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APPLICATION NOTE I No. 451

Production of Recombinant Human Phosphoserine Phosphatase Using the Innova® S44i Shaker and the Centrifuge Harvesting Bundle 4 x 1.5 L

Natascha Weiß[1], Vincent Dufey[2], Silvia Tejerina[2], Aurélie Tacheny[2]
[1]Eppendorf SE, Hamburg, Germany
[2]Eppendorf EAT, Namur, Belgium

Abstract

In the production of recombinant proteins, the goal is to generate a high yield of functional protein. An important basis for a successful experiment is the cultivation of the cells as well as centrifugation as the basic technique for isolation and purification of the protein. In this Application Note, the production of a recombinant human phosphoserine phosphatase (hPSP), which is used as a proof of concept system, is described. Starting with the growth of bacteria culture in the Innova® S44i Shaker, these were harvested in 1.5 L bottles in the 6 L rotor of the high-speed Centrifuge (CR30NX or CR22N). This Harvesting Bundle can be complemented by a "High-Speed Pelleting Kit for Conicals" (Rotor with corresponding 50 mL conical tubes), which was employed for the separation after the lysis step. It was shown that this product combination offers an efficient and at the same time flexible solution as the basis of the workflow for large-scale production of recombinant proteins.

Introduction

The production of recombinant proteins is a fundamental biotechnology technique which is applied in research, medicine and industry. The proteins are produced by genetically modified organisms. Depending on the type of protein and its purpose, a wide variety of expression systems (e.g. bacteria, yeasts, mammalian cells, insect cells) are used.
In the process, the desired DNA sequence (genetic information encoding the protein) is introduced into the host organism via molecular cloning. After selection of the clones expressing the recombinant protein, they are cultured. In the first step, the cells are propagated and in the second step gene expression is induced. Usually, a combination of different techniques is applied to isolate and purify the desired protein. Initially, proteins are separated from other cell components by cell disruption (lysis). The protein of interest is then separated from the remaining proteins of the cell using its specific properties and subsequently enriched.

Especially when an unknown protein needs to be characterized, i.e. to study its structure and function, larger quantities are required. In particular, two process steps are affected by this: Cultivation and harvesting of the cells must be performed on a large scale (several liters). This has an impact on the equipment used for these purposes, as well as the potentially higher workload (compared to smaller volumes).

The bacterium *Escherichia coli* is a popular choice as an expression system since it can be easily genetically modified, and cultivation is fast. To achieve both sufficient cell growth and excellent protein expression, a robust shaker capable of shaking cultures at high rpm on a liter scale is required. Centrifugation is another important technique in the production of recombinant proteins, as it is often involved in several process steps. It is used to harvest the cells, to clarify the cell lysate and (if necessary) to concentrate the protein solution (Figure 1). The following demands have to be considered: Harvesting several liters of culture is time-consuming as a result of numerous manual steps required for each individual vessel (1). Variable culture volumes on the one hand and additional centrifugation steps with smaller volumes together with different parameters for isolation, purification and concentration of the protein on the other hand require additional equipment in terms of vessels, rotors and possibly also devices.

