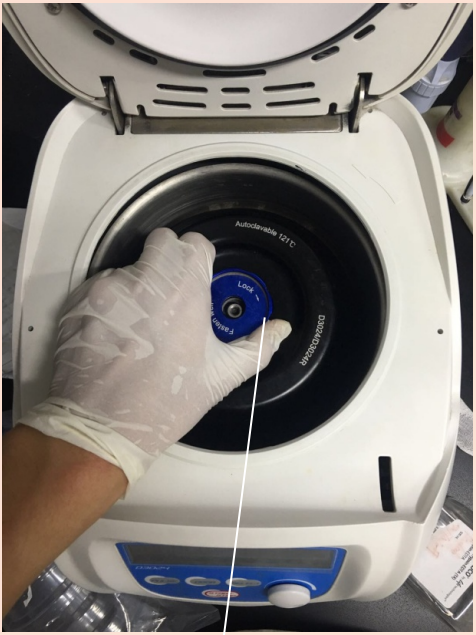
Centrifuges



You must place the centrifugal tubes symmetrically.
It there is only one centrifugal tube, it must be balanced by a centrifugal tube with the same volume of water.

Turn this button to set parameters.

These are buttons to confirm parameter, start, and pause.
For example, the parameter in the picture shows 13000 revolutions, 33 ℃, and 2 minutes.
When using the centrifuge, wait until it reaches its highest speed. When the centrifuge starts to shake or make loud noises, you must shut down it.



You must make sure that this lid is closed firmly. After screwing the lid, you may start to use the machine.

When the machine is working, if it begins to squeal, it's probably because you didn't screw the lid firmly. You must pause the machine immediately.

After finishing the work, the main lid of the machine will open automatically.

Vortex

Press the bottom of the tube in the bottom of the rubber part. Stop pressing the centrifuge tube when the reagent is readied to use.

Metal Bath (65 ℃)

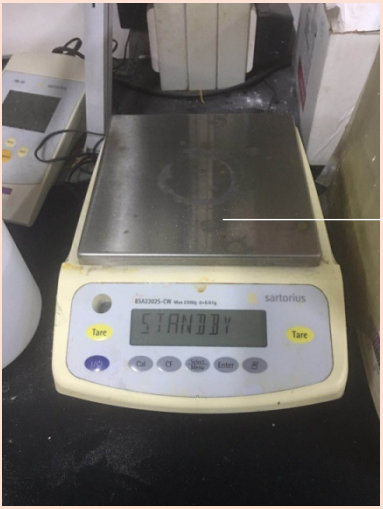
The black part is hot. Be careful.

Current temperature is shown here.

You must shut down the machine

Pre-set temperature
This machine works at constant temperature. The pre-set temperature is 65 °C
After working, the machine will gradually increase its temperature to 65 °C.
If time is limited, you are recommended to set the temperature to 65 °C before starting.

Electronic Balance



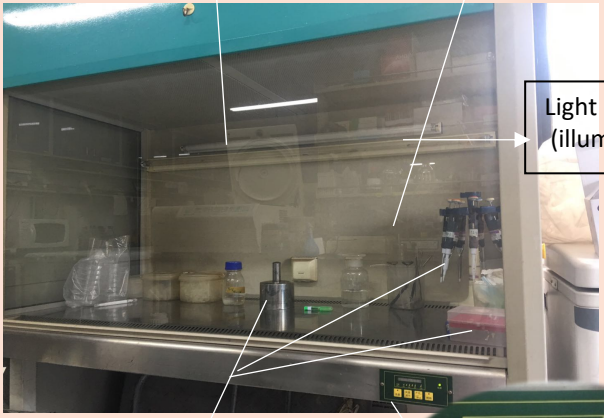
Do not put anything on the platform before turning on the balance. Before using, please press “tare” button. When using, you must place a piece of folded paper on the platform and place the specimen you want to weigh on that paper. After using the balance, if there are some spilled specimen on the plat form, you must clean them.

You will find weighing paper and spoon for holding reagent in the drawer behind you.

Super Clean Bench

UV Sterilization
Normally, you should keep it working for 15 minutes. But this lab doesn't require us to open the UV light, so we don't need to do so.

When opening the glass cover, be as gentle as possible. Use both of your hands and do not exert too much force on the cover. **Also, open the cover until you can put your hands in. Do not open the cover too much.**



Light (illumination)

You must not take out anything inside the super clean bench. (ex. Spirit burners, pipettes)
After your experiments, you must place everything back to where they originally are.

