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APPLICATION NOTE No. 478

High Throughput Purification of Plasmid DNA and *in vitro* Transcribed mRNA Using the High-Speed Centrifuge CR22N

Authors

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Abstract

The generation of large amounts of plasmid DNA is important for various applications including the downstream processing of *in vitro* transcribed mRNA. This workflow is central to instant vaccine research, where plasmids are stored in large DNA library and the inserted target gene on the plasmid can be used as a template for, for example, *in vitro* transcription, to generate mRNA. To obtain consistent results, an optimized process is important. In this Application Note, a high throughput method is shown to harvest bacteria with the unique 4 x 1.5 L capacity of Rotor R9A2 in the Centrifuge CR22N. Also using Centrifuge CR22N, plasmid DNA is purified using the 10 x 50 mL and 10 x 15 mL capacity of Rotor R15A and the 30 x 2 mL capacity of Rotor R22A4. This results in the generation of a high quality and consistent DNA library that can be used for the production of mRNA by *in vitro* transcription, which can then be purified on the same device.

Introduction

Optimal plasmid production requires efficient processes to produce high-yield and high-purity products. This process has recently gained much attention because of its central role in the biotechnology and pharmaceutical industries. Here, high throughput and high-quality plasmid production are important for the development of mRNA therapeutics and vaccines for the pharmaceutical industry. In this workflow, it is important to grow large numbers of bacteria containing plasmid to generate a unique, consistent, and pure library of purified DNA for subsequent steps, to the final product. Previously, we have shown that the Centrifuge CR22N with its unique 1.5 L bottle can save up to 32% handling time when processing large bacterial batches[1–4].

In this Application Note, we show how the Centrifuge CR22N can be further used to purify plasmid DNA and PCR products, which serves as a first step in the mRNA production workflow of *in vitro* transcription. To this end, we harvested a large bacterial culture containing the pluripotency-associated transcript target gene, LIN28A, on a plasmid DNA, grown in the Shaker Innova® S44i. Rotor R9A2 was used to pellet 3 L (2 x 1.5 L) of bacterial culture. The DNA purification of the entire bacterial pellet obtained from one 1.5 L bottle (1500PP bottle) was performed using a combination of the Rotor R15A, which can hold up to 10 x 50 mL and 10 x 15 mL, and the Rotor R22A4 which can hold up to 30 x 2 mL. This combination allows a reduced number of spins due to their high capacity. Finally, we show that high-quality transcription processes can produce the mRNA by *in vitro* transcription (IVT)[5,6].

Focus: High Throughput Production of Plasmid DNA and Purification Using High-speed Centrifuge CR22N

