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# EVALUATION OF MINIMALLY INVASIVE MUSCLE BIOPSY METHOD FOR GENETIC ANALYSIS IN HORSE*

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**Abstract**

**In horses, muscle biopsy is widely used in veterinary practice for routine diagnosis of various muscle disorders. Development of genetic methods such as gene expression measurements using microarrays, RNA-seq, and real-time PCR leads to searching an alternative, less invasive biopsy method in order to obtain an appropriately representative group of animals for genetic testing. In the present study, we proposed a minimally invasive method of muscle sample biopsy using Pro-Mag™ Ultra Automatic Biopsy Instrument (Surgical Specialties Corporation, US, Inc.), which is commonly utilized in human medicine. This approach does not require skin incisions and usage of stitches. The quantity of muscle sample gained in accordance with presented procedure was sufficient to obtain RNA with a good quality. Furthermore, application of the automatic biopsy instrument allows obtaining a sufficient amount of biological material for genetic analysis from a wide variety of tissues. Moreover, samples acquired according to this method could be used in other analyses.**

**Key words: muscle biopsy, horse, automatic biopsy, genetic analysis**

Muscle biopsy is widely used in veterinary practice for routine diagnosis of various muscle disorders such as: exertional myopathies (equine rhabdomyolysis syndrome ERS, polysaccharide storage myopathy EPMS) (Valberg et al., 1992), equine

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motor neuron disease (Valentine et al., 1994), myotonic dystrophy (Montagna et al., 2001), immune mediated myositis (Lewis et al., 2007) or atypical myoglobinuria (Cassart et al., 2007). Moreover, muscle samples obtained through biopsy can be utilized to diagnose muscle disorders using immuno- and histochemistry methods (Ledwith and McGowan, 2004).

In horses, a muscle biopsy method has evolved since the 1970s (Lindholm and Piehl, 1974; Snow and Guy, 1980). This technique was associated with using a 6-mm diameter needle and required a 10-mm skin incision. Muscle samples obtained by this method were commonly used to identify muscle fiber sizes, shapes, contractile and metabolic properties, as well as neuromuscular junctions, nerve branches, connective tissue, and blood vessels (Valberg and Borgia, 2009). Recently, the biopsy samples have also been widely used for genetic analyses including the whole transcriptome evaluation in selected tissues (Eivers et al., 2012; Martin et al., 2010). The new techniques of molecular biology such as cDNA microarrays or RNA-seq methods allow detecting global cell gene expression profiles in specific developmental stages or physiological conditions. Thus, genetic analyses enable broadening the knowledge about development and molecular etiology of the majority of diseases (Wang et al., 2009). The evolution of new genetic methods requires a new less invasive and simpler approach to collect biological samples. Moreover, the material obtained for nucleic acid isolation should be sufficient and suitably protected until analysis. In horses, the most common method for muscle sampling is biopsy with the use of Bergstrom needles, also known as surgical biopsy (Ledwith and McGowan, 2004). In the present study, we propose an alternative, minimally invasive method for tissue sampling using Pro-Mag™ Ultra Automatic Biopsy Instrument (Surgical Specialties Corporation, US, Inc.), which is standardly used in the human biopsy.

## Material and methods

### Animals

In our study, the *gluteus medius* muscle biopsy was performed on 15 Arabian horses (7 mares and 8 stallions), which were maintained in a private stud (Poland). The *gluteus medius* muscle has been widely studied because it plays a major propulsive role in the locomotion (Valberg and Borgia, 2009). All of the used procedures were carried out under legal and ethical requirements (agreement no. 00665). The horses included in the experiment had a day of rest from their training. The feeding of horses was withheld at least 4 hours before treatment and shortly after surgery horses were given hay. Water was available *ad libitum* prior to and after treatment.

