003 - Plasmid production and purification using HiSpeed® Plasmid Midi Kit

This protocol describes the procedure to generate medium quantities of pure plasmid DNA using HiSpeed® Plasmid Midi Kit (Qiagen, Cat.# 12643). This protocol is based on the HiSpeed® Plasmid Midi Kit Quick-Start Protocol (March 2016) available on the Qiagen website.

Before starting the procedure in the lab, it is highly advised to review the detailed HiSpeed® Plasmid Purification Handbook (May 2012) available on the Qiagen website.

1. Important safety notes

1.1. Always be aware of the safety risks posed by the different chemicals and instruments used while preparing the solutions described in this guide.

1.2. Before starting to work with the chemicals listed in this guide, review the safety data sheet provided with each chemical. If a copy of the safety data sheet is not provided with the chemical, check the website of the manufacturer.

1.3. Always wear the appropriate personal protective equipment (PPE) when handling the chemicals and executing the procedures described in this guide. Examples of PPE that should be worn at all times are:

1.3.1. Safety goggles

1.3.2. Disposable rubber gloves

1.3.3. Face mask (if required)

1.3.4. Laboratory coat

1.4. When handling hot containers use appropriate protective equipment and judgement to avoid injuries.

1.5. When handling chemicals use appropriate protective equipment and judgement to avoid:

1.5.1. Direct contact with the skin or mucosal membranes.

1.5.2. Inhaling powders or vapours.

1.6. When using a gas burner, keep flammable substances at a safety distance from the flame. If using ethanol to sanitise equipment, make sure the ethanol has evaporated before bringing the equipment near the flame.

2. List of abbreviations

Abbreviation	**Full form**
SDS	Sodium dodecyl sulphate
KDS	Potassium Dodecyl Sulphate
RT	Room temperature
rpm	rotations per minute
g	Gravitational force (see rcf below)
rcf	Relative centrifugal force (value is same as *g* above)
h	hour(s)

3. Storage

HiSpeed Plasmid Kits should be stored dry and at room temperature (15–25°C). HiSpeed Tips, QIAfilter Cartridges, and QIAprecipitator Modules can be stored for at least two years without showing any reduction in performance, capacity, or quality of separation. After addition of RNase A, Buffer P1 should be stored at 8°C and is stable for six months. Other buffers and RNase A stock solution can be stored for two years at room temperature.

4. Things to do before starting

4.1. Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle Buffer P1 for a final concentration of 100 µg/mL.

4.2. Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.

4.3. Pre-chill Buffer P3 at 4°C.

4.4. Optional: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use 1 vial LyseBlue reagent per bottle Buffer P1 for a final dilution of 1:1000 (e.g., 10 µL LyseBlue into 10 mL Buffer P1). 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.

5. Equipment and Reagents to Be Supplied by User

5.1. QIArack or equivalent holder (see “6.1 Setup of HiSpeed Tips” below)

5.2. Isopropanol

5.3. 70% ethanol (Molecular Biology Grade)

5.4. 5 mL serological pipettes

5.5. 10 mL serological pipettes

5.6. 25 mL serological pipettes

5.7. Pipette aid

5.8. 1.5 mL tubes

5.9. 1.5 mL tube rack

5.10. 50 mL conical tubes

5.11. 50 mL tube rack

5.12. 10 µL mechanical pipette and tips

5.13. 20 µL mechanical pipette and tips

5.14. 200 µL mechanical pipette and tips

5.15. 1000 µL mechanical pipette and tips

5.16. LB broth (sterile)

5.17. 100 mg/mL ampicillin solution

5.18. 50 mg/mL kanamycin solution

5.19. Shaker incubator

5.20. Gas burner

5.21. Sharpie marker

6. Procedure

6.1. Setup of HiSpeed Tips

HiSpeed Tips can be held upright in a suitable collection vessel such as a tube or fl TV ask, using the tip holders provided with the kit (Figure 2A). Alternatively, HiSpeed

Tips can be placed in the QIArack (cat. no. 19015), which has a removable collection tray for collecting liquid flow-through (Figure 2B).

Figure 1. HiSpeed Midi and Maxi Tips.

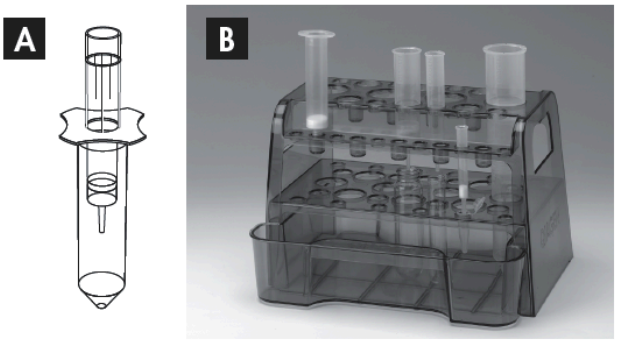

Figure 2. Setup of HiSpeed Tips A with tip holder or B with the QIArack.

