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7.13 Experimental Microbial Genetics

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Bootcamp Day 2 – Purification, digestion and dephosphorylation of pMQ64

A. PLASMID DNA PURIFICATION OF pMQ64 USING HISPEED PLASMID MAXI KIT


This protocol is for preparation of up to 750 µg of high- or low-copy plasmid or cosmid DNA using the HiSpeed Plasmid Maxi Kit. A final DNA concentration of up to 1.5 µg/µl can be expected, if eluting a high-copy plasmid with 500 µl of Buffer TE.

Maximum Recommended Culture Volumes*

	HiSpeed Plasmid Midi Kit	HiSpeed Plasmid Maxi Kit
High-copy plasmids	50 ml	150 ml
Low-copy plasmids	150 ml	250 ml

* For high-copy plasmids, expected yields are 300–750 µg for the HiSpeed Plasmid Maxi Kit. For low-copy plasmids, expected yields are 50–250 µg for the HiSpeed Plasmid Maxi Kit using these culture volumes.

Important notes before starting

- New users are advised to familiarize themselves with the detailed protocol provided in the HiSpeed Plasmid Purification Handbook posted at the 7.17 Stellar website. In addition, extensive background information is provided on the Qiagen plasmid resource page: www.qiagen.com/goto/plasmidinfo.
- Optional: Remove samples at the steps indicated with the symbol  in order to monitor the procedure on an analytical gel (see page 29 of the HiSpeed Plasmid Purification Handbook for analytical procedure)

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use (check for P1 + RNaseA in the fridge first). Use one vial of RNaseA (centrifuge briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use one vial LyseBlue (centrifuge briefly before use) per bottle of Buffer P1 to achieve a 1:1000 dilution (Check to see if LyseBlue has already been added). LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see “Using LyseBlue reagent” on page 16 of the HiSpeed Plasmid Purification Handbook.

Procedure (during bootcamp, proceed to step 3):

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).

Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate • 150 ml medium. For low-copy plasmids, inoculate • 250 ml medium. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).

Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately $3\text{--}4 \times 10^9$ cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter.

3. Harvest the pMQ64 containing *E.coli* cells by centrifugation at 6000 x g for 15 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. Save the culture flask to serve as a waste receptacle in subsequent steps.

*If you wish to stop the protocol and continue later, freeze the cell pellet at –20°C.

4. Resuspend the bacterial pellet in • 10 ml Buffer P1.

For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. Add • 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.

Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO₂ in the air. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

During the incubation prepare the QIAfilter Cartridge: Screw the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge. Place the QIAfilter Cartridge into a convenient tube or a QIArack. SEE VISUAL AIDS AT THE END OF THIS PROTOCOL FOR HELP DETERMINING WHAT KITS COMPONENT IS WHAT!

6. Add • 10 ml chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4–6 times. Proceed directly to step 7. Do not incubate the lysate on ice.

Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and KDS becomes visible. The buffers must be mixed completely. If the mixture appears still viscous and brownish, more mixing is required to completely neutralize the solution. It is important to transfer the lysate into the QIAfilter Cartridge immediately in order to prevent later disruption of the precipitate layer. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10 min. Do not insert the plunger!

Important: This 10 min incubation at room temperature is essential for optimal performance of the QIAfilter Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging. If, after the 10 min incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipette tip around the walls of the cartridge to dislodge it.

8. Equilibrate a ● **HiSpeed Maxi** Tip by applying ● **10 ml** Buffer QBT and allow the column to empty by gravity flow.

Set the tip up to drip into the flask you grew your culture in using one of the blue rings in the kit. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the HiSpeed Tip to drain completely. HiSpeed Tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

9. Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the ● **QIAfilter Maxi** Cartridge and filter the cell lysate into the previously equilibrated HiSpeed Tip.

Filter until all of the lysate has passed through the QIAfilter Cartridge, but do not apply extreme force. Approximately ● **25 ml** of the lysate is generally recovered after filtration.

☞ Remove a ● **120 µl** sample of the filtered lysate and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

10. Allow the cleared lysate to enter the resin by gravity flow.

☞ Remove a ● **120 µl** sample of the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

11. Wash the ● **HiSpeed Maxi** Tip with ● **60 ml** Buffer QC.

Allow Buffer QC to move through the HiSpeed Tip by gravity flow.

☞ Remove a ● **240 µl** sample of the wash fraction and save for an analytical gel (sample 3).

12. Elute DNA with ● **15 ml** Buffer QF.

Collect the eluate in a tube with a minimum capacity of ● **30 ml**.

☞ Remove a ● **60 µl** sample of the eluate and save for an analytical gel (sample 4).

* If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding ● **10.5 ml** (0.7 volumes) room temperature isopropanol to the eluted DNA. Mix and incubate at room temperature for 5 min.

All solutions should be at room temperature in order to minimize salt precipitation.

14. During the incubation remove the plunger from a • 30 ml syringe and attach the • QIAprecipitator Maxi Module onto the outlet nozzle. Do not use excessive force, bending, or twisting to attach the QIAprecipitator!

Important: Always remove the QIAprecipitator from the syringe BEFORE pulling up the plunger!

15. Place the QIAprecipitator over a waste bottle, transfer the eluate/isopropanol mixture into the • 30 ml syringe, and insert the plunger. Filter the eluate/isopropanol mixture through the QIAprecipitator using constant pressure.

