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ARTICLE

Extraction of RNA from tough tissues with high proteoglycan content by cryosection, second phase separation and high salt precipitation

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Abstract Reverse-transcription quantitative PCR (RT-qPCR) is a powerful technique for quantification of gene expression. Extraction of RNA directly from human and large animal intervertebral disc (IVD) tissue is technically challenging due to its tough nature, low cell-to-matrix ratio and high proteoglycan content. IVD is consisted of nucleus pulposus (NP) and annulus fibrosis (AF). We compared the yield and quality of RNA extracted from cultured cells using TRIzol and combined TRIzol-column method (TRIspin) with and without bovine NP tissue added for high proteoglycan content simulation. Higher yields were obtained using the TRIzol method compared to the TRIspin method and the quality of RNA extracted using both methods was comparable. Cryosectioning was by far the most effective homogenization method for the tough bovine NP tissue. The typical modification to TRIzol extraction by the use of high salt solution in a previously reported study for tissue with high proteoglycan was insufficient for bovine NP tissue, where undesirable precipitates were formed during the RNA precipitation step. This necessitates other modifications to the protocol. In our study, the combination of additional phase separation and high salt precipitation was shown to be effective to avoid the formation of the undesirable precipitates. The modified TRIzol method was compared with the TRIspin method and shown to give higher RNA yield which resulted in smaller Ct values in RT-qPCR. In addition, we showed that the cryosectioning method was applicable to bovine muscle, mouse IVD and rat NP tissues. This method of RNA preparation also allows simultaneous preparation of tissue for histological study and quantitative gene expression analysis.

Keywords: RNA extraction, cryosection, nucleus pulposus, proteoglycan, TRIzol

INTRODUCTION

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is a powerful method to assess the gene expression at the transcript level. In many applications, especially for tissues, it requires the extraction of RNA prior to reverse transcription. Extraction of RNA directly from intervertebral disc (IVD) tissue or cartilage has been difficult due to its low cell density and high proteoglycan content [1,2]. An IVD is consisted of a nucleus pulposus (NP) core surrounded by a thick outer ring of fibrous annulus fibrosus (AF) and is sandwiched between cartilage endplates [3]. Cells in bovine NP tissue and AF tissue only occupy around 0.19% and 0.82% volume of the tissues respectively while there is 33.9% dry weight of proteoglycan in bovine NP and 9.7% in AF from adult animals of 18–36 month old [4]. There were studies assessing the relative mRNA levels of IVD tissues using cells isolated from these tissues by collagenase treatment [5-8]. Typically, the enzymatic cell isolation method requires the dicing of tissues into small pieces and enzymatic digestion for at least two hours. This may result in changes in relative mRNA levels, especially for those with short half-lives. Sharova et al. have shown that mRNA for all genes in mice has a median half-life of 7.1 h and the half-life for mRNA for some genes (<100 genes) may be less than 1 h [9]. This suggests the possible necessity of quenching RNA degradation before RNA extraction for some short lived genes in some studies. A comparison of different tissue processing methods for RNA extraction from tissues was given in **Figure 1** and **Table 1**.

We tested different methods to homogenize the bovine NP tissue using an electric pestle and mortar system but it was difficult to break the tough tissue when it was in frozen state. Cryosection was also tested since bovine NP tissue sections have been successfully prepared for histology. In laser capture microdissection (LCM), cryosectioning has been reported in preparing sections on glass or membrane slides [10-15]. With LCM, a specific region of interest can be selected for RNA extraction. This can be a viable option for extracting RNA from bovine or human NP tissue which has high proteoglycan content and low cellularity as LCM can exclude the high proteoglycan region of the tissue in RNA extraction. However, LCM is expensive and not readily accessible for every laboratory. The staining procedures required for cell visualization may also induce RNA degradation. Therefore, we explored other methods to extract RNA from NP tissue. There are only a few studies which extract RNA directly from NP tissue (summarized in **Table 2**) and none of the reported studies used cryosections as a means to break the tissue for RNA release. On the other hand, RNA was successfully extracted from kidney cryosections using a RNeasy

isolation kit [16]. Compared to kidney tissue, bovine and human NP tissues have high proteoglycan content with low cellularity. Hence a higher extraction efficiency in terms of RNA yield is desirable.

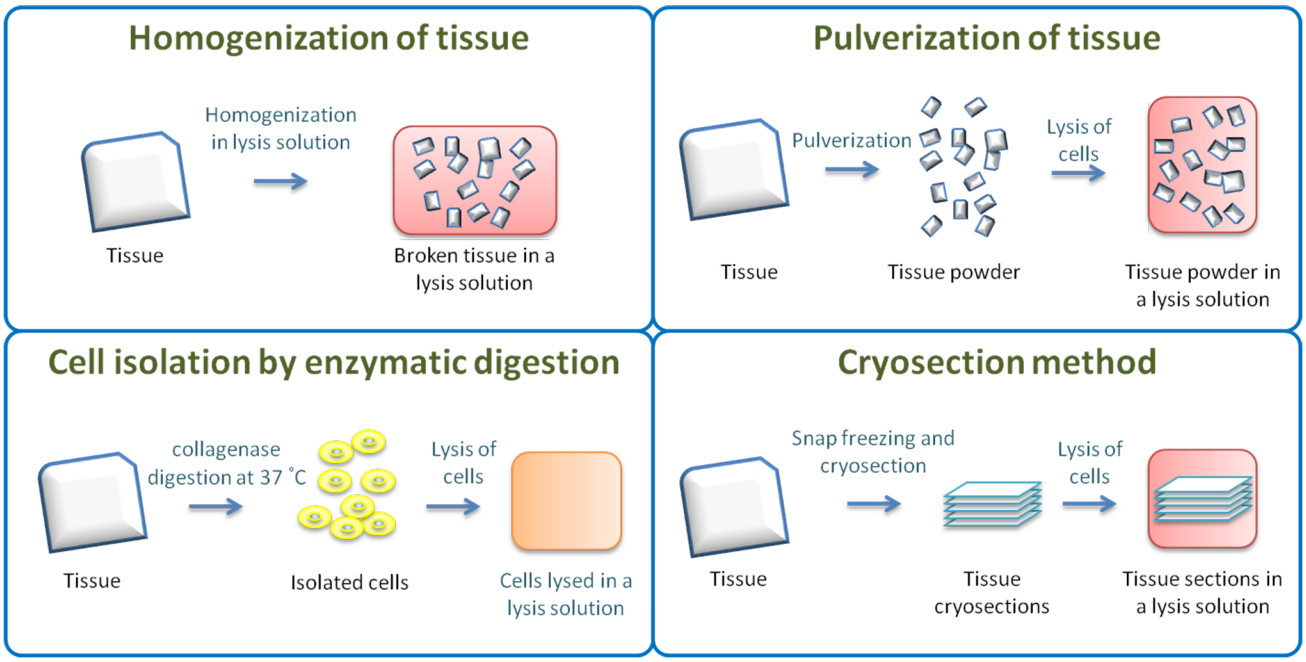

Figure 1. Schematics showing different tissue processing methods for RNA extraction from tissues.

Table 1. Comparison of the advantages and disadvantages of different tissue processing methods for RNA extraction.

	Homogenization/ pulverization	Enzymatic method	Cryosection
√	• No cell isolation procedure	• Can concentrate the cells and remove the extracellular matrix, facilitating RNA extraction • Can also obtain cells for culturing	• Can prepare cryosections for histological study at the same time • Minimal time of cells at room temperature or elevated temperature • No cell isolation procedure
×	• Heat may be generated, resulting in RNA degradation • May be difficult to break the tough IVD tissues • Requires a homogenizer/ pulverizer	• Relative mRNA levels may change during the cell isolation • Extra time is required to isolate cells before RNA extraction	• Requires a cryostat

Table 2 Studies that investigated the gene expression in IVD tissues through direct RNA extraction from the tissues.

Tissue	Tissue processing	Homogenizer used	Extraction	Tissue amount per sample	Reference
Human NP tissue	Homogenized in TRIzol on ice	Omni TH Homogenizer	TRIzol extraction followed by RNeasy mini clean up	100–300 mg	Wang *et al.* [17]
Human NP and AF tissue	Frozen at -80°C and pulverized on dry ice	Stainless steel mortar	Aurum Total Mini Kit	Not mentioned	Capossela *et al.* [18]
Rat NP tissue	Pulverized under liquid nitrogen	Not specified	RNeasy mini kit	Not mentioned	Yurube *et al.* [19]
Rabbit NP and AF tissue	Pulverized after freezing in liquid nitrogen	Not specified	Not specified	Not mentioned	Mwale *et al.* [20]
Rat NP and AF	Flash-frozen in liquid nitrogen, pulverized and homogenized in TRIzol	Not specified	RNeasy mini kit (TRIspin)	Discs from four rats	Tang *et al.* [21]

Table 3. Comparison of the advantages and disadvantages of different RNA extraction methods.

	GITC based method (eg. TRIzol, TRI-reagent)	TRIspin (Combined GITC-column based method) (eg., TRIzol + RNeasy)	Column based method (eg. RNeasy)
√	• Possible higher yield for a small number of cells	• Easy and quick	• Easy and quick
×	• Longer time • Demands more techniques	• The RNA yield may be low for a smaller number of cells • Higher cost	• The RNA yield may be low for a smaller number of cells • May not be effective for some tissues

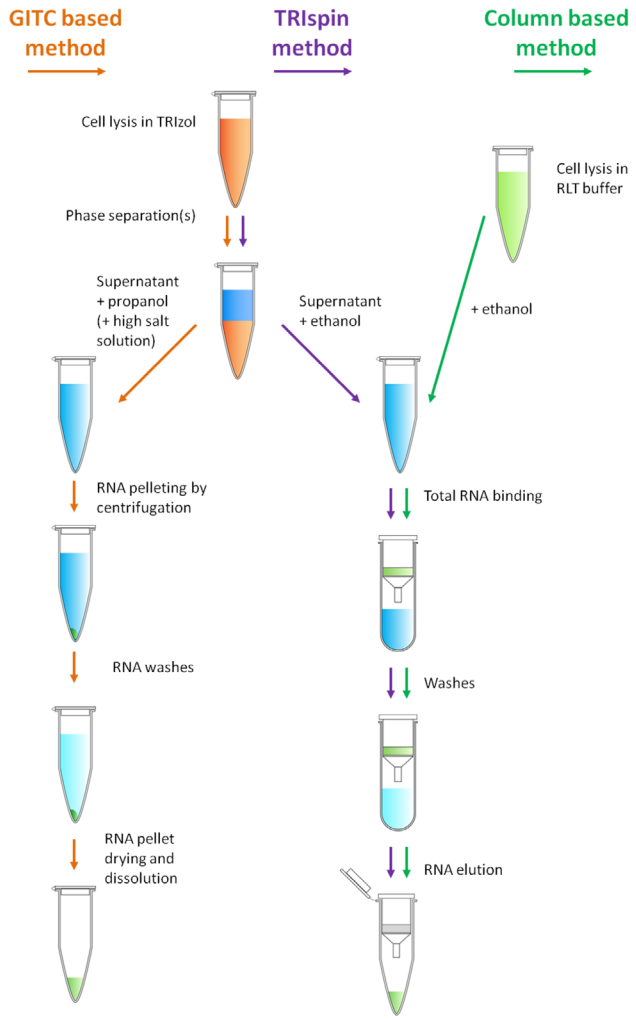

Figure 2. Schematics of RNA extraction procedures for GITC based method (orange arrows, e.g., TRIzol), combined GITC-column based (TRIspin) method (purple arrows, e.g., TRIzol + RNeasy) and column based method (green arrows, e.g., RNneasy kit).

Table 3 compares three commonly used methods for RNA extraction. They are the guanidinium thiocyanate (GITC) based method (e.g., TRIzol, TRI Reagent, TRIsure, STAT-60), the column based method (e.g., RNeasy, Aurum Total Mini Kit) and the TRIspin method which is a combination of the GITC based method and the column based method. The procedures for these 3 methods are summarized in the schematics in **Figure 2**. Some studies have suggested the yield of RNA obtained using the GITC-based method can be higher than the column based method for some samples [22-25].

For extracting RNA from bovine NP tissue directly using TRIzol, undesirable precipitates were observed even with the use of high salt solution as recommended in the manufacturer's instruction and a reported study for samples with high proteoglycan content [26]. Therefore further modifications of the protocol were explored. The lack of reported study using TRIzol extraction with NP cryosections prompted us to modify the typical TRIzol extraction procedure for bovine NP cryosections with an attempt to obtain a higher RNA yield than column based methods. The summary of experiments in this report and the associated rationale of experimental design are given in **Figure 3**. An overview of the suggested experimental procedure for extraction of RNA from tissues rich in extracellular matrix with low or high cellularity is given in **Figure S1**, Section A of the supplementary information.

MATERIALS AND METHODS

Tissue harvest

Bovine NP, AF and muscle tissues were harvested from bovine tails of young adult animals (18 - 36 month old). Rat NP tissue was harvested from tails of Lewis rats. Mouse IVDs were harvested from tails of NOD/SCID mice. The tissues were snap frozen in liquid nitrogen to quench RNA degradation as early as possible. Before further processing, the harvested tissue was kept at -80°C, which most enzymatic activities are very slow.

Tissue sectioning and cell lysis in TRIzol or RLT buffer

The tissues were put in a cryostat set at around -20°C before cryosectioning into 10–30 µm sections. The details of the procedures were given in the supplementary information. The tissue sections were kept frozen in the cryostat or liquid nitrogen during transfer before the addition of TRIzol to avoid RNA degradation. The cryosections were lysed with 0.5 ml of TRIzol or 0.35 ml of RLT buffer supplied in the RNeasy kit with β-mercaptoethanol added to 1%. The lysates were stored at -80°C before RNA extraction. The tissue was removed by centrifugation before RNA extraction.

Extraction of RNA from bovine NP tissue using TRIspin

For the combined TRIzol-column based (TRIspin) method, an extra 0.5 ml of TRIzol was added to lysate in TRIzol as described in Wang et al [27]. In brief, the aqueous phase after phase separation of the TRIzol protocol was mixed with 0.5 volume of 100% ethanol. The mixture was then applied to the RNeasy spin columns followed by purification according to the manufacturer's instruction.

Extraction of RNA from bovine NP tissue using RNeasy kit

For the RNeasy method, an extra 0.35 ml of RLT buffer was added to the sample and the extraction was performed according to the manufacturer's protocol.

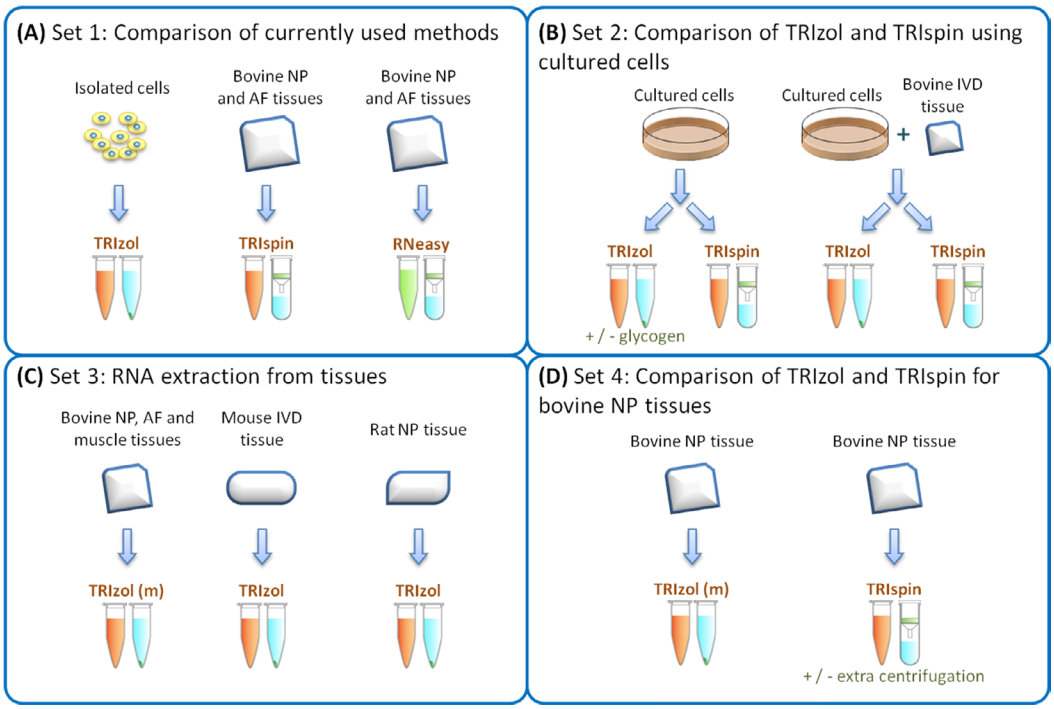

Figure 3. Summary of experiments in this study. A. Set 1: Comparison of currently used methods. We first compared the extraction of RNA from NP tissue sections using RNeasy and using TRIspin and from isolated cells using TRIzol since TRIzol could not be used with bovine NP tissue directly. **B.** Set 2: Comparison of TRIzol and TRIspin using cultured cells. In order to test whether TRIzol could give a higher yield for a small number of cells, we tested the extractions with TRIspin and TRIzol using a small number of cells with and without the addition of NP tissue extract. Our result showed that the TRIzol method gave a higher RNA yield. **C.** Set 3: RNA extraction from tissues. We investigated different approaches to extract RNA from NP tissue directly using TRIzol. With the modified protocol, RNA could be extracted from bovine NP tissue. We tested the modified procedures for bovine NP, AF and muscle tissues. RNA extraction was also performed with mouse IVD and rat NP cryosections. **D.** Set 4: Comparison of TRIzol and TRIspin for bovine NP tissues. The RNA extracted using the modified TRIzol method was compared with that using the TRIspin method by UV absorbance and RT-qPCR. ["TRIzol (m)" denotes the modified TRIzol extraction with an extra phase separation and use of high salt solution, and "TRIzol" denotes the typical TRIzol extraction with the use of high salt solution].

Extraction of RNA from bovine NP isolated cells using TRIzol

Bovine NP and AF tissues were cut into small pieces of 1–2 mm. The weights of the tissue were estimated based on the weights of tubes with and without the tissue. Approximately 500 mm^3 of tissue was used per sample. The tissue pieces were digested with 0.5% pronase (Roche) in serum free DMEM (Gibco) for 1 h, washed twice with DMEM and subsequently digested with 0.5% collagenase (Worthington, Type 2) in DMEM with 10% fetal bovine serum (FBS) for 4 h for NP and 30 h for AF. The cells were then passed through tailor made cell strainers of approximately 23 µm sieve size and washed with DMEM. The cells were lysed with 0.5 ml of TRIzol and stored at -80°C before RNA extraction.

Comparison of RNA extracted using TRIzol versus TRIspin with cultured cells

We compared the TRIzol extraction and the TRIspin extraction with cultured cells. The details of the methods are given in Section B of the supplementary information and **Figure S2** gave a simplified flow of the procedures in the TRIzol method and the combined TRIzol-column (TRIspin) method. Glycogen was used as a co-precipitant in the RNA precipitation step. In order to simulate the extraction of RNA from cells with high proteoglycan content, we also prepared cells in TRIzol with high proteoglycan content by adding IVD tissue to cell lysate in TRIzol. Some components such as proteoglycan dissolved from the IVD tissue into the TRIzol and increased the proteoglycan content in it. The extraction of RNA using TRIzol was performed according to the manufacturer's instruction and with a high salt solution when proteoglycan was involved according to the manufacturer's recommendation. For the TRIspin method, a published protocol was used [27].

Extraction of RNA from bovine IVD tissue

When RNA was extracted according to the manufacturer's instruction without any modification, a large white precipitate of proteoglycan was resulted. Based on manufacturer's instruction and a published study [26], high salt solution was added during the RNA precipitation step but this modification is not sufficient for bovine NP tissue which has a very high proteoglycan content. After different trials, the following modifications were made: (i) additional centrifugation of the initial homogenate to remove the residual tissue; (ii) extra phase separation step when necessary; (iii) use of high salt solution (0.8 M sodium citrate and 1.2 M sodium chloride) in the RNA precipitation step and (iv) an extra RNA wash using 75% ethanol to remove salts more completely.

The details are given in the Section C of the supplementary information. Some technical notes about the use of high salt solution and the handling of small RNA pellets or precious samples are described in Section D of the supplementary information, Some cautionary notes are included in the supplementary information where **Figure S3** shows a schematic of a bubble that may be formed when high salt solution is used and **Figure S4** shows a schematic of how the microcentrifuge tubes may be oriented during the centrifugation. Some troubleshooting notes are given in **Table S1** under Section E. **Figure S5** shows the UV spectra of RNA before and after re-extraction with TRIzol and before and after ethanol precipitation of RNA.

Extraction of RNA from mouse and rat tissue

In addition to bovine NP and AF tissues, RNA from cryosectioned mouse IVD and rat NP tissues were also extracted using a slightly different method. The details are given in Section F of the supplementary information where **Figure S6** shows a schematic of the procedures of embedding small tissue samples in DEPC treated water prior to cryosectioning.

Quantification of RNA yield and quality

RNA concentration and quality were estimated by Nanodrop 1000 or 2000c (Thermo Scientific). The integrity of RNA was assessed using an Agilent 2200 Bioanalyzer. There were overestimations of concentrations for RNA from bovine NP tissue, bovine AF tissue and rat NP tissue due to their low concentrations and a minute amount of phenol present. Thus the concentrations were corrected using a reported computational method developed in our group [28].

RT-qPCR

Total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara, cat. # HRR036A) using a themocycler. The cDNA was diluted five times by adding autoclaved water. qPCR was performed with a total reaction volume of 10 µl, containing 1 µl of single stranded cDNA, 5 µl of SYBR Green PCR Master Mix (Applied Biosystems, part # 4309155) and 500 nM each of forward primer and reverse primer (sequences given in **Table 4**), in 96 well reaction plates using a standard PCR protocol for SYBR green reactions (Applied Biosystems, StepOnePlus) with melt curve analysis. Autoclaved water was used as a negative control and RNA before reverse transcription was used to estimate the level of genomic DNA contamination. DNaseI treatment by Amplification Grade DNaseI (Invitrogen, cat. # 18068-015) was also included when necessary. The relative gene expression was calculated based on comparative Ct method.

Table 4. Sequences of bovine, rat and mouse primers used in this study. Sequences of bovine genes were from [6], mouse *Gapdh* and *Sox9* were from [29], and other rat and mouse primers were by design. Sequences were written in 5' to 3' for both forward and reverse primers.

Primer	Sequence (forward)	Sequence (reverse)
Bovine *GAPDH*	TGCCGCCTGGAGAAACC	CGCCTGCTTCACCACCTT
Bovine *ACAN*	GGGAGGAGACGACTGCAATC	CCCATTCCGTCTTGTTTTCTG
Bovine *COL2A1*	CGGGCTGAGGGCAACA	CGTGCAGCCATCCTTCAGA
Bovine *CDH2*	GCCATCAAGCCAGTTGGAA	TGCAGATCGAACCGGGTACT
Bovine *KRT18*	TTGAGCTGCTCCATCTGCAT	AAGGCCAGCTTGGAGAACAG
Bovine *KRT19*	CGGTGCCACCATTGAGAACT	CAAACTTGGTGCGGAAGTCA
Mouse *Gapdh*	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
Mouse *Sox9*	TGGCAGACCAGTACCCGCATCT	TCTTTCTTGTGCTGCACGCGC
Mouse *Mia1*	GACCGATCAATGGGATTTCT	CATAGAGCCGGAAGGGTAAC
Mouse *Runx1*	GGAGCGGTAGAGGCAAGA	ATGGTAGGTGGCAACTTGTG
Mouse *Acan*	GCAATTACCAGCTGCCCTTC	TCTTCTGCCCGAGGGTTCTA
Mouse *Col2a1*	AGAAGGCCTTGCTCATCCAG	GACGGTCTTGCCCCACTTAC
Mouse *Cdh2*	GACGAGAGGCCTATCCATGC	GGGTCGTTGTCAGCAGCTTT
Mouse *Krt19*	AGGGCCTTGAGATTGAGCTG	GCTCAGCTGGGCTTCAAAAC
Rat *Gapdh*	GTGAAGCTCATTTCCTGGTATG	AACTGAGGGCCTCTCTCTTG
Rat *Sox9*	AGGAAGTCGGTGAAGAATGG	TGGCGTTAGGAGAGATGTGA
Rat *Acan*	GGGCGTGAGAACTGTCTACC	ACTGACACACCTCGGAAGC
Rat *Col2a1*	GATGGCTGCACGAAACAC	GCCCTATGTCCACACCAAAT
Rat *Cdh2*	TTAATGAGGGCCTTAAAGCTG	GCTGGAGGAGTTGAGAGAGC
Rat *Krt19*	TCTCAGCTCAGCATGAAAGC	ACTGCTGATCACACCTTGGA

RESULTS

Processing of bovine IVD tissues

We found that the bovine NP tissue cannot behomogenized by an electric ceramic pestle and mortar system. Thus cryosectioning was used to cut the bovine NP or AF tissues into sections so that RNA can be released in TRIzol when TRIzol is added later.

Comparison of RNA extracted using TRIspin and using RNeasy from bovine IVD tissues and using TRIzol from bovine isolated cells (Experiment set 1 in Figure 3)

Among the three methods, the extraction of RNA from isolated cells using TRIzol gave the highest yield and lowest Ct values in qPCR amplification (**Table 5**) with peaks at 260 nm in the UV spectra (**Figure 4A** and **4B**). RNA from the TRIspin method gave a peak at 270 nm (**Figure 4C** and **4D**) which is a typical peak of phenol contamination. There was no peak of RNA (260 nm) observed for the RNeasy method (**Figure 4E** and **4F**), probably due to the low RNA concentrations. The measured RNA concentrations of the TRIspin method were higher than those of the RNeasy method but this was due to the overestimation of RNA concentrations caused by residual phenol present. Thus the concentrations were corrected using a mathematical formula developed in our group [28]. The corrected concentrations were indeed much lower than the measured concentrations. Instead of comparing the yield of RNA from NP tissues using the TRIspin method and that using the RNeasy method, the Ct values of the amplification in qPCR were compared. Both methods yield comparable Ct values (**Table 5**). Among the three methods, the use of TRIzol with isolated cells gave the highest cell yield but the mRNA levels may change during the enzymatic cell isolation where the cells were treated in an environment different from that *in vivo*. Therefore we further investigated the optimization of RNA extraction from NP tissue directly using TRIzol.

Table 5. RNA extracted from bovine NP cells using TRIzol and NP tissues using TRIspin or RNeasy and the corresponding Ct values of *GAPDH* amplification. Concentration, yields and quality of RNA extracted from bovine NP tissue or cells estimated by Nanodrop (mean ± SD; n = 3). [a]About 500 mm^3 of NP tissue was used for cell isolation and about 50–100 mm^3 of tissue was used to prepare one 1.5 ml tube of cryosectioned tissue. [b]The concentrations were corrected with a mathematical formula [28]. The RNA was resuspended in 12.5 µl of DEPC treated water for TRIzol extracted RNA and the column was eluted with 12.5 µl of DEPC treated water twice (total of 25 µl) for the TRIspin method and the RNeasy method. [c]The Ct values were adjusted based on 100 mg of tissues and 4 µl of RNA used in DNaseI treatment. [d]The concentration correction using the mathematical formula reported in [28] was not applicable for RNA extracted using the RNeasy kit as the RNA was not contaminated with phenol.

	Tissue weight (mg)[a]	A260/A280	A260/A230	Measured conc. (ng/µl)	Corrected conc. (ng/µl)[b]	Yield (ng RNA/ mg tissue)	Adjusted Ct[c]
NP cells	624.7 ± 80.3	2.01 ± 0.03	1.51 ± 0.05	294.3 ± 36.1	298.7 ± 36.7	6.1 ± 1.4	20.5 ± 2.4
NP TRIspin	78.4 ± 4.3	1.60 ± 0.16	0.46 ± 0.16	12.7 ± 8.1	1.5 ± 0.3	0.5 ± 0.1	26.8 ± 0.7
NP RNeasy	64.7 ± 6.5	-3.57 ± 3.56	0.06 ± 0.06	0.9 ± 0.1	[d]	[d]	26.3 ± 0.6
AF cells	497.9 ± 26.2	1.97 ± 0.05	2.15 ± 0.09	308.3 ± 43.1	305.2 ± 44.9	7.7 ± 1.3	23.0 ± 2.1
AF TRIspin	83.2 ± 16.5	1.55 ± 0.09	0.65 ± 0.20	20.8 ± 19.6	4.0 ± 4.5	1.3 ± 1.6	26.1 ± 1.7
AF RNeasy	64.3 ± 3.8	2.81 ± 0.40	0.17 ± 0.21	4.2 ± 1.1	[d]	[d]	26.1 ± 1.1

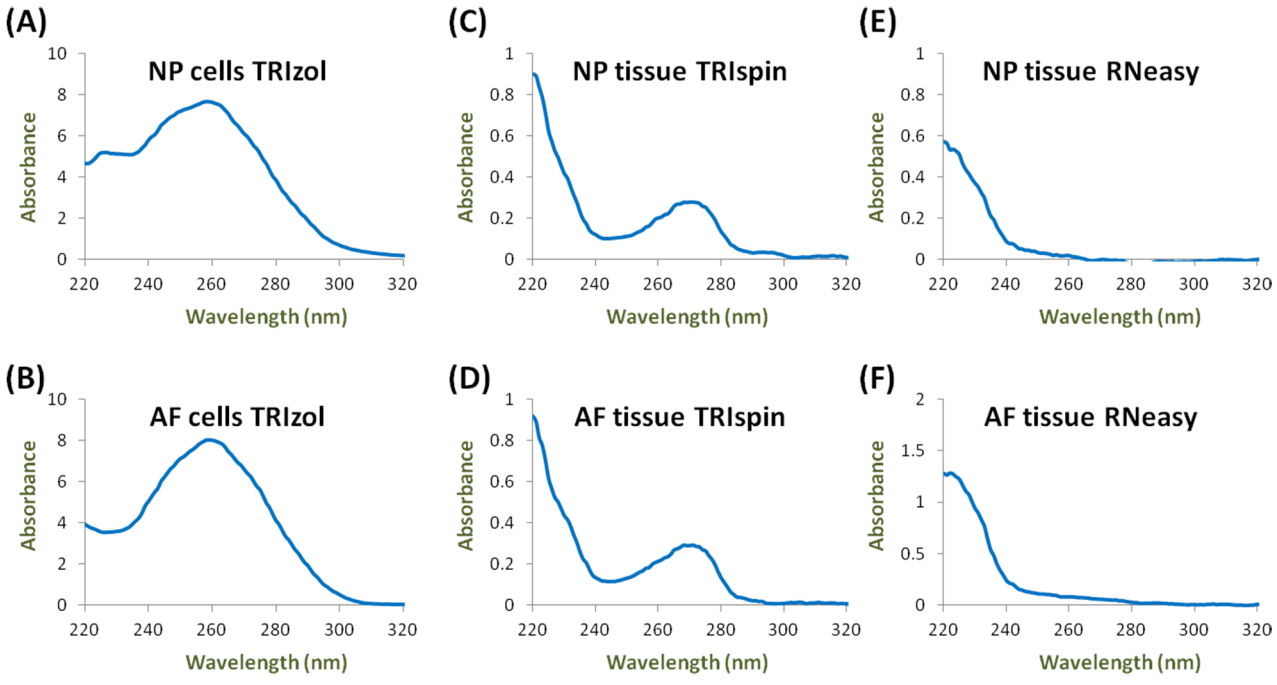

Figure 4. UV spectra of RNA extracted from bovine NP and AF cells using TRIzol and from tissues using TRIspin or RNeasy. A-F. UV spectra of RNA extracted using TRIzol from (A) isolated NP cells, (B) isolated AF cells; extracted using TRIspin from (C) NP tissue cryosections, (D) AF tissue cryosections; extracted using RNeasy kit from (E) NP tissue cryosections and (F) AF tissue cryosections.

Comparison of RNA extracted using TRIzol and using TRIspin with cultured cells (Experiment set 2 in Figure 3)

As shown in **Figure 5A**, all the samples showed peak absorbance at 260 nm in Nanodrop measurements and the peaks for TRIzol extracted RNA were sharper due to the higher concentrations of RNA compared to the ones from the TRIspin method. The 28S and 18S bands were observed in the electropherograms of the samples (**Fig. 5B**). Bands of small molecular weights were observed for RNA extracted from cells with the TRIzol methods (CT, CTG) but not for the TRIspin method (CC). This may be due to the low binding affinity of low molecular weight RNA to the column silica membrane which can get discarded during the isolation process. Stronger bands were observed for RNA extracted from cells with proteoglycan using the TRIzol method (HPT) than that using combined method (HPC). The smear present in the HPT sample may represent mRNA of different molecular weights or partially degraded RNA.

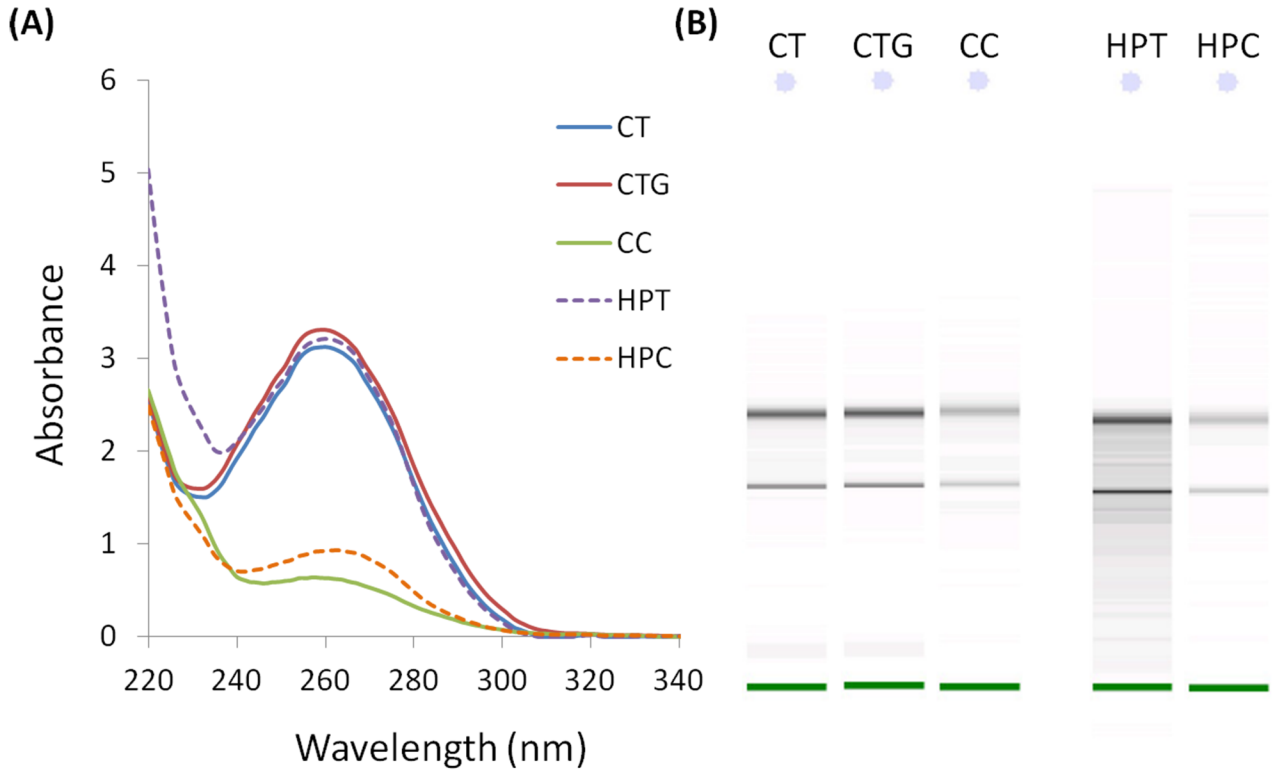

Figure 5. UV spectra and electropherograms of RNA extracted using TRIzol or combined TRIzol-column (TRIspin) method. A. UV spectra of RNA extracted from bovine cultured cells, and from lysed cultured cells mixed with IVD tissue using TRIzol or TRIspin methods. **B.** Electropherograms of RNA extracted with different methods (CT, from cells using TRIzol without the use of glycogen; CTG, from cells using TRIzol with the use of glycogen; CC, from cells using the combined TRIspin method; HPT, from cells and high proteoglycan content using TRIzol; HPC, from cells and high proteoglycan content using the combined TRIspin method).

Table 6 shows the Nanodrop measurements and Bioanalyzer results of RNA extracted with the TRIzol method or the TRIspin method. For the cells without extra proteoglycan added, the concentration of RNA extracted using the TRIzol method (101.6 or 125.9 ng/µl) was four times more than that of using the TRIspin method (23.3 ng/µl). The use of glycogen increased the yield of RNA obtained but is not statistically significant. In our study, RNA pellets could be observed, indicating the precipitation of RNA, even when glycogen was not added. When the cell number was even lower, glycogen was required as a co-precipitant to visualize the RNA pellet. For the cells with higher proteoglycan content, the extraction of RNA using TRIzol procedures were also four times more (171.7 ng/µl) than the TRIspin method (38.8 ng/µl). There were no significant differences in the A260/A280 values of RNA obtained. Consistent with the data from Nanodrop, RNA samples extracted with the TRIzol method estimated with Bioanalyzer were of higher concentrations compared with the TRIspin method. RNA extracted using different methods yielded RNA of RNA integrity number (RIN) > 5, indicating their suitability for RT-qPCR.

We tested the RT-qPCR with the RNA extracted with different methods and all samples have amplification efficiency within 90–110% in qPCR calibration curves (**Table 6**), which is a criterion for reliable qPCR. These indicate the RNA extracted with these methods was compatible with the qPCR system used.

Modification of RNA extraction methods for bovine IVD tissues

In standard extraction of RNA using TRIzol, the supernatant after the first phase separation is clear (**Fig. 6A**). However, when RNA was extracted from bovine NP tissue, the supernatant was milky and this led to undesirable precipitates in the subsequent RNA precipitation step. Using high salt solution in RNA precipitation step was suggested to avoid the precipitation of contaminating proteoglycans [26]. We tested this approach with the bovine NP tissue but contaminating precipitates were still observed, probably due to the high proteoglycan content of the

tissue (approximately 60% of dry weight) [3,30] and a low cell content (0.19%) [4] which made using a small volume of tissue insufficient. We further explored different modifications to overcome this problem and found that using a second phase separation could yield a clear supernatant (**Fig. 6B**) which would not give the undesirable precipitates. The modifications made were summarized in **Figure 6C**. Using the cryosection method, sections could also be obtained for histology study simultaneously (**Fig. 6D**).

Table 6. Concentration, yield and quality of RNA extracted from cultured cells using TRIzol or combined TRIzol-column (TRIspin) method. Upper part: Concentrations, yield and quality of RNA measured using Nanodrop (mean ± SD; 3 samples per group; single measurement per sample for cells and double measurements per sample for cells with tissue); Lower part: Concentrations and quality of RNA assessed using Bioanalyzer and the corresponding qPCR efficiency in RT-qPCR (mean ± SD; 3 samples per group for Bioanalyzer; amplification efficiency was estimated from 4 standard curves from 2 samples per group) [a]For cells with and without tissues, the yield was based on the cells which were approximately equivalent to 2 wells of bovine cells in a 96 well plate (estimated number of cells of around 10^5). The RNA was resuspended in 12 µl of DEPC treated water for TRIzol extracted RNA and the column was eluted with 12 µl of DEPC treated water twice (total of 24 µl) for the TRIspin method.

Samples	**Concentration (ng/µl)**	**Yield (µg)[a]**	**A260/A280**	**A260/A230**
By Instrument	**Nanodrop**	**Nanodrop**	**Nanodrop**	**Nanodrop**
Cells				
TRIzol, w/o glycogen	101.6 ± 24.6	1.22 ± 0.29	1.84 ± 0.05	2.10 ± 0.04
TRIzol, w glycogen	125.9 ± 6.2	1.51 ± 0.07	1.80 ± 0.01	1.96 ± 0.12
TRIspin	23.3 ± 4.1	0.56 ± 0.10	1.87 ± 0.05	0.88 ± 0.39
Cells mixed with tissue (high proteoglycan)				
TRIzol	171.7 ± 5.9	2.06 ± 0.07	1.99 ± 0.02	1.28 ± 0.23
TRIspin	38.8.± 6.1	0.93 ± 0.15	1.92 ± 0.02	0.84 ± 0.46
Samples	**Concentration (ng/µl)**	**RIN**	**28s/18s**	**qPCR efficiency**
By Instrument	**Bioanalyzer**	**Bioanalyzer**	**Bioanalyzer**	**StepOnePlus**
Cells				
TRIzol, w/o glycogen	91.0 ± 33.1	9.67 ± 0.25	1.87 ± 0.15	99% ± 3%
TRIzol, w glycogen	105.0 ± 23.8	9.90 ± 0.10	1.97 ± 0.12	97% ± 11%
TRIspin	15.7 ± 2.3	8.33 ± 0.45	2.43 ± 0.93	107% ± 10%
Cells mixed with tissue (high proteoglycan)				
TRIzol	192.0 ± 10.8	6.93 ± 0.81	1.60 ± 0.44	95% ± 6%
TRIspin	51.0 ± 7.9	9.07 ± 0.15	1.83 ± 0.12	101% ± 5%

RNA extracted from bovine NP, AF and muscle tissues (Experiment set 3 in Figure 3)

We tested the modified TRIzol protocol with bovine NP, AF and muscle tissues. Muscle was included as a non-IVD control as it has a high cell density with low proteoglycan content, to which typical extraction should be applicable. As shown in **Table 7**, the concentrations and yields of RNA from NP and AF were lower than that of muscle, probably due to the higher density of cells in muscle. The RNA isolated directly from NP tissue and AF tissue had lower A260/280 ratios compared to muscle, which was probably due to the contamination by phenol. This was suggested by the UV absorption spectra that there was a shift of peak towards 270 nm for the RNA isolated from NP or AF tissue (**Fig. 7A**). The RNA from bovine muscle using the same extraction method yielded a sharp peak at 260 nm (**Fig. 7A**). The presence of phenol from TRIzol in the samples can cause over-estimations of RNA concentrations due to the absorbance contributed by phenol at 260 nm. Therefore, we corrected the concentrations using a reported computational method developed in our group [28].

Extraction of RNA from mouse IVD and rat NP tissues (Experiment set 3 in Figure 3)

In order to extend the method to other species, we also tested the extraction of RNA from mouse and rat IVDs. The details of the procedures are given in Section F of the supplementary information. Unlike bovine NP tissue and AF tissue, the tissues obtained from mouse and rat IVDs were rather small. It was difficult to mount the mouse or rat tissue directly for cryosection. Thus a method to embed the disc in DEPC-treated water was used. In brief, small aluminum containers were made to hold DEPC-treated water. Then the containers were dipped into liquid nitrogen to partially freeze the water, leaving the central top part of the water unfrozen. The pre-frozen mouse or rat disc tissues were then put in the top part of the unfrozen water and fixed quickly by freezing the remaining unfrozen water using liquid nitrogen. Afterwards, the procedures of cryosection and RNA extraction were similar to those of bovine tissues.

The measurements of the RNA extracted from mouse IVD and rat NP by Nanodrop are shown in **Table 7**. Concentrations of RNA extracted

from a mouse IVD was higher than that from NP from half a rat tail. NP was isolated from the rat disc but not mouse discs since the mouse discs were much smaller in sizes which made separating NP from the disc more difficult. There was a slight shift of the peak in UV spectrum from 260 nm to 270 nm for the rat NP (**Fig. 7B**). The 28S and 18S bands were visible (**Fig. 7C**) and the RINs were 6.9 and 7.5 for mouse IVD and rat NP respectively. Amplifications were detected for different genes for the extracted RNA as shown in **Figure S7** (Section G).

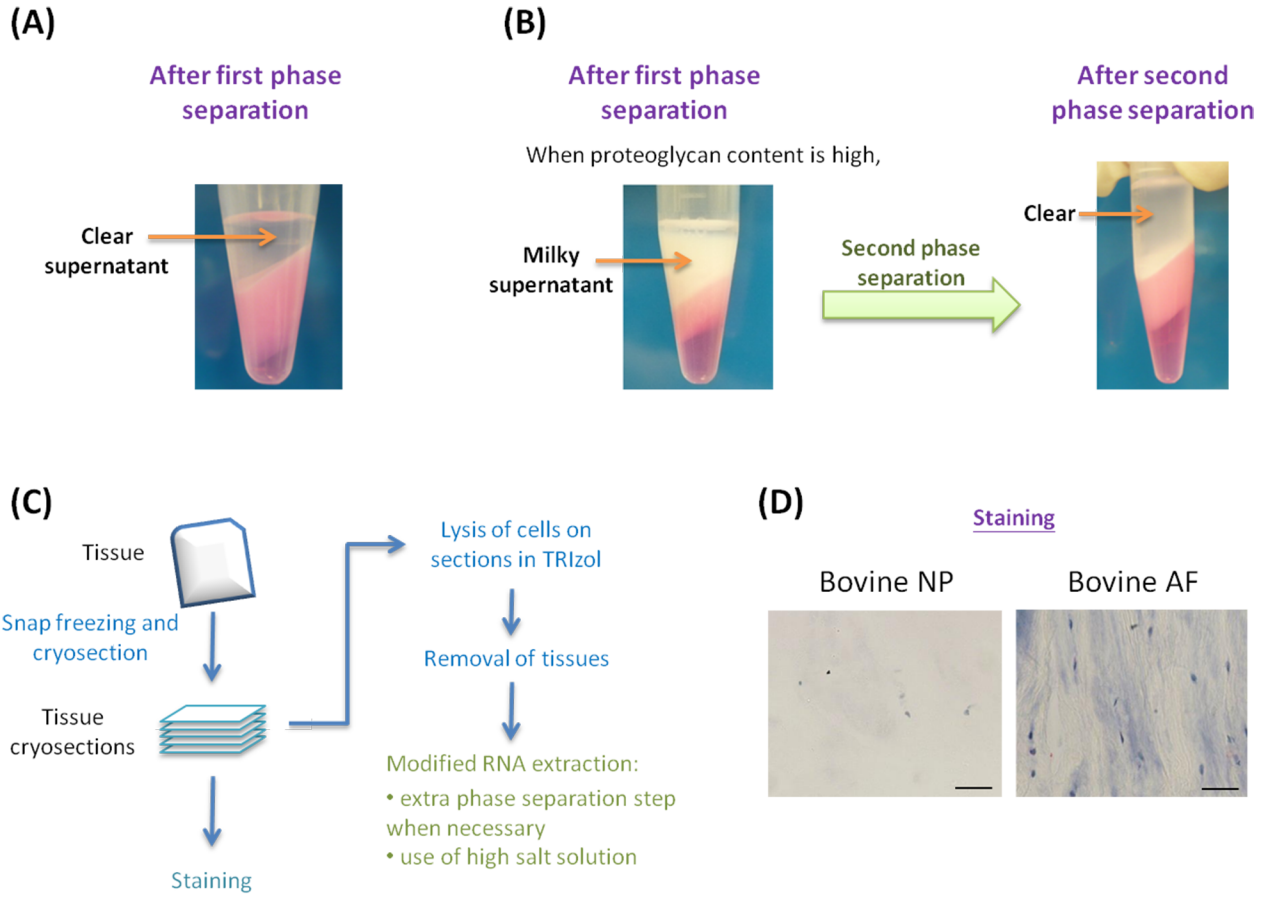

Figure 6. RNA extraction from bovine IVD tissues using TRIzol. A. Representative photo of a sample after the first phase separation in the TRIzol extraction protocol. In standard extraction, the supernatant should be clear. **B.** Representative photo of a sample with high proteoglycan content after the first phase separation in the TRIzol extraction protocol. The supernatant was milky and when the supernatant was re-extracted using TRIzol, the supernatant after the second phase separation became clear. **C.** Schematic showing the procedure of RNA extraction from IVD tissue. **D.** Micrographs of sections of bovine IVD tissues with nuclei stained purple with hematoxylin (scale bar = 50 µm).

Table 7. RNA extracted from bovine, mouse and rat tissues using TRIzol. Concentration, yields and quality of RNA extracted from bovine NP, AF and muscle tissues, mouse IVD tissue and rat NP tissue estimated by Nanodrop. [a]Concentrations of bovine NP, bovine AF and rat NP were corrected with a mathematical formula [28]. [b]The yield was based on one 1.5 ml tube of cryosectioned bovine tissue prepared from 100–250 mm^3 of tissues, one mouse disc or rat NP tissue from half a rat tail. (mean ± SD; n = 3 for bovine tissues, n = 7 for mouse IVDs, and n = 2 for rat NP).

Samples	Concentration (ng/µl)[a]	Yield (µg)[b]	A260/A280	A260/A230
By Instrument	**Nanodrop**	**Nanodrop**	**Nanodrop**	**Nanodrop**
Bovine NP	30.2 ± 1.5	0.33 ± 0.02	1.76 ± 0.04	0.74 ± 0.10
Bovine AF	42.8 ± 5.5	0.47 ± 0.06	1.81 ± 0.05	0.50 ± 0.28
Bovine muscle	372.1 ± 33.0	4.09 ± 0.36	2.07 ± 0.01	1.55 ± 0.19
mouse IVD	325.5 ± 110.4	3.25 ± 1.10	1.88 ± 0.03	1.48 ± 0.34
rat NP	107.6 ± 15.7	1.35 ± 0.20	1.78 ± 0.01	0.97 ± 0.01

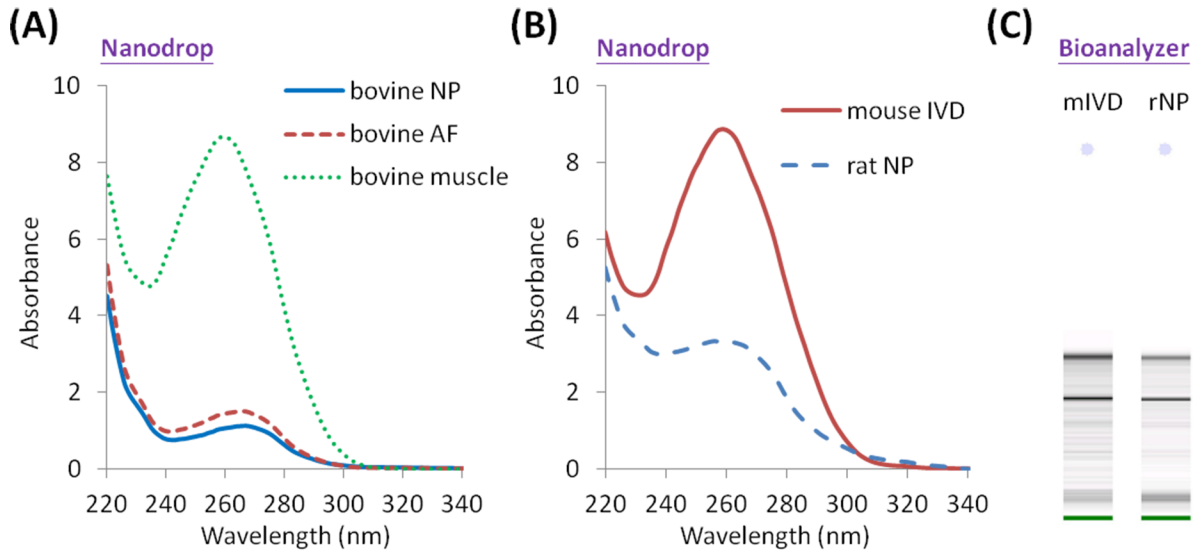

Figure 7. RNA extracted from bovine, mouse and rat tissues. A. UVspectra of RNA extracted from bovine NP, AF and muscle tissues. **B.** UVspectra of RNA extracted from mouse IVD and rat NP tissues. **C.** Electropherograms of RNA extracted from mouse IVD (mIVD) and rat NP tissues (rNP).

Comparison of RNA extracted using TRIzol and using TRIspin from bovine NP tissue (Experiment set 4 in Figure 3)

The yields and quality of RNA extracted from bovine NP cryosections using TRIzol were compared with those using TRIspin. For the TRIspin method, milky supernatant was occasionally observed after the phase separation. Therefore we further investigated whether further centrifugation of the supernatant after phase separation before ethanol addition could improve the RNA yields or quality (**Fig. S8**, Section H). After the centrifugation, a small pink layer (about 5 µl) with a white layer on top was observed at the bottom of the tubes. **Table 8** shows the quality and yields of RNA obtained from the three methods. The yields of RNA obtained using the modified TRIzol method was higher than those using the TRIspin method (**Table 8**) and the corresponding Ct values were smaller (**Table 9**) for the three housekeeping genes (*GAPDH*, *18S rRNA*, *YWHAZ*) and two extracellular matrix genes (ACAN, *COL2A1*). **Figure 8** shows the corresponding melt curves of the qPCR amplification. Both TRIzol and TRIspin methods gave single major peaks for *GAPDH*, *18S rRNA* and *COL2A1*. No peak was observed for YWHAZ and a less defined peak for *ACAN* for RNA extracted using the TRIspin method. This was probably due to the low concentrations of RNA extracted. There was no significant difference between the TRIspin samples prepared with or without the extra centrifugation but one out of the three samples without an extra centrifugation showed a UV absorbance curve without any peak in the 220–350 nm range (**Fig. S9**, Section I), which may be due to proteoglycan contamination.

Table 8. RNA extracted from bovine NP tissues using modified TRIzol or TRIspin. Concentration, yields and quality of RNA extracted from bovine NP tissue estimated by Nanodrop (mean ± SD; n = 3). [a]One 1.5 ml tube of cryosectioned bovine tissue prepared from about 50 mm^3 of tissue (about 30–50 cryosections of 14 µm thick and about 1 cm^2 large) was used per sample. [b]The concentrations were corrected with a mathematical formula [28]. The RNA was resuspended in 12.5 µl of DEPC treated water for TRIzol extracted RNA and the column was eluted with 12.5 µl of DEPC treated water twice (total of 25 µl) for the TRIspin method. [c]"TRIspin 1" refers to TRIspin extraction without an extra centrifugation of supernatant after TRIzol phase separation before ethanol addition and "TRIspin 2" refers to that with the extra centrifugation (refer to **Fig. S8**). [d]For the corrected concentration and yield calculation, the values from the sample with a high level of contamination were excluded.

	Tissue weight (mg)[a]	A260/A280	A260/A230	Measured conc. (ng/µl)	Corrected conc. (ng/µl)[b]	Yield (ng RNA/ mg tissue)
TRIzol	68.8 ± 15.3	1.67 ± 0.11	0.41 ± 0.36	25.6 ± 13.6	16.8 ± 12.9	2.80 ± 1.66
TRIspin 1[c, d]	72.8 ± 5.5	1.55 ± 0.08	0.50 ± 0.08	13.8 ± 13.9	0.9 ± 0.6	0.30 ± 0.22
TRIspin 2[c]	80.5 ± 24.2	1.46 ± 0.32	0.46 ± 0.08	6.5 ± 2.3	1.2 ± 1.4	0.52 ± 0.71

DISCUSSION

Homogenization methods for IVD tissues

Homogenization is a general term to describe the dispersion of minute fragments of tissue evenly throughout a mixture by mechanical action such as macerations or crushing or by chemicals such as detergents or organic solvents. There are a wide variety of homogenizers available to achieve such a purpose. In our study, bovine NP tissue could not be homogenized by an electric ceramic pestle and mortar system. However, there were some studies which reported the use of homogenizer for extraction of RNA from NP tissues (**Table 2**) [17-21]. Only two out of the five studies have mentioned the types homogenizers used.

Wang et al. used an Omni TH homogenizer [17] which used vibration to break the tissues. The use of Omni tissue homogenizer may be a viable option for bovine NP tissue but this requires the purchase of a specialized equipment. Simple pestle and mortar systems may be a less expensive option. Capossela et al. used a stainless steel mortar to pulverize human NP and AF tissue on dry ice [18] but the size and type of mortar was not mentioned precisely. In our attempts to homogenize the bovine NP tissue by a pestle and mortar system, it was difficult to pulverize the tissue as the bovine NP tissue from an adult animal was hard yet not brittle when frozen. In contrast, the bovine NP tissue could be cut without much difficulty when preparing cryosections for histology. The cryostat was available in our institute as a shared equipment. Thus we tested cryosectioning to cut the tissues into thin sections so that the cells can be exposed to TRIzol and RNA can be released from them.

Table 9. Adjusted Ct values of RT-qPCR for the RNA extracted from bovine NP tissues using modified TRIzol or TRIspin after DNase1 treatment. [a]TRIspin samples had lower RNA concentrations than the TRIzol samples and thus a larger volume of RNA solution was used for TRIspin (4 µl of RNA for TRIspin and 0.5 µl for TRIzol were used for DNaseI treatment). The Ct values were adjusted based on 4 µl of RNA used for DNaseI treatment for equal volume basis of starting materials (raw Ct values and values with adjustment to the same RNA amount are given in **Table S2** and **Table S3** respectively). [b]"TRIspin 1" and "TRIspin 2" refers to TRIspin extraction without and with an extra centrifugation of supernatant after TRIzol phase separation before ethanol addition (refer to **Fig. S8**). [c]Only 1 out of the 3 samples has detectable amplification. (*GAPDH*, *18S rRNA*, *YWHAZ* are housekeeping genes and *ACAN* and *COL2A1* are extracellular matrix genes highly expressed in NP cells and chondrocytes) (mean ± SD; n = 3).

	GAPDH	*18S rRNA*	*YWHAZ*	*ACAN*	*COL2A1*
TRIzol[a]	23.3 ± 2.2	14.6 ± 1.7	27.5 ± 2.5	20.3 ± 0.2	18.3 ± 0.6
TRIspin 1[a, b]	32.5 ± 0.6	25.4 ± 0.6	33.5[c]	31.0 ± 0.7	28.2 ± 0.5
TRIspin 2[a, b]	32.2 ± 1.3	24.6 ± 0.9	Undetectable	30.7 ± 1.2	28.1 ± 1.6

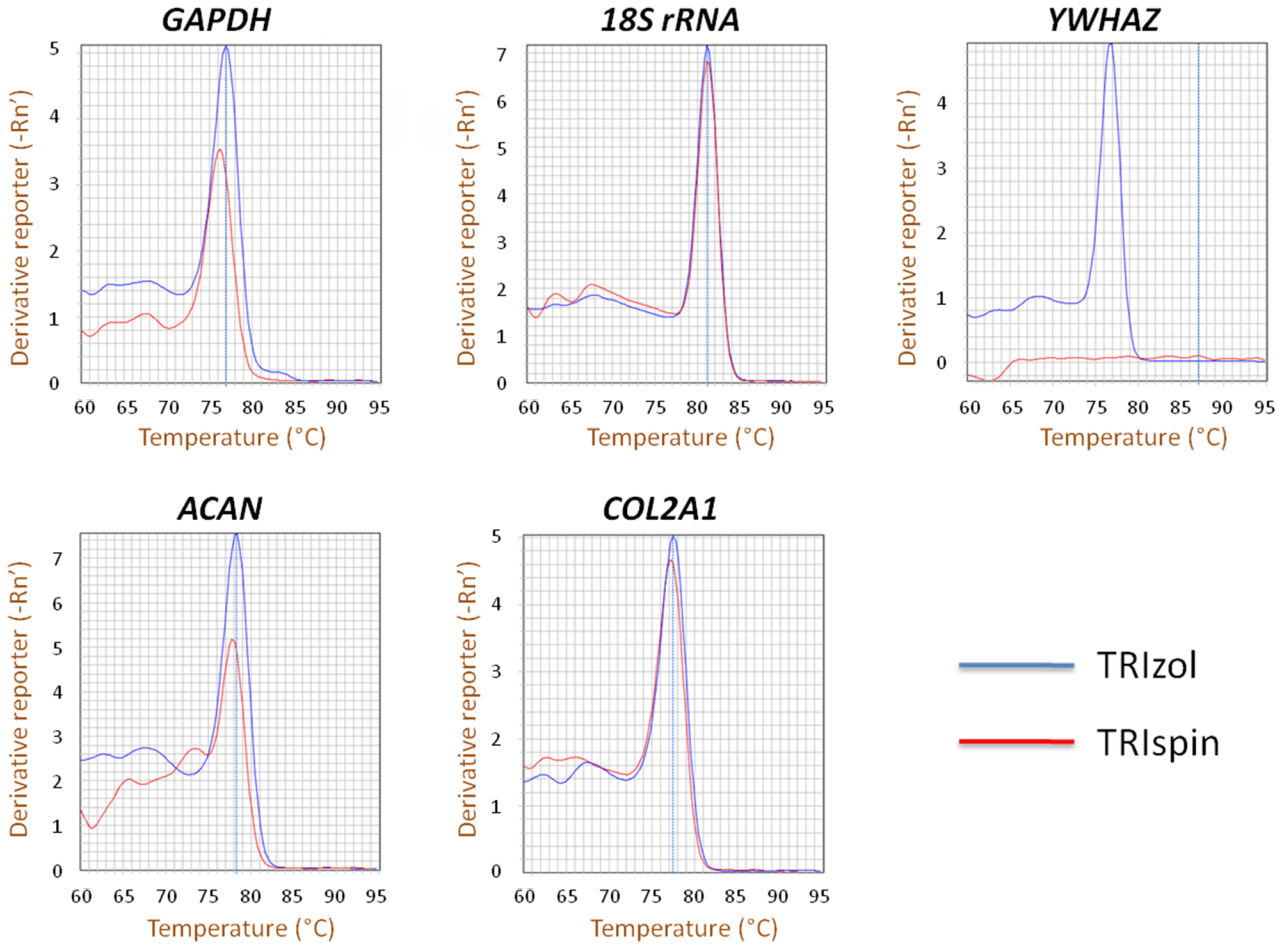

Figure 8. Melt curves of *GAPDH, 18S rRNA, YWHAZ, ACAN and COL2A1* PCR products fromRNA extracted with the TRIzol method and the TRIspin method. *GAPDH*, *18S rRNA*, *YWHAZ* are housekeeping genes and *ACAN* and *COL2A1* are matrix genes highly expressed in NP cells and chondrocytes

Yields of RNA from rat NP tissue

In our study, RNA precipitation method using propanol and a high salt solution was used. The studies reported by other groups used column based kits for RNA extraction [17-21]. Tang et al. extracted RNA from rat NP and AF for cDNA microarray analysis and RT-qPCR. After harvesting the tissue, they immediately flash-freezed the tissues in liquid nitrogen, then pulverized and homogenized in TRIzol reagent, followed by total RNA extraction using a RNeasy mini kit. For each sample in microarray, tissue was pooled from all discs across four rats to collect sufficient RNA for one sample [21]. The amount and quality of RNA used was not mentioned in their study. For Affymetrix GeneChip Expression analysis service provided by the Centre for Genomic Sciences of the University of Hong Kong, 2.5 μg total RNA of RIN > 8 is recommended. In our study, 1.35 μg of total RNA was obtained from NP tissue from half a rat tail. In other words, RNA from one tail (> 2.5 μg) was sufficient for microarray analysis. The RIN was 7.5 which was lower than 8, indicating that the procedures may need to be improved for better RNA quality. However, in an article about quality control of RNA for microarray, RIN of 7.0 was used as a cut-off value for deciding whether the RNA is suitable for microarray analysis [31]. The authors selected this value because Thompson et al. showed that there was a substantial increase in the rate of false positives on the microarray when the starting RNA had a RIN value of <7.0 [32]. This may suggest the RNA obtained in our study which has RIN value of 7.5 may also be suitable for microarray analysis. Only one rat is sufficient to obtain RNA from rat NP tissue for microarray analysis using our modified protocol compared to four rats required in the study by Tang et al. Fewer animals may be needed to be sacrificed for each microarray analysis.

CONCLUSIONS

In summary, higher yields were obtained from a small number of cells using the TRIzol method compared to the combined TRIzol-column (TRIspin) method. We have developed a method to isolate RNA from bovine IVD tissues directly by integrating cryosectioning, additional phase separation and high salt precipitation into conventional GITC-based RNA extraction method. The cryosectioning approach of sample preparation was also applicable to other tissues such as bovine muscle, mouse IVD and rat NP tissues. This study suggests an alternative means of homogenization using a cryostat which is more easily available as a shared equipment. The possible higher yield of TRIzol extraction may reduce the number of animals sacrificed for obtaining sufficient amount of RNA.

Authors' contributions

In this report, J.T.Y.L. designed and performed the experiments, and wrote the paper; K.M.C.C. and V.Y.L.L. were involved in experiment design, supported the study and helped in the revision of this paper. All authors have read and approved the final submitted manuscript.

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Supplementary information

Figure S1. Suggestion of experiment procedures for extraction of RNA from tissues rich in extracellular matrix with low or high cellularity.

Figure S2. Simplified procedures in the TRIzol method and the combined TRIzol-column (TRIspin) method.

Figure S3. Schematic showing that a bubble may be formed after the 75% ethanol addition when the mixture of supernatant with propanol and high salt solution is not sufficiently removed.

Figure S4. Schematic showing how the microcentrifuge tubes may be oriented during the centrifugation .

Figure S5. UV spectra of RNA (A) before and (B) after re-extraction with TRIzol; and (C) before and (D) after ethanol precipitation of RNA.

Figure S6. Schematic showing the procedures of embedding small tissue samples in DEPC-treated water prior to cryosectioning.

Figure S7. (A) qPCR amplification curves of RNA extracted from mouse IVD and (B) from rat NP.

Figure S8. Simplified procedures for TRIspin 1 and TRIspin 2.

Figure S9. UV spectra of RNA extracted with the TRIspin method without an extra centrifugation of supernatant after TRIzol phase separation before ethanol addition.

Table S1. Troubleshooting notes for extracting RNA from tissues with high proteoglycan content with low cellularity.

Table S2. Raw Ct values of the RNA extracted from bovine NP tissues using modified TRIzol or TRIspin after DNase1 treatment and RT-qPCR.

Table S3. Adjusted Ct values of the RNA extracted from bovine NP tissues using modified TRIzol or TRIspin after DNase1 treatment and RT-qPCR.

Supplementary information of this article can be found online at http://www.jbmethods.org.