Ice Box

In some experiments, reagents must be kept in a low temperature (such as various enzymes). So, we need an ice box to keep those reagents at a low temperature. If you are not using the ice box, please put it in the refrigerator with a temperature of -20 °C. Please place the ice box with its holes facing down.

Thermostat Water Bath

The sensitivity of the machine is low. The temperature will rise very slowly. You are recommended to turn on the machine and rise the temperature in advance.

Incubator

It is used to cultivate bacteria. Bacteria grow at the fastest rate when living in a condition with 37 °C.

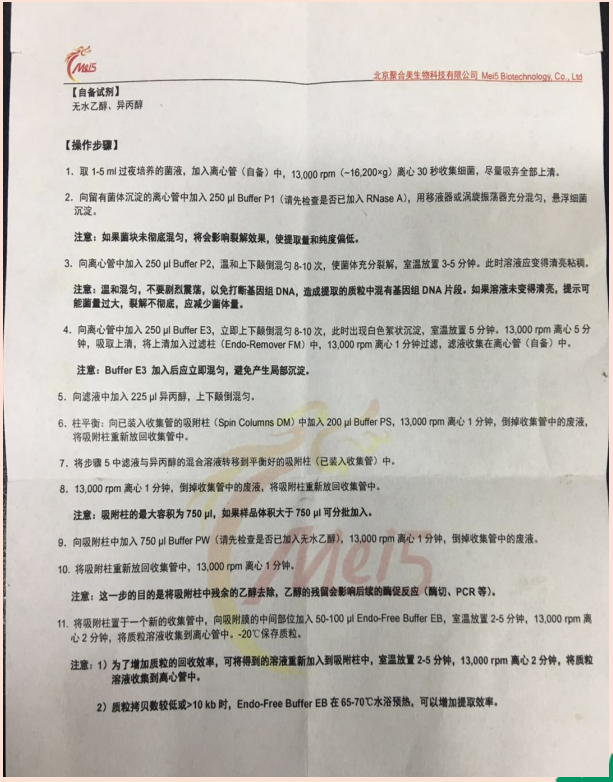
The temperature is shown here. This machine works at 37 °C constantly.

Switch
The main power is controlled by the main switch. After using the machine, please shut it down if there are no other culture plates with bacteria.

You do not deed to shake the bacteria (culture plates do not need to be shaken, but liquid culture medium do). That's why we place our plate on the first layer instead of the second layer, which shakes the plates.

Molecular cloning experiment

Plasmid Extraction



Mei5 北京聚合美生物科技有限公司 Mei5 Biotechnology, Co., Ltd

【自备试剂】
无水乙醇、异丙醇

【操作步骤】

1. 取 1-5 ml 过夜培养的菌液，加入离心管（自备）中，13,000 rpm（~16,200×g）离心 30 秒收集细菌，尽量吸弃全部上清。

2. 向留有菌体沉淀的离心管中加入 250 µl Buffer P1（请先检查是否已加入 RNase A），用移液器或涡旋振荡器充分混匀，悬浮细菌沉淀。

   注意：如果菌块未彻底混匀，将会影响裂解效果，使提取量和纯度偏低。

3. 向离心管中加入 250 µl Buffer P2，温和上下颠倒混匀 8-10 次，使菌体充分裂解，室温放置 3-5 分钟。此时溶液应变得清亮粘稠。

   注意：温和混匀，不要剧烈震荡，以免打断基因组 DNA，造成提取的质粒中混有基因组 DNA 片段。如果溶液未变得清亮，提示可能菌量过大，裂解不彻底，应减少菌体量。

4. 向离心管中加入 250 µl Buffer E3，立即上下颠倒混匀 8-10 次，此时出现白色絮状沉淀，室温放置 5 分钟。13,000 rpm 离心 5 分钟，吸取上清，将上清加入过滤柱（Endo-Remover FM）中，13,000 rpm 离心 1 分钟过滤，滤液收集在离心管（自备）中。

   注意：Buffer E3 加入后应立即混匀，避免产生局部沉淀。

5. 向滤液中加入 225 µl 异丙醇，上下颠倒混匀。

6. 柱平衡：向已装入收集管的吸附柱（Spin Columns DM）中加入 200 µl Buffer PS，13,000 rpm 离心 1 分钟，倒掉收集管中的废液，将吸附柱重新放回收集管中。