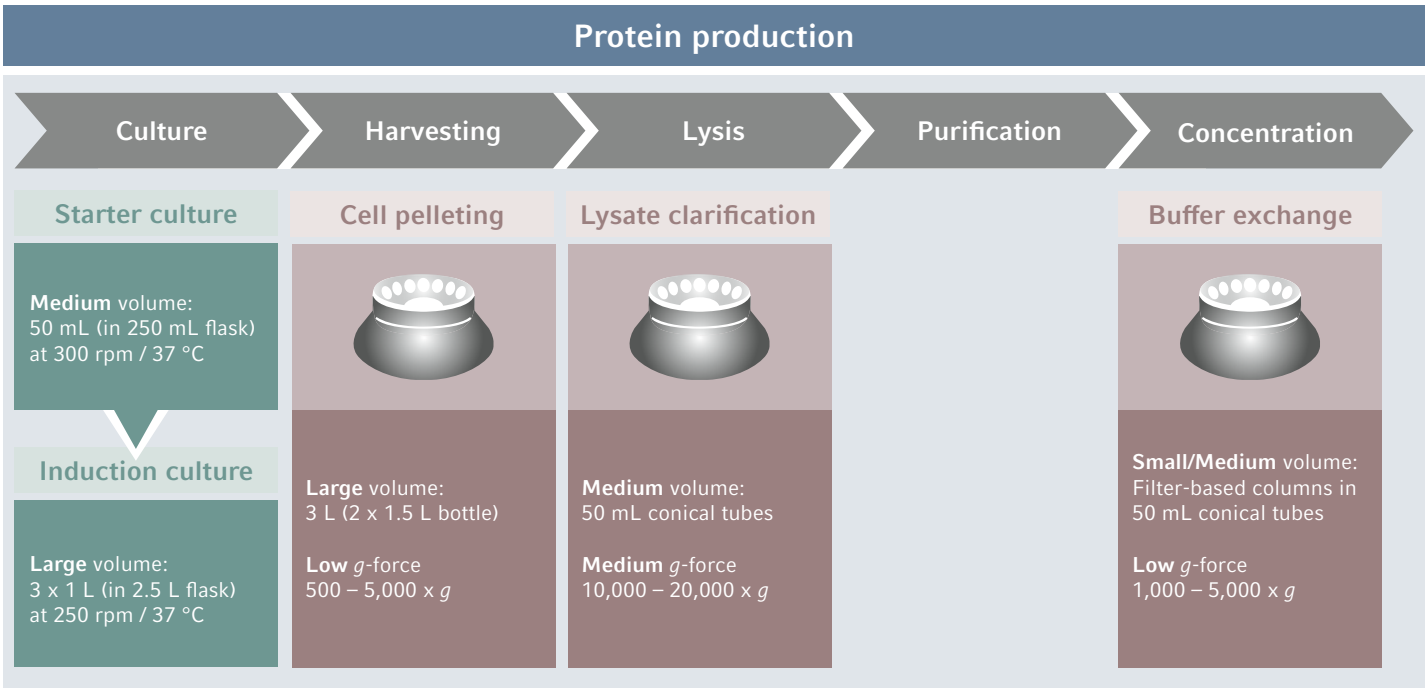

Figure 1: Example of a protein production process based on a culture volume of 3 liters including the required culture and centrifugation steps.

In this Application Note, the production of a recombinant protein is presented using a workflow for culturing and harvesting bacteria and purifying proteins. The aim is to demonstrate that a high yield of functional recombinant protein can be realized by using the Innova S44i shaker and the high-speed Centrifuge CR30NX. For this purpose, the Centrifuge Harvesting Bundle is used and supplemented with a High-Speed Pelleting Kit. This equipment is meant to help carry out the individual process steps in a way that is both user-friendly and effective.

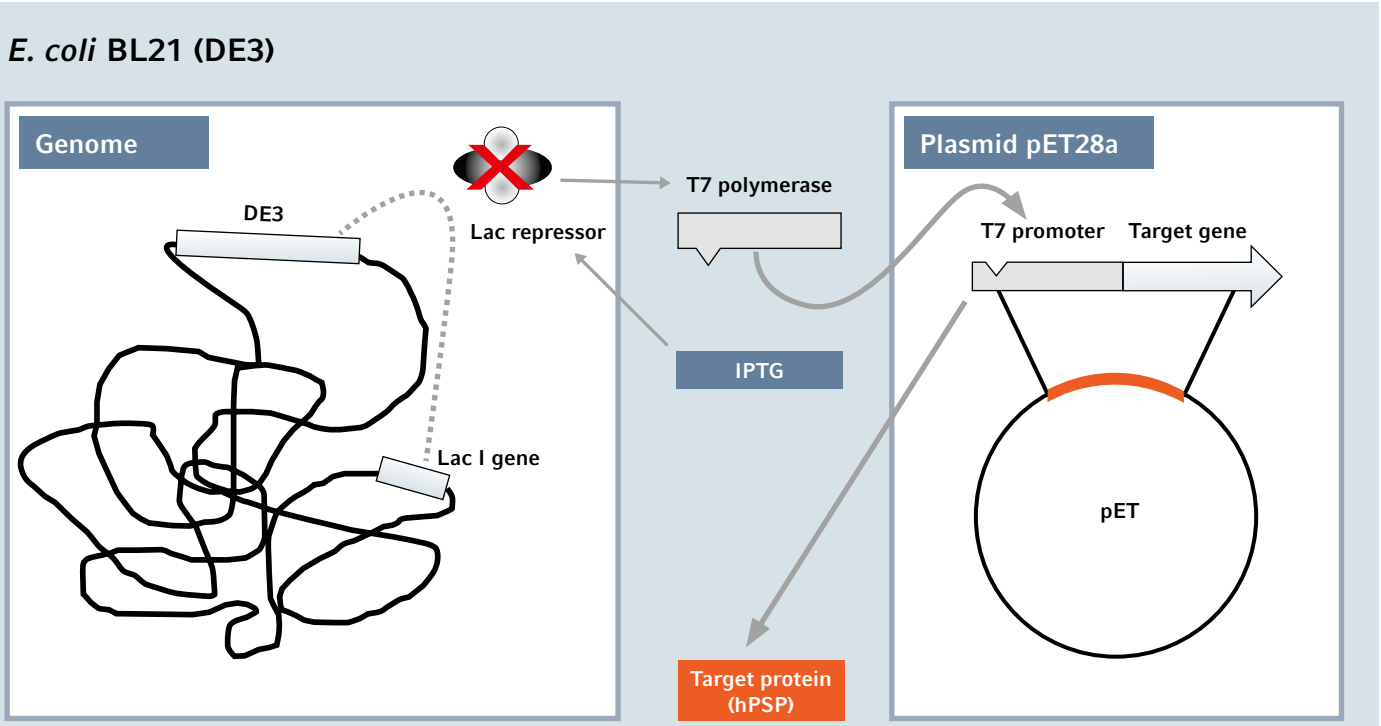

Figure 2: Recombinant protein expression system: The strain *E. coli* BL21 (DE3) containing the T7 RNA polymerase gene under the control of lacUV5 promoter was transformed with the plasmid pET28a. This carries the target gene for human phosphoserine phosphatase (hPSP) which is under control of the T7 polymerase. IPTG (Isopropyl β-d-1-thiogalactopyranoside) induces the lac promoter, leading to the expression of T7 polymerase and indirectly to the expression of the hPSP gene.

Materials

Expression system
E. coli BL21 (DE3) transformed with pET28a plasmid containing the gene coding for the His-tagged protein human phosphoserine phosphatase (hPSP) (Figure 2).

Culture system
For cultivation of bacteria the Innova S44i (refrigerated, orbit diameter 2.5 cm) has been used. This incubator shaker has a large capacity and enables efficient shaking of heavy loads at high speed. It is therefore well suited for the cultivation of cultures in liter scale.

Centrifugation system
The high-speed Centrifuge CR30NX is available in combination with a unique 6 L rotor (Rotor R9A2) and matching 1.5 L triangular bottles as a so-called Harvesting Bundle designed for harvesting large culture volumes (Figure 3). This system is supplemented by the High-Speed Pelleting Kit which consists of the Rotor R19A2 including matching 50 mL conical tubes.

Alternatively, the Centrifuge CR22N can be used. For this, a Harvesting Bundle and complementary kits are also available.

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Detailed information on reagents, consumables and other equipment is found in the Appendix 1 for this Application Note.

