Benchmark

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Optimized RNA isolation of FFPE uterine scar tissues for RNA expression analyses delineated by laser microdissection

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ABSTRACT

Samples for histological analyses are often formalin-fixed paraffin-embedded (FFPE) and slide-mounted, which complicates RNA extraction for many downstream molecular applications. Furthermore, when the region of interest is extremely small due to isolation with laser microdissection (LMD), extracting RNA of adequate quality and quantity is difficult. We describe an optimized protocol for maximizing RNA output from FFPE tissue devised to identify and analyze gene expression of human maternal uterine scar tissue obtained from uterotomy scars resulting from prior cesarean deliveries. Gomori trichrome staining allowed for region identification for LMD. Successful RNA isolation, reverse transcription and, importantly, quantitative real-time PCR (qRT-PCR) were performed. This report provides an optimized step-by-step protocol yielding sufficient RNA for qRT-PCR analyses from challenging tissue/LMD-FFPE samples.

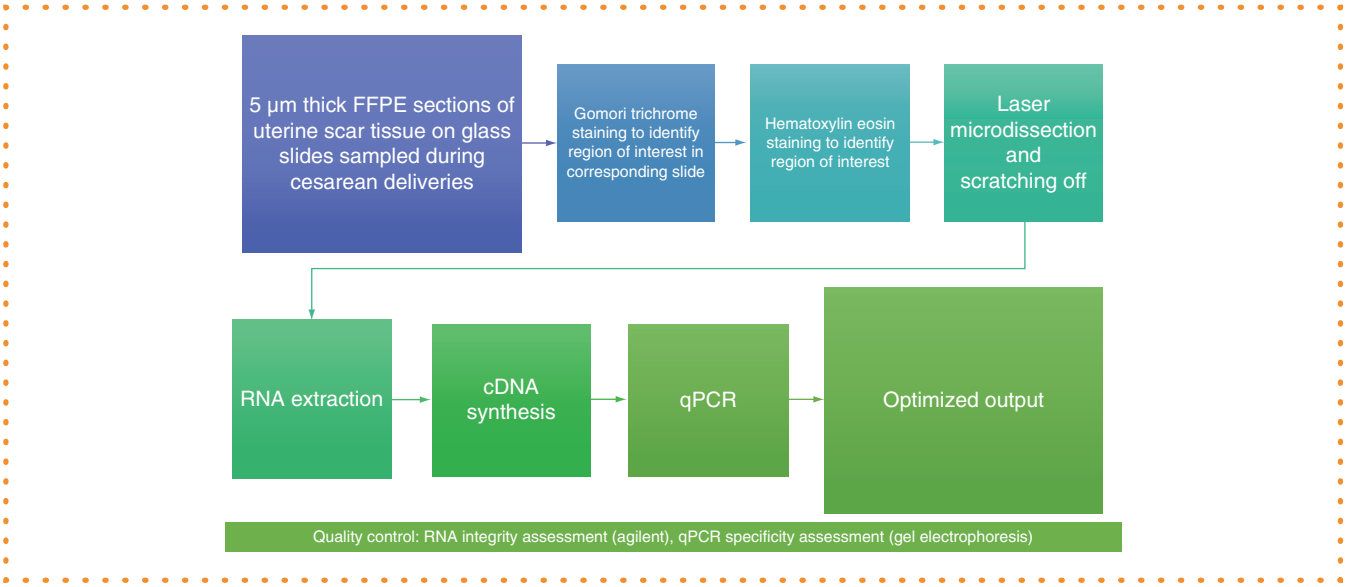
METHOD SUMMARY

FFPE sample sections were mounted on glass slides and stained with hematoxylin and eosin (HE). A corresponding slide was stained according to Gomori trichrome for orientation. Regions of interest in HE-stained samples were marked using laser microdissection (LMD) and subsequently scratched off the slide with a sterile scalpel. RNA extraction and cDNA synthesis were carried out, and quantitative real-time PCR with TaqMan probes was performed.

TWEETABLE ABSTRACT

We describe a protocol for maximized RNA output from FFPE tissue delineated by laser microdissection devised to analyze gene expression of human maternal uterine scar tissue obtained from uterotomy scars of prior cesarean deliveries.

GRAPHICAL ABSTRACT



KEYWORDS:
FFPE ● laser microdissection ● LMD ● qRT-PCR ● RNA expression ● RNA isolation ● uterine scar tissue

Formalin fixation and paraffin embedding (FFPE) is a practical method for long-term storage at room temperature and for a large amount of samples. However, extracting RNA of adequate quality and quantity from slide-mounted FFPE sections for downstream analyses, such as quantitative real-time PCR (qRT-PCR) is challenging because formalin treatment damages RNA, resulting in fragmentation and crosslinking [1]. It becomes even more complex when only small areas of the sections contain the region of interest (ROI). One example of such challenging tissue samples are specimens of the uterine wall, which were excised during cesarean delivery with the aim of examining the uterine scar tissue from prior cesarean deliveries. Due to the difficulty of locating the scarred area intraoperatively, samples often contain mostly large parts of unscarred myometrium, whereas the scar itself occupies only a small area of the sample. Laser microdissection (LMD) is the optimal method to specifically target these regions. However, generating sample material with LMD is significantly more laborious with samples on glass slides compared with membrane slides. Therefore, LMD and a subsequent manual transfer of the ROI is the ideal combination to protect the tissue from damage by lengthy processing time. Furthermore, human myometrium is a complex tissue that undergoes constant changes in its hormonal profile, cell size and differentiation during pregnancy, making gene expression analysis additionally difficult [2].

Despite the aforementioned challenges, we have successfully developed a step-by-step protocol for maximizing RNA output from slide-mounted FFPE tissue to identify and analyze gene expression of human maternal uterine scar tissue obtained from uterotomy scars from prior cesarean deliveries [3]. The samples were taken during the QUWACS study (Quantifying Uterine Wound Healing after Cesarean Section) that was performed at the Charité University hospital Berlin, Germany during 2018–2020. All women had provided signed informed consent under protocols approved by the Institutional Review Board at Charité – Universitätsklinikum Berlin, Berlin, Germany (EA4/159/16). Gomori trichrome staining was established as a suitable method to identify the ROI for LMD. Immediately after collection, samples were cut using anti-RNase treated surfaces and equipment to a thickness of 1 cm and fixed in 4% neutrally buffered formalin. Samples were left in the fixant for at least 24 h and subsequently dehydrated, paraffinized (Citadel 1000, Shandon GmbH, Frankfurt am Main, Germany), embedded in paraffin (Histocentre2, Shandon GmbH, Frankfurt am Main, Germany) and stored at room temperature. Sections were cut to 5 μm using a microtome (Microm HM 340E, Thermo Scientific, Walldorf, Germany). Subsequently, sections were mounted on glass slides (SuperFrost Plus; R. Langenbrinck, Emmendingen, Germany), dried overnight at 37°C in an incubator (Heraeus, Hanau, Deutschland) and then stored at room temperature until further use. To identify the scarred ROI with increased collagen content, one slide was stained according to Gomori trichrome, resulting in green collagen fibers and pink muscle cells (Figure 1A–C). Collagen content in the scarred and unscarred regions was analyzed with the software Fiji (NIH, MD, USA) (Figure 1D). Sample sections for RNA extraction were stained with hematoxylin eosin (H&E) to identify basic structures necessary for orientation during LMD resulting in dark pink collagen fibers and light pink muscle cells (Figure 1E). For this purpose, a rapid H&E staining protocol was implemented with 30-s steps to reduce RNA degradation by RNases activated by liquids. Additionally, water used in this protocol was treated with diethylpyrocarbonate (DEPC), and final dehydration steps were carried out with undenatured ethanol. For the last step, 100% ethanol was used instead of xylene because the latter can interfere with RNA extraction and integrity [4]. The remaining alcohol evaporated under the hood for 30 s, and the slide was immediately transferred under the laser microscope. The remaining stained glass slides were stored at 4°C in a sterile falcon tube. Subsequently, the ROI was outlined using laser under the microscope (LMD5 Leica Microsystems, Wetzlar, Germany) based on the scarred area defined in the corresponding Gomori trichrome-stained slide. Laser power was set to maximum to enable macroscopic identification of the ROI (Figure 1E). The ROI was then scratched from the slide with a sterile scalpel and transferred into digestion buffer (supplied with the kit) together with an RNase inhibitor (Sigma Aldrich, Merck, Darmstadt, Germany). When sufficient tissue ($\geq$400 mm^2) had been collected, the samples were stored at 4°C for up to 24 h. To obtain the necessary amount of tissue, the procedure was performed on up to 64 slides per patient and collected the tissue in a 1.5-ml tube. To dissolve the muscle tissue more easily, the samples were then briefly frozen at -20°C and thawed again right before the RNA extraction. RNA extraction of was carried out using the RNeasy FFPE kit (Qiagen, Hilden, Germany) [5]. Importantly, proteinase K treatment incubating at 56°C overnight up to 24 h in a thermomixer (Eppendorf, Hamburg, Germany) instead of the recommended incubation period of 10 min proved necessary to yield optimized ratios. Genomic DNA (gDNA) digestion with DNase was carried out during RNA extraction. RNA concentration was measured (230:260 and 260:280 ratios) with a Nanodrop spectrophotometer (Thermo Fisher Scientific, MA USA).

Subsequently, RNA strand integrity was measured with the Agilent Bioanalyzer (Agilent 2100 Bioanalyzer, CA, USA). Reverse transcription was carried out in Eppendorf Mastercycler epgradient S (Eppendorf) using 1-μg RNA per sample (iScript cDNA Synthesis Kit, BioRad, Feldkirchen, Germany), resulting in a cDNA concentration of 50 ng/μl. cDNA was stored at -20°C. Negative controls without reverse transcriptase or without RNA template were examined to rule out contamination by gDNA or other substances.

qRT-PCR was carried out with 50 ng cDNA starting material in singleplex using TaqMan probe technology (Thermo Fisher Scientific) with an ABI 7500 PCR machine (Applied Biosystems, Thermo Fisher Scientific). Starting cDNA amount was determined by a tenfold dilution series, as the most efficient amount of cDNA with which all assays worked reliably and provided enough remaining limited material for all experiments. *COL1A2* was analyzed as a gene of interest due to its significant role in scarring [6]. *ATP5B* and *EIF2A*

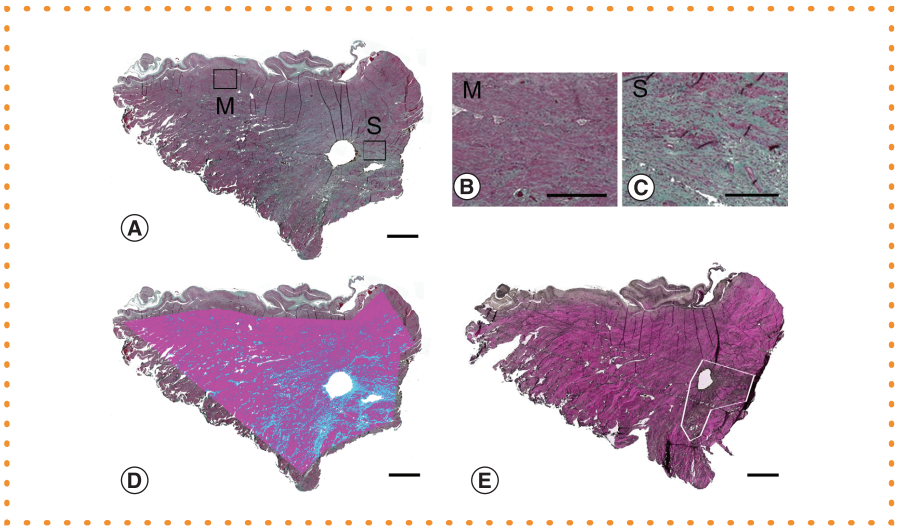


Figure 1. Gomori trichrome staining of a scarred myometrial sample. (A) M denotes an exemplary region of unscarred myometrial tissue, S denotes an exemplary region of scarred myometrium. **(B)** Magnification of unscarred myometrial region (M in **[A]**). **(C)** Magnification of the scarred region (S in **[A]**). **(D)** Fiji analysis of Gomori trichrome stained sample, magenta indicates healthy myometrium and cyan indicates scarred myometrium. **(E)** Corresponding section with hematoxylin and eosin staining, region outlined by laser, for demonstration purposes repainted with GIMP (open source program, https://www.gimp.org/). Scale bars in **(A, D & E)**: 2 mm. Scale bars in **(B & C)**: 0.5 mm.

were reference genes based on previous studies in the literature testing reference genes for myometrium [7–9]. Applied Biosystems TaqMan primer assays were used (ID: *COL1A2*: Hs01028956_m1; *ATP5B*: Hs00969569_m1; *EIF2A*: Hs00230684_m1). Assays had short amplicon length to amplify as many of the short RNA fragments caused by formalin fixation as possible [10] and exon-spanning design to prevent gDNA amplification. Before stability of the reference genes across the sample population could be tested, we validated that the expression abundance was enough to enable amplification from FFPE tissue.

Because of the limited amount of scratched tissue, efficiencies were tested in pooled matched fresh frozen (FF) myometrial tissue samples [11]. For plate setup, the sample maximization method was implemented with one assay in singleplex on each plate and qRT-PCR runs of 45 cycles of alternating denaturation and annealing-elongation steps to ensure amplification. The amplified qRT-PCR products sizes and banding were validated with agarose gel (5%) electrophoresis.

RNA was obtained from six patient samples, using a median amount of 520 mm^2 (interquartile range [IQR]: 380–1050 mm^2) from up to 64 sections of 5-μm thickness per patient. This yielded a median amount of 1184.05 ng/mm^3 tissue (IQR: 628.45–2178.52) with excellent ratios for FFPE tissue (optical density [OD] 260:280 ratio: median 1.96 [IQR: 1.93–1.96], OD 230/260 ratio: median 1.96 [IQR: 1.91–1.96]). The obtained RNA amounts are comparable those in the publication of Choi *et al.*, who gained 2762.94 ng/mm^3 (2039.94–4197.73) from an average area of 235.35 mm^2 from 10-μm sections of FFPE breast cancer tissues [12]. Another study reported only one-hundredth of the RNA quantity from stored FFPE tissue [13]. qRT-PCR showed exponential amplification in FFPE tissue with *COL1A2* as early as Cq = 28 (range: 28.27–33.66, standard deviation SD: 2.03) compared with matched samples of FF tissue (range: 20.65–21.53, SD: 0.29) (Figure 2A). Cq-values for reference genes were as follows: *ATP5B* in FFPE tissue (range: 34.33–40.33, SD: 1.93) and in FF tissue (range: 23.46–24.92, SD: 0.51) (Figure 2B); *EIF2A* in FFPE tissue (range: 36.70–39.80, SD: 1.10) and in FF tissue (range: 24.53–25.42, SD: 0.32) (Figure 2C).

Ratios in FF tissue differed only slightly from FFPE tissue (OD 260/280 ratio: median 2.03 [IQR: 1.98–2.04], OD 230/260 ratio: median 1.78 [IQR: 1.66–1.98]). Exponential amplification started later (approximately eight cycles for *COL1A2*, 11 cycles for *ATP5B* and 12 cycles for *EIF2A*) in FFPE tissue than in FF tissue possibly due to RNA fragmentation during formalin fixation [14], suggesting that fewer gene copies are transcribed in FFPE tissue versus intact FF tissue. RNA integrity number of FFPE tissue reflected heavily fragmented RNA (range: 1.8–2.4, SD: 0.21), which can be expected after formalin fixation [15]. Gel electrophoresis showed one band per product with correct length (*COL1A2* 71 base pairs [bp], *ATP5B* 96 bp, *EIF2A* 106 bp; Figure 2D) and no bands detected in negative controls.

In conclusion, this is the first study to demonstrate that appropriate-quality RNA can be obtained for successful qRT-PCR analyses in challenging FFPE tissues mounted on glass slides, even with a limited ROI and especially in this difficult, sparsely researched tissue. Moreover, this work shows that a precisely defined ROI using Gomori trichrome staining and Fiji analysis makes subsequent LMD and scratching off the ideal method for efficient use of mounted FFPE sections for downstream analyses. The described combination of the two methods has proven to be the optimal, time-saving approach that protects RNA to obtain greater ROI samples, integrating the precision of LMD and the speed of scratching off. This would not be possible for much smaller areas such as single-cell isolation in which the classic method of using LMD alone for lasering the cells from the glass slide or scratching the entire section from the slide would be implemented, as has been successfully shown in previous studies using qRT-PCR [16,17].