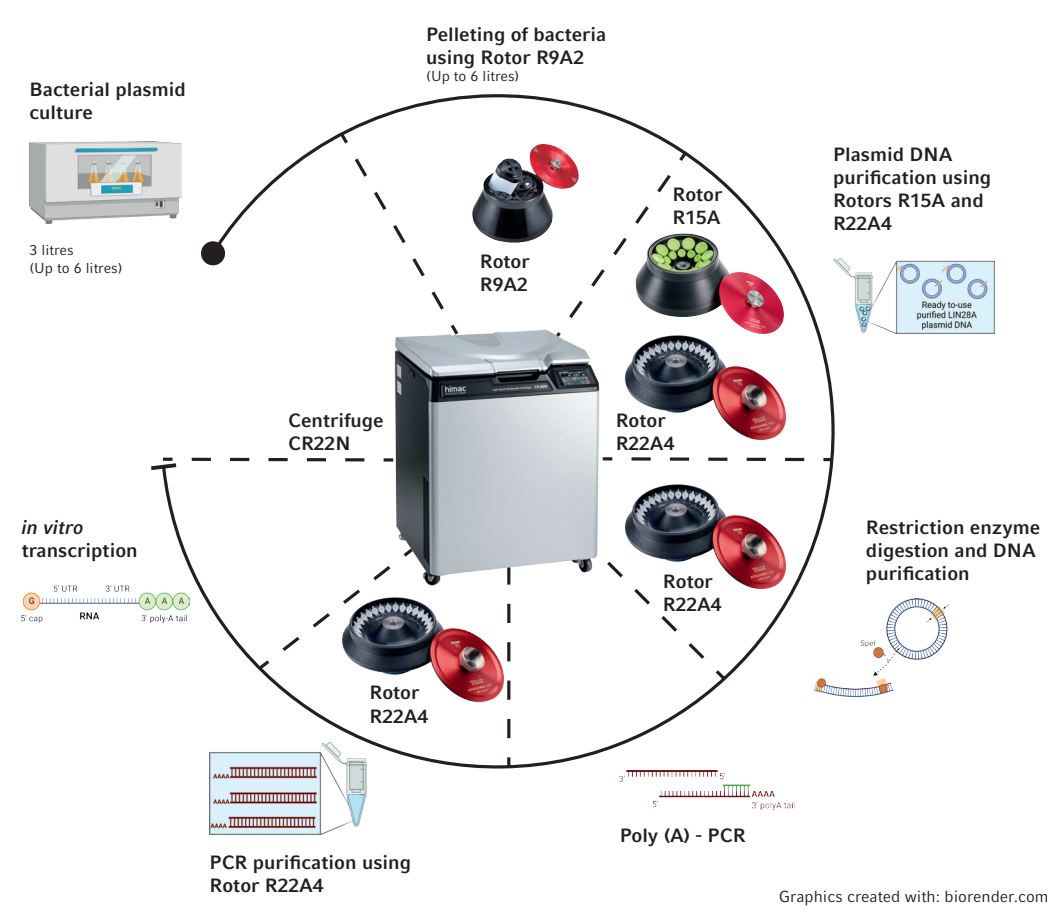

Fig. 1: Workflow from the bacterial culture to *in vitro* transcription using the Centrifuge CR22N and a combination of Rotor R9A2, Rotor R15A, and Rotor R22A4.

Materials and Methods

The entire procedure followed the protocol and recommendations of the research papers by Rossi et al [7,8].

Plasmid

A plasmid encoding human LIN28A was purchased from the Addgene plasmid repository (Addgene plasmid #*26819; http://n2t.net/addgene:26819; RRID: Addgene_26819). The plasmid construct was designed by Derrick Rossi in the DH5alpha growth strain. It contained the LIN28A sequence (813 bp), the 5′ sequencing primer "CMV Forward" and the 3′ sequencing primer "TK polyA reverse" (5′UTR-LIN28A-3′UTR). The total plasmid backbone size with the insert was 6213 bp.

* # is the symbol for „order number"

Culturing of bacteria

Amplification in dish and cryopreservation in glycerol

Bacteria were inoculated on agar (Invitrogen™, #11518916) containing ampicillin at 100 µg/mL (Sigma-Aldrich, #A5354) in a 10 mm dish and grown for 24 h at 37 °C in an incubator. A single colony was picked and transferred to a 15 mL conical tube filled with 10 mL of LB Broth (Sigma Aldrich, #L2897) containing ampicillin at 100 µg/mL and amplified overnight at 37 °C and 200 rpm in an Innova® S44i shaker (Eppendorf, #S44I311001). Bacteria were spun at 1,400 × *g* for 5 min and the pellet was resuspended in 10 mL of LB Broth and glycerol 100% (v/v). Aliquots of 500 µL were frozen in cryovials at -80 °C for further experiments.

Pre-Culture in a 250 mL flask
LB Broth (50 mL) containing ampicillin at 100 µg/mL was poured into an Ultra Yield 250 mL flask (CliniScience, #931144) with 50 µL of defrosted bacteria in glycerol and 250 µL of 1% Antifoam 204 (Sigma-Aldrich, #A8311). The flask was tightly closed with AirOtop Enhanced Seal (CliniSciences, #899423). Bacteria were then cultured for 24 h at 37 °C with 300 rpm agitation in an Innova S44i shaker.