7. 将步骤 5 中滤液与异丙醇的混合溶液转移到平衡好的吸附柱（已装入收集管）中。

8. 13,000 rpm 离心 1 分钟，倒掉收集管中的废液，将吸附柱重新放回收集管中。

   注意：吸附柱的最大容积为 750 µl，如果样品体积大于 750 µl 可分批加入。

9. 向吸附柱中加入 750 µl Buffer PW（请先检查是否已加入无水乙醇），13,000 rpm 离心 1 分钟，倒掉收集管中的废液。

10. 将吸附柱重新放回收集管中，13,000 rpm 离心 1 分钟。

    注意：这一步的目的是将吸附柱中残余的乙醇去除，乙醇的残留会影响后续的酶促反应（酶切、PCR 等）。

11. 将吸附柱置于一个新的收集管中，向吸附膜的中间部位加入 50-100 µl Endo-Free Buffer EB，室温放置 2-5 分钟，13,000 rpm 离心 2 分钟，将质粒溶液收集到离心管中。-20℃保存质粒。

    注意：1）为了增加质粒的回收效率，可将得到的溶液重新加入到吸附柱中，室温放置 2-5 分钟，13,000 rpm 离心 2 分钟，将质粒溶液收集到离心管中。

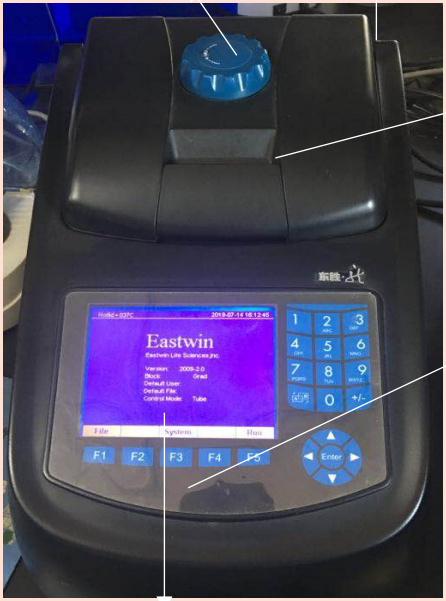
    2）质粒拷贝数较低或>10 kb 时，Endo-Free Buffer EB 在 65-70℃水浴预热，可以增加提取效率。

PCR

The system is 50 μl

The knob adjusts the lid's tightness and make sure to tighten it before you begin until there is a "kata" sound.

The switch is at the back



Turn the handle to open the lid Remember to cover it when PCR is finished because the dust, which fell into the machine, will affects the performance of the machine.

Remember to press the stop button first before opening the lid, otherwise the inner part will remain the hot temperature, which brings a lot of harm to the machine.

There is a setting of pcr in rdfzigem called pcr vector. （parameter ：annealing temperature:68 ℃, time:90s）
Gibson assembly （parameter：50 ℃ for 2h and 12 ℃ forever ）

System（50μl）： This year the team unified using the mix to match the system, so as to minimize errors. This regulation is suitable for novices, which is relatively simple.

materials：pcr mix (contains DNA polymerase, bases /dntps, buffer contains Mg2+)

forward & reverse primer

target gene's template

ddH2O

Pcr tube

（Taq）pcr mix	25 μl
F primer	1 μl 10mM
R primer	1 μl 10mM
Template	1 μl less than200ng
ddH2O	22 μl（make sure total volume is 50 μl）

Mix together the following on the ice box.

Always adding mix last and adding ddH2O first to ensure solvents are in.

5000rpm 10s centrifuging after adding all the substances to make sure that there's no remaining substances on the wall of the tube.

PCR temperature：

The setting is already in the machine, no more operations needed：

98℃ 5min

98 ℃ 30s

30 cycles

68 ℃ 30s（annealing time，our annealing temperature is about 68 degrees centigrade）

72 ℃ 90s

（annealing time，parameter，it differs according to different length of the gene. Using 60s/kb as a standard is conservative. However, it is fine to have a longer annealing time. Based on our target gene, which is around 1500bp, we set 90s）

72 °C 5min

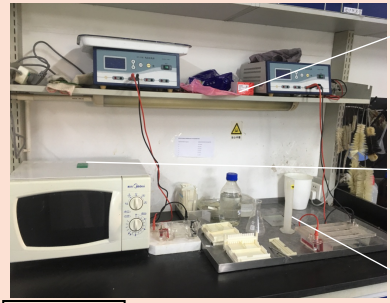
4 °C forever

Electrophoresis*

BOOKLET

highly recommend doing this accompanied by someone for the first time!

material

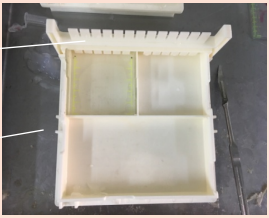


EB dyeing agent is in the red box. Do not forget!

Microwave
Medium heat heating 2min
Notice to wear gloves

Cylinder to measure TAE

Bigger mold



Gel forming mold
First put the rectangle cell, then put the mold

Agarose is in the locker

spoon for holding reagent and weighing paper

Electronic balance
For weighing the agarose
Remember to press the tare before using.
Always turn off it after using.

Gel formation（30ml protocol）

materials：

30ml TAE buffer （in contaminated area）

0.3g agarose(in the penultimate cupboard)

1μl DNA dyeing agent

Gel formation mold（white box and transparent square cell）&bigger comb

process：

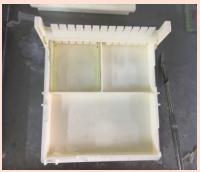
First use cylinder to measure 30ml TAE buffer（could have more）

Put the solution into the flask and add 0.3g agrose,shake

Medium heat heating for 2 min in microwave（take it out with cloth gloves, careful it is hot）

If you observe any particle sediments please keep heating for 30s.

Add 1μl DNA dyeing agent, shake



Pour the configured solution evenly into the mold and verify that the gel should have sufficient thickness.

20-30 minutes for cooling

Be careful when removing the comb. Do not use too much force or take the comb with one hand, otherwise the hole will be broken.

Gel electrophoresis

material：solidified gel
Electrophoresis instrument
PCR product
Loading buffer is in the secone 4 ℃ fridge
DNA marker（2kb）is also in that fridge
First we estimate whether there is loading in pcr product, if it has then no need to add again.

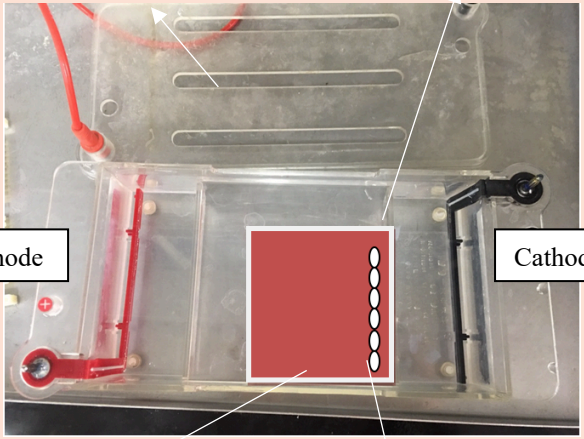
Process：
Add 10 μl loading buffer to every tube that contain 50 μl pcr product
Put the cell with gel into the right end of the pool，use your fingers to press the cell in order to prevent it floating
The side with holes needs to face the cathode（because DNA has negative polarity, so it need to swim from cathode to anode）
Make sure that electrophoresis fluid is higher than the surface of gel
Put 10 μl 2kb DNA marker to the right end lane
Inject the prepared product mixture into the gel hole in turn (not shake)

Set the parameters of the electrophoresis system: 140V, 20min
Make sure the lid is tightly closed, then begin electrophoresis
If you want to determine whether the electrophoresis starts, you can see the cathode. The process begins if there are some bubbles.
Notice that the instrument will not automatically turned off. You need to turn it off after the bell ringing.
Switch off the power and get the gel

electrophoresis

After preparation close the lid tightly switch on the power and begin the process.

Put marker+PCR product +loading buffer



Make sure that electrophoresis fluid is higher than the surface of gel. If not, then keep adding.

When you inject the mixture, use both hands to hold the micropipette in order to prevent the diffusion caused by shaking.

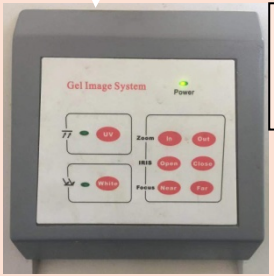
Cut gel* highly recommend doing this accompanied by someone for the first time!

