APPLICATION NOTE | No. 437

Standardized and Water-free Cell Thawing using the Eppendorf ThermoMixer® C with the Eppendorf SmartBlock™ cryo thaw

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Abstract

Cryopreservation allows for continuous access to functional cells and is an indispensable technique in cell-based research and production. Successful cryopreservation depends on multiple factors, like selection of cryoprotectant, storage conditions, and freezing and thawing procedures. Since cells are the basis for many downstream applications, consistent quality is critical. Standardization of processes can help to maintain this. While standardization in terms of media composition and freezing is often well established, the thawing procedure is still, in most cases, neglected. The water bath is the most common technique for thawing. Variances in handling can hardly be eliminated, resulting in inhomogeneous vial-to-vial conditions between thawing processes. Moreover, the risk of introducing a contamination is high. The Eppendorf ThermoMixer C in combination with the Eppendorf SmartBlock cryo thaw has been optimized for cell thawing. We show here that it is well suited to thaw cell lines and sensitive stem cells and provides more reproducible thawing conditions compared to existing methods. The method eases handling with the pre-set program 'Thawing cells' and minimizes the risk of contamination.

Introduction

Cryopreservation is a technology for long-term storage of living cells at cryogenic temperatures (usually utilizing liquid nitrogen at -196 °C) while maintaining the cells structurally and functionally intact. At these ultra-low temperatures, the metabolic activity normally occurring at cellular level can remain suspended for an extended period of time [1]. Thus, establishing a master cell bank and working stocks, for example, can provide an inexpensive source of viable cells without significant deterioration in quality over years.

Cryopreservation includes freezing, storage, and recovery. Best practices for successful cryopreservation have been established [2, 3, 4]. Cells should be frozen at early passage numbers, in an actively growing state (log phase) and at an appropriate concentration, typically 1×10^6 to 5×10^6. Cells should be tested for absence of contaminants. The type of cryoprotectant and final concentration in the freezing medium, as well as potential additives to improve cell survival (e.g. serum), should be selected according to recommendations for the specific cell type.

The principle of successful cryopreservation is to freeze slowly and thaw rapidly. A controlled slow cooling at a rate of -1 °C/min down to -80 °C before transferring to ultra-low temperature storage is required to prevent cell death by intracellular ice formation. Specialized containers paired with reliable ULT freezers, or even programmable cell-freezing equipment, allow for standardization of the freezing procedure [5, 6, 7].

In comparison, the most common method for thawing is still submerging the cryovial in a 37 °C water bath for 2–3 minutes, prior recovery in pre-warmed media, and removal of cryoprotectant by centrifugation. A standardization is hardly possible. Immersion depth and amount of movement of cells in the water varies from time-to-time and person-to-person. Besides that, the warm, moist water bath can be a source of sample contamina-

tion. Careful handling and disinfection of each tube after removal is important to avoid the risk of cross-contamination. The person is, in most cases, integrated into the process of thawing by holding the tube, and handling more than one tube per time is hardly possible. The bead bath and simple hand warming are alternatives, but also are not optimal. Pros and cons of different thawing methods have been described in detail [8].

The Eppendorf ThermoMixer C in combination with the new Eppendorf SmartBlock cryo thaw provides an integrated, pre-set, optimized program for thawing cells. We examine and compare the standard thawing methods with the 'Thawing cells' program in terms of cell thawing performance and reproducibility of the thawing process. As cells sensitivity to cryopreservation varies, testing includes not only cell lines but also human induced pluripotent stem cells (hiPSCs), which are one of the cell lines most sensitive to cryopreservation [9].

Materials and Methods

Cell tests

Cells lines and hiPSCs tested: S16 (ATCC no. CRL-2546), HEK293 (DSMZ no. ACC 305), CHO-K1 (DSMZ no. ACC 110), episomal hiPSC line A18944, Lot 1992258 (Thermo Fisher Scientifc) and hiPSC line AISC-0011 (Coriell Institute).

Cell lines and hiPSCs were frozen using liquid nitrogen according to standard procedures given for the specific cell type using 2 mL cryovials filled to 1 mL (Eppendorf CryoStorage Vials 2.0 mL or Corning® cryogenic vial 2.0 mL (430659)). For detailed protocols for freezing and thawing and media composition please refer to the cell specific performance sheets on www.eppendorf.com/ThermoMixer.