Culture in 2.5 L flasks and pelleting of bacteria
Each Ultra Yield 2.5 L flask (CliniScience, #931136-B), containing LB Broth (500 mL) with ampicillin at 100 µg/mL was supplemented with 2.5 mL of 1% Antifoam 204 (Sigma-Aldrich, #A8311) and subsequently inoculated with 2.5 mL of pre-cultured bacteria. Flasks were tightly closed with AirOtop Enhanced Seal (CliniSciences, # 899425). Bacteria were then cultured for 24 h at 37 °C with 300 rpm agitation in an Innova S44i shaker. To pellet the bacteria and proceed to the purification step, media were transferred into two 1.5 L bottles under a hood. The bottles were then closed using the bottle vise and cap opener and centrifuged at 3,340 × *g* for 30 min at 4 °C using the Rotor R9A2 in the Centrifuge CR22N. Each centrifugation bottle held the media from three Ultra Yield 2.5 L flasks. Following centrifugation, the media were discarded, and the bacterial pellet was resuspended for DNA purification. Of note, in this Application Note, only one bottle was used to generate data in the entire process. The other pellet was frozen at -80 °C for further analysis.

Plasmid purification
To measure the bacterial pellet weight and use the correct amount of reagent from the purification kit, each bottle was weighed first empty and again after centrifugation and removal of the media. To purify the plasmid DNA, the CompactPrep Plasmid kit (Qiagen, #12863) was used according to the manufacturer's instructions with slight modifications. Briefly, the pellet was resuspended with 25 mL of buffer P1 and distributed to 5 x 50TC (Eppendorf, #5721411170) tubes.
Buffer P2 (5 mL) was added and incubated for 3 min before adding Buffer P3 (5 mL). Tubes were centrifuged directly at 20,000 × *g* for 30 min at 4 °C using Rotor R15A in the Centrifuge CR22N. Each supernatant was transferred to three CompactPrep extenders rather than into one (as mentioned in the manufacturer's protocol) because the DNA concentration was saturating the filter at one-third of the volume.
Each CompactPrep extender was attached to a column linked to a vacuum source to draw the solution through.
Buffer PE (700 µL) was added to the column membrane to wash it, and the column was centrifuged twice at 20,000 × *g* for 1 min at 4 °C using Rotor R22A4 in the Centrifuge CR22N. DNA was eluted with 200 µL of water using a centrifugation step at 20,000 × *g* for 1 min at 4 °C using Rotor R22A4 in the Centrifuge CR22N. The nucleic acid concentration was measured using the Nanodrop™ (Thermo Fisher, #ND-2000) and samples were stored at -20 °C for further analysis.

Plasmid linearization and purification
Linearization of the plasmid eliminates circular templates that could potentially generate run-on transcripts during the IVT reaction. Plasmid was linearized using the restriction enzyme *SpeI* (Promega, #R6591). Briefly, a single 50 µL restriction digest, containing the plasmid (500 ng/µL), 5 µL of buffer (100x), 0.5 µL of BSA (10x), *SpeI* (0.1 U/µL), and water, was incubated in a ThermoMixer® C (Eppendorf, #5382000015) for 2 h at 37 °C. The enzyme was then inactivated for 20 min at 80 °C in the ThermoMixer® C.
The linearized plasmid was purified using the QIAquick PCR Purification Kit (Qiagen, #28106) following the manufacturer's instructions. Briefly, 5 volumes of Buffer PB were mixed with 1 volume of the sample. The mix was transferred to a QIAquick spin column and centrifuged at 17,900 × *g* for 1 min at 4 °C using Rotor R22A4 in the Centrifuge CR22N. DNA was washed with 0.75 mL of Buffer PE and centrifuged twice at 17,900 × *g* for 1 min at 4 °C using Rotor R22A4. DNA was finally eluted with 50 µL of water at 17,900 × *g* for 1 min at 4 °C in Rotor R22A4. The nucleic acid concentration was measured using the Nanodrop™ (Thermo Fisher, #ND-2000) and stored at -20 °C for further analysis.

