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Technical note: Efficient protocol for isolation of total ribonucleic acid from lyophilized fat and muscle pig samples

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ABSTRACT: Isolation of total RNA from frozen muscle and fat samples typically results in small yields due to the presence of connective tissue between muscle fibers, which impairs complete tissue homogenization, and the excess of fat and relatively small cellularity of adipose tissue. Meat quality studies involve determination of fatty acid composition and content from muscle and subcutaneous fat samples, a process that may produce an excess of lyophilized tissue samples. The purpose of this work was to investigate the stability of total RNA in lyophilized tissue samples generated during the routine detection of fatty acid content of pig muscle and fat tissues, stored at room temperature or at −20°C. The protocol described here results in increased yields of total RNA from freeze-dried samples stored at −20°C, which facilitates the homogenization step. The isolated RNA is suitable for common gene expression techniques such as final point and quantitative reverse transcription-PCR.

Key words: freeze-dry, gene expression, lyophilization, meat quality, pig

INTRODUCTION

Fat composition and content are important traits influencing sensory and nutritional meat quality properties. Thus, there is a growing interest in the study of the molecular mechanisms underlying subcutaneous and intramuscular fat deposition events. Many of these studies focus on global or candidate gene expression characterization in muscle and fat tissues. Isolation of RNA from muscle and fat samples is largely inefficient because connective tissue within the muscle samples is difficult to homogenize, even with mechanical rotors, and the large fat content of adipose tissue interferes with the isolation process, whereas its relatively small cellularity results in decreased RNA concentrations.

Meat quality studies often rely also on muscle and fat chemical composition analysis, some of which involve previous sample lyophilization. Freeze-drying or lyophilization is a widely used technique for sample preservation commonly applied to store vaccines, microorganisms, and plant (Jaiprakash et al., 2003) and virus (Vaughan et al., 2006) samples. Although RNA is an unstable molecule, easily degraded by enzymes and at alkaline environments, viral RNA has been detected from a wide range of freeze-dried biological samples. The RNA is rapidly degraded in shelf-preserved freeze-dried biopsies, but it can still be used for some reverse transcription-PCR (RT-PCR) analyses (Matsuo et al., 1999). Despite these reports, the use of lyophilized animal samples is nearly anecdotic in molecular assays and particularly restricted to protein detection by Western blotting (Salomon et al., 1994) and tissue immunostaining (Louis et al., 2000).

The protocol presented here results in large yields of isolated total RNA from freeze-dried samples generated during the ordinary detection of fatty acid content of pig muscle and fat tissues. Additionally, the stability of total RNA in lyophilized tissue samples, stored at room temperature or at −20°C, and the suitability of this RNA as a start-up material for commonly used molecular techniques have been investigated.

MATERIALS AND METHODS

All the experimental procedures were approved by the Ethics Committee for Animal Experimentation of the University of Lleida.
Sample Collection and Processing

Samples of semimembranosus muscle and subcutaneous fat from a Duroc commercial line were collected at slaughter, snap frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analyzed. Once defrosted, muscle and adipose tissue samples were freeze-dried at \(-20^\circ\text{C}\), 0.01 hectopascals for 72 h using a HETO FDS system (Heto-Holten A/S, Allerod, Denmark) and thoroughly homogenized with sand using a glass stirring rod. Lyophilized samples were stored at room temperature for 5 d or at \(-20^\circ\text{C}\) before being used for RNA isolation.