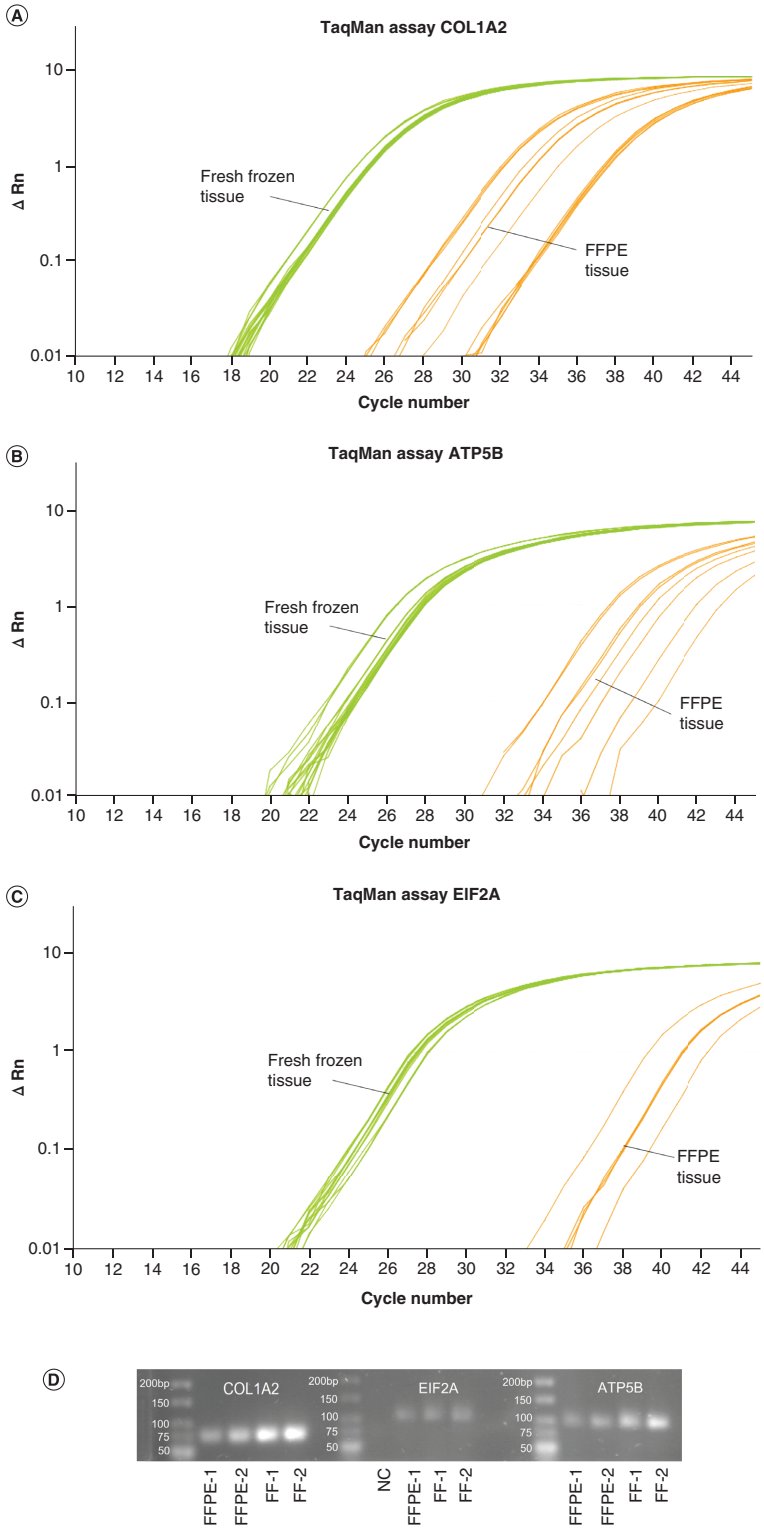


Figure 2. Output from TaqMan quantitative real-time PCR (qRT-PCR) in matched fresh frozen (FF) and formalin fixation and paraffin embedding (FFPE) uterine tissues. (A) Cq values of *COL1A2* in a subset of FFPE samples (n = 6, orange curves) compared with matched FF tissue samples (n = 6, green curves). **(B)** Cq values of *ATP5B* FFPE samples (n = 6, orange curves) compared with matched FF tissue (n = 6, green curves). **(C)** Cq values of *EIF2A* in FFPE samples (n = 4, orange curves) compared with matched FF tissue samples (n = 4, green curves). Every qRT-PCR had the same cDNA concentration per well for each gene shown in A, B & C. **(D)** Example of 5% agarose gel validating product size of FFPE and FF tissues qRT-PCRs shown in **(A, B & C)**; expected product sizes: 71 bp-*COL1A2*, 106 bp-*EIF2A* and 96 bp-*ATP5B*, negative control (NC), (GeneRuler Ultra Low Range DNA Ladder, Thermo Fisher Scientific).

Author contributions

A Paping: study design, sample collection, performing cesarean deliveries, sample analyses, data analysis, writing of the manuscript and revision of the manuscript. C Basler: study design, sample collection, sample analyses, data analysis, writing of the manuscript and revision of the manuscript. RC Rancourt: study design, laboratory analyses, data analysis and revision of the manuscript. L Ehrlich: study design, sample collection, laboratory analyses, data analysis and revision of the manuscript. K Melchior: study design, sample collection, laboratory analyses, data analysis and revision of the manuscript. W Henrich: study design, performing cesarean deliveries and revision of the manuscript. T Braun: study design, sample collection, performing cesarean deliveries, sample analyses, data analysis, revision of the manuscript. All authors reviewed and approved the final submitted version of the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All women provided signed informed consent under protocols approved by the Institutional Review Board at Charité – Universitätsklinikum Berlin, Berlin, Germany (EA4/159/16). The data are available on request.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Chung JY, Braunschweig T, Williams R *et al.* Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* 56(11), 1033–1042 (2008).
• **This article describes the impact of tissue fixation and processing on the quantity and quality of the RNA in formalin-fixed paraffin-embedded samples.**
2. Bethin KE, Nagai Y, Sladek R *et al.* Microarray analysis of uterine gene expression in mouse and human pregnancy. *Mol. Endocrinol.* 17(8), 1454–1469 (2003).
3. Paping A, Basler C, Rancourt RC *et al.* Optimized protocol for the RNA isolation of laser microdissected formalin-fixed paraffin-embedded uterine scar tissues for RNA expression analyses. *protocols.io* **https://doi.org/10.17504/protocols.io.byhgpt3w** (2022).
4. Amini P, Ettlin J, Opitz L *et al*. An optimised protocol for isolation of RNA from small sections of laser-capture microdissected FFPE tissue amenable for next-generation sequencing. *BMC Mol. Biol.* 18(1), 22 (2017).
5. Seiler C, Sharpe A, Barrett JC *et al*. Nucleic acid extraction from formalin-fixed paraffin-embedded cancer cell line samples: a trade off between quantity and quality? *BMC Clin. Pathol.* 16, 17 (2016).
• **This article compares different RNA extraction kits for formalin-fixed paraffin-embedded samples analyzing RNA quality and quantity.**
6. Chai CY, Song J, Tan Z *et al*. Adipose tissue-derived stem cells inhibit hypertrophic scar (HS) fibrosis via p38/MAPK pathway. *J. Cell Biochem.* 120(3), 4057–4064 (2019).
7. Almeida TA, Quispe-Ricalde A, Montes De Oca F *et al*. A high-throughput open-array qPCR gene panel to identify housekeeping genes suitable for myometrium and leiomyoma expression analysis. *Gynecol. Oncol.* 134(1), 138–143 (2014).
8. Arenas-Hernandez M, Vega-Sanchez R. Housekeeping gene expression stability in reproductive tissues after mitogen stimulation. *BMC Res. Notes* 6, 285 (2013).
9. Arrowsmith S. Identification and validation of suitable reference genes for quantitative real-time PCR gene expression analysis in pregnant human myometrium. *Mol. Biol. Rep.* 48(1), 413–423 (2021).
•• **This is the first article on reference genes in pregnant human myometrium.**
10. Antonov J, Goldstein DR, Oberli A *et al.* Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization. *Lab. Invest.* 85(8), 1040–1050 (2005).
• **This article describes that fragmented RNA can be reliably analyzed in qRT-PCR using primers with short amplicon length and optimal reference genes.**
11. Svec D, Tichopad A, Novosadova V *et al*. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. *Biomol. Detect. Quantif.* 3, 9–16 (2015).
12. Choi Y, Kim A, Kim J *et al*. Optimization of RNA extraction from formalin-fixed paraffin-embedded blocks for targeted next-generation sequencing. *J. Breast Cancer* 20(4), 393–399 (2017).
13. Chung JY, Hewitt SM. An optimized RNA extraction method from archival formalin-fixed paraffin-embedded tissue. *Methods Mol. Biol.* 611, 19–27 (2010).
14. Sanchez-Navarro I, Gamez-Pozo A, Gonzalez-Baron M *et al.* Comparison of gene expression profiling by reverse transcription quantitative PCR between fresh frozen and formalin-fixed, paraffin-embedded breast cancer tissues. *Biotechniques* 48(5), 389–397 (2010).
• **This article analyzes the performance of formalin-fixed paraffin-embedded and fresh-frozen tissues in qRT-PCR.**
15. Wehmas LC, Wood CE, Chorley BN *et al*. Enhanced quality metrics for assessing RNA derived from archival formalin-fixed paraffin-embedded tissue samples. *Toxicol. Sci.* 170(2), 357–373 (2019).
• **This article evaluates adequate methods to measure RNA quality in formalin-fixed paraffin-embedded samples.**

16. Gjerdrum LM, Sorensen BS, Kjeldsen E *et al*. Real-time quantitative PCR of microdissected paraffin-embedded breast carcinoma: an alternative method for HER-2/neu analysis. *J. Mol. Diagn.* 6(1), 42–51 (2004).
17. Yang W, Maqsodi B, Ma Y *et al.* Direct quantification of gene expression in homogenates of formalin-fixed, paraffin-embedded tissues. *Biotechniques* 40(4), 481–486 (2006).