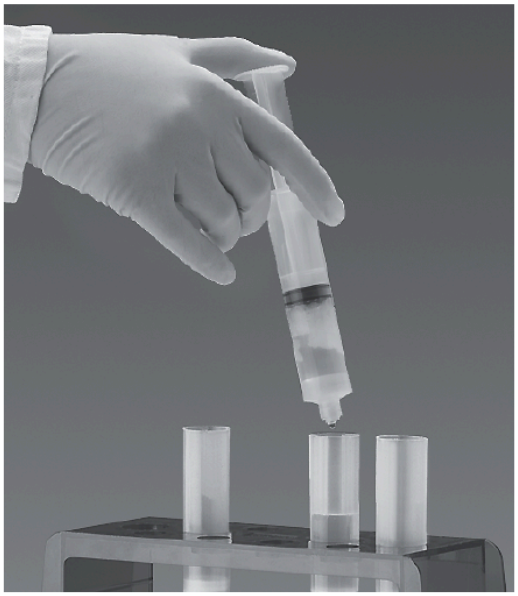

Figure 3. The syringe format QIAfilter in use.

Figure 4. The QIAprecipitator in use.

6.2. Day 1

6.2.1. Pick a single colony from a selective LB-agar plate and inoculate a starter culture of 50 mL LB medium containing the appropriate selective antibiotic in a 250 mL Erlenmeyer flask:

6.2.1.1. 50 µL of 100 mg/mL ampicillin stock solution, mar the flask with tape and a marker as LB-ampicillin and the name of the plasmid.

6.2.1.2. 50 µL of 50 mg/mL kanamycin stock solution, mar the flask with tape and a marker as LB-kanamycin and the name of the plasmid.

6.2.2. 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.

6.3. Day 2

6.3.1. Harvest the bacterial cells by centrifugation at 6000 × *g* for 15 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. If you wish to stop the protocol and continue later, freeze the cell pellet at –20°C.

6.3.2. Resuspend the bacterial pellet in 6 mL Buffer P1.

NOTE: 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.

6.3.3. Add 6 mL Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at RT 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 localised colourless 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 Midi. Place the QIAfilter Cartridge into a convenient tube or a QIArack.

6.3.4. Add 6 mL chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4–6 times. Proceed directly to step 4.2.5. 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 neutralise the solution. It is important to transfer the lysate into the QIAfilter Cartridge immediately to prevent later disruption of the precipitate layer.

If LyseBlue reagent has been used, the suspension should be mixed until all traces of blue have gone and the suspension is colourless. A homogeneous colourless suspension indicates that the SDS has been effectively precipitated.

6.3.5. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at RT for 10 min. Do not insert the plunger!

Important: This 10 min incubation at RT 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 pipet tip around the walls of the cartridge to dislodge it.

6.3.6. Equilibrate a HiSpeed Midi by applying 4 mL Buffer QBT and allow the column to empty by gravity flow.

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 the buffer will stop when the meniscus reaches the upper frit in the column.

6.3.7. Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the QIAfilter Midi 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 15 mL of the lysate is generally recovered after filtration.

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

6.3.9. Wash the HiSpeed Midi Tip with 20 mL Buffer QC. Allow Buffer QC to move through the HiSpeed Tip by gravity flow.

6.3.10. Elute DNA with 5 mL Buffer QF. Collect the eluate in a tube with a minimum capacity of 10 mL.

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

6.3.11. Precipitate DNA by adding 3.5 mL of RT isopropanol to the eluted DNA. Mix and incubate at RT for 5 min.

All solutions should be at RT to minimise salt precipitation.

6.3.12. During the incubation remove the plunger from a 20 mL syringe and attach the QIAprecipitator Midi 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!

6.3.13. Place the QIAprecipitator over a waste bottle, transfer the eluate/isopropanol mixture into the 20 mL syringe, and insert the plunger. Filter the eluate/isopropanol mixture through the QIAprecipitator using constant pressure.

Important: Complete the QIAprecipitator procedure (steps 4.2.14. - 4.2.19.) 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.

6.3.14. Remove the QIAprecipitator from the 20 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.

6.3.15. Remove the QIAprecipitator from the 20 mL syringe and pull out the plunger. Attach the QIAprecipitator to the 20 mL syringe again, insert the plunger, and dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step.

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

6.3.17. 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. With a marker, mark the content of the collection tube.

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: Buffer TE 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.

6.3.18. Remove the QIAprecipitator from the 5 mL syringe, pull out the plunger, and reattach the QIAprecipitator to the 5 mL syringe.

6.3.19. Transfer the eluate from step 4.2.17. 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.

6.4. Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm.