Abstract

Biopsy of equine embryos at the early/expanded blastocyst stage is a rather complex procedure, essentially due to the presence of a distinctive capsule in the perivitelline space that is extraordinarily resistant and difficult to penetrate using conventional micromanipulation methods. In addition, this embryonic capsule has been considered essential for the embryo survival and, until recently, many doubts persisted about whether it could be breached without impairing the viability of the biopsied embryo. Recent studies, however, suggested that encapsulated equine blastocysts could be biopsied using a piezo-actuated micropipette that allows creating a small hole in the capsule to collect trophectoderm cells for genetic analysis without compromising their further viability. In this Userguide, the biopsy procedure of encapsulated equine embryos is described using a device from Eppendorf for piezo-assisted manipulation, the Eppendorf PiezoXpert, in combination with the Eppendorf TransferMan® 4r micromanipulation workstation.

Introduction

Establishing an efficient clinical method for preimplantation genetic diagnosis (PGD) in the horse could represent a very valuable tool for the equine breeding industry, as it would allow the elimination of devastating genetic diseases or the selection of the desired gender, coat color, or other genetic traits in the offspring produced [1-4]. Traditionally, the most popular method to collect equine embryos has been performed by uterine flush of inseminated mares on Day 6 – Day 8 after ovulation, when the embryos are generally at the early/expanded blastocyst stages, respectively [5,6]. In this sense, to make a PGD program practical, the flushed embryos would have to be biopsied at these developmental stages, vitrified, and later transferred into synchronized recipients, once the results from the genetic analysis had been assessed. A problem related to this program is that the early equine embryo, on late Day 5 after ovulation, starts developing a distinctive capsule in the perivitelline space that is composed of mucin-like glycoproteins and which is extraordinarily resistant to chemical or enzymatic digestion [7-9]. The physiological role of the equine capsule remains unclear, but it is believed that it may constitute a physical barrier to protect the conceptus development in the uterus until about Day 21 of gestation [10]. The presence of the capsule on late morula and early/expanded blastocysts makes the embryo biopsy procedure particularly difficult, as the capsule has to be penetrated to collect enough cells for the genetic diagnosis. In fact, until so far, there have been only few reports on embryo biopsy in horse and it is unclear whether the capsule of the equine embryo could be breached without impairing the viability of the biopsied embryo [2,10,11].
In a recent study, however, Choi et al., [12] demonstrated that morulae (Day 6) and early/expanded (Day 7/8) encapsulated blastocysts could be biopsied using a piezo-actuated micropipette that allows creating a small opening in the capsule to collect trophectoderm cells for genetic analysis, without compromising their further viability [12]. These findings evidenced the importance of the piezo-assisted micromanipulation on the establishment of a valuable method for PGD in horse. Eppendorf has developed a device for piezo-assisted manipulation, the Eppendorf PiezoXpert, and this userguide describes its application for the biopsy of encapsulated equine embryos. The evaluation of the PiezoXpert was carried out at Mr. Paul Schockemöhle’s farm (Lewitz, Germany) in collaboration with Eppendorf, Olympus Europa Holding GmbH and NidaCon International AB.

**Materials and Methods**

**Materials**

- Binocular stereo microscope (SZH, Olympus® or similar)
- Inverted microscope with up to 40x, Modulation contrast (IX 71, Olympus or similar)
- 2x Microinjectors for holding and biopsy (e.g., CellTram 4r Air or CellTram 4r Oil, Eppendorf)
- 2x Micromanipulators (e.g., TransferMan 4r, Eppendorf)
- Piezo-drill unit (Eppendorf PiezoXpert, Eppendorf)
- Borosilicate Pasteur pipettes for embryo manipulation
- Hamilton microliter syringe (Hamilton Company)
- Piezo Drill Tip ES (ID 15 µm, angle 25°, blunt end, Eppendorf)
- VacuTip I (ID 15 µm, OD 100 µm, angle 35°, Eppendorf)
- Petri culture dishes 90 mm (e.g., Eppendorf)
- Holding medium (Minitub)
- Mercury (215477, Sigma-Aldrich)
- Mineral oil (Nidoil, Nidacon)
- Polyvinylpyrrolidone (PVP, PVP360, Sigma-Aldrich)
- Perfluor (FC770, Sigma-Aldrich)

**Equipment setup**

All micromanipulation procedures should be carried out with a micromanipulation system installed on an inverted microscope and using the Eppendorf PiezoXpert. Figure 1 shows the mounting of the actuator of the PiezoXpert onto the motor module of the TransferMan 4r. The injection tube of the microinjector is connected with the actuator. Any air bubble is removed out of the oil-filled system by moving the oil forward up to the mounted grip head of the actuator using the coarse rotary knob of the CellTram 4r Oil.

Fig. 1: Actuator of Eppendorf PiezoXpert mounted onto the manipulator (Eppendorf TransferMan 4r).
Preparation of the micromanipulation chamber
The arrangement of the micromanipulation chamber depends on personal preferences. As an example, the lid of a 90 mm Petri dish can be used as a micromanipulation chamber as, compared to the bottom dish, the lid has a lower rim that will not interfere with the movement of the microcapillaries. Several droplets (10 µL) of holding medium, for embryo biopsy, and of holding medium supplemented with 12 % PVP, for pipette washing, should be distributed as shown in Figure 2 and immediately covered with mineral oil. Each droplet of holding medium can then be numbered according to the reference previously attributed to each embryo after collection, to guarantee that the biopsied cells for genetic diagnosis will match the corresponding embryo.

Collection of the equine embryos
Equine embryos can be collected from inseminated mares, as described elsewhere [6]. After collection, each embryo should be extensively washed and held in holding medium at room temperature or in cultivation medium in an incubator at 38.3 °C and 5 % CO₂ until being processed. It is strongly recommended to assign a reference number to each collected embryo to guarantee the identification of the corresponding biopsied cells throughout all steps of the PGD.

Preparation of microcapillaries
Holding and biopsy microcapillaries can be purchased from Eppendorf. Whenever possible, the holding capillary should have an outside diameter (OD) slightly smaller than that of the embryo. 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 when piezo impulses are applied and reduce the efficiency of the drilling. The biopsy capillary should have a blunt-end, an ID of about 15 µm at the tip and a flat angle (<25°) of the bend capillary for more direct transfer of the piezo impulses. Before connecting the biopsy capillary to the Eppendorf PiezoXpert, the capillary should be backfilled with a small amount of mercury (approximately 2 µL, i.e. 4 mm inside the capillary) using a Hamilton Syringe.
This is an important step to enhance the transmission of the piezo impulses and to ensure an optimal performance when drilling the zona pellucida and the capsule. As an alternative to mercury, Perfluor can be used. Please consult [13] for a detailed description on how to connect the biopsy capillary to the Eppendorf PiezoXpert actuator.

Eppendorf PiezoXpert parameters
Pre-installation of Eppendorf PiezoXpert parameters (intensity, speed and number of piezo impulses) can significantly speed up the biopsy procedure and at the same time shorten the time-period in which the embryos are exposed to stressful conditions. Thus, this will improve the final results. Up to 3 sets of optimized programs can be stored in the Eppendorf PiezoXpert. Each program consists of parameter sets A and B, see Figure 4. Usually, parameter set A is used with strong parameters for the penetration of the zona pellucida and breaching of the capsule. Set B is used afterwards in exceptional situations when cells to be biopsied cannot be isolated from the embryos by gentle suction with the biopsy capillary: In these rare cases, additional gentle piezo impulses can help achieve successful cell isolation. Both sets A and B can be triggered via either the button on the control unit or the foot control.

Fig. 2: Layout of the micromanipulation chamber used in the embryo biopsy procedure. The chamber is divided in two sections: the right side is composed by several droplets (approximately 20 µL) of holding medium supplemented with 12 % PVP for pipette washing and lubrication of the piezo-actuated capillary. This can be done by dispensing microscopic mercury droplets and by aspiring and dispensing PVP several times; the left side of the chamber contains individual droplets of holding medium for embryo biopsy.
Optimization of parameters

1. Set the parameters for speed, intensity and number of piezo impulses to 1.
2. Gradually increase the value for intensity until the piezo impulses are strong enough to penetrate the zona pellucida and the capsule.
3. Fine tune the speed and pulse parameters.
4. Use the lowest parameter settings that work.

Usually, the parameter settings for Perfluor may be slightly higher than for mercury.

NOTE: Users should optimize the parameters for their own experiments as the settings always depend on individual laboratory protocols. As a guideline, if non-cryopreserved embryos are used, the Eppendorf PiezoXpert parameters using mercury recommended for morula, early and expanded blastocysts are shown in Table 1. These parameters can be saved in the Eppendorf PiezoXpert control unit as different programs according to the embryonic developmental stage of the embryos to be biopsied.

Embryo biopsy procedure

After all equipment has been set-up, a single embryo should be moved to a droplet of holding medium in the micromanipulation chamber under a stereo microscope. The chamber should then be placed onto the inverted microscope and the embryo should be morphologically inspected at 20x and 40x magnifications.

The embryo should be fixed for biopsy with the holding capillary by gentle suction. The biopsy capillary should next be washed and lubricated in the PVP containing medium droplets, and then moved back to the droplet of holding medium where the embryo to be biopsied was previously placed. Embryo biopsy can then be performed by application of piezo impulses (see Table 1) combined with gentle suction in the biopsy capillary to allow the drilling of a small hole in the zona pellucida and the embryonic capsule, and thus facilitate the access of the blunt-end pipette to the trophectoderm cells, as explained in detail in Figure 3. An acceptable number (5 to 10) of trophectoderm cells should be removed as quickly as possible by applying gentle suction with the biopsy capillary after penetrating the capsule.

In some cases, when simple suction with the biopsy pipette is not efficient to isolate the cells from the embryo, a few weak piezo impulses facilitate the access of the blunt-end pipette to the trophectoderm cells, as detailed in Figure 3B-C. After the biopsy, the removed cells should be released from the capillary in the vicinity of the biopsied embryo and then aspirated and placed in a minimum volume (5 µL) of phosphate buffered saline (PBS) in a 0.2 mL PCR tube using a fine-bore glass pipette. The tubes with the biopsied cells can then be kept at 4 °C or be frozen directly in liquid nitrogen until the genetic analysis is assessed by PCR, as described elsewhere [1,14].

Table 1: Parameter settings using a capillary backfilled with mercury for biopsy of equine embryos at morula, early- and expanded-blastocysts stages.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morula</th>
<th>Early Blastocyst</th>
<th>Expanded Blastocyst</th>
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<tr>
<td></td>
<td>Set A</td>
<td>Set B</td>
<td>Set A</td>
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<tr>
<td>Intensity</td>
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<td>8</td>
</tr>
<tr>
<td>Pulse</td>
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Fig. 3: Biopsy procedure in an early equine blastocyst (Day 7). A) Embryo firmly immobilized with the holding capillary, while piezo impulses combined with gentle suction are applied with the biopsy capillary to allow the drilling of a hole through the zona pellucida and the capsule and the access of the blunt-end pipette to the trophectoderm cells. B, C) After aspiration of a few cells of the trophectoderm, the biopsy capillary is slowly pulled away from the embryo. In some cases a few weak Piezo impulses have to be applied to detach the cells from the embryo before the pipette is completely removed. D) The biopsied cells are finally expelled from inside the pipette and the embryo is released from the holding capillary.

Fig. 4: Setting of parameter sets A and B
Literature


### Ordering Information

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<thead>
<tr>
<th>Description</th>
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<tr>
<td><strong>Eppendorf PiezoXpert®,</strong> For piezo-assisted micromanipulation, incl. actuator II, foot control and grip head 4 size 0</td>
<td>5194 000.016</td>
</tr>
<tr>
<td><strong>TransferMan® 4r,</strong> Micromanipulator with DualSpeed™ joystick for direct and dynamic movement control</td>
<td>5193 000.012</td>
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<td><strong>CellTram® 4r Air,</strong> Manual pneumatic microinjection, with gears 1:1 and 1:10, for holding and injection</td>
<td>5196 000.013</td>
</tr>
<tr>
<td><strong>CellTram® 4r Oil,</strong> Manual hydraulic microinjector, with gears 1:1 and 1:10, for holding and injection</td>
<td>5196 000.030</td>
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<td><strong>VacuTip II,</strong> Holding capillary, 35° tip angle, 60 μm inner diameter, sterile, set of 25</td>
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<td><strong>Microloader,</strong> Pipette tip for back-filling microinjection capillaries, set of 2x 96 pcs.</td>
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<td><strong>Microscope Adapter,</strong> Adapter for micromanipulators, available for different inverse microscopes of major brands</td>
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<td><strong>Galaxy® 48 R,</strong> CO₂ incubator, 48 L, 230 V/50/60 Hz, High-Temp Disinfection, 0.1–19 % O₂ Control</td>
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