For testing cell thawing performance, cryopreserved cells where thawed in parallel with three methods:

1. Eppendorf Thermomixer C with Eppendorf SmartBlock cryo thaw and program 'Thawing cells'
2. Water bath immersion 2-3 min.
3. Room temperature for 3 h as negative control (data not shown)

Tests were run in triplicates (three vials)

Recovery for cell lines: After thawing, cell lines where diluted with 10 mL medium, centrifugated, resuspended in 2 mL fresh medium, and counted with the CASY cell counter (Innovatis). Cells were seeded in the Eppendorf Cell Culture Plates, 96-Well and incubated at 37 °C with 5 % CO_2 in the CellXpert® CO_2 incubator. After 96 hours, the cells morphology was analysed by phase-contrast microscope and a CellTiter-Blue® Cell Viability Assay (Promega, G8081) was done according to supplier's recommendations to check the cell viability.

Recovery for hiPSCs: After thawing, episomal human iPSCs were cultivated in Eppendorf Cell Culture Plates, 6-well on Matrigel®-coated surface (Corning Matrigel hESC-Qualified Matrix, 354277) in a feeder-free adapted culture medium of Essential 8™ Flex Medium (Thermo Fisher Scientific, A2858501) and RevitaCell™ Supplement (Thermo Fisher Scientific, A2644501) according to supplier's instructions and checked for cell morphology, spontaneous differentiation, and cell growth 3 days post-thawing and during two successive passages post-thawing. In addition, immunostaining was performed to confirm the maintenance of pluripotency after 4 successive passages post-thawing for one hiPSC line (AISC-0011).

Briefly, after thawing, cells were recovered by adding 9 mL medium drop-wise. Cryoprotectant was removed by centrifugation at 200x g for 5 min at room temperature. Cell pellet was resuspended in 2 ml of medium supplemented with RevitaCell. 0.5 ml of the iPSC suspension was added drop-wise to the Matrigel well containing 1.5 ml of the supplemented medium. Cell aggregates were incubated at 37 °C and 5 % CO_2. From 24 hours post seeding, a microscopic inspection and a medium refreshment without RevitaCell was performed each 24 or 48 hours. About 3-4 days were required between two successive passages. Cells were maintained during four successive passages. Cell counting was performed with the Vi-CELL™ XR Cell Counter (Beckman Coulter). Immunostainings was performed using the Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Thermo Fisher Scientific, A24881) according to manufacturer's recommendations. Fluorescent-stained cells were observed with the EVOS™ FL Cell Imaging System (Thermo Fisher Scientific, AMF 4300).

Thawing reproducibility

For comparing reproducibility of temperature conditions of the Eppendorf Thermomixer C with the water bath and hand warming, the sample temperature in the cryovial was analysed during thawing using an external sensor (Omega 5SRTC-TT-KI-36-2M). Briefly, the vials were prepared with a micro hole in the cap, the sensor was passed through the hole to measure the temperature in the sample. The vials were filled with 1 mL freezing media (70 % media, 20 % FBS, 10 % DMSO) and the vials were quick frozen in liquid nitrogen. The temperature was documented over 15 min from the start of thawing. To determine the average thawing time, the finish was defined at the condition when a small bit of ice is still left in the vial, which occurs between -5 and 5 °C. For the water bath, three scenarios were tested, immersion by hand without moving the vial, with moving the vial, and by use of a floater. For the hands, three different hands (persons) were used. All experiments were done in triplicates.

Results and Discussion

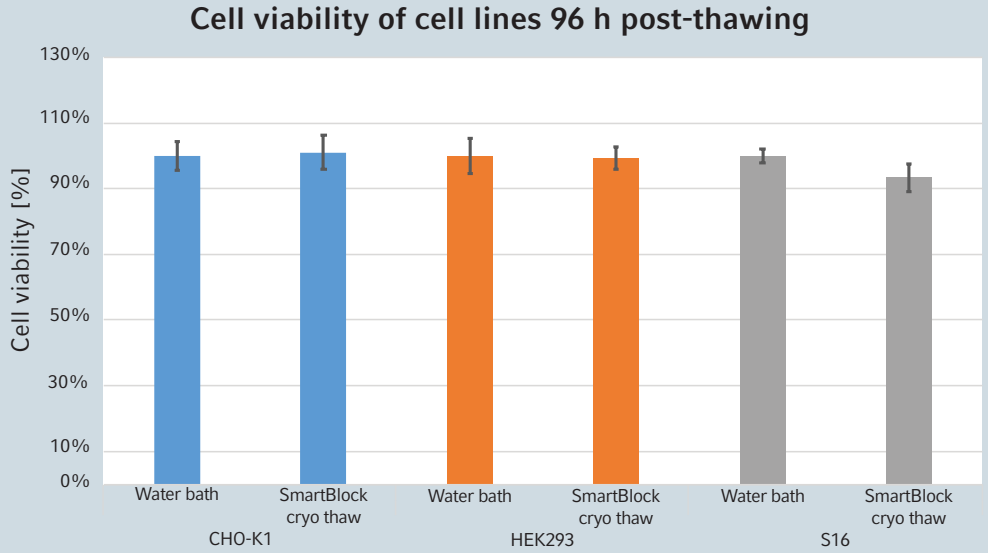

Figure 1: Percentage viability of cell lines 96 hours after thawing analyzed with the CellTiter blue assay. Water bath set as 100 % reference. Results represent a mean of three vials. Data of negative control (thawing at room temperature) resulted in lower cell viability (data not shown).

Cell tests

Sensitivity to cryopreservation varies between cell types. Thus, testing involves cell lines CHO-K1, HEK293, and S16, as well as one of the most sensitive cells to cryopreservation, human induced pluripotent stem cells.

The cell tests show clearly, that the 'Thawing cells' program of the Eppendorf Thermomixer C in combination with the Eppendorf SmartBlock cryo thaw is well suited for all tested cells. As depicted in Figure 1, for all cell lines, the same high percentage of viability could be observed after 4 days as in the common water bath procedure, which was set as 100 % reference. All negative controls resulted in a lower percentage viability, between 77 and 92 % (data not shown) compared to the water bath, demonstrating the robustness of cell lines during the thawing procedure.

The cell lines also displayed a normal morphology and similar confluence levels with the two thawing methods as depicted in Figure 2. The cells thawed at room temperature showed a slower proliferation after 96 hours (data not shown).

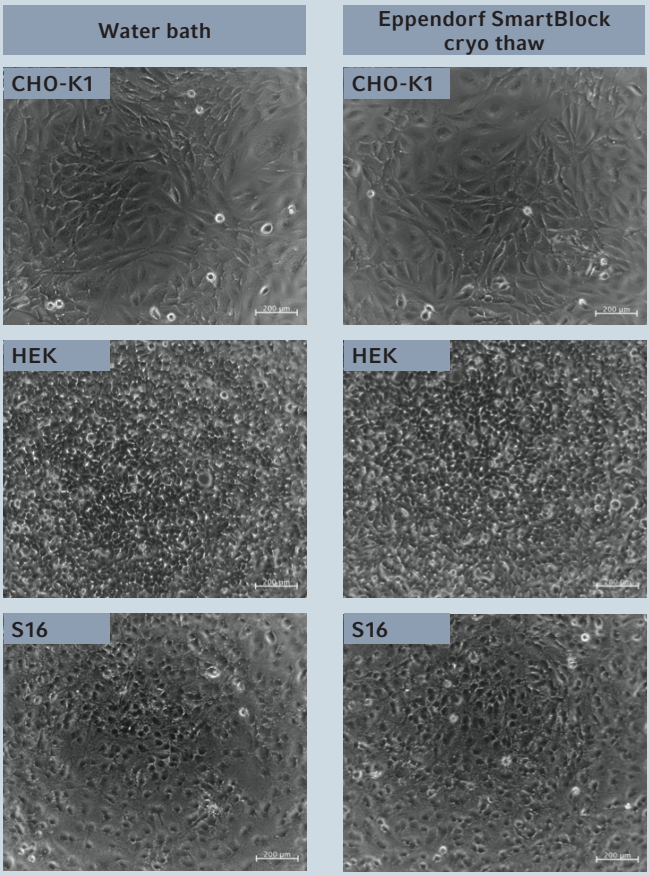

Figure 2: Microscopic analysis of morphology of cell lines 96 hours after thawing with the water bath or the Eppendorf ThermoMixer C. Cells show normal morphology and similar confluences (Magnification 100x) .

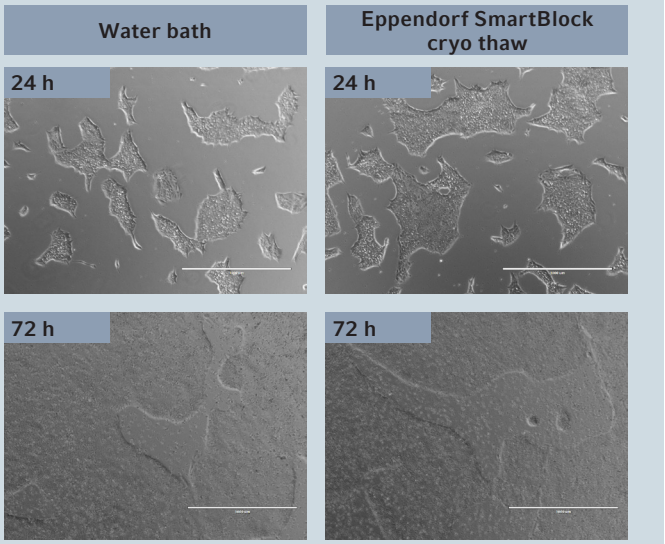

Figure 3: hiPSCs 24 and 72 hours after thawing show the typical and expected morphology with both methods. Depicted is the hiPSC (AISC-0011) (magnification 100x). The other hiPSC line (A18944) showed similar results (data not shown). Thawing at room temperature resulted in smaller clumps and lower cell surface (data not shown).

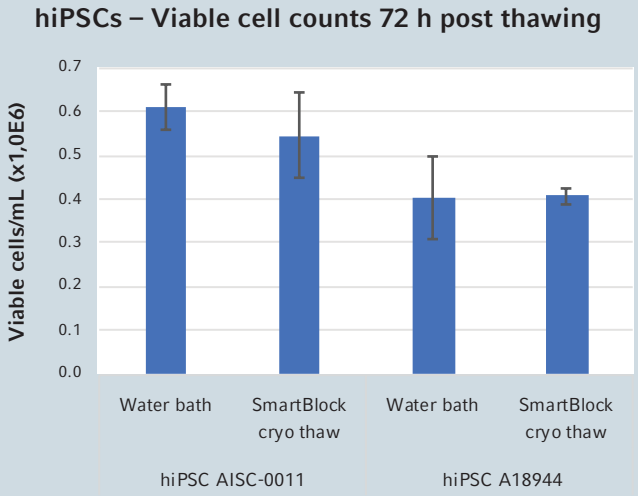

Figure 4: Viable cell counts of hiPSCs 72 hours after thawing. Results represent a mean of three vials. Data of negative control (thawing at room temperature) was lower at all times (data not shown).

For the stem cells, two different clones were tested. Both showed the typical and expected hiPSC morphology for one clone after 24 hours, as depicted in Figure 3. No abnormalities in shape or densities could be observed. Thawing at room temperature (negative control) resulted in smaller clumps and lower cell surface (data not shown). 72 hours post-thawing, cells thawed with the Eppendorf Thermomixer C formed a confluent monolayer as well as the cells thawed with the water bath. The cells thawed at room temperature proliferated but formed a less dense cell layer (data not shown). No spontaneous differentiation or spontaneous detachment was observed during all conditions.

Cells were passaged until passage 4, a normal proliferation rate and morphology was observed during all passages (data not shown). After these four successive passages, immunostaining was performed to confirm the maintenance of pluripotency for one line in addition, the hiPSC line (AISC-0011). Immunostaining results confirmed the maintenance of pluripotency for the cells thawed with the Eppendorf Thermomixer C (Figure 5) and the water bath (data not shown).

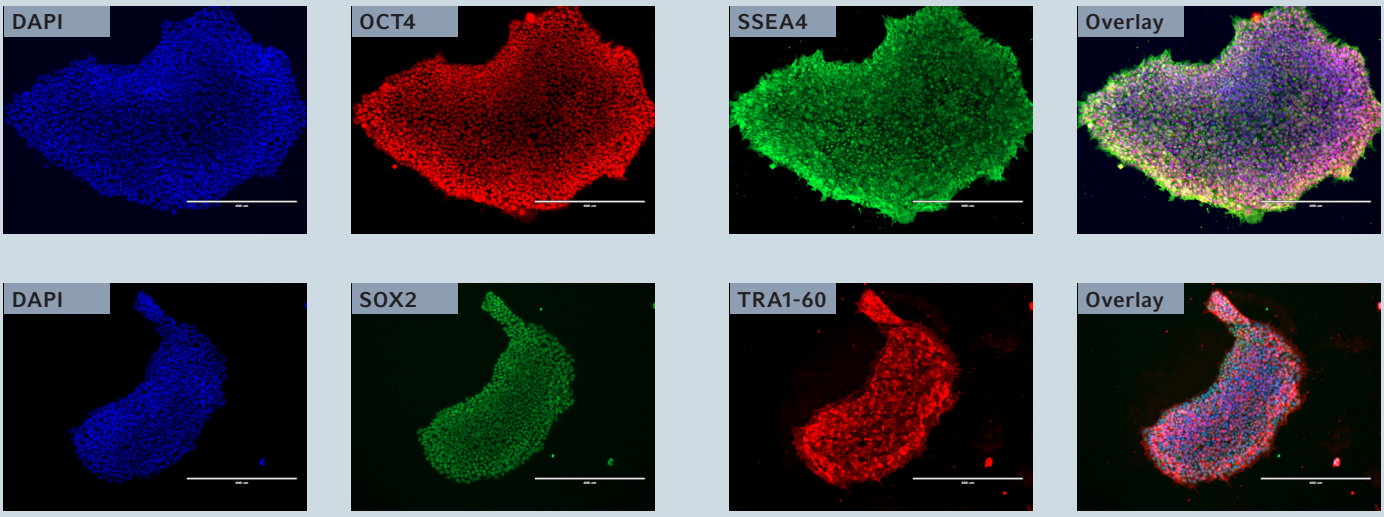

Figure 5: Immunostaining results of hiPSC thawed with the Eppendorf Thermomixer C confirm the maintenance of pluripotency after 4 successive passages post-thawing (magnification 100x).

In sum, all cell types thawed with the Eppendorf ThermoMixer C showed similar fast recoveries, cell viability, and growth patterns as cells thawed with the water bath. In addition, it was shown that pluripotency was maintained for stem cells. The results confirm that the 'Thawing cells' program is well-suited to thaw cells.

Thawing reproducibility

The aim of standardization is to minimize variations between samples. Consistency of temperature profiles can be beneficial in maintaining a constant thawing results. To examine temperature reproducibility, the in vial sample temperature (1 mL freezing media) was measured during the process of thawing. As depicted in Figure 6, the in-vial temperature profile of the samples thawed with the 'Thawing cells' program showed the most consistent and reproducible temperature conditions, whereas thawing with the water bath and hand thawing were far less reproducible. It is also visible that different handling procedures within the water bath, immersing the vial with or without moving, or using a floater, had an additional negative impact on thawing reproducibility. The same is true for hand thawing, where different hands resulted in different temperature profiles, as hand size and hand temperature varies between persons. All methods resulted in thawing times ≤ 5 minutes with the water bath being the fastest, 'Hand 3' being the slowest.

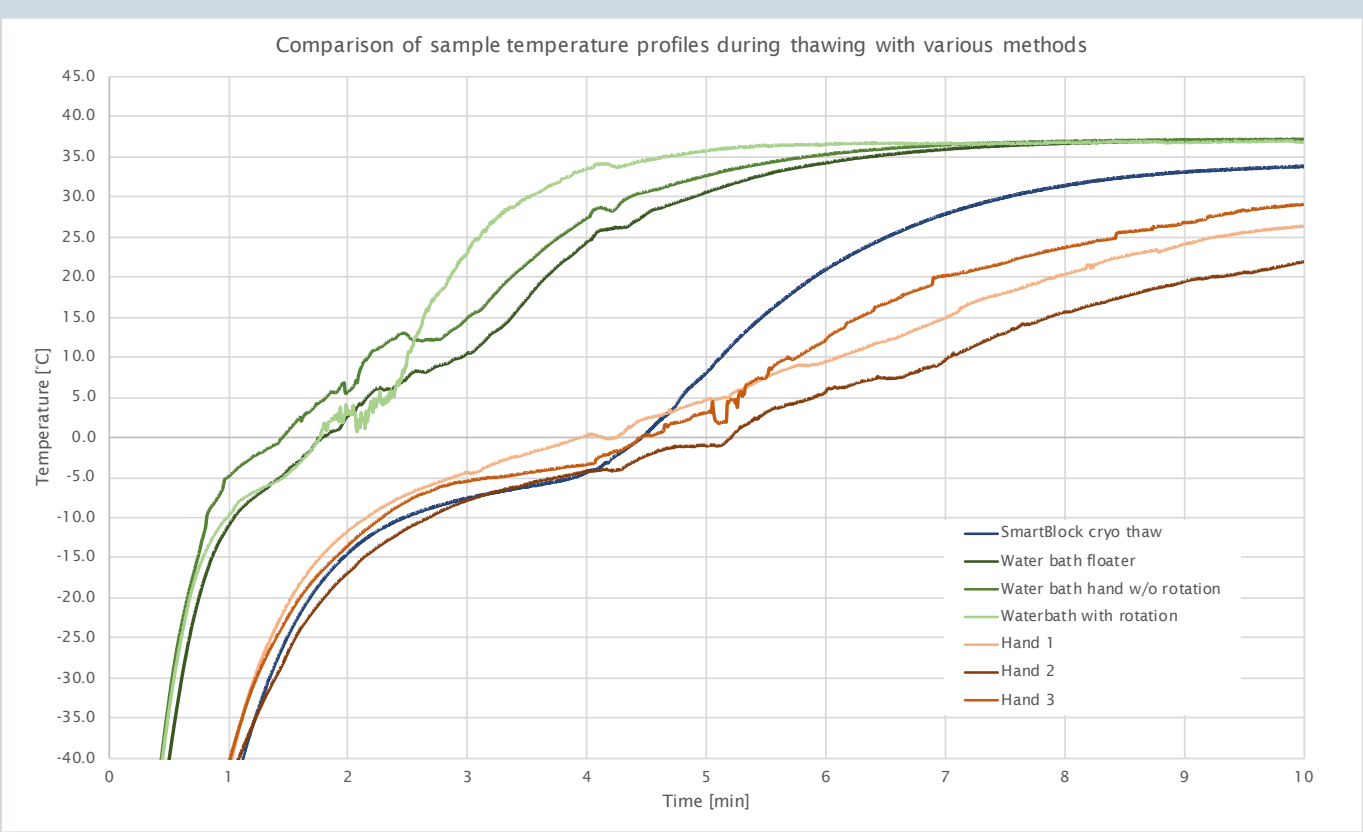

Figure 6: Comparison of sample temperature profiles during thawing with the water bath, Eppendorf Thermomixer C, and hand warming. Each curve represents a mean of three vials.

The Eppendorf SmartBlock cryo thaw provides the most reproducible thawing conditions and still thaws quickly in <5 min. The 'Thawing cells' program is pre-set for 3 min and 3–4 min worked for all cells tested. The Eppendorf SmartBlock cryo thaw allows standardized thawing of up to 24 cryovials in parallel. In comparison to the water bath, the risk of introducing a contamination is reduced to a minimum due to the absence of water. Also, as the process of thawing does not require a person to hold the tube as hand-warming or the water bath do (if not using a floater), preparations for subsequent procedures can run in parallel. Having the Eppendorf Thermomixer C placed beside the biosafety cabinet, for example, makes this even eassier. Last but not least, the Eppendorf Thermomixer C is a multifunctional device. With the compact size and the easily interchangeable Eppendorf SmartBlock, the device can be used for other applications at the bench or in the lab if thawing is not a daily procedure.

Conclusion

The results demonstrate that the Eppendorf ThermoMixer C with the Eppendorf SmartBlock cryo thaw is well suited to thaw cell lines and even sensitive hiPSCs with the same high retrieval rate as a water bath. It offers highly reproducible thawing procedures and can handle multiple vials in parallel. The handling is easy with the pre-set program and the device can be integrated into flexible in workflows with the interchangeable Eppendorf SmartBlock system. The risk of contamination is reduced to a minimum.

Literature

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Ordering information

Description	International Order no.	North American Order no.
Eppendorf ThermoMixer® C, basic device without Eppendorf SmartBlock™, 220 V–240 V	5382 000.015	–
Eppendorf ThermoMixer® C, basic device without Eppendorf SmartBlock™, 110 V–130 V	–	5382000023
Eppendorf SmartBlock™ cryo thaw, thermoblock for 24 cryo tubes, 2.0 mL, all base shapes	5318 000.001	5318000001
Eppendorf CryoStorage Vial 2.0 mL, pre-capped, pre-racked, 10x48 vials, 2D SafeCode, sterile	0030 079.485	0030079485

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