The place where your hands reach into the machine. Be careful that the place where the get is cut is very dirty, so the lab coat is a must here.

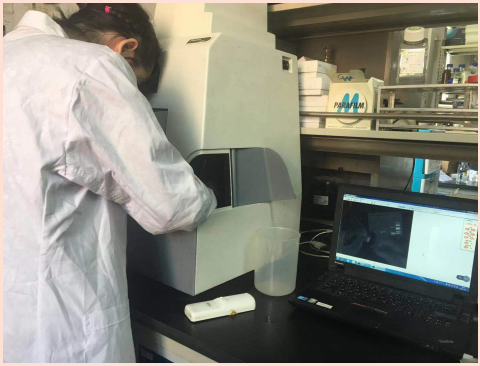
Pull out the bottom platform. Place the gel on the liquid crystal region. Adjust the position to the gel at the center of computer screen.

Judging the target strip by observing the portion with UV light

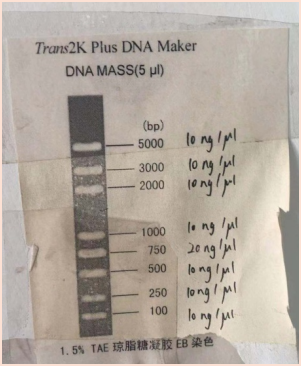
The computer will synchronize the real-time image of the gel and conserve the image.



Switch panel
We only use the UV switch in the upper left corner.

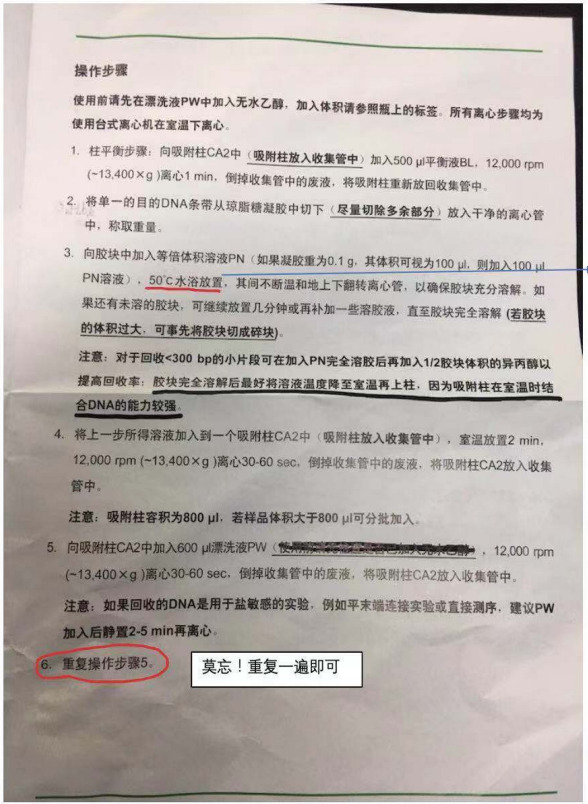


The correct posture. The principle of cutting gel is that you need to use the smallest agarose plug possible, which is exactly the region of the right stripe. The less agarose in solution, the more efficient the extraction will be. This requires you to cut carefully as well as decidedly. Consider that the image we observed in upper window is not as clear as that of computer, we can let one people watch the computer and instruct another to operate. After judging the position, try to cut it along the edge once, which is more efficient.



2K DNA marker schematic
The picture on the left shows that the position of marker in gel, and the mass-to-charge ratio will decrease in stripe from top to bottom.
The size of each strip is estimated by comparing the position of the marker on the gel with the strip, so that the correct strip is screened and the corresponding gel is cut down.

Gel Extraction purification



操作步骤

使用前请先在漂洗液PW中加入无水乙醇，加入体积请参照瓶上的标签。所有离心步骤均为使用台式离心机在室温下离心。

1. 柱平衡步骤：向吸附柱CA2中（吸附柱放入收集管中）加入500 μl平衡液BL，12,000 rpm (~13,400×g)离心1 min，倒掉收集管中的废液，将吸附柱重新放回收集管中。
2. 将单一的目的DNA条带从琼脂糖凝胶中切下（尽量切除多余部分）放入干净的离心管中，称取重量。
3. 向胶块中加入等倍体积溶液PN（如果凝胶重为0.1 g，其体积可视为100 μl，则加入100 μl PN溶液），50℃水浴放置，其间不断温和地上下翻转离心管，以确保胶块充分溶解。如果还有未溶的胶块，可继续放置几分钟或再补加一些溶胶液，直至胶块完全溶解（若胶块的体积过大，可事先将胶块切成碎块）。