Addition of the poly-(A) tail by Polymerase Chain Reaction (PCR)
The primers used were synthesized by and purchased from Integrated DNA Technologies. Primer Xu-T120 was synthesized as Ultramer oligos at a 4-nmol scale.

Xu-F1: 5'-TTGGACCCTCGTACAGAAGCTAATACG-3'

Xu-T120 : 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTCTTCCTACTCAGGCTTTATTCAAAGAC-
CA-3'

The PCR master mix contained 100 µL of KAPA HiFi Hot-Start ReadyMix (Roche, #KK2602), 6 µL of each primer (10 µM), and 8 µL of digested plasmid (400 pg/µL).

The master mix was aliquoted into 8 x 25 µL PCR strips and run on a MasterCycler® X50 Aluminum (Eppendorf, #6313000018) with the following conditions: 1 cycle (Denaturation: 95 °C, 3 min) – 30 cycles (Denaturation: 98 °C, 20 s / Annealing: 60 °C, 15 s / Extension: 72 °C, 60 s) – 1 cycle (Denaturation: 72 °C, 3 min). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, #28106) as described above. Tailed PCR products were measured using the Nanodrop™ (Thermo Fisher, #ND-2000) and stored at -20 °C for further analysis.

In vitro Transcription

Before starting the synthesis of RNA, the working area and pipettes were cleaned with RNaseZap™ RNase Decontamination Solution (Invitrogen™, #10708345). RNA was synthesized with the MEGAscript™ T7 Transcription Kit (Invitrogen™, #10065754), with 40 ng/µL (final concentration) of purified tailed PCR product to template each 40 µL reaction. A custom ribonucleoside blend was added comprising 4 µL of 3´-O-Me-m7G(5')ppp(5')G RNA Cap Structure (New England Biolabs, #S1411S), 4 µL of adenosine triphosphate, and 0.8 µL of guanosine triphosphate (from the MEGAscript T7 kit), 3 µL of each 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies, #N-1014 and #N-1019). The custom ribonucleoside blend was vortexed and spun briefly before adding the tailed PCR product, 4 µL of each the T7 Buffer and T7 enzyme (from the MEGAscript T7 kit). Water was added to reach 40 µL final volume. The solution was incubated for 4 h at 37 °C in a MasterCycler® X50 Aluminum (Eppendorf, #6313000018). After incubation, 2 µL of Turbo DNase (from the MEGAscript T7 kit) was added to the sample and incubated for 15 min at 37 °C. RNA was purified using the MEGAclear™ Transcription Clean-Up Kit (Invitrogen™, #AM1908) following the manufacturer's instructions. Briefly, samples were mixed with 60 µL of Elution buffer, 350 µL of Binding solution, and 250 µL of 100 % ethanol, transferred to a cartridge, and centrifuged at 10,000 × *g* for 1 min at 4 °C using the Centrifuge 5702R (Eppendorf, #5703000010). Cartridges were washed in 2 steps consisting of 500 µL of Wash solution and centrifugation at 10,000 × *g* for 1 min at 4 °C. RNA was collected in 2 steps consisting of 50 µL of pre-heated (95 °C) Elution solution and centrifugation at 10,000 × *g* for 1 min at 4 °C. Purified RNA was treated with 11 µL of 10 × Antarctic phosphatase buffer and 2 µL of Antarctic phosphatase (New England Biolabs, #M0289S) for 1 h at 37 °C using a ThermoMixer® C (Eppendorf, #5382000015). Phosphatase-treated RNA was then purified again using the MEGAclear™ Transcription Clean-Up Kit (Invitrogen™, #AM1908) as described above. RNA was measured with the Nanodrop™ (Thermo Fisher, #ND-2000) and stored at -80 °C for further analysis.