Figure 3: A) Centrifuge CR30NX, B) Centrifuge CR22N, C) Rotor R9A2 (4 x 1.5 L rotor) with triangular bottle 1,500 mL, D) Rotor 19A2 with 50 mL conical tubes

Methods

Bacteria proliferation and induction

Preparation of starter culture: To 50 mL of medium in a 250 mL flask 50 µL of *E. coli* (with pET28a) in glycerol were added. The flasks were incubated in the Innova S44i at 300 rpm and 37 °C overnight until an OD600 of 15-25. Preparation of 3 flasks of induction culture: To 1 L medium in a 2.5 L flask 5 mL of the starter culture were added. The flasks were incubated in the S44i at 250 rpm and 37 °C until the OD600 reaches approx. 2.0 (~4-5 h). After adding 10 mL of IPTG and incubation was continued at 250 rpm at 37 °C for 3 h.

Harvesting

At the end of the induction the suspension has been recovered in two 1.5 L triangular bottles (previously weighted) and spun down in the Centrifuge CR30NX with Rotor R9A2 at 4,000 rpm for (7,100 x *g*) 30 min at 4 °C. The supernatants were discarded. (Bottles were weighted before adding the cell culture suspension and after centrifugation, to have the weight of the pellet).

Lysis

Per gram bacteria pellet 20 mL of lysis buffer has been added and resuspended with a serological pipet. After stirring for 30 min at RT or 4 °C the suspension was sonicated on ice for 10 min total ON time (15 sec ON, 45 sec OFF). Then the lysate was clarified by centrifugation at 12,500 rpm (32,900 x *g*) for 1 h at 4 °C in the Centrifuge CR30NX and Rotor R19A2 using 50 mL conical tubes. The supernatant was recovered and filtered through a 0.2 µm filter.

Purification by affinity chromatography

The HisTrap® FF crude column was washed with 3 to 5 column volumes of distilled water and equilibrated with at least 5 column volumes of binding buffer. The clarified lysate (400 mL) was applied under continuous stirring (flow rate 10 mL/min). The column was washed with binding buffer until the absorbance reached a steady baseline (approximately 15 column volumes). The elution was done by a one-step procedure: The first 10 fractions were collected with the elution buffer at 20 % Imidazole while the last 10 fractions were collected with the elution buffer at 100 % Imidazole at a flow rate of 5 mL/fraction/min.

Concentration

A concentration step was not performed because preliminary tests had shown that the protein solutions had a sufficiently high concentration.

Analysis

> Photometric total protein quantification (absorbance measurement at 280 nm).
> Protein identification by SDS-Page 4-12 % in reduced and denaturing conditions and Coomassie® blue staining.
> Enzyme activity measurement by Malachite green phosphate assay.

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Detailed information on analysis methods is found in the Appendix 1 for this Application Note.

Results and Discussion

A total of 10 fractions were obtained from the affinity column using an elution buffer at 20 % Imidazole. This part was intended to remove impurities. The subsequent elution of 10 fractions with a buffer at 100 % Imidazole was intended to retrieve the purified protein. To identify the relevant fractions, their absorbance at 280 nm was measured.

Figure 4 shows the results of the absorbance measurement, which provides information about the total protein concentration per eluted fraction. It can be clearly seen that the elution buffer based on 100 % Imidazole dissolves a large proportion of proteins from the column. The highest proportion of protein is in the second fraction with 100 % Imidazole (= fraction 12).