### Biopsy procedure

Fifteen minutes before biopsy, the animals were sedated for standing procedure with 10 $\mu g/kg^{-1}$ detomidine hydrochloride (Domosedan, Pfizer Ltd, UK) by intravenous injection (Taylor et al., 2014). The biopsy site was clipped, shaved, disinfected with antiseptic and locally anesthetized by injection of 5 ml lidocaine hydrochloride

(Lurocaine; Vetoquinol N-A, Princeville, Québec, Canada). The biopsy *ProMag*™ Ultra Automatic *Biopsy* Instrument has been prepared according to manufacturer's instruction. For the present study, the biopsy needle had a length of 16 cm and diameter of 2 mm. The biopsy points were determined approximately 15 cm caudodorsal to the tuber coxae on line from the tuber coxae to the first caudal vertebra. Immediately before procedure, the skin was punctured with the use of 2.2 mm diameter injection needle (Polfa, Lublin, Poland) in order to generate a point of entry for the biopsy needle. The biopsy needle was introduced through approx. a 2 mm skin incision to a depth of 80 mm and the sampling was performed by a single shot.

**Isolation of RNA**

In the present study, tissue samples were collected into cryotubes, frozen in liquid nitrogen and finally stored at –80°C. The alternative way of tissue stabilization could be used such as RNAlater solution (Ambion, Life Technologies) and then samples should be stored at –20°C.

The total RNA was isolated from muscle samples using TriReagent (Ambion, Life Technologies) according to the method described previously by Chomczyński (1993). The tissue samples were homogenized with the use of zirconium oxide beads (diameter 0.5 mm) and BulletBlender homogenizer (Next Advance Inc., USA). The quality and quantity of obtained RNA was evaluated on TapeStation 2200 (Agilent Technologies) using RNA Screen Tape and also by NanoDrop 2000 spectrophotometer (Thermo Scientific).

## Results

The muscle samples obtained by proposed biopsy weighed approx. 14 mg (Figure 1).

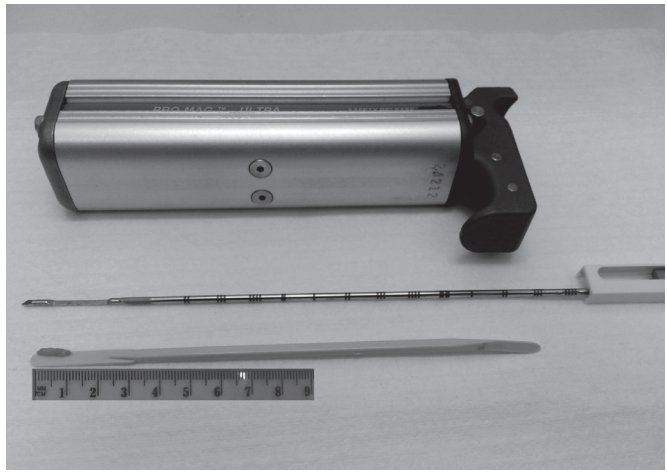

Figure 1. The size of muscle samples obtained according to described biopsy procedure. The sterile tweezers were used to extract the muscle from the hub of Pro-MagTM Ultra biopsy needle

The average RNA concentration isolated with the use of TriReagent from muscle samples obtained by biopsy was 148.3 ng/µl, and the range of RNA concentration was 56.8–301.2 ng/µl (coefficient of variation – 11.8%) (Figure 2). Furthermore, the 260 nm: 280 nm absorbance ratio was in the range of 1.79 to 1.94 and the average RIN coefficient (RNA Integrity Number) amounted to 7.4 (Figure 3).

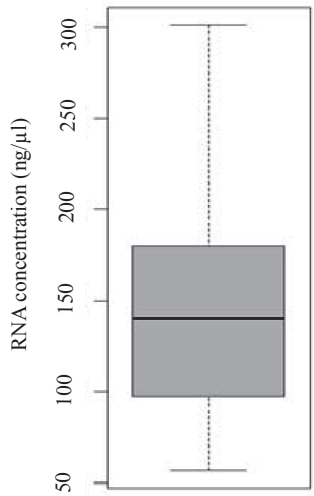


Figure 2. The basic statistical characteristics of obtained RNA concentration (n=15)