Electrophoresis

The analysis of linearized plasmid, PCR products, and RNA was performed using the automated platform TapeStation 4150 system (Agilent, #G2992AA) according to the manufacturer's instructions. Digested plasmid and PCR products were run through the D5000 ScreenTape System (Agilent, #5067-5588, #5067-5589). The RNA ScreenTape System (Agilent, #5067-5576, #5067-5577, #5067-5578) was used to visualize the RNA.

Result and Discussion

Bacterial culture in a volume of up to 6 liters

To increase the culture volume and quantity of bacteria in order to generate a DNA library, we used Ultra Yield 2.5 L flasks and Rotor R9A2 in the Centrifuge CR22N. In this Application Note, six Ultra Yield 2.5 L flasks were used to collect 3 liters of media to fill two bottles. Of note, users can increase the bacterial culture to up to twelve Ultra Yield 2.5 L flasks as the rotor can accommodate up to four 1.5 L bottles, which represents 6 liters of media in one run. The 1.5 L bottles have a wide mouth which makes it easy to transfer the media from Ultra Yield 2.5 L flask to the bottle. Bottles were closed using the included tools such as bottle vise and cap opener. The bottles were loaded with the bottle setter. After the centrifugation, the pellet was easy to resuspend and collect due to the unique triangular shape and the wide mouth of the bottle (Figure 1). In this Application Note, two bottles were used to harvest bacteria. Nevertheless, only one bottle was used to generate data for DNA purification, PCR, and IVT.

Plasmid DNA purification

A pellet from one bottle was used to generate a library of DNA, enough to run multiple PCRs and IVTs. The pellet was resuspended, mixed with a buffer from the CompactPrep Plasmid kit from Qiagen, and distributed to 5 x 50TC tubes. The final volume of 15 mL was centrifuged at 20,000 × *g* for 30 min at 4 °C using the Rotor R15A. Each supernatant was then distributed to 3 extenders attached to the CompactPrep column and to the vacuum. Each column was then centrifuged at 20,000 × *g* for 1 min at 4 °C using Rotor R22A4. The average DNA concentration at the end of the purification in columns was 1.5 µg/µL. Taking into consideration the total elution volume and the number of columns, an estimated amount of 7.23 mg of DNA can be purified from a single bottle.

Moreover, the unique designs of these two fixed-angle rotors (Rotor R15A: 10 x 15TC and 10 x 50TC conical tubes - Rotor R22S4: 30 x 2 mL) would allow the processing (the first steps) of four bottles in a single run using Rotor R15A, with the final steps in two runs using Rotor R22S4.

Fig. 2: Representative workflow from bacterial culture to bacterial pellet. Media were transferred to the 1.5L bottle and spun at 3,340 × *g* for 30 min at 4 °C using the Rotor R9A2 in the Centrifuge CR22N. Media was discarded, and the pellet was resuspended and collected for DNA purification

The bacterial pellet weights for both bottles were 8.92 g and 8.55 g. Considering the capacity of Rotor R9A2 and its 4 x 1.5 L bottles, users can harvest around 35 g of total bacteria to proceed to DNA purification and later IVT.

This drastically reduces operation time, and it also reduces any differences that might appear when samples are in line for the next centrifugation run when there is not enough space in the rotor. From the entire 6 L bacterial culture, users would be able to generate and purify around 29 mg of DNA (Figure 2).