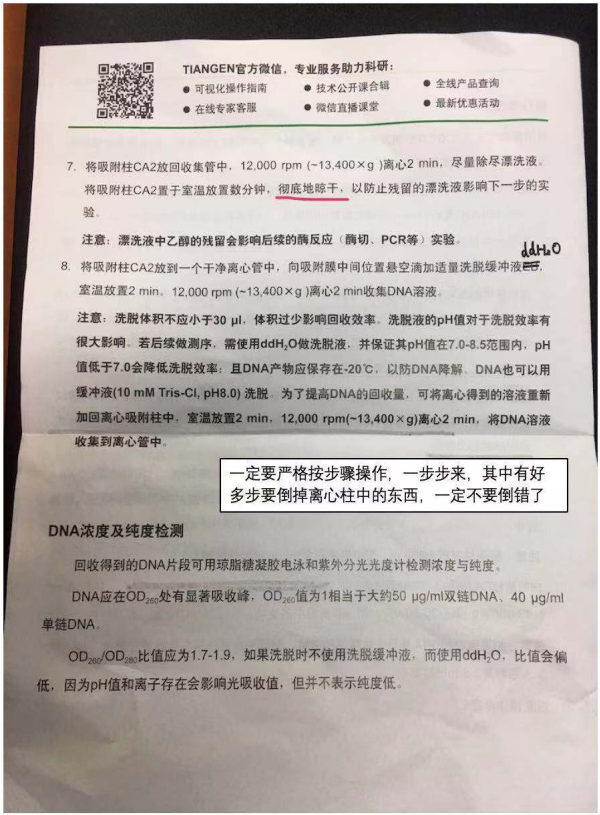
   注意：对于回收<300 bp的小片段可在加入PN完全溶胶后再加入1/2胶块体积的异丙醇以提高回收率；胶块完全溶解后最好将溶液温度降至室温再上柱，因为吸附柱在室温时结合DNA的能力较强。
4. 将上一步所得溶液加入到一个吸附柱CA2中（吸附柱放入收集管中），室温放置2 min，12,000 rpm (~13,400×g)离心30-60 sec，倒掉收集管中的废液，将吸附柱CA2放入收集管中。

   注意：吸附柱容积为800 μl，若样品体积大于800 μl可分批加入。
5. 向吸附柱CA2中加入600 μl漂洗液PW（[redacted]），12,000 rpm (~13,400×g)离心30-60 sec，倒掉收集管中的废液，将吸附柱CA2放入收集管中。

   注意：如果回收的DNA是用于盐敏感的实验，例如平末端连接实验或直接测序，建议PW加入后静置2-5 min再离心。
6. 重复操作步骤5。

放入B区55°C金属浴机器中即可

莫忘！重复一遍即可



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7. 将吸附柱CA2放回收集管中，12,000 rpm (~13,400×g)离心2 min，尽量除尽漂洗液。将吸附柱CA2置于室温放置数分钟，彻底地晾干，以防止残留的漂洗液影响下一步的实验。

注意：漂洗液中乙醇的残留会影响后续的酶反应（酶切、PCR等）实验。

8. 将吸附柱CA2放到一个干净离心管中，向吸附膜中间位置悬空滴加适量洗脱缓冲液ddH$_2$O，室温放置2 min。12,000 rpm (~13,400×g)离心2 min收集DNA溶液。

注意：洗脱体积不应小于30 μl，体积过少影响回收效率。洗脱液的pH值对于洗脱效率有很大影响。若后续做测序，需使用ddH$_2$O做洗脱液，并保证其pH值在7.0-8.5范围内，pH值低于7.0会降低洗脱效率；且DNA产物应保存在-20℃，以防DNA降解。DNA也可以用缓冲液(10 mM Tris-Cl, pH8.0) 洗脱。为了提高DNA的回收量，可将离心得到的溶液重新加回离心吸附柱中，室温放置2 min，12,000 rpm(~13,400×g)离心2 min，将DNA溶液收集到离心管中。