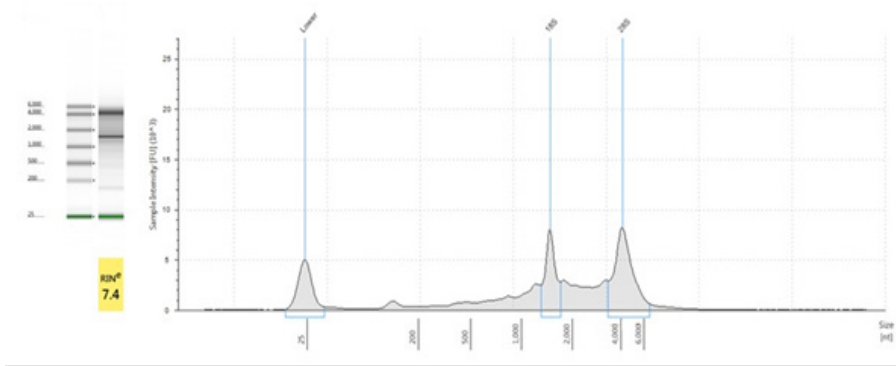


Figure 3. The quantity of one of the isolated RNA samples estimated on TapeStation 2200 (Agilent)

The horses after biopsy treatment were inspected twice a day. There were no reports of any disconcerting symptoms of inflammation or locomotive disorders. Due to the absence of stitching in the incision site, all the investigated horses had no scars or other signs of biopsy.

## Discussion

The Pro-Mag™ Ultra Automatic Biopsy Instrument is a reusable device which utilizes Pro-MagTM Ultra biopsy needles. This appliance was constructed to obtain high quality of histological preparations in human medicine. The biopsy needles are available in different sizes, in the range of 0.9–2.0 mm diameter and the length of 10 cm to 20 cm, the numerical markings facilitate the needle placement at a precise depth. Moreover, each needle has an echogenic tip to work under ultrasound guidance. The hub of needle was designed in the way to prevent sample contamination during the transfer of the probe. In the present study, the collected amount of muscle tissues was lower (an average of 15 mg) in comparison to sample capacity obtained by Bergstrom biopsy needle (an average of 250 mg), which was associated with the diameter of used biopsy needle (2 mm and 6 mm, respectively). On the other hand, the presented approach is much less invasive. While the biopsy with the use of Bergstrom needles requires skin incisions on the length of 3–4 cm (Ledwith and McGowan, 2004), the presented procedure avoids scarification of the skin and sutures. Thus, the biopsy site is less susceptible to infections, which prevents complications. In the present study, horses involved in sampling procedures were carefully observed and examined. There have been no reports of any complication such as hemorrhage, infection, petechiae or persistent soreness. Therefore, the horses could be taken to normal training regime and no loss of condition, lameness or markings were observed. The muscle biopsy using Pro-Mag™ Ultra biopsy needles was previously performed by Votion et al. (2010), who estimated the quantitative changes in muscle mitochondrial respiration of endurance horses. According to the authors, the amount of muscle obtained by needle aspiration biopsy was sufficient to determine the high-resolution respirometry (OXPHOS and ETS capacities). Furthermore, this microbiopsy technique was well tolerated by all investigated animals and also no stitches were required at the sampling site.

Since the skeletal muscle biopsy has been increasingly used to collect material for the genetic assays (Hill et al., 2010; Eivers et al., 2010; Park et al., 2012), there is a need to develop new methods which will be less invasive. In this study, the quantity of muscle samples gained in accordance with presented procedure was sufficient to obtain good quality of RNA. The average RNA concentration estimated by TapeStation 2200 (Agilent) was 148.3 ng/µl, while the minimum and maximum RNA concentrations were 56.8 and 301.2 ng/µl. The ratio of absorbance at 260 nm and 280 nm ranged from 1.79 to 1.94. Good quality of obtained RNA samples was confirmed by high RIN coefficient (RNA Integrity Number). RNA that has the above parameters is characterized by suitable quality and could be used in most genetic analyses.

The proposed type of biopsy extends the possibility to obtain an appropriate number of animal samples for genetic testing (microarrays, RNA-seq, real-time PCR) and moreover it could widen our knowledge of gene expression studies of methylome, miRNAome or proteome analysis in horses. Furthermore, user-friendliness, some technical specification of presented automatic biopsy instrument and minimal invasiveness for animal will probably in future enable securing other tissues and broadening the application to other diagnostic methods like immuno- or histochemistry.

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