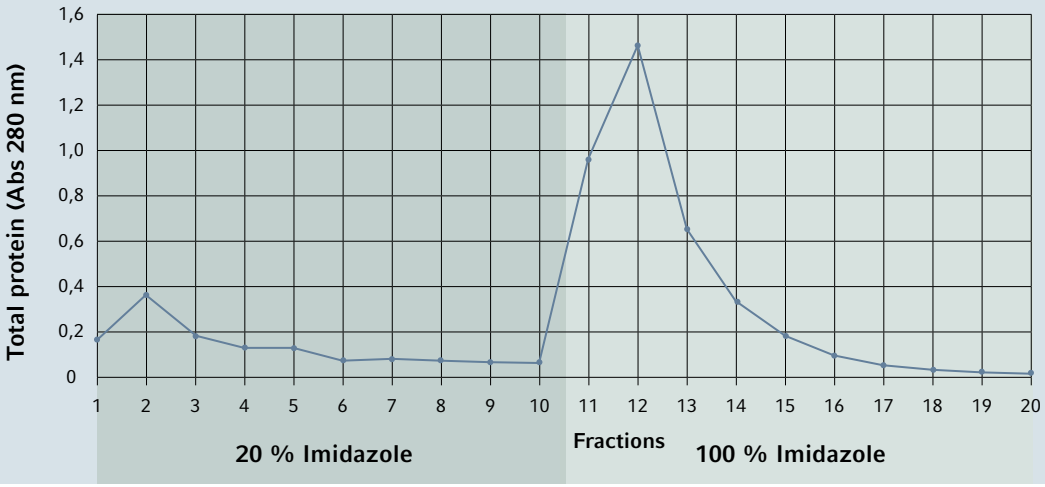

Figure 4: Absorbance at 280 nm of eluted fractions (fractions 1 – 10 at 20 % Imidazole, fractions 11 – 20 at 100 % Imidazole).

To determine whether and in which amount the target protein human phosphoserine phosphatase is present in the individual fractions and whether it is functional, the individual fractions were separated by SDS PAGE and a Malachite green phosphate assay was performed to measure the enzyme activity.

The SDS gel stained with Coomassie blue confirms that a large amount of protein was solubilized from fraction 11 (start of elution with 100 % Imidazole) (Figure 5). The position of the band showing the highest density indicates that it is the target protein hPSP, which has a size of 25 kDa. Contaminants were mainly eluted at the beginning using the buffer at 20 % Imidazole.

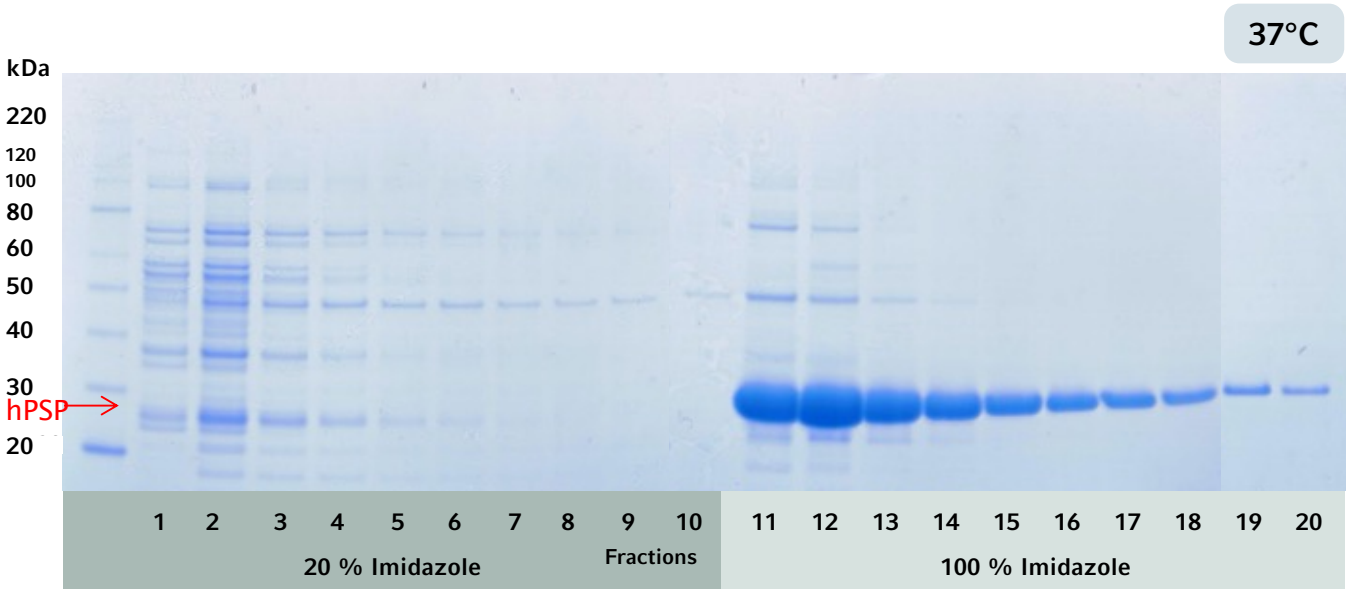

Figure 5: Coomassie blue staining of SDS gel electrophoresis showing the total protein elution profile (25 kDa).