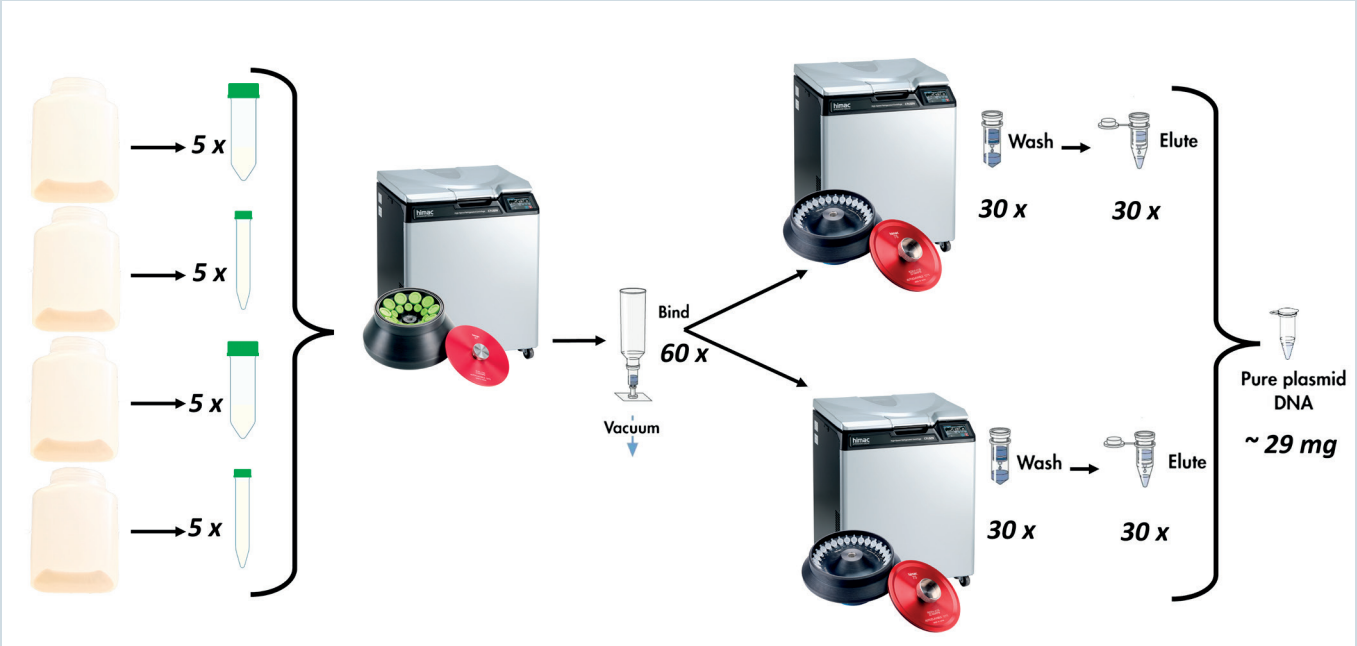

Fig. 3: Representative workflow of the maximum capacity of all rotors used in the Centrifuge CR22N to isolate bacterial plasmid DNA. Using the Rotor R9A2, four pellets can be obtained from 6 L of bacterial culture. Each pellet is resuspended and distributed into 5 different conical tubes (either 15 or 50 mL). The 20 tubes can be spun all at once using Rotor R15A. Each supernatant from the tubes is divided into 3 extenders and columns. The CompactPrep Plasmid kit (Qiagen, #12863) was used as it is designed for large volumes thanks to its extenders. DNA is eluted from each column using Rotor R22S4, in two spins, generating around 29 mg of DNA. Nevertheless, in this Application Note, two bottles were spun and only one was used to purify the DNA.

Plasmid linearization and purification

Linearization of the plasmid was performed using the *SpeI* restriction enzyme. This enzyme was used as it cut the plasmid in two places creating a short (~684 bp) and a long fragment containing the inserted gene (~5,529 bp). One cut was situated a few bp downstream of the inserted gene. After purification using the R22A4 fixed-angle rotor, the digestion was verified using the Agilent D5000 ScreenTape in the Tapestation. The band at the expected size confirmed the proper digestion by the enzyme (Figure 4-A). The mean DNA concentration was 70 ng/µL.

Amplification of the target gene by PCR

To generate the template for the IVT, the addition of a poly-(A) tail to the template DNA (target gene) by tail-PCR was performed using primers Xu-F1 and Xu-T120 in the MasterCycler® X50 Aluminum. PCR products were purified using Rotor R22A4. The expected length of PCR products, based on data generated previously by Mandal et al.[7], was confirmed using Agilent D5000 ScreenTape in the Tapestation (Figure 4-B). The mean concentration of the PCR products was 686 ng/µL.