Important: Complete the QIAprecipitator procedure (steps 16–21) within 10 min. To prevent detachment of the QIAprecipitator and subsequent loss of DNA and alcohol, do not use excessive force when pushing liquid through the QIAprecipitator.

16. Remove the QIAprecipitator from the • 30 ml syringe and pull out the plunger. Re-attach the QIAprecipitator and add 2 ml 70% ethanol to the syringe. Wash the DNA by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.

17. Remove the QIAprecipitator from the • 30 ml syringe and pull out the plunger. Attach the QIAprecipitator to the • 30 ml syringe again, insert the plunger, and dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step.

18. Dry the outlet nozzle of the QIAprecipitator with absorbent paper to prevent ethanol carryover.

19. Remove the plunger from a new 5 ml syringe and attach the QIAprecipitator onto the outlet nozzle. Hold the outlet of the QIAprecipitator over a 1.5 ml collection tube. Add 1 ml of Buffer TE to the 5 ml syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure.

Ensure that the outlet of the QIAprecipitator is held over the collection tube when Buffer TE is poured into the syringe, as eluate can drip through the QIAprecipitator before the syringe barrel is inserted.

Be careful, as residual elution buffer in the QIAprecipitator tends to foam when expelled.

Alternatively, if a higher DNA concentration is desired and a reduction in yield of up to 10% is acceptable, elute with 500 µl Buffer TE. Lower volumes of elution buffer are not recommended, since incomplete wetting of the QIAprecipitator membrane will lead to reduced DNA yields.

Water or buffers commonly used to dissolve DNA (e.g., Tris, may also be used for elution).

Note: TE Buffer contains EDTA which may inhibit downstream enzymatic or sequencing reactions.

Note: Store DNA at –20°C when eluted with water as DNA may degrade in the absence of buffering and chelating agents.

20. Remove the QIAprecipitator from the 5 ml syringe, pull out the plunger and reattach the QIAprecipitator to the 5 ml syringe.

21. Transfer the eluate from step 19 to the 5 ml syringe and elute for a second time into the same 1.5 ml tube.

This re-elution step ensures that the maximum amount of DNA in the QIAprecipitator is solubilized and recovered. Be careful, as residual elution buffer in the QIAprecipitator tends to foam when expelled.

22. Label tube such that it is clear that this is maxi-prepped pMQ64!!!!!!

B. LINEARIZATION OF PMQ64 WITH BAMHI

pMQ64 is the vector you will be using to clone a library of fragments of the *Pseudomonas aeruginos* genome (a map of this vector is posted on the Stellar site). In order to do that, you must first linearize the circular plasmid using the restriction enzyme BamHI, and phosphatase treat it to prevent it from religating to itself in next week's ligation reactions.

Note: Restriction enzymes are heat sensitive! Keep your reagents and reactions on ice as much as possible and add the restriction enzyme last!

0. Set your heat block to 65°C

1. Set up a 50uL reaction that includes the following:

- NEB Buffer 3 (from 10x stock)
- BSA (from 10x stock)
- 5ug plasmid (your maxiprep yield will be around 250ng/uL)
- 1uL (20 units) BamHI
- Water (to a final volume of 50uL)

Component	Volume to add
pMQ64 (maxi prep)	
dH ₂ O	
NEB Buffer 3 (10X)	
BSA (10x)	
BamHI	

Total Volume	50 ul
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2. Mix well and spin down briefly. Then place in a tube rack and leave in the 37°C room for 1 hour. **After incubation, proceed to dephosphorylation step.**

C. DEPHOSPHORYLATION OF LINEARIZED PMQ64

To prevent the plasmid from self-ligating in next week's ligation reactions, we will treat it with Antarctic Phosphatase. More information about the use of this enzyme can be found in the NEB Catalog. The protocol below is specific to our purposes for bootcamp. Refer to the product insert posted on the Stellar website if you are using this enzyme for your project to ensure proper usage.

1. Add Antarctic Phosphatase buffer to a final concentration of 1x (from 10x stock), plus 1uL of Antarctic Phosphatase per ug of plasmid. Return to 37°C for 30 minutes.

2. Heat inactivate the Antarctic Phosphatase by placing your reaction at 65°C for 5 minutes (or as required to inactivate the restriction enzyme).

Note: Many restriction enzymes can be heat inactivated, however BamHI cannot. When working with other enzymes, check the NEB catalog for more information, as the procedures for inactivating them can vary.

3. LABEL YOUR TUBE SUCH THAT IT IS CLEAR THAT IT IS BAMHI CUT & PHOSPHOTASED pMQ64!!! Save tube in your -20°C freezer box until day 4 when you will gel purify the linearized and phosphatase-treated plasmid.

D. START AN OVERNIGHT CULTURE OF *PSEUDOMONAS AERUGINOSA* PA14

1. Inoculate 5 mls of LB with a single colony of *Pseudomonas aeruginosa* PA14 from the plate you struck out yesterday.

Be sure to use sterile technique!! Since this strain is not antibiotic resistant we will not use antibiotic in the media, which means anything can grow!

2. Incubate the culture overnight at 37°C on the roller drum. BALANCE THE TUBES!

Maxi Prep Procedure Visual Aids:

