APPLICATION NOTE No. 351

Trophectoderm biopsy of blastocysts using the Eppendorf TransferMan® 4m micromanipulators assisted by a laser system

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Abstract

In recent years, the enhancement of assisted reproductive technologies (ART) has been joined by the development of advanced genetic tests for pre-implantation genetic diagnosis (PGD) or screening (PGS). Oocyte polar bodies or single blastomeres from embryos at the cleavage stage (Day 3 post-fertilization) have been the primary sources used in PGD/S cycles for many years. Unfortunately, the limited amount of material (cells) obtained for genetic analysis with these two biopsy techniques may impair the accuracy of the results. At present, IVF centers are increasingly using trophectoderm biopsy in their clinical practice. This strategy, among other advantages, has demonstrated to be less traumatic for the embryos and to provide a higher number of biopsied cells for molecular analysis, which may increase the sensitivity and reliability of the genetic tests.

In this application note, we describe the so-called "pulling" and "flicking" methods that we routinely use to perform trophectoderm biopsies on hatching or fully hatched blastocysts. Both methods require practice and micromanipulation skills, however, once mastered in the mouse model these techniques can be successfully transferred to human blastocysts with excellent results.

Introduction

Since its first introduction in 1989, pre-implantation genetic diagnosis (PGD) has been used to infer the genomic characteristics of human embryos and to prevent the transmission of inheritable diseases [1]. In general, PGD analysis comprises primer-based DNA amplification for single gene disorders, such as Huntington disease, cystic fibrosis and myotonic dystrophy, among others [2]. Additionally, in human IVF practice, pre-implantation genetic screening (PGS) has also been applied as another valuable tool in the identification of aneuploid embryos. Aneuploid embryos have little potential to result in a viable pregnancy, however they cannot be distinguished from normal embryos using standard morphological evaluation [3].

The genetic analysis of oocyte polar bodies was primarily used for aneuploidy screening and less commonly for the detection of maternal transmission of single gene defects [3]. In contrast, embryo biopsy at cleavage stage, which traditionally involves the removal of one blastomere from pre-compaction Day 3 embryos, has been a preferred practice for both PGD and PGS [3]. Unfortunately, the limited amount of material (one cell) obtained for genetic analysis with these two biopsy techniques may impair the accuracy of the results. To overcome these technical limitations and eventually increase the sensitivity and reliability of the genetic diagnosis, another biopsy technique based on the excision of ideally between 4 and 8 trophectoderm cells from a blastocyst was proposed [4].

This technique was first reported in 1990 [4] and subsequently improved with the development of laser systems that have greatly simplified the excision of the trophectoderm cells [5]. Apart from providing a higher number of cells for the genetic analysis, blastocysts have been described as stronger and with higher implantation potential than cleavage stage embryos [5]. Despite these advantages, blastocyst biopsies are usually performed on Day 5 or Day 6 of development, and the time needed to obtain the results of the genetic tests may not allow a fresh embryo transfer to be performed [6]. Therefore, when using this method, it is essential that IVF labs have a well-implemented and robust cryopreservation program to guarantee the biopsied blastocysts can be vitrified and subsequently thawed for successful transfer in a natural cycle [6-8]. Technical difficulties during the trophectoderm cell excision may vary depending on the morphology and quality of the blastocysts, expansion grade, and the position of the inner cell mass (ICM). Consequently, it is recommended that the practitioners in the different IVF centers acquire extensive experience first in animal models, such as the mouse, before the technique is implemented in the human IVF routine in order to ensure consistent results.

In this application note, we describe two different blastocyst biopsy techniques, the so called "pulling" and the "flicking" methods that we usually use to perform trophectoderm biopsies on hatching or fully hatched blastocysts, respectively. By using the Eppendorf micromanipulators, in combination with an OCTAX® Navilase™ system, we were able to simplify and reduce the technical difficulties normally associated with these procedures, and achieve excellent results both in mouse and human blastocysts.

Materials and Methods

Equipment setup

> Stereo microscope, e.g. SZH, OLYMPUS® (Japan)
> Inverted microscope with up to 40x magnification, e.g. IX73, OLYMPUS (Japan)
> OCTAX Adaptive Electronic Condenser™, MTG GmbH (Germany)
> OCTAX NaviLase™ laser system, MTG GmbH (Germany)
> CellTram® Air microinjector for holding the blastocysts, Eppendorf (Germany)
> CellTram vario microinjector for removal of cells, Eppendorf (Germany)
> Two TransferMan 4m micromanipulators, Eppendorf (Germany)
> Antivibration pads, Eppendorf (Germany)

An example of a laser-assisted micromanipulation station is illustrated in Figure 1.

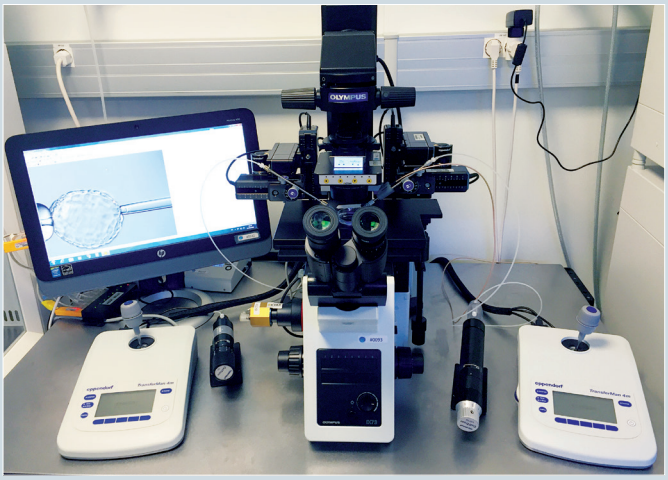

Figure 1: The micromanipulation station composed of an inverted microscope, the OCTAX NaviLase™ laser system, two TransferMan® 4m micromanipulators, a CellTram vario microinjector and a CellTram® Air microinjector.

Materials

> Holding capillary, for holding of oocytes (recommended: 15-20 µm inner diameter, 100 µm outer diameter, 35° tip angle)
> Biopsy capillary, for laser-assisted biopsy of cells (recommended: 19 µm inner diameter, 35° tip angle)
> Embryo tested 60 mm dishes
> Embryo tested mineral oil
> Hepes-buffered medium
> E-tubing™ adapter for Eppendorf PCR Tubes, Embryotools (Spain); Order no. 19310/4200 at mail@mtg-de.com)
> Eppendorf PCR Tubes, 0.2 mL, Eppendorf (Germany)

Methods

Preparation of the micromanipulation chamber

The arrangement of the micromanipulation chamber depends on personal preferences. In our practice, we use a 60 mm dish as a micromanipulation chamber, prepared with 5 µL droplets of a Hepes-buffered medium placed in the central area of the dish and covered with approximately 14 mL of mineral oil. Each droplet of manipulation medium is numbered for unambiguous identification of each embryo to guarantee that the biopsied cells for genetic analysis will match with the corresponding embryo.

Preparation of microcapillaries

The Eppendorf holding and biopsy capillaries are set in the holders of the Eppendorf microinjectors, CellTram Air and CellTram vario, respectively. Whenever possible, the holding microcapillary should have an outer diameter (OD) slightly smaller than that of the blastocyst and an inner diameter (ID) of about 15–20 µm. If the OD of the holding capillary is too small in proportion to the diameter of the embryo, it can cause the embryo to move during the procedure. The biopsy microcapillary installed in the CellTram vario microinjector has a blunt-end, an angle identical to that of the holding pipette, and an ID of about 19 to 25 µm, depending on the degree of expansion of the blastocyst.

The "pulling" method for the biopsy of hatching blastocysts

The pulling method is recommended for blastocysts with few trophectoderm (TE) cells herniating through the zona pellucida. To induce the hatching of the TE cells, a hole of approximately 20 µm is usually induced in the zona pellucida (assisted hatching, AH) using a laser pulse when the embryo is still at the morula stage, normally on Day 3 (post-fertilization) of pre-implantation development (Figure 2). After the AH has been performed, the morula stage embryo is returned to the incubator and cultured in optimal conditions until it develops into a blastocyst at which time it is possible to clearly distinguish the TE and inner cell mass (ICM) cells.

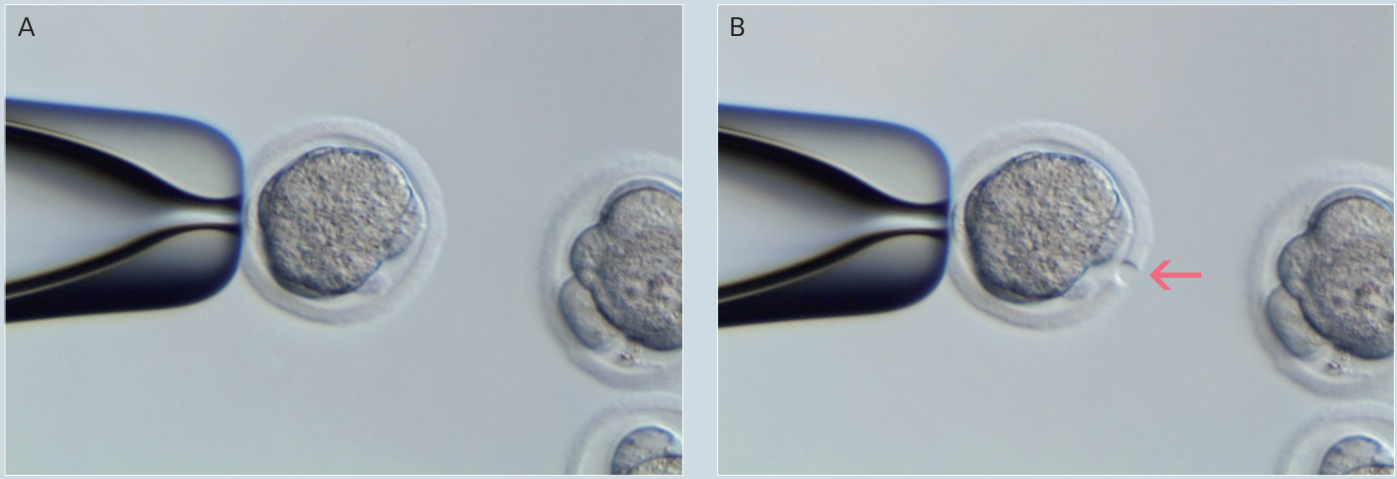

Figure 2: A mouse embryo at a compacted morula stage immobilized with the holding pipette before (A) and after (B) an assisted hatching hole (arrow) performed with a laser system. This hole will facilitate the herniation of trophectoderm cells once the embryo has reached the blastocyst stage.

Once at the blastocyst stage, embryos must be examined under the inverted microscope and rotated until the ICM is clearly visible. The blastocysts should then be positioned so that the TE cells herniating through the zona pellucida hole performed on Day 3 are facing the biopsy pipette. At this point, the blastocyst is held firmly with the holding pipette and the biopsy needle is brought near the TE cells, which can then be drawn carefully into the biopsy pipette by applying gentle suction. Subsequently, the TE cells should be gently pulled away from the blastocyst, while laser pulses are applied.

The detachment of the TE cells should normally be achieved with less than five laser pulses. It is important to be careful with the amount of pressure exerted inside the biopsy capillary so not to lose control of the TE cells post-biopsy. For a successful excision of the cells, laser pulses should be applied at the cell junctions, so they can easily be pulled away from the blastocyst while minimizing cell damage. An example of a trophectoderm biopsy performed using the "pulling" method is illustrated in Figure 3.

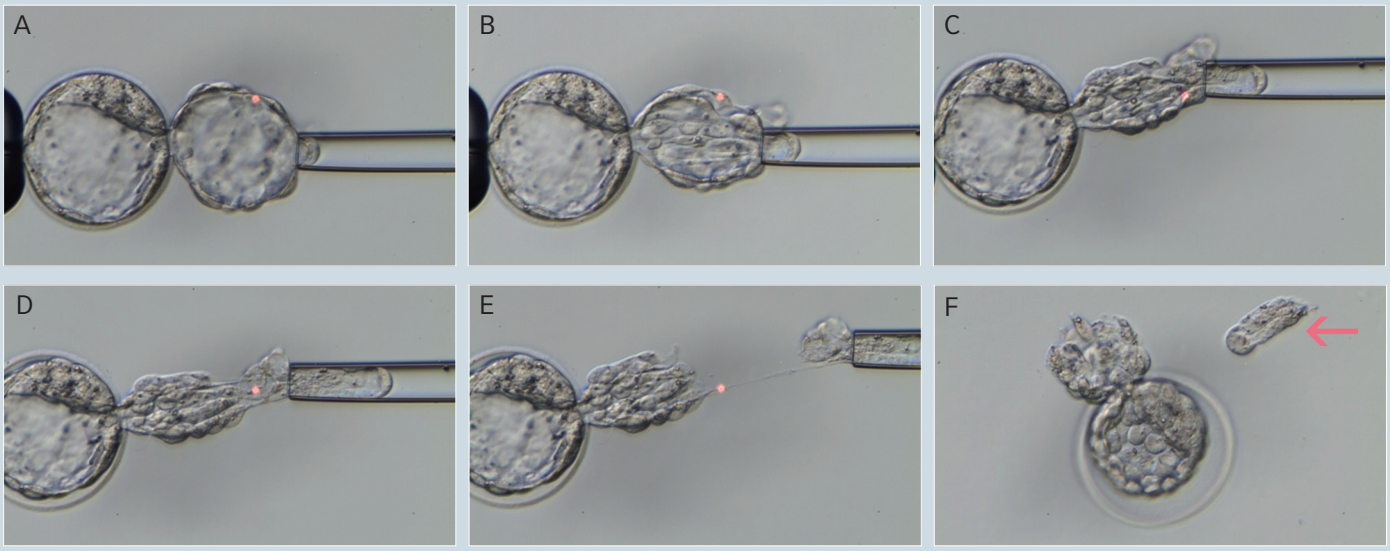

Figure 3: Trophectoderm biopsy using the "pulling" technique, indicated for an early hatching blastocyst. A-B) A blastocyst is firmly immobilized with the holding capillary. Note the ICM positioned away from the biopsy side. Gentle suction is applied to aspirate the trophectoderm cells facing the biopsy needle. C-E) While applying laser pulses, the biopsy pipette is slowly pulled away from the embryo. Note the importance of the correct internal diameter of the biopsy pipette, so that, after embryo collapse, trophectoderm cells are well controlled inside the pipette and are not lost after detaching from the blastocyst. F) The blastocyst together with the excised trophectoderm cells (arrow) after the biopsy procedure. Note the importance of having a good microscope optics and electronic condenser to be able to have an optimal image of the nuclei and the cell junctions.

The "flicking" method - Biopsy of fully hatched blastocyst

The "flicking" method is recommended for blastocysts with TE cells herniating through a wide opening in the zona pellucida or for those completely hatched. As described above, all blastocysts must be assessed morphologically under an inverted microscope and rotated if necessary until the ICM is clearly identified. The blastocyst should be positioned so the ICM is next to the holding pipette (Figure 4). TE cells located on the opposite side of the ICM can then be carefully aspirated into the biopsy pipette. Immediately after, a first laser pulse is applied to one edge of the trophectoderm, followed by two or three more laser pulses. While laser pulses are applied, the blastocyst will collapse, allowing more TE cells to be drawn inside the biopsy pipette. The collapsed blastocyst is then gently released from the holding pipette, while the biopsy pipette holds the embryo through the TE cells. Immediately, the holding and biopsy pipettes should be aligned on the same focal plan of the microscope, while a tension is applied by moving one pipette against the other (Figure 4). The TE cells can then be detached from the blastocyst with a quick flicking movement of the biopsy pipette against the holding pipette.

An example of a trophectoderm biopsy performed with the "flicking" method is illustrated in Figure 4.

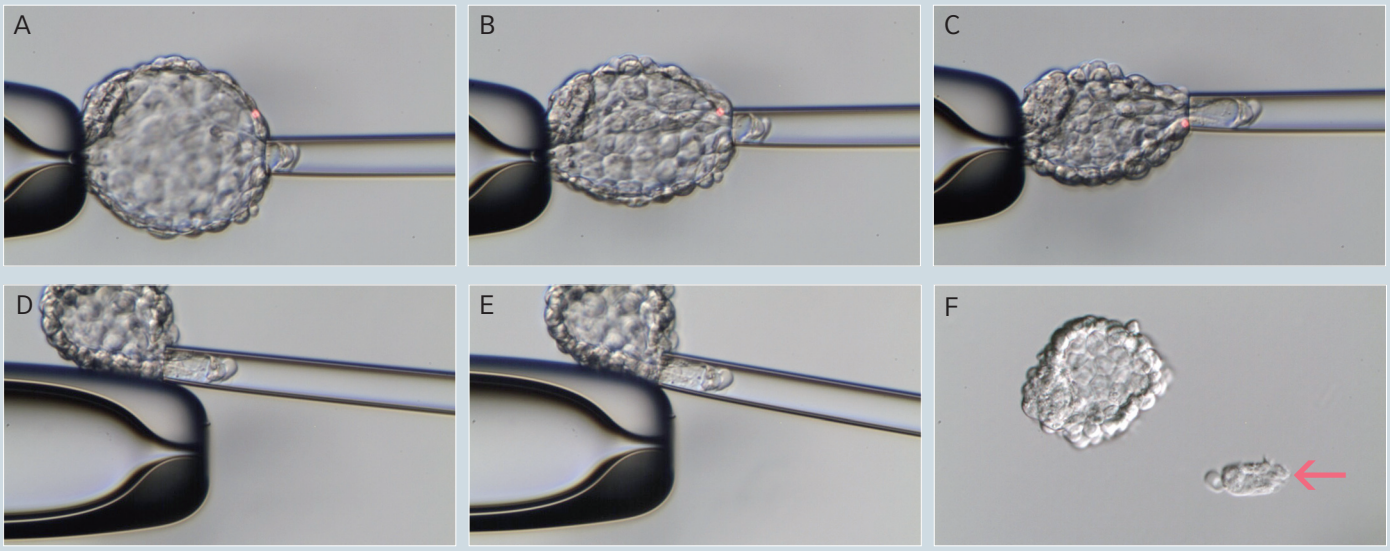

Figure 4: Biopsy procedure using the so called "flicking" method. A-C) A fully expanded hatched blastocyst gently immobilized with the holding capillary. Trophectoderm cells sited on opposite side of the ICM are brought into the fine biopsy pipette, while applying low-intensity laser pulses. D, E) The collapsed blastocyst is released from the holding pipette and maneuvered to the rim of the biopsy pipette. A quick flicking movement from the biopsy pipette against the holding pipette is performed in order to excise the biopsied cells from the blastocyst. F) The blastocyst and the sample of trophectoderm cells (arrow) after biopsy. Note the importance of having good microscope optics and electronic condenser to produce an optimal image of the nuclei and the cell junctions.

Vitrification of biopsied blastocysts

After the biopsy, blastocysts can be put back in culture and evaluated later on or the next day to ensure they have re-expanded and were not damaged by the biopsy procedure. Alternatively, the blastocysts can be cryopreserved immediately after the biopsy, while still collapsed, and kept cryopreserved until use.

"Tubing" of cells for genetic analysis

The process of transferring the biopsied cells from the micromanipulation dish into an Eppendorf PCR Tube is generally known as "tubing of cells". This step is usually performed in a flow hood cabinet at room temperature, under a stereo microscope. Using an appropriate sterile capillary or pipette, the biopsied TE cells are taken from the micromanipulation dish and moved onto a clean droplet of washing medium not covered with oil. The cluster of biopsied cells can then be washed two or three times in clean droplets of washing medium and then loaded again using a clean capillary or pipette for transfer into a small droplet of lysis buffer placed inside the bottom of a 0.2 mL Eppendorf PCR Tube. To ensure the biopsied cells are correctly deposited inside the PCR tube, the sample must be viewed coming out of the capillary or pipette under the stereo microscope. This is a critical step in the process. A common problem that embryologists face is having to focus on the tip of the capillary while also needing to focus on the cells being deposited into the PCR tube.

Consequently, to solve this problem we recommend using an adaptor (E-tubing™). This adapter has been developed to hold the Eppendorf PCR Tubes at different angles and simplify the technique, which has demonstrated to greatly assist embryologists on this step (Figure 5).

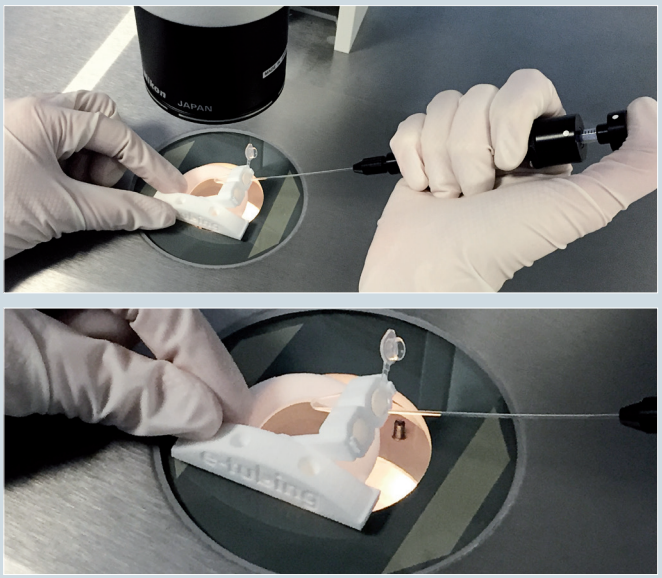

Figure 5: The "tubing" process of transferring the biopsied cells into an Eppendorf PCR Tube under a stereo microscope.

Results and Discussion

Table 1 shows typical results from operators with micromanipulation knowledge, but no previous experience with blastocyst biopsy or tubing. Increasingly successful results for both the "pulling" and "flicking" techniques are typically achieved after participating in an intensive hands-on program on mouse blastocysts. After performing a minimum of 50 biopsies, practitioners are usually proficient in both methods, as well as tubing of the biopsied cells with consistent results. Practitioners should extensively wash blastocysts in culture medium and keep them overnight in culture at 37°C under an optimal % of CO_2/O_2. The next morning, blastocysts will be evaluated under the inverted microscope to confirm they were able to re-expand and were not damaged by the biopsy procedure. Similarly, the efficiency of the tubing of the biopsied cells can be evaluated by confirming whether the cells have not been lost during biopsy or tubing, and are seen coming out of the capillary or pipette when transferred to the bottom of the Eppendorf PCR Tube.

Table 5: Expected results for operators without previous experience on blastocyst biopsy.

		No. of blastocysts			
	Biopsy Technique	**Biopsied**	**Expanded after culture (%)**	**Lysed or non-expanded (%)**	**No. of successful "tubings" (%)**
Operator without previous experience	Pulling	50	45 (90.0)	5 (10.0)	35 (70.0)
	Flicking	50	42 (85.0)	8 (15.0)	35 (70.0)
Operator after intensive hands-on	Pulling	30	30 (100.0)	0 (0.0)	30 (100.0)
	Flicking	30	29 (96.7)	1 (3.3)	30 (100.0)

Conclusion

In this application note, we describe two different trophectoderm biopsy techniques, the so called "pulling" and the "flicking" methods performed on hatching or fully hatched blastocysts, respectively. The use of Eppendorf micromanipulators, in combination with the OCTAX NaviLase™ system, helps to simplify these procedures and to reduce technical challenges.

With the appropriate equipment and the correct technique for blastocyst culture and cryopreservation, blastocyst biopsy can represent a practical and preferable path to preimplantation genetic testing of embryos compared with polar body or cleave stage embryo biopsy.

Trophectoderm biopsy is likely to become the gold standard to obtain material for pre-implantation genetic testing. Choosing the right tools is an essential pre-requisite for obtaining consistent results. However, fully mastering this technique requires appropriate training and micromanipulation skills. In order to achieve excellent results, we recommend the described techniques to be first mastered in an animal model, such as the mouse. Once learned, both methods can be applied successfully in clinical practice in the biopsy of human blastocysts.

Literature

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Ordering information

Description	Order no. international
TransferMan® 4m, Micromanipulator with DualSpeed™ joystick for direct and dynamic movement control[1]	5191 000.015
Microscope adapters, Adapter for micromanipulators, available for different inverse microscopes of major brands	Available upon request
CellTram® 4m Air, Manual pneumatic microinjector, with gears 1:1 and 1:10, for holding and injection[1,2]	5196 000.021
CellTram® 4m Oil, Manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection[1,2]	5196 000.048
VacuTip I, Holding capillary, 35° tip angle, 15 µm inner diameter, 1 mm flange, sterile, set of 25[3]	5195 000.036
Biopsy Tip I, Transfer capillary for laser-assisted cell biopsy, 35° tip angle, 19 µm inner diameter, 1.9 mm flange, sterile, set of 25[3]	5195 000.052
Eppendorf PCR Tubes, 0.2 mL, colorless, certified free from human DNA, DNase, RNase and PCR inhibitors	0030 124.332

[1] This product is registered in the European Union as a medical device (according to Medical Device Directive MDD 93/42/EEC). For availability in your country, please contact your local sales organization.
[2] The CellTram 4m Air and CellTram 4m Oil replace the CellTram Air and CellTram vario.
[3] For research use only (mouse and other animal models).

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