一定要严格按步骤操作，一步步来，其中有好多步要倒掉离心柱中的东西，一定不要倒错了

DNA浓度及纯度检测

回收得到的DNA片段可用琼脂糖凝胶电泳和紫外分光光度计检测浓度与纯度。

DNA应在OD$_{260}$处有显著吸收峰，OD$_{260}$值为1相当于大约50 μg/ml双链DNA、40 μg/ml单链DNA。

OD$_{260}$/OD$_{280}$比值应为1.7-1.9，如果洗脱时不使用洗脱缓冲液，而使用ddH$_2$O，比值会偏低，因为pH值和离子存在会影响光吸收值，但并不表示纯度低。

Please follow the instruction step by step

Gibson assembly

material：PCR tube
Mix
Backbone（product in Gel Extraction purification）
Target gene
ddH2O,

PCR system（total volume--10 μl）：

Mix	5 μl
Backbone	need to calculate
target gene	need to calculate
ddH2O	add until the overall volume is 10 μl

Attention:
Mix together the following on ice
always adding mix last and adding ddH2O first
First deforest backbone and target gene
Please make sure you successfully add the solvent to the pcr tube because the dosage of each solvent is small.

PCR condition :

50℃ 2hours
12℃ forever
Inside the PCR machine is an account named rdfzigem,
folder name: gibsonassem

Conversion

Material：50 μl competence （delivered within 3-5days ）
2 μl Gibson product
LB culture solution
K-resistance plate
Sterilized loop (use bunsen burner to sterilize before and after the transformation）

Procedure：
It must be carried out in strict accordance with the parameters and in the specified time!
Take 50μl competence from 80°C fridge and put it on icebox（competence on ice, otherwise it will in failure mode）
In super clean bench， put 2 μl Gibson product to the competence, Flick the bottle to make it evenly mixed
（don't touch the nozzle and the inner wall of ep tube）
Put the solvent in to -20°C fridge, stay 20min. At this time, the water bath can be opened in advance and adjusted to 42 ℃.
Packed in float and placed in a 42°C water bath for 90 seconds.
(When waiting for the water bath to reach 42 ° C, you can put it at -20 ° C for a while)
Place in a -20 ° C refrigerator for 2 min
Add 400 μl of LB culture solvent to each tube in a clean bench
40 min in 37 °C shaker box
Pour the mixture onto the plate
Sterilize the inoculating loop by placing it in the flame of the Bunsen burner(remember to turn off). Pass the lower half of the loop through the flame until it glows red.
Allow the loop to cool. You can touch it to the sterile agar on the plate to make sure it has cooled down. (Do not place the loop on the table or let it contact anything other than sterile agar or the desired culture)
Dip the loop into the E. coli culture and then remove it.
Open the agar plate and gently glide the loop back and forth across the surface of one section of the agar. Take care to not scratch through the agar with the loop.
Put the top back onto the agar plate. Turn the plate upside down and place it into the incubator set to 37 ℃ (98.6 ℉).

Colony PCR

Colony PCR is used to determine if inserts are present, so the gel we make is 20 μl. Remember to conserve a copy of each colony.

PCR system（20 μl）：

material：

pcr mix (contains DNA polymerase，base/dntps，and buffer contains Mg2+)

forward & reverse primer（a middle primer will be needed if the length of target gene is big）

ddH2O

PCR tube

Mix together the following on ice

（Taq）pcr mix 10 μl

F primer 1 μl 10mM
R primer 1 μl 10mM
（other primers 1 μl 10mM）
Template. put clone strain in the tube using anti-bacterial tips
ddH2O the rest of 20 μl

Procedure:

Always adding mix last and adding ddH2O first to ensure solvents are in

After all the operation, the PCR tube can be loaded into the collection tube and centrifuged at 5000 rpm for 10s to sink substances on the wall.

- Picking up a monoclonal strain with a sterile gun head in a clean bench
- Soak the gun head in the system and mix it properly.
- Draw grids on a new plate cover and mark it
- Squat it on the surface and keep
- Retained and placed colony in the incubator

PCR temperature:

Colony p's temperature is already set up in the machine.

96 degrees centigrade 10min

96 degrees centigrade 30s ⇨ 25 cycles

58 degrees centigrade 30s（annealing temperature，parameter，our team use 68 degrees centigrade ）

72 degrees centigrade 90s（annealing time ，parameter，it differs according to different length of the gene. Using 60s/kb as a standard is conservative. However, it is fine to have a longer annealing time. Based on our target gene, which is around 1500bp, we set 90s）

72 degrees centigrade 5min

4 degrees centigrade forever