The Malachite green phosphate assay provides a colorimetric method to determine the activity of a phosphatase via the amount of phosphate released. The result of the assay shows that a large amount of functional hPSP was leached out by the elution buffer based on 100% Imidazole, with a maximum in fraction 12 which correlates with the absorbance measurement (Figure 6).

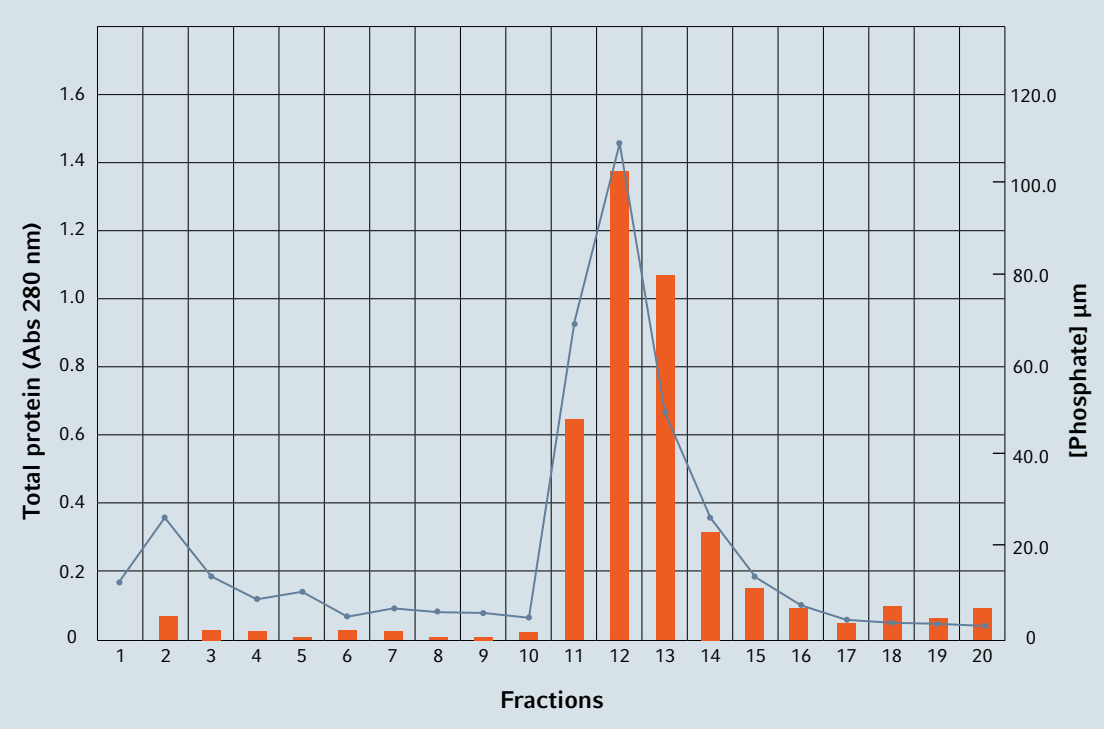

Figure 6: Plot of absorbance measurement at 280 nm (blue dots/line) in combination of enzyme activity (orange bars) per eluted fraction.