RNA Isolation

Isolation of RNA was performed in parallel from frozen and freeze-dried samples. Muscle samples (60 mg if frozen or 30 mg if lyophilized) were homogenized in 1 mL of TRI Reagent (Sigma-Aldrich, Madrid, Spain) using a mechanical rotor (IKA Werke, Staufen, Germany) following the manufacturer’s instructions. For adipose samples, the protocol was modified as follows: fat tissue (0.3 g) was homogenized in 3 mL of TRI Reagent and centrifuged at 3,000 \(\times\) \(g\), 4°C, 20 min. The lower (aqueous) layer was vigorously mixed with 1 mL of chloroform for 30 s, incubated at room temperature for 10 min, and spun at 3,000 \(\times\) \(g\), 4°C, 20 min. From the aqueous layer, RNA was precipitated with ice-cold isopropanol and resuspended in 100 \(\mu\)L of diethyl-pyrocarbonate-treated water and quantified in a Nanodrop-1000 spectrophotometer (Nanodrop, Wilmington, DE). Integrity of the isolated RNA was checked by electrophoresis in a 1.2% formaldehyde-agarose gel using standard methods (Sambrook and Russell, 2001) and in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) where RNA quality was evaluated by the RNA integrity number calculated with the Agilent 2100 expert software (Schroeder et al., 2006).

First-Strand cDNA Synthesis

Total RNA (1 \(\mu\)g) was treated with Turbo DNA-free DNase (Ambion, Austin, TX) according to the manufacturer’s protocol and retrotranscribed with 0.5 pmol of random hexamers using 100 U of MuMLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) at 37°C for 1 h.

Final Point RT-PCR

The PCR reactions for the porcine low-density lipoprotein receptor (LDLR) mRNA were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA) as in Pena et al. (2009). Primer sequences are described in Supplemental Table 1 (http://jas.fass.org/content/vol88/issue2/).

Real-Time Quantitative RT-PCR

Single-strand cDNA was diluted 1:10 in diethyl-pyrocarbonate-treated water before real-time quantitative RT-PCR (qRT-PCR) analysis of 3 porcine genes [stearyl-CoA desaturase (SCD), acetyl-CoA carboxylase \(\alpha\) (ACACA) and hypoxanthine phosphoribosyltransferase 1 (HPRT)] Primer sequences and TaqMan probes are described in Supplemental Table 1 (http://jas.fass.org/content/vol88/issue2/). For the SCD and ACACA genes, real-time qRT-PCR assays were carried out in triplicate in an ABI-7500 device (Applied Biosystems, Foster City, CA) in a final volume of 5 \(\mu\)L containing 1 \(\times\) SYBRgreen Master mix (Applied Biosystems) and 200 nM of each primer. For the endogenous control (HPRT), reactions contained 300 nM primers, 200 nM TaqMan probe, and 1 \(\times\) Universal Taqman Master Mix (Applied Biosystems). The following thermal profile was used for all reactions: 10 min at 95°C, 40 cycles of 15 s at 93°C, and 1 min at 60°C. Mean expression measures from the endogenous control were used as an internal reference to normalize and quantify the expression data from the other 2 genes as in Yuan et al. (2006).

RESULTS AND DISCUSSION

In the present work, the use of freeze-dried pig muscle and adipose samples has been investigated as a more efficient source of total RNA for gene expression studies. For this, total RNA was isolated from tissues frozen at \(-80^\circ\text{C}\) and freeze-dried tissues that were then stored at \(-20^\circ\text{C}\) (Lyoph.+Frozen) or kept at room temperature for 5 d (Lyoph.+RT). The isolation protocol was slightly modified for the adipose samples to eliminate the excess of fat previous to RNA separation by the phenol acid method. Lyophilization of pig muscle and fat samples resulted in double the yield of isolated RNA per milligram of tissue without the need to scale up the TRI Reagent volume (Supplemental Table 2; http://jas.fass.org/content/vol88/issue2/). Although RT-PCR and many qRT-PCR studies do not require large amounts of RNA as a starting material, other gene expression applications such as microarrays require greater amount of total RNA at a concentration of >500 \(\mu\)g/\(\mu\)L. Isolating high yields from small samples would also be advantageous for the analysis of muscle and fat biopsies from time-course experiments (Bosch et al., 2009). Yields did not differ between lyophilized samples stored at \(-20^\circ\text{C}\) or at room temperature for 5 d. However, RNA integrity was lost in the latter (Figure 1 and Supplemental Figure 1; http://jas.fass.org/content/vol88/issue2/). Electrophoresis of total RNA gave comparable integrity results, as measured by the RNA integrity number, for frozen and Lyoph.+Frozen samples (Figure 1 and Supplemental Figure 1; http://jas.fass.org/content/vol88/issue2/), indicating that RNA remained stable during the freeze-drying process.
The suitability of RNA isolated from freeze-dried samples as a starting material for common gene expression techniques was tested by final point RT-PCR and by real-time qRT-PCR. Two fragments of 1,150 and 600 bp of the coding region of the pig \textit{LDLR} gene were successfully amplified from frozen and Lyoph.+Frozen samples in both tissues (Figure 2A). In contrast, only the smaller fragment could be amplified at a reduced efficiency from the Lyoph.+RT degraded RNA. Some reports indicate that RNA integrity does not affect amplification efficiency for many PCR-based applications on the range of 70 to 250 bp (Fleige and Pfaffl, 2006). Thus, qRT-PCR assays, which are usually based on very small amplicons (range of 75 to 150 bp), should not be influenced by degradation of starting RNA. Two genes related to the metabolism of fatty acids (\textit{SCD} and \textit{ACACA}) and 1 gene commonly used as a reference gene (\textit{HPRT1}) were analyzed by qRT-PCR. Identical amplification curves were obtained for the 3 genes in frozen and Lyoph.+Frozen samples (Supplemental Figure 2; \url{http://jas.fass.org/content/vol88/issue2/}). Thus, relative quantification of \textit{SCD} and \textit{ACACA} expression gave similar results in samples treated either way (Figure 2B). In contrast, a delayed amplification curve was obtained with fat Lyoph.+RT samples, indicating a less efficient amplification (Supplemental Figure 2B; \url{http://jas.fass.org/content/vol88/issue2/}). Consequently, quantification values differed from the 2 other sample types (Figure 2B), indicating that RNA obtained after storing of freeze-dried samples at room temperature might not always be suitable for quantitative methods such as qRT-PCR. It is important to remark that, in both tissues, amplification plots and quantification measures were undistinguishable between frozen and Lyoph.+Frozen samples, validating the use of freeze-dried samples for gene expression analysis.

In conclusion, the method presented here represents an alternative to isolate large yields of total RNA from freeze-dried muscle and fat samples such as those obtained after processing meat and subcutaneous fat for fatty acid content determination. Freeze-drying facilitates RNA isolation from tissues difficult to homogenize (Tsuka et al., 1997), whereas integrity of RNA, end point, and quantitative RT-PCR results are not impaired. Although results for a limited number of samples are presented here, we routinely use lyophilized samples in gene expression assays with consistent results. In the practicalities of meat quality projects, this new protocol eliminates the need to collect additional tubes for DNA/RNA/protein studies, unburdening the tissue-collecting protocol at the abattoir.
Figure 2. Isolated RNA was used in 2 common gene expression assays. A) Two fragments of 1,150 and 600 bp from the coding region of pig low-density lipoprotein receptor (LDLR) gene were successfully amplified by reverse transcription-PCR (RT-PCR) from frozen samples and samples lyophilized and then frozen at −20°C (Lyoph.+Frozen), whereas samples lyophilized and then stored at room temperature (Lyoph.+RT) amplified only the smaller fragment; B) quantitative RT-PCR analysis of 2 genes [stearoyl-CoA desaturase (SCD) and acetyl-CoA carboxylase α (ACACA)] gave identical results in frozen and Lyoph.+Frozen samples, whereas Lyoph.+RT estimations differed in adipose fat. Bars represent mean of 3 replicates. Error bars indicate SE.

LITERATURE CITED


**Supplementary Material**  
Supplementary material can be found at:  
http://jas.fass.org/content/suppl/2010/01/28/jas.2009-2298.DC1.html

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