IVT and purification of the mRNA

The final steps, which consist of the production of the mRNA, were carried out using the MasterCycler® X50 Aluminum. After DNase and phosphatase treatment, the mRNA was purified and then analyzed using the Agilent RNA ScreenTape. A single band below 1,000 bp was observed which confirmed the IVT process and the single amplification of the targeted gene. The mean concentration of the mRNA was 1,023 µg/µL. The RNA integrity number (RIN) assigns a value of 1 to 10, with 1 being strongly degraded and 10 being the least degraded (intact). The RIN score here was shown to be 10, meaning pure and intact mRNA was produced.

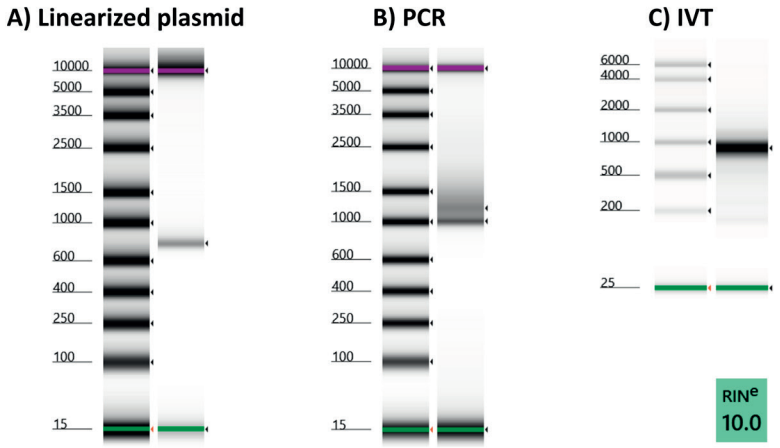

Fig. 4: A) Digested plasmid, B) PCR amplifying the target gene, C) IVT producing single and intact mRNA.

Conclusion

Here, we have shown that the Centrifuge CR22N can be used to consistently produce high-quality DNA from large bacterial cultures. Using different fixed-angle rotors, Centrifuge CR22N allows harvesting from 6 L of bacterial culture and obtaining 35 g of bacteria in a single centrifuge run with Rotor R9A2 and the 4 x 1.5 L triangular bottles. This rotor reduces the number of vessels to be handled, therefore greatly improving process efficiency without impacting pelleting efficacy. The bottles are designed for specific harvesting applications with many advantages described and explained in the Eppendorf white paper N°64[4]. After resuspension, the pellet from four bottles can be redistributed into 50TC and 15TC mL tubes to start the purification steps using the Rotor R15A. This rotor allows the use of both 10 x 15TC and 10 x 50TC conical tubes, holding up to 650 mL in a single run. The first steps of the DNA purification of the four pellets can therefore be achieved using only one run. To finish the purification in 1.5/2 mL tubes using a column, the Rotor R22A4 was used as it can hold up to 30 samples. The subsequent PCR product was also purified using the Rotor R22A4.

This combination of rotors, all compatible with the Centrifuge CR22N, reduces drastically the time of sample handling and processing by one-third[4]. It simultaneously enables fast and efficient DNA purification from a single bacterial culture on a single, versatile device, under consistent conditions, thus reducing variability and inconsistencies between DNA libraries and allowing the production of high quantity and quality mRNA.

Literature

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Ordering information

Description	Order No.
Centrifuge CR22N	**Inquire***
Rotor R15A	**5721 221 007**
Rotor R22A4	**5721 221 003**
Rotor R9A2	**5721 221 014**
1500PP bottle	**5721 411 030**
Innova® S44i	**S44I 300 001**
Mastercycler® X50	**6313 000 018**
50TC tubes	**5721 411 170**
15TC tubes	**5721 411 049**

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