The results demonstrate that the Innova S44i shaker and Centrifuge CR30NX, together with the previously described accessories, can be successfully used for the production and purification of a recombinant protein in high yield.
With its robust design and high vessel capacity, the Innova S44i enables the culture of microorganisms at high speeds, even with heavy loads (2). These are basic requirements for optimal cell growth and high protein expression.
The high-speed Centrifuges CR30NX and CR22N offer excellent versatility by numerous rotors and tubes (including a continuous flow rotor for scaling up options). The Harvesting Bundle consisting of the 4 x 1.5 L Rotor and corresponding 1.5 L bottles represents a matching system for harvesting cells. The unique triangular format of the 1.5 L bottle makes pellet resuspension, supernatant recovery and cleaning very easy. In addition, the ability to process 1.5 liters of culture volume per bottle used, saves time during each handling step. These steps include filling, balancing, closure, rotor loading, decanting, pellet resuspension and recovery as well as washing of each bottle. In Whitepaper 64 it is shown that compared to handling 6 bottles of 1 L volume each (as used as standard for 6 x 1 L capacity rotors), this results in a time saving of 32 % of the process time (1). Another point that illustrates the high flexibility of the system is the fact that the triangular 1.5 L bottles are not limited by a necessary minimum volume (as is the case with conventional bottles).
The Harvesting Bundle can be extended by additional Centrifugation Kits (combination of rotors and tubes) depending on the application. Only one additional Centrifugation Kit (High-Speed Pelleting Kit for conical tubes) is required to cover all centrifugation steps to produce a recombinant protein from harvesting to concentration. This means that cell culture, harvest and subsequent purification can be realized with the Innova S44i Shaker and a high-speed Centrifuge.

Conclusion

In this Application Note, the successful use of the Shaker Innova S44i and the Centrifuge CR30NX including rotors and vessels to produce a recombinant protein is shown. In addition to a high yield and the proven functionality of the product, the process steps could be carried out efficiently. Contributing significantly to this is the combination of products, which complement each other perfectly with their respective accessories, as they provide the basic framework for executing essential workflow steps.
Especially worth mentioning are the user-friendly and time-saving handling of the unique 1.5 L centrifugation bottles and the flexible Bundle and Kit system of the high-speed Centrifuges which offers simple solutions for a wide range of applications.

Acknowledgement

We thank Professor Johan Wouters (Laboratoire de Chimie Biologique Structurale (CBS), Namur Medicine and Drug Innovation Center (NAMEDIC), Namur Research Institute for Life Sciences (NARILIS), University of Namur (UNamur), B-5000 Namur, Belgium) for providing the hPSP plasmid.

Literature

[1] Tacheny A. Unique 4 x 1.5 L Capacity Rotor for High-Speed Centrifuges CR22N and CR30NX, Eppendorf White Paper No. 64
[2] Hartmann I, Jarvis J. The New Eppendorf X-Drive – Maximum Performance, Flexibility and Longevity for Demanding Shaker Tasks. Eppendorf White Paper No. 47

Ordering information

Description	Order no.
Innova® S44i, refrigerated, orbit 2.5 cm (1 in), Europe, 230 V, 50/60 Hz	S44I310001*
Innova® S44i, refrigerated, orbit 2.5 cm (1 in), USA, 120 V, 50/60 Hz	S44I210005
Innova® S44i, refrigerated, orbit 2.5 cm (1 in), Japan, 100 V, 50/60 Hz	S44I010006
CR30NX Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, China/APA, 220 V	5721 351 511
CR30NX Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, EU/India, 220 V	5721 351 512
CR30NX Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, EU/India (three-phase four-wire)	5721 351 514
CR30NX Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, US, 208 V	5721 351 513
High-Speed Pelleting Kit for Centrifuge CR30NX: Rotor R19A2 (fixed-angle) 8 x 50 mL, incl. 100 x 50 mL high-speed conical tubes plus caps	5721 302 107
CR22N Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, China/APA, 220 V	5721 261 411
CR22N Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, EU/India, 220 V	5721 261 412
CR22N Harvesting Bundle (4 × 1,5 L) + Rotor R9A2 incl. 4 x 1.5 mL triangular bottles, incl. caps and tube vise, US, 208 V	5721 261 413
High-Speed Pelleting Kit for Centrifuge CR22N: Rotor R15A (fixed-angle) 10 x 50 mL, incl. 100 x 15/50 mL high-speed conical tubes plus caps	5721 221 007

*Last digit is country dependent. For UK/HKG, change 1 to 2; for Australia, change 1 to 3; for China, change 1 to 4; for Argentina, change 1 to 8; for Brazil, change